EFFECT OF c-Ha-*ras*(V12) ON PROTEASE TRAFFICKING IN INVASIVE BREAST CANCER CELLS.

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ABSTRACT

Effect of c-Ha-ras(V12) on cathepsin trafficking in invasive breast cancer cells.

Various mutations of Ha-*ras* together with lack of p53-related control over cell cycle progression, result in an immortal, tumorigenic phenotype in 50% human epithelial cancers. Unmutated Ha-Ras transiently mediates external growth factor-related signaling, initiating downstream kinase activity that is normally terminated by p53. This protects the cell from immortalization, i.e. uncontrolled proliferation.

An MCF10A breast epithelial cell line, derived from a fibrocystic breast mastectomy specimen, spontaneously immortalized in culture, due to a chromosomal deletion (*9p21-/-)*. This gave rise to a non-malignant and non-invasive cell line in which the effects of deletion of upstream control of both p53 and the cell cycle and c-Ha-*ras*(V12) transfection may be studied. Transfection of this cell line with the c-Ha-*ras*(V12) oncogene gave rise to the invasive MCF10AneoT premalignant derivate, in which distribution of cathepsin B (CB), cathepsin L (CL) and cathepsin D (CD), membrane-type 1 matrix metalloprotease (MT1-MMP), a membrane-bound collagenase, is altered. The possible role of these proteases in the premalignant invasive phenotype, as well as the role of the V12 mutation and the effect of p53 on vesicle trafficking, was explored.

In the MCF10AneoT cell line lack of negative feedback by p53 and other Ha-Ras effectors such as Rac, Rho and CDC42, seems to result in lack of control over the cytoskeleton and thus cell polarity during growth stimulus-related migration. Luminal alkalinization, especially of vesicles distant from the perinuclear region, as well as degradative efficiency seem affected, possibly as functional assembly of the acidifying vacuolar-ATPase proton pump on these vesicles is compromized. In normal cells CB and CD seem discretely located, while a spread of proteases was noted in transfected cells, from a perinuclear position to along the basal plane. Increased association of CB with lysosome-associated membrane protein-2 (LAMP-2), and of CD with an acidic juxta-nuclear structure (JNS) was also noted, while this structure was observed in two sites in transfected cells, compared to only one in normal cells. In invasive cancers increased levels of both CB and MT1-MMP have been found to correlate with accelerated pathological degradation and invasion of the underlying basement membrane (BM) barrier and extracellular matrix (ECM). MT1-MMP is known to regulate BM turnover, while the manner in which the association of CB with the plasma membrane (PM) supports such turnover, ECM degradation and migration, is not yet clear. The current investigation showed altered distribution of PM-associated CB and MT1-MMP in transformed cells, compared to normal. This phenotype seems explained in terms of the effects of the mutationally activated c-Ha-Ras(V12) on its downstream effectors, Rac and PI3K and their effectors, on cytoskeletal organization and vesicle trafficking, increased calcium and, via Rho, cytoplasmic alkalinization due to proton extrusion by an activated NHE-1 membrane-associated proton pump.

PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg from June 2004 to December 2009, under the supervision of Dr Edith Elliott.

These studies represent original work by the author and have not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

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Celia Snyman

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Dr Edith Elliott

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May this work glorify the God and Creator of the cosmos Who, through this experience revealed some of the splendour of the microscopic universe.

LIST OF ABBREVIATIONS

3D	three dimentional
Ab	antibody
AJ	adherent junction
AKT/PKB	AK mouse transforming retrovirus / protein kinase B
AP	adaptor protein
ARF	ADP-ribosylation factor
BAD	Bcl-2 member and agonist of cell death
BafA1	bafilomycin A1
Bcl	beclin
BM	basement membrane
BRCA-1	breast cancer gene-1
BRCA-2	breast cancer gene-2
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CANSA	Cancer Association of South Africa
CAPRI	calcium-promoted Ras inhibitor/ inactivator
Cat	catalytic domain
cav-1	caveolin-1
CB	cathepsin B
CCV	clathrin-coated vesicle
CD	cathepsin D
CD44	cyclin dependent surface glycoprotein 44
CDC25	cell division cycle protein 25
CDC42	cell division cycle protein 42
CDK	cyclin D-dependent kinase
CD-MPR	cation-dependent mannose-6-phosphate receptor
CDP	CCAAT-displacement protein
CI-MPR	cation-independent mannose-6-phosphate receptor
CL	cathepsin L
Cl^-	chloride ion
col-IV	collagen-IV
ConA	concanamycin A
COP	coatomer proteins
DAG	diacylglycerol
DAMP	*N*-(3-[(2,4-dinitrophenyl)-amino]-propyl)-*N*-(3-aminopropyl-methylamine)dihydrochloride
DCIS	ductal carcinoma *in situ*
DNP	2,4-dinitrophenyl
ECM	extracellular matrix
ECV	endosomal carrier vesicle
EE	early endosome
EEA1	early endosomal antigen 1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ER	endoplasmatic reticulum
ERK	extracellular-signal-related kinase
ERM	ezrin / radixin / moesin / merlin proteins
ESCRT	endosomal sorting complex required for transport
EsR	estrogen receptor
FA	focal adhesion

FAK	focal adhesion kinase
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GEF	guanosine nucleotide exchange factor
GGA	Golgi-localized, γ ear-containing ARF protein
Grb-2	growth factor receptor-binding protein-2
GRF	guanine nucleotide-releasing factor
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
H^+	hydrogen ion
HA	hyaluronic acid
HEPES	N-2-hydroxy-piperazine-N'-2 ethane sulfonic acid
HER-2/ERB-2	human estrogen receptor-2 / estrogen receptor -2
HMEC	human mammary epithelial cell
HRP	horseradish peroxidase
HS	heparan sulfate
Hsc70	70 kDa heat shock cognate protein
Hsp90	90 kDa heat shock protein
JNK	c-*jun* N-terminal kinase
jun	avian sarcoma virus 17 ['ju-nana' means '17' in Japanese]
JNS	juxta-nuclear structure
LAMP-1, -2, or -3	lysosome associated membrane protein-1, -2 or -3
LAP	lysosomal acid phosphatase
LC3-II	Light chain 3-II
LDL	low density lipoprotein
LE	late endosome
Lgp	lysosomal glycoprotein
LIMP	lysosomal integral membrane protein
LIMK	LIM domain kinase
LRP	low density lipoprotein receptor-related protein
M-I	myosin-I
M-II	myosin-II
M6P	mannose-6-phosphate
MAPK	mitogen-activated protein kinase
MDM-2	murine double minute-2
MEF	mouse embryo fibroblasts
MEK	MAPK-ERK kinase
MEKK	MAP kinase kinase kinase
Mg^{2+}	magnesium
MLC	myosin light chain
MLCK	MLC kinase
MMP	matrix metalloprotease
MPR	mannose-6-phosphate receptor
MT1-MMP	membrane type-1matrix metalloprotease
MTCBP-1	membrane-type 1 matrix metalloproteinase cytoplasmic tail-binding protein-1
MTOC	microtubular organizing centre
MVB	multi-vesicular body
Na^+	sodium ion
NCL	neuronal ceroid-lipofuscinosis (also known as Batten disease)
pH_i	intracellular pH / acidity
NHE-1	Na^+,H^+-exchanger 1

NPC	Niemann-Pick C disease
p130CAS	130 kDa crack associated substrate
PAK	p21-activated kinase
PCP	planar cell polarity
Pex	hemopexin domain
PG	progesterone receptor
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PIPES	1,4-piperazinediethanesulfonic acid
PKB/AKT	protein kinase B (AKT)
PKC	protein kinase C
PLC	phospholipase C
PM	plasma membrane
PMT	photomultiplier tube
PS	phosphatidylserine
PTEN	phosphatase and tensin homologue deleted in chromosome ten
Rab	Ras-like protein derived from rat brain
Rac	Ras-related C3 botulinum toxin substrate
Rad	Ras associated with diabetes
Ras	Rat sarcoma-derived transforming factor (Ha-, K-, N-, or R-Ras)
RAVE	regulator of the H^+-ATPase of vacuolar and endosomal membranes
Rb	retinoblastoma protein
RECK	reversion inducing cysteine-rich protein with Kazal motifs
RGB	red-green-blue image
Rho	Ras homologue gene
RILP	Rab-interacting lysosomal protein
ROCK	Rho-associated coiled-coil-containing protein kinase
SAPK	stress-activated protein kinase
SASD	sialic acid storage disease
SH2	Src homology 2 domain
Shc	SH2-domain-containing α2-collagen-related
Src	steroid receptor coactivator
SNARE	soluble *N*-ethylmaleimide-sensitive factor receptor protein
SOS	sons of sevenless
TB	mycobacterium tuberculosis
Tfn	transferrin
TGF-β	transforming growth factor-β
TGN	*trans*-Golgi network
TIAM	T-lymphoma invasion and metastasis inducing protein
TIMP-2	tissue inhibitor of metalloproteases-2
TIP47	47 kDa tail-interacting protein
TNF	tumour necrosis factor
uPA	urokinase-like plasminogen activator
uPAR	urokinase-like plasminogen activator receptor
V12	glycine mutationally replacement with valine at codon 12
V-ATPase	vacuolar H^+-type ATPase
Wnt	Drosophila wingless protein / mammalian int-1 version

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CHAPTER 1.

1.1. Breast cancer in South Africa

In accordance with a worldwide trend, the Cancer Association of South Africa (CANSA) reports that, in South Africa, breast cancer has become a leading cancer in women (Spandidos *et al.*, 1999). It is ranked the most common cancer in women of European descent (17.9%) and the second most common cancer (after cervical cancer) in mixed-race- (18.2%) and black African women (13.4%) in South Africa (Vorobiof *et al.*, 2001). (Aivazian *et al.*, 2006)

While 73% of European women are diagnosed only after menopause, on average 50% black African and mixed-race patients are diagnosed with the disease premenopause (Vorobiof *et al.*, 2001). European women also present with advanced breast cancer in 30.7% of cases. This is in clear contrast to the 80% of black African women who present at such a stage, and for whom the illness is terminal, in most cases. It is believed that the adoption of a Western diet and lifestyle by black African women are the main contributing factors to a rising number of breast cancer cases (Vorobiof *et al.*, 2001). The severity of the disease noted in black African women may, however, be due to late initial diagnosis, a factor that may be attributed to cultural predisposition to support herbalists and traditional medical practitioners before resorting to Western medical treatment (Vorobiof *et al.*, 2001). In general, known risk factors for breast cancer include familial/genetic predisposition, hormonal factors, obesity, physical inactivity, smoking and high alcohol consumption (American Cancer Society, 2005).

1.1.1. Known mutations in human breast cancer

Cancer usually develops slowly over a period of time during which cells overcome several genetic barriers and change from mortal to immortalized cells and finally to cells that form cancer growths or tumours (Rao *et al.*, 2006; Li *et al.*, 2007). A tumour growth is a collection of cells which may be either benign (non-pathogenic) or malignant (highly pathogenic). The pathogenic or malignant tumour is known as a cancer.

Although the course to malignancy (cancer) is highly variable for different cell types, one of the early steps in most common types of tumours is the acquisition of

immortality (infinite lifespan), or resistance to programmed cell death (apoptosis). These initial events may occur via genetic or mutational inactivation of the p16 (Thullberg *et al.*, 2000) or p53 tumour suppressors (Elenbaas *et al.*, 2001). These conditions result in loss of control over the cell cycle, lack of DNA repair and genetic instability (Vousden, 2000; Zhou *et al.*, 2001) (Section 1.8).

Under normal conditions p53 regulates the cell cycle and apoptosis, with death being induced in response to genetic instability. Two other tumour suppressor genes, *BRCA-1* (breast cancer gene-1) and *BRCA-2* (breast cancer gene-2), when functioning normally, help repair damage to DNA (a process that also prevents tumour development). However, women who carry mutations of *BRCA-1* or *BRCA-2* are at higher risk of developing both breast and ovarian cancer than women who do not have these genetic mutations. Malignant transformation of normal cells may also occur when, due to *BRCA-1* and *BRCA-2* mutations, cells suffer further genetic instability that leads to uncontrolled growth and loss of differentiation (Gao *et al.*, 2004; Houle *et al.*, 2006). Currently, women with *BRCA-1* mutations account for 5% of all breast cancer cases, with an increasing risk to developing breast cancer, as they get older (20% by age forty, 51% by age fifty, and 87% by age sixty) (Spandidos *et al.*, 1999).

1.1.2. Oncogenes

An unrepaired mutation in a key signaling gene constitutes one of the early steps in the progression to a malignant phenotype (Rao *et al.*, 2006). These mutated, cancer-promoting genes are referred to as oncogenes and may give rise to mutated proteins or oncoproteins, e.g. the Ras oncoprotein involved in growth signal transduction, of interest in the current study. A combination of the Ras oncoprotein and inactivation of tumour suppressor genes, such as *p53* or *p16* (Hanahan *et al.*, 2000) (Section 1.8), results in a genetically unstable cell and may cause normal cells to begin to progress towards an increasingly malignant phenotype (Malumbres *et al.*, 1998; Fürst *et al.*, 2002). How tumour progression is facilitated by a particular *ras* mutation together with a p53- or p16-associated mutation, has only just begun to become clear (Gao *et al.*, 2004; Paradiso *et al.*, 2004) and forms a focus of the current study.

Before discussion of the breast epithelial cell line model used in the current study and justification of its choice (Section 1.7), some background will be given in the following section on the various closely-related *ras* members that exist, the role of

Ras guanosine triphosphatases (GTPases) and the Ras family members that play a key role in downstream signaling. Various Ras-GTPase products will be discussed in order to explain the effects of specific *ras* mutations found in cancer and how these mutations affect the function of the various Ras members, cell phenotype, trafficking of proteases and invasion, with specific focus on cancer of the breast.

1.2. Cell proliferation and migration mediated by small GTPases

Cell proliferation and migration is tightly controlled by factors such as growth factors (Weber *et al.*, 2000; Mo *et al.*, 2007) and adhesion to the extracellular matrix (ECM) (Aplin *et al.*, 1998). These stimuli modify the normal behaviour of the cell and affect cytoskeletal activity, protein transcription and ion trafficking. A range of small GTPases mediates these fundamental cellular processes (Santos *et al.*, 1989; Denhart, 1996; Malumbres *et al.*, 1998; Walker *et al.*, 2003). The Ras GTPases is a major mediator in normal and cancer cell phenotypes. In order to study such phenotypes it is necessary to outline the normal and abnormal roles of the various Ras stages.

Ras proteins belong to a large super-family of related proteins known as low-molecular weight G-proteins (20 to 29 kDa) and are present in all eukaryotic organisms (Bos, 1989). All G-proteins bind guanine triphosphate (GTP) in order to affect hydrolysis to guanine diphosphate (GDP) and are, therefore, known as GTPases. A specific group of small GTPases plays critical roles in cellular biogenesis, with the more than 80 mammalian members comprizing several distinct branches, including Ras, Rho/Rac, Rab, Arf, Ran and Rad/Gem as well as the Rheb and Rin/Rif families (Vojtek *et al.*, 1995; Denhart, 1996; Van Aelst *et al.*, 1997; Bishop *et al.*, 2000; Reuther *et al.*, 2000) (Table 1.1). Mammalian cells express four closely related Ras proteins, namely Ha-Ras and K-Ras (cellular counterparts of the viral Harvey strain of rat sarcoma and the Kirsten strain of rat sarcoma, respectively), N-Ras (first identified in a neuroblastoma cell line) and R-Ras (Ras-related Ras) (Table 1.1). These consist of either 188- (Ha-Ras, K-RasA and N-Ras) or 189 (K-RasB) amino acids (Grand *et al.*, 1991; Reuther *et al.*, 2000). The Rho/Rac group is involved in cytoskeletal dynamics, while the Rab/Arf group is involved in vesicle dynamics (Table 1.1). These two groups will be further discussed in chapter 3 on vesicle trafficking, while the other members are not relevant in this study and will not be further discussed. The small GTPases subgroup is based primarily on common structural features. Sequence comparison between subfamilies reveals ~30%

similarity, while sequence comparison between members within the same subfamily reveals 40% similarity in identity.

Table 1.1. Various Ras GTPases.
The various Ras GTPase families: origin of nomenclature and cellular functions and date of discovery (Vojtek *et al.*, 1995; Reuther *et al.*, 2000).

GTPase	**Origin of name (year)**	**Functions**
Ha-Ras K-Ras N-Ras R-Ras	The transforming factor in Harvey and Kirsten strains of rat sarcoma (1964), or of a neuroblastoma cell line Ras-related protein.	Play prominent roles in cell growth and development.
Rab	Ras-like protein derived from rat brain (1987).	Monitor and direct the movements of vesicles within the cell.
Arf	ADP ribosylation factor, a cofactor for cholera toxin-dependent ADP-ribosylation of Gα (1984).	Monitor and direct the movements of vesicles within the cell.
Ran		Required for nuclear protein import.
Rac Rho	Ras-related C3 botulinum toxin substrate (1989). Ras homologue gene (1985).	Play dynamic roles in the regulation of cytoskeletal dynamics and thus cell shape and migration.
Rad Gem	Ras associated with diabetes (1993). GTP-binding protein induced by mitogens (1994).	

The G-proteins have several common characteristics. They have a similar functional mechanism, based on the ability to cycle between inactive (GDP-bound) and active (GTP-bound) states, and have an intrinsic enzymatic activity that allows them to act as timed switches to precisely control cellular processes (Section 1.2.4). They are modulated by many external regulatory factors, such as growth factors, hormones, cytokines and neurotransmitters, and, therefore, play important roles in health and disease.

While the various forms of Ras, such as Ha-Ras and K-Ras (Table 1.1), generally seem to signal via a set of common major pathways (Figure 1.3), differences in activation, signaling efficiency, control and cross-talk between these major pathways result in different cellular outcomes (Section 1.5.5). Where detail such as the type of Ras is unknown, the literature generally refers to 'Ras' and, therefore, facts were taken as applying to all 'Ras' types. Knowledge of which form of Ras is involved in a

given type of cancer, due to either upregulation or mutation of a specific *ras* (Table 1.2), may assist in understanding the dominant downstream pathway that may, in turn, result in differences in cellular outcome. It is also important to note that the site of mutation in the Ras protein may confer a specific phenotype, and that such differences contribute to the reported confusion in Ras signaling pathways and differences in malignant phenotypes. Mutations in Ha-*ras,* such as c-Ha-*ras*(V12) (Section 1.2.1), for example, are often associated with invasion (Mo *et al.*, 2007) and a group of proteases highly active against the ECM and hence are important in invasion. Such proteases include secreted matrix metalloproteases (MMPs) such as MMP-2 and MMP-9 (Shin *et al.*, 2002; Shin *et al.*, 2005) and the membrane-associated membrane type-1 MMP (MT1-MMP) (Kim *et al.*, 2009) as well as the urokinase-type plasminogen activator (uPA) cascade (Andreasen *et al.*, 1997) (Section 1.6.4) and cathepsin B (CB) (Roshy *et al.*, 2003; Schraufstatter *et al.*, 2003; Cavallo-Medved *et al.*, 2005). These will be further discussed in Chapter 4. Cancer related to a mutationally activated K-Ras seems less invasive, but preferentially stimulates proliferation, resulting in more aggressive growth (Bissonnette *et al.*, 2000; Caron *et al.*, 2005; McCubrey *et al.*, 2006) (Section 1.3).

1.2.1. c-Ha-*ras*(V12) mutation in cancer

Key hallmarks in malignant cancers are constitutive signaling for growth, without an appropriate stimulus, loss of contact inhibition and metastasis or invasion into the blood stream and into distant organs (Hanahan *et al.*, 2000). Over expression of growth factor receptors such as epidermal growth factor (EGF) and its co-receptor HER-2/ERB-2 was reported in 20-40% of all breast cancers (Bissonnette *et al.*, 2000) and seems to give rise to transcriptional activation of *ras,* as these receptors activate and signal through either normal K-Ras (Bissonnette *et al.*, 2000), or Ha-Ras (Eckert *et al.*, 2004). In breast cancer biopsies from women of unspecified ethnic origin, a high percentage of samples showed activation of at least one of the *ras* gene family, with Ha-Ras activation reported in 37% of cases (Spandidos *et al.*, 1999). Whereas normal receptor-mediated Ras stimulation is transient (Santos *et al.*, 1989) (Section 1.8.5), mutated Ras is usually constitutively active and gives rise to constant, though perhaps low level, stimulation and signaling (Gideon *et al.*, 1992). Mutational activation of Ha-Ras itself has, however, been shown in about 10% of human breast cancers (Bos, 1989; Hollestelle *et al.*, 2007). The normal human Ha-*ras* gene located on human chromosome 11, has a GGC sequence that encodes the amino acid glycine

at codon 12 of the amino acid sequence (Ruta *et al.*, 1986; Moerkerk *et al.*, 1994). A point mutation in the Ha-*ras* oncogene, that leads to the loss of the glycine at codon 12 and instead encodes valine (a V12 mutation) (Bos, 1989), results in the V12 constitutively activating c-Ha-*ras*(V12) mutation (Moerkerk *et al.*, 1994; Scwab *et al.*, 1994; Bouras *et al.*, 1998) that gives rise to a mutated Ras protein that is a major predisposing factor in the development of many human cancers (Gideon *et al.*, 1992; Denhart, 1996). Besides a relatively low association with breast cancer, this mutation is frequently associated with keratoacanthomas (30%), squamous cell skin carcinoma (12%) and with bladder cancer (6%) (Bos, 1989) (Table 1.2). It seems that any mutational change that leads to a loss of the glycine at codon 12 can change normal *ras* gene into a gene that may cause tumours (Bos, 1989). Mutations at codons 12 as well as 61 give rise to forms of Ha-Ras that are constitutively active and have been shown to morphologically transform established cells in culture (Basolo *et al.*, 1991) and mouse liver epithelial cells (Kanda *et al.*, 1993).

Table 1.2. Mutations and incidence of *ras* genes found in human tumours. [Adapted from (Bos, 1989)].

Tumor Type	**Incidence**	***ras* oncogene**
Keratinoacanthoma	30%	Ha-*ras*
Squamous cell line carcinoma	12%	Ha-*ras*
Breast	8%	Ha-*ras*
Bladder carcinoma	6%	Ha-*ras*
Pancreatic carcinoma	90%	K-*ras*
Colon adenoma	50%	K-*ras*
Colon adenocarcinoma	50%	K-*ras*
Seminoma	40%	K-*ras,* N-*ras*
Lung adenocarcinoma	30%	K-*ras*
Myelodisplastic syndrome	30%	N-*ras*
Acute myelogenous leukemia	30%	N-*ras*
Thyroid carcinoma	25%	N-*ras*
Melanomas	20%	N-*ras*
Cervical carcinoma, Esophageal carcinoma, Glioblastoma, Lymphocytic leukemia	<5%	

While the normal Ras is a molecular switch (therefore indicates regulated activation), the mutated c-Ha-Ras(V12) structure is altered, prolonging the lifetime of the active GTP-bound form. Even though such a change also decreases the activity of the GTPase, it generates a constitutive signal that is influencial in the development of many human cancers (Gideon *et al.*, 1992; Denhart, 1996) (Table 1.3). The ratio of Ras-GDP to Ras-GTP is also changed, so that more active than inactive Ras collects

in the cell. The mutation does not allow the replacement of GTP, making the protein constitutively active (Gideon *et al.*, 1992; Hwang *et al.*, 1996). Because the signal delivered by the Ras oncoprotein is continuously delivered, the cell is constantly under pressure to make DNA, proliferate and grow. This unabated growth, together with loss of tumour suppressor proteins such as p16 or p53, may lead to the development of cancer (Pestell *et al.*, 1999; Lin *et al.*, 2001).

Several mutations of the Ras protein exist (such as in amino acid positions 12, 13, 59, 61 and 62 of the phosphate-binding region [Table 1.3]). Some of these have been experimentally generated to study the various binding and activating domains of the protein, in order to investigate the interaction of Ras with its downstream effectors or its own activators and deactivators. The onset of cancer depends, however, on cellular context and cooperation events (Mo *et al.*, 2007).

A focus of this study is invasion and the effect of the V12 mutation in c-Ha-Ras on protease trafficking in invasive breast cancer. The broad aim of this study is to understand and attempt to explain how malignant progression and invasion may come about largely through the mutation of a single oncogene in combination with immortality, or an ineffective or silenced cell cycle control system (p53/p16) (Section 1.8). Both these proteins control the progression of the cell cycle from G1 to the S phase (Thullberg *et al.*, 2000; Alarcon-Vargas *et al.*, 2002). Studies of this nature are most easily carried out in cell lines, and although several cell lines exists, this work was facilitated by the availability of a spontaneously immortalized, otherwise normal, MCF10A breast epithelial cell line, established from a woman with fibrocystic disease and its c-Ha-*ras*(V12) transformed invasive derivative, the MCF10AneoT cell line (Soule *et al.*, 1990; Tait *et al.*, 1990; Basolo *et al.*, 1991) (Table 1.3 and Section 2.1).

Table 1.3. *ras* oncogenes in human and mouse tumours and cell lines.

Ras mutation	Cell type transfected & other information	Altered activity	Reference
H-Ras	Small GTPase	Normal membrane-bound 'switch' protein in signal transduction	(Reuther *et al.*, 2000)
H-Ras(V12)	MCF10AneoT, a transfected HMEC	Low, but constitutive GTPase activity	(Basolo *et al.*, 1991)
H-Ras(V12,S35)	NIH 3T3(UNC) cells (have low rate of spontaneous transformation)	Does not activate Raf1/MAPK pathway or PI3K-PAK	(Koshravi-Far *et al.*, 1996; Tang *et al.*, 1999; Tscharntke *et al.*, 2005)
H-Ras(V12,G37)	NIH 3T3 fibroblasts	Activates Ral, but not Raf or PI3K (or PAK); causes focus formation; forms aggressive, infiltrative metastasis	(Koshravi-Far *et al.*, 1996; Tang *et al.*, 1999; Ward *et al.*, 2001; Tscharntke *et al.*, 2005)
H-Ras(V12,C40)	NIH 3T3 fibroblasts	Effector domain defective for Raf binding; activates PI3K; causes focus formation	(Koshravi-Far *et al.*, 1996; Tang *et al.*, 1999; Tscharntke *et al.*, 2005)
H-Ras(V12,R186)		Defective for membrane localization and as a result is biologically inactive	(Joneson *et al.*, 1999)
H-Ras(L61)	HMEC immortilized through *hTERT*	Some cytogenetic abnormalities, but not tumorigenicity	(Rao *et al.*, 2006)
c-H-Ras(G60A)	Glycine residue substituted by alanine	Decreases GTPase activity of H-Ras without significantly affecting GTP/GDP binding	(Hwang *et al.*, 1996)
H-Ras(17Asn)	NIH 3T3 fibroblasts	Ras mutant with preferential affinity for GDP over GTP; used as a dominant negative Ras	(Scheele *et al.*, 1995; Koshravi-Far *et al.*, 1996)
R-Ras(38V)	MCF10A – normal immortal HMEC MDA-MB-231 – HBEC, highly metastatic	Constitutively active; enhances adhesion and inhibits migration in these cells	(Jeong *et al.*, 2005)
R-Ras(43N)	MCF10A – normal HMEC MDA-MB-231 – HBEC, highly metastatic	Dominant-negative form of R-Ras; no effect on adhesion or motility	(Jeong *et al.*, 2005)
Other p21 mutant proteins	Mutations of residues in: Loop L1 (Gly-12 and Gly-13), Loop L2 (Thr-35 and Asp-38), Loop L4 (Gln-61 and Glu-63)	Variably altered GAP binding capacities	(Gideon *et al.*, 1992)

Abbreviations: HBEC: human breast epithelial cells; HMEC: human mammary epithelial cells; *hTERT*: human telomerase reverse transcriptase; MDA-MB-231: metastatic breast cell line 231, established at M. D. Anderson Hospital and Tumour Institute; MCF: Michigan Cancer Foundation; NIH 3T3(UNC): spontaneously immortalized fibroblast cells established from primary NIH Swiss mouse embryonic tissue, transferred (T) every 3 days (3) and inoculated at a rigid density of 3 x 10^5 cells per 20-cm² dish (3). The specific strain from University of North Carolina (UNC) does not transfect as easily as the original cell line.

The influence of Ras mutations in signaling and the importance of control via p53 or p16 tumour suppressors will be covered in the following sections. Though the various

forms of Ras signal via a common pathway, the activation of either a more aggressive of invasive phenotype seems to depend on hyperstimulation or mutation of a particular type of Ras (Table 1.3). Before the relevance of such effects is considered, however, the general or generic 'Ras' pathway needs to be described.

1.2.2. Importance of the Ras molecule above the other G-proteins

In health, the Ras protein is a key signaling component in many intracellular pathways. Ras transmits incident-related extracellular signals from activated growth factor receptors, such as hormones (Pethe *et al.*, 1999) and EGF and conveys the signal intracellularly to downstream cytoplasmic Ras effectors (Reuther *et al.*, 2000). These downstream targets are also involved in other cellular functions and may thus be activated via pathways that are independent of Ras activity (Aplin *et al.*, 1998). However, when Ras is activated, stimulation of these downstream effectors leads to a transient increase in growth and migration by influencing proliferation (through cyclin D, etc) (Bissonnette *et al.*, 2000), cytoskeletal integrity (through Rac and Rho), cell migration (through MMPs), adhesion (through focal adhesion kinase [FAK]) (Section 1.7 and Section 1.8) and apoptosis (through p53) (Nepveu, 2001) (Section 1.8). Ras-related growth is controlled through negative feedback mechanisms that counteract these proliferative effects and which allows arrest of the cell cycle in a non-proliferative state, or senescence (Lin *et al.*, 2001) (Section 1.8.5).

1.2.3. The Ras protein, its ribbon structure and biosynthesis

The ribbon structure of the Ras protein is composed of 5 α-helices and six β-sheets, which are all connected by ten loops, L1-10 (Santos *et al.*, 1989; Ma *et al.*, 1997; Mello *et al.*, 1997)] (Figure 1.1). The arrangement of loops 8 and 10 form a guanine nucleotide binding area and loops 1 2, and 4 (consisting of amino acids 10-16, 26-36 and 59-64, respectively), participate in phosphate binding and form 2 switches that are involved in regulation of the GTPase activity (Denhart, 1996; Ma *et al.*, 1997). Proteins that catalyse the release of GDP/GTP bind to Ras in the C-terminal hypervariable effector region. Although the amino acids in these regions may differ among the various members of the Ras superfamily, the order and the approximate spacing between these regions in the protein is approximately the same for all known small GTP-binding proteins.

The first 86 amino acids of Ras proteins are 100% identical, with 85% homology in the next 80 amino acids (Santos *et al.*, 1989; Bar-Sagi, 2001). The remaining carboxyl-terminal sequence consists of residues 166–185 and forms the highly divergent effector or hypervariable domain (Santos *et al.*, 1989; Ritter *et al.*, 1997; Laude *et al.*, 2008).

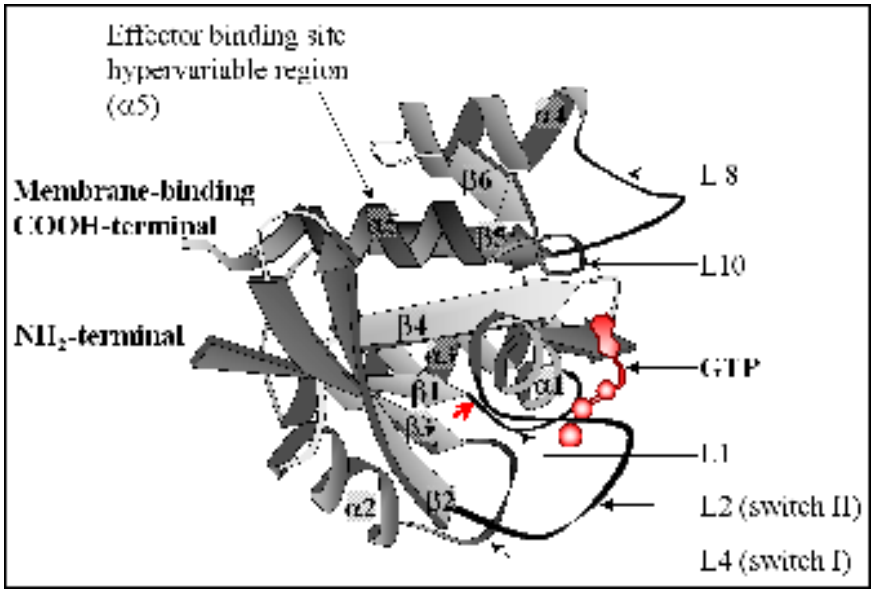


Figure 1.1. The ribbon structure of the Ras protein.
This schematic presentation illustrates the various α-helices, β-sheets and loops of the Ras GTPase structure. The location of the GTP molecule indicates the guanine nucleotide binding site. Red arrow indicates position of the V12 mutation on loop 1. This image is an adaptation of various sources (Santos *et al.*, 1989; Ma *et al.*, 1997; Mello *et al.*, 1997).

The final C-terminal region consists of a four amino acid motif with a CAAX sequence (C = cysteine; A = aliphatic residue; X = any residue) (Santos *et al.*, 1989; Bar-Sagi, 2001). A series of posttranslational modifications to this region is required for the precursor to become functional. Initially a farnesyl group is added to cysteine residue 186 of the CAAX sequence. Subsequently, the AAX residues are proteolytically cleaved, and the now C-terminal farnesylcysteine is methylated (Reuther *et al.*, 2000). These steps are critical for further maturation of the precursor, and subsequent localization to and activity at the plasma membrane (PM) (Apolloni *et al.*, 2000; Fürst *et al.*, 2002).

Ras proteins are synthesized as inactive precursors that translocate to the PM. The various forms of Ras, i.e. Ha-Ras, N-Ras and K-Ras proteins, exhibit differences in their post-translational modification of the C-terminals (Grand *et al.*, 1991; Bar-Sagi, 2001). This allows for variations in trafficking of the various Ras isoforms to- and

positioning at the inner face of the PM (Laude *et al.*, 2008), thereby affecting accessibility to effectors or activators (Hancock *et al.*, 1991; Voice *et al.*, 1999; Apolloni *et al.*, 2000; Bar-Sagi, 2001; Prior *et al.*, 2001; Jaumot *et al.*, 2002). A lipid modification via addition of palmitate to the cysteine residues 181 and 184 of the Ha-Ras C-terminus in the Golgi (palmitoylation) (Grand *et al.*, 1991; Baker *et al.*, 2000) increases its hydrophobicity (Basu, 2004), and allows it to be compartmentalized to and stably associate with, caveolin-1 (cav-1)-containing, cholesterol-rich PM domains or lipid rafts (Melkonian *et al.*, 1999; Baran *et al.*, 2007; Greaves *et al.*, 2007; Laude *et al.*, 2008). In contrast, K-Ras is not palmitoylated, but contains a polybasic sorting signal that allows it to be trafficked directly from the ER to a more general distribution over the PM (Grand *et al.*, 1991; Apolloni *et al.*, 2000; Reuther *et al.*, 2000; Prior *et al.*, 2001).

While palmitoylation is required for membrane localization, full activation of function of the Ras protein, is dependent on replacement of GDP with GTP (Baker *et al.*, 2000; Prior *et al.*, 2001; Jaumot *et al.*, 2002) (Section 1.5.3). Due to the resultant conformational change activated Ras is released from the caveolae (Baker *et al.*, 2000; Prior *et al.*, 2001), a process that may allow interaction of its downstream effector proteins with the hypervariable binding sites (Jaumot *et al.*, 2002; Greaves *et al.*, 2007) (Section 1.5.4). The importance of membrane association and specificity of localization is demonstrated in the relatively low biological activity of the mutated Ha-Ras(V12,R186) that changes the C-terminal membrane binding region, results in a generalized or ectopic PM localization (Joneson *et al.*, 1999) and less effective activation of downstream effectors, due to ineffective binding to lipid rafts (Jaumot *et al.*, 2002). This process is relevant in invasive cancers (Cavallo-Medved *et al.*, 2005), playing a central role in the transforming activity of Ras oncoproteins (Kohl *et al.*, 1994), since mutated Ras may produce low level, but consistent, down-stream signaling. Inhibition of membrane translocation is also exploited in therapeutic regimes such as the farnesyl transferase inhibitor, stimvastatin, which counteracts the transforming ability of c-Ha-Ras(V12) (Fürst *et al.*, 2002) by preventing farnesylation and hence, membrane association (Reuther *et al.*, 2000).

1.2.4. Ras as a molecular switch

All wild-type Ras proteins act as timed molecular switches at the PM, cycling between two conformations: an inactive (GDP-bound) and an active (GTP-bound)

conformation (Denhart, 1996; Ma *et al.*, 1997; Mello *et al.*, 1997; Wieland *et al.*, 2005) (Figure 1.2). Under normal conditions Ras-GDP to Ras-GTP is in a 1:1 ratio, even with experimental over-expression of the protein (Sheele *et al.*, 1995). As for most GTPases, the GDP/GTP cycle of Ras is controlled by two kinds of regulators. A guanine nucleotide exchange factor (GEF) such as sons of sevenless (SOS) interacts partly with the switch II region (Denhart, 1996) and allows the replacement of GDP with GTP to generate an activated protein (Figure 1.2). Ras-GTP interacts with and activates effector proteins, which leads to further regulated preferential and consecutive phosphorylation of downstream kinase complexes (Figure 1.2).

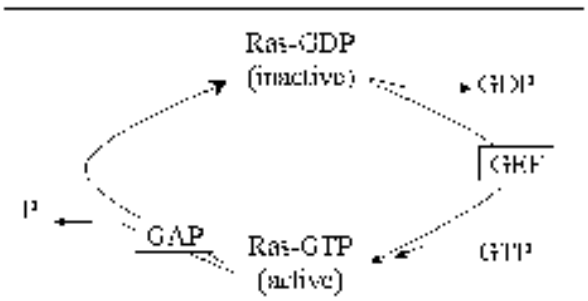


Figure 1.2. The activation and deactivation cycle of Ras.
With the assistance of a GEF, GDP is replaced with GTP to activate the Ras protein, while a GAP forces hydrolysis of the GTP and returns the Ras to an inactive state.

Low-level intrinsic Ras-GTPase activity can be greatly accelerated by interaction with a GTPase activating protein (GAP) (Santos *et al.*, 1989; Giglione *et al.*, 1997; Malumbres *et al.*, 1998), which forces hydrolysis of the GTP and returns the Ras to an inactive state (Figure 1.2). GAP competes with the downstream effectors for binding with Ras, and could, therefore, be seen as a negative regulator of Ras (Denhart, 1996). Another function of GAPs may be the protection of the activated Ras from unproductive replacement of GTP with another GTP under the direction of GEF, and, as such, it may have regulatory significance (blocking) regarding the Ras-GEF interaction (Giglione *et al.*, 1997). This interaction and its effect is prohibited by the V12 mutation (Gideon *et al.*, 1992; Hwang *et al.*, 1996).

1.2.5. The main Ras effector pathways

Active GTP-bound Ras turns on distinct and well-defined cascades (Figure 1.3. [Since reference is made to Figure 1.3 throughout the text, this Figure is reproduced as a fold-out in Appendix V at the back of the thesis, for ease of reference]). Ras-related pathways include the Raf, Ral, phosphatidylinositol 3-kinase (PI3K) and

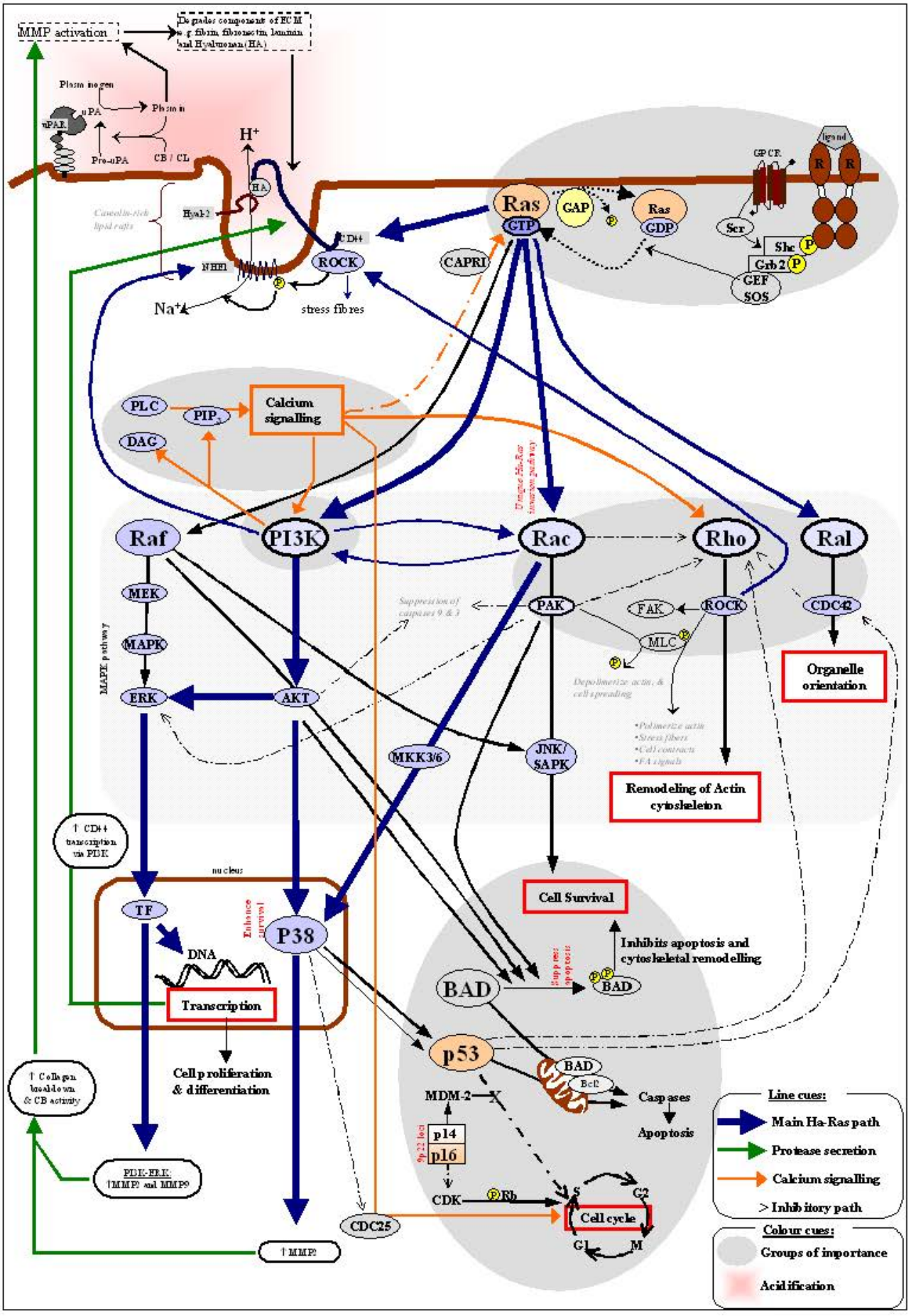
Rac/Rho pathways (Joneson *et al.*, 1999; Jaumot *et al.*, 2002), with each contributing in a unique way, yet in concert with one another, to the ability of the cell to proliferate and migrate following growth factor stimulation.

The best-known pathway involves the Raf- mitogen-activated protein kinase (MAPK)- extracellular-signal-related kinase (ERK) kinase cascade that is typically stimulated strongly by mitogens and growth factors (Figure 1.3 on fold-out). Activated Raf phoshorylates MAPK and MAPK-ERK kinase (MEK) (Herrmann *et al.*, 1996; Joneson *et al.*, 1998) and ERK. ERK translocates to the nucleus and stimulates protein transcription including that of growth factors involved in proliferation and differentiation. One such example is cyclin D, a cell cycle regulator which stimulates the progression of the cell cycle into the S-phase and through the remainder of the cell cycle (Herrmann *et al.*, 1996; Giglione *et al.*, 1998).

PI3K is another key effector involved in many functions of the Ras pathway. Activation of PI3K leads to cell proliferation via protein kinase B (PKB, also known as AK mouse transforming retrovirus or AKT), and p38 (Bulavin *et al.*, 2003; Tscharntke *et al.*, 2005), and assists the migration process via upregulation of extracellular proteases that are involved in the remodelling of the ECM during migration (Shin *et al.*, 2005; Song *et al.*, 2006) (Figure 1.3 on fold-out).

Figure 1.3. The Ha-Ras downstream pathway.

This schematic presentation illustrates the Ha-Ras downstream pathway through the initial effectors Raf, PI3K, Rac and Ral and the involvement of calcium in stimulation of Ha-Ras signaling. It clarifies the relationship between growth stimulation (ERK and AKT paths), migration (Rac and Rho-ROCK), cell survival (Rac-PAK and JNK/SAPK) and cell cycle control and the apoptotic paths via p16 and p53, respectively. This drawing also illustrates events such as extracellular acidification, transcription and extracellular activation of the gelatinases, such as MMP-2 and MMP-9 and ECM degradation. This illustration was compiled from references mentioned throughout the text. Since reference is made to this Figure throughout the text, it is reproduced as a fold-out on the last page of this thesis for ease of reference.


MMP activation
Degrades components of ECM
e.g. fibrin, fibronectin, laminin
and Hyaluronan (HA)
Plasminogen
Plasmin
uPA
uPAR
Pro-uPA
CB / CL
H+
HA
Hyal-2
Caveolin-rich lipid rafts
CD44
ROCK
NHE1
Na+
stress fibres
CAPRI
Ras
GTP
GAP
Ras
GDP
GPCR
ligand
R
R
Scr
Shc
P
Grb2
P
GEF
SOS
PLC
PIP2
Calcium signalling
DAG
Raf
PI3K
Rac
Rho
Ral
MEK
MAPK
ERK
AKT
MAPK pathway
PAK
FAK
ROCK
MLC
CDC42
Organelle orientation
Depolimerize actin, & cell spreading
•Polimerize actin
•Stress fibers
•Cell contracts
•FA signals
Remodeling of Actin cytoskeleton
MKK3/6
JNK/ SAPK
↑ CD44 transcription via PI3K
nucleus
TF
DNA
Transcription
P38
Enhance survival
Cell Survival
Inhibits apoptosis and cytoskeletal remodelling
BAD
Suppress apoptosis
BAD
p53
BAD
Bcl2
Caspases
Apoptosis
MDM-2
p14
p16
9p21 loci
CDK
Rb
S
G2
Cell cycle
G1
M
CDC25
↑ Collagen breakdown & CB activity
PI3K-ERK: ↑MMP2 and MMP9
↑ MMP2
Cell proliferation & differentiation
Line cues:
Main Ha-Ras path
Protease secretion
Calcium signalling
> Inhibitory path
Colour cues:
Groups of importance
Acidification

PI3K also forms diacylglycerol (DAG) and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) from phosphatidylinositol (PI) on the PM. PIP_3 is cleaved from PI by phospholipase C (PLC) and subsequently translocates to the endoplasmatic reticulum (ER) membrane. Here it binds to a PIP_3 receptor. This releases calcium from the ER store (Reuther *et al.*, 2000; Karp, 2002) (Figure 1.3 on fold-out). Through these actions PIP_3 plays a pivotal role in the calcium homeostasis of the cell and subsequent cell responses. The relevance of this pathway is discussed in the context of the influence of Ha-Ras signaling on intracellular calcium concentrations and mitosis (Section 1.4.5).

Following Ras activation Rac may be activated in either a PI3K-dependent (Rodriguez-Viciana *et al.*, 1994), or a PI3K-independent path (Tscharntke *et al.*, 2005). Activated Rac induces membrane ruffling and cytoskeletal rearrangements (Van Aelst *et al.*, 1997; Tscharntke *et al.*, 2005) (Figure 1.3 on fold-out). Rac is also involved in cell-cycle progression and cell survival (Bishop *et al.*, 2000) by activating p21-activated kinase (PAK). Active PAK promotes cell survival during growth-related stress conditions via the stimulation of the c-*jun* N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, key in cell survival (Tang *et al.*, 1999) (Figure 1.3 on fold-out). Rac and another very similar GTPase, Rho, are both uniquely stimulated by Ras (Van Aelst *et al.*, 1997) and exist in a unilateral inhibitory balance that controls the organization of the cytoskeletal elements and thus cell shape, polarity and migration (Joneson *et al.*, 1998, 1999; Sander *et al.*, 1999; Tang *et al.*, 1999; Ory *et al.*, 2000; Paradiso *et al.*, 2004).

A central role in Ras-related signaling is played by p38, which mediates both the growth promoting (Buchsbaum *et al.*, 2002; Frey *et al.*, 2004) and differentiating activities of Ha-Ras (Figure 1.3 on fold-out). Increased calcium-related Ha-Ras-PI3K activation (Fan *et al.*, 1998) initially stimulates mitosis (Santos *et al.*, 1989). Subsequently, competition between cell division cycle protein 25 (CDC25), a Ras-guanine nucleotide-releasing factor (GRF), and Raf (both binding to Ras to the exclusion of the other) leads to increased levels of p38 and p53. Cell cycle arrest in G1 may occur via p38 (Giglione *et al.*, 1998; Katunuma *et al.*, 1998; Erster *et al.*, 2004; Uetake *et al.*, 2007) and a downstream effector (Bulavin *et al.*, 2003) that

initiates p53 activation (Bulavin et al., 2003; Uetake et al., 2007). p38 also causes cell cycle arrest in a p53-independent manner. It blocks mitosis by phosphorylation of CDC25, thus inhibiting this phosphatase (Savitsky *et al.*, 2002; Bulavin *et al.*, 2003) (Figure 1.3 on fold-out).

Stimulation of downstream Ral effectors such as cell division cycle protein 42 (CDC42), remodels the actin cytoskeleton to form filopodia at the leading front of cells during cellular migration (Van Aelst *et al.*, 1997), as well as invasion (Herrmann *et al.*, 1996; Reuther *et al.*, 2000) (Figure 1.3 on fold-out). During migration filopodia formation is controlled by coordinating levels of p38-p53 and CDC42 (Gadea *et al.*, 2004), which is also important in the maintenance of cell polarity and correct orientation of the Golgi apparatus and the nucleus in the cellular space during migration and terminal differentiation (Nobes *et al.*, 1999) (Figure 1.3 on fold-out). In addition, p38 also upregulates levels of MMP-2 and MT1-MMP (Kim *et al.*, 2003; Shin *et al.*, 2005; Song *et al.*, 2006; Zhong *et al.*, 2006) and expression of uPA (Behren *et al.*, 2005), proteases involved in migration and invasion.

Finally, Rho stimulates Rho-associated coiled-coil-containing protein kinase (ROCK) downstream to cause cell contraction through the formation of stress fibres and assist in migration through its role in focal adhesions (FA) (Le Boeuf *et al.*, 2006; Pirone *et al.*, 2006) (Figure 1.3 on fold-out). The Rac-Rho interaction that controls cell migration is further discussed in Section 1.4.3.

1.2.6. Cross talk between major pathways

The phosphorylation cascades mediating many biological pathways are complex and interwoven, with frequent cross talk and co-operation between various existing effector pathways (Reuther *et al.*, 2000). For example, Ras-GRF-1 and -2, which are calcium-stimulated Ras GEFs, have been shown to be bi-functional signaling proteins that are able to bind and activate both Ras and Rac, and thereby coordinate the activation of ERK, a known part of the Ras-Raf protein transcription pathway, as well as JNK/SAPK survival path, downstream from Rac (Fan *et al.*, 1998; Buchsbaum *et al.*, 2002) (Figure 1.3 on fold-out). In turn, JNK/SAPK can effect Rac in an activity-mediating loop (Fan *et al.*, 1998), while Raf stimulates JNK activity via an autocrine mechanism in bi-directional cross talk between the Raf-MEK-ERK pathway

downstream from N-Ras and the Rac-JNK or RhoA pathway (Aplin *et al.*, 1998; Gupta *et al.*, 2000) (Figure 1.3 on fold-out).

1.3. Different functions of various forms of Ras

As mentioned, the interaction of Ras with its effectors is mediated by the effector-binding loop or hypervariable region, which spans residues 32–40 (Figure 1.1). This region shows the greatest variability amongst Ras members (4% homology) (Reuther *et al.*, 2000). These variations allow for differences in cellular outcomes (Reuther *et al.*, 2000; Caron *et al.*, 2005) such as focus formation, anchorage-independent growth and cell migration amongst the different types of Ras (Voice *et al.*, 1999). This occurs through differential activation of downstream effectors such as the Raf-1, PI3K and Ral branches of the generic Ras downstream stimulation pathway (Figure 1.3 on fold-out). These hierarchies appear to result, at least in part, from differences in the mechanisms by which the three Ras isoforms attach to membranes. This may be due to the post-translational modifications to the Ras C-terminal domains (Section 1.2.3). A covalently added palmitate fatty acid moiety added to cysteine residues, may target Ha-Ras proteins to the cav-1-containing, cholesterol-rich pits (caveolae), while a lysine-rich sequence added to K-Ras provides a second signal that may result in a more general localization with the PM (Hancock *et al.*, 1991; Yan *et al.*, 1998). Such compartmentalized localization appears to be important in the efficiency of signaling by the Ras proteins, and will be further discussed in Section 4.3.3 and Section 4.7.3.

K-Ras is largely associated with cell survival and aggressive cancers (Takakura *et al.*, 2000; Mareel *et al.*, 2003; Caron *et al.*, 2005). Lack of K-Ras is lethal, so the K-Ras downstream Raf/MEK/MAPK pathway is important for survival (Ninomiya *et al.*, 2004). This is possibly since K-Ras activates Raf-1 and, therefore, cell growth, more effectively than Ha-Ras and N-Ras (Voice *et al.*, 1999). It also phosphorylates beclin (Bcl)-2 member and agonist of cell death (BAD) (Caron *et al.*, 2005), that protects the mitochondrion and thus guards against apoptosis. Similarly, cross-talk from Raf to AKT, or PAK activation supports survival via BAD phosphorylation (Menard *et al.*, 2005) (Section 1.5.1). Mutation of K-Ras is, however, not sufficient for-, nor conducive to, increased motility and invasion (Ward *et al.*, 2001).

Lack of Ha-Ras and N-Ras does not seem to change the cellular phenotype (Bar-Sagi, 2001), whereas mutations of Ha-Ras is often associated with invasive cancers (Mo *et*

al., 2007). Increased levels of c-Ha-Ras(V12) induced apoptosis in 38% of the cells, while K-Ras(V12) had no apparent negative effect on cell viability (Walsh *et al.*, 2001). While K-Ras activation of Raf is more associated with proliferation and the prevention of apoptosis, Ha-Ras is a more potent activator of PI3K than K-Ras (Yan *et al.*, 1998) and Ha-Ras-stimulated AKT is associated with survival (Menard *et al.*, 2005). This is probably due to inhibition of caspases by AKT and/or PAK and phosphorylation of BAD (Reuther *et al.*, 2000; Menard *et al.*, 2005) (Figure 1.3 on fold-out), and is regulated by downstream p38-p53 related programmed cell death or cell cycle progression (Bulavin *et al.*, 2003; Uetake *et al.*, 2007).

Downstream stimulation by Ha-Ras, resulting in alterations to the cytoskeleton, may be one of the main influences in an invasive phenotype (Ward *et al.*, 2001). Ha-Ras seems to promote cell motility more effectively than N-Ras, while both Ha-Ras and N-Ras effectively activate ERK-1 and ERK-2 (Kim *et al.*, 2003), the growth-related pathway (Figure 1.3 on fold-out). It has also been shown that V12 mutated Ha-Ras [c-Ha-Ras(V12)] promotes cell migration and Rac activation better than mutated K-Ras(V12) (Walsh *et al.*, 2001).

1.4. An integrated approach to migration and survival

Physical linkage between the ECM and the actin cytoskeleton is important in growth (or Ras)-stimulated migration and survival of most cell types (Buchsbaum *et al.*, 2002; Pirone *et al.*, 2006). Survival is dependent on extracellular and intracellular stimuli and there is always a balance between migration and elongation or adhesion and spreading of the cell, as well as between cell proliferation and differentiation or senescence or apoptosis. These factors are part of the subtle control system of stimulus-related changes in the cell and are relevant in cancer, since disturbance of these balances may give transformed cells the opportunity to become progressively more tumorigenic and proliferate unabated.

1.4.1. Focal adhesions and turnover

The physical linkage between the ECM and the actin cytoskeleton is established through FAs, which are formed by integrin receptors that span the PM and recruit integrins and numerous other cellular proteins. FAK, a tyrosine kinase that localizes prominently to the FAs (Aplin *et al.*, 1998) is stimulated via phosphorylation, by integrin-mediated adhesion (Le Boeuf *et al.*, 2006; Pirone *et al.*, 2006), growth factors

(Fan *et al.*, 2005) and oncogene transformation (Hamadi *et al.*, 2005; Siesser *et al.*, 2006). FAK is activated via phosphorylation of several distinct serine and tyrosine residues (Fan *et al.*, 2005; Le Boeuf *et al.*, 2006). The degree of FAK phosphorylation at these various residues regulates the retention of activated FAK at adherence points, downstream signaling to e.g. microtubular organization and leads to rapid disassembly of FAs required for cell spreading and migration (Hamadi *et al.*, 2005; Le Boeuf *et al.*, 2006; Siesser *et al.*, 2006). In addition, increased localized calcium signaling prolongs the association of FAK with FAs, supporting release of FAs and thus, migration (Giannone *et al.*, 2004). Downstream signaling from integrins clustered at FAs, via phosphorylated FAK, is relevant in the anterior-posterior polarization of the migrating normal and invasive cell, and is thus further discussed in Section 4.1.4.2.

1.4.2. The process of migration

During migration FAs are established and released at various points along the base of the cell (Machesky *et al.*, 1997; Nobes *et al.*, 1999) to assist in traction over the underlying matrix. The shape of cells changes, with cells becoming polarized, i.e. develop leading and trailing edges via extension of lamellipodia at the leading end and retraction of the trailing end (Machesky *et al.*, 1997; Nobes *et al.*, 1999). The physical alterations in cell shape are manipulated by the actin and myosin components of the cytoskeleton (Buchsbaum *et al.*, 2002). Polymerization of actin allows the formation of protrusions (Ridley *et al.*, 1992; Machesky *et al.*, 1999; Vicente-Manzanares *et al.*, 2007), while interaction with myosin forms stress fibres that cause cell contraction (Vicente-Manzanares *et al.*, 2007). Myosin-II (M-II) is a bipolar, contractile protein (Karp, 2002) that integrates these processes through two isoforms, M-IIA and M-IIB, based on variations in the myosin light chain (MLC). M-IIA controls the dynamics of focal adhesions in the cell centre and retraction from the rear, while M-IIB establishes front-back polarity and orientate organelles such as the centrosome, the Golgi and the nucleus (Vicente-Manzanares *et al.*, 2007). Migration is a highly dynamic process that is affected by wounding (Tscharntke *et al.*, 2005; Buth *et al.*, 2007), extracellular chemotactic (Machesky *et al.*, 1997) or growth-related factors (Machesky *et al.*, 1997; Haase *et al.*, 2003) and, as a result, the direction of migration, position of leading and trailing ends and, therefore, cell shape, may change often over the course of time (Machesky *et al.*, 1997).

The role of Ha-*ras* mutation in increasing migration and invasion has been evident for some time (Kim *et al.*, 2003) and may be explained in terms of the effect of c-Ha-*ras*(V12) on Rac, Rho and FAK.

1.4.3. Molecular control of migration by Rac, Rho and FAK

During cell migration interplay between Rac and Rho GTPase signal transduction pathways (Figure 1.4) result in alterations in the cell shape at opposing ends and assists forward flow (Ridley *et al.*, 1992). At the same time the organelles are orientated within the intracellular space by CDC42 (Cau *et al.*, 2005). These actions are clearly evident during wound healing experiments (or scratch assays), which mimic changes that occur when cells migrate away from a confluent epithelial cell layer. Such assays have demonstrated the importance of a balance in dominance of either Rac or Rho, at various sites in the cell during the migratory process (Nobes *et al.*, 1999; Ory *et al.*, 2000; Hall, 2005).

During cell proliferation, increased intracellular calcium levels allow Rho to activate downstream ROCK (Masiero *et al.*, 1999) (Figure 1.4). Rho-ROCK activates polymerization of actin, stimulating the formation of protrusions and a rounded spindle-shaped cell (Ridley *et al.*, 1992; Jeong *et al.*, 2005) (Figure 1.4). Increased levels of Rho activate ROCK-LIM kinase (LIMK) that phosphorylates cofilin, an actin depolymerizing factor. Since phosphorylated cofilin cannot bind to actin, actin is allowed to polymerize and stress fibers form, causing cell contraction and ultimately an elongated mesenchymal cell shape (Nishimura *et al.*, 2004). Rho has also been shown to permit cell cycle progression past G1 by allowing ubiquitination-dependent degradation of a G1 inhibitory protein (Mammoto *et al.*, 2004). Rho-ROCK also allows the phosphorylation of MLC by MLC kinase (MLCK) and thus formation of contractile actin-myosin stress fibers, leading to retraction of the rear of the migrating cell (Machesky *et al.*, 1997; Oliveira *et al.*, 2003; Vicente-Manzanares *et al.*, 2007) (Figure 1.4). Rho is involved in the turnover of focal adhesions that assists in the controlled release of the cell from the ECM at the cell periphery (Totsukawa *et al.*, 2002; Pirone *et al.*, 2006). Increased levels of local intracellular calcium prolong the association of FAK with the FA sites, thereby leading to increased activation of its effectors involved in FA disassembly (Giannone *et al.*, 2004).

At the leading edge activated Rac, downstream from Ras-PI3K, results in ruffling of the PM and formation of broad peripheral lamellipodia (Nobes *et al.*, 1999; Pankov *et al.*, 2005) (Figure 1.4). This process is achieved through the dephosphorylation of MLC via downstream stimulation of PAK (Machesky *et al.*, 1997; Sander *et al.*, 1999) and causes random migration that slows the directional movement of the cell (Oliveira *et al.*, 2003; Siesser *et al.*, 2006) (Figure 1.4). In a stationary/non-migratory cell increased overall levels of Rac inhibit phosphorylation of Rho. This leads to relaxation of the cellular framework, sustained FAs and its components and a spreading of the cell, thus allowing the cell to become adherent and differentiate (Quinlan, 1999; Sander *et al.*, 1999; Bishop *et al.*, 2000; Ory *et al.*, 2000; Zondag *et al.*, 2000; Siesser *et al.*, 2006).

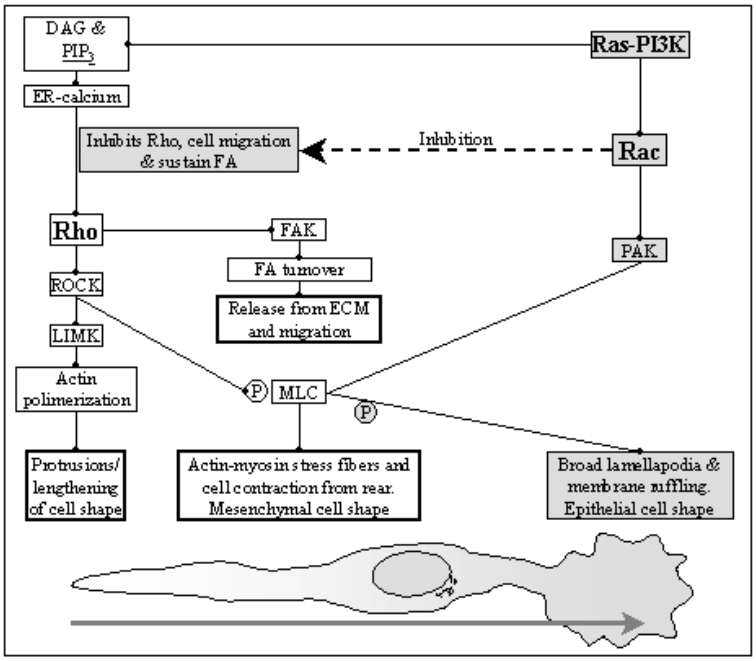


Figure 1.4. The balance between the Rac and Rho GTPases in a migratory cell.
Activation of Rac at the leading front affects the development of the broad ruffling front, while activation of Rho to the rear of the cell results in formation and contraction of actin stress fibres. This illustration was compiled from references mentioned in Section 1.4.3.

Following growth factor stimulation, therefore, the Ha-Ras interactions with downstream Rac and Rho coordinate cell migration in a stepwise manner that alternately allow front extension of membrane protrusions and rear retraction. This controls directional 'flow' while maintaining proper cell shape and polarity through CDC42. Together with FA turnover this process efficiently directs speed and migration in a specific direction with minimal diversion (Zondag *et al.*, 2000). Hence, constant signalling by c-Ha-Ras(V12) may, in response, cause changes in the cell

shape and motility and support the invasive capability of c-Ha-*ras*(V12)-transfected cells (Parsons *et al.*, 2005).

1.5. An integrated approach to signaling

As described in the sections above, Ha-Ras integrates various events during proliferation through a selective continuation of specific signaling activities, negative feedback loops and the endocytosis and subsequent degradation of the EGF receptor (EGFR) complex, in order to control Ras-related growth stimulus (Haugh *et al.*, 1999) (Figure 1.3 on fold-out). Other effects include those on survival, adhesion, cell polarization, and migration. In addition, it influences intracellular calcium levels and influences endocytic vesicles via its effect on the proton pumps and vesicle pH. These are important factors in the current study on vesicle trafficking.

1.5.1. Influence of PAK and BAD on cell survival

Activated PAK is a downstream effector of the Ras signaling pathway that plays a key role in cell survival on many levels. PAK regulates survival signals via downstream JNK/SAPK (Figure 1.3 on fold-out), stimulating increased cell proliferation and DNA synthesis (Auer *et al.*, 1998; Menard *et al.*, 2005) (Figure 1.3 on fold-out). As already described, PAK supports cell spreading through disassembly of stress fibres (Maruta *et al.*, 1999) and allows better adherence to the underlying matrix (Tang *et al.*, 1999). Via inhibitory cross-talk to ERK on the MAPK path (Shin *et al.*, 2005) (Figure 1.3 on fold-out) PAK indirectly inhibits the secretion of matrix MMP-2 (Ward *et al.*, 2001; Eblen *et al.*, 2002), an ECM remodelling protease that facilitates migration, as would be anticipated in a cell that is not migrating.

PAK supports cell survival through phosphorylation of BAD, preventing its binding to the mitochondrial death molecules (Joneson *et al.*, 1999; Menard *et al.*, 2005) (Figure 1.3 on fold-out). Non-phosphorylated BAD promotes cell death, as it heterodimerizes with pro-death molecules such as Bcl-2 on the mitochondrial membrane, thereby neutralizing their protective effect (Zha, 1996; Martin *et al.*, 2001). This results in destabilization of the mitochondrial membrane and the activation of pro-apoptotic caspases 3 and 9 (Menard *et al.*, 2005) and cell death. Other Ras effectors that also support BAD phosphorylation (and guard against apoptosis) are AKT and Raf (Wang *et al.*, 1998; Menard *et al.*, 2005; Shin *et al.*, 2005) (Figure 1.3 on fold-out).

1.5.2. Influence of ERK and Ral signaling paths on migration

During adhesion of untransformed cells to the ECM, Rac-PAK facilitates the formation of MEK-ERK complexes required for ERK activation (Eblen *et al.*, 2002) (Figure 1.3 on fold-out). This shows that the MAPK-ERK transcription pathway is coordinated with cellular adhesion. On the other hand, both the ERK and Ral-GEF signal paths make critical but distinct contributions in the promotion of migration, or invasion and metastasis in the case of transformed cells. ERK activity is necessary for c-Ha-*ras*(12V,37G)-transformed cells to express matrix-degrading activity, possibly via MMP production, while the Ral pathway allows for the cytoskeleton to play a role in the actual migratory process (Ward *et al.*, 2001). Fibroblasts that are transformed by c-Ha-*ras*(12V,37G) activate a Ral-GEF but not Raf or P13K, and form aggressive, infiltrative metastasis (Table 1.3). This demonstrates that, in such Ha-Ras mutations, cellular migration and invasion may be coordinated via the Ral effector pathway (Ward *et al.*, 2001). It seems, therefore, as if a compliant cytoskeletal network is more important for infiltrative metastasis than the capacity to degrade the underlying matrix (Ward *et al.*, 2001).

1.5.3. Influence of CD44, ROCK, and NHE-1 and extracellular acidity

Cyclin dependent surface glycoprotein-44 (CD44) is a transmembrane receptor for the ECM component hyaluronic acid (HA), but also binds collagen and the BM components fibronectin and laminin (Goodison *et al.*, 1999), and it serves as a PM anchor for intracellular actin filaments (Naor *et al.*, 2003). In normal migrating cells CD44 is spatially restricted to the membrane of the leading-edge lamellipodia (Denker *et al.*, 2002; Misra *et al.*, 2003; Cardone *et al.*, 2005). Here it is recruited into lipid rafts by HA together with ROCK and Na^+,H^+-exchanger-1 (NHE-1) (Bourguignon *et al.*, 2004) (Figure 1.3 on fold-out). ROCK is a substrate for CD44 and during migration CD44-ROCK activates NHE-1, thereby ensuring localized extracellular acidification of the leading edge microenvironment, through ion translocation (Orlowski *et al.*, 1997). Such localized acidification may create a favourable environment for ECM degradation by secreted or membrane-bound lysosomal protease such as CB, mostly active at low pH, and may be a stimulus for further secretion of MMPs (Cardone *et al.*, 2005). Acidification of the cell microenvironment also induces stress fibre formation and assists in migration (Denker *et al.*, 2002; Ponta *et al.*, 2003).

1.5.4. Influence of calcium on Ras-related cell functions

Ras activity is controlled by calcium, a universal intracellular signal / second messenger that is responsible for controlling a host of cellular processes. Increased extracellular calcium (in the growth medium) will support the attachment of cells. When detached from the ECM for long periods, cells will otherwise undergo p53-induced anoikis (programmed cell death due to detachment) (Menard *et al.*, 2005).

As explained before, during growth stimulus Ras activates PI3K and PLC, which activates membrane PI and gives rise to membrane-associated DAG and cytoplasmic PIP_3 (Section 1.2.5). These two breakdown products have contradicting effects on Ras activity. DAG inhibits protein kinase C (PKC) and terminal differentiation (Yuspa *et al.*, 1991; Lin *et al.*, 2001). Under conditions of high calcium concentrations PLC-DAG also phosphorylates a GRF (a GEF that is calcium-calmodulin-dependent), possibly CDC25 that inhibits Ras, to release it from Ras and allow its replacement with, for example, Rac (Fan *et al.*, 1998). This process activates Ras and stimulates the cell to enter mitosis (Santos *et al.*, 1989; Fan *et al.*, 1998; Walker *et al.*, 2003). The second breakdown product, PIP_3, diffuses into the cytosol, binds to a specific PIP_3 receptor on the ER. This receptor forms a calcium channel and allows the release of calcium from the internal ER stores (Reuther *et al.*, 2000; Karp, 2002). Increases in intracellular calcium regulate a diverse set of cellular processes such as endothelial cell proliferation via MAPK (Soltoff, 1998b). Increased intracellular calcium levels activate the RhoA pathway and assists in endothelial cell migration by yielding faster development of actin stress fibers and faster and prolonged turnover of FA (Masiero *et al.*, 1999; Giannone *et al.*, 2004) (Figure 1.3 on fold-out). At the same time PLC-PIP_3 exerts a negative feedback to Ras via a calcium-promoted Ras inhibitor/ inactivator (CAPRIs), that is a Ras-GAP (Yuspa *et al.*, 1991; Fan *et al.*, 1998; Cullen *et al.*, 2002; Walker *et al.*, 2003) Figure 1.3 on fold-out). CAPRI has low basal activity in resting cells, but after elevation of calcium levels it translocates to the PM to deactivate Ras (Cullen *et al.*, 2002). It is, however, virtually absent in cells transformed by oncogenic Ras, that is resistant to GAP activity (Walker *et al.*, 2003). Such lack of negative feedback may allow the continuation of signaling by mutated c-Ha-Ras(V12).

1.5.5. Influence of Ras on vesicle trafficking

Various populations of endocytic vesicles that are involved in processing of cargo, such as internalized BM and ECM components, have not been well characterized. These endosomal populations include early endosomes (EE) into which cargo is internalized and sorted, which in turn mature into degradative vesicles, or late endosomes (LEs) (Clague, 1998). It is important to note, however, that factors involved in fusion and secretion of vesicles on the endocytic routes are also regulated by Ras effectors, such as intracellular calcium and PI3K (Soltoff, 1998b) (Section 3.3.7). Newly produced precursor cathepsins, of interest in this study, are transported via the ER to the Golgi, before maturation in- and further trafficking to, specialized intracellular storage compartments (Section 3.3.4.1). This process is facilitated by specialized recognition sequences on the precursors, and the assistance of proteins involved in vesicle formation and trafficking between these stations. The fusion of these cathepsin-containing storage vesicles with endosomes carrying internalized cargo is under the influence of PI3K, a downstream Ras effector (Figure 1.3 on fold-out), that controls the sequestration, release and replacement of a group of proteins facilitating vesicle-specific fusion, called Rabs, during the maturation, movement and acidification of these organelles (Grosshans *et al.*, 2006) (Section 3.3.7.2). Membrane-bound MT1-MMP, also of importance in this study on invasion, is, on the other hand, trafficked to the PM along a secretory route under the guidance of Rab8, but with little recorded influence from Ras or its downstream effectors (Section 4.1.3).

The distribution of endocytic vesicles relies on a functional cytoskeletal system along which several motor proteins convey these vesicles (Ichikawa *et al.*, 2000) (Section 3.3.7.1). The interplay between the Ras effectors, the Rac and Rho GTPases, affect both cell shape and possibly the distribution of vesicles that are attached to the cytoskeletal network via several motor proteins (Bananis *et al.*, 2000a; Ichikawa *et al.*, 2000). The careful balance between Rac and Rho activities ensures effective migration in response to a stimulus. Extracellular acidity and intracellular cytoplasmic alkalinity, due to the Ras-related stimulation of NHE-1 activity, alter the organization of the cytoskeletal elements and may affect vesicle movement (Bourguignon *et al.*, 2004). Constant c-Ha-*ras*(V12) may, however, upset this balance and may be a leading influence in the invasive capacity of transformed cells due to a possible effect on secretion of cathepsins (Section 3.8.1.2 and Section 3.8.1.7).

Invasion requires alterations in the migratory ability of the cell, as well as the assistance of certain proteases trafficked to the PM in order to digest the basement membrane (BM) and ECM, an intact network underlying epithelial and blood vessel endothelial cells (Leblond *et al.*, 1989; Mignatti *et al.*, 1993; Karp, 2002), and other protein barriers to allow invasive cells to move to other parts of the body. In addition, our interest in proteases involved in this process was due to the reported secretion of specifically a group of normally intracellular proteases called cathepsins, into the bloodstream and contribution to invasiveness in human breast cancer (Thomssen *et al.*, 1995; Lah *et al.*, 2000; Harbeck *et al.*, 2001), as well as membrane-associated MT1-MMP. Transfection of the MCF7 breast epithelial cancer cell with MT1-MMP was shown to result in invasive behaviour (Hotary *et al.*, 2006) and was upregulated in the MCF10A cell line transfected with an activated Ha-Ras mutation (Kim *et al.*, 2007), similar to the cell model used in this study. It was, therefore, important due to our interest in the c-Ha-Ras(V12) mutation that results in invasion as well as the proteases involved in this process (Section 1.6). In order to understand the role that proteases play in invasion, a description of the major barriers to invasion, the BM and the ECM, is required.

1.6. The BM, ECM and invasion

The BM and ECM consist of a rich and diverse complex of proteins and polysaccharides in various layers (Figure 1.5), composed of collagen and laminin, that provide a scaffold for a range of other structural proteins (Leblond *et al.*, 1989; Mignatti *et al.*, 1993; Karp, 2002). Laminin is the major component in the first layer, the lamina lucida, with type IV collagen and various proteoglycans, including fibronectin, occupy the lamina densa. These two layers form the BM. Collagen types I-III and V are the major components in the lamina reticularis. The interstitial stroma is similar to the lamina reticularis, except less dense and contains elastin (Mignatti *et al.*, 1993). The BM and ECM play major roles in the growth and adherence of cells and are not merely passive environments in which biochemical exchanges between cells occur (Leblond *et al.*, 1989). When cells become malignant, however, invasion of the BM and ECM and metastasis may occur (Zhuge *et al.*, 2001; Bok *et al.*, 2003).

Invasion and metastasis are two processes that distinguish a malignant- from a benign tumour. During metastasis a primary tumour cell may detach, breach BMs due to

expression and release of elevated levels of proteolytic enzymes (Koblinski *et al.*, 2000; Yan *et al.*, 2003; Blum *et al.*, 2005; Cavallo-Medved *et al.*, 2005; Sloane *et al.*, 2005) and disseminate to a distant site. Neoplastic cells usually achieve this as they invade into and out of the vascular system to establish secondary tumours, distant from the original tumour (Hanahan *et al.*, 2000). Such a tumour is known as a metastatic tumour, from the Greek "meta" and "stasis", which means, "to stand somewhere else". Before such a process may be understood, it is necessary to look at normal migration and the proteases involved.

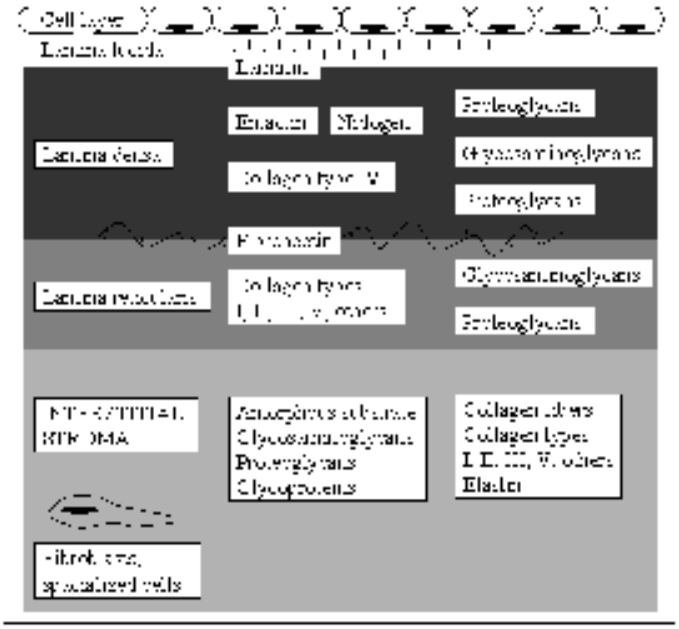


Figure 1.5. Structural components and layers of the BM and ECM.
A combination of various structural components constitute the different layers of the BM (lamina lucida and lamina densa) and ECM (lamina reticularis and interstitial stroma). Illustration was redrawn from Mignatti *et al.* (1993).

1.6.1. Proteases in migration

Cell migration is a natural event during wound healing (Nobes *et al.*, 1999; Buth *et al.*, 2007), in migratory osteoclasts during resorption of bone matrix (Sun-Wada *et al.*, 2004) and when embryonic cells and macrophages cross intact tissue barriers during implantation and inflammation, respectively (Mignatti *et al.*, 1993). These cells focus proteolytic activities at their cell surface to create a migratory path for the cell through ECM reorganization and to promote detachment (Lund *et al.*, 1999; Murphy *et al.*, 1999; Takino *et al.*, 2004) and it is a key factor during angiogenesis by normal endothelial cells (Lund *et al.*, 1999; Murphy *et al.*, 1999). These processes are limited in duration and subject to the cooperation and strict regulation by extracellular proteolytic cascades.

Intracellular lysosomal proteases usually assist in the degradation of endocytosed materials, including the ECM components (Jager *et al.*, 2004; Boya *et al.*, 2005; Demarchi *et al.*, 2006; Sato *et al.*, 2007) and the normal degradative capacity of intracellular lysosomal proteases is supported by these specialized conditions. Various characteristics of endocytic vesicles, such as the lowered luminal pH and a reducing environment, facilitate specific enzyme requirements (Sun-Wada *et al.*, 2004). Numerous pathological conditions arise due to inadequacies in cargo degradation if such enzyme requirements are not satisfied (Fratti *et al.*, 2003).

1.6.2. Cathepsin B

CB is a cysteine protease with a broad pH optimum of 5-6.5 (Linebaugh *et al.*, 1999; Pillay *et al.*, 2002b). Mature CB is capable of degrading constituents of the ECM and BM, such as type IV collagen (Coetzer *et al.*, 1991; Buck *et al.*, 1992; Tu *et al.*, 2008), fibronectin and laminin at both acid pH and neutral pH (Buck *et al.*, 1992) and seems to normally be involved in the degradation of endocytosed BM and ECM components (Sameni *et al.*, 2008). It is not highly active against components of the ECM and, therefore, may have other roles such as key receptor or an indirect role in MT1-MMP activity. Secreted, membrane-associated CB can activate both the soluble and tumour cell receptor-bound forms of uPA (Figure 1.6) and other latent proteases that are involved in the degradation of the ECM, and thereby facilitate migration (Mignatti *et al.*, 1993; Bok *et al.*, 2003; Cavallo-Medved *et al.*, 2005). An increase in CB activity has been seen to result in a comparable increase in invasiveness in a poorly metastatic mouse melanoma (Szpaderska *et al.*, 2001), for example. This is an indication that CB may somehow be involved in invasion, which is the subject of many reviews (Koblinski *et al.*, 2000; Yan *et al.*, 2003; Kroemer *et al.*, 2005; Sloane *et al.*, 2005; Tardy *et al.*, 2006). Though the actual role of CB is still not clear, possible functions are proposed in Section 1.6.5.

1.6.3. Cathepsin L

CL, a cysteine protease, is mostly trafficked to the PM and secreted, and is thus known as the "major excreted protein" (Fujishima *et al.*, 1997; Kirschke *et al.*, 1997). It has a maximal proteolytic capacity at a slightly acidic pH of 5.5-6 (Maciewicz *et al.*, 1990; Turk *et al.*, 1999). CL seems to be involved in a number of normal and pathological processes. Secreted CL is involved in osteoclastic bone collagen

degradation and bone resorption (Katunuma *et al.*, 1998), and the normal turnover of periodontal tissue (Nishimura *et al.*, 2002) and the myocardium, since deficiencies result in interstitial fibrosis (Petermann *et al.*, 2006). Intracellular CL is involved in the maturation of neurotransmitters (Yasothornsrikul *et al.*, 2003), the degradation of EGF (Reinheckel *et al.*, 2005) and the generation of epitopes in antigen-presenting macrophages (Nepal *et al.*, 2006) CL has also been shown to play a role in the nucleus, through proteolytic maturation of relevant transcription factors (Goulet *et al.*, 2004) that regulate entry into the S-phase of the cell cycle (Nepveu, 2001; Sansregret *et al.*, 2006). In highly metastatic human melanoma, secreted CL interferes with the efficiency of the immune system by cleaving C3, the third component of complement (Jean *et al.*, 1996; Frade *et al.*, 1998; Rousselet *et al.*, 2004), while its level of secretion is used as a prognostic indicator in metastatic breast cancers (Lah *et al.*, 2000; Troy *et al.*, 2004).

1.6.4. Cathepsin D

CD is the major mammalian aspartyl endopeptidase and is mainly involved in high-grade degradation of proteins and peptides e.g. insulin (Authier *et al.*, 2002), within an acidic compartment. Deficiency in CD may lead to ineffective degradation and increased autophagy, similar to what is observed in Batten disease (Koike *et al.*, 2005). Defective acidification of intracellular organelles also results in the secretion of CD, especially in cancer cells (Davidson, 1995). Increased expression of CD has been noted in MCF7 breast cancer cells (Montcourrier *et al.*, 1997; Kokkonen *et al.*, 2004) as well as in primary cultures of human breast cancers cells, compared to primary cultures of normal mammary epithelial cells (Montcourrier *et al.*, 1990).

1.6.5. Extracellular proteases in migration– MMPs, CB and uPa

In a controlled cascade of proteolytic activation the PM, uPA, an extracellular protease, colocalizes with integrins at focal contacts (Andreasen *et al.*, 1997; Murphy *et al.*, 1999). It is secreted as an inactive pro-enzyme (pro-uPA) by stromal cells (Figure 1.6). Pro-uPA binds to its cell surface receptor (uPAR) and is activated by intracellular proteases that are secreted or become membrane associated under certain conditions. Such proteases include serine proteases (e.g. plasmin) or cysteine proteases (e.g. CB or CL) (Andreasen *et al.*, 1997; Bok *et al.*, 2003). Activated uPa converts the plasminogen precursor to plasmin. This degrades numerous components of the ECM. Plasmin may also be a physiological activator of latent MMPs, thereby

switching on collagen degradation (Mignatti *et al.*, 1993; Rabbani *et al.*, 1998; Murphy *et al.*, 1999; Collette *et al.*, 2004).

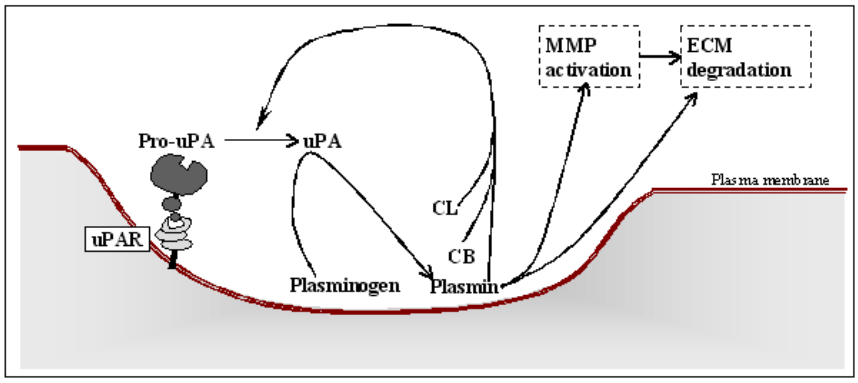


Figure 1.6. The uPA-cathepsin cascade on the PM.
The activation of pro-uPA by CB, CL and plasminogen to plasmin, leads to MMP activation and degradation of the ECM. This illustration was generated from references in Section 1.6.5.

Whereas the extracellular MMPs function at a neutral pH, cathepsins require a decreased extracellular pH. Such an acidic environmental pH is observed in many tumour microenvironments, such as at the migration front, is usually due to the activity of the NHE-1 proton pump (Montcourrier *et al.*, 1997; Bourguignon *et al.*, 2004; Cardone *et al.*, 2005).

1.6.6. Proteases in c-Ha-*ras*(V12)-related cancer and invasion

Metastatic tumour cells acquire the ability to continuously and excessively degrade the ECM and pass through this barrier, leading to increased tumour growth, migration, invasion, angiogenesis and metastasis (Montcourrier *et al.*, 1997; Foekens *et al.*, 1998; Dohchin *et al.*, 2000; Troy *et al.*, 2004; Fehrenbacher *et al.*, 2005; Nomura *et al.*, 2005; Mohamed *et al.*, 2006). This phenomenon is mainly thought to be due to increased membrane association or release of normally intracellular proteases (Rousselet *et al.*, 2004; Sloane *et al.*, 2005) and the resultant amplification of extracellular proteolytic activity (Koblinski *et al.*, 2000; Sloane *et al.*, 2005). Neutral proteases such as the extracellular surface-bound MT1-MMP (Sabeh et al., 2004) and MMP-2 and MMP-9, activated by uPa (Figure 1.6) (Siesser et al., 2006), are also believed to be central to the acquisition of invasive and metastatic ability (Gupta et al., 2000) since they can degrade various components of the connective tissue matrix (Takino *et al.*, 2004; Cavallo-Medved *et al.*, 2005; Hotary *et al.*, 2006; Siesser *et al.*, 2006) to the extent that the intact BM and ECM barriers are compromised, resulting in

cancer cell exit of an organ and invasion (Dohchin *et al.*, 2000; Szpaderska *et al.*, 2001; Tu *et al.*, 2008). In oral cancer (Nomura *et al.*, 2005) and in breast cancer (Thomssen *et al.*, 1995) a strong relationship between the expression levels of CB and CD and local invasive and metastatic growth patterns has been observed. Overexpression, but not secretion of CD, seems to increase the growth of cancer cells to a higher density via a proteolysis-independent mechanism (Liaudet *et al.*, 1995; Nomura *et al.*, 2005). This may possibly occur via a mechanism that interferes with the secretion of growth inhibitor(s) (Liaudet *et al.*, 1995). Together, CB, CD and CL have been found to be highly elevated in carcinoma homogenates and plasma of patients with metastatic breast cancers, compared to normal tissue and have, therefore, been used prognostically in primary human breast cancer (Thomssen *et al.*, 1995; Foekens *et al.*, 1998; Lah *et al.*, 2000; Troy *et al.*, 2004).

Following Ras activation or transfection with a mutated *ras* oncoprotein (Bervar *et al.*, 2003; Collette *et al.*, 2004), the distribution of vesicles containing proteases, known to characteristically function intracellularly, such as CB, CD and CL, is altered from a perinuclear to a more peripheral and PM-associated distribution and excessive secretion of these proteases was reported in the c-Ha-*ras*(V12)-transfected MCF10AneoT cell line (Sloane *et al.*, 1994; Linebaugh *et al.*, 1999), as well as in invasive cancer (Szpaderska *et al.*, 2001). The manner in which these proteases are involved in invasion of c-Ha-Ras(V12) transfected cells, is as yet undefined. Under certain conditions, MT1-MMP was also upregulated in the MCF10AneoT cell line (Kim *et al.*, 2007). Inhibition of the MMPs may also stay, or prevent such metastasis (Mills et al., 2002) and is, therefore, often employed in therapeutic regimes (Rabbani *et al.*, 1998; Murphy *et al.*, 1999; Mills *et al.*, 2002; Stoppelli, 2002; Bok *et al.*, 2003; Cavallo-Medved *et al.*, 2005; Hotary *et al.*, 2006; Siesser *et al.*, 2006). During a parallel research project done in our laboratory it was determined, with the use of a chicken invasion model system (Zijlstra *et al.*, 2008) that inhibition of MMPs in the c-Ha-*ras*(V12)-transfected MCF10AneoT cell line inhibited metastasis to the lower part of the chick embryo chorioallantoic membrane (CAM) by approximately 97%. On the other hand, inhibition of CB and other lysosomal cysteine proteases inhibited invasion by 40% (van Rooyen *et al.*, 2008) (Gordon conference 2008 Poster, Appendix IV). This is an indication that, while CB does play a role in invasion, MMPs play a major role in c-Ha-*ras*(V12)-related invasive potential. Correlation of redistribution, membrane association and secretion of intracellular proteases,

especially lysosomal enzymes, with the effects of activation of the downstream effector pathways of a mutationally activated c-Ha-*ras*(V12), transfected into the immortal MCF10A breast epithelial cell line, is therefore, relevant.

1.7. The MCF10A model cell system

In order to attempt to study the influence of c-Ha-*ras*(V12) on protease trafficking and possible relevance in invasive breast cancer, a representative cell line is required. Currently available commercial cancer cell lines suffer from the disadvantage that they do not have a 'normal' equivalent against which their behaviour may be assessed. The most frequent genetic mutations in commercial cancer cell lines are those in the tumour suppressor genes, e.g. phosphatase and tensin homologue deleted in chromosome ten *(PTEN),* phosphatidylinositol 3-kinase *(PI3K), Raf* and *Ras* genes (Hollestelle *et al.*, 2007). In this study *ras* mutations are of the greatest interest, since the Ras protein is usually directly or indirectly upregulated in cancer (Spandidos *et al.*, 1999; Eckert *et al.*, 2004), and Ha-Ras mutations are associated with acquisition of an invasive phenotype (Mo *et al.*, 2007). Since invasion and the role of proteases is a focus in this study, the MCF10A cell line and its c-Ha-Ras(V12) expressing derivative was an ideal model.

The MCF10A cell line was first reported in 1990 from mastectomy tissue from a 36-year-old, parous, premenopausal woman suffering from fibrocystic disease. This was a genetically 'normal' diploid, but immortal, breast epithelial cell line, that acquired spontaneous immortality, ascribed to lack of a p16 protein (Brenner *et al.*, 1995) due to deletion of the *9p21* locus (Zientek-Targosz *et al.*, 2008) (Section 1.8.4). While loss of p53 was found in more that 50% of breast cancers (Ozbun *et al.*, 1995; Elenbaas *et al.*, 2001), the MCF10A cell line did express a wild-type p53 (Mello *et al.*, 2008) and it did not form tumours (Soule *et al.*, 1990; Tait *et al.*, 1990; Basolo *et al.*, 1991).

Since it was known at the time that almost 30% of cancers in general harboured a V12 mutated *ras* oncogene (Bos, 1989), the MCF10A cell line was stably transfected with a mutated c-Ha-*ras*(V12) oncogene, to give rise to the MCF10AneoT cell line (Basolo *et al.*, 1991). This produced a cell that is constantly and not transiently, activated. In addition to being immortal, cells are invasive and have enhanced growth rates compared to the parent cells (Koshravi-Far *et al.*, 1996; Shin *et al.*, 2005).

Transforming c-Ha-*ras*(V12) in addition to a faulty p16/p53 signaling system (Section 1.8.6) causes crucial alterations in cellular events, such as independence of growth factor stimulation, cell proliferation, increased calcium uptake, cytoskeletal remodelling, intracellular alkalinization and increased cell volume (Fürst *et al.*, 2002). The reported increase in calcium uptake and cytoplasmic alkalinization are also both mitogenic stimulators (Ritter *et al.*, 1997). Due to the fact that p53 causes genetic instability, great care must, however, be taken to keep passage numbers as low as possible so that changes observed after transfection with the c-Ha-*ras*(V12) oncogene (Sloane *et al.*, 1994; Sameni *et al.*, 1995; Linebaugh *et al.*, 1999) are strictly due to the over expressed, mutated Ras oncoprotein, especially in a cell that had been immortalized due to direct or indirect inactivation of the p53 tumour suppressor system. The influence of p53 suppression in immortality and the masked influence it may have in tumorigenicity, is explored in the following section.

1.8. Homeostasis - proliferation vs. senescence

Normal growth and cell cycle progression is under the tight control of both positive stimulators (growth factors) and negative regulators. The intricate balance between these 2 opposing forces is not static, but is rather quite dynamic and includes significant safety mechanisms such as senescence- and apoptosis-inducing molecules and tumour suppressors. These proteins maintain homeostasis of the cellular replicative life span (Malumbres *et al.*, 2001) and defends the cell against an inappropriately extended response to growth stimuli, or illegitimate activation of oncogenic stimuli that may lead to an incorrect cellular response such as uncontrolled growth or tumour formation. The main tumour suppressor barriers are the p53, p16 (Vousden, 2000; Sharpless *et al.*, 2004) and retinoblastoma protein (Rb) pathways (Sharpless *et al.*, 2004) (Figure 1.7).

1.8.1. Homeostasis through the tumour-suppressor pathway of p14, MDM-2 and p53

In response to growth-related Ras-p38 levels (Bulavin *et al.*, 2003), increased levels of p53 down-regulates the expression of several genes involved in cell-cycle progression and blocks G1-S phase progression (Sharpless *et al.*, 2004) (Section 1.2.5). In response to DNA damage p53 acts as an "emergency brake" by reversibly inducing arrest of the proliferative cycle in G1 (Malumbres *et al.*, 2001; Zhou *et al.*, 2001; Uetake *et al.*, 2007), without causing senescence (Merlo *et al.*, 1995; Ferbeyre

et al., 2002) (Figure 1.3 on fold-out and Figure 1.7 Lane 2). During programmed cell death (apoptosis) (Merlo *et al.*, 1995) (Figure 1.3 on fold-out), or in response to death stimuli such as hypoxia or irreparable DNA damage (Chang *et al.*, 1999), p53 forms inhibitory complexes at the mitochondrion, with anti-apoptotic Bcl-2 proteins (Vousden, 2000; Pattingre *et al.*, 2006). This process results in caspase-3 activation and apoptosis (Erster *et al.*, 2004) (Figure 1.7 Lane 2).

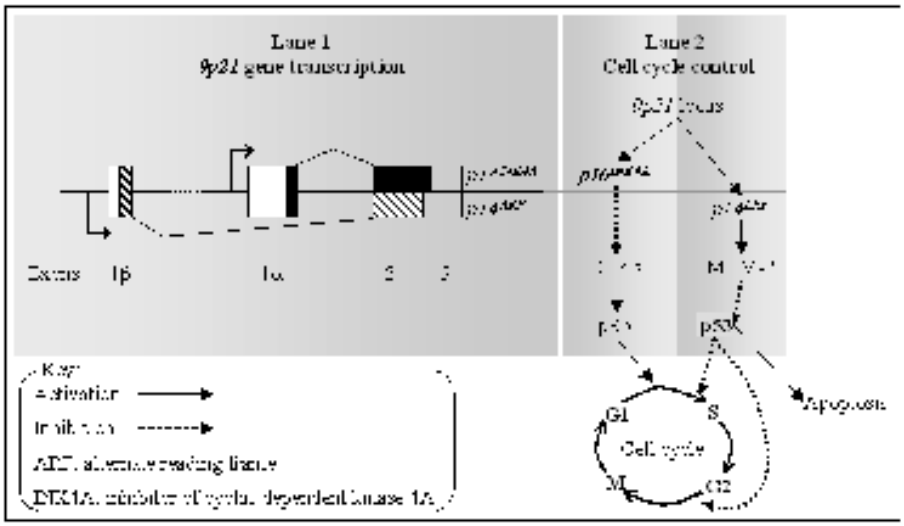


Figure 1.7. Control of mortality and progression from G1 to S phase of the cell cycle.

The INK4A-alternate reading frame locus, located on *9p21*, codes for two different proteins, p16 and p14, both involved in cell-cycle regulation (Lane 1). Unbound p53 inhibits progression of the cell cycle past G1 and G2, or may lead to apoptosis. MDM-2 binds p53, regulating its degradation and allowing G1-S phase transition. P14 binds MDM-2, and thus allows levels of free p53 to increase in response to Ras-p38 signaling (Lane 2). The inhibition of CDKs by p16 inhibits phosphorylation of Rb, so that the cell cycle does not move past G1 (Lane 2). Since both p16 and p14 negatively regulates the cell cycle, loss of either transcription or activity of these proteins may result in immortality or cancer. This illustration was generated from references in Section 1.8.

In normal cells p53 is not necessary for cell viability. To ensure growth and proliferation, p53 is constantly counteracted by murine double minute-2 (MDM-2) (Alarcon-Vargas *et al.*, 2002) (Figure 1.7 Lane 2), that constitutively binds to p53 in the nucleus (Pestell *et al.*, 1999; Zhou *et al.*, 2001), to oppose p53 activity (Lin *et al.,* 2001) and facilitate its degradation (Sharpless *et al.*, 2004) in the proteosome (Merlo *et al.*, 1995; Ferbeyre *et al.*, 2002). This process occurs in the absence of the MDM-2 inhibitor p14 (the human version of p19) (Alarcon-Vargas *et al.*, 2002; Ferbeyre *et al.*, 2002) (Figure 1.7 Lane 2). The p14 protein is induced in response to an oncogenic signal such as activated *ras* (Pestell *et al.*, 1999). The association of p14 with MDM-2 thus prevents MDM-2-related p53 degradation, and p53 levels are allowed to

increase and the cell cycle is halted or it may cause apoptosis through its translocation to the mitochondrion (Erster *et al.*, 2004).

The p53 system is, therefore, an important regulator of cell growth (Alarcon-Vargas *et al.*, 2002; Sharpless *et al.*, 2004; Shehata, 2005) and provides a mechanism of protecting cells against malignant transformation (Bulavin *et al.*, 2003).

1.8.2. Mortality is maintained through the p16 and Rb pathway.

In addition to p53, cell proliferation is controlled by the cyclin D-dependent kinases (CDKs), that are stimulated by mitogens and Ha-Ras (Ries *et al.*, 2000). In turn, these kinases phosphorylate Rb, which leads to the activation of genes whose products are required for the replication of DNA as well as for the progression of the cell cycle into S phase (Buchmann *et al.*, 1998) (Figure 1.3 on fold-out and Figure 1.7 Lane 2). The p16 proteins regulate the CDKs through inhibition (Pestell *et al.*, 1999; Thullberg *et al.*, 2000; Holland, 2001; Sharpless *et al.*, 2004), and thus maintain Rb in its unphosphorylated state to stop cell cycle progression (Dyson, 1998). Similar to p53, p16 levels increase acutely in response to DNA damage or some oncogenic stimuli, to halt the cell cycle in G1 (Pavey *et al.*, 1999; Serrano, 2000).

1.8.3. p16 and p14 are encoded from alternate reading frames on 9p21

It is clear that the p14-p53 and p16-phosphorylated Rb signaling paths are key checkpoints in the mechanism of survival vs. death signaling by promoting exit from the proliferation cycle and maintaining a finite life span (Lin *et al.*, 1998), as explained above (Figure 1.3 on fold-out and Figure 1.7 Lane 2). Both p16 and p14 guard against malignant transformation of primary cells (Thullberg *et al.*, 2000; Sharpless *et al.*, 2004) and may, therefore, be seen as anti-tumour mechanisms (Thullberg *et al.*, 2000; Sharpless *et al.*, 2004). It is then interesting, and important, to note that the p16 and p14 proteins are both encoded by an alternate reading frame locus on chromosome *9p21* (Brenner *et al.*, 1995; Ruas *et al.*, 1998; Tannapfel *et al.*, 2001) (Figure 1.7 Lane 1). These two proteins are characterized by two distinct promotors and first exons, spliced to a common exon 2 in different reading frames, i.e. *p16INK4A* (that encode p16, inhibitor of CDKs) and *p14ARF* (that encode human p14 on alternate reading frame) (Ruas *et al.*, 1998; Serrano, 2000).

Loss of function of either p14 or p16 may allow the cell to overcome an important senescence barrier (Foster *et al.*, 1998; Pavey *et al.*, 1999) and gain an infinite life span (Huschtscha *et al.*, 1998; Li *et al.*, 2007). Cells that lack p16 signaling due to deletion of the *p16INK4A* genetic sites on chromosome *9p21* (*9p21-/-*), or mutation of p16, may allow unrestricted CDK-related phosphorylation of Rb and, thus, constant proliferation (Sharpless *et al.,* 2004). Such cells acquire an infinite life span and become immortal (Brenner *et al.*, 1995; Foster *et al.*, 1998; Ruas *et al.*, 1998; Sharpless *et al.*, 2004), as was the case with the MCF10F cell line, from which the MCF10A cell line, used as a normal control in this study, was derived (Brenner *et al.*, 1995; Rao *et al.*, 2006). Most deletions affecting the *p16INK4A* gene on chromosome *9p21* also affect the rest of the alternate reading frame and may, therefore, also have an impact on *p14ARF* gene transcription (Ruas *et al.*, 1998; Serrano, 2000). The resultant lack of the p14 transcriptional product of this locus may indirectly allow unbound MDM-2 to constantly remove p53 from circulation (Ries *et al.*, 2000), effectively silencing this senescence control system (Figure 1.7 Lane 2). Such simultaneous deletion of p16 and inactivation or silencing of the p14 downstream p53 control, may allow proliferation to continue unrestricted, especially after DNA damage (Brenner *et al.*, 1995; Ries *et al.*, 2000; Zientek-Targosz *et al.*, 2008). Under such conditions immortality, in combination with time-dependent expression/accumulation of DNA mutations, may lead to cancer (Zhou *et al.*, 2001). Links between tumorigenesis in humans and genetic alterations of the *INK4A/ARF* locus on *9p21* have often been described (Ruas *et al.*, 1998; Elenbaas *et al.*, 2001; Rao *et al.*, 2006), with transcriptional silencing of p16 and immortality occurring in up to 25% of established human tumour cell lines (Zhou *et al.*, 2001)

Normally, the initial growth factor stimulation of the Ras GTPase may cause controlled, migratory and proliferative cellular reactions, but this response is of a transient nature due to the upregulation of p16 and p53, that leads to senescence (Lin *et al.*, 1998). Oncogenic c-Ha-Ras(V12) signaling, however, increases the expression of p53 (Ries *et al.*, 2000; Ferbeyre *et al.*, 2002), p16 or p14 (Lin *et al.*, 2001; Rao *et al.*, 2006), that may cause the cell to exit mitosis and terminally differentiate. Lack of p53, on the other hand, may cause hyperplasia (Ozbun *et al.*, 1993; Gao *et al.*, 2004; Zientek-Targosz *et al.*, 2008). Silencing of this cell cycle control in conjunction with a mitogenic oncogene, such as c-Ha-*ras*(V12), may, therefore, allow transformation of the cell (Ruas *et al.*, 1998; Gao *et al.*, 2004; Rao *et al.*, 2006; Zientek-Targosz *et*

al., 2008). This allows immortal cells lacking an effective p16/p53 senescence system, such as found in the MCF10A cell line, to be transformed on transfection with an oncogenic c-Ha-*ras*(V12) (Rao *et al.*, 2006).

1.8.4. Loss of p53/p16 senescence system in immortal MCF10A cells

As described above, loss of p53 function due to mutation of lack of upstream control, leads to immortality. The simultaneous loss of p16 and p14, due to deletion of the *9p21* locus, as found in the MCF10A normal cell line used in the current study, removes the protective constraint on cell division and regulated cell death, leading to immortality of the cell (Li *et al.*, 2007). This may allow further mutations to occur, often resulting in a more malignant or lethal phenotype.

p53 and other negative feedback systems control cell proliferation and migration, stimulated by growth factors and the EGFR-Ras pathways. Immortal tumour cells lacking a functioning p53/p16 system and that acquired the ability to metastasis, couple consistent extracellular proteolysis with immortality to invade and proliferate, at times and places that would be inappropriate for normal cells. This combined with a capacity to select for more and more malignant cells gives rise to run-away instability of the cell, allowing malignant progression. It also facilitates/enhances the effect of the c-Ha-*ras*(V12) oncogene in an unanticipated manner.

1.8.5. Establishment of a c-Ha-ras(V12)-transfected cell line (MCF10AneoT)

It had become clear that *ras* genes with mutations at several codons, including codon 12, are responsible for abnormal behaviour of breast cancer cells (Kasid *et al.*, 1987; Bos, 1989) (Section 1.2.1). Experimental transfection of the immortal MCF10A cell line with the c-Ha-*ras*(V12) oncogene and a neomycin resistance gene, resulted in the MCF10AneoT cell line, that had an altered mesenchymal shape and formed metastases in the liver of test animals (Russo *et al.*, 1991). While the MCF10A cells grew as monolayers, the transfected cells formed multicellular foci and cells exhibited enhanced migratory activity and an increased capability to invade the BM (Ochieng *et al.*, 1991; Russo *et al.*, 1991; Sloane *et al.*, 1994). Xenographs of the MCF10AneoT cell line resulted in further cell lines representing cells of increasing malignancy and hence, a model of cancer cell progression (Upadhyay *et al.*, 2001; Hu *et al.*, 2008; Mello *et al.*, 2008; Imbalzano *et al.*, 2009). It is thus clear that, in human breast

epithelial cells, such a genetic alteration in addition to immortality, allowed transformation and further tumour progression (Wang *et al.*, 2004a).

Before the establishment of the MCF10A and MCF10AneoT cell lines, and the other subsequently established derivative malignant cell lines (Miller *et al.*, 1993), all immortal breast epithelial cancer cells lines, that were available for research on tumorigenicity and invasion, had malignant origins. These cell lines, e.g. the highly metastatic, estrogen-independent MDA-MB breast cancer cell lines and the estrogen-dependent MCF7 cell line (Kasid *et al.*, 1987; Couissi *et al.*, 1997; Montcourrier *et al.*, 1997; Glunde *et al.*, 2003; Nomura *et al.*, 2005), suffered from the disadvantage that they had no available non-malignant, immortalized parental cell lines against which the progression towards malignancy could be assessed. The advantage of the MCF10A model set of cell lines is that the normal and transfected cells are of the same genetic background and are available for direct comparison and assessment of the effect of any inserted oncogene. Therefore, these cell lines may be uniquely used to investigate the changes that occur in the cell due to a mutated Ras(V12) oncoprotein.

Many of the downstream effectors that are stimulated by Ha-Ras contribute distinct properties to the metastatic c-Ha-*ras*(V12) phenotype. These were considered in the context of the MCF10A breast epithelial cell line in the context of combined immortality and transfection with a transforming c-Ha-*ras*(V12) oncogene.

1.8.6. Immortality, transformation and malignancy in the c-Ha-*ras*(V12) cells

Raised levels of p53 in the cell has two possible outcomes: cell cycle arrest (senescence) or apoptosis (Erster *et al.*, 2004). However, a cell expressing a mutated p53 may become hyperplastic (Ozbun *et al.*, 1993), but it may still take some time to progress towards malignancy (Rao *et al.*, 2006). However, the loss of functional p53 may promote genetic instability, allowing the acquisition of the complex changes required for transformation (Vousden, 2000; Pattingre *et al.*, 2006).

As mentioned, even cells with increasing levels of the constantly active c-Ha-Ras(V12) mutated oncoprotein, upregulate Rb and p53 in order to regain cell-cycle control, as increased p53 levels normally counteract oncogenic cell proliferation

(Katunuma *et al.*, 1998; Joneson *et al.*, 1999; Ferbeyre *et al.*, 2000; Gao *et al.*, 2004; Mo *et al.*, 2007), restricts Rho and thus cell motility (Xia *et al.*, 2007) and promotes apoptosis in response to DNA damage (Erster *et al.*, 2004). This usually prevents progression to the malignant phenotype (Katunuma *et al.*, 1998). In immortal cells, c-Ha-*ras*(V12) together with the silenced tumor suppressor p16/p53 system (Ruas *et al.*, 1998; Gao *et al.*, 2004; Rao *et al.*, 2006; Zientek-Targosz *et al.*, 2008) (Section 1.8.4), however, fails to control mitogenesis and may thus allow the genetic instability and many other complex changes required for malignant transformation (Santos *et al.*, 1989; Gao *et al.*, 2004; Rao *et al.*, 2006). An inactive p16/p53 senescence system not only promotes immortality, but in addition results in an unbalanced Rac-Rho ratio [due to constant signaling from mutated c-Ha-Ras(V12)] that facilitate migration and invasive behaviour (Santos *et al.*, 1989).

The use of the immortal MCF10A cell lines have assisted in research on phenotypical changes and effect on signaling following c-Ha-*ras*(V12)-related transformation (Basolo *et al.*, 1991; Russo *et al.*, 1991; Reshkin *et al.*, 2000a) and on the intracellular localization and distribution (Sloane *et al.*, 1994) and the secretion of cathepsins in pathology (Linebaugh *et al.*, 1999). Much in these studies remains incomplete. Further studies on the mechanism by which such alterations in organelle trafficking occurs, its molecular control and the final identification of the organelles and routes, may assist to better understand the influence of constant Ras-related growth signaling. In addition, understanding the roles of various proteases in the premalignant phenotype may refine our approach to therapy for invasive cancer. The difference between simple migration and invasion is often difficult to ascertain. The ability to compare the 'normal' MCF10A cell line to its c-Ha-*ras*(V12)-transfected premalignant cell line, however, may assist in unravelling some of these questions.

Correlation of methods via which homeostasis in proliferation is controlled in normal, non-tumorigenic cells with possible loss of control over Ha-Ras downstream effectors, may assist in understanding the invasive MCF10AneoT phenotype.

1.8.7. Homeostasis through Ras signal modulation and negative feedback

While activated Ras may indirectly control the cell cycle, degradation of the initial growth factor stimulus and negative feedback loops from downstream Ras effectors continually and increasingly counteract the initial mitogenic effect of Ras signaling.

These mechanisms ensure a transient response to growth signaling and arrest, preventing oncogene-induced proliferation and migration (Figure 1.3 on fold-out, Figure 1.4). These feedback mechanisms may include binding of a GAP that facilitates the return of the enzyme to its GDP-bound inactive state (Santos *et al.*, 1989), or increased levels of PAK, that limits transcription via inhibitory cross-talk to ERK (Section 1.5.1.). In addition, p53 inhibits CDC42 (Cau et al., 2005) and its related polarization during cell division, localization of lamellipodia at the leading edge and the reorientation of the Golgi apparatus in the direction of movement (Nobes et al., 1999). In addition, CDC42 (Sander et al., 1999) and p53 (Xia et al., 2007) inhibits Rho, that affects the cytoskeletal organization. These changes, in combination with many others, allow a proliferative, migratory phenotype with extended podocytes to become a stable, confluent cell phenotype that spreads, adheres and acquires epithelial-like characteristics (Sander et al., 1999).

Inhibition of Ras signaling in response to stimulus from the original EGF-EGFR complex may occur on endocytosis of this complex (Haugh *et al.*, 1999; Ceresa *et al.*, 2006). While endocytosed receptors such as the transferrin receptor are returned to the PM (Thilo *et al.*, 1995), the internalised EGFR is targeted for subsequent degradation. This happens in a number of steps. After binding with a ligand and internalization into an EE, the EGFR cytoplasmic tail signaling domain recruits the tyrosine-phosphorylated adaptor protein Src homology-2 (SH2)-domain-containing α2-collagen-related protein (Shc) (Authier *et al.*, 1999a) and SOS that subsequently activate downstream effectors (Haugh *et al.*, 1999) (Figure 1.3 on fold-out). Ubiquitin (Alwan *et al.*, 2003) and annexin-I binding (White *et al.*, 2006) cause the EGF-EGFR complex to be concentrated into clathrin-coated domains of the initially formed EE (Sachse *et al.*, 2002; White *et al.*, 2006) (Section 1.8.7). This is followed by dephosphorylation and isolation of the EGFR cytoplasmic tail from the cytoplasm through annexin-I-related secondary inward budding into the EE, giving rise to a structure containing many intraluminal vesicles, the multivesicular body (MVB) (Section 3.3.2). This process terminates signaling and through fusion with LE or lysosomes, the receptor-ligand complex is degraded.

Failure of these safeguard mechanisms (that counteract proliferation and growth) may lead to abnormal cellular growth, or cancer, as well as inappropriate migration, or invasion (Bulavin *et al.*, 2003; Gao *et al.*, 2004; Rao *et al.*, 2006; Mo *et al.*, 2007).

1.8.8. Rho-ROCK in cancer and in the MCF10A c-Ha-*ras*(V12)-transfected cells

Most cancers have their origin in cells that would normally differentiate into a specialized cell type. Differentiated cells are neither programmed genetically for motility, nor are their basic skeletal structure conducive to this function. During the course of tumour progression, most cancer cells acquire the capacity to invade surrounding tissues and metastasize to distant sites, which implies alterations in cellular signaling pathways that, in normal cells, control cell adhesion and motility.

Normally, virtually all aspects of cell migration and invasion are controlled by the actin and microtubule components of the cytoskeleton (Machesky *et al.*, 1999; Anguelov, 2000). The Rho GTPases (Rac, Rho and CDC42) are best known as regulators of these elements (Machesky *et al.*, 1997; Bishop *et al.*, 2000). They confer the metastatic phenotype, to a large extent, due to changes in cytoskeletal organization that perturb cell-cell and cell-substrate adhesion and alterations in the cell shape (Menard *et al.*, 2005) (Section 1.4.3 and Figure 1.4). In transfected or mutated cells the sustained signaling of Ras to downstream Rho-ROCK can interfere with the reciprocal balance between Rac and Rho activity (Sander *et al.*, 1999), permanently down-regulating Rac activity. This may allow sustained elevation of Rho activity (Zondag *et al.*, 2000). Expression of constitutively active RhoA or ROCK alone can induce proliferation even in the presence of a low amount of cell adhesion (Pirone *et al.*, 2006), since several distinct peptides or drugs that block Rho-mediated pathways (i.e. PAK, CDC42 and Rho) have been found to suppress the Ras-induced malignant phenotype (Maruta *et al.*, 1999). The over-expression of ROCK has been reported in MCF7 human breast cancer cells (Nishimura *et al.*, 2003). The imbalance between Rac and ROCK may result in morphological transformation of epithelial cells into a spindled-shaped mesenchymal phenotype, and faster migration (Nishimura *et al.*, 2003). Such a phenotype that includes malignancy and migration, is associated with the c-Ha-*ras*(V12)-transfected invasive MCF10AneoT cell line.

1.8.9. Protease secretion in MCF10A cells in relation to Ha-Ras signalling

An altered cytoskeletal organization under the spatial influence of Rac and Rho may be one of the factors that promote both migration and pathological invasion. In combination with these changes, though, spatially controlled degradation of the

matrix and cell adhesion to the underlying matrix via e.g. integrins (Section 4.1.4.2) and CD44 (Section 4.1.7.1), are important factors in epithelial cell mobility. CD44 links the ECM to the intracellular cytoskeleton (Ponta *et al.*, 2003), but also stimulated calcium influx, which in turn may activate of the NHE-1 PM proton pump (Yuspa *et al.*, 1991; Ritter *et al.*, 1997; Bourguignon *et al.*, 2004) (Figure 1.3 on fold-out). This may allow pericellular acidification that contributes to a favourable environment for maturation of secreted or membrane-associated CB (Bourguignon *et al.*, 2004). Secretion of proteases such as CB and MT1-MMP are related to the migratory ability of many cells (Section 1.6), and both these proteases have been shown to be elevated in migrating MCF10A cells (Gilles *et al.*, 2001; Bervar *et al.*, 2003). It seems as if various components of the matrix may influence secretion of these, and other proteases. In normal MCF10A cells type I collagen, for example, elevates levels of MMP-2 (Kim *et al.*, 2007) that is matured by MT1-MMP, while the laminins may influence the distribution of MT1-MMP to the leading front in these cells (Kim *et al.*, 2007). In turn, cleavage of laminin-5 shown in the BM of MCF10A cells (Imbalzano *et al.*, 2009) by MT1-MMP and MMP-2 (Koshikawa *et al.*, 2000; Gilles *et al.*, 2001) may generate fragments that may further stimulate growth, due to its binding to EGFR (Schenk *et al.*, 2003). CB may act in a cascade with other membrane-bound proteases (Section 1.6.5 and Section 4.1.2) that create a path for migration through the ECM (Murphy *et al.*, 1999). In invasive cancers, elevated pericellular levels of these proteases and the potential excessive proteolytic degradation of matrix components may unmask fragments or cryptic sites in the BM or ECM that may act as further migratory stimuli. Apart from matrix degradation, pericellular proteases, especially MT1-MMP, may be involved in the cleavage of cell-cell adhesion or cell-matrix proteins, such as E-cadherin (Cao *et al.*, 2008) or CD44 (Marrero-Diaz *et al.*, 2008). These factors may negatively influence adherence to the matrix and may allow these cells to overcome contact inhibition. It has been suggested that cleavage of CD44 in c-Ha-*ras*(V12)-transfected epithelial NIH-3T3 (Kawano *et al.*, 2000a) and MCF10A cells is due to MMPs (Kawano *et al.*, 2000b), possibly MT1-MMP and that this activity is under the influence of the downstream Ha-Ras effectors PI3K, Rac and CDC42.

Ha-Ras also uniquely stimulates the Rac-p38 pathway that elevates the transcription of MT1-MMP and MMP-2, that are involved in remodeling of the ECM during the initial stages of migration, and increase the metastatic ability of the transformed cell

(Murphy *et al.*, 1999; Shin *et al.*, 2005). In addition, PI3K-related cross-talk from AKT to ERK results in the increased transcription of both MMP-2 and MMP-9 (Shin *et al.*, 2005), that increases its assistance in the invasive capability of transformed cells (Hotary *et al.*, 2006). Surface expression of MT1-MMP through inhibition of its endocytosis (Siesser *et al.*, 2006; Zhong *et al.*, 2006), was shown to be promoted via FAK signaling, usually involved in contact turnover and migration (Section 4.7.1). In addition FAK inhibits apoptosis by suppressing components of the cell death machinery such as the caspases via a PI3K-AKT pathway (Menard *et al.*, 2005).

1.9. Aim of this thesis

CB and MT1-MMP are known to regulate BM turnover (Cao *et al.*, 2004). Altered trafficking of intracellular cathepsins such as CD, CL and especially CB, and the association of these proteases and MT1-MMP with the PM, supports BM degradation and migration in c-Ha-*ras*(V12)-transformed MCF10A breast epithelial cells, and thus seems to implicate involvement in invasion. Protease inhibitor investigation by our research group indicated a key role for MT1-MMP in invasion in the MCF10AneoT cell line (van Rooyen, 2009) (Gordon conference 2008 Poster, Appendix IV). The mechanisms behind these observations are not yet clear, and form the focus of the current investigation.

In this project we investigated the relationship between signaling by a mutated c-Ha-*ras*(V12) oncoprotein in an immortal cell, and the underlying factors that may support protease-related invasion of transformed breast epithelial cells. In order to set the framework and anticipate, and subsequently explain, possible outcomes, a general pathway for Ras signaling was compiled in Chapter 1, with specific mention of the influence of Ha-Ras on migration. Acquired immortality of the parental MCF10A normal cell line was described in the context of homeostasis and transformation of the c-Ha-*ras*(V12)-transfected MCF10AneoT invasive derivate, and the implied influence on trafficking of proteases by a mutationally activated c-Ha-Ras(V12) oncoprotein in conjunction with immortality.

In general, in addition to using an indicator for acidity, antibodies to cathepsins B, D and L, lysosome-associated membrane proteins (LAMP-2) and MT1-MMP were used to fluorescently localize and, in some instances, quantify, the proteases of interest. MT1-MMP antibodies were characterized for use in the MCF10A model cell system.

Light- and electron microscopy results assisted in studies on cellular morphology and ultrastructure. In Chapter 2, general information on all reagents used is supplied and background descriptions and the general protocols outlined. Relevant detailed information is presented in each individual chapter.

In order to find a new perspective on the role of the cathepsins in c-Ha-*ras*(V12)-related invasion, proteins involved in endosomal vesicle maturation and trafficking and the association of precursor and mature, active cathepsins with these various structures was reviewed in Chapter 3, Our objectives for further experimental work included the clarification of sets of characteristics and possible trafficking routes of cathepsin-containing endosomal vesicles. Redistribution of these proteases was correlated with the effects of activation of the downstream effector pathways of the mutationally activated c-Ha-*ras*(V12). The relevance and negative impact of constitutive signaling by c-Ha-Ras(V12) in the trafficking of these structures was presented at hand of reported differences in digestive efficiency, and alterations in distribution of immunocytochemically labeled intracellular cathepsins and their colocalization with a membrane protein characteristic of degradative vesicles, as well as with an indicator of luminal acidity.

Lastly, we investigated the contribution that alterations in distribution on the PM of membrane-associated CB and membrane-bound MT1-MMP may have on the metastatic character of the c-Ha-*ras*(V12)-transfected MCF10AneoT cell line. To this end a review of reported CB and MT1-MMP trafficking routes and control mechanisms was presented in Chapter 4. Antibodies recently raised to various domains of the MT1-MMP protein were characterized and used to clarify the membrane distribution of various processing forms of MT1-MMP and indicated c-Ha-*ras*(V12)-related alterations in anterior-posterior polarity of transfected cells. Possible differences in the distribution of membrane-associated CB and MT1-MMP in the MCF10AneoT cell line were explored and correlated with the invasive phenotype.

In Chapter 5 altered intracellular trafficking and membrane-related localization of the cathepsins and MT1-MMP were correlated to the transition from an epithelial- to a mesenchymal morphology, a process that is transient in normal migrating cells, but persists in invasive cells. The influence of Wnts (so called after the wingless protein

in Drosophila, and its mammalian version, int-1) in both these processes, and the possible direct impact of changes in PM location of transforming c-Ha-Ras(V12) on formation of a leading front, cell polarity in general and PM-associated distribution of CB and MT1-MMP is discussed. While knowledge obtained from experiments done on monolayers may not be directly applicable in 3D culture models or *in vivo* ductal homeostasis and transformation, it may at least provide some appreciation of the polarization of proteolytic activities and the turmoil that may be caused due to alterations in signaling by mutated c-Ha-Ras(V12) and related protease distribution.

CHAPTER 2.

Materials and methods

2.1. Materials

Glycerol was from AR–Associated Chem. Enterprises (Glenvista, South Africa). Acrylamide, ammonium persulfate, calcium chloride, microbiological grade gelatin (porcine skin), glycine, H_2O_2 35% (v/v), hydrochloric acid, Na_2HPO_4, $Na_2HPO_4.H_2O$, paraformaldehyde (PFA), potassium chloride, sodium azide, sodium chloride and sodium hydroxide were from BDH (Poole, England). Sodium dodecyl sulfate (SDS) was from Boehringer Mannheim (Mannheim, Germany). Protein A-gold (PAG) was from the Department of Cell Biology, University of Utrecht, Netherlands. Horse serum, Fungizone was from Gibco (Paisley, UK). Ponceau S was from Searle (High Wycombe, Bucks, United Kingdom). Donkey anti-IgY Cy3 conjugate was from Jackson ImmunoResearch Pharmaceuticals (West Grove, PA). Elite fat free milk powder was from Clover SA (Pty) Ltd (Roodepoort, South Africa). Medium grade L R White was from the London Resin Company (Reading, UK). Glutaraldehyde (25% v/v solution) was from Merck (Midrand, RSA). 2-Mercapto ethanol was from Merck Schuchardt OHG (Munich, Germany). (2,4-Dinitroanilino)-3'-amino-*N*-methyldipropylamine (DAMP) and LysoTracker Red DND-99 were from Molecular Probes Inc. (Eugene, Or). 2-Amino-2-(hydroxymethyl)-1,3-propandiol (Tris) was from MP Biomedicals (Eschwege, Germany). Magnesium chloride and potassium digydrogen orthophosphate were from Saarchem (Wadeville, South Africa). Bisacrylamide (N,N'-methylene-bisacrylamide), bovine serum albumin (BSA) (Mr 68 kDa), carbonic anhydrase (Mr 30 kDa), Coomassie brilliant blue G, diaminobenzidine/3,3',4,4'-tetraaminobiphenyl (DAB), Dulbecco's minimal essential medium (DMEM): Ham's F-12 medium, ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*,*N*',*N*'-tetra acetic acid (EGTA), fish skin gelatin (FSG), gelatin (porcine skin), goat anti-rabbit IgG (whole molecule) FITC conjugate, goat anti-rabbit IgG (whole molecule) tetramethyl rhodamine isothiocyanate (TRITC), goat anti-rabbit IgG (whole molecule) Cy3 conjugate, Hanks' balanced salts (HBSS), horseradish peroxidase (HRP), hydrocortisone, insulin, lysozyme (Mr 14 kDa, N-2-hydroxy-piperazine-N'-2 ethane sulfonic acid (HEPES), ovalbumin (OVA, Grade V) (Mr 45 kDa), phosphorylase B (Mr 97.4 kDa), piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), rabbit anti-chicken IgG (whole molecule) fluorescein isothiocyanate (FITC)

conjugate, saponin, sodium bicarbonate, soybean trypsin inhibitor (Mr 21.5 kDa), trypsin-EDTA solution (x 10) (Sigma Chemical Co) and trypsin-ethylenediaminetetra-acetic acid (EDTA) were from Sigma (St. Louis, Mo). Epidermal growth factor was from Upstate Biotechnology. Mouse anti-LAMP-2 was a gift from Dr. T August (Developmental Studies, Hybridoma Bank, University of Iowa, IA). Chicken anti-human liver CB was provided by Dr E Elliott. Rabbit anti-host CL was provided by Dr R Pike. Chicken anti-porcine CD was provided by F Fortgens. Chicken anti-proCat was provided by C Crouch. Chicken anti-EA and chicken anti-HPX were provided by D van Rooyen (Department of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Pietermaritzburg).

2.2. MCF10A and MCF10AneoT culture

The MCF10 is an immortal diploid human breast epithelial cell line, derived from mastectomy tissue of a patient with fibrocytic breast disease. This line underwent spontaneous immortalization in culture and grows floating (MCF10F) in the absence of calcium or attached in the presence of calcium (MCF10A) (Soule *et al.*, 1990). Transfection of the MCF10A with a plasmid containing the neomycin resistance gene as a selection marker and a mutated oncogenic c-Ha-*ras*(V12) resulted in the MCF-10AneoT cell line. While the normal MCF10A cells are normal, non-tumorigenic and non-invasive, the MCF10AneoT cells formed tumours when injected under the skin of mice and showed metastases in the liver (Basolo *et al.*, 1991; Ochieng *et al.*, 1991).

The MCF-10A and MCF-10AneoT cell lines were supplied collaboratively by Prof. Sloane (Department Pharmacology, Wayne State University, Detroit, MI). Both cell lines were grown in DMEM-Ham's F12 nutrient mixture, containing 5% equine serum, supplemented with insulin, hydrocortisone, antibiotics and epidermal growth factor medium in a humidified atmosphere containing 5% CO_2. This protocol was the same than what had been used for the original cultures to ensure that the cell phenotype maintained consistent with that of the laboratory of origin as this protocol differs slightly from the routine (Soule *et al.*, 1990; Basolo *et al.*, 1991; Sloane *et al.*, 1994).

2.2.1. Reagents

Hanks' balanced salt solution (HBSS)

Powdered HBSS and sodium hydrogen carbonate (1.2 g) were dissolved in about 900 ml of dd.H_2O, adjusted to pH 7.3 with NaOH and made up to 1 litre. HBSS was filtered through a 0.22 μm filter into sterile autoclaved bottles under sterile conditions and stored at 4°C.

Decomplemented horse serum

Aliquots of commercially available horse serum were incubated at 56°C for 30 min to denature the complement component. Aliquots were stored at -20°C.

Epidermal growth factor (EGF, 50 μg/ml)

EGF (100 μg) was dissolved in dd.H_2O (2 ml) and stored at -20°C.

Insulin (600 μg/ml)

Insulin (10.2 mg) was dissolved in HBSS (16.5 ml) with the addition of 0.1M NaOH (500 μl) and stored at -20°C.

Hydrocortisone (3.33 mg/ml)

Hydrocortisone (10 mg) was dissolved in absolute ethanol (3 ml) and stored at -20°C.

Fungizone (250 μg/ml)

Fungizone (5 mg) was dissolved in dd.H_2O (20 ml) and stored at -20°C.

Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 complete medium, hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), EGF (20 ng/ml), horse serum (5%, v/v), fungizone (0.25 μg/ml), pH 7.3)

Powdered medium and sodium hydrogen carbonate (1.2 g) were dissolved in about 900 ml dd.H_2O, adjusted to pH 7.3 with NaOH and made up to 1 litre. Medium was filtered through a 0.22 μm filter into sterile, autoclaved bottles under sterile conditions. The base solution was supplemented with horse serum (50 ml), EGF (400 μl 50 μg/ml EGF), insulin (17 ml 600 μg/ml), hydrocortisone (150 μl 3.33 mg/ml), and fungizone (10 ml 250 μg/ml). Complete medium was stored at 4°C.

2.2.2. Procedure

Cells were cultured at 37°C in 25 or 75 cm^2 flasks in complete medium in a humidified atmosphere containing 5% CO_2, fed every 3-4 days. Upon reaching 80% confluence cells were passaged by washing with HBSS, followed by just enough trypsin-EDTA to cover the monolayer and allow the cells to detach. Detached cells were diluted in complete medium, split in a ratio of 1:3 for each passage and seeded into new flasks.

For intra- and extracellular immunolabeling and pH studies, cells were prepared as described above, seeded onto sterile 12 mm round coverslips (in 24 well Terasaki plates) and grown to 70% confluence in DMEM-Ham's F12 medium supplemented with 10% decomplemented horse serum.

2.3. Polyacrylamide gel electrophoresis (PAGE) and preparation of marker proteins

In gel electrophoresis protein molecules are coated with sodium dodecylsulfate (SDS), an ionic detergent, which denatures and unfolds the proteins and binds to most proteins to 1.4 g SDS/g protein. This imparts a negative charge-to-mass ratio and proteins are forced through a sieving gel, driven by an electrical current to achieve separation of sample components according to their molecular weights. The speed of migration and, therefore, sample separation, takes place on the basis of physical properties such as size and electric charge and determine how rapidly an electric field can move the molecule through a gelatinous medium. In such a system an inverse relationship exists between the molecular weight of a protein and the distance migrated in a gel.

Laemmli (Laemmli, 1970) formulated the discontinuous Tris-glycine buffer and gel system that is most often used. Due to the pH of 6.8 of the stacking gel, a narrow zone of steep discontinuous voltage gradient develops between the leading chloride and the trailing glycine ions, focusing all proteins in the sample into a tight band at the interface of the stacking and running gel. Here, at the top of the running gel the change in pH to 8.8 increases the mobility of the glycine so that it moves past the proteins. As they both migrate, the higher matrix density of the running gel allows the concentrated sample proteins to be sieved and separated on the basis of their molecular size.

The inclusion of β-mercaptoethanol in the preparation sample treatment buffer prevents oxidation of cysteines and breaks up disulfide bonds. Glycerol, being denser than water is added so that the sample sinks to the bottom of the sample well, while bromophenol blue is added to track the leading migration front.

2.3.1. Reagents

Solution A: Monomer Solution [30% (m/v) acrylamide, 2.% (m/v) bis-acrylamide]

Acrylamide (73 g) and bis-acrylamide (2 g) were dissolved and made up to 250 ml with d.H_2O and stored in an amber coloured bottle at 4°C.

Solution B: 4 x Running gel buffer [1.5 M Tris-HCl, pH 8.8]

Tris (45.37 g) was dissolved in approximately 200 ml of d.H_2O, adjusted to pH 8.8 and made up to 250 ml.

Solution C: 4 x Stacking gel buffer [500 mM Tris-HCl, pH 6.8]

Tris (3 g) was dissolved in 40 ml d.H_2O, adjusted with HCl to pH 6.8 and made up to 50 ml.

Solutions A, B and C were filtered through Whatman No 1 filter paper before use.

Solution D: 10% (m/v) SDS

SDS (10 g) was dissolved in 100 ml d.H_2O.

Solution E: Initiator [10% (m/v) ammonium persulfate]

Ammonium persulfate (0.1 g) was made up to 1 ml just before use.

Reducing treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoehanol, pH 6.8]

Buffer C (2.5 ml), SDS (4 ml solution D), glycerol (2 ml) and 2-mercaptoethanol (1 ml) were made up to 10 ml with d.H_2O.

0.1% (m/v) Bromophenol blue

Bromophenol blue (0.01 g) was made up in 1 ml dH_2O.

Tank buffer (250 mM Tris-HCl, 192 mM glycine, 0.1% (m/v) SDS, pH 8.3)

Tris (15 g) and glycine (72 g) were dissolved and made up to 5 litres with d.H_2O. Prior to use, 2.5 ml of SDS stock (solution D) was added to 250 ml for use in the Mighty Small II electrophoresis apparatus.

Coomassie Brilliant blue G250 (0.02% (m/v) Coomassie Brilliant Blue G250, 2% (w/v) phosphoric acid, 5% (m/v) aluminium sulfate, 10% (v/v) ethanol) (Coomassie Blue 250)

Coomassie Brilliant blue G250 dye (0.2 g) and $Al_2(SO_4)_3.(H_2O)_{15}$ (50 g) were dissolved in 86% (v/v) phosphoric acid (24 ml) and ethanol (100 ml) and made up to 1 liter with d.H_2O (876 ml). The solution was stirred (5 min) and stored in a glass reagent bottle at room temperature.

Molecular weight marker proteins (5 mg/ml)

Each marker protein (phosphorylase B from rabbit muscle, bovine serum albumin, ovalbumin from chick egg, carbonic anhydrase from bovine erythrocyte, soybean trypsin inhibitor and lysosyme from chicken egg white) (1 mg) was individually made up in 200 µl solution C.

2.3.2. Molecular weight marker protein stock

A stock solution (5 mg/ml) of phosphorylase B from rabbit muscle, bovine serum albumin, ovalbumin from chick egg, carbonic anhydrase from bovine erythrocyte, soybean trypsin inhibitor and lysosyme from chicken egg white was individually prepared in stacking gel buffer.

A sample of each individual protein marker was prepared for testing. Each stock solution (16 µl) (made up of a solution with 5 mg/ml made up to 100 µl with 83 µl reducing treatment buffer and 1 µl bromophenol blue) was boiled (120 seconds) and loaded into the wells of the stacking gel. During the electrophoretic protein separation procedure (18 mA per gel, unlimited voltage) the gels were cooled using a circulating water bath (4°C) and were subsequently processed immediately for western blotting or stained using Coomassie G (Section 2.3.1) (Figure 2.1).

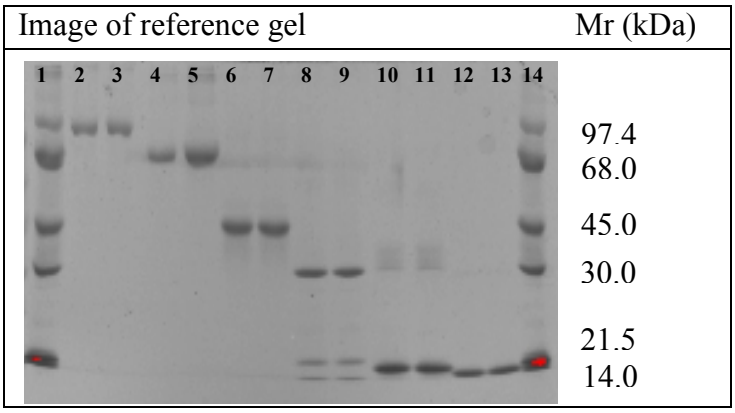


Figure 2.1. Molecular weight marker proteins.
Proteins with known molecular weights that were used as molecular weight reference markers. The reference gel indicates the relative electrophoretic mobility of each of the individual markers that were used in the set of molecular weight markers.

Molecular weight marker working solutions

Combined sets of reference markers were made up by combining each of the 6 marker stock solutions (80 µl, 0.8 mg/ml) made up in reducing treatment buffer (final volume 500 µl with 15 µl reducing treatment buffer), containing bromophenol blue (5 µl). The samples were boiled (120 seconds), stored (–20°C) and thawed just before loading onto the polyacrylamide gel.

A sample of the combined set of reference markers (5 µl) per electropheresis gel, was loaded into a well of the stacking gel, giving a final concentration of 4 µg/ml of each protein. During the electrophoretic protein separation procedure (18 mA per gel, unlimited voltage) the gels were cooled using a circulating water bath (4°C) and were subsequently processed immediately for western blotting or stained using Coomassie G (Section 2.3.1).

2.3.3. Procedure

The acrylamide-bisacrylamide monomer and SDS stock solutions, running gel buffer and distilled H_2O were mixed with ammonium persulfate initiator solution and TEMED as indicated for the 12.5% running gel (Table 2.1). This solution was loaded into the gel caster, overlaid with distilled H_2O to exclude oxygen and allowed to polymerize (1 h). After polymerization the distilled H_2O was poured off, the 4% stacking gel solution was prepared (Table 2.1), layered on top of the separating gel and a plastic 15 well comb inserted into the stacking gel. After the gel had polymerized (30 min), the combs were removed and the gels placed into the Mighty Small II electrophoresis apparatus, which was filled with the tank buffer.

Table 2.1. The volumes of various stock solutions used in the preparation of a 12.5% 15 well electrophoresis gel.

Reagent	**Running gel (12.5%)**	**Stacking gel (4%)**
A. (poly-acrylamide stock solution)	3.123 ml	0.47 ml
B. (Running gel buffer)	1.875 ml	
C. (Stacking gel buffer)		0.875 ml
D. H_2O	2.375 ml	2.15 ml
D. (SDS stock solution)	75 µl	35 µl
E. (Initiator)	37.5 µl	17.5 µl
TEMED	3.75 µl	7.5 µl

Cultured cells were washed, scraped from the flask base, centrifuged and the supernatants were discarded. The pellets were resuspended, prepared for reducing electrophoresis, loaded into a polyacrylamide gel and electrophoretically separated. The gel was washed and the proteins were electrophoretically transferred onto nitrocellulose paper.

2.4. Western blot

Western blotting or immunoblotting is useful to assess the presence of a specific protein or peptide in a test sample, based on its molecular weight. Antigen detection relies on the specificity of the antigen-primary antibody interaction. Samples are prepared from homogenized tissues or cells and proteins in the sample are electrophoretically separated using SDS-PAGE and transferred to a nitrocellulose membrane for detection with a specific primary antibody and a secondary antibody-peroxidase conjugate, followed by chemiluminescent detection, based on the oxidation of a luminol-based substrate (peroxide) by horseradish peroxidase. Light emission is captured and visualized by exposing the blot to film.

2.4.1. Reagents

Tris buffered saline [20 mM Tris buffer, 150 mM NaCl and 0.05% Tween 20 (v/v) pH 7.5] (TBS-Tween)

NaCl (8 g), KCl (0.2 g) and Tris base (3 g) were dissolved in 800 ml of d.H_2O, the pH was adjusted to 7.5 with 1 M HCl and made up to 1 liter.

Blocking agent [5% (m/v) non-fat milk powder (Elite) in TBS-Tween]

Non-fat milk powder (Elite) (3 mg) was dissolved in 60 ml TBS-Tween.

Luminol-peroxidase substrate (luminol)

Luminol enhancer solution (50 µl per strip) and peroxide solution (50 µl per strip) were mixed immediately before use.

2.4.2. Procedure

The nitrocellulose membrane strips containing reduced cell samples were placed in the incubation vessel, blocked, incubated in chicken antibodies, washed, incubated in peroxidase-tagged secondary antibody, incubated in luminol and the blots were exposed to film. Blots were photographed in a VersaDoc 4000 Imager (BioRad, California, USA) and analysed.

2.5. Tissue processing for ultrastructural analysis

Aldehyde-based fixatives are commonly used in microscopy studies, with its main purpose to cross link proteins in order to stabilize and preserve the ultrastructure. The cross-linking reaction of paraformaldehyde (PFA) with proteins occurs slowly, while glutaraldehyde is a small molecule that rapidly penetrates tissue and has high protein cross-linking potential. Since fixation may also mask antigen sites on proteins and inhibit antibody binding during immunocytochemistry, free aldehyde groups introduced by aldehyde fixation should be removed or blocked. A combination of a low percentage of glutaraldehyde for rapid penetration, and PFA for reversible cross-linking, is often used in specimen processing protocols for immunocytochemistry (Kiernan, 2000).

Schliwa *et al* (1981) identified the PHEM buffer system (Pipes, Hepes, EGTA and magnesium chloride) that gave excellent morphological results in tissue samples prepared for immunocytochemical investigations. It has been used by others to achieve high quality structural preservation of fibrous components of the cytoskeleton as well as other intracellular structures for cryo-immunocytochemistry (Schliwa *et al.*, 1981; Santama *et al.*, 1998). The high EGTA molarity preserves structural integrity of all components, while the buffer components provide a buffering effect against a wide range of variable pH. This buffer system was, therefore, selected for this study.

Most biological tissues are not sufficiently firm to be cut into thin sections and have to be embedded in a material of sufficient strength to allow a thin section to be cut.

These embedding media should preserve the fine structure, with little extraction of cellular constituents or loss of biochemical activity in specimens used for immunocytochemical studies. The sectioning quality of embedded specimen relies on its hardness and plasticity. Most embedding media are not miscible with water, with strong dehydration agents required prior to embedding and high curing temperatures. LR White resin can tolerate 12% water by volume and can be infiltrated with resin after dehydration to 80% ethanol and cured at temperature as low as 56°C. This resin is not cross-linked, and ultrathin sections are therefore easily permeated by cytochemical reagents such as stains and antibodies.

2.5.1. Reagents

Double strength PHEM [130 mM PIPES, 60 mM HEPES, 20 mM EGTA, 4 mM $MgCl_2$, pH 7.3] (2x PHEM Buffer)

PIPES (18 g), HEPES N-2-hydroxy-ethylpiperezine N'-2-ethanesulfonic acid (5.36 g), EGTA (3.75 g) and $MgCl_2.6H_2O$ (0.326 g) were dissolved in 360 ml of $d.H_20$, adjusted to pH 7.3 with NaOH and made up to 400 ml with $d.H_2O$. Aliquots (50 ml) were stored at –20°C.

Single strength PHEM [65 mM PIPES, 30 mM HEPES, 10 mM EGTA, 2 mM $MgCL_2$, pH 7.3] (1x PHEM Buffer)

2x PHEM (50 ml) was diluted with $d.H_20$ (45 ml), adjusted to pH 7.3 and made up to 100 ml.

1% (v/v) glutaraldehyde in PHEM, pH 7.3

Glutaraldehyde (100 µl) (25% (v/v)) was added to 2x PHEM buffer (1.25 ml) and made up to 2.5 ml with $d.H_2O$.

1% Osmium tetroxide

Osmium tetroxide (10 µl) was added to 90 µl $d.H_2O$.

10% (m/v) Gelatin in PHEM, pH 7.3

Gelatin (10 g) was added to 100 ml 1x PHEM and dissolved by heating. The volume was made up to 100 ml with d.H_2O if necessary.

2% Formvar

Formvar [2% (w/v)] was dissolved in chloroform and stored in a sealed glass bottle.

2% (m/v) uranyl acetate in distilled water

Uranyl acetate (1 g) was dissoled in d.H_2O (50 ml) and stored at 4°C.

Lead citrate

Pb-citrate (0.1 g, 0.2% (m/v) in d.H_2O) was dissolved in 50 ml d.H_2O and stored in a sealed glass bottle at 4°C (Parton et al., 1989).

2.5.2. Procedure

Monolayers of MCF10A and MCF10AneoT cells were grown to 70% confluence in 25 or 75 cm^2 flasks as described in Section 2.2. Cells were fixed *in situ* by addition of 1% glutaraldehyde in equal volumes of PHEM buffer and complete medium (2 h at RT). Cells were scraped from the flask with a rubber scraper, collected into a 15 ml tube and centrifuged (1000 x g, 2 min). The supernatant in each tube was discarded and the pellet was washed with PBS (3 x 2 min) and infiltrated with gelatin (30 min, 36°C). The fixed cell pellets were divided into small pieces using a scalpel blade, osmicated (10 min), washed in d.H_2O (3 x 2 min) and dehydrated in a graded series of alcohols (25%, 50%, 70%, 90%, 100%). After processing through increasing concentrations of resin diluted in alcohol (1:2 resin to EtOH, 1:1 resin to EtOH, 2 changes of fresh L R White resin), cell pellets were embedded in L R White resin. Curing was carried out in resin-filled gelatin capsules (48 h at 56°C) in a hot air oven.

Formvar-coated copper grids (100 mesh) were prepared in advance. A clean glass slide was immersed into formvar, lifted out and left to air dry. The film of formvar that formed on the glass slide was scored with a blade for ease of removal. The glass slide was slowly lowered into dish containing distilled water, so that the formvar film floated onto the water surface. Copper grids were placed onto the formvar film with the dull sides of the grids facing the formvar film. The film was picked up with filter

paper and left in a petri dish to dry and subsequently coated with a thin layer of carbon to stabilize the hydrophilic L R White resin in the EM column.

Ultrathin sections were cut on a Reichert-Jung Ultracut ultramicrotome using a MicroStar diamond knife and were picked up onto formvar- and carbon-coated copper grids and counterstained with UA and lead citrate. Sections were viewed with a Philips CW120 Biotwin TEM Electron Microscope.

2.6. Fluorescence immunolabeling for confocal microscopy

Laser scanning confocal microscopy is a powerful imaging tool in the investigation of fluorescent-labeled subcellular molecules, using laser beams of set wavelengths as sources of excitation light. It allows for the control of depth of field, elimination of information that is not in the focal plane and collection of optical serial sections from a thick specimen. To these ends, the optical path of a confocal microscope is uniquely adjusted so that only an illuminated point in the specimen is detected by the photomultiplier. This is the result of a pinhole, placed in front of the laser light source, allowing only a limited passage of beams (Figure 2.2 Lane 1), that will illuminate only a specific depth of tissue optical slice. Light emitted by the excited fluorophore at this point passes through a second pinhole in front of the detector system, which rejects stray light or glare from areas not in the focal plane (Figure 2.2 Lane 1). The beam scans along an x-y grid in order to construct an image of the entire focal plane. This process is called “optical sectioning”. The signal may be amplified by manipulating the voltage (gain) on the photomultiplier tubes (PMT), or the background signal may be removed by adjusting the threshold (offset) on the PMT and scan speed may be altered to reduce electrical ‘noise’ in the signal.

The thickness of a focal plane, or confocal detection volume, is determined by the size of the pinhole. For optimal imaging of a focal plane, the diameter of the pinhole should be the size of the central disk of the Airy diffraction pattern of the image of the illuminated point, or 1 Airy unit (Figure 2.3). The Airy unit is dependent on the excitation wavelength and the numerical aperture, or resolving power, of the objective used. When the pinhole is set to 1 Airy unit, then both specimen illumination and recording of the image by the detectors are performed according to only the size of the Airy disk.

A stepping motor is used to record sets of images of a pre-determined number of focal planes along the z-axis of a tissue sample (i.e. optical rather than mechanical sectioning). These images may be presented as a z-stack or 3D reconstruction, while individual images may be used as representative of the basal, middle and apical layers of the tissue sample.

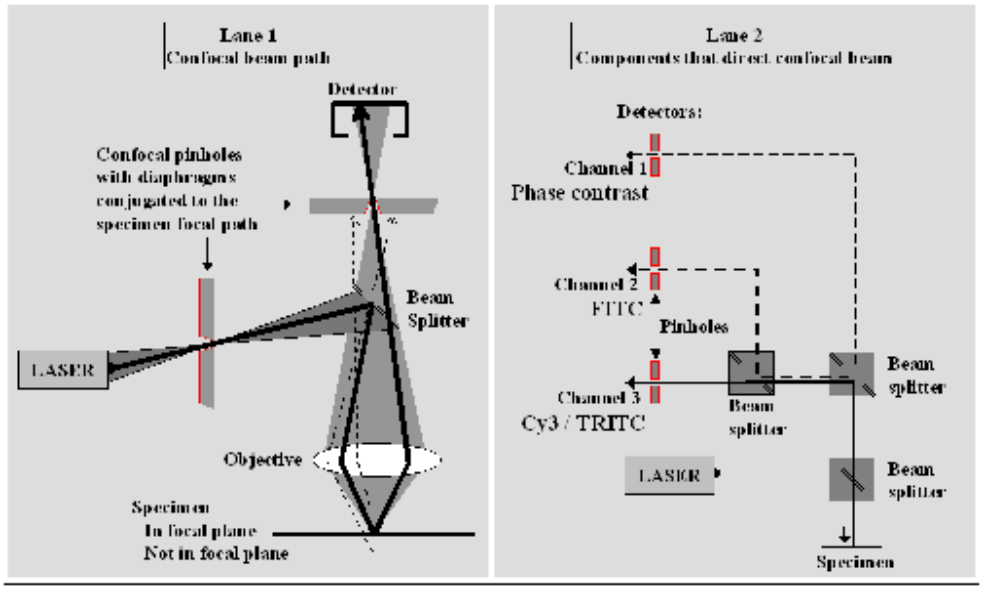


Figure 2.2. A schematic presentation of the beam path in a confocal microscope. The laser beam in a confocal microscope passes through a pinhole in front of the laser source to illuminate a single point in the specimen. A second pinhole in front of the detector system limits the recorded signal to light emitted from the probe in a narrow, focal plane. The pinholes and the specimen focal plane are conjugated to the focal path, or confocal (Lane 1). A set of mirrors and beam splitters in various focal planes direct the beams (limited bands of wavelengths) to the PMTs (Lane 2).

The beam path is directed by sets of beam splitters and mirrors. Mirrors deflect all light, while beam splitters deflect wavelengths shorter than-, and are transparent to wavelengths longer than their predetermined settings. Images from different fluorophores may be recorded due to the directing of a specific light emitted from the specimen, onto different PMTs, while mirrors will deflect all light (Figure 2.2 Lane 2). This allows all emitted light to be split into wavelength bands with set lower and upper thresholds, which may then be detected by different photomultipliers (Figure 2.2 Lane 2). During consecutive image acquisition, each fluorophore is individually excited and the emitted light recorded. Digital images of single-, double- and triple-labeled fluorescent samples may, therefore, be collected from the same focal planes throughout a specimen (Figure 2.2 Lane 2).

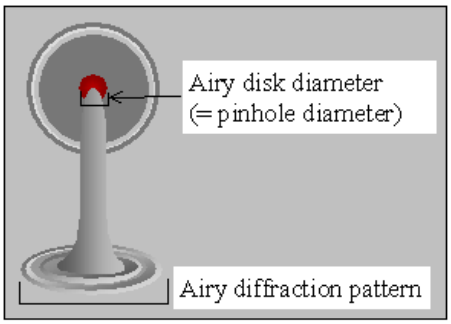


Figure 2.3 A schematic presentation of the Airy diffraction pattern and Airy disk. For optimal image acquisition, the diameter of the pinhole should be similar to the diameter of the Airy disk, which is the middle, highest intensity curve of the Airy diffraction pattern.

In fluorescence microscopy the term 'colocalization' describes the degree to which various fluorophores can be detected in the same volume. ImageJ is a public domain Java image-processing program widely used in the scientific field. Plug-ins, or macros created by other users of the software, are made available on the ImageJ website for downloading by others. The 'co-localization' plugin was used in this study for analysis image, in order to calculate relative levels of individual fluorescence or to calculate and illustrate colocalization of various probes (Section 2.7).

2.6.1. Reagents

25 nM LysoTracker Red DND-99

LysoTracker Red DND-99 (1 µl of 1 mM stock solution) was made up in sterile serum-containing medium (40 ml).

Phosphate buffered saline [8 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2O$, pH 7.4] (PBS)

$Na_2HPO_4.2H_2O$ (1.42 g) and KH_2PO_4 (0.20 g) were first dissolved in d.H_2O (200 ml). NaCl (7.99 g), KCl (0.20 g), $CaCl_2.2H_2O$ (0.15 g) and $MgCl_2.6H_2O$ (0.10 g) were added and the solution made up to 1 l with d.H_2O. The solution was filtered and stored at 4°C.

16% (m/v) PFA stock solution

(see Section 2.5.1)

3.7% (m/v) PFA in PBS, pH 7.4 (PFA fixative)

16% PFA stock solution (23.13 ml) was added to PBS, pH 7.4 (76.87 ml).
The solution was made up just before use.

1% (m/v) Bovine serum albumin in PBS, pH 7.4 (PBS-BSA)

BSA (1 g) was dissolved in PBS, pH 7.4 (100 ml) just before use.

0.1% (m/v) Saponin in PBS, pH 7.4 (PBS-saponin)

Saponin (0.1 g) was dissolved in PBS (100 ml) and filtered through Whatman No. 1 just before use.

Tris buffer (0.2 M) pH 8.5 (Tris)

Tris (0,8 g) was dissolved in d.H_20 (20 ml) and adjusted to pH 8.5.

10% (m/v) Moviol and 23 M glycerol in Tris (0.1M, pH8.5) (Moviol)

Moviol (2.4 g) was dissolved in 0.2 M Tris (12 ml) in a closed container, wrapped in foil and stirred over night. Glycerol (6 g) and d.H_20 (6 ml) was added and stirred over night. The solution was centrifuged (500 x g, 15 min) (Ono *et al.*, 2001) and 100 µl aliquots were stored in small containers at -20°C.

2.6.2. Procedure

MCF10A and MCF10AneoT cells were seeded onto sterile round 12 mm coverslips in 24 well Tersaki plates and cultured over night in DMEM-Ham's F12 medium supplemented with 10% decomplemented horse serum, as described in Section 2.2.2.

For labeling of surface antigens the cells were cooled down (21^{o}C), washed in PBS without saponin, blocked with PBS-BSA (30 min), washed in PBS without saponin, incubated in primary antibodies diluted in PBS-BSA without saponin (1 h), fixed with PFA fixative (10 min), washed in PBS with saponin (5 x 5 min) and incubated in secondary antibodies, diluted in PBS-saponin containing BSA (1% m/v).

For single intracellular immunolabeling, cells were washed with PBS (3 x 5 min), fixed with PFA fixative (10 min), washed in PBS (5 x 5 min), permeabilized in PBS-saponin (10 min), blocked with PBS-BSA (30 min), incubated in primary antibodies,

diluted in PBS-saponin containing BSA (1% m/v) and incubated in secondary antibodies, diluted in PBS-saponin containing BSA (1% m/v).

For intracellular double labeling the protocol for single labeling was repeated using the appropriate primary and secondary antibodies, diluted as described above.

For studies on acidity, subconfluent cells grown on coverslips, prepared as described in Section 2.2.2, were washed in HBSS, incubated in Lysotracker Red DND-99 (30 min), washed in PBS, fixed (10 min), permeabilized in PBS-saponin (10 min), and immunolabeled with primary and secondary antibodies as described above for single intracellular immunolabeling.

Finally, coverslips were washed in PBS (3 x 5 min), fixed (10 min), mounted with Moviol anti-fade reagent and observed on a Zeiss LSM 510 META or a Zeiss 710 confocal laser scanning microscope (Heidelberg, Germany).

In controls, the primary antibody was substituted for pre-immune serum (either rabbit or chicken), diluted to the same concentration as in the test, or primary antibody was replaced with PBS-BSA.

Phase contrast images of subconfluent MCF10A and MCF10AneoT cells, fixed and mounted for confocal microscopy as described above, were recorded using a 40 x objective on an Olympus AX 70 light microscope fitted with a F-View CCD camera and analySis software.

Single confocal images were generally captured with a scanning mode format of 1024 X 1024 pixels in line-averaging mode and a scanning speed of 6, with a pinhole setting of 1 Airy unit (AU). A series of images over the apical-basal planes of the cells (z-stacks) were similarly obtained at 0.5 μm intervals. Images were saved in a database. Images from each z-stack representing the apical, middle and basal planes were selectively exported as RGB images and for further image analysis.

2.7. Image analysis procedure

ImageJ (http://rsbweb.nih.gov/ij/) was used in this study for enhancement of phase contrast images and post acquisition processing and analysis of fluorescence

distribution and colocalization of combinations of immunolabeled proteins. The colour balance of phase contrast images were adjusted to improve contrast. For confocal images, individual areas of high gray scale values (180 to 255), representing intense fluorescence, were accepted to resemble immunolabeled vesicles. Analysis of the fluorescence signal over areas in specifically the basal plane that resembled vesicular structures was used to get a concept of the size of vesicles of interest. The size of these vesicles was measured on scaled images and represented on graphs to indicate the relevance of the vesicle size in the route of endocytic structures. Such vesicles represented the volume within which colocalization was to be determined. Colocalization referred to comparison of the extent to which two individual markers were restricted to a specific volume. Relative fluorescence intensities were calculated to compare the distribution and colocalization of cathepsins, membrane proteins and acidity indicators between the normal and cancer cell lines

Exported Red-Green-Blue (RGB) images were scaled using the magnification bars on each image and split into its red, green and blue components. The blue image component was used to normalize the background in red and green image components, which represented captured fluorescence from the two different channels. These images were converted to 256 gray scale images. Pixels with gray scale values between 80 and 256 on the gray scale were determined to represent fluorescence. The total area represented by these pixels was calculated and represented the relative amount of fluorescence in that image. Each cell was outlined and the area also calculated.

Two images representing the red and green channels were analysed using the co-localization highlighter plug-in to emphasize points that correlated in terms of their intensity ratio. Two points were considered as colocalized if their respective intensities were higher than the set lower fluorescence threshold of their channels (80) and if their intensity ratio was higher than the ratio setting value (50% by default). For ease of recognition colocalized pixels were accentuated by being displayed as white pixels (default display value = 255). The three original images were then combined in an RGB image.

Data points referring to the area of each cell analysed, the values representing area of fluorescence of the individual markers and the calculated points that referred to the

colocalization between these markers were exported into Microsoft Excell. From these data the relative distribution across the apical-basal axis of the cell, as well as the degree of colocalization between each set of markers were calculated and graphically presented.

The fluorescence signal was used to assess the size of vesicles in both the MCF10A and MCF10AneoT cells that had certain combinations of characteristics. Using the fluorescence signal of LAMP-2 and for the specific cathepsin, the size of vesicular structures and their position relative to the nucleus was noted. Measurements were done on only the basal layers, since this is where the cell is in contact with the matrix, and where degradation of most of the internalized matrix components may occur. The difference between the visual observations of almost complete colocalization between the fluorescent markers over individual structures, compared to the figures obtained through image analysis need to be explained. Due to a probable lower intensity of detected immunofluorescence along the border of a vesicle, such fluorescence / low value pixels might have been eliminated during the image capturing and/or image manipulation process. However, since equivalent microscope settings for capturing of the fluorescence signal were applied throughout, and the same gray scale values were subsequently used during image analysis, comparison of the images with its digitally analyzed results is justifiable. The efficiency of each antibody in recognition of its antigen, the use of a secondary, tagged antibody for each primary antibody as well as the extent of signal amplification for individual fluorescent markers should be remembered, especially when measuring ‘vesicle’ sizes.

CHAPTER 3.

c-Ha-*ras*(V12) alters the trafficking of cathepsin-containing vesicles

ABSTRACT

Various vesicle populations were classified according to a distinctive combination of characteristics such as luminal acidity, cathepsin content and membrane proteins such as LAMPs. These characteristics were used to monitor the effect of mutationally activated c-Ha-Ras(V12) signaling on the colocalization, localization and distribution of CB, CD and CL-labeled vesicles in the cell. c-Ha-*ras*(V12)-transfected breast epithelial cells show altered polarity and motility and mesenchymal characteristics associated with the invasive phenotype. The increased internalization and degradation of ECM products associated with Ras transfection seem to elevate the load on the intracellular degradative system. The late endosomes and storage lysosome in the degradative path have been described by similar sets of characteristics, e.g. cathepsin content, acidity and membrane proteins, leading to confusion in classification and roles. These markers have now been utilized to show that various combinations of these may illustrate the function of each type of endocytic vesicle in the overall degradative system. These vesicles could be considered as heterogeneous populations, each with a unique set of characteristics that supports its function. In this chapter we discussed the possible implications of our findings in the context of signaling by a constitutively activated c-Ha-Ras(V12) and the implication for vesicle trafficking and acidity of the degradative vesicles in premalignant cells. The mutated c-Ha-Ras(V12) oncoprotein stimulates cell proliferation and migration due to constant activation of downstream effectors that could directly, or indirectly, be responsible for changes observed in premalignant cells. Luminal acidity of degradative vesicles, LAMP-2 and cathepsins distribution is altered and a decrease in the acidity of CB, CL and CD-containing endosomes (and their association with LAMPs) and cell cytoplasm seems to have a negative effect on vesicle trafficking and capacity to degrade ECM components internalized after ECM degradation.

3.1. Introduction

The c-Ha-*ras*(V12) oncogene has been noted in up to 30% of invasive cancers (Bos, 1989) and seems to affect proliferation, cell shape and proteolytic enzyme (protease) production and distribution, factors that directly contribute to an invasive phenotype (Basolo *et al.,* 1991; Kim *et al.,* 2003; Shin *et al.,* 2005; Song *et al.,* 2006). Cathepsins have similarly been identified as proteases possibly involved in the increased invasive potential of colon, prostate and metastatic breast cancers (Couissi *et al.,* 1997; Dohchin *et al.,* 2000; Szpaderska *et al.,* 2001), due to elevated levels and potential for degradation of the ECM (Buck *et al.,* 1992; Koblinski *et al.,* 2000; Bok *et al.,* 2003; Sloane *et al.,* 2005). In addition to signaling for growth, Ras and its downstream effectors may also affect the trafficking and maturation of cathepsin-containing vesicles via an effect on PI3K, and Ras-like vesicle fusion GTPases (such

as Rab5 and Rab7 and others) (Duclos *et al.,* 2003; Grosshans *et al.,* 2006; Schwartz *et al.,* 2007). Due to these influences c-Ha-Ras(V12) activation may affect expression and trafficking of cathepsins and influence invasion, and for these reasons, formed a focus for the first investigation reported here.

Together with membrane-related proteases such as the MMPs and the plasminogen-activator system (Lund *et al.,* 1999), membrane-associated cathepsins seem to participate in normal cell migration (Buth *et al.,* 2007), e.g. after wound healing. It is also generally considered that the increased invasive activity of Ha-*ras*-related metastatic cancers is related to the movement of cathepsin-containing vesicles towards the invasive front, implying involvement in invasion and malignancy, as previously seen in the MCF10AneoT cells transfected with the mutated c-Ha-*ras*(V12) oncogene (Sloane *et al.,* 1994; Sameni *et al.,* 1995). In addition, the association of cathepsin-containing vesicles with the PM has been observed and correlated with increased invasion. Movement of proteases, if correlated with an altered phenotype, may indicate a possible role in that phenotype. CB and L, the major cathepsins previously reported to be altered by c-Ha-*ras*(V12) transfection (Sloane *et al.,* 1994; Sameni *et al.,* 1995) together with CD, seem to enhance the metastatic capacity of cancer cells (Lorenzo *et al.*, 2000). MT1-MMP, a PM-bound protease, also seems to be involved in migration and invasion (Hofmann *et al.,* 2000; Hotary *et al.,* 2006), and since these proteases are upregulated in the MCF10A cell line transfected with c-Ha-*ras*(V12) (Kim *et al.*, 2009), this cell line and these proteases form a focus. MT1-MMP has to be processed and moved from the Golgi to the PM via regulated secretory vesicles (Cao *et al.*, 1996), while the cathepsins are first trafficked from the Golgi to intracellular compartments for maturation (Section 3.3.4.1) and subsequently to storage vesicles. These processes involve the endosome-lysosome system, as described later.

3.2. Vesicle populations and trafficking

Even though various morphologically distinct endocytic vesicles have been described along the endocytic degradative route, confusing classification abounds in the literature. This hampers the investigation and understanding of observed alterations in protease distribution. Clarification of the nomenclature used in describing normal vesicular pathways and defining the nomenclature that will be used throughout this study and choice of markers for identification of such vesicles, was thus required.

This proved a challenge, however, due to the lack of well defined and conflicting terminology historically used for the various vesicle populations. Certain organelles are, however, easier to define.

One of the major difficulties that hamper the study of the endosome-lysosome degradative route is the fact that these different organelles vary only slightly in the composition of their membrane and marker components (Clague, 1998). This slight change is due to the recycling of components (Thilo *et al.,* 1995) and the addition of new ones over the life span of a vesicle (Thilo *et al.,* 1995; Mullock *et al.,* 1998; Scianimanico *et al.,* 1999; Luzio *et al.,* 2000). Endosomes, formed by inward budding of the PM and known as EEs, bring receptors and other cargo into the cell (Tjelle *et al.,* 1996). The LE is possibly the final destination of internalized cargo destined for degradation, while the lysosomal enzymes are stored in what is known in the literature as the lysosome (Luzio *et al.,* 2000). The question of whether these are all pre-existing or dynamically changing, structures is a matter of more debate. The early Palade paradigm proposed that endocytosed cargo is transferred between pre-existing structures (Gruenberg *et al.,* 1989). Recent studies tend to support this view (Griffiths, 1996). The current, more plausible, but contentious view (or a combination of both models) supports the "maturation" of cargo-carrying vesicles derived from the PM, i.e. evolution of outer membrane components via dynamic fission and fusion with the concurrent recycling of many lipid and protein components. Such events are believed to transform EEs into degradative LE structures (Clague, 1998; Eskelinen, 2006).

These processes occur under the guidance of reversible complexes of cytoplasmic proteins sequestered to the vesicle membranes (Denhart, 1996; Duclos *et al.*, 2003), with trafficking of these vesicles, along the cytoskeletal components, under the tight control of specialized motor protein complexes (Johansson *et al.,* 2007). Such membrane fluidity and the proteins involved in this process, is reviewed in Section 3.3. In spite of the fluidity of the various cellular membrane components, the degradative path seems to have a range of vesicular compartments, each of which has a combination of distinct morphological and biochemical characteristics (Gruenberg *et al.,* 1989). These characteristics include pH and acidity, vesicle size and markers such as receptors that define a specific pathway, e.g. mannose-6-phosphate receptor (MPR) and marker proteins such as the LAMPs.

3.2.1. Distinguishing characteristics of vesicles along the degradative route

Major vesicles in the endosomal or degradative route can be distinguished on the basis of their pH or the presence or absence of specific proteins such as specific proton pumps or specific LAMPs. Various non-degradative processes rely on an acidic environment for processes such as the release of ligands from their receptors in the mild acidity of the EE (Ludwig *et al.,* 1991; Mousavi *et al.,* 2001), that has a pH of 6 (Ciechanover, 2005). pH or the presence or absence of specific receptors e.g. transferrin (Tfn) receptor may be used to identify the EE or sorting endosome. Separation of precursor-carrier complexes (Cain *et al.,* 1989; Stoorvogel *et al.,* 1989) and the maturation of most newly-synthesized hydrolases (Gieselmann *et al.,* 1985) by separation from their MPR, also require an acidic environment, such as in the LE (pH ~5-5.5). On the other hand, alkalinization of the recycling vesicle that returns receptors and structural elements to the PM, has been reported (Mousavi *et al.,* 2001).

Degradation of internalized material and protein processing by acidic proteases such as cathepsins (Xiao *et al.,* 2007) is regulated by restricting this function to vesicles with variable (Mignatti *et al.,* 1993; Grabe *et al.,* 2001; Pillay *et al.,* 2002b) acidities (Ciechanover, 2005), maintained by a range of proton pumps (Cain *et al.*, 1989; Forgac, 1999; Counillon *et al.*, 2000). This process supplies the cell with nutrients (Jager *et al.,* 2004; Boya *et al.,* 2005; Demarchi *et al.,* 2006; Sato *et al.,* 2007) as well as components such as cholesterol, that support the structural integrity and fluidity of its membranes (Hoekstra *et al.,* 2000). Formation of the degradative LE and its progressive acidification to as low as 5-5.5 (Beyenbach *et al.,* 2006) ensures an environment that supports such enzymatic degradation of internalised material (Butor *et al.*, 1995; van Weert *et al.*, 1995; Eskelinen *et al.*, 2003; Ciechanover, 2005).

3.2.1.1. Vesicle acidity

Various proton pumps that adjust the vesicle luminal acidity have been associated with various endocytic vesicles (Figure 3.1 Lane 1, Figure 3.2 Lane 3 and Figure 3.3 Lane 2). These bring hydrogen (H^+) ions into endocytic vesicles against a gradient (Cain *et al.*, 1989; Forgac, 1999; Counillon *et al.*, 2000) and possibly regulate the H^+ concentration to suit the activity of specific enzymes at specific times for various functions in the compartment. Identification of relevant proton pumps that supports a

specific organelle function may assist in distinguishing some populations of endosomes.

3.2.1.2. NHE-1 proton pump

Eukaryotic cells maintain cytosolic pH close to neutrality (Orlowski *et al.,* 1997; Putney *et al.,* 2002; Cardone *et al.,* 2005). The NHE-1 proton pump on the cell membrane (Figure 3.1 Lane 1) is normally activated in response to cytosolic acidity (Putney *et al.,* 2002; Haworth *et al.,* 2003), osmotic stress and also cell spreading (Orlowski *et al.,* 1997; Counillon *et al.,* 2000). It eliminates excess acid from actively metabolizing cells to establish electroneutrality (Putney *et al.,* 2002; Haworth *et al.,* 2003; Lacroix *et al.,* 2004).

Apart from NHE-1 on the PM, the Na^+,K^+-ATPase and vacuolar- H^+-ATPase (V-ATPase) proton pumps has been described on membranes of internal vesicles (Grabe *et al.,* 2001). Subsequent endocytic or secretory vesicle acidity may be controlled by varying the activity or the number of proton ATPase units in the vesicle membrane to achieve the desired luminal pH (Anderson *et al.,* 1988; Grabe *et al.,* 2001; Sun-Wada *et al.,* 2003) (Figure 3.1 Lane 1).

3.2.1.3. Na^+,K^+-ATPase

The Na^+,K^+-ATPase is the main regulatory element of mostly the early endocytic vesicle acidity. Ligands that bind to specific cell-surface receptors are internalized together with the Na^+,K^+-ATPase, into the EE (Cain *et al.,* 1989; Cardone *et al.,* 2005) (Figure 3.1 Lane 1). The EE pH of approximately 6.0-6.5 (Cain *et al.*, 1989; Grabe *et al.*, 2001; Pillay *et al.*, 2002b) is sufficient to allow dissociation of ligands such as Tfn from its receptor and the release of iron from Tfn (Cain *et al.,* 1989). The pH in EEs is balanced by the interplay between the proton transfer by the proton pumps and the movement of another charged species through the Na^+,K^+-ATPase ion channel and transporters that maintain an interior-positive membrane potential (Beyenbach *et al.,* 2006). This is further affected by the cytoplasmic pH, that is affected by the NHE-1 pump on the PM (Counillon *et al.,* 2000). Removal of the Na^+,K^+-ATPase (by recycling or degradation) relieves the membrane potential and permits effective further acidification by a H^+-type proton pump to pH 5, a luminal acidity characteristic of maturing vesicles (Grabe *et al.,* 2001; Beyenbach *et al.,* 2006).

3.2.1.4. Vacuolar type H^+-ATPase (V-ATPases)

The V-ATPases are a family of ATP-dependent proton pumps modelled on the yeast V-ATPase (Kane, 2006). These proton pumps are present in membranes of most intracellular structures such as lysosomes, EEs and LEs, the Golgi apparatus, chromaffin granules and coated vesicles. V-ATPases transport ions across membranes through the hydrolysis of ATP (Ciechanover, 2005) to maintain an acidic milieu (Sun-Wada *et al.*, 2003; Ciechanover, 2005; Beyenbach *et al.*, 2006; Kane, 2006), a prerequisite for the degradative activity of lysosomal acidic hydrolases (Barile *et al.,* 1990; Kirschke *et al.,* 1998; Schmid *et al.,* 1999). They also pump protons across the PMs of specialized cells including osteoclasts and epithelial cells in kidneys and acrosomes on sperm (Sun-Wada *et al.,* 2004; Beyenbach *et al.,* 2006).

In the LE vesicles the V-ATPase seems to be solely responsible for creating the acidic environment (Grabe *et al.,* 2001) (Figure 3.1 Lane 1). Since it is limited to a maximum pH difference of 4.5 units (Beyenbach *et al.,* 2006), calcium and chloride channels alongside the V-ATPase collaborate to create a very negative membrane potential that helps to further drive protons into the lumen and keep acidity to an appropriate and distinct level (Beyenbach *et al.,* 2006).

The V-ATPase is a multi-subunit complex (Orlowski *et al.,* 1997) (Figure 3.1 Lane 2 and Table 3.1) that is composed of two main individual domains. The V0-domain is an integral membrane protein found on most endocytic vesicle membranes, that is composed of six different subunits and is responsible for proton translocation across the membrane (Forgac, 1999; Grabe *et al.*, 2001; Beyenbach *et al.*, 2006; Kane, 2006). The complimentary V1 domain is a catalytic soluble complex that consists of eight different subunits (A–H) (Figure 3.1 Lane 2). It is associated with the cytoskeletal framework via its C-component and contains the catalytic site responsible for ATP hydrolysis (Kane, 2006).

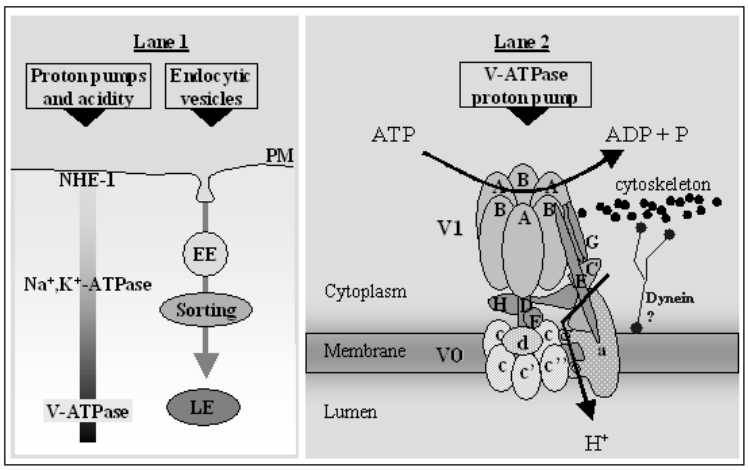


Figure 3.1. Proton pumps that modify luminal pH along the endocytic route and strudture of the V-ATPase proton pump.
(Lane 1) The NHE-1 on the PM modulates cytoplasmic pH, while the Na^{+}-K^{+} proton pump maintains a slight acidity in the EE. (Lane 2) The assembled V-ATPase proton pump consists of the cytoplasmic/cytoskeletal V1 ATPase domains, the integral membrane-bound V0 proton translator that ensures structural integrity during the rotary action and the C molecule that ensures binding between the V0 and V1 domains. This schematic diagram (Lane 2) was adapted from various sources (Inoue *et al.,* 2005; Beyenbach *et al.,* 2006; Xiao *et al.,* 2007). (PM = plasma membrane)

In a 'resting' state the V0 integral proton transporting domain of the V-ATPase is not assembled with the V1 ATPase domain, that is independently located on the cytoskeletal framework via its C-component (Sumner *et al.*, 1995; Beyenbach *et al.*, 2006; Kane, 2006). This implies that no proton transfer can take place, limiting the overall level of luminal acidification. During such disassembly the active sites are blocked by the H- and a-subunits on V1 and V0 sectors respectively (Kane, 2006). On activation of the proton pump, the V1 component connects to V0 via several components of a stalk region (Beyenbach *et al.*, 2006; Kane, 2006; Ohira *et al.*, 2006; Xiao *et al.*, 2007). Assembled V-ATPases operate by a rotational mechanism that involves V0 and V1 that turn in relation to one another, a motion driven by ATP hydrolysis (Qi *et al.*, ; Forgac, 2000). In this dynamic equilibrium subunit C plays a central role by forming a link between the subunits E and G of the V1 domain and subunit a of the V0 domain (Forgac, 2000; Iwata *et al.*, 2004; Inoue *et al.*, 2005) to form an assembled functional unit (Figure 3.1) and initiate proton translocation (Landolt-Marticorena *et al.,* 1999; Inoue *et al.,* 2005). This unique, reversible dissociation regulate activity of vacuolar acidification (Forgac, 1999; Inoue *et al.*, 2005; Kane, 2006). V-ATPase proton pump inhibitors (van Weert *et al.,* 1995; van Weert *et al.,* 2000; Alwan *et al.,* 2003; Xiao *et al.,* 2007), such as the antibiotics bafilomycin A1 (BafA1) and concanamycin A (ConcA), interfere with the proton

translocation system (Zhang *et al.*, 1994; Forgac, 1999) and induces alkalinization of specifically the degradative vesicles.

Table 3.1. Functions of various V1 and V0 subunits of the V-ATPase proton pump.

V-ATPase subunit	**Function**	**Reference**
V0 subunit a	Dictates cell-specific expression during development, transports protons across the membrane and blocks the active site when V1 and V0 are disassembled.	(Oka *et al.*, 2001; Beyenbach *et al.*, 2006; Kane, 2006)
V0 subunits c	Assists in rotary motion during proton translocation.	(Beyenbach *et al.,* 2006)
V1 subunits A and B	Both participate in nucleotide binding, with the catalytic site located on the A subunit.	(Forgac, 1999; Crider *et al.*, 2003; Kane, 2006)
V1 subunit B	Contain binding sites for F-actin on the amino-terminal domains of both isoforms of the B subunit, and likely are responsible for the interaction between V-ATPase and actin filaments in vivo.	(Holliday *et al.,* 2000)
V1 Subunit C	Acts as an anchor protein that regulates the linkage between V-ATPase V1 and the actin-based cytoskeleton.	(Vitavska *et al.,* 2003; Iwata *et al.,* 2004)
V1 subunits D, E and G	V1 stalk units that link V1 to V0 into an assembled, active pump.	(Kane, 2006; Ohira *et al.*, 2006)
V1 subunit E	Associates with SOS, a Ras GEF (Ras activator). E1 isoform localizes to acrosomes acidic structures found in testicular tissue.	(Sun-Wada *et al.,* 2003).
V1 subunit H	Involved in assembly of V1 and V0 domains. Blocks active site on subunit A when V1 and V0 are disassembled.	(Crider *et al.,* 2003) (Kane, 2006)

Activity of the V-ATPase proton pump relies on correct assembly of the integral membrane and cytoskeleton-associated units. Any disruption of, or changes in the actin cytoskeleton, therefore, may affect the possible association of these subunits and hence may alter vesicle acidification. Cytoplasmic alkalinization, induced by an overactive NHE-1 pump on the cell surface, may be a root cause of such a phenomenon (Reshkin *et al.*, 2000b). These are factors that may play important roles in the control of activity of the V-ATPase pump, and will later be considered in the light of c-Ha-*ras*(V12) transfection.

3.2.1.5. LysoTracker Red DND-99 as an indicator of organelle acidity

A variety of weakly basic amines have been used to study and distinguish low pH compartments (Anderson *et al.*, 1988; Cain *et al.*, 1989; Niemann *et al.*, 2000; van Weert *et al.*, 2000). At neutral pH these weak bases are only partially protonated, can diffuse through hydrophobic membranes in this state and become protonated and trapped due to their positive charge in vesicles with a high proton concentration (acidotropic). Due to its selective accumulation in such low pH cellular compartments, it may give an indication of the acidity of these compartment (Anderson *et al.*, 1988).

Common base models such as (2,4-dinitroanilino)-3'-amino-*N*-methyldipropylamine (DAMP) (Niemann *et al.*, 2000) or acridine orange, have been used. Since DAMP is not flourescent, it has to be visualized with tagged antibodies to a 2,4-dinitrophenyl (DNP) group contained in the base. Often, swelling of these structures have been reported, possibly due to osmotic effects (Cain *et al.*, 1989; Niemann *et al.*, 2000). It may also be due to activity of the V-ATPase proton pump (Papini *et al.*, 1996), which continually tries to decrease the pH (van Weert *et al.*, 2000). The high concentrations of DAMP required for successful immunolabeling may, thus, affect the morphology, acidity and function of these structures.

A range of LysoTracker probes are available that also consist of a weak base, but that is linked to any one from a range of fluorophores and may, therefore, be used at nanomolar concentrations, according to instructions from the supplier. From this range LysoTracker Red DND-99, with an absorption maximum of 577 nm and a fluorescence emission maximum of 590 nm was selected for use in the current study, since it could be used together with a green fluorescing probe in a double labeling protocol. It is often used in live cell studies (Bucci *et al.*, 2000; Sameni *et al.*, 2000), without apparent harmful effects on the cell, but it is also well-retained after aldehyde fixation (Mukhopadhyay *et al.*, 2007). Concentration of fluorescence in vesicles may, therefore, be an indication of an acidic lumen, without any negative influences on the function or structure of the vesicle.

While the range of acidity within which the pH of LysoTracker Red DND-99-containing vesicles fall, cannot be accurately assessed, a vesicle with intense

fluorescence may be considered as having a low luminal pH. Comparative alterations in vesicle luminal acidity may be determined by decreased accumulation of LysoTracker Red DND-99 (Bucci et al., 2000). In this study references such as the altered distribution and the size of acidic vesicles, was used to assess the impact of c-Ha-*ras*(V12) transfection on MCF10A breast epithelial cells.

3.2.2. Membrane-bound proteins in limiting membranes

Retention of the degradative acidic environment to dedicated vesicles is the function of several types of integral membrane-bound proteins found in the limiting membranes of LEs and lysosomes (Ogata *et al.*, 1994; Eskelinen, 2006). Lysosome-associated membrane proteins (LAMPs), together with lysosome integral membrane proteins (LIMPs) (Granger *et al.,* 1990; Ogata *et al.,* 1994) and other lysosomal glycoproteins (lgp) (Granger *et al.,* 1990; Harter *et al.,* 1992), form a barrier that assists in the isolation of mature lysosomal enzymes into an acidic environment suitable for digestive activity, while preventing leakage of lysosomal enzymes into the cytoplasm (Granger *et al.,* 1990; Kundra *et al.,* 1999). These proteins have intraluminal domains that are extensively glycosylated, protecting the core structure of the protein from degradation by its digestive environment (Fukuda, 1991). The sialic acid residues on the glycosylated side chains of the core LAMP, LIMP and lgp proteins may also assist in maintaining the acidity of the degradative vesicle lumen (Granger *et al.,* 1990; Eskelinen *et al.,* 2003). These residues make e.g. the mature lgp110 protein extremely acidic, with an isoelectric point of between 2 and 4 (Granger *et al.,* 1990).

3.2.2.1. LAMPs

LAMPs represent more than 50% of the total membrane components of lysosomes (Eskelinen, 2006). Various isoforms, i.e. LAMP-1, LAMP-2 (Furuta *et al.*, 1999; Eskelinen, 2006) and LAMP-3 (Astarie-Dequeker *et al.,* 2002) are known, each with its own function and controlling conditions.

In antigen presenting cells LAMP-3/CD63 may play a role in initial antigen uptake (Salik *et al.,* 1999), in the secretory / recycling pathways to the PM (Peden *et al.*, 2004a), and may partner with integrins on the membrane to establish polarity (Yunta *et al.,* 2003). Therefore, low levels of LAMP-3 may possibly lead to loss of cellular polarization (Jung *et al.,* 2006) and increase migration in cancer (Kanao *et al.,* 2005),

an interesting, but unanticipated role. LAMP-3 (Ayala *et al.,* 1998; Astarie-Dequeker *et al.,* 1999) as well as LAMP-1 and -2 (Huynh *et al.,* 2007) assist in the maturation of phagosomal vesicles and processing of the internalized pathogenic microorganisms.

LAMP-2 (but not LAMP-l) (Eskelinen *et al.,* 2004; Höltta-Vuori *et al.,* 2005) is involved in vesicle-mediated transfer of esterified cholesterol from the degradative compartment to the PM (Eskelinen *et al.,* 2004). In Niemann-Pick C disease (NPC) (Neufeld *et al.,* 1999) a mutated NPC lipid-binding protein that should escort low density lipoprotein (LDL) into a digestive site, results in high levels of unestrified cholesterol in the LE (Willenborg *et al.,* 2005). Unesterified cholesterol in the LE of these patients stabilizes Rab9, a GTPase protein that controls retrograde trafficking of MPR, the lysosomal enzyme receptor (Hirst *et al.,* 1998; Barbero *et al.,* 2002; Ganley *et al.,* 2004; Aivazian *et al.,* 2006; Ganley *et al.,* 2006) (Section 3.3.3.1 and Figure 3.3 Lane 4). The receptor can, therefore, not recycle to the TGN and is degraded (Ganley *et al.,* 2006). This may lead to a shortage of lysosomal enzymes in degradative organelles, since the precursor lysosomal enzymes may be secreted from the Golgi, due to a lack of available MPR.

LAMPs also have a function during autophagy (cytoplasmic degradation) or phagosome formation, or endocytosis of large particles. Lack of LAMP-1 or LAMP-2 could reduce phagosome fusion with lysosomes, and impair formation of a degradative vesicle. Through recognition by its cytoplasmic binding site (Section 3.2.2.2) LAMP-2 in the limiting membrane of the degradative vesicle binds unfolded cytoplasmic substrate proteins under the influence of an autophagy-related chaperone protein, heat shock cognate protein 70 (Hsc70) (Eskelinen, 2006; Kaushik *et al.*, 2006; Massey *et al.*, 2006). Subsequently, these LAMP-2 proteins are clustered under the influence of heat shock protein 90 (Hsp90) in the membrane of the endosomal structure, with subsequent internalization of these substrate proteins into the vesicle lumen (Bandyopadhyay *et al.,* 2008) for degradation (Massey *et al.,* 2006). This function of LAMP-2 is increased during starvation (Jager *et al.,* 2004; González-Polo *et al.,* 2005), when LAMP-2 may also assist in the fusion of lysosomes with autophagic vesicles (Jager *et al.,* 2004; González-Polo *et al.,* 2005) to contribute to the supply of nutrients to the cell (Klionsky *et al.,* 2000). In Danon disease defective LAMP-2 interferes with autophagy, causing accumulation of unprocessed lipids in

muscle and brain (Eskelinen, 2006). This leads to starvation and cell death (Klionsky *et al.*, 2000; Nishino *et al.*, 2000; Eskelinen *et al.*, 2003; Eskelinen, 2006).

Similarly, in lymphoblasts LAMP-2 influences transport of arginine into the LE, a process that may also lower the pH of these organelles (Ramirez-Montealegre *et al.,* 2005). This is confirmed by conditions of LAMP-2 deficiency, such as Batten disease (González-Polo *et al.,* 2005), in which lack of arginine transport results in higher lysosomal pH (Holopainen *et al.,* 2001).

In addition to their protective role LAMPs could affect vesicle acidity and be pilot proteins, delivering/guiding phagosomes and macromolecules to the LE (Jager *et al.*, 2004; Huynh, 2007). As a result, it seems to play a wide range of functions that are all involved in the function of degradative sites.

LAMP-2 has been used as a marker for the main degradative vesicle, the LE (Nakamura *et al.,* 2000; Furuta *et al.,* 2001), a large, acidic degradative structure (Granger *et al.*, 1990; Fukuda, 1991) (Section 3.3.3) and will be used in this study to distinguish such a structure. A LAMP-2-containing structure with similar morphology has, however, mistakenly been called the lysosome (Eskelinen *et al.,* 2002), while the lysosome, originally described by de Duve (1955) as a small electron-dense structure, has also been described as a carrier or storage vesicle for active hydrolases, suggesting that it is not a site of active enzyme activity (Griffiths, 1996). While it is not impossible that LAMP-2 proteins could be incorporated into all these structures, in this study LAMP-2 will specifically be used as a LE vesicle marker.

3.2.2.2. LAMP-2 cytoplasmic signal sequence in directed vesicle trafficking

The cytoplasmic tails of integral membrane proteins such as LAMPs (Fukuda, 1991), MPRs (Ghosh *et al.,* 2004), lgp120 (Harter *et al.,* 1992) and epidermal growth factor receptors (EGFRs) (Authier *et al.,* 1999a) contain specialized tyrosine residues (Fukuda, 1991). These signal sequences are responsible for the diversity of trafficking of these molecules. Evidence of the role of LAMPs in directed vesicle trafficking is provided in Neisseria infections, where the Neisseria pathogen destroys the intraluminal hinge region of LAMP-1 (Ayala *et al.,* 1998), indirectly affecting the trafficking directed via the cytoplasmic tail recognition motif. In this way it escapes

fusion of the phagosome with lysosomes, and thus destruction. Sorting that relates to signaling domains occurs during clathrin-dependent sorting and endocytosis into transport vesicles, at donor structures such as the PM or TGN. These recognition domains are also important during subsequent delivery of these vesicles to target organelles (Fukuda, 1991) along the endocytic and secretory pathways (Ohno *et al.,* 1995). Lysosomal acid phosphatase (LAP) and LAMP-2 is trafficked to large, acidic degradative structures (Granger *et al.*, 1990; Fukuda, 1991), while LAMP-1 may be associated with cathepsin-containing vesicles that migrate to the PM during wound healing. Since the release of acidic content at the wound edge could be detrimental to the tissue, and it is, therefore, considered that LAMP-1 is possibly involved in targeting secretory vesicles with a higher luminal pH (Furuta *et al.,* 2001). In this study lysosomal enzyme-containing vesicles that are non-acidic (i.e. LysoTracker Red DND-99 negative) will, therefore, be considered as possibly containing LAMP-1. In comparison, LAMP-2 labeled structures would be anticipated to be acidic LEs (LysoTracker Red DND-99 positive).

During this study the association between the membrane-bound LAMP-2 and the size, luminal acidity and the type of cathepsin present in endocytic vesicle populations was investigated in order to establish sets of distinguishing features of immunolabeled vesicles. Such information may assist in establishing the position of a vesicle on the endocytic or secretory grid and further to understand the effect that c-Ha-Ras(V12) signaling may have on vesicle trafficking in cancer cells.

3.3. Vesicle formation and classification

Through pinocytosis (or pinching off of small vesicles containing liquid) and receptor-mediated endocytosis (receptors and ligands), fluids or external macromolecules respectively, are internalized into the cell (Tardy *et al.,* 2006). Through phagocytosis, on the other hand, large molecules such as bacteria, are ingested from the extracellular environment when the PM extends around and engulfs the foreign particle (May *et al.,* 2001). Since phagocytosis is not relevant to cancer, it will not be further described.

3.3.1. The early endosome

During endocytosis material is initially concentrated in specialized areas of the PM (Keyel *et al.,* 2006), which is distorted to form a reversible (Sachse *et al.,* 2002)

inward curvature into the cytoplasm of the cell under the influence of clathrin (Janvier *et al.*, 2005). These proteins coat and deform a focused area on the membrane (Haucke, 2006). The neck of the bud is then restricted by dynamin, a GTPase, to form small, internalized coated vesicles (Sachse *et al.*, 2002; Heuser, 2004). Similar processes of cargo selection (Barois *et al.*, 2005) and vesicle formation are known to occur at other donor membranes, such as the ER and trans Golgi network (TGN) (Karlsson *et al.*, 1998) and may be involved in isolation of synthesized cargo, such as degradative enzymes, and subsequent trafficking to relevant destinations throughout the cell (Ghosh *et al.,* 2003), or in the retrograde trafficking between e.g. the LE and Golgi (Nicoziani *et al.*, 2000).

The EE is the first membrane-bound structure that forms within about 5 min after endocytosis (Gruenberg *et al.,* 1989) (Figure 3.2 Lane 3). It is often characterised by the presence of the Tfn receptor and ligand, and a minimum luminal pH of about 6.0-6.5 (Cain *et al.*, 1989; Grabe *et al.*, 2001; Pillay *et al.*, 2002b) that is managed by the Na^+,K^+-ATPase proton pump (Section 3.2.1.3). After loss of the initial clathrin coat, and dissociation from the ligand in the EE, most receptors are recycled via recycling endosomes that pinch off and return to the PM (Figure 3.2 Lane 3). This occurs while cargo to be digested is sorted (Sachse *et al.,* 2002) in sorting, or early carrier vesicles (ECVs) (Figure 3.2 Lane 3, sorting) (Griffiths, 1996) through homotypic fusion and fission vesicle interactions, termed 'kiss and run' (Desjardins, 1995; Duclos *et al.*, 2003; Kalia *et al.*, 2006) (Figure 3.2 Lane 2). The actin cytoskeleton controls the distribution of these EEs (Gruenberg *et al.,* 1989; Kaksonen *et al.,* 2003) along the perimeter of the cell (Gruenberg *et al.,* 1989; Ludwig *et al.,* 1991; Tjelle *et al.,* 1996; Aschenbrenner *et al.,* 2004) and possibly the final stages of secretory vesicle fusion with the PM (Blott *et al.,* 2002) (Figure 3.2 Lane 1).

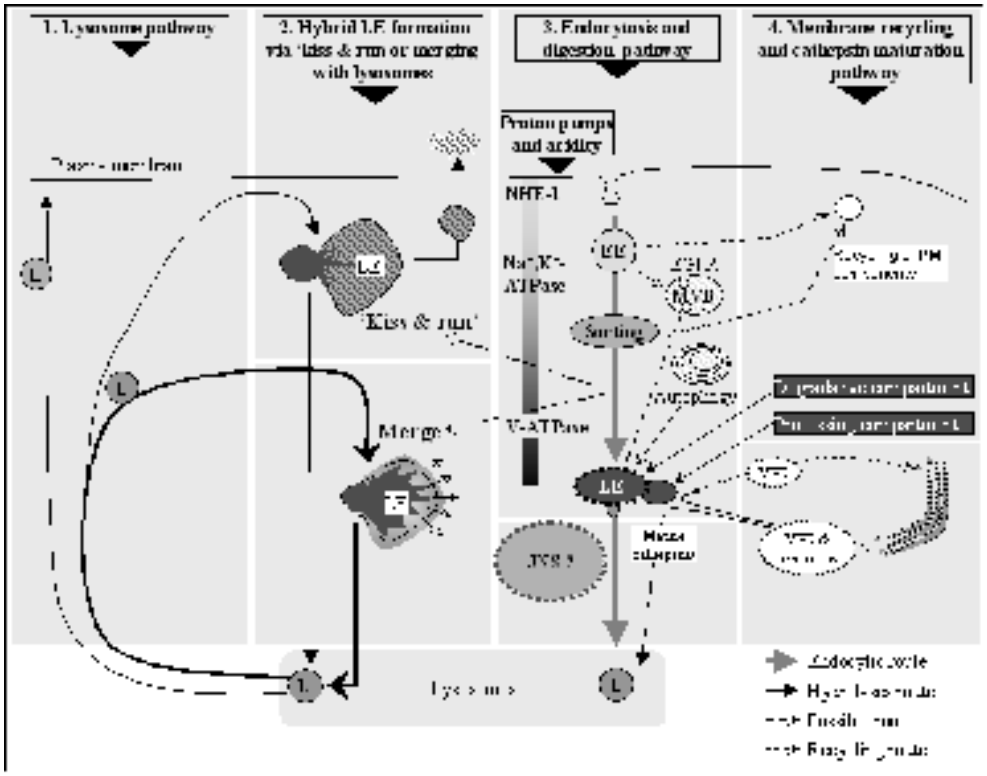


Figure 3.2. A schematic presentation of the various vesicle types in the degradative pathway, to which reference will be made in this study.

The EE and sorting vesicles are the dynamic early endocytic vesicles, and the LE is the hybrid, degradative vesicle (Lane 3). A main digestive body (JNS) is included in this illustration as a separate, stable compartment, but could form part of an extensive interconnected LE compartment. The MVB and multilamellar bodies are indicated but their positions and functions have not yet been clarified (Lane 3). Two suggested vesicle integration pathways via which the LE is formed, is 'kiss and run' or merging between the lysosome and cargo-carrying EE (Lane 2). The various stations along the degradative route, form due partly to vesicle maturation involving recycling of PM components (Lane 4). The lysosome may carry mature hydrolytic enzymes towards the incoming cargo. This structure may reform after completion of digestion (Lane 1). The various proton pumps that are involved in acidification of the various compartments are also illustrated, while a graded scale bar indicates known decrease in vesicle pH. Dotted vesicle outlines indicate possible LAMPs in the limiting vesicle membranes (Lane 3). Precursor lysosomal enzymes are carried from the Golgi to the processing compartment of the LE (Lane 4), where these are released from the mannose-6-phosphate receptor (MPR), and subsequently mature. This diagram was generated from references mentioned in Section 3.2 and Section 3.3.

3.3.2. The multi-vesicular body and autophagy

Another organelle in this pathway is the MVB, a vesicle that invaginates its limiting membrane to give rise to its characteristic intraluminal smaller vesicle content (Duclos *et al.*, 2003; Razi *et al.*, 2006). Such inward budding may be controlled by the activity of membrane binding proteins, the annexins (White *et al.*, 2006; Futter *et al.*, 2007). These vesicles may contain cholesterol and low levels of lysosomal enzymes (Möbius *et al.,* 2003). The MVB is reported to be responsible for either further cargo sorting or downregulating signaling of certain receptors targeted for degradation by collection in inwardly budding domains of the limiting membrane.

During the degradation of EGFR, for example, such inward budding of the EE membrane (Sachse *et al.,* 2002; White *et al.,* 2006) serves to isolate the cytoplasmic signaling domain and terminate cytoplasmic signaling (Haugh *et al.,* 1999) giving rise to multivesicular bodies.

Via the process of autophagy (Cuervo, 2004; Levine *et al.*, 2005; Juhasz *et al.*, 2006), several proteins and components may be brought into the degradative systems via inward vesiculation of the limiting membrane of endosomal vesicles (Klionsky *et al.*, 2000; Cuervo, 2004; Demarchi *et al.*, 2006; Juhasz *et al.*, 2006). These merge with the LE for degradation (Bampton *et al.,* 2005), known as microautophagy (Cuervo, 2004). Light chain 3-II (LC3-II) is an associated, ubiquitin-like marker protein that represents the first known protein covalently attached to the autophagic membranes and seem to be involved in such a process (Bampton *et al.,* 2005; Juhasz *et al.,* 2006). Macroautophagy, on the other hand, occurs to remove larger, damaged organelles such as mitochondria for digestion, by envelopment in membranes derived from the endoplasmic reticulum (Cuervo, 2004; Levine *et al.*, 2005; Cao *et al.*, 2006; Juhasz *et al.*, 2006).

Microautophagic vesicles morphologically resemble enlarged late endosomal structures, containing multiple vesicles with limiting membranes, forming MVBs (Koike *et al.,* 2005; Levine *et al.,* 2005; Demarchi *et al.,* 2006; Juhasz *et al.,* 2006). It is possible that gold colloid, observed in these structures after multiple chases (Bright *et al.,* 1997), is regarded by the cell as an indigestible component that remains after the BSA coating has been completely removed, by digestion. If such a gold-containing lysosome were to be targeted for digestion via autophagy, this could explain the appearance of membrane-bound gold colloid in MVB-like-organelles (Bright *et al.,* 1997).

Under nutrient-rich conditions autophagy is suppressed (Klionsky *et al.,* 2000), but could be enhanced during starvation (Jager *et al.,* 2004), to generate metabolites for cellular survival (Eskelinen *et al.,* 2002). Starvation caused by a lack of proteolytic enzymes and the resulting defective degradation could also induce autophagy (Koike *et al.,* 2005) .

Experimental restriction of microautophagy (through inhibition of the PI3K family that includes LC3) and pathological disruption of microautophagy, such as in Batten disease (Cao *et al.,* 2006) or Danon disease (Klionsky *et al.,* 2000; Nishino *et al.,* 2000), leads to cell death. Uncontrolled autophagy, however, has been reported in neurodegenerative diseases including Parkinson and Alzheimer diseases (Levine *et al.,* 2005). Cancer cells may have an increased metabolic rate (Weber *et al.*, 1971; Partin *et al.*, 1989; Mazurek *et al.*, 1997) and it has been suggested that cancer cells may exploit autophagy (Cuervo, 2004) to adapt to hypoxic and nutrient-limiting environments (Sato *et al.,* 2007).

When studying the various organelles normally present, therefore, it is important not to induce the formation of autophagic bodies by starvation, or to be aware that some vesicles present could be autophagic in nature.

3.3.3. The late endosome

The next vesicle along the degradative route is the LE (Figure 3.2 Lane 3 and Figure 3.3 Lane 3). This has multiple roles and is responsible for degradation of cargo molecules and has been identified as a processing site for lysosomal proenzymes, due to the presence of the MPR (Kornfeld, 1986; Gruenberg *et al.*, 1989; Ludwig *et al.*, 1991; Griffiths, 1996; Tjelle *et al.*, 1996). This receptor delivers mannose-6-phosphate (M6P)-labeled precursor lysosomal enzymes from the TGN to the acidic processing LE for maturation before storage in the lysosome.

In order to define the possible connection between cathepsin protease secretion and the Ha-Ras(V12) mutations found in invasive cancer, and vesicle trafficking, it is important to look now at the biosynthetic pathway and activity of cathepsins, the enzymes reported to influence invasive capacity of cancer cells (Couissi *et al.,* 1997; Dohchin *et al.,* 2000; Szpaderska *et al.,* 2001).

3.3.4. The cathepsins

Cathepsins, from the Greek word kathepsin (to digest), represent the main class of lysosomal acidic proteases (Kirschke *et al.,* 1998). This group of proteases, structurally related to papain, is subdivided into three subgroups according to the amino acid residues in their active site responsible for their catalytic activity: cysteine (cathepsins B, C, F, H, K, L, N, O, S, T, U, W and X), aspartic (CD and CE) and

serine cathepsins (CA and CG) (Tardy *et al.,* 2006). The cathepsins were originally reported to be contained in the lysosome (de Duve *et al.,* 1955), but are also found in late endosomes (Nakamura *et al.,* 2000). Here they are key enzymes responsible for normal intracellular degradation and turnover of proteins endocytosed or removed from circulation via autophagy (Wang *et al.*, 1988; Shuja S *et al.*, 1996; Brix, 2005). The end products are amino acids and dipeptides, which diffuse through the vesicle membrane and are available again for protein synthesis in the cell (Storer *et al.,* 1994). Since all endocytic vesicles involved in cathepsin-related degradation are under the control of the vesicle-forming proteins such as adaptor proteins (APs) and Rabs (Section 3.3.6), the influence of these components on the biosynthetic route and sites of activity of the cathepsins will be discussed in the following sections.

Even though CB, CD and CL are all categorized as acidic proteases, it seems that their processing from precursor to mature forms occurs at different pHs and their trafficking may occur via different mannose-6-phosphate receptors (MPRs) and they may possibly have different final destinations. After synthesis on the ER and export from the TGN, the latent 45 kDa CB precursor is processed in a slight acidic pH of 5.5-6 into a mature 33 kDa protease, after the removal of the inhibitory propeptide region (Schmid *et al.,* 1999). The 53-kDa CD proenzyme is processed at a pH of 5.5, (Gieselmann *et al.*, 1985) in a processing compartment, that, judging by the pH given, is possibly a LE. Here the propeptide is removed to form a 47-kD intermediate, that is subsequently autocatalytically activated at a pH as low as 3.7 (Wittlin *et al.,* 1999) to form the mature 31-kD enzyme (Davidson, 1995; Schwartz, 1995; Wittlin *et al.*, 1999). Mature CD is subsequently trafficked to the lysosome. Unlike other cathepsins, proCL does not bind effectively to the regular carrier receptor and is secreted, rather than trafficked to the lysosome (Lazzarino *et al.*, 1990; Yeyeodua *et al.*, 2000). ProCL (38 kDa) may autocatalytically process itself to a mature form of 28 kDa (Yeyeodua *et al.*, 2000) at a pH of about 5.5 (Menard *et al.*, 1998), or may be processed by CD or extracellular matrix metalloproteases (MMPs) (Kirschke *et al.*, 1998).

While each of the cathepsins have optimal activity at a characteristically acidic pH, many of them can function at neutral pH, albeit with a decreased stability and/or altered specificity (Pillay *et al.,* 2002b; Turk *et al.,* 2002; Brix *et al.,* 2008). Intracellular CB acts in an acidic environment (Musil *et al.*, 1991; Authier *et al.*,

2005) (pH of 4.5-5.5) to break down macromolecules for cell survival (Felbor *et al.,* 2001), but is also significantly stable at physiological pH (Dehrmann *et al.*, 1995). It degrades EGF that is complexed to EGFR as a control mechanism during growth stimulation (Authier *et al.,* 1999a; Authier *et al.,* 1999b). When secreted, or PM associated, CB may be involved in cell migration, e.g. during wound closure (Buth *et al.*, 2007). In relation to other intracellular proteases CB was shown to be involved in the processing of CL (Felbor *et al.,* 2001) and of CD (Gieselmann *et al.,* 1985).

CL is stable at a physiological pH range between 6.8 and 7.2 (Dehrmann *et al.*, 1995). Intracellular CL plays a role in EGF and collagen degradation (Reinheckel *et al.,* 2005) and contributes to cell cycle regulation, when it is involved in proteolytic processing in the neutral environment of the nucleus (Goulet *et al.,* 2004). Secreted CL is important in bone resorption (Katunuma *et al.*, 1998) and is a gelatinase, maintaining e.g. periodontal and myocardial tissue (Nishimura *et al.*, 2002). Deficiencies in cellular CL result in interstitial fibrosis (Nishimura *et al.*, 2002; Petermann *et al.*, 2006), abnormal endosomal ultrastructure and insufficient cellular and mitochondrial catabolism and oxidative phosphorylation (Petermann *et al.*, 2006).

CD has optimal catalytic activity at pH 2.8-5.0, and needs an acidic environment to function optimally (Capony *et al.,* 1994; Pillay *et al.,* 2002b). CD is, therefore, often used as marker for this high grade degradative compartment, (Gieselmann *et al.*, 1985; Davidson, 1995; Fusek *et al.*, 2005). Most ingested proteins are degraded by cysteine proteinases (e.g. CB and CL) in the less acidic early endosomal vesicles, after which CD may be involved only in final degradation in the most acid vesicles. CD also may degrade other cathepsins like CB and CL (Linebaugh *et al.*, 1999; Turk *et al.*, 1999) that may have been inactivated in the acidic environment of the degradative vesicles (Pillay *et al.,* 2002b).

Some cells such as macrophages, fibroblasts and transformed cells may secrete the precursors of the enzymes for extracellular activation. Such proteolysis outside the lysosomes is regulated by physiological pH (Dehrmann *et al.*, 1995) and natural inhibitors (Storer *et al.,* 1994). An alteration of this balance in favour of enzyme activity may lead to uncontrolled protein breakdown (Dehrmann *et al.*, 1995), as seen in several disorders (Koike *et al.*, 2005). Secreted CD could play a negative role since it stimulates high-density cancer cell growth by inactivating secreted growth

inhibitors (Liaudet *et al.*, 1995). Pathologic degradation by CL may lead to osteo- and rheumatoid arthritis such as inflammation, arthritis (Maciewicz *et al.,* 1990) and tumour growth (Storer *et al.,* 1994; Lah *et al.,* 2000). Invasive malignancies that excessively degrade their ECM (Sloane *et al.*, 2005; Mohamed *et al.*, 2006) have an elevated requirement for intracellular degradation (Mignatti *et al.*, 1993; Dohchin *et al.*, 2000). Under normal and pathological conditions proteolytically degraded collagen from the ECM is internalised (Baricos *et al.,* 1988; Barile *et al.,* 1990; Katunuma *et al.,* 1998) and digested by CB (Arora *et al.,* 2000), CL (Nishimura *et al.,* 2002) and CD (Montcourrier *et al.,* 1990).

3.3.4.1. Cathepsins biosynthesis

In general, newly synthesized lysosomal enzymes are trafficked to the lysosome, a storage vesicle, after the activating or processing in a processing compartment (Figure 3.2 Lane 4 and Figure 3.3 Lane 4). Understanding this path in the normal cell is important in order to distinguish what constitutes altered trafficking of these enzymes under the influence of the c-Ha-Ras(V12), and which organelles are involved. Therefore, apart from the maturation steps of the lysosomal enzymes, information on components known to be involved in trafficking of carrying vesicle between the Golgi, ER and LE processing compartments, will also be included.

Most lysosomal enzyme precursors acquire a unique M6P signal sequence (Gabel, 1983; Goldberg *et al.*, 1983; Ludwig *et al.*, 1991; Pohlmann *et al.*, 1995) that is added exclusively to the N-linked oligosaccharides in the *cis*-Golgi (Ren *et al.,* 1996). In the lumen of the Golgi two enzymes act sequentially to catalyze the addition of M6P groups. The first is a phosphotransferase that recognizes a conformation-dependent signal patch on the hydrolase, while a separate catalytic site adds the phosphate (GlcNAc-PO_4). A second enzyme cleaves off the GlcNAc residue creating the M6P marker recognized by MPR (Pillay *et al.,* 2002b).

Subsequently, the precursor lysosomal enzyme-MPR complexes are transported from the TGN to a LE-associated processing compartment (Stoorvogel *et al.,* 1989; Hirst *et al.,* 1998) (Figure 3.2 Lane 4 and Figure 3.3 Lane 4). Such sorting and shuttling require various trafficking molecules and signaling domains to be present. In the TGN, the cytoplasmic tail of the MPRs initially bind to the Golgi-localized, γ ear–containing, ADP-ribosylation factor (ARF) (GGA) vesicle coat protein (Ghosh *et al.,*

2003). Subsequently, the MPR may be associated with AP-1 (Ghosh *et al.*, 2004) or AP-4 (Barois *et al.,* 2005), that may act as secondary (destination-related) sorting determinants (Figure 3.3 Lane 4 and Section 3.3.6.1). Binding of AP-1 to any of 4 binding or sorting motifs on the MPR cytoplasmic tail, is regulated by variations in phosphorylated conditions and conformational changes of these motifs (Peters *et al.,* 1990; Harter *et al.,* 1992; Ogata *et al.,* 1994; Ghosh *et al.,* 2004). The precursor enzyme-MPR complexes are concentrated into selected membrane domains that bud off, and are transported to tubular endosomal structures, possibly the processing compartment (Waguri *et al.,* 2003). This compartment is reported to consist of a dynamic network of tubules and vesicles that are separate from and peripheral to, the degradative LE (Nicoziani *et al.*, 2000; Waguri *et al.*, 2003), described as a pre-lysosomal station (Hirst *et al.,* 1998) (Section 3.3.5). While the Golgi has a pH of 7, the pH of 5-6 in the acidified LE (Schmid *et al.,* 1999; Authier *et al.,* 2005) facilitates precursor dissociation from their MPR (Anderson *et al.*, 1988; Stoorvogel *et al.*, 1989; Bresciani *et al.*, 1996; Clague, 1998; Orsel *et al.*, 2000), the cleavage of the pro-sequences (Kornfeld, 1986) and targeting of activated cathepsins to the lysosome for storage (Stoorvogel *et al.*, 1989; Griffiths, 1996). On the other hand, association of the precursor enzyme-MPR complexes at the TGN with AP-4 may traffic lysosomal enzymes to LAMP-2 negative, immature secretory vesicles (Bonifacino *et al.,* 1999; Barois *et al.,* 2005) (Figure 3.3 Lane 4, Table 3.4 and Section 3.3.6.1), where these enzymes may take part in processing of proteins prior to their secretion (Arvan *et al.*, 1998; Brix, 2005). During subsequent granule maturation, MPRs are removed from immature granules by AP-1 in clathrin-coated vesicles (Klumperman *et al.,* 1998) (Figure 3.3 Lane 4).

Subsequent to ligand release in the processing LE the MPRs do not proceed to the degradative path or the lysosome (Barbero *et al.,* 2002). The degradative organelle and lysosomal structures are, therefore, devoid of MPRs (Stoorvogel *et al.,* 1989; Ludwig *et al.,* 1991; Shepherd *et al.,* 1996; Tjelle *et al.,* 1996; Ghosh *et al.,* 2003) and the MPR is found only occasionally in the MVB (Hirst *et al.,* 1998) or the LE, along with enzymes such as acid phosphatase (Gruenberg *et al.,* 1989; Ludwig *et al.,* 1991). Instead, MPRs are retrieved into vesicles that bud from the tubular processing LE (Nicoziani *et al.*, 2000) and cycled back to the TGN (Nicoziani *et al.*, 2000; Barbero *et al.*, 2002; Kalia *et al.*, 2006) for reuse (Gabel, 1983; Goldberg *et al.*, 1983; Kornfeld, 1986; Ludwig *et al.*, 1991; Ohno *et al.*, 1995; van Weert *et al.*, 1995; Hirst

et al., 1998). This retro-transport process is facilitated by the recognition and interaction of tail-interacting 47-kDa adaptor protein (TIP47) with the cytoplasmic signaling domain on MPR (Orsel *et al.,* 2000) under the influence of Rab9 (Barbero *et al.,* 2002) and vesicle isolation by clathrin or a coatomer (Sachse *et al.,* 2002) and by the dynamin GTPase (Nicoziani *et al.*, 2000). While the MPR remains intact during exposure to the low pH in the LE processing compartment, receptor oligosaccharides possibly become partially degraded and require reglycosylation after recycling to the Golgi (Goldberg *et al.,* 1983). Receptors, therefore, return to the Golgi not only to be repaired, but also to bind and transport further lysosomal precursor enzymes.

Mammalian cells express two different MPRs**,** a cation-dependent (CD) MPR of 46 kDa and a cation-independent (CI) MPR of 300 kDa. Both mediate targeting of M6P-containing lysosomal proteins from the TGN, yet cater for heterogeneity in lysosomal proteases, neither fully able to replace the function of the other (Pohlmann *et al.,* 1995; Sohar *et al.,* 1998). Unlike the CI-MPR (Gabel, 1983), the CD-MPR does not need an acidic environment to release its ligand (Probst *et al.,* 2006). Deficiencies of both receptors lead to missorting or secretion of newly synthesized lysosomal enzymes. This may result in lack of a degradative function in the cell (Pohlmann *et al.,* 1995).

3.3.4.2. Degradation in the LE

Degradation or processing of endocytosed cargo in the LE seems to occur only after the introduction of enzymes from lysosomal storage compartment, or lysosome (de Duve *et al.*, 1955; Griffiths, 1996). Lysosomes intermittently fuse with the LE (Desjardins, 1995; Duclos *et al.*, 2003). Therefore, the morphologically distinct LEs are often regarded as hybrid vesicles that form upon fusion of containing cargo vesicles, such as MVB and digestive enzymes. This was initially demonstrated using a combination of acid phosphatase cytochemistry (Holtzman *et al.,* 1968) and gold chase studies (Gruenberg *et al.,* 1989). It was illustrated that lysosome-like vesicles in which an initially chased gold marker accumulates after a prolonged 20 hour chase, may fuse with incoming EE vesicles containing gold from a second, more recent pulse (Ludwig *et al.,* 1991; Bright *et al.,* 1997). This was taken as an indication that final cargo degradation takes place in the LE (Tjelle *et al.,* 1996) and not the lysosome, as is often stated (Duclos *et al.,* 2003; Eskelinen *et al.,* 2003) and that gold-containing lysosomes possibly re-form from hybrid LE vesicles (Bright *et al.,* 1997) to again fuse

with newly-formed EEs. This may occur after "kiss and run" fusion and fission events and exchange of gold contents of the LE and storage lysosomes (Desjardins, 1995; Duclos *et al.*, 2003).

The endosomal system may also consist of a set of tubular, interconnected structures (Griffiths, 1996) or compartments / domains that have varying enzymatic activities and thus may have varying degradative capabilities (Gruenberg *et al.*, 1989; Ludwig *et al.*, 1991; Griffiths, 1996; Tjelle *et al.*, 1996). The LE lumen may acquire its low luminal pH of 5-5.5 due to V-ATPase proton pumps carried in its limiting membranes (Beyenbach *et al.,* 2006) (Section 3.2.1.4, and Figure 3.2 Lane 3). If the endocytic system does consist of a continuum of vesicles, it is difficult to imagine how an acid microenvironment may be maintained in a specific region, unless due to the presence of specialized proteins, such as LAMPs (Section 3.2.2).

Structures with a combination of characteristics of both MVBs, with internal vesicles with single containing membrane, and autophagosomes, with internal vesicles with more than one limiting membrane, have also been observed (Raposo *et al.,* 1996; Bright *et al.,* 1997). The morphology of these structures may be difficult to preserve by fixation and it has been suggested that the structures of the MVB and sorting vesicles could be artefacts of fixation (Griffiths, 1996; Sachse *et al.*, 2002) and that the structure of aldehyde-fixed organelles, such as the EE and LE, may be deformed to give rise to such structures due to shrinkage, probably as a result of hyperosmolarity of the fixative (Murk *et al.,* 2003b). This, however, has been shown to be not true, using freeze-fracture of unfixed unprocessed tissue (Griffiths *et al.,* 1984). Preservation of the ultrastructure of these organelles, however, relies on adequate preservation during sample processing for electron microscopical investigations, a requirement extremely difficult to achieve.

3.3.5. The lysosome

While the degree of luminal acidity in the lysosome and LE may vary over a wide range, both structures may contain variable combinations of the same membrane and luminal components often used in the description of either vesicle types (Table 3.2). This is possibly because the LE acquires degradative capacity after acquisition of lysosomal enzymes via "kiss and run" or intermittent vesicle fusion with lysosomal storage organelles. Discrimination between the LE and the lysosome is thus more

easily made on a morphological basis of size and electron density, in combination with luminal acidity than on the basis of lysosomal enzyme content, for example. The lysosome was first described in 1955 by de Duve *et al.* (1955) to be small, electron-dense and approximately 0.2-0.4 μm in diameter, containing hydrolases or lysosomal proteolytic enzymes, including the cathepsins, which are characterised by an acid pH optimum (de Duve *et al.,* 1955; Peters *et al.,* 1990). Most lysosomal enzymes were seen to hydrolyse appropriate substrates only after the enzymes were released by treatments that disrupted the limiting lysosomal membrane (Tjelle *et al.,* 1996).

Due to the dynamic nature of the vesicle maturation process, vesicle classification should be considered on the basis of a combination of characteristics, such as the relative size of various vesicle populations, electron density of the lumen and membrane markers such as MPR, LAMPs, as well as comparative luminal acidity.

3.3.5.1. Difference in vesicles sizes

Due to the highly dynamic nature of the endocytic vesicle system, the size or diameter of structures such as the EE or lysosome is usually mentioned in reference to other related structures, or compared to similarly labeled structures following alteration to cell function (Bucci *et al.*, 2000), chemical treatment of the cell (Fratti *et al.*, 2001) or genetic transfection of e.g. signaling proteins (Rosenfeld *et al.*, 2001). In addition, recognition on the basis of morphology only may be misleading, since the endocytic system has been described as an interconnected tubular, or tubular-vesicular system with smaller vesicles budding off (Gruenberg *et al.*, 1989; Nicoziani *et al.*, 2000). In a previous electron microscopy-based study in our laboratory the sizes of vesicles identified with markers for the EE [early endosomal antigen 1 (EEA1)] (Tuma *et al.*, 2001), the degradative LE (LAMP-2) or the processing LE (MPR) were recorded. For the current study, vesicle sizes have been recorded, and were used in combination with other markers, such as specific cathepsins, luminal acidity and LAMP-2, in order to investigate the effect of c-Ha-*ras*(V12) transfection on cathepsin-containing structures with that of un-transfected cells. This will be discussed together with immunolabeling results.

3.3.6. Vesicle classification in relation to the lysosome

Reference is often made to endosomal structures on the endocytic route according to their spatial relationship with the LE and lysosome and their content (Table 3.2). The

primary lysosome or terminal lysosome describes the unfused lysosome containing mature lysosomal enzymes. The acidic 'pre-lysosomal compartment' to which reference is made is possibly a late endosomal compartment where lysosomal precursor enzymes are processed, or released from their receptors (Figure 3.2 Lane 3). A 'mature lysosome' (or degradative LE, according to the nomenclature used in this study (Table 3.2) is formed when a 'primary lysosome' fuses with a cargo-containing early endosomal vesicle to form a late endosome or degradative body (Gruenberg *et al.*, 1989; Ludwig *et al.*, 1991; Griffiths, 1996; Clague, 1998; Farquhar *et al.*, 1998) (Figure 3.2 Lane 2).

While the 'pre-lysosomal compartment' or processing LE is the site of initial release of lysosomal enzymes from its Golgi-derived carrier receptor, and subsequent maturation, the 'lysosome', 'primary' or 'terminal' lysosomes are regarded as the carrier or storage, or secretory vesicles of the active enzymes (Griffiths, 1996) (Table 3.2). The 'secretory lysosome' may release its content at the PM in a regulated fashion and is hence usually known as a regulated secretory lysosome (Figure 3.2 Lane 1). The main intracellular site of degradation, though, seems to be the LE (Mizuno *et al.*, 2003; Ceresa *et al.*, 2006; Progida *et al.*, 2007).

Often a structure in a relatively fixed position adjacent to the nucleus, or a juxta-nuclear structure (JNS), is described in the literature (Figure 3.2 Lane 3). It has been called the perinuclear late-endosomal-lysosomal cluster (Bucci *et al.*, 2000; Harrison *et al.*, 2003) or the perinuclear recycling compartment (Jordens *et al.,* 2001; Johansson *et al.,* 2007). The JNS has also been characterized by a low pH (Harrison *et al.*, 2003) and may be the mature endosome with a digestive role. In this study reference will be made to this structure as the JNS, mainly since its formation or a clear set of characteristics has not yet been established. The classification of this structure as either a distinct pre-existing digestive body or a LE, is important as it may represent a separate, unique digestive organelle. The JNS is often associated with the presence of mature CD, an enzyme that is active only in an acidic environment (Liaudet *et al.,* 1995), and has been described as being involved in high-grade macromolecule degradation and may be a key organelle in autophagy. It is our hypothesis that specific forces maintain the JNS in a close relationship with the microtubular organizing centre, the nucleus and trans-Golgi network (Nobes *et al.*, 1999; Wang *et al.*, 2004a; Cau *et al.*, 2005) and that it is a specialized LE, forming a

stable area of intense digestive activity, in contrast to the more dynamic EE and LE (Section 3.8.1.2 for further discussion).

Table 3.2 Terminology for various vesicles classified as lysosomes in this study.
The various types of lysosomes to which reference is made in a number of sources, their characteristics and the markers used in identification are correlated with nomenclature to be used in this study (basis for such correlation indicated in brackets). Where uncertainty exists about the type of marker, it is indicated with a (?) (compiled from references given below).

Type of 'lysosome' referred to in source reference	**Source reference**	**Vesicle description in source reference**	**Characteristic markers and vesicle size used for structure identification**	**Corresponding terminology as used in this study**
Primary lysosome	(Kornfeld, 1986; Huynh *et al.*, 2007)	A small vesicle containing enzymes after their release from the MPR.	Small vesicle Hydrolases + MPR-	Lysosome (circulating, storing newly-matured enzyme)
Lysosome	(Shepherd *et al.,* 1996)	A small vesicle containing lysosomal enzymes and specific membrane proteins such as LAMPs and LIMPs, but lack the precursor carrier MPR.	Small Hydrolases + MPR- LIMPs + LAMP ? Acidity ?	Lysosome (primary / circulating vesicle storing mature enzyme)
Terminal lysosome	(Tjelle *et al.,* 1996)	A small electron dense storage body for acid hydrolases that may be sequestered when needed.	Hydrolases + LIMPs LAMPs Acidity ? Dense, small	Lysosome (storage or circulating)
Secretory or mature lysosome	(Linebaugh *et al.*, 1999; Furuta *et al.*, 2001; Astarie-Dequeker *et al.*, 2002; Blott *et al.*, 2002; Huynh *et al.*, 2005)	Small secretory granules in e.g. macrophages, neutrophils and melanosomes. Contains material that is stored after production and can be secreted at the PM under certain conditions, such as altered [Ca^{2+}] and wound healing.	Not acidic Hydrolase +/- Small, electron dense LAMP-1	Secretory lysosome

The nomenclature used for the various vesicles in this study is illustrated in Figure 3.2 and outlined in Table 3.3, together with some of the characteristics that define and are involved in trafficking of these organelles and that may be used as markers for these organelles in the current study. The nomenclature that will be adopted for these organelles from here on is now given in the first column of Table 3.3, with a summary

of their major characteristics, while the last column indicates the markers that will be used in the current study to identify these organelles (Table 3.3).

Table 3.3. Summary of vesicle description and nomenclature used in this study.
In this table the nemenclature adopted for this study is outlined, as well as a description of the vesicle and markers that will be used to identify these structures. Where uncertainty exists about the type of marker, it is indicated with a (?) (compiled from references given below).

Name of structure.	**Description of structure.**	**Markers used in identification.**
Circulating, storage or primary lysosome	A small, electron dense vesicle containing mature hydrolytic enzymes (cathepsins) (de Duve *et al.,* 1955) which may merge with cargo-containing vesicles (Bright *et al.,* 1997).	Acidic ? LAMP-2 ? Lysosomal enzymes
EE and ECV	The first endocytic vesicles that form subsequent to cargo uptake and are spatially close to the PM. The cargo content is possibly sorted in the ECV (Gruenberg *et al.*, 1989; Desjardins, 1995).	Non-acidic Lysosomal enzymes ?
Processing compartment of the LE (prelysosomal compartment)	An intermediate compartment of the LE with slightly acidic lumen where protease precursors dissociate from MPR (Shepherd *et al.*, 1996; Hirst *et al.*, 1998; Huynh *et al.*, 2007). It may contain both hydrolase precursors and their carrier proteins (MPR). The receptor is known to cycle back to the Golgi (Orsel *et al.,* 2000), while the precursor undergoes further maturation in this structure.	Acidic LAMP-2 +? Lysosomal enzymes
Degradative compartment of the LE (mature lysosome)	A hybrid vesicle that is formed through fusion with a lysosome. These are the large structures that may be positive for many of the lysosomal characteristics, such as membrane proteins, lgps, cathepsins and low luminal acidity and may be involved mainly in cargo degradation (Bakker *et al.*, 1997; Schmid *et al.*, 1999; Bucci *et al.*, 2000; Luzio *et al.*, 2000).	Acidic LAMP-2 + Lysosomal enzymes
The JNS	An acidic structure containing CD and characterized by a low luminal acidity and LAMP-2 (Baravalle *et al.,* 2005). This may be the major degradative structure of the cell.	CD Acidic LAsMP-2 +
Multi-vesicular or multi-lamellar body	Contains many small intraluminal vesicles or membranes wrapped around each other (Haugh *et al.*, 1999; Duclos *et al.*, 2003). These structures could be part of the degradative and/or autophagic route, depending on the number of membrane layers.	Non-acidic Intraluminal vesicles within limiting membrane
Secretory lysosomes	Contain cargo that is destined for secretion at the PM (Arvan *et al.*, 1998; Martinez *et al.*, 2000; Huynh *et al.*, 2005). These may form subsequent to limited digestion (in antigen presenting cells) or contain specialized secretory products (melanosomes in the skin, secretory granules in the neutrophils or proteases for ECM degradation).	Non-acidic LAMP-2 neg Lysosomal enzymes (CB, CL)

Directed trafficking and maturation of these vesicles throughout the cell relies on the cytoskeletal tract and various specialized cytoplasmic pilot molecules and membrane-related proteins, however. These coordinate interaction between donor and target vesicle membranes and the maturation process of vesicles along both the biosynthetic and endocytic paths. Detail of the influence of these components and the cytoskeletal network on vesicle trafficking and luminal acidity, will be given in the following sections. In order to explain and predict possible effects that expression of the c-Ha-Ras(V12) oncoprotein may have on these factors and the resultant trafficking and luminal acidity of cathepsin-containing vesicles.

3.3.7. Factors possibly relevant in membrane trafficking that may be affected by c-Ha-*ras*(V12) transfection

The membranes of each of the donor, target and hybrid compartments along the endosomal vesicle maturation route have characteristics relevant to each of these stations, while their morphology and limiting membrane composition differs greatly. The PM contains 80% of cholesterol, accumulated in specialized lipid rafts (Hoekstra *et al.,* 2000; Bourguignon *et al.,* 2004) where it facilitates membrane budding and vesicle formation (Mukherjee *et al.,* 1997) and the association of endosomes with the actin cytoskeleton (Höltta-Vuori *et al.,* 2005). About 50% of all proteins of the lysosome/LE membrane, though, consists of highly glycosylated LAMPs (Eskelinen, 2006) (Section 3.2.2.1). Such distinct membrane compositions are achieved through highly dynamic vesicle budding, fusion and fission and recycling of membrane components during endosome maturation. These processes are under strict molecular control (Figure 3.3 Lane 4). As cargo progresses down the degradative path, the limiting membranes of maturating EEs and carrier vesicles shed integral protein, coat components and receptors such as the Tfn receptor (Stoorvogel *et al.,* 1989) that are recycled or returned to the PM (van Weert *et al.,* 1995; Sachse *et al.,* 2002) through recycling vesicles (Thilo *et al.,* 1995) (Figure 3.3 Lane 3). During formation of late endosomal degradative bodies, EEs merge with storage or circulating lysosomes (Bright *et al.,* 1997; Mullock *et al.,* 1998) and the hybrid structure gains components such as LAMPs (possibly LAMP-2) and degradative hydrolases (Figure 3.3 Lanes 1,2). Secretory vesicles take processed cargo, such as cholesterol derived from LDL processed in the LE, from the LE to the PM (Möbius *et al.,* 2003). Similarly, retrograde vesicles from the LE return MPRs to the TGN after release of newly-formed hydrolases (Ganley *et al.,* 2004) (Figure 3.2 Lane 4 and Figure 3.3 Lane 4).

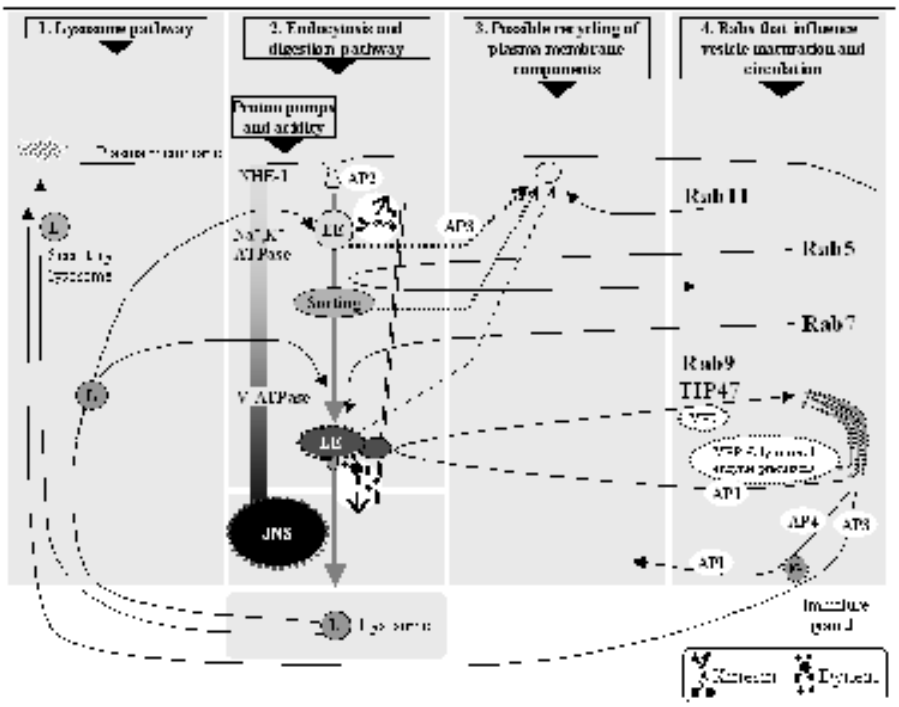


Figure 3.3. A schematic illustration of possible mechanisms that control vesicle trafficking along the degradative and secretory pathway.
The endocytic pathway has several definite morphological and biochemical stations and associated proton pumps (Lane 2). Recycling of PM components (Lane 3) and fusion with lysosomes (Lane 1) form part of the various maturation processes. Small Rab GTPases energize these processes (Lane 4) and adaptor proteins (APs) guide target-specific membrane trafficking during formation of EEs (Rab5 and AP2), LEs (Rab7), secretory lysosomes (Rab11 & AP4), MPR carrier vesicles (AP1, Rab9, TIP47 and AP4) and PM recycling (AP3). This illustration was generated from references in Section 3.3 and illustrates predictions for vesicle trafficking routes as discussed in the final discussion.

During vesicle biogenesis initial membrane curvature and budding are controlled by coat proteins (Heuser, 2004). A range of APs concentrate molecules into these domains (Ohno *et al.*, 1995; Sachse *et al.*, 2002; Ohno, 2006) (Section 3.3.6.1). Motor proteins, such as dynein (Jordens *et al.,* 2001) and kinesin (Santama *et al.,* 1998; Ichikawa *et al.,* 2000), allow the association of vesicles with the cytoskeletal network in a direction-specific way (Section 3.3.6.1), while membrane-membrane attachment proteins and their receptors control membrane recognition and tethering (Section 3.3.6.2) and annexins assist in membrane fusion during integration of vesicles (Nakamura *et al.,* 2000; Brandhorst *et al.,* 2006). Rabs are the energy-supplying switches that control these fission, fusion and trafficking processes (Section 3.3.6.2). Together, these factors provide several levels of control in donor and target organelle-specific trafficking. These biochemical forces that drive vesicle formation and the principles that guide vesicle trafficking will be highlighted in the following

section and their relevance in cells that express a mutationally active c-Ha-Ras(V12) will be indicated and finally discussed in the light of our experimental results in this chapter.

3.3.7.1. Adaptor proteins (AP), coat proteins (COPs) and motor proteins in directed vesicle trafficking

APs are least likely to be affected by c-Ha-*ras*(V12) transfection, unless they are indirectly affected by altered trafficking of other components of vesicles such as the presence of LAMPs or other vesicle trafficking proteins. APs are recruited to- and recognize several different signaling domains, located on the cytoplasmic domains of integral membrane receptors, such as LAMPs (Bonifacino *et al.*, 1999; Ghosh *et al.*, 2004) (Section 3.2.2.2). They, therefore, do mediate selection of cargo, such as protease precursors (Section 3.3.3.1), and incorporation into vesicles budding off along the endocytic and secretory pathways (Ohno *et al.,* 1995) (Figure 3.3 and Table 3.4). They may determine the destination for delivery of these precursors, and may, therefore, possibly determine variations in trafficking routes. An example is the tyrosine signal on EGFR, which recruits the AP, Src homology (SH)-2-domain-containing α2-collagen-related protein (Shc), that facilitates its incorporation into degradative vesicles and termination of downstream growth signaling (Authier *et al.,* 1999a) (Section 1.9.5). At the TGN AP-1 facilitates the sorting and trafficking of CI-MPR, one of the receptors for newly synthesized M6P-labeled precursor lysosomal enzymes, to the LE or EE (Gabel, 1983; Ludwig *et al.*, 1991; Ohno *et al.*, 1995; Waguri *et al.*, 2003; Ghosh *et al.*, 2004) (Section 3.3.3.1, Figure 3.3 Lane 4 and Table 3.4). In the pancreas, AP-1 assists in the removal of processing enzymes from immature granules before the mature secretory vesicles are translocated to the PM (Klumperman *et al.,* 1998). Retrograde trafficking of CI-MPR to the Golgi after release from its precursor cargo (Stoorvogel *et al.,* 1989) depends on its recognition by TIP47 adaptor protein (Aivazian *et al.,* 2006) (Section 3.3.3.1).

In clathrin coated vesicles at the PM AP-2 facilitates the incorporation, by receptor-mediated endocytosis, of ligands, such as LDL, Tfn (Styers *et al.,* 2004; Janvier *et al.,* 2005) and LAMP-2 (Bonifacino *et al.*, 1999; Janvier *et al.*, 2005; Haucke, 2006) (Figure 3.3 Lane 2 and Table 3.4). AP-2 may also facilitate assembly of the V-ATPase late endosomal proton pump (Sun-Wada *et al.,* 2004) (Section 3.2.1.4), thus

indirectly influencing the acidification of endocytic vesicles, a factor relevant in efficient degradation (Section 3.2.1).

AP-3, involved in specific recognition and sorting of proteins with tyrosine-based motifs (e.g. LAMPs) (Clague, 1998; Peden *et al.*, 2004a), interacts with intermediate filaments (Styers *et al.,* 2004) and is associated with recycling of transmembrane receptors from the EE to the PM (Styers *et al.,* 2004) (Figure 3.3 Lane 4 and Table 3.4). It is also found at the TGN. Here it controls the exit of precursor molecules from the Golgi to immature granules, e.g. platelet dense bodies (Helip-Wooley *et al.,* 2005), and melanosomes. Both these structures assist in secretion of proteins at the PM (Peden *et al.*, 2004a; Bossi *et al.*, 2005; Helip-Wooley *et al.*, 2005).

Table 3.4 Members of the AP family are heterotetrameric cytosolic complexes that are involved in the intracellular trafficking of cargo proteins.
Information was compiled from references given below.

APs	**Function**	**Reference**
AP-1	Targets precursor hydrolases on CI-MPR from TGN to processing LE.	(Waguri *et al.,* 2003; Ghosh *et al.,* 2004; Barois *et al.,* 2005)
AP-2	Required for uptake/recycling of Tfn and LAMP-2 at the PM.	(Bonifacino *et al.*, 1999; Janvier *et al.*, 2005; Haucke, 2006)
AP-3	AP-3 regulates the recycling route between the EE or the TGN, secretory vesicles and PM.	(Peden *et al.*, 2004a; Styers *et al.*, 2004; Helip-Wooley *et al.*, 2005)
AP-4	Possibly involved in the trafficking of cathepsins on the MPR to vesicles containing precursors *(destined for secretion).*	(Bonifacino *et al.,* 1999; Barois *et al.,* 2005)

At the TGN membranes (Bonifacino *et al.,* 1999), AP-4 alternatively regulates the isolation of enzymes that will assist in the maturation of precursor proteins retained in immature secretory vesicles or granules (Figure 3.3 Lane 4 and Table 3.4), such as in melanosomes (Barois *et al.,* 2005).

At the ER or Golgi donor vesicles form under the influence of clathrin or coatomer proteins (COPs) (Sohn *et al.,* 1996; Donaldson *et al.,* 2000). COP-II-coated vesicles bud off the ER *en route* to the Golgi (Stephens *et al.,* 2000), while COP-I-coated vesicles bud from the Golgi (Donaldson *et al.,* 2000; Dell'Angelica *et al.,* 2001). These are responsible for retrieval (retrograde transport) of ER and previous Golgi

stack membrane proteins, returning these to their respective Golgi stack as these mature, or to the ER from where they originally came (Sohn *et al.,* 1996; Cosson *et al.,* 1998; Donaldson *et al.,* 2000). Subsequent release of clathrin and COP components is under the influence of hydrolyses of ARF, a Ras-like small GTPase that controls vesicle trafficking in the cell (Waguri *et al.,* 2003; Hurtado-Lorenzo *et al.,* 2006). This process can be inhibited by brefeldin A, that negatively affects ARF (Dell'Angelica *et al.,* 2001), and may inhibit the progression of vesicles along the secretory route (Fernandez *et al.*, 1997).

After cargo isolation into relevant vesicles, the motor proteins kinesin and dynein, attach these vesicles to the cytoskeletal network and move them along the microtubule tract (Machesky *et al.*, 1999; Bananis *et al.*, 2000b) (Figure 3.3 Lane 2), In general, dynein regulates the centripetal movement of maturing endosomes along microtubules towards the nucleus, i.e. movement towards the microtubule organizing centre (MTOC) (Jordens *et al.,* 2001). Centrifugal movement by secretory vesicles, i.e. away from the nucleus towards the microtubule plus end and the cell periphery, is due to their association with kinesin (Santama *et al.,* 1998; Ichikawa *et al.,* 2000) (Figure 3.2 Lane 2). Both kinesin and dynein use ATP as an efficient source of energy to move vesicles (Blocker *et al.*, 1997; Ichikawa *et al.*, 2000) and are recruited to the vesicle membrane and activated by a range of small GTPases, the Rabs and their related effectors.

3.3.7.2. Rabs in endocytic vesicle formation and maturation

Rab5 and Rab7 are most relevant in vesicle maturation and trafficking, and may be more directly affected by c-Ha-*ras*(V12) transfection. These Rabs form part of a range of small GTPases in the Ras-related protein family (Table 1.1), that act as biological switches. In a process similar to Ras the Rabs undergo a conformational change when activated through binding to GTP (Ma *et al.,* 1997) (Section 1.5.3). Rab5 and Rab7 are recruited from the cytoplasm (Scianimanico *et al.,* 1999; Vieira *et al.,* 2003; Jager *et al.,* 2004) to distinct vesicle membranes (Kalia *et al.,* 2006). Activated Rabs supply energy for vesicle fusion and fission (Bucci *et al.,* 2000; Wilcke *et al.,* 2000; Rosenfeld *et al.,* 2001; Deneka *et al.,* 2003) (Figure 3.3 Lane 4) and recruit cytosolic fusion and attachment proteins, such as soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) from the cytosol onto distinct cellular compartments to permit subsequent membrane fusion

(Barr, 2000; Heuser, 2004; Kalia *et al.*, 2006). SNAREs form complexes that correctly guide and mediate membrane fusion between vesicles (Clague, 1998; Pelham, 2001; Brandhorst *et al.*, 2006). This process is mediated through pairing between unique sets of donor vesicle-associated SNARE (v-SNARE) and target membrane SNARE (t-SNARE) (Clague, 1998) that form a complex transmembrane bridge between these membranes (Brandhorst *et al.,* 2006).

The homotypic EE fusion-fission interactions ('kiss and run' for EEs) (Figure 3.2 Lane 2), during which cargo is sorted, requires membrane fusion of homotypic EEs (Vieira *et al.,* 2003; Kalia *et al.,* 2006), and involves Rab5 (Duclos *et al.,* 2003) (Figure 3.3 Lane 4) and its association with the EE marker, EEA-1 (Rosenfeld *et al.,* 2001). Rab5 binds EEs to the kinesin motor protein, keeping these vesicles at the cell perimeter (Ichikawa *et al.,* 2000). It is released from the EE by activated PI3K (and formation of PIP_3), during which process Rab7 is acquired (Vieira *et al.,* 2003) (Figure 3.3 Lane 4).

Rab7 is used as a marker for the degradative LE. It acts at the EE / LE interface by guiding the fusion of cargo-carrying endosomes with hydrolytic enzyme-carrying lysosomes, thus regulating the formation of LEs (Bucci *et al.,* 2000; Cantalupo *et al.,* 2001). In addition, Rab7 assists in acidification of these vesicles (Harrison *et al.*, 2003) and regulates the positioning of the LE / lysosome hybrid vesicle through the formation of a complex with with several different molecules e.g. a Rab-interacting lysosomal protein (RILP), Rabring-7, cytoskeletal elements and molecular motors, such as myosin, dynein, kinesin and KIF-3A, (Santama *et al.*, 1998; Bananis *et al.*, 2000b; Ichikawa *et al.*, 2000; Vieira *et al.*, 2003; Ceresa *et al.*, 2006). The formation of this complex accounts for LE motility towards the nucleus (Harrison *et al.*, 2003; Johansson *et al.*, 2007). The process via which components from the EE are recycled to the PM, such as integral PM receptors, is under the control of Rab11 (Fernandez *et al.,* 1997; Wilcke *et al.,* 2000; Moore *et al.,* 2004) (Figure 3.3 Lane 4).

The relevance of Rabs in this study Rabs is their important role in vesicle maturation and trafficking (Wilcke *et al.,* 2000; Grosshans *et al.,* 2006; Schwartz *et al.,* 2007). Rabs are under the influence of PI3K (Shepherd *et al.,* 1996; Chen *et al.,* 2001; Rosenfeld *et al.,* 2001; Vieira *et al.,* 2003; Kalia *et al.,* 2006), that is a direct downstream effector of Ha-Ras during proliferative signaling (Giglione *et al.,* 1998;

Pacold *et al.,* 2000; Jaumot *et al.,* 2002) (Section 1.6.4 and Figure 1.3 on fold-out). The influence of Ha-Ras on vesicle dynamics is further described in Section 3.3.7.

Normal Ha-Ras, transiently activated by growth stimulus-related factors, signals down a cascade of downstream GTPases and kinases to support processes such as survival and protein transcription during proliferation (Figure 1.3 on fold-out). However, these activated kinases also allow relocation of membrane-bound proteins to the leading migratory front, such as the NHE-1 proton pump (Denker *et al.,* 2002), contraction of cytoskeletal stress fibers to aid such motility (Section 1.7.3), and may, therefore, influence the distribution of endocytic vesicles.

3.3.8. Ha-Ras influences on cytoskeletal organization and vesicle dynamics

Motility of endocytic vesicles through the cell is aided by direction-specific motor proteins, that are associated with the cytoskeletal network (Section 3.3.6.1 and Figure 3.3 Lane 2). The actin filaments and microtubules that make up this network are affected by Ha-Ras downstream effectors, especially during migration (Figure 1.3 on fold-out), when the cell shape is altered to an elongated meshechymal shape in response to activated Ha-Ras-related signaling.

The Ha-Ras downstream effectors, including the Rho family (Rac, Rho and CDC42) (Denhart, 1996; Koshravi-Far *et al.*, 1996; Cau *et al.*, 2005) and Ral (Ward *et al.,* 2001; Caron *et al.,* 2005) are all involved in organization of cytoskeletal components. CDC42 is also involved in membrane trafficking (Donaldson *et al.,* 2000) and formation of protrusions at the leading edges of the cell (Cerione, 2004; Cau *et al.*, 2005; Parsons *et al.*, 2005) (Figure 1.4). Malignant phenotypes induced by c-Ha-Ras(V12) can be suppressed by drugs that block the Rho family GTPases-mediated pathway (Maruta *et al.,* 1999), an indication of the importance of cytoskeletal organization in the invasive process. The microtubular network can be depolarized by nocodazole (Matteoni *et al.*, 1987) via its inhibition of PI3K (Wang *et al.*, 2004a), also an Ha-Ras downstream effector (Kodaki *et al.,* 1994; Rodriguez-Viciana *et al.,* 1994; Khoshavi-Far *et al.,* 1996; Ward *et al.,* 2001). Another function of PI3K is its involvement in vesicle trafficking. PI3K controls the sequestration to- and release of the GTPase Rab5 from EEs (Vieira *et al.,* 2003; Grosshans *et al.,* 2006; Kalia *et al.,* 2006) and its replacement with Rab7 (Vieira *et al.,* 2003; Grosshans *et al.,* 2006; Schwartz *et al.,* 2007) (Figure 3.3 Lane 4). Rab7 manages the fusion of late

endosomes with lysosomes (maturation into a degradative vesicle) (Press *et al.,* 1998), movement (connection with motor proteins) (Cantalupo *et al.,* 2001) and acidification (and thus enzymatic cargo degradation) of these organelles (Papini *et al.,* 1996). This process is cleverly by-passed by e.g. the Salmonella bacterium (Manuel *et al.,* 2001) and other pathogens such as *Mycobacterium tuberculosis* (TB) (Deretic *et al.,* 1997). Phagocytosed live TB survives in macrophages due to its evasion of Rab7 recruitment, and thus inhibits phagosome-lysosome fusion and subsequent vesicle acidification and degradation (Scianimanico *et al.,* 1999; Malik *et al.,* 2001; Gruenberg *et al.,* 2006).

In contrast, trafficking of MPR-carrying vesicles (relying on adaptor proteins, COPs and clathrin) on the biosynthetic route that shuttle between the Golgi to LE, or possibly to the processing compartment of the LE where precursor enzymes are thought to be matured (Martys *et al.,* 1996; Nakajima *et al.,* 1997), is not affected by Ha-Ras-PI3K (thus by factors that influence Rab5/7-related maturation) (Shepherd *et al.,* 1996). The acidification of these vesicles is also not affected by BafA1, and therefore, does not depend on V-ATPases (van Deurs *et al.,* 1996) (Section 3.2.1.4). Therefore, it seems that routine precursor trafficking from the TGN to their site of maturation (Figure 3.3 Lane 4) is not affected by downstream Ras stimulation. The MCF7 breast cancer cells, however, cannot successfully acidify their late endocytic vesicles (Kokkonen *et al.,* 2004), including the processing compartment (Montcourrier *et al.,* 1997). This may lead to the secretion of proCD from the MCF7 cells (Capony *et al.*, 1994; Davidson, 1995), since the maturation of proCD requires an acidic environment (Wittlin *et al.,* 1999).

In invasive cancer cells, therefore, that express a mutated, constitutively activated c-Ha-Ras(V12) oncoprotein, such as the c-Ha-*ras*(V12)-transfected MCF10AneoT breast cancer cell line used in the current study, may have negative effect on cytoskeletal organization. This may, in turn, lead to a distribution of endocytic vesicles more distant from the nucleus, in contrast to the normal perinuclear position. Such redistribution may be the result of cytoplasmic alkalinization due to constant proton secretion by the NHE-1 proton pump, activated by c-Ha-Ras(V12) signaling (Ritter *et al.*, 1997). This may in turn affect the cytoskeleton and cause inefficient assembly of individual V-ATPase proton pump components (Section 2.5.1.4), resulting in alkalinization or inefficient acidification, of the lumen of vesicles

involved in degradation. Hence, degradative capacity of cargo processing organelles, such as the LE, and possibly also processing efficiency of compartments involved in precursor maturation, may be negatively affected. This may, in addition, have a deletariously effect on nutrient supply, especially in cancer cells that generally have an increased metabolism and, thus, increased nutrient requirement (Chiaradonna *et al.*, 2006; Xu *et al.*, 2007).

The influence of c-Ha-*ras*(V12) transfection on intracellular distribution of cathepsin-containing vesicles was explored in relation each other, as well as to luminal acidity and the presence of LAMP-2, the membrane protein usually associated with acidic vesicles, and the sizes of different vesicle populations. It was hoped that such information may assist in characterization of various populations of cathepsin-containing vesicles, and, in combination with the Ha-Ras downstream pathway as discussed in Chapter 1 (Figure 1.3 on fold-out), to better understand the influences of c-Ha-*ras*(V12) transfection on distribution nd insufficient acidification of these vesicles in invasive cancer cells.

3.4. Distribution of CB, CD and CL in both normal and c-Ha-*ras*(V12)-transfected cells

Cathepsin distribution and secretion is altered after c-Ha-*ras*(V12) transfection of the MCF10A cell line, possibly contributing to the invasive behaviour of these cells (Sloane *et al.*, 1994; Sameni *et al.*, 1995). Elevated levels of CB, CD and CL in MCF10AneoT cells, compared to non-transformed MCF10A cells, were also seen in a parallel study in our laboratory (van Rooyen, 2009) (Extracts from thesis in Appendix III). In the current study, cells were immunolabeled consecutively with sets of two antibodies against either CB, CD or CL in order to compare the localization of such cathepsins relative to each other and assess any change in polarized distribution after c-Ha-*ras*(V12) transfection. Sets of images of optical planes along the apical-basal plane from double-labeled cells were captured. These sets of images were analyzed independently to assess the apparent role of c-Ha-*ras*(V12)-transfection on the distribution of the individual cathepsins of interest, i.e. CB, CD and CL. Images from the individual cathepsins on selected planes on the apical-basal axis were also analysed with reference to each other, to assess the degree of colocalization in both normal MCF10A and c-Ha-*ras*(V12)-transfected MCF10AneoT cells.

3.4.1. Reagents

Reagents used for cell culture were described in Section 2.2.1.

Reagents used for double fluorescence immunolabeling for confocal microscopy were described in Section 2.6.1.

3.4.2. Procedure

For studies on intracellular immunolabeling of cathepsins, subconfluent cells were grown on coverslips, as described in Section 2.2.2, i.e. were washed, fixed, permeabilized and immunolabeled with primary and fluorescently tagged secondary antibodies, as described in Section 2.6.2.

For intracellular labeling, coverslips were initially incubated in either a chicken antibody to CB (20 μg/ml, diluted in PBS-BSA, 1h), a chicken antibody to CD (20 μg/ml, diluted in PBS-BSA, 1 h) or a rabbit antibody to CL (100 μg/ml, diluted in PBS-BSA, 1 h), washed and incubated with either a secondary donkey anti-chicken antibody conjugated to Cy3 (diluted 1:1500 in PBS-BSA, 1 h), a secondary goat anti-rabbit antibody conjugated to TRITC (diluted 1:300 in PBS-BSA, 1 h), a secondary donkey anti-chicken antibody conjugated to FITC (diluted 1:400 in PBS-BSA, 1 h), or a secondary goat anti-rabbit antibody conjugated to FITC (diluted 1:200 in PBS-BSA, 1 h). Coverslips were washed, fixed and washed and mounted in Moviol as described in Section 2.6.2.

In double labeling, the protocol for single labeling was repeated as described in Section 2.6.2.

Images representing the apical, middle and basal planes were analysed as described in Section 2.7 (Figure 3.4 I).

3.4.3. Results and discussion

In c-Ha-*ras*(V12)-transfected MCF10AneoT cells, CB was spread along entire base of the cell where the cell is in contact with the underlying matrix, and in a perinuclear medial position (Figure 3.4 D red), compared to what was seen in the normal MCF10A cells, where CB was positioned predominantly in a basal position and in the

medial position (Figure 3.4 A, B red). Here CB was seen polarized mostly to a thin, discreet region, possibly close to the PM, on one side of the cell (Figure 3.4 A lower insert), implying membrane association, or in a single discreet area at the opposite pole in relation to the nucleus (Figure 3.4 A upper insert). Figure 3.4 A illustrates the presence of more CB in the basal region than the apical region. Image analysis of 4 representative cells (not shown), on the other hand, taking the cytoplasmic area in each image into consideration, seems to indicate that most CB is found in the medial layer of the normal cell (Figure 3.4 G). This illustrates the difficulty in selecting a single representative image. For this reason it is important to realize that, what is illustrated in the selected images of fluoresence, and the compared to the analysis described by the graphs may not seem to be representative. The images included here are generally representative of the difference in distribution of cathepsins between normal MCF10A and c-Ha-*ras*(V12)-transfected MCF10AneoT cells, however. In the case of CB and CD the distribution seen in Figure 3.4 is largely representative of the majority of normal cells labeled for CB and CD (Figure 3.4 A, B and C). The opposite is true in the c-Ha-*ras*(V12)-transfected cell, where CB seemed to be equally distributed on either side of the nucleus (Figure 3.4 E, open arrow). An image of a trail of immunolabeled CB behind a seemingly migrating cell was captured at the basal level of the cultured cells (Figure 3.4 J, green). Such a finding seems to indicate that CB is secreted and subsequently associated with the PM, and may be involved in the extracellular degradation of the BM that is laid down during their attachment to the coverslips. The distribution and role of CB in the invasive behaviour of the MCF10AneoT cells will be discussed in Chapter 4.

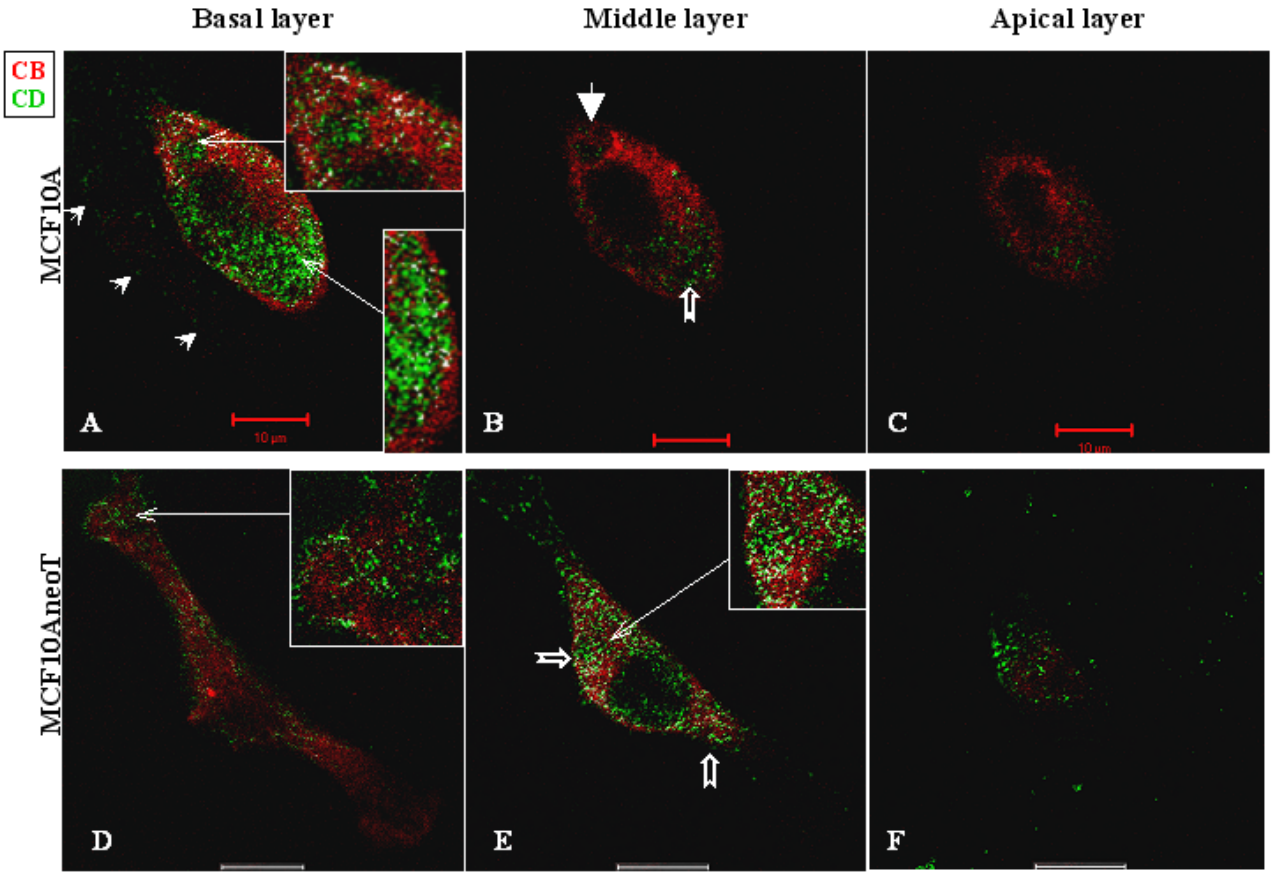
Colocalization analysis of CB and CD in 4 cells of the normal MCF10A cell line seems to support the view that colocalization is greatest at the base of these cells (Figure 3.4 A, H). The opposite is true after transfection of MCF10A cells with the c-Ha-*ras*(V12) oncogene (Figure 3.4 D).

Variations in confocal results between different sets of images, may be attributed to differences in cell shape and the leading fronts of the normal and transformed cells. This may be evidence that reflect the dynamic nature of these cells while they are in a subconfluent, migratory phase (Section 1.4).

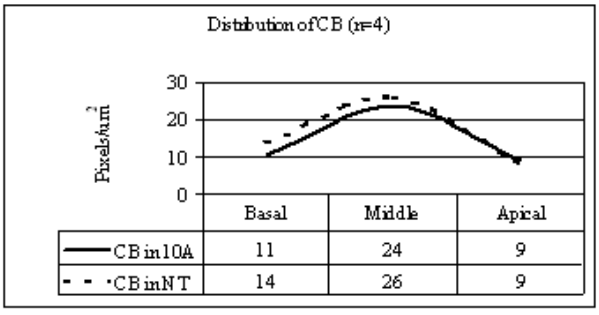
In normal cells, CD was mostly found in a perinuclear basal-to-medial region, polarized towards the opposite side of the nucleus, compared to CB (Figure 3.4 A, B, green and Figure 3.6 A, B red), with a few isolated immunolabeled vesicles spread along the base at the leading edge of membrane ruffles (arrowheads in Figure 3.4 A red and Figure 3.6 A red). Though the images of the MCF10A cells indicated more CD in discrete regions of the basal region (Figure 3.4 A green), in c-Ha-*ras*(V12)-transfected cells, a marked spread of was noted in non-colocalized CD-positive vesicles at the cell base and forward extending lamellipodia (Figure 3.4.D green and Figure 3.6 D red). The concentrated immunolabeling for CD in a relatively fixed position polarized towards one side of the nucleus in the basal to medial layers in normal cells was seen (Figure 3.4 A, open arrow in B, and Figure 3.6 B), possibly correlating with the position of the JNS (Section 3.3.5). In c-Ha-*ras*(V12)-transfected cells, however, this concentration of CD could be seen mainly at both poles of the nucleus, with some discreet smaller areas and less pronounced than in the normal (Figure 3.4 E, open arrows) or pronounced, large areas (Figure 3.6 E, open arrows). The possible JNS seen in labeling of MCF10AneoT cells for CD and CL (Figure 3.6 D, E) appears as a large area of CD-labeled vesicles that possibly form an interconnected network in normal cells, the size of which may vary depending upon the degradative activity at the time (compare Figure 3.4 A, open arrow in B and Figure 3.6, insets in D, E). An interesting, previously undescribed small, rounded area was observed, mostly not labeled for CD (Figure 3.4 B, large arrow head) or any other cathepsin labeled in this study (Figure 3.5 A, Figure 3.6 B, D and E). This structure was noted to be non-acidic (arrowheads in Figure 3.8 A, B and D, Figure 3.9) and to contain no CD (Figure 3.8 A) or CL (Figure 3.9 A).

Figure 3.4. The distribution and colocalization of CB with CD over the basal-apical axis in MCF10A and MCF10AneoT cells.

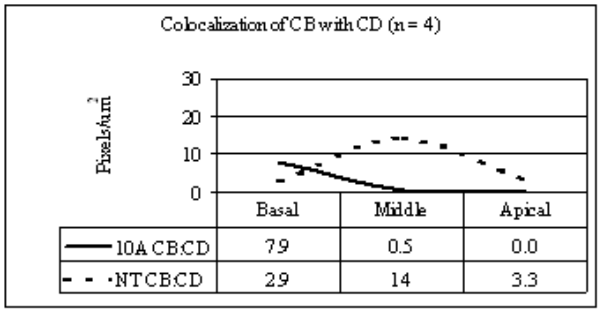
The distribution and colocalization of Cy3-labeled CB (red) with FITC-labeled CD (green) was illustrated over the basal-apical axis of MCF10A (A-C) and MCF10AneoT cells (D-F). Specific areas of interest as described in the text, were enlarged in the insets, to highlight altered localization and colocalization of cathepsins. Arrowheads indicate CD along the base of membrane ruffles (A) and open arrows indicate areas of intense CD labeling. Graphs illustrate the distribution of CB (G) and the ratio of colocalization of CB with CD (H) over representative planes of the basal, middle and apical layers of the basal-apical axis (I). FITC-labeled CB (green) was observed in the degraded trail (J), illustrating that CB may play a role in remodelling of the ECM during the migratory process. A small, rounded unlabeled area is indicated by a large arrowhead. (Bars = 10 μm)


Basal layer
Middle layer
Apical layer
CB
CD
MCF10A
MCF10AneoT
A
B
C
D
E
F
10 µm

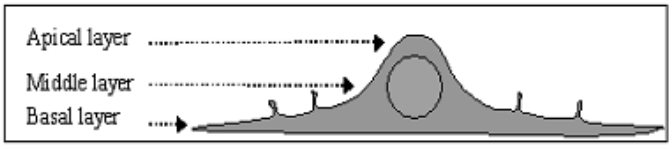
G


Distribution of CB (n=4)
Pixels/µm²
30
20
10
0
Basal
Middle
Apical
CB in 10A 11 24 9
CB in NT 14 26 9

H


Colocalization of CB with CD (n = 4)
Pixels/µm²
30
20
10
0
Basal
Middle
Apical
10A CB:CD 7.9 0.5 0.0
NT CB:CD 2.9 14 3.3

I


Apical layer
Middle layer
Basal layer

J

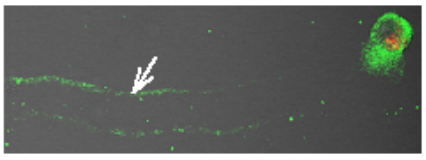
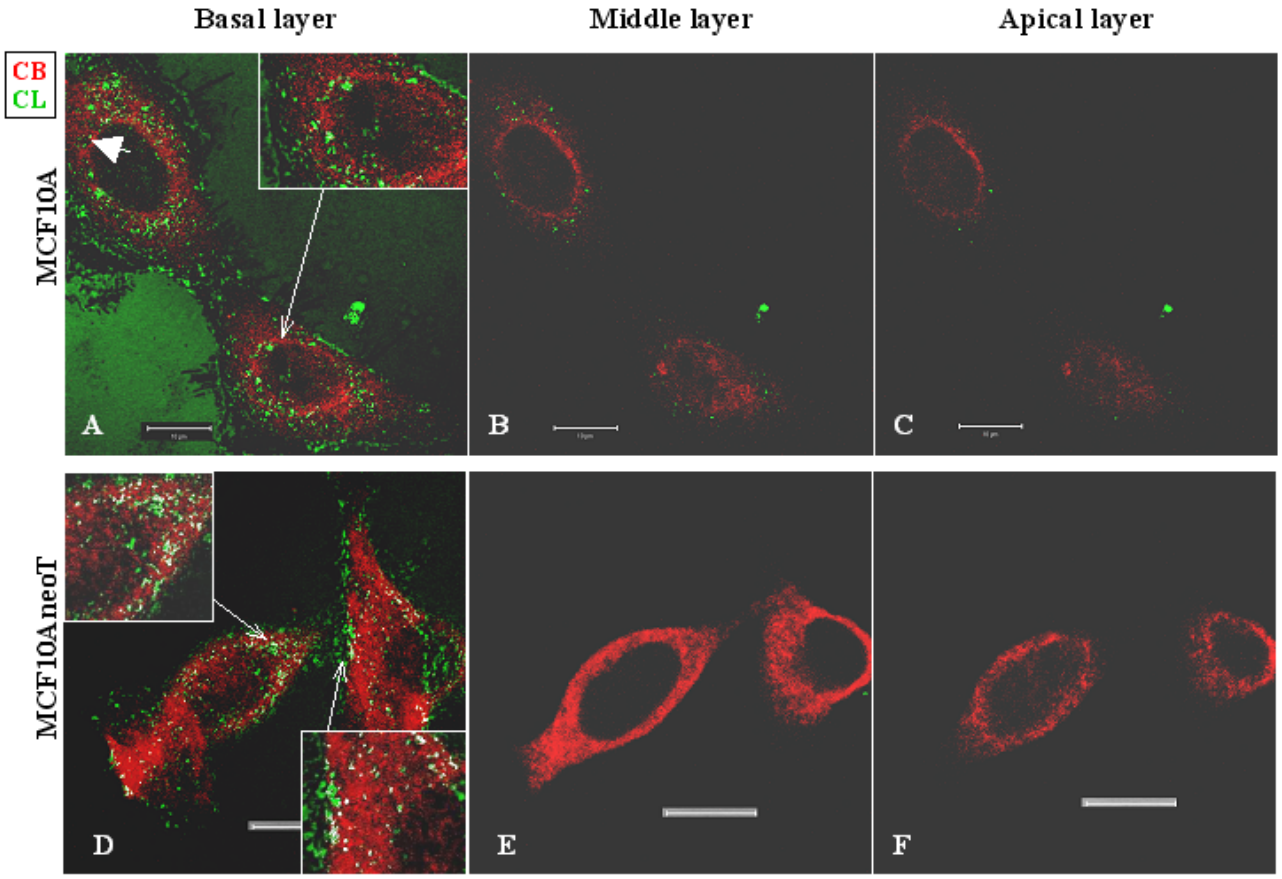
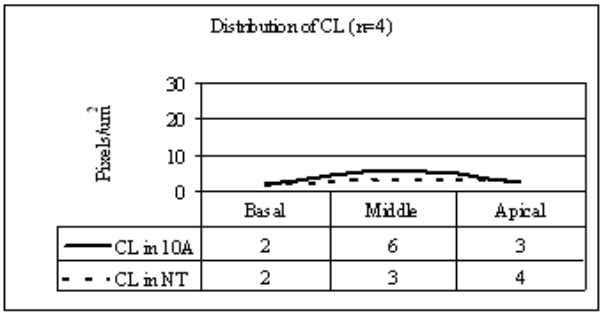


Figure 3.5 The distribution and colocalization of CB with CL over the basal-apical axis in MCF10A and MCF10AneoT cells.

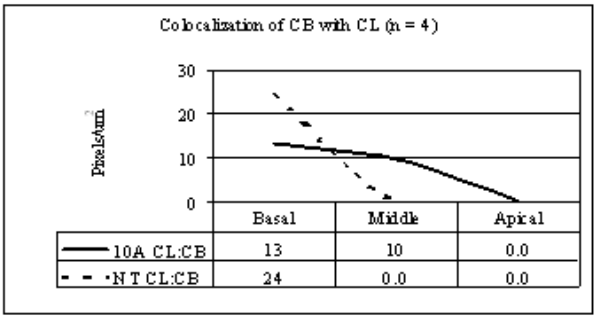
The distribution and colocalization of Cy3-labeled CB with FITC-labeled CL was illustrated over the basal-apical axis of MCF10A (A-C) and MCF10AneoT cells (D-F). Insets illustrated altered localization and colocalization. Graphs illustrate the distribution of CL (G) and the ratios of colocalization of CB with CL or CL with CB (H) over the basal-apical axis. Immunolabeled CL (green) was also observed in the nucleus of dividing cells that was double labelled with LysoTracker Red DND-99 (I). A small, rounded unlabeled area is indicated by a large arrowhead. (Bars = 10 μm)


Basal layer
Middle layer
Apical layer
CB
CL
MCF10A
MCF10AneoT
A
B
C
D
E
F

G


Distribution of CL (n=4)
Pixels/um2
30
20
10
0
Basal
Middle
Apical
CL in 10A 2 6 3
CL in NT 2 3 4

H


Colocalization of CB with CL (n = 4)
Pixels/um2
30
20
10
0
Basal
Middle
Apical
10A CL:CB 13 10 0.0
NT CL:CB 24 0.0 0.0

I

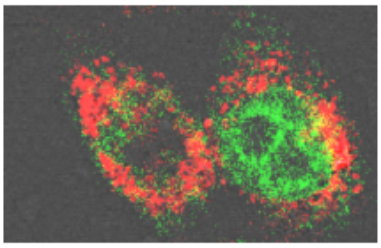
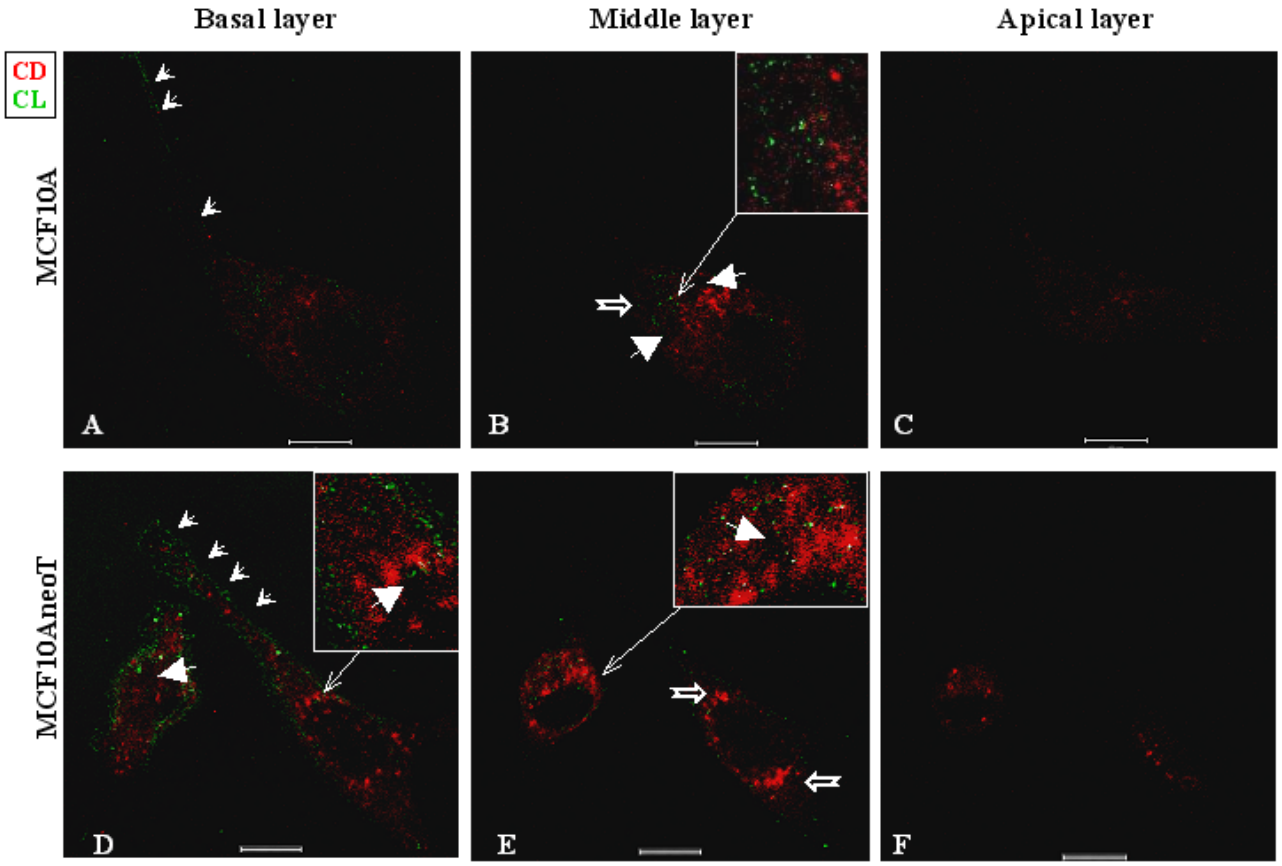
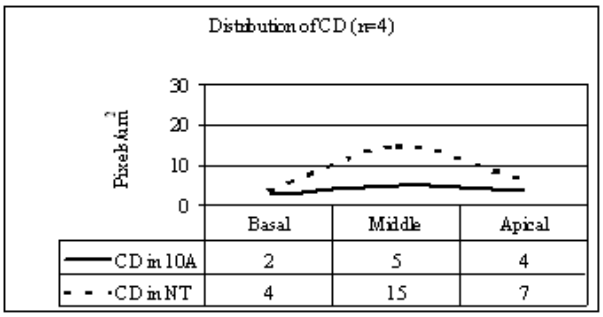


Figure 3.6 The distribution and colocalization of CD with CL over the basal-apical axis in MCF10A and MCF10AneoT cells.

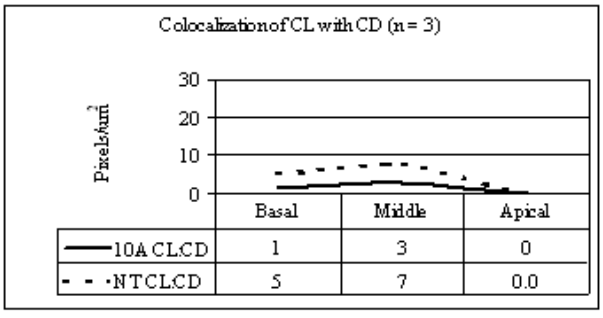
The distribution and colocalization of Cy3-labeled CD with FITC-labeled CL was illustrated over the basal-apical axis of MCF10A (A-C) and MCF10AneoT cells (D-F). Insets illustrate altered localization and colocalization, and open arrows indicate areas of intense CD labeling. Arrowheads indicate punctate areas of CL labeling along the podocytes. Graphs illustrate the distribution of CD (G) and the ratio of colocalization of CL with CD (H) over the basal-apical axis. A small, rounded unlabeled area is indicated by a large arrowhead. (Bars = 10 μm)


Basal layer
Middle layer
Apical layer
CD
CL
MCF10A
MCF10AneoT
A
B
C
D
E
F

G


Distribution of CD (n=4)
Pixels/um2
30
20
10
0
Basal
Middle
Apical
CD in 10A 2 5 4
CD in NT 4 15 7

H


Colocalization of CL with CD (n = 3)
Pixels/um2
30
20
10
0
Basal
Middle
Apical
10A CL:CD 1 3 0
NT CL:CD 5 7 0.0

Overall, the amount of colocalization of CB and CD in MCF10A and MCF10AneoT cells is similar (Figure 3.4 A-H) and relatively low. The major difference is that the vesicles are widely dispersed into 2 extending invadopodia (lamellipodia) in the c-Ha-*ras*(V12)-transfected cells, especially in the basal aspect of the cell, though reflecting similar amounts of colocalization (Figure 3.4 D). Such spread may be due to alkalinization of the cytoplasm by c-Ha-*ras*(V12) transfection (Ritter *et al.*, 1997) (Section 3.8.2.1).

Sparse immunolabeling for CL was seen, mostly in the basal or middle aspects of the MCF10A cell Figure 3.5 A, B, green and Figure 3.6 A, B, green), and in mostly the basal region of c-Ha-*ras*(V12)-transfected cells (Figure 3.5 D, green and Figure 3.6 D, green). Such labeling was noted more specifically at contact points with the underlying matrix (Figure 3.5 D and Figure 3.6 arrowheads in A, D). Generally CL levels in cells is low, making graphical analysis difficult (Figure 3.6 G). It was noted that, especially in cells that seemed to be dividing, CL was observed in the nucleus (Figure 3.5 I, green). Increased metabolic activity during G1 has been reported to induce the cell cytoplasm to become more reducing and alkalinized as it enters the S-phase (Menon *et al.,* 2003). Since c-Ha-Ras(V12) may constantly signal to the transfected cell to proliferate, CL is possibly involved in the proteolytic control of gene transcription components during the mitotic process (Nepveu, 2001; Goulet *et al.*, 2004).

CL seems to be generally secreted from the normal cell (Fujishima *et al.*, 1997; Kirschke *et al.*, 1998). It may, therefore, be present in non-acidified secretory vesicles and not colocalize with CD (Figure 3.6 A-C, H), which is mostly active only in acidic vesicles. Colocalization between CB and CL was observed mostly at the base in both the normal MCF10A and c-Ha-*ras*(V12)-transfected MCF10AneoT cells (Figure 3.5 A, D). A significant increase in colocalization of CL with CB was noted, however, after c-Ha-*ras*(V12) transfection, compared to normal cells (Figure 3.5 A, D, insets and Figure 3.5 H). This may arise to an need to digest increased endocytosed BM (Turk *et al.*, 1999; Sameni *et al.*, 2000), since it was shown that CB and CL both degrade cartilage collagens at different rates, giving rise to different sizes in end product (Maciewicz *et al.,* 1990).

A similar, though lower, colocalization trend between CB and CL was observed (Figure 3.5 A-C and D-F) than seen between CB and CD (Figure 3.4 A-C and D-F), with cathepsin-containing vesicles apparently carrying mainly one of the cathepsins labeled. This may imply storage in separate 'vesicles', one for each lysosomal enzyme type, except in the basal aspect of the normal cell MCF10A cells, where CB and CD showed an increased colocalization (Figure 3.4 A), and similarly for CB and CL in the transfected MCF10AneoT cells (Figure 3.5 D).

It seems from our immunolabeling results that, while CB, CD and CL in normal, non-transfected cell were distributed generally along the basal plane of the cell, these cathepsins were located mostly separately, except for CB and CD in the MCF10A cells (Figure 3.4 A and H) and CB and CL in the MCF10AneoT cells (Figure 3.5 H). Colocalization of CD with either CB or CL was, however, very limited (Figure 3.4, Figure 3.5 and Figure 3.6). The concentrated CD in the vesicles mainly distributed towards the middle and discrete regions of the cell (Figure 3.6 B, E) could be an indication that this enzyme is involved in a secondary level of degradation of BM components that were not completely digested by CB and CL in vesicles in the basolateral part of the cell. Transfection with c-Ha-*ras*(V12), however, clearly influences the distribution of each of these cathepsins differently. While the colocalization of CB and CL becomes more pronounced in transfected cells, and seemed to be restricted largely to the base of the cell (Figure 3.5 D, H), the colocalization of CB with CD was mainly limited to the perinuclear region (Figure 3.4 E, H). In addition a clear duplication of the CD-containing compartments previously speculated to be the JNS network on either side of the nucleus, was noted after transfection (Figure 3.4 E, Figure 3.5 E and Figure 3.6 D). In collaborative studies an increase in the levels of CB, CD and CL was shown in c-Ha-Ras(V12) expressing MCF10AneoT cancer cells in our laboratory (van Rooyen, 2009) (Extracts from thesis in Appendix III). The suggested increased invasive potential of c-Ha-*ras*(V12) transfected cells may be facilitated by the degradation of the underlying matrix and digestion load increase, enhancing the requirement for higher cell levels of CB and CL at the base of the transfected cell to cope with the increased levels of BM-derived breakdown products endocytosed in a cell where the pH of vesicles may not be sufficiently acidic, due to c-Ha-*ras*(V12) transfection (Ritter *et al.*, 1997) (as described in Section 3.8.2.1). The requirement for CB and CL instead of CD could possibly be due to the substrate specificity of CB and CL, but also possibly as these

enzymes may function at a higher pH (Dehrmann *et al.*, 1995; Pillay *et al.*, 2002a). This possibility was tested in the next study (Section 3.5). These findings lend weight to the theory that these cathepsins, especially during normal cell migration and metastasis, each seems to have specific functions, and each may be activated and regulated individually. There may thus be a range of late endosome-like degradative structures, assembled when digestion is required, each with its own functionally related characteristics. These may include variation in the luminal acidity, as will be discussed in the following section.

The shift noted in the current study (CB and CL shift from a perinuclear location into a peripheral pattern due to c-Ha-*ras*(V12) transfection) could be in response to both increased ECM degradation and endocytosis, but may also be due to over-expression of downstream Ha-Ras effectors in human epithelial and cancer cells, such as Rho, ROCK (Nishimura *et al.,* 2003), LIMK-1 (Nishimura *et al.,* 2004) and Rac (Shin *et al.*, 2005). All these kinases and GTPases may be activated during normal migration due to growth factor stimulation, but these signals are probably elevated in transfected cells due to the mutationally activated c-Ha-Ras(V12). These signaling proteins influence cytoskeletal organization and the cell shape (Tscharntke *et al.*, 2005) (Section 1.4.3 and Figure 1.3 on fold-out), factors that probably affect vesicle distribution. Since the factors causing the vesicle distribution were still not clear at this stage, we assessed vesicle acidity next and finally give possible reasons for this in the final discussion of this study.

Since transfected cells also acidify the extracellular space as previously described (Section 1.5.3), this may also play a role in influencing the trafficking of secretory vesicles, especially CB as reported in MDA-MB-435 breast cancer cells, where they are secreted in a positive feedback loop as a result of an acidified extracellular environment (Cardone *et al.,* 2005).

3.5. Variable acidity of cathepsin-containing vesicles

While enzymatic activity of cathepsins theoretically requires a low luminal pH (Section 3.3.4), a variable pH, maintained by a range of proton pumps, has been reported for endocytic vesicles (Section 3.2.1) (Cain *et al.*, 1989; Forgac, 1999; Counillon *et al.*, 2000). The LE is formed via fusion of cargo-carrying early endosomes with either existing degradative LEs, or with cathepsin-carrying storage

lysosomes. In an earlier study in our laboratory (Jackson *et al.*, 1999, Appendix I; Jackson *et al.*, 2003) it was shown that the acidity of specifically the later endocytic vesicles is decreased in c-Ha-*ras*(V12)-transfected MCF10A cells, compared to the untransfected MCF10A cells. In order to assess which vesicles are acidic and gain some insight into the relative acidity of the cathepsin-containing vesicles, both normal and transfected cells were incubated with LysoTracker Red DND-99, a fluorescently conjugated weak base that accumulates in proton-rich (H^+-rich) structures. These cells were subsequently immunolabeled for individual cathepsins to assess the frequency with which cathepsins occur in vesicles with an acidic lumen. While the pKa of LysoTracker Red DND-99 and pH indicator range is not well defined, it is reported to be an indicator of luminal acidity or pH that is characteristic of especially LE-type degradative vesicles (approximately pH 5.5) and vesicles which have a pH below this (Bucci *et al.,* 2000). In addition, it has been used to indicate alkalinization of compartments, such as during hypoxia (Mukhopadhyay *et al.*, 2007) or with increased oxidative activity (Chen, 2002). In this study LysoTracker Red DND-99 will similarly be used as an indicator of the relative alkalinization of vesicles that may occur after c-Ha-*ras*(V12) transfection, and the associated changes in cathepsin content. Since LysoTracker Red DND-99 emits light in the red range, the cathepsins were immunofluorescently labeled with a FITC-tagged secondary antibody, for clear separation and detection of fluorescence labeling.

3.5.1. Reagents

Reagents used for cell culture were described in Section 2.2.1.

25 nM LysoTracker Red DND-99 in Ham's F12 complete medium (LysoTracker)
1 mM LysoTracker stock (100 µl) was diluted in 4 ml serum-containing complete Ham's F12 culture medium containing 10% (v/v) horse serum.

Reagents used for fluorescence immunolabeling of CB, CD and CL for confocal microscopy were described in Section 2.6.1.

3.5.2. Procedure

MCF10A and MCF10AneoT cells were seeded onto sterile round coverslips in 24 well Tersaki plates and cultured over night in DMEM-Ham's F12 medium supplemented with 10% decomplemented horse serum as described in Section 2.2.2.

For studies on vesicle acidity in combination with intracellular immunolabeling of cathepsins, live, unfixed cells were washed with HBSS (1 x), incubated in LysoTracker Red DND-99 (30 min, 37°C in 10% CO_2), washed with PBS (3 x 5 min), fixed with PFA fixative (10 min) and washed with PBS (5 x 5 min).

Cells were subsequently immunocytochemically labeled for intracellular cathepsins, as described in Section 2.6.2 and Section 3.4.2. Coverslips were incubated in either a chicken antibody to CB (20 μg/ml, diluted in PBS-saponin, 1h), a chicken antibody to CD (20 μg/ml, diluted in PBS-saponin, 1 h) or a rabbit antibody to CL (100 μg/ml, diluted in PBS-saponin, 1 h). Coverslips were washed in PBS-BSA (5 x 5 min) and incubated with a secondary donkey anti-chicken antibody tagged with FITC (diluted 1:400 in PBS-saponin, 1 h), or with a secondary goat anti-rabbit antibody tagged with FITC (diluted 1:200 in PBS-saponin, 1 h). Coverslips were subsequently washed with PBS (3 x 5 min), fixed with PFA (10 min) and washed in PBS (5 x 5 min). Finally, cells were washed, fixed and mounted with Moviol anti-fade reagent and observed on a Zeiss LSM 510 META confocal laser scanning microscope (Heidelberg, Germany).

Images were analysed as described in Section 2.7.

3.5.3. Results and discussion

Compared to normal MCF10A cells (Figure 3.7 A-C, Figure 3.8 A-C and Figure 3.9 A-C), LysoTracker Red DND-99 uptake and distribution in c-Ha-*ras*(V12)-transfected cells (Figure 3.7 D-F, Figure 3.8 D-F and Figure 3.9 D-F) was decreased in general on all apical-to-basal representative planes (Figure 3.7 G), an indication that c-Ha-*ras*(V12) transfection of MCF10A cells is accompanied by considerable alkalinization of initially acidic compartments in the transformed cell, as described earlier by our co-workers laboratory (Jackson *et al.*, 1999) (Abstracts in Appendix II). The low level of uptake of LysoTracker Red DND-99 by the transformed cell also make it impossible to perform meaningful statistical analysis of the cathepsin distribution relative to the distribution of LysoTracker in the MCF10AneoT cells.

In normal cells CB (Figure 3.7 A-C) and CL (Figure 3.9 A-C) were largely unassociated with LysoTracker Red DND-99. It was assumed that these proteases were localized in storage vesicles or lysosomes with a pH higher than the optimum for

activity of most lysosomal cathepsins. The pKa of each of CB and CL can be used as an indication of activity at different luminal acidities, such as has been shown for both CB and CL (Pillay *et al.*, 2002a; Turk *et al.*, 2002; Brix *et al.*, 2008). Colocalization of CB, CD and CL with LysoTracker Red DND-99 was noted at the base of the normal cell, however, where these enzymes are probably involved in degradation of matrix components, such as Col-IV (Sameni *et al.*, 2000) (Figure 3.7 A, Figure 3.8 A and Figure 3.9 A respectively). Such association may occur in digestive organelles, such as the LE.

After c-Ha-*ras*(V12) transfection both CB (Figure 3.7, green) and CL (Figure 3.9 D, E, green) show a general decreased association with the little LysoTracker in LysoTracker Red DND-99-positive vesicles at various levels in cells. In the transfected cells, however, CB and LysoTracker Red DND-99 colocalization was observed in the juxta nuclear basal position (Figure 3.7 D). CL in transfected cells, on the other hand, had a very low association with an acidic juxta nuclear area, where colocalization with LysoTracker Red DND-99 was limited to a very small area next to the nucleus in the medial aspect of the cell (Figure 3.9 E), with very little association at the base of the cell (Figure 3.9 D). CB has a broad pH optimum (Buck *et al.*, 1992; Linebaugh *et al.*, 1999; Pillay *et al.*, 2002a) and CL has maximal proteolytic capacity at a pH that is only slightly acidic (Dehrmann *et al.*, 1995; Turk *et al.*, 1999). Even though both CB and CL can be enzymatically active at higher pH (Kirschke *et al.,* 1998; Pillay *et al.,* 2002a), an increase in pH could severely prejudice proteolytic activity and the build-up of collagen could trigger the compensatory intracellular enzyme levels apparent in the basal aspect of the cell in the case of CL (Figure 3.9 D).

In normal cells almost all immunolabeled CD colocalized with acidic vesicles, especially in one juxta nuclear focal area (Figure 3.8.A, B). This was expected, since CD, mainly involved in high-grade degradation of proteins and peptides and has optimal catalytic activity at pH 2.8-5.0 (Authier *et al.,* 2002). Since CD proteolytic activity is reliant on a low pH environment, this area could possibly be considered as a focused site of optimal degradative activity for CD specifically, and possibly for the cell in general.

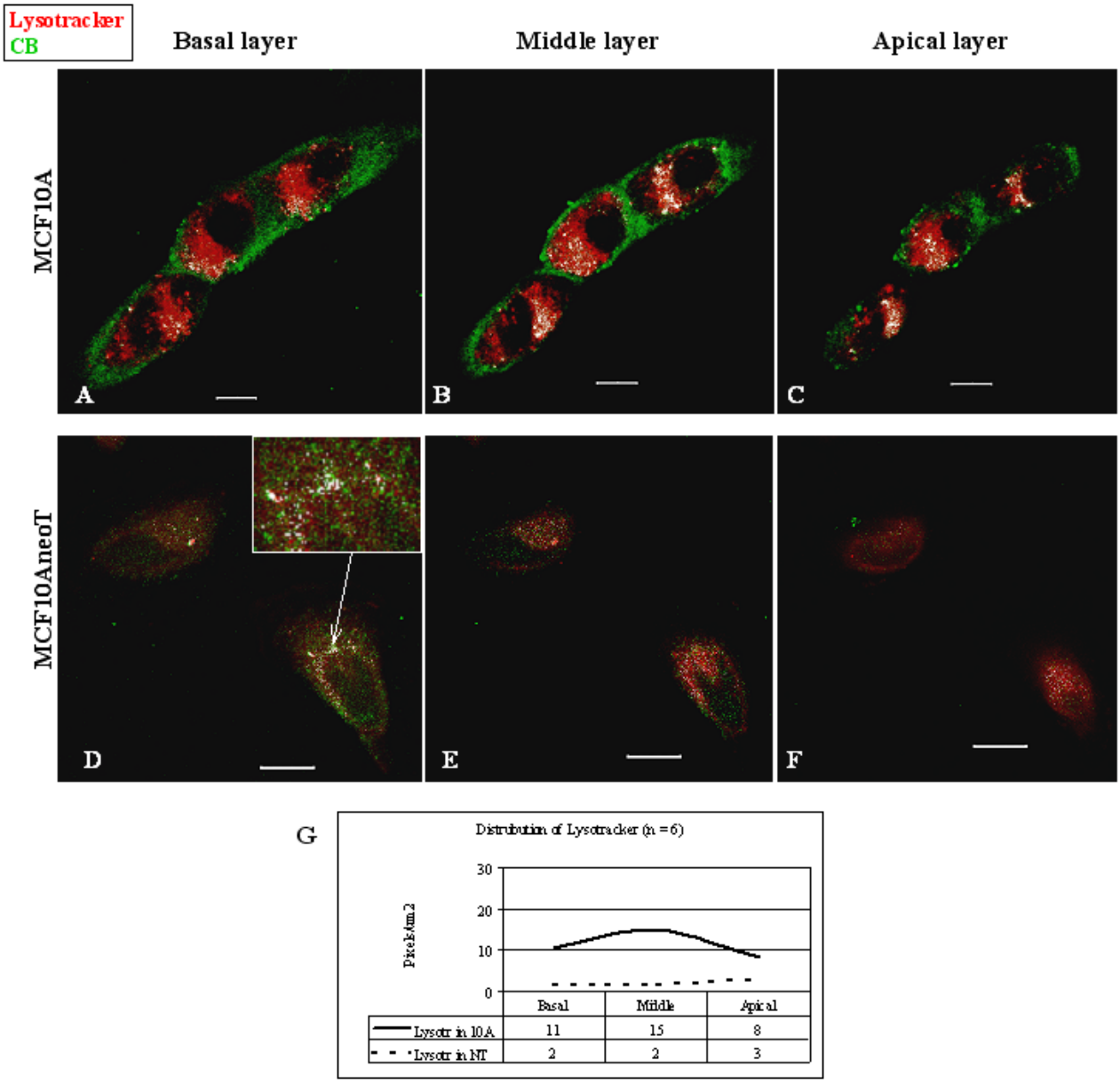


Figure 3.7. The distribution of LysoTracker Red DND-99-labeled acidic vesicles and colocalization with CB, over the basal-apical axis in MCF10A and MCF10AneoT cells.

The distribution and colocalization of FITC-labeled CB (green) with LysoTracker Red DND-99 (red fluorescence) was illustrated over the basal-apical axis of MCF10A (A-C) and MCF10AneoT cells (D-F). A specific area of interest was enlarged to illustrate altered localization and colocalization. The distribution of acidic, LysoTracker Red DND-99 positive vesicles over the basal-apical axis of both MCF10A and MCF10AneoT cells was illustrated on a graph (G). (Bars = 10 µm)

After transfection, however, CD was generally found in two acidic sites at opposite poles of cells, though this is not well shown in the micrograph presented (Figure 3.8 D). The rest of the CD was distributed in vesicles that largely do not seem to colocalize with LysoTracker Red DND-99 and were also spread further away from the nucleus into the invadopodia (lamellipodia) (Figure 3.8 A, D). The required low pH for CD activity may mean that these vesicles, which are not acidic, could possibly

be on the secretory path, or could contain CD that had been endocytosed from the extracellular spaces. Alternatively, such CD may also be present in storage lysosomes. These structures may be distinguished using specific biochemical markers not used in the current study, as will be discussed in the final discussion of this chapter.

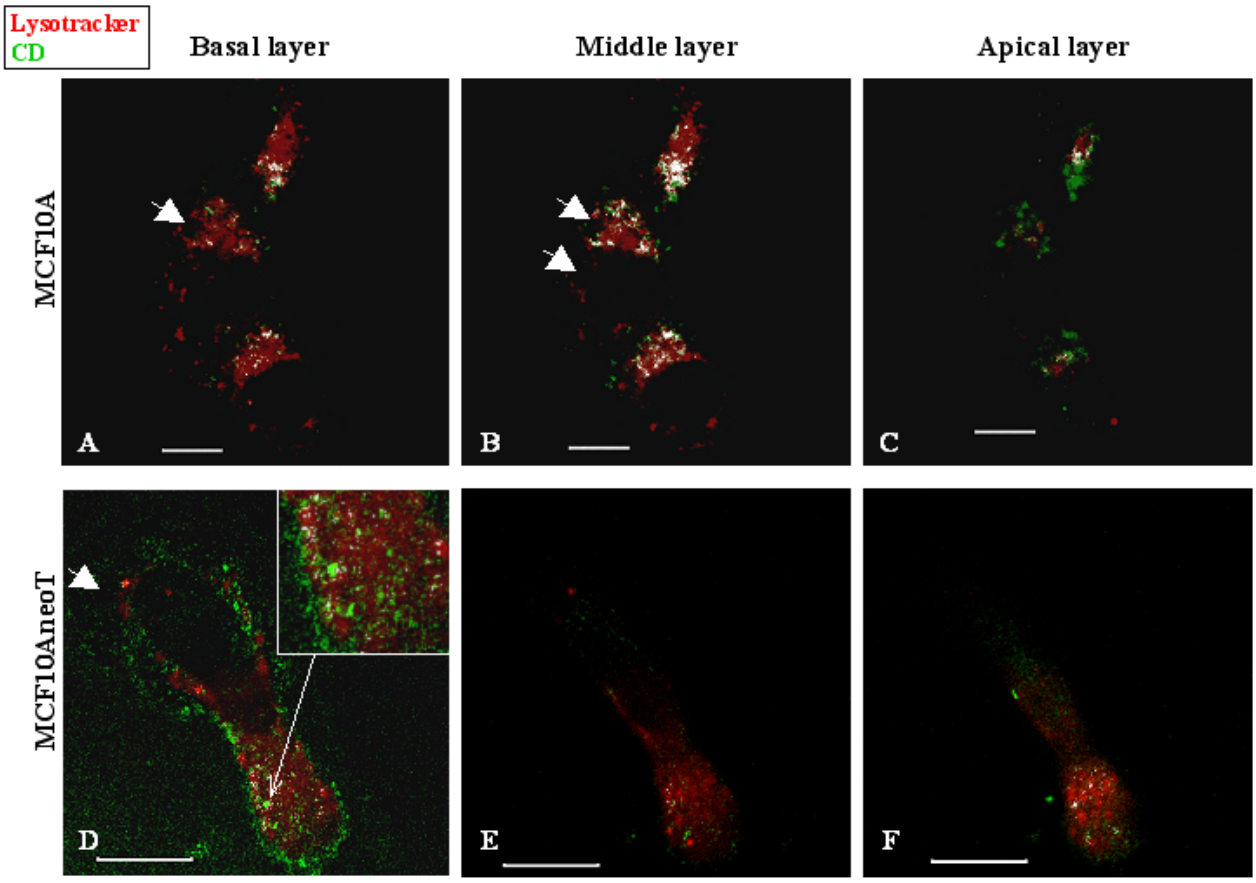


Figure 3.8. The distribution and colocalization of CD in relation to LysoTracker Red DND-99-labeled acidic vesicles, over the basal-apical axis in MCF10A and MCF10AneoT cells.

The distribution and colocalization of FITC-labeled CD (green) with LysoTracker Red DND-99 (red fluorescence) was illustrated over the basal-apical axis of MCF10A (A-C) and MCF10AneoT cells (D-F). A specific area of interest was enlarged to illustrate altered localization and colocalization. A small, rounded unlabeled, non-acidic area is indicated by a large arrowhead. (Bars = 10 µm)

Even though cathepsins are described as acidic hydrolases, it is clear that various vesicle populations exist that vary in their enzyme content and acidity. The separate location of the different cathepsins in both normal and transfected cells could indicate that there may be more than one type of lysosomal storage body, i.e. lysosomal enzymes are stored largely in distinct lysosomes, while colocalized in various degradative sites, various late endosomal populations or lysosomal enzyme-containing

digestive vesicles that form during the endocytic process. This idea is supported by studies on the sorting of cargo in the EE (Duclos *et al.,* 2003) and the different times and way in which variety of endocytosed cargo is degraded. Newly-endocytosed vesicles interact with intracellular vesicles on an ongoing basis (Desjardins, 1995; Bright *et al.*, 1997; Arora *et al.*, 2000; Duclos *et al.*, 2003) and ECM components e.g. collagen are degraded in progressive degrees by CB (Buck *et al.,* 1992) over time (Arora *et al.,* 2000) and at varying acidity in EEs as they mature into acidic LEs (Authier *et al.,* 2005).

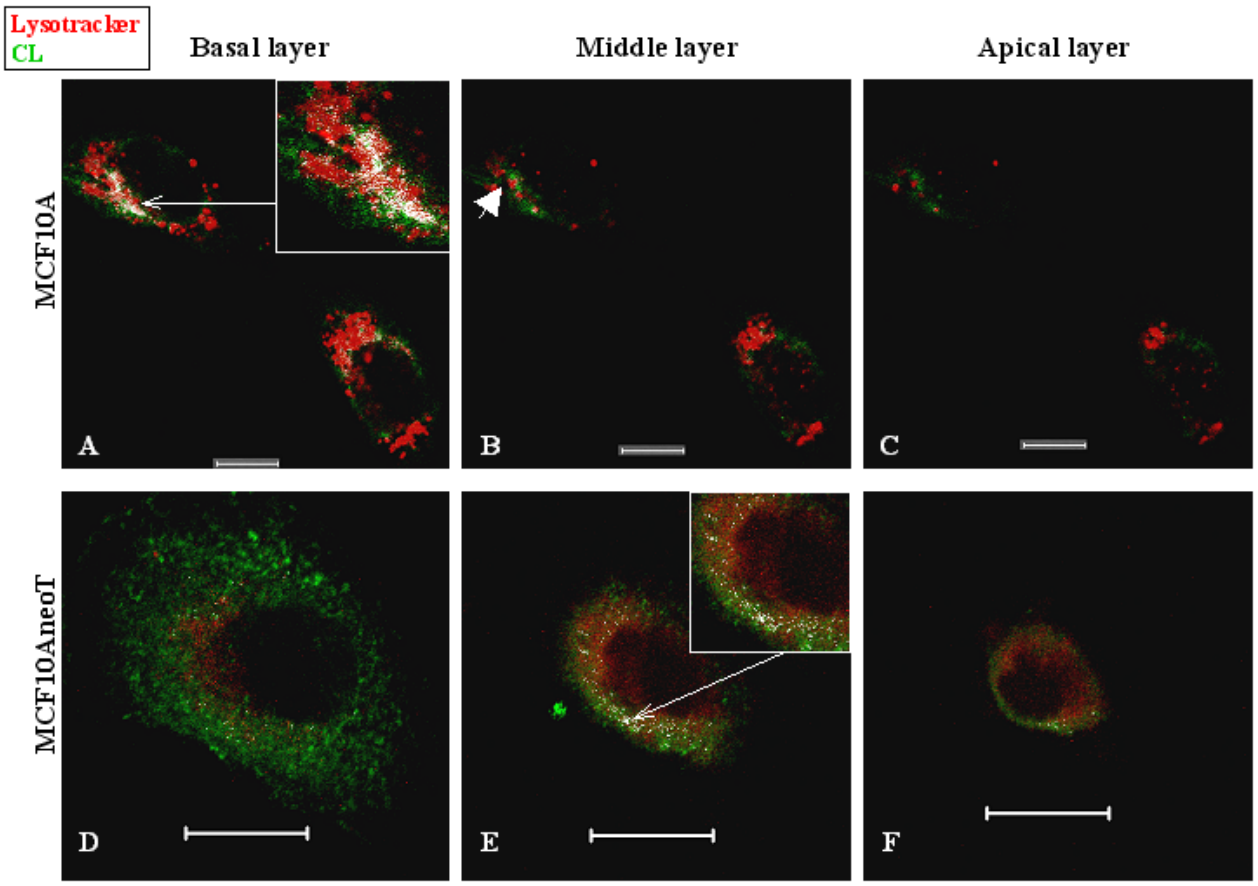


Figure 3.9. The distribution and colocalization of CL in relation to LysoTracker Red DND-99-labeled acidic vesicles, over the basal-apical axis in MCF10A and MCF10AneoT cells.

The distribution and colocalization of FITC-labeled CL (green) with LysoTracker Red DND-99 (red fluorescence) was illustrated over the basal-apical axis of MCF10A (A-C) and MCF10AneoT cells (D-F). A specific area of interest was enlarged to illustrate altered localization and colocalization. A small, rounded unlabeled, non-acidic area is indicated by a large arrowhead. (Bars = 10 µm)

The effect that c-Ha-*ras*(V12) transfection has on the efficiency of the proton pump or the components, that are known to acidify the late endosomal vesicle population, (Section 3.2.1.4), and the influence on distribution of the cathepsins, will be more

comprehensively explained in Section 3.8.2.7. Such changes may, however, affect the degradative efficiency of vesicle populations. The effect of c-Ha-ras(V12) transfection will be investigated in terms of the effect on colocalization of cathepsins with LAMP-2 or LysoTracker Red DND-99 with LAMP-2 in the following section.

3.6. Colocalization of LAMP-2 with cathepsins or LysoTracker Red DND-99

The best-known function of the endocytic system is the turnover of endocytosed cargo assisted by enzymes such as the cathepsins. These are involved in both the extracellular and intracellular degradation of ECM components during both migration and invasive cancer. From results reported in the preceding sections, only some cathepsin-positive vesicles seem to be acidic, a characteristic necessary for cathepsin proteolytic activity. Markers often used for the distinction of various endocytic vesicles are the cathepsins, luminal acidity, indicated by LysoTracker Red DND-99, and LAMP-2, an integral membrane component reported for both the LE and the lysosome (Section 3.2.2.1).

The importance of LAMP-2 in degradative vesicles, in addition to maintaining the structural integrity of these vesicles, is that it has been considered to be in indicator of low luminal acidity (Eskelinen *et al.*, 2003), since it may serve to isolate an acidic, hydrolytic environment from the rest of the more alkaline cytoplasm and the proteins contained in it (Pillay *et al.*, 2002b). In addition, it may play a role in vesicle maturation along the degradative route (Section 3.2.2.1). To further characterize cathepsin-containing and low acidity vesicles, their association with specifically LAMP-2, was investigated. This combination of characteristics has been assigned in the literature to both the LE and lysosomes. Therefore, colocalization of LAMP-2 with cathepsins and a marker for acidity, could indicate that the cathepsin-containing vesicles are on the degradative route. In addition, the size of vesicles in combination with positive or negative LAMP-2 labeling, may assist in distinguished vesicles as being part of a degradative population, from those which are classical primary lysosomes involved in cathepsin storage (Gruenberg *et al.*, 1989). Alterations in the combination of characteristics may also assist in assessing the influence of c-Ha-*ras*(V12) on these vesicle populations.

3.6.1. Reagents

Reagents used for cell culture were described in Section 2.2.1.

Reagents used for LysoTracker Red DND-99 incubation was described in Section 3.5.1.

Reagents used for intracellular immunolabeling for confocal microscopy were described in Section 2.6.1.

3.6.2. Procedure

For intracellular double labeling of LAMP-2 and cathepsins, cells were double labeled, as described in Section 2.6.2 and Section 3.4.2. Coverslips with fixed, permeabilized cells were incubated in either a chicken antibody to CB (20 μg/ml, diluted in PBS-BSA, 1h), a chicken antibody to CD (20 μg/ml, diluted in PBS-BSA, 1 h), or a rabbit antibody to CL (100 μg/ml, diluted in PBS-BSA, 1 h), washed, blocked with PBS-BSA and incubated with either a secondary donkey anti-chicken antibody conjugated to Cy3 (diluted 1:1500 in PBS-BSA, 1 h) or a secondary goat anti-rabbit antibody conjugated to TRITC (diluted 1:300 in PBS-BSA, 1 h), washed, fixed and washed. Coverslips were subsequently incubated in a mouse anti-LAMP-2 (diluted 1:400 in PBS-BSA, 1h), washed in PBS (3 x 5 min), and incubated with a FITC-conjugated secondary donkey anti-mouse antibody (diluted 1:150 in PBS-BSA, 1 h).

For studies on intracellular immunolabeling of LAMP-2 in combination with vesicle acidity, subconfluent cells grown on coverslips were prepared, as described in Section 2.2.2. Live, non-fixed cells were washed, incubated with LysoTracker Red DND-99, as described in Section 3.5.2, washed, fixed, permeabilized and immunolabeled for LAMP-2 with primary and fluorescently tagged secondary antibodies, as described for single labeling in Section 2.6.2. Coverslips were incubated in a mouse anti-LAMP-2 (diluted 1:400 in PBS-BSA, 1h), washed in PBS (3 x 5 min), blocked with PBS-BSA (30 min) and incubated with a FITC-conjugated secondary donkey anti-mouse antibody (diluted 1:150 in PBS-BSA, 1 h).

Finally, coverslips were mounted with Moviol anti-fade reagent and observed on a Zeiss LSM 510 META confocal laser scanning microscope (Heidelberg, Germany).

Variations in the size of vesicles that contained either the cathepsins, and/or LAMP-2 were calculated as described in Section 2.7.

3.6.3. Results and discussion

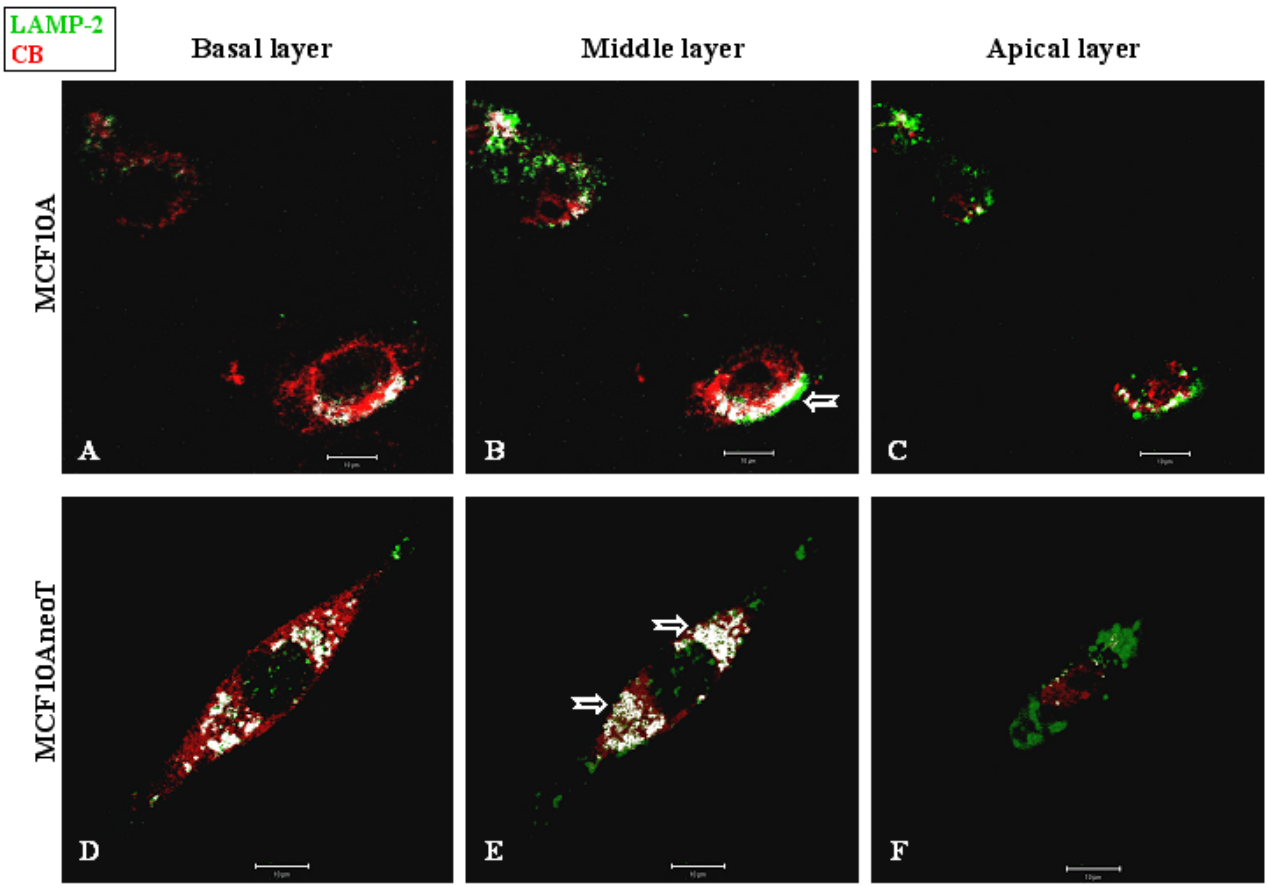
In normal MCF10A cells colocalization between LAMP-2 and CB was limited to a discreet juxta nuclear area, which could be followed through all apical to basal levels on one side of the nucleus (Figure 3.10 B, open arrow). In the basal and medial areas of the c-Ha-*ras*(V12)-transfected MCF10AneoT cells two areas of colocalization on either side of the nucleus was observed (Figure 3.10 D, E). This colocalization occurred in the basal, but mainly the medial region (Figure 3.10 G) in both the normal and transfected cells. On the other hand, in both cell types, many CB-positive vesicles in the basal region of the cell did not contain LAMP-2 (Figure 3.10 A, D). In the normal cells CB immunolabeling was limited to the perinuclear area, while in the MCF10AneoT cells, labeling was spread further from the perinuclear area, similar to the results obtained in Section 3.4 (Figure 3.4 A, D). In MCF10AneoT cells LAMP-2 –positive vesicles were spread into cell protrusions and the tips of lamellipodia (Figure 3.10 D, E), with only a few isolated LAMP-2-positive vesicles in the normal cells spread into less marked protrusions (Figure 3.10A, B).

Analysis of vesicle sizes gave valuable insight into the existence of different vesicle populations, and clearly showed the altered association of LAMP-2 and the cathepsins of interest, in c-Ha-*ras*(V12)-transfected cells compared to that in non-transfected cells (Figure 3.10 H, I). In general it seemed that, in MCF10A cells, CB not colocalized with LAMP-2, was located in small vesicles of approximately 0.7 μm^2 in diameter (Figure 3.10 H, red line). As these vesicles were spread furthest from the nuclear area (Figure 3.10 A, B) and possibly not acidic (Section 3.5.3, Figure 3.7 A) and could, therefore, be storage or carrier lysosomes. Vesicles in which CB did colocalize with LAMP-2 were in a perinuclear position of the MCF10A and c-Ha-*ras*(V12)-transfected MCF10AneoT cells (Figure 3.10 D, E) and were, on average, almost double the size of single-labeled vesicles, with an approximate diameter of 1.1 μm (Figure 3.10 H, I, yellow line), ranging from 1-1.4 μm in MCF10A cells (Figure 3.10 H, yellow line) and from 0.6 to 1.7 μm in transfected cells (Figure 3.10 I, yellow line).

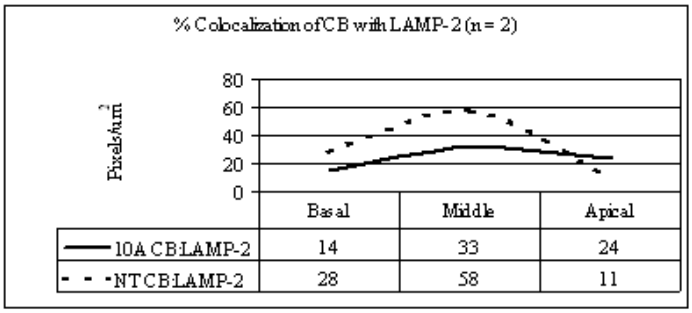
Since LAMP-2 is often used as a marker for the LE, these structures were considered to be LEs. In addition, due to their juxta nuclear position, these vesicles were also considered to be involved in degradation. Relative to the low number of individual, clearly distinguishable vesicles positive for both CB and LAMP-2 in the MCF10A cells, an increase of almost 5-fold was observed in the number of such labeled vesicles in the MCF10AneoT cells (Figure 3.10 H, I yellow line). In addition, the number of vesicles that contained LAMP-2 only also increased (Figure 3.10 green line). This may be an indication of increased autophagy or increased ECM uptake due to elevated metabolism and thus nutrient insufficiency, in cancer cells (Section 3.3.2), as well as the possible presence in these structures of proteolytic enzymes other than CB. We speculated that the level of CB was increased in transfected cells (Figure 3.10 G and Figure 3.10 I, red and yellow lines) to assist in degradation of endocytosed ECM components, or possibly also, as with LAMP-2 association, during nutrient stress related autophagy (Section 3.3.2). The increased association of CB with LAMP-2 (Figure 3.10 G) and the effect of apparent altered vesicle pH deducted from the LysoTracker Red DND-99 in previous experiments (Figure 3.7 A-F) on the degradative capacity of digestive vesicles supported this theory.

Figure 3.10. The distribution and colocalization of CB in relation to LAMP-2, over the basal-apical axis in MCF10A and MCF10AneoT cells.

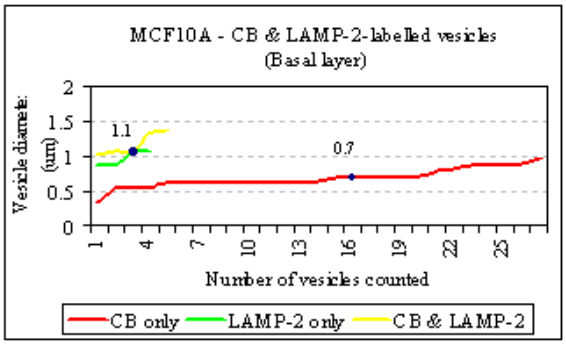
The distribution and colocalization of Cy3-labeled CB (red) with FITC-labeled LAMP-2 (green) was illustrated over the basal-apical axis of MCF10A (A-C) and MCF10AneoT cells (D-F). Open arrows indicate the position of either one (in the MCF10A [B]) or two (in the MCF10AneoT [E[) degradative areas in the cell next to the nucleus. The ratio of colocalization of CB with LAMP-2 over the basal-apical axis was illustrated on a graph (G). The sizes of vesicle containing either CB [red lines] or LAMP-2 [green lines], and vesicles containing both these markers [yellow lines], for either the MCF10A (H) or the MCF10AneoT cells (I) were illustrated on graphs. A filled circle indicates the average vesicle size for each data range. (Bars = 10 μm)


LAMP-2
CB
Basal layer
Middle layer
Apical layer
MCF10A
MCF10AneoT
A
B
C
D
E
F

G


% Colocalization of CB with LAMP-2 (n = 2)
Pixels/um2
80
60
40
20
0
Basal
Middle
Apical
10A CB:LAMP-2
14
33
24
NT CB:LAMP-2
28
58
11

H


MCF10A - CB & LAMP-2-labelled vesicles
(Basal layer)
Vesicle diameter (um)
2
1.5
1
0.5
0
1.1
0.7
1
4
7
10
13
16
19
22
25
Number of vesicles counted
CB only
LAMP-2 only
CB & LAMP-2

I

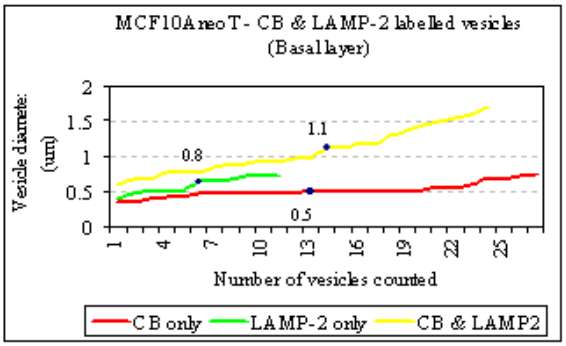
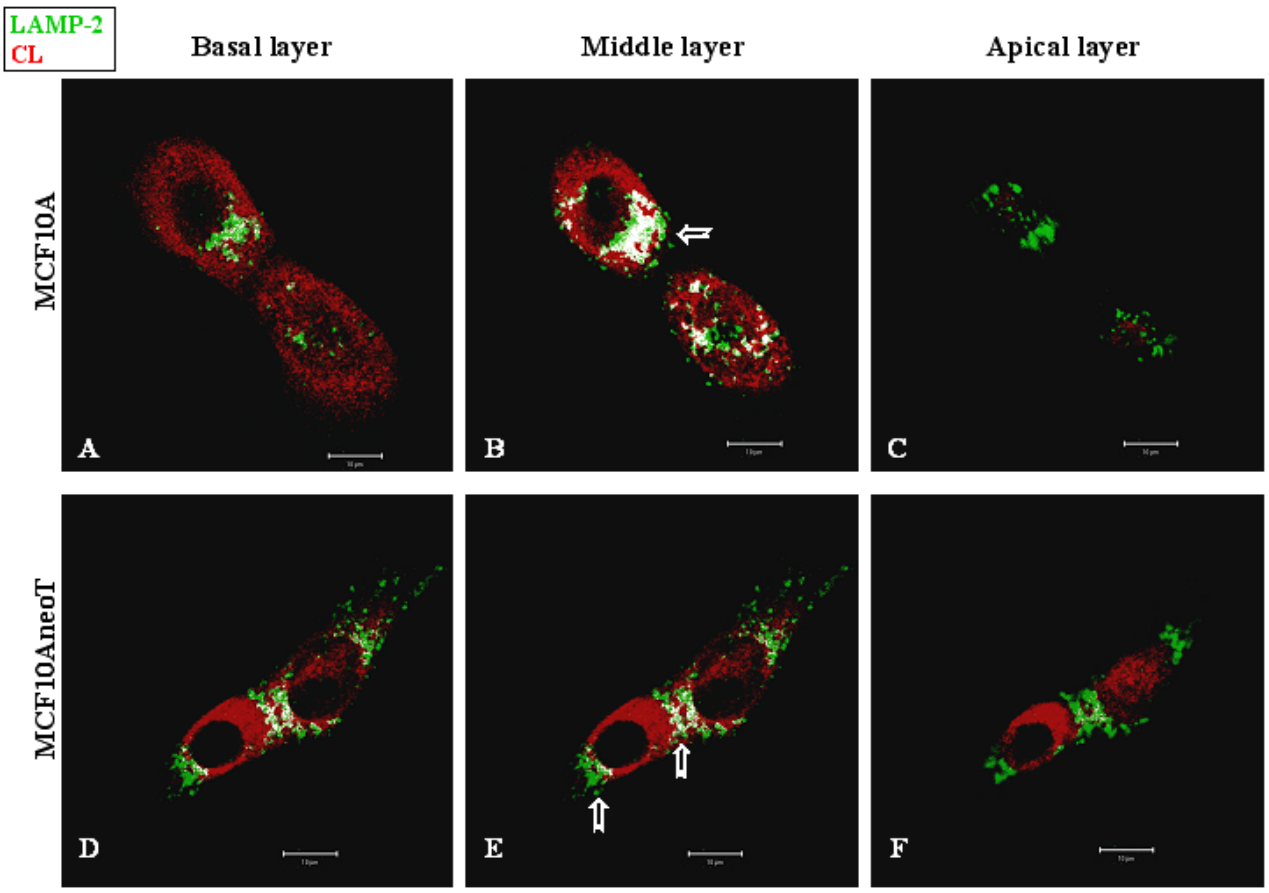
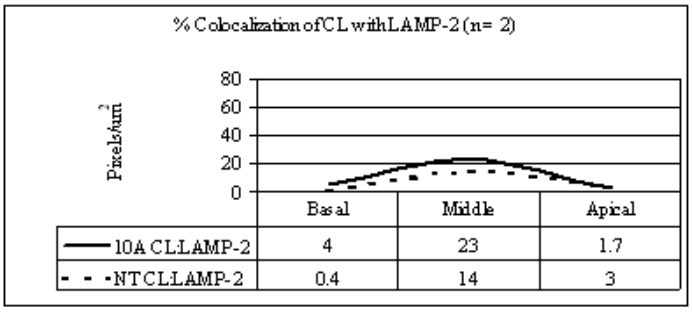

MCF10AneoT - CB & LAMP-2 labelled vesicles
(Basal layer)
Vesicle diameter (um)
2
1.5
1
0.5
0
0.8
1.1
0.5
1
4
7
10
13
16
19
22
25
Number of vesicles counted
CB only
LAMP-2 only
CB & LAMP2

Figure 3.11. The distribution and colocalization of CL in relation to LAMP-2, over the basal-apical axis in MCF10A and MCF10AneoT cells.

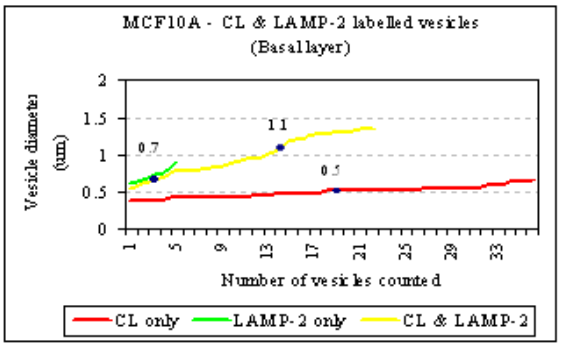
The distribution and colocalization of Cy3-labeled CL with FITC-labeled LAMP-2 was illustrated over the basal-apical axis of MCF10A (A-C) and MCF10AneoT cells (D-F). Open arrows indicate the position of either one (in the MCF10A [B]) or two (in the MCF10AneoT [E]) degradative areas in the cell next to the nucleus. The ratio of colocalization of CL with LAMP-2 (G) over the basal-apical axis was illustrated on a graph. The sizes of vesicle containing either CL [red line in H] or LAMP-2 [green line in H and I], and vesicles containing both these markers [yellow line in H and I] in the MCF10A cell line (H) were illustrated on a graph. A filled circle indicates the average vesicle size for each data range. (Bars = 10 μm)


LAMP-2
CL
Basal layer
Middle layer
Apical layer
MCF10A
MCF10AneoT
A
B
C
D
E
F

G


% Colocalization of CL with LAMP-2 (n= 2)
Pixels/um²
80
60
40
20
0
Basal
Middle
Apical
10A CL LAMP-2
4
23
1.7
NTCL LAMP-2
0.4
14
3

H


MCF10A - CL & LAMP-2 labelled vesicles
(Basal layer)
Vesicle diameter (um)
Number of vesicles counted
CL only
LAMP-2 only
CL & LAMP-2

I

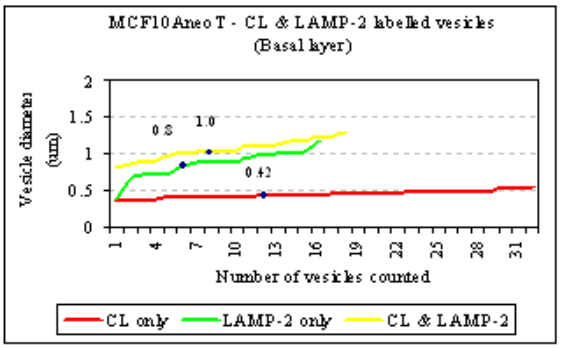
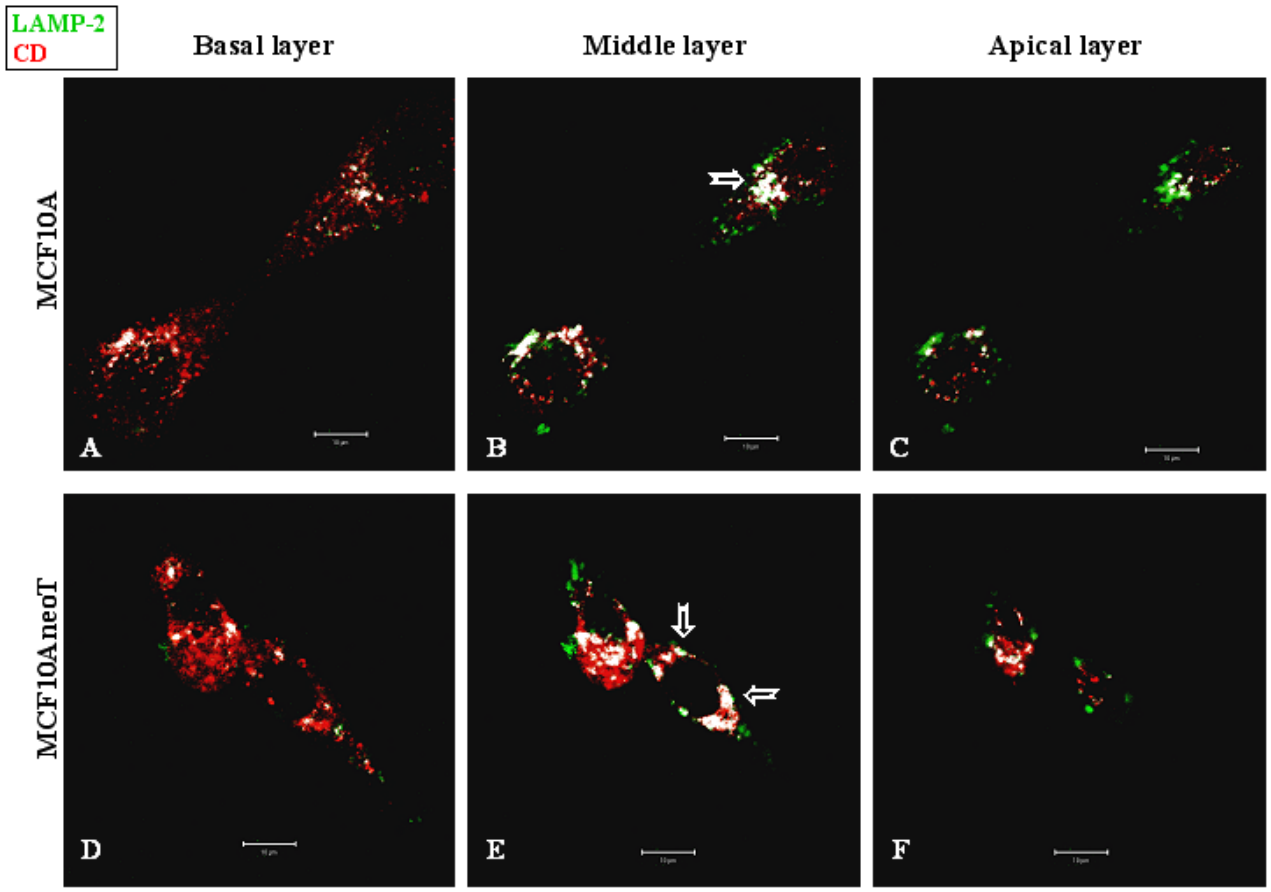
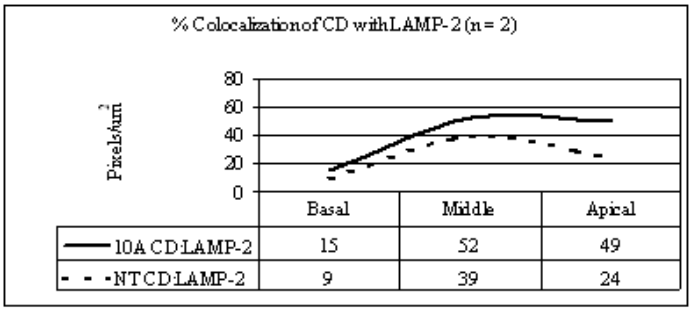

MCF10AneoT - CL & LAMP-2 labelled vesicles
(Basal layer)
Vesicle diameter (um)
Number of vesicles counted
CL only
LAMP-2 only
CL & LAMP-2

Figure 3.12. The distribution and colocalization of CD in relation to LAMP-2, over the basal-apical axis in MCF10A and MCF10AneoT cells.

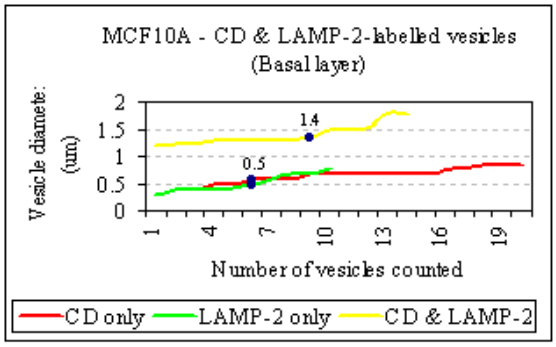
The distribution and colocalization of Cy3-labeled CD with FITC-labeled LAMP-2 was illustrated over the basal-apical axis of MCF10A (A-C) and MCF10AneoT cells (D-F). Open arrows indicate the position of either one (in the MCF10A [B]) or two (in the MCF10AneoT [E]) degradative areas in the cell next to the nucleus. The ratio of colocalization of CD with LAMP-2 over the basal-apical axis was illustrated on a graph (G). The sizes of vesicle containing either CD [red lines in H and I] or LAMP-2 [green lines in H and I], and vesicles containing both these markers [yellow lines in H and I], for either the MCF10A (H) or the MCF10AneoT (I) were illustrated on graphs. A filled circle indicates the average vesicle size for each data range. (Bars = 10 μm)


LAMP-2
CD
Basal layer
Middle layer
Apical layer
MCF10A
MCF10AneoT
A
B
C
D
E
F

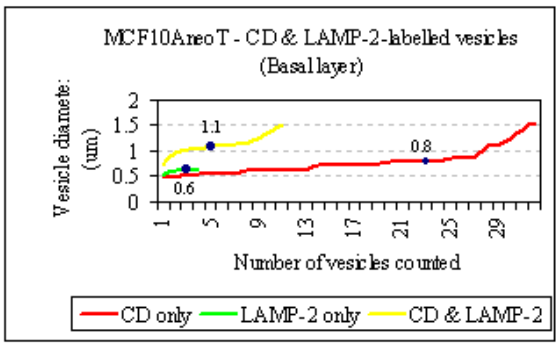
G


% Colocalization of CD with LAMP-2 (n = 2)
Pixels/um2
80
60
40
20
0
Basal
Middle
Apical
10A CD:LAMP-2 15 52 49
NT CD:LAMP-2 9 39 24

H


MCF10A - CD & LAMP-2-labelled vesicles
(Basal layer)
Vesicle diameter (um)
2
1.5
1
0.5
0
1.4
0.5
1
4
7
10
13
16
19
Number of vesicles counted
CD only
LAMP-2 only
CD & LAMP-2

I


MCF10AneoT - CD & LAMP-2-labelled vesicles
(Basal layer)
Vesicle diameter (um)
2
1.5
1
0.5
0
1.1
0.8
0.6
1
5
9
13
17
21
25
29
Number of vesicles counted
CD only
LAMP-2 only
CD & LAMP-2

Though the cells selected for presentation of colocalization of CL with LAMP-2 (Figure 3.11) seem to be dividing, they are nonetheless prepresentative of the distribution of CL and LAMP-2 seen in MCF10AneoT cells. CL (Figure 3.11 A-F, red), a collagenase similar to CB, was also found to have a decreased association with LAMP-2 (Figure 3.11 A-F, green) after transfection (Figure 3.11 D, E and G), compared to the MCF10A cells (Figure 3.11 A, B and G), with large double labeled vesicles (an average diameter of 1.1 μm or 1.0 μm in normal or transfected, respectively) being clustered in a single juxta-nuclear position in normal cells, mostly along the middle plane (Figure 3.11 B, open arrow, G) and in two clearly separate sites in the transfected cells (Figure 3.11 D, E open arrows). The size of LAMP-2 only vesicles remained similar before (an average diameter of 0.7 μm) or after c-Ha-*ras*(V12) transfection (an average of 0.8 μm). The small CL-labeled vesicles (an average diameter of 0.5 μm or 0.4 μm in the MCF10A and MCF10AneoT cells, respectively) (Figure 3.11 H, red line) could be storage circulating lysosomes (Table 3.2) or secretory vesicles transferring unprocessed precursor CL to the PM for extracellular release. In the MCF10AneoT cells (Figure 3.11 D-F) the colocalization of CL with LAMP-2 was lower, with limited CL associated with the LAMP-2-positive structures (Figure 3.11 G).

Immunolabeled CD-LAMP-2 positive vesicles were found in a single juxta nuclear site in normal cells (Figure 3.12 B, open arrow), while largely separated into two sites after transfection (Figure 3.12 E, open arrows). While in the MCF10A cells vesicles labeling for CD and LAMP-2 were observed (an average diameter of 1.4 μm) (Figure 3.12 A-C and H, yellow line), not all CD positive vesicles in non-transfected cells labeled for LAMP-2 and vesicles were largely smaller (an average diameter of 0.6 μm) (Figure 3.12 A-C and H, red line). Further, there seems to be a decrease in LAMP-2 after transfection (Figure 3.12 D-F), especially on the apical layer (Figure 3.12 F), though this is not very evident in the micrograph presented (Figure 3.12 A-F). The graphic presentation of vesicles sizes showed that c-Ha-*ras*(V12)-transfected MCF10A cells contained fewer large vesicles that were positively labeled for CD than in the normal (Figure 3.12 H and I, yellow lines). Since it was demonstrated that the vesicular pH could be associated with the wider distribution of CD positive vesicles (Figure 3.8 D, E), it could be speculated that these vesicles were affected by alkalinization associated with a NHE-1 proton pump (Section 3.2.1.2) activation by

Ha-*ras*(V12) (Ritter *et al.,* 1997). Alternatively, more CD could be on a secretory route, since it is reported that extracellular levels of CD may play a role in cancer progression and invasion and are secreted post transfection and resultant cytoplasmic alkalinization (Liaudet *et al.*, 1995; Couissi *et al.*, 1997).

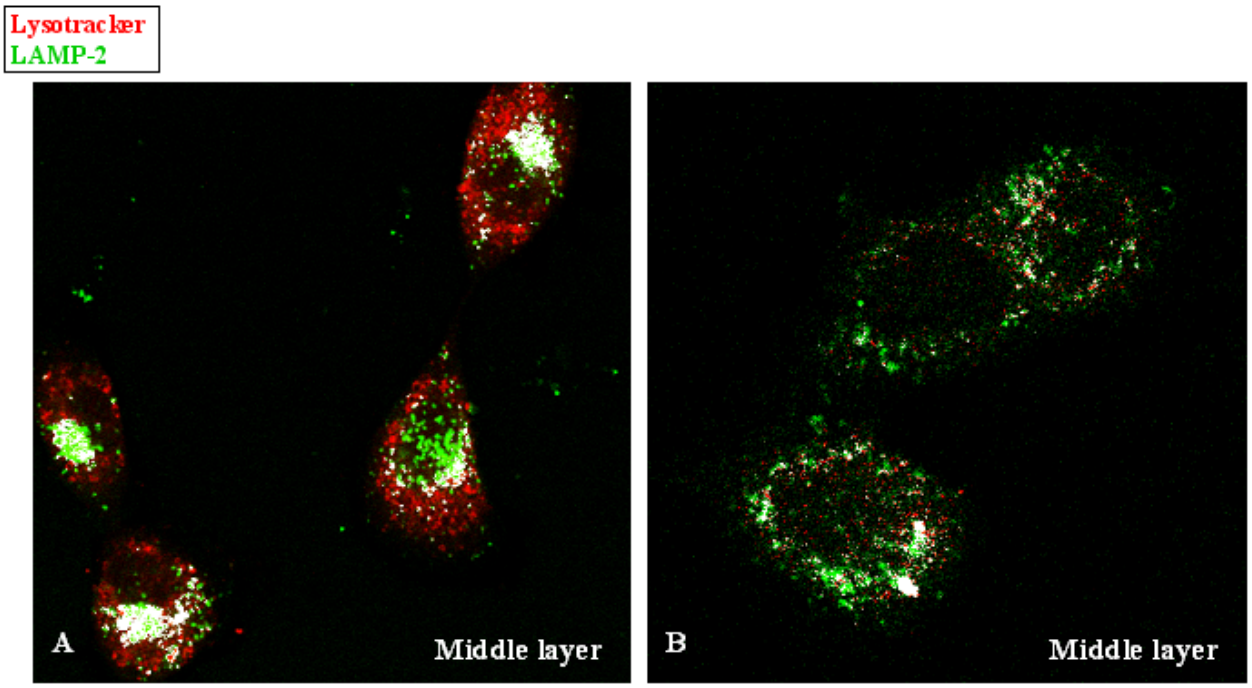


Figure 3.13. The distribution of LAMP-2 in relation with LysoTracker Red DND-99-labeled acidic vesicles, on representative middle layers in MCF10A and MCF10AneoT cells.
The colocalization of FITC-labeled LAMP-2 with LysoTracker Red DND-99-labeled acidic vesicles was illustrated to one site on middle layers of MCF10A (A) and to two sites on either side of the nucleus, in MCF10AneoT cells (B). The level of acidity seemed to be higher in the transfected cells (B).

Lastly, LAMP-2 labeling for colocalization with LysoTracker Red DND-99, in order to assess the degree of association of this membrane protein with vesicles that have an acidic lumen, showed colocalization in the non-transfected MCF10A cells in a medial juxtanuclear site clearly visible at a polarized position, on the one side of the cell (Figure 3.13 A). In c-Ha-*ras*(V12)-transfected cells, a duplication of this site could be seen (Figure 3.13 B), similar to what has been described above for CD (Figure 3.12). It was also clear that the transfected cells had a lower level of acidic vesicles (Figure 3.13, compare A to B).

LAMPs have been described as lysosomal membrane proteins, protecting limiting membranes from its hydrolytic content (Section 3.2.2.1). However, we found that not all LAMP-2-positive vesicles in non-transfected cells had an acidic lumen, as

demonstrated by LysoTracker Red DND-99 incubation (Figure 3.13 B). The low pH of the LAMP-2-containing JNS together with its incidental/altering cathepsin content over the basal-apical axis, as described above, may indicate areas of high proteolytic activity (either protein turnover or degradation) in normal cells. In the transfected cells two acidic sites were observed that double labeled with LAMP-2 (Figure 3.10 E open arrows and Figure 3.12 E open arrows). The LAMP-2-positive structures in transfected cells that were remote from the JNS seem more alkaline. The vesicles containing CB or CL that did not colocalize with LAMP-2 in both the normal (Figure 3.10 A, B and Figure 3.11 A, B) and transfected cells (Figure 3.10 D, E and Figure 3.11 D, E) could possibly be associated with LAMP-1, that may be involved in targeting of secretory vesicles with a higher luminal pH for release from the cell (Section 3.2.2) and LAMP-2 could possibly be involved in carrying mature enzymes towards incoming cargo as has been described (Ludwig *et al.,* 1991; Bright *et al.,* 1997). This happens in the macrophage, where CB and LAMP-2 were trafficked to early endosomes in a time-dependent manner (Arora *et al.,* 2000). The requirement for the acquisition of both Rab7 (vesicle maturation and microtubular interaction) and LAMP-2, before degradation can proceed in phagolysosomes (Huynh *et al.,* 2007) may indicate that the acquisition of LAMP-2 is an important step in LE formation and subsequent lowering of luminal pH, a function of enzymatic degradation in the LE.

Small CB- or CL labeled vesicles in the vicinity of the PM negative for both LysoTracker Red DND-99 and LAMP-2 may be secretory vesicles, that are directed to the leading front of migrating cells, as described during wound healing (Huynh et al., 2005) where CB, for example, is required during wound closure (Buth et al., 2007). Due to their cathepsin content and elevated luminal pH, these vesicles may have LAMP-1 on their membranes (Huynh et al., 2005) (Table 3.2), but this was not tested.

The specialized signaling sequences on the LAMP-2 cytoplasmic tail (Section 3.2.2.2) allow these proteins to not only play a protective role, but also act as piloting molecules, assisting in correct targeting of endosomes in fusion processed in the biogenesis of degradative vesicles. Increased association of LAMP-2 with CB in transfected-, compared to non-transfected cells (Figure 3.10) seems an interesting effect of c-Ha-*ras*(V12) transfection. With the general decreased acidity this increased association with bodies of lesser acidic pH may possibly reflect the

increased role of CB in digestion at a higher pH, where CD activity may possibly be highly sub-optimal. Autophagy, however, may also take place and require LAMP-2 and CD, but may be less pronounced and effective than CB-mediated digestion. This may be due to the decrease in LAMP-2 and LysoTracker Red DND-99 association in transfected cells. Such upregulation as part of a survival strategy (Cao *et al.,* 2006) has previously been indicated in cancer cells (Sato *et al.,* 2007), and could mediate the nutrient supply to the cell through degradation of cytoplasmic macromolecules and assist invasion by removal of endocytosed BM and ECM components. This may be required due to the increased metabolism and demand for intracellular nutrients, but, as shown in our results, the elevated endocytic vesicle acidity may not allow sufficient degradation in digestive sites. In addition, LAMP-2 is known to assist in the fusion of autophagic vesicles and lysosomes during conditions of starvation (González-Polo *et al.,* 2005).

The clusters of LAMP-2-containing vesicles that observed may consist of a compilation of interconnected vesicles in a single area wrapped around one side of the nucleus in the normal cell (Figure 3.11 A-C and Figure 3.12 A-C). When images of individual layers through the cell were investigated, however, smaller LAMP-2^{+} vesicles that were more widely distributed, were noted to be individually located vesicles that did not seem to be connected to their neighbours, or to this interconnected structure. This, however, is best checked using electron microscopy. Structures that contain both cathepsins and LAMP-2 and are acidic, most probably are the areas of higher enzymatic activity in the degradative compartment. In the normal cells immunolabeled CD seems to occur in large vesicles clustered into a focal area next to the nucleus, with comparatively minimal other smaller vesicles distributed around the nucleus. CL and CB, both with a pKa above 5.5, seem to colocalize minimally with CD and this structure. The distribution of LAMP-2- and LysoTracker Red DND-99 positive vesicles and the increased level of low pKa CD in this area, as well as the position relative to the nucleus and size of these vesicles, seems to indicate that a focal area of acidic, CD- and LAMP-2-associated high-grade digestive activity next to the nucleus where the position of the MTOC is described (Matteoni *et al.,* 1987). This structure, possibly the JNS, could either be an extension of the interconnected tubulo-vesicular LE, since it contains characteristics of the degradative LE, such as LAMP-2, CD and acidity detectable by LysoTracker Red DND-99. It could also be a separate, more stable structure where material that has not been fully

degraded by CL and/or CB could possibly be trafficked here for final breakdown. The changes observed in this study to such a degradative JNS, i.e. two smaller acidic CD-containing sites instead of only one larger structure, in addition to an increased association of CB with acidic, LAMP-2 containing vesicles, may assist in assessing the impact that c-Ha-*ras*(V12) has on the degradative capacity of the c-Ha-*ras*(V12)-transfected cell.

3.7. Degradative capacity is decreased after c-Ha-*ras*(V12) transfection

MCF10A immortal cells transfected with the c-Ha-*ras*(V12) oncogene are reported to be tumorigenic and invasive (Soule *et al.,* 1990; Tait *et al.,* 1990; Basolo *et al.,* 1991; Sloane *et al.,* 1994), aided by excessive degradation of BM and ECM components (Elenbaas *et al.,* 2001; Bervar *et al.,* 2003; Debnath *et al.,* 2003) by elevated levels of extracellular proteases such as the MMPs (Munoz-Najar *et al.,* 2005; Siesser *et al.,* 2006) and CB (Linebaugh *et al.,* 1999; Premzl *et al.,* 2003; Zajc *et al.,* 2003). An altered acidity in endocytic and other cathepsin-containing vesicles in general and fewer vesicles containing cathepsins showing an acidic lumen (Section 3.5 and Section 3.6) after c-Ha-*ras*(V12) transfection may have a negative effect on the degradative capacity of digestive vesicles. To assess such a possible influence and confirm the sizes of vesicles and possible interconnectivity mentioned in confocal studies (Section 3.6), electron microscopy ultrastructural assessment was performed on the MCF10A and MCF10AneoT cells.

3.7.1. Reagents

Reagents used for cell culture were described in Section 2.2.1.

Reagents used for cell pellet embedding for ultrastructural investigation of degradative capability were described in Section 2.5.1.

3.7.2. Procedure

Monolayers of MCF10A and MCF10AneoT cells were grown to 70% confluence in 25 cm^3 dishes as described in Section 2.2 and fixed *in situ* (2 h at RT). Cells were scraped from the dish, collected into a 15 ml tube, centrifuged (1000 x g, 2 min), the supernatant was discarded and the pellet was infiltrated with gelatin (30 min, 36°C), divided into small pieces using a scalpel blade, osmicated, washed in d.H_2O, dehydrated in a graded series of alcohols. After processing through increasing

concentrations of resin diluted in alcohol cell pellets were finally embedded in L R White resin. Curing was carried out in resin-filled gelatin capsules in a hot air oven.

Cells were prepared for electron microscopic investigation as described in Section 2.5.2.

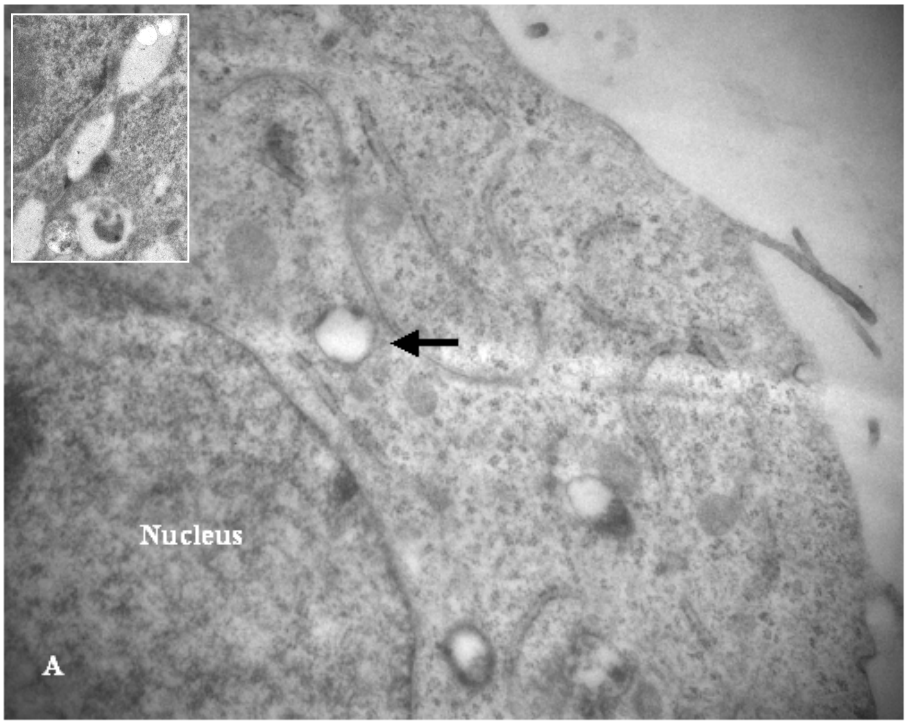
3.7.3. Results and discussion

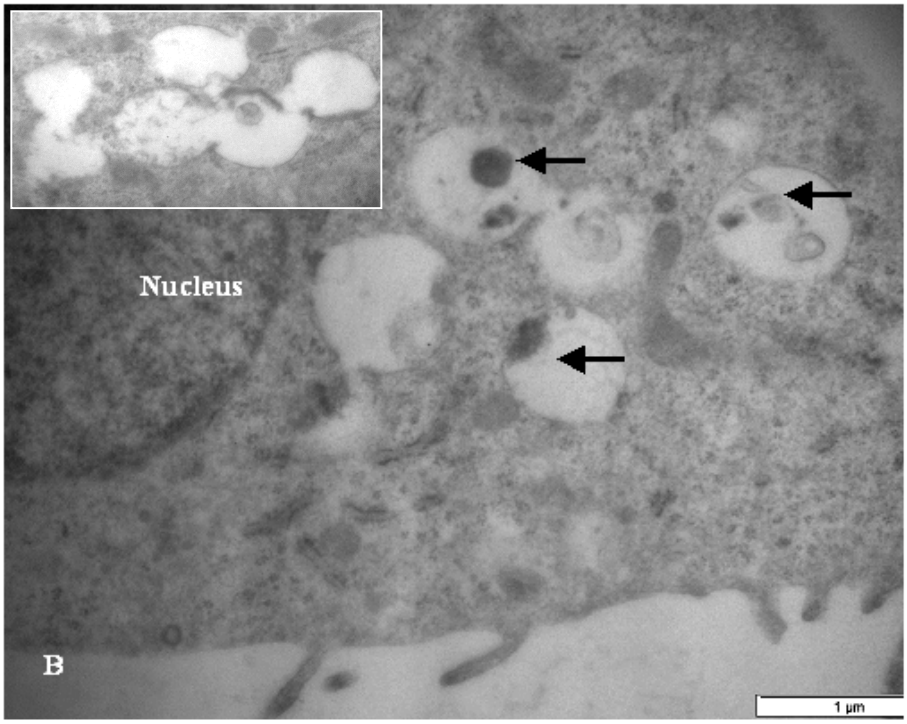
In this study, non-degraded electron dense matter was clearly visible in enlarged LE-like vesicles of c-Ha-*ras*(V12)-transfected cells (Figure 3.14 B) compared to non-transfected MCF10A cells (Figure 3.14 A). In both the normal and transfected cells these structures were in a perinuclear position, similar to where later endosomes were expected, from earlier confocal studies on CB- and LAMP-2 colocalization (Figure 3.10 B, E) and CD- and LAMP-2 colocalization (Figure 3.12 B, E). In the MCF10A cells the average diameter of larger vesicles that contain non-degraded matter was on average 0.4 μm (n = 8), while the enlarged vesicles in the transfected MCF10AneoT cells were on average 0.8 μm in diameter (n = 33). In both the MCF10A and MCF10AneoT cells these structures seemed to be interconnected (Figure 3.14 A, B inserts). Such non-degraded matter was an indication that the degradative capacity of these cells was affected, since using ultramicroscopy, non-degraded matter in vesicles (Bright *et al.*, 1997; Vergarajauregui *et al.*, 2008) in close vicinity to the nucleus may point to inefficiency of cargo degradation. In pathological conditions such as Batten disease degradative deficiency is due to a shortage of the cathepsins (Koike *et al.,* 2005). However, activated c-Ha-Ras(V12) increases cathepsin expression and secretion (Sloane *et al.,* 1994; Sameni *et al.,* 1995; Yan *et al.,* 2003; Sloane *et al.,* 2005). In spite of such increases, our results seem to show diminished association of CB and CL with acidic vesicles and the fewer vesicles seem to have an acidic lumen. It was, therefore, clear that transfection of MCF10A cells with the c-Ha-*ras*(V12) oncogene changed the intraluminal conditions of vesicles involved in degrading endocytosed matter, were significantly and negatively changed, so that degradation was impaired.

The affect of altered cathepsin content and the manner in which acidification of later endosomal vesicles occurs, as well as the underlying influence of c-Ha-Ras(V12) signaling, will be explored in the following section.

Figure 3.14. Inefficient degradation in c-Ha-ras(V12)-transfected cells.

Compared to few, small structures (average vesicle diameter 0.4 μm; n = 8) in positions close to the nucleus in untransfected MCF10A cells (A), an increase in the number of enlarged LEs (average vesicle diameter 0.8μm; n = 33) that still contained non-degraded cargo (arrows) was observed in a similar position in a representative c-Ha-*ras*(V12)-transfected MCF10AneoT cell (B). Insets in A and B indicate the interconnectivity of these vesicular structures in both cell types, but more pronounced in the transfected cells. (Bars = 1 μm)


Nucleus
A


Nucleus
B
1 µm

3.8. Final discussion

Many different endocytic stations, their maturation (Section 3.3) and general controlling proteins involved in trafficking (Section 3.3.6.1) have been described. While the EE is easy to distinguish in short pulse-chase uptake studies (Gruenberg *et al.*, 1989; Ludwig *et al.*, 1991; Desjardins, 1995; Thilo *et al.*, 1995; Tjelle *et al.*, 1996; Bright *et al.*, 1997; Duclos *et al.*, 2003), the LE compartment may consist of a tubular, interconnected set of structures close to the nucleus (Figure 3.13 A, B insets) with varying degradative or processing domains (Nicoziani *et al.*, 2000). Fixation with aldehydes only may destroy such interconnections, as aldehyde fixation does not fix lipids. The use of calcium and magnesium in buffers and osmium tetroxide, as used in the current study, may assist in stabilizing microtubules and hence, may preserve such structures. Such an integrated system may allow a selection of enzymatic activities over the basal-apical axis of the polarized cell (Gruenberg *et al.*, 1989; Ludwig *et al.*, 1991; Griffiths, 1996; Tjelle *et al.*, 1996), as described in our results on both normal MCF10A and c-Ha-*ras*(V12)-transfected MCF10AneoT cells and discussed in the following sections.

While a range of distinguishing characteristics has been highlighted, identification of these stations has been problematic, due to the dynamic nature of cells and confusing nomenclature (Section 3.2.6), which may hamper interpretation and insight of the function and possible trafficking routes, especially in fixed samples. In this study, an attempt to standardize the nomenclature, and highlight characteristics that may facilitate classification of cathepsin-containing endosomes along the degradative route, such as degradative compartments and circulating storage lysosomes, has been attempted. Markers chosen, including vesicle size, and luminal conditions such as enzyme content, acidity or degraded cargo contents, visible in ultrahigh magnification may be useful markers for later endosomes. These may provide a reasonable frame of reference for assessment of, for example, the impact of mutation of- or transfection with, the c-Ha-*ras*(V12) oncogene, as in the current study .

Both lysosomes and LEs have been reported to contain a range of acidic hydrolytic enzymes (de Duve *et al.,* 1955; Baccino *et al.,* 1971; Butor *et al.,* 1995; Luzio *et al.,* 2000), while their limiting membranes provide a barrier between proteolytic hydrolases and the environment (Granger *et al.*, 1990; Fukuda, 1991; Eskelinen *et al.*,

2003). The LE has been confirmed to be the site of digestion, if LAMP-2 and acidity are used as markers, while the lysosome is possibly only a transient storage vesicle in this very dynamic system, even though it is still often called the end structure where final degradation is considered to take place.

In the slightly acidic processing LE compartment precursor cathepsins carried from the Golgi are released from MPR and processed (Figure 3.3 Lanes 3 and 4), and may, therefore, be identified due to the presence of MPRs (Nicoziani *et al.*, 2000). The processing LE may contain LAMP-1 (Nicoziani *et al.*, 2000), but not necessarily any LAMP-2 proteins, a marker for a digestive organelle. In this study such juxtanuclear cathepsin-containing structures were negative for LysoTracker Red DND-99 and LAMP-2. The lysosome and digestive LE are devoid of MPRs (Ludwig *et al.,* 1991), making them structures downstream from the LE processing compartment along the cathepsin maturation route (Section 3.3.3 and Figure 3.2 Lane 3, 4). Isolation of a lysosome with its content of newly-matured proteases, its subsequent trafficking and fusion with a degradative structure, such as the LE, relies on a range of specific trafficking proteins.

Small circulating storage lysosomes with a high pH and mature cathepsins (Section 3.3.5 and Table 3.3) are possibly formed from the processing compartment under the influence of specific cargo-selecting APs (Section 3.3.3.1). Association of such APs with the cytoplasmic tails of LAMP-1 or -2 and MPR (Nicoziani *et al.*, 2000) (Section 3.2.2.2) in a protein complex, may direct movement towards either specific target organelles or the Golgi (Aplin *et al.,* 1998; Bonifacino *et al.,* 1999) (Section 3.3.6.1). In the current study small vesicles containing either CB or CD were noted, with little colocalization between these two cathepsins in the normal MCF10A cells. Most of these small vesicles were scattered through the cytoplasm away from the nucleus and not associated with LysoTracker Red DND-99, and were, therefore, considered to be storage, circulating vesicles. In the c-Ha-*ras*(V12)-transfected MCF10AneoT cells, these CB- or CD-containing storage vesicles were spread further from the nucleus than in normal MCF10A cells, and seemed to be directed to the basal plane of the cell, where it is in contact with underlying matrix.

From our results it is clear that several lysosome-type vesicle populations exist with differing combinations of cathepsin content, luminal acidity and LAMPs**,** with these

vesicles varying in size and location. These LEs and lysosomes seem to be in a dynamic cycle over a period and during specific cellular activities, such as excessive collagen uptake during migration. While it is not possible to label a specific vesicle population in this dynamic system at a given point with all the relevant markers, factors that may distinguish endosomes actively involved in the digestive process from e.g. storage vesicles are a low luminal pH that creates an environment favourable for proteolytic enzyme activity and the acquisition of protective LAMP-2 in the limiting membrane. A LE may have a low luminal pH that creates a suitable digestive environment, due to the activity of a functionally assembled V-ATPase pump (Section 3.2.1.4). The pH in the LEs could possibly fluctuate to accommodate protease activity over a wide range of pH optima for efficient macromolecule and endocytosed matrix component degradation (Butor *et al.*, 1995; Sameni *et al.*, 2000). After digestion, it may then be possible that the mature, active cathepsins are again pinched off into a small storage vesicle that may initially have a relatively low pH, but may quickly dissipate protons due to inactivation or loss of the proton pump activity. Correlation of the distribution patterns of cathepsins, LAMP-2 and LysoTracker Red DND-99, gave a clear indication that there were mature, active enzymes located in large vesicles that had all the characteristics supporting degradation, i.e. LAMP-2 membrane proteins, low vesicle acidity and cathepsin content. Such structures were observed in both the MCF10A cells as well as the MCF10AneoT cells, with reduction of this degradative vesicle type in the latter. However, whether these are LEs remains difficult to prove using the current markers.

A specialized degradative structure close to the nucleus, the JNS, has been suggested (Section 3.3.6), but a function significantly different to the LE, or clear set of characteristics has not yet been established. In our study such a structure has been observed (Section 3.6.3), and was characterized to contain CD, LAMP-2 , and an acidic lumen. Such a structure may supply a favourable environment for high-grade degradative activity by CD, which needs an acidic environment for optimal activity. The JNS could be a localized section of the interconnected LE network, specialized to perform high-grade degradation. In MCF10AneoT cells, CB was noted in this area. It could be that, since the pH of the degradative vesicles is elevated, conditions characteristic of the JNS has been compromised, with CB now more involved in degradation than CD, as found in the non-transfected cell. This is supported by the observed increase in association of CB with LAMP-2 and the increase in size of

LAMP-2-CB positive structures (Figure 3.10 G and I), as well as the location of these structures in a juxta-nuclear position (Figure 3.10 E and Figure 3.14). On the other hand, the decrease in association of CD with this structure and its restriction to small, juxta nuclear areas (Figure 3.11 E), may be another indication that the acidity of the JNS is compromized in the c-Ha-*ras*(V12)-transfected MCF10AneoT cells, resulting in CD remaining in small, non-acidic (Figure 3.8 D) LAMP-2 negative (Figure 3.11 D, G and I) circulating storage vesicles spread further away from the nucleus. While the JNS is reported to maintain a low pH during migration, during mitosis (Matteoni *et al.*, 1987; Nobes *et al.*, 1999) or experimental alkalinization of the cytoplasm (Probst *et al.*, 2006) it is briefly disrupted into 2 sites. Compared to the normal MCF10A cells, a similar pattern of two degradative sites were observed in this study, in the MCF10AneoT cancer cells, in which control over c-Ha-Ras(V12)-related growth was compromised. Treatment with nocodazole was shown to disrupt the microtubules (Matteoni *et al.*, 1987) and arrangement of the Golgi, while inhibition of the V-ATPase proton pump with BafA1 resulted in a focusing of these structures to a juxta-nuclear position (Wang *et al.,* 2002). This is a clear indication of the influence of the microtubular network on the retention of the organelle organization around the nucleus. c-Ha-Ras(V12) stimulates cytokinesis and progression through the cell division cycle (Thullberg *et al.*, 2007). During such mitosis CDC42 is closely involved in the disruption and re-orientation of juxtanuclear structures, such as the Golgi (Altan-Bonnet *et al.*, 2003; Yasuda *et al.*, 2006). The influence of c-Ha-Ras(V12) on CDC42 could explain the two sites of concentrated acidic, LAMP-2-positive areas. The observation of two such distinct focal sites compared to one only in the normal, slower proliferating cell, could be under the influence of constant c-Ha-Ras(V12) stimulation that affects CDC42 (Teramoto *et al.,* 2003), a cytoskeletal organizing GTPase (Cau *et al.,* 2005) that is under the control of Ras-Ral (Figure 1.3 on fold-out). During migration CDC42 together with Rab34 (Wang *et al.,* 2002), formation of a Rab34-RILP complex (Wang *et al.,* 2002) (Section 3.3.1.6) and the phosphorylation of myosin (Vicente-Manzanares *et al.,* 2007) may control the spatial orientation of organelles such as the Golgi (Nobes *et al.*, 1999; Gadéa *et al.*, 2002) and possibly manipulate late endocytic structures and JNS in relation to the nucleus (Nobes *et al.,* 1999; Donaldson *et al.,* 2000). We suggest that this JNS (Hirst *et al.,* 1998; Santama *et al.,* 1998) could be a specialized LE compartment that has mechanisms such as additional ion channels (e.g. for chloride) that may assist in lowering the acidity by dissipating the membrane potential (Forgac, 1999). It may

carry out this function even in the face of ineffective acidification of the rest of the interconnected, degradative LE.

It seems then, that it may be important and possible, to distinguish between the LE, the lysosome and a focal JNS next to the nucleus, using combinations of several characteristics. The JNS resembles both LE and lysosomes in individual characteristics, since they may all be components of the same degradative system. The specialized degradative function of the JNS and the combination of characteristics that support high-level degradation, however, sets it apart from the LE or lysosome, while the lysosome may be a circulating storage vesicle with no enzymatic activity. In this study it was interesting to find that the acidity of the JNS, contrary to other endosomes, was not compromized in the MCF10AneoT c-Ha-*ras*(V12)-transfected cells. This finding will be included in the following discussion, where we suggest that possible altered cytoskeletal organization may influence the trafficking and spatial organization of these structures.

3.8.1.1. Simplistic model of possible 'basic normal' cathepsin-containing vesicle trafficking

Endocytic vesicles, including the cathepsin-containing vesicles, are relocated along the microtubules by kinesin towards the PM, while dynein transports vesicles towards the MTOC (Blocker *et al.*, 1997; Bananis *et al.*, 2000b; Ichikawa *et al.*, 2000; Jordens *et al.*, 2001), located close to the nucleus and Golgi complex (Ichikawa *et al.,* 2000; Johansson *et al.,* 2007) (Figure 3.3 Lane 2). As a result mature, degradative LEs are normally concentrated around the nucleus (Matteoni *et al.,* 1987; Sloane *et al.,* 1994; Clague *et al.,* 2006) and secretory vesicles may be located close to the PM in normal cells (Andrews, 2000; Huynh *et al.*, 2005). This is an important point in this study, since the location of cathepsin-containing vesicles seems to be altered in cancer cells (Cavallo-Medved *et al.*, 2005) and after c-Ha-*ras*(V12) transfection (Sameni *et al.*, 1995), and is suggested to support an invasive phenotype. The underlying mechanisms of such altered trafficking are still largely unclear, however. Direction of vesicle migration is largely pre-determined by many pilot proteins, including the dynein and kinesin motor proteins (Section 3.3.6.1). Factors that affect these motor proteins, such as the cytoskeletal organization (Ichikawa *et al.,* 2000) and cytoplasmic pH (Blocker *et al.*, 1997; Probst *et al.*, 2006) may then influence the movements of

these vesicles and both these factors may be influenced by Ha-Ras and c-Ha-Ras(V12) downstream signaling (Section 3.3.7).

Using strictly the information gathered on endosomal vesicle formation (Section 3.3) and trafficking proteins involved in this process (Section 3.3.6.1), we propose a model for possible vesicle kinetics of especially cathepsins. With the use of a combination of various markers we observed several different endosomal vesicle populations and now propose a summary of several theoretical routes along which the newly formed cathepsin-containing endosomes may be transported (Figure 3.15). These may be directly under the influence of Ras signaling, as was mentioned in the previous section.

Route 1. Newly-formed hydrolases that are carried to the MPR-containing processing compartment (Pohlmann *et al.,* 1995) are matured (Kornfeld, 1986; Schmid *et al.*, 1999) and could subsequently be incorporated into a storage lysosomes that fuse with early endosomal vesicles containing endocytosed products for digestion, to form the hybrid LE(s) (Figure 3.15 Route 1). These vesicles may contain mature CB or CL, after it has been matured in the acidic environment (Anderson *et al.,* 1988; Stoorvogel *et al.,* 1989; Bresciani *et al.,* 1996; Orsel *et al.,* 2000) of the processing compartment of the LE (Gabel, 1983; Ludwig *et al.*, 1991). It may subsequently be trafficking to a circulating lysosome for storage.

Route 2. The isolation of colloidal gold in 'lysosomes' subsequent to BSA digestion (Bright *et al.,* 1997) in the LE suggests that such a vesicle is a recycling or a storage 'lysosome' budded off from the LE and provides evidence for the 'storage lysosome' hypothesis. It is possible that lysosomal integral membrane proteins could bud off from the digestive sites after cargo degradation and reform into a separate lysosomal vesicle (Figure 3.15 Route 2). Hence, such vesicles could contain LAMP-2 or LAMP-1, depending upon the compartment from which these vesicles bud off. If CB and CL are not destroyed in the initial degradative process due to the lowered luminal acidity, these enzymes could possibly be included into the newly formed storage lysosome.

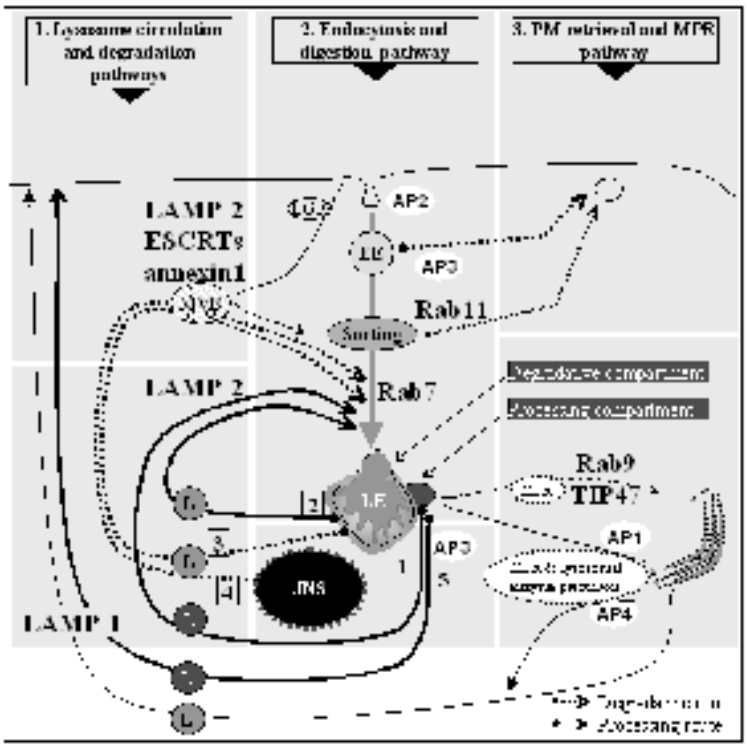


Figure 3.15. Possible trafficking routes for cathepsin-containing vesicles.
Membrane budding off at processing or degradative LE or JNS sites (Lane 2) could be routed to target membranes, incorporating newly-synthesized (Lane 3), mature or denatured cathepsins to their sites of action (Lane 2), or their sites of storage or degradation (Lane 1). (1) A (primary) lysosome containing newly synthesized cathepsins pinches off and fuses with incoming EEs under the guidance of LAMPs and Rab7. (2) The lysosomal membrane components that were incorporated into degradative LE during the merge between the EE and lysosomes could be pinched off and recycled to maintain membrane ratios. This vesicle could possibly re-join the degradative route by merging with incoming EEs. Such a vesicle could possibly contain hydrolases that are still active such as CB or CL, if the pH of the LE did not denature these proteases. (3) Alternatively, this lysosome could be on the degradative route and possibly gets incorporated into a MVB (Tjelle *et al.*, 1996; Bright *et al.*, 1997; Hirst *et al.*, 1998; Duclos *et al.*, 2003) or multi-lamellar body. Inward budding into MVB may be manipulated by clathrin (Murk *et al.,* 2003a), annexin I receptor (White *et al.,* 2006) and endosomal sorting complex required for transport (ESCRTs) (Razi *et al.,* 2006; Shim *et al.,* 2006). Since these structures are considered types of LEs, these vesicles and their cathepsin content could be degraded. (4) The stable JNS is considered to be a compartment of low acidity and mostly CD activity, but turnover of its membrane components could result in vesicle budding. (5) Secretory lysosomes could be involved in maturation of precursors produced in the Golgi prior to presentation on the PM. This illustration was generated to demonstrate predictions mentioned in Section 3.8.1.1.

Route 3 & 4. Proteins partially digested in the acidic environment of LEs (Figure 3.15 Route 3) may be completely digested in the low pH environment of the JNS (Figure 3.15 Route 4). Cathepsins such as CL performing functions in the cytoplasm may be degraded via inward endosomal membrane budding to form MVBs. This may be a function of LAMP-2 during the autophagic process. These cathepsins would be observed in the lumen of vesicles in a MVB, as previously reported (van Deurs *et al.*, 1996; Bright *et al.*, 1997).

Route 5. Secretory lysosomes containing mature enzymes seem to pinch off the LE processing compartment (Figure 3.15 Route 5). In contrast to Route 1, however, these storage lysosomes may fuse with vesicles that originate from the Golgi, possibly containing products such as prohormones or precursor molecules that have to be proteolytically matured before secretion at the PM, as is the case with immature secretory granules (Fernandez *et al.*, 1997) (Section 3.3.1.2). Cathepsins and LAMPs have been observed to be removed from these immature granules after proteolytic activity (Klumperman *et al.,* 1998).

3.8.1.2. Factors that possibly affect normal distribution of cathepsin-containing vesicles.

The mutated c-Ha-Ras(V12) oncoprotein, even though it has a lower level of activity (Gideon *et al.,* 1992), alters proliferation and polarity of the cells (Teramoto *et al.*, 2003; Bourguignon *et al.*, 2004), increases invasiveness of breast epithelial cells (Ochieng *et al.*, 1991; Ward *et al.*, 2001; Kim *et al.*, 2003; Shin *et al.*, 2005). This may occur via alters in the intracellular cytoskeleton of MCF7 (Nishimura *et al.*, 2003; Nishimura *et al.*, 2004) and MCF10A breast epithelial cells (Nishimura *et al.*, 1998), resulting in the distribution of CB- and CD-containing vesicle to the cell periphery (Sameni *et al.*, 1995), altering normal vesicle distribution. This may be due to overexpression of ROCK and its downstream effector, LIMK-1, both involved in calcium-stimulated Ha-Ras signaling to cytoskeletal components (Section 1.7.3 and Figure 1.3 on fold-out). Similarly, experimental depolymerization of the microtubules with nocodazole (Gruenberg *et al.,* 1989; Cantalupo *et al.,* 2001; Mizuno *et al.,* 2003) and disruption of the cytoskeletal organization by alkalinization of the cytoplasm with ammonium chloride (Probst *et al.,* 2006), also results in spread of vesicles (Gruenberg *et al.*, 1989; Orlowski *et al.*, 1997; Baravalle *et al.*, 2005) from their usual perinuclear position at the MTOC (Matteoni *et al.*, 1987) to the cell perimeter (Ichikawa *et al.,* 2000; Cantalupo *et al.,* 2001). This illustrates that the interaction between vesicles and cytoskeleton is dependent on cytoplasmic pH (Blocker *et al.,* 1997).

Cytoplasmic pH is an important factor in the establishment of cellular polarity in migrating cells and is controlled by NHE-1 (Section 3.2.1.2), which is normally activated in response to intracellular osmotic stress, cell spreading (Orlowski *et al.,* 1997; Counillon *et al.,* 2000) or intracellular pH and is activated when cells become

acidic (Putney *et al.,* 2002; Haworth *et al.,* 2003) (Section 1.9.3, Figure 1.4). In cancer cells increased mitosis and metabolism (Brand *et al.,* 1997; Xu *et al.,* 2007) could cause intracellular alkalinization and swelling of the cell, both of which are Ras-related mitogenic signals (Fürst *et al.,* 2002). In support of this theory, cancer cell lines with progressively increasing metastatic ability such as the highly metastatic MDA-MB cell lines, show a related increase in activity of NHE-1 and alkalinization of the cytoplasm (Reshkin *et al.*, 2000b; Paradiso *et al.*, 2004; Cardone *et al.*, 2005). In cancer cells activation of NHE-1 is due to its increased affinity for protons, but since the kinetics of the sodium co-element is not altered (Reshkin *et al.*, 2000a), this process leads to acidification of the micro-environment of the cell that assists in activation of membrane-associated proteases such as CB (Bourguignon *et al.,* 2004; Cardone *et al.,* 2005) and excessive ECM degradation and invasion (Ritter *et al.*, 1997; Reshkin *et al.*, 2000a; Reshkin *et al.*, 2000b; Denker *et al.*, 2002). In the MCF10AneoT invasive cancer cell line used in this study, NHE-1 may indirectly be activated due to the constant downstream signaling by activated c-Ha-Ras(V12). Normal Ha-Ras is activated at the site of growth stimulus, or the leading front, where its downstream effector, PI3K, increases calcium release that indirectly activates NHE-1 (Ritter *et al.*, 1997; Reshkin *et al.*, 2000a) via increased levels of ROCK. Constant c-Ha-Ras(V12) signaling may, however, result in elevated levels of calcium influx and ROCK activation (Hofmann *et al.*, 1993b; Kawano *et al.*, 2000a) (Figure 1.3 on fold-out), supporting formation of stress fibers and cell-matrix adhesions that in turn promotes motility and invasion (Denker *et al.*, 2002; Paradiso *et al.*, 2004; Cardone *et al.*, 2005).

From the discussion above, it would seem that even though acidification of the extracellular microenvironment is relevant in migration, the general cytoplasmic alkalinization by over-stimulation of the NHE-1 proton pump allows disorganization of the cytoskeletal network. This in turn may allow the distribution of various cathepsin-containing endosomes further away from the nucleus than in a normal cell. We suggest, therefore, that the altered shape of the MCF10AneoT cancer cells may be the reason for the 'lysosomal spread' reported by many workers. The connection between these factors, constant c-Ha-*ras*(V12) signaling and degradative capacity, will be considered in the next section.

3.8.1.3. Correlation of altered cathepsin-containing vesicle distribution and LE acidity

In addition to altered vesicle spreading, we showed in unpublished work previously done in this laboratory that acidification of the degradative vesicles has been affected by c-Ha-*ras*(V12) transfection (Jackson *et al.*, 1999, Appendix I) and that the degradative ability of these vesicles has been compromized (Section 3.7). Similar insufficient acidification of the processing compartments where precursor enzymes are matured, may be the reason for secretion of the CD precursor reported in the colorectal CaCo2 cancer cells and the MCF7 breast cancer epithelial cells (Capony *et al.*, 1994; Couissi *et al.*, 1997; Kokkonen *et al.*, 2004). In the current study intracellular CB and CD to a lesser extent, were still observed in large late endosomes and small, electron dense lysosomes, but colocalization with LysoTracker Red DND-99, indicating luminal acidity, was reduced. Mature CD, on the other hand, seemed to largely accumulate in the acidic JNS (Section 3.6.3). It has been shown that, under experimental alkalinization of the cytoplasm by ammonium chloride, CD trafficking was not influenced (Capony *et al.,* 1994; Isidoro *et al.,* 1997). This implies that the JNS manages to maintain a level of acidity that will allow CD activity, as seems to be the case in this study in the JHS of the MCF10AneoT cells. These cells may digest endocytosed matter, even if the degradative system has been compromized by the effect of c-Ha-*ras*(V12) on vesicle pH. This effect is similar to what is seen in some pathologies such as Batten disease, or neuronal ceroid-lipofuscinosis (NCL) (Holopainen *et al.*, 2001; Aula *et al.*, 2002; Nishimura *et al.*, 2002) and pathological accumulation of non-degraded cholesterol (Holopainen *et al.,* 2001; Koike *et al.,* 2005; Ganley *et al.,* 2006) and may be enhanced during treatment with pH altering chemicals. This may affect trafficking of the MPR cathepsin transporting receptor (Koike *et al.,* 2005), insufficient levels of CB, CD and CL and incorrect intracellular degradation. This may affect trafficking of the MPR cathepsin transporting receptor, leading to secretion of cathepsins, reduced levels of CB, CD and CL and compromized cellular degradation (Koike *et al.*, 2005).

In I-cell disease (Nishimura *et al.,* 2002) similar low levels of CB and CL are observed due to a lack of M6P (Gabel, 1983) and, thus compromised trafficking of lysosomal precursor enzmes to the degradative vesicles (Dittmer *et al.,* 1999; Tardy *et al.,* 2006) (Section 3.3.3.1).

In sialic acid storage diseases (SASD) cleaved sialic acids accumulate in the LE due to a defective sialin receptor and export of the cleaved products from the LE (Aula *et al.*, 2002). Since the addition of sialic acid to membrane proteins of the processing compartment normally lowers the luminal pH (Granger *et al.,* 1990), removal of the C-terminal extension of pro-CB is inhibited and proteolytic degradation is negatively affected in this pathological condition (Schmid *et al.,* 1999). Disruption of lysosomal enzyme trafficking or processing is usually evident as build-up of undegraded materials in digestive bodies, as in the c-Ha-*ras*(V12)-transfected MCF10AneoT cells (Section 3.7) and may result in cell death, if severe enough.

Collectively, this information indicates that alkalinization of the later endosomal vesicle system negatively influences the maturation process of the cathepsin precursors, resulting in lack of dissociation of cathepsin precursors from their MPR carrier. This, in turn, may restrict recycling of MPR to the Golgi for delivery of precursor lysosomal enzymes (Section 3.3.3.1), and as a consequence, default secretion of lysosomal enzyme precursors (Lazzarino *et al.,* 1990; Yeyeodua *et al.,* 2000) as previously described in MCF-7 cells (Capony *et al.,* 1994; Kokkonen *et al.,* 2004). While the cell may attempt to compromize for the lack of nutrients by increasing autophagy (Cuervo, 2004; Sato *et al.*, 2007), this process, too, may be negatively affected by alkalinization of degradative vesicles. This is also seen with ammonium chloride (Syntichaki *et al.*, 2005) and treatment with V-ATPase proton pump inhibitors, such as the antibiotics BafA1 and ConcA (van Weert *et al.,* 1995; van Weert *et al.,* 2000; Alwan *et al.,* 2003; Xiao *et al.,* 2007), where alkalinization affects the activity of the V1 cytoplasmic component of the intact proton pump (Figure 3.1). This may in turn decrease acidification in the LE (Syntichaki *et al.,* 2005) and inhibit degradation of endocytosed cargo (Probst *et al.,* 2006). Also, enzyme precursors may be released from the MPRs (van Deurs *et al.,* 1996; Xiao *et al.,* 2007) and may be secreted (van Deurs *et al.,* 1996; Xiao *et al.,* 2007), while oxidative metabolism is also disrupted (Barile *et al.,* 1990). BafA1 and ConcA interfere with the proton translocation system (Zhang *et al.*, 1994; Forgac, 1999) by binding to the 95-kDa subunit c (Landolt-Marticorena *et al.,* 1999; Huss *et al.,* 2002) on the V0 domain (Zhang *et al.,* 1994) and specifically inhibit acidification of the degradative vesicles (Yoshimori *et al.,* 1991; Mousavi *et al.,* 2001; Alwan *et al.,* 2003) (Section 3.2.1.4, Figure 3.1 and Table 3.1).

Both BafA1 (Zhang *et al.*, 1994; Forgac, 1999) and ConcA (Arora *et al.,* 2000) inhibit degradation of internalised cargo such as EGF and bovine serum albumin (BSA). Release of receptor-bound ligands, such as Tfn from its receptor, is reliant on an appropriate acidic environment (Ludwig *et al.,* 1991; Mousavi *et al.,* 2001). This process is blocked by BafA1 vesicle alkalinization, and results in the recycling of the receptor-ligand complex to the PM with the ligand still attached (Mousavi *et al.,* 2001). BafA1 also inhibits macroautophagy (Boya *et al.,* 2005), possibly interfering with the association of the proton pump and trafficking components on the phagosome, which may interfere with the ability of the cell to defend itself against invasive pathogens. Elevation in vesicle pH may interfere with the supply nutrients, and may eventually lead to starvation. However, while both BafA1-treated cells (Duclos *et al.,* 2003) and cells treated with an alkalinizing treatment such as ammonium chloride (van Weert *et al.,* 1995; Donaldson *et al.,* 2000), showed inefficient cargo degradation, it is important to note that maturation of EEs into LE-like structures that contained digesting proteases was not affected (Duclos *et al.,* 2003). Hence, the actual proteolytic activity of the LE appears to rely on a functioning acidification system (Anderson *et al.,* 1988; Yoshimori *et al.,* 1991; van Weert *et al.,* 1995; Mousavi *et al.,* 2001).

The combination of altered vesicle acidification, the enlarged morphology and reduced degradative capacity of the later endosomal vesicles shown here in c-Ha-*ras*(V12)-transfected cells (Section 3.7), was similar to the effect of inhibition of the V-ATPase proton pump in BafA1-treated cells. Since c-Ha-*ras*(V12) transfection affected mostly the later endosomal vesicles (Jackson *et al.*, 1999) that are acidified by V-ATPases (Grabe *et al.*, 2001), this was now considered to be the underlying mechanism in lack of degradative capacity in cancer cells, and we thus investigated factors that may interfere with the functions of this proton pump and related it to behaviour of cancer cells.

3.8.1.4. The V-ATPase proton pump regulates LE acidification

Mainenance of a low LE acidity and thus proton translocation (Landolt-Marticorena *et al.*, 1999; Kane, 2006), relies on the stable assembly of two individual components of the V-ATPase proton pump (Orlowski *et al.,* 1997) (Figure 3.1) and on an organized cytoskeletal system (Section 3.2.1.4). The V0 integral membrane component of the V-ATPase proton pump is located in most cellular membranes (Sun-Wada *et al.,*

2004), with the V1 component usually anchored to the actin-based cytoskeleton via its subunit C (Vitavska *et al.,* 2003). V-ATPase assembly was, therefore, examined, in order to investigate possible influences that may affect it. The question we asked was: "What could the influence be of the mutated c-Ha-Ras(V12) downstream effectors?" Mechanisms are outlined in Section 3.8.1.5 and illustrated in Figure 3.16 Lane 2.

3.8.1.5. Factors/components that control the V-ATPase proton pump activity

Biological membranes contain phospholipids that provide the matrix in which V0 components are embedded. The phosphatidylserine (PS) content of these membranes is especially important in regulating luminal acidity by activating proton transport (Crider *et al.*, 2003; Kane, 2006) and is essential for the function of subunit H (part of the V1 stalk) which inhibits ATP hydrolysis by blocking the active site on subunit A when V1 and V0 are disassembled (Kane, 2006) (Figure 3.1). Phospholipid content of vesicles may regulate the number of active, assembled proton pumps present in a membrane, possibly accounting for the variability in internal pHs of different types of organelles along the endocytic route (Crider *et al.,* 2003).

Disulfide bonds between conserved cysteine residues at the catalytic site of the V1 subunit A (Figure 3.1) may allow the proton pumps to exist in a reversible inactivated state. This may regulate activity of the V-1 component *in vivo* and at a less acidic pH, such as in the EE, these bonds may inhibit the activity of the proton pump. A mutant that is unable to form such inhibitory disulfide bonds, and the mitochondrial F-ATPase that has a similar conformation, are both constitutively active (Forgac, 2000).

At physiological concentrations, glucose induces V-ATPase-dependent acidification of the intracellular vesicular system (Su *et al.,* 2003; Sautin *et al.,* 2005). The assembly of the V0 and V1 components into a functioning unit is stabilized by a glucose-specific regulator of the V-ATPase of vacuolar and endosomal membranes (RAVE), through its interaction with subunit C on the proton pump (Smardon *et al.,* 2007). On the other hand, starvation due to serum or glucose deprivation causes a decrease in levels of ATP and inactivates the V-ATPase via temporary disassembly of its two domains. This mechanism ensures a link between cytosolic ATP levels (energy supply) and the acidification of degradative vesicles (Forgac, 1999; Sautin *et al.*, 2005; Xiao *et al.*, 2007). In hyper-proliferating cancer cells, increased cell

division and enhanced biosynthesis may cause increased oxidative stress (Section 3.3.2). Under such conditions, limited oxygen levels may cause cells to increase their glucose metabolism to protect the cancer cells against the resultant reactive oxygen species (Brand *et al.,* 1997; Xu *et al.,* 2007). Over expression of activated Ras that causes hyperproliferation (Gao *et al.*, 2004), mimics, therefore, insulin-mediated stimulation of Ras. This condition may promote basal glucose uptake (Kozma *et al.,* 1993) by trafficking glucose transporters (GLUT) to the PM (Valverde *et al.,* 1998).

Vesicle acidification is affected by cytoskeletal organization and activity of the V-ATPase proton pump. Both these two factors may be influenced by constant signaling by the mutated c-Ha-Ras(V12), leading to ineffective degradation in the MCF10AneoT cancer cells.

3.8.1.6. Effect of c-Ha-Ras(V12) and PI3K on vesicle acidification

Downstream effectors of Ha-Ras are not only involved in cytoskeletal organization (Figure 1.3 on fold-out), but are also involved in vesicle kinetics (Figure 3.16). Signaling during endosome maturation by activated PI3K (Shepherd *et al.,* 1996), a direct downstream effector of Ha-Ras (Figure 3.16 Lane 2) supports the release of Rab5 from the early endosomes and sequestration of Rab7 (Vieira *et al.,* 2003; Schwartz *et al.,* 2007). Rab7 guides the fusion and maturation process of cargo-carrying EEs with hydrolase-containing lysosomes (Bucci *et al.,* 2000) to form the LE (Ceresa *et al.,* 2006) (Figure 3.16 Lane 1). The association of RILP with Rab7 (Cantalupo *et al.*, 2001; Jordens *et al.*, 2001; Harrison *et al.*, 2003) prevents further cycling of Rab7 and stabilizes the vesicle structure, or prevent further fission events (Jordens *et al.,* 2001). Subsequently, the association of the Rab7-RILP complex with Rabring7 (Mizuno *et al.,* 2003) forms a complex that incorporates the LE membrane, the cytoskeleton scaffold (Bucci *et al.,* 2000; Johansson *et al.,* 2007) and the dynein motor protein (Ichikawa *et al.*, 2000; Jordens *et al.*, 2001; Johansson *et al.*, 2007). After formation of this complex, dynein transports vesicles along the microtubules towards their minus end (Cantalupo *et al.*, 2001; Harrison *et al.*, 2003; Johansson *et al.*, 2007) and thereby effectively detains these vesicles in close association with the MTOC at the cell center (Ichikawa *et al.,* 2000; Jordens *et al.,* 2001). In addition, the recruitment of Rab7 (Papini *et al.,* 1996; Vieira *et al.,* 2003) and the presence of Rabring7 is essential for late endocytic vesicle acidification, since their over expression causes increased lysosomal acidity (Mizuno *et al.,* 2003). In support,

inhibition of PI3K with Wortmannin and, therefore, lack of Rab7 sequestration, restricts vesicle maturation (Vieira *et al.,* 2003) and acidification (Davidson, 1995) (Section 3.3.7.2). This may be the underlying cause of secretion of a precursor CD after Wortmannin treatment (Davidson, 1995), since the precursor probably is not properly matured (Wittlin *et al.,* 1999).

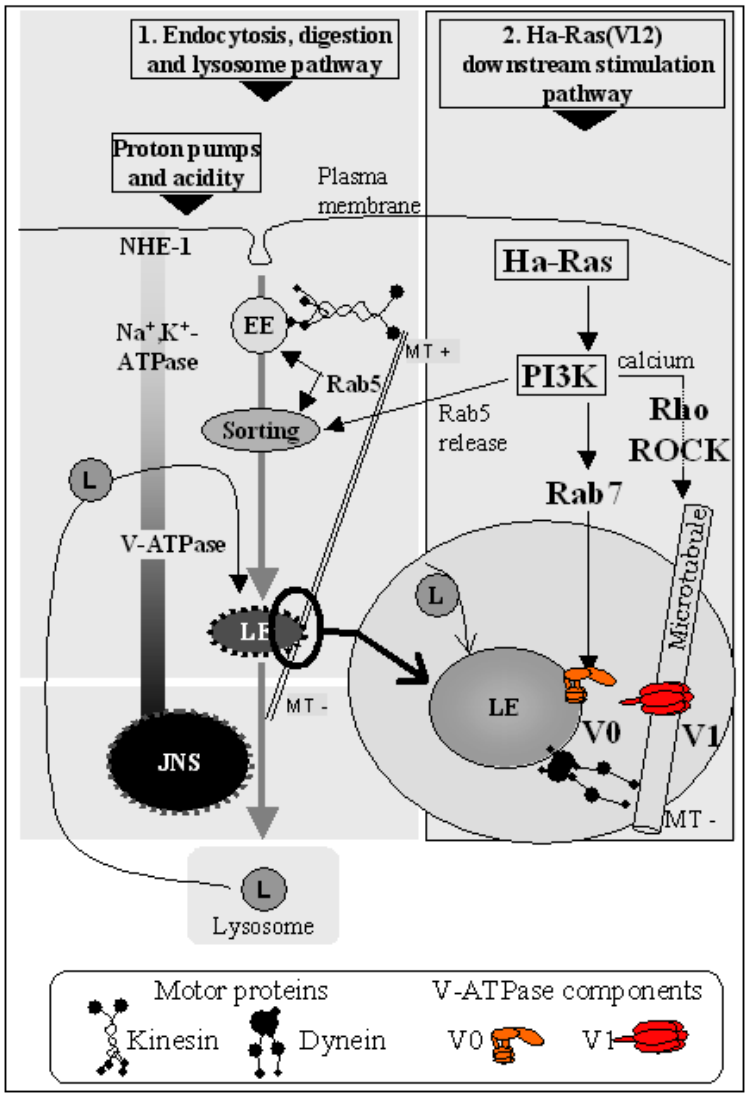


Figure 3.16. A schematic illustration of the influence of Ha-Ras on LE acidification. Ha-Ras has an influence on PI3K downstream signaling, Rab5 and Rab7 sequestration and the assembly of the V0 and V1 components of the V-ATPase proton pump. This interaction plays a role in the initiation of LE acidification after fusion of the sorting and lysosomal vesicles. The orientation of the late endosomal vesicles in terms of the MTOC and the positions in which motor proteins kinesin and dynein could be involved is shown to illustrate the possible direction in which the various vesicle types could be mobile. PI3K could also be involved in the cytoskeletal organization via calcium signaling to Rho-ROCK. This illustration was generated to demonstrate predictions mentioned in Section 3.8.1.6.

It is our theory, as illustrated by the model described above and illustrated in Figure 3.16, that constant PI3K signaling due to the mutated c-Ha-Ras(V12) and the assumed

associated increase in Rab7 recruitment, should cooperate to lower the acidity of the later endocytic vesicles to levels lower than in normal cells and, in this way, support enhanced degradative ability. In addition, the constant pressure on the endocytic path should also be a positive signal for lowering of the degradative vesicle luminal pH to assist in creating conditions conducive to degradation. Some factors that associate endocytosis with acidification of the degradative vesicles have been suggested. AP-2, e.g. that is internalized with the clathrin-coated vesicles in association with specific cargo (Sun-Wada *et al.,* 2003), recognizes tyrosine signals on the LAMP-2 cytoplasmic tail (Bonifacino *et al.,* 1999). It may, however, activate the V-ATPase proton pump through phosphorylation of a B-unit (Sun-Wada *et al.,* 2003) (Figure 3.1 and Table 3.1) and may be an important link in the sequestration of LAMP-2 during autophagic degradation.

However, even though all these signals are theoretically increased due to the constant signaling by the constitutively active c-Ha-Ras(V12), as described above, in this study the intraluminal acidity of the degradative vesicles and their digestive capacity was shown to be decreased in transfected cells (Figure 3.14). In c-Ha-*ras*(V12)-transfected cells downstream effectors other than PI3K have to account for the restricted acidification of degradative vesicles subsequent to LE formation. Since disruption of the cytoskeleton is one of the factors that are relevant in defective V-ATPase proton pump assembly and vesicle acidification, the influence of c-Ha-Ras(V12) on cytoplasmic alkalinity and cytoskeletal organization, will now be discussed.

3.8.1.7. The role of NHE-1 in c-Ha-Ras(V12) –related vesicle dynamics and alkalinization

NHE-1 stabilizes cellular volume and establishes an acidic microenvironment through ion translocation (Reshkin *et al.*, 2000a) (Section 3.2.1.4). In addition, it is spatially restricted to the leading edge of the cell (Denker *et al.,* 2002; Putney *et al.,* 2002) under the influence of activated Ras. Here it functions as a membrane anchor for the actin-based cytoskeleton, assisting in anterior-posterior polarity due to direct action on focal adhesion remodeling and also organelle orientation (Denker *et al.,* 2002). CD44 is also associated with the leading front, where it binds to both matrix components such as hyaluronan (HA) and intracellular cytoskeletal proteins. Through its ROCK-related activation of NHE-1 (Mammoto *et al.,* 2004), CD44 is associated with

extracellular acidification and polarization of BM degradation (Reshkin *et al.*, 2000a; Ponta *et al.*, 2003; Bourguignon *et al.*, 2004) during normal migration and as part of the invasive process of cancer cells (Hofmann *et al.*, 1993b; Kawano *et al.*, 2000a; Cichy *et al.*, 2003; Ponta *et al.*, 2003). ROCK is a downstream effector of activated Ras that is also associated with cytoskeletal components, indicating a direct connection between adhesion and maintanance of cytoplasmic pH (Counillon *et al.,* 2000; Lacroix *et al.,* 2004).

Mutated c-Ha-Ras(V12) constantly activates NHE-1 (Ritter *et al.*, 1997). This may promote intracellular alkalinity, and disrupt the microtubular cytoskeleton, along which endocytic vesicles migrate (Cantalupo *et al.,* 2001; Höltta-Vuori *et al.,* 2005) in a way similar to treatment with nocodazole (Wang *et al.*, 2004a) or ammonium chloride (Ritter *et al.,* 1997). It has been shown that such alkalinization of the cytoplasm by NHE-1 affects the rate of vesicle endocytosis, secretion and redistribution within cells (Orlowski *et al.,* 1997) and may result in a wider than normal spread of endosomes. NHE-1 activity may also be modulated by the increase in calcium levels due to c-Ha-*ras*(V12) transfection (Section 3.8.1.8), or other factors including cAMP and the products of PI3K such as PIP_3, all of which are known to alter the rates of endo- or exocytosis or effect redistribution of vesicles within cells (Orlowski *et al.,* 1997).

Inadequate degradation of the LE and, therefore, inadequate acidification of the later endocytic vesicles may possibly be due to decreased activity of the V-ATPase proton pump, or its failure to assemble, since both PI3K and Rab7, required for degradative vesicle maturation and acidification, are actually more consistently activated in c-Ha-*ras*(V12)-transfected cells (Figure 3.16). Alkalinization of the cytoplasm and disruption of the cytoskeletal components in transfected cells, however, may be the result of upregulation of a heparan sulfate-binding isoform of CD44 (Paradiso *et al.,* 2004; Cardone *et al.,* 2005) by c-Ha-*ras*(V12) and the activation of the NHE-1 proton pump (Counillon *et al.,* 2000; Lacroix *et al.,* 2004) (Figure 1.3 on fold-out). As a result, limited V0-V1 alignment may in turn restrict the number of proton pumps that are assembled into functioning units. In this way acidification of the later degradative endosomes could be negatively affected, as was illustrated in this study. This theory is strengthened given that disorganization of the cytoskeleton by nocodazole (Wang *et al.*, 2004a) or ammonium chloride (Ritter *et al.,* 1997) negatively affect the

maturation of lysosomal protease precursors, since it possibly results in an elevation of the processing endosomal pH and thus inhibits dephosphorylation of their mannose-phosphate trafficking signal (Bresciani *et al.,* 1996), a process that requires an acidic environment. In addition, cytoplasmic alkalinization inactivates the A-subunit of the V-ATPase proton pump through the formation of disulfide bonds over the catalytic site that inhibits ATP hydrolysis (Qi *et al.*, ; Forgac, 1999). Assembly of the V-ATPase may also be regulated by glucose levels and the RAVE regulator (Section 3.8.1.5), suggesting that starvation, such as has been suggested for cancer cells (Sato *et al.*, 2007), may also play a temporary role in disassembly of the V-ATPase, and thus reduction of degradative efficiency of the cell (Forgac, 1999; Sautin *et al.*, 2005; Xiao *et al.*, 2007).

It seems, therefore, that over-stimulation of the NHE-1 proton pump, may lead to extracellular acidification, relevant and possibly essential, in migration. On the other hand, such c-Ha-*ras*(V12)-related effect on NHE-1 in the MCF10AneoT cell line possibly results in cytoplasmic alkalinization and the proposed disorganization of the cytoskeletal elements, which may influence spread of the later endosomal vesicles from their tight perinuclear concentration (Blocker *et al.,* 1997). It may also negatively affect the stable assembly, and, thus, activity of the V-ATPase proton pump into a functioning enzyme (Capony *et al.,* 1994), restricting luminal acidification, and allowing elevation of the pH of the degradative vesicles. In addition, distribution of cathepsins to these structures may be changed, with CB and CL still retaining activity, even at the elevated pH of poorly acidified LEs. However, activity of CD, requiring an acidic environment, may be limited to the JNS. Its tight association with the MTOC lends stability to this structure, supporting stable V-ATPase assembly and acidification of this structure (Section 3.8.1.3) and necessitate the presence of LAMP-2 in its limiting membranes (Section 3.6).

3.8.1.8. Calcium influences Ras-related functions and secretion of cathepsin-containing vesicles

Fluctuations in intracellular calcium (Bakker *et al.*, 1997; Andrews, 2000; Malik *et al.*, 2001) due to the effect of Ras-PI3K downstream to PLC (Karp, 2002; Walker *et al.*, 2003) (Figure 1.3 on fold-out) is involved in the heterotypic fusion process between vesicles that carry incoming cargo and hydrolase-containing lysosomes (Lemons *et al.,* 1991; Luzio *et al.,* 2000; Malik *et al.,* 2001), and allows homotypic

fusion of LE structures (Bakker *et al.,* 1997). These fusion processes are mediated through calcium-binding transmembrane proteins (Andrews, 2000; Martinez *et al.*, 2000), possibly the annexin family (Mollenhauer, 1997; Gerke *et al.*, 2002; Futter *et al.*, 2007). The importance of cytoplasmic calcium was illustrated when killed TB induced an elevation in cytoplasmic calcium concentrations as part of the phagosome-lysosome fusion and degradation processes, while the live pathogen interfered with the calcium homeostasis and managed to evade degradation (Malik *et al.,* 2001; Vergne *et al.,* 2003). Calcium is also relevant in subsequent lysosomal reformation from the LE (Luzio *et al.,* 2000) and lysosomal exocytosis in normal cells (Rodríguez *et al.*, 1997; Andrews, 2000; Martinez *et al.*, 2000) and during wound healing (Huynh *et al.,* 2005), but was also shown to support the tumorigenic and metastatic phenotype (Soule *et al.,* 1990; Tait *et al.,* 1990; Basolo *et al.,* 1991).

The internalization process may also be supported by elevated calcium levels related to activated Ras. Clathrin-coated pit formation at the PM under the influence of calcium may be initiated by the interaction of annexin II with phosphatidylinositol 4,5 biphosphate (PIP_2), which in turn facilitates interaction with a tyrosine internalization motif on AP-2 (Futter *et al.*, 2007). Annexin II also interacts with actin and may thus link CCVs to the actin cytoskeleton (Hayes *et al.,* 2004) to regulate the trafficking of recycling vesicles in a manner that is independent of calcium (Zobiack *et al.,* 2003). Annexin VI plays a role in endosome-late endosome fusion and has been shown to be localized on LAMP-1 positive lysosomal structures during starvation-induced autophagy, as part of the chaperone-mediated lysosomal pathway (Futter *et al.,* 2007).

During proliferation elevated levels of calcium released from the ER could have both a stimulatory effect on Ras, as well as negative feedback influence on Ras signaling via PLC, and is, therefore, a significant factor in cancer progression (Walker *et al.,* 2003). Calcium is required during stimulation of the NHE-1 proton pump on the PM, by mutated Ras (Ritter *et al.,* 1997), which results in alkalinization of the cytoplasm and affects vesicle dynamics. Intracellular calcium levels, in addition to the Rabs, affect the formation (Mousavi *et al.,* 2001) and luminal acidification (thus digestion) (Lemons *et al.,* 1991) of degradative LEs (Vergne *et al.,* 2003) (Section 3.3.1.5), as well as trafficking or exocytosis of e.g. LAMP-1-positive vesicles (Rodríguez *et al.,* 1997; Kima *et al.,* 2000). Therefore, calcium affects cytoplasmic alkalinity and vesicle trafficking. In this way it may play a general role in the budding/fusion

machinery and co-ordinate calcium-dependent changes in vesicle formation and trafficking after c-Ha-*ras*(V12) transfection.

3.9. Altered p53/p16 synescence system a mutated c-Ha-Ras(V12), and cathepsin isoforms supports tumorigenicity

During normal migration Ras-induced CDC42 controls the polarization of membrane protrusions (Cau *et al.,* 2005) and the reorientation of the Golgi apparatus in the direction of movement (Nobes *et al.,* 1999). Increased levels of CDC42 (Wang *et al.,* 2007) and p38 will elevate cytoplasmic levels of p53, sending the cell into senescence (Gadéa *et al.,* 2002) as part of a negative feedback control of Ras-related mitosis (Section 1.8.7). However, due to the total deletion of the *9p21* locus, the control parental MCF10A cell line has a compromized p53 senescence system. This is as a result of lack of both p16 and p14, an upstream regulator of p53 (Section 1.8). Such a silenced p53/p16 system occurs in many immortalized, non-invasive breast cancer cell lines (Li *et al.,* 2007) and p53 may be absent in many cancers (Vousden, 2000). This condition may lead to uncontrolled CDC42 not regaining control over organelle distribution and is not able to control the orientation of one Golgi complex and MTOC adjacent to the nucleus.

However, altered vesicle distribution does not necessarily imply enzyme secretion, a function that may assist in invasion. c-Ha-Ras(V12) does, however, sustains proliferation that may also affect the transcription of the cathepsins investigated in the current study. It has been shown that each of the cathepsins may have various isoforms, resulting in a range of molecular weights. These were initially thought to be various processing forms (Sloane *et al.,* 2005). While CB is a lysosomal enzyme that is also found in the LE, such alternatively spliced isoforms of the CB may be trafficked to the nucleus during mitosis (Riccio *et al.*, 2001; Brix *et al.*, 2008) or, under conditions of stress, to the mitochondrion (Bestvater *et al.,* 2005), leading to apoptosis (Gyrd-Hansen *et al.,* 2006). A mature, active isoform is secreted by cancerous cells (Mehtani *et al.,* 1998; Linebaugh *et al.,* 1999). Interestingly, a splicing variant of CL devoid of the signal peptide also appears associated with the nucleus during S-phase (Goulet *et al.*, 2004; Sansregret *et al.*, 2006; Brix *et al.*, 2008). Precursor CL does not bind effectively to the MPR that carry most hydrolytic enzymes to the lysosome, but information on the cytoplasmic tail regulates trafficking of the mature enzyme towards the PM (Lazzarino *et al.,* 1990; Yeyeodua *et al.,* 2000).

It seems that, under conditions of stress, the cell will regulate the synthesis of proteases with or without the appropriate signaling sequences in order to direct it to its required site of action, i.e. either the lysosome, mitochondrion, nucleus or PM.

Secretion of cathepsins could also be as a result of increased levels of calcium due to c-Ha-ras(V12) stimulation. Secreted, membrane-associated cathepsins may, therefore, be responsible for the degradation of the BM and ECM at focal points at the leading edges of lamellipodia, or the invasive front during metastasis. During this process the increased level of endocytosed cargo in addition to the increased cytoplasmic pH, as well as the decreased degradative capacity of the LE, could have an influence on the distribution of the cathepsins contained in separate degradative vesicles, or the circulating storage lysosome. We suggest that it is not the direction of trafficking of the carrier vesicle, or lysosome *per se* that is altered, but that the distribution of CL and CB (to either storage lysosome or LE) is altered to cope with the increased levels of ingested collagen and the reduced degradative capacity due to alkalinization of the digestive vesicles and that secretion of these enzymes is the result of the migratory, or invasive drive in c-Ha-*ras*(V12)-transfected MCF10AneoT cells.

In addition to an underlying silenced p53, constant signaling by c-Ha-Ras(V12) results in the alkalinization of the cytoplasm by NHE-1 and loss of control over increased levels of CDC42 during mitosis. Increased levels of calcium and ROCK that disrupts the cytoskeleton of the cell may support the invasive phenotype. Such disruption may also alter the normal distribution of intracellular vesicles from a perinuclear to a more peripheral location. In addition, we suggest, as described above, that the altered organization of the cytoskeleton may negatively affect the efficiency of assembly of the V-ATPase proton pump and result in alkalinization of the degradative and precursor processing vesicles. This may, in turn, affect the luminal mature cathepsin content and supporting vesicle characteristics, such as luminal acidity and protective membrane proteins.

At this stage, it was known that CB, usually an intracellular protease, may be associated with the PM during normal processes such as wound healing (Buth *et al.*, 2007), but also in invasive cancer cells (Cavallo-Medved *et al.*, 2005) and that, in invasive cancers increased levels of both CB (Zajc *et al.*, 2003) and MT1-MMP (Hotary *et al.*, 2006) have been found to correlate with accelerated pathological

degradation of the underlying BM barrier and ECM. Both these proteases have been reportedly associated with the invasion and possible invasion front in many cell lines and in the MCF10AneoT model. They, therefore, must have some role in migration and or invasion. Our hypothesis is that, due to the effect of the c-Ha-Ras(V12) oncoprotein on cell polarity and the change from an epithelial to the mesenchymal shape, the distribution pattern of CB and MT1-MMP in the MCF10AneoT cells may be altered, compared to the normal MCF10A cells. We, therefore, investigated the distribution of CB on the PM of normal MCF10A cells and c-Ha-*ras*(V12)-transfected MCF10AneoT cells, as reported in the next chapter. In addition, it has more recently become clear that metalloproteases may also be involved in the migration process, especially MT1-MMP. This membrane-bound protease is involved in degradation of the BM at the leading front of migrating cells, but also matures a range of additional secreted proteases to form a digesting cascade during migration. The effect of c-Ha-*ras*(V12)-transfection on the distribution of CB and MT1-MMP will be investigated in the next chapter and correlated with the formation of a leading front during normal and invasive migration.

Since the association of MT1-MMP and CB with the PM that supports ECM degradation and migration, is largely unknown and complicated, a review of what is known about the function and role of these membrane-associated enzymes, was required, and alterations in the morphology were analyzed. Alterations in the distribution pattern of PM-associated CB during migration of both MCF10A and MCF10AneoT cells, was investigated. In order to similarly investigate the distribution of MT1-MMP, a set of antibodies previously raised to various combinations of the MT1-MMP ectodomains, was used. However, characterization of recognition in the MCF10A model cell line has not previously been done. The reviews and experimental work reported in the following chapter is, therefore, setting a baseline for further studies.

CHAPTER 4.

Spatial distribution of extracellular proteases CB and MT1-MMP in invasive c-Ha-*ras*(V12)-transfected MCF10A cells

ABSTRACT

CB, an acidic lysosomal protease, located intracellularly under normal conditions, has been implicated in tumour invasion and is elevated on the PM and in the plasma of patients with metastatic breast cancer. MT1-MMP, on the other hand, is a membrane-bound extracellular protease that is involved in remodeling of the ECM and cell migration. While CB and MT1-MMP seem to play separate roles and are controlled independently, both seem to be part of an extracellular enzyme activation cascade that supports the migratory capacity and invasion of cells.

Transfection of a c-Ha-*ras*(V12) mutated oncogene, present in 20-30% of metastatic cancers, into a control breast epithelial cell line (MCF10A) (spontaneously immortal due to a lack of a functional p16/p53 senescence system) results in constant stimulation by the mutationally activated c-Ha-Ras(V12) oncoprotein. This seems to lead to elevated levels of total cellular CB and MT1-MMP, increased growth, migration and ultimately invasion and metastasis. Altered extracellular distribution of CB and MT1-MMP seems to correlate with altered signaling after transfection of a *9p/21(-/-)* immortal breast epithelial cell (MCF10A) with a c-Ha-*ras*(V12) oncogene, as reviewed in Chapter 1 of this thesis. Caveolae, representing centers of assembly of inactive signaling molecules, kept inactive until activation via growth factors, are also reduced. The possible reason for the enhanced MT1-MMP and CB proteolytic activity and their possible role in the excessive degradation of the ECM during c-Ha-Ras(V12)-related invasion, is explored.

Compared to control cells, transformed cells extensively degrade artificial basement membranes. Using confocal microscopy and antibodies raised to various domains of MT1-MMP, a wide distribution of immunofluorescence labeling for MT1-MMP was observed on the PM of non-permeabilized transfected cells compared to control cells. MT1-MMP was concentrated mostly in the invasive front and intracellular MT1-MMP levels, in permeabilized, transfected cells, were elevated compared to the non-transfected cells. Both the control and transfected cells had immunolabelled CB on the membrane, lateral to the nucleus, with an increase in the level of CB at the invasive front of the transfected cells.

MT1-MMP may be involved in extracellular proteolytic activity at the leading edge of the migrating cell, due to transient association with phosphorylated cav-1, and the adhesion molecules CD44 and integrins at leading edge focal adhesion sites. Through the cleavage of CD44 at the rear of the cell and its internalization and representation on the PM, MT1-MMP may also facilitate motility. These processes are key to migration and are under the control of kinases and GTPases downstream from normal Ha-Ras.

We propose that extracellular proteolytic activity of CB, where it forms part of an extracellular BM-degrading enzyme cascade, may be retained by signaling that originate from the underlying matrix. On the other hand, initial activation of MT1-

MMP in the control cell may be guided by intracellular factors related to growth factor signaling. Lack of control over the distribution of MT1-MMP may be due to a lack of cav-1 and lack of association of mutated c-Ha-Ras(V12) with the caveolae.

4.1. Extracellular proteases involved in migration

Cell migration is associated with physiological processes such as angiogenesis (vascularization) (Roy *et al.*, 2006) and wound healing (Piccard *et al.*, 2007). During these processes, the BM, an uninterrupted barrier that separates epithelia from connective tissues (Leblond *et al.*, 1989; LeBleu *et al.*, 2007) and contains mostly collagen-type IV and laminins (LeBleu *et al.*, 2007), is dynamically modified in a highly regulated manner (Mignatti *et al.*, 1993). This process involves the participation of a large number of enzymes located on the plasma membrane (PM), or secreted at the invasion front. These play pivotal roles in the degradation of the surrounding matrix. While low levels of matrix-degrading proteases may give rise to arthritis and connective tissue diseases (Somerville *et al.*, 2003), continuous secretion of high levels may promote metastases (Hotary *et al.*, 2006; Nishida *et al.*, 2008).

As mentioned previously (Sections 1.8.4), in breast cancers of epithelial origin, initial immortality is often acquired due to silencing of p16, or inactivation of p53 by constant degradation or mutation (Elenbaas *et al.*, 2001; Li *et al.*, 2007). The wild-type p53 regulates the fidelity of DNA replication (Zhou *et al.*, 2001) and apoptosis (Vousden, 2000). It also suppresses growth or Ras-related phenotype changes, via the induction of cell cycle arrest and terminal differentiation (Lee *et al.*, 2000b; Lin *et al.*, 2001; Li *et al.*, 2007). Cancers that originate from cells that harbour a mutated p53, however, may progress towards the malignant phenotype due to secondary mutations such as the V12 mutation of c-Ha-*ras* (Gao *et al.*, 2004; Houle *et al.*, 2006). Expression of the c-Ha-*ras*(V12) oncogene in experimentally transfected cells, such as the MCF10A control, immortal breast epithelial cells, results in a migratory, invasive, premalignant phenotype (Basolo *et al.*, 1991) and protease trafficking to the cell surface or secretion from the transfected cells (Sloane *et al.*, 1994; Sameni *et al.*, 1995; Shin *et al.*, 2005).

In the previous chapters the generic downstream effector pathways of Ras proteins were reviewed (Section 1.6 to Section 1.10) in order to explain the role, feedback controls, and the effect of the oncogenic c-Ha-Ras(V12) mutations and to illustrate the mechanisms via which mutated c-Ha-Ras(V12) oncoprotein may affect intracellular

cathepsin-containing vesicle acidity and trafficking. In this chapter, the focus shifts towards the extracellular proteases, their function, trafficking and role in invasion.

CB is part of a group of usually intracellular lysosomal acidic proteases (Kirschke *et al.*, 1997), functions in processing or degradation in the acidic late endosome and is stored in lysosomes (Schmid *et al.*, 1999; Brix *et al.*, 2008). In the previous chapter changes to the characteristics of CB-containing vesicles and its intracellular distribution were investigated. These changes highlighted the influence of c-Ha-*ras*(V12) transfection in immortal cells on intracellular control of protease trafficking, and its possible secretion and membrane association. CB (Buth *et al.*, 2007), as well as the membrane-bound protease, membrane type-1 matrix metalloprotease (MT1-MMP) (Okada *et al.*, 1997; Galvez *et al.*, 2002), may be associated with the plasma membrane during normal processes such as wound healing. In invasive cancers, however, increased levels of both CB (Zajc *et al.*, 2003; Cavallo-Medved *et al.*, 2005) and MT1-MMP (Hotary *et al.*, 2006) have been found to correlate with accelerated pathological degradation of the underlying BM barrier and ECM. The cellular location of CB (Roshy *et al.*, 2003) and MT1-MMP (Itoh *et al.*, 2006b) and related functions have recently been investigated in order to understand their role and develop effective therapeutic regimes (Ueda *et al.*, 2003; Cavallo-Medved *et al.*, 2005). MT1-MMP is known to regulate BM turnover (Cao *et al.*, 2004), while the manner in which the association of CB with the PM, supports ECM degradation and migration, is not yet clear. These unknowns form the focus of the current investigation on the distribution of CB and MT1-MMP on the PM of normal MCF10A and c-Ha-*ras*(V12)-transfected MCF10AneoT breast epithelial cells. In order to identify markers for future studies on the role of membrane-associated MT1-MMP and CB in invasion formed a focus, as well as the individual distribution of CB and MT1-MMP as evidence of function.

Using protease inhibitors and other investigations into invasion by our research group, a key role of MT1-MMP in invasion seems to be indicated. The invasive MCF10AneoT cell line showed expression of high levels of MT1-MMP after transfection, with these inhibitors completely blocking invasion (van Rooyen *et al.*, 2008) (Gordon conference 2008 Poster, Appendix IV). Our aim was to verify the established distribution of CB and MT1-MMP and to investigate the influence of c-

Ha-*ras*(V12) transfection on their distribution, since this information may also be relevant in invasion. Antibodies to different domains of the MT1-MMP protein were previously raised by members of our laboratory, and were characterized with my assistance (Section 4.5) (Gordon conference 2008 Poster, Appendix IV).

While it is still unclear how the association of MT1-MMP and CB with the PM, supports ECM degradation and migration, it is necessary to review what is known about the function and role of these enzymes, though in both cases the literature is difficult to follow and full of unknowns.

4.1.1. Association of CB with the plasma membrane

CB may be associated with the plasma membrane at the leading front of normal migrating cells, but has previously been seen at the plasma membrane of cancer cells (Roshy *et al.*, 2003; Cavallo-Medved *et al.*, 2005) and more specifically the MCF10AneoT cells (Sloane *et al.*, 1994; Sameni *et al.*, 1995). Here it may be associated with the uPA, an extracellular protease, and its related enzyme cascade (Section 4.1.2).

CB functions mainly as an exopeptidase (Schmid *et al.*, 1999) in protein degradation and macromolecule processing (Therrien *et al.*, 2001) in a low pH environment (Turk *et al.*, 2000; Turk *et al.*, 2002). When it is secreted from cells or become cell surface-associated (Sloane *et al.*, 1994; Sameni *et al.*, 1995; Linebaugh *et al.*, 1999) CB may act as an endopeptidase at neutral pH values of the extracellular environment (Linebaugh *et al.*, 1999). In pathological conditions such as arthritis, therefore, both membrane-associated and secreted CB can degrade cartilage collagens at pH values near neutrality (Maciewicz *et al.*, 1990). Mature CB protein contains a unique flexible occluding loop structure that partially obscures one end of the substrate binding cleft and supports the propeptide structure (Musil *et al.*, 1991; Turk *et al.*, 1996; Illy *et al.*, 1997; Quraishi *et al.*, 1999). This structure appears to play an important role in the binding of substrates that are cleaved by CB, serving as an anchor to the C- or N-terminus of substrates and facilitating the exopeptidase activity of CB, while it interferes with the binding of larger extended peptides, a prerequisite for endopeptidic activity (Nagler *et al.*, 1999). At normal physiological pH, however, this loop contributes to the instability of CB (Almeida *et al.*, 2001a). Heparin and heparan sulfate (HS) proteoglycan, components of the lamina densa of the ECM

(Mignatti *et al.*, 1993) binds to the occluding loop of extracellular CB and may thus protect CB from denaturation at the extracellular, more alkaline pH (Almeida *et al.*, 2001a).

4.1.2. Plasma membrane binding partners for CB

At the plasma membrane a lipid raft-associated cluster of enzymes that includes CB (Figure 4.1), create a path for migration through the ECM (Murphy *et al.*, 1999). uPA, secreted as a precursor that binds to its cell surface receptor (uPAR), is activated by cysteine proteases, e.g. CB or CL (Andreasen *et al.*, 1997; Bok *et al.*, 2003) (Figure 4.1). Activated uPA converts a plasminogen precursor to plasmin, degrades numerous components of the ECM (Andreasen *et al.*, 1997; Takino *et al.*, 2004; Cavallo-Medved *et al.*, 2005; Hotary *et al.*, 2006; Siesser *et al.*, 2006) and may activate latent matrix metalloproteases (MMPs), such as MMP-2 and MMP-9 (Siesser *et al.*, 2006). In this way these proteases may sustain local degradation of the BM (Mignatti *et al.*, 1993; Rabbani *et al.*, 1998; Murphy *et al.*, 1999; Collette *et al.*, 2004). This seems to occur via an association of CB and its precursor with annexin-II and p11 (Mai *et al.*, 2000; Cavallo-Medved *et al.*, 2005), two proteins that exist in a heterotetrameric complex (Zobiack *et al.*, 2003) and which is associated with caveolae on the PM. Caveolae are flask-shaped, 50–100-nm invaginations that form on association of a protein known as caveolin-1 (cav-1), with lipid rafts on the PM (Reuther *et al.*, 2000; Razani *et al.*, 2001; Thomsen *et al.*, 2002). Cav-1 anchors these caveolae to the actin cytoskeleton through filamin, making these domains immobile (Stahlhut *et al.*, 2000; Thomsen *et al.*, 2002; Navarro *et al.*, 2004). In addition, cav-1 gathers and regulates endocytosis-related fusion molecules, e.g. annexin-II and -VI (Schnitzer *et al.*, 1995).

The adaptor protein p11 also plays a role in trafficking of PM proteins, such as trafficking of PM-associated ion channels from the ER (Girard *et al.*, 2002; Svenningsson *et al.*, 2007). At the PM, p11 is relevant in successful recycling of e.g. the PM-bound transferrin receptor in Rab11-positive recycling endosomes (Zobiack *et al.*, 2003). An annexin-II-p11 complex is suggested to be involved in clathrin-mediated, calcium-dependent endocytosis (Gerke *et al.*, 2002; Futter *et al.*, 2007; Morel *et al.*, 2007). Annexin-II is a member of the family of Ca^{2+}- and phospholipid-binding proteins (Rescher *et al.*, 2004). As a monomer, annexin-II is a major component of lipid rafts and caveolae (Futter *et al.*, 2007), and interacts with actin to

serve as a link between endosomal sorting vesicles and the actin cytoskeleton (Hayes *et al.*, 2004). Dimeric annexin-II, on the other hand, binds PIP_2, facilitating its interaction with the tyrosine internalization motif on AP-2 (Futter *et al.*, 2007) (Section 3.3.4.2). While both p11 and phospholipid-bound annexin-II, contain distinct binding sites for plasmin, the C-terminal of p11 forms uPa- (Choi *et al.*, 2003), t-Pa- and plasminogen-binding sites (MacLeod *et al.*, 2003) (Figure 4.1). In this way cell surface expression of p11 may assist in the accumulation of plasmin and increase in cellular plasmin production and, together with focused localization of CB (Figure 4.1) lead to enhanced ECM degradation and invasiveness (Choi *et al.*, 2003) (Figure 4.8 Lane 2).

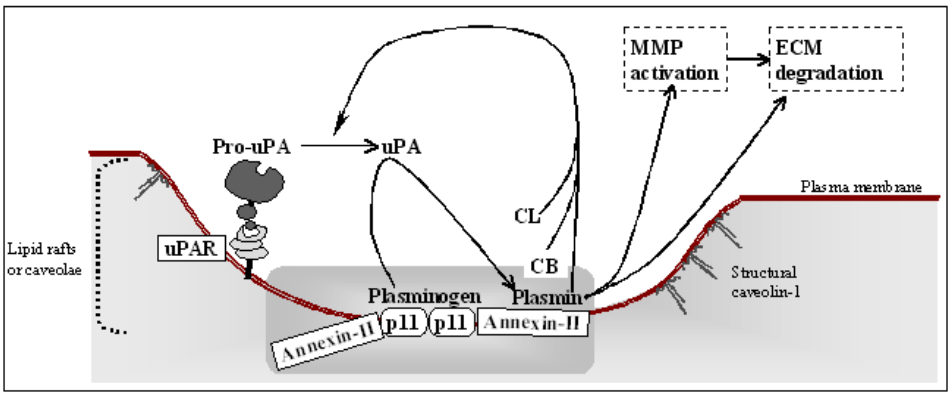


Figure 4.1. Possible CB, CL, uPA and MMP activation cascade leading to degradation of the ECM.
uPA binds to its cell surface receptor (uPAR) and is activated by cysteine proteases, e.g. CB or CL. Activated uPA initiates activation cascades that assist in further uPA activation, MMP activation and ECM degradation. The annexin-II-p11 heterotetrameric complex may act as membrane receptors for plasminogen, plasmin and possibly CB. These processes may occur in caveolae, specialized lipid rafts shaped by a structural protein, caveolin-1. This illustration was generated from references mentioned in Sections 4.1.2.

Membrane-bound CB, associated with the p11-annexin-II complex (Mai *et al.*, 2000; Cavallo-Medved *et al.*, 2005), may, therefore, be part of the uPa cascade, involved in the gelatinolytic degradation of ECM components (Andreasen *et al.*, 1997; Mort *et al.*, 1997). It may also form part of regular migratory mechanisms in normal polarized epithelial cells (Linke *et al.*, 2002), controlling chemotaxic migration (Schraufstatter *et al.*, 2003) and wound healing (Buth *et al.*, 2007). Elevated levels of secreted and membrane-associated CB, as found in metastatic breast cancers (Hirai *et al.*, 1999), seems, therefore, to be implicated in tumour invasion (Cavallo-Medved *et al.*, 2005; Sloane *et al.*, 2005). For this reason, elevated levels of CB in the plasma of metastatic breast cancer patients, may be used as a negative prognostic predictor for

survival (Lah *et al.*, 2000). MMPs, on the other hand, are zinc-dependent extracellular neutral proteolytic enzymes that also seem to participate in the turnover of ECM and invasion. It is our hypothesis that interaction between CB and MT1-MMP is key in migration and invasion and we would expect to find them closely associated on the PM. It is not yet known, however, which processing form is involved and whether antibodies to MT1-MMP would recognize this form. To this end, antibodies raised to combinations of the MT1-MMP ectodomains were used in the current study to investigate the localization of intracellular and PM-associated MT1-MMP in MCF10A and c-Ha-*ras*(V12)-transfected MCF10AneoT cells.

4.1.3. MT1-MMP structure and processing

MT1-MMP, the most common membrane-bound MMP, is distinguished from other MMPs by the presence of a single hydrophobic transmembrane domain. Unique to most membrane-type MMPs, this anchors the protein to the cell surface (Folgueras *et al.*, 2004) (Figure 4.2).

The precursor domain of MT1-MMP, like most proteases, has an N-terminal signal peptide and a propeptide (Pro) domain (Figure 4.2). The prodomain contains a cysteine (C-93), which interacts with histidines in the active site, allowing the prodomain to shield the catalytic domain from autocatalytic cleavage (Van Wart *et al.*, 1990; Massova *et al.*, 1998). A haemopexin (Pex) domain, consisting of four haemopexin-like repeats, gives rise to the substrate-binding specificity of the enzyme (Hofmann *et al.*, 2000; Tam *et al.*, 2002; Piccard *et al.*, 2007) (Figure 4.2). This domain is connected to the catalytic (Cat) domain by a flexible proline-rich hinge region (Somerville *et al.*, 2003) and to a transmembrane domain and the short cytoplasmic C-terminal domain, which has a highly conserved 20 amino acid segment (Somerville *et al.*, 2003) (Figure 4.2).

MT1-MMP is produced as an inactive precursor (approximate Mr 66 kDa) (Figure 4.2). The prodomain is intracellularly cleaved by furin (Zhuge *et al.*, 2001; Mazzone *et al.*, 2004) at an N-terminal $RRKRY^{111}$ furin cleavage sequence, with cleavage occuring between R^{110} of the precursor and Y^{111} of the catalytic domain (Sato *et al.*, 1996; Tellier *et al.*, 2007). Cleavage at this site allows the mature, active 54-58 kDa protein (Remacle *et al.*, 2006a) to subsequently be trafficked to the PM under the guidance of Rab8 (Peranen *et al.*, 1996; Bravo-Cordero *et al.*, 2007) (Figure 4.2).

In addition to the mature 54-58 kDa protein, a partially processed 63 kDa form has been described. Initial cleavage within the prodomain was shown to be required prior to the furin cleavage step (Remacle *et al.*, 2006b) and may allow a short amino acid sequence of propeptide domain to remain attached to the mature sequence (Cao *et al.*, 1998) (Figure 4.3 Lane 2). This process may give rise to a furin–independent 63 kDa intermediate form of MT1-MMP (Yana *et al.*, 2000; Golubkov *et al.*, 2007) (Figure 4.2), that may form may form part of the catalytic triad involved in MT1-MMP processing of MMP-2 (Cao *et al.*, 1998; Pavlaki *et al.*, 2002; Golubkov *et al.*, 2007), as discussed below (Section 4.1.4.1). Such a two-step mechanism may facilitate initial processing of the inhibitory prodomain and the subsequent release of the activated, mature 58 kDa MT1-MMP enzyme (Golubkov *et al.*, 2007). It has also been suggested, that either plasmin (Hofmann *et al.*, 2000) or uPA (Kazes *et al.*, 1998) on the PM may be responsible for subsequent maturation of the partially processed 63 kDa intermediate form of MT1-MMP to the mature form (Figure 4.3 Lane 3).

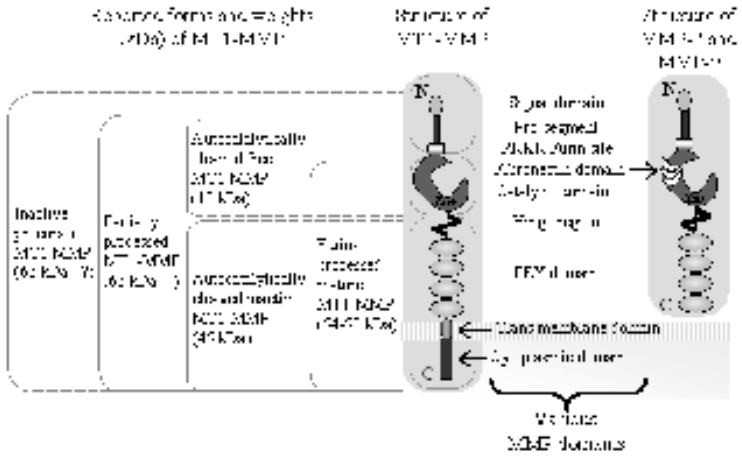


Figure 4.2. Diagramatic summary of various processing forms of MT1-MMP domains compared to MMP-2 and MMP-9.
MT1-MMP is produced as an inactive 66 kDa precursor, that is matured by furin to a 54-58 kDa protein. The 63 kDa protein may be an intermediate form that contains a partially processed precursor domain, required for TIMP-2 binding during proMMP-2 processing. Autocatalytic processing produces the inactive 45 kDa and soluble 18 kDa forms. References for the various molecular weight forms are documented in Sections 4.1.3 and 4.1.4.

Finally, autocatalytic processing may give rise to the inactive 45 kDa and the soluble 18 kDa forms of MT1-MMP (Remacle *et al.*, 2006a) (Section 4.1.6, Figure 4.2 and Figure 4.4 Lane 3).

4.1.4. MT1-MMP specificity and role

MT1-MMP is active at neutral pH (Vu *et al.*, 2000), and regulates a wide range of other molecules in the PM microenvironment, by proteolytic processing or degradation (Ueda *et al.*, 2003). It activates MMP precursors such as pro-MMP-2 (Butler *et al.*, 1998; Fernandez-Catalan *et al.*, 1998; Morrison *et al.*, 2001) (Section 4.1.4.1) and proteolytically processes many cell surface proteins at the front of the cell, such as integrins (Section 4.1.4.2). In addition, at the rear of the cell MT1-MMP may cleave CD44, a cell attachment protein, which allows release of the cell and facilitates migration (Rozanov *et al.*, 2004b; Rozanov *et al.*, 2004c) (Section 4.1.7.1 and Figure 4.4 Lane 3). (Okamoto *et al.*, 2001; Ratnikov *et al.*, 2002; Piccard *et al.*, 2007).

Table 4.1. MT1-MMP-, MMP-2- and CB activity on components of the ECM.

MMP	**ECM component as substrate**	**References**
MT1-MMP (membrane-bound)	Collagen types I, II, III, IV, and gelatin Aggrecan, dermatan sulphate proteoglycan, fibrin, fibronectin, laminin and laminin-5, nidogen, perlecan, tenascin and vitronectin	(d'Ortho *et al.*, 1998; Koshikawa *et al.*, 2000; Somerville *et al.*, 2003; Cao *et al.*, 2004; Sabeh *et al.*, 2004; Wu *et al.*, 2004; Hotary *et al.*, 2006; Munoz-Najar *et al.*, 2006)
MMP-2 (secreted)	Collagen types I, IV, V, VII, X, XI, XIV, and gelatin Aggrecan, elastin, fibronectin, laminin and laminin-5, nidogen, proteoglycan link protein and versican.	(Koshikawa *et al.*, 2000; Somerville *et al.*, 2003; Sabeh *et al.*, 2004; Hotary *et al.*, 2006; Munoz-Najar *et al.*, 2006)
CB (membrane associated)	Collagen type IV Fibronectin and laminin.	(Buck *et al.*, 1992; Sloane *et al.*, 2005; Buth *et al.*, 2007; Daley *et al.*, 2008)

Remodelling of the basement membrane is key in cell motility, and through its proteolytic activity MT1-MMP degrades a number of these ECM proteins (Zhuge *et al.*, 2001; Sabeh *et al.*, 2004; Lafleur *et al.*, 2005) (Table 4.1). Degradation of collagen and its gelatin components is an important step in this process. Due to their unique composition and structure, collagens of the ECM are resistant to cleavage by most proteinases except specific collagenases such as MT1-MMP (Somerville *et al.*, 2003). MT1-MMP, homodimerized via its Pex domain, makes a single cleavage in most collagen strands (Itoh *et al.*, 2006b; Itoh *et al.*, 2008) (Figure 4.3 Lane 1 and

Table 4.1). This denatures the triple helix polypeptide chain structure and allows further degradation of the individual subunits by gelatinases, such as MMP-2 or CB, or other broad-spectrum proteinases (Mignatti *et al.*, 1993). Together with MMP-2, MT1-MMP can cleave almost every component of the BM (mostly type IV collagen and laminins) and ECM, including type I (Sabeh *et al.*, 2004; Lafleur *et al.*, 2005), II, III and VI collagens, gelatin, laminins 1 and 5, fibronectin, vitronectin, aggrecan and fibrin (Pei *et al.*, 1996; d'Ortho *et al.*, 1998; Nagase *et al.*, 1999; Zhuge *et al.*, 2001; Somerville *et al.*, 2003; Folgueras *et al.*, 2004; Itoh *et al.*, 2006a) (Table 4.1). MT1-MMP on its own, however, may be sufficient to cause destruction of the ECM and gives rise to inflammatory diseases e.g. in the lung (Maisi *et al.*, 2002). In many cancer types, moreover, a strong correlation has also been made between elevated MT1-MMP expression and invasion (Caenazzo *et al.*, 1998; Seiki *et al.*, 2003; Annabi *et al.*, 2004; Sabeh *et al.*, 2004).

4.1.4.1. Processing of proMMP-2 and MT1-MMP degradation

Proteolytically active MMP-2 also plays an important role in BM destruction and cell migration associated with tumour metastasis (Munoz-Najar *et al.*, 2006). MT1-MMP proteolytically matures proMMP-2 to its active form. During this process, mature 58 kDa MT1-MMP forms a homodimer via its Pex domain, with the 63 kDa partially activated form of MT1-MMP and tissue inhibitor of metalloproteases-2 (TIMP-2) (Butler *et al.*, 1998; Atkinson *et al.*, 2006; Piccard *et al.*, 2007), in the proMMP-2 activation complex (Kazes *et al.*, 1998; Lehti *et al.*, 2000; Zhuge *et al.*, 2001; Morgunova *et al.*, 2002; Tam *et al.*, 2002) (Figure 4.3 Lane 2). The C-terminal of TIMP-2 electrostatically binds to- and functions as a receptor of the C-terminal of proMMP-2 (Butler *et al.*, 1998; Morgunova *et al.*, 2002). Via the N-terminal of TIMP-2, this complex subsequently binds to the N-terminal of the partially cleaved precursor tail of the 63 kDa MT1-MMP, which contains a TIMP-2 binding motif (Yana *et al.*, 2000) and facilitates the activation of proMMP-2 (Butler *et al.*, 1998; Hofmann *et al.*, 2000; Morgunova *et al.*, 2002; Atkinson *et al.*, 2006; Nishida *et al.*, 2008) (Figure 4.3 Lane 2). Mature MMP-2, released in this manner, may degrade laminin-5 (Koshikawa *et al.*, 2000), or may further degrade various collagens, cleaved by homodimeric MT1-MMP (Figure 4.3 Lanes 1 and 2, Table 4.1).

Degradation of the partially cleaved prodomain on the 63 kDa intermediate form, by either plasmin (Okumura *et al.*, 1997) or uPa, may subsequently release the activated,

mature 54 and 58 kDa MT1-MMP enzyme (Golubkov *et al.*, 2007) (Figure 4.3 Lane 3). The activity on the plasma membrane of both MMP-2 and the mature 58 kDa MT1-MMP may be regulated by TIMP-2 (Bernardo *et al.*, 2003).

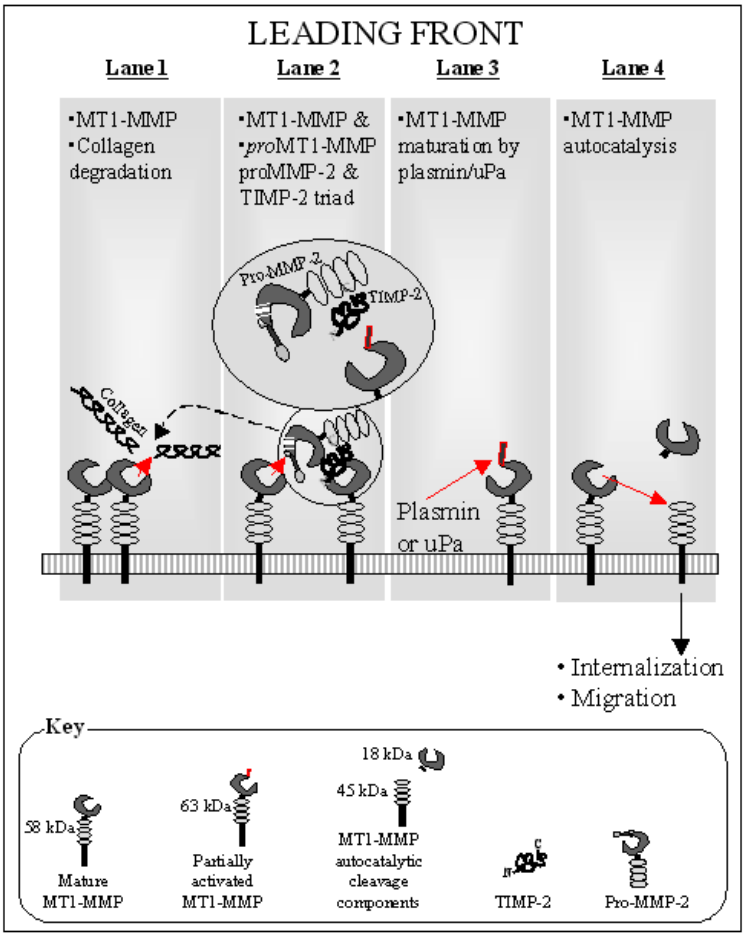


Figure 4.3. Processing of MT1-MMP and proMMP-2 at the plasma membrane.
A schematic illustration of the plasma membrane-associated processing steps involved in maturation and autocatalysis of MT1-MMP at the leading front and its role in MMP-2 maturation. This illustration was generated from references mentioned in Section 4.1.4.1.

4.1.4.2. Integrin processing and signalling via FAK

Integrins regulate the attachment of cells to ECM and are key in interaction between the extracellular environment and intracellular signaling cascades. Such signaling may occur in either direction between the ECM and the intracellular cytoskeleton (Takagi *et al.*, 2002; Humphries *et al.*, 2006). "Inside-out signaling" results in cell adhesion (Somanath *et al.*, 2007), while "outside-in signaling" results in cytoskeletal rearrangements, gene expression and cellular differentiation (Humphries *et al.*, 2006) (Figure 4.5, Figure 4.6 and Figure 4.7 F) and migration via focal adhesion kinase (FAK) (Figure 4.5).

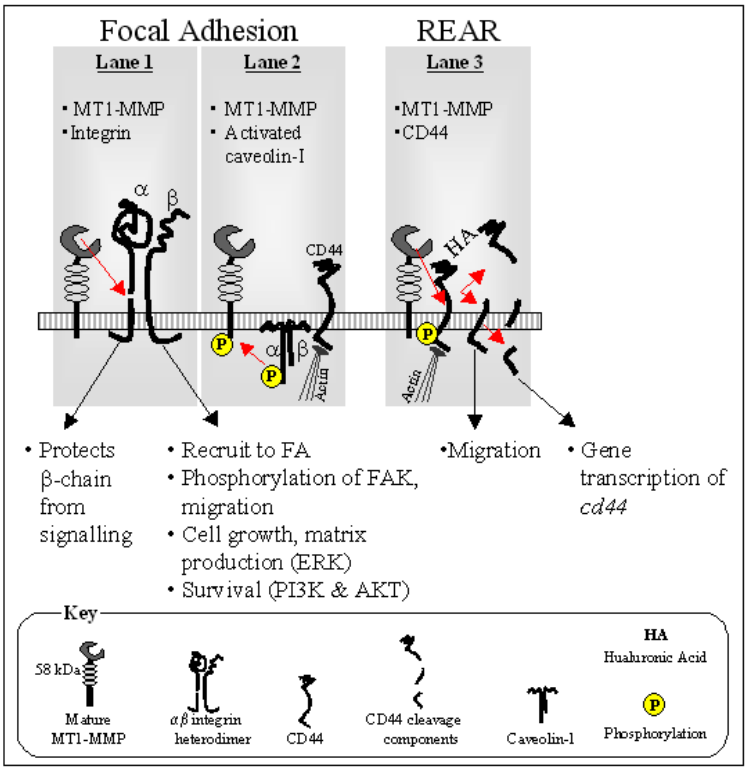


Figure 4.4. MT1-MMP and its associated membrane partners at focal adhesions.
In this schematic presentation the MT1-MMP molecule and its various known membrane partners are grouped in lanes to illustrate the relationship between MT1-MMP and integrins at FAs that may lead to various downstream signaling paths (Lane 1), the phosphorylation of MT1-MMP by cav-1 and its association with CD44 at the leading front (Lane 2), cleavage of phosphorylated CD44 by MT1-MMP at the rear of the cell and subsequent internalization of the CD44 endodomain and its role in migration and gene transcription. This illustration was generated from references mentioned in Section 4.1.4

Integrins are heterodimeric molecules containing non-covalently linked type I transmembrane α and β protein subunits (Humphries *et al.*, 2006) (Figure 4.4 Lane 1) and are one of the major families of cell adhesion receptors that mediate cell migration and adhesion (Plow *et al.*, 2000; Humphries *et al.*, 2006). In general, the extracellular domains of the combination of particular α and β subunits determines the ligand specificity of the integrin complex (Guan, 1997) for many ECM proteins such as collagens, laminins, vitronectin, fibronectins and fibrinogen (Aplin *et al.*, 1998; Plow *et al.*, 2000; Humphries *et al.*, 2006). The affinity for ligands is increased due to a change in conformation from a closed, to an open form (Marcantonio *et al.*, 1997; Takagi *et al.*, 2002). This process is controlled by N-glycosylation (Luo *et al.*, 2003), while the level of activation is regulated by low affinity bound cations, i.e. Mg^{2+} and Ca^{2+} in the substrate binding domains (Plow *et al.*, 2000).

MT1-MMP plays a key role in integrin processing leading to the formation of FAs, since it is involved in processing the pro-αv integrin into a heavy and a light chain connected via a disulphide bridge (Deryugina *et al.*, 2002; Ratnikov *et al.*, 2002) (Figure 4.4 Lane 1). This process improves cell adhesion by elevating or increasing "outside-in" signaling by the integrin as the processed integrin begins to bind the ECM at focal adhesion sites (Deryugina *et al.*, 2002; Ratnikov *et al.*, 2002) Figure 4.5).

Non-phosphorylated paxillin correlates with adhesion assembly and stability. On integrin association with FAs, the cytoplasmic domains of the β-integrins recruit inactive tyrosine kinase FAK (Aplin *et al.*, 1998; Hamadi *et al.*, 2005; Le Boeuf *et al.*, 2006; Pirone *et al.*, 2006), facilitated by the scaffold proteins talin and phosphorylated paxillin that bind both integrin and the C-terminal of FAK (Guan, 1997; Wary *et al.*, 1998; Mitra *et al.*, 2006; van Nimwegen *et al.*, 2007; Iwanicki *et al.*, 2008) (Figure 4.5 Lane 1). FAK is activated by activated growth factor receptors and G protein-coupled receptors on the cell surface, that recruit and activate Src kinases and other signaling proteins (Fan *et al.*, 2005; Lietha *et al.*, 2007). The N-terminal domain of FAK blocks substrate access to the FAK catalytic domain to keep it in an inactive state, but is displaced by binding to integrins and growth factors. This allows autophosphorylation of FAK Tyr-397 (Navarro *et al.*, 2004; Lietha *et al.*, 2007) and initial stabilization of FAK within the FA (Hamadi *et al.*, 2005; Goetz *et al.*, 2008). Subsequently, Src that has been activated by phosphorylated cav-1 (Williams *et al.*, 2005), may associate with FAK (Playford *et al.*, 2004; Siesser *et al.*, 2006) (Figure 4.5 Lane 2) and contribute to FAK activation by phosphorylation of FAK at Tyr-576 and Tyr-577 (Mitra *et al.*, 2006).

The activated FAK/Src complex may lead to phosphorylation of FAK at several serine and tyrosine residues (Aplin *et al.*, 1998) (Figure 4.5), with the degree of phosphorylation regulating specific downstream signaling paths (Hamadi *et al.*, 2005; Le Boeuf *et al.*, 2006; Siesser *et al.*, 2006; van Nimwegen *et al.*, 2007). G-protein-coupled- and growth receptor signaling rapidly increases FAK phosphorylation at Ser-843 and at Ser-910, with signaling paths that include downstream Ras activation and increased intracellular calcium levels (Fan *et al.*, 2005). In this way FAK may regulate further downstream cellular responses including cell adhesion, migration,

survival, matrix production and proliferation (Fan *et al.*, 2005) (Figure 4.4 Lane 1 and Figure 4.5).

Engagement of integrins with components of the ECM such as fibronectin (Murillo *et al.*, 2004; Siesser *et al.*, 2006), vitronectin (Deryugina *et al.*, 2002; Ratnikov *et al.*, 2002) or laminin (Fiucci *et al.*, 2002a; Liu *et al.*, 2007), upregulates association of PI3K with FAK Tyr-397 via the β-integrin cytoplasmic domain (Guan, 1997) and activation of downstream AKT (Guan, 1997; Khwaja *et al.*, 1997), which may assist in survival of migrating cells (Figure 4.5 Lane 2), protect detached cells from cell death (anoikis) and allow survival of cancer cells (Joy *et al.*, 2003; Siesser *et al.*, 2006) (Figure 4.5 Lane 2).

Subsequent to phosphorylation of Tyr397, phosphorylation of Tyr-925 may lead to the association at its C-terminal proline-rich domains, with 130 kDa crack associated substrate (p130CAS), that in turn binds to the growth factor receptor-binding protein-2 (Grb-2) (Aplin *et al.*, 1998; Playford *et al.*, 2004; Mitra *et al.*, 2006) (Figure 4.5 Lane 1). This complex permits interactions with a number of different signaling effectors, e.g. extracellular signal-regulated protein kinase-2 (ERK-2) (Schlaepfer *et al.*, 1997; Barberis *et al.*, 2000) that in turn activates the myosin light chain by myosin light chain kinase (MLCK) (Moissoglu *et al.*, 2006) to facilitate contraction of actin-myosin stress fibers (Figure 4.5 Lane 2). This may lead to turnover of adhesions, release and retraction of the rear and migration (Machesky *et al.*, 1997; Oliveira *et al.*, 2003; Vicente-Manzanares *et al.*, 2007; Zaidel-Bar *et al.*, 2007) (Figure 4.5 Lane 2 and Section 1.8). ERK is also a transcription factor that can translocate to the nucleus (Figure 4.5 Lane 1), resulting in elevated transcription of proteins involved in proliferation, migration and promotion of G1-S transition in the cell cycle (Bissonnette *et al.*, 2000; Goetz *et al.*, 2008). Phosphorylation of FAK at Tyr-576 and Tyr-577 by Src, may contribute to maximal FAK activation (Mitra *et al.*, 2006; van Nimwegen *et al.*, 2007).

FAK/Src signaling through p130CAS may also lead to the expression of MMPs, and its activation, possibly through enhanced expression of MT1-MMP on the cell surface (Siesser *et al.*, 2006) (Figure 4.5 Lane 1). Such association of MT1-MMP with focal adhesion signaling proteins, such as p130CAS in the FAK complex gives rise to increased MT1-MMP-related invasion of collagen barriers at the leading edge, where

these proteases are part of the proteolytic cascade that degrades the BM during migration and invasion (Nyalendo *et al.*, 2007; Zaidel-Bar *et al.*, 2007; Gingras *et al.*, 2008; Goetz *et al.*, 2008; Ouyang *et al.*, 2008b) (Figure 4.7). Together the facilitation of anchorage-independent growth, MT1-MMP allows movement of the cell past the G1-phase of the cell cycle in cancer cells (Nyalendo *et al.*, 2008), possibly via initial maturation of integrins and further signaling via ERK.

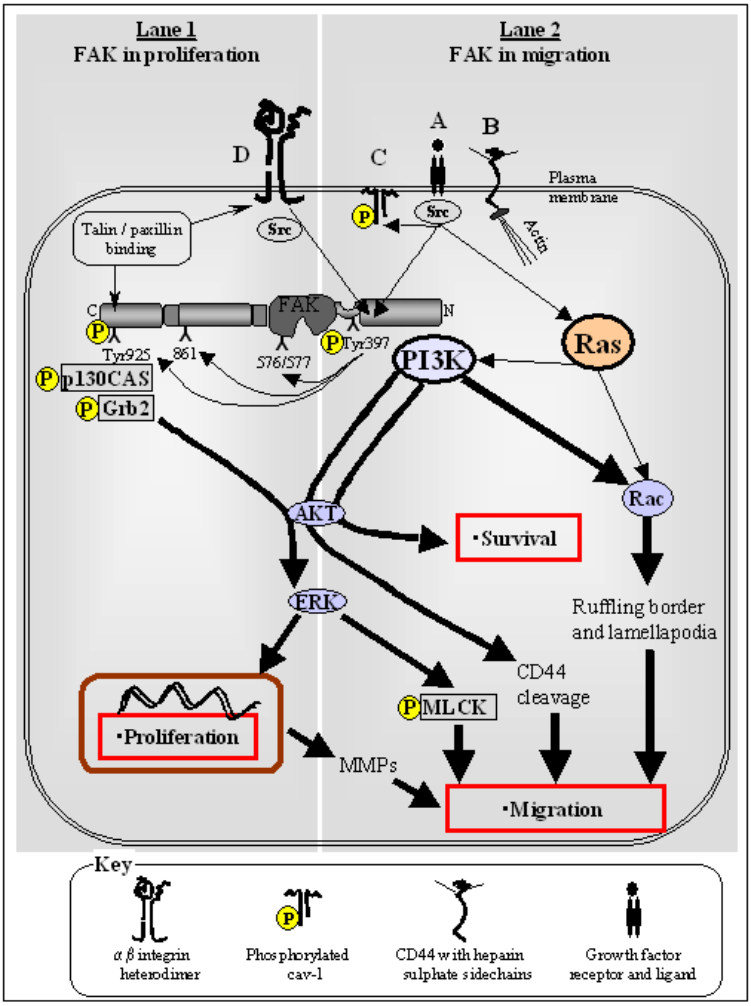


Figure 4.5. Schematic illustrations of FAK downstream signaling.
Phosphorylated cav-1 stabilize FAK within the FA and promote FA disassembly and turnover. FAK initially binds to integrins at its C-terminal via paxillin and talin, and may be phosphorylated at various residues. Several downstream effectors, such as p130CAS and PI3K may bind to phosphorylated sites on FAK, and signal downstream to Rac, that is involved in formation of a leading front, while signaling via AKT ensures survival, allows CD44 cleavage and MLCK phosphorylation and MMP transcription via ERK, all of which are factors that assist in migration. This illustration was generated from references mentioned in Section 4.1.4.2.

Polarization of signals at the leading front or the rear is necessary for migration. Control of FAK activity by GTPases (Rac, Rho) (Siesser *et al.*, 2006) and kinases (Src, ROCK, etc) that also affect the cytoskeletal elements (Aplin *et al.*, 1998; Nobes

et al., 1999; Ory *et al.*, 2000; Wehrle-Haller *et al.*, 2002; Mammoto *et al.*, 2004) (Figure 4.7 A, D), co-ordinate cell adherence and release, and, therefore, migration. At the leading front of the cell activated Rac uni-directionally inhibit Rho (Moissoglu *et al.*, 2006) (Figure 4.12 Lane 2). At the trailing edges, though, inactivation of Rac and growth factor-related elevation of intracellular calcium levels (Soltoff, 1998a; Masiero *et al.*, 1999; Giannone *et al.*, 2004) leads to Rho- and ROCK-II-related phosphorylation of FAK (Playford *et al.*, 2004; Moissoglu *et al.*, 2006) at Ser732 and subsequently at Tyr407 (Fan *et al.*, 2005; Le Boeuf *et al.*, 2006). Therefore, at these points, FAK may lead to Rho-ROCK-related disassembly of focal adhesions and release of the cell from the matrix. Together with Rho-related stress fiber formation and contraction (Figure 4.12 Lane 1), the cell flows forward (Masiero *et al.*, 1999; Fan *et al.*, 2005), supporting mobility.

The partners interacting with MT1-MMP seems to be important in a number of events, such as migration and invasion. Many details still seem to be unclear. What is known on the trafficking and association partners, is reviewed in the following sections and possible hypothesis on the implied interactions between these various partners are explored.

4.1.5. MT1-MMP trafficking

Distribution of MT1-MMP to specialized membrane domains called caveolae (Labrecque *et al.*, 2004; Anilkumar *et al.*, 2005), subsequent internalization (Jiang *et al.*, 2001; Remacle *et al.*, 2003; Galvez *et al.*, 2004) and recycling to the PM via EEs (Remacle *et al.*, 2005), are all activities suggested to be a prerequisite for the invasive phenotype of cancer cells (Figure 4.3, Figure 4.4, Figure 4.5 and Figure 4.7). Much work has been done on these various components of MT1-MMP trafficking, but the relationship with all its interacting partners and the exact mechanism by which active MT1-MMP is recycled, is still unclear and the importance of some of these events may, therefore, still not be clearly evident. An accurate understanding of these processes may better explain the effect of altered c-Ha-Ras(V12) downstream signaling on the various functions of MT1-MMP, especially its role in the invasion of *ras*-transformed breast cancer cells. Hence an attempt is made to review what is known this stage.

4.1.5.1. Palmitoylation of the MT1-MMP cytoplasmic tail, its internalization and recycling

The post-translational lipid modification via addition of a palmitate to the cysteine residue (Cys-574) sulphydryl group of the MT1-MMP cytoplasmic domain, results in its incorporation into organized, cholesterol-rich lipid rafts and subsequent internalization, a process that is required for cell motility (Anilkumar *et al.*, 2005) (Figure 4.6 Lane 1 and Figure 4.7 A). This reversible process may possibly be facilitated by the proximity of the protein to a hydrophobic surface, such as a lipid raft (Dietrich *et al.*, 2004). The ability of the cysteine residue to modulate the surrounding conditions (such as the pKa) may also facilitate this process, that usually does not occur at the physiological pH of the surrounding cytoplasm (Tian *et al.*, 2008).

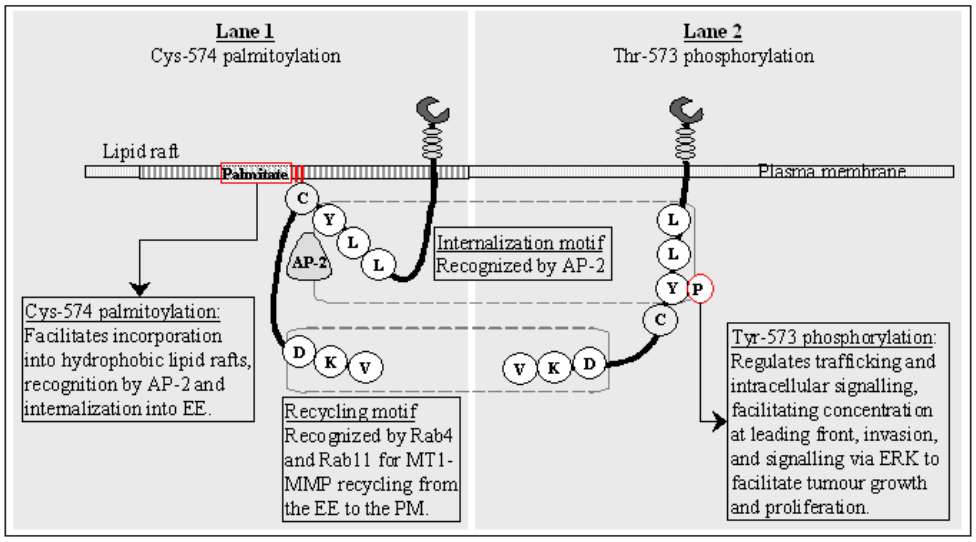


Figure 4.6. Signaling sequences on the cytoplasmic tail of MT1-MMP regulate its trafficking.
Palmitoylation of the cysteine-574 residue on the MT1-MMP cytoplasmic domain, and recognition of the LLY internalization motif by AP-2, are important components of the MT1-MMP endocytosis process (Anilkumar *et al.*, 2005) (Lane 1). The terminal DKV motif allows recycling in Rab-11 positive vesicles (Wang *et al.*, 2004b). Phosphorylation of the tyrosine-573 residue inhibits internalization, possibly by masking the LLY internalization motif from recognition by AP-2 (Nyalendo *et al.*, 2007) (Lane 2). This illustration was generated from references mentioned in Section 4.1.5.1.

Palmitoylation of the MT1-MMP cytoplasmic tail occurs adjacent to a tyrosine-based LLY internalization signaling motif, found to be essential for adaptor protein-2 (AP-2) associated MT1-MMP endocytosis (Wang *et al.*, 2004b; Anilkumar *et al.*, 2005) (Figure 4.6 Lane 1 and Figure 4.7 A). Prior to MT1-MMP endocytosis, AP-2 binds to PIP_2 (Traub, 2003) at the PM under ADP-ribosylation factor (ARF) stimulation (Gardinier, 2003). The AP-2 μ2 heavy chain is phosphorylated at Thr-156 by

clathrin, causing a conformational change and increases the affinity of AP-2 for cargo (Jackson *et al.*, 2003). This process may allow MT1-MMP collection from the lipid rafts on the cell surface and subsequent internalization into a clathrin-coated vesicle (Remacle *et al.*, 2003; Galvez *et al.*, 2004). After inward budding, the vesicle neck is restricted by dynamin (a GTPase) (Jiang *et al.*, 2001; Galvez *et al.*, 2004), resulting in the formation of an early endosome (EE) (Jiang *et al.*, 2001; Remacle *et al.*, 2005) (Figure 4.7 A). If the association between MT1-MMP and AP-2 is maintained, it may be degraded (Janvier *et al.*, 2005) (Figure 4.7 B). However, MT1-MMP is usually sorted out of the EEs and recycled to the PM (Remacle *et al.*, 2003) in both normal epithelial (Galvez *et al.*, 2004) and tumor cells (Remacle *et al.*, 2003) (Figure 4.7 C). This process is facilitated by Rab4 and Rab11 (Remacle *et al.*, 2005). These are markers for vesicles that contain isolated surface receptors, such as the transferrin receptor (TfR), that have been separated from their ligands, and are being returned to the PM from EEs or sorting vesicles (Lapierre *et al.*, 2001; Deneka *et al.*, 2003; Peden *et al.*, 2004b; Grosshans *et al.*, 2006). This process is facilitated by a DKV recognition motif at the end of the MT1-MMP cytoplasmic tail (Figure 4.6), as deletion of this sequence restricts recycling (Wang *et al.*, 2004b). Restriction of recycling after endocytosis may, therefore, affect the proteolytic potential of MT1-MMP and hence, migration and invasion.

4.1.5.2. Phosphorylation of the MT1-MMP cytoplasmic tail regulates its catalytic activity on the PM

Palmitoylation of MT1-MMP, which is a reversible process, may be part of an intricate mechanism that maintains a balance between internalization and reduction of MT1-MMP activity and retention of proteolytically active MT1-MMP on the plasma membrane. Phosphorylation of the tyrosine residue (Tyr-573) immediately upstream of Cys-574 may result in depalmitoylation and dissociation of the C-terminus from the plasma membrane (Anilkumar *et al.*, 2005) (Figure 4.6). This process may interfere with the endocytosis of MT1-MMP into EEs, since it may mask the LLY internalization motif from recognition by adaptor protein-2 (AP-2) (Nyalendo *et al.*, 2007), a process that has been explained in the previous paragraph. It seems, then, that, the internalization or retention on the PM of MT1-MMP may be controlled, depending on palmitoylation of Cys-574, or the phosphorylation state of Tyr-573. A similar interactive control process of either palmitoylation of a cysteine residue, or phosphorylation of neighbouring residues (termed the phoshorylation gate) as

suggested here, has been illustrated in the control of potassium channel function in the plasma membrane (Tian *et al.*, 2008) and control of internalization of CTLA-4, a T-cell receptor (Bradshaw *et al.*, 1997; Shiratori *et al.*, 1997), where dephosphorylation of the cytoplasmic tail allowed its endocytosis (Bradshaw *et al.*, 1997).

Stimulation by chemotaxic factors (Nyalendo *et al.*, 2007; Gingras *et al.*, 2008) or growth factors, allows direct, but reversible phosphorylation by Src kinase of Tyr-573 on the MT1-MMP cytoplasmic tail (Nyalendo *et al.*, 2007). Such phosphorylation of MT1-MMP is facilitated by its associated with activated cav-1, phosphorylated at a Tyr-14 residue (p-cav-1) (Labrecque *et al.*, 2004; Nyalendo *et al.*, 2007) (Figure 4.4 Lane 2 and Figure 4.7.D) and may facilitate clustering and oligomerization of– and proteolytic activity and pro-MMP-2 activation by MT1-MMP at the leading front PM of migrating cells (Lehti *et al.*, 2000) (Figure 4.7.F).

In addition to its role in lipid raft morphology (Thomsen *et al.*, 2002) and endocytosis-related annexins (Schnitzer *et al.*, 1995) (Section 1.4.2), non-phosphorylated, inactive cav-1 is associated with signal transduction via other lipid modified signaling molecules (thus membrane-bound) such as kinases (e.g. Src) (Sotgia *et al.*, 2002) and GTPases (e.g. Ras, Rac and CDC42) (Li *et al.*, 1996; Grande-García *et al.*, 2007b) at the PM. It interacts preferentially with inactive Ras and its related kinases, such as Src (Song *et al.*, 1996; Razani *et al.*, 2001) and possibly regulates the activation state of these signaling proteins. Stimulation, via lipid raft-anchored growth factor receptors (Navarro *et al.*, 2004), allows activation of these signaling proteins and activates subsequent downstream paths relevant in survival, proliferation and migration of growth factor signaling (Labrecque *et al.*, 2004; Navarro *et al.*, 2004) (Figure 4.5 and Figure 4.7 D). p-Cav-1, phosphorylated at Tyr-14 by Src, are translocated to the FAs at sites where a leading signaling front is formed (Lee *et al.*, 2000a), and in this way may be involved in the establishment of cell polarity that assists directional migration. This function is managed through coordination of the signaling of activated Src kinase (Lee *et al.*, 2000a) and Rho/Rac GTPases (del Pozo *et al.*, 2000; Grande-García *et al.*, 2007a) (Figure 4.7 D). Evidence of such a role is found in cells that lack cav-1 and is visible in loss of cell polarity and impaired directional migration (Grande-García *et al.*, 2007a) and in

hyperproliferation due to constant signaling via ERK and gene transcription (Razani *et al.*, 2001) (further discussed in Section 4.4.1.3 and Figure 4.7 E).

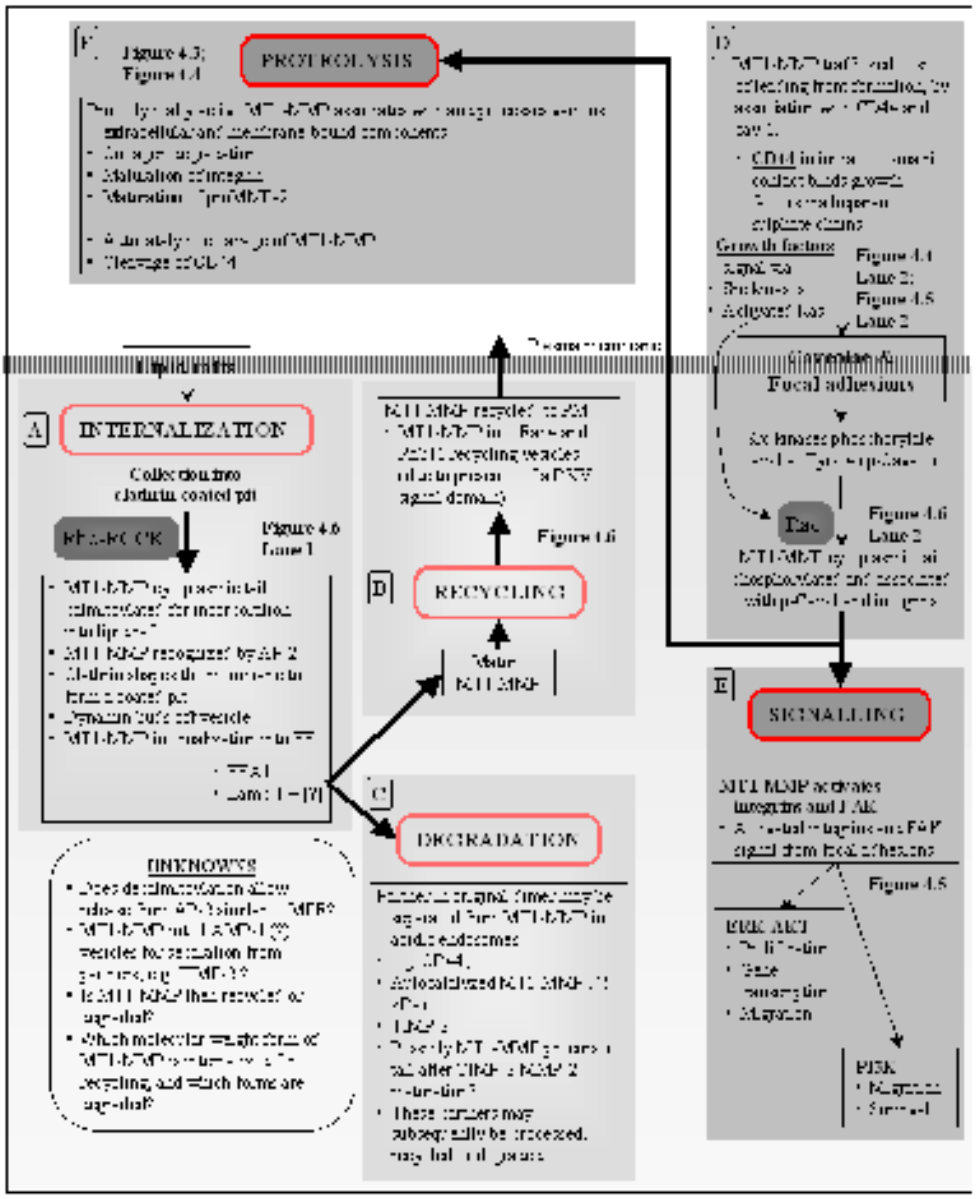


Figure 4.7. The various processing pathways of MT1-MMP.
This diagram illustrates the pathway via which MT1-MMP is palmitoylated and incorporated into lipid rafts and internalized (A). It may subsequently be recycled to the PM (B), or degraded (C). Alternatively, phosphorylation of its cytoplasmic tail results in its association with integrins and FAK (D) in which way it may influence intracellular proliferative and migratory signaling via ERK-AKT and PI3K (E). Phosphorylated MT1-MMP remains on the PM, where it exerts its proteolytic functions (F). References are made to other Figures in the text that provide more detailed information. This illustration was generated from references mentioned in Section 4.1.5.

Src-related phosphorylation of MT1-MMP at Tyr-573 (Nyalendo *et al.*, 2007) and its association with p-cav-1 regulates MT1-MMP trafficking to the leading front. Such phosphorylation possibly inhibits internalization (Figure 4.6 Lane 2 and Section

4.1.5.1) and rather supports its retention on the PM and its roles in both extracellular proteolysis and intracellular signaling (Lehti *et al.*, 2000; Nyalendo *et al.*, 2007; Nyalendo *et al.*, 2008) (Figure 4.7 A, B and C and Figure 4.6 Lane 2). Phosphorylated MT1-MMP may interact with integrins and FAK-related p130CAS (Gingras *et al.*, 2008) to facilitate proliferative and migratory signaling via ERK-AKT and PI3K (Nyalendo *et al.*, 2007) (Figure 4.7 and Figure 4.6). Retention of such phosphorylated MT1-MMP on the PM may facilitate invasion in cancer cells (Lehti *et al.*, 2000; D'Alessio *et al.*, 2007). The role of this process will later be discussed in context with constantly active c-Ha-Ras(V12).

4.1.6. Regulation of MT1-MMP activity

Various components of ECM, such as collagen-I (Lafleur *et al.*, 2005), fibronectin, fibrinogen, gelatin and vitronectin, may diffentially regulate MT1-MMP localization on the PM, its association with integrins and may also modulate its internalization and activity (Galvez *et al.*, 2002). MT1-MMP specificity for substrates such as collagen-I (Zhuge *et al.*, 2001; Tam *et al.*, 2002; Lafleur *et al.*, 2005; Cao *et al.*, 2008) and homodimerization of the 58- and 63 kDa forms in the proMMP-2 activation complex, is regulated by the extracellular Pex domains (Butler *et al.*, 1998; Hofmann *et al.*, 2000; Lehti *et al.*, 2000; Zhuge *et al.*, 2001; Lehti *et al.*, 2002; Morgunova *et al.*, 2002; Tam *et al.*, 2002; Itoh *et al.*, 2008; Nishida *et al.*, 2008) and are unaffected by deletion or absence of the cytoplasmic tail (Lafleur *et al.*, 2005).

TIMP-2, an endogeneous inhibitor of MT1- MMP (Vu *et al.*, 2000), controls the activity of MT1-MMP (d'Ortho *et al.*, 1998), by forming a stable complex with activated 58 kDa MT1-MMP (Sato *et al.*, 1997). TIMP-2 controls MT1-MMP-related ERK-2 activation, a function which is dependent on phosphorylation of the MT1-MMP cytoplasmic tail (D'Alessio *et al.*, 2007). In addition, while low levels of TIMP-2 is involed in initial proMMP-2 maturation by MT1-MMP (Morgunova *et al.*, 2002) (Section 4.1.4.1), increased TIMP-2 levels may inhibit this function of MT1-MMP (Sato *et al.*, 1997; d'Ortho *et al.*, 1998). Glycosylation of the hinge region adjacent to the Pex domain, at 5 possible sites seems to be a prerequisite for TIMP-2 binding and MT1-MMP homodimerization in the MT1-MMP/TIMP-2/proMMP-2 activation complex, that occurs via the transmembrane domain (Wu *et al.*, 2004; Itoh *et al.*, 2008). The strongly negatively charged terminal sialic acid of the glycosidic addition restricts access of the catalytic domain of most likely the 58 kDa MT1-MMP

to the hinge region of the MT1-MMP 63 kDa intermediate form in the homodimer. In so doing MT1-MMP is protected from autocatalysis on the cell surface (Remacle *et al.*, 2006a).

It would seem that glycosylation may not completely prevent autocatalysis of MT1-MMP after activation of proMMP-2, as the 58 kDa form of MT1-MMP may be involved in cleaving the 63 kDa form of MT1-MMP, that was possibly part of the proMMP-2-TIMP-2 triad (Figure 4.3 Lane 2). After MMP-2 activation such cleavage may occur at the hinge region (Remacle *et al.*, 2003; Osenkowski *et al.*, 2005; Remacle *et al.*, 2006a) and removes the catalytic domain from the 63 kDa form (Tam *et al.*, 2002; Toth *et al.*, 2002) (Figure 4.3 Lane 4 and Figure 4.7.C). This process may give rise to the inactive 45 kDa and the soluble 18 kDa autocatalytic forms (Stanton *et al.*, 1998) (Figure 4.2 and Figure 4.3 Lane 4). Degradation of the rest of triad seems to subsequently occur via internalization and degradation, or via autocatalysis (Figure 4.7.C). This process may be triggered after activation of proMMP-2, when MT1-MMP is not bound to its inhibitor, TIMP-2 (Lehti *et al.*, 2000) (Figure 4.3 Lane 4). This may be a controlling step for MT1-MMP proteolytic activity (Remacle *et al.*, 2006a), as the the cleaved residual 45 kDa ectodomain disrupts MT1-MMP-related native type-I collagen digestion, which inhibits further ECM degradation and cell invasion (Lehti *et al.*, 2002; Tam *et al.*, 2002).

Besides being regulated by autocatalytic cleavage, an extracellular membrane-anchored glycoprotein, reversion-inducing cysteine-rich protein with Kazal motifs (RECK), inhibits MT1-MMP-related processing of secreted proteases, MMP-2 and MMP-9 (Oh *et al.*, 2001). RECK may, therefore, regulate the activity of MMPs such as MT1-MMP, MMP-2 and MMP- 9 during e.g. angiogenesis (Oh *et al.*, 2001). Downregulation of this protein seems to be involved in tumour spread, as in c-Ha-Ras(V12) expressing cells in which this protein is downregulated, allowing increased tumour vascularization (Chang *et al.*, 2006).

In addition, an intracellular cytoplasmic protein, MT1-MMP-cytoplasmic tail-binding protein-1 (MTCBP-1), may control the function of MT1-MMP by forming a complex with the cytoplasmic tail of MT1-MMP. While the conditions under which such a complex may form, are not known, it may disrupt the essential role of the cytoplasmic tail in trafficking of the MT1-MMP during cell migration and invasion (Uekita *et al.*,

2004). Since a lack of the cytoplasmic tail does not affect the ability of MT1-MMP to process proMMP-2 (Lehti *et al.*, 2002), the cytoplasmic tail region of MT1-MMP seems, therefore, mainly important in trafficking of this protein.

Extracellular proteases such as MMP-2 and MMP-9 may be bound to- and internalized with low-density lipoprotein receptor-related protein (LPR), regulating these MMPs and their activity. MT1-MMP may degrade LPR, however, and thus maintain extracellular gelatinase activity (Rozanov *et al.*, 2004a). Regulation of MT1-MMP levels on the PM is, therefore, an important factor in cell migration and ECM degradation.

In the following section the involvement of MT1-MMP in migration and development of an invasive front, i.e. signaling for anterior-posterior polarity rather than apical-basal polarity, or epithelial to mesenchymal transition (EMT), is explored.

4.1.7. EMT, polarity and cell-matrix contact via adhesion molecules

A migrating cell not only strategically degrades the basement membrane at the leading front, but it has to adhere to the underlying matrix and alter its shape/polarity into a characteristically leading front and trailing rear (Vicente-Manzanares *et al.*, 2007). During this process even normally apical-basolaterally polarized cells, such as epithelial cells, acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility. Such EMT is a crucially important, transient, developmental phase, especially in multicellular organism development (Vincent-Salomon *et al.*, 2003). This process is accompanied by a decrease in epithelial cell markers such as the E-cadherin adhesion molecule and enhanced expression of mesenchymal markers, such as Wnts (Behrens *et al.*, 2004; Cao *et al.*, 2008) [so named due to a combination of the Drosophila wingless protein, and its mammalian version, int-1 (Fischer *et al.*, 2006)], involved in cell proliferation and polarized developmental processes (Mareel *et al.*, 2003; Behrens *et al.*, 2004). Upregulation of Wnt (Taki *et al.*, 2003; Cao *et al.*, 2008) as well as increased MT1-MMP catalytic activity (Cao *et al.*, 2008) has, however, been implicated in EMT-related changes in cancers, with MT1-MMP expression in cancer cells leading to disorientated migration and morphologic changes, that resemble EMT. When the EMT process is not reversed to allow epithelial cell spreading and adherence it may become a hallmark of oncogenesis (Grille *et al.*, 2003; Vincent-Salomon *et al.*, 2003).

Initial polarization of the cell into an anterior, leading edge that adheres to the underlying matrix, and a posterior end, that is subsequently pulled forward via cell-spanning stress fibers (Machesky *et al.*, 1997), is facilitated by adhesion proteins such as integrins (Aplin *et al.*, 1998; Plow *et al.*, 2000; Humphries *et al.*, 2006) (Section 4.1.4.2) and CD44.

4.1.7.1. CD44

During normal cell migration several adhesion molecules such as integrins (Section 4.1.4.2) and CD44, a heparan sulfate (HS) proteoglycan that binds mainly hyaluronic acid (HA) in the matrix (Cichy *et al.*, 2003; Naor *et al.*, 2003; Sillanpaa *et al.*, 2003), localize to lipid rafts on the plasma membrane (Bourguignon *et al.*, 2004) and mediate adhesion to the underlying matrix under protrusions that extend in the direction of intended movement, or a leading front (Wehrle-Haller *et al.*, 2002). In the presence of high calcium levels due to growth factor stimulation, the cytoplasmic tail of CD44 (phosphorylated at Ser-325) indirectly interacts with the actin cytoskeleton through ezrin/radixin/moesin (ERM) proteins, supplying cell-matrix attachment (Ponta *et al.*, 2003; Thorne *et al.*, 2004) (Figure 4.5 Lane 2). On incorporation of integrins, these sites are strengthened into tight FAs (Nobes *et al.*, 1999; Ory *et al.*, 2000). Bundles of actin-myosin stress fibers are anchored at these FAs**,** traverse the cell to the trailing posterior ends and contract to pull the cell forward (Wehrle-Haller *et al.*, 2002) and assist in migration (Ballestrem *et al.*, 2001). As the cell subsequently 'flows' forward, FAs along the cell edge and towards the rear of the cell, are disassembled to allow release from the matrix and migration (Ballestrem *et al.*, 2001; Laukaitis *et al.*, 2001). Disengagement of the ERM proteins and loss of cytoskeletal association occurs due to the dephosphorylation of Ser-325 on CD44 and phosphorylation of Ser-291 (Thorne *et al.*, 2004). Subsequent cleavage of the CD44 ectodomain, possibly by MT1-MMP (Ueda *et al.*, 2003) at the rear of the cell (Somerville *et al.*, 2003; Marrero-Diaz *et al.*, 2008), releases the cell-matrix hold and thus facilitates cell migration and invasion, with MT1-MMP essential for such migration (Seiki *et al.*, 2003). CD44 appears critical for directed movement, as cells lacking CD44 form fewer FAs and stress fibers spanning the cell and even though these cells may migrate with high velocity, migration seems disorganized and directionless (Acharya *et al.*, 2008). In invasive tumor cells CD44 is usually upregulated (Wielenga *et al.*, 2000; Bourguignon *et al.*, 2003; Wanga *et al.*, 2005), and together with integrins, is involved in positioning

activated MMPs and MT1-MMP at the migrating front in normal cells (Mori *et al.*, 2002) (Figure 4.7 D) and at the invasive front in tumour cells (Hofmann *et al.*, 2000; Mori *et al.*, 2002).

4.1.7.2. Ras-related anterior-posterior polarization and NHE-1 activity during EMT

The location of extracellular growth factor stimulus mainly determines the site where a leading front is established. During this process a broad ruffling border is formed and firm focal adhesions are established on incorporation of matrix binding CD44 (Naor *et al.*, 2003) and integrins such as αvβ3 (Wehrle-Haller *et al.*, 2002) (Section 4.1.7.1). Growth factors are further gathered by heparin sulfate side chains on CD44 (Wielenga *et al.*, 2000; Thorne *et al.*, 2004) and presented to their respected receptors (Wielenga *et al.*, 2000; Naor *et al.*, 2003), thereby mediating intracellular signaling by receptor tyrosine kinases (Ponta *et al.*, 2003) (Figure 4.5 Lane 2 and Figure 4.7 D, E). During this process EGF binds to EGFR and allows dimerization and activation of the EGFR through autophosphorylation of its cytoplasmic tail (Klein *et al.*, 2004; Dawson *et al.*, 2005). This EGFR domain acts as a docking-site for signaling molecules such as the adaptor protein Shc (Authier *et al.*, 1999a) and the Ha-Ras GEF, SOS (Haugh *et al.*, 1999). SOS subsequently activates Ha-Ras which signals down the transduction pathways of the Rho GTPase family (Rho, Rac, PAK and CDC42) (Denhart, 1996). These signaling proteins cooperate to rearrange the cytoskeleton that alters cell shape to a more mesenchymal shape (Machesky *et al.*, 1997), establish anterior-posterior polarity and ultimately promote cell movement (Denhart, 1996; Khoshavi-Far *et al.*, 1996; Maruta *et al.*, 1999; Nobes *et al.*, 1999; Lee *et al.*, 2000b; Walsh *et al.*, 2001; Bulavin *et al.*, 2003) (Section 1.9.4, Figure 4.5 Lane 2, Figure 4.8 and Figure 1.3 on fold-out) (Sameni *et al.*, 2008)and survival (Ninomiya *et al.*, 2004; Menard *et al.*, 2005). Alterations in the Rac:Rho gradient over the anterior-posterior cell axis to control the cytoskeletal architecture and the migatory process (Moissoglu *et al.*, 2006). At the leading front the activated Rac-PAK complex induces actin polymerization to form lamellipodia or a ruffling border (Cau *et al.*, 2005; Vicente-Manzanares *et al.*, 2007) (Figure 4.8 and Figure 1.3 on fold-out), while Rac-CDC42 activation is associated with the spatial control of cellular structures that are affected by migration, especially the Golgi and nucleus (Donaldson *et al.*, 2000; Cau *et al.*, 2005) (Figure 4.8 Lane 1). Such changes in the actin cytoskeleton may, in turn, lead to changes in the clustering behaviour of β3 integrin and, therefore, affects mobility of

the various FAs spread over the basal cell surface (Ballestrem *et al.*, 2001). While Rac downregulates Rho at the leading edge, in the rear of the cell Rac is inhibited, followed by activation of Rho (Figure 4.8 Lane 1). Activated Rho triggers the extention of contractile stress fibers that are anchored to the anterior FA sites via cytoskeletal components, and traverse the cell to the rear (Machesky *et al.*, 1997; Moissoglu *et al.*, 2006). When these stress fibers contract, the cell assumes an elongated shape (Figure 4.8 Lane 1). In addition, at the rear of the cell Rho increases the tyrosine phosphorylation of FAK and dissociation of the focal contacts resulting in disassembly of FAs and release of the trailing back of the cell from the matrix and the cell "flows" forward (Nobes *et al.*, 1995; Navarro *et al.*, 2004) (Section 4.1.4.2).

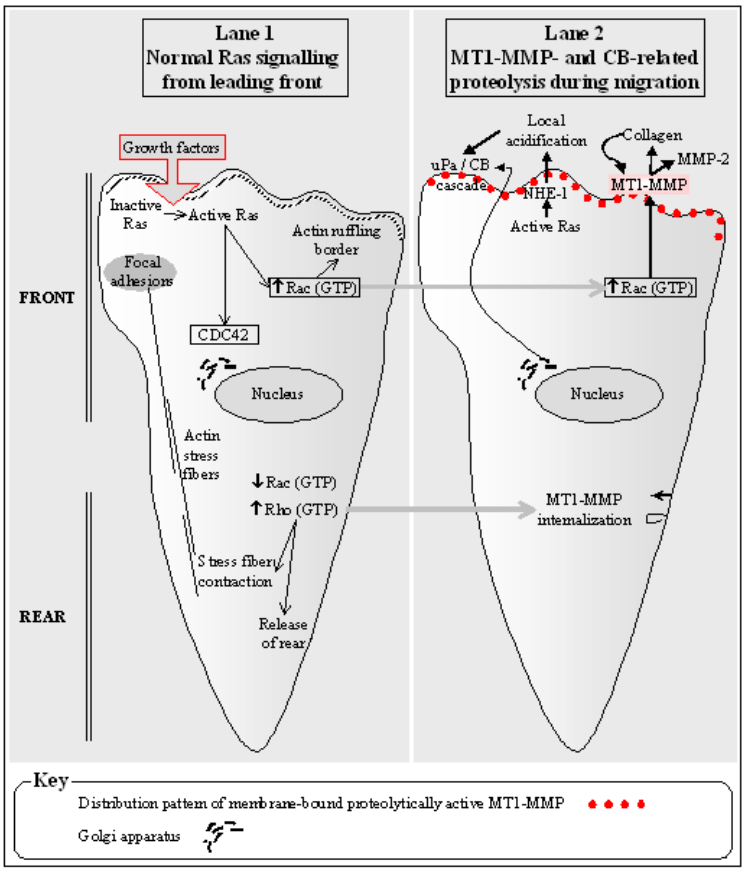


Figure 4.8. Ha-Ras downstream signaling affects distribution of CB and MT1-MMP.

In this illustration the influence of downstream Ha-Ras affectors, Rac, Rho and CDC42 on the formation of the leading front ruffling border, position of the Golgi and possible distribution of CB and MT1-MMP during migration. This illustration was generated from references mentioned in Section 4.1.7.2 and demonstrates predictions mentioned in Section 4.1.7.2.

Ras-activated PI3K, AKT and ERK signaling affects transcription of metalloproteases during cell migration, which would facilitate ECM digestion (Takino *et al.*, 2004; Shin *et al.*, 2005; Zhong *et al.*, 2006) (Section 4.1.4.2 and Figure 1.3 on fold-out). An

important consequence of AKT activation, often detected in human carcinomas, is the acquisition of an invasive phenotype (Grille *et al.*, 2003), which may be due to elevated transcription of MT1-MMP and MMP-2 (Siesser *et al.*, 2006). In addition, outside-in signaling by integrins via the interconnected FAK and Ras pathways and intracellular kinase activation cascades, regulate survival of the cell via Jnk/STAT during these altering stressful conditions, while signaling via AKT/ERK controls protein transcription (Shepherd *et al.*, 1996; Menard *et al.*, 2005) (Figure 4.5). These are the Ras-related cellular signals for establishment of anterior-posterior polarity, migration and proliferation (Guan, 1997; Aplin *et al.*, 1998; Le Boeuf *et al.*, 2006).

Adhesion to ECM components, such as fibronectin, raises the intracellular pH by activating the plasma membrane NHE-1 proton pump (Aplin *et al.*, 1998) (Section 3.2.1.2). During migration NHE-1 is relocated to the leading-edge of lamellipodia (Cardone *et al.*, 2005), where it is connected to the cytoskeletal network through the binding of ERM proteins, and may be associated with focal adhesions (Aplin *et al.*, 1998). Cytoplasmic alkalinity is a mitogenic stimulus (Cardone *et al.*, 2005), while localized NHE-1 activity integrates signals such as those from Src kinases, and from diverse membrane receptors, such as integrins and G protein–coupled receptors (GPCR), to coordinate early polarity signals and promote FA remodeling (Cardone *et al.*, 2005). These two functions of NHE-1 may, therefore, promote both mitosis and cell polarity during migration. The localized adjustment of the acidity in the microenvironment of the plasma membrane due to proton secretion by activated NHE-1, may also assist in generating a sufficiently acidic environment for some proteolytic lysosomal enzymes, such as CB, which has been found associated with p11 and cav-1 on the PM (Cavallo-Medved *et al.*, 2005) (Section 4.1.2 and Section 4.1.5.2.), as proposed in Figure 4.8 Lane 2. As mentioned before, here CB may function in activating plasmin, which may activate uPa (Figure 4.1 and Figure 4.8 Lane 2). The extracellular acidity in the microenvironment of the leading plasma membrane of malignant tumours may be related to activity of the NHE-1 (Montcourrier *et al.*, 1997; Bourguignon *et al.*, 2004; Cardone *et al.*, 2005) (Figure 4.8 Lane 2). In such an acidified environment CB may activate uPa, which in turn may in turn lead to activation of plasminogen (Figure 4.1). One of the proposed functions of activated plasmin is the activation of MT1-MMP at the cell surface (Figure 4.3 Lane 2). For this reason, a close association between CB and MT1-MMP at the leading front of the cell, is anticipated. At the rear of the cell ion translocation

is required for de-adhesion of cell-matrix attachments (Denker *et al.*, 2002), a function which may be assisted by ROCK (Ponta *et al.*, 2003) and PI3K (Reshkin *et al.*, 2000a), a downstream Ha-Ras effector (Figure 1.3 on fold-out and Figure 4.8 Lane 1).

While Ha-Ras activated by growth factors, may lead to proliferation and migration (Van Aelst *et al.*, 1997), it acts as an internal control, or switch, in the cellular signaling processes, since termination of Ras signaling ensures that such proliferation is a transient process. Such inhibition is via guanine activating proteins (GAPs) (Walker *et al.*, 2003; Grosshans *et al.*, 2006), through negative feedback to Ras (Giglione *et al.*, 1998; Katunuma *et al.*, 1998; Erster *et al.*, 2004) (Figure 1.3 on fold-out), or by internalization of the activated growth receptor complex (Ceresa *et al.*, 2006) (Section 1.9.4).

4.1.8. The effect of Ras and immortality (*9p21*-/-) on CB and MT1-MMP distrubution

Cellular response to Ras activation may be negatively influenced by elevating levels of p53, an apoptosis regulator (Ries *et al.*, 2000; Lin *et al.*, 2001). The p53/p16 senescence signaling molecules regulate genetic integrity, cell cycle progression and differentiation, while p53 also negatively controls the activity of Rho-ROCK (and stress fiber formation and contraction) and CDC42 (and organelle orientation during migration) (Figure 1.3 on fold-out). However, elevated signaling from a mutationally activated c-Ha-Ras(V12) oncoprotein in combination with a downregulated p16/p53 signaling system (Section 1.9) allows uncontrolled growth and migration of transformed cells (Tannapfel *et al.*, 2001; Bulavin *et al.*, 2003; Gao *et al.*, 2004; Houle *et al.*, 2006). These processes and the downstream pathways from especially Ha-Ras stimulation are reviewed in Chapter 1, while the effect of mutationally activated c-Ha-*ras*(V12) on cathepsin-containing vesicles and degradative vesicle acidity is described in Chapter 3. From this review we know that normal activated Ha-Ras stimulates proliferation via many downstream pathways, affecting cell polarization and migration, including transcription and secretion of proteases, including CB and MMPs, required in BM degradation. This process is normally transient, and controlled by negative feedback mechanisms (Section 4.1.7.2). In 'normal' cells that lack the p53 signaling system, such as the MCF10A cell line, anterior-posterior cell polarization may be compromized to some extent, due to

insufficient control of p53 over CDC42 and, therefore, spatial orientation of organelles such as the Golgi complex (Gadéa *et al.*, 2002), as well as actin dynamics (Mareel *et al.*, 2003). In these immortal, but 'normal' cells the wild-type Ha-Ras will only signal downstream under conditions that normally stimulate growth. Here we wish to investigate the effect of signaling by a mutationally activated c-Ha-Ras(V12) on cell polarity and the distribution of proteases on the PM to assess its role in the invasion process.

In the preceding chapters the effect of constant downstream signaling by a mutated c-Ha-Ras(V12) in a c-Ha-*ras*(V12)-transfected immortal breast epithelial cell line was analysed, including a review on alteration in spatial distribution and acidity of vesicles hosting mature CB and other cathepsins that are suggested to be involved in breast cancer metastases. In this part of the study the focus is on the effect of c-Ha-Ras(V12) and the lack of full p53 function, on the PM distribution of CB and MT1-MMP, both proteases that degrade the ECM. An aim is to show a possible relationship at the invasive front of c-Ha-*ras*(V12) transfected cells, between position and functions of these membrane-bound proteases in the transformed phenotype. More specifically, the observed changes related to polarity and protease distribution in the c-Ha-*ras*(V12)-transformed MCF10AneoT cell lines compared to the non-transformed MCF10A cells, will be correlated with possible effects predicted for the mutationally activated c-Ha-Ras(V12) signaling and deficient p53 function, as suggested in the literature (Chapter 1 and subsequent chapters).

An altered distribution pattern of CB and MT1-MMP in the MCF10AneoT cells due to the effect of the c-Ha-Ras(V12) oncoprotein on cell polarity and the change from an epithelial to the mesenchymal shape, was anticipated (Sameni *et al.*, 1995; Vincent-Salomon *et al.*, 2003). This hypothesis was mostly due to the known enhanced invasive and metastatic capacity of c-Ha-*ras*(V12)-transfected, invasive cells. Since CB is known to associate with the PM in both CB precursor and mature forms (Sloane *et al.*, 1994; Sameni *et al.*, 1995), we anticipated an increase in PM CB association at the invasive front of the transformed cell. Upregulation of both CB and MT1-MMP in the c-Ha-*ras*(V12)-transfected MCF10AneoT cell line, compared to the normal MCF10A cell line, has previously been seen in our laboratory (Preliminary manuscript attached) (van Rooyen *et al.*, 2008). An increase in expression of MT1-MMP on the PM, also concentrated at the leading edge of the cell, was thus

anticipated in the transfected cells. Such upregulation would support the migratory activity previously seen and reported and would result in the increased, focused proteolysis of the BM (Takino *et al.*, 2007).

While both mature CB and proteolytically active MT1-MMP have been localized to specialized lipid rafts, or caveolae (Sowa *et al.*, 2001), at the leading front of the cell, the reduction in caveolae in c-Ha-*ras*(V12)-transfected cells may have an influence on these enzymes. As an initial step, therefore, alteration in the number of caveolae on the PM of both the control and transfected cells was investigated.

PM-related cav-1 serves as a negative controller of growth signaling transduction (Razani *et al.*, 2001; Williams *et al.*, 2005) and may influence Ha-Ras signaling (Section 4.1.5.2). Cav-1 transcription and expression is regulated by p53. Cells in which p53 is constantly removed, such as in the immortilized MCF10A cell line, may lead to an ineffective p53/p16 senescence system (Section 1.8) and may, therefore, express reduced levels of cav-1 (Williams *et al.*, 2005). Inactive normal Ha-Ras is associated with cav-1, while expression of the mutated c-Ha-*ras*-(V12) reduces cav-1 expression in an ERK-related pathway (Song *et al.*, 1996; Engelman *et al.*, 1998a; Engelman *et al.*, 1998b). This may, thus, lead to a lack of control over the downstream signaling of the mutated c-Ha-Ras(V12), supporting the tumour-causing downstream cellular outcome. Ultrastructural assessment of the number and distribution of caveolae with electron microscopy may indicate the effect of c-Ha-*ras*(V12) transfection on the control of the downstream signaling and hence such a study was performed.

Antibodies for immunnolabeling comparison were previously raised against various domains of human MT1-MMP (Figure 4.7) expressed in *E-coli* (Gordon conference 2008 Poster, Appendix IV). These were characterized and western blot analysis was used to interpreted recognition pattern of specific processing forms. This information may be useful to gain possible clues on the forms found intracellularly, as well as the processing form of MT1-MMP expressed on the PM during migration. This may indicate the form of MT1-MMP that is relevant in invasion. First, the differences in migrational pattern seen post c-Ha-*ras*(V12) transfection was examined using phase contrast microscopy.

4.2. c-Ha-*ras*(V12) transfection affects the anterior-posterior polarity of migrating MCF10AneoT cells

During activation of Ha-Ras due to growth factor stimulation normal epithelial cells may adopt mesenchymal characteristics, typical of EMT (Section 4.1.7). During this transition epithelial cells adopt a motile phenotype, altering their shape into an elongated, polarized mesenchymal shape (Christiansen *et al.*, 2006). Under these migratory conditions several activities seem to be localized to the leading front. Polymerization of actin allows formation of a ruffling border along the edge of leading, wide lamellipodia, while cell-matrix adhesion proteins such as CD44 (Mori *et al.*, 2002) and integrins become concentrated at the leading front (Deryugina *et al.*, 2001). Enzymes associated with the leading front proteolytically degrade the underlying BM (Deryugina *et al.*, 2001; Mori *et al.*, 2002; Takino *et al.*, 2007), while NHE-1 assists in both polarized cytoskeletal anchoring at the front (Denker *et al.*, 2002) and local acidification of the pericellular microenvironment, supplying a favourable environment for proteases that require a pH lower than the alkaline extracellular pH (Section 4.1.7.2). The rear of the cell seems to contract to allow forward movement (Figure 4.8). Such anterior-posterior polarization assures efficient migration (Munevar *et al.*, 2001). Since constitutive c-Ha-Ras(V12) signaling is known to be involved in invasive cancer (Kim *et al.*, 2003; Shin *et al.*, 2005; Mo *et al.*, 2007), we investigated the shape of migrating c-Ha-*ras*(V12)-transfected cells compared to that of normal MCF10A cells. It was expected that the cell shape or anterior-posterior polarity, would be altered to facilitate migration, that, together with proteolytic degradation of the BM, may support the invasive behaviour of these cells.

4.2.1. Reagents

Reagents used for cell culture as described in Section 2.2.1.

Reagents used for mounting of coverslips were described in Section 2.6.1.

4.2.2. Procedure

Subconfluent cells were grown on coverslips, as described in Section 2.2.2, washed, fixed and mounted with Moviol anti-fade reagent. Phase contrast images were recorded using the 40 x objective on an Olympus AX 70 light microscope and analySis software. Images were stored in the JPEG format and the contrast was improved using ImageJ free software (Section 2.7).

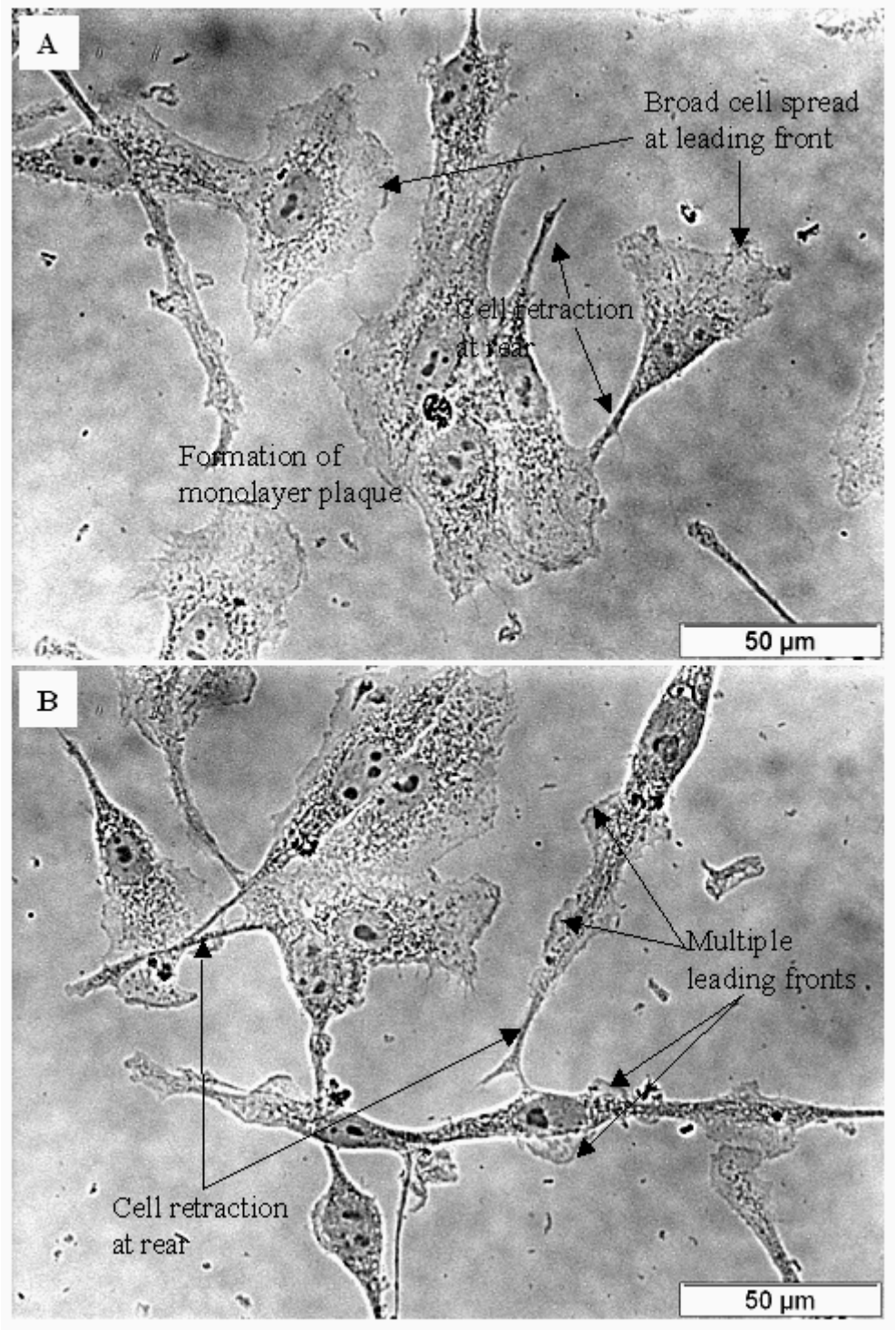
4.2.3. Results and discussion

Most of the subconfluent migrating, non-transfected MCF10A cells had a clearly anterior-posterior polarized shape, with a broad leading front ruffling border, or lamellipodium and a narrow, clearly retracting or trailing rear (Figure 4.9 A). In all the cells the nucleus was located in the middle of the cell, or medial to the anterior-posterior axis, between the broad front and the trailing rear. Where cells were in contact with one another over wide areas of the lateral edges, a plaque started to form. This could be described as a monolayer of broad, spread cells that were demonstrating clear inhibition of migration on contact (contact inhibition) at that side, resembling an *in vivo* epithelial layer (Debnath *et al.*, 2003). These cells formed lamellipodia at the edges that were at sides opposing those that were in contact with other cells in the plaque (Figure 4.9 A). In MCF10A 3D cultures these cells will form the polarized epithelial cell lining of the mammary duct lumen (Debnath *et al.*, 2003). Under the same conditions a few of the c-Ha-*ras*(V12)-transfected MCF10AneoT cells had polarized shapes similar to the normal cells, while most of these transfected cells had an elongated, narrow shape (Figure 4.9 B). The retracting rear end could be recognized in most cells, while the leading lamellipodia were generally much smaller than in the normal cells. In addition, most of these transfected cells had small, ectopic ruffling lamellipodia that could be observed along the lateral side of what seems to be the trailing part, or around the nucleus, indicating aberrant anterior-posterior polarity. The nucleus in these cells still seemed to be located to the medial of the anterior-posterior axis. Lastly, while contact inhibition could be observed in the MCF10A cell sample, many of the c-Ha-*ras*(V12)-transfected cells seemed to be extended over those alongside them. When left to further proliferate, these cultures eventually formed a multi-layered growth, resembling cancer (Figure 4.9 B).

During anterior-posterior polarization of a migrating front, the formation of the leading front relies on transduction of the extracellular growth signal to intracellular pathways, that focus proteolytic activity to the leading front and affect the cytoskeletal organization that facilitate the migratory process. During such signaling, phosphorylated cav-1 plays a role in cell polarization and directional migration (Lee *et al.*, 2000a; Parat *et al.*, 2003; Grande-García *et al.*, 2007a; Goetz *et al.*, 2008). The effect of c-Ha-*ras*(V12) transfection on caveolae will be discussed in the following section.

Figure 4.9. Differences in anterior-posterior polarity of MCF10A and MCF10AneoT monolayer cell cultures.

(A) Most MCF10A epithelial cells in monolayer culture had a polarized shape, with broad anterior ruffled lamellipodia, narrow trailing retracting posterior ends and the nucleus located between these two poles. Plaques formed due to contact inhibition of migration. (B) The c-Ha-*ras*(V12)-transfected cells had narrow trailing backs, but, contrary to the normal cells, presented multiple small, ectopic lamellipodia along the lateral edge of the cell, in addition to the leading front. The nuclei of these transfected cells were generally still located to the cell median. (Bar = 50 μm)


A
Broad cell spread
at leading front
Cell retraction
at rear
Formation of
monolayer plaque
50 µm
B
Multiple
leading fronts
Cell retraction
at rear
50 µm

4.3. c-Ha-*ras*(V12) transfection affects the number of caveolae on the PM

Cav-1 is the main structural component of PM caveolae, shaping these structures from lipid rafts (Section 4.1.2). In addition, it serves as a negative controller of growth-related signaling proteins (Razani *et al.*, 2001; Williams *et al.*, 2005) with overexpression of cav-1 reported to halt progression of the cell cycle into the S-phase (Galbiati *et al.*, 2001) (Section 4.1.5.2). During growth factor signaling phosphorylated cav-1 plays a role in directional migration and cell polarization by supporting formation of a leading front (Lee *et al.*, 2000a; Grande-García *et al.*, 2007a; Goetz *et al.*, 2008), focussing proteases such as CB (Roshy *et al.*, 2003; Cavallo-Medved *et al.*, 2005) (Section 4.1.2) and MT1-MMP (Labrecque *et al.*, 2004; Nyalendo *et al.*, 2007) at the invasive front. Since caveolae are small, dense lipid raft-derived indentations on the PM, it may easily be observed with an electron microscope (Thomsen *et al.*, 2002). Ultrastructural assessment of the number and distribution of caveolae with electron microscopy may, therefore, be a relevant indicator of the level of cav-1 expression, especially when an antibody to either cav-1 itself, or, of more importance, its Tyr-14 phosphorylated form, is not available, as in this study. For this study, cells were fixed and processed to retain the ultrastructure for recognition of dense caveolae on the plasma membrane.

4.3.1. Reagents

Reagents used for cell culture as described in Section 2.2.1.

Reagents used for cell pellet embedding for ultramicroscopical investigation of caveolae as described in Section 2.5.1.

4.3.2. Procedure

Monolayers of MCF10A and MCF10AneoT cells were grown to 70% confluence in 75 cm^3 dishes as described in Section 2.2. Cells were fixed *in situ* by addition of 1% v/v) glutaraldehyde in equal volumes of PHEM buffer and complete medium (2 h at RT). Cells were scraped from the dish with a rubber scraper, collected into a 15 ml tube and centrifuged (1000 x g, 5 min). The supernatant in each tube was discarded and the pellet was washed with PBS (3 x 2 min) and infiltrated with gelatin (30 min, 36°C).

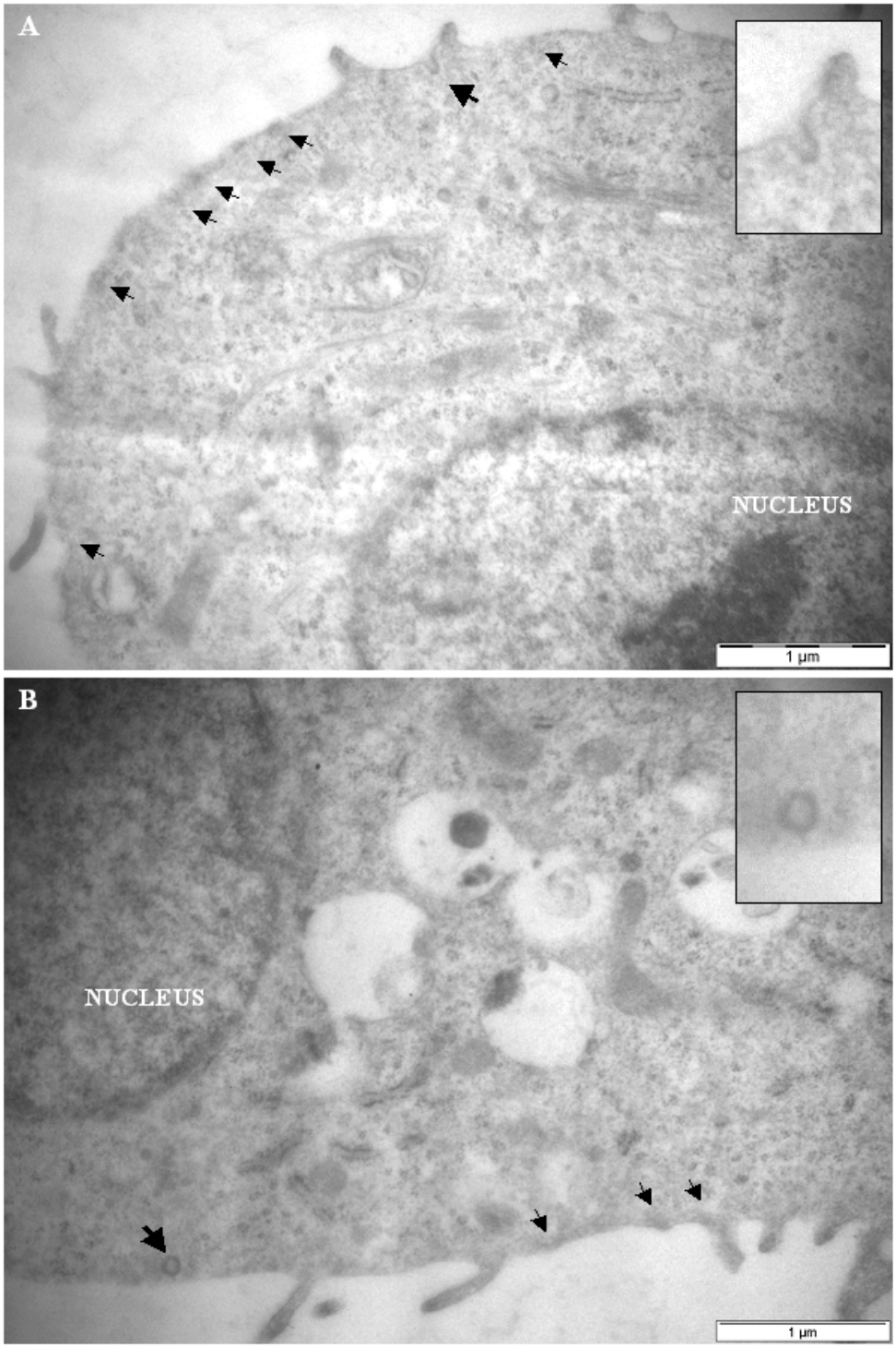
The gelatin-infiltrated cell pellets were divided into small pieces using a scalpel blade, osmicated (10 min), washed in d.H_2O (3 x 2 min) and dehydrated in a graded series of alcohols (25%, 50%, 70%, 90%, 100% [v/v in d.H_2O]). After processing through increasing concentrations of resin diluted in alcohol (1:2 resin to EtOH, 1:1 resin to EtOH, 2 changes of fresh L R White resin), cell pellets were embedded in L R White resin. Curing was carried out in resin-filled gelatin capsules (56°C, 48 h) in a hot air oven.

Cells were prepared for electron microscopic investigation as described in Section 2.5.2.

4.3.3. Results and discussion

c-Ha-*ras*(V12)-transfected cells clearly showed a reduced number of curved, electron dense domains, or caveolae on the PM (Figure 4.10 B), compared to the control MCF10A cells (Figure 4.10 A). This seems to confirm reports that c-Ha-Ras(V12) downregulates cav-1, the main protein that shapes these dense caveolae (Song *et al.*, 1996; Williams *et al.*, 2005) and hence downregulates the formation of caveolae from lipid rafts.

Figure 4.10. c-Ha-*ras*(V12) alters the number of caveolae on the PM.
Compared to the untransfected MCF10A cells (A), the c-Ha-*ras*(V12)-transfected MCF10AneoT cells (B) had reduced number of curved, electron dense caveolae (arrows and inserts). The large arrows indicate the area that was enlarged in the insert. (Bars = 1 μm)


A
NUCLEUS
1 µm
B
NUCLEUS
1 µm

After synthesis, inactive, palmitoylated Ha-Ras is trafficked to lipid rafts on the PM (Baker *et al.*, 2000; Reuther *et al.*, 2000). On EGF stimulation and EGFR cytoplasmic tail dimerization, normal Ha-Ras is activated by GTP loading (Reuther *et al.*, 2000), is released from the lipid rafts and may then in turn activate downstream kinases and GTPases such as Src, Rac and Rho (Figure 1.3 on fold-out). The inhibitory function of cav-1 is indicated in the overexpression of cav-1, which is related to decreased migration, even with EGF stimulation of cancer cells (Zhang *et al.*, 2000). An increase in cav-1 also results in decreased proliferation of MCF-7 breast epithelial cancer cells (Fiucci *et al.*, 2002b; Ravid *et al.*, 2005) and in NIH-3T3 fibroblast cells (Galbiati *et al.*, 2001), indicated by an exit of S-phase and an increased number of cells in G0 and G1. Elevated levels of cav-1 also suppresses anoikis, or detachment-induced cell death, by suppressing p53-related activation of caspases (Ravid *et al.*, 2005). On the other hand, an apparent decrease in caveolae, as shown in this study via ultrastructural investigation, may be an indication of a decrease in cav-1 expression, confirmed in c-Ha-Ras(V12) expressing NIH-3T3 fibroblasts (Koleske *et al.*, 1995; Engelman *et al.*, 1998b; Le *et al.*, 2001), where it appeared to increase migration (Zhang *et al.*, 2000). This may occur since lack of cav-1 possibly removes its inherent inhibition of downstream Ha-Ras-related signaling proteins, such as ERK-2 (Section 4.1.5.2). Constant c-Ha-Ras(V12) activation of EKR-2 (Fiucci *et al.*, 2002a) and further downstream signaling to ROCK and myosin light chain kinase (MLCK), may allow actin polimerization, the formation of acto-myosin stress fibers (Figure 4.12) and cell contraction during migration (Section 1.7.3). Lack of cav-1 may allow hyperproliferation, but does not lead to cancer (Razani *et al.*, 2001). Synergistic enhancement, however, of c-Ha-Ras(V12) signaling and downregulation of the p53/p16 apotosis system due to loss of the *9p21* gene (Section 1.9), such as in c-Ha-*ras*(V12)-transfected MCF10AneoT mammary epithelial cells, may enhance oncogene-induced cell proliferation and cancer formation (Williams *et al.*, 2004). While cav-1 seems to be downregulated in MCF10AneoT cancer cells, compared to the normal MCF10A cells (Figure 4.8 A, B), it may still be involved in the accumulation of proteases such as CB (Cavallo-Medved *et al.*, 2005) (Section 4.2.1) and MT1-MMP (Labrecque *et al.*, 2004) (Section 4.1.5.2) at the leading front of migrating cells, and in this way may add to the invasive potential of c-Ha-*ras*(V12)-transfected cells. The distribution on the PM of these proteases, related to reduction

in the number of caveolae after c-Ha-*ras*(V12) transfection and increased invasive potential, now needed to be further investigated.

4.4. CB distribution in control and c-Ha-*ras*(V12)-transfected cells

Intracellular distribution, characteristics of CB-containing vesicle and the influence of c-Ha-Ras(V12) signaling have been discussed in Chapter 3. CB may, however, also be secreted and become associated with the PM, where it is reported to be involved in the gelatinolytic degradation of ECM components during growth factor-related (Linke *et al.*, 2002), chemotaxic cell migration (Schraufstatter *et al.*, 2003) and during migration or wound healing (Brix *et al.*, 2008; Daley *et al.*, 2008). Elevated levels of proteases, including CB, was reported in plasma of patients with invasive breast cancer (Thomssen *et al.*, 1995; Lah *et al.*, 2000), and was associated with invasive behaviour of pancreatic and colon cancers (Sinha *et al.*, 1998; Troy *et al.*, 2004). In our laboratory, transcription of CB was reported to be increased in c-Ha-*ras*(V12)-transfected MCF10AneoT breast epithelial cancer cells (Preliminary manuscript attached). More relevant to this part of the study, though, is the connection between PM-associated CB in the MCF10AneoT cells (Sameni *et al.*, 1995; Pillay *et al.*, 2002b) and invasive behaviour of these cells (Premzl *et al.*, 2001).

CB on the PM seems to be associated with caveolae and the resident uPa proteolytic cascade system (Cavallo-Medved *et al.*, 2005; Sloane *et al.*, 2005), which supports BM degradation (Section 1.3.5 and Section 4.1.2). In this study, immunolabeling of mature CB on non-permeabilized cells was performed to explore the influence of c-Ha-Ras(V12) in conjunction with the observed loss of caveolae (Section 4.2) on the distribution of this protease along the extracellular PM surface. Such information may further illucidate the contribution that CB makes to BM and ECM degradation during invasion.

4.4.1. Reagents

Reagents used for cell culture were described in Section 2.2.1.

Reagents used for intracellular CB immunolabeling for confocal microscopy were described in Section 2.6.1.

4.4.2. Procedure

For studies on surface immunolabeling of CB, subconfluent cells were grown on coverslips, as described in Section 2.2.2. Live, non-fixed cells were washed with warm HBSS, cooled down on the bench to about 20^{o}C before continuing with the immunolabeling procedure, to prevent internalization of the antibodies (Peters *et al.*, 1990; Sameni *et al.*, 1995; Sameni *et al.*, 2000; Lafleur *et al.*, 2006). Cells were blocked with PBS-BSA without saponin (30 min), incubated in a chicken antibody to CB (20 μg/ml, diluted in PBS-BSA, 1 h), washed with PBS without saponin, fixed with PFA (10 min) and washed in PBS-saponin (3 x 5 min). All subsequent antibody and wash solutions contained 0.1% saponin. Coverslips were incubated with a secondary donkey anti-chicken antibody tagged with Cy3 (diluted 1:1500 in PBS-BSA, 1 h), washed with PBS-saponin (3 x 5 min), fixed with PFA (10 min) and washed in PBS (5 x 5 min). Finally, coverslips were washed, fixed and mounted with Moviol anti-fade reagent and observed on a Zeiss 710 confocal laser scanning microscope (Heidelberg, Germany).

4.4.3. Results and discussion

Routinely in immunolabeling procedures cells are fixed and treated with a detergent, such as saponin, in order to improve penetration of chemicals for intracellular antigen recognition. Using a similar protocol, we immunolabelled CB both in the cytoplasma and on the PM of fixed cells. In order to also investigate the effect of c-Ha-Ras(V12) on the distribution of the CB protein on the PM only, the fixation and permeabilization steps had to be performed only after incubation in primary antibody. For that reason non-fixed, live cells were incubated with the primary antibody, and internalization of the antibodies during this initial step was limited by cooling the cells to RT, as previously described (Section 2.6.2). We did find that cooling of these live, non-fixed cells to 4^{o}C resulted in all the cells pulling into a contracted, small, rounded shape, and therefore, it was decided to cool cells to only RT. Cells treated (cooled) in this way did have an altered morphology to untreated, fixed cells, and were only slightly more rounded and less elongated than the fixed counterparts, but still resembled a migratory shape, as described in Section 4.2.3. Still, we were sure that only PM-located CB was visualized using this protocol, which was confirmed by optical confocal sections.

An important novel finding in our study was the spatial distribution of CB in the control (Figure 4.11 A, B) and c-Ha-*ras*(V12) transfected cells (Figure 4.11 C, D) to regions lateral to the nucleus along the anterior-posterior axis. In transfected cells CB was also observed at the basal layer of the cell in leading invadopodia (Figure 4.11 C, D). These results could clearly be observed in galleries of sets images generated from consecutive focal planes throughout both the control (Figure 4.11 B) and transfected cells (Figure 4.11 D). The sites of CB localization lateral to the nucleus in both control and transfected cells, were similar to those shown to be positive for cav-1 in normal fibroblasts (Stahlhut *et al.*, 2000) and heterotetrameric p11 in migrating HT1080 epithelial cells (Choi *et al.*, 2003). In addition, proteolytically active CB, p11 [a receptor for plasminogen (MacLeod *et al.*, 2003)], annexin-II [binds phospholipids and a p11 partner in a heterotetramer (Rescher *et al.*, 2004)], uPA and the uPAR may all be located to these distinct PM domains (Mai *et al.*, 2000; Choi *et al.*, 2003; Cavallo-Medved *et al.*, 2005) (Figure 4.1). This may indicate a site of activity of the plasmin proteolytic cascade (Section 1.3.5). Here CB may play a PM-related role in the maturation of precursors involved in the uPA proteolytic cascade (Mignatti *et al.*, 1993; Murphy *et al.*, 1999; Cavallo-Medved *et al.*, 2005) (Figure 4.8 Lane 2). Such spatial restriction of proteases possibly limits proteolytic degradation to the invasive front during cancer invasion and metastasis (Cavallo-Medved *et al.*, 2005; Vasiljeva *et al.*, 2006).

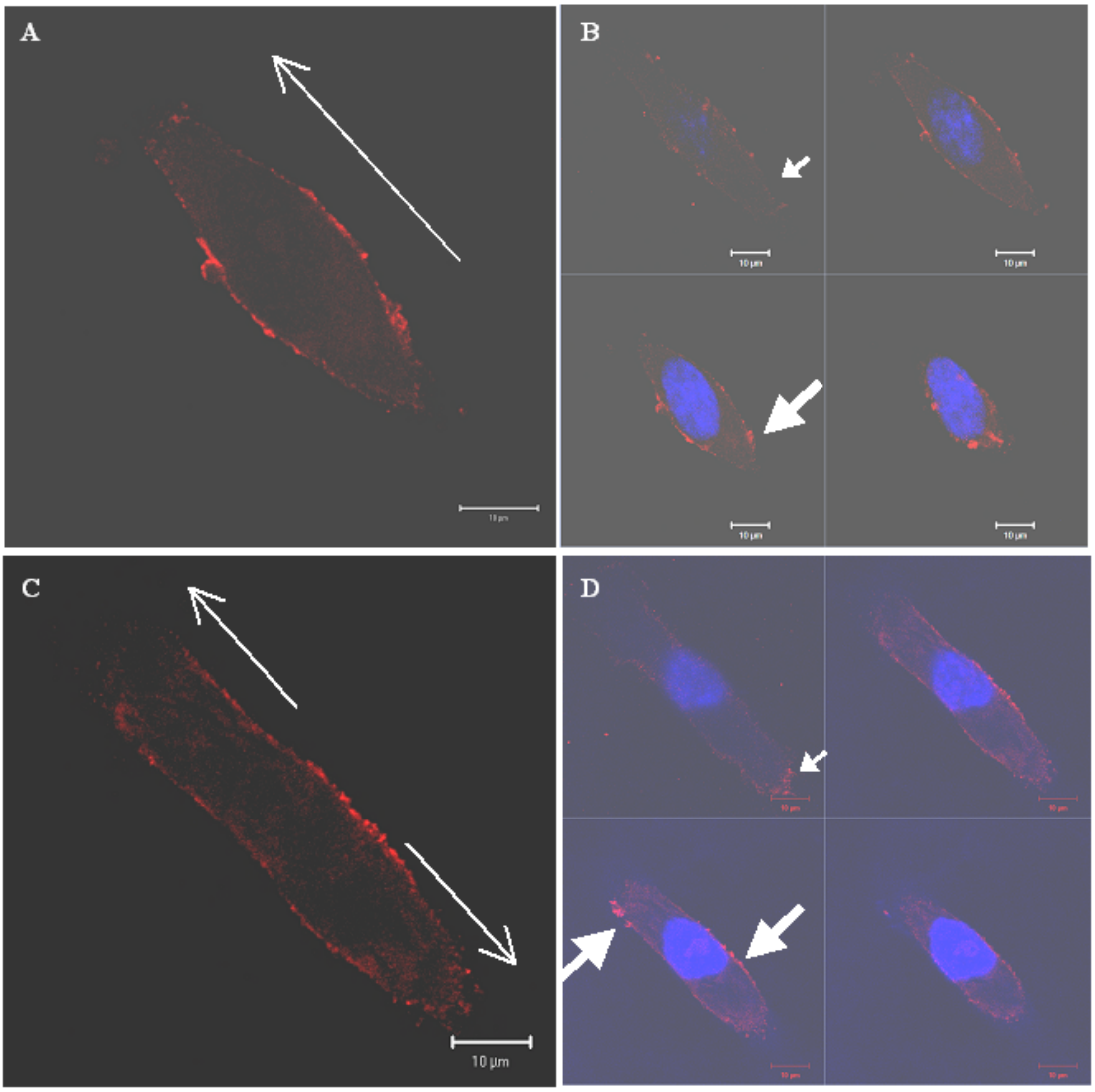


Figure 4.11. Localization of mature CB on the PM of breast epithelial cells.
Mature CB was immunolocalized on the PM of non-permeabilized, non-transfected MCF10A (A, B) and c-Ha-ras(V12)-transfected MCF10AneoT cells (C, D). The direction of the leading, or ruffling front is indicated with a long arrow in A and C. A gallery of each image serves to indicate the lateral distribution of CB in both control (B) and transfected cells (D) along the middle plane of the cells (thick arrows), and its relocation to basal layer and the invasive front in the transfected cells (D) (small arrows). (Bars = 10 µm)

While the components of the uPa-plasmin cascade usually functions in an alkaline extracellular milieu, activity of mature CB generally requires an acidic environment and may be inactivated at physiological pH. Under these conditions CB may be protected from inactivation by heparin sulphate (Almeida *et al.*, 2001a) and may be capable of degrading constituents of the ECM and basement membrane, such as type IV collagen (Coetzer *et al.*, 1991), fibronectin and laminin at both acid pH and neutral pH at the surface of malignant tumour cells (Buck *et al.*, 1992). The NHE-1 PM-related proton pump causes slight acidification of the local microenvironment (Denker *et al.*, 2002). It was shown that NHE-1, initially located in a similar position next to

the nucleus in normal cells, is actively relocated to invasive podocytes in transformed and invasive cells during starvation-related migration (Cardone *et al.*, 2005). The similar translocation to the invasive front of CB in c-Ha-*ras*(V12)-transfected cells (Figure 4.11 C, D), may illustrate that the more acidic extracellular conditions, reported in malignant tumours (Montcourrier *et al.*, 1997), and required for proteolytic activity of membrane-associated CB, could be attained and may adequately support CB function on the PM.

PM-bound MT1-MMP has two endogeneous inhibitors, TIMP-1 and TIMP-2 that restrict its degradative ability and its maturation of proMMP-2 (Sato *et al.*, 1997; d'Ortho *et al.*, 1998; Vu *et al.*, 2000) (Section 4.1.6). An important factor that has not yet been associated with the CB to the invasive ability of cancer cells, is the little known fact that CB may cleave both TIMP-1 and TIMP-2 (Kostoulas *et al.*, 1999). Degradation of TIMPs by CB may relieve the TIMP inhibitory constraint on MT1-MMP, allowing matrix degradation (Kostoulas *et al.*, 1999). While no reference has been made to such cleavage of TIMP-2 by CB, in conjunction with invasion, we now suggest that it may also play a role at the leading front of invasive cells, to inhibit TIMP-2 and retain the proteolytic functions of MT1-MMP, such as collagen degradation, or proMMP-2 processing and subsequent gelatinolytic activity. This interaction will be further investigated during intended future research.

The accumulation of CB at the invasive front in breast cancer (Thomssen *et al.*, 1995), colon cancer (Troy *et al.*, 2004; Cavallo-Medved *et al.*, 2005) and in invasive cancer in general (Koblinski *et al.*, 2000; Szpaderska *et al.*, 2001), and now also shown in this study of c-Ha-*ras*(V12)-transfected cells (Figure 4.11 C, D), supports the relevance of membrane-associated CB in the invasive process.

4.5. Characterization of chicken antibodies raised to various domain combinations of MT1-MMP

While it is well established that CB participates in normal cell migration, wound closure and in cancer invasion, MT1-MMP has recently been shown to support migration in normal, non-transformed MCF10A breast epithelial cells (Gilles *et al.*, 2001). In addition, from invasion assays in our laboratory using RT-PCR and inhibitor- or immunoinhibition (Gordon conference 2008 Poster, Appendix IV), MT1-MMP has been shown to be possibly the main extracellular protease involved in the

invasive behaviour of c-Ha-*ras*(V12)-transfected MCF10AneoT breast epithelial cancer cells (Gordon conference 2008 Poster, Appendix IV). It also seems that CB and MT1-MMP may collaborate in some way in such invasive activity, as these inhibitor studies indicated that general inhibition of MMP activity decreases invasion to about 90%, whereas cysteine protease inhibitors appear to reduce invasion only 40% (Gordon conference 2008 Poster, Appendix IV).

The proteolytically active Cat- and Pex extracellular regions of MT1-MMP (Figure 4.12 A) seem involved in proteolytic degradation of many BM components and determine specificity for these substrates (Section 4.1.4). MT1-MMP is anchored to the PM via its transmembrane domain, while an intracellular cytoplasmic domain plays a major role in its trafficking, internalization and redistribution the PM. Through this domain the enzyme is also involved in intracellular signal transduction (Section 4.1.5). The 68 kDa prodomain peptide maintains MT1-MMP latency by obstructing the catalytic site. Processing of the precursor domain reportedly occurs in several steps, either along the secretory route, or on the PM (Section 4.1.3), with a 63 kDa intermediate form, containing part of the prodomain, said to be involved in processing of other proteases, such as proMMP-2.

A mature 58 kDa MT1-MMP form is known as the main protease involved in the invasive behaviour of c-Ha-*ras*(V12)-transfected MCF10A breast epithelial cells (Kim *et al.*, 2009). An autocatalytically cleaved, inactive 45 kDa form, without the catalytic domain and autocatalytic processing produces an inactive 45 kDa and a soluble 18 kDa form (Section 4.1.3). Raising of antibodies to such specific domains was extremely important, as, due to highly conserved domains in MMPs, antibodies raised against MT1-MMP aften cross-react with many other MMPs, rendering them of little use. Many also recognize only specific processing forms, preventing the detection and study of all forms of the enzyme. Hence, a set of antibodies raised to specific combinations of the proteolytically active extracellular domains of the MT1-MMP protein, is vital for the study currently proposed, i.e. to distinguish between various processing forms of MT1-MMP and ascertain MT1-MMP distribution.

As part of a related immuno-inhibition project three peptides that represent various combinations of the MT1-MMP protein domains (Figure 4.12 A) were previously expressed in *E. coli* and purified (Crouch, 2009; van Rooyen, 2009). These peptides,

representing various combinations of the MT1-MMP domains (Figure 4.12 A) were expressed in *E. coli* and purified in a parallel study in our laboratory. These included a 33 kDa peptide to the precursor and catalytic domains (the ProCat peptide, representing the precursor form of MT1-MMP, truncated before the hinge region [Ala^{21}-Ile^{318}]) (Lichte *et al.*, 1996), a 48 kDa peptide representing the haemopexin and catalytic domains (the PexCat peptide, identical to the Pro^{96}-Gly^{511} MT1-MMP with the inclusion of 6 histidine residues flanking the C-terminus) and a 24 kDa Pex peptide, representing the haemopexin domain only. Expressed, unrenatured peptides were injected into chickens, IgY antibodies were isolated after 8 weeks and characterized. Cross-reactivity of this set of antibodies was previously established, but not its reactivity in the MCF10A cell system. Such characterization was, therefore, done in the current study and the presence of processed forms of MT1-MMP in a reduced sample of c-Ha-*ras*(V12)-transfected MCF10AneoT cell lysates was establish by western blot. It was anticipated that antibodies raised to the ProCat and PexCat peptides would recognize of the 68 kDa MT1-MMP precursor, the intermediate, partially processed 63 kDa form and the catalytically active 58 kDA form of MT1-MMP. Antibodies raised to the Pex peptide were expected to detect all the forms of MT1-MMP, as described above, including the cleaved, catalytically inactive 45 kDa form of MT1-MMP. These various antibodies to MT1-MMP domains were subsequently applied in the current fluorescent immunocytochemical studies as indicators of most of the MT1-MMP forms, as reported in Section 4.6. .

4.5.1. Reagents

Reagents used for cell culture were described in Section 2.1.1.

Reagents used for SDS-PAGE were described in Section 2.3.1.

Reagents used for western blots were described in Section 2.4.1.

4.5.2. Procedure

Monolayers of MCF10AneoT cells were grown to 70% confluence in 75 cm^3 dishes as described in Section 2.2. The medium was discarded, cells were washed in HBSS (3 x), scraped into fresh HBSS and centrifuged (1000 x g for 2 min) and the supernatant discarded. Each pellet sample was resuspended in Solution C (100 μl), reducing treatment buffer (50 μl) was added and the samples were boiled (120 sec)

and stored (–20°C). Samples were thawed just before use and loaded onto the prepared polyacrylamide gel (Section 2.3).

An aliquot of the combined set of molecular weight markers (3 µl) and aliquots of the prepared cell samples (10 µl) were loaded into a 15 well (12% [m/v]) polyacrylamide gel. During the electrophoretic protein separation procedure (18 mA per gel, unlimited voltage) the gels were cooled using a circulating water bath (4°C). Electrophoresed experimental samples were transferred onto a nitrocellulose membrane by western blotting on a Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell (unlimited voltage, 25 mAmp, 16 h). The nitrocellulose membrane strips were placed in the incubation vessel and blocked with non-fat milk powder (5% m/v) in TBS-Tween (5 ml for 1 h, RT). The blots were incubated in chicken anti-ProCat antibody (5 µg/ml), chicken anti-PexCat antibody (5 µg/ml) or chicken anti-Pex antibody (5 µg/ml), all diluted in TBS-Tween (5 ml ea, 1h). The blots were washed in TBS-Tween (5 x 10 min), incubated in peroxidase-tagged rabbit anti-chicken IgY (diluted 1:15 000 in TBS-Tween, RT), washed in TBS-Tween (6 x 10 min) and incubated in luminol (5 min). X-ray film was exposed to the treated blots, developed, fixed, washed and dried. Blots were photographed in a VersaDoc 4000 Imager (BioRad, California, USA) and analysed.

4.5.3. Results and discussion

Chicken anti-ProCat seemed to recognize 58 kDa mature MT1-MMP processing forms in a reduced MCF10AneoT cell pellet sample (Figure 4.12 B Lane 1) as well as several additional bands (at about 45, 35, 33 and 24 kDa). These appeared to be processed forms of MT1-MMP.

The chicken anti-PexCat antibody, raised against a peptide that represented the catalytic and haemopexin domains, seemed to recognize the 58 kDa mature MT1-MMP protein, as well as the additional bands labeled by the anti-ProCat antibody (Figure 4.12 B Lane 2). It was expected that the anti-PexCat antibody would, in addition, recognize a band that may represent the 45 kDa autocatalytically cleaved domain, since it was raised to an additional domain. However, no such band was seen. This suggested that both these two antibodies recognize mainly the catalytic domain of the MT1-MMP protein

The chicken anti-Pex antibody, raised against a peptide that represented only the haemopexin domain, recognized a band of protein at 58 kDa, which could possibly be indicating that it recognized the mature form of MT1-MMP (Figure 4.12 B Lane 3). In addition, high molecular bands with approximate weights of 80-97 kDa, was recognized. This may indicate cross-reactivity with the haemopexin domain of pro-MMP-2 or proMMP-9, which is also secreted by the transformed MCF10AneoT cancer cells. Such cross-reactivity was also reported in the previous characterization of this peptide (Gordon conference 2008 Poster, Appendix IV).

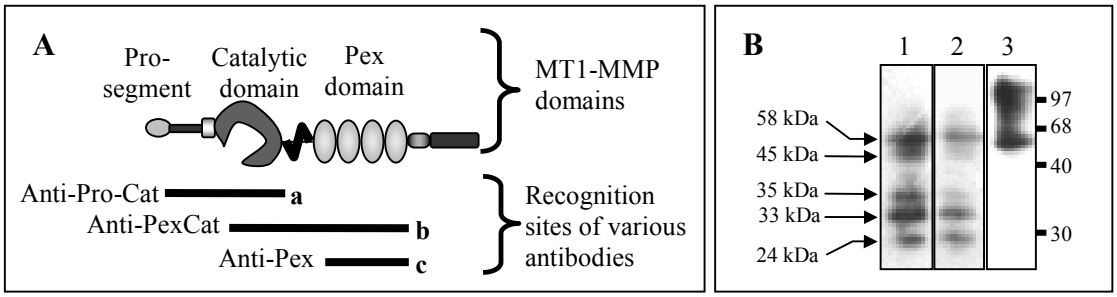


Figure 4.12. Characterization of antibodies to extracellular domains of MT1-MMP against MCF10AneoT homogenates.
(A) Antibodies raised in chickens to constructs that represent combinations of the extracellular MT1-MMP domains were raised in chickens. The chicken anti-ProCat antibody was raised to the precursor and catalytic domains (a), chicken anti-Pex-Cat was raised to the Pex and catalytic domains (b) and chicken anti-Pex antibody was raised to the Pex domain only (c). These antibodies, raised and characterized in a parallel study (Gordon conference 2008 Poster, Appendix IV) were used to detect the cellular distribution of MT1-MMP by confocal microscopy (Crouch, 2009; van Rooyen, 2009). (B) Using chicken anti-ProCat (Lane 1), chicken anti-PexCat (Lane 2) or chicken anti-Pex antibodies (Lane 3) processed forms of MT1-MMP, including the 58 kDa mature form, were detected in a reduced cell pellet sample of MCF10AneoT cells. The chicken anti-Pex antibody seemed to cross-react with a 72 kDa MMP-2 precursor and a 92 kD MMP-9 precursor (Lane 3).

These antibodies to the MT1-MMP protein were then employed to immunolabel MT1-MMP in permeabilized cells, as well as on only the PM of non-permeabilized cells. It was anticipated that results from these experiments might assist in predicting sites of proteolytic activity on the PM and give an indication of the trafficking or recycling route of MT1-MMP.

4.6. MT1-MMP distribution in breast epithelial cells

CB may be one of the main proteases involved in the invasive ability of transfected and cancer cells. However, investigations into metastatic melanoma and prostate cancers (Szpaderska *et al.*, 2001) and work currently being done in our laboratory on c-Ha-*ras*(V12)-transfected breast epithelial cells (van Rooyen *et al.*, 2008), illustrated

that, using a chicken CAM invasion assay, invasion is abrogated with inhibition of enzymatic ability of MT1-MMP (van Rooyen *et al.*, 2008). It is, therefore, important to investigate the distribution of extracellular proteolytically active MT1-MMP and to consider the mechanisms that are at play to retain this activity on the PM, specifically in c-Ha-*ras*(V12)-transfected MCF10A breast epithelial cells. Such correlations have not yet been found in the literature. In this part of the study antibodies that had been raised in a parallel immune-inhibition study (Section 4.4, preliminary manuscript attached), were used to immunofluorescently label MT1-MMP in control and c-Ha-*ras*(V12)-transfected cells, either intracellularly, or on the PM only. It was hoped that these results might clarify the influence that this oncogene, often mentioned in connection with metastatic cancers, has on expression and distribution of the proteolytically active enzyme on the PM, that may support invasion. In addition, labeling of permeabilized cells would indicate the level of expression of total MT1-MMP, including the proportion of enzyme retained within the cytoplasm.

4.6.1. Reagents

Reagents used for cell culture were described in Section 2.2.1.

Reagents used for fluorescence immunolabeling for confocal microscopy were described in Section 2.6.1.

4.6.2. Procedure

For intracellular and surface immunolabeling MCF10A and MCF10AneoT cells were seeded onto sterile round 12 mm coverslips in 24 well Tersaki plates and cultured over night in DMEM-Ham's F12 medium supplemented with 10% (v/v) decomplemented horse serum as described in Section 2.2.2.

Immunolabeling of MT1-MMP on the surface of live, non-permeabilized cells was done as described in Section 4.4.2. Coverslips were incubated in a chicken antibody to proCat (20 μg/ml, diluted in PBS, 1 h), an antibody to PexCat (20 μg/ml, diluted in PBS, 1 h), or an antibody to Pex (20 μg/ml, diluted in PBS, 1 h), washed with PBS, fixed with PFA (10 min), washed in PBS (3 x 5 min). All subsequent antibody and wash solutions contained 0.1% saponin. Coverslips were incubated with a secondary donkey anti-chicken antibody tagged with Cy3 (diluted 1:1500 in PBS-BSA, 1 h),

washed with PBS-saponin (3 x 5 min), fixed with PFA (10 min) and washed in PBS (5 x 5 min).

Immunolabeling of MT1-MMP in fixed, saponin-permeabilized cells was done as described in Section 2.2.2. Coverslips were washed, fixed, permeabilated and incubated in either a chicken antibody to proCat (20 μg/ml, diluted in PBS-saponin, 1 h), an antibody to PexCat (20 μg/ml, diluted in PBS-saponin, 1 h), or an antibody to Pex (20 μg/ml, diluted in PBS-saponin, 1 h). Coverslips were washed in PBS-saponin (3 x 5 min), incubated with a secondary donkey anti-chicken antibody tagged with Cy3 (diluted 1:1500 in PBS-BSA, 1 h) and washed with PBS-saponin (3 x 5 min), fixed with PFA (10 min) and washed in PBS (5 x 5 min).

Finally, cells were fixed and mounted with Moviol anti-fade reagent and observed on a Zeiss LSM 510 META or a Zeiss 710 confocal laser scanning microscope (Heidelberg, Germany).

4.6.3. Results and discussion

Using the three available antibodies to MT1-MMP on fixed, saponin-permeabilized control MCF10A cells, different patterns were observed for MT1-MMP distribution throughout the cytoplasm (Figure 4.13). The anti-ProCat antibody detected high levels of intracellular MT1-MMP, as well as distinct areas on the leading ruffling border or the normal MCF10A (Figure 4.13 A) and c-Ha-*ras*(V12)-transfected MCF10AneoT cells (Figure 4.13 D). This could represent the 58 kDa mature form of the MT1-MMP protein. Even though this antibody did not detect any of the intermediately processed precursor forms in western blots (Figure 12 B Lane 1), in a tissue sample it may recognize the native 68 kDa precursor, the 63 kDa intermediate and 58 kDa mature forms, i.e. all active or precursor forms, since all these forms of MT1-MMP contain the Pro and Cat domains to which the anti-ProCat antibody was raised (Figure 4.12 A). This antibody may, however, not recognize the autocatalytically cleaved 45 kDa form of MT1-MMP, which does not contain the precursor or catalytic domains (Figure 4.2 and Figure 4.3 Lane 4). With the anti-PexCat antibody, on the other hand, very little labeling was found on the leading front of normal cells (Figure 4.13 B), while intracellular MT1-MMP was clearly immunolabelled in an area around the nucleus. Since western blot results indicated

that the anti-PexCat antibody had a lower efficiency in recognition of most forms, but gave similar bands as the anti-ProCat antibody, the labeling given by the anti-PexCat antibody may reflect its inefficient recognition of even the native form of the protein (Figure 4.13 B, E). Also, since it was raised to a peptide that, in addition to the catalytic domain, contained regions similar to both the Cat and Pex domains, it should give an indication of the distribution of most of the MT1-MMP processing forms present (Figure 4.12 A). Since immunolabeling was relatively restricted to a perinuclear region (Figure 4.13 B), this may be an indication that recognition of newly-produced 68 or 65 kDa forms of the protein may be better in denatured (cellular) forms of MT1-MMP, even though these forms were not indicated in the blots of reduced protein, where MT1-MMP is denatured (Figure 4.12 B Lane 2). In the c-Ha-*ras*(V12)-transfected cells widely-distributed labeling was observed throughout the cytoplasm, and was not limited to the perinuclear area (Figure 4.13 E). The anti-Pex antibody did not clearly detect PM-bound or intracellular MT1-MMP in the normal cell, but for distinct, small areas that could be seen lateral to the nucleus (Figure 4.13 C). This is strange, as there is far more recognition in the c-Ha-*ras*(V12)-transfected cell (possibly indicating a higher presence of the 58 kDa form of MT1-MMP) (Figure 4.13 F). This antibody was shown to recognize the 58 kDa mature MT1-MMP protein in western blots (Figure 4.12 B Lane 3), as well as possible cross-reactivity with precursor MMPs of approximately 78 and 98 kDa and may, therefore, represent such MMPs in addition to MT1-MMP. These results may indicate that these antibodies are possibly recognizing denatured forms of the MT1-MMP protein and, therefore, should possibly be checked with non-denatured blots.

Compared to the normal, in permeabilized, transfected MCF10AneoT cells, chicken anti-ProCat antibody-labeled MT1-MMP showed minimal difference in cytoplasmic distribution (Figure 4.13 A, D). Chicken anti-PexCat antibody-labeled MT1-MMP showed a slight increase in cytoplasmic levels and was distributed to the ruffling borders (Figure 4.13 B, E). Increased levels of intracellular MT1-MMP immunolabelled with the chicken anti-PEX antibody was observed compared to the control, non-transfected MCF10A cells described above (Figure 4.13 C, F). Immunolabeling was distributed throughout the cell, as well as on the ruffling borders. Such labeling on the ruffling border could be seen on several small leading fronts (Figure 4.13 F), which were also described in Section 4.2 (Figure 4.9 B). Such a general intracellular increase, compared to the normal, strongly suggested that

transcription of MT1-MMP has been elevated after c-Ha-*ras*(V12) transfection. Newly-produced MT1-MMP protein presented on the PM, forms part of a complicated route of internalization from the PM and subsequent representation during the course of its catalytic functions on the PM, with many different trafficking proteins involved in this process (Section 4.1.5.1). A fraction of immunolabeled MT1-MMP seen in this study may represent newly-synthesized protein, especially in the transfected cells, where an increase in levels of the enzyme was clearly illustrated (Figure 4.13 D-F). It was suggested that such newly-produced proteins be identified via double labeling with Rab8 (Huber *et al.*, 1993; Bravo-Cordero *et al.*, 2007). Another portion of the immunolabelled MT1-MMP protein may be on the internalization-representation route and should, therefore, be positive for Rab4 and/or Rab11 (Peden *et al.*, 2004b; Remacle *et al.*, 2005) (Section 4.1.5.1). Further studies could assess this possibility. After processing of either proMMP-2 or autocatalysis, the processed Pex and cytoplasmic tail domains most probably have to be separated from the complex in slightly acidic endosomes (Yunta *et al.*, 2003). While the whole molecule may be recycled to the PM for further activity, the released ligands may now be further degraded (Remacle *et al.*, 2003; Itoh *et al.*, 2006a), and may be associated with LAMP-1, possibly LAMP-2 (Section 3.2.2.1) or Rab7-positive structures (Section 3.3.7.2), that form part of the degradative route signaling components. This too, could be a useful investigation for the future. In addition, the autocatalytically cleaved 45 kDa inactive form of MT1-MMP may also be degraded along this path. Due to the lack of appropriate antibodies to the markers mentioned above, such as Rab4, Rab11 or Rab7, however, we can presently not distinguish between these routes, but it will form part of studies planned for the near future.

The proteolytically active form of MT1-MMP, located to the PM, is reported to be involved in the invasive behaviour of cancer cells. In order to also investigate the effect of c-Ha-Ras(V12) on the distribution of the MT1-MMP protein on the PM only, live, non-fixed cells were cooled to RT and immunolabelled for PM-located MT1-MMP, as described for CB labeling (Peters *et al.*, 1990; Sameni *et al.*, 1995; Sameni *et al.*, 2000; Lafleur *et al.*, 2006) (Section 4.3.3). Similarly, we were sure that only PM-located MT1-MMP was visualized using this protocol, which was confirmed by optical confocal sections that showed labeling along the PM only, with no labeling observed in the cytoplasm.

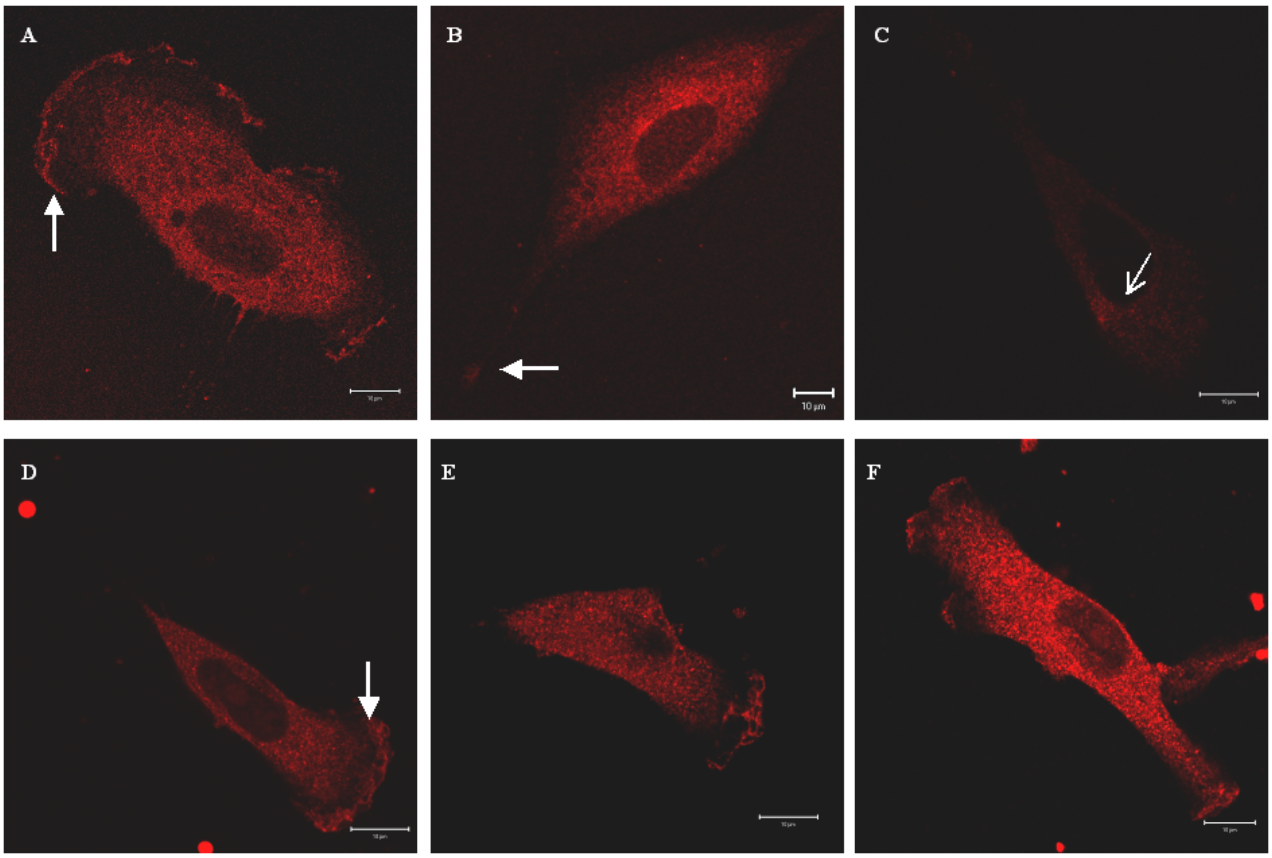


Figure 4.13. Intracellular immunolabeling of MT1-MMP.
Three antibodies raised in chickens to various domains of the MT1-MMP protein structure were employed to immunolocalize MT1-MMP in fixed, saponin-permeabilized cells, with the focus on the basal plane of the cell where it is in contact with the BM. Anti-ProCat (A, D) anti-PexCat (B, E) and anti-Pex (C, F). In the control MCF10A cells both the anti-ProCat and anti-PexCat antibodies detected MT1-MMP in the cytoplasm (A, B), while the anti-ProCat antibody also immunolabelled MT1-MMP in the leading ruffling border (thick arrows in A, B) and the anti-Pex antibody detected MT1-MMP at small, distinct sites next to the nucleus (thin arrow in C). In the transfected MCF10AneoT cells all three antibodies localized MT1-MMP in the cytoplasm and on the ruffling borders (arrow in D), with a slight increase shown with the anti-ProCat antibody (D) and visible increase shown in intracellular levels of the enzyme and on the ruffling border in the transfected cells with the anti-PexCat and anti-Pex antibodies (E-F), compared to the control cells (A-C). (Ruffling border: closed arrow; site of possible internalization: thin arrow.) (Bars = 10 μm)

Using the anti-ProCat antibody on non-permeabilized control MCF10A cells, a high intensity of immunolabelled MT1-MMP was observed mostly to the leading front ruffling border of the control cells (Figure 4.14 A). These findings are in agreement with other workers who showed the localization of MT1-MMP to the ruffling border of migrating cells, where it is suggested to facilitate matrix remodelling, specifically of collagen-I degradation (Zhuge *et al.*, 2001; Tam *et al.*, 2002). This was also demonstrated in MCF10A cells grown on laminin (Gilles *et al.*, 2001) that showed the overexpression of MT1-MMP in epithelial cells can be associated with the migratory

status of the cells (Gilles *et al.*, 2001). The transfected cells, on the other hand, did not show the same level of concentration of label to the front, using the anti-ProCat antibody, but instead fluorescence was low and relatively evenly spread to distinct, small sites over the PM (Figure 4.14 D). The anti-PexCat antibody showed slight labeling over the plasma membrane of lamellipodia of the control cells (Figure 4.14 B), but in the transfected cells fluorescence was mostly restricted to small areas next to the nucleus, with only slight labeling over the rest of the PM (Figure 4.14 E). Labeling with anti-Pex antibody showed labeling in a concentrated area lateral to the nucleus in both control cells (Figure 4.14 C) and transfected cells (Figure 4.14 F), as well as in small areas over the PM. It was noted that the anti-proCat antibody did not label similar small, laterally located areas (Figure 4.14 A, D). This could possibly be an indication of cross-recognition of proMMP-2 or proMMP-9.

The individual polyclonal antibodies used during these experiments were raised in chickens to combinations of various domains of the MT1-MMP pro-form (Section 4.5). It has been described above, though, that the precursor and catalytic domains are involved in the catalytic activity of the enzyme at the migrating front, most specifically during migration (Section 4.1.4.1). The Pex domain, on the other hand, is essentially involved in substrate specificity (Section 4.1.4), which may not necessarily be limited to a specific pole of the cell. In addition, the autocatalytically degraded form of MT1-MMP consists of only the Pex domain, and labeling seen could represent this degraded form of MT1-MMP (Section 4.1.6). This could explain the variation in localization of essentially the same protein with these 3 different antibodies, as described above and illustrated in Figure 4.13 and Figure 4.14. These differences also seem to indicate abbarent control over trafficking of MT1-MMP either due to c-Ha-Ras(V12), or its downstream signaling effectors.

MT1-MMP localized to the leading edge of the cells was immunolabelled best with the antiProCat antibody, which did show recognition for the 58 kDa mature form previously seen at invasion front. Since this polyclonal antibody may contain antibodies to both precursor and catalytic domains, the MT1-MMP protein on the cell membrane immunolabelled by this antibody may most probably be part of the complex formed by catalytically active MT1-MMP-TIMP-2-proMMP-2 complex that assist in proMMP-2 maturation (Section 4.1.4.1). On the other hand, MT1-MMP could be associated with cav-1 or adhesion molecules such as CD44 and integrins,

with which the phosphorylated MT1-MMP protein was said to form a complex (Rozanov *et al.*, 2006). This may account for the localization of MT1-MMP along the PM in the control and (Figure 4.14 A) transfected cells (Figure 4.14 D).

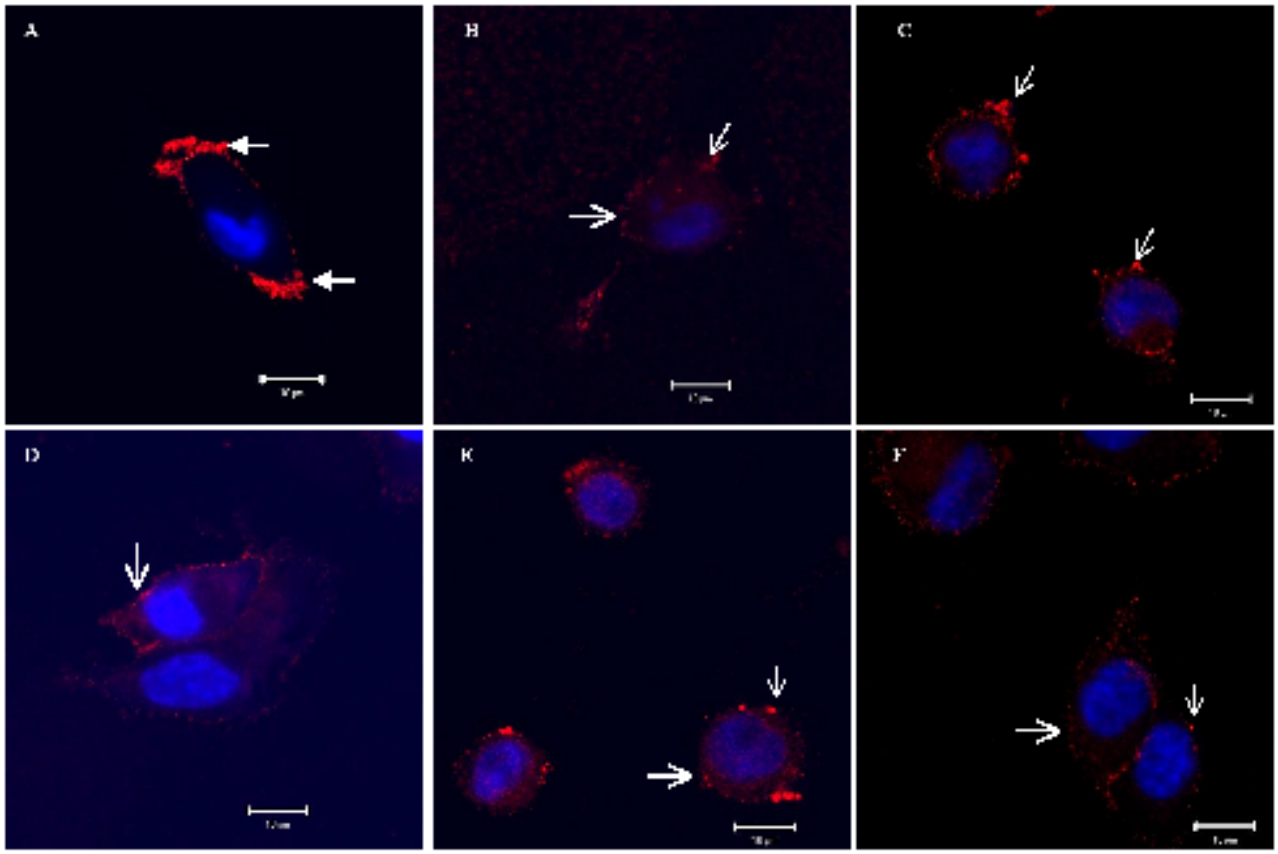


Figure 4.14. Extracellular immunolabeling of MT1-MMP.
Three antibodies raised in chickens to various domains of the MT1-MMP protein structure were employed to immunolocalize MT1-MMP in live, non-permeabilized cells. Anti-ProCat (A,D) anti-PexCat (B,E) and anti-Pex (C,F). (Ruffling border - closed arrow; labeling away from the leading front, along the PM – open arrow; site of possible internalization – thin arrow.) (Bars = 10 μm)

The discrepancy in immunolabeling of effectively the same protein, using antibodies raised to various domains of the protein, could possibly also be explained by the presence of glycosylation of MT1-MMP in its intact, active form. Such glycosylation could be inhibiting immunolabeling of the Pex region of active, intact molecules, while low levels of glycosylation was reported to allow the cleavage of the catalytic site from the Pex to cytoplasmic domains, just above the hinge region. It is possible that the non-glycosylated Pex domain of the autocatalysed 45 kDa form of MT1-MMP is easily recognized by the antibody, while it hinders recognition of the native molecule. The clusters of enhanced MT1-MMP labeling next to the nucleus, shown by immunolabeling with both the anti-PexCat and anti-Pex antibodies (Figure 4.13 C

and Figure 4.14 C) could indicate the presence of all the processing forms of MT1-MMP, i.e. the intermediately processed 63 kDa form, the mature 58 kDa enzyme, as well as the autocatalytically degraded 45 kDa protein, since all these forms may be recognized by the anti-Pex domain antibodies. However, since these two antibodies may mainly label the Pex domain, it will probably label the majority of the MT1-MMP proteins that are internalized for degradation, and may explain why labeling is found at focal adhesion sites at the rear of the cell during migration. During release of the rear of the cell, proteins involved in FAs are moved along the PM from the rear towards the nuclear area and are removed from the PM in a gradient-dependent manner, assisting in establishing polarity (Ballestrem *et al.*, 2001). MT1-MMP, through its Pex domain, associates tightly with the tetraspan CD151 (Liu *et al.*, 2007) and its associated partner αvβ1 integrin in endothelial cells (Sincock *et al.*, 1997). This association with CD151 stimulates its endocytosis together with integrins, and presumably thus limits further proteolytic degradation of collagen (Yanez-Mo *et al.*, 2008). On the basis of this information we would speculate that the association and endocytosis of MT1-MMP together with integrins and later the integrin partner in lipid rafts, CD151, could possibly also remove MT1-MMP from the PM. This led us to believe that these restricted areas next to the nucleus may be the sites of internalization of degraded or denatured MT1-MMP in both the control and transfected cells. This is possibly why the anti-ProCat antibody did recognize these sites, as it may only recognize the Pro and Cat domains, which may be absent in denatured forms.

4.7. Final discussion – CB and MT1-MMP in migration and c-Ha-*ras*(V12)-related invasion

On growth stimulation, well-defined anterior-posterior polarity of the cell changes to form a leading front, and distribution of matrix adherence molecules and the cytoskeletal organization is altered to assist in adhesion at the front and contraction from the rear (Section 4.1.7.2).

The radially scattered small leading fronts observed in the c-Ha-*ras*(V12)-transfected MCF10AneoT cells, was similar to those observed in monolayers of migrating, Ha-*ras*(V12)-transfected NIH-3T3 fibroblasts (Munevar *et al.*, 2001). This is in contrast to the well-defined anterior-posterior polarization of the normal MCF10A cells, and may lead to inefficient migration due to aberrant tracking along the underlying matrix.

While these processes are important in the mechanism of migration, focusing proteolytic activity at the front is important during the normal process, but when this process becomes unrestrained, it seems to assist in pathological invasion by cancer cells. Transfected immortal cells that express the c-Ha-Ras(V12) oncoprotein (the MCF10AneoT cell line), form cancers due to uncontrolled proliferation, and are also invasive, compared to their untransfected control counterparts (the MCF10A cell line) (Basolo *et al.*, 1991). The invasive ability of tumour cells is dependent on both their ability to constantly migrate and elevated activity of proteases at the cell surface. Elevated levels of CB (Sameni *et al.*, 1995; Sinha *et al.*, 1998; Hirai *et al.*, 1999; Dohchin *et al.*, 2000; Szpaderska *et al.*, 2001; Zajc *et al.*, 2003; Troy *et al.*, 2004; Sloane *et al.*, 2005) as well as for MT1-MMP (Hotary *et al.*, 2006; Itoh *et al.*, 2006a; Rozanov *et al.*, 2006; Cao *et al.*, 2008; Hotary *et al.*, 2008; Nishida *et al.*, 2008) are often reported in transformed cells and cancers. Both these proteases have been shown to be actively involved in the tumorigenic degradation of the ECM, such as in the MCF10AneoT c-Ha-*ras*(V12)-transfected breast epithelial cells and thus were the proteases of interest in this part of the study. While the mechanical processes of extention and retraction that are involved in migration may be understood, the biochemical control of protease trafficking is, in contrast, not always appreciated. From our cumulative results it was clear that transcription of MT1-MMP was increased following c-Ha-*ras*(V12) transfection (Figure 4.13). It is known, though, that both CB and MT1-MMP exerts its catalytic activity when expressed on the PM. As a result, we immunolabelled PM-associated MT1-MMP, using antibodies to different domains, as well as mature CB in order to investigate the relationship between c-Ha-Ras(V12) signaling (Figure 1.3 on fold-out) and the invasive behaviour of these cells.

4.7.1. Factors that retain MT1-MMP on PM

In contrast to secreted MMPs, the transmembrane domain of MT1-MMP restricts it to the PM (Folgueras *et al.*, 2004; Sabeh *et al.*, 2009). This focuses proteolytic activity to the pericellular microenvironment. Physiological regulation of the catalytic function of MT1-MMP is attained by factors such as association with its endogeneous inhibitors, the TIMPs (Sato *et al.*, 1996) and other mechanisms (Section 4.6.1). It includes clearance from lipid rafts on the PM due to palmitoylation of a residue on its cytoplasmic domain (Section 4.1.5.1). While the original stimulus that either directs

MT1-MMP back to the PM, or allows it to be degraded (Figure 4.7 B,C), is unsure, it is known that phosphorylation of the residue next to the palmitoylation site, interferes with internalization and retains the enzyme on the PM (Section 4.1.5.2). Such phosphorylated MT1-MMP is proteolytically active and it is concentrated to the leading front in control (Labrecque *et al.*, 2004) and invasive, non-c-Ha-*ras*(V12)-related cancer cells (Nyalendo *et al.*, 2007), where it may be involved in the proteolytic clearance of the BM or ECM during migration or invasion (Section 4.1.5.2). On the contrary, impaired phosphorylation of MT1-MMP reduces proliferation and growth of tumour cells (Nyalendo *et al.*, 2008). We propose that such reversible phosphorylation, known as a phosphorylation gate, serves as a control of MT1-MMP function, as it has been described for many other regulatory proteins (Dietrich *et al.*, 2004; Tian *et al.*, 2008). Additional support for this theory is that proteolytic activity of MT1-MMP is inhibited by MTCBP-1, since it binds to the MT1-MMP cytoplasmic tail and possibly inhibits phosphorylation and proteolytic activity, but still allows retention of MT1-MMP on the PM. Interestingly, MTCBP-1 is downregulated in invasive cancer (Uekita *et al.*, 2004), supporting opposing functions for either the proteolytic activity or internalization of MT1-MMP. In the following discussion we will attempt to correlate retention of proteolytic activity and its abbarent distribution on the PM, with downstream signaling of c-Ha-Ras(V12) and the connection with phosphorylation of the MT1-MMP cytoplasmic tail.

It is our theory that several factors known to elevate the MT1-MMP activity on the PM indirectly affect phosphorylation of the cytoplasmic tail. MT1-MMP surface levels are elevated by EGF (Ouyang *et al.*, 2008a) and hypoxic conditions in the microenvironment of the cell (Munoz-Najar *et al.*, 2005), both proliferative signals that possibly signal via Ras. Such signals promote presentation of proteolytically active MT1-MMP to newly formed anterior membrane ruffles (Deryugina *et al.*, 2001; Gingras *et al.*, 2008). Collagen-I stimulates such enzyme production and transport to the PM (Huber *et al.*, 1993; Zhuge *et al.*, 2001; Bravo-Cordero *et al.*, 2007), possibly since collagen-I inhibits endocytosis of MT1-MMP and thus its clearance from the PM (Lafleur *et al.*, 2005). In this way it may assist in the invasiveness of melanomas (Kazes *et al.*, 1998). Restraint of endocytosis via inhibition of dynamin (Section 3.2.3) due to migration-related FAK signaling, also retains MT1-MMP on the PM. As a result, it may allow pro-MMP-2 maturation by MT1-MMP (Siesser *et al.*, 2006). These factors may enhance proteolytic degradation

of the BM at the leading front during migration, while internalization of MT1-MMP from the PM could represent a rapid response mechanism used by the cell for representing active MT1-MMP at the leading edge during migration (Remacle *et al.*, 2003). In contrast, low levels of matrix or substrate may allow MT1-MMP to be autocatalytically cleaved (Osenkowski *et al.*, 2005), suggesting a control measure for enzymatic activity, e.g. at the rear of the cell. The cleaved 45 kDa membrane-bound processing form of MT1-MMP is catalytically inactive, but may interfere with further native collagen-1 degradation (Tam *et al.*, 2002). This processed form may be internalized into an EE and subsequently degraded (Figure 4.7 C).

No specific position along the PM for such internalization was mentioned in any previously published work. We believe, however, as discussed in Section 4.6.1, that the sites lateral to the nucleus on the PM, which immunolabelled with the two antibodies that recognize the Pex domain of MT1-MMP, may indicate a site of internalization of MT1-MMP. In this way in could be assisting in controlled removal of this protease from the PM in an effort to reduce proteolytic activity on the membrane at positions other than the leading front.

4.7.2. Distribution of MT1-MMP related to its PM binding partners, related to cell-cell or cell-matrix contact. Formation of a leading front during migration

The anterior-posterior distribution of MT1-MMP noted in our control or normal, non-permeabilized MCF10A cells (Figure 4.14) seems to be related to its association with, and the trafficking of, its membrane-bound partners. These partners, such as CD44, integrins and cadherins, are mostly involved in cell-matrix or cell-cell contact, respectively. We did note from the literature, though, that trafficking of all these proteins is under the control of the signaling cascade that initiates the formation of the leading front. It is then important to appreciate the process of migration as a temporally and spatially regulated event (Moissoglu *et al.*, 2006). Migration is associated with initial rapid forward membrane extension, formation of firm cell-matrix adhesions and localized clearance of the ECM of the leading front. Formation and contraction of cell spanning stress fibers, with disassembly of FAs and cleavage of cell-matrix adhesion molecules at the opposite cellular pole, is subsequently required for effective forward migration (Hamadi *et al.*, 2005; Le Boeuf *et al.*, 2006; Pirone *et al.*, 2006). These actions require stimulus-related, polarized signaling by

growth receptors or chemotaxins. This may occur as a result of signaling via downstream activation of Ras, Rac, RhoA and a cascade of kinases along the PM (Alwan *et al.*, 2003; Moissoglu *et al.*, 2006; White *et al.*, 2006) to the adhesion and cytoskeletal elements along the PM (Figure 4.8 A and Figure 4.15 Lanes 1, 2). During this process the PI3K-ERK pathway related to growth and transcription, is also triggered (Figure 4.15).

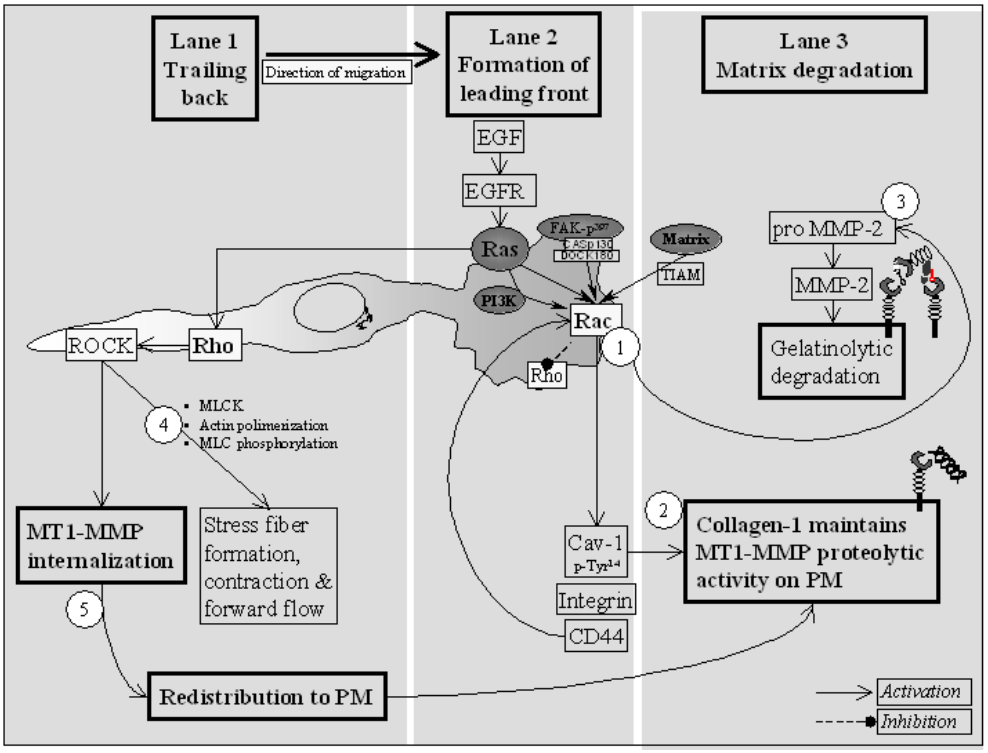


Figure 4.15 Rac and Rho affects MT1-MMP differently at the front and rear of the normal cell.

At the position of the cell where a leading front is formed, due to e.g. EGF stimulation (Lane 2), Rac is activated (1) by Ras-GTP, phosphorylated FAK as well as by a matrix-related Ras GEF, TIAM (Lane 3). The association of CD44, integrins and phosphorylated cav-1 with phosphorylated MT1-MMP, leads to collagen degradation (2), proMMP-2 maturation (3) and gelatinolytic degradation (Lane 3). While Rho is unidirectionally inhibited by activated Rac at the leading front (Lane 2), such inhibition is lifted at the rear of the cell (Lane 1), allowing actin-myosin stress fiber extention and contraction (4), clearance of MT1-MMP from the PM and possible redistribution to the PM (5) for continued proteolysis (Lane 1). (This diagram was generated to illustrate conclusions drawn from references mentioned in this chapter.)

In normal cells the association of proteases, such as MT1-MMP with adhesion molecules such as CD44, limit activity to the leading edges, ensuring polarity and specifically directing the cell towards a stimulus (Hofmann *et al.*, 2000; Deryugina *et al.*, 2001) and inducing EMT phenotypic changes, including cell elongation (Cao *et al.*, 2008).

The maturation of cell receptors including αvβ3 integrins at the leading front, may be an important function of MT1-MMP in migrating cells (Ratnikov *et al.*, 2002), since cleavage of the pro-αv subunit at the invasive invadopodia enhances the binding property of the integrin pair, and, as a result, improves traction. During this maturation process the inhibitory association of the pro-αv subunit on the β-chain may be released (Takagi *et al.*, 2002), allowing enhanced Tyr-397 autophosphorylation of FAK (Figure 4.5 Lane 1 and Figure 4. 15 Lane 2), a process that supports both adhesion and migration of cells (Deryugina *et al.*, 2002). A specific function of integrin αvβ8 is to attract latent TGF-β to the cell surface, where it may be activated by MT1-MMP, leading to cell growth and matrix production (Mu *et al.*, 2002; Illman *et al.*, 2006; Acharya *et al.*, 2008). In addition, at these sites MT1-MMP is associated with the FA complex and the adaptor protein p130Cas in the signaling cascade (Gingras *et al.*, 2008) (Section 4.1.4.2 and Figure 4. 15 Lane 2). In this way MT1-MMP, phosphorylated due to growth factor activity and formation of the leading front, may be involved in this signaling process to promote proliferation and migration (Section 4.1.4.2 and Figure 4. 15 Lane 2).

CD44 is another cell-matrix adhesion protein that binds both matrix and engages with cytoskeletal elements (Section 4.1.7.1 and Figure 4. 15 Lane 2). MT1-MMP is associated with CD44, phosphorylated at Ser-325 (Ponta *et al.*, 2003; Thorne *et al.*, 2004), at the leading front via its haemopexin domain (Kawano *et al.*, 2000b; Mori *et al.*, 2002), an association which may allow MMP-dependent activation of latent TGF-β, as described above. In addition, c-Ha-Ras(V12) upregulates a variably spliced form of CD44 (CD44v) that has an altered ectodomain (Hofmann *et al.*, 1993a) and that may, due to its ability to bind heparin sulphate, enhance EGF-related binding to its receptor (Figure 4.5 Lane 2 and Figure 4. 15 Lane 2). This process sustains late Ras signaling and possibly cell cycle progression, in a subsequent positive feedback loop (Cheng *et al.*, 2006) to enhance the strength of signaling during formation of the leading front. The importance of association of MT1-MMP with CD44 is that it may facilitate the relationship between MT1-MMP and cav-1 (Section 4.1.4.2 and Figure 4.5 Lane 2), and ultimately the phosphorylation of the MT1-MMP cytoplasmic tail by Src kinases, activated by Ha-Ras, or c-Ha-Ras(V12). This process is further discussed below. Once the cell has 'flowed' forward over the initial front area, the rear has to be released. Due to disengagement of the ERM proteins at the rear of the cell, and the

changed phosphorylation pattern of CD44 to Ser-291, which is in close vicinity to the PM (Thorne *et al.*, 2004), CD44 may now interact with MT1-MMP at its haemopexin-like domain (Marrero-Diaz *et al.*, 2008). It is subsequently cleaved and the ectodomain is endocytosed (Kawano *et al.*, 2000b). Therefore, the MT1-MMP-CD44 interaction now becomes one of shedding of the CD44 ectodomain, allowing release of the cell-matrix connection that promotes cell migration (Hofmann *et al.*, 1993a; Piccard *et al.*, 2007) (Section 4.1.7.1). Excessive cleavage of CD44 by ectopic MT1-MMP, on the other hand, may possibly lead to disruption of cell-matrix adhesion, especially since it was shown that loss of CD44 disrupts cytoskeletal organization and directional migration of cells, resulting in inefficient migration (Acharya *et al.*, 2008). The increased and ectopic expression of MT1-MMP over an area of the PM wider than the leading front may clearly have other negative impacts on the cell in addition to excessive BM degradation.

As part of the inhibition of migration on cell-cell contact, E-cadherin, via its intracellular signaling mechanisms, inhibits transcription of MT1-MMP (Nawrocki-Raby *et al.*, 2003). This may form the foundation of downregulation of protease expression during mesechymal-to-epithelial transition and differentiation. In cells that express elevated levels of MT1-MMP, however, this enzyme is shifted to cell-cell contacts, where it sheds E-cadherin (Cao *et al.*, 2008). Under conditions such as normal growth or during wound healing, and especially during ischaemia, this process leads to disruption of the adherent junctions (AJs) or reduced cell adhesion (Covington *et al.*, 2006) and may form part of the preparation of the cell for migration. Excessive proteolytic degradation of E-cadherin, on the other hand, may overcome the inhibitory control that cell-cell contact has over migration and proliferation (contact inhibition), and may be one of the early steps in transition from an epithelial cell to a polarized mesenchymal-type transformed cell (Cao *et al.*, 2008).

4.7.3. Altered polarity

Since anterior-posterior polarity of the cell is the result of distribution of adhesion and related protein complexes along this axis, the question is whether the altered distribution of MT1-MMP in the c-Ha-*ras*(V12)-transfected cells is related to the loss of polarization in general, of MCF10AneoT cells. Initial phosphorylation of MT1-MMP cytoplasmic tail (required for its proteolytic activity) is related to phosphorylation of cav-1 at the leading front (Gilles *et al.*, 2001; Nyalendo *et al.*,

2007). This event is usually associated with initiation of a signaling node by EGFR dimerizing due to ligand binding. The unexpected wide distribution of proteolytically active MT1-MMP in transformed cells, contrary to the normal, as illustrated in this study, and the known excessive levels of BM degradation by invasive cancer cells, has to be correlated to the effect of c-Ha-Ras(V12) on intracellular signalling components and control mechanisms (Figure 4.16). The advantage of our model set of cell lines was that expression of the mutationally activated c-Ha-*ras*(V12) was the single delineating factor between the control, non-invasive MCF10A cells and the invasive, premalignant MCF10AneoT cell line.

The mutated c-Ha-Ras(V12) oncoprotein is constantly active, and was shown to not be bound to cav-1 (Song *et al.*, 1996), and does not require to be membrane-bound for it to be active (Joneson *et al.*, 1999). In addition, c-Ha-Ras(V12) downregulates cav-1. We speculated that the more random distribution of the constantly active c-Ha-Ras(V12) may result in the activation of cav-1 not specifically at the front, as is reported in control cells, but rather at random sites over the plasma membrane. This may, in turn, allow phosphorylation of MT1-MMP and subsequent ECM degradation to be widely distributed over the PM. So, due to a lack of cav-1, inactive Src may exhibit altered subcellular localization (Sotgia *et al.*, 2002). Therefore, in cells that lack cav-1, active palmitoylated Ha-Ras may be targeted to PM glycosylphosphatidyl inositol (PI), but not necessarily to lipid rafts. In some breast cancers PAK, a signaling protein downstream from Ras-Rac (Figure 1.3 on fold-out), is also constitutively active and is misdirected to not bind to FA, thus allowing FA turnover and cell migration (Stofega *et al.*, 2004).

Cav-1 plays an important controlling role in intracellular signaling. It binds to inactive signaling proteins such as GTPases (e.g. Rac) and kinases (e.g. Src) (Li *et al.*, 1996; Ponta *et al.*, 2003) and palmitoylated, normal inactive Ha-Ras (Song *et al.*, 1996; Baran *et al.*, 2007). This interaction may inhibit the kinase activity of the ERK-MAPK downstream Ras cascade in an anti-proliferative, tumour-suppressing manner (Engelman *et al.*, 1998b). In addition, cav-1 compartmentalizes these signaling cascades downstream from Ras, to caveolae along the PM (Venkatesha *et al.*, 2006). Activated, phosphorylated cav-1 no longer binds these signaling proteins, making it part of the formation of the leading front. Since MT1-MMP is associated with Src-activated cav-1 via its cytoplasmic tail (Labrecque *et al.*, 2004; Navarro *et al.*, 2004),

alterations in the expression of cav-1 may also affect the activation and trafficking of MT1-MMP. Loss of cav-1 expression, as seen in loss of caveolae in this study, may alter intracellular signaling, an effect that may be due to mislocalization of signaling proteins, such as c-Ha-Ras(V12) (Baran *et al.*, 2007).

Mutated c-Ha-Ras(V12) is transcribed with a valine residue that has replaced a glycine, leading to an altered conformation, which occurs in the normal protein only due to GTP loading during growth-related activation. Inactive normal Ha-Ras is associated with caveolae, which is activated on stimulus from growth factors, and result in the formation of a leading edge. The mutated c-Ha-Ras(V12) protein is constitutively activated and even though the mutated protein is palmitoylated, it is suggested to no longer require incorporation into lipid-rich caveolae prior to activation. Therefore, the mutationally activated c-Ha-Ras(V12) oncoprotein may possibly signal to these downstream effectors, from sites distributed over the whole of the PM, and such signaling no longer needs to be limited to caveolae, or a leading or invasive front. Constant signaling from c-Ha-Ras(V12) could theoretically activate Src and cav-1 and also, therefore, MT1-MMP (Section 4.1.6), over a wider area of the plasma membrane. In addition, downregulation of cav-1 as reported in immortal c-Ha-Ras(V12)-containing mouse embryonic fibroblasts (Williams *et al.*, 2005), and observed in our c-Ha-*ras*(V12) transfected MCF10AneoT cancer cells (Figure 4.10), possibly compromising caveolin-related anti-tumorigenicity. This may be due to a limited number of docking sites for inactive kinases (Williams *et al.*, 2005; Venkatesha *et al.*, 2006) and may, in principle, allow increased signaling downstream of mutated Ras. In this study it was noted that MT1-MMP was widely distributed over the PM with few leading edge focal sites, and small focal areas seen laterally to the nucleus (Figure 4.16 Lane 3). This pattern correlated with the degree of BM degradation by the invasive MCF10AneoT cells that was of such a nature that these cancer cells could easily migrate through this intact barrier. The related ectopic distribution of active MT1-MMP (Figure 4.14 D and Figure 4.16 Lane 3) may assist in degradation of initially the underlying BM and later components of deeper ECM layers, as the cell invades into these layers. The altered pattern of MT1-MMP PM distribution could be an indication that cav-1, which sequesters phosphoryolated MT1-MMP to the leading edge (Labrecque *et al.*, 2004) and induces tumour cell proliferation (Nyalendo *et al.*, 2007), could also be more randomly distributed. Our results did indicate that the c-Ha-Ras(V12) transfected cells had fewer caveolae

(Figure 4.10) confirming that c-Ha-Ras(V12) downregulates cav-1 (Song *et al.*, 1996; Williams *et al.*, 2005).

Both normal and oncogenic c-Ha-Ras(V12) specifically affect growth and migration (Teramoto *et al.*, 2003) (Section 1.2 to Section 5, Figure 1.3 on fold-out). We, therefore, attempted to correlate the effect of this mutation and its downstream signaling effectors associated with proliferation and migration, e.g. Rac and Rho-ROCK, on the distribution of CB and MT1-MMP, or their PM-related binding partners.

During a normal cellular response, these processes are under the control of the Ras growth signaling switch and its downstream effectors. Normal Ras, activated at the site of external stimulation, spatially restricts activation of Rac-dependent actin polymerization and the formation of a ruffling border at the leading front of the migrating cells (Cau *et al.*, 2005). Rac is also activated by T-lymphoma invasion and metastasis inducing protein (TIAM), a matrix-related calcium-dependent Rac activator (Van Aelst *et al.*, 1997; Zondag *et al.*, 2000) (Figure 4.15 Lane 2). Rac may also be activated from the FAs by FAK-DOCK180 signaling (Côté *et al.*, 2005) (Figure 4.15 Lane 2). Activated Rac increases levels of MT1-MMP expression on the PM (Zhuge *et al.*, 2001) (Figure 4.15 Lane 2 and Figure 4.16 Lane 1) and retains MT1-MMP extracellular proteolytic activity at the leading edge by inhibiting its internalization. In contrast, at the rear, downregulation of intracellular Rac activation, may allow Rho and downstream ROCK activation (Sander *et al.*, 1999) (Figure 4.15 Lane 1 and Figure 4.16 Lane 1. This, in turn, may be involved in the formation of stress fibers that pull the cell forward, as well as the internalization of MT1-MMP (Galvez *et al.*, 2004) (Figure 4.15 Lane 1 and Figure 4.16 Lane 1). These findings further strengthen our suggestion that the internalization site for MT1-MMP is to the rear of the cell.

4.7.4. CB associated with the PM in invasion

While phosphorylated, proteolytically active MT1-MMP seems to be the main protease involved in the invasive behaviour of c-Ha-*ras*(V12)-transfected cells, CB, which was originally found to be a lysosomal cysteine protease, is also an important matrix-digesting protease. During normal migration CB may be directed to caveolae at the leading front, together with its partners, e.g. components of the uPa cascade

enzymes and their precursors (Cavallo-Medved *et al.*, 2005; Sloane *et al.*, 2005), and NHE-1 (Figure 4.16 Lane 2). NHE-1, stimulated by c-Ha-Ras(V12)-PI3K signaling (Reshkin *et al.*, 2000a; Cardone *et al.*, 2005) (Figure 4.16 Lane 1), progressively acidifies and optimizes the local extracellular microenvironment (Bourguignon *et al.*, 2004; Cardone *et al.*, 2005) to support ECM degradation by elevated CB (Figure 4.16 Lane 2). At the same time NHE-1 induces cytoplasmic alkalinization (Reshkin *et al.*, 2000b; Haworth *et al.*, 2003) and cytoskeletal changes involving reciprocal RhoA and Rac1 signaling (Figure 4.16 Lane 1), factors that promote motility and invasion (Paradiso *et al.*, 2004). Under these conditions, extracellular CB assists in BM and ECM degradation. In addition, it has been reported that extracellular CB degrades TIMPs, the endogeneous inhibitors of MMPs (Kostoulas *et al.*, 1999).

Therefore, control of MT1-MMP distribution and activation seems to be closely associated with Ha-Ras activation (Figure 4.16 Lane 1). This is possibly in contrast to the factors that control the distribution of CB.

The expression of proteases such as cysteine cathepsins, plasminogen and metalloproteases is an important part of epithelial migration in e.g. wound healing (Lund *et al.*, 1999; Buth *et al.*, 2007) and in cancer cell invasion and relies on the expression of proteases in migrating cells. However, consistant, ectopic expression on the PM due to constitutive signalling from mutationally activated c-Ha-Ras(V12) (Figure 4.16 Lane 3), may lead to cleavage of adhesion molecules such as CD44 and E-cadherin, that may increase the efficiency of migration, and inhibit growth and migration on cell-cell contact. Taken together, these findings support the theory that migrating or invading cells initially retain MT1-MMP at the plasma membrane in order to support the extracellular proteolytic degradation of the ECM. Cells that have been immortalized due to *9p21* deletion, such as the MCF10A breast epithelial cells, may merely become hyperproliferative, but do not form cancers, unless further mutations occur. With introduction of the c-Ha-*ras*(V12) oncogene, however, such as with the MCF10AneoT transformed cells, enzymes such as CB and MT1-MMP are upregulated, and control over migration is possibly lost, all due to a lack of negative feedback mechanisms from p53 to Ha-Ras. We suggest, therefore, that the retention of MT1-MMP on the PM and its wider distribution in transformed cells expressing the c-Ha-Ras(V12) oncoprotein (Figure 4.14 and Figure 4.16 Lane 3) allows degradation

of ECM not only at the leading edge, but over a much wider area of the PM (Figure 4.16 Lane 3). Such constant, and possibly excessive proteolysis of the underlying BM and later, of the ECM, possibly facilitates invasion in c-Ha-*ras*(V12)-induced cancers.

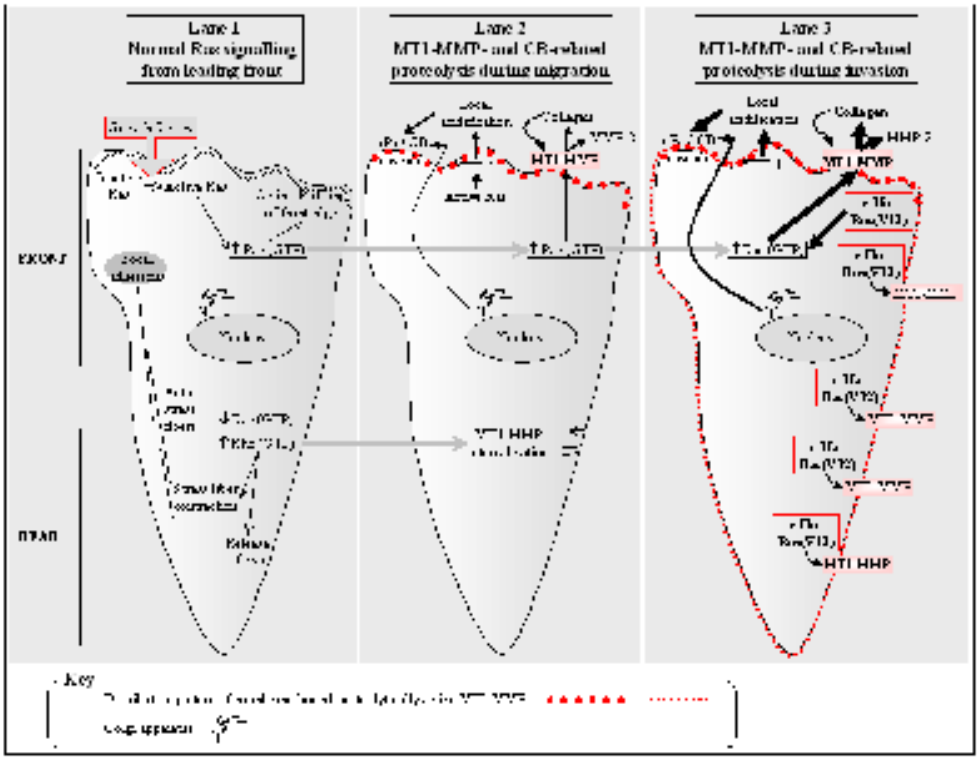


Figure 4.16. Ras signaling and MT1-MMP proteolytic activity in control and transformed cells.

This illustration is an extention of Figure 4.8, with additional illustration of the possible effect of c-Ha-Ras(V12) signaling on CB and MT1-MMP distribution. In the control cell the activation of Ras-Rac signaling by growth factors results in the formation of a leading front (Lane 1) and localized proteolytic activity by e.g. CB and MT1-MMP (Lane 2). The release of the rear and internalization of MT1-MMP in this vacinity allows migration. Altered PM distribution of the constitutively active c-Ha-Ras(V12) oncoprotein may allow retention of proteolytically active MT1-MMP over a wide area of the cell surface (Lane 3), facilitating increased degradation of the BM and ECM, that supports invasion.

4.8. Conclusion

The link between membrane proteases, adhesion proteins and intracellular signaling systems is key in understanding the role and regulation of ECM proteolysis in cell migration. However, considering the importance of MT1-MMP catalytic activity in invasion, the role that phosphorylation of its cytoplasmic tail and its association with adhesion and signaling molecules plays in polarity, has mostly been overlooked. The intracellular domain is involved under stimulatory conditions, in the activation of the molecule localized in the vicinity of stimulation, and/or its trafficking to sites on the

PM where catalytic activity is required. Its internalization may be due to its dephosphorylation, subsequent palmitoylation, after which it may be recycling to the PM, or degraded.

The c-Ha-Ras(V12) mutation could also limit the amount of cav-1 presented on the PM, thus changing the docking area for inactive kinases, as well as for MT1-MMP. Conditions in cancer cells that may be responsible for the wide distribution of proteolytically active MT1-MMP over the cell membrane, may include the downregulation of cav-1 and elevated levels of CDC42, especially due to a lack of control due to constant removal of p53 by MDM-2 (Section 1.8).

On the other hand, trafficking of CB to the invasion front may take advantage of the acidic and, hence favourable conditions for CB activity and its role in invasion, created by the sequestered NHE-1 proton pump.

In normal cells inactive Ras is located with cav-1. In stimulated cells activation of Ras may be restricted to the leading front, where it forms part of the downstream signaling from growth factor receptors. Activated cav-1 and Src has been colocalized with phosphorylated MT1-MMP to these sites, an action that promotes proteolytic degradation of the ECM at the leading edge.

In contrast, the mutated, constantly active c-Ha-Ras(V12) may be active over the whole of the PM, since it is not bound to cav-1. It may, therefore, allow the continuous phosphorylation of MT1-MMP cytoplasmic tail by Src kinases, and consequently its retention on the PM and subsequent degradation of the ECM over a wider area.

Due to the altered distribution of CB and MT1-MMP degradation of the ECM possibly occurs not only at the leading edges but over the whole PM. This possibly disturbs the integrity of the PM to such an extent that the cell migrate through with more ease than proteolytic removal of the ECM only at the leading edges. These events may be the underlying factors in the widespread that support escape from the original tumour.

In addition, the mutated Ras(V12) could assist in EMT due to increased CD44 production and possibly its cleavage by elevated levels of MT1-MMP and continued formation of stress fibers by the elevated levels of Rho-ROCK.

The establishment of polarity, that regulates the cell's ability to migrate in a specified direction, is a combination of these two functions – proteolytic degradation and the gradient in signaling intensity. The mutated c-Ha-Ras(V12) oncoprotein, therefore, elevates membrane association and secretion of proteases, compromizes the cell's polarity, promotes EMT-like changes and ECM degradation and thus facilitates invasion.

CB and MT1-MMP are both proteases that have been implicated in the metastatic cascade of migrating cancer cells. Since CB has been reported to be located to the leading edge of the invasive gliomas (Rempel *et al.*, 1994), and MT1-MMP is known to play different roles at opposite poles of the migrating cell, the distribution of these molecules on monolayers could provide a base for the understanding their expression, trafficking and specific localization on the PM. In future this information could be used to predict events that could occur when these cells are grown on various ECM components as well as in a 3D model.

CHAPTER 5.

Overview

5.1. Ras in cancer

The p53 and p16 tumour suppressors (Sharpless *et al.*, 2004) are vital parts of the inhibitory feedback to Ras signaling (Giglione *et al.*, 1998; Katunuma *et al.*, 1998; Bulavin *et al.*, 2003; Erster *et al.*, 2004) (Section 1.8). Low expression levels of a constitutively active Ras may merely induce hyperproliferation (Mo *et al.*, 2007), as will a silenced p53 system in normal cells (Russo *et al.*, 1991; Gao *et al.*, 2004), as found in the MCF10A cell line due to a *9p21* deletion (Brenner *et al.*, 1995; Rao *et al.*, 2006). Overexpression of Ha-Ras leads to apoptosis (Joneson *et al.*, 1999; Gao *et al.*, 2004), possibly due to a concurrent elevation of p53 levels which counteracts tumour growth (Lin *et al.*, 1998; Vousden, 2000; Gao *et al.*, 2004). In immortal cells lacking p16, p53 or its upstream regulators, activated oncogenic c-Ha-Ras(V12) forces uncontrolled mitogenesis and transformation (Lin *et al.*, 1998), with cells unable to go into apoptosis (Bulavin *et al.*, 2003). This may allow oncogenic mutations of a second gene, such as *ras*, to go undetected and unchecked, resulting in immortality, malignant progression and uncontrolled proliferation. c-Ha-Ras(V12) implicated in the transformation of epithelial cells (Russo *et al.*, 1991; Kanda *et al.*, 1993) is more specifically related to invasiveness of breast cancers (Ward *et al.*, 2001; Kim *et al.*, 2003; Shin *et al.*, 2005). About 50% to 60% of breast cancers (Elenbaas *et al.*, 2001; Houle *et al.*, 2006) have an ineffective p53 senescence system as an underlying initial genetic alteration and thus acquire immortality (Santos *et al.*, 1989). Ha-Ras(V12) mutations, in combination with defective p16/p53 signaling, may potentially result in an immortal, invasive cancer and malignant progression (Miller, 2000). This was demonstrated in transformed MCF10AneoT cells used in this study (Russo *et al.*, 1991; Miller, 2000), as well as in cancer of the bladder (Gao *et al.*, 2004) and breast (Houle *et al.*, 2006).

The focus of this study was to investigate the relevance of proteases in metastatic cancer, and the role of mutated c-Ha-Ras(V12) in events that may lead to aberrant protease trafficking and membrane-association in migration, growth and invasive ability.

5.1.1. The unique model system used in this study

Alterations giving rise to invasive breast cancer may be studied in primary human mammary epithelial cells (HMECs) through the introduction of known mutations (Elenbaas *et al.*, 2001). While almost all commercially available breast cell lines are derived from cancers, the MCF10A cell line is part of a series of human mammary epithelial cell lines that was derived from a non-malignant growth from a young woman with fibrocystic disease. Transfection with c-Ha-*ras*(V12) in addition to a loss of function of p53 [due to a *9p21* deletion (Section 1.8)] has given rise to a sequence of histological changes via xenografts in nude mice, that resembles various stages of progression in breast cancer, from infiltrating duct carcinoma to invasive carcinomas (Ochieng *et al.*, 1991; Miller *et al.*, 1993; Miller, 2000; Polyak, 2001). The uniqueness of this set of cell lines is that it has the same genetic background and thus may allow the effect of a single oncogene, e.g. c-Ha-*ras*(V12), to be comparatively studied in an immortal, p16/p14 deficient, but phenotypically 'normal' parental MCF10A cell line. Three-dimensional (3D) cultures would better represent what occurs *in vivo*, but may be difficult to interpret due to the diversity of influences from cell-cell or cell-matrix contact, or other environmental influences found *in vivo*. It is also known that various individual BM and ECM components differently influence cellular responses such as proliferation, adhesion, polarity and acini formation (Hu *et al.*, 2008; Hattar *et al.*, 2009; Schnitt, 2009) (Section 5.1.2). In order to limit the number of variables and inputs in this study, these cells were, therefore, plated onto uncoated coverslips and cultured as monolayers, with cells allowed to put down their own BM, even though it is most probably not what will be found in the body.

In the current study we wanted to establish whether (1) the altered intracellular trafficking of cathepsins known to be elevated in invasive cancer patients, i.e. CB, CL and CD (Lah *et al.*, 2000), is as a result of alkalinization of the degradative endosomes (Jackson *et al.*, 1999) (Appendix II), and/or as a result of c-Ha-Ras(V12) and its downstream effectors (Section 3.8.1.6) and are illustrated in Figure 5.1. To summarize, c-Ha-Ras(V12) activates CD44 and NHE-1, both increasing the acidity of the extracellular microenvironment, that in turn may support the role of CB in activation of uPa. Downstream signalling by c-Ha-Ras(V12) via PI3K also increases MT1-MMP and other MMP expression on the PM, that, together with the CB cascade, elevates BM and ECM degradation. Elevated calcium, together with

increased levels of ROCK and cytoplasmic alkalinization may cooperate to affect the cytoskeletal organization, that in turn, may change vesicle distribution. In combination, these factors support an altered cell shape, vesicle distribution and invasion due to altered proteolytic activity.

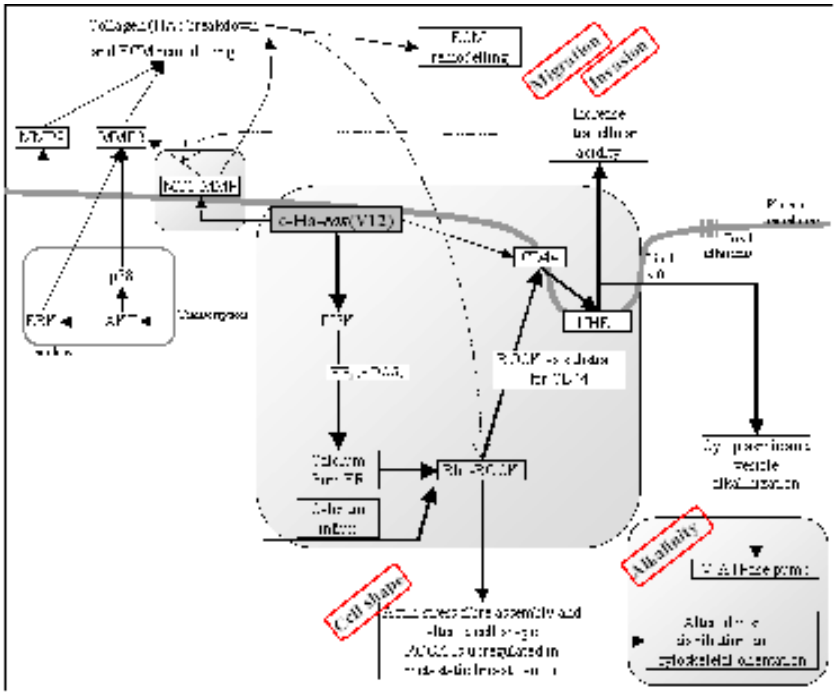


Figure 5.1 A general description of the effect of c-Ha-Ras(V12) on the cell.
Constant c-Ha-Ras(V12) signaling influences cell shape, cytoplasmic and degradative vesicle alkalinity, as well as migration and invasion due to secretion and maturation of proteases that degrade components of the BM.

These c-Ha-Ras(V12)-related effects are comparable to the influence of chemical treatments on vesicle maturation and migration, as described in Figure 5.2. In fibroblasts c-Ha-Ras(V12) has been shown to activate NHE-1, the proton pump normally activated by cytoplasmic acidosis (Haworth *et al.*, 2003). The resultant cytoplasmic alkalinization (Ritter *et al.*, 1997) may be mimicked by NH_4Cl treatment (Figure 5.2, Lane 2). Depolymerization of the cytoskeleton by nocodazole produces similar cell spreading and peripheral 'shift' of endocytic vesicles, and replicates calcium influx related to Ha-Ras signaling (Martinez *et al.*, 2000; Beatty *et al.*, 2001; Huynh *et al.*, 2005) (Figure 5.2, Lane 2). A similar trend was observed in our study on the mutated c-Ha-Ras(V12), suggesting that alkalinization of the cytoplasm affects cytoskeletal organization. In addition, alkalinization may indirectly and negatively influence assembly of the various components of the LE-related V-ATPase proton pump into a working unit, and thus impede acidification of these degradative vesicles, as shown in this study (Section 3.8.1.7). It is in these acidic degradative and

processing compartments where the cathepsins CB, CL and CD are matured and are optimally active. Changes to the acidity of these vesicles seem to lead to altered colocalization of CD and CB, and of CB and CL, as well as alterations in the size, location and cathepsin content of the JNS.

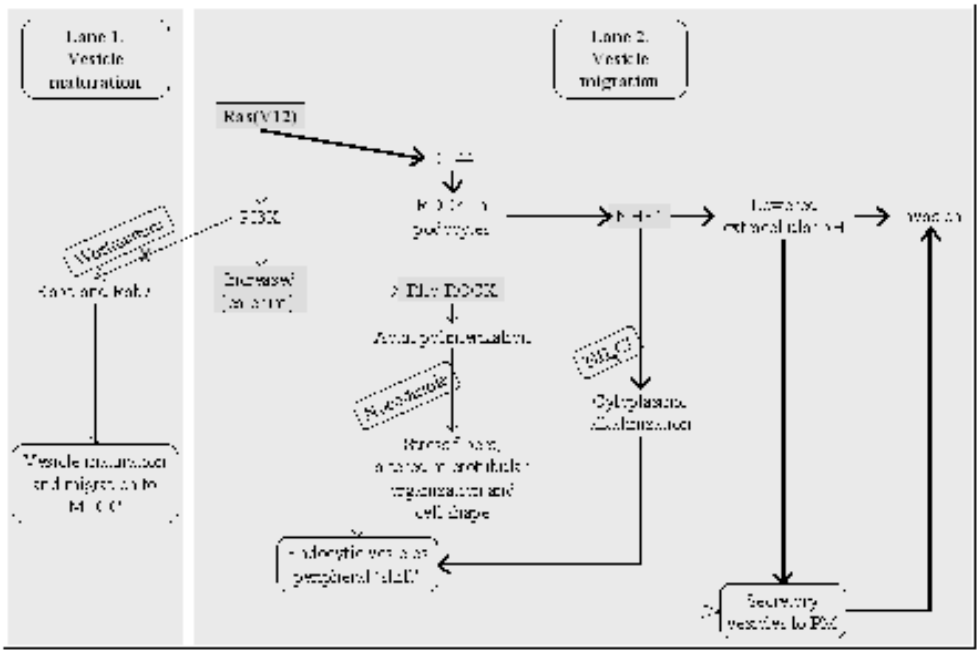


Figure 5.2. The effect of c-Ha-Ras(V12) and various drugs on endocytic vesicles. This figure illustrates interesting similarities between the effect of c-Ha-Ras(V12) downstream effectors and the influence of various drug treatments on vesicle maturation (Lane 1) and migration (Lane 2). Inhibition of Rab5 and Rab7 by wortmannin inhibits vesicle maturation, while the depolymerization of the cytoskeleton with either nocodazole or via alkalinization of the cytoplasm by NH_4Cl, may lead to the apparent redistribution of vesicles towards the cell periphery. This may be as a result of the altered cytoskeletal organization. c-Ha-Ras(V12) negatively affects vesicle acidification, an essential component of maturation.

The JNS in the vicinity of the MTOC is usually at the minus end of microtubules. Its position, acidity and CD content, was of interest as this is possibly the main acidic, degradative body of the cell (Section 3.6.3). In the transformed MCF10AneoT epithelial cells that have generally elevated LE pH (Jackson *et al.*, 1999) (Appendix II), the duplication and more pronounced nature of the JNS was seen as an indication of the mitotic stresses due to c-Ha-Ras(V12) and reduced control over intracellular organelle organization. Even so, it seems as if these cells have the ability to persistently maintain an acidic, but limited, CD-containing environment, suitable for high-grade degradation.

From the wider distribution of the PM-associated proteases, i.e. CB (Section 4.4) and MT1-MMP (Section 4.6) on the PM of MCF10AneoT cells, we deducted that

extracellular degradation and subsequent endocytosis of BM components (Sameni *et al.*, 2008) is increased along the whole of the BM, and possibly not only in the vicinity of the leading edges.

Constant proliferative signaling by c-Ha-Ras(V12) could be one of the main influences in elevated levels of CB and CL, since both are implicated in assisting the cell in preparation for entering S-phase. In proliferating cells active CB, noted in the nucleus (Mehtani *et al.*, 1998; Riccio *et al.*, 2001) may be an isoform transcribed without a signaling sequence that is related to proliferation of tumorigenesis (Mehtani *et al.*, 1998). Similarly, an isoform of CL without a regular signaling tail (Goulet *et al.*, 2004; Sansregret *et al.*, 2006) is involved in the proteolytic processing (Maitra *et al.*, 2006) of CCAAT-displacement protein (CDP), a nuclear transcription factor (Nepveu, 2001) that accelerates the G1-S phase transition (Sansregret *et al.*, 2006). CDP expression is significantly increased in high-grade carcinomas (Michl *et al.*, 2005) and it also upregulates Wnt5a (Michl *et al.*, 2005), which in turn is implicated in- and has in itself been shown to be responsible for MT1-MMP upregulation in cancer (Cao *et al.*, 2008). The relevance of Wnts in cancer progression will be further discussed in Section 5.1.3. CB and CL also seem to compensate for possible decrease in CD activity due to altered vesicle acidity.

The influence of immortality in many cells currently being used in studies on signaling effects downstream from oncoproteins, may be an important, but often disregarded factor, since it may have concealed influences on cellular response to stimulation.

5.1.2. The masked influence of immortality (loss of p16 or the *9p21* locus)

Most cell lines grown in culture have a pre-existing genetic defect. The spontaneously immortalized MDCK cell line (Mareel *et al.*, 2003) and MCF7 breast cancer cells, do not express endogeneous MT1-MMP (Rozanov *et al.*, 2004b), while the deficiency of MEF cells in the central regulating protein p38 (Bulavin *et al.*, 2003), or its downstream effectors (Gadea *et al.*, 2004) may negatively impact on p53 activity during senescence and apoptosis (Joneson *et al.*, 1999). The HCT116 and other colon cancer cells express mutated K-Ras known to stimulate proliferation, resulting in aggressive cancers (Bissonnette *et al.*, 2000; Caron *et al.*, 2005; Sloane *et al.*, 2005), compared to Ha-Ras of the MCF10A epithelial cell line. Information

deducted from these cells may not pertain to Ha-Ras-related downstream signaling, that is rather involved in migration and invasive cancer. This confirms the relevance of the downstream path for Ras proteins in general, that was assembled for this study (Figure 1.3 on fold-out). Using this figure, the activities of Ras, its downstream effectors and p53 could be correlated. Since it is known that p53 cooperates with oncogenic Ras to induce senescence (Alarcon-Vargas *et al.*, 2002; Ferbeyre *et al.*, 2002; Li *et al.*, 2007), and that many cancers develop after initial immortalization (Huschtscha *et al.*, 1998), the influence on cellular outcome of a constantly active c-Ha-Ras(V12) oncoprotein, in a cell that has a defective p16-p53 growth regulatory system (Section 1.8) could be better interpreted.

While Ras proteins in general may stimulate growth-related proliferation, the cell cycle is under the tight, complicated control of p16 and p53 (Pestell *et al.*, 1999; Holland, 2001; Tannapfel *et al.*, 2001) (Section 1.8 and Figure 1.3 on fold-out). The MCF10A cells used in the current study were immortalized due to complete deletion of the *p16ARF4A* locus (*9p21-/-*) (Brenner *et al.*, 1995; Rao *et al.*, 2006). This genetic alteration resulted in the associated loss of p16 protein control over CDKs, phosphorylation of Rb and the cell cycle (Brenner *et al.*, 1995; Cowell *et al.*, 2005). Simultaneous loss of the product of the *p14ARF* gene (Ruas *et al.*, 1998; Serrano, 2000) that usually inhibits MDM-2, may, however, result in constant removal of the normal wild-type p53 found in MCF10A cells (Zientek-Targosz *et al.*, 2008). The immortal MCF10A cells used in this study are, however, still diploid and reacted similarly to a normal epithelial cell, in terms of alterations to its cell shape and polarity during migration and contact inhibition of proliferation and may, therefore, be used as a non-invasive 'prototype'. Our focus was the cellular response of the immortal MCF10A cell line to transfection with the c-Ha-*ras*(V12) oncogene that resulted in an invasive phenotype, with prior knowledge of the existing limitations of this cell line, as discussed above.

In the normal cell upregulation of p53 levels initiates conclusion of proliferation and migration to return the cell to its epithelial phenotype, a function that is controlled via negative control of e.g. CDC42 (Cau *et al.*, 2005) and Rho (Xia *et al.*, 2007). These are both signaling proteins downstream from Ha-Ras, that contribute to cell polarity and cytoskeletal organization that support a migratory phase (Kawano *et al.*, 2000a). Lack of negative feedback from p53 may fail to control these functions, promoting an

irreversible state of EMT and migration, characteristics of invasive cancer. During normal and invasive migration CDC42 focuses Rac activity to the leading front of the cell (Nobes *et al.*, 1999) that modulates microtubule dynamics (Wittmann *et al.*, 2001) and formation of membrane filopodia (Gadéa *et al.*, 2002). It concentrates PAK activity to these membrane protrusions in a survival strategy (Cau *et al.*, 2005; Parsons *et al.*, 2005) that supports anchorage-independent survival (Nguyen *et al.*, 2002). During migration CDC42 also spatially orientates intracellular structures to the front of the cell, such as the Golgi (Nobes *et al.*, 1999), which, via Rab34, also influences the spatial orientation of the MTOC and distribution of e.g. the MPR-containing processing LE (Wang *et al.*, 2002). It was noted in this study that the JNS, normally positioned close to the nucleus and MTOC, was duplicated in proliferating MCF10AneoT cancer cells. This may be due to a combination of constant c-Ha-Ras(V12) proliferative signaling and constant unchecked CDC42 activity that may alter organelle orientation and vesicle trafficking. Rho signaling, together with CDC42 collaborate to maintain the stable polarization of a directionally migrating cell (Wittmann *et al.*, 2001) and lack of control may support invasive behaviour. Ha-Ras-related stimulation of CDC42 and Rac-1 may contribute to cleavage of CD44, that results in loss of cell-matrix adhesion.

In Figure 5.3 the degradation pattern of matrix underlying cultured MCF10A and MCF10AneoT cells is indicated (Fortgens, 1996). It shows the extent of matrix degradation in the c-Ha-*ras*(V12)-transfected cell line, compared to much smaller, and less pronounced areas of matrix digestion by the MCF10A cells. This may be an indication of the alteration in directional movement in MCF10AneoT, compared to that of the MCF10A cells. Such matrix digestion may be the function of CB and MT1-MMP, and possibly illustrate the suggested effect of the alteration in spread of these proteases on the PM, in terms of cell polarity and the formation of leading fronts (illustrated in Figure 4.9). It may also be an indication of the influence of Wnts signalling (Section 5.2) and persistent EMT-related changes post transfection.

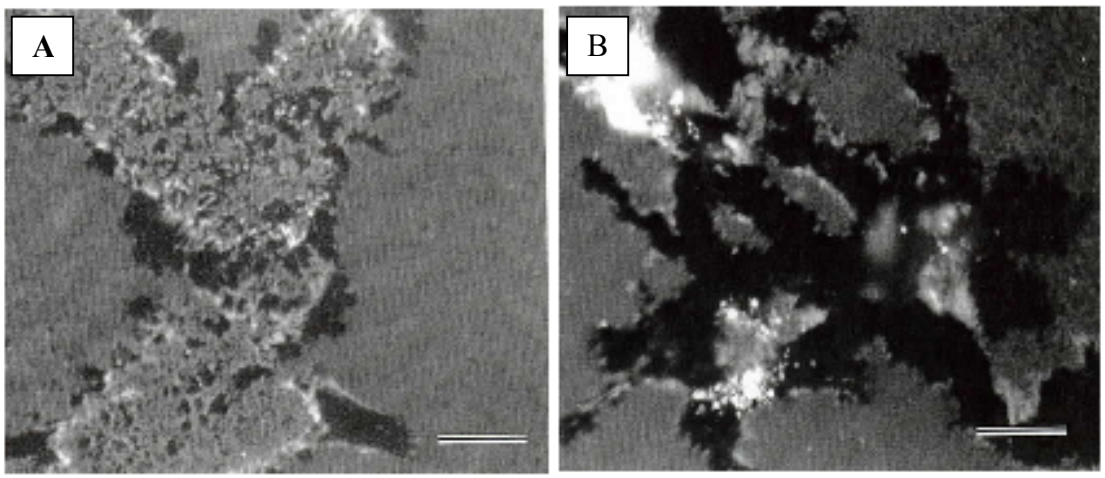


Figure 5.3. Comparative BM degradation by MCF10A and MCF10AneoT cells.

Compared to small areas of matrix digestion by normal MCF10A cells (A), c-Ha-*ras*(V12)-transfected MCF10AneoT cells show more pronounced and extensive degradation of underlying matrix (Fortgens, 1996).

5.2. EMT and proteolytic degradation during migration and invasion

EMT refers to the global alterations in cell activity during a migratory phase (Grille *et al.*, 2003). The process of EMT creates the integrated mechanism that facilitates the epithelial cell in changing its polarity and adopting a morphology, which includes extension of filopodia from the basal surface (Hay, 2005), supporting migration across the surrounding matrix (Vincent-Salomon *et al.*, 2003; Hay, 2005). During this process intracellular signaling is aimed at survival and resistance to differentiation. Migration-related EMT, such as during wound healing, involves the disassembly of cell-matrix contacts and reduced cell-cell contacts and AJs (Vincent-Salomon *et al.*, 2003). A central component of both migration and invasion is stimulus-related, focused degradation of the underlying matrix associated with the leading edges of the cell (Vincent-Salomon *et al.*, 2003; Hay, 2005; Christiansen *et al.*, 2006). This highly regulated activity is facilitated by proteases such as the membrane-bound collagenase, MT1-MMP (Koshikawa *et al.*, 2000; Remacle *et al.*, 2003), the membrane-associated gelatinases, CB (Buth *et al.*, 2007), secreted MMP-2 (Koshikawa *et al.*, 2000) and many others. In combination MT1-MMP, CB and MMP-2 can degrade all the components of the BM, as well as almost all the components of the ECM (Table 4.1), that need to be traversed during invasion. Loss of control over this function is what differentiates physiologically normal migration from cancer cell invasion, with carcinoma cells degrading the BM to such an extent (Chapter 4 and Figure 5.3), that these cells perforate and migrate across the normally intact BM structure.

5.2.1. The Wnt pathway in EMT and cancer

Wnts [so called after the wingless protein in Drosophila, and its mammalian version, int-1 (Fischer *et al.*, 2006)] and transforming growth factor-β (TGF-β) are growth factors that form concentration-dependent gradients along the anterior-posterior axis to regulate cellular polarity (Fischer *et al.*, 2006). They are closely linked to a wide range of developmental (Cadigan *et al.*, 2006) and regenerative processes during tissue repair (Jiang *et al.*, 2007), including cell migration and mitogenic, secretory and inflammatory activities (Ma *et al.*, 2006). Extracellular Wnts bind to their receptors and co-receptors (Cadigan *et al.*, 2006), and intracellularly the signal is indirectly continued via β–catenin. When associated with scaffold components, β–catenin is phosphorylated by various kinases, ubiquitinated and subsequently degraded in the proteosome (Garcia-Rostan *et al.*, 1999; Cadigan *et al.*, 2006). Activation of Wnts results in competitive binding of β–catenin to an intracellular Wnt receptor complex, which inhibits its degradation. β-Catenin is subsequently transported into the nucleus (Perez-Moreno *et al.*, 2006), where it associates with transcription factors (Christofori, 2006) to regulate expression of specific target genes related to proliferation, such as Cyclin D-1 (Hirohashi, 1998; Behrens *et al.*, 2004; Christofori, 2006). Such nuclear transport was shown in both regenerating muscle stem cells (Otto *et al.*, 2008) an in MCF10A untransformed cells (Graham *et al.*, 2004).

During cell-cell adhesion, on the other hand, β–catenin may be associated with intercellular contact points (Aplin *et al.*, 1998). As cells become confluent and their lateral sides come into contact with one another, homotypic intercellular recognition sites or AJs, are formed (Conacci-Sorrell *et al.*, 2002). In epithelial cells, AJs are composed of membrane-spanning, calcium-regulated E-cadherins, with β-catenins (Takaishi *et al.*, 1997) and α-catenins (Nagafuchi, 2001; Drees *et al.*, 2005; Bajpai *et al.*, 2008), forming the intracellular connection of E-cadherin to the actin filaments (Nagafuchi, 2001; Conacci-Sorrell *et al.*, 2002; Perez-Moreno *et al.*, 2006; Yap *et al.*, 2007; Bajpai *et al.*, 2008). Through these cell-cell adhesion sites and a process known as contact inhibition, migration and proliferation is restricted, i.e. the cytoskeletal organization is altered to acquire the distinguished flat shape of a normal, confluent epithelial layer, while epithelial proliferation into multi-layer growths, is inhibited.

Without this contact, cells will usually die through a signaling cascade that induces anoikis (apoptosis due to loss of contact with the ECM (Menard *et al.*, 2005).

The calcium-related Wnt pathway plays a role during migration (Behrens *et al.*, 2004) via calmodulin and intracellular calcium release (Vergne *et al.*, 2003; Walker *et al.*, 2003). Calmodulin may activate GRF2, a Ras-GEF, that in turn may also activate both Ras-PI3K and Ras-Rac downstream signaling to coordinate proliferation (via ERK) and survival (via SAPK) (Fan *et al.*, 1998; Cullen *et al.*, 2002) (Figure 1.3 on fold-out). Rac destabilizes AJs and its components, thus promoting epithelial cell migration (Quinlan, 1999). Calmodulin may indirectly promote phosphorylation of FAK (Fan *et al.*, 2005), that allows reorganization of the cytoskeletal components, and facilitate migration (Fan *et al.*, 2005) as part of the EMT-related change in anterior-posterior polarity.

The non-canonical Wnt pathway (not related to β-catenin) is associated with polarized cell shape changes, called planar cell polarity (PCP), and involves cytoskeletal changes. During this process Rho GTPases may integrate Wnt-induced signals spatially and temporally to regulate both morphological (via cytoskeletal dynamics and β-catenin at the AJs) and transcriptional changes (via β-catenin-related signaling pathways) that affect cell behaviour/migration and tissue homeostasis (Settleman, 2001; Perez-Moreno *et al.*, 2006).

Activation of the intracellular α- and β-catenin complex associated with the Wnt receptor allows RhoA-ROCK activation and formation of stress fibers that assists in the migration process and β-catenin translocation to the nucleus to stimulate proliferation. In addition, inhibition of α-catenin, that assists in the isolation of β-catenin to the AJs, as explained above, results in hyperproliferation (Vasioukhin *et al.*, 2001). This may possibly be due to β-catenin alternatively being transported to the nucleus (Perez-Moreno *et al.*, 2006), and increased MAPK activity (Vasioukhin *et al.*, 2001). Normal growth factor related migration and proliferation is a transient process due to negative feedback from the initial downstream Ras effectors to switch activated Ras-GTP to inactive Ras-GDP (Section 1.2.4) and restraint of proliferation via tumour suppressor proteins such as p53 (Section 1.8).

While all the Wnt pathways are related to proliferation and migration during normal processes, the Wnt pathway may also be involved in the control of patho-physiological processes such as cancer since acquisition of an invasive cancer stem cell phenotype is associated with induction of Wnt-mediated EMT (Jiang *et al.*, 2007). Down-regulation of Wnt-4 and up-regulation of Wnt-5a expression, has, for example been was shown in EMT of human squamous cells giving rise to carcinoma cells (Taki *et al.*, 2003). Of interest in this study is that, during migration, Wnts may be involved in the induction of expression of MMPs such as MT1-MMP (Conacci-Sorrell *et al.*, 2002; Nawrocki-Raby *et al.*, 2003).

There seems to be co-operation between Wnt signaling, Rac and Rho-ROCK and protease expression, all factors related to migration (cytoskeleton and BM degradation). In order to explain how migration occurs and using the knowledge gained during this project, we present a comparative summary of the processes involved in migration and invasion. In order to gain insight into events such as polarity and protease trafficking, that may, due to c-Ha-Ras(V12) activity, change migration into invasion, we must first consider how migration is initiated via growth factor-related EMT or acquisition of a mesenchymal anterior-posterior polarity, instead of an apical-basolateral polarity.

5.2.2. CB and MT1-MMP in c-Ha-Ras(V12)-related invasion

At the leading front of normal migrating cells elevated levels of CB (Section 4.4) and MT1-MMP (Section 4.6), may assist in controlled degradation of BM components. Via ‘outside-in’ integrin-related transmembrane signaling these components may provide signals required to support cell polarization, direction of migration and cell survival during this process (Section 4.1.4.2). We proposed that the distribution and activity of membrane-associated CB during these processes seems to rely on factors already located to the PM (Section 4.1.2 and Section 4.7), while the distribution and activity of MT1-MMP seems to be directly under the influence of Ha-Ras, transiently activated as part of the formation of a leading front (Section 4.7). We proposed that the altered distribution of active MT1-MMP in transfected cells is indirectly due to the possible altered distribution of mutationally activated c-Ha-Ras(V12). However, these suggestions still need to be confirmed experimentally. Since the integrins at focal adhesions play a major role in outside-in signaling, the distribution of FAK that

signals down PI3K and Ras-related pathways in relation to MT1-MMP, should also be investigated. This, however, fell outside of the scope of this project.

During cell-cell contact laminin, an epithelial BM component (Hotary *et al.*, 2006), stimulates assembly of E-cadherin-containing AJs and desmosomes (Li *et al.*, 2003). E-cadherin subsequently suppresses proliferation by binding β-catenin to its adherence site and limits its nuclear transfer (Section 5.1.3). During mammary gland formation in a 3D culture, caspase-3 is activated in cells that lack contact with a BM and thus binding to laminin (Zhao *et al.*, 2006) which, in turn, results in apoptosis of interior cells and the formation of the glandular lumen (Li *et al.*, 2003; Imbalzano *et al.*, 2009). In confluent cells E-cadherin mediates the downregulation of MT1-MMP and other MMPs, possibly as part of inhibition of migration and related gradation (Almeida *et al.*, 2001b; Nawrocki-Raby *et al.*, 2003; Bonnomet *et al.*, 2008). Under normal conditions there may be a functional link between MT1-MMP and E-cadherin, since E-cadherin-positive cells are less invasive, showing downregulation of transcription and translation of β-catenin and MMPs (including MT1-MMP) (Nawrocki-Raby *et al.*, 2003). On the other hand, detection of β-catenin in the nucleus, such as during lack of intercellular contact (Section 5.1.3), was correlated with decreased of E-cadherin expression (Mareel *et al.*, 2003), and loss of E-cadherin protein expression, indicating reduced ability to form cell-cell contacts, was observed in invasive lobular breast carcinoma (Droufakou *et al.*, 2001).

5.2.3. Speculations on the role of MT1-MMP in invasive behaviour

It has been demonstrated in this study(Fortgens, 1996) (Chapter 4) and by others (Gilles *et al.*, 2001), that MT1-MMP in non-tumorigenic migrating MCF10A cells is spatially and temporally regulated, with its distribution specifically to the lamellipodia and basal surfaces at focal adhesion sites (Mori *et al.*, 2002; Gingras *et al.*, 2008) (Section 4.6). Here MT1-MMP may proteolytically cleave the γ2 chain of laminin-5 (Gilles *et al.*, 2001; Baumgartner *et al.*, 2008) and may, therefore promote migration by contributing to release of cell-matrix contacts. Controlled, limited MT1-MMP-related cleavage of E-cadherin (Covington *et al.*, 2006) and CD44 at the rear of the cell (Marrero-Diaz *et al.*, 2008) may support EMT during the normal migratory phase, while this function has also been demonstrated in invasive cancer. In this study catalytically active MT1-MMP in MCF10AneoT invasive cancer cells was observed

over an area wider than merely the leading edges. During tumour progression (Illman *et al.*, 2006) ectopic expression of MT1-MMP on the PM may then cleave E-cadherin (Covington *et al.*, 2006; Illman *et al.*, 2006; Bonnomet *et al.*, 2008; Cao *et al.*, 2008). This may disrupt E-cadherin function, leading to reduced inhibition of proliferation on cell-cell contact, loss of characteristic monolayer formation by epithelial cells, and rather multilayered growths. This scenario may be reflected in initial infiltrative cancer, where abarently-growing cells fill a ductal structure, without the formation of a lumen. As the cancer progress through various stages, these cells may acquire the ability to also digest the surrounding limiting BM, resulting in metastases, all characteristics of metastatic cancer. Also, in metastatic cancers E-cadherin gene expression is silenced (Hirohashi, 1998; Ferguson *et al.*, 2000), which may further limit regulatory intracellular signaling (for regular reversal of EMT and migration) during cancer progression and invasion (Vasioukhin *et al.*, 2001; Conacci-Sorrell *et al.*, 2002). In support of this theory, cancer cells with a functioning E-cadherin system are less invasive, and form larger clusters of cells (Nawrocki-Raby *et al.*, 2003).

From the current study on invasive c-Ha-*ras*(V12)-transfected MCF10A breast epithelial cells the expression of MT1-MMP that extended beyond only the leading front, compared to non-transfected MCF10A cells (Figure 4.14), may lead to excessive proteolytic activity, wide-spread BM and ECM degradation, perforation of the BM and invasion by c-Ha-Ras(V12) transfected- or cancer cells. Included in the list of MT1-MMP substrates related to invasive behaviour, may thus be adherence proteins CD44 and E-cadherin, with resultant multilayered cancer growth,

5.3. From monolayer to 3D cultures, DCIS and invasive cancer and environmental influences

Current studies on monolayers of a set of cells derived from a single genetic background, allowed the monitoring of changes to the behaviour of cells following a single mutation, such as the c-Ha-*ras*(V12) oncogene. The observations made in this study have assisted in establishing an experimental system to investigate possible biological changes due to transformation of normal MCF10A cells with the c-Ha-*ras*(V12) oncogene that may result in the invasive behaviour of the MCF10AneoT cell line. Since a probable mechanism for trafficking, distribution and association with membrane partners has been established in this thesis, the proteases involved in

intra- and extracellularly proteolysis and their role in invasion may now more easily be considered in cultures that grow in 3D. Malignant growths seem to loose polarity, and without the baseline understanding of the normal roles that Ras and proteases play in the normal, migrating epithelial cell, as we have attempted to establish with this project, unraveling the complex combination of oncogenes, their signaling and the effect on the cell as a whole, may be a near impossible task.

The normal mammary duct consists of epithelial cells surrounded by a microenvironment, such as a BM and ECM. Interactions in the ductal microenvironment supports BM production, cell-matrix and cell-cell interactions (Hu *et al.*, 2008; Mailleux *et al.*, 2008; Imbalzano *et al.*, 2009). The arrangement of the functional mammary gland or duct suggests that changes in the function of epithelial cells may be a consequence of changes in mammary stroma. In the normal breast inhibition of growth may be due to the menstrual phase and hormone levels, since inhibition of estrogen mimics the non-proliferative phase, with the epithelial cells in senescence (Hattar *et al.*, 2009).

The 3D MCF10A model (Baumgartner *et al.*, 2008) and its transfected xenographs cultured in 3D (Hu *et al.*, 2008) may, therefore, be more appropriate to mimic the *in vivo* transition, or morphological alterations, from abnormal epithelial proliferation, through progression into non-invasive ductal carcinoma *in situ* (DCIS) to infiltrative and invasive carcinomas (Castro, 2008). DCIS is an accumulation of non-invasive malignant cells within the lumens of the mammary duct, while invasive carcinoma manages to break free from the enclosing BM. While this progression is still poorly understood, it has been shown to be due to pre-existing genetic alterations and associated alterations in the microenvironment, such as alterations in myoepithelial cell differentiation and fibroblast signaling (Castro, 2008; Hu *et al.*, 2008; Schnitt, 2009).

Inhibition of MT1-MMP, MMPs and the membrane-associated and membrane-associated cathepsins in 3D models may better reveal the effect of elevated levels extracellular proteases in conditions that better resemble the *in vivo* situation . Such culture in 3D may also allow the investigation of factors such as contact inhibition, a function that is lost in cancer cells and that may impact on the formation of glandular structures.

Work is currently being done to investigate the effect of the microenvironment by studying multi-cell type cultures, such as epithelial cells and ECM-producing fibroblasts.

In epithelial cells, opposing effects were seen on E-cadherin-mediated cell-cell adhesion and migration, with invasion-inhibitory signals from contact with fibronectin or laminin and invasion-stimulatory signals from collagen (Mareel *et al.*, 2003; Loughran *et al.*, 2005). Binding of the cell to laminin by integrins (Liu *et al.*, 2007) facilitates migration and induces activation of ERK1/2 and Akt in breast cancer cells (Fiucci *et al.*, 2002a), an activity that is blocked by cav-1(Fiucci *et al.*, 2002a).

Cell cultures grown on a reconstituted BM results in formation of three-dimensional (3D), and may form polarized, growth-arrested acini-like spheroids (Debnath *et al.*, 2003). Matrigel, a commercially available matrix of reconstituted BM derived from Engelbreth–Holm–Swarm tumour (Debnath *et al.*, 2003), is often used to investigate aspects of glandular architecture in cancerous cell cultures (Szpaderska *et al.*, 2001; Cavallo-Medved *et al.*, 2005; Sloane *et al.*, 2005). It may also be used to investigate the invasive ability of cancerous cells (Sounni *et al.*, 2002; Ueda *et al.*, 2003). Mixing the epithelial tumor cells with Matrigel or primary human mammary fibroblasts substantially increased the efficiency of tumor formation and decreased the latency of tumor formation, demonstrating a significant influence of the stromal microenvironment on tumorigenicity (Elenbaas *et al.*, 2001).

Proteolytic fragments of ECM proteins may regulate a variety of critical processes in cell biology, and a balance that is changed may mean the difference between migration during e.g. wound healing and (increased BM and ECM degradation) or invasive behaviour of transformed cells (Shapiro, 1998). While intact components of the ECM inhibited migration, proteolysis, or degraded collagen or fibronectin, stimulated migration of macrophages (Hattar *et al.*, 2009).

Epithelial cells migrate along the BM, and discreetly digest the BM at the leading edge of the migrating cells. However, these cells replace the BM in the wake of the leading edge. Integrins that have these BM and ECM components as ligands, and that

subsequently signals via the FAK pathway, may play an important role in polarity, or the loss thereof (Deryugina *et al.*, 2002).

However, the individual ECM components have been shown to affect the migratory and proliferative ability of cells and to affect the trafficking of MT1-MMP. Therefore, these components have been isolated and used to coat culture surfaces to investigate the role that these individual ECM components play in signaling to the migrating cell, such as HA (hyaluronan) (Ueda *et al.*, 2003). Cells grown in culture lay down a BM that may contain collagen IV, as shown by specific immunolabeling (Buth *et al.*, 2007). DQ-collagen IV allows the observation of actual collagen cleavage by components secreted by the cell during glandular formation (Cavallo-Medved *et al.*, 2005), or invasion. Comparison in the migratory speed of cells on collagen, laminin and fibronectin and the involvement of FAK at the focal adhesion sites (Guan, 1997).

Several cell lines from breast tumours that have the ability to perforate (extensively degrade) and therefore invade ECM gels, mimic metastatic behavior in culture (Hotary *et al.*, 2006). This was also shown in vitro 3D assays for invasion by cancer cells (Mareel *et al.*, 2003).

The elucidation of the role of proteases in normal cells and subsequently also in cancer, will allow us to design more effective inhibitors and novel protease-based drugs for clinical use. While it is becoming clear that the extracellular MMPs and MT1-MMPs are critical factors in invasive pathology, mechanisms to regulate the elevated intracellular cathepsin levels should also be explored as additional or supporting therapeutic protocols. It may not be possible to overcome the immortal nature of tumorigenic cells, but where possible the incorrect and elevated expression of proteases that occurs as a result of transformation, should be addressed during therapy. In conclusion, MMPs in general and some of the cathepsins may support the invasive capability of tumour cells. CB, CD and especially CL, however, allow desensitization of the immune system to cancer cells *in vivo*, which may facilitate evasion of these cells from detection by the immune system. Further studies will include investigation of processing and distribution of CD44, colocalization of CB and MT1-MMP and their endogeneous inhibitors as well as inhibition studies and protein expression in live cells.

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Appendix

Appendix I List of abstracts

List of abstract from this thesis prepared for presentation at conferences, 2004-2009.

Appendix II Jackson *et al.* Abstracts

Abstracts from Electron Microscopy of Southern Africa Conferences

1. Jackson, A. J., V. H. Bandu, C. Dennison and E. Elliott (1999). "In vitro pH determination of acidic vesicles using 3-(2.4-dinitroanilino)-3'-amino-n-methyldipropylamine (DAMP)." Proceedings of the Microscopy Society of Southern Africa **29**: 89.

2. Jackson, A. J., C. Dennison, M. Ariatti and E. Elliott (2003). "Identity and role of peripheral organelles in invasive breast epithelial cells." Proceedings of the Microscopy Society of Southern Africa **33**: 81.

Appendix III Extract from thesis - CB, CD, CL blots

Preliminary manuscript of western blots of cathepsin B, cathepsin D and cathepsin L.

Membrane type-1 matrix metalloproteinase (MT1-MMP) in c-Ha-ras-induced breast cancer cell invasion

D van Rooyen MSc thesis (2009).

Appendix IV Gordon Conference Poster

Gordon Conference, June 2008

Membrane type-1 matrix metalloproteinase (MT-1 MMP) as a target for preventing invasion.

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Appendix V Fold-out of Figure 1.3