ENDOPLASMIC RETICULUM ASSOCIATED DEGRADATION (ERAD) OVERFLOW PATHWAYS

by
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PREFACE

The experimental work described in this dissertation was carried out in the Department of Biochemistry, University of KwaZulu Natal, Pietermaritzburg, from January 2006 to February 2007, 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 (except for Fig. 3.3 and 4.14 A and C, previously produced by honours student Clyde Cossey illustrating minor ultrastructural details for discussion).

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ABSTRACT

Accumulation of misfolded proteins in the endoplasmic reticulum (ER) causes numerous human pathologies. Biochemical evidence suggests that soluble misfolded proteins are retrotranslocated out of the ER, via the endoplasmic reticulum associated degradation (ERAD) pathway, for proteosome-mediated cytoplasmic degradation. Excess, misfolded- or insoluble proteins, are suggested to cause induction of "overflow" degradation pathways. For soluble proteins, overflow to vacuole-mediated destruction is suggested to occur via two Golgi-to-vacuole (Gvt) routes, the alkaline phosphatase (ALP), direct route, or, a carboxypeptidase Y- (CPY-), prevacuolar compartment-vacuole, indirect route, though only the CPY route is thought to degrade soluble proteins. Insoluble aggregate-containing structures are suggested to be degraded by engulfment by membranes of unknown origin and trafficking to the vacuole for destruction, via an autophagic pathway. To confirm biochemical evidence, wild-type (BY4742), autophagosome- (W303/ΔATG14), CPY- and autophagy pathway- (W303/ΔVPS30), and proteosome (WCG/2) mutants of *S. cerevisiae* yeasts were transformed with a high expression pYES plasmid and mutant (Z) human alpha-1-proteinase inhibitor (A1PiZ), giving rise to the derivatives cells BY4742/Z, W303/ΔATG14/Z, W303/ΔVPS30/Z and WCG/2/Z, respectively. Electron microscopy using gold labeling for A1PiZ, markers for the ER, the ERAD ER channel protein, Sec61, or the chaperone, binding protein (BiP), ALP for the ALP pathway, and CPY for the CPY pathway, was used. Overexpression of A1PiZ seems to result in targeting to the vacuole via a prevacuolar, CPY-like compartment (PVC, 200-500 nm), though CPY and A1PiZ appears not to colocalise, unconvincingly confirming collaborative biochemical data. Large amounts of A1PiZ localise in the cytosol, possibly indicating a largely proteasome-mediated degradation. ER-resident A1PiZ targeting to the vacuole seems also to occur by the budding of the ER and peripheral plasma membrane or ER membrane only. This occurs in all cells, but especially in *ATG14* gene (*ΔATG14*) mutants, possibly indicating autophagosome-mediated degradation independence, in the latter mutants. The *ATG14* mutation gave rise to crescent-shaped, initiating membrane-like (IM-like) structures of approximately Cvt vesicle-diameter, possibly indicating that *ΔATG14* blocks autophagosome- (500-1000 nm) and Cvt vesicle (100-200 nm) enclosure, after core IM formation.

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ABBREVIATIONS

A1PiZ	alpha-1 protease inhibitor Z-variant
ADP	adenosine diphosphate
ALP	alkaline phosphatase
Ape1	aminopeptidase 1
Apg	autophagy
ATF6	activating transcription factor 6
ATP	adenosine triphosphate
BiP	binding protein
BSA	bovine serum albumin
BSA-PBS	bovine serum albumin in phosphate buffered saline
CD	casein amino acids/dextrose
cER	cytoplasmic endoplasmic reticulum
COP I	coat protein I
COP II	coat protein II
CPY	carboxypeptidase Y
Cvt	cytoplasm-to-vacuole targeting
CW	cell wall
d.H_2O	distilled water
dd.H_2O	double distilled water
DER3	degradation in endoplasmic reticulum
DNA	deoxyribonucleic acid
EGTA	ethylene glycol-bis(β-aminoethylether)-*N*, *N*, N',N'-tetraacetic acid
eIF2a	eukaryote translation initiation factor 2a
EM	electron microscopy
ER	endoplasmic reticulum
ERA	ER-containing autophagosomes
ERAD	endoplasmic reticulum associated degradation
EtOH	ethanol
Fab	fragment antigen bind

FBG	fish skin gelatin, bovine serum albumin, glycine
Fc	fragment, crystallisable
G	Golgi
g	gram
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
Gvt	golgi-to-vacuole targeting
h	hour
HALLT	L-histidine HCl, adenine sulfate, L-lysine HCl, L-leucine and L-tryptophan
HCl	hydrogen chloride
HECT	homologous to the E6-AP carboxyl terminus
HEPES	*N*-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid
HRD1	HMG-CoA reductase protein 1
Hsp40	heat shock protein 40
Hsp70	heat shock protein 70
I	invaginating body
IgG	gamma immunoglobulin
IM	isolation membrane
IRE1	inositol requiring transmembrane kinase
kDa	kiloDalton
L	litre
LR White	London Resin White
M	mitochondria
M	molar
m/v	mass per volume
mCPY	mature carboxypeptidase Y
mg	milligram
Min	minute
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
nm	nanometer

OD	optical density
PAG	protein A-gold
PAS	pre-autophagosomal structure
PBS	phosphate buffered saline
PCD	programmed cell death
pER	peripheral endoplasmic reticulum
PERK	protein kinase R-like ER kinase
PHEM	PIPES, HEPES, EGTA and $MgCl_2$
PIPES	piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)
PtdIns	phosphatidylinositol
PtdIns3P	phosphatidylinositol 3-phosphate
PVC	prevacuolar compartment
PX	Phox homology
RING	really interesting new protein
RIP	regulated intermembrane proteolysis
RT	room temperature
SD	synthetic drop-out
sec	secretory
SNARE	N-ethylmaleimide sensitive factor-attachment protein receptor
TEM	transmission electron microscopy
TGN	*trans* Golgi network
Tor	target of rapamycin
UBC	ubiquitin conjugating enzyme
UPR	unfolded protein response
V	vacuole
v/v	volume per volume
vam	vacuolar morphology
Vps	vacuolar protein sorting
YPD	yeast extract, peptone, dextrose
µl	microlitre

Chapter 1

1.1 Introduction

Soluble or small extracellular protein aggregates are imported into eukaryotic cells via constitutive pinocytosis or receptor-mediated endocytosis, and are degraded by the mammalian lysosome or the vacuole of the yeast (Clague, 1998; Geli and Reizman, 1998; Lemmon and Traub, 2000). Newly synthesised misfolded intracellular proteins, on the other hand, may be degraded via three major pathways. These are the endoplasmic reticulum associated degradation (ERAD) pathway, where misfolded proteins are fed into the cytoplasm and are degraded by the proteasome (Fig. 1.1 A), and two ERAD "overflow" pathways, the autophagic pathway **(**Fig. 1.1 C, pathway D will be explained later) and one of the Golgi-to-vacuole (Gvt) pathways (Fig. 1.1 B). The Gvt pathway consists of two pathways, the carboxypeptidase Y (CPY) and alkaline phosphatase (ALP) pathways (Fig. 1.1 B), so-named due to the enzymes they carry from the Golgi to the vacuole. Under normal circumstances the Gvt pathways facilitate the maturation of ALP and CPY, as both enzymes mature upon reaching the vacuole. Transport into the ALP pathway is signal-mediated, whereas transport into the CPY pathway is receptor mediated (Bonangelino *et al.*, 2002; Conibear and Stevens, 1998). Under certain circumstances, the CPY pathway, however, may be responsible for the degradation of soluble protein aggregates formed in the ER and transported through the Golgi (Kruse *et al*., 2006b). Hence the CPY pathway is termed an "ERAD overflow" pathway.

1.1.1 The ER and overview of ER associated degradation (ERAD)

In the cell, the endoplasmic reticulum (ER) is responsible for the folding, quality control and trafficking of proteins. Proteins are translated from mRNA by the ribosomal machinery in the cytoplasm and subsequently targeted to the ER by a signal sequence. The heterotrimeric trans-membrane complex Sec61 facilitates the entry of newly

synthesised proteins into the ER (Kleizen and Braakman, 2004; Lord *et al.*, 2000; Romisch, 1999). Once proteins enter the ER they are bound by chaperones such as binding protein (BiP) and calnexin. These chaperones ensure the correct folding and success of posttranslational modifications to the nascent polypeptides (Hirsch *et al.*, 2004; Kruse *et al.*, 2006a) as misfolded proteins disrupt the homeostasis of the ER, potentially leading to cell death. Misfolded proteins are recognised as ERAD substrates and are retrotranslocated out of the ER and into the cytoplasm. Here they are targeted to the 26S proteasome for degradation via a process known as ubiquitination (Fig. 1.1, A) (Lord *et al.*, 2000; Schmitz and Herzog, 2004; Werner *et al.*, 1996). The ER-associated proteasomal degradation system constitutes the default pathway for protein degradation in normal cells.

1.1.2 Overview of ERAD overflow pathways

When the ERAD (proteasome) pathway becomes saturated with misfolded proteins or is compromised, the cell is forced to degrade aberrant proteins via the "overflow" Gvt and autophagic pathways. These may both target their contents to the vacuole for degradation (Fig. 1.1, B and C, respectively). The CPY route of the Gvt pathway is believed to degrade soluble misfolded proteins (to date, the ALP pathway is not known to be involved in degradation) (Kruse *et al.*, 2006b). The autophagic pathway, on the other hand, degrades insoluble aggregates of misfolded proteins located in the cytoplasm or in organelles such as the ER by a process known as autophagy.

By definition, autophagy (self-eating) is the generic name for the sequestration of long-lived/misfolded proteins, organelles or cytosol within a double membrane structure termed the autophagosome (Deretic, 2006). Other names for this structure are the macroautophagosome. To distinguish this, the major form of autophagy to which reference is made when autophagy is mentioned (two lesser known and described forms will be subsequently described in Section 1.4 and Fig. 1.7). The origin of the membrane used for the formation of these macroautophagosomes or autophagosomes is, however, still unclear but is thought to be predominantly ER-derived. The outer membrane of the double membrane of the autophagosome is incorporated into the vacuolar membrane

during the vesicle-vacuole fusion event, while the subvacuolar vesicle, known as the autophagic body is released into the vacuolar lumen for degradation (Reggiori and Klionsky, 2005) (Figure 1.1, C).

The Cvt pathway is structurally and functionally similar to the autophagic pathway, but instead of functioning in the degradation of protein aggregates, it is involved in the biosynthetic processing of proteins such as aminopeptidase 1 (APE1). This is achieved by sequestering the unprocessed cytoplasmic precursor APE1 in a double membraned vesicle and delivering APE1 to the vacuole where it is processed to the mature form (Fig. 1.1 D) (Reggiori and Klionsky, 2005). This pathway resembles the autophagic pathway closely and has to be distinguished from it. The easiest way to achieve this is to hyperexpress a malfolded protein, saturate the ERAD-proteasomal system and induce the autophagic pathway to remove the insoluble protein aggregates which occur in organelles such as the ER or in the cytoplasm. The autophagic or autophagosomal route is the major "overflow", last resort route known to be induced when the ERAD pathway is overwhelmed. The autophagic, and not the overflow pathway, is perhaps best known for its association with various diseases.

When the ERAD pathway, i.e. the ubiquitin proteasome pathway and overflow pathways are overwhelmed and organelle or major protein aggregates in damaged organelles need removing, autophagy is induced by a number of severe cellular events such chemical toxicity, pathogens, major organelle damage, severe genetic mutation and nutrient deprivation. Therefore, the autophagic pathway is associated with a number of stress-inducing cellular events and is not specifically associated with the removal of minor protein aggregates or minor soluble proteins via the ERAD and Gvt CPY pathways (Fig. 1.2).

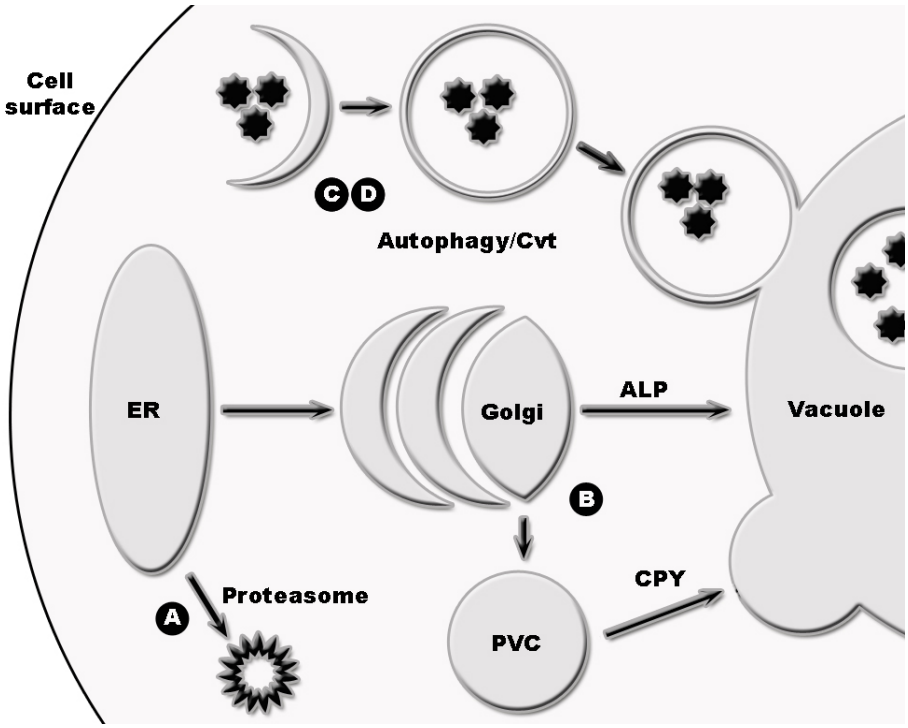

Figure 1.1 **Overview of protein degradation mechanisms in eukaryote cells.**

The ERAD pathway (A) targets aberrant proteins to the proteasome. The Gvt pathway (B) targets soluble mutants to the vacuole through the Golgi and is made up of two constituent pathways, the ALP and CPY pathways. The major difference between these two pathways is that the CPY pathway uses the prevacuolar compartment (PVC) as an intermediate organelle. The autophagic and Cvt pathways (C and D) both sequester cargo in the cytoplasm by enwrapping it in a double membrane vesicle which fuses with the vacuole.

1.1.3 "Autophagy" in disease

In addition to maintaining cellular homeostasis by removing malfolded proteins, autophagy is possibly best known for the elimination of aberrant structures. It has also been implicated in the defense against pathogens and tumours, and Type II programmed cell death (PCD) (Fig 1.2). Type II PCD is morphologically dissimilar to Type I PCD and is characterised by the autophagic degradation of Golgi cisternae, polyribosomes and ER. Degradation of these proceeds nuclear destruction, the primary event in Type I PCD (Cuervo, 2004; Reggiori and Klionsky, 2005). Besides the fact that such an event is responsible for the elimination of malfolded protein, it would be classified as "non-overflow" autophagy.

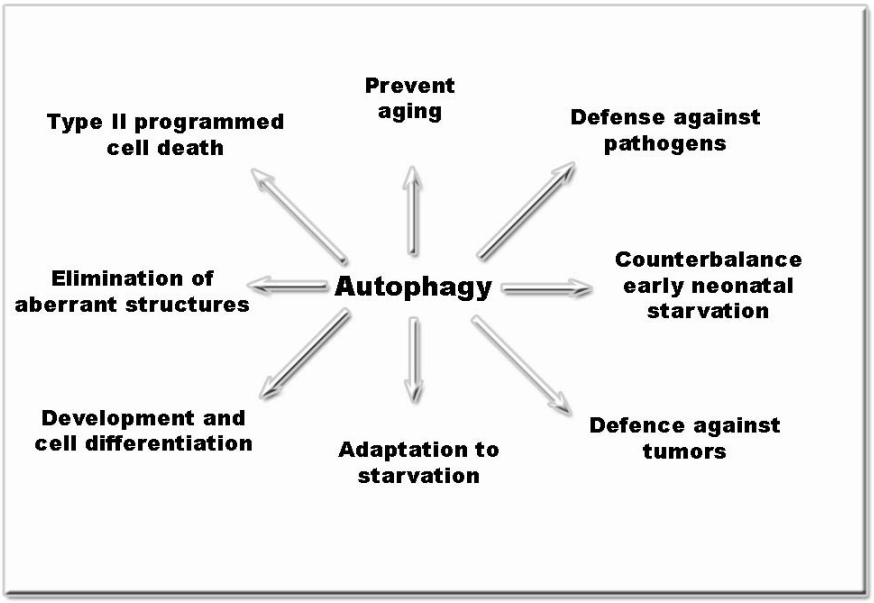

Figure 1.2: **Summary of the roles of autophagy in maintaining cellular homeostasis and in health and disease (Reggiori and Klionsky, 2005).**

A non-ERAD-associated role of autophagy is the degradation of certain toxins and pathogens. The activation of non-ERAD associated or ERAD-associated autophagy is believed to play a 'protective' role in early stages of several diseases. Recent findings, however, have suggested that autophagy may also play either a protective or detrimental role, depending of the stage of the disease, the surrounding cellular environment or the method of treatment employed (Cuervo, 2004; Deretic, 2006).

1.1.3.1 Autophagy in cancer

The number of autophagosomes in cancer cells compared with that in normal cells seems to give variable results were seen leading to the conclusion that both activation and inactivation of autophagy can benefit cancer cells (Table 1.1). After confluence is reached, the growth of normal cells is retarded. This is accomplished by a switch from an anabolic to a catabolic state. If cells cannot activate autophagy, then protein synthesis is dominant over degradation, resulting in continued cellular growth (a typical

characteristic of tumour cells) (Meijer and Codogno, 2006). Such cells would also be immune to any events which usually trigger cell death through autophagy.

Activation of autophagy in early stages of cancer, however, may block tumour growth but can also prove advantageous for cancer cells. It can facilitate their survival under extreme conditions (e.g. an under-vascularised tumour, where nutrient availability is poor), and after cancer cells have undergone radiation treatment or chemotherapy, it may remove damaged organelles, preventing the onset of apoptosis and removal of cancer cells. Activation of autophagy in late stages of tumour development may also favour cancer cell survival, however (Cuervo, 2004) (Table 1.1).

Table 1.1: Possible outcomes of the activation of autophagy in different pathologies.

Red text indicates harmful outcomes and blue text indicates beneficial outcomes. (Cuervo, 2004).

Disease	Activation of autophagy	Inactivation of autophagy
Cancer		
Early stages	Blocks tumour growth	Favors tumour growth, Makes cells unable to enter autophagic cell death after exposure to anticancer treatments
Late stages	Favours survival of cells in low-vascularized tumours Favours removal of damaged intracellular macromolecules after anticancer treatments	Prevents survival of cells in low-vascularized tumours Increases efficiency of anticancer treatments because damaged macromolecules cannot be eliminated
Vacuolar myopathies		
	Promotes elimination of the cytosolic autophagic vacuoles If hyperactivated, could result in muscle waste	Results in the accumulation of autophagic vacuoles that weaken skeletal and cardiac muscle
Neurodegeneration		
Early stages	Favours the removal of cytosolic protein aggregates	Increases the accumulation of cytosolic protein aggregates
Late stages	Destroys irreversibly damaged neurons by autophagic cell death	
Axonal Injury		
	Favours removal of neurotransmitter vesicles and damaged organelles Provides energy and membranes for regeneration	Prevents removal of damaged organelles and neurotransmitter vesicles Cytosolic release of neurotransmitters induces apoptosis Slows down regeneration
Infectious disease		
	Contributes to the elimination of bacterial and viral particles	Offers a survival environment for the bacteria that are able to inhibit autophagosome maturation Facilitates viral infection

1.1.3.2 Autophagy in the pathogen-to-host response

Autophagy plays a crucial role in the elimination of most infectious agents present in the cytoplasm. It is activated in most cases of bacterial infection, resulting in the degradation of these microorganisms (Table 1.1). Certain bacteria, however, are able to use autophagosomes as "shelter", by preventing fusion of the autophagosome with the lysosome and other organelles containing antimicrobial factors. This protects the bacteria from the acidic, proteolytic and bacteriocidal conditions associated with the killing potential of these organelles. *Legionella pneumophila* secrete a product which activates autophagy and use autophagosomes as a shelter in which to proliferate. Autophagy is also activated in response to viruses, to eliminate viral particles. Studies on bacteria and viruses which activate autophagy could provide useful insight into how autophagy may be activated for therapeutic purposes (Cuervo, 2004).

1.1.3.3 Autophagy in neurodegenerative disorders

Autophagy has been implicated in a number of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and prion protein diseases (Cuervo, 2004; Shastry, 2003). The accumulation and aggregation of aberrant proteins which are deposited in inclusion bodies, plaques, or in extracellular spaces, can transmit "toxic responses" which trigger ERAD-associated autophagy. Whether inside or outside the ER, these aberrant aggregates, if inadequately removed, cause the malfunction of a distinctive set of neurons and ultimately lead to cellular failure and neuronal death (Meijer and Codogno, 2006; Nixon, 2006).

This leads to inhibition or destruction of synaptic function, interference with numerous signal transduction pathways, alteration of calcium homeostasis, release of free radicals and consequent oxidative damage, dysfunction of the protein degradation through the ubiquitin-proteasome system and the activation of cell-death proteases leading to programmed cell death (PCD) through ER stress. Activation of autophagy, in such cells, is aimed to improve cellular homeostasis and prevent or delay neuronal death (Table

1.1). Many other examples of malfolding and removal of such proteins are known (Table 1.2). Unfortunately, however, autophagic degradation mechanisms do not always cope with the high levels of accumulated malfolded protein and disease results.

As new autophagy-related genes and proteins are discovered and characterised, new links between disease, the misfolding and aggregation of aberrant proteins in the ER and autophagy are discovered. Some of the diseases resulting from the failure of ERAD and autophagy have been mentioned and are outlined in Table 1.2. The cell responds to misfolded proteins in a number of ways but the initial and most prevalent reaction to this problem is known as the unfolded protein response (UPR). In order to describe the UPR, normal protein folding and the recognition of unfolded proteins must be first described.

Table 1.2: Human diseases caused by the misfolding and aggregation of proteins (Schroder and Kaufman, 2005).

Type/disease	Class	Affected protein
Cystic fibrosis	I. A.	CFTR
Diabetes mellitus	I. A.	Insulin receptor
Albinism/tyrosinase defficiency	I. B.	Tyrosinase
alpha 1-Antitrypsin deficiency without liver disease	I. B.	α1-Antitrypsin
Cardiovascular disease	I. B.	Lipoprotein(a)
Congenital hypothyroidism	I. B.	Thyroglobulin
	I. B.	Thyroid peroxidase
	I. B.	Thyroxin binding globulin
Familial hyperchylomicronemia	I. B.	Lipoprotein lipase
Familial isolated hypoparathyroidism	I. B.	Preproparathyroid hormone
Global polyendocrinopathy associated with obesity and infertility	I. B.	Carboxypeptidase E
Hemophilia A	I. B.	Factor VIII
Hypercholesterolemia	I. B.	LDL receptor
Laron dwarfism	I. B.	Growth hormone receptor
Diabetes insipidus	I. B./I. D.	Arginine vasopressin (AVP)
	I. B.	AVP receptor 2
	I. B.	aquaporin-2
Obesity	I. B.	Prohormone convertase 1
Osteogenesis imperfecta	I. B./I.D.	Type 1 procollagen
	I. B.	Decorin
Parkinsonism, autosomal recessive juvenile	I. B.	Pae 1 receptor
Protein C deficiency	I. B.	Protein C
Spondyloepiphyseal dysplasia due to hyperchondrogenesis	I. B.	Type II procollagen
von Willebrand disease	I. B.	von Willebrand factor
Spondyloperipheral dysplasia	I. B./I.D.	Type II collagen
β-Amyloid toxicity	I. D	β-Amyloid
alpha 1-Antitrypsin deficiency with liver disease	I. D	α1-Antitrypsin
Charcot-Marie-Tooth disease	I. D	Peripheral myelin protein PMP22
Pelizaus-Merzbacher leukodystrophy	I. D	Proteolipid protein
Pre-senile dementia/mycolonus	I. D	Neuroserpin
Abetalipoproteinemia	II.	Apolipoprotein B/microsomal triglyceride transfer protein
Combined coagulation factors V and VII deficiency	II.	Factor V, factor VIII/LMAN1
	II.	Factor V, factor VIII/MCFD2
Bipolar disorder	III.	XPB-1
Colitus (mouse model)	III.	IRE1β
Diabetes mellitus (mouse model)	III.	PERK
Hypoglycemia (mouse model)	III.	eIF2α
Wollcott-Rallison syndrome	III.	PERK
Polyglutamine diseases (dentatorubral-pallidoluysian atrophy, Huntingtons disese, spinobalbar muscular atrophy, spinocerebellar ataxia)	IV.	Proteasome

1.2 The unfolded protein response (UPR) and ER stress

The ER is the "primary compartment" through which secretory proteins enter the secretory pathway. It is vital in ensuring the correct synthesis, modification, folding and delivery of proteins to their target sites (Schroder and Kaufman, 2005). The ER is particularly sensitive to endogenous and exogenous stress which may affect protein folding. Pathogenic infection, chemical toxicity, genetic mutation and nutrient deprivation may all have negative effects on protein folding, resulting in ER stress. Increased cellular and ER stress is handled by a process known as the unfolded protein response (UPR) (Rutkowski and Kaufman, 2004).

The UPR describes the eukaryote intracellular signaling pathways which relay information from the ER lumen to the nucleus and activate specific target genes in response to errors in protein folding and assembly (Kaufman, 1999; Rutkowski and Kaufman, 2004; Shen *et al.*, 2004). The UPR regulates over 300 genes as well as the ER-associated degradation (ERAD) pathway (Xu *et al.*, 2005). Biological and physiological stimuli which change the homeostasis of the ER impose stress which leads to the accumulation of misfolded or aberrant proteins in the ER lumen (Kaneko and Nomura, 2003; Xu *et al.*, 2005). In order to remove aggregated protein from the ER, the ERAD or proteasome response is initially activated (Kaneko and Nomura, 2003). The UPR is also linked to various aspects of ER function, such as protein translocation and glycosylation (Ng *et al.*, 2000; Patil and Walter, 2001).

In order to explain the mechanism of the UPR it is necessary to take a step-wise approach and consider each step of the induction of the various pathways, beginning with how proteins are folded in the ER and the affect of ER stress.

ER stress has a threefold effect. The rate of protein synthesis is attenuated, there is an up regulation in the genes which encode chaperones which facilitate peptide folding as well as prevent polypeptide aggregation, and ER proteins are retrotranslocated into the cytoplasm where they are degraded (Fig. 1.3) (Kaneko and Nomura, 2003; Rao and Bredesen, 2002; Shen *et al.*, 2004). The aim of such a response is to minimise ER stress by reducing the amount of aggregated protein, while at the same time increasing the

capacity of the ER for folding and for cytoplasmic degradation (Rao and Bredesen, 2002; Rutkowski and Kaufman, 2004; Schroder and Kaufman, 2005). In order to understand such a process, a more detailed description of protein folding in the ER is necessary.

1.2.1 Protein folding in the ER.

Proteins are folded into their native conformations in the ER and undergo a number of post-translational modifications. These include *N*-linked glycosylation, disulfide bond formation, lipidation, hydroxylation and oligomerisation. N-linked glycosylation and the formation of disulfide bonds are the most prevalent and are common to the majority of secreted proteins (Hampton, 2000; Schroder and Kaufman, 2005). Misfolded proteins disrupt ER homeostasis, potentially leading to cell death. It is, therefore, important that quality control mechanisms are in place to efficiently remove and degrade misfolded proteins.

The quality control machinery consists of three classes of proteins: foldases, molecular chaperones and the lectins calnexin and calreticulin (Table 1.3). Foldases are enzymes which catalyze the steps in protein folding, increasing the rate and efficiency of folding. Molecular chaperones function by shielding unfolded regions of a peptide from surrounding proteins, but do not increase the rate of protein folding (Schroder and Kaufman, 2005). These are classified into a number of classes. The Heat shock protein 70 (Hsp70) class is the most important in dealing with misfolded proteins and consists of BiP/GRP78/Kar2p, Lhs1p (Cer1p/Ssi1p) and GRP170 (Table 1.3). The Hsp40 class function as co-chaperones, regulating the ATPase activity of BiP, while the GrpE-like chaperones function as nucleotide exchange factors for BiP (Schroder and Kaufman, 2005) (Table 1.3). BiP plays a central role in the recognition and processing of misfolded proteins.

Table 1.3: Molecular chaperones, foldases and lectins present in the ER (Schroder and Kaufman, 2005).

Class and name	Function
Chaperones, HSP70 class	
BiP/GRP78/Kar2p	Chaperone, translocation, folding sensor
Lhs1p/Cer1p/Ssi1p/GRP170	Chaperone
Chaperones, DNA-J-like, HSP40 class	
ERdj1/MTJ1	Co-chaperone regulating ATPase activity of BiP
ERdj3/HEDJ/Scj1p	Co-chaperone regulating ATPase activity of BiP
ERdj4	Co-chaperone regulating ATPase activity of BiP
ERdj5	Co-chaperone regulating ATPase activity of BiP
Jem1p	Co-chaperone regulating ATPase activity of BiP
Sec63p	Co-chaperone regulating ATPase activity of BiP, translocation
Chaperones, GrpE-like	
BAP	Nucleotide exchange factor for BiP
SIs1p/SiI1p	Nucleotide exchange factor for BiP
Chaperones, HSP90 class	
GRP94/endoplasmin	Chaperone
Lectins	
Calnexin	Glycoprotein quality-control
Calreticulin	Glycoprotein quality-control
Mn11p/Htm1p/EDEM	Glycoprotein degradation
Foldases, subclass disulfide isomerases	
PDI	Oxidoreductase
ERp72	Oxidoreductase
ERp61	Oxidoreductase
ERp57	Oxidoreductase
ERp44	Retention of Ero1α in ER
Ero1p/Ero1α/Ero1β	Oxidoreductase for PDI
Erv2p	Oxidoreductase for PDI
Foldases, subclass FAD dependent oxidases	
Fmo1p	FAD-dependent oxidase

1.2.2 Recognition of unfolded proteins by BiP

The BiP/Kar2p/GRP78 complex (hereafter referred to as BiP) proteins are upregulated by the UPR and bind to solvent-exposed or hydrophobic segments of the unfolded proteins to preserve the "folding competence" of the nascent chain (Fig. 1.3) (Hirsch *et al.*, 2004; Kimata *et al.*, 2003; Lord *et al.*, 2000). This association with hydrophobic domains is also believed to retain the solubility of retro-translocating peptides during their movement from the ER to the cytoplasm through the ER-membrane channel Sec61. Mutations in the KAR2 gene, encoding BiP cause the aggregation of ERAD

substrates and directly compromises the efficiency of ERAD in yeast (Kabani *et al.*, 2003; Nishikawa *et al.*, 2001). The regulation of protein flux through the ER as well as the integration of the UPR and ERAD is shown in Fig. 1.3.

It is believed that BiP has an ATPase domain and a peptide binding domain that facilitates the cyclic process of ATP hydrolysis and ADP exchange, bringing about the binding and release of the native protein. Co-chaperones such as Hsp40 influence this cycle by modulating the ATP hydrolysis (Fink, 1999; Kleizen and Braakman, 2004). This association of BiP with the ER makes it a useful marker for this organelle for immunocytochemical studies and for distinguishing ER-associated processes (Hirsch *et al.*, 2004; Schroder and Kaufman, 2005). The Sec61 ER membrane complex (Section 1.1) is also a useful marker for the ER, as it is not found in any other cellular structures. Golgi-associated pathways, on the other hand, may be distinguished using CPY or ALP markers which may be used for their respective pathways in the Gvt-associated pathways.

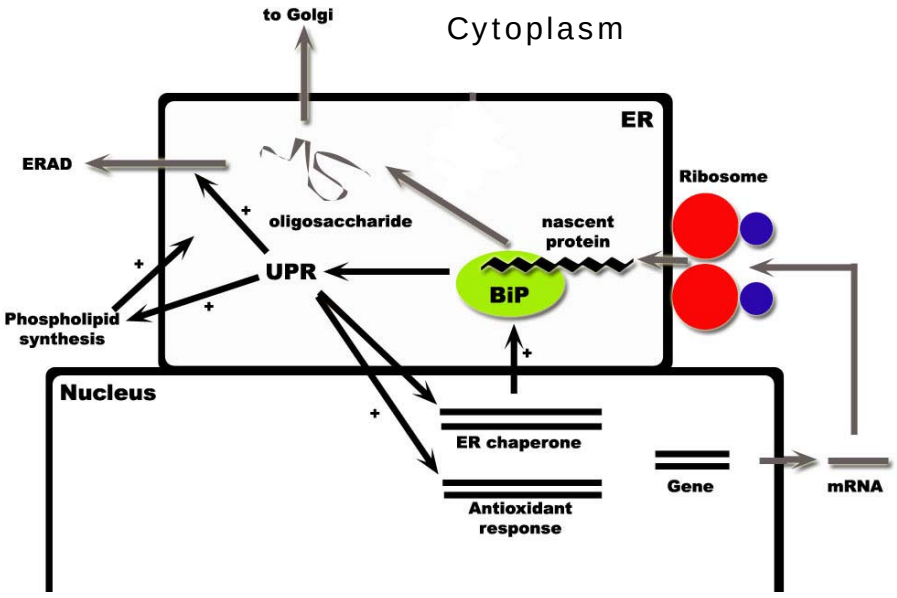

Figure 1.3: **Protein flux through the ER (grey) and the principal activities of the UPR in response to the protein folding burden in the ER (black).**

Abbreviations: ER-endoplasmic reticulum, ERAD-endoplasmic reticulum associated degradation. UPR-unfolded protein response, BiP-binding protein (Schroder and Kaufman, 2005).

1.2.3 The UPR in *Saccharomyces cerevisiae*

The yeast *S. cerevisiae* was the first organism in which all the molecules involved in the UPR were described (Shen *et al.*, 2004). The UPR is initiated by the ER-resident transmembrane serine/threonine kinase Ire1p (inositol-requiring transmembrane kinase) which is thought to sense the presence of aberrant proteins in the ER (Bertolotti and Ron, 2001; Cassagrande *et al.*, 2000; Kaufman, 1999; McCracken and Brodsky, 2000; Patil and Walter, 2001). The activation of Ire1p is brought about by its oligomerisation, which results in the *trans*-autophosphorylation of its cytosolic kinase domain (Fig. 1.4). This results in the activation of the Ire1p carboxy terminal domain which is a "site specific endoribonuclease" (Kaufman, 1999; Patil and Walter, 2001).

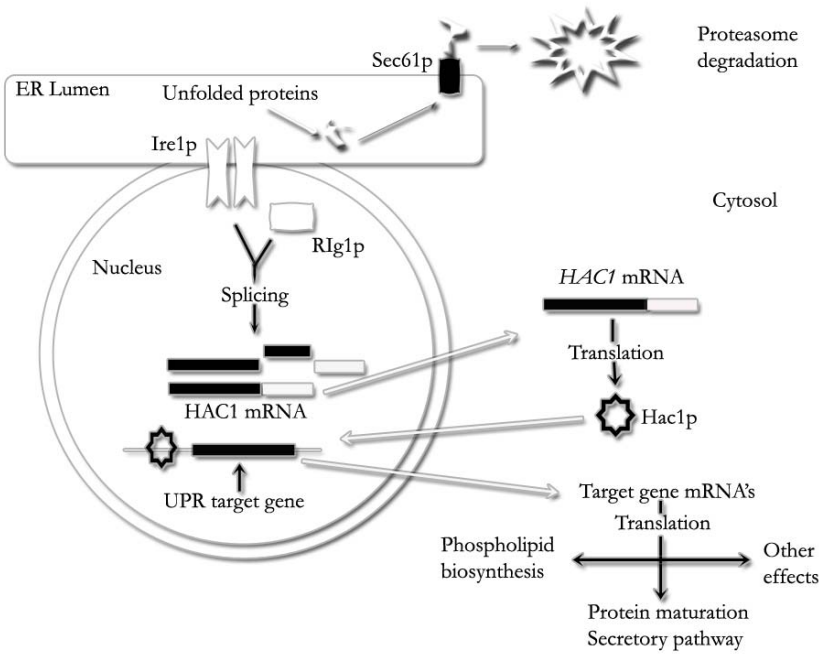

Figure 1.4: **Unfolded proteins in the ER activate the unfolded protein response (UPR) and the endoplasmic reticulum associated degradation (ERAD) pathways in yeast.**

Misfolded proteins are retrotranslocated to the cytoplasm through the Sec61p channel where they are degraded by the proteasome. Misfolded proteins also activate the transmembrane kinase Ire1p which works together with tRNA ligase to splice the HAC1 primary transcript. *HAC1* mRNA then passes to the cytoplasm for translation and the newly synthesised transcript, Hac1p enters the nucleus where it triggers the transcription of the UPR target genes (McCracken and Brodsky, 2000).

The activated Ire1p facilitates the removal of a 10 amino acid intron from the mRNA transcript of the Hac1p transcription factor (Kaufman, 1999; McCracken and Brodsky, 2000; Pahl, 1999). Subsequent ligation of the two remaining mRNA strands is facilitated by the tRNA ligase, RIg1p (Fig. 1.4). Once ligated, the Hac1p transcription factor RNA is transported to the cytosol where it is translated. The Hac1p protein product is transported back into the nucleus where it acts as an initiator for UPR related genes (Kaufman, 1999; Pahl, 1999; Patil and Walter, 2001).

1.2.4 The UPR in mammals

Many details of the yeast UPR have been conserved in higher eukaryotes. In mammals the UPR is controlled and regulated by three sensory proteins, protein kinase R (PKR)-like ER-kinase (PERK), inositol-requiring transmembrane kinase (IRE1) and activating transcription factor 6 (ATF6). The presence of aberrant proteins in the ER triggers the oligomerisation and autophosphorylation of PERK. The activated PERK subsequently phosphorylates the eukaryote translation initiation factor 2α (eIF2α) which brings about its inactivation. This in turn stops protein translation, stopping the inflow of nascent polypeptides into the ER lumen, lowering the protein load (Bertolotti and Ron, 2001; Patil and Walter, 2001; Rao and Bredesen, 2002) (Fig. 1.5).

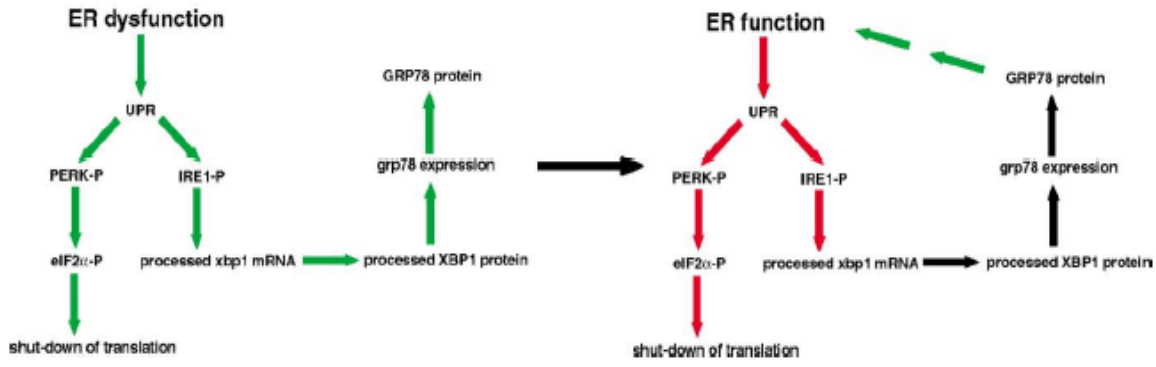

Figure 1.5: **Overview of ER stress-induced unfolded protein response (UPR).**
Conditions of ER stress bring about the activation of the UPR which in turn activates two ER resident kinases, PERK and IRE1, through phosphorylation. This results in the shut down of protein translation and the expression of proteins such as GRP78 (BiP) (green arrows). Once GRP78 levels have sufficiently increased, normal ER functioning resumes. This results in the down-regulation of the UPR (red arrows) (Paschen, 2003).

Accumulation of aggregated protein also leads to the translocation of ATF6 to the Golgi where it is processed through regulated intermembrane proteolysis (RIP) by the proteases S1P and S2P. This yields a free cytosolic domain, which, after translocation into the nucleus triggers the transcriptional upregulation of ER chaperone protein GRP78 (BiP) (Fig. 1.5) (Rutkowski and Kaufman, 2004; Shen *et al.*, 2004). One of the first and most important effects of the UPR is the activation of the ERAD pathway.

1.3 Endoplasmic reticulum associated degradation (ERAD)

The protein folding process, as previously mentioned, is carefully monitored and controlled by chaperones (Kabani *et al.*, 2003). ERAD substrates (misfolded proteins) are retrotranslocated out of the ER, a process mediated by chaperones such as BiP, where they are targeted to the proteasome by a process known as ubiquitination (Hirsch *et al.*, 2004).

1.3.1 Ubiquitination and the proteasome

Ubiquitination is a cytoplasmic process used to target proteins to a large number of biological processes including proteosomal degradation, endocytosis, viral budding and vacuolar protein sorting (Vps). Ubiquitin, a small 76 amino acid protein found in all eukaryotes, is ligated through its C-terminus to the lysine side chains of acceptor proteins (Plemper and Wolf, 1999). It is a highly stable protein, with resistance to heat as well as acidic and alkaline conditions, aiding in its defensive role against “stress induced accumulation of abnormal proteins”. Monoubiquitinylation refers to the process where one ubiquitin molecule is bound to an acceptor protein. Polyubiquitylation is a process where ubiquitin labels are themselves ubiquitylated and at least tetraubiquitination is required for proteasome targeting. Monoubiquitinylation, on the other hand, is sufficient to mark proteins for both endocytosis and lysosomal/vacuolar trafficking (Fisher *et al.*, 2003).

The process of ubiquitination is controlled by ubiquitin activating (E1), ubiquitin conjugating (E2) and ubiquitin ligase (E3) enzymes (Fig. 1.6). The ubiquitin activating enzyme (E1) forms a thioester bond with the C-terminal glycine residue of ubiquitin. This facilitates the transfer of ubiquitin to the ubiquitin conjugating enzyme E2 (Fig. 1.6). The transfer of ubiquitin to the substrate is subsequently either mediated by E2 or by the E3 ubiquitin ligase (Fig. 1.6) and substrates may be monoubiquitinated or polyubiquitinated. The ubiquitin label targets substrate proteins to the 26S proteasome where they are degraded, whereas the ubiquitin molecules are recycled for further rounds of substrate conjugation (Balakirev *et al.*, 2003; Fisher *et al.*, 2003; Plemper and Wolf, 1999) (Fig. 1.6).

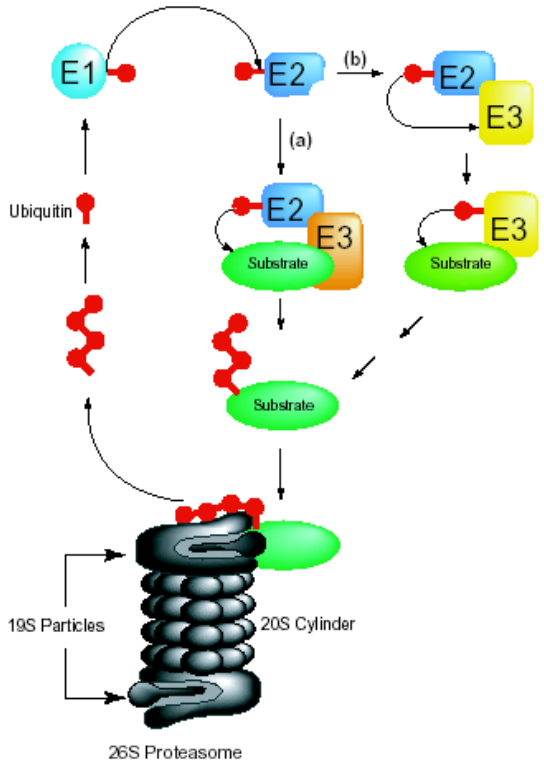

Figure 1.6: **Summary of the ubiquitin-proteasome system.**
The ubiquitin activating enzyme (E1) forms a thioester bond with the c-terminal glycine residue of ubiquitin. The ubiquitin residue is subsequently transferred to the active cysteine residue of an ubiquitin conjugating enzyme (E2). The ubiquitin ligase (E3) then facilitates the attachment of ubiquitin to the lysine side chains of the substrate protein. This can occur by the direct transfer of ubiquitin to the substrate by E2 (a) or through the E3 ubiquitin ligase (b). Polyubiquitination is achieved through the repeated addition of ubiquitin to lysine residues of bound ubiquitin. The ubiquitinated substrate is recognised and degraded by the 26S proteasome. Prior to hydrolysis, the ubiquitin chains are released from the complex and recycled (Plemper and Wolf, 1999).

Evidence for the ubiquitination process was first seen with the identification of the yeast membrane-anchored, ubiquitin-conjugating enzyme, Ubc6p. Ubiquitin protein ligases (E3s) (Fig. 1.6) determine the substrate specificity for ubiquitinylation and can be divided into HECT (homologous to the E6-AP carboxyl terminus) and RING (really interesting new protein) finger families. The RING-type ubiquitin ligase Hrd1p/Der3p has subsequently been found to be an integral membrane protein, working in conjunction with the ubiquitin-conjugating enzymes, Ubc7p and Ubc1p. Doa10p is another membrane spanning E3 enzyme which participates in the degradation of ER proteins and uses Ubc6p and Ubc7b to ubiquitinate substrate proteins. Substrate selection has been found to occur independently of the ubiquitin system, (Hirsch *et al.*, 2004; Hitchcock *et al.*, 2003). Ubiquitination has been found to enhance the rate of ERAD, but is not essential as examples of ubiquitin-independent ERAD have been found (Lord *et al.*, 2000).

1.3.2 Links between the UPR and ERAD

There is a high level of cooperation between the ERAD and UPR. Many of the specific components in the ERAD pathway are induced by the UPR, indicating a tight physiological link (Ng *et al.*, 2000). The *IRE1* pathway, described in Section 1.2.3, regulates chaperone induction, ERAD and the expansion of the ER in response to stress (Schroder and Kaufman, 2005). It has been found that ERAD is less efficient in strains which are *IRE1*-deficient and that induction of the UPR increased the efficiency of ERAD. If the UPR was upregulated, or the concentration of aberrant proteins increased to very high levels, ERAD was found to be compromised. This indicates a link between the UPR and ERAD but suggests that once the mass of aberrant proteins reaches a certain level, ERAD is reduced through saturation, resulting in the induction of the overflow pathways (McCracken and Brodsky, 2000).

The connection between ERAD and the UPR was further established when it was found that the deletion of ERAD-essential genes induced the UPR. Strains lacking certain ERAD genes and *IRE1* were found to be non-viable at elevated temperatures. This

suggests that cells are able to survive a certain level of aberrant proteins in their ER (i.e. ERAD deficiency), or the inability to respond to these aberrant proteins (UPR deficiency), but are unable to survive when both are compromised. Loss of any one of several ERAD genes also makes cells dependent on the UPR for normal growth under mild stress (Shen *et al.*, 2004).

The saturation or failure of ERAD has far reaching consequences, resulting in severe cellular stress and possible cell death. An alternative or "overflow" mechanism for the degradation and removal of aberrant proteins is, therefore, important and constitutes the second major pathway activated by the UPR.

1.4 Alternatives to ERAD: autophagy

The vacuole is a prominent organelle in the yeast cell. It is involved in a number of physiological processes such as cytosolic ion and pH homeostasis, as well as being a storage site for various metabolites such as basic amino acids and divalent cations such as calcium. A large number of membrane-bound and soluble hydrolases which facilitate the degradation and turnover of proteins are found in the vacuole (Klionsky, 1998). Once ERAD becomes saturated, mutant proteins are targeted to the vacuole for degradation via the Gvt and autophagic pathways. The autophagic and Cvt pathways will be discussed first.

1.4.1 Autophagy and the relationship with the cytoplasm-to-vacuole (Cvt) biosynthetic pathway

There are three autophagic pathways in which the vacuole is involved in. These are the chaperone-mediated, micro and macroautophagic pathways (Klionsky, 2005) (Fig. 1.7). Chaperone-mediated autophagy differs from the other two forms of autophagy as it is not vesicle-dependent and it allows the direct translocation of a targeted protein across the vacuolar membrane, a process conserved in mammalian cells (Klionsky, 2005; Massey *et al.*, 2004). Microautophagy, the least characterised of the processes,

sequesters cytoplasm by invagination or deformation of the vacuolar membrane to engulf cytosolic substrates (Fig. 1.7) (Cuervo, 2004; Levine and Klionsky, 2004). Macroautophagy, the most widespread of the processes, occurs by the formation of a membrane bilayer of non-vacuolar origin, creating an autophagic vacuole or autophagosome. This engulfs portions of the cytoplasm containing organelles or major aggregates to be removed (Fig. 1.7).

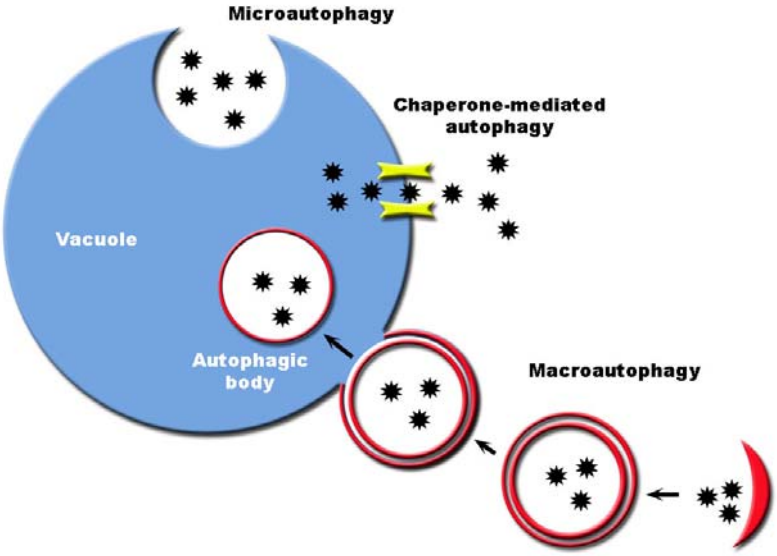

Figure 1.7: **Three ways in which autophagy mediates transport to the vacuole.** Microautophagy occurs by the invagination of the vacuolar membrane. Chaperone-mediated autophagy is independent of vesicle formation and allows for the direct translocation of proteins across the vacuolar membrane. Macroautophagy occurs by the formation of a double stranded membrane of non-vacuolar origin (autophagosome) which fuses with the vacuole, releasing the single membrane autophagic body into the vacuolar lumen (Teter and Klionsky, 2000).

These autophagic processes need to be distinguished from the cytoplasm-to-vacuole targeting (Cvt) biosynthetic pathway which is involved in precursor aminopeptidase 1 (Ape1) and alpha-mannosidase processing. This pathway overlaps mechanistically and genetically with autophagy (Fig.1.1 C and D)**.** Cvt vesicles (100 - 200 nm), however, are significantly smaller than macroautophagosomes (300 - 900 nm) and deliver cytoplasmic proteins to the vacuole for processing in order to effect their maturation (Kim *et al.*, 2002; Levine and Klionsky, 2004; Scott *et al.*, 1996). The membrane dynamics involved in the autophagic and Cvt pathways are unique from other cellular trafficking pathways and needs to be distinguished from that of the Gvt pathways.