UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

Whole transcriptome analysis to elucidate the role of *mtp* in gene regulation of pulmonary epithelial cells infected with *Mycobacterium tuberculosis*

By

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DECLARATION

I. Mr Mlungisi Thabiso Dlamini (208500070), declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. That my contribution to the project was as follows:

a) Infection of A549 epithelial cells with the wild type and *mtp*-knockout mutant strain of *M. tuberculosis* V9124 isolate.
b) Extraction of RNA from uninfected A549 epithelial cells (control) and cells infected with the wild type and mutant strain.
c) Analysis of RNA-sequencing data.
d) Dissertation write-up.

3. That the contributions of others to the project were as follows:
a) Professor Manormoney Pillay: Supervisor of the study.
b) Professor Balakrishna Pillay: Collaborator of the study.
c) Johns Hopkins University Deep Sequencing and Microarray Core: RNA-sequencing.
d) Dr Alecia Pillay: Guidance with upstream analysis of RNA-sequencing data.
e) Dr Nontobeko Mvubu: Guidance with downstream analysis of RNA-sequencing data.

4. Signed  Date: November 2016

Supervisor (Professor Manormoney Pillay)

DEDICATION

This work is dedicated to my late uncles, Mr Commando and Musa Nyawo. I would also like to thank my mother, Mrs Sibongile Goba, my aunt, Miss Thandazile Nyawo and my grandmother, Mrs Phumzile Grace Nyawo for their endless support, love and motivation.

PRESENTATIONS

Conferences:

- **Dlamini MT**, Mvubu NE, Pillay B, Christoffels A, Pillay M. (16 July 2016). Whole Transcriptome Analysis to Elucidate the Role of *M. tuberculosis* Curli Pili (MTP) on Host Gene Regulation in a Pulmonary Epithelial Cell Model. TB 2016 International Conference, Durban, South Africa. Poster Presentation.

Symposiums:

- **Dlamini MT**, Mvubu NE, Pillay B, Christoffels A, Pillay M. (16 July 2016). Whole Transcriptome Analysis to Elucidate the Role of *M. tuberculosis* Curli Pili (MTP) on Host Gene Regulation in a Pulmonary Epithelial Cell Model. University of KwaZulu-Natal College of Health Sciences Research Symposium 2016. Oral Presentation.

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LIST OF ABBREVIATIONS

AECOM	Albert Einstein College of Medicine
AES	Allelic Exchange Substrate
Ag85	Antigen 85 Complex
ANOVA	Analysis of Variance
APC	Antigen-presenting Cell
ART	Antiretroviral Therapy
BCG	Bacilli Calmette-Guerin
cDNA	Complementary DNA
CFU	Colony Forming Unit
CR	Complement Receptor
DEG	Differentially Expressed Gene
ECM	Extracellular Matrix
ELISA	Enzyme-linked Immunosorbent Assay
EMB	Ethambutol
EMEM	Eagle's Minimal Essential Media
ESAT 6	Early Secretory Antigen Target 6
FBS	Fetal Bovine Serum
FcR	Fc Receptor
FPKM	Fragments Per Kilobase of Transcript per Million Mapped Reads
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GPI	Glycophosphatidylinositol
HBHA	Heparin-binding Hemagglutinin Adhesin
HIV	Human Immunodeficiency Virus
ICT	Immunochromatographic Test
IF	Immunofluorescence
IFN	Interferon
IL	Interleukin
INH	Isoniazid
IPA	Ingenuity Pathway Analysis
KZN	KwaZulu-Natal
LAM	Lipoarabinomannan
LBP	Laminin-binding Protein
LM	Lipomannan
LPA	Line-probe Assay
LPS	Lipopolysaccharides

LTBI	Latent Tuberculosis Infection
ManLAM	Mannose-capped Lipoarabinomannan
MDR-TB	Multidrug-resistant Tuberculosis
MeV	MultiExperiment Viewer
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
MOPS	3-(N-Morpholino) Propanesulfonic Acid
MR	Mannose Receptor
MS	Malate Synthase
MTBC	*M. tuberculosis* Complex Strains
MTP	*Mycobacterium tuberculosis* Curli Pili
NAA	Nucleic Acid Amplification
NAAT	Nucleic Acid Amplification Test
OADC	Oleate-Albumin-Dextrose-Catalase
PAMP	Pathogen-associated Molecular Pattern
PAS	Para-aminosallicylate sodium
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PILAM	Phosphor-myo-inositol-capped Lipoarabinomannan
PIM	Phosphatidyl-Myo-Inositolmannosides
PMA	Phorbol-myristate Acetate
POC	Point of Care
PRR	Pattern Recognition Receptor
PZA	Pyrazinamide
RIF	Rifampin
RIN	RNA Integrity
RNA-seq	RNA Sequencing
RT	Room Temperature
RT-PCR	Real-time Polymerase Chain Reaction
SA	South Africa
SDEG	Significantly Differentially Expressed Gene
SPR	Surfactant Protein Receptor
SR	Scavenger Receptor
TA	Toxin-Antitoxin
TB	Tuberculosis
TDR-TB	Totally Drug-resistant TB
TEM	Transmission Electron Microscopy

TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
WHO	World Health Organisation
XDR-TB	Extensively Drug-resistant Tuberculosis

ABSTRACT

Background: Bacterial adhesins, including *Mycobacterium tuberculosis* curli pili (MTP), are the first point of contact with host cells. MTP bind laminin of the extra cellular matrix and are viewed as bacterial virulence factors due to their key role in pathogenesis. Since the seminal paper describing the presence of pili in *M. tuberculosis*, research in this field, has been steadily gaining momentum to further characterize this pathogen's curli pili to interrogate its potential as a biomarker for rapid diagnostics and therapeutic interventions. In this study, we performed global transcriptomics in a pulmonary epithelial cell infection model to investigate the role of MTP as a potential biomarker for TB diagnostics and vaccine and drug development. The epithelial cell model was chosen due to the increasing body of evidence demonstrating that *M. tuberculosis* is able to invade and survive inside these cells.

Materials and Methods: RNA was extracted from A549 human alveolar epithelial cells infected with *M. tuberculosis* wild type (WT) and *mtp*-knockout mutant strains 4 hours post-infection and sequenced using the Illumina HiSeq RNA Sequencing Platform. Sequencing reads were aligned to the human reference genome (*Homo sapiens* UCSC hg38) using *TopHat* and differential gene expression profiles were generated with *Cuffdiff*. An FPKM pairwise scatter plot comparison was performed in order to investigate global gene expression profiles of uninfected A549 epithelial cells relative to A549 cells infected with the mutant and wild type strain. Differentially expressed genes were filtered using a cut-off point of ≥ 2 and a Venn diagram was created using the VENNY software. Gene Ontology analysis was performed using the Comparative Toxicogenomics Database. Heat maps of gene expression profiles in A549 epithelial cells infected with *M. tuberculosis* V9124 wild type and *mtp*-knockout strains were created using a MultiExperiment Viewer (MeV). Changes in canonical pathways, network and transcription factor enrichment were investigated with Ingenuity Pathway Analysis (IPA). The significance of differences between the two strains was analysed by one-way analysis of variance (ANOVA) SPSS 24.0 statistical software, $p < 0.05$ was considered significant.

Results: In this study, the number of invaded bacteria observed in A549 epithelial cells infected with the wild type strain (2.47×10^3 CFU/ml) did not differ significantly (p=0.121) when compared to the *mtp*-knockout mutant strain (1.67×10^3 CFU/ml). Our results further showed that, as a result of *mtp* deletion, the number of significantly differentially expressed genes and canonical pathways induced in A549 epithelial cells was reduced, suggesting that MTP regulates gene expression in epithelial cells in vitro. The WT and Δ*mtp*- strains uniquely induced 307 (89 up-regulated and 218 down-regulated) and 115 (42 up-regulated and 73 down-regulated) genes respectively while 610 (345 up-regulated and 265 down-regulated) genes were induced by both strains. The loss of MTP resulted in a lower number, 115 (27%) of genes induced only by the mutant strain compared to 307 (73%) by the wild type strain.

The wild type strain enriched a higher number of canonical pathways in epithelial cells i.e. 44 (59%) compared to 30 (41%) by the mutant strain. A total of 27 pathways was unaffected by MTP as these were enriched by both strains. The majority of these pathways, 23 (85%), were highly enriched by the mutant strain compared to the wild type strain. Cell surface receptors such as *CD14*, *CD24*, *CD38*, *CD96* and *CD99* were found to be more up-regulated by the wild type strain when compared to the mutant strain.

Whilst the majority of genes associated with cytokine receptors and tumor necrosis factor (*TNF*) receptors were down-regulated by both strains, the wild type strain highly up-regulated genes coding for chemokine receptors compared to the mutant strain. Our findings show an up-regulation of chemokines compared to cytokines by both strains. *CCL2, CCL26, CCL28, CCL5, CXCL1, CXCL3, CXCL5, CXCL6* and *CXCL9* chemokines and *IL-11, IL-15*, and *IL-32* cytokines were highly up-regulated by the wild type strain compared to the mutant strain. Genes associated with antigen presentation were down-regulated by both strains in epithelial cells except for *HLA-DMA* and *HLA-DMB*. Our network analysis showed that the wild type strain enriched a similar number of networks when compared to the mutant strain. However, the wild type strain enriched top ranking networks compared to those that were enriched by the mutant strain.

Discussion and Conclusion: In this study, the number of invaded bacteria in A549 cells infected with the *mtp*-mutant strain did not differ significantly when compared to wild type-infected A549 cells, 4 hours post-infection. Our gene ontology and canonical pathway analyses strongly suggest that *M. tuberculosis* invades epithelial cells with the aid of one of its major adhesins, MTP, which induces differential host gene expression leading to the cellular and host immune response against the pathogen.

The wild type strain induced the highest total number (917) of genes when compared to the mutant (725) while 610 genes were expressed by both strains. This reduction by 12%, in the number of genes expressed by the mutant strain shows that MTP plays an important role in gene regulation of alveolar epithelial cells. The enrichment of a high number of pathways by the wild type strain suggests that MTP plays an important role in gene regulation of alveolar epithelial cells. Our findings also show that the deletion of MTP resulted in down-regulation of some of the genes that were up-regulated in the wild type infection. These findings suggest that the presence of the MTP adhesin in the wild type strain plays an important role in the launch of a strong immune response against this strain, while the knocking out of the *mtp* gene negatively affects the ability of epithelial cells to respond to *M. tuberculosis* infection. MTP highly induced the production of chemokines in A549 epithelial cells compared to cytokines. This is supported by previous findings in our group that MTP did not largely influence cytokine production in *M. tuberculosis*-infected epithelial cells (Ramsugit *et al.*, 2015). *IL-*

1, IL-4, IL-6, IFN-Y, and *G-CSF* were produced at low concentrations by epithelial cells at 24, 48 and 72 hours post infection (Ramsugit *et al.*, 2015).

Our findings add to the growing evidence that *M. tuberculosis* uses MTP as one of its major invasins and virulence factors during the infection of host cells. Our data confirms that epithelial cells play an important role in host immune response to infections. In the absence of MTP, the number of significantly differentially expressed genes and canonical pathways induced in A549 alveolar epithelial cells was reduced. Our findings further showed that alveolar epithelial cells infected with the wild type strain launched a stronger host immune response when compared to those infected with the *mtp*-knockout mutant strain. Taken together, these findings suggest that MTP plays an important role in gene regulation of A549 alveolar epithelial cells and could be a potential TB vaccine candidate and antimicrobial drug target. Thus, the development of rapid antimicrobial drugs and vaccines that target MTP would play a huge role in the treatment and prevention of TB infections as these structures are mainly involved in adherence to and invasion of host cells, and have been shown to have an ability to induce a strong immune response in epithelial cells in vitro.

INTRODUCTION

Tuberculosis (TB) is an infectious bacterial disease caused by *Mycobacterium tuberculosis* which usually affects the lungs as well as the respiratory system (Mishra *et al.*, 2014). Despite a major reduction in TB cases and deaths in the past decade, the disease continues to be a major public health problem (WHO, 2015). In 2014, about 9.6 million people developed TB and 1.5 million people died from the disease, making TB a leading cause of death in humans (WHO, 2015). One of the challenges facing TB treatment includes human immunodeficiency virus (HIV) co-infection, emergence of drug-resistant tuberculosis (WHO, 2014) as well as the lack of biomarkers for early diagnosis and treatment (Murray, 2007). The design and development of rapid TB diagnostic tools and novel drugs is hampered by the shortage of suitable biomarkers that can serve as drug targets (Murray, 2007). Adhesins, due to their surface localisation, may serve as potential novel targets for rapid TB diagnosis as well as antimicrobial drugs and vaccine development (Kumar *et al.*, 2013).

Attachment of pathogenic bacteria to any mucosal surfaces is an essential step in the pathogenesis of many infections. In order to achieve this, bacteria make use of an array of adhesins displayed at their cell surfaces. These highly specific proteins are usually expressed at different stages during infection (Kumar *et al.*, 2013). Interactions between infecting bacteria and host cells are aided by specialized microbial adhesins that modulate binding to the surface of host cells (Klemm and Schembri, 2000). Adhesins allow pathogenic bacteria to attach to host cells and resist physical removal from the surface and that leads to invasion and induction of the host immune response (Niemann *et al.*, 2004). Microbial adhesins usually appear as polymeric organelle structures as well as non-organelle structures attached to the surface of bacterial cells as monomers or oligomers (Klemm and Schembri, 2000).

M. tuberculosis expresses a number of different essential adhesins including heparin-binding hemagglutinin adhesin (HBHA), Apa, malate synthase (MS), 19 kDa antigen, laminin binding protein (LBP) (Kumar *et al.*, 2013) and curli pili (MTP) (Alteri *et al.*, 2007). HBHA is involved in binding of bacteria to lungs and in extrapulmonary dissemination of the bacteria (Kumar *et al.*, 2013). Curli pili (MTP) bind laminin and are produced during the infection of host cells (Alteri *et al.*, 2007). MTP, encoded by the *mtp* gene (Rv3312A), is viewed as a bacterial virulence factor due to its key role in pathogenesis and is therefore an important target for vaccine and drug development (Finlay and Falkow, 1997). MTP is associated with biological activities such as colonization of mucosal surfaces, agglutination of human and animal red blood cells, biofilm formation and bacterial adherence (Finlay and Falkow, 1997). *M. tuberculosis* interacts with and invades different animal and human epithelial cells. However, very little is known about the interactions that take place between the microbe and host cells before bacterial survival and replication (Lin *et al.*, 1998). Previous studies have indicated

that alveolar epithelial cells play a significant role in immune response by recruiting immune cells such as lymphocytes, neutrophils and monocytes to the site of infection through cytokine and chemokine production (Lin *et al.*, 1998).

Different technologies used to study host-pathogen interactions have been developed and some of these include hybridization-based techniques such as microarrays (Mantione *et al.*, 2014). This is a high throughput technique that has been widely used in whole genome studies in the past due to its inexpensive nature (Wang *et al.*, 2009). However, microarrays have high levels of background noise owing to cross-hydridization and have a limited range of detection (Mantione *et al.*, 2014). RNA sequencing, a recently developed technique for whole transcriptome profiling, can achieve a higher resolution and has a much lower limit of detection compared to microarrays (Wang *et al.*, 2009). In this study, we used whole transcriptome analysis in order to investigate the role of MTP in gene regulation of A549 pulmonary epithelial cells infected with *mtp*-gene knockout mutant and wild type strains of *M. tuberculosis* V9124, a clinical isolate of the F15/LAM4/KZN (KZN) family.

Dissertation Outline

The first chapter presents a review of the relevant literature. Chapter 2 describes all the methods that were used in order to carry out objectives of the study. Chapter 3 presents the results that we obtained in the study while chapter 4 provides a discussion which integrates the findings of chapter 3 and highlights conclusions and future recommendations.

CHAPTER 1: LITERATURE REVIEW

1.1. Epidemiology of TB

Mycobacterium tuberculosis is a causative agent of tuberculosis (TB), a deadly disease that caused about 1.5 million deaths while 9.6 million new cases were reported in 2014 and is now ranked as the leading cause of death in human population (WHO, 2015). *M. tuberculosis* is a Gram-positive bacillus with a cell wall rich in mycolic acid, an unusual waxy coating that makes the cells impervious to gram staining and its physiology requires high levels of oxygen and Mg (2+) in a mildly acidic environment (Kassim, 2004). Inhalation of airborne *M. tuberculosis* bacilli, followed by adherence and invasion of cells located at the surface of the lungs leads to a successful establishment of a TB infection (Hickey *et al.*, 2010). *M. tuberculosis* primarily infects the lungs where it causes pulmonary TB infections. However, it can also disseminate to other body parts such as lymphatic, central nervous and genitourinary systems, where it causes extra pulmonary TB infections which are more common in children and immune compromised individuals (WHO, 2015). About one third of the world's population harbours *M. tuberculosis* in a latent form that shows no clinical symptoms and is difficult to diagnose (WHO, 2015).

Despite a slight decline in global TB rates over the years, South Africa (SA) remains one of the worst hit countries by TB. Among high TB-burden countries, SA has the third and fifth highest number of reported TB cases and undiagnosed active TB cases respectively (Churchyard *et al.*, 2014). The growing burden of MDR-TB coupled with the emergence of XDR-TB in 2006 added a further load to overstretched health facilities (Churchyard *et al.*, 2014). The increase and decline in cure rates and treatment default respectively, has increased TB treatment success rate in SA, for both new smear positive and smear negative TB patients (WHO, 2015). The high mortality rate even after treatment completion could be attributed to the HIV disease (Abdool Karim *et al.*, 2009). Therefore, scaling up antiretroviral therapy (ART) in TB patients infected with HIV is needed in order to reduce HIV-related deaths (Churchyard *et al.*, 2014). SA has made notable progress in enhancing TB control, however, the TB burden remains very high.

A study conducted by Hassim *et al.* (2010) revealed that about 20% of people with TB/HIV co-infection in SA were infected with MDR-TB strains, introducing a major stumbling block in fighting the worsening TB/HIV pandemic. The KwaZulu-Natal (KZN) province in SA is the epicenter of TB/HIV co-epidemic with an HIV prevalence of about 40% and TB notification rate of 1094/100 000 population (Houlihan *et al.*, 2010; Health Systems Trust, 2011). KZN experienced a well-documented MDR and XDR-TB outbreak and remains one of the provinces with the highest burden of these types of TB infection (Gandhi *et al.*, 2006; Bateman, 2010). Absent or delayed TB diagnosis leads to the

delay of TB treatment, therefore increasing transmission and mortality and morbidity rates (Kasprowicz *et al.*, 2011). Autopsy studies in SA have revealed that up to 50% of patients with HIV infection or AIDS have TB infection at their time of death (Ansari *et al.*, 2002; Cohen *et al.*, 2010). Furthermore, 17% of HIV-related TB cases at death were due to MDR strains (Cohen *et al.*, 2010).

1.2. Drug resistance

1.2.1. *Multidrug-resistant TB (MDR-TB)*

M. tuberculosis is a very slow growing intracellular organism, therefore, TB treatment requires the use of multiple antimicrobial drugs such as isoniazid (INH), rifampin (RIF), ethambutol (EMB) and pyrazinamide (PZA) which are considered first-line anti-tuberculosis drugs and aminoglycosides, fluoroquinolones, polypeptides, thioamides, cycloserine and *p*-aminosalicylic acid which are second-line drugs (WHO, 2015). TB infection caused by *M. tuberculosis* resistant to at least INH and RIF is referred to as multidrug-resistant TB (MDR-TB). Globally, 5% of TB cases were estimated to have had MDR-TB in 2014. Drug resistance surveillance data revealed that about 480 000 people were infected with MDR-TB in 2014 and 190 000 people died as a result (WHO, 2015). An estimation of about 1.8% of new TB cases and 6.7% of previously treated TB cases in SA are multi drug resistant (WHO, 2014). However, the majority of new MDR-TB cases are as a result of transmission of already resistant strains (Dheda *et al.*, 2010). Unpublished data from the Western Cape department of Health revealed that half of new MDR-TB cases diagnosed every year have never received treatment (Hughes and Osman, 2014). MDR-TB surveillance in KZN, especially Tugella Ferry, revealed that an extraordinary high incidence of TB in the area. Among MDR and XDR-TB patients, the rate of HIV co-infection was reported to be more than 90% (Giffin and Robinson, 2009).

1.2.2. *Extensively drug-resistant TB (XDR-TB)*

TB infection resistant to INH and RIF as well as to any member of the fluoroquinolone family and one of the aminoglycosides or polypeptides is known as extensively drug-resistant TB (XDR-TB) (WHO, 2015). In 2014, XDR-TB was reported by 105 countries globally, with an estimated average of 9.7% people with MDR-TB having XDR-TB (WHO, 2015). This type of TB infection is associated with the development of resistance towards drugs in virulent *M. tuberculosis* strains as well as with poor treatment outcomes, especially among individuals infected with HIV (Tsara *et al.*, 2009; Shah *et al.*, 2011). XDR-TB strains arise when MDR-TB is treated poorly, which allows amplification of resistance towards second-line drugs. Inadequate treatment for XDR-TB may result in additional resistance which could limit options for effective TB treatment (Shah *et al.*, 2011). Early diagnosis and treatment of infectious patients with active pulmonary TB is considered crucial in decreasing the transmission and possibly the elimination of *M. tuberculosis* (Tsara *et al.*, 2009). Moreover, early identification and treatment of individuals with latent TB infections is important for disease control

due to their high risk to develop active TB infection. In SA, 10% of MDR cases are XDR (WHO, 2014) and initial treatment of these cases with MDR drugs is suspected to contribute to amplified drug resistance as a result of selective pressure of treatment with one or two effective drugs (Muller *et al.*, 2012). In 2007, XDR had been reported by 60 health care facilities in KZN. A total of 47 000 case of TB had been reported with 6% of them being XDR. According to the WHO 2013 TB report, treatment success rate was 48% and 22-28% for MDR and XDR respectively, with a death rate of 44-49% in individuals with XDR-TB (Muller *et al.*, 2012).

1.2.3. *Totally drug-resistant TB (TDR-TB)*

Totally drug-resistant TB (TDR-TB) is acquired as a result of resistance to all first- and second line drugs used to treat TB (Parida *et al.*, 2014). TDR-TB was first discovered in two patients in Italy in 2003 and subsequently reported in 2007 (Migliori *et al.*, 2007). Iran reported second TDR-TB cases where 10.3% *M. tuberculosis* isolates from 146 patients with MDR-TB were found to have TDR-TB infection (Velayati *et al.*, 2009). Other TDR-TB cases have been reported in India while 92% of 236 MDR-TB strains collected in SA belonged to an atypical Beijing strain genotype that is resistant to 10 first- and second line anti-TB drugs. Some of these strains were reported to be resistant to para-aminosallicylate sodium (PAS), suggesting the emergence of TDR-TB in SA (Klopper *et al.*, 2013). TDR-TB has only been reported in four countries (Italy, Iran, India and SA) so far, however, factors such as limited laboratory access to in resource-limited countries with high TB prevalence could lead to the under-representation of the real extent of drug resistance in *M. tuberculosis* isolates (Parida *et al.*, 2014).

1.3. Vaccines

New TB treatment strategies are needed, but the most effective tool would be a vaccine that prevents infections (Barker *et al.*, 2011). Current TB research strategies have mainly focused on the development of an improved bacilli Calmette-Guerin (BCG) vaccine to be given at birth and a booster vaccine to be administered after BCG (Checkley and McShane, 2011). In case-control studies, the BCG vaccine given to newborns has often been associated with reduced severity of childhood TB, including brain-affecting meningeal TB and disseminated miliary TB (Barker *et al.*, 2011). However, administering the vaccine to newborns does not seem to offer protection against adult pulmonary TB which is accountable for the global TB morbidity and mortality burden (WHO, 2014). The reactivation of latent TB infection to active TB and re-infection with *M. tuberculosis* after successful TB treatment indicates that immune response to TB infection is complex, therefore, multiple vaccines may need to be developed (Barker *et al.*, 2011). A major stumbling block in the development of an effective vaccine against TB infection is the lack of understanding of the host immune response to *M. tuberculosis* that is associated with protection (Barker *et al.*, 2011). However, studies on CD4, CD8

and Th17 T cells have revealed that these cells can specifically target *M. tuberculosis* and produce multiple signalling molecules that offer protection to host cells (Barker *et al.*, 2011).

Apart from not being effective against adult active TB, some studies have shown that the BCG vaccine enhances the risk of *M. tuberculosis* infection (D'Arcy and Sutherland, 1977; Baily, 1979; Sadoff, 2009). A South African TB Vaccine Initiative in the Western Cape province of SA conducted a trial in >10 000 infants over 18 months and found a 4.5% incidence of the disease despite routine BCG vaccination (Hawkridge *et al.*, 2008). The spread of HIV infection, which is concentrated in areas with high TB burden, offers an additional risk when the BCG vaccine is administered to HIV-infected infants. Despite being a highly attenuated live vaccine, the BCG vaccine causes extra-pulmonary TB in HIV-infected children at rates as high as 1% (Beresford and Sadoff, 2010) and it is for this reason that the WHO has recommended that the vaccine should not be administered to HIV-infected infants. Most infants are not tested for HIV infection and this provides a major problem to policy makers in allowing vaccination and risk diseases in HIV-infected children or not to allow vaccination and risk the development of different forms of the TB disease which children infected with HIV are susceptible to (Hesseling *et al.*, 2009). The effectiveness of the BCG vaccine coupled with the risk of adverse effects demands the development of better vaccines to offer protection to everyone.

1.4. Anti-TB drugs

Current TB treatment is based on different combinations of drugs with high cure rates for drug-susceptible strains of *M. tuberculosis* when lengthy and complex treatment protocols are adhered to (Laurenzi *et al.*, 2007). However, the lack of adherence to treatment protocols, incorrect prescribing of drugs, emergence of drug-resistant TB and drug-to-drug interaction that interferes with optimal treatment of HIV and TB co-infection have generated a need for improved TB treatment interventions (Laurenzi *et al.*, 2007). Current drugs used to treat TB were developed decades ago when both drug development processes and regulatory environments significantly differed from today's processes (Laurenzi *et al.*, 2007). Success rates achieved by these drugs are high but still need complex and more recent processing protocols. Drugs with different mechanisms of action are likely to kill various populations of bacteria (Ying, 2003). Isonaizid is the most active of the current TB drugs against actively replicating *M. tuberculosis* while rifampicin is active against both actively replicating and non-replicating mycobacteria through the inhibition of RNA synthesis (Ying, 2003).

Problems associated with current TB drugs include the longevity and the complex nature of treatment protocols which impact negatively on patient adherence and drive the emergence of drug-resistant TB (Laurenzi *et al.*, 2007). Reasons for inadequate TB treatment include incomplete implementation of regimens especially the duration of treatment, drug quality and number of drugs and their dosages

(van den Boogard *et al.*, 2009). Another challenge for the successful treatment of TB is the high prevalence if HIV and TB co-infection as these two infections are synergistic and the risk of progression from latent TB to active TB is estimated to be 50-fold higher in HIV-infected individuals compared to HIV negative patients (WHO, 2003). Current TB drugs have been reported to have side effects such as liver disfunctioning, hepatotoxicity, ototoxicity, neuropsychiatric manifestations and hyperuricemia (Gulbay *et al.*, 2006). There's an urgent need to improve current TB treatment strategies by either introducing new drugs on the pipeline or enhancing the application of existing ones. Potential new drugs should have reduced treatment duration, be active against MDR and XDR-TB, be applicable in TB patients infected with HIV, have a good and acceptable profile of tolerability, be readily available in all healthcare facilities, have a reduced cost for better access in developing countries with high TB burden and they must also be active against latent TB infections (van den Boogard *et al.*, 2009). Novel biomarkers are urgently needed to satisfy these criteria for the development of effective drugs.

1.5. Immunotherapeutics for TB Treatment

Granulomas, a hallmark of the host response to *M. tuberculosis* infection, are employed by host cells in order to physically contain infections that cannot be removed by host cell defences (Wallis, 2005). The sequential recruitment of host immune cells towards the site of infection creates a barrier that prevents dissemination of *M. tuberculosis* and provides a hostile environment with reduced pH, micronutrients and oxygen tension (Wallis, 2005). *M. tuberculosis* undergoes alterations in biosynthesis, metabolism and replication when faced with this hostile environment and this adaptation forms the basis of latency in TB infection (Wallis, 2005). The reactivation of *M. tuberculosis* latency to active TB which has poor treatment outcomes has given rise to the development of adjunct immunotherapy to treat TB (Uhlin *et al.*, 2012). Dampening of pro inflammatory response to TB could be of value in the treatment of individuals with non-productive TB infection (Uhlin *et al.*, 2012). The use of interleukin 2 (*IL-2*), interferon ϒ (*IFN-ϒ*) and tumor necrosis factor (*TNF*) in immunotherapy may increase success rates in the treatment of MDR-TB and could shorten the time it takes to treat drug-susceptible TB by improving the immune system of infected individuals through the elimination of *M. tuberculosis* to prevent TB recurrence (Wallis, 2005; Uhlin *et al*, 2012).

IFN-ϒ plays an essential role in host immune response against mycobacteria (Jouanguy *et al.*, 1996). In mice, it increases the mycobactericidal ability of macrophages through the production of reactive nitrogen intermediates (Flesch and Kaufmann, 1987; Chan *et al.*, 1992). Condos *et al.* (1997) conducted the first trial of therapeutic *IFN-ϒ* in patients with TB where they found no defects in *IFN-ϒ* production or responsiveness. In this study, 500µg of *IFN-ϒ* was administered 3 times a week to patients with MDR-TB and their sputum smear results were negative while the number of colony forming units (CFUs) decreased. *IL-2* is essential for host immune cell functioning and also promote

T cell replication (Wallis, 2005). In a study conducted in 1997, the administration of 2 low-dose *IL-2* regimens per day to patients with MDR-TB was found to have decreased CFU counts (Johnson *et al.*, 1997). The *TNF* cytokine is expressed by T cells and macrophages as a cell surface-associated cytokine or soluble homotrimer (Wallis *et al.*, 1990; Black *et al.*, 1997). *TNF* is also involved in the stimulation of cytokine and chemokine release, as well as endothelial adhesion molecules (Wallis *et al.*, 1990). *TNF* is expressed at the site of infection in patients with newly diagnosed TB and its levels increase after the initiation of anti-tuberculosis therapy (Bekker *et al.*, 1998) and decrease when the mycobacterial burden is reduced by treatment (Ribeiro-Rodrigues *et al.*, 2002). Animals that lack *TNF* have been found to be more susceptible to granulomatous infections (Flynn *et al.*, 1995). This makes *TNF* a suitable candidate for immunotherapeutic treatment of TB infections. Due to challenges associated with current TB diagnostic tools anti-TB drugs, an area of TB treatment using immunotherapy needs to be investigated further. *M. tuberculosis* adhesins, that have been identified to play a significant role in modulating host response, could potentially contribute to this type of adjunct therapy, and therefore, should be included in such investigations.

1.6. TB Diagnostic Techniques and Challenges

TB can be diagnosed through a number of diagnostic tests e.g. tuberculin skin tests, chest x-rays, sputum smear microscopy and sputum culture. The most common method for TB diagnosis worldwide is sputum smear microscopy (WHO, 2013). It is a cost-effective test with low sensitivity and can miss up to 40% of all TB cases especially in regions with high HIV/AIDS incidence (Maertzdorf *et al.*, 2007). The sputum culture test is costly but more sensitive. However, it takes several weeks to produce results and this could lead to the transmission of TB during the waiting period.

In order to achieve higher sensitivity and specificity compared to the traditional microscopic sputum examination and culture based methods, new rapid and accurate diagnostic methods need to be put into place (Tsara *et al.*, 2009). Current TB diagnostic methods are characterized by low specificity, reduced sensitivity as well as false results that can even occur in severely ill patients. Substantial research has led to the development of diagnostic tests such as nucleic acid amplification (NAA) tests that can be applied directly to sputum samples (Tsara *et al.*, 2009).

The Genotype MTBDR*plus* test, a line-probe assay (LPA), is used for rapid detection of *Mycobacterium tuberculosis* and mutations that result to resistance to rifampin and isoniazid in smear-positive sputum specimens (Chainani, 2013). Genotype MTBDR*plus* is used in the rapid diagnosis of MDR-TB in known, smear-positive TB cases in which drug-resistance is suspected and in culture isolates. However, it has a sensitivity of 84.3% and 98.1% for INH and RIF respectively (Chainani, 2013). This means that it can miss TB cases in patients with low mycobacterial load therefore, further

testing is always needed. The Line Probe Assay uses multiplex PCR amplification and reverse hybridization to identify *M. tuberculosis* complex and mutations associated with rifampicin and isoniazid resistance and has been recommended by WHO for rapid screening of MDR-TB in low and middle income settings (Albert *et al.*, 2010). It is highly specific, sensitive and can be performed directly from culture isolates or smear-positive sputum providing results in 1-2 days. However, false results have been reported (Albert *et al.*, 2010).

In December 2010, the World Health Organization introduced a novel rapid test for tuberculosis, the GeneXpert (Xpert MTB/RIF). This cartridge based machine is a fully automated nucleic acid amplification test (NAAT) capable of diagnosing TB and resistance to rifampicin (an indicator for MDR-TB) within two hours (WHO SA, 2011). However, it is not regarded as a point of care test due to its expensive nature and the machine can only be operated by trained personnel (WHO, 2011). Amplicor MTB test and the Amplifier Mycobacterial Tuberculosis Direct test are characterized by high specificity in positive sputum smear and short times needed for the diagnosis (Tsara *et al.*, 2009). However, these NAA tests need to be confirmed by sputum culture and they have low specificity and sensitivity for extra-pulmonary TB (Tsara *et al.*, 2009). In addition, molecular-based methods are laboratory based and require expensive equipment as well as specialized personnel (WHO, 2013).

The need to facilitate early TB treatment and disease transmission has led to the discovery of potential point of care tests such as the Immunochromatographic Test (ICT) (Gounder *et al.*, 2002), Capilia TB Assay (Muyoyeta *et al.*, 2010), Urine Lipoarabinomannan (LAM) (Peter *et al.*, 2013) and the MPB64 Antigen Assay (Nakamura *et al.*, 1998). ICT is a rapid, card-based immunochromatographic test that is used to detect antibodies directed against *M. tuberculosis* antigens. Since target proteins are secreted by actively growing mycobacteria, ICT has the potential to have a higher degree of specificity for active TB infections and the use of multiple antigens may increase its sensitivity (Gounder *et al.*, 2002). However, serologically based tests are often associated with cross reactivity which leads to low sensitivity (Pai and Pai, 2012).

Muyoyeta *et al.* (2010) evaluated the performance and cost of the Capilia TB Assay in South Africa and Zambia for its use in resource-limited settings. The assay was found to have a specificity and sensitivity of 99.5% and 99.6% respectively. These findings indicate that the Capilia TB Assay is an inexpensive, rapid and simple *M. tuberculosis* identification test that can be applied in resource-limited settings. However, its use has been validated on culture and not on sputum specimens (Muyoyeta *et al.*, 2010). Urinary LAM has recently been studied for diagnosis of TB in HIV-infected patients (Peter *et al.*, 2010). The presence of LAM in patients has been associated with higher mycobacterial burden and mortality rate (Talbot *et al.*, 2012). LAM is detected by a low cost and

simple point of care lateral assay that provides results in 25 minutes (Peter *et al.*, 2010). However, it remains unclear whether this test would be useful in high HIV prevalence settings (Peter *et al.*, 2013).

MPB64, an antigen specific to the *M. tuberculosis* complex, was first described as MPT64 and has been well studied in laboratories (Nakamura *et al.*, 1998). This antigen is used as a skin test reagent for the diagnosis of TB in patients. The use of the MPB64 Patch Assay as a point of care test to detect active TB is promising, however, it is only applicable in patients with active TB infections (Kumar *et al.*, 2011). The design and development of rapid TB diagnostics, novel drugs and an effective vaccine is hampered by the lack of suitable biomarkers that can serve as targets, despite the considerable amount of research that has been conducted in this area.

1.7. *M. tuberculosis* Characteristics

M. tuberculosis is classified as a Gram-positive bacterium, due to its unique cell wall composition which causes poor staining with crystal violet (Ducati *et al.*, 2006). The bacterium is rod-shaped, non-sporulating and does not have a flagellum or capsule (Ghosh *et al.*, 2009). Its waxy and complex cell wall gives the bacillus acid-fast properties, making it resistant to decolourization by acids during staining procedures (Welin, 2011). *M. tuberculosis* is a very slow-replicating bacterium with a doubling time of about 24 hours (Welin, 2011). It has a diameter of 0.5 μm and a length of about 1-4 μm and is a facultative anaerobic intracellular pathogen (Ducati *et al.*, 2006). Its uniquely composed cell wall consists of long chains of fatty acids called mycolic acids (Figure 1) that are linked to a peptidoglycan-attached arabinogalactan (Welin, 2011). The cell wall comprises several lipoglycans such as lipoarabinomannan (LAM), its precursor lipomannan (LM) and phosphatidyl-myo-inositolmannosides (PIM) (Welin, 2011). All these components are non-covalently attached to the plasma membrane through glycophosphatidylinositol (GPI) anchors and extend to the exterior of the cell wall (Ducati *et al.*, 2006; Briken *et al.*, 2004). LAM is made up of a phosphatidyl-myo-inositol anchor, a D-mannan polymer attached to the inositol ring, D-arabinose chains and capping motifs that are situated at the end of arabinose residues (Vergne *et al.*, 2003).

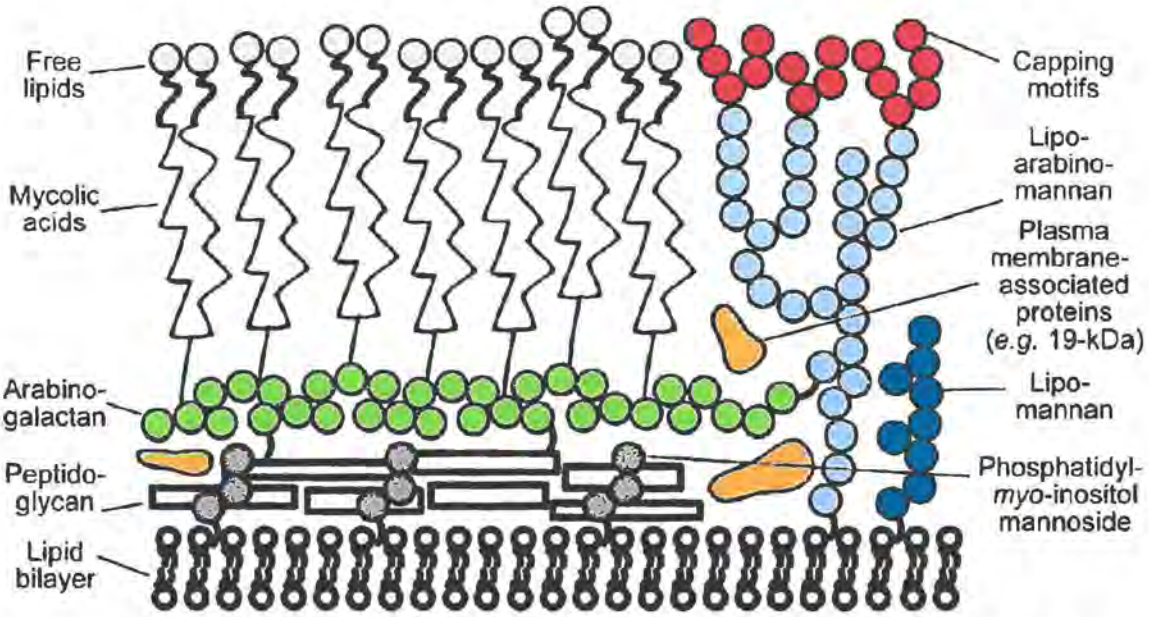


Figure 1: *M. tuberculosis* cell wall. Arabinogalactan is attached to the peptidoglycan through phosphatidyl-*myo*-inositolmannoside. Mycolic acids and glycolipids extend through the cell wall. (Welin, 2011).

M. tuberculosis uses LAM as one of its virulence factors to inhibit functions of macrophages that are important for killing the pathogen (Briken *et al.*, 2004). These virulence factors inhibit phagosomal maturation and disrupt cell signalling and pro inflammatory response through cytokine production (Briken *et al.*, 2004; Pathak *et al.*, 2005; Vergne *et al.*, 2005). Slow-growing and virulent mycobacteria consist of mannose-capped LAM (ManLAM) in their cell wall while fast-growing and non-virulent mycobacteria such as *M. smegmantis* consist of non-capped AraLAM or phosphor-myo-inositol-capped LAM (PILAM). It is this type of capping that is essential for the virulence of mycobacteria (Dao *et al.*, 2004). The cell wall of *M. tuberculosis* also consists of 19-kDa lipoprotein with no known function but has been reported to be involved in virulence through a role it plays in host cell death and manipulation of bactericidal pathways (Ciaramella *et al.*, 2000). This lipoprotein, as well as LM and AraLAM of fast-growing mycobacteria induce an immune response in host cells by binding to Toll-like receptors (TLRs) on the host cell surface (Means *et al.*, 1999; Ciaramella *et al.*, 2004).

1.8. TB Pathogenesis

The inhalation of mycobacterium bacilli which are released from an infected individual through coughing or sneezing is one of many ways that cause TB transmission (Welin, 2011). After inhalation, the bacterium is taken up by phagocytic alveolar macrophages and dendritic cells (Fogel, 2015). The majority of individuals who inhale mycobacterium bacilli have the ability to launch an effective immune response in the lungs which leads to the inhibition of mycobacterial growth inside

host cells (Thillai *et al.*, 2014). Growth inhibition leads to the bacteria becoming dormant and this type of infection is referred to as latent TB infection (LTBI). Immunocompetent individuals infected with LTBI do not show TB symptoms and are unable to transmit it (WHO, 2014). In this case, the growth of *M. tuberculosis* is controlled by antimicrobial peptides or keeping mycobacteria within the granuloma allowing the adaptive immunity to prevent the development of an infection to a disease (Goldman and Schafer, 2011). However, even after years of latency, resuscitation-promoting factor (RPF) proteins can be activated allowing mycobacteria to revert from a latent to an active state and resume cell division (Chao and Rubin, 2010). In addition, toxin-antitoxin (TA) gene pairs and plasmid maintenance factors encoded by *M. tuberculosis* also play a huge role in the switch from latency to reactivation through the production of a cell-killing toxin which gets neutralized by an antitoxin that is degraded later (Chao and Rubin, 2010).

Approximately 5-10% of individuals infected with LTBI are at risk of developing active (primary) TB (Cruz-Knight and Blake-Gumbs, 2014). These include immunocompromised individuals such as those infected with HIV, cancer and persons taking immunosupressing medication (Cruz-Knight and Blake-Gumbs, 2014). Granulomas have always been viewed as beneficial only for host cells, but studies have indicated that mycobacteria use granulomas for their own benefit. The recruitment of host cells to the granuloma allows an easy spread of mycobacteria to new host cells once they've killed their primary host cells (Davis and Ramakrishnan, 2009). In active TB, the granuloma contains necrotic macrophages which form cavities in the lungs (Russell, 2007). Rapturing of cavity structures causes the spillage of infectious bacilli into the airways, allowing the spread of mycobacteria to surrounding host cells (de Chastellier, 2009). Pulmonary TB which results in symptoms such as weight loss, dry and bloody cough and night sweats is the most common form of TB infection (Ducati *et al.*, 2006). However, the spread of mycobacteria through lymph nodes can cause extra-pulmonary TB infection which affects the brain and the spine (Donoghue, 2009).

1.9. TB Immunology

The inhalation of mycobacterium bacilli results in their interaction with macrophages which are immune cells with the ability to phagocytose and kill bacteria (Fogel, 2015; Peters and Ernst, 2003). If bacilli are not killed during this interaction, they proliferate within macrophages and dendritic cells at a rapid rate leading to the production of pro-inflammatory cytokines such as IL-1β and IL-1α (Fogel, 2015). *M. tuberculosis* expresses pathogen-associated molecular patterns (PAMPs) on its surface and these are recognized by pattern recognition receptors (PRRs) located at the surface of macrophages and dendritic cells (Hossain and Norazmi, 2013). The uptake of *M. tuberculosis* is mediated by toll-like receptors (TLRs) of host cells (Figure 2) leading to cytokine production due to the induction of intracellular signalling cascade (Sakomoto, 2012; de Martino *et al.*, 2014). *M. tuberculosis*

proliferates within host cells during early innate immune response and induces death via the ESX1 type VII secretion system (de Martino *et al.*, 2014).

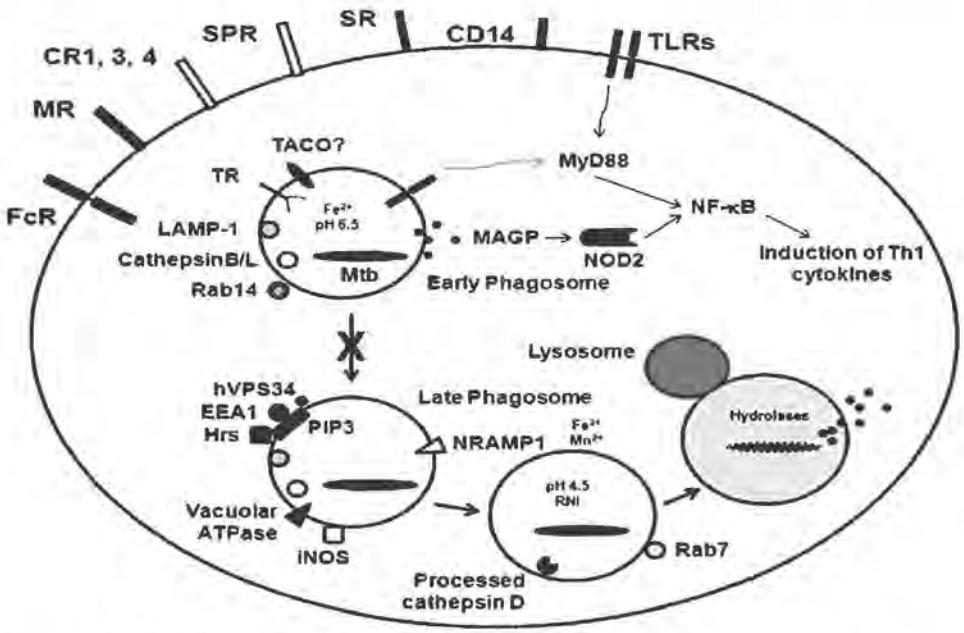


Figure 2: Cell biology of *M. tuberculosis* infection. The bacterium interacts with macrophages through a wide range of receptors located at the surface of the host cell. Phagocytic PRRs include the Fc receptor (FcR), complement receptors (CR1, CR3 and CR4), mannose receptor (MR), scavenger receptors (SR) and surfactant protein receptors (SPR). The CD14 receptor is involved in TLR signalling. TLRs are expressed at phagosomal and plasma membranes where they induce cytokine production via the MyD88 signalling pathway. (Sakomoto, 2012).

Dendritic cells transport mycobacteria to the lymph nodes (Figure 3) where T cells are activated by antigen-presenting cells (APCs) (Peters and Ernst, 2003) allowing mycobacteria to be loaded onto major histocompatibility complex (MHC) class II (Shaler *et al.*, 2013). Activated T helper cells move to the lungs where they secrete IFN-Y cytokines and activate the adaptive immune response, including CD4+ and CD8+ T cells (Shaler *et al.*, 2013). *M. tuberculosis* accumulates mutations during this period and can change the expression of some of its genes and surface antigens in order to evade T-cell recognition (Shaler *et al.*, 2013).

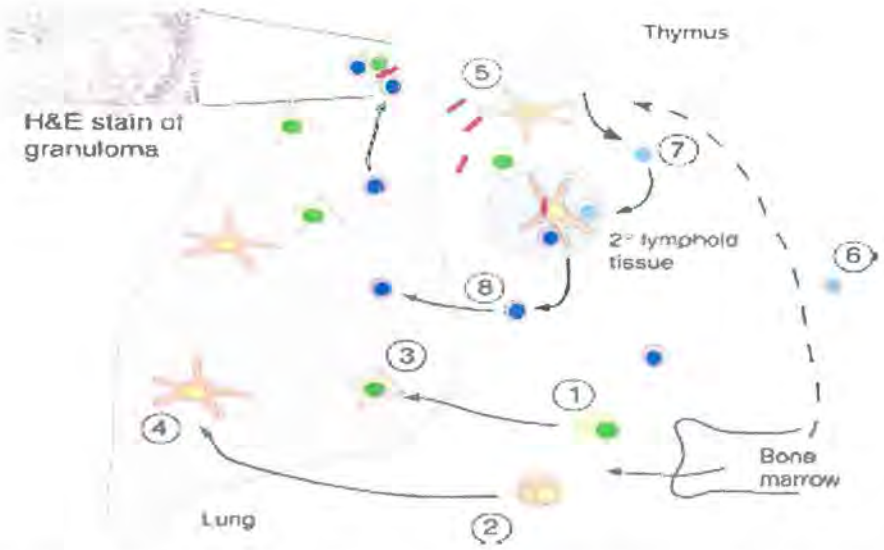


Figure 3: Cell trafficking during host immune response to *M. tuberculosis*. Host immune cells involved in immune response are indicated by numbers: (1) monocyte, (2) dendritic cell precursor, (3) tissue macrophages, (4) immature dendritic cell, (5) mature dendritic cell, (6) T-cell progenitor, (7) naïve T cell, (8) effector T cell. (Peters and Ernst, 2003).

After continuous cytokine production, T cells limit the movement of macrophages and activate them for granuloma formation (Figure 4) which is composed of macrophages, neutrophils, monocytes, dendritic cells and T cells (Peters and Ernst, 2003; Shaler *et al.*, 2013). Some studies have shown that granuloma formation might control the growth of *M. tuberculosis*, however, mycobacteria can manipulate granulomas for their own benefit by altering the immune response through IL-10 which suppresses the activation of T cells and macrophages (de Martino *et al.*, 2014). Knockout studies in mice have indicated a small reduction in bacterial load suggesting that *M. tuberculosis* might make use of multiple pathways to manipulate granulomas for its own benefit (de Martino *et al.*, 2014).

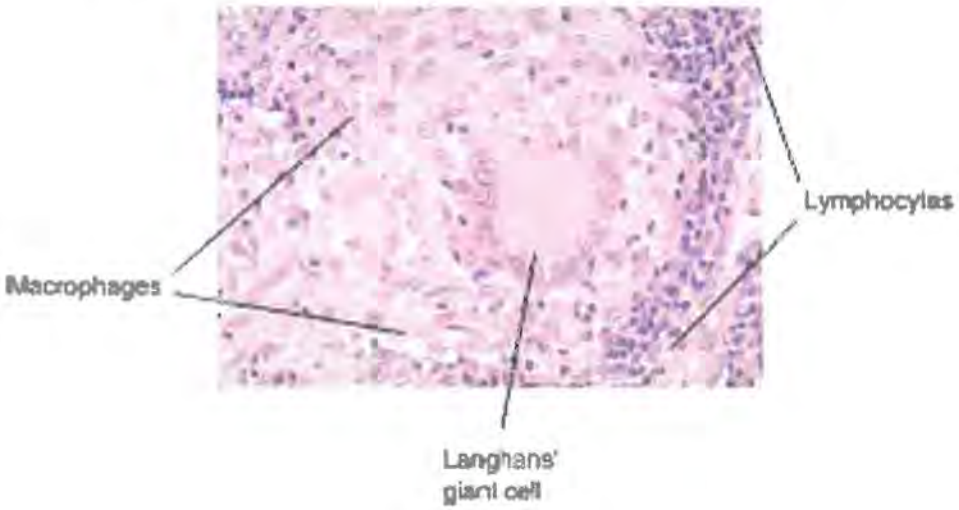


Figure 4: Granuloma formation in response to *M. tuberculosis* infection. T cells limit the movement of macrophages and activate them for granuloma formation which is composed of macrophages, neutrophils, monocytes, dendritic cells and T cells. (Peters and Ernst, 2003).

Mechanisms of *M. tuberculosis* survival inside macrophages has been studied extensively, however, there's about 30 000 alveolar epithelial cells and 50 macrophages per alveolus (Crandall and Kim, 1991; Crystal, 1991; Schneeberger, 1991). Recent studies have demonstrated the presence of viable mycobacteria and *M. tuberculosis* DNA in non-macrophage cells from lungs, kidneys and spleen of latently infected subjects (Barrios-Payan *et al.*, 2012). Studies in *M. tuberculosis*-infected mice showed an extensive replication of *M. tuberculosis* in non-migrating compartments of epithelial cells which do not present antigens to CD4+ cells during the first 2-3 weeks after infection (Wolf *et al.*, 2008). However, Cunningham *et al.* (1994) reported that alveolar epithelial cells most likely play a role in antigen presentation via MHC class I molecules. Mvubu *et al.* (2016) demonstrated an increased expression of *HLA-B*, *HLA-C*, *HLA-F* and *HLA-H* genes in alveolar pulmonary epithelial cells infected with various *M. tuberculosis* strains for 48 hours. These studies revealed that post inhalation, apart from macrophages, *M. tuberculosis* invades and replicate in alveolar epithelial cells which provide a suitable environment for rapid replication and protect mycobacteria from phagocytic cells (Ryndak *et al.*, 2015). Mehta *et al.* (1996) reported that A549 alveolar epithelial cells offer a suitable environment in which *M. tuberculosis* replicates about more than 55-fold in a 7-day period compared to 3-fold in macrophages.

Epithelial cells may be the first point of contact with the pathogen due to their over-representation in the alveolar space compared to macrophages (Li *et al.*, 2012). *M. tuberculosis* uses cell surface-bound adhesins in order to adhere to, and invade epithelial cells (Ryndak *et al.*, 2015), and is known to produce multiple adhesins in order to enhance its entry into host cells via extracellular matrix proteins (Ryndak *et al.*, 2015). Recent studies have suggested that MTP may be a major adhesin and invasin of macrophages (Ramsugit and Pillay, 2014) and epithelial cells (Ramsugit *et al.*, 2016) by binding to the laminin protein of the extracellular matrix in vitro (Alteri *et al.*, 2007) but does not largely influence the overall cytokine response in epithelial cells (Ramsugit *et al.*, 2016). *M. tuberculosis* has been known to induce immune response in macrophages by eliciting cytokine and chemokine production which leads to the recruitment of neutrophils, dendritic and natural killer cells towards the site of infection (Welin, 2011; Peters and Ernst, 2003; Fogel, 2015). Previous studies conducted using RT-PCR have implicated human alveolar epithelial cells in the production of *IL-8* and *MCP-1* (Lin *et al.*, 1998), *CXCL-10*, *TNF-α* and *CCL-5* (Lam *et al.*, 2010) and IL-1β, *IL-6*, *TNF-α* and *MCP-1* (Savard *et al.*, 2002) contributing to the activation of immune cells and pro inflammatory response (DiMango *et al.*, 1995; Jung *et al.*, 1995; Lin *et al.*, 1998).

1.10. TB Biomarkers

One of the main problems associated with current TB diagnostic tests is the lack of biomarkers, which are defined as biological characteristics that can be evaluated and measured as indicators of pathological processes e.g. a response to a therapeutic drug or preventive vaccine (Maertzdorf *et al.*, 2007). Biomarkers reliably diagnose active TB, predict risk of disease progression and could facilitate rapid diagnosis and treatment of patients (Maertzdorf *et al.*, 2007). Moreover, biomarkers have a potential to speed up clinical trials with novel vaccine and drug candidates in the pipeline. This could overcome the problem of the lack of newer drugs to treat MDR-TB and XDR-TB, therefore a simple and low cost biomarker-based point of care test is of essentiality (Maertzdorf *et al.*, 2007). An effective TB vaccine could have a significant impact on the current TB pandemic.

Studies on *M. tuberculosis* biomarkers have mainly focused on predicting treatment efficacy and cure of active TB, activation of LTBI and induction of protective immune response by vaccination (Wallis *et al.*, 2013). Inflammation-based biomarkers such as the C-reactive protein lack predictive value for clinical use due to their non-specific nature (Wallis *et al.*, 2013). The most-robust biomarkers measure essential factors associated with pathological processes of the disease being treated in order to capture effects of different of types of interventions on clinical outcomes (Maertzdorf *et al.*, 2007). The detection of *M. tuberculosis* DNA in sputum correlates with a mycobacterial burden at the time of TB diagnosis but cannot distinguish between live and dead mycobacteria as the treatment progresses (Hellyer *et al.*, 1999). High levels of neopterin, which is a non-specific marker for macrophage activation, that persist during TB treatment are associated with an increased rate of relapse and LTBI activation in patients infected with HIV-1 (Hosp *et al.*, 1997x). PCR-based methods for the quantification of active mycobacteria have been successfully applied in the development of TB biomarkers thus far. The GeneXpert MTB/RIF assay, a highly sensitive, automated and rapid molecular diagnostic tool for active TB detection amplifies DNA from both live and dead mycobacteria in sputum samples (Weyer *et al.*, 2012; Lawn *et al.*, 2013).

Increased concentrations of interleukin 18 (*IL-18*) and high expression of chemokine (c-c motif) receptor (CCR7) accompanied by low expression of Bcl2 in RNA extracted from blood samples have been shown to differentiate persons who developed active TB from controls (Sutherland *et al.*, 2011). Increased *IL-18* concentrations in patients with active TB have been reported to be in direct proportion with the extent of the disease (Yamada *et al.*, 2000). In TB and other inflammatory diseases, *IL-18* induces T-cell activation, as well as interferon Ɣ while CC chemokines are responsible for the recruitment of lymphocytes to the site of infection which is an essential step in immune response to *M. tuberculosis* infection (Wallis *et al.*, 2013). Some of the pathogen-specific TB biomarkers that have been previously identified include the early secretory antigen target 6 (ESAT 6),

antigen 85 complex (Ag85) and the *M. tuberculosis* cell wall component, lipoarabinomannan (LAM) (Mukundan *et al.*, 2012).

ESAT6 is used as a biomarker for TB diagnosis using the interferon-Ɣ release assay (Zwerling *et al.*, 2012). Talaat *et al.* (2010) developed immunoassays for the rapid detection of ESAT6 antibodies in TB patients which were associated with 97.6% sensitivity and 75% specificity. The production of antibodies to ESAT6 has also been reported to be a sensitive strategy for early bovine TB infection detection (Koo *et al.*, 2005). Antibodies to the Ag85 complex have been shown to be produced in the blood of patients with central nervous system TB (Kashyap *et al.*, 2001; Miki *et al.*, 2004) and tuberculous meningitis (Kashyap *et al.*, 2005). The direct detection of LAM, a lipoglycan component of *M. tuberculosis* cell wall, for the diagnosis of TB was evaluated by Reither *et al.* (2009) using a plate-based sandwich ELISA (enzyme-linked immunosorbent assay) immunoassay in blood samples of patients with pulmonary TB in Tanzania. They demonstrated the assay's sensitivity of 87.8% and a specificity of 66.7% and 62% in women and patients with HIV co-infection, respectively, when compared to the sputum-smear microscopy. Adhesins, due to their surface localisation, may serve as potential biomarkers and novel targets for antimicrobial drugs.

1.11. Bacterial Adhesins

Binding of pathogenic bacteria to any mucosal surfaces is an essential step in the pathogenesis of many infections. To achieve this, bacteria make use of an array of adhesins displayed at their cell surfaces and these highly specific proteins are usually expressed at different stages during infection (Kumar *et al.*, 2013). Interactions between infecting bacteria and host cells are aided by specialized microbial adhesins that mediate attachment to the surface of host cells (Figure 5). Adhesins allow pathogenic bacteria to attach to host cells and resist physical removal from the surface and that leads to invasion and induction of signalling cascades such as cytokine production (Niemann *et al.*, 2004). Microbial adhesins usually appear as polymeric organelle structures as well as non-organelle structures attached to the surface of bacterial cells as monomers or oligomers (Klemm and Schembri, 2000).

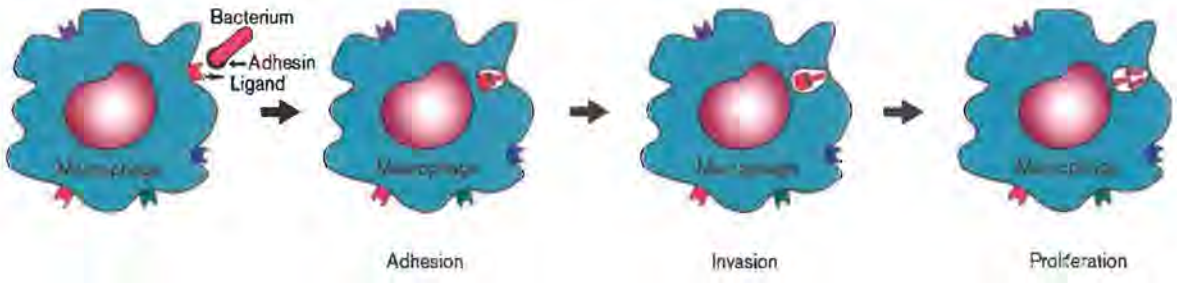


Figure 5: The role of bacterial adhesins in the infection of host cells. Adhesins located at the surface of the bacteria bind to host cell ligands which leads to the invasion of the macrophage. Once they've invaded, bacteria survive and replicate within the host cells (Govender *et al.*, 2014).

1.11.1 *M. tuberculosis Adhesins*

M. tuberculosis expresses a number of different essential adhesins (Figure 6) such as heparin-binding hemagglutinin adhesin (HBHA), Apa, malate synthase (MS), 19 kDa, laminin binding protein (LBP) (Kumar *et al.*, 2013) and *M. tuberculosis* pili (MTP) (Alteri *et al.*, 2007). HBHA is a major adhesin of epithelial cells (Pethe *et al.*, 2001) involved in bacterial attachment to lungs and in extrapulmonary dissemination of the bacteria (Pethe *et al.*, 2001; Kumar *et al*, 2013). MTP are curli pili that bind laminin and are produced during the infection of host cells (Alteri *et al.*, 2007). LBP is involved in cytoadherence through the recognition of laminin (Kumar *et al.*, 2013). *M. tuberculosis* also expresses essential enzymes such as CPN60.2, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and MS that are encoded by *cpn60.2*, *gap* and *glcB* respectively and are believed to have potential adhesion function (Dumke *et al.*, 2011). In 2010, Hickey *et al* reported the involvement of CPN60.2 in bacterial attachment to macrophages. *M. tuberculosis* adheres to and invades animal and human epithelial cells and macrophages via various entry mechanisms (Alteri *et al.*, 2007). Inadequate knowledge has been generated with regards to host-microbe interactions as well as a series of events that take place before bacteria invade and replicate in host cells. The specific bacterial adhesins involved in the interplay are also largely unknown (Alteri *et al.*, 2007).

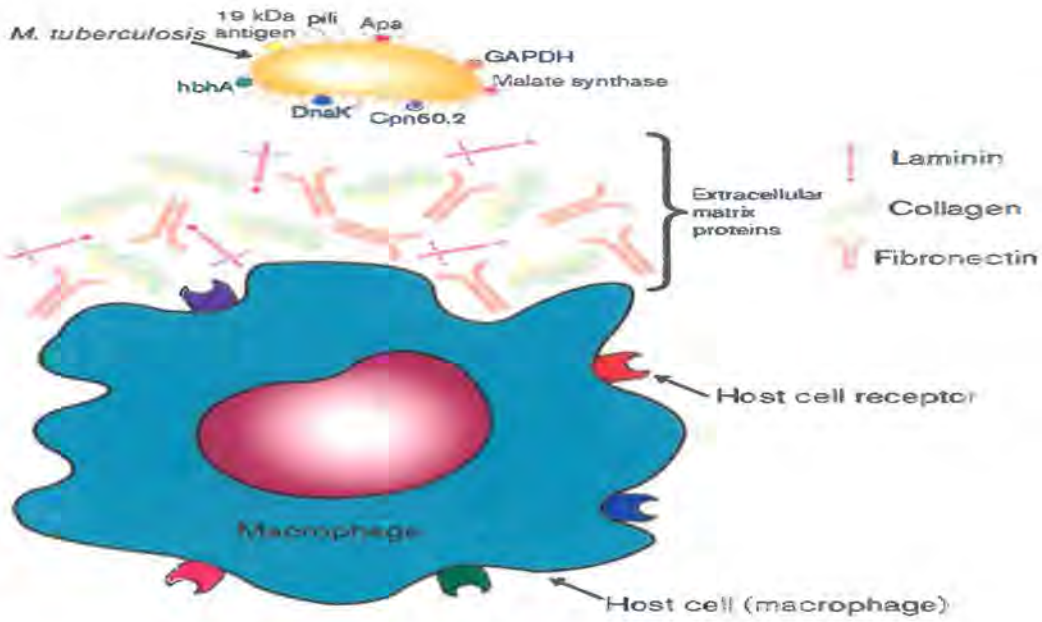


Figure 6: *M. tuberculosis* adhesin-mediated host-pathogen interaction. Adhesins use specialized pathways to initiate host interaction and colonization. (Modified from Kumar *et al.*, 2013; by Govender *et al.*, 2014).

1.12. Bacterial Pili

Adhesins, including pili, are the first bacterial point of contact with host cells, where they help with the overcoming of net repulsive forces on the two cell surfaces (Proft and Baker, 2009). Pili are composed of major repeated pilin subunits as well as a tip-associated adhesin unit. A variety of genes are needed for pili biogenesis and these encode proteins involved in membrane translocation, major pilin subunit, chaperone, minor subunit, prepilin peptidase, channel-forming proteins and nucleotide-binding proteins (Strom and Lory, 1993; Thanassi *et al.*, 1998; Klemm and Schembri, 2000). They're viewed as bacterial virulence factors due to their key role in pathogenesis and therefore, they are important targets for vaccine and drug development (Finlay and Falkow, 1997). Pili are associated with biological activities such as colonization of mucosal surfaces, agglutination of human and animal red blood cells and bacterial adherence (Finlay and Falkow, 1997).

Gram-negative bacteria possess pili that are formed as a result of non-covalent homopolymerization of pilins (Proft and Baker, 2009). Moreover, pilins may be added to the fiber where they function as adhesion molecules to host cells. Pili found in Gram-negative bacteria are mainly associated with DNA uptake, phage transduction, biofilm formation, as well as twitching which is a special form of bacterial movement (Proft and Baker, 2009). In contrast, Gram-positive pili are formed by covalent polymerization of pilin subunits which is attributed to the sortase enzyme (Telford *et al.*, 2006). The addition of minor pilins to the fiber leads to host cell colonization by Gram-positive bacteria (Proft and Baker, 2009). Gram-negative bacteria produce different types of pili in S pili, P pili, Type I pili, Type IV pili and Curli Pili (Telford *et al.*, 2006; Proft and Baker, 2009). Type IV pili are differentiated into Type IVa and Type IVb pili (Proft and Baker, 2008). Gram-positive bacteria produce Spa pili, PI-1 pilus, PI-2 pilus, Type I pili and Type II pili, Type IV pili and Curli Pili (Telford *et al.*, 2006; Alteri *et al.*, 2007; Proft and Baker, 2009).

1.12.1. *Type IV Pili*

Type IV pili are found in *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Vibrio cholera*, *Aeromonas hydrophila* (Giron *et al.*, 1991; Strom and Lory, 1993; Giron *et al.*, 1994) and in *Legionella pneumophila*. They're involved in virulence and intracellular infection of human macrophages (Liles *et al.*, 1998; Stone and Abu, 1998), as well as biofilm formation and development, DNA transformation, colonization of host tissues and bacterial aggregation (Strom and Lory, 1993; Bieber *et al.*, 1998). Type IV pili have hydrophobic amino terminus and a signal peptide. They appear under the electron microscope as long filaments with rope-like structures.

1.12.2. *Curli Pili*

Pathogens such as *Escherichia coli* and *Salmonella typhimurium* (Romling *et al.*, 1998), Gram-positive pathogens Group B *Streptococcus* and *Corynebacterium diptheriae* (Lauer *et al.*, 2005) possess curli pili, with the latter being closely related to *Mycobacterium* in terms of phylogeny and the complexity of the cell wall (De Sousa-D'Áuria *et al.*, 2003). Curli pili are associated with colonization of abiotic surfaces, internalization into epithelial cells and persistence in poultry infections (Kluftinger *et al.*, 1989; Chen and Dubnau, 2003). They're also involved in the induction of cytokine activation during human sepsis (Bian *et al.*, 2000) and development of biofilms by binding to host proteins such as plasminogen, major histocompatibility complex class I (MHC I) molecules and extracellular matrix (ECM) proteins (Olsen *et al.*, 1989; Bian *et al.*, 2000; Olsen *et al.*, 2002).

1.12.2.1. *M. tuberculosis curli pili (MTP)*

Using negative staining and transmission electron microscopy (TEM), Alteri *et al.* (2007) provided genetic, biochemical and ultrastructural data which revealed that *M. tuberculosis* produces two types of pili, Type IV and curli pili, during human infection. *M. tuberculosis* curli pili (MTP) encoded by the *mtp* gene (Rv3312A), are highly sticky, aggregative and insoluble fibers that bind laminin (Alteri *et al.*, 2007).

The role of MTP as adhesive structures was supported by their data which showed that purified MTP has an ability to bind to laminin, a component of the extracellular matrix. To determine whether the ability of MTP to bind to laminin was attributed to the *mtp* gene, *M. tuberculosis* mutants lacking the *mtp* gene were generated and their binding affinity was compared to that of a wild type strain. Their findings showed that the mutants demonstrated a 40-fold reduction in laminin-binding capacity when compared to the wild type strain, suggesting that MTP mediates adhesion to the extracellular matrix. *M. tuberculosis* produces molecules that are recognized as antigens by the host during infection and this leads to the production of antibodies which their presence is a powerful indicator that an antigen was produced during a natural infection (Alteri *et al.*, 2007). To determine if *M. tuberculosis* produces MTP during infection, Alteri *et al.* (2007) applied immunofluorescence (IF) microscopy in sera from TB patients to detect the presence of anti-MTP IgG antibodies. Their findings showed that among the individuals that were tested, 60% produced a positive MTP-IgG reaction.

Recent studies have suggested that MTP may be a major adhesin and invasin of macrophages (Ramsugit and Pillay, 2014) and epithelial cells (Ramsugit *et al.*, 2016). Ramsugit *et al.* (2016) revealed that MTP-deficient strain of *M. tuberculosis* demonstrated a significant reduction in its ability to adhere to and invade A549 pulmonary epithelial cells when compared to the wild type strain while the complementation of the gene restored its functions to the wild type levels (Figure 7). Furthermore, the wild type and *mtp*-mutant strains induced similar concentrations of cytokine and

chemokines in A549 epithelial cells, suggesting that MTP does not largely influence the cytokine response in epithelial cells.

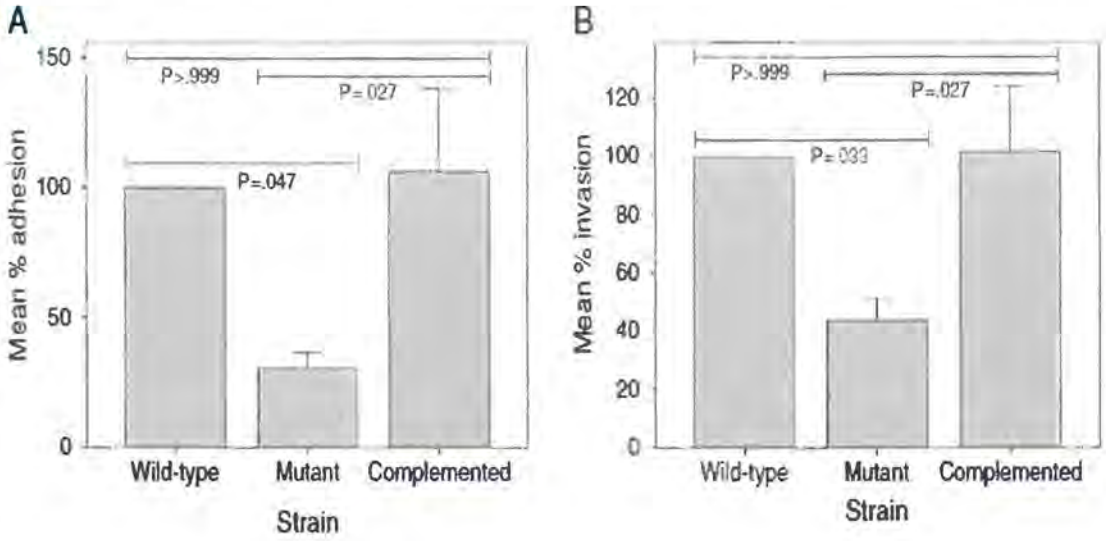


Figure 7: The role of MTP in adhesion (A) to and invasion (B) of A549 alveolar epithelial cells. A549 epithelial cells were infected with the wild type, *mtp*-mutant and *mtp*-complemented strains of *M. tuberculosis* for 1 hour (adhesion) and 2 hours (invasion). (Ramsugit *et al.*, 2016).

In addition to epithelial cells, loss of MTP was also shown to decrease adhesion to, and invasion of THP-1 monocyte derived macrophages (Figure 8), whilst complementation of MTP resulted in an increased expression of the *mtp* gene compared to the wild type (Ramsugit and Pillay, 2014). The role of MTP in *M. tuberculosis* biofilm formation has been identified and documented (Ramsugit *et al.*, 2013), indicating that they may present an important target for the development of therapeutics. Naidoo *et al.* (2014) investigated the role of MTP as a diagnostic biomarker. BLAST analysis on publically available genome databases and sequencing was performed in order to detect the presence of the *mtp* gene in *M. tuberculosis* complex strains (MTBC). The *mtp* gene and its encoded protein product, MTP, were found to be unique to MTBC strains and their absence in non-tuberculous mycobacteria suggested their potential as suitable biomarkers for a point of care TB test.

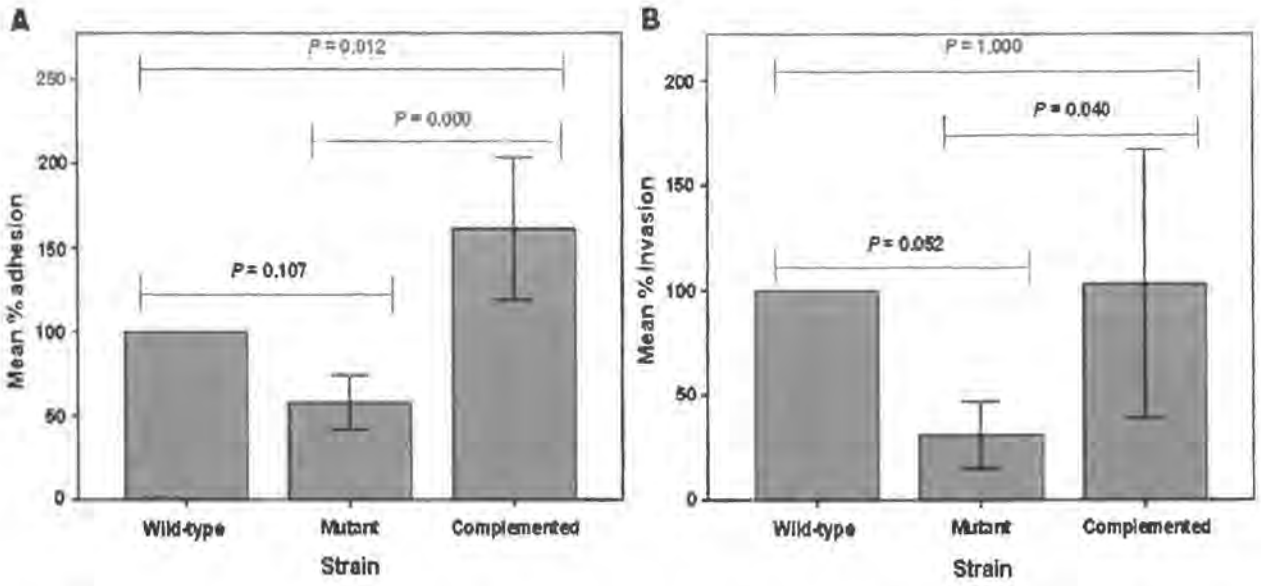


Figure 8: The role of MTP in adhesion (A) to and invasion (B) of macrophages. THP-1 macrophages were infected with the wild type, *mtp*-mutant and *mtp*-complemented strains of *M. tuberculosis* for 1 hour (adhesion) and 2 hours (invasion). (Ramsugit *et al.*, 2014).

1.13. Whole Transcriptome Analysis

Advances in *M. tuberculosis* genetics have allowed for the development of existing techniques and establishment of new techniques that are aimed at improving the analyses of *in vivo* and *in vitro M. tuberculosis* gene expression. Techniques such as subtractive hybridisation, real-time polymerase chain reaction (RT-PCR) and DNA microarray technology have been employed in studying events that take place at gene expression level in host tissues after they've been infected with *M. tuberculosis* (Waddell and Butcher, 2007).

1.13.1. *Real time PCR*

Before the development of hybridization and sequence-based techniques that are used in gene expression studies nowadays, RT-PCR had emerged as a robust method of choice due to its ability to detect and amplify small amounts of nucleic acids (Valasek and Repa, 2005). In gene expression studies, RT-PCR has been widely applied in order to accurately and rapidly detects changes in the expression levels of specific genes in a very short space of time (Deepak *et al.*, 2007).

RT-PCR differs from conventional PCR as it allows for the monitoring of DNA while it's being amplified and this allows researchers to be able to determine the amount of starting DNA in the sample before amplification (Valasek and Repa, 2005). RT-PCR amplifies specific nucleic acid sequences in a sample and monitors the progress of DNA amplification using fluorescent dyes such as ethidium bromide and SYBR green (Deepak *et al.*, 2007). One of major restrictions for conventional

PCR is that it can only use DNA as a template whereas RT-PCR makes use of a reverse transcriptase enzyme which has an ability to generate complementary DNA (cDNA) from an RNA template (Valasek and Repa, 2005). RT-PCR limitations include susceptibility to inhibition by components (haemoglobin and urea) of certain biological samples such as haemoglobin and urea. RT-PCR cannot be used to target the entire genome of a particular species and as a result of recent technological advancements it has been limited to being used as a confirmatory tool for RNA sequencing and microarray findings (Deepak *et al.*, 2007).

1.13.2. *DNA Microarray Analysis*

DNA microarray technology has been one of the most widely used techniques for the investigation of transcriptional profiles on a genome-wide level in recent years (Talaat *et al.*, 2002). This technology has been largely applied in studies that are aimed at developing new antimicrobial drugs and vaccines by exploring transcription profiles of infectious pathogens on a genome-wide level (Talaat *et al.*, 2002). Microarray technology makes use of nucleic acids probes that are bound to glass slides (Mantione *et al.*, 2014). Target sequences (fluorescently labelled) are hybridized to the probes, scanned and the images are converted to signal intensities followed by data analysis with an aid of a software specific to the platform in use (Mantione *et al.*, 2014). Microarray technology is high throughput and has been widely used in whole genome studies in the past due to its reliability and inexpensive nature (Wang, 2009). In the past ten years, microarray analysis has become easier owing to small raw data files, user friendliness of the software packages as well as protocols that are comparable across all platforms and are universally applicable (Mantione *et al.*, 2014).

According to Kendall *et al.* (2004), microarrays are seen as tools that are used to generate hypothesis and can also be used to sort and compare data from different studies which then can be analysed in detail using more definitive methods. Microarrays have proven to be more useful when applied in gene expression studies based on the hypothesis that changes in gene expressions levels are biologically significant e.g. it could be predicted that *M. tuberculosis* genes that are up-regulated after macrophage infection are more likely to be essential for intracellular survival of the pathogen (Kendall *et al.*, 2004). Waddell (2004) further stated that monitoring of whole genome expression will allow a precise definition of differentially expressed genes that are involved in host-pathogen interactions. Microarray technology has been applied in gene expression studies that are aimed at investigating *M. tuberculosis* response to different environmental conditions such as low iron (Rodriguez *et al.*, 2002), carbon starvation (Betts *et al.*, 2002), heat shock (Stewart *et al.*, 2002) and hypoxia (Park *et al.*, 2003). Ryndak *et al.* (2015) investigated the transcriptional profile of *M. tuberculosis* replicating in A549 alveolar epithelial cells using microarray technology. Up-regulated genes were associated with tryptophan synthesis and increased virulence, whereas, down-regulated genes were associated with stress and heat shock response and DosR regulon (Ryndak *et al.*, 2015).

However, this technique has a number of limitations that include spotting efficiency, transcript representation, high levels of background noise owing to cross-hydridization, low hybridization reproducibility (Talaat *et al.*, 2002) as well as limited range of detection (Mantione *et al.*, 2014).

1.13.3. *RNA-Sequencing (RNA-Seq)*

RNA sequencing is a recently developed deep sequencing-dependent approach for transcriptome profiling that provides more precise measurements of gene expression levels than any other method (Wang *et al.*, 2009). The technique involves fragmenting of RNA which is followed by reverse transcription and labelling with adapter sequences (Mantione *et al.*, 2014). Unlike microarray methods, sequence-based approaches are high throughput, provide precise gene expression levels and they can directly determine the cDNA sequence (Wang *et al.*, 2009). RNA sequencing remains advantageous over existing approaches and is believed to have a huge potential to revolutionize the manner in which trascriptomes are analysed (Nagalakshmi *et al.*, 2010). It has an ability to achieve a much higher resolution of genes that are differentially expressed and has a low limit of detection when compared to microarrays (Zhao *et al.*, 2014). Chen *et al.* (2015) reported that recent developments in high-throughput next generation sequencing technologies has gradually promoted RNA-seq to be the main method of choice for whole transcriptome profiling and analysis.

RNA-seq can carry out transcription fragment sequencing in an unbiased manner (Wang *et al.*, 2009). Unlike microarrays, RNA-seq has an ability to detect novel sequences in a genome and has a very low background noise (Wang *et al.*, 2009). RNA-seq has its own limitations which include working with large data files and therefore, making data analysis, management, sharing and storage difficult (Trapnell *et al.*, 2012). RNA-seq data analysis requires extensive experience and bioinformatics skills essential for the handling of big data files (Trapnell *et al.*, 2012). Numerous data analysis methods can be applied but there's no standard protocol (Trapnell *et al.*, 2012). Technological and software developments will allow for the mitigation of the complicated nature of RNA-seq data analysis. It is believed that its high cost will eventually drop over time, therefore the method has a bright future and a huge role to play in gene expression studies and bioinformatic data collection (Mantione *et al.*, 2014).

Some work that has been done on RNA sequencing for *M. tuberculosis* was by Zhang *et al.* (2014). They evaluated the potential of microRNAs as biomarkers for TB where they applied Solexa sequencing to examine their expression in the serum of patients with active disease, individuals with latent TB and healthy individuals with or without BCG inoculation. The microRNA expression profile obtained showed 24 and 6 microRNAs up and down regulated respectively in patients with active TB, 75 and 11 in patients with latent TB and 134 differentially expressed between BCG inoculated and un-inoculated individuals. Mvubu *et al.* (2016) demonstrated, using RNA-sequencing, that different

strains of *M. tuberculosis* induce differential gene expression and strain-specific molecular signatures in A549 alveolar epithelial cells.

Rienksma *et al.* (2015) applied dual RNA sequencing with an aim of getting an insight into transcriptional adaptation of attenuated *M. tuberculosis* surrogate *M. bovis* Bacillus Calmette–Guérin (*M. bovis* BCG) in a monocyte derived THP-1 cell line. Microbial enrichment and specific ribosomal RNA depletion was used to simultaneously analyze host and pathogen transcriptional responses. Their findings showed that genes involved in aspartate metabolism, cholesterol biosynthesis and recycling of mycolic acids were up-regulated. RNA sequencing has been employed to investigate bovine macrophage response to *M. bovis* infection *in vitro* over a 24-hour period (Nalpas *et al.*, 2013). Their gene ontology analysis of differentially expressed genes showed an induction of cell signalling, apoptotic and immune response genes. The number of differentially expressed genes identified using RNA-sequencing was greater (2584 vs 2015) than the number of genes they had identified in their previous study using microarray technology. Furthermore, their data revealed a greater range of detection and gene transcript quantification for RNA-sequenced compared to microarrays.

Before RNA-seq, it was assumed that much greater fractions of *Drosophila melanogaster* (Bertone *et al.*, 2004) and the human genome were transcribed and a large number of distinct isoforms had been found for many genes belonging to these species. However, the starts and ends of exons and transcripts had not been fully explored and resolved therefore, high resolution and sensitivity of RNA-seq has helped reveal many novel genes, transcribed regions as well as more splicing isoforms of known genes (Bertone *et al.*, 2004). Chen *et al.* (2015) used RNA-sequencing to elucidate the response of lung epithelial cells infected with *Aspergillus fumigatus*. Using a cut off fold change of 1.5 or greater, they found 302 (66%) up-regulated and 157 (34%) down-regulated genes. Their gene ontology enrichment analysis showed that up-regulated genes were associated with immune response, chemotaxis, cell activation and response to bacterium whereas down-regulated genes were mainly enriched for skeletal system development, hemopoiesis, ion transport and immune response.

1.14. Infection Models

Numerous gene expression studies aimed at better understanding the survival mechanisms of *M. tuberculosis* inside the host cells have been conducted and published since its first sequencing in 1998 (Kendall *et al.*, 2004; Waddell and Butcher, 2007). The data that has been generated from these expression studies needs to be utilized in order to understand the mechanisms that are employed by *M. tuberculosis* during infection establishment and evasion of the host immune response (Ward *et al.*, 2010). One of the challenges facing TB research is the difficulty of working with the bacteria, an extremely low infectious dose and the severity of the disease which means that research must always be carried out within specialized facilities (Ward *et al.*, 2010).

The lengthy doubling time of *M. tuberculosis* coupled with its lipid-rich cell wall makes extraction of bacterial RNA difficult and this is one of the main reasons why many gene expression studies have been conducted *in vitro* (Ward *et al.*, 2010). These *in vitro* models have been developed in a way that mimics the intracellular conditions (low oxygen levels, low nutrients, low pH and heat shock) that *M. tuberculosis* faces inside the host cells (Fontán *et al.*, 2008). However, *in vitro* infection models are unable to fully mimic the conditions found in an *in vivo* system. It is very unlikely for these models to represent normal surroundings of intracellular pathogens and form granulomas which are very much involved in the control of *M. tuberculosis* infection (Ward *et al.*, 2010). Despite all these limitations, *in vitro* models have been widely used in gene expression studies using macrophages and epithelial cells (Fontán *et al.*, 2008).

Genes coding for bacterial survival, intracellular replication and virulence are the main targets for novel antimicrobial drugs, especially those located at cell surfaces and are easily accessible to antibiotics (Triccas and Gicquel, 2000). Analysis of complete gene expression profiles in mouse macrophages showed that *M. tuberculosis* exposed to a fatty acid rich and iron deficient environment could damage the cell envelope, as well as bacterial DNA (Schnappinger, 2003). Biological experiments conducted in broth culture simulating conditions in the macrophage environment (hypoxia, starvation and low pH) in lung mouse models, mouse macrophages and cell line models have generated much information on *M. tuberculosis* gene expression (Fontán *et al.*, 2008). Transcriptional profiles of *M. tuberculosis* infecting human macrophages and macrophage-like THP-1 cell lines provided evidence that this organism regulated genes involved in the evasion of the immune system (Fontán *et al.*, 2008).

Cell lines such as the THP-1 and A549 have been widely used in studies involving the analyses of interactions that take place between mycobacteria and human host cells (Fontán *et al.*, 2008). The use of cell lines overcomes difficulties presented by human primary cells e.g. genetic variability and other donor to donor differences (Iona *et al.*, 2012). The treatment of THP-1 cells with phorbol-myristate acetate (PMA) differentiates them into mature macrophages, allowing interactions such as bacterial uptake, replication and survival to be analysed (Fontán *et al.*, 2008). Most infection studies have focused on macrophage models, despite the presence of a greater proportion of epithelial cells compared to the former in the human lung alveolus (Iona *et al.*, 2012). Thus, it is likely that epithelial cells may be the first cells to encounter *M. tuberculosis* adhesins rather than macrophages. Epithelial cells have also been shown to play an important role in inducing host immune response upon infection with *M. tuberculosis* (Castro-Garza *et al.*, 2002).

1.15. Gene Knockout Experiments

Emerging gene knockout/knockin technologies are promising future prospects for the evaluation of the effects of single gene inactivation on the *M. tuberculosis* transcriptome during infection. These should increase our understanding of natural *M. tuberculosis* infections and contribute towards the development of novel antimicrobial drugs and vaccines (Waddell and Butcher, 2007). A swift development of functional genomics for pathogenic mycobacteria is needed in order to generate an understanding on genetic basis for their persistence and virulence in the host (Bardarov *et al.*, 2002). Gene transfer strategies have been widely used for fast growing bacteria such as *Mycobacterium smegmatis*, however, the construction of strains with defined single or multiple mutations has always been difficult with slow-growing mycobacteria (Bardarov *et al.*, 2002). The usage of DNA fragments as allelic exchange substrates for homologous recombination has been successfully applied for gene disruption in slow-growing *M. tuberculosis* and *M. bovis* BCG (Azad *et al.*, 1996).

A 'suicide'vector approach and a two-step selection method have both been reported to be successful in achieving allelic exchange in both slow and fast- growing mycobacteria, however, these methods lack an efficient homologous recombination and the 'suicide' vector method is dependent upon the delivery of allelic exchange substrates by electroporation (Bardarov *et al.*, 2002). These limitations would be overcome by a highly efficient, reproducible and phage-based genetic method called specialized transduction, which is used to generate targeted gene deletions in both fast and slow-growing mycobacteria by allelic exchange with the aid of in vitro-generated specialized transducing mycobacteriophages (Bardarov *et al.*, 2002).

Gene knockout studies have been largely and successfully used to elucidate gene function in *M. tuberculosis*. The effectiveness and reproducibility of the specialized transduction method were demonstrated by the generation of seven auxotrophic *M. smegmatis* strains, three *M. bovis* BCG substrains and three *M. tuberculosis* strains (Bardarov *et al.*, 2002). The use of phagemids in gene replacement experiments with *M. tuberculosis* H37Rv was found to increase levels of homologous recombination and decrease illegitimate recombination. This method has also been successfully used to generate gene mutations in the *M. tuberculosis* H37Rv, Erdman and Beijing F2 strains (Lamrabet *et al.*, 2012). Previous studies have indicated that genes coding for *M. tuberculosis* virulence and survival are up regulated during the infection of host cells (Triccas and Gicquel, 2000).

Like any other intracellular pathogen, *M. tuberculosis* induces a cascade of genes in response to different conditions it encounters inside the host cells (Fontán *et al.*, 2008). However, it is not clear whether the expression of one gene influences expression of other genes or not. *M. tuberculosis* genes that have been studied by gene knockout include *lysA, nadBC, panC, panCD, leuCD, Rv3291c Rv0867c* (Bardarov *et al.*, 2002) and *rpf* (Russell-Goldman *et al.*, 2007). Russell-Goldman *et al.* used

specialized transduction to construct single (*rpfA* and *rpfB*) and double knockouts (*rpfAB*) of the *rpf* gene. The *rpf* gene codes for resuscitation-promoting factor (Rpf), a peptidoglycan hydrolase responsible for the reactivation of bacteria (Russell-Goldman *et al.*, 2007). They demonstrated that the deletion of *rpfB* impaired the reactivation of *M. tuberculosis* in mouse model. Since *M. tuberculosis* encodes five Rpf paralogues, a series of *rpf* double knockouts were generated and results showed a further increased impairment of bacterial reactivation.

1.16. Significance of Study

The role of adhesin genes, in particular *mtp*, in *M. tuberculosis* and host gene regulation is of special interest. Adhesins, including MTP, are regarded as one of the most important virulence factors in pathogenic bacteria (Klemm and Schembri, 2000). Therefore, this study would contribute to a better understanding of the role of MTP in TB pathogenesis and further elucidate its potential as a biomarker for the development of new drugs and vaccines. Furthermore, whole transcriptome studies during infection of host cells could prove to be important in the identification of other novel biomarkers (Klemm and Schembri, 2000). This study will evaluate the effects of *mtp* in gene regulation of A549 pulmonary epithelial cells using a *M. tuberculosis mtp* mutant and full transcriptome RNA sequencing.

1.17. Aims and Objectives

Aim:

To perform full transcriptome analysis by RNA sequencing to determine host gene regulation in A549 epithelial cells infected with an *mtp* knockout mutant.

Objectives:

To achieve the above aim, the following objectives were established:

1. To infect A549 cells (human alveolar basal epithelial cells) with *mtp* gene knockout mutant and wild type strains of *M. tuberculosis* V9124.
2. To extract RNA from A549 epithelial cells infected with *mtp* gene knockout mutant and wild type strains of *M. tuberculosis* V9124.
3. To perform RNA sequencing for the whole transcriptome analysis of infected A549 cells.

CHAPTER 2: METHODOLOGY

2.1. Ethics Clearance

The study was approved by the Biomedical Research Ethics Committee, University of KwaZulu-Natal (BREC Reference Number: BE280/14).

2.2. Strains and Growth Conditions

M. tuberculosis wild type V9124, a clinical isolate of the F15/LAM4/KZN (KZN) family, isolated from the Tugela Ferry (KwaZulu-Natal, South Africa) and its Δ*mtp* mutant were obtained from the culture collection of Medical Microbiology and Infection Control (University of KwaZulu-Natal). The *mtp* mutant was constructed in V9124 by specialized transduction with allelic exchange substrate (Ramsugit *et al.*, 2013). Strains were cultured in 10 mL Middlebrook 7H9 broth (Difco) containing 10% Oleate-Albumin-Dextrose-Catalase (OADC; Becton Dickinson), 0.5% glycerol (Sigma), and 0.25% Tween-80 (Sigma) at 37°C with shaking for 7 days to an OD_{600} = 1.0, equivalent to approximately $1x10^8$ colony forming units (CFU)/mL (Larsen *et al.*, 2007).

2.3. Revival of Frozen A549 Epithelial Cell Stocks

A volume of 1 mL of thawed A549 epithelial cells (ATCC CCL-185) was added to 20 mL of E10 medium [Eagle's Minimal Essential Media (EMEM) with L-glutamine (Lonza) and 10% Fetal Bovine Serum (FBS; Biowest)] in a 250 mL tissue culture flask (Lasec) and incubated for 5 days in a humidified 37°C incubator with 5% CO_2. The cells were washed with phosphate buffered saline (PBS; Oxoid) and fresh E10 media was added after 24 hours in order to get rid of the DMSO, non-adherent and dead cells. The cells were incubated in a humidified 37°C incubator with 5% CO_2 for 7 days.

2.3.1. *Recovery of A549 Epithelial Cells*

Confluent A549 epithelial cells were washed thrice with 10 mL of PBS. A volume of 2 mL of trypsin (Biowest) was added and cells were incubated in a humidified 37°C incubator with 5% CO_2 for 5 minutes, allowing them to detach (by tapping flask vigorously). Trypsinization was stopped by adding 2 mL FBS against the cell layer. Detached A549 cells were removed and placed in a 15 mL tube.

2.3.2. *Enumeration of A549 Epithelial Cells*

A cell count was performed using the Trypan Blue Exclusion Assay. A coverslip was placed on the hemocytometer and an aliquot of the cell suspension (20 µL cells with 20 µL trypan blue) was applied by capillary action. The hemocytometer was placed under the microscope for the viewing of the cells over a grid. Cells in four big squares (each containing 16 small squares) were viewed and counted.

Only the viable, white cells were counted, as the blue cells were dead. The total number of cells/mL was obtained by multiplying the average number of cells in the four big squares by a dilution factor of 2 and 10^4 in order to determine the number of cells/mL in a diluted sample.

2.3.3. *Seeding of A549 Epithelial Cells*

A volume of $5x10^5$cells/mL was added to 7 x 250 mL tissue culture flasks each containing 20 mL of E10 medium. Flasks were incubated to confluency in a humidified 37°C incubator with 5% CO_2 for 5 days. Thereafter, the spent medium was discarded, cells were washed once with 10 mL of PBS and 20 mL of fresh E10 medium was added to each flask. Cells were now ready for infection. A cell count was performed using one flask in order to determine the number of A549 epithelial cells to be infected.

2.4. Construction of *mtp*-knockout mutant and *mtp*-complemented strains

The construction of the *mtp*-knockout mutant and *mtp*-complemented strains was carried out as described in Ramsugit *et al.* (2013). An allelic exchange substrate (AES) designed to replace the *mtp* gene with a hygromycin-resistance (hyg^R)-sacB cassette was donated by W. R. Jacobs, Jr., from the Albert Einstein College of Medicine (AECOM). Briefly, upstream and downstream arms were digested with BstAPI andVan91I, respectively, and cloned into Van91I-digested pYUB1471 vector arms. The resulting AES was digested with PacI and ligated with PacI-digested phAE159, in vitro packaged using a MaxPlax packaging extract (Epicentre Biotechnologies), and transduced in the *E. coli* HB101 strain (Invitrogen Life Technologies) for construction of shuttle phasmids. The phasmids were electroporated into *Mycobacterium smegmatis* mc^2155 (gift from W. R. Jacobs, Jr.) and incubated at 30°C for phage propagation. Specialized transduction of the *M. tuberculosis* V9124 wild-type strain was performed. A 188-bp region of the *mtp* gene was deleted. Genomic DNA of the deletion mutant was isolated and screened using PCR. The *mtp* gene is not operonic, the deletion was in frame and confirmed by sequencing as part of a previous study (Ramsugit *et al.*, 2013). For complementation, the *mtp* gene was cloned into the pMV261 vector. The sequence was then amplified using PCR and its products were sequenced for confirmation.

2.5. Infection of A549 Epithelial Cells

The cells were transferred to the BSL2+ laboratory and all the subsequent steps were performed in the class II safety cabinet. A volume of 5 mL of each of the *M. tuberculosis* V9124 wild type and *mtp* knockout mutant cultures (OD_{600} = 1) was pelleted at 2,000*g* for 20 minutes. Supernatants were discarded and pellets re-suspended in 50 mL E10 by vortexing. An OD_{600} was measured in order to determine the number of organisms, as loss of cells can occur during centrifugation and to bring all cultures to an OD_{600} = 0.1. A549 epithelial cells were then infected in triplicate with *M. tuberculosis* V9124 wild type strain and *mtp* knockout mutant strain at a multiplicity of infection (MOI) of 10:1

and incubated at 37°C with 5% CO_2, and 95% humidity for 1, 2, 4 and 24 hours respectively. Experiments were repeated thrice for all the time intervals. Uninfected A549 cells were used as a control.

2.5.1. *Determination of Colony Forming Units/mL*

Serial dilutions of the initial bacterial suspensions were plated out in order to determine an accurate bacterial count. Eppendorf tubes (Merck) were used to make 10^{-5} dilutions by transferring 100 µL suspensions into 900 µl 7H9 broth. Aliquots of 100 µL were plated onto 7H11 agar plates in triplicate, sealed in CO_2 permeable plastic bags and incubated at 37°C for 3 weeks, after which the colony counts were read. Colony forming units (CFU)/mL were calculated according to the formula: Number of Bacterial Colonies Counted x Dilution Factor/Volume Plated. Similarly, CFU/mL was determined at each interval post infection, after epithelial cells were lysed with buffer RLT to release invaded bacilli.

2.6. Extraction of RNA from Infected A549 Epithelial Cells

The spent medium was removed after incubation and the cells were washed thrice with 10 mL of PBS in order to remove non-adherent cells. Cells were trypsinized using 2 mL of trypsin and incubated for 5 minutes in a humidified 37°C incubator with 5% CO_2. A volume of 2 mL FBS was added to stop trypsinization. Cells were transferred to 15 mL tubes and centrifuged at 300 x g for 5 minutes. The supernatant was discarded and RNA was extracted from the pellet using the RNeasy Mini Kit (RNeasy Mini Handbook, 4th Edition, 2012, Qiagen). Briefly, 350 µL of lysis buffer RLT (Qiagen) with 3.5 µL of beta-mercaptoethanol (Sigma) was added to lyse epithelial cells. Cells were transferred to 2 mL eppendorf tubes (Merck) and passed 10 times through a blunt 20 gauge needle (Becton Dickinson) fitted to an RNase-free syringe (Becton Dickinson). A volume of 350 µL 70% ethanol (Sigma) was added and the lysate was transferred to an RNeasy spin column (Qiagen) placed in a 2 mL collection tube (Qiagen). The lysate was centrifuged for 15 seconds at 12 000 rpm, the flow-through was discarded and 700 µL of buffer RW1 (Qiagen) was added to the spin column. After centrifugation for 15 seconds at 12 000 rpm, the flow-through was discarded and 500 µL of buffer RPE (Qiagen) was added to the spin column. This wash step was repeated with 500 µL of buffer RPE. The lysate was centrifuged for 2 minutes at 12 000 rpm and the flow-through was discarded. The spin column was placed in a new 2 mL collection tube and centrifuged at 12 000 rpm for 1 minute. The spin column was placed in a new 1.5 mL collection tube (Qiagen), 30 µL of RNase-free water (Qiagen) was added and centrifuged for 1 minute in order to elute RNA. RNA extracted from uninfected A549 cells was used as a control.

2.7. Assessment of RNA Quality

RNA concentration and integrity in each sample were determined using a Nanodrop 2000 spectrophotometer (Thermofisher Scientific) and a 3-(N-Morpholino) propanesulfonic acid (MOPS, Sigma) gel respectively. The RNA quality was also assessed at Johns Hopkins University Deep Sequencing and Microarray Core Facility using the Agilent 2100 BioAnaylzer machine with an RNA Integrity (RIN) value of 7 being used as a cut-off point.

2.8. cDNA Library Preparation and Sequencing

A total of 6 x RNA samples (2 x control samples, 2 x samples each A549 cells infected with *M. tuberculosis* V9124 wild type strain and *mtp*-knockout mutant strain) from the 4 hour post-infection interval were couriered on dry ice to Johns Hopkins University Deep Sequencing and Microarray Core Facility. The 4 hour time interval was chosen because the aim of this study was to determine the role of MTP in initiating host-pathogen interactions by adhering to and invading epithelial cells and inducing early innate immune response against *M. tuberculosis* upon invasion through the induction of changes on host gene regulation. A cDNA library was prepared using the Illumina ScriptSeq Complete Gold kit for Epidemiology according to manufacturer's instructions (http://www.illumina.com/products/scriptseq-epidemiology.html). Briefly, 225 µL of magnetic beads were added to an RNase-free tube and placed on a magnetic stand for 1 minute at room temperature (RT). Beads were washed with 225 µL of RNase-free water and place on a magnetic stand. The wash step was repeated after which beads were re-suspended in 65 µL of the Re-suspension Solution. For rRNA removal, each sample was treated with 40 µL of rRNA Removal Solution, incubated for 10 minutes at 68°C and then for 5 minutes at RT.

Magnetic beads were added and the mixture was vortexed and incubated at RT for 5 minutes and then at 50°C for another 5 minutes. The mixture was placed on a magnetic stand and the supernatant (rRNA-free sample) was transferred to an RNase-free tube and precipitated with ethanol. For RNA fragmentation, 1 µL of RNA Fragmentation Solution was added and the mixture was incubated at 85°C for 5 minutes in a thermocycler and then placed on ice. For cDNA synthesis, a cDNA master mix of 4 µL was added to the mixture, mixed by pipetting and incubated at 25°C for 5 minutes followed by 42°C for 20 minutes and cooling to 37°C. A Finishing Solution of 1 µL was added and the mixture was incubated at 37°C for 10 minutes followed by 95°C for 3 minutes and cooling to 25°C. A volume of 8 µL of a Terminal Tagging master mix (for adaptor tagging) was added and the mixture was incubated at 25°C for 15 minutes followed by 3 minutes at 95°C and cooling to 4 °C. The mixture was eluted in 22.5 µL of Agencourt AMPure or Qiagen MinElute solution for cDNA purification. cDNA was amplified with Polymerase Chain Reaction (PCR) and the library was purified using AMPure purification. Sequencing was carried out using the Illumina HiSeq 2000

Sequencing Software System (www.illumina.com/support). Samples were pooled and sequenced in duplicate as paired-ends with a length of 100 base pairs and coverage of 25-35 million per sample.

2.9. Bioinformatics Analysis

2.9.1. *Read Quality Control, Mapping and Generation of Gene Expression Profiles*

The quality of the reads was assessed using the FastQC toolkit (v. 0.10.1). All reads had a quality score of 34/38. Sequencing reads were then mapped to the *Homo sapiens* UCSC hg38 reference genome (support.illumina.com/sequencing/sequencing_software/igenome.html) using *TopHat* (v. 2.1.0) (Trapnell *et al.*, 2012). All samples had an overall mapping rate > 69% (Table 2). Differential gene expression profiles and fold changes between infected and uninfected epithelial cells were generated with *Cuffdiff* (v. 2.2.1) (Trapnell *et al.*, 2012).

2.9.2 *Global Gene Expression and Statistical Analysis*

An FPKM pairwise scatter plot comparison was performed in order to investigate global gene expression profiles of uninfected A549 epithelial cells relative to A549 cells infected with the mutant and wild type strain. The significance of differences between the two strains was analysed by one-way analysis of variance (ANOVA) using SPSS 24.0 statistical software. $p < 0.05$ was considered significant.

2.9.3. *Differential Gene Expression, Gene Ontology and Ingenuity Pathway Analysis*

Differentially expressed genes were filtered using a cut-off point of 2 and a Venn diagram was created using VENNY (http://bioinfogp.cnb.csic.es/tools/venny_old/venny.php) software in order to determine the number of genes that were shared as well as genes that were unique to A549 epithelial cells infected with the wild type or *mtp*-knockout mutant strain. Gene Ontology analysis was performed using the Comparative Toxicogenomics Database (http://ctdbase.org/). Biological processes, molecular functions and cellular components were enriched using a corrected *p*-value of 0.05 and a highest Gene Ontology level of 3. Heat maps of gene expression profiles in A549 epithelial cells infected with *M. tuberculosis* V9124 wild type and *mtp*-knockout strains were created using a MultiExperiment Viewer (MeV, v. 4.9.0) software. Differentially expressed genes were used to investigate canonical pathways, networks and transcriptional factors induced by the *M. tuberculosis* V9124 wild type and *mtp*-knockout strains in A549 epithelial cells. Changes in canonical pathways, network and transcription factor enrichment were investigated with Ingenuity Pathway Analysis (IPA v. 01-07, Ingenuity Systems, USA).

CHAPTER 3: RESULTS

3.1. Infection of A549 Epithelial Cells

A549 epithelial cells were infected in triplicate with wild type and *mtp*-knockout mutant strains of *M. tuberculosis* V9124 at an MOI of 10 for 4 hours. The initial bacterial inoculum for the wild type and *mtp*-knockout mutant strain was $1.03x10^4$ CFU/mL and $1.028x10^4$ CFU/mL respectively (Figure 9). There were no significant differences in the bacterial initial inoculum between both strains (p=0.374).

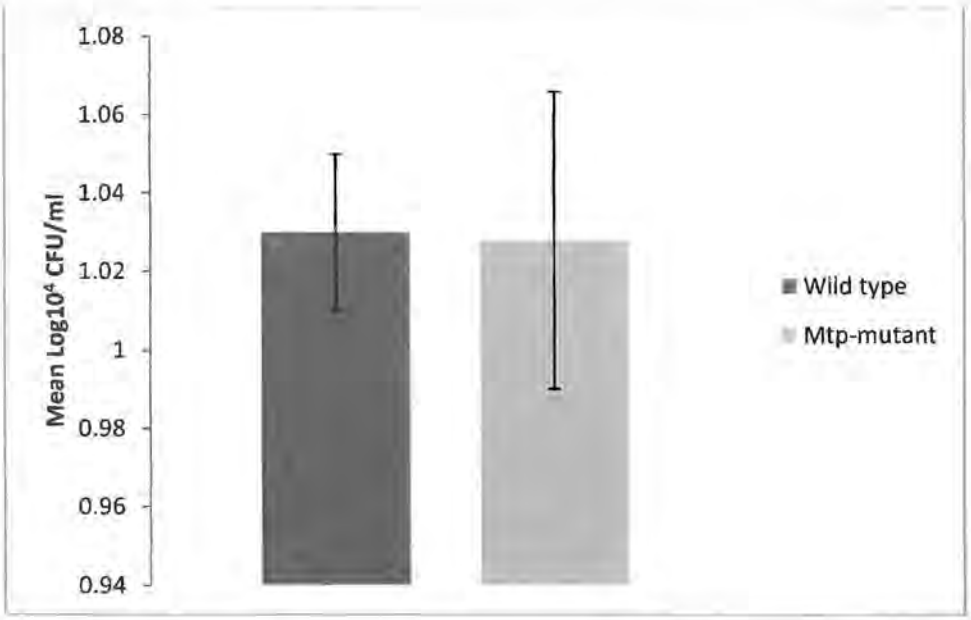


Figure 9: Initial bacterial inoculum used to infect A549 epithelial cells. A549 epithelial cells were infected with the wild type and *mtp* knockout mutant strain. CFU/ml was determined in order to obtain the initial bacterial inoculum for each strain before incubation.

After 4 hours of incubation, epithelial cells were washed thrice with PBS and cells were lysed with TritonX-100. The lysate was plated out for CFU/ml enumeration in order to determine the number of *M. tuberculosis* cells that were able to invade epithelial cells. The number of invaded bacteria observed in A549 epithelial cells infected with the wild type strain ($2.47x10^3$ CFU/ml) did not differ significantly (p=0.121) when compared to the *mtp*-knockout mutant strain ($1.67x10^3$ CFU/ml) (Figure 10).

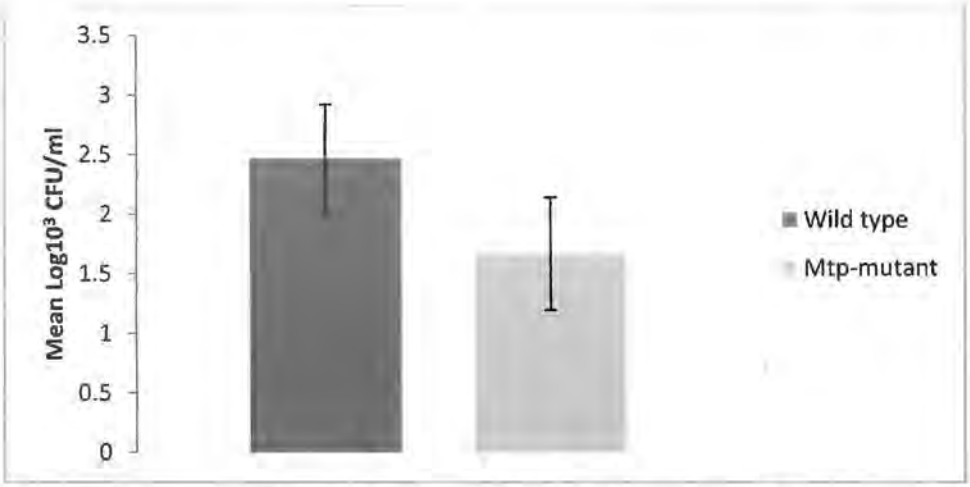


Figure 10: Number of bacteria infecting A549 epithelial cells. After 4 hours of incubation, infected epithelial cells were washed 3 times with PBS and lysed with TritonX-100. CFU/ml was determined in order to obtain the final number of bacteria that were able to invade epithelial cells.

3.2. RNA Concentration and Integrity

Table 1: NanoDrop and BioAnalyzer (Johns Hopkins University, Deep Sequencing and Microarray Core) results of RNA extracted from uninfected A549 epithelial cells (control) and A549 epithelial cells infected with *M. tuberculosis* wild type and *mtp*-mutant strains.

Number	Sample	Concentration (ng/µl)	260/280	260/230	RNA (RIN)	Integrity
1	Uninfected	957.94	2.10	2.08	10	
2	Uninfected	1045.18	2.11	2.20	10	
3	Wild type-infected	1397.15	2.09	2.07	9.7	
4	Wild type-infected	1402.05	2.09	2.11	10	
5	Wild type-infected	1733.81	2.09	2.23	10	
6	Mutant-infected	1303.61	2.10	2.13	9.8	
7	Mutant-infected	1612.83	2.08	2.23	10	
8	Mutant-infected	1594.78	2.08	2.21	9.8	

The yield, purity and integrity of RNA extracted from infected and uninfected A549 epithelial cells were all high as shown by the concentrations, 260/280 and 260/230 ratios and RNA Integrity (RIN) values (Table 1). RNA samples 1, 2, 4, 5, 7 and 8 were sent out for sequencing. A cut off value of 2.0 was used for 260/280 and 260/230 ratios while 7.0 was used for RIN.

After RNA extraction, a MOPs gel (Figure 11) and virtual gel (Figure 12) (Johns Hopkins, University Deep Sequencing and Microarray Core) were performed in order to further confirm the integrity of the RNA samples.

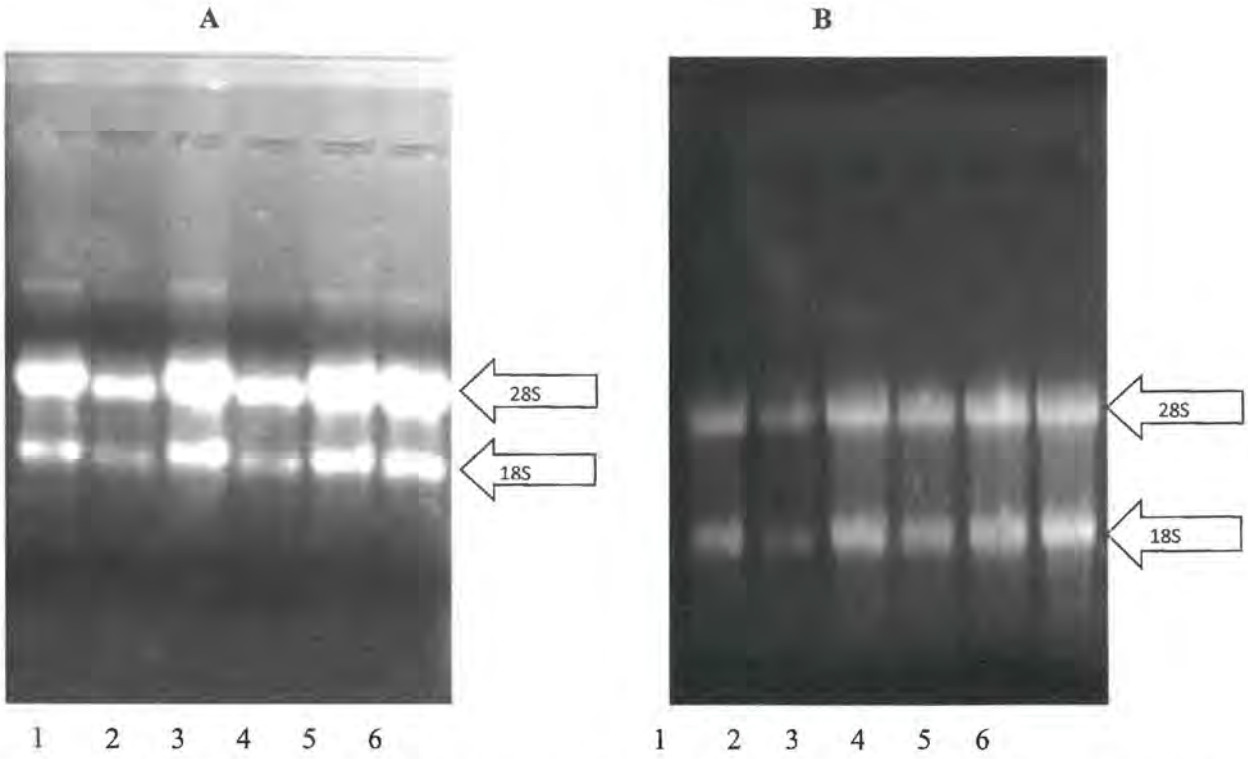


Figure 11: MOPS gels showing 28S and 18S bands of RNA extracted from infected and uninfected A549 epithelial cells. **X-axis**: **A**: Lanes: 1(RNA extracted from uninfected A549 cells), 3 (RNA extracted from uninfected A549 cells), 5 (RNA extracted from A549 cells infected with the wild type strain), and 6 (RNA extracted from A549 cells infected with the wild type strain) and **B**: Lanes: 1(RNA extracted from A549 cells infected with *mtp*-knockout strain) and 3 (RNA extracted from A549 cells infected with *mtp*-knockout strain) represent RNA samples that were sent out for sequencing.

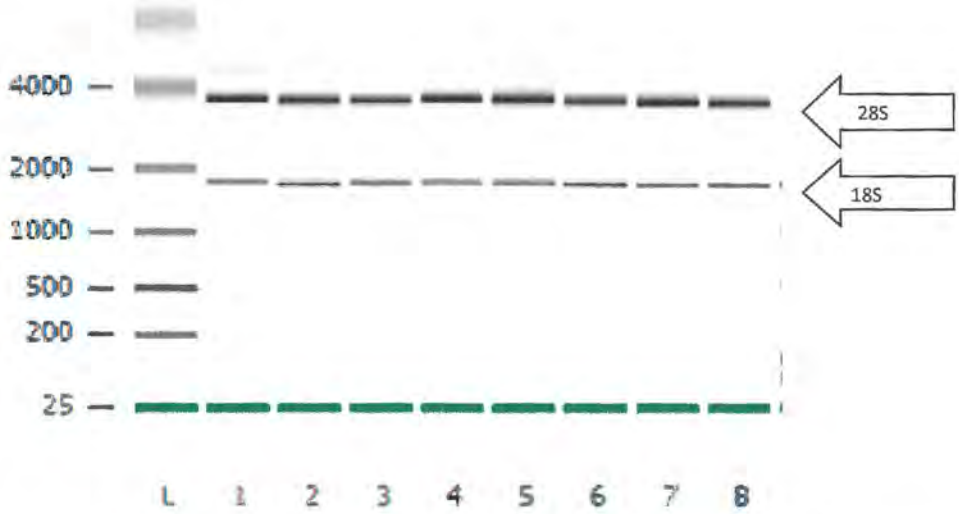
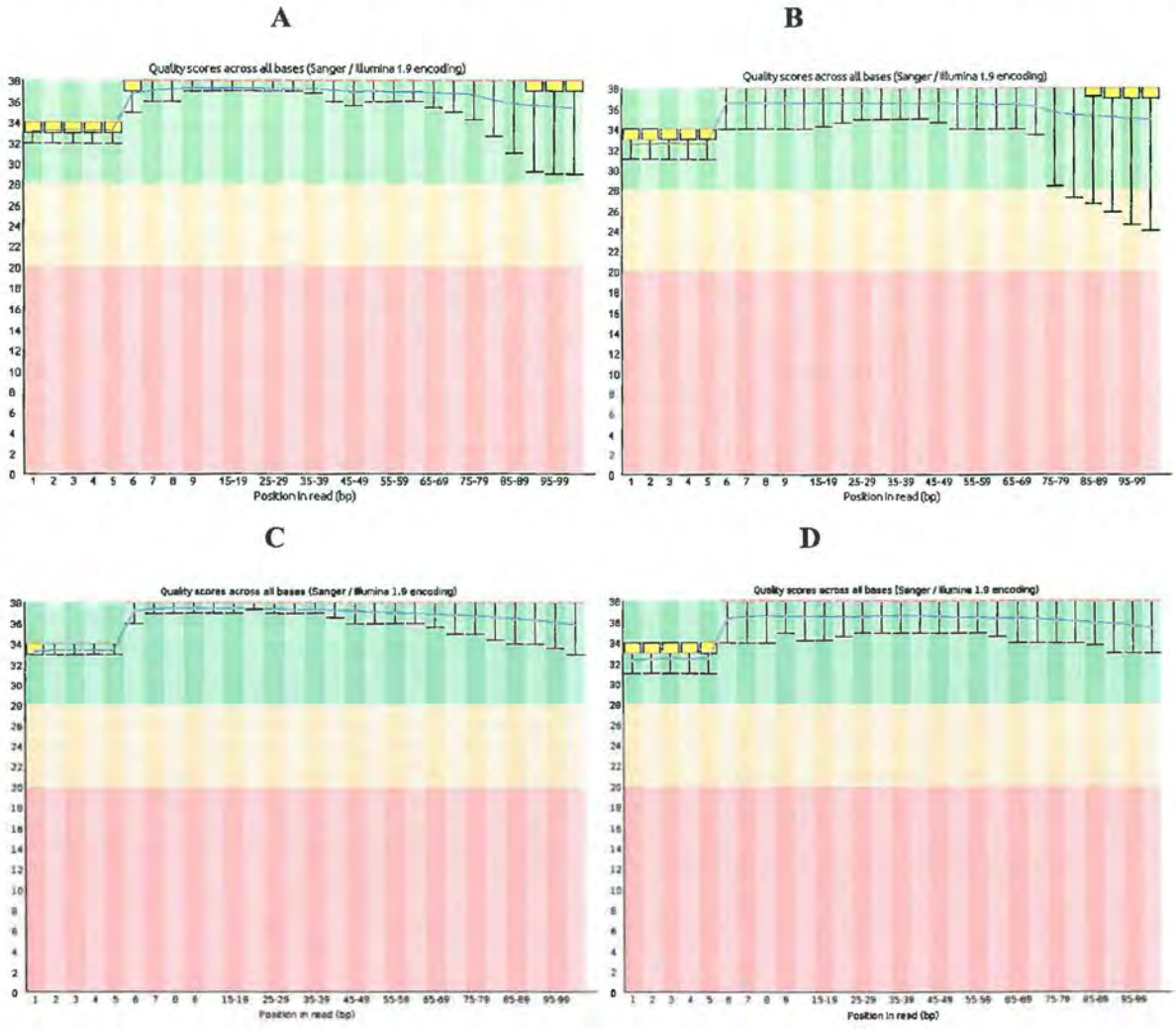


Figure 12: A virtual gel image showing 28S and 18S bands of RNA extracted from infected and uninfected A549 epithelial cells. **X-axis**: Lane L: Bands of a molecular weight marker (bp). Lanes 1-

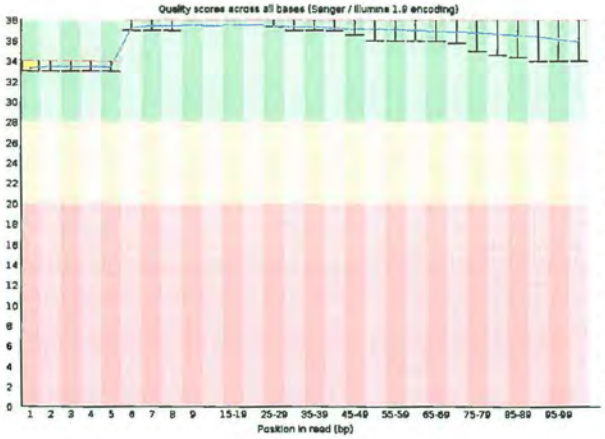
8: RNA samples shown in Table 1. **Y-axis**: Molecular weight size. RNA samples formed 18S and 28S rRNA bands at 1800kb and 3800kb, respectively.

3.3. RNA-Sequencing Read Quality Control

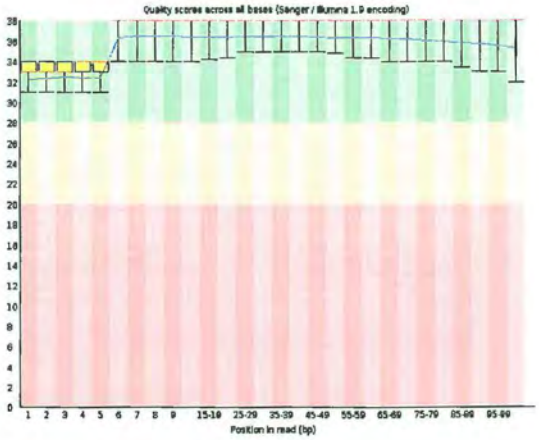
RNA samples were sequenced using the HiSeq Illumina 2000 platform as paired ends at a length of 100 bp per end. Quality control was performed on sequenced reads using the FastQC software and all samples had a high read quality score (Figure 13). In all samples, the first 5 reads had a mean (indicated by the red line) quality score of 33-34 whereas reads 6-100 had a mean quality score of 38.



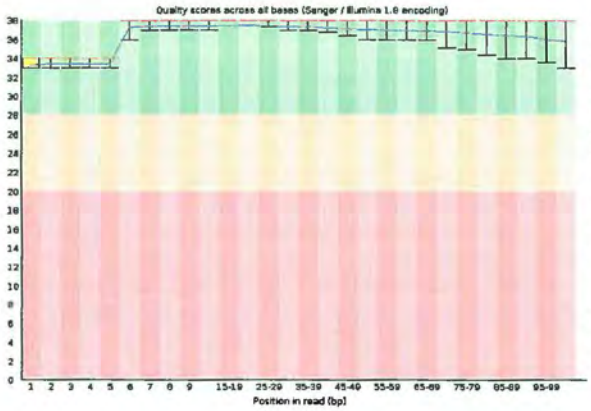
E


Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

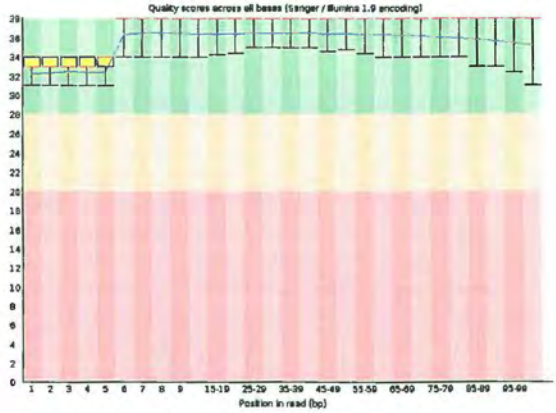
F


Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

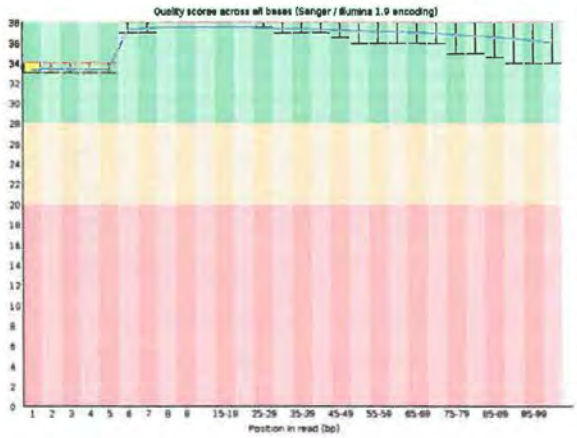
G


Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

H


Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

I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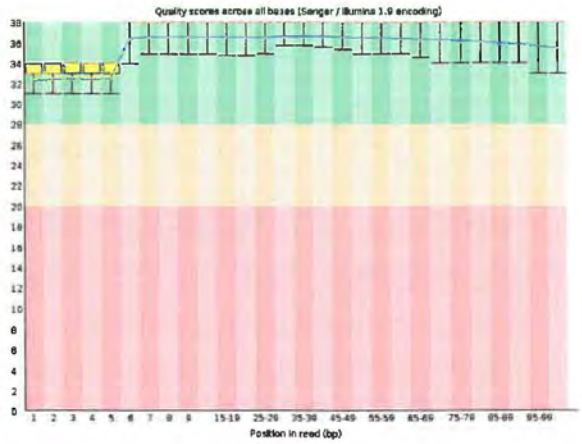
Position in read (bp)

J


Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

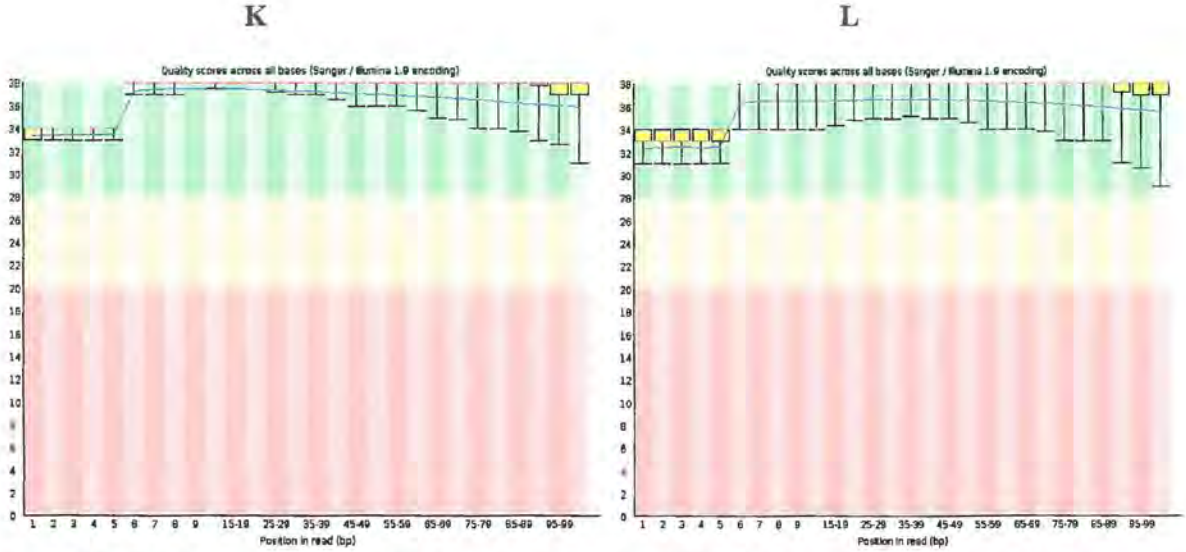


Figure 13: Read quality scores of left and right end of RNA sequences of samples: RNA extracted from uninfected A549 cells (**A** and **B**), RNA extracted from uninfected A549 cells (**C** and **D**), RNA extracted from A549 cells infected with the wild type strain (**E** and **F**), RNA extracted from A549 cells infected with the wild type strain (**G** and **H**), RNA extracted from A549 cells infected with *mtp*-knockout mutant strain (**I** and **J**) and RNA extracted from A549 cells infected with *mtp*-knockout mutant strain (**K** and **L**). **X-axis**: Position in Read. **Y-axis**: Read Quality Score Rating. RNA samples were sequenced using the HiSeq Illumina 2000 platform as paired ends at a length of 100 bp per end. The red lines and blue line represent the mean quality and median value, respectively**. Green:** Good quality reads. **Orange:** Average quality reads**. Red:** Poor quality reads**.**

3.4. Mapping of RNA-Sequencing Reads

After quality control on sample sequences, reads were mapped to a human reference genome (*Homo sapiens* UCSC hg38) using *TopHat* where an overall read mapping rate for each sample was generated. The read mapping rate of RNA from A549 epithelial cells ranged from 69.4 to 84%, with the highest from A549 epithelial cells infected with the wild type strain and lowest from uninfected A549 epithelial cells (Table 2).

Table 2: Overall read mapping rate for all the RNA samples that were sequenced.

Sample Number	Sample	Overall Read Mapping Rate (%)
1	Uninfected	84
2	Uninfected	69.4
4	Wild type-infected	83.3
5	Wild type-infected	82.4
7	Mutant-infected	82.4
8	Mutant-infected	79.8

3.5. Global Gene Expression Analysis

The role of MTP on gene regulation in A549 human alveolar epithelial cells was investigated using genome-wide whole transcriptome analysis on A549 epithelial cells infected with the wild type and *mtp*-knockout mutant strain at 4 hours post *M. tuberculosis* infection, relative to uninfected A549 cells. After the alignment of sequenced reads to the human reference genome (*Homo sapiens* UCSC hg38) using *TopHat*, *Cuffdiff* was used to generate gene expression profiles of differentially expressed genes (DEGs) induced in A549 epithelial cells in response to infection with the two *M. tuberculosis* strains. An overview of transcript abundance, expressed as Fragments Per Kilobase of Transcript per Million Mapped Reads (FPKM), induced in infected A549 epithelial cells, relative to uninfected cells, was investigated using pairwise scatter plots.

An FPKM pairwise scatter plot comparison was performed in order to investigate global gene expression profiles of uninfected A549 epithelial cells relative to A549 cells infected with the mutant (Figure 14) and wild type strain (Figure 15). The majority of genes induced in A549 cells as a result of the *mtp*-mutant infection had FPKM values in the region of 0-500 in relation to uninfected cells. *CYP3A7-CYP3A51P* and *EEF1A1* genes had the lowest (0.000153292) and highest (4182.27) FKPM values respectively.

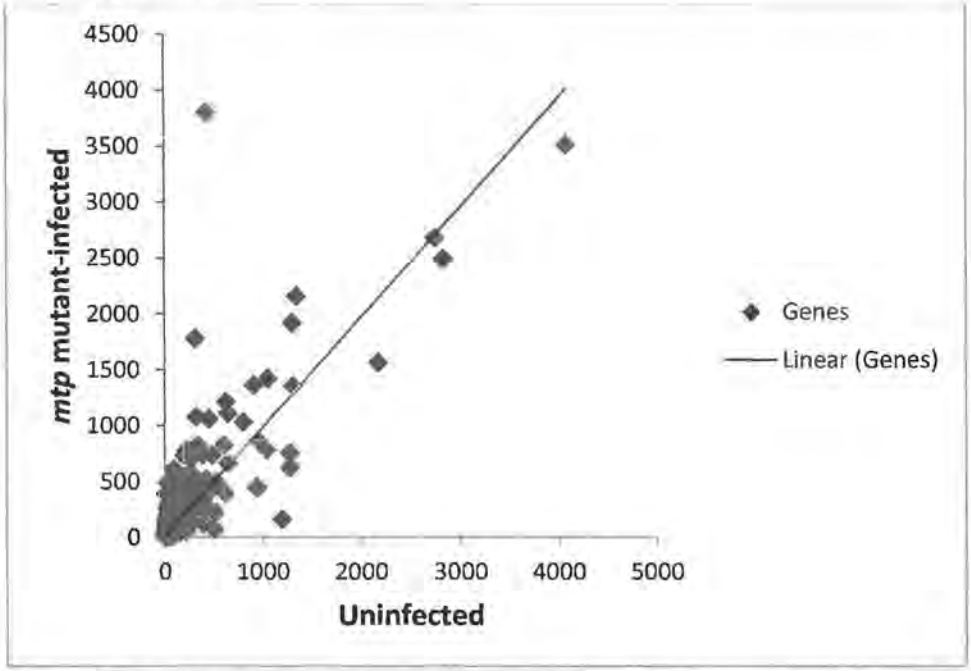


Figure 14: FPKM pairwise comparison of global gene expression profiles of A549 cells infected with the *mtp*-mutant strain relative to uninfected A549 epithelial cells. X and Y axis represent FPKM values of genes induced in A549 epithelial cells in response to infection with the *mtp*-mutant strain, relative to uninfected cells. Genes with similar FPKM values are closer to the line of regression. Genes below the regression line had low FPKM values while genes above had high FPKM values.

Unlike in the mutant infection, a majority of genes induced in A549 epithelial cells as a result of the wild type infection had higher FPKM values, ranging from 0-1000, in relation to uninfected cells (Figure 15). The *BAGE5* gene had the lowest (0.000288809) FPKM value while *FTL* had the highest (3872.75).

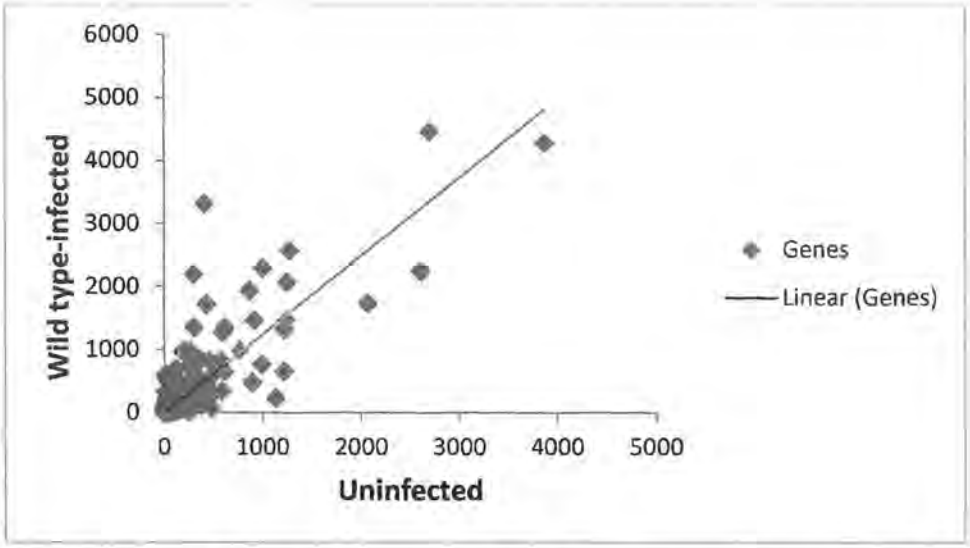


Figure 15: FPKM pairwise comparison of global gene expression profiles of A549 cells infected with the wild type strain relative to uninfected A549 epithelial cells. X and Y axis represent FPKM values of genes induced in A549 epithelial cells in response to infection with the wild type strain, relative to uninfected cells. Genes with similar FPKM values are closer to the line of regression. Genes below the regression line had low FPKM values while genes above had high FPKM values.

3.6. *M. tuberculosis* Induces Differential Gene Expression in Epithelial Cells

After the generation of gene expression profiles in A549 epithelial cells infected with the *mtp*-mutant and wild type strains of *M. tuberculosis*, relative to uninfected A549 epithelial cells, significantly differentially expressed genes (SDEGs) were obtained by filtering DEGs based on significance and using a Log2 fold change cut-off point of 2. The wild type strain induced a total of 917 SDEGs compared to 725 by the *mtp*-knockout mutant strain in A549 epithelial cells, relative to uninfected cells. A total of 307 and 115 SDEGs were exclusively induced by the wild type and *mtp*-knockout mutant strain, respectively, while 610 genes were induced by both strains (Figure 16). The induction of a high number of total and unique SDEGs by the wild type strain compared to the *mtp*-mutant strain is indicative of the important role that MTP plays in gene regulation of A549 epithelial cells.

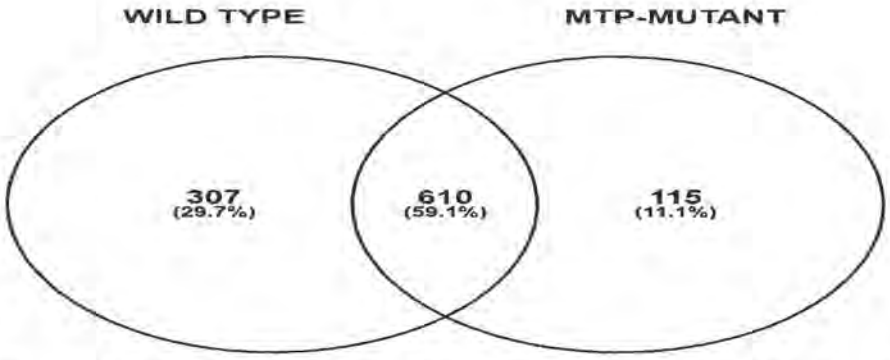


Figure 16: Significantly differentially expressed genes induced by the wild type and *mtp*-knockout mutant strains of *M. tuberculosis* in A549 pulmonary epithelial cells, relative to uninfected cells. The wild type strain induced the expression of a high number of total and unique genes in A549 epithelial cells compared to the *mtp*-mutant strain.

The number of up and down-regulated SDEGs in all 5 conditions is shown in Table 3. The wild type strain induced a higher number of down-regulated compared to up-regulated SDEGs. In contrast, the *mtp*-knockout mutant strain induced a high number of up-regulated compared to down-regulated genes. The number of up-regulated genes was higher than down-regulated genes amongst those that were shared by both strains, whilst genes that were uniquely induced by both strains were mostly down-regulated genes.

Table 3: Significantly differentially expressed genes that were up- and down-regulated by both strains in A549 epithelial cells, relative to uninfected cells.

	ALL WT	ALL MUTANT	SHARED	UNIQUE WT	UNIQUE MUTANT
Genes	917	725	610	307	115
Up-regulated	434	387	345	89	42
Down-regulated	483	338	265	218	73

ALL WT: All wild type-induced SDEGs.
ALL MUTANT: All *mtp* mutant-induced SDEGs.
UNIQUE WT: All SDEGs exclusively induced by the wild type strain.
UNIQUE MUTANT: All SDEGs exclusively induced by the *mtp*-mutant strain.

All top 10 most up-regulated SDEGs were shared by both strains except for *FXYD2* and *NEK2* which were exclusive to the wild type and *mtp*-mutant strain, respectively (Table 4). The fold change of the top 10 most up-regulated genes by the wild type and mutant strains ranged from 6.30766 (*FXYD2*) to 8.18454 (*TK1*) and 6.46674 (*KIAA0101*) to 8.33365 (*TK1*), respectively. All most up-regulated genes

that were shared by both strains were highly induced by the mutant strain compared to the wild type, except for *KIAA0101*.

The top 10 down-regulated genes were exclusively induced by each strain except for *AMTN* and *SCG5*, which were induced by both strains. Both these genes were highly down-regulated by the wild type strain compared to the mutant strain. The top 10 most down-regulated genes by the wild type strain ranged from a fold change of -5.83867(*RPLPOP2*) to -8.12269 (*UQCRHL*) while those down-regulated by the mutant strain ranged from -5.15085 (*FAM49A*) to -5.84926 (*CD163L1*). All top 10 most down-regulated genes were highly suppressed by the wild type strain compared to the mutant strain.

Table 4: The 10 most up- and down-regulated genes induced by the wild type and *mtp*-mutant strain in A549 pulmonary epithelial cells.

	Wild type		***mtp*-mutant**	
Up-regulated	**Gene**	**Fold Change**	**Gene**	**Fold Change**
	TK1	8.18454	*TK1*	8.33365
	MKI67	7.59066	*MKI67*	8.25201
	BIRC5	7.41559	*MYBL2*	7.75614
	MYBL2	7.25921	*BIRC5*	7.6842
	MCM10	7.1523	*MCM10*	7.5441
	DLGAP5	7.14504	*DLGAP5*	7.42044
	KIAA0101	6.6899	*UBE2C*	6.94315
	UBE2C	6.63154	*CCNB2*	6.68863
	CCNB2	6.38028	*NEK2*	6.65515
	FXYD2	6.30766	*KIAA0101*	6.46674
Down-regulated	**Gene**	**Fold Change**	**Gene**	**Fold Change**
	UQCRHL	-8.12269	*CD163L1*	-5.84926
	KRT34	-7.09732	*AMTN*	-5.79149
	RPL13AP5	-6.59157	*PAPPA2*	-5.68869
	AMTN	-6.44063	*SCG5*	-5.66398
	PIPSL	-6.38781	*DMGDH*	-5.42477
	FAM45B	-6.26196	*FABP6*	-5.34328
	ATP6V0D2	-6.25275	*PTPRR*	-5.30236
	RPS18P9	-6.15522	*LCP1*	-5.26313
	SCG5	-6.06841	*IL1RL1*	-5.19347
	RPLP0P2	-5.83867	*FAM49A*	-5.15085

3.7. Gene Ontology Analysis

The generation of gene expression profiles using *Cuffdiff* in A549 epithelial cells infected with the wild type and *mtp*-mutant strains of *M. tuberculosis*, in relation to uninfected cells, yielded 917 and 725 SDEGs, respectively. A total of 610 SDEGs were commonly induced by both strains while 307 and 115 genes were exclusively induced by the wild type and *mtp*-mutant strain, respectively. SDEGs were then enriched for biological functions using the Comparative Toxicogenomics Database with a corrected *p*-value of 0.05 and a highest Gene Ontology level of 3.

3.7.1. *Gene Expression Profiles of Common SDEGs Induced by M. tuberculosis*

A total of 610 SDEGs were commonly induced by both wild type and *mtp*-mutant strains in A549 pulmonary epithelial cells, relative to uninfected cells. These genes were then enriched for biological functions according to their level of expression (up- or down-regulated) using gene ontology analysis. Up-regulated genes were enriched for DNA replication, cell cycle and cell division, regulation of cell-to-cell adhesion, as well as host immune response functions such as complement activation, signal transduction and response to wounding and antigen presentation via MHC class II (Table 5). Some up-regulated genes were involved in multiple biological functions e.g. *C3* and *C4BPA* were associated with complement activation and signal transduction and response to wounding, *HLA-DMB*: antigen presentation and regulation of cell-to-cell adhesion, *CCL2*: signal transduction and response to wounding and regulation of cell-to-cell adhesion, *CDK1*: signal transduction and response to wounding and DNA replication, *BRCA1*: DNA replication and cell cycle and cell division.

Table 5: Biological functions associated with up-regulated SDEGs induced by both strains in A549 pulmonary epithelial cells, relative to uninfected cells.

	Gene Ontology Functions	Genes
Up-regulated	Complement Activation	*C3, C4BPA, CFB, CFH, CFI, SUSD4, VTN*
	Antigen Presentation via MHC Class II	*AP1M2, HLA-DMB, KIF11, KIF15, KIF18A, KIF23, KIF2C, KIF4A*
	Signal Transduction and Response to Wounding	*ANGPT1, ARRB1, C3, C4BPA, CCL2, CCNB1, CDK1, CFB, CFD, CFH, CFI*
	DNA Replication	*BRCA1, CDC25A, CDC45, CDC6, CDK1, CDT1, CHEK1, CLSPN*
	Cell Cycle and Cell Division	*ANLN, ASPM, AURKA, AXIN2, BIRC5, BRCA1, BRIP1, BUB1, BUB1B, CASC5, CCNA2, CCNB1, CCNB2, CCND3*
	Regulation of Cell-to-Cell Adhesion	*CCL2, CD24, EGR3, FGA, FGB, FGG, HES1, HLA-DMB, MAP3K8, MYB, NR4A3, SOX2*

Down-regulated genes were associated with cell communication, enzyme binding, regulation of cellular metabolic processes as well as host response-related functions such as the cell surface receptor signalling pathway and cell and lymphocyte activation (Table 6). Some down-regulated genes were also associated with multiple biological functions e.g. *ACKR3*: cell communication, cell surface receptor signalling pathway and regulation of cellular metabolic processes, *ARHGEF2*: cell communication, enzyme binding, cell surface receptor signalling pathway and regulation of cellular metabolic processes, *CCBE1*: cell communication, enzyme binding and cell surface receptor signalling pathway, *CD8A*: cell communication, cell surface receptor signalling pathway, lymphocyte and cell activation and enzyme binding, *IL12A* and *IL6*: cell communication, cell surface receptor signalling pathway, lymphocyte and cell activation and regulation of cellular metabolic processes, *CD274*: cell surface receptor signalling pathway and lymphocyte and cell activation, *IRF4*: cell surface receptor signalling pathway, lymphocyte and cell activation and regulation of cellular metabolic processes.

Table 6: Biological functions associated with down-regulated SDEGs induced by both strains in A549 pulmonary epithelial cells, relative to uninfected cells.

	Gene Ontology Functions	Genes
Down-regulated	Cell Communication	*ACKR3, ARHGAP25, ARHGDIB, ARHGEF2, BMF, CCBE1, CD8A, IL12A, IL6*
	Enzyme Binding	*ARHGEF2, BHLHE41, BRSK1, CACNB1, CACNB2, CCBE1, CD8A, CSTA*
	Cell Surface Receptor Signalling Pathway	*ACKR3, AKAP3, ARHGEF2, CCBE1, CD274, CD8A, DACT1, FOXL1, G0S2, GBP1, GDF6, GREM1, HDAC9, IGFBP1, IL12A, IL6, IRF4*
	Lymphocyte Activation and Cell Activation	*CD274, CD8A, DOCK2, HDAC9, IL12A, IL20RB, IL6, IRF4, LAT, LCP1*

To display gene expression profiles of the SDEGs that were induced by both strains in A549 epithelial cells, expression levels of top 50 most up- and down-regulated genes were investigated using a heat map (Figure 17). Amongst top 100 genes, both strains significantly up-regulated DNA replication genes such as *CDC45* and *CDC6* while cell surface receptors such as *CD22* and *CD163L1* were down-regulated. *HIST1H* genes (*HIST1H2AL*, *HIST1H3B* and *HIST1H3C*) were up-regulated by both strains while *PTP* genes (*PTPRH*, *PTPRO* and *PTPRR*) were down-regulated. *SERPIN* genes were differentially expressed with *SERPINB3* and *SERPINB4* down-regulated and *SERPINA6* up-regulated by both strains. A differential expression by both strains was also observed with *FAM* genes where *FAM111B* and *FAM64A* were up-regulated while *FAM49A* was down-regulated. *TK1*, *MKI67*, *BIRC5*, *MYBL2*, *MCM10* and *DLGAP5* were the most up-regulated genes by both strains whereas *AMTN*, *SCG5*, *PTPRR*, *SERPINB4* and *SPON1* were the most down-regulated genes.

Figure 17: Gene expression profiles of top 50 (out of 345) up-regulated and top 50 (out of 265) down-regulated genes induced by both strains in A549 pulmonary epithelial cells at 4 hr post-infection, relative to uninfected cells. Sequenced reads were mapped to the reference genome using *TopHat*. *Cuffdiff* of the RNA-sequencing Tuxedo pipeline was used to identify differentially expressed genes between uninfected and infected epithelial cells.

3.7.2. *Strain-specific SDEG Expression Profiles*

One hundred and fifteen (115) SDEGs were uniquely induced by the *mtp*-mutant strain in A549 alveolar epithelial cells, with 43 and 72 genes up- and down-regulated, respectively. Our gene ontology analysis showed that up-regulated genes were enriched for biological functions such as protein refolding, cellular response to heat, programmed cell death as well as positive regulation of tumor necrosis factor-mediated pathways (Table 7). Heat shock proteins *HSPA1A* and *HSPA1B* were associated with all the above-mentioned functions whereas *HSPA6* was only involved in protein refolding and cellular response to stress and heat. *DDIAS*, *EEF2K* and *PRDX2* were all associated with programmed cell death and cellular response to stress and heat.

Table 7: Biological functions associated with up-regulated SDEGs induced by the *mtp*-mutant strain in A549 pulmonary epithelial cells, relative to uninfected cells.

	Gene Ontology Functions	**Genes**
Up-regulated	Protein Refolding	*HSPA1A, HSPA1B, HSPA2, HSPA6*
	Cellular Response to Stress and Heat	*CHAF1A, DDIAS, EEF2K, HIST1H4D, HSPA1A, HSPA1B, HSPA6, KLF10, KLF2, PRDX2, RECQL4, SNAI1*
	Programmed Cell Death	*APLP1, DDIAS, EEF2K, HSPA1A, HSPA1B, ID1, LMNB1, PRDX2, SNAI1, TNS4*
	Positive Regulation of Tumor Necrosis Factor-Mediated Pathways	*HSPA1A, HSPA1B*

Down-regulated genes induced by the *mtp*-mutant strain in A549 epithelial cells were enriched for cellular processes, binding and ion binding (Table 8). *ABAT*, *ADRB2*, *AIFM3*, *ALOX5AP*, *BBS10*, *CDK14*, *CYP27B1*, *EIF4A2* and *HACR2* were associated with all the above mentioned biological functions whereas *ACOT4*, *BBC3*, *FYB* and *GSAP* were associated with cellular processes and binding. *HFM1* was involved in both cellular processes and ion binding while *ADAM32*, *ADAMTS6* and *AMY2B* were associated with binding and ion binding

Table 8: Biological functions associated with down-regulated SDEGs induced by the *mtp*-mutant strain in A549 pulmonary epithelial cells, relative to uninfected cells.

	Gene Ontology Functions	Genes
Down-regulated	Cellular Processes	*ABAT, ACOT4, ADRB2, AIFM3, ALOX5AP, BBC3, BBS10, CDK14, CYP27B1, EIF4A2, FYB, GSAP, HAVCR2, HES6, HFM1, HOXD8, HYDIN*
	Binding	*ABAT, ACOT4, ADAM32, ADAMTS6, ADRB2, AIFM3, ALOX5AP, AMY2B, BBC3, BBS10, CDK14, CYP27B1, EIF4A2, FYB, GSAP, HAVCR2*
	Ion Binding	*ABAT, ADAM32, ADAMTS6, ADRB2, AIFM3, ALOX5AP, AMY2B, BBS10, CDK14, CYP27B1, EIF4A2, HAVCR2, HFM1, MAT1A, NELL2*

The wild type strain uniquely induced 307 SDEGs in A549 alveolar epithelial cells, with 89 and 218 genes up- and down-regulated respectively. Up-regulated genes were enriched for cell communication, signal transduction, response to lipids and cellular response to chemical stimulus (Table 9). All genes involved in cell communication, except for *ANG*, *LDHA*, *MAPRE2* and *NMU*, were also associated with signal transduction. Moreover, *ADCY5* was involved in all above mentioned biological functions while *ADM* and *EDN1* also played a role in signal transduction and response to lipids. *ERBB3*, *IFITM3* and *IL6ST* were associated with cell communication, signal transduction and cellular response to chemical stimulus while *NR2F1*, *NR5A2*, *PTGER2* and *UCP2* were involved in response to lipids and cellular response to chemical stimulus.

Table 9: Biological functions associated with up-regulated SDEGs induced by the wild type strain in A549 pulmonary epithelial cells, relative to uninfected cells.

	Gene Ontology Functions	Genes
Up-regulated	Cell Communication	*ABCC9, ADCY5, ADM, ANG, ANXA4, BCAM, CALY, CAMK1, EDN1, EPHA6, ERBB3, GSG2, HIST1H4B, HIST1H4C, IFITM3, IGF2, IL6ST, LDHA, MAPRE2, NMU*
	Signal Transduction	*ABCC9, ADCY5, ADM, ANXA4, BCAM, CALY, CAMK1, EDN1, EPHA6, ERBB3, GSG2, HIST1H4B, HIST1H4C, IFITM3, IGF2, IL6ST*
	Response to Lipids	*ADCY5, ADM, APOH, AQP3, EDN1, IGF2, LDHA, NR2F1, NR5A2, PTGER2, UCP2*
	Cellular Response to Chemical Stimulus	*ACY3, ADCY5, ADH6, CXCL3, EDN1, ERBB3, IFITM3, IGF2, IL6ST, MT1F, NR2F1, NR5A2, POU5F1, PTGER2, STOX1, TBXAS1, UCP2, WNT4*

Down-regulated genes induced by the wild type strain in A549 epithelial cells were enriched for cell communication, signal transduction, cell differentiation and regulation of biosynthetic processes (Table 10). It is important to note that down-regulated genes that were enriched for cell communication and signal transduction were completely different from the up-regulated genes that were enriched for the same functions. However, the trend that was observed in up-regulated genes associated with these functions was also observed with down-regulated genes where all genes associated with cell communication, except for *CNTN4* and *IL23A,* were also involved in signal transduction. Moreover, *EDA2R* was associated with cell differentiation and regulation of biosynthetic processes while *ADRA2C, CDKN1C, CNT4* and *DNER* were involved in cell differentiation. *CRLF1* was involved in cell communication and regulation of biosynthetic processes while *FRMD6* was only associated with cell differentiation and regulation of biosynthetic processes.

Table 10: Biological functions associated with down-regulated SDEGs induced by the wild type strain in A549 pulmonary epithelial cells, relative to uninfected cells.

Down-regulated	Cell Communication	*ADGRD1, ADRA2C, AKAP12, ARHGEF4, ASIC2, ATP6V0D2, CALCB, CAPN3, CDKL2, CDKN1C, CLEC2D, CNTN4, COL16A1, CRLF1, DISP1, DNER, ECM1, EDA2R, IL23A*
	Signal Transduction	*ADGRD1, ADRA2C, AKAP12, ARHGEF4, ASIC2, ATP6V0D2, CALCB, CAPN3, CDKL2, CDKN1C, CLEC2D, COL16A1, CRLF1, DISP1, DNER, ECM1, EDA2R*
	Cell Differentiation	*ADRA2C, ARID5A, CAPN3, CATSPER3, CCDC85B, CDH4, CDKN1C, CEBPG, CNTN4, CRTAC1, DNER, EDA2R, FHL2, FRMD6, GAB2*
	Regulation of Biosynthetic Processes	*CRLF1, EDA2R, FOXD1, FRMD6, HES7, HEY1, KCNMA1, KRT34, LAMB3, NKX2-5, PGF, PODXL*

3.8. Ingenuity Pathway Analysis

The role of MTP in enrichment of canonical pathways was investigated with knowledge-based IPA using datasets of SDEGs induced in A549 alveolar epithelial cells by the wild type strain, *mtp*-knockout mutant strain, shared SDEGs as well as SDEGs that were unique to the wild type strain and *mtp*-knockout mutant strain (Table 11).

The wild type strain enriched the highest number of canonical pathways compared to the *mtp*-mutant strain. SDEGs that were exclusively induced by the wild type also enriched the highest number of canonical pathways than those that were unique to the *mtp*-mutant strain (Table 11). These findings suggest that MTP affects gene regulation of A549 epithelial cells through differential gene expression.

A pathway for Cell Cycle Control of Chromosomal Replication was the highest enriched pathway by both strains. The Role of IL-17A in Arthritis and LPS/IL-1 Mediated Inhibition of RXR Function pathways were the lowest enriched pathways by the wild type and *mtp*-mutant strain, respectively.

Table 11: Canonical pathways enriched by SDEGs induced by wild type and *mtp*-knockout mutant strains in A549 epithelial cells, relative to uninfected cells.

	ALL WT	ALL MUTANT	SHARED	UNIQUE WT	UNIQUE MUTANT
Genes	917	725	610	307	115
Pathways	44	30	27	17	3

ALL WT: All wild type-induced SDEGs.
ALL MUTANT: All *mtp* mutant-induced SDEGs.
UNIQUE WT: All SDEGs exclusively induced by the wild type strain.
UNIQUE MUTANT: All SDEGs exclusively induced by the mutant strain.

3.8.1. *Canonical Pathway Analysis*

A total of 27 canonical pathways were enriched by both strains while 3 and 17 pathways were enriched uniquely by the *mtp*-mutant and wild type strains, respectively (Figure 18).

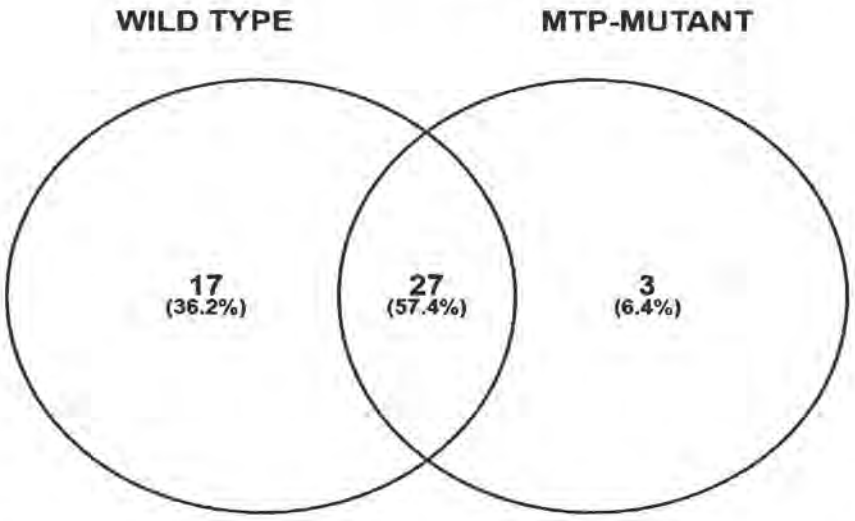


Figure 18: The number of canonical pathways that were enriched by significantly differentially expressed genes induced by the wild type and *mtp*-knockout mutant strains of *M. tuberculosis* in A549 epithelial cells after 4 hours of infection. A three-way Venn diagram was created using the VENNY software.

3.8.2. *Pathways Commonly Induced by the wild type and mutant strain*

A total of 27 canonical pathways were enriched by both wild type and *mtp*-mutant strain in A549 epithelial cells, relative to uninfected cells (Figure 19). Our canonical pathways analysis showed that 23 of the 27 shared pathways were highly enriched by the *mtp*-mutant strain compared to the wild type strain (Figure 19). The wild type strain highly enriched 4 pathways (FXR/RXR Activation, Ethanol Degradation II, Ethanol Degradation IV and LPS/IL-1 Mediated Inhibition of RXR Function) compared to the *mtp*-mutant strain and all these were low ranking pathways. All top 10 high ranking pathways were highly enriched by the *mtp*-mutant strain, compared to the wild type strain. Both strains induced pathways involved in normal cellular processes and early host immune response. The Cell Cycle Control of Chromosomal Replication pathway was the highest enriched pathway by both strains, however, this pathway was highly enriched by the mutant strain. LPS/IL-1 Mediated Inhibition of RXR Function pathway was the lowest enriched pathway by both strains (Figure 19).

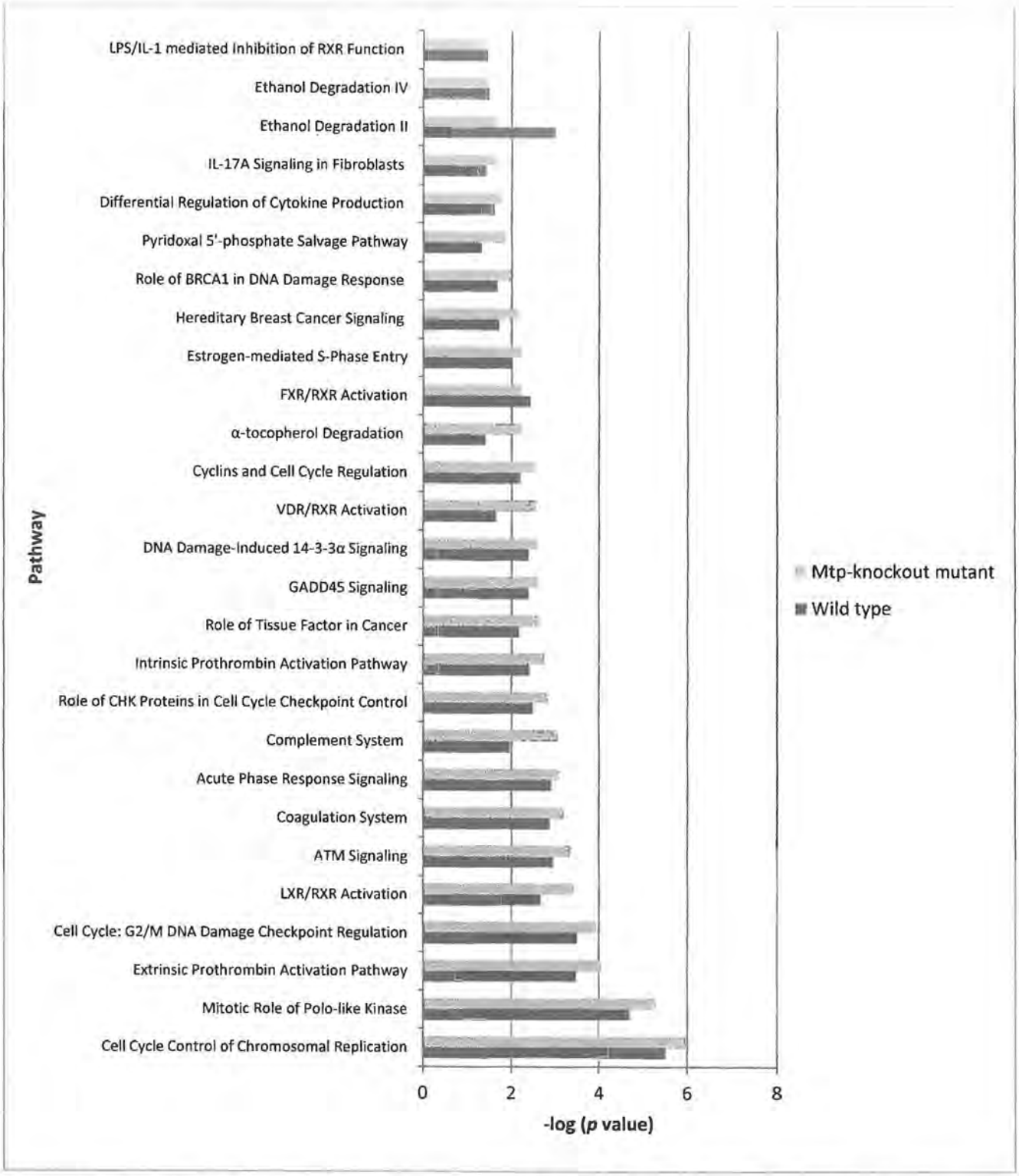
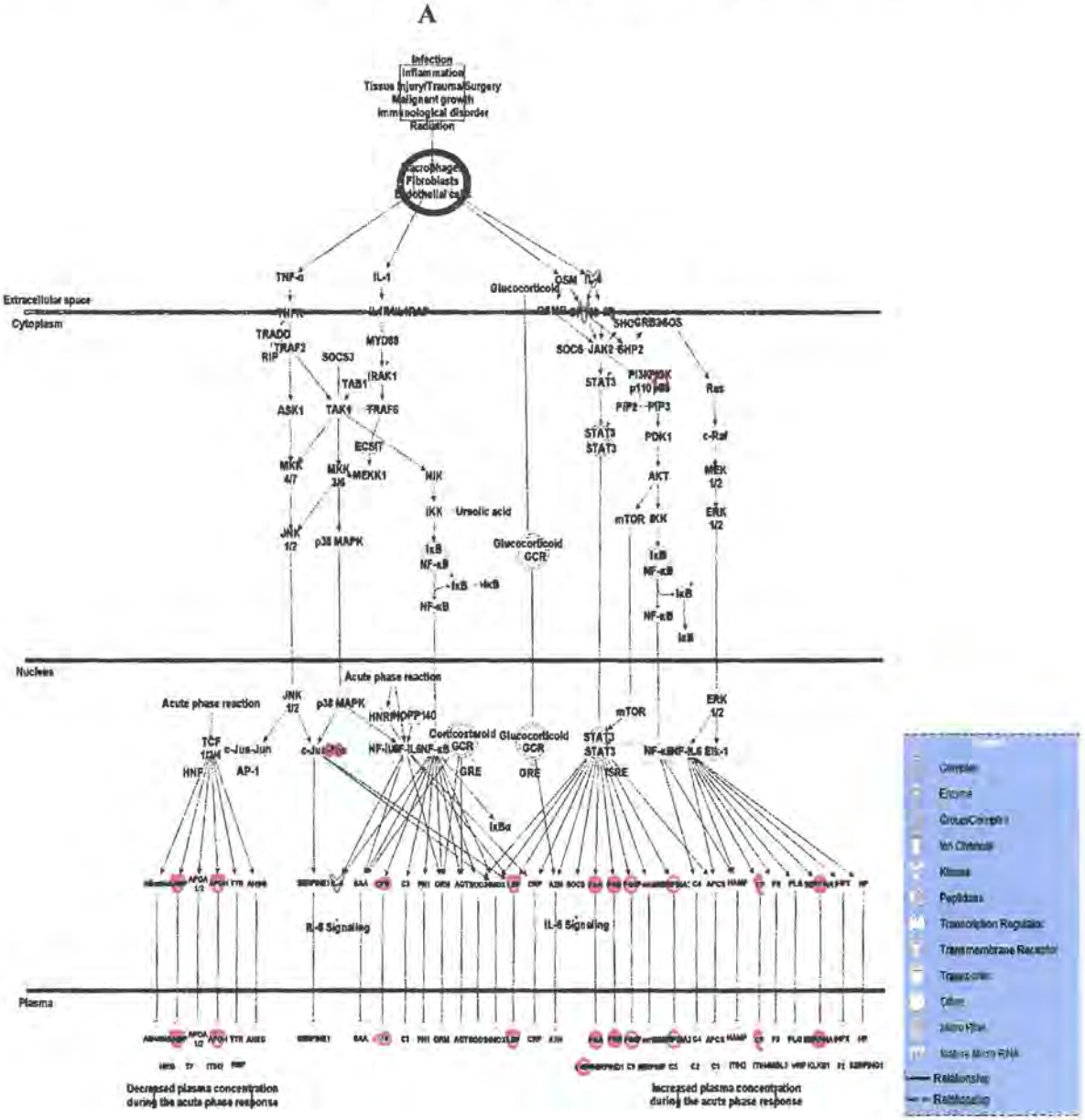


Figure 19: A total of 27 canonical pathways were enriched by both wild type and *mtp*-knockout mutant strains in A549 epithelial cells. –log p values (which correlate with the level of enrichment) were used to compare the enrichment of these pathways in each infection condition. A total of 23 canonical pathways were highly enriched by the *mtp*-mutant strain compared to the wild type strain.

3.8.3. *Host Immune Response-related Pathways Commonly Induced by both strains*

Both strains enriched pathways associated with host immune response e.g. Acute Phase Response Signaling, Complement System and Differential Regulation of Cytokine production in Macrophages and T helper cells.

Genes involved in Acute Phase Response Signaling Pathway were differentially induced by both strains (Figure 20). However, this pathway was highly enriched by the *mtp*-mutant strain compared to the wild type. Both strains up-regulated *PL3K/P85* complex, *SERPINA1, CP, SERPINA3, FGG, FGB, FGA, LBP, CFB, AMBP, CFOS* and *C4BP* genes while *IL-6* was the only down-regulated gene. The wild type strain exclusively up-regulated *GP130* and *APOH* genes whereas *C3*, which is very essential for the acute phase response, was exclusively up-regulated by the *mtp*-mutant strain.



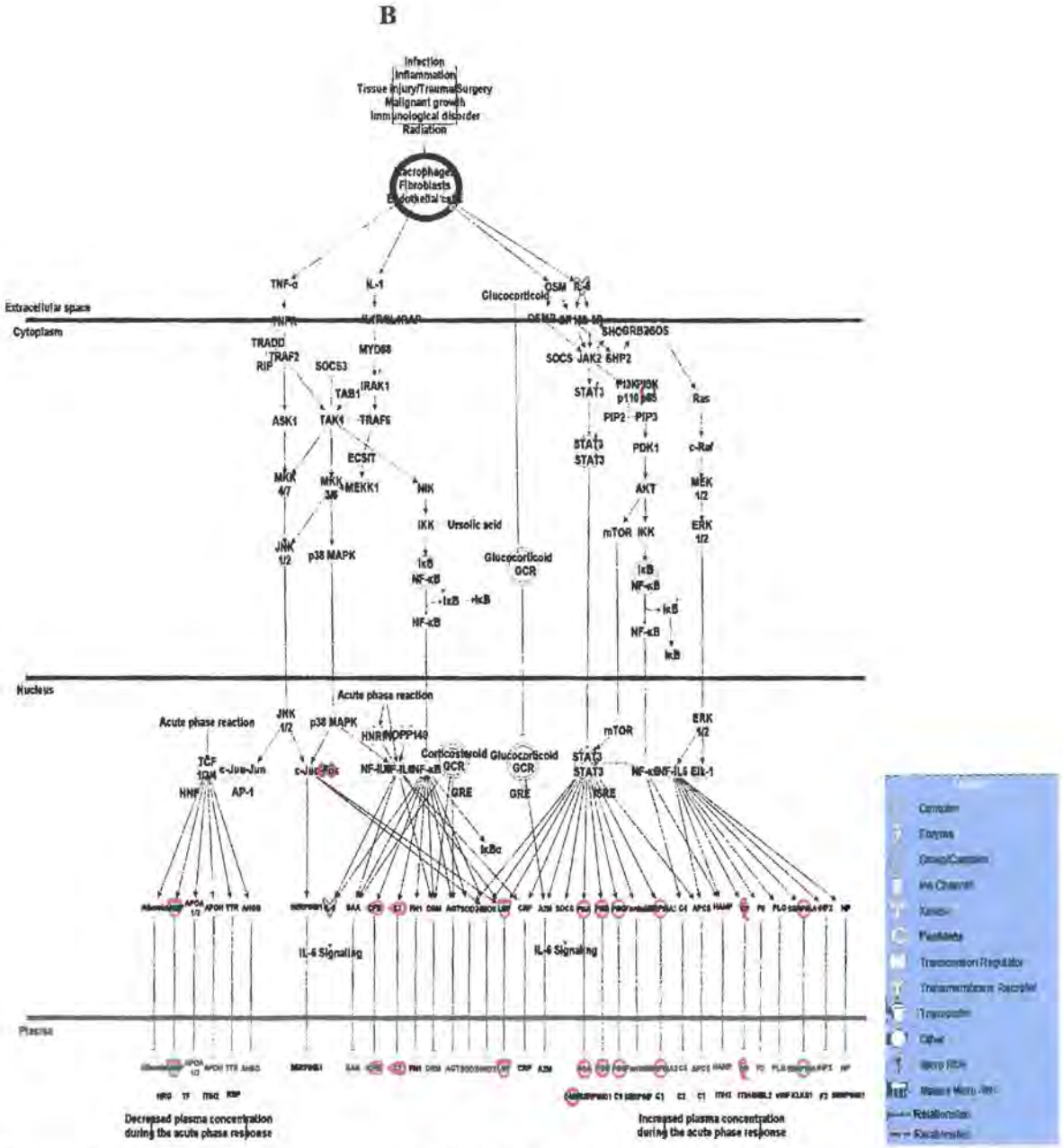


Figure 20: **Acute Phase Response Signaling Pathway**. Canonical pathways enriched by wild type strain (A) and *mtp*- mutant strain (B). SDEGs associated with this pathway are displayed with red and green which indicate up- and down-regulated genes. The brighter the red or green colour, the more up- and down-regulated the gene in A549 epithelial cells, relative to uninfected cells. Genes shaded in white are part of the knowledge-based IPA software and were not expressed in our datasets.

In addition to the Acute Phase Response Signalling Pathway, the Complement System Pathway, especially the alternate pathway, was more highly enriched by the *mtp*-mutant compared to the wild type strain in A549 epithelial cells, relative to uninfected cells (Figure 21). Both strains up-regulated *C4BP, BF, IF, HF1, DF, BFA* and *BFB* genes while the *mtp*-mutant strain exclusively up-regulated *C3, C3A* and *C3B* genes.

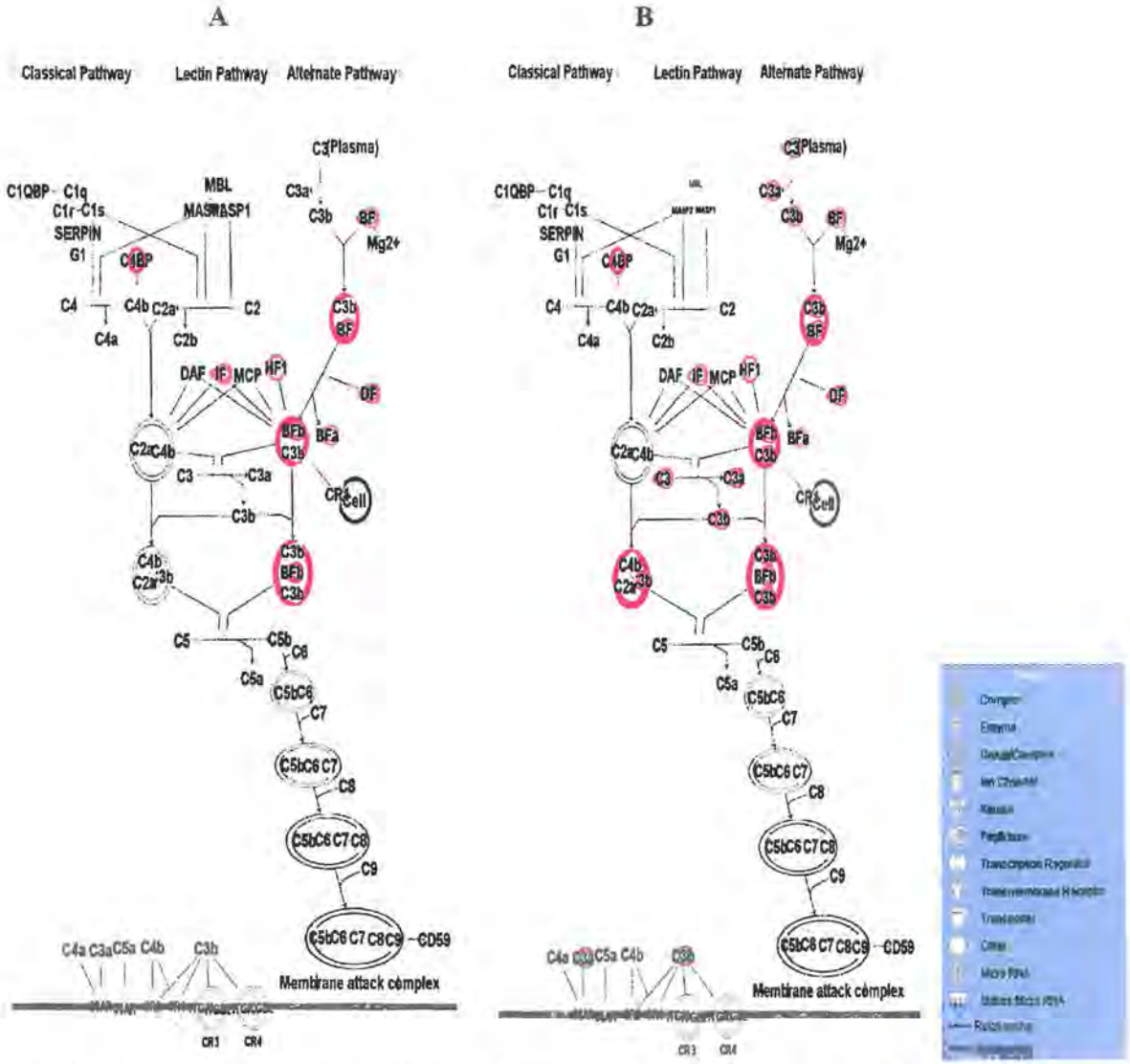


Figure 21: **Complement System Pathway**. Canonical pathways enriched by wild type strain (A) and *mtp*- mutant strain (B). SDEGs associated with this pathway are displayed with red and green which indicate up- and down-regulated genes. The brighter the red or green colour, the more up- and down-regulated the gene in A549 epithelial cells, relative to unifected cells. Genes shaded in white are part of the knowledge-based IPA software and were not expressed in our datasets.

3.8.4 *M. tuberculosis Exclusively Enriches Three Pathways in the Absence of MTP*

In addition to canonical pathways that were commonly enriched by both strains in A549 epithelial cells, pathways that were exclusively enriched by the *mtp*-mutant strain were investigated further. In the absence of the *mtp* gene, only three pathways were uniquely enriched by the *mtp*-mutant strain (Figure 18). Amongst the three, p53 Signaling Pathway was the highest enriched pathway in *mtp*-mutant-infected A549 epithelial cells while Glioma Invasiveness Signalling Pathway was the lowest enriched pathway (Figure 22).

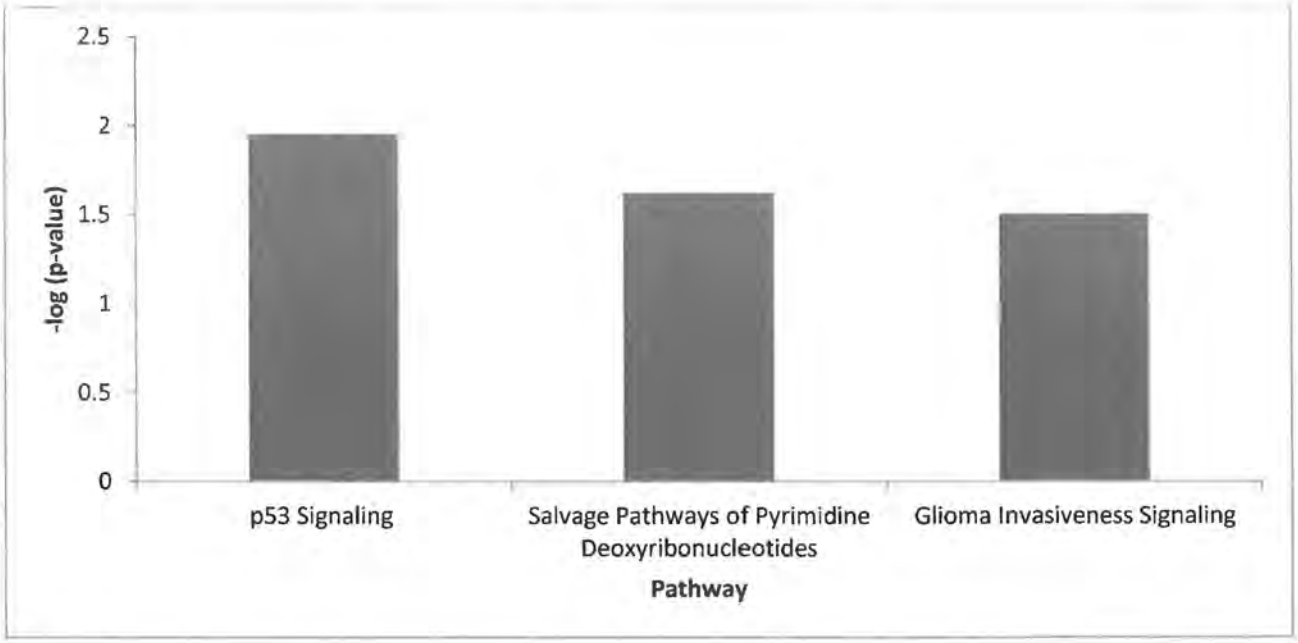


Figure 22: Pathways that were exclusively enriched by the *mtp*-knockout mutant strain in infected A549 epithelial cells. MTP did not have any effect on these pathways.

A majority of genes involved in the p53 Signalling Pathway were up-regulated (Figure 23). *PUMA* molecules and *HDAC9* and *Slug* transcription regulators were down-regulated whereas *PL3K, CHK1, E2F1, BRCA1*, *E2F1* and the *Survivin* molecule were up-regulated.

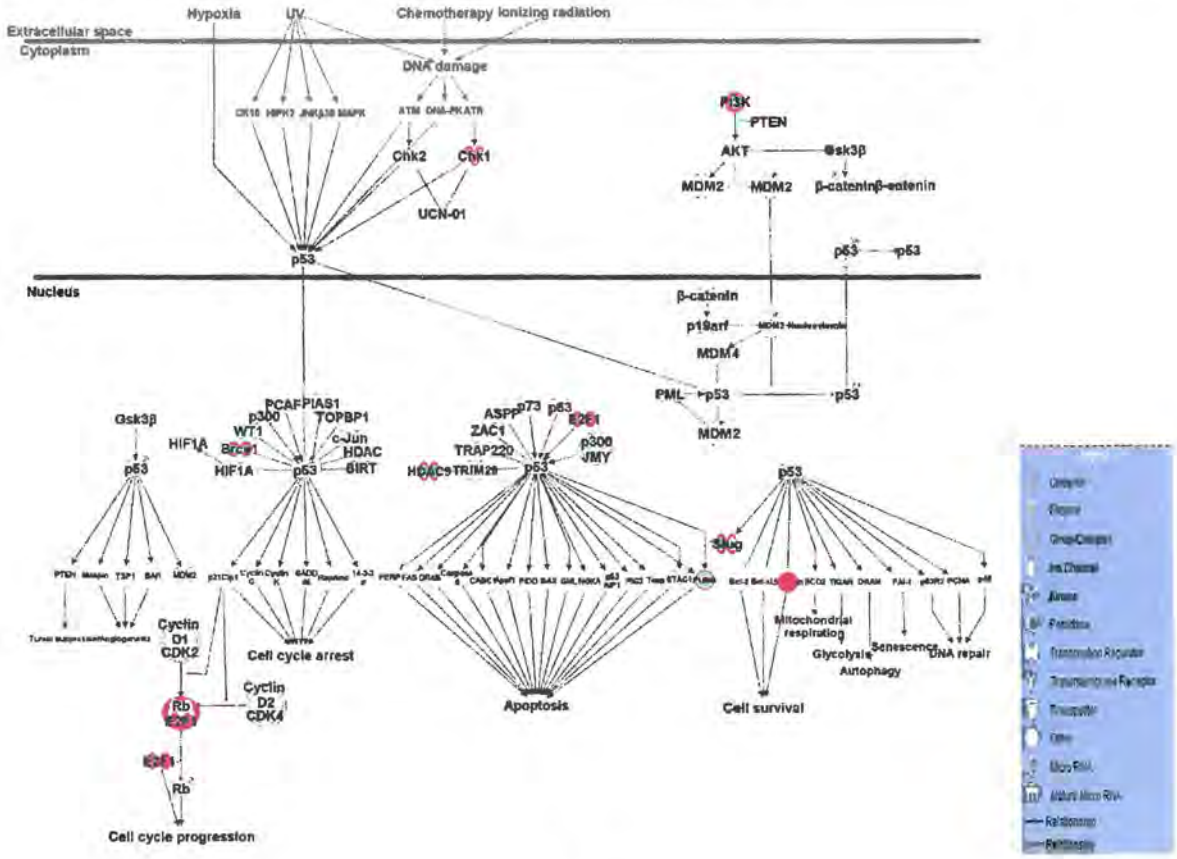


Figure 23: **p53 Signaling Pathway**. Canonical pathway exclusively enriched by *mtp*- mutant strain. SDEGs and molecules associated with this pathway are displayed with red and green which indicate up- and down-regulated genes. The brighter the red or green colour, the more up- and down-regulated the gene in A549 epithelial cells, relative to unifected cells. Genes shaded in white are part of the knowledge-based IPA software and were not expressed in our datasets. The legend on the right hand side shows different molecules displayed on the pathway.

The pathway for Salvage Pathways of Pyrimidine Deoxyribonucleotides was the second highest exclusively enriched pathway by the *mtp*-mutant strain (Figure 24). Deoxycytidine deaminase and thymidine kinase were the only up-regulated molecules in this pathway while other molecules were not expressed at all.

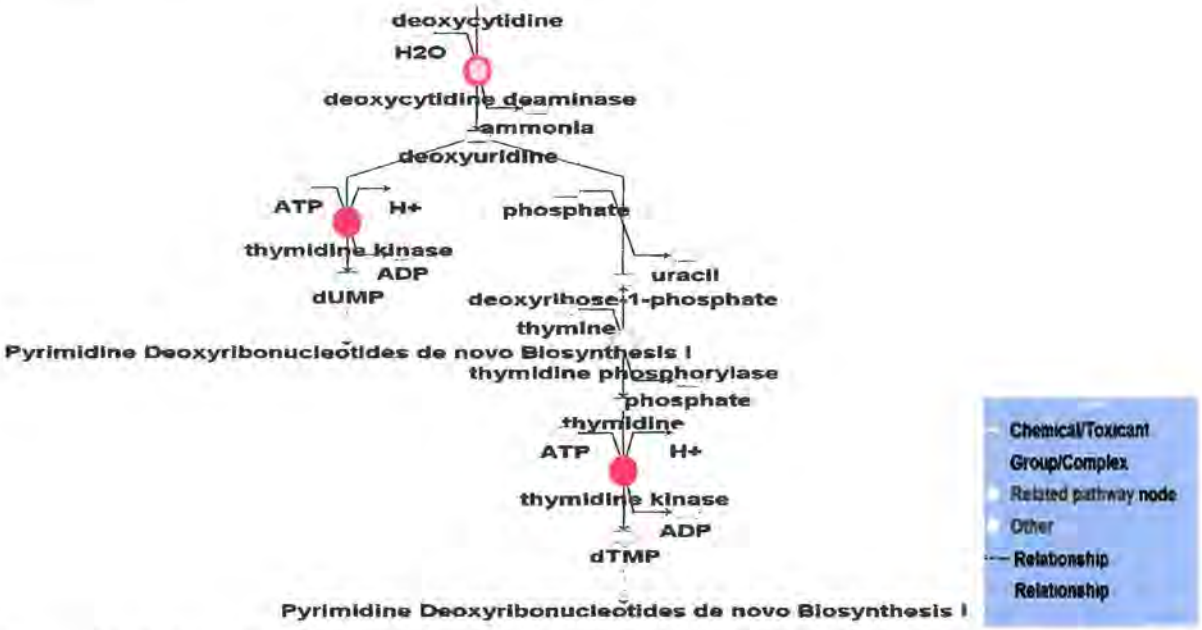


Figure 24: **Salvage Pathways of Pyrimidine Deoxyribonucleotides Pathway**. Canonical pathway exclusively enriched by *mtp*- mutant strain. Molecules associated with this pathway are displayed with red and green which indicate up- and down-regulated genes. The brighter the red or green colour, the more up- and down-regulated the molecule in A549 epithelial cells, relative to unifected cells. Molecules shaded in white are part of the knowledge-based IPA software and were not expressed in our datasets.

The Glioma Invasiveness Signalling pathway was the lowest exclusively enriched canonical pathway by the *mtp*-mutant strain in A549 epithelial cells (Figure 25). Cell surface receptors involved in this pathway were both up- and down-regulated, with the ITG receptor being down-regulated and the RHAMM receptor being up-regulated. *Vibronectin, RHO* and *PL3K* molecules were up-regulated, whereas, the expression of other genes and molecules associated with this pathway were not induced by the *mtp*-mutant strain in A549 epithelial cells.

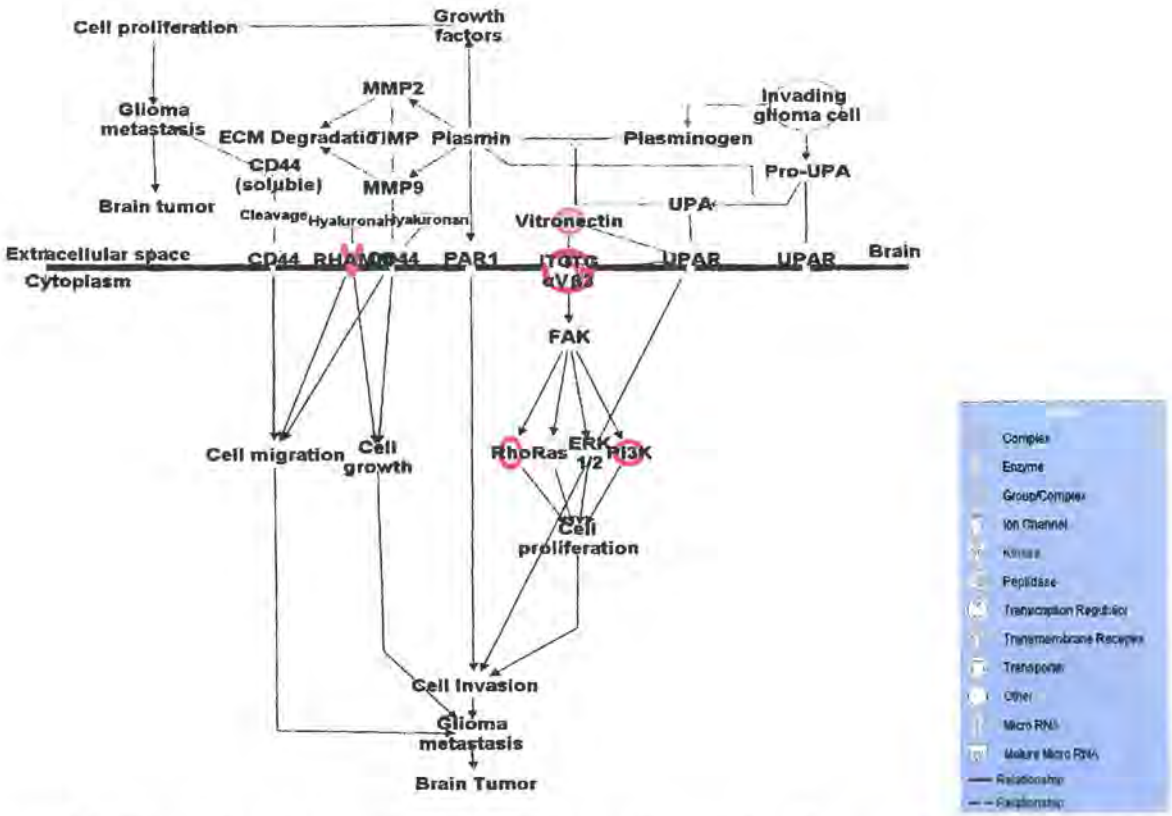


Figure 25: Glioma Invasiveness Signalling Pathway. Canonical pathway exclusively enriched by *mtp*- mutant strain. Molecules associated with this pathway are displayed with red and green which indicate up- and down-regulated genes. The brighter the red or green colour, the more up- and down-regulated the gene in A549 epithelial cells, relative to unifected cells. Molecules shaded in white are part of the knowledge-based IPA software and were not expressed in our datasets. The legend on the right hand side shows different molecules displayed on the pathway.

3.8.5 *M. tuberculosis Exclusively Enriches 17 Pathways in the Presence of MTP*

Our canonical pathway analysis also showed that 17 of the 44 pathways that were enriched by the wild type strain were not enriched by the mutant strain (Figure 26). Amongst these, the Human Embryonic Stem Cell Pluripotency pathway was the highest enriched pathway whereas the Role of IL-17A in Arthritis pathway was the lowest enriched. The majority of these pathways are associated with normal cellular biochemical processes, with Fatty Acid α-oxidation Pathway being the only host immune response-related pathway (Figure 27).

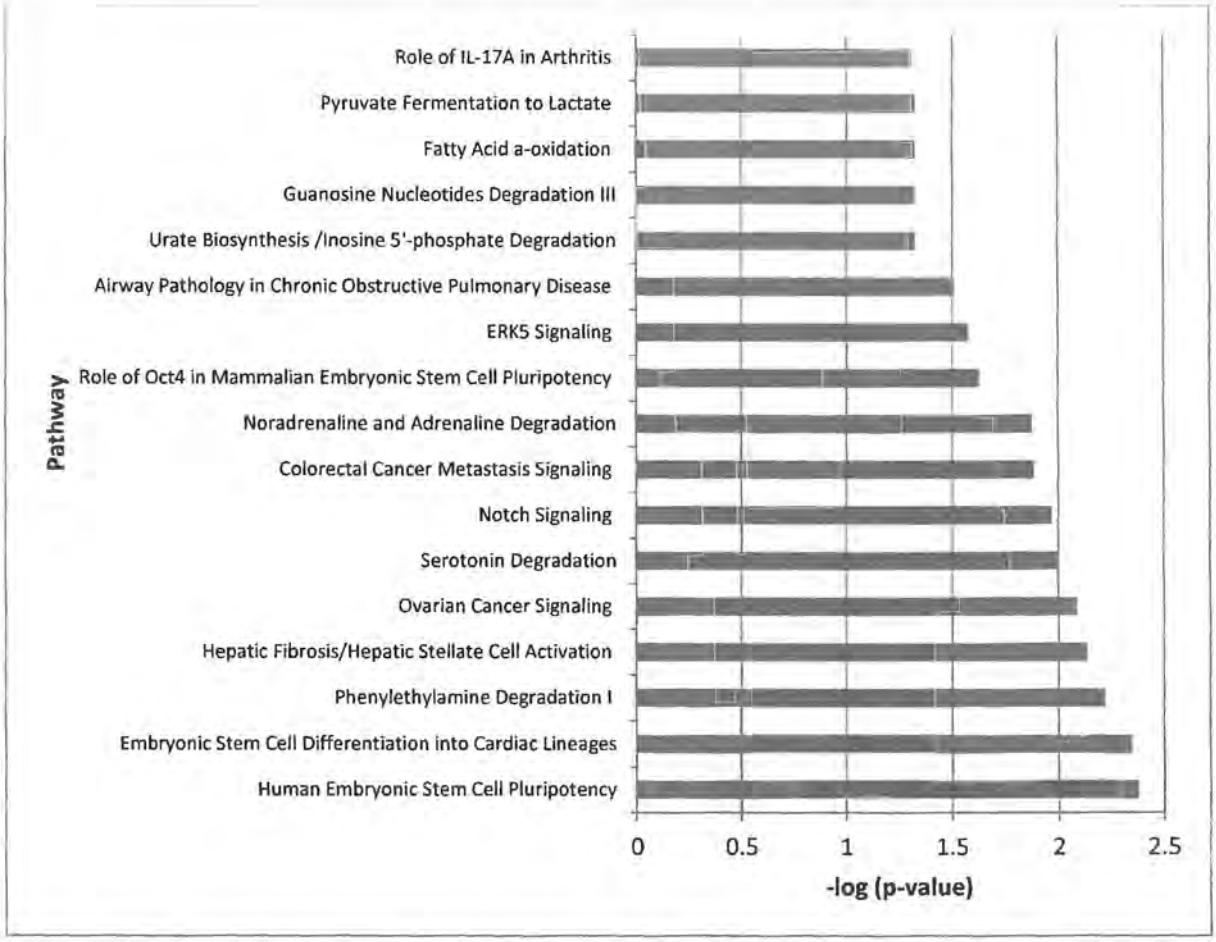


Figure 26: Pathways that were only enriched as a result of the wild type infection. MTP caused the enriched of these pathways in infected A549 epithelial cells.

NAD+, aldehyde dehydrogenase and oxygen oxidoreductase molecules were the only expressed molecules in the Fatty acid α-oxidation pathway (Figure 27).

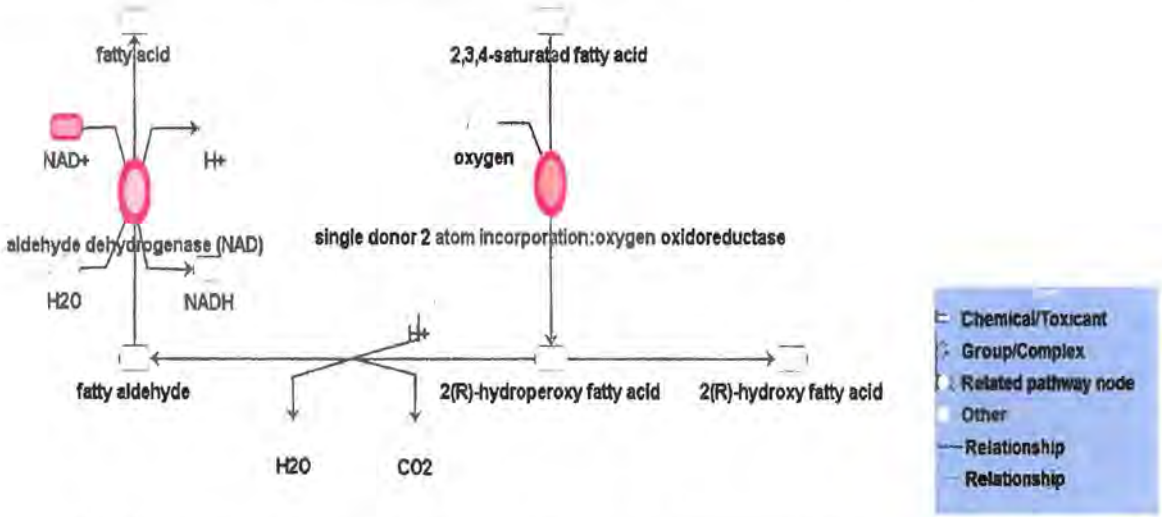


Figure 27: **Fatty acid α-oxidation Pathway**. A low ranking canonical pathway associated with the host immune response that was exclusively enriched as a result of wild type infection. Molecules

associated with this pathway are displayed with red and green which indicate up- and down-regulated genes. The brighter the red or green colour, the more up- and down-regulated the molecule in A549 epithelial cells, relative to unifected cells. Molecules shaded in white are part of the knowledge-based IPA software and were not expressed in our datasets.

3.9. The Role of MTP in Host Immune Response

Gene expression profiles of DEGs associated with the host immune response were further investigated using a heat map (Figure 28). These included genes coding for various functions such as host-pathogen interactions, immune and inflammatory response and immune cell activation. The level of up-regulation for all up-regulated genes was higher in genes induced by the wild type strain when compared to those induced by the *mtp*-mutant strain, while the level of down-regulation varied between the two conditions. The heat map also shows that the deletion of MTP results in down-regulation of some of the genes that were up-regulated by the wild type strain. These findings further reveal the essential role that MTP plays in gene regulation of A549 epithelial cells.

Both strains up-regulated genes associated with the complement system e.g. *C1S, C3, C4BPA, C5, C6* and *C7*. However, the level of up-regulation of these genes was highly induced by the wild type strain compared to the mutant. There was no significant up-regulation of *C4BPB* and *C5AR1* by the *mtp*-mutant strain while the wild type strain significantly up-regulated these genes. The majority of cell surface markers were down-regulated by both strains except for *CD14, CD24, CD302, CD38, CD99, CD9* and *CD96*. Moreover, *CD14, CD24, CD302, CD38* and *CD99* were highly induced by the wild type strain compared to the *mtp*-mutant while the up-regulation of *CD9* and *CD99L2* by the *mtp*-mutant strain was not significant.

All genes associated with the ability of epithelial cells to present antigens (HLA genes) were down-regulated except for *HLA-DMA* and *HLA-DMB*. The wild type strain highly up-regulated *HLA-DMA* when compared to the *mtp*-mutant strain. Both strains did not induce the up-regulation of cytokines in A549 alveolar epithelial cells except for *IL-15* and *IL-32*. The wild type strain significantly up-regulated these two cytokines while the *mtp*-mutant strain did not affect their expression. Both strains highly up-regulated the expression of chemokines in epithelial cells except for *CCL20* and *CXCL8* for the wild type strain and *CCL5, CXCL6, CXCL8* and *CCL20* for the *mtp*-mutant strain. These findings suggest that, in the absence of MTP, cytokine and chemokine response is negatively affected in A549 epithelial cells. There was also differential expression in genes associated with tumor necrosis factor (*TNF*) receptors and cytokine receptors as well as *STAT* signalling and *TNF* response.

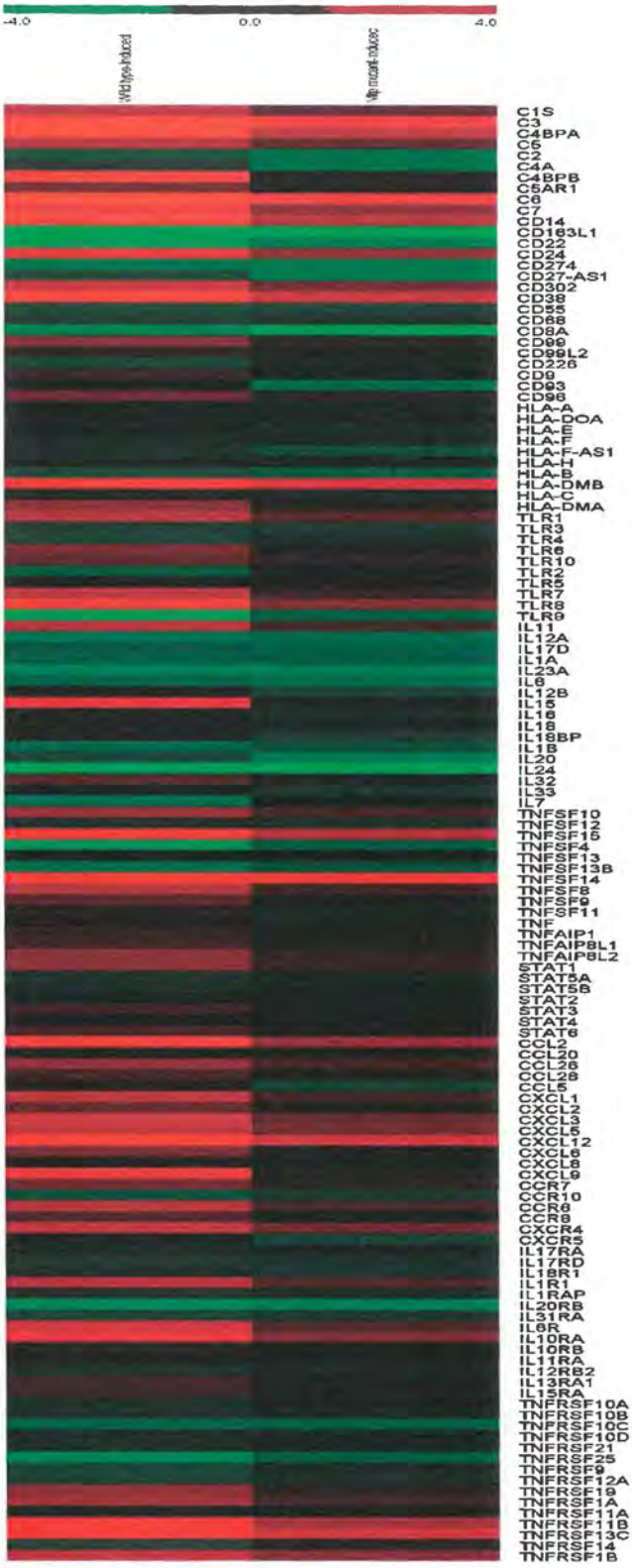


Figure 28: Host immune response-related DEGs induced as a result of infection of A549 epithelial cells with wild type and *mtp*-knockout mutant strains at 4 hr post-infection, relative to uninfected cells. Sequenced reads were mapped to the reference genome using *TopHat*. *Cuffdiff* of the RNA-sequencing Tuxedo pipeline was used to identify DEGs between uninfected and infected epithelial cells. Red indicates up-regulated genes while green indicates down-regulated genes.

3.9.1. *MTP Plays a Limited Role in Inducing Antigen Presentation by Epithelial Cells*

Our gene expression profiles indicate that the majority of genes involved in antigen presentation were down-regulated by both strains in A549 pulmonary epithelial cells, relative to uninfected cells. Up-regulated genes (*HLA-DMA* and *HLA-DMB*) were highly up-regulated by the wild type strain compared to the mutant strain (Figure 29).

Figure 29: **Antigen Presentation Signaling.** The induction of DEGs involved in antigen presentation in A549 epithelial cells infected with the wild type and *mtp*-mutant strain for 4 hours. Sequenced reads were mapped to the reference genome using *TopHat*. *Cuffdiff* of the RNA-sequencing Tuxedo pipeline was used to identify DEGs between uninfected and infected epithelial cells. Red indicates up-regulated genes while green indicates down-regulated genes.

3.9.2. *MTP Activates the Expression of Cell Surface Receptors in Epithelial Cells*

Cell surface receptors such as *CD14*, *CD24*, *CD302*, *CD38*, *CD96*, *CD99* and *CD99L2* were all up-regulated by both strains. However, the level of up-regulation was higher in the wild type infection compared to the mutant infection (Figure 30). There was no significant expression of *CD9* by the mutant strain while the wild type strain, up-regulated this surface marker.

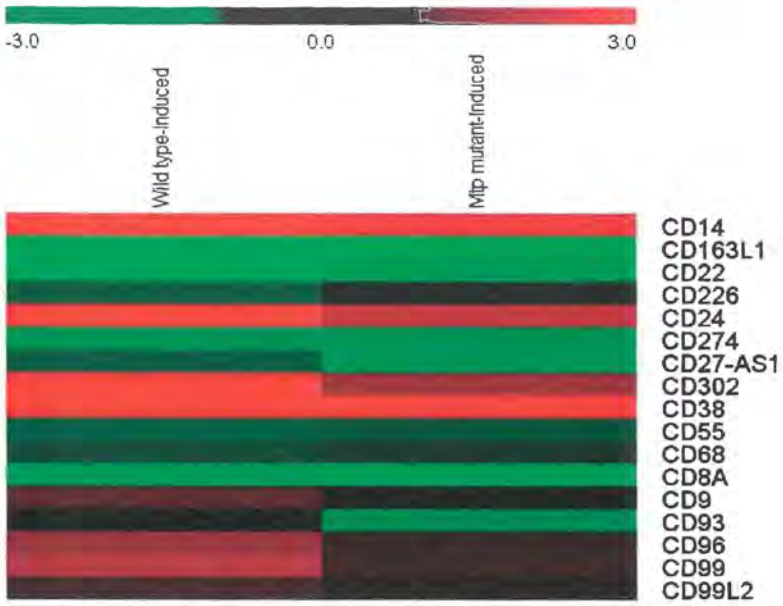


Figure 30: Cell Surface Receptor Signaling. The induction of DEGs associated with cell surface receptor signalling in A549 epithelial cells infected with the wild type and *mtp*-mutant strain for 4 hours. Sequenced reads were mapped to the reference genome using *TopHat*. *Cuffdiff* of the RNA-sequencing Tuxedo pipeline was used to identify DEGs between uninfected and infected epithelial cells. Red indicates up-regulated genes while green indicates down-regulated genes.

3.9.3. *MTP Highly Induces Antibody Response via the Complement System in Epithelial Cells*

All DEGs involved in the complement system were up-regulated by both strains except for *C2* and *C4A* (Figure 31). All up-regulated genes were highly induced by the wild type strain compared to the mutant strain. There was no significant differential expression for *C4BPB* and *C5AR1* by the *mtp*-mutant strain while the wild type strain highly up-regulated these genes.

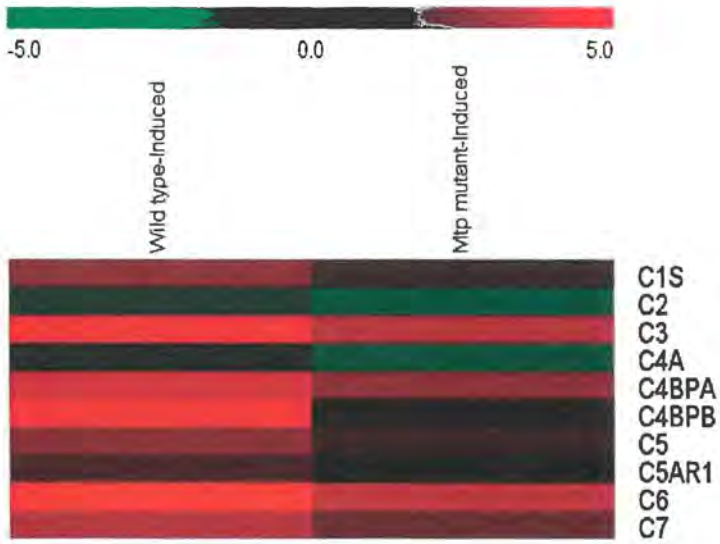


Figure 31: **Complement System Signalling.** The induction of DEGs involved in the complement system in A549 epithelial cells infected with the wild type and *mtp*-mutant strain for 4 hours. Sequenced reads were mapped to the reference genome using *TopHat*. *Cuffdiff* of the RNA-sequencing Tuxedo pipeline was used to identify DEGs between uninfected and infected epithelial cells. Red indicates up-regulated genes while green indicates down-regulated genes.

3.9.4. *MTP Induces Toll-like Receptor Signalling in Epithelial Cells*

Our gene expression profile analysis demonstrated the induction of TLR expression in infected epithelial cells. TLRs are host cell receptors that recognize microbial components as non-self, leading to the activation of immune responses to eliminate them (Takeda and Akira, 2005). These receptors play a huge role in the recognition of microbial components, which initiates signal transduction pathways and the activation of different genes in host cells (Takeda and Akira, 2005). *TLR1*, *TLR6*, *TLR7*, *TLR8* and *TLR10* were up-regulated by both strains while *TLR3*, *TLR4* and *TLR9* were down-regulated. The wild type strain highly expressed up-regulated TLRs when compared to the *mtp*-mutant strain (Figure 32). There was no significant expression of *TLR2* by the mutant strain while the wild type strain down-regulated the expression of this receptor. Both strains did not induce significant expression of *TLR5* in A549 alveolar epithelial cells.

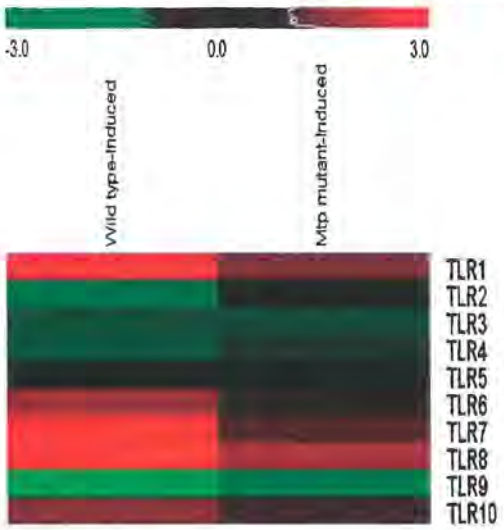


Figure 32: **Toll-like Receptor Signaling.** The induction of DEGs associated with toll-like receptor signalling in A549 epithelial cells infected with the wild type and *mtp*-mutant strain for 4 hours. Sequenced reads were mapped to the reference genome using *TopHat*. *Cuffdiff* of the RNA-sequencing Tuxedo pipeline was used to identify DEGs between uninfected and infected epithelial cells. Red indicates up-regulated genes while green indicates down-regulated genes.

3.9.5. *MTP Regulates Genes Associated with Immune Cell Activation*

DEGs involved in host immune cell activation were further investigated and displayed in Figure 33A and 33B below. These included genes coding for the activation of cytokine and chemokine receptors

and TNF receptor signalling. Both strains up-regulated the expression of genes coding for cytokines (*IL10RA*, *IL13RA1*, *IL15RA*, *IL1R1* and *IL6R*) chemokines (*CCR6*, *CCR7* and *CXCR4*) and tumor necrosis factor (*TNFRSF11B*, *TNFRSF13C*, *TNFRSF19*, *TNFRSF1A* and *TNFRSF1B*) receptors while other receptors were down-regulated. The wild type strain highly induced the expression of all up-regulated genes in epithelial cells compared to the mutant strain.

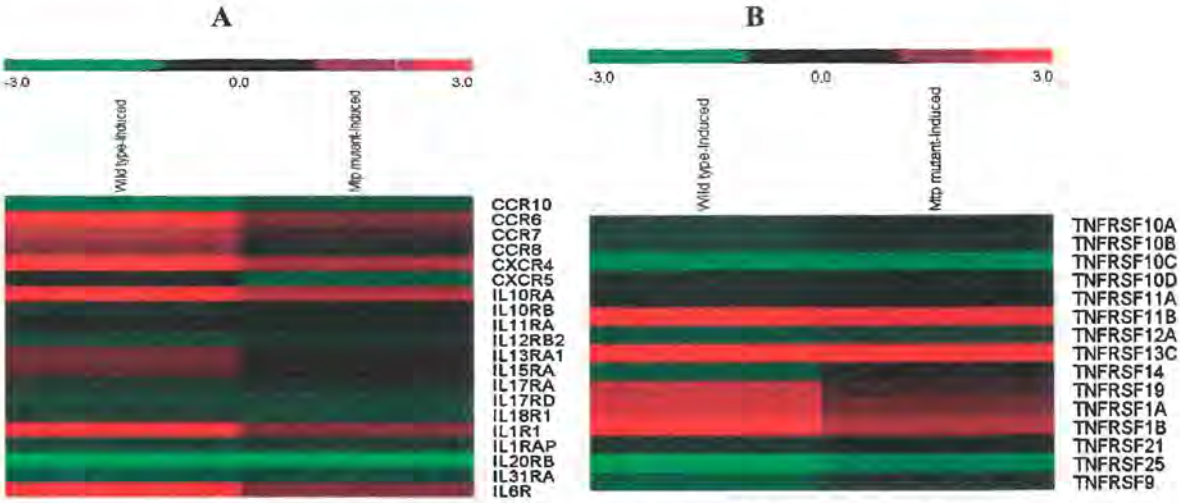
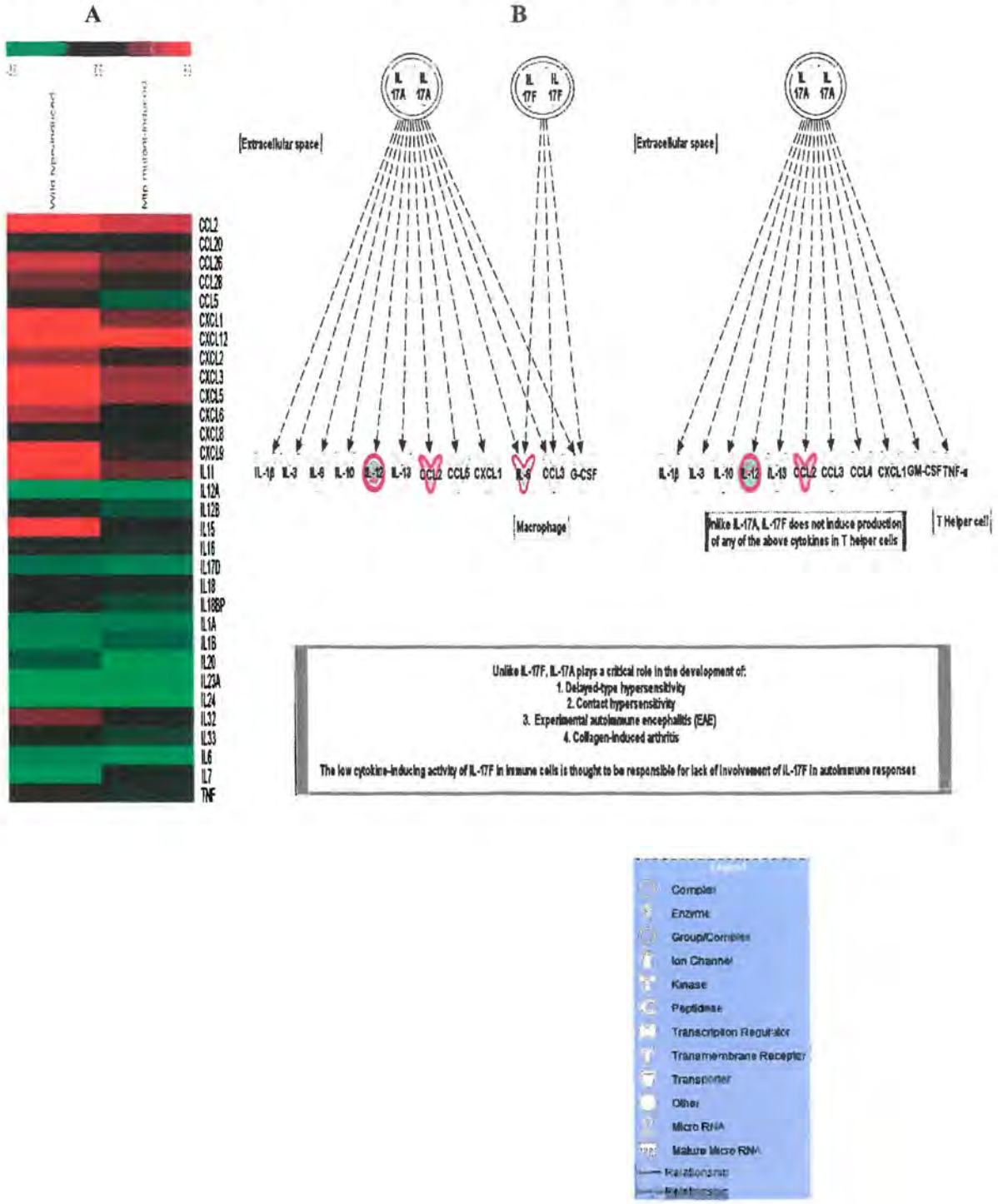


Figure 33: Gene expression profiles of DEGs associated with Cytokine and Chemokine Receptor Signalling (A) and *TNF* Receptor Signalling (B) in A549 epithelial cells infected with the wild type and *mtp*-mutant strain for 4 hours. Sequenced reads were mapped to the reference genome using *TopHat*. *Cuffdiff* of the RNA-sequencing Tuxedo pipeline was used to identify DEGs between uninfected and infected epithelial cells. Red indicates up-regulated genes while green indicates down-regulated genes.

3.9.6. *MTP Highly Induces the Expression of Chemokines in Epithelial Cells*

The role of MTP in inducing DEGs associated with the expression of chemokines in A549 epithelial cells was further investigated. Our gene expression analysis showed that the wild type strain up-regulated the expression of all chemokines induced except for *CCL20* while the *mtp*-mutant strain also up-regulated these chemokines except for *CCL5* and *CXCL8* chemokines. The level of expression in up-regulated chemokines was highly induced by the wild type strain compared to the *mtp*-mutant strain (Figure 34A). The induction of cytokines in A549 epithelial cells was largely down-regulated by both strains. The wild type strain up-regulated the expression of *IL11*, *IL15*, and *IL32* while the mutant strain up-regulated *IL11* only. There was no significant expression of *IL18*, *IL18BP* and *TNF* cytokines by the wild type strain while the same was true for *IL15* and *IL32* by the mutant strain (Figure 34A). The pathway associated with differential regulation of cytokine production in macrophages and T-helper cells was highly enriched by the *mtp*-mutant strain in A549 epithelial cells

but the expression of DEGs involved in the pathway was similar for both strains (Figure 34B) while the *IL6* upstream regulator was enriched by the wild type strain only (Figure 34C).



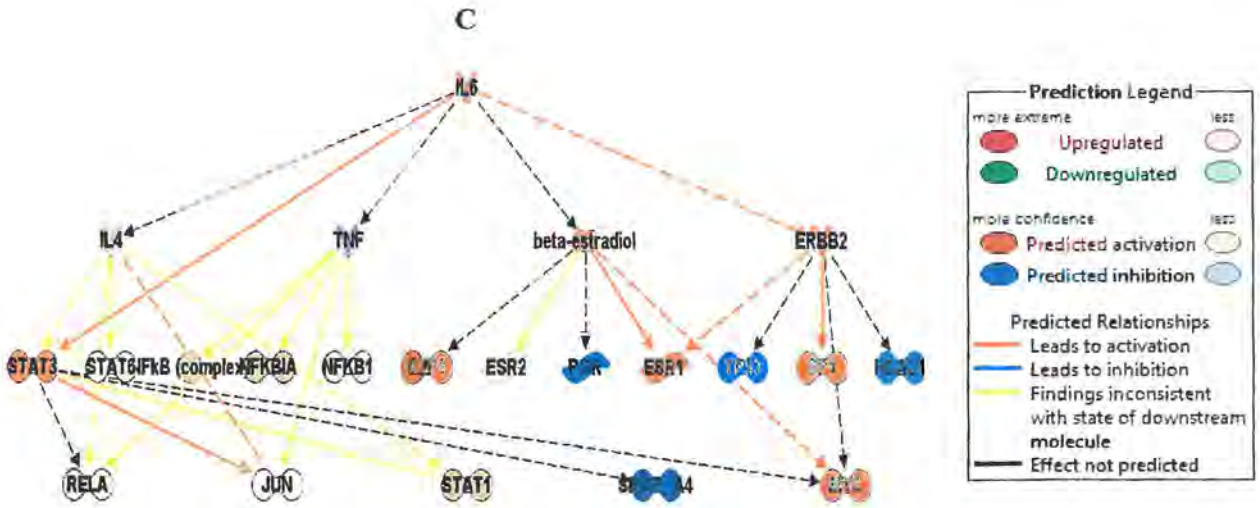


Figure 34: **Cytokine and Chemokine Response Signalling. A:** The induction of DEGs associated with cytokine and chemokine production in A549 epithelial cells infected with the wild type and *mtp*-mutant strain for 4 hours. Sequenced reads were mapped to the reference genome using *TopHat*. *Cuffdiff* of the RNA-sequencing Tuxedo pipeline was used to identify DEGs between uninfected and infected epithelial cells. Red indicates up-regulated genes while green indicates down-regulated genes. **B**: Differential Regulation of Cytokine Production in Macrophages and T-helper Cells by IL-17A and IL-17F Pathway. This Canonical pathway was equally enriched by both strains. Expression profiles of DEGs involved in the pathway were also similar for both strains. Molecules associated with this pathway are displayed with red and green which indicate up- and down-regulated genes. The brighter the red or green colour, the more up- and down-regulated the gene in A549 epithelial cells, relative to unifected cells. Molecules shaded in white are part of the knowledge-based IPA software and were not expressed in our datasets. The legend below the image shows different molecules displayed on the pathway. **C**: A mechanistic network involving *IL6* upstream regulator that was only enriched by the wild type strain in A549 epithelial cells. The legend on the right hand side of the image shows different molecules represented and different inter-relationships they have with *IL6* and with one another. Molecules shaded in white are part of the knowledge-based IPA software and were not expressed in our datasets.

3.9.7. *MTP Expresses STAT Response Signalling in A549 Epithelial Cells*

Our gene expression profiles further revealed that the wild type strain highly induced the expression of genes coding for *STAT 1*, *STAT3* and *STAT6* compared to the *mtp*-mutant strain while *STAT5A* and *STAT5B* were down-regulated by both strains (Figure 35A). There was no significant expression of *STAT2* and *STAT4* genes by the wild type strain and *STAT2* and *STAT6* by the *mtp*-mutant strain. A mechanistic network involving *STAT1* upstream regulator (Figure 35B) was only induced by the *mtp*-mutant strain.

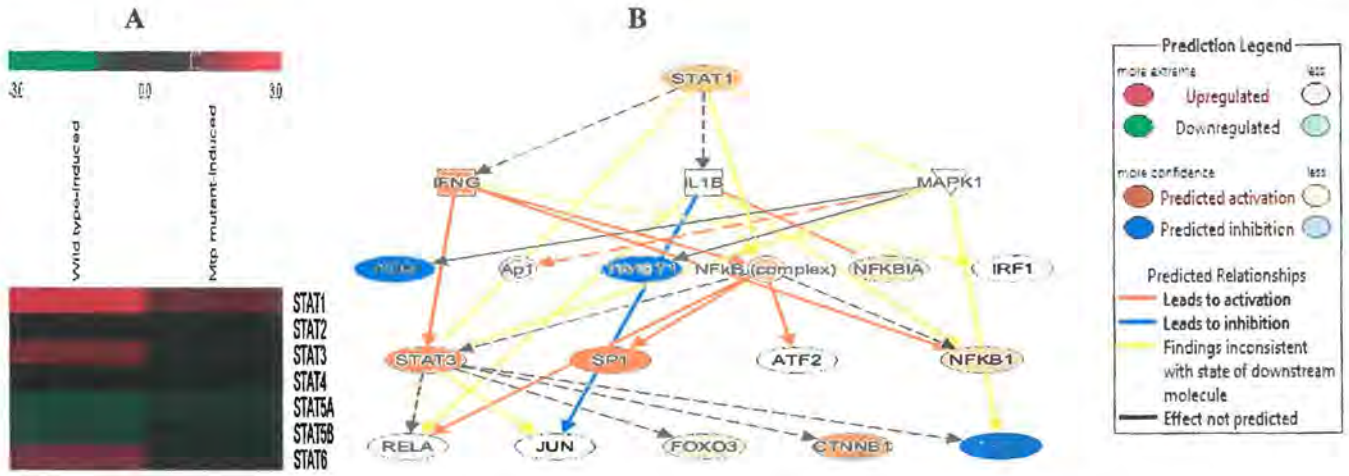


Figure 35: ***STAT* Response Signalling. A**: The induction of DEGs associated *STAT* response signalling in A549 epithelial cells infected with the wild type and *mtp*-mutant strain for 4 hours. Sequenced reads were mapped to the reference genome using *TopHat*. *Cuffdiff* of the RNA-sequencing Tuxedo pipeline was used to identify DEGs between uninfected and infected epithelial cells. Red indicates up-regulated genes while green indicates down-regulated genes. **B**: A mechanistic network involving *STAT1* upstream regulator that was only enriched by the *mtp*-mutant strain in A549 epithelial cells. The legend on the right hand side shows different molecules represented and different inter-relationships they have with *STAT*1 and with one another.

3.9.8. MTP Induces *TNF* Response Signalling in Epithelial Cells

The impact MTP has on inducing cell death in A549 epithelial cells in response to wild type and *mtp*-mutant infection was also investigated where the majority of the genes associated with *TNF* Response Signalling were found to be up-regulated by both strains. However, the level of up-regulation for these genes differed between the two infection conditions. All the up-regulated genes were highly enriched by the wild type strain when compared to the mutant strain (Figure 36). *TNFSF11* was up-regulated by the wild type strain while it was down-regulated by the mutant strain. There was no significant expression of *TNFSF13* in A549 alveolar epithelial cells by both strains.

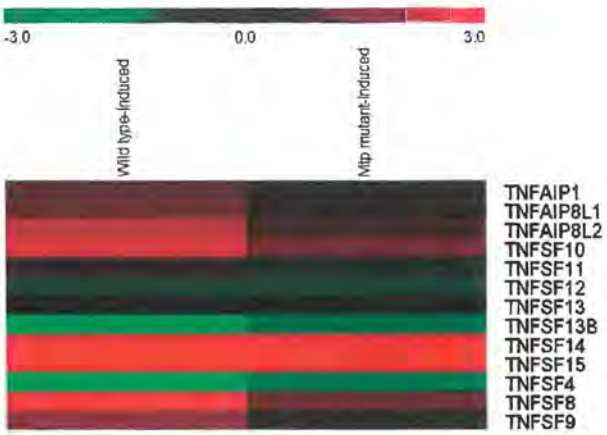


Figure 36: ***TNF* Response Signaling.** The induction of DEGs associated *TNF* response signalling in A549 epithelial cells infected with the wild type and *mtp*-mutant strain for 4 hours. Sequenced reads were mapped to the reference genome using *TopHat*. *Cuffdiff* of the RNA-sequencing Tuxedo pipeline was used to identify DEGs between uninfected and infected epithelial cells. Red indicates up-regulated genes while green indicates down-regulated genes.

3.10. Network Analysis

To further understand the biology encoded by SDEGs induced in A549 epithelial cells in response to wild type and *mtp*-mutant infection, knowledge-based software, IPA, was used to investigate networks enriched by these genes in infected cells, relative to uninfected cells. SDEGs elicited in A549 epithelial cells infected with the wild type and *mtp*-mutant strain enriched an equal number (25) of networks (Table 12). Further network analysis showed that both strains enriched 5 networks that were associated with similar top diseases and biological functions. SDEGs that were uniquely induced as a result of wild type and *mtp*-mutant infection in A549 epithelial cells enriched 21 and 11 networks, respectively. Uniquely induced SDEGs did not enrich similar networks in A549 epithelial cells, relative to uninfected cells (Table 12).

Table 12: Biological networks enriched by SDEGs in A549 epithelial cells in response to wild type and *mtp*-mutant infection, relative to uninfected cells.

	Uninfected vs WT	**SHARED**	**Uninfected vs MUTANT**	**UNIQUE WT**	**UNIQUE MUTANT**
Networks	25	5	25	21	11

3.10.1. *Network Enrichment by the mtp-mutant strain*

The *mtp*-mutant strain enriched a total of 25 networks in A549 pulmonary epithelial cells, relative to uninfected cells. The top scoring network (Network 1, Table 13) enriched by the *mtp*-mutant strain had a score of 55 with 34 interacting molecules involved in cellular assembly and organisation, DNA replication, recombination, and repair and post-translational modification (Figure 37). Network 1 and Network 13 were associated with similar top diseases and biological functions. However, the latter was lowly ranked and had less interacting molecules when compared to the former (Table 13). This trend was also observed in Network 5 and Network 10.

Table 13: Top 15 networks enriched by SDEGs induced by the *mtp*-mutant strain in A549 pulmonary epithelial cells, relative to uninfected cells.

Rank	Score	Top Diseases and Functions	Number of Interacting Molecules
1	55	Cellular assembly and organisation, DNA replication, recombination, and repair, post-translational modification.	34
2	42	Connective tissue disorders, developmental disorder, hereditary disorder.	29
3	35	Cell cycle, cellular assembly and organisation, DNA replication, recombination, and repair.	26
4	35	Cell cycle, cellular development, cell death and survival.	26
5	35	Cell cycle, cancer, organismal injury and abnormalities.	26
6	33	Neurological disease, connective tissue development and function, embryonic development.	25
7	33	Gene expression, cellular development, cell cycle.	25
8	31	Cardiovascular disease, cell death and survival, connective tissue disorder.	24
9	31	Connective tissue disorders, developmental disorder, gastrointestinal disease.	24
10	31	Cancer, cell cycle, organismal injury and abnormalities.	24
11	28	Cancer, endocrine system disorders, organismal injury and abnormalities.	22
12	28	Cellular assembly and organization, cell cycle, post-translational modification.	22
13	28	Cellular assembly and organisation, DNA replication, recombination, and repair, post-translational modification.	22
14	26	Immunological disease, cellular function and maintenance, cellular development.	21
15	22	Cell-to-cell signalling and interaction, cellular growth and proliferation, haematological system development and function.	19

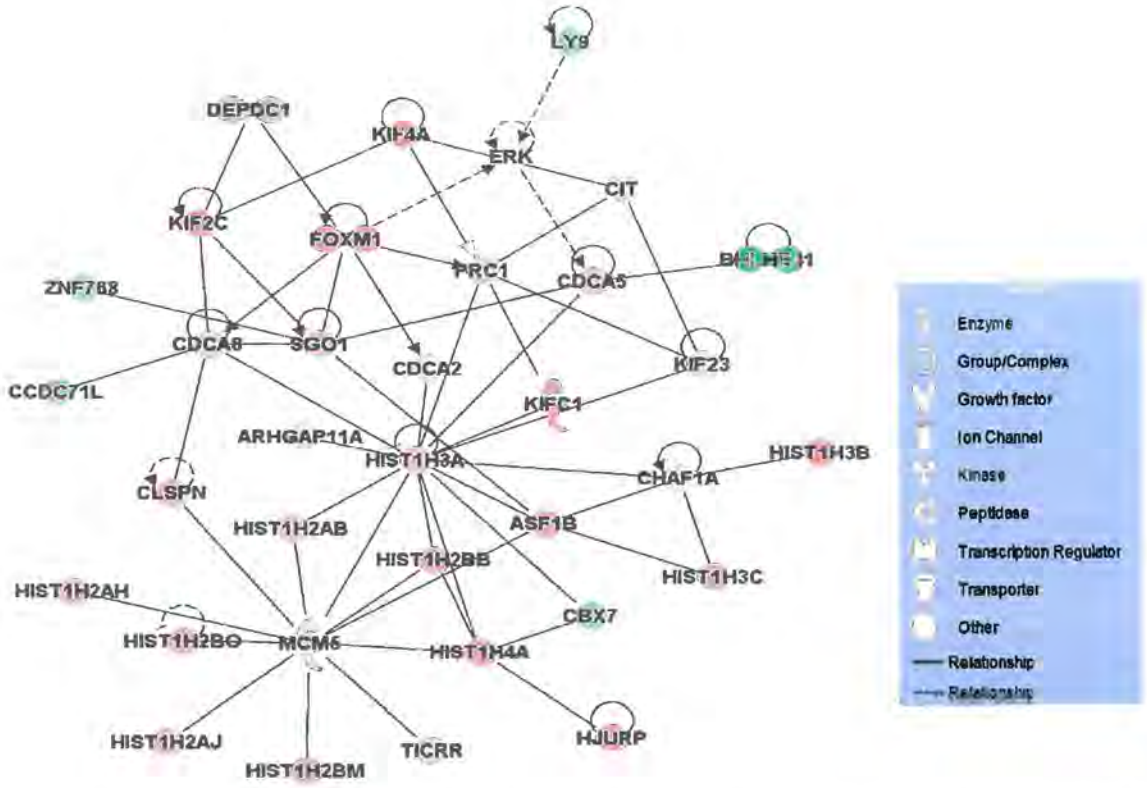


Figure 37: Top scoring (55) network of most up- and down-regulated SDEGs that were induced by the *mtp*-mutant strain in A549 epithelial cells, in relation to uninfected cells. The network was associated with top biological functions such as cellular assembly and organisation, DNA replication, recombination, and repair, post-translational modification. The legend represents different types of molecules present on the network. Red represents up-regulated while green represents down-regulated SDEGs. The brighter the red or green colour, the more up- or down-regulated the gene. *DEPDC1, FOXM1, KIF2C, KIF4A* and *HIST1H3B* were the most up-regulated molecules, whereas *BHLHE41*was the most down-regulated.

3.10.2. *Network Enrichment by the wild type strain*

The wild type strain enriched a similar number of networks (25) in A549 epithelial cells when compared to the mutant strain. The top scoring network (Network 1, Table 14) had a ranking score of 42 and 30 interacting molecules associated with cell cycle, cellular development, cell death and survival (Figure 38). Network 3 and Network 4 were associated with similar top diseases and biological functions. However, the latter was lowly ranked and had one less interacting molecules when compared to the former (Table 14).

Table 14: Top 15 networks enriched by SDEGs induced by the wild type strain in A549 pulmonary epithelial cells, relative to uninfected cells.

Rank	Score	Top Diseases and Functions	Number of Interacting Molecules
1	42	Cell cycle, cellular development, cell death and survival.	30
2	40	Connective tissue disorders, developmental disorder, gastrointestinal disease.	29
3	40	Cell cycle, cellular assembly and organisation, DNA replication, recombination, and repair.	29
4	37	Cell cycle, cellular assembly and organisation, DNA replication, recombination, and repair.	28
5	37	Cellular development, cellular growth and proliferation, reproductive system development and function.	28
6	35	Energy production, small molecule biochemistry, endocrine system development and function.	27
7	35	Cell cycle, cellular movement, cellular assembly and organization.	27
8	33	Hereditary disorder, metabolic disease, organismal injury and abnormalities.	26
9	31	Connective tissue disorders, developmental disorder, hereditary disorder.	25
10	31	Cell cycle, DNA replication, recombination, and repair, cardiovascular disease.	25
11	29	Cell-to-cell signalling and interaction, cellular growth and proliferation, connective tissue development and function.	24
12	27	Immunological disease, inflammatory disease, inflammatory response.	23
13	26	Carbohydrate metabolism, lipid metabolism, molecular transport.	22
14	24	Cell-to-cell signalling and interaction, haematological system development and function, cancer.	21
15	24	Cellular assembly and organisation, DNA replication, recombination, and repair, post-translational modification.	21

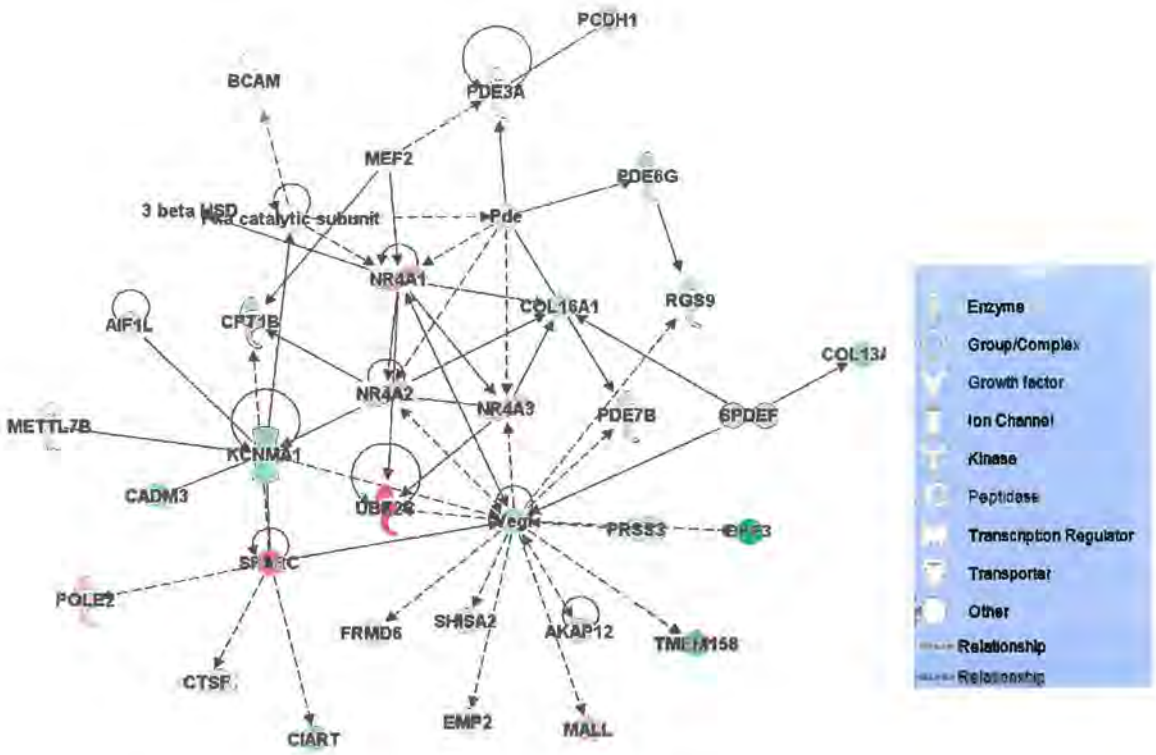


Figure 38: Top scoring (42) network of most up- and down-regulated SDEGs that were induced by the wild type strain in A549 epithelial cells, in relation to uninfected cells. The network was associated with cell cycle, cellular development, cell death and survival. The legend represents different types of molecules present on the network. Red represents up-regulated while green represents down-regulated SDEGs. The brighter the red or green colour, the more up- or down-regulated the gene. There was an overexpression of *UBE2C* while *DPF3* was highly down-regulated.

3.10.3. ***Network Enrichment by SDEGs Uniquely Induced by the wild type and mtp-mutant strain***

Further network analysis showed that SDEGs uniquely induced by the wild type strain in A549 epithelial cells enriched 21 networks (Table 16) while those that were uniquely induced by the *mtp*-mutant strain enriched 11 networks (Table 15), suggesting the importance of MTP in gene regulation of epithelial cells. The top scoring network (Network 1, Table 15) enriched by SDEGs uniquely induced by the *mtp*-mutant strain in A549 epithelial cells had a ranking score of 39 with 19 interacting molecules associated with cellular function and maintenance, connective tissue development and function and tissue morphology (Figure 39) while the top scoring network (Network 1, Table 16) enriched by SDEGs uniquely induced by the wild type strain had a ranking score of 45 with 25 interacting molecules involved in cell signalling, molecular transport and nucleic acid metabolism (Figure 40). SDEGs that were uniquely induced by the *mtp*-mutant strain in A549 epithelial cells enriched low ranking networks with fewer interacting molecules when compared to those that were enriched by SDEGs that were exclusively elicited by the wild type strain. This analysis reveals that the absence of MTP affects the regulation of gene expression in epithelial cells in vitro.

Table 15: Networks enriched by SDEGs uniquely induced by the *mtp*-mutant strain in A549 pulmonary epithelial cells, relative to uninfected cells.

Rank	Score	Top Diseases and Functions	Number of Interacting Molecules
1	39	Cellular function and maintenance, connective tissue development and function, tissue morphology.	19
2	36	Post-translational modification, protein folding, cellular compromise.	18
3	29	Cellular development, embryonic development, haematological system development and function.	15
4	24	Cell death and survival, neurological disease, cellular development.	13
5	24	Embryonic development, lymphoid tissue structure and development, organ development.	13
6	21	Cell death and survival, cellular compromise, gene expression.	12
7	2	Amino acid metabolism, cancer, carbohydrate metabolism.	1
8	2	Endocrine system disorders, hereditary disorder, organismal injury and abnormalities.	1
9	2	Cancer, connective tissue disorders, dermatological diseases and conditions.	1
10	2	Inflammatory response.	1
11	2	Post-translational modification, carbohydrate metabolism, gene expression.	1

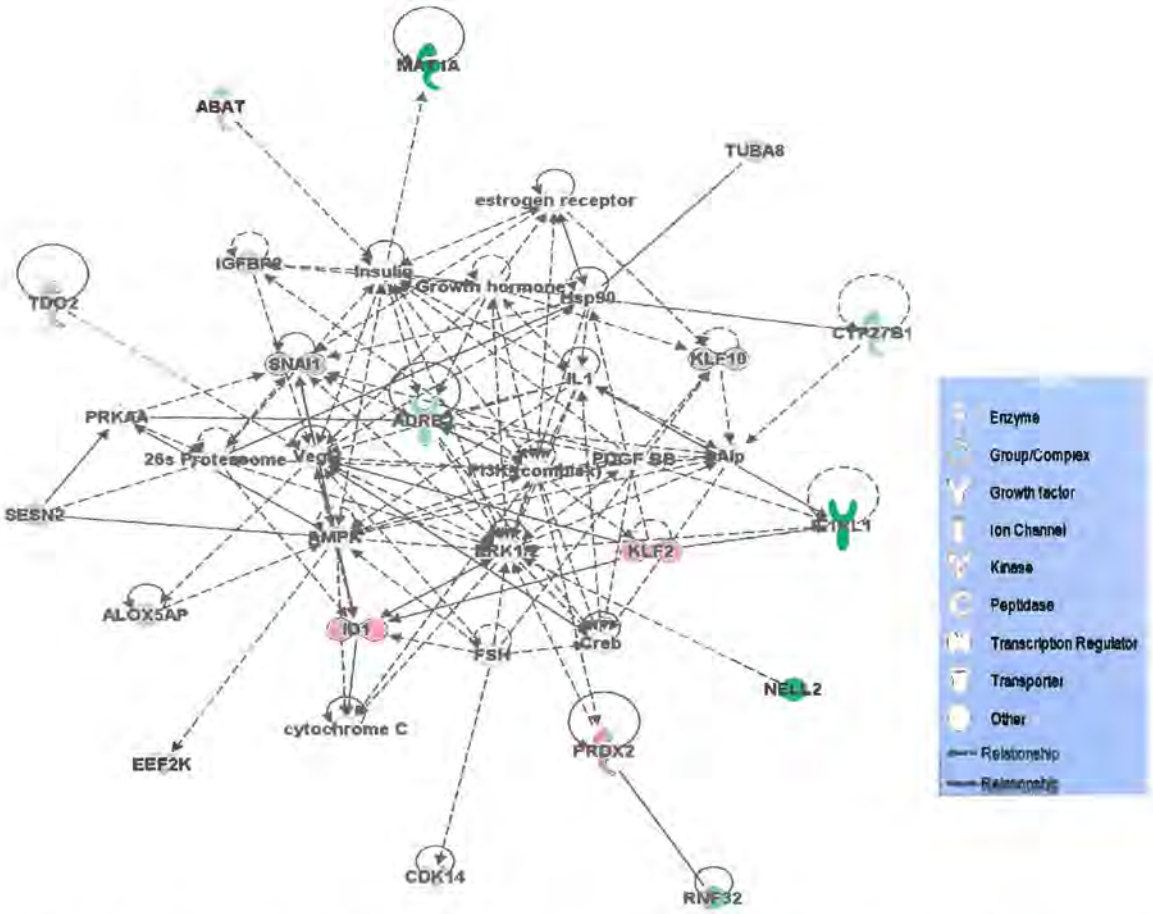


Figure 39: Top scoring (39) network of most up- and down-regulated SDEGs that were uniquely induced by the *mtp*-mutant strain in A549 epithelial cells, in relation to uninfected cells. The network was associated with cellular function and maintenance, connective tissue development and function, tissue morphology as a result of elevated up- and down-regulation of molecules such as *KLF2* and *IL1RL1*. The legend represents different types of molecules present on the network. Red represents up-regulated while green represents down-regulated SDEGs. The brighter the red or green colour, the more up- or down-regulated the gene.

Table 16: Top 15 networks enriched by SDEGs uniquely induced by the wild type strain in A549 pulmonary epithelial cells, relative to uninfected cells.

Rank	Score	Top Diseases and Functions	Number of Interacting Molecules
1	45	Cell signalling, molecular transport, nucleic acid metabolism.	25
2	31	Cell-to-cell signalling and interaction, lymphoid tissue structure and development, tissue morphology.	19
3	31	Molecular transport, energy production, embryonic development.	19
4	26	Lipid metabolism, small molecule biochemistry, infectious diseases.	17
5	22	Connective tissue disorders, developmental disorder, hereditary disorder.	15
6	22	Developmental disorder, organismal injury and abnormalities, reproductive system disease.	15
7	20	Reproductive system development and function, cellular function and maintenance, organ development.	14
8	20	Cellular development, haematological system development and function, hematopoiesis.	14
9	18	Nervous system development and function, organ morphology, organismal development.	13
10	18	Cancer, cell cycle, cell-to-cell signalling and interaction.	13
11	18	Cell death and survival, haematological system development and function, lymphoid tissue structure and development.	13
12	16	Cell death and survival, cellular development, cellular function and maintenance.	12
13	16	Neurological disease, gene expression, RNA damage and repair.	12
14	16	Cell cycle, carbohydrate metabolism, lipid metabolism.	12
15	15	Cellular growth and proliferation, haematological system development and function, cell-to-cell signalling and interaction.	11

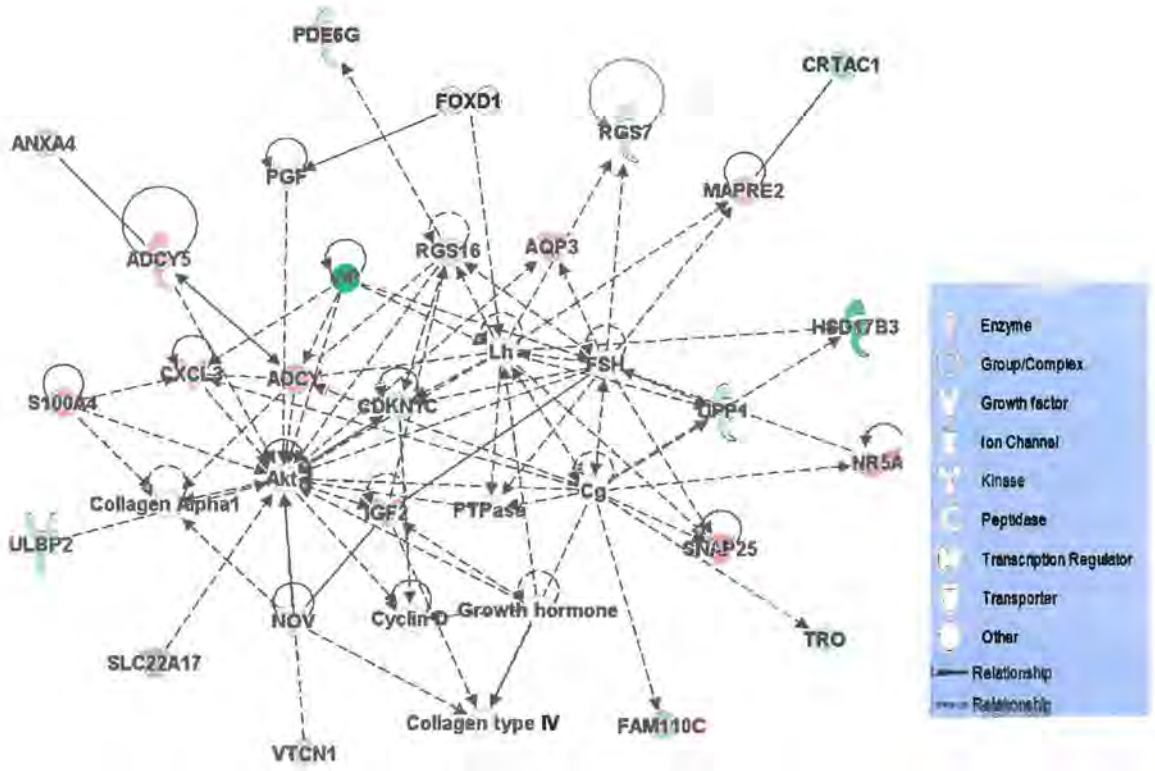


Figure 40: Top scoring (45) network of most up- and down-regulated SDEGs that were uniquely induced by the wild type strain in A549 epithelial cells, in relation to uninfected cells. The network was associated with cell signalling, molecular transport, nucleic acid metabolism. The legend represents different types of molecules present on the network. Red represents up-regulated while green represents down-regulated SDEGs. The brighter the red or green colour, the more up- or down-regulated the gene. *VIP* was the most down-regulated molecule while *NR5A, AQP3* and *ANXA4* were most up-regulated.

3.10.4. *Common Networks Enriched by SDEGs Induced by the wild type and mtp-mutant strain*

Our network analysis showed that SDEGs elicited by both wild type and *mtp*-mutant strains in A549 epithelial cells enriched 5 networks with similar top diseases and biological functions. However, there was a variation in the network ranking and score, as well as the number of interacting molecules involved in each shared network (Table 17). Overall, the wild type strain enriched top scoring common networks in A549 epithelial cells compared to those enriched by the *mtp*-mutant strain. These findings suggest that the presence of MTP in *M. tuberculosis* leads to the enrichment of top scoring networks with a high number of interacting molecules that are up- and down-regulated by A549 epithelial cells in response to infection.

Table 17: Common networks enriched by SDEGs induced by the wild type strain and *mtp*-mutant strain in A549 pulmonary epithelial cells, relative to uninfected cells.

Strain	Rank	Score	Top Diseases and Function	Interacting Molecules
wild type	15	24	Cellular assembly and organisation, DNA	21
mtp-mutant	1	55	replication, recombination, and repair,	34
	13	28	post-translational modification.	22
wild type	3	40	Cell cycle, cellular assembly and	29
	4	37	organisation, DNA replication,	28
mtp-mutant	3	35	recombination, and repair.	26
wild type	1	42	Cell cycle, cellular development, cell death	30
mtp-mutant	4	35	and survival.	26
wild type	2	40	Connective tissue disorders, developmental	29
mtp-mutant	9	31	disorder, gastrointestinal disease.	24
wild type	23	19	Organismal injury and abnormalities,	18
mtp-mutant	18	20	developmental disorder, haematological	18
			disease.	

3.11. Upstream Regulator Analysis

To further understand the effects and role of MTP in gene regulation of A549 epithelial cells, knowledge-based IPA software was used to investigate the enrichment of upstream regulators in A549 epithelial cells infected with the wild type and *mtp*-mutant strain. IPA upstream regulator analysis enables the identification of a cascade of upstream transcriptional regulators that can explain observed gene expression changes in our datasets and elucidate biological activities taking place in A549 epithelial cells infected with the wild type and *mtp*-mutant strain. The up- or down-regulation of these regulators may not always correlate with the information contained in our datasets as IPA gets updated on a regular basis. The wild type and *mtp*-mutant strain enriched 18 and 32 upstream regulators in A549 epithelial cells, respectively, relative to uninfected cells. A total of 10 (Appendix 8) and 24 (Appendix 9) upstream regulators were exclusively enriched by wild type and *mtp*-mutant strain, respectively (Figure 41) while 8 were enriched by both strains (Appendix 7). The enrichment of a high number of upstream regulators by the *mtp*-mutant strain in A549 epithelial cells, compared to the wild type, suggests that the loss of MTP in *M. tuberculosis* could result in an elevated response of A549 epithelial cells to TB infections.

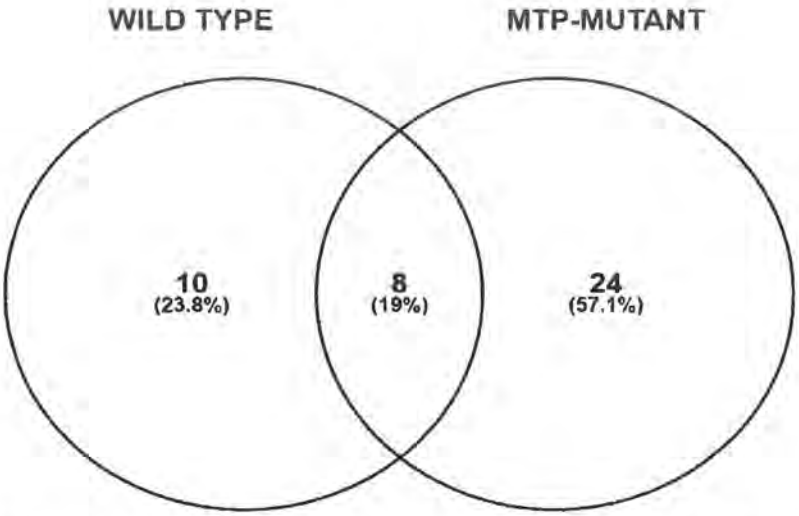


Figure 41: Upstream regulators that were induced by the wild type and *mtp*-mutant strain in A549 pulmonary epithelial cells. A 2-way Venn diagram shows that 18 and 32 upstream regulators were enriched by the wild type and mtp-mutant strain, respectively. The wild type and mtp-mutant strain exclusively enriched 10 and 24 upstream regulators in epithelial cells, respectively, while 10 upstream regulators were commonly enriched by both strains.

CEBPD, *E2F1* and *FOXM1* upstream regulators were up-regulated by both strains. However, *CEBPD* was highly enriched by the wild type strain compared to the mutant strain while *STAT3* was up-regulated by the wild type strain and not significantly expressed by the mutant strain (Figure 42). *CCND1*, *NUPR1* and *SMARCA4* were down-regulated by both strains while there was no significant expression for *TP53*.

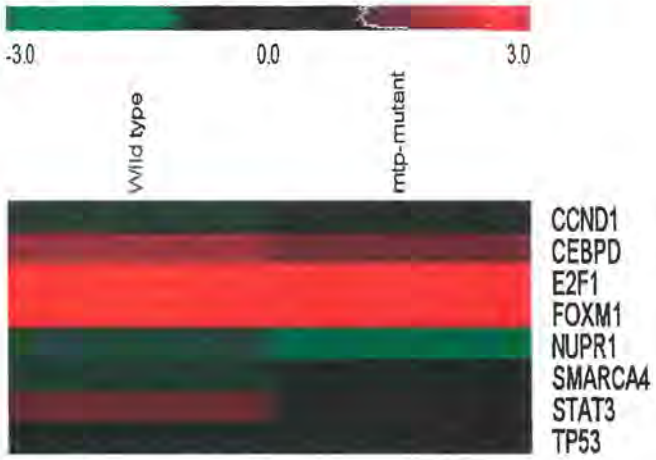


Figure 42: Gene expression profiles showing upstream regulators that were commonly induced by both strains in A549 pulmonary epithelial cells, relative to uninfected cells. Red represents up-regulated while green represents down-regulated. The more intense red or green colour, the higher up-regulation and down-regulation.

3.11.1 *Upstream Regulators Exclusively Enriched by the mutant strain*

The *mtp*-mutant strain exclusively enriched 24 upstream regulators in A549 pulmonary epithelial cells, with 13 and 11 being up and down-regulated, respectively (Table 18). In our dataset, *MYBL2* was the most up-regulated upstream regulator with a fold change of 7.75 while other up-regulated regulators had fold changes ranging from 0 to 1.5. A similar trend was also observed in down-regulated upstream regulators where *MYC* was the only highly down-regulated regulator with a fold change of -2.30 while other down-regulated regulators had fold changes ranging from –0.02 to -0.83.

Table 18: Upstream regulators exclusively induced by the *mtp*-mutant strain in A549 pulmonary epithelial cells, relative to uninfected cells.

	Upstream Regulator
Up-regulated	*MYBL2*
	RBL1
	HNF1B
	TP63
	STAT1
	NFKBIA
	TBX2
	DACH1
	CTNNB1
	YAP1
	EP300
	CREBBP
	CDKN2A
Down-regulated	*YY1*
	NFKB1
	KDM5B
	TCF3
	MED1
	FOSL1
	RELA
	CEBPB
	MITF
	TP73
	MYC

3.11.2 *Upstream Regulators Exclusively Enriched by the wild type strain*

The wild type strain exclusively enriched 10 upstream regulators in A549 pulmonary epithelial cells (Table 19). A total of 6 and 4 upstream regulators were up and down-regulated, respectively. *HGF* was the highest up-regulated upstream regulator whereas *ESR1* was the lowest up-regulated. *CSF2* and *IRF1* were the highest and lowest down-regulated upstream regulators respectively.

Table 19: Upstream regulators exclusively induced by the wild type strain in A549 pulmonary epithelial cells, relative to uninfected cells.

	Upstream Regulator
Up-regulated	*HGF*
	IL6
	TGFB1
	ELF3
	ERBB2
	ESR1
Down-regulated	*IRF1*
	E2F4
	CDKN1A
	CSF2

CHAPTER 4: DISCUSSION

Since the seminal paper describing the presence of pili in *M. tuberculosis* (Alteri *et al.*, 2007), research in this field, has been steadily gaining momentum to further characterize this pathogen's curli pili (MTP) to interrogate its potential as a biomarker for rapid diagnostics and therapeutic interventions (Govender *et al.*, 2014; Naidoo *et al.*, 2014; Ramsugit and Pillay, 2015). However, this research area is still in its infancy, and its complete role in host pathogen interaction remains unknown. In this study, for the first time RNA-Seq was used to investigate the global transcriptomics of infected pulmonary epithelial cells to elucidate the *in vitro* role of MTP in gene regulation and pathway and network analysis using a *mtp* gene knockout mutant of *M. tuberculosis*.

The epithelial cell model was chosen due to the increasing body of evidence demonstrating that *M. tuberculosis* is able to invade and survive inside these cells (McDonough and Kress, 1995; Bermudez and Goldman, 1996; Lin *et al.*, 1998; Dobos *et al.*, 2000; Castro-Garza *et al.*, 2002; Mvubu *et al.*, 2016; Ramsugit *et al.*, 2016). Epithelial cells may be the first point of contact with the pathogen due to their over-representation in the alveolar space compared to macrophages (Li *et al.*, 2012). *M. tuberculosis* uses cell surface-bound molecules called adhesins, in order to adhere to, and invade epithelial cells (Ryndak *et al.*, 2015), and is known to produce multiple adhesins in order to enhance its entry into host cells via extracellular matrix proteins (Ryndak *et al.*, 2015). Recent studies have suggested that MTP may be a major adhesin and invasin of macrophages (Ramsugit and Pillay, 2014) and epithelial cells (Ramsugit *et al.*, 2016) by binding to the laminin protein of the extracellular matrix in vitro (Alteri *et al.*, 2007) but does not largely influence the overall cytokine response in epithelial cells (Ramsugit *et al.*, 2016).

In this study, the number of invaded bacteria observed in A549 cells infected with the wild type strain did not differ significantly when compared to the mutant strain after 4 hours of infection. However, our results showed that, as a result of *mtp* deletion, the number of significantly differentially expressed genes and canonical pathways induced in A549 epithelial cells was reduced, suggesting that MTP regulates gene expression in epithelial cells in vitro. These findings suggest that the differences in the gene expression levels and canonical pathways are not attributed to significant differences in the bacterial count but are due to deletion of the *mtp* gene.

Curli pili have been shown to play a role in invasion of human cervical epithelial cells (Gophna *et al.*, 2001) and Hep-2 cells by *E. coli* (Uhlich *et al.*, 2002) as well as in adhesion of cultured mouse small intestine epithelial cells by *Salmonella typhimurium* (Sukupolvi *et al.*, 1997). In addition to epithelial cells, loss of MTP in *M. tuberculosis* was also shown to decrease adhesion to, and invasion of THP-1

monocyte derived macrophages, whilst complementation of MTP resulted in an increased expression of the *mtp* gene compared to the wild type (Ramsugit and Pillay 2014).

4.1. MTP Induces Differential Gene Expression in A549 Pulmonary Epithelial Cells

The role of the MTP adhesin of *M. tuberculosis* in gene regulation of human alveolar epithelial cells was elucidated by investigating the number of significantly differentially expressed host genes due to infection by the wild type and mutant strain. The wild type strain induced the highest total number (917) of genes when compared to the mutant (725) while 610 genes were expressed by both strains. This reduction by 12%, in the number of genes expressed by the mutant strain shows that MTP plays an important role in gene regulation of alveolar epithelial cells.

The expression of 610 genes by *M. tuberculosis* infection remained unaffected by MTP as these were induced by both strains, 345 (57%) of which were up-regulated while 265 (43%) were down-regulated Gene ontology analysis showed that up-regulated genes were associated with essential biological functions such as cell cycle and cell division, regulation of cell-to-cell adhesion, DNA replication, signal transduction, complement system as well as antigen presentation via MHC Class II. Down-regulated genes were associated with cell communication, enzyme binding, regulation of cellular metabolic processes, lymphocyte activation and cell surface receptor signalling pathway.

Chen *et al.* (2015) also demonstrated a higher number of up-regulated than down-regulated genes when RNA-sequencing was used to elucidate the response of lung epithelial cells infected with *A. fumigatus*. Using a cut off fold change of 1.5 or greater, they found 302 (66%) up-regulated and 157 (34%) down-regulated genes. Their gene ontology enrichment analysis showed that up-regulated genes were associated with immune response, chemotaxis, positive regulation of macromolecule processes, cell activation, response to bacterium, regulation of transcription and response to stimulus. Down-regulated genes were mainly enriched for skeletal system development, hemopoiesis, ion transport and immune response. Collectively, these two sets of findings suggest a general response induced in pulmonary epithelial cells as a result of bacterial infection.

Differences in the number of differentially expressed genes as well as biological functions they are associated with are most likely attributed to the different infecting pathogens. Ryndak *et al.* (2015) used microarray technology to investigate the transcriptional profile of *M. tuberculosis* H37Rv replicating in A549 alveolar epithelial cells compared to H37Rv actively growing in broth. After 72 hours of replication inside A549 cells, 261 *M. tuberculosis* genes were differentially expressed, with 186 and 75 genes up- and down-regulated respectively. Up-regulated genes were associated with tryptophan synthesis and increased virulence whereas down-regulated genes were associated with stress and heat shock response and DosR regulon (Ryndak *et al.*, 2015).

In this study, loss of MTP resulted in a lower number, 115 (27%) of genes induced only by the mutant strain compared to 307 (73%) by the wild type strain. However, both strains induced a higher number of up-regulated genes compared to down-regulated genes. The wild type strain uniquely up-regulated genes associated with cell communication (*ERBB3, GSG2, HIST1H4B, HIST1H4C, IL6ST, IFITM3, IGF2*), signal transduction (*ABCC9, ADCY5, ADM, ANXA4, BCAM, CALY, IL6ST*), response to lipids (*ADCY5, ADM, APOH, AQP3*) and chemical stimulus (*ACY3, ADCY5, ADH6, CXCL3, EDN1, ERBB3, IFITM3*) (Table 9). These included host immune response-related *IL6ST* cytokine and *CXCL3* chemokine. *IL6ST* is a major signal inducer that plays an essential role in increasing the binding affinity of the *IL6* receptor (Waetzig *et al.*, 2010). It is also involved in the activation of the *STAT3* transcriptional factor (Schutt *et al.*, 2013) and mediates signals which regulate pain control, hematopoiesis and immune response (Waetzig *et al.*, 2010).

The *CXCL3* chemokine, together with *CCL2, CCL20, CXCL1* and *CXCL2*, have been reported to be expressed by infected epithelial cells in response to *Girdia lamblia* infection (Roxstrom-Lindguist *et al.*, 2005). The up-regulation of these chemokines is essential for the induction of both the innate and adaptive immunity (Roxstrom-Lindguist *et al.*, 2005). *CXCL1, CXCL2, CXCL3* and *CXCL5* are induced in lung tissue upon *M. tuberculosis* infection (Nouailles *et al.*, 2014). The up-regulation of *CXCL3* and *IL6ST* by epithelial cells in the presence of MTP only, implies the essential role it plays in inducing pro inflammatory response in host cells.

M. tuberculosis has been known to induce immune response in macrophages by eliciting cytokine and chemokine production which leads to the recruitment of neutrophils, dendritic and natural killer cells towards the site of infection (Welin, 2011; Peters and Ernst, 2003; Fogel, 2015). Previous studies conducted using RT-PCR have implicated human alveolar epithelial cells in the production of *IL-8* and *MCP-1* (Lin *et al.*, 1998), *CXCL-10*, *TNF-α* and *CCL-5* (Lam *et al.*, 2010) and *IL-1β*, *IL-6*, *TNF-α* and *MCP-1* (Savard *et al.*, 2002) contributing to the activation of immune cells and pro inflammatory response (DiMango *et al.*, 1995; Jung *et al.*, 1995; Lin *et al.*, 1998). Genes associated with cell differentiation and regulation of biosynthetic processes were down-regulated by the wild type strain. Interestingly, genes involved in cell communication and signal transduction were both up and down-regulated by the wild type strain. This could mean that these biological functions are coded for by different sets of genes which are significantly down and up-regulated in pulmonary epithelial cells in vitro.

The mutant strain exclusively up-regulated genes associated with biological functions such as protein refolding, cellular response to stress and positive regulation of tumor necrosis factor associated-pathways. All these biological functions were driven by heat shock proteins. Van Eden *et al.* (2005) reported that immune response to heat shock proteins develops in almost all inflammatory diseases,

resulting in the prevention of cell damage due to inflammation. These proteins are up-regulated by host cells in order to arrest inflammatory damage and their peptides have been implicated in promoting the production of anti-inflammatory cytokines, indicating a potential ability to regulate the immune system (Van Eden *et al.*, 2005). Inflammatory response to infections leads to the up-regulation of tumor necrosis factor pathways which often results in disruption of the epithelial cell barrier (Mashukova *et al.*, 2010). Therefore, the expression of genes coding for cytokines and tumor necrosis factor response in mutant infected-epithelial cells could be attributed to the up-regulation of heat shock proteins by these cells. By inference, MTP prevents disruption to epithelial cells, most likely in order to protect its replication niche. Genes associated with cellular processes and ion binding were down-regulated by the mutant strain. This could suggest that MTP affects essential cellular processes once it invades epithelial cells.

4.2. MTP Enriches a High Number of Canonical Pathways in Epithelial Cells

Changes in canonical pathways were investigated with IPA using datasets of significantly differentially expressed genes. The wild type strain enriched a higher number of pathways i.e. 44 (59%) compared to 30 (41%) by the mutant strain. These results suggest that MTP plays an important role in gene regulation of alveolar epithelial cells which is evident from its involvement in the enrichment of a high number of canonical pathways and the lower number of pathways enriched in its absence. A total of 27 pathways was unaffected by MTP as these were enriched by both strains. The majority of these pathways, 23 (85%), were highly enriched by the mutant strain compared to the wild type strain. A possible explanation for this may be that the mutant strain compensates for the loss of the *mtp* gene by up-regulating genes coding for other adhesins which, in turn, could lead to elevated levels of pathway enrichment by the mutant strain.

Mvubu *et al.* (2016) reported that hyper-virulent strains induced lower protective response as displayed by low enrichment of immune related canonical pathways when compared to less virulent strains. Similarly, this could explain the low pathway enrichment by the wild type strain used in our study compared to the mutant strain which may present with reduced virulence upon loss of the *mtp* gene. Clinical *M. tuberculosis* strains representing major strain families in South Africa have also been shown to induce specific molecular pathways in A549 epithelial cells after 48 hours of infection (Mvubu *et al.*, 2016). In this study, after 4 hr, pathways such as Role of IL-17A in Athritis and Fatty Acid Oxidation pathways were exclusively induced by the wild type strain of the F15/LAM4/KZN family compared to Dendritic Cell Maturation, Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses and Antigen Presentation pathways that were enriched by the same strain at 48 hr (Mvubu *et al.*, 2016).

The Acute Phase Response Signalling pathway was enriched by both wild type and mutant strains in our study, with –log (p value) of 2.908 and 3.095, respectively. The Hepatic Fibrosis/Hepatic Stellate Cell Activation pathway was only enriched by the wild type strain. Mvubu *et al.* (2016) reported the enrichment of the Hepatic Fibrosis/Hepatic Stellate Cell Activation pathway by F15/LAM4/KZN, H37Rv, Unique, F28, F11 and Beijing strains of *M. tuberculosis* 48 hr post-infection while the Acute Phase Response Signalling pathway was enriched by F15/LAM4/KZN, Beijing, Unique and F28 strains. While the role of the MTP adhesin in adhesion to and invasion of macrophages (Ramsugit *et al.*,2015) and epithelial cells (Ramsugit *et al.*, 2016) has been previously demonstrated, to our knowledge, no published data is available that elucidates the role of this and other bacterial adhesins in host gene regulation and canonical pathway enrichment.

MTP did not affect certain host responses as both strains enriched pathways associated with host immune response e.g. acute phase response signalling pathway (Figure 20), complement system (Figure 21) and a pathway for differential regulation of cytokine production in macrophages and T helper cells (Figure 34). All these pathways were highly enriched by the mutant strain compared to the wild type strain. In the acute phase response signalling pathway, both strains up-regulated *PL3K/P85* complex, *SERPINA1, CP, SERPINA3, FGG, FGB, FGA, LBP, CFB, AMBP, CFOS* and *C4BP* genes while *IL-6* was the only down-regulated gene. The wild type strain exclusively up-regulated *GP130* and *APOH* genes whereas *C3*, which is very essential for the acute phase response, was exclusively up-regulated by the *mtp*-mutant strain. The up-regulation *C3* by the *mtp*-mutant strain only, may be responsible for the high enrichment of the acute phase response signalling pathway in this strain compared to the wild type. The acute phase signaling pathway is involved in immune response to infection, tissue injury and inflammation (Moshage, 1997). This pathway has been implicated in the induction of acute phase proteins which restore homeostasis and is mediated by cytokines (Moshage, 1997).

Both strains up-regulated *C4BP, BF, IF, HF1, DF, BFA* and *BFB* genes of the complement system pathway, implying that the absence of MTP did not affect this pathway. The mutant strain exclusively up-regulated *C3, C3A* and *C3B* genes of the alternate pathway wing in the complement system pathway. The high enrichment of the complement system pathway by the mutant strain, compared to the wild type strain, could be attributed to the exclusive up-regulation of *C3, C3A* and *C3B*. The complement system uses a cascade of soluble proteins and antibody complexes that function by opsonising invading bacteria, clumping and neutralization of bacterial antigens, as well as lysis of bacterial cells (Welin, 2011). The enrichment of the Differential Regulation of Cytokine Production in Macrophages and T-helper Cells by IL-17A and IL-17F Pathway by both wild type and mutant strains correlated with the findings of Ramsugit *et al.* (2016) that demonstrated the ability of *M. tuberculosis* V9124 wild type and *mtp*-knockout mutant strain to elicit cytokine production in A549 alveolar

epithelial cells. The interaction of pili with host cell receptors was reported to play an important role in the induction of inflammation through the production of inflammatory factors such as cytokines and chemokines (Sauer *et al.*, 2000). Our results suggest that epithelial cells also play a role in the regulation of cytokine production in other immune cells such as macrophages and T helper cells as shown by the enrichment of the Differential Regulation of Cytokine Production in Macrophages and T-helper Cells by IL-17A and IL-17F Pathway. In this pathway, the expression of *IL-12* and *CCL2* was down- and up-regulated respectively in both macrophages and T-helper cells whereas *IL-6* was down-regulated in the former and not expressed in the latter.

In the absence of the *mtp* gene, only 3 unique canonical pathways were enriched compared to 17 when the gene was present. These findings suggest that the presence of MTP leads to the regulation of 17 cellular and immune response-related processes that are not regulated when the adhesin is absent. The mutant strain uniquely enriched the p53 Signaling Pathway, Salvage Pathways of Pyrimidine Deoxyribonucleotides and Glioma Invasiveness Signalling Pathway. The p53 Signalling Pathway is involved in response to stress that can disrupt DNA replication and cell division (Harris and Levine, 2005) whereas the Glioma Invasiness Pathway is associated with the invasion of normal brain cells by tumor cells (Rao, 2003).

Among 17 pathways that were uniquely enriched by the wild type strain were the Fatty Acid Oxidation Pathway, Role of *IL-17A* in Arthritis and the Pyruvate Fermentation to Lactate Pathway. According to Simon and Hertig (2015), epithelial cells alter the oxidation of fatty acids under biological stress. Therefore, it is tempting to speculate that MTP may be associated with the presence of the abundance of fatty acids in the cell membrane of *M. tuberculosis*. The enrichment of the Pyruvate Fermentation to Lactate Pathway by the wild type strain only could suggest that MTP may be responsible for the induction of the Warburg effect in epithelial cells which allows mycobacterial proliferation. Microorganisms proliferate using fermentation, an equivalent of aerobic glycolysis, which allows the metabolism of glucose to lactate and provides sufficient energy, in a form of ATP, for cell proliferation (Vander Heiden *et al.*, 2009). Kirkham *et al.* (2014) reported the involved of *IL-17A* in autoimmune diseases such as psoriasis, psoriatic arthritis and rheumatoid arthritis. The enrichment of the Role of IL-17A in Arthritis pathway by the wild type strain could imply the involvement of MTP in the induction of autoimmune diseases in the host.

4.3. MTP Induces a Strong Immune Response in A549 Epithelial Cells

Differentially expressed genes associated with the host immune response as a result of infection of A549 pulmonary epithelial cells were induced by both strains. These included genes coding for various functions, such as host-pathogen interactions, immune and inflammatory response and immune cell activation. The level of up-regulation for all up-regulated genes was higher in the wild

type infection compared to the mutant infection. The level of down-regulation varied between the two conditions. Our findings also show that the deletion of MTP resulted in down-regulation of some of the genes that were up-regulated in the wild type infection. These findings suggest that the presence of the MTP adhesin in the wild type strain plays an important role in the launch of a strong immune response against this strain, while the knocking out of the *mtp* gene negatively affects the ability of epithelial cells to respond to *M. tuberculosis* infection.

4.3.1. *MTP Activates the Expression of Cell Surface Receptors in Epithelial Cells*

Cell surface receptors such as *CD14*, *CD24*, *CD38*, *CD96* and *CD99* were found to be more up-regulated by the wild type strain when compared to the mutant strain. These surface-bound molecules function as essential cell surface co-receptors (alongside TLRs) for the detection of bacterial lipopolysaccharides (Jersmann, 2005), lipoteichoic acid, glycolproteins (Jaggupilli and Elkord, 2012), proteins and carbohydrates. TLRs are intracellular and surface-bound receptors that are involved in the recognition of lipoproteins, lipids and extracts of *M. tuberculosis* and other pathogens (Welin, 2011). In our study, genes coding for *TLR1*, *TLR6*, *TLR7*, *TLR8* and *TLR10* were highly up-regulated by the wild type strain than the mutant strain. Takeda and Akira (2005) reported *TLR2* as one of the most important TLRs, playing a crucial role in the recognition of pathogens where it binds to lipoproteins/lipopeptides, peptidoglycan from Gram-positive bacteria and lipoarabinomannan from mycobacteria.

In our study, *TLR2* was down-regulated by the wild type strain and was not significantly expressed by the *mtp*-mutant strain. However, this did not affect the recognition of *M. tuberculosis* as *TLR1* and *TLR6*, which both functionally associate with *TLR2* (Takeda and Akira, 2005), were up-regulated by both strains. *TLR3* has been reported to be mainly involved in the recognition of double-stranded RNA produced by viruses during viral replication in host cells (Alexopoulou *et al.*, 2001), therefore, this may explain why *TLR3* was down-regulated by both strains in our study. The possible explanation for the down-regulation of *TLR4* by both strains could be that these receptors interact with CD14 cells in order to mediate signal transduction events initiated by lipopolysaccharides (LPS) found in most Gram-negative bacteria (Hoshino *et al.*, 1999). Moreever, LPS binds to the LPS-binding protein which is present in serum, the LPS-LPS-binding protein complex forms and is subsequently recognized by CD14 which is preferentially expressed in neutrophils and macrophages (Takeda and Akira, 2003). LPS stimulation increases a physical proximity between *TLR4* and CD14 molecules, suggesting that *TLR4* and CD14 may interact in LPS signalling (Jiang *et al.* 2000, Da Shilva Correia *et al.*, 2001). *TLR7* and *TLR8* were up-regulated by both strains in our study, however, both were highly expressed in epithelial cells infected with the wild type strain. These findings could suggest that the presence of MTP in *M. tuberculosis* increases its recognition by host cells as *TLR7* and *TLR8* have been predicted to recognize nucleic acid-like components of pathogens, especially

guanosine or uridine-rich single-stranded RNA from viruses such as HIV and influenza virus (Heil *et al.*, 2004; Lund *et al.*, 2004). An increased expression of TLRs and CD surface receptors by the wild type compared to the mutant strain suggests that MTP activates the induction of these receptors in epithelial cells which could lead to an increased recognition of the pathogen and induction of a strong immune response.

4.3.2. *MTP Induces the Expression of Chemokine Genes in Epithelial Cells*

Whilst the majority of genes associated with cytokine receptors and tumor necrosis factor (TNF) receptors were down-regulated by both strains, the wild type strain highly up-regulated genes coding for chemokine receptors compared to the mutant strain. MTP significantly affected differentially expressed genes associated with cytokines and chemokines as well as a pathway of differential regulation of cytokine production in macrophages and T helper cells, which were highly expressed by the wild type strain compared to the mutant strain. An *IL6* upstream regulator was only enriched by the wild type strain.

Our findings show an up-regulation of chemokines compared to cytokines by both strains. *CCL2, CCL26, CCL28, CCL5, CXCL1, CXCL3, CXCL5, CXCL6* and *CXCL9* chemokines and *IL-11, IL-15,* and *IL-32* cytokines were highly up-regulated by the wild type strain compared to the mutant strain. These findings suggest that MTP highly induced the production of chemokines in A549 epithelial cells compared to cytokines. This is supported by previous findings in our group that MTP did not largely influence cytokine production in *M. tuberculosis*-infected epithelial cells (Ramsugit *et al.*, 2015). *IL-1, IL-4, IL-6, IFN-Y,* and *G-CSF* were produced at low concentrations by epithelial cells at 24, 48 and 72 hours post infection (Ramsugit *et al.*, 2015). In our study, at 4 hr post infection, these cytokine genes were not expressed, except for *IL-4* which was down-regulated. Ramsugit *et al.* (2015) speculated that MTP-mediated invasion of epithelial cells may be advantageous to *M. tuberculosis* by suppressing cytokine response which plays an essential role in the control of infections. This also supports the belief that the less harsh environment of epithelial cells may be a temporary safe haven for the pathogen to replicate in (Bermudez and Goodman, 1996; Mehta *et al.*, 1996; Dobos *et al.*, 2000; Ryndak *et al.*, 2015).

In a previous study by our group, RNA-sequencing showed that *IL1A, IL1B, IL8* and *IL23A* cytokines and *CCL2, CCL5, CCL20, CXCL1* and *CXCL5* chemokines were up-regulated by A549 epithelial cells infected with the F15/LAM4/KZN strain of *M. tuberculosis* at 48 hours post-infection (Mvubu *et al.*, 2016). Similarly, in the present study, *CCL2, CCL5, CCL20, CXCL1* and *CXCL5* were highly up-regulated by the wild type strain compared to the mutant strain. In contrast, *IL1A, IL1B* and *IL23A* cytokines were down-regulated by both strains. This difference could have been as a result of the study being conducted at an earlier time point (4 hours) and the previous study being aimed at the

regulation of canonical pathways, networks and transcriptional factors by different *M. tuberculosis* strains. These findings further suggest that MTP strongly mediates the immune response in A549 epithelial cells through the induction of chemokine production.

The role of epithelial cells in the expression of genes coding for cytokines (*IL-6, IL-3* and *PTX3*) and chemokines (*IL-8, CXCL2, CCL5, CCL2, MCP-1, CXCL10* and *CXL1*) had been demonstrated using microarray technology (Kim *et al.*, 2013; Guerra-Laso *et al.*, 2015) at 1, 2 and 4 hours post infection and RT-PCR (Yang *et al.*, 2002; Miller *et al.*, 2003) at 2, 9, 24, 48 and 72 hours post infection) previously. In our study, 4 hr post infection, these cytokines and chemokines were differentially expressed except for *IL-3, IL-8, MCP-1* and *CXCL10* which were not expressed at all. In comparison to F11, Beijing, Unique and H37Rv strains of *M. tuberculosis*, F15/LAM4/KZN strain induced a higher production of Rantes, IL-8, GM-CSF, IFN-γ, G-CSF and TNF-α in A549 epithelial cells 48 and 72 hr post infection (Mvubu *et al.*, 2013). However, in our study, none of these cytokine and chemokine genes were expressed in epithelial cells 4 hours post infection, except for TNF-α.

4.3.3. *MTP Induces STAT Response and Cell Death in Epithelial Cells*

Our data shows that the presence of MTP elevated the up-regulation of genes coding for *STAT1, STAT3* and *STAT6* in epithelial cells compared to the mutant strain. These transcription regulators play an essential role in mediating gene expression as they are activated by cytokines, leading to phosphorylation and migration to the nucleus where they bind specific sequences that activate gene expression (Murray, 2007). Furthermore, activation of *STAT1* (Sharma *et al.*, 2007) and *STAT2* (Stockinger and Decker, 2009) transcriptional factors lead to an increased protective immune response in epithelial cells via the production of cytokines such as IFN-Y, which plays an essential role in antibacterial removal at the site of infection (Sharma *et al.*, 2007). The increased expression of *STAT1, STAT3* and *STAT6* by the wild type strain suggests that MTP plays an essential role in regulating gene expression in epithelial cells which could lead to an increased protective immune response against pathogens.

Our gene expression profiles show that genes associated with tumor necrosis factor signalling were highly up-regulated in epithelial cells infected with the wild type strain compared to the mutant strain. These genes play a major role in controlling cell death which is either programmed by host cells in an attempt to deprive pathogens their replication niche or by pathogens themselves due to infection-associated stress they impose on host cells (Welin, 2011). The induction of cell death by MTP in epithelial cells could suggest that *M. tuberculosis* uses this adhesin as one of its virulence factors during infection in order to infect, replicate in and disseminate and colonize other host cells, upon cell death.

4.4. MTP does not Induce Antigen Presentation by Epithelial Cells

Genes associated with antigen presentation were down-regulated by both strains in epithelial cells except for *HLA-DMA* and *HLA-DMB*. These findings are in line with the data that has been reported by Lin *et al* (1998), Peters and Ernst (2003) and Welin (2011) so far about the role of epithelial cells in antigen presentation. Epithelial cells are known to be involved in launching an immune response through the recognition of pathogens by surface-bound toll-like receptors (TLRs) and cytokine and chemokine production, which leads to the activation and recruitment of other immune cells towards the site of infection (Lin *et al.*, 1998). Welin (2011) reported that presentation of antigens from extracellular pathogens is achieved through the uptake by antigen-presenting cells (APCs) such as macrophages, dendritic cells, natural killer cells and B cells. These antigens are then presented to CD8+ or CD4+ cells via MHC class I and II respectively, leading to the activation of the adaptive immune response.

Our study showed that MTP plays no role in inducing the presentation of antigens by epithelial cells, as seen by the down-regulation of genes associated with this function. These findings do not correlate with those of Cunningham *et al.* (1994) who reported that alveolar epithelial cells most likely play a role in antigen presentation via MHC class I molecules. However, it is important to mention that the lack of similarity may be due to this study being conducted on uninfected alveolar pulmonary epithelial cells. Mvubu *et al.* (2016) reported an increased expression of *HLA-B*, *HLA-C*, *HLA-F* and *HLA-H* genes in alveolar pulmonary epithelial cells infected with various *M. tuberculosis* strains for 48 hours, in contrast to our findings. These differences could be attributed to the fact that our cells were sampled after 4 hours, suggesting that the expression of these genes occurs later in the infection process.

4.5. MTP Induces the Expression of Networks in Alveolar Pulmonary Epithelial Cells

Our network analysis showed that the wild type strain enriched a similar number of networks (25) when compared to the *mtp*-mutant strain. *UBE2C* and *NR5A2* were the most up-regulated genes in biological networks induced by the wild type strain. *UBE2C* has been reported to be highly expressed in cancer cells where it causes chromosome missegregation and alters the cell cycle (Fujita *et al.*, 2009). This protein is also associated with aggressive thyroid (Guerriero *et al.*, 2010), ovarian (Troncone *et al.*, 2009) and breast cancer (Berlingieri *et al.*, 2007). The overexpression of the gene coding for this protein in infected host cells may be indicative of a host cell response under stressful conditions. *NR5A2* has been implicated in the reprogramming of somatic cells to pluripotent cells (Heng *et al.*, 2010), an event which could be advantageous to host cells under bacterial or viral attack. The overexpression of both *UBE2C* and *NR5A2* in epithelial cells infected with the wild type strain suggests that the presence of MTP may induce protective host cell response against *M. tuberculosis*.

The *mtp*-mutant strain highly up-regulated *DEPDC1* and *KLF2* in infected epithelial cells. Kanehira *et al.* (2007) reported the overexpression of *DEPDC1* in bladder cancer cells. The overexpression of the gene coding for this protein in epithelial cells infected with the mutant strain could suggest a general host cell response to infection. The overexpression of *KLF2* in monocytes inhibits LPS-mediated activation of cytokines, chemokines and other pro-inflammatory factors (Das *et al.*, 2006). The overexpression of this gene in epithelial cells infected with the mutant strain explains the reduced cytokine and chemokine response in these cells. Overall, the wild type strain enriched top scoring common networks in A549 epithelial cells compared to those enriched by the *mtp*-mutant strain. These findings further suggest that the absence of MTP in *M. tuberculosis* does affect gene regulation in A549 epithelial cells, in response to mycobacterial infection.

4.6. MTP Induces the Expression of Upstream Regulators in Epithelial Cells

Our analysis of the enrichment of upstream regulators in infected epithelial cells revealed that the wild type strain activated the expression of essential upstream regulators in A549 epithelial cells, relative to uninfected cells. The *STAT3* upstream regulator was up-regulated by the wild type strain and not significantly expressed by the *mtp*-mutant strain. Tkach *et al.* (2013) reported that in response to interferons, growth factors and cytokines, *STAT3* transcriptional factors are phosphorylated and translocated to the nucleus where they activate transcription which leads to the expression of a variety of genes in response to cell stimuli. *STAT3* has also been reported to play a key role in cellular growth and apoptosis (Yuan *et al.*, 2004), which plays a protective role against pathogens in host cells. Therefore, the high expression of *STAT3* by the wild type strain could suggest the involvement of MTP in activating epithelial cells to induce a stronger host immune response against *M. tuberculosis*, through transcription activation.

The *CEBPD* upstream regulator codes for a protein which is responsible for the regulation of genes involved in immune and inflammatory response and has been shown to strongly regulate *IL-1* and *IL-6* cytokines (Szpirer *et al.*, 1992). Epithelial cells infected with *M. tuberculosis* have been reported to produce *IL-1β* and *IL-6*, even though these cytokines are produced at low concentrations before 72 hour post infection (Ramsugit *et al.*, 2016). The up-regulation of *CEBPD* by the wild type strain suggests the involvement of MTP in the activation of cytokine production in epithelial cells.

The mutant strain exclusively enriched 24 upstream regulators in A549 pulmonary epithelial cells. *MYBL2* was the only highly up-regulated upstream regulator. A similar trend was also observed in down-regulated upstream regulators where *MYC* was the only highly down-regulated regulator. The *MYC* upstream regulator codes for proteins involved in cell cycle division, cellular transformation and apoptosis (Finver *et al.*, 1988). The wild type strain exclusively enriched 10 upstream regulators in A549 pulmonary epithelial cells. *HGF* was the highest up-regulated upstream regulator followed by

IL6 whereas *ESR1* was the lowest up-regulated. *HGF* is a growth factor that is produced in stromal cells and has been reported to be associated with epithelial cell proliferation, motility and morphogenesis (Nakamura and Mizonu, 2010). It is also responsible for the self- repair of injured lungs, kidneys and livers (Nakamura and Mizonu, 2010), which could explain its up-regulation by the wild type strain in alveolar pulmonary epithelial cells. These findings could imply that the presence of MTP may lead to increased cytotoxicity of *M. tuberculosis* to pulmonary epithelial cells.

4.7. Significance of the Findings

One of the main problems associated with the development of rapid point of care (POC) TB diagnostic tests and therapeutic interventions is the lack of biomarkers that can be targeted for this purpose. Our findings confirm previous reports that support MTP as a biomarker for the potential development of anti-TB drugs and POC diagnostic test, as they're surface-located and have been shown to play an important role in adherence to and invasion of host immune cells. Our findings also strongly suggest that MTP has the ability to induce host immune response in epithelial cells, making it an essential antigen and immunogen for *M. tuberculosis* and thus, a potential vaccine candidate or immunotherapeutic agent as adjunct therapy for TB.

4.8. Limitations of the Study

The cost of RNA-sequencing technology was the major limiting factor of our study. The number of biological and technical replicates is an important factor of the study design in RNA-sequencing experiments. In this study, only biological replicates were included due to the expensive nature of RNA-seq. In addition, samples from only one (4 hours) of the four time intervals investigated were sequenced. Sequencing of a sample at a later time interval would have allowed for the comparison of two sets of gene expressions profiles in epithelial cells during the early innate response and early adaptive immune response *in vitro*. Samples had to be sequenced using the multiplexing method which involves processing a large number of samples at once and can sometimes decrease the overall read mapping rate to the reference genome. However, these sequencing-related limitations were compensated for by employing deep core sequencing with a large coverage which permitted higher transcript detection and increased quantification. The lack of bioinformatics skills was also a major limiting factor as it caused major delays in the analysis of huge amounts of data. RNA-seq data has not yet been validated using RT-PCR. However, a previous study by our research group (Mvubu *et al.*, 2016), as well as other studies (Arnvig *et al.*, 2011; Uplekar *et al.*, 2012; Chen *et al.*, 2015; Rienksma *et al.*, 2015) reported good correlation of RNA-Seq data with RT-qPCR.

4.9. Recommendations for Future Studies

The RNA-Seq data will be validated by RT-qPCR of a selected number of genes in the near future. Since RNA-Seq is expensive, gene expression profiles of host immune response-related genes such as

genes coding for cytokines, antigen presentation and granuloma formation should be interrogated at different time intervals using RT-qPCR to understand the kinetics of expression. RT-qPCR will also be applied in RNA extracted from A549 epithelial cells infected with the *mtp*-complemented strain in order to confirm if the observed phenotypes are reversed to wild type levels. Northern blot hybridization could also be applied to study and confirm gene expression profiles. According to Ding *et al.* (2007), northern blotting is a gold standard method for confirming gene expression data generated by microarrays when compared to RT-qPCR. It does not involve enzymatic manipulation of RNA and this is critical as it eliminates artificial changes that are introduced during RNA processing. Whole transcriptome analysis of the *mtp* gene deletion mutant relative to the wild type *M. tuberculosis* infecting host cells such as A549 epithelial cells, THP1-derived macrophages as well as mouse organs would elucidate the pathogen genes that are regulated by MTP and further inform on how different host environments respond to *M. tuberculosis* infection.

4.10. Conclusion

This is the first study to investigate the role of MTP in gene regulation of epithelial cells using RNA-sequencing technology. In the absence of MTP, the number of significantly differentially expressed genes and canonical pathways induced in A549 alveolar epithelial cells was reduced. Our findings further showed that alveolar epithelial cells infected with the wild type strain launched a stronger host immune response when compared to those infected with the *mtp*-knockout mutant strain. Taken together, these findings suggest that MTP affects gene regulation of A549 alveolar epithelial cells and could be a potential TB vaccine candidate and antimicrobial drug target. The presence of MTP also allowed the enrichment of a high number of pathways compared to its absence, including those associated with the host immune response such as the complement system, cell surface receptor signalling, *STAT* signalling, *TLR* signalling, *TNF* response signalling and cytokine and chemokine production. These findings add to the growing evidence that *M. tuberculosis* uses MTP as one of its major invasins and virulence factors during the infection of host cells. Our data also confirms that epithelial cells do not provide just a physical barrier against extracellular objects, but they also play an important role in host immune response to infections.

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APPENDICES

1. **Bacterial colony forming units (CFUs) obtained before (initial inoculums) and after (final) infection of A549 epithelial cells with the wild type and mutant strain for 4 hours. Experiments were conducted in triplicate.**

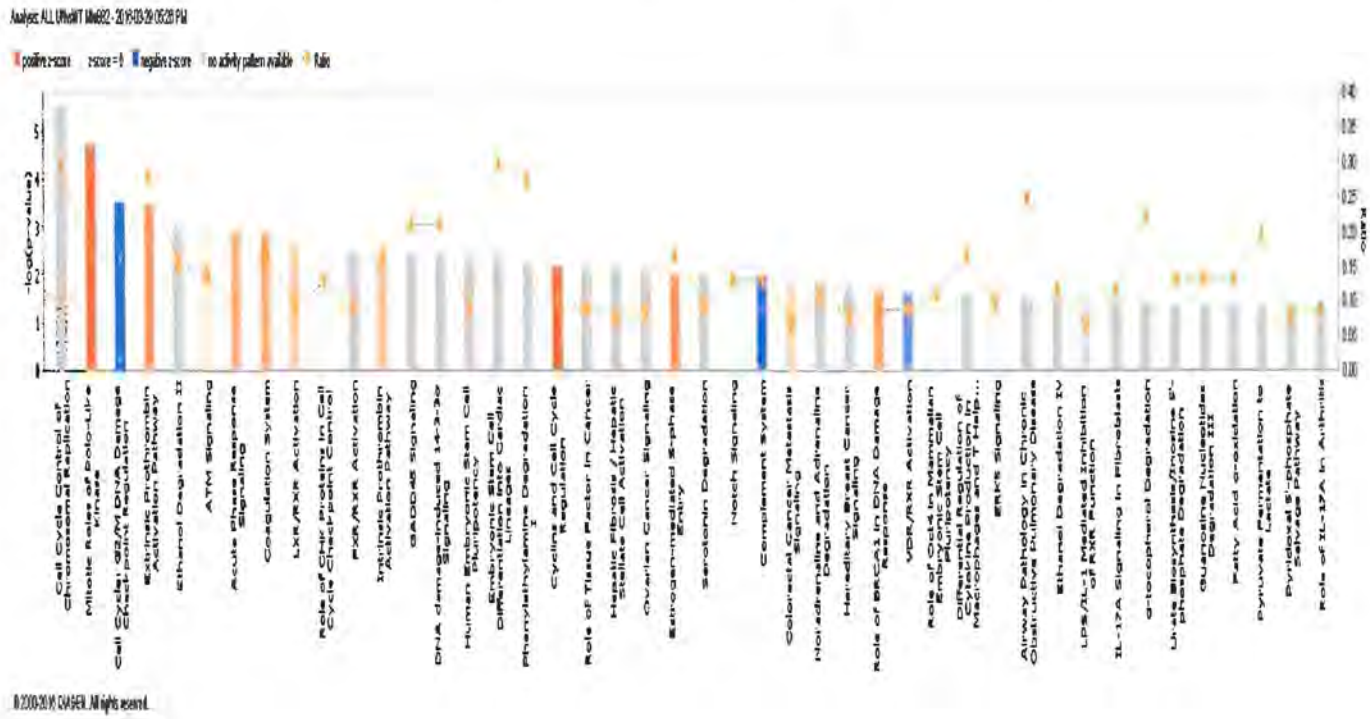
WILD TYPE

Replicate	CFU/ml (Initial Inoculum)	CFU/ml (Final)
1	1.01×10^4	2.2×10^3
2	1.03×10^4	2.8×10^3
3	1.05×10^4	2.4×10^3
Average CFU/ml	$= 1.03 \times 10^4$	$= 2.47 \times 10^3$

MUTANT

Replicate	CFU/ml (Initial Inoculum)	CFU/ml (Final)
1	1.03×10^4	1.3×10^3
2	1.044×10^4	2.4×10^3
3	1.01×10^4	1.3×10^3
Average CFU/ml	$= 1.028 \times 10^4$	$= 1.67 \times 10^3$

2. **Canonical pathways enriched by the wild type strain in A549 epithelial cells.**

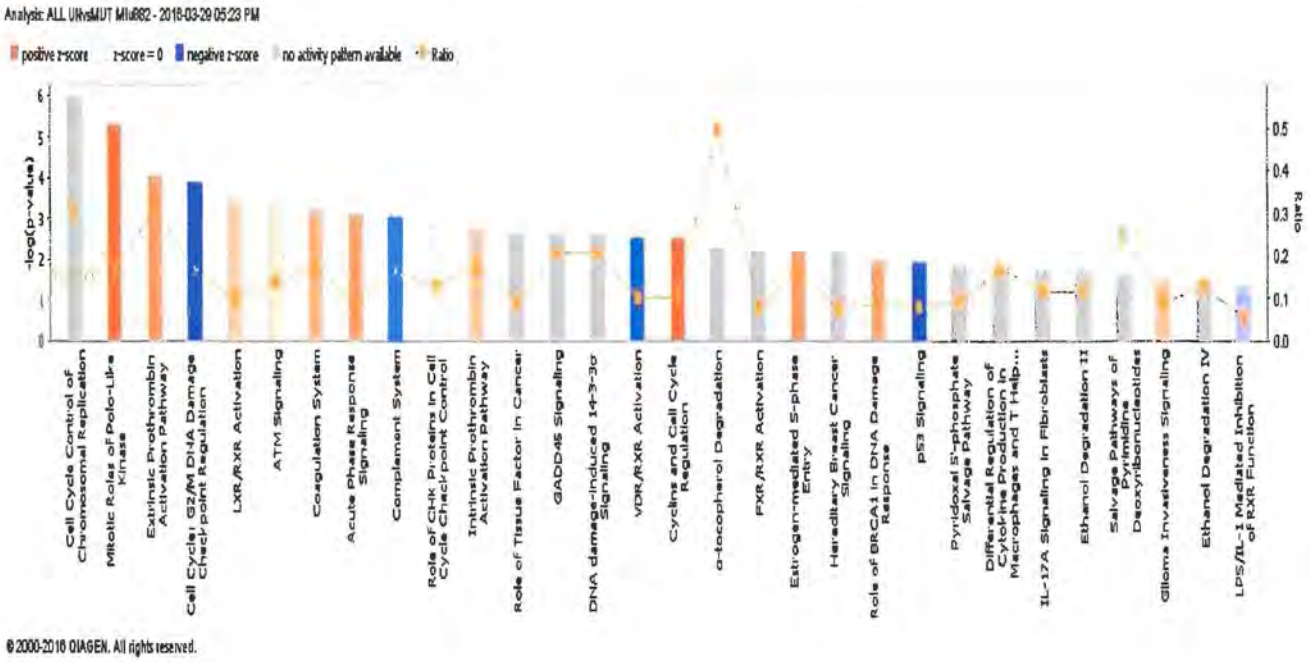


3. **–log (p value) of canonical pathways enriched by the wild type strain in A549 epithelial cells. Pathways in red were uniquely enriched by the wild type strain.**

Pathway	-log (p-value)
Cell Cycle Control of Chromosomal Replication	5.513
Mitotic Role of Polo-like Kinase	4.702
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	3.506
Extrinsic Prothrombin Activation Pathway	3.48
Ethanol Degradation II	3.002
ATM Signaling	2.95
Acute Phase Response Signaling	2.908
Coagulation System	2.869
LXR/RXR Activation	2.675
Role of CHK Proteins in Cell Cycle Checkpoint Control	2.485
FXR/RXR Activation	2.431
Intrinsic Prothrombin Activation Pathway	2.412
GADD45 Signaling	2.392
DNA Damage-Induced 14-3-3α Signaling	2.392
Human Embryonic Stem Cell Pluripotency	2.38
Embryonic Stem Cell Differentiation into Cardiac Lineages	2.347
Phenylethylamine Degradation I	2.221
Cyclins and Cell Cycle Regulation	2.198
Role of Tissue Factor in Cancer	2.178
Hepatic Fibrosis/Hepatic Stellate Cell Activation	2.135
Ovarian Cancer Signaling	2.089
Estrogen-mediated S-Phase Entry	2.015
Serotonin Degradation	1.993
Notch Signaling	1.967
Complement System	1.967
Colorectal Cancer Metastasis Signaling	1.884
Noradrenaline and Adrenaline Degradation	1.877
Hereditary Breast Cancer Signaling	1.721
Role of BRCA1 in DNA Damage Response	1.674
VDR/RXR Activation	1.647
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	1.631

Differential Regulation of Cytokine Production in Macrophages and T helper Cells	1.607
ERK5 Signaling	1.58
Airway Pathology in Chronic Obstructive Pulmonary Disease	1.511
Ethanol Degradation IV	1.498
LPS/IL-1 mediated Inhibition of RXR Function	1.466
IL-17A Signaling in Fibroblasts	1.432
a-tocopherol Degradation	1.412
Urate Biosynthesis /Inosine 5'-phosphate Degradation	1.327
Guanosine Nucleotides Degradation III	1.327
Fatty Acid a-oxidation	1.327
Pyruvate Fermentation to Lactate	1.325
Pyridoxal 5'-phosphate Salvage Pathway	1.315
Role of IL-17A in Arthritis	1.311

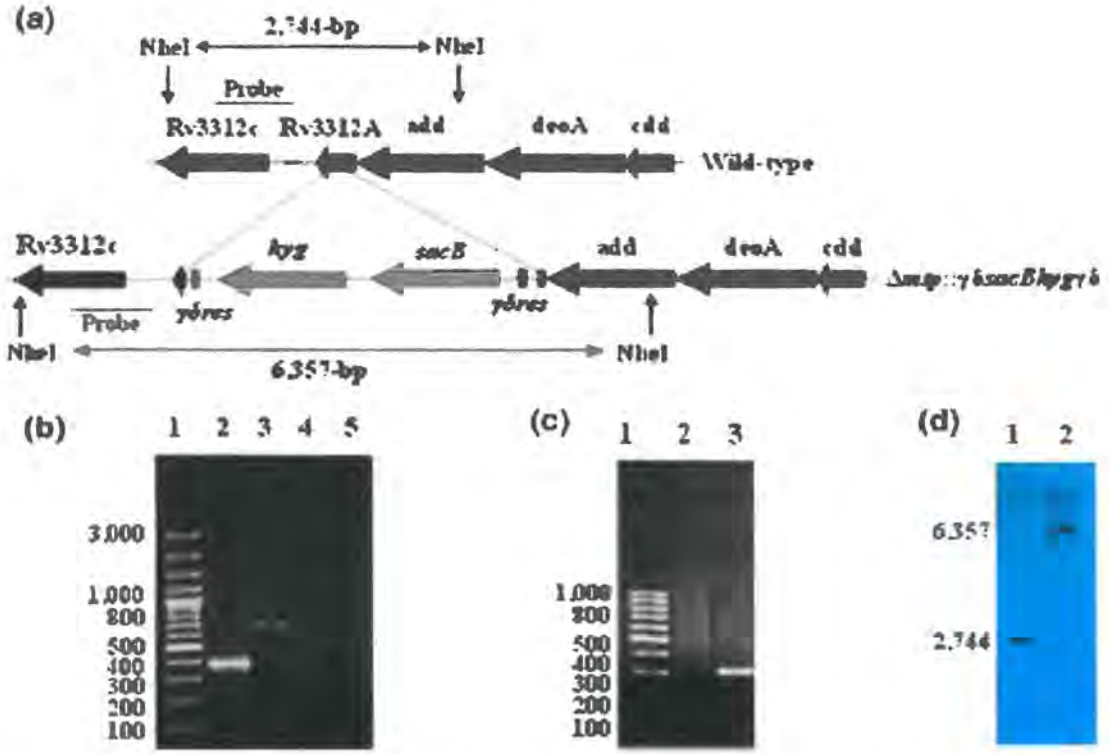
4. Canonical pathways enriched by the mutant strain in A549 epithelial cells.



5. **–log (p value) of canonical pathways enriched by the mutant strain in A549 epithelial cells. Pathways in red were uniquely enriched by the mutant strain.**

Pathway	log (p-value)
Cell Cycle Control of Chromosomal Replication	5.973
Mitotic Role of Polo-like Kinase	5.281
Extrinsic Prothrombin Activation Pathway	4.036
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	3.926
LXR/RXR Activation	3.423
ATM Signaling	3.352
Coagulation System	3.188
Acute Phase Response Signaling	3.095
Complement System	3.055
Role of CHK Proteins in Cell Cycle Checkpoint Control	2.832
Intrinsic Prothrombin Activation Pathway	2.747
Role of Tissue Factor in Cancer	2.658
GADD45 Signaling	2.614
DNA Damage-Induced 14-3-3α Signaling	2.614
VDR/RXR Activation	2.551
Cyclins and Cell Cycle Regulation	2.551
a-tocopherol Degradation	2.259
FXR/RXR Activation	2.234
Estrogen-mediated S-Phase Entry	2.229
Hereditary Breast Cancer Signaling	2.164
Role of BRCA1 in DNA Damage Response	1.981
p53 Signaling	1.956
Pyridoxal 5'-phosphate Salvage Pathway	1.851
Differential Regulation of Cytokine Production in Macrophages and T helper Cells	1.769
IL-17A Signaling in Fibroblasts	1.652
Ethanol Degradation II	1.652
Salvage Pathways of Pyrimidine Deoxyribonucleotides	1.626
Glioma Invasiveness Signaling	1.505
Ethanol Degradation IV	1.482
LPS/IL-1 mediated Inhibition of RXR Function	1.368

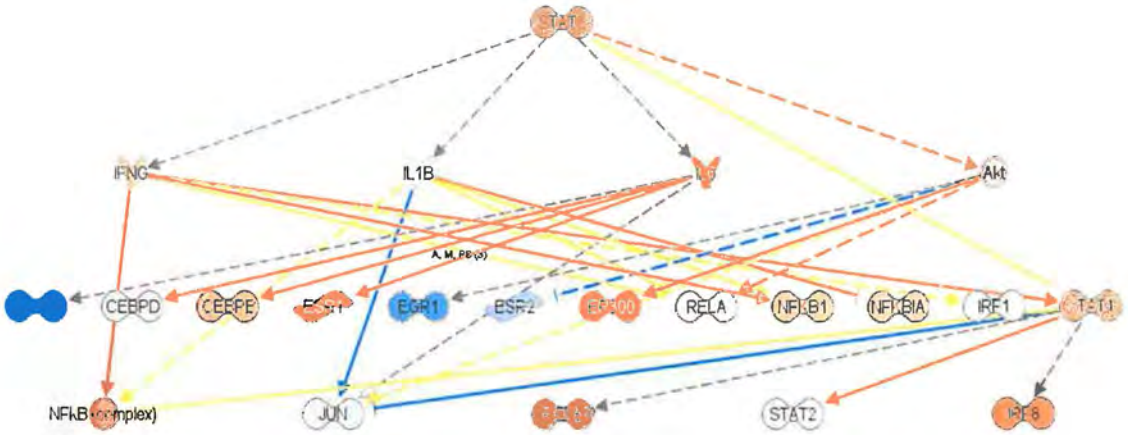
6. **Confirmation of *mtp* deletion by PCR (Ramsugit *et al.*, 2013).**



Construction of the *mtp* deletion mutant by allelic replacement and its confirmation. **(a)** Chromosomal organization of the wild type strain (upper) and *mtp*-mutant strain (lower), in which the *mtp* gene has been disrupted by a hyg^R-*sac*B cassette. **(b, c)** Confirmation of the *mtp*-knockout mutant by PCR. **(d)** Southern blot showing digested genomic DNA of the wild type (Lane 1) and *mtp*-mutant (Lane 2).

7. **Mechanistic networks of upstream regulators that were commonly induced by both strains in A549 pulmonary epithelial cells, relative to uninfected cells.**

STAT3



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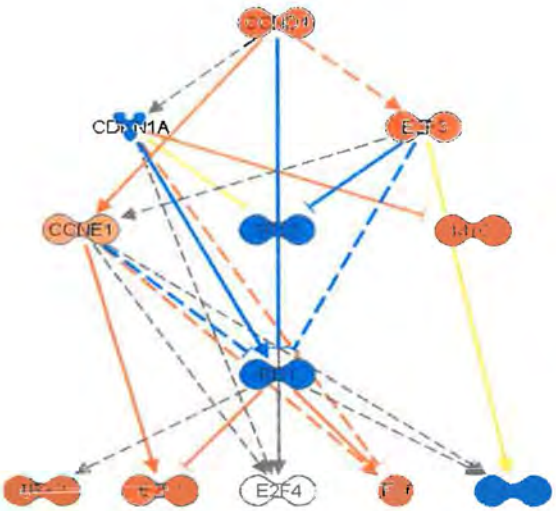

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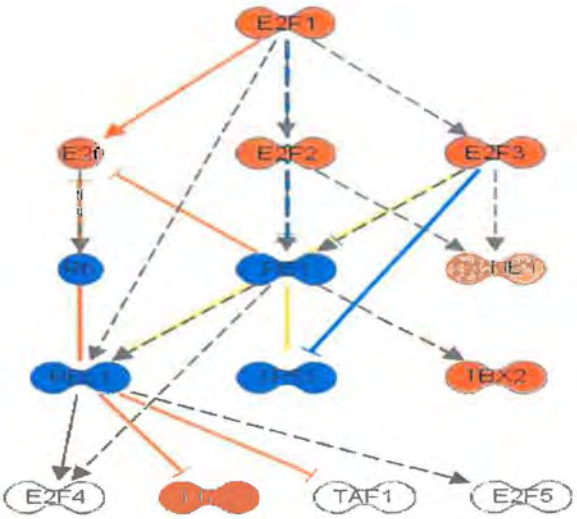

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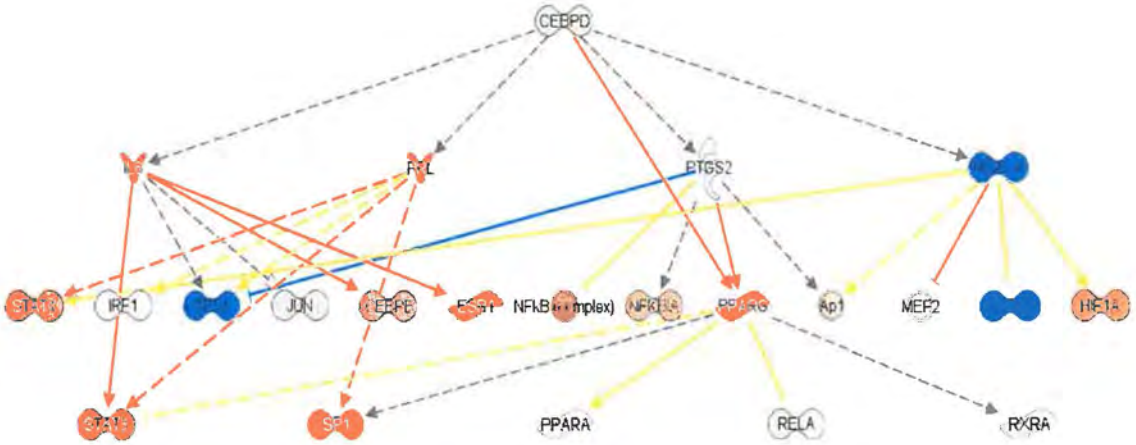
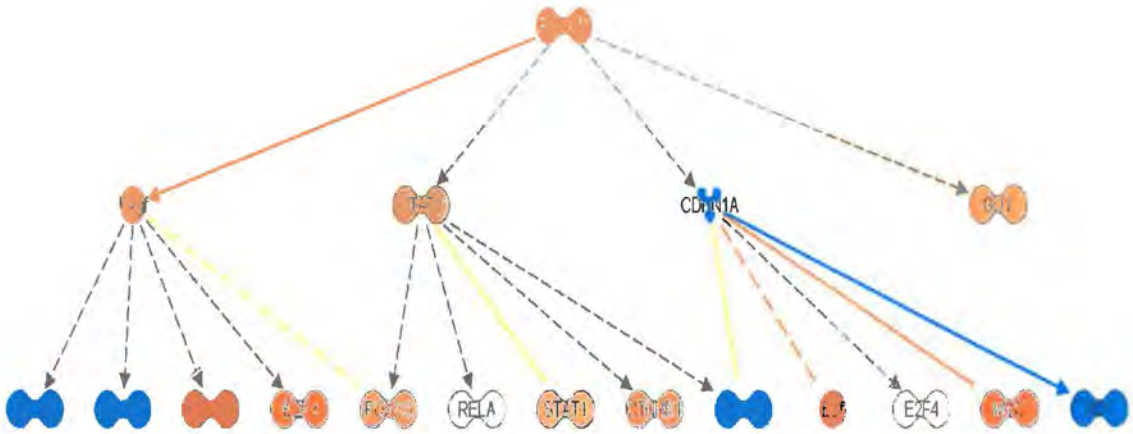
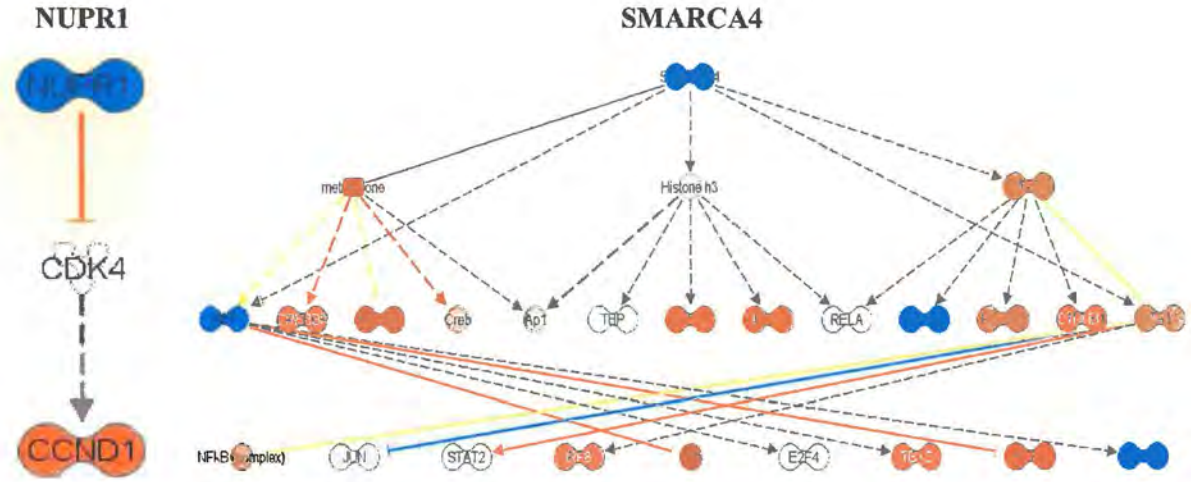
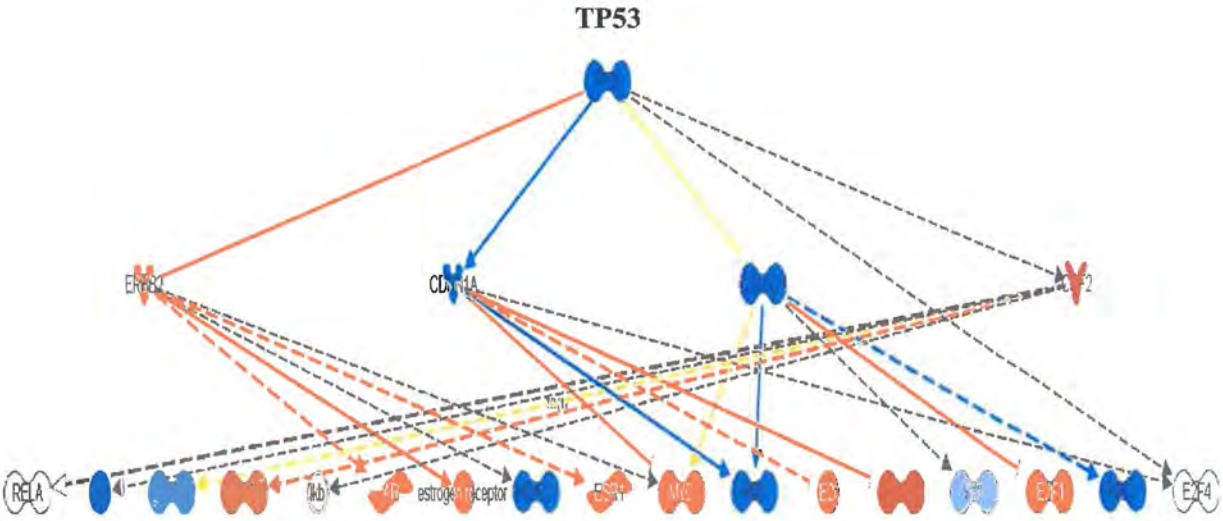


FOXM1

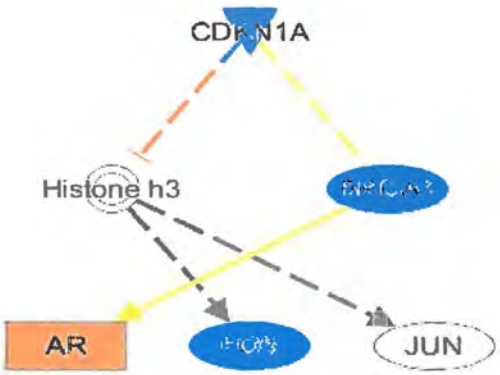




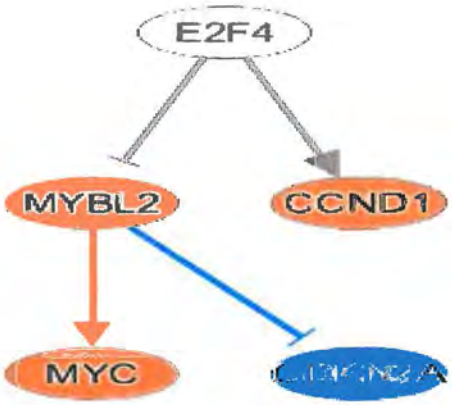


8. Mechanistic networks of upstream regulators that were uniquely induced by the wild type strains in A549 epithelial cells, relative to uninfected cells.

CDKN1A



E2F4



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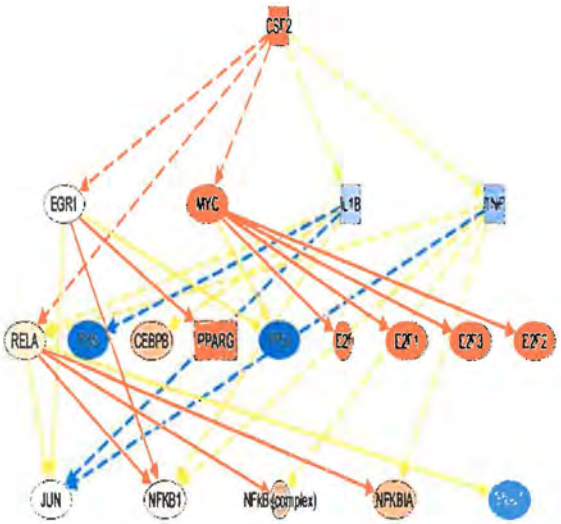
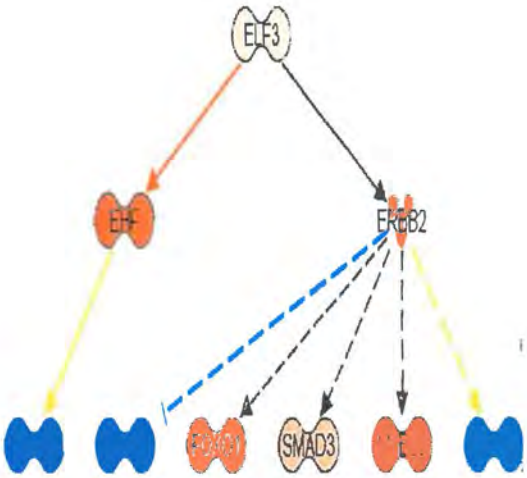
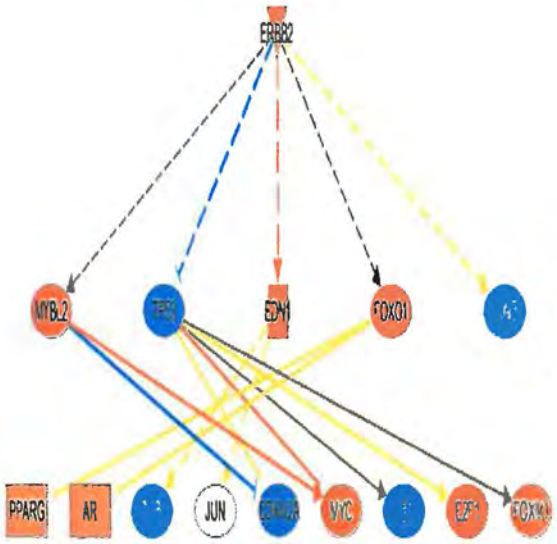


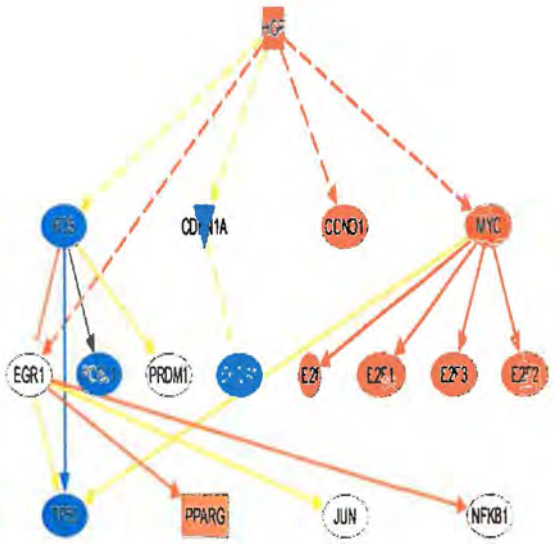
ELF3



ERBB2



HGF



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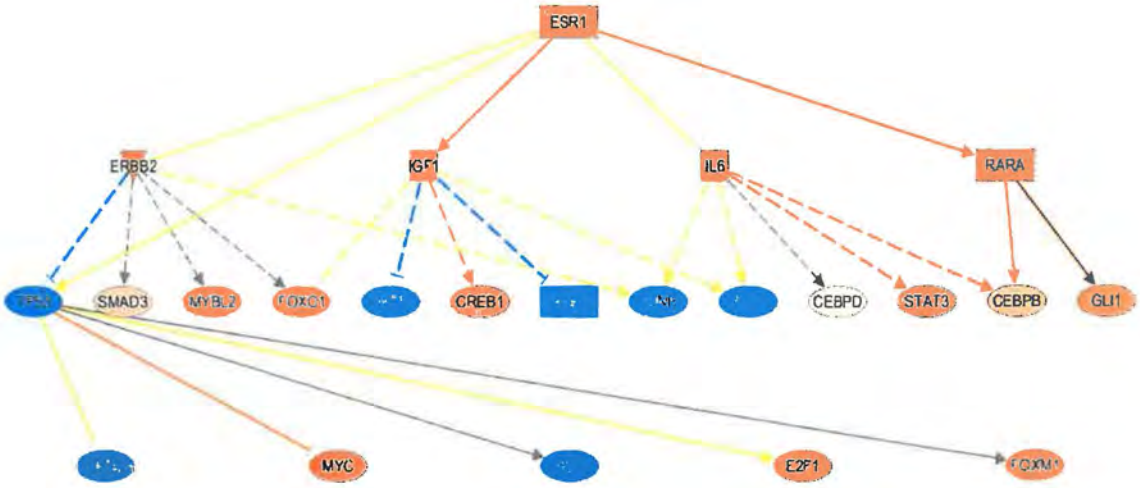

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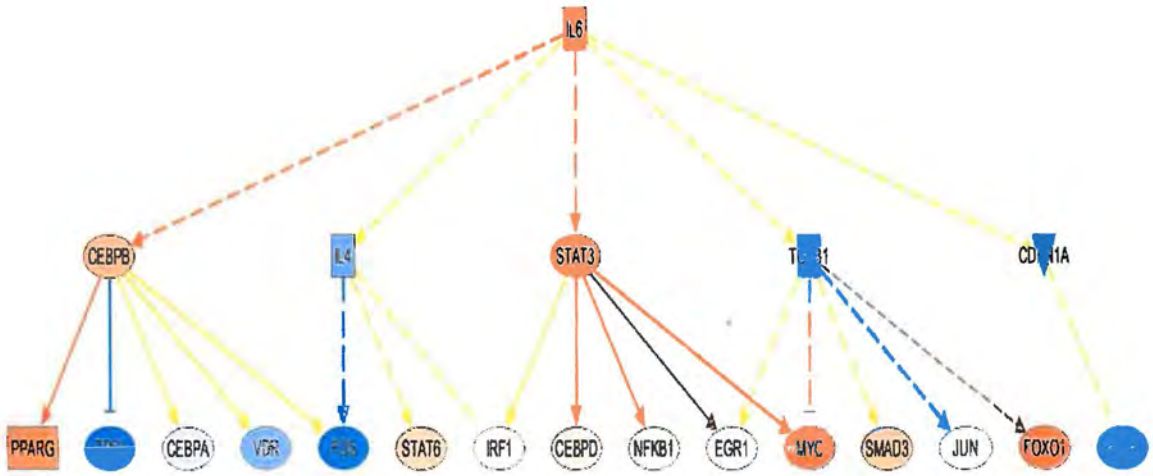

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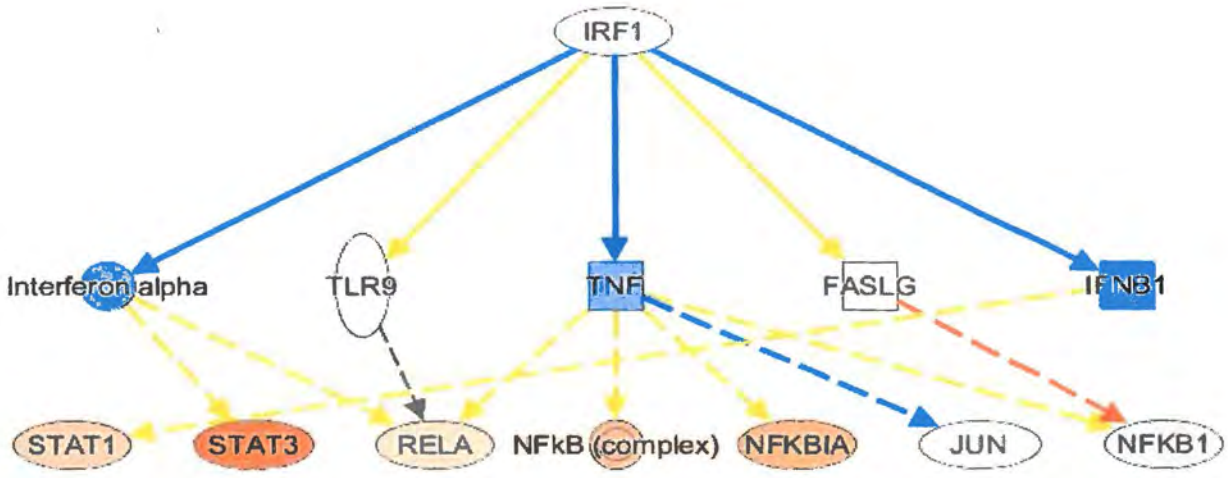

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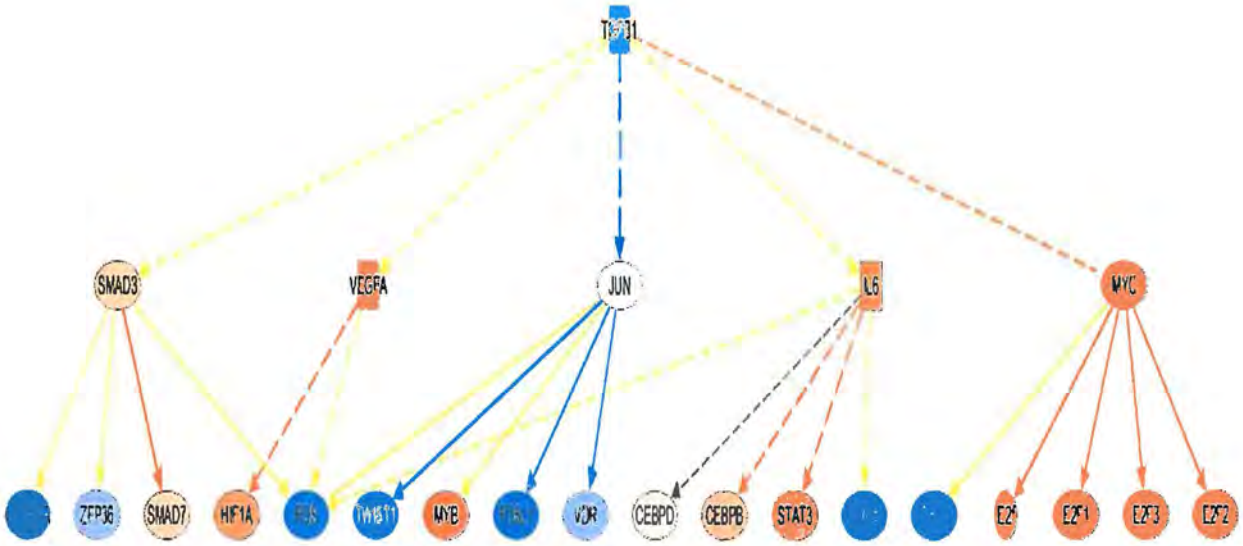
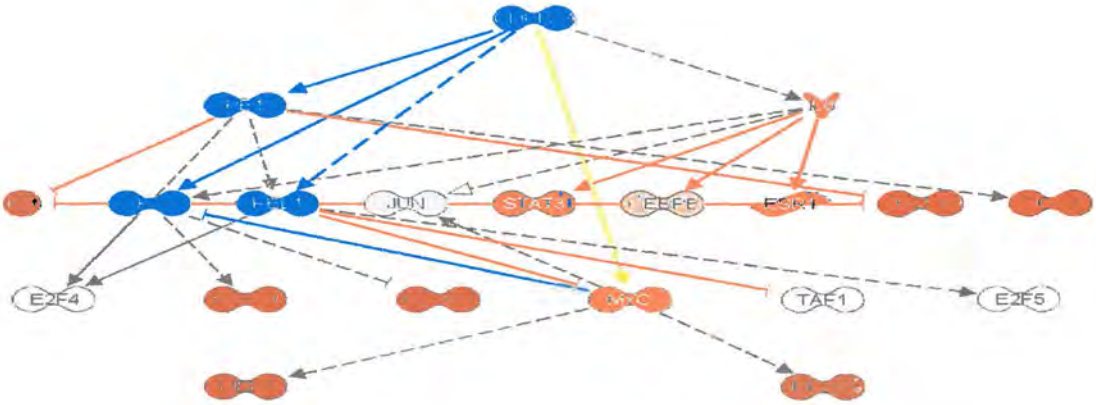


9. **Mechanistic networks of upstream regulators that were uniquely induced by the wild type strains in A549 epithelial cells, relative to uninfected cells.**

CDKN2A



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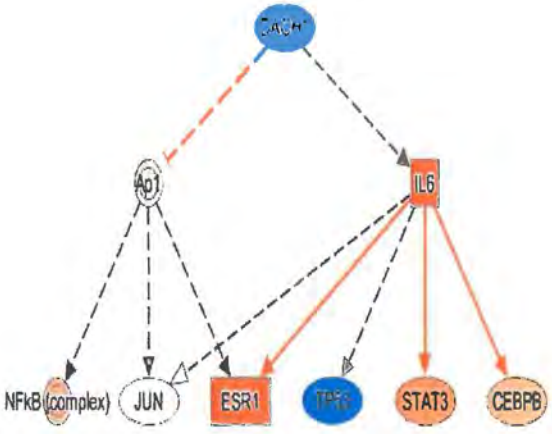
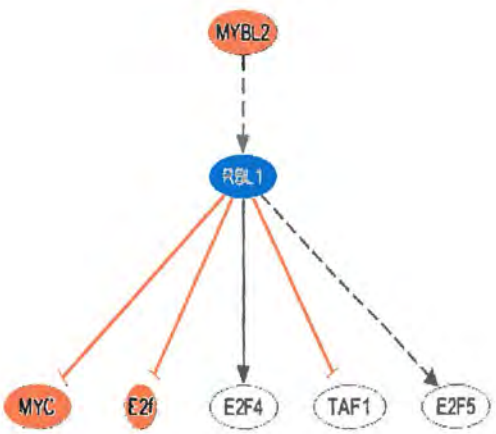


MYBL2



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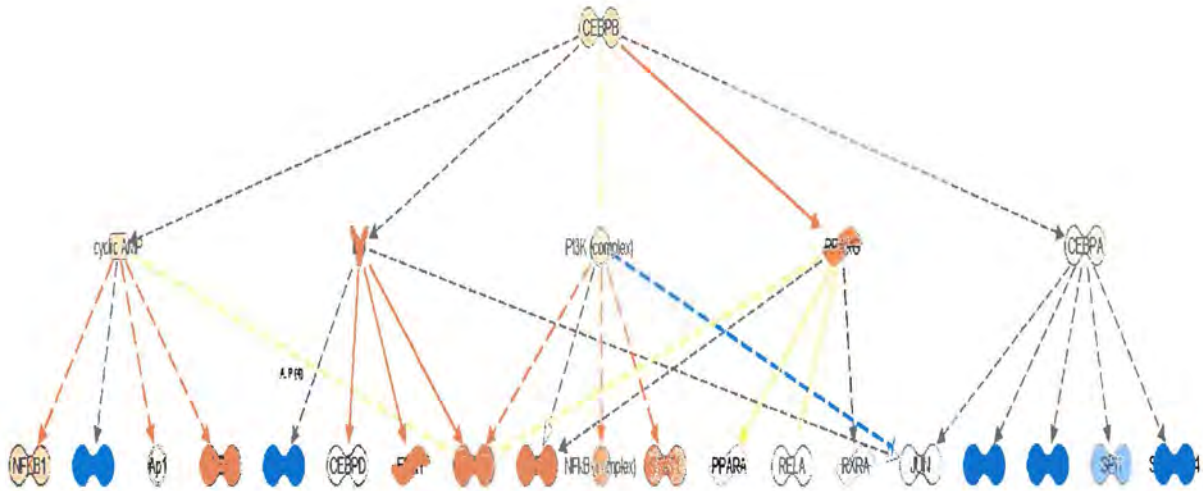

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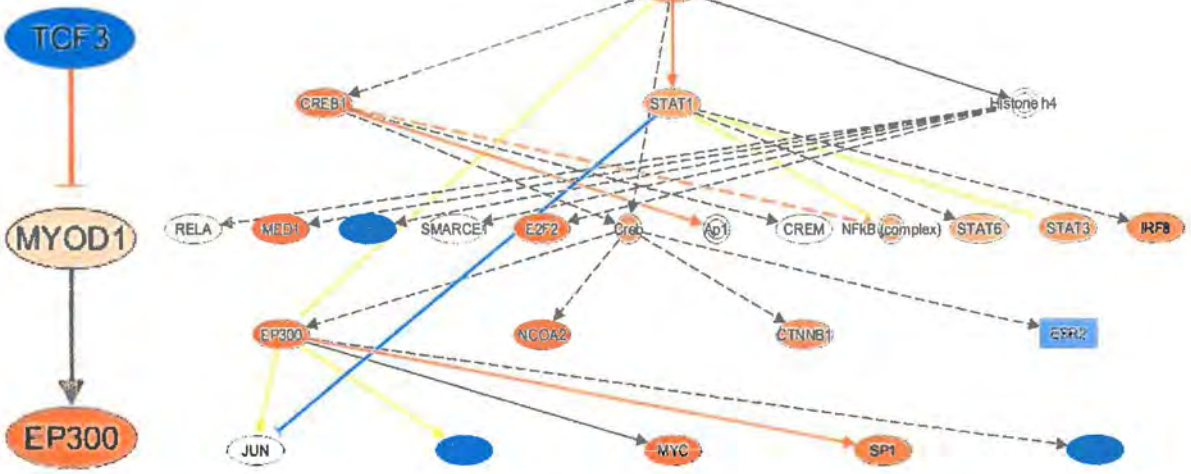

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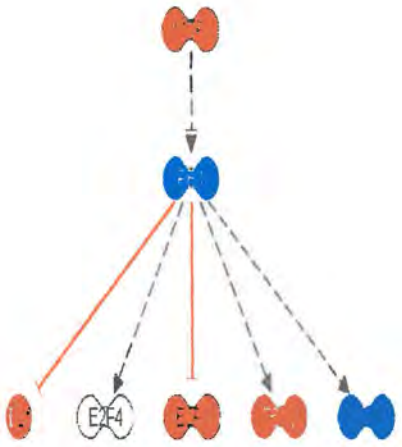

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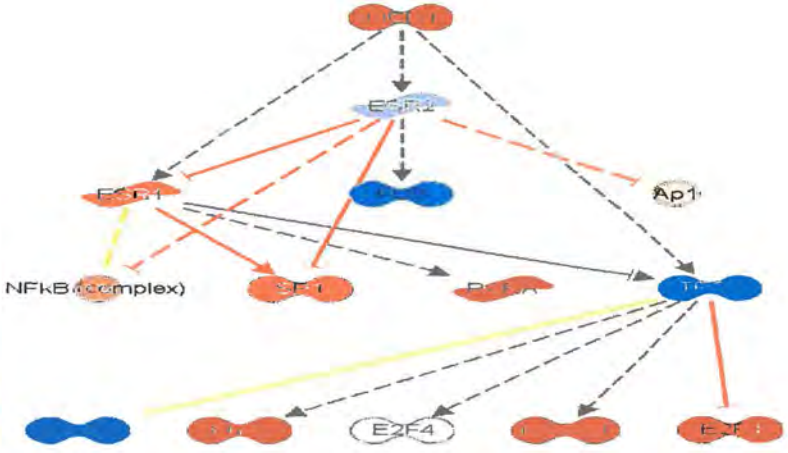

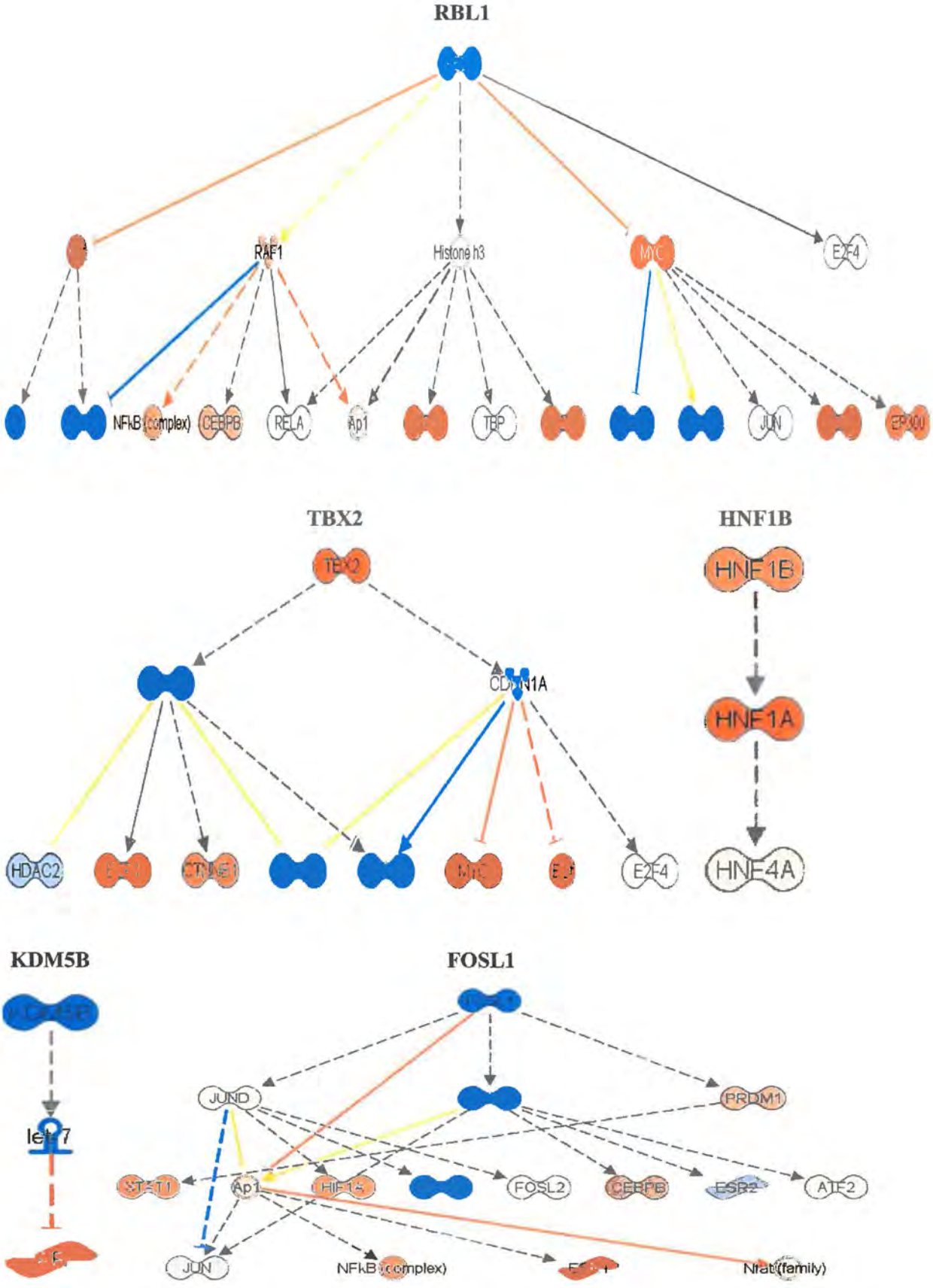
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Histone h3
MYC
E2F4
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CEBPB
RELA
Ap1
TBP
JUN
TBX2
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HNF1B
HNF1A
HNF4A
HDAC2
E2F4
KDM5B
FOSL1
let-7
JUND
PRDM1
STAT1
Ap1
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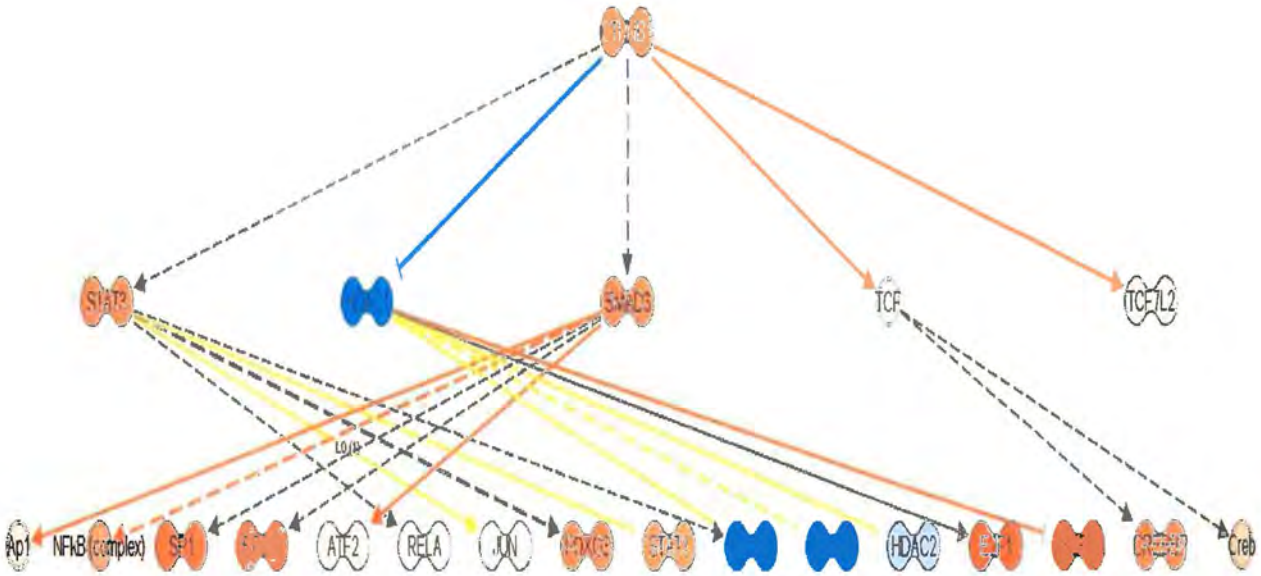
ESR2
ATF2
JUN
NFkB (complex)

CTNNB1



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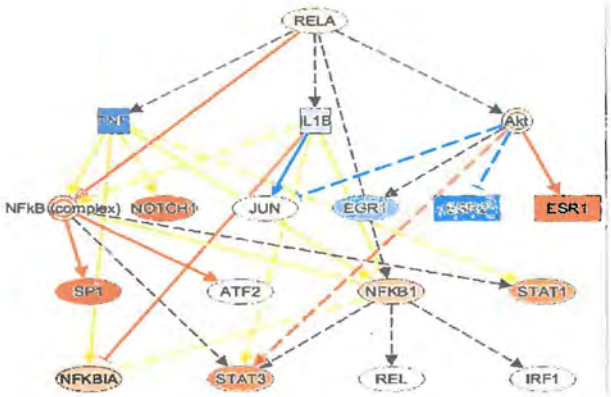

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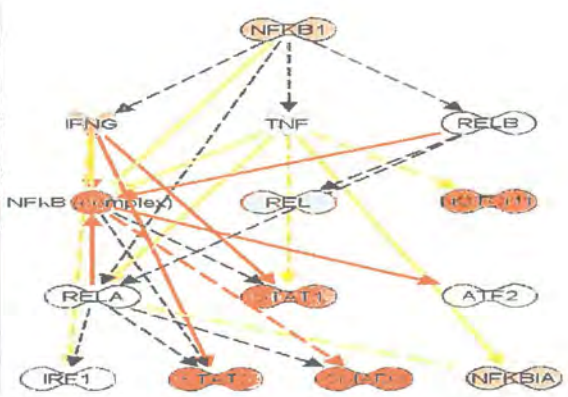

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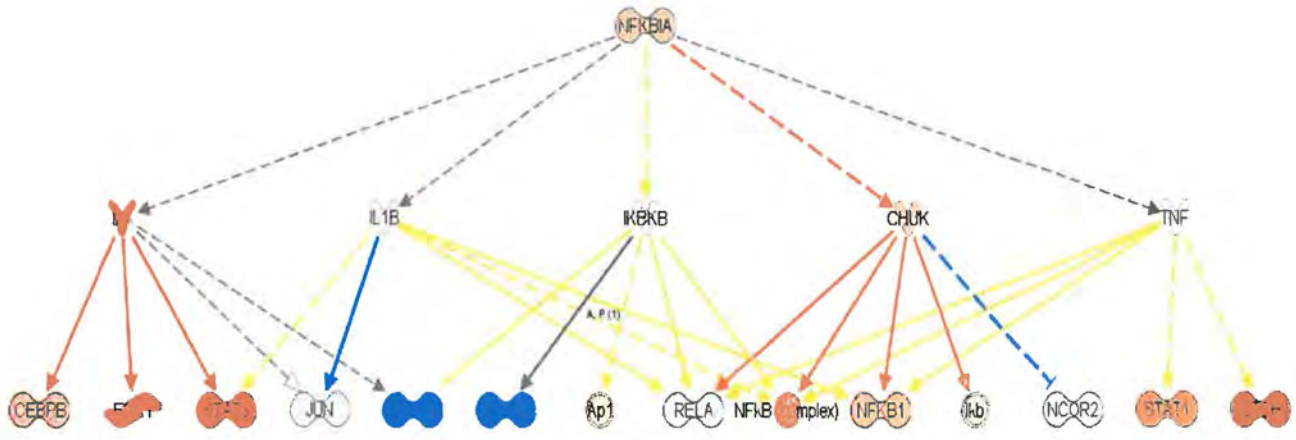

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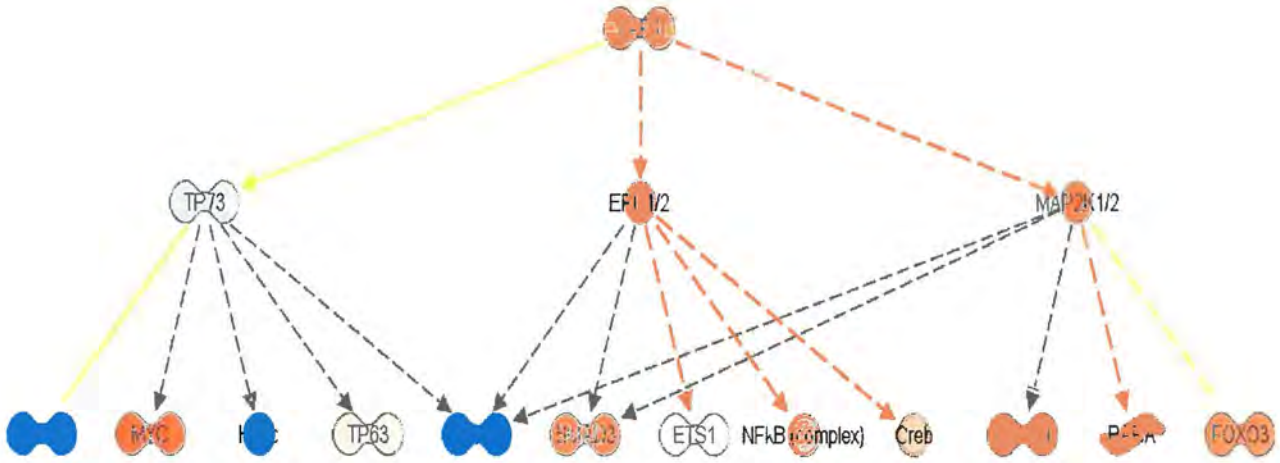
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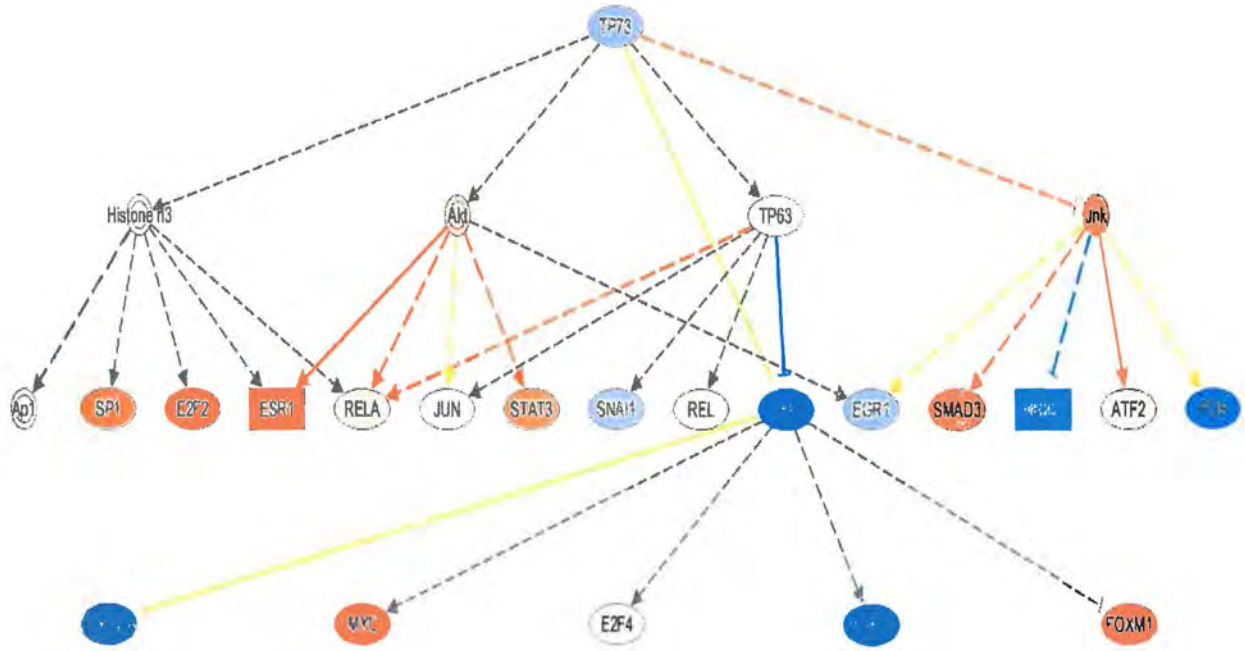
TP63
ETS1
NFkB (complex)
Creb
FOXO3

TP73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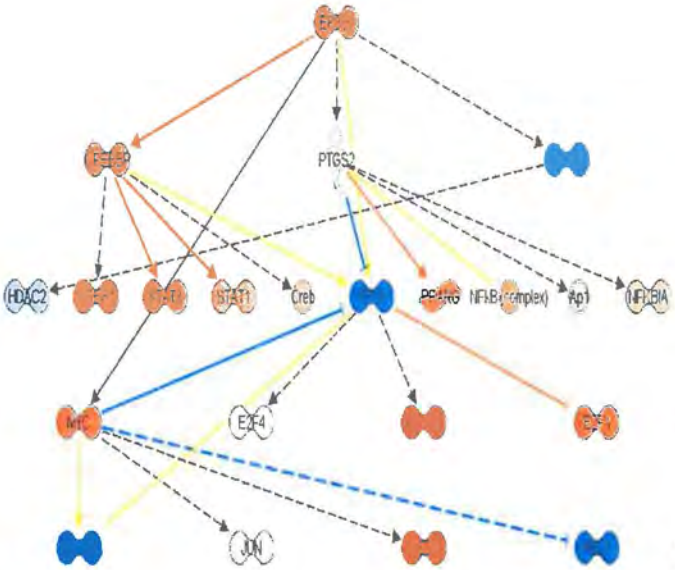
EP300



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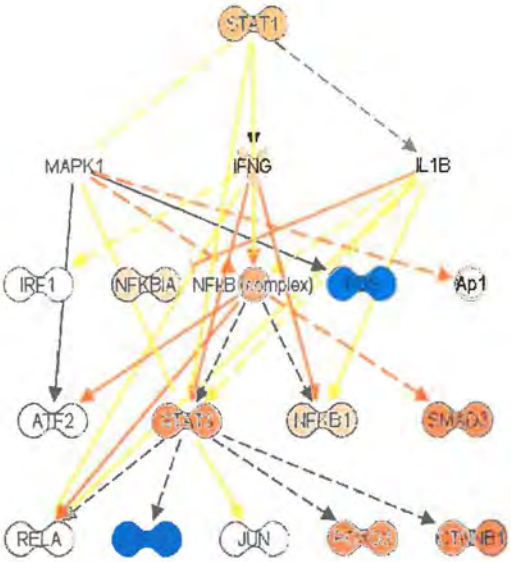

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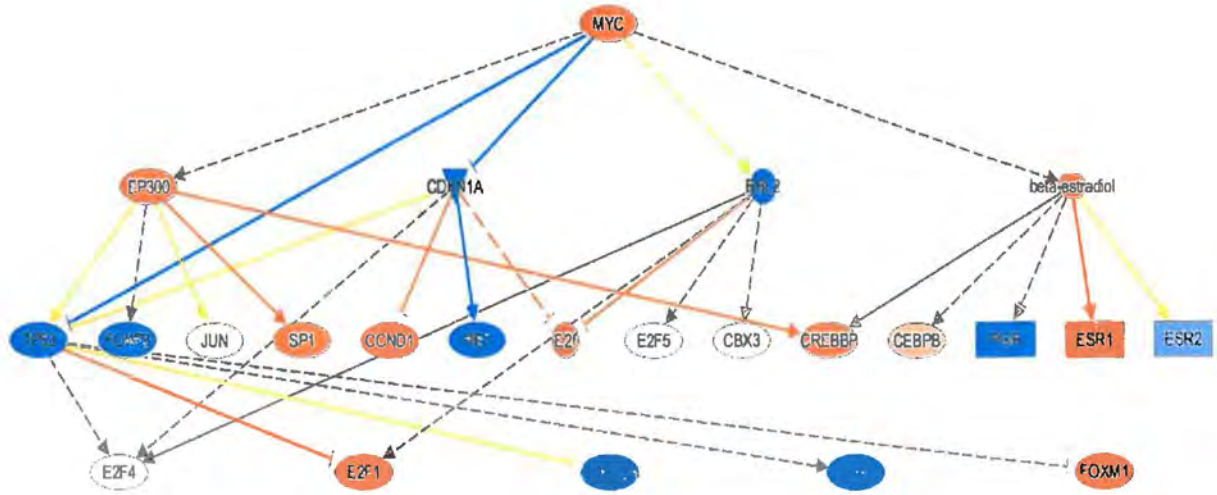

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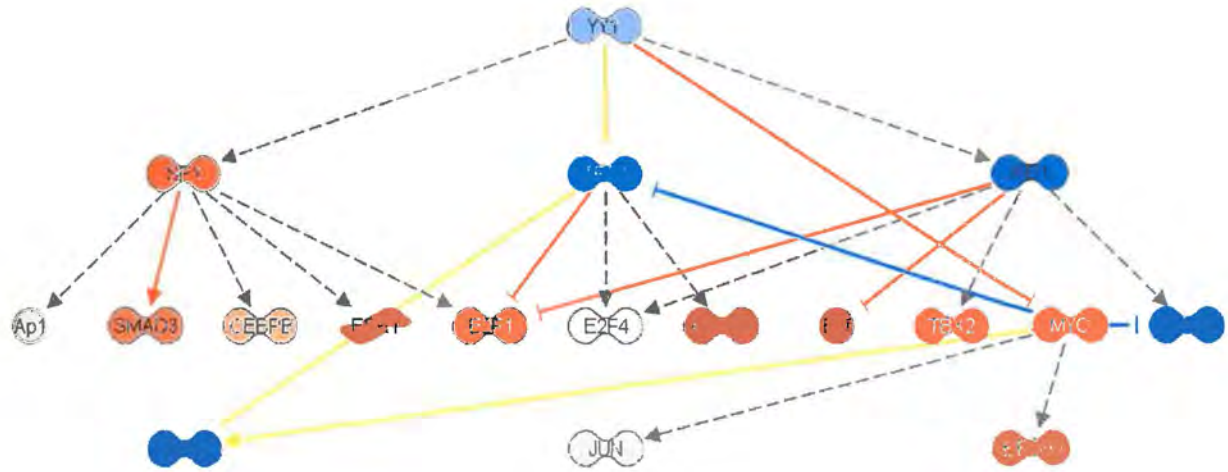
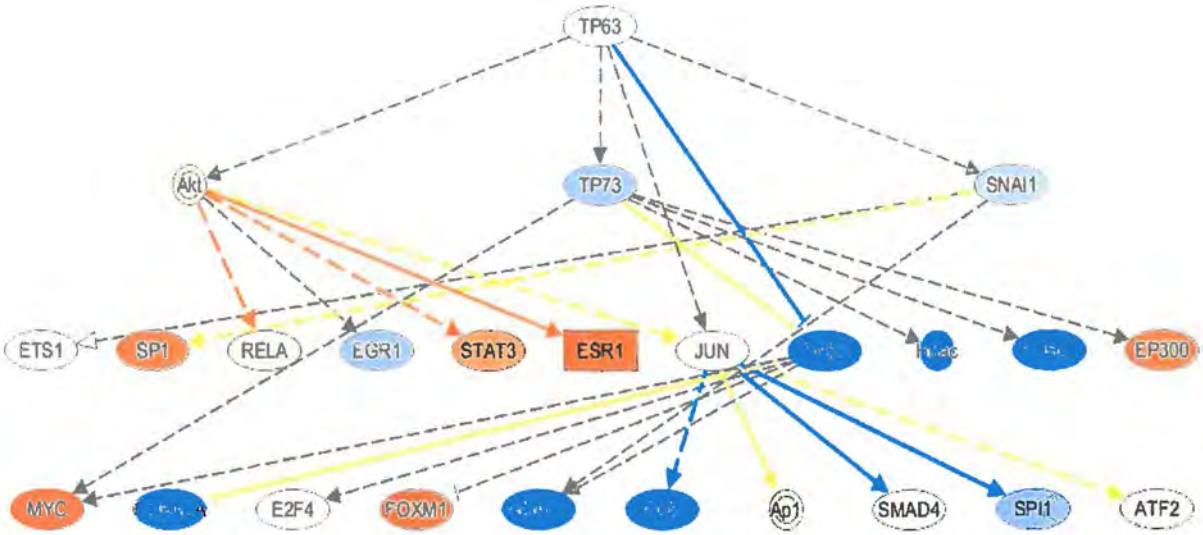


TP63


TP63
Akt
TP73
SNAI1
ETS1
SP1
RELA
EGR1
STAT3
ESR1
JUN
EP300
MYC
E2F4
FOXM1
Ap1
SMAD4
SPI1
ATF2