STUDIES IN PHAGOSOME MATURATION: MODULATION BY BOTH THE HOST PHOSPHATIDYLINOSITOL 3-KINASE p110α AND THE MYCOBACTERIAL PROTEIN PE-PGRS62

by

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Phagosome maturation is a key innate immune response involving interactions of phagosomes with the endosomal system. These interactions result in the creation of a destructive, antimicrobial phagolysosome compartment. How this process is regulated is not entirely known, and intracellular pathogens such as *Mycobacterium tuberculosis* (Mtb) inhibit phagosome maturation as a survival strategy. We examined phagosome maturation from the perspective of two factors, one host- and one pathogen-derived. First, we determined whether the class IA phosphatidylinositol 3-kinase (PI3K), p110α, contributes to maturation regulation. Of the various PI3Ks, only hVps34 is known to regulate phagosome maturation. During studies of phagosome maturation in THP-1 cells deficient in p110α, we discovered that this PI3K isoform controls maturation processes beyond Rab7 acquisition, leading to delivery of lysosomal markers. Phagosomes from p110α knockdown cells were markedly deficient in LAMP-1, LAMP-2 and β-galactosidase, and could not fuse with lysosomes. Despite lacking lysosomal components, p110α deficient phagosomes recruited Rab7 and its effectors RILP and HOPS components Vps16 and Vps41, suggesting that in addition to Rab7, p110α is required for phagolysosome formation.

We also examined how Mtb mediates phagosome maturation arrest by screening an Mtb genomic library for factors able to disrupt yeast vacuolar protein sorting (VPS). Since VPS is homologous to mammalian endosomal trafficking, factors that inhibit VPS might also arrest phagosome maturation. Four proteins able to disrupt yeast VPS were identified in this screen, two hypothetical proteins, Rv0900 and Rv1268c, the P-type ATPase Rv0425c, and PE-PGRS62.
To study its effects on macrophage function, we generated *M. smegmatis* able to express PE-PGRS62. Murine macrophages infected with this construct had arrested phagosome maturation, displaying decreased Rab7 and LAMP-1 recruitment. Infected macrophages also expressed 2-3 fold less iNOS protein when compared to cells infected with control bacteria.

Loss of PE-PGRS62 expression in *M. marinum* resulted in greater iNOS levels, and complementation of the mutant with PE-PGRS62 restored the ability to inhibit iNOS expression. Marked differences in colony morphology were also seen in *M. smegmatis* expressing PE-PGRS62 and in the *M. marinum* PE-PGRS62 transposon mutant. Our results suggest that PE-PGRS62 supports mycobacterial virulence via inhibition of phagosome maturation and iNOS expression.
PREFACE


A version of Chapter 3 has been published. Thi, E.P., Lambertz, U. and Reiner, N. (2012) Class IA Phosphatidylinositol 3-kinase p110α Regulates Phagosome Maturation. *PLoS ONE* 7(8): e43668. I conducted all of the experiments and wrote all of the manuscript. Lambertz, U. contributed to one of the experiments.

A version of Chapter 5 has been submitted as a manuscript. Thi, E.P., Hong, C., Sanghera, G. and

iv
Reiner, N. (2012) Identification of the PE-PGRS62 Protein of *Mycobacterium tuberculosis* as a Novel Effector That Functions to Block Phagosome Maturation and Inhibit iNOS Expression. I conducted two thirds of the experiments and wrote all of the manuscript. Hong, C. assessed iNOS expression upon *M. smegmatis* infection. Sanghera, G. helped with the construction of PE-PGRS62-expressing *M. smegmatis*.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>APPL1</td>
<td>Adaptor protein containing pleckstrin homology domain, PTB phosphotyrosine-binding domain, and leucine zipper/bin-amphiphasin-rvs domain 1</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CORVET</td>
<td>Class C core vacuole-endosome tethering complex</td>
</tr>
<tr>
<td>CR</td>
<td>Complement receptor</td>
</tr>
<tr>
<td>EEA-1</td>
<td>Early endosomal antigen 1</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fragment, crystallizable region gamma receptor</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>HOPS</td>
<td>Homotypic vacuole fusion and proteins sorting complex</td>
</tr>
<tr>
<td>hVps34</td>
<td>Human vacuolar protein sorting 34</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin class G</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine based inhibitory motif</td>
</tr>
<tr>
<td>LAMP-1, -2</td>
<td>Lysosome associated membrane protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>ManLAM</td>
<td>Mannosylated lipoarabinomannan</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose binding lectin</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>Mtb</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3- kinase</td>
</tr>
<tr>
<td>PI-PLC</td>
<td>Phosphoinositol phospholipase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>Rab GTPase</td>
<td>Ras analog in brain guanosine triphosphatase</td>
</tr>
<tr>
<td>Rac GTPase</td>
<td>Member of Rho family of Ras-like GTPases</td>
</tr>
<tr>
<td>Rho GTPase</td>
<td>Family of Ras-like GTPases</td>
</tr>
<tr>
<td>RILP</td>
<td>Rab interacting lysosomal protein</td>
</tr>
<tr>
<td>SHIP1</td>
<td>SH2 domain containing phosphatidylinositol polyphosphate 5-phosphatase</td>
</tr>
<tr>
<td>SLAMF</td>
<td>Signalling lymphocyte-activation molecule family</td>
</tr>
<tr>
<td>SNAREs</td>
<td>Soluble N-ethylmaleimide sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>v-ATPase</td>
<td>Vacuolar ATPase</td>
</tr>
</tbody>
</table>
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For my mother,

Thuy Van Thi

October 20, 1959 - December 6, 2007

Slow Blue Nights
CHAPTER 1: INTRODUCTION

1.1 Phagosome Maturation Models

Macrophages are differentiated cells descended from a myeloid precursor. These cells play an essential role in both innate and adaptive immunity through microbicidal mechanisms and antigen presentation. The ability of macrophages to contribute to immunity stems in large part from their ability to phagocytose prey. Phagocytosis is a cellular process involving the formation and extension of pseudopods that envelop prey greater than 0.5 µm in diameter. Once ingested, prey is encapsulated within a vacuole termed the phagosome. These organelles then undergo a dynamic process of maturation resulting ultimately in fusion with lysosomes, and the creation of a highly destructive, antimicrobial environment that plays a key role in the innate immune response as well as cellular processes such as autophagy, antigen processing and presentation. At its most basic, phagosome maturation is a sequential, fluid process involving interactions of the maturing vacuole with diverse vesicles from the endosomal system.

Two general models detailing how maturing phagosomes interact with the endosomal system and ultimately become phagolysosomes have been proposed (Figure 1.1.1). The first model, which we will call the complete fusion model, posits that phagosomes mature by fusing entirely with whole vesicles of the endosomal system (2). After each fusion event, the phagosome takes on the characteristics of the endosome with which it has fused. This model presents several difficulties, however, in terms of phagosome composition and size. It would be expected that successive fusions with entire endosomes would result in the generation of larger and larger...
Figure 1.1.1 Phagosome maturation models. The complete fusion model posits that the maturing phagosome undergoes sequential, complete fusion events with early and late endosomes, and lysosomes. With each fusion event, the phagosome acquires the characteristics of the fusion partner. Excess phagosomal membrane resulting from these fusion events is recycled throughout the maturation process. The "kiss and run" model of maturation theorizes that the maturing phagosome undergoes transient and localized fusion interactions with the endosomal system, whereby only a limited amount of the content of early and late endosomes and lysosomes are exchanged.

phagosomes unless significant membrane recycling occurs, which would seem to be energetically costly for the macrophage. Whole endosome fusion events would also be expected to result in the rather abrupt and simultaneous phagosomal acquisition of endosomal membrane and luminal contents, a fact that is not borne out by studies examining phagosomal composition during the course
of maturation. These indicate gradual changes in the maturing phagosome's composition over time. In order to account for these observations, the "kiss and run" model of phagosome maturation was proposed (3). This model accounts for the gradual changes in phagosome composition by hypothesizing maturation not as a series of whole fusion events, but rather as a compendium of sequential and transient fission and fusion events between the maturing phagosome with endosomes, in which there is limited exchange of content through a fusion pore (3). These interactions would result in the specific and gradual transfer of components to the phagosome, and are consistent with time lapse microscopic observations which describe transient interactions between phagosomes and endosomes.

Through these exchanges, the phagosomal environment takes on some of the characteristics of the endosomes with which it has interacted. As such, phagosome maturation stages are defined by the acquisition of protein markers used to characterize specific endosome types (4,5). For example, the small GTPase Rab5, the transferrin receptor, and early endosomal antigen 1 (EEA-1) define an early maturation stage, whereas acquisition of Rab7, cathepsin D, and late endosomal/lysosomal membrane proteins such as LAMP-1 and -2 indicate a more advanced, late stage of maturation. The markers most commonly used to denote phagosome maturation stage are illustrated in Figure 1.1.2. Progression through each of these maturation stages is dependent upon the interplay between signalling cascades and regulatory proteins involved in the dynamics of vesicular trafficking and membrane fusion.
1.2 Phosphatidylinositol 3-Kinases Regulate Phagosome Maturation

Starting with the receptors used for phagocytosis, and up until fusion of phagosomes with lysosomes, phosphatidylinositol 3-kinases (PI3Ks) and their lipid products play important roles in regulating many aspects of the maturation process. PI3Ks catalyze the phosphorylation of the 3' position on the inositol ring. Three classes of PI3Ks have been identified and grouped according to enzyme structure, substrate specificities, and products formed (summarized in Table 1.2). The heterodimeric Class I PI3Ks are divided into two subgroups; Class IA enzymes share one of several common regulatory subunits (p85α and β, p50α, and p55α and γ) and different catalytic subunits (p110α, β, and δ). The lone class IB enzyme consists of a p101 or p84 regulatory subunit and contains the p110γ catalytic subunit (6,7). Class I PI3Ks have been shown to catalyze the production of PI(3,4)P₂ and PI(3,4,5)P₃. In contrast, class II PI3Ks lack a regulatory subunit, and contain three isoforms (C2α, C2β, and C2γ). These enzymes catalyze the production of PI3P and PI(3,4)P₂ from PI and PI4P, respectively. The class III enzyme is hVps34, which exists as a heterodimer along with its regulatory subunit, p150/Vps15. This PI3K produces PI3P from phosphatidylinositol.
Figure 1.1.2. Phagosome maturation markers used to indicate maturation stage. Early phagosomes acquire Rab5, EEA-1 and transferrin receptor. In late phagosomes, Rab5 is exchanged for Rab7 and its effector RILP, which serves to couple the maturing phagosome to dynein/dynactin motors which bring it into the vicinity of lysosomes. LAMPs are acquired at this stage, and also upon phagolysosome fusion. The destructive phagolysosomes contain hydrolases, which render the compartment microbicidal. Reprinted with permission from Annual Reviews.
Table 1.2. PI3K isoforms

<table>
<thead>
<tr>
<th>PI3K Class</th>
<th>Subunits</th>
<th>Substrate</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Class I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class IA</td>
<td>Catalytic p110α, p110β, p110δ Regulatory p85α, p85β, p50α, p55α, p55γ</td>
<td>PI4P PI(4,5)P_2</td>
<td>PI(3,4)P_2 PI(3,4,5)P_3</td>
</tr>
<tr>
<td>Class IB</td>
<td>Catalytic p110γ Regulatory p101, p84</td>
<td>PI4P PI(4,5)P_2</td>
<td>PI(3,4)P_2 PI(3,4,5)P_3</td>
</tr>
<tr>
<td><strong>Class II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class II</td>
<td>Catalytic C2α, C2β, C2γ Regulatory None</td>
<td>Phosphatidylinositol PI4P</td>
<td>PI3P PI(3,4)P_2</td>
</tr>
<tr>
<td><strong>Class III</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class III</td>
<td>Catalytic HVps34 Regulatory p150/Vps15</td>
<td>Phosphatidylinositol</td>
<td>PI3P</td>
</tr>
</tbody>
</table>

PI3K activity regulates phagosome maturation downstream of diverse phagocytic receptors and this involves the activation and recruitment of multiple small Rab guanosine triphosphatases (GTPases) which modulate endosomal trafficking. The activities of these small GTPases, part of the Ras GTPase superfamily, play important roles in allowing the maturing phagosome to interact with early and late endosomes, and lysosomes. In addition to Rab activity, Ca^{2+} dependent signalling cascades are also needed to ensure that maturation proceeds towards the formation of a degradative compartment (8-10). Like the Rabs, certain Ca^{2+} dependent signalling events depend on the function of PI3Ks, which serve to link effector proteins activated by Ca^{2+} to the phagosomal membrane through the formation of lipid effectors. The contributions of the specific PI3K classes to phagosome maturation initiated downstream of distinct phagocytic receptors and how these lipid kinases regulate the activities of the Rab GTPases and Ca^{2+} signalling cascades involved in the
maturation process, is described in detail over the next few sections.

1.2.1 Role of PI3Ks in the phagocytosis of prey by receptors

Uptake of prey by a cell involves the engagement of phagocytic receptors located on the plasma membrane. Two broad groups of phagocytic receptors have been characterized, and the roles that PI3Ks play in their function is summarized in Table 1.2.1. Opsonic receptors include the antibody-binding FcγR, which serves to mediate the uptake of prey opsonized with immunoglobulin, and the complement receptors CR1 through CR4, which initiate the uptake of prey coated with C3b, iC3b, and C4b (19). Non-opsonic receptors include C-type lectin receptors (CLRs), scavenger receptors, and other pattern recognition receptors such as TLRs and the microbial sensing SLAM family (18,20,21).
Once ingested, maturation of the newly formed vacuole is influenced by the particular phagocytic receptors that were engaged. An excellent example of how phagosome maturation can differ according to the receptors used for ingestion, was provided by a study that examined maturation of phagosomes containing the human pathogen, *Mycobacterium tuberculosis* (Mtb). Upon uptake by a macrophage in the absence of added opsonins (presumably by CR1-4 and by MR (22)), Mtb blocks maturation at a stage before phagolysosome generation (4). In striking contrast, however, IgG opsonization of virulent Mtb and uptake via FcγR vitiates the ability of Mtb to bring about phagosome maturation arrest (23). These findings indicate: (1) that the ability of Mtb to block phagosome maturation requires that its uptake is independent of FcγR, and of more general importance to cell biology, (2) that the dynamics of phagosome maturation are dependent on the phagocytic receptors involved.

Once phagocytosis has been initiated, PI3Ks are involved in modulating the actin dynamics that are necessary for the membrane remodelling needed for uptake. During both FcγR- and CR3-mediated phagocytosis, Class IA PI3Ks are activated (24-27), and their activity is needed for closure of phagosomes containing particles greater than 3 µm in diameter (28,29). PI(3,4,5)P₃ has been shown to accumulate on the phagocytic cup, and to disappear quickly upon closure (11). The presence of PI(3,4,5)P₃ promotes pseudopod extension and contraction events required for phagosome closure through its recruitment of the unconventional, PH-domain containing protein, myosin X (29-31). In addition, PI(3,4,5)P₃ also mediates the sequential activation and deactivation of Rho GTPases that occur at the phagocytic cup. It does this by promoting deactivation of the Rho-family GTPases Cdc42 and ARF6 which are involved in actin polymerization during early stages of phagocytosis. In parallel to this, PI(3,4,5)P₃ also brings about the activation of late stage Rac2 and ARF1 which function in phagosome closure (32-34). PI(3,4,5)P₃ mediated deactivation of Cdc42 is thought to be necessary to
allow polymerized actin around the nascent phagosome to dissipate, thus allowing phagosome interactions with the endocytic system (28,35). The role that class IA PI3Ks play in the actin dynamics involved in phagocytosis is summarized in Figure 1.2.1.

As is the case for FcyR phagocytosis, both PI(3,4)P$_2$ and PI(3,4,5)P$_3$ appear on the phagocytic cup during integrin (CR) mediated phagocytosis where they function to trigger Ca$^{2+}$ signalling to promote cup closure (36). Ca$^{2+}$ release may result from activation of phosphoinositide-specific phospholipase C (PI-PLC), which hydrolyzes PI(4,5)P$_2$ to initiate an inositol -1,4,5- trisphosphate (InsP$_3$) mediated release of Ca$^{2+}$ from ER stores (37). In addition to phagocytic cup closure, Ca$^{2+}$ triggers downstream events that are needed for phagosome maturation, such as promoting hVps34 recruitment to phagosomes through its interaction with calmodulin (38-41).
Figure 1.2.1. PI3K regulation of actin dynamics during phagocytosis. Levels of PI(4,5)P₂ increase at the site of phagocytosis. Using this substrate, class IA PI3Ks catalyze the production of PI(3,4,5)₃, which mediates the activation of the Rho GTPases Cdc42 and Arf6, resulting in actin polymerization. PI(3,4,5)₃ then deactivates Cdc42 and Arf6 and activates Rac2, Arf1, and Myosin X, which provides the contraction event required for phagosome closure. Upon closure, PI(3,4,5)₃ disappears, and PI3P is found on the nascent phagosome membrane. In the case of CR-mediated phagocytosis, PI(3,4,5)₃ reappears on the phagosome to instigate actin tail formation.
1.2.2 Phosphoinositide products and their contributions to phagosome maturation

**PI3P**

Upon closure of the nascent phagosome, PI3P first appears on the vacuole due to the activity of the class III enzyme, hVps34 (42), which may be recruited by the activation of Ca\(^{2+}\) signalling and its interaction with calmodulin (38-41). However, PI3P can also conceivably be formed via the action of lipid phosphatases on PI(3,4,5)P\(_3\). The 3’-inositol phosphatase PTEN has been implicated in the breakdown of phagosomal PI(3,4,5)P\(_3\), as its overexpression abrogates uptake of IgG opsonized sheep red blood cells (43). However, PTEN does not appear to be recruited to the nascent phagosome, and is thus thought to exert its effects in a global manner (44). It is known that the 5’ phosphoinositide phosphatase SHIP1 is recruited to the nascent phagosome at a time when PI(3,4,5)P\(_3\) levels drop dramatically, and its presence is thought to contribute to the disappearance of PI(3,4,5)P\(_3\) upon phagocytic cup closure (11). The PI(3,4)P\(_2\) formed as the result of SHIP1 activity could then be further degraded by the activity of PI 4- and 5-phosphatases, resulting in PI3P formation on the phagosome membrane, independent of hVps34. PI3P formation from PI(3,4,5)P\(_3\) could also conceivably come from Rab5 interaction with the class IA PI3K p110β, followed by subsequent interactions with PI-4- and 5-phosphatases (45). Although this sequence of events has been postulated for endosomes (46), no experimental data exists to support the hypothesis that Rab5 recruitment of class IA PI3Ks and PI-4- and 5-phosphatases to the phagosome membrane results in the formation of PI3P.

Notwithstanding any potential role for class I PI3Ks in contributing to phagosomal PI3P levels, a recent report studying apoptotic corpse removal in the nematode *Caenorhabditis elegans* implicated the class II PI3K PIKI-1 in forming PI3P seen on nascent phagosomes, with hVps34 responsible for PI3P production at later stages of maturation (47). Whether class II PI3Ks also contribute to phagosomal PI3P production in mammalian cells remains to be determined.
As mentioned previously, Rab5 is a member of the Rab subfamily of small GTPases, which includes over 60 proteins that are involved in the regulation of endosomal and exocytic traffic (48,49). Rabs function as molecular switches, being in an active state when bound to GTP, and inactive when GTP is hydrolyzed to GDP upon activation of their intrinsic GTPase activity by a GTPase activating protein (GAP) (50). This cycling between active and inactive states is essential to Rab function and enables the attachment of active Rab proteins to the phagosomal membrane via C-terminal lipid geranylgeranyl tails (51). Inactive, GDP-bound Rabs are cytosolic and complexed with GDP dissociation inhibitors (GDIs). Although many models have been proposed to explain the mechanism of Rab activation and membrane attachment, a recent study that examined Rab membrane targeting using semi-synthetic fluorescent Rab probes, supports a model in which spontaneous Rab dissociation from GDIs and subsequent replacement of GDP with GTP by guanine nucleotide exchange factors (GEFs) allows membrane attachment (Figure 1.2.2). GTP-bound Rab is unable to be bound by the Rab GDI, thereby favouring membrane attachment and allowing active Rabs to migrate to the phagosome membrane (52). In addition to the importance of Rab GEFs in activating Rab activity, the negatively charged phosphoinositides PI(4,5)P$_2$ and PI(3,4,5)P$_3$ may also contribute to Rab membrane targeting by binding to certain Rabs which contain C-terminal polybasic clusters (53). Following membrane recruitment, subsequent GAP activation of Rab GTPase activity brings about the conversion of GTP to GDP. This results in binding of the GDI to Rab-GDP, dissociation from the phagosomal membrane, and the completion of a cycle. This cycling is essential for Rab function in regulating phagosome maturation.
Figure 1.2.2. Models of Rab GTPase membrane targeting. A) Accepted model of Rab GTPase extraction from membranes. GTP hydrolysis is promoted by a GTPase activating protein (GAP). The resulting GDP-bound Rab is then bound by a Rab GDP dissociation inhibitor (RabGDI). B) Model I: Classical model for Rab membrane attachment. GDI displacement factor (GDF) dissociates Rab from RabGDI, allowing guanine nucleotide exchange factor (GEF) to mediate exchange of GDP for GTP. The resulting Rab-GTP binds to the membrane. Model II: Proposed model of the direct interaction of GEF with RabGDI, resulting in the exchange of GDP for GTP, RabGDI dissociation, and Rab-GTP attachment to the membrane. Model III: Proposed model confirmed by (52). Spontaneous dissociation of Rab from RabGDI allows subsequent interaction with GEF, resulting in Rab-GTP and membrane attachment. Reprinted with permission from Nature Publishing Group, Nature Chemical Biology, Wu Y et al. 2010. 6: 534-540.
Rab5 mediates the fusion of nascent phagosomes with early endosomes. Although Rab5 has been shown to interact with hVps34 (45), phagosomal acquisition of Rab5 can occur independently of PI3K, as wortmannin treatment during the course of FcγR phagosome maturation did not inhibit Rab5 (or late phagosomal Rab7) recruitment (54). This study did note, however, that inhibition of PI3K activity resulted in prolonged retention of Rab5 on phagosomes (54). This is particularly interesting given that several studies have found that the p85α regulatory subunit of class IA PI3Ks displays GAP activity towards Rab5 (in addition to Rab4, Cdc42, and Rac1) (55-57) and p85 has been shown to be present on early to late phagosomes during FcγR and CR3 mediated phagosome maturation (12). It may be that the presence of the class IA PI3K p85 regulatory subunit on the phagosome is necessary for Rab5 dissociation from the membrane. However, as wortmannin acts by irreversibly binding to the ATP-binding pocket of the p110 catalytic subunit (58,59), the inhibitor should not in principle affect the GAP activity of p85, which would be free to exert its effects on Rab5. Thus, if p85 were indeed a GAP for Rab5, wortmannin-treated cells should presumably show normal Rab5 displacement from the phagosome. One explanation for this paradox would be an allosteric effect by wortmannin on p85 GAP activity resulting from p110 catalytic inhibition. Alternatively, it may be that wortmannin, in addition to inhibiting p110 catalytic activity, also affects phagosomal recruitment of the p85 subunit. This would explain the retention of phagosomal Rab5 seen in wortmannin treated cells (54). Further experiments to directly test p85 recruitment in wortmannin- treated cells should help to clarify the biological relevance of p85 GAP activity in the context of phagosome maturation.

Notwithstanding any contribution from class IA PI3Ks, the class III enzyme hVps34 is a well established Rab5 effector (42,45). This is a critical function since PI3P is needed for phagosomal recruitment of EEA-1 which serves as a tethering factor to promote fusion of early endosomes with the phagosome (60-65).
During the course of maturation, Rab5 is subsequently replaced on the phagosomal membrane by Rab7, in a process termed "Rab conversion" (66). The mechanism for this conversion event has recently been elucidated in yeast and in the nematode worm Caenorhabditis elegans. It is initiated by class C core vacuole-endosome transport (CORVET) binding to active Rab5 (67), followed by the recruitment of Mon1/Ccz1 (or SAND-1 in C. elegans) (68). SAND-1/Mon1 then acts as a GEF for Rab7, mediating its membrane binding (68-70) and this is followed by a conversion from CORVET to the homotypic vacuole fusion and protein sorting (HOPS) complex (67). Because the multisubunit CORVET and HOPS complexes share four of the same class C vacuolar protein sorting (Vps) proteins (Vps11, Vps16, Vps18, and Vps33), conversion of CORVET to HOPS only requires exchange of the CORVET Vps3 and Vps8 with the HOPS components Vps39 and Vps41 (67). Vps39 and Vps41 have been shown to bind Rab7, with Vps41 binding specifically to Rab7-GTP (71). By binding to both active Rab7 and soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNAREs), HOPS serves to bridge Rab7 activation with the SNARE complex primed in its proper orientation (see below) (71). In addition to Rab7 activity, HOPS membrane recruitment may also be dependent on phosphoinositides, as purified, active HOPS can bind to PI(4,5)P₂, PI(3,5)P₂, and PI4P on liposomes (72).

The SNAREs that are recruited via HOPS are membrane bound proteins that drive fusion events by interacting in trans on opposite membranes. Late endosome/lysosome membrane fusion requires the assembly of a SNARE complex consisting of three Q-SNAREs on one membrane (containing glutaminyl residues essential for fusion) and one R-SNARE (containing an arginyl residue) on the opposing membrane. HOPS has been shown to proofread the assembled SNAREs to ensure their correct formation into a 3Q + 1R complex (73). In addition to HOPS tethering, PI(4,5)P₂ and PI3P are essential for enhancing SNARE-mediated membrane fusion (74-76). PI3P is specifically required for membrane targeting of the soluble SNARE Vam7p, which contains a phox (PX) homology domain.
that binds to PI3P (77,78). These studies, and others suggest that in addition to active Rab7, PI3P formation is needed for proper targeting of HOPS and SNAREs required for phagolysosome fusion.

\[
\text{PI(3,4,5)P}_3
\]

In terms of FcγR-mediated phagocytosis, aside from the phagocytic cup, no evidence has been found for PI(3,4,5)P₃ on completed phagosomes formed around IgG-coated beads, although class I PI3K are recruited to these phagosomes (12). The failure to detect PI(3,4,5)P₃ on these completed phagosomes may reflect the lack of PI(4,5)P₂ substrate resulting from PLC-mediated degradation (79-81). In addition, recent work from the laboratory of Sergio Grinstein has identified the Rab5 effectors and inositol 5- phosphatases OCRL and Inpp5B as being involved in PI(4,5)P₂ removal from the sealing phagosome (82). Studies done with FcγRIIA-transfected, p85α and p85β double knockout fibroblasts indicate that hVps34 plays a major role in phagosome maturation downstream of FcγR- and CR-mediated phagocytosis by allowing the recruitment of the Rab5 effector, EEA-1 (62,63,83). hVps34 and its adaptor protein Vps15/p150 have also been demonstrated to interact with Rab7, thus promoting subsequent phagolysosome formation (42,84). In this model system, the class I PI3Ks were found to be required for mediating formation and closure of the phagocytic cup and not maturation (42). However, the contributions of PI3K to phagosome maturation, particularly with regards to phagolysosome fusion, likely involve more than just simply mediating the recruitment of Rab5 effectors and Rab7. This inference is based upon several related findings. For instance, wortmannin-treated murine macrophages (RAW264.7) fed IgG-coated latex beads showed an approximate 50% reduction in Rab7 recruitment to phagosomes, but this Rab7 was still in an active state, and was able to recruit the downstream effector RILP (54). Furthermore, wortmannin treatment of phagocytic cells
routinely disrupts phagolysosome fusion by upwards of 90%. It seems unlikely then that a 50% decrease in Rab7 phagosomal recruitment alone could account for this dramatic phenotype of maturation arrest (54). This finding suggests that other PI3K-dependent mechanisms exist which are necessary for phagosome maturation, and that Rab7 recruitment alone is insufficient for phagosomes to mature to completion.

Unlike phagosomes formed after FcγR engagement, CR3 phagosomes display a second wave of PI(3,4,5)P₃ formation after phagosome cup closure. This, along with the presence of PI3P, leads to the formation of actin tails that propel phagosomes through the cell (12). The consequences of this propulsion of CR3 phagosomes are unknown although the increased motility of these vesicles may hinder their interactions with endosomes, thereby causing a delay in phagosome maturation (12). Such a delay may promote the survival of intracellular pathogens such as Mtb which is taken up primarily through CR3 (22,85), and M. leprae which enters macrophages via both CR3 and CR1 (86). However, no experimental studies have been done to date which examine the contribution of class I PI3Ks to phagosome maturation, a situation which is addressed by this dissertation.

1.3 The Human Pathogen *Mycobacterium tuberculosis* Arrests Phagosome Maturation as an Intracellular Survival Strategy²

*M. tuberculosis* (Mtb) is part of the Mtb complex that causes tuberculosis in humans. One third of the world’s population is estimated to be latently infected with Mtb, with eight million cases

² A portion of this section has been submitted for publication
of reactivation disease and three million deaths occurring each year (87). Mtb infects macrophages and has evolved multiple mechanisms through which it avoids macrophage microbicidal activity. Within the macrophage Mtb resides in an early stage phagosomal vacuole, which is known to be arrested between the early endocytic and late phagosome stages (4,5,88,89). Mycobacteria-containing phagosomes display a higher pH (6.2-6.3) compared to normal endosomes (pH 5.3-5.4) due to exclusion of the vacuolar proton ATPase (v-ATPase) from the phagosomal membrane (90). Phagosomes containing virulent mycobacteria display early endosomal markers such as transferrin receptors and the glycosphingolipid GM1 ganglioside (91), but not late endosomal or lysosomal markers such as mannose-6-phosphate receptors, cathepsin D, and the proton ATPase (4,89,90). Mtb phagosomes also fail to recruit EEA-1, which is required for fusion of the maturing phagosome with late endosomes. Although Rab5 is present on mycobacterial phagosomes, Rab7 recruitment may be impaired (92-95), suggesting that Rab exchange, in which Rab5 is replaced with Rab7 (66,96) is arrested. However, other studies have suggested that Mtb phagosomes do indeed acquire Rab7, but that this Rab7 is inactive (97,98). The reasons for this confusion as to the acquisition of Rab7 by Mtb phagosomes are unclear, but may be due to the use of different model systems, particularly with regard to Rab7 overexpression. The Mtb mediated phagosome maturation block is also associated with perturbation of intracellular Ca$^{2+}$ levels and reduced phagosomal acquisition of the hVps34 product, PI3P (99). Both Rab22a and Rab14, which function in early endosomal and recycling pathways, accumulate on Mtb phagosomes and this is thought to contribute to maturation arrest (92,100). The ability of Mtb to block phagosome maturation and phagolysosome fusion allows this pathogen to reside unmolested in a protected vacuole (4,89).

The mechanisms responsible for maturation arrest of the mycobacterial phagosome are not fully defined; however, several candidate Mtb lipid and protein effectors have been identified. Live
Mtb blocks a rise in intracellular Ca\(^{2+}\) which is thought to be necessary for maturation by inhibiting sphingosine kinase activity (39,41). The Mtb lipoglycan ManLAM has been implicated in preventing increases in intracellular Ca\(^{2+}\), which are thought to promote phagosome maturation by allowing recruitment of hVps34 and the production of PI3P (38-41). The resulting PI3P formed may then mediate the phagosomal recruitment of Syntaxin 6, a SNARE which is needed for TGN to endosome trafficking (101,102). Recruitment of Syntaxin 6 to mycobacterial phagosomes would thus allow delivery of lysosomal components from the TGN to the phagosome (38). Although ManLAM has been hypothesized to be responsible for the Mtb mediated block in Ca\(^{2+}\) signalling, dead Mtb cannot prevent the increase in Ca\(^{2+}\) that is required for maturation, and ManLAM would presumably still be present on the surface of dead mycobacteria. Thus, other Mtb factors, in addition to ManLAM, likely play a role in preventing Ca\(^{2+}\) signalling. The Mtb phosphatidylinositol mannoside (PIM) has also been proposed to be involved in maturation arrest by promoting phagosomal interactions with early endosomes bearing transferrin receptors and iron (103). In addition, mycobacterial phenolic glycolipid, which is found in the hypervirulent W-Beijing family of Mtb strains that contain an intact polyketide synthase gene (104-106), has also been implicated in phagosome maturation arrest, potentially through inhibition of TLR signalling which would otherwise promote maturation (See Table 1.2.1) (15-17,20,107). In terms of protein effectors, the mycobacterial serine/threonine kinase PknG has been suggested to play a role in inhibiting phagosome-lysosome fusion (108). In addition, the secreted Mtb SapM phosphatase targets PI3P (109). However, phagosome maturation arrest by Mtb is likely to involve more than just simply modulation of PI3P levels in mycobacteria-containing phagosomes (110). Recently, the mycobacterial protein tyrosine phosphatase PtpA has been implicated in preventing phagosome maturation and acidification by dephosphorylating Vps33B, a component of the class C Vps complex, and by excluding assembly of the v-ATPase complex on
mycobacterial phagosomes (111,112). In addition, mycobacterial nucleoside diphosphate kinase has been shown to exhibit GAP activity towards Rab5 and Rab7, thereby contributing to maturation arrest (98). Taken together, accumulating evidence points to a role for both lipid and protein mycobacterial effectors in bringing about the phagosome maturation block. The known effectors that appear to contribute to Mtb mediated phagosome maturation arrest are listed in Table 1.3.

Table 1.3. Known Mtb effectors for phagosome maturation arrest.

<table>
<thead>
<tr>
<th>Effector</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
<td></td>
</tr>
<tr>
<td>Trehalose dimycolate (TDM)</td>
<td>Arrests maturation at early phagosome stage, may involve interaction with receptors or intercalation into phosphatidylinositol-containing membranes (113,114)</td>
</tr>
<tr>
<td>Mannosylated lipoarabinomannan (ManLAM)</td>
<td>Arrests lysosomal delivery by preventing increases in intracellular Ca^{2+}, leading to null recruitment of hVps34 and no PI3P (38-41)</td>
</tr>
<tr>
<td>Phosphatidylinositol mannosides (PIMs)</td>
<td>Promotes interactions with early endosomes (103)</td>
</tr>
<tr>
<td>Phenolic glycolipid</td>
<td>Unknown, but may involve inhibition of TLR signalling (107)</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
</tr>
<tr>
<td>Protein kinase G (PknG)</td>
<td>Serine/threonine kinase, mechanism of preventing maturation unknown (108) but kinase is involved in glutamate metabolism (115)and may interact with PKCα (116)</td>
</tr>
<tr>
<td>SapM</td>
<td>Phosphatase that targets PI3P (109)</td>
</tr>
<tr>
<td>Protein Tyrosine Phosphatase A (PtpA)</td>
<td>Dephosphorylates Vps33B, a component of the class C Vps complex, and excludes assembly of v-ATPase on mycobacterial phagosomes (111,112)</td>
</tr>
<tr>
<td>Nucleoside Disphosphate Kinase (Ndk)</td>
<td>GAP activity towards Rab5 and Rab7 (98)</td>
</tr>
</tbody>
</table>
1.4 Evasion and Detoxification of the Macrophage Nitric Oxide Response is Key to *M. tuberculosis* Survival

In addition to phagosome maturation, nitric oxide (NO) production is a crucial macrophage microbicidal effector mechanism that plays a role in the elimination of intracellular pathogens. NO is produced through the activity of inducible nitric oxide synthase (iNOS or NOS2) which catalyzes the oxidation of L-arginine to produce NO (illustrated in Figure 1.4). The expression of iNOS and production of reactive nitrogen intermediates (RNIs) is crucial to host immunity against *M. tuberculosis*, as NOS2\(^{-/-}\) deficient mice display enhanced susceptibility to infection, and treatment of wild-type mice with the NOS2 inhibitors \(N^6\)-(1-iminoethyl)-L-lysine (L-NIL), aminoguanidine (AG), and \(N^G\)-monomethyl-L-arginine (NMMA) increase bacillary burden and mortality (118,119). The need for NO-mediated host protection is also linked to the inability of IFN-\(\gamma\)-knockout mice to control *M. tuberculosis* infection (120). Indeed, the formation of RNIs has been identified as a principal factor in IFN-\(\gamma\)-mediated killing of *M. tuberculosis* (121).

*M. tuberculosis* may resist NO-mediated killing by several mechanisms. Macrophage iNOS competes for its substrate, L-arginine, with Arginase 1 which promotes the production of urea. A recent study indicated that *M. tuberculosis* may upregulate arginase expression via autocrine cytokine signaling in infected macrophages, in order to reciprocally limit NO production (122). In addition, the pathogen may also block iNOS recruitment to phagosomes by targeting the actin scaffolding protein EBP50, which links iNOS to the actin cytoskeleton surrounding the phagosome (123,124). However, it is unclear whether prevention of iNOS localization in the vicinity of the *M. tuberculosis* phagosome would be sufficient for the bacterium to escape the effects of NO production. Although excluded from phagosomes, iNOS might still be active elsewhere in the infected cell and readily diffusible NO

\[^3\] Some of the material in this section has been published in ref. (117)
would likely access the *M. tuberculosis* vacuole. This has been shown for *Pseudomonas aeruginosa*-infected alveolar macrophages, where iNOS is not recruited to *P. aeruginosa* phagosomes, yet NO-dependent killing is still observed (126). Clearly, *M. tuberculosis* must use other means to avoid the microbicidal effects of iNOS expression and NO production.

**Figure 1.4. The synthesis of nitric oxide by nitric oxide synthase.** The catalytic domains of iNOS are shown above. iNOS uses L-arginine, NADPH, and oxygen as substrates to mediate the synthesis of L-citrulline and NO. FMN, FAD, tetrahydrobiopterin, and calmodulin serve as cofactors. Calmodulin is tightly associated with iNOS, as a result, the activity of the enzyme does not depend on elevated calcium levels (125). Modified and reprinted with permission from Elsevier Publishing, *Digestive and Liver Disease*. Cirino, G. 2003. 35(S2): S2-S8.

To protect itself from RNIs, the catalase-peroxidases of Mtb can also act as peroxynitritases that deactivate peroxynitrite formed when NO [produced by inducible nitric oxide synthase (iNOS)] reacts with superoxide (127,128). In addition, the truncated hemoglobins of Mtb (129), *M. bovis* (130), and *M. leprae* (131) may aid in the detoxification of NO via its conversion to nitrate by oxygenated heme in a manner similar to that of *E. coli* flavohemoglobin (132).
1.5 Mycobacterial PE/PPE Proteins are Linked to Virulence

One protein family that has close associations with virulence in mycobacteria is the PE/PPE multigene family. PE proteins are characterized by highly conserved N-terminal domains containing a proline and glutamate residue at positions 8 and 9 (PPE proteins contain an additional proline residue at position 9 followed by a glutamate at position 10). The PE/PPE protein family is further subdivided into those proteins containing an additional C-terminal domain. This can be either unique or fall within two groups, those possessing either a PGRS domain (Polymorphic GC-rich Repetitive Sequence, for PE proteins), or a MPTR domain (Major Polymorphic Tandem Repeat, for PPE proteins). These subgroupings of the PE/PPE protein family are illustrated in Figure 1.5. PE-PGRS and PPE-MPTR proteins have only been found in pathogenic mycobacteria. Genetic expansion of the PE/PPE protein family has been linked to that of the ESX gene clusters which encode the Type VII secretion system, an important mycobacterial virulence determinant (133-138). This close linkage of ESX transport with PE/PPE family proteins was demonstrated in a study that found that the ESX-5 transport system was necessary for the secretion of PPE41, a *M. marinum* protein into macrophages upon infection (139). In addition, PPE and PE-PGRS proteins have been shown to be secreted through the ESX-5 secretion system in *M. marinum* (140).

Although the PE/PPE protein family is thought to be involved in virulence, definitive functions have not been assigned (141-143). The expression of several PE/PPE proteins is upregulated during macrophage infection and in lung granulomas (144-148). Accumulating evidence obtained from proteolytic digestion of surface proteins, high throughput mass spectrometric analysis of culture filtrate proteins, and specific protein functional studies point to the localization of these proteins on the mycobacterial cell wall (139,140,149-156). Many of these proteins may be passively or actively secreted through Type VII secretion mechanisms (ESX-1 or ESX-5-mediated), or through the general
Sec transportation machinery, as some PE/PPE proteins have been shown to possess functional N-terminal signal peptide cleavage sites (153). Thus, PE/PPE proteins are able to gain access to, and possibly interact with, host cellular components. Indeed, several proteins, such as PE-PGRS62, PPE41, and PPE46 (amongst a host of other PE/PPE proteins) have been shown to elicit T-cell responses (157-160), indicating that during the course of infection these proteins are visible to the host immune response. In terms of aiding mycobacterial virulence, the *M. marinum* homologs for PE-PGRS62 and PE-PGRS30 contribute to bacterial survival in macrophages and in frog granulomas (148). In addition, PE-PGRS33 has been shown to upregulate TNF-α release in infected macrophages through TLR-2 activation, leading to host cell apoptosis or necrosis (161-163).

**Figure 1.5. PE/PPE protein family organization.** PE and PPE proteins each contain a conserved PE or PPE domain respectively located at their N-termini. PE and PPE proteins are further subdivided based upon their C-terminal regions. PE/PPE proteins may consist of only their respective PE or PPE N-terminal domains, or they may contain unique C-termini. The PPE-SVP proteins contain a characteristic SVP motif located at position 350 in their C-termini. PE-PGRS and the PPE-MPTR proteins contain a glycine-rich C-terminal domain, which can be highly variable among Mtb complex members. Figure taken from Sampson, S. *Clinical and Developmental Immunology*, Article ID 497203, 2011. doi:10.1155/2011/497203. Reprinted with permission from Hindawi Publishing Corporation.
PE-PGRS proteins have been suggested to play a role in preventing phagosome acidification, as several of these genes (PE-PGRS5, PE-PGRS28, PE-PGRS44, PE-PGRS59) were identified in a *M. bovis* BCG transposon mutant library screen for mutants enriched in acidified phagosomes (164). In terms of functions other than being potentially involved in phagosome maturation arrest, expression of PE-PGRS11 may promote resistance against oxidative stress (165). PE-PGRS17 and PE-PGRS33 may also inhibit antigen processing, in a manner similar to the Epstein Barr Virus EBVNA protein (166,167). However, given that microarray data of PE-PGRS gene expression has shown that individual proteins of this family are variably regulated by different growth conditions and do not appear to be expressed as a group (168), it is likely that specific PE-PGRS proteins may have discrete functions and contribute to mycobacterial virulence in different ways.

### 1.6 Thesis Rationale and Objectives

Phagosome maturation plays a significant role in innate immunity; however, the factors which serve to regulate this process are still unclear. In addition, inhibition of phagosome maturation is a survival strategy used by pathogens such as Mtb, but the mechanisms involved have not been fully elucidated, and are likely to be multifold. The overall goal of this thesis was to examine the phagosome maturation process from two different yet complementary perspectives, namely the influences of both host factors that drive this process forward and pathogen effectors that may act to impede it. The **FIRST** objective was to examine the contribution of the class IA PI3K p110α to phagosome maturation, and by so doing evaluate whether PI3Ks other than the class III enzyme hVps34, play a role in regulating this process. The **SECOND** objective of this thesis was to identify novel candidate mycobacterial virulence factors through a genomic screen in yeast designed to
identify factors able to disrupt yeast vacuolar protein sorting. The THIRD, and final objective was to select one of the candidate virulence factors identified from the yeast screen and evaluate its effects on phagosome maturation in macrophages. In addition to phagosome maturation, other aspects of macrophage function were also examined, such as cytokine production and iNOS expression in infected cells.

1.6.1 Objective 1: To examine the contribution of the Class IA PI3K, p110α, to phagosome maturation

Rationale

As discussed (section 1.3), while PI3Ks are known to play a major role in regulating phagosome maturation, only the contribution of the class III enzyme, hVps34, has been extensively characterized. Notably, an accumulating body of recent evidence suggests that other classes of PI3Ks are likely to be involved as well. For example, class IA PI3Ks have been shown to be involved in actin polymerization around maturing CR3-generated phagosomes (12), and Akt (Protein kinase B, which is recruited to membranes by the class I PI3K product PI(3,4,5)P₃) is found on phagosomes and endosomes (82,169). Given these indications that class IA PI3Ks may play a role in regulating phagosome maturation, we evaluated whether the class IA PI3K, p110α, affected maturation. We did this by silencing p110α expression in the human monocytic cell line THP-1 using lentivirally introduced short hairpin RNA (shRNA) sequences, and assessed the phagosome maturation phenotype of these cells.
1.6.2 Objective 2: Identification of novel candidate Mtb virulence factors by Pathogen Effector Protein Screening in Yeast (PEPSY)

Rationale

Genomic screens identifying Mtb effectors able to inhibit phagosome maturation have been done previously using Mtb or BCG transposon mutant libraries searching for mutants unable to resist phagosome acidification or phagolysosome fusion (164,170,171). Although informative, these previous approaches to identify pathogen-encoded virulence factors have not always provided valuable information as to the specific functions of the candidate factors identified. For example, studies identifying Mtb mutants unable to block phagosome acidification (164,170,171) failed to distinguish whether the overall effect (phagosome acidification) was attributable directly to the action of the specific protein in preventing this process, or whether the phenotype could be an indirect result due to the inability of the mutant bacteria to survive intracellularly. This problem arises from the use of mutant bacteria, which are grown up in vitro and then used to infect cells and assessed for the phenotype of interest. Thus, a particular mutant may be lacking a protein that may have nothing to do with phagosome maturation inhibition, but is necessary for overall bacteria intracellular survival. The result, upon infection, would be dead bacteria, and dead mycobacteria cannot prevent phagosome acidification or block maturation. To address the question directly, ideally one would like to use a system that would allow the assessment of the ability of a protein to block phagosome maturation/interfere with endosomal trafficking in the absence of confounders. In pursuit of this objective we constructed a Mtb genomic library and screened it in yeast to identify proteins able to disrupt yeast vacuolar protein sorting. This provides an advantage over previous strategies on at least two levels. First, theoretically we will be able to screen all potential pathogen-derived proteins. Second, for any candidates we are able to identify we can attribute their function directly to an ability
to disrupt yeast VPS trafficking.

 Trafficking of protein cargo from the Golgi to the yeast vacuole – the vacuolar protein sorting (VPS) pathway as illustrated in Figure 1.6.2- is a highly regulated and well characterized system with many similarities to trafficking in the endosomal pathways in mammalian cells (172,173). The latter is not surprising since nearly all of the more than 70 proteins involved in yeast VPS have mammalian orthologs. Notably, the yeast vacuole is the analog of the mammalian lysosome, which fuses with phagosomes as they reach full maturity. Yeast is also an ideal model organism in which to study the effects of pathogen effector proteins since many bacterial effectors involved in mammalian infection have been shown to retain their function in yeast (174). In fact, two studies used a genetic

![Figure 1.6.2. Yeast vacuolar protein sorting pathways.](image) Overview of protein trafficking pathways from the late Golgi apparatus to the yeast vacuole. Two pathways exist for protein traffic to the plasma membrane, one involves direct movement via vesicles while the other
involves (1) trafficking to early endosomes (EE) and then (2) secretion. Proteins that are destined for the vacuole may be trafficked in several ways. The CPY pathway (3) (named after the vacuolar hydrolase carboxypeptidase Y, its most well characterized cargo) involves sorting from the late Golgi to late endosomes or the multivesicular body (MVB). It is at the MVB where the endocytic and CPY pathways convevne and cargo sorted. Those proteins destined for the vacuole lumen are sorted into luminal vesicles of the MVB (4). Proteins are also cycled back to the early endosomes via retrograde transport vesicles. Those proteins (such as the vacuolar proton ATPase) that are destined for the vacuolar membrane remain on the MVB membrane for transport. A more direct route from the Golgi to the vacuole which bypasses the endosomal system is the (5) ALP (alkaline phosphatase) pathway.

screening system in *Saccharomyces cerevisiae* and identified candidate secretory proteins of *Legionella pneumophila* that disrupted the VPS pathway (175,176). Thus, yeast is an ideal model in which to screen for pathogen effectors that may influence mammalian phagosome trafficking as they are also likely to interfere with the yeast VPS pathway. We constructed a Mtb genomic library in a yeast expression vector and transformed it into yeast. We then screened these yeast clones for VPS inhibition, which allowed us to identify candidate Mtb virulence effectors based upon their ability to disrupt VPS trafficking in yeast.

### 1.6.3 Objective 3: Role of Mtb protein PE-PGRS62 in modulation of macrophage phagosome maturation, cytokine production, and iNOS expression

**Rationale**

We identified several candidate Mtb virulence factors from Objective 2. In order to determine whether these candidates were able to modulate macrophage function during infection, one protein candidate, PE-PGRS62, was selected to assess for effects on macrophage phagosome maturation, cytokine production, and iNOS expression.

PE-PGRS62 was an ideal candidate for further study as this protein was previously implicated
as contributing to *M. marinum* virulence. PE-PGRS62 is expressed within granulomas in infected frogs, and the *M. marinum* transposon mutant for PE-PGRS62 displays reduced intracellular survival within macrophages (148). Despite its link to virulence however, the exact role played by this protein in promoting Mtb pathogenesis is unknown. Furthermore, PE-PGRS62 is a member of the PE/PPE protein family, which is linked to virulence but specific functions for members of this family are unclear. Thus, it seemed likely that an assessment of how PE-PGRS62 modulates macrophage function would provide novel information as to how this protein contributes to virulence, and may also expand knowledge about PE/PPE protein function.

PE-PGRS62 was able to disrupt yeast VPS, as assessed by the yeast screen conducted for Objective 2. In order to validate the translational ability of this yeast screen towards mammalian endosomal trafficking, PE-PGRS62 was evaluated for its ability to inhibit phagosome maturation in mammalian cells. In addition, the effects of PE-PGRS62 on cytokine production and on iNOS expression were also examined. Previous studies had implicated other PE-PGRS proteins in modulating macrophage cytokine expression (162,177). In addition, expression of iNOS and the production of reactive nitrogen intermediates is a key host immune response to mycobacterial infection (118-121). PE-PGRS62 was evaluated to determine whether this protein played a role in modulating both of these important macrophage responses.
CHAPTER 2: MATERIALS AND METHODS

2.1 Determining the Role of Class IA PI3K p110α in Phagosome Maturation

2.1.1 Creation of THP-1 cells with p110α expression knockdown

Lentivirus production and transduction of THP-1 cells

THP-1 cells were purchased from the American Type Culture Collection (ATCC TIB-202). The generation of constitutively silenced p110α knockdown and control THP-1 cells was described previously (178). Briefly, the shRNA sequences for control and p110α knockdown were cloned into the pSHAG-1 entry vector developed by Dr. Greg Hannon (Cold Spring Harbour Laboratory, Cold Spring Harbour, NY). This allows shRNA expression under the control of the RNA polymerase III U6 promoter. Using Gateway Cloning Technology (Invitrogen), the U6 promoter and shRNA sequences were then inserted into the lentiviral pHR'-CMV-EGFP vector which had been modified to enable Gateway cloning (178). Lentivirus was produced using the produced pHR'-CMV-EGFP plasmids, the packaging vector pCMVΔR8.2, and the VSV envelope vector pMD.G as described (178). In order to generate an inducible system for p110α knockdown using distinct shRNA sequences, a pTRIPZ construct encoding a shRNA targeting p110α was purchased from Open Biosystems (Thermo Scientific). This vector allows for the expression of shRNA after doxycycline induction. Table 2.1.1 shows the shRNA sequences used for constitutive and inducible p110α knockdown. pTRIPZ lentivirus was produced and THP-1 cells were transduced as described previously (178).
Table 2.1.1. Short hairpin sequences used to generate p110α knockdown cells

<table>
<thead>
<tr>
<th>Sequence for Silencing p110α Expression</th>
<th>shRNA Sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>red = sense</td>
<td>green = loop</td>
</tr>
<tr>
<td>blue = antisense</td>
<td></td>
</tr>
<tr>
<td><strong>Constitutive Knockdown</strong></td>
<td><strong>CAGCACGAGGAAGATCAGGAATGTATATGAAGCTTGATATGCATTTCCTTCTTTTCTGTTGCTCTTTTT</strong></td>
</tr>
<tr>
<td><strong>Constitutive Control</strong></td>
<td><strong>CCCCACAGTTCACCTGATGTGGTCTTTGAAGCTTGCGAGACCCTCATCGGGGTAGCTGTTGCCCATTTTTT</strong></td>
</tr>
<tr>
<td><strong>pTRIPZ Inducible Knockdown (Black = miR30 backbone)</strong></td>
<td><strong>TGCTGTGACAGTGAGCGCAGCCAATCTCCTCATAAATCTTTAGTGAAGCCACAGATGTAAGATTTATGAAGAGATTGGCA</strong></td>
</tr>
<tr>
<td></td>
<td><strong>TGCTACTGCTCGGA</strong></td>
</tr>
</tbody>
</table>
**Cell culture and induction of p110α knockdown**

All cells were maintained in RPMI media (Stem Cell Technologies) supplemented with 10% fetal bovine serum (Gibco), and 2 mM L-glutamine (Stem Cell Technologies). Twenty-four hours prior to phagosome maturation assays, constitutively silenced p110α knockdown and control cells were given 10 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and seeded onto 6-well culture plates (2 x 10^6/well) for differentiation for 16 h at 37°C/ 5% CO₂. Three hours prior to feeding prey, cells were washed once with warm media and allowed to rest for 4 h. For pTRIPZ transduced THP-1, cells were seeded into 6-well culture plates with 10 ng/mL of PMA and 2 µg/mL of doxycycline. Cells were allowed to differentiate for 24 h, then washed once with warm media, and subsequently given fresh media with 2 µg/mL of doxycycline for an additional 24 h prior to experimentation.

2.1.2 Phagocytosis assays

**Alexafluor 633 labelling of M. smegmatis and infection**

*Mycobacterium smegmatis* mc²155 was cultured from frozen glycerol stocks in LB media. Cultures were shaken overnight at 37°C to log phase, then the optical density at 580 nm was measured to determine cell titer. The required number of bacteria for an MOI of 20:1 was taken from this culture, and washed three times with Middlebrook 7H9 media (BD Biosciences) supplemented with 0.05% Tween-80, then resuspended in 200 µL of Middlebrook 7H9 with Tween-80. A final concentration of 30 µg/mL of Alexafluor 633 succinimidyl ester (-SE,
Molecular Probes) was added, and the mixture was incubated at 37°C for 1 hour with rotation. Bacteria were centrifuged at 13000 x g for 1 minute.

For phagocytosis assays, bacteria were then washed three times with RPMI, resuspended to give a final cell titer of 1.6E6/µL and used to infect PMA-differentiated THP-1 in culture plates. Phagocytosis was synchronized by placing cells at 4°C for 20 minutes prior to incubation at 37°C/5% CO₂. Timepoints were taken by washing cells three times with PBS (without Mg²⁺/Ca²⁺, Stem Cell Technologies), resuspending cells in 1 mL of PBS with 0.2% trypan blue (used to quench fluorescence of extracellular bacteria), then pelleting the cells by centrifugation at 1500 x g for 2 minutes. Cell pellets were washed three times with PBS, then resuspended and fixed in 2% paraformaldehyde (Electron Microscopy Sciences) in PBS and stored at 4°C. For quantification of bacteria uptake, samples were washed once, resuspended in PBS, and run on a flow cytometer (FacsCalibur, Becton Dickinson). Data were analyzed using FlowJo software (v8.7, Tree Star, Inc.).

**BSA-coupling and Alexafluor 633 labelling of magnetic beads and bead-treatment**

Carboxylic acid functionalized 3 µm diameter magnetic beads (COMPEL™, Bangs Laboratories) were coupled with bovine serum albumin (Fisher Scientific) as per manufacturer's protocol (Bangs Laboratories). Washed coupled beads were then labelled with Alexafluor 633-SE as stated above for *M. smegmatis* labelling, but instead of centrifugation, beads were pulled away from supernatants with the use of a magnetic tube holder (Bangs Laboratories). PMA-differentiated THP-1 cells were treated with RPMI-washed and resuspended beads at an MOI of
5:1, phagocytosis was synchronized and timepoints were taken as described. Quantification of bead uptake was done with a flow cytometer (FacsCalibur, Becton Dickinson).

2.1.3 Phagosome marker staining and analysis

Phagosome isolation

Phagosomes containing BSA-coupled 3 µm magnetic beads were isolated from bead-fed THP-1 cells chased at the timepoints indicated. Culture plate wells containing cells were washed three times with PBS (without Mg^{2+}/Ca^{2+}), then cells were resuspended in PBS and pelleted at 330 x g at 4°C for 10 minutes. Cell pellets were resuspended in ice-cold homogenization buffer (0.25M sucrose, 10 mM HEPES, pH 7.4, protease inhibitor cocktail (Roche) in PBS), and then lysed by passage through a 27 1/2 gauge needle 80 times. Cell lysis was confirmed by placing a sample of the homogenate under a light microscope and determined to be >99%. For isolation of bead phagosomes, homogenates were transferred to 12 x 75mm round bottom culture tubes (VWR) and placed on ice into a cold magnetic cell separating block (EasySep®, Stem Cell Technologies) for 3 minutes. Isolated bead phagosomes were washed three times with ice-cold PBS, then fixed with 2% paraformaldehyde in PBS. Phagosomes were stored at 4°C until staining.

Markers staining

Antibodies against LAMP-1 and LAMP-2 were obtained through the Developmental Studies Hybridoma Bank. Anti-Rab5B, EEA-1, Vti1p, and VAMP7, Actin, and N-WASP were purchased from Santa Cruz Biotechnology. Antibodies against PI3P and PI(3,4,5)P3 were
purchased from Echelon Biosciences Inc. Cy5-conjugated secondary antibodies were from
Applied Biological Materials, and mouse, goat, and rabbit IgG isotype controls were purchased
from Sigma-Aldrich. Briefly, phagosomes were permeabilized and blocked with blocking buffer
(1% BSA in PBS containing 0.05% Tween-20 and 0.2% saponin) for 30 minutes at room
temperature, followed by pelleting at 600 x g for 5 minutes. Then phagosomes were incubated
with primary antibodies and isotype controls in blocking buffer for 1-3 hours at room
temperature. Phagosomes were then washed three times with PBS containing 0.05% Tween-20
(PBS-T), and incubated with secondary antibody in blocking buffer for 1 hour at room
temperature. After washing three times with PBS-T, phagosomes were resuspended in PBS,
then analysed on a FacsCalibur flow cytometer. The resulting data was analyzed with FlowJo
software (v8.7, Tree Star, Inc). Confocal microscopy was done with fixed cells on coverslips.

2.1.4 β-galactosidase assays

Assays were done as per Yates et al. (179). The red fluorescence β-galactosidase
substrate, C₁₂RG, was used for experiments done with constitutive p110α knockdown and
control cells, while the green substrate, C₁₂FDG was used for pTRIPZ transduced cells
(Molecular Probes). M. smegmatis and 3 μm BSA-coupled magnetic beads that had been
previously labelled with Alexafluor 633-SE were subsequently incubated with C₁₂RG or C₁₂FDG
for 1 h at 37°C, then washed three times with RPMI. Prey was then resuspended in RPMI and
used to infect PMA-differentiated THP-1 at their respective MOIs (20:1 for M. smegmatis, and
5:1 for beads).
2.1.5 Bead-phagosome lysate preparation, immunoprecipitation and Western blotting

Four hour phagosomes containing magnetic beads were isolated from PMA-differentiated THP-1 cells as described above, with the exception that instead of fixation, PBS-T was used to resuspend phagosomes, followed by centrifugation at 1900 x g for 5 minutes. Pellets were resuspended in 1x SDS loading buffer (63 mM Tris-Cl, pH 6.8, 715 mM 2-mercaptoethanol, 2% SDS, 20% glycerol) and boiled prior to running in 10% or 12.5% SDS-PAGE. Following transfer onto nitrocellulose, blots were probed with antibodies against Rab7 (Abcam), Cathepsin D (Santa Cruz Biotechnology), Vps16 and Vps41 (Novus Biologicals), and p110α (Cell Signaling Technology). For immunoprecipitation experiments, phagosomes were isolated as described, but were then lysed in lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 1% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, and protease inhibitor cocktail mix (Roche) as described (180). Phagosomes were kept on ice for 10 min, followed by centrifugation at 13,000 x g for 10 min at 4°C. All subsequent steps were conducted either on ice or at 4°C. Lysates was precleared by incubation with Protein A agarose (Santa Cruz Biotechnology) for 10 min, then centrifuged at 13,000 x g for 1 min to remove the Protein A agarose. Cleared lysates were incubated with 2 μg/mL of antibody against RILP (Santa Cruz Biotechnology) overnight, followed by incubation with Protein A agarose for an additional 3 h. Lysates were then centrifuged at 13,000 x g for 1 min, and washed five times with lysis buffer. Samples were then boiled in 2x SDS loading buffer and loaded onto 10% SDS-PAGE for immunoblotting with an antibody against Rab7. All blots were developed using Supersignal West Pico and Femto Chemiluminescent Substrate (Thermo Fisher Scientific).
2.1.6 Assessment of acidification

*M. smegmatis* was grown in Middlebrook 7H9 medium supplemented with ADC enrichment overnight until an O.D.₆₀₀ reading of 0.8. Bacteria were then washed three times with PBS, then incubated with 1 mM pHrodo™-succinimidyl ester dye as per manufacturer's instructions. After 2 h of incubation at 37°C, bacteria were washed three times with RPMI, then used to infect cells.

2.1.7 Statistical analyses

All statistical analyses were done with GraphPad Prism software. Paired t-tests were done on all data to compare p110α knockdown with control cells at each timepoint. Results were considered significant at p < 0.05.

2.2 Identification of Candidate *M. tuberculosis* Virulence Factors by Pathogen Effector Protein Screening in Yeast (PEPSY)

2.2.1 PEPSY plasmid genomic library construction

The genomic library used for PEPSY screening was constructed in a similar manner as described previously for *Legionella pneumophila* (176). Briefly, genomic DNA from *M. tuberculosis* strain H37Rv was isolated and subjected to incomplete digestion with the restriction enzyme Sau3Al for 15 minutes at 37°C. Digested DNA was then run out on an agarose gel and fragments between 0.8 - 5 kb were gel extracted and purified. These fragments were then ligated into the pWS93 yeast expression vector (a kind gift from Dr. Marion Carlson, Columbia University) that had been previously linearized by BamHI/BglIII digestion. This vector...
allows the constitutive expression of inserts under the alcohol dehydrogenase (ADH1) promoter. The resulting ligation reactions were then transformed into the *Escherichia coli* strain DH5α, and the resulting colonies were pooled together and a plasmid maxiprep done to isolate plasmids. This resulted in a collection of heterogenous plasmids, which was then used to transform the yeast strain NSY01.

*Yeast transformation*

*Yeast strain*

The yeast strain NSY01 was a kind gift from Dr. Howard Shuman (Columbia University). This yeast strain expresses a carboxypeptidase Y/invertase (CPY/Inv) fusion protein. Carboxypeptidase Y (and CPY/Inv) is normally trafficked from the late golgi apparatus to the yeast vacuole (see Figure 1.6.2). Disruption of this vacuolar protein sorting pathway (VPS) in the yeast by ectopic expression of a pathogen virulence factor results in altered protein sorting and release of CPY/Inv into the extracellular medium, where its presence can be detected by overlaying yeast colonies with agar containing glucostat reagent (125 mM sucrose, 100 mM sodium acetate (pH 5.5), 0.5 mM *N*-ethylmaleimide, 10 µg/mL horseradish peroxidase, 8 units/mL glucose oxidase, and 2 mM *O*-dianisidine). A description of the biochemical reactions that occur when invertase enzyme is given glucostat reagent as substrate is illustrated in Figure 2.2.1).
Figure 2.2.1. Glucostat reagent biochemistry and the detection of yeast VPS disruption. 

(1) Exogenous sucrose is used as a substrate by invertase, which is secreted from yeast upon disruption of VPS. (2) The glucose that is formed as the result of invertase activity is then used by glucose oxidase to produce H\(_2\)O\(_2\). (3) Horseradish peroxidase (HRP) then uses this substrate to catalyze the oxidation of the compound O-dianisidine, resulting in the generation of a brown-red product. Thus, yeast clones which have had VPS disrupted exhibit brown-red colouration when overlaid with agar containing glucostat reagent.

**Transformation**

NSY01 was streaked onto a yeast-peptone-fructose (YPF) agar plate from glycerol stock and incubated at 30°C for 3 days until colonies appeared. One of these colonies was selected and grown in 5 mL of YPF media supplemented with 50 µg/mL of adenine hemisulfate (YPAF) at 30°C with shaking at 200 rpm overnight. An optical density reading at 600 nm (O.D.600) of the overnight culture was then taken and 1.25E08 cells were then taken to inoculate 25 mL of YPAF fresh culture to give a cell titer of 5E06 cells/mL. This culture was incubated at 30°C with shaking at 200 rpm for 2 h, or until cells had doubled. Cells were then harvested by centrifugation at 4500 x g for 6 min. The cell pellet was washed with 12.5 mL of sterile ddH\(_2\)O, and centrifuged again. The resulting pellet was then resuspended in 0.5 mL of sterile ddH\(_2\)O and transfered to a sterile 1.5 mL microcentrifuge tube. This cell suspension was then centrifuged at maximum speed in a microcentrifuge for 30 seconds, after which the supernatant was discarded and the cell pellet resuspended in 0.5 mL of sterile ddH\(_2\)O by vigorous vortexing.
µL of this cell suspension was then aliquoted into microcentrifuge tubes (one per transformation reaction), which were then centrifuged at maximum speed in a microcentrifuge for 30 seconds and the supernatant discarded. Cell pellets were resuspended in a transformation mix made up of 240 µL PEG 3350 (50% w/v), 0.1 M lithium acetate, 278 µg/mL of boiled salmon testes denatured DNA, 300 ng of plasmid, and sterile ddH₂O to make up a total transformation volume of 360 µL. Empty vector and control transformations without vector were included. Cells were resuspended in transformation mixes by vigorous vortexing, and tubes were incubated at 42°C for 40 min, after which tubes were centrifuged at maximum speed in a microcentrifuge for 30 seconds and the supernatant removed. Cell pellets were resuspended in 1 mL of sterile ddH₂O, and 150 µL was plated onto synthetic complete medium lacking uracil (SC-Ura⁻) with fructose added as a carbon source and incubated at 30°C for ~ 3 days until colonies appeared.

### 2.2.2 Yeast library screening

Positive colonies were selected based on brown colouration when plates were overlaid with glucostat reagent. Brown colonies were picked, then grown in 5 mL of SC-Ura⁻ liquid media with fructose added as the carbon source at 30°C with shaking at 200 rpm overnight. A yeast plasmid miniprep was then done on these liquid cultures (Omega Biotek). The isolated plasmids were then used to transform DH5α *E. coli* in order to obtain adequate plasmid quantities for a second yeast transformation. Plasmids were isolated from transformed DH5α and used to transform NSY01 yeast, which were then screened again with glucostat reagent to confirm that
the trafficking defect phenotype seen in these yeast clones was attributable to the plasmids they were carrying, and not to spontaneous mutations in VPS trafficking.

**Sequencing to identify *M. tuberculosis* inserts**

Positive candidates that were identified by plasmid isolation and re-transformation into yeast, as well as by counterselection, then had their plasmids sequenced with pWS93 vector specific primers flanking the cloning site (forward primer 5'-CATTGCAGGAGTAGCAT-3', reverse primer 5'-GATTGTATGCTTGGTATAGC-3') to identify inserts. Sequences thus obtained were then blasted against *M. tuberculosis* sequences found in the TB Database (http://www.tbdb.org/).

2.3 **Assessment of PE-PGRS62 for Effects on Macrophage Function**

2.3.1 **Construction of PE-PGRS62- expressing *Mycobacterium smegmatis***

The full length gene for Rv3812 was initially amplified from *M. tuberculosis* genomic DNA using the forward primer 5'- GTGTCGTTGTCGTCAGGACATGC-3' and reverse primer 5'-AGCCGCCCAGTGATTGC-3'. After obtaining the 1539 bp PCR product corresponding to the full length gene, subsequent PCRs were done which allowed the Rv3812 gene to be cloned into various expression vectors (primers and vectors into which the Rv3812 gene was cloned are
summarized in Table 2.3.1). The mycobacterial expression plasmid allowing for Rv3812 expression was then transformed into *M. smegmatis* mc²155 by established methods (181).

**Expression testing**

A 6 x histidine tag was placed at the N-terminus of full length Rv3812 in order to facilitate protein detection. To confirm expression of fusion protein, *M. smegmatis* clones were grown in 7H9/ADC/0.02% Tween 80 media with 25 µg/mL kanamycin overnight at 37°C with shaking at 150 rpm. Bacteria were then pelleted by centrifugation at 13000 x g for 1 min, and washed three times with 7H9 media with 0.02% Tween 80. The resulting pellets were then resuspended in 7H9/Tween 80 and divided evenly into two cultures, which contained 7H9/Tween 80 supplemented with either 0.2% dextrose or 0.2% acetamide to induce fusion protein expression. These cultures were grown overnight at 37°C with shaking at 150 rpm, after which bacteria were pelleted, washed twice with PBS, and resuspended with denaturing buffer containing 100 mM NaH₂PO₄, 10 mM Tris-Cl, and 8 M urea. Resuspended bacteria were then sonicated on ice for 10 seconds followed by 10 second rests for a total of ten times. Following sonication, cell debris was then pelleted by centrifugation at 13000 x g for 1 min, and SDS loading buffer added to the isolated supernatant. This was then boiled for 7 min prior to loading onto a 10% SDS-PAGE. After separation, proteins were transferred to nitrocellulose (Biorad) and an immunoblot was done with an antibody specific for the histidine epitope tag (Applied Biological Materials).
Subcellular fractionation of *M. smegmatis*

To examine the subcellular localization of Rv3812 fusion protein, a 150 mL culture of 7H9/ADC/Tween 80 was inoculated with *M. smegmatis* expressing Rv3812 and was grown overnight at 37°C with shaking at 150 rpm. Bacteria were then pelleted, washed three times with 7H9/Tween 80, resuspended in 10 mL of 7H9/Tween 80 and divided evenly between two 75 mL cultures of 7H9/Tween 80 supplemented with either 0.2% dextrose or 0.2% acetamide. These cultures were then grown overnight at 37°C with shaking at 150 rpm after which bacteria were harvested by centrifugation at 5000 x g for 10 min. Subsequent steps were done on ice or at 4°C. Bacterial pellets were resuspended in 2 mL of breaking buffer (PBS, 1 mM PMSF, 0.6 ug/mL each of DNase and RNase) and sonicated with 10 x 10 sec pulses, with 10 sec rests in between pulses. Lysates were then pelleted at 3000 x g for 20 in to generate clarified whole cell lysates. The supernatants were then removed and centrifuged at 27,000 x g for 30 min to obtain the cell wall pellet. The supernatant from this was then centrifuged again at 100,000 x g for 2 h to separate the membrane fraction from the cytosolic fraction. Pellets from the cell wall and cell membrane centrifugation steps were washed once with PBS. Samples from each of the fractions were then resuspended in SDS loading buffer, boiled for 7 min, and loaded onto 10% SDS-PAGE. After separation, proteins were transferred to nitrocellulose membrane and an immunoblot was done with an antibody specific for the histidine tag epitope (Applied Biological Materials).
2.3.2 Cytokine profiling of J774A macrophages infected with *M. smegmatis* expressing PE-PGRS62

J774A cells were infected with *M. smegmatis* induced to express Rv3812 or not in order to compare the expression of IL-1β, IL-10, and IL-12p40 upon infection. 0.5E6 cells were placed in each well of a 6-well culture plate and incubated overnight at 37°C/5% CO₂ to allow plate attachment. 5 mL cultures of *M. smegmatis* in 7H9/Tween 80 supplemented with either 0.2% dextrose (for uninduced, control bacteria) or 0.2% acetamide (to induce Rv3812 expression) were made and incubated overnight at 37°C with shaking at 150 rpm. The next day, O.D.600 readings of the bacterial cultures were taken, and the number of bacteria required to infect J774A cells at a multplicity of infection (MOI) ratio of 20:1 was calculated (O.D.600 of 0.1 = 0.7 x 10⁸ cells/mL). The required amount of bacteria was then washed three times with plain DMEM media without fetal bovine serum and then resuspended in an adequate volume of complete DMEM media to allow 1 mL of this resuspension to be given to each well of the 6-well plates. Cells were allowed to phagocytose bacteria for 2 h, then were washed in warm PBS and given complete media with 50 µg/mL gentamycin. Cells were incubated for the required times, after which they were washed three times with cold PBS, scraped off, and centrifuged at 3000 x g for 2 min. The supernatant was pipetted off, and the cell pellets were resuspended in lysis buffer supplied with the E.Z.N.A total RNA isolation kit (Omega Biotek). RNA was subsequently isolated as per manufacturer's protocol. Equal amounts of RNA were then used in first strand cDNA synthesis reactions using the cDNA synthesis kit from Invitrogen. The cDNA produced was then used as template for PCR reactions using primers specific for IL-1β (forward primer 5' - AAGGAGAACCAAGCAAGCAGAAA-3', reverse primer 5' TGGAGCAACAAGTGGTGTTCTCCA3'), IL-
10 (forward primer 5' - AGAAGCATGGCCAGAAATCA -3', reverse primer 5' - GGCTTGACACCTTTGGT -3'), IL-12p40 (forward primer 5' - TGGTTTGCCATCGTTTTGCTG -3', reverse primer 5' - AGAAACAGTGAACCTCACCTGT -3'), and TNF-α (forward primer 5' - CTGAACCTTCGAGGTGATCGG -3', reverse primer 5' - GGCTTGCTCAGAATTMTGAGA -3'). Signals were normalized to that obtained for mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer 5' - TGACCACAGTCCATGCGATC -3', reverse primer 5' - GACCGACATTTGGGGGTAG -3').

2.3.3 Determination of iNOS levels in infected cells

*Examining levels of iNOS protein*

Cells were infected with *M. smegmatis* expressing Rv3812 as described in the preceding section. At the required times, cells were washed three times with cold PBS, and scraped into 240 µL of ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/mL leupeptin, and 1 mM PMSF). Cell suspensions were incubated on ice for 5 min, then passaged through a 27 1/2 gauge needle 10 times. 80 µL of 4X SDS loading buffer was then added to each suspension, which were vortexed and centrifuged at 13,000 x g for 10 min at 4°C. Supernatants were removed and loaded onto 10% SDS-PAGE for separation. Proteins were then transferred to nitrocellulose membrane and an immunoblot was done with an antibody against iNOS protein (Santa Cruz Biotechnology).
For infections with the *M. marinum* transposon mutant for Rv3812, as well as complemented and wildtype strains, bacteria were grown in Sauton’s media supplemented with 0.2% Tween 80 and 30 µg/mL kanamycin (for complemented and Rv3812 transposon mutant strains) until an O.D.600 reading of ~0.8-1.0 was reached. The required amount of bacteria needed to achieve an MOI of 20:1 was then taken, washed three times with PBS, and resuspended in complete DMEM medium. Cells were infected for 4h, after which they were washed three times with warm PBS and given fresh media supplemented with 20 U/mL murine IFN-γ and 20 µg/mL amikacin. Cells were then incubated for the required times, harvested, and processed as described above for *M. smegmatis* infections.

**Examining levels of iNOS transcript**

Cells were infected as described and were harvested as described previously in section 2.3.2. The resulting cDNA products were used in PCR reactions using primers specific for murine iNOS (forward primer 5'- ACATCGACCCGTCCACAGTAT -3', reverse primer 5'-CAGAGGGGTAGGCTTGTCCTC -3'). Again, signals were normalized to that of murine GAPDH.

2.3.4 Phagosome maturation studies

Phagosome isolation and immunoblotting

Phagosomes were isolated following a protocol which has been previously described (182). Cells were infected as described above, and were harvested by washing three times with cold PBS. J774A cells were then isolated by scraping into cold PBS and centrifugation at 600 x g for 10 min. Supernatants were decanted and cell pellets were resuspended in homogenization
buffer (10 mM HEPES, 25 mM sucrose) with protease inhibitor mix cocktail (Roche). Cells were homogenized by passage through a 27½ gauge needle 80 times and cell debris was pelleted by centrifugation at 2000 x g for 5 min at 4°C. Supernatants were transferred to new tubes and the centrifugation step repeated a total of three times. Cleared supernatants were then layered on top of a 27% percoll gradient made up in homogenization buffer and ultracentrifuged in a swinging bucket rotor 36,000 x g for 1 h at 4°C. The bottommost 3 mL were then taken and centrifuged for 2 min at 13,000 x g to pellet phagosomes, which were then resuspended in SDS loading buffer and boiled for 7 min. Phagosome lysates were loaded onto 12% SDS-PAGE for protein separation, transferred to nitrocellulose, and immunoblotting was done for Rab7 (Abcam), Rab5 (Santa Cruz Biotechnology), and Cathepsin D (Santa Cruz Biotechnology).

Confocal analysis of LAMP-1 colocalization

J774A were grown on coverslips and infected as described above and at the required times cells were washed three times with cold PBS and fixed in 2% paraformaldehyde overnight at 4°C. Cells fixed onto coverslips were then washed three times with PBS, permeabilized with 0.1% Triton X-100 for 10 min, washed three times with PBS, and blocked with 1% BSA, 0.2% saponin in PBST for 30 min, followed by an overnight incubation with an antibody against murine LAMP-1 (Developmental Hybridoma Bank) at 4°C. After washing with PBS, an anti-rat secondary antibody conjugated to FITC was used to detect antibody binding, and coverslips were mounted on Prolong® Gold antifade reagent with added DAPI stain (Invitrogen) to allow detection of bacteria within cells. Z-stacks of cells were imaged using a Leica DMIRE2 inverted microscope equipped with a SP2 AOBS laser scanning head. Images were processed using the
MacBiophotonics version of the Image J software (National Institutes of Health) and colocalization analysis was done using the Colocalization Highlighter plugin (P. Bourdoncle, Institute Jacques Monod, Service Imagerie, Paris, France). Quantification was done by counting the number of complete colocalization events; at least 300 phagosomes were counted for each of three experiments (~900-1000 phagosomes in total).

2.3.5 Colony morphology and SDS resistance assay

Analysis of colony morphology

7H9/Tween 80 cultures of *M. smegmatis* containing empty vector as well as the plasmid for Rv3812 expression were induced as described previously, and grown overnight at 37°C with shaking at 150 rpm. Cultures were diluted to O.D.600 of 0.01, and 5 and 10 µL of each culture was spotted onto 7H10 agar plates supplemented with 25 µg/mL kanamycin, and either 0.2% dextrose or 0.2% acetamide. To one set of plates, 0.05% Tween 80 was added. Plates were then incubated at 37°C until colonies appeared (~2 days).

To examine the colony morphology of *M. marinum* strains, bacteria were grown in Sauton's media supplemented with 0.2% Tween 80. Cultures were then diluted to O.D.600 of 0.01, and 5 and 10 µL of each culture was spotted onto 7H10 agar plates supplemented with 0.1% Middlebrook OADC (Becton Dickinson), with or without 0.05% Tween 80. Plates were incubated at 30°C until colonies appeared (~7-10 days).
**SDS resistance assay**

*M. marinum* strains were grown in Middlebrook 7H9 media supplemented with ADC and 0.1% SDS. Cultures were grown at 30°C with shaking at 100 rpm. Timepoints were taken, diluted and plated onto Middlebrook 7H10 agar plates supplemented with Middlebrook OADC, and 30 µg/mL kanamycin (for transposon mutant plates). Plates were incubated at 30°C until colonies appeared (~7-10 days).
CHAPTER 3: DETERMINING THE ROLE OF THE PI3K P110α IN PHAGOSOME MATURATION

3.1 Introduction

PI3Ks play an essential role in phagosome maturation. However, only the class III enzyme, hVps34, has been clearly shown to be involved, and other PI3K isoforms were thought to contribute little. In particular, the class IA PI3Ks were previously characterized as having a role in phagocytosis of prey (42,79). Recent evidence suggests that the class IA PI3Ks may play additional roles in regulating maturation, as PI(3,4,5)P3 was found to be needed for actin formation around late stage phagosomes taken up by complement receptors (12), and Akt has been found on phagosomes and endosomes (82,169). Previous work by our laboratory had shown that 1α,25-dihydroxyvitamin D3 treatment is able to reverse the phagosome maturation inhibition imposed by Mtb lipoarabinomannan, and that this involves PI3K activity (183). Furthermore, it is known that 1α,25-dihydroxyvitamin D3 is able to activate the class IA PI3K p110α in THP-1 cells (184). Given these previous studies, we wanted to assess whether the class IA PI3K p110α contributed to phagosome maturation per se. We did this by specifically silencing the p110α catalytic unit in THP-1 cells by lentivirus transduction of silencing shRNA to generate a doxycycline-inducible system for p110α knockdown. We then took these inducibly silenced cells, and a cell line which had been constructed previously in which p110α expression had been constitutively silenced (178), and assessed for effects on phagosome maturation phenotype. Our results showed that cells in which p110α expression had been abrogated show marked reductions in the acquisition of LAMP-1 and LAMP-2, as well as of the lysosomal
hydrolase β-galactosidase. Cells in which p110α expression had been silenced also displayed a significant decrease in the ability to fuse with fluorescent dextran-loaded lysosomes. These results, using a variety of methodologies to evaluate phagosome maturation state, reveal a novel role for the class IA PI3K p110α in regulating phagosome maturation.

3.2 **Knockdown of the Class IA PI3K p110α Does Not Impair Phagocytosis of Prey Less Than or Equal to 3μm in Diameter**

Selective, stable knockdown of expression of the class IA PI3K p110α in the THP-1 human monocytic cell line was done by lentiviral transduction as described previously (178). It has been documented that inhibition of PI3K activity impairs phagocytic uptake of prey larger than 4 μm (29). To test the ability of constitutive p110α knockdown cells to take up prey less than or equal to 3μm in diameter, phorbol-12-myristate-13-acetate (PMA)-differentiated control and p110α knockdown cells were fed BSA-coupled 3μm magnetic beads. Alternatively, parallel cell cultures were infected with the minimally virulent, opportunistic organism *M. smegmatis*. Prey, both beads and bacteria, were covalently surface labelled with the fluorescent dye Alexafluor 633 succinimidy ester (-SE) to allow quantitation of uptake by flow cytometry. Both control and p110α constitutively deficient cells displayed equivalent levels of uptake (Fig. 3.2), thereby indicating that knockdown of p110α did not impair phagocytosis of the prey involved in this study.
Figure 3.2. Knockdown of p110α expression does not impair uptake of 3 µm magnetic beads or *M. smegmatis*. Cells were either fed Alexafluor 633-SE labelled BSA-coupled 3µm magnetic beads or infected with Alexafluor 633-SE labelled *M. smegmatis*. Flow cytometric analysis was done to determine uptake. Solid circles = control cells, empty squares = p110α knockdown cells. Data are means +/- s.e.m., from three independent experiments. No significant differences were observed.

3.3 Phagosomes from p110α Knockdown Cells Have Less p110α and PI(3,4,5)P3 but Similar Levels of PI3P

We examined whether p110α is recruited to phagosomes and assessed phagosomal levels of its phosphoinositide product, PI(3,4,5)P3. PMA-differentiated control and constitutive knockdown cells were fed BSA-coupled 3 µm magnetic beads and 4 hour phagosomes were isolated by magnetic pulldown. Phagosomes were then either fixed for staining with antibodies against PI3P and PI(3,4,5)P3, or lysed for determination of p110α phagosomal recruitment by
immunoblotting. Our results showed that the PI3K p110α was associated with phagosomes isolated from THP-1 cells, and that the class IA PI3K phosphoinositide product, PI(3,4,5)P3 was also present on these vesicles. Phagosomes isolated from p110α knockdown cells displayed significantly less recruitment of this PI3K (Fig. 3.3A), and a decreased amount of PI(3,4,5)P3, though levels of PI3P were unaffected (Fig. 3.3B). Flow organellometry of bead phagosomes indicated that PI3P levels appeared to be unchanged throughout phagosome maturation (Fig. 3.3C).

**Figure 3.3 (next page). Phagosomes isolated from PMA-differentiated THP-1 cells contain the class IA PI3K p110α, and this is decreased in knockdown cells.** Late stage 4 h bead phagosomes were isolated by magnetic pulldown, lysed, and immunoblotted for p110α. Actin was done as a loading control. Densitometry of four independent experiments indicates a significant decrease in phagosomal p110α in phagosomes isolated from knockdown cells. Empty bars = control cell phagosomes, solid bars = phagosomes from p110α knockdown cells. p < 0.05. **B) Phagosomes from p110α knockdown cells contain less PI(3,4,5)P3, but similar amounts of PI3P as control cells.** Bead phagosomes were isolated by magnetic pulldown, fixed, stained for PI3P and PI(3,4,5)P3, and flow organellometry was done. A representative dot plot of SSC vs FL4 fluorescence channel depicting how the phagosomes were gated is shown. The data show PI(3,4,5)P3 staining of 4 h phagosomes isolated from control and p110α knockdown cells from one experiment. Empty bars = control cell phagosomes, solid bars = phagosomes from p110α knockdown cells. Data are means +/- s.e.m., from four independent experiments. The levels of PI(3,4,5)P3 were found to be significantly decreased in phagosomes isolated from p110α deficient cells. p < 0.05. No significant differences were observed for PI3P levels. **C) Kinetics of PI3P acquisition onto phagosomes.** Phagosomes were isolated at 30 min and 2 h and stained for PI3P. The graph shows geometric means normalized to isotype control. Open bars = phagosomes isolated from control cells, Solid bars = phagosomes isolated from p110α knockdown cells. No significant differences were observed, and PI3P levels appeared to remain consistent throughout phagosome maturation.
3.4 Flow Organellometry of Phagosomes from p110α Knockdown Cells Shows Similar Levels of Rab5B and EEA-1, But a Defect in Acquisition of Lysosomal Marker Proteins

Control and constitutive p110α knockdown cells were fed BSA-coupled 3μm magnetic beads and phagosomes were isolated at various timepoints. Vacuoles were then stained for various phagosome maturation markers and analyzed by flow organellometry (Fig. 3.4). In comparison to wild-type cells, p110α knockdown cells acquired similar levels of the early endosomal small GTPase, Rab5B, and early endosomal autoantigen 1 (EEA-1) (Fig. 3.4A). However, acquisition of LAMP-1 and LAMP-2 by p110α deficient cells was markedly impaired (Fig. 3.4B). Nevertheless, both cell types were found to express similar levels of these markers at the whole cell level (indeed, the knockdown cells appear to express increased cellular levels of LAMP-1) (Fig. 3.4C). Furthermore, confocal fluorescence microscopy showed no defects in biosynthetic sorting of LAMP-1 from the trans-Golgi network to the endosomal system (Fig. 3.4D). Thus, deficient acquisition of these lysosomal membrane proteins most likely was the result of reduced frequency of fusion events between maturing phagosomes, endosomes and lysosomes. Our kinetic studies which consisted of a relatively long time course (up to 6 hours) also indicate that this defect in acquiring LAMP proteins was not simply due to delayed kinetics, but an actual deficiency in marker acquisition.
Figure 3.4. Magnetic bead phagosomes from p110α knockdown cells show defective acquisition of the lysosomal proteins LAMP-1 and LAMP-2. Kinetic analysis of phagosome maturation by flow organellometry shows A) normal acquisition of the early endosomal marker Rab5 and EEA-1, and B) marked defects in the acquisition of the lysosomal membrane proteins LAMP-1 and LAMP-2 by p110α knockdown cells. Solid circles = control cells, empty squares = p110α knockdown cells. Timepoints were taken at 30 minutes, 1 hour, 2 hours, 4 hours, and 6
Confocal Analysis of p110α Knockdown Cells Showed Impaired Phagosomal Acquisition of LAMP-1 and a Defect in Phagosome Fusion with Dextran-Loaded Lysosomes

In order to confirm a defect in LAMP-1 acquisition, cells were fed beads and assessed for LAMP-1 recruitment to bead-containing phagosomes. Confocal microscopy showed that p110α deficient cells had a significant reduction in LAMP-1 acquisition, in close agreement to our flow organellometry results (Fig. 3.5A). In addition, phagosomes from p110α knockdown cells showed a significant decrease in fusion with lysosomes that had been loaded with Texas Red dextran, thus indicating impaired phagolysosome fusion (Fig. 3.5B). This result correlated well with our LAMP-1 confocal microscopy data, and strongly suggests that phagosomes from p110α deficient cells have markedly reduced access to lysosomal components.
Figure 3.5. Phagosomes from p110α show marked decreases in LAMP-1 acquisition and phagolysosome fusion. **A** Confocal microscopy of control and p110α knockdown cells shows reduced phagosomal LAMP-1 acquisition. Cells were fixed 2 h post-phagocytosis. The graph to the right shows counts of phagosomes colocalized with LAMP-1 staining. At least 100 phagosomes in each of three independent experiments were counted. Open bar = Control cells, Solid bar = p110α knockdown cells. Means +/- s.e.m. are shown. ** p< 0.01. **B** Confocal microscopy of control and p110α knockdown cells shows reduced phagosome-lysosome fusion. Cells were loaded with Texas Red dextran (10,000 MW, lysine fixable), which was then chased into lysosomes. Cells were fixed 2 h post-phagocytosis. The graph to the right shows the numbers of phagosomes that were positive for a dextran signal. At least 100 phagosomes in each of three independent experiments were counted. Open bar = control cells, Solid bar = p110α knockdown cells. Means +/- s.e.m. are shown. * p< 0.05. Size bar: 10 µm.
3.6 Phagosomes from p110α Knockdown Cells Display Decreased Acquisition of the Lysosomal Hydrolase, β-galactosidase

In light of the deficient delivery of lysosomal membrane marker proteins to phagosomes isolated from p110α knockdown cells, it was of interest to know whether this was indicative of a broader defect extending to the delivery of active lysosomal enzymes. To examine this question, control and constitutive knockdown cells were assessed for the phagosomal acquisition and activity of the lysosomal hydrolase β-galactosidase in an assay developed by Yates et al. (186). PMA-differentiated knockdown and control cells were fed either BSA-coupled 3μm magnetic beads or infected with M. smegmatis. In both cases, prey had been previously labelled with Alexafluor 633-SE and a fluorescent β-galactosidase substrate, C12RG or C12FDG. Cleavage of the C12RG/C12FDG substrate on the surface of internalized prey by β-galactosidase and subsequent release of fluorescence occurs only when active lysosomal hydrolase is delivered to phagosomes containing labelled prey. Flow cytometric analyses of β-galactosidase activity of control and constitutive p110α knockdown cells showed that the latter displayed a marked reduction in the acquisition of β-galactosidase to phagosomes, and this was true for both beads and M. smegmatis prey (Fig. 3.6A). This defect in β-galactosidase delivery was also seen in cells in which p110α expression had been knocked down by a different, inducible shRNA sequence (Fig. 3.6B). The reduced delivery and activity of lysosomal β-galactosidase to phagosomes in p110α knockdown cells correlated well with our lysosomal marker data, and provided further evidence that p110α-deficient cells display a deficiency in phagosome-lysosome fusion.
Figure 3.6. Delivery of the lysosomal enzyme β-galactosidase is impaired in p110α knockdown cells. A) Knockdown of p110α results in decreased acquisition of the lysosomal enzyme β-galactosidase by phagosomes containing either BSA coated beads or M. smegmatis. Cells were fed prey that had been previously surface labelled with Alexafluor 633-SE and the fluorescent β-galactosidase substrate, C_{12}RG. Cleavage of C_{12}RG releases fluorescence which is read in the FL2 channel of the flow cytometer. This was then normalized to the Alexafluor 633 signal (FL4) for phagocytic uptake. Solid circles = control cells, empty squares = p110α knockdown cells. Timepoints were taken at 1 hour, 2 hours, 4 hours, and 6 hours post bead treatment or infection. * p < 0.05, ** p < 0.001, *** p < 0.0005. Data are means +/- s.e.m., from at least four independent experiments. B) Inducible p110α knockdown via a distinct shRNA sequence also showed defects in β-galactosidase acquisition. Western blot showing knockdown was induced by doxycycline treatment prior to experimentation, which also induces expression of TurboRFP which is linked to shRNA production. The histogram on the left shows induction of red fluorescence when cells were given doxycycline. Cells were also fed the green fluorescent β-galactosidase substrate, C_{12}FDG, which is read in the FL1 channel. Empty bars = Control cells (no doxycycline induction), Solid bars = Cells induced for p110α knockdown. * p < 0.05, ** p < 0.01. Data are means +/- s.e.m. from five independent experiments.
3.7 Cathepsin D Delivery and Processing is Unaffected in Phagosomes Isolated from p110α Deficient Cells

Delivery of cathepsin D is used as a marker of phagosome maturation (187-190). To examine cathepsin D delivery to and processing in phagosomes isolated from p110α deficient cells, we probed 4 and 6 hour phagosome lysates by immunoblotting for cathepsin D using an antibody that recognizes all the processed forms. Compared with control cells, the results showed similar levels of both the intermediate and mature forms of cathepsin D, although there was a statistically non-significant trend towards lower levels of cathepsin D in the p110α knockdown cells. These results indicate that this protease is normally conveyed to and processed by phagosomes from p110α deficient cells (Fig. 3.7). The fact that cathepsin D was processed normally in p110α deficient cells suggested that phagosomal acidification was taking place, as acidification is needed to initiate proper processing of this pre-proenzyme (191).
In order to assess whether phagosomes from p110α cells acidified normally, we surface labelled *M. smegmatis* with the acidotropic dye, pHrodo™. Although non-fluorescent under neutral conditions, the dye fluoresces red at acidic pH. We fed control and p110α knockdown cells *M. smegmatis* which had been previously labelled with pHrodo, then assessed acidification by running cells through a flow cytometer. Our data (Figure 3.8) revealed that although...
Phagosomes in p110α deficient cells do eventually acidify to the same extent as control cells, they do so with delayed acidification kinetics. It also appears that the acidification that does occur in p110α knockdown cells is sufficient for cathepsin D processing, although a trend towards lower levels of mature cathepsin D is seen in phagosomes from these cells (Figure 3.7).

**Figure 3.8.** Phagosomes from p110α deficient cells acidify with slower kinetics when compared to control cells. Cells were infected with *M. smegmatis* which had been previously surface-labelled with the acidotropic dye, pHrodo™. Cells were fixed at specific times during maturation (as indicated by the different histogram colours), and were assessed by flow cytometry. The production of red fluorescence indicates acidification of *M. smegmatis*-containing phagosomes. The bar graph depicts the mean fluorescence intensities of three independent experiments.
3.9 Phagosomes from p110α Knockdown Cells Acquire Active Rab7 That is Competent in Mediating Recruitment of RILP and HOPS Components

Reduced acquisition of LAMPs and lysosomal hydrolase activity by p110α knockdown cells pointed towards a defect in phagosome-lysosome fusion. In light of this, it was important to assess whether this could be explained by a block in Rab7 activation and recruitment to the maturing phagosome membrane. To address this, lysates from 4 hour phagosomes were prepared from control and constitutive p110α knockdown cells and immunoblotting was done to assess phagosomal translocation of Rab7. Surprisingly, rather than revealing a defect, Western blotting of late stage phagosomes from p110α knockdown cells showed an approximate two-fold increase in Rab7 levels compared to control cells (p= 0.0529, Fig. 3.9A). Rab7 is thought to be recruited from a cytosolic pool to membranes only when activated (in GTP-bound form) (192). Furthermore, a recent study examining Rab membrane targeting highlighted a model in which insertion of Rab proteins into membranes is mediated by membrane-bound GEFs (guanine nucleotide exchange factors). Upon spontaneous dissociation of a Rab GDI (GDP dissociation inhibitor) from a Rab protein, Rab-GTP is generated which then inserts into the membrane (52). In light of this model, we sought to determine the activation state of the Rab7 found on phagosomes from p110α deficient cells. This was done by examining the ability of phagosome associated Rab7 to recruit its downstream effectors, RILP, and the HOPS (homotypic vacuole fusion and protein sorting) complex to phagocytic vacuoles. RILP binds to GTP-bound Rab7 and recruits dynein-dynactin motor complexes which allow phagosomes to migrate from the cell periphery towards perinuclear lysosomes (193-195).
Figure 3.9. Phagosomes from p110α deficient cells recruit Rab7 and its effectors, RILP and HOPS. A) Phagosomes from p110α knockdown cells are not defective in the recruitment of Rab7. Western blots of bead phagosome lysates (4 hours) isolated from control and p110α
knockdown cells show no significant differences in recruitment of Rab7. Actin is shown as a loading control. Empty bars = control cells, solid bars = p110α knockdown cells. Data are means +/- s.e.m., from four independent experiments. B) Phagosomes from p110α knockdown cells show similar levels of Rab7-RILP interaction as control cells, confirming that the Rab7 present is active. 4 h bead phagosome lysates were immunoprecipitated with an antibody against RILP, then probed for Rab7 and RILP. Isotype IgG was added as a control for non-specific binding. Results are representative of three independent experiments. C) Phagosomes isolated from p110α deficient cells are not defective in the recruitment of HOPS proteins. Western blots of bead phagosome lysates (4 hours) were probed for the HOPS components Vps41 and Vps16. Densitometry is of means +/- s.e.m., from four independent experiments. D) Flow organellometry staining of isolated phagosomes (2 h and 4h) shows kinetics of Rab7 and Vps41 acquisition. Phagosomes were stained for Rab7 and Vps41 and analyzed by flow cytometry. The graph shows geometric means +/- s.e.m. derived from three independent experiments. Open bars = Phagosomes isolated from control cells, Solid bars = Phagosomes from p110α knockdown cells. No significant differences in Rab7 and Vps41 recruitment were seen between phagosomes isolated from control and p110α knockdown cells. E) Phagosomes from p110α deficient cells do not show alterations in the levels of SNAREs Vti1p and VAMP-7. Phagosomes were stained for Vti1p and VAMP-7 and analyzed by flow cytometry. Open bars = Phagosomes from control cells, Solid bars = Phagosomes from p110α knockdown cells. No significant differences were observed.

As for the HOPS complex, it is a key Rab7 effector that functions to bridge Rab7 activation and SNARE-mediated endosomal membrane fusion (71,196-199). A recent study examining subunit arrangement within the HOPS tethering complex found that the HOPS component Vps41 interacts exclusively with Rab7 in an active state (71). In addition, Vps16 forms a subcomplex with Vps33, the HOPS component that putatively binds SNAREs (71,196-198). To examine phagosomal recruitment of RILP, we isolated 4 h late stage bead phagosomes from control and p110α deficient cells, and immunoprecipitated phagosome lysates with an antibody against RILP. Probing of the immunoprecipitated samples with an antibody against Rab7 revealed that this protein formed complexes with RILP in phagosomes isolated from p110α knockdown cells in a manner that was indistinguishable from that of phagosomes from control cells (Fig. 3.9B).
When phagosome lysates were immunoblotted for the HOPS components Vps16 and Vps41, we observed no significant differences in the recruitment of either Vps16 or Vps41 to phagosomes from p110α deficient cells when compared with control vacuoles (Fig. 3.9C). Taken together, these findings indicate that the Rab7 present on phagosomes from p110α knockdown cells was in an active state competent to engage downstream effectors essential for phagolysosome fusion. In addition, flow cytometric staining of phagosomes isolated from both control and p110α knockdown cells showed no significant differences in the levels of Rab7 or Vps41 (Fig. 3.9D), or in the cellular or phagosomal levels of the t-SNARE Vti1p and the v-SNARE VAMP7 (Fig. 3.9E). These SNAREs are part of the trans-SNARE complexes which were previously shown to be involved in mediating homo- and heterotypic late endosome-lysosome fusion (76,200-207). The presence of normal levels of key HOPS components and SNAREs indicates that the deficiency in delivery of lysosomal components to phagosomes in p110α knockdown cells is not due to a lack of membrane fusion machinery, but rather to an upstream event regulated by the PI3K p110α.

### 3.10 Discussion

While there are clear lines of evidence pointing to a role for the class III PI3K, Vps34, in mediating phagosome maturation events, recent studies have suggested that other classes of these lipid kinases may contribute to this process and to endosomal trafficking in general (45,46,54,55,64,183,208-216). Regulation of vacuole maturation is thought to be influenced by the ligands and receptors engaged in phagocytosis, although only very recently have there been
detailed studies examining the effects of specific ligand-receptor interactions on the evolving vacuole (12, 217, 218).

Previous work had indicated a need for class IA PI3Ks in phagocytosis per se of IgG-opsonized prey, and for hVps34 in mediating phagosome maturation (42). However, there are few reports to date examining the role PI3Ks play in the phagosome maturation of prey taken up by other means. A recent article directly comparing phagosomal actin dynamics resulting from uptake by FcyR or CR3 receptors noted the formation of phagosomal PI(3,4,5)P₃. However, the contribution of class IA PI3Ks to actin polymerization on late stage phagosomes was observed only in the case of CR3 (12). These results illustrate differential requirements for PI3K, dependent upon the specific phagocytic receptors that are engaged. In particular, this study highlighted the role of PI(3,4,5)P₃ in phagosome trafficking and the importance of class IA PI3K in development of the CR3 phagocytic vacuole. Our results indicate the involvement of a class IA PI3K in phagosome maturation, and show that the class IA isoform, p110α, plays a role in this process. In particular, we have shown that PI3K p110α is required for the delivery of lysosomal components to late stage phagosomes in the case of prey taken up by CR3 and non-specific phagocytic receptors. This advances the novel concept that multiple PI3K classes are involved in regulating phagosome maturation, with a requirement for class IA PI3K activity in regulating the delivery of late endosomal/lysosomal components.

At first glance, it might appear that some of our data were collected at relatively late time points, ranging from 2 to 6 h in some cases. In fact, this was not very unusual, as multiple studies using magnetic or latex beads have reported protracted phagosome maturation times
which may be due to the indestructible nature of the prey. Nonetheless, it is important to note that we observed significant differences in the acquisition of lysosomal LAMPs, β-galactosidase, and lysosome-associated fluorescent dextran in our p110α knockdown cells between 1-2 h post phagocytosis, and that these differences were only enhanced further at later time points.

Our data indicate that phagosomes from p110α deficient cells progress normally through early maturation, as they acquire normal levels of Rab5B and EEA-1 (Figure 3.4A). As it is known that EEA-1 recruitment to phagosomes is dependent on both the presence of Rab5 (62-64,83,220) and a phagosomal pool of PI3P, it is thus not surprising that EEA-1 recruitment was observed in phagosomes from p110α knockdown cells as these cells displayed normal levels of Rab5B and showed levels of phagosomal PI3P that were similar to control cells (Figure 3.3B). EEA-1 is thought to function as an early endosomal tethering molecule through its interactions with a proposed endosome fusion complex consisting of calmodulin, syntaxins 6 and 13 to bring about homotypic early endosome fusion events (60,61,63,65,221). In addition to normal Rab5B and EEA-1 recruitment, phagosomes from p110α deficient cells also showed abundant levels of Rab7 (Figure 3.8A), as well as the Rab7 effectors RILP and HOPS components Vps16 and Vps41 (Figure 3.8B and C). Despite this evidence for phagosomal accumulation of Rab7 competent to recruit its downstream effectors, p110α deficient cells showed clear defects in the delivery of late endosomal/lysosomal components (LAMPs, β-galactosidase, lysosome-associated fluorescent dextran). These findings indicated that Rab7 recruitment alone is insufficient to drive phagosome maturation towards phagolysosome fusion. Given that our results indicate that Rab7 by itself cannot bring about phagolysosome fusion in the absence of
p110α, clearly more work needs to be done to examine the possibility that additional factors modulated by this PI3K isoform also contribute to phagosome-lysosome interactions. Although we saw a modest but statistically significant decrease in PI(3,4,5)P₃ production in our p110α deficient cells (Figure 3.3B), this did not appear to be sufficient to account for the magnitude of the deficit in late endosome/lysosome interactions we observed (Figures 3.4B, 3.5, 3.6). This suggests that the presence of p110α itself may be required at the phagosome membrane in order to mediate its effects on maturation. In support of this, we found that p110α was recruited to phagosomes during the course of normal maturation, and that this was significantly abrogated in p110α knockdown cells (Figure 3.3A).

Defective delivery of LAMP-1 and LAMP-2 to phagosomes in p110α deficient cells (Figures 3.4B and 3.5A) indicated a block in interactions with late endosomal/lysosomal compartments. This block was not limited to membrane proteins alone, as recruitment of the luminal hydrolase, β-galactosidase, was also significantly impaired (Fig. 3.6). Confocal fluorescence microscopic analysis of endosomal trafficking in p110α knockdown cells showed no defects in endosomal localization or biosynthetic trafficking of LAMP-1 (Figure 3.4D) thus excluding the possibility of defective recruitment of LAMPs to phagosomes. Furthermore, these imaging results were consistent with a previous report showing that LAMP sorting from the TGN to late endosomes/lysosomes was not sensitive to wortmannin, leading to the conclusion that this process is PI3K independent (222). While we hypothesize that the abrogation of LAMP-1 and LAMP-2 delivery to phagosomes in p110α deficient cells reflected a defect in phagosome-late endosome/lysosome interactions, we cannot rule out the possibility that LAMP-1 and LAMP-2 themselves may also contribute to the maturation process per se. Indeed, recent
studies have indicated that these late endosomal/lysosomal membrane glycoproteins may be required for the fusion of lysosomes with phagosomes (223,224). Thus, it may be that the defect of phagosome-lysosome fusion resulting from p110α deficiency may be exacerbated by the lack of initial delivery of LAMP proteins that promote further phagosome-lysosome interactions. Future work involving the overexpression and engineered phagosomal delivery of LAMP proteins in p110α knockdown cells would clarify the precise contributions of these membrane glycoproteins to phagosome maturation, and how this correlates with PI3K p110α activity.

Notably, defective phagosomal acquisition of β-galactosidase did not coincide with a defect in cathepsin D delivery, despite the fact that these hydrolases share the mannose-6-phosphate receptor mediated pathway for endosomal targeting (225-229). Taken together, these findings suggest that the defective delivery of lysosomal proteins to phagosomes is most likely explained by a selective defect in the fusion of lysosomes with the phagosome, rather than by abnormal biosynthetic sorting of these proteins. This conclusion is supported by our confocal microscopy imaging results which showed that phagosomes in p110α knockdown cells were unable to interact with dextran-loaded lysosomes (Figure 3.5B), thus indicating defective phagolysosome fusion.

Despite defects in LAMP protein and lysosomal β-galactosidase acquisition, phagosomes from p110α cells still acquired and processed the protease cathepsin D, and acidified, albeit with slower kinetics as compared to control cells (Figures 3.7 and 3.8). These findings prompt us to propose that the phagosomal acquisition of cathepsin D and the v-ATPase required for
acidification likely take place at a late phagosome stage, but prior to bulk fusion with lysosomes. Consistent with such a model, previous studies reported that v-ATPase subunits may be delivered to phagosomes early via tubular extensions of lysosomal compartments (179,186,230,231). Alternatively, phagosomes may obtain cathepsin D and v-ATPase subunits through interactions with vesicles from the trans-Golgi network. In this regard, it has been observed that latex bead and mycobacterial phagosomes can acquire cathepsin D through vesicles from the biosynthetic pathway (232). Consistent with this, it has previously been shown that latex bead phagosomes also contain syntaxin 6 and cellubrevin (VAMP3), two SNAREs known to be involved in TGN to phagosome trafficking (38,233). Thus it is likely that the cathepsin D we found on p110α deficient phagosomes came directly from the TGN.

Cathepsin D is a major component of lysosomes, and its biosynthetic transport involves cycling through acidic late endosomes where proteolytic removal of the pro-peptide to produce the active intermediate form is believed to take place (227,229,234). We detected normal levels of active intermediate cathepsin D in late phagosome lysates from p110α knockdown cells (Figure 3.7). This suggests that despite a deficiency in p110α, phagosomes from these cells recruited cathepsin D either through direct TGN to phagosome transport, or by interactions with late endosomes. The finding that phagosomes from p110α deficient cells were capable of interacting readily with late endosomes was supported by the abundant presence of Rab7 on these phagosomes (Figure 3.9A) which has been shown to be required for cathepsin D delivery to these vesicles (93,94). Moreover, the Rab7 that we detected on phagosomes from p110α deficient cells was in an active conformation since both RILP and Vps41 were recruited to these organelles (Figure 3.9B and C), and both of these effectors
exclusively recognize GTP-bound Rab7 (71,193,195). However, despite our observation of the presence of cathepsin D in phagosomes from p110α deficient cells, we detected markedly reduced amounts of β-galactosidase and LAMPs, which also share a similar late endosome/lysosome subcellular localization (Figures 3.4B, 3.5A, and 3.6). In addition, we also saw a significant decrease in phagosome interactions with lysosomes loaded with fluorescent dextran (Figure 3.5B). Taken together, these results indicate that p110α is required for phagosome interactions with late endosomes/lysosomes, and that the presence of cathepsin D in these phagosomes most likely comes directly from the TGN.

Noteworthy, is the fact that Vps41 is a component of the HOPS complex. The multisubunit HOPS complex is made up of the class C Vps proteins Vps11, Vps16, Vps18, and Vps33 (which are also found in the endosomal CORVET complex), as well as the Rab7 effectors Vps39 and Vps41 (only found in the HOPS complex) (235,236). This complex serves to tether membranes together for fusion, and recent evidence points to a specific role in bridging Rab7 activation and SNARE binding (71,196-199). Our immunoblot data of HOPS protein recruitment specifically addresses recruitment of the vacuolar HOPS complex to phagosomes as opposed to endosomal CORVET, as we probed for Vps41, which is only found in the HOPS complex. As shown in Figure 3.9C, we found no significant differences in recruitment of HOPS complex to phagosomes from p110α deficient cells.

Although we did not detect any changes in HOPS complex recruitment to phagosomes in p110α knockdown cells, our data does not indicate whether this complex was in an active state competent to mediate SNARE activation. Interestingly, it has been documented in yeast
that increasing the levels of Ypt7-GTP (the yeast homologue of mammalian Rab7), results in inhibition of Yck3, a vacuolar casein kinase. Yck3 positively regulates HOPS complex mediation of membrane fusion by phosphorylating the HOPS component, Vps41 (237-239). Our immunoblot data of late stage phagosomes from p110α deficient cells showed nearly a two-fold increase in Rab7 levels compared to control cells (Figure 3.9A). This suggests the possibility of a negative feedback loop involving inhibition of the mammalian ortholog of Yck3 by excess Rab7 and downregulation of HOPS activity. The Western blotting and flow organellometry data showing the presence of both Vps41 and Vps16 on phagosomes from p110α deficient cells (Figure 3.9C and D) suggests that there is no deficiency in HOPS complex recruitment; however, whether this complex was active and capable of promoting SNARE assembly remains to be elucidated. The apparent normal recruitment of HOPS components, and our observations of similar levels of SNARE proteins on phagosomes isolated from control and knockdown cells, suggests that p110α deficient phagosomes should be at least partially competent to undergo membrane fusion events with late endosomes/lysosomes. On the other hand, defective delivery of LAMPs, β-galactosidase, and lysosome-associated fluorescent dextran to these phagosomes clearly indicates that there was a defect in late endosomal/lysosomal content delivery in p110α knockdown cells. Whether this was related to a lack of HOPS complex activation and SNARE priming for membrane fusion is presently unclear. Future work studying membrane fusion capability involving reconstitution of SNARE, HOPS, and phagosome components isolated from p110α cells would clarify how each of these specific factors contribute to the deficient phagolysosome fusion phenotype in these cells.
The findings reported above identify a specific role for the PI3K p110α in influencing phagosome-lysosome fusion. The phenotype of p110α deficient cells appears not to involve abnormal interactions with the TGN or abnormal biosynthetic sorting and most likely involves a defect in the fusion of late phagosomes with late endosomes/lysosomes. The regulatory node controlled by PI3K p110α is likely located parallel to Rab7 acquisition on the phagosome membrane. This conclusion is based upon our findings that although phagosomes isolated from p110α knockdown cells contained Rab7 and were capable of recruiting important effectors for membrane fusion (RILP, and HOPS component Vps41), a block in the delivery of late endosomal/lysosomal components (LAMPs, β-galactosidase, fluorescent dextran) was nevertheless observed. This interpretation is supported further by a previous finding that phagosomes in cells treated with the class I and III PI3K inhibitor wortmannin also had normal recruitment and activation of Rab7 and RILP, despite showing a profound inability to fuse with late endosomes/lysosomes (54). The latter findings and those reported here provide strong evidence that phagosome maturation requires an additional PI3K-mediated step in addition to Rab7 activity, and that Rab7 acquisition and activation are not sufficient to ensure complete maturation, i.e. fusion of phagosomes with lysosomes. In addition, we found that the delivery of cathepsin D and acidification of phagosomes occurred independently of the acquisition of LAMPs and β-galactosidase. Thus, cathepsin D and v-ATPase subunit recruitment may occur from direct TGN to phagosome transport. Taken together, these findings suggest a model of phagosome maturation in which phagosomes formed by ingestion of prey recruit Rab5 and EEA-1 independently of p110α PI3K activity. Rab7 and its downstream effectors are then recruited to phagosomes. However, acquisition of functional Rab7 is by itself not sufficient for
mediating fusion of the maturing phagosomes with late endosomes/lysosomes, which requires p110α activity. In conclusion, the data show that PI3K p110α plays a pivotal role in this process, and in its absence, completion of phagosome maturation does not occur.
CHAPTER 4: IDENTIFICATION OF CANDIDATE VIRULENCE FACTORS BY PATHOGEN EFFECTOR PROTEIN SCREENING IN YEAST (PEPSY)

4.1 Introduction

The intracellular pathogen Mtb disrupts phagosome maturation upon uptake into host macrophages, and this process plays a key role in the establishment of infection. Much work has been done to characterize the Mtb effectors that play a role in mediating the phagosome maturation block, and some Mtb protein and lipid effectors have been identified (Table 1.4). In addition to individual studies of particular proteins, three genomic screens have been done using BCG or Mtb transposon mutant libraries to look for mutants unable to resist phagosome acidification or phagolysosome fusion (164,170,171). However, it is not clear whether all of the proteins identified by these screens are directly involved in phagosome maturation arrest, as any effects on the intracellular viability of the bacteria would render them unable to resist phagosome acidification or phagolysosome fusion. Independent assessments for direct functionality against phagosome maturation are required for any candidates isolated from these screens. These may involve the coating of recombinant protein onto latex beads which are then used in maturation studies, or, should a mycobacterial context be required, ectopic expression in M. smegmatis, which normally cannot resist phagosome maturation. In order to screen for proteins with direct functionality against phagosome maturation we conducted a Mtb genomic screen in yeast to search for Mtb factors able to disrupt yeast VPS, a system that is highly homologous to mammalian endosomal sorting pathways (172,173). Previous studies...
have used yeast as a model in which to screen for *L. pneumophila* pathogen effectors that may influence mammalian phagosome trafficking as they may also interfere with the yeast VPS pathway (175,176). To determine whether this methodology would work to identify candidate Mtb factors, we screened our Mtb genomic library for proteins able to disrupt yeast VPS, and identified four candidate effector proteins which were able to recapitulate the phenotype of yeast VPS disruption when they were retransformed back into yeast.

### 4.2 Construction of H37Rv Genomic DNA Library in Yeast

Genomic DNA was isolated from the virulent Mtb strain H37Rv. This genomic DNA was then subjected to incomplete digestion with the restriction enzyme *Sau3A*I, and digested products were run out on an agarose gel (Figure 4.2A). Fragments between 550-6000 bp were excised and used for a ligation reaction with previously linearized yeast expression vector pWS93. The ligation reaction was then transformed into *E. coli* TOP10 cells. Approximately 6800 colonies were produced after this transformation reaction, which represents about three genome equivalents of the Mtb genome (see Figure 4.2B for calculation). These colonies were scraped, pooled together, and plasmids were isolated. Using this pool, the yeast strain NSY01 was transformed following the protocol described previously for *L. pneumophila* (176).
TO CALCULATE NUMBER OF CLONES TO SCREEN FOR EXHAUSTIVE LIBRARY SCREENING:

To find any one clone:

\[ N = \frac{\ln (1 - p)}{\ln (1 - f)} \]

where:
- \( N \) = Number of recombinants to screen to find any one clone
- \( p \) = Probability of finding any one clone
- \( f \) = Frequency of insert to genome size

Average size of DNA fragment in library = 2000 bp
Size of Mtb genome = 4.4 x 10^6 bp

\[ N = \frac{\ln (1 - 0.59)}{\ln (1 - (1/2000))} = \frac{\ln (1 - 0.59)}{\ln (4.4 \times 10^6/2000)} = \sim 10,130 \text{ colonies must be screened} \]

TO CALCULATE SIZE OF LIBRARY:

Obtained \sim 6800\ colonies

\[ \frac{6800 \times \text{average bp size of 2000}}{4.4 \times 10^6} = \sim 3 \text{ genomes' worth} \]

**Figure 4.2. Size of Mtb genomic DNA that was selected for library construction.** A) Mtb genomic DNA was partially digested with the restriction enzyme Sau3A1. Fragments between 550 - 6000 bp were excised and ligated into the yeast expression vector. B) Calculations to find the number of clones needed to be screened if the entire library were to be exhaustively screened. Approximately 67% of the library was screened, representing 3 genomes' worth of the Mtb genome.
4.3 Identification of Mtb Factors Able to Disrupt Yeast VPS

Transformed yeast clones were assessed for defects in VPS by overlaying plates with glucostat reagent. The yeast strain NSY01 expresses an invertase-Carboxypeptidase Y (Inv-CPY) fusion protein which is normally trafficked to the yeast vacuole (176). Disruption of the trafficking of Inv-CPY results in the protein being secreted extracellularly, and this can be detected by overlaying colonies with glucostat reagent containing sucrose, the substrate for invertase. The resulting glucose that is formed is utilized by glucose oxidase present in glucostat reagent to form a lactone and H$_2$O$_2$. The latter is used by horseradish peroxidase to oxidize the compound O-dianisidine, resulting in the formation of a brown-red product (176). Thus, yeast which display a protein trafficking defect to the yeast vacuole exhibit brown-red colouration when given glucostat reagent. Four candidate Mtb effector proteins were identified which were able to disrupt VPS upon re-transformation back into yeast (Figure 4.3). These are summarized in Table 4.3, and are discussed in the subsequent sections.
Figure 4.3. Yeast transformed with plasmids containing Mtb genomic DNA fragments display brown-red pigmentation indicative of a VPS trafficking defect. Plasmids were extracted from each yeast clone and were sequenced. Fragments bearing portions of the Mtb genes for Rv0425c, Rv0900, Rv1268c, and PE-PGRS62 were identified. The Mtb phosphatase SapM, previously characterized as being able to disrupt phagosome maturation in macrophages, was cloned into the yeast expression vector and transformed into yeast to ascertain whether this protein could also disrupt yeast VPS. VipD = *L. pneumophila* effector protein previously identified as being able to disrupt yeast VPS (176). pWS93 = Empty vector negative control.

### 4.4 CtpH (Rv0425c), Metal Cation Transporting P-Type ATPase

A 1206 bp fragment that encompasses amino acids 548-948 of the 1539 amino acid sequence predicted to make up the putative metal cation transporting P-type ATPase CtpH was identified through the yeast genomic screen. Although predicted to be a metal cation transporter, functional studies of this protein have not been done, and the identity of the metal cation that is transported is unknown. CtpH is considered essential for *in vitro* growth by the TraSH transposon mutagenesis studies done by Sassetti *et al.* (240).
4.5  Rv1268c, Secreted Hypothetical Protein

A 345 bp fragment corresponding to amino acids 117-232 of the 232 amino acid sequence predicted to make up the hypothetical protein Rv1268c was identified. This protein is considered non-essential for \textit{in vitro} growth based upon Himar1-based transposon mutagenesis in H37Rv and CDC1551 strains (240,241). An analysis of the predicted amino acid sequence of this hypothetical protein by the prediction program SignalP (v4.0,(242)) suggests that this protein contains an N-terminal secretion signal and signal peptide cleavage site between amino acids 32 and 33. To assess whether this protein is indeed expressed and secreted, we generated a \textit{M. smegmatis} construct able to express a Rv1268c-GFP fusion protein. Trichloroacetic acid precipitation of the culture filtrate proteins and subsequent probing with an antibody against the C-terminal GFP epitope suggested that this protein is expressed and secreted (Figure 4.5). However, in order to unequivocally confirm secretion, control experiments with \textit{M. smegmatis} expressing GFP fused to a non-secreted protein (such as the cytoplasmic protein GroEL) is required.
Table 4.3. Candidate virulence factors identified by PEPSY.

<table>
<thead>
<tr>
<th>Gene in H37Rv</th>
<th>Annotation</th>
<th>Proposed Function</th>
<th>Essentiality for in vitro Culture*</th>
<th>Location in the Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv0425c</td>
<td>CtpH</td>
<td>Possible metal cation transporting P-type ATPase</td>
<td>Yes (H37Rv), no (CDC1551)</td>
<td>Mycobacterial membrane (243)</td>
</tr>
<tr>
<td>Rv0900</td>
<td>N/A</td>
<td>Possible membrane protein, function unknown</td>
<td>Yes (H37Rv)</td>
<td>Unknown (membrane?)</td>
</tr>
<tr>
<td>Rv1268c</td>
<td>N/A</td>
<td>Hypothetical unknown protein</td>
<td>No (H37Rv, CDC1551)</td>
<td>Unknown, possible secretion signal at N-terminus</td>
</tr>
<tr>
<td>Rv3812</td>
<td>PE_PGRS62</td>
<td>PE_PGRS protein Function unknown, thought to be involved in virulence</td>
<td>No (H37Rv, CDC1551)</td>
<td>Unknown, some PE_PGRS proteins shown to be localized in cell wall or cell membrane (142,143), may be secreted (ESX-5) (140)</td>
</tr>
</tbody>
</table>

* Essentiality as determined by high density mutagenesis studies (240,241)
Figure 4.5. The protein encoded by Rv1268c is expressed and secreted from *M. smegmatis*. *M. smegmatis* expressing GFP-tagged Rv1268c protein was grown in Middlebrook 7H9 medium supplemented with 2% dextrose. Bacteria were pelleted, lysed, and the cell lysates were probed with an antibody against GFP. Cleared culture filtrates were TCA precipitated and also probed with anti-GFP. N = GFP fused at N-terminal end of Rv1268c, C = GFP fused at C-terminal end of Rv1268c. Numbers denote different *M. smegmatis* clones. Expected size of the fusion protein is approximately 52 kDa.

**4.6 Rv0900, Hypothetical Membrane Protein**

A 66 bp fragment corresponding to amino acids 27-49 of the predicted 50 amino acid sequence corresponding to the hypothetical membrane protein Rv0900 was found to disrupt yeast VPS. Rv0900 is predicted to belong to an operon containing the outer membrane protein OmpA and Rv0901. This protein is predicted to be essential for *in vitro* growth by TraSH high density mutagenesis (240). Although the transmembrane portion is predicted to span across amino acids 4-26 (TMHMM server, v2.0), the fragment isolated from the PEPSY screen does not contain this, and is thus predicted to be soluble.
4.7 PE-PGRS62 (Rv3812)

A 852 bp fragment corresponding to amino acids 89 to 373 of the predicted 504 amino acid sequence belonging to PE-PGRS62 was found to disrupt yeast VPS. This protein has been found to be non-essential for \textit{in vitro} growth by TraSH high density mutagenesis (240). PE-PGRS62 has been previously implicated in virulence, as its deletion from the pathogenic mycobacterium \textit{M. marinum} attenuated virulence (148). The protein is also expressed in infected macrophages and in frog granulomas during infection (148). Despite its links to virulence, the exact function of this protein is unknown. PE-PGRS proteins are thought to be secreted through the ESX-5 Type VII secretion system (140), and have been documented as cell wall proteins (141-143,149).

4.8 Discussion

Using a Mtb genomic screen in yeast, four candidate effector proteins were identified that were able to disrupt yeast VPS trafficking. The genes for these proteins are spread out along the Mtb genome, suggesting that an adequate amount of coverage was achieved. In addition, a range of fragment lengths were identified, ranging from 66 - 1206 bp, suggesting that the insert lengths in the plasmid library were of variable lengths. It should be noted that although we did not identify from our yeast screen any of the Mtb effectors known to play a role in disrupting phagosome maturation (see Table 1.3), we conducted only a partial screen of the H37Rv genomic library. In order to screen the library with 99% probability that any particular insert would be found, approximately 10,130 clones would have had to be screened.
(Figure 4.3). This is a conservative estimate, as this calculation is dependent on an average insert length of 2000 bp. If a large percentage of the library were to consist of insert lengths shorter than 2000 bp, the number of clones that would be needed to be screened in order to find any one particular sequence would be larger than 10,130. In actuality, approximately 6800 colonies were screened. Thus, it would seem unlikely that we would identify known Mtb effectors from our partial screen.

*L. pneumophila* is a gram negative bacterium that disrupts phagosome maturation as a pathogenic strategy (244). Upon entry into the macrophage, the *L. pneumophila*-containing vacuole is modified, resulting in its being isolated from the endosomal system and surrounded by endoplasmic reticulum (244-246). This mode of phagosome maturation disruption is different from that of Mtb. The Mtb vacuole interacts readily with early endosomes, as shown by its acquisition of transferrin receptors (247). The different modes of maturation arrest by *L. pneumophila* and Mtb begs the question as to whether the PEPSY screen would be able to detect Mtb effectors which may not function in a similar manner as those of *L. pneumophila*. The fact that we were successful in identifying Mtb candidates from our PEPSY screen [one of which, PE-PGRS62, was able to disrupt phagosome maturation in macrophages (described in Chapter 5)] confirms that PEPSY is able to detect Mtb effectors despite the dissimilarity between Mtb and *L. pneumophila*-mediated modes of arrest. Furthermore, expression of the known Mtb effector SapM (109) resulted in a positive signal for VPS disruption (Figure 4.3), suggesting that some Mtb effectors may retain their function in yeast and are able to disrupt VPS accordingly.
The four candidates identified from the PEPSY screen include a secreted protein (Rv1268c), a membrane protein (Rv0900), a metal cation-transporting P-type ATPase (Rv0425c), and a PE-PGRS protein (Rv3812). Our work with a M. smegmatis construct able to express Rv1268c suggests that this protein is indeed expressed and is secreted into culture filtrates. Further work exploring its secretion into the macrophage cytosol and its contribution to phagosome maturation inhibition may reveal whether this protein is a bona fide virulence factor in the context of mycobacterial infection.

Of interest was the identification of a mycobacterial P-type ATPase as an effector able to disrupt VPS trafficking in yeast. P-type ATPases (also known as E1-E2 ATPases) are a large, ubiquitous superfamily of integral membrane proteins involved in the transport of all the known biologically relevant cations (248). They are expressed in prokaryotes as well as eukaryotes and share several biochemical characteristics such as utilizing the energy harnessed from the hydrolysis of the terminal pyrophosphate bond in ATP for the transport of cations, possessing a high energy aspartyl phosphate intermediate, as well as existing in either of two intermediate conformations designated E1 and E2 (249). P-type ATPases are grouped into 4 major groups: 1.) Ca\textsuperscript{2+} transporting ATPases, 2.) Na\textsuperscript{+}/K\textsuperscript{+} ATPases and H\textsuperscript{+}/K\textsuperscript{+} ATPases, 3.) plasma membrane proton pumps of plants, fungi, lower eukaryotes, and 4.) all bacterial P-type ATPases except Salmonella typhimurium Mg\textsuperscript{2+} P-type ATPases MgtA and MgtB, which share closer similarities to mammalian Ca\textsuperscript{2+} ATPases. P-type ATPases may directly act to sequester essential cations from the phagosome lumen. It has also been suggested that P-type ATPases may play a role in maintaining the transmembrane electrochemical gradients required for the function of
other divalent cation transporters. A P-type ATPase has been previously hypothesized to play such a role in Mtb intraphagosomal survival by supporting the activity of the mycobacterial transporter Mramp. Mramp is the mycobacterial homologue of Nramp (natural resistance associated macrophage protein), a mammalian divalent cation transporter which has been shown to be recruited and maintained on the phagosome membrane during phagosome maturation (250). Mramp displays activity primarily against divalent transition metal cations that are used in redox reactions catalyzed by bacterial superoxide dismutase and catalase. Superoxide dismutase catalyzes the dismutation of superoxide to form hydrogen peroxide and oxygen, and catalase further breaks down hydrogen peroxide to form water and oxygen. As such, these two enzymes, when expressed by intracellular bacteria, function to protect bacteria against the reactive oxygen species formed by the macrophage during the oxidative burst. It is hypothesized that Nramp may carry out its protective role against intracellular bacterial infection by actively removing these necessary transition metal cations from the phagosome lumen, thereby preventing their use by intracellular pathogens such as mycobacteria. Mramp is speculated to compete with Nramp by allowing mycobacteria to actively uptake these same cations (251), and the activity of a yet unidentified P-type ATPase is thought to be necessary for Mramp function.

The mycobacterial protein PE-PGRS62 was also identified from our PEPSY screen. This protein has been linked to virulence (148), although a definitive function for it has not yet been assigned. Our results identifying this protein as being able to disrupt yeast VPS trafficking suggests that PE-PGRS62 may play a role in disrupting endosomal trafficking during infection. Given its previous links to virulence, PE-PGRS62 was selected for further characterization of its
effects on macrophage function during mycobacterial infection, which is the subject of the next chapter.
5.1 Introduction

Of the candidate Mtb factors found to be able to disrupt yeast VPS (Table 4.3), PE-PGRS62 was selected for further study of its effects on macrophage function. PE-PGRS62 was found to be expressed during macrophage infection and in infected frog granulomas, and the *M. marinum* transposon mutant for this protein is attenuated for virulence (148). Furthermore, patients with latent or active tuberculosis display strong antibody responses to PE-PGRS62, indicating that this protein is expressed and recognized by the host immune system during infection (252). However, while it is highly likely that PE-PGRS62 plays some essential role for mediating Mtb virulence, the mechanisms of its contribution to pathogenicity are unknown.

We identified PE-PGRS62 as a Mtb factor able to disrupt yeast VPS, a protein trafficking system that is highly homologous to mammalian endosomal sorting (Figure 4.3). Thus, it was logical to ask whether the ability of this protein to disrupt yeast VPS was predictive of its ability to disrupt phagosome maturation in macrophages. The ability of Mtb to block phagosome maturation is one of the key hallmarks of Mtb pathogenesis, and is essential for this pathogen's intracellular survival. Our identification of PE-PGRS62 as an Mtb factor able to disrupt yeast VPS suggested that this protein may also play a role in preventing phagosome maturation.

In addition to blocking phagosome maturation, Mtb also nullifies nitric oxide production by inhibiting macrophage activation. The expression of iNOS protein and the production of RNIs
constitute an important immune response to mycobacterial infection. Indeed, abrogation of this response by pharmacological inhibitors (118-120) or genetic deletion of the iNOS gene renders mice highly susceptible to Mtb infection (253,254). Although human macrophages were previously thought to be deficient in iNOS expression and the NO response, numerous studies have made it clear that human macrophages express iNOS protein and transcript in response to tuberculosis infection (255-258), and that iNOS activity suppresses Mtb growth in human macrophages (259). As part of our assessment of the ability of PE-PGRS62 to affect macrophage function, we also examined iNOS expression in infected murine macrophages to determine whether PE-PGRS62 expression influenced the ability of the infected macrophage to generate a nitric oxide response.

Cytokine expression has previously been shown to influence both phagosome maturation and the initiation of an effective nitric oxide response. Activation of macrophages by IFN-γ treatment results in iNOS expression and the maturation of mycobacterial phagosomes (260-262). In addition, the proinflammatory cytokines IL-6 and IL-12 have been shown to promote the expression of Rab5 and Rab7, respectively (263), and TNF-α treatment promotes the maturation of Mtb-containing phagosomes (264). The mycobacterial zinc metalloproteinase Zmp1 inhibits inflammasome activation and the production of IL-1β, which is processed to an active form after inflammasome activation (265). Deletion of the zmp1 gene in Mtb results in IL-1β production and promotes phagosome maturation (265). On the other hand, anti-inflammatory IL-10 has been shown to block maturation of Mtb-containing phagosomes (266,267). Both IL-6 and IL-10 production by mycobacterial-infected macrophages also induce the expression of Arginase 1, which serves to funnel arginine substrate away from iNOS,
resulting in less NO production (122). Given the mounting evidence that cytokine production can influence both phagosome maturation and the nitric oxide response in mycobacterial infections, we assessed whether PE-PGRS62 also played a role in modulating the macrophage cytokine response.

In order to examine the effects of PE-PGRS62 on phagosome maturation, iNOS expression, and cytokine production, we constructed an *M. smegmatis* transformant able to express PE-PGRS62 when induced with acetamide. We then examined whether this *M. smegmatis* was capable of expressing PE-PGRS62 for gain-of-function in terms of specific effects on phagosome maturation, iNOS expression, and cytokine modulation. We observed that macrophages infected with *M. smegmatis* expressing PE-PGRS62 show decreased Rab7 and LAMP-1 phagosomal acquisition, thus suggesting that this protein contributes to mycobacterial-mediated phagosome maturation inhibition. In addition, macrophages infected with *M. smegmatis* expressing PE-PGRS62 showed 3-4 fold less iNOS compared to cells infected with control bacteria. These effects on macrophage function did not appear to be related to cytokine expression, as no significant differences in the expression of IL-12, IL-10, TNF-α, or IL-1β were observed. Examination of a *M. marinum* transposon mutant for PE-PGRS62 for loss-of-function effects upon infection of macrophages revealed that macrophage infections with the transposon mutant elicited 3-4 fold greater iNOS expression than those cells infected with wildtype or complemented bacteria, thus supporting a role for PE-PGRS62 in downregulating iNOS expression in infected macrophages. These results suggest that PE-PGRS62 contributes significantly to inhibition of phagosome maturation and iNOS expression during mycobacterial infection.
5.2 Construction of *M. smegmatis* Expressing PE-PGRS62

The ordinarily non-pathogenic *M. smegmatis* does not contain a PE-PGRS62 ortholog. We took advantage of this deficiency to study the effects of PE-PGRS62 on macrophage function and generated an *M. smegmatis* strain that expressed histidine-tagged PE-PGRS62 upon induction with acetamide (Figure 5.2A). Moreover, subcellular fractionation of these clones identified the location of PE-PGRS62 as being on the cell wall (Figure 5.2B), in agreement with previous studies which identified PE-PGRS proteins as being cell wall proteins (141-143,149).

5.3 *M. smegmatis* Expressing PE-PGRS62 is Able to Block Phagosome Maturation

When J774A cells were infected with PE-PGRS62-expressing *M. smegmatis*, immunoblotting of phagosome lysates showed normal recruitment of the small GTPase Rab5 (Figure 5.3A). However, probing with an antibody against the late endosomal GTPase Rab7 showed a 50% reduction in phagosomal recruitment of Rab7 (Figure 5.3A). Phagosomes isolated from cells infected with *M. smegmatis*-PE-PGRS62 also showed low levels of Cathepsin D, although this was not found to be statistically significant. Additional confocal microscopic analysis of infected cells showed a significant decrease in LAMP-1 colocalization to
Figure 5.2. Induction of PE-PGRS62 expression in *M. smegmatis* transformants. A) *M. smegmatis* transformed with pJAK2.D-Rv3812 expresses histidine tagged fusion protein when induced with acetamide. Two different concentrations of acetamide were tested. The same clones were grown in normal dextrose-containing medium, and did not show expression of PE-PGRS62 fusion protein. B) Subcellular fractionation of *M. smegmatis* induced to express PE-PGRS62 show localization to the cell wall fraction. No signal was seen for fractions isolated from uninduced bacteria. The blot was also probed for the mycobacterial cytosolic chaperone GroEL2 to confirm fractionation efficacy.
phagosomes containing PE-PGRS62-expressing bacteria (Figure 5.3B, white highlighting indicates colocalization of bacterial containing phagosomes with LAMP-1 as determined by the Colocalization Highlighter plugin in Image J software, described in Materials and Methods). These findings established that expression of PE-PGRS62 in macrophages is linked to a phenotype of phagosome maturation arrest, and were consistent with the genetic screen in yeast showing disruption of VPS by PE-PGRS62. Moreover, *M. smegmatis* expressing PE-PGRS62 had increased survival in infected macrophages when compared to non-expressing bacteria (Figure 5.3C), thus suggesting that the ability of PE-PGRS62 to disrupt phagosome maturation significantly increases *M. smegmatis* persistence within cells.
Figure 5.3. Macrophages infected with *M. smegmatis* expressing PE-PGRS62 show phagosome maturation arrest. A) Phagosomes isolated from J774A cells infected with *M. smegmatis*
induced to express PE-PGRS62 showed normal acquisition of Rab5, but significantly decreased Rab7 acquisition when compared to phagosomes isolated from cells infected with uninduced control bacteria. Phagosomes containing *M. smegmatis* expressing PE-PGRS62 also show a trend towards lower Cathepsin D acquisition, although this was not found to be statistically significant. Blots are representative of three independent experiments. Densitometry shows mean +/- s.e.m. of three independent experiments. * p < 0.05. White bars = Uninduced, black bars = Induced. **B**) Cellular levels of Rab5, Rab7, and Cathepsin D do not change upon PE-PGRS62 expression, suggesting that differences seen in phagosome lysates are due to differential recruitment, and not because of changes to the expression of these markers. **C**) LAMP-1 colocalization was significantly decreased for phagosomes containing *M. smegmatis* induced to express PE-PGRS62 when compared to those infected with uninduced control bacteria. White arrowhead indicates the area of interest which is depicted in the enlarged panel. Colocalization is highlighted in white using the Colocalization Highlighter plugin from ImageJ. DAPI images have been pseudo-coloured red to allow colocalization analysis. Images are representative of three independent experiments and depict cells 6 h and 24 h post-infection. Bar graph to the right shows mean +/- s.e.m of colocalization of three independent experiments. * p < 0.05. White bars = Uninduced, black bars = Induced. **D**) *M. smegmatis* expressing PE-PGRS62 shows increased survival in macrophages. Lysates from 24 h-infected macrophages were plated onto Middlebrook 7H10 agar supplemented with OADC and colonies were counted after incubation at 37°C for two days. Shown are log of means +/- s.e.m of four independent experiments. * p < 0.05. White bars = Uninduced, black bars = Induced.

### 5.4 Macrophages Infected with Either *M. smegmatis* Expressing PE-PGRS62 or the *M. marinum* PE-PGRS62 Transposon Mutant Display Similar Cytokine Levels

The *M. marinum* ESX-5 mutant, which displays defective secretion of PGRS proteins (140), elicits increased levels of the proinflammatory cytokines TNF-α, IL-12p40, and IL-6, and decreased levels of IL-1β in infected macrophages (177). Because these findings suggested that PGRS proteins may influence cytokine responses, we examined whether our PE-PGRS62-expressing *M. smegmatis* could modulate the macrophage cytokine response. Assessment of transcript levels for TNF-α, IL-1β, IL-12p40, and IL-10 in infected macrophages showed no significant differences in cells infected with either *M. smegmatis* expressing PE-PGRS62 or the *M. marinum* PE-PGRS62 transposon mutant when compared to control or complemented or
wild-type strains (Figure 5.4). Moreover, these results do not appear to reflect a saturated response to MOI, as infection with a lower MOI (3:1) also did not reveal any significant differences in cytokine expression. Thus, barring any regulatory effects on cytokine protein levels, while other members of the PE-PGRS family may modulate macrophage cytokine responses, our results do not appear to support such a role for PE-PGRS62.
Figure 5.4. PE-PGRS62 expression does not modulate the cytokine responses of infected macrophages. Cytokine analysis of TNF-α, IL-12p40, IL-10, and IL-1β of J774A cells infected with either _M. smegmatis_ expressing PE-PGRS62 or _M. marinum_ deficient for PE-PGRS62 show no significant differences when compared to cells infected with control uninduced bacteria or other control strains. Tn = Transposon mutant for PE-PGRS62, Cm = Complemented mutant, Wt = wildtype.
5.5 PE-PGRS62 Modulates iNOS Protein Expression in Infected Macrophages

The expression of iNOS and the formation of RNIs is a potent anti-mycobactericidal strategy utilized by macrophages to combat infection. To this end, we sought to determine if iNOS levels are differentially regulated in macrophages infected with *M. smegmatis* expressing PE-PGRS62. Immunoblotting of cell lysates at different times post infection indicated a significant decrease in the levels of iNOS protein in J774A cells infected with *M. smegmatis* expressing PE-PGRS62 (Figure 5.5A). Moreover, these differences in iNOS expression appeared to be restricted to the protein level, as the levels of iNOS transcripts in cells infected with *M. smegmatis* expressing PE-PGRS62 were not significantly different from those found in control-infected cells (Figure 5.5B). Notably, J774A cells infected with the *M. marinum* transposon mutant for PE-PGRS62 showed 2-3 fold greater iNOS protein than did cells infected with either the wild-type or complemented strains (Figure 5.5C). This difference in iNOS expression was again restricted to the protein level, as analysis of iNOS transcript levels showed no significant differences between the treatment groups (Figure 5.5D). Taken together, these results show that PE-PGRS62 is able to down regulate iNOS expression in infected macrophages, and it does this at a post-transcriptional level.
Figure 5.5. Murine macrophages infected with mycobacteria expressing PE-PGRS62 display inhibited protein iNOS expression. A) J774A cells infected with M. smegmatis expressing PE-PGRS62 showed significantly reduced iNOS protein expression when compared to cells infected with control uninduced bacteria. Blot is representative of five independent experiments. PE-PGRS62\(^+\) = bacteria induced to express PE-PGRS62, PE-PGRS62\(^-\) = uninduced bacteria. The bar graph to the right shows densitometry of 24h and 30h time points from five independent
experiments.** p < 0.01. B) Analysis of iNOS transcript levels from cells infected with *M. smegmatis* expressing PE-PGRS62 showed no significant differences when compared to cells infected with control uninduced bacteria. C) J774A cells infected with the *M. marinum* transposon mutant for PE-PGRS62 expressed 2-3 fold more iNOS protein when compared to cells infected with wild-type or complemented strains. Blot is representative of three independent experiments. Tn = PE-PGRS62 transposon mutant, Cm = Complemented strain, Wt = Wild-type strain. The bar graph to the right shows densitometry from three independent experiments. * p < 0.05. D) Analysis of iNOS transcript levels from cells infected with the *M. marinum* transposon PE-PGRS62 mutant shows no significant differences when compared to cells infected with wild-type or complemented strains.

5.6 *M. smegmatis* Expressing PE-PGRS62 and the *M. marinum* PE-PGRS62 Transposon Mutant Display Altered Colony Morphology

A previous study found that overexpression of the PGRS domain of Rv1818c in *M. smegmatis* and *M. tuberculosis* modified bacterial cell structure (143). To assess any changes in colony morphology related to PE-PGRS62 expression, we spotted equal amounts of *M. smegmatis* induced to express PE-PGRS62 and uninduced control bacteria onto 7H10 agar plates supplemented with Middlebrook OADC enrichment. The results showed significant (Figure 5.6A) colony morphology differences in *M. smegmatis* induced to express PE-PGRS62, and these disappeared upon growth on solid media in which Tween-80 had been added. The latter suggests that these differences may be attributable to changes in cell wall lipid composition. In addition, the *M. marinum* transposon mutant for PE-PGRS62 displayed a marked sensitivity to the addition of Tween-80 in the solid media, as colony growth was significantly impaired (Figure 5.6B) when compared with the wild-type and complemented strains. Sensitivity to detergent was demonstrated in a SDS resistance assay where the *M. marinum* wildtype, complemented, and PE-PGRS62 transposon mutant were grown in
Middlebrook 7H9 media supplemented with ADC and 0.1% SDS. The PE-PGRS62 transposon mutant displayed decreased survival when grown in medium containing SDS (Figure 5.6C), suggesting that lack of PE-PGRS62 expression resulted in increased fragility of the cell wall. Such modifications to cell wall structure may be due to changes in lipid composition.
Figure 5.6. Expression of PE-PGRS62 alters mycobacterial colony morphology. A) *M. smegmatis* induced to express PE-PGRS62 and the *M. marinum* transposon mutant for PE-PGRS62 show similar growth in liquid culture compared to their wild-type and complemented counterparts. B) *M. smegmatis* expressing PE-PGRS62 shows differences in colony morphology when compared to bacteria containing empty vector. Induced = plates containing acetamide, which induces PE-PGRS62 expression, Uninduced = plates containing dextrose, which does not
induce PE-PGRS62 expression. Bacteria grown on plates containing Tween-80 do not display appreciable differences in colony morphology. C) The *M. marinum* transposon mutant for PE-PGRS62 (Tn PE-PGRS62) shows growth retardation on plates containing Tween-80 when compared to wild-type (wt) or complemented strains (Cm PE-PGRS62). D) The *M. marinum* transposon mutant for PE-PGRS62 shows increased sensitivity to SDS. *M. marinum* strains were grown in Middlebrook 7H9 media supplemented with ADC and 0.1% SDS. Means +/- s.e.m. of percent survival relative to wildtype bacteria from three independent experiments are shown. *p < 0.05.
5.8 Discussion

Interest in the potential functions of mycobacterial PE-PGRS proteins is currently the focus of active research. PE/PPE proteins are estimated to make up approximately 10% of the coding potential of the *M. tuberculosis* and *M. marinum* genomes and have been implicated in virulence (141-143,148); however, detailed information regarding the specific functions of these proteins is scarce. The high sequence variability between members of the PE/PPE family has been suggested to act as a form of antigenic variation to aid in mycobacterial immune evasion (141,268,269). Microarray data of PE/PPE gene expression has shown that individual proteins of this family are variably regulated by different growth conditions and do not appear to be regulated as a group (168). The authors of this study viewed this as support for PE/PPE protein function in antigenic variation. However, an alternative explanation is that variability in expression could also reflect the fact that specific PE/PPE proteins may have discrete functions and are regulated by different environmental conditions. Recent studies have indicated specific roles for PE-PGRS proteins such as PE-PGRS11 (implicated in mediating mycobacterial resistance to oxidative stress) (165) and PE-PGRS63 (lipase activity) (270). Beyond this, there is very limited information available about the functional activities of this group of proteins and their roles in host-pathogen interaction. In the present study, we identified two novel roles for PE-PGRS62 specifically in mediating phagosome maturation arrest and in inhibition of macrophage iNOS expression.

Our results show that *M. smegmatis* expressing PE-PGRS62 was able to inhibit phagosome maturation post Rab5 acquisition by blocking the acquisition of Rab7 (Figure 5.3A).
A trend towards lower levels of Cathepsin D was also observed in phagosomes from cells infected with *M. smegmatis* expressing PE-PGRS62 (Figure 5.3A), although this was not found to be statistically significant. Interestingly, direct transport of Cathepsin D to phagosomes from the TGN has been previously observed (38). The acquisition of Cathepsin D by phagosomes containing *M. smegmatis* expressing PE-PGRS62 suggests that TGN to phagosome transport of Cathepsin D is unaffected by PE-PGRS62 expression, and that PE-PGRS62 may specifically affect endosome/lysosome-phagosome interactions. This is supported by the observation of a corresponding deficit in LAMP-1 recruitment to phagosomes containing PE-PGRS62 positive *M. smegmatis* (Figure 5.3B). The phenotype of the arrested *M. tuberculosis* phagosome may involve either a block at the junction between Rab5 loss and Rab7 acquisition onto the phagosome membrane (92-95) or the inactivation of Rab7 function (97,98). Thus, it is striking that the sole expression of PE-PGRS62 in *M. smegmatis* was sufficient to recapitulate one of the phenotypes of maturation arrest brought about by infection with *M. tuberculosis*. Moreover, expression of PE-PGRS62 promoted the survival of non-pathogenic *M. smegmatis* within infected macrophages (Figure 5.3D). This suggests that the inhibition of phagosome maturation or iNOS expression (see below) both by PE-PGRS62 is sufficient to confer a significant (nearly two orders of magnitude) survival advantage to *M. smegmatis* during macrophage infection. Regarding potential underlying mechanisms, subcellular fractionation analysis showed localization of PE-PGRS62 in the mycobacterial cell wall (Figure 5.2B), which renders it accessible to macrophage host proteins. Thus, it is possible that PE-PGRS62 may interact with host phagocytic receptors or phagosomal proteins thereby disrupting critical interactions between endosomal vesicles.
In addition to its role in disrupting phagosome maturation, macrophages infected with PE-PGRS62 expressing *M. smegmatis* showed a deficiency in induction of iNOS relative to cells infected with control bacteria (Figure 5.5A). This implicates PE-PGRS62 in modulating the macrophage nitric oxide response. In support of this, macrophages infected with the *M. marinum* transposon mutant for PE-PGRS62 expressed 2-3 fold more iNOS protein when compared to cells infected with wild-type bacteria (Figure 5.5C). Reinstatement of PE-PGRS62 expression to the transposon mutant resulted in restoration of decreased iNOS levels comparable to those of macrophages infected with the complemented and wildtype strains (Figure 5.5C). Remarkably, these differences in iNOS protein levels did not appear to be due to changes in iNOS gene transcription. Assessment of transcript levels in macrophages infected with the *M. marinum* PE-PGRS62 transposon mutant, wild-type or complemented strains showed no significant differences in levels of iNOS transcripts. Nor could we detect any differences in cytokine expression profiles (Figure 5.4) which could be expected to affect iNOS gene expression. Macrophages infected with *M. smegmatis* expressing PE-PGRS62 also displayed similar levels of iNOS and cytokine transcripts when compared to cells infected with control bacteria (Figures 5.5B and 5.4 respectively). These results suggest that PE-PGRS62 modulates iNOS expression at a post-transcriptional stage, possibly through iNOS protein proteasomal degradation. The mechanism of PE-PGRS62 mediated inhibition of iNOS protein expression is currently being investigated.

Subcellular localization analysis identified PE-PGRS62 as being present on the cell wall of *M. smegmatis* when it was ectopically expressed (Figure 5.2B). Although ESX-5- mediated secretion has been identified as being necessary for PGRS protein secretion in *M. marinum, M.*
smegmatis lacks the ESX-5 locus (139,140). This suggests that PE-PGRS62 may be one of several PGRS proteins which do not require ESX-5 for secretion to the cell wall. Other precedents for this include: PE-PGRS11, PE-PGRS33 (Rv1818c) and Rv1917c (143,156,165). In silico analysis of the amino acid sequence for PE-PGRS62 using SignalP (version 4.0 (242)) did not indicate the presence of an N-terminal peptide signal sequence for Sec-dependent translocation (data not shown), suggesting that PE-PGRS62 may be delivered to the mycobacterial cell surface through an alternative translocation system yet to be identified.

In the context of any discussion regarding PE-PGRS62-mediated effects on phagosome maturation arrest and inhibition of iNOS expression, it is important to note that both M. smegmatis expressing PE-PGRS62 and the M. marinum transposon mutant displayed significant differences in colony morphology when compared to control, wild-type, and complemented strains. The M. marinum transposon mutant for PE-PGRS62 showed significant growth impairment on solid media containing Tween-80 (Figure 5.6B). This sensitivity to the addition of detergent is similar to that seen for the mmaA4 M. tuberculosis mutant lacking the ability to impart distal oxygen-containing modifications to its mycolic acids (271). In addition, the M. marinum mutant for PE-PGRS62 showed significantly increased sensitivity to SDS when added to growth medium (Figure 5.6C), suggesting increased cell wall permeability. Thus, sensitivity to detergents suggests significant changes to cell wall structure and lipid composition that may be dependent on PE-PGRS62 expression. Expression of PE-PGRS62 in M. smegmatis also resulted in notable differences in colony morphology when compared to control (Figure 5.6A). It was observed previously that expression of other PE-PGRS proteins in M. smegmatis (PE-PGRS11, PE-PGRS33) also resulted in changes to colony morphology (143,165), although the qualitative
morphological changes observed in each case were distinct. It is tempting to speculate that PE-PGRS62 (and PE-PGRS11 and 33) modulate some aspects of mycobacterial cell wall lipid composition which may contribute to virulence. In the case of PE-PGRS62, the expression of this protein was sufficient to confer upon *M. smegmatis* the ability to block phagosome maturation and impair iNOS expression. Whether the mechanism behind these two phenotypes is the result of differential cell wall lipid composition is the subject of ongoing investigation.

In our studies, we have used *M. marinum* as a model for a virulent mycobacterium. We did not study an *M. tuberculosis* mutant for PE-PGRS62; although a transposon mutant for PE-PGRS62 has been generated, we have found that this mutant is not viable in culture. *M. marinum* is genetically very similar to Mtb, with 3000 orthologs being shared between these two mycobacterial species (272). *M. marinum* is documented to escape from phagosomes within cells as part of its pathogenic strategy (273,274), and although Mtb was thought to reside exclusively within phagosomes in infected cells, there have been reports of Mtb escape from phagosomes (275-277). A recent study, using quantitative FRET-based measurements to track cytosolic access of Mtb, appears to confirm that Mtb does indeed access the macrophage cytosol, and that like *M. marinum*, this is dependent on ESX-1 expression (278).

Notwithstanding debate on the similarities between *M. marinum* and *M. tuberculosis* pathogenic strategies, our work with *M. smegmatis* expressing PE-PGRS62 from Mtb strongly suggests that this protein is involved in phagosome maturation arrest and the inhibition of iNOS protein expression (Figures 5.3 and 5.5), and confers a gain-of-function advantage to this non-pathogenic mycobacterium. It is thus likely that PE-PGRS62 is one of many Mtb effectors which contribute to mycobacterial pathogenesis.
In conclusion, our results assign novel roles for PE-PGRS62 in mediating mycobacterial virulence. PE-PGRS62 is able to inhibit phagosome maturation in infected macrophages and in parallel, impair iNOS expression, thereby defusing two important microbicidal strategies in the macrophage arsenal. This dynamic combination of phagosome maturation block and nitric oxide evasion is all the more remarkable in that it appears to require only the expression of this one PE-PGRS protein to bring about these phenotypes. PE-PGRS62 thus represents a potentially important and novel mycobacterial virulence factor.
CHAPTER 6: CONCLUSIONS AND DISCUSSION

6.1 The Class IA PI3K p110α Regulates Phagosome Maturation

PI3K-mediated regulation of phagosome maturation was thought to be restricted to the activity of the class III isoform hVPS34. Our results with two human monocytic cell lines deficient for p110α expression indicate that this class IA PI3K also plays a role in regulating the maturation process. We showed that phagosomes acquire p110α, and contain the class I PI3K product, PI(3,4,5)P3 (Figure 3.3). Cells deficient in p110α displayed a marked decrease in phagosomal LAMP-1 and LAMP-2 acquisition (Figures 3.4B and 3.5A), and showed a reduction in lysosomal β-galactosidase activity (Figure 3.6). Moreover, phagosomes lacking p110α were impaired in their ability to fuse with lysosomes loaded with fluorescent dextran, suggesting that phagolysosome fusion was defective (Figure 3.5B). However, phagosomal recruitment of cathepsin D was not affected by p110α knockdown, and acidification, although delayed, still occurred, suggesting that phagosomes from p110α deficient cells may acquire cathepsin D and vacuolar ATPases through pathways other than from lysosomes, most likely from the TGN (Figures 3.7 and 3.8). Our results show a novel function for p110α in regulating phagosome maturation in parallel to Rab7 acquisition, and suggest that an additional PI3K-dependent step is needed for phagolysosomal fusion. Interestingly, such a step was predicted from a previous study of PI3K regulation of phagosome maturation. This study found that wortmannin treatment did not preclude Rab7 recruitment to phagosomes or its activation, yet a defect in phagolysosome fusion was still observed (54). This result suggested that an additional PI3K-
dependent step was needed, in conjunction with Rab7 activity, to enable phagolysosome fusion (54). We propose that p110α mediates such a step, and that both p110α activity and Rab7 acquisition are needed for phagosome maturation completion. These findings represent a significant advance to our understanding of phagosome maturation regulation, and may provide insight into mechanisms used by pathogens to curtail this microbicidal process.

6.2 Possible Mechanisms of Action

Our results indicate a role for p110α in regulating phagosome maturation. How this class IA PI3K accomplishes this task is at present unclear, and is the subject of ongoing studies. Some insight into the potential mechanism of p110α-mediated regulation of the maturation process may be gleaned from current studies of Akt involvement in phagosome maturation. Constitutively active Akt promotes phagolysosome fusion and LAMP-1 acquisition of Francisella tularensis phagosomes, while bone marrow derived macrophages from SHIP−/− mice show greater numbers of phagolysosome fusion and LAMP-1 acquisition events when compared to cells isolated from SHIP+/+ mice (279). These results suggest that Akt may promote phagosome maturation in macrophages. It is thus highly probable that p110α mediates Akt activation through production of PI(3,4,5)P3 to drive maturation towards completion.

Further studies of Akt involvement in phagosome maturation suggest a role for this serine/threonine kinase in Rab GTPase activation. In addition to Rab5 and Rab7, other Rab GTPases have also been implicated in regulating phagosome maturation. Rab14, for example, has been shown to promote phagosome maturation by recruiting lysosomes to phagosomes
containing apoptotic cell corpses in *C. elegans* (280). Recruitment of Rab14 is dependent on Rab5 and PI3P, as loss of Rab5 or hVps34 function abrogated Rab14 recruitment to phagosomes (280). More evidence for a role for Rab14 in regulating phagosome maturation was shown in a study that found that its retention on phagosomes contributed to maturation arrest of the Mtb vacuole. This study attributed Rab14 regulation of phagosome maturation to the persistent presence of Rab14 promoting prolonged early endosome-phagosome interactions, thus retarding maturation (100). Although numerous studies have implicated Rab14 as having a role in promoting biosynthetic/recycling sorting between the Golgi and early endosomes in mammalian cells (281-283), RabD, the Rab14 homolog in *Dictyostelium discoideum*, appears to regulate homotypic phagosome and lysosome interactions in this single-celled eukaryote, suggesting that this Rab may participate in different areas of the endosomal network depending on the model system being investigated (284).

Although its recruitment appears to be dependent on both Rab5 and PI3P (280), Rab14 function is also regulated by Akt. PI3K-induced activation of Akt has been shown to bring about phosphorylation of the Akt substrate Rab GAP AS160 (285). This results in the inactivation of AS160 and prevents it from binding to membranes (286). As AS160 is the GAP for Rab14 (286), inactivation of the former prolongs both the activation state of Rab14 and its retention on phagosomes, thereby contributing to phagosome maturation arrest. In support of these roles for Akt and AS160 in preventing effective phagosome maturation and the development of an optimal microbicidal vacuole, it was found that Akt inhibitors reduced the intracellular survival of *S. typhimurium*. It was inferred from this result that by preventing Akt mediated inactivation of AS160, dissociation of phagosomal Rab14 was no longer impaired and phagosome
maturation leading to fusion with lysosomes was able to proceed (287). Although this study did not observe Akt on *S. typhimurium* phagosomes (287), Akt has been found on EEA-1 positive endosomes (169), and FcγR phagosomes (82). Akt on FcγR phagosomes is inhibited by recruitment of the Rab5 effector and Akt interactor, APPL1 (adapter protein containing pleckstrin homology domain, PTB phosphotyrosine-binding domain, and leucine zipper/bin-amphiphysin-rvs domain 1), which inhibits Akt activity by promoting the recruitment of inositol 5- phosphatases (82). Although the latter study did not examine the consequences of Akt phagosomal recruitment and deactivation, it seems plausible that active phagosomal Akt may promote and prolong Rab14 mediated early endosome- phagosome interactions by inactivating Rab14 GAP AS160. Conversely, the coordinate or sequential inactivation of Akt by APPL1 via inositol 5-phosphatase removal of the class I PI3K substrate PI(4,5)P₂ might be expected to release AS160 from Akt-mediated inhibition, thereby promoting both Rab14 dissociation from the phagosome membrane and phagolysosome fusion. Thus, completion of phagosome maturation appears to be dependent on the coordinate activation and inactivation of Akt, AS160 and Rab14. Under conditions in which Akt recruitment to phagosomes is impaired, this might be expected to result in constitutively active AS160, which would prevent Rab14 activation and acquisition onto phagosomes. The lack of Rab14 would thus impede the recruitment of factors yet to be identified from the early endosomal network which are needed to drive the phagosome down the maturation pathway. Thus, Rab14 acquisition to and its subsequent loss from the phagosome are both needed to ensure completion of phagosome maturation. With respect to recruitment of Akt to phagosomes, APPL1 has been documented to bind to both p110α and inactive Akt in a trimolecular complex (288). Given this interaction, it is
reasonable to consider that loss of p110α would impact Akt phagosomal recruitment, leading to an inhibitory effect on phagosome maturation. This may explain why our results with p110α deficient cells show a profound decrease in late endosomal/lysosomal marker delivery that does not appear to be explained by the comparatively modest decrease in PI(3,4,5)P3 we observed on phagosomes isolated from these cells (Figure 3.3). If APPL1, acting as an adaptor, interacts specifically with p110α, and serves to physically link Akt activation with p110α activity by interacting with both of these proteins, loss of p110α expression should significantly affect Akt activation and its contribution to phagosome maturation. In support of this mechanism of action, reprobing our phagosome immunoblots for Akt revealed that phagosomes from p110α deficient cells show less Akt recruitment when compared to phagosomes isolated from control cells (Figure 6.2B). With regards to APPL1 levels, we have not yet probed our phagosome lysates for APPL1. However, APPL1 recruitment is dependent on active Rab5 (82), and because phagosomal Rab5 levels do not appear to be affected by p110α knockdown (Figure 3.4A), we would expect to see normal APPL1 recruitment to p110α deficient phagosomes. If this is confirmed, then the question arises as to why, despite the presence of APPL1 and phagosomal PI(3,4,5)P3 in p110α knockdown cells (Figure 3.3B), Akt recruitment is specifically impaired. Presumably, the PI(3,4,5)P3 that is generated on the phagosomal membrane by the activity of the other class IA PI3K isoforms would be sufficient to promote Akt recruitment. However, the significant decrease in Akt in phagosomes from p110α knockdown cells (Figure 6.2B) suggests that this is not the case, and implies that p110α is specifically required for Akt recruitment. Thus, one potential explanation for this may be that Akt activation on the vacuole strictly requires APPL1 interacting with p110α and this cannot be achieved simply by the presence of
PI(3,4,5)P3. Consistent with this hypothesis, only p110α has been documented to interact with APPL1 (288). The inability of either p110β or p110δ to interact with APPL1 may explain why they do not compensate for the absence of p110α. Taken together, these findings suggest that p110α modulation of Akt activity may serve as a regulatory point for phagosome maturation. Future work aimed at dissecting the mechanisms involved may include the identification of novel effectors that interact selectively with p110α. In addition, examining the recruitment and activation of phagosomal Akt in cells expressing catalytically inactive p110α should allow us to determine whether the activity of this isoform is specifically required for these events. These types of approaches should further our understanding of the processes that regulate phagosome maturation.

6.3 Significance of p110α Regulation of Phagosome Maturation

The identification of the class IA PI3K p110α as a regulator of phagosome maturation represents a novel and fundamental advance in our understanding of this key innate immune response. It demonstrates unambiguously that PI3K-dependent regulatory nodes that are independent of hVps34 activity influence the maturation process. This is a significant observation that challenges the tenet that amongst all PI3Ks, only the class III isoform hVps34 regulates phagosome maturation. This novel finding is noteworthy on multiple levels. Many intracellular pathogens, such as Mtb, inhibit phagosome maturation in order to survive within the host cell. Although the phagosome maturation defect mediated by Mtb ManLAM has been attributed in part to its modulation of hVps34 activity and PI3P levels, (40,109), there are indications that Mtb modulation of p110α activity may also play a role in preventing
phagosome maturation. For example, our laboratory has previously found that 1α,25-
dihydroxyvitamin D₃ treatment reversed the phagosome maturation inhibition imposed by
ManLAM, and rescue was PI3K dependent (183). Indeed, the class IA PI3K p110α was found to
be activated by 1α,25-dihydroxyvitamin D₃ treatment (184) and we have shown in this
dissertation that p110α regulates phagolysosome formation. Taken together, these results
suggest that the phagosome maturation defect imposed by Mtb may also involve upstream
disruption of p110α activity, and that restoration of p110α activity by 1α,25-dihydroxyvitamin
D₃ treatment overrides maturation inhibition. Further studies examining how changes in p110α
activity, subcellular localization or both, contribute to inhibition of phagosome maturation by
Mtb and other pathogens such as *L. pneumophila, Salmonella,* and *Chlamydia* may provide new
insights into virulence mechanisms targeting class IA PI3K. This in turn will likely lead to the
discovery of novel pathogen effectors that may serve as therapeutic targets for the treatment
of tuberculosis or other infectious diseases. In addition, should disruption of p110α activity be
found to play a key role in microbial pathogenesis, stimulation of this PI3K isoform by
pharmacological agents may prove to be beneficial in activating phagosome maturation. This
would result in decreased pathogen survival, and may enhance antigen presentation
Figure 6.2. Potential mechanism of action for p110α regulation of phagosome maturation. A) Schematic illustrating putative p110α involvement in phagosome maturation. (1) In wild-type cells, the adaptor protein APPL1 is recruited by active Rab5, and interacts with both p110α and Akt on the phagosome membrane. PI(3,4,5)P₃ is produced, which further secures Akt on the phagosomal membrane and allows for its activation. In p110α knockdown cells, Rab5 is present and APPL1 is recruited. However, p110α is not present and PI(3,4,5)P₃ is not generated. (2) In wild-type cells, active Akt inhibits the Rab14 GAP AS160. In p110α deficient cells, Akt is lost from the phagosome, and cannot inhibit the activity of AS160. (3) In wild-type cells, the inhibition of AS160 allows GTP-Rab14 to be recruited to the phagosome membrane. In p110α
knockdown cells, AS160 is active, and inhibits Rab14 activation. (4) In wild-type cells, GTP-bound Rab14 mediates interactions of the maturing phagosome with early endosomes (EE), thus allowing the recruitment of as yet unidentified factors that function in mediating interactions between the maturing phagosomes with lysosomes. In p110α knockdown cells, Rab14 is GDP-bound and is inactive. This abrogates interactions of the phagosome with EE, and factors are not recruited. (5) In wild-type cells, acquisition of factors from EE, in addition to Rab7 activity, are all needed in order for phagosomes to mature into phagolysosomes. Phagosomes from p110α knockdown cells lack these factors, and phagosome maturation is arrested. B) Phagosomes from p110α deficient cells show decreased Akt recruitment. Western blotting of phagosome lysates revealed a significant reduction in Akt associated with phagosomes from p110α knockdown cells when compared to control cells. The bar graph to the left represents densitometry of three different blots. Open bar = phagosomes from control cells, solid bar = phagosomes from p110α knockdown cells. A representative blot is shown (reprobed blot from Figure 3.7).

capabilities and cell mediated immunity. All of these factors are needed to ensure effective and lasting immunity against intracellular pathogens such as Mtb.

### 6.4 Pathogen Effector Protein Screening in Yeast Allows for the Identification of Novel Candidate Mtb Virulence Factors

Using PEPSY, we were able to identify several Mtb proteins that appeared to disrupt yeast vacuolar protein sorting. Subsequently, we demonstrated that one of these factors, PE-PGRS62, was able to impart a phenotype of phagosome maturation arrest in macrophages infected with *M. smegmatis* expressing PE-PGRS62 (Figure 5.3). This result confirms that pathogen factors that are able to disrupt yeast VPS are able to reproduce this phenotype and block trafficking pathways in mammalian cells, thereby validating the use of PEPSY as a methodology for identifying candidate effector proteins. The advantages of using yeast as a model system in which to identify candidate factors include the genetic tractability of the
organism, potential for high-throughput assays with low biosafety requirements, and the possession of highly characterized, fundamental pathways (such as protein trafficking) that are conserved amongst all eukaryotic organisms. Most importantly, one is able to identify potential pathogen effectors directly based on their functionality, independent of any indirect effects on the robustness of pathogens that confound screens that use mutagenized organisms. As of this dissertation's writing, three independent screens have been done searching for Mtb or BCG mutants unable to resist phagolysosome fusion or phagosome acidification (164,170,171). Although informative, all of these screens suffer from the same caveat - they were unable to distinguish whether the proteins thus identified were functionally responsible for modulating phagosome maturation, or whether the abnormal phenotype (phagosome acidification or phagolysosome formation) resulted simply from the inability of the mutants to survive upon infection. On the other hand, screening Mtb proteins in yeast allows for the identification of effectors solely based on their functionality, without the possibility of confounding results due to reduced viability.

As with all genomic screens, candidates identified from PEPSY must be validated in the pathogenic organism of interest and in mammalian cells. However, the playing field can be narrowed down considerably by prior screening with PEPSY. In addition, the use of PEPSY allows for the identification of novel candidate effectors, and thus opens up new exploratory avenues for study.
6.5 **Mycobacterial PE-PGRS62 is a Virulence Factor That Disrupts Phagosome Maturation and Inhibits iNOS Expression**

Although it had been previously implicated in mediating mycobacterial virulence (148), the exact contribution of PE-PGRS62 to pathogenicity was unknown. We have identified PE-PGRS62 as a novel mycobacterial virulence factor whose expression impairs phagosome maturation (Figure 5.3) and decreases iNOS expression (Figure 5.5). This represents a considerable advance in the general understanding of PE-PGRS proteins, as the roles played by this large mycobacterial protein family in virulence have been elusive. PE-PGRS proteins were proposed to function as a form of antigenic variation in order to escape immune system recognition (141,268,269). However, instead of passively acting as a tool for immune evasion, our research shows that PE-PGRS62 performs specific roles in pathogenesis by subverting phagosome maturation prior to Rab7 acquisition, and by dampening RNI formation by inhibiting the expression of iNOS. The significant survival advantage conferred by PE-PGRS62 expression to *M. smegmatis* during macrophage infection (Figure 5.3D) strongly suggests that this virulence factor contributes to mycobacterial pathogenicity.

The inhibition of iNOS expression by PE-PGRS62 represents a novel virulence mechanism for mycobacteria. Up to now, mycobacterial evasion of the nitric oxide response has been characterized as involving the depletion of arginine usage by Arginase I (122), and disrupted trafficking of iNOS protein to mycobacteria-containing phagosomes (123,124). Our results indicate yet another layer of evasion exists, one that involves inhibition of iNOS protein expression. This appears not to involve modulation of iNOS transcription, but rather an effect
on iNOS protein translation or stability. This post-transcriptional regulation of iNOS expression is an entirely novel virulence mechanism which, to our knowledge, has not been previously observed in other studies of microbial pathogenesis. The post-transcriptional regulation of iNOS expression by PE-PGRS62 may involve the modulation of arginine levels that results in prevention of iNOS translation (289) or by increasing the expression of miRNA-939, which has been shown to downregulate iNOS expression by binding to the 3′-UTR of iNOS mRNA (290). Given that PE-PGRS62 expression appears to profoundly affect the lipid composition of the mycobacterial cell wall, it is likely that the modulation of iNOS expression, and that of phagosome maturation inhibition, may be attributable to the presence of some as yet unidentified lipid effectors. Lipid effectors, such as ManLAM (40) and phenolic glycolipid (107), have been previously identified as negative regulators of phagosome maturation. Quantitative or qualitative alterations in particular lipids on the cell wall of PE-PGRS62-expressing mycobacteria may also activate signalling pathways that serve to negatively regulate iNOS protein translation or enhance its proteasomal degradation. Future work with lipidomics and mass spectrometry will allow us to identify specific changes in lipid species present in PE-PGRS62-expressing mycobacteria and to examine how they may contribute to the phenotypes of phagosome maturation arrest and inhibition of iNOS expression.

Our work with *M. smegmatis* expressing PE-PGRS62 indicates that this protein is able to be translocated to the cell wall (Figure 5.2). Although PE-PGRS proteins have been previously characterized to be secreted through the ESX-5 Type VII secretion system (140), *M. smegmatis* does not possess an ESX-5 locus (139,140). This indicates that PE-PGRS62 is secreted through alternative secretion systems other than ESX-5. Recently, a signal motif for Type VII secretion
has been identified. It was observed that PE/PPE proteins with a YxxxD/E motif located at the C-terminus were secreted through either ESX-1 or ESX-5 systems (291). *In silico* analysis of the amino acid sequence of PE-PGRS62 identified the presence of such a motif at position 87, located at the end of the conserved PE domain near the N-terminal end of the protein. Given that PE-PGRS proteins all possess a conserved PE domain, but variable C-terminal PGRS domains, it is possible that the YxxxE motif in PE-PGRS62 may allow it to be secreted through a Type VII secretion system distinct from ESX-5, such as ESX-1, which is present in *M. smegmatis* (292,293). Future work studying the mechanisms of PE-PGRS62 export to the cell surface will be of special interest given the effect this protein apparently has on mycobacterial cell wall lipid composition (Figure 5.6).

Because of the localization of PE-PGRS62 to the mycobacterial cell wall, how this protein mediates its effects on disrupting phagosome maturation and iNOS expression through the phagosome membrane needs to be addressed. It is possible that during uptake, PE-PGRS62 may interact with plasma membrane receptors which would then trigger signalling cascades resulting in the downregulation of phagosome maturation and iNOS expression. For example, uptake of mycobacteria through mannose receptors has been shown to contribute to phagosome maturation arrest (294). We have conducted experiments blocking the uptake of *M. marinum* with cytochalasin D treatment, and have determined that phagocytosis of bacteria is needed to induce iNOS expression in infected macrophages (data not shown). This suggests that PE-PGRS62 modulates its effects upon uptake into phagosomes. Within phagosomes, the protein may alter signalling by intraphagosomal receptors to bring about its inhibitory effects on maturation and iNOS expression. Indeed, the recently characterized SLAM receptor
promotes phagosome maturation and NADPH oxidase activity from within \textit{E. coli} containing phagosomes \cite{18}, and it is possible that phagosome-bound PE-PGRS62 may interact with other as yet uncharacterized SLAM receptors to modulate maturation and iNOS expression. Alternatively, PE-PGRS62 or its active proteolytically cleaved fragments may be shed into the phagosomal lumen, where they may be transported out of the phagosome. A recent study has determined that Mtb phagosomes are permeabilized by the ESX-1 secreted protein ESAT-6, and this allows host cytosolic proteins to access phagosomal bacteria \cite{295}. In such a scenario, shed PE-PGRS62 (either whole protein or fragments) could also access the macrophage cytosol, where this candidate virulence factor could mediate its effects on macrophage function.

6.6 Significance of PE-PGRS62 as a Mycobacterial Virulence Factor

Given our finding that PE-PGRS62 is localized to the mycobacterial cell wall, and that its expression profoundly affects cell wall morphology, prevention of PE-PGRS62 expression or its transport to the mycobacterial cell wall may be an effective antimycobacterial strategy. Indeed, \textit{in silico} analysis of metabolic pathways in mycobacteria has identified cell wall lipid biosynthesis as a promising target area for new drug discovery \cite{296}. Targeting the lipid biosynthetic pathways has been proven to be a highly effective antimycobacterial strategy. This is exemplified by the first line drugs isoniazid and ethambutol, both of which target cell wall lipid biosynthesis and which have been used successfully for more than fifty years to treat tuberculosis \cite{297}. The finding that PE-PGRS62 is expressed throughout both latent and active tuberculosis \cite{252} suggests that any new drugs with activity against this protein or which prevent its expression will most likely be effective throughout the stages of the disease. Such
pharmaceutical agents are in high demand, as current antimycobacterial drug therapy suffers from its ineffectualness during the dormant stage of the disease. This is the primary reason why current drug treatment regimens take months or years to complete. Indeed, the rise of drug resistant forms of Mtb may be attributable to ineffective treatment against the different metabolic populations of bacteria that exist during infection and patient non-compliance (298). Thus, the addition of novel drugs that show potency even during latent tuberculosis is a significant step towards reducing treatment times and retarding the emergence of drug resistant Mtb. As our results indicate that PE-PGRS62 plays a role in mediating phagosome maturation inhibition as well as decreasing iNOS expression, drug targeting of PE-PGRS62 expression, activity, or transport to the cell wall may negate these essential mycobacterial survival strategies and render the bacterium particularly sensitive to drug treatment. Thus, drug targeting of PE-PGRS62 may prove to be particularly effective for ensuring the elimination of the intracellular mycobacteria population present during the course of the disease.

6.7 Conclusions and Final Summary

The overall goal of this dissertation was to examine the effects of both host and pathogen factors on phagosome maturation. The first aim was to examine whether a host factor, the class IA PI3K p110α, contributes to maturation regulation. Using cells in which p110α expression was silenced by both constitutive and inducible systems, we determined that this PI3K isoform does indeed play a role in regulating phagosome maturation. p110α activity is
needed to ensure the effective delivery of the late endosomal/lysosomal LAMPs, as well as the lysosomal hydrolase β-galactosidase. Cells in which p110α expression had been silenced also displayed a significant decrease in phagolysosome fusion. Despite the lack of lysosomal components, phagosomes from p110α deficient cells acquired active Rab7 and the Rab7 effectors, RILP and HOPS. These results suggest that p110α activity is needed to mediate a PI3K-dependent step that acts in parallel to Rab7 activity in order to promote phagolysosome formation, and reveal that another PI3K, in addition to hVps34, regulates phagosome maturation.

The second aim of this dissertation was to examine how the intracellular pathogen, *Mycobacterium tuberculosis*, disrupts phagosome maturation by screening in yeast for effectors able to disrupt vacuolar sorting pathways (VPS). The latter pathways are highly homologous to mammalian endosomal trafficking, and pathogen factors able to disrupt yeast VPS may retain their function in blocking phagosome maturation in macrophages. A partial screen yielded four candidate Mtb proteins that were able to disrupt yeast VPS, the hypothetical proteins Rv0900 and Rv1268c, the metal cation-transporting P-type ATPase Rv0425c, and PE-PGRS62 (Rv3812).

The third aim of this dissertation was to examine in detail one of the candidate factors identified from the yeast screen for effects on macrophage function. PE-PGRS62 was chosen as this protein had been previously implicated in virulence (148), but its function was unknown. We generated a *M. smegmatis* construct which was able to express PE-PGRS62 when induced with acetamide, then used this construct to infect murine macrophages. Cells that had been infected with *M. smegmatis* expressing PE-PGRS62 displayed a significant decrease in Rab7 and
LAMP-1 acquisition, thus indicating that this protein played a role in mediating phagosome maturation arrest. This result validated the yeast screen and suggested that candidates shown to disrupt yeast VPS could also inhibit phagosome maturation in macrophages. *M. smegmatis* expressing PE-PGRS62 also showed increased survival within macrophages, suggesting that this protein is important for mycobacterial persistence during cellular infection. Further examination of the effect of PE-PGRS62 expression on macrophage function showed that in addition to mediating phagosome maturation arrest, this PE-PGRS protein also inhibited the expression of iNOS by a factor of 2-3 fold. Correspondingly, cells infected with a *M. marinum* PE-PGRS62 transposon mutant showed 2-3 fold greater iNOS expression when compared to cells infected with wildtype or complemented bacteria. These results suggest that PE-PGRS62 inhibits iNOS expression, and it does this at a post-transcriptional stage, as iNOS transcript levels were unchanged. Bacteria expressing PE-PGRS62 showed significant changes in their colony morphology, suggesting that PE-PGRS62 may alter the presence of immunomodulatory cell wall lipids or structure. Taken together, our results confirm that PE-PGRS62 is a *bona fide* virulence factor able to contribute to two essential mycobacterial immune evasion strategies, the inhibition of phagosome maturation and the macrophage nitric oxide response.
REFERENCES


