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Defining the function of the Chediak-Higashi Syndrome related protein, LvsB, in *Dictyostelium discoideum*: Functional interactions that antagonize vesicle fusion

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Dedication

This work is dedicated to my family.

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Defining the function of the Chediak-Higashi Syndrome related protein, LvsB, in *Dictyostelium discoideum*: Functional interactions that antagonize vesicle fusion

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Lesions in the human Lyst gene are associated with the lysosomal disorder Chediak Higashi Syndrome. The absence of Lyst causes the formation of enlarged lysosome related compartments in all cells. This defect results in severe immunodeficiency, neurological dysfunction, and ultimately in death. Despite decades of research, the mechanism for how these enlarged compartments arise is not well established. Two opposing models have been proposed for Lyst function. The fission model describes Lyst as a positive regulator of fission from lysosomal compartments, while the fusion model identifies Lyst as a negative regulator of fusion between lysosomes. To date, a consensus on which model is correct has not been reached.

This thesis details my investigation of Lyst function using *Dictyostelium discoideum*. To establish a definitive model for the function of the *Dictyostelium* Lyst ortholog, LvsB, we used assays that distinguish between defects in vesicle fusion versus fission. We compared the phenotype of cells defective in LvsB with that of two known fission defect mutants (µ3 and WASH null mutants). The temporal localization characteristics of the post-lysosomal marker vacuolin, as well as vesicular acidity and fusion dynamics of LvsB null cells are distinct from those of both fission defect mutants. These distinctions are predicted by the fusion defect model and implicate LvsB as a negative regulator of vesicle fusion.

This work also presents evidence that LvsB antagonizes the function of two fusion regulatory proteins, Rab14 and dLIP5. The *Dictyostelium* Rab14 GTPase is known to stimulate lysosome fusion, and here we implicate dLIP5 as a promoter of Rab14 activity. Constitutive activation of Rab14 increases vesicle fusion in wild type cells but not in dLIP5 mutant cells. Thus, Rab14 activity is dependent on dLIP5. Additionally, the aberrant vesicle morphology and fusion phenotypes of LvsB mutant cells are suppressed

by expression of dominant inactive Rab14 or disruption of dLIP5. This suppression suggests that LvsB antagonizes Rab14 activity to negatively regulate vesicle fusion.

These studies validate the fusion model for LvsB function and provide new insights into the relationships that dictate vesicle fusion regulation. By extension, we propose that Lyst negatively regulates vesicle fusion by antagonizing the activity of a RabGTPase.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

1.1 Chediak Higashi Syndrome

This dissertation describes my efforts to dissect the functional contribution of the *Dictyostelium discoideum* LvsB protein along the endo-lysosomal pathway and the interplay that LvsB may have with other proteins along that pathway. My hope is that these studies will provide the basis for a better understanding of the cellular defect contributing to the manifestation of the devastating genetic disorder, Chediak Higashi Syndrome.

Chediak Higashi syndrome (CHS) is a lethal autosomal recessive disorder that was first described in 1943 and later defined by Chediak (1952), and Higashi (1954). Both studies described patients with similar symptomatic attributes including hypopigmentation, recurrent infections, and hepatosplenomegaly. Even before the identification of lysosomes, Higashi described the disorder as being characterized by a general enlargement of peroxisomes. CHS has since been characterized as a lysosomal trafficking disorder, and true to this designation, CHS presents with a multitude of symptoms resulting from defects in processing along the endo-lysosomal pathway. All cells of CHS patients are characterized by the presence of greatly enlarged lysosomes or lysosome related compartments (Dell'Angelica et al., 2000). However, the most deleterious effects of these mutant compartments are observed in specialized cells such as lymphocytes (Stinchcombe et al., 2000), neutrophils (Kjeldsen et al., 1998), and melanocytes (Introne et al., 1999). The resulting sub-functionality of these specialized cells culminates in symptoms including partial oculocutaneous albinism (OCA), immune deficiency and neutropenia as hallmarks of the CHS phenotype (Introne et al., 1999).

Most patients that are diagnosed with CHS succumb to one of many recurrent infections, or to the onset of a lymphoproliferative infiltration of their major organs called the “accelerated phase”. Currently, it is unclear what causes the accelerated phase. To date the only treatment options available to CHS patients are administration of prophylactic antibiotics, or hematopoietic cell transplantation (HTC). While HTC is sufficient to ameliorate immune system complications as well as defective clotting in

these patients, it does not prevent the late onset neurological dysfunction that is also associated with the disorder.

1.2 Identification of Lyst and the BEACH family of proteins

Cloning of the human CHS locus and the mouse ortholog, *beige*, allowed the molecular analysis of the Lyst protein encoded by these genes (Barbosa et al., 1996; Nagle et al., 1996). The Lyst protein is the product of a single gene and is highly conserved in both sequence and function in mammals. Lyst is the founding member of the BEACH (Beige and Chediak Higashi) family of proteins which are found across all eukaryotes (Wang et al., 2002). Sequencing of the *lyst* gene in CHS patients that presented with variable symptom penetrance revealed that severe childhood onset CHS is associated with either nonsense, or frameshift mutations while milder cases are the product of missense mutations (Nagle et al., 1996; Karim et al., 2002). The degree of functional conservation of Lyst orthologues was demonstrated by the ability of the mouse beige gene to rescue the enlarged lysosomal morphology in human CHS cells (Barbosa et al., 1996; Nagle et al., 1996). However, despite decades of research, the characterization of mammalian Lyst has proven to be a monumental task due to its large size (~430 KDa) and minimal expression level (Perou et al., 1997; Wang et al., 2002). Currently, the localization and molecular function of human Lyst remains unknown.

1.2.1 Common structural features of BEACH family proteins

Members of the BEACH family share common features in their structure as well as proposed function. Structurally, these proteins have a divergent N-terminal domain adjacent to a highly conserved BEACH domain (enriched in tryptophan, isoleucine, aspartic acid, and leucine) that is followed by multiple WD-40 (tryptophan-aspartic acid) repeats. WD-40 repeats are commonly known to participate in protein-protein interactions, but the function of the BEACH domain is yet undefined. More recently, a region upstream of the BEACH domain in multiple BEACH proteins was described as being structurally similar to a pleckstrin-homology (PH) domain (Jogl et al., 2002).

Classical PH domains are involved in binding to phospholipids or in protein-protein interactions.

One study found that the PH like domain of the human BEACH protein, FAN (factor associated with neutral sphingomyelinase activation), had binding affinity for phosphoinositide (4,5) phosphate (PI-(4,5)-P), and is required for the localization of FAN to the plasma membrane (Haubert et al., 2007). However, this PI-(4,5)-P binding property of the FAN PH domain requires basic residues in the canonical PIP binding fold, and this cluster of basic residues is missing from the PH domains of other BEACH proteins (Gebauer et al., 2004; Lemmon, 2004; Haubert et al., 2007). Accordingly, a PIP binding panel showed that the PH domains of the BEACH family proteins NBEA (neurobeachin) and LRBA did not have binding affinity for any of the Phospho-inositides tested (Gebauer et al., 2004). Alternatively, a structural analysis of the BEACH protein PH-like domain suggests that it is capable of protein-protein interactions and is specifically suggested to interact with the adjacent BEACH domain (Jogl et al., 2002; Gebauer et al., 2004). Consistent with these structural predictions, in vitro binding studies demonstrated the binding of the PH domain to the BEACH domain of FAN is essential for its function *in vivo* (Haubert et al., 2007). Despite the highly conserved nature of the BEACH and PH domains amongst BEACH family proteins, they have done little more than provide inklings as to how these proteins might function.

1.2.2 Functional characterization of BEACH family proteins

Functional studies of BEACH family proteins seem to indicate a common theme with respect to their cellular activities. Though all of the characterized BEACH proteins have different expression patterns and localizations, they all seem to be involved in regulating membrane trafficking. Apart from Lyst, the most extensively studied BEACH domain-containing protein is neurobeachin (NBEA). NBEA has a predominantly neuronal expression pattern and loss of NBEA appears to correlate with autism (Castermans et al., 2003; Medrihan et al., 2009; Castermans et al., 2010; Nuytens et al., 2013). Sub-cellular localization studies in rats showed that NBEA localizes to pleiomorphic vesicular structures and is also found to concentrate on a sub-population of post-synaptic membranes (Wang et al., 2000). Studies in multiple model systems have

implicated NBEA in regulating the secretion of vesicles at the neuro-muscular junction (de Souza et al., 2007; Medrihan et al., 2009; del Pino et al., 2011; Niesmann et al., 2011; Nair et al., 2013).

Another BEACH family protein that is very similar in domain organization to NBEA is LRBA (lipopolysaccharide-responsive, beige-like anchor protein). Like Lyst, LRBA has a more ubiquitous expression pattern, and mutations in LRBA are associated with deleterious alterations in immune cell function (Lopez-Herrera et al., 2012). Initial *in vivo* characterization in macrophages demonstrated that LRBA localizes to the membranes of lysosomes, TGN, ER, and endocytic vesicles in response to lipopolysaccharide stimulation (Wang et al., 2001). This localization pattern is indicative of a functional role in vesicular trafficking, and assessment of immune cells from patients with defects in LRBA provided evidence that LRBA plays an important role in the process of autophagy associated with B-cell activation (Lopez-Herrera et al., 2012).

The only other human BEACH family protein that has been functionally characterized is ALFY. Unlike its other BEACH domain containing relatives, ALFY contains a FYVE zinc finger domain at its C-terminus (Simonsen et al., 2004). Though canonical FYVE domains target their respective proteins to PI(3)P enriched endosomal membranes, ALFY is not found on endosomes. Instead, it is localized on autophagosomes and co-localizes with clusters of ubiquitylated protein aggregates under stress conditions (Simonsen et al., 2004). As with the other described BEACH domain proteins, this localization pattern is indicative of the regulatory role that ALFY plays in selective autophagy of ubiquitylated protein aggregates (Filimonenko et al., 2010). The function of homologous BEACH proteins in lower eukaryotes is surprisingly well conserved. Characterization of the NBEA/LRBA homologue, sel-2, in *C. elegans* reported co-localization with lysosomal structures in non-neuronal cells. Similar to the vesicular trafficking defects described for both NBEA and LRBA, the disruption of sel-2 in these cells induced a delay in trafficking to late endosomes and lysosomes (de Souza et al., 2007). The *Drosophila* ALFY homologue, Bchs (blue cheese), has also been found to have significant functional similarities to its human counterpart. Bchs expression is most prominent in neural tissues, and it co-localizes with late endo-lysosomal markers in motor neurons during specific stages of embryonic development (Lim and Kraut, 2009).

A genetic modifier screen done by Simonsen et al (2007) identified multiple genetic interactions between *bchs* and genes for lysosomal trafficking proteins, and *in vivo* vesicle kinetics studies demonstrated that Bchs mutant neurons have defects in anterograde axonal vesicle trafficking (Lim and Kraut, 2009). As with many vesicle trafficking mutants, the defects in trafficking associated with abrogation of Bchs ultimately lead to neural degeneration (Finley et al., 2003).

Though each of these BEACH proteins has a unique vesicle trafficking role, all of the compartments that are influenced by these trafficking events intersect with the endo-lysosomal pathway. The protein of focus in this dissertation, Lyst, does not waver from this membrane trafficking regulatory trend. In fact, just as it was the founding member of the BEACH family of proteins, Lyst was also the first to be implicated in regulating membrane traffic along the endo-lysosomal pathway.

1.2.3 BEACH Protein interactions with RabGTPases

Though the involvement of BEACH proteins in the regulation of membrane trafficking is well established, the mechanisms and protein interactions that contribute to their functions are largely unknown. In more recent studies, there have been reports of functional interactions between BEACH family proteins and Rab GTPases. Rab GTPases are master regulators of membrane trafficking, and they are known to participate in vesicle formation as well as vesicle docking and fusion. The specific role of Rab proteins in vesicle fusion is elaborated below.

The first report of a possible functional relationship between a BEACH family protein and a Rab GTPase was in *Drosophila*. In a screen for genetic modifiers of the *Drosophila* Alfy orthologue, Bchs, Rab11 GTPase was identified as an enhancer of the Bchs overexpression phenotype (Khodosh et al., 2006). Rab11 functions as a molecular switch to regulate vesicular recycling from the perinuclear recycling compartment as well as exocytosis of biosynthetic cargo through interaction with the exocyst tethering complex (Ren et al., 1998; Zhang et al., 2004; Beronja et al., 2005; Satoh et al., 2005). These Rab11 functions are required for *Drosophila* development and the absence of Rab11 is lethal. The few flies that survive to adulthood despite the absence of Rab11 have defective neuromuscular junction (NMJ) and sensory bristle formation. Both of

these processes require exocyst mediated membrane addition. The antagonistic nature of the interaction between Rab11 and Bchs was elucidated by the suppression of all three of these Rab11 mutant phenotypes in response to loss of Bchs. Additionally, Bchs is found exclusively in the membrane fraction and co-localizes with Rab11 on vesicles near the synapse (Khodosh et al., 2006).

Similarly, the *Dictyostelium* BEACH family protein, LvsA, is reported to functionally interact with a Rab GTPase that participates in osmoregulation. LvsA is known to localize to the membrane of a specialized osmoregulatory organelle called the contractile vacuole (CV) and is required for its function (Gerald et al., 2002). The CV is a membranous system of interconnected tubules and bladders that is exclusively found in protozoa and *Dictyostelium*, and is used to maintain cell integrity under hypo-osmotic conditions. When *Dictyostelium* cells encounter a hypo-osmotic environment, ions are pumped into the lumen of the CV which causes the subsequent influx of excess water from the cytosol. Once the CV bladder is full, it forms a temporary fusion pore with the plasma membrane and expels its contents. This expulsion causes the CV bladder to collapse and form a temporary patch at the plasma membrane before fragmenting and reforming tubules (Heuser et al., 1993). Similar to fusion of exocytic vesicles with the plasma membrane, the *Dictyostelium* equivalent to the exocyst complex is involved in emptying of CV bladders by pore formation at the plasma membrane (Zanchi et al., 2010). As mentioned above, the assembly of exocyst at sites of exocytosis is a Rab GTPase dependent process, and in *Dictyostelium* the localization of exocyst to CV bladders requires Rab8a in its activated, GTP bound, form (Essid et al., 2012). The BEACH family protein, LvsA, also localizes to CV bladders and is essential for their function (Gerald et al., 2002). A link between LvsA and Rab8a was indicated by the ability of constitutively active Rab8a to significantly suppress the formation of aberrant CV structures in LvsA null cells (Essid et al., 2012).

These reported functional interactions of BEACH proteins with Rab GTPases are intriguing because Rab proteins are important regulators of the membrane trafficking processes associated with BEACH family proteins. This raises the possibility that all BEACH family members influence their respective membrane trafficking events by interacting with Rab proteins.

1.3 Rab GTPases and vesicle fusion

Rab GTPases interact with a diverse set of effector proteins to coordinate many aspects of membrane trafficking including endocytosis, vesicle uncoating, vesicle movement along cytoskeletal filaments, as well as vesicle docking and fusion. Rab proteins accomplish this diverse set of functions by reversibly associating with their respective membranes and directing the localization and activity of proteins that are required for each of these processes. Of particular interest is the role that Rab proteins play in vesicle fusion, as much of the current data implicate BEACH proteins in fusion mediated processes. Initial contact between a vesicle and its target membrane is made by elongated proteins and protein complexes called tethering proteins. This connection must be made before the complementary SNARE proteins from each membrane can form a complex (Ungermann et al., 1998).

The importance of tether recruitment by Rabs for downstream fusion events was elucidated by investigation of the early endosomal (EE) associated GTPase Rab5 in both yeast and mammalian cells. Active (GTP bound) Rab5 physically interacts with EE tethering proteins, and is required for their recruitment to the membrane (Horazdovsky et al., 1996; Simonsen et al., 1998; Nielsen et al., 2000). The now "activated" tethers form a bridge between the membranes that are destined for fusion and also perpetuate the fusion process by interacting with SNARE regulatory proteins as well as SNARE complex proteins (Simonsen et al., 1998; McBride et al., 1999; Simonsen et al., 1999; Morrison et al., 2008). In a similar scenario, Rab27a promotes SNARE complex formation for docking of exocytic vesicles with the plasma membrane indirectly through its effector proteins (Fukuda, 2005; Gomi et al., 2005; Tsuboi and Fukuda, 2005; Tsuboi and Fukuda, 2006).

The transition between Rab proteins along the endo-lysosomal pathway establishes changes in fusion properties that promote maturation of vesicles. This transition is often choreographed by the Rabs themselves. A well characterized and eloquent example of this is the transition of Rab5 to Rab7 as early endosomes become late endosomes. Rab5 elicits homotypic fusion amongst early endosomes by recruiting the CORVET tethering complex (Gorvel et al., 1991; Horazdovsky et al., 1996; Rubino et al., 2000; Balderhaar et al., 2013). Several of the core subunits of CORVET are shared with its successor, the

HOPS complex, which tethers late endosomes to lysosomes (Solinger and Spang, 2013). As early endosomes mature, Rab5 initiates a transition in their fusion specificity by recruiting the HOPS specific sub-units to replace CORVET. HOPS then participates in the recruitment of Rab7 (Rink et al., 2005). To complete the transition, Rab7 is suggested to recruit a Rab5 GAP for the deactivation and removal of Rab5 from the membrane (Del Conte-Zerial et al., 2008). The evidence for interaction of BEACH family proteins as antagonists of Rab function warrants the question of how they fit into these Rab activation and deactivation processes.

1.4 Lysosome related organelles in specialized secretory cells

Interestingly, while the majority of mammalian cells have an endosomal pathway that terminates at the lysosomal stage, the cells types that are most severely affected in CHS patients contain specialized compartments that mature beyond the lysosomal stage. The classical symptoms of CHS arise due to dysfunction of cells that rely heavily on regulated secretion of lysosome related organelles (LRO). Furthermore, sub-cellular characterization indicates that all of the currently well characterized LROs are perturbed in CHS patients including the significant enlargement of melanosomes, lytic granules, MHC (major histocompatibility complex) class II compartments, and Azurophil granules as well as the absence of platelet dense granules (Dell'Angelica et al., 2000).

Unlike the secretory granules found in endocrine cells which mature as distinct compartments, LROs have extensive linkage with the endo-lysosomal system. This property is demonstrated by the presence of lysosomal proteins as well as the acidic luminal environment of LROs (Griffiths, 1996; Dell'Angelica et al., 2000). The maturation of LROs begins with the sorting of proteins destined for regulated secretion into immature granules that bud off of the TGN. Sorting signals in many of these proteins are known to target them either directly to the lysosome or to the earlier multivesicular body (MVB) (Blagoveshchenskaya et al., 1998; Honing et al., 1998; Zuccato et al., 2007). Mature LROs constitute a population of post-lysosomal storage compartments that exists only in a subset of specialized secretory cells. Upon receiving the proper extra-cellular signal, mature LROs move to the cell periphery in a microtubule dependent manner and fuse with the plasma membrane. The general enlargement of

acidic compartments in all CHS cells coupled with the specific enlargement or absence of LROs in CHS patients indicates that Lyst is likely involved in regulating vesicle trafficking events at the lysosomal and post-lysosomal stage.

1.5 Models for Lyst function

Under normal conditions, lysosomes maintain a typical morphology and proteomic identity despite continual rounds of fusion and exchange of membrane and luminal material with late endosomes (Deng et al., 1991). This conservation of identity is accomplished by fission mediated recycling of membrane from lysosomes (Luzio et al., 2000). The balance of fusion and fission is integral for the maintenance of distinct endosomal populations (Storrie and Desjardins, 1996). Tipping this balance in either direction has the potential to produce changes in lysosomal morphology like those described in CHS cells.

Characterization studies of the cellular defects contributing to CHS and homologous disorders in multiple model systems have coalesced into two opposing models for Lyst function. Proponents of the fusion regulatory model for Lyst function propose that disruption of Lyst leads to an increase in vesicle fusion events and the subsequent formation of enlarged lysosome related compartments. Alternatively, a fission regulatory model hypothesizes that Lyst positively regulates vesicle biogenesis by promoting fission events that are required for modifying the identity of endosomes as they mature. In this model, defects in Lyst function would reduce fission mediated removal of vesicular membrane as well as specific protein markers causing the formation of enlarged endosomes with markers from multiple maturation stages. Both of these models are strongly supported by results in multiple publications, and currently no consensus on which of these models is correct has been reached.

1.5.1 The fusion model

Investigation into the cellular mechanism underlying CHS began long before the CHS/lyst gene was identified. Conclusions derived from these studies introduced the

"increased fusion" model for how aberrant vesicles arise in CHS cells. Morphological analysis of CHS cells by electron microscopy revealed an increase in the frequency of secretory vesicles that appeared to be in various stages of fusion (Oliver and Essner, 1975; Rozenszajn et al., 1977; Collier et al., 1985). This increased fusion hypothesis was bolstered by a study that used time lapse microscopy to show vesicles with lysosomal characteristics fusing to form large compartments in beige (CHS) mouse fibroblasts (Willingham et al., 1981).

Further support for the fusion model was obtained by using the uniquely inducible lytic granules of cytotoxic T lymphocytes (CTLs) to determine which point of LRO maturation requires Lyst function (Stinchcombe et al., 2000). CTLs that have not come into contact with an external stimulus exist in a resting state in which they are devoid of lytic granules (LROs that are specific to CTLs). Upon stimulation the CTLs enter an activated state which initiates the biogenesis of lytic granules. Thus, the formation and maturation of lytic granules can be synchronized and followed over time in order to pinpoint where a defect occurs. Using this method, the initial stages of lytic granule formation and merging with lysosomes were found to be normal in cultured CHS CTLs. It was at a later maturation stage that the LROs from CHS CTLs acquired an enlarged morphology in comparison the LROs of normal CTLs. This phenotypic change could arise from the increased fusion of LROs or from the inability of membranes to separate via fission after the mixing of immature lytic granules with lysosomes. However, in support of the fusion model, the authors reported seeing clusters of fully formed LROs that may have been in the process of preparing to fuse in CHS, but not in wild type, CTLs.

The secretory lysosomes (LROs) from other Lyst deficient secretory cells also have mutant characteristics that are indicative of increased fusion. Hammel (1987; 2010) used a mathematical modeling approach to assess the fusion characteristics of secretory lysosomes (LROs) in mouse mast and pancreatic acinar cells. Previous studies in human and mouse secretory cells showed that the distribution of mature LRO volume and luminal content correspond to multiples of unit granule addition (Dvorak et al., 1984; Hammel et al., 1985; Mroz and Lechene, 1986; Hammel et al., 1987). Unit granules form through the fusion of pro-granules that arise directly from the TGN and subsequent

condensation of their contents (Tooze et al., 1991; Lew et al., 1994). The fusion of pro-granules is balanced with condensation such that the volume of mature unit granules is tightly constrained (Hammel and Anaby, 2007). The unit granule addition distribution presented in these studies support a model where the mature unit granule is able to fuse with LROs of any size, but that random fusion between LROs does not occur in wild type mast and pancreatic acinar cells. In contrast, the volumetric distribution observed for LROs of corresponding cell types in beige (Lyst deficient) mice support a mathematical model where random fusion between LROs does occur and the fusion between these larger compartments leads to the formation of the giant LROs that are a defining characteristic of CHS.

Disruption of the *Drosophila* Lyst orthologue, Mauve, leads to phenotypic attributes that are similar to those of CHS cells including increased pigment granule (an LRO) size in primary pigment cells of the eye, decreased survival upon infection with E. coli, and a corresponding decrease in the degradation of bacteria by primary hemocytes (Rahman et al., 2012). Interestingly, while early phagosome maturation appeared normal in *mv* mutant macrophages, late phagosomes were often found to contain >3 bacteria which was an infrequent occurrence in wild type cells. The formation of these multi-particulate phagosomes was attributed to increased homotypic phagosome-phagosome fusion. These giant multi-particulate phagosomes were also found to mix with dextran labeled lysosomes at an earlier time point than the phagosomes of wild type macrophages, suggesting a defect in the regulation of fusion of phagosomes with lysosomes.

The hypothesis that Lyst is a regulator of vesicle fusion is also supported by the identity of its proposed binding partners. In a yeast two hybrid screen for Lyst interacting proteins, Tchernev et al (Tchernev et al., 2002) identified multiple proteins that are known to be important for fusion regulation. The most prominent of these were 14-3-3τ, HRS, CALM, and CK2β. The interaction of Lyst with 14-3-3τ, HRS, and CK2β were verified using *in vitro* binding assays with purified proteins but were not tested with endogenous proteins or by any other physiological test. Similar to the proposed fusion inhibition function of Lyst, HRS is known to inhibit vesicle exocytosis through inhibitory interactions with the snare complex regulator SNAP-25 (Ungermann et al., 1998;

Tsujimoto and Bean, 2000). CALM plays multiple signaling roles, but is also found to be required for exocytosis of secretory vesicles (Peters and Mayer, 1998). Similar to the importance of CALM for exocytosis, 14-3-3τ prepares vesicles for exocytosis through its inhibitory influence on PKC (Toker et al., 1990; Morgan and Burgoyne, 1992; Jones et al., 1995). The interaction between Lyst and 14-3-3τ is of particular interest due to the reported inhibitory relationship between Lyst and PKC (discussed in detail below). Finally, CK2β is a serine threonine kinase that influences vesicle trafficking and fusion indirectly through phosphorylation of substrates such as PKC, CALM, synaptobrevin and synaptotagmin amongst others (Allende and Allende, 1995). Once again the relationship that CK2β and Lyst share with PKC is noteworthy. The combination of these biochemical, genetic, and cell biological studies has created a platform for a fusion regulatory model for Lyst function. However, compelling evidence for alternative models of Lyst function have also appeared in the CHS literature.

1.5.2 The fission model

An increase in vesicle fusion is not the only mechanism that can lead to the formation of enlarged lysosome related compartments. Decreased fission mediated recycling from lysosomal compartments could also be responsible for the formation of aberrant compartments in CHS cells. This fission model for Lyst function was not formally introduced until 1997 (Perou et al.). This study showed that the overexpression of Lyst/Beige in wild type or beige mutant mouse fibroblasts elicited a significant decrease in the size of lysosomes. This phenotypic change could have been derived from decreased lysosomal fusion capacity in the presence of excessive fusion inhibition by Beige. However, because the fusion competence of lysosomes in CHS/Beige mutant cells were previously shown to be comparable to their wild type counterparts *in vivo*, the authors argued against a dose dependent change in lysosomal fusion rates correlating with increased levels of Beige (Perou and Kaplan, 1993). Additionally, increased levels of Beige did not induce a concurrent decrease in fusion amongst lysosomes *in vitro* (Perou et al., 1997).

Results from a recently published study that tested for both defective fusion and fission in Beige null (*beige*$_j$) macrophages reinforce the fission regulatory model for Lyst

function and contest the fusion model (Durchfort et al., 2012). Following artificial fragmentation of lysosomes, the rate of lysosome fusion mediated recovery in *beige$_j$* macrophages was indistinguishable from that in wild type cells. In contrast, fission mediated recovery after drug induced enlargement of lysosomes was much slower in the *beige$_j$* cells than in wild type, and overexpression of Beige prompted a faster recovery rate. These authors also used time-lapse microscopy to directly assess individual fission events from tubulated vesicles, and reported that *beige$_j$* macrophages had fewer fission events and the duration of those events was significantly longer than for normal macrophages. These studies have challenged the fusion model for Lyst function and have provided compelling evidence suggesting that Lyst is a positive regulator of lysosomal fission.

1.5.3 Lyst and PKC activity

Another model that has prompted much analysis is one where Lyst influences the activity of protein kinase C (PKC). This model is not entirely removed from the fusion model for Lyst function. PKC is known to regulate the docking and fusion of exocytic vesicles by modulating calcium levels (Chen et al., 2004; Korogod et al., 2007; Xue et al., 2009). Initial investigation into PKC activity in CHS cells was prompted by studies reporting reduced natural killer cell activity in response to inhibitors of PKC (Jondal et al., 1986; Ito et al., 1988). This effect on immune cell function is very similar to the reduced functionality of like cells in human and mouse CHS models. In normal cells, PKC can be activated by the addition of phorbol esters. Activated PKC relocates from the cytosol to the membrane fraction. This activation of PKC, as represented by redistribution to the membrane fraction, was significantly diminished in *beige$_j$* mutant mouse cells (Ito et al., 1988). This result initiated multiple studies of how PKC activity contributes to different aspects of the CHS phenotype. PKC is subject to proteolysis by the protease calpain, and the thiol protease inhibitor E-64-d prevents calpain mediated PKC degradation. Many studies have demonstrated the ability of E-64-d to restore normal PKC activation, increase natural killer activity, and prevent giant granule formation in *beige$_j$* mutant cells (Ito et al., 1989; Tanabe et al., 2000; Morimoto et al., 2007). Tanabe et al (2000) also found that inhibitors of PKC activity can induce the

formation of giant granules in normal mouse fibroblasts. The ability of E-64-D to ameliorate many of the cellular defects associated with CHS suggested that it might be a good candidate for therapeutic administration. The effectiveness of E-64-d as a course of treatment for CHS was supported by the increased survival of Staphylococcus aureus infected beigej mutant mice following a 3 day oral administration of E-64-d (Morimoto et al., 2007).

1.6 The endosomal pathway in Dictyostelium discoideum

The simple soil amoeba *Dictyostelium discoideum* has emerged as a useful model system for the study of proteins that regulate the endo-lysosomal pathway. Many of the proteins that are required for endosomal trafficking in mammalian cells have homologues in *Dictyostelium* with conserved function (Maniak, 2011). *Dictyostelium* also provides an easily genetically tractable venue for the study of protein function owing to its haploid genome and short, 8 hour, doubling time. The genome of *Dictyostelium* has been fully sequenced, and a plethora of genetic, biochemical, and cell biological tools have been developed for the characterization of protein function (Bozzaro, 2013). In this respect *Dictyostelium* rivals yeast as an ideal model organism for the study of protein function, but it also has the distinct advantage of demonstrating higher homology to the cellular processes of mammalian cells.

Dictyostelium is especially suited for the investigation of Lyst function, as the progression of its endo-lysosomal pathway closely resembles that of specialized secretory immune cells. This is not surprising since *Dictyostelium*, like macrophages, are professional phagocytes. Unlike the mammalian secretory cells, the primary function of the endo-lysosomal pathway in *Dictyostelium* is to serve as a "digestive tract" for the sustenance of the cell. However, similar to the fate of LROs in mammalian secretory cells, biosynthetic cargo from the TGN is known to merge with the endo-lysosomal pathway in *Dictyostelium* and is likewise secreted by a post-lysosomal compartment (Maniak, 2002).

In *Dictyostelium*, the endocytic pathway begins with the uptake of extracellular media by macropinocytosis, or of bacterium by phagocytosis (Fig. 1.1 A). As in

mammalian systems, both of these processes are driven by actin and myosin (Maniak et al., 1995; Hacker et al., 1997; Schwarz et al., 2000), and after internalization, the nascent endosome sheds its actin coat and its lumen begins to acidify (Maniak et al., 1995; Hacker et al., 1997; Peracino et al., 1998; Maniak, 1999; Maniak, 2001). Just as described for mammalian cells, acidification occurs through the fusion mediated delivery of v-ATPase proton pumps (Clarke et al., 2002). *Dictyostelium* has a unique, tubulated recycling compartment where a sub-set of proteins are sorted into small recycling vesicles and returned to the PM (Neuhaus and Soldati, 2000; Neuhaus et al., 2002; Charette et al., 2006). As early endosomes mature, they fuse with transport vesicles carrying lysosomal enzymes. Interestingly, specific enzymes are delivered in a sequential order and they remain in distinct endosomal populations despite constant fusion and exchange of luminal material (Souza et al., 1997). This partitioning of luminal contents during endosomal maturation is attributed to fission mediated recycling (Buczynski et al., 1997; Souza et al., 1997).

Equivalent to the process in mammalian cells, the delivery of lysosomal enzymes along with a significantly reduced luminal pH marks the lysosomal stage where digestion occurs. Homotypic fusion between early endosomes and also between lysosomes is directed by Rab GTPase tethering and snare complex formation as outlined above. The Rab and snare proteins that function along the endo-lysosomal pathway in *Dictyostelium* are homologous in sequence and function to those in mammalian systems (Laurent et al., 1998; Bogdanovic et al., 2000; Weidenhaupt et al., 2000; Bogdanovic et al., 2002; Harris and Cardelli, 2002). Not all of the material that enters the lysosome can be digested. Indigestible remnants must be shuttled out of the cell by a distinct post-lysosomal compartment. Post-lysosomes mature from lysosomes through the fission directed removal of vATPase from the membrane (Fig. 1.1 C), and the arrival of proteins, such as vacuolin, that prime the post-lysosome for fusion with the plasma membrane and concurrent exocytosis (Nolta et al., 1994; Rauchenberger et al., 1997; Jenne et al., 1998; Carnell et al., 2011). A simplified diagram of the endo-lysosomal pathway in *Dictyostelium* is shown in Figure 1.1.

The neutral nature of the post-lysosome is most analogous to that of LROs found in mammalian cells. This characteristic was demonstrated by studies showing that the

release of secretory lysosomes (LROs) in macrophages can be induced by artificially raising their luminal pH, and that resting secretory vesicles of pancreatic acinar cells have a higher luminal pH than typical lysosomes (Tapper and Sundler, 1990; Yang et al., 2007). The similarity between the endo-lysosomal pathway in *Dictyostelium* and specialized mammalian secretory cells is also evinced by multiple examples where genetic endosomal trafficking disease defects are recapitulated by disruption of homologous genes in *Dictyostelium*. This trend holds true for the *Dictyostelium* Lyst homologue, LvsB.

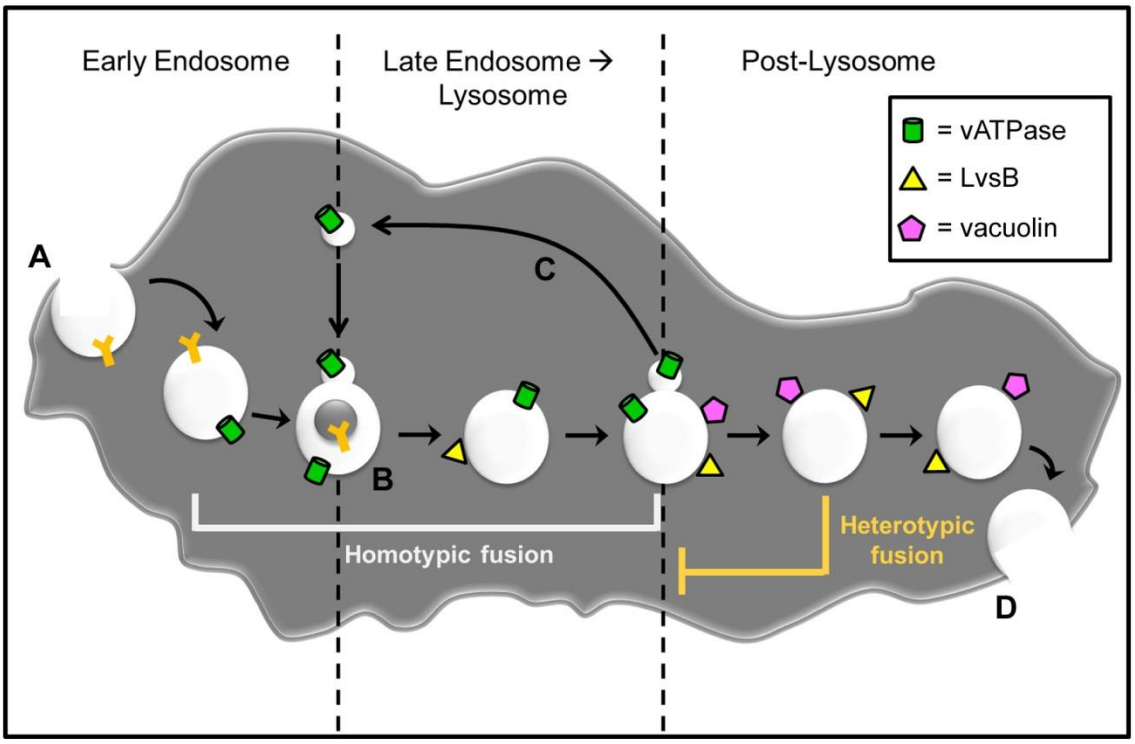


Figure 1.1: The endo-lysosomal pathway in *Dictyostelium discoideum*. (A) Extracellular material as well as plasma membrane and its components are taken into the cell and initially housed in early endosomal compartments. These early endosomes are quickly acidified by the delivery of vATPase proton pumps. Early endosomes then mature through the sequential delivery of hydrolytic enzymes into late endosomes and then lysosomes. This maturation process is choreographed by highly regulated fusion and fission events. **(B)** During the maturation of endosomes, some trans-membrane proteins that are destined for degradation by the lysosome are packaged into small vesicles that pinch off from the endosomal membrane and end up in the lumen of the endosome. This intraluminal vesicle (ILV) formation is accomplished by the sequential activity of ESCRT pathway proteins, and is essential for complete digestion of ILV contents by lysosomal hydrolases. The lysosome serves to digest any material that is delivered to its lumen. This digestion occurs through the activity of hydrolytic enzymes that are active only in the acidic lumen of the lysosome. In *Dictyostelium* and specialized mammalian cells, not all material that is delivered to the lysosome can be digested and the indigestible material needs to be excreted out of the cell. The first step in this process is the de-acidification of the lysosome **(C)** Lysosomes de-acidify by removal of vATPase proton pumps from their membranes by the fission of small vesicles carrying the proton pumps. The resulting neutral post-lysosomal compartments then carry indigestible remnants to the cell periphery for exocytosis **(D)**. The fusion events that occur between early endosomes, late endosomes, and lysosomes are referred to as homotypic fusion and are integral to the maturation process and for exchange of luminal material. Conversely, post-lysosomes are prevented from undergoing heterotypic fusion with earlier endosomal stages. This sequestration is essential for efficient processing of endocytosed material. The reported localization characteristics of vATPase proton pump, LvsB, and vacuolin are shown (Jenne et al., 1998; Clarke et al., 2002; Kypri et al., 2007).

1.7 LvsB in Dictyostelium

Similar to higher eukaryotes, *Dictyostelium* have multiple BEACH family proteins. Six BEACH proteins, designated Lvs (large volume sphere) A through F, were identified and characterized using knock out analysis (Wang et al., 2002). Sequence alignment of the BEACH and WD domains using the ClustalW algorithm showed that of the six *Dictyostelium* BEACH proteins, LvsB shared the most sequence similarity to Lyst (Wang et al., 2002). Consistent with this, the loss of LvsB caused an enlargement of lysosomes similar to that of mammalian Lyst mutant cells (Cornillon et al., 2002; Harris et al., 2002; Wang et al., 2002). Importantly, the disruption of LvsB in *Dictyostelium* does not disturb cell growth, making *Dictyostelium* a suitable venue for the study of LvsB function along the endo-lysosomal pathway. The tools available in *Dictyostelium* have prompted new insights into the sub-cellular physical and functional attributes of Lyst/LvsB. Like Lyst, the substantial size of LvsB (430 KDa) precludes the typical cloning method for tagging and assessing sub-cellular localization. However, using a GFP knock in approach, LvsB was found to localize on the membranes of lysosomes and post-lysosomes (Kypri et al., 2007) (Fig. 1.1). This localization pattern is in contrast to the reported cytosolic immuno-localization pattern of mammalian Lyst (Perou et al., 1997), and is in accord with the described importance of Lyst for maintenance of lysosomal and LRO morphology.

As with Lyst, characterization of LvsB has elicited evidence for both the fusion and fission regulatory models for LvsB function. Initial characterization of LvsB null cells showed a significant increase in the amount of homotypic lysosome and homotypic phagosome fusion *in vivo*. In a later study the extended localization of LvsB on post-lysosomes aroused interest in the implications of LvsB dysfunction on post-lysosomal integrity. As mentioned above, under wild type conditions, the post-lysosomal population remains distinct from the earlier endosomal and lysosomal populations (Fig. 1.1). This results in the partitioning of lysosomal and post-lysosomal markers on disparate pools of vesicles. However, disruption of LvsB leads to the frequent co-localization of lysosomal and post-lysosomal markers insinuating a disturbance in the mechanism that maintains the mutual exclusivity of these compartments (Kypri et al., 2007). In support of this hypothesis, LvsB null cells are reported to have an increased

level of heterotypic fusion between lysosomes and post-lysosomes compared to a negligible amount in wild type cells (Kypri et al., 2007). The inference from these studies is that LvsB is a negative regulator of homotypic lysosome, homotypic phagosome, and heterotypic lysosome-post-lysosome fusion. However, and alternate conclusion was drawn by Charette and Cosson (2007). In this study, the absence of LvsB caused an increase in lysosome size, a decrease in post-lysosome number, and a concurrent delay in the delivery of latex beads from lysosomes to post-lysosomes. Together, these observations implicated LvsB as an important regulator of secretory lysosome (post-lysosome) biogenesis. This hypothesis is confounded by the normal temporal delivery of fluid phase to post-lysosomes in LvsB null cells that was reported by Kypri et al (Kypri et al., 2007). The conflicting observations reported in these studies are likely due to differences in the methods used to assess endo-lysosomal identities and dynamics. The potential consequences of these differences will be discussed in chapter 2.

1.8 The goals of this thesis

My work described in this thesis has used characterization studies of the *Dictyostleium* LvsB protein to provide key functional information relating to the homologous Chediak Higashi Syndrome related protein Lyst. In chapter 2, I detail novel attributes of the LvsB null phenotype that both distinguish it from vesicle fission defect mutants and support the fusion model for LvsB function. In chapter 3, I provide evidence that LvsB negatively regulates vesicle fusion by antagonizing the function of the GTPase Rab14. Chapter 4 describes the first characterization study of the *Dictyostelium* Lip5 homologue (dLip5), and provides evidence that it promotes Rab14 mediated vesicle fusion in opposition to LvsB function. Finally, in chapter 5, I expound upon the implications of my work and how we might approach the new questions that have been raised by the conclusions drawn in this thesis.

Chapter 2: Comparison of the LvsB null phenotype against endosomal fission defect mutants in *Dictyostelium* suggests a fusion regulatory role for LvsB

2.1 Introduction

The endo-lysosomal pathway is comprised of a complex network of traffic from the golgi, plasma membrane, and autophagosomes that converge through intricately regulated fusion and fission events. As early endosomes mature into lysosomes, they utilize fusion and fission events to acidify, sort cargo and acquire the lysosomal enzymes needed for the digestion of intra-luminal material (Ferris et al., 1987; Deng and Storrie, 1988; Mullock et al., 1998; Ward et al., 2000b; Maniak, 2003; Mesaki et al., 2011). In professional phagosomes, like macrophages and *Dictyostelium discoideum*, indigestible material is exocytosed by a third, neutral post-lysosomal compartment (Padh et al., 1993; Sundler, 1997) (a simplified diagram of the endo-lysosomal pathway is shown in Fig. 1.1). The tight regulation of fusion and fission events along the endosomal pathway is integral for the correct processing of cargo that transits the endo-lysosomal pathway. Disruption of any of these steps can lead to devastating defects at the cellular and organismal level. This is evinced by the severe phenotypes that are associated with lysosomal trafficking disorders such as Hermansky-Pudlak, Griscelli, and Chediak Higashi syndromes (CHS) amongst others (Huizing et al., 2001). While the underlying basis of these disorders is well understood, a consensus on the lysosomal defect contributing to the pathophysiology of CHS has yet to be reached.

CHS is a lethal autosomal recessive disorder that is characterized by the presence of abnormally large lysosome-related compartments in all cells types (Introne et al., 1999). Studies in human (CHS) and mouse (beige) cells revealed that lesions in the mammalian *lyst* (lysosomal trafficking regulator) gene are responsible for the manifestations of CHS (Barbosa et al., 1996; Nagle et al., 1996). This discovery prompted the use of several model systems to investigate the molecular function of Lyst and its orthologs in order to understand the pathophysiology of CHS. These functional studies produced two distinct models for Lyst function. Initial characterization of beige

mice suggested that Lyst functions to limit the rate of homotypic lysosome fusion (Oliver and Essner, 1975). This model for Lyst function was subsequently supported by many studies in humans (Stinchcombe et al., 2000), *Dictyostelium* (Harris et al., 2002; Kypri et al., 2007), mice (Willingham et al., 1981; Hammel et al., 1987; Hammel et al., 2010), cats (Collier et al., 1985), and *Drosophila* (Rahman et al., 2012). An alternative model suggested that Lyst functions to control lysosomal fission instead of fusion (Burkhardt et al., 1993). Studies in mice (Perou et al., 1997; Durchfort et al., 2012), and *Dictyostelium* (Charette and Cosson, 2007; Charette and Cosson, 2008) have attributed beige and LvsB mutant defects, respectively, to decreased fission in lysosomes. Despite decades of research across a plethora of model systems, a unifying model for Lyst function has not been established.

Our research has focused on understanding the cellular mechanisms of the Lyst orthologue LvsB (Large vacuolar sphere B), in the simple soil amoebae *Dictyostelium discoideum*. Disruption of LvsB results in the accumulation of large acidic compartments, much like those observed in cells of CHS patients (Harris et al., 2002; Wang et al., 2002). Characterization of the LvsB null phenotype has provided definitive evidence of an important role for LvsB during progression of the endo-lysosomal pathway (Kypri et al., 2007). However, similar to the conundrum of deciphering Lyst function in other systems, studies in *Dictyostelium* proposed both the fusion and fission models for LvsB function. Studies published by Harris (2002) and Kypri (2007) proposed a fusion regulatory role for LvsB. In contrast, Charette (2007; 2008) described LvsB as a positive regulator of lysosomal fission. This discrepancy exists because many of the LvsB null phenotypes described in these studies can be explained by either the fusion or fission regulatory model, and are therefore subject to interpretation.

The ambiguity of the LvsB null phenotype is exemplified by its characteristic changes in endosomal membrane protein composition and luminal pH. In *Dictyostelium*, as a lysosome matures into a post-lysosome, the exocytic fotillin like protein vacuolin accumulates on the vesicular membrane as vATPase is removed and the lumen begins to de-acidify (Rauchenberger et al., 1997; Jenne et al., 1998; Carnell et al., 2011) (Fig. 1.1). Thus, vacuolin predominantly labels the membranes of neutral, post-lysosomal compartments (Rauchenberger et al., 1997; Kypri et al., 2007). Under normal conditions,

lysosomes undergo homotypic fusion to exchange their contents, but are prevented from undergoing heterotypic fusion with post-lysosomes to preserve the identity of these distinct stages (Maniak, 2003). Due to the coordination of lysosome to post-lysosome maturation, vacuolin is rarely observed on vesicular membranes until late stages of endosome maturation in wild type cells, and the majority of vacuolin labeled vesicles have a neutral luminal pH (Jenne et al., 1998; Kypri et al., 2007). In contrast, vacuolin frequently labels acidic vesicles that contain early endocytic markers in LvsB null cells (Kypri et al., 2007). Both of these observations are used to support a model where LvsB inhibits heterotypic fusion between lysosomes and post-lysosomes, and the absence of LvsB allows fusion of neutral vacuolin-positive post-lysosomes with earlier acidic lysosomes. However, these observations can also be explained using the fission model for LvsB function. In this model, LvsB acts as a positive regulator of fission events that are necessary for the maturation of lysosomes into post-lysosomes. Thus, the absence of LvsB would cause delayed transition of lysosomes to post-lysosomes. This delay could, in theory, cause an accumulation of vacuolin on acidic late lysosomes that are still competent to undergo homotypic fusion with earlier endosomal compartments. Unfortunately, because fusion and fission events are so tightly interwoven during the progression of the endo-lysosomal pathway, most of the phenotypes described for LvsB null cells and Lyst mutant cells are subject to inherent ambiguity. Consequently, it has been very difficult to ascribe a mechanism of function to Lyst or LvsB based on the currently published studies.

Without a consensus on the regulatory function of LvsB and Lyst, it is not possible to advance our understanding of how the loss of Lyst leads to the manifestation of CHS. Here we have used a direct comparison of the LvsB null phenotype against that of two known fission defect mutants to distinguish between the fusion and fission model for LvsB function. Our results reveal that the LvsB null phenotype is distinct from that of both fission defect mutants, and supports a role for LvsB as a negative regulator of fusion. Additionally, we showed that the phenotypic defects in cells expressing dominant active Rab14 diverge from those of the fission defect mutants in a manner similar to what we found for LvsB null cells. This is consistent with

the proposed antagonistic relationship of LvsB with Rab14 that is detailed in chapter 3 and further substantiates the fusion regulatory role of LvsB.

2.2 Results

2.2.1 Fission defect mutants have a similar phenotype to LvsB null cells

To distinguish between the two models for LvsB function, we wanted to compare specific aspects of the LvsB null phenotype with two well characterized fission defect mutants, the μ3 null and WASH (WASP and SCAR homologue) null cell lines. The μ3 protein is a subunit of the Adaptor protein 3 (AP3) complex in *Dictyostelium*. AP3 is a clathrin adaptor complex found on endosomal compartments (Bonifacino and Traub, 2003). Absence of *Dictyostelium* μ3 results in fission mediated recycling defects during early stages of endosome maturation (Charette et al., 2006; Charette and Cosson, 2008). The WASH protein is required for the removal of vATPase from late lysosomes that are transitioning to the post-lysosomal stage (Fig. 1.1 C). This WASH dependent step occurs through actin driven fission of small recycling vesicles (Carnell et al., 2011). Both of these mutant cell lines have a reported delay in the maturation of lysosomes into post-lysosomes as conjectured in the fission model for LvsB function.

To begin our comparative studies, we first determined the phenotype of these fission mutants with the same assays used to characterize the LvsB null phenotype. As previously described, the characteristics and dynamics of vacuolin labeled vesicles are perturbed in LvsB null cells. These aspects of the LvsB null phenotype can be visualized using the post-lysosomal marker, GFP-vacuolin, in conjunction with fluid phase markers or the acidophilic dye Lysotracker red, which preferentially accumulates in acidic vesicles (Wubbolts et al., 1996). Consistent with previous studies, GFP-tagged vacuolin accumulated on dextran-labeled vesicles at early time points in LvsB null cells compared to wild type cells (Fig. 2.1 A-A”, B-B”, E).

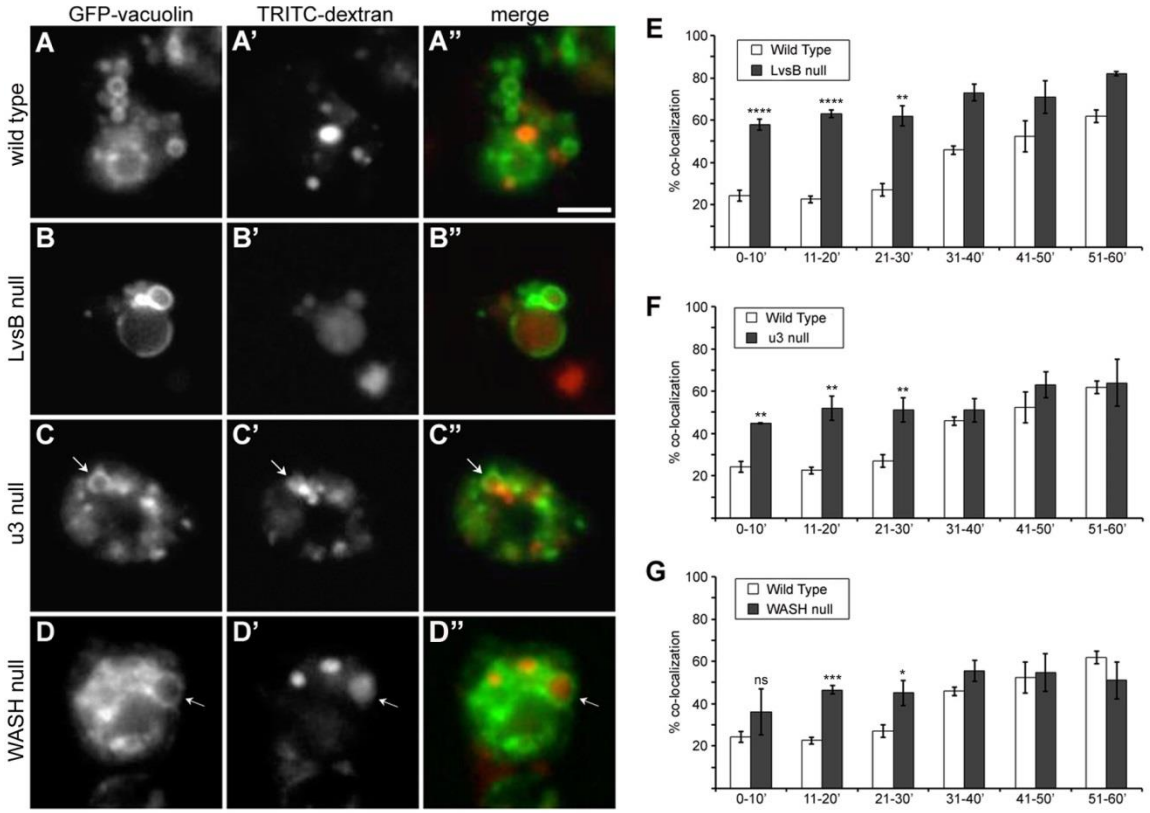


Figure 2.1: Vacuolin localizes to dextran labeled vesicles at early time points in both LvsB null and fission defect mutants. Cells were transfected with GFP-vacuolin B and then given a 5 minute pulse of TRITC-dextran to label a subpopulation of pinocytic vesicles. **(A-D)** Cells were then washed and imaged continuously for 60 minutes. **(E)** The percent of cells (>20 cells per experiment) containing GFP-vacuolin positive TRITC-dextran vesicles was quantified for each 10 minute interval and plotted as the mean +/- s.e.m. (n=3). Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, P>0.05; *, P< 0.05; **, P< 0.01). During the first 30 minutes of imaging, the majority of wild type cells maintained separation of their dextran and GFP-vacuolin labeled vesicle populations **(A-A")**. In contrast, GFP-vacuolin was found to significantly localize on TRITC-dextran labeled vesicles at earlier time intervals in the LvsB null **(B-B")**, μ3 null **(C-C")**, and WASH null **(D-D")** cell lines.

LvsB null cells also contained a large proportion of acidic vesicles labeled by GFP-vacuolin (47.8% +/- 1.45 s.e.m.) (Fig. 2.2 B-B", E) compared to wild type cells (11.1% +/- 2.33 s.e.m.) (Fig 2.2 A-A", E). The fission defect model predicts that vacuolin should accumulate on late acidic lysosomes that are delayed in their maturation to the post-lysosomal stage. These vacuolin-labeled lysosomes should still be competent to fuse with earlier endosomes. In agreement with this model we found that both μ3 null and WASH null cells contained GFP-vacuolin labeled vesicles at earlier times than wild type

cells (Fig. 2.1 C-C" and F, D-D" and G). In both cell lines we also observed a significant increase in the percentage of acidic vesicles labeled by GFP-vacuolin (40.6% +/- 0.3 s.e.m. for μ3 null; 30.1% +/- 1.45 s.e.m. for WASH null) (Fig. 2.2 C-C", D-D", E) over wild type cells. These observations show that the phenotype of LvsB null cells demonstrated with these assays is similar to that of known fission mutants and that while the LvsB null phenotype could be attributed to a defect in fusion (Kypri et al., 2007), it could also be interpreted as being caused by a defect in fission. Thus, our results emphasize the importance of studies that can distinguish between defects in fission and fusion.

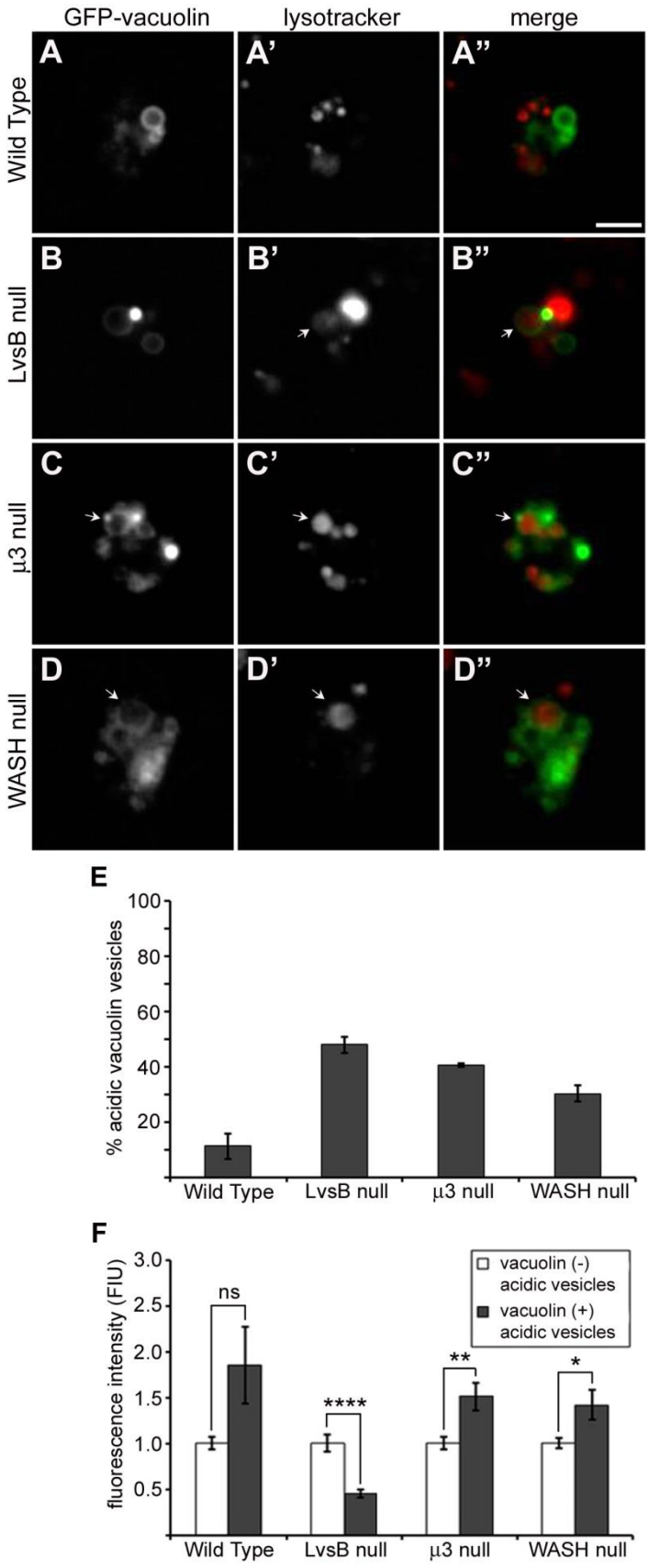


Figure 2.2: LvsB null and fission defect mutants have an increased occurrence of acidic vacuolin-labeled vesicles but the characteristics of acidic vesicles in LvsB null cells are distinct from those in fission defect mutants. **(A-D)** GFP-vacuolin B expressing cells were incubated with Lysotracker red then imaged live to visualize acidic compartments with and without GFP-vacuolin membrane localization. **(E)** Using similar live cell images, we quantified the percent of GFP-vacuolin vesicles with Lysotracker red fluorescence and plotted the mean +/- range for two experiments. As previously described, there was an increased occurrence of acidic GFP-vacuolin vesicles in LvsB null cells **(B-B")** compared to wild type cells **(A-A")**. The fission defects in µ3 null cells **(C-C")** and WASH null cells **(D-D")** also resulted in a higher percentage of acidic GFP-vacuolin vesicles similar to that observed in the LvsB null cell line. **(F)** The fluorescence intensity of lysotracker red was measured in GFP-vacuolin positive (late lysosomal/hybrid organelle) and in GFP-vacuolin negative (lysosomal) populations and used as an indicator of relative vesicle acidity. The fluorescence intensity of Lysotracker red for each population was normalized within each cell line to the average fluorescence of GFP-vacuolin negative lysosomes and plotted as the mean +/- s.e.m. Notice that the relative acidity of vacuolin-labeled compared with unlabeled vesicles in LvsB-null cells is very different from that seen in wild type, µ3 null, and WASH null cells. The higher acidity of vacuolin-labeled vesicles in µ3 null, and WASH null cells is consistent with their role in vesicle fission during lysosomal maturation. In contrast, the fluorescence of GFP-vacuolin positive vesicles is significantly reduced when compared to GFP-vacuolin negative vesicles in the LvsB null cells. This is consistent with a role for LvsB in reducing fusion between acidic lysosomal and neutral post-lysosomal compartments. For description of statistical analysis, see figure legend for figure 2.1. Bar is 5 µm.

2.2.2 Acidity characteristics of mutant compartments suggest a fusion regulatory role for LvsB

Although an increase in the number of acidic vacuolin positive vesicles can be explained by defects in either fusion or fission, the different mechanisms for how these vesicles arise should produce predictable differences in their characteristics. One such difference is the relative luminal acidity of vacuolin positive acidic vesicles (hereafter referred to as hybrid vesicles) when compared to the acidity of vacuolin negative acidic vesicles (hereafter referred to as the normal lysosomal population). In the fission defect model, we propose that vacuolin accumulates on very late lysosomes due to a delay in their maturation. This should result in hybrid vesicles that have higher luminal acidity than the normal lysosomal population because they retain their proton pumps. In contrast, the hybrid vesicles described in the fusion defect model arise from inappropriate fusion between acidic lysosomes and neutral, vacuolin positive, post-lysosomes. This fusion should produce hybrid vesicles with lower luminal acidity compared to the normal lysosomal population. To obtain a comparative measure of hybrid vesicle acidity within each cell line, we incubated GFP-vacuolin expressing cells with Lysotracker red and then quantified the average red fluorescence signal of acidic vesicles with (hybrid vesicles) or without (normal lysosomal population) GFP-vacuolin. The fluorescence of Lysotracker red is not directly sensitive to pH changes, but its retention and concentration is known to increase in response to increases in vesicle acidity (Chen, 2002). Thus, we used concentration dependent differences in Lysotracker red signal to gauge pH differences. To test the ability of this assay to produce reliable results we first looked at wild type cells. In wild type cells the acidity of hybrid vesicles was slightly but not significantly higher than the normal lysosomal population (Fig. 2.2 A-A", F). This result agrees with the described transition of lysosomes to post-lysosomes, where vacuolin arrives on the late lysosomal membrane as vATPase is being removed (Carnell et al., 2011). The results observed in μ3 null and WASH null cells were consistent with their defect in fission. The acidity of hybrid vesicles in both μ3 null and WASH null cells was significantly higher than their respective normal lysosomal populations (Fig. 2.2 C-C", D-D", F). These results support our model of how these hybrid vesicles form in fission defect mutants, but more importantly, they provide a baseline for comparison with the

LvsB null phenotype. In contrast to both wild type and the fission defect mutants, the hybrid vesicles in LvsB null cells had significantly lower acidity compared to the normal lysosomal population (Fig. 2.2 B-B", F). This decrease in fluorescence is not a function of increased vesicle size in these mutants. We compared fluorescence of hybrid vesicles with similar diameter from μ3 null and LvsB null cells and found that the LvsB null hybrid vesicles had significantly lower average fluorescence (Fig. 2.3). This result not only sets the LvsB null phenotype apart from the fission defect mutants, but correlates with the predicted characteristics of hybrid vesicles formed by inappropriate heterotypic fusion.

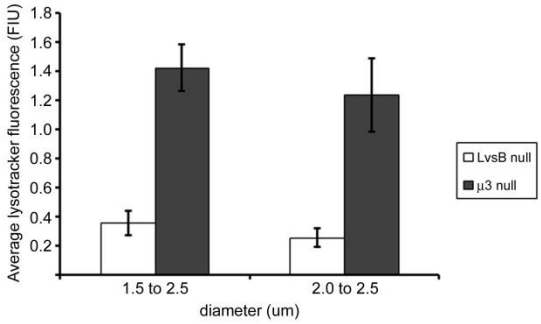


Figure 2.3: Reduced acidity of GFP-vacuolin vesicles in the LvsB null cell line is not a function of vesicle diameter. Acidic compartments were labeled with Lysotracker red in cells expressing GFP-vacuolin. Live images were taken and the diameter and lysotracker red fluorescence was quantified for acidic GFP-vacuolin labeled vesicles. All fluorescence values are normalized to the average fluorescence of the lysosomal population (acidic compartments not labeled by GFP-vacuolin) for each cell line. The Lysotracker red fluorescence of acidic GFP-vacuolin vesicles with similar diameter from the LvsB null and μ3 null cell lines was plotted as the mean +/- s.e.m. for >7 vesicles. Despite a similar range of diameters, the vesicles from the μ3 null cell line have a significantly higher average fluorescence than those from the LvsB null cell line suggesting that fluorescence is not diluted by larger vesicle volume.

2.2.3 Formation of mutant compartment is different in LvsB null cells than in fission defect mutants

To further compare the phenotypes of the LvsB null and fission defect mutants, we modified a previously described 2 dextran pulse chase assay (Kypri et al., 2007). In this assay, cells were given a pulse of FITC-dextran followed by a thirty minute chase and then a second pulse with TRITC-dextran. In wild type cells these maturation times resulted in FITC-labeled post-lysosomes and TRITC-labeled lysosomes (Padh et al., 1993) with very minimal co-localization of the two signals (5.6% +/- 0.35 s.e.m.) (Fig. 2.4 A-A", F). Similar to previous results, LvsB null cells had a significant increase in the number of vesicles containing both FITC- and TRITC-dextran (31.4% +/- 0.99 s.e.m.) (Fig. 2.4 B-B", F). This phenotype can, again, be explained by a defect in either fusion or fission. In the fission model, delayed maturation of lysosomes into post-lysosomes would result in the delayed progression of the first pulse of FITC-dextran to post-lysosomes. Under these conditions, co-localization of the two pulses of dextran is indicative of normal homotypic fusion events between lysosomes. To validate this interpretation we showed that the fission defects in both μ3 null and WASH null cells also resulted in an increased co-localization of the two dextran pulses (16.9% +/- 0.12 s.e.m. for μ3 null and 21.3% +/- 0.88 s.e.m. for WASH null) (Fig. 2.4 C-C", D-D", F). Alternatively, in the fusion defect model, the absence of LvsB would result in the inappropriate heterotypic fusion of FITC-dextran labeled post-lysosomes with earlier TRITC-labeled lysosomes.

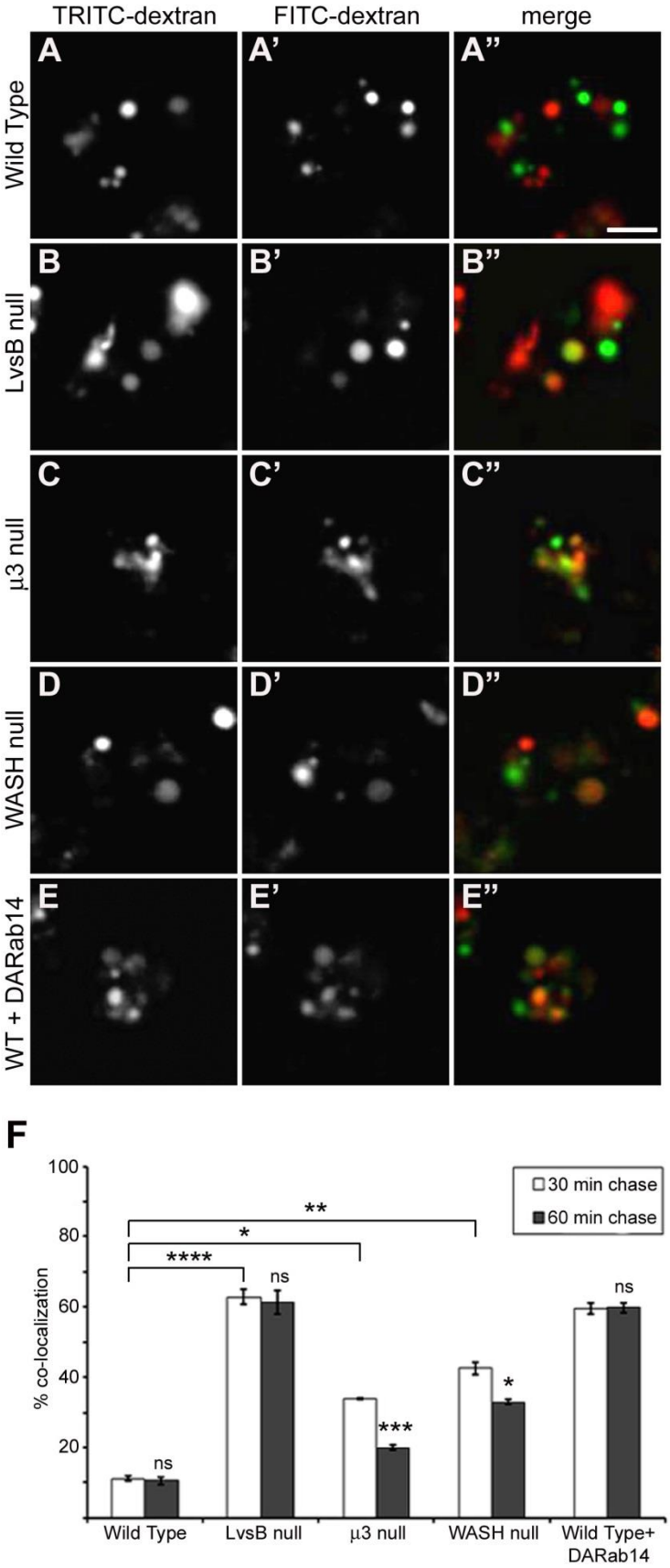


Figure 2.4: Longer maturation time causes reduced fusion of early and late endosome populations in fission defect mutants, but not in the LvsB null mutant. (A-E) Cells were given a pulse of FITC-dextran followed by a 30 or 60 minute chase then given a second pulse with TRITC-dextran. Co-localization of the two dextran signals was used as an indicator of fusion events between the two dextran labeled populations. **(F)** The fraction of vesicles containing both fluid phase markers was quantified for three independent experiments and shown as the mean +/- s.e.m. Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, $P>0.05$; *, $P< 0.05$; **, $P< 0.01$). We observed significantly more fusion between dextran populations separated by a 30 minute chase in the LvsB null cells **(B-B")** compared to wild type cells **(A-A")**. Less dramatic increases in fusion were also evident in both the µ3 null **(C-C")** and WASH null **(D-D")** fission defect mutants. This increased fusion was partially relieved in both the µ3 and WASH null cell line with a 60 minute chase. This reduction is indicative of a delay in the maturation of late lysosomes into post-lysosomes. In opposition to the fission defect model, LvsB null cells experienced no reduction in fusion levels with a 60 minute chase. This pattern of maturation independent increased fusion is mimicked in constitutively active (DA) Rab14 expressing cells **(E-E")**.

To determine whether LvsB is a regulator of fusion or fission, we modified the dextran co-localization experiment to compare the amount of co-localization of the two dextran pulses using a 30 minute or 60 minute chase time. Each model predicts a different outcome when using longer chase times. If a defect in fission causes delayed delivery of the first pulse of FITC-dextran to post-lysosomes, then a longer chase time should allow more FITC-dextran to reach the post-lysosomes and result in decreased co-localization of the two dextran pulses. Consistent with this hypothesis, both the μ3 null and WASH null mutants exhibited a significant decrease in co-localization of the two dextran pulses with a 60 minute chase compared to a 30 minute chase (decreased to 10% +/- 0.29 s.e.m. for μ3 null and 16.5% +/- 0.29 s.e.m. for WASH null) (Fig. 2.4 F). In the case of the fusion model, the 30 minute chase time is sufficient to allow the first pulse of FITC-dextran to reach the post-lysosomes and co-localization is the result of inappropriate fusion between early and late compartments. Applying a longer chase time should not affect the stages of the endo-lysosomal pathway that are labeled by each dextran. Therefore, there should be no change in the amount of co-localization of the two markers. As predicted by the fusion defect model, in the LvsB null cell line we observed no significant change in co-localization of the 2 dextran pulses with a 60 minute chase (30.7% +/- 1.68 s.e.m.) (Fig. 2.4 F). These results show another important dissemblance of the LvsB null phenotype from known fission defect mutants and implicate LvsB as a regulator of fusion rather than fission.

2.2.4 Endosome size is more severely perturbed in LvsB null cells than in WASH null cells

The fission model for Lyst and LvsB function predicts that the delayed maturation of lysosomes should cause an increase in vesicle size at the stage where maturation is delayed (Durchfort et al., 2012). Delaying the maturation of lysosomes allows the continued flow of endosomes that are competent to undergo homotypic fusion. This would result in the "dumping" of earlier endosomal contents into the delayed compartments and a consequential increase in their volume. Alternatively, the fusion model for LvsB function postulates that promiscuous fusion of post-lysosomes with

earlier compartments in LvsB null cells causes an increase in endosome size that is evident at the early endosomal stage and persists through the post-lysosomal stage. To test this theory, cells were given a 5 min pulse of TRITC-dextran and then chased for 15, 35, or 60 minutes. In wild type cells, these chase times label lysosomes, the point of transition from lysosomes to post-lysosomes, and post-lysosomes, respectively (Padh et al., 1993; Temesvari et al., 1996b). The WASH null mutant was selected for comparison because of its reported severe delay in maturation of lysosomes at the same stage that LvsB is suggested to function (Charette and Cosson, 2007; Carnell et al., 2011). The dextran endosomes in WASH null cells showed no significant change in diameter when compared to wild type cells after a 15 minute chase, but displayed a significant increase in diameter over wild type endosomes after a 35 or 60 minute chase time (Fig. 2.5 A-A", C-C", D). The change in morphology observed at the 35 minute time point is in agreement with the homotypic fusion and "dumping" of earlier compartments into stagnated lysosomes at the point of blocked maturation. In contrast to the WASH null fission mutant, the LvsB null defect produced a significant increase in the average diameter of dextran labeled endosomes at all three time points when compared to wild type cells (Fig. 2.5 A-A", B-B", D). This increase was markedly more dramatic than that observed for the WASH null cell line at the 35 and 60 minute time points. The broad increase in endosome size that we observed in the LvsB null cell line does not fit the fission model for how these morphological changes arise. However, promiscuous fusion of post-lysosomes with lysosomes explains the increase in size that persists along the entire endo-lysosomal pathway. Additionally, the increase in endosome size induced by the WASH null defect was much less severe than that observed in the LvsB null cell line, despite the much longer delay in maturation that is reported for WASH null cells (Charette and Cosson, 2008; Carnell et al., 2011). This, again, opposes the fission model for how enlarged endosomes arise in LvsB null cells.

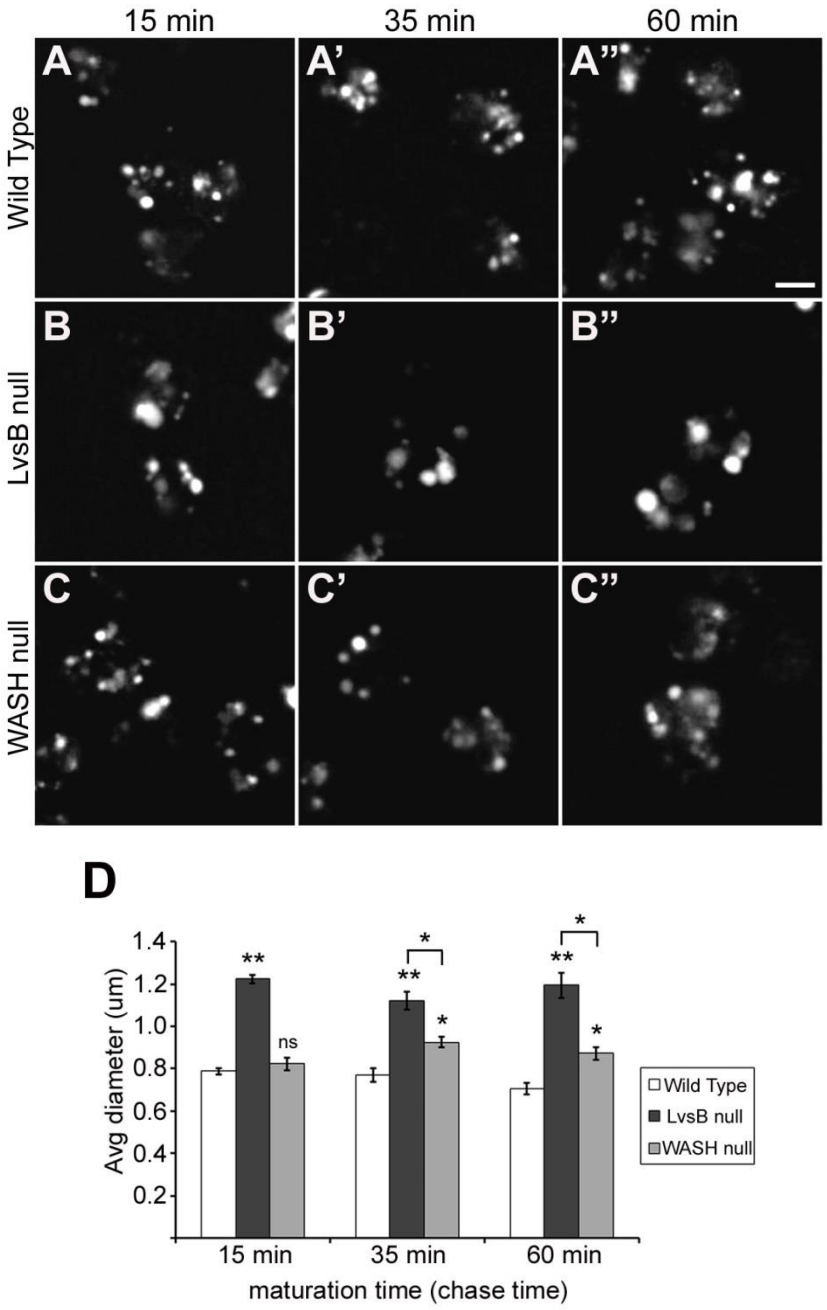


Figure 2.5: Increased endosome size is evident earlier and is more severe in LvsB null cells compared to WASH null cells. (A-C) Endosomes were pulse labeled with TRITC-dextran then imaged after 15 (lysosomal stage), 35 (lysosome to post-lysosome transition), and 60 (post-lysosomal stage) minutes of chase. **(D)** Dextran vesicle diameter for >80 vesicles/experiment was quantified from images similar to A-C and plotted as the mean +/- s.e.m. of 3 experiments. Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, $P>0.05$; *, $P< 0.05$; **, $P< 0.01$). The WASH null vesicles **(C-C")** did not demonstrate a significant increase in diameter over those in wild type cells **(A-A")** until the 35 minute chase. This result is congruous with the delayed maturation of lysosomes that is intrinsic to the WASH null phenotype. A significant increase in vesicle diameter is evident at all three time points in LvsB null cells **(B-B")** compared to wild type **(A-A")**. The increase in vesicle diameter observed for LvsB null cells is 2 fold greater than that of WASH null cells at the 35 minute time point and 3 fold greater at the 60 minute time point. Both the magnitude and temporal pattern of increased endosome size in LvsB null cells differs from the WASH null fission defect mutant. These differences are consistent with the fusion model for LvsB function.

2.2.5 Cells expressing Dominant active Rab14 have fusion defects similar to LvsB null cells

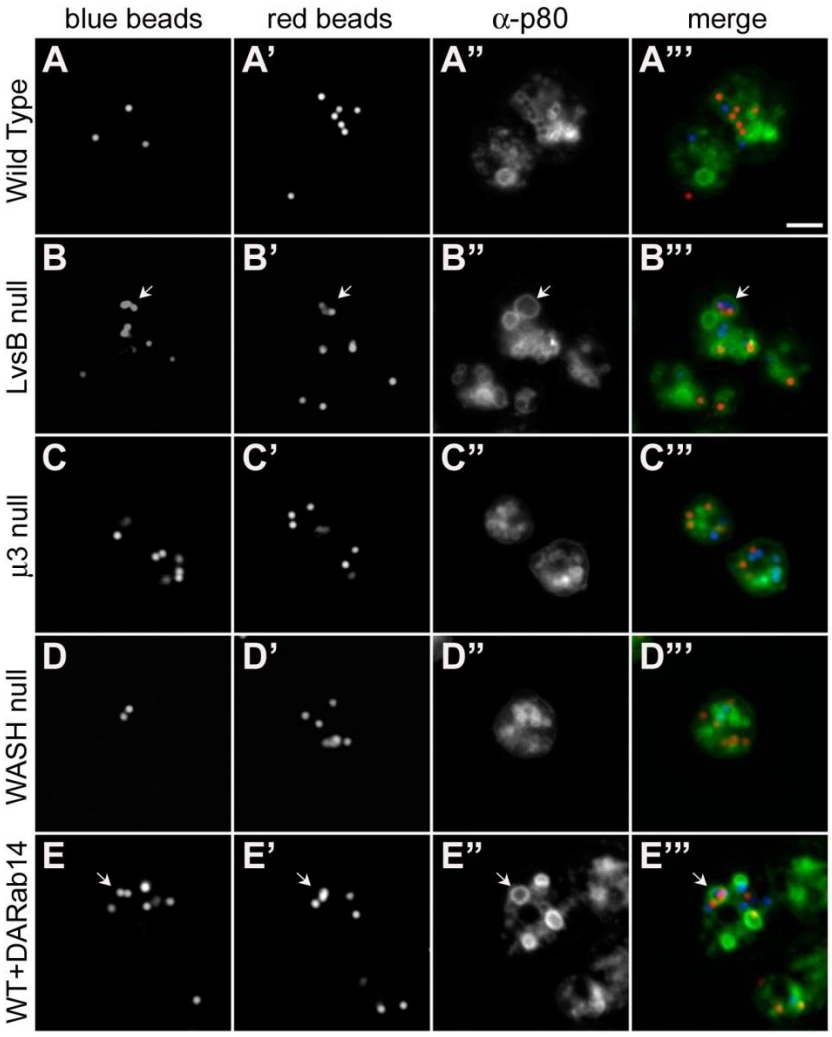
When in its active, GTP bound state, the small GTPase Rab14 associates with endosomal membranes, and acts as a promoter of homotypic fusion between lysosomes (Harris and Cardelli, 2002). A mutant form of Rab14 that is not able to hydrolyze bound GTP into GDP (Rab14Q67L) was cloned and characterized by Harris et al. (Harris and Cardelli, 2002). This constitutively active form of Rab14 (DA-Rab14) was shown to induce increased vesicle fusion when expressed in wild type cells. As will be extensively detailed in chapter 3, the LvsB null cell line shares many phenotypic similarities with cells expressing DA-Rab14 (Kypri et al., 2013). Among these similarities, the expression of DA-Rab14 is implicated in causing increased heterotypic fusion of lysosomes with post-lysosomes. However, we have shown here that many of the assays previously used to diagnose increased heterotypic fusion cannot reliably rule out defects in fission. Because of the strong evidence supporting a relationship between LvsB and Rab14 as well as the known role of Rab14 in promoting homotypic vesicle fusion, we were interested in comparing the vesicle fusion characteristics of the DA-Rab14 phenotype with the LvsB null and fission defect mutants. To this end, we subjected wild type cells expressing DA-Rab14 to the modified 2 dextran pulse chase experiments described above. Consistent with the importance of Rab14 for promoting vesicle fusion, expression of DA-Rab14 caused a significant increase in the fusion of FITC labeled vesicles with TRITC labeled vesicles using a 30 minute chase (29.8% +/- 0.76 s.e.m.) (Fig. 2.4 F) when compared to wild type cells (5.6% +/- 0.35 s.e.m.). This increased level of co-localization in DA-Rab14 expressing cells was not significantly reduced when a 60 minute chase was applied (29.9% +/- 0.76 s.e.m.) (Fig. 2.4 E-E", F). This observation supports the role of Rab14 as a promoter of fusion. Importantly, the pattern of increased fusion levels in response to prolonged maturation time that we reported for LvsB null cells mirrors that for fusion enhanced DA-Rab14 expressing cells. This phenotypic parallel reinforces the role LvsB as a negative regulator of vesicle fusion.

2.2.6 Defects in fission do not recapitulate the phagosome defect of LvsB null cells

An additional phenotypic similarity shared by LvsB null cells and wild type cells expressing DA-Rab14 is the increased occurrence of multi-particulate phagosomes (Harris and Cardelli, 2002; Harris et al., 2002). It has been suggested that Rab14 promotes the fusion of phagosomes with lysosomes for the digestion of phagocytosed material and the homotypic fusion of phagosomes (Harris and Cardelli, 2002). As described in chapter 3, we propose that LvsB is an antagonist of Rab14 function. With this in mind, it is not surprising that loss of LvsB leads to an increase in phagosome fusion (Harris et al., 2002). However, as previously discussed for lysosomes, the formation of multi-particulate phagosomes could be induced by a fission defect. There is a significant amount of interplay between the phagocytic and pinocytic pathways in *Dictyostelium* and many proteins that are important for regulating the endo-lysosomal pathway are shared with maturing phagosomes. These proteins include Rab GTPases such as Rab14 and Rab7, lysosomal hydrolytic enzymes, and the vATPase proton pump (Rupper et al., 2001; Gotthardt et al., 2002; Harris and Cardelli, 2002). Many of these shared proteins are delivered to phagosomes through fusion and intermingling of endo-lysosomal compartments with nascent and maturing phagosomes (Rupper and Cardelli, 2001; Clarke et al., 2010). Under these conditions, the fission defect model described above would predict that delayed progression of lysosomes to the post-lysosomal stage could also block progression of phagosomal compartments. A block of this nature has the potential to increase homotypic fusion of phagosomes to form multi-particulate phagosomes.

To investigate this possibility, we used a phagosome fusion experiment in which cells were given a short pulse of blue fluorescent beads followed by a second pulse of red fluorescent beads. After a 30 minute incubation time to allow the phagosomes to mature through fusion with endo-lysosomal compartments, the cells were fixed and immunostained for the p-80 protein which indiscriminately labels phagosomal, endo-lysosomal, and plasma membranes (Ravanel et al., 2001). Immunostaining of p-80 labeled phagosomal membranes allowed us to distinguish between phagosomes containing multiple beads, and clusters of closely adjacent phagosomes. Only phagosomes containing both blue and red beads were counted as multi-particulate to

ensure that they arose through fusion events rather than by uptake of multiple beads in a single phagocytic cup. In agreement with previous studies (Harris and Cardelli, 2002; Harris et al., 2002), both the LvsB null cell line and wild type cells expressing DA-Rab14 displayed a significantly larger population of cells containing multi-particulate phagosomes (47.4% +/- 1.65 s.e.m. for LvsB null and 37.7% +/- 1.84 s.e.m. for DA-Rab14 expressing cells) (Fig. 2.6 B-B‴, E-E‴, F) when compared to wild type cells (19.2% +/- 2.34 s.e.m.) (Fig. 2.6 A-A‴, F). In contrast, we observed no significant difference in the occurrence of cells containing multi-particulate phagosomes in either the μ3 or WASH null mutant lines compared to wild type cells (22.5% +/- 2.79 s.e.m. for μ3 null and 27.3% +/- 0.78 s.e.m. for WASH null) (Fig. 2.6 C-C‴, D-D‴, F). This disparity between the LvsB null cell line and the fission defect mutant phenotypes coupled with the striking similarities that the LvsB mutant shares with cells expressing DA-Rab14 further validates the role of LvsB as an antagonist of Rab14 mediated fusion events. Furthermore, the absence of a phenotypic change in either of the fission mutants contends that the LvsB null phagosome phenotype cannot be directly attributed to a fission defect along the endo-lysosomal pathway.



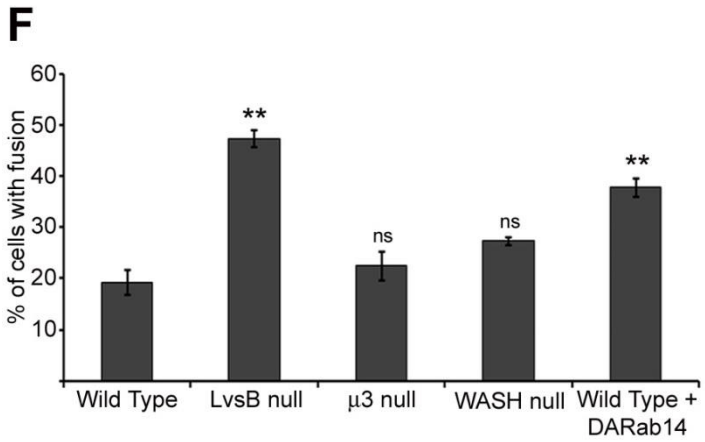


Figure 2.6: The phagosome defect of LvsB null and dominant active (DA) Rab14 expressing cells is not evident in the fission defect mutants. (A-E) Cells were given a pulse with blue fluorescent beads followed by a second pulse with red fluorescent beads to label adjacent populations of phagosomes. After a 30 minute incubation to allow the labeled phagosomes to mature, cells were fixed, flattened, and immunostained with anti-p80 antibody to label the limiting membranes of phagosomes. **(F)** The fraction of cells (>33 cells/experiment) with multi-particulate phagosomes containing both blue and red beads was plotted as the mean +/- s.e.m. of 3 experiments. Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, $P>0.05$; *, $P< 0.05$; **, $P< 0.01$). The increased multi-particulate phagosome formation observed in both LvsB null **(B-B"')** and DA-Rab14 **(E-E"')** expressing cells over wild type **(A-A"')** is consistent with previous studies and supports a fusion regulatory role for both proteins. The μ3 null **(C-C"')** and WASH null **(D-D"')** fission defects did not induce significant increases in multi-particulate phagosome formation. This result suggests that a fission defect alone is not sufficient to cause the formation of multi-particulate phagosomes.

2.2.7 Inappropriate heterotypic fusion is the major contributor to enlarged vesicle size in LvsB null cells

The results of these comparative studies as well as our previously published data have centered on defects in heterotypic fusion between lysosomes and post-lysosomes. However, many studies have attributed the enlarged vesicular phenotype of Lyst or LvsB mutant cells to increased homotypic fusion between lysosome related organelles (Oliver and Essner, 1975; Willingham et al., 1981; Hammel et al., 1987; Stinchcombe et al., 2000; Harris et al., 2002; Hammel et al., 2010). This possibility is bolstered by the reported antagonistic relationship between LvsB and Rab14 in *Dictyostelium* (Kypri et al., 2013). Since Rab14 functions to promote homotypic fusion amongst lysosomes, the absence of LvsB could result in unrestrained lysosome-lysosome fusion, producing enlarged lysosomal compartments. Previously published data showed that when the entire endo-lysosomal pathway is labeled with dextran, LvsB null cells display a marked increase in average vesicle diameter compared to wild type cells (Harris et al., 2002). Increased diameter of vesicles labeled by the post-lysosomal marker, vacuolin, in LvsB null cells along with the data reported in this study implicate deleterious heterotypic fusion as responsible for the enlarged vesicle phenotype (Kypri et al., 2007). These studies cannot, however, exclude increased homotypic lysosome fusion as an additional factor promoting the generation of enlarged vesicles. To determine whether increased homotypic lysosome fusion also contributes to the LvsB null phenotype, we compared the morphology of the lysosomal population and the hybrid vesicle/post-lysosomal population in LvsB null and wild type cells. For this, we used dextran to label the entire endo-lysosomal pathway in cells expressing GFP-vacuolin. The average diameter of lysosomes (dextran vesicles without GFP-vacuolin) in the LvsB null cell line was not significantly different than that of wild type cells (1.04 μm +/- 0.14 s.e.m. for LvsB null and 0.62 μm +/- 0.04 s.e.m. for wild type; p-value 0.08656) (Fig. 2.7 A). In contrast, the average diameter of hybrid vesicles/post-lysosomes (dextran vesicles labeled with GFP-vacuolin) was significantly larger in LvsB null cells compared to wild type cells (1.93 μm +/- 0.07 s.e.m. for LvsB null and 0.87 μm +/- 0.09 s.e.m. for wild type; p-value .000858) (Fig. 2.7 A). This drastic increase in GFP-vacuolin labeled vesicle size alongside a statistically insignificant difference in lysosome size of LvsB null cells indicates that

homotypic lysosome fusion contributes minimally, if at all, to the enlarged vesicle phenotype in LvsB null cells.

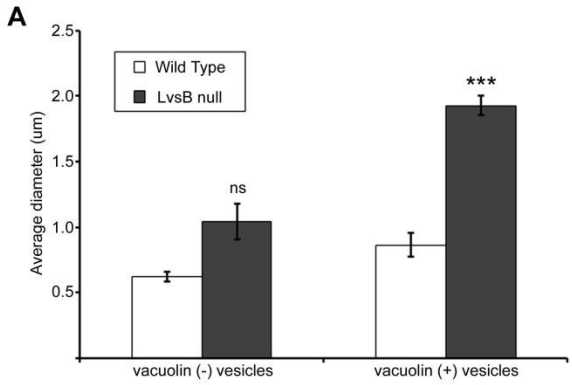


Figure 2.7: The morphology of GFP-vacuolin labeled vesicles is more severely affected than the lysosomal population in LvsB null cells. (A) The entire endosomal pathway was labeled in cells expressing GFP-vacuolin. Live cell images were used to measure the diameter of GFP-vacuolin negative vesicles (lysosomes) and GFP-vacuolin positive vesicles (hybrid vesicles/post-lysosomes). Results were plotted as the mean +/- s.e.m. (>30 vesicles for each population/experiment for 3 experiments). Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, $P>0.05$; *, $P< 0.05$; **, $P< 0.01$). The average lysosome diameter was not significantly elevated in the LvsB null cell line compared to wild type, while the hybrid vesicle/post-lysosomal population demonstrated a 2 fold increase in average diameter. The increased size of only the GFP-vacuolin labeled population coupled with the observed increase in endosomes before the post-lysosomal stage (figure 4) in LvsB null cells indicate that heterotypic fusion between lysosomes and post-lysosomes rather than homotypic lysosome fusion is responsible for the enlarged vesicles in LvsB null cells.

2.3 Discussion

The complex intermingling of regulated fusion and fission events required for the maturation of vesicles along the endo-lysosomal pathway has hampered our understanding of the regulatory role of mammalian Lyst and its *Dictyostelium* orthologue, LvsB. Studies in several model systems have led to two differing models for Lyst/LvsB function. Both the fusion and fission models are well supported in the literature. We attribute this discrepancy in interpretation of Lyst function to the inability of the methods used in most of the currently published studies to decipher between abnormalities caused by defects in fusion or fission.

In this study we used two well-characterized fission defect mutants to show that defects in fission can produce similar phenotypic characteristics to those that were previously attributed to increased heterotypic fusion in LvsB null cells. These results attest to the importance of designing studies that can reliably distinguish between defects in fusion and fission when characterizing regulators of endo-lysosomal trafficking. To this point, we also demonstrated four properties of the LvsB null cells that distinguish them from fission defect mutants and are consistent with a fusion regulatory model for LvsB function. First, the hybrid organelles found in the LvsB null cell line are significantly less acidic than the normal lysosomal population suggesting that they arise from fusion of acidic and neutral compartments and not from delayed maturation of lysosomes to the post-lysosomal stage. This conclusion is supported by the reported normal lysosome de-acidification dynamics in LvsB mutant cells (Kypri et al., 2007). Another study in *Dictyostelium* documented the delayed delivery of phagocytosed beads to post-lysosomal compartments along with a reduced number of post-lysosomes and suggested that these phenotypes signify the delayed biogenesis of post-lysosomes (Charette and Cosson, 2007). However, post-lysosomes were distinguished from lysosomes in this study by the absence of vATPase proton pump. This method of post-lysosome identification does not account for post-lysosomes that have fused with vATPase positive lysosomes. Therefore, the delayed delivery of beads to vATPase negative vesicles does not necessarily indicate delayed delivery to post-lysosomes. Second, we have shown here that the amount of fusion between pulse-labeled vesicles decreases with increased maturation time in the fission defect mutants but not in LvsB

null cells. This observation is indicative of increased heterotypic fusion between lysosomes and post-lysosomes in the absence of LvsB. Third, the WASH-null fission defect does not produce the same pattern or severity of increased endosome size that we observed in LvsB null cells. As expected for a defect in fission, the lysosomes of WASH null cells enlarge at the transition point between lysosomes and post-lysosomes. In contrast, LvsB null cells contain much larger vesicles at early and late times after endocytosis, again consistent with uncontrolled fusion between early and late compartments. Finally, we showed that the fission defect mutants do not have the multi-particulate phagosome phenotype that characterizes both LvsB mutant and DA-Rab14 expressing cells. These phenotypic distinctions suggest that the primary cause of abnormal endo-lysosomal vesicles in LvsB mutant cells is inappropriate heterotypic fusion of post-lysosomes with earlier acidic vesicles. This study cannot, however, determine whether this aberrant fusion is a primary or secondary effect of the loss of LvsB function.

It is entirely possible that the function of LvsB/Lyst is far more complex than what is described by either the fusion or fission model. This possibility is suggested by the results of a two hybrid screen that was used to identify Lyst binding partners (Tchernev et al., 2002). Many of the proposed Lyst interacting proteins identified in this study, such as HRS, CALM, and CK2β, are implicated in the regulation of vesicle fusion (Allende and Allende, 1995; Peters and Mayer, 1998; Schekman, 1998; Tsujimoto and Bean, 2000). This "regulator of fusion-regulators" model for Lyst function is further supported by our recently published results that are outlined in chapter 3 showing an antagonistic relationship between LvsB and the fusion promoting GTPase Rab14 in *Dictyostelium* (Kypri et al., 2013). We have shown here that the kinetics of increased fusion are common to both the dominant active Rab14 and LvsB mutant phenotypes. This characteristic is predictably distinct from the fission defect mutants, and correlates with opposing fusion regulatory roles for LvsB and Rab14. We also showed that homotypic lysosome fusion contributes very minimally to the LvsB null enlarged vesicle phenotype. Coupled with our findings presented in chapter 3, this result suggests that LvsB most likely antagonizes Rab14 function only at the late lysosomal/early post-lysosomal stage and not when Rab14 is needed to promote homotypic lysosome fusion. Based on these

studies, it is intriguing to consider LvsB and Lyst as a hub for regulating fusion events at the transition of lysosomes to the post-lysosomal stage. The localization of LvsB to late lysosomes and post-lysosomes in *Dictyostelium* puts it in the right place at the right time to mediate fusion regulation during this important transition (Kypri et al., 2007).

Though our comparative analyses have shown strong evidence that the LvsB mutant phenotype arises from inappropriate fusion, Durchfort et al (2012) presented evidence arguing that the mouse Lyst homologue promotes fission rather than fusion. While they convincingly show that endosomal fission is disrupted in the absence of Lyst, the experimental approaches used to refute the fusion model for Lyst function have inherent flaws that render their results inconclusive. Two lines of evidence were used to demonstrate normal fusion in the absence of Lyst. In one case, endosomes were artificially fractured in order to measure fusion mediated recovery. Under these conditions, no difference in recovery rate was documented upon disruption of Lyst. However, our results indicate that it is vesicle fusion promiscuity rather than rate that is altered in the absence of the *Dictyostelium* Lyst homologue. Durchfort et al. (2012) also reported that the *in vitro* fusion competency of purified endosomes was not affected by the absence of Lyst. However, all vesicles used in these experiments were collected from wild type cells and fusion was assessed in cytosol from either *beige*$_j$ (Lyst mutant) or wild type cells. Though immuno-localization of Lyst has shown only a cytosolic localization, microscopic examinaiton of *Dictyostelium* cells expressing GFP-tagged LvsB show it localized primarily on endosomal membranes. If Lyst localization mimics that shown for LvsB, then the Lyst that is associated with purified endosomal membranes would mitigate their fusion competency regardless of the cytosolic environment. Based on this possibility, the currently reported *in vitro* fusion results cannot be regarded as reliable. While the contention that Lyst does not influence vesicle fusion presented by Durchfort et al (2012) is unfounded, they did document direct visual evidence of defective fission from drug-induced enlarged endosomal compartments in Lyst mutant cells. In reconciling these findings with the fusion model, we postulate that Lyst may also regulate fusion through fission mediated recycling of fusion machinery as a lysosome matures into a fusion restricted post-lysosome. The idea that both phagosome and lysosome fusion dynamics could be perturbed by a defect in recycling of fusion

machinery is bolstered by the fact that phagosomes and lysosomes share multiple snares (Gotthardt et al., 2002). This could explain why studies which focus specifically on fission show evidence that Lyst promotes fission, but inappropriate fusion defects are implicated in studies using Lyst mutant cells where exocytosis is the rate limiting step. In agreement with this hypothesis, the strongest phenotypic effects of CHS arise due to decreased or aberrant function of specialized secretory cells that function through regulated exocytosis of post-lysosomal secretory vesicles (Introne et al., 1999). Additionally, a study comparing cells of beige and wild type mice showed an 18 fold increase in mast cell secretory granule size, which can be retained for months before exocytosis, but only a moderate, 23%, increase in secretory granule size of pancreatic acinar cells which are retained for just hours (Hammel et al., 1987; Hammel et al., 2010). With this model in mind, it would be interesting to investigate the recycling dynamics of snares that function along both the endosomal and phagosomal pathways, such as vamp7a and vti1, in the LvsB null cell line.

By homology with LvsB, we suggest that the enlarged lysosome related compartments in CHS patients are a product of inappropriate fusion. This fusion regulatory model for Lyst function is not a new one, but has been widely disputed. Taking the vast number of functional Lyst homologue studies into account with our own comparative analysis of LvsB, we believe that Lyst is a negative regulator of fusion events, and helps to stop fusion of vesicles as they mature beyond the lysosomal stage. Though a few studies have provided insight into how Lyst might influence fusion regulation, the degree of separation between Lyst and the fusion machinery is yet undefined. As we collaborate to understand the mechanisms contributing to Lyst mediated regulation, it will be interesting to see if Lyst prevents fusion directly, indirectly, or both.

Chapter 3: Antagonistic control of lysosomal fusion by Rab14 and the Lyst-related protein LvsB.

3.1 Introduction

The endo-lysosomal system is a complex collection of pleomorphic organelles that traffic a wide range of molecules and receive input from multiple sources including the TGN, phagocytosis and endocytosis (Bonifacino and Glick, 2004). To accomplish their function, endo-lysosomal vesicles must control their composition by undergoing multiple fusion and fission events. In this way, one molecule internalized by endocytosis may eventually reach the lysosome while another one may be recycled back to the plasma membrane. To achieve proper sorting of different cargo molecules, the fusion between different compartments of this system must be precisely regulated. Thus, it is not surprising that a large number and diversity of regulatory proteins have been identified in different compartments of the endolysosomal system, including Rabs, SNAREs, HOPS, etc (Wickner, 2010; Yu and Hughson, 2010). A major challenge in this field is understanding how these and other components collaborate to accomplish the tightly regulated sorting necessary for the elaborate functions of the endo-lysosomal system.

The importance of the endo-lysosomal system is evinced by the severe hereditary diseases that are caused by defects in its regulation. Many lysosomal storage diseases have been identified that impinge on important regulatory mechanisms (Ward et al., 2000a). Among them, Chediak-Higashi syndrome has been a difficult case to dissect in detail. The gene affected in patients with this disorder was identified as one encoding a 430KDa protein named LYST (lysosomal trafficking regulator) whose function remains unknown (Kaplan et al., 2008). Cells from these patients contain grossly enlarged lysosomes that fail to function properly and lead to defects in skin pigmentation, blood clotting and immune defense. To date, the localization of LYST along the endo-lysosomal pathway has not been established and no binding partner has been identified *in vivo*. Thus, it has not been possible to postulate any mechanism by which LYST may regulate lysosomal size and function.

We have shown that *Dictyostelium* LvsB protein is the ortholog of human LYST and, like LYST, is also required for the proper function of the lysosome (Kypri et al., 2007). Loss of LvsB results in the enlargement of acidic lysosomal compartments and causes secretory defects (Cornillon et al., 2002; Harris et al., 2002). These observations suggest that the *Dictyostelium* LvsB-null mutant represents an excellent single-cell model system for the study of the cellular defects that cause Chediak-Higashi Syndrome.

The endo-lysosomal system of *Dictyostelium* consists of multiple compartments that rapidly process endocytosed materials and excrete indigestible substances. Endocytic and phagocytic vesicles are quickly acidified and receive lysosomal enzymes to digest their contents. The acidic lysosomal vesicles subsequently mature into post-lysosomes, neutral secretory vesicles that are destined for exocytosis (Rauchenberger et al., 1997; Jenne et al., 1998) (simplified endo-lysosomal pathway shown in Fig. 1.1). Consequently, the *Dictyostelium* lysosome is not a terminal organelle as in most mammalian cells, but is most similar to the secretory lysosomes of specialized mammalian cells (Blott and Griffiths, 2002).

Previously, we showed that LvsB localizes on late lysosomes and post-lysosomes (Fig. 1.1). Moreover, in LvsB-null cells lysosomes fuse inappropriately with post-lysosomes; a rare occurrence in wild type cells (Kypri et al., 2007) (also see chapter 2). A consequence of the inappropriate fusion between compartments is that the maturation of secretory competent post-lysosomes is delayed (Charette and Cosson, 2007). These results suggested that the function of LvsB (and of LYST) is to act as a negative regulator of vesicle fusion and that the enlarged lysosome phenotype of Chediak-Higashi Syndrome patients could result from uncontrolled LRO fusion.

To better understand how LvsB controls vesicle fusion events it is important to determine whether LvsB interacts with any of the known components that promote vesicle fusion. In mammalian cells the Rab family of GTPases plays a major role in the regulation of vesicular trafficking. Rabs have been implicated in the control of both fusion and fission events that are required for the proper maturation of endosomes as well as phagocytic compartments (Grosshans et al., 2006). This Rab mediated control of vesicular trafficking seems to be conserved across many species including *Dictyostelium*. Currently, 54 putative Rab related GTPase genes have been identified in *Dictyostelium*.

Characterization studies have shown that the diversity of these proteins extends to their functional capacities along many membrane trafficking pathways. Similar to the complexity of mammalian cells, a number of *Dictyostelium* Rab proteins including Rab21, Rab7, and Rab14 serve to regulate distinct maturation steps along the endocytic and/or phagocytic pathways (Bush et al., 1994; Bush et al., 1996; Buczynski et al., 1997; Harris and Cardelli, 2002; Khurana et al., 2005). Interestingly, studies of the *Dictyostelium* small GTPase Rab14 showed that activation of Rab14 induced a phenotype reminiscent of that shown in LvsB-null cells (Bush et al., 1994; Bush et al., 1996; Harris and Cardelli, 2002). *Dictyostelium* Rab14 was shown by fluorescence microscopy as well as by cell fractionation to localize on lysosomes and on the membranes of contractile vacuoles (Bush et al., 1994). Expression of constitutively active (DA) Rab14 (Rab14Q67L) enhanced the fusion of lysosomal vesicles leading to the formation of enlarged lysosomes. Similarly, constitutive activation of Rab14 caused phagosomes to fuse together, forming large vesicles containing multiple engulfed bacteria (Harris and Cardelli, 2002), a phenotype shared with LvsB null cells (Kypri et al., 2007). These studies suggested that LvsB and Rab14 may control similar fusion events along the endo-lysosomal pathway. However, this possibility has not been examined as the studies of Rab14 focused solely on the early homotypic fusion of lysosomes whereas our LvsB studies centered on the control of heterotypic fusion between lysosomes and post-lysosomes.

In the present study, Dr. Elena Kypri and I collaborated to provide evidence that LvsB acts as an antagonist to the fusion-promoting activity of the GTPase Rab14. Dr. Kypri's studies also show that the function of LvsB is restricted to the regulation of fusion between lysosomes and post-lysosomes and not of other compartments. These results provide a mechanistic framework to understand the function of LvsB, and by extension, of human LYST. My contributions to this study are distinguished from those of Dr. Kypri in the following results section, and all data contributed by Dr. Kypri can be found in her previously published thesis.

3.2 Results

3.2.1 Wild-type Rab14 mis-localizes on post-lysosomes in LvsB-null cells

To explore a possible functional interaction between Rab14 and LvsB, we initially determined whether the localization of wild-type Rab14 was affected by the loss of LvsB. GFP-Rab14, a previously characterized reporter of endogenous Rab14 localization in *Dictyostelium* (Harris and Cardelli, 2002), was expressed in wild-type and LvsB-null cells. The expression of wild-type GFP-Rab14 did not alter the size or morphology of endo-lysosomal vesicles in wild-type or LvsB-null cells (data not shown). In both cell lines GFP-Rab14 brightly labeled the membranes of the contractile vacuole (Figure 3.1 A-B) (contractile vacuole data contributed by Dr. Kypri). This organelle was readily identified by its dynamic contractile behavior in time-lapse movies (data not shown). The presence of Rab14 on acidic endo-lysosomal vesicles has been previously demonstrated using immuno-electron microscopy and cell fractionation experiments (Bush et al., 1994). To determine whether Rab14 is also localized on the neutral post-lysosomal compartment we stained cells with antibodies against vacuolin, a flotillin-like protein that associates primarily with the post-lysosome (Rauchenberger et al., 1997) (Fig. 1.1). We found that wild-type cells contained few vesicles labeled by both GFP-Rab14 and vacuolin (5.8%, n=86) (Figure 3.1 C-C''). In contrast, LvsB-null cells contained many large vesicles that were clearly labeled by both GFP-Rab14 and vacuolin (60%, n=40) (Figure 3.1 D-D''). Thus LvsB null cells, which suffer from inappropriate lysosomal fusion, also exhibit abnormal co-localization of lysosomal (Rab14) and post-lysosomal (vacuolin) markers. Images of anti-vacuolin staining in GFP-Rab14 expressing cells were collected by Dr. Kypri, and quantification of co-localization from these images was performed by me.

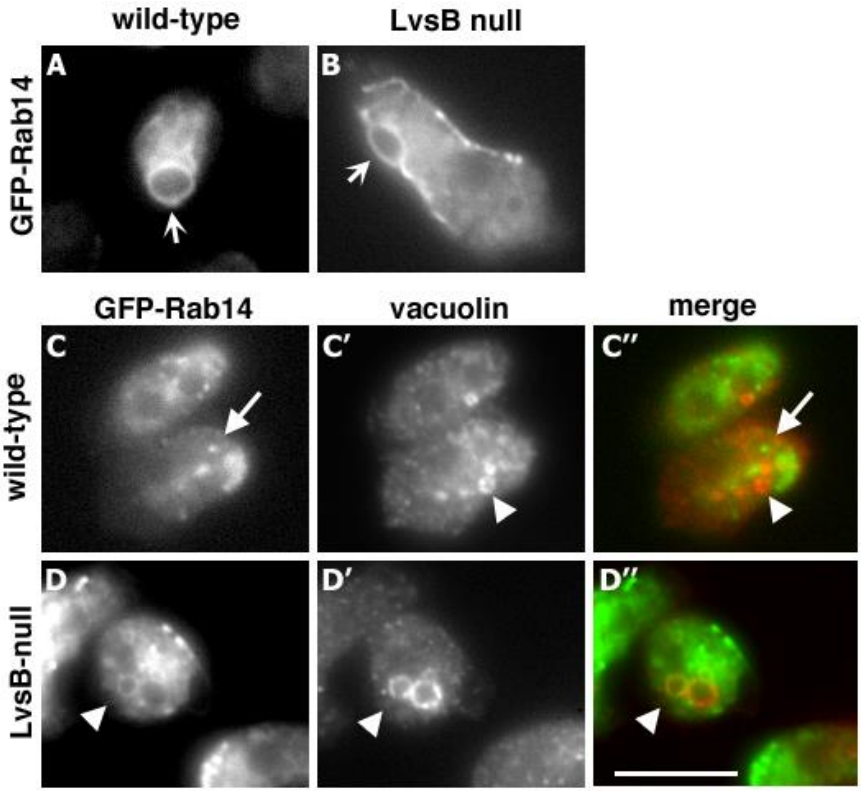


Figure 3.1: Loss of LvsB induces mis-localization of wild-type Rab14 on post-lysosomes but does not affect its localization on the contractile vacuole. Wild-type and LvsB-null cells were transfected with wild-type GFP-Rab14. The localization of wild-type GFP-Rab14 on the contractile vacuole was indistinguishable in wild-type **(A)** and LvsB-null cells **(B)** (arrows). Contractile vacuoles were identified using time-lapse microscopy by their characteristic expanding and contracting behavior (data not shown). Cells expressing GFP-Rab14 were also stained with anti-vacuolin antibody to visualize the post-lysosomal compartment. In wild type cells **(C)**, Rab14 was detected in small vesicles (arrow) that did not co-localize with vacuolin **(C')** (arrowhead). In contrast, LvsB null cells contained large vesicles that were labeled by both GFP-Rab14 **(D)** and vacuolin **(D')** (arrowheads). This cell also shows an adjacent vacuolin-labeled post-lysosome that did not contain GFP-Rab14. Merged images are shown in C" and D". Focal planes in C & D were different from those in A & B to avoid imaging the contractile vacuoles. Bar,10 µm.

3.2.2 Activation of Rab14 mimics the phenotype of LvsB-null cells.

The abnormal co-localization of Rab14 and vacuolin in LvsB-null cells may be due to one of two possible scenarios. First, since lysosomes fuse with post-lysosomes in LvsB null cells, it is possible that Rab14 on the lysosomal membrane is passively mixed with post-lysosomal markers when such heterotypic fusion events occur. Alternatively, the activity of Rab14, which promotes fusion among lysosomes (Harris and Cardelli,

2002), may be antagonized by LvsB when late lysosomes become post-lysosomes. In this case, loss of LvsB would allow Rab14 to remain active on a lysosome as it matures into a post-lysosome and would then promote heterotypic fusion between post-lysosomes and earlier compartments. A major difference between these two models is that in one, Rab14 activity is not responsible for the heterotypic fusion while in the other, Rab14 is the cause of heterotypic fusion.

To distinguish between these models we compared the effect of introducing constitutively active (DA) Rab14 (Rab14Q67L) into wild-type and LvsB-null cells. This mutant form of Rab14 cannot hydrolyze bound GTP to GDP (Harris and Cardelli, 2002). We expressed flag-tagged DA-Rab14 in both cell lines and evaluated the morphology of their entire endo-lysosomal system by incubating cells with TRITC-dextran for 1 hour (Figure 3.2 A-D). Western blot analysis demonstrated that the flag-DA-Rab14 protein was equally expressed in both cell lines (data not shown). As reported previously, relative to wild type control cells, LvsB-null cells contained fewer but greatly enlarged dextran-labeled vesicles (Figure 3.2 A, B and G) (Harris et al., 2002; Kypri et al., 2007). Similarly, the expression of flag-DA-Rab14 in wild-type cells caused the enlargement of dextran-labeled vesicles (Figure 3.2 C, G). The expression of DA-Rab14 in LvsB-null cells caused additional enlargement of their dextran-labeled vesicles to the same diameter as those in wild-type cells expressing DA-Rab14 (Figure 3.2 D, G). Dextran vesicle images for all cell lines were contributed by Dr. Kypri. Quantification of dextran diameter was done by me using images collected by Dr. Kypri.

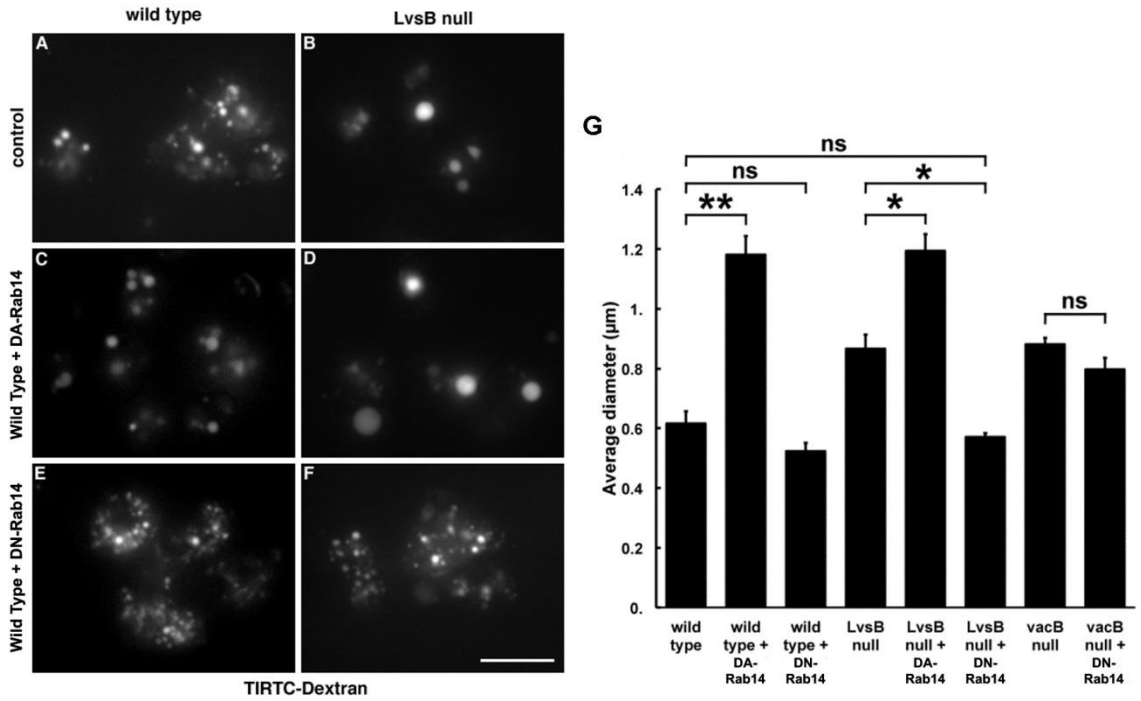


Figure 3.2: Expression of mutant forms of Rab14 alters the endolysosomal morphology of wild-type and LvsB-null cells. Wild-type and LvsB-null cells were transfected with the plasmid for expression of constitutively active flag-DA-Rab14 or constitutively inactive flag-DN-Rab14 (Rab14N121I). **(A-F)** The endosomal morphology was evaluated by incubating with TRITC-dextran for 1 hour. Untransfected wild-type and LvsB-null cells were used as controls. **(G)** The size of >30 vesicles/experiment were measured from micrographs similar to those shown in A-F and plotted as the mean +/- s.e.m. (n=3). Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, P>0.05; *, P< 0.05; **, P< 0.01). Expression of the active form of DA-Rab14 in wild-type cells **(C)**, resulted in the accumulation of significantly enlarged vesicles **.** Expression of the active form of DA-Rab14 in the LvsB-null cells **(D)** also caused a significant enlargement of endosomes compared to the vesicles in the LvsB-null untransfected cell line **(B)**. The expression of inactive DN-Rab14 did not cause a significant decrease in the size of labeled endosomes in wild-type cells **(E)**. In contrast, DN-Rab14 greatly decreased the size of endosomes in LvsB-null cells **(F)** to a size similar to those found in wild-type cells **(A)**. **(G)** The size of Dextran labeled endosomes were also measured in vacuolin B mutant cells with or without expression of DN-Rab14. Vacuolin B mutant cells contain enlarged endosomes similar to those seen in LvsB null cells. Expression of DN-Rab14 in vacuolin B-null cells did not reduce the size of their endosomes to a wild-type size. Thus, while activation of Rab14 in wild-type cells mimics the phenotype of LvsB-null cells, the inactivation of Rab14 suppresses the phenotype of LvsB-null cells but not vacuolin B null cells. These results suggest an antagonistic relationship between LvsB and Rab14 in controlling endo-lysosomal vesicle size. Bar, 10µm

We next determined the effect of DA-Rab14 expression on the vacuolin-labeled post-lysosome. LvsB null cells contained enlarged vacuolin-labeled vesicles compared to those found in wild type cells (Figure 3.3 A, B, and G). Again, DA-Rab14 expression

caused an enlargement of post-lysosomes in wild type cells to a size comparable to those in LvsB null cells (Figure 3.3 C, G), and a further enlargement in LvsB null cells (Figure 3.3 D, G). Thus, expression of constitutively active (DA) Rab14 in wild type cells seems to replicate the effect of loss of LvsB function suggesting an active role for Rab14 in promoting heterotypic fusion in LvsB-null cells. Representative images were contributed by Dr. Kypri. Quantification of GFP-vacuolin labeled vesicles was performed by me using images collected by both Dr. Kypri and myself.

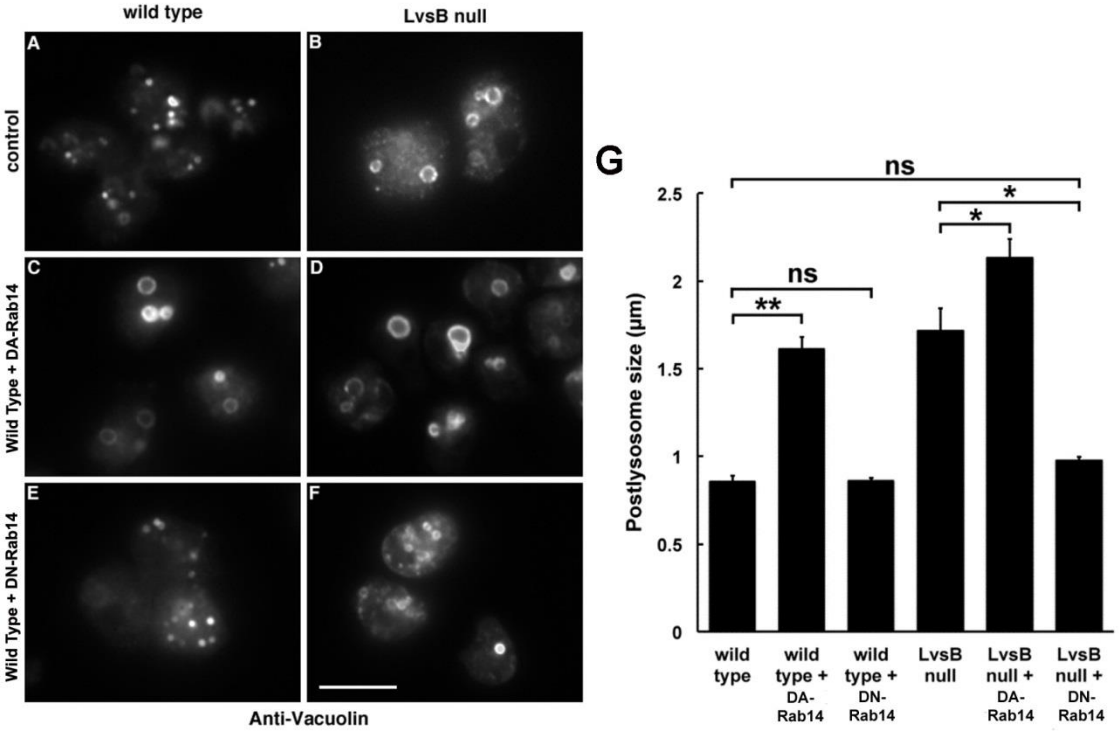


Figure 3.3: Expression of mutant forms of Rab14 alters the size of vacuolin-labeled post-lysosomes in wild-type and LvsB-null cells. (A-F) Wild-type and LvsB-null cells expressing mutant forms of Rab14 as in Figure 2 were fixed and stained with antibodies against vacuolin B. **(G)** The size of >30 vesicles/experiment were measured and plotted as the mean +/- s.e.m. (n=3). Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, P>0.05; *, P< 0.05; **, P< 0.01). Expression of the active form of Rab14 (DA-Rab14) caused the enlargement of post-lysosomes in wild-type cells **(C)** so that they resembled those in LvsB-null cells **(B)**. Active Rab14 (DA-Rab14) slightly increased the size of post-lysosomes in LvsB-null cells **(D)**, compared to those in non-expressing cells **(B)**. On the other hand, while expression of the inactive form of Rab14 (DN-Rab14) in wild-type cells did not significantly alter the size of their post-lysosomes **(E)**, its expression drastically reduced the size of post-lysosomes of LvsB-null cells **(F)** to a size similar to wild-type control **(A)**. Bar, 10µm

To explore this phenotypic similarity in more detail, we tested the effect of active Rab14 (DA-Rab14) expression on the rates of heterotypic fusion between lysosomes and post-lysosomes (Figure 3.4). We used an *in vivo* fusion assay of vesicles labeled by endocytosis of two differently labeled dextrans (Kypri et al., 2007). A 30 minute chase period allows the first dextran pulse to reach the post-lysosomal compartment before the second dextran pulse is internalized by the cells. In wild type cells, the early and late compartments remained distinct and, as a result, did not display significant co-localization between the two dextran markers (4.3%, n=310) (Figure 3.4 A and F). In contrast, the two markers co-localized to a great extent in LvsB null cells (41.2%, n=136) indicating inappropriate heterotypic fusion between early and late labeled vesicles (Figure 3.4 C and F). Remarkably, the expression of active Rab14 (DA-Rab14) caused a five-fold increase in the percentage of heterotypic fusion in wild-type cells (20.5%, n=241) but only a slight increase in LvsB-null cells (49.8%, n=130) (Figure 3.4 B, D and F).

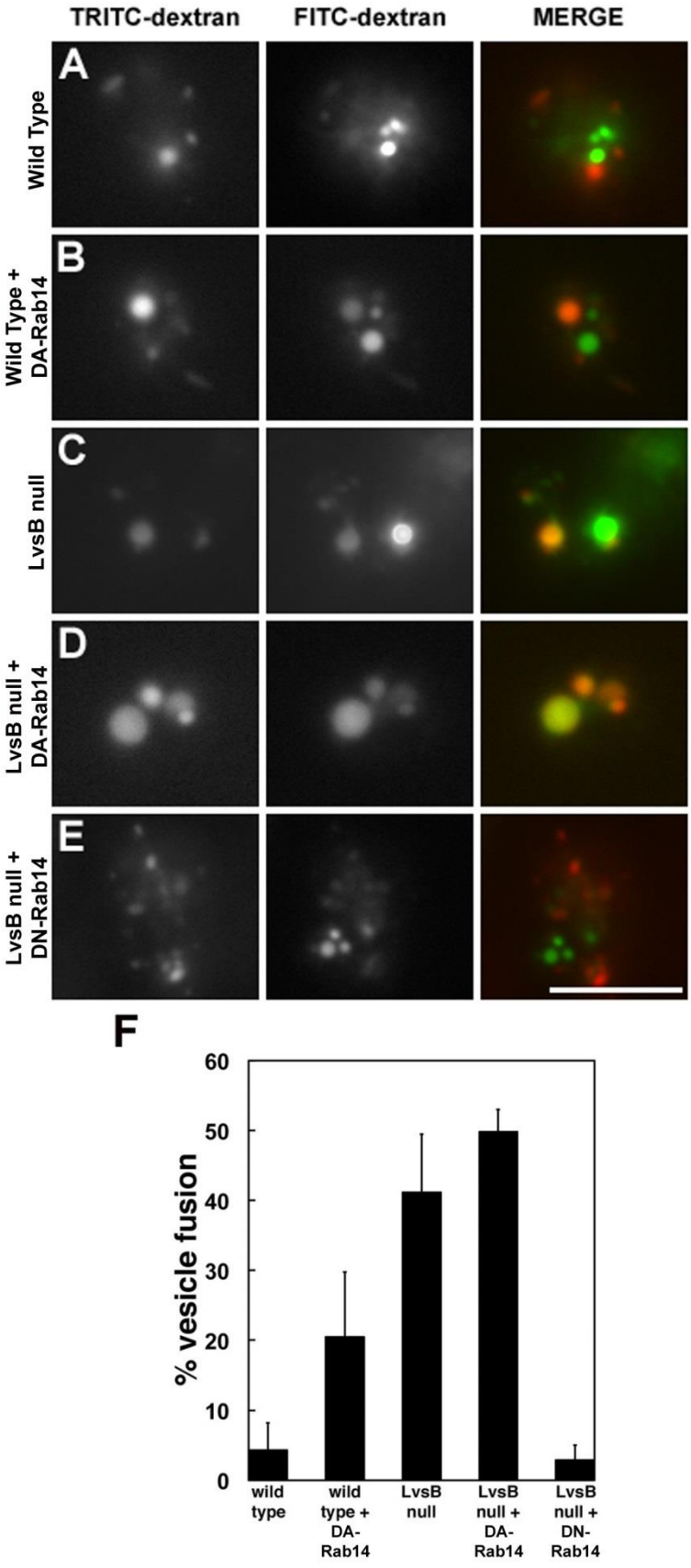


Figure 3.4: Expression of mutant forms of Rab14 influences the fusion of early and late endocytic vesicles in wild-type and LvsB-null cells. Cells were given a pulse of FITC dextran followed by a 30 minute chase and then a second pulse with TRITC dextran. The fusion of endosomal vesicles was determined by colocalization of the two differently labeled dextrans from images similar to those shown for **(A)** Wild-type control cells; **(B)** Wild-type cells expressing active flag-DA-Rab14; **(C)** LvsB-null cells; **(D)** LvsB-null cells expressing active flag-DA-Rab14; **(E)** LvsB-null cells expressing inactive flag-DN-Rab14. **(F)** The fraction of vesicles containing both fluid phase markers were quantified in two independent experiments and shown as the mean +/- range. Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, $P>0.05$; *, $P< 0.05$; **, $P< 0.01$). Expression of the active form of Rab14 (DA-Rab14) caused a five-fold increase in the heterotypic fusion in wild-type cells but not in LvsB-null cells. Significantly, the expression of the inactive form of Rab14 (DN-Rab14) suppressed the heterotypic fusion shown in LvsB-null cells. Bar 10 μm.

Lastly, we determined whether the constitutively active (DA) Rab14 protein remained associated with post-lysosomes, thereby affecting heterotypic fusion. We visualized the localization of DA-Rab14 in cells co-expressing flag-DA-Rab14 and GFP-vacuolin B. Active (DA) Rab14 localized mainly on the contractile vacuole membranes in both wild-type and LvsB-null cells (data not shown). Unlike wild-type Rab14, we found that active (DA) Rab14 co-localized with vacuolin on post-lysosomes of wild type cells (Figure 3.5, A-C). This localization could account for the increase in heterotypic fusion in wild type cells expressing constitutively active (DA) Rab14. As expected, in LvsB-null cells the active form of Rab14 (DA-Rab14) was also found on post-lysosomes (Figure 3.5, D-F). All dextran co-localization data presented in this section were contributed by Dr. Kypri.

Taken together, these results show that enhanced activation of Rab14 promotes heterotypic fusion between early and late endo-lysosomal compartments leading to the formation of grossly enlarged hybrid vesicles, a phenotype identical to that observed in LvsB null cells.

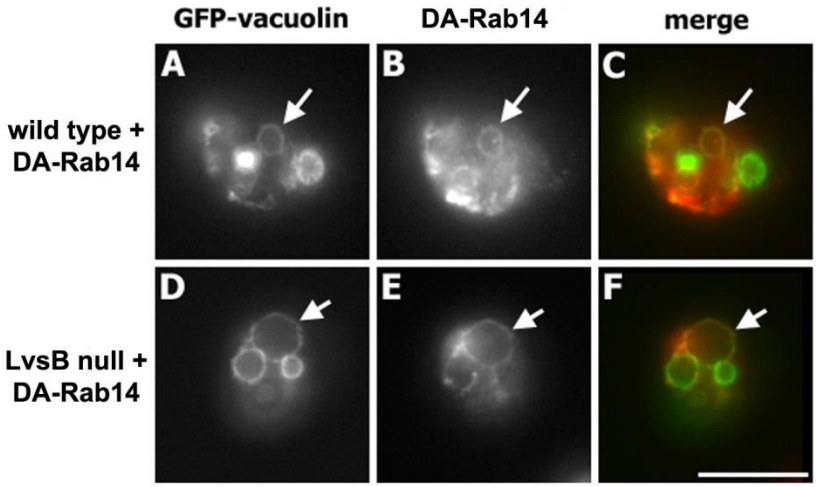


Figure 3.5: The active form of Rab14 (DA-Rab14) associates with post-lysosomes in wild-type and LvsB-null cells

Wild-type and LvsB-null cells were transfected with GFP-Vacuolin B and flag-DA-Rab14 and stained with anti-flag monoclonal antibody. Active Rab14 (DA-Rab14) co-localized with vacuolin B in both wild-type **(A - C)** and LvsB-null **(D - F)** cells (arrows). Bar, 10µm

3.2.3 Inactivation of Rab14 suppresses the lysosomal defect of LvsB-null cells

Our data suggested that the phenotype of LvsB null cells could be caused by the inability to inactivate Rab14 at the appropriate time during the maturation of lysosomes into post-lysosomes. If this scenario is correct, then we would expect that lowering Rab14 activity should compensate for the defect of LvsB null cells. To test this idea, we examined the effect of expressing constitutively inactive (DN) Rab14 (Rab14N121I) on the phenotype of wild type and LvsB-null cells. This mutant form of Rab14 is unable to bind to GTP or GDP and is therefore non-functional (Bush et al., 1996). Overexpression of this non-functional DN-Rab14 construct is shown to have a dominant negative effect on vesicle size and fusion. Western blot analysis confirmed that the flag-DN-Rab14 protein was expressed at similar levels in both cell lines and staining with anti-flag monoclonal antibodies showed that the protein had the expected cytosolic localization (data not shown). We then determined the morphology of dextran-labeled vesicles and vacuolin-stained post-lysosomes in these cell lines.

Remarkably, expression of inactive Rab14 (DN-Rab14) was able to completely suppress the phenotype of LvsB null cells. Both the morphology of their dextran labeled vesicles (Figure 2F, G) and the size of their vacuolin-labeled post-lysosomes (Figure 3.3 F, G) were similar to those observed in wild type cells (Figure 3.2A, G and 3.3A, G). I quantified dextran vesicle and vacuolin labeled vesicle diameter from images contributed by Dr. Kypri. Representative images were also provided by Dr. Kypri. Furthermore, expression of inactive Rab14 (DN-Rab14) in LvsB null cells caused a dramatic reduction in their rates of heterotypic fusion to only 2.9% (n=326), close to that observed in wild-type controls (Figure 3.4 E and F). In contrast, expression of DN-Rab14 caused only a small change in the size of dextran-labeled vesicles in wild-type cells (Figure 3.2 E, G), and did not seem to affect their post-lysosome size (Figure 3.3 E, G). Therefore, the reduction of Rab14 activity in LvsB null cells restored normal size and segregation of their lysosomes and post-lysosomes. All heterotypic fusion data detailed in the previous section was collected and contributed by Dr. Kypri.

3.2.4 The phenotype of LvsB-null cells is not suppressed by changes in Rab7 activity.

While our results show that LvsB antagonizes the function of Rab14, it is possible that LvsB has a broad role in regulating the activity of Rab GTPases along the endocytic pathway. Of the *Dictyostelium* Rab GTPases that have been characterized, Rab7 emerged as the best candidate for investigating this possibility. *Dictyostelium* Rab7 is known to localize to lysosomes, post-lysosomes, and phagosomes (Buczynski et al., 1997). This localization pattern is similar to that of LvsB, and we previously showed that Rab7 co-localizes with LvsB on post-lysosomal vesicles (Kypri et al., 2007). In contrast to Rab14, expression of constitutively active (DA) Rab7 (Rab7Q67L) causes a reduction in the size of acidic vesicles by promoting the retrograde traffic from late lysosomes to earlier compartments (Buczynski et al., 1997). Consistent with this, dominant negative (DN) Rab7 (Rab7T22N) blocks retrograde traffic and causes an enlargement of acidic lysosomal compartments (Buczynski et al., 1997). This enlargement of acidic compartments is reminiscent of both the LvsB null, and dominant active (DA) Rab14 phenotypes, though the reported causal defect is different (Harris and Cardelli, 2002; Harris et al., 2002). Based on these localization and phenotypic similarities, we wanted to test the possibility of a functional interaction between Rab7 and LvsB. Therefore, we compared the effect of activating and inactivating mutations of Rab7 on the phenotype of LvsB null cells and contrasted those with our previous observations using Rab14 mutants. Figure 3.6 A shows the size of dextran-labeled vesicles in LvsB null cells expressing the different Rab mutant proteins. In contrast to the suppression of the LvsB null phenotype caused by inactive Rab14 (DN-Rab14), neither active Rab7 (DA-Rab7) nor inactive Rab7 (DN-Rab7) reduced the size of dextran labeled vesicles in these cells. As expected, impairing retrograde traffic with inactive Rab7 (DN-Rab7) caused a further enlargement of the dextran-labeled vesicles in LvsB null cells. Importantly, the enhancement of retrograde traffic with DA-Rab7 did not suppress the large endosomal phenotype of LvsB null cells. This suggests that the functional interaction between LvsB and Rab14 is specific to these two proteins. All Rab7 data detailed in this section were collected and analyzed by me.

These results support the model that, in contrast to Rab14 and LvsB, Rab7 does not control the fusion between lysosomes and post-lysosomes. Since the active and inactive mutations of Rab7 produce effects opposite to those of the corresponding Rab14 mutations we wanted to ask if the dominant active form of DA-Rab7 could suppress the inappropriate fusion phenotype of LvsB null cells. Indeed, using the *in vivo* fusion assay we found that expression of DA-Rab7 did not suppress the high rate of heterotypic vesicle fusion in LvsB null cells (Figure 3.6 B). Taken together our data indicates that the functional interaction between LvsB and Rab14 seems specific to these two proteins and does not extend to additional GTPases like Rab7.

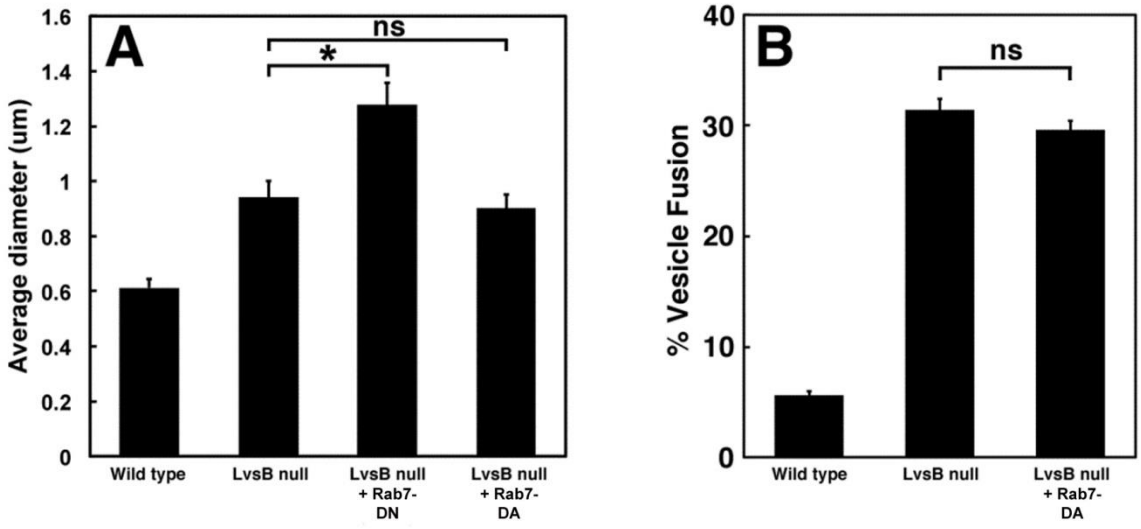


Figure 3.6: Changes in Rab7 activity do not suppress the defects of LvsB-null cells. (A) Mutant forms of Rab7 do not suppress the enlarged endosome phenotype of LvsB-null cells. LvsB-null cells expressing mutant forms of Rab7 were labeled with TRITC-dextran as in Figure 3.2. The size of >30 vesicles/experiment were measured and plotted as the mean +/- s.e.m. of at least three experiments (ns, not significant, P>0.05; *, P< 0.05). In contrast to the suppression of the LvsB null phenotype by the inactive form of Rab14 (DN-Rab14) (Figure 2), neither activating nor inactivating mutants of Rab7 caused any reduction in the size of LvsB null endosomes. **(B)** Expression of active Rab7 (DA-Rab7) does not suppress the abnormal fusion of early and late endocytic vesicles in LvsB null cells. The fusion of early and late endosomal vesicles was determined by co-localization of two differently labeled dextrans as described in Figure 3.4 and plotted as the mean +/- s.e.m. as in (A). Expression of the active form of Rab7 (DA-Rab7) did not alter the high rate of heterotypic fusion in LvsB-null cells.

3.2.5 Inactivation of Rab14 does not suppress the post-lysosomal defects of Vacuolin-null cells.

The suppression of the LvsB null phenotype by expression of inactive Rab14 (DN-Rab14) is consistent with our hypothesis that LvsB acts as an antagonist to Rab14. However, it is also possible that expression of DN-Rab14 causes a general block of membrane fusion that would reduce the size of all endo-lysosomal vesicles in LvsB null cells. To distinguish between these possibilities we determined the effect of inactive Rab14 (DN-Rab14) expression in vacuolin B-null cells. Vacuolin B mutant cells display an enlarged post-lysosomal morphology similar to that observed in LvsB null cells (Jenne et al., 1998). However, unlike LvsB null cells, the enlarged post-lysosome phenotype of vacuolin B null cells arises from a defect in the exocytosis of post-lysosomes. We found that expression of DN-Rab14 caused only a minimal reduction in the size of dextran-labeled vesicles in vacuolin B null cells (Figure 3.2 G). This decrease in size was of similar magnitude to that caused by DN-Rab14 expression in wild type cells and quite different from its effect in LvsB null cells. Hence, expression of DN-Rab14 specifically suppresses the phenotype of LvsB null cells supporting the notion that LvsB is an antagonist of Rab14. I collected and processed all data presented in the above results section.

The observation that DN-Rab14 did not suppress the phenotype of vacuolin B null cells implies that the enlarged post-lysosomes in these cells do not experience heterotypic fusion as it occurs in LvsB null cells. Indeed, using the two-dextran fusion assay we found that heterotypic fusion occurs with a low frequency in vacuolin B null cells (4.9%) similar to wild type cells (3.3%). In addition, staining with Lysotracker red, a probe that accumulates in acidic compartments, showed that vacuolin B null cells contain small acidic lysosomes similar to those in wild type cells (Figure 3.7). Therefore, the enlargement of post-lysosomes in vacuolin B and LvsB null mutants arise by different mechanisms. In vacuolin B null cells they arise by a defect in exocytosis and in LvsB null cells they arise by a Rab14-mediated pathway. Characterization of acidic vesicle morphology and heterotypic fusion levels in vacuolin B null cells was performed by Dr. Kypri.

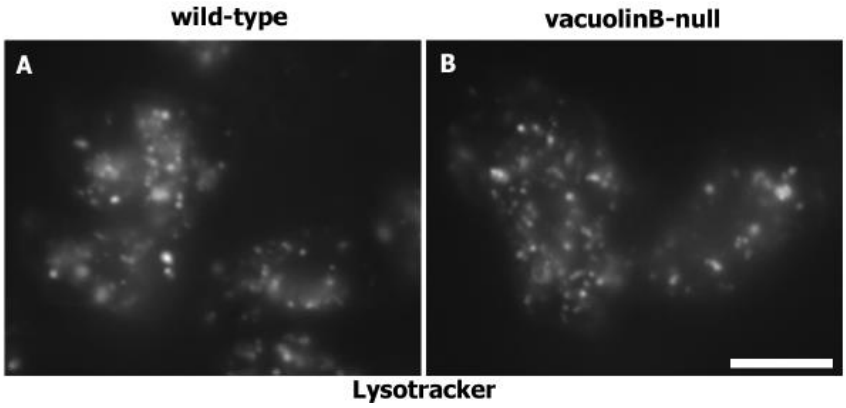


Figure 3.7: Loss of vacuolin B does not affect the morphology of acidic lysosomes.
Acidic compartments of wild-type **(A)** and vacuolin B-null **(B)** cells were assessed using Lysotracker red, a dye that accumulates in acidic compartments. Vacuolin B-null cells contained acidic compartments that were similar in size to those found in wild-type cells. Bar, 10μm

3.2.6 LvsB does not control fusion of post-lysosomes with early endosomes or with the contractile vacuole.

While we have shown that LvsB inhibits fusion between lysosomes and post-lysosomes, it is possible that LvsB also controls fusion of post-lysosomes with other compartments. This possibility is particularly relevant for early endosomes and the contractile vacuole since they also contain Rab14 (Bush et al., 1994; Bush et al., 1996; Harris and Cardelli, 2002).

To test the possibility that post-lysosomes fuse with early endosomes in LvsB null cells we tested the localization of the integral membrane protein p25, a marker for the plasma membrane and early endosomes. The protein p25 is internalized together with endocytic cargo in early endosomes, and shortly retrieved to a recycling compartment before recycling back to the plasma membrane (Charette et al., 2006). Accordingly, p25 is absent from lysosomes and post-lysosomes in wild type cells. We immuno-localized p25 in wild type and LvsB-null cells expressing GFP-Vacuolin B (Figure 3.8). In both cell lines p25 localized normally to the plasma membrane and the recycling compartment and did not co-localize with vacuolin B. This result suggests that early endosomes do not fuse inappropriately with post-lysosomes in the absence of LvsB.

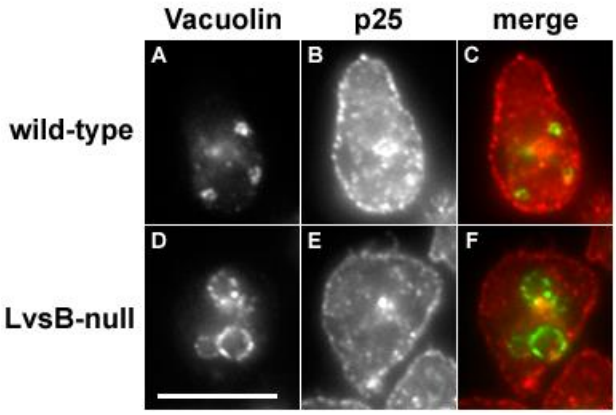


Figure 3.8: Early endocytic traffic is not impaired in the absence of LvsB. Control **(A - C)** and LvsB-null cells **(D - F)** expressing GFP-Vacuolin B were fixed and stained with antibodies against p25. p25 is internalized together with endocytic cargo in early endosomes, then retrieved to a recycling compartment before final recycling back to the plasma membrane (Charette et al., 2006). In control cells, p25 localized on the plasma membrane and the juxtanuclear recycling compartment and did not co-localize with vacuolin on post-lysosomes **(A-C)**. Similarly, p25 did not co-localize with vacuolin in the absence of LvsB **(D - F)**. This result suggests that early endosomes and post-lysosomes did not fuse in the absence of LvsB. Bar, 10µm.

We also explored whether LvsB may have a role in restricting the fusion of endo-lysosomal vesicles with the membranes of the contractile vacuole. The *Dictyostelium* contractile vacuole is a dynamic osmoregulatory organelle composed of a reticular network of tubules and bladders. As mentioned before, Rab14 localizes prominently on the membranes of the contractile vacuole and could potentially regulate traffic between lysosomes and the CV. In fact, the contractile vacuole and the lysosomal compartment share several other markers including golvesin (Schneider et al., 2000), the vacuolar-ATPase (Temesvari et al., 1996a) and SNARE proteins (Wen et al., 2009). We determined the distribution of Rh50, a resident integral membrane protein of the contractile vacuole, and p80, an integral membrane protein found on lysosomes and post-lysosomes in our different cell lines. We did not find any co-localization of these markers in either wild type or LvsB-null cells, indicating that the two compartments remained distinct in both cell types (Figure 3.9). We also found that expression of constitutively active Rab14 (DA-Rab14) in wild-type cells did not induce co-localization of Rh50 and p80, demonstrating that the post-lysosome and the contractile vacuole did not fuse (data not shown). All data pertaining to the co-localization of Rh50 with p80 and of p25 with GFP-vacuolin B was collected and contributed by Dr. Kypri.

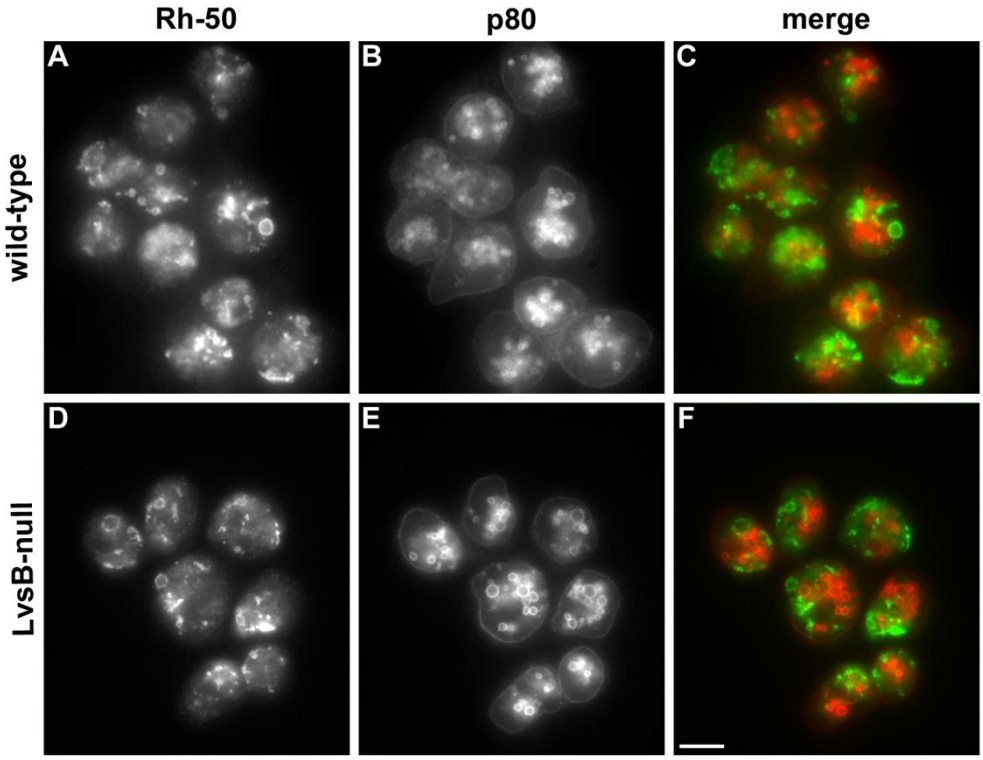


Figure 3.9: The absence of LvsB does not cause inappropriate fusion between endosomes and the contractile vacuole. Control and LvsB-null cells were fixed and stained with antibodies against Rh50, a contractile vacuole marker, and p80, a marker for lysosomes and post-lysosomes (Benghezal et al., 2001; Ravanel et al., 2001). In both wild-type **(A, B)** and LvsB-null cells **(C, D)**, Rh50 is distributed normally on the membranes of the contractile vacuole and p80 distributed normally on the plasma membrane and endocytic vesicles. Thus, the lack of LvsB did not cause inappropriate fusion between endosomes and the contractile vacuole. Bar, 10 μm.

3.3 Discussion

While the protein Lyst, defective in patients with Chediak Higashi Syndrome, has been implicated in lysosomal trafficking, the mechanism by which it contributes to lysosomal function is not known. Previous studies indicated that the *Dictyostelium* Lyst orthologue, LvsB, is a negative regulator of heterotypic fusion, repressing the fusion of lysosomes with post-lysosomes (Kypri et al., 2007) (chapter 2).

We have shown here three lines of evidence that suggest LvsB acts as a functional antagonist for the GTPase Rab14. First, Rab14 is mis-localized to post-lysosomes in LvsB null cells. Second, activation of Rab14 in wild type cells causes the same heterotypic fusion phenotype observed in LvsB null cells. Third, inactivation of Rab14 suppresses the mutant phenotype of LvsB null cells. In addition, we have shown that the function of LvsB is restricted to controlling the fusion between lysosomes and post-lysosomes since loss of LvsB does not affect other cellular compartments.

Our studies were prompted by previous reports that *Dictyostelium* Rab14 is localized on lysosomes and promotes the fusion of lysosomes when activated. We have extended those studies by showing that Rab14 is absent from post-lysosomes since it does not co-localize with the post-lysosomal marker vacuolin. These results indicate that Rab14 is present exclusively during the lysosomal stage consistent with a role in promoting homotypic fusion among lysosomes (Harris and Cardelli, 2002). In order for a lysosome to mature into a post-lysosome it has to cease fusion with other lysosomes to allow for the removal of proton pumps and lysosomal hydrolases (Maniak, 2003). Those pumps and enzymes are then recycled by delivery to newly internalized endosomes. We propose that the inactivation and removal of Rab14 from lysosomes is necessary to allow them to mature into post-lysosomes.

We showed previously that the localization of LvsB on the lysosome coincides precisely with this transition phase to a post-lysosome (Kypri et al., 2007) (Fig. 1.1). Consequently, LvsB arrives on the lysosome at the right time to antagonize Rab14 and allow post-lysosomal maturation. Here we have shown that in LvsB-null cells Rab14 co-localizes with the post-lysosomal marker vacuolin. One interpretation of this result is that in the absence of LvsB, Rab14 is not antagonized and remains on the lysosome as it begins to mature into a post-lysosome. The presence of active Rab14 on the post-

lysosome would then allow it to fuse inappropriately with an earlier lysosome. Consistent with this interpretation we found that constitutive activation of Rab14 in wild type cells causes a phenotype indistinguishable from that of LvsB null cells, including an increase in heterotypic fusion, enlargement of post-lysosomes, and the presence of constitutively active (DA) Rab14 on post-lysosomes. Expression of DA-Rab14 in LvsB null cells further increases the activity of Rab14 in these cells causing a concomitant increase in post-lysosome size.

In support of this model we also showed that inactivation of Rab14 by overexpression of dominant negative (DN) Rab14 blocks heterotypic fusion and therefore suppresses the phenotype of LvsB null cells. This effect is specific to Rab14 since alterations in Rab7 activity did not suppress the LvsB null phenotype. Importantly, the dominant negative (DN) Rab14 construct does not affect the size of post-lysosomes in wild-type cells or in vacuolin-null cells since, according to our model, Rab14 does not contribute to the regulation of post-lysosomal function. Since inactivation of Rab14 seems crucial for the maturation of lysosomes into post-lysosomes it would imply that a RabGAP protein may also be important for this process. The *Dictyostelium* genome encodes 25 putative RabGAP proteins that have not been characterized in detail. It will be interesting to determine whether one of these proteins is involved in the interaction between Rab14 and LvsB shown here.

While Rab14 is found on lysosomes, it is most prominently observed on the contractile vacuole (Bush et al., 1994; Bush et al., 1996; Harris and Cardelli, 2002). In addition to Rab14, lysosomes and contractile vacuoles also share other proteins, including SNAREs and proton pumps (Clarke et al., 2002; Wen et al., 2009). These similarities suggest that vesicle traffic may occur between these compartments. In fact, it has been suggested that the vacuolar proton pumps are delivered to the endosomal pathway through the contractile vacuole system (Padh et al., 1991). More importantly, another study showed that contractile vacuole markers can traffic to the contractile vacuole membranes via early endosomes and the recycling compartment (Mercanti et al., 2006). Hence, it would seem possible that LvsB may also be involved in restricting the fusion of post-lysosomes with these compartments. However, we have shown that this is not the case. We did not detect any fusion of post-lysosomes with contractile vacuole

membranes or with early/recycling endosomes in the LvsB null cell line. These results further support that LvsB functions specifically in regulating fusion between lysosomes and post-lysosomes.

While the distribution of Lyst has not been determined in any metazoan it seems likely that, like LvsB, Lyst may be localized on lysosomes and lysosome-related organelles. Some of the tissues most drastically affected in patients with Chediak Higashi Syndrome are those that form lysosome-related organelles, such as melanosomes and secretory lysosomes. These organelles undergo a process of maturation that may be analogous to the maturation of the post-lysosome in *Dictyostelium*. For example, secretory lysosomes mature from lysosomes upon stimulation of cytotoxic T lymphocytes. In lymphocytes from CHS patients the initial formation of lysosomes is normal but subsequent fusion of lysosomes is abnormally high *in vivo* causing the enlargement of secretory lysosomes (Stinchcombe et al., 2000). A later study using mast cells and pancreatic acinar cells also indicated a role for Lyst in regulating the fusion of secretory vesicles (Hammel et al., 2010). The results outlined in chapter 2 support this fusion regulatory model for Lyst function. However, a recent study in fibroblasts and macrophages demonstrated that a defect in lysosomal fission was responsible for lysosomal enlargement in CHS mutant cells (Durchfort et al., 2012). Clearly, we are far from understanding the molecular function of Lyst and related proteins. It is possible that the different results from these papers are due to the different cell types used. It is also possible that Lyst plays a role in both fusion and fission events and that the different assays used in these studies highlight one role over the other.

Based on our results we hypothesize that, in analogy with *Dictyostelium* LvsB, mammalian Lyst may regulate lysosomal fusion by antagonizing the activity of a lysosomal Rab protein. This antagonist function may be a general mechanism of action for other Lyst-related proteins. Lyst and LvsB are members of the BEACH family of proteins with representatives in all eukaryotes. Among them, the *Drosophila* BEACH protein Bchs antagonizes the activity of Rab11 in membrane trafficking during development (Khodosh et al., 2006). In *Dictyostelium*, a functional link was observed between the BEACH protein LvsA and the contractile vacuole GTPase, Rab8a (Essid et al., 2012). In addition, the nematode BEACH protein SEL-2 is a negative regulator of

membrane traffic during Notch signaling (de Souza et al., 2007). However, the mechanism by which a BEACH protein, like LvsB, could antagonize the activity of a specific Rab, like Rab14, has yet to be discovered. An analysis of proteins co-precipitating with TAP-tagged LvsB did not reveal any potential RabGAP or other regulator of membrane traffic (Kypri et al., 2007). Unfortunately, none of the BEACH-related proteins have yet been identified in any of the interactome databases from various organisms. A yeast 2-hybrid screen with human Lyst protein did identify a large number of potential interacting proteins, but none of them have been confirmed in physiological conditions (Tchernev et al., 2002). Importantly, none of those potential interacting proteins could explain the antagonism with Rab proteins. We believe that understanding the mechanism of action of LvsB/Lyst is important not just for a better understanding of the Chediak-Higashi Syndrome, but for the discovery of novel mechanisms of membrane traffic regulation.

Chapter 4: *Dictyostelium* LIP5 antagonizes LvsB function by promoting the vesicle fusion activity of Rab14

4.1 Introduction

The endo-lysosomal pathway serves as a transit hub for biosynthetic cargo from the golgi, autophagosomes, as well as endosomes and phagosomes carrying cargo from the plasma membrane and extracellular environment (Mellman, 1996). With such a vast and diverse influx, it is imperative that incoming cargo is properly processed and sorted to arrive to the correct final destination. Many of the biosynthetic cargo, such as lysosomal hydrolases, as well as many receptors from the plasma membrane that are destined for delivery to lysosomes must first be sorted from the limiting membrane into small vesicles inside the endosomal lumen in order to be properly processed or degraded in the lysosome (Reggiori and Pelham, 2001; Katzmann et al., 2002; Gruenberg and Stenmark, 2004) (Fig 1.1 B). This sorting of cargo into intra-luminal vesicles (ILVs) is accomplished by a set of protein complexes called ESCRT (Endosomal Sorting Complexes Required for Transport) -0 through –III along with a number of accessory proteins (Babst, 2005). The coalescence of ESCRT complex function results in the formation of multivesicular bodies (MVB) that will fuse with lysosomes for the digestion and/or processing of their contents (Piper and Katzmann, 2007). The final scission step of ILV formation along with recycling of the ESCRT-III complex proteins into the cytosol is attributed to the activity of the AAA (ATPases associated with diverse cellular activities) type ATPase SKD1 (and its stimulatory binding partner LIP5 (Lyst Interacting Protein 5) (Henne et al., 2011). The LIP5 protein as well as its yeast homologue, vta1p, is known to bind to and assist in the oligomerization of SKD1/vps4p (Fujita et al., 2004; Azmi et al., 2006). Additionally, *in vitro* studies show that vta1p increases the ATPase activity of vps4p by 6 to 8 fold in yeast (Lottridge et al., 2006).

Despite strong evidence of the stimulatory effect that LIP5/vta1p has on SKD1/vps4p, LIP5/vta1p is not required for SKD1/vps4p ATPase activity or for its localization to the endosomal membrane *in vivo* (Azmi et al., 2006; Azmi et al., 2008; Shestakova et al., 2010). Furthermore, the absence of LIP5/vta1p has only a mild ILV

formation phenotype when compared to SKD1/vps4p and most of the other ESCRT mutants (Shiflett et al., 2004; Ward et al., 2005; Azmi et al., 2006). This has raised the possibility that LIP5 may function in a capacity beyond MVB formation. One aspect of the LIP5 null phenotype that has been reported in multiple studies is the delayed delivery of fluid phase as well as internalized plasma membrane and biosynthetic cargo to the lysosome or the comparable vacuole in yeast (Yeo et al., 2003; Shiflett et al., 2004). One possible explanation for this delay in trafficking is that LIP5 mutants are defective in the fusion of MVBs with lysosomes. In support of this concept, recent studies have provided evidence that some of the proteins that function during late stages of ILV formation also regulate fusion of the MVB with lysosomes in mammalian cells or with the equivalent vacuolar compartment in yeast (Balderhaar et al., 2010; Metcalf and Isaacs, 2010; Urwin et al., 2010; Russell et al., 2012). These proteins are in the right place at the right time to mediate fusion of mature MVBs with lysosomes, and this fusion regulatory model for late acting ESCRT and accessory proteins is gaining traction. Currently, our understanding of how ESCRT and ESCRT accessory proteins might mediate fusion is incomplete, and the possible contribution of many accessory proteins, like LIP5, to fusion regulation has not been explored.

Preliminary evidence for a link between LIP5 and fusion regulation came in the form of a yeast two hybrid screen that identified LIP5 as a possible binding partner of the fusion regulatory protein Lyst (Tchernev et al., 2002). In support of the possible interaction of Lip5 and Lyst, expression of an ATPase deficient form of the LIP5 binding partner, SKD1, in mammalian cells causes the redistribution of both endogenous LIP5 and Lyst from the cytosol to the membranes of aberrant class E compartments (Fujita et al., 2004). Disruption of human Lyst is associated with the devastating genetic disorder Chediak Higashi Syndrome. Defects in Lyst result in the formation of enlarged acidic lysosome related compartments and subsequent problems in endo-lysosomal trafficking at the cellular level (Introne et al., 1999; Huizing et al., 2001). Studies of Lyst homologues in mice (Oliver and Essner, 1975; Willingham et al., 1981; Hammel et al., 1987; Hammel et al., 2010), humans (Stinchcombe et al., 2000), cats (Collier et al., 1985), *Drosophila* (Rahman et al., 2012), and *Dictyostelium* (Harris et al., 2002; Kypri et al., 2007; Kypri et al., 2013) have provided strong evidence that Lyst functions as a

negative regulator of vesicle fusion, and that the enlarged acidic compartments that form in the absence of Lyst are a product of inappropriate fusion events. Interestingly, many characteristics of the Lyst mutant phenotype are strikingly similar to those in cells that have perturbations in ESCRT or ESCRT accessory protein function. Both Lyst mutant and vps/ESCRT mutant cells are characterized by the presence of abnormally large vesicular compartments and the increased secretion of lysosomal/vacuolar hydrolases (Garrus et al., 2001; Kranz et al., 2001; Bache et al., 2003; Doyotte et al., 2005; Razi and Futter, 2006). It has also been reported that over-expression of large epitope tagged CHMP5 (Charged multi-vesicular body protein) results in the formation of hybrid vesicles that contain both early and late endosomal markers (Ward et al., 2005). Hybrid vesicular compartments containing a mixture of vacuolar, endocytic, and late Golgi markers are reported in vps4 mutant yeast (Babst et al., 1997). Similarly, disruption of the Lyst homologue, LvsB, in *Dictyostelium* cells is marked by the formation of hybrid vesicles with characteristics of both early and late endosomes (Harris et al., 2002; Kypri et al., 2007). Interestingly, CHMP5 and vps4 are both strong binding partners of LIP5/vta1p (Yeo et al., 2003; Fujita et al., 2004; Shiflett et al., 2004; Scott et al., 2005; Ward et al., 2005; Azmi et al., 2006) and the localization of CHMP5 to endosomal membranes is LIP5 dependent (Azmi et al., 2008). These phenotypic parallels between Lyst and ESCRT/ESCRT accessory mutants coupled with the potential physical interaction of Lyst with LIP5 allude to a possible functional relationship between LIP5 and Lyst for the regulation of vesicle fusion.

Our previous studies in *Dictyostelium* have shown that LvsB localizes to the membranes of lysosomes as well as neutral post-lysosomal compartments (Kypri et al., 2007) (Fig. 1.1). While degradation in acidic lysosomes is the terminal step of the endo-lysosomal pathway in most cells, *Dictyostelium* cells require a neutral post-lysosomal compartment to carry indigestible material to the plasma membrane for exocytosis (Padh et al., 1993) (Fig 1.1). This extended endo-lysosomal pathway is most similar to that of specialized secretory cells and some immune cells (Sundler, 1997; Dell'Angelica et al., 2000). While the post-lysosomal population is distinct from the earlier lysosomal population in wild type *Dictyostelium* cells, disruption of LvsB results in an increased occurrence of inappropriate heterotypic fusion between lysosomes and post-lysosomes

(Kypri et al., 2007). This inappropriate heterotypic fusion leads to the formation of acidic hybrid compartments that represent a defining characteristic of the LvsB null phenotype. While the mechanisms contributing to LvsB mediated fusion regulation remain largely unknown, in chapter 3 we showed an antagonistic relationship between LvsB and the lysosomal fusion promoting GTPase, Rab14 (Kypri et al., 2013). Because of the evidence for a possible physical interaction between LIP5 and Lyst as well as mounting support for the involvement of ESCRT complex proteins in lysosome fusion, we were interested in exploring whether LIP5 participates functionally with LvsB and Rab14 to regulate vesicle fusion.

We identified a single protein with homology to LIP5 in *Dictyostelium* (Mattei et al., 2006). Sequence alignment showed that this putative *Dictyostelium* Lip5 protein has >35% identity and >55% similarity to the human Lip5 protein sequence. The present study marks the first functional characterization of the *Dictyostelium* Lip5 homologue, and the first investigation into a possible functional relationship between Lip5 and Lyst/LvsB. In this study, we show that knock down of *Dictyostelium* LIP5 (dLIP5) protein expression partially rescues the LvsB null heterotypic fusion phenotype. Additionally, our dLIP5 knock out studies indicate a functional interaction with the Rab14 GTPase to promote endosome as well as phagosome fusion. These studies provide further insight into possible Lyst interactions that contribute to the manifestation of CHS.

4.2 Results

4.2.1 The *Dictyostelium* LIP5 homologue co-localizes with LvsB on vesicle membranes

In concordance with the described function of LIP5 during MVB formation, the yeast LIP5 homologue, vta1p, is known to localize to late endosomal membranes as well as to the cytosol (Shiflett et al., 2004). However, despite the similarity of the mammalian LIP5 mutant phenotype to that described in vta1p deficient yeast, multiple studies have shown that mammalian LIP5 has a strictly cytosolic localization under wild type conditions (Fujita et al., 2004; Ward et al., 2005). Given these differences in localization characteristics, we first evaluated the intracellular localization of the *Dictyostelium* LIP5 homologue, dLIP5. Immuno-staining of endogenous dLIP5 protein in agarose flattened and fixed cells revealed a primarily diffuse localization for dLIP5 with some staining that was indicative of dLIP5 localized on vesicle membranes (Fig 4.1 A-B).

In this study, we were interested in exploring the relationship between dLIP5 and LvsB. Thus, we wanted to determine how dLIP5 membrane localization corresponded to LvsB labeled vesicles. Immuno-staining of endogenous dLIP5 protein in cells expressing GFP-tagged LvsB revealed a significant overlap of dLIP5 with LvsB on vesicle membranes. We found that 45% of 53 dLIP5 labeled vesicles were also labeled by GFP-LvsB (Fig 4.1 C-C'). GFP-LvsB was previously shown to localize on the membranes of late lysosomes and all post-lysosomes such that LvsB likely stays on the post-lysosomal membrane until exocytosis (Kypri et al., 2007) (Fig 1.1). This suggests that the dLIP5 labeled vesicles that are not labeled by LvsB likely represent an earlier lysosomal population and that dLIP5 arrives at the endosomal membrane before LvsB.

To determine whether dLIP5 is present on post-lysosomes we immuno-stained for both dLIP5 and the post-lysosomal marker, vacuolin. Vacuolin arrives at the lysosomal membrane just as it matures into a neutral post-lysosome, and then remains on the membrane until exocytosis (Rauchenberger et al., 1997; Jenne et al., 1998; Carnell et al., 2011) (Fig 1.1). We found that 11.7% of 76 dLIP5 labeled vesicles were labeled by vacuolin and only 6.9% of 128 vacuolin labeled vesicles were labeled by dLIP5 (Fig 4.1

D-D'). Interestingly, we previously showed that 11.1% of vacuolin labeled vesicles in wild type cells were acidic (Kypri et al., 2007). This population likely represents lysosomes that are on the cusp of becoming post-lysosomes. Because dLIP5 labeled a smaller percentage of post-lysosomes than the percentage previously found to be acidic, we infer that dLIP5 likely leaves the lysosomal membrane before it has completed the transition into a post-lysosome.

The co-localization characteristics of dLIP5 with LvsB and vacuolin suggest that dLIP5 precedes LvsB on the lysosomal membrane and then leaves at the transition of lysosomes into post-lysosomes. The presence of dLIP5 on lysosomal membranes is very similar to the localization reported for vta1p in yeast, and supports the likelihood that it functions in a homologous manner along the endo-lysosomal pathway. Most importantly, the overlap of dLIP5 with LvsB localization on lysosomal membranes puts dLIP5 in the right place at the right time to interact with LvsB during the regulation of lysosomal fusion.

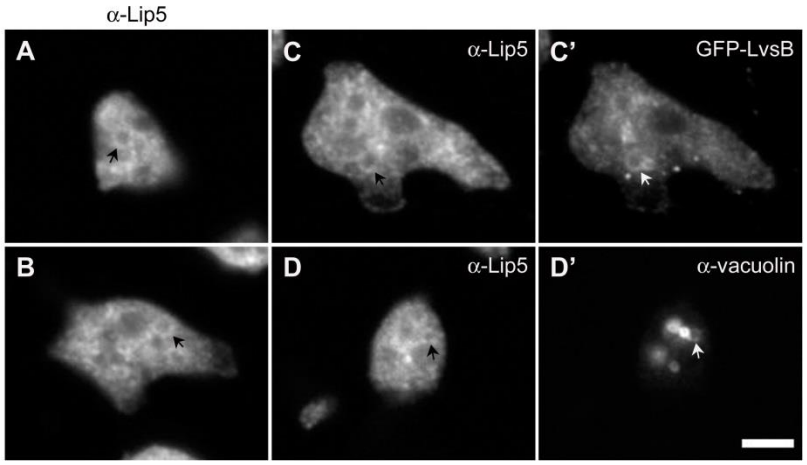


Figure 4.1: dLIP5 co-localizes with LvsB and vacuolin on endosomal membranes. (A-B) Endogenous dLIP5 protein localization was assessed using polyclonal antibodies against dLIP5 in flattened wild type cells. dLIP5 demonstrated a primarily cytosolic distribution with occasional localization on the membranes of vesicular structures. **(C-C')** These dLIP5 labeled vesicles were often labeled by GFP-LvsB, which is known to localize to both lysosomal and post-lysosomal membranes. **(D-D')** A smaller population of dLIP5 labeled vesicles was labeled by the post-lysosomal marker vacuolin. The pattern of dLIP5 co-localization with both LvsB and vacuolin combined with the known distribution of these proteins along the endo-lysosomal pathway indicates that dLIP5 precedes LvsB on the endosomal membrane, and that dLIP5 leaves the endosomal membrane at the transition of lysosomes to the post-lysosomal stage. Bar is 5 μm.

4.2.2 Knock down of dLIP5 expression relieves the severity of the LvsB null phenotype

The co-localization of dLIP5 with LvsB on endosomal membranes is in agreement with the possibility of a functional interaction between the two. As a negative regulator of heterotypic fusion between lysosomes and post-lysosomes, the absence of LvsB is marked by the formation of enlarged hybrid vesicles that contain features of both lysosomes and post-lysosomes (Harris et al., 2002; Kypri et al., 2007; Kypri et al., 2013). This increased vesicle size can be visualized using the fluid phase marker, TRITC-dextran, or by immuno-staining of the post-lysosomal marker vacuolin.

To explore the possible functional relationship between dLIP5 and LvsB, we knocked down dLIP5 protein expression and assessed subsequent changes in LvsB null vesicle morphology. To achieve dLIP5 knock down, we used an RNA interference hairpin construct that yields an antisense product covering 59% of the *lip5* coding sequence (Fig 4.2 A). Using a blast search of the *Dictyostelium* genome we found no other sequence with significant homology to this hairpin construct. Using this method we were able to achieve >79% knock down of dLIP5 protein in LvsB null and wild type cells in one set of transformants (Fig 4.2C), and >99% knock down in a second set (Fig 4.2B) as determined by western blot analysis. Cells transformed with empty shuttle vector were used as a control for subsequent experiments. Experiments done in both sets of transformants were combined to produce the final results shown in Figures 4.3 and 4.4.

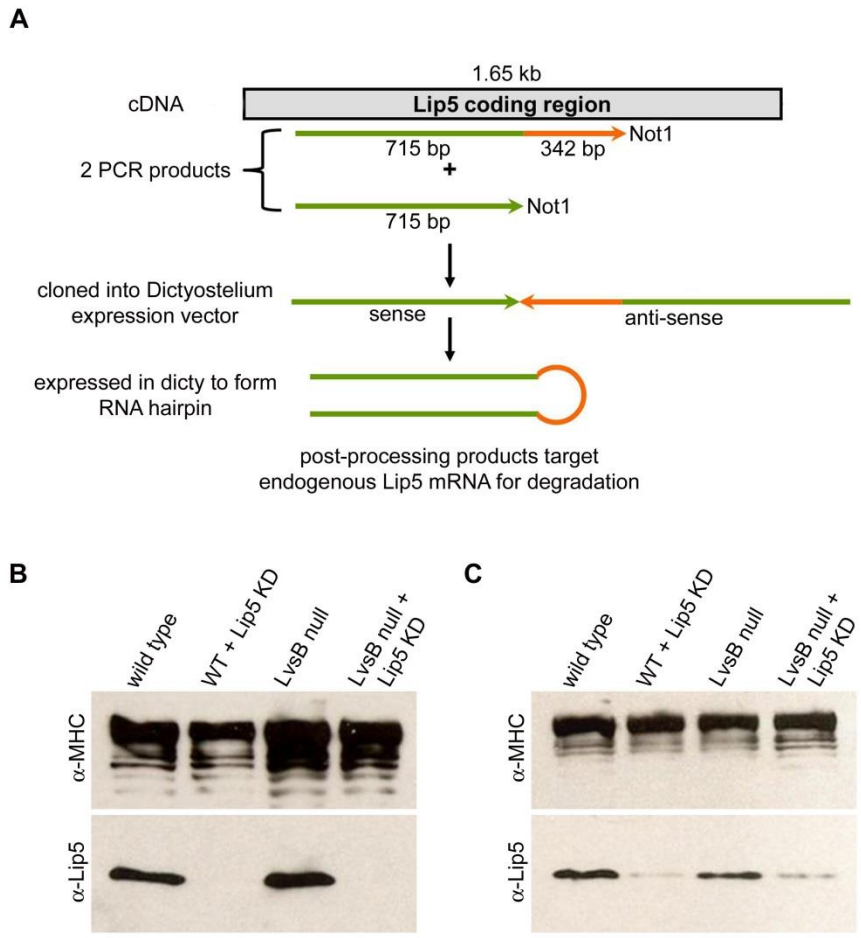


Figure 4.2: Knock down of dLIP5 protein expression in wild type and LvsB null cell lines. (A) Endogenous dLIP5 mRNA was targeted for degradation using an RNA hairpin construct containing anti-sense sequence with homology to 59% of the dLIP5 coding sequence. Both wild type and LvsB null cell lines showed minimal to no expression of the dLIP5 protein upon expression of the dLIP5 hairpin construct. **(B)** Our initial trans-formants displayed a >99% decrease in dLIP5 protein as shown by western blot analysis. **(C)** A second set of trans-formants also demonstrated a significant knock down of dLIP5 protein expression with a >89% decrease of dLIP5 protein in wild type cells and a >79% decrease in LvsB null cells. Both sets of dLIP5 knock down trans-formants were used to characterize the effects of reduced dLIP5 protein expression.

Knock down of dLIP5 expression caused a significant reduction of dextran labeled vesicle size (33.6% decrease in average diameter +/- 3.8 s.e.m.) and vacuolin vesicle size (25.5% decrease in average diameter +/- 1.4 s.e.m.) in the LvsB null cell line (Fig 4.3 G-H and J-K). In contrast, we observed no significant change in either dextran or vacuolin labeled vesicle size upon knock down of dLIP5 expression in wild type cells (Fig 4.3 C-D and J-K). Transformation of either wild type or LvsB null cells with vector alone caused no significant change in dextran or vacuolin vesicle diameter (Fig 4.3 J and K, and

unpublished data). The decreased vesicle size associated with reduced dLIP5 protein expression in LvsB null cells but not in wild type cells suggests that dLIP5 may be acting in opposition to the function of LvsB. Alternatively, knock down of dLIP5 may reduce vesicle size in LvsB null cells indirectly through a fusion independent mechanism.

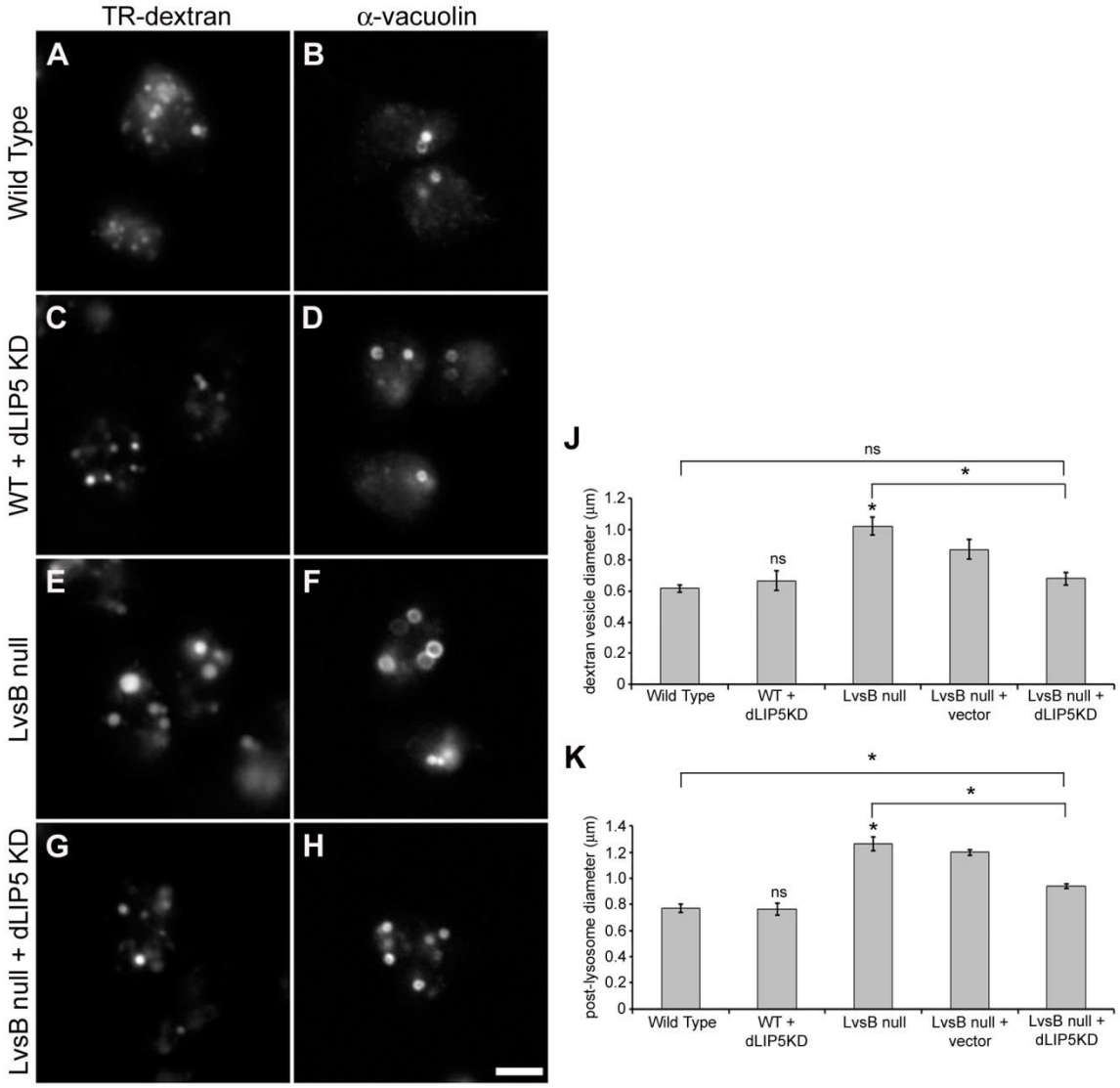


Figure 4.3: Knock down of dLIP5 protein expression partially rescues both dextran and vacuolin labeled vesicle size in the LvsB null cell line. The endo-lysosomal pathway was labeled with TRITC-dextran for one hour in parent **(A & E)**, vector control, and dLIP5 knock down **(C & G)** cell lines. **(J)** Live cell images were used to measure the diameter of >50 vesicles per experiment and plotted as the mean +/- s.e.m. for 3 experiments. Parent **(B & F)**, vector control, and dLIP5 knock down **(D & H)** cells were immuno-stained for the post-lysosomal marker vacuolin. **(K)** Post-lysosome diameters were quantified from fixed cell images for >40 vesicles per experiment and the mean +/- s.e.m. for 3 experiments using two independent transformants and parental strains is shown. As previously described, both dextran vesicle and vacuolin labeled post-lysosome diameter is dramatically increased in LvsB null cells. However, our results show that this increase is markedly reduced in response to decreased dLIP5 protein expression. Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, $P>0.05$; *, $P< 0.05$; **, $P< 0.01$). Bar is 5 µm.

4.2.3 The dLIP5 knock down induced reduction in LvsB null vesicle size is a product of reduced heterotypic fusion between lysosomes and post-lysosomes

In previous studies, we used a two dextran pulse chase assay as a gauge for the increased heterotypic fusion that defines the LvsB null phenotype (Kypri et al., 2007). In order to determine if dLIP5 is a functional antagonist of LvsB, we used the same two dextran assay to examine the effect of dLIP5 knock down on heterotypic fusion levels in the LvsB null cell line. In this assay, cells were given a pulse of FITC-dextran followed by a 30 minute chase and then a second pulse with TRITC-dextran. The 30 minute chase time results in FITC labeled post-lysosomes and TRITC labeled lysosomes (Padh et al., 1993). In wild type cells, lysosomes are inhibited from undergoing heterotypic fusion with post-lysosomes. This creates a distinct separation between the lysosomal and post-lysosomal populations that is integral for proper processing along the endo-lysosomal pathway. Consequently, we observed a very minimal number of vesicles containing both FITC- and TRITC-dextran in wild type cells (5.6% +/- 0.36 s.e.m.) (Fig 4.4 A-A" and E). Conversely, LvsB null cells exhibited a significant increase in heterotypic fusion of lysosomes with post-lysosomes (33% +/- 0.36 s.e.m.) (Fig 4.4 C-C" and E). These results are consistent with previously published data, and illustrate the function of LvsB as an inhibitor of heterotypic lysosomal fusion (see Chapter 2).

If dLIP5 is an antagonist of LvsB function, then reduced dLIP5 expression should relieve inappropriate heterotypic fusion in LvsB null cells. In accordance with this prediction, we found that knock down of dLIP5 expression in LvsB null cells caused a significant reduction in heterotypic fusion to near wild type levels (11.9% +/- 1.08 s.e.m.) (Fig 4.4 D-D" and E). This level of fusion was not significantly higher than that observed in wild type cells transformed with vector control (8% +/- 0.68 s.e.m.; data not shown). Knock down of dLIP5 expression caused a slight increase in dextran co-localization in wild type cells (7.8% +/- 0.41 s.e.m.) (Fig 4.4 B-B" and E), but this increase was comparable to that observed for wild type cells transformed with the vector control. The reduced heterotypic fusion that results from knock down of dLIP5 protein expression in LvsB null cells is demonstrative of a functional interaction between dLIP5 and LvsB, and suggests a role for dLIP5 in promoting vesicle fusion.

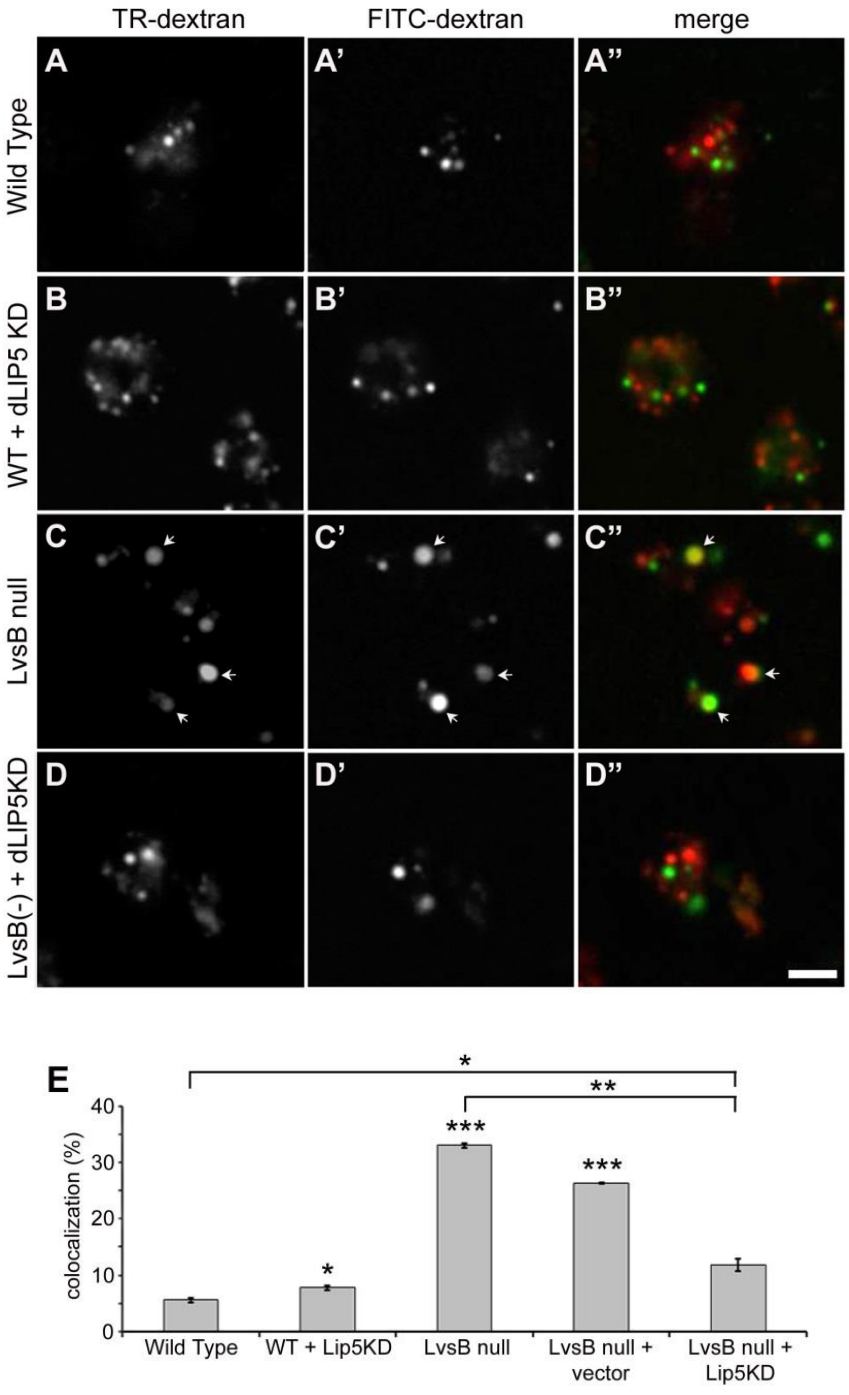


Figure 4.4: Knock down of dLIP5 protein expression significantly rescues inappropriate heterotypic fusion between lysosomes and post-lysosomes in LvsB null cells. Cells were given a short pulse of FITC-dextran, chased for 30 minutes, and then given a second pulse with TRITC-dextran. This pulse chase application results in FITC labeled post-lysosomes and TRITC labeled lysosomes. Thus the co-localization of FITC-dextran with TRITC-dextran is indicative of heterotypic fusion between lysosomes and post-lysosomes. **(A-D)** Live cell images were taken for ten minutes following the second dextran pulse. **(E)** Images similar to those shown in A-D were used to quantify the percentage of >68 vesicles/experiment that were labeled with both TRITC- and FITC-dextran. The mean percentage +/- s.e.m. of three experiments using two independent transformants and parental strains is shown. Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, $P>0.05$; *, $P< 0.05$; **, $P< 0.01$). Consistent with previous studies, the amount of co-localization between FITC- and TRITC-dextran was significantly increased in LvsB null compared to wild type cells. Upon knock down of dLIP5 protein expression in the LvsB null cell line, the amount of co-localization between FITC- and TRITC-dextran was markedly decreased to near wild type levels. Bar is 5 μm.

4.2.4 dLIP5 promotes fusion through a functional interaction with Rab14

The mechanism by which LvsB inhibits heterotypic vesicle fusion is not clearly understood. We reported recently that LvsB antagonizes the fusion promoting function of the small GTPase Rab14 (Kypri et al., 2013) (See Chapter 3). *Dictyostelium* Rab14 is known to promote homotypic phagosome as well as homotypic lysosome fusion (Bush et al., 1996; Harris and Cardelli, 2002). The overexpression of a constitutively active (DA) form of Rab14 that is unable to hydrolyze GTP to GDP, induces an increased level of heterotypic fusion between lysosomes and post-lysosomes and a concurrent increase in post-lysosome size (Kypri et al., 2013). Both of these phenotypes parallel those of the LvsB null cell line, suggesting opposing fusion regulatory roles for Rab14 and LvsB. This functional antagonism between Rab14 and LvsB is directly demonstrated by the ability of dominant negative (DN) Rab14 expression to rescue the LvsB null heterotypic fusion phenotype (Kypri et al., 2013). DN-Rab14 is a mutant form of Rab14 that is unable to bind either GTP or GDP and is therefore inactive. Expression of this inactive form of Rab14 has a dominant negative effect on vesicle fusion and size (Bush et al., 1996). Since my results presented above show a similar rescue of the LvsB null phenotype upon knock down of dLIP5 protein expression it is possible that dLIP5 and Rab14 function together to promote vesicle fusion. If this hypothesis is correct, then the absence of dLIP5 should suppress the phenotypic effects of DA-Rab14 expression.

To test this hypothesis we expressed DA-Rab14 in dLIP5 null cells and assessed consequential changes in post-lysosome morphology by immuno-staining of vacuolin. The dLIP5 null cell line was generated using homologous recombination and knock out of *lip5* was confirmed by western blot analysis (Fig 4.5). A non-homologous recombinant sibling cell line was used as a control for all subsequent assays. Consistent with previous results, when DA-Rab14 was expressed in either wild type cells or the sibling control cell line, we observed a significant increase in post-lysosome size (51% increase in diameter +/- 8.56 s.e.m. in wild type and 32.9% increase in diameter +/- 3.33 s.e.m. in sibling) (Fig 4.6 A-D and G). In contrast, expression of DA-Rab14 did not induce a significant increase in post-lysosome size in the dLIP5 null cell line (Fig 4.6 E-F and G). This observation was reproducible in a second dLIP5 null clonal line (data not shown). These results, coupled with the antagonism that we demonstrated between

dLIP5 and LvsB, are suggestive of a functional relationship between dLIP5 and Rab14. However, the inability of DA-Rab14 to induce the formation of enlarged post-lysosomes in the dLIP5 null cell line did not directly implicate dLIP5 in regulating Rab14 mediated fusion events.

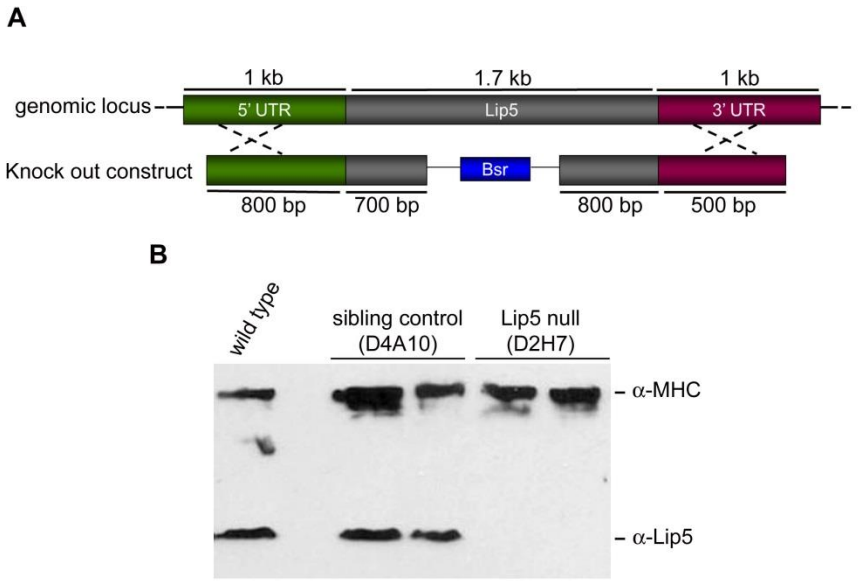


Figure 4.5: Generating the dLIP5 null cell line. (A) Homologous recombination was used to generate a dLIP5 null cell line. A linearized DNA construct containing a blasticidin-resistance cassette (Bsr) flanked by two >1 kb sequence fragments with homology to portions of the dLIP5 coding and up- or down-stream un-translated chromosomal regions was used to induce recombination and "looping out" of the endogenous dLIP5 gene under selective pressure. **(B)** Western blot analysis using the dLIP5 antibody was used to select a non-homologous recombinant sibling (D4A10) and a dLIP5 null (D2H7) clonal line. A second sibling (D2E1) and dLIP5 null (D8E10) line were also selected by western blot analysis (data not shown) and used to verify the reproducibility of our results.

In order to elucidate the relationship between dLIP5 and Rab14 with respect to vesicle fusion, we revisited the 2 dextran heterotypic fusion assay described above. As reported for the LvsB null cell line, expression of DA-Rab14 in wild type cells elicits a significant increase in heterotypic fusion between lysosomes and post-lysosomes (Kypri et al., 2013). This increased heterotypic fusion serves as an indicator of the fusion promoting capacity of Rab14. If dLIP5 is important for promoting vesicle fusion by influencing Rab14 activity, then the absence of dLIP5 should suppress the heterotypic fusion phenotype in cells expressing DA-Rab14. Consistent with previous studies, expression of DA-Rab14 in both wild type and the sibling control cell line prompted a

significant increase in heterotypic fusion between FITC-dextran labeled post-lysosomes and TRITC-dextran labeled lysosomes (5 fold increase in wild type and 4.4 fold increase in sibling) (Fig 4.6 H). This increase in heterotypic fusion was significantly less dramatic in LIP5 null cells expressing the DA-Rab14 construct (1.8 fold increase) (Fig 4.6 H). The moderate increase in heterotypic fusion that we observed when DA-Rab14 was introduced into the dLIP5 null background suggests that dLIP5 is not required for Rab14 vesicle fusion activity, but that it acts to enhance the efficiency of Rab14 to promote vesicle fusion.

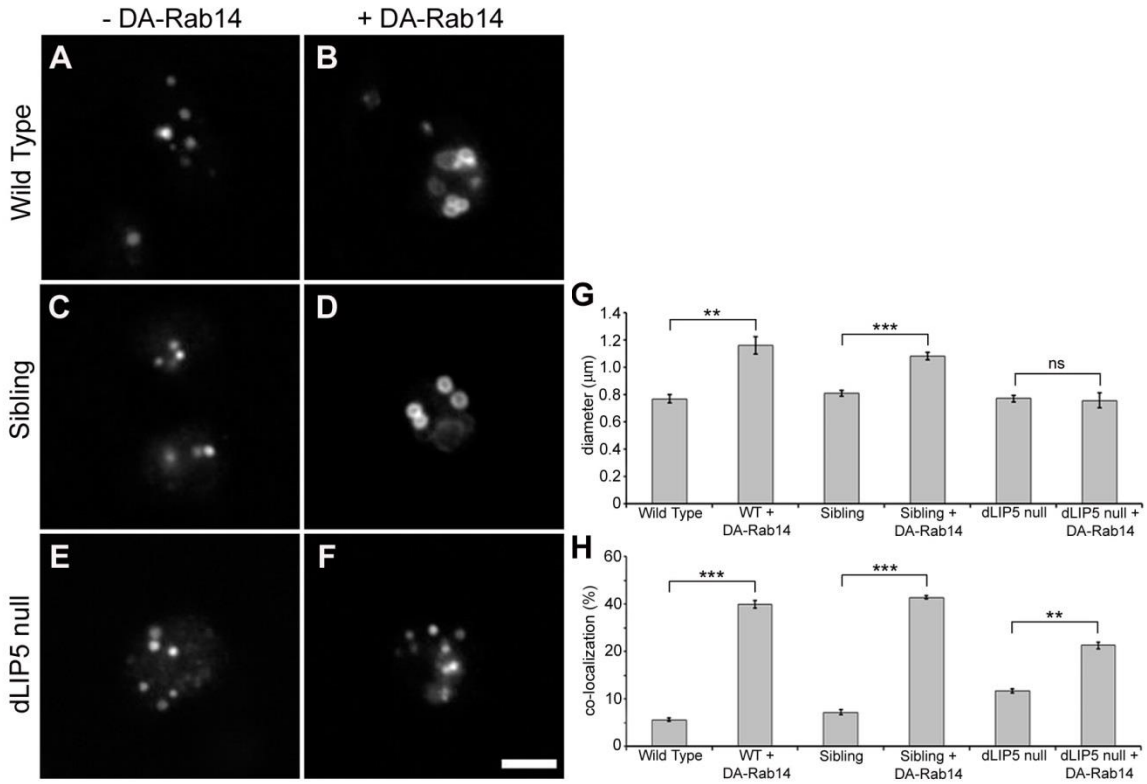


Figure 4.6: Dominant active (DA)-Rab14 induced post-lysosome morphology and heterotypic fusion defects are partially suppressed in dLIP5 null cells. (A-C) Cells were flattened, fixed and immunostained for the post-lysosome specific marker vacuolin. (G) The diameter of >43 vesicles per experiment were measured from images similar to those shown in A-C, and plotted as the mean +/- s.e.m. of three experiments. The expression of DA-Rab14 caused a significant increase in vacuolin vesicle (post-lysosome) size in both the wild type (A-B) and the non-homologous recombinant sibling control (C-D) cell lines. In contrast, vacuolin vesicle size was not affected in response to DA-Rab14 expression in the dLIP5 null cell line (E-F). (H) Heterotypic fusion between lysosomes and post-lysosomes was assessed as described in figure 3. The fraction of vesicles containing both FITC- and TRITC- fluid phase markers was quantified for >3 experiments and is shown as the mean +/- s.e.m.. Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, $P>0.05$; *, $P< 0.05$; **, $P< 0.01$). In agreement with previous studies, the percentage of vesicles containing both fluid phase markers significantly increased in response to DA-Rab14 expression in both wild type and sibling control cells. While the expression of DA-Rab14 did elicit an increased occurrence of heterotypic fusion between lysosomes and post-lysosomes in dLIP5 null cells, this affect was significantly less dramatic than that observed in either wild type or sibling control cells. Bar is 5 µm.

4.2.5 The enlarged vesicle phenotype of cells expressing dominant negative Rab7 is not suppressed in the dLip5 null cell line

Rab14 is not the only GTPase that localizes to and functions along the endo-lysosomal pathway. Rab7 GTPase is found on the membranes of lysosomes and post-lysosomes and is known to regulate recycling of lysosomal proteins at the transition to the post-lysosomal stage (Buczynski et al., 1997). In contrast to Rab14, it is the expression of dominant negative (DN) Rab7 that induces the formation of enlarged vesicles. Because Rab7 functions at a stage along the endo-lysosomal pathway that coincides with dLIP5 endosomal membrane localization, we were interested in exploring the possibility that dLIP5 may also influence Rab7 function and thus have a more generalized interaction with Rab GTPases during the maturation of lysosomes. For this, we expressed DN-Rab7 in the dLIP5 null cell line and assessed subsequent changes in endosome size. Cells were incubated with TRITC-dextran for one hour to label the entire endo-lysosomal pathway and then live cell images were used to quantify average vesicle diameter (Padh et al., 1993). In wild type and sibling control cells, average dextran vesicle size was significantly increased in response to expression of the DN-Rab7 construct (48.2% increase in diameter +/- 1.73 s.e.m. in wild type and 34% increase in diameter +/- 4.42 s.e.m. in sibling) (Fig 4.7). Unlike the capacity of dLIP5 null cells to attenuate the effects of DA-Rab14, DN-Rab7 provoked a comparably significant increase in vesicle size when expressed in dLIP5 null (35% increase in diameter +/- 4.35 s.e.m.), wild type, or sibling control cells (Fig 4.7). This result is indicative of a specific functional interaction between dLIP5 and Rab14 for promoting vesicle fusion.

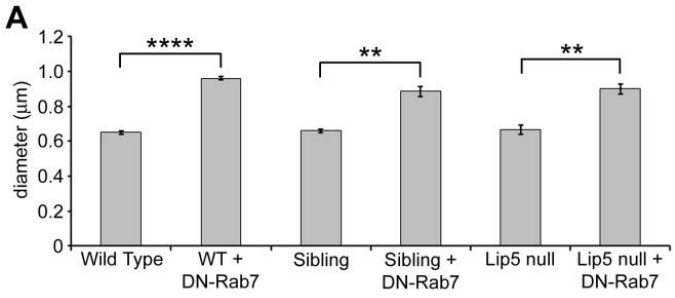


Figure 4.7: The absence of dLIP5 does not suppress the increased endosome size that is induced by dominant negative (DN) Rab7 expression. The entire endosomal pathway was labeled using a 1 hour pulse with TRITC-dextran. **(A)** The diameters of >125 vesicles per experiment were measured from live cell images and plotted as the mean +/- s.e.m. for three experiments. The expression of DN-Rab7 caused a similar increase in average endosome diameter in wild type, sibling control, and dLIP5 null cells.

4.2.6 The DA-Rab14 induced phagosome fusion phenotype is suppressed in dLIP5 null cells

In addition to regulating fusion along the endo-lysosomal pathway, both LvsB and Rab14 are known to have opposing fusion regulatory roles during the maturation of phagosomes (Harris and Cardelli, 2002; Harris et al., 2002). This is understandable, as phagosome maturation and processing relies heavily on fusion driven intermingling with the endo-lysosomal pathway (Rupper and Cardelli, 2001; Clarke et al., 2010). Thus, many proteins that are found to function along the endo-lysosomal pathway including specific snares, Rab14 and Rab7, as well as the vATPase proton pump also play important roles along the phagosomal pathway (Rupper et al., 2001; Gotthardt et al., 2002; Harris and Cardelli, 2002). Because Rab14 is a positive regulator of both lysosome, and phagosome fusion, and our results indicate that dLIP5 has a functional interaction with Rab14, we were interested in the possibility that dLIP5 also promotes phagosome fusion. This hypothesis is bolstered by studies of multi-vesicular body formation in *Dictyostelium.* Under axenic growth conditions where phagocytosis is dormant, *Dictyostelium* contain simple multi-vesicular bodies containing minimal amounts of intra-luminal membrane (Neuhaus et al., 2002). However, EM images show the presence of significantly more complex multi-lamellar compartments in bacterially

fed, actively phagocytic, cells (Marchetti et al., 2004). This suggests that *Dictyostelium* ESCRT complex proteins, like dLIP5, likely function along the phagosomal pathway.

To assess whether the fusion promoting relationship of dLIP5 with Rab14 contributes to phagosome fusion dynamics, we used a fluorescent bead pulse chase assay. Cells were given a 10 minute pulse of blue fluorescent beads, washed briefly, and then given a 10 minute pulse of red fluorescent beads followed by a 30 minute chase. We immuno-stained the cells with antibody against the p-80 protein which labels endosomal, phagosomal, and plasma membranes (Ravanel et al., 2001) such that single phagosomes containing multiple beads (multi-particulate phagosomes) could be distinguished from clusters of phagosomes containing single beads. Only phagosomes containing both blue and red beads were qualified as multi-particulate to ensure that they arose by fusion rather than uptake of bead clusters. Under these conditions, expression of DA-Rab14 in both wild type (Fig 4.8 A-B”’) and sibling control (Fig 4.8 C-D”’) cells prompted a significant increase in the percentage of cells containing multi-particulate phagosomes (~2 fold increase in both wild type and sibling control cells) (Fig 4.8 G). In marked contrast, the expression of DA-Rab14 failed to induce the formation of multi-particulate phagosomes in the dLIP5 null cell line (Fig 4.8 E-F”’ and G). The suppression of the DA-Rab14 phagosome fusion phenotype by loss of dLIP5 implies that the functional interaction between dLIP5 and Rab14 extends to promoting fusion between phagosomes as well as lysosomes.

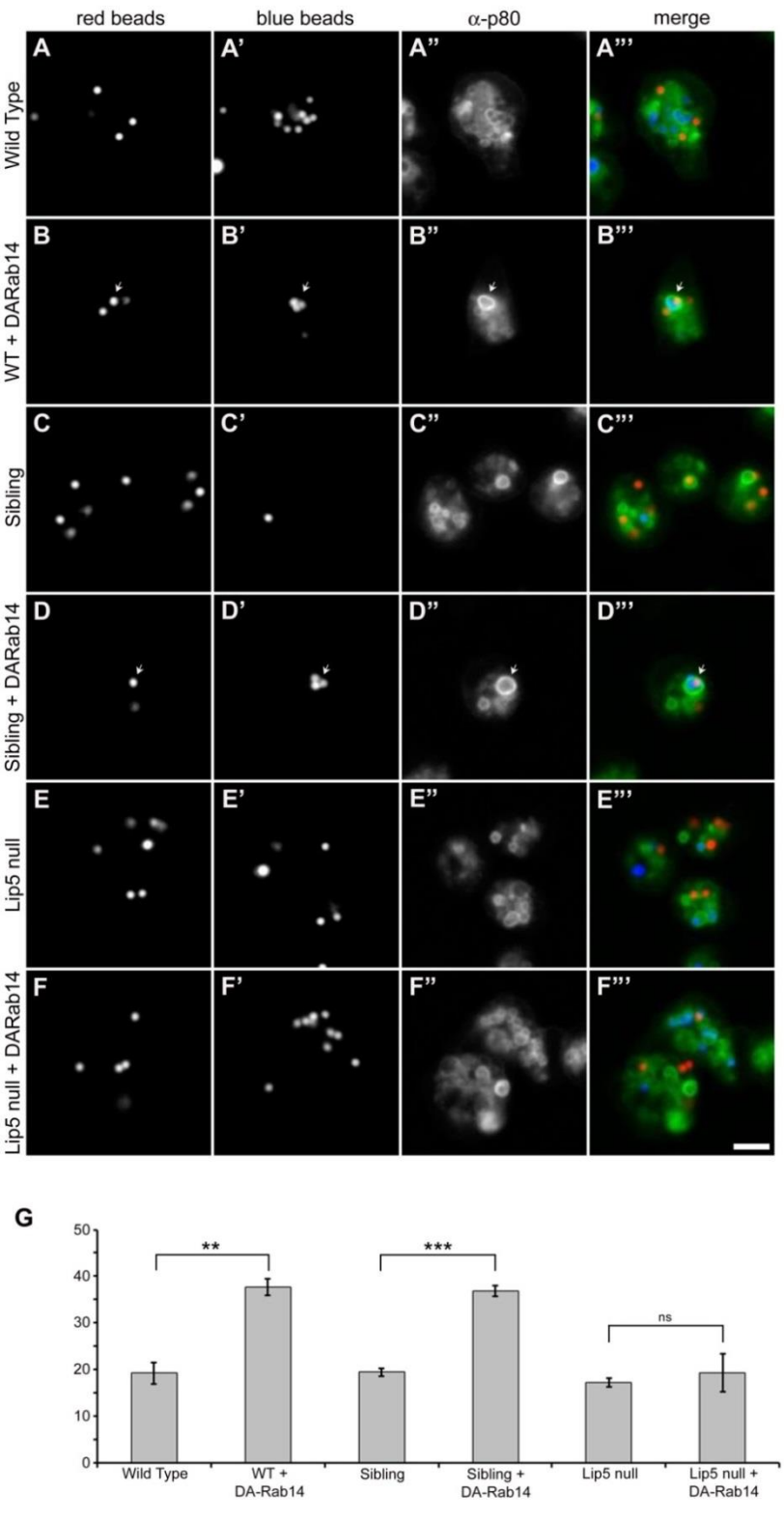


Figure 4.8: The multi-particulate phagosome phenotype associated with DA-Rab14 expression is suppressed in dLIP5 null cells. (A-F) Cells were given a pulse with blue beads followed by a pulse with red beads, then immunostained for the p-80 protein which labels plasma, endosomal, and phagosomal membranes. Phagosomes containing both blue and red beads were considered multi-particulate and are presumed to arise through phagosomal fusion events. **(G)** The percentage of cells containing multi-particulate phagosomes was established from images similar to those shown in A-F, and plotted as the mean +/- s.e.m. for three experiments. Statistical significance by two-tailed Student's t-test is indicated among relevant pairs (ns, not significant, $P>0.05$; *, $P< 0.05$; **, $P< 0.01$). Congruent with previous studies, expression of DA-Rab14 in wild type and non-homologous recombinant "sibling" control cells elicited a significantly increased fraction of cells containing multi-particulate phagosomes. In contrast, we observed no significant change in the fraction of cells containing multi-particulate phagosomes when DA-Rab14 was expressed in the dLIP5 null cell line. Bar is 5 μm.

4.3 Discussion

LIP5 has been extensively studied in the context of its relationship with the ESCRT-III complex and the AAA type ATPase SKD1 during MVB formation, yet only a minor supportive role has been described for LIP5 during this process. Mounting evidence has emerged supporting a secondary, fusion regulatory function for the ESCRT complex family of proteins which may serve as a cue for the fusion of mature MVBs with lysosomes. The temporal positioning of LIP5 during the final step of ILV formation places it in an ideal position to coordinate the switch from active MVB formation to fusion with the lysosome for degradation of luminal contents. Many characteristics of the described LIP5 null phenotype allude to a possible defect in fusion. The most prominent of these is the delayed delivery of internalized fluid phase, plasma membrane, and MVB cargo to the lysosome (Yeo et al., 2003; Shiflett et al., 2004; Ward et al., 2005). The identification of LIP5 as a possible binding partner of the fusion regulatory protein, Lyst, further insinuates that LIP5 possesses some fusion regulatory function (Tchernev et al., 2002).

In the present study, we presented evidence for the functional interaction of *Dictyostelium* LIP5 (dLIP5) with two proteins that serve opposing roles in the regulation of vesicle fusion. First, functional antagonism between dLIP5 and the *Dictyostelium* Lyst homologue, LvsB, was demonstrated by the significant rescue of increased vesicle size and inappropriate heterotypic fusion between lysosomes and post-lysosomes upon knock down of dLIP5 protein expression in the LvsB null cell line. The ability of dLIP5 perturbation to attenuate the LvsB null phenotype suggests that dLIP5 promotes vesicle fusion upstream of LvsB fusion inhibition. This proposed order of activity is reinforced by our localization studies showing that dLIP5 is present on endosomal membranes before the arrival of LvsB.

The antagonism shown between dLIP5 and LvsB is reminiscent of the relationship between LvsB and the vesicle fusion promoting GTPase Rab14. Rab14 is a positive regulator of lysosome fusion, and we previously showed that LvsB antagonizes Rab14 function to inhibit heterotypic fusion between lysosomes and post-lysosomes (Harris and Cardelli, 2002; Kypri et al., 2013). In light of our evidence for the opposing functions of

dLIP5 and LvsB, we proceeded to show that dLIP5 and Rab14 work in a concerted manner to promote vesicle fusion. This collaboration was evinced by the observed quenching of dominant active Rab14 induced heterotypic fusion in the absence of dLIP5. Once again, this epistatic phenotypic suppression suggests that dLIP5 functions upstream of Rab14 to promote vesicle fusion. The inability of the dLIP5 null cell line to suppress increased vesicle size upon expression of dominant negative Rab7 speaks to the specificity of the dLIP5 interaction with Rab14.

Finally, we showed that the functional interaction between dLIP5 and Rab14 influences phagosomal as well as endosomal fusion. Similar to their function along the endo-lysosomal pathway, Rab14 and LvsB are known to serve opposing fusion regulatory roles along the phagosomal pathway (Harris and Cardelli, 2002; Harris et al., 2002; Kypri et al., 2013). Consistent with concerted fusion regulatory mechanisms for both endosomes and phagosomes, the absence of dLIP5 was able to suppress the aberrant formation of multi-particulate phagosomes associated with dominant active Rab14 expression. The coalescence of our findings in this study represents the first direct evidence that the ESCRT accessory protein LIP5 plays a role in regulating vesicle fusion. Furthermore, the functional interplay that we demonstrated here between dLIP5, Rab14, and LvsB implicates a novel player in the mechanism of how LvsB inhibits vesicle fusion. In this respect we hope to elicit a better understanding of how Lyst malfunction contributes to the manifestation of CHS.

Although the fusion regulatory function of dLIP5 presented here is novel, the idea of ESCRT pathway proteins participating in vesicle fusion regulation is not unfounded. Recent studies in yeast and mammalian cells have implicated a functional tethering of ESCRT proteins to Rab7 GTPase mediated fusion of late endosomes with the lysosome/vacuole (Balderhaar et al., 2010; Urwin et al., 2010; Russell et al., 2012). As detailed in chapter 1, when an MVB is mature and is prepared for delivery of its cargo to the lysosome, Rab5 regulated fusion switches off and Rab7 mediated fusion becomes active (Rink et al., 2005; Woodman and Futter, 2008; Poteryaev et al., 2010; Balderhaar et al., 2013). Rab7 is known to promote the fusion of late endosomes or, in this case, mature MVBs with lysosomes (Vanlandingham and Ceresa, 2009). Urwin et al (2010) showed that absence of the ESCRT-III protein CHMP2B in human neural cells caused a

reduction in the recruitment of Rab7 to endosomal membranes. A second study in yeast showed that the vps4 ATPase mutant is not able to make the switch from vps21 (Rab5) regulated fusion to Ypt1(Rab7) regulated fusion with the vacuole (Russell et al., 2012). Our results demonstrating the importance of dLIP5 to the fusion promoting capacity of Rab14 further validate the relationship between late acting ESCRT pathway proteins and Rab7, as *Dictyostelium* Rab14 has homologous function to mammalian Rab7 (Harris and Cardelli, 2002; Vanlandingham and Ceresa, 2009).

In yeast, the failure to switch from Rab5 to Rab7 directed fusion gives rise to aberrant compartments called "class E compartments" that fail to fuse with the vacuole (Raymond et al., 1992; Shiflett et al., 2004; Russell et al., 2012). Though "class E" compartments are common to all ESCRT and ESCRT accessory protein mutants, it is unlikely that all ESCRT family proteins participate in regulating fusion through Rab GTPases. Because the ESCRT complexes function in sequence, the most likely scenario is one where proteins that act late in the ESCRT pathway dictate when the switch to Rab7 mediated fusion is made. LIP5 is brought to the MVB membrane in a complex with SKD1 and the arrival of this hetero-oligomeric complex marks the final step of ILV formation (Azmi et al., 2006; Wollert et al., 2009). Interestingly, the disruption of any ESCRT-I, -II, or –III complex protein as well as disruption of the yeast SKD1 homologue, vps4, results in the loss of vta1p, the yeast LIP5 homologue, localization to vesicular membranes (Shiflett et al., 2004). Thus, fusion disruption in any of these mutants could be indirectly attributed to the subsequent loss of vta1p/LIP5 localization. This possibility is supported by our results showing that the absence of dLIP5 reduces the fusion promoting activity of dominant active Rab14. As the latest acting member of the ESCRT pathway proteins shown to influence vesicle fusion, we propose that LIP5 promotes the switch from MVB formation to fusion with lysosomes.

Our results showed that dLIP5 antagonizes LvsB function in a similar manner to what we previously described for the GTPase Rab14. The intermingling functional relationships that we have shown for these three proteins add a new dimension to our understanding of the functional relationships contributing to LvsB mediated fusion inhibition. Based on the data presented in this study as well as the previously described dynamics between LvsB and Rab14 (Kypri et al., 2013), we propose that dLIP5 acts

upstream of Rab14 to stimulate its fusion promoting activity either directly or indirectly. LvsB is later recruited to the lysosomal membrane as it prepares to mature into a post-lysosome. At this stage, studies of mammalian Lyst localization in SKD1 mutant cells suggest that Lyst/LvsB vesicular localization characteristics might be influenced by the SKD1/LIP5 oligomeric complex (Fujita et al., 2004). When a lysosome matures into a post-lysosome it must first cease Rab14 mediated fusion with earlier lysosomes. We previously showed that LvsB antagonizes Rab14 function and that this antagonism likely occurs as a lysosome becomes a post-lysosome (Kypri et al., 2013). Additionally, studies in yeast and mammalian cells indicate that ESCRT proteins regulate vesicle fusion dynamics through a synergistic relationship with the Rab14 functional homologue Ypt7/Rab7 (Balderhaar et al., 2010; Urwin et al., 2010; Russell et al., 2012). As mentioned earlier, the expression of CHMP5 fused to a large epitope tag causes the formation of hybrid compartments that contain both early and late endosomal markers (Ward et al., 2005). This phenotype likely arises due to inappropriate fusion and is strikingly similar to the formation of hybrid compartments in LvsB null cells (Kypri et al., 2007). Given this phenotypic similarity and the relationships that LIP5 has with CHMP5 and LvsB (Shiflett et al., 2004; Ward et al., 2005; Azmi et al., 2008), it will be interesting to see how CHMP5 fits into the LvsB regulatory picture. It is our hope that by elucidating the interactions that LvsB has with vesicle fusion regulatory proteins along the endo-lysosomal pathway, we will begin to understand how LvsB influences vesicle fusion. By extension, we anticipate that the interactions we define for LvsB will translate to a better understanding of Lyst function.

Chapter 5: Summary & Future Directions

To understand the cellular mechanisms underlying the devastating genetic disorder Chediak Higashi Syndrome we must decipher the functional role that Lyst plays during endo-lysosomal maturation. Studies in mammalian systems have proven difficult, and have produced conflicting vesicle fusion and fission regulatory models for Lyst function. Although significant strides have been made toward characterizing the Lyst homologue, LvsB, in *Dictyostelium discoideum*, previous investigations have been unable to define it as a regulator of vesicle fusion or fission. The studies described in this dissertation have demonstrated that disruption of LvsB elicits specific phenotypic characteristics along the endo-lysosomal pathway that are both disparate from those of fission defect mutants, and consistent with a fusion regulatory role for LvsB. We have also identified two functional relationships that LvsB has with known endo-lysosomal trafficking proteins. These interactions begin to define the mechanisms controlling negative regulation of endosomal fusion by LvsB. Furthermore, a novel fusion regulatory function for dLIP5 is presented here that supports the hypothesis that proteins related to the ESCRT complex not only provoke the formation of MVBs, but also contribute to the fusion of mature MVBs with lysosomes. Our results have not only provided clarity to the mechanism of how LvsB exerts its functions along the endo-lysosomal pathway, but have also brought to light new questions whose answers may further our understanding of the mechanisms contributing to this function.

5.1 Future Directions

5.1.1 Defining the specific endo-lysosomal maturation stage that requires the fusion inhibitory function of LvsB

The presence of LvsB on the membranes of lysosomes as well as post-lysosomes has raised the question of which compartment is involved in the heterotypic fusion that arises in the absence of LvsB. This question could be addressed using *in vitro* fusion assays by mixing vesicle populations from LvsB null and wild type cells. Previous *in vitro* fusion assays using mammalian cell derived endosomes have produced results that dispute the role of Lyst in fusion regulation (Durchfort et al., 2012). However, as discussed in chapter 2, the experimental parameters used for these studies were intrinsically flawed such that the published results are inconclusive. To accurately assess the fusion capacities of lysosomes and post-lysosomes from LvsB null cells, lysosomes collected from LvsB null cells could be mixed with post-lysosomes from wild type cells and vice versa. This would be done *in vitro* in the absence of cytosolic LvsB. Because LvsB seems to antagonize Rab14 function at the transition from lysosome to post-lysosome, we hypothesize that post-lysosomes aberrantly retain fusion competency with earlier lysosomes. If this is the case then we would expect to see an increase in fusion between LvsB null post-lysosomes and wild type lysosomes but no increase in the fusion of LvsB null lysosomes with wild-type post-lysosomes. Defining the identity of the vesicles that cause aberrant fusion in the LvsB null cell line will help to specify potential fusion regulatory mechanisms that are defective in LvsB null cells, and hone in on the specific role of LvsB in preventing heterotypic fusion.

5.1.2 Investigating the possible contribution of aberrant SNARE recycling to the LvsB null fusion phenotype

By comparing the LvsB null phenotype with that of known fission defect mutants, we have shown that the enlarged hybrid compartments of LvsB null cells are the product of promiscuous fusion rather than fission. However, this does not preclude aberrant

fission as an upstream factor that contributes to defective fusion. Durchfort et al (2012) used time lapse microscopy to record the fission of small vesicles from tubulated lysosomes, and reported a decreased number and increased duration of fission events in Lyst deficient mouse macrophages. This observation reinforces the possibility that Lyst/LvsB does influence vesicle fission at some level. Additionally, the functional homologues of *Dictyostelium* Rab14 in yeast and mammalian cells are suggested to mediate retromer driven recycling from endosomal compartments as a prelude to their role in promoting the fusion of late endosomes with lysosomes (Rojas et al., 2008; Balderhaar et al., 2010; Liu et al., 2012). Thus, it is not unfounded that a regulator of vesicle fusion might also dictate recycling events from endosomal compartments. Fission of small vesicles from endosomal compartments is frequently used to recycle integral membrane components that are specific to a particular stage as an endosome matures to the next stage. A well characterized example of this in *Dictyostelium* is the removal of vATPase via fission of actin coated recycling vesicles as a lysosome matures into a post-lysosome. One way that a defect in fission mediated recycling could perturb fusion dynamics is by changing the distribution of SNAREs (Soluble N-ethylmaleimide-sensitive factor attachment protein receptors) along the endo-lysosomal pathway.

Apart from selective association of Rab proteins, fusion specificity is achieved through the association of specific trans-membrane SNAREs. Membrane fusion is dependent upon the formation of a tetrameric complex consisting of specific and complementary SNAREs present on the membranes that are destined to fuse. The identity of the SNAREs present on an endosomal compartment deciphers its ability to fuse with specific target membranes. As a late endosome matures, it must change its fusion specificity by altering its SNARE identity. In mammalian cells, late endosomes participate in homotypic fusion using a tetrameric SNARE complex that consists of syntaxin 8, syntaxin 7, Vti1b, and VAMP8 (Antonin et al., 2002). The fusion specificity of fully matured late endosomes is altered in preparation for the delivery of their contents to lysosomes. This is achieved by the specific exchange of VAMP8 for VAMP7 while the other 3 SNAREs remain the same (Pryor et al., 2004). The resulting complex is specific for heterotypic fusion of late endosomes with lysosomes as well as homotypic lysosome fusion (Advani et al., 1999; Ward et al., 2000b; Pryor et al., 2004).

The interaction that we have described between Rab14 and LvsB suggests that LvsB is important for preventing the heterotypic fusion of post-lysosomes with lysosomes, and it likely accomplishes this by antagonizing the factors that contribute to homotypic lysosome fusion as a vesicle matures to the post-lysosomal stage. This transition would presumably require the removal of VAMP7 from the lysosomal membrane. A similar mechanism was demonstrated to regulate the fusion of contractile vacuole membranes by the snare Vamp7B. The clathrin adaptor protein AP-180 is used to retrieve Vamp7B from contractile vacuole membranes to limit their homotypic fusion (Wen et al., 2009). In agreement with this model for altering vesicle fusion specificity, VAMP7 is absent from the membranes of post-lysosomes (Bennett et al., 2008). Characterization of VAMP7 recycling dynamics identified a role for clathrin associated AP (adaptor protein) complexes in VAMP7 retrieval from the plasma membrane, but not from post-lysosomes (Bennett et al., 2008). Given the direct visual evidence for Lyst participation in fission from lysosomes (Durchfort et al., 2012), and our evidence that LvsB inhibits the fusion of lysosomes with post-lysosomes, it would be interesting to determine whether LvsB is important for recycling of VAMP7 as a lysosome becomes a post-lysosome. This could be studied using endosomal maturation stage specific markers to assess the distribution of *Dictyostelium* VAMP7 along the endo-lysosomal pathway in LvsB null versus wild type cells. If VAMP7 is found to localize inappropriately on post-lysosomes in the absence of LvsB, then the contribution of this altered distribution to the LvsB null fusion phenotype could be investigated using an *in vitro* fusion assay, similar to the one described above, in the presence and absence of antibodies against VAMP7. The results from this type of study have the potential to integrate the fusion and fission models for LvsB/Lyst function into a single mechanistic composition.

5.1.3 Understanding how dLIP5 promotes Rab14 activity

Our results implicate dLIP5 in regulating Rab14 mediated fusion which, by homology with Rab7 driven fusion in mammalian systems, results in the delivery of MVB contents to the lysosome for digestion. In support of this model, recent studies in yeast as well as mammalian cells have linked ESCRT protein dysfunction with decreased

localization and activation of Rab7 and its yeast orthologue Ypt7 (Balderhaar et al., 2010; Urwin et al., 2010; Russell et al., 2012). Consistent with our findings that loss of dLIP5 suppressed the increased fusion phenotype caused by DA-Rab14, Russell et al (2012) found that disruption of the vta1p binding partner vps4 in yeast resulted in decreased activation of Ypt7. Decreased Ypt7 activity was attributed to the absence of the Ypt7 GEF, Ccz1, on endosomes in vps4 mutant cells. A second study in mammalian cells demonstrated a reduced presence of Rab7 on vesicular membranes upon disruption of the ESCRT-III complex protein CHMP2B (Urwin et al., 2010). Interestingly, while LIP5 requires the function of all upstream ESCRT proteins as well as vps4 for its localization to the endosomal membrane (Shiflett et al., 2004; Azmi et al., 2006), LIP5 is not known to be necessary for the localization or function of any ESCRT or ESCRT accessory protein that precedes it at the membrane (Azmi et al., 2006; Azmi et al., 2008; Wollert et al., 2009; Shestakova et al., 2010). This non-reciprocal relationship coupled with the influence that dLIP5 has on Rab14 function suggests that the previously described disturbances to Ypt7 and Rab7 activity and localization may be the result of the mis-localization of LIP5. If this is the case, then we would expect the localization of Rab14 on vesicular membranes in dLIP5 null *Dictyostelium* to be perturbed. This hypothesis could be tested by comparing the fraction of Rab14 present on magnetically purified endosomes in the presence and absence of dLIP5.

Similar to the factors contributing to reduced activation of Ypt7 in vps4 mutant yeast, dLIP5 might also influence Rab14 GEF localization to the endosomal membrane in *Dictyostelium*. Although a definitive GEF for Rab14 has not been identified in *Dictyostelium*, a blast search of the *Dictyostelium* proteome identified a single putative protein with >30% identity and >50% identity to the human Rab7 GEF, Ccz1. Due to the functional similarity that *Dictyostelium* Rab14 shares with human Rab7, and their common sensitivity to disruptions in the ESCRT pathway machinery, the putative *Dictyostelium* Ccz1 protein is a reasonable Rab14 GEF candidate. Due to this possibility, it will be interesting to explore the spatial and functional relationship that Ccz1 has with both dLIP5 and Rab14.

5.1.4 Investigating how LvsB antagonizes Rab14 activity

While our studies indicate that dLIP5 assists in the activation of Rab14 driven fusion during lysosomal maturation, we have also shown that this fusion activity is antagonized by LvsB as a lysosome matures to the post-lysosomal stage. We hypothesize that LvsB might be important for inactivating Rab14 at the transition to the post-lysosomal stage. With this hypothesis in mind, it would be interesting to investigate the relative levels of endogenous GTP-bound Rab14 in the presence and absence of LvsB. This could be done using antibodies specific for the GTP- bound form of Rab14. Additionally, the antagonism of Rab14 function by LvsB may occur through a direct interaction, or via relationships with Rab14 GAP or GEF proteins. If Ccz1 is identified as a Rab14 GEF, then we might also investigate the possibility that LvsB antagonizes Rab14 activity by governing the availability or localization of Ccz1. Additionally, mammalian Rab7 activity is shut off by its interaction with the Rab7 specific GAP TBC1D15 (Peralta et al., 2010). A blast search of the *Dictyostelium* proteome identified multiple protein sequences with homology the C-terminal half of human TBC1D15. Of these, the putative protein DDB0233124 demonstrated the highest homology with >40% identity, >60% similarity, and an E value of $9e^{-66}$. Interestingly, the region of TBC1D15 that shared homology with this putative *Dictyostelium* protein covers the TBC GAP domain. In the future it would be pertinent to investigate the relationship of this putative Rab-GAP with Rab14. Once again, if DDB0233124 is identified as a Rab14 specific GAP, then the effects of LvsB disruption on its localization dynamics may provide insight into how LvsB antagonizes Rab14 activity at the lysosome to post-lysosome transition. These avenues of investigation into how both dLIP5 and LvsB contribute to Rab14 activity have the potential to delineate novel mechanisms that dictate how specific vesicle fusion capabilities are switched on and off at particular maturation stages along the endo-lysosomal pathway.

5.1.5 Assessing the role of CHMP5

Our demonstration that dLIP5 functions upstream of Rab14 to promote vesicle fusion does not preclude the possibility that intermediary proteins link dLIP5 indirectly to

Rab14. The idea that consecutive activity of ESCRT pathway proteins leads to the activation of Rab14 is supported by the reported reduction of Rab7 and Ypt7 activity upon disruption of ESCRT pathway proteins that precede and are required for the localization of LIP5 on endosomal membranes (Balderhaar et al., 2010; Urwin et al., 2010; Russell et al., 2012). In this study we have shown that dLIP5 represents the most distally acting member of the ESCRT pathway proteins to affect Rab14 activity, and by extension, Rab7/Ypt7 activity. However, LIP5 may represent another intermediary between MVB formation and Rab7 activation, as its localization is required for the recruitment of the downstream ESCRT related protein, CHMP5 (Shiflett et al., 2004; Ward et al., 2005; Azmi et al., 2008). Although no definitive function has been assigned to CHMP5, its disruption in mice causes the formation of aberrantly large MVBs packed with intraluminal vesicles and the delayed delivery of MVB cargo to lysosomes or the vacuole in yeast (Kranz et al., 2001; Bache et al., 2003; Bache et al., 2004; Shiflett et al., 2004; Ward et al., 2005; Shim et al., 2006). These phenotypic attributes are similar to those of Ypt7 deficient yeast and are indicative of defective fusion between MVBs and lysosomes (Russell et al., 2012). Due to the requirement of LIP5 for CHMP5 localization and the similarity of the CHMP5 mutant phenotype to that of cells deficient in Ypt1 activity, it would be interesting to evaluate the importance of CHMP5 for Rab14 mediated fusion. Additionally, the *in vivo* localization dynamics between LIP5 and CHMP5 indicate that some sort of dynamic balance exists between the two. While LIP5 is required for CHMP5 vesicle localization, CHMP5 is reported to antagonize LIP5 membrane localization (Shiflett et al., 2004; Azmi et al., 2008). Given the nature of how these proteins influence the localization characteristics of one another, it is possible that the LIP5 to CHMP5 transition coincides and likely drives the transition from MVB formation to MVB-lysosome fusion. *Dictyostelium* has a single putative CHMP5 homologue (Mattei et al., 2006) that could be investigated in relation to dLIP5, Rab14 and LvsB in a similar manner as we have done for dLIP5 as described in chapter 4. Understanding how all of these proteins interact can provide important information about how MVB formation is coordinated with the delivery of MVB contents to the lysosome for digestion.

5.2 Potential impact of this work

Our understanding of how lesions in *lyst* contribute to the manifestation of CHS has long been stunted by the absence of a definitive model for how Lyst functions along the endo-lysosomal pathway. Here we have used *Dictyostelium discoideum* to distinguish between the vesicle fusion and fission models for the Lyst homologue, LvsB. Beyond this, we have shown that LvsB exerts its function by antagonizing two upstream lysosomal fusion promoting proteins, dLIP5 and Rab14. This study also marks the first investigation of *Dictyostelium* LIP5 (dLIP5) function, and has provided the first evidence that a LIP5 homologue participates in the initiation of Rab GTPase specific vesicle fusion. The functional interactions described here outline regulatory steps that correlate with transitions in Rab14 activity. These transitions ultimately impart fusion specificity at precise maturation stages along the endo-lysosomal pathway. Our localization and functional studies indicate that dLIP5 is important for directing Rab14 driven fusion activation, likely during lysosomal maturation, and that LvsB shuts Rab14 driven fusion off as lysosomes mature to the post-lysosomal stage. By homology, we suggest that mammalian Lyst and LIP5 likely participate in a similar pathway to regulate vesicle fusion competency by influencing Rab7 activity. While the process of vesicle fusion itself is well described, our understanding of the regulatory mechanisms that dictate when fusion occurs is still in its infancy. While it is clear that both dLIP5 and LvsB assist in this regulation, the experiments described above have the potential to expand upon the intricate intermingling of opposing interactions that drive vesicle fusion at specific endosomal maturation stages. This interplay between fusion regulators potentiates the precise and eloquent process of trafficking endo-lysosomal cargoes from a multitude of origins to their final destinations. We hope that future studies on the function of LvsB and its interacting proteins will contribute important insights into how Lyst dysfunction elicits the devastating lysosomal trafficking defects associated with CHS.

Chapter 6: Experimental Methods and Materials

6.1 Methods and materials

6.1.1 Cell lines and culture conditions

Dictyostelium discoideum cell lines were maintained in HL-5 media supplemented with 0.6% Penicillin/Streptomycin at 18°C on petri dishes. The media for maintenance of mutant and plasmid transfected lines were supplemented with 5ug/ml of blasticidin, and/or 10-15ug/ml gentamycin accordingly. The wild type strain NC4A2 was used for all controls, for expression of constructs, and for previous generation of the LvsB null cell line (Kypri et al., 2007) and generation of the LIP5 null cell line. The WASH null cell line was generated in AX2 and generously gifted by Dr. Thierry Soldati (Université de Genève, Switzerland). The AP3 μ3 null cell line was derived from DH1-10 and generously gifted by Dr. Pierre Cosson (Centre Medical Universitaire de Geneve, Switzerland). Vacuolin B-null cell line was derived from AX2 and was a gift from Dr. Markus Maniak (Universitaet Kassel, Germany) (Jenne et al., 1998).

6.1.2 Knock down of dLIP5 protein expression

The dLIP5 RNA hairpin construct was sub-cloned into and expressed from the previously described vector pLD1A15SN (Robinson D, Spudich JA (2000 JCB); Girard KD 2005). The anti-sense fragment of the hairpin was cloned using the forward primer AO-722 [5'-TGCGGCCGCTGACCAAAATGAAGAAGGTGGAGC-3'] containing a Not1 restriction site, and the reverse primer AO-723 [5'-TGTCGACCTGTTGTTGAATTTGTTTTTGAAGTAGTTGG-3'] containing a Sal1 restriction site. These primers generate an anti-sense fragment that covers bases 486 to 1462 of the dLIP5 coding sequence. The sense fragment of the hairpin construct was generated from bases 829 to 1462 using the forward primer AO-724 [5'-TGCGGCCGCCCACCAACAACAACCAATTCTGATAGC-3'] containing a Not1 restriction site, and the reverse primer AO-725 [5'-

GACGCGTTGTTGTTGAATTTGTTTTTGAAGTAGTTGG-3'] containing a Mlu1 restriction site. The resulting plasmid (pLD1A15SN-Lip5hp) does not contain a *Dictyostelium* origin of replication (REP) and was transformed into wild type (NC4A2) and LvsB null (B1B11) *Dictyostelium* cells with and without a pREP helper plasmid to achieve either extra-chromosomal or chromosomal integrant expression respectively. We found that expression from integrated plasmid resulted in greater knock down of dLIP5 protein expression. Therefore all dLIP5 knock down experiments were done using transformants with pLD1A15SN-LIP5hp integrants. Cells transformed with empty pLD1A15SN vector were used as a control for all subsequent experiments.

6.1.3 Generating the dLIP5 null cell line

Homologous recombination was used to disrupt the *lip5* gene in the NC4A2 wild type cell line as previously described (Harris et al., 2002). The blasticidin-resistance cassette of pSP72-Bsr was flanked by sequence fragments generated from the coding and adjoining untranslated regions of the *lip5* genomic locus. Upper primer AO-778 [5'-CTCGAGTACGTGTCACACTTTTCCTTATGGTCC-3'] in combination with lower primer AO-779 [5'-GAAGCTTCTTCAGTTTCTCCACTTGCTCCACCT-3'] amplified 799bp of 5' untranslated region upstream of the lip5 gene and 705bp into the beginning of the *lip5* gene. This fragment was sub-cloned into the pSP72-Bsr vector in reverse orientation using the xhoI and HindIII restriction sites. Upper primer AO-780 [5'-GGATCCAACCAATTCTGATAGCGCACCTTCTTTC-3'] in combination with lower primer AO-781 [5'-GAATTCGCAGCTGGTGTTGGTGTTGGTATGTTAG-3'] were used to amplify the last 811 bp of the *lip5* gene and 492 bp into the 3' untranslated region downstream of the lip5 gene. This fragment was sub-cloned in reverse orientation upstream of the Bsr cassette in pSP72-Bsr using EcoRI and BamHI restriction sites. The final construct was linearized using the EcoRI and xhoI then 10ug was introduced into both wild type (NC4A2) and LvsB null (B1B11) cells by electroporation. Electroporated cells were then diluted and plated in 96 well culture plates (Nalge-Nunc Int., Rochester, NY). Clonal integrants were selected for in HL-5 media supplemented with 5ug/ml

blasticidin. Resulting clonal populations were screened for the absence of dLIP5 protein by western blot analysis.

6.1.4 Western blot analysis

dLIP5 protein expression was assessed in the dLIP5 knock down cell lines as well as in possible *lip5* null clonal populations using western blot analysis. For all screening blots, $1x10^6$ cells were loaded and run on a 10% SDS polyacrylamide gel, then transferred over night at 4°C to nitrocellulose using 80 mAmp current for small gels and 200 mAmp for large gels. dLIP5 protein was detected using 1:1000 diluted guinea pig polyclonal antibody against dLIP5 and HRP conjugated goat anti-guinea pig secondary antibody (SouthernBiotech, Birmingham, AL, USA). Myosin heavy chain (MHC) protein was used as a reference for protein loading and was detected with a 1:4000 dilution of rabbit polyclonal antibody against MHC followed by HRP conjugated goat anti-rabbit secondary antibody (SouthernBiotech, Birmingham, AL, USA). HRP signal was detected by ECL (Pierce Biotechnology, Rockford, IL, USA). Relative dLIP5 protein levels were assessed for dLIP5 knock down cell lines using the imageJ program.

6.1.5 Plasmid expression and Antibodies

The GFP-vacuolin B expression vector was constructed and provided by Dr. Marcus Maniak (Universitaet Kassel, Germany). The wild-type GFP-Rab14 and mutant GFP-Rab7Q67L as well as the mutant Rab14Q67L, Rab14N121I, and Rab7T22N template vectors were kindly provided by Dr. Cardelli (LSU Med. Ctr., LA) (14,1997 paper) (Buczynski et al., 1997; Harris and Cardelli, 2002). We then constructed the pTXflag-Rab14Q67L and pTXflag-Rab14N121I vectors by amplifying the corresponding coding regions and cloning them into the pTX-flag expression vector (Levi et al., 2000) at the BamHI and XbaI sites. Similarly, we amplified the coding regions of wild-type Rab7 and Rab7T22N and cloned them into the pTX-GFP expression vector at the BamHI and XbaI sites.

Anti-vacuolin monoclonal antibody 264-79-2 (postlysosomes) was a kind gift from Dr. Maniak (Jenne et al., 1998). Monoclonal anti-Flag antibody (M2) was purchased from Sigma-Aldrich, St. Louis, MO. Monoclonal antibodies against p25 (H72) (early endosomes), p80 (H161) (lysosomes, postlysosomes, and phagosomes) and polyclonal anti-RH50 (contractile vacuole) were a generously provided by Dr. Pierre Cosson (Centre Medical Universitaire de Geneve, Switzerland) (Benghezal et al., 2001; Ravanel et al., 2001). Antibodies against Myosin Heavy Chain (MHC) and LIP5 were generated by our lab in collaboration with Cocalico Biologicals, Reamstown, PA, USA.

6.1.6 Endosome labeling

For morphological studies, live cell images were taken after labeling of the entire endo-lysosomal pathway. Cells ($1x10^6$ cells/ml) were allowed to attach in well chambers (Nalge-Nunc Int., Rochester, NY) for 20 minutes and then switched to low-fluorescence media for 1 hour at 18°C. Cells were then incubated in 2 mg/ml TRITC-dextran (mw 64kDa; Sigma- Aldrich Inc. St Louis, MO, USA) for 1 hour at 18°C. The cells were washed 4 times with low fluorescence media and imaged using GFP and Texas Red filters on a Nikon inverted epi-fluorescence microscope TE200 (Nikon Instruments, Dallas, TX, USA). A Photometrics cooled CCD camera driven by Metamorph software was used to collect acquisitions of different fields of cells continuously for 10 minutes. ImageJ software was used to generate merged images when applicable and to measure dextran vesicle diameter.

For temporal localization of GFP-vacuolin on endosomes, visualization of maturing endosomes was done using pulse labeling and subsequent live cell imaging of *Dictyostelium* in well chambers. GFP-vacuolin expressing cells ($1x10^6$ cells/ml) were allowed to attach in chambers as above and then switched to low-fluorescence media for 1 hour at 18°C. Cells were then pulsed with 2 mg/ml TRITC-dextran for 5 minutes. The cells were then washed 4 times with low fluorescence media and imaged using a Nikon inverted epi-fluorescence microscope. Images were taken of different fields of cells over the course of 60 minutes using GFP and Texas Red filter sets. ImageJ software was subsequently used for quantification of co-localization.

6.1.7 Labeling of acidic compartments

To visualize the acidity characteristics of different populations of vesicles, live cell imaging was done in GFP-vacuolin B expressing cells exposed to a membrane permeable acidophilic fluorophore. Cells ($1x10^6$) were allowed to attach in chambers for 20 minutes and then switched to low-fluorescence media and incubated for 1 hour at 18°C. Attached cells were then incubated with 1μM Lysotracker red DND-99 (Invitrogen, Eugene, OR, USA) for 10 minutes at room temperature. After being washed twice with low-fluorescence media, cells were imaged using an inverted Nikon epi-fluorescence microscope to collect multi-dimensional aqcuisitions as previously described. Different fields of cells were imaged continuously for only 10 minutes after being washed to prevent Lysotracker red from artificially raising the alkalinity of acidic compartments. ImageJ software was used to generate merged images for quantification of co-localization as well as to quantify fluorescence intensity of acidic compartments. Background subtraction of fluorescence was done on a cell by cell basis.

6.1.8 Fixation and Immuno-localization studies

Cells ($2x10^6$cells/ml) were allowed to attach on coverslips for 15 minutes at 18 °C and washed briefly with PDF buffer (2mM KCl, 1.1 mM K2HPO4, 1.32 mM KH2P04, 0.1mM CaCl2, 0.25mM MgSO4, pH 6.7) and then overlaid with a thin layer of 2% PCR agarose (BioRad, Hercules, California). Cells were then fixed with 1% formaldehyde in methanol for 5 minutes at -20°C followed by a wash with phosphate-buffered saline (PBS), rinsed briefly with distilled water and mounted on microscope slides with mounting media (MOWIOL, Calbiochem, EMD Biosciences Inc. La Jolla, CA). The slides were allowed to dry in the dark and analyzed.

For immunolabeling of specific membranes, cells were incubated with either anti-p80 (labels plasma, endosomal, and phagosomal membranes), anti-RH50 (contractile vacuole), anti-p25 (early endosomes), or anti-vacuolin (labels post-lysosomal membranes) antibody for 1 hour at 37°C in the dark. Cells were washed three times with PBS and incubated with Texas-Red or FITC conjugated goat-anti mouse antibody or with Texas-Red conjugated goat anti-rabbit (30μg/ml; Molecular Probes, Eugene,OR)

accordingly for 1 hour at 37°C in the dark. Following incubation with secondary antibody, cells were again washed 3 times in PBS then rinsed briefly with ddH_2O and mounted on microscope slides with mounting media (MOWIOL, Calbiochem, EMD Biosciences Inc.). The slides were dried in the dark at 4°C overnight and then imaged with a Nikon inverted epi-fluorescence microscope TE200 (Nikon Instruments, Dallas, TX, USA). Images were collected using a Photometrics cooled CCD camera driven by Metamorph software. ImageJ software was used to generate merged images, to subtract background fluorescence, and to measure vacuolin labeled vesicle diameter.

The sub-cellular localization of endogenous LIP5 protein was also assessed by immuno-fluorescence. Following fixation, cells were incubated in a 1:500 dilution of the LIP5 antibody for 1 hour at 37°C. Cells were then washed 3 times with PBS and before incubation with alexafluor-594 conjugated goat anti-guinea pig secondary antibody (Invitrogen, Eugene, OR, USA) for 1 hour at 37°C. Cells were then washed, mounted on slides, and imaged as described above.

6.1.9 *In vivo* endosome fusion assay

To assess fusion between different temporal populations of vesicles, cells were subjected to two pulses of differently labeled dextran separated by a specified chase time. Cells ($1x10^6$) were allowed to attach in chambers for 20 minutes and then switched to low-fluorescence media and incubated for 1 hour at 18°C. Attached cells were given a 5 minute pulse of 4 mg/ml FITC-dextran (mw 59 kDa; Sigma- Aldrich Inc. St Louis, MO, USA) and then washed 3 times before chasing for 30 or 60 minutes in low-fluorescence media. Following the chase, cells were given a 5 minute pulse of 2 mg/ml TRITC-dextran then washed 4 times in low-fluorescence media before being imaged. Continuous live images of different fields were taken for 10 minutes using an inverted Nikon epi-fluorescence microscope to collect multi-dimensional acquisitions as previously described. Co-localization of the two dextran signals was quantified using imagej software.

6.1.10 Phagosome fusion assay

Phagosome fusion was assessed by pulse labeling of consecutive phagosomal populations with blue or red fluorescent beads. Cells were collected and re-suspended to $1.5x10^6$ cells/ml then 5 ml of re-suspension was allowed to recover in HL-5 media by shaking at 160 rpm for 15 minutes at 18°C. Cells were given a 10 minute pulse of 1um blue (350/440) conjugated Carboxylate FluoSpheres (Invitrogen, Eugene, OR, USA) diluted 1:2500 in the shaking culture. Cells were then collected by centrifugation and washed by re-suspending in 5 ml of ice cold HL-5 media and shaking for 2 minutes at 4°C. Following the wash, cells were collected, resuspended in 5 mL of room temperature HL-5 media, and given a 10 minute pulse of 1:2500 diulted 1um red (580/605) conjugated Carboxylate FluoSpheres (Invitrogen, Eugene, OR, USA) while shaking at 160 rpm. Cells were collected and washed twice in ice cold HL-5 media before being re-suspended in 2.5 mL of room temperature HL-5 media. 200ul of the cell re-suspension was pipetted onto a coverslip and cells were allowed to attach for 30 minutes at 18°C. Cells were briefly flattened using a thin 2% PCR agarose (Biorad) overlay before being fixed for 5 minutes at -20°C with 1% formaldehyde in MeOH. Following fixation, cells were washed briefly in phosphate-buffered saline (PBS) and then immunostained.

For immuno-labeling of phagosomal membranes, cells were incubated with α-p80 primary antibody for 1 hour at 37°C in the dark. Cells were washed 3 times with PBS before being incubated with FITC conjugated goat-anti mouse secondary antibody (30 μg/ml; Molecular Probes) for 1 hour at 37°C in the dark. Cells were washed 3 times in PBS then rinsed briefly with ddH_2O and mounted on microscope slides with mounting media (MOWIOL, Calbiochem, EMD Biosciences Inc.). The slides were dried in the dark at room temperature and imaged the following day. Phagosome fusion was quantified from merged images generated using imagej software.

6.2 Plasmids

Table 6.1: Plasmids used in this study.

Plasmid	Description
GFP-vacuolin B	Vacuolin B coding region starting at amino acid 25 amplified from *Dictyostelium* cDNA and cloned into a pDNeoII vector containing both GFP and gentamycin resistance cassette. Expression product is vacuolin B with an N-terminal GFP tag.
dLIP5 knock down plasmid	Sense (633 residues) and anti-sense (976 residues) fragments were amplified from *Dictyostelium* dLIP5 cDNA and constructed into the pLD1A15SN that contains a gentamycin resistance cassette, but no *Dictyostelium* origin of replication. Note: when transformed without pREP helper, construct integrates into genome, and we documented more efficient knock down of dLIP5 protein expression than when the pREP helper was co-transformed.
dLIP5 knock out plasmid	Upstream 5' (starting 799 bases upstream of the *lip5* gene, and terminating 705 bases into the *lip5* gene) and downstream 3' (starting at base 811 of the *lip5* gene and terminating 492 bases into the 3' untranslated region) DNA fragments were amplified from *Dictyostelium* genomic DNA and cloned into the pSP72-Bsr vector such that the 5' and 3' regions are in reverse orientation with regards to the direction of the Bsr (Blasticidin resistance) cassette. Knock out construct can be linearized with EcoRI and XhoI for transformation into *Dictyostelium*.

Flag-Rab14Q67L (DA-Flag-Rab14)	Full length *Dictyostelium* Rab14 gene with a mutation in the codon for amino acid 67 changing glutamine (CAA) to leucine (CTA). Cloned into the pTX-Flag vector that contains a gentamycin resistance cassette. Expression product has an N-terminal flag tag.
Flag-Rab14N121I (DN-Flag-Rab14)	Full length *Dictyostelium* Rab14 gene with a mutation in the codon for amino acid 121 changing asparagine (AAC) to isoleucine (ATC). Cloned into the pTX-Flag vector that contains a gentamycin resistance cassette. Expression product has an N-terminal flag tag.
Flag-Rab7Q67L (DA-Flag-Rab7)	Full length *Dictyostelium* Rab7 gene with a mutation in the codon for amino acid 67 changing glutamine (CAA) to leucine (CTA). Cloned into the pTX-Flag vector that contains a gentamycin resistance cassette. Expression product has an N-terminal flag tag.
Flag-Rab7T22N (DN-Flag-Rab7)	Full length *Dictyostelium* Rab7 gene with a mutation in the codon for amino acid 22 changing Threonine (ACA) to asparagine (AAT). Cloned into the pTX-Flag vector that contains a gentamycin resistance cassette. Expression product has an N-terminal flag tag.

Appendix

Miscellaneous experiments:

Early stages of fruiting body formation are perturbed in the dLIP5 null cell line.

Under favorable growth conditions, *Dictyostelium* exist in a vegetative single cell state. However, when *Dictyostelium* are stressed by starvation they will transition into a developmental state where cells aggregate to form a fruiting body which houses spores that will remain dormant until the environment once again becomes favorable for growth. Upon starvation, development is initiated by cAMP motivated aggregation of *Dictyostelium* cells toward an aggregate center to form a mound containing up to $1x10^5$ cells. Once tightly aggregated into a mound, cell signaling cascades perpetuate the differentiation of ~20% of the cells into pre-stalk cells which segregate to the tip of the mound, and ~70% of the cells into pre-spore cells which make up the remainder of the aggregate. These partially differentiated cell populations then reorganize into a slug like form and the entire slug can migrate in order to position the aggregate in a favorable environment before final differentiation and fruiting body formation. Ultimately, the fruiting body consists of a large head which contains the dormant spore population supported by an elongated stalk. This developmental process requires cell signaling cascades that are initiated by cell surface receptors at every stage (Aubry and Firtel, 1999). Because the endo-lysosomal pathway and MVB formation are important for establishing differential sensitivity of cells to extracellular signals (Luttrell, 2006; Piper and Lehner, 2011), we were interested in observing the developmental process of the dLIP5 null cell line.

dLIP5 null, sibling, and wild type cells (2x107 cells) were collected by centrifugation, resuspended in starvation buffer, and a 20 µl dot of cell suspension was applied to a lawn of E. coli B/R that was previously grown on an SM-5 plate. Plates were then stored in the dark at 18°C for two weeks to allow plaque formation. The edge of the plaque was used to assess early stages of the developmental pathway while the center of the plaque was used to assess fruiting body morphology. We were able to distinguish morphologically normal formation of all developmental stages for the wild type and the

sibling control cell lines (Fig A.1 A-B, and Fig A.2 A-B). However, while fruiting bodies formed by the dLIP5 null cells appeared similar to those formed by wild type and sibling control cells (Fig A.2 A-C), we observed some aberrant morphological features of early developmental structures for the dLIP5 null cell line (Fig A.1 C). At the loose aggregate phase (mound phase) when cells begin to differentiate into pre-stalk and pre-spore populations, we observed multiple peak like structures in mounds formed by dLIP5 null cells compared to single peaks in mounds formed by wild type, or sibling cells (Fig A.1 A-C arrowheads). Additionally, we often observed multiple slug like structures extending from a single aggregate (possibly multiple tips extending from a single mound) at the edge of the dLIP5 null cell plaque (proximal to the aggregate mounds at the edge of the plaque) (Fig A.1 C arrows). These aberrant structures were not observed in plaques formed by wild type or sibling control cells. The morphological perturbations that we observed for early developmental structures in the dLIP5 null cell line could be indicative of problems with cell signaling. However, because final fruiting body morphology appears to be normal in the dLIP5 null cell line, the perturbations observed during early development seem to resolve by the terminal stages of fruiting body formation. While the dLIP5 null fruiting bodies are morphologically intact, the implications of dLIP5 disruption on proper spore formation and viability have not been assessed.

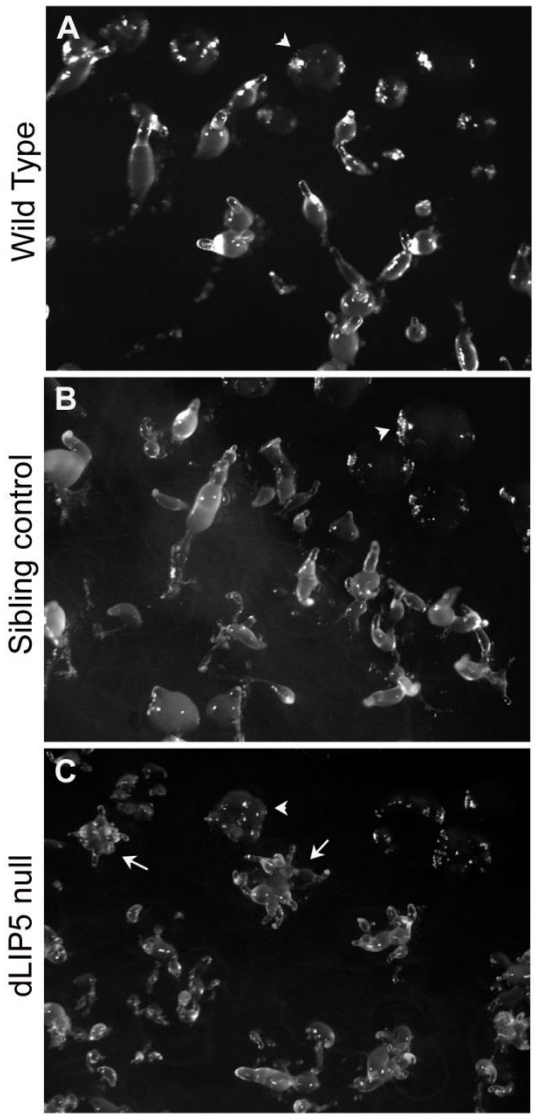


Figure A.1: dLIP5 null cells form aberrant early developmental structures. Wild type, sibling control, and dLIP5 null cells were dot plated on a lawn of E. coli B/R to allow plaque formation and development behind the feeding front of the forming plaque. The morphology of early developmental structures **(A-C)** was assessed by imaging the edge of the plaques. Early developmental structures formed by dLIP5 null cells appeared abnormal compared to those formed by wild type and sibling control cells. Many aggregate mound structures appeared to have multiple apexes in the dLIP5 null plaque **(C)** compared to aggregates with a single apex formed by both sibling **(B)** and wild type cells **(A)** (arrowheads). At a slightly later developmental stage, the dLIP5 null aggregates appear to have multiple slug like structures extending from a single base (**C)** (arrows). These structures were not apparent in the plaques formed by either sibling control or wild type cells.

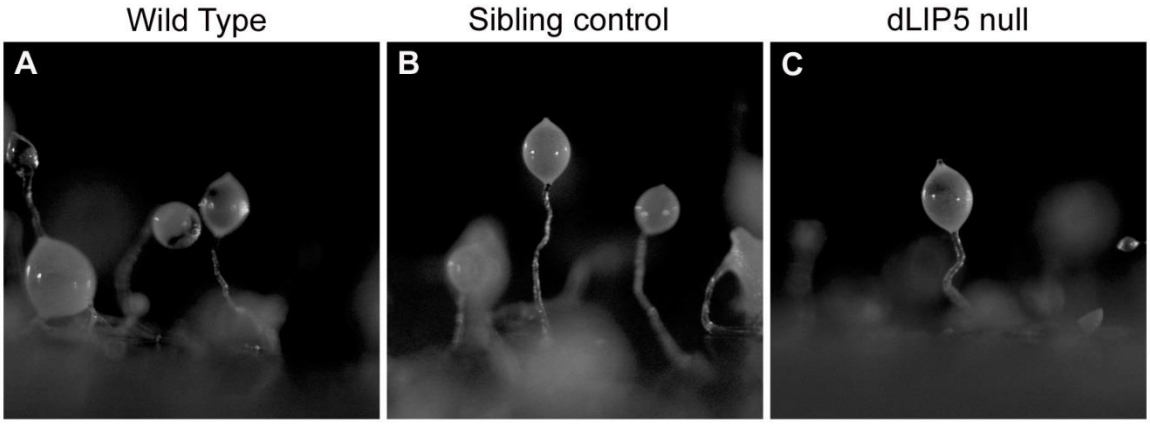


Figure A.2: dLIP5 null cells form fruiting bodies with normal morphology. Mature fruiting body morphology **(A-C)** was assessed by imaging structures towards the center of the plaques formed by either wild type, sibling control, or dLIP5 null cells on a lawn of E. coli B/R. The morphology mature fruiting bodies in the dLIP5 null plaque **(C)** looked similar to those formed in both the sibling control plaque **(B)** and the wild type plaque **(A)**.

RFP-dLIP5 co-localizes with LvsB and vacuolin on vesicle membranes.

In order to assess the effects of dLIP5 overexpression, we cloned the dLIP5 gene from a *Dictyostelium* cDNA library with the upper primer [CGCGGATCCGCTGCTGCTGCTGCTGCTATGGATTTAGCAAGTTTACCACCAG CATTA] paired with the lower primer [CGGAATTCTTATGAATCTTCGCCAGTTAAATATTTGAGAG] and inserted it into the pTXRFP vector using BamHI and EcoR1 restriction sites. We then transformed this construct into wild type cells as well as cells expressing GFP- tagged LvsB, and used fluorescence co-localization studies to establish the vesicular localization characteristics of the RFP-dLIP5 construct. Like endogenous dLIP5, the RFP-dLIP5 construct was found to localize primarily to the cytosol with some vesicular membrane localization (Fig A.3 A and B). We found that 45% of 77 RFP-dLIP5 labeled vesicles were labeled by GFP-LvsB (Fig A.3 A-A'). This result correlated with the co-localization of endogenous dLIP5 with GFP-LvsB that we reported in chapter 4. We next investigated the assessed the localization of the RFP-dLIP5 construct on post-lysosomal membranes by immuno-staining RFP-dLIP5 expressing cells with antibodies against the post-lysosomal marker vacuolin. Less than 1% of 89 RFP-dLIP5 labeled vesicles were also labeled by vacuolin (Fig A.3 B-B'). This result is in contrast to the 11.7% of endogenous dLIP5 labeled

vesicles that were labeled by vacuolin (see chapter 4). Together with the co-localization of RFP-dLIP5 with GFP-LvsB, these results suggests that the RFP-dLIP5 construct is present on a similar population of vesicles as we reported for endogenous dLIP5, but that it may have reduced stability as late lysosomes mature to the post-lysosomal stage. Alternatively, the RFP-dLIP5 construct may perturb the localization of vacuolin to vesicular membranes.

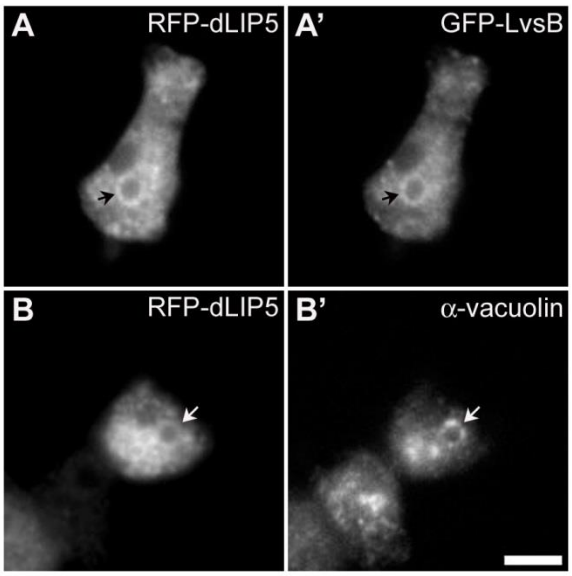


Figure A.3: RFP-dLIP5 co-localizes with GFP-LvsB and vacuolin on vesicular membranes. (A-A') Cells expressing both RFP-dLIP5 and GFP-LvsB were flattened and fixed for fluorescence imaging. Co-localization was assessed using imageJ software, and we found that 45% of 77 RFP-dLIP5 labeled vesicles were also labeled by GFP-LvsB. **(B-B')** Cells expressing RFP-dLIP5 were flattened, fixed, and immuno-stained with antibodies against the vacuolin. A minimal population (<1%) of 89 RFP-dLIP5 labeled vesicles were labeled by vacuolin. Bar is 5μm.

Overexpression of RFP-dLIP5 has a dominant negative effect on post-lysosome morphology and heterotypic fusion.

We reported in chapter 4 that the disruption of dLIP5 results in reduced post-lysosome size as well as reduced heterotypic fusion between lysosomes and post-lysosomes in LvsB null cells. These results led to the conclusion that LvsB antagonizes the fusion promoting function of dLIP5. To further investigate the antagonistic

relationship between dLIP5 and LvsB, we wanted to ask if the overexpression of dLIP5 would elicit a further increase in both post-lysosome size and in heterotypic vesicle fusion in LvsB null cells. RFP-dLIP5 expression relative to endogenous dLIP5 was assessed by western blot analysis using the dLIP5 antibody generated by our lab. The western blot image shown in Figure A.3 A shows a much larger and darker band corresponding to RFP-dLIP5 fusion protein compared to the band corresponding to endogenous dLIP5 protein in LvsB null cells (Fig A.4 A). This result is indicative of significant overexpression of the RFP-dLIP5 fusion construct compared to endogenous dLIP5 protein.

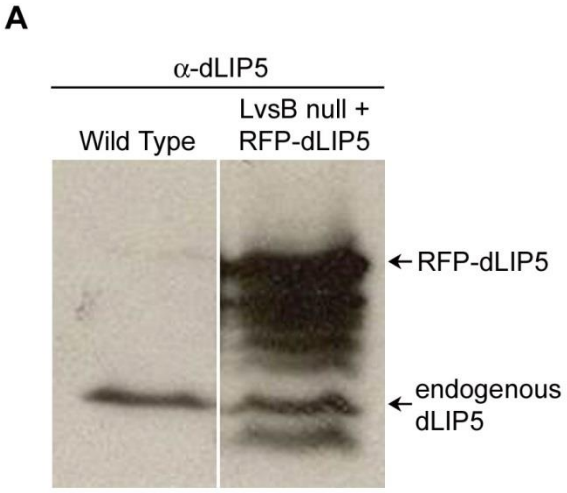


Figure A.4: Overexpression of RFP-dLIP5 in the LvsB null cell line. (A) The relative expression level of the RFP-dLIP5 fusion protein was compared to that of endogenous dLIP5 by western blot analysis using a polyclonal dLIP5 antibody generated by our lab. We found that the RFP-dLIP5 fusion protein was expressed at much higher levels than the endogenous dLIP5 protein.

To investigate the effect of RFP-dLIP5 overexpression on the LvsB null phenotype, we first compared the average post-lysosome size of wild type, LvsB null, and RFP-dLIP5 expressing LvsB null cells. As previously reported (Kypri et al., 2007), average post-lysosome size was significantly increased in LvsB null cells when compared to wild type cells (Fig A.5 A-B and D). In contrast to our prediction, when RFP-dLIP5 was overexpressed in LvsB null cells we observed a significant decrease in post-lysosome size (Fig A.5 B-C and D). The rescue of aberrant post-lysosome size in LvsB

null cells by RFP-dLIP5 overexpression is similar to what we observed when dLIP5 protein expression was disrupted (see chapter 4). This result suggests that overexpression of the RFP-dLIP5 fusion protein might have a dominant negative effect. To further explore this possibility, we next used the previously described two dextran pulse-chase assay to determine the effect of RFP-dLIP5 overexpression on heterotypic fusion between lysosomes and post-lysosomes in the LvsB null cell line. Consistent with previous studies, we observed a significant increase in heterotypic fusion between lysosomes and post-lysosomes in LvsB null cells compared to wild type cells. Overexpression of the RFP-dLIP5 fusion protein resulted in a significant decrease in inappropriate heterotypic fusion in the LvsB null cell line. Once again, this result mimics the effect of disrupting dLIP5 in the LvsB null cell line and further suggests that overexpression of the RFP-dLIP5 construct has a dominant negative effect.

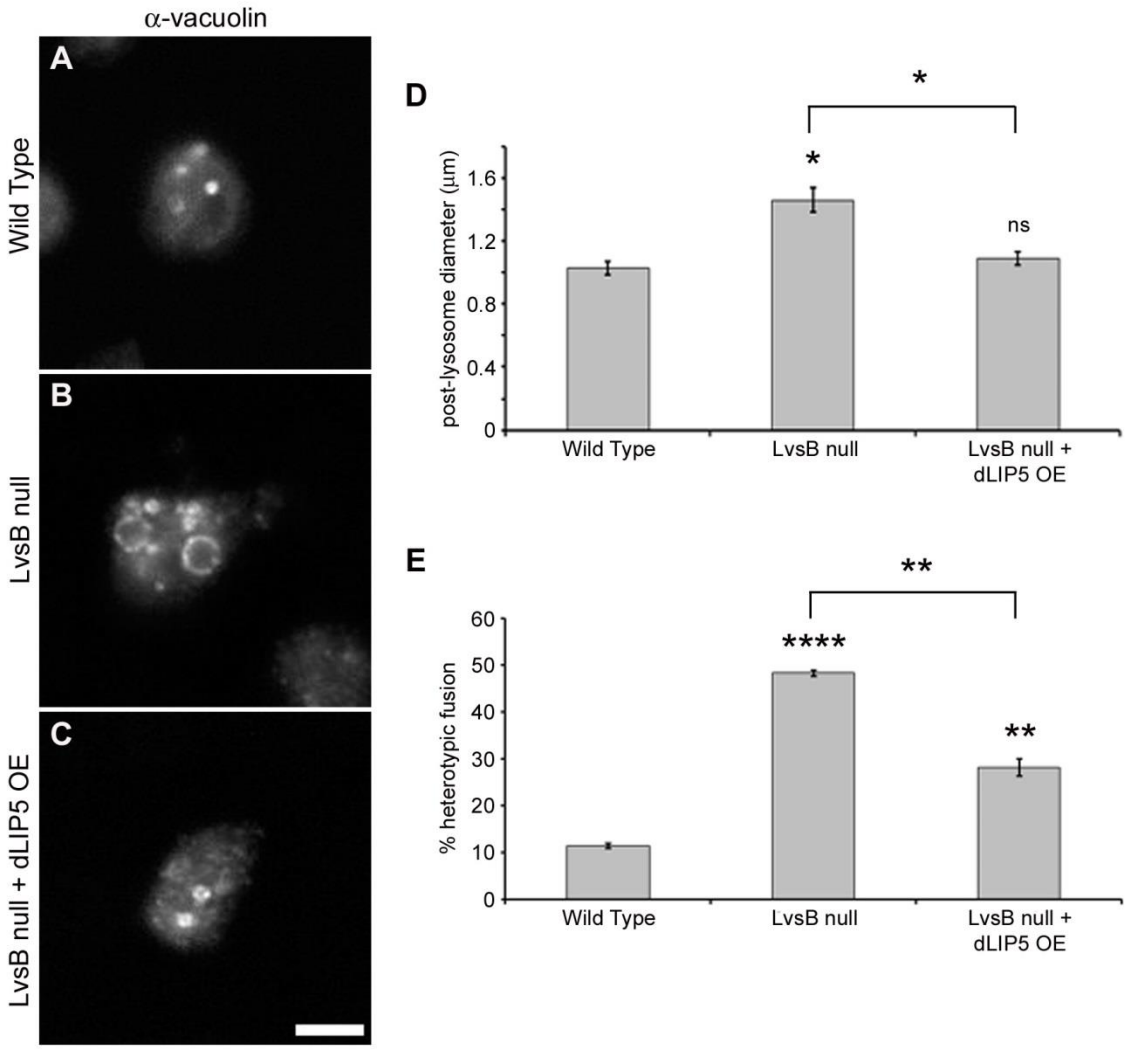


Figure A.5: Overexpression of the RFP-dLIP5 fusion protein in LvsB null cells reduces aberrant post-lysosome size and heterotypic fusion. (A-C) Cells were flattened, fixed and immuno-stained with antibodies against vacuolin. Average post-lysosome size was quantified from images similar to those shown in A-C using imageJ software. **(D)** The mean of >66 vacuolin labeled vesicles per trial is plotted +/- s.e.m. for three experiments. As previously reported, the average vacuolin labeled post-lysosome size of LvsB null cells was significantly larger than that of wild type cells. Overexpression of RFP-dLIP5 in LvsB null cells elicited a significant decrease in post-lysosome size. **(E)** Heterotypic fusion between lysosomes and post-lysosomes was assessed using the two dextran in vivo fusion assay described in chapter 6, and plotted as the mean +/- s.e.m. for three experiments. Disruption of LvsB caused a significant increase in heterotypic fusion, and overexpression of the RFP-dLIP5 fusion protein partially relieved this aberrant fusion. The effects of overexpression of RFP-dLIP5 on the LvsB null phenotype are similar to those observed when we disrupted dLIP5 protein expression. Bar is 5 μm.

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Vita

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