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Study of LvsB in *Dictyostelium discoideum* provides insights into the Chediak-Higashi syndrome

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Study of LvsB in *Dictyostelium discoideum* provides insights into the Chediak-Higashi syndrome

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Dedication

To my family

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Study of LvsB in *Dictyostelium discoideum* provides insights into the Chediak-Higashi syndrome

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The Chediak-Higashi Syndrome is a disorder affecting lysosome biogenesis. At the cellular level, the Chediak-Higashi syndrome is characterized by the presence of grossly enlarged lysosomes in every tissue. Impaired lysosomal function in CHS patients results in many physiological problems, including immunodeficiency, albinism and neurological problems. The Chediak-Higashi syndrome is caused by the loss of a BEACH protein of unknown function named Lyst.

In this work, I have studied the function of the *Dictyostelium* LvsB protein, the ortholog of mammalian Lyst and a protein that is also important for lysosomal function. Using a knock-in approach we tagged LvsB with GFP and expressed it from its single chromosomal locus. GFP-LvsB was observed on endocytic and phagocytic compartments. Specific analysis of the endocytic compartments labeled by LvsB showed that they represented late lysosomes and postlysosomes. The analysis of LvsB-null cells revealed that loss of LvsB resulted in enlarged postlysosomes, in the abnormal localization of proton pumps on postlysosomes and their abnormal acidification. This

work demonstrated that the abnormal postlysosomes in LvsB-null cells were produced by the inappropriate fusion of lysosomes with postlysosomal compartments.

Furthermore, this work provided the first evidence that LvsB is a functional antagonist of the GTPase Rab14 in vesicle fusion events. In particular, we demonstrated that reduction of Rab14 activity suppressed the LvsB-null phenotype by reducing the enlarged post-lysosomes and the enhanced rate of heterotypic fusion. In contrast, expression of an active form of Rab14 enhanced the LvsB-null phenotype by causing an even more severe enlargement of endosome size.

The results provided by this work support the model that LvsB and Lyst proteins act as negative regulators of fusion by limiting the heterotypic fusion of early with late compartments and antagonize Rab GTPases in membrane fusion. The LvsB localization studies and the functional assessment of the LvsB-null phenotype helped make unique contributions to the understanding of the molecular function of Lyst proteins.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

1.1 THE CHEDIAK-HIGASHI SYNDROME

This dissertation focuses on the study of LvsB in the *Dictyostelium discoideum* model system. The main aim of this dissertation is to uniquely contribute to the understanding of a severe immune disorder, the Chediak-Higashi syndrome (CHS) that is currently not well understood and has no potential therapies available.

The Chediak Higashi syndrome (CHS) is a hereditary disorder first described in 1943 and is one disorder for which there has been little progress in understanding its etiology. This disorder belongs to a group of diseases affecting lysosome biogenesis and secretion. The Chediak-Higashi syndrome (CHS) is a rare, autosomal recessive immunodeficiency disorder. This human disorder is characterized by severe immunologic defects, albinism, susceptibility to bacterial infections and abnormal platelet function. The diagnostic feature of CHS is the presence of enlarged intracellular vesicles. Affected vesicles include lysosomes, melanosomes, and platelet dense granules. This disorder is lethal, and patients die from recurrent bacterial infections or from a lymphoproliferative infiltrate known as the accelerated phase. Currently, the only treatment available is bone marrow transplantation (Introne et al., 1999; Ward et al., 2000). However, patients that receive bone marrow transplantation show a progressive neuropathy (Misra et al., 1991), indicating that the pathology of this disorder is complex.

A homologous disorder with variability in clinical severity has been found in other mammalian species. These include mice (Lutzner et al., 1967), rats (Nishimura et al., 1989), cows (Padgett et al., 1967), cats (Kramer et al., 1977), mink (Padgett et al., 1967) and a reported case in killer whale (*Orca*) (Ridgway, 1979). Currently, the mouse is the best-studied animal model.

A major breakthrough in the study of this immune disorder is the identification of the gene affected in CHS patients. Cloning of the *CHS* locus was greatly facilitated by the finding that Beige mutant mice have the same genetic defect (Barbosa *et al.*, 1996; Nagle *et al.*, 1996a). The protein encoded by the *CHS/beige* genes is named Lyst for (lysosomal-trafficking-regulator). Unfortunately, even though the *CHS/beige* gene was identified more than ten years ago, the function of the Lyst protein is still not known. In part, progress is made difficult because the *CHS/beige* gene is enormous and encodes a protein of approximately 3800 amino acids (Perou et al., 1997). The mRNA transcript from the *CHS/beige* genes is reported to be 12.0kb and is expressed in all cell types at very low levels (Perou et al., 1996). In the majority of cases studied, to date, the *CHS/Beige* mutations result in the generation of a premature stop codon and the production of a truncated protein (Certain et al., 2000).

Many cell types are affected by mutations in the *CHS/beige* genes, resulting in symptoms such as albinism, bleeding and immunodeficiency. Melanocytes from patients with CHS show abnormally large melanosomes, containing different varying of melanin (Windhorst et al., 1968) that are clustered abnormally in the juxtanuclear area. It is unclear whether there is a defect in transfer of melanosomes to the periphery of the melanocyte or transfer of melanosomes to keratinocytes. Additionally, platelets from CHS patients show irregular dense bodies that result in prolonged bleeding times (Buchanan and Handin, 1976; Rendu et al., 1983). *CHS/beige* mutations also cause severe defects in cells of the immune system. Activated cytotoxic T lymphocytes (CTLs) are involved in destroying infected target cells. CHS/Beige mutant cells show decreased CTLs activity due to a defect in lytic granule exocytosis ((Baetz et al., 1995). Additionally, CHS/ Beige mutant B-cells show delayed loading and peptide presentation (Faigle et al., 1998). Work in immune cells also indicates that CHS/Beige mutant cells

abnormally accumulate several lysosomal markers in early endosomes and at the plasma membrane, suggesting that LYST functions in trafficking (Faigle et al., 1998). Interestingly, a recent study showed that while CHS giant lysosomes, contain low levels of lysosomal enzymes, they contain increased levels of ER resident proteins when compared to control lysosomes (Zhang *et al.*, 2007). The precise trafficking event affected is not well understood.

Biochemical analysis of CHS/beige mutants also indicated several biochemical alterations in these cells. Beige mutant cells exhibit abnormally decreased PKC activity. E-64-d (a thiol proteinase inhibitor that protects PKC from proteolysis) and phorbol myristic acid (PMA) were shown to reduce lysosome size in CHS/Beige cells by reversing the decrease in PKC activity (Ito *et al.*, 1989; Sato *et al.*, 1990; Tanabe *et al.*, 2000; Cui *et al.*, 2001). Additionally, it was reported that the levels of sphingomyelinase activity and the degradative product ceramide are significantly increased in the CHS/Beige cells (Tanabe et al., 2000). Exogenously added ceramide induces the formation of abnormally large lysosomes (Li et al., 1999). Furthermore, ceramide has been shown to affect PKC levels (Liu, 1996). These data suggest a correlation between the activities of PKC, sphingomyelinase and CHS/Beige proteins, but this connection is currently unclear. The complexity of the CHS/Beige phenotype is also indicated by alterations in nuclear phosphoinositides specifically PtdIns(4,5)P2. CHS/Beige mutants exhibit a dramatic reduction in nuclear PtdIns(4,5)P2 (Ward et al., 2003). The role of Lyst in the regulation of nuclear lipids is not currently known.

1.2 BEACH PROTEINS

1.2.1 Structural organization of BEACH proteins

Very little is currently known about the function of Lyst, no significant binding partners have been identified and no molecular mechanism of its function has been proposed. An exciting development in the study of Lyst is the identification of homologous proteins in different model systems. Many proteins that share similar structural organization as Lyst, have been identified in multiple organisms and are grouped together in the BEACH family (Beige and Chediak-Higashi) (Wang et al., 2002). All BEACH proteins share a similar structural organization. At the C-terminus they have multiple WD motifs. The sequence similarity among the WD motifs of BEACH proteins is low (~20%), but are all predicted to fold into a beta-propeller structure that creates a protein-protein interaction domain. The most highly conserved domain among these proteins is the BEACH domain (~50-60% identity) that is found adjacent to the WD motifs. The function of the BEACH domain is currently unclear. Given the high degree of similarity of BEACH domains across species it is thought that this domain may play an important functional role. Unfortunately, the crystal structure of the BEACH domain did not provide any insights into the role of this domain (Jogl *et al.*, 2002; De Lozanne, 2003).The crystal structure revealed that the BEACH domain has a unique structure, never seen in any other protein. The majority of the conserved amino acids are found in the interior of the molecule. Next to the BEACH domain is a novel domain that folds into a structure similar to that of PH domains, although it does not share sequence similarities to PH domains from other proteins. The remaining portion at the N-terminus of BEACH proteins is unique to each BEACH protein (Jogl *et al.*, 2002; Wang *et al.*, 2002; De Lozanne, 2003).

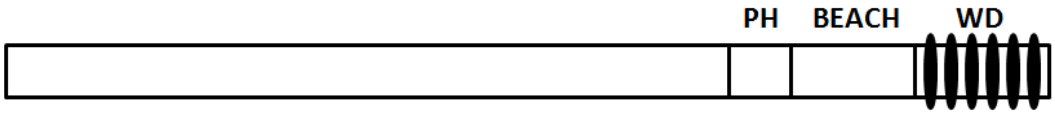

Figure 1.1: General structural organization of BEACH proteins

This diagram shows the general structural organization of BEACH proteins. At the C-terminus they contain multiple WD motifs (the number of the WD motifs varies between four-six). The most highly conserved domain among BEACH proteins is the BEACH domain, found adjacent to the WD motifs. Next to the BEACH domain is a novel PH-like domain. The N-terminus of BEACH proteins is unique to each BEACH protein (Wang *et al.*, 2002; De Lozanne, 2003).

1.2.2 Classification of BEACH proteins

The phylogenetic analysis of BEACH proteins using the conserved BEACH and WD domains, indicates that they can be clustered into five distinct classes (Figure 1.2) (De Lozanne, 2003). This analysis showed that both budding and fission yeast have only one BEACH protein each, named BPH1 (Beige Protein Homologue 1) (De Lozanne, 2003; Shiflett et al., 2004) while *Dictyostelium discoideum* contains six BEACH proteins named LvsA-LvsF. *A. thaliana* contains five uncharacterized BEACH proteins. *D. Melanogaster* contains five BEACH proteins and *H. sapiens* has six. Some of these classes have distinct roles in the cell and proteins in each class share additional regions of similarity. This suggests that each class of BEACH proteins may have a distinct cellular role. Only few of the BEACH proteins have a known function and most of them have only been identified as predicted proteins in genomic sequences. Currently the molecular mechanism of action of any of these proteins is not known.

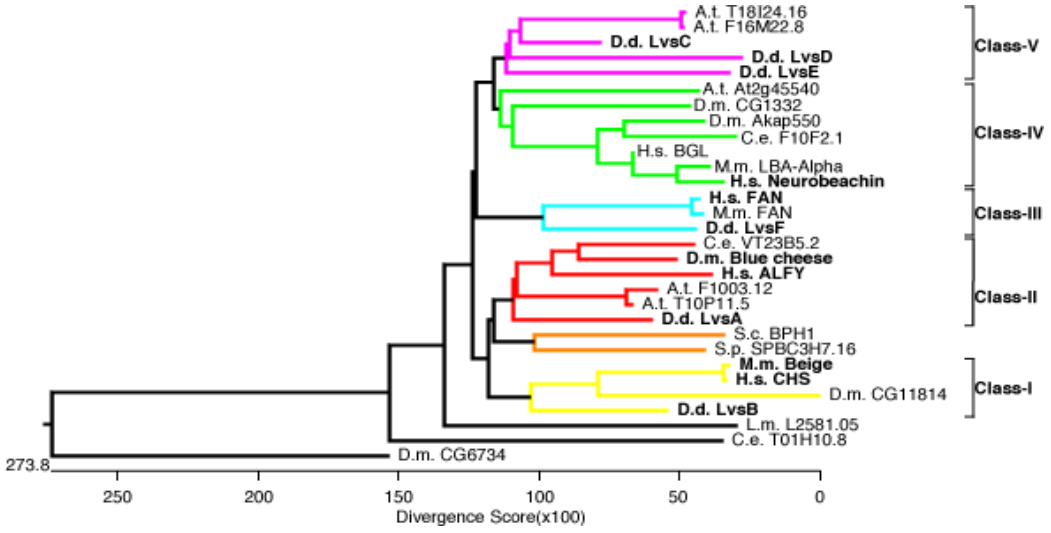

Figure 1.2: Phylogenetic tree of the BEACH family of proteins

The BEACH and WD domains are used for the alignment of the phylogenetic tree. (De Lozanne, 2003). The different classes of BEACH proteins are indicated by the brackets on the right. (Figure adapted from De Lozanne 2003).

The different classes are represented by the most well characterized proteins from each class. The most well characterized proteins in Class I is the human CHS, murine Beige and the *Dictyostelium discoideum* LvsB. These proteins function in lysosome biogenesis by controlling lysosome fusion events. A more detailed discussion of CHS and LvsB proteins is found later in this section. The single BEACH protein found in yeast Bph1 was recently characterized in *S. cerevisiae* (Shiflett *et al.*, 2004). *BPH1* gene is not essential and the encoded Bph1 protein was shown to fractionate as a soluble cytosolic protein and peripherally bound to membranes. Unfortunately, the disruption or overexpression of *BPH1* did not affect vacuole morphology, the equivalent of lysosomes in yeast. However, Bph1 is shown to be involved in protein sorting of several vacuolar hydrolases and cell wall formation (Shiflett et al., 2004).

The best characterized protein in Class II is *Dictyostelium* LvsA. Recently the role of *Drosophila* Blue cheese (Bchs) was also reported (Gerald et al., 2001; Khodosh et al., 2006). LvsA was the first BEACH protein identified in *Dictyostelium* during a screen for identification of cytokinesis mutants (Kwak et al., 1999). LvsA mutants fail at a late stage of cytokinesis specifically at the ingression of the cleavage furrow. Interestingly, LvsA has a separate function in the regulation of an osmoregulatory organelle, the contractile vacuole (Heuser et al., 1993). *Dictyostelium* cells can withstand a hyposmotic environment by disposing excess water through the contractile vacuole system. LvsA is the first BEACH protein found to localize on a membranous compartment. Due to the large size of the protein, a knock-in approach was employed that generated a GFP fusion protein. LvsA associates with the membrane of the contractile vacuole when the vacuole is at its maximum diameter and stays on until the vacuole has contracted completely (Gerald et al., 2002) . The mechanism of regulation of the expulsion of the contractile vacuole by LvsA is still not well understood.

A different study on LvsA revealed the requirement of different portions of this BEACH protein for its function (Wu et al., 2004). A series of N-terminal and C-terminal deletion mutants revealed that deletion of only the small WD at the extreme C-terminus, results in complete loss of LvsA function. Deletion of only 688 amino acids from the N-terminus yields a protein that is partially functional. Further deletions of the N-terminus, result in completely inactive protein (Wu et al., 2004). Further studies are required to determine the exact portions required for the localization and function of LvsA and other BEACH proteins.

The mammalian protein ALFY and the *Drosophila* protein Blue Cheese (Bchs) are two recently described proteins that belong to Class II. These two proteins are characterized by an additional FYVE domain at their C-termini. Defects associated with

mammalian ALFY have not been described but Bchs mutants exhibit progressing neural degeneration (Simonsen *et al.*, 2004; Khodosh *et al.*, 2006; Simonsen *et al.*, 2007). Bchs mutants exhibit premature adult death and contain protein inclusions throughout the CNS. These inclusions contain insoluble ubiquinated protein aggregates. Additionally Bchs mutants show extensive neuronal apoptosis. This study suggests that Bchs may be involved in trafficking of proteins but the exact molecular mechanism in unclear (Finley et al., 2003).

In a later study, Bchs was found highly expressed in the nervous system, where it is associated with vesicles and concentrated in synaptic regions(Khodosh *et al.*, 2006). More importantly Bchs was shown to antagonize the GTPase Rab11 in synapse morphogenesis and multiple other developmental events. In fact, Bchs colocalizes with Rab11 at the neuromuscular junction. At the neuromuscular junction, Rab11 is known to be important for synaptic growth and morphogenesis, as reductions in Rab11 function increases bouton density and branching. The *bchs*, *rab11* double mutant partially suppresses the defects in synapse morphogenesis. In fact, this genetic interaction is evident also in multiple developmental contexts. Reduction or loss of *bchs*, restores viability and normal bristle development in animals with reduced *rab11* function. Also, reductions in *rab11* exacerbate the defects in eye development caused by the overexpression of *bchs* (Khodosh et al., 2006). Rab11 is an important regulator of membrane traffic and the genetic interaction between these two genes suggest that Bchs protein functions similarly. It can be speculated that Bchs negatively regulates Rab11 activity. This study provides the first evidence of a genetic interaction that may help discover the mechanism of action of Bchs as well as other BEACH proteins.

BEACH proteins of Class III are represented by the mammalian FAN (factor associated with n-Smase), and its unique ortholog in *Dictyostelium*, LvsF. Interestingly

there seems to be no orthologs in *Drosophila* or *C. elegans*, although it is present in the genome databases from bees, beetles and paramecium. FAN and the other members of the Class III BEACH proteins are the smallest of all BEACH proteins (~ 100 kDa) and consist mainly of the PH, BEACH and WD domains. FAN was initially discovered as a binding partner of tumor necrosis factor receptor (TNF-R55). This receptor mediates a variety of cellular effects ranging from proliferation, to apoptosis, as a result of engaging to different signaling cascades. Upon ligand stimulation, the WD domain of FAN interacts with the cytoplasmic tail of the receptor and subsequently activates the Neutral Spingomyelinase (n-Smase) at the plasma membrane. This activation of n-Smase results in hydrolysis of sphingomyelin and production of ceramide which initiates various signaling cascades (Adam-Klages et al., 1996). Biochemical analysis of FAN also revealed that the PH and BEACH domains of this protein show extensive interactions and that both domains are important for the signaling ability of FAN (Jogl et al., 2002).

In a separate study, FAN was shown to be crucial for the formation of filopodia and actin cytoskeleton reorganization induced by tumor necrosis factor (TNF). FAN was shown to localize on the plasma membrane, where it colocalizes with TNF-R55 and is important for activation of the Rho GTPase Cdc42. Interestingly, the PH domain of FAN is required to target the protein to the membrane where it binds specifically to phosphatidylinositol-4,5-bisphosphate (Haubert et al., 2007). Perhaps FAN regulates signaling pathways by modulating membrane lipid composition but the exact mechanism remains to be investigated. This last study provides new information about the ability of PH domains in BEACH proteins to bind lipids and contradicts previous observations that suggested that the PH domain of both Neurobeachin and FAN do not bind to phospholipids (Jogl *et al.*, 2002; Gebauer *et al.*, 2004).

Class IV includes the mammalian Neurobeachin, the *Drosophila* AKAP550 and their related proteins. Neurobeachin is most prominently expressed in neural tissues. EM studies localized neurobeachin to the cytoplasmic surfaces of membranous compartments near the *trans* Golgi stacks (Wang *et al.*, 2000c). This localization suggests that neurobeachin may modulate post-Golgi membrane targeting and vesicle trafficking. In addition to the conserved continuous PH-BEACH-WD containing C-terminus, neurobeachin contains a centrally located binding site for the regulatory subunit of protein kinase A (PKA). Neurobeachin may function as an adaptor protein for PKA. Different lines of evidence implicate PKA and their adaptor proteins in synaptic function. The presence of the PKA anchoring domain in neurobeachin, may play a role in targeting PKA to specific membranous compartments in neurons and the synapses (Colledge and Scott, 1999). In fact, neurobeachin (*nbea*) null mice have severe defects in neuromuscular synaptic transmission but the precise mechanism by which neurobeachin controls neurotransmitter release is unclear (Su et al., 2004). *Drosophila* AKAP550 also binds the regulatory subunit of PKA but the significance of this interaction needs further investigation (Han et al., 1997).

A recent study characterized the neurobeachin homolog SEL-2 in *C. elegans* (de Souza *et al.*, 2007). *Sel-2* mutants exhibit compromised traffic in polarized epithelial cells. These mutants show aberrant basolateral protein accumulation that may result from problems in endocytic sorting (de Souza et al., 2007). The precise step regulated by Sel-2 is currently not understood.

Class V encompasses proteins that are only found in plants and *Dictyostelium* proteins (LvsC-LvsE). Up to date, no function of any of these proteins is discovered.

The field of BEACH proteins is a new and exciting field that contains a multitude of questions to be addressed. Overall, it seems that BEACH proteins associate

with different types of membranous compartments. But is there a common mechanism of action to all these proteins? Do they share common effectors? What is the significance of the conserved BEACH domain they share? And why are their N-termini is so unique to each protein? The establishment of good model systems is imperative to the understanding of the molecular function of BEACH proteins but more importantly in the understanding of the Chediak-Higashi syndrome.

1.2.3 Lysosomal Trafficking Regulator (LYST)

The founding member of the BEACH family of proteins and the best characterized protein that represents class I is the protein lysosomal trafficking regulator (Lyst), encoded by the *CHS/beige* genes. Lyst consists of a similar C-terminal structural organization to other BEACH proteins. The amino terminus contains a large stretch of alpha helices termed ARM/HEAT repeats. ARM motifs are thought to mediate membrane association and HEAT repeats, such as the ones found in Huntington's disease protein, are thought to mediate vesicle transport (Peifer *et al.*, 1994; Andrade and Bork, 1995). The precise role of these motifs in Lyst is currently unknown. Lyst is a very large protein of approximately 430 kDa that is expressed at very low levels in cells. The large size of Lyst makes the biochemical characterization very difficult (Perou et al., 1996). Murine Lyst is expressed in most mouse tissues, with the highest level of expression in brain, spleen and lung (Perou et al., 1997). Immunofluorescence microscopy studies failed to reveal the localization of murine Lyst. However fractionation experiments also indicated that this protein associates with different types of membranous compartments (Perou et al., 1997). As indicated above, impaired function of Lyst leads to enlargement of lysosome-related organelles. Two predominant models have been offered to explain the enlarged lysosomal phenotype presented by the

CHS/Beige mutant cells. The first model suggests that Lyst may regulate lysosomal fission (Perou et al., 1997) This model is suggested by the observation that when Lyst is overexpressed using a YAC containing the *beige* gene, lysosomes become much smaller compared to control cell lines. A second model suggests that Lyst regulates lysosome fusion. The second model is supported by evidence utilizing secretory lysosomes from cytotoxic T lymphocytes. This study showed that in CHS secretory lysosomes, while early maturation events such as the targeting of lysosomal and lytic enzymes, proceeds normally, late maturation events are impaired. After early maturation, CHS secretory lysosomes aggregate, fuse together, grow in size and decrease in numbers (Stinchcombe et al., 2000). Further studies are required to distinguish which model represents the Lyst function and to explore the molecular mechanism of how Lyst may regulate fission or fusion. To understand the molecular mechanism of Lyst function it will be crucial to identify binding partners. In a recent yeast two hybrid screen a multitude of binding partners have been identified that includes many proteins of unknown function and some proteins involved in membrane fusion and vesicular transport. Unfortunately none of these interactions is further characterized (Tchernev et al., 2002).

The overexpression of different domains of Lyst cause a dominant negative effect on lysosomal size, suggesting that Lyst interacts with at least two binding partners (Ward et al., 2003). The smallest regions required to produce the dominant negative phenotype is a construct that contains 674 amino acids close to the N-terminus and a construct that includes part of the BEACH domain and the WD motifs (Ward et al., 2003).

The role of Lyst is mostly studied in relationship to lysosome biogenesis. However, the phenotypes and biochemical alterations exhibited by CHS/Beige mutant

cells suggest that Lyst may be involved in multiple cellular processes. Further studies are required to determine the precise cellular functions of Lyst and its potential interactors.

1.2.4 LvsB in *Dictyostelium discoideum*

An exciting development in dissecting the function of Lyst and the understanding of the Chediak-Higashi syndrome is the identification of the Lyst ortholog, LvsB, in *Dictyostelium discoideum* (Wang et al., 2002). Interestingly, disruption of *lvsB* gene produces a phenotype similar to that presented by CHS/Beige mutant cells (Harris *et al.*, 2002b). LvsB mutant cells contain enlarged endosomal compartments that are acidic and show secretory defects (Cornillon *et al.*, 2002; Harris *et al.*, 2002b). In fact, the enlarged acidic compartments appear to form as a result of an increase in the rate of vesicle fusion (Harris *et al.*, 2002b) . Therefore, LvsB is suggested to negatively regulate vesicle fusion, a model that seems to agree with one of the proposed models for Lyst function. The defects in the morphology of the endolysosomal compartments do not affect the rates of fluid phase endocytosis, exocytosis and particle phagocytosis. Also, LvsB mutant cells display normal growth and development (Harris *et al.*, 2002b). A recent study proposed that LvsB regulates the biogenesis of secretory lysosomes. In fact, they observed that the number of secretory lysosomes (post-lysosomes) is decreased and the maturation of lysosomes to post-lysosomes is defective (Charette and Cosson, 2007). However, this last model cannot account for the normal rates of fluid phase exocytosis and the enhanced secretion of lysosomal enzymes presented by the LvsB mutant cells in earlier studies. Further studies are required to

determine the precise function of LvsB and to account for the differences between different studies. This is the subject of this thesis.

1.3 REGULATION OF THE ENDOCYTIC PATHWAY BY RAB PROTEINS

Localization studies and the study of loss of function phenotypes suggest that BEACH proteins are found on specific membranous compartments and are regulators of membrane trafficking. Different studies suggest that mammalian Lyst and the *Dictyostelium* LvsB negatively regulate fusion. Alternatively, Lyst may regulate vesicle fission. Master regulators of membrane trafficking including fusion and fission events are the Rab proteins. It is possible that functional interactions exist between Rab and BEACH proteins.

1.3.1 Role of Rab proteins and their effectors in membrane traffic

It is crucial that each intracellular organelle maintains its characteristic structure, biochemical composition and function, which represents a big challenge for the organelles of endocytic and exocytic pathways, given the continuous flow of protein and membrane along these pathways. The exocytic pathway sorts newly synthesized proteins from the endoplasmic reticulum, through the Golgi apparatus to their final destination at the lysosome or the plasma membrane. Conversely, the endocytic pathway is required for the uptake of nutrients and the internalization of receptors. Master regulators of organelle identity are the Rab proteins that regulate four major steps in membrane traffic: vesicle formation, vesicle delivery, vesicle tethering and fusion of the vesicle membrane with that of the target compartment. These different tasks are carried out by a diverse collection of Rab effector molecules.

Rab proteins are small (20-29kDa), ubiquitously expressed and constitute the largest branch of the Ras GTPase superfamily (Chavrier and Goud, 1999). To date, more than 60 Rabs have been identified in mammalian cells, a number that reflects the complexity of transport events in higher eukaryotes (Pfeffer, 2001) . Like other GTPases, the Rab proteins cycle between an active (GTP-bound) and in inactive (GDP-bound). In addition, Rab GTPases are post-translationally modified with the addition of a carboxy-terminal geranyl-geranyl groups that allows the tight association with membranes (Kinsella and Maltese, 1992). Inactive, prenylated Rab GTPases are bound to GDP dissociation inhibitors (GDI), which mask the isoprenyl anchor and keeps the Rab in a soluble cytosolic form (Garrett *et al.*, 1994; Shapiro and Pfeffer, 1995; Shisheva *et al.*, 1999). Membrane attachment of Rabs requires the function of GDI displacement factor (GDF) that dissociates the GDI and allows the prenyl anchor to be inserted into the membrane (Pfeffer and Aivazian, 2004). Subsequently, specific guanine exchange factors (GEF) stimulate the exchange of GDP with GTP, thereby activating the Rabs. The active, membrane bound Rabs are then able to fulfill various functions in membrane traffic by binding to specific effector proteins. Lastly, specific GDP activating proteins (GAPs) inactivate the Rabs by accelerating the hydrolysis of the bound GTP to GDP. The inactive, GDP-bound Rabs can then be extracted from the membrane by GDI and recycled for another round of function (Pfeffer, 2001; Segev, 2001).

Rab effectors are proteins that respond to a specific Rab GTPases and mediate variety of downstream effects. Specific Rab signal transmission requires specificity of effector recognition. This specificity if achieved by specific changes in the Rab structure upon GTP binding (Ostermeier and Brunger, 1999; Fukuda, 2003; Zhu *et al.*, 2004; Eathiraj *et al.*, 2005). Rabs and their effector are implicated into four major steps of

membrane traffic. These steps include: vesicle formation, vesicle delivery utilizing motor proteins, vesicle tethering and membrane fusion. Due to the large list of known Rabs and their effectors, only selected examples will be discussed.

1.3.2 Regulation of vesicle formation

In forming a transport vesicle the correct cargo and the appropriate fusion machinery must be incorporated before scission from the donor membrane. Different lines of evidence implicate Rabs in cargo selection and vesicle formation/fission. Initial evidence for Rab involvement in vesicle formation and budding is suggested by the absence of accumulated vesicles when membrane transport is inhibited by expression of dominant negative Rab proteins (Nuoffer *et al.*, 1994; Riederer *et al.*, 1994). Subsequently, a different study suggests that active Rab5 may facilitate cargo selection in clathrin-coated-pits (McLauchlan *et al.*, 1998). Rab9 and its effector tail-interacting protein (TIP47) are clearly implicated in the process of vesicle formation (Diaz and Pfeffer, 1998). Mannose-6-phosphate receptors (MPRs) transport newly synthesized lysosomal hydrolases from the trans-Golgi network to late endosomes. MPRs must be transported back to the Golgi for additional rounds of transport a process that requires TIP47 that binds to the cytoplasmic tail of MPR. GTP-Rab9 localizes on late endosomes and stimulates the capture of MPRs by increasing the affinity of binding with TIP47 (Carroll *et al.*, 2001). Additional studies are needed to determine whether this type of mechanism is used at different stages of membrane traffic.

1.3.3 Regulation of cytoskeletal transport

Membrane vesicles are dynamically transported within the cell towards their target membrane by using either actin-dependent motors (myosins) or microtubule-dependent motors (kinesin or dyneins) to allow for the delivery and recycling of protein and lipids. A number of studies have implicated Rabs and their effectors in this transport step. Various Rab proteins recruit either directly or indirectly specific microtubule or actin-based motor proteins to their target membranes.

The first evidence that Rab proteins are directly coupled to motor proteins is the identification of the effector of the Golgi-associated Rab6. This Rab6 effector, Rabkinesin-6 (RB6K) is a new kinesin-like protein (Echard et al., 1998). RB6K specifically interacts with GTP-Rab6, and overexpression results in dispersion of the Golgi towards the plus end of microtubules. In fact, two related dynactin-binding proteins BicD2 and BicD1 also bind to active Rab6, thereby linking Rab6 with the dynein-dynactin complex (Matanis et al., 2002). In addition, a link between Rab6 and the dynactin subunit p150 ^{Glued} was observed *in vitro* (Short et al., 2002). Thus, active Rab6 can interact with both kinesin and the dynein/dynactin compex and regulates both plus-end and minus-end directed transport of Golgi compartment. How the relative activity of both motors is controlled is yet unclear.

Furthermore, Rab4 and Rab5 regulate exocytosis and endocytosis via co-ordinated interactions with kinesin and dynein motors. The transport of the insulin-responsive glucose transporter GLUT-4 in adipocytes is a clear example that demonstrates the interplay between these Rab proteins and motors. Upon insulin stimulation GLUT-4 proteins are translocated from the perinuclear area to the plasma membrane due to enhanced exocytosis and reduced endocytosis (Czech and Corvera, 1999; Pessin et al., 1999). Rab4 is identified as a major regulator of GLUT-4 exocytosis.

Insulin enhances the association of active Rab4 with a member of the kinesin-2 family (KIF3B) that results in enhanced plus-end directed transport and exocytosis (Imamura et al., 2003). In contrast, reduction in GLUT-4 endocytosis is controlled by Rab5. Upon insulin stimulation, levels of active Rab5 decrease which results in reduction with dynein association and inhibition of minus-end transport (Huang *et al.*, 2001).

Rab7 GTPase is present on late endosomes and lysosomes that move on microtubules in a bidirectional manner due to the alternating activities of dynein and kinesin motors. Rab7 requires the effector protein Rab7-interacting lysosomal protein (RILP) in order to recruit the dynein/dynactin to endosomes and lysosomes resulting in the accumulation of these structures in the perinuclear area (Cantalupo *et al.*, 2001; Jordens *et al.*, 2001). Overexpression of the C-terminal Rab7 binding portion of RILP prevents the recruitment of the dynein/dynactin complex and results in the relocation of late endosomal compartments towards the cell periphery by kinesin (Cantalupo *et al.*, 2001; Jordens *et al.*, 2001).

There are also many examples of Rab proteins interacting with actin-based myosin motors. Perhaps the best-studied example is the recruitment of myosin-Va to melanosomes by Rab27a. Rab27a localizes to the pigment containing melanosome granules and is essential for the retention at the periphery of the melanocytes (Bahadoran *et al.*, 2001). The Rab27 effector melanophilin links Rab27a positive melanosomes to the actin motor myosinVa (Fukuda *et al.*, 2002; Nagashima *et al.*, 2002; Strom *et al.*, 2002). Without myosinVa-dependent capture of these organelles on the actin filaments, melanosomes are not retained in the cell periphery and therefore cannot be transferred to neighboring keratinocytes (Wu *et al.*, 1998). As a result, mouse mutants of Rab27, melanophilin or myosinVa display a pigmentary dilution of their

skin. In fact, genetic alterations of human Rab27a lead to a pigmentation disorder named Griscelli syndrome (Moore *et al.*, 1988; Matesic *et al.*, 2001)

Rab8 and Rab11 are also shown to couple to myosins. Rab11 is associated with the recycling compartment and regulates the recycling of the transferrin receptor and the recycling of several G-protein coupled receptors (Chen *et al.*, 1998; Wang *et al.*, 2000d; Volpicelli *et al.*, 2002). A direct interaction is found between Rab11 and myosinVb in a yeast-two hybrid screen (Lapierre *et al.*, 2001). In addition, the Rab11 effector family-interacting protein-2 (FIP2) also interacts with myosin VI (Hales *et al.*, 2002). Rab8 regulates the biosynthetic pathway from the Golgi towards the plasma membrane (Huber *et al.*, 1993; Ang *et al.*, 2003). Recently, myosin VI is shown to interact with optineurin, an interaction partner of Rab8 (Hattula and Peranen, 2000; Sahlender *et al.*, 2005).

1.3.4 Regulation of vesicle tethering

Another crucial step in the targeting of vesicle to the correct destination is the tethering of the vesicle to the target membrane. This tethering process restrains the vesicle at or near their cognate target membranes allowing for SNARE pairing. Rab proteins in their GTP-bound form appear to facilitate the recruitment of tethering factors to their specific locations. In fact, Rab proteins bind various tethering factors. Tethering factors can be divided into two groups: long coiled-coil proteins and large subunit complexes (Sztul and Lupashin, 2006). Examples in the former group are proteins such as the p115 (in yeast Uso1p) and the early endosome antigen 1 (EEA1). Examples in the latter group include the yeast exocyst and the TRAPP complexes.

Perhaps the best characterized tethering factor is p115 (yeast Uso1p), a peripheral Golgi membrane protein. The interaction of p115 with a Golgi residing complex (GM130/GRASP65) is thought to tether endoplasmic reticulum-derived

vesicles to the Golgi (Sztul and Lupashin, 2006). Interestingly, both tethers p115 and GM130/GRASP65 have been shown to be effectors of Rab1 (Allan *et al.*, 2000; Moyer *et al.*, 2001). In fact, active Rab1 is required for the recruitment of p115 into transport vesicles. Rab1 may also regulate the assembly and/or activity of the GM130/GRASP65 complex on the Golgi (Allan *et al.*, 2000; Moyer *et al.*, 2001).

In addition, several groups have shown that the early-endosome-antigen 1 (EEA1) is important for the early endosome tethering and fusion. Active Rab5 transiently binds to the hVPS34 lipid kinase and generates a local microdomain that recruits EEA1 (Patki *et al.*, 1997; Christoforidis *et al.*, 1999). Following membrane recruitment, EEA1 becomes part of a high molecular mass oligomer that includes Rabaptin, Rabex and NEM-sensitive factor (NSF) (McBride *et al.*, 1999). EEA1 both tethers the vesicle and mediates the incorporation of a t-SNARE essential for the fusion event (McBride *et al.*, 1999).

The exocyst is the first large multisubunit tethering complex to be identified as a Rab effector (Guo *et al.*, 1999). The exocyst complex is an octameric complex, localizes to sites of polarized growth in yeast and is required to tether post-Golgi and recycling vesicles to the plasma membrane (Guo *et al.*, 1999). The exocyst component Sec15 is found to associate specifically with secretory vesicles and to interact with active RabSec4, which is present on the vesicular membrane (Guo *et al.*, 1999). This Sec4-GTP-Sec15 interaction seems to trigger further interactions between Sec15 and other exocytic components, eventually leading to fusion with specific domains on the plasma membrane.

One interesting idea about the role of Rabs in tethering is that they may link the functions of coiled-coil tethers with those of the multisubunit tethering complexes. An example is indicated by the large macromolecular complex , named TRAPP (for

transport protein particle) that is also required for ER-Golgi transport in yeast (Sacher *et al.*, 1998). TRAPP complex resides on the Golgi and has been shown to activate the Rab GTPase Ypt1p (mammalian Rab1) which is present on ER-Golgi vesicles (Wang *et al.*, 2000a). Ypt1p would then be able to recruit its effector Uso1p (mammalian p115) although the direct sequence of events is yet not clear (Cao *et al.*, 1998).

1.3.5 Regulation of vesicle fusion

The last step in vesicle-mediated transport is the fusion of the vesicle with its target membrane. Fusion is thought to occur by the pairing of SNARES (soluble NSF attachment protein receptor where NSF stands for N-ethyl-maleimide-sensitive fusion protein). A SNARE on a transport vesicle (v-SNARE) pairs with its cognate SNARE on the target membrane (t-SNARE) forming, a *trans*-SNARE complex that leads to the opening of a fusion pore and final fusion of vesicle and organelle membrane (Chen and Scheller, 2001). The ATPase NSF (N-ethylmaleimide-sensitive factor) can disassemble SNARE complexes in the presence of a soluble co-factor SNAP (soluble NSF attachment protein). Such unpairing would make free t-SNARES available, which would then engage in subsequent rounds of membrane fusion. Rabs are thought to influence vesicle fusion through their indirect effects on SNARES, although the precise mechanisms are currently not well understood.

Evidence suggests that Rabs indirectly regulate SNARES, through the activity of their effector molecules. This is supported by evidence that show that tethering factors interact with SNARES. The complex formed between the Rab5 effector, EEA1 and two t-SNARES, syntaxin 13 and syntaxin 6, is required for homotypic early endosome fusion. Interestingly, the formation of an oligomeric complex of EEA1 with Rabaptin and Rabex requires the activity of NSF (McBride *et al.*, 1999). Additionally, Ypt7p

(yeast Rab7) effector proteins, VPS/HOPS, have also been found in complexes with the SNARE Vamp3p complex that is required for vacuole fusion (Price *et al.*, 2000; Sato *et al.*, 2000; Seals *et al.*, 2000) . The interaction of the Ypt7p effector VPS/HOPS and SNARES requires the presence of NSF (Sec18p in yeast) (Price *et al.*, 2000). The significance of the incorporation of NSF with the tethering factors is currently unclear. It is possible that NSF action couples SNARE disassembly with tethering and *trans*-SNARE pairing. Additionally, through directly binding to syntaxin-5 complexes, the Rab1 effector p115 might stimulate their incorporation into vesicles (Allan *et al.*, 2000).

1.4 ENDOCYTIC PATHWAY IN *DICTYOSTELIUM DISCOIDEUM*

1.4.1 *Dictyostelium* as a model system

Dictyostelium discoideum is the only unicellular model system that contains the full complement of BEACH proteins found in metazoans (LvsA-LvsF) (Wang *et al.*, 2002; De Lozanne, 2003). With the extensive array of tools available to dissect the function these novel proteins, *Dictyostelium* is a powerful model system for the study of the function of LvsB in membrane trafficking and the understanding of the Chediak-Higashi syndrome.

Dictyostelium discoideum is a social amoeba that lives in the soil and feeds on bacteria. Since the discovery of *Dictyostelium discoideum* in 1935, its fascinating biology has made it a very popular model system for studying the molecular basis of cell and developmental biology. *Dictyostelium* cells grow by mitotic division every 8-10 hours. Single cells in vegetative stage, feed by phagocytosis on bacteria or by macropinocytosis on simple axenic liquid medium, making it possible to grow many cells for analysis. The term ‘social amoeba’ derives from the behavior of cells when

their food supply is exhausted or removed. Upon onset of starvation, undifferentiated single cells immediately stop division and enter what is now a well characterized developmental program of gene expression and morphogenesis. The steps of the developmental cycle are well defined and the whole process, that lasts approximately 24 hours, can be easily reproduced and visualized in the laboratory. When *Dictyostelium* cells starve, cAMP is secreted to signal neighboring cells to start chemotaxis and about 10^5 cells polarize and migrate towards an aggregation center to form a slug (Parent and Devreotes, 1999; Firtel and Chung, 2000). The cells that make up the slug differentiate into stalk or spore cells. Subsequently, a fruiting body forms which is composed of a stalk and a sorus. When nutrients become available, dormancy ends and the spores germinate into amoeboid cells (Kessin, 2001). The life cycle of *Dictyostelium* is ideal to study fundamental developmental processes such as cell polarity, chemotaxis, cell migration and differentiation.

Dictyostelium discoideum has six chromosomes and its entire 35Mb genome is sequenced (Eichinger *et al.*, 2005). The cells are haploid and the mutant phenotypes are therefore easily observable. Many molecular tools are available including the ability to make rapid homologous gene replacements, random insertional mutagenesis (REMI), multiple gene deletions and RNA interference (RNAi) (De Lozanne and Spudich, 1987; Kuspa and Loomis, 1992; Faix *et al.*, 2004; Popova *et al.*, 2006).

Dictyostelium is also suitable for morphological studies. The 10 micron diameter and morphology, allows for the visualization of dynamic processes such as cell division, chemotaxis, macropinocytosis and organelle morphology. *Dictyostelium* provides unique advantages for the investigation of endocytosis. Vegetative cells exhibit phagocytosis and pinocytosis at rates higher than professional phagocytes (Thilo, 1985). In addition, with an array of fluorescent tags and markers available, many features of the

endocytic pathway can be studied in real time. In fact many aspects of mammalian endocytosis are highly conserved in *Dictyostelium* (Maniak, 2002).

1.4.2 Endosomal uptake and maturation

Endocytosis is a highly conserved cellular process that involves the uptake of particles (phagocytosis), macromolecules, and solutes (pinocytosis) from the surrounding environment via plasma membrane-derived invaginations and the subsequent digestion of ingested material. The endocytic pathway in *Dictyostelium* serves primarily a nutrititive function. Nutrients within the endocytosed fluid are digested by lysosomal enzymes in a very dynamic process that involves a complex series of fusion and fission events. Finally undigested remnants are released by exocytosis.

The main route for fluid phase uptake in *Dictyostelium* is macropinocytosis that leads to a bulk fluid-phase uptake through actin driven membrane ruffles protruded in the surrounding medium. These surface protrusions called crowns are filled with F-actin and actin-binding proteins. The membrane protrusions finally constrict to engulf the newly formed endosome in a process that depends on the activity of motor proteins. In fact, a variety of unconventional myosin molecules localize to the surface protrusions (Fukui *et al.*, 1989; Schwarz *et al.*, 2000). The newly formed endosome, remains stable for 1 minute and thereafter, loses its protective actin shell and becomes available for fusion processes (Maniak, 1999, , 2001).

Within minutes of endosome internalization early recycling events take place that facilitate recycling of plasma membrane components by budding of small tubular vesicular-transport intermediates (Neuhaus *et al.*, 2002). This pathway depends on myosin IB and has been identified following the path of lipid probes and biotinylated

surface proteins (Aguado-Velasco and Bretscher, 1999; Neuhaus and Soldati, 2000). Interestingly, following the plasma membrane marker p25 revealed the identification of a juxtanuclear recycling compartment. P25 was shown to be excluded from early endosomes shortly upon endocytosis and to traffic to the recycling compartment in a process that requires the adaptor protein AP-3. Whether all plasma membrane markers traffic through the recycling compartment and finally back to the plasma membrane is currently not clear (Charette *et al.*, 2006).

In addition to early recycling, the actin uncoating event occurs concomitantly with acidification of the vesicle lumen and the maturation into the lysosomal stage (Maniak, 2001). The enzyme responsible for the acidification is the vacuolar-ATPase, while the maintenance of the low pH depends on the transmembrane transporters of the ABC type (Brazill *et al.*, 2001; Clarke *et al.*, 2002a). Concomitantly with the vacuolar-ATPase, the small GTPase Rab7 also associates with the endosomal membrane (Rupper *et al.*, 2001c). Early lysosomes are competent to undergo homotypic fusion. Fusion is possible among endosomes which are formed sequentially and within a few minutes of each other (Clarke *et al.*, 2002b). The small GTPase Rab14 is known to associate with lysosomes at this stage and regulates homotypic fusion (Bush *et al.*, 1994; Bush *et al.*, 1996). In fact, expression of a constitutively active Rab14 mutant protein results in an enhanced rate of homotypic fusion (Harris and Cardelli, 2002). *In vitro* assays for homotypic membrane fusion at this stage of endocytic transit revealed the requirement for homologs of syntaxin7, SNAP, NSF, and Rab7 (Laurent *et al.*, 1998; Weidenhaupt *et al.*, 1998; Bogdanovic *et al.*, 2000; Weidenhaupt *et al.*, 2000).

Within the acidic phase that lasts approximately 30 minutes, lysosomal enzymes carrying different modifications appear sequentially in endosomes. Among the enzymes delivered within the first minutes is the protease cathepsin D (Journet *et al.*, 1999).

Thereafter, in another wave of vesicle fusion, Glc-Nac-1-P modified cysteine proteases fuse with the lysosomes (Souza *et al.*, 1997). In addition, another set of Man-6-P-OCH_3 modified enzymes are also delivered to the lysosomes (Souza *et al.*, 1997).

About 30 minutes after internalization, the acidic lysosomes begin a process of neutralization and maturation into a secretory lysosome (post-lysosome). The neutralization is brought about by the budding of transport intermediates that remove the vacuolar-ATPase from the membrane of lysosomes and possible recycling to newly endocytosed vesicles. *Dictyostelium* Rab7 is thought to regulate retrograde transport and may regulate the retrieval of lysosomal enzymes and the vacuolar-ATPase from post-lysosomes. In fact, expression of an inactive form of Rab7 results in oversecretion of lysosomal enzymes (Buczynski *et al.*, 1997). The sites where recycling vesicles are formed are identified by the patchy distribution of dynamin. Accordingly, disruption of dynamin affects the kinetics of endocytic transit, but whether the retention of lysosomal enzymes is affected, is not known (Wienke *et al.*, 1999). After the retrieval of the vacuolar-ATPase and maturation into post-lysosomes the vesicles may become competent to undergo fusion. In fact, artificial inhibition of the vacuolar-ATPase results in the artificial formation of large vacuoles in the cell (Temesvari *et al.*, 1996). Homotypic fusion at this stage is best studied during phagocytosis and fusion events at this stage of endosomal maturation are currently not clear.

In subsequent stages post-lysosomes acquire the cytoskeletal proteins coronin, Scar and Arp2/3, which act together to provide the post-lysosome with a coat of filamentous actin (Rauchenberger *et al.*, 1997; Insall *et al.*, 2001; Seastone *et al.*, 2001). In fact, cells deficient in Scar lack an actin coat around late endocytic compartments and are defective in neutralization. The role of actin coat is controversial but it may prevent fusion. Subsequently, the peripheral protein vacuolin associates with the post-lysosomes

(Rauchenberger *et al.*, 1997; Jenne *et al.*, 1998). Vacuolin is a protein with an HflC domain, a domain found in many proteins similar to mammalian flotillins, proteins known to bind lipid rafts (Liu *et al.*, 2005). Cells lacking vacuolin show enlarged post-lysosomes that are severely reduced in numbers compared to control cells (Jenne *et al.*, 1998). The precise role of vacuolin is currently not understood. It is possible that vacuolin controls fusion or exocytosis.

Exocytosis releases the contents of post-lysosomes into the surrounding medium, which include indigestible particulate material, as well as small amounts of lysosomal enzymes (Dimond *et al.*, 1981). At the site of exocytosis, the empty vesicle, the surrounding actin coat and a patch of vacuolin, remain on the plasma membrane (Jenne *et al.*, 1998; Lee and Knecht, 2002; Neuhaus *et al.*, 2002). The final dissociation of these proteins from the plasma membrane allows for a new round of endocytic traffic.

Chapter 2: The BEACH protein LvsB is localized on lysosomes and post-lysosomes and limits their fusion with early endosomes

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2.1 INTRODUCTION

Despite its long history, the Chediak Higashi syndrome (CHS) remains a poorly understood genetic disorder (Ward *et al.*, 2000; Shiflett *et al.*, 2002; Ward *et al.*, 2002). First described in 1943, this disorder was recognized as a unique lysosomal disease that causes the gross enlargement of lysosomes in all tissues from CHS patients. Defects in lysosomal function are at the root of the physiological problems that develop in these patients, including delayed blood clotting, albinism, immunodeficiency and neurological problems. Unfortunately, after more than 60 years of research, the molecular basis of this lethal disease is still not understood and no potential therapies are available.

A major breakthrough in the study of this disease was the identification of the gene affected in CHS patients. Cloning of the human CHS locus was greatly facilitated by the finding that *beige* mutant mice have the same genetic defect (Barbosa *et al.*, 1996; Nagle *et al.*, 1996b). The protein encoded by the CHS/beige gene has been named Lyst for lysosomal-trafficking regulator (Barbosa *et al.*, 1996).

Lyst was the founding member of the novel family of BEACH proteins. With the exception of yeast, which has only a single BEACH protein, all eukaryotes contain multiple BEACH proteins that can be grouped into different functional classes (Wang *et al.*, 2002). All BEACH proteins share the conserved BEACH domain of unknown function and several WD motifs at their C-termini. BEACH proteins tend to be large (> 400 kDa) and expressed at low levels (Perou *et al.*, 1997; De Lozanne, 2003). This has made the biochemical characterization of BEACH proteins a difficult enterprise. The genetic analysis of BEACH proteins has been more fruitful and has revealed that several

BEACH proteins have important roles in membrane trafficking pathways, although their precise role has not been defined. In mammals, the protein neurobeachin seems to be localized in vesicles close to the Golgi and is important for neuronal function (Wang *et al.*, 2000b). In *Drosophila*, AKAP550 and blue-cheese are also important for neuronal function (Han *et al.*, 1997; Finley *et al.*, 2003). Recent studies in *Drosophila* and *C. elegans* suggest that some BEACH proteins may antagonize the function of Rab11 and Lin-12 in development (Khodosh *et al.*, 2006; de Souza *et al.*, 2007).

We have shown previously that *Dictyostelium* cells contain six distinct BEACH proteins, two of which have separate functions in different membrane compartments (De Lozanne, 2003). *Dictyostelium* LvsA is a protein that associates transiently with the contractile vacuole and is essential for the osmoregulatory function of this organelle (Gerald *et al.*, 2002). LvsA is also required for the separation of daughter cells during cytokinesis (Kwak *et al.*, 1999). In contrast, the *Dictyostelium* LvsB protein, the ortholog of mammalian Lyst, is important for lysosomal function. LvsB-null cells contain enlarged lysosomal compartments similar to those found in CHS and beige mutant cells. Characterization of LvsB-null cells suggested that the enlarged lysosomes arose by rapid homotypic fusion of endosomes (Harris *et al.*, 2002a).

Attempts to localize Lyst in mammalian cells have not been successful, probably due to the small amount of this protein in the cell. We present here a knock-in approach to tag the *Dictyostelium* Lyst ortholog, LvsB with GFP and its localization on postlysosomes. Functional assessment of the endosomal pathway of LvsB-null cells shows that the loss of LvsB leads to the inappropriate heterotypic fusion of different compartments. Our data supports the model that Lyst proteins act as negative regulators of fusion and suggest that Lyst may provide specificity for endosomal fusion.

2.2 RESULTS

2.2.1 Labeling of LvsB by homologous recombination

Based on our previous experience with LvsA, we used a knock-in approach to insert the TAP and GFP tags at the amino terminus of the LvsB protein. A construct containing these two tags was fused in frame with the 5' end of the LvsB coding sequence and this construct was introduced into wild-type *Dictyostelium* cells (Figure 2.1A). After the construct inserted into the LvsB gene by homologous recombination, the fusion protein was expressed from the single copy in its normal chromosomal locus. Cells containing the correct insertion were identified by PCR analysis (data not shown) and by Western Blot analysis (Figure 2.1B).

Since this tagging method completely replaced the endogenous LvsB protein with the fusion protein, we could determine whether the fusion protein was functional. We compared the phenotype of our tagged cell lines with the parental wild-type cells and with LvsB-null cells. We labeled the entire endocytic pathway of these cells by internalization of TRITC-dextran as a fluid phase marker. As shown before, LvsB-null cells contained enlarged vesicles (Figure 2.1E) (Harris *et al.*, 2002a). The GFP-tagged LvsB cell lines contained vesicles similar in both number and size to those found in wild-type cells (Figure 2.1C-D). The tagged cell lines did not display any abnormal phenotype in growth or development. Thus, similar to our results with the LvsA protein, addition of a tag at the amino terminus of LvsB does not seem to disturb its function.

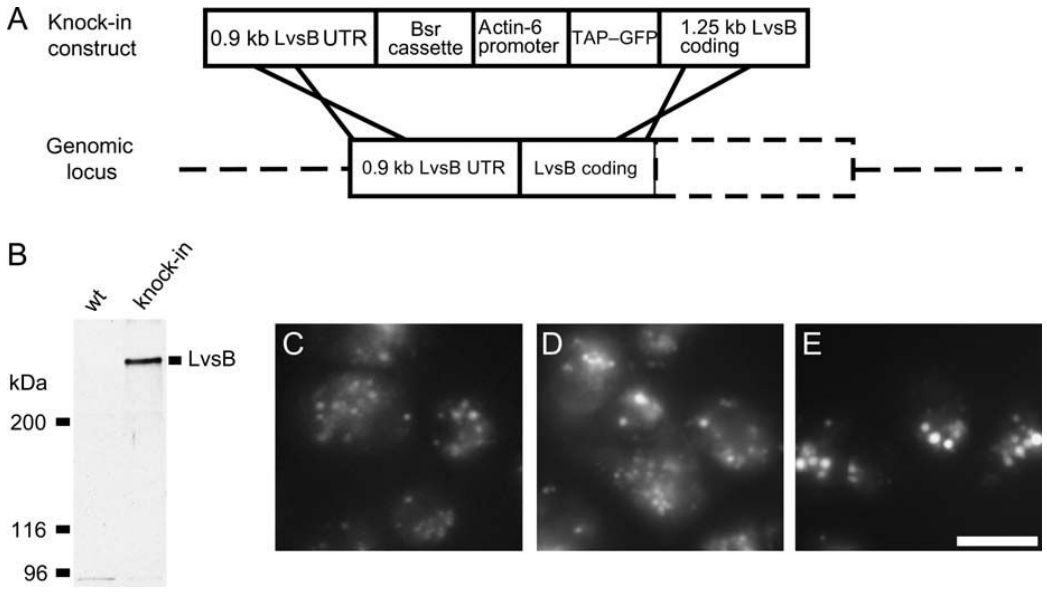

Figure 2.1: Knock-in strategy to label LvsB with GFP

A) Diagram indicating the construct used to insert the GFP coding region at the 5' end of the LvsB coding region. The construct contained 0.9 kb of LvsB 5' untranslated region (UTR), a blasticidin-resistance marker, an actin 6 promoter that drives the expression of the fusion protein and the TAP/GFP coding region fused in frame to the initial 1.25 kb of the LvsB coding region. Knock-in cell lines were screened by PCR. B) Knock-in cell lines express full length tagged LvsB protein. Western blot analysis using an anti-GFP antibody detects a single >400 kDa band in a knock-in cell line that is absent in a wild type control cell line. C-E) The inserted tag does not alter LvsB function. The endolysosomal compartments of wild type (C), knock-in (D), and LvsB-null (E) cells were labeled by internalization of TRITC-dextran for 1 hour. The knock-in cell lines contain labeled endosomes similar in size to those found in wild type cells and lack the enlarged compartments found in LvsB-null cells. Bar, 10 μm

2.2.2 LvsB is localized on lysosomes and post-lysosomes

Imaging of the GFP-tagged LvsB cells by fluorescence microscopy revealed that the fluorescence levels in these cells was so low that we were unable to image the tagged protein above the autofluorescence background in the live cells. This is consistent with the low levels of expression of LvsB and its homologs in mammalian cells (Perou *et al.*, 1997). However, fixation of the expressing cells allowed us to determine (with prolonged exposures) that the tagged-LvsB protein was associated with vesicles of various sizes (Figure 2.2). These vesicles were a part of the endocytic pathway of *Dictyostelium* since they were labeled by endocytic markers such as plastic beads (Figure 2.2 A-B).

To determine more precisely the identity of the vesicles marked by GFP-LvsB we stained these cells with markers found on different endolysosomal compartments. The endocytic pathway of *Dictyostelium* traffics endocytosed material through a system of vesicles that end in the secretion of undigested material (Maniak, 2003). Soon after internalization, early endosomes receive proton pumps that acidify their contents. Lysosomal enzymes are subsequently delivered to form early and then late lysosomes in a process that takes 30-40 min. Proton pumps and lysosomal enzymes are then retrieved from late lysosomes to mature into a neutral compartment called the postlysosome. This compartment is a secretory organelle that eventually exocytoses its contents. *Dictyostelium* Rab7 is a protein known to localize on lysosomes and postlysosomes (Rupper *et al.*, 2001b). It has been shown that expression of wild-type GFP-Rab7 does not cause an increase in the size of lysosomes and postlysosomes and acts as a reliable marker of these compartments (Buczynski *et al.*, 1997). We expressed RFP-tagged Rab7 in our GFP-tagged LvsB cells and found that all vesicles marked by GFP-LvsB were also labeled by RFP-Rab7 (Figure 2.2 C-D). Not all Rab7-labeled vesicles

contained tagged-LvsB, indicating that LvsB has a more restricted localization compared to Rab7.

In addition, we found that a large fraction (62 %, n=58) of LvsB-containing vesicles were also labeled by the protein vacuolin (Figure 2.2 E-F, arrowheads). Vacuolin is a cytosolic protein known to associate with postlysosomes, the neutralized vesicles that are destined for exocytosis (Jenne *et al.*, 1998). While all vacuolin labeled vesicles were also labeled by GFP-LvsB, many LvsB-positive vesicles were not labeled by vacuolin (Figure 2.2 E-F, arrows). This distribution suggested the possibility that LvsB associates with lysosomes that are about to become postlysosomes. To explore this possibility we stained GFP-tagged LvsB cells with an antibody against the 70kDa A-subunit of the proton pump v-ATPase, a known marker of lysosomes that is absent from postlysosomes (Jenne *et al.*, 1998). We found that 44% (n=70) of GFP-LvsB labeled vesicles were also stained by the proton pump antibodies. Thus, we conclude that LvsB is a cytosolic protein that associates with late lysosomes and postlysosomes.

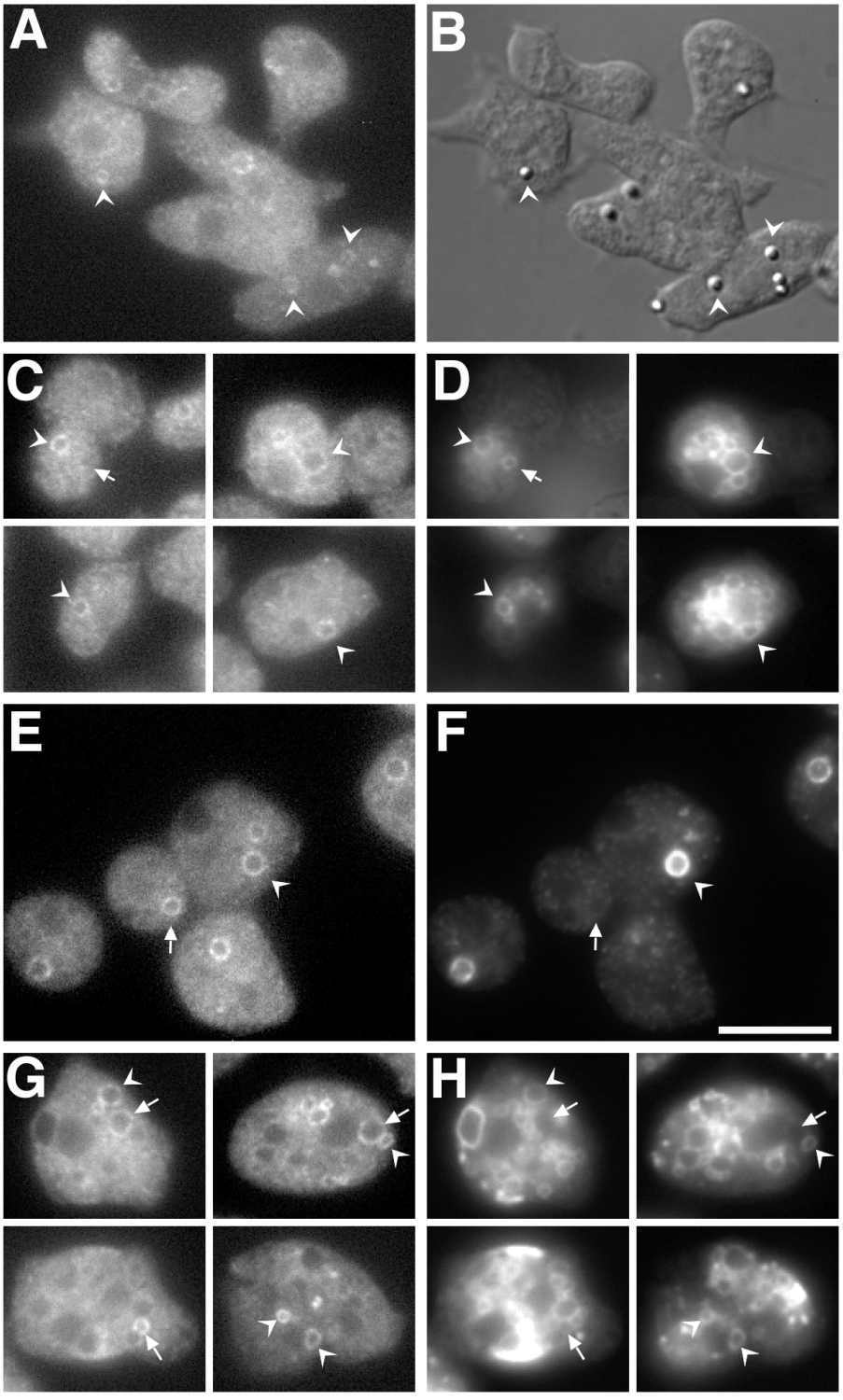
A
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Figure 2.2: GFP-LvsB is localized on vesicles including phagosomes, lysosomes and post-lysosomes

(A-B) GFP-LvsB is localized on phagosomes. Cells were allowed to internalize 0.9μm latex beads before being fixed and imaged by microscopy. GFP-LvsB was clearly visible on phagosomes containing internalized beads (arrowheads). (C-D) GFP-LvsB colocalizes with Rab7 a marker of lysosomes and postlysosomes. GFP-LvsB cells were transfected with an RFP-Rab7 expression vector and imaged by fluorescence microscopy. All GFP-LvsB labeled vesicles (C) were also labeled by RFP-Rab7 (D) (arrowheads). In contrast, many RFP-Rab7 labeled vesicles were not labeled by GFP-LvsB (arrows). This indicates that LvsB is found on a limited subset of endolysosomal vesicles. (E-F) GFP-LvsB colocalizes with vacuolin. Cells were fixed and stained with an anti-vacuolin monoclonal antibody. Vacuolin is a cytosolic protein known to label the neutral postlysosomal compartment (Jenne et al., 1998). All vacuolin-labeled vesicles (F) were also labeled by GFP-LvsB (E) (Arrowheads). While the majority (62%, n=58) of GFP-LvsB vesicles were labeled by vacuolin there were several vesicles that were not labeled by vacuolin (arrows). (E-F) GFP-LvsB colocalizes with proton pumps. Cells were fixed and stained with an antibody against the proton pump v-ATPase, a marker of lysosomal vesicles. About 44% (n=70) of GFP-LvsB labeled vesicles were also labeled by the v-ATPase (arrowheads). The rest of GFP-LvsB labeled vesicles did not contain proton pumps (arrows). These results suggested that GFP-LvsB labels late lysosomes that are about to become postlysosomes. Bar, 10 μm

2.2.3 LvsB-null cells have enlarged, abnormal post-lysosomes.

The presence of LvsB on postlysosomes suggested that LvsB may play an important role on that compartment. To explore this possibility we determined the localization of GFP-vacuolin expressed in LvsB-null cells. As shown before for wild type cells (Rauchenberger *et al.*, 1997; Jenne *et al.*, 1998), GFP-vacuolin labeled several postlysosomal vesicles (Figure 2.3) that were neutral in pH since they did not stain with lysotracker, an acid compartment stain (Figure 2.3A-C). In contrast, in LvsB-null cells, GFP-vacuolin was found on a few enlarged postlysosomal vesicles (Figure 2.3D-F). Besides their abnormal size, these mutant postlysosomes differed from wild-type postlysosomes in their acidity. Whereas postlysosomes in wild type cells are always neutral, the huge postlysosomes in LvsB cells were frequently acidic (~40%) (Figure 2.3 D-F). Interestingly, the intensity of the GFP-vacuolin staining appeared to be inversely proportional to the acidity of postlysosomes in the mutant cells.

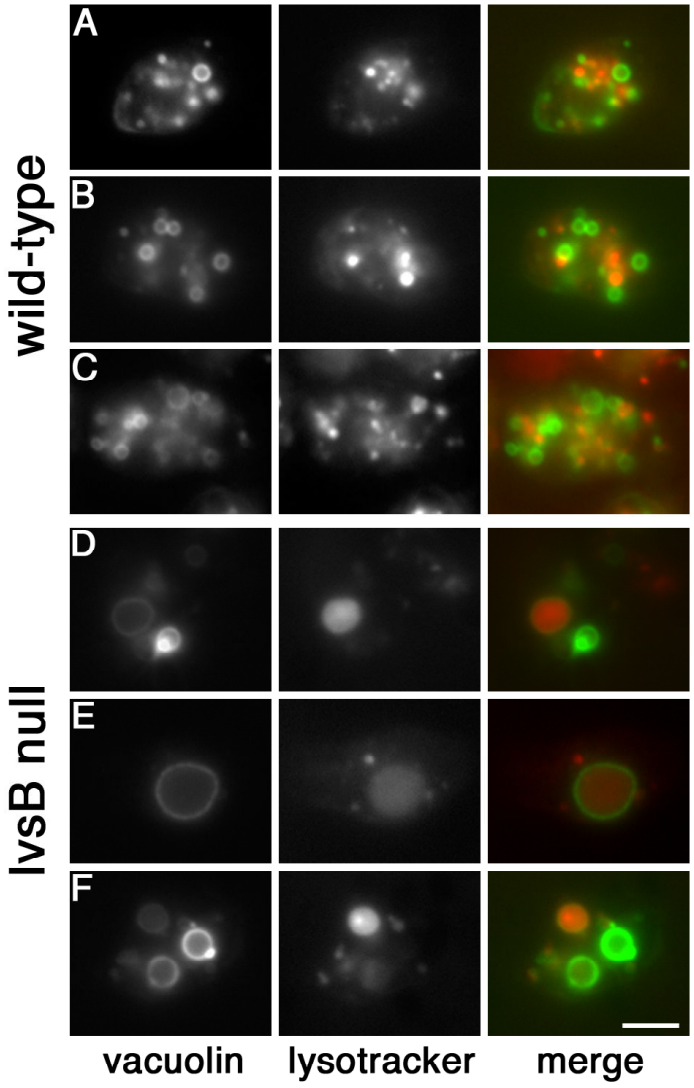


Figure 2.3: LvsB-null cells display abnormally large and acidic post-lysosomes

Wild-type and LvsB-null cells were transformed with a GFP-vacuolinB expression vector and stained with Lysotracker Red, a dye that accumulates in acidic compartments. (A-C) Wild-type cells contain normal post-lysosomes with normal pH. In wild-type cells, the cytosolic protein vacuolin is known to associate with post-lysosomal vesicles that have neutral pH and are destined for excretion (Rauchenberger *et al.*, 1997). These three wild-type cells show that vacuolin does not colocalize with lysotracker-red. (D-F)

LvsB-null cells contain enlarged and acidic postlysosomes. While LvsB-null cells do have a few small acidic vesicles, most acid compartments are grossly enlarged in these mutant cells. Importantly, some of these large acidic vesicles are labeled by GFP-vacuolin suggesting their possible postlysosomal identity. Bar, 5 μm

The abnormal acidity of postlysosomes in LvsB-null cells suggested the possibility that the vacuolar H^+-ATPase (v-ATPase) was missorted to this compartment. In wild-type cells, the v-ATPase is incorporated into endosomes within a minute after internalization (Maniak, 1999, , 2001, , 2003); subsequently the v-ATPase is removed from late lysosomes to allow their neutralization and maturation into postlysosomes (Nolta *et al.*, 1994; Maniak, 2003). Accordingly, when we stained wild type cells expressing vatM-GFP with an antibody against vacuolin we observed almost no co-localization of these two markers (Figure 2.4A-C). The fraction of wild type cells that contained at least one vesicle labeled by the two markers was 19% (n= 68). In contrast, a large fraction of LvsB-null cells (80%, n= 49) contained vesicles labeled with both markers (Figure 2.4D-F). Thus, the loss of LvsB results in both an enlargement of acid lysosomes (Harris *et al.*, 2002a) and the formation of abnormally large and acidic postlysosomes.

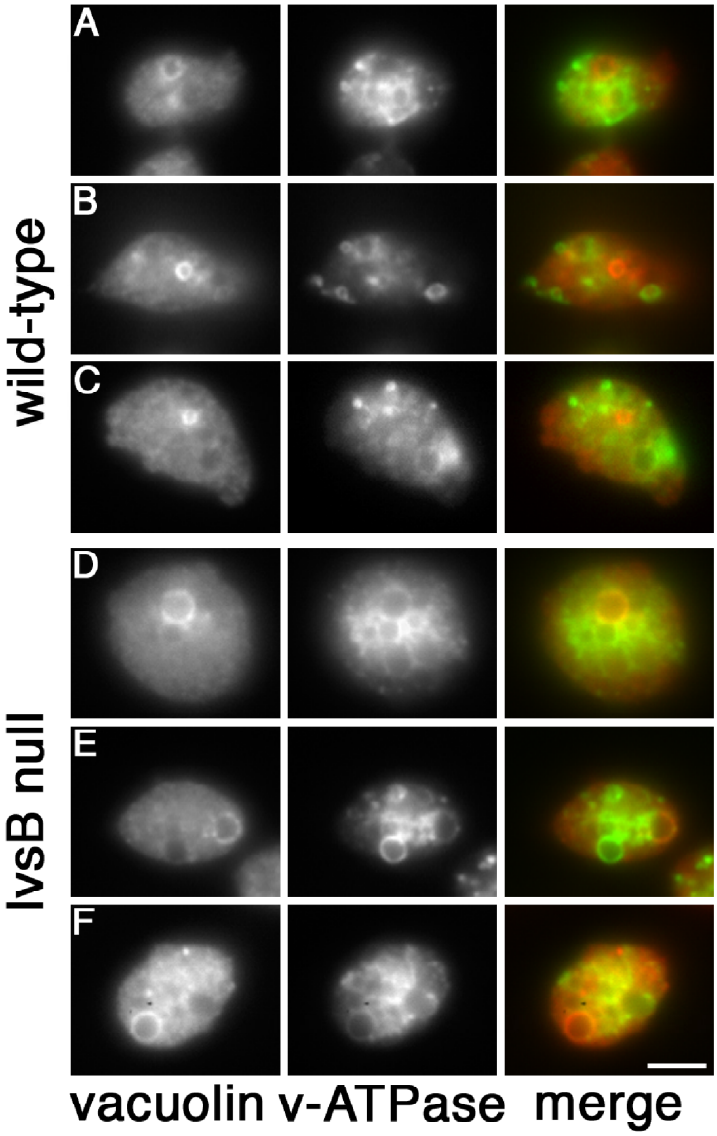


Figure 2.4: The proton pump v-ATPase is abnormally found on post-lysosomes of LvsB-null cells

Wild type and LvsB-null cells were transfected with a VatM-GFP to determine the localization of their v-ATPase proton pumps. The cells were fixed and stained with an anti-vacuolin monoclonal antibody. In wild type cells (A-C), proton pumps are normally distributed in early endosomes, lysosomes and in the contractile vacuole

(Clarke *et al.*, 2002a). These three wild type cells show that the vacuolin-labeled postlysosomes do not contain proton pumps. In contrast, the vacuolin-labeled postlysosomes of LvsB-null cells (D-F), also contain proton pumps. These results are in agreement with the observation that wild type postlysosomes have a neutral pH while mutant postlysosomes have a low pH (see Figure 2.3). Bar, 5 μm

2.2.4 Inappropriate fusion of endosomes in LvsB-null cells.

Initial studies of LvsB-null cells suggested that early endosomes fused with each other faster than early endosomes from wild type cells (Harris *et al.*, 2002a). Those observations seemed contrary to the localization and role of LvsB on postlysosomes described here. However, a model consistent with all observations is that the function of LvsB is to inhibit the fusion of early endosomes with late compartments like the postlysosomes. To test this model, we measured the time required for delivery of a pulse of endocytosed labeled dextran to the vacuolin-labeled postlysosome. We gave cells expressing GFP-vacuolin a 10 minute pulse of media containing TRITC-labeled dextran. We then determined the fraction cells that contained dextran-labeled vesicles that colocalized with GFP-vacuolin at different times after a chase. In wild-type cells, the endocytosed dextran was delivered to the vacuolin-labeled postlysosome ~30 minutes after the pulse (Figure 2.5). During early time points the dextran did not colocalize with vacuolin, but after 30 minutes the majority of dextran-labeled vesicles were also labeled by vacuolin. Remarkably, in LvsB-null cells a substantial fraction of endocytosed dextran was found in vacuolin labeled vesicles soon after the pulse (Figure 2.5). Examples of images used for the quantification are also shown in Figure 2.6

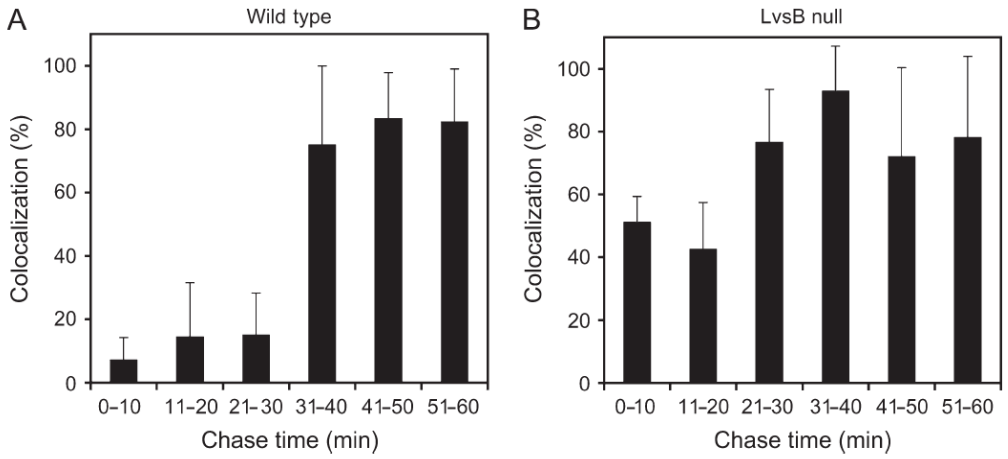


Figure 2.5: The loss of LvsB leads to abnormal fusion of early endosomes with post-lysosomes

Wild type (A) and LvsB-null (B) cells expressing GFP-vacuolinB were labeled for 10 min with TRITC-Dextran, chased in buffer, and imaged at different times after initiation of chase. The percentage of the cell population that contained vesicles labeled by both TRITC-Dextran and GFP-vacuolin was quantified (see images in Figure 2.6). In wild type cells the internalized dextran did not colocalize with GFP-vacuolin until after 30 minutes of chase. This indicates that the newly internalized marker normally needs more than 30 minutes to reach the postlysosomal compartment. In contrast, LvsB-null cells displayed extensive colocalization of dextran and GFP-vacuolin from early time points. This mutant phenotype could be the result of abnormal fusion of newly internalized endosomes with vacuolin-labeled postlysosomes. Error bars represent standard deviation.

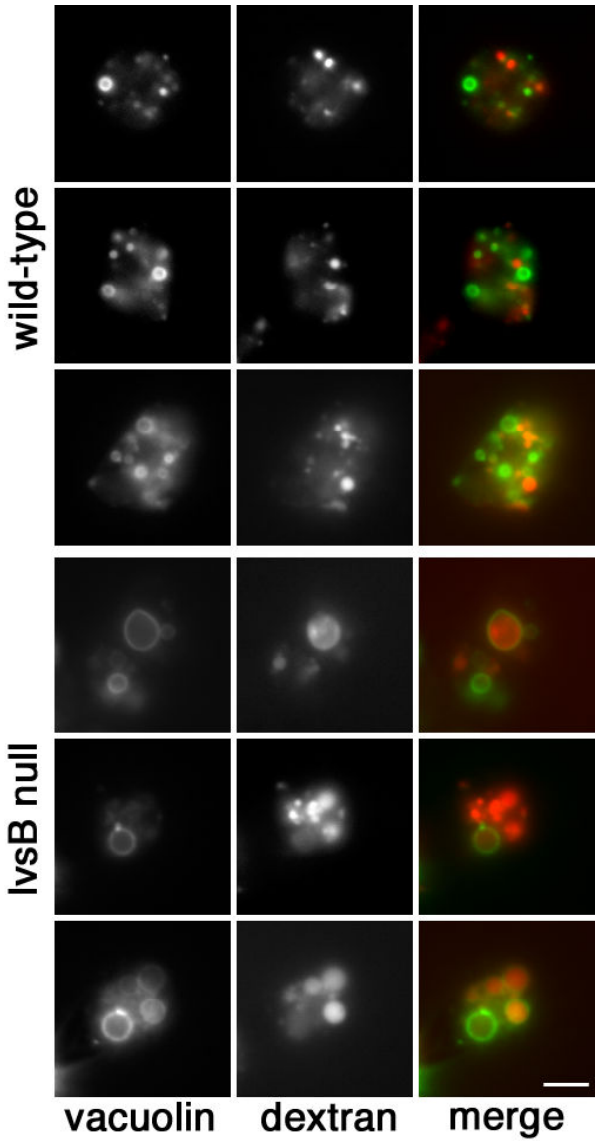


Figure 2.6: The loss of LvsB leads to abnormal fusion of early endosomes with post-lysosomes

Wild type (A) and LvsB-null (B) cells expressing GFP-vacuolinB were labeled for 10 min with TRITC-Dextran, chased in buffer, for 20-30 minutes and imaged by fluorescence microscopy. The three wild type cells shown here do not have any colocalization of the two markers. In wild type cells the internalized dextran did not

colocalize with GFP-vacuolin during the first 30 minutes of chase. This indicates that newly the internalized marker normally needs more than 30 minutes to reach the postlysosomal compartment. In contrast, the three LvsB-null cells shown here illustrate colocalization of dextran and GFP-vacuolin in less than 30 minutes of chase. This mutant phenotype could be the result of abnormal fusion of newly internalized endosomes with vacuolin-labeled postlysosomes. (See Figure 2.5 for quantification at different time points after chase). Bar, 5 μm

Ten minutes after the pulse ~50% of cells contained dextran-labeled vesicles that were also labeled by vacuolin and this fraction increased over time. The speed with which dextran entered vacuolin-positive compartments in the LvsB-null cells, suggests that early endosomes fuse inappropriately with postlysosomal compartments.

An alternative interpretation of our results is that vacuolin is mislocalized on early compartments in LvsB-null cells. Since vacuolin is a peripheral membrane associated protein, vacuolin could associate with early endosomes in the absence of LvsB. In this case, the observation of vacuolin-labeled vesicles containing proton pumps and early endosomal markers would not represent inappropriate fusion events but, rather, mislocalization of vacuolin. To distinguish between these alternatives, we employed two differently labeled dextrans to follow the luminal contents of endocytic vesicles (Figure 2.7). We gave wild type and LvsB mutant cells a short (5 min) pulse of FITC-Dextran and then chased with buffer for 30 minutes to allow the label to reach late compartments. We then gave the cells a second 5 minute pulse of TRITC-Dextran, washed the cells, and imaged them within 10 minutes after chase. In wild type cells the green and red dextrans mixed minimally; only 7.2% of their vesicles contained both markers. In contrast, the LvsB mutant cells contained a high fraction (49.6%) of

vesicles labeled by both markers. Examples of images used for quantification of the colocalization of the two dextrans are shown in Figure 2.8. Therefore, LvsB-null cells are unable to constrain the inappropriate fusion of their early and late compartments.

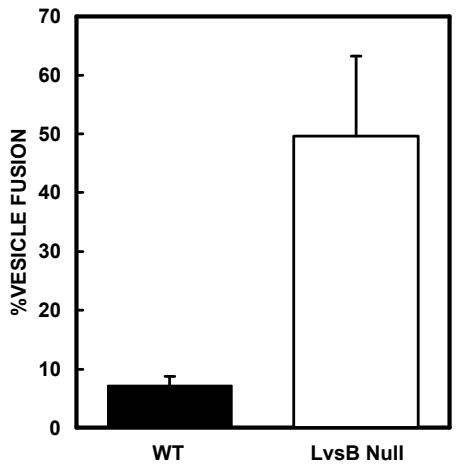


Figure 2.7: Fusion of early and late endosomal compartments is increased in LvsB-null cells

Wild type and LvsB-null cells were labeled with FITC-dextran for 5 minutes and then chased in buffer for 30 minutes to allow the internalized marker to reach late compartments in the cells. Cells were then labeled with a 5 minute pulse of TRITC-dextran, washed and imaged by fluorescence microscopy within 10 minutes (See Figure 2.8). The fraction of vesicles containing both fluid phase markers was quantified in two independent experiments. Error bars represent standard deviation.

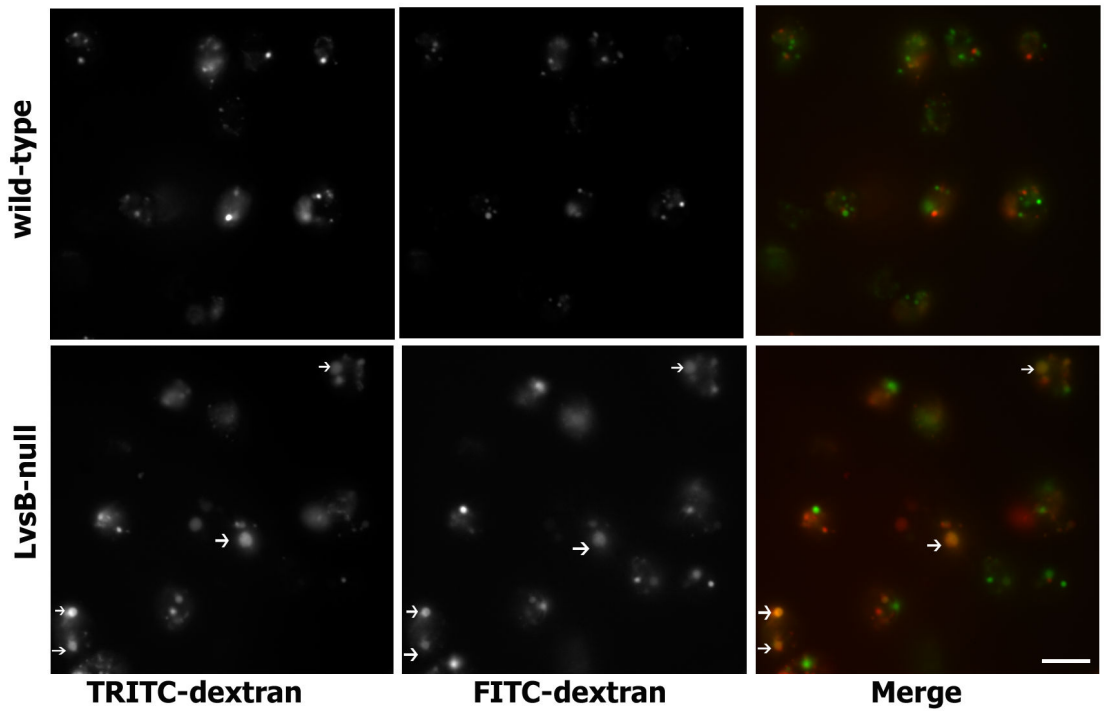


Figure 2.8: Fusion of early and late endosomal compartments is increased in LvsB-null cells

Wild type and LvsB-null cells were labeled with FITC-dextran for 5 minutes and then chased in buffer for 30 minutes to allow the internalized marker to reach late compartments in the cells. Cells were then labeled with a 5 minute pulse of TRITC-dextran, washed and imaged by fluorescence microscopy within 10 minutes. In wild type cells the two pulses of dextran remain in separate compartments. In contrast, the two pulses of dextran rapidly merge in LvsB-null cells. Examples of fusion are indicated with arrows (See Figure 2.7 for quantification). Bar, 10 μm

As previously shown by Maniak *et al*, the loss of vacuolinB results in the formation of enlarged post-lysosomes, the enhanced secretion of lysosomal enzymes and

increased transit time of endocytic markers (Jenne *et al.*, 1998). These phenotypes presented by the vacuolinB-null cells were very similar to the phenotypes seen in our LvsB-null cells. Thus, we tested whether vacuolin also controls heterotypic fusion events. As described above, the absence of LvsB resulted in formation of enlarged acidic compartments that represented mixed post-lysosomes with earlier acidic compartments. Using Lysotracker, a probe that accumulates in acidic compartments, we assessed that the morphology of acidic compartments in vacuolinB-null cells (Figure 2.9B) was normal, similar to wild-type cells (Figure 2.9A). This result suggested that the enlarged post-lysosomes found in the vacuolinB-null cells did not arise from fusion of post-lysosomes with earlier acidic compartments. The *in vivo* fusion assay using two color dextrans was performed in vacuolinB-null cells as described above, further supported that the heterotypic fusion of post-lysosomes with earlier compartments was normal (4.9%) compared to controls (3.3%) (data not shown). Therefore, we concluded that the mechanism by which vacuolin controls post-lysosome morphology is different than LvsB. This result further supported that the increase in the rate of heterotypic fusion, described above, was a specific phenotype caused by the absence of LvsB.

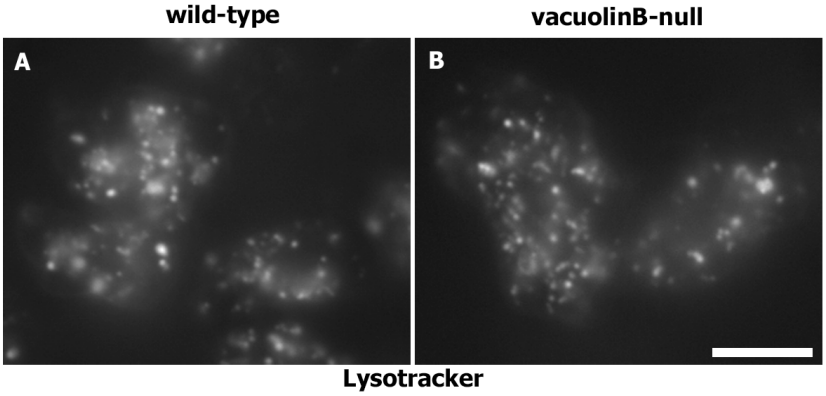


Figure 2.9: Loss of vacuolinB does not affect the morphology of acidic lysosomes

Acidic compartments of wild-type (A) and vacuolinB-null (B) cells were assessed using Lysotracker, a dye that accumulates in acidic compartments. VacuolinB-null cells contained acidic compartments that were similar in size to those found in wild-type cells. Bar, 10μm

2.3 DISCUSSION

We have shown here that the *Dictyostelium* BEACH protein LvsB is localized on vesicles of the endolysosomal system and that it plays a role in the regulation of fusion among vesicles of this compartment. Our results represent the first direct evidence that orthologs of the beige and Chediak-Higashi Syndrome proteins localize on specific vesicles of the endolysosomal system. Moreover, functional studies of LvsB-null cells show that this protein is required to prevent the fusion of postlysosomes with early compartments of the endolysosomal system. By analogy, we suggest that Lyst prevents the mixing of compartments in mammalian cells.

A knock-in approach allowed us to tag LvsB with GFP and express the tagged protein from its single chromosomal locus. While a fraction of soluble GFP-LvsB was observed in the cytoplasm, GFP-LvsB also associated with vesicles of various sizes. The LvsB-labeled vesicles were in the endolysosomal pathway since they also labeled with Rab7, a small GTPase known to associate with multiple compartments of the endolysosomal system (Buczynski *et al.*, 1997; Rupper and Cardelli, 2001; Rupper *et al.*, 2001a; Rupper *et al.*, 2001b). Importantly, about 40% GFP-LvsB colocalized with v-ATPase-labeled vesicles, while the other 60% colocalized with vacuolin, a marker of postlysosomes (Jenne *et al.*, 1998; Wienke *et al.*, 1999). In wild-type cells, recycling of v-ATPase proton pumps from late lysosomes allows these organelles to be neutralized

and converted into postlysosomes (Nolta *et al.*, 1994; Maniak, 2003). Vacuolin normally associates with vesicles after the removal of the proton pumps and the two do not normally colocalize (Jenne *et al.*, 1998). Thus, our results suggest that LvsB associates with late lysosomal vesicles that subsequently mature into postlysosomes.

LvsB was also found associated with phagosomes. This result is in agreement with previous observations that LvsB mutant cells exhibit enhanced rates of phagosome fusion (Harris *et al.*, 2002a). *Dictyostelium* endocytic and phagocytic pathways share many common features and effectors (Cardelli, 2001). This suggests that LvsB may function in both pathways with a similar mechanism.

The presence of the LvsB on late lysosomes and postlysosomes suggested that LvsB exerts its function in these compartments. Indeed, we found that the morphology of postlysosomes is severely altered in LvsB-null cells. In these mutants, vacuolin-stained postlysosomes are significantly enlarged and fewer in number than in wild type cells. In addition to their abnormal morphology, the mutant postlysosomes frequently exhibit abnormally acidic pH. We found that this abnormal pH is correlated with the abnormal presence of v-ATPase proton pumps in the mutant postlysosomes. A possible interpretation of these observations is that LvsB is required for the retrieval of proton pumps from late lysosomes. However, the fact that LvsB mutant cells contain some neutral postlysosomes indicates that proton pump retrieval is still active in these cells.

Earlier studies of beige, CHS, and LvsB mutant cell lines suggested a role for LYST proteins as negative regulators of endosome homotypic fusion (Jones *et al.*, 1992; Harris *et al.*, 2002a). This role was suggested by pulse-chase experiments showing that early endosome fusion occurred faster in LvsB-null cells than in wild type cells (Harris *et al.*, 2002a). A role for LvsB on early endosomes may seem puzzling given the localization of LvsB on late lysosomes and postlysosomes described here. However,

these data can be explained by a model where the role of LvsB is to inhibit the inappropriate fusion between early endosomes and postlysosomal compartments. Two different observations provide support for this model. First, we determined the time course of fluid phase marker delivery to postlysosomes, as labeled by vacuolin. We showed that, in wild type cells, a short 10 minute pulse of fluorescent dextran reached the vacuolin-labeled postlysosomes 30 minutes after chase. In contrast, in LvsB-null cells, the endocytosed marker was found in vacuolin-labeled postlysosomes within 10 minutes of chase. Second, we used an *in vivo* endosome fusion assay to directly quantify the fusion between early and late compartments. In wild type cells the contents of early compartments rarely mixed with those of late compartments. However, in the LvsB mutant cells, almost half of the early endosomes fused with late compartments. Together, these two approaches strongly indicate that in the absence of LvsB there is a loss of specificity of vesicle fusion resulting in the mixing of early and late compartments of the endolysosomal system. The specificity of this LvsB-null phenotype was further supported by the lack of this phenotype in vacuolinB null cells, which also contain enlarged post-lysosomes.

The model presented here can also explain the observation of acidic postlysosomes and the missorting of proton pumps in LvsB-null cells. In the absence of LvsB protein, fusion between an acidic early endosome and a postlysosome will deliver proton pumps to the postlysosome and cause its acidification. In addition, unregulated fusion is probably the cause of the severe enlargement and reduction in number of lysosomes and postlysosomes. Finally, the intermixing of compartments helps explain the enhanced secretion of lysosomal enzymes observed in beige and LvsB mutant cells (Tanaka, 1980; Takeuchi *et al.*, 1986; Harris *et al.*, 2002a).

An alternative early model proposed that the formation of large lysosomes in beige and CHS mutant cell lines was due to a reduction in the rate of fission from maturing lysosomes. This model was suggested by the observation that overexpression of LYST in fibroblasts causes the formation of smaller than normal lysosomes (Perou *et al.*, 1997). However, this model cannot account for the observations described here. On the other hand, our model for LvsB function can explain why the overexpression of LYST results in smaller than normal lysosomes. If the function of LvsB/LYST/Beige is to inhibit the fusion between endosomal compartments, an increase in Lyst activity would decrease the rate of fusion of endosomes with lysosomes, resulting in smaller lysosomes.

It is possible that the mechanism of LvsB action involves interactions with known regulators of vesicle fusion such as the Rab and SNARE proteins. LvsB and other LYST-like proteins may control specific SNARE interactions and control the heterotypic fusion between late and early endosomal compartments. It is not uncommon that certain adaptor proteins selectively inhibit the formation of specific SNARES complexes. For example, it was recently shown that the endosome-associated hepatocyte responsive serum phosphoprotein (Hrs) specifically inhibits the homotypic fusion of early endosomes without having a significant effect on late endosomes or lysosomes (Sun *et al.*, 2003). Interestingly, a possible interaction between Hrs and Lyst was detected in a yeast two hybrid experiment (Tchernev *et al.*, 2002). Furthermore, deletion or mutation of Hrs results in an enlarged endosomal phenotype in mouse, fly and yeast (Raymond *et al.*, 1992; Komada and Soriano, 1999; Lloyd *et al.*, 2002). This is clearly a possible pathway that needs to be dissected further.

In conclusion, we have demonstrated that LvsB localizes on late endocytic compartments, and provides an additional level of specificity by controlling fusion

events between late and early compartments. Future studies will focus on defining the precise mechanism of LvsB function by identifying potential binding partners. It will also be crucial to determine the domains that control the subcellular localization and function of LvsB and other BEACH proteins in *Dictyostelium*. This will help us understand how the different BEACH proteins have specialized to work in different cellular contexts.

Chapter 3: The BEACH protein LvsB antagonizes the Rab14 GTPase in vesicle fusion

3.1 INTRODUCTION

The Chediak Higashi syndrome (CHS) remains a poorly understood genetic disorder. The identifying feature is the presence of enlarged lysosomes in all tissues from CHS patients. Impaired lysosomal function in these patients results in many physiological problems, including immunodeficiency, albinism and neurological problems. The gene affected in humans with CHS encodes a 430KDa protein named LYST (lysosomal trafficking regulator), expressed in very low levels in cells (Ward *et al.*, 2000). The beige mouse has been used as an animal model for the study of CHS since the clinical and pathological features of the CHS and the beige mouse are very similar (Barbosa *et al.*, 1996; Nagle *et al.*, 1996a; Perou *et al.*, 1996).

LYST belongs to the novel family of BEACH proteins. BEACH proteins are conserved in all eukaryotes. All proteins in this family have a similar structural organization and share the conserved BEACH domain of unknown function and multiple WD motifs at the C-termini. Localization studies of several BEACH proteins, as well as loss of function phenotypes suggest that these proteins may play an important role in membrane trafficking. Unfortunately after many years of research the mechanism by which BEACH proteins regulate vesicle trafficking is not understood.

Dictyostelium discoideum has been proven to be a very useful system to study the function of BEACH proteins. *Dictyostelium* contains six BEACH domain-containing proteins termed Lvs (large volume sphere) A-F (Wang *et al.*, 2002; De Lozanne, 2003). LvsA and LvsB are studied in detail and seem to have different roles in membrane compartments in the cell (Kwak *et al.*, 1999; Gerald *et al.*, 2002; Harris *et al.*, 2002b).

Dictyostelium LvsA is proven to be essential for cytokinesis and the function of the contractile vacuole, an osmoregulatory organelle. In fact, LvsA associates transiently with the contractile vacuole (Kwak *et al.*, 1999; Gerald *et al.*, 2002). *Dictyostelium* LvsB is the ortholog of LYST and recent data shows that LvsB functions similarly to LYST. LvsB-null cells show enlargement of acidic compartments and present with secretory defects (Cornillon *et al.*, 2002; Harris *et al.*, 2002b). Additionally, characterization of the LvsB-null cells suggests that the enlarged acidic compartments arise by enhanced homotypic fusion of lysosomes (Harris *et al.*, 2002b). Recently, we have demonstrated that LvsB-null cells also contain enlarged post-lysosomes that are abnormally acidic. Post-lysosomes are terminal secretory vesicles, marked by the association of vacuolin that are destined for exocytosis (Rauchenberger *et al.*, 1997; Jenne *et al.*, 1998). In fact we have shown that LvsB localizes on late lysosomes and post-lysosomes and provides an additional level of vesicle fusion specificity by controlling heterotypic fusion events between late and early endocytic compartments.

In the present study, with a more precise functional assessment of the endosomal pathway in the LvsB-null cells, we showed that the early endosomes and the recycling compartment function normally in the absence of LvsB and the function of this protein is more restricted in regulating the fusion between lysosomes and post-lysosomes. In addition, we found that LvsB antagonizes with the *Dictyostelium* lysosomal GTPase Rab14.

Rab proteins are small (20-29kDa), ubiquitously expressed and constitute the largest branch of the Ras GTPase superfamily (Chavrier and Goud, 1999). Like other GTPases, the Rab proteins cycle between an active (GTP-bound) and an inactive (GDP)-bound state. Numerous studies have established that Rab proteins are distributed to distinct intracellular compartments. Activated Rab proteins on their corresponding

membranous compartments interact with effector molecules that mediate a variety of downstream effects. Rab GTPases and their effectors are implicated into four major steps of membrane traffic such as vesicle formation, vesicle delivery utilizing motor proteins, vesicle tethering and membrane fusion (Zerial and McBride, 2001).

Dictyostelium Rab14 is a novel GTPase related to mammalian Rab14. *Dictyostelium* Rab14 is shown to be a positive regulator of homotypic lysosome fusion. Rab14 localizes primarily on the contractile vacuole, an organelle important in osmotic regulation and to a lesser extent on lysosomal vesicles. The localization of Rab14 on lysosomal membranes is shown more clearly using subcellular fractionation experiments. When Rab14 is expressed in a constitutively active form (Rab14Q67L), cells accumulate enlarged acidic vesicles similar to the enlarged vesicles seen LvsB-null cells (Bush *et al.*, 1994; Bush *et al.*, 1996; Harris and Cardelli, 2002). Cells expressing a constitutively inactive Rab14 (Rab14N121I) accumulate many endosomes of smaller size compared to control cell lines. Furthermore, the neutralization kinetics of lysosomes is delayed. In addition, cell lines expressing Rab14(N121I) show deficient contractile vacuole activity (Bush *et al.*, 1996).

In this study, we provide the first evidence for a functional interaction between LvsB and Rab14. Expression of the inactive form of Rab14(N121I) suppressed the LvsB-null phenotype by reducing the enlarged post-lysosomes and the enhanced rate of heterotypic fusion. In contrast, expression of the active form of Rab14(Q67L) enhanced the LvsB-null phenotype by causing an even more severe enlargement of endosome size.

This functional link between LvsB and Rab14 provides the initial mechanistic insights into the regulation of membrane fusion events that are controlled by LvsB. Interestingly in a recent study, the *Drosophila* BEACH protein Blue cheese (Bchs) is shown to antagonize the GTPase Rab11 in multiple developmental events and during

synapse morphogenesis (Khodosh *et al.*, 2006). Thus, it is likely that BEACH proteins localize to specific membranous compartments to control membrane fusion by antagonizing specific Rab GTPases.

3.2 RESULTS

3.2.1 The absence of LvsB does not cause inappropriate fusion between the contractile vacuole and endosomes.

We have shown previously that in the absence of LvsB, endosomes of different maturation stages fused inappropriately (Kypri *et al.*, 2007). It is currently not known whether LvsB also acts to prevent fusion of membranes derived from other organelles. Interestingly, a recent study shows that lysosomes derived from beige mice contained high amounts of endoplasmic reticulum proteins (Zhang *et al.*, 2007). Thus, the absence of beige/LvsB may cause abnormal fusion with membranes derived from other organelles. In *Dictyostelium*, the contractile vacuole is a very dynamic osmoregulatory organelle composed of a reticular network of tubules and bladders. This organelle is highly dynamic and is set in motion as soon as the osmotic environment changes. As the hyposmotic media, such as water, enters the cell, the tubules collect the excess water and feed it to the main bladder that expands. When the bladder reaches its maximum capacity it fuses briefly with the membrane and expels its contents to the extracellular milieu (Gerisch *et al.*, 2002). The membranes of the contractile vacuole and the endocytic pathway are distinct, and no apparent trafficking of the bulk flow of membranes occurs between these organelles (Gabriel *et al.*, 1999). Despite this, the proteins Rab14, golvesin and the vacuolar-ATPase, are found to associate with both the contractile vacuole and endosomes (Bush *et al.*, 1994; Temesvari *et al.*, 1996; Schneider *et al.*, 2000) In addition, we recently identified that endosomes and the contractile

vacuole also share SNARE proteins (Kevin Bersuker, unpublished data). To test whether in the absence of LvsB there is loss of fusion specificity between endocytic membranes and membranes of the contractile vacuole, we tested whether two integral membrane proteins, Rh50 a resident protein of the contractile vacuole, and p80 a resident protein of endosomes colocalize in LvsB-null cells (Benghezal *et al.*, 2001; Ravanel *et al.*, 2001). In fact, p80 localizes on the plasma membrane and both early and late endocytic vesicles (Ravanel *et al.*, 2001), thus making it a good marker for vesicles of all maturation stages. Immunofluorescence using antibodies directed against Rh50 and p80 revealed that the localization of both proteins appeared normal in both control (Figure 3.1 A.B) and LvsB-null cells (Figure 3.1C,D). Rh50 displayed a normal localization characteristic of the contractile vacuole tubules and bladders (Benghezal *et al.*, 2001). P80 localized normally on the plasma membrane and endocytic vesicles, although p80 was detected on enlarged vesicles in the LvsB-null cells (D). This can be explained by the enlarged endosomes found in LvsB-null cells. Therefore, both Rh50 and p80 localized to their respective compartments and no intermixing occurred, suggesting that the absence of LvsB does not cause fusion of membranes between these compartments.

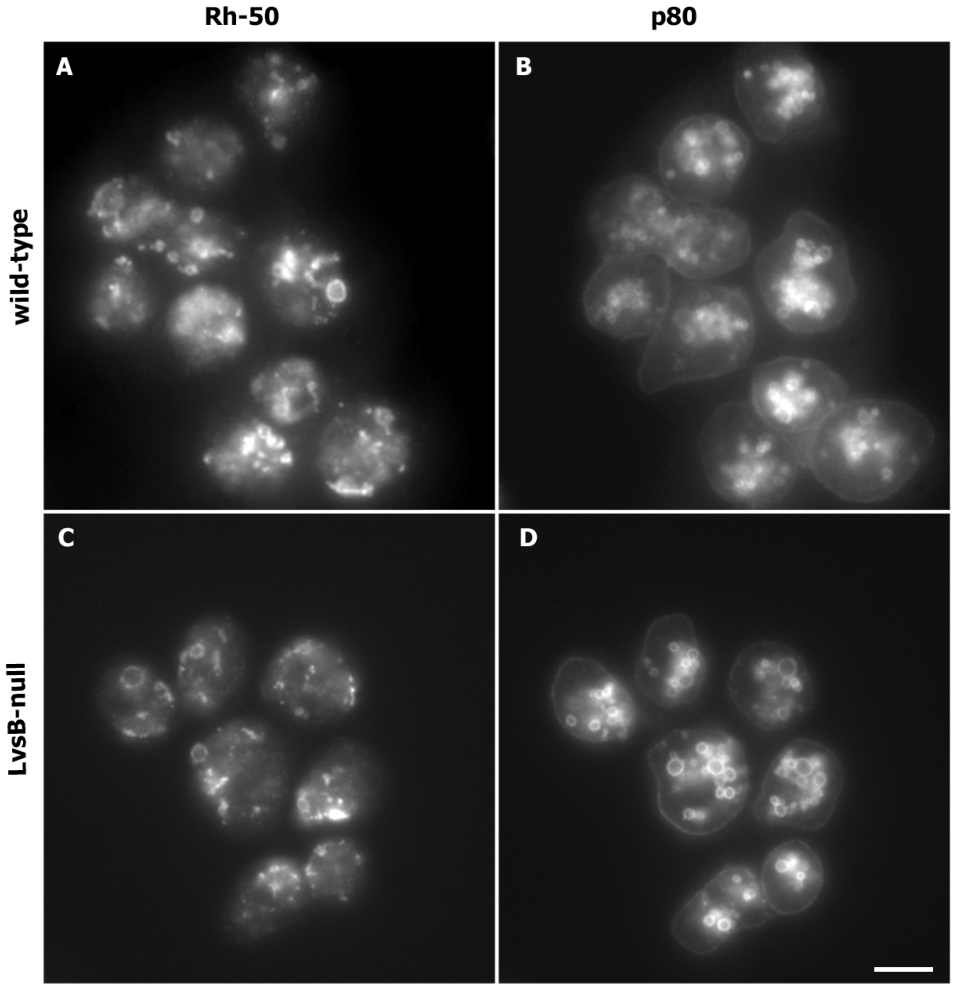


Figure 3.1: The absence of LvsB does not cause inappropriate fusion between endosomes and the contractile vacuole

Control and LvsB-null cells were fixed and stained with antibodies against Rh50, a contractile vacuole marker and p80, a marker for endosomes. 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 endocytic vesicles. Thus, the lack of LvsB did not cause inappropriate fusion between endosomes and the contractile vacuole. Bar,10 μm

3.2.2 Early endosomes and post-lysosomes do not fuse inappropriately in the absence of LvsB

We previously showed that LvsB regulates post-lysosome morphology. Post-lysosomes are the terminal secretory organelles formed by the fusion of lysosomes. The maturation of lysosomes into post-lysosomes is marked by the association of vacuolin (Rauchenberger *et al.*, 1997; Jenne *et al.*, 1998). In fact, we showed that in the absence of LvsB, lysosomes containing V-ATPase proton pumps fuse with post-lysosomes In addition, using an *in vivo* fusion assay we showed that earlier endosomes fuse with later endosomes in LvsB-null cells compared to control cells (Kypri *et al.*, 2007). The *in vivo* fusion assay limited our ability to distinguish whether the newly formed early endosomes also fused with post-lysosomes in the absence of LvsB. Therefore, we tested the trafficking of the integral membrane protein p25, a marker for the plasma membrane and early endosomes in control and LvsB-null cells. P25 is internalized together with endocytic cargo in early endosomes, and shortly retrieved to a recycling compartment before final recycling back to the plasma membrane (Charette *et al.*, 2006). Thus, p25 is absent from lysosomes and post-lysosomes. We colocalized p25 and vacuolin in control and LvsB-null cells (Figure 3.2). Similar to control cells (A,B,C), in LvsB-null cells (D,E,F), p25 localized normally to the plasma membrane and the recycling compartment and did not colocalize with vacuolin. This result suggested that early endosomes did not fuse inappropriately with late endocytic compartments in the absence of LvsB.

The normal localization of p25 suggested that early endosome trafficking proceeded normally in LvsB-null cells. Thus, the lysosome to post-lysosome fusion did not affect the dynamics of trafficking to the recycling compartment. In fact, the association of another protein of the recycling compartment, the *Dictyostelium* GTPase Rab5A was normal in LvsB-null cells (Figure 3.3E). Rab5A was found to associate with

the juxtanuclear recycling compartment and showed extensive colocalization with p25, a marker for this early endocytic compartment (A,B,C). Therefore, we concluded that the composition of the recycling compartment was not perturbed by the lack of LvsB. Interestingly Rab5A was also found to associate with vesicles that differed in size in LvsB-null cells compared to the control (D,E). This can be explained by the presence of enlarged endosomes in LvsB-null cells. This functional assessment of the early steps in the endosomal pathway in LvsB-null cells, helped specify the function of LvsB in regulating fusion only between lysosomes and post-lysosomes.

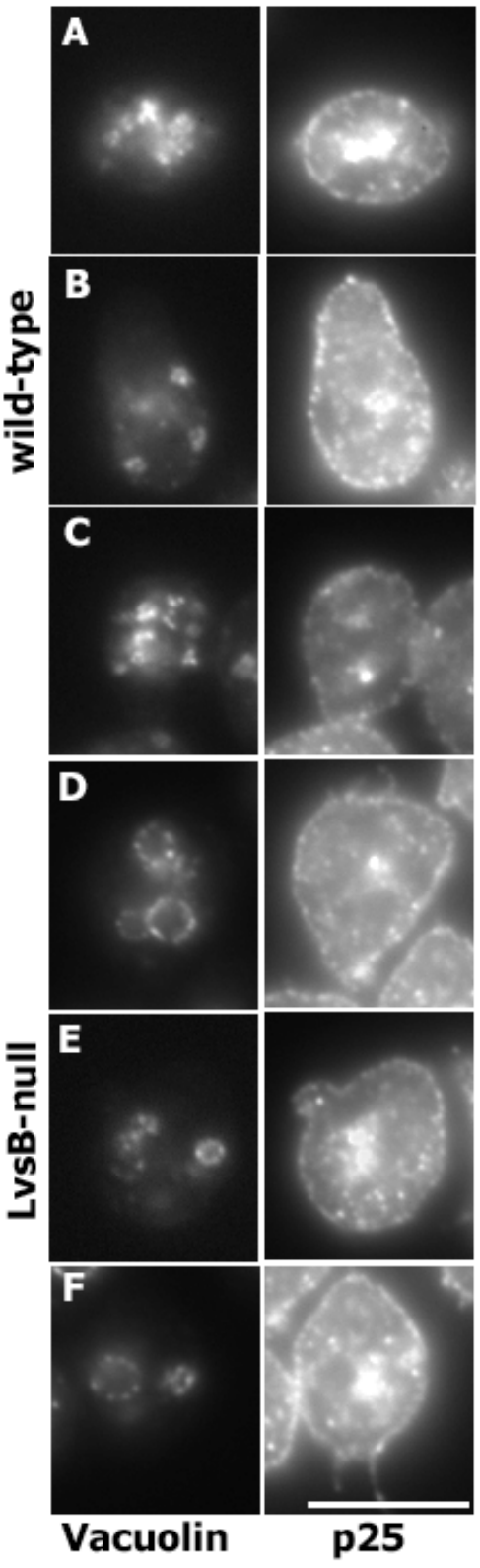

A
B
C
D
E
F
wild-type
LysB-null
Vacuolin
p25

Figure 3.2: Early endosomes and post-lysosomes do not fuse inappropriately in the absence of LvsB

Control and LvsB-null cells expressing GFP-VacuolinB were fixed and stained with antibodies against p25. P25 is internalized together with endocytic cargo in early endosomes, and shortly retrieved to a recycling compartment before final recycling back to the plasma membrane (Charette *et al.*, 2006). As previously described, in control cells, p25 localized normally on the plasma membrane and the juxtanuclear recycling compartment and did not colocalize with vacuolin on post-lysosomes (A,B,C). Similarly, p25 did not colocalize with vacuolin in the absence of LvsB (D,E,F). This result suggests that early endosomes and post-lysosomes did not abnormally fuse in the absence of LvsB. This limits the function of LvsB as a negative regulator of heterotypic fusion only between lysosomes and post-lysosomes. Bar,10µm

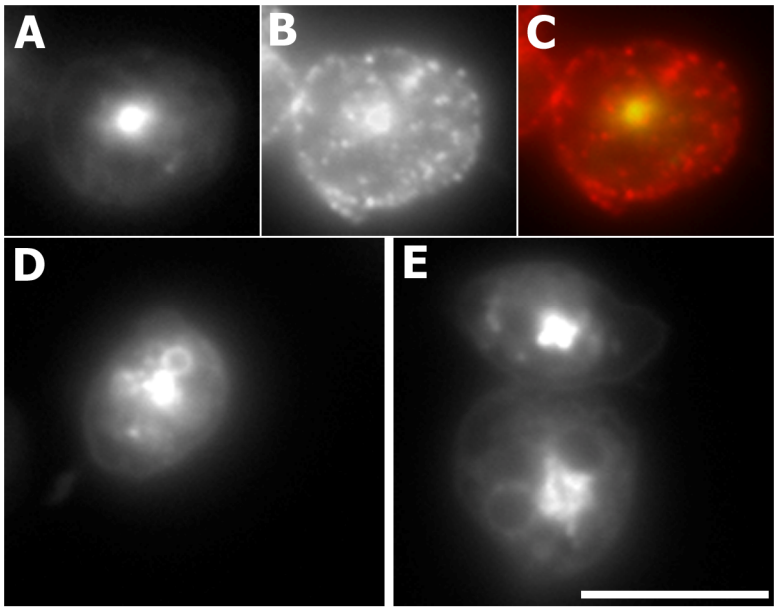


Figure 3.3: The association of Rab5A with the recycling compartment remains normal in LvsB-null cells

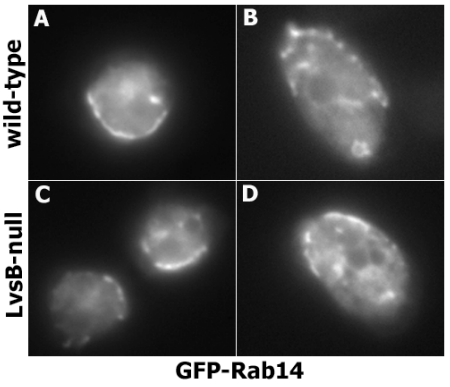
The GTPase Rab5A was found to associate with the juxtanuclear recycling compartment (A,B,C) as indicated by colocalization with p25, a marker previously shown to associate with the recycling compartment (Charette *et al.*, 2006). Wild-type cells expressing GFP-Rab5A (A), fixed and stained with p25 mab (B) showed extensive colocalization of the two proteins (C). The association of Rab5A with the juxtanuclear compartment remained normal in LvsB-null cells (E). Interestingly, Rab5A was also found to associate with vesicles in both wild-type (D) and LvsB-null cells (E). Bar,10μm

3.2.3 Wild-type Rab14 abnormally colocalizes with vacuolin in LvsB-null cells

Our results showed that vesicles, specifically of the lysosomal stage, fuse abnormally with post-lysosomes in the absence of LvsB. This suggested that lysosomes contained markers, different from markers on early endosomes that allowed the vesicles to be competent for fusion. In fact, lysosomes in wild-type cells show a very dynamic behavior and undergo homotypic fusion (Maniak, 2001). An important positive regulator of homotypic lysosome fusion is the *Dictyostelium* GTPase Rab14. Rab14 is a small GTPase shown previously to localize primarily on the contractile vacuole, and on lysosomal membranes. When Rab14 is expressed in a constitutively active form, cells accumulate enlarged acidic vesicles (Bush *et al.*, 1994; Bush *et al.*, 1996; Harris and Cardelli, 2002). The Rab14 constitutively active phenotype was very similar to the phenotype seen in LvsB-null cells. This suggested the possibility that LvsB and Rab14 may exhibit an antagonistic relationship in vesicle fusion.

To explore a possible functional interaction between Rab14 and LvsB, we initially tested the localization of wild-type Rab14 in the absence of LvsB (Figure 3.4). GFP-Rab14 was expressed in wild-type (A,B) and LvsB-null (C,D) cells. Rab14

exhibited normal localization on the contractile vacuole membranes in both wild-type (A,B) and LvsB-null cells (C,D). *Dictyostelium* lysosomes mature into a neutral secretory compartment called the post-lysosome This compartment is marked by the association of a peripheral membrane protein vacuolin (Rauchenberger *et al.*, 1997; Jenne *et al.*, 1998). Since Rab14 is shown to be a lysosomal marker, it did not colocalize with vacuolin on post-lysosomes (E,F,G). Interestingly in LvsB-null cells, while Rab14 retained the normal localization on the contractile vacuole membranes, it was found to abnormally colocalize with vacuolin (H,I,J). The localization of Rab14 on post-lysosomes in LvsB-null cells is likely to occur because of the abnormal heterotypic fusion of lysosomes and post-lysosomes. Alternatively, the removal of Rab14 from lysosomes upon maturation to post-lysosomes may be impaired.


A
B
wild-type
C
D
LvsB-null
GFP-Rab14

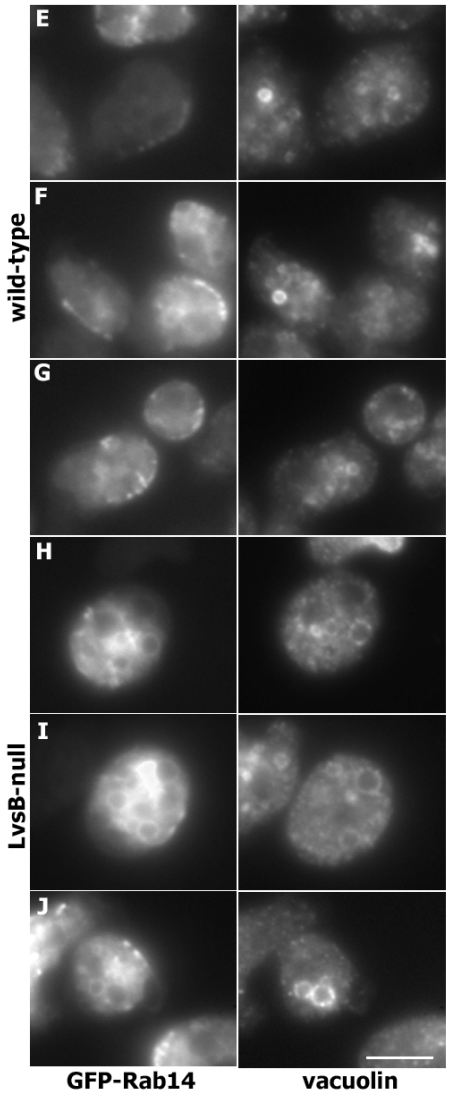

E
F
wild-type
G
H
I
LvsB-null
J
GFP-Rab14
vacuolin

Figure 3.4: Rab14 GTPase abnormally colocalizes with vacuolin in LvsB-null cells

Wild-type and LvsB-null cells were transfected with GFP-Rab14 expression vector (provided by Cardelli lab) to determine the localization of Rab14. Cells were also fixed and stained with anti-vacuolin mab. In the examples shown, for wild-type (A,B) and LvsB-null cells (C,D), Rab14 localized normally on the membranes of the contractile vacuole. While Rab14 was not detected to colocalize with the post-lysosomal marker vacuolin in control cells (E,F,G), Rab14 was frequently found to colocalize with vacuolin in LvsB-null cells (H,I,J). This suggested a mislocalization of Rab14 in the absence of LvsB. Bar, 10μm

3.2.4 Inactive form of Rab14 suppresses the endocytic defects of LvsB-null cells

Loss of LvsB results in enlarged late endocytic compartments with abnormal acidity that is caused by the inappropriate fusion of lysosomes with post-lysosomes (Kypri *et al.*, 2007). Since Rab14 regulates trafficking events at the acidic stage, we examined the effects of the expression of the inactive form of Rab14(N121I) on the LvsB-null phenotype. It has been shown previously that cell lines expressing the inactive form of Rab14(N121I) accumulate smaller vesicles compared to wild-type cells and show some delay in the neutralization kinetics of vesicles (Bush *et al.*, 1996). The endocytic morphology was evaluated in control and LvsB-null cells expressing flag-Rab14(N121I) that were incubated with TRITC-dextran for 1 hour (Figure 3.5A,B,C,D). Our flag-Rab14(N121I) tagged protein was detected at the predicted weight in western blot analysis and showed a cytosolic localization when stained with anti-flag monoclonal antibodies (data not shown). Importantly, similar to wild-type cells (A), LvsB-null cells expressing the inactive form of Rab14(N121I) (D), contained normal

size endosomes similar to endosomes in wild-type cells (A). This result suggested that the expression of a mutant form of Rab14 that was unable to associate with lysosomes caused a phenotypic rescue in LvsB-null cells. As previously described, the expression of Rab(N121I) in wild-type cells (C) caused an increased accumulation of small vesicles compared to non-transfected cells (A), although the effect was not as dramatic as in LvsB-null cells.

We showed previously that LvsB-null cells exhibited post-lysosomes that were abnormally enlarged, compared to control cells. The effects of the inactive form of Rab14(N121I) on post-lysosomal morphology was tested with vacuolin staining (Figure 3.6 A,C,D,F). Immunostaining with vacuolin revealed that the size of post-lysosomes was reduced in LvsB-null cells expressing the inactive Rab14 construct (F). Thus, the expression of a cytosolic form of Rab14 suppressed the enlarged post-lysosomal size in LvsB-null cells. While a small decrease in overall endosome size was visible in wild-type cells expressing Rab14(N121I) that were incubated with dextran, (Figure3.5C), the effect on post-lysosome size was not easily distinguished. It is likely that the small changes on post-lysosome size were not easily detected. Interestingly, the small delay in the in neutralization kinetics of lysosomes measured previously (Bush *et al.*, 1996) did not seem to affect maturation of lysosomes and the association of vacuolin, since the number of post-lysosomes in cells expressing Rab14(N121I) (Figure 3.6C) was very similar to control cells (Figure 3.6A).

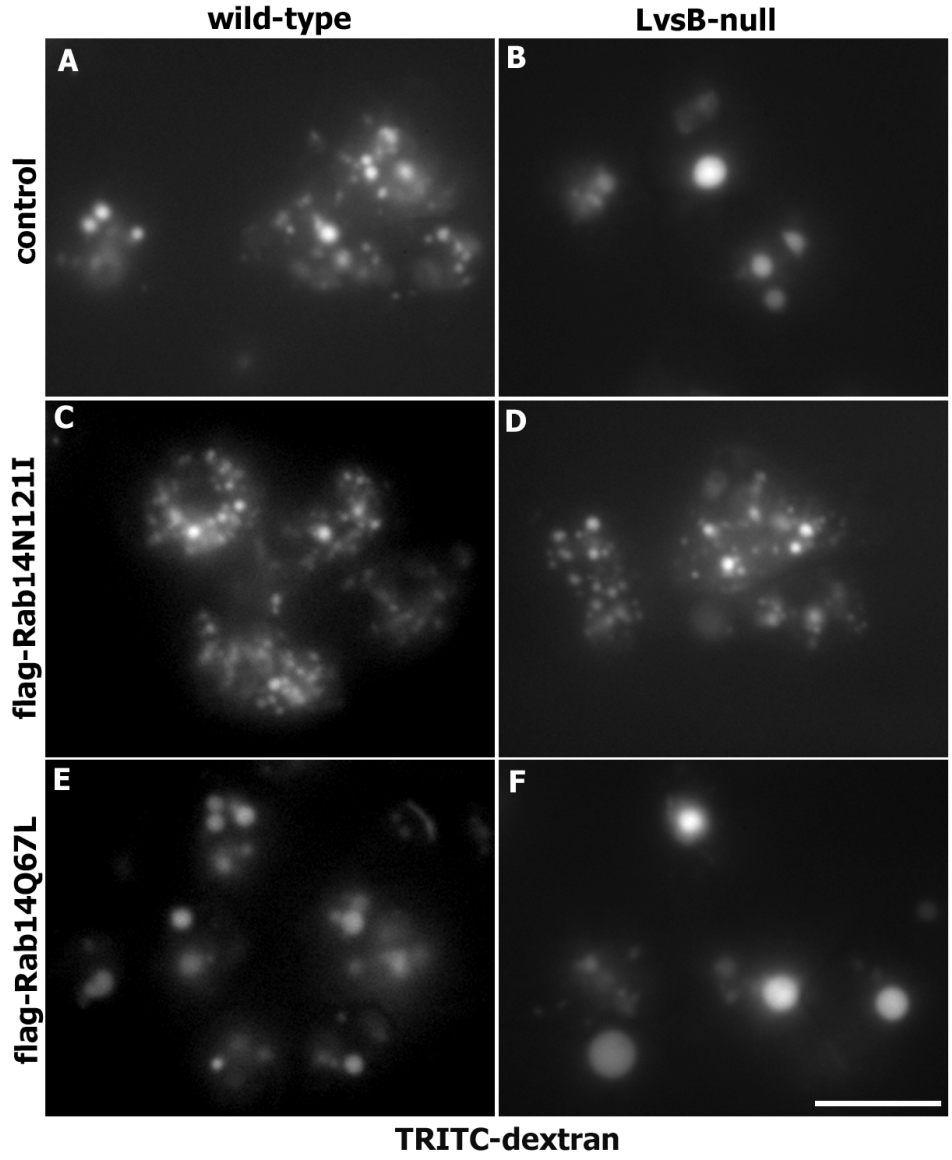


Figure 3.5: Expression of the mutant forms of Rab14 alters the LvsB-null endosomal morphology

Wild-type and LvsB-null cells were transfected with the constructs flag-Rab14-N121I or flag-RabDQ67L and the endosomal morphology was evaluated by incubating with TRITC-dextran for 1 hour. Unstranfected wild-type and LvsB-null cells were used as controls (A,B). As previously described, the expression of the inactive form of

Rab14(N121I) in wild-type cells (C) caused the accumulation of many small size vesicles compared to the endosomes in wild-type control cells (A) (Bush *et al.*, 1996). LvsB-null cells expressing the inactive form of Rab14(N121I) (D) showed endosomes that were similar in size to wild-type cells. Thus, the expression of the inactive form of Rab14(N121I) rescued the LvsB-null phenotype. As shown previously, expression of the active form of Rab14(Q67L) in wild-type cells (E), resulted in the accumulation of enlarged vesicles, similar to the vesicles seen in the LvsB-null cells (B) (Harris and Cardelli, 2002). Expression of the active form of Rab14(Q67L) in the LvsB-null cells (F) caused an even greater enlargement of endosomes compared to the vesicles in the LvsB-null untransfected cell line (B). These results suggested that LvsB and Rab14 act antagonistically to control vesicle size. Bar, 10µm

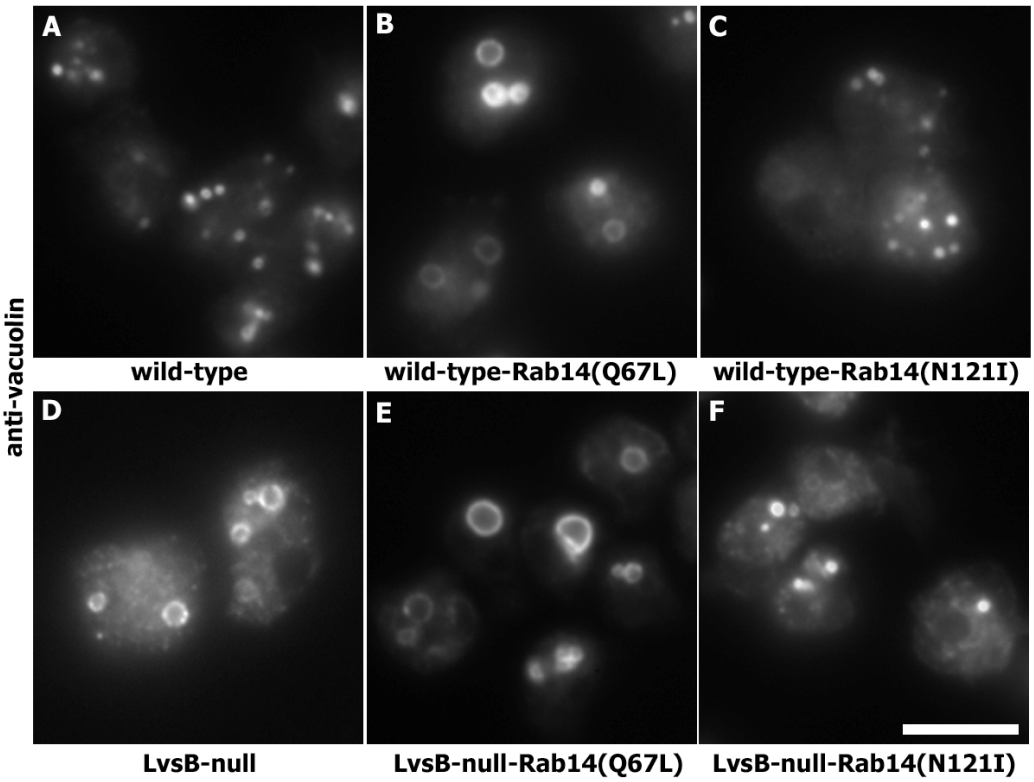


Figure 3.6: Expression of the mutant forms of Rab14 alters the LvsB-null post-lysosomal morphology

The morphology of the post-lysosomes was evaluated in wild type cells (A), wild-type expressing Rab14(Q67L) (B), wild-type expressing Rab14(N121I) (C), LvsB-null (D), LvsB-null expressing Rab14(Q67L) (E) and LvsB-null expressing Rab14(N121I) (F). The expression of the active form of Rab14(Q67L) caused enlargement of post-lysosomes in both wild-type (B) and null cells (E), compared to post-lysosomes in non-expressing cells (A,D). Importantly, LvsB-null cells expressing the inactive form of Rab14(N121I) (F), exhibited normal size post-lysosomes similar to wild-type control (A) While the Rab14(N121I) inactive construct caused significant changes when expressed in LvsB-null cells it did not significantly alter the post-lysosome size when expressed in wild-type cells (C). It is possible that the difference in post-lysosome size is too small to detect. Bar, 10μm

We showed previously that the enlarged size of post-lysosome size in LvsB-null cells is caused by the inappropriate fusion of lysosomes with post-lysosomes (Kypri *et al.*, 2007). To determine whether the expression of the inactive Rab14 rescued the heterotypic fusion of lysosomes with post-lysosomes we performed in an *in vivo* fusion assay as previously described (Kypri *et al.*, 2007) (Figure 3.7A,C,E). Control (A), LvsB-null (C) and Rab14(N121I) (E) expressing cell lines were given a short pulse (5 min) of fluorescein isothiocyanate (FITC)-dextran and then chased with buffer for 30 min to allow the label to reach late compartments. Subsequently, cells were given a second short pulse (5min) of TRITC-dextran, washed and imaged within 10 minutes of the chase. In wild-type cells (A) the green and red dextran mixed minimally; 4.2% (n=310) of the vesicles contained both markers. LvsB-null cells (C) contained a high percentage

of vesicles that labeled by both markers (41.2%, n=136). In contrast, LvsB-null cells expressing the inactive Rab14(N121I) (E) contained a low percentage of fused vesicles that were similar to wild-type numbers (2.9% , n=326). Therefore, the reduction in Rab14 activity on lysosomes restored normal endosome fusion in LvsB-null cells. This result suggests that Rab14 and LvsB work in the same pathway and compete in endosome fusion events.

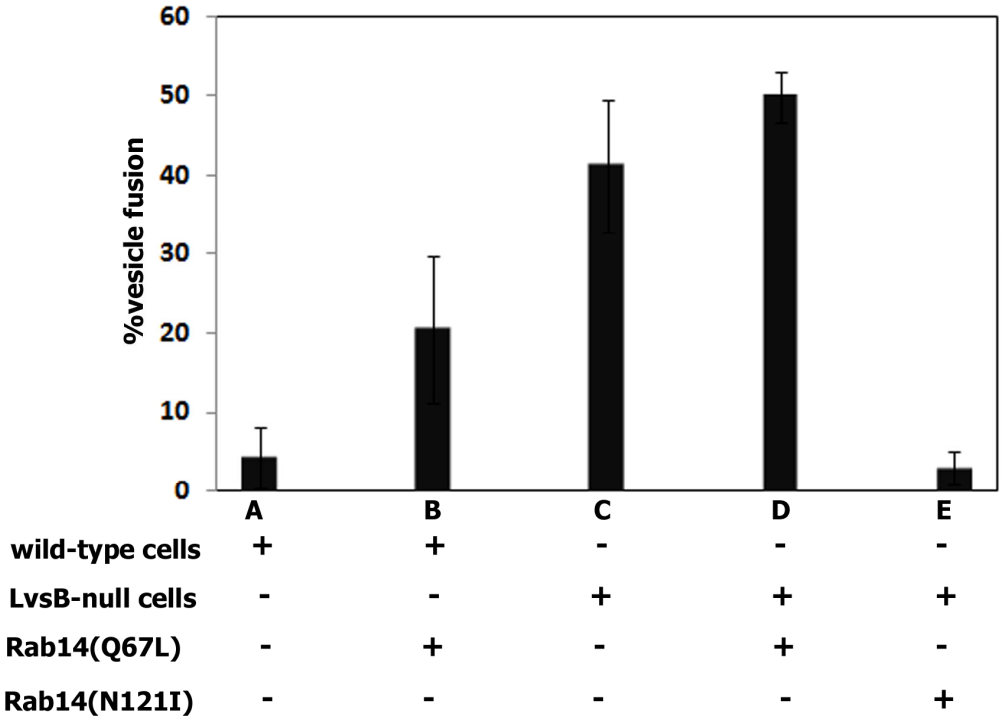


Figure 3.7: The expression of the mutant inactive Rab14(N121I) rescues the increased heterotypic fusion in the LvsB-null cells

An *in vivo* fusion assay was performed as previously described (Kypri *et al.*, 2007) in the following cell lines: wild type (A), LvsB-null (C), LvsB-null expressing Rab14(N121I) (E), wild-type expressing Rab14(Q67L) (B) and LvsB-null expressing Rab14(Q67L) (D). Briefly, control and expressing cell lines were labeled with FITC-

dextran for 5 minutes and then chased in buffer for 30 minutes to allow the internalized marker to reach late compartments in the cells. Cells were then labeled with a 5 minute pulse of TRITC-dextran, washed and imaged by fluorescence microscopy within 10 minutes. The fraction of vesicles containing both fluid phase markers was quantified in two independent experiments. Error bars represent standard deviation. While the expression of the active form of Rab14(Q67L) caused a five-fold increase in the heterotypic fusion in wild-type (B) cells it did not significantly change the heterotypic fusion in LvsB-null cells (D) Significantly, the expression of the inactive form of Rab14(N121I) rescued the increased heterotypic fusion shown by the LvsB-null cells (E).

3.2.5 Active form of Rab14 enhances the LvsB-null phenotype

Our experiments described above established a functional interaction between LvsB and Rab14. To further understand how Rab14 and LvsB coordinate to regulate vesicle fusion, we studied the effects of the expression of the active form of Rab14(Q67L) in control and LvsB-null cells. Previously published data showed that when Rab14 is expressed in a constitutively active form (Rab14Q67L) causes the formation of enlarged acidic compartments that arise by enhanced fusion rates (Harris and Cardelli, 2002).

We expressed a flag-Rab14(Q67L) in control and LvsB-null cells and evaluated the endosome size by incubating with TRITC-dextran for 1 hour (Figure 3.5 A,B, E,F). The flag-Rab14(Q67L) protein was highly expressed at the predicted weight in western blog analysis (data not shown). As reported previously, LvsB-null cells (Figure 3.5B) contained enlarged vesicles (Harris *et al.*, 2002b; Kypri *et al.*, 2007). The expression of the flag-Rab14(Q67L) in wild-type cells (Figure 3.5E) caused the formation of enlarged

vesicles that were similar in size with vesicles in the LvsB-null cells (B). Interestingly, the expression of the Rab14(Q67L) construct in the LvsB-null cells caused an even greater enlargement of endosomes.(Figure 3.5F).

To study whether these enlarged vesicles acquired vacuolin normally we stained control, LvsB-null and Rab14(Q67L) cell lines with anti-vacuolin antibody (Figure 3.6 A,B,D,E). Staining with vacuolin revealed that the post-lysosomes of wild-type cells expressing the constitutively active Rab14 (Q67L) are enlarged (Figure 3.6B) and similar in size to LvsB-null cells (D). In contrast, expression of the constitutively active form of Rab14(Q67L) (Figure 3.6 E) caused an even more severe enlargement post-lysosome size, compared to non-expressing LvsB-null cells (D) . Thus, the expression of the active form of Rab14(Q67L) did not prevent vesicle maturation and the association of vacuolin.

The enhancement of the LvsB-null phenotype suggested that the two proteins may act independently in regulating vesicle fusion events. The localization and the functional phenotypes suggested that Rab14 positively regulates homotypic lysosome fusion whereas LvsB negatively regulates heterotypic lysosomes to post-lysosome fusion.

We also tested the effects of the expression of the active Rab14(Q67L) on the rates of heterotypic fusion between late and early endosomal vesicles, utilizing the *in vivo* fusion assay with two differently labeled dextrans (Figure 3.7 A,B,C,D). As discussed previously, LvsB-null cells contained a high fraction of vesicles labeled by both markers (41.2%, n=136) (C). While the expression of the active Rab14(Q67L) in wild-type cells caused a small but significant increase in the percentage of heterotypic fusion (20.5%, n=241) (B) it did not significantly enhance heterotypic fusion when expressed in LvsB-null cells (49.8%, n=130) (D) This suggested that in the LvsB-null

cells the fusion machinery has reached saturation and the low vesicle numbers prevent further fusion.

Interestingly, the effect of the active Rab14(Q67L) on heterotypic fusion of post-lysosomes with earlier endosomes seemed contradictory to the localization of wild-type Rab14 (Figure 3.7B). Thus, we tested the possibility that the active form of Rab14(Q67L) remained associated with post-lysosomes, therefore affecting heterotypic fusion. We visualized the localization of active Rab14(Q67L) in cells co-expressing flag-Rab14(Q67L) and GFP-vacuolinB, fixed and stained with anti-flag antibody in both wild-type and LvsB-null cells (Figure 3.8). Active Rab14(Q67L) mainly localized on the contractile vacuole membranes (data not shown) in both wild-type and LvsB-null cells. In wild-type, we also detected Rab14(Q67L) to colocalize with vacuolin on post-lysosomes (Figure 3.8 A,B,C). This localization could account for the increase in heterotypic fusion in control cells expressing active Rab14(Q67L). As expected, in LvsB-null cells the active form of Rab14(Q67L) was found also on post-lysosomes (D,E,F). At this time we cannot distinguish the percentage of active Rab14 that occurs on post-lysosomes in LvsB-null cells that is caused by fusion or failure of dissociation.

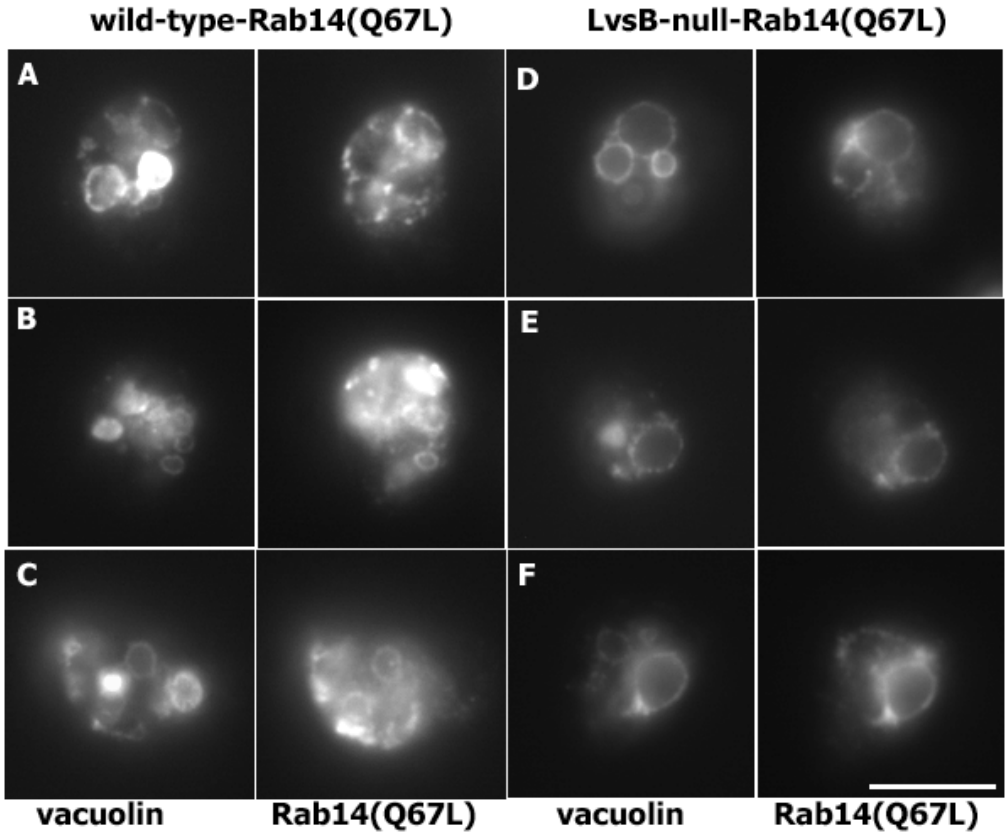


Figure 3.8: The active form of Rab14(Q67L) frequently associates with post-lysosomes in wild-type and LvsB-null cells

Wild-type and LvsB-null cells were transfected with GFP-VacuolinB and flag-Rab14(Q67L) and stained with anti-flag monoclonal antibody. Active Rab14(Q67L) was frequently found to colocalize with vacuolin in both wild-type (A,B,C) and LvsB-null (D,E,F) cells. The association of Rab14(Q67L) with post-lysosomes may explain the significant increase in heterotypic fusion seen in wild-type cells expressing the active form of Rab14(Q67L) (Figure 3.7B). Bar, 10µm

The enlarged vacuolin labeled post-lysosomes caused by the expression of active Rab14(Q67L) (see Figure 3.8) often localized near the edge of the cell and resembled

structures similar to the contractile vacuole bladders. Since active Rab14(Q67L) localized both on the contractile vacuole (data not shown) and frequently on post-lysosomes, we investigated the possibility that the membranes of the contractile vacuole and post-lysosomes fused inappropriately, when the active Rab14 is expressed. Thus, we tested whether Rh50 a resident protein of the contractile vacuole, and vacuolin a post-lysosome associated protein colocalized in cells expressing active Rab14(Q67L) (Figure 3.9). Immunofluorescence in wild-type (B,C,D) and LvsB-null cells (F,G,H) expressing Rab14(Q67L), with antibodies directed against Rh50 and vacuolin revealed that these proteins localized to their respective compartments and no intermixing occurred. This suggested that expression of active Rab14(Q67L) did not cause fusion of membranes between these two compartments. Interestingly, the localization of Rh50 and the structure of the contractile vacuole seemed to be disrupted in cells expressing the active form of Rab14(Q67L) (Figure 3.9 B,C,D,F,G,H) compared to control cells (A,E). It appeared that the localization of Rh50 was more diffused in the cytoplasm and the contractile vacuole bladders were enlarged. While cells expressing inactive Rab14(N121I) are unable to handle osmotic stress, the role of Rab14 in contractile vacuole regulation is still not clear (Bush *et al.*, 1996). More careful analysis of the contractile vacuole morphology in cells expressing the mutant forms of Rab14 is required to understand the function of Rab14 in the regulation of this organelle.

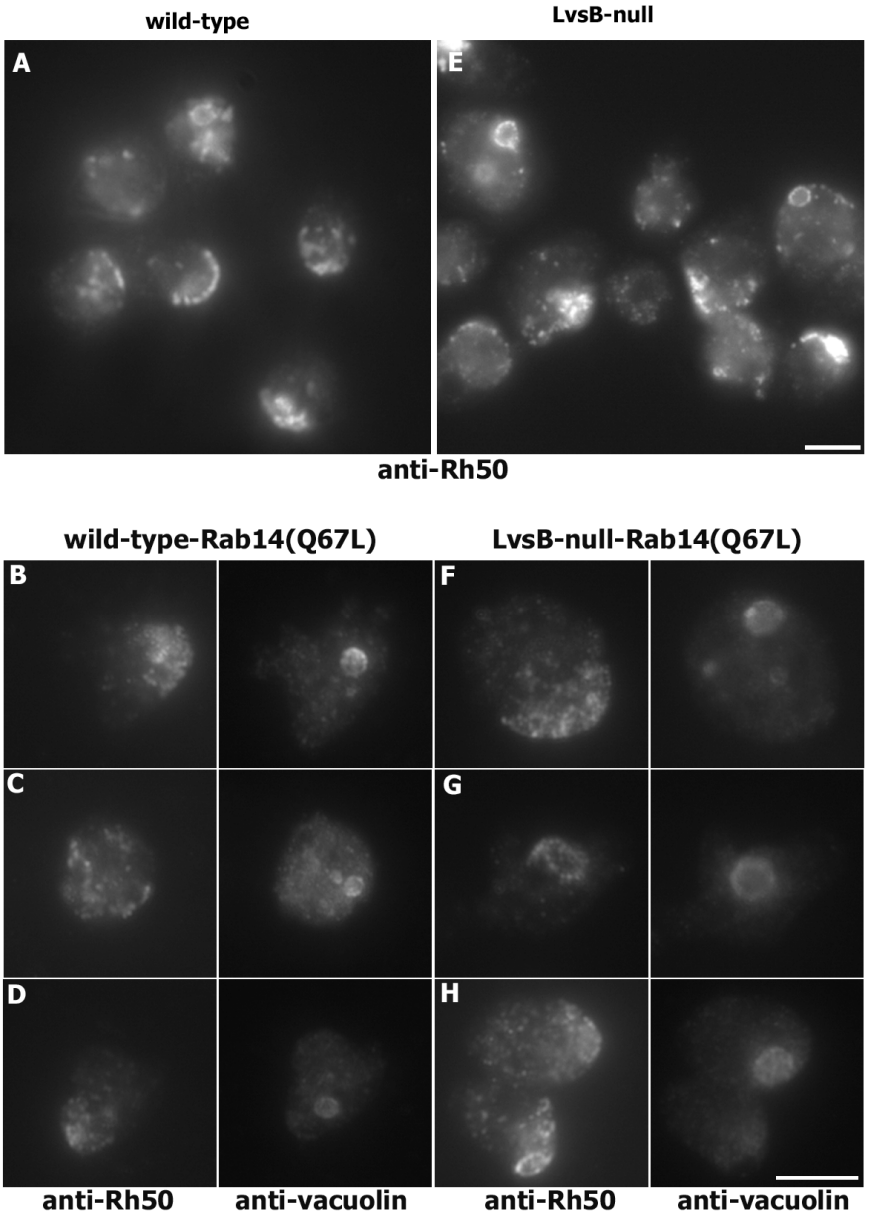


Figure 3.9: The expression of the active Rab14(Q67L) does not enhance fusion between post-lysosomes and the contractile vacuole

Control and LvsB-null cells expressing the active form of Rab14(Q67L) were fixed and stained with antibodies against Rh50, a contractile vacuole marker and vacuolin, a peripheral protein that associates with post-lysosomes. Vacuolin did not appear to

colocalize with Rh50 in wild-type (B,C,D) or LvsB-null (F,G,H) cells expressing the active Rab14(Q67L). Thus, we concluded that the expression of the active form of Rab14(Q67L) did not cause inappropriate fusion between post-lysosomes and the contractile vacuole. Interestingly, the expression of the active formed of Rab14(Q67L) seemed to cause changes in the contractile vacuole structure in both wild-type (B,C,D) and LvsB-null cells (F,G,H) compared to the contractive vacuole morphology in non-expressing cells (A,E). Bar, 10μm

3.3 DISCUSSION

While several BEACH proteins have been implicated in vesicle trafficking, the mechanism through which they may regulate this process is unknown. In this study we showed that a *Dictyostelium* BEACH protein, LvsB, is a negative regulator of heterotypic fusion, specifically the fusion of post-lysosomes with lysosomes. More importantly, we provide the first evidence that LvsB is a functional antagonist of the GTPase Rab14. In particular, reduction of Rab14 activity suppressed the LvsB-null phenotype by reducing the enlarged post-lysosomes and the enhanced rate of heterotypic fusion. In contrast, expression of an active form of Rab14 enhanced the LvsB-null phenotype by causing an even more severe enlargement of endosome size. By analogy we suggest that mammalian Lyst also prevents mixing of specific endocytic compartments and functionally interacts with members of the Rab family.

Following the dynamic trafficking of p25 from the plasma membrane to early endosomes and subsequently to the recycling compartment, we showed that these steps proceeded normally in LvsB-null cells. This was further supported by the normal association of *Dictyostelium* Rab5A with the recycling compartment in cells lacking LvsB. Upon internalization p25 transiently resides in early endosomes/macropinosomes.

Within 5 minutes of internalization p25 is retrieved from early endosomes and is visible in the punctuate juxtanuclear recycling compartment. P25 is absent from maturing endosomes and does not colocalize with the vacuolar-ATPase, a marker for lysosomes. In fact, the clathrin adaptor protein AP-3 is essential for the retrieval of p25 from early endosomes (Charette *et al.*, 2006). As shown previously, cells lacking LvsB are unable to control heterotypic fusion between vacuolin-labeled post-lysosomes and earlier endocytic compartments (Kypri *et al.*, 2007). Results in this study showed that in LvsB-null cells, p25 traffics normally to the recycling compartment and does not colocalize with vacuolin on post-lysosomes, suggesting that post-lysosomes and early p25 positive endosomes did not fuse. These results defined a role of LvsB in specifically regulating fusion between lysosomes and post-lysosomes. These results also suggested that lysosomes contained markers, different from markers on early endosomes that allowed the vesicles to be competent for fusion. In fact, lysosomes in wild-type cells show a very dynamic behavior and undergo homotypic fusion (Maniak, 2001).

Many lines of evidence suggest trafficking between the contractile vacuole and the endocytic pathway. Initially, the GTPase Rab14 is shown to localize both on the contractile vacuole and on lysosomes. In fact, the expression of mutant forms of Rab14 causes defects in both the contractile vacuole regulation and in lysosome formation(Bush *et al.*, 1994; Bush *et al.*, 1996; Harris and Cardelli, 2002). We also recently identified a SNARE protein that localizes on endosomes and the contractile vacuole (unpublished data by Kevin Bersuker). In addition, clathrin and adaptor proteins are also important for the regulation of endocytic trafficking and the contractile vacuole formation (O'Halloran and Anderson, 1992; Lefkir *et al.*, 2003; Stavrou and O'Halloran, 2006). Furthermore, a study by Padh *e al.* suggested that the vacuolar proton pumps are delivered to the endosomal pathway through the contractile vacuole system (Padh *et al.*,

1991). More importantly, in a recent study Cosson *et al.* showed that contractile vacuole markers can traffic to the contractile vacuole membranes via the recycling compartment (Mercanti *et al.*, 2006). While the absence of LvsB disrupted fusion between endocytic compartments it did not affect the morphology of the contractile vacuole as detected by the localization of Rh50 and did not cause an abnormal fusion between the contractile vacuole and endocytic membranes. These results further support that LvsB functions specifically in regulating fusion between lysosomes and post-lysosomes.

In a process of understanding the mechanism by which LvsB regulates membrane fusion we investigated the interaction between *Dictyostelium* Rab14, a GTPase known to regulate lysosome fusion. Even though the precise mechanism by which Rab14 regulates lysosome fusion is not known, we can speculate that is by a similar mechanism used by other known Rab GTPases. While Rab GTPases may also regulate direct SNARE interactions it is well established that Rabs regulate vesicle tethering. This tethering process restrains the vesicle at or near their cognate target membranes allowing for SNARE pairing. *Dictyostelium* Rab14 localizes mainly on the contractile vacuole and on lysosomal membranes and does not colocalize with vacuolin on post-lysosomes. In the absence of LvsB, Rab14 was detected to colocalize with vacuolin on post-lysosomes. The presence of the wild-type Rab14 on post-lysosomes did not cause any changes on post-lysosome morphology, suggesting that GTPase-activating proteins (GAP) proteins functioned normally to keep the Rab14 in its inactive state. Based on the fact that LvsB-null cells exhibit enhanced fusion between lysosomes and post-lysosomes we can assume that the presence of Rab14 on post-lysosomes was caused by fusion.

A prominent phenotype of LvsB-null cells is the presence of enlarged endocytic vesicles that arise by enhanced heterotypic fusion of post-lysosomes with lysosomes.

Importantly, this phenotype is completely rescued by the expression of the inactive form of Rab14(N121I). In particular, we showed that reduction of Rab14 activity suppressed the LvsB-null phenotype by reducing the enlarged post-lysosomes. The reduction of the post-lysosome size was caused by a significant reduction in the enhanced rate of heterotypic fusion. These results established a functional interaction between LvsB and Rab14. The localization of wild-type Rab14 on lysosomes and not on post-lysosomes, coupled with the previously characterized role of Rab14 in homotypic lysosome fusion, suggested that that Rab14 acts upstream of LvsB (Harris and Cardelli, 2002). The rescue of the LvsB-null phenotype by the expression of a cytosolic inactive Rab14(N121I) suggested that the activity of this GTPase on lysosomal membranes is required for fusion events to occur.

As previously characterized, the expression of the active Rab14(Q67L) in wild-type cells caused the formation of enlarged acidic compartments that was produced due to an enhanced rate of homotypic fusion (Harris and Cardelli, 2002). Here we provide the first evidence that the increase in size produced by of the expression of the active form of Rab14(Q67L) may also be caused by an increase in the heterotypic fusion of post-lysosomes with lysosomes. The increase in heterotypic fusion may be caused by active Rab14(Q67L) that remained on post-lysosomes, as shown by the colocalization with vacuolin. It is likely the factors such as GAP proteins that are required to inactivate and remove the active Rab14(Q67L) from the lysosomal membranes are depleted. We can speculate that the active Rab14(Q67L) on post-lysosomes recruits tethering factors allowing for heterotypic fusion.

The expression of the active Rab14(Q67L) in LvsB-null cells produced a synergistic effect. The size of post-lysosomes in null cells expressing active Rab14(Q67L) was even more enlarged, compared to post-lysosomes in LvsB-null cells.

This synergistic effect on the LvsB-null phenotype suggested that the two proteins have an antagonistic relationship. The reciprocity of these functional phenotypes also suggested that the two proteins participate in the same process. While the expression of the active form of Rab14 in the LvsB-null cells caused a significant increase in the size of post-lysosomes it did not alter the rate of heterotypic fusion. This suggested that in the LvsB-null cells the fusion machinery has reached saturation and the low vesicle numbers prevented further fusion.

Interestingly, although the significance of the Rab14 function in contractile vacuole regulation was highlighted by defects caused by the inactive form of Rab14(N121I), the effects of the active mutant Rab14(Q67L) were not tested. Expression of the inactive Rab14(N121I) caused osmosensitivity and mislocalization of contractile vacuole markers such as the vacuolar-ATPase and calmodulin (Bush *et al.*, 1996). In this study we showed initial evidence that the expression of the active Rab14(Q67L) also caused alterations in the structure of the contractile vacuole in both wild-type and LvsB-null cells. The bladders of the contractile vacuole seemed to be enlarged and the tubular membranes appeared fragmented as detected by the localization of Rh50. It is likely that changes in the localization of the vacuolar-ATPase on the membranes of the contractile vacuole could account for the morphological changes. More careful investigation of the contractile vacuole structure and localization studies of other regulators of contractile vacuole activity will allow assessing the function of Rab14 in the regulation of the contractile vacuole morphology. While Rab14 seems to regulate trafficking in endocytic pathway as well as the contractile vacuole, the expression of the active form of Rab14(Q67L) did not cause fusion between the two pathways suggesting that additional levels of regulation exist to maintain the unique composition of each pathway.

The link between Rab14 and LvsB provided the initial mechanistic insights into the regulation of membrane fusion controlled by LvsB and GTPases. Our results favor the model where Rab14 and LvsB are involved in competing intracellular pathways. Wild-type Rab14 may promote homotypic lysosome fusion, thus allowing for the mixing of contents during the acidic stage. Subsequently, LvsB associates with late lysosomes and post-lysosomes to inhibit their heterotypic fusion and allow for the maturation of the secretory vesicles before exocytosis. This model is supported by the localization of both proteins as well as the synergistic effect on post-lysosome size of active Rab14 expression in LvsB-null cells. Our results at this time, cannot do not distinguish whether LvsB negatively regulates Rab14 activity. Thus, the possibility that LvsB negatively regulates the activity of Rab14 or a different Rab GTPase on post-lysosomes still exists.

Our finding that *Dictyostelium* LvsB antagonizes Rab14 in addition to the finding that *Drosophila* Bchs antagonizes Rab11 raises the possibility that other BEACH proteins might also functionally interact with Rab family members. It will be interesting to determine whether alterations in Rab function could ameliorate defects caused by the absence of Lyst.

Chapter 4: Conclusions and future directions

Understanding the function of Lyst is crucial to develop therapies for the Chediak-Higashi syndrome. While the study of the localization and function of Lyst has not been successful, the study of LvsB, the Lyst ortholog in *Dictyostelium,* has provided many new insights into the function of Lyst-like proteins and further advanced the understanding of the Chediak-Higashi syndrome. Specifically, in this study we localized LvsB on endocytic and phagocytic compartments. This was the first evidence of a localization of a Lyst-like protein. In addition, we presented with a new model for LvsB function as a negative regulator of heterotypic fusion of post-lysosomes with lysosomes. Furthermore, we identified an antagonistic relationship between LvsB and the GTPase Rab14, providing the initial mechanistic evidence of how LvsB regulates membrane fusion. This study raises a lot of interesting questions about the precise role of LvsB and other BEACH proteins in regulating membrane fusion.

4.1 FUTURE DIRECTIONS

4.1.1 Domain analysis of LvsB

This study found that LvsB, the Lyst ortholog localizes on endocytic vesicles. This finding further supports the hypothesis that BEACH proteins function by localizing to specific membranous compartments. These findings also raise the question whether specific LvsB domains confer its specific function and localization. By extension, an LvsB domain analysis will also help understand how other BEACH proteins are targeted and specialized to work in different cellular contexts. Our preliminary data showed that the PH-Beach-WD domains of LvsB did not include a sufficient membrane localization

signal (Appendix Figure A-2) and showed mainly a cytosolic localization. The lack of a sufficient membrane localization signal on the C-terminal region that is most conserved between BEACH proteins, suggested that the membrane localization signal of LvsB resides on the N-terminus. Thus, we started to make deletions at the C-terminus to begin understanding the function of the remaining portion of LvsB. Unfortunately, the cell line lacking the WD domain (Appendix, Figure A-5) expressed the truncated LvsB protein in very low levels and showed an LvsB-null phenotype. Alternatively, to study the function of the N-terminus we will generate deletion cell lines tagged with GFP, lacking portions of the N-terminus, using a knock-in approach. The deletion cell lines will allow assessing whether the mutant truncated proteins are able to localize on endocytic vesicles. In addition, they will allow assessing whether the mutant proteins are able to regulate vesicle fusion. Interestingly, the N-terminus of LvsB contains two additional regions of similarity with mammalian Lyst. Of particular interest is the fact that a human CHS missense mutation has been mapped in one of these regions of similarity. These two regions provide the initial sequences to be studied within the N-terminus.

4.1.2 Perform *in vitro* endosome fusion assays

Our *in vivo* fusion assay utilizing dextrans provided the initial evidence that LvsB regulates heterotypic fusion between postlysosomes and lysosomes. However, our *in vivo* fusion assay failed to detect whether LvsB also controls homotypic fusion events. In addition, the assay failed to detect the precise timing of LvsB action due to the requirement for broad chase times. An *in vitro* endosome fusion assay will allow us to answer these issues more specifically and will be performed in control and LvsB-null cells, as previously described by Laurent *et al* (Laurent *et al.*, 1998). The endosome

fusion assay utilizes the ability of avidin and biotin to bind each other. The fusion will be quantified by the formation of a complex between avidin and biotin-HRP loaded endosomes, in the presence of cytoplasm. After fusion, endosomes will be loaded onto an anti-avidin-coated enzyme-linked immunoabsorbent assay plate and the optical density will be measured upon addition of HRP-substrate. Initially, we will confirm that LvsB regulates heterotypic fusion between post-lysosomes and lysosomes. Subsequently, we will assess both homotypic and heterotypic fusion rates by testing fusion using specific internalization and chase times. This assay will allow the precise assessment of the maturation stage of endosomes that are abnormally regulated in the absence of LvsB. In addition, by modifying the contents of the cytoplasm we can determine additional factors that affect fusion of endosomes originating from LvsB-null background.

4.1.3 Determine the molecular partners of LvsB

Very little is currently known about the function of Lyst, no significant binding partners are identified and the molecular mechanism of its function is unknown. A major drawback in the biochemical characterization of Lyst and other mammalian BEACH proteins has been the low expression level and the difficulty in manipulating their large genes. To make a reasonable progress in the understanding of the molecular mechanism of function of Lyst and other BEACH proteins, we must understand some of the basic biochemical and structural properties of these proteins. Are they elongated or globular molecules? Are they monomeric or multimeric? Do these proteins interact with other proteins known to function in membrane trafficking? *Dictyostelium* has been proven a good system to manipulate these genes. In this study, using homologous

recombination we inserted the GFP and TAP tags at the amino termini of LvsB (Figure 2.1). Following a large scale TAP purification protocol, the full length LvsB in denatured form was purified and confirmed by mass spectrometry (Appendix-FigureA-6). Unfortunately many contaminants and large amounts of IgG co-purified with LvsB, which made the identification of binding partners very difficult (Figure A-6). The full length purification of LvsB presents an exciting development towards the biochemical characterization of Lyst-like proteins. In the future, to enable the purification of the full length non-denatured form of LvsB, we will insert a different tag at the N-terminus of LvsB. For example, maltose binding protein (MBP) has been successfully used to purify proteins in *Dictyostelium* (Graf, 2001). Isolated non-denatured LvsB, subjected to electron microscopy can provide some initial evidence about the general structure of the protein. Upon optimization of the purification protocol we will also identify binding partners of LvsB. Since our data suggest that LvsB is a novel regulator of fusion, identification of binding partners that are known regulators of membrane trafficking will provide insights into the mechanism of how LvsB regulates fusion.

4.1.4 Test the functional interaction between LvsB with Lip5 and SKD1

To understand the regulatory role of LvsB in membrane fusion it is important to investigate whether LvsB exhibits functional interactions with other proteins that are known regulators of endocytic traffic. Of particular interest is the ATPase SKD1 because when altered produces a phenotype similar to LvsB-null.

Mammalian SKD1 is an ATPase that belongs to the AAA family of ATPases and is crucial for the formation of multivesicular bodies (MVB). Multivesicular bodies are late endocytic compartments that contain intravesicular vesicles. These intravesicular

vesicles contain cargo destined for degradation. Central to the selection of cargo are the protein complexes ESCRT-I,II,III that assembly on the membrane of the MVBs. The ATPase activity of SKD1 is crucial for the disassembly of ESCRT-III from the MVB and the final formation of the intravesicular vesicles. Interestingly, expression of a dominant negative form of SKD1(E235Q) causes the accumulation of enlarged acidic compartments that bear features of both lysosomes and late endosomes due to inappropriate fusion (Fujita *et al.*, 2003). In addition, the expression of the mutant SKD1 significantly influences the membrane association of Lyst (Fujita *et al.*, 2004) The SKD1 mutant phenotype is close to the phenotype presented by LvsB-null cells. Thus, we can speculate that a functional interaction exists between these two proteins. The *Dictyostelium* genome encodes one protein with significant homology to mammalian SKD1, thus it will important to study the functional interaction with LvsB. In fact, we tagged the full length *Dictyostelium* SKD1 with RFP and showed that while it is mainly cytosolic, it also localized on vesicles and colocalized with LvsB (data not shown). A mutant form of SKD1(E235Q) was generated (with Kristin Benson) and the effect of the overexpression of this construct on endosomal morphology and the LvsB-null phenotype is currently under investigation.

A binding partner of mammalian SKD1 is the novel protein Lip5 (Lyst Interacting Protein 5) (Fujita *et al.*, 2004). While the precise function of Lip5 is currently unknown, the yeast homologue Vta1 has been shown to stimulate the ATPase activity of SKD1(Azmi *et al.*, 2006). Interestingly, Lip5 was identified, independently by a different group, as a novel binding partner of Lyst (Tchernev *et al.*, 2002). Thus, it is possible that Lip5 also directly interacts with LvsB. In fact, *Dictyostelium* contains a mammalian Lip5 homolog. *Dictyostelium* Lip5 was shown to associate with endocytic vesicles and colocalized with LvsB (by Kristin Benson, unpublished data). While the

connection between LvsB, SKD1 and Lip5 are currently not clear, further exploration of functional interactions will help understand how LvsB inhibits membrane fusion.

We can postulate that LvsB binds to Lip5 to promote the ATPase activity of SKD1. Unpublished data suggests that Lip5 localizes on endosomes earlier than LvsB and the overexpression of Lip5 seems to partially rescue the LvsB-null phenotype (by Kristin Benson, unpublished data). It will be important to test whether Lip5 recruits LvsB on late lysosomes. This can be tested by the generation of Lip5 knockout cell lines. A direct interaction of LvsB, SKD1 and Lip5 will also be tested. Subsequently, stimulation of SKD1 activity could facilitate the formation of intravesicular vesicles and the retrieval of factors important for fusion. We can postulate that in the absence of LvsB the formation of intravesicular vesicles and the retrieval of fusion factors is impaired, resulting in abnormal heterotypic fusion. The requirement of LvsB for the SKD1 ATPase activity could be tested using an *in vitro* ATPase assay, which requires the purification of full length LvsB.

While known factors that affect fusion such as the SNARE proteins are not yet detected in intravesicular vesicles, the accumulation and degradation of the lipid messenger phopshatidylinositol-3-phosphate in intravesicular vesicles is described in yeast vacuole (Wurmser and Emr, 1998). The importance of this lipid messenger is highlighted during early endosome fusion in mammalian cells, thus, is tempting to speculate that the removal of this lipid from the lysosomal membrane into intravesicular vesicles is crucial for the regulation of fusion.

4.1.5 Future implications of this study to the understanding of the function of Lyst and the Chediak-Higashi syndrome

The results accomplished in this thesis as well as the future directions outlined in this section aim to help understand the role of Lyst-like proteins in higher organisms and ultimately a better understanding of the Chediak-Higashi syndrome. The endocytic localization of LvsB, as well as the detailed characterization of endocytic compartments in LvsB-null cells suggested that Lyst may also localize on specific endocytic vesicles to control heterotypic fusion events between vesicles of specific stages. Our current model of the function of LvsB in *Dictyostelium* can also be tested in CHS/beige cells using the *in vitro* endosome fusion assay, an assay that is well characterized in mammalian systems (Diaz *et al.*, 1988; Gruenberg *et al.*, 1989). Importantly, the identification of an antagonistic relationship between LvsB and Rab14 suggested that a similar interaction may exist between Lyst/beige and a mammalian Rab GTPase. Interestingly the mammalian Rab7 GTPase seems to function similarly to the *Dictyostelium* Rab14 in controlling lysosome fusion. In fact, Rab7 associates mainly with lysosomes and the expression of an active mutant Rab7(Q67L) causes enlargement of lysosome size and enhances fusion (Bucci *et al.*, 2000). We can postulate that the overexpression of the inactive Rab7(T22N) mutant will rescue the phenotypes presented by CHS and beige cells.

The identification of LvsB binding partners will also suggest possible binding partners of Lyst and will contribute to the elaboration of a model that explains the molecular function of Lyst-like proteins. As described earlier in this section, the recent evidence in mammalian cells as well as preliminary data from *Dictyostelium* studies (Kristin Benson, unpublished data) suggest that Lyst proteins may regulate fusion through functional interactions with the proteins SKD1 and Lip5. We can speculate that

the overexpression of SKD1 and Lip5 in CHS/beige cells will result in phenotypic rescue.

The information we receive from this study of LvsB in *Dictyostelium,* will provide valuable insights into the understanding of Chediak-Higashi syndrome. We hope that the possible modifications of the activities of the endocytic regulators Rab7, SKD1 and Lip5 will provide a path in identifying potential therapies for CHS patients.

Chapter 5: Experimental Procedures

5.1 MATERIALS AND METHODS

5.1.1 Strains and culture

Dictyostelium discoideum wild type cells, NC4A2 and DH1 and AX2, were grown axenically in HL-5 medium supplemented with 0.6% penicillin-streptomycin (PS) (GIBCO BLR, Gaithersburg,MD) at 18^0C on Petri dishes. Mutant or transfected cell lines were grown in medium supplemented with 5μg/ml blasticidin (Calbiochem, EMD Biosciences, Inc. La Jolla, CA) or 10μg/ml G418 (Geneticin, Gibco,BRL, Grand Island, NY,USA)

5.1.2 Tagging LvsB with a knock-in construct

The LvsB knock-in construct was designed similarly to the GFP-LvsA construct (Gerald et al., 2002). In this construct, in addition to the GFP coding sequence, the TAP tag sequence was also fused to the *lvsB* gene. The vector used to make the LvsB knock-in construct is the pTAP-GFP (constructed by Joe Mireles). The TAP-GFP coding sequence was fused in frame with approximately 1.25kb of the *lvsB* open reading frame. For the amplification of the *lvsB* coding sequence starting at amino acid 48 we used the following primers AO-483 (5'CGGATCCTGGAATACGTATACACAAACAGTAGTTTATCAAG3') and AO-484 (5'CCTCGAGGATAAACATGATTTTGACATTATTTGAGCG3'). The *lvsB* coding sequence was inserted into the BamHI and XhoI sites of the pTAP-GFP vector. The fusion construct was placed under the control of the *Dictyostelium* actin-6-promoter. The construct also contained 0.9kb of *lvsB* untranslated sequence. For the amplification of

the untranslated region the primers AO-493 (5'CGCGGCCGCGTTTCCAAATGCTTTCTTTTTGAGACTCTG3') and AO-494 (5'GTCTAGAGTCGACACTAGTGGAATTTTCTATTGCCCCTTATTAATTTTG 3') were used. The untranslated region was inserted into the NotI and XbaI sites of the pTAP-GFP vector. A blasticidin-resistance cassette was included as a selectable marker and was inserted into the SalI and SpeI sites that were included with the amplification of the *lvsB* untranslated sequence.

The final construct was linearized using NotI, XhoI sites and was introduced into wild-type (DH1) cells by electroporation using a gene pulser (75kv, 25μF) (BioRad, Hercules,CA) Transformants were selected in HL-5 medium containing 5μg/ml blasticidin. Resulting clones were screened with the polymerase chain reaction (PCR) and western blot analysis.

For PCR screening, the following primers were used: A0-488 (5' GTTTCCAAATGCTTTCTTTTTGAGACTCTG 3'), located in the 5'UTR of *lvsB* gene, AO-293 (5'CCATTACCTGTCCACACAATCTGCCC3') found in the GFP sequence, AO-484 (5'CCTCGAGGATAAACATGATTTTGACATTATTTGAGCG 3'), AO-492 (5'GAATCTTGATAAACTACTGTTTGTGTATACGTATTCC3'), both located in the *lvsB* coding region and lastly AO-486 (5' TTGTTGATGGTATTGATAGCCGTGG3') found in the beginning of the *lvsB* coding region. The primers were used in the following combinations: A0-488 with AO-486 (set1), A0-488 with AO-484 (set2), AO-293 with AO-484 (set3), and AO-488 with AO-492 (set4).

5.1.3 Western blot analysis

Positive cells lines expressing the TAP-GFP-LvsB were screened by western blot analysis. *Dictyostelium* cell lysates ($2x10^6$cells/lane) were run on a 7.5% SDS polyacrylamide gel. The gel was transferred onto a nitrocellulose membrane and blotted

with 1.1000 dilution of an anti-GFP polyclonal antibody developed in our laboratory. The blot was probed with an HRP-conjugated goat anti-rabbit secondary and the signal was detected using an ECL kit (Pierce Biotechnology, RockFord, IL, USA).

The expression level of the C-terminal domains of LvsB (GFP-BEACH-WD, GFP-PH-BEACH-WD) was detected by western blot analysis using an anti-GFP polyclonal antibody developed in our laboratory using the same conditions as described above.

5.1.4 Labeling of endosomes and phagosomes

For visualization of LvsB on phagosomes, expressing cells ($2x10^6$ cells/ml), were allowed to attach to coverslips and were incubated with 200µl of a 1:4000 dilution of 0.9µm carboxylate-modified polystyrene beads (Sigma-Aldrich, Inc. St Louis, MO, USA) in PDF. Cells were allowed to phagocytose the beads for 90 minutes, briefly washed in PDF for 2 times, followed by methanol fix.

For evaluating the morphology of endosomes images were taken in live cells. Cells ($1x10^6$cells/ml) were allowed to adhere to well chambers (Nalge-Nunc Int., Rochester, NY) and were incubated in low fluorescence media (http://dictybase.org/techniques/media/lowflo_medium.html) to reduce the background for 1 hour at room temperature. Cells were then incubated with 1mg/ml TRITC-dextran (mw 64kDa;Sigma- Aldrich Inc. St Louis, MO, USA) diluted in low fluorescence media. Cells were washed twice with the low fluorescence media and were imaged.

For the visualization of acidic compartments and for the colocalization of vacuolin with the acidic endosomes, control, LvsB-null, vacuolinB-null or cells expressing GFP-vacuolinB ($1x10^6$cells/ml), were allowed to attach on well chambers and were incubated in low fluorescence media as described above. Cells were then incubated with 1µM Lysotracker Red (Molecular Probes, Eugene, Oregon, USA) diluted

in low fluorescence media. Cells were washed once with the low fluorescence media and were imaged.

5.1.5 Colocalization of vacuolin with the fluid phase marker

Expressing cells ($1x10^6$cells/ml) were allowed to adhere to well chambers and were incubated in low fluorescence media, as described above. Cells were incubated with 1mg/ml TRITC-dextran diluted in low fluorescence media for 10 minutes followed by and then briefly rinsed twice in low fluorescence media. Images of living cells were taken for 60 minutes after the initiation of the chase in both the GFP and Texas Red Filters. Images were separated for analysis according to the chase time. Cells that contained vacuolin positive vesicles that were colocalized with the fluid phase marker were counted in both control cells and mutant cell lines. Data points from three different independent experiments were taken.

5.1.6 Cell fixation

Cells expressing ($2x10^6$cells/ml) were allowed to attach on coverslips for 15 minutes at 18^0 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 -20^0 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 immunolocalization studies the following antibodies were used: anti-vacuolin monoclonal antibody (264-79-2), anti-v-ATPase A subunit monoclonal antibody (221-35-2), monoclonal anti-p25(H72) (Charette *et al.*, 2006), monoclonal

anti-DdCP224 (Graf *et al.*, 2000; Koch *et al.*, 2006), monoclonal anti-flag(M2) (Sigma-Aldrich, St. Louis, MO) and polyclonal anti-Rh-50 (Benghezal *et al.*, 2001). For immunocolocalization studies, primary antibody was added to the fixed cells and incubated for 1 hour at 37 ^{0} C in the dark. Cells were washed four times with PBS and incubated with Texas-Red conjugated goat-anti mouse antibody or Texas-Red conjugated goat anti-rabbit or FITC conjugated goat anti-mouse (30µg/ml; Molecular Probes, Eugene,OR) for 1 hour at 37 ^{0} C in the dark. Cells were washed four times with PBS, rinsed briefly in ddH_20 and mounted on microscope slides as described above.

For nuclear staining, expressing cells were fixed as described previously and incubated with 4,6-diamidino-phenylindole (DAPI) at concentration 0.1µg/ml in PBS for 10 minutes followed by four washes with PBS. Lastly cells were mounted on microscope slides are described above.

Cells were imaged on an inverted Nikon Microscope TE200 (Nikon Instruments, Dallas, TX, USA). GFP, Texas Red and DAPI filters were used. Images were acquired on a Photometrics cooled CCD camera and processed using the Metamorph 5.0 software. When visualizing TAP-GFP-LvsB due to the low fluorescence levels, prolonged exposures of 800ms were used.

5.1.7 Construction of Rab plasmids

The r*ab7* gene was amplified using *Dictyostelium* cDNA and using primers selected from sequence (DDB0191507; http://dictybase.org) (Buczynski *et al.*, 1997). The primers used for this amplification were the following: AO-579 (5'CGCGGATCCGCTGCTGCTGCTGCTGCTATGGCCACAAAGAAAAAGGTTTT ATTAA 3') and AO-580 (5' CGCTCTAGATTAACAACAACCTGATTTAGCTGGTTG 3'). The resulting product was subcloned into the pTX-GFP expression vector (Levi *et al.*, 2000) at the BamHI,

XbaI sites. Using an EcoRI site from the pTX-GFP vector and the XbaI site, the *rab7* gene was also subcloned into the mRFP-mars vector (Fischer *et al.*, 2004).

The *rab5A* gene was amplified using Dictyostelium cDNA and using primers selected from sequence (DDB0229401; http://dictybase.org). The forward primer used for the amplification of the *rab5A* gene was AO-581(5"CGCGGATCCGCTGCTGCTGCTGCTGCTATGAATAATAATAATAAGATA TTTCAATTTAAACTTGTA 3'). The reverse primer used was AO-582 (5' CGCTCTAGATTAGTTCAACATTTGTTTTTCTTTCCAGTG 3'). The resulting product was subcloned into pTXGFP at the BamHI, Xba1 sites (Levi *et al.*, 2000).

The pvEII-Rab14Q67L and pvEIIRab14N121I plasmids that contain the mutant forms of Rab14 (provided by Cardelli's lab) were used for the construction of the pTXflag-Rab14Q67L and pTXflag- Rab14N121I vectors. The primers used for the amplification of the *rab14* were the following: AO-685 (5' GCGGATCCATGTCATTTCCATATGAATATATATTTAAATACATCATTATTGGT G3') and AO-686 (5'GCTCTAGATTAACAAGAACATTTACTGGCATCTTGAGGTTTATC3'). The resulting product of approximately 0.6kb was subcloned into the pTX-flag expression vector (Levi *et al.*, 2000) at the BamHI, XbaI sites.

5.1.8 Construction of plasmids for LvsB domain analysis

The BEACH-WD fragment of LvsB was amplified using the following primers: AO-465 (5'GGATCCGTTGATTATGCAGAGGTTCATGG3') and AO-547 (5' CGGATCCTTAACCAAGAACAATTGAAGAATAACCATCA3'). The resulting fragment of approximately 2kb plasmid was subcloned in the pTX-GFP plasmid at the

single BamHI site. The PH-BEACH-WD fragment of LvsB was cloned in the pTX-GFP plasmid by Carter. A. Mitchell.

A construct was designed to replace the WD domain at the genomic locus of *lvsB* with the G418 resistance cassette using homologous recombination. A sequence starting upstream of the BEACH domain and ending at the end of the BEACH domain (~1.1kb) was amplified using the primers AO-670 (5'GCGGATCCCTCGAGATGATATAATTGAAATTCATAAGAGAAGACATGTTT TAAAGAATAATGC 3') and AO-671 (5' GCTCTAGAGGGGCCCTAGGCGGCCGCTGACCAATTCTTTTTTGGATGTGGGT TTGTAAAGATTTG 3'). This sequence was cloned into the pA15TX vector at the BamHI and XbaI sites. The sequence of the forward primer AO-670 contained a BamHI and an XhoI site to be used at subsequent cloning steps. The sequence for the reverse primer AO-671 contained a NotI site, a stop codon, an ApaI site and an XbaI site. Subsequently, a sequence within the WD domain (~ 1kb) was amplified using the primers AO-672 (5' GCGGATCCGTCGACAACCTCTTACATTATGTTTCAATATCGTTCACCC 3') and AO-673 (5' GCCTCGAGCAAGAACAATTGAAGAATAACCATCAAAAGAACGAC 3'). The AO-672 primer included a BamHI and a SalI site. The primer AO-673 included an XhoI site. The resulting WD product was subcloned upstream of the BEACH region into the pA15TX vector into the BamHI and XhoI sites. The final construct was linearized using the single XhoI site and was introduced into the TAP-GFP-LvsB cell line (7D1).

The resulting clones were selected with PCR for the absence of the WD-domain. The primers used for the screening of clones containing the deletion were the following: AO-546 (5' CGATAACTGGATTGATTTACTATTTGGTTATAAACAACAAGGTGA

3') located in the BEACH domain, AO-547 (5' CGGATCCTTAACCAAGAACAATTGAAGAATAACCATCA 3'), located at the end of the WD domain, AO-553 (5'TCCATACCAGTTATTAAAAGATTAGTATTAACACCTTG 3'), located within the WD domain, AO-461 (GAATTCAAACTTGGTGAAAAAGTAAAAGAAGTTTTTAAATG 3'), located at the beginning of the pH domain, and AO-633 (TGTACCACCTGATAAGACGACATTACCG 3'), located in the pA15TX plasmid. The primers were used in the following combinations: AO-356, AO-359 (Set1, PCR positive control), AO-546, AO-553 (Set2), AO-461, AO-633 (Set3) and AO-546, AO-547 (Set4).

5.1.9 Endosome fusion assay using two fluid phase markers

Control and null cells ($1x10^6$cells/ml) or expressing cell lines were allowed to adhere to well chambers and were incubated in low fluorescence media, as described above. Cells were incubated with 4mg/ml FITC-dextran (mw 77kDa; Sigma- Aldrich Inc. St Louis, MO, USA) diluted in low florescence media for 5 minutes. Cells were then briefly rinsed two times in low fluorescence media followed by a chase for 30 minutes. Subsequently cells were incubated with 2mg/ml TRITC-dextran diluted in low fluorescence media for 5minutes washed two times in low fluorescence media and visualized immediately. Images of living cells in different fields were taken continuously for 10 minutes in both the GFP and Texas Red Filters. Fused (yellow) vesicles were counted and compared to the total vesicles (red and green) in both control, null or expressing cells. Vesicle counts from two different independent experiments were taken.

5.1.10 Tandem affinity purification of LvsB

Cells (2000ml) expressing TAP-GFP-LvsB were grown to a concentration of $3x10^6$cells/ml. Cells were harvested by centrifugation at 3000rpm for 10 minutes. The resulting pellet was washed with isolation buffer pH 6.5 (100mM MES pH 6.5, 500mM KoAc pH6.5, 0.5mM MgCl2, 1mM EGTA, 1mM DTT, 0.02% NaN3). Cells were centrifuged at 3000rpm for 3 minutes and resuspended to a concentration of $2x10^8$cells/ml into modified isolation buffer pH 7.5 (20mM TES pH 7.5, 500mM KoAc pH6.5, 0.5mM MgCl2, 1mM EGTA, 1mM DTT, 0.02% NaN3). Protease inhibitor (1%) (Fungal Protease Inhibitor cocktail, Sigma-Aldrich, St. Louis, MO) and leupeptin (0.1%) were added to the resulting supernatant. Lysis was performed using a nebulizer (BioNeb cell and DNA disruptor) for 3 times. Cell-debris was recovered by centrifugation at 5000rpm for 3 minutes and supernatant was collected. NaCl and NP40 were added to the supernatant at 100mM concentration and 0.1% respectively. IgG Sepharose 6 Fast flow beads (GE Health Care Bio-Sciences, Uppsala, Sweden) (200µl) were prewashed with IPP50 buffer (Tris-HCL 10mM, pH 8.0, NaCl 150mM, NP-40 0.1%) and incubated with supernatant for 2 hours at 4 degrees. Upon incubation supernatant was allowed to pass through a Poly-Prep chromatography column (BioRad, Hercules, CA). Beads were washed with modified IPP50 buffer (Tris-HCL 10mM, pH 8.0, NaCl 175mM, NP-40 0.1%). The resulting IgG beads were removed to an eppendorf tube and the protein was eluted by boiling in hot sample buffer for 5 minutes.

5.2 PLASMIDS AND CELL LINES

Table 5.1: Plasmids and cell lines used in this study

Plasmids	Description

TAP-GFP-LvsB	0.9 kb of *lvsB* untranslated sequence cloned upstream of the blasticidin resistance cassette and 1.25 kb of *lvsB* coding sequence cloned downstream of the TAP-GFP sequence, used for LvsB knock-in construct
RFP-Rab7	Full length Rab7 subcloned from PTX-GFP plasmid into the mRFP-mars-pBSRH plasmid (Fischer *et al.*, 2004), BSR resistance
GFP-vacuolinB	*Dictyostelium* vacuolin cDNA starting at amino acid 25 cloned into a pDNeoII based vector, N-terminal GFP tag, G418 resistance (Jenne *et al.*, 1998)
VatM-GFP	Large subunit of *Dictyostelium* v-ATPase proton pump, cloned into pDXA-3H, C-terminal GFP tag, G418 resistance (Clarke *et al.*, 2002a)
GFP-Rab14	Full length *Dictyostelium* Rab14 cloned into pVEII, N-terminal GFP tag, G418 resistance (provided by Cardelli lab)
FLAG-Rab14Q67L	Full length *Dictyostelium* Rab14 with amino acid 69 mutated, (CAA to TAA), N-terminal flag tag, G418 resistance

FLAG-Rab14N121I	Full length *Dictyostelium* Rab14 with amino acid 121 mutated, (AAC to ATC), N-terminal flag tag, G418 resistance
GFP-BEACH-WD	*Dictyostelium* LvsB BEACH and WD domains cloned into the pTXGFP vector, N-terminal GFP tag, G418 resistance
GFP-PH-BEACH-WD (by Carter.Mitchell)	*Dictyostelium* LvsB PH, BEACH, WD domains cloned into the pTXGFP vector, N-terminal GFP tag, G418 resistance
pA15TX-BEACH-WD	Region including the BEACH domain (1.1kb) and WD domain (0.9kb) of LvsB cloned upstream of G418 sequence in the pA15TX vector, used for WD-deletion construct.

Cell lines	**Description**
NC4A2	Wild-type axenic strain, grows in HL-5
AX2	Wild-type axenic strain, grows in HL-5
LvsB-null (B1B11)	Derived from NC4A2 parent strain, *lvsB* gene disrupted by the blasticidin gene, cassette (Harris *et al.*, 2002b), grows in HL-5 supplemented with 5µg/ml Blasticidin

VacuolinB-null	Derived from AX2 parent strain, *vacuolinB* gene disrupted by the blasticidin gene cassette (Jenne *et al.*, 1998), grows in HL-5 supplemented with 5µg/ml Blasticidin
DH1	Derived from Ax3 wild-type axenic strain, with the pyr5-6 gene deleted, uracil oxotroph, grows in HL-5 and FM media
TAP-GFP-LvsB (7D1)	Derived from DH1 parent strain, TAP-GFP sequence recombined into the N-terminus of lvsB coding sequence, grows in HL-5 supplemented with 5µg/ml Blasticidin
TAP-GFP-LvsB(-WD)(2H6)	Derived from 7D1 parent strain, WD domain is disrupted by a G418 resistance cassette, grows in HL-5 supplemented with 5µg/ml Blasticidin and 10µg/ml G418.

Appendix

MISCELLANEOUS EXPERIMENTS

The BEACH-WD domains of LvsB localize to the cytosol

The most conserved region among BEACH proteins is the C-terminus that contains the BEACH-WD domains. It is possible that this region contains the LvsB membrane localization signal. To understand whether the BEACH and WD domains of LvsB are necessary for the localization of the protein on late lysosomes and post-lysosomes we generated a GFP fusion construct that contained both domains. This construct was transformed in both wild-type and LvsB-null cells and was expressed at high levels as indicated by western blot analysis (Figure A-1 A,B). This construct expressed uniformly in cells and exhibited cytosolic localization in both wild-type cells and LvsB-null cells (Figure A-2 A,B). Thus, we concluded that the BEACH and WD domains are not sufficient to localize LvsB on vesicles.

The PH-BEACH-WD domains of LvsB localize to the cytosol, nuclear envelope and the MTOC

Since the BEACH-WD domain did not target LvsB to endocytic compartments we investigated the contribution of the PH domain to LvsB localization. To accomplish that, we generated a GFP-fusion construct (made by Carter A. Mitchell) that included the three C-terminal domains of LvsB (PH, BEACH,WD). This construct was expressed at high levels and exhibited a cytosolic localization in both wild-type and LvsB-null cells (Figure 2 C,D). In addition, cells expressing GFP-PH-BEACH-WD fixed and stained with 4'-6-Diamidino-2-phenylindole (DAPI) showed that the C-terminal domains of LvsB localized on the nuclear envelope (Figure A-3 A,B). In fact, this

construct also localized on the microtubule organizing center (MTOC) as indicated by colocalization with the centrosome marker DdCP224 (Figure A-3 C,D) (Graf *et al.*, 2000; Koch *et al.*, 2006). Few reasons can account for the mislocalization of this construct to the nuclear envelope and the MTOC. It is likely that this mislocalization is caused by the overexpression and possibly degradation, as indicated by western blot analysis (Figure A-1 C). Alternatively, it is possible that the exposed PH domain exhibits affinity for nuclear lipids. More experiments are required to understand this mislocalization, including the expression of the PH domain alone.

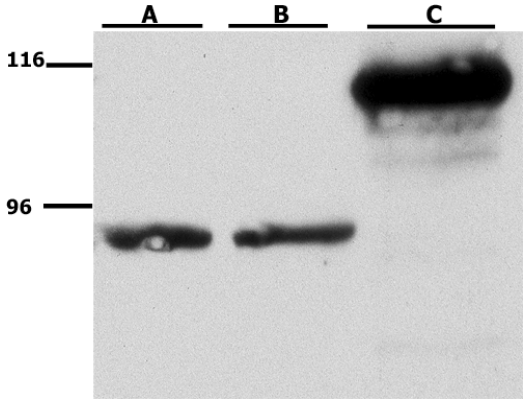


Figure A-1: Expression levels of the BEACH-WD and PH-BEACH-WD domains

Western blot analysis with anti-GFP antibody of wild-type (A) and LvsB-null (B) cells expressing the BEACH-WD domains detects a single band of the predicted molecular weight. Western blot analysis of wild-type (C) cells expressing the PH-BEACH-WD domains detects a band of the predicted molecular weight. Degradation bands are also visible. The expression of the PH-BEACH-WD construct is much higher compared to the BEACH-WD construct.

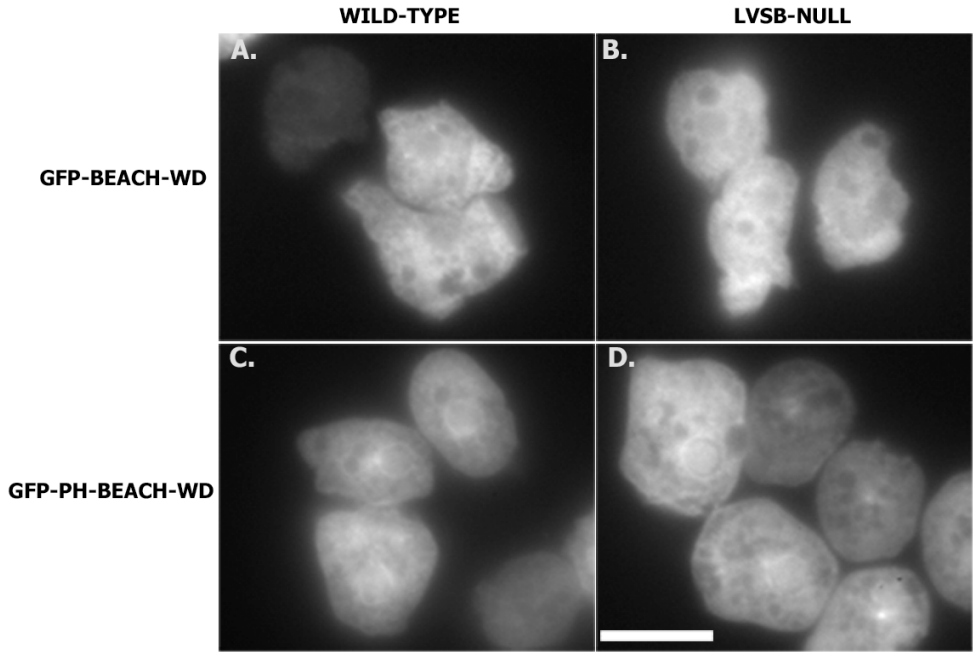


Figure A-2: C-terminal domains of LvsB exhibit a cytosolic localization

The constructs GFP-BEACH-WD (A, B) or GFP-PH-BEACH-WD (C, D) were expressed in wild-type and LvsB-null cells. Both constructs exhibited cytosolic localization suggesting that the C-terminal region of LvsB is not sufficient to target LvsB to the endocytic compartments. Bar, 10µm

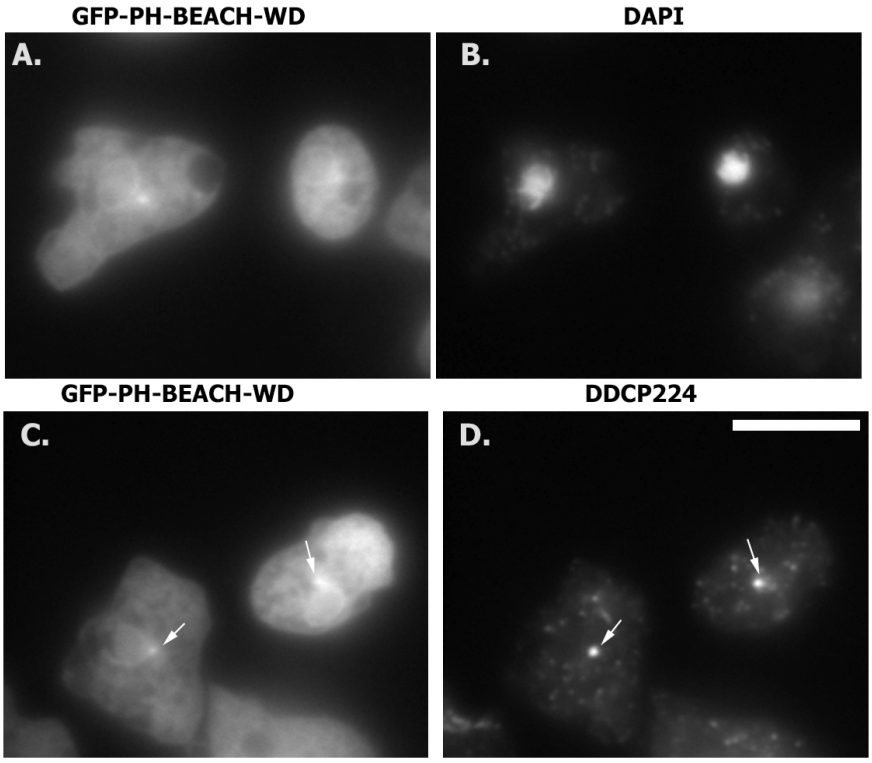


Figure A-3: The PH-BEACH-WD domains localize on the nuclear envelope and the MTOC

Wild-type cells expressing the PH-BEACH-WD domains were fixed and stained with 4'-6-Diamidino-2-phenylindole (DAPI) (A,B) and with a monoclonal antibody against the *Dictyostelium* centrosomal marker DdCP224 (C,D) (Graf *et al.*, 2000; Koch *et al.*, 2006). GFP-PH-BEACH-WD construct localized on the nuclear envelope (A,B) and colocalized with the centrosomal marker on the microtubule organizing center (MTOC) (C,D). Bar,10μm

Expression of the C-terminal domains of LvsB does not rescue the LvsB-null phenotype

To test the effects of the overexpression of the C-terminal domains of LvsB on endosomal morphology, we expressed the two C-terminal constructs in wild-type cells and LvsB-null cells and examined the morphology of the endosomes using TRITC-dextran in living cells. The visualization of the PH-BEACH-WD domain on the nuclear envelope and the MTOC in living cells is more difficult due to the very high cytosolic signal. Wild-type cells expressing GFP-BEACH-WD or GFP-PH-BEACH-WD that were incubated with TRITC-dextran for 1 hour exhibited endosomes of normal size and morphology (Figure A-4 A,B,E,F). This suggested that the expression of these two constructs did not cause a dominant negative effect when expressed in wild-type cells. It is possible that these two constructs did not cause a dominant negative effect due to their inability to be targeted on endocytic vesicles. In addition, the expression of GFP-BEACH-WD or GFP-PH-BEACH-WD did not cause any changes to the LvsB-null phenotype (Figure A-4 C,D,G,H). Therefore, we concluded that the C-terminal region of LvsB is not sufficient for the localization and function of LvsB.

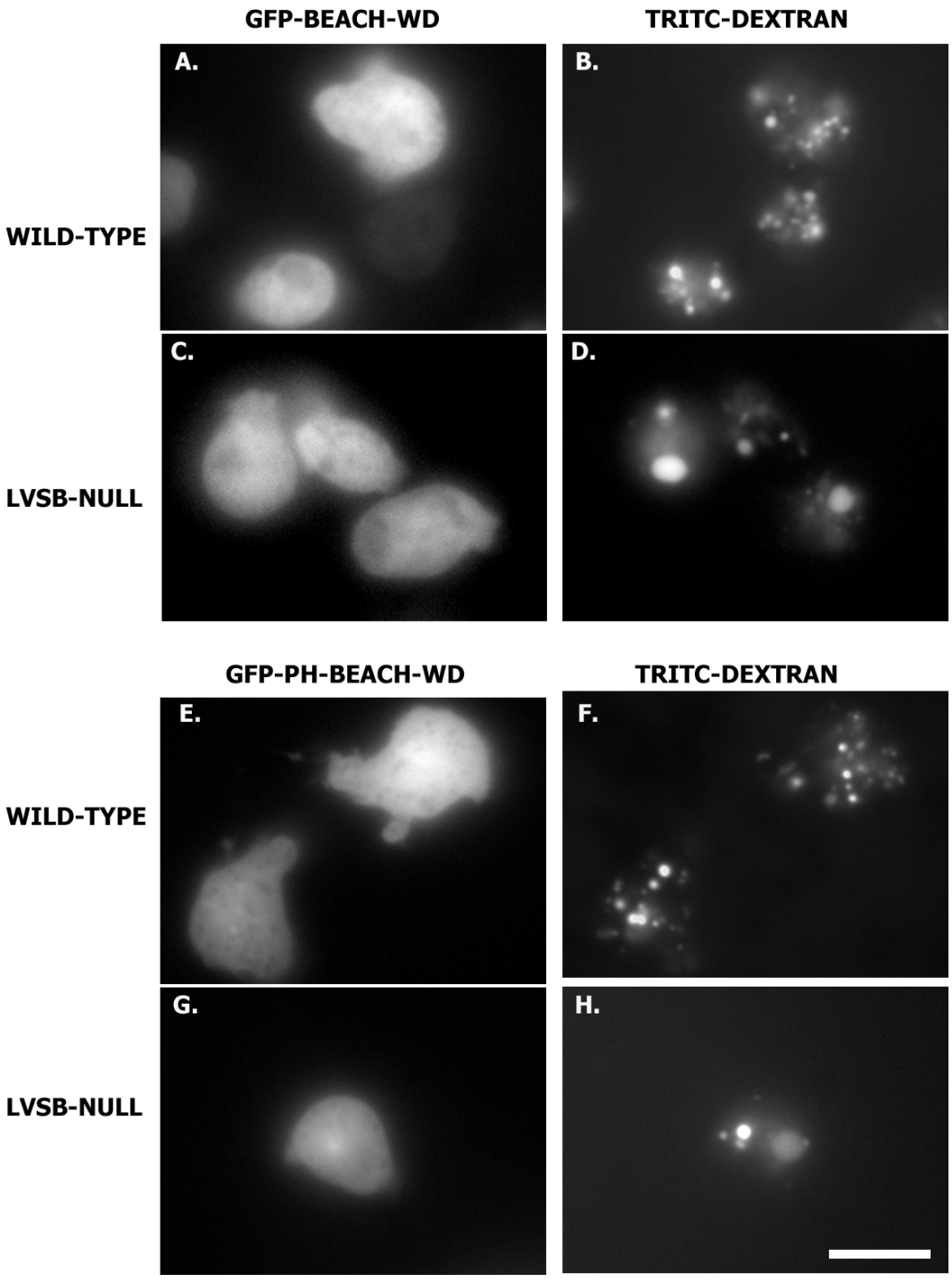


Figure A-4: Overexpression of the C-terminal region of LvsB does not alter the endosome morphology in wild-type and LvsB-null cells

The constructs containing the GFP-BEACH-WD and or GFP-PH-BEACH-WD were expressed in wild-type and LvsB-null cells and incubated with TRITC-dextran for 1 hour to evaluate the endosome morphology. Wild-type cells expressing GFP-BEACH-WD (A) or GFP-PH-BEACH-WD (E) exhibited endosomes of normal morphology (B,F respectively) that were indistinguishable from endosomes in non-expressing cells (data not shown). In addition, LvsB-null cells expressing GFP-BEACH-WD (C) or GFP-PH-BEACH-WD (G) showed enlarged endosomes (D,H respectively) that were similar to endosomes found in LvsB-null cells (data not shown). Bar,10μm

Disruption of the C-terminal WD domain of LvsB leads to an unstable protein

The lack of the LvsB localization signal on the C-terminal region that is most conserved between BEACH proteins, suggested that the localization signal resides on the N-terminus. To test this possibility we generated a construct lacking the WD motif at the genomic locus of *lvsB* gene. To achieve that, we made a construct where the BEACH and WD domains flanked the G418 resistance cassette. We targeted this construct for recombination into the GFP-LvsB knock-in cell line (7d1 in materials and methods). Deletion of the WD domain in this cell line allows for visualization of the truncated protein. Cell lines that contained the correct disruption of the WD domain were selected by polymerase chain reaction (PCR). Many clones have been identified lacking the WD region. Clone 2H6 gave us the strongest PCR reactions with all sets of primers (see Materials and Methods).

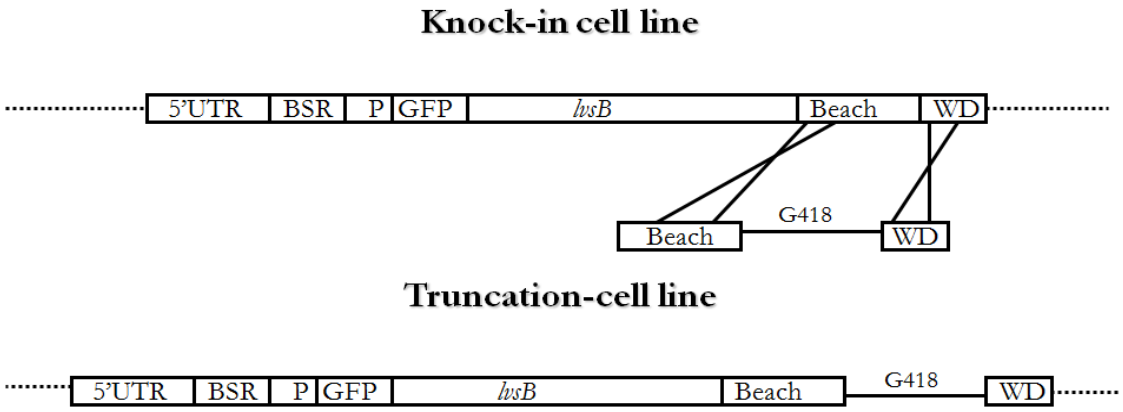


Figure A-5: Strategy to produce a stable cell line lacking the WD domain of LvsB

Diagram indicating the construct used to disrupt the WD domain of LvsB at the genomic locus with the G418 resistance cassette. A construct that contained regions of the BEACH (~ 1.1kb) and WD (~1kb) domains flanking a G418 resistance cassette was introduced into the GFP-LvsB knock-in cell line. The cell lines containing the WD deletion were selected by PCR.

Western blot analysis of the deletion clones, using anti-GFP antibody, revealed that the expression level of the TAP-GFP-LvsB(-WD) truncated protein is much lower that the TAP-GFP-LvsB full length (data not shown). In fact, the GFP-signal of the deletion clones was undetectable, even after prolonged exposures, thus preventing the visualization of the localization of the truncation construct. Evaluation of the endosome morphology by incubating with TRITC-dextran for 1 hour revealed that the deletion clone (2H6) contained large endosomes similar to endosomes seen in LvsB-null cells. This is likely caused by the low expression level of the truncation construct. Since the C-terminal deletion lead to the production of unstable protein, N-terminal deletions will be

more useful for the study of the role of the LvsB N-terminal region in the localization and function.

Tandem affinity purification of LvsB

Currently, the molecular mechanism underlying Lyst function is not known and no significant binding partners have been identified, probably due to the large size and the low expression levels of the protein. Since *Dictyostelium* presents an easier system for manipulation of BEACH proteins, identifying binding partners of LvsB is crucial to help understand the mechanism of the regulation of membrane fusion by LvsB and Lyst-like proteins. In order to accomplish the purification of full-length LvsB, we established a cell line (7D1, see Materials and Methods) that expressed the TAP and GFP tags at the amino terminus of the LvsB protein. Using cells expressing TAP-GFP-LvsB, we performed a large scale tandem affinity purification (TAP). A control experiment with cells expressing TAP-GFP was performed in parallel. Cell extract was passed through an IgG column and LvsB was detected by western blot analysis to bind to the IgG beads successfully (data not shown). Unfortunately, the second step of the TAP-tag purification that involves the cleavage of LvsB from the IgG beads using TEV protease was unsuccessful. It is likely that LvsB remained bound to the IgG beads because the TEV cleavage site was masked. Since LvsB remained bound to the IgG beads we optimized the washing conditions (see Materials and Methods) and eluted the protein from the IgG beads using reducing hot sample buffer. By performing a large-scale purification the full length LvsB was purified in an amount that was visible in a Coomassie stain. The eluate from the IgG beads, containing the full length LvsB and potential binding partners was analyzed by mass spectrometry (Figure A-6). The eluate recovered from the IgG beads was not very pure and only few distinct bands were

visible that were not present in the negative control. These bands were cut out from the gel and analyzed (bands 1,2,3 Figure A-6). Additional areas 4,5,6,7 (Figure A-6) were also cut out from the gel and analyzed for potential binding partners. The eluate from the negative control experiment was not analyzed due to the increased cost of the mass spectrometry.

Bands 1 and 2 were identified as LvsB. Band 2 most likely represents a degradation product. This result suggested that our purification procedure was successful in isolating the full-length LvsB. This purification is significant because this is the first time that a full-length BEACH protein was isolated from any system. Band 3 was identified as putative fatty acid synthase (entry DDB0230068 in http://dictybase.org).

The abundant protein below band number 3 indicated by the arrow represents myosin II heavy chain, a non-specific contaminant of this preparation, which was also present in the negative control (data not shown). In the gel area 4 we identified myosin II heavy chain (entry DDB0191444 in http://dictybase.org) that most likely represents a degradation product. In area 4 we also identified pyr1-3 (entry DDB0201646 in http://dictybase.org). In gel area 5 we identified a putative protein (entry DDB0215923 in http://dictybase.org) and clathrin heavy chain (DDB0185029 in http://dictybase.org). In area 6 we could not identify any proteins clearly. Lastly, in area 7 we identified a phosphoribosylformylglycinamide synthase (DDB0230086 in http://dictybase.org) and a Pleckstrin (PH) homology domain-containing protein (DDB0191188 in http://dictybase.org). The areas below area 7 could not be analyzed due to the large amounts of background IgG.

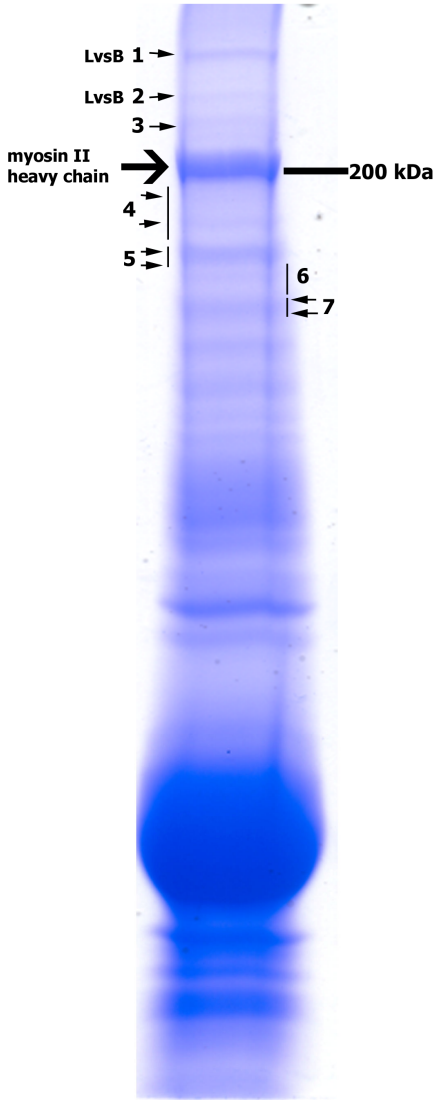


Figure A-6: TAP-tag purification of LvsB

Full length TAP-LvsB was eluted from the IgG beads using reducing hot sample buffer. The final elution was resolved on a 7.5% gel and stained followed by coomasie blue. Specific bands (1,2,3) and gel areas (4,5,6,7) were subjected the mass spectrometry following in-gel digestion. Band 1 was identified as LvsB (410kDa). Band 2 was identified as LvsB and probably represents a degradation product. Band 3 was identified as a putative fatty acid synthase of approximate molecular weight 290kDa. The

abundant protein present below band number 3, indicated by the arrow represents myosin II heavy chain, a non specific contaminant of this preparation. In area 4 two proteins were identified that included myosin II-heavy chain and Pyr-1-3 of size 240kDa and 243kDa respectively. Area 5 included clathrin heavy chain (194kDa) and a putative protein (188kDa). No proteins were identified in area 6. Lastly, area 7 included a phosphoribosylformylglycinamide synthase (150kDa) and a Pleckstrin-homology (PH) domain-containing protein (139kDa).

Western blot analysis using antibodies against myosin II and clathrin heavy chain, of the eluate in Figure A-6 and the negative control identified that these proteins were also present in the negative control (data not shown). This suggested that our eluate analyzed by mass spectrometry was not pure enough for identification of true binding partners. Further optimization of the washing conditions will be necessary to obtain a pure eluate for analysis.

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