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## MOLECULAR MECHANISMS OF INWARD AND OUTWARD BUDDING FROM MULTIVESICULAR ENDOSOMES

Monica Gireud Goss

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# MOLECULAR MECHANISMS OF INWARD AND OUTWARD BUDDING FROM MULTIVESICULAR ENDOSOMES

by

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**MOLECULAR MECHANISMS OF INWARD AND OUTWARD  
BUDDING FROM MULTIVESICULAR ENDOSOMES**

A

DISSERTATION

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

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Houston, Texas

May 2017

## **Dedication**

To God for all the wonderful blessings in my life and to my parents for all the support, guidance, and love you have provided for me my entire life. I also want to dedicate this to my grandparents, for being the pillars of my family.

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First and foremost, I would like to thank my family for the unconditional love and support. My parents taught me to dream big and have been my biggest support system, even when my career goals have changed. They have taught me to be independent, career-driven, and most importantly, family focused. I also want to thank my brothers, Fer and Alberto. They have always been there for me, through good and bad and have always supported my dreams. You are my role models. I want to thank my sister in law, Bonnie, for showing me what it means to have a supportive sister.

I would not be where I am without the love and support of my husband, Dayne. We started dating my first week of graduate school, and ever since that day you have provided so much love and happiness. I can't thank you enough for going through this entire process with me (for better or worse, right? 😊) and for lifting me up when I felt defeated. I look forward to spending my life with you (and our growing family!).

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# **MOLECULAR MECHANISMS OF INWARD AND OUTWARD BUDDING FROM MULTIVESICULAR ENDOSOMES**

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Regulating the residence time of membrane proteins (e.g. transporters, ion channels, receptors) on the cell surface can modify their response to extracellular cues and allow for cellular adaptation to environmental conditions. The fate of membrane proteins that are internalized from the plasma membrane and arrive at the limiting membrane of the late endosome/multivesicular body (MVB) is dictated by whether they remain on the limiting membrane, bud into internal MVB vesicles, or bud outwardly from the membrane. The molecular details underlying the disposition of membrane proteins that transit this pathway and the mechanisms regulating these trafficking events are unclear. We established a cell-free system that reconstitutes budding of membrane protein cargo into internal MVB vesicles and onto vesicles that bud outwardly from the MVB membrane. Both budding reactions are cytosol-dependent and supported by *Saccharomyces cerevisiae* (yeast) or *Drosophila melanogaster* (fly) cytosol, providing a system amenable to genetic manipulation. We observed that inward and outward budding are mechanistically distinct but may be linked, such that inhibition of inward budding enhances outward budding.



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## **Abbreviations**

Adaptor Protein-2 complex (AP-2)

ADP-ribosylation factor (ARF)

Angiotensin Type 1 Receptor (AT1R)

Adenosine Tri-Phosphate (ATP)

Bin-amphiphysin-rvs (BAR)

Clathrin Coated Vesicles (CCV)

Coatomer (COPI)

Dominant Negative Rab11 (Rab11S25N)

Endosomal sorting complexes required for transport (ESCRT)

Endocytic Recycling Compartment (ERC)

Epidermal Growth Factor Receptor (EGFR)

Fibroblast Growth Factor Receptor 4 (FGFR4)

Guanosine Tri-Phosphate (GTP)

Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs)

Homogenization Buffer (HB)

Major histocompatibility complex class I (MHC-1)

Multivesicular Body (MVB)

Nanosight Tracking Analysis (NTA)

Phosphatidylinositol 3-phosphate (PI3P)

Phosphatidylinositol-4,5-bi-sphosphate (PtdIns(4,5)P<sub>2</sub>)

Signal-transducing adaptor molecule (Madsen et al.)

Vacuolar protein sorting (Vps)

# Chapter 1. Introduction

## *1.1 Endocytosis of membrane proteins*

Membrane proteins (e.g. receptors, ion channels, and transporters) reside on the plasma membrane and respond to signals from the extracellular environment that affect global cellular processes such as growth, proliferation, and differentiation. The removal of membrane proteins from the cellular surface tunes environmental signaling by preventing their continued activation by extracellular molecules, although the termination of their signaling activity may not occur until their lysosomal degradation (Gruenberg, 2001; Sorkin and von Zastrow, 2009; Sun et al., 2010).

The canonical endocytic pathway for cell surface transmembrane proteins begins with their internalization into vesicles that bud from the plasma membrane and fuse with each other or with early endosomes (Fig. 1.1) (Gruenberg, 2001; Sorkin and von Zastrow, 2009). Early endosomes may mature into late endosomes and subsequently into multivesicular bodies (MVBs). MVBs are formed when the endosomal limiting membrane invaginates producing vesicles that separate from the limiting membrane resulting in an organelle containing internal vesicles (Eden et al., 2012; Futter et al., 1996; Gruenberg, 2001; Lemmon and Traub, 2000; Sorkin and von Zastrow, 2009; Sun et al., 2010). The fate of membrane proteins that move through this pathway depends on whether they enter the internal vesicles or remain on the limiting MVB membrane (Fig. 1.1) (Eden et al., 2012; Felder et al., 1990; Futter et al., 1996; Sirisaengtaksin et al., 2014). Membrane proteins that move from the limiting membrane into internal vesicles of MVBs are degraded upon MVB-lysosome fusion or are secreted into the extracellular space as exosomes upon



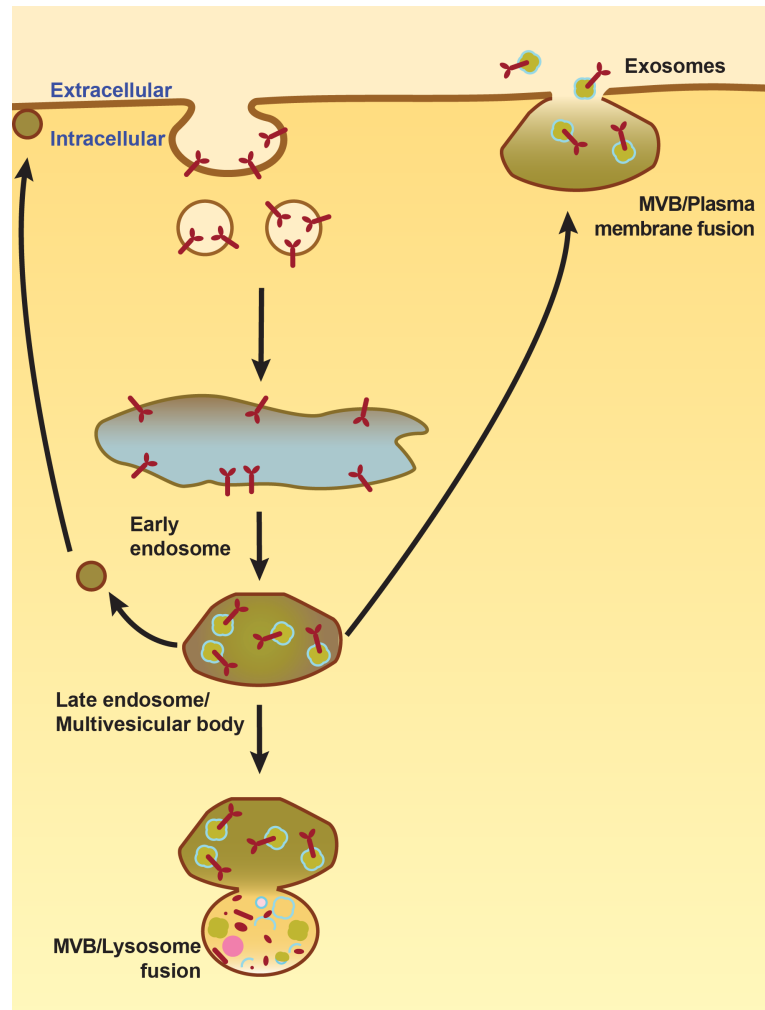
MVB-plasma membrane fusion (Corrado et al., 2013; Hurley and Odorizzi, 2012; Kowal et al., 2014). Membrane proteins that are not sorted into internal MVB vesicles can remain on the limiting membrane and become incorporated into the lysosomal membrane upon MVB-lysosome fusion (Fig. 1.1) (Eden et al., 2012; Futter et al., 1996; Katzmann et al., 2002; Piper and Luzio, 2001; Raymond et al., 1992a) or may bud from the limiting endosomal membrane for recycling to various cellular compartments (e.g. the plasma membrane, Golgi apparatus, endoplasmic reticulum) (Felder et al., 1990; Gruenberg, 2001; Sun et al., 2010). Coordination of membrane protein degradation and recycling regulates cell surface protein composition and signaling (Grant and Donaldson, 2009).

### *1.2. Epidermal Growth Factor Receptor Trafficking*

The Epidermal Growth Factor Receptor (EGFR), is a type I transmembrane protein that is a prototypical cell-surface signaling receptor (Cohen, 1962; Goh and Sorkin, 2013; Tomas et al., 2014). The EGFR contains an extracellular ligand-binding domain, a single membrane-spanning domain, and a cytoplasmic tyrosine kinase-containing domain (Olayioye et al., 2000; Riese and Stern, 1998). Binding of EGFR to its ligand promotes its dimerization and auto-phosphorylation of the tyrosine residues on the cytoplasmic tail (Chung et al., 2010). The auto-phosphorylation activates the downstream signal transduction pathways (Olayioye et al., 2000; Riese and Stern, 1998). EGFR activation results in its internalization via clathrin-mediated endocytosis and subsequent movement through the endocytic pathway resulting in lysosomal degradation. An alternative pathway for EGFR

following internalization allows escape from lysosomal trafficking and recycling back to the plasma membrane for further signaling.

Alterations in membrane protein trafficking can alter cellular responses to ligand activation. For example, a truncated EGFR mutant, EGFR<sub>vIII</sub>, is expressed in 50% of glioblastoma tumors (Frederick et al., 2000; Furnari et al., 2007; Gan et al., 2009; Heimberger et al., 2005; Sugawa et al., 1990). EGFR<sub>vIII</sub> lacks the receptor ectodomain, and is internalized independent of ligand binding and moves through the endocytic pathway (Grandal et al., 2007). It follows the same itinerary as wild-type EGFR, but constitutively recycles to the plasma membrane (Grandal et al., 2007). The enhanced recycling of EGFR<sub>vIII</sub> results in increased surface expression, and uncontrolled cell proliferation and signaling, behaviors that may underlie glioblastoma pathogenesis (Grandal et al., 2007). The mechanisms regulating the intracellular trafficking of EGFR<sub>vIII</sub> are unclear.



**Figure 1.1: The Endocytic Pathway.** Internalized membrane proteins are initially transported to early endosomes. From the early or sorting endosomes, protein cargo can be recycled and/or transported to multi-vesicular bodies (MVBs). At the MVB, a membrane protein may bud outwardly for recycling to other cellular compartments (e.g. plasma membrane), or inwardly forming the internal vesicles of the MVB whose contents will be degraded upon MVB-lysosome fusion, or secreted into the extracellular space (exosomes) upon MVB-plasma membrane fusion.

### *1.3. Mechanisms of Membrane Budding Events.*

Vesicle-mediated trafficking is a process in which cargo and membrane are transported in membrane-bounded compartments and requires the generation of membrane transport vesicles that bud from a donor compartment and fuse with an acceptor membrane. Ultimately, vesicle budding allows for the selective incorporation of cargo proteins into newly synthesized vesicles (Bonifacino and Glick, 2004). Understanding the requirements for proteins that regulate membrane budding events have largely been advanced using reconstitution experiments (Bremser et al., 1999; Matsuoka et al., 1998; Wollert and Hurley, 2010). Vesicle budding can occur with or without the aid of coat proteins, however little is known about non-coated vesicle transport. Three classical coat complexes have been identified that are composed of cytosolic proteins that associate with budding membranes. Clathrin is a coat that binds indirectly to the donor membrane through adapter proteins that bind directly to cargo (Farsad and De Camilli, 2003; Honing et al., 2005), whereas some other coats bind directly to donor membranes to facilitate cargo clustering and membrane deformation (Bonifacino and Glick, 2004; Harter et al., 1996; Hurley et al., 2010). I will briefly summarize the current view of coated-vesicle membrane trafficking mechanisms.

#### *1.3.1. Cargo selection and membrane deformation*

Cargo selection and membrane deformation must act in concert if the budded vesicles are carrying cargo. Thus, the mechanism of membrane budding requires, recognition and clustering of selected cargo for incorporation into regions of the membrane that will vesiculate (Bonifacino and Glick, 2004). The best understood

mechanism of cargo selection and membrane deformation occurs in clathrin-containing vesicle (CCV) budding where the adaptor protein-2 complex (AP-2), directly binds to activated receptors and the plasma membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) (Honing et al., 2005). After binding of AP-2, the bin-amphiphysin-rvs (BAR) domain superfamily scaffolding proteins, amphiphysin, endophilin, and epsin, are recruited (Blood and Voth, 2006; Castillo et al., 2002; Farsad and De Camilli, 2003; Hurley et al., 2010; Takei et al., 1999). BAR-domain containing proteins possess intrinsic curvature sensing and producing properties (Cui et al., 2013; Madsen et al., 2010). Membrane budding is an energetically unfavorable event (Farsad and De Camilli, 2003; Hurley et al., 2010) and thus the BAR domain family proteins are required for membrane bending, curvature, and scission (Farsad and De Camilli, 2003). The BAR domain proteins bind to PtdIns(4,5)P<sub>2</sub> (Lemmon, 2008) and initiate convex curvature and bending of the membrane to provide a platform for recruitment of the clathrin coat.

### *1.3.2. Coat Proteins.*

Clathrin is a soluble protein that does not bind cargo or membranes and is not sufficient to generate membrane curvature and bending (Dell'Angelica, 2001; Nossal, 2001). Therefore, after binding of adaptor proteins that can simultaneously bind membrane and cargo, clathrin is recruited to the membrane. Polymerizing clathrin forms a lattice around the forming pits and stabilizes the membrane curvature that ultimately regulates vesicle size (60-100nm) (McMahon and Boucrot, 2011). Regulatory proteins can alter the polymerization rate of clathrin to keep up with cellular demand. In contrast to clathrin, that is necessary but not sufficient for

budding, COP coats directly bind to membranes and, along with associated GTPases, are both necessary and sufficient to mediate vesicle budding (see section 1.3.4 for a detailed description) (Bethune et al., 2006). Upon coat protein binding, the vesicles are ready for detachment from donor membranes.

### *1.3.3. Vesicle Scission.*

The final step of vesicle budding requires vesicle fission, the detachment of vesicles from donor membranes. In CCV, the vesicle fission step is mediated by the large GTPase dynamin (Sweitzer and Hinshaw, 1998; Yoshida et al., 2004). As the CCV vesicle invaginates, amphiphysin recruits dynamin, (Yoshida et al., 2004). Dynamin assembles around the neck of the budding vesicle and GTP hydrolysis induces a conformational change in dynamin that results in membrane fission and vesicle release (Ferguson and De Camilli, 2012; Sweitzer and Hinshaw, 1998). Following vesicle release, an ATPase, heat shock cognate 70 (Hsc70), and its cofactor, auxilin, remove the clathrin coat before the vesicle is able to fuse with an acceptor membrane (Eisenberg and Greene, 2007). Transport vesicle fusion with acceptor membranes is regulated by the Soluble NSF Attachment Protein Receptor (SNARE) proteins, as vesicle-SNARES on transport vesicles bind to target-SNAREs on target organelles (Sollner et al., 1993). In CCV, the SNARE proteins together with the small GTPase Rab5 are required for membrane fusion (Bucci et al., 1992; Gorvel et al., 1991).

#### *1.3.4. Distinct coats mediate different trafficking steps.*

Clathrin coated-vesicles (CCV) are involved in budding transport vesicles from the plasma membrane (Kirchhausen, 2000). The Coatamer (Coat protein complex I (COPI)) complex plays a role in budding retrogradely-directed transport vesicles from the Golgi to the Endoplasmic Reticulum (ER), while the COPII complex is involved in the budding of anterogradely-directed transport vesicles from the Golgi that are involved in retrograde trafficking to the ER (Kirchhausen, 2000). Thus, in contrast to the clathrin-mediated process described above, retrograde transport in the secretory pathway requires the Coatamer (COPI). The Coatamer complex consists of 7 subunits ( $\alpha$ ,  $\beta$ ,  $\beta'$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$ -COP) and additional cofactors. COP-mediated trafficking differs from the clathrin-dependent process described above (Bonifacino and Lippincott-Schwartz, 2003). Initially, the small GTPase ADP-ribosylation factor (ARF) is recruited to the membrane. Activated ARF provides the link between cargo binding and coat protein recruitment. ARF recruits the intact coatamer complex from the cytosol and the GTPase activating protein, ARFGAP1. The Coatamer complex is preassembled in the cytoplasm into a heptameric complex consisting of two layers. One layer forms the base of the coat (similar to AP-2 in CCV) and further binds to membrane through  $\gamma$ -COP (Harter et al., 1996). The second layer is a cage-like trimeric subcomplex (Yu et al. 2012) that is responsible for determining vesicle size and initiates polymerization of the coat, leading to deformation of the membrane. Deactivation of ARF by ARFGAP1 along with regulatory proteins that enhance ARFGAP1 function are required for vesicle fission (Goldberg; Hsu and Yang, 2009; Lanoix et al., 2001; Yang et al., 2005). Following

vesicle fission, ARF deactivation destabilizes the coatomer and leads to vesicle uncoating before vesicles are competent to fuse with the ER (Spang, 2013).

#### *1.4. The ESCRT Machinery regulates inward budding at MVBs.*

The molecular mechanisms underlying membrane protein trafficking have been illuminated with the help of genetic and cell-free model systems. Studies using the yeast, *Saccharomyces cerevisiae*, enabled isolation of proteins required for inward budding of membrane proteins into the vacuole, the yeast degradative organelle (Babst et al., 2011; Babst et al., 2002b; Bilodeau et al., 2003; Bowers et al., 2004; Hurley and Emr, 2006; Katzmann et al., 2001; Piper and Luzio, 2001; Raymond et al., 1992b; Schmidt and Teis, 2012; Teis et al., 2010). A study by Rothman et al, (Rothman et al., 1989), revealed the identification of at least 40 genes, the Vacuolar protein sorting (Vps) genes, that when mutated were defective for the sorting of proteins to the vacuole (Rothman et al., 1989). However it was not until 1992, when Raymond et al identified a subset of the Vps proteins, known as the class E Vps proteins, that when mutated accumulated a large, prevacuolar endosomal compartment and were defective in the formation of MVB internal vesicles (Raymond et al., 1992a). Characterization of the Class E Vps genes resulted in identification of the Endosomal Sorting Complexes Required for Transport (ESCRTs) (Babst et al., 2011; Babst et al., 2002a; Babst et al., 2002b; Bilodeau et al., 2003; Bowers et al., 2004; Hurley and Emr, 2006; Katzmann et al., 2001; Katzmann et al., 2003; Kostelansky et al., 2006; Lemmon and Traub, 2000; Malerod et al.; Piper et al., 1995; Piper and Luzio, 2001; Teis et al., 2010). The ESCRTs are four cytosolic protein complexes that are recruited to endosomal



membranes and enable the sorting of membrane proteins into internal vesicles (Futter et al.; Hurley and Emr; Katzmann et al., 2001; Lemmon and Traub; Raiborg and Stenmark, 2009; Wegner et al., 2010). However, we lack a complete biochemical and molecular understanding of mechanisms that underlie regulation of ESCRT function, membrane protein movement, and vesicle formation/budding from the limiting MVB membrane.

The sorting of membrane proteins into the internal vesicles of the MVB requires their ubiquitination to enable engagement with the sorting machinery (Bache et al., 2003; Katzmann et al., 2001; Saksena et al., 2007; Sirisaengtaksin et al., 2014). A protein lacking ubiquitin remains on the limiting membrane and may remain on the limiting membrane for incorporation into the lysosomal membrane upon MVB-lysosome fusion or be recycled to the plasma membrane. Membrane protein cargo that has been ubiquitinated can be recognized by the ESCRT proteins and sorted into internal vesicles that will be degraded upon MVB-lysosome fusion (Fig. 1.2a). ESCRT complex formation occurs sequentially such that Vps27 is the first ESCRT component recruited to endosomal membranes through its second coiled-coil domain (Raiborg et al., 2001) and its membrane-lipid targeting domain, FYVE (Fab1, YGL023, Vps27, EEA1) (Gaullier et al., 1998; Gillooly et al., 2000; Raiborg et al, 2001; Urbe et al, 2000) which binds to the membrane phospholipid, phosphatidylinositol 3-phosphate (PI3P) (Gaullier et al., 1998; Gillooly et al., 2000; Raiborg et al, 2001; Urbe et al, 2000; Williams and Urbe, 2007; Katzmann et al, 2001). After recruitment to endosomal membranes, Vps27 binds to cargo via its ubiquitin-interacting motif (UIM) (Bilodeau et al., 2002; Shih et al., 2002). Vps27 acts

as a scaffold to recruit Hse1 to form the initial ESCRT complex (ESCRT-0) and in turn, sequester cargo into clusters. After formation of ESCRT-0, Vps27 binds to Vps23, a component of ESCRT-I that also binds ubiquitinated cargo (Babst et al., 2002a; Bache et al., 2003; Hurley and Emr, 2006; Katzmann et al., 2001; Katzmann et al., 2003). Vps23 recruits the remaining ESCRT-I components (Vps28, Vps37, and Mvb12a) from the cytoplasm to the endosome. ESCRT-I initiates ESCRT-II formation (Vps36, Snf8, Vps25) (Babst et al., 2002a; Schmidt and Teis). The function of the ESCRT-II complex is not well defined but it is believed that ESCRT-II initiates oligomerization of small coiled-coiled proteins to form ESCRT-III (Vps20, Snf7, Vps24, and Did4) (Babst et al., 2002a; Hurley and Emr, 2006; Schmidt and Teis, 2012). The composition of ESCRT-III is not clearly defined, though it is thought to be composed of two major complexes, Vps20/Snf7 and Vps24/Did4 that act to concentrate cargo and initiate MVB vesicle formation (Babst et al., 2002a; Hurley and Emr, 2006; Hurley and Hanson, 2010). Vps20 couples ESCRT-II to ESCRT-III, binds to the endosomal membranes and triggers oligomerization of Snf7. The oligomerization of Snf7 is capped by the Vps24/Did4 complex. The ESCRT-III machinery may be responsible for the fission of invaginated endosomal membrane that becomes internal MVB vesicles (Fig. 1.2) (Hurley and Emr, 2006; Schmidt and Teis, 2012). Finally, the AAA ATPase Vps4, acts to hydrolyze ATP and disassemble the ESCRT machinery (Babst et al., 1998).

The function of the ESCRT components in MVB biogenesis is highly conserved across multiple organisms, including yeast, flies, worms, and humans. The importance of the ESCRT machinery was revealed when mutations in the

ESCRT components rendered a phenotype characterized by enlarged endosomal compartments (vacuoles/MVBs) and failed transportation of proteins into the internal vesicles of the vacuole (Bilodeau et al., 2002; Hurley and Hanson, 2010; Schmidt and Teis). Deletion of some of the class E Vps genes (Vps27, Vps23, and Vps4) result in impaired MVB biogenesis. The functions of these genes appear to be highly conserved in mammalian cells (Gruenberg, 2001; Rieder et al., 1996). The depletion of the mammalian homologs of Vps27, Hse, and Vps4 (Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), Signal-transducing adaptor molecule (Madsen et al.), and Vps4 respectively) significantly decreased the number of MVB internal vesicles formed (Bilodeau et al., 2002; Katzmann et al., 2001; Sirisaengtaksin et al., 2014; Sun et al., 2010). Additionally, many ESCRT mutations (Vps27, Hse, Snf7, Vps23) are embryonically lethal in mice (Kanazawa et al., 2003; Komada and Soriano, 1999; Lloyd et al., 2002; Raymond et al., 1992a), highlighting the essential nature of the ESCRT machinery.

Two competing models describe the roles of the ESCRT complexes in cargo sorting. The conveyor belt model, suggests that cargo molecules are handed off sequentially from one ESCRT complex to the next in a linear manner and is based on sequential ESCRT protein interactions (Fig. 1.2a) (Hurley and Emr, 2006). The concentric ring model suggests that a supercomplex of ESCRT-0, ESCRT-I, and ESCRT-II complexes simultaneously form, cluster, and bind multiple ubiquitinated cargoes (Fig. 1.2b) (Nickerson et al., 2007). Evidence exists to support both theories and both models agree that the ESCRT-0 proteins, Hrs and STAM, bind to ubiquitinated cargo and initiate ESCRT complex formation (Hurley and Emr, 2006;

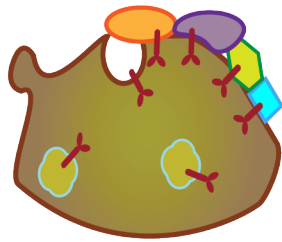
Nickerson et al., 2007). A major point of divergence between the two competing models is how ESCRT complexes bind to ubiquitinated cargo proteins. In contrast to the conveyor belt model, the concentric ring model assumes that multiple ESCRT components bind ubiquitinated cargo simultaneously (Nickerson et al., 2007). However the assumption that ESCRT complexes simultaneously bind ubiquitinated cargo has not been tested. If the concentric ring model is correct, disruption of an ESCRT component, or the ubiquitin binding site in an individual ESCRT component, should reduce, but not completely inhibit MVB internal vesicle formation (Nickerson et al., 2007). Another point of divergence between the two models is the reliance on linear formation of ESCRT complexes. In this regard, there is evidence that ESCRT-II is not required for MVB internal vesicle formation, at least under some conditions, arguing against a strict sequential order of ESCRT complex engagement (Bowers et al., 2006). However, it is possible that the ESCRTs proteins may have redundant functions allowing for enough flexibility to overcome disruptions in ESCRT-cargo engagement (Nickerson et al., 2007). Understanding whether all ESCRT complexes are generally required for cargo sorting and how the ESCRT complexes assemble on endosomes would aid in clarifying whether either of these models is correct.

The ESCRT machinery regulates two other key processes: viral budding and cytokinetic abscission (Fig. 1.3). ESCRT-I (TSG101, Vps28, Vps37, and Mvb12a) and ESCRT-III (Snf7, Did4, Vps24), along with Vps4 and the ESCRT-associated protein, Apoptosis-Linked Gene 2-Interacting Protein X (Alix), are required for the release of enveloped retroviruses, including HIV-1 and Ebola, at the plasma membrane (Hurley and Hanson, 2010; McDonald and Martin-Serrano, 2009). The

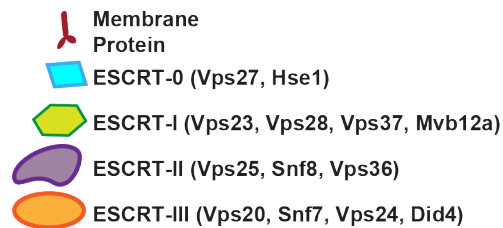
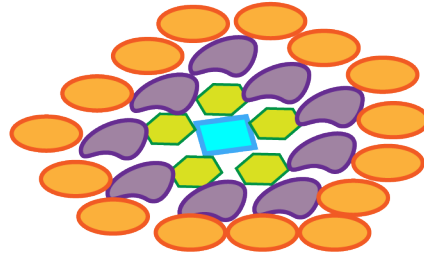
viral HIV-1 GAG protein can assemble and drive vesicle formation, but not scission in the absence of the ESCRT machinery (Jouvenet et al., 2009). The ESCRT-I component, TSG101, and Alix are recruited to the plasma membrane by binding to the HIV-1 Gag protein L-Domain (Demirov et al., 2002; Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001). The L-domain is a 4 amino acid motif in the HIV-1 Gag protein, that when mutated results in inhibited release of viral proteins from the plasma membrane (Gottlinger et al., 1991; Huang et al., 1995). Upon binding to the Gag protein L-domain, Tsg101 then recruits Vps28, Vps37, and MVB12a while Alix binds to Snf7 and recruits Did4, Vps24 and lastly Vps4. Binding of the ESCRT components triggers membrane scission and release of viral proteins into the extracellular environment (Fisher et al., 2007; Martin-Serrano et al., 2003; McCullough et al., 2008; Strack et al., 2003; Usami et al., 2007; von Schwedler et al., 2003; Zhai et al., 2008). In similar fashion, ESCRT-I (TSG101, Vps28, Vps37, and Mvb12a) and ESCRT-III (Snf7, Did4, Vps24), along with Vps4 and Alix are required for abscission during cytokinesis, the final step of the cell cycle following mitosis (Carlton and Martin-Serrano, 2007; McDonald and Martin-Serrano, 2009). The ESCRT machinery is recruited to the membrane neck/midbody between dividing cells by the binding of Alix and Tsg101 to the midbody component, centrosome protein 55 kDa (Cep55). Disruption of this interaction results in cytokinesis failure (Carlton and Martin-Serrano, 2007). Tsg101 recruits the ESCRT-I components (Vps28, Vps37, and Mvb12a) while Alix binds to the ESCRT-III component, Snf7, for the recruitment of additional ESCRT-III components (Did4 and Vps24) and Vps4 (Carlton et al., 2008; Carlton and Martin-Serrano, 2007; Morita et al., 2007). The

recruitment of ESCRT-III to the midbody area results in abscission and release of the daughter cells (Carlton et al., 2008; Carlton and Martin-Serrano, 2007; Morita et al., 2007). These data suggest a unique ability for ESCRT-I and ESCRT-III proteins to drive membrane budding and scission events in the absence of ESCRT-0 and ESCRT-II.

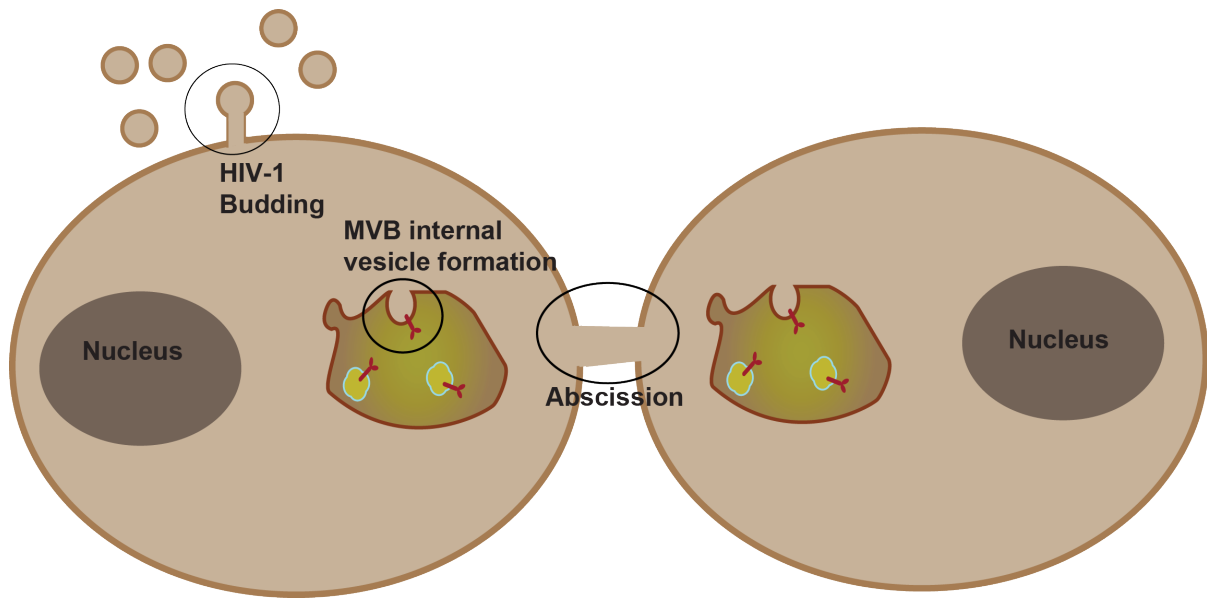
### a. Conveyor Belt Model



### b. Concentric Ring Model



**Figure 1.2: ESCRT function.** a) Conveyor belt model of ESCRT function. According to this model, ESCRT complexes are recruited sequentially to the endosome and recognize ubiquitinated transmembrane proteins, passing cargo from one complex to the next to facilitate sorting to MVB vesicles. b) Concentric circle model of ESCRT function. ESCRT-0, -I and -II mediate cargo recognition, lipid binding and complex assembly, resulting in formation of an ESCRT-0/I/II supercomplex on the endosomal membrane with MVB cargo proteins concentrated beneath. Subunits of ESCRT-III assemble to form a perimeter and promote disassembly by Vps4. Dissociation of the ESCRT- 0/I/II core precedes vesicle formation, making sequestered MVB cargoes available for deubiquitination before vesicle scission. a,b) Adapted from Nickerson, D.P., M.R. Russell, and G. Odorizzi. 2007. A concentric circle model of multivesicular body cargo sorting. *EMBO Rep.* 8:644-650. (Nickerson et al., 2007).



**Figure 1.3. The ESCRT machinery regulates multiple budding events.** The ESCRT machinery regulates the formation of internal vesicles within multivesicular bodies (MVBs), viral budding and abscission during cytokinesis. Adapted from McDonald, B., and J. Martin-Serrano. 2009. No strings attached: the ESCRT machinery in viral budding and cytokinesis. *Journal of cell science*. 122:2167-2177. (McDonald and Martin-Serrano, 2009).



### *1.5. The Mechanism of Outward Vesicle Budding from Endosomes.*

Endosomes are dynamic organelles that deliver material to other cellular compartments via membrane budding (Grant and Donaldson, 2009; Gruenberg, 2001; Maxfield and McGraw, 2004; Tanowitz and von Zastrow; Thompson et al., 2007; Weigert et al., 2004). The separation and sorting of cargo proteins on endosomal membranes allows for the selective incorporation of membrane proteins into newly formed vesicles (Bonifacino and Glick, 2004). Membrane budding into the cytosol usually requires coat proteins (Kirchhausen, 2000), however the role of coat proteins in outward budding from late endosomes is unclear. The cytosolic GTPases are known to regulate outward vesicle budding from early endosomes and the trans-Golgi (Stenmark, 2009). A non-hydrolysable analogue of GTP, GTP $\gamma$ S, inhibits budding of cargo between the golgi cisternae (Melancon et al., 1987), from the ER to Golgi (Baker et al., 1990; Beckers and Balch, 1989; Ruohola et al., 1988), and from the trans-Golgi network (Tooze et al., 1990). These results led to the identification of the Ras related proteins in brain (Rab) and suggested that GTPases are regulators of membrane budding in the endocytic pathway (Segev et al., 1988; Stenmark, 2009). Subsequently, multiple types of GTPases, both small (Rab family) and large (dynamin) have been implicated in membrane budding events (Pryer et al., 1992; Robinson, 1994; Salminen and Novick, 1987; Zerial and Stenmark, 1993).

Rab GTPases are the largest family of small GTPases and were originally implied to regulate vesicle docking and fusion (Gorvel et al., 1991), uncoating (Semerdjieva et al., 2008) and organelle identity (Stenmark, 2009; Zerial and McBride, 2001). Rab GTPases regulate vesicle formation (Plutner et al., 1991; Ren

et al., 1998) and transport between organelles (Chavrier et al., 1990). Inactivation of Rab homologs results in enlargement of the Golgi, but not in accumulation of Golgi-derived vesicles (Benli et al., 1996; Jedd et al., 1997). Thus, Rab proteins regulate many membrane trafficking stages (Pfeffer, 2001). Like most GTPases, Rab proteins function between two alternate conformational states: the active GTP-bound “on” state and the inactive GDP-bound “off” state (Bourne et al., 1990). For membrane trafficking events the GTP-bound Rabs are often membrane associated by virtue of their ability to bind receptors when GTP-bound (Stenmark, 2009; Pfeffer, 2001). Active Rab proteins are required for transport vesicle formation however; it is still unclear how Rab proteins regulate vesicle budding events.

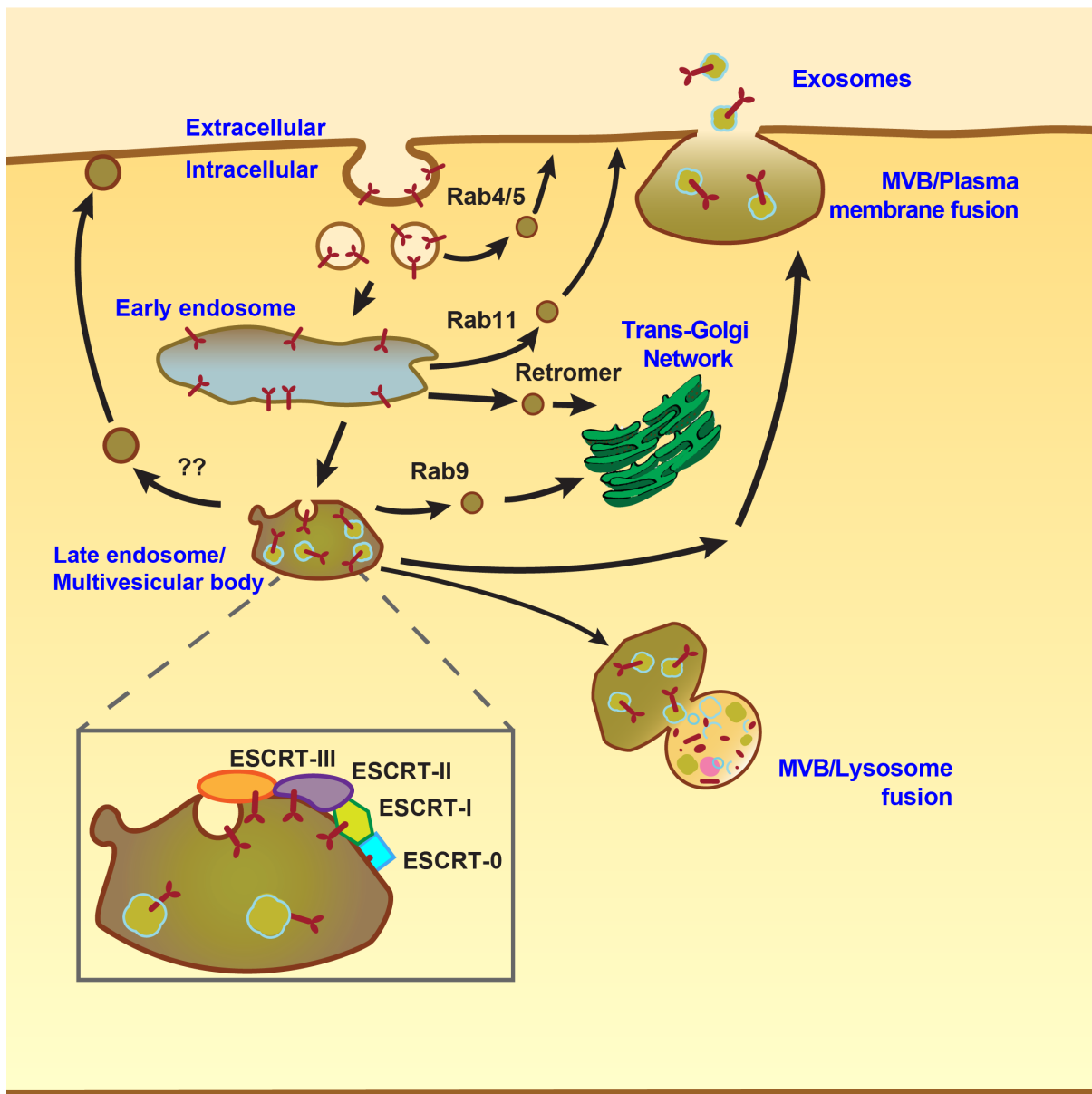
Similar to small GTPases, the large GTPase, dynamin is known to regulate membrane budding events. Dynamin was initially implicated in vesicle budding due to its role in vesicle scission of newly formed vesicles via its GTP hydrolysis capability, both at the plasma membrane and at the Golgi (Urrutia et al., 1997). Expression of a dominant negative form of Dynamin (DynaminK44A) impaired vesicle release from the plasma membrane and from endosomes to the Golgi (Robinson, 1994). Therefore, understanding the role of GTPases in vesicle budding events is key to understanding membrane trafficking pathways.

GTPases regulate most of the outward budding pathways from endosomes. There are three main outward budding routes from early endosomes: a direct “fast” route, an indirect, “slow” route, and an endosome to Trans Golgi network route (Fig. 1.4) (Grant and Donaldson, 2009; Maxfield and McGraw, 2004; Thompson et al., 2007; Weigert et al., 2004). The fast route is dependent on the small GTPases Rab4

and Rab5 and can occur prior to fusion of internalized vesicles with the early endosomes, or through the perinuclear and tubular vesicles that bud off from early endosomes to form the endosomal recycling compartment (ERC) (Grant and Donaldson, 2009; van der Sluijs et al., 1992; Xie et al., 2016). In this regard, overexpression of a dominant negative Rab4, Rab4S22N, results in vesicle accumulation in the perinuclear area of early endosomes (McCaffrey et al., 2001). The slow route requires the small GTPase Rab11, and movement through the ERC (Grant and Donaldson, 2009). The ERC plays a key role in the recycling of the Transferrin receptor (TfR) back to the plasma membrane for reuse (Ullrich et al., 1996). Expression of a dominant negative form of Rab11, Rab11S25N, inhibits the movement of TfR from early/sorting endosomes to the ERC (Ren et al., 1998). Lastly, the non-GTPase retromer protein complex regulates trafficking of the mannose-6-phosphate (M6PR) receptor from endosomes to the trans-Golgi network (Arighi et al., 2004). Depletion of the retromer components, Vps26 or Vps35, decreases recycling and enhances degradation of M6PR (Arighi et al., 2004). Therefore, regulation of outward budding from early endosomes is dependent on Rab GTPases and retromer components. If a receptor does not bud out through one of the three routes described above, it will be transported to late endosomes, although the mechanisms of outward budding from late endosomes is not well understood.

There are two outward budding routes from the late endosomes (Fig. 1.4). One trafficking route depends on the small GTPase, Rab9 and the large GTPase, Dynamin. Rab9 and Dynamin regulate the trafficking of M6PR from endosomes to

the trans-Golgi network (Lombardi et al., 1993; Nicoziani et al., 2000; Riederer et al., 1994). Lack of Rab9 impairs transport of M6PR between endosomes and the trans-Golgi network by inhibiting vesicle budding (Lombardi et al., 1993). Similarly, dynamin is required for transport of M6PR. Dynamin plays a role in vesicle fission and thus expression of a dominant negative mutant form of Dynamin, DynaminK44A, impairs the release of vesicles containing M6PR from endosomes to the trans-Golgi network (Nicoziani et al., 2000). Lastly, transport vesicles can bud from MVBs prior to MVB-lysosome fusion and may carry cargo to the plasma membrane for reuse (Felder et al., 1990). Ultrastructural studies of an EGFR mutant lacking kinase activity, EGFR<sub>K721A</sub>, revealed that EGFR<sub>K721A</sub> is found on the limiting membranes of MVBs prior to budding outwardly into vesicles destined for the plasma membrane (Felder et al., 1990). The increased recycling of the EGFR<sub>K721A</sub> results in an increase in cell-surface expression (Felder et al., 1990). However, the molecular mechanisms underlying this outward vesicle budding from the MVB is not well understood and is the focus of this study.



**Figure 1.4. Membrane Protein Trafficking through the Endocytic Pathway.**

Internalized cargo is transported to early endosomes. From the early or sorting endosomes, cargo can be recycled through two different pathways. The 'Fast' recycling pathway requires the activity of Rab4 and Rab5, and the 'slow' recycling pathway is dependent on the activity of Rab11. If a membrane protein is not recycled at the early endosome, it is transported to late endosomes/multivesicular bodies

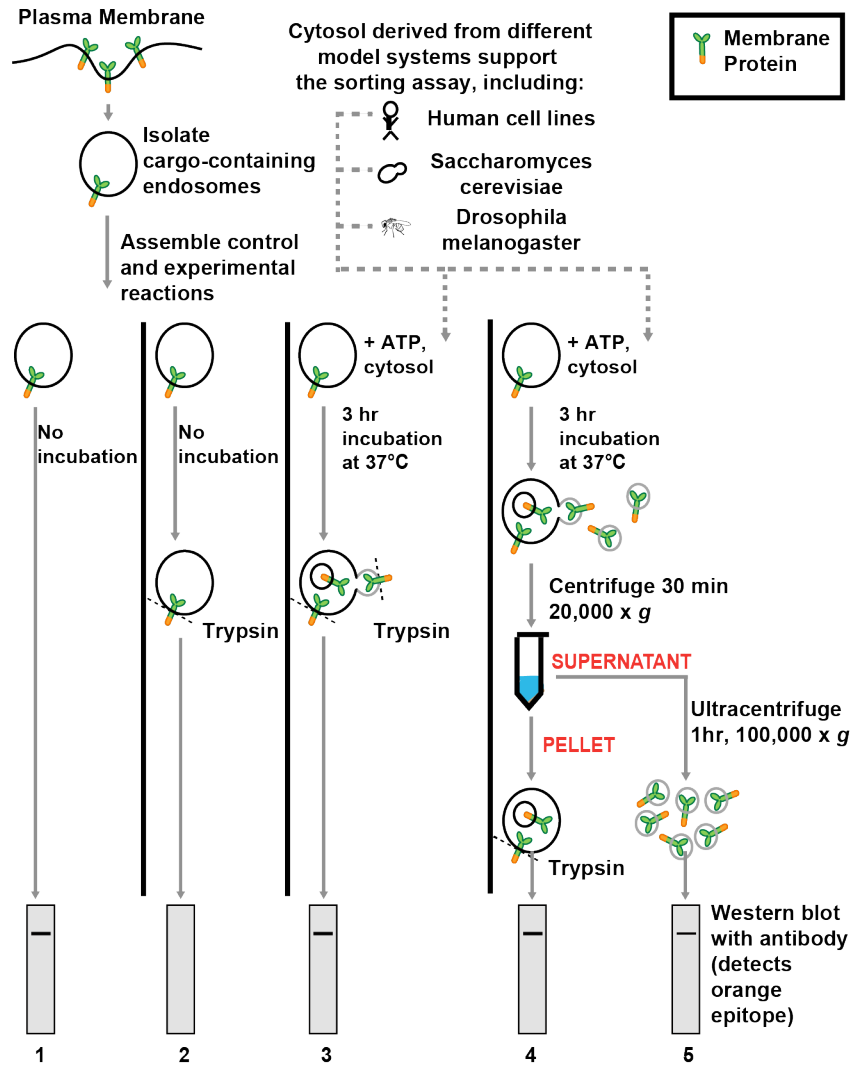
(MVBs). At the MVB, a membrane protein may bud outwardly for recycling to other cellular compartments in a Rab9 dependent manner (e.g. plasma membrane or Golgi), or it will be internalized into the internal vesicles of the MVB for eventual degradation upon MVB-lysosome fusion, or for secretion into the extracellular space as exosomes upon MVB-plasma membrane fusion. To facilitate sorting of proteins into the internal vesicles of the MVB, most protein cargo must be ubiquitinated to enable binding by the endosomal sorting complexes required for transport (ESCRTs) that enable protein sorting.

### 1.6. Cell-Free Sorting Assay

The purpose of this study was to identify the molecular machinery that regulates outward budding from the MVB and determine whether the machinery is distinct from the inward budding machinery. ***We hypothesize that distinct molecular mechanisms mediate inward versus outward budding from the endosomal membrane.*** We used a cell-free assay that reconstitutes both morphological formation of internalized and budded vesicles (using ultrastructural methods) and quantitatively measures the amount of membrane protein cargo sorted into the endosomal lumen or present on vesicles that bud from that membrane (using biochemical methods) (Fig. 1.5). To measure cargo internalization, an intracellular epitope of a transmembrane cargo protein is detected using an epitope-specific antibody (Fig. 1.5) (Sirisaengtaksin et al., 2014; Sun et al., 2010). If the intracellular domain of the transmembrane protein is sorted into internal MVB vesicles, it will no longer be accessible to exogenously added trypsin and is protected from digestion, (Fig. 1.5) (Sirisaengtaksin et al., 2014; Sun et al., 2010). Both the protease protection of a transmembrane cargo protein and the formation of internal endosomal vesicles are dependent on cytosol, ATP, temperature, and an intact proton gradient (Sirisaengtaksin et al., 2014; Sun et al., 2010). During the cell-free reaction, the number of internal vesicles formed, quantified by electron microscopy, and protease protection of a membrane protein cargo (EGFR) are correlated, suggesting that this assay measures endosomal cargo sorting and MVB formation (Sun et al., 2010). I have modified this protease protection assay in two important ways. First, I have shown that cytosol isolated from yeast and fly strains

are sufficient to support the inward budding of EGFR, thus enabling the use of genetics to examine the factors in cytosol that are required to support membrane protein sorting and MVB formation (Chapters 3-4). Second, I have modified the assay to capture membranes that may bud outwardly from the donor membranes (endosomes) during the reactions (Chapter 5-7). To isolate the outwardly budded vesicles, differential centrifugation of reaction supernatant following the 3-hour incubation enabled me to obtain outwardly budded vesicles. Using this approach, I have identified molecules regulating both inward and outward budding from the MVB.





**Figure 1.5. Cell-free Reconstitution of MVB Sorting.** Serum-starved cells are stimulated to induce internalization of a membrane protein receptor from the plasma membrane (e.g. EGFR), resulting in movement of ligand-receptor complex into endosomes. Isolation of partially purified endosomes (1) that contain the receptor can be detected by immunoblotting using an intracellular epitope-specific antibody. Incubation of these endosomes with trypsin (2) removes the C-terminal epitope of the receptor that protrudes from the plasma membrane, resulting in a loss of signal for that epitope on an immunoblot. Incubation of endosomes with ATP and cytosol,

at 37°C results in formation of internal vesicles and protection of the C-terminal EGFR epitope from subsequent trypsin cleavage (3). Incubation of endosomes with ATP and cytosol for 3 hours at 37°C [as in (3)] followed by centrifugation results in separation of MVBs (in pellet) and outwardly budded vesicles (in supernatant). The MVB pellet (4) is subsequently digested with trypsin while supernatant (5) is centrifuged again to concentrate budded vesicles for collection.

## Chapter 2. Materials and Methods

### 2.1. Materials

*Materials*— Antibodies were purchased from the following commercial sources: EGFR (Invitrogen), V5-tag (Invitrogen), c-Myc (9E10, Santa Cruz Biotechnologies), EEA1 (Thermo Fisher), LAMP1 (H4A3 clone, Developmental Studies Hybridoma Bank), Rab11 (Millipore), Rab 7 (Invitrogen), Transferrin Receptor (TfR) (Abcam). Reagents were purchased from the following commercial sources: Methyl- $\beta$ -Cyclodextrin (M $\beta$ CD, Sigma), Monensin (Sigma), and soluble cholesterol (Sigma).

*Constructs*— The pCMV-AT1R-Myc construct was kindly provided by Dr. Guangwei Du (UTHealth). The pcDNA3.1-hisB-V5-R4-FGFR4<sub>Gly388</sub> construct was kindly provided by Dr. Michael Ittmann (Baylor College of Medicine). The pcDNA-DEST40 V5-tagged Kv4 construct was kindly provided by Dr. Susan Tsunoda (Colorado State University). The pcDNA6a myc-tagged EGFR<sub>K721A</sub> construct was kindly provided by Dr. Mien-Chie Hung (M.D. Anderson). The PCMV-intron myc Rab11 S25N construct was purchased from Addgene.

*Cell Culture*— HeLa cells (ATCC) were cultured as a monolayer in 10-cm plastic plates in Dulbecco's Modified Eagle Medium (DMEM, Mediatech) containing 10% Fetal Bovine Serum (FBS, Sigma) under 5% CO<sub>2</sub> at 37°C. Before each experiment, cells were split by removing them from the plate using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and seeded into 10-cm tissue culture plates. SH-SY5Y cells were cultured as a monolayer in 10-cm plastic plates in

Roswell Park Memorial Institute (RPMI, Mediatech) containing 10% FBS and 5% L-glutamine under 5% CO<sub>2</sub> at 37°C. Before each experiment, cells were split by removing them from the plate using 0.25% trypsin/EDTA and seeded into 10-cm tissue culture plates. U87 cells were cultured as a monolayer in 10-cm plastic plates in Modified Eagle Medium (MEM, Mediatech) containing 10% FBS and 5% Non-essential amino acids (Sigma) under 5% CO<sub>2</sub> at 37°C. Before each experiment, cells were split by removing them from the plate using 0.05% trypsin/EDTA and seeded into 10-cm tissue culture plates.

*Recombinant proteins*— Hrs and STAM were produced in insect cells as previously described (Sirisaengtaksin et al., 2014; Tsujimoto et al., 1999). Recombinantly produced Dynamin 1 protein was kindly provided by Dr. Sandra L. Schmid.

## *2.2 Cytosol preparation*

*Mammalian:* HeLa cells were placed on ice, washed with ice-cold PBS (2x with 5 mL), scraped from the plate, and centrifuged (2000 x *g* for 15 min) at 4 °C. The cell pellet was resuspended in 100 µL of homogenization buffer (HB) (20 mM HEPES pH 7.4, 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, and 0.1 mM DTT) containing a protease inhibitor cocktail (112 µM PMSF, 3 µM aprotinin, 112 µM leupeptin, 17 µM pepstatin). Cells were sonicated 5 times (5 pulses of 1 second at output control 3) (Branson Sonifier 250, VWR Scientific). The lysate was centrifuged (2000 x *g* for 10 min) at 4°C, and the supernatant was further centrifuged (100,000 x *g* for 1 hour) at

4°C. The supernatant was collected and protein concentration was calculated using a Bradford assay (Sirisaengtaksin et al., 2014).

*Saccharomyces cerevisiae*: *Saccharomyces cerevisiae* strains were plated on YPD plates (500 mL ddH<sub>2</sub>O containing: 10 g bactopectone, 5 g yeast extract, 8 g agar, 25 mL 40% dextrose) and incubated for 48 hours on a shaker at 30°C. YPD media (5 mL) was inoculated with various *Saccharomyces cerevisiae* strains and incubated overnight on a shaker at 30 °C. Cultures were transferred into a secondary culture of YPD media (50 mL) and were grown until OD<sub>600</sub> reached 0.8-1.0. Cells were collected (3000 x g for 3 min) and washed twice, first with 500 µL of ddH<sub>2</sub>O followed by 500 µL TP buffer (20 mM Tris, pH 7.9; 0.5 mM EDTA; 10% glycerol; 50 mM NaCl, 112 µM leupeptin, 3 µM aproptinin, 112 µM PMSF, and 17 µM pepstatin). The cells were recollected (3000 x g for 3 min) and resuspended into 130 µL of TP buffer. Acid-washed beads (50 µL) were added to the cells and the cells were lysed (1 min vortex-1 min incubation on ice, 5X). Cells were centrifuged (3000 x g for 10 min) and the supernatant was collected. Protein concentration was calculated using a Bradford assay. The supernatant was divided into 70 µg aliquots and stored at -80 °C. For *Saccharomyces cerevisiae* deletion strains (Table 1), we inoculated strains in YPD media containing G418 (500 µg/mL).

*Drosophila melanogaster*: Frozen whole head homogenates of approximately 1000 fly heads were centrifuged (100,000 x g for 60 min) to pellet total membranes and the supernatant was collected. Protein concentration was calculated using a Bradford assay. Supernatants were stored in 25 µg aliquots at -80°C.

### 2.3 Cell Transfection

*Cell Transfection*—Plasmid DNA was prepared (Qiagen), and HeLa cells were transiently transfected using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol. The constructs used in each transfection are as indicated. Briefly, cells were plated in 6-well plates and grown until they reached 80-90% confluence. In each well of the plate, DNA (3  $\mu$ g) was added to Opti-MEM reduced serum medium (250  $\mu$ L), mixed, and incubated for 5 min at room temperature. Lipofectamine 2000 reagent (3.5  $\mu$ L) was added to Opti-MEM reduced serum medium (250  $\mu$ L), mixed, and incubated for 5 min at room temperature. The tubes were combined, mixed gently, and incubated for 20 min at room temperature before adding 500  $\mu$ L to each well. After 48 hours, the cells were used in the cell-free sorting assay described below.

### 2.4 Cell-Free Sorting Assay

*Cell-free reconstitution of inward budding from MVB membranes*— The reconstitution of inward budding was performed as described (Gireud et al., 2015; Sirisaengtaksin et al., 2014; Sun et al., 2010). In experiments where EGFR was the membrane protein cargo, HeLa cells were grown to 75-80% confluence. Before harvesting, cells were serum starved (2 hours at 37°C) and stimulated with EGF (100 ng/mL; 10 min at 37°C or 2ng/mL; 10 min at 37°C). Endosomal membranes were isolated as previously described (Sirisaengtaksin et al., 2014; Sun et al., 2010) and resuspended in HB buffer (volume dependent on number of reactions; 10  $\mu$ L for

control reactions and 15  $\mu\text{L}$  per experimental reaction), and used for reconstitution reactions.

Endosomal membranes (starting material) were either incubated on ice or were trypsin-treated (6  $\mu\text{L}$  of 0.27  $\mu\text{g}/\mu\text{L}$  trypsin; 4°C for 30 minutes). For reactions containing mammalian cytosol, a standard reaction (50  $\mu\text{L}$ ) contained 15  $\mu\text{L}$  endosomal membranes, 6  $\mu\text{L}$  ATP regeneration system (2 mM MgATP, 50  $\mu\text{g}/\text{mL}$  creatine kinase, 8 mM phosphocreatine and 1 mM DTT of final concentrations), 25  $\mu\text{g}$  of Hela cytosol and HB to a total reaction volume of 50  $\mu\text{L}$ . For the *Saccharomyces cerevisiae* cytosol reactions, a standard reaction (50  $\mu\text{L}$ ) contained 15  $\mu\text{L}$  membranes, 6  $\mu\text{L}$  ATP regeneration system, 70  $\mu\text{g}$  of *Saccharomyces cerevisiae* cytosol and HB to a total reaction volume of 50  $\mu\text{L}$ . For the *Drosophila melanogaster* cytosol reactions, a standard reaction (50  $\mu\text{L}$ ) contained 15  $\mu\text{L}$  membranes, 6  $\mu\text{L}$  ATP regeneration system, 25  $\mu\text{g}$  of *Drosophila melanogaster* (fly) cytosol, and HB to a total reaction volume of 50  $\mu\text{L}$ .

All experimental reactions were incubated for 3 hours at 37°C, followed by trypsin-treatment (6  $\mu\text{L}$  of 0.27  $\mu\text{g}/\mu\text{L}$  trypsin; 30 min at 4°C). Experimental reactions were centrifuged (20,000  $\times g$ ; 30 min at 4°C) while control reactions remained on ice. Control reactions were resuspended in sample buffer for SDS-PAGE. For experimental reactions, supernatant was aspirated and pellet was resuspended in sample buffer for biochemical examination by SDS-PAGE. Resultant blots were probed with an antibody that recognizes amino acids 1190-1210 (C-terminal epitope) of EGFR (1:200 dilution in 5% nonfat milk with PBS, overnight at 4 °C).

To examine the dependence of cargo sorting on the presence of ESCRT proteins, yeast cytosol was prepared from strains listed in Table 1 and used in place of wild-type yeast cytosol. All reactions were normalized to wild-type controls. For the FGFR4 experiments, transfected cells were serum-starved in media containing cycloheximide (30 µg/mL) for 2 hours and stimulated with bFGF (50 ng/mL) for 5 hours. Following the bFGF stimulation, subsequent experimental conditions were as described for EGFR. The resulting blots were probed with an antibody that recognized the intracellular V5-tag of FGFR4 (1:5000 dilution in 5% nonfat milk with PBS-Tween, overnight at 4°C). For the AT1R experiments, transfected cells were starved in serum-free media (2 hours at 37°C) and stimulated with angiotensin II (1 mg/mL) (30 minutes at 37°C). Following the angiotensin II stimulation, subsequent experimental conditions were as described for EGFR. The blots were probed with an antibody that recognized the intracellular Myc-tag of AT1R (1:500 dilution in 5% nonfat milk with PBS-Tween, overnight at 4°C). For the Kv4 experiments, transfected cells were not serum-starved or stimulated. Instead, transfected cells were collected and partially purified endosomes isolated. Experimental conditions were then performed as described for EGFR experiments. The blots were probed with an antibody that recognizes the V5-tag that was fused to the COOH-terminus of the Kv4 clone used in these studies (1:5000 dilution in 5% nonfat milk with PBS-Tween, overnight at 4°C). For the EGFR<sub>K721A</sub> experiments, transfected cells were starved in serum-free media (2 hours at 37°C) and stimulated with EGF (100 ng/mL) (10 minutes at 37°C). Experimental conditions were then performed as described for EGFR experiments. The blots were probed with an antibody that recognized the



intracellular Myc-tag that was fused to the COOH-terminus of the EGFR<sub>K721A</sub> clone used in these studies EGFR<sub>K721A</sub> (1:500 dilution in 5% nonfat milk with PBS-Tween, overnight at 4°C). For the EGFR<sub>VIII</sub> experiments, HeLa transfected cells, or U87 cells stably expressing EGFR<sub>VIII</sub>, were serum-starved (2 hours at 37°C) and stimulated with EGF (100 ng/mL) (10 minutes at 37°C). Experimental conditions were then performed as described for EGFR. The resulting blots were probed with an antibody that recognized the intracellular V5-tag that was fused to the COOH-terminus of the EGFR<sub>VIII</sub> clone used in these studies (1:5000 dilution in 5% nonfat milk with PBS-Tween, overnight at 4°C).

*Isolation of outwardly budding vesicles from cell-free reactions*—Experimental conditions were performed as in EGFR inward budding experiments described above, including 70 µg of *Saccharomyces cerevisiae*, ATP, and the 3-hour reaction incubation. Following the 3-hour incubation, experimental reactions were centrifuged (20,000 x *g* for 30 min at 4°C). The supernatant was collected and further centrifuged (150,000 x *g* for 1 hour at 4°C). After ultracentrifugation, the resulting pellet was either resuspended in sample buffer for SDS-PAGE, or subjected to Nanosight Tracking Analysis.

*Pharmacological treatments*—To examine the role of cholesterol in EGFR sorting, crude endosomal membranes were treated with 15 mM MβCD for 15 min at 37°C. Treated endosomal membranes were collected by centrifugation (15 min at 1500 x *G*) and added into inward budding reactions (described above). Soluble cholesterol

was added (50 µg/mL) to some reactions that also contained M $\beta$ CD-treated membranes, and incubated as described. For all drug experiments, membranes were collected after 3 hours of incubation in assay, and analyzed as described. The effect of monensin, a sodium ionophore that interferes with pH changes in the endosome {Ju et al, 2004; Mesbah et al, 2011; Mora-Montes et al, 2008}, was examined by adding either 10 µM or 60 µM monensin into experimental reactions. Control reactions were performed using equal concentrations of the monensin diluent, methanol.

## *2.5. Nanosight Tracking Analysis*

*NanoSight Tracking Analysis (NTA)*— NTA measurements were performed on membranes isolated from cell-free reactions in which the reaction supernatant had been centrifuged to isolate outwardly budded vesicles using a NanoSight NS300 instrument following the manufacturer's instructions. NTA is performed by measuring the rate of Brownian motion of particles in a low volume light scattering system (NanoSight Ltd., Amesbury, United Kingdom). Results are presented as mean size of vesicles (x-axis) and concentration of particles per mL of solution (y-axis). Samples were examined in triplicate.

## *2.6. Electron Microscopy*

*Electron Microscopy (EM)*— Vesicle size was visualized using TEM on membranes isolated from supernatant obtained from MVB sorting reactions. 5 µL of vesicles were placed on glow discharged carbon formvar grids (TedPella) for approximately 5

min. Grids were rinsed 3x with 5  $\mu$ L of water, using blotting paper to wick away excess liquid between rinses. Finally, grids were rinsed quickly with 50% mixture of NanoW (Nanoprobes) stain, wicked, and then stained for approximately 30 min before wicking excess liquid and allowing grids to dry for at least 30 min prior to imaging. Micrographs were collected on a JOEL 1400 electron microscope operated at 120 kV using a Gatan ultrascan camera.

### *2.7. Mass Spectrometry*

*Mass spectrometry (MS)*— Membranes isolated from supernatant obtained from MVB sorting reactions were isolated for biochemical examination by SDS-PAGE as described above. Following gel electrophoresis, gels were stained with coomassie blue. Gel slices were submitted to the Taplin Mass Spectrometry Facility at Harvard Medical School for analysis.

### *2.8. OptiPrep Gradient*

*OptiPrep Gradient*— After HeLa cells were serum-starved and stimulated (100 ng/mL EGF; 20 min at 37°C), post-nuclear supernatant was isolated and loaded on top of a continuous Opti-prep gradient (Sigma, 10-20%) and centrifuged (150,000  $\times$   $g$  for 10 hours at 4°C) in a swinging bucket rotor (TLS 55, Beckman). Fractions (200  $\mu$ L) were collected and diluted in of HB (200  $\mu$ L) followed by centrifugation (150,000  $\times$   $g$  for 1 hour at 4°C). The resulting pellet was resuspended in sample buffer for biochemical examination. If the fractions were to be used in the cell-free assay, fractions 3 & 4 containing late endosomal membranes were collected and fractions 8

& 9 containing early endosomal membranes were collected and diluted in HB (400 uL) followed by centrifugation (150,000 x g for 1 hour at 4°C). The resulting pellets were resuspended in HB and membranes were used in cell-free reactions.

## *2.9. Generation of yeast double knock out strains*

*Generation of Saccharomyces cerevisiae heterozygous double knock out strains—*

*Conversion of snf7::KANMX6 and did4::KANMX6 strains: snf7::KANMX6*

(Dharmacon: MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 snf7Δ::KANMX6) and did4::KANMX6 (Dharmacon: MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 did4Δ::KANMX6) strains were transformed as previously described (Gietz and Woods) with Sall-ClaI digested plasmid pAG32 (HPHMX6) (Goldstein and McCusker, 1999). Briefly, cells were grown in 5 mL of YPD and incubated (overnight at 30°C). The next day, cells were re-inoculated in 20 mL of YPD at a starting OD<sub>600</sub> of 0.2. Once cells had reached a final OD<sub>600</sub> of 0.8, they were collected (1000 x g for 5 minutes) and washed in 500 μL of 100 mM LiAc. The cell suspension was then collected (1000 x g for 5 minutes) and resuspended in transformation solution (50% PEG, 0.1M LiAc, 10 μg carrier DNA, ddH<sub>2</sub>O) and 1 μg of digested pAG32. Cells were incubated for 30 minutes at 30 °C followed by incubation at 42 °C for 20 minutes. Next, cells were collected (3300 x g for 15 seconds), resuspended in 100 μL of ddH<sub>2</sub>O and plated on YPD plates containing 500 μg/mL Hygromycin B. Transformants were selected based on sensitivity to G418 and resistance to Hygromycin B. The resulting strains were yMG1 and yMG2 (see Table 1).

Creation of heterozygous double knock out strains: To create the *snf7 vps20* and *did4 vps24* strains, the yMG1 and yMG2 strains were crossed with the *vps20Δ* and *vps24Δ* strains, respectively. G418R Hygromycin B<sup>R</sup> heterozygous diploid knock out strains were selected for further studies.

## 2.10. Statistical Analysis

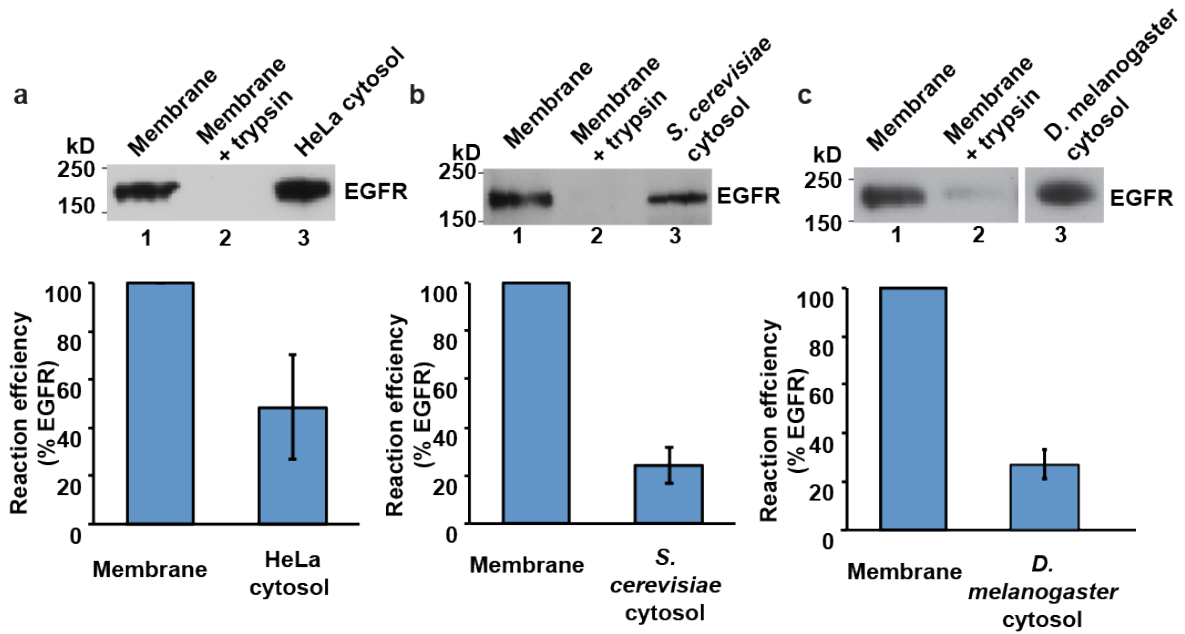
*Statistical Analysis*— Statistical significance was determined using either a Paired t-test or one-way ANOVA for independent samples and post-hoc analysis (Tukey test). All data were tested for normality using the Shapiro-Wilk test prior to analysis. A *p*-value of <0.05 was considered statistically significant with an *n*≥3.

## Chapter 3. Mechanisms of Inward Budding

*Rationale:* Membrane proteins that move from the limiting membrane into internal vesicles of MVBs may be degraded upon MVB-lysosome fusion. The process of protein movement into internal vesicles is thought to involve protein aggregation on the membrane to sort the proteins to be internalized into a domain of the membrane, followed by invagination of that part of the membrane and fission of the invaginated membrane resulting in formation of an internal vesicle. Using our cell free assay (described in Chapter 1 and Figure 1.6), I can measure internal vesicle formation and movement of cargo proteins from the limiting endosomal membrane into internal vesicles.

### *3.1. Saccharomyces cerevisiae (yeast) and Drosophila melanogaster (fly) cytosol are sufficient to support the mammalian endosomal sorting of the EGFR.*

To determine whether yeast or fly cytosol could substitute for cytosol obtained from mammalian sources in our cell-free assay (Fig. 1.3) (Sirisaengtaksin et al., 2014; Sun et al., 2010), reactions were incubated with mammalian, yeast, or fly cytosol. The efficiency of the cell-free reactions as judged by the EGFR protected from protease cleavage after the cell-free reactions using yeast and fly cytosol was 24 $\pm$ 7% and 32 $\pm$ 1%, respectively, compared to what I observed with mammalian cytosol, 48 $\pm$ 9% (Fig. 3.1a). These data suggest that while quantitative differences exist between the mammalian, yeast, and fly systems, all three systems contain essential proteins required for the inward budding event.



**Figure 3.1 Cytosol isolated from HeLa cells, *Saccharomyces cerevisiae*, and *Drosophila melanogaster* support the protease protection of EGFR.** Partially purified HeLa endosomal membranes containing EGFR were isolated. (a-c) Endosomal membranes (5  $\mu$ L, lane 1) and endosomal membranes (5  $\mu$ L) digested with trypsin to remove the C-terminal epitope of the receptor (lane 2), as well as partially purified HeLa endosomal membranes (15  $\mu$ L) that had been incubated in reactions containing ATP and cytosol derived from: HeLa cells (25  $\mu$ g, a), *Saccharomyces cerevisiae* (70  $\mu$ g, b), or *Drosophila melanogaster* (25  $\mu$ g, c), at 37°C prior to trypsin treatment (lane 3) are shown. Data represents the mean  $\pm$  S.E. (n=3) normalized to membrane control.

### 3.2 The protease protection of EGFR, or inward budding, is dependent on ESCRT proteins.

To examine whether ESCRT proteins (Bache et al., 2003; Futter et al., 1996; Hurley and Emr, 2006; Katzmann et al.; Lemmon and Traub; Saksena et al., 2007; Sun et al., 2010) are required for inward budding, measured by protease protection of the EGFR intracellular epitope, cytosol derived from yeast strains deleted of ESCRT proteins (Table 1) was used in cell-free reactions in place of wild-type yeast cytosol. Reactions containing cytosol derived from ESCRT-0 deficient yeast strains (*vps27Δ*, *hse1Δ*) decreased EGFR epitope protease protection (Fig. 3.2a). If I added recombinant mammalian ESCRT-0 homologs (Hrs and STAM, 8μg) into reactions that include cytosol isolated from *vps27Δ* or *hse1Δ* strains, the inhibition of inward budding was rescued (Fig. 3.2b, 3.2c). Thus, deletion of individual ESCRT-0 components decreased inward budding of EGFR into internal endosomal vesicles, an effect that was rescued by addition of exogenous orthologous mammalian recombinant proteins.

Deletion of ESCRT-I, ESCRT-II, or ESCRT-III decrease MVB internal vesicle formation and result in impaired MVB biogenesis (Bilodeau et al., 2002; Hurley and Hanson, 2010; Schmidt and Teis). To determine whether we are able to reproduce the ESCRT-dependence of inward endosomal budding and cargo sorting in our assay, cytosol isolated from yeast strains deleted of ESCRT genes (Table 1) was used in place of cytosol isolated from the parental strain. Cytosol derived from ESCRT-I deficient yeast strains (*vps23Δ*, *vps28Δ*, *vps37Δ*, or *mvb12Δ*) or ESCRT-II deficient yeast strains (*vps36Δ*, *snf8Δ*, *vps25Δ*) significantly decreased the protease

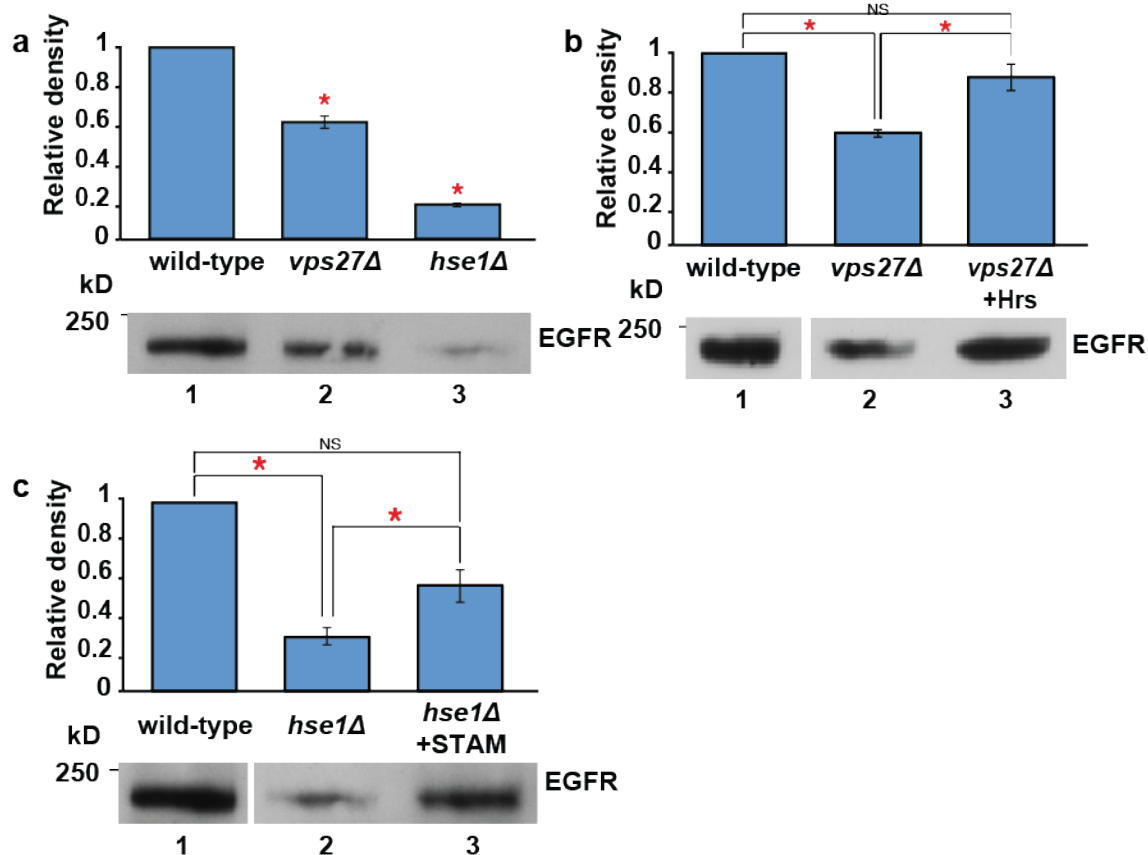


protection of the EGFR epitope (Fig. 3.3a and Fig. 3.3b respectively), suggesting that ESCRT-I and ESCRT-II components are required for inward endosomal membrane budding.

I observed that protease protection of the EGFR is dependent on Vps4 as well as the ESCRT-III components Snf7 and Vps24 (Fig. 3.3c, lanes 4, 5, 7). However, cytosol derived from yeast strains deficient in the ESCRT-III genes, *VPS20* and *DID4*, did not significantly impair protease protection of the EGFR (Fig. 3.3c, lanes 3, 6). Haplo-insufficient yeast strains of the two major ESCRT-III complexes (Snf7/Vps20 and Vps24/DID4, yMG3 and yMG4, respectively) were generated to determine whether the components of these complexes might have overlapping or redundant roles (Table 1). Cytosol derived from yMG3 and yMG4 yeast strains significantly decreased the protease protection of the EGFR epitope compared to cytosol isolated from parental strains (Fig. 3.3c, lanes 8-9 compared to lane 1). However, the effect of yMG3 (47 $\pm$ 5%) compared to the Snf7 single deletion strains (44 $\pm$ 12%), or yMG4 (55 $\pm$ 1%) compared to the Vps24 single deletion strains (51 $\pm$ 12%), on the protease protection of the EGFR epitope was not significantly different (Fig. 3.3c, Lane 8 compared to lane 4, and lane 9 compared to lane 5). The cytosol isolated from ESCRT-III double deletion strains appears additive, suggesting that Vps20 and Did4 are not required for inward budding and that Snf7 and Vps24 are sufficient to support inward budding of the EGFR.

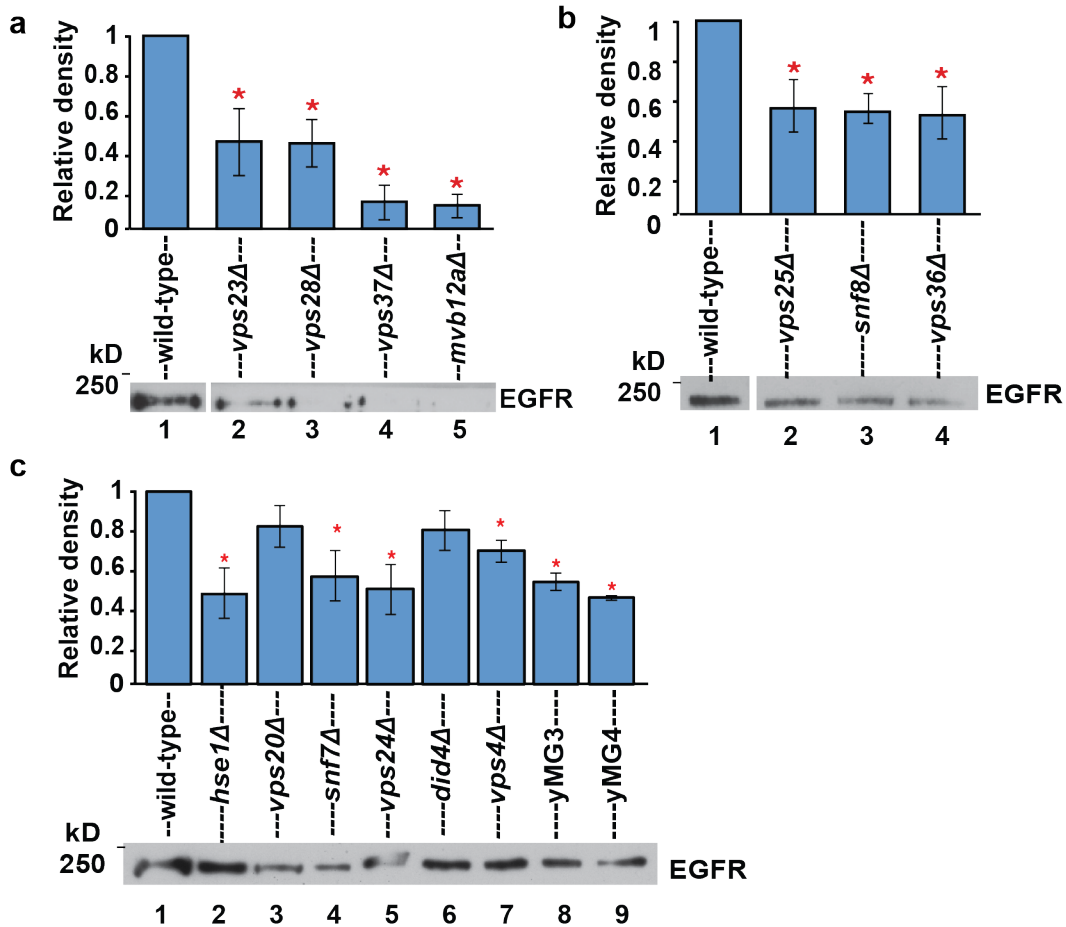
Name	Genotype	Source Reference	or
<i>vps27Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 vps27::KANMX6</i>	Dharmacon	
<i>hse1Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hse::KANMX6</i>	Dharmacon	
<i>vps23Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 vps23::KANMX6</i>	Dharmacon	
<i>vps28Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 vps28::KANMX6</i>	Dharmacon	
<i>vps37Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 vps37::KANMX6</i>	Dharmacon	
<i>mvb12aΔ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 mvb12a::KANMX6</i>	Dharmacon	
<i>vps36Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 vps36::KANMX6</i>	Dharmacon	
<i>snf8Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 snf8::KANMX6</i>	Dharmacon	
<i>vps25Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 vps25::KANMX6</i>	Dharmacon	
<i>snf7Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 snf7::KANMX6</i>	Dharmacon	
<i>did4Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 did4::KANMX6</i>	Dharmacon	
<i>vps20Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 vps20::KANMX6</i>	Dharmacon	
<i>vps24Δ</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 vps24::KANMX6</i>	Dharmacon	
yMG1	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 snf7::HPHMX6</i>	This study	
yMG2	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 did4::HPHMX6</i>	This study	
yMG3	<i>MATa/MATα his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 SNF7/snf7::HPHMX6 VPS20/vps20::KANMX6</i>	This study	
yMG4	<i>MATa/MATα his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 DID4/did4::HPHMX6 VPS24/vps24::KANMX6</i>	This study	

**Table 1: *Saccharomyces cerevisiae* strains used in this study.** Yeast strains were kindly provided by Dr. Kevin Morano but are commercially available. Yeast strains yMG1-yMG4 were generated for use in this study.



**Figure 3.2. ESCRT-0 proteins are required for inward budding of EGFR.**

Partially purified HeLa endosomal membranes were isolated as described in Fig. 1.6. a) Reactions incubated with cytosol isolated from ESCRT-0 deficient yeast strains (*vps27Δ*, *hse1Δ*) significantly decrease protease protection of EGFR compared to cytosol isolated from a parental strain (Lane 2 and 3 compared to lane 1). b) Recombinant human Hrs rescues the inhibition of EGFR protease protection (b, lane 3). c) Recombinant human STAM rescues the inhibition of EGFR protease protection (c, lane 3). Data represents the mean  $\pm$  S.E (n=3) normalized to the wild-type control. \*Denotes  $P < 0.05$ .

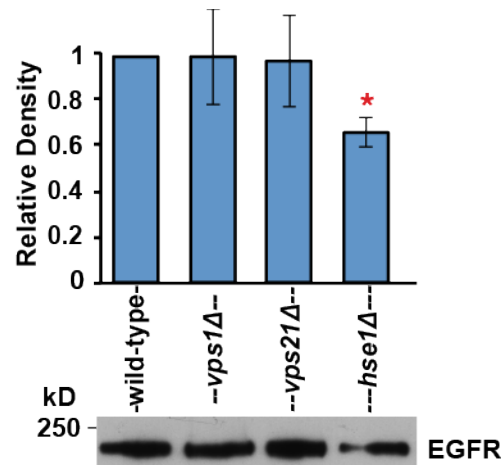


**Figure 3.3: ESCRT proteins are required for inward budding of EGFR.** Partially purified HeLa endosomal membranes were isolated as described in Fig. 1.6. a) Reactions incubated with cytosol isolated from ESCRT-I deficient yeast strains (*vps23Δ*, *vps28Δ*, *vps37Δ*, *mvb12Δ*) significantly inhibit EGFR protease protection (lanes 2-5) compared to cytosol isolated from a parental strain (lane 1). b) Reactions incubated with cytosol isolated from ESCRT-II deficient yeast strains (*vps25Δ*, *snf8Δ*, *vps36Δ*) significantly inhibit EGFR protease protection (lanes 2-4) compared to cytosol isolated from a parental strain (lane 1). c) Reactions incubated with cytosol isolated from yeast strains deleted of the ESCRT-III genes (*snf7Δ*, *vps24Δ*) or the AAA ATPase *VPS4* gene (*vps4Δ*) significantly inhibit EGFR protease

protection (lanes 4, 5, 7), compared to cytosol isolated from a parental strain (lane 1). Reactions incubated with cytosol isolated from *vps20Δ* or *did4Δ* strains prior to trypsin treatment, did not alter EGFR protease protection (lane 3, 6), compared to cytosol isolated from a parental strain (lane 1). Cytosol isolated from the yeast strain deficient of *HSE1* was used as a control in these experiments (lane 2). Reactions incubated with cytosol isolated from ESCRT-III haplo-insufficient yeast strains (yMG3 and yMG4) significantly inhibit EGFR protease protection compared to cytosol isolated from a parental strain (lanes 8-9). However, the EGFR protected was not significantly different from the single deletion strains (lane 4-5). Data represents the mean  $\pm$  S.E (n=3) normalized to the wild-type control. \*Denotes  $P < 0.05$  (t-test for a, d-f; Anova for b,c).

### 3.3. *Vps1* and *Vps21* do not affect EGFR sorting.

Control experiments were performed using cytosol derived from yeast strains in which non-ESCRT cytosolic proteins were deleted. Cytosol from yeast strains in which *Vps1*, a dynamin-like yeast homolog, or *Vps21*, the yeast homolog of Rab5, were deleted and were examined in our cell-free assay. Neither *Vps1* nor *Vps21* have been implicated in MVB biogenesis and cytosol from yeast strains in which *Vps1* or *Vps21* were deleted resulted in no significant alteration in protease protection of the EGFR (Fig 3.4).



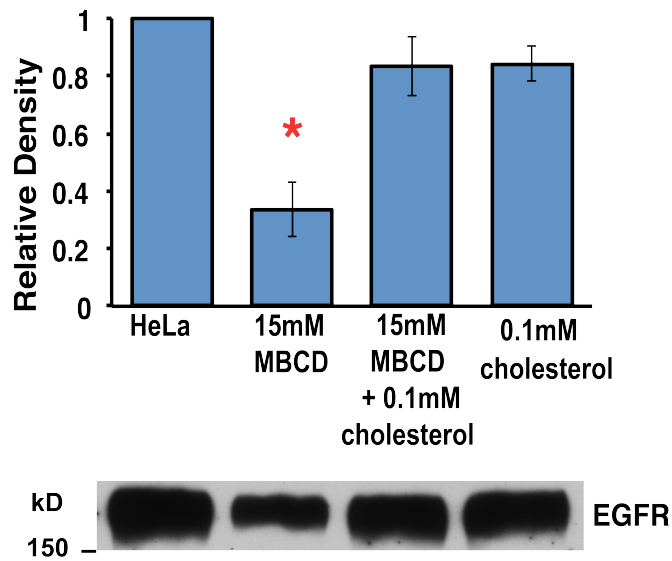
**Figure 3.4: Deletion of *VPS1* and *VPS21* genes does not impair EGFR sorting.**

Partially purified HeLa endosomal membranes were isolated as described in Fig. 1.6. a) Endosomes incubated with cytosol derived from yeast strains that are deficient of the genes *VPS1* or *VPS21* did not alter the protease protection of EGFR compared to cytosol isolated from a parental strain (lanes 2 and 3 compared to lane 1). Cytosol isolated from *hse1Δ* strains was used as a control in these experiments (lane 4). Data represents the mean  $\pm$  S.E (n=3) normalized to control. \*denotes  $p < 0.05$ .

#### *3.4. Cholesterol is required for inward budding of EGFR at MVBs.*

Cholesterol is enriched in lipid rafts and lipid rafts have been implicated in various membrane budding events (Babst, 2011; Bissig and Gruenberg, 2013). Moreover, cholesterol is concentrated on internal MVB vesicles (Mobius et al., 2003) suggesting that cholesterol may play a role in inward budding events at the MVB. To determine whether cholesterol is required for MVB sorting of transmembrane cargo, endosomes were depleted of cholesterol using Methyl- $\beta$  CycloDextrin (M $\beta$ CD). Endosomal membranes treated with 15 mM M $\beta$ CD had significantly decreased sorting of EGFR into internal vesicles compared with vehicle treated cells (Fig. 3.5, lane 2 compared to lane 1). The inhibition of EGFR protease protection in M $\beta$ CD-treated endosomes was rescued by the addition of soluble cholesterol (Fig. 3.5, lane 3), whereas soluble cholesterol had no effect on EGFR protease protection (Fig 3.5, lane 4). These data suggest that cholesterol is required for protease protection of EGFR and imply that inward vesicle budding is dependent on the availability of cholesterol in the endosomal membrane.

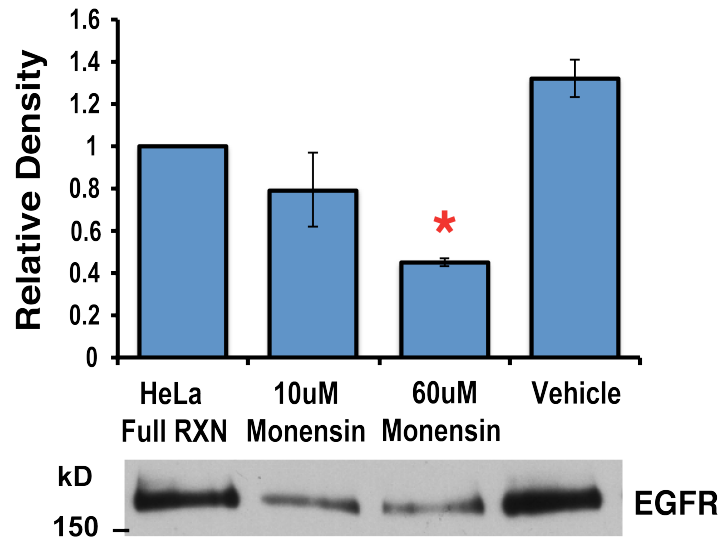




**Figure 3.5: Cholesterol is required for EGFR sorting at the MVB.** Partially purified HeLa endosomal membranes were isolated as described in Fig. 1.6. Endosomal treatment with MβCD decreased protease protection of EGFR (lanes 2) compared to vehicle control (lane 1). Soluble cholesterol rescues the inhibition of EGFR protease protection (lane 3). Soluble cholesterol had no effect on its own (lane 4). Data represents the mean  $\pm$  S.E (n=3) normalized to the control. \*denotes  $P < 0.05$ . These experiments were performed by Sahily Reyes and used with permission.

### *3.5. Inward budding of EGFR is dependent on pH at MVBs.*

Previous studies from our laboratory reported that when the ionophore nigericin was incubated in cell-free reactions protease protection of the EGFR was inhibited, suggesting that intra-endosomal pH is an important factor in inward budding (Sun et al., 2010). We examined whether the ionophore monensin would inhibit protease protection of the EGFR. Partially purified endosomes were isolated and incubated with either vehicle or Monensin (10  $\mu$ M or 60  $\mu$ M), ATP and cytosol for 3 hours at 37°C. Significant inhibition of EGFR protease protection was observed in reactions incubated with 60  $\mu$ M Monensin (Fig. 3.6, lane 3 compared to lane 1 and lane 4) compared to vehicle control. A lower concentration of Monensin, 10  $\mu$ M, had no significant effect on EGFR sorting (lane 2 compared to lane 1 and lane 4). These data suggest that intra-endosomal pH regulates endosomal sorting.



**Figure 3.6. EGFR sorting into internal vesicles is dependent on pH.** Partially purified HeLa endosomal membranes were isolated as described in Fig. 1.6. Endosomes incubated with 60  $\mu$ M Monensin decreased protease protection of EGFR (lane 3) compared to vehicle controls (lane 1 and 4). Endosomes incubated with 10  $\mu$ M Monensin (lane 2) had no effect on protease protection of EGFR compared to vehicle controls (lane 1 and 4). Data represents the mean  $\pm$  S.E (n=3) normalized to the control. \*denotes  $P < 0.05$ . These experiments were performed by Sahily Reyes and used with permission.

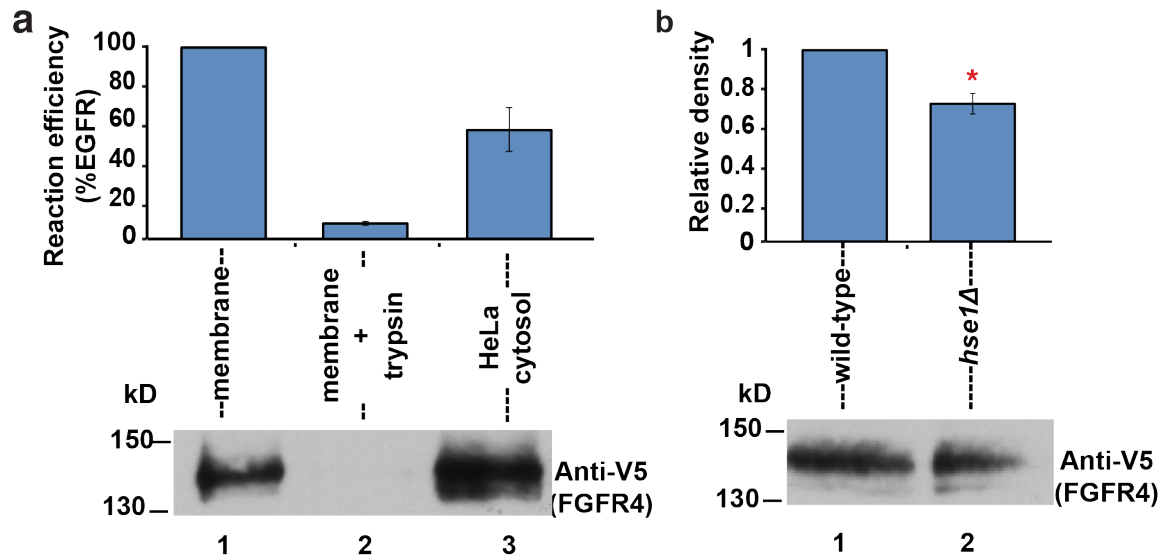
## **Chapter 4. Different types of cargo proteins are internalized into MVBs in an ESCRT-dependent manner**

*Rationale:* For the majority of my experiments, EGFR was used as a proxy for transmembrane cargo proteins that are internalized and pass through the endocytic pathway prior to degradation in the lysosome. I considered the possibility that our reaction conditions are specific for EGFR movement from the endosomal membrane but may not reflect a variety of membrane proteins thought to transit this pathway *en route* to lysosomes. To determine whether our assay may be generally applicable to understanding trafficking of membrane proteins in the late endocytic pathway I examined the movement of other types of membrane proteins from the endosomal membrane.

### *4.1. FGFR4 is sorted into MVBs in an ESCRT-dependent manner.*

To determine whether other single pass transmembrane proteins can be examined using our approach, we determined whether protease protection of the Fibroblast Growth Factor Receptor 4 (FGFR4) would occur during our cell-free reaction. I observed that under our assay conditions, the intracellular epitope of FGFR4 was protected from protease cleavage, suggesting that during the cell-free reactions FGFR4 budded inwardly into internal vesicles of MVBs (Fig. 4.1a). Cytosol derived from a yeast strain deficient in the ESCRT-0 component, *HSE1*, significantly decreased the FGFR4 protease protection compared to reactions containing parental yeast cytosol (Fig. 4.1b, lane 1). These data suggest that FGFR4

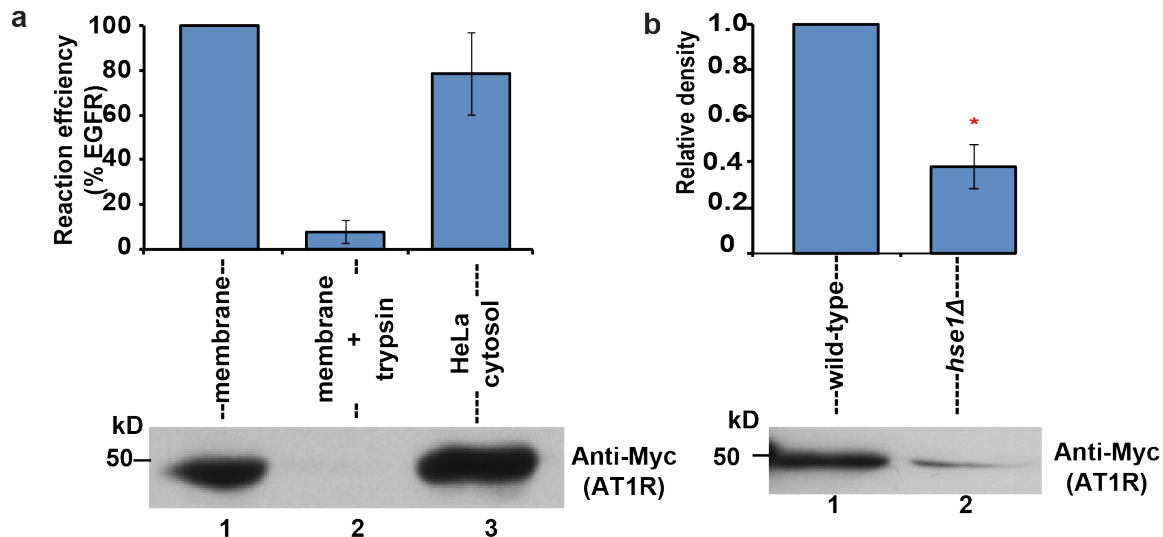
undergoes ESCRT-dependent transport into MVB internal vesicles and imply that FGFR4 follows the canonical MVB degradation pathway to the lysosome.



**Figure 4.1. FGFR4 is sorted into MVBs in an ESCRT-dependent manner.** HeLa cells were transfected with an FGFR4 construct containing a C-terminal V5 epitope tag. Partially purified HeLa endosomal membranes were isolated as described in Fig. 1.6. a) FGFR4 containing endosomes incubated with mammalian cytosol (25  $\mu$ g) result in the protease protection of the FGFR4. b) Endosomes incubated with cytosol isolated from *hse1Δ* strains significantly decrease protease protection of FGFR4 (lane 2) compared to cytosol isolated from a parental strain (lane 1). Data represents the mean  $\pm$  S.E (n=3) normalized to the control. \*denotes  $P < 0.05$ .

#### *4.2. AT1R is sorted into MVBs in an ESCRT-dependent manner.*

I next examined whether the endosomal internalization of a G-protein coupled receptor, the Angiotensin II Type 1 Receptor (AT1R) could be measured in the protease protection assay. AT1R is thought to enter MVBs prior to lysosomal degradation (Hunyady et al., 2002). An intracellular epitope of AT1R was protected from protease cleavage during our cell-free reaction suggesting that during the reaction the AT1R buds inwardly into internal vesicles of MVBs (Fig. 4.2a). Cytosol derived from a yeast strain deficient in *HSE1* significantly decreased protease protection of the AT1R compared to reactions containing parental yeast cytosol (Fig. 4.2b). These data suggest that the AT1R requires ESCRT protein sorting on endosomal membranes and can be internalized into MVB luminal vesicles.

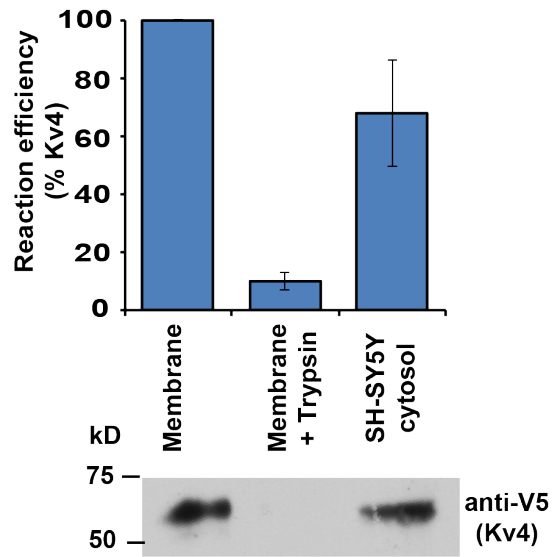


**Figure 4.2. AT1R is sorted into MVBs in an ESCRT-dependent manner.** HeLa cells were transfected with an AT1R construct containing a C-terminal myc epitope tag. Partially purified HeLa endosomal membranes were isolated as described in Fig. 1.6.a) AT1R containing endosomes incubated with mammalian cytosol (25  $\mu$ g) result in the protease protection of the AT1R. b) Endosomes incubated with cytosol isolated from *hse1Δ* strains significantly decrease protease protection of AT1R (lane 2) compared to cytosol isolated from a parental strain (lane 1). Data represents the mean  $\pm$  S.E (n=3) normalized to the control. \*denotes  $p < 0.05$  (t-test). These experiments were performed in part by Kimiya Memarzadeh and used with permission.

#### *4.3. Kv4 is sorted into MVBs.*

Ion channels are another category of membrane protein that signal when present on the plasma membrane and are degraded in the lysosome (Abriel and Staub, 2005; Staub et al., 1997). To examine the endosomal trafficking of the neuronal A-type potassium channel Kv4 (Ping et al., 2015) SH-SY5Y neuronal cells were transfected with V5 epitope-tagged Kv4. The intracellular epitope of Kv4 is protected from protease cleavage during the cell-free reaction, suggesting that Kv4 can bud inwardly into MVB internal vesicles (Fig. 4.3). These data suggest that the Kv4 channel transits through the MVB during its endosomal trafficking route and that endosomal trafficking of multiple categories of membrane proteins can be examined under cell-free conditions.





**Figure 4.3. Kv4 is sorted into MVBs.** SH-SY5Y cells were transfected with Kv4 constructs containing a C-terminal V5 epitope tag. Partially purified SH-SY5Y endosomal membranes were isolated as described in Fig. 1.6. Kv4 containing endosomes incubated with mammalian cytosol (25  $\mu$ g) result in the protease protection of the Kv4. Data represents the mean  $\pm$  S.E (n=3) normalized to the control.

## Chapter 5. Characterization of outwardly budded vesicles from MVBs

*Rationale:* Cargo that is not sorted into regions of the endosomal membrane that invaginate and bud inwardly forming internal MVB vesicles can remain on the limiting endosomal membrane for incorporation into the lysosomal membrane upon MVB-lysosome fusion. Alternatively, membrane proteins may bud outwardly on vesicles that would enable transport to other cellular compartments, a mechanism that could limit cargo accumulation on the MVB limiting membrane (Adell and Teis; Babst, 2011; Baumgart et al., 2007; Hurley and Hanson, 2010), and ensure consistent MVB size (Babst, 2011; Hurley and Hanson, 2010). However, these hypothesized transport vesicles have never been isolated and therefore requirements for this budding reaction are unknown. I modified our cell-free assay to attempt to isolate vesicles that may bud outwardly from MVBs to characterize these vesicles and elucidate the requirements for this budding step.

### *5.1. Isolation and characterization of vesicles that bud outwardly from the MVB compartment.*

To isolate the outwardly budded vesicles, partially purified endosomes were isolated as previously described in Figure 1.6 (Gireud et al., 2015; Sun et al., 2010; Sirisaengtaksin et al., 2014) and incubated with ATP and *Saccharomyces cerevisiae* (70 µg) cytosol for 3 hours at 37°C. Following the 3-hour incubation, experimental reactions were centrifuged and the resulting supernatant was collected for additional

high speed centrifugation to obtain outwardly budded vesicles. I isolated vesicles that are liberated from the endosomal membrane during our cell-free reactions (Fig. 5.1). The outward budding of vesicles in our reactions is dependent on cytosolic factors (Fig. 5.1c, lane 2 compared to lane 1). I characterized this vesicle population using light scattering and Brownian motion analysis (Nanosight Tracking Analysis), electron microscopy, immunoblotting and mass spectrometry. NTA analysis revealed that vesicles recovered from cell-free reaction supernatant were found in a major peak with a hydrodynamic diameter of  $118 \pm 13.6$  nm (Fig. 5.1a). When the isolated vesicles were visualized using electron microscopy they were approximately 100 nm in size (Fig. 5.1b). In addition, the isolated vesicles were immunoreactive for EGFR (Fig. 5.1c). The percentage of total EGFR recovered in the outwardly budded vesicles is  $6.5 \pm 1.5\%$  (Fig. 5.1c) compared to  $39 \pm 7\%$  of total EGFR that is protected from protease cleavage (inwardly budded) (Fig. 5.1c). The intracellular epitope of the EGFR was cleaved from isolated outwardly budded vesicles by trypsin incubation (Fig. 5.1d, lane 2 compared to lane 1), confirming that the tail domain of the EGFR is present on the outside of isolated vesicles as would be expected from vesicles that have budded outwardly from the endosomal limiting membrane. Mass Spectrometry analysis of proteins found on the isolated outwardly budded vesicles revealed proteins associated with these vesicles that have previously been implicated in protein trafficking (see Table 2), including adaptor proteins, Rab proteins, coat proteins, retromer proteins, SNAREs, sorting nexins, and large GTPases (see Table 2). My studies are the first to isolate and identify the cargo

constituents of the transport vesicles from the MVB, and will enable identification of the molecular mechanisms regulating outward budding from the MVB.

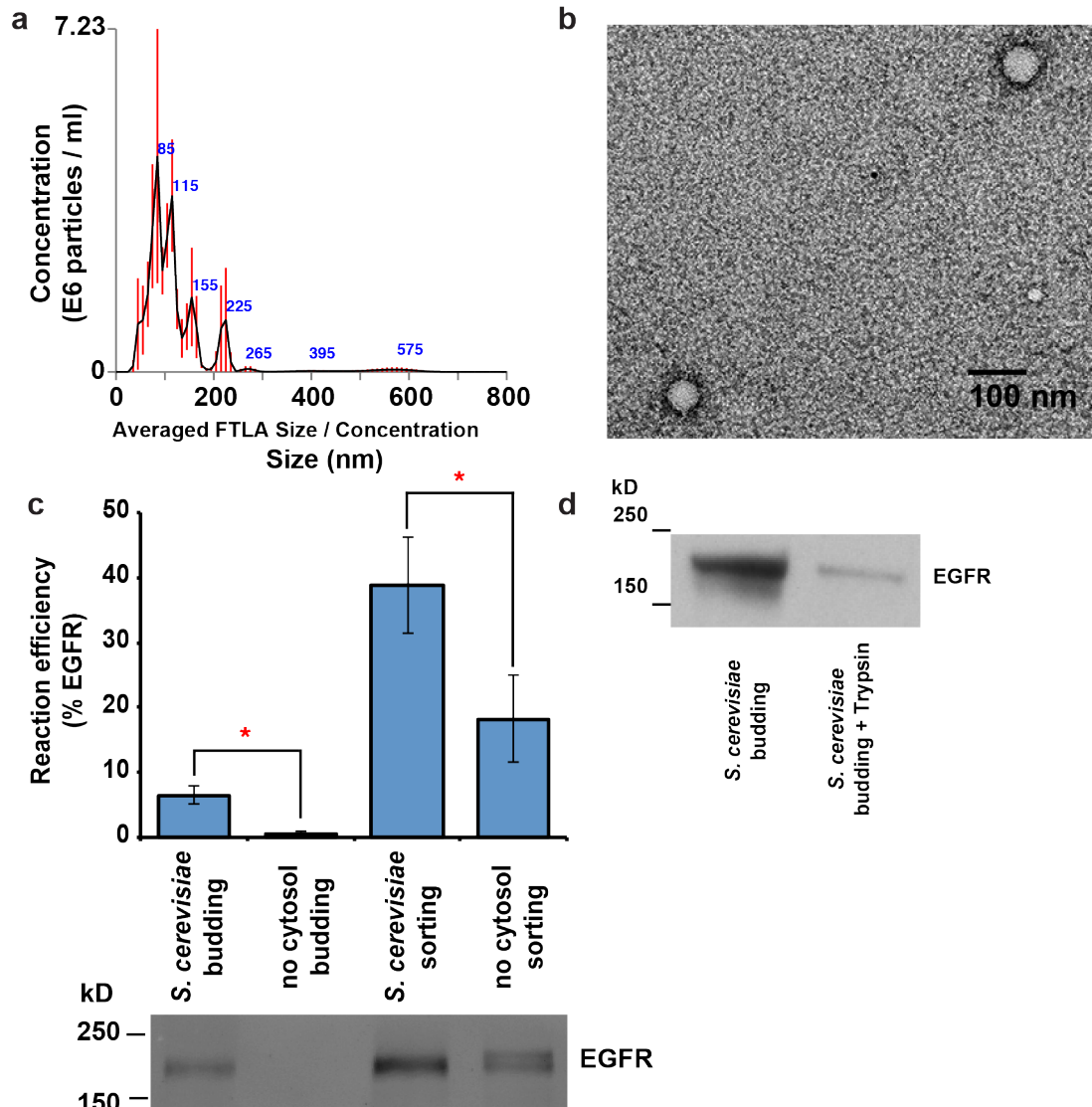
*5.2. A subset of the outwardly budding vesicles may be targeted to the plasma membrane.*

An EGFR mutant lacking kinase activity, EGFR<sub>K721A</sub>, is internalized from the plasma membrane and is transported to the limiting membranes of MVBs prior to budding outwardly into vesicles, some of which reach the plasma membrane (Felder et al., 1990). I examined whether EGFR<sub>K721A</sub> is found in outwardly budded vesicles isolated from our cell-free assay. I found that 5+/-1.3% of the total EGFR<sub>K721A</sub> is recovered in the isolated vesicles (Fig. 5.2) and 19+/-4.8% of total EGFR<sub>K721A</sub> is protected from trypsin digestion (inwardly budded) (Fig. 5.2). These data suggest that some of the outwardly budding vesicles I isolate from the cell-free reactions may be targeted to the plasma membrane.

*5.3. Differential amounts of EGF do not alter EGFR trafficking.*

The trafficking of EGFR can vary under different ligand stimulation conditions (Harris et al., 2003; Henriksen et al., 2013; Sigismund et al., 2008; Wilson et al., 2012). For example, low EGF stimulation results in internalization through clathrin-mediated endocytosis and decreased degradation of EGFR whereas high EGF stimulation results in internalization through clathrin-mediated endocytosis or clathrin-independent endocytosis and enhances EGFR degradation (Sigismund et al., 2008). I examined whether the amount of EGFR found in inwardly and outwardly budded vesicles was different under different stimulation conditions (Fig. 5.3). When stimulated with low EGF concentrations (2ng/ml) conditions, 2+/-0.2% of EGFR is

found in the outwardly budded vesicles (Fig. 5.3, lane 1) and  $20 \pm 6.3\%$  of EGFR is protected from trypsin digestion (inwardly budding) (Fig. 5.3, lane 3). Following high EGF stimulation (100ng) conditions, approximately  $6 \pm 1.7\%$  of EGFR is found in the outwardly budded vesicles (lane 2) and  $35 \pm 4.3\%$  of EGFR is protected from protease cleavage (inwardly budded) (lane 4). While there was a trend towards an increase in the outward budding:inward budding ratio, there is no significant difference between EGFR disposition under low or high EGF stimulation conditions ( $9.7\%$  and  $16.2\%$ ), respectively. These data suggest that the trafficking of EGFR at the MVB is similar under different stimulation conditions and perhaps the differences in internalization of EGFR may affect EGFR trafficking during early parts of the endocytic pathway.



**Figure 5.1: Isolation and characterization of outwardly budded vesicles from endosomal membranes.** Outwardly budded vesicles were isolated as described in Fig. 1.6. a) Nanosight tracking analysis revealed the vesicles were  $118 \pm 13.6$  nm in size. b) Vesicles visualized using electron microscopy were approximately 100 nm in size. Scale bar = 100 nm. c) The amount of EGFR found in the budded vesicles is  $6.5 \pm 1.5\%$  of the total added into the reactions (c, lane 1). The percentage of EGFR that is protease protected (internalized) is  $39 \pm 7\%$  (c, lane 3). Both outward budding

and inward budding are dependent on cytosolic components (c, lane 2 compared to lane 1, and lane 4 compared to lane 3). d) The intracellular epitope of the EGFR was cleaved from isolated outwardly budded vesicles by trypsin incubation. Data represents the mean  $\pm$  S.E (n=3) normalized to the control. \*denotes  $P < 0.05$ . NTA analysis was performed in part by Shinji Yamashita and used with permission. The EM experiments were performed in part by Madeline Farley and used with permission.

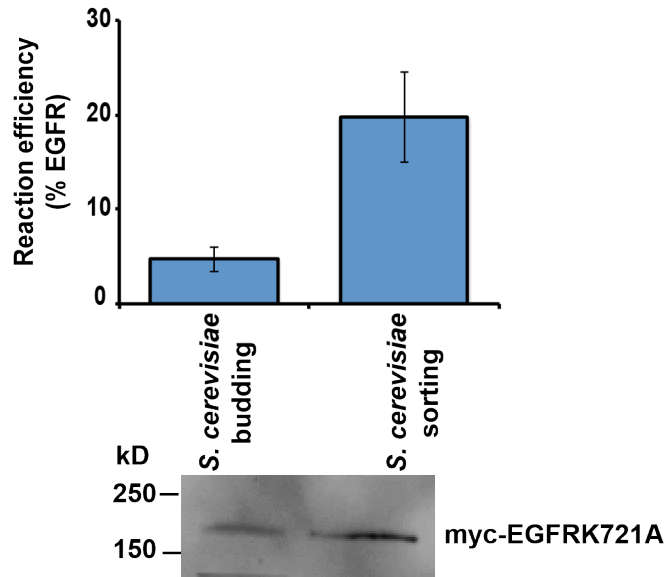
Protein Class	Gene symbol	Protein	Unique	Total	Reference	Molecular weight (kDa)	Average
Adaptor Proteins	AP1B1	Adaptor Related Protein Complex 1 Beta 1 Subunit	11	11	Q10567_AP1B1_HUMAN	104.57	2.8713
	AP1G1	Adaptor Related Protein Complex 1 Gamma 1 Subunit	3	3	O43747_AP1G1_HUMAN	91.29	2.7409
	AP1M1	Adaptor Related Protein Complex 1 Mu 1 Subunit	2	2	Q9BXS5_AP1M1_HUMAN	48.56	2.7235
	AP1S1	Adaptor Related Protein Complex 1 Sigma 1 Subunit	2	2	P61966_AP1S1_HUMAN	18.72	3.6144
	AP1S2	Adaptor Related Protein Complex 1 Sigma 2 Subunit	1	1	P56377_AP1S2_HUMAN	18.6	4.4323
	AP2A1	Adaptor Related Protein Complex 2 Alpha 1 Subunit	12	12	O95782_AP2A1_HUMAN	107.48	2.6643
	AP2A2	Adaptor Related Protein Complex 2 Alpha 2 Subunit	2	2	O94973_AP2A2_HUMAN	103.89	3.2007
	AP2B1	Adaptor Related Protein Complex 2 Beta 1 Subunit	8	8	P63010_AP2B1_HUMAN	104.49	2.9398
	AP2M1	Adaptor Related Protein Complex 2 Mu 1 Subunit	4	4	Q96CW1_AP2M1_HUMAN	49.62	2.9861
ARF Proteins	ARF1	ADP Ribosylation Factor 1	4	4	P84077_ARF1_HUMAN	20.68	2.413
	ARF4	ADP Ribosylation Factor 4	2	2	P18085_ARF4_HUMAN	20.5	2.5135
	ARF5	ADP Ribosylation Factor 5	2	2	P84085_ARF5_HUMAN	20.52	2.8499
Rab Proteins	RAB1A	RAB1A, Member RAS Oncogene Family	3	3	P62820_RAB1A_HUMAN	22.66	3.3149
	RAB5A	RAB5A, Member RAS Oncogene Family	2	2	P20339_RAB5A_HUMAN	23.64	2.7698
	RAB5C	RAB5C, Member RAS Oncogene Family	2	2	P51148_RAB5C_HUMAN	23.47	2.7439
	RAB6A	RAB6A, Member RAS Oncogene Family	1	1	P20340_RAB6A_HUMAN	23.58	2.7129
	RAB7A	RAB7A, Member RAS Oncogene Family	6	7	P51149_RAB7A_HUMAN	23.47	3.0881
	RAB8A	RAB8A, Member RAS Oncogene Family	2	2	P61006_RAB8A_HUMAN	23.65	3.5539
	RAB11A	RAB11A, Member RAS Oncogene Family	9	9	P62491_RB11A_HUMAN	24.38	3.1934
	RAB11B	RAB11B, Member RAS Oncogene Family	1	1	Q15907_RB11B_HUMAN	24.47	5.614
	RAB14	RAB14, Member	3	3	P61106_RAB	23.88	2.121



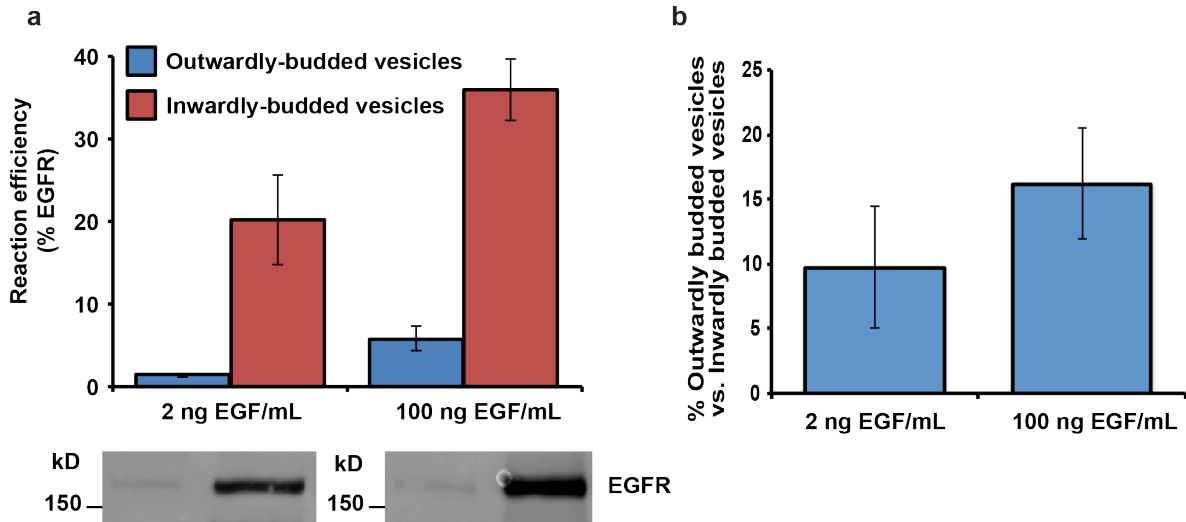
		RAS Oncogene Family			14_HUMAN		
	RAB32	RAB32, Member RAS Oncogene Family	1	1	Q13637_RAB32_HUMAN	24.98	2.3859
	CLTC	Clathrin Heavy Chain	31	31	Q00610_CLH1_HUMAN	191.49	3.4423
	COPA	Coatome Protein Complex Subunit Alpha	1	1	P53621_COPA_HUMAN	138.26	2.5796
	COPB1	Coatome Protein Complex Subunit Beta 1	3	3	P53618_COPB_HUMAN	107.07	2.6352
<b>Coat Proteins</b>	COPB2	Coatome Protein Complex Subunit Beta 2	2	3	P35606_COPB2_HUMAN	102.42	2.4587
	COPG1	Coatome Protein Complex Subunit Gamma 1	2	2	Q9Y678_COPG1_HUMAN	97.66	2.8014
	COPZ1	Coatome Protein Complex Subunit Zeta 1	2	2	P61923_COPZ1_HUMAN	20.19	3.5031
	SEC23A	Sec23 Homolog A, Coat Complex II Component	5	5	Q15436_SC23A_HUMAN	86.11	2.7646
	SEC24C	SEC24 Homolog C, COPII Coat Complex Component	5	5	P53992_SC24C_HUMAN	118.25	2.7165
	SNX2	Sorting Nexin 2	1	1	O60749_SNX2_HUMAN	58.43	2.3274
	VPS29	VPS29 Retromer Complex Component	1	1	Q9UBQ0_VPS29_HUMAN	20.49	3.1522
	VPS35	VPS35 Retromer Complex Component	5	5	Q96QK1_VPS35_HUMAN	91.65	3.0096
<b>Retromer proteins</b>	SEC22B	SEC22 Homolog B, Vesicle Trafficking Protein	2	2	O75396_SC22B_HUMAN	24.58	3.7099
	SNAP91	Synaptosome Associated Protein 91kDa	2	2	O60641_AP180_HUMAN	92.44	3.1687
	VAMP2	Vesicle Associated Membrane Protein 2	3	3	P63027_VAMP2_HUMAN	12.65	4.0905
<b>SNARE proteins</b>	VAMP3	Vesicle Associated Membrane Protein 3	1	1	Q15836_VAMP3_HUMAN	11.3	4.41
	SNX3	Sorting Nexin 3	3	3	O60493_SNX3_HUMAN	18.75	2.3169
	SNX12	Sorting Nexin 12	3	3	Q9UMY4_SNX12_HUMAN	19.72	2.7626
	DNM2	Dynamin 2	1	1	P50570_DYN2_HUMAN	98	2.3105
<b>Sorting Nexin Proteins</b>	A2M	Alpha-2-Macroglobulin	2	3	P01023_A2MG_HUMAN	163.19	2.0934
	ACTA2	Actin, Alpha 2, Smooth Muscle,	23	26	P62736_ACTA_HUMAN	41.98	2.512

		Aorta					
<b>Large GTPase</b>	ACTN1	Actinin Alpha 1	11	11	P12814_ACTN1_HUMAN	102.99	3.5522
<b>Other Proteins</b>	ACTN4	Actinin Alpha 4	14	14	O43707_ACTN4_HUMAN	104.79	3.0338
	ANXA1	Annexin A1	9	9	P04083_ANXA1_HUMAN	38.69	3.7527
	ANXA2	Annexin A2	35	37	P07355_ANXA2_HUMAN	38.58	3.1317
	ANXA3	Annexin A3	5	5	P12429_ANXA3_HUMAN	36.35	2.5467
	ANXA5	Annexin A5	13	13	P08758_ANXA5_HUMAN	35.91	2.9349
	CLIC1	Chloride Intracellular Channel 1	4	4	O00299_CLIC1_HUMAN	26.91	2.6256
	LAMTOR1	Late Endosomal/Lysosomal Adaptor, MAPK And MTOR Activator 1	1	1	Q6IAA8_LTOR1_HUMAN	17.73	4.6145
	M6PR	Mannose-6-Phosphate Receptor, Cation Dependent	1	1	P20645_MPRD_HUMAN	30.97	3.9622
	TFRC	Transferrin Receptor	14	15	P02786_TFR1_HUMAN	84.82	3.5202
	UBE2D2	Ubiquitin Conjugating Enzyme E2 D2	1	1	P62837_UBE2D2_HUMAN	16.72	2.8667
	UBE2V1	Ubiquitin Conjugating Enzyme E2 V1	5	5	Q13404_UBE2V1_HUMAN	16.48	2.3619
	VCP	Valosin Containing Protein	45	59	P55072_VCPA_HUMAN	89.27	2.9445

**Table 2: Analysis of Outwardly Budded Endosomal Vesicles.** Mass Spectrometry analysis reveals multiple types of protein classes associated with outwardly budded vesicles. Gene name, protein name, reference, molecular weight, and average are listed (n=2).



**Fig. 5.2. A subset of outwardly budded endosomal vesicles may target the plasma membrane.** HeLa cells were transfected with EGFR<sub>K721A</sub> containing a C-terminal myc epitope tag. Outwardly budded vesicles were isolated as described in Fig. 1.6. Outwardly budding vesicles containing EGFR<sub>K721A</sub> were 5 $\pm$ 1.3% of total EGFR<sub>K721A</sub> isolated following the cell-free reaction (e, lane 1) whereas 19 $\pm$ 4.8% of total EGFR<sub>K721A</sub> is protected from trypsin digestion (e, lane 2). Data represents the mean  $\pm$  S.E (n=3) normalized to the control. \*denotes  $p < 0.05$  (t-test).



**Figure 5.3. Varying EGF stimulation does not alter endosomal EGFR trafficking.** Outwardly budded vesicles were isolated as described in Fig. 1.6. a) Stimulation with low concentrations of EGF (2ng/ml) revealed that 2 $\pm$ 0.2% of EGFR is found in outwardly budded vesicles (lane 1) while 20 $\pm$ 6.3% of EGFR is protected from protease digestion (lane 3). Stimulation with high concentrations of EGF (100ng/ml) revealed that 6 $\pm$ 1.7% % of EGFR is found in outwardly budded vesicles (lane 2) while 35 $\pm$ 4.3% of EGFR is protected from trypsin digestion (inwardly budded) (lane 4). b) The ratio between outwardly and inwardly budded vesicles under different stimulation conditions is not significantly different. Data represents the mean  $\pm$  S.E (n=3) normalized to the control. \*denotes  $p < 0.05$ .

## Chapter 6. Mechanisms of outward vesicle budding from MVBs.

*Rationale:* My results show that outward budding from MVBs is dependent on cytosolic components (Fig. 5.1c). However, the cytosolic molecules that regulate outward budding from MVBs have not been elucidated. I have taken both candidate and genetic approaches to discover cytosolic factors required for this budding event.

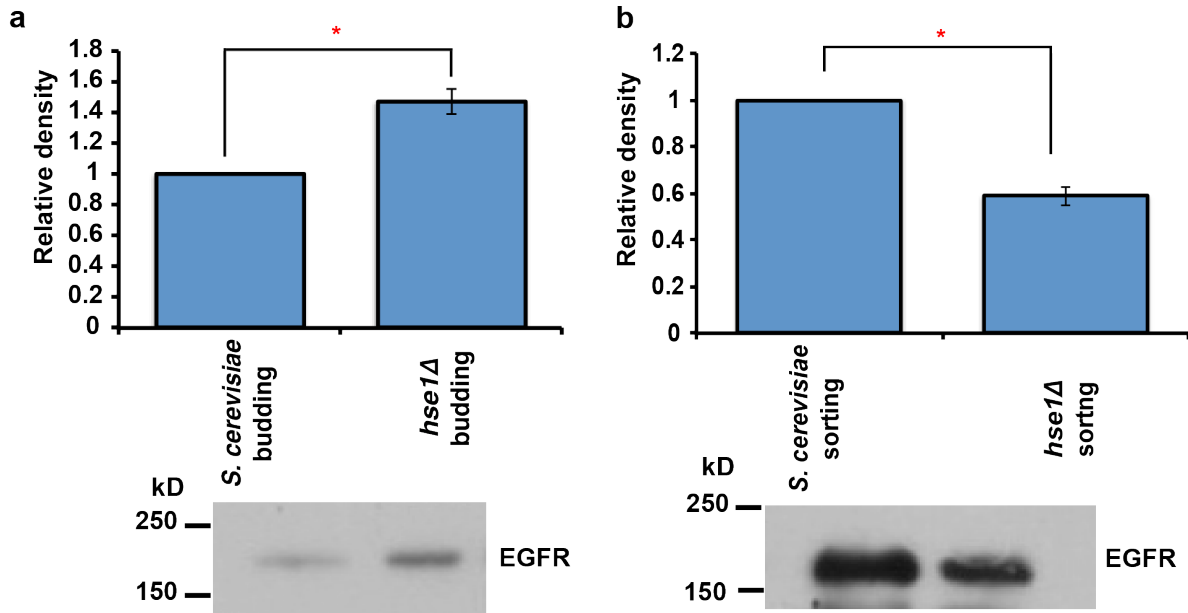
### *6.1. Distinct molecular machineries regulate outward vesicle budding and inward vesicle budding.*

To identify factors that may play a role in outward budding, I isolated cytosol from yeast strains lacking various genes. To examine whether ESCRT proteins may play a role in outward vesicle budding, cytosol derived from a yeast strain lacking *HSE1* (*hse1Δ*) was used in place of cytosol isolated from a parental yeast strain in our cell-free assay. Interestingly, EGFR immunoreactivity is significantly increased in outwardly budded vesicles that were isolated from *hse1Δ* reaction supernatant compared to cytosol isolated from the parental strain (Fig. 6.1a, lane 2 compared to lane 1). The EGFR was less protected from protease cleavage in reactions containing *hse1Δ* cytosol compared to its protease protection in reactions containing cytosol isolated from parental strains (Fig. 6.1b, lane 2 compared to lane 1). Thus, while the ESCRT proteins do not appear to be required for outward vesicle budding, inhibition of inward budding by deleting HSE1 results in increased outward budding, suggesting that inward and outward budding are linked processes.

To interrogate the cytosol further, I screened cytosol isolated from yeast deletion mutants for its ability to support outward budding. Interestingly, cytosol derived from a yeast strain deficient in a dynamin-like yeast ortholog, VPS1 (*vps1Δ* cytosol), inhibited the amount of EGFR-immunoreactivity in isolated vesicles compared to cytosol isolated from the parental strain (Fig. 6.2a, lane 2 compared to lane 1). *Vps1Δ* cytosol had no significant effect on the protease protection of EGFR (Fig. 6.2b) suggesting that inward budding was not altered in the absence of VPS1. Rescue experiments in which I added recombinant mammalian Dynamin1 to reactions containing *vps1Δ* cytosol, rescued the inhibition of outward budding (Fig. 6.2a, lane 3 compared to lane 2). These data suggest that dynamin is required for outward budding of EGFR, but not inward budding into internal MVB vesicles.

Aside from dynamin, I identified multiple GTPases that are associated with outwardly budded vesicles isolated from our cell-free reaction supernatant (Table 2). GTPases have been implicated in multiple membrane budding events (Nicoziani and van Deurs, 2000; Stenmark 2009; Traub, 2010; Grant and Donaldson, 2009; Maxfield and McGraw, 2004; Thompson et al., 2007; Weigert et al., 2004; Kobayashi, 2013; McCaffrey and Bucci, 2001; Yamashiro and Maxfield, 1984; Hopkins and Trowbridge, 1994; Ghosh and Maxfield, 1995). In this regard, the GTPase, Rab11 regulates outward budding from early endosomes (Kobayashi and Fukuda, 2013; van Dam and Stoorvogel, 2002). However, Rab11 or Transferrin Receptor (TfR, which is known to traffic in Rab11 compartments) were not detected on vesicles isolated from our reaction supernatant suggesting that Rab11 does not play a role in outward budding from MVBs. Nevertheless, I expressed dominant-

negative Rab11 (Rab11S25N) (Fig. 6.3c) in cells and examined whether its expression would affect outward vesicle budding from MVBs. EGFR immunopositive outwardly budded vesicles were isolated from reactions and there was no significant difference in EGFR levels observed in reaction supernatant when cells expressed Rab11S25N (Fig 6.3a, lane 1), suggesting that Rab11 is not required for EGFR-positive outwardly budded vesicles. As I previously observed (Fig. 6.1) reactions containing cytosol isolated from an ESCRT-0 deficient yeast strain (*hse1Δ*) produced significantly higher levels of EGFR-immunoreactivity in the reaction supernatant compared to cytosol isolated from a parental yeast strain (Fig. 6.3a, lane 2 compared to lane 1). By contrast, decreased EGFR-immunoreactivity in reaction supernatant (outwardly budded vesicles) was observed in reactions containing dynamin-deficient (*vps1Δ*) cytosol (Fig. 6.3b, lane 3 compared to lane 1) compared to control in cells expressing Rab11S25N.

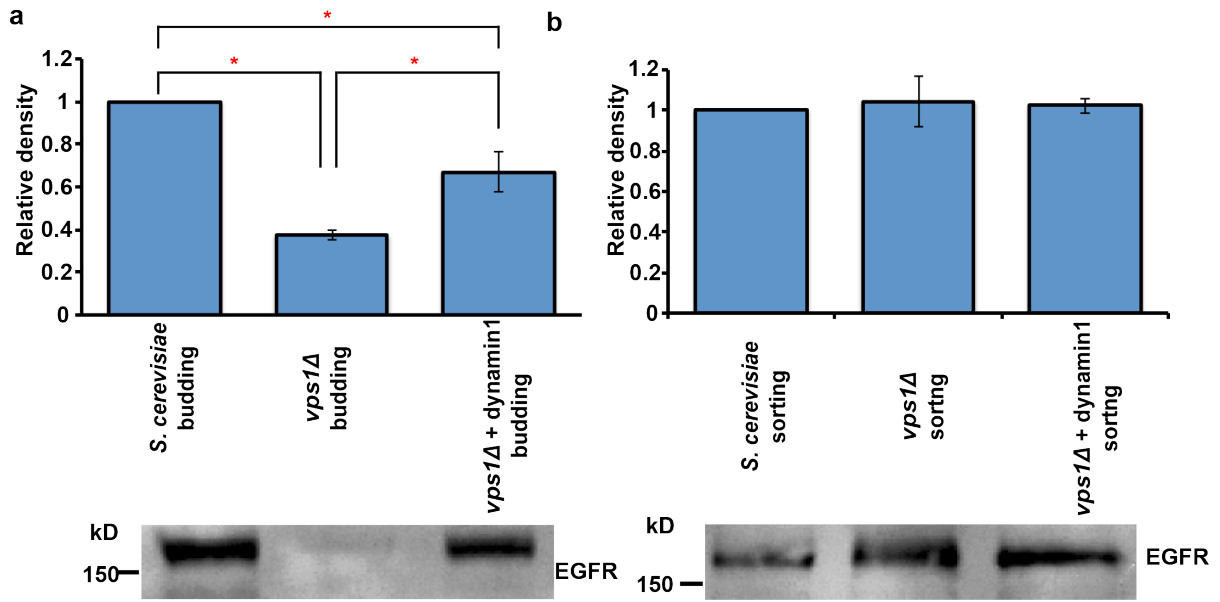


**Figure 6.1. Deletion of ESCRT machinery increases outward vesicle budding.**

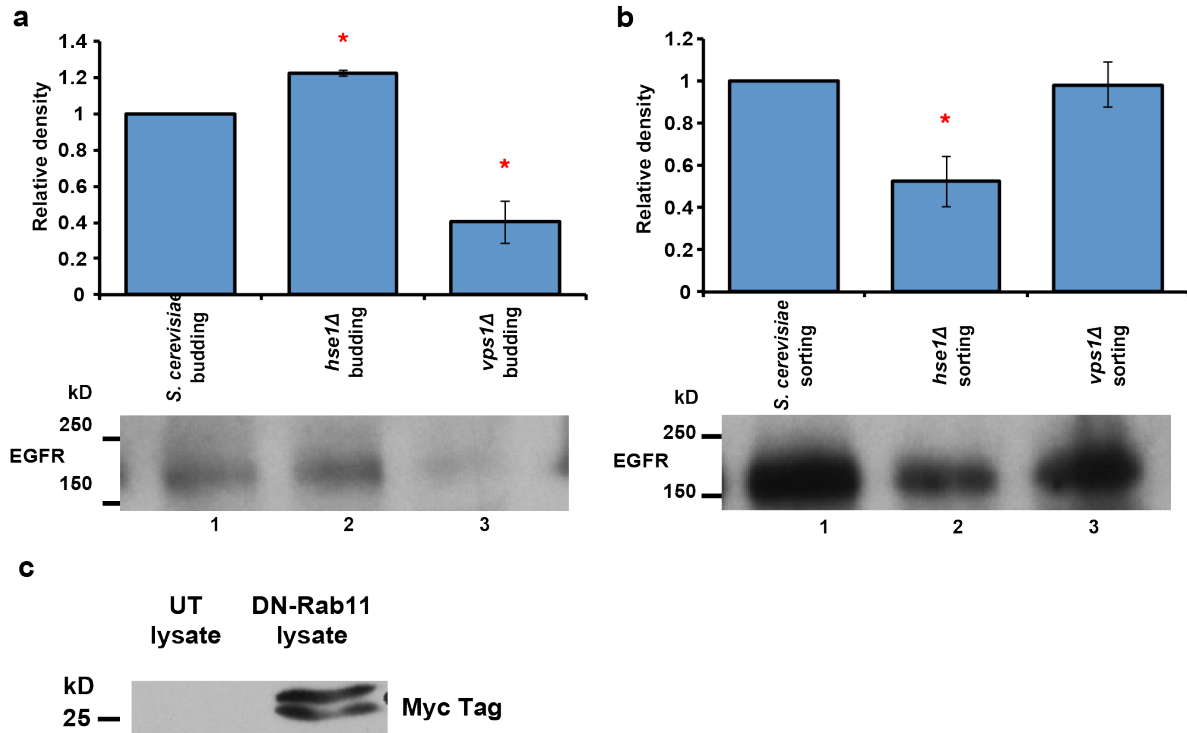
Outwardly budded vesicles were isolated as in Figure 1.6. a) Vesicles isolated from *hse1Δ* reaction supernatant significantly increase EGFR immunoreactivity compared to cytosol isolated from a parental strain (lane 2, compared to lane 1). b) Reactions incubated with cytosol isolated from *hse1Δ* strains significantly inhibit protease protection of EGFR compared to cytosol isolated from parental strains (lane 2 compared to 1). Data represents the mean  $\pm$  S.E (n=3) normalized to the control.

\*denotes  $p < 0.05$ .





**Figure 6.2. Dynamin is required for outward vesicle budding.** Outwardly budded vesicles were isolated as in Figure 1.6. a) Vesicles isolated from *vps1Δ* reaction supernatant significantly decrease EGFR immunoreactivity compared to cytosol isolated from a parental strain (lane 2 compared to lane 1). Recombinant human dynamin1 (1μm) rescues the inhibition of EGFR immunoreactivity (lane 3 compared to lane 1). b) Reactions incubated with cytosol isolated from *vps1Δ* strains or *vps1Δ* strains plus dynamin1 did not alter protease protection of EGFR compared to cytosol isolated from a parental strain (lane 2 and 3 compared to lane 1). Data represents the mean  $\pm$  S.E (n=3) normalized to the control. \*denotes  $p < 0.05$  (t-test for a,b; ANOVA for c,d).



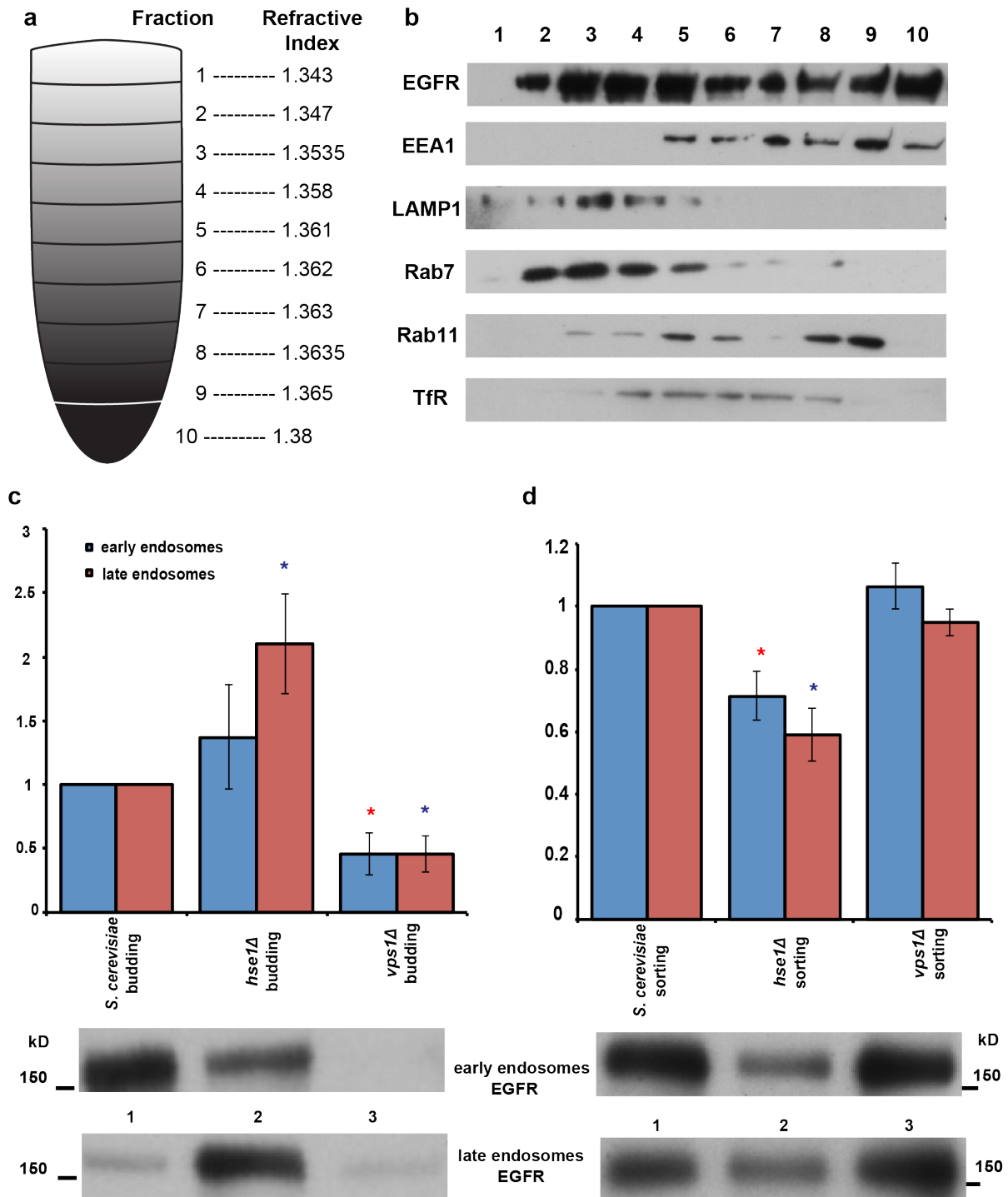
**Figure 6.3. EGFR buds outwardly from endosomal membranes in a Rab11 independent manner.** HeLa cells were transfected with a dominant negative Rab11 (Rab11S25N) construct containing a C-terminal myc epitope tag. Outwardly budded vesicles were isolated as in Figure 1b. a) Vesicles isolated from *hse1Δ* reaction supernatant significantly increase EGFR immunoreactivity compared to cytosol isolated from a parental strain (lane 2, compared to lane 1). Vesicles isolated from *vps1Δ* reaction supernatant significantly decrease EGFR immunoreactivity compared to cytosol isolated from a parental strain (lane 2 compared to lane 1). b) Reactions incubated with cytosol isolated from *hse1Δ* strains significantly inhibit protease protection of EGFR compared to cytosol isolated from parental strains (lane 2 compared to 1). Reactions incubated with cytosol isolated from *vps1Δ* strains or *vps1Δ* strains plus dynamin1 did not alter protease protection of EGFR compared to cytosol isolated from a parental strain (lane 2 and 3 compared to lane 1). c) HeLa

cells express Rab11S25N. Data represents the mean  $\pm$  S.E (n=3) normalized to the control. \*denotes  $p < 0.05$ .

## 6.2. *EGFR buds outwardly from late endosomal compartments.*

My previous experiments were performed using endosomes that contain a mixture of early and late endosomal populations. While early endosomes are thought to mature into late endosomes by acquiring and discarding peripheral membrane proteins, it is not clear when endosomes are competent to sort membrane proteins and invaginate their limiting membranes to produce MVBs. To determine whether early and late endosomal populations can bud EGFR-containing vesicles, I separated these populations for use in our cell-free reactions. Post nuclear supernatant from lysed cells was separated using Optiprep gradients and gradient fractions were examined for the presence of markers for endosomal compartments (EEA1 for early endosomes, LAMP1/Rab7 for late endosomes, RAB11 for recycling endosomes) as well as EGFR and TfR (Fig 6.4b). Early endosomal membranes (fractions 8 and 9) and late endosomal membranes (fractions 3 and 4) were collected and incubated in separate reactions containing ATP and yeast cytosol. I observed that inward and outward budding of EGFR-immunoreactivity occurred from both endosomal populations (Fig. 6.4c and Fig. 6.4d). Reactions containing cytosol isolated from the *hse1Δ* yeast strain produced significantly increased EGFR immunoreactivity in supernatant from reactions containing late endosomal membranes compared with cytosol isolated from the parental yeast strain, suggesting that ESCRT-0 deletion increased outward budding from late endosomes. In contrast, there was no increase in EGFR-immunoreactivity in supernatant from reactions containing early endosomal reactions and incubated with *hse1Δ* cytosol compared to the parental yeast strain, suggesting that outward

budding from the early endosome is not altered by ESCRT-0 protein deletion (Fig. 6.4c, lane 2 compared to lane 1). The protease protection of the EGFR was significantly decreased in reactions containing either early or late endosomal membranes and incubated with *hse1Δ* cytosol (Fig. 6.4d, lane 2 compared to lane 1) suggesting that both membrane populations I isolated can invaginate and protect EGFR in an ESCRT-dependent manner. Reactions containing cytosol isolated from the *vps1Δ* yeast strain produced significantly decreased EGFR immunoreactivity in supernatant from reactions containing either early or late endosomal membranes, suggesting that dynamin deletion decreases outward budding from both membrane populations (Fig. 6.4c, lane 3 compared to lane 1). *Vps1Δ* cytosol had no significant effect on protease protection of EGFR in either population (Fig. 6.4d, lane 3 compared to lane 1), suggesting it is not required for internal vesicle formation of MVBs. In summary, our results suggest dynamin facilitates outward budding from the late endosome/MVB.



**Figure 6.4. EGFR buds outwardly from late endosomal membranes in a dynamin dependent manner.** Post-nuclear supernatant was loaded onto a continuous 10-20% Optiprep gradient. a) Fractions were collected and the refractive

index was measured. b) Fractions were immunoblotted for endosomal markers (EEA1 for early endosomes, LAMP1/Rab7 for late endosomes, RAB11 for recycling endosomes, EGFR, and TfR). c) Early endosomes (fractions 8 and 9) incubated with cytosol isolated from *hse1Δ* strains did not alter EGFR immunoreactivity on vesicles isolated from reaction supernatant compared to cytosol isolated from a parental strain (lane 2 compared to lane 1). Late endosomes (fractions 3 and 4) incubated with cytosol isolated from *hse1Δ* strains significantly increase EGFR immunoreactivity on vesicles isolated from reaction supernatant compared to cytosol isolated from a parental strain (lane 2 compared to lane 1) ( $p=0.042$ ). Vesicles isolated from reaction supernatant obtained from either early or late endosomes and incubated with cytosol isolated from *vps1Δ* strains significantly decrease EGFR immunoreactivity compared to cytosol isolated from a parental strain (lane 3, compared to lane 1). d) Early and late endosomes incubated with cytosol isolated from *hse1Δ* strains significantly decreased protease protection of EGFR compared to compared to cytosol isolated from a parental strain (lane 2 compared to 1). Early and late endosomes incubated with cytosol isolated from *vps1Δ* strains did not alter protease protection of EGFR compared to cytosol isolated from a parental strain (lane 3 compared to lane 1). Data represents the mean  $\pm$  S.E ( $n=3$ ) normalized to the control. \*denotes  $p < 0.05$  (t-test).

## Chapter 7. EGFR<sub>VIII</sub> inward and outward budding from the MVB

### *Rationale:*

Altered intracellular trafficking of membrane proteins may alter their cell surface expression, modulate their signaling, and can be a significant driver of disease. For example, an EGFR mutant, EGFR<sub>VIII</sub>, is internalized from the plasma membrane but is not efficiently degraded compared to the wild-type receptor. This pathogenic EGFR mutant constitutively recycles resulting in increased surface expression, signaling, and proliferation, and is expressed in 50% of glioblastoma cases (Frederick et al., 2000; Furnari et al., 2007; Gan et al., 2009; Heimberger et al., 2005; Sugawa et al., 1990) where its presence correlates with aggressive disease (Shinojima et al., 2003). Thus, elucidating the mechanism by which the endocytic itinerary of EGFR<sub>VIII</sub> differs from that of the wild-type EGFR may provide an understanding of a trafficking pathway important for disease.

### *7.1. EGFR<sub>VIII</sub> is sorted into internal vesicles of MVBs.*

Although EGFR<sub>VIII</sub> is internalized from the plasma membrane, inhibition of its degradation rate may result from slowed movement through the endocytic pathway. This inhibited trafficking of EGFR<sub>VIII</sub> may occur at multiple steps during its movement to the lysosome (Fig. 1.5). EGFR<sub>VIII</sub> is found on endosomes (Grandal et al., 2007) suggesting that it passes through those organelles *en route* to lysosomes and may therefore take the canonical MVB pathway to the lysosome. Initially, I determined that EGFR<sub>VIII</sub> is internalized into internal vesicles of MVBs (Fig. 7.1a) by examining



protease protection of an intracellular epitope as I had done previously for the wild-type receptor. I determined that in the absence of EGF stimulation, EGFR<sub>VIII</sub> is present on endosomes and a portion of the receptor is protected from digestion into internal vesicles of MVBs under basal conditions, suggesting that a pool of the EGFR<sub>VIII</sub> moves through the endocytic pathway (Fig. 7.1b, lanes 4-6) into internal MVB vesicles. In contrast, WT EGFR was not detected on endosomes in the absence of EGF stimulation (Fig. 7.1b, lanes 1-3). Upon EGF stimulation, the intracellular epitope of WT EGFR was protected from protease cleavage suggesting that the WT EGFR has entered endosomes (Fig. 7.1b, lanes 7-9). These data suggest that a pool of EGFR<sub>VIII</sub> can be sorted into internal vesicles of MVBs in a ligand-independent manner.

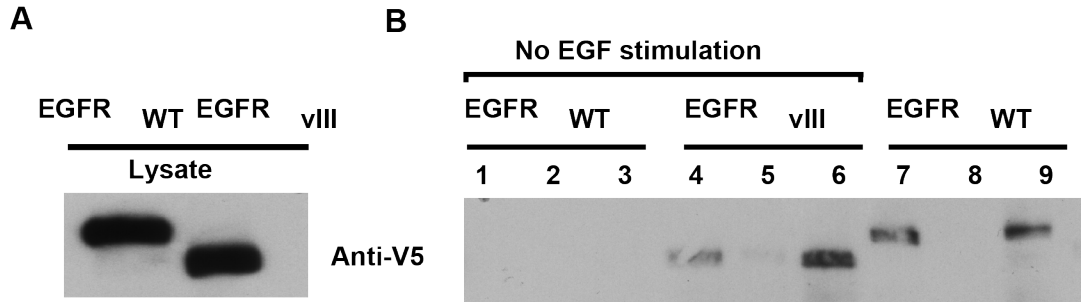
## *7.2. Isolation of mutant EGFR outwardly budding vesicles.*

To determine whether EGFR<sub>VIII</sub> buds outwardly from endosomal membranes, partially purified endosomal membranes isolated from U87 cells expressing either WT-EGFR or EGFR<sub>VIII</sub> were incubated with ATP and *Saccharomyces cerevisiae* (70 µg) cytosol for 3 hours at 37°C. Following incubation, reactions were centrifuged to obtain outwardly budded vesicles. Characterization of this vesicle population using light scattering and Brownian motion analysis (NTA) revealed that vesicles containing WT-EGFR were found in a major peak with a hydrodynamic diameter of 107.8 +/- 12.4 nm (Fig. 7.2a). Similarly, NTA analysis revealed that vesicles recovered from EGFR<sub>VIII</sub> expressing cells were found in a major peak with a hydrodynamic diameter of 124 +/- 11.1 nm (Fig. 7.2b) suggesting that EGFR<sub>VIII</sub> vesicles can be isolated from cell-free reactions and that there is no significant

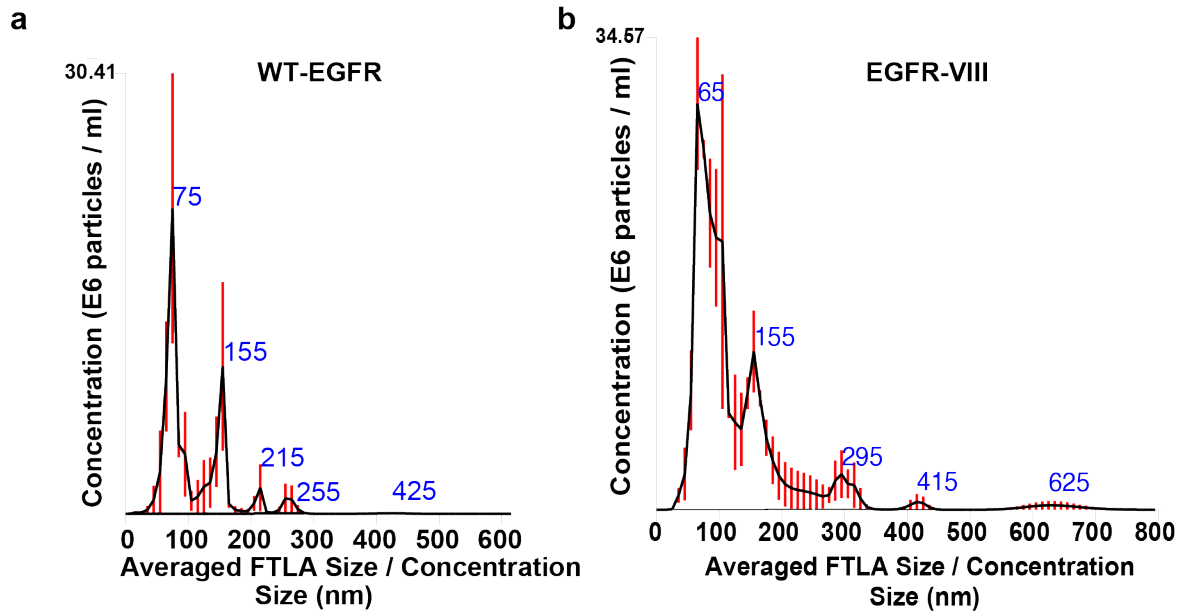
difference between the diameter of WT-EGFR and EGFR<sub>VIII</sub> outwardly budded vesicles.

### *7.3. MVB Budding of EGFR and EGFR<sub>VIII</sub> is dynamin-dependent.*

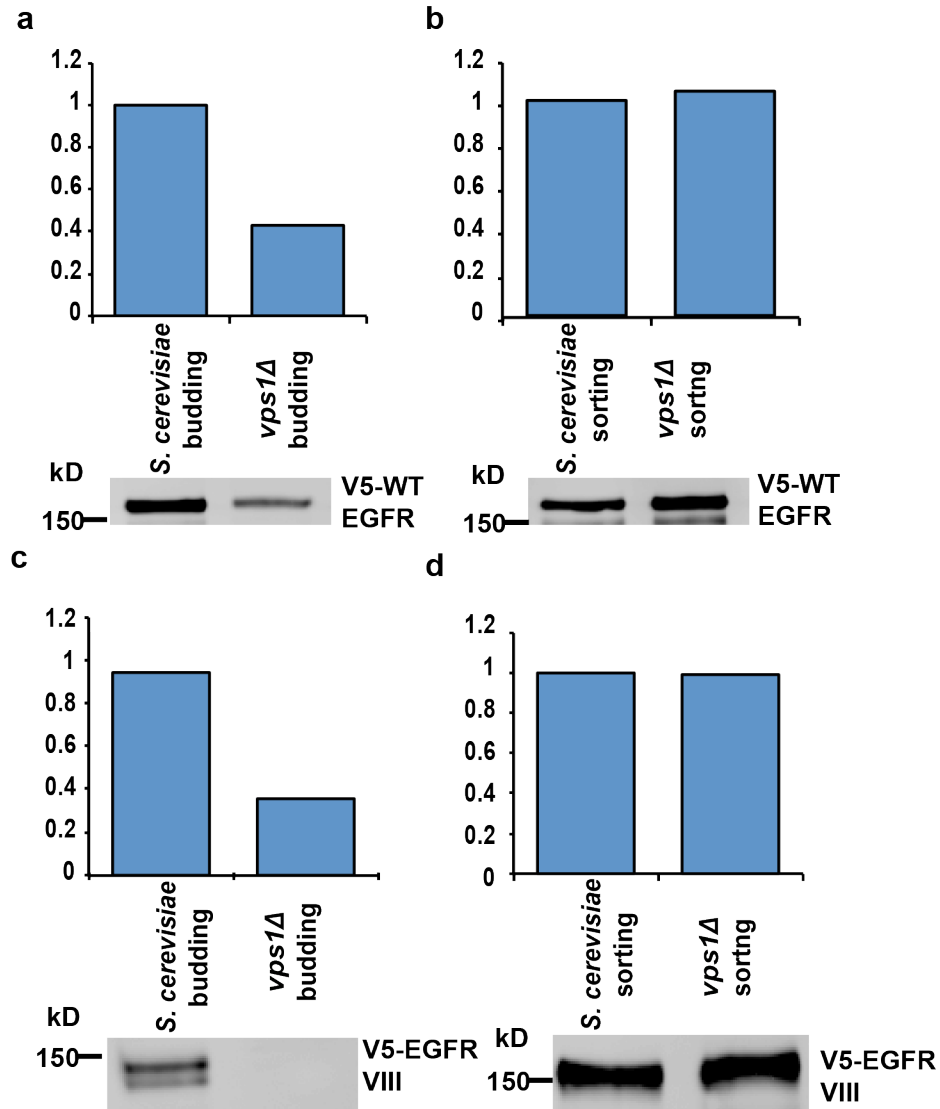
My previous results suggest that dynamin is required for outward vesicle budding of EGFR from late endosomes (Fig. 6.2 and Fig. 6.4). To determine whether dynamin is required for endosomal budding of EGFR<sub>VIII</sub>, partially purified endosomal membranes isolated from U87 cells expressing either WT-EGFR or EGFR<sub>VIII</sub> were incubated with ATP and *Saccharomyces cerevisiae* (70 µg) cytosol for 3 hours at 37°C. Following incubation, reactions were centrifuged and supernatant was collected for additional centrifugation to obtain outwardly budded vesicles. The budding of WT-EGFR and EGFR<sub>VIII</sub> was inhibited in vesicles isolated from reaction supernatant in reactions lacking dynamin (*vps1Δ*) compared to parental strains (Fig. 7.3 a and c, lane 2 compared to lane 1). Protease protection of WT-EGFR or EGFR<sub>VIII</sub>, a measure of inward budding, was not affected by the lack of VPS1 (Fig. 7.3 b and d, lane 2 compared to lane 1). Thus, both WT-EGFR and EGFR<sub>VIII</sub> are internalized into endosomes and can bud from endosomal membranes in a dynamin dependent manner, suggesting that EGFR<sub>VII</sub> can follow a similar endocytic trafficking pathway to the EGFR.



**Figure 7.1. EGFR<sub>vIII</sub> can bud inwardly into internal vesicles of the MVB.** a) HeLa cells were transfected with WT-EGFR or EGFR<sub>vIII</sub> constructs. b) Endosomal membranes (5  $\mu$ L, lanes 1, 4, and 7) and endosomal membranes (5  $\mu$ L) digested with trypsin to remove the C-terminal epitope of the receptor (lanes 2, 5, and 8) as well as partially purified endosomal membranes (15  $\mu$ L) incubated in reactions containing ATP and mammalian cytosol (25  $\mu$ g) (lanes 3, 6, and 9) were treated in the absence of EGF stimulation (lanes 1-6) or presence of EGF stimulation (lanes 7-9). In absence of EGF stimulation, WT-EGFR is not present on endosomal membranes and is not sorted into internal vesicles (lanes 1-3). In contrast, EGFR<sub>vIII</sub> is sorted into internal vesicles in the absence of EGF stimulation (lanes 4-6). WT-EGFR is sorted into internal vesicles when stimulated with EGF (lanes 7-9). n=2



**Figure 7.2. WT-EGFR and EGFR<sub>VIII</sub> outwardly budded vesicles are similar in size.** Partially purified endosomal membranes isolated from U87 cells were isolated as described in Fig. 1.6. Following incubation, reactions were centrifuged and supernatant was collected for additional centrifugation to obtain outwardly budded vesicles. Nanosight tracking analysis was performed on isolated vesicles to measure mean vesicle size. a) WT-EGFR isolated vesicles are 108 $\pm$ 12.4% nm in size. b) EGFR<sub>VIII</sub> isolated vesicles are 124 $\pm$ 11.1 nm in size. There was no significant difference in vesicles size and distribution between WT-EGFR and EGFR<sub>VIII</sub>. N=3 with representative analysis shown for both.



**Figure 7.3. EGFR and EGFR<sub>VIII</sub> bud outwardly in a dynamin-dependent manner.**

Partially purified endosomal membranes isolated from U87 cells were isolated as described in Fig. 1.6. Following incubation, reactions were centrifuged and supernatant was collected for additional centrifugation to obtain outwardly budded vesicles. a) Vesicles isolated from *vps1Δ* reaction supernatant of WT EGFR endosomes, the amount of EGFR on outwardly budded vesicles was decreased

compared to a parental strain (lane 2 compared to lane 1). b) WT EGFR reactions incubated with cytosol isolated from *vps1Δ* strains did not alter the protease protection of EGFR compared to a parental strain (lane 2 compared to lane 1). c) Vesicles isolated from *vps1Δ* reaction supernatant of EGFR<sub>VIII</sub> endosomes, the amount of EGFR<sub>VIII</sub> on outwardly budded vesicles was decreased compared to a parental strain (lane 2 compared to lane 1). EGFR<sub>VIII</sub> reactions incubated with cytosol isolated from *vps1Δ* strains did not alter the protease protection of EGFR<sub>VIII</sub> compared to a parental strain (lane 2 compared to lane 1). Data represents the mean (n=2) normalized to the control. These experiments were performed in part by Natalie Sirisaengtaksin and used with permission.

## Chapter 8. Discussion and Future Directions

The number of signaling membrane proteins on the cell surface, and the time they spend in an activated state, are critical determinants for cellular responses to extracellular cues that can regulate homeostasis, plasticity, growth, and differentiation (Maxfield and McGraw, 2004; Gruenberg and Stenmark, 2004; Katzmann, 2002; Tanowitz and von Zastrow, 2003; Donaldson and Dutta, 2016; Grant and Donaldson, 2009). Internalization and movement through the endocytic pathway is required to tune the signaling responses of various membrane proteins. After movement through multiple, morphologically defined, compartments of the endocytic pathway, membrane protein signaling is attenuated upon their lysosomal degradation (Gruenberg, 2001; Sorkin and von Zastrow, 2009; Sun et al., 2010; Yang et al., 2005).

A membrane protein that transits the endocytic pathway and arrives at the limiting membrane of the MVB either buds inwardly into the internal vesicles for eventual degradation in the lysosome, remains on the limiting membrane for incorporation into lysosomal membranes upon MVB-lysosome fusion, or is transported into vesicles that bud outwardly from the MVB for movement to various cellular compartments (Grant and Donaldson, 2009; Gruenberg, 2001; Maxfield and McGraw, 2004; Tanowitz and von Zastrow; Thompson et al., 2007; Weigert et al., 2004). While outward budding from the late endosome has been suggested (Felder et al., 1990), my data provides the first reported isolation of vesicles that bud outwardly from MVBs. These outwardly budding vesicles could provide a mechanism by which membrane proteins are transported out of the late endocytic

pathway to other cellular compartments (Felder et al., 1990). The ability to isolate outwardly budding vesicles allowed an examination of the molecular machinery regulating the budding event and to characterize these vesicles. These studies also allowed comparison of the mechanisms underlying inward and outward budding from the same endosomal membrane and resulted in an appreciation of the differences in molecular machinery required for these unique budding events. Interestingly, while differences in the mechanisms of the budding events were evident, I also observed that the budding processes are linked such that inhibition of inward budding enhances outward budding.

### *8.1. Mechanisms of inward budding at the MVB*

Since both budding events I measured are dependent on cytosolic components, and cytosol isolated from yeast and fly are able to support these budding events, I used genetic approaches to interrogate the cytosol for molecules involved in the regulation of MVB budding. I found that ESCRT complex components are required for the protease protection of a cargo protein (EGFR), confirming that these molecules are involved in inward MVB budding in our reconstituted system. In this regard, cytosol isolated from yeast strains deleted of either of the ESCRT-0 proteins impaired membrane protein budding into internal vesicles, an effect that was rescued by the addition of the soluble mammalian orthologous ESCRT proteins. While EGFR was used as a proxy for membrane protein cargo, I found that multiple types of membrane proteins follow the same internal budding pathway [e.g. tyrosine kinase receptors (Fig. 4.1), GPCRs (Fig. 4.2), ion channels (Fig. 4.3) in an ESCRT-0-dependent manner. Thus, reconstitution of this budding event allows examination



of a variety of cargo proteins and the underlying mechanisms by which this budding event occurs.

Similar to ESCRT-0 components, deletion of ESCRT-I impaired EGFR budding into internal MVB vesicles. These data are consistent with previous studies reporting that ESCRT-I is required for membrane protein movement into internal vesicles (Bache et al., 2004; Doyotte et al., 2005). However, the role of ESCRT-II in membrane protein movement into internal vesicles is controversial. Previous studies suggest that ESCRT-II is dispensable for inward budding of cargo including the EGFR and major histocompatibility complex class I (MHC-I) (Bowers et al., 2006; Malerod et al., 2007). However, others have found that ESCRT-II is required for EGFR and ferroportin degradation, and not required for MHC-I degradation (Langelier et al., 2006; Williams and Urbe, 2007). To address the controversy, I measured inward budding of the EGFR and used cytosol lacking ESCRT-II proteins, allowing a direct and unambiguous examination of the role of these molecules. Our results suggest that all components of that ESCRT-I and ESCRT-II are required for inward budding of the EGFR into internal MVB vesicles.

Interestingly, deletion of only two of the four ESCRT-III genes, *SNF7* and *VPS24* inhibit the movement of EGFR from the endosomal membrane into internal vesicles. These data are consistent with previous studies reporting that deletion of *SNF7* or *VPS24* inhibit degradation of EGFR (Bache et al., 2006; Shim et al., 2006). Somewhat surprisingly, deletion of the ESCRT-III genes *VPS20* and *DID4* failed to inhibit protease protection of the EGFR. To determine whether components of the two major ESCRT-III complexes (Vps20/Snf7 and Vps24/Did4) may have redundant

or overlapping roles (Babst et al., 2002a) I examined EGFR protease protection in reactions using cytosol from yeast strains in which double deletions were made in ESCRT III genes. Double deletions (Vps20/Snf7 or Did4/Vps24) did not inhibit the protease protection of EGFR significantly more than cytosol from single deletion (*snf7Δ* or *vps24Δ*) strains, suggesting that the ESCRT-III components Vps20 and Did4 are not required for the inward budding of EGFR-containing vesicles. The ESCRT-III complex has been suggested to drive membrane fission events that allow internal vesicle formation within MVBs, as well as viral budding events, and cytokinesis (Adell and Teis, 2011; Hurley and Hanson, 2010; McDonald and Martin-Serrano, 2009). It has been hypothesized that Vps20 binds to ESCRT-II and recruits Snf7, which in turn recruits the remaining ESCRT-III components for MVB budding (Adell and Teis, 2011; Hurley and Hanson, 2010). In cytokinesis and viral budding events Vps20 is dispensable and Snf7 binds to an ESCRT-associated protein, Bro1, which directly activates Snf7 and the remaining ESCRT-III components (Carlton and Martin-Serrano, 2007; Martin-Serrano et al., 2003; Wemmer et al., 2011). In a manner similar to cytokinesis and viral budding, my results show that Vps20 is not required for membrane protein budding into internal vesicles of MVBs, suggesting that Snf7 may be directly activated through binding of other proteins (e.g. Bro1). After formation of the Vps20/Snf7 complex, the Vps24/Did4 complex is thought to act as a cap for Snf7 (Adell and Teis, 2011; Hurley and Hanson, 2010). Previous studies suggested that Did4 is not required for membrane scission, but instead is required for disassembly of ESCRT complexes, by initiating the recruitment of Vps4 (Williams and Urbe, 2007; Wollert et al., 2009).

My results are consistent with the hypothesis that Did4 is not required for membrane protein sorting at the MVB.

The ESCRT machinery plays a role in cargo sorting and MVB vesicle formation however increasing evidence suggests that lipids may play a key role in regulating internal vesicle formation (Babst et al, 2010). ESCRT-0 localizes to lipid rafts through binding of Hrs to phosphatidylinositol 3-phosphate (PI3P) (Gaullier et al., 1998; Petiot et al., 2003; Schmidt and Teis, 2012; Stenmark et al., 2002) and may therefore help to sort cargo into these lipid domains. Interestingly, ESCRT deletion inhibits cargo sorting but not internal vesicle formation (Babst; Sun et al., 2010), suggesting that ESCRTs regulate cargo formation but lipids may play a larger role in regulating internal vesicle formation. Lipid clustering causes formation of lipid rafts that may help membrane deformation, ultimately leading to vesicle formation (Babst, 2011; Bissig and Gruenberg, 2013). Cholesterol is enriched in lipid rafts and is concentrated on internal MVB vesicles (Mobius et al., 2003), suggesting that cholesterol may be required for internal vesicle formation. We observed that cholesterol depletion inhibits protease protection of the EGFR implying that cholesterol is required for inward vesicle budding. Cholesterol is required for formation of highly curved structures such as synaptic vesicles (Mobius et al., 2003; Wang et al., 2007), suggesting that a similar cholesterol-dependent mechanism might underlie inward budding of similar sized endosomal vesicles.

## *8.2. Characterization of outwardly budded vesicles from MVBs*

Transport from MVBs to other cellular compartments, including the Golgi and plasma membrane, likely require an outward vesicle budding event to cluster cargo

and allow for targeted movement (Felder et al, 1990; Nicoziani and van Deurs, 2000). I have isolated and characterized vesicles that bud outwardly from MVBs during our cell-free reactions. The vesicles are approximately 100nm in size and contain EGFR. To isolate these outwardly budding vesicles I used yeast cytosol (that does not contain detectable EGFR) in place of mammalian cytosol, to enable definitive measurement of the amount of EGFR that is transported from the endosome. Analysis of these vesicles by mass spectrometry identified many proteins known to be involved in protein trafficking steps (Table 2). Clarifying the role these proteins play in the outward budding step from MVBs may provide insight into the trafficking of these vesicles. For example, the SNARE proteins mediate vesicle fusion events (Sudhof and Rothman, 2009), and identification of 4 SNARE proteins (e.g. Vamp2, Vamp3, Sec22B, SNAP91) in our analysis provides clues to potential fusion mechanisms and destinations for these vesicles. Vamp2 regulates the fusion of intracellular vesicles with the plasma membrane through binding to the plasma membrane protein syntaxin1 (Salaun et al., 2004). Vamp3 mediates fusion of the Rab11 recycling compartment with the plasma membrane by binding to Syntaxin4 and SNAP23 (Veale et al., 2010). SEC22B binds to syntaxin18, a resident SNARE on the Endoplasmic Reticulum (ER) (Hatsuzawa et al., 2009) and is required for Golgi-ER and ER-Golgi Transport (Chatre et al., 2005). SNAP91 binds to the clathrin coat protein and regulates clathrin-mediated endocytosis (McMahon and Boucrot, 2011). Therefore, vesicles may be transported to the plasma membrane, ER or the Golgi. Similarly, the mass spectrometry results identified three retromer protein components (e.g. SNX2, Vps29, Vps35). The retromer complex regulates recycling

of receptors to the trans-Golgi and the plasma membrane (Arighi et al., 2004; Seaman, 2004; Steinberg et al., 2013) and consists of 5 components (e.g. VPS35, VPS29, VPS26, SNX1, and SNX2) (Carlton et al., 2004; Haft et al., 2000; Steinberg et al., 2013). Identification of retromer proteins suggests that a subset of vesicles may be transported to the plasma membrane or Golgi in a retromer-dependent manner. Additional proteins identified in our analysis include coat proteins (e.g. Clathrin, COPs), adaptor proteins (e.g. AP1, AP2) and Dynamin2. Thus, the vesicle components identified by Mass spectrometry provide guidance for understanding the mechanisms and destination of the outwardly budded vesicles.

My studies isolated and identified the cargo constituents of outward budding vesicles, however the destination of these vesicles was still unclear. Therefore, I took advantage of the known itinerary of an EGFR mutant lacking kinase activity, EGFR<sub>K721A</sub>, that traffics to the limiting membranes of MVBs prior to budding onto vesicles that ultimately fuse with the plasma membrane (Felder et al., 1990). I found EGFR<sub>K721A</sub> on the outwardly budding vesicles, suggesting that these vesicles were budding from the MVB and therefore, that at least some proportion of the outwardly budding vesicles I isolate are targeted to the plasma membrane. Interestingly, Felder et al, 1990 found 25% of the EGFR<sub>K721A</sub> on internal endosomal vesicles, 42% on recycling vesicles, and 33% on the limiting endosomal membrane (Felder et al., 1990). By comparison, I found a similar percentage of EGFR<sub>K721A</sub> present on internal vesicles, however I observed a lower percentage of EGFR<sub>K721A</sub> on outwardly budded vesicles perhaps due in part to a low recovery of outwardly budded vesicles in our biochemical assay.

### *8.3. Endosomal trafficking of EGFR in response to varying ligand concentration*

Challenging tumor cells with high concentrations of EGF results in internalization of the ligand-receptor complex by both clathrin-mediated endocytosis and clathrin-independent pathways (Sigismund et al., 2008). In contrast, stimulation of the same cells with low EGF concentrations results in internalization only via clathrin-mediated endocytosis (Sigismund et al., 2008). Thus, engagement of different internalization pathways has been hypothesized to be necessary to tune cellular responses of enhanced levels of extracellular EGFR (Chandra et al., 2013; Sigismund et al., 2008). High extracellular EGFR concentrations increase cellular proliferation and signaling (Chandra et al., 2013; Sorkin, 2001). I observed that stimulation of tumor cells with either low or high EGF concentrations results in both inward and outward endosomal vesicle budding. However, the ratio of inward to outward budding from the MVB membrane did not differ based on ligand concentration used for EGFR stimulation. My results are in agreement with a report suggesting that 25-30% of EGFR recycles and 40-50% of EGFR degrades when cells are treated with a range of EGF (5-200ng) (Sorkin et al., 1991) suggesting that any differences in internalization of EGFR may play a role in EGFR trafficking during early parts of the endocytic pathway (Fig. 1.1 and Fig. 1.4) but do not result in differences at the MVB membrane.

### *8.4. Mechanisms of outward budding from MVBs*

By reconstituting both inward and outward budding from the same endosomal membranes I was able to compare these budding events. As I observed for inward budding, I found that outward vesicle budding from MVBs was also dependent on

cytosolic components. To identify the specific cytosolic components that are required for the outward budding event I have taken multiple approaches. Initially, I took a candidate approach and chose to examine selected GTPases as it has been appreciated for many years that these molecules are required for various vesicle budding steps (Baker et al., 1990; Beckers and Balch, 1989; Melancon et al., 1987; Ruohola et al., 1988; Tooze et al., 1990). In this regard, outward budding from early endosomes is dependent on the GTPases Rab4, Rab5, and Rab11 (Kobayashi and Fukuda, 2013; van Dam and Stoorvogel, 2002). Rab11 regulates the slow recycling pathway (Grant and Donaldson, 2009; Ren et al., 1998; Ullrich et al., 1996), and thus is more indicative of a mechanism that may regulate late endosomal outward budding. However, my results suggest that outward budding from the MVB is Rab11 independent. This suggests a different molecular mechanism regulates MVB budding. I examined whether the ESCRTs were involved in outward budding in addition to an unbiased approach to examine proteins that may regulate outward budding from the MVB.

A relationship between inward MVB budding and recycling was suggested by Babst et al, (Babst et al., 2000) who found that deletion of ESCRT proteins resulted in an increase in EGFR recycling. In these experiments, EGFR was measured at the plasma membrane although the compartment from which it was recycled (e.g. early or late endosomes) was unresolved (Babst et al., 2000). In the reconstituted system, I found that deletion of an ESCRT-0 component increased outward vesicle budding from the limiting membrane of the MVB, but not from early endosomes. My result is consistent with Babst et al (Babst et al., 2000) but identifies the MVB as an

endosomal compartment from which EGFR may originate for plasma membrane recycling.

The increased outward vesicle budding that occurs when inward budding is inhibited provides insight into the role cargo proteins may play in vesicle formation and the linkage between cargo proteins and the ESCRT machinery. One hypothesis (elevator hypothesis) (Robinson, 2004; Santini and Keen, 1996; White et al., 2006) suggests that vesicle formation/budding is dependent on the presence of membrane protein cargo, while another hypothesis (escalator hypothesis) (Santini and Keen, 1996) posits that vesicles form/bud regardless of whether cargo is present (Robinson, 2004; Santini and Keen, 1996). Our data suggest that cargo plays a role in vesicle formation, because inhibition of inward budding enhances outward budding. If vesicle budding/formation occurs constitutively, one might expect that outward budding would not be affected by inhibition of inward budding events.

Lastly, I took an unbiased approach to screen for genes that are required for outward budding. I observed that cytosol isolated from a dynamin-deficient yeast strain (*vps1Δ*) inhibited outward budding in our cell-free assay, an effect that could be partially rescued by mammalian dynamin 1. The partial rescue may be a result of using the mammalian protein to rescue the yeast deletion or that other dynamin isoforms are required to fully rescue the inhibition of outward budding. In this regard, dynamin 1 and 2 have been speculated to have overlapping functions in endocytosis (Ferguson et al., 2009; Liu et al., 2008) and we identified dynamin 2 as a constituent of outwardly budded vesicles (Table 2). Dynamin has been localized on late endosomal membranes and regulates the late endosome-Golgi recycling of the



mannose-6-phosphate receptor (Nicoziani et al., 2000). Therefore, a subset of our isolated vesicles may be transported to the trans-Golgi network or dynamin may regulate outward vesicle budding from late endosomes to the plasma membrane.

#### *8.5. ESCRT assembly and cargo sorting may provide a clue to the link between inward and outward budding from MVBs*

My data also makes a contribution to understanding how ESCRT complexes enable cargo sorting at the endosomal membrane. The conveyor belt model (Hurley and Emr, 2006) suggests that cargo molecules are handed off sequentially from one ESCRT complex to the next in a linear fashion. The concentric ring model (Nickerson et al., 2007) suggests that multiple cargoes are clustered beneath an ESCRT supercomplex. Our data are in favor of the concentric ring model. If membrane cargo is clustered as suggested by the concentric ring model and inward budding is inhibited, the cargo clusters would begin to accumulate on MVB membranes, leading to an increase in endosome size. Therefore, to limit cargo accumulation on the MVB limiting membrane and ensure consistent MVB size, cargo clusters are transported to other cellular compartments, leading to an increase in outward budding from MVBs, consistent with what I have observed. The linkage between inward and outward budding may be due in part, to the localization of cargo clusters and the machinery regulating inward and outward budding on the MVB membrane. The ESCRT-0 component, Hrs, is found clustered in areas that contained EGFR (Sachse et al., 2002; Tsujimoto et al.). Interestingly, coat proteins are known to regulate budding events, and one such coat protein, clathrin, is found in clusters adjacent to the Hrs clusters (Sachse et al., 2002), suggesting that ESCRT

and Clathrin clusters are adjacent but not overlapping on the endosomal membrane in membrane domains that may correspond those undergoing inward and outward budding. It is therefore possible that under conditions that inhibit inward budding, cargo is budded outwardly due to the close spatial arrangement of the machinery that regulates these distinct budding events.

#### *8.6. EGFR<sub>VIII</sub> can be internalized into internal vesicles of the MVB*

Mutations in membrane proteins can result in aberrant trafficking that results in disease. For example, EGFR<sub>VIII</sub> is a pathogenic variant of EGFR found in a high percentage of glioblastoma tumors (Padfield et al., 2015), and results in aggressive disease. Interestingly, increased EGFR<sub>VIII</sub> recycling that results in increased downstream signaling is thought to underlie glioblastoma tumorigenesis (Grandal et al., 2007). My results suggest that at least a portion of EGFR<sub>VIII</sub> can bud inwardly into internal vesicles of MVBs, suggesting that at least a pool of this mutant receptor can be degraded in the lysosome. However, I observed that EGFR<sub>VIII</sub> is also found on outwardly budding endosomal vesicles and that dynamin is required for the outward budding of EGFR<sub>VIII</sub>. Thus, EGFR<sub>VIII</sub> buds inwardly and outwardly from endosomal membranes suggesting that a therapeutic strategy might involve shifting the balance of degraded/recycled EGFR<sub>VIII</sub>. One caveat to this interpretation is that internalization of EGFR<sub>VIII</sub> into internal vesicles of the MVB does not always imply that the receptor will be degraded in the lysosome. MVBs can also fuse with the plasma membrane and release the internal vesicles into the extracellular space as “exosomes” (Corrado et al., 2013; Hurley and Odorizzi, 2012; Kowal et al., 2014). In glioblastoma patients, EGFR<sub>VIII</sub> has been found on exosomes (Skog et al., 2008).

These results suggest that MVBs containing EGFR<sub>vIII</sub> may fuse with the plasma membrane instead of the lysosome.

### *8.7. Future Directions*

#### **1. To further characterize vesicles that bud outwardly from isolated late endosomal membranes.**

Analysis of the outwardly budding vesicles by Mass Spectrometry revealed the identity of proteins known to be involved in protein trafficking steps. Future experiments will take advantage of this proteomic information to try to understand whether there are multiple populations of budding vesicles that may carry distinct cargo or may be targeted to different compartments. For example, the SNARE proteins identified by Mass Spectrometry (e.g. Vamp2, Vamp3, Sec22B, SNAP91) could be used to define potential target membranes and future experiments could try to understand whether outwardly budded MVB vesicles with different SNAREs target different compartments. Moreover, the cargo carried by different populations of vesicles could be identified. The role of vesicle SNAREs in the fusion of recycling vesicles with plasma membrane and/or other cellular compartments could also be addressed.

#### **2. To identify cytosolic components that regulate MVB budding.**

My studies took advantage of a yeast deletion library in a limited screen for genes that are required for outward budding. Future experiments could include a large-scale screen using yeast cytosol that could identify proteins that regulate inward and outward budding events.

### **3. To determine the mechanistic link between inward and outward budding from MVBs.**

Inhibition of inward budding resulting from deletion of the ESCRT machinery increases outward vesicle budding from MVBs, suggesting that the two mechanisms of budding may be linked. It is possible that outward budding is a bulk-flow process that is enhanced when inward budding is inhibited. Ubiquitination may trigger cargo clustering and assembly of ESCRT complexes, as suggested in the concentric ring model. In the absence of ESCRT recruitment, cargo that is ubiquitinated may become deubiquitinated, not able to enter inwardly budding vesicles, and be transported to other cellular compartments. This hypothesis could be examined by determining whether inhibition of inward budding by blocking ubiquitination (or enhancing deubiquitination) results in increased outward budding.

### **4. To determine the trafficking pathway of EGFR<sub>VIII</sub>.**

Our results suggest that a percentage of EGFR<sub>VIII</sub> can bud inwardly into internal vesicles of MVBs. MVBs can fuse with the lysosome to degrade the contents of the internal vesicles, or they can fuse with the plasma membrane and release the internal vesicles as exosomes. The destination of EGFR<sub>VIII</sub> containing MVBs is unknown but the lack of considerable EGFR<sub>VIII</sub> degradation suggests that these MVBs may fuse with the plasma membrane. Comparing the cargo constituents of MVBs containing EGFR<sub>VIII</sub> with MVBs containing wild-type EGFR may allow understanding of differences in cargo and fusion machinery required for MVB-lysosome versus MVB-plasma membrane fusion.

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## **Vita**

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