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The Role of ESCRT-III-like Subunit IST1 in Membrane Trafficking Pathways
by
Amy K. Clippinger

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Amy Clippinger

Washington University in St. Louis

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Dedicated to my husband.
ESCRTs (Endosomal Sorting Complex Required for Transport) are a modular set of proteins with membrane remodeling activities that include the formation and release of intraluminal vesicles (ILVs) to generate multivesicular endosomes. ESCRT-III filaments have an established role in membrane fission for ILV formation and the topologically related processes of viral budding and cytokinesis. Among the 12 ESCRT-III proteins most have established roles in ILV formation, but the cellular roles of IST1 remain elusive. We found that IST1 and another ESCRT-III subunit CHMP1B form filaments that spiral around the outside or cytoplasmic surface of membrane tubules. Consistent with a role in tubule-based sorting processes—and a possible role in fission—CHMP1B and IST1 are present in puncta along endosomal tubules and live-cell imaging showed that depleting either one stabilized elongated Rab5 endosomes. We also identified a novel IST1 interacting protein SNX15, which promotes the recruitment of IST1 to a microdomain distinct from those established for cargo degradation and cargo recycling. Proximity biotinylation studies (BioID2) using IST1 and SNX15 established that both proteins...
associate with a common set of factors involved in endosomal recycling including clathrin and branched actin regulators, while IST1 has additional unique partners that bind uniquely via ESCRT-III and MIT domain interactions. Using live-cell microscopy we found that SNX15 and CHMP1B alternately control IST1 distribution and its relationship to clathrin and branched actin—as well as how IST1 affects the distribution of transferrin-positive recycling tubules within the cell. Using the transferrin receptor (TfnR) as an endosomal cargo, we established that IST1 regulates sorting and trafficking from the early/sorting endosome to the tubular endocytic recycling compartment (ERC) present in the perinuclear region. Loss of IST1 impaired the sorting of TfnR by this pathway and led to its accumulation in abnormal peripheral endosomes that were positive for the endosomal clathrin adaptor AP-1. These results demonstrate that IST1 is a multifunctional adaptor involved in clathrin-based recycling on the endosome either by interacting with clathrin-binding SNX15 or endo-tubule membrane constricting CHMP1B filaments—and possibly functions with other actin-based sorting mechanisms on the endosome.
Chapter 1: Introduction

1.1 Overview of the ESCRTs

1.1.1 ESCRTs: the machinery required for sorting into ILVs

The ESCRT (Endosomal Sorting Complex Required for Transport) machinery was first identified for its role in intraluminal vesicle (ILV) formation in yeast (1). The ESCRT machinery is modular with distinct complexes implicated in recruiting ubiquitinated cargo (ESCRT-0 – ESCRT-II) and membrane deformation and scission (ESCRT-III and VPS4, sometimes referred to as ESCRT-IV). ESCRT-III proteins, in particular, are known to be cytosolic monomers that transiently form polymeric structures on membranes, and studies of both yeast and mammalian ESCRT-III polymers have elucidated how this ESCRT complex deforms membranes and is disassembled (returned to monomeric state) by the AAA ATPase Vps4 (VPS4A and VPS4B in mammalian cells) (2, 3). There are 8 ESCRT-III proteins in yeast (12 in mammalian cells), of which 4 (Vps20, Vps32/Snf7, Vps2, and Vps24) were first identified as “class E” vacuolar protein sorting (Vps) mutants necessary for ILV biogenesis in yeast (4, 5). Unlike the so called “core” ESCRT-III subunits, the remaining 4 ESCRT-III subunits, Vps46/Did2, Vps60, Ist1, and Chm7 appear to be not essential for ILV formation (6, 7). For example, Vps46/Did2 (also known as CHMP1A and CHMP1B in mammalian cells) was identified in a screen along with Vps32/Snf7, Vps24, Vps2 (all “core” subunits) and was found to be structurally related to ESCRT-III. However loss of Vps46/Did2 does not greatly impair multivesicular body (MVB) trafficking and does not prevent intraluminal vesicle (ILV) formation (5, 6, 8). Ist1 (IST1 or CHMP8 in mammalian cells) was not identified in any screen with other ESCRT-III proteins, and instead was discovered as a positive modulator of MVB sorting that forms a complex with
Vps46/Did2 and regulates Vps4 (9, 10). Crystal structures of the N-terminal domain of IST1 revealed structural homology with ESCRT-III proteins (11, 12). However, loss of Ist1 also does not impair MVB trafficking or ILV biogenesis (6, 10, 12, 13). The apparent decrease or lack of the ILV biogenesis defects associated with deleting these “non-core” ESCRT-IIIIs has led to the interpretation that Ist1 and Vps46/Did2 have regulatory roles in ESCRT machinery function.

In recent years, known cellular functions of the ESCRT machinery have expanded to include virus budding, nuclear envelope closure and cytokinetic abscission, which are all types of membrane transformations that are topologically similar to ILV formation. Since the modular ESCRT machinery is capable of forming a diverse collection of heteropolymeric ESCRT-III filaments, it is not difficult to imagine that this list of functions will only grow.

1.2 Endosomal sorting through the decades

1.2.1 The ESCRTs were originally implicated in both degradation and retrograde trafficking of cargo

ESCRT-III proteins were initially identified as vps mutants that secrete the vacuolar/lysosomal soluble hydrolase carboxypeptidase Y (CPY), and are therefore involved in the lysosomal biosynthetic pathway (these included the “core” ESCRT-III proteins: Vps2 (CHMP2), Vps20 (CHMP6), Vps24 (CHMP3), Vps32 (CHMP4) (14). (Note hereafter that mammalian homologs will be listed in parentheses, or separated by “/” if there is evidence of conserved function.)

Identified vps mutants that secrete CPY were initially sorted into classes based on their vacuole morphology. ESCRT-III vps mutants were initially found to have normal vacuole morphology (Class A mutants) in comparison with other vps mutants that had fragmented vacuoles (Class B mutants) (14). The Class B mutants include Vps17 (SNX5/SNX6 and SNX32), Vps5 (SNX1/SNX2), and Vps1 (Dynamin) which have been shown to associate with membrane
tubules in the topology opposite that of “canonical” ESCRT machinery (14). This classification system was later revisited and expanded, and the “core ESCRT-III” mutants were found to have a novel prevacuolar-like organelle (Class E compartment) distinct from the vacuole in which vacuolar proteins accumulated (15). Thus the “core” ESCRT-III subunits as well as other components of the ESCRT machinery were placed into Class E (Note: “Class E” is another term to describe ESCRT machinery components in the literature).

However, the assignment of ESCRTs to Class E remained incomplete. For example, Vps46/Did2 (CHMP1B) was retained in Class A since at the time of its classification structural electron microscopy (EM) analysis was not used to inspect the morphology of the class E compartment and thus the fragmented vacuole was not included as criteria for classification. In addition to the class E compartment lacking ILVs, it consists of flattened and stacked endosomal cisternae. The aberrant morphology of this endosomal structure seems highly related, but also separable from ILV formation, as ultra-structural analysis has revealed that both Vps1 (Dynamin) and Vps46/Did2 (CHMP1B) have “Class E-like” compartments (6, 16) (Odorizzi, personal communications).

In addition to Vps46/Did2, Class A also contains components of the retromer cargo-selection complex (CSC) Vps29 (VPS29) and Vps35 (VPS35), which interacts with SNX-BAR proteins Vps17 (SNX5/SNX6 and SNX32) and Vps5 (SNX1/SNX2). The SNX-BAR proteins themselves—Vps17 (SNX5/SNX6 and SNX32) and Vps5 (SNX1/SNX2)—were retained in class B, but Vps1 (Dynamin) was placed into class F—along with the third component of retromer CSC Vps26 (VPS26)—as they displayed a mild phenotype characterized by a less
fragmented vacuole. Curiously, while reports in yeast indicate that the trimeric Vps29, Vps35, Vps26 and the heterodimerized SNX-BARs (Vps17 and Vps5) are an obligate pentameric complex, the fact that mutants of different subunits have different phenotypes may suggest otherwise (17, 18). In support of this, the mammalian retromer CSC (VPS35, VPS29, VPS26) co-purifies with SNX3 but not the two different sets of heterodimerized SNX-BARs, (SNX1 with either SNX5 or SNX6 and SNX2 with either SNX5 or SNX6). Thus, the retromer appears to be comprised of several modular components, the CSC and a membrane binding module that can be comprised of several different combinations of SNXs. Regardless, in current literature the term retromer in yeast refers to the entire pentameric complex, while it refers to only the trimeric CSC complex in mammalian cells (19)

Additional characterizations of how vps mutants affect the trafficking of Vps10 (M6PR)—and therefore lead to CPY secretion—have implicated ESCRT in retrograde trafficking from the endosomal system to the Golgi. The original “retromer” complex was identified in yeast as the complex directly responsible for the retrieval of Vps10 from the endosome (18). However, similar to Vps1 (Dynamin) and Vps26 (VPS26) mutants, class E mutants exhibited increased degradation of Vps10 (20). Further, the overexpression of Vps10 rescued the vps phenotype (CPY secretion) in class E mutants, indicating that Vps10 was not being returned to the Golgi (21). The authors concluded (without knowing about involvement of ESCRT machinery in ILV biogenesis) that these class E proteins control traffic from the endosome to the vacuole, as well as retrograde traffic from the endosomal pre-vacuolar compartment to the Golgi. While the former assertion has been verified, the latter has largely been ignored. Thus it is clear that
ESCRTs are involved in recycling and retrograde trafficking—whether direct or indirect—however, the mechanism remains unknown.

1.2.2 The retromer associated sorting nexins were originally implicated in the degradative trafficking of cargo
The original sorting nexin, SNX1, was identified in mammalian cells as a yeast two-hybrid partner of the epidermal growth factor receptor EGFR. This study mistakenly concluded that SNX1 enhances the degradative sorting of EGFR, because they observed that overexpression of SNX1 leads to a decrease in cell surface EGFR levels and an increase in constitutive and ligand-induced degradation of EGFR (22–25). Furthermore, parallel characterization of the mammalian SNXs had also found that SNXs are important regulators of receptor degradation. It is important to note, that up until this point all studies of SNX1 were based on overexpression paradigms. By contrast, subsequent knockdown studies revealed that depleting SNX1 and SNX2 had no effect on EGFR trafficking (25, 26), but did affect the trafficking of the mammalian homolog of Vps10, M6PR (26, 27). This supports a role for both yeast and mammalian homologs of SNX1/2 in retrograde trafficking rather than degradation and thus results of earlier overexpression studies were dismissed as artificial (28). However, while depletion of SNX1, SNX2 has no effect on EGFR levels, depletion of SNX5 resulted in decreased degradation of EGFR and SNX6 was found to interact with GIT1 to promote the degradation of EGFR (29–31). This indicates that the SNX-BAR retromer is modular, and depending on the associated SNX-BARs can lead to either recycling or degradative trafficking of EGFR.

1.2.2 Retromer and the sorting nexins promote tubule-based endosomal sorting
In mammalian cells, several sorting nexins have been identified to interact and mediate cargo sorting together with retromer. Retromer along with its associated sorting nexins, can recognize
cargo proteins, but requires interactions with the sorting nexins or Ypt7/Rab7 for membrane recruitment (18, 32–37). Several of these sorting nexins, like Vps5 and Vps17, contain Bin/Amphiphysin/Rvs (BAR) domains that are capable of sensing and stabilizing membrane curvature (38), and suggest that the retromer is also sensitive to the topology of the endosomal membrane. Despite this only Vps5, and its mammalian homologs of SNX1/SNX2 have been found to tubulate liposomes, while Vps17 and its mammalian homologs of SNX5, SNX6, and SNX32 do not (38). In mammalian cells, it is not clear if SNX1/2 and SNX5/6 form a complex with the retromer, since the knockout of either SNX1/2 or SNX5/6, but not VPS35 or VPS29, leads to the accumulation of M6PR in endosomes and inhibits its localization to the trans Golgi network (TGN) (39). This is surprising, since VPS35 and SNX1 do overlap on endosomal subdomains (39).

SNX3 does not have a BAR domain, but is known to form a complex with the retromer CSC. In yeast, Snx3 mediates the endosome-to-Golgi transport of the iron permease complex, Fet3p-Ftr1p, by direct interaction of Snx3 with Ftr1p, and is dependent on all of the components of the pentameric SNX-BAR retromer (40). In mammalian cells, SNX3 and the CSC interact with the transferrin receptor (TfnR) (41). SNX3 therefore represents another retromer-associated SNX, but it does not inherently associate with curved membranes. We will refer to SNX1/2, SNX5/6, and SNX3 as retromer-associated sorting nexins that mediate endosome-to-PM recycling or endosome-to-Golgi retrograde trafficking.

1.2.3 Mechanisms of Tubular based sorting
Tubule-based sorting involves several steps that include (1) membrane curvature induction/stabilization, (2) cargo recognition, (3) scission of the tubule from the parent endosome and (4) molecular motor recruitment for transport. SNX5/SNX6 have been demonstrated to interact with dynein, which likely explains the retrograde transport of cargo along microtubules (MTs) from endosomes to the perinuclear destination of Golgi and/or recycling endosomes (42–45). As for the recruitment of scission machinery, Mvp1 (SNX8 in mammalian cells) has been shown to interact with the Dynamin-like GTPase implicated in fission, Vps1 (46)—and depletion of either Mvp1 (SNX8) or Vps1 (Dynamin) prevents efficient cargo transport in yeast. It is not clear if dynamin or a dynamin-like protein is involved in tubule fission in mammalian cells.

While decades of work have elucidated how dynamin mediates fission, it is clear it does so in cooperation with other binding partners—but coordination among these machineries is less understood (47). The best studied example of membrane fission occurs during clathrin-mediated endocytosis (CME), where the neck of clathrin-coated vesicles emerging from the luminal side of the plasma membrane undergoes fission to generate an early endosome. As of date, most of the protein components involved in CME have been identified and their functions within CME have been mapped with exquisite temporal and spatial precision (48). Amongst all these proteins, dynamin, BAR domain proteins, epsin, and actin, have been shown in cells and in vitro to have unique effects on membranes that when combined lead to membrane fission. Both dynamin and BAR domain proteins have the ability to assemble into oligomeric scaffolds on membrane tubules, but only dynamin has the ability to constrict the membrane in a nucleotide-dependent way. In the case of dynamin it has been demonstrated that fission occurs at the edge of the oligomerized protein (49). This highlights the need for both constriction of the membrane, but
also the need for sufficient disordered of the lipids outside of the stabilized and constricted membranes in within the dynamin neck.

BAR domain containing proteins contain a crescent-shaped fold that uniquely mediates both dimerization (and possibly oligomerization) and membrane association. The inherent curvature of the BAR domain allows BAR-domain containing proteins to stabilize and induced both positive and negative membrane curvature, depending on whether the membrane binding interface is on the convex or concave side of the fold. In addition to binding to membranes via there positively-charged surfaces, a specific subset of BAR-domains (N-BAR domains) also have an N-terminal amphipathic helix that inserts into the membrane, which importantly includes the SNX-BARs(50). BAR domain proteins can also assemble in large helical arrays on membranes that not only stabilize and/or stimulate membrane tubulation(51), but can also facilitate friction-driven or steric pressure-driven fission(52, 53). In addition, many of these BAR domain containing proteins contain one or more SH3 domains that can bind directly to the proline-rich-regions (PRD) that are frequently found in actin cytoskeleton and membrane associated proteins (54, 55), such as dynamin(47). Thus, BAR domain proteins can simultaneously coordinate both the actin assembly machinery with the membrane scission machinery.

Epsins and the ARF GTPases are also known to promote fission (56, 57). These proteins have an N-terminal amphipathic helices that shallowly insert into the membrane and are known to initiate membrane curvature that is required for membrane fission as well as participate directly in membrane fission (56–58). Epsins, unlike dynamin and BAR domain containing proteins, do not
form oligomeric arrays on membranes, but can still deform liposomes into tubules with narrow diameters of 20 nm (58). In a cellular context, epsins are recruited to clathrin-coated structures through a direct interaction with clathrin, Eps15 and AP-2 (59–61), and have been shown to localize throughout the entire clathrin-coated structure (62). The early recruitment of epsins to sites of CME, allows for a rapid induction of membrane curvature, which in itself facilitates the recruitment of BAR domain proteins and dynamin. Interestingly, this Epsin-induced membrane curvature can also drive membrane fission in vitro, even in the absence of dynamin (56). Due to this positive cooperativity in membrane curvature generation, Epsin can rapidly accumulate on the curved membrane. At a sufficiently high Epsin concentration, this can cause runaway process leading to membrane fission (56). Presumably, the concerted actions of dynamin and epsin lead to fission at the CCV neck, however both proteins are also known to cooperate with actin (63, 64).

Actin dynamics is another potent driver for endocytic fission in yeast (65) and in mammalian cells (66), where actin association with the plasma membrane is believed to be necessary to maintain the high-membrane tension needed to sever the membrane at the neck (67, 68). Pharmacological inhibition of actin assembly leads to excessive neck tubulation (66), which indicates impaired membrane fission. In addition to pushing on the PM to maintain high-membrane tension, actomyosin networks have also been implicated in CME (69, 70).

Another type of membrane fission can be found in the formation of intraluminal vesicles (ILVs) within a late endosome. Unlike CME-dependent endosome formation, the fission machinery must assemble on the opposite membrane (the surface of the late endosome) and push the
membrane inward towards the lumen form a tubular pocket that is eventually detached from the endosomal membrane. While the fission of tubules is topologically opposite from ILV formation, there are many parallels between factors involved in CME and the ESCRT machinery. The ESCRT-III CHMP4/Snf7 can polymerize on membranes and has an N-terminal amphipathic helix that inserts into the membrane—similar to epsin and ARF-family proteins (71). A heteropolymer of CHMP2A/Vps2 and CHMP3/Vps24 can form a helical tubular structure within membrane tubules of ~50nm in width (72)—similar to BAR-domain proteins but with an inverted topology. Snf7 is known to recruit the Vps2/Vps24 complex to the membrane (5, 73). Vps2/CHMP2A and Vps24/Chmp3, in turn, recruit and are constricted, in an energy-dependent manner, by the AAA ATPase VPS4 (74)—similar to the energy dependent constriction of the dynamin GTPase. In addition, though not fully explored, ALIX, which functions as an ESCRT-I/-II independent way to recruit ESCRT-III assemblies via Snf7/CHMP4, is known to regulate branched actin and actomyosin networks within the cell (75–77).

By comparison to CME, the mechanisms that underlie the fission of endosomal tubules are less understood. The process likely recapitulates many of the principals involved in CME and thus involves an interplay between proteins involved in membrane scaffolding, membrane constricting, and actin cytoskeleton dynamics. For example, endosomal tubules are shown to be coated by the SNX-BARs, which with their ability to hetero-oligomerize with other SNX-BAR proteins, are able to give rise to a menagerie of different membrane tubule diameters. Moreover, several of the SNX-BAR proteins are known to associate with the DNAJ protein RME-8, and together these proteins can directly recruit the pentameric WASH complex(78), an essential
activator or the Arp2/3 complex (79). Thus, much like the case for CME, membrane scaffolding is intrinsically coupled to actin cytoskeleton dynamics.

Membrane constriction of endo-tubules also follows that of CME and involves nucleotide-dependent enzymatic activities of dynamin and/or dynamin-related proteins. While the role of dynamin in endo-tubule fission remains unclear, the dynamin family member, EHD1, localizes and functions at the tubular endocytic recycling compartment (ERC), and while its ability to hydrolyze ATP is required for endocytic recycling, however unlike dynamin, it is proposed to promote fission by membrane bulging instead of membrane constricting (80). Additionally, there is an endosome/TGN associated epsin-related protein (EpsinR) that binds clathrin and the intracellular clathrin adaptor AP-1 (81–84). The membrane affinity of EpsinR is much weaker than epsin1 (81), and thus its recruitment may require additional factors. For example, in zebrafish a tetraspanin protein plasmolipin has been shown to recruit EpsinR to the endosome and also to regulate the formation of recycling tubular compartments (85). Despite the identification of these different players on the endosome, it is not certain how and if they interact in a concerted way to drive fission similarly to at the plasma membrane.

1.3 Endosomal microdomains involved in degradation and recycling

1.3.1 Tubule-based sorting and ILV formation are not independent processes
ESCRTs and SNXs share overlapping functions outside of their “canonical” roles in degradative and retrograde/recycled cargo trafficking, respectively. For example, In addition to preventing EGFR degradation, SNX5 depletion was also shown to prevent the biogenesis of ILVs (29). A
similar effect on ILV formation was observed upon depletion of the ESCRT-0 subunit HRS (86). Further, SNX5 and HRS were found to co-purify, and this interaction was found to be phosphoinositide dependent (29). It is unclear how this interaction relates to the activities of SNX5 in tubule-based sorting and that of HRS in sorting into ILVs, but this suggests that these two processes are coupled.

1.3.2 The ESCRT and SNX microdomains
As it is becoming increasing difficult to distinguish the different sorting pathways on the “enigmatic” endosome (see review (87)) it is, instead, becoming increasing relevant to understand the spatial relationship of microdomains that form on the endosome. A current understanding of microdomains centers around 2 domains: the degradation and retrieval domains. The degradation domain is used to identify the location of cargoes that are sorted into ILVs and is populated mainly by the ESCRT machinery complexes 0-III. In contrast, the retrieval domain is used to identify the tubule profiles that store cargoes that ultimate escape degradation and are populated by the retromer, its associated sorting nexins, and branched actin nucleated by the WASH complex (19). Indeed, While the ESCRTs and retromer-associated sorting nexins have overlapping effects on protein degradation and recycling, they are known to localize to distinct domains. IST1 localizes to a domain distinct from, but seemingly adjacent to, SNX1 (88, 89). In addition, HRS has been shown to localize to a distinct and adjacent domain to SNX1 on enlarged endosomes in C. elegans (90).

Branched actin assembly on endosomes has been ascribed roles in the formation of sorting tubular profiles emanating from the endosomes, sorting of cargos into these tubules and the scission of cargo-filled tubules into vesicle carriers (91). This branched actin is mainly believed
to be nucleated by WASH complex-induced activation of the Arp2/3 complex. Indeed, initial reports of its involvement in fission were drawn from the observation that depleting WASH led to accumulation of endosomal elongated tubules and impaired recycling of TfnR (79). WASH was also shown to localize with retromer and its loss affected trafficking of mannose 6-phosphate receptor (M6PR) to perinuclear regions, while promoting localization to exaggerated tubules (92). It is important to note that the effects on specific cargo and tubule morphology seem to vary depending on cell type and on whether WASH is depleted (knockdown) or ablated (knocked out) (93). More recently it was shown that HRS, though in a distinct structural domain, can also regulate the localization and activity of WASH (94), inviting the possibility that WASH complex plays a role in ILV formation as well.

1.3.3 The WASH-HRS axis involved in recycling and degradation
The “HRS domain” on the endosome has been characterized as a flat clathrin domain, a clathrin “bilayer coat”, or simply a “flat” dense region (19, 95). It is not clear if clathrin is always present, and—if it is—whether it assembles similar to flat clathrin lattices on the PM, or due to the drastically different PI composition on early endosomes (PI3P) into a different structure all together. Regardless, HRS has been shown to recruit clathrin to the endosome (96). HRS is reported to bind to SNX1 (97), and also has a functional link to RME-8, another SNX1 binding partner (90, 98). RME-8 contains a J-domain which recruits and activates hsc70-family chaperones (99). This is of significance because J-domain proteins are known to drive clathrin lattice rearrangements, and loss of RME-8 leads to the accumulation of clathrin on internal membranes (98, 100, 101). Both loss of RME-8 and hsc70 lead to identical defects in the retrieval of cargos, indicating that the function of hsc70 is coupled with that of RME-8 function (98). There is evidence that RME-8 and cargo destined for retrieval are transient HRS domain
residents, as they require both require SNX1 for their removal from the HRS domain (90, 98).

Due to the ability of SNX1 to associated with, and possibly induce, curved membranes via its BAR domain, this chain of evidence demonstrates how retrieval proteins can transiently localize to the flat HRS domain but their final or more prominent location to the tubular retrieval domain is favored by their preferred geometry.

Similarly, the well-studied ESCRT-III protein CHMP4B, which appears to be critical for membrane deformation away from the cytoplasm to form ILVs, is also a transient resident of the HRS domain (102). A model proposed recently suggests that ESCRT-III assembly helps to corral the HRS domain, thereby containing ubiquitinated cargo and allowing de-ubiquitinases to remove ubiquitin before cargos are sorted into an ILV (103). Evidence from the kinetics of HRS and CHMP4 at the endosome revealed that HRS is required for the recruitment of CHMP4, but that the HRS domain abruptly diminishes as CHMP4B accumulates (102). Using EM, each wave of HRS and CHMP4B was found to correlate with the formation of a single ILV. Therefore, if CHMP4 polymers do corral the HRS domain, this can only be in the final stages of the HRS domain. While it has been speculate that loss of the HRS might be due to the loss of PI3P, or the loss of ubiquitinated cargos (due to the action of DUBs) during ILV biogenesis, there is evidence that speaks to the contrary. In fact, the retrieval proteins RME-8 and SNX1, as well as the ability of HRS to bind clathrin seem to be critical to regulating the lifetime of HRS on the endosome.

Specifically, ablating either RME-8, SNX1, or clathrin, as well as interfering with the ability of HRS to bind clathrin, all seem to stabilize HRS on the endosome (90, 102). Moreover, clathrin depletion had no effect on CHMP4 recruitment dynamics but ILV formation was still impaired. Intriguingly in C. elegans, loss of IST1 leads to the incorporation of HRS into abnormal ILVs,
which is a similar phenotype to the stabilization/impaired removal of the HRS (104). So while HRS regulates ILV formation, the HRS domain can regulate and be regulated by components of the retrieval domain.

However, in addition to sorting nexins acting as cargo adaptors for sorting into tubules, actin has been thought to play a sorting role on the endosome, which has been clearly illustrated for multiple cargos. The v-ATPase has been shown to bind directly to actin and this is responsible for its retrieval (105). Further two cargos manipulated to prevent their retrieval, TfnR that is stably ubiquitinated and beta-adrenergic receptor missing its sorting signal, can be rescued from their degradative fate by the addition of an actin binding domain (94, 106). In particular the latter cargo ub-TfnR, without the addition of the actin binding domain, was used to initially demonstrate how HRS sorted ubiquitinated proteins for degradation (107). It therefore seems that while ubiquitination is a signal for degradative sorting into ILVs it can be outcompeted by binding directly to actin, or to adaptors that bind actin.

On top of this, it is very curious that HRS has been reported to affect the residence of WASH on endosome, as its loss leads to less WASH localization, as well as less actin, on the endosome (94). The loss of actin on endosomes due to HRS depletion was also accompanied impaired the trafficking of cargo to the PM. While it is not clear by what mechanism HRS regulates WASH, other studies on what regulates the recruitment and activity of the WASH complex can provide insight.
The WASH complex subunit FAM21 binds to RME-8, indicating it could also be a transient HRS domain resident, in addition to the ability of the WASH complex to be recruited to the endosome via VPS35 (108). Interestingly, WASH activity is known to be regulated by two factors (1) ubiquitination of the nucleation promoting factor (NPF) itself, which is regulated by a DUB-E3 complex of ESP7 and MAGE-L2-TRIM27 and (2) PI4P (109–111). The WASH complex NPF requires K63-linked ubiquitination in order to activate Arp2/3 ((109, 111). Due to the high avidity of self-associated HRS for ubiquitinated proteins on the early endosome, it seems possible that in addition to the regulation of WASH by transient HRS-domain resident RME-8, HRS might also bind directly to ubiquitinated WASH. It is tempting to speculate that the ability of HRS to bind ubiquitinated proteins also allows the linking of ubiquitinated cargos to cargo adaptors that bind actin to a source of actin filaments polymerized by ubiquitinated WASH.

While WASH’s activity is positively regulated by ubiquitination, it also depends on PI4P (110). Loss of the ER-Endosome contact sites that enable the conversion of PI4P to PI leads to over-activity of WASH. Interestingly, in addition to the enzyme involved in PI4P metabolism, OSBP, and the ER-contact site tethering protein VAP, SNX2 was also shown to be important for the negative regulation of WASH activity through its binding to VAP. So curiously, it seems that HRS positively regulates WASH activity (via unclear mechanisms) but retromer-associated sorting nexins actually negatively regulate WASH activity.

Complete loss of actin polymerization leads to a coalescence of WASH domains as well as increased WASH recruitment, indicating that the ability of WASH to polymerize actin
contributes to its localization to discreet domains, as well as its removal from the endosome (112). Since maximum force generation would be obtained by the restriction and specific orientation of the active NPF, as presumably achieved by retromer and its associated sorting nexins, this also provides a way to not only control but also restrict pushing force generated by actin polymerization. This feedback loop on WASH activity, beginning with positive regulation at the HRS domain and ending with the negative regulation at the retrieval domain, provides an interesting model on how cargo sorting into tubules can be affected by both the degradative and retrieval domains.

There have been contradictory results from studies of IST1 function in both recycling and degradation. As such, the localization of degradative and retrieval domains—and the components they include—relative to IST1 is a central focus of this thesis with the aim of providing new mechanistic insight into the role of IST1 on the endosome.

1.4 The diverse physiological functions of ESCRT-III-like IST1

1.4.1 IST1 is involved in PM recycling in yeast

Ist1 in yeast was identified in the PM recycling screen using a synthetic cargo (113) and shown to regulate the membrane recycling of an arginine transporter Can1 (114). ESCRT mutant strains exhibit increased sensitivity to the toxic arginine analogue canavanine due to the blocked degradation of Can1 via the MVB pathway and increased recycling of Can1 back to the PM. However, it was shown that Ist1 mutants were able to prevent the PM localization of Can1 and rescue the canavanine-sensitive phenotype of ESCRT-mutant cells. This implies that Ist1
regulates a recycling pathway that is independent from ILV formation and unaffected by depletion of “core” ESCRT proteins.

1.4.2 IST1 in mammalian cells
The first studies of IST1 in mammalian cells had identified a role in cytokinesis. This was mainly due the prominent localization of IST1 to mitotic cell bridges and that depletion of IST1 in HeLa cells lead to mitotic dysfunction (13, 88). Coincidently, cytokinesis involves a scission reaction that is topologically identical to ILV formation—though orders of magnitude larger. IST1 was also found to localize to endosomes, and it has been shown to affect the trafficking of TfnR and M6PR (88, 115, 116). Intriguingly the loss of IST1 phenocopies the loss of the AAA ATPase spastin, which can bind directly to both IST1 and the IST1-interacting partner CHMP1B. However, it remains unclear how IST1 and CHMP1B can differentially recruit spastin in order to regulate endosomal sorting and cytokinesis (117).

1.4.2.1 IST1 function in cytokinesis
More recent work has better described ESCRT-III helical polymeric assemblies (118–120) and demonstrated how cytokinetic abscission requires the coordinated action of the ESCRT machinery with microtubule, actin and septin cytoskeletons (117, 121, 122). Importantly, ESCRT-III assemblies at the cell bridge (CB), notably that contain IST1, have been shown to be negatively regulated by actin (122), which needs to be cleared from the cell bridge before abscission can separate the daughter cells. In contrast, septins are important for the recruitment of ESCRT-III to the cell bridge, thus ESCRT-III assembly during cytokinesis is tightly coupled to actin and septin dynamics. Two mechanisms have been shown to both reduce actin present at the CB and are required for ESCRT assembly at the CB: (1) fusion of Rab35 and Rab11 positive recycling endosomes and (2) recruitment of the MICAL proteins by Rab35 and Rab11 (122–125).
to the abscission site. Recycling endosomes marked by Rab11 and Rab35 are well known to regulate cytokinesis. Specifically, the dynamin-like protein EHD1 is found on Rab11 and Rab35 tubular compartments in interphase and has also been found to localize to and function at the cell bridge during cytokinesis (126). Furthermore, Rab35 and Rab11 have been shown to direct TfR-positive endosomes to the midbody region during cytokinesis (127), which recapitulates their roles, along with Rab4, in the recycling of TfR back to the cell surface during non-mitotic phases of the cell cycle. The accumulation of Rab35 and Rab11 endosomes at the midbody is coupled to the recruitment of MICAL proteins, which are mono-oxygenases that specifically oxidizes actin monomers within the actin filament. Oxidized actin is exceptionally fragile and, as a consequence, disassembles rapidly and cannot be readily reassembled into actin filaments. Additionally, Rab35 can negatively regulate actin polymerization by recruiting OCRL phosphatase to hydrolyze PI(4,5)P₂ at the cell bridge, as it also does during CME (128, 129). This results in the loss and/or inactivation of PM associated actin assembly factors, most of which are activated either directly or indirectly by PI(4,5)P₂.

1.4.2.2 IST1 function at ER-endosome contact sites
Considerable work on the interaction of IST1 with the microtubule-severing AAA ATPase spastin has revealed a role for IST1 in the formation of ER-endosome contact sites and endosomal fission (130). Indeed, loss of either IST1 or spastin has been shown to lead to the accumulation of elongated tubules that are positive for BAR-domain proteins SNX1 and SNX4. Importantly expression of the ER-associated isoform of spastin (M1 spastin) was able to rescue the accumulation of tubules in spastin depleted cells (115). Depletion of spastin alone leads to an increase in the longevity of SNX1 coated tubules before eventual fission (which occurred along
the ER more than 80% of the time) and/or collapse. Moreover, mutations in spastin were shown to decreased the number of ER-endosome contacts in MEFs (116).

The defects in tubule resolution due to loss of IST1 or spastin were also accompanied by the following impairments in the sorting of TfR and M6PR: (1) an increase in the overlap of TfR and M6PR, but only TfR was observed in elongated tubules, (2) an increase in the degradation of TfR and (3) impaired sorting of endocytosed M6PR to, or proximal to, the Golgi (115, 116). The authors also attempted to determine if the spastin-IST1 interaction was required for tubule fission, However, their approach to rescuing the effect of IST1 depletion with an IST1 construct lacking the two MIM motifs of IST1 (116) was flawed, since the IST1 MIM motifs (as well as the “core ESCRT-III” domain) are known to interact with a wide variety of proteins essential to the regulation of endosomal sorting and degradation, including VPS4 which disassembles ESCRT-III filaments from membranes. Thus, it remains unclear if an interaction of IST1 and M1 spastin is necessary for endosomal fission. Complicating matters, how spastin interacts with IST1 for endosomal fission is still unclear, since IST1, as well as CHMP1B, is also recruited by M1 spastin to lipid droplets, which are know to grow from, and form several different kinds of contact sites with the ER (131).

1.4.2.3 IST1 function at lipid droplet-peroxisome contact sites
In addition to ER-endosome contact sites, Chang et al also showed that IST1 and spastin are also involved in LD-peroxisome contact sites (131). Interestingly, formation of this contact site does not depend on microtubules but does depend on spastin’s ATPase activity (131). IST1 was found to regulate fatty acid transport from lipid droplets to peroxisomes, but the mechanism is unclear (131). Adding to this confusion, another MIT domain containing protein, spartin, but not spastin,
was identified in proximity labeling studies on lipid droplets (132, 133). Spartin is also shown to interact with the ubiquitous LD coat protein perilipin 3 (134). What is more, the MIT domain of spartin specifically interacts with the MIM1 motif of IST1, and not any other ESCRT III protein (135). Spartin is also known to localize to much of the same cellular real estate as IST1, including: endosomes, lipid droplets, and the cytokinetic cell bridge (135–137).

The cellular functions for IST1 discussed thus far are just the tip of iceberg. In addition to the interacting proteins mentioned previously, and there are more with even less well understood functions such as MITD1 and calpain 7 (138, 139). It is clear that detailed analysis of IST1 at many locations is needed, and the purpose of this thesis work was to do this for IST1 on the endosome and determine how the function of IST1 is distinct from that of other ESCRT-III proteins on the endosome.

1.5 Endosomal trafficking from the plasma membrane to the lysosome—or back to the plasma membrane

1.5.1 Rab-GTPases regulate distinct domains and endosomal trafficking pathways

Rab GTPases have long been used to describe intracellular compartments as well as trafficking pathways. Rabs function as molecular switches that can regulate the activities of many different effector proteins and, as a consequence, can act as hubs that control entire trafficking pathways (140). Rab5 has been shown to be a master regulator of endosome biogenesis and trafficking originating from the PM (via CME) and the TGN (141–143). However, as an early endosome matures, the composition of the endosomal membrane changes and so do the Rabs. Specifically, the conversation from Rab5 to Rab7, marks the transition of the early endosomes into late
endosome, and is accompanied with many important trafficking steps, both involved in
degradation and recycling (144). For example, SNX-BAR sorting tubules most frequently
localize to endosomes with both Rab5 and Rab7 (38), and Rab7 is required for the recruitment of
the core retromer protein VPS35 (34, 145). In addition, a key step in degradation, ILV
formation, is also tightly coupled with the Rab5 to Rab7 transition (146).

In addition to a Rab5 to Rab7 conversion, Rab5 endosomes can also undergo a transition
to Rab4 and this has been shown to mediate the recycling of TfnR into a tubular compartment
(147–149). Rab4 regulates the exit of cargo from Rab5 endosomes and their return to the PM, as
it can directly promote the exocytosis of cargo itself (fast pathway) or the conversion of the
compartment to Rab11 which promotes the slower exocytosis of cargo (slow pathway) (147,
150–152). While this mode of recycling undoubtedly requires the action of the retromer, it is
therefore surprising that the retromer associated sorting nexin, SNX1, does not localize to Rab4
and Rab11 positive endosomes (50). On the other hand, the WASH complex, which is tightly
associated with the retromer, localizes to Rab5, Rab4, Rab11, and Rab7 positive endosomes and
therefore seems important in tubular sorting from all endosomal domains (79, 153). How or if
retromer or any of its associated-sorting nexins relates to the Rab5 to Rab4 transition critical for
cargo return to the PM remains unknown.

1.5.2 The role of the ESCRT machinery in the Rab5 to Rab7 transition
As noted in section 1.1, 4 of the 7 ESCRT-III subunits in yeast are essential for ILV biogenesis
(4, 5). Mutant yeast strains of these so “core” ESCRT-III’s, as well as components of the
ESCRT-0, -I, and –II complexes, could be distinguished among other vps mutants as having a
novel prevacuolar-like organelle (Class E compartment). The Class E compartment consists of
abnormally stacked cisternae, distinct from the vacuole, in which vacuolar proteins accumulate
(15). This Class E compartment phenotype was found to be dissociable from ILV formation, as the expression of constitutively active Rab5 and loss of Rab7 also results in the formation of the stacked cisternae observed in class E mutants, however they contained ILVs (146). The penetrance of this phenotype was low, which indicates that despite the block in the Rab5 to Rab7 transition, ILV formation can still drive the maturation of the prevacuolar endosomal (PVE) compartment. As noted above, loss of the other 3 ESCRT-III proteins in yeast, Did2/Vps46 (CHMP1A/B), Vps60 (CHMP5) and Ist1 (IST1), does not prevent ILV formation (6, 154). Despite this, 2 of these non-core ESCRT-III proteins Did2/Vps46 (CHMP1A/B) and Vps60 (CHMP5) mutants do display impaired MVB trafficking, which indicates that these subunits are still involved in the maturation of the PVE compartment, but by a different mechanism than ILV formation (5). IST1, on the other hand, does not affect MVB trafficking, and may even antagonize ILV formation (6, 155) by negatively regulating the activity of Vps4 (10, 155). Instead, IST1 has been uniquely implicated in recycling to the PM and would therefore could act upstream of the Rab5-to-Rab7 transition.

1.5.3 The role of the ARF-GTPases that regulate clathrin and clathrin-adaptors at the Rab5 to Rab4 transition
The earliest “endosome” is the endocytic vesicle pinched off from the PM, and while there are many forms of endocytosis, CCVs from the PM have been shown to fuse with larger endosomal compartments—and this is regulated by Rab5 (156). Rab5 localizes to both CCVs and PI3P endosomes, while the Rab5 effector EEA1 only localizes to PI3P endosomes (156). Correspondingly, Rab5 can mediate the homotypic fusion of endosomes (i.e both contain EEA1), or heterotypic fusion between CCVs and EEA1 positive endosomes. Curiously, Rab5 was identified as a necessary component in the maturation of clathrin coated pits (157). The localization of Rab5 to CCV is, at least in part, due to the effector proteins that regulate its
membrane association. Rab5 effector Rap6 or hRME-6 was found to bind to the clathrin adaptor protein AP-2 (via the alpha-adaptin subunit), and in doing so displaces the AP-2 associated kinase 1 (AAK1) by competitive binding (158, 159). Phosphorylation of AP-2 by AAK1 critically regulates the ability of AP-2 to bind cargo and associate with the nascent endosome. Thus by displacing AAK1, hRME-6 enables the uncoating of AP-2 and allows the endocytic vesicle to undergo fusion with the early endosome.

Rab5 has other effectors including rabaptin that binds clathrin adaptors AP-1 and GGAs (via the GAE domain of the GGAs and gamma appendage of AP-1) (160, 161). Rabaptin is also an effector for Rab4, and has distinct binding sites for Rab4 and Rab5 (162). Depending on which of these Rabs are bound, Rabaptin can function in either endosomal maturation (Rab 5) (163), or recycling to the PM (Rab 4) (164). Recycling of cargoes from endosomes is further fine-tuned by Rab4 isoforms. The Rab4a isoform has been found to regulate the rapid recycling of TfnR to either the PM as well as trafficking of other cargos to lysosomes and lysosomal related organelles (165–167), while Rab4b regulates the sorting of cargo to the slow recycling compartment (151). In either case, regulation of recycling by Rab4a and Rab4b requires direct or indirect binding to clathrin adaptor proteins AP-1 (151, 168) and AP-3 (169) (albeit whether the latter acts with clathrin is controversial). One potential function of Rab4 in recycling is that it helps to recruit Arf GEFs and GAPs to the endosome, which are essential in the generation of PIP4 and loading of AP-1 adaptors onto cargo (170). While a similar Arf-family GTPase cascade is required (and best understood) for AP-1 function at the TGN, Rab4 directs this process on the endosome and offers an explanation for how AP-1 can localize to endosomal compartments thought to be exclusively PI3P positive (171, 172). In support of a role of AP-1 in
endosomal recycling, depletion of AP-1 and some accessory proteins, such as NECAP-2 (173), a protein that can bind both AP-2 and AP-1, affects the fast recycling of TfnR while depleting of other AP-1 accessory proteins, such as aftiphilin leads to impaired sorting of Tfn to the perinuclear compartment (associated with slow recycling) and increased fast recycling for peripheral compartments (174).

Rab4 is not only involved in the recycling of cargoes and has been found to regulate the degradation of certain cargo. For example, the Rab4 and Rab5 effector, rabenosyn, is similar to EEA1 in that is has FYVE domain that binds specifically to PI3P, but has been reported to localize to a distinct set of endosomes (175). Rabenosyn was found to regulate the sorting TfnR down recycling or degradative pathways (175). Interestingly, rabenosyn has also been reported to be required for TfnR to reach the perinuclear recycling compartment—a phenotype similar to the loss of aftiphilin (174, 176). This suggests that the recycling and degradative fates of proteins can also be determined at the Rab4/Rab5 positive endosome instead of at the Rab5/Rab7 transition associated with the formation of ILVs (the defining characteristic of the late endosome). While the roles of Rab5 and Rab4 in clathrin-based trafficking pathways on the endosome are not well understood, the fact that many of their effectors also bind directly to clathrin adaptor proteins AP-2 and AP-1, they can be effectively considered accessory proteins of clathrin coats.

1.5.4 Clathrin coats with diverse functions on the endosome
Clathrin was originally identified as the component responsible for the distinct pentagonal and hexagonal lattices found at invaginations on the PM and isolated vesicles (177), which were similar to those found associated with the TGN. Another key component of the clathrin coat was
found to be adaptor protein complex (APs) which link the clathrin lattice to membrane associated proteins (178). Clathrin can form vesicular cages in vitro, but this ability to assemble could be enhanced by what where later called adaptor proteins (AP-1 and AP-2) and their so-called accessory proteins. The adaptor proteins are composed of two similar large subunits, the first of which interacts with accessory proteins, gamma adaptin (for AP-1) and alpha adaptin (for AP-2), and the second which interacts with clathrin and accessory proteins, beta-1 (for AP-1) and beta-2 (for AP-2). Accessory protein binding via specific motifs to the ear domains of alpha (AP-2) or gamma (AP-1) adaptin have been well described (179–181), and are important for their specific functions at the PM and TGN, respectively. The large adaptins have hinge regions that connect the ear domain to the trunk region, and the hinge region of the beta subunits is known to bind clathrin via a motif called a “clathrin box.” The trunk domains of the large subunits and other two subunits (µ and σ) form the “core” that binds specific membrane associated cargo motifs. This multi-functional ability of AP-1 and AP-2 to simultaneously interact with cargo, clathrin and accessory proteins makes them ideal hubs for clathrin-mediated carrier processes.

AP-1/2 are not the only clathrin cargo-binding adaptors. Dab2 and ARH are monomeric adaptors that bind both cargo and clathrin and mediate the sorting of LDL receptor in CCVs (182, 183). Similarly, stonin is a monomeric adaptor for synaptotagmin, and may recruit clathrin independently of AP-2 (184, 185). Interestingly, all these monomeric adaptors can also bind AP-2 and may serve as an extra layer of cargo recognition. Similarly the GGA proteins, originally identified based on their homology to the gamma appendages of AP-1 (186), are monomeric adaptors that can also bind lyosomal hydrolase receptors, such as cation-independent mannose-6-phosphate receptor (CI-M6PR), and clathrin. In addition to their homology to the AP-1 gamma
appendage, GGAs also have the ability to bind the gamma appendage of AP-1 via a ϕXXϕ motif in their hinge domain. This mode of binding seems to block the binding of other accessory proteins to AP-1, like p56 (187). Conversely, AP-1 has a CK-2 associated kinase that phosphorylates and inactivates GGA1 and GGA3, but not GGA2, from binding to cargo (188). This, in part, explains why that only GGA2 can be found in CCVs. It is then curious that dynamic GGA1 and clathrin has been visualized on tubular carriers from the TGN as well as on endosomes (189–191)—as GGA1 is not incorporated into the final CCV. Thus it remains unclear if GGAs are a part of a sub population of CCVs distinct from AP-1 (192), or are dynamically associated with AP-1 during canonical CCV maturation.

Clathrin adaptor proteins have also been shown to interact with ESCRT proteins and therefore may play a key role in the function and assembly of the ESCRT machinery on endosomes. For example, GGAs via their GAT domain, also bind rabaptin5, ubiquitin and ESCRT machinery component Tsg101 (193). Further GGA3 depletion was found to impair the degradative sorting of EGFR, similar to loss of “upstream” ESCRT proteins HRS, STAM1, STAM2, and Tsg101 (194–196). Similarly a second isoform of AP-1 gamma adaptin (gamma2 adaptin) was found to bind to ubiquitin (unlike gamma1 adaptin) and similarly its loss leads to impaired degradation of EGFR (197, 198). Finally, Another “upstream” ESCRT protein Alix, has also been demonstrated to interact with another AP-2 accessory protein EHD1, and has been found to regulate PM recycling in C. elegans (199, 200).

Supporting the notion that clathrin may coordinate the ESCRT machinery, the degradative domain where ILV formation occurs is known to both contain and to be regulated by clathrin
The “HRS domain” on the endosome that is responsible for ILV formation has been characterized as either a “flat” clathrin domain, a clathrin “bi-layer” coat, or a “flat” dense region as it is still present in the absence of clathrin. Several questions have yet to be resolved about this domain, including: if clathrin is necessary to form this domain and if its clathrin-coat resembles flat clathrin lattices on the PM. Regardless, HRS has been shown to recruit clathrin to the endosome (201). Specifically, HRS was originally identified as recruiting clathrin to the endosome through a clathrin box motif that is similar to that found in beta 1 (AP-1) and beta 2 (AP-2) (201). Over-expression of HRS led to increased clathrin recruitment and impaired degradation of EGFR, whereas its loss impairs the recycling of several other cargos (202–204)—indicating it is a clathrin adaptor that can promote both degradation or recycling. HRS can bind Eps15, and a specific Eps15 isoform that lacks an EF-hand was shown to associate with HRS on the endosome and to be required for EGFR degradation (205). In contrast, HRS is reported to bind to SNX1 and VPS35 (97, 98), and also has a functional link to RME-8, also a SNX1 binding partner (90, 98), which recruits and activates hsc70-family chaperones via its J-domain (99). This is significant because J-domain proteins are essential regulators of clathrin lattice dynamics, and loss of RME-8 leads to the accumulation of clathrin on internal membranes (98, 100, 101). Both loss of RME-8 and hsc70 lead to identical defects in the retrieval of cargos, suggesting that the function of hsc70 is critical for RME-8 function (98). Furthermore, there is evidence that RME-8 and retrieval cargo can transiently localize to the HRS domain, as they await for the arrival of SNX1 for removal (90, 98). Another Thus, the HRS domain, may act as a central hub for sorting, that transiently stores proteins that are either sorted down SNX1 positive tubules or ILVs for recycling and degradation, respectively.
Much remains to be understood about what specific clathrin-interacting proteins are present in this HRS domain. For example, dynamin, which associates with AP-2 (206), was also shown to be dramatically recruited to the endosome and co-localized with over-expressed HRS (201). However, the role of dynamin at these sites is unknown. In contrast, AP-1 was shown not to localize to this HRS-clathrin domain (201), identifying a clear difference between the clathrin HRS domain and budding clathrin coats. Despite this distinction, the requirement of an endosome-specific isoform of Eps15 for EGFR degradation, highlights not only a protein common to both ILV and CCV (Eps15) but also the ubiquitin sorting motif (UIM) it, as well as HRS and STAM1/2, contains (205). While it is appreciated that ubiquitin-based sorting is important in endocytosis and ILV formation, it remains unclear how clathrin adaptors and accessory proteins contribute to degradative sorting. AP-2, for instance, is essential for the internalization of TfR, but not for EGFR. This is because CME of EGFR depends on CCVs containing either AP-2 or the combination of epsin1 and Eps15 (207). There appears to be competition between the two flavors of CCVs, as AP-2 has been shown to displace Eps15 (208), and these 2 distinct sets of clathrin adaptors differently regulate the degradative sorting of EGFR. EGFR endocytosed via AP-2 showed increased increased ability to signal, whereas EGFR endocytosed via Eps15 and epsin1 showed decreased ability to signal and enhanced degradation. The likely mechanism of EGFR incorporation into a non-AP-2 clathrin coat is via the ubiquitin interaction motifs (UIMs) of Eps15 and epsin1 binding to ubiquitinated EGFR. This suggests that clathrin and ubiquitin-based sorting from the PM into a CCV may have several parallels with ubiquitin sorting from the endosome into an ILV, despite having opposing membrane topology.
There remains much to be learned about the role of clathrin in endosomal sorting. While two decades of research has firmly cemented a role for AP-1 in endosomal recycling, our mechanistic understanding of how AP-1 and its accessory proteins regulate endosomal recycling and degradative sorting remains primitive, especially when compared to what is known for AP-2 in CME.

1.5.5 Clathrin and retromer mediated sorting on the endosome—linked or not?
Both clathrin and SNX-BAR/retromer are required for the retrograde trafficking of the cargos CI-M6PR and the β subunit of Shiga toxin (98, 209–213). This lead to the proposal that clathrin and retromer act sequentially, either by clathrin initiating membrane deformation followed by recruitment of the SNX-BARs to the tubule neck (similar to CME) or by acting to organize cargo into a flat domain that is then tubulated by the SNX-BARs (214, 215). This was examined in live-cell imaging experiments of clathrin and sorting nexins, which revealed no relationship of clathrin along the length of SNX-BAR tubular carriers, albeit clathrin was present at the base of SNX1 tubules (166). Furthermore, SNX-BAR tubules could be seen when either dominant negative clathrin was expressed or clathrin was knocked-down, however the dynamics of these tubules was not assessed by live cell (166). In addition, the clathrin construct used for live cell imaging experiments (clathrin light chain tagged with DsRed), has been shown to act in a dominant-negative fashion, inhibiting both clathrin dynamics on endosomes and clathrin light chain-dependent recycling pathways of integrins and TfnR (216). Since this study, little progress has been made in understanding the relationship of clathrin- and retromer-based recycling on endosomes (19).
1.6 Sorting nexin 15 is a PI3P- and clathrin-binding protein with a MIT domain

1.6.1 The known roles of SNX15 in endosomal sorting
Sorting nexin 15 was identified in a screen looking for sorting nexins able to regulate the degradation of EGFR (30). However, its depletion did not inhibit, but simply delayed, the degradation of EGFR. In addition, loss of SNX15 did not affect the endocytosis of EGFR upon stimulation with EGF, which suggested that SNX15 aids in the recycling of EGFR from early endosomes back to the PM in response to EGF stimulation. In support of this, SNX15 was found to localize to EEA1 positive early endosomes, as well as to CCVs juxtaposed to the PM (30). This is localization is in part due to the the N-terminal PX domain found in SNX15 that binds specifically to PI3P enriched in early endosomes, but also because it can bind directly to clathrin (30). The authors of this study conclude that SNX15 links clathrin-mediated endocytosis with the PI3P endosome, and thereby directs EGFR internalized by CCV to the PI3P endosome where it can be sorted for either recycling or degradation. Finally, SNX15 is the only sorting nexin known possess a MIT domain that shares is ~30% homology with the MIT domain of VPS4, and was therefore was speculated to interact with the ESCRT machinery (217). However, no interaction between SNX15 and any component of the ESCRT machinery was identified using a yeast-2-hybrid, although it is important to note, that IST1 was not tested in this screen (218).

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Chapter 2: ESCRT-III-like helical polymers of IST1 and CHMP1B adopt an “inverse” topology

2.1 Forward and Hypothesis
ESCRT-III subunits assemble from soluble monomers into membrane-associated polymers that mediate the membrane remodeling required for ILV formation. Work previously from the Hanson lab demonstrated how membrane binding and polymer assembly of ESCRT-III proteins is favored by deletion of C-terminal autoinhibitory sequences or the subunit’s fifth alpha helix (1). This fifth alpha helix packs against the N-terminal alpha helical hairpin and is responsible for the “closed” confirmation that soluble monomers adopt in the cytoplasm and its removal instead favors the “open” confirmation in which polymers form on membranes. Additionally, the Hanson lab demonstrated that ESCRT-III CHMP4A/B (Snf7) form polymers that spiral on membranes and, when subunits lack the C-terminal autoinhibitory sequence, are able to deform membranes away from the cytoplasm (2). ILV formation also requires CHMP3 and CHMP2A, which are recruited to endosomes by CHMP4 (3, 4). In vitro, CHMP2A and a similarly C-terminally truncated CHMP3 are known to form a tight helical co-polymer within membrane tubules of ~50nm diameter (5). Since the diameter of PM buds formed by filaments of truncated CHMP4 is ~80nm, this suggests that ability of the CHMP3/CHMP2A to assemble with CHMP4 drives further constriction of the membrane neck.
Beyond the “core” ESCRT-III proteins above, ESCRT-III related proteins CHMP1B and IST1 were also found to co-polymerize, as well as form homopolymers in isolation (6). In contrast to CHMP3, which required relief of autoinhibition to polymerize with CHMP2A, IST1 co-polymerized with CHMP1B in an autoinhibited or “closed” state. Mutation analysis revealed that these pairs (CHMP2A/CHMP3 and CHMP1B/IST1) assemble into structurally distinct complexes.

The genesis of this chapter was our observation that CHMP1B, alone or together with IST1, associates with and tubulate cellular membranes with a topology that is distinct from other ESCRT-III proteins—lining the outer positively curved surface of ~100 nm diameter tubules. This finding and its potential implications for the function of these ESCRT-III proteins, complemented observations made independently by Adam Frost and colleagues, who had obtained a cryo-EM structure of the CHMP1B-IST1 co-polymer. Their structure demonstrated how CHMP1B subunits interact through a specific “open” conformation which ultimately drives the formation of a helical macromolecular assembly. Importantly, the strongly basic interior of the CHMP1B/IST1 copolymer predicted potential membrane association inside the copolymer could induce membrane tubulation, which is opposite to that expected based on prevailing models of ESCRT function in ILV formation (and previously seen for the CHMP2A/CHMP3 copolymer). We proposed that their polymers might be expected to tubulate membranes as seen in our images. They tested this hypothesis and found this to indeed be the case. We therefore collaborated with the Frost and Sundquist labs to develop deeper mechanistic understanding as to how CHMP1B and IST1 associate regulate membrane topology. This led to publication of the paper that comprises this chapter.


2.2 Acknowledgments
The work presented in this chapter has been previously published as the following:


The four main figures (Figure 2.1-2.4) are included in this chapter, and the supplemental figures and methods of experiments performed by the Frost and Sundquist labs can be found online at https://science.sciencemag.org/content/suppl/2015/12/02/science.aad8305.DC1?_ga=2.216904726.1014177659.1591757453-1237467521.1574726789

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2.3 Abstract
The endosomal sorting complexes required for transport (ESCRT) proteins mediate fundamental membrane remodeling events that require stabilizing negative membrane curvature. These include endosomal intralumenal vesicle formation, HIV budding, nuclear envelope closure, and cytokinetic abscission. ESCRT-III subunits perform key roles in these processes by changing
conformation and polymerizing into membrane-remodeling filaments. Here, we report the 4 angstrom resolution cryogenic electron microscopy reconstruction of a one-start, double-stranded helical copolymer composed of two different human ESCRT-III subunits, charged multivesicular body protein 1B (CHMP1B) and increased sodium tolerance 1 (IST1). The inner strand comprises “open” CHMP1B subunits that interlock in an elaborate domain-swapped architecture and is encircled by an outer strand of “closed” IST1 subunits. Unlike other ESCRT-III proteins, CHMP1B and IST1 polymers form external coats on positively curved membranes in vitro and in vivo. Our analysis suggests how common ESCRT-III filament architectures could stabilize different degrees and directions of membrane curvature.

2.4 Results & Discussion
The endosomal sorting complexes required for transport (ESCRT) pathway is best known for facilitating membrane remodeling and fission for processes such as the endosomal intralumenal vesicle (ILV) formation, enveloped virus budding, nuclear envelope closure, and cytokinetic abscission (1–3). In these reactions, the ESCRT machinery assembles on the interior of a negatively curved, cytoplasm-filled membrane neck and pulls the membrane toward itself to the fission point. These fission reactions are topologically distinct from reactions in which cytoplasmic BAR domain-containing proteins and dynamin-family guanosine triphosphatases assemble around and constrict positively curved membrane tubules.

ESCRT components are recruited to different membranes by site-specific adaptors that ultimately recruit ESCRT-III subunits and their binding partners, including VPS4-family adenosine tri-phosphatases (ATPases). ESCRT-III assemblies promote membrane constriction and fission, possibly in concert with VPS4. Humans express 12 related ESCRT-III proteins, called charged multi-vesicular body proteins (CHMPs) 1A-7 and increased sodium tolerance 1
Crystal structures of CHMP3 and IST1 show a common structure in which the first two helices form a long hairpin, the shorter helices 3 and 4 pack against the open end of the hairpin, and helix 5 folds back and packs against the closed end of the helical hairpin (4–6). This “closed” conformation appears to autoinhibit ESCRT-III membrane binding and oligomerization (4, 7, 8). ESCRT-III subunits can also adopt a second, more extended “open” conformation that has been characterized biochemically, but not visualized in molecular detail. The open conformation appears to be the active, polymerization-competent state because mutations or solution conditions that favor this conformation typically promote polymerization (1–3). Many ESCRT-III subunits form spiraling homo- and heteromeric filaments, both in vitro (4, 9–15) and in cells (10, 16–19), but the structural basis for filament assembly is unclear.

We used cryogenic electron microscopy (cryo-EM) to determine the molecular structure of a helical copolymer comprising human IST1 and CHMP1B. Full-length IST1 and CHMP1B spontaneously coassembled under low ionic strength conditions into well-ordered helical tubes. Helical order was further enhanced by using a truncated IST1 construct that spanned residues 1 to 189, hereafter termed IST1NTD, and by including small, acidic unilamellar vesicles (SUVs) to nucleate polymer formation. The resulting IST1NTD-CHMP1B tubes were long, straight, and 24 nm in diameter (Fig. 2.1A). The three-dimensional (3D) structure of IST1NTD-CHMP1B assemblies was determined to a resolution of ~4 Å by real-space helical re-construction (supplementary materials, materials and methods, and figs. S1 to S4). Each tube comprised a right-handed one-start helical filament that packed with an interfilament spacing of 5.1 nm per turn (Fig. 2.1, B to D, and movie S1). Each filament was double-stranded, with distinct inner and outer strands (at 7.7 and 10.2 nm radii, respectively). Segmented densities from sub-units in the outer strand corresponded well to
the crystal structure of IST1<sub>NTD</sub> in its closed conformation [Protein Data Bank (PDB) 3FRR] (5), with only minor refinements required to optimize the position of helix A (Fig. 2.1E and movie S2). In contrast, the CHMP1B subunits of the inner strand adopted a very different, open conformation. These subunits were almost entirely α-helical, and side chain densities were clearly evident in the EM density (Fig. 2.1, B, D, and F; and movie S3). The open CHMP1B conformation resembled an arm, with helices 1 to 3 forming the upper arm and biceps, helix 4 and helix A forming the forearm, and helix 5 forming the hand. Joints between helices 3 and 4 and between helices A and 5 correspond to the elbow and wrist, respectively (Fig. 2.1, F and G).

High-ionic-strength conditions (4, 8) typically favor the monomeric, closed ESCRT-III subunit conformation (4, 8, 20, 21), and CHMP1B also remained monomeric under high-ionic-strength conditions (fig. S5). Lowering the ionic strength triggered coassembly of IST1 and CHMP1B, implying that CHMP1B subunits are captured as they open. To visualize this conformational change, we generated a structure-based homology model for the CHMP1B closed state (supplementary materials, materials and methods). In comparison with the modeled closed state, the helix 5 hand is displaced by ~100 Å when CHMP1B opens. This global reorganization requires only three local rearrangements: The elbow angle between helices 3 and 4 must change, and the loops that connect helix 2/3 and helix 4/A must become helical to create the longer, continuous helices that extend the upper arm to the elbow and create the fore-arm in the open state (Figs. 2.1, F and G, and 2A and movie S4).

In the filament, the open CHMP1B conformation is stabilized by extensive intersubunit interactions along the inner strand (Fig. 2.2B). Each CHMP1B molecule interacts with four other CHMP1B subunits that pack together and cross the forearm of the original subunit. In addition, the helix 5 hand grasps the shoulder of the hair-pin four subunits away, making a domain-
swapped contact that is analogous to the intrasubunit interaction between the hairpin and helix 5 in the closed ESCRT-III conformations (Fig. 2.2, A and B). Opening and assembly reduces the total solvent-accessible surface area of CHMP1B from ~10,720 to ~6,350 Å². The IST1\textsuperscript{NTD}-CHMP1B assembly is further stabilized by three additional types of interactions, which differ completely from crystallized contacts for soluble IST1-CHMP1B heterodimers (Fig. 2.2, C to F, and figs. S6 and S7) (6).

A final notable feature of the IST1-CHMP1B tube is the remarkably cationic interior. This surface is created by a series of conserved basic residues from helix 1 of CHMP1B (Fig. 2.2, D and F), which forms the principal membrane-binding site in other ESCRT-III proteins (5, 22). Its position inside the IST1-CHMP1B copolymer was unexpected because well-characterized ESCRT-mediated membrane remodeling events require that membranes interact with the exterior surface of coiled ESCRT-III filaments, such as in the neck of a nascent ILV or viruses. The functional roles of IST1 and CHMP1B in such canonical ESCRT activities have been enigmatic, however, because neither protein is required for ILV biogenesis (23–26) or virus budding (27). Moreover, IST1 functions in the resolution of endosomal tubules that project into the cytoplasm and recycle cargoes back to the plasma membrane (28). Looking in cells, we found that moderately over-expressed CHMP1B polymerized into elongated cytoplasmic structures (fig. S8). Deep-etch EM of the plasma membranes revealed filaments that were similar to those of CHMP4A and other well-studied ESCRT-III proteins (17), except that CHMP1B filaments coated tubules ~35 to 60 nm in diameter that extended into rather than away from the cytoplasm (Fig. 2.3, A to D) (17, 18). Replicas of cells transfected and immunolabeled for CHMP1B alone, or with IST1, also revealed immunodecorated organelles and tubules that were not attached to the plasma membrane but again had recognizable striations (Fig. 2.3, E to K). The resemblance of
these organelles to early endosomes (29), including the occasional presence of clathrin-coated buds (Fig. 3, J and K), indicates that CHMP1B and IST1 can coat and potentially remodel endosomal tubules.

To determine whether distinct membrane re-modeling topologies are intrinsic properties of different ESCRT-III filaments, we compared the structures induced by spirals of CHMP1B with those induced by the prototypical ESCRT-III protein CHMP4A. In earlier studies, CHMP4 proteins only deformed the membrane when bound to ATPase-deficient VPS4B (17) or an activated CHMP2A mutant (18). We found that deleting C-terminal sequences yielded a mutant CHMP4A that formed tight, membrane-deforming spirals (Fig. 2.4A). The deformations induced by CHMP4A spirals were directed away from the cytoplasm, as expected (17, 18). Comparable views of cells transfected with full-length and C-terminal truncations of CHMP1B confirmed that CHMP4A and CHMP1B induced cellular membranes to tubulate in opposite directions (Fig. 2.4B and fig. S9).

We also tested how CHMP1B polymers bind and remodel liposomal membranes in vitro. Under physiological solution conditions, CHMP1B formed single- and double-stranded one-start helices and spirals around membrane tubules, with interstrand spacing of 4.7 ± 0.1 nm (Fig. 2.4C and figs. S10 and S11). Adding IST1 to CHMP1B-induced membrane tubules generated copolymeric helices that were structural analogs of the membrane-free IST1-CHMP1B assemblies described above, as judged by their interstrand spacing (5.1 ± 0.1 nm versus 5.2 ± 0.1 nm) and by the similarities of 2D class averages of the two assemblies (Fig. 2.4D and figs. S10 and S11). The positively curved membrane tubules within the protein coats could be visualized with negative staining of the CHMP1B and IST1-CHMP1B assemblies (Fig. 2.4, C and D, and fig. S10), in cryo-EM images, and in 2D class averages of the molecules along the tangential.
surface of the bilayer (fig. S11). Thus, CHMP1B and IST1 form external coats on positively
curved membranes in vitro and in cells.

Our analyses also provide insight into how ESCRT-III filaments can assemble conical spirals of
decreasing diameter, as might be required to draw membranes together to the fission point. In
addition to uniform 24-nm helices, we frequently observed conical spirals of membrane-
associated CHMP1B and IST1^{NTD}-CHMP1B (Fig. 2.4, C and D, and figs. S10 and S11), as well
as conical IST1-CHMP1B filaments assembled in the absence of nucleating vesicles (fig. S12).
One class of membrane-free IST1^{NTD}-CHMP1B cones was sufficiently common to be
reconstructed at low resolution, which confirmed that the cones are composed of the same
double-stranded filaments as those seen in the IST1^{NTD}-CHMP1B helices (Fig. 2.4D; figs. S1,
S11, and S12; and movies S5 and S6). Within the conical spiral, both the degree of filament
curvature and the lateral interactions between adjacent filaments varied continuously. Small
changes in filament curvature are likely accommodated by altering the angles of the “elbow” and
“wrist” joints. Larger changes could, in principle, be accommodated (or even driven) by
ratcheting the buttressing intersubunit interactions made by the “forearm” and “hand.” For
example, changing the connectivity from i+4 to i+5 would tend to straighten the CHMP1B
filament. The required continuum of differing lateral interactions in the IST1^{NTD}-CHMP1B
spirals is apparently accommodated by small shifts in ionic interstrand interactions, which is
plausible because the basic charges are distributed almost uniformly along one edge of the
double strand (Fig. 2.2, C and D). Wider IST1-CHMP1B spirals will tend to propagate toward
their preferred 24-nm diameter. At this diameter, the narrow lumen (∼10 nm) would force
internal opposing lipid bilayers (each ∼4.7 nm wide) to-ward hemi-fission (30, 31), potentially
providing a driving force for membrane constriction and fission. Last, we note that other
ESCRT-III subunits, such as CHMP4A, tubulate membranes in the opposite direction to that seen with CHMP1B. Such stabilization of negative, rather than positive, membrane curvature could be achieved simply by altering the intrinsic degree and direction of filament curvature while retaining an analogous membrane-binding surface and subunit connectivity.
2.5 Figures

Fig. 2.1. IST1NTD and CHMP1B copolymerized into helical tubes comprising polar, double-stranded helical filaments. (A) Electron cryomicrograph showing IST1NTD-CHMP1B tubes (white arrows) assembled by incubating equimolar IST1NTD and CHMP1B in the presence of polymer-nucleating small acidic unilamellar vesicles (SUVs; yellow arrows). (Inset) End-on view of a short IST1NTD-CHMP1B tube. Scale bars, 40 nm (A) and 20 nm (inset). (B) End-on view of the reconstructed IST1NTD-CHMP1B tube highlighting single subunits of IST1NTD (light green, outer strand) and CHMP1B (dark green, inner strand). (C) External view of the reconstructed helix with a highlighted IST1NTD subunit. (D) Internal cutaway view of the reconstructed helix, with a highlighted CHMP1B subunit. (E) Ribbon diagram of the modeled IST1NTD subunit (closed conformation). (F) Ribbon diagram of the modeled CHMP1B subunit (open conformation). (G) Secondary structure diagrams for closed IST1NTD (top), open CHMP1B (middle), and closed CHMP1B (bottom).
Fig. 2.2. CHMP1B opening, strand structure, and electrostatic surface potentials of the IST1NTD-CHMP1B assembly. (A) Superposition of the open and closed CHMP1B conformations. (B) Five interlocked CHMP1B molecules from the inner strand of the filament. (C) “Top-end,” electrostatic surface view of the IST1NTD-CHMP1B tube, highlighting the acidity of the CHMP1B inner strand (including Glu$^{130}$, Asp$^{131}$, Asp$^{147}$, Glu$^{152}$, Asp$^{155}$, Glu$^{156}$, and Asp$^{160}$) and the IST1NTD outer strand (including Asp$^{49}$, Glu$^{50}$, Glu$^{57}$, Glu$^{163}$, Glu$^{168}$, Glu$^{178}$, Asp$^{180}$, and Glu$^{186}$). (D) “Bottom-end,” electrostatic surface view of the IST1NTD-CHMP1B tube, highlighting the strongly basic characters of the CHMP1B inner strand (including Lys$^{3}$, Lys$^{87}$, Lys$^{94}$, Lys$^{101}$, Lys$^{107}$, and Lys$^{114}$) and the IST1NTD outer strand (including Lys$^{7}$, Arg$^{10}$, Lys$^{90}$, Arg$^{109}$, Lys$^{118}$, Lys$^{127}$, Lys$^{130}$, Lys$^{134}$, and Arg$^{137}$). (E) Exterior, electrostatic surface view of the IST1NTD-CHMP1B tube, revealing the modestly basic character of the IST1NTD outer strand. (F) Internal cutaway electrostatic surface view of the IST1NTD-CHMP1B tube, revealing the strongly basic character of the lumenal surface, contributed primarily by basic residues in CHMP1B helix 1 (arrows), including Lys$^{3}$, Lys$^{13}$, Arg$^{17}$, Lys$^{20}$, Lys$^{21}$, Lys$^{24}$, Lys$^{32}$, and Lys$^{35}$.
Fig. 2.3. CHMP1B and IST1 tubulated cellular membranes. (A) Survey view of the cytoplasmic surface of the plasma membrane in an unroofed COS-7 cell expressing FLAG-CHMP1B. Tubular invaginations extending into the cell interior are apparent along the exposed plasma membrane and as stabilized openings at the edges of the cell. Use view glasses for 3D viewing of anaglyphs (left eye, red). (B) Higher magnification view of tubular invaginations induced and coated by FLAG-CHMP1B filaments. (C) Immunodecoration confirmed the presence of CHMP1B around and along a tubule in a cell expressing untagged CHMP1B. Antibody detected with 12 nm gold is white in these contrast-reversed EM images. (D) Higher magnification view of FLAG-CHMP1B filament spirals on exposed plasma membrane. (E) CHMP1B immunoreactive organelle in an unroofed COS-7 cell expressing untagged CHMP1B. Antibody detected with 12 nm gold is white in these contrast reversed EM images; a representative gold particle is circled in blue. (F to I) Representative internal tubules from cells coexpressing untagged CHMP1B (12 nm gold; example circled in blue) and IST1-myc (18 nm gold; examples circled in red). (J and K) Clathrin-coated bud capping the end of (J) CHMP1B and (K) IST1-myc immunolabeled tubules from cotransfected cells. Measurements of filament diameter (and interstrand spacing) showed that when apparently unitary filaments were resolvable, their diameter varied from 5 to 10 nm, including platinum. These measurements are generally consistent with the dimensions of IST1-CHMP1B and CHMP1B filaments formed in vitro. Scale bars, 500 nm (A) and 100 nm (B) to (K).
Fig. 2.4. Topology of ESCRT-III membrane deformation in cells and in vitro. (A) Series of filament spirals on the plasma membrane of COS-7 cells expressing CHMP4A1-164 show development of the outwardly directed protrusions previously associated with ESCRT-III filaments (15, 16). Drawing highlights relationship between a CHMP4A conical spiral and a negatively curved plasma membrane tubule. (B) Series of filament spirals on the plasma membrane of COS-7 cells expressing FLAG-CHMP1B show development of invaginations directed into the cell. Drawing highlights relationship between a CHMP1B conical spiral and a positively curved plasma membrane tubule. (C) Negative stain electron micrograph showing that CHMP1B tubulates liposomes and forms a filamentous coat on the outside of the tubule. White arrows highlight regions coated by the CHMP1B helices, and the yellow arrow highlights a break in the coat where the internal lipid is visible. (D) Negative stain electron micrograph showing that the IST1NTD-CHMP1B copolymer forms on the outside of membrane tubules. White arrows highlight regions coated by the IST1NTD-CHMP1B copolymer, and the yellow arrows highlight breaks in the helical coat or uncoated regions of the liposome where the internal membrane is visible. Scale bars, 100 nm (A) and (B), 50 nm (C) and (D).
2.6 Materials & Methods

Plasmids:

pcDNA3.1-FLAG-CHMP1B (full length, 1-181, and 1-168) are as previously described (33). CHMP1B cDNA (NM_020412.4) was recloned into pcDNA4 to create untagged CHMP1B. CHMP4A(1-164) was amplified by PCR and recloned into pcDNA3.1 as described (34). IST1 isoform A (NM_001270975.1, NP_001257904.1) was subcloned into pcDNA4/TO following addition of EcoRI and XhoI sites to generate IST1-myc.

Cell culture and Transfection:
U2OS and COS-7 cells originally derived from ATCC were cultured in DMEM (Invitrogen) with 10% FBS (Atlanta Biologicals). Cells plated on coverslips (#1.5 for immunofluorescence, #1 poly-L-lysine coated BioCoat coverslips (BD Biosciences) for EM) were transfected with the indicated plasmid(s) using Lipofectamine 2000 (Thermo Fisher) following the manufacturer’s instructions and were used for experiments within 18–24 h. Plasmids used include pcDNA/TO IST1

Antibodies:
Antibodies used include rabbit polyclonal against CHMP1B (Proteintech, 14639-1-AP) and mouse monoclonal against c-myc (9E10, Developmental Studies Hybridoma Bank). Goat anti-rabbit and mouse secondary antibodies conjugated to Alexa 488 or 555 were from Molecular Probes (Thermo Fisher, Waltham MA) and the 12 or 18 nm gold were from Jackson ImmunoResearch (West Grove, PA).
Immunofluorescence Microscopy:
Cells were fixed and stained essentially as described (21). Widefield epifluorescence imaging was performed using an Olympus IX81 microscope with 60x 1.42 NA objective and FLASH 2.8 camera (Hamamatsu Photonics). Brightness and contrast adjustments were made using Fiji (69).

Deep-etch Electron Microscopy:
Samples were prepared as previously described (17, 18). Briefly, coverslips were washed in 30 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM CaCl2, dipped into an intracellular buffer (30 mM HEPES, pH 7.2, 70 mM KCl, 5 mM MgCl2, and 3 mM EGTA) and subjected to a brief pulse of ultrasound before transfer into the same buffer containing fixative (2% glutaraldehyde or 2% paraformaldehyde if immunostaining was planned). The area of coverslip with the highest yield of plasma membranes was identified by phase contrast microscopy and trimmed with a diamond knife to ~3 x 3 mm. Replicas were prepared as previously described (17, 18). Replicas were viewed on a JEOL 1400 transmission electron microscope at two different tilt angles (+/- 5°) and images were captured using an AMT XR111 camera. Digital image pairs were made into anaglyphs as described (70).

2.7 References
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31. Materials and methods are available as supplementary materials on Science Online.


Chapter 3: The role of IST1 and CHMP1B in “inverse” topology fission

3.1 Forward and Hypothesis
Building on the work described in the previous chapter, in which we found that filaments composed of ESCRT-III subunits CHMP1B and IST1 form extended tubules rather than invaginations on membranes (1), we set out to explore the cellular implications of this challenge to the central dogma of ESCRTs. In particular, the discovery that ESCRT-III function is not limited to a single topology expands the range of possible functions that ESCRT-III filaments can accomplish across the endosomal system and the work in this and the next chapter lays the groundwork for broadly defining and understanding the contributions of this fundamental membrane remodeling machinery.


3.2 Acknowledgments
I would like to thank again the members from the Hanson lab and those affiliated with Washington University that were foundational to this and the previous chapter. As some of the figures in this manuscript refer to images ultimately published in chapter 2, they are referred to in the previous chapter. Data included in figures in this chapter were contributed by Michael Skowyra (Fig. 3.1), Phyllis Hanson and Robyn Roth (Figs. 3.2, 3.4, S.3.2), Teri Naismith (Fig. 3.4, S3.9), and myself (Fig. 3.3, 3.4, S3.1, S3.3-3.10, Movies S1-3).
Sources of funding: R01GM076686 (P.I.H.), and R01NS050717 (P.I.H.); and NSF fellowship DGE-1143954 (A.K.C.).

3.3 Abstract
ESCRT-III filaments are established as the executors of membrane fission for intraluminal vesicle formation on endosomes and the topologically related processes of viral budding and cytokinesis. These filaments are thought to coil on the inside surface of membrane tubules, constricting them to drive membrane fission. Here we find that two ESCRT-III proteins – CHMP1B and IST1 – previously thought to play regulatory roles, instead form filaments that spiral around the outside or cytoplasmic surface of membrane tubules. CHMP1B and IST1 are present in puncta along endosomal tubules and live-cell imaging shows that depleting either one stabilizes elongated Rab5 endosomes consistent with a role in endosome fission. Parallel deficiencies in cargo sorting confirm that CHMP1B and IST1 are important for endosomal function but are not essential for membrane protein degradation. We conclude that “inverse ESCRT-III” spirals are attractive candidates for previously unsuspected roles in endosome fission.

3.4 Results & Discussion
Pioneering studies in yeast cells defined the ESCRT (Endosomal Sorting Complex Required for Transport) machinery as a set of interacting protein complexes that act in forming intraluminal vesicles (ILVs) inside endosomal multivesicular bodies (MVBs) (1). Evolutionary conservation and the discovery that ESCRTs are required for topologically equivalent processes including viral budding and cytokinetic abscission led to the now widely accepted concept that ESCRTs
are uniquely involved in membrane fission reactions that share this topology (1, 2). Recently described roles in plasma membrane repair, dendrite pruning, and nuclear envelope closure further expand the ESCRT machinery’s repertoire (3). Studies of which ESCRTs are needed for these different processes define varying requirements, indicating that the machinery is modular (4). Importantly, however, all reactions appear to converge on and require ESCRT-III polymers and the remodeling AAA+ ATPase Vps4 for fission.

While it remains unclear exactly how ESCRT-III filaments drive membrane fission, their activity is connected to assembly of filament spirals on the inside surface of constricting membrane necks (5-9). There are 7 structurally related ESCRT-III proteins in yeast and 12 in humans. A major roadblock to understanding ESCRT-III function has been defining individual role(s) for this large number of mostly non-redundant proteins. The clearest subdivision is between “core” (Vps20/CHMP6, Snf7/CHMP4, Vps2/CHMP2, and Vps24/CHMP3) and “regulatory” (Did2/CHMP1, Vps60/CHMP5, and IST1) ESCRT-III proteins, based on whether or not each is essential for MVB biogenesis in yeast (10-12). Core ESCRT-III proteins including the most abundant ESCRT-III protein CHMP4/Snf7 polymerize into filaments able to coil inside membrane necks or tubules, providing the topologic foundation for current models of ESCRT-III function (5, 6, 8). Regulatory ESCRT-III proteins are proposed to interact with the core proteins to modulate remodeling or disassembly by Vps4 (12-14). Notably, both core and regulatory ESCRT-III proteins are present throughout evolution indicating that all play functionally important roles.
Here we turned our attention to two regulatory ESCRT-III proteins, CHMP1B and IST1. Deleting their orthologs from yeast has little (Did2/CHMP1B) (15) or no (Ist1/IST1) (12, 14, 16) effect on MVB biogenesis and neither protein is required in mammalian cells for viral budding (4). Their roles in endosomal trafficking are incompletely defined, but IST1 is not required for EGF receptor degradation (17, 18) and has instead been connected to receptor recycling in mammalian cells (19) and to regulating plasma membrane protein turnover in yeast (20). Both CHMP1B and IST1 are required along with many other proteins for cytokinesis (17, 21) although their exact contributions remain to be defined.

Similar to other ESCRT-III proteins, CHMP1B assembles into homo-polymeric filaments and tubes in vitro (22, 23) but because it is not one of ESCRT-III proteins essential for characterized fission reactions, the functional significance of these polymers has not been explored. For insight into this, we turned to analyses in cells where we found that even moderately overexpressed CHMP1B formed rod-shaped and filamentous structures (fig. S3.1). Because these structures were frequently on or adjacent to the plasma membrane, we reasoned they would be amenable to visualization by deep-etch electron microscopy and prepared replicas for analysis. On inspection, CHMP1B filaments were immediately apparent along the plasma membrane (Fig. 2.3A). We were, however, surprised to see that while CHMP1B filaments were similar to those formed by the core ESCRT-III protein CHMP4A (5), the effect of these filaments on plasma membrane morphology was strikingly different. CHMP1B filaments induced and stabilized tubules extending into the cytoplasm (Fig. 2.3A,B) rather than the oppositely directed tubular protrusions seen with core ESCRT-III proteins (5, 9). Openings into CHMP1B-induced tubules were visible from the cell surface (Fig. 2.3A,B), confirming that they arise on both dorsal and ventral surfaces.
of the cell. Tubules were ~30-50 nm in diameter, and striations visible in side views suggested coating by fine CHMP1B filaments (Fig. 2.3B). Immunogold labeling localized CHMP1B both around and along the tubules (Fig. 2.3C), paralleling the distribution of filaments in undecorated samples. Importantly, at least some of the C-terminal MIM motifs needed for CHMP1B to engage MIT domains in known interacting proteins, including VPS4, are accessible as shown by efficient binding of coexpressed VTA1/LIP5 (Fig. 3.1A). Given an appropriate ratio of VPS4 or other ATPase, these filaments could thus be dynamically remodeled. En face views of the plasma membrane highlighted a range of CHMP1B spirals that are sometimes flat but more frequently feature a central deformation directed into the cytoplasm (Fig. 2.3D). To determine if something intrinsic to the filaments constrains how different ESCRT-III spirals deform the membrane, we wanted to compare spirals of CHMP1B and the core ESCRT-III protein CHMP4A. In earlier studies, CHMP4A only deformed the membrane when bound to ATPase-deficient VPS4 (5) or an activated CHMP2A mutant (9). Here we found that deleting C-terminal autoregulatory sequences (including α5 and α6) yielded a mutant CHMP4A that formed tight, membrane-deforming spirals (Fig. 2.4A). However, consistent with previous studies and different from CHMP1B spirals (Fig. 2.4B), central deformations in CHMP4A spirals were directed away from the cytoplasm (Fig. 2.4A). Deleting similar C-terminal sequences from CHMP1B did not change its oppositely directed topology (fig. S3.2). While unanticipated, the difference between conventional “internal coil” and inverse “external coil” ESCRT-III topology lies in whether the filaments associate with positively or negatively curved membrane surfaces (Fig. 2.41A,B).
Regardless of the orientation, the total area occupied by each filament spiral of overexpressed ESCRT-III is approximately constant while the length of the centrally emerging tubule is variable (Fig. 2.3 and (5)). This is consistent with a model in which the diameter of the tubule is indicative of the filament’s preferred curvature (24). In this paradigm, a central tubule arises when an expanding spiral on the membrane reaches a size at which buckling occurs to relieve filament strain at the perimeter. How subunits interact with each other to define filament curvature and orientation on the membrane remains to be established, but it is clear that a wide range of interactions within ESCRT-III filaments are needed to enable their diverse functions. Importantly, McCullough et al. provide the first high-resolution description of these interactions and demonstrate that CHMP1B assembles similarly on membranes in vitro (personal communication).

Cells disrupted to expose cytoplasmic membrane surfaces for deep-etch EM retain variable amounts of plasma membrane associated cytoskeleton and internal organelles (25). In replicas of cells transfected and immunolabelled for CHMP1B, we noted that in addition to labeling along the plasma membrane there were immunodecorated tubules deeper inside the cell including some that clearly emerged from larger vacuolar domains (Fig. 3.2A). Although partially occluded by immunodecoration, striations consistent with helical spirals of CHMP1B were recognizable on some of these tubules. The resemblance of decorated organelles to images of early endosomes (25, 26) along with the occasional presence of clathrin-coated buds (Fig. 2.3J) suggests that at least a subset of the CHMP1B decorated organelles correspond to endosomes.
Genetic studies in yeast suggest that the ortholog of CHMP1B, Did2, functions together with a second regulatory ESCRT-III protein IST1 (14, 16). Human IST1 also has a special relationship to CHMP1B based on their robust binding to each other and a shared but unique complement of binding partners including Vps4, the related AAA+ ATPase spastin, the deubiquitinase USP8, and others (17, 21, 27). We therefore wondered about the topology of IST1-containing polymers. Overexpressed full-length IST1 did not polymerize as readily as CHMP1B, and instead ranged from diffusely distributed to partially concentrated on internal structures (fig. S3.3). IST1-labeled structures were not readily apparent in proximity to the plasma membrane in replicas of disrupted cells (data not shown). Looking instead at detergent extracted whole cells we did, however, find occasional immuno-decorated tubules, indicating that IST1 also has a propensity to assemble with “inverse ESCRT-III” topology (Fig. 3.2B).

Based on its limited polymerization, we hypothesized that IST1 might – like many ESCRT-III proteins – be autoinhibited and depend on interacting factor(s) for efficient assembly. Consistent with this, we found that co-expressing IST1 with CHMP1B dramatically redistributed IST1 onto tubular and occasionally reticular structures (fig. S3.4). As expected based on this light microscopy, replicas of cells co-transfected and immunolabelled for CHMP1B and IST1 revealed striking decoration for both proteins along intracellular tubules (Fig. 2.3E-K). Interestingly, consistent with the pattern apparent by light microscopy, tubules in co-transfected cells were most frequently decorated with antibodies against one or the other protein (Fig. 2.3F-H) indicating a preference for homotypic interactions, although occasional tubules were immunoreactive for both (Fig. 2.3I). Overall, although the tubules varied somewhat in diameter, length, and eventual tapering, both CHMP1B and IST1 are clearly able to surround and perhaps
extend tubular structures in the cell. To confirm that organelles decorated by IST1 and/or CHMP1B are, at least in part, derived from endosomes, we transfected and immunostained Rab5BGFP expressing cells and found that a fraction of tubules decorated with co-transfected CHMP1B and/or IST1 coincide with structures marked by Rab5 (fig. S3.5).

To assess functional implications of these proteins’ propensity to assemble on cytoplasmic tubules with “inverse ESCRT-III” topology, we next explored the localization of endogenous CHMP1B and IST1. We found, as previously reported (17), IST1 both diffusely distributed in the cytoplasm and concentrated in puncta adjacent to or overlapping with endosomal compartments, with robust juxtaposition to Rab5 on the early/sorting endosome (fig. S3.6). We found that CHMP1B puncta were similarly juxtaposed with early endosomal compartments (fig. S3.6). As an additional marker to differentiate vacuolar and tubular domains of the early endosome, we used SNX1. SNX1 is a BAR domain containing sorting nexin associated with retromer and localized to Rab5 endosomes and in particular to associated tubules (28). We confirmed that SNX1 is associated with Rab5-labeled endosomes, and further found that IST1 and CHMP1B puncta localized with or adjacent to SNX1 (fig. S3.7). Additionally, a fraction of U2OS cells contain extended SNX1-labelled tubules under normal culture conditions. Strikingly, both IST1 and, particularly, CHMP1B puncta localized along, to breaks of, or to the ends of these SNX1-decorated tubules (Fig. 3A-D). This localization suggests that CHMP1B and IST1 filament spirals could participate in endosome remodeling and even endosomal fission. Notably, it has previously been reported that depleting IST1 leads to accumulation of abnormally long SNX1- and SNX4-labelled tubules in fixed cells, supporting involvement of IST1 in membrane fission (19).
To directly assess endosomal dynamics and in particular endosome fission, we developed a live cell assay to monitor changes in Rab5 endosome morphology focusing on the transient appearance of tubules which resolve by homotypic fission. We chose Rab5 as a marker because it is associated with early endosomes through which most cargo passes and that are known to undergo fusion and fission as they mature (29, 30). Rab5 endosomes typically appear as puncta by fluorescence microscopy and any tubular structures are typically short-lived. As shown (fig. S3.8), we were able to see tubules in cells stably expressing Rab5Bgfp and assess their length and duration. We predicted that if depleting IST1 or CHMP1B (fig. S3.9) slowed or otherwise impaired fission, this would be reflected in increased tubule length and/or duration. We quantified tubule length and duration, counting Rab5 tubules that remained longer than 5 mm for more than 2 seconds (fig. S3.10). The number of these tubules seen in each cell over 30 seconds is clearly increased after depleting CHMP1B or IST1 compared to control (Fig. 3.3E) and these tubules are also longer-lived (Fig. 3.3F). Importantly, despite the transient appearance of tubules, Rab5 labeled structures were otherwise indistinguishable from those in control cells in terms of overall size, number, and distribution. We conclude that depleting CHMP1B or IST1 delays homotypic fission of Rab5-labeled endosomes.

An obvious question is whether changes in Rab5 compartment dynamics are specific to depleting CHMP1B and IST1 or instead are a general and potentially indirect result of interfering with ESCRT machinery function in ILV biogenesis and accompanying defects in endosome maturation. We were unable to directly compare the effect on Rab5 endosomes of depleting the core ESCRT-III proteins CHMP4A/4B to that of depleting IST1 and CHMP1B because –
different from IST1 and CHMP1B – depleting CHMP4A/B led to global changes in the Rab5-labeled endosomal compartment which included the appearance of smaller and more numerous endosomes with a dispersed localization (supplemental movie S3). So while there are clear differences in the effects of interfering with known core components of the ESCRT machinery vs. those we found to adopt the “inverse ESCRT-III” external topology, we could not directly assess involvement of the core ESCRT machinery in endosome fission. We next turned to assessing more generally the effects of depleting different ESCRT-III proteins on cargo processing in the endosomal system.

If endosomal fission is impaired by depleting CHMP1B or IST1, we expect to see perturbations in the distribution, processing and/or turnover of proteins traversing the endosomal system that are independent of the cell’s general ability to degrade endosomal cargo. To assess this, we measured the steady state levels of various proteins by immunoblotting. Immunoblots of the lysosomal protease cathepsin D revealed that depleting CHMP1B, IST1, or CHMP4A/B impairs its processing (Fig 3.4A). This could result from inefficient sorting and recycling at the early/sorting endosome and/or impaired proteolysis in the late endosome/lysosome. To distinguish between these explanations, we wanted to directly assess the effects of depleting ESCRT-III proteins on membrane protein turnover. We found that U2OS cells plated in the absence of fibronectin maintain low levels of α5 integrin, consistent with constitutive rapid turnover. Depleting CHMP1B and/or IST1 did not change the amount of α5 integrin while depleting CHMP4A/B strikingly increased it (Fig. 3.4A). This indicates that the effects of depleting CHMP1B and IST1 on cathepsin D processing are not the result of globally impaired membrane protein degradation, and are instead attributable to impaired endosomal recycling.
Our unexpected finding that CHMP1B and IST1 filaments spiral around the cytoplasmic surface of endosomal tubules, similar to helical spirals of proteins responsible for well-studied membrane fission reactions such as dynamin at the plasma membrane, expands the range of processes in which ESCRT-III-like filaments and their associated AAA+ ATPases may function to include endosome remodeling and potentially endosome fission. Most of the CHMP1B and IST1 containing spirals that we saw on intracellular tubules were narrow – but not narrow enough to drive membrane fission. We speculate that interactions with known and unknown remodeling factors could drive further constriction and eventual fission (Fig. 3.4B). Indeed, the combination of membrane remodeling ESCRT-III filaments with AAA+ ATPases such as Vps4 may create a dynamin or FtsZ like membrane fission machine.

Importantly, the new roles we explore for IST1 and CHMP1B are not mutually exclusive with previously proposed function of these proteins as regulatory factors in heteropolymeric ESCRT-III assemblies operating with canonical ESCRT-III topology (12, 13). Differentiating involvement in MVB biogenesis from other perturbations in endosomal homeostasis is challenging. However, reexamining earlier studies provides support for the idea that these proteins participate in events other than ILV formation. IST1 is implicated in endosomal fission and recycling in human cells (19) and is reported to play no role in creating MVBs or delivering cargo to the vacuole in yeast (12, 14, 16). It does, however, regulate trafficking and turnover of plasma membrane proteins (20) and was independently identified in a genetic screen for transporter recycling (31). Yeast cells deleted of the CHMP1B ortholog Did2 contain abnormal vesicular tubular clusters in lieu of MVBs, indicative of perturbed endosomal organization but
potentially more consistent with a problem in organelle fission than in ILV biogenesis (15). How specific ESCRT-III proteins are recruited and assembled to create homo- or hetero-polymers with appropriate topology for different cellular functions will be an important question for the future.

Endosome fission is one of the least well-understood organelle remodeling events. Studies over the last several years have established an important role for the actin nucleating WASH complex as well as retromer associated proteins in endosomal fission (32). Beyond this, however, mechanistic insight is limited. It will be important to explore potential roles for inverse topology ESCRT-III spirals in these and other reactions.
3.5 Figures

Fig. 3.1 CHMP1B filaments create tubular membrane invaginations on the plasma membrane with “inverse ESCRT-III” topology. (A) Tubules from cell expressing CHMP1B together with GFP-VTA1/LIP5. Scale bar 100 nm.
Fig. 3.2 CHMP1B and IST1 on intracellular membrane tubules. (A) CHMP1B-immunoreactive organelle in an unroofed COS-7 cell expressing CHMP1B. Antibody detected with 12 nm gold is white in these contrast reversed EM images; a representative particle is circled in blue. Use view glasses for 3D viewing of anaglyphs (left eye = red). Scale bar 100 nm. (B) IST1-immunoreactive organelle in detergent extracted whole COS-7 cell expressing IST1-myc. Antibody detected with 18 nm gold; a representative particle is circled in red. Scale bar 500 nm.
Fig. 3.3 Role for CHMP1B and IST1 in endosome fission. (A-D) U2OS Cells fixed and stained for SNX1 and either CHMP 1B or IST1. (A) Endogenous CHMP1B puncta localize along SNX1 tubules, particularly at a tubule break (bottom panel shows magnification of region boxed in yellow). (B) Endogenous IST1 puncta localize along SNX1 tubules (bottom panel shows magnification of region boxed in yellow). (C and D) More examples (from different cells) of the conspicuous juxtaposition of CHMP1B and IST1 puncta along SNX1 tubules. All images are maximum intensity projections from 5 confocal slices except for (B), which is from a single plane. (E-G) Results from live cell experiments monitoring Rab5b-GFP dynamics. (E) Each dot in the scatter plots shows the number of tubules in a single cell longer than 5 µm and present for >2 sec over a 30 sec recording. Shown is comparison of depleting CHMP1B, IST1, or the unrelated protein LAP1. (F) Bar graph totals tubules counted in (E) and further shows how long each was present (tubule duration). Not only did CHMP1B and IST1 KD cells have more tubules, but only 5 tubules observed in control KD cells persisted for over 4 seconds while 43 and 14 tubules observed in CHMP1B and IST1 KD cells, respectively, persisted for over 4 seconds. (G) Representative single frames from live cell movies. Note the presence of long tubules (inset) in cells depleted of CHMP1B and IST1. Scale bars = 10µm
**Fig. 3.4 Cargo sorting defects in cells depleted of ESCRT-III proteins support speculative model of inverse ESCRT-III function.** (A) Immunoblots showing cathepsin D, integrin α5, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in whole cell lysates of U2OS cells depleted of CHMP1B, IST1, or CHMP4A and CHMP4B by siRNA as indicated. Blots are representative of three independent experiments. (B) Schematic model showing canonical ESCRT-III topology (grey) and proposed inverse ESCRT-III topology (red). Speculative role for inverse ESCRT-III filament spirals in membrane fission is shown in deep-etch EM views of two atypical filament assemblies seen along the plasma membrane in cells expressing CHMP1B and IST1. Scale bars 50 nm.
3.7 Supplemental Figures

Fig. S3.1. Overexpressed CHMP1B forms tubules proximal to the plasma membrane. (A) Tubular CHMP1B structures in a U2OS cell expressing untagged CHMP1B. Similar structures were commonly observed with moderate to high expression of untagged or FLAG-CHMP1B. (B) Another cell (left) with tubules next to a cell (right) in which CHMP1B remains diffuse. Relative expression differences suggest that efficient tubule formation is concentration dependent. Epifluorescence imaging, scale bar = 10 µm

Fig S3.2. Filament spirals formed by CHMP1B with C-terminal deletions. Filament spirals selected from COS-7 cells expressing FLAG-CHMP1B lacking a6 (CHMP1B 1-181) or α5 and α6 (CHMP1B 1-168) are indistinguishable from those of full-length CHMP1B shown in Fig. 1. Use view glasses for 3D viewing (left eye = red). Scale bar 100 nm.
Fig. S3.3. Distribution of overexpressed IST1. (A) Diffuse distribution of IST1myc in transfected U2OS cell. (B) Punctate and diffuse distribution of IST1myc in transfected U2OS cell. Epifluorescence imaging, scale bar = 10 µm.

Fig. S3.4. Coexpressed CHMP1B changes IST1 distribution. Image of U2OS cell cotransfected with CHMP1B and IST1myc shows dramatic redistribution of IST1 to tubular and reticular structures. Epifluorescence imaging, scale bar = 10 µm.
Fig. S3.5. Polymers of coexpressed CHMP1B and IST1 localize in part to Rab5 labeled endosomes. U2OS cell stably expressing Rab5bGFP, co-transfected with, and stained for, IST1myc and CHMP1B. Bottom panels show higher magnification of regions boxed in yellow. Arrows and arrowheads highlight the relationship of IST1 and CHMP1B with Rab5. Laser scanning confocal imaging, scale bar = 5 µm.
Fig. S3.6. Distribution of endogenous IST1 and CHMP1B relative to markers of the early/sorting endosome. (A) Immunostaining of endogenous IST1 in U2OS cells expressing Rab5Bgf. (B) Immunostaining of endogenous CHMP1 along with EEA1, CI-M6PR, and Rab5Bgf. Spinning disc confocal imaging, scale bar = 10 µm.
Fig. S3.7. Distribution of SNX1 relative to Rab5Bgfp, IST1 and CHMP1B in U2OS cells. (A) U2OS cells expressing Rab5Bgfp immunostained for SNX1. (B) U2OS cell immunostained for endogenous CHMP1B and SNX1. (C) U2OS cell immunostained for endogenous IST1 and SNX1. Spinning disc confocal imaging, scale bar = 10 µm.
**Fig. S3.8. Rab5Bgfp compartment dynamics.** Still images captured in an example time series monitoring Rab5bgfp endosome morphology. (A and B) The entire frame at 0 and 10 seconds time points, respectively. (C) Boxed region enlarged and shown at 2 second intervals. Note apparent fission events between 6 and 8 seconds, and also between 8 and 10 seconds. Spinning disc confocal imaging, scale bar = 10 µm.
Fig. S3.9. Effect of siRNA on targeted ESCRT-III proteins. Representative immunoblots demonstrating degree of depletion of the proteins as indicated.
Figure S3.10. Analysis of Rab5gfp compartment dynamics. Movies collected for analysis were each a total of 15 frames, with a frame taken every 2 seconds. (A-G) Image processing and analysis done for each frame of a movie (the first frame in Supplemental Movie 1 is shown as an example): (A) the original image, (B) the background determined with a rolling ball window size of 10 pixels, (C) the background subtracted image (B subtracted from A), (D) image cropped to contain single cell, (E) the image model produced from the SQUASSH workflow, range of pixel
values is 0 to 1, (F) a binary image created by setting the threshold to include the top 3% of pixel values, (G) ‘Tubule candidates’ or ROIs with an eccentricity greater than 0.7 and a major axis length greater than 20 pixels (5µm) shown in green. A blind scorer determined if a ‘tubule candidate’ was an actual tubule (boxed in red, to be scored) or a cluster of endosomes (not to be scored). (H-J) show results of A-G for all frames of Supplemental Movie 1. The first 4 rows of the montage show the areas of the boxed tubules identified in G, and each column is each area for a single frame of the movie. (H) The same montage from the SQUASH image model. (I) The montage from the green ‘tubule candidate’ ROIs. For final scoring, H and I were overlayed to produced (J) and identify ‘tubule candidates’ (any object that appeared green) which could then either be ignored or counted as a tubule by a blinded scorer. (J) Blue boxes outline the number of frames that a tubule 5µm or longer remained 5µm or longer. (K) Table shows the number of frames each tubule (labeled in J) remained 5 µm or longer. Note that any tubules lasting for a single frame (i.e. less than 2 seconds) were not counted in the final quantification in Fig. 3.

Supplemental Movies 1-3. (S1) Example movie of Rab5bGFP cell depleted of CHMP1B (scored in Figure S10) and (S2) a corresponding control cell. (S3) Example movie of Rab5bGFP cell depleted of CHMP4A/4B. Note the global change in morphology of the Rab5bGFP compartments which precluded analysis. Movies can be viewed at https://wustl.box.com/s/73driz2rf5umf84vkgxg10cjh16pd0zz4
3.6 Material & Methods

Plasmids

GFP-Rab5a, GFP-Rab5bGFP, and GFP-Rab5c were a kind gift from Dr. Philip Stahl.

pcDNA3.1-FLAG-CHMP1B (full length, 1-181, and 1-168) are as previously described (33).

CHMP1B cDNA (NM_020412.4) was recloned into pcDNA4 to create untagged CHMP1B.

CHMP4A(1-164) was amplified by PCR and recloned into pcDNA3.1 as described (34). IST1 isoform A (NM_001270975.1, NP_001257904.1) was subcloned into pcDNA4/TO following addition of EcoRI and XhoI sites to generate IST1-myc.

Cell culture and Transfection

U2OS and COS-7 cells originally derived from ATCC were cultured in DMEM (Invitrogen) with 10% FBS (Atlanta Biologicals, Atlanta GA). Cells were transfected with the indicated plasmid(s) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and were used for experiments within 18–24 h. Stable cell lines expressing GFP-Rab5 constructs were generated by selection in 500µg/mL Geneticin (Invitrogen).

Antibodies

Antibodies used include rabbit polyclonals against Integrin α5 (Santa Cruz, sc-10729), IST1 (Proteintech, 19842-1-AP), CHMP1B (Proteintech, 14639-1-AP), LAP1 (35), CHMP4A (36), CHMP4B (37) and mouse monoclonals against EEA1 (BD Transduction Labs, 610456), SNX1 (BD Transduction Laboratories, 611482), Cathepsin D (BD Transduction Laboratories, C47620), and GAPDH (Millipore, MAB374). Goat anti-rabbit and mouse secondary antibodies conjugated
to Alexa 488, 555, and 647 were from Molecular Probes. 12 and 18 nm gold-conjugated goat anti-rabbit or anti-mouse antibodies were from Jackson Immunoresearch.

siRNA treatment

All siRNA duplexes used have been previously validated as indicated below. Successful knockdown was verified as shown in fig. S9. The following sequences and concentrations were used:

IST1 (50nM) 5′-GCAAAUACGCCUUUCUCAUdTdT-3’ (19)
CHMP1B (25nM) 5′-UGGACAAAUUCGAGCACCAdTdT-3’ (38)
CHMP4A (25nM) 5′-AAGUAUGGGACCAAGAAUAdTdT-3’ (38)
CHMP4B (25nM) 5′-CGAUAAAGUUGAUGAGUUAdTdT-3’ (38)
LAP1 (25nM) 5′-CGUCUUUCCUCUAGUACUA-3’ (35)

All siRNA duplexes were synthesized by Dharmacon. Cells at ~70% confluence in 35 mm dishes were transfected with indicated siRNA duplexes using DharmaFECT#1 according to manufacturer guidelines. Cells were re-plated for imaging in No. 1.5 glass bottom dishes (MatTek Corp.). Cells were imaged or harvested for immunoblotting 62-71 hrs after transfection (CHMP1B, CHMP4A/B) or 101-113hrs after transfection (IST1, LAP1 control). For combined depletion of CHMP1B and IST1, cells were first transfected with IST1 siRNA and then 48 hrs later with CHMP1B siRNA 48hrs later.

Immunofluorescence Microscopy

Cells were fixed and stained essentially as described (39). Spinning disk confocal imaging was performed on an Olypmus 1X81 microscope using a 60x 1.42NA objective, and images were
captured by a Cascade:512B digital imaging system (Photometrics). Laser scanning confocal imaging was performed on a Nikon A1Rsi confocal microscope using a 100x 1.45 NA objective. Widefield epifluorescence imaging was performed using a 60x 1.42 NA objective and a FLASH 2.8 camera (Hamamatsu). Maximum intensity projections as well as brightness and contrast adjustments were made using Fiji.

Immunoblotting

Whole cell lysates were quantified and subjected to immunoblotting as described (33). Imaging was on a ChemiDoc MP imaging system (BioRad).

Deep-etch EM (electron microscopy)

Samples were prepared as previously described (5, 9). Briefly, coverslips were washed in 30 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM CaCl2 and then dipped into an intracellular buffer (30 mM Hepes, pH 7.2, 70 mM KCl, 5 mM MgCl2, and 3 mM EGTA) and subjected to a brief pulse of ultrasound before transfer into the same buffer containing fixative (2% glutaraldehyde or 2% PFA if immunostaining was planned). To extract fixed samples with detergent (Fig. 2B), fixed coverslips were incubated for 2 hr in buffer containing 1% Triton X-100 and 0.1% saponin. The area of coverslip with the highest yield of plasma membranes was identified by phase contrast microscopy and trimmed with a diamond knife to ~3 x 3 mm. Replicas were prepared as previously described (9). Replicas were viewed on a JEOL 1400 transmission electron microscope at two different tilt angles (+/- 5°) and images were captured using an AMT camera. Digital image pairs were made into anaglyphs as described (9).

Live Cell Microscopy
During imaging, cells were incubated in a Tokai Hit live cell chamber at 37°C with 5% CO2. Movies of GFP-Rab5b cells were acquired using a Yokogawa CSU-10 spinning disc confocal system using a 488 nm laser, a 60x 1.42NA objective and a Cascade:512B digital imaging system (Photometrics). 33ms exposures were collected every 2s over a total of 30 sec. In order to quantify tubule length and duration, it was necessary to segment Rab5bgfp positive endosomes in each frame of the movies. We used a model-based segmentation algorithm (40) implemented in the Fiji plugin Squassh (41) to generate a segmentation mask. As described (41), the segmentation mask assigns each pixel a value (0-1) based on its probability of belonging to an object and is the result of simultaneous image denoising, deconvolution, and segmentation. Using Squassh, background was subtracted from the original image with a rolling ball window of 10 pixels. Segmentation was determined using a theoretical PSF model calculated for our spinning disk microscope, a regularization parameter of 0.05, and a Poisson noise model. The next steps in image processing were then done using MATLAB (MathWorks). The resulting segmentation mask was converted into a binary image by thresholding to include only the top 3% of pixel values (the top 3% of pixels most likely to belong to an object). ROIs were determined from connected thresholded regions, and only ROIs with an eccentricity of greater than 0.7 and a major axis length of greater than 20 pixels (5µm) were considered, empirically chosen to eliminate the majority of ROIs corresponding to non-tubular or short tubular endosomes. Note that this length criterion provides a conservative estimate of tubule length as tubules that are not linear (i.e. bent or curved) and are actually longer than 5µm are excluded. A blind scorer then determined if the remaining ROIs, or ‘tubule candidates’, were actual tubules (to be scored) or a cluster of endosomes (not to be scored) for every frame analyzed. See fig. S10 for further details and example of quantification.
3.8 References

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Chapter 4: The ESCRT-III related protein IST1 functions as an adaptor to regulate endosomal trafficking together with SNX15 and CHMP1B

4.1 Forward and Hypothesis
Work in the previous chapter demonstrated a potential role of IST1 and CHMP1B in fission, with data similar to that published at around the same time and again a year or so later by others (1, 2). We set out to determine what endosomal pathways required the function of IST1 and CHMP1B and how IST1 and CHMP1B related to other trafficking machinery such as retromer and the WASH complex.

We subsequently found that in addition to its effects on fission, CHMP1B but not IST1 had a role in degradative sorting, similar to Did2/Vp46 in yeast, and so we focused our experiments on studying the role and relationships of IST1 in trafficking of specific cargoes. Since the initial publication by Reid and colleagues (1), they went on to establish a role for spastin and IST1 in generating specific ER contact sites (2). They documented, similar to our observations in Chapter 3, a juxtaposed localization of IST1 to SNX1 tubules and impaired fission of SNX1 tubules from endosomes following depletion of IST1. While our results establishing contributions of IST1 and CHMP1B to Rab5-labeled tubule fission was similar to their reports, Rab5 has been reported to exclusively localize to the vacuolar domain of endosomes (3, 4)—making the physiological relevance of impaired scission of Rab5 tubules and what, if any, cargo trafficking this facilitated unclear. Reid and colleagues also linked their observed defect in SNX1
tubule fission with a deficiency in the retrograde trafficking of M6PR from the PM to the Golgi (2). Overall, we concluded that additional work was needed to fully understand and extend the observations made in Chapter 3 and by Reid and coworkers (1, 2).

We selected TfnR as a cargo to study, as its trafficking is restricted to recycling back to the PM, though its recycling is also known to be mediated by retromer- and clathrin-associated processes. TfnR is also a well-known marker of endosomal recycling tubules, and is amenable to kinetic assays using labeled Tfn to assess the trafficking of endogenous TfnR. While Reid and colleagues had reported that loss of IST1 leads to increased degradation of TfnR (1), this was clearly not the case in the cell types we used—highlighting challenges in understanding and generalizing cargo-sorting defects seen in different cell types, particularly in the absence of clear mechanistic insight into what pathways IST1 effects.

What became clear was that IST1 was on a distinct endosomal domain, that unlike WASH, did not overlap with the retromer/SNX-BAR cargo retrieval domain. We therefore set out to define when and where IST1 acted in the trafficking of endocytosed TfnR, and also to identify other IST1-interacting proteins that are present on endosomes and their relationship to other factors implicated in endosomal sorting including clathrin and branched actin. These studies uncovered a new IST1-SNX15 marked microdomain on early endosomes and explored the role of these proteins in transferrin recycling. We conclude that IST1 acts alternately with CHMP1B and SNX15 to promote different aspects of plasma membrane cargo recycling.


### 4.2 Acknowledgments

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### 4.3 Abstract

ESCRTs (Endosomal Sorting Complex Required for Transport) are a modular set of with membrane remodeling activities that include the formation and release of intralumenal vesicles (ILVs) to generate multivesicular endosomes. Among 12 ESCRT-III proteins most have established roles in ILV formation, but the cellular roles of IST1 remain elusive. We found that IST1 colocalizes and interacts with the MIT and PX domain containing SNX15 previously implicated in early endosomal recycling. Kinetic and spatial examination of endosomal protein recruitment showed that SNX15 and IST1 occupy an endosomal microdomain distinct from that
marked for recycling by retromer or degradation by HRS. Proximity biotinylation studies using IST1 and SNX15 established that both proteins associate with a common set of factors involved in endosomal recycling including clathrin and branched actin regulators, while IST1 has additional unique partners that bind uniquely via ESCRT-III and MIT domain interactions. Using live-cell microscopy we find that SNX15 and CHMP1B alternately control IST1 distribution and its relationship to clathrin and branched actin—as well as how IST1 effects the distribution of transferrin-positive recycling tubules within the cell. Using the transferrin receptor as an endosomal cargo, we show that IST1 is a multi-functional protein that regulates sorting and trafficking from the early/sorting endosome to the tubular endocytic recycling compartment (ERC) present in the perinuclear region. Loss of IST1 impairs the sorting of Tfn by this pathway and leads to its accumulation in abnormal peripheral endosomes that are positive for the endosomal clathrin adaptor AP-1. These results demonstrate that IST1 is a multifunctional adaptor involved in clathrin-based recycling on the endosome either by interacting with clathrin-binding SNX15 or endo-tubule membrane constricting CHMP1B filaments—and possibly functions with other actin-based sorting mechanisms on the endosome.

4.3 Results & Discussion

4.3.1 IST1 localizes and functions on early endosomes
ESCRT function in the endosomal system is generally associated with degradative trafficking, with roles in sorting cargo as well as generating intraluminal vesicles (ILVs) that ultimately enable lysosomal degradation. In preliminary studies described in the previous chapter, we observed distinct effects of depleting IST1 vs. the “core” ESCRT-III CHMP4B on the trafficking of representative endosomal cargos (Fig. 3.4, previous chapter), suggesting that loss of CHMP4 but not IST1 impaired sorting into ILVs. As degradative cargos are generally marked by
ubiquitination, we used an antibody that recognizes mono- and poly-ubiquitinylated proteins (FK2) to examine how depleting CHMP4B and IST1 affects the distribution of ubiquitinated proteins within the cell. As expected for its role in degradative sorting, depleting CHMP4B led to a dramatic increase in FK2 staining on swollen, vacuolar structures in the cytoplasm (Fig. 4.1A arrow), while depleting IST1 had little to no effect on normal, predominantly nuclear, FK2 staining (Fig. 4.1A). This confirmed that unlike CHMP4B, IST1 is not required for normal ubiquitinylated cargo processing.

Due to the unclear role of IST1 on the endosome, we set out to better define its distribution and function within the mammalian endosomal system in two cultured human cell lines, T24 and U2OS. An antibody recognizing endogenous IST1 confirmed its localization throughout the cytoplasm as well as its punctate accumulation on organelles in the perinuclear region and on cell bridges and midbodies of both cell lines, consistent with previous reports in HeLa cells (1, 2) (Fig. 4.1B, Sup Fig. 4.1). IST1 localizes to early endosomes in HeLa cells (1, 3), and the perinuclear organelles labelled with IST1 in T24 cells were correspondingly frequently stained with EEA1 (Fig S4.1). The relatively large size of endosomes in T24 cells allowed us to resolve protein distribution on individual organelles without the need for swelling (Fig S4.1-2). We decided to use a kinetic pulse assay to assess the temporal recruitment and spatial localization of IST1 on early endosomes. A pulse of fluorescently labeled transferrin (Tfn) mixed with epidermal growth factor (EGF) can be used to monitor distinct and divergent trafficking pathways into and out of early endosomes (4–7). While the duration of the pulse used can vary, we found that a 30 second pulse, similar that utilized in (5), was sufficient to resolve and track
endocytosed EGF and Tfn in EEA1- and, surprisingly, IST1-labelled endosomes with as little as 2-3 minutes of chase (Fig 4.1C-D).

Temporally, the recruitment of IST1 to vesicles/organelles containing endocytosed EGF is more similar to that of the ESCRT-0 protein HRS than that of the ESCRT-III protein CHMP4B, with HRS reported to co-localize with EGF within 2-5 minutes after endocytosis, while CHMP4B only began to co-localize at 5 minutes and peaked after 15 minutes following endocytosis (8, 9). EGF and its receptor are sorted into ILVs for degradation, and a single wave of ESCRT recruitment (HRS followed by CHMP4B) correlated with formation of a single ILV containing EGFR (9). In contrast, Tfn and its receptor are mainly recycled, allowing for the continued uptake and delivery of iron, although like all membrane proteins TfnR can be ubiquitinylated, sorted into ILVs, and degraded (10, 11). Loss of IST1 has been previously shown to increase degradation of TfnR in HeLa cells (12), but we found that depleting IST1 in T24 cells did not have this effect (Fig S4.3). The effects of depleting IST1 on TfnR trafficking may be cell type specific, as has been noted for the many factors regulating TfnR trafficking (13). However, TfnR is known to be sorted into tubule protrusions from early endosomes, and we noted that on some of the larger endosomes in T24 cells we could see that EEA1 was not present homogeneously on the surface, and in fact, Tfn and IST1 localized to domains lacking EEA1 and, occasionally, overlapped (Fig 4.1C-D, Fig S4.1).

Based on this spatial and temporal localization of IST1, we decided to determine if, and if so how, IST1 regulates the trafficking of TfnR and EGFR. We depleted T24 cells of IST1 using siRNA (Fig. S4.3) and monitored the distribution of a 30 second cargo pulse containing both Tfn
and EGF as a function of time. We found that both EGF (Fig. 4.1E) and Tfn (Fig. 4.1F) normally moved from being dispersed throughout the cell after 2 min to a more clustered localization in the juxtanuclear region after 4 min, and this was substantially impaired in cells deplete of IST1 (Fig. 4.1E-F and Fig. S4. 4). However, we found that there was no clear difference in the amount of Tfn and EGF internalized (FigS4.5), indicating that the deficiency observed in cargo trafficking in IST1 depleted cells is due to post-endocytic sorting processes.

Endocytic vesicles undergo heterotypic fusion with early endosomes marked by the Rab5 effector EEA1. As EEA1 is only found on early endosomes that contain PI3P (and not on clathrin coated vesicles (CCVs)), the coincidence of Tfn and EGF with EEA1 indicates successful fusion of endocytic vesicles with early endosomes. We looked at the distribution of EEA1 to ask if the stalling of cargo in the periphery in IST1 depleted cells is due to impaired fusion of endocytic vesicles with early endosomes. We noted that the total concentration of EEA1 on endosomes was increased in IST1 depleted cells (Fig. 4.1G), indicating that some aspect of sorting or maturation on early endosomes was impaired. Tfn did, however, reach EEA1 containing endosomes with similar kinetics whether or not IST1 was present (Fig. 4.1H).

Another possible explanation for decreased juxtanuclear cargo concentration could be impaired microtubule organization and function. To assess this, we checked the organization and distribution of the Golgi apparatus as another organelle that depends on microtubules. We found that depleting IST1 had no effect on Golgi organization, as assessed by TGN associated protein p230/Golgin-245 or the TGN transmembrane protein TGN46, both of which remained clustered.
in the perinuclear region (Fig. S4.6). This suggests that the overall organization of microtubule dependent processes is not affected by loss of IST1.

We did note that IST1 depleted cells had an increased concentration of p230/Golgin-245, a protein known to be recruited to the TGN by the Rab GTPase ARL1 (Fig. S4.6) This may parallel the increase in EEA1 on endosomes, and could imply a relationship between IST1 and release of small GTPases from both endosomal (Rab5) and secretory (ARL1) membranes. IST1 has also been reported to affect the trafficking of the mannose-6-phosphate receptor (M6PR) (3). M6PR has a complicated trafficking itinerary which includes anterograde export from TGN to endosomes, as well as the plasma membrane, and retrograde transit back to the TGN. Similar to what others have seen, we found that cells depleted of IST1 contained abnormally dispersed M6PR (Fig S4.7A). We also compared the localization of M6PR and EEA1, and found that while tubules of M6PR could be seen originating from EEA1 endosomes in both IST1 depleted and control cells, clear overlap of M6PR and EEA1 on vacuolar endosomes was apparent only in IST1 depleted cells (Fig S4.7B). As M6PR trafficking from the TGN or from the PM are the only ways to track M6PR kinetically (via the RUSH system or M6PR antibody uptake), M6PR sorting from the endosome can only be assessed by steady state immunostaining (Fig. S4.7).

Due to the complexities of M6PR retrograde trafficking pathways from the endosome, and the possibility that IST1 depletion impairs function at the TGN (i.e. increased p230 recruitment) as well as the endosome, we did not investigate the trafficking of M6PR further.

As TfnR recycles through the endosomal system and is readily visualized using labeled Tfn, we sought to better understand how IST1 contributes to endosomal sorting and Tfn trafficking. We
(Chapter 2) and others (3, 14, 15) have proposed that IST1 may be involved in or even directly mediate the fission of endosomal tubules involved in recycling, based on its ability to co-assemble with the ESCRT-III protein CHMP1B to promote constriction of membrane tubules. IST1, like other ESCRT-III proteins, has C-terminal “MIM” motifs that bind to MIT domains to mediate interaction with a variety of other factors including the AAA ATPase VPS4A/B that mediates ESCRT-III disassembly and deubiquitinases (DUBs) involved in endosomal ubiquitin dynamics. Different from other ESCRT-III proteins, IST1 has two MIM motifs (Fig. 4.2D) that interacts with a unique set of MIT-domain containing proteins, including calpain 7 (via MIM1 & MIM2) (2) and spartin (via MIM1) (16). Curiously, a MIT domain containing protein, sorting nexin 15 (SNX15), is reported to play a role in EGF trafficking to perinuclear endosomes, similar to what we found above for IST1. Interestingly, despite predictions that SNX15 would interact with ESCRT-III proteins via its MIT domain, no such interactions were observed in previously reported yeast two-hybrid analysis (17). Notably, however, IST1 was not tested. We next set out to see if IST1 interacted with SNX15.

4.3.2 IST1 associates with sorting nexin 15
Sorting nexin 15 is recruited to endosomal membranes containing phosphatidyl-inositol-3-phosphate (PI3P) via its PX domain and is also known to bind clathrin using a nonstandard clathrin box motif (17, 18) (Fig. 4.2D). It may additionally bind phosphatidyl-inositol-3,4-bisphosphate (PI(3,4)P2) (19). SNX15 has a C-terminal MIT domain, which shares ~30% homology with MIT domains in VPS4A/VPS4B. To ask if IST1 interacts with SNX15 we used co-overexpression in transfected U2OS cells to assess potential interaction in the context of cellular membranes. In agreement with previous observations using yeast two-hybrid analyses,
we found that all ESCRT-III proteins do not coassemble with SNX15 (data not shown), however, IST1, which was not previously tested, shows striking colocalization with SNX15 (Fig. 4.2A-C). Importantly, we found that internalized Tfn was present within SNX15 and IST1 decorated endosomes, as well as within tubules protruding from these endosomes (Fig. 4.2C). Over expressed SNX15 and IST1 showed nearly perfect co-localization (inset Fig. 4.2C).

If SNX15 helps bring IST1 to the membrane, we predicted that its overexpression would promote recruitment of endogenous IST1 to the endosome. Indeed, overexpressing SNX15 lead to a striking redistribution and recruitment of IST1 (Fig. 4.2B). IST1 accumulations, both in control and SNX15 over expressing cells, were more readily visualized after fixation with methanol and BS$_3$ (20) than with paraformaldehyde (Fig S4.8). The ability of SNX15 to recruit IST1 to the membrane presents a new mechanism for bringing this distinctive ESCRT-III like protein to the endosomal membrane, which may be ESCRT-independent.

Notably, while IST1 shares structural homology to other ESCRT-III proteins, it interacts with only a subset of them (CHMP1A and CHMP1B (21)) and how these interactions compare with those between other ESCRT-III proteins remains to be fully defined. Current understanding of ESCRT-III function involves a soluble and auto-inhibited or “closed” state, that upon interaction with an upstream activating factor (ESCRT-I/II in the canonical pathway) is relieved of auto-inhibition and “opened” to allow polymerization and filament assembly on the membrane. High resolution structures of CHMP3, IST1, CHMP1B and Snf7/shrub/CHMP4 filaments have defined “closed” and “open” conformations. Notably, however, IST1 polymerized either alone or in conjunction with CHMP1B has only been found in the “closed” conformation (22–24).
To determine how IST1 polymerization affects its engagement with SNX15, we tested if removing its core “ESCRT-III” domain required for polymerization affected assembly with SNX15 (Fig. 4.2D). We found that the C-terminal fragment (CTD) of IST1 lacking its “ESCRT-III” core was still recruited to domains of over-expressed SNX15 on the endosome, but to a lesser extent than full length IST1 (Fig. 4.2E). Based on this, we conclude that the interaction of IST1 with SNX15 is at least in part mediated by a sequence within its CTD of IST1 (consistent with a possible MIM-MIT interaction) and that IST1 polymerization enhances interaction with SNX15. Future studies will be required to determine if IST1-SNX15 coassembly is affected by conformational differences in either protein.

To more specifically define motifs responsible for binding between these two proteins, we deleted the C-terminal MIM1 motif from the IST1 CTD fragment and found that this abolished SNX15-mediated recruitment of this fragment to endosomes (Fig. 4.2E). Conversely, we removed the MIT domain from SNX15 and found that this reduced the ability of SNX15 to deplete soluble IST1mApple from the cytoplasm (Fig. 4.2F). These changes establish that an IST1 MIM1-SNX15 MIT domain interaction provides a novel mechanism by which IST1 can be recruited to PI3P containing early endosomes.

### 4.3.3 IST1 and SNX15 localize to an endosomal domain that is distinct from HRS
We noted that SNX15 seemed similar to HRS in that both bind PI3P and clathrin, localize to newly formed endosomes, and potentially recruit or engage other ESCRT machinery. While HRS is a key component of ESCRT-0 with a known role in ESCRT-mediated degradation, it also
has been found to play a role in recycling of some cargos (25–28). As the diversity of machinery involved in sorting on early endosomes expands, the concept of segregating cargo into distinct but potentially interrelated microdomains has emerged as a framework to explain how cargo fate can be controlled (29, 30). HRS and SNX15 have been reported to localize to the same early endosomes, but the precise relationship between them, and between them and IST1, has not been explored. Furthermore, given its apparent lack of involvement in cargo sorting for degradation, the relative role of IST1 within the hierarchy of the ESCRT pathway needs to be clarified.

Despite their presence and function on newly formed endosomes proximal to the cell surface, the predominant localization of HRS and SNX15 (as well as IST1) is to endosomes clustered in the juxtanuclear region of the cell. In T24 as well as U2OS cells, we found that IST1 appeared to co-localize better with SNX15 than with HRS (Fig. 4.3A), although the predominant cytoplasmic IST1 signal made correlation methods of co-localization uninformative. In addition, the poor spatial resolution on centrally clustered endosomes made it difficult to compare the relative localization of endogenous IST1 with other endosomal proteins including EEA1, SNX15, and HRS with any quantitative detail.

In order to robustly quantify the relationship between IST1 and other endosome-associated proteins, we wanted (1) a cargo-independent endocytic marker and (2) a way to disperse endosomes normally clustered around the nucleus. As the clustering of endosomes is mediated by microtubules, depolymerizing microtubules with nocodozole (NOC) leads to dispersal of endosomes thought the cell and enables robust quantitation on isolated single endosomes (31, 32).
To obtain kinetic resolution, we turned to the lectin wheat germ agglutinin (WGA) which binds glycoconjugates (including broadly distributed sialic acid and N-acetylglucosamine (GlcNAc) moieties) and is commonly used to label the plasma membrane (33), and robustly labels newly forming endosomes (34).

We confirmed that fluorescently labelled WGA delivered into NOC-treated cells would allow us to spatially isolate a population of endosomes containing recently endocytosed material in U2OS cells. As outlined schematically in Fig. 4.3B, cells were incubated with WGA for 30 min on ice, and then immediately fixed or allowed to internalize bound WGA for 5 min at 37°C (Figure 4.3C). Internalized WGA was clearly detected above background signal after 5 min and partially overlapped with EEA1 (Figure 4.3C, insets). For quantitation, we reduced plasma membrane PM associated WGA by washing with an excess of unlabeled GlcNAc at 4°C after uptake intervals at 37°C (Figure 4.3B, cell shown in Fig. 4.3D), which then enabled us to automate delineating WGA-containing puncta (regions of interest, ROIs) (Fig. 4.3D). We could then compare immunostained signals within individual ROIs to define the spatial relationship to each other on the endosome. We noted that not all EEA1-marked endosomes acquired WGA in this time period (Fig. 4.3D filled arrow heads), and when quantified approximately 60% of the total EEA1 positive endosomes area contained WGA. Conversely, not all WGA labelled endosomes contained EEA1 (Fig. 4.3D empty arrow head, Fig. S4.9A). Notably, IST1 localized to both EEA1-positive and -negative endosomes in these 5-10 min kinetic experiments (Fig. 4.3D). Analysis of the relative distribution of IST1 with HRS and SNX15 revealed that these are present together on a subset of kinetically early endosomes (Fig. 4.3E). Importantly, the localization of IST1 to an EEA1-negative domain was apparent, indicating that this assay yields endosomes
large enough resolve domains (Fig. 4.3D-E). We noted that IST1 also did not appear to co-localize with HRS on kinetically defined early endosomes, showing the same relationship noted at steady state. However, IST1 was co-localized with SNX15.

Building on these observations, we wanted to quantitatively interrogate the relationships among these proteins on WGA-containing endosomes, asking about the correlation or co-distribution among signals. A commonly used approach for assessing correlation in light microscopy is to determine if there is a linear relationship between the intensity of the two signals within each pixel (i.e. Spearman’s rank coefficient or Pearson’s Correlation Coefficient). This pixel based correlation is less sensitive to signals that exhibit a correlated, but juxtaposed, relationship—such as IST1 with EEA1 or HRS, as we could observe non-overlapping, but adjacent signals that were on the same endosome. These pairs of signals exhibited poor pixel-based correlation compared to that of IST1 and SNX15 (Fig. 4.3F, x axis). Note that the Spearman’s rank coefficient is shown, but similar results were obtained using Pearson’s correlation analysis (Fig. S4.9B). When we instead assessed the correlation of mean intensities between the two signals within each WGA ROI (on the same endosome), we found that both IST1/EEA1 and IST1/HRS were well correlated (Fig 4.3F y axis). We also found that while IST1 and SNX15 signals were largely co-localized at 5 minutes, they exhibited both pixel-based and ROI-based correlation at 10 minutes. This demonstrates that while IST1 and SNX15 signals are co-localized on endosomes after a 5 minute uptake, they exhibit both co-localized and juxtaposed relationships at 10 minutes. We also observed increased SNX15 staining within WGA ROIs after a 10 minute uptake (Fig S4.9A). Of note, two isoforms of SNX15 have been reported: the one represented in Figure 4.2D,
and an isoform that lacks the C-terminal MIT domain. Differential engagement of these isoforms could impact IST1 recruitment, generating domains marked only by SNX15.

While we found that IST1 did not co-localize with HRS, a marker of the degradative domain, it also did not co-localize with a marker of the retromer and actin-containing retrieval/recycling domain, VPS35. Comparing VPS35 to EEA1, HRS, and SNX15, revealed that VPS35 had the same juxtaposed relationship to HRS and SNX15 (Fig. S4.10)—confirming other reports that HRS is adjacent to VPS35 and the WASH complex (28, 30) and IST1 is adjacent to SNX1 (3).

To directly examine the relation of these three domains with each other, we immunostained U2OS cells stably expressing low levels of SNX15GFP with antibodies recognizing HRS and another marker of the retrieval/recycling domain, a component of the WASH complex FAM21. We again saw striking juxtaposition of HRS and FAM21, and it was clear that SNX15, as well as IST1, localized to a third district domain (Fig. S4.10), that is distinct from HRS and the WASH complex. Taken together, these studies define what appear to be three separable protein domains on early endosomes. This extends earlier characterizations of retrieval/recycling vs. degradative domains (30, 35) with addition of a third domain uniquely characterized by the presence of IST1 and SNX15.

4.3.4 Microscopy and proximity labeling indicate that SNX15 and IST1 are close to actin and clathrin-coat accessory proteins on the endosome.

Having established that SNX15 and IST1 together define what appears to be a new microdomain on early endosomes distinct from previously characterized retrieval and degradative microdomains (29), we set out to understand the composition of this domain. To develop a comprehensive overview of proteins within a ~15 nm radius of these markers, we fused the promiscuous BiolID2 biotin ligase (36) to the C-terminus of IST1 and SNX15 and generated
U2OS cell lines stably expressing each fusion protein. We used mass spectrometry to identify proteins biotinylated both with and without addition of excess biotin. While adding exogenous biotin increased the number of biotinylated proteins, it also has the potential to increase nonspecific labeling. We therefore compared the enrichment of labelled proteins relative to control samples (lysates from cells not expressing BioID2 fusion constructs) prepared with or without the addition of exogenous biotin.

Strikingly, the list of specifically labelled proteins identified in association with IST1 and SNX15 was largely superimposable (Figure 4.4A), with the exception of known IST1 specific interactors and in the case of SNX15 labeling itself supporting a recent report that SNX15 forms a dimer (19). IST1 specific interactors detected included Spartin (16) a and Calpain 7 (2) and the AAA ATPase Spastin. We also detected both isoforms of VPS4, known to bind and disassemble ESCRT-III proteins, and at least in S. Cerevisiae thought to be regulated by IST1 (37).

Among the many proteins enriched in both the IST1 and SNX15-BIOID2 samples were retromer associated SNX and SNX-BAR proteins SNX3 and SNX1, as well as components of the WASH complex (Strumpellin, FAM21). Among ESCRT proteins, STAM1&2 (ESCRT-0 components) as well as the ESCRT-III proteins CHMP5 and CHMP4B were detected. Since SNX15 is reported to bind clathrin (38), we were interested in known clathrin interactors and identified EpsinR and AP-2 activating kinase 1 (AAK1). These proteins bind to clathrin adaptor proteins AP-1 gamma adaptin and AP-2 alpha adaptin, respectively (39–42). Additional AP-1 accessory proteins including gamma synergin, Eps15, EpsinR, SNX9, and actophilin were also labeled.
Another high ranking clathrin associated protein in our data set, CD2AP, binds to AP-2 but also contains SH3 domains which bind cortactin, which itself was one of the most highly labeled hits and is a multifunctional actin binding protein and class II Arp2/3 nucleation promoting factor (43). It should be noted that SH3 domain mediated interactions are commonly employed by proteins that modulate actin dynamics, and that a proline rich linker in IST1 as well as the proline containing MIM2 motif (PXXPXXP) are similar to what is found in ZO-1, Dynamin2, and other proteins that are bonafide interactors of the cortactin SH3 domain (44). Overall, this dataset establishes that IST1 and SNX15 are proximal to much of the machinery implicated in endosomal sorting and especially endosomal retrieval and recycling and suggests many interesting relationships for further exploration.

4.3.5 **SNX15 localizes to clathrin-coated domain that is distinct from HRS and the actin-rich domain**

Prevailing models explaining how cargo destined for recycling or retrograde transport is segregated from that targeted for degradation highlight key contributions of retromer and associated machinery to recycling and ESCRT to degradation (35, 45, 46). Branched actin is involved in domain segregation and also in fission of recycling tubules (32, 47–50). Clathrin, in conjunction with HRS/ESCRT-0 on vacuolar endosomes, contributes to establishing and maintaining the degradative domain (9, 10, 51–53). The framework of machinery segregating cargo into distinct domains with different destinations/fates is a general one, but much remains to be done to understand what seem likely to be many additional levels of sorting and regulation. One set of machinery not yet incorporated into this framework are endosome-derived clathrin coated vesicles containing AP-1 (and other intracellular clathrin adaptors, e.g. GGAs, EpsinR). Given the identification of clathrin-associated proteins within SNX15 and IST1 associated
microdomains in our BioID2 analysis, we set out to explore the relationship between clathrin and these factors from the vantage point of the SNX15-IST1 microdomain.

Using U2OS cells stably expressing SNX15GFP, we confirmed the nearly perfect colocalization of SNX15 with IST1 (Fig S4.10D, Fig. 4.4C), and further explored the relationship of the domain occupied by these proteins to that of retromer and actin associated proteins as well as clathrin. We found that while SNX1, FAM21, WASH, and cortactin were present in discrete puncta/domains on SNX15GFP labelled endosomes, i.e. clear evidence of juxtaposition but also lack of overlap (Fig. 4.4 D-F). We also noted that despite the reported ability of SNX15 to bind clathrin (38) and the presence of clathrin and clathrin associated proteins in the BioID2 data, there was limited direct overlap between SNX15GFP and clathrin heavy chain (Fig. 4.4C) but instead a similar adjacent relationship as observed between SNX15-IST1 and the branched actin-rich domain. We found this curious and chose to explore the relationship of SNX15GFP with clathrin and actin on endosomes in live cells.

To do this, we transfected cells with mRFP clathrin light chain A (CLCa) and SNX15GFP and found that similar to what we had seen with stably expressing cells and endogenous protein, there was incomplete overlap of SNX15GFP and mRFP CLCa. While less intense that the tubule associated clathrin, we did resolve clathrin localizing to the vacuolar domain together with SNX15GFP (Fig. 4.5A arrow). We also faintly detected SNX15GFP on bright mRFP CLCa tubular and punctate structures originating from the endosome (Fig. 4.5A arrow heads). A movie sequence of SNX15GFP marked endosome is shown in the bottom panel (Fig 4.5A). We confirmed that Tfn was present in these clathrin-marked tubules protruding from SNX15GFP
endosomes (Fig. 4.5B). The presence of clathrin-containing tubules at the TGN is well established (54), and despite the lack of a clear understanding of incorporation of similar structures on the sorting endosome into current models, there have been other reports similar to ours. Ultrastructure of recycling tubules containing clathrin coated buds as well as TfnR were visualized decades ago (55), and more recently populations of dynamic or “gyrating” peripheral clathrin and GGA1 (presumed to be clathrin buds on waving tubules) that are also positive for Tfn have been imaged in live cells but are not well understood (32, 56–58). Rab4 marked endosomes have Tfn positive tubules that are also decorated with AP-1 and GGA adaptors (59). Overexpression of SNX15 enables the clear visualization of both the clathrin-coated tubule and the originating endosome. We set out to use this approach to explore/define the spatio-temporal relationship between SNX15GFP and actin as well as the relationship between both of these and clathrin-marked tubules to actin in order to define the relative contributions of these different machineries to sorting and cargo trafficking out of the early endosome.

To explore the dynamics and localization of actin on SNX15-delineated endosomes, we co-expressed SNX15GFP with mCherry-cortactin and mTagBFP2-LifeAct. SNX15-marked endosomes typically had a bright dot of cortactin that also contained varying levels of LifeAct (Fig. 4.5C). All three fluorescent proteins were subject to photobleaching under our imaging conditions; quantifying this allowed us to explore each of their dynamics. Both mTagBFP2-LifeAct and SNX15GFP were consistently photobleached, as shown for a single endosome (Fig. 4.5D, rate of SNX15GFP in Fig. 5F). The mCherry-cortactin signal fluctuated, with bright dots appearing and disappearing over time (Fig. 4.5D). Although SNX15GFP and mCherry-cortactin
sometimes overlapped, most often SNX15GFP appeared to be anti-correlated with mCherry-cortactin (Fig. 4.5D, asteriks in linescan plots). To explore the basis for this, we acutely ablated branched actin assembly on the endosome using the Arp2/3 complex inhibitor CK666 (Fig. 4.5E). Adding CK666 immediately eliminated mCherry-cortactin from the endosome (Fig. 4.5E). Surprisingly, CK666 treatment rescued the photobleaching of SNX15GFP (Fig. 4.5F-H, rates shown in Fig S4.11) and allowed uniform expansion of SNX15 to surround the entire endosome.

We also noted that although SNX15GFP localized primarily to the vacuolar domain, it was occasionally also present on endosomal tubules (Fig. 4.5I). Strikingly, appearance of branched actin at the base of the tubule (highlighted by asterisk) preceded the loss of SNX15GFP on the tubule and on bordering regions of the vacuolar endosome (framed in yellow). These observations suggest one way in which branched actin regulates and possibly creates distinct domains on the early endosome, negatively regulating recruitment of SNX15 and possibly restricting its localization to vacuolar regions from which tubules emerge.

We next examined the distribution of clathrin and branched actin in cells overexpressing unlabeled SNX15 (Figure 4.5J). Distinct SNX15 coated endosomes could still be detected in cells by the dim vacuolar presence of clathrin on many large endosomes. Branched actin could be visualized at the base of clathrin containing tubules (Figure 4.5J), similar to what has been described for actin localizing and functioning in the scission of TGN or melanosome tubules. We previously reported that IST1 can polymerize with CHMP1B on intracellular tubules that end in clathrin coated vesicles (Chapter 2). We wondered what the relationship of CHMP1B to the
SNX15/IST1 microdomain might be. As SNX15 only localized to the vacuolar domain and the base of tubules—due to antagonism of the branched actin domain also present at the base of tubes—this sharply contrasts the localization of IST1 and CHMP1B assembled on tubules. We then explored the location of CHMP1B relative to SNX15, actin, and clathrin.

4.3.6 IST1 is recruited by CHMP1B to dynamic tubules that associate with clathrin
By staining SNX15GFP expressing cells for CHMP1B and cortactin, and we could observe that while endogenous CHMP1B did not co-localize with SNX15, it could localize to the actin rich domain on endosomes (Fig. 4.6A). As IST1 coassembled with CHMP1B might have a role in the scission of recycling endosomes, we reexamined the assemblies of CHMP1B and IST1 on tubules, previously only imaged via deep-etch, in living cells. Similar to SNX15, overexpressed CHMP1B dramatically redistributed IST1GFP onto short tubular structures, similar to what we reported by deep-etch (Fig. S4.13A-B). Live cell imaging of these tubules allowed us to observe them originating from actin rich domains, which could be clearly associated with endosomes. These tubule were dynamic (Fig. S4.13C) and demonstrated both overlap and continuous association with the branched actin domain, and could also be observed to have a branched morphology, again similar to what we observed by EM (Fig. S4.13B), and what has been observed for recycling tubules with clathrin-coated buds (60). We could also see branched actin usually associated with one end of a tubular polymer structure even in the absence of a clear endosome. These experiments demonstrate that IST1 can border the branched actin domain from the tubular domain when coassembled with SNX15 and from the vacuolar domain when assembled with SNX15. (See model in Fig 4.6F).
To see if IST1 assembled with CHMP1B was linked to the unique clathrin domain associated with co-assembled SNX15/IST1, we compared the localization of IST1GFP with mRFP CLCa when it was assembled with either SNX15 or CHMP1B (Fig 4.6C). IST1GFP showed colocalization to clathrin in both complexes, but it was much higher in when assembled with SNX15 (Fig. 4.6C). We found that smaller amount of colocalization of IST1GFP/CHMP1B with mRFP CLCa was eliminated when IST1 lacked its CTD (Fig. 4.6C). The dependence of the CTD of IST1 for its localization with clathrin might be explained by the MIT-MIM1 interaction of IST1 and endogenous SNX15, or by the ability of the CTD region to interact with a clathrin interaction factor, such as those identified by proximity labeling. We also found that the CTD of IST1 contributed to this dynamic movement of these tubular polymers (Fig S4.13D-F), providing further evidence of the physiological importance of CTD of IST1 and the need to explore its role in greater detail. Regardless, our studies with ISTNTD clearly indicated that the slight colocalization of IST1GFP/CHMP1B with clathrin is not an artifact of expressing a tubulating protein complex (Fig 4.6C).

While IST1 mainly co-localized with clathrin on the vacuolar domain when assembled with SNX15, it could also be seen, albeit weakly, along the base of clathrin positive tubules (Fig 4.6D). In contrast, IST1 coassembled with CHMP1B showed weak localization to the vacuolar domain and was enhanced on tubules that would transiently co-localize with CLCa (Fig 4.6E). Our live cell-imaging of IST1GFP in different complexes strongly suggests their location to and possible involvement in the generation of clathrin coated tubular carriers on endosomes (Fig 4.6F).
4.3.7 Distinct roles for IST1 on endosomal tubules controlled by binding partners
To test the involvement of IST1 in the generation of recycling tubules, we incubated cells expressing IST1GFP together with either SNX15 or CHMP1B with Tfn for 1 hr to allow Tfn to reach steady state localization in cells. As above, IST1GFP recruited by SNX15 predominantly localized to vacuolar endosomes with protruding Tfn tubules, while IST1GFP recruited by CHMP1B localized to dynamic tubules associated with Tfn positive endosomes and tubules (Fig. 4.6E). In addition to SNX15 and CHMP1B concentrating soluble IST1GFP at distinct locations (Fig. 4.7A-B), we found SNX15 also had an effect on the distribution of Tfn within cells. Specifically, when IST1 was in a complex with SNX15, Tfn positive tubules remained associated with IST1 coated endosomes and showed a dispersed localization—which contrasted with the tight clustering of Tfn positive tubules at the MTOC when IST1 was complexed with CHMP1B (Fig. 4.7A-B,D). Additionally, while cells overexpressing IST1-GFP had a similar Tfn distribution to IST1/CHMP1B cells, they also had increased steady-state levels of internal Tfn that are similar to SNX15/IST1-GFP cells (Fig 4.7D). As IST1 depletion impaired the ability of Tfn/TfnR to cluster at the perinuclear ERC, these over-expression studies indicated two separate complexes by which IST1 affects the trafficking of TfnR within the cell. Having gained new insight into how IST1 associates with clathrin-based recycling domain on the endosome, we next re-examined the identity of the peripheral compartments from which the sorting of Tfn was impaired in IST1 depleted cells.

4.3.8 Loss of IST1 impairs the sorting of Tfn/TfnR from abnormal peripheral AP-1 positive compartments
As the effects of IST1 on TfnR tubule distribution were potentially relevant to the impaired cluster of Tfn that we observed upon IST1 depletion (Fig. 4.1), we returned to this assay and
used AP-1 as a specific marker of endosomal clathrin coats. While using CLCa was informative as a live cell marker for studying clathrin on endosomes, immunostaining of clathrin (clathrin heavy chain) provided a mostly PM associated signal, from which endosomal recruitment was difficult distinguish. However, the clathrin adaptor AP-1, likely best known for its role in CCV formation at the trans-Golgi network (TGN), is also known to localize to endosomes, as well as to regulate the trafficking of TfnR (59, 61–63).

We therefore compared AP-1 γ-adaptin distribution to that of a pulse of Tfn in control and IST1 depleted cells (Fig. 4.8A). We observed a striking accumulation of the Tfn pulse in abnormal, peripheral, and AP-1 positive endosomes in IST1 depleted cells (Fig. 4.8B). In contrast, in control cells the pulse concentrated at the perinuclear region that is also positive for AP-1. Due to the poor spatial resolution in this perinuclear region, it is not clear if the perinuclear AP-1 in control cells is associated with Tfn within the ERC, or simply with the TGN which shares the same region. However, it is clear that in addition to impaired clustering of Tfn in IST1 depleted cells, these cells also have increased and more peripheral recruitment of AP-1 (Fig 4.8E)—and the increased spatial resolution of the peripheral region clearly demonstrates that this abnormal population of AP-1 is positive for the mis-trafficked Tfn (Fig. 4.8B). In the previous report of IST1 depletion on Tfn/TfnR trafficking the authors observed a dramatic increase in Tfn-positive tubules (in HeLa cells). In our experiments we did not observe such a dramatic tubulation phenotype, though exaggerated Tfn-positive tubules could be observed in some IST1 depleted cells, such as shown in Fig. 4.8D, and these tubules were also positive for AP-1.
This rapid sorting of Tfn from peripheral endosomes to the ERC (within 2.5 minutes) observed in control cells, and absent in IST1 depleted cells is consistent with reports of AP-1 function on the early endosome (61, 64, 65). AP-1 is involved in (1) recycling to the PM and (2) sorting to the slow recycling compartment (usually located at the ERC). Cargo transport to both of these locations has been described to have an identical half-life of ~2 min (at least in CHO cells), and to be regulated by Rab4 (65–67). We then proceeded to see if the recycling, as well as the sorting to the ERC was dependent on IST1.

4.3.9 IST1 regulates the retention of TfnR in intracellular compartments

To assess the role of IST1 in recycling we used a kinetic load-chase assay that measures the exocytosis of internalized transferrin (Tfn) bound to transferrin receptor (TfnR) to determine if the loss of IST1 had an effect on the exocytic pathways that TfnR/Tfn uses to return to the plasma membrane. We loaded the cells with labeled Tfn Alexa555 for 1 hr, and then washed and chased cells in a media of excess unlabeled Tfn (Fig. 4.9A). We used live cell imaging to measure the decrease in the intensity of Tfn within the cell every 2.5 minutes for 30min. As there was variability in the intensity of internalized Tfn, we normalized all cells to their original Tfn intensity. This kinetic assay allowed us to measure the rate of Tfn exocytosis for individual cells, in addition to monitoring the distribution of Tfn (Fig 4.9B). We found that while the distribution of Tfn was identical in cells treated with control or IST1 siRNA, the total amount of Tfn present was dramatically reduced in IST1 depleted cells (Fig 4.9B-C). The rate of Tfn exocytosis was the same, if not faster in IST1 depleted cells (Fig 4.9D-E), indicating that the main difference between control and IST1 siRNA treated cells was not the rate by which internalized Tfn was exocytosis, but how much internalized Tfn was present at steady state.
Our previous pulse studies had demonstrated that the amount of Tfn internalized during a short period of time was not reduced upon depletion of IST1, just sorting of Tfn into a centralized ERC typically associated with the “slow” recycling. Our exocytosis studies supports impaired sorting of TfnR into the “slow” compartment, as sorting to this compartment would increase the life-time (and total intensity) of Tfn within the cell. In addition, the “fast” and the “slow” pathways of exocytosis are known to be upregulated when the other is impaired, which fits with the slightly faster rate of exocytosis in IST1 depleted cells. So while AP-1 has been reported to effect the sorting of Tfn from early endosomes directly to the PM as well as to the slow sorting compartment, we found that IST1 primarily effects the latter. Curiously, this has been reported for AP-1-interacting protein, alphophilin (also found in our proximity labeling dataset), indicating that the role of AP-1 in sorting on the endosome is as complicated as its many accessory proteins.

The role of clathrin and clathrin-interacting proteins on the endosomes has remained an enigma both in recycling, as well as degradative sorting. This is mainly because we have a limited understanding of how protein domains are arranged on the endosome. Our studies in the function of IST1 have begun to sort these different processes out as we have identified IST1 as a central scaffold that links SNX15 and an endosomal clathrin domain that distinct from that from HRS to the membrane constricting properties of CHMP1B filaments.
4.4 Figures

**Fig. 4.1.** IST1 functions in endosomal cargo sorting shortly after endocytosis. (A) T24 cells individually treated with control or indicated ESCRT-III siRNA and then mixed and plated together and stained for FK2 (shown) as well as CHMP4B or IST1, as shown in smaller insets. (B) U2OS cell stained for endogenous IST1, asterisk denote IST1 at cytokinetic cell bridges and mid-bodies, inset ROI shows perinuclear puncta. (C-D) T24 cells allowed to internalize a 30 second pulse of EGF 488 or Tfn 555 and were fixed after 3 min or 2.5 min, respectively, and stained for IST1 and EEA1. (E-F) Fluorescence of EGF (E) and Tfn (F) in IST1 siRNA treated and control T24 cells that were fixed (at the indicated times) after internalizing a 30 second pulse of both EGF 488 and Tfn 555. To compare the localization of EGF and Tfn signals, the distribution of an equivalent area (1µm² or EGF, 4 µm² for Tfn) of the highest intensities was assessed by K-means clustering. The area and determined center is shown for cells in the insets, and the distances of each pixel from the center was plotted in a histogram. The histograms shown are an average of n=18, 21, and 17 for control and n=17,12, and 12 for IST1siRNA treated cells at 2, 3, and 4min, respectively. Similar results were obtained for EGF-only and Tfn-only pulses. (G) Immunostaining of EEA1 in control and IST1 siRNA treated T24 cells, and the recruitment of EEA1 is quantitated to the right, n= 17 for control, n=19 for IST1siRNA treated. Statistical analysis with Mann Whitney U test showed significant increase in EEA1 in IST1 depleted cells.
(H) Recruitment of EEA1 to the Tfn areas used in the K-means clustering assay (F) for control and IST1 depleted cells. (I) Average histogram of intensity-independent distribution of EEA1 as quantified using K-means clustering on an equivalent area (7 µm²) of the highest EEA1 intensites for each cell. The data shown in (I) is of cells quantified in (G) and is one representative experiment of three independent experiments with similar results. Scale bars in the whole cells and insets are as follows: (A) 25 µm and 5 µm, respectively; (B) 25 µm and 25 µm, respectively (C,D) 25 µm and 1 µm, respectively; (E-F) 25 µm and 25 µm, respectively; (G) 25 µm.
Fig 4.2. IST1 uniquely associates with sorting nexin 15. (A) Comparison of the colocalization of ectopically expressed SNX15 and IST1 in U2OS cells. (B) Redistribution of endogenous IST1 upon ectopic expression of SNX15 in U2OS cells, colocalization of IST1 with SNX15. (C) IST1 and SNX15 co-assemble on Tfn-containing endosomes in living U2OS cells. (D) Domain diagram of IST1 (top), SNX15 (bottom), and constructs used to examine the interaction between IST1 and SNX15. (E) Fixed cells expressing SNX15myc with GFP-tagged IST1 constructs shown in D, in the inverted magenta-green images overlapping signal is black and captures dynamic range of IST1 constructs recruited to SNX15. (F) Fixed U2OS cells expressing IST1mApple and SNX15GFP or SNX15ΔMIT. Scale bars in the whole cells and insets are as follows: (C) 25 µm and 5 µm, respectively; (E) 25 µm and 1 µm, respectively; (F) 25 µm.
Fig. 4.3. Endogenous IST1 co-localizes with SNX15 on early endosomes. (A) T24 cells stained for IST1 and either HRS (left) or SNX15 (right) (B) Schematic of the WGA assay. (1) U2OS cells are incubated on ice with WGA-TRITC for 30 min and either fixed in PFA (C, left), or (2) allowed to internalize bound WGA for 5 min at 37 °C in the presence of nocodazole (C, right), and then stained for EEA1. (D) Cells treated identically as D, but (3) washed in ice cold GlcNAc prior to fixation to remove remains plasma membrane associated WGA, and then stained for EEA1 and IST1. WGA is shown in first panels, which was used to generate WGA ROIs (green outlines) shown with EEA1 and IST1 in the right panels. (E) WGA ROIs are shown with cells treated identically as D but stained for HRS (left) or SNX15 (right). (F) A plot of the Spearman’s rank coefficient of pixel-based vs ROI-based correlations of IST1 relative to EEA1 (5 min n=10, 10min n =10), HRS (5min n=12, 10min n =10) or SNX15 (5 min n=10, 10 min, n=10). Scale bars in the whole cells and/or insets are as follows: (A) 25 µm and 1 µm, respectively; (C) 25 µm and 5 µm, respectively; (D) 25 µm, 5 µm, and 1 µm respectively; (E) 5 µm, and 1 µm respectively.
Fig. 4.4. SNX15 and IST1 interact with clathrin-coat assembly and the actin cytoskeleton proteins. (A) Select biotinylated proteins detected by mass spectrometry (MS) using the BIOID2 proximity labeling system for IST1 and SNX15. Average log2 fold change in biotinylated peptides relative to the peptides found in a parent U2OS (non-BIOID2) cell lysate. 2 replicates were done for each condition. (B) The correlation of all biotinylated proteins detected in IST1 BIOID2 or SNX15 BIOID2 cell lines treated without exogenous biotin. (C-E) Validation of targets found from proximity ligation assay. SNX15GFP stable cells stained for IST1 and clathrin (C) or FAM21 and SNX1 or WASH and Cortactin (E). (F) Model of relationship of SNX15 and IST1 microdomain relative to other endosomal domains. Scale bars in the whole cells and insets for (C-F) 25 µm and 1 µm, respectively.
Fig. 4.5. SNX15 localization to endosomal clathrin-coated tubules is antagonized by actin. (A) U2OS cells expressing SNX15GFP and mRFP CLCa. Lower panels show separate and merged channels of ROI 1 (the relationship of SNX15 and clathrin on the vacuolar domain is denoted by arrow and on the tubular domain by arrow heads), and a movie of the endosome in ROI2 demonstrates the localization of tubular and vesicular clathrin structure to SNX15GFP coated endosome. (B) Cells over-expressing SNX15GFP and mRFP CLCa allowed to
endocytosed Tfn647 for 1 hr (C,D) Cells over-expressing SNX15GFP, BFP LifeAct, and mcherry cortactin. Movie for a single endosome is shown in D, with the lower panels displaying a line scan through the actin rich domain and across the endosome, asterisks denote that when there is a large difference in the intensity of SNX15GFP between the two sides of the endosome, actin is present on the lower SNX15GFP peak. (E) Endosome from cell shown in C treated with branched actin inhibitor CK666 after the first frame. (F,G) Intensity plot of SNX15GFP on the endosomes shown in D and E and the total intensity plot across the cell. (J,K) Time-dependent changes in the plotted intensities of SNX15GFP from all endosomes in cell shown in C before (J) and after (K) CK666 treatment. (H) The plotted intensity of SNX15GFP on all endosomes from 10 cells before CK666 treatment (left) and after (right). (I) Time series demonstrating the relationship of SNX15GFP and branched actin to a tubular domain. Asterisks denote the presence of SNX15GFP signal on a tubule or at the base of a tubule that is also proximal to branched actin is subsequently decreased in the next frame (outlined in yellow). (J) Cells expressing unlabeled SNX15myc with mEmerald-CLCb, mTagBFP2-LifeAct, and mCherry-cortactin. Asterisks denote clear clathrin-coated tubules associated with and without the actin-rich domain. Scale bars in the whole cells and/or insets are as follows: (A) 5 µm and 1 µm, respectively; (B) 5 µm (C) 5 µm (D-E & I-J) 1 µm.
Fig. 4.6. IST1 is recruited to distinct clathrin-positive locations on the endosome by SNX15 and CHMP1B. (A) SNX15GFP U2OS stable cells stained for Cortactin and CHMP1B. Insets show 2 examples of the localization of these proteins on endosomes. (B) U2OS cell expressing exogenous IST1gfp, untagged CHMP1B, BFP LifeAct, and mcherry cortactin. Insets show IST1gfp polymerized, due to the presence of CHMP1B, on tubular structures associated with branched actin on endosomes (identified by the absence of cytosolic cortactin). (C) U2OS cells expressing IST1GFP/SNX15myc, IST1GFP/CHMP1B, or IST1NTDGFp with mRFP CLCa. The extent of colocalization between GFP tagged IST1 constructs and CLCa when assembled with SNX15myc or CHMP1B is quantified on the right. (D-E) Insets from C show time series of IST1GFP and clathrin colocalization when either SNX15myc (D) or CHMP1B (E) is also expressed. (F) Model of IST1 localization to endosomal microdomains when coassembled with SNX15 or CHMP1B. Scale bars in the whole cells and/or insets are as follows: (A-B) 25 µm and 1 µm, respectively; (C) 25 µm; (D-E) 1 µm.
Fig. 4.7. IST1 has distinct effects on the distribution of Tfn positive tubules depending on whether it is assembled with SNX15 or CHMP1B. (A-C) U2OS cells expressing IST1GFP with SNX15myc (A), CHMP1B (B), or alone (C) loaded with Tfn 555 for 1hr prior to imaging. (D) Average histogram of the intensity-independent distribution of Tfn within cells similar to those shown in (A-C), and the intensity-dependent mean of Tfn within cells is shown in the inset plot, n=35 (IST1gfp), n=18 (SNX15myc/IST1gfp), n=34 (CHMP1B/IST1gfp). Statistical analysis using Kruskal-Wallis test followed with Dunn’s multiple comparison and found that Tfn intensities in IST1GFP cells showed no significant differences with SNX15myc/IST1gfp and a significant difference with CHMP1B/IST1GFP. Scale bars in the whole cells and insets are as follows: (A-C) 25 µm and 1 µm, respectively.
Fig. 4.8. Dispersed Tfn positive compartments in IST1 depleted cells are associated with abnormal accumulations of the endosomal clathrin adaptor AP-1 (A) Diagram of the Tfn pulse experiment (B) Control (upper panel) or IST1 depleted T24 cells (lower panel) were treated with 30 second pulse of Tfn555, washed and allowed to continue endocytosis of bound Tfn for 1.5 minutes before being chased with unlabeled Tfn. Cells were fixed at 30 seconds (1st panel), 1.5 minutes (2nd panel), 2.5 minutes (3rd panel) or 4 minutes (4th panel). (C) Total amount of Tfn internalized for the time point shown at 2.5 minutes in (B, 3rd panel), n=14 (control) and n=21 (IST1 KD). Statistical analysis using Mann Whitney U test showed Tfn uptake was not impaired in IST1 depleted cells. (D) IST1 depleted cell subject to similar pulse assay in (B) shows AP-1 localization along abnormal peripheral Tfn tubules. (E) Average histogram of the intensity-independent distribution of AP-1, and the intensity-dependent mean of membrane-associated AP-1 of the cells is shown in the inset plot, n=15 (control) and n=14 (IST1 siRNA). Statistical analysis using Mann Whitney U test showed AP-1 recruitment is increased in IST1 depleted cells. Scale bars in the whole cells and insets are as follows: (B) 25 µm; (D) 25 µm and 5 µm, respectively.
Fig. 4.9. **TfnR retention within the cell is impaired in IST1 depleted cells.** (A) Diagram of the Tfn load-chase assay. (B) T24 cells were incubated with Tfn Alexa 555 for 1 hr, washed with PBS, and chased in media containing excess unlabeled Tfn. (C) The mean intensity of Tfn prior to the chase, n=24 control siRNA and n =25 IST1 siRNA treated cells. Statistical analysis using Mann Whitney U test showed significant decrease in Tfn levels in IST1 depleted cells. (D) Normalized time-dependent decay of Tfn fluorescence, measured at 2.5 min intervals after the start of the chase, n=26 for control siRNA and n=25 IST1 siRNA cells. The rate of exocytosis was determined by curve-fitting with a one-phase decay model (solid lines). (E) The half-lives of the two fits were calculated from the fit of the averaged data from n=26 control siRNA and n=25 IST1 siRNA treated cells; Error bars represent 95% confidence interval of the fit. Scale bars in the whole cells of (B) are 25 µm. (F) Model of the location and function of IST1 on recycling tubules.
4.5 Supplemental Figures

Fig. S4.1. U2OS and T24 cells have similar distributions of IST1 on endosomes. U2OS and T24 cells immunostained with IST1 and EEA1. Scale bars in the whole cells and insets are 25 µm and 1 µm, respectively.

Fig. S4.2. T24 cells have large endosomes. TEM of high-pressure frozen and freeze-substituted cells T24 cells. Smaller image (1X) is at same magnification as insets in (Fig.S1). Scale bars are 1µm.

Fig. S4.3. U2OS and T24 cells do not show increased degradation of TfnR upon IST1 depletion. Immunoblots from lysates of cells depleted of CHMP1B, IST1, or LAP1 (control) siRNA.
Fig. S4.4. Loss of IST1 impairs the trafficking of endocytosed cargo, as assessed by k-means clustering. Cell masks are shown in grey, and red pixels show the locations of the highest Tfn or EGF pixel intensities of the cell. The X denotes the center identified by k-means clustering. This intensity-independent assay enables a quantitative way to detect dispersed endocytosed Tfn and EGF after 2 min of endocytosis and the efficiency of cargo clustering after 4 min.

Fig. S4.5. Loss of IST1 does not impair uptake of a pulse of Tfn or EGF within 3 min. The mean Tfn intensity of T24 cells harvested at various points in the pulse assay.
Fig. S4.6. TGN localization is unimpaired in IST1 depleted cells. (A) Immunostaining of the TGN marker p230 in control and IST1 depleted cells. (B-C) Average histograms show the intensity-independent distribution of p230 and TGN46, and the intensity-dependent mean of membrane-associated p230 and TGN46 of the cells is shown in the inset plot. Statistical analysis using Mann-Whitney U test showed significant increase in p230 intensity for IST1 depleted cells in three independent experiments, one experiment being shown in B. Similar statistical analysis in TGN46 intensity for three independent experiments, one experiment being shown in C, yielded inconsistent results. For p230 staining n = 28 control cells, n = 16 IST1 depleted cells, and for TGN46 staining n = 6 control cells, n = 6 IST1 depleted cells. Scale bar is 25 µm.

Fig S4.7. IST1 depletion causes M6PR to accumulate in EEA1-labeled early endosomes. (A) Average histograms show the intensity-independent distribution of M6PR, and the intensity-dependent mean of M6PR in cells is shown in the inset plot. Statistical analysis in M6PR intensity for three independent experiments, one experiment being shown in A, yielded inconsistent results, n = 11 control cells, n = 10 IST1 depleted cells. (B) Immunostaining of M6PR and EEA1 in control and IST1 depleted cells. Images are maximum projections of z-stacks obtained on Zeiss LSM 880 with Airyscan and subsequent Airyscan processing. Contrast adjustments on M6PR channel are equivalent for both conditions, but are different for the EEA1 channel, due to increased EEA1 recruitment in IST1 depleted cells. Scale bars in the whole cells and insets are as follows: (A) 10 µm and 1 µm, respectively.
Fig S4.8. Methanol + BS3 fixation greatly improves detection of endogenous IST1. (A) U2OS cells expressing exogenous SNX15myc were harvested, stained for endogenous IST1 and imaged in parallel. Top cell was frozen in methanol and fixed in BS3 (see methods) and bottom cell was fixed in PFA (see methods). Redistribution of endogenous IST1 due to expression of IST1 was evident in both fixation types, but much more dramatic with methanol + BS3.
Fig. S4.9. Localization of early endosomal proteins to internalized WGA. (A) Total intensity of EEA1 (n=20, 15), HRS (n=20, 19), and SNX15 (n=17, 20) within a cell's WGA ROIs at 5 min and 10 min, respectively. Statistical analysis using 2-way ANOVA showed an increase in SNX15 recruitment to WGA ROIs. (B) Pixel-based and ROI-based Pearson’s correlations for IST1 and VPS35 co-stained with either SNX15, HRS, or EEA1 in WGA assay. p-values were calculated using multiple t-tests with Holm-Sidak correction.
Fig. S4.10. SNX15 and IST1 localize to a domain that is juxtaposed to both HRS and VPS35. Cells subjected the WGA assay (Fig. 3) were stained for VPS35 and either HRS (A) or SNX15 (B), and the extent of colocalization of SNX15 and HRS with is quantified in (C). (D) Fixed cells stably expressing SNX15GFP shows clear co-localization with endogenous IST1. (E) Immunostained SNX15GFP stable cells show the juxtaposed relationship of endogenous HRS, the WASH complex subunit FAM21, and SNX15GFP.
Fig. S4.11. SNX15GFP rates of photobleaching on endosomes affected by branched actin polymerization. (A) Plots of intensities shown with linear fits before CK666 (1) up to 9 min of CK666 treatment (2) and after 9 min of CK666 treatment (3). (B) The value of the slopes is plotted for each period in the bar graph.

Fig. S4.12. Clathrin-coated vesicular and tubular structures originate from SNX15 and SNX15/IST1 coated endosomes. (A) U2OS cell expressing exogenous SNX15GFP and mRFP CLCa (B) U2OS cells expressing exogenous SNX15myc, IST1mApple, and mEmerald CLCb. Scale bars are 1 µm.
Fig. S4.13. Coassembled IST1GFP and CHMP1B localize to dynamic tubules (A) U2OS cell expressing exogenous IST1GFP and untagged CHMP1B. (B) Unroofed and immunostained COS-7 cell coexpressing untagged CHMP1B and IST1-myc. Antibodies detected are white in these contrast reversed EM images, untagged CHMP1B (12 nm gold) and IST1-myc (18 nm gold). Arrows denote possible clathrin buds with a diameter of ~60nm. (C) Movie of copolymer shown in ROI 1 in (A). The highest speed of acquisition (~60ms per frame) was used to capture the dynamics of tubules. Similar to reports of “gyrating” clathrin, these tubules demonstrate high speeds but little displacement. Red lines denote the “waving” behavior of tubules, and minimal displacement after 2 seconds of imaging. (D-E) U2OS cell expressing exogenous IST1GFP or IST1NTD-GFP and untagged CHMP1B. Overlays of two sequential images acquired at high speeds (62.5ms apart) are shown in different channels and demonstrate how the CTD of IST1 contributes to the dynamic behavior of the polymer. (F) Quantification the Pearson’s correlation coefficient between sequential images (as shown in D-E). Scale bars are as follows: (A) 2µm; (B) 0.5µm; (C) 1 µm; (D) 2µm.
4.7 Materials & Methods

Reagents:

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. Compounds were used at the following concentrations unless explicitly stated: 50 µM CK666; 10 µM Nocodozole. Concentrated stock solutions of all compounds were prepared in dimethyl sulfoxide (DMSO) and stored at -80°C in single-use aliquots. Conjugates of EGF to Alexa Fluor 488 (Cat # E13345) and Transferrin to Alexa Fluor 5565 (Cat # T35352) were from Invitrogen, and unlabeled Holo-Transferrin was from Sigma, and were used at the stated concentrations. IST1 isoform A (NM_001270975.1, NP_001257904.1) and SNX15 full length or truncations as indicated were subcloned into pEGFP-N1 and pcDNA4TO. pcDNA4 CHMP1B is as previously described (Chapter 3). LifeAct was a gift from David Kast (Washington University in St. Louis) and has been described (68). mEmerald-Clathrin-15 was a gift from Michael Davidson (Addgene plasmid # 54040 ; http://n2t.net/addgene:54040 ; RRID:Addgene_54040) (69). mRFP-Clec was a gift from Ari Helenius (Addgene plasmid # 14435 ; http://n2t.net/addgene:14435 ; RRID:Addgene_14435) (70). Cortactin-pmCherryC1 was a gift from Christien Merrifield (Addgene plasmid # 27676 ; http://n2t.net/addgene:27676 ; RRID:Addgene_27676) (71).

Antibodies:

Table 1.1 The following antibodies were used for immunofluorescence at the listed concentration.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Company</th>
<th>Catalog #</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>CHMP1B</td>
<td>Rabbit (polyclonal)</td>
<td>Proteintech</td>
<td>14639-1-AP</td>
<td>1:200</td>
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<tr>
<td>EEA1</td>
<td>Mouse (monoclonal)</td>
<td>BD Transduction Laboratories</td>
<td>610456</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
Goat anti-rabbit and mouse secondary antibodies conjugated to Alexa 488, 555 or 647 were from Molecular Probes (Thermo Fisher) and the 12 or 18 nm gold were from Jackson Immunoresearch.

**Cell culture:**

U2OS, T24, and COS-7 cells originally derived from ATCC were cultured in DMEM or McCoy’s (for T24 cells) (Invitrogen) with 8-10% FBS (Atlanta Biologicals). All cells were maintained at 37°C and supplemented with 5% CO2.
**Plasmid Transfections:**

Cells were transfected with the indicated plasmid(s) using Lipofectamine 2000 following the manufacturer's instructions and were used for experiments within 16–24 hour. For light microscopy immunostaining and live cell imaging, U2OS cells were reverse transfected (150,000 cells per 6 well dish) with Lipofectamine (i.e. plated and transfected at the same time). Cells were plated onto glass slips (#1.5 for immunofluorescence, Electron Microscopy Sciences) or 35 mm glass bottom live cell dishes with #1.5 cover glass live (Cellvis) and were imaged or fixed 12-18hrs later.

**siRNA treatment**

All siRNA duplexes used have been previously validated as indicated below. Successful knockdown was verified as shown in Chapter 2. The following sequences were synthesized by Dharmacon and used at the following concentrations:

- IST1 (50nM) 5’-GCAAAUACGCCUUCUAUdTdT (14)
- CHMP4B (25nM) 5’-CGAUAAAGUUGAUGUUAdTdT (72)
- Control (non-targeting firefly luciferase) (25nM) 5’-AUGUAUUGCCUGUAAUAG-dTdT-3’

Cells were plated at 90,000 (T24) or 150,000 (U2OS) per 6 well dish, and 3-5 hrs after plating cells were transacted using DharmaFECT 1 (Dharmacon) according to manufacturer’s instructions. Cells were replated after 24 hrs on to slips or live cell dished, either uncoated or coated with fibronectin (10µg/mL Fibronectin for 1hr at RT, washed 2X in PBS, then covered in media before cells added), as indicated. The cells were then imaged or fixed after 48 hrs of...
siRNA treatment. Alternatively, after 24 hrs IST1 siRNA treated cells were replated and transfeected with a second IST1 siRNA transfection. These cells were replated onto slips after 24 hrs, and then fixed after a total of 96 hrs of siRNA treatment. If ESCRT-III siRNA treated cells were mixed with control siRNA cells before plating on slips (as indicated), the ESCRT-III antibody IST1 or CHMP4B was used to identify conditions.

**Immunofluorescence Microscopy:**

Cells were fixed using using two approaches: either cross-linking fixative alone (paraformaldehyde) followed by detergent to permeabilize cell membranes, or precipitation fixation to remove membranes (-20°C methanol) followed by rehydration in cross-linking fixative (bis(sulfosuccinimidyl)suberate) as described in (20). PFA fixed + permeabilized cells were incubated in 3% PFA, 4% sucrose for 20 minutes, and were subsequently permeabilized with either 0.1% saponin or 0.1% Triton for 10 min (as indicated). Methanol + BS3 fixed cells were process as described in (20) with the slight modification that 12mm slips were dropped in 5mLs of methanol in a 15mL conical tube, cooled to -80°C to ensure that the methanol was < -20°C when slips were added. All subsequent IF steps were identical for both processes, starting with inverting the slips on blocking buffer (1hr at RT), and then in primary antibodies in block (overnight at 4°C), washing, as described in (20), and then inverting in secondary antibodies in block (30min at RT), washing, and mounting in gelvatol.

**Microscopy:**

**Table 1.1** The following light microscope platforms were used for imaging.

<table>
<thead>
<tr>
<th>Microscope Platform</th>
<th>1 Widefield fluorescence</th>
<th>2 Spinning disk confocal</th>
<th>3 Spinning disk confocal</th>
<th>4 Spinning disk confocal</th>
<th>5 Airy Scan confocal</th>
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</thead>
<tbody>
<tr>
<td>Microscope</td>
<td>Olympus IX-81</td>
<td>Olympus IX-81 (same as Platform 1)</td>
<td>Nikon Ti-E inverted</td>
<td>Nikon Eclipse Ei2 inverted</td>
<td>Zeiss LSM 880</td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------</td>
<td>-------------------------------------</td>
<td>---------------------</td>
<td>---------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Objective(s)</td>
<td>60X, 1.42 NA oil immersion (PLAPON)</td>
<td>60X, 1.42 NA oil immersion (PLAPON Apochromat)</td>
<td>60X1.4 NA oil immersion (CFI Plan Apochromat Lambda)</td>
<td>60X1.4 NA and 100X 1.45 NA oil immersion (CFI Plan Apochromat Lambda)</td>
<td>63X, 1.40 oil immersion Plan Apochromat</td>
</tr>
<tr>
<td>Light Source/Lasers</td>
<td>Ex-Cite 120Q (Excelitas)</td>
<td>Two-line (488 and 561nm) Sapphire laser module (Coherent)</td>
<td>Four-line (405, 488, 561, 647nm)</td>
<td>Four-line (405, 488, 561, 647nm)</td>
<td>Six-line (405, 488, 561, 647nm used)</td>
</tr>
<tr>
<td>Spinning disk</td>
<td>N/A</td>
<td>CSU10 (Yokogawa)</td>
<td>CSU-X1 (Yokagawa)</td>
<td>CSU-W1 (Yokogawa)</td>
<td>N/A</td>
</tr>
<tr>
<td>Camera</td>
<td>ORCA-Flash 2.8 scientific CMOS (Hamamatsu Photonics)</td>
<td>Cascade B12 (Photometrics)</td>
<td>Zyla 4.2-megapixel scientific CMOS camera (Andor)</td>
<td>ORCA-Flash 4.0 scientific CMOS (Hamamatsu Photonics)</td>
<td>Airy Scan Detector</td>
</tr>
<tr>
<td>Acquisition software</td>
<td>Metamorph Advanced software</td>
<td>µMανγερ</td>
<td>Nikon Elements</td>
<td>Nikon Elements</td>
<td>Zeiss ZEN</td>
</tr>
<tr>
<td>Live cell imaging chamber</td>
<td>N/A</td>
<td>Tokai Hit INU series stage-top incubator</td>
<td>N/A</td>
<td>Tokai Hit STX stable top incubator</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Image Analysis and Quantification**

Cell ROIs were traced to define masks for individual cells. For quantification of signal within masked area, image stacks were subjected to 50pixel rolling background subtraction (unless otherwise noted) in FIJI prior to taking a maximum intensity projection. Max projections were further background subtracted to set cytoplasmic and/or dark noise to zero, and all images within the same experiment were treated identically. Image processing and analysis was completed with custom-written scripts in FIJI that are available upon request. Alternatively, max projections and masks were imported into Matlab using Bio-Formats. Custom-written scripts, available upon
request, using the Matlab Image Processing Toolbox were used for image analysis to measure
intensities, channel correlations, and dispersal using K-means clustering. K-means clustering
analysis was performed on the highest intensity pixels within a given cell—with the number of
pixels depending on the area to be counted and the resolution of the image. The distances of each
pixel from the identified K-means center for the cell was then plotted in a histogram. Equivalent
binning was used for replicate cells so that the pixel count per bin could be averaged for all cells
of a particular condition.

**WGA Assay and Analysis**

U2OS cells were washed in ice cold PBS and inverted on drop containing 2.5µg/mL TRITC
labeled WGA (2.5µg/mL) in serum-free pulse media containing 1:1 HBSS and McCoy’s Media
to maintain pH for the incubation period (on ice). WGA-TRITC was allowed to bind for 30
minutes, then slips were washed in ice-cold serum free media for 1 minute. Note that the total
time for ice-induced MT disassembly was greater than 30 minutes. Slips were either fixed in
PFA to visualize WGA binding or allowed to endocytose WGA-TRITC labeled proteins at 37°C.

Slips were transferred to serum free DMEM with or without 10uM nocodazole at 37°C (in
incubator) for different intervals (5-15 minutes). Slips were either fixed in PFA (retaining PM
associated WGA) or were washed in ice-cold PBS with 0.1M GlcNAc (Sigma) for 2 minutes and
then fixed in PFA (to compete off WGA-TRITC bound to PM). Cells were then permeabilized
(as described) and stained with antibodies.

Cells were images using Platform 3. A z-stack was acquired for each image, at increments in z at
niquist sampling. Background subtraction (with rolling ball radius of 3 pixels or 270nm) was
performed in image J for each slice. The maximum intensity projection of the stack was then taken. This had little to no effect on the location of IST1 to an HRS-negative domain (compare Figure 2A, single slice and 2H, max projection), or to an EEA1-negative domain (Figure 2G).

Background intensity levels of non-cell area was confirmed to be zero, and additional background (150) was subtracted across all pixels and all channels to remove diffuse signal within the cell. WGA and EEA1 ROIs were then defined by using an additional threshold of 150, so as to exclude smaller, less bright WGA or EEA1 signal. Once these ROIs were defined they were dilated by two pixels (180nm) so as to include any signal immediately adjacent to them (as can be seen in Figure 2F, larger WGA puncta were often surrounded by domains of EEA1, likely due to their lumenal and cytoplasmic locations respectively). Nocodazole treatment is critical to the ability to enlarge the “sphere of influence” of endocytosed material and to assign any signal within that region to that endosome. Non-treated cells exhibit clusters of endosomes at the nucleus, so that a similar processing results in many endosomes within a single ROI so that the assumption that any signals within the ROI are on the same endosome is not valid (Supplemental figure). To determine if WGA or EEA1 ROIs were positive for a signal, the following procedure was used: if a WGA or EEA1 ROI contained any non-zero signal (from the background subtracted image) it was counted at EEA1, WGA, HRS, or SNX15 positive.

**Statistical Analysis**

For all measures of correlation coefficient’s (Pearson’s or Spearmen’s) in the paper values were transformed using Fisher’s Transformation \((1/2*\ln(1+r))/(1-r)\) to obtain an approximately normal sampling distribution. Data was graphed and statistical analysis was performed in Prism. Statistical tests were used as described.
Proximity labeling with BIOID2

Parent U2OS, stable SNX15-BIOID2, and stable IST1-BIOID2 cells in 1 or 2 p150 plates were either incubated in the absence or presence 50uM Biotin for 16 hrs and were dissociated in trypsin-free cell dissociation buffer (CellStripper, Corning), pelleted and frozen. 2 p150 plates were used for each replicate of parent or stable cell lines grown in the absence of exogenous Biotin, and 1 p150 was used for each replicate of parent or stable cell lines grown with exogenous Biotin. Two replicates were done for each condition. Cells were lysed using denaturing conditions by adding 1.5 or 3 mLs of TSD buffer (50mM Tris-Cl pH 7.5, 1% SDS, 5mM DTT) with 1mM PMSF and benzonase (1:1000) directly to the frozen pellet. Sample was mixed by pipetting and then boiled for 10 minutes. Then TSD buffer was diluted by adding 4 or 8 mLs of TNN Buffer (50mM Tris-Cl pH 7.5, 250mM NaCl, 5mM EDTA, 0.5% NP-40) with protease inhibitor (Pierce) and 1mM PMSF and benzonase (1:40,000) and samples were allowed to incubate in cold room for 20 minutes (with rocking). Lysates were cleared by centrifugation at 20,000 x g at 4C for 10 minutes. Supernatant was removed, added to an additional 8 or 16 mLs of TNN Buffer (with protease inhibitor and benzonase), and incubated with 20 or 40uL of strepavidin-agarose beads (S1638, Sigma) for 12 hrs in a cold room (with rocking). Beads were spun down, supernatant was removed and washed 3 times with 1mL TNN Buffer for 10 minutes, and then 3 times with 1mL of TNN Buffer without detergent for 10 minutes. Buffer was removed and bead were covered in MS buffer (6M Guanidium Chloride, 10mM TCEP, 40mM Chloroacetamide, 100mM Tris pH8.5) and boiled for 10min at 95C. Tubes were sealed with parafilm and stored a room temperature.
Deep Etch Electron Microscopy:

U2OS Cells with exogenous IST1 and CHMP1B were prepared as previously described (17, 18). Briefly, cells plated on coverslips (#1.5 for immunofluorescence, #1 poly-L-lysine coated BioCoat coverslips (BD Biosciences) prior to transfection. After transfection for 16-24 hrs, coverslips were washed in 30 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM CaCl2, dipped into an intracellular buffer (30 mM HEPES, pH 7.2, 70 mM KCl, 5 mM MgCl2, and 3 mM EGTA) and subjected to a brief pulse of ultrasound before transfer into the same buffer containing fixative (2% glutaraldehyde or 2% paraformaldehyde if immunostaining was planned). The area of coverslip with the highest yield of plasma membranes was identified by phase contrast microscopy and trimmed with a diamond knife to ~3 x 3 mm. Replicas were prepared as previously described (17, 18). Replicas were viewed on a JEOL 1400 transmission electron microscope at two different tilt angles (+/- 5°) and images were captured using an AMT XR111 camera. Digital image pairs were made into anaglyphs as described (70).

Transmission Electron Microscopy

T24 cells were plated onto fibronectin coated sapphire disks. Cells were high pressure frozen using a Leica EM High-Pressure Freezer (WUCCI core). Prior to immediate freezing cells were kept at 37°C in 1:1 McCoy’s and HBSS to maintain pH. Samples were freeze substituted using Leica EM AF2S Freeze Substitution System (WUCCI core) and subsequently sectioned and imaged using the TEM described above.

4.7 References


49. T. S. Gomez, D. D. Billadeau, A FAM21-containing WASH complex regulates retromer-


Chapter 5: Discussion and Future Directions

ESCRT-III proteins are essential mediators of ILV formation that use their inherent ability to self-assemble into spiral-like configurations to invaginate membranes. As a consequence, however, recent advances in understanding ESCRT-III filament structure and biochemistry (Chapter 2), along with the new cellular activities that are presented in this thesis (Chapter 3-4), have forced us to revise the central dogma surrounding ESCRT-III function. Specifically, we have found that the modular design of ESCRT-III proteins allows them to form homo and heteropolymers that can deform membranes either through invagination or extrusion. These properties enable ESCRT-III to function in a menagerie of cellular functions that not only includes the historical cellular functions of ILV formation and cytokinesis, but also newly discovered activities in membrane trafficking.

In the continued exploration of the CHMP1B and IST1 heteropolymer, Frost and colleagues have demonstrated that while CHMP1B readily associates with, polymerizes on, and tubulates membranes, IST1 does not (1). However, addition of IST1 to CHMP1B-coated tubules, and the resulting copolymer that forms, constricts membranes tubules to a smaller diameter than CHMP1B alone (1). We had observed this similar phenomenon in cells (Chapter 2&3), and therefore seemed like a simple recipe for endotubule fission. However, work in this thesis has highlighted the complexity of IST1 function by showing that IST1 can also form a distinct endosome-associated complex with SNX15. This highlights a unique multifunctional role for IST1 and since SNX15 and CHMP1B are only two members of an already large group of IST1 interacting proteins, much more is left to be uncovered in IST1 function.
One outstanding question is if IST1 only polymerizes in the “closed” state or if it can also assemble in the “open” state. If it is the former, IST1 could be considered an “ESCRT-III-related” protein that is similar in structure but functionally distinct from other ESCRT-IIIIs. If on the other hand, it adopts an open confirmation when assembled with other ESCRT-III subunits—or with SNX15—it could be considered a “true” ESCRT-III protein with expanded functionality in its closed confirmation. Similar to IST1, CHMP1B has also been described as an “ESCRT-III-related” protein due to its lack of canonical ESCRT-III function, however its assembly on membranes occurs through an open confirmation and makes it structurally analogous to Snf7 (CHMP4), but functionally very different (Chapter 2). Another difference between Snf7 (CHMP4) and CHMP1B is membrane association in the open state. While Snf7 (CHMP4) requires an N-terminal amphipathic helix (α0) to associate with membranes (as well as to possibly promote membrane curvature and fission) (2), the residues of CHMP1B in the copolymer are adjacent, but not inserted, into the lipid headgroups (1). The CHMP1B filaments, constricted by IST1 copolymerization, do however thin the outer leaflet of the lipid bilayer and, under the in vitro conditions used, still form stable tubules (1). While this could be consistent with a role in tubule fission, narrowing the tubule diameter does not seem to be sufficient for fission, since even the super-constricted dynamin GTPase transition-state-defective mutant can achieve a stable tubule lumen of 3.7nm but is unable to mediate fission (3). Given that membrane fission by dynamin also requires the actin cytoskeleton, it is a reasonable hypothesis that the actin cytoskeleton maybe required for ESCRT-dependent fission as well.
The AAA ATPase Vps4 has been shown to regulate the dynamic turnover of ESCRT-III subunits present at the midbody during cytokinesis. This dynamic turnover allows ESCRT-III s to reassemble with new geometries that appear to be integral for their ability to constrict membranes. VPS4 interacting with “inverse” ESCRT-III s via their MIM motifs could be somewhat analogous to dynamin in the nucleotide-dependent fission of endosomal tubules, but in this case ATP is consumed in order to drive constriction. Interestingly, IST1 binds the MIM of CHMP1B and in the copolymer structure (4, 5), thereby preventing an interaction between VPS4 and CHMP1B. In contrast, IST1 has two MIM motifs that would hypothetically be able to interact with VPS4 in the copolymer, albeit this unclear from the structure since the CTD of IST1 was not resolved (4, 5). Having said that, IST1 has been demonstrated to inhibit the hydrolysis activity of VPS4 (6, 7), making it possible that IST1 copolymerized in a closed confirmation can affect the dynamics of VPS4 and the assembly and/or membrane remodeling activities of other ESCRT-III proteins.

While further work will be needed to understand the role of IST1 in ESCRT-III assemblies, we were able to identify a critical role of the CTD of IST1 in linking the tubule forming properties of CHMP1B to clathrin on the endosome. While this could be due to the interaction of IST1 with the clathrin-binding SNX15 via the MIM-MIT interaction that we identified, it could also be due to another interaction mediated by the CTD of IST1. The arrangements of the MIMs in IST1 are atypical for ESCRT-III proteins as the MIM motifs are not adjacent to the ESCRT-III core domain. In addition to the tandem MIM motifs, IST1 also contains a proline-rich domain (PRD) with an SH3 domain-binding consensus sequence (PXXPXXP) that links the ESCRT-III core with the MIMs. This SH3-PRD interaction is one of the most common interaction found
amongst cytoskeleton and membrane-associated proteins. In particular, the IST1 SH3 binding motif is also similar to the sequence found in dynamin and ZO-1, both of which bind the SH3 domain of cortactin (8). Notably, we have also found that both cortactin and ZO-1 were enriched by proximity labeling of both SNX15 and IST1. While this interaction demands further exploration, a potential direct interaction with the cytoskeleton would place IST1 as a central player in coordinating endosomal membrane dynamics with actin cytoskeleton dynamics. These distinct features of IST1 may explain its ability to assemble onto unique organelles that are not typically bound by ESCRT-IIIIs. For example, we found IST1 on the PI3P containing endosomes (via SNX15) and endosomal tubules (via CHMP1B), but it also has been reported to bind lipid droplets (via Spastin) (9).

Clathrin is a scaffold that regulates and is regulated by actin and suggests another possible link between the SNX15 microdomain and the branched actin domain, as well as the antagonism we observed between these domains. Clathrin recruited by HRS to the endosome regulates the membrane association of HRS as well as ILV formation (10). Consequently, HRS regulates the membrane association of the WASH complex, which in turn regulates branched actin polymerization by the Arp2/3 complex on the endosome (11). While HRS and SNX15 both bind to clathrin and PI3P, we found they localize to separate and distinct domains. What leads to the formation of these distinct clathrin subdomains will be important for understanding their distinct functions. Candidate proteins include accessory proteins, such as Eps15 and epsins. This possibility is supported by the fact that a specific isoform of Eps15 binds HRS on endosomes and promotes EGFR degradation. Another possibility is the clathrin triskelions themselves, as there are two isoforms of clathrin in mammalian cells CHC17 and CHC22, which have been shown to
be required for the retrograde transport of TGN46 and M6PR, respectively (12–14). A clear
distinction in the two isoforms is the ability of clathrin light chains (CLCs) to assemble with the
cloathing triskelion. While CHC17 is involved in both CME and endosomal trafficking and
coassembles with CLCs. By contrast, CHC22 does not coassemble with CLCs and it is not
involved in CME but does mediate distinct endosomal trafficking processes. Importantly, CLCs
facilitate the interaction of clathrin with actin. Both CLCa and CLCb can bind directly to HIP1R,
which in of itself directly binds actin filaments and negatively regulates actin polymerization (15,
16). More recently, it was shown that myosin VI can to bind directly to CLCa, to the same site
that binds HIP1R (17). This is particularly important because myosin VI is required for the
fission of recycling tubules from melanosomes as well as CME (17, 18). At endosomes,
specifically melanosomes, WASH complex driven branched-actin networks and myosin VI work
together to constrict the necks of tubule protrusions that ultimately mediate the recycling of
certain SNAREs (19). In this way, CLCs can fine tune actin dynamics through a competitive
interplay of actin binding proteins in order to drive membrane fission.

This raises the possibility that the ability of endosomal clathrin scaffolds to interact with actin
may differentially affect the sorting of cargos (TGN46 vs M6PR). As the visualization of clathrin
in live cells, as we have done, depends not only on CLC labeling but also which isoform is
expressed, the roles of clathrin and the isoforms (CHC17 with CLCa/CLCb or CHC22) that
populate the unique domains of SNX15/IST1 and HRS warrants further investigation.

A key result from our work, using mEmerald- and mCherry-tagged CLCs, was the visualization
of clathrin-positive tubules with SNX15 and IST1 at their base. This is of particular importance
because previous live cell imaging of DsRed-tagged CLC did not reveal clathrin association with endotubules that were positive for retromer components (20). It has since been shown that the DsRed tag impairs the dynamic behavior of clathrin on endosomes and the recycling of β1 integrin (21). Given the results of our imaging studies, the role of clathrin in retromer tubule formation and its localization relative to retromer tubules deserves to be re-examined.

Clathrin assembly on endosomes and involvement in tubule formation has been established. AP-2 and clathrin coats have been shown to assemble on lysosomes (22), and this has been shown to be critical for the generation and resolution of long lysosomal tubules (23). Specifically, work on clathrin-mediated sorting on lysosomes has highlighted the importance of phosphoinositides in localizing clathrin. In particular, proteomic analysis and siRNA screens have identified that both clathrin-dependent tubule formation and for the pinching off of small vesicles at the tip of the tubule require PI(4,5)P2 kinases. (23). While PI(4,5)P2 is well known to be required for this process, it was not widely appreciated that this phosphoinositide, usually associated with the PM, exists on lysosomes. Similarly on endosomes, where the accumulation of PI3P is responsible for the recruitment of sorting nexins (and the retromer), AP-1 localization depends on an often overlooked phosphoinositide of the early endosome, PI4P. The importance of PI4P metabolism on the early endosome is gaining recognition. Exocytosis of Tfn is mediated by a phosphoinositide conversion from PI3P to PI4P (24, 25). This maybe in part because PI4P is a potent regulator of the WASH complex. Curiously, PI4P catabolism requires transfer of PI4P from an endosome to the ER through a specialized ER-endosome contact sites that involves the retromer associated SNX2 (24). IST1 is also thought to be involved in the formation of ER-endosome contact sites, and we have coincidently observed an accumulation of AP-1 upon IST1
depletion. This suggests that IST1 may play a fundamental role in regulating PI4P turnover by regulating the contact between endosomes and the ER.

In addition to elucidating the function of IST1 in recycling processes on the endosome, this thesis work establishes several methods that can be used by researchers to assess the locations of microdomains relative to one another on endosomes either in fixed (WGA assay) or living cells expressing SNX15 (either stably or transiently). The latter provides a valuable way to assess clathrin on a tubular domain. Live cell imaging of highly dynamic “gyrating” clathrin on endosomes has been established, and while this is inferred to be clathrin on waving recycling tubules attached to endosomes, clear tubular morphology has not been evident (21, 26, 27). In addition to improved ways to visualize domains in fixed and living cells, this work also provides new approaches for visualizing Tfn compartments and quantifying Tfn-based trafficking in both endocytosis (pulse assay) and exocytosis (load-chase assay). While this work specifically focused on the role of IST1 trafficking, it also provides a necessary frame of reference for future work on assessing the impact of clathrin adaptors and accessory proteins, as well as Rab proteins.

Lastly, this thesis provides foundational work on the role of IST1 at the endosome but provides a narrow sampling of IST1 interactors. As such, much remains to be understood about the function of this unique ESCRT-III protein.

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