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The role of ubiquitination within the endocytic pathway

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THE ROLE OF UBIQUITINATION WITHIN THE ENDOCYTIC PATHWAY

by

Daniel Kenneth Stringer

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Molecular Physiology and Biophysics
in the Graduate College of
The University of Iowa

December 2010

Thesis Supervisor: Professor Robert C. Piper

ABSTRACT

Ubiquitination is a post-translational modification that mediates sorting of integral membrane proteins to lysosomes for their degradation. ESCRTs (Endosomal Sorting Complex Required For Transport) bind and sequester ubiquitinated membrane proteins and direct them into multivesicular bodies (MVBs). ESCRTs themselves become covalently ubiquitinated, simply by virtue of non-covalently binding Ub. However, it is unclear whether this regulates a critical aspect of ESCRT function. In yeast, many MVB cargo proteins are ubiquitinated by the HECT-type Ub-ligase Rsp5, sometimes via the action of Rsp5 adaptor proteins. While many Rsp5 targets are modified by polyubiquitination, it remains unclear whether polyubiquitination is a necessary signal for their incorporation into MVBs. Despite years of research, these and related questions have been difficult to resolve because it is technically quite challenging to control the level of a given protein's ubiquitination. The aim of this research was to develop a novel technique, which can render proteins resistant to ubiquitination. The technique involved the fusion of the Ub-peptidase to a protein of interest via a flexible linker, essentially creating a "DUb module". The intent of this module would be to cleave any Ub form the target protein, essentially immunizing it from the effects of ubiquitination. This novel method was used in combination with several conventional methods to examine the role of ubiquitination within the endocytic pathway and in particular focus on the questions of what type of ubiquitin signal was sufficient for sorting into MVB vesicles and whether ubiquitination of ESCRTs was required for their sorting activity. We found that a single Ub was sufficient for membrane protein entry into MVBs in the absence of ESCRT ubiquitination.

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To Mom and Dad. I find joy in what I do because you taught me to be who I am.

We choose to go to the moon. We choose to go to the moon in this decade and do the other things, not because they are easy, but because they are hard, because that goal will serve to organize and measure the best of our energies and skills, because that challenge is one that we are willing to accept, one we are unwilling to postpone, and one which we intend to win, and the others, too.

-John Fitzgerald Kennedy

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Ubiquitination is a post-translational modification that mediates sorting of integral membrane proteins to lysosomes for their degradation. ESCRTs (Endosomal Sorting Complex Required For Transport) bind and sequester ubiquitinated membrane proteins and direct them into multivesicular bodies (MVBs). ESCRTs themselves become covalently ubiquitinated, simply by virtue of non-covalently binding Ub. However, it is unclear whether this regulates a critical aspect of ESCRT function. In yeast, many MVB cargo proteins are ubiquitinated by the HECT-type Ub-ligase Rsp5, sometimes via the action of Rsp5 adaptor proteins. While many Rsp5 targets are modified by polyubiquitination, it remains unclear whether polyubiquitination is a necessary signal for their incorporation into MVBs. Despite years of research, these and related questions have been difficult to resolve because it is technically quite challenging to control the level of a given protein's ubiquitination. The aim of this research was to develop a novel technique, which can render proteins resistant to ubiquitination. The technique involved the fusion of the Ub-peptidase to a protein of interest via a flexible linker, essentially creating a "DUb module". The intent of this module would be to cleave any Ub form the target protein, essentially immunizing it from the effects of ubiquitination. This novel method was used in combination with several conventional methods to examine the role of ubiquitination within the endocytic pathway and in particular focus on the questions of what type of ubiquitin signal was sufficient for sorting into MVB vesicles and whether ubiquitination of ESCRTs was required for their sorting activity. We found that a single Ub was sufficient for membrane protein entry into MVBs in the absence of ESCRT ubiquitination.

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CHAPTER 1: INTRODUCTION

Lysosomes

At the turn of the 20th century, a biologist named Elie Metchnikoff was studying the development of starfish larvae. In a classic experiment, he introduced a thorn from a rose plant into the larvae and noted a migration and accumulation of mesodermal cells near the extracellular agent. This phenomenon as well as the observations of others eventually lead Metchinikoff to the Phagocytic theory and a type of cell known as a "phagocyte", or a cell that can "eat" (4, 22, 112).

It would take biologists another half century to begin to understand the specific cellular components required for this "cell eating". In the mid 1950's, a group lead by Christian De Duve used centrifugal fractionation and was able to isolate a membrane bound factor (granules) from rat liver that was enriched in acid specific proteases (24, 25). He noted that the proteases had latent enzyme activity, meaning they were more active after several days in the refrigerator, mechanical disruption, freeze-thawing or osmotic stress. De Duve named these granules lysosomes. Within the next decade, De Duve's group and others established that lysosomes were well conserved within eukaryotes and that they had similar function, to digest or to "eat" macromolecules. In a review published in 1966, De Duve noted that lysosomes and related vacuolar organelles were the cellular equivalent to the animal digestive tract. He also noted that not all lysosomes were identical, that prelysosomes containing what he referred to as "unattacked" material were also present in cells (26). It would be several more decades until cell biologists had a better understanding of the endocytic pathway first described by De Duve. Several groups began to study the internalization of ligand bound plasma membrane receptors, which were eventually degraded by

lysosomes. De Duve's work was key to understanding how cells are able to degrade macromolecules and he received the Nobel Prize in medicine in 1974 for his discovery of lysosomes.

Lysosomes and lysosome related organelles (LROs), share several features in common including their lipid composition and their relatively low pH. The pH within lysosomes is around 5 whereas the pH in the cytoplasm is around 7.2. This low pH is essential for many of the lipases and proteases within lysosomes to function optimally and is achieved by an ATPase that utilizes the energy liberated by ATP hydrolysis to pump protons into the lumen of the lysosome (111). The two major functions of the lysosomes are the degradation of biological macromolecules at a low pH and the storage of biological molecules/materials. These functions are required to maintain cellular homeostasis. Using these two basic criteria, lysosomes are found within all eukaryotic cells (4).

In plants and fungal cells, the lysosome is referred to as the vacuole. Vacuoles are similar to lysosomes in that they have a relatively low pH and contain many hydrolytic enzymes that degrade macromolecules. Vacuoles also act as a storage site for waste products and nutrients. In plants, vacuoles help to maintain the turgor pressure of plant cells as a means to regulate cell size and can occupy as much as 90% of the total cell volume (66). These mechanisms are critical for maintaining plant cell homeostasis since plants undergo drastic variations in their environment (117). Like plants, yeast vacuoles also have many versatile roles from protein degradation to amino acid and metal ion storage. They also function in the detoxification of toxic molecules taken up by the yeast. This versatility allows yeast to survive under extreme conditions of nutrient depravation or starvation during the normal life cycle (82, 94).

Lysosomes in mammalian cells are also versatile and carry out several distinct functions. Mammals have specialized LROs (lysosome related organelles) that carry out very specialized functions within specific cell types. These LROs share many common features with lysosomes such as protein content, trafficking pathways and an acidic lumen. Some examples of LROs include melanosomes, which are required for pigment production, and platelet dense granules, which play a major role in immune response. Other examples include MHC II compartments, which are involved in antigen presentation and Lameller bodies, which store and secrete surfactant in lungs (27, 138, 152). There are also specialized cells, such as macrophages, whose major function is the degradation of macromolecules or foreign particles. These cells use a specific type of endocytosis to internalize very large particles, which will eventually be degraded in phagosomes, another LRO (22). Despite their remarkable versatility and differentiation in mammalian cells, the major role for lysosomes is the degradation of macromolecules. Lysosomes contain over 50 soluble hydrolases such as proteases, lipases, phosphatases and nucleases as well as over 120 lysosomal membrane proteins (14, 85, 152). Materials that are degraded by lysosomes include endocytosed receptors, channels and other protein, extracellular material, intracellular organelles, oligosaccharides and complex lipids (117). Transport proteins on the limiting membrane of lysosomes export catabolites generated by lysosomal degradation into the cytosol, so the catabolites can be re-used by the cell (151, 153).

Sorting to Lysosomes

From the Plasma Membrane

There are several different routes that proteins can take to lysosomes Proteins can traffic from the plasma membrane to lysosomes, from the Golgi to lysosomes and large organelles such as mitochondria or proteins can enter the lysosomes via autophagy (98). Materials from the plasma membrane that are destined for lysosomal sorting are internalized by endocytosis. During this process, a portion of the plasma membrane becomes invaginated and forms a vesicle that visits several intracellular compartments before reaching the lysosome. Two major types of endocytosis have been described. They are termed phagocytosis and pinocytosis.

Phagocytosis is the endocytosis of large particles (typically particles larger than 0.5 μ in diameter) and is carried out by specific cell types in mammals such as macrophages or neutrophils (110). These cells ingest macromolecules such as invading microorganisms or dead cells. Remarkably, over 50% of the surface area of the plasma membrane of a macrophage can be utilized during uptake of a phagocytic load (190). Phagocytosis is a regulated process carried out by specific receptors such as the Fc receptor. The Fc receptor recognizes the Fc region of antibodies that bind to foreign bodies (110, 113, 172). Other receptors recognize particular sugar moieties found on the surface of microorganisms or particular lipid compositions on the surface of apoptotic cells.

Pinocytosis, sometimes called “cell drinking”, refers to the constitutive or the regulated endocytosis within a process that uses vesicles that are typically less then 0.2 μ in diameter (110). These vesicles usually contain extracellular fluid or macromolecules bound to the plasma membrane. In many instances, pinocytosis is initiated via clathrin-mediated endocytosis (104). Clathrin is a coat protein that binds to specific sequences of the cytosolic portion of integral

membrane proteins. It was discovered by Barbara Pearse, who purified coated vesicles from pig brain and showed that the coat was composed of a 180 kDa protein that she named clathrin (129). Other work established a link between endocytosis of plasma membrane receptors and clathrin-coated vesicle formation (17). Clathrin has a tri-skeleton shape, and when it polymerizes on membranes, forms a polyhedral lattice (118). Assembly of clathrin subunits on membranes facilitates vesicle formation and integral membrane protein internalization from the plasma membrane as well as vesicle budding at other locations in cells.

Incorporation of integral membrane proteins into clathrin-coated vesicles (CCVs) is facilitated by clathrin adaptor proteins (14, 130). Some examples of clathrin adaptors are the AP1-4 (Adaptor protein complexes 1-4), epsins and GGA proteins. Some clathrin adaptors bind to specific hydrophobic motifs within the cytosolic domains of transmembrane proteins and to clathrin (14). These motifs include di-leucine and tyrosine based motifs. While clathrin functions as a coat protein for vesicle formation at many locales within cells, adaptor proteins appear to be specific for different types of trafficking events and different cargo proteins. For instance, AP-2 promotes CCV formation at the plasma membrane (12, 34). GGA proteins and the AP1 complexes are thought to facilitate vesicle formation at the Golgi apparatus (11, 181). Once CCVs have formed, they pinch away from the membrane by the action the GTPase dynamin, which is required for clathrin-mediated endocytosis (114, 186). Once CCV formation is complete and the vesicle has pinched away from the plasma membrane, the clathrin coat proteins are shed from the vesicle (29).

Not all vesicle formation is mediated by clathrin. Between the ER and Golgi, COPI and COPII act as the primary coat proteins (168). Also, endocytic vesicles can form at the plasma membrane without clathrin. Direct evidence for this came from experiments in yeast where the gene that encodes clathrin heavy

chain was deleted (128). In mammalian cells, the most described clathrin-independent internalization mechanism involves small bud like structures named caveolae. The molecular mechanisms for caveolae are still being elucidated. Three mammalian caveolin proteins have been discovered, but the major protein thought to direct caveolae formation is caveolin1 (29).

Transmembrane proteins destined for lysosomal degradation, are delivered to a compartment known as an early endosome, sometimes referred to as “sorting endosomes”. Next, they are incorporated into intralumenal vesicles (ILVs) that bud inward from the limiting membrane of the early endosome. This is so that the protein is eventually confined within a vesicle inside the lumen of the endosome. As these ILVs accumulate, the endosome is thought to exchange its protein content and becomes known as a multivesiclated body (MVB) or a “late endosome”. Ultimately, the intralumenal vesicles are delivered to lysosomes where the proteases and lipases have complete access to the luminal, cytosolic and the transmembrane portion of the entire integral membrane. This ensures the complete and efficient degradation of the entire integral membrane protein. Proteins at the early endosome that are not incorporated into MVBs are free to recycle back to the plasma membrane or to other intracellular compartments/organelles and are usually sequestered away from the sites of MVB formation in tubular sections of the endosome (Fig. 1-1). ILVs are degraded by lysosomal lipases but the limiting membrane of the lysosome is not because of the unique lipid composition of ILVs. Phosphatidylinositol 3-phospahte (PI3P), cholesterol and LBPA are all enriched in ILVs of MVBs (133).

A classic cargo protein of the MVB pathway is Epidermal Growth Factor Receptor (EGFR), a receptor tyrosine kinase that is degraded in lysosomes after activation (37, 39, 44). This protein becomes activated when bound to the

extracelluar ligand Epidermal Growth Factor (EGF) or other EGFR ligands and rapidly becomes phosphorylated. Activated EGFR is most often incorporated into a CCV and internalized from the plasma membrane, although EGFR is not exclusively internalized by CCVs (165). The coat proteins are quickly shed and the EGFR is sorted to the early endosome and either incorporated into an ILV or recycled back to the plasma membrane (165). In most cases, once proteins like EGFR are incorporated into ILVs, they are committed to eventual degradation by lysosomes. Thus, incorporation into ILVs at the early endosome is considered to be the critical decision step in determining the fate of integral membrane proteins.

Similar examples in yeast include several amino acid and carbohydrate permeases such as Gap1, Mup1, Hxt1 and the mating factor receptor Ste3 (90). For example, under conditions of low methionine, the Mup1 transporter is expressed and localized to the plasma membrane. Exposure to methionine causes Mup1 to rapidly internalize from the plasma membrane, ultimately sorting to the interior of the yeast vacuole where it is degraded (67, 95). Like mammalian cells, the yeast endocytic pathway is composed of an early and late endosome as well as a vacuole, which is analogous to mammalian lysosomes (8, 135).

Finally, some of the resident lysosomal proteins must first traffic to the plasma membrane so that they can be internalized and sent through the early/late endosomal pathway to lysosomes. For example, acid phosphatase is first sent to the plasma membrane and undergoes several rounds of internalization and recycling back to the plasma membrane, however during each round of internalization and recycling a portion of the protein is diverted to lysosomes where it is active (16, 132).

From the Golgi

While many polytopic membrane proteins like EGFR are transported from the plasma membrane to lysosomes, others are capable of direct sorting from the Golgi apparatus to endocytic pathway. Many of the soluble and membrane lysosomal proteases take this direct route, sometimes via the early endosome or sometimes directly through the late endosome (14). Proteins that exchange between the endosomal pathway and the Golgi, usually include a tyrosine or di-leucine sorting signal in their cytosolic portions, which are recognized by adaptor proteins including AP1, AP3 and GGAs. One protein that facilitates the trafficking of many soluble lysosomal proteins is the homo-dimer Mannose-6-phosphate receptor. Many lysosomal resident proteins are modified with mannose-6-phosphate groups, which are recognized by one of two mannose-6-phosphate receptors (M6PR) at the Golgi. These receptors consist of the larger cation-independent M6PR or the smaller cation-dependent M6PR (14, 41). Once bound to their cargo, these proteins bud into vesicles and traffic to early or late endosomes where the relatively lower pH causes their disassociation with their cargo. The cargo then continues on to the lysosome and the M6PR recycles back to the Golgi for another round of trafficking. In addition to direct sorting from the Golgi to endosomes, M6PR can also traffic to endosomes after first visiting the plasma membrane (15, 41).

Some proteins traffic directly from the Golgi to lysosomes independent of M6PR. In yeast, Vps10 is involved in the direct sorting of Carboxypeptidase Y (CPY) to vacuoles (21). Vps10 delivers proCPY to the PVC and while CPY is delivered to the vacuole, Vps10 is able to recycle back to the Golgi (103). Once proCPY reaches the vacuole, the CPY becomes active after cleavage by protease B. Protease B is cleaved and activated by protease A, which is encoded by the *PEP4* gene in yeast. Thus, in *PEP4* mutants, CPY is inactive

and is secreted by the cell (147, 173). In mammalian cells, sortilin facilitates the trafficking of sphingomylelinase to lysosomes (119). Also, lysosomal integral membrane protein 2 (LIMP-2), a lysosomal integral membrane protein that is involved in the trafficking of β- glucocerebrosidase to lysosomes (140).

In addition to the biosynthetic pathway, cells can divert plasma membrane proteins from their trafficking pathway to the plasma membrane to the endocytic pathway to maintain cellular homeostasis. The yeast general amino acid permase Gap1 is one of these proteins. Under rich nitrogen conditions, Gap1 can be diverted from the plasma membrane to endosomes where it is incorporated into MVBs and subsequently degraded (90).

Autophagy

Cells are capable of another trafficking pathway to lysosomes known as autophagy. During autophagy, cells sequester the material destined for lysosomal degradation within a membrane and the autophagic vesicle membrane fuses with the lysosome, delivering its contents into the lysosome for degradation (80, 152). This process is very common in cells like phagosomes, which routinely ingest and degrade large macromolecules. It is also common as a general mechanism in cells to clear large intracellular debris such as large protein aggregates and organelles like mitochondria and peroxisomes. Autophagy is a highly regulated process and takes place in all eukaryotic cells. Genetic studies in yeast have revealed many proteins involved in autophagy, collectively named Atg proteins (80, 199). Autophagy is induced under stress conditions such as cell starvation or ER stress. Detection of either of these stress conditions by sensors like TOR (target of rapamycin) or Ire1 for ER stress, induces the autophagic response (122, 199). An isolation membrane is

generated, which expands and eventually fuses to form an autophagosome. The autophagosome then fuses with the vacuole, spilling its contents into the vacuolar lumen where they are degraded (175).

Ubiquitin

In 1975, an 8.5 kDa protein was discovered that was conserved in all eukaryotic cells and thus was named ubiquitin for its ubiquitous expression in cells. During the next decade, the protein was characterized as a signal added as a covalent post-translational modification to other proteins, directing them for degradation in an ATP dependent manner (51, 53). Only 76 residues long, Ub is generally added to lysine residues as a covalent linkage between the epsilon amine group of lysine and the C-terminal glycines of Ub. Proteins can be monoubiquitinated (a single Ub on one lysine), multiubiquitinated (several lysines each with one Ub) or polyubiquitinated (a chain of Ubs linked by one of the lysines on Ub). There are seven lysine residues in Ub. Proteomics studies from yeast revealed that all seven lysines of Ub can be ubiquitinated, however the polyubiquitin chains that are most commonly described are lysine-48 or lysine-63 linkages (131). Many proteins are polyubiquitinated on lysine-48 of ubiquitin, leading to their degradation by the proteasome. Ubiquitination of integral membrane proteins also leads to their degradation in lysosomes. In 2004, Aaron Ciechanover, Avram Hershko and Irwin Rose were awarded the Nobel Prize in Chemistry for their discovery and characterization of ubiquitin.

Ub is a highly conserved protein, encoded by four genes in both yeast and mammals. *UBI1, UBI2, UBI3* and *UBI4* encode yeast Ub, with *UBI*1-3 encoding Ub with the C-terminus of Ub fused to the N-terminus of the 40S or 60S ribosome subunits (38). The *UBI4* gene is expressed as a fusion of five Ub molecules form

head to tail (124). To liberate free Ub from these fusions, the activity of deubiquitinating enzymes (DUbs) is required (reviewed in chapter 3). Since proteins that are ubiquitinated are generally degraded, cells have mechanisms to ubiquitinate and deubiquitnate proteins as a way to regulate homeostasis. Ubiquitination of proteins is carried out in three steps (52). E1 enzymes, also known as the ubiquitin activating protein, use the energy from ATP to covalently bind ubiquitin through a thiol esther linkage on the N-terminus of the E1 protein. E2 enzymes, also known as ubiquitin conjugating enzymes, also bind ubiquitin via a thiol esther linkage with specific cysteine residues within E2 enzymes. Ub is added to substrates via the action of E3 ubiquitin ligases. Ub ligation can be mediated by a direct transfer of ubiquitin to the E3 as is the case with HECT domain ubiquitin ligases and sometimes with the ligase acting as an intermediate bridge with the substrate as is the case with RING finger ubiquitin ligases (74). In yeast, there is one gene encoding an E1 enzyme, *UBA1*. There are approximately 13 E2 enzymes in yeast. E2 enzymes combined with E3 Ub ligases provide substrate specificity. Some ubiquitin ligases generate specific ubiquitin chain linkages, which is important, as some cellular processes require specific linkages. DUbs catalyze the removal of ubiquitin from substrates and do not require ATP. There are five different families of DUbs and many of the different types of DUbs have different specificity for ubiquitin and polyubiquitin chains (1). Ubiquitination is involved in many processes including protein expression, protein quality control, stress response, DNA repair, cell cycle progression, autophagy and signal transduction. Thus, this reversible dynamic process plays a critical role in maintaining cellular homeostasis .

Ubiquitination signals are transmitted by proteins that contain one or more ubiquitin-binding domains (UBDs). These conserved domains facilitate the non-covalent binding of proteins to ubiquitin. In most cases, UBDs recognize a

hydrophobic patch on Ub, consisting of three residues L8, I44 and V70 (Fig. 1-2). At the proteasome, Rpn10 contains a UIM (Ub Interaction Motif) domain and Rpn13 also binds Ub (65). Several ubiquitin ligases such as Rsp5 contain UBDs within their ubiquitin ligase (HECT) domain and DUbs also contain UBDs within their catalytic domains and sometimes on other portions of the protein. Several of the proteins involved in the trafficking of integral membrane protein to lysosomes also have UBDs. For example, the GGA proteins contain GAT (GGA and Tom1) and VHS (Vps27, HRS, STAM) which both bind Ub (144). Many clathrin adaptor proteins also contain ubiquitin-binding domains. ENTH domain containing proteins such as Ent1 and Ent2 in yeast contain tandem UIM domains. Ede1, an EH domain clathrin adaptor in yeast also has a UBA domain (31). Finally, a set of protein complexes known as the Endosomal Sorting Complexes Required for Transport (ESCRT) associate with early endosomes at the sites of MVB formation also contain several UBDs.

ESCRTs and Intralumenal Vesicles

The ESCRTs were first discovered in yeast as part of a screen that uncovered genes that were important for the transport of receptors to the yeast vacuole. Genetic screens uncovered mutants that gave rise to abnormal localization to ALP protein and the fluorescent dye quinacrine, both of which accumulated in a compartment (termed the “class E” compartment) that was separate from the vacuole (139). The ESCRTs consist of five protein complexes, ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and the Vps4 complex, which are conserved from Archaea to mammals (197). The ESCRT-0 proteins Vps27 (Hrs in mammals) and Hse1 (STAM in mammals) are recruited to endosomes by virtue of the FYVE domain within Vps27 that binds to PI(3)P, a phospholipid that

is enriched on the cytosolic face of endosomes (133). The ESCRT-I and ESCRT-II complexes are recruited by virtue of domains that interact directly with ESCRT-0 as well as the GLUE domain within Vps36, an ESCRT-II protein, that also has PI(3)P binding ability (197). Vps22, another component of ESCRT-II, as well as ESCRT-III subunit Vps24, also contain an alpha helix that can interact with acidic lipids of endosomes with no apparent specificity (191). Also, the ESCRT-III subunit Vps20 is myristoylated, which can facilitate Vps20 interaction with lipids in the absence of ESCRT-II (178). Clathrin is also enriched at the sites of ILV formation and is recruited via a clathrin-binding domain Hrs and STAM (133, 150). Clathrin is concentrated in flat subdomains on endosomes and is required for the efficient degradation of EGFR (137). However, a clear role for Clathrin during ILV formation has yet to be established, with current models favoring Clathrin acting as a “protein scaffold” at the sites of ILV formation on endosomes (150).

At the sites of ILV formation on endosomes, the ESCRTs serve two purposes. The first is to bind and sequester ubiquitinated cargo at the sites of ILV formation. The ESCRT-0 proteins house several UBDs including two UIM domains and a VHS domain within Vps27/Hrs as well as a VHS and one UIM domain within Hse1/STAM (8, 137). The ESCRT-I protein Vps23 has a UEV domain and the ESCRT-II protein Vps36 has a GLUE domain (an NZF domain in yeast Vps36), both capable of binding to Ub (73, 155). The ESCRT-I protein Mvb12 also has a UB (161). Interestingly, many UBD containing proteins also get ubiquitinated themselves by virtue of their UBD. This process is called “coupled ubiquitination” and has been proposed as a potential regulatory mechanism for ESCRTs and other UBDs (Discussed in Chapter 4).

The other major function of ESCRTs is that they promote the inward budding of vesicles into the lumen of the endosomes. However it is still unclear

whether the ESCRTs are directly responsible for making ILVs. ESCRT-III consists of Vps20, Snf7, Vps2 and Vps24. Current models suggest that the ESCRT-II protein Vps36 acts as a "hub" to catalyze the recruitment and activation of ESCRT-III (100, 178, 179). In addition to Vps36, ESCRT-II consists of one subunit of Vps22 and two subunits of Vps25. Vps25 binds to the ESCRT-III subunit Vps20, which binds to another ESCRT-III subunit Snf7. Snf7 can oligomerize, forming a "ring-like" filament that is approximately 10-20 Snf7 monomers long (179). While Vps20 can associate with membranes independently of ESCRT-II, Snf7 oligomers only form when the ESCRT-II subunit Vps20 is present. The Snf7 oligomer is then "capped" by the ESCRT-III subunit Vps24 and then Vps2. Finally, the Vps2 protein is capable of recruiting the Vps4 complex AAA-ATPase complex to disassemble ESCRT-III (179). Recent studies use *in-vitro* ILV reconstitution assays which both suggest that ESCRT-III subunits are sufficient to drive vesicle formation and scission (156, 195, 196). These data provide strong evidence that ESCRTs function to both concentrate Ubiquitinated cargo at the sites of ILV formation at to serve as the machinery that drives ILV formation and scission. *In vivo* data are necessary to confirm these findings. Doa4, a DUb, can also interact the ESCRT-III subunit Snf7. During MVB formation, Ub is removed from the cargo protein once the cargo is committed to an ILV. Deubiquitination serves to maintain levels of free Ub in cells (155).

In summary, for ILV formation ESCRTs appear to mediate processes that are topologically opposite to those mediated by coat proteins like clathrin or scission proteins like dynamin. Clathrin mediates vesicle budding towards the cytosol whereas ESCRTs mediate budding away from the cytosol. Dynamin mediates vesicle scission from the outer surface of membranes whereas ESCRTs direct scission from the inner surface of membranes. ESCRTs are recruited to their sites of action by different means, but appear to mediate similar

processes for ILV formation, viral budding and membrane scission during cell division.

In addition to participating in MVB formation, ESCRTs also play a role in other membrane budding events within cells. During cytokinesis in mammalian cells, a “membrane neck” connects the two daughter cells along with a microtubule rich structure called the midbody. The protein CEP55 recruits ESCRT subunits to the midbody, which in combination with a protein called spastin that severs microtubules, is thought to facilitate membrane scission so that the two daughter cells can separate (150, 197). ESCRT proteins are also used by several retroviruses to facilitate budding from the plasma membrane. The Gag protein of HIV contains a PTAP motif that binds the UEV domain from the ESCRT-I subunit Tsg101. Tsg101 is recruited to the sites of viral budding and subsequently recruits ESCRT-III subunits, which are essential for viral budding and scission. In addition to the ESCRT-I subunit Tsg101, viral Gag proteins can also recruit ALIX, an ESCRT associated protein. Current models suggest that the major role for both Tsg101 and ALIX in viral budding is the recruitment of ESCRT-III (155, 197).

Related Disease

Protein trafficking to lysosomes and protein degradation by lysosomes is a highly regulated process. Disruption in lysosomal function or the trafficking of proteins to lysosomes can lead to disease. Diseases where lysosomes cannot normally degrade macromolecules are commonly referred to as lysosomal storage disorders. Over 50 lysosomal storage disorders have been described, all of them due to a disruption in the activity, processing or trafficking of lysosomal hydrolases (127). This usually leads to an accumulation of partially degraded

material, mainly in cell types or tissues where substrate turnover is high. In one of the first lysosomal storage characterized, Pompe disease is caused by the accumulation of glycogen in lysosomes. It is caused by genetic mutations within the gene that encodes acid apha-glucosidase, which is a lysosomal hydrolase that normally degrades alpha-1,4 and alpha-1,6 linkages in glycogen (50, 127). This leads to a buildup of glycogen in both lysosomes and the cytosol, which likely leads to secondary effects and abnormal lysosomal function. Infantile Pompe disease usually manifests shortly after birth, with myopathy of both cardiac and skeletal muscle tissue. The disease progresses quickly, usually resulting in death within the first year of life due to cardiac arrest. Recently, enzyme replacement therapy has been developed to treat Pompe disease. Recombinant enzyme is given to patients intravenously and is taken up by cells using mannose-6-phosphate receptors (136). Several studies have demonstrated that this treatment clearly prolongs survival for the infantile forms of the disease and has also been used to treat the late onset form of the disease (127).

Work in our lab mainly focuses on how integral membrane proteins are marked for degradation in lysosomes and how cells mediate that degradation. Disruption of these processes can lead to disease. EGFR has been extensively studied and prolonged signaling from this RTK is associated with a variety of human carcinomas. EGFR degradation by lysosomes is promoted by the RING-like Ub-ligase c-Cbl. Upon ligand binding, Cbl binds to and ubiquitinates Cbl. EGFR that is not ubiquitinated does not get incorporated into MVBs and can continue signaling by virtue of its activated cytosolic domain. Thus, mutations in EGFR that prevent Cbl binding lead to a strong mitogenic response. Interestingly, the most oncogenic EGFR family member Erb2/Her2/Neu2, does not contain a Cbl binding site (154, 183). Another example is the HECT-type Ub

ligase Nedd4-2, which ubiquitinates the ENaC channel. Ubiquitination leads to the internalization and lysosomal degradation of ENaC. Genetic mutations in the PY motifs that facilitates ENaC interaction with Nedd4-2, lead to hypertension in a disease called Liddles syndrome (167).

Many human diseases are also associated with the ESCRT machinery. The ESCRT-I subunits Tsg101 (Tumor Suppressor Gene 101) and Vps37A were originally identified as tumour-suppressor genes. Reduced expression of either of these two genes is common in several types of cancer including leukemia, prostate cancer and hepatocelluar carcinomas. Many ESCRT subunits are also involved in neurodegenerative disease (154). Autosomal dominant mutations within the ESCRT-III subunit CHMP2B are found within a subset of patients with Alzheimers disease. Since depletion of ESCRT subunits causes the accumulation of protein aggregates, many of the neurodegenerative diseases associated with the ESCRTs are thought to be due to a defect in the autophagy pathway. ESCRT-I and ESCRT-III subunits are also involved in the replication of many viruses (discussed earlier). Recent studies also suggest that Mycobacteria (*Mycobacterium tuberculosis*) are able to evade degradation by lysosomes by interfering with ESCRT function. This could be due to a defect in phagosome maturation or because components of lysosomes are not delivered properly in the absence of ESCRT function, resulting in a more hospitable environment for the bacterium (174).

Project Scope

My work has focused on the development and application of a novel tool to make proteins resistant to ubiquitination. It involves the simple idea of fusing that catalytic domain from a de-ubiquitinating enzyme to a protein of interest,

thus making that protein immune to ubiquitination. This technique has several advantages over conventional methods. The first is that it provides specificity. Many studies make use of drastic manipulations to cells to examine one process related to ubiquitination. For example, to examine the role of ubiquitination on the localization of the general amino acid permease Gap1, many studies manipulate Ub-ligases to prevent Gap1 ubiquitination (discussed in chapter 5). While these types of studies are powerful, they make it difficult to draw conclusions about specific processes like Gap1 trafficking to vacuoles. Many Ub-ligases, like the ligase responsible for Gap1 ubiquitination Rsp5, have multiple targets. Thus, manipulation of Rsp5 activity will presumably also manipulate the ubiquitin status of every single Rsp5 target in yeast. The other advantage to my technique is that it provides a control. A direct means of assessing a role for ubiquitination of a particular protein of intrest, is to alter the residues that contribute to the ubiquitination of that protein and assess whether the behavior of that protein is altered. However, this approach can be very difficult, time consuming and lacks a control since manipulation of the ubiquitinatable residues could potentially perturb aspects of protein function that are unrelated to ubiquitination. Most DUbs rely on a catalytically conserved cysteine residue for their activity (discussed in chapter 3). Hence, a single point mutation of the catalytic cysteine renders DUbs inactive. Inactivation of a DUb fused to a ubiquitinated protein of interest should restore the ubiquitination of that protein, thus providing a negative control. These advantages allow for a more direct assessment of cellular processes mediated by ubiquitination.

The yeast *Saccharomyces cerevisiae* (hereafter referred to as “yeast”) is a powerful model system for studying biological processes. Compared to other model organisms, genetic and biochemical manipulations are relatively easy and less time consuming in yeast. The yeast secretory and endocytic pathways are

good model systems for studying protein sorting to lysosomes since these pathways are well conserved between yeast and mammals. Many of the genetic manipulations that are lethal to multicellular organisms are possible in yeast using a variety of techniques.

Using both conventional and the novel technique of fusing a DUb to a protein of interest, I examined the role of ubiquitination within the endocytic pathway in yeast. My study has revealed critical aspects of sorting events within cells that require ubiquitination. I tested a model for the function of Ub-sorting receptors that are modified by ubiquitination. I examined the ubiquitination requirements for integral membrane protein entry into MVBs. I made an interesting observation about the Ub-ligase Rsp5 and its ability to function as a dimer. Finally, I examined a proposed MVB cargo that reaches the vacuole without ubiquitination.

Figure 1-1. The Endocytic Pathway.

This diagram depicts the trafficking pathway(s) for integral membrane proteins. They are translated in the Endoplasmic Reticulum and move through the Golgi apparatus. Proteins from the Golgi or from the plasma membrane that are destined for degradation in lysosomes traffic to the early sorting endosome. Here they are incorporated into intralumenal vesicles which accumulate until the vesicle becomes a Mutli-vesculated Body (MVB) and are finally delivered to lysosomes where the cytosolic, luminal and transmembrane portions of the protein can be efficiently degraded.

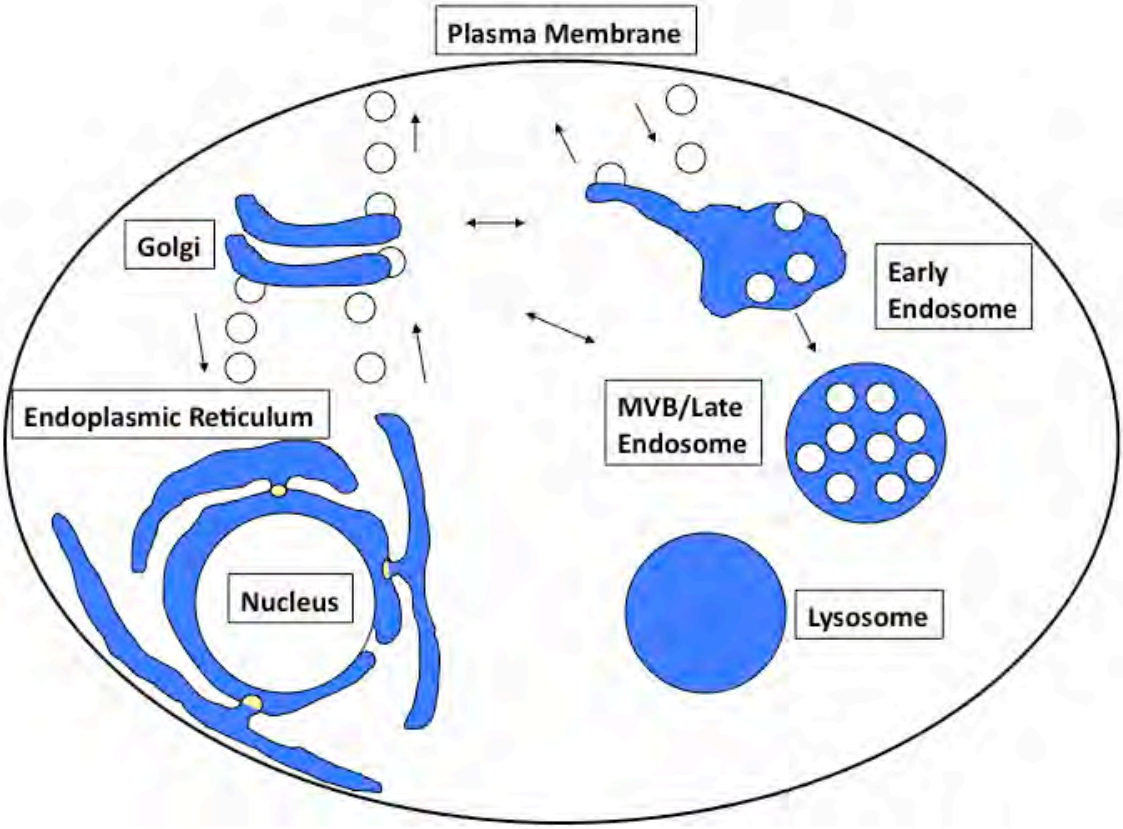

Plasma Membrane
Golgi
Early Endosome
Endoplasmic Reticulum
MVB/Late Endosome
Nucleus
Lysosome

Figure 1-2. Ubiquitination and Deubiquitination.

Ubiquitination of integral membrane proteins is carried out in three distinct steps, requires ATP and is reversible. In the first step, ubiquitin is “activated” in a process that requires ATP by an E1 enzyme. The second step involves the transfer or “conjugation” of ubiquitin from the E1 to an E2. In the third step, ubiquitin is ligated to substrates directly or through an adaptor protein in the case of “HECT” E3 ubiquitin ligases or the E3 is used as an adaptor protein in the case of “RING” E3 ubiqutin ligases. Finally, ubiquitin can be removed from substrates via the action of a deubiquitinating enzyme (DUb). Adapted from (133)

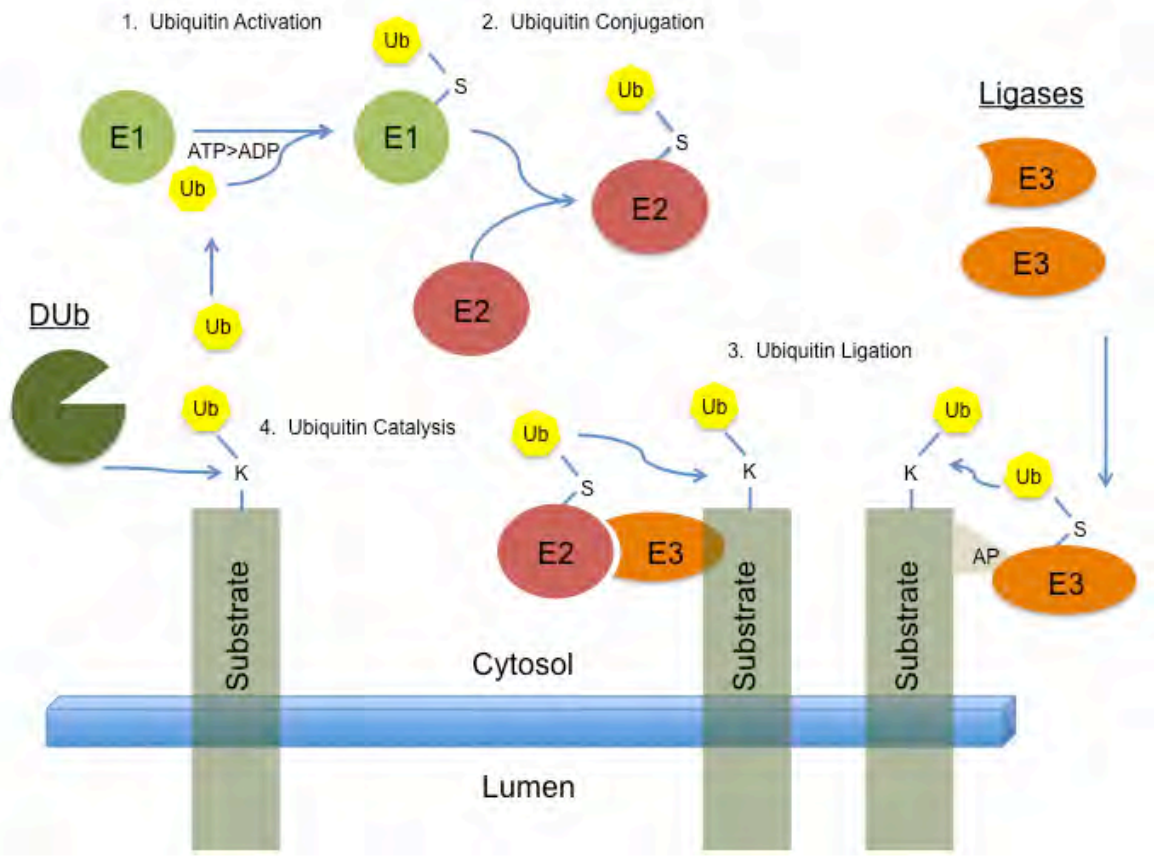

1. Ubiquitin Activation
2. Ubiquitin Conjugation
Ligases
E1
ATP>ADP
Ub
S
E2
E3
DUb
3. Ubiquitin Ligation
4. Ubiquitin Catalysis
K
Substrate
Cytosol
Lumen
AP

Figure 1-3. The Structure of Ubiquitin.

Ubiquitin is a 76 amino acid, 8.5 kDa protein and is depicted in 3 orientations. Key features include 3 residues labeled in yellow which make up a hydrophobic patch on the surface of Ub (L8, I44, V70) and two C terminal glycine residues labeled in blue which allow for the covalent attachment of Ub to other proteins. The two most prominent residues involved in polyubiquitination, K63 and K38 are labeled in orange. (A) Surface view. (B) Ribbon view. PDB::1UBQ

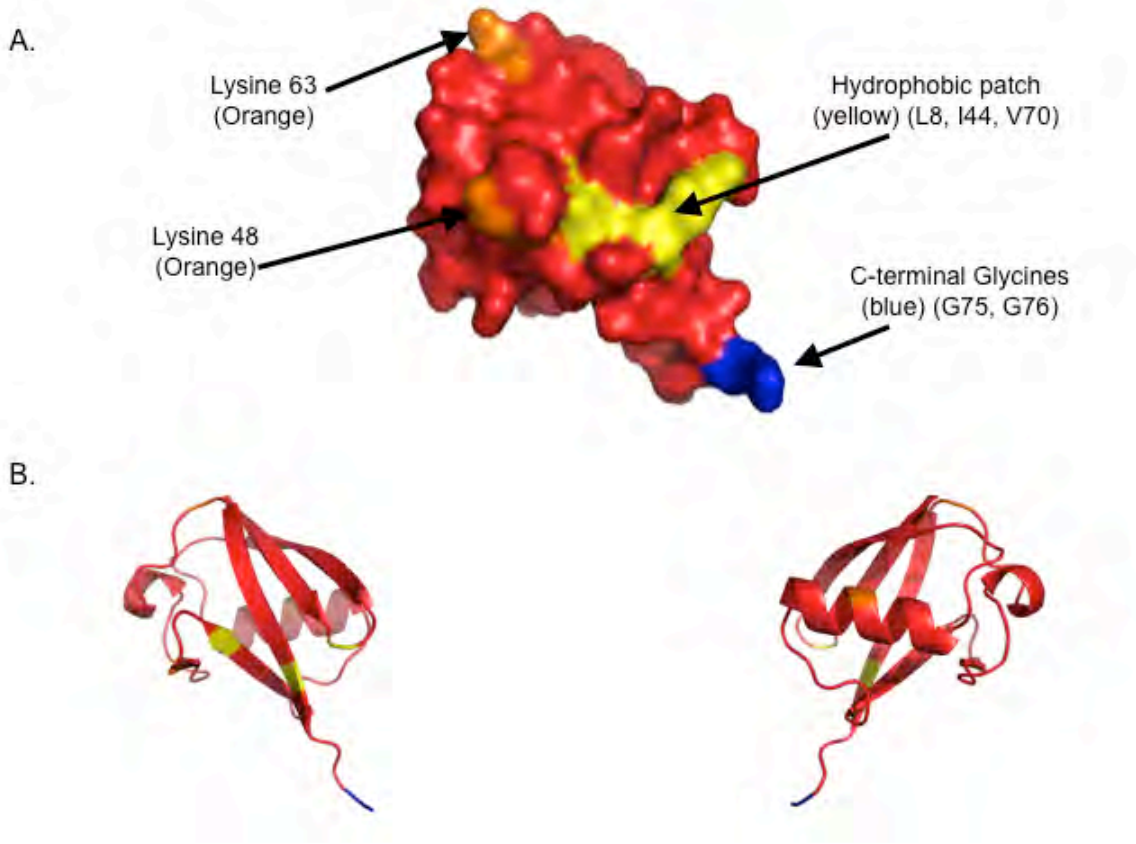
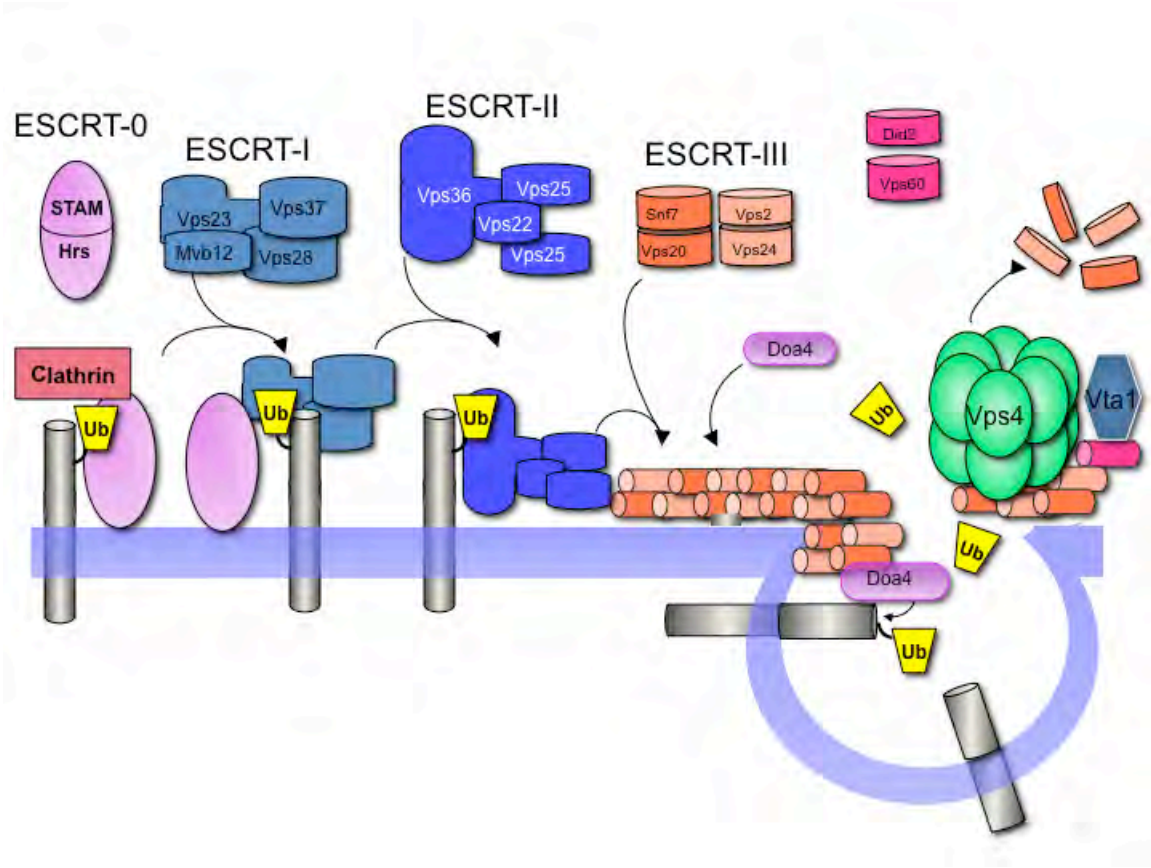

A.
Lysine 63
(Orange)
Hydrophobic patch
(yellow) (L8, I44, V70)
Lysine 48
(Orange)
C-terminal Glycines
(blue) (G75, G76)
B.

Figure 1-4. The ESCRTs.

The ESCRT complexes consist of 5 different multi-subunit complexes, ESCRT 0, ESCRT I, ESCRT II, ESCRT III and the Vps4 complex. ESCRT 0, I, and II are thought to bind to, sequester and concentrate ubiquitinated membrane cargo at the sites of MVB formation on endosomes while ESCRT III and the Vps4 complex are thought to mainly be responsible for MVB formation and disassembly of the ESCRT machinery. Adapted from (133)


ESCRT-0
ESCRT-I
ESCRT-II
ESCRT-III
STAM
Hrs
Vps23
Vps37
Mvb12
Vps28
Vps36
Vps25
Vps22
Vps25
Snf7
Vps2
Vps20
Vps24
Did2
Vps60
Clathrin
Ub
Ub
Ub
Doa4
Ub
Vps4
Vta1
Ub
Doa4
Ub

CHAPTER 2. MATERIALS AND METHODS

Materials, yeast strains and plasmids.

Chemicals, growth methods, and other general procedures were used as described previously (161). Growth on plates in the presence and absence of copper was done with plates containing 100μM $CuCl_2$ or Bathocuproine disulfonate (BCS) respectively. Anti-CPY, GFP, HA and Pho8 were used as described (142). Purchased antibodies were: anti-myc monoclonal (A00704: GenScript), anti myc polyclonal (18826-01: QED biosciences, SanDiego, CA), anti-Ub (P4D1, sc-8017, Santa Cruz Biotechnology, Santa Cruz, CA) anti-HA monoclonal (MMS-101R: Covance). Strains and plasmids used in this study are described in Table 1 and 2 respectively. The *vps27 ubp2* strain was by inserting the *HIS5* gene (47) into *VPS27* within the *ubp2*Δ mutant from the BY4742 MATalpha yeast deletion collection (42). The *hse1Δ vps27Δ* strain was made by inserting the *TRP1* gene into the *HSE1* locus in SEY6210 cells (142). The *rsp5*Δ null strains were made from a parental W303 strain, which contained a disruption of the genomic *RSP5* gene and contained a *URA3*-based low copy plasmid housing the wildtype *RSP5* gene (pRS416-*RSP5*). This strain was transformed with a low copy plasmid expressing a truncated Mga2 protein (19), and an *ADE2* selectable plasmid expressing a GFP-tagged reporter protein. Stains that lost the plasmid bourne *RSP5* gene where selected on plates containing 5-Fluoroorotic Acid and oleic acid that were incubated at 25°C (187). DUb catalytic domains were residues 519-926 of yeast Doa4, residues 561-1071 of yeast Ubp7, residues 401-805 of Ubp5, residues 240-424 of mouse AMSH, the N-terminal residues 15-260 (UL36) of the Type I Herpes virus VP1/2 tegument protein (45) and residues 4-234 of M48 Ub-specific protease (159). Rsp5 HECT domain mutants were previously described (70). The mutation within the *rps5-2*

allele was determined by PCR amplification of *rsp5-2* followed by sequencing. The mutation was S535N.

Microscopy

Cells were grown to mid-log phase, briefly centrifuged and re-suspended in 0.2% NaN_3, 0.2% NaFl, 100mM Tris pH 8.0 For cells containing copper-inducible expression plasmids (containing the *CUP1* promoter), cells were grown overnight, diluted in copper free media for 2-3 hours, and then induced with 100μM $CuCl_2$. Induction of *CUP1*-dependent expression *rsp5-1* cells was similar except cells were divided and grown at permissive (30°C) and non-permissive temperature (37°) during copper-triggered induction. Electron microscopy and cell processing were repeated as described previously(8).

Immunoblotting and Immunoprecipitation

Whole cell denatured lysates were generated by resuspending pelleted cells in 0.2M NaOH. After 2 min, cells were pelleted and resuspended in 8M Urea, 5% SDS, 10% Glycerol, 50 mM Tris pH 6.8 and heated at 70°C for 5 min as outlined previously (143). For immunoprecipitations, whole cell denatured lysates were diluted with 10 volumes of 100 mM Tris pH 7.5, 0.4% Triton X-100. Samples were incubated with polyclonal anti-HA for 90 min at 25°C and. Immune complexes were captured on fixed *Staphylococcus aureus* cells (IgGSorb) purchased from the Enzyme Center (Walden, MA). Beads were washed thrice in 0.1M Tris pH 7.5, 0.4% Triton X-100, 0.1% SDS and analyzed by SDS-PAGE and immunoblotting. Cells carrying the *CUP1*-myc-Ub plasmid were grown overnight in 50μM $CuCl_2$ prior to experiment. Immunoprecipitation from *rsp5-1* cells was performed as above except that cell cultures were divided

and grown at 30°C and 37°C for 2 hrs prior to lysis. CPY secretion assay was performed as previously described (21, 142).

Protease Treatment

Recombinant UL36^{CD} was generated by expressing UL36^{CD} from pET151 in BL21 (DE3) star cells. Enzyme was purified from bacterial lysates (French press, Slm-Aminco) using Cobalt Talon resin (Clontech, Mountain View, CA). Invitro de-ubiquitinating assays were carried out at 37°C for 30 min in PBS containing 0.2 mM DTT. Ub chains (1µG of K48 or K63 purchased from Boston Biochemical, Cambridge, MA) were mixed with recombinant UL36^{CD} in the presence and absence of 1µM Ub-Aldehyde (Boston Biochemical, Cambridge, MA). Reactions were terminated by adjusting samples to 2% SDS and heating at 100°C for 10 min.

For protease protection assays, cells (~5 OD) were grown to mid-log phase. Cells were spheroplasted in 1.4M Sorbitol, 50 mM Tris pH 8, 10 mM NaN_3, 1mM DTT containing Zymolyase (Amsbio, Abingdon, UK) for 1hr. Cells were purified over a cushion of 1.4 M Sucrose by centrifugation and lysed at 4°C with 12 passes through a 26g needle in Sorbitol, 50 mM Tris pH 8.0. Debris was removed from lysates by centrifugation at 1000 x g for 5 min. The resulting supernatant was then incubated in the presence and absence of Trypsin (Pierce, Rockford, IL). Samples were adjusted to 2.5% SDS, 4 M Urea and heated to 100°C for 5 min to halt protease activity.

Table 2-1. Plasmids Used in this Study.

Plasmid	**Description**	**Source**
pRS315	Centromere containing low copy yeast shuttle plasmid (*LEU2*)	(166)
pRS316	Centromere containing low copy yeast shuttle plasmid (*URA3*)	(166)
pRS313	Centromere containing low copy yeast shuttle plasmid (*HIS3*)	(166)
pAC376	Yep351 (2µ *LEU2*) expressing myc-tagged-Ubiquitin from *CUP1* promoter	This Study
pRSmga2NΔTM	*HIS3* low copy plasmid Containing truncated MGA2.	(19)
pYM-N36	Nourseothricin-resistance marker natMX	(68)
Genomic DNA	HSV-1 (Strain F)	(35)
pPL967	pRS315 expressing Ste3-GFP from *STE3* promoter	(184)
pPL991	pRS313 expressing Ste3-GFP from *STE3* promoter	(184)
pPL3484	pRS315 expressing Ste3-GFP-Ub from *STE3* promoter	This Study
pPL3876	pRS316 expressing Ste3-GFP-Ubp7^{CD} from *STE3* promoter.	This Study
pPL3609	pRS316 expressing Ste3-mCherry-Ubp7^{CD} from *STE3* promoter.	This Study
pPL2334	pRS315 expressing Gap1-GFP from *CUP1* promoter	This Study
pPL3797	pRS315 expressing Fur4-GFP from *CUP1* promoter	This Study
pPL3584	pRS316 expressing Mvb12-Ubp7^{CD}-3xHA from *CUP1* promoter. Residues 561-1071 of Ubp7.	This Study
pPL3643	pRS316 expressing Ste3-GFP- ubp7^{CD*} from *STE3* promoter. Residues 561-1071 of Ubp7 with C618S mutation.	This Study
pPL3669	pRS316 expressing Fur4-GFP-Ubp7^{CD} from *CUP1* promoter.	This Study
pPL3722	pRS316 expressing His3-Ubp7^{CD}-3xHA from *CUP1* promoter. Residues 561-1071 of Ubp7 followed by 3xHA epitope.	This Study
pPL3723	pRS316 expressing Hse1-Ubp7^{CD}-3xHA from *CUP1* promoter.	This Study

Table 2-1 continued

pPL3724	pRS316 expressing Vps23-Ubp7^{CD}-3xHA from *CUP1* promoter.	This Study
pPL3746	pRS316 expressing Gga2-Ubp7^{CD}-3xHA from *CUP1* promoter.	This Study
pPL3776	pRS316 expressing Mvb12- ubp7^{CD*}-3xHA from *CUP1* promoter.	This Study
pPL3742	pRS316 expressing Rsp5-Ubp7^{CD}-3xHA from *CUP1* promoter.	This Study
pPL3810	pRS316 expressing Fur4-mCherry-Ubp7^{CD} from *CUP1* promoter.	This Study
pPL3887	pRS315 expressing Ste3-GFP-Ubp7^{CD} from *STE3* promoter.	This Study
pPL3900	pRS316 expressing Gap1-GFP-Ubp7^{CD} from *CUP1* promoter.	This Study
pPL3901	pRS316 expressing Gap1-GFP- ubp7^{CD*} from *CUP1* promoter.	This Study
pPL3994	pRS316 expressing Ste3-GFP-UL36^{CD} from *STE3* promoter. Contains: 561-591 of Ubp7 (Ubp7 linker)-and residues 15-260 of HSV1 UL36	This Study
pPL3962	pRS315 expressing Hxt1-GFP from *HXT1* promoter	This Study
pPL3964	pRS316 expressing Hxt1-GFP-ubp7^{CD*} from *HXT1* promoter.	This Study
pPL3986	pRS316 expressing Ste3-GFP-Ubp7$^{CD(G>S)}$ -3xHA from *STE3* promoter. Residues 561-1071 of Ubp7 with mutations G932S.	This Study
pPL4012	pRS316 expressing Hse1-UL36^{CD}-3xHA from *CUP1* promoter	This Study
pPL4013	pRS316 expressing Mvb12-UL36^{CD}-3xHA from *CUP1* promoter	This Study
pPL4014	pRS316 expressing His3-UL36^{CD}-3xHA from *CUP1* promoter	This Study
pPL4247	pRS316 expressing Vps23-UL36^{CD}-3xHA from *CUP1* promoter	This Study
pPL4015	pRS316 expressing Vps23-GFP-UL36^{CD} from *VPS23* promoter	This Study
pPL4017	pRS316 expressing Gga2-UL36^{CD}-3xHA from *CUP1* promoter	This Study
pPL4143	pRS315 expressing Fur4-mCherry from the *CUP1* promoter	This Study

Table 2-1 continued

pPL4144	pRS315 expressing Fur4-mCherry-Ub from *CUP1* promoter	This Study
pPL4058	pRS316 expressing Ste3-GFP-ul36^{CD*} from *STE3* promoter. Contains: 561-591 of Ubp7 (Ubp7 linker)-and residues 15-260 of HSV1 UL36 with mutation of C40S in UL36.	This Study
pPL4131	pRS316 expressing Hse1-ul36^{CD*}-3xHA from *CUP1* promoter	This Study
pPL4140	*HIS3* conversion of pPL4012 expressing Hse1-UL36^{CD}-3xHA from *CUP1* promoter	This Study
pPL4141	*HIS3* conversion of pPL4131 expressing Hse1-ul36^{CD*}-3xHA from *CUP1* promoter	This Study
pPL4146	pRS315 expressing Mup1-GFP from *CUP1* promoter	This Study
pPL4147	pRS315 expressing Mup1-GFP-Ub from *CUP1* promoter	This Study
pPL4149	pRS316 expressing HA-Vps27 from *MET25* promoter	This Study
pPL4150	pRS316 expressing HA-Vps27$^{K>R}$ (where all K residues are altered to R) from *MET25* promoter.	This Study
pPL4215	*ADE2* conversion of pPL2334 (*ADE2* replacement of *LEU2*) expressing Gap1-GFP from *CUP1* promoter	This Study
pPL4214	*ADE2* conversion of pPL967 (*ADE2* replacement of *LEU2*) expressing Ste3-GFP from *STE3* promoter	This Study
pPL4216	*ADE2* conversion of pPL4146 (*ADE2* replacement of *LEU2*) expressing Mup1-GFP from *CUP1* promoter	This Study
pPL4214	*ADE2* conversion of pPL3484 (*ADE2* replacement of *LEU2*) expressing Ste3-GFP-Ub from *STE3* promoter	This Study
pPL4217	*ADE2* conversion of pPL4147 (*ADE2* replacement of *LEU2*) expressing Mup1-GFP-Ub from *CUP1* promoter	This Study
pPL4252	natMX conversion of pPL4146 (*natMX* replacement of *LEU2*) expressing Mup1-GFP from *CUP1* promoter	This Study
pPL4253	natMX conversion of pPL4146 (*natMX* replacement of *LEU2*) expressing Mup1-GFP Ub rom *CUP1* promoter	This Sutdy

Table 2-1 continued

pPL4320	pRS315 expressing Mup1-GFP-Ub^{K63R} from *CUP1* promoter	This Study
pPL4342	natMX conversion of pPL4320 (*natMX* replacement of *LEU2*) expressing Mup1-GFP-Ub^{K63R} from *CUP1* promoter	This Study
pPL4218	pRS316 expressing Ste3-GFP-M48^{CD} from *STE3* promoter. Contains: 561-591 of Ubp7 (Ubp7 linker)-AGDRDIV-mCMV M48(residues 4-234)-VAITHPLR.	This Study
pPL4219	pRS316 expressing Ste3-GFP-m48^{CD*} from *STE3* promoter. Contains linker residues 561-591 of Ubp7, residues 15-21 of UL36) and residues 4-234 mCMV M48 and the mutations C23A, L110A, Y113A, Y192A followed by residues (253-260 of UL36)	This Study
pPL3974	pET151D expressing UL36^{CD} (residues 15-260 of HSV1 UL36)	This Study
pPL3667	pRS316 expressing Sna3-GFP	
pPL4064	pRS316 expressing Sna3-mCherry	This Study
pPL4066	pRS316 expressing Sna3-mCherry-Ubp7^{CD}	This Study
pPL4065	pRS316 expressing Sna3^{PY}-mCherry	This Study
pPL3959	pRS316 expressing HA-Rsp5	(70)
pPL4033	pRS316 expressing HA-Rsp5^{C777S}	(70)
pPL4036	pRS316 expressing HA-Rsp5^{L771D}	(70)
pPL4037	pRS4316 expressing HA-Rsp5$^{L771A, L796A}$	(70)
pPL4038	pRS316 expressing HA-Rsp5$^{L771D, L796D}$	(70)
pPL4502	pRS316 expressing Rsp5-ubp7^{CD*}	This Study
pPL4500	pRS316 expressing Ste3-GFP-M48^{CD}-3xHA from *STE3* promoter.	This Study
pPL4513	pRS316 expressing Ste3-GFP-m48^{CD*}-3xHA from *STE3* promoter.	This Study
pPL4515	pRS316 expressing Ste3-GFP-ubp7*-3xHA from *STE3* promoter	This Study

Table 2-2. Yeast Strains used in this Study.

Strain	Genotype	Source
BY4742	MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(13)
SF838-9D	MATα *leu2-3,112 ura3-52 his4-519 ade6 pep4-3*	(139)
SEY6210	MATα *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 mel*	(5)
PLY3909	MATα *hse1Δ::TRP1 vps27Δ::LEU2 ura3-52 his3-Δ200 lys2-801 pep4*	This Study
GW004	MATα *rsp5Δ::LEU2, pRS416-RSP5 leu2-3112,ade2-,his3-11,trp1-1,can1-100. Derived from W303*	(187)
PLY441 vpl23-5 (vps27Δ)	MATα *leu2-3,112, ura3-52, his4, ade6 vpl23-5 (vps27Δ) pep4-3*	(139)
BY4742 YOR124C	MATα *ubp2Δ:: Kan^r his3Δ1, leu2ΔO, lys2ΔO, uraΔO*	(192)
PLY3827	MATα *ubp2Δ:: Kan^r vps27Δ::HIS3 leu2ΔO, lys2ΔO, uraΔO*	This Study
RH1800	MATα *leu2 his4 ura3 bar1*	(116)
LHY23	MATα *rsp5-1 leu2 ura3 his3 trp1 lys2 bar1 GAL*	(33)
PLY2667	MATα rsp5Δ::HIS3 leu2::rsp5-2::LEU2 ura3 his3 trp1 lys2 bar1 GAL	(33)
KLY201	MATα *end3Δ::Kan^r his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(96)
BY4742 YJL204C	MATα *rcy1Δ::Kan^r his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(192)
vpl4-21	MATα *vps4Δ leu2-3,112, ura3-52, his4, ade6 (SF838-9D)*	(139)
PLY3623	MATα *mvb12Δ vps36-ΔNZF::TRP1::vps36Δ::HIS vps23-ΔUb (F52Q117W125) leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 mel*	(161)
BY4742 YCL008C	MATα *vps23Δ:: KanR his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(192)

Table 2-2 continued

BY4742 YHL002W	MATα *hse1Δ::KanR his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(192)
EN67	MATa *ecm21::G418 csr2::G418 bsd2 rog3::natMX rod1 ygr068c aly2 aly1 ldb19 ylr392c bul1 bul2::HIS his3 ura3 leu2*	(121)
SUB492	MATα *lys2-801 leu2-3, 112 ura3-52 his3-200 trp1-1*[am] *ubi1-1::TRP1 ubi2-2::ura3 ubi3-Δ ub-2 ubi4-Δ::LEU2* [pUB39][pUB100]. pUB39 is 2μ plasmid with *CUP1-Ub* (WT). pUB100 is plasmid expresses *RPL40A tail.*	(169)
SUB493	Isogenic to SUB492 except pUB197 replaces pUB39 pUB197 is 2μ plasmid with *CUP1*-Ub (K63R)	(169)

CHAPTER 3: PROTEIN “UPGRADE”

Abstract

Despite years of research on ubiquitinated proteins, the technology used to study them is still very limited and has several disadvantages. Once it is established that the protein of interest is ubiquitinated using western blot, IP or Mass Spectrometry analysis, one is left with time consuming and relatively difficult experiments to understand the biological consequences of that protein’s ubiquitination. Mutational analysis, usually of lysine residues within the protein of interest, can be difficult as it is hard to predict which lysines are potentially ubiquitinated. Lysine mutation can lead to non-specific effects such as loss of protein folding or protein-protein interactions. In some cases other residues including cysteines, serines, threonines and even the N-terminus of some proteins can also be ubiquitinated (18, 30, 188). Another disadvantage to mutational analysis is that while changing lysines within a protein of interest may reduce or eliminate the ubiquitination of that protein, there is no negative control experiment that can be performed for a protein that has been mutated to show other unknown aspects of protein function are unaltered. This makes it difficult to base conclusions on the lack of ubiquitination, when in fact the changes resulting from the mutational analysis could have nothing to do with that particular proteins ubiquitination status.

To render proteins resistant to ubiquitination, we devised a system where a catalytic domain of a DUb would be attached by a flexible linker to a protein of interest. This would allow the catalytic domain to attack any portion of the protein of interest that is potentially ubiquitinated. Fusion of structurally identical but catalytically inactive DUbs provided our experiments with a powerful negative control. Our data indicate this strategy is very effective at preventing

ubiquitination of proteins as well as ubiquitin dependent protein trafficking and degradation. This novel technique to render proteins resistant to ubiquitination was named protein “upgrade”, the opposite of degrade.

Introduction

Deubiquitinating enzymes are found throughout nature in both Eukaryotic cells as well as many viruses. Approximately 100 DUbs have been discovered within the human genome (20, 89) whereas only 19 have been characterized within yeast (194). There are five different families of DUbs: USPs being the most frequent (ubiquitin specific proteases), UCHs (ubiquitin C-terminal hydrolases), OTUs (ovarian tumour proteases) and JD (Josephin domain containing) encoding cysteine based “papain-like” proteases and one JAMM (JAB1/MPN/MOV34) metalloprotease which requires zinc (Figure 3-1)(1). While some DUbs are not specific to a particular type of Ub linkage, many chain-specific DUbs do exist but this level of specificity is not confined to a particular DUb family (89). Like many ubiquitin-binding proteins, DUbs interact with Ub, mainly via the hydrophobic patch on Ub located near Ile44 via their catalytic domain (Figure 3-1). Some DUbs have additional Ub-binding domains such as the USP family DUbs USP37 and USP5 that contain UIM or UBA domains respectively or the USP family member USP25 which contains both a UBA and UIM domains (89). Interestingly, DUbs are able to recognize Ub rather than other UBLs such as SUMO, by recognition of the C-terminus of Ubiquitin which is different than the C-terminus of UBLs making DUbs specific towards ubiquitin rather then other Ub-like post-translational modifications (32). Two exceptions to this rule are the DUbs USP18, which has specificity for the UBL ISG15, and USP21, which has duel specificity for the UBL NEDD8 and Ub (43, 99). As four

of the five families are papain like “cysteine based” proteases, many of them share sequence similarity and a common enzymatic mechanism. Upon ubiquitin binding, many DUbs undergo a conformational change that allows the c-terminal residues of ubiquitin to fit into a catalytic site, which consists of a catalytic triad including cysteine, histidine and sometimes an aspartic acid or aspargine residue. The molecular rearrangement upon Ub binding moves the histidine residue into close proximity with the cysteine, allowing for a nucleophilic attack on isopeptide linkages (89), although for some DUbs such as Usp14 and UbpY, no molecular rearrangement is necessary as the catalytic residues are already aligned for activity (3, 62). In these cases a surface loop, which moves upon ubiquitin binding, usually blocks the catalytic site.

DUbs serve several purposes within cells. Ubiquitin is expressed as a linear fusion and requires the activity of DUbs to cleave multi-ubiquitin fusions into single Ub moieties. For instance *UBC*, one of the four genes which encodes ubiquitin in mammalian cells, expresses nine tandem ubiquitin repeats as in-frame fusion proteins and in yeast, the *UBI4* gene, also one of four genes which encodes ubiquitin in yeast, expresses five tandem ubiquitin repeats, both requiring the activity of DUbs to generate mono-ubiquitin from the tandem repeats (89, 124, 125). The major function of DUbs is to reverse the ubiquitination of proteins, which in turn can stabilize proteins destined for degradation by lysosomes or the proteasome. Several DUbs associate with the ESCRT machinery at the sites of MVB formation. For example, the cysteine based DUb USP10, associates with the early endosome and regulates the ubiquitination and relative stability of CFTR (10). DUbs can also trim back ubiquitin chains altering the type or number of ubiquitin molecules on a particular protein of interest. Once a protein is committed to degradation, DUbs at the proteasome and the sites of MVB formation can also cleave the ubiquitin off of a

protein as a means to recycle the ubiquitin, maintaining the ubiquitin homeostasis within a cell(89). At the sites of MVB formation, the yeast DUbs Doa4, Ubp2, and Ubp7 along with their mammalian counterparts UbpY and AMSH, all have been described as contributing to ubiquitin homeostasis as well as cargo stability (20, 101, 120, 142, 164). At the proteasome complex the yeast DUbs Rpn11 and Ubp6 and in mammalian cells POH1, USP14 and UCH37 are all thought to contribute to ubiquitin homeostasis by recycling ubiquitin molecules from cargo proteins destined for degradation (49, 89, 92).

Over the past three decades, several methods have been devised to examine how ubiquitination affects proteins, ranging from experiments involving the deletion of ubiquitinatible residues on proteins to the deletion, over-expression or chemical inhibition of Ub-ligases or DUbs. Each of these techniques has advantages and drawbacks. For example, mutating lysine residues potentially reduces or eliminates the ubiquitination of a given protein of interest, however it could potentially lead to side effects unrelated to ubiquitination of the protein of interest. For instance, it could potentially perturb a protein-protein interaction or disturb the protein folding. Also, inhibiting, deleting or over-expressing a particular ubiquitin ligase or de-ubiquitinating enzyme could have global consequences that are unrelated to the particular process being studied. These relatively simple manipulations to cells and proteins can be informative but can also lead to an artifacts that confound understanding the role ubiquitination plays in cells. To really understand what ubiquitination could potentially be doing to a particular protein or process there needs to be a way to both inhibit or prevent the ubiquitination as well as a control that does not completely change the protein being studied.

To render a protein resistant to the effects of ubiquitination, we fused the catalytic domain from a deubiquitinating enzyme as well as a flexible linker to a

protein of interest. This will detach any Ub from the protein, essentially immunizing it from the effects of ubiquitination. To control for non-specific effects, we mutated the critical catalytic portions of the DUbs, which would render the DUb domain inactive generating a method to control for the non-specific effects of the DUb cassette while maintaining the primary sequence of the protein itself. The idea was named “protein upgrade”, the opposite of degrade.

Results

Validation of Protein Upgrade

To assess whether protein upgrade could block Ub-dependent sorting, we used the yeast G protein coupled receptor Ste3. Ste3 is the a-factor mating receptor and after exiting the Golgi apparatus it traffics to the plasma membrane where it is rapidly phosphorylated, recruits the Ub-ligase Rsp5 which ubiquitinates Ste3 as the protein is internalized, incorporated into an MVB and sent to the yeast vacuole where it is degraded. When fused to GFP, the steady state distribution of Ste3-GFP is inside the yeast vacuole with relatively very little signal at the plasma membrane (Fig. 3-5)(8, 58). Thus, Ste3 makes an excellent candidate to test the feasibility of protein upgrade (Fig. 3-4). Initially four catalytic domains from four different DUbs, three from yeast including Ubp7, Doa4 and Ubp5 and one from mammalian cells AMSH were chosen as candidates for protein upgrade for several reasons. First we examined the crystal structure of HAUSP in complex with Ubiquitin, a DUb that shares sequence similarity with many cysteine based DUbs in yeast (Fig. 3-2), which allowed us to exclude any DUbs with bulky unstructured loops, regions or protein-protein interaction domains as ideal candidates would have relatively small and compact catalytic domains that would not interact with other proteins. DUbs that required other

proteins or associations to function properly such as Ubp6 found at the proteasome complex were excluded (49, 92) and a predicted “flexible linker” portion, 30 residues in size, between the catalytic domain and the N-terminus from each of the three yeast DUbs was also included to allow the DUb to have sufficient access to any portion of Ste3 that might be ubiquitinated. The linker portion from the Ubp7 cassette was used in the AMSH DUb cassette. Each of the four cassettes tested were able to stabilize Ste3-GFP at the plasma membrane to varying degrees (Fig. 3-4). Ubp7 and Doa4 cassettes were initially the most effective at stabilizing Ste3 with majority of the GFP signal at the plasma membrane and in dots, which likely correspond with endosomes or a PVC (pre-vacuolar compartment). The Ubp5 cassette partiallystabilized Ste3 with signal at the cell surface, on the limiting membrane of the vacuole and inside the lumen of the vacuole. Interestingly, AMSH, a DUb that is specific to K63 Ubiquitin linkages (Fig. 3-1)(107) was the least effective at stabilizing Ste3. To test the possibility that something non-specific about the DUb cassettes conferred localization to the cell surface, the catalytic cysteine residues in the Ubp7 and Doa4 cassettes were changed to serines which resulted in the vacuolar localization of both Ste3-GFP-Ubp7$^{C>S}$ and Ste3-GFP-Doa4$^{C>S}$ (Fig.3-7).

While the initial results from the Ubp7 and Doa4 upgrade cassettes were encouraging, they could potentially mediate unknown protein-protein interactions. Also, they are relatively large. Two other DUb cassettes were also designed and engineered from viruses, UL36 from the Herpes Simplex Virus and M48 from Murine Cyomegalovirus (MCMV) (Fig.3-5). These two DUbs are approximately half the size of Ubp7 and Doa4 and as both are from viruses, we could be reasonably assured that they would not interact with other proteins in yeast. Both of these viral DUb cassettes were generated by placing the catalytic

domains from the DUbs behind the linker portion of Ubp7 (Fig. 3-5). Each of the viral DUb cassettes were able to stabilize Ste3-GFP at the plasma membrane and in dots, preventing it from trafficking to the vacuole, which suggests that the protein lacks ubiquitination (Fig. 3-6). Mutation of a single conserved catalytic cysteine residue within the UL36 DUb cassette restored trafficking of Ste3-GFP to vacuoles (Fig. 3-7). To examine the ubiquitination status of Ste3-GFP fused to active UL36 and inactive ul36, those two proteins were expressed in *end3Δ* cells, which allow for protein trafficking to the plasma membrane but prevent protein internalization (96). Both Ste3-GFP-UL36 and Ste3-GFP-ul36* are localized the plasma membrane in *end3Δ* cells (Fig. 3-8A) and both proteins are detectible by western blot for GFP with one band for Ste3-GFP-UL36 and several bands for Ste3-GFP-ul36, suggesting that the Ste3-GFP-ul36* is ubiquitinated (Fig. 3-8B). PGK (3-phosphogycerate kinase) was also blotted from those lysates as a loading control (Fig. 3-8B). An enzymatically inactive m48* DUb cassette was engineered slightly differently then the rest. Despite inactivation of the DUb cassettes for Doa4, Ubp7 and UL36, the Ub-binding domains from those DUbs were left intact which could potentially allow them to bind to and to mask the ubiquitin on the protein that they are fused to. For the M48 DUb cassette we mutated the catalytic cysteine (C23A in the gene, C56A in the cassette) as well as several residues (L110A, Y113A, Y192A in the gene, L143, Y146, Y225 in the cassette) that were predicted to bind to Ubiquitin (Fig. 3-9). Ste3-GFP-m48* was expressed and was localized to the vacuole, suggesting that the inactive m48* was not capable of de-ubiquitinating and stabilizing Ste3-GFP (Fig. 3-10). Cells expressing Ste3-GFP-M48-3xHA or Ste3-GFP-m48*-3xHA were treated with cycloheximide and lysates were generated every 20 minutes for one hour. Western blot analysis revealed that the active M48 cassette stabilized Ste3 with similar levels at one hour after cycloheximide addition. In contrast, the Ste3-

GFM-m48*-3xHA was almost undetectable by 40 minutes and completely undetectable by 1 hour (Fig. 3-17A). Taken together, these data provide strong evidence that the viral DUb cassettes confer a strong resistance to ubiquitination and that the enzymatically inactive viral DUb cassettes restored the normal ubiquitination of Ste3.

The feasibility of protein upgrade was also tested on several other cell surface proteins. The active and inactive Ubp7 cassette was fused to Gap1, a general amino acid transporter, Hxt1 a hexose transporter and the active Ubp7 cassette was fused to Fur4, a uracil transporter. Each of these proteins has a steady state distribution in the yeast vacuole, which is dependent on ubiquitination as demonstrated by previous work (9, 90) (Fig. 3-11). When each of these proteins were fused to the active Ubp7 DUb cassette, their localization pattern was changed to the plasma membrane indicating that protein upgrade is sufficient to prevent the ubiquitination and subsequent trafficking to the yeast vacuole. Inactive ubp7* DUb cassette fused to either Gap1 or Hxt1 allowed sorting to the vacuole demonstrating that the DUb fusions stabilize the protein because of their ability to de-ubiquitinate the protein and not something about the upgrade cassette itself (Fig.3-11).

To investigate the possibility that DUb fusions could be de-ubiquitinating other unintended targets, Ste3-RFP-Ubp7 or Fur4-RFP-Ubp7 were co-expressed with Gap1-GFP or Ste3-GFP. We found that in each case, even in cells expressing Ste3-RFP-Ubp7 and Ste3-GFP, the RFP tagged cargo modified with the upgrade cassette was localized at the plasma membrane, without disrupting the normal vacuolar localization of the GFP tagged cargo, suggesting that these proteins are ubiquitinated and can undergo normal ubiquitin dependent trafficking (Fig. 3-12).

Autocatalysis of Ubp7

To measure the stability of proteins fused to DUb cassettes, we performed a cycloheximide chase analysis. A 3xHA tag was cloned on to the C-terminus of Ste3-GFP-Ubp7 and subjected to western blot analysis for HA. A long exposure to film revealed bands corresponding to the correct size of approximately 140kDa for Ste3-GFP-Ubp7-3xHA, in addition to several smaller molecular weight bands with one prominent band at approximately 17 kDa (Fig. 3-13). Previous work suggested that some de-ubiquitinating enzymes are able to self cleave via the activity of their catalytic domain (63). Active and inactive Ubp7cassettes were fused to the ESCRT-1 subunit Mvb12, generating Mvb12-Ubp7-3xHA and Mvb12-ubp7*-3xHA respectively. Interestingly, the lower molecular weight band at 17 kDa, was only present when the Ubp7 DUb was active, suggesting that DUb activity was required and that the protein is likely undergoing autocatalysis (Fig. 3-14). Since self-cleavage of the DUb proteins could potentially function as a regulatory mechanism for DUbs (63), we wanted to generate a Ubp7 cassette that was resistant to autocatalysis.

Since DUbs recognize di-glycine motifs at the C-terminus of ubiquitin, we examined the primary structure of the Ubp7 looking for di-glycine motifs and found two such motifs. We reasoned that it was likely one or both of these di-glycine motifs that was contributing to the autocatalysis of the Ubp7 cassette. Using the crystal structure from the USP DUb HAUSP in complex with ubiquitin, we examined the conserved domains within Ubp7 and found that one of the di-glycine motifs was located between two conserved regions of homology and according to the crystal structure from HAUSP, that di-glycine motif likely lies within an unstructured loop of Ubp7 (Fig. 3-15)(198). One of the two glycines within that di-glycine motif was mutated to a serine and the protein was expressed in wild type cells. While the altered DUb cassette was still able to

confer stability to Ste3-GFP resulting in localization to the plasma membrane and in endosomes (Fig. 3-16B), the autocatalyzed cleavage product was undetectable, suggesting that the protein no longer undergoes autocatalysis (Fig. 3-16A). Cells expressing either Ste3-GFP-HA or Ste3-GFP-Ubp7$^{G>S}$-3xHA were treated at time zero with cycloheximide and lysates were generated every 30 minutes after cycloheximide addition for a total of 90 minutes. Western blot analysis of the lysates revealed that Ubp7 cassette prevented the degradation of Ste3-GFP whereas Ste3-GFP alone was completely degraded by 60 minutes after cycloheximide addition (Fig. 3-17B).

Discussion

Protein upgrade provides a novel method to render proteins resistant to the effects of ubiquitination. It has several advantages over conventional methods. First, it provides specificity as many conventional methods require global changes to cells. Deletion of a ubiquitin ligase or DUb to prevent the ubiquitination or de-ubiquitination of a protein of interest could be informative with regards to that protein but might also have unintended consequences as every target of that particular ligase or DUb could potentially have an altered ubiquitination status. Protein upgrade can prevent the ubiquitination of specific proteins of interest without altering the ubiquitination of unintended targets as demonstrated by the data in figure 3-13. However, it is impossible to know if the DUb cassettes are completely confined to the protein that they are fused to and they could be affecting the ubiquitination status of proteins that are closely associated with the upgrade target. A second major advantage of protein upgrade is that it provides a negative control in that the catalytic domain from the DUb cassettes can be inactivated. The conventional method requires that the

protein of interest be mutated to prevent ubiquitination but mutation of proteins can have unintended consequences such as preventing protein-protein interactions, aberrant protein folding and even can prevent ubiquitin-binding(200) making it difficult to assign the observed physiological consequences to ubiquitination within those mutant proteins. While the fusion of a DUb cassette to protein could likewise result in those unintended consequences, the inactive upgrade cassettes allow one to examine whether those consequences are due to a lack of ubiquitination or because the protein has been modified by the DUb fusion. Finally, protein upgrade is considerably less time consuming the conventional methods. Mutational analysis of ubiquitinated proteins can be difficult and may require several rounds of cloning and characterizing whereas upgrade is relatively simple and can usually be carried out in one or two cloning steps. While it is important to know which particular residues of a ubiquitinated protein are ubiquitinated, a rapid method to test the functional consequences of that proteins ubiquitination is convenient and could provide the impetus for more involved studies such as mutational analysis or ligase manipulation.

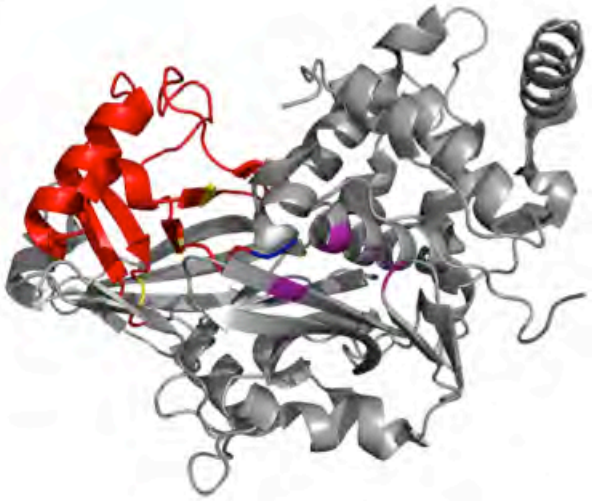
Some of the caveats described above could be addressed with future studies. For instance, if DUb fusions are affecting the ubiquitination status of unintended targets, the linker portion of the cassettes could be altered to try and confine the DUb activity to the protein of interest. Every DUb cassette generated thus far has been a c-terminal fusion protein and in some instances it would be necessary to fuse the DUb portion to the n-terminus. If the integral membrane protein of interest has a luminal c-terminus or if there is a conserved domain on the c-terminus that might be affected by the placement of a fusion protein, an amino-terminal DUb cassette would be necessary to apply protein upgrade to those situations. Several DUbs have catalytic domains on the n-terminus including UL36 which was used in this study and they would make good

candidates to begin examining the feasibility n-terminal upgrade. Also, many of the proteins tested with protein upgrade were integral membrane proteins and many of the ubiquitinated proteins in cells are cytosolic proteins so the feasibility of the technique should be tested on cytosolic proteins as well. Like the DUb AMSH, many DUbs have specificity to specific types of ubiquitin chains or towards specific types of ubiquitination. This could provide a means to test the consequence of specific types of ubiquitination. For instance, if a particular poly-ubiquitin linkage is suspected to have a functional consequence towards a protein of interest, an upgrade cassette using a DUb specific for that type of linkage could be used to prevent that type of ubiquitination. Furthermore, as ubiquitination of proteins is a dynamic process and occurs at different times in the lifetime of a protein of interest, inducible and repressible upgrade could be a powerful tool to promote a wave of ubiquitination or deubiquitination. FKBP and FRB are two proteins that dimerize in the presence of a drug rapamycin. This chemically induced de-ubiquitination could be used to chemically dimerize a DUb cassette to a protein of interest (Fig. 3-18). Also, protease cleavage sites could be engineered on the linker portions of a DUb cassette so that when a particular protease is present or expressed within cells, the DUb portion could be clipped away (Fig.3-18). Thus, inducible or reversible upgrade is possible and could be very useful for kinetic studies so that a wave of ubiquitinated or stabilized protein could be analyzed or perhaps as a control.

Figure 3-1. Families of DUbs.

Structures (grey) of the catalytic domains from a member of each of the five families of DUbs and conserved catalytic site in purple. Ubiquitin (45) is depicted in complex with each of the DUbs with the hydrophobic patch (L8,I44,V70) depicted in yellow and the two c-terminal glycine residues of Ubiquitin depicted in blue. (A) The structure of the USP family member Usp7/Hausp in complex with ubiquitin (PDB identifier 1nbf). (B) The structure of the OTU family member yeast homologue Otu1 in complex with ubiquitin (PDB identifier 3by4). (C) The structure of the UCH family member UCH-L3 in complex with ubiquitin (PDB identifier 1xd3). (D) The structure of the JD family member ataxin 3 in complex with ubiquitin (PDB identifier 2jri). (E) The structure of the JAMM family member AMSH in complex with two ubiquitin molecules with a lysine 63 linkage (PDB identifier 2znv). The side chain from lysine 63 on the distal ubiquitin molecule is depicted in orange as spheres. Adapted from (1, 89)

A.



B.

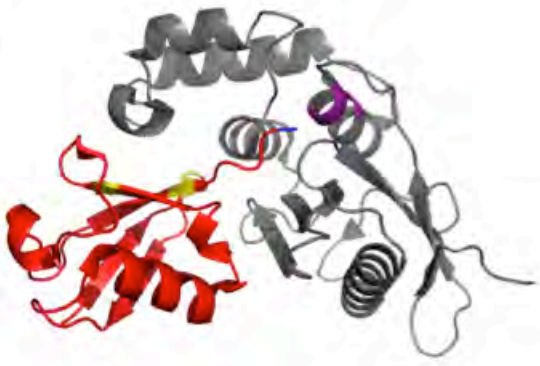
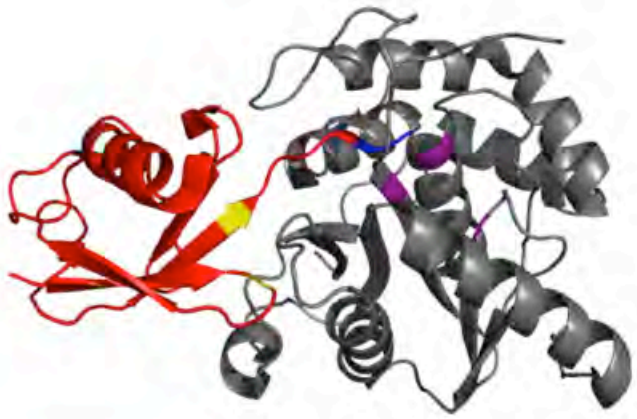


Figure 3-1 continued

C.



D.

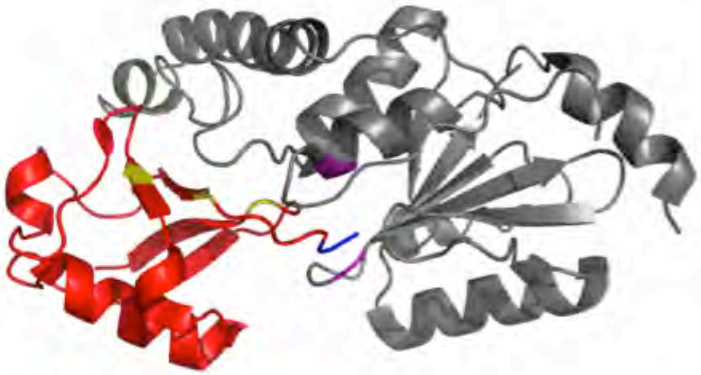


Figure 3-1 continued

E.

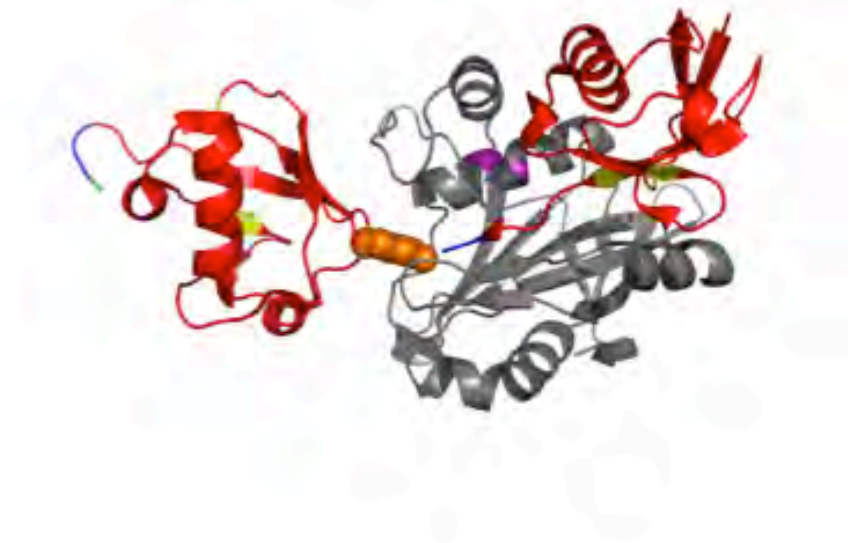


Figure 3-2. Structure of USP14 with Ub.

Ubiquitin is shown in red, USP14 in grey and the conserved USP residues that make contact with Ub are indicated in green. USP4 shares close sequence homology to the yeast DUbs Doa4 and Ubp7, two of the initial DUbs tested for protein upgrade and the crystal structure from USP14 was used to help define both the catalytic domain and the important residues which make contact with Ub. (PDB Idenifier 2J7Q)

Figure 3-3. Schematic of Protein Upgrade.

Proposed model for the fusion of an active or inactive catalytic domain from a DUb to an integral membrane protein. Proteins that undergo normal ubiquitination are depicted on the left, proteins that are resistant to ubiquitination by protein “upgrade” are in the center and proteins fused to an inactive upgrade cassette are on the right.

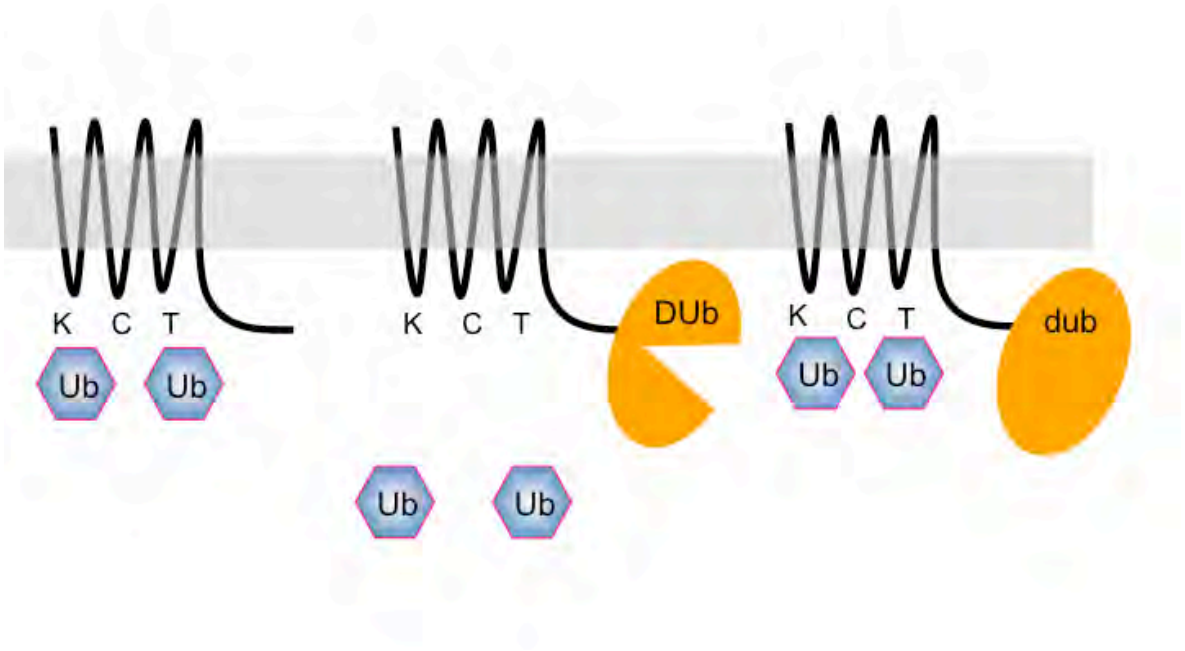

K C T
Ub
Ub
K C T
DUb
Ub
Ub
K C T
Ub
Ub
dub

Figure 3-4. Upgrade of Ste3.

Modification of Ste3-GFP with different catalytic domains from four DUbs; Ubp7, Doa4, Ubp5 or AMSH. In comparison to Ste3-GFP, the Ubp7 and Doa4 cassettes were the most successful at stabilizing Ste3 with localization at the plasma membrane and in puncta. Ubp5 as able to stabilize Ste3 to a lesser degree with localization at the cell surface, in puncta and inside the vacuole and AMSH was the least effective with the majority of GFP inside the vacuole.

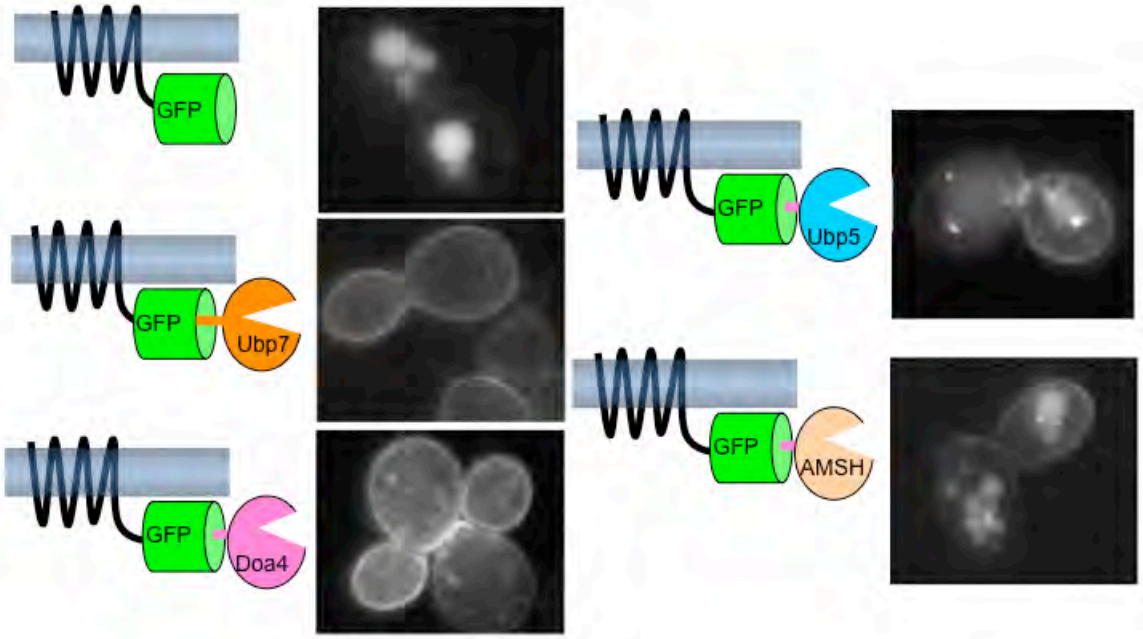

GFP
GFP
Ubp7
GFP
Doa4
GFP
Ubp5
GFP
AMSH

Figure 3-5. Design of Upgrade Cassettes from Virus DUbs.

Upgrade cassettes were generated from two different DUbs from viruses, UL36 (A) (blue) from the HSV-1 virus and M48 (B) (yellow) from the MCMV virus. The catalytic domains from each of these enzymes were cloned behind the linker portion from the Ubp7 DUb cassette (orange). Note the M48 cassette was engineered with small pieces from the UL36 DUb cassette flanking the 5' and 3' portions of the defined M48 catalytic domain.

A. The UL36 Upgrade Cassette

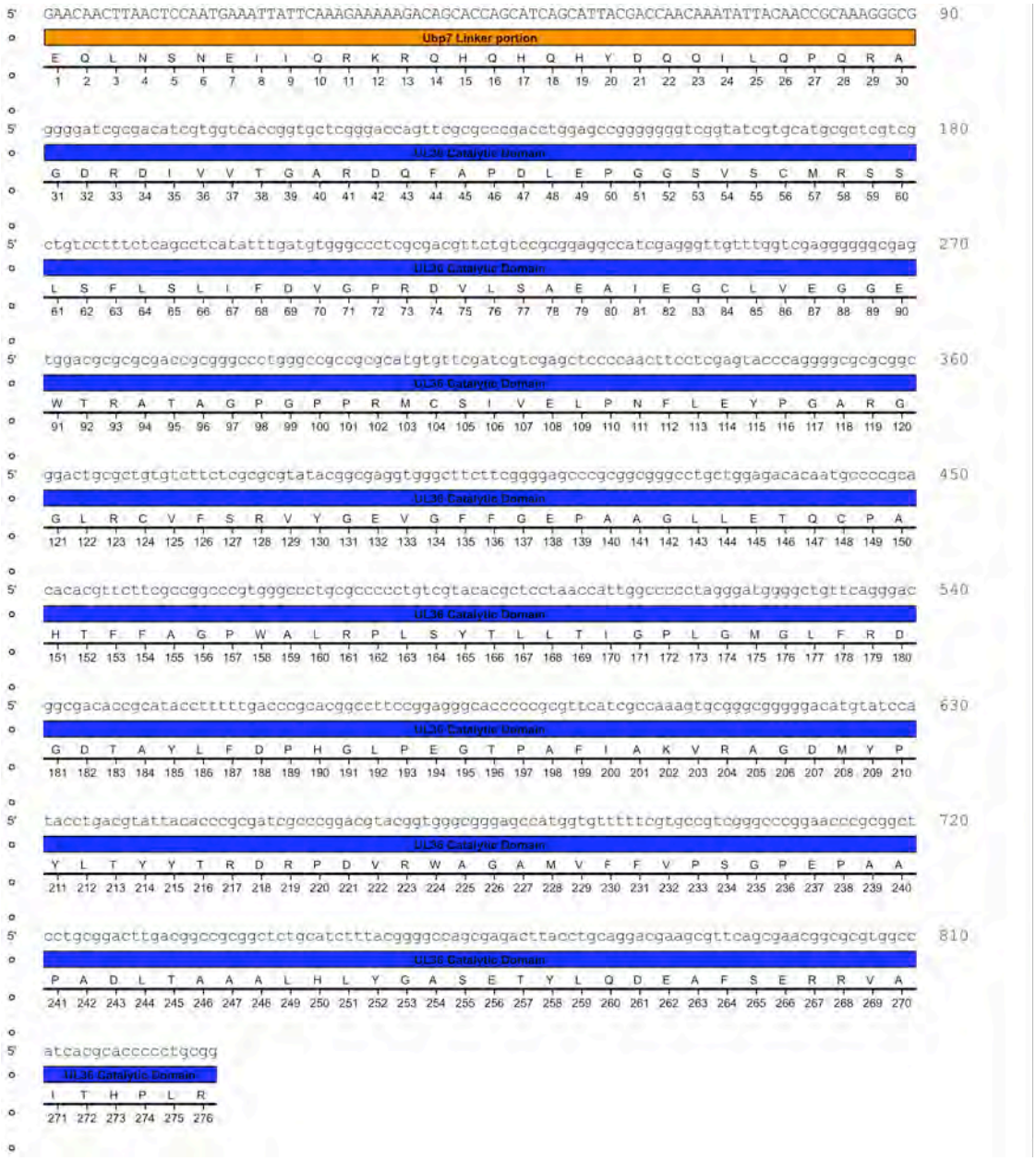


Figure 3-5 continued

B. The M48 Upgrade Cassette

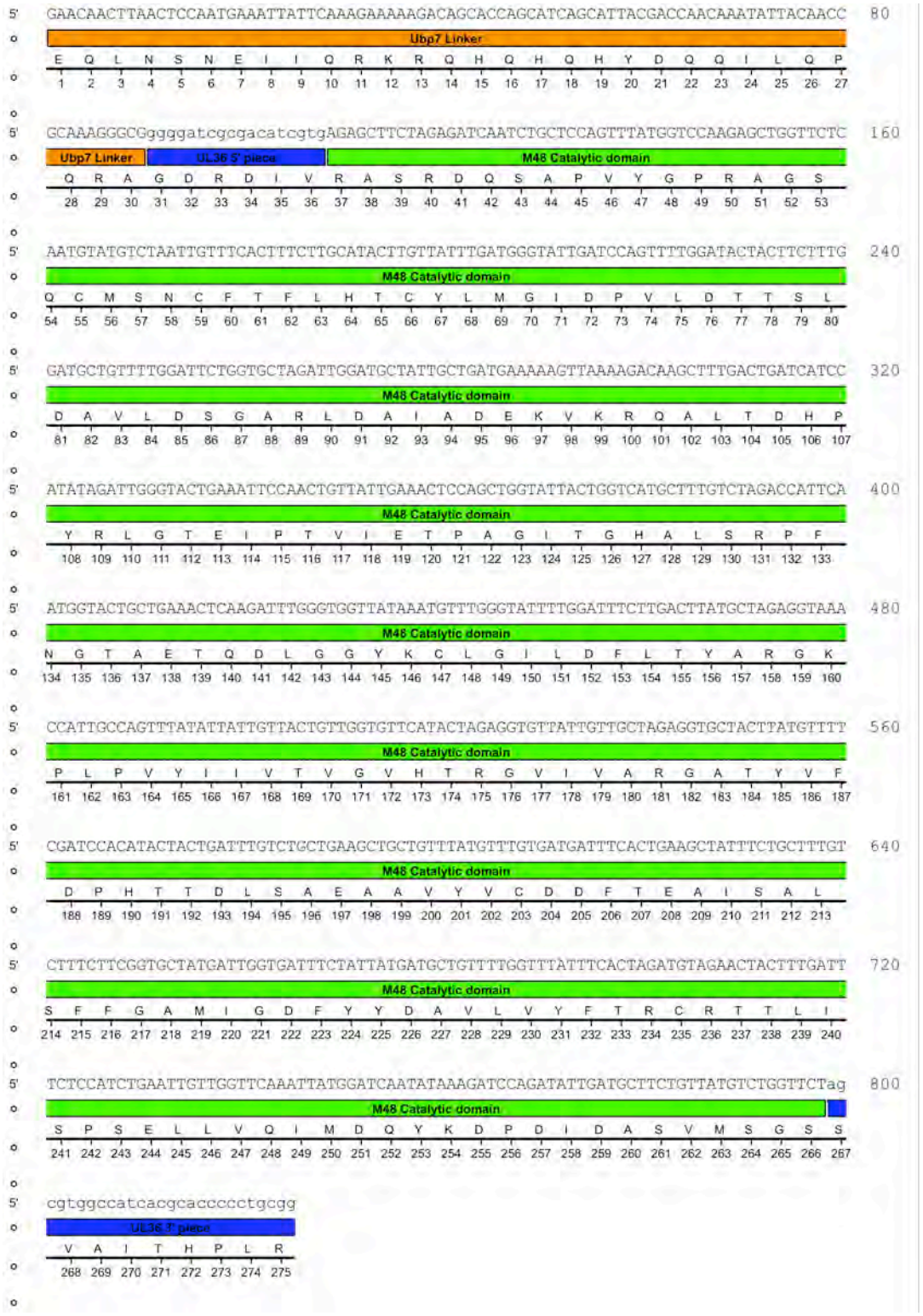


Figure 3-6. The Effect of Upgrade Cassettes from Virus DUbs.

Viral upgrade cassettes render Ste3 resistant to ubiquitination. The viral upgrade cassettes each render Ste3-GFP resistant to ubiquitination, with localization at the plasma membrane and in puncta.

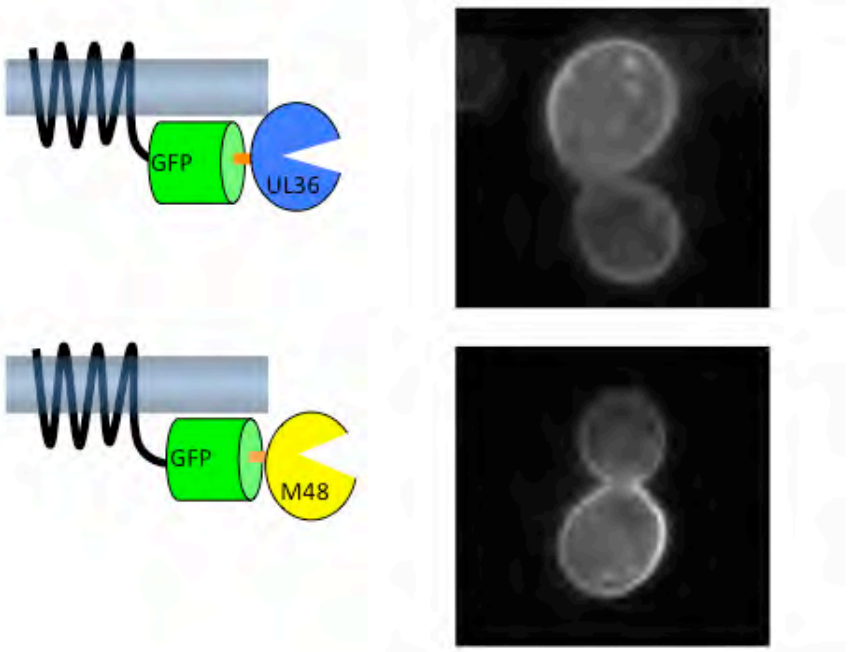

GFP
UL36
GFP
M48

Figure 3-7. Catalytically Inactive Upgrade Cassettes.

Inactivation of the catalytic domain restores ubiquitin dependent sorting. A single point mutation that converts the conserved catalytic cysteine to a serine restores the Ub-dependent localization of Ste3-GFP to the vacuole.

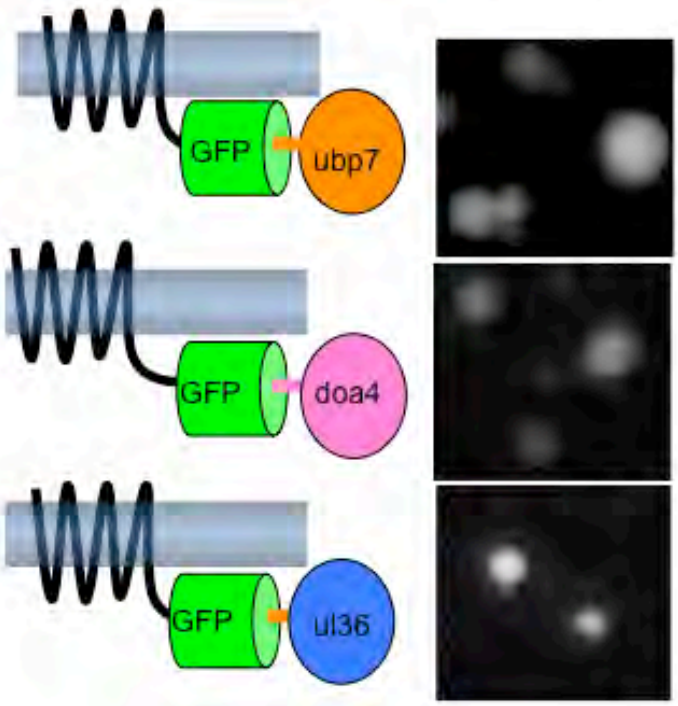

GFP
ubp7
GFP
doa4
GFP
ul36

Figure 3-8. Biochemical Analysis of UL36 Upgrade Cassettes.

Ste3-GFP-UL36 and Ste3-GFP-ul36* were expressed in *end3Δ* cells. (A) Localization by fluorescence microscopy. (B) Lysates were generated and western blot analysis was performed for GFP. PGK levels were assessed as a loading control.

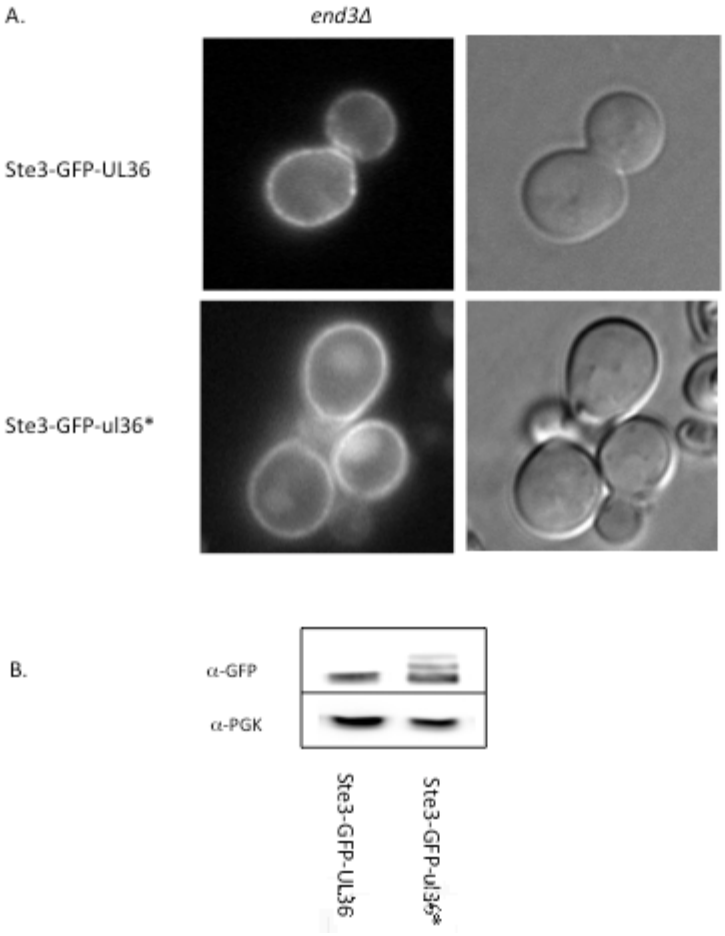

A.
end3Δ
Ste3-GFP-UL36
Ste3-GFP-ul36*
B.
α-GFP
α-PGK
Ste3-GFP-UL36
Ste3-GFP-ul36*

Figure 3-9. Engineering an Inactive M48 Upgrade Cassette.

The inactive m48* upgrade cassette was designed to inactivate the catalytic domain and the ub-binding domain on m48. (A). Sequence map of defined m48* inactive domain. The m48* is depicted in yellow with the conserved cysteine (C56 purple) as well as the hydrophobic residues of m48 that are predicted to contact Ub (L143, Y146, Y225) all converted to alanine. (B) Crystal structure of the catalytic domain from M48 in complex with Ubiquitin (45). The hydrophobic patch (L8, I44, V70) are depicted in yellow with the mutated Ub-binding residues (L143, Y146, Y225) with visible side chains (black spheres) and catalytic cysteine (C56) depicted in purple.

A.

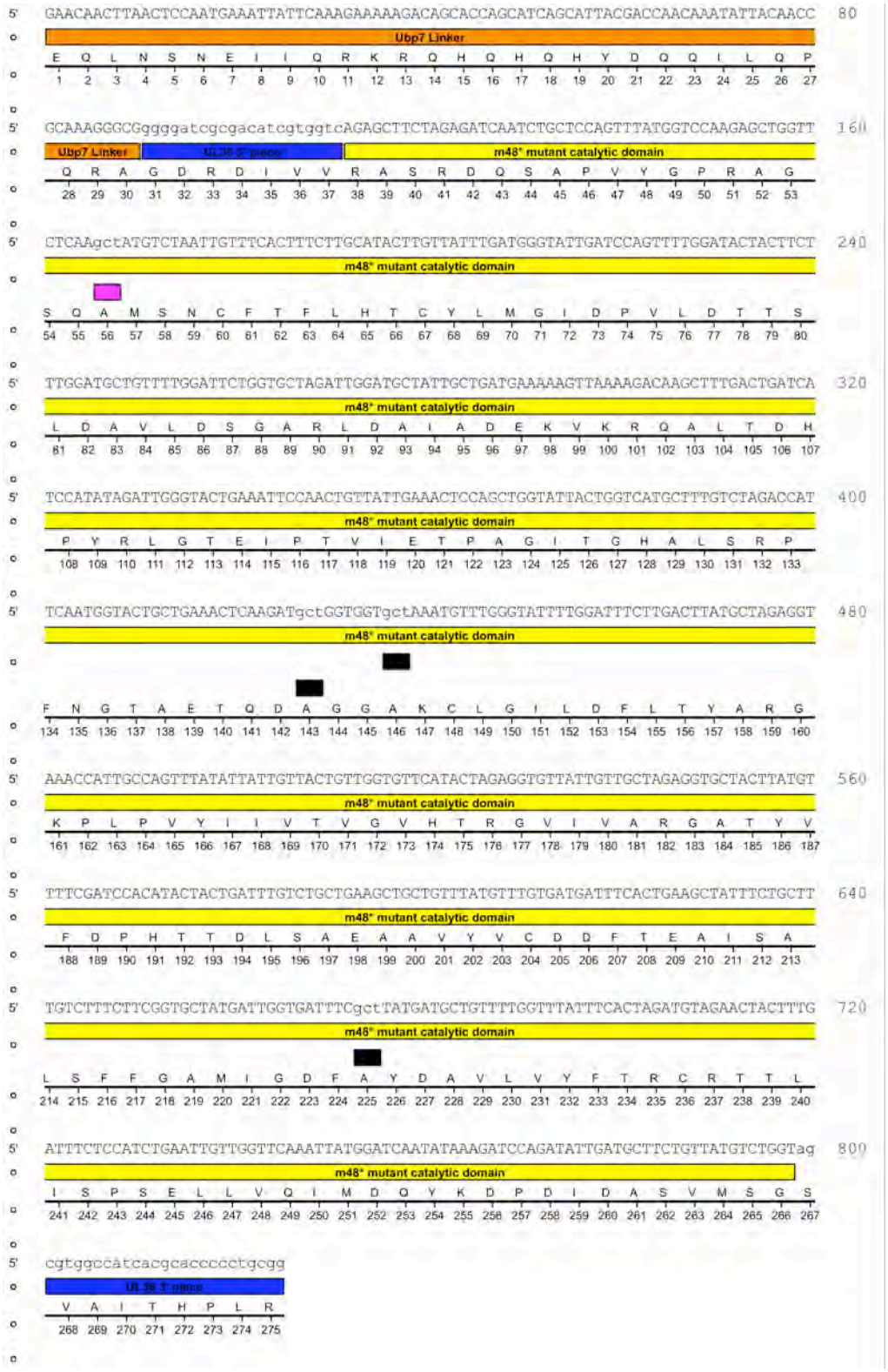

5' GAACAACTTAACTCCAATGAAATTATTCAAAGAAAAAGACAGCACCAGCATCAGCATTACGACCAACAAATATTACAACC 80
Ubp7 Linker
E Q L N S N E I I Q R K R Q H Q H Y D Q Q I L Q P
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
5' GCAAAGGGCGggggatcgcgacatcgtggtcAGAGCTTCTAGAGATCAATCTGCTCCAGTTTATGGTCCAAGAGCTGGTT 160
Ubp7 Linker
m48* mutant catalytic domain
Q R A G D R D I V V R A S R D Q S A P V Y G P R A G
28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53
5' CTCAAgctATGTCTAATTGTTTCACTTTCTTGCATACTTGTTATTTGATGGGTATTGATCCAGTTTTGGATACTACTTCT 240
m48* mutant catalytic domain
S Q A M S N C F T F L H T C Y L M G I D P V L D T T S
54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80
5' TTGCATGCTGTTTTGGATTCTGGTGCTAGATTGGATGCTATTGCTGATGAAAAAGTTAAAAGACAAGCTTTGACTGATCA 320
m48* mutant catalytic domain
L D A V L D S G A R L D A I A D E K V K R Q A L T D H
81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107
5' TCCATATAGATTGGGTACTGAAATTCCAACTGTTATTGAAACTCCAGCTGGTATTACTGGTCATGCTTTGTCTAGACCAT 400
m48* mutant catalytic domain
P Y R L G T E I P T V I E T P A G I T G H A L S R P
108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133
5' TCAATGGTACTGCTGAAACTCAAGATgctGGTGGTgctAAATGTTTGGGTATTTTGGATTTCTTGACTTATGCTAGAGGT 480
m48* mutant catalytic domain
F N G T A E T Q D A G G A K C L G I L D F L T Y A R G
134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160
5' AAACCATTGCCAGTTTATATTATTGTTACTGTTGGTGTTCATACTAGAGGTGTTATTGTTGCTAGAGGTGCTACTTATGT 560
m48* mutant catalytic domain
K P L P V Y I I V T V G V H T R G V I V A R G A T Y V
161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187
5' TTTCGATCCACATACTACTGATTTGTCTGCTGAAGCTGCTGTTTATGTTTGTGATGATTTCACTGAAGCTATTTCTGCTT 640
m48* mutant catalytic domain
F D P H T T D L S A E A A V Y V C D D F T E A I S A
188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213
5' TGTCTTTCTTCGGTGCTATGATTGGTGATTTCgctTATGATGCTGTTTTGGTTTATTTCACTAGATGTAGAACTACTTTG 720
m48* mutant catalytic domain
L S F F G A M I G D F A Y D A V L V Y F T R C R T T L
214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240
5' ATTTCTCCATCTGAATTGTTGGTTCAAATTATGGATCAATATAAAGATCCAGATATTGATGCTTCTGTTATGTCTGGTag 800
m48* mutant catalytic domain
I S P S E L L V Q I M D Q Y K D P D I D A S V M S G S
241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267
5' cgtggccatcacgcacccccctgcgg
UL36 3' piece
V A I T H P L R
268 269 270 271 272 273 274 275

Figure 3-9 continued

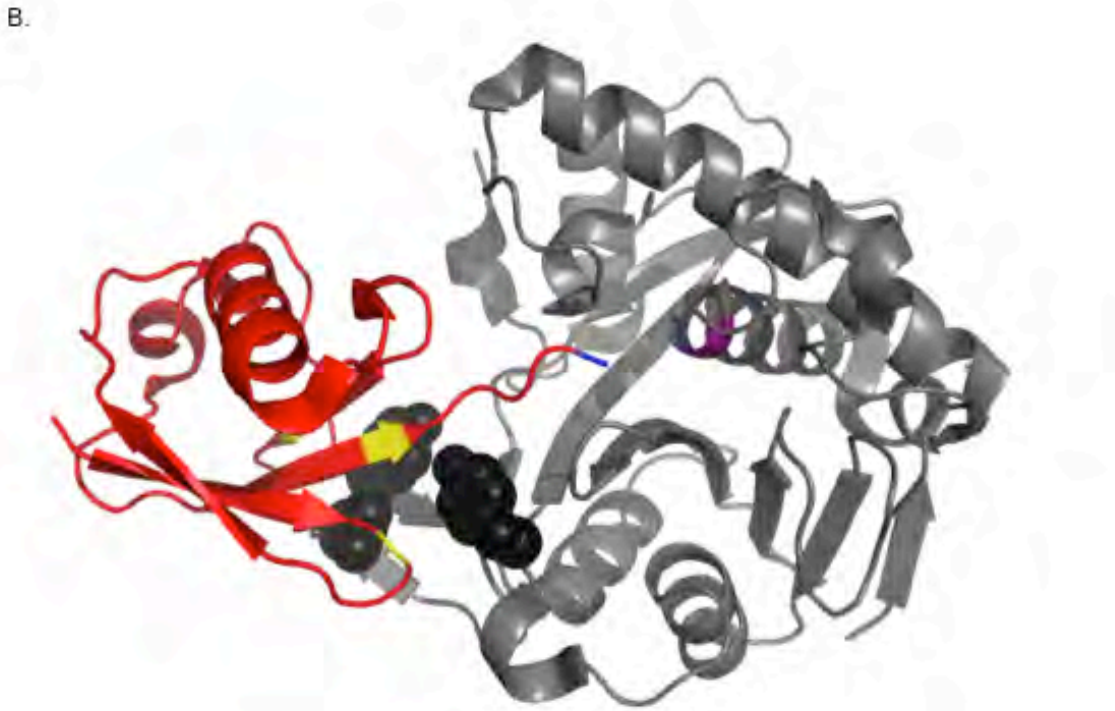


Figure 3-10. The Effect of Catalytically Inactive m48* on Ste3.

The m48* cassette was mutated (C56,L143, Y146, Y225) to inactivate both catalytic activity and Ub binding (see Fig. 3-9). Mutant m48* was fused to Ste3-GFP and expressed in WT cells. Vacuolar localization indicates that the cassette is no longer active.

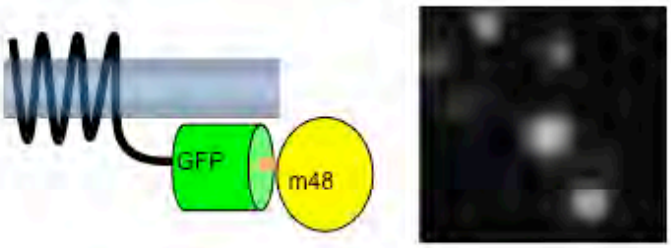

GFP
m48

Figure 3-11. Protein Upgrade of Yeast Permeases.

Upgrade was tested using the general amino acid permease Gap1, the hexose transporter Hxt1 and the uracil transport Fur4. Gap1-GFP, Hxt1-GFP and Fur4-GFP all have a steady state distribution in the yeast vacuole. The Ubp7 cassette was able stabilize each of the permeases at the plasma membrane. The inactive ubp7* cassette restored Gap1 and Hxt1 trafficking to the yeast vacuole.

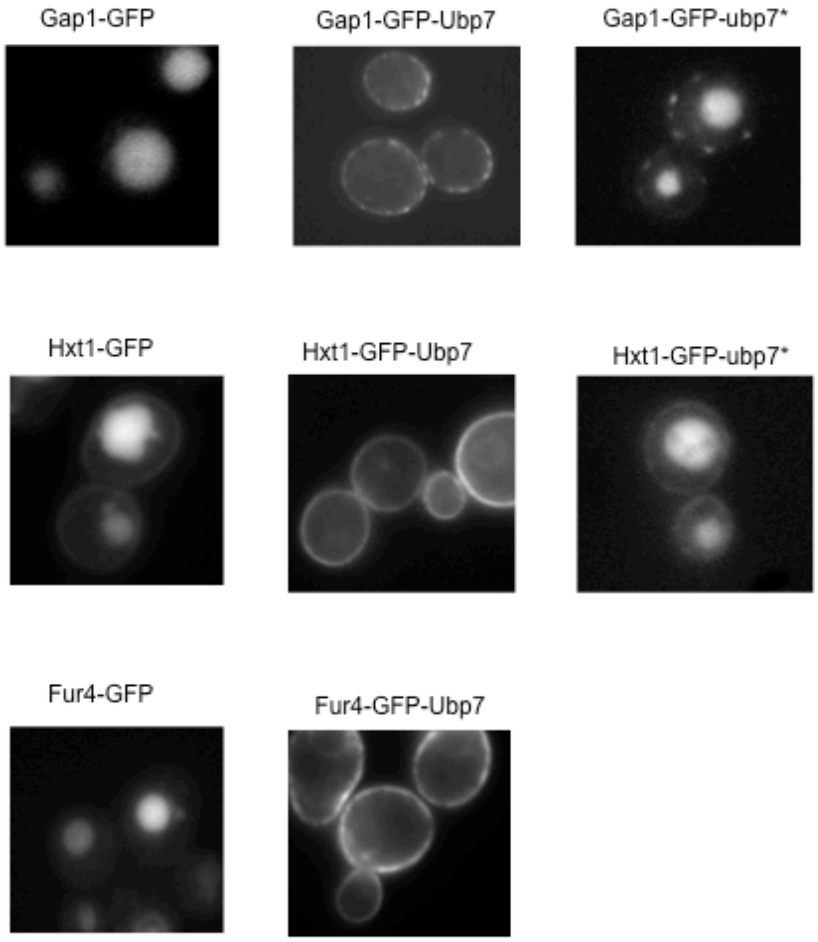

Gap1-GFP
Gap1-GFP-Ubp7
Gap1-GFP-ubp7*
Hxt1-GFP
Hxt1-GFP-Ubp7
Hxt1-GFP-ubp7*
Fur4-GFP
Fur4-GFP-Ubp7

Figure 3-12. Specificity of Protein Upgrade.

Ste3-RFP-Ubp7 and Fur4-RFP-Ubp7 were co-expressed in cells with Ste3-GFP or Gap1-GFP to examine whether upgrade of one protein could block the Ub-dependent trafficking of another protein. Merged images (center right) and DIC images (far right) are as indicated.

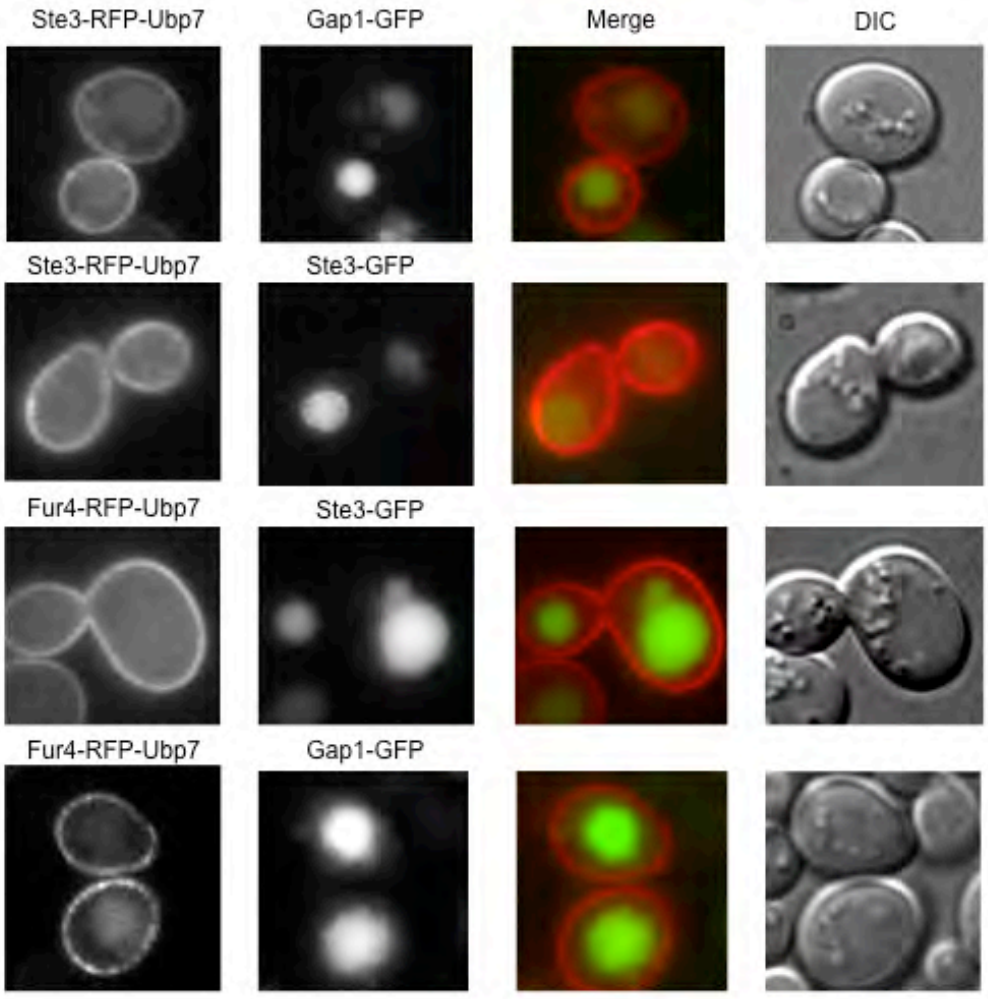

Ste3-RFP-Ubp7
Gap1-GFP
Merge
DIC
Ste3-RFP-Ubp7
Ste3-GFP
Fur4-RFP-Ubp7
Ste3-GFP
Fur4-RFP-Ubp7
Gap1-GFP

Figure 3-13. Autocatalysis of Ubp7 Upgrade Cassette.

Cells expressing Ste3-GFP-Ubp7-3xHA were subjected to western blot analysis for HA. Long exposure to film shows Ste3-GFP-Ubp7-3xHA at the predicted size of approximately 140 KDa but also smaller bands at 72 KDa, 36 KDa and a prominent band at 17 KDa.

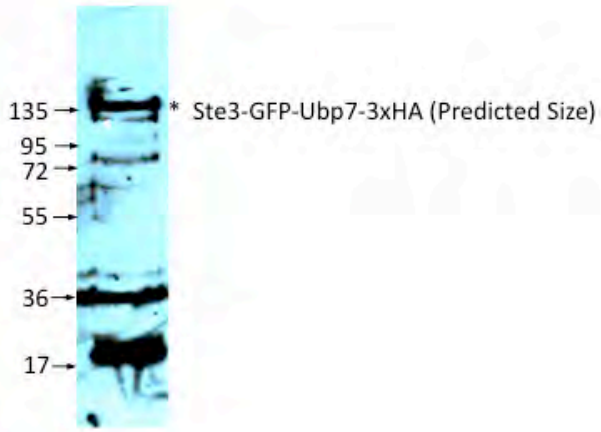

135
95
72
55
36
17
* Ste3-GFP-Ubp7-3xHA (Predicted Size)

Figure 3-14. Inactivation of Ubp7 Prevents Autocatalysis.

Inactivation of catalytic domain from Ubp7 upgrade cassette renders the protein non-cleavable. Fusion of the active and inactive Ubp7 cassettes to the ESCRT-I subunit Mvb12.

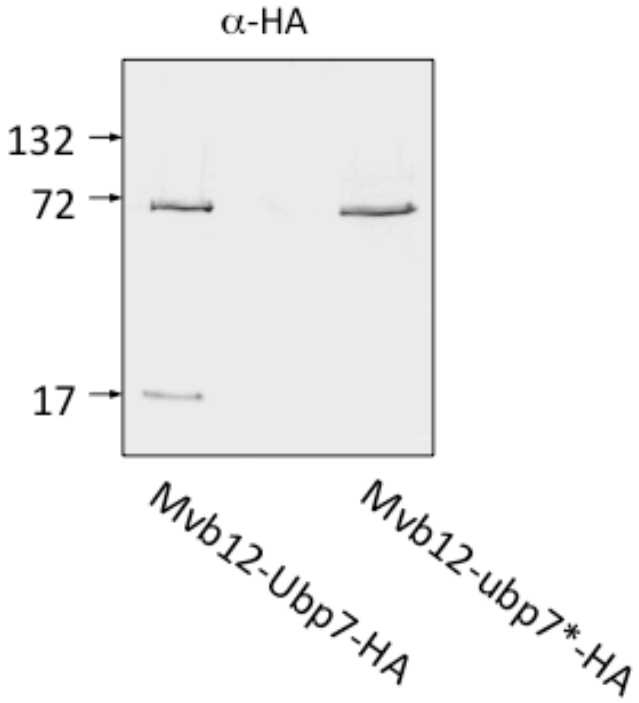
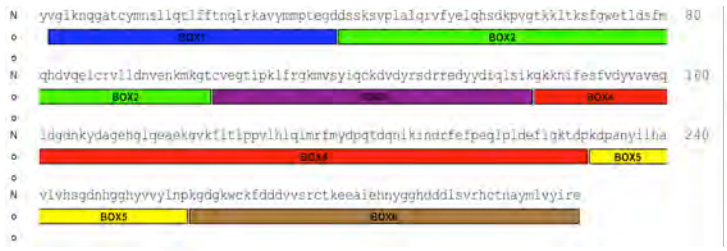
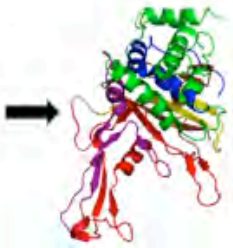

α-HA
132
72
17
Mvb12-Ubp7-HA
Mvb12-ubp7*-HA

Figure 3-15. Sequence Position of Di-glycine Motifs in Ubp7 Catalytic Domain. Conserved domains of HAUSP/USP14 and Ubp7 upgrade cassette (left) and the crystal structure of HAUSP (PDB identifier 2F1Z) (164). Conserved “boxes” are as indicated in blue (box1), green (box2), purple (box3), red (box4), yellow (box5) and brown (Box6). Black domains/arrow indicate a di-glycine motif in an unstructured loop between box4 and box5.

HAUSP/USP14 Catalytic Domain

HAUSP/USP14 Catalytic Domain Structure



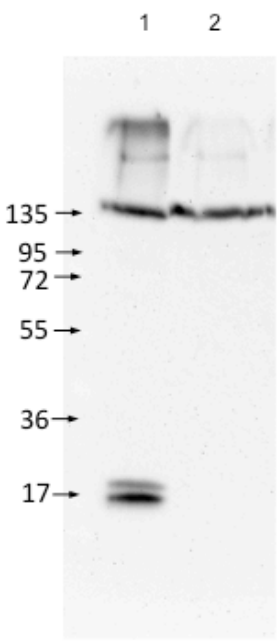


UBP7 Upgrade Cassette

Figure 3-16. Alteration of Di-Glycine Motifs in the Ubp7 Cassette.

The predicted di-glycine motif within the Ubp7 upgrade cassette was mutated from GG to GS. (A) Western blot analysis of (1) Ste3-GFP-Ubp7-3xHA and (2) Ste3-GFP-Ubp7$^{G>S}$-3xHA. The 17kDa band is no longer present indicating that mutation of the di-glycine motif no longer results in autocatalysis. (B) Localization of Ste3-GFP-Ubp7$^{G>S}$-3xHA in WT cells.

A.



B.

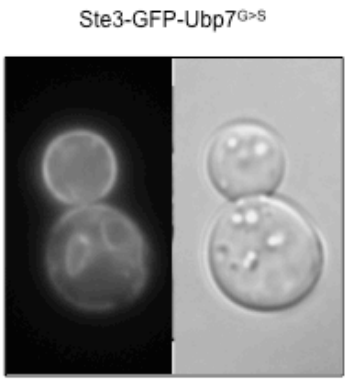
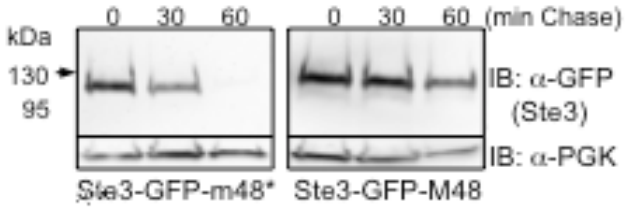


Figure 3-17. Stability of Proteins Modified by Upgrade.

A cycloheximide chase was performed with WT cells expressing Ste3-DUb-HA fusions. (A). Ste3-GFP fused to catalytically active and inactive (*) M48, (B). The active and inactive (*) catalytic domain from Ubp7 (note: The active Ubp7 cassette was ΔGG mutant described previously in Fig. 3-16 to prevent autocatalysis) or Ste3-GFP alone. Fusion of active catalytic domains stabilizes Ste3-GFP, while fusion of inactive catalytic domain does not.

A.


0 30 60 0 30 60 (min Chase)
kDa
130
95
IB: α-GFP
(Ste3)
IB: α-PGK
Ste3-GFP-m48*
Ste3-GFP-M48

B.

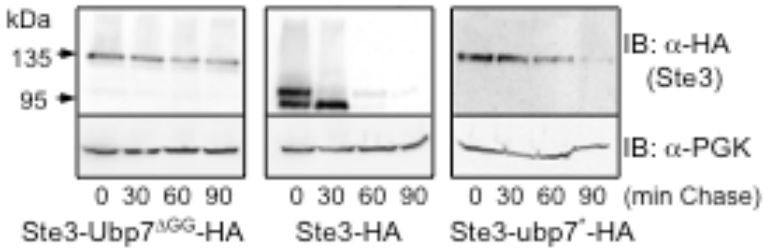
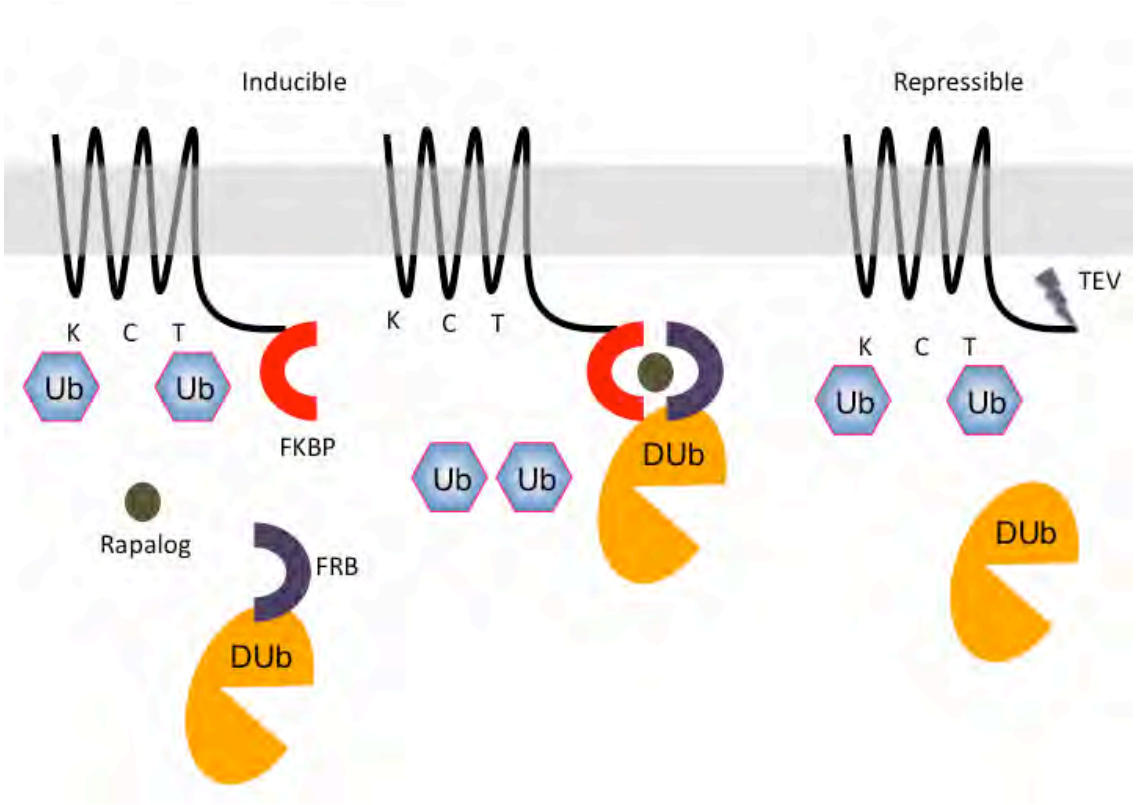

kDa
135
95
IB: α-HA
(Ste3)
IB: α-PGK
0 30 60 90 0 30 60 90 0 30 60 90 (min Chase)
Ste3-Ubp7ΔGG-HA
Ste3-HA
Ste3-ubp7*-HA

Figure 3-18. Schematic of Inducible and Repressible Upgrade Cassettes. Schematic depicting chemically inducible upgrade using FRB and FKBP with a rapalog (left) or repressible upgrade using a protease cleavage site such as TEV (164). FRB and FKBP dimerize in the presence of rapamycin or a rapalog. By fusing these proteins to a DUb cassette and to a target for protein upgrade, the addition of a rapalog would make the fusion of an upgrade cassette chemically inducible. TEV sites between the upgrade cassette and the protein of interest could also be generated. Here, upgrade could be repressed by the expression or addition of a protease like TEV.


Inducible
Repressible
K C T
Ub
Ub
FKBP
Rapalog
FRB
DUb
TEV

CHAPTER 4: COUPLED UBIQUITINATION

Abstract

As a general rule, ubiquitination of proteins leads to their degradation by the proteasome or lysosome. However, sometimes ubiquitination of a protein causes other changes that are unrelated to protein degradation. Virtually all proteins that contain Ub-binding domains also get ubiquitinated themselves, which has lead to speculation as to the potential role of the ubiquitination of Ub-binding proteins. In mammalian cells, the ESCRT-0 subunits Hrs and STAM get ubiquitinated as well as the ESCRT-I subunit Tsg101. While much is known about the molecular mechanisms responsible for ubiquitination of Ub-binding proteins, very little data exists as to the physiological role of ESCRT ubiquitination. We used both conventional methods as well as protein upgrade to determine whether ESCRT ubiquitination played a central role in how ESCRTS operate. We found that while several ESCRT subunits do get ubiquitinated in yeast, preventing their ubiquitination or increasing their ubiquitination does not affect their ability to direct ubiquitinated cargo into MVBs for eventual degradation by the yeast vacuole.

Introduction

Many ESCRT proteins have UBDs including the ESCRT-0 subunits Hse1 and Vps27 (STAM and Hrs in mammalian cells), which contain VHS and UIM domains. In addition, ESCRT-I has two subunits that bind Ub, Mvb12 and Vps23, while ESCRT-II has one subunit that binds Ub, Vps36 (133, 150, 161, 197). Like other UBD containing proteins, ESCRTs can be ubiquitinated by a

variety of mechanisms that are mediated by the UBD itself in a process known as “Coupled Ubiquitination” (48, 59, 115, 193).

Several models have been proposed for how UBD containing proteins are ubiquitinated in cells. Some Ub-ligases get ubiquitinated, which could potentially mediate interactions between the Ub-ligase and UBD containing proteins, leading to the ubiquitination of the UBD (Fig. 4-2) (48, 163, 193). Alternatively, some UBD containing proteins, like the ESCRT-0 proteins, associate with several Ub-ligases and DUbs that could potentially regulate the ubiquitination status of ESCRTs (2, 71, 77, 106, 142). For instance, the HECT-type Ub-ligase Rsp5, interacts with ESCRT-0 in yeast. Some UBD proteins, including the ESCRT-0 subunit Hrs, can also be ubiquitinated without an E3 enzyme (60). This observation mainly comes from *in vitro* studies where UBD proteins were mixed with E2 enzymes. However the authors of this study also knock down two E3 ligases in cells Nedd4 and AIP4 which both interact with the endocytic machinery, and demonstrate that Hrs is ubiquitinated to similar levels as control cells (102, 193).

Coupled ubiquitination has been proposed as a mechanism that regulates critical aspects of ESCRT function by altering intramolecular interactions between ESCRTs with UBDs and ubiquitinated ESCRTs (31, 54, 97, 163). Alternatively, ubiquitination might provide a mechanism to dislodge the ESCRT proteins from the cargo itself (Fig. 4-2) (56, 87, 133, 134, 150, 191). One other possibility is that ubiquitination of ESCRTs and other UBD containing proteins does not regulate their activity at all, and is simply an artifact. Definitive evidence that coupled ubiquitination is a necessary process for ESCRTs to function properly remains elusive. Many studies describe the Ub ligases and DUbs that might be contributing to ESCRT and UBD-containing protein ubiquitination (2, 31, 69, 101, 105, 164). AMSH is a DUb that associates with ESCRT-0. Two recent

studies show depletion of AMSH can result in MVB sorting defects for the chemokine receptor CXCR4 (101, 164). The exact role of AMSH here is unknown, but the authors conclude that because more ubiquitinated Hrs is detected in cells depleted for AMSH, that ubiquitination of Hrs mechanistically controls CXCR4 degradation. Another interpretation is that AMSH contributes to the MVB sorting of membrane proteins in some other way. For instance, MVB cargo could potentially be "overubiquitinated" for efficient incorporation into ILVs by ESCRTs. AMSH could potentially be modifying the ubiquitination status of every membrane protein at the sites of MVB formation. Even if depletion of AMSH does not appear to affect the ubiquitination of the CXCR4 receptor directly, other MVB cargos that are potentially overubiquitinated in cells lacking AMSH could overwhelm the MVB sorting machinery. Other work suggests that similar depletion of AMSH leads to enhanced degradation of EGFR and that AMSH deubiquitnates EGFR directly, thus rescuing EGFR from an otherwise very efficient Ub-mediated MVB sorting fate (106). Does depletion of AMSH lead to an enhancement or reduction in MVB cargo sorting? It is simply too difficult to answer without tracking which proteins are getting deubiquitinated and whether AMSH has other functions apart from its DUb activity. Studies like these where many proteins are potentially affected by the manipulation of a DUb or E3 ligase are common, but make it difficult to draw conclusions about specific processes like ubiquitination of ESCRTs.

To really understand if coupled ubiquitination is affecting ESCRT function, ubiquitination of Ub-binding proteins must be "uncoupled". It is very difficult to "uncouple" coupled ubiquitination, as a functional UBD is required for coupled ubiquitination to take place (54, 115, 193). Inactivation of UBDs is likely to lead to physiological consequences that are unrelated to ubiquitination of UBD containing protein. To circumvent this problem, some studies make use of in-

frame fusions of Ub to Ub-binding proteins (59, 105). This approach is a common technique used to recapitulate ubiquitination of proteins. The advantage of an approach like this one is that the ubiquitination status of a single protein can be manipulated. One study reported that overexpression of Hrs-Ub fusion protein was unable to recapitulate a phenotype that results upon overexpression of Hrs alone (59). These studies followed the trafficking of a Ub-TfR chimeric protein. Transferrin Receptor (TfR), normally cycles between the plasma membrane and endosomes. A Ub-TfR fusion diverts the receptor into the MVB pathway (137). Overexpression of Hrs enhances that effect (59, 137). However, overexpression of Hrs-Ub does not enhance Ub-TfR the effect. This is likely because the UIM domains of Hrs are bound to the fused Ub and therefore cannot bind Ub-TfR. The authors of this study conclude that ubiquitination of Hrs modulates Hrs activity. These data are also open to other interpretations. Several studies demonstrate that overexpression of Hrs causes abnormal endosome morphology and function (86, 88, 182, 185). Hrs has a FYVE domain which binds to PI(3)P on endosomes, as do several other proteins associated with endosomes. One possibility is that Hrs overexpression causes a redistribution of endosomal proteins because all of the PI(3)P on endosomes is bound by Hrs.

One study on the ESCRT-I subunit Tsg101 describes an entirely different physiological outcome for the ubiquitination of Tsg101, where the ubiquitin ligase Tal1 contributes to the ubiquitination of Tsg101 leading to its degradation by the proteasome. Tsg101 is also required for the release of HIV and the authors found no difference in the amount of virus released from cells expressing either WT or Tsg101 lacking several lysines that contribute to the ubiquitination of Tsg101. The authors conclude that ubiquitination of Tsg101 is not required for ESCRT-1 function (108). However, it is difficult to make conclusions about

ESCRT function related to MVB sorting using an assay for viral release. Also, some residual ubiquitination is still visible for Tsg101 lacking lysines. Finally, this study uses a pulldown to examine ubiquitination of Tsg101. The assay is performed under non-denaturing conditions. Many other studies make use of non-denaturing pulldowns or immunoprecipitations to examine ubiquitination of Ub-binding proteins (59, 60, 101, 105, 106, 164). Thus, for many of these studies it is impossible to determine which ubiquitinated protein is being examined.

Taken together, it is clear that coupled ubiquitination as a regulatory mechanism for UBD containing proteins, especially the ESCRT complex proteins, merits further study. We employed both conventional techniques and the protein upgrade technique to explore the physiological role of coupled ubiquitination as it relates to the function of ESCRT complex proteins during MVB formation. Our data reveal that coupled ubiquitination is dispensable for ESCRT function and for cargo release by ESCRT subunits.

Results

Mutational Analysis of Vps27

To determine if Vps27 gets ubiquitinated in yeast, Vps27 and Myc-Ub were expressed in *WT*, *vps27Δ, and vps4Δ* strains. Lysates from those strains were subjected to western blot analysis for Vps27, which indicated that Vps27 does get ubiquitinated in yeast (Fig. 4-3). To further analyze the ubiquitination of Vps27, WT cells expressing HA-Vps27 and Myc-Ub were subjected to IP under denaturing conditions. Western blot analysis of the IPs confirmed that Vps27 does get ubiquitinated with majority of the Vps27 migrating at ~96kDa, with a less prominent band at ~105kDa corresponding to ubiquitinated Vps27 (Fig. 4-3).

Myc blots confirmed that that the higher molecular weight bands were in fact ubiquitination (Fig 4-3). Next, to generate a version of Vps27 that could not be ubiquitinated, each of the 47 lysine residues (Fig. 4-4A) within Vps27 was converted to an arginine and IP analysis from cells lacking Ubp2, a DUb that antagonizes the activity of the yeast HECT Ub ligase Rps5 (75, 76, 148). A sizable proportion of Vps27 (HA-Vps27) was ubiquitinated in *ubp2Δ* cells as ~20% was found at ~105kDa corresponding to ubiquitinated Vps27 (Fig. 4-4B). In contrast, no detectible ubiquitination for Vps27 (HA-Vps27$^{K>R}$) lacking lysine residues was found in *ubp2Δ* (Fig4-4B).

With a version of Vps27 that does not get ubiquitinated in hand, cells lacking endogenous Vps27 (*SF838-9D* background) were co-transformed with plasmids that express either HA-Vps27^{WT} or HA-Vps27$^{K>R}$ along with the G-protein coupled receptor Ste3-GFP (described in Chapter 3). In cells without any Vps27, Ste3-GFP and Ste3-GFP-Ub (an in-frame fusion of Ub) failed to sort to the yeast vacuole with localization on the limiting membrane of the vacuole, in dots and at the plasma membrane (Fig. 4-5). Interestingly, both HA-Vps27^{WT} and HA-Vps27$^{K>R}$ were sufficient to complement for the loss of endogenous Vps27 as both Ste3-GFP and Ste3-GFP-Ub as both proteins were visualized in the yeast vacuole. HA-Vps27^{WT} and HA-Vps27$^{K>R}$ were also tested with Ste3-GFP in a strain lacking Ubp2 and Vps27 (*BY4742* background) and both versions of Vps27 restored sorting of Ste3-GFP to the vacuole (Fig. 4-6).

In mammalian cells the Hse1 homolog STAM gets ubiquitinated (149, 164), raising the possibility that Hse1 and not Vps27 is regulated by coupled ubiquitination for the ESCRT-0 complex. To test this we took advantage of a strain background (*SEY6210*) that requires Vps27 but not Hse1 for MVB sorting. Thus, *hse1Δ, vps27Δ* cells expressing Vps27$^{K>R}$ would eliminate all lysines from

ESCRT-0. Here we found that Ste3-GFP soring was normal in cells expressing either WT or the K>R mutant Vps27 as their sole source of ESCRT-0.

The affect of Rsp5 on ESCRT ubiquitination

Hrs associates with HECT-type Ub ligases such as Nedd4 and AIP/Itch, which mediate Hrs ubiquitination both *in vivo* and *in vitro* (72, 102, 193). The homolog of Nedd4 in yeast is Rps5, which interacts with ESCRT-0 (142). Based on these observations, we wanted to assess whether Rsp5 was responsible for ubiquitinating Vps27. If Rsp5 was responsible for Vps27 ubiquitination, we could eliminate Rsp5 activity and determine of MVB sorting would still occur. *RSP5* is an essential gene as Rsp5 activity is required for the ubiquitination of two transcription factors, Mga2 and Spt23, which are subsequently cleaved by the proteasome and translocate from the ER to the nucleus where they facilitate oleic acid synthesis (19, 61, 201).

One way to circumvent the necessity for *RSP5* is to use the temperature sensitive allele *rsp5-1*, which has reduced Rsp5 activity at the non-permissive temperature of 37°C. IP analysis from cells expressing HA-Vps27 and Myc-Ub at 37°C revealed that ubiquitination of Vps27 was virtually undetectable in *rsp5-1* cells but occurred normally in the WT counterpart (Fig. 4-8A). Many studies have shown that Rsp5 activity is required for the incorporation of a variety of cargo proteins into MVBs (70, 148). These findings were confirmed for Gap1-GFP and Fur4-GFP, which had severe vacuolar sorting defects upon induction and temperature shift (Fig. 4-8B). An in-frame fusion of Ub to Mup1-GFP restored the vacuolar localization of Mup1, indicating that MVB sorting can proceed normally in cells lacking ESCRT ubiquitination (Fig. 4-8B).

Unfortunately, *rsp5-1* cells still harbor a low level of activity at the non-permissive temperature, which could potentially drive a low level of ESCRT ubiquitination (76). One way to bypass the essential requirements for Rsp5 activity is to express a truncated version of either Spt23 or Mga2 (19, 61). Using the truncated form of Mga2, cells lacking *RSP5* were generated which grew slowly at 30°C and were inviable at 37°C (Fig. 4-9B). Sorting was defective for Ste3-GFP, Gap1-GFP and Mup1-GFP in *rsp5Δ* cells. This result was expected since all of these proteins require Rsp5. However, fusion of Ub to Mup1-GFP and Ste3-GFP completely restored the vacuolar localization of those cargos, indicating that Rsp5-dependent ESCRT ubiquitination are not required for MVB sorting (Fig 4-9B).

Protein Upgrade of ESCRT proteins

Thus far, two conventional methods to make proteins resistant to ubiquitination have demonstrated that ESCRT ubiquitination is not required for ESCRTs to function as MVB sorting receptors. However, each of these approaches has caveats. While Vps27$^{K>R}$ was sufficient to complement for the loss of endogenous Vps27 in multiple strain backgrounds, other ESCRT proteins could still be getting ubiquitinated. Mutation of every potential ubiquitinatable residue within every one of the ESCRT subunits would likely lead to uninterpretable results. Moreover, attenuating Rsp5 activity might not be sufficient to eliminate ESCRT ubiquitination as other Ub-ligases could be compensating for the loss of Rsp5 and there are also instances where UBDs can be ubiquitinated without the activity of an E3 ligase (60).

Protein upgrade was employed to make ESCRT proteins resistant to ubiquitination. Two different DUb catalytic domains were fused to Hse1, Vps23, Mvb12 and the Golgi/ESCRT associated protein Gga2 (Fig. 4-10). First, upgrade

was tested by IP analysis to see if ESCRTs could be rendered resistant to ubiquitination. Catalytically active and inactive UL36 fused to Hse1. Fusing DUb catalytic domains to ESCRTs should not only prevent the ubiquitination of the ESCRTs themselves, but if suitably robust, the DUb should also strip Ub from cargo proteins and thus blocking their entry into the MVB lumen. Furthermore, catalytically inactive DUb fusions should not prevent the ubiquitination of ESCRTs or block MVB sorting. Cells expressing Hse1-UL36-3xHA (active) or Hse1-ul36*-3xHA (inactive), HA-Vps27 and myc-Ub were subjected to IP for HA. Western blots for HA and myc indicated that when fused to the active upgrade cassette, neither Hse1 nor Vps27 are ubiquitinated whereas when Hse1 was fused to the inactive cassette, both proteins are ubiquitinated (Fig. 4-11). Next, ESCRT-DUb fusions were tested in cells to see if they could block cargo proteins from entering the vacuole. The Ubp7 and UL36 upgrade cassettes were fused to Hse1, Mvb12, Vps23, Gga2 and His3, with an HA tag on the C-terminus of each fusion protein and expressed in WT cells (Fig. 4-12). The inactive ubp7* and ul36* cassettes were also fused to Mvb12 and Hse1 respectively (Fig. 4-12). Ste3-GFP, Fur4-GFP, Gap1-GFP and Mup1-GFP were co-expressed with ESCRT-DUb fusions and Hse1-DUb, Vps23-DUb and Mvb12-DUb were all capable of preventing each cargo protein from reaching the vacuole with localization at the plasma membrane, in dots and on the limiting membrane of the vacuole (Fig. 4-13). This indicates that ESCRT-DUb fusions were not only robust enough to block ubiquitination of themselves, but were also robust enough to block MVB cargo that they come into close contact with. We also tested the effect of fusing a DUb catalytic domain to GGA2, a Ub-binding clathrin adaptor protein thought to sort ubiquitinated proteins from the TGN to endosomes (160). GGA proteins may also contribute to Ub-dependent transport of proteins from the cell surface to endosomes in yeast, however, evidence for a clear role in this

pathway is obscured since ESCRT-0 also operates in this pathway in parallel (28, 36, 91). Interestingly, Gga2-DUb was capable of blocking Fur4-GFP, Gap1-GFP and Mup1-GFP but not Ste3-GFP, potentially uncovering a cargo specific role for Gga2 at the sites of MVB formation. Importantly, the block in cargo sorting for all cargo was alleviated when the catalytic domain from either DUb cassette was inactivated (Fig. 4-13). This indicates that a protein fusion to the C-terminus of ESCRTs does not simply block cargo, but that the deubiquitinating activity of the DUb specifically blocks ubiquitinated cargo from entering the vacuole. To examine whether cargo proteins could still reach the vacuole in the absence of ubiquitinated ESCRT subunits, Mup1-GFP-Ub and Gap1-GFP-Ub were used, as these cargo proteins should not be susceptible to de-ubiquitination by DUbs. For each of the ESCRT-DUb fusions tested in Figure 4-13, Mup1-GFP-Ub and Gap1-GFP-Ub were properly sorted to the vacuole (Fig. 4-14). These data indicate that MVB sorting can proceed in the absence of ESCRT ubiquitination. His3-DUb had no affect on any of the cargo proteins tested (Fig. 4-13, Fig. 4-14) and none of the ESCRT-DUb fusions caused CPY secretion (Fig. 4-20).

ESCRT-DUb fusions were also tested to determine if they could substitute for their wildtype counterparts. Cells lacking either Hse1 or Vps23 failed to sort Ste3-GFP or Gap1-GFP into the lumen of the vacuole. However, both cell types were able to sort Ste3-GFP-Ub or Fur4-RFP-Ub (Fig. 4-15) when Hse1-UL36 or Vps23-UL36 was expressed. Both Ste3-GFP and Ste3-GFP-Ub were able to reach the vacuole in *hse1Δ* cells when Hse1-ul36* was expressed. Previous data demonstrated that Mvb12 sorting defects are only revealed when the Ub-binding domains within Vps23 and Vps36 are inactive (161). Ste3-GFP and Ste3-GFP-Ub expressed in cells harboring these mutations failed to enter the lumen of the vacuole, consistent previous findings (Fig. 4-15). Expression of

Mvb12-Ubp7-3xHA or Mvb12-UL36 allowed Ste3-GFP-Ub to enter the vacuole. However, Ste3-GFP, which is vulnerable to the ESCRT-DUbs, was blocked (Fig. 4-15). Inactivation of the catalytic domain from Ubp7 allowed for both Ste3-GFP and Ste3-GFP-Ub to reach the vacuole (Fig. 4-15).

Upgrade of Rps5 was also employed as a method to block the ubiquitination of Rsp5 substrates as well as the ESCRTs since Rsp5 interacts directly with ESCRT-0. Active and inactive Ubp7 catalytic domains were fused to the C-terminus of Rsp5. A slight growth defect at 30°C and a more severe growth defect 37°C in WT cells expressing Rsp5-Ubp7-3xHA but not His3-Ubp7-3xHA (Fig. 4-17). These data are consistent with previous studies (described earlier) indicating that ubiquitination by Rsp5 is necessary for viability. Expression of Rsp5-Ubp7 also blocked Ste3-GFP, Fur4-GFP, Gap1-GFP and Mup1-GFP from reaching the vacuole (Fig. 4-16A). However, both Ste3-GFP-Ub and Mup1-GFP-Ub reached the vacuole (Fig. 4-16A). These data indicate that fusion of a DUb to Rsp5 prevents the ubiquitin-dependent sorting of Rsp5 substrates, but that MVB cargo, which was not susceptible to a DUb could sort normally. The HECT domain of Rsp5 is on the C-terminus, and expression of Rsp5 with a C-terminal fusion could potentially block cargo from reaching the vacuole (reviewed in Chapter 6). To test this possibility, a catalytically inactive DUb fused to Rsp5. Rsp5-ubp7* (inactive) allowed both Ste3-GFP and Ste3-GFP-Ub to reach the vacuole and did not lead to growth defects like those for Rsp5-Ubp7 (active) (Fig. 4-16B, Fig. 4-17). These data indicate that the dominant effect exerted by Rsp5-Ubp7 was not due to simple protein fusion to the C-terminus of Rsp5.

ESCRT Cargo Release

Coupled ubiquitination of ESCRTs could potentially regulate ESCRTs by forcing them to release the ubiquitinated cargo that they are bound to during MVB formation. This would likely result in the ESCRT subunits themselves being incorporated into MVBs and eventually into vacuoles. To test this possibility, lysates were subjected to protease treatment. This experiment was performed in cells lacking the vacuolar protease Pep4. This was to ensure that if Vps27 did reach the vacuole, it would not be degraded and thus, undetectable by western blot. Lysates from *pep4* or *pep4 vps27Δ* cells expressing either HA-Vps27^{WT} or HA-Vps27$^{K>R}$ were subjected to trypsin treatment. Both HA-Vps27^{WT} and HA-Vps27$^{K>R}$ were susceptible to protease treatment, with no signal for either protein compared to bands from control lysates that were not subjected to trypsin (Fig. 4-18). Alkaline phosphatase (ALP), a protein that localizes to the limiting membrane of the vacuole, was also examined by western blot. ALP (*PHO8* gene product) is a type-II integral membrane protein, with the N-terminus of ALP within the lumen of the vacuole and the last 8 residues of ALP in the cytosol. A previous study demonstrates that the C-terminus of ALP is susceptible to protease treatment, resulting in a lower molecular weight fragment of ALP which is detectable by western blot (81). A western blot for ALP showed that the luminal portion of ALP was protected from protease treatment, and that the cytosolic portion was susceptible to protease treatment (Fig. 4-18).

Discussion

Both conventional techniques and protein upgrade were used to examine how coupled ubiquitination might regulate ESCRT protein function. All of the data indicated that ubiquitination of ESCRT subunits is not necessary for cargo protein entry into vacuoles. This study, as well as others, have revealed that

endogenous levels of ubiquitination of ESCRTs are very low and are virtually undetectable by western blot unless both ubiquitin and the ESCRT subunit are overexpressed (Fig. 4-3)(2, 164, 193). It seems unlikely that a key regulatory process in cells would be so difficult to detect and in many instances. The immunoprecipitation analysis performed in these studies are done under non-denaturing conditions which makes it difficult to know which ESCRT subunits or associated proteins are being ubiquitinated (59, 101, 164, 193). Mutation of all 47 lysine residues within Vps27 eliminated all detectable ubiquitination of that protein, which could functionally substitute for loss of endogenous Vps27. This was true even in a *ubp2Δ* strain where ESCRTs and other Rsp5 substrates are overubiquitinated (Fig. 4-4)(75, 76). Elimination of Rsp5 activity using several approaches decreased ESCRT ubiquitination but allowed for MVB cargo trafficking to the vacuole, so long as the ubiquitination of cargo was satisfied. These data further validate the idea that ESCRT ubiquitination is dispensable for cargo protein entry into MVBs.

Protein upgrade provides a more comprehensive approach to prevent ESCRT ubiquitination. Many of the ESCRTs contain UBDs and virtually all UBD containing proteins are themselves ubiquitinated. Even if ubiquitinatable residues could be mutated in all of the proteins known to harbor UBDs, other ESCRT subunits could still harbor an unknown UBD and could potentially compensate as the “coupled ubiquitination” substrate. Moreover, if all of the ubiquitinatable residues could be eliminated within ESCRTs, and if that manipulation were to block cargo sorting to vacuoles, the data would be uninterpretable since mutation of ubiquitinatable residues could perturb other ESCRT processes such as Ub-binding or ESCRT interactions. Upgrade circumvents these caveats since fusion of an active upgrade module prevented the ubiquitination of Vps27 and Hse1 as well as the vacuolar sorting of every

cargo protein tested (Fig. 4-13). These data provide strong evidence that upgrade robustly blocks the ubiquitination of the proteins modified by upgrade as well as the proteins closely associated with the ESCRT-DUb fusions. However, MVB cargo could still reach the vacuole, as long as the cargo had an in-frame fusion of Ub, which is not susceptible to the activity of a DUb (Fig. 4-14). Thus, ubiquitination of the ESCRT machinery is not necessary for cargo protein entry into MVBs. Previous data from chapter 3 as well as the His3-Ubp7 growth assay from this chapter (Fig. 4-16) show that upgrade is specific to proteins and protein complexes that the DUb modules are fused to. ESCRT-DUb fusions were able to functionally substitute for their wildtype counterparts, indicating that they still harbored Ub-binding activity and could still function as MVB sorting receptors. Using this technique has essentially allowed us to uncouple “coupled ubiquitination” because preventing the ubiquitination of ESCRTs did not require inactivation of their UBDs (Fig. 4-15). Perhaps the greatest advantage to this technique is that it provided both specificity in that the de-ubiquitination could be localized to a particular process at a particular location within cells but also that a negative control so that other unrelated phenotypes could be ruled out.

Since the lack of ubiquitination of ESCRTs had no affect on MVB sorting of cargo proteins and also did not prevent ESCRT subunit release from cargo proteins, the current models for the regulation of ESCRT proteins in the endocytic pathway are incorrect. Ubiquitination of ESCRTs could modulate ESCRT activity at different places in cells or could potentially be a mechanism to regulate the stability of ESCRTs as ubiquitination of so many other proteins is. “Coupled ubiquitination” could also simply be an artifact and have no functional role in cells.

Figure 4-1. Molecular Mechanisms of Coupled Ubiquitination.

Schematic of potential mechanisms that drive coupled ubiquitination. (A) Ubiquitinated E3 ligase binding by UBD containing proteins. Many E3 ligases are themselves ubiquitinated. This has lead to some models where UBD-containing proteins are ubiquitinated by E3 ligases through UBD-dependent interaction with ubiquitinated E3 ligases. (B) E3 ligase interaction with a protein or protein complex. Some E3 ligases interact directly with complexes that contain UBD proteins. In this model, E3 ligases contribute to the ubiquitination of UBD proteins by indirect interactions with protein complexes, or through direct interactions other then UBD binding to ubiquitinated E3. Adapted from (193).

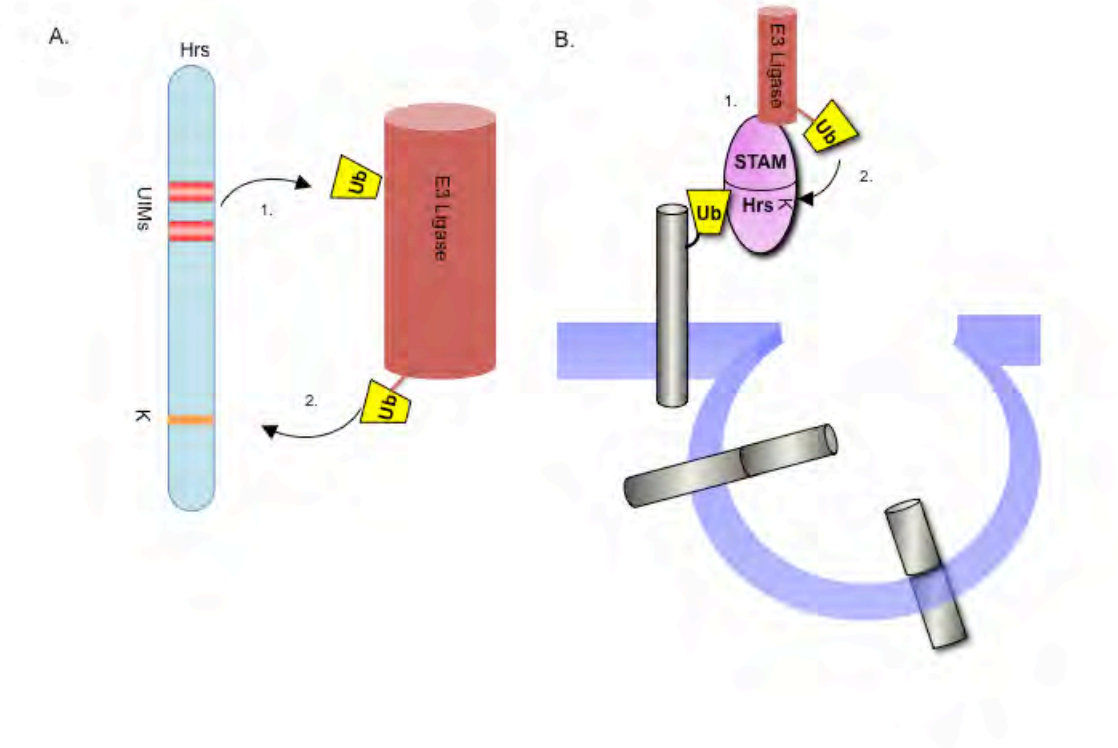

A.
Hrs
UIMs
K
1.
Ub
E3 Ligase
2.
Ub
B.
E3 Ligase
1.
Ub
STAM
2.
Ub
Hrs

Figure 4-2. Potential Regulatory Mechanisms of ESCRT proteins by Coupled Ubiquitination.

Schematic of two models for the coupled ubiquitination of ESCRT complex proteins. (A). Intermolecular interactions between UBDs from ESCRTs and ubiquitinated ESCRTs could contribute to their ability to assemble at the sites of MVB formation. In this model, ubiquitinated ESCRTs recruit other UBD-containing ESCRTs. (B). Intermolecular interaction between the UBDs within ESCRTs and ubiquitin on the ESCRT with the UBD could contribute to their ability to dislodge from bound cargo. Here, ubiquitination of UBD proteins causes a molecular rearrangement where the UBD binds to the ubiquitinated portion of itself, dislodging the UBD protein from bound Ub-cargo. Adapted from (31, 97)

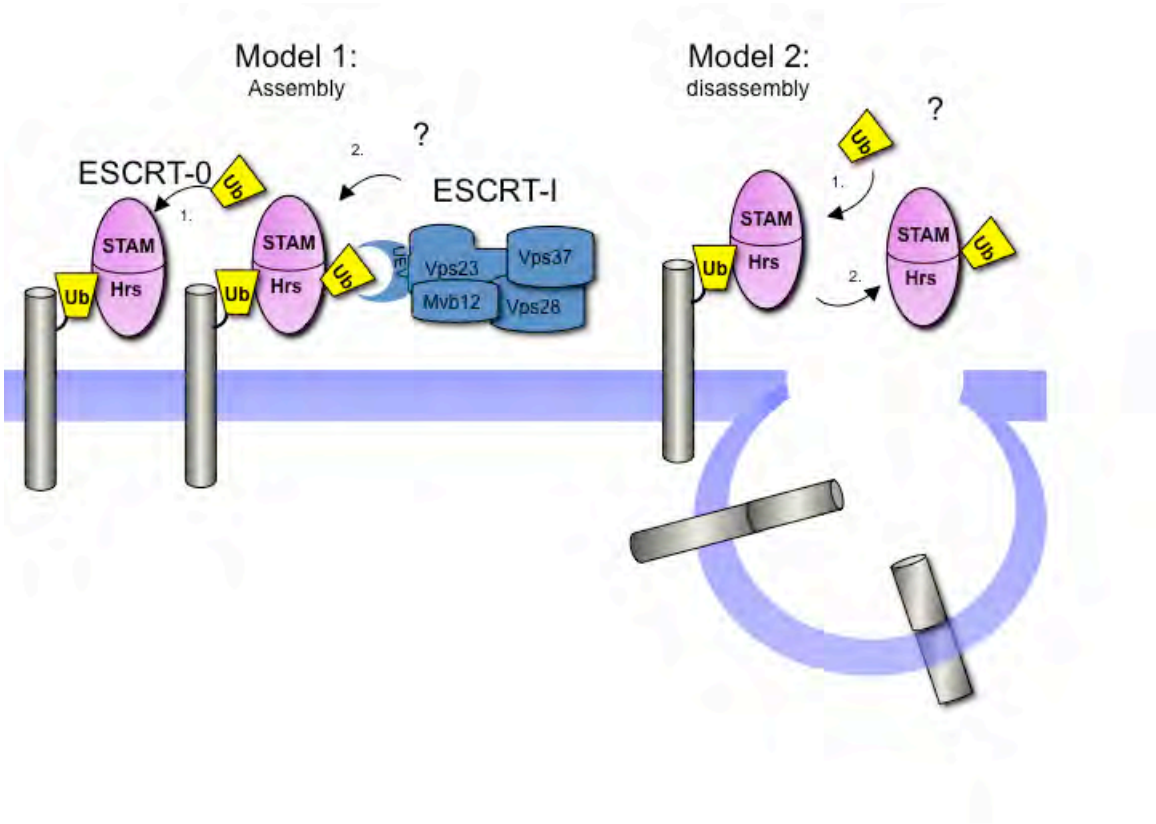

Model 1:
Assembly
Model 2:
disassembly
ESCRT-0
ESCRT-I
?
?
1.
2.
1.
2.
Ub
STAM
Hrs
UEV
Vps23
Vps37
Mvb12
Vps28

Figure 4-3. Ubiquitination of Vps27.

(A) To examine ESCRT ubiquitination in yeast, *vps27Δ* or *vps4Δ* cells expressing vector control, Vps27 alone, Myc-Ub alone or both Myc-Ub and Vps27 were subjected to western blot analysis for Vps27. (B). IP analysis from WT cells expressing HA-Vps27 and Myc-Ub. Lysates were Immunoprecipitated for HA (Vps27) and immunoblotted for HA and Myc. Input represents a 5% equivalent.

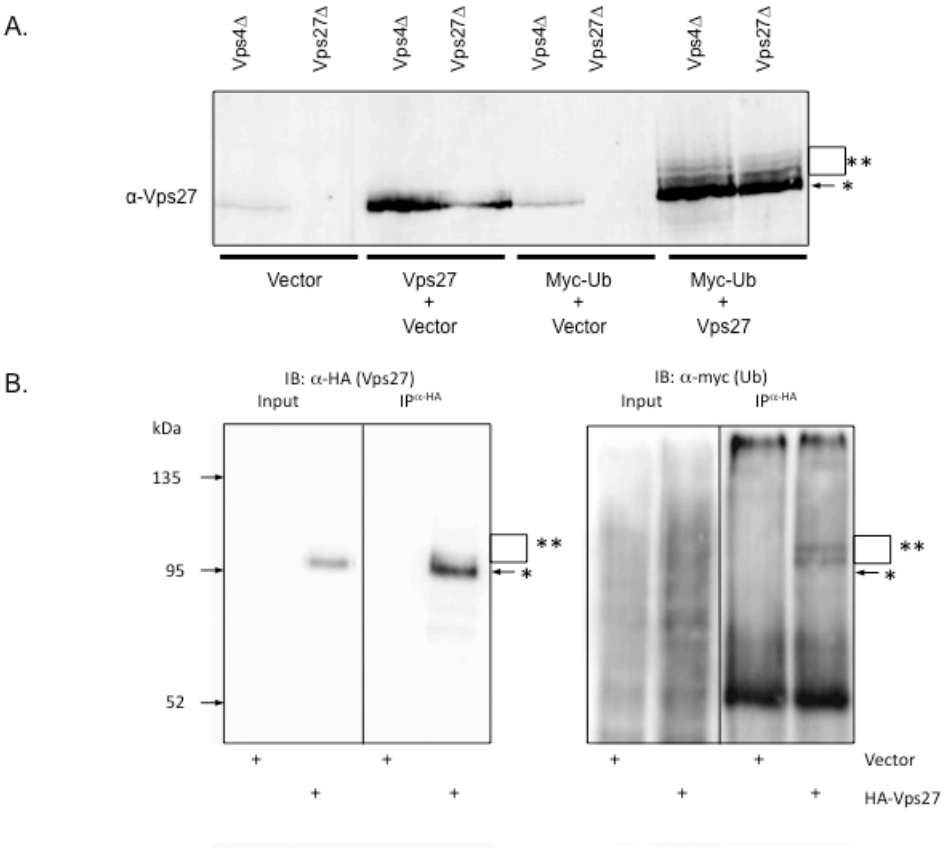

A.
Vps4Δ
Vps27Δ
Vps4Δ
Vps27Δ
Vps4Δ
Vps27Δ
Vps4Δ
Vps27Δ
α-Vps27
**
*
Vector
Vps27 + Vector
Myc-Ub + Vector
Myc-Ub + Vps27
B.
IB: α-HA (Vps27)
Input
IP α-HA
IB: α-myc (Ub)
Input
IP α-HA
kDa
135
95
52
**
*
**
*
+
+
+
+
+
+
+
+
Vector
HA-Vps27

Figure 4-4. Lysines within Vps27 are required for Vps27 Ubiquitination.
A) Schematic of Vps27 with indicated domains and a vertical line representing the location of each of the 47 lysines within Vps27. (B) IP analysis from *ubp2Δ* cells expressing either HA-Vps27^{WT} or HA-Vps27$^{K>R}$ along with Myc-Ub. Lysates were immunoprecipiated for HA and immunoblotted for HA and Myc.

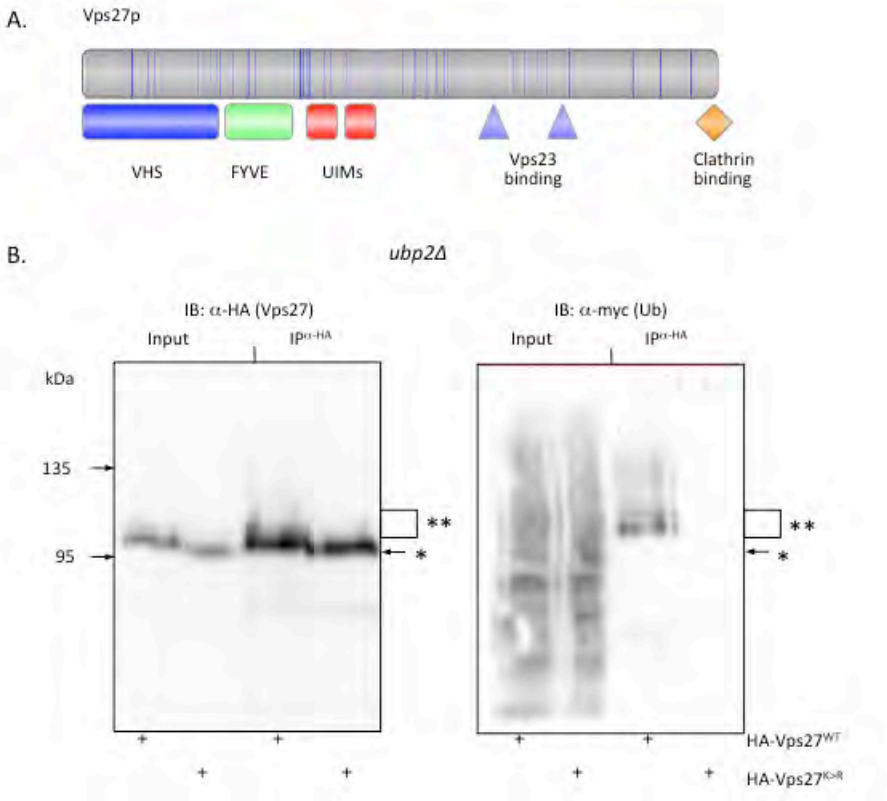

A.
Vps27p
VHS
FYVE
UIMs
Vps23 binding
Clathrin binding
B.
ubp2Δ
IB: α-HA (Vps27)
IB: α-myc (Ub)
Input
IPα-HA
kDa
135
95
**
*
HA-Vps27WT
HA-Vps27K>R

Figure 4-5. Vps27 Lacking Lysines Can Functionally Substitute for WT Vps27. *SF838-9D* yeast lacking Vps27 expressing either Ste3-GFP (A) or Ste3-GFP-Ub (B) with vector only, $Vsp27^{WT}$ or $Vps27^{KR}$.

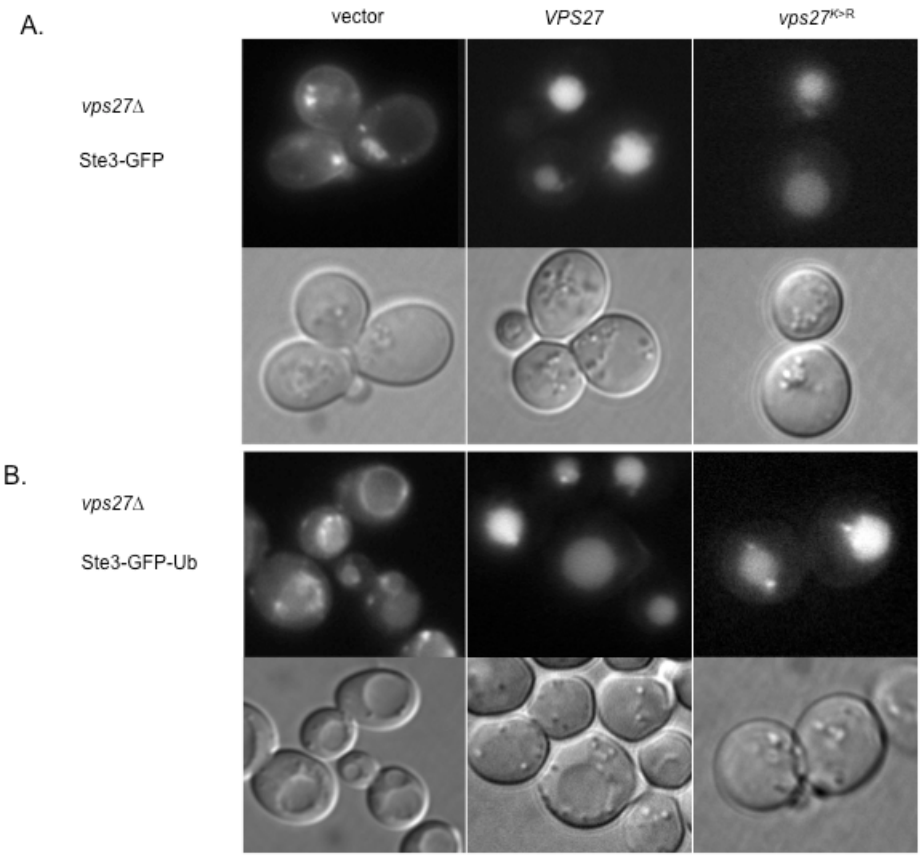

A.
vector
VPS27
vps27K>R
vps27Δ
Ste3-GFP
B.
vps27Δ
Ste3-GFP-Ub

Figure 4-6. Vps27 Without Lysines can Functionally Substitute for Vps27 in Cells Lacking Ubp2.

BY2742 cells lacking endogenous Vps27 and Ubp2 expressing Ste3-GFP with Vector, $Vsp27^{WT}$ or $Vps27^{KR}$.

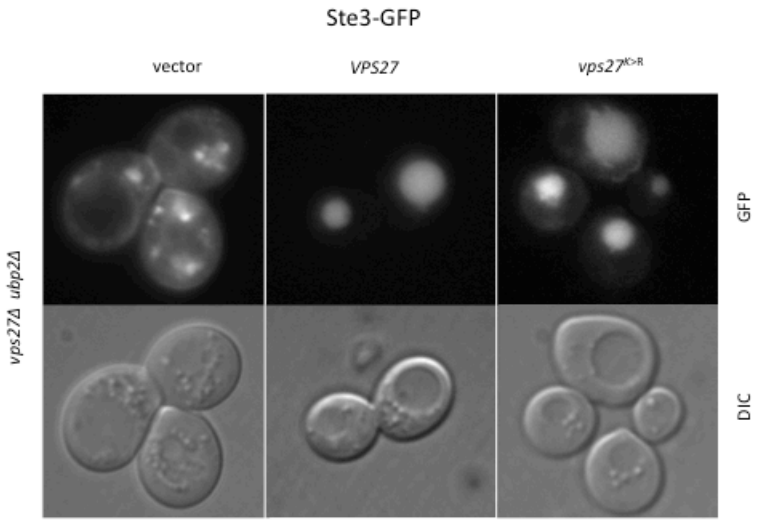

Ste3-GFP
vector
VPS27
vps27K>R
vps27Δ ubp2Δ
GFP
DIC

Figure 4-7. ESCRT-0 Without Lysines can still Function in MVB Sorting. *SEY6210* cells do not require the activity of Hse1 to sort MVB cargo into vacuoles. $Vsp27^{WT}$ or $Vps27^{KR}$ were expressed in a version of this strain lacking Hse1 and Vps27 with Ste3-GFP.

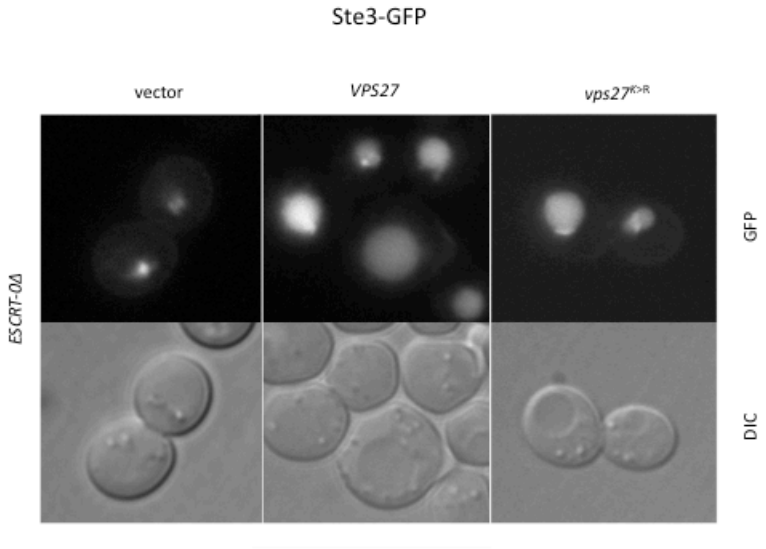
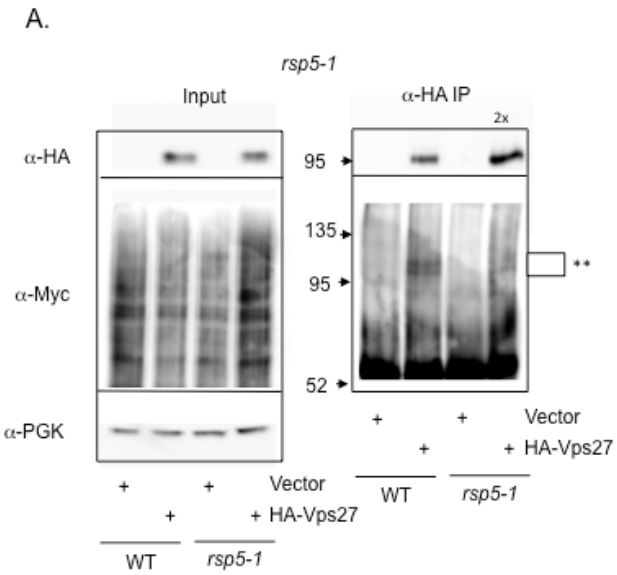

Ste3-GFP
vector
VPS27
vps27K>R
ESCRT-0Δ
GFP
DIC

Figure 4-8. Inactivation of Rsp5 Activity using the TS Allele *rps5-1*.

(A) HA-Vps27 and Myc-Ub were expressed in cells harboring *rsp5-1* cells or in WT cells and subjected to Immunoprecipitation for HA (Vps27) and immunoblotting for HA and Myc. (B) MVB cargo sorting was tested using three reporter proteins, Gap1-GFP, Mup1-GFP and Mup1-GFP-Ub at the permissive and non-permissive temperature as indicated.


A.
rsp5-1
Input
α-HA IP
2x
α-HA
α-Myc
α-PGK
95
135
95
52
**
+ + Vector
+ + HA-Vps27
WT
rsp5-1
+ + Vector
+ + HA-Vps27
WT
rsp5-1

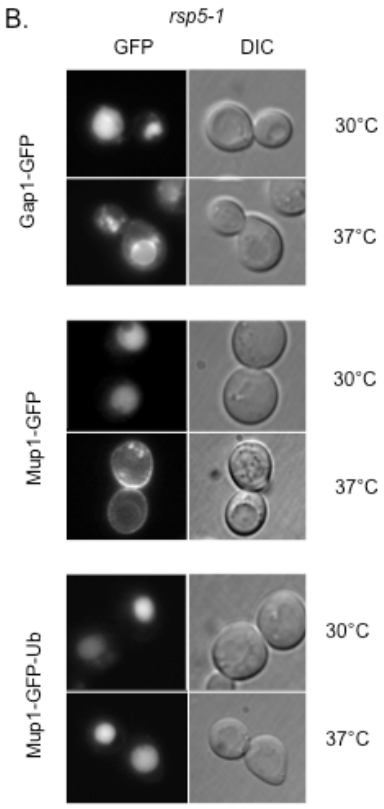
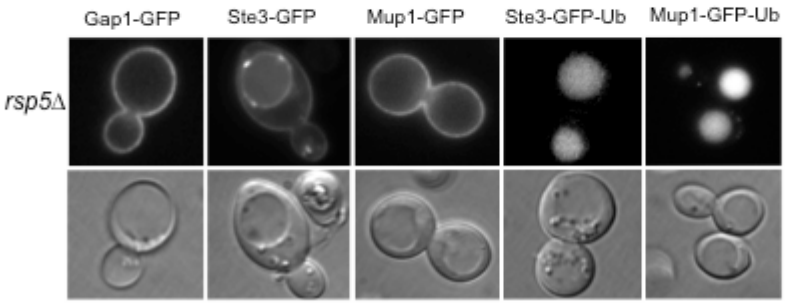

B.
rsp5-1
GFP
DIC
Gap1-GFP
Mup1-GFP
Mup1-GFP-Ub
30°C
37°C
30°C
37°C
30°C
37°C

Figure 4-9. Deletion of Rsp5 Does Not Block Sorting of MVB Cargo.

(A) Null rsp5Δ cells expressing Gap1-GFP, Ste3-GFP, Mup1-GFP, Ste3-GFP-Ub or Mup1-GFP-Ub. (B). WT or rsp5Δ cells parental strain grown at 30°C and 37°C .

A.


Gap1-GFP
Ste3-GFP
Mup1-GFP
Ste3-GFP-Ub
Mup1-GFP-Ub
rsp5Δ

B.

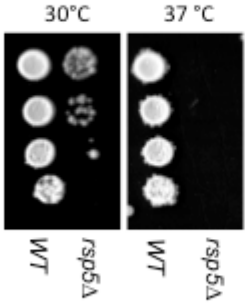

30°C
37 °C
WT
rsp5Δ
WT
rsp5Δ

Figure 4-10. Schematic of Protein Upgrade of ESCRTs.

Schematic depicting active (A) and inactive or control (B) DUb modules fused to ESCRT complex subunits. Active DUb modules should prevent ubiquitination of ESCRTs and cargo. Inactive DUb modules should allow for ESCRT ubiquitination and normal cargo sorting.

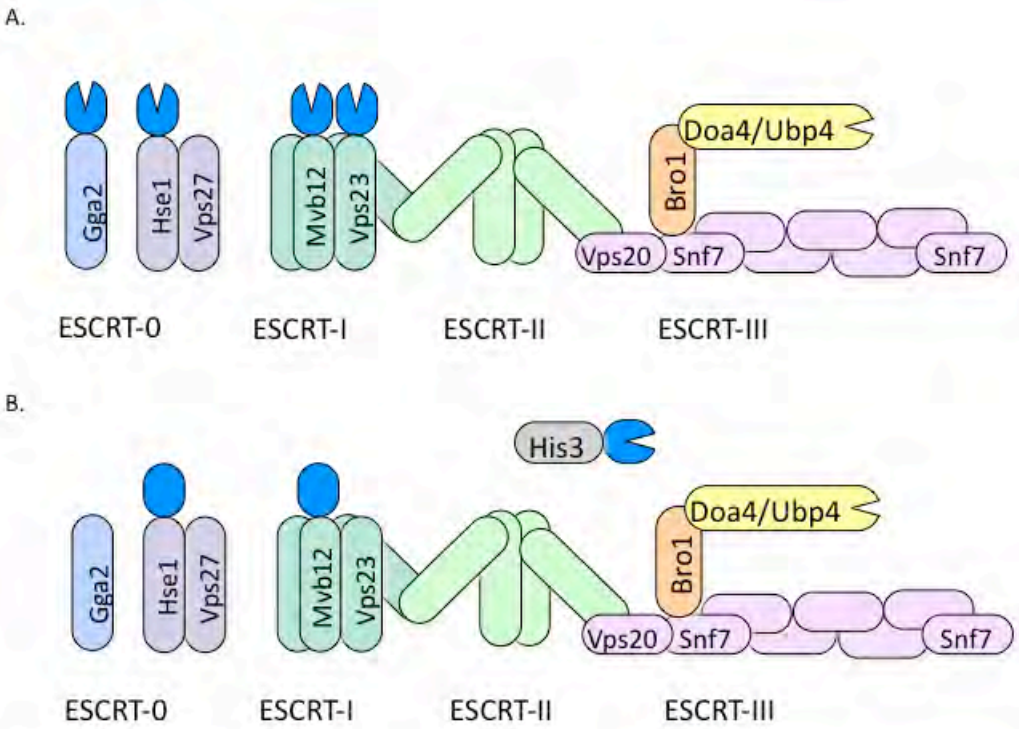

A.
Gga2
Hse1
Vps27
Mvb12
Vps23
Bro1
Doa4/Ubp4
Vps20
Snf7
Snf7
ESCRT-0
ESCRT-I
ESCRT-II
ESCRT-III
B.
His3
Gga2
Hse1
Vps27
Mvb12
Vps23
Bro1
Doa4/Ubp4
Vps20
Snf7
Snf7
ESCRT-0
ESCRT-I
ESCRT-II
ESCRT-III

Figure 4-11. Upgrade Renders ESCRTs Resistant to Ubiquitination. Anti-HA immunoprecipitates from lysates of cells expressing myc-Ub, HA-Vps27 and either Hse1-UL36 or Hse1-ul36* (inactive) were immunoblotted for HA and myc to assess ubiquitination status.

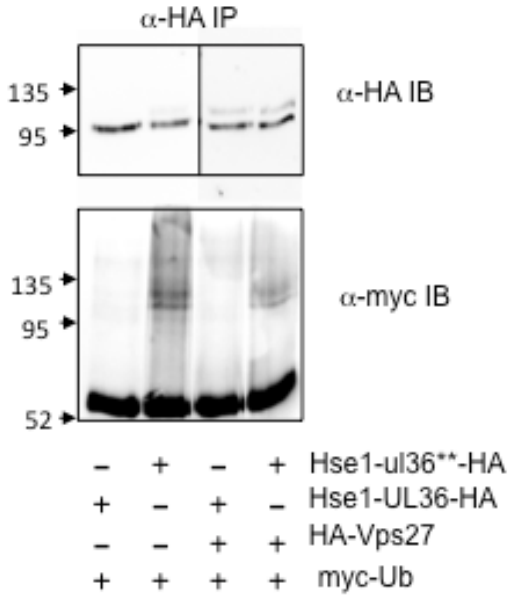

α-HA IP
135
95
α-HA IB
135
95
52
α-myc IB
– + – + Hse1-ul36**-HA
+ – + – Hse1-UL36-HA
– – + + HA-Vps27
+ + + + myc-Ub

Figure 4-12. Western Blot Analysis of Cells Expressing ESCRT-DUb Fusions. Lysates from cells expressing ESCRT-DUb-3xHA fusions and control modules (as indicated), were subjected to western blot analysis for HA and for PGK.

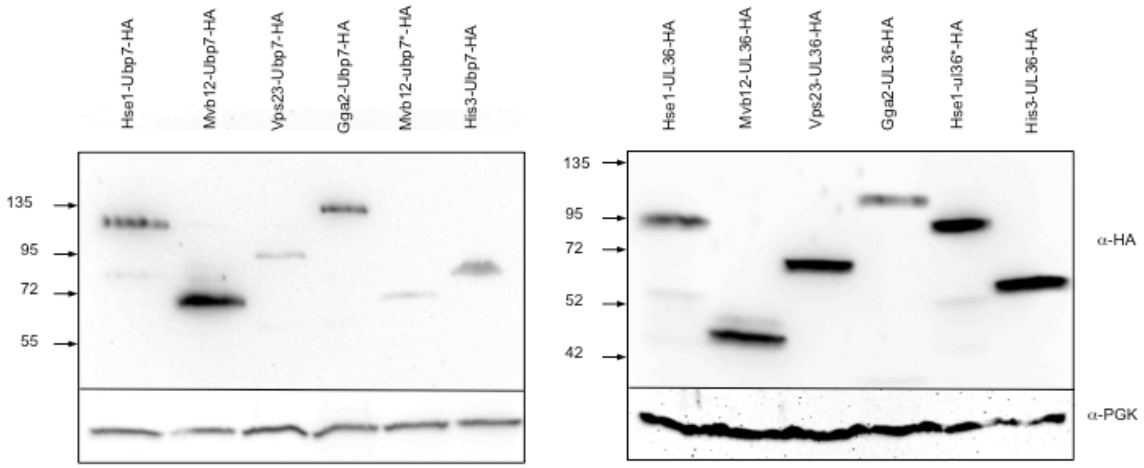

Hse1-Ubp7-HA
Mvb12-Ubp7-HA
Vps23-Ubp7-HA
Gga2-Ubp7-HA
Mvb12-ubp7*-HA
His3-Ubp7-HA
135
95
72
55
Hse1-UL36-HA
Mvb12-UL36-HA
Vps23-UL36-HA
Gga2-UL36-HA
Hse1-ul36*-HA
His3-UL36-HA
135
95
72
52
42
α-HA
α-PGK

Figure 4-13. Upgrade of ESCRT Subunits Blocks MVB Cargo Sorting. ESCRT subunits, GGA, or His3 fused to the indicated active (Ubp7 or UL36) or inactive (ubp7* and ul36*) catalytic domains were expressed in WT cells. Sorting of GFP-tagged MVB cargo (as indicated) was assessed by fluorescence microscopy.

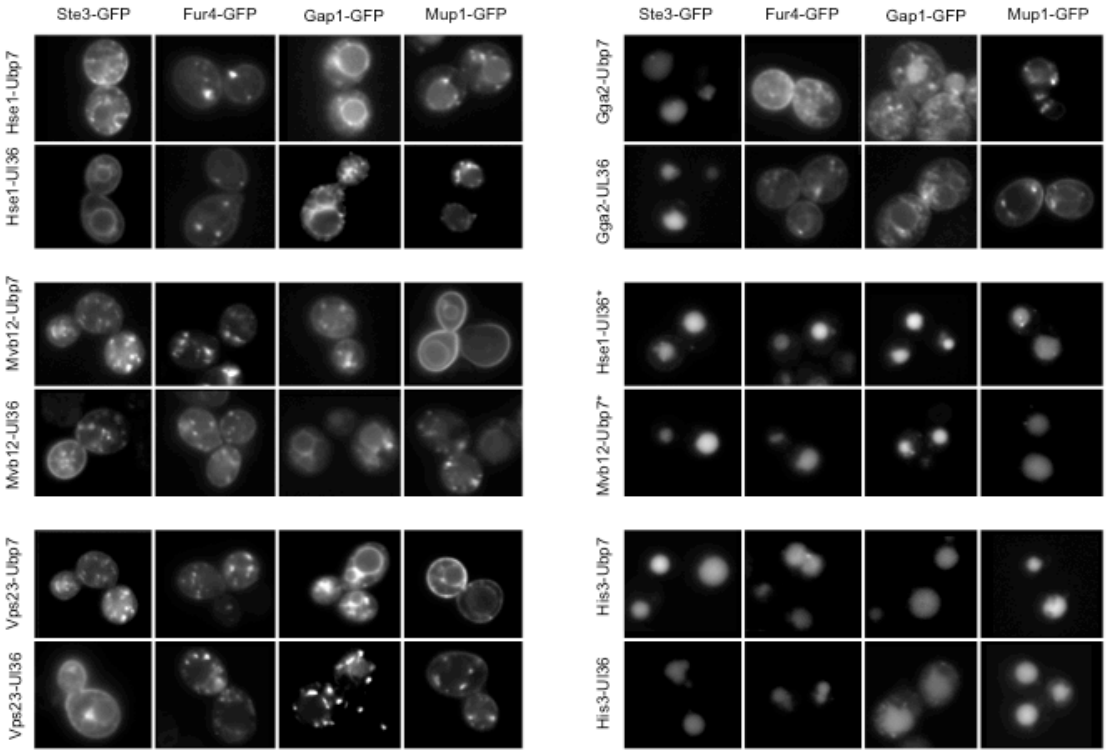

Ste3-GFP
Fur4-GFP
Gap1-GFP
Mup1-GFP
Hse1-Ubp7
Hse1-UI36
Mvb12-Ubp7
Mvb12-UI36
Vps23-Ubp7
Vps23-UI36
Ste3-GFP
Fur4-GFP
Gap1-GFP
Mup1-GFP
Gga2-Ubp7
Gga2-UL36
Hse1-UI36*
Mvb12-Ubp7*
His3-Ubp7
His3-UI36

Figure 4-14. Upgrade of ESCRT Subunits Allows for Ub-Cargo sorting to Vacuoles.

ESCRT subunits, GGA, or His3 fused to the indicated active (Ubp7 or UL36) or inactive (ubp7* and ul36*) catalytic domains were expressed in WT cells. Sorting of Mup1-GFP-Ub or Ste3-GFP-Ub was assessed by fluorescence microscopy.

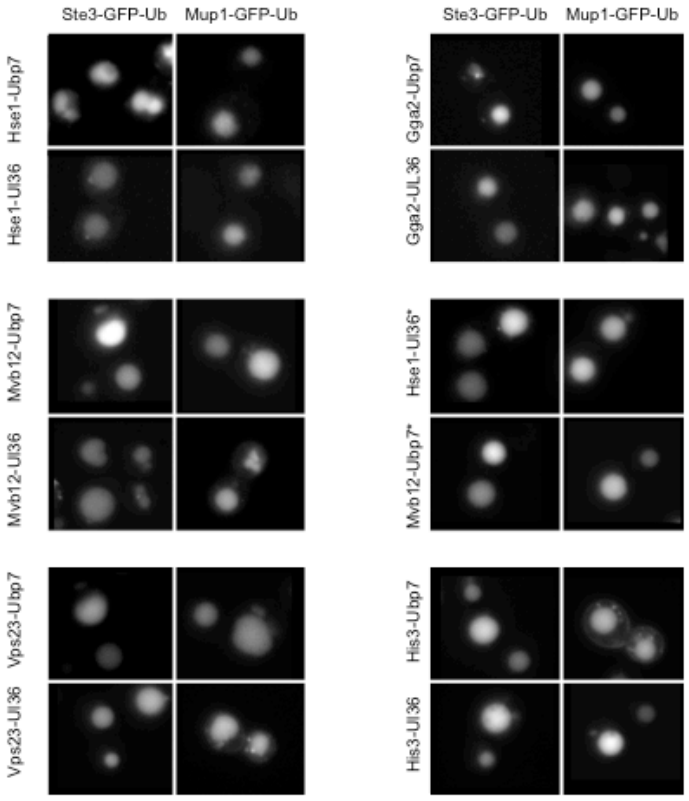

Ste3-GFP-Ub
Mup1-GFP-Ub
Hse1-Ubp7
Hse1-Ul36
Mvb12-Ubp7
Mvb12-Ul36
Vps23-Ubp7
Vps23-Ul36
Ste3-GFP-Ub
Mup1-GFP-Ub
Gga2-Ubp7
Gga2-UL36
Hse1-Ul36*
Mvb12-Ubp7*
His3-Ubp7
His3-Ul36

Figure 4-15. Functional Substitution by ESCRT-DUbs.

(A) Localization of Gap1-GFP or Fur4-RFP-Ub in vps23Δ cells or cells expressing vps23-UL36 as their sole copy of Vps23. (B) Localization of Ste3-GFP or Ste3-GFP-Ub in hse1Δ cells expressing Hse1-UL36 or Hse1-ul36* as their sole copy of Hse1. (C) Localization of Fur4-GFP or Ste3-GFP-Ub in mvb12Δ vps23ΔUb vps36ΔUb cells with Mvb12-UL36, Mvb12-Ubp7 or Mvb12-ubp7* as their sole copy of Mvb12.

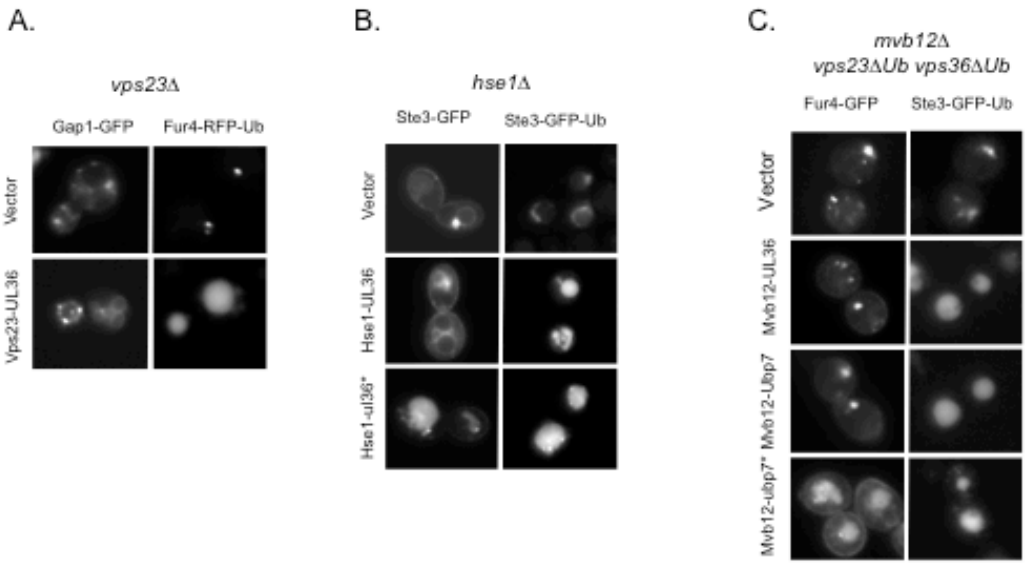
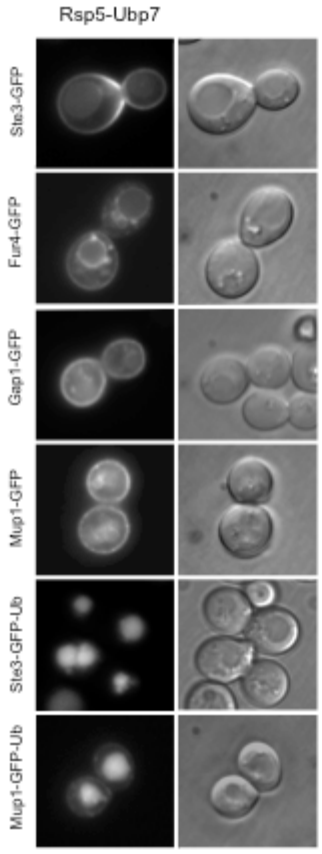

A.
vps23Δ
Gap1-GFP
Fur4-RFP-Ub
Vector
Vps23-UL36
B.
hse1Δ
Ste3-GFP
Ste3-GFP-Ub
Vector
Hse1-UL36
Hse1-ul36*
C.
mvb12Δ
vps23ΔUb vps36ΔUb
Fur4-GFP
Ste3-GFP-Ub
Vector
Mvb12-UL36
Mvb12-Ubp7
Mvb12-ubp7*

Figure 4-16. Upgrade of the HECT-type Ub-ligase Rsp5.

(A) Localization of the indicated GFP-tagged reporters in cells expressing Rsp5-Ubp7(active). (B) Localization of Ste3-GFP or Ste3-GFP-Ub in cells expressing Rsp5-ubp7*(inactive).

A.


Rsp5-Ubp7
Ste3-GFP
Fur4-GFP
Gap1-GFP
Mup1-GFP
Ste3-GFP-Ub
Mup1-GFP-Ub

B.

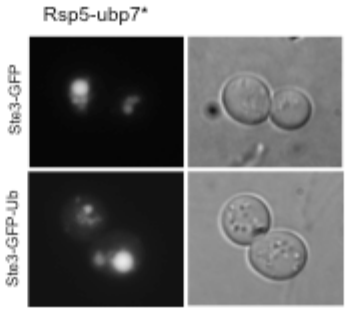

Rsp5-ubp7*
Ste3-GFP
Ste3-GFP-Ub

Figure 4-17. Expression of Rsp5-Ubp7 Leads to Growth Defects.

(A). Cells grown on plates with $CuCl_2$ or BCS (copper chelator) grown at 30°C and 37°C expressing Rsp5-Ubp7 or His3-Ubp7 from the copper inducible *CUP1* promoter. (B). Cells grown on plates with $CuCl_2$ grown at 30°C expressing Rsp5-Ubp7(active) or Rsp5-ubp7*(inactive).

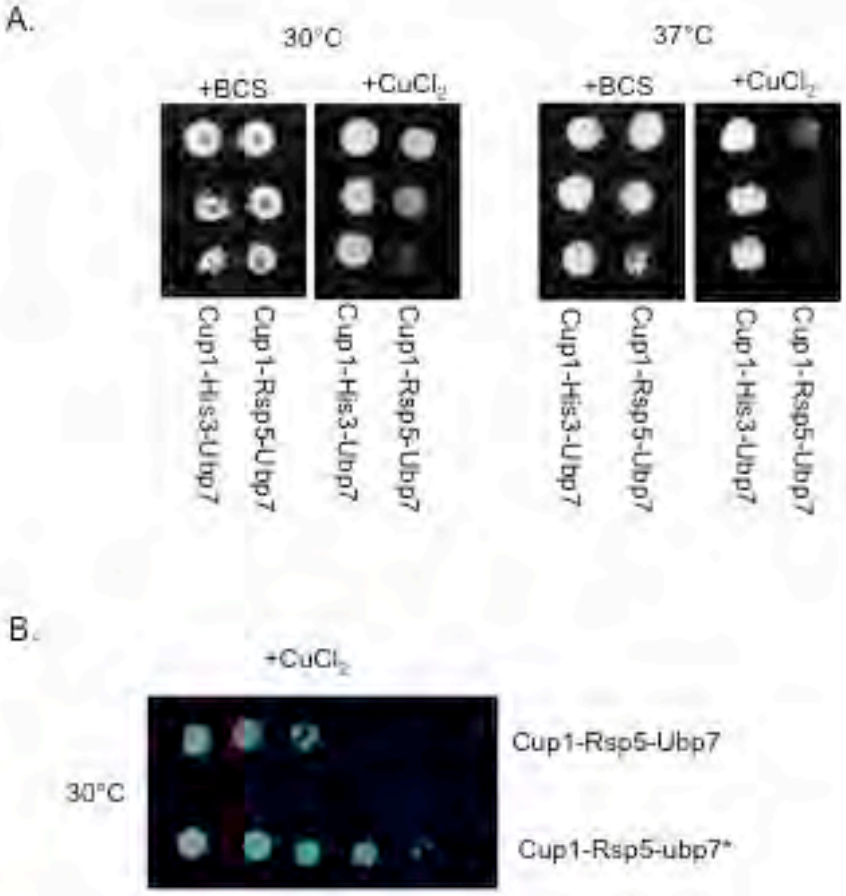

A.
30°C
37°C
+BCS
+CuCl2
+BCS
+CuCl2
Cup1-His3-Ubp7
Cup1-Rsp5-Ubp7
Cup1-His3-Ubp7
Cup1-Rsp5-Ubp7
Cup1-His3-Ubp7
Cup1-Rsp5-Ubp7
Cup1-His3-Ubp7
Cup1-Rsp5-Ubp7
B.
+CuCl2
30°C
Cup1-Rsp5-Ubp7
Cup1-Rsp5-ubp7*

Figure 4-18. Coupled Ubiquitination of ESCRTs in Not Required for Cargo Release.

HA-Vps27^{WT} or HA-Vps27$^{K>R}$ were expressed in *pep4Δ* mutant cells or *vps27Δ pep4*Δ mutant cells. Lysates were incubated in the presence and absence of trypsin. Samples were immunoblotted for HA (Vps27), and the alkaline phosphotase (Pho8) protein that localizes to the limiting membrane of the vacuole.

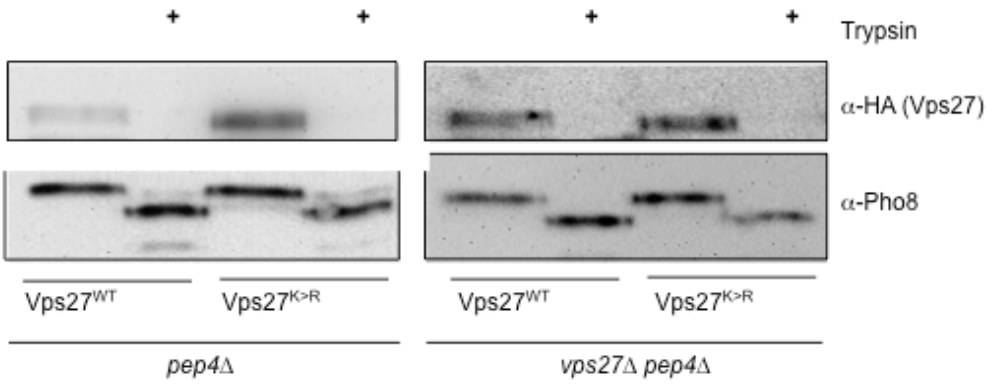
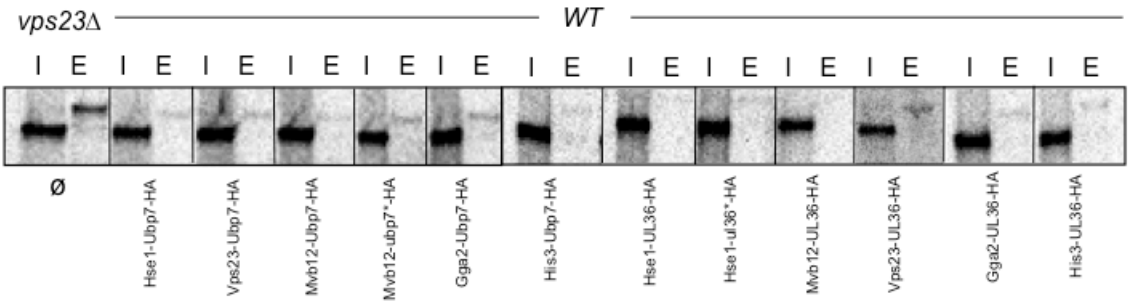

+
+
+
+
Trypsin
α-HA (Vps27)
α-Pho8
Vps27WT
Vps27K>R
Vps27WT
Vps27K>R
pep4Δ
vps27Δ pep4Δ

Figure 4-19. Upgrade of ESCRTs Does Not Cause a Synthetic Class E Phenotype.

Mutant *vps23Δ* cells or WT cells expressing the indicated DUb fusion proteins were grown in $CuCl_2$ overnight, diluted, pulse labeled with ^{35}S-Methionine and chased with cold Methionine for 60 minuts. CPY was immunoprecipitated from intracellular (I) and extracellular (E) fractions and analyzed by SDS-PAGE.


vps23Δ
WT
I E I E I E I E I E I E I E I E I E I E I E I E I E
ø
Hse1-Ubp7-HA
Vps23-Ubp7-HA
Mvb12-Ubp7-HA
Mvb12-ubp7*-HA
Gga2-Ubp7-HA
His3-Ubp7-HA
Hse1-UL36-HA
Hse1-ul36*-HA
Mvb12-UL36-HA
Vps23-UL36-HA
Gga2-UL36-HA
His3-UL36-HA

CHAPTER 5: UBIQUITINATION REQUIREMENTS FOR CARGO PROTEIN ENTRY INTO VACUOLES

Abstract

Ubiquitination plays a critical role in determining the fate of integral membrane proteins. Ubiquitination of membrane proteins mediates their incorporation into MVBs and their delivery to lysosomes. Whether monoubiquitination or K63-linked polyubiquitination is the operative MVB sorting signal remains an important question. Recent work demonstrates that the cargo protein Gap1 cannot undergo sorting into MVBs in cells lacking ability to form K63 polyubiquitin chains. We used both conventional approaches and protein "upgrade" to test the hypothesis of K63-linked polyubiquitination as a necessary signal for MVB sorting. Our data indicate that K63 polyubiquitination is not necessary for cargo protein entry into MVBs and that a single ubiquitin is sufficient for entry into the vacuole. Upgrade was also used to determine if ILV formation could occur in the absence of ubiquitinated cargo. Our data indicate ubiquitinated cargo is necessary for the formation of ILVs.

Introduction

Integral membrane proteins destined for degradation by lysosomes must first be incorporated into intralumenal vesicles that bud inward from the limiting membrane of early endosomes. Ubiquitination of integral membrane proteins is required for this intralumenal budding step (9, 133). Proteins that are not ubiquitinated at the sites of MVB formation are free to recycle, thus escaping degradation by lysosomes. This makes ubiquitin the key signal that determines the fate of integral membrane proteins (133). Ubiquitination is a dynamic process that can occur on multiple lysine residues with multiple linkages. DUbs

can edit Ub chains or remove them entirely from cargo proteins (Introduction and Chapter 3) (Fig. 5-1). Some of the E3 ubiquitin ligases and DUbs have a preference towards particular types of ubiquitin linkages. For instance, the yeast ubiquitin ligase Rsp5 prefers to make K63-linked chains *in-vitro*, although it can also make K48-linked chains *in-vitro* as well (75). The yeast DUb Ubp2, which associates with Rsp5 through the adaptor protein Rup1 (142), antagonizes Rsp5 activity and also shares a preference for K63 Ub linkages (75, 76). In mammalian cells the DUb AMSH, which interacts with the Hse1 homolog STAM, and also shares a preference for K63 linkages (Fig. 3-1).

The major ubiquitin ligase that contributes to membrane protein ubiquitination in the yeast endocytic pathway is Rsp5, which prefers to make K63 linkages. Thus, many membrane proteins that undergo MVB sorting are modified by K63-linked polyubiquitination. For instance, the general amino acid permease Gap1 is ubiquitinated by Rsp5 with K63 poly-ubiquitin chains and degradation of that protein is delayed but not completely eliminated when Ub with a K63R mutation is overexpressed (170). When mutant UbK63R, which cannot make K63-linked chains is the sole source of ubiquitin in cells, there are defects in the MVB sorting of Gap1, the lactate transporter Jen1, the Iron transporter Sit1, and the uracil permease Fur4 (36, 40, 91, 126). One idea for the role of K63 linkages is that K63 polyubiquitination is a necessary signal for cargo protein incorporation into MVBs (90). Moreover, some adaptor proteins found within the yeast endocytic pathway, such as Ent1 and Ent2, contain tandem UIM domains which are predicted to bind more efficiently to K63 polyubiquitin linkages (157). Vps27 also contains tandem UIM domains and its mammalian homologue also shows a binding preference towards K63 poly-ubiquitin linkages (6). While compelling, the finding that expression of a K63R Ub as the sole source of Ub in cells prevents many cargo proteins from entering the vacuole does not

necessarily mean that K63-linked Ub chains are the precise signal on MVB cargo that determines their entry into an intralumenal vesicle. Expression of mutant Ub as the sole source of Ub in cells could potentially perturb any number of important processes from the biosynthetic pathway to gene expression, making it difficult to draw conclusions about one specific process like the requirements of a particular cargo protein. In addition, while Ent1, Ent2 and mammalian Hrs show a binding preference towards K63-linked chains, tandem UIM domains and yeast Vps27 can also bind to a single Ub (162) (8, 160). Also, while Rsp5 prefers to make K63 linkages, it also makes K48 linkages by virtue of the Rsp5 dependent ubiquitination of Mga2 and Spt23, two proteins that are processed by the proteasome (19, 61). Rsp5 is also capable of monoubiquitination of target proteins such as the G protein α subunit Gpa1, which undergoes both mono and polyubiquitination by Rps5. Furthermore, some of the DUbs associated with the ESCRT machinery, such as Doa4 in yeast and Usp8/UbpY in mammalian cells can facilitate deubiquitination on both K48 and K63 polyubiquitin linkages. Finally, other studies have demonstrated that a single Ub is sufficient for entry into MVBs (141, 145, 184). These studies employ either a single Ub fused in-frame to the protein of interest. These studies also have caveats as they use reporter proteins such as GFP, which could potentially become ubiquitinated, perhaps by K63-linked polyubiquitination.

While it is clear that proteins destined for degradation by lysosomes can be, and perhaps might preferentially be ubiquitinated by K63 linked poly-ubiquitin chains, it is still unclear if K63 poly-ubiquitin linkages are a necessary signal for protein sorting into the MVB pathway. We used both conventional methods as well as protein “upgrade” to determine if K63-linked polyubiquitination of integral membrane proteins was a necessary signal for entry into MVBs. Our data indicate that K63-linked chains are dispensable for entry into MVBs and that a

single ubiquitin is a sufficient signal for incorporation into MVBs. We also used protein upgrade of the ESCRT-0 subunit Hse1 to block ubiquitinated cargo proteins from entering the vacuole. Under these conditions we find that ILV formation is severely decreased but that ILVs are present when the DUb cassette is inactivated and when a cargo with an in-frame fusion to Ub is used. These data indicate that MVB formation cannot proceed in the absence of ubiquitinated cargo proteins.

Rationale and Results

The data in Chapter 4 provide strong evidence that a single Ub is sufficient for entry into MVBs. ESCRT-DUb fusions had a robust effect on cargo sorting. GFP-tagged reporter proteins were completely blocked from the vacuole. These data suggest that the ESCRT-DUbs were able to deubiquitinate any portion of the protein that was ubiquitinated. Fusion of single Ub that was not susceptible to deubiquitination was sufficient to restore the vacuolar localization of Ste3 and Mup1. Since the ESCRT-DUb fusions were capable of blocking Ub-dependent MVB sorting of GFP-tagged cargo by virtue of an active DUb, it is likely that the active ESCRT-DUb fusions also removed any polyUb from the GFP-Ub cargo. Moreover, an *in vitro* DUb assay using recombinant UL36 indicated that the UL36 DUb cassette had activity against both K48 and K63 polyubiquitin chains (Fig. 5-2). A recently published report confirms this result (78). These data further support the idea that “upgrade” cassettes are capable of deubiquitinating any Ub linkage. In addition, the Rsp5-DUb fusions blocked sorting of every GFP-cargo tested, unless a single Ub was fused to cargo. These data also suggest that DUb fusions are robust and that a single Ub is sufficient for entry into MVBs. Chapter 4 also describes two separate

approaches that successfully inactivated Rsp5, the major Ub ligase that ubiquitinates MVB cargo and can make K63 linkages. In cells expressing the TS allele *rsp5-1* and in *rsp5Δ* cells, vacuolar sorting could proceed normally as long as the need for cargo ubiquitination was satisfied. To further examine the idea that a single Ub is a sufficient signal for vacuolar localization, we used two more conventional approaches.

Many Ub ligases use accessory proteins or adaptors, which facilitate the ubiquitination of specific cargo proteins (Chapter 6) (7, 93). In yeast, Rsp5 uses these adaptor proteins to ubiquitinated a wide variety of membrane cargo (90, 121). We took advantage of a strain lacking all of the Arrestin Related Trafficking (ARTs) as well as Bul1/2 and Bsd2, all of which have been described as Rsp5 adaptor proteins (Chapter 6) (121). Mup1-GFP and Mup1-GFP-Ub were expressed in these cells. Mup1-GFP was completely localized to the plasma membrane in cells lacking many Rsp5 adaptor proteins (*en67*) (Fig. 5-3). However, the in-frame fusion of Ub to Mup1-GFP restored sorting to the vacuole. Similar to our other experiments that make use of Rsp5 inactivation, this approach also allows for vacuolar sorting of MVB cargo, as long as the need for cargo ubiquitination is satisfied (Fig. 5-3). Nevertheless, another E3 ligase could be compensating for the loss of Rsp5 activity or Rsp5 connectivity to cargo.

To investigate K63-linked polyubiquitination directly, we used a strain expressing UbK63R as the sole source of Ub. Mup1-GFP was expressed the UbK63R strain and like Gap1 we found that Mup1 was localized to the plasma membrane and on the limiting membrane of the vacuole (Fig. 5-4). One explanation for this result is that Rsp5 prefers to make K63-linked ubiquitin chains and that by depleting the cell of all K63 Ub, Rsp5 has severely reduced activity in this strain. To bypass the need for cargo ubiquitination by Rsp5, we used an in-frame Ub fusion. Mup1-GFP-Ub^{K63R}, containing a single in-frame Ub

also lacking K63, was expressed in UbK63R cells. This protein sorted normally to the vacuole interior, demonstrating that K63 linkages are not required for MVB sorting to proceed normally (Fig. 5-4).

Discussion

This study provides a comprehensive examination of the specific Ub requirements for cargo protein entry into MVBs. Preventing the ubiquitination of a particular protein of interest to investigate particular types of ubiquitination that may drive certain processes in cells can be very difficult. While E3 ligases like Rsp5 and DUbs such as AMSH and Ubp2 show a preference towards a certain type of Ub linkage, that does not necessarily mean that particular Ub linkages are necessary for the subsequent Ub-dependent process. Approaches using a mutant Ub as the sole source of Ub in cells can be informative but do not control for non-specific effects. It is impossible to know what is being affected by such a large manipulation to cells, which makes it difficult to draw conclusions about a specific sorting event like cargo sorting into MVBs. The study that concludes that K63 polyubiquitin linkages are a necessary signal for MVB sorting uses Gap1 as the MVB cargo protein. We tested whether an in-frame fusion of Ub to Gap1 could be employed in our assays. However, an in-frame fusion of Ub to the C-terminus of Gap1, lead to partial Gap1 localization in ER (Fig. 5-5). Thus, we chose Mup1 in our assays because like Gap1, it is an amino acid permease that is internalized from the plasma membrane after substrate addition.

Protein “upgrade” provides a more robust means to make proteins resistant to ubiquitination. Upgrade cassettes are confined to a protein of interest and closely associated proteins but do not change the ubiquitination status of every ubiquitinated protein in cells. In addition, protein upgrade provides a

control in that the activity of the DUb can be inactivated, which is lacking under circumstances where proteins have to be mutated or deleted. Since epitope and reporter tags such as GFP are a routinely used to follow the trafficking of ubiquitinated cargo in cells, upgrade provides the only known method to make those proteins resistant to ubiquitination. While it appears that a single Ub is sufficient for entry into MVBs, K63-linked polyubiquitin chains or perhaps other polyubiquitin chains might enhance the rate at which proteins are trafficked to lysosomes. Future studies should include an examination of the kinetics of ubiquitinated cargo protein trafficking to lysosomes. Ste2 internalization rates and degradation can be measured using labeled α-mating factor (55). These experiments could be performed in cells expressing K63R as their sole source of Ub, with in-frame fusions of Ub or UbK63R to Ste2. Alternatively, they could be performed in cells expressing ESCRT-DUb fusions. Degradation rates could also be measured using cycloheximide treatment followed by western blot for MVB cargo with and without Ub at specific time points after cycloheximide treatment. These studies could likewise be performed in cells expressing UbK63R as their sole source or Ub, or in cells expressing ESCRT-DUb fusions.

Figure 5-1. Ubiquitination of Membrane Proteins.

A schematic depicting the different types of ubiquitination of integral membrane proteins including monoubiquitination, multiubiquitination and polyubiquitination by K48 or K63 linkages (from left to right).

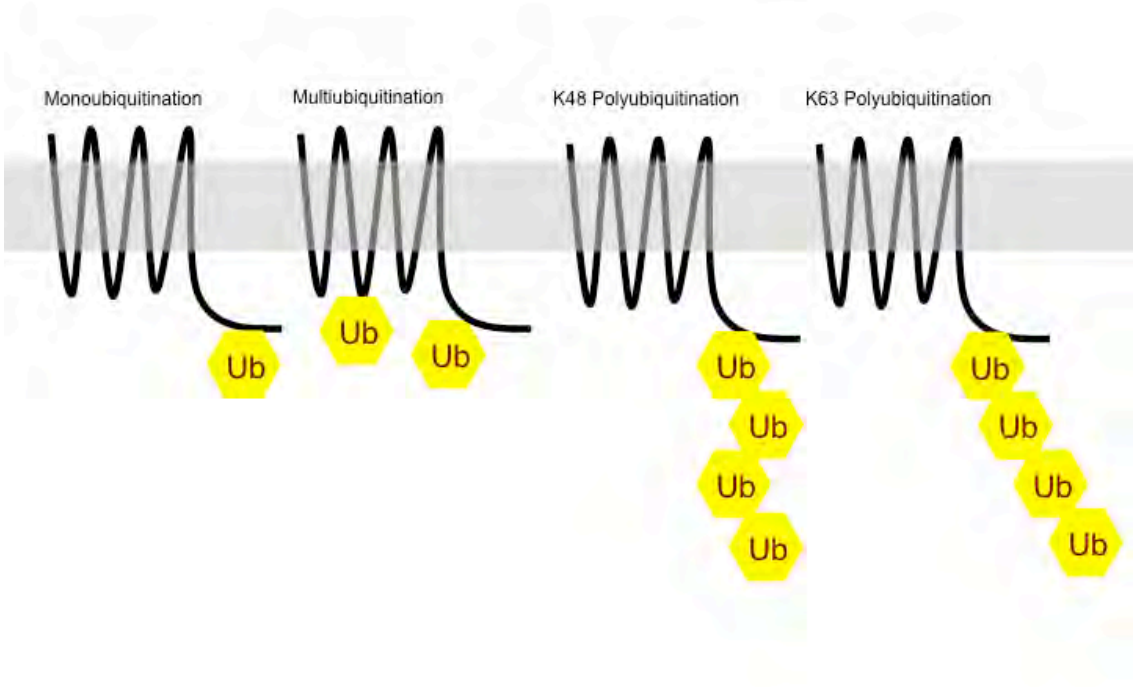

Monoubiquitination
Multiubiquitination
K48 Polyubiquitination
K63 Polyubiquitination
Ub
Ub
Ub
Ub
Ub
Ub
Ub
Ub
Ub
Ub
Ub

Figure 5-2. UL36 has activity against K63 and K48 Polyubiquitin Linkages. K-48 and K-63 Ub chains were incubated with increasing amounts of recombinant UL36. Equivalent dilutions of recombinant enzyme were added to the last two lanes and Ub-aldehyde was also added to the last lane. Recombinant enzyme had activity against both K63 and K48 polyUb linkages.

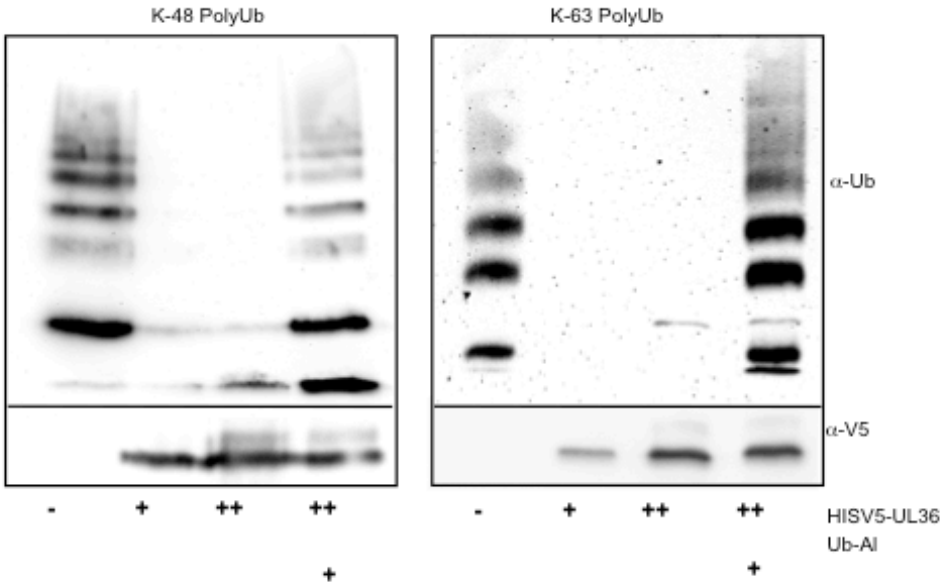

K-48 PolyUb
K-63 PolyUb
α-Ub
α-V5
-
+
++
++
+
-
+
++
++
+
HISV5-UL36
Ub-Al

Figure 5-3. Elimination of Rsp5 Adaptor Proteins does Not Prevent MVB Cargo from Reaching the Vacuole.

Localization of Mup1-GFP and Mup1-GFP-Ub in *en67* cells lacking multiple Rsp5 adaptor proteins. In this strain, Mup1-GFP fails to reach the vacuole, indicating that Rsp5 adaptors are necessary for the vacuolar localization and likely the ubiquitination of Mup1. Vacuolar localization of Mup1-GFP is restored in this strain when a single Ub is fused in frame.

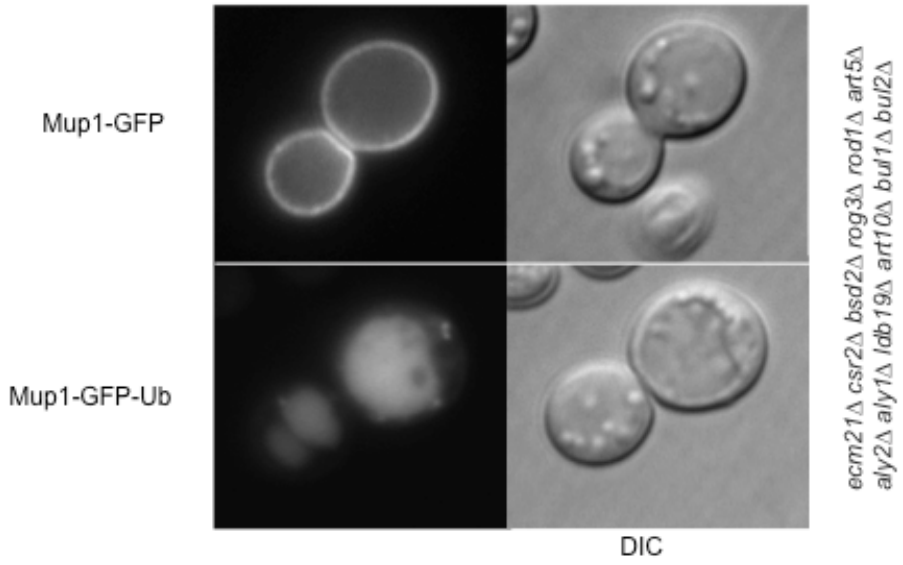

Mup1-GFP
Mup1-GFP-Ub
DIC
ecm21Δ csr2Δ bsd2Δ rog3Δ rod1Δ art5Δ
aly2Δ aly1Δ ldb19Δ art10Δ bul1Δ bul2Δ

Figure 5-4. Polyubiquitination on Lysine 63 is Not Required for Vacuolar Sorting. Localization of Mup1-GFP and Mup1-GFP-Ub^{K63R} in cells expressing WT Ub (*SUB492*) or K63R Ub (*SUB493*) as their sole source of Ub.

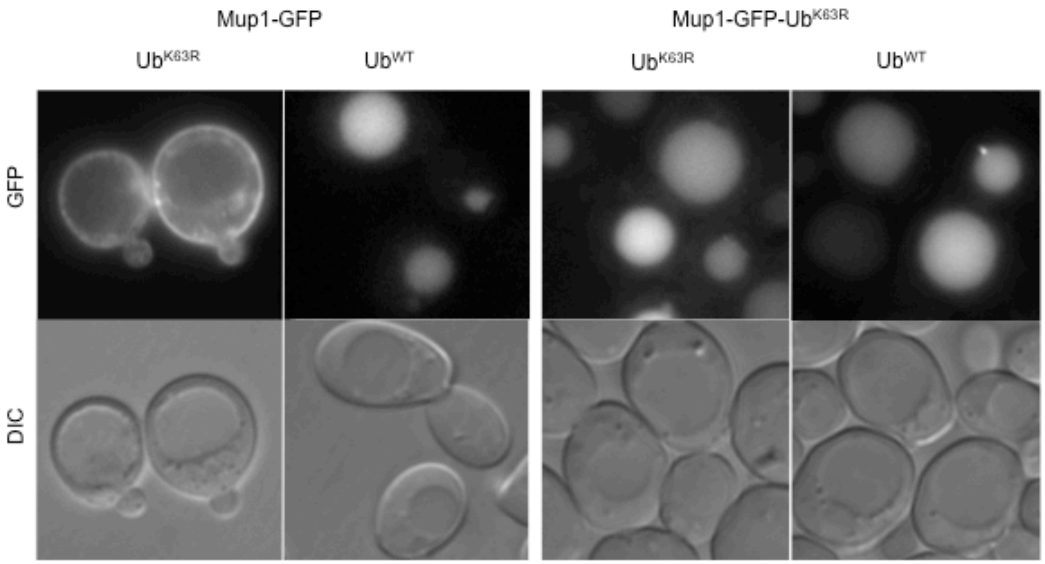
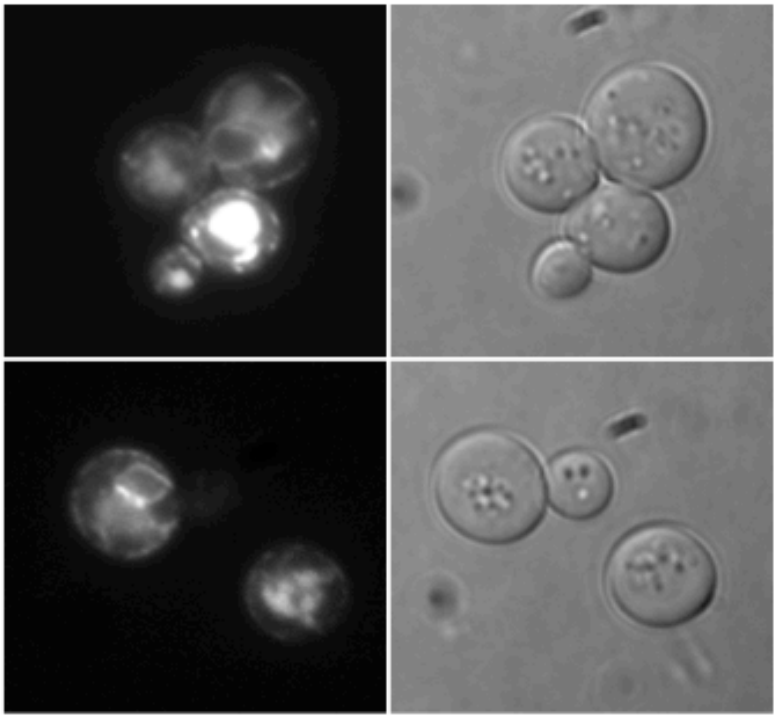

Mup1-GFP
Mup1-GFP-Ub^K63R
Ub^K63R
Ub^WT
Ub^K63R
Ub^WT
GFP
DIC

Figure 5-5. The Effects of an In-frame Fusion of Ub to Gap1-GFP.

Gap1-GFP-Ub expressed in WT cells localizes to the ER and vacuole. This phenotype makes it impossible to use Gap1-GFP-Ub as a reporter for vacuolar sorting assays.

Gap1-GFP-Ub



CHAPTER 6: RSP5 AND THE ROLE OF SNA3 AS A POTENTIAL RSP5 ADAPTOR PROTEIN

Abstract

The Nedd4 homolog Rsp5, a HECT type ubiquitin ligase in yeast, facilitates the ubiquitination of many proteins. In the endocytic pathway Rsp5 plays a major role in the ubiquitination and subsequent vacuolar delivery of a variety of proteins that include many permeases and the G-protein coupled receptor Ste3. Rsp5 is able to mediate Ub-dependent sorting events at multiple places within cells, mainly through the action of Rsp5 adaptor proteins. Adaptor proteins interact with Rsp5 through a conserved PY motif and are also ubiquitinated by Rsp5. Their role is to bridge interaction between Rsp5 and substrates to which they bind. Sna3, a membrane protein with unknown function in yeast, also has a PY motif that is necessary for it to interact with the WW domains of Rsp5. Like many Rsp5 adaptor proteins, Sna3 is also ubiquitinated by Rsp5. Sna3 localizes to the vacuole but fails to enter the vacuole if it cannot interact with Rsp5. Several studies have shown that mutation of the lysines within Sna3 prevents ubiquitination but not vacuolar localization of Sna3. From such studies, several groups have concluded that Sna3 is sorted into MVBs by a mechanisms that is independent of ubiquitination. This model has important implications however, whether Sna3 actually uses an Ub-independent pathway remains unclear. We used protein “upgrade” to examine whether Sna3 could localize to the vacuole, independent of ubiquitination. Our data indicate that Sna3 actually does require ubiquitination to reach the vacuole and further experiments support the idea that Sna3 works as an adaptor proteins for Rsp5 and may sort to the vacuole by virtue of its association with its ubiquitinated substrates.

We also examined aspects of how Rsp5 works as an E3 enzyme. Previous studies indicated that Rsp5 may be able to dimerize. Using temperature-senstive alleles of Rsp5, we tested the ability of catalytically altered Rsp5 mutants to complement for the loss of Rsp5 activity. Interestingly, we found that two Rsp5 alleles housing mutations on the same lobe of the HECT domain did not support viability, but that mutations on opposite lobes of the HECT domain could support viability. These data suggest that HECT domains may be able to function in tandem, and provide evidence for an entirely novel mode for how HECT-type ligases work and imply that heterodimerization of HECT ligases may play a role physiologically.

Introduction

The functional link between ubiquitination of plasma membrane proteins and their degradation in lysosomes was established about 16 years ago. The turnover rate of the yeast **a**-factor transporter Ste6 was reduced approximately 3 fold in cells lacking the E2 enzymes ubc4 and ubc5, suggesting that ubiquitination was somehow involved. In *end4* mutants deficient for internalization, ubiquitinated Ste6 accumulated at the plasma membrane, suggesting that the target of ubiquitination was the endocytosed protein itself and that such ubiquitination preceeded internalization (83, 84). Other work had already established the product of the gene *NPI1* (nitrogen permease inactivator) was involved in the downregulation of Gap1, a general amino acid permease in yeast. Mutation of NPI1 stabilized Gap1 at the cell surface under conditions such as growth in high nitrogen, where it is normally internalizaed and degraded in the vacuole (46, 170). *NPI1* turned out to encode the HECT-type ubiquitin ligase Rsp5, which is responsible for the ubiquitination and subsequent degradation of

many yeast membrane proteins (Table 6-1) including Ste2 and Ste3, two G-protein coupled receptors (54, 64, 90, 146).

Rsp5 is a versatile ubiquitin ligase with many targets in the endocytic pathway as well as the biosynthetic pathway (148). Rsp5, is a HECT-type Ub ligase that is thought capable of forming homodimers (33). HECT-type Ub ligases are distinguished from RING finger Ub ligases in that they form an E3-Ub intermediate with an active cysteine on the HECT domain, whereas RING finger E3s simply facilitate the transfer of Ub from a charged E2 enzyme to the substrate (Fig. 6-1)(158). The HECT domain is always found on the C-terminus of HECT E3 ligases. It is made up of two lobes, the N-lobe and the C-lobe, separated by a flexible “hinge” portion (Fig. 6-2A)(Fig. 6-6)(79). The N-lobe, which is more N-terminal, binds to the E2-Ub complex. The E2-bound Ub interacts with the C-lobe and this interaction helps bring the N-lobe into close proximity with the C-lobe. The conformational change that results is thought to facilitate the transfer of Ub from the E2 onto the catalytic cysteine of the HECT domain (Fig. 6-2B)(70). Different HECT domain E3 ligases will facilitate different types of polyubiquitination. A recent report suggests that substituting the C-lobe of Rsp5 with the C-lobes from Nedd4 homologs can change the chain specificity from K63 to K48 or other linkages (70). Rsp5 also contains a C2 domain that facilitates interactions with lipids, as well as three WW domains that facilitate interaction with other proteins (Fig. 6-6)(187).

Rsp5 is recruited to many permeases by specific adaptor proteins, some of which share common features with mammalian arrestin proteins (Fig. 6-1)(Fig. 6-3)(58, 95, 121). These adaptors share a common feature in that they all contain a PY motif which facilitates the adaptor proteins interaction with Rsp5 via the WW motifs on Rsp5 (Fig. 6-3). Many of them are also ubiquitinated by virtue of their interaction with Rsp5 which may influence their ability to bind cargo or

their localization (93). Sna3, a protein with unknown function in yeast, has two transmembrane domains, a PY motif and gets ubiquitinated by Rsp5 (Fig.6-4)(109, 123, 133, 141, 171). Interestingly, deletion of the lysines within Sna3 attenuates the ubiquitination of Sna3 but does not prevent Sna3 from reaching the vacuole, leading to speculation that Sna3 could potentially reach the vacuole independent of ubiquitination (123, 141, 189). This is relevant because other studies in animal cells suggest other proteins sorted into MVBs may also do so in a manner that does not require their ubiquitination (57, 176, 180). However, in that absence of a PY motif, Sna3 fails to reach the vacuole, indicating that interaction with Rsp5 plays a role in Sna3 trafficking to vacuoles (189). Also, Sna3 fails to reach the vacuole in the absence of Mvb12, an ESCRT-1 subunit, suggesting an ESCRT dependent role for the vacuolar localization of Sna3 (161). Using several “upgrade” blocks we demonstrate that Sna3 does infact rely on ubiquitination for entry into the vacuole, although it remains unclear whether ubiquitination of Sna3 itself or perhaps one of the proteins that Sna3 interacts with is responsible. These data suggest a potential role as an Rsp5 adaptor protein for Sna3.

Results

Analysis of HECT domain Rsp5 mutants.

During Ub transfer from E2 enzymes to the catalytic cysteine on HECT domains, the HECT domain comes into contact with Ub. Several mutations were tested in the HECT domain of Rsp5 that were predicted to bind Ub in cells harboring ts (temperature-sensitive) alleles of Rsp5. At the non-permisive temperature, inactivation of ts alleles in cells expressing Rsp5 HECT Ub-binding mutants, should not support viability. This was first tested using an Rsp5 lacking

its catalytic cysteine within the HECT domain that accepts Ub during transfer from the E2 enzyme to Rsp5. Temperature sensitive *rsp5-1* and *rsp5-2* cells were transformed with a plasmid encoding a mutant version of Rsp5 ($Rsp5^{C777S}$). Growth assays were performed at the permissive (37°C) and non-permissive temperature (37°C). As expected, only the WT version of Rsp5 was capable to suppress the growth defect at 37°C for the *rsp5-1* strain (Fig 6-5). Surprisingly, the WT and the $Rsp5^{C777S}$ were both capable of suppressing the growth defect of the *rsp5-2* cells grown at 37C. The *rsp5-1* strain carries an allele of Rsp5 that exhibits a ts growth phenotype because of a mutation in the C-lobe of the HECT domain at L733 (Fig. 6-6). We found the mutation within the mutant Rsp5 in the *rsp5-2* strain using PCR and DNA sequencing and found that the mutation in the HECT domain was located in the N-lobe of the HECT domain, with an amino acid substitution of S535 (Fig. 6-6). Importantly, Rsp5 has previous data has shown that Rsp5 can form a homodimer, both in-vitro and in-vivo (33). We reasoned that perhaps the two mutant versions of Rsp5, one harboring a mutation in the N-lobe and the other in the C-lobe, could stabilize each other since the *rsp5-2* allele has an intact C-lobe and the $Rsp5^{C777S}$ has an intact N-lobe.

Next, we tested several other Rsp5 alleles, all of them harboring mutations in the C-lobe of the HECT domain changing residues which are predicted to make contact with Ub. Each of the mutants had an HA tag fused the N-terminus of Rsp5 and were expressed in *rsp5-1* and *rsp5-2* cells. A western blot for HA confirmed expression (Fig. 6-7). In the *rsp5-1* strain, none of the mutants tested were able to support growth at the non-permissive temperature, suggesting that the mutations in C-lobe combined with the *rsp5-1* mutant could not support Rsp5 activity (Fig. 6-8A). We also assessed whether Rsp5 mutants were capable of mediating ubiquitin-dependent Gap1 sorting in *rsp5-1* cells. Cells were shifted to the non-permissive temperature (37°C) and Gap1-GFP expression was induced.

While the wildtype Rsp5 mediated sorting of Gap1-GFP to the vacuole, all of the C-lobe HECT domain mutants tested showed defective sorting. We further characterized the C-lobe mutants in *rsp5-2* cells. The Rsp5 mutants were expressed in *rsp5-2* cells and like the catalytically dead C777S mutant, the C-lobe mutants were all capable of suppressing the growth defect at the non-permissive temperature (37°C)(Fig. 6-9)(Fig. 6-5). These data support the idea that the two alleles are Rsp5 are forming a dimer and that the two HECT domains can function together.

Analysis of the ubiquitin-dependent trafficking of Sna3

Several reports have proposed that Sna3 enters the yeast vacuole, independent of ubiquitination. We used protein upgrade to assess whether ubiquitination plays a role in Sna3 trafficking to vacuoles. The Ubp7 upgrade cassette was fused to the C-terminus of Sna3-mCherry and expressed in WT cells. Sna3-mCherry was localized to the yeast vacuole but surprisingly, fusion of the catalytic domain from Ubp7 prevented Sna3 trafficking to the vacuole (Fig. 6-8). These data suggest a role for ubiquitination in Sna3 trafficking to vacuoles. Next, we confirmed previous data that the PY motif which facilitates the interaction between Sna3 and the WW domains of Rsp5. Sna3$^{\Delta PY}$-mCherry was expressed in cells and as expected, Sna3ΔPY-mCherry was localized in the cytosol in small puncta and was excluded from the vacuole (Fig. 6-9). This result was consistent with previous data suggesting Rsp5 binding plays a role in Sna3 trafficking to the vacuole (109, 171).

Deletion of the ESCRT-I subunit Mvb12, leads to a defect in vacuolar sorting for Sna3 (161). This suggests that ESCRT proteins play a role in Sna3 sorting to vacuoles. To further determine whether the vacuolar sorting of Sna3 to

vacuoles was a ubiquitin-dependent sorting step catalyzed by ESCRTs, Hse1-DUb fusions were expressed in cells with Sna3-GFP. Here, Hse1-DUb should prevent the sorting proteins that require ubiquitination for entry into the MVB but should not block proteins that do not require ubiquitination for entry (Chapter 4). Expression of Hse1-Ubp7 or Hse1-UL36 prevented Sna3-GFP from reaching the vacuole (Fig. 6-10). These data are similar to what was observed in cells lacking Mvb12, suggesting that Sna3 enters the vacuole by coming into contact with ESCRTs and that ubiquitination plays a role in Sna3 vacuolar targeting (161). As a control, we found that the catalytically inactive DUb fusion Hse1-ul36* had no affect on Sna3-GFP localization (Fig. 6-10).

Interaction with Rsp5 is necessary for Sna3 ubiquitination and trafficking to the vacuole and our data and those of previous studies agree on these points. Previous studies concluded that it was the physical association of Rsp5 and not the ligase activity of Rsp5 ubiquitinating Sna3 that was the key to Sna3 sorting into the vacuole (189). In contrast, our above data suggest that indeed ubiquitination of Sna3 or a Sna3 associated protein is required for MVB sorting and that Sna3 is not an example of a Ub-independent MVB cargo. As a further test, we tested determined Rsp5-Ubp7 could prevent Sna3 from reaching the vacuole (109, 189). The Rsp5-Ubp7 fusion is Rsp5 fused to the catalytic domain of the Ubp7 DUb, which essentially turns Rsp5 into a specific deubiquitinating enzyme targeted to endogenous Rsp5 substrates. Expressing Rsp5-Ubp7 partially blocked the delivery of Sna3-GFP to the vacuole, resulting in significant localization to non-vacuolar puncta (Fig. 6-11). This localization was similar to that Sna3-GFP displayed in cells expressing the Hse1-DUb fusion protein or by the Sna3$^{\Delta PY}$-mCherry protein that is defective in its ability to bind Rsp5 (Figures 6-9 and 6-10). These results indicate that ubiquitination plays a role in Sna3 sorting to vacuoles. This could be due to direct ubiquitination of Sna3 itself, or

because Sna3 binds to another Rsp5 substrate that undergoes ubiquitin-dependent vacuolar localization.

Sna3 has several features that are common to many of the Rsp5 adaptor proteins listed in Table 6-1 and depicted in Figure 6-3. An *in-vivo* screen for protein-protein interactions based on bi-molecular complementation in yeast showed an interaction between Sna3 and the methionine permease Mup1 (177). This led us to hypothesize that perhaps Mup1 was a possible Sna3 substrate in the sense that Sna3 could help recruit Rsp5 to Mup1 and facilitate the latter's ubiquitination and vacuolar delivery. To test this idea, we determined whether Sna3-DUb could prevent the ubiquitin-dependent sorting of the Mup1. Cells were transformed with Mup1-GFP and either Sna3-mCherry or Sna3-mCherry-Ubp7 and grown under conditions lacking methionine, a condition where Mup1 is found solely at the plasma membrane. Addition of methionine caused Mup1-GFP to rapidly change localization from the plasma membrane to the vacuole in in wildtype cells in the absence or the presence of exogenously expressed Sna3-mCherry. Sna3-mCherry itself was localized in the vacuole before and after methionine addition as is expected for Sna3 (Figure 6-14 A). In contrast, expressing Sna3-mCherry-Ubp7 dramatically slowed the progression of Mup1-GFP to the vacuole upon addition of methionine. In addition, Sna3-mCherry-Ubp7 itself was stabilized at the plasma membrane. These data suggest that Sna3 is interacting with Mup1 or a Mup1 binding protein, possibly as an Rsp5 adaptor protein.

Discussion

Rsp5 is a versatile HECT-type ubiquitin ligase in yeast that facilitates the ubiquitination and subsequent trafficking of many proteins including permeases,

G-protein coupled receptors and transcription factors. Since Rsp5 is an essential gene, temperature-sensitive alleles of Rsp5 have been used to study the effects of Rsp5 dependent ubiquitination. In this study we used two ts alleles of Rsp5 to characterize the activity of several clones of Rsp5 housing mutations in the C-lobe of the HECT domain. All of these mutants are incapable of complementing rsp5 null mutants. However, we found that these Rsp5 mutants could provide Rsp5 function when present along side of the *rsp5-2* mutant allele. The Rsp5-2 mutant is a TS protein with a mutation in the N-lobe of the HECT domain, which on its own does not support viability at 37°C. Previous studies have demonstrated that Rsp5 can work as an oligomer and that the *rsp5-2* mutation is semidominant, exerting an effect on WT Rsp5 through oligomerization (33). Our data suggest that Rsp5 oligomers can suppress HECT domain mutations that result in a loss of function.

Interestingly, cells expressing two Rsp5 mutations housing mutations on the C-lobe of the HECT domain were not able to suppress the growth defect at 37°C, suggesting that for a functional Rsp5 oligomer, at least one intact N-lobe and one intact C-lobe are required. In our model, the *rsp5-2* allele makes contact with Ub due to its intact C-lobe, and the $Rsp5^{C777S}$ mutant provides the functional N-lobe, which together form a functional HECT domain by virtue of the ability of Rsp5 to form a homodimer. The N-lobe of the HECT domain makes contacts with E2 enzyme so that Ub can be transferred from the E2 to the catalytic cysteine in the C-lobe of the HECT domain. One possibility is that the $Rsp5^{C777S}$ mutant binds to the E2 enzyme, which allows for the transfer of Ub from the E2 to the *rsp5-2* allele. We began some preliminary work to explore this possibility further. Mutations in Rsp5 were generated using crystal structure of the HECT domain from Nedd4-2 in complex with an E2 enzyme (Fig. 6-13). Two Rsp5 mutants were generated, each with one mutation of a conserved residue (Asp608 or

Trp597) predicted to disrupt Rsp5 interaction with E2 enzyme. The mutants were expressed alone or in combination with catalytically inactive Rsp5^{C777A}, or WT Rsp5 in the ts *rsp5-1* strain and grown at 30°C or 37°C. Both of the E2 binding mutants were able to support growth at the non-permissive temperature when expressed alone or with WT or catalytically inactive Rsp5 (Fig. 6-13). These data could mean one of two things. The first possibility is that the mutants are able to function cooperatively, with the *rsp5-1*, WT or catalytically inactive providing the E2 binding and the E2 binding mutants providing the catalytic activity. Another possibility is that the E2 binding mutants are still able to bind to E2 and the mutations were not sufficient to prevent E2 binding. This can be examined with future studies. Before biochemical verification of the mutants, we could first determine this by a simpler genetic test. If the new *rsp5* mutants really do not bind E2, they should not work as the sole source of *RSP5* within cells and only function when another *rsp5* allele is present. Thus, the E2 binding mutants should be expressed in *rsp5Δ* cells. As the sole copy of Rsp5 in cells, if the E2 binding mutants can support viability, it is likely that they can still bind to E2 enzyme.

Sna3 has emerged in the literature several times over the past decade as a “ubiquitin-independent” MVB cargo. The Sna3 data in chapter 6 demonstrate an advantage of protein upgrade over conventional methods, which were used previously to block Sna3 ubiquitination. One problem with these studies is that they used GFP-tagged Sna3 and only eliminated lysines within Sna3 and not GFP. The GFP tag that we used in our studies contains 15 lysine residues and the mCherry tag contains 20 lysine residues. Since Sna3 interacts directly with Rsp5, it is likely that the lysine residues contained within the fluorescent tags are ubiquitinated. Consistent with our study is another that used cell fixation and immunohistochemistry to show that Sna3 lacking lysine residues cannot traffic to

the yeast vacuole and instead is trapped on the limiting membrane of the vacuole. However the data presented in that study are difficult to interpret because the immunofluorescent images are not very clear (171). Nevertheless, our data suggest the ubiquitination plays a critical role in the trafficking of Sna3 to the vacuole. One possibility is that Sna3 is capable of entering the vacuole via binding to other ubiquitinated cargo protein, which would mean Sna3 can traffic to vacuoles independent of its own ubiquitination, but dependent on the ubiquitination of another protein or proteins.

Further studies are needed to validate our findings with Sna3. Since Sna3-mCherry-Ubp7 partially blocked Mup1-GFP trafficking to vacuoles, it is possible that Sna3 is an Rsp5 adaptor for Mup1. In this model, Sna3 facilitates interactions between Rsp5 and MVB cargo like Mup1. Many MVB cargos share multiple adaptor proteins. Many MVB cargos are associated with multiple Rsp5 adaptors (Table. 6-1). If Sna3 is an Rsp5 adaptor, the MVB cargo Sna3 associates with is most likely associated with other Rsp5 adaptors in parallel. To determine the particular targets of Rsp5 adaptors, many studies make use of Rsp5 adaptor deletion strains where several adaptors are deleted in the same strain so that the targets of a single adaptor can be determined. This approach should be applied here. Also, the experiment in Figure 6-14 should be repeated with several MVB cargo proteins to determine if there is specificity for Sna3-DUb or if Sna3-DUb is simply blocking ubiquitination of all Rsp5 target proteins. If Sna3-DUb is cargo specific, the fusion of a DUb to an E3 ligase adaptor protein could be used in the future as a novel tool to reveal the targets of E3 ligases and their adaptors.

Figure 6-1. HECT Ub Ligases.

Schematic of ubiquitination mediated by HECT-type Ub ligases. HECT-type E3s mediate the direct ubiquitination of substrates (A) or can mediate ubiquitination via an adaptor molecule (B).

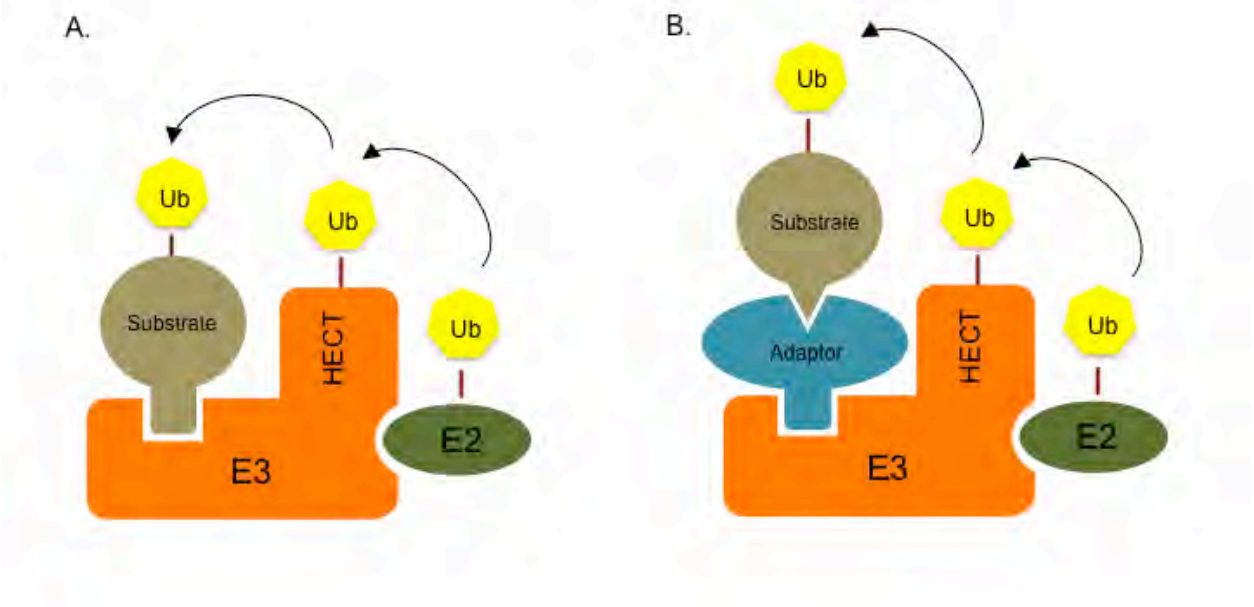

A.
Ub
Ub
Substrate
HECT
Ub
E2
E3
B.
Ub
Substrate
Ub
Adaptor
HECT
Ub
E2
E3

Figure 6-2. The Struture of the HECT Domain.

The crystal stucture of the HECT domain in complex with an E2 enzyme alone (A)(PDB 2ONI) or in complex with Ub (B) (PDB 3JW0). The E2 is in blue, the N-lobe in green, the C-lobe in grey and Ub in Red. The hinge portion of the HECT domain is indicated by the arrow.

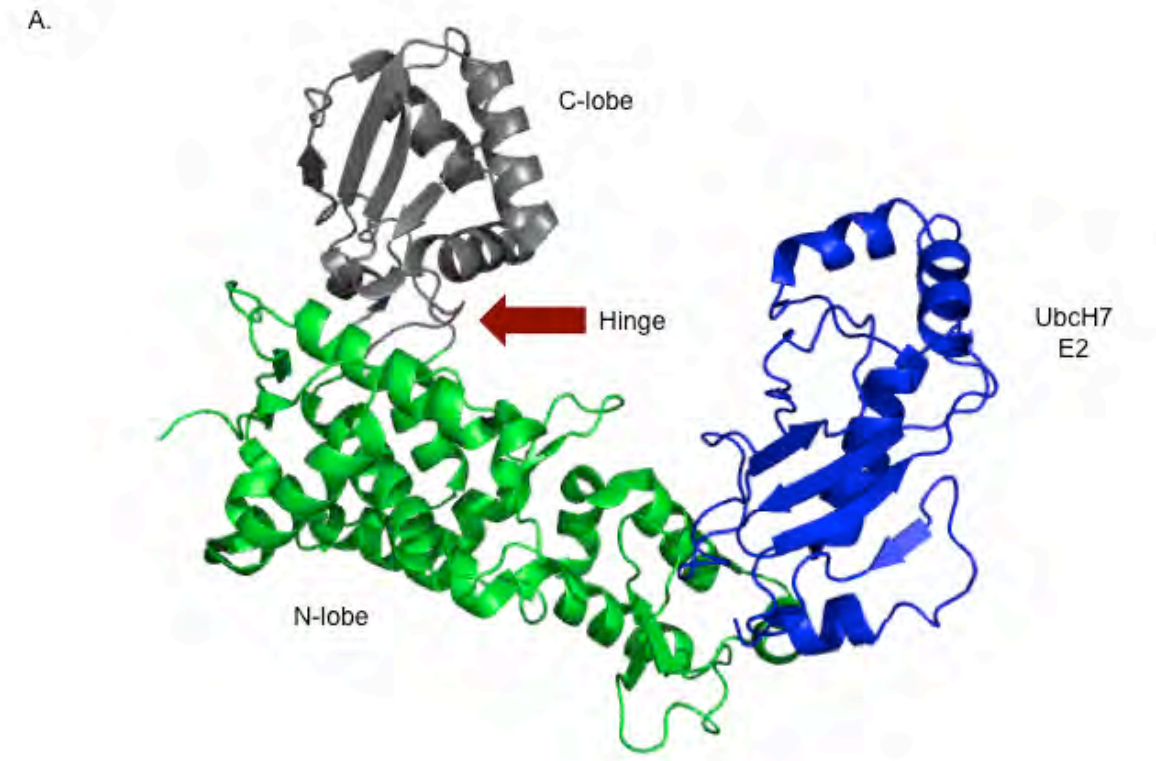

A.
C-lobe
Hinge
UbcH7
E2
N-lobe

Figure 6-2 continued

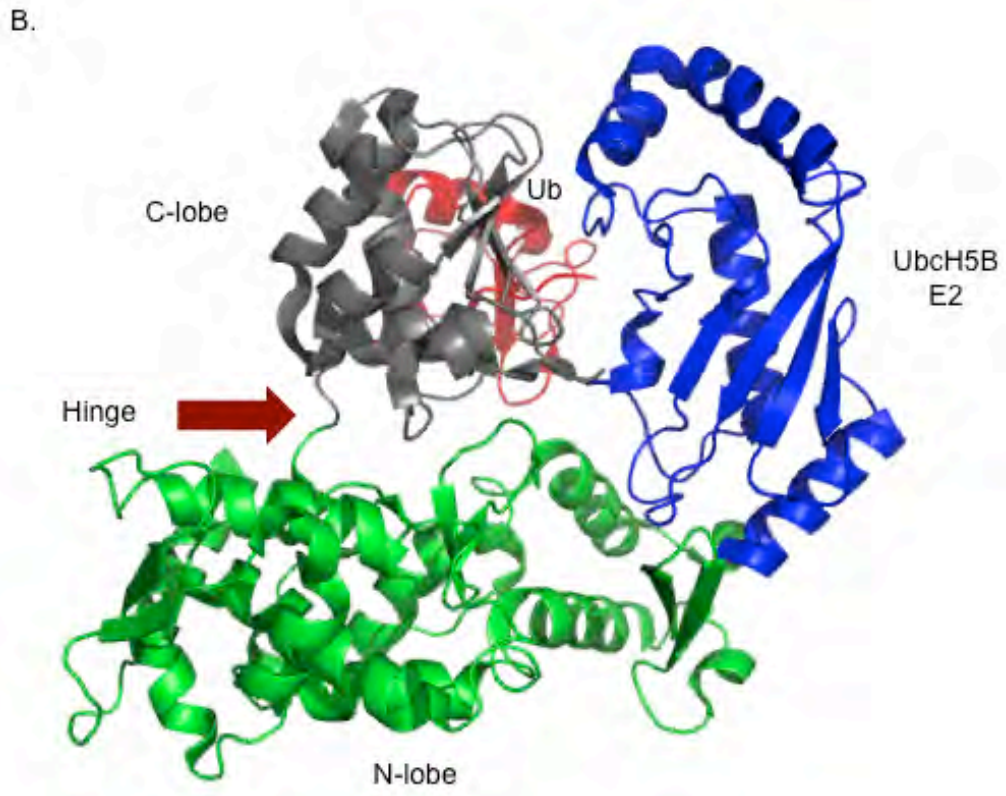


Figure 6-3. Features of Rsp5 Adaptor Proteins.

Schematic representation of the Rsp5 adaptor proteins Bul1, Art1, Art2, Art8, Bsd2, Tre1 and Ear1. Sna3, a protein with unknown function that binds to Rsp5 is also depicted. PY motifs are as indicated. Conserved arrestin motifs (AM) are in blue.

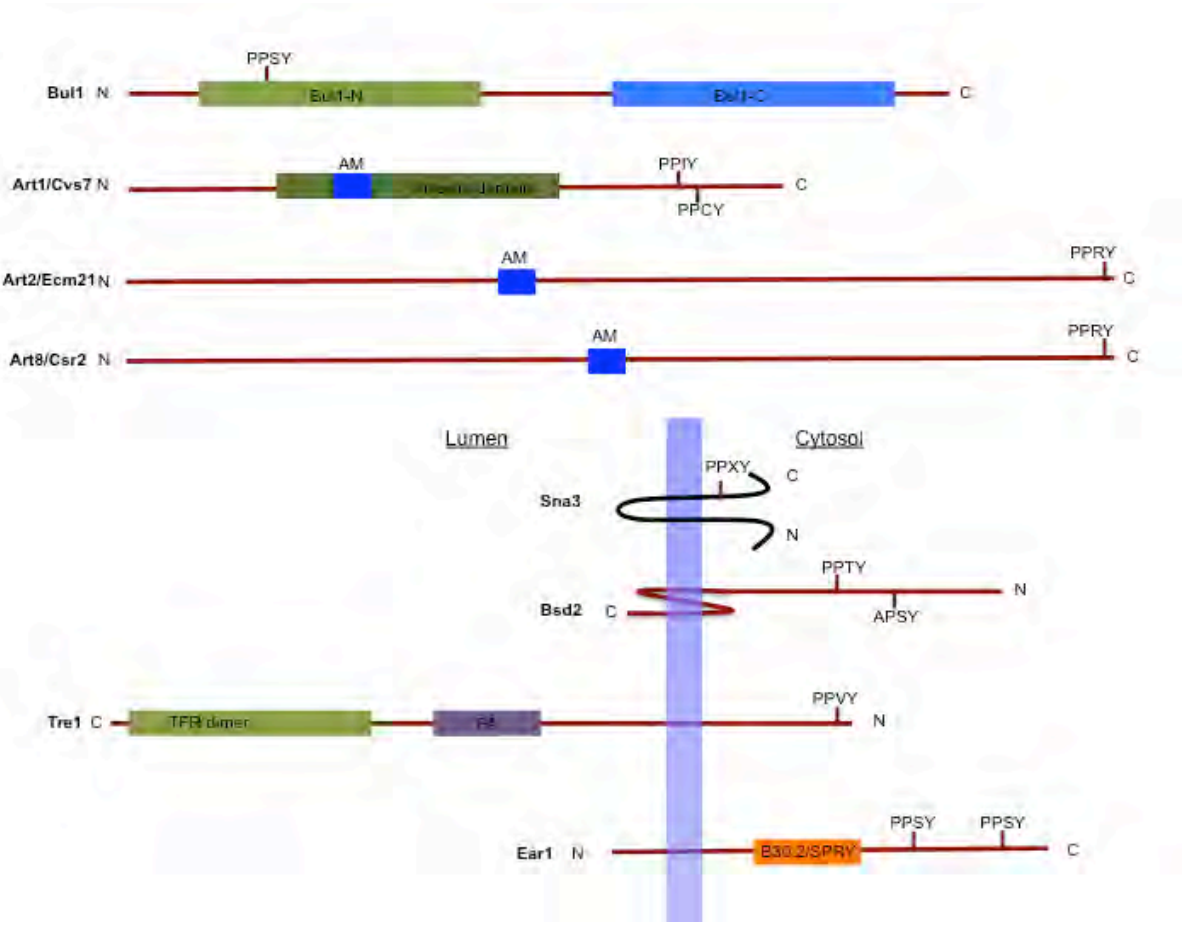

PPSY
Bul1 N
Bul1-N
Bul1-C
C
AM
PPIY
Art1/Cvs7 N
C
PPCY
AM
PPRY
Art2/Ecm21 N
C
AM
PPRY
Art8/Csr2 N
C
Lumen
Cytosol
PPXY
C
Sna3
N
PPTY
N
Bsd2 C
APSY
PPVY
Tre1 C
TFR dimer
N
PPSY
PPSY
Ear1 N
B30.2/SPRY
C

Figure 6-4. Rsp5 Growth Defects in TS Strains.

Vector, HA-Rsp5 or catalytically inactive Rsp5^{C777S} expressed in *rsp5-1* or *rsp5-2* strains grown at 30°C or 37°C. Catalytically inactive Rsp5^{C777S} lead to growth defects in *rsp5-1* but not *rsp5-2* TS strains.

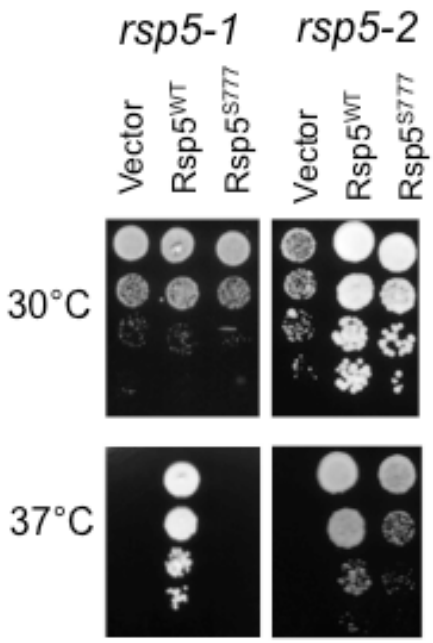

rsp5-1
rsp5-2
Vector
Rsp5WT
Rsp5S777
Vector
Rsp5WT
Rsp5S777
30°C
37°C

Figure 6-5. Schematic of Rsp5.

Schematic of the domains and mutations within Rsp5 (Orange). The C2 domain (blue), WW domains (red), N-lobe (green) and C-lobe (grey) and hinge portion (black) are as indicated. Mutations are indicated by label and marked by black arrows.

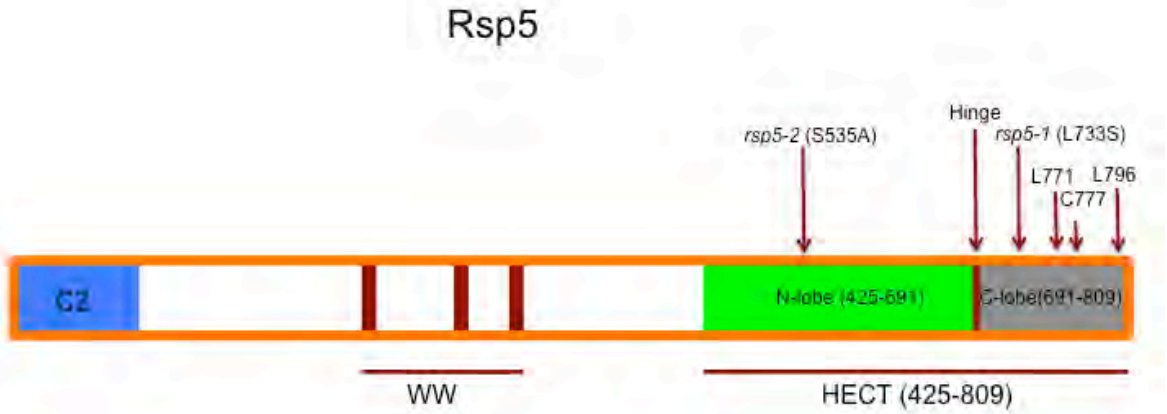

Rsp5
rsp5-2 (S535A)
Hinge
rsp5-1 (L733S)
L771
C777
L796
C2
N-lobe (425-691)
C-lobe(691-809)
WW
HECT (425-809)

Figure 6-6. Expression of HA-Rsp5 Mutants in Cells.

Cells expressing Vector, or HA-tagged Rsp5 mutants (as indicated) were subjected to western blot analysis for HA.

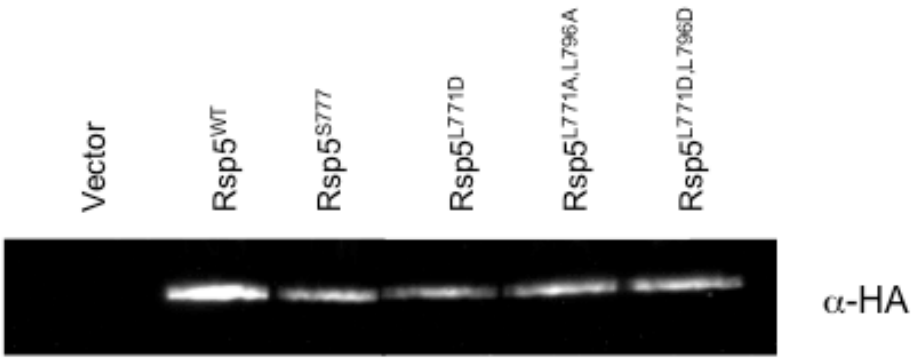
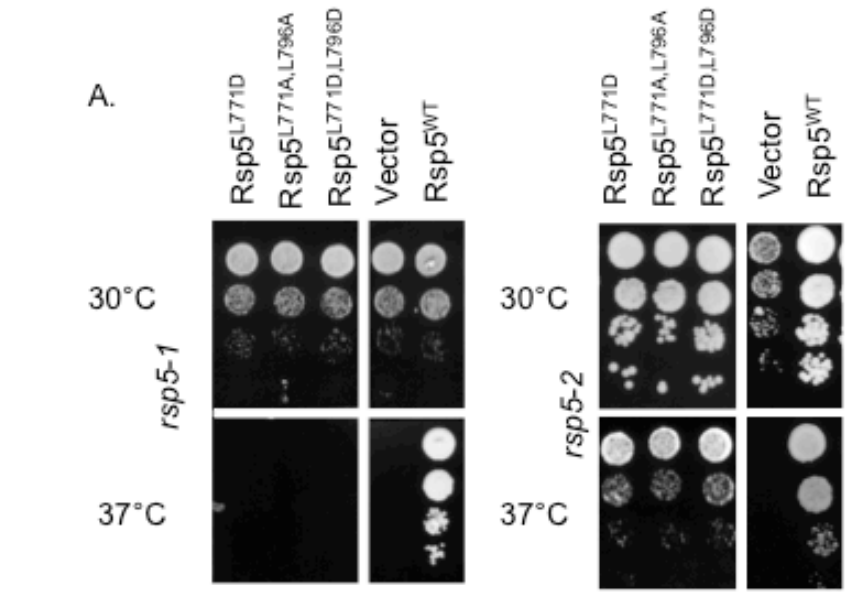

Vector
Rsp5WT
Rsp5S777
Rsp5L771D
Rsp5L771A,L796A
Rsp5L771D,L796D
α-HA

Figure 6-7. Analysis of Rsp5 TS Strains Expressing Rsp5 Mutants.

(A). Rsp5 activity was assessed in *rsp5-1* and *rsp5-2* cells expressing the indicated Rsp5 mutants grown at 30°C or 37°C. (B). Gap1-GFP was expressed by CuCl2 addition after shift to the non-permissive temperature in rsp5-1 cells expressing the indicated Rsp5 mutants.


A.
Rsp5L771D
Rsp5L771A,L796A
Rsp5L771D,L796D
Vector
Rsp5WT
30°C
rsp5-1
37°C
Rsp5L771D
Rsp5L771A,L796A
Rsp5L771D,L796D
Vector
Rsp5WT
30°C
rsp5-2
37°C

B.

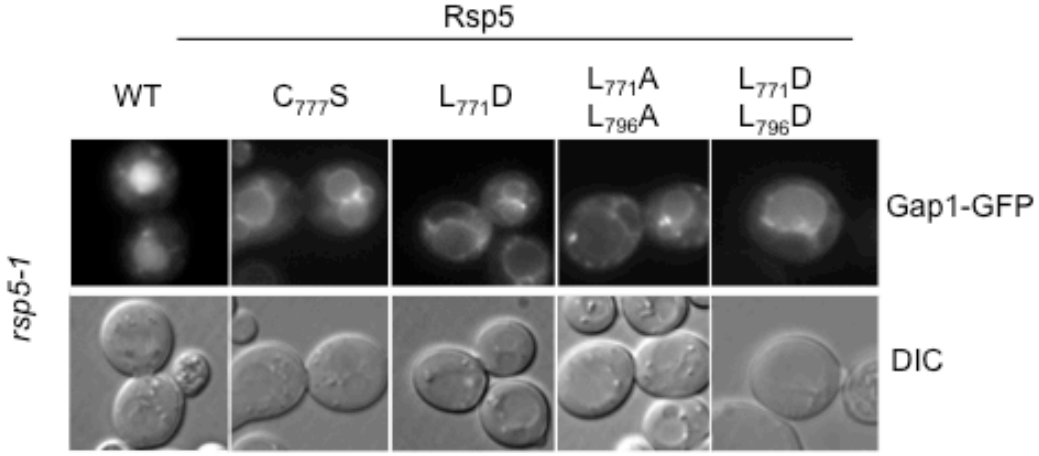

Rsp5
WT
C777S
L771D
L771A
L796A
L771D
L796D
rsp5-1
Gap1-GFP
DIC

Figure 6-8. Upgrade Prevents Sna3 Localization in Vacuoles.

Cells expressing Sna3-mCherry or Sna3-mCherry-Ubp7. Active DUb prevented Sna3 vacuolar targeting, suggesting that the vacuolar localization of Sna3 is dependent on ubiquitination.

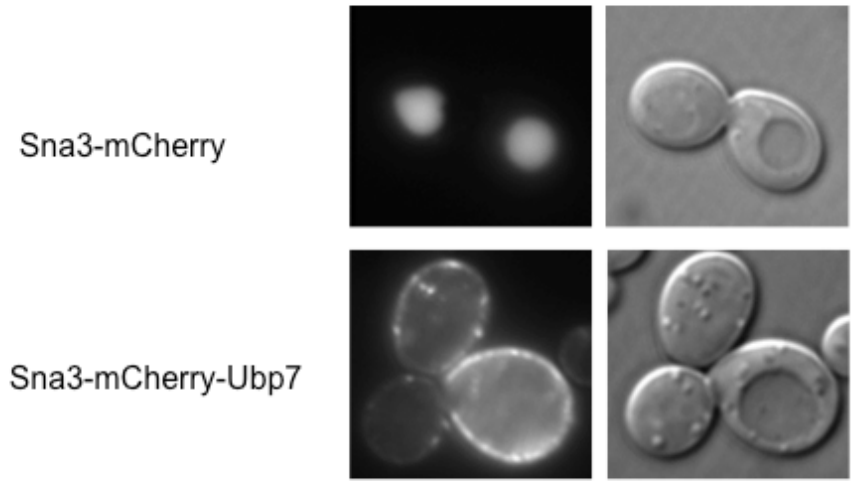

Sna3-mCherry
Sna3-mCherry-Ubp7

Figure 6-9. The PY Motif of Sna3 is Necessary for Vacuolar Localization. Cells expressing Sna3PY-mCherry with localization in puncta, outside the vacuole. These data confirm previous studies that an intact PY motif on Sna3 is necessary for vacuolar targeting.

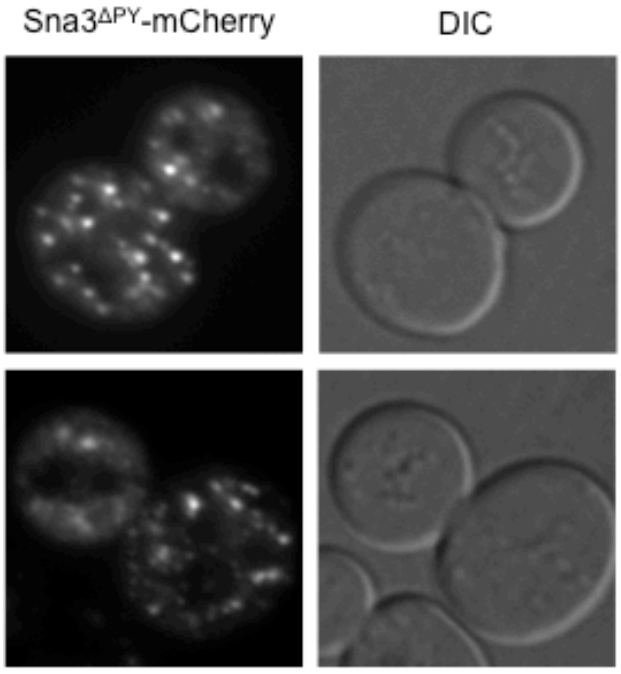

Sna3ΔPY-mCherry
DIC

Figure 6-10. Hse1-DUb Expression Prevents Sna3 Vacuolar Localization. Cells expressing Sna3-GFP and Hse1-Ubp7, Hse1-UL36 or Hse1-ul36*. Hse1-Ubp7 blocks the vacuolar localization of Sna3-GFP, indicating that ubiquitination is necessary for Sna3 trafficking to vacuoles. Inactivation of the DUb cassette restored the vacuolar localization of Sna3-GFP.

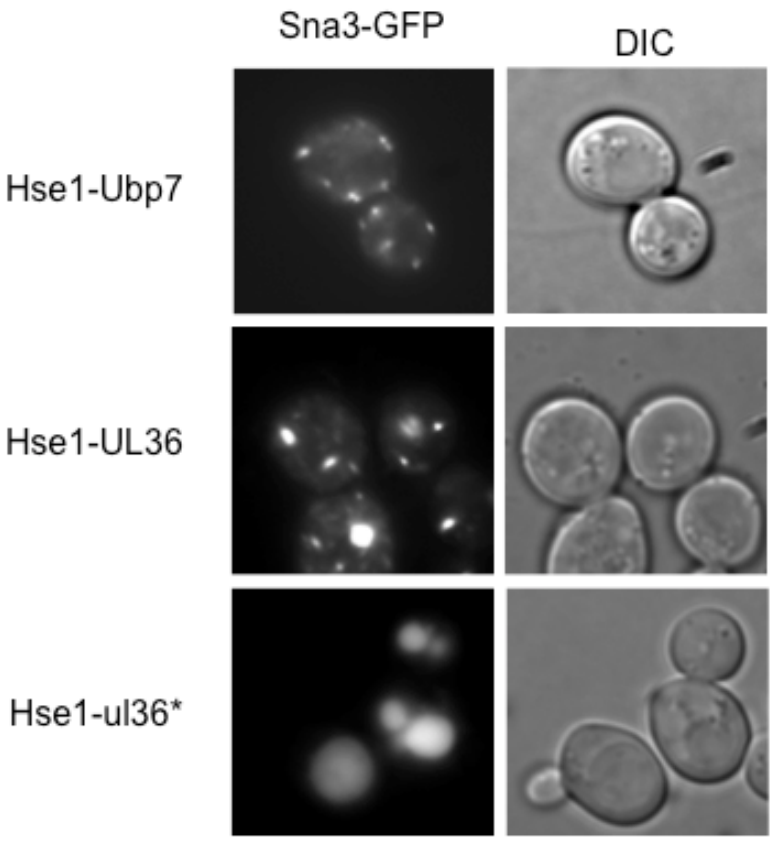

Sna3-GFP
DIC
Hse1-Ubp7
Hse1-UL36
Hse1-ul36*

Figure 6-11. Upgrade of Rsp5 Prevents Sna3 Vacuolar Localization.
Cells expressing Sna3-GFP and Rsp5-Ubp7 (4 hour induction). Expression of Rsp5-Ubp7 prevents Sna3-GFP localization to vacuoles, indicating that ubiquitination is required for Sna3 vacuolar targeting.

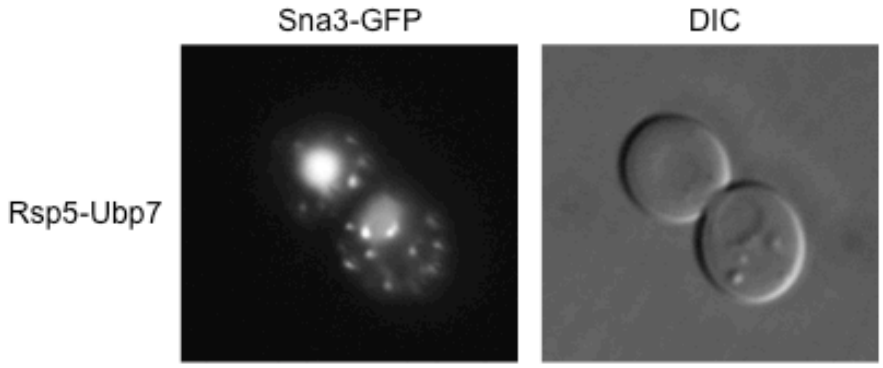
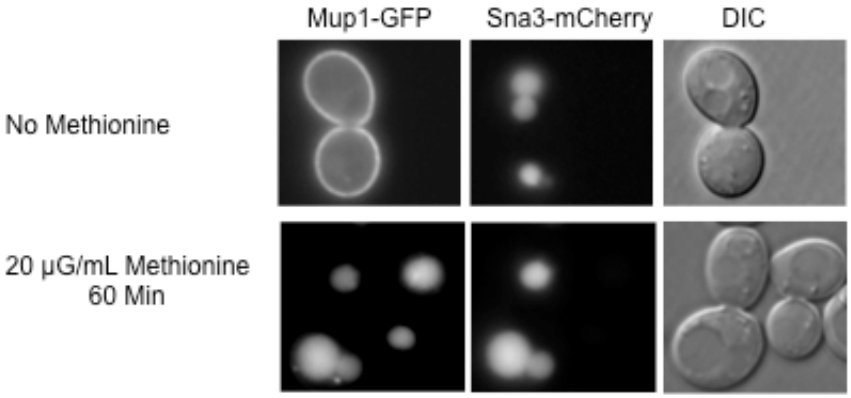

Sna3-GFP
DIC
Rsp5-Ubp7

Figure 6-12. Potential Role for Sna3 as an Rsp5 Adaptor Protein. Localization of Mup1-GFP before and 60 minutes after methionine addition in cells co-expressing Sna3-mCherry (A) or Sna3-mCherry-Ubp7 (B). Expression of Sna3-Ubp7 partially blocked the vacuolar sorting of Mup1-GFP, indicating that Sna3-Ubp7 can block the Ub-dependent trafficking of Mup1.

A.


Mup1-GFP
Sna3-mCherry
DIC
No Methionine
20 µG/mL Methionine
60 Min

B.

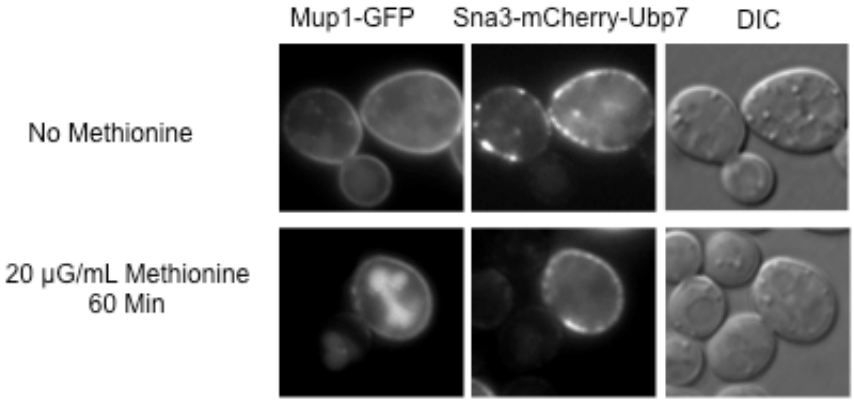
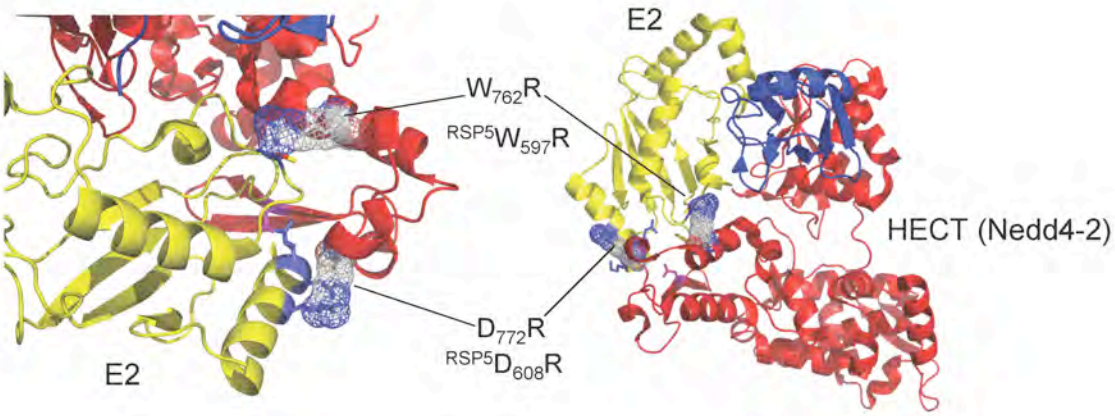

Mup1-GFP
Sna3-mCherry-Ubp7
DIC
No Methionine
20 µG/mL Methionine
60 Min

Figure 6-13. Mutation of the E2 Binding Domain of Rsp5.

Schematic of the Rsp5 homolog Nedd4-2 with an E2 enzyme with mutations to Rsp5 as indicated (A) (23). Rsp5-WT, C>A mutant, or potential E2 binding mutants (indicated) expressed in *rsp5-1* cells at 30°C and 37°C (B).

A.


E2
W762R
RSP5W597R
HECT (Nedd4-2)
D772R
RSP5D608R
E2

B.

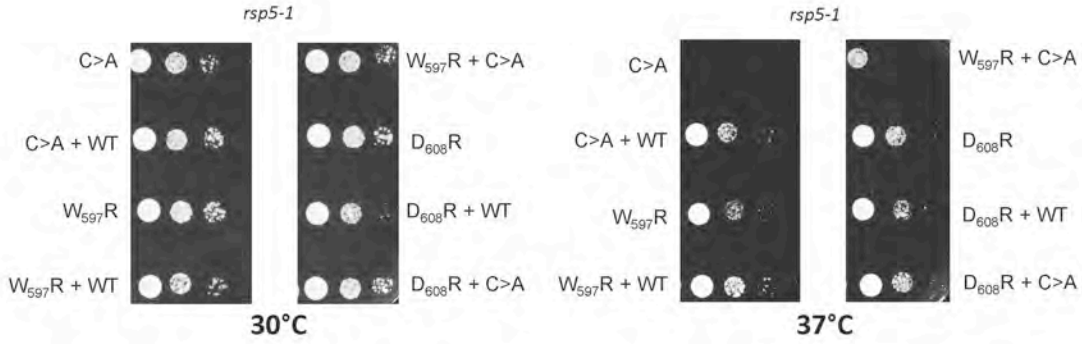

rsp5-1
C>A
C>A + WT
W597R
W597R + WT
W597R + C>A
D608R
D608R + WT
D608R + C>A
30°C
rsp5-1
C>A
C>A + WT
W597R
W597R + WT
W597R + C>A
D608R
D608R + WT
D608R + C>A
37°C

Table 6-1. Rsp5 Adaptors and Their Targets.

Permease	Adaptor(s)
Can1 (arginine)	Art1
Ctr1 (copper)	Bul1, Bul2
Fur4 (uracil)	Bul1, Bul2, Art1, Art2, Art9, Bsd2, Ear1,Ssh4
Gap1 (amino acids)	Bul1, Bul2, Ear1, Ssh4
Hxt6 (hexoses)	Art8, Art4
Itr1 (inositol)	Art5, Bsd2
Lyp1 (lysine)	Art1, Art2
Mup1 (methionine)	Art1
Sit1 (ferroxiamines)	Ssh4, Ear1, Tre1, Tre2
Smf1 (manganese)	Bsd2, Tre1, Tre2, Art2, Art8, Ear1, Ssh4
Tat2 (tryptophan)	Bul1, Bul2, Art1, Art2, Art8, Bsd2

CHAPTER 7: DISCUSSION

Ubiquitination is a versatile process that is involved in many cellular processes. Here, we have developed a novel technique, upgrade, that renders proteins resistant to ubiquitination. We used this technique as well as others to examine how ubiquitination controls protein sorting in the endocytic pathway. Virtually all UBD containing proteins are themselves ubiquitinated. This has lead to a vast literature regarding the potential role of ubiquitination of Ub-binding proteins. We found that ESCRT proteins, which house many UBDs, do not require ubiquitination to facilitate cargo sorting into vacuoles. This is the first study that comprehensively examines whether this process is necessary for MVB formation to proceed normally. Ubiquitination of ESCRTs could modulate other ESCRT related processes in cells, or it could just be an artifact.

Our work also examines two other controversial subjects in the field. Many studies support the idea that K63-linked Ub plays a role in MVB trafficking. One recent study suggests that K63-linked Ub is a necessary signal for MVB sorting (90, 91). We used similar approaches and protein upgrade to test this hypothesis. Our data clearly show that K63 Ub linkages are dispensable for MVB sorting to occur. ESCRT-DUb fusions also provided strong evidence that a single Ub is sufficient for entry into MVBs. This technique is powerful because the DUb should remove any Ub on the protein of interest, but cannot remove in-frame fusions of Ub that are not susceptible to deubiquitination. Many studies make use of GFP or other reporter proteins to follow the trafficking of proteins like Mup1 and Gap1. GFP and other epitopes often have lysine or other residues that are potentially ubiquitinated. Many of the studies claiming that Sna3 is a ubiquitin-independent cargo make use of GFP tags on Sna3 (141) (109) (123)

(189). In fact, one of the only studies that attempted to study Sna3 vacuolar targeting without GFP, found that Sna3 was not localized to vacuoles in the absence of Ub (171). Whether direct ubiquitination of Sna3, indirect Ubiquitination of Sna3 binding proteins or a mixture of both, contribute to Sna3 vacuolar localization, protein “upgrade” suggests that ubiquitination must play some role in Sna3 trafficking to vacuoles.

Future directions

Other methods for making proteins resistant to ubiquitination are not very comprehensive. This is because ubiquitination is a robust process, and in many instances redundant Ub ligases contribute to the ubiquitination of a single substrate. This fact has made it difficult to understand the precise role for the ubiquitination of proteins. Also, since many proteins are ubiquitinated by multiple E3 ligases, it has been difficult to determine the molecular targets for E3 ligases. Upgrade could potentially solve some of these complicated questions. Upgrade of proteins has a dominant effect in that the DUb attached to the protein of interest because it deubiquitinates the protein itself and also the associated proteins (Chapters 3-6). Fusion of the Ubp7 DUb cassette to Rsp5 reversed the vacuolar cargo sorting of every Rsp5 substrate tested. Also, expression Rsp5-Ubp7 but not Rsp5-ubp7* lead to a growth defect, consistent with growth defects observed in Rsp5 mutant strains. This is likely due to a defect in the ubiquitination of Spt23 and Mga2, both substrates of Rsp5 (19, 61, 201). It is likely that this technology could be used in a similar fashion with other E3 ligases. By essentially creating a reversal enzyme, every target of an E3 ligase fused to a DUb should have impaired ubiquitination. Ubiquitination by redundant Ub ligases would also be reversed by the activity of the E3-DUb fusion. This would make it

possible to make the targets of orphan E3 ligases using proteomics. Using mass spectrometry, the ubiquitination status of every protein within cells can be examined. E3 ligases could potentially make good targets for therapeutic drugs. Thus, determining the protein targets of E3 ligases is very important.

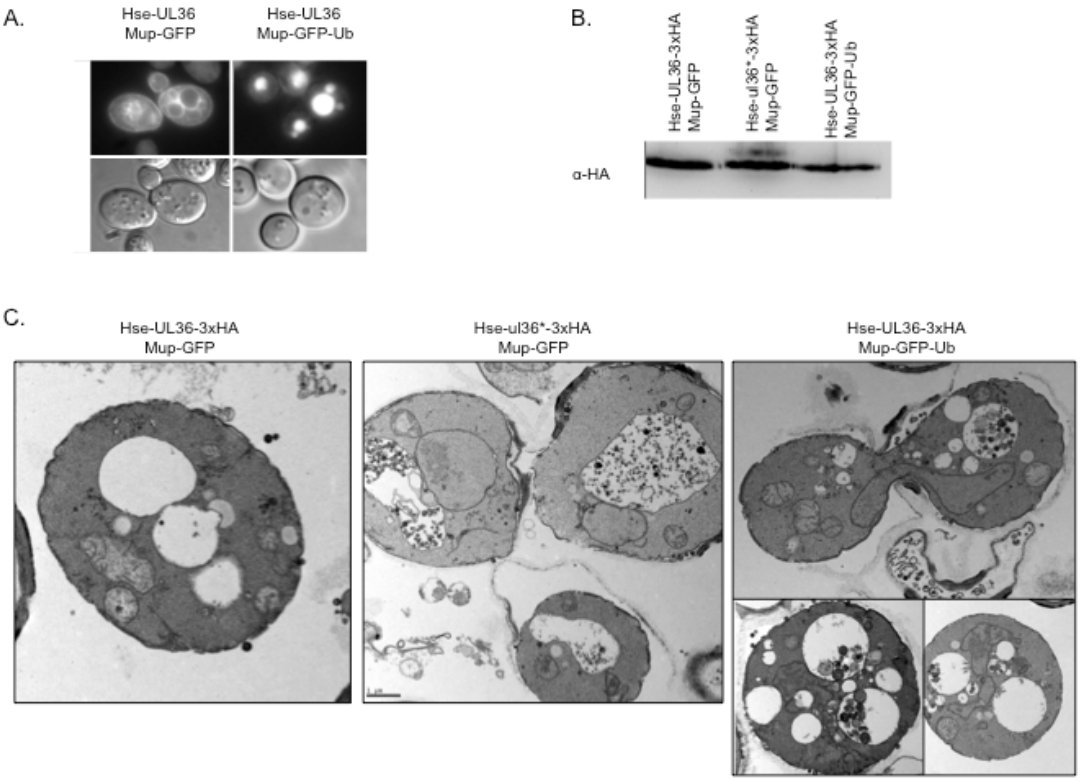
Much work has been done over the past 10 years in determining the molecular functions of the ESCRTs at the sites of MVB formation. A clear role for Ub-binding and ILV formation has emerged. However, it is still unclear whether these ILVs form in the absence of cargo. Many studies have revealed that Ub is the key signal in determining whether a protein gets incorporated into an MVB. But can ILVs still form without Ub-Cargo? We have begun some of the preliminary work to answer this question. ESCRT-DUb fusions blocked the vacuolar localization of every MVB cargo protein we tested (Chapter 4). Since the effect on vacuolar localization was so robust, it is likely that ESCRT-DUb fusions could be used to block every cargo protein that requires Ubiquitination from reaching the vacuole. Hse1-UL36 or Hse1-ul36* was expressed with Mup1-GFP or Mup1-GFP-Ub in cells lacking the *PEP4*. Electron microscopy was performed to examine if ILVs could still form in the absence of Ub-cargo. Very few ILVs were detectable when Hse1-UL36 was expressed with Mup1-GFP (Fig. 7-1). However, ILVs were detectable in vacuoles from cells expressing Hse1-UL36 and Mup1-GFP-Ub and in cells expressing Hse1-ul36*. These preliminary data suggest that ILVs cannot form in the absence of Ub-Cargo. Conceptually, this is consistent with observations for other cellular processes. For instance, cargo proteins recruit AP complexes and coat proteins like clathrin to promote vesicle formation. The process occurs when a signal, such as a tyrosine or dileucine motif, is recognized by AP complexes. Clathrin is recruited to the AP complex and vesicle formation begins. In our hypothesis, Ub is the signal analogous to the tyrosine motif. Ub on cargo is recognized by the UBDs of

ESCRTs, which in turn promote ILV formation. Studies should be performed to determine whether ESCRT-II and ESCRT-III are recruited to the sites of ILV formation in the absence of Ub-cargo when ESCRT-0-DUb or ESCRT-I-DUb is expressed.

My work has demonstrated that protein upgrade is a powerful technique, capable of reversing ubiquitination. Compared with conventional methods, it is fast, effective and provides a negative control. I have used this method to answer complicated questions about how ubiquitination affects the endocytic pathway. Hopefully, this technique can be applied to other complicated questions regarding the role of ubiquitination in cells.

Figure 7-1. Ubiquitinated Cargo Proteins Promote ILV Formation.

(A). Localization of Mup1-GFP and Mup1-GFP-Ub in *pep4Δ* cells expressing Hse1-UL36-3xHA. (B). Western blot analysis (anti-HA) for indicated Hse1-DUb-3xHA expression. (C). Vacuoles from *pepΔ* wildtype cells expressing Hse1-DUb fusions with Mup1-GFP or Mup1-GFP-Ub.


A.
Hse-UL36
Mup-GFP
Hse-UL36
Mup-GFP-Ub
B.
Hse-UL36-3xHA
Mup-GFP
Hse-ul36*-3xHA
Mup-GFP
Hse-UL36-3xHA
Mup-GFP-Ub
α-HA
C.
Hse-UL36-3xHA
Mup-GFP
Hse-ul36*-3xHA
Mup-GFP
Hse-UL36-3xHA
Mup-GFP-Ub

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