A Dissection of Lysosomal Membrane Protein Regulation in *Saccharomyces cerevisiae*

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April 20th, 2021
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Under the Guidance of Dr. Ming Li
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Acknowledgements

There is a general understanding of science I greatly appreciate: we stand on the shoulders of giants. Modern progress is only accessible thanks to the actions of those who worked on the novel discoveries we take as inherent teachings. While this mindset is usually applied to explaining the power of the scientific community, it applies to a scenario as simple as a research team mentoring an undergraduate. With this, I would like to acknowledge and express my appreciation to every member of the Ming Li laboratory for the teachings, the development, and the benevolence they have imparted unto me these four years. To Dr. Li, thank you for always seeing the potential in any situation: research projects, opportunities, and especially people. To Felichi Mae "Peach" Arines, thank you for your never-ceasing kindness, willingness to listen, and for having one of the kindest hearts on this globe. To Varsha Venkatarangan, thank you for always bringing a ray of kindness and empathy to any situation, usually accompanied with a perfect comedic articulation or stylish graphic tee. To Weichao Zhang, thank you for showing me the power behind a direct force of intention and for your inclusion of myself within your mammalian project. To Liang Chen, thank you for your friendship and continuous happiness no matter the hour of the morning. To Dominic Chomchai and past fellow undergraduate, thank you for your partnership and collaboration throughout our years as a research team. Ultimately, thank you to Dr. Xi Yang. As iron sharpens iron, so one person sharpens another; thank you for your guidance, your commitment, and for allowing me to revel in your passion for science alongside you from the laboratory benchside.

I owe everything I am as a scientist to the people listed above and to all who have had a part in showing me firsthand what it means to stand on the shoulders of giants.

Abstract

Subcellular proteome regulation is a necessity to promote cellular efficiency across various growth and stress conditions. The lysosomal organelle of *Saccharomyces cerevisiae*, the vacuole, employs such regulation for management of its membrane bound proteome. This advantageous governance requires proper functionality of a host of machinery responsible for roles like trafficking, signaling, and internalization of the vacuole membrane to the degradative environment of the vacuole lumen. The research presented in this thesis investigates a variety of topics related to the cellular activities surrounding the vacuole. A discrepancy between internalization mechanisms was discussed in which evidence is presented in support of the ubiquitin- and ESCRT-dependent process of targeting and sequestering VM proteins for degradation. Along with this, investigations into the possible existence of a homologous system of internalization within mammalian systems were completed through ectopic expression of human lysosomal membrane protein, RNF152, in yeast to study its degradation dynamics when present in the vacuole system. A final consideration of the power of interactome cataloging is presented through identifying the general trafficking path of the vacuole membrane protein, Fth1p, throughout the cell. This profiling identified required proteomic interactions and localization pathways, showcasing the necessary hierarchical cascade of cellular activities needed to localize a sole protein. In total, this work amalgamates various investigations of cellular regulation in an attempt to model the complexities of cellular activity through considerations of the discrete sum of necessary biochemical processes.

Introduction

The diversity of biochemical life is arguably only rivaled by the complexity of the underlying cellular unit[1]. The scope of activities and responses the cell must accomplish through its molecular machinery is incredibly extensive, ranging from simple metabolic processes to inducing specialized regulatory behavior during stress conditions[1,2]. How such a dynamic nature is projected from the simplicity of biomolecular interactions is only explainable through complexity. Cellular processes and biochemical pathways are intertwined in a branching and layered manner to allow simple fluctuations in either the system or environmental surroundings to invoke profound and compounded effects. While this perspective of biological life is quite general, the accurate modeling of such systems is only limited by the depth of research devoted to defining the interactomes of cellular machinery and their interconnected activities and regulations.

The degradative processes of cellular machinery stand as a prime area of investigation for the principal goal of an encompassing model of biochemical life[3]. Standing as a perfect example of the necessity of complexity, degradation underlies the compounding systems of cellular signaling and targeting in which the cell can dynamically regulate its proteome in response to diverse environmental conditions and triggers to promote homeostasis and growth[2,3]. Along with this, an immense collection of preliminary research is currently available for practically all known macrosystems of degradation[3,4]. Of these systems, the lysosome is an exciting research focus given it exists as an organelle specifically evolved to handle the degradation and recycling of numerous types of cellular components, even its own membrane-bound proteome[2,3].

Research involving the lysosomal organelle of the fungi *Saccharomyces cerevisiae*- the vacuole- have showcased the dynamic regulation of lysosomal membrane proteins due to

selective degradation treatments[5]. For example, Ypq1p, a lysine transporter localized to the vacuole membrane (VM), experiences selective internalization into the vacuolar lumen for degradation upon lysine withdrawal. This simple step-wise process is a hallmark example of cellular complexity in which a simple trigger invokes a hierarchical cascade of effects to induce an advantageous adaptive response, such as lowering the transport of lysine into the vacuole for storage when available sources of said nutrient are limited and would be better utilized within the Endoplasmic Reticulum for protein synthesis. The vacuole has been the focus for a wide range of such research, given the advantages *Saccharomyces cerevisiae*- Baker's yeast- provide as a model organism allows for advanced genetic manipulation[6]. Besides the underlying procedural advantages yeast entails as a genetically well-defined unicellular organism, the vacuole is the only degradative organelle present in the yeast and its coupled functions in nutrient storage allow the vacuole to acquire an immense size in regards to the rest of the cell[7]. Such characteristics allow for ease in visualizing the dynamic nature of its proteome and membrane while ensuring the only degradative activity of the cell is linked to either the cytosolic proteasome or the vacuole protease.

Overall, the vacuole provides a powerful tool in which to connect genetically defined cellular machinery to regulatory processes in which underlying biochemical interactions can be elucidated, like the dynamic mixed nature of lipid and protein populations[8]. Continued identification of homology between the machinery behind such interactions across the yeast vacuole and mammalian lysosome cements research of this focus as a fundamental physiological interest[9]. The fundamental importance of degradative organelles is increasingly becoming apparent given Lysosomal Storage Diseases (LSDs) encompass an immense range of medical conditions like Cystinosis, skeletal dysplasia, Niemann-Pick, and Gaucher's disease[10].

Through collaborative efforts between Dr. Li, Dr. Xi Yang, Mr. Weichao Zhang, fellow undergraduate Dominic Chomchai, myself, and all other members of our team, the Ming Li laboratory has provided multiple advancements in our understanding of these dynamic organelles. The research presented in this thesis stands as a dissection of a multitude of research interests surrounding the yeast vacuole I have investigated under the guidance and collaboration of the aforementioned list of fellow laboratory personnel. The vacuole has provided a manifold of investigative topics, including identification of necessary machineries responsible for proper degradative functionality of the organelle, insights into the homology of such machinery across mammalian and fungi systems, and the power of degradative and trafficking profiling of proteins of interest in aidance of the principal objective of modeling biochemical life in its entirety.

Chapter 1: The Degradative Profiling of Vacuolar Membrane Proteins

Introduction

While the substrates of vacuolar degradation extend throughout the cell, the selective regulation of the vacuolar membrane proteome provides an interesting subset of cargoes given this process is of self-management[5,8]. To handle this undertaking, the cell employs a variety of machinery responsible for the various necessary cellular and biochemical processes[5,8,11]. Studying how exactly this regulation is managed provides intensive insight into the driving factors of cellular activity. In general, degrading a membrane protein entails signaling of targets in need of recycling through ubiquitination, internalization of tagged substrates in which the cargoes and their surrounding lipid bilayer are sequestered into the vacuolar lumen, and ultimately degradation by the vacuolar proteases. This process of vacuolar membrane regulation can be induced by a selective triggering of degradation, such as Ypq1p downregulation due to lysine withdrawal, or at the global level in which a vacuole membrane proteome is majorly downregulated or upregulated through a fundamental cellular sensing mechanism, like the protein complex Target of Rapamycin Complex 1 (TORC1)- mTORC1 for mammalian cells.

Recently, two opposing mechanisms of internalization have been proposed for this process of vacuolar membrane protein regulation[5,12]. Besides differing in the method of sequesterment and the necessary machinery responsible, these methods report on conflicting conditions of global proteome downregulation while simultaneously claiming responsibility for the internalization of similar VM targets. The first proposed method of internalization cites the necessity of a series of cellular machinery titled the Endosomal Sorting Complexes Required for Transport (ESCRT). ESCRT is a group of protein complexes (ESCRT-0 through ESCRT-III) which act in tandem to target ubiquitinated membrane cargoes and sequester them into the lumen

of the respective membrane structure the cargo originated upon[5]. ESCRT has been shown to act both on the endosomal membrane, where cargoes for degradation are sorted into internalized vesicles of Multivesicular Bodies (MVBs), and directly upon the vacuolar membrane. This method of internalization is ubiquitin and ESCRT-dependent. Any deletion or loss of function in a single member of the ESCRT machinery has been shown to halt the degradation of targeted vacuolar membrane proteins, and thus this mechanism is referred to as the ESCRT-dependent pathway. The second method proposed is the Intraluminal Fragment pathway (ILF pathway)[12]. The ILF pathway entails a mechanism of internalization in which targeted cargoes are sequestered into the vacuole lumen through the process of homotypic vacuole fusion in which two vacuoles fuse to conjoin membrane structures and lumenal contents[13,14]. In brief, membrane proteins are internalized at the site of fusion, which produces an intraluminal fragment (ILF) of the membrane vertex. It has been proposed that cargoes are selectively sorted to this vertex site of fusing vacuoles, so that the produced ILF is concentrated with machineries to be recycled. The machinery or cellular processes responsible for this selective sorting has yet to be identified, but is reported to be decisively independent of the ESCRT machinery. Instead, the only identified necessary machinery for the ILF pathway to date is vacuole fusion machinery.

Besides differing in the necessary machineries for internalization, these two competing models report on different TORC1 activity states as global downregulators of the vacuole membrane proteome. Research behind the ESCRT-dependent pathway describes a global downregulation caused by the inactivation of the TORC1 complex through conditions like the addition of rapamycin[11]. Contrary to this, research behind the ILF pathway report conditions like cycloheximide addition, in which TORC1 activity is hyperactivated through the accumulation of

available amino acids due to blockage of translation, trigger a global downregulation of vacuole membrane proteins[12].

Both of these proposed mechanisms could hypothetically occur independent of one another, however a stark competition has arisen based on this disagreement in the effect of TORC1 activation and inactivation. Along with this, the overlap between proposed cargoes of each mechanism stands as a direct conflict of modeling. The degradation of Fth1p, a subunit of a putative iron transporter complex of the vacuole membrane, has been reported to be dependent on both pathways, an impossibility due to the ESCRT dependency of one and the independence of the other.

Due to these discrepancies, the exact mechanism of internalization has become blurred. Lysosomal, and interconnectedly vacuolar, research is increasingly being connected with translational science for the purposes of treating medical conditions[15-20]. Because of this, this controversy must be addressed; to do so, a careful degradative profiling of reported cargoes for both mechanisms was performed in considerations of the conditions which induce degradation and the related necessary machineries.

Results

Rapamycin-induced TORC1 inactivation leads to Fth1-GFP and Fet5-GFP degradation

TORC1 acts as a master regulator of proteome populations due to its nutrient and stress sensory functions having interconnected roles in signaling cascades that affect transcription[21]. Rapamycin inactivates TORC1 via binding to its FRB domain while the protein synthesis inhibitor, cycloheximide, indirectly causes TORC1 activation through a generated abundance of free amino acids. As previously discussed, differing degradative conditions have been proposed across the ILF and ESCRT-dependent pathways. To begin to unravel the discrepancies between these two mechanisms, the degradation of two key target proteins were compared across rapamycin and cycloheximide treatment. Fth1p was chosen as a candidate of interest given degradation of Fth1p was reported to be dependent on both mutually exclusive pathways. The co-subunit of Fth1p, Fet5p, was also studied given its degradation was reported as reliant only upon the ESCRT-dependent pathway. These proteins' degradation was investigated through microscopy and western blotting while in a fusion construct with Green Fluorescent Protein (GFP). Free GFP is relatively stable within the vacuole lumen despite its acidity and proteases, therefore, upon cleavage of a protein-GFP construct, the observable full length population diminishes while free GFP accumulates. This accumulation is observable through both western blotting and microscopy in which signal is generated as an approximately 27 kDA sized band or as fluorescence in the vacuole lumen, respectively[5].

Beginning with the investigations of the effect of cycloheximide upon Fth1-GFP and Fet5-GFP constructs, a decisive lack of degradation was observed for both cargoes through western blot analysis (Figure 1A-B). In contrast to this, eight hour rapamycin treatment caused degradation for both Fth1-GFP and Fet5-GFP as seen through the diminishment of full length

construct and the paired accumulation of approximately 27 kDa free GFP (Figure 1C-D). This result showcased the degradation of Fth1-GFP and Fet5-GFP populations through TORC1 inactivation. This observation was confirmed through fluorescence microscopy in which GFP signal transitioned from the vacuole membrane to the vacuole lumen following rapamycin but not cycloheximide treatment (Figure 1E). These results showcase the degradative effects of TORC1 inactivation, not activation, for the reported cargoes of the two methods of internalization. This was in direct conflict with the research underlying the ILF pathway, thus continuing investigations were completed in an attempt to replicate and check this pathway's research[12].

Another reported degradative treatment of the ILF pathway was 37℃ heat shock. The inducing capabilities of this treatment was thus analyzed for Fth1-GFP and Fet5-GFP and a third reported cargo of the ILF pathway, Vph1-a subunit of the vacuolar V-ATPase proton pump. Upon 37℃ incubation for the exact length reported as degradation-inducing, no degradation was observable for any of these target proteins (Figure 1F). Based on these discrepancies between the claims and the actual observed results of degradation triggers, the independence of established degradation machinery, as reported on through the ILF pathway proposal, required confirmation. Fth1, as the key overlapped cargo between the two methods of internalization, was chosen as the primary cargo of interest for these further studies with rapamycin as the chosen degradation-inducement treatment based on these initial results of degradative dynamics.

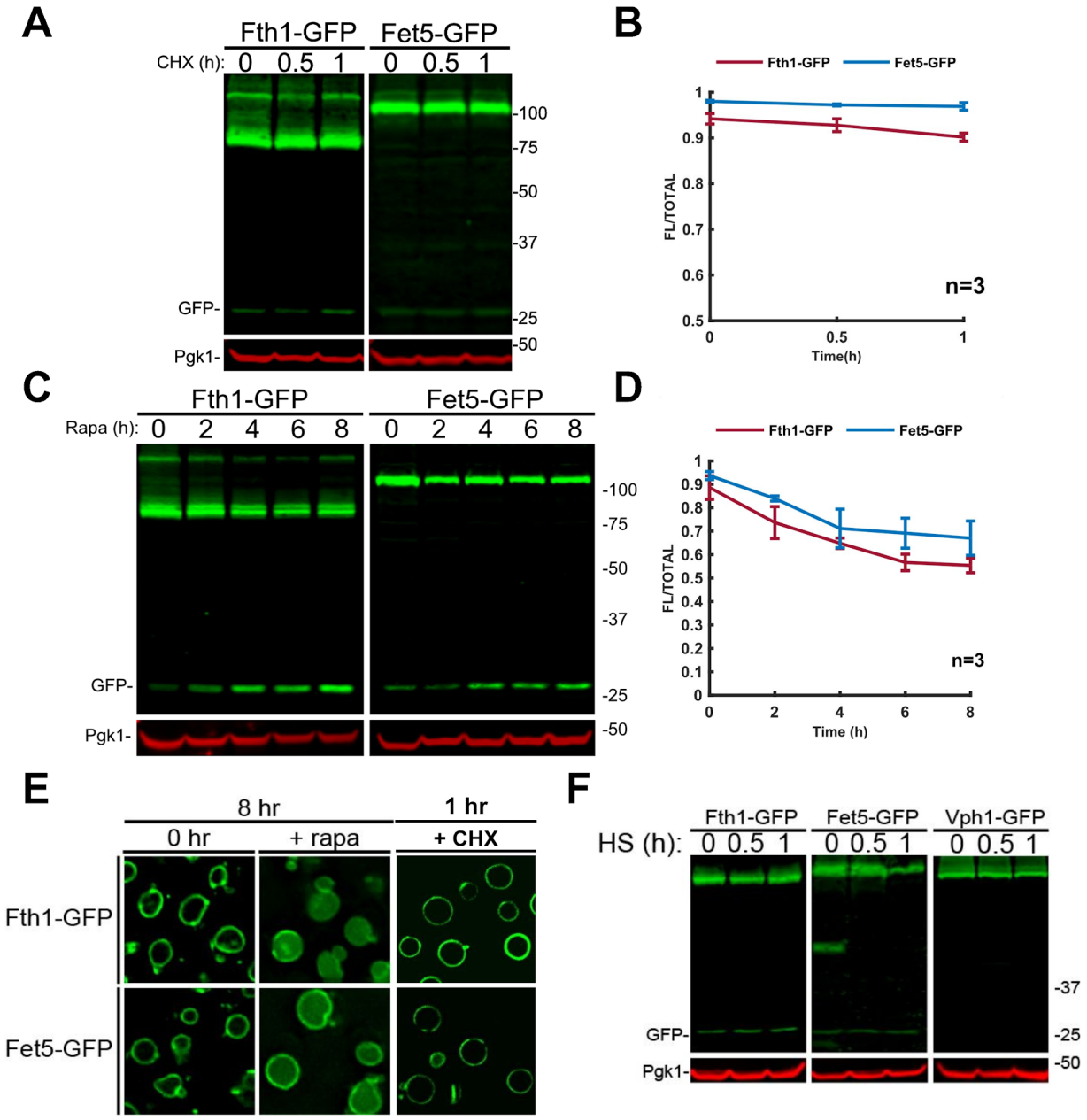

Figure 1: **Rapamycin-induced TORC1 inactivation leads to Fth1-GFP and Fet5-GFP degradation.** **(A)** Western blot of Fth1-GFP and Fet5-GFP samples induced to a 1hr cycloheximide treatment. Significant accumulation of free GFP or diminishment of the full length construct was not observable. **(B)** Quantification of full length levels in A. Relative full length proportions calculated as Full Length/(Free GFP+Full Length). Error bars represent ∓SD (n=3) unless stated otherwise. **(C)** Western blot showing the downregulation of Fth1-GFP and Fet5-GFP following rapamycin treatment. **(D)** Quantification of full length levels in C. **(E)** Fluorescent microscopy images of Fth1-GFP and Fet5-GFP following either 8hr rapamycin or 1hr cycloheximide treatment. Significant internalization of GFP signal for both Fth1-GFP and Fet5-GFP was only observable in rapamycin treated samples. **(F)** Western blots testing the degradative capabilities of 1hr 37℃ heat shock treatment for the following three vacuole membrane proteins: Fth1-GFP, Fet5-GFP, and Vph1-GFP. Significant cellular response was not observable. Intermediate bands for the starting time point of the Fet5-GFP blot are postulated to be initial degradation fragments.

Fth1-GFP Degradation is Ubiquitin-Dependent

A well-established precursor of a majority of biological degradation is the addition of a ubiquitin tag upon lysine residues of targeted substrates[22]. Ubiquitin is an omnipresent 8.6 kDa protein whose signaling effects can be regulated through the number of ubiquitins present on the targeted substrate (polyubiquitination) and the position of the ubiquitinated lysine residue within the polypeptide. The process of attaching ubiquitin to substrates involves a chain of ubiquitin transferring enzymes[23]. The first protein, E1, to transfer the ubiquitin tag is referred to as the ubiquitin-activating enzyme whose function is to attach a free ubiquitin to a sulfur-containing cysteine residue within itself (Figure 2A). The E1 enzyme then transfers the tag to a E2 ubiquitin-conjugating enzyme. The transfer of ubiquitin from the E2 enzyme to the actual substrate is then mediated by an E3 ubiquitin ligase. This ligase activity can also be mediated by the substrate itself through the self-ubiquitination

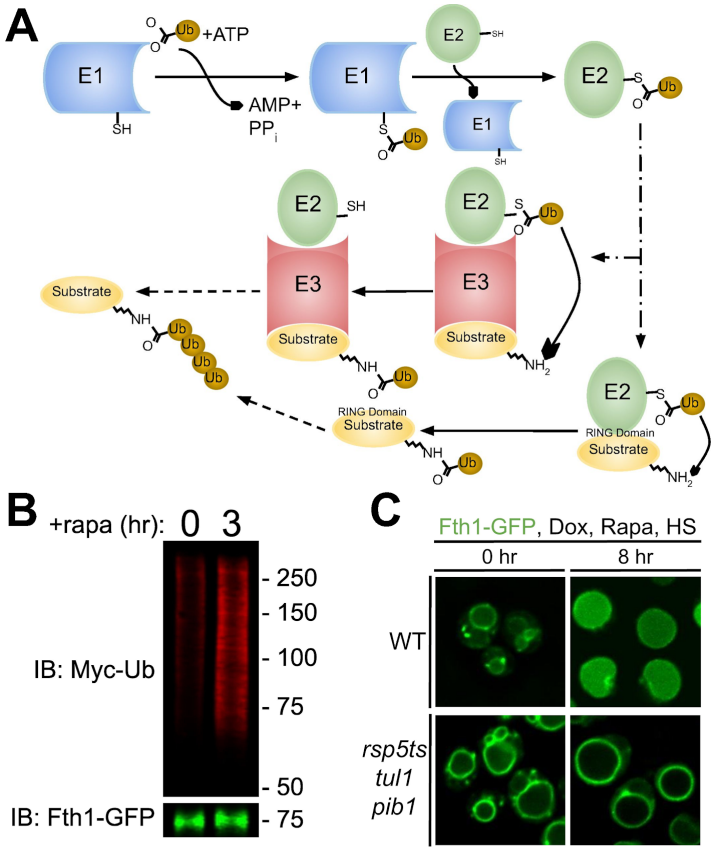

Figure 2: **Fth1-GFP Degradation is Ubiquitin-dependent.** **(A)** An overview graphic of the E1, E2, E3 ubiquitination machinery. **(B)** A ubiquitin blot of Fth1-GFP immunoprecipitated samples across a 3hr rapamycin treatment. Multiple polyubiquitination states were upregulated upon treatment (second lane). **(C)** Fluorescent microscopy of Fth1-GFP in wildtype and a triple E3 ligase mutant strain. Samples were treated with 1hr doxycycline to trigger the tet-off system and halt nascent Fth1-GFP production and 8hr rapamycin treatment to trigger degradation. Cultures were grown during 37°C heat treatment to stop functionality of the temperature sensitive *rsp5p*.

properties of a RING domain[24]. This organization of a hierarchy of transfers allows for a cascade of specificity from a handful E1 enzymes to increasingly diverse populations of E2, E3 ligases, and ultimately substrates themselves. Overall, ubiquitination machinery not only exists as prime necessary enzymes for degradation but also a model example of evolutionary cell development to diversify regulatory capabilities.

In aim to establish a degradative profiling of Fth1-GFP, any involvement with ubiquitin began with investigating its inherent availability to ubiquitination. Following three hour rapamycin treatment, multiple states of ubiquitination were observable for Fth1-GFP, as seen through immunoprecipitation of cell lysates (Figure 2B), in which an observable smear of detectable ubiquitin existed in treated samples. With this understanding, the dependence on the ubiquitination of Fth1-GFP for its degradation was analyzed through comparing free GFP accumulation in the vacuole lumen following degradation treatment across wildtype cells and a strain harboring mutations for three different E3 ligases. These three E3 ligases- Rsp5, the DSC complex whose function was eliminated following the deletion of the core subunit Tul1, and Pib1- have all previously been identified to act primarily on vacuole membrane targets[5,25]. Eliminating the functionality of these vacuole-orientated ubiquitination machinery prevented the degradation of Fth1-GFP despite eight hour rapamycin treatment (Figure 2C). From these results, the degradation of Fth1-GFP was inferred to be ubiquitination-dependent.

Fth1-GFP Degradation is ESCRT-dependent

With an established understanding of the degradative properties for Fth1p, the dependency of ESCRT for degradation was chosen as the next topic of investigation to ultimately settle the dispute of the conflicting methods of internalization, in regards to at least Fth1p. Vps4p is a core component of the ESCRT machinery whose function is to disassemble the

membrane-locked ESCRT-III complex following internalization of cargoes[26]. Without vps4, ESCRT machinery becomes entrapped on membranes and is not able to operate at normal efficiencies thus essentially losing functionality after a minimal amount of time. Such mutations accumulate vesicle punctae around the vacuolar membrane given Class E trafficking vesicles are stalled from being internalized into the lumen for degradation[27]. Following deletion of vps4, free GFP accumulation and downregulation of full length Fth1-GFP is halted in a similar fashion to deletion of the vacuolar protease itself (*pep4*) despite degradative treatment (Figure 3A). In contrast, deletion of Atg1, a necessary component of the degradative pathway of autophagy, caused no significant impact in the degradation of Fth1-GFP as compared to wildtype. Autophagy is an extensive pathway for recycling unnecessary or dysfunctional cellular components, yet was shown to be independent from the disruption of Fth1-GFP degradation[28]. From these observations, the degradation of Fth1-GFP was postulated to be ESCRT-dependent. In congruence with this result, microscopy showcased the dependency of functional Vps4p for GFP signal accumulation within the vacuole lumen (Figure 3B). Under *vps4* deletion, signal accumulates as puncta surrounding the vacuole membrane, reminiscent of a lack of function for the necessary internalization machinery to traffick degradation-bound vesicles across the vacuole membrane. However, Fth1-GFP was effectively invaginated into the vacuole lumen for wildtype, *pep4* mutant, and *atg1* mutant strains. In further support, directly deleting a core subunit of the ESCRT-I complex (vps23) produced the same effect of stabilizing the full length construct and stopping the accumulation of free GFP when analyzing various timepoints of rapamycin treatments through western blot (Figure 3C). From these results, Fth1-GFP degradation was determined to be in requirement of the ESCRT-dependent pathway process of internalization, against the reportings of the ILF pathway.

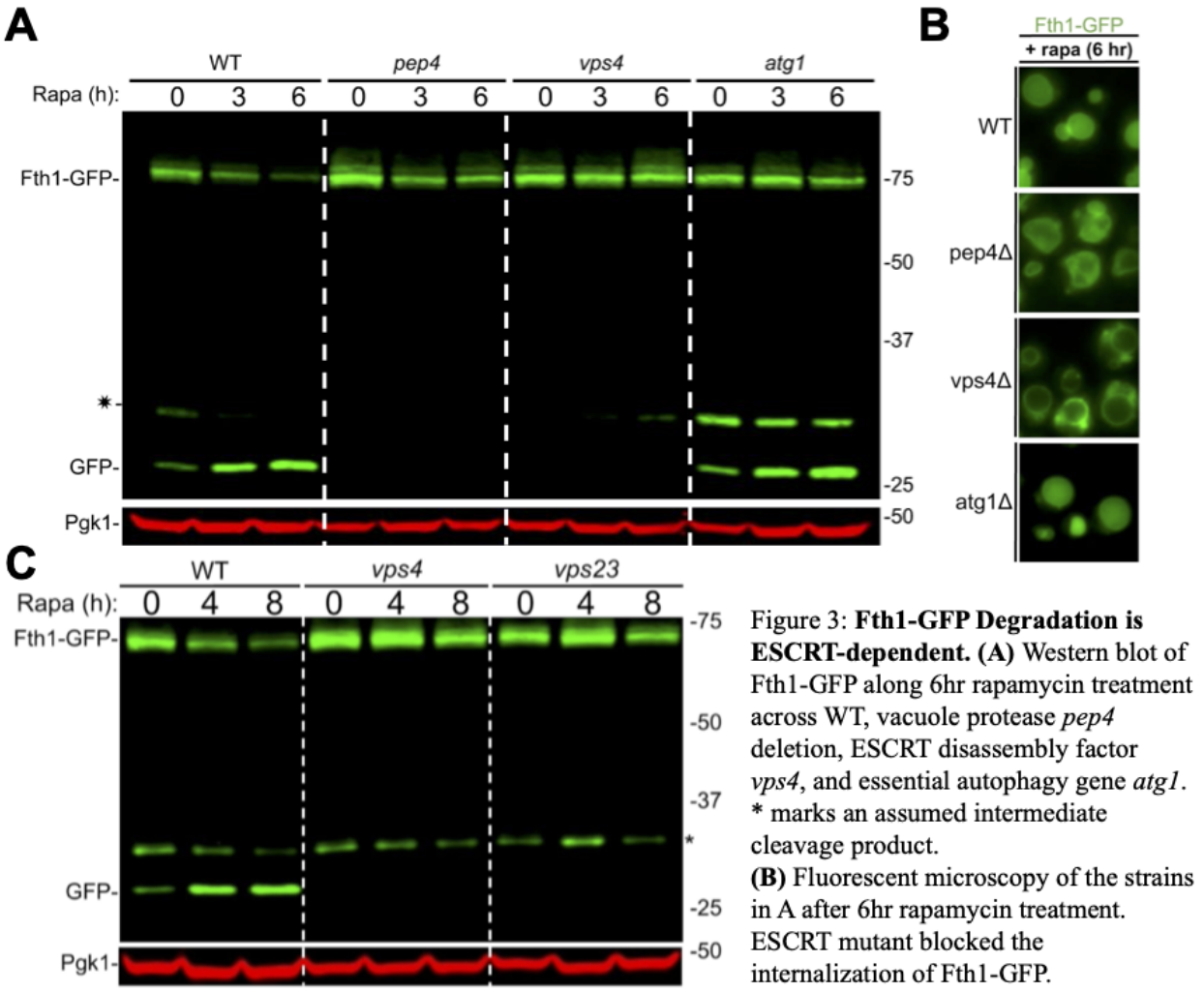

Figure 3: **Fth1-GFP Degradation is ESCRT-dependent. (A)** Western blot of Fth1-GFP along 6hr rapamycin treatment across WT, vacuole protease *pep4* deletion, ESCRT disassembly factor *vps4*, and essential autophagy gene *atg1*. * marks an assumed intermediate cleavage product. **(B)** Fluorescent microscopy of the strains in A after 6hr rapamycin treatment. ESCRT mutant blocked the internalization of Fth1-GFP. *Pep4* mutant showcased mosaicing of GFP signal in the vacuole lumen, indicating the cell's inability to degrade invaginated macrostructures. **(C)** Western blot of Fth1-GFP along 6hr rapamycin across WT, and two ESCRT mutant strains, *vps27* and *vps36*. * marks an assumed intermediate cleavage product from the cytosolic proteasome. Free GFP accumulation is blocked by ESCRT function disruption.

Plasma Membrane Protein Degradation is Dependent on ESCRT

An additional reported cargo of the ILF pathway is the plasma membrane localized glucose transporter, Hxt3p[13]. Given the degradation of Fth1 was identified to be ESCRT-dependent, it was postulated if Hxt3p degradation is also dependent on this machinery for its internalization into the vacuole lumen following trafficking from the plasma membrane. Previous research has reported on a multitude of degradative treatments for Hxt3p. Depleting the cellular environment of glucose emplaces such a condition given the cell would inherently

require less machinery devoted to transporting glucose as this nutrient is in limited supply. Three hour glucose depletion does in fact trigger the degradation of Hxt3, observable through the accumulation of free GFP and downregulation of full length Hxt3-GFP for wildtype cells (Figure 4A-B). However, deleting any component of the ESCRT machinery (*vps4*, disassembly factor; *vps27*, ESCRT-0 subunit; *vps23*, ESCRT-I subunit; *vps36*, ESCRT-II subunit; *snf7*, ESCRT-III subunit) completely blocks the degradation of Hxt3-GFP. This result was observable under two hour rapamycin treatment as well (Figure S1A-B). This ESCRT-dependency was observable through microscopy as well, following either three hour glucose depletion or two hour rapamycin treatment (Figure 4C). Wildtype cells are visualized as trafficking Hxt3-GFP populations from the plasma membrane to the vacuole lumen for degradation, while deleting any component of ESCRT blocks the internalization processes of trafficked vesicles thus causing the accumulation of GFP signal in class E compartments marooned around the vacuole membrane.

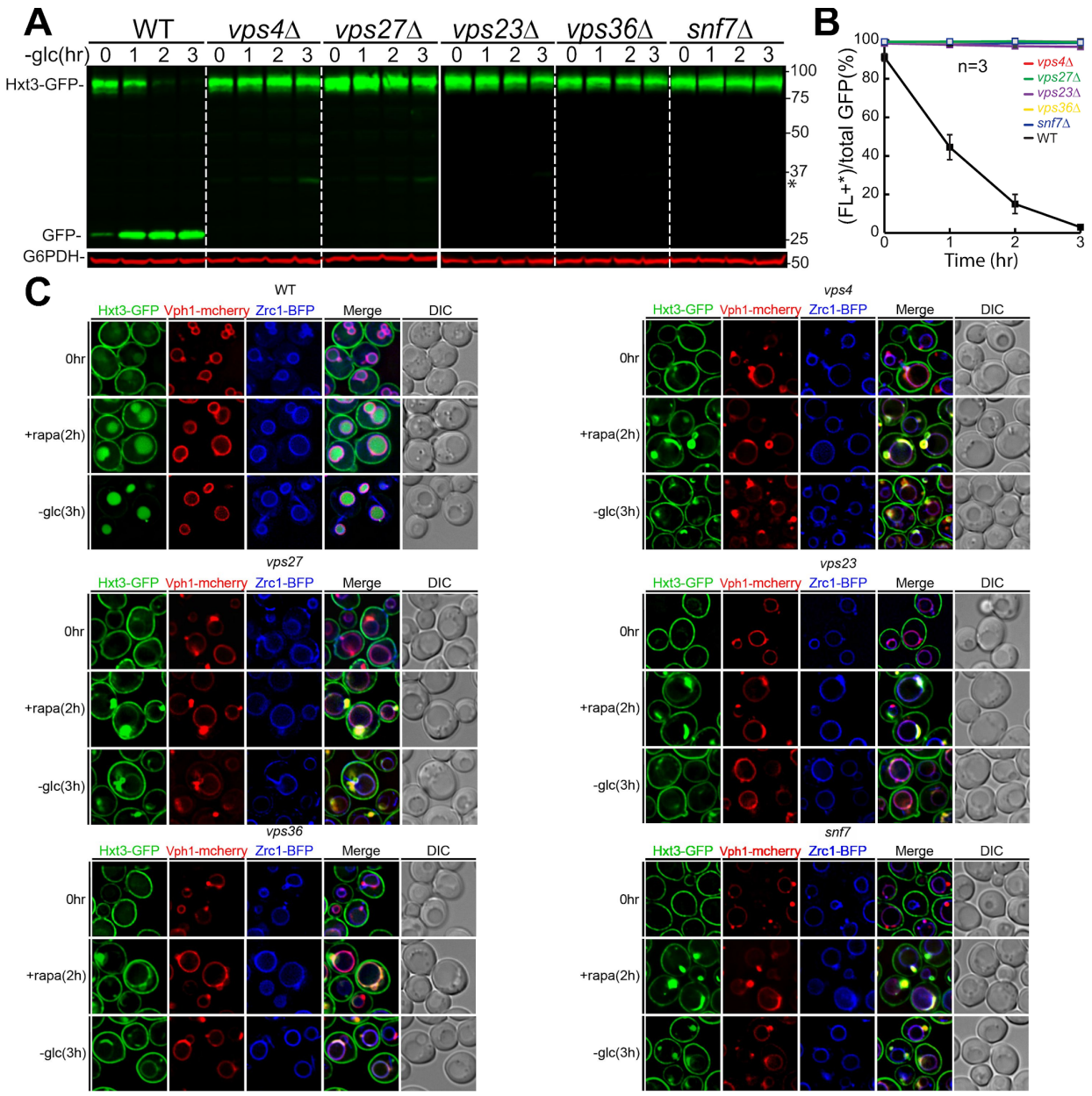

Figure 4: **Plasma Membrane Protein Degradation is Dependent on ESCRT. (A)** Western blot of Hxt3-GFP samples across WT, and five ESCRT mutant strains, *vps4, vps27, vps23, vps36, and snf7* along 3hr glucose depletion treatment. ESCRT mutation blocked any observable degradation. * marks an assumed intermediate cleavage product from cytosolic proteasome. **(B)** Quantification of A showcasing any diminishment of full length construct. Relative full length levels calculated as Full Length/(Free GFP+Full Length). **(C)** Fluorescent microscopy images of 2hr rapamycin or 3hr glucose depletion treatment of Hxt3-GFP, Vph1-mCherry (VM MVB pathway marker), and Zrc1-BFP (VM AP3 pathway marker) across WT, and all five ESCRT mutant strains. ESCRT mutants blocked the internalization of Hxt3-GFP across all treatments.

The research reporting upon the ILF pathway additionally discussed the treatments of cycloheximide and 2-deoxyglucose- a toxic glucose analog (2-DG) as triggers for Hxt3p degradation, thus ESCRT-dependency was investigated under these treatments as well. Following either cycloheximide treatment or 2-DG treatment, Hxt3-GFP was targeted for degradation under wildtype conditions (Figure 5A-D). However, deletion of ESCRT components blocked said degradation as observable through western blot analysis (Figure 5A-D) and microscopy (Figure 5E). Similar results and conclusions were observed through microscopic analysis of Hxt3-GFP degradation following two hour 37℃ heat shock treatment as well (Figure S2). Through these results, the degradation of the plasma membrane transporter Hxt3-GFP was concluded to be dependent on the ESCRT-dependent pathway mechanism of internalization.

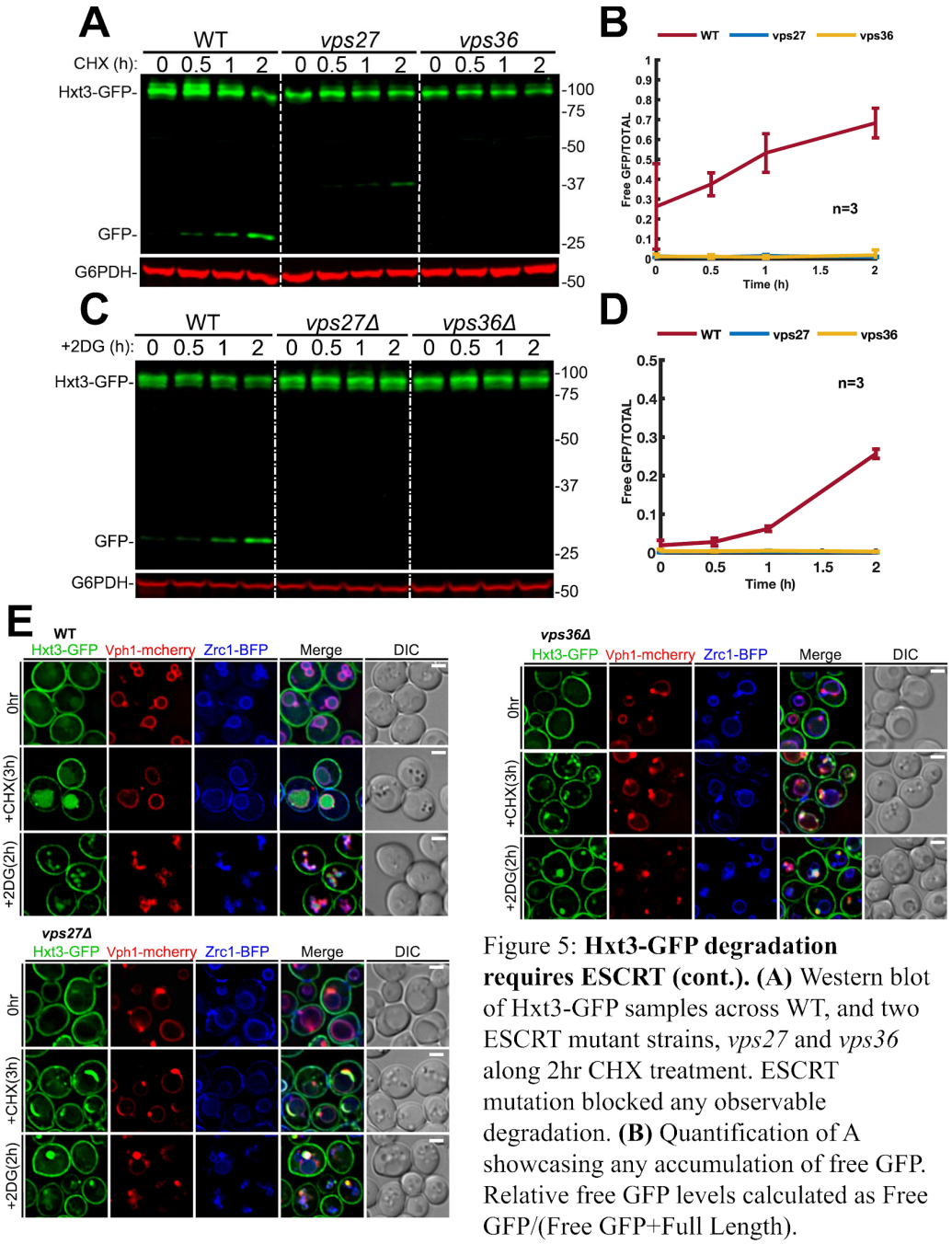

Figure 5: **Hxt3-GFP degradation requires ESCRT (cont.). (A)** Western blot of Hxt3-GFP samples across WT, and two ESCRT mutant strains, *vps27* and *vps36* along 2hr CHX treatment. ESCRT mutation blocked any observable degradation. **(B)** Quantification of A showcasing any accumulation of free GFP. Relative free GFP levels calculated as Free GFP/(Free GFP+Full Length). **(C)** Repeated experiment of A except with 2hr 2-deoxyglucose treatment. **(D)** Quantification of C. **(E)** Fluorescent microscopy images of 3hr CHX or 2hr 2-DG treatment of Hxt3-GFP, Vph1-mCherry (VM MVB pathway marker), and Zrc1-BFP (VM AP3 pathway marker) across WT, and two ESCRT mutant strains, *vps27* and *vps36*. ESCRT mutants blocked the internalization of Hxt3-GFP across all treatments.

Discussion

Differing Mechanisms of Internalization

The observable decisive dependency of functional ESCRT machinery for the degradation of all studied cargoes supported the ESCRT-dependent pathway process of internalizing degradation targets. The dispute these two differing mechanisms are currently fueling must be settled through the scientific process in hopes of generating a representative model of the actual biology; this research stands as a significant resolvement for clarifying this disagreement. While vacuole fusion is a highly regulated process capable of administering multiple cellular impacts, the reportings of a completely ESCRT-independent pathway of internalization is in complete disagreement from the observed *in vivo* dynamics and thus likely requires reevaluation.

For an encompassing analysis of these two processes, furthering research exists in consideration of blocking functionality of the proposed ILF pathway. Effective blockage of the ILF pathway is possible through inhibition of a regulator protein of a core fusion component. Ypt7 is a Rab-GTPase essential for vacuole fusion whose post-fusion recycling from the vacuole membrane for further activity relies upon its formation into a chaperone protein complex with rGdi1. Inhibitors of rGdi1 exist whose optimized addition could allow effective investigation despite lowered vacuole fusion efficiency. This methodology could create an *in vivo* system to study the degradation dynamics of cargoes under effective ILF pathway loss of function. If the degradation of Fth1p or Hxt3p still proceeds despite this blockage, it could be argued the dispute between these differing mechanisms would be fully resolved.

Evaluating Interactomes through Degradative Profiling

The careful investigations of necessary degradative inducements and machineries for Fth1-GFP provided a recognizable start of outlining its individual interactome. As previously

discussed, a next-generational approach to the modeling of biochemical systems lies within the complete accountment of all biomolecular interactomes for all cellular components. This undertaking is immense, but is possible through elegant conjunctions of biochemistry and genetics featuring careful step-wise analysis of necessary components for general cellular processes, as done in this research through the degradative profiling of Fth1p.

Interactomes are inherently highly complex given the immense intricacies biology has evolved to survive and adapt throughout changing temporal and environmental conditions. Because of this, extending research will undoubtedly entail a great deal of settling initially conflicting observations. One such example of this arose through the observed effects of TORC1 activation and inactivation. As discussed, TORC1 is a master regulator whose extent of susceptibility and impacts are incredibly diverse[11]. As observed through the identification of degradation-inducement of Fth1-GFP and Fet5-GFP (Figure 1), TORC1 inactivation caused degradation. In contrast to this, both TORC1 inactivation and activation was observed to induce degradation for the plasma membrane cargo Hxt3-GFP from cycloheximide and rapamycin treatment respectively (Figure 5). Ongoing investigations continually report on added intricacies of the activity of TORC1 across its many activation states, thus this initial conflict of observations is possibly explainable following continued research into this master regulator. Overall, a complete perspective of the activities of these cellular components is possible, yet only fully representative if enough effort is put towards elucidating the extent of activities and interactions.

Chapter 2: Identifying Possible Homology for Internalization Machinery

Introduction

While an immense amount of research has been completed in focus of the activity and machinery of the yeast vacuole, the respective systems for the homologous mammalian organelle of the lysosome still largely remain to be investigated. Due to the increasing awareness of the significance behind proper lysosomal functionality, these machineries and cellular processes stand as a prime research focus. Through preliminary lysosomal investigations, a collection of internalization machinery similar to the yeast ESCRT system has been postulated to exist in human cells[29]. The coexistence of these homologous systems would provide insight into the evolutionary development of cellular recycling processes while simultaneously interconnecting congruences of scientific information across model organisms. Functional homeostatic mechanisms stand as a hallmark of all life, thus machineries like ESCRT could provide insight into the evolutionary complexing of the cell's ability to manage resources and environmental stresses[30]. Ultimately, these understandings could guide the treatment of LSDs[15-20].

A variety of investigative questions are currently underway to determine the existence of a mammalian ESCRT system and its homology with the established vacuolar machinery. One such consideration is the postulation if a mammalian lysosomal membrane protein could be targeted for degradation by the yeast invagination machinery, inferring similar processes of membrane regulation. This ectopic expression of a human protein would provide the same conditions for degradative profiling as done previously with yeast proteins and would provide excellent insight if any homology does exist between internalization machinery. The human protein for this investigation was chosen from a collection of lysosomal membrane candidates that experience relatively high natural turnover within the mammalian cell to provide ease in

studying its degradation. From this collection, RNF152 was chosen. RNF152 is an E3 ubiquitin-protein ligase whose functionality is connected to the regulation of the mTORC1 complex[31]. Based on its short half life and general involvement with degradative processes, RNF152 stood as a prime candidate and thusly was expressed within yeast cells through a plasmid vector for the following studies.

Results

GFP-RNF152 Internalizes into the Vacuole Lumen

To begin the degradative profiling of GFP-RNF152 within yeast cells, a viable treatment needed to be identified for further studies of the degradation dynamics. Based on its relatively short half life within its endogenous system, simply inhibiting nascent protein synthesis was hypothesized to allow observation of the constitutive degradation of GFP-RNF152 . Microscopy assays confirmed such postulations in which three hour treatment with cycloheximide caused accumulation of GFP signal within the vacuole lumen (Figure 6). With this preliminary result, cycloheximide treatment was chosen to visualize the degradation of GFP-RNF152 in consecutive mutant assays.

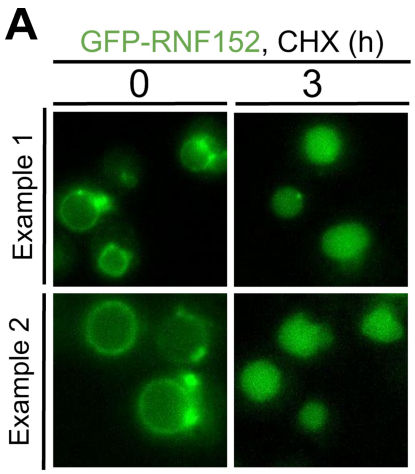

Figure 6: **GFP-RNF152 Internalizes into the Vacuole Lumen. (A)** Fluorescent microscopy of vacuole lumen accumulation of GFP-RNF152 signal following three hour cycloheximide treatment.

GFP-RNF152 Degradation Involves ESCRT Activity and Ubiquitination

Considerations into the activity of ESCRT and ubiquitination were investigated to continue the degradative profiling of GFP-RNF152. To establish the role of GFP-RNF152 degradation to the vacuole recycling process and not the cytosolic proteasome, the effect of deleting the vacuolar protease, *pep4*, was observed through western blotting (Figure 7A-B). Removing the degradative capabilities of the vacuole blocked all GFP-RNF152 degradation as seen from the stabilization of the full length construct and lack of free GFP accumulation. Under the considerations of the possible role of ubiquitination in the degradation of GFP-RNF152, three mutants dysfunctional for ubiquitin-related machineries and a self-ubiquitination RING domain

were generated. The first strain (CS) contained a loss-of-function in the RING domain of RNF152 by mutating the available cysteine residues to actively dead serine residues (C12S, C15S, C30S, C34S), removing the fundamental sulphur residue for ubiquitin binding. The second strain (*triple*) contained a triple mutation for vacuolar E3 ligase ubiquitination machinery: *tul1* deletion for loss-of-function in the DSC complex, *pib1* deletion for loss-of-function in the pib1 E3 ligase, and *ssh4* deletion which is an adaptor protein for the rsp5 E3 ligase. A direct deletion of rsp5 in conjunction with the other two E3 ligases caused too low of cell survivability for retention of the GFP-RNF152 plasmid. The final strain was a conjunction of the CS and triple E3 ligase mutant (CS/*ligase*). Following one hour cycloheximide treatment, the CS mutant was observed to stabilize the starting population of full length GFP-RNF152, showcasing the dependence of self ubiquitination for the relatively quick, inherent turnover (Figure7 A-B). Similar degradation kinetics through the one hour treatment in terms of free GFP accumulation and full length diminishment were observed in comparison to WT. In stark contrast to this similar ratio of degradation, the *triple* mutant showcased similar partial stabilization of starting full length populations but displayed markedly upregulated degradation of GFP-RNF152 due to CHX addition. The amount of full length GFP-RNF152 after treatment was congruent to WT yet the full length pool began at a much larger proportion. This aligns with the understanding that deleting pools of E3 machinery could inherently cause diminishment in the trafficking of ubiquitin-charged E2 ubiquitin-conjugating enzymes near the vacuole membrane given the sequential interacting machinery of the E3 ligases are not present and trafficked themselves. This would explain the partial stabilization before treatment given the functional RING domain of RNF152 would have less charged E2 enzymes to interact with. Following CHX addition however, the cell could be considered to be in a stressed state which could potentially pressure an

inherent upregulation in trafficking of charged E2 enzymes to the vacuole membrane surroundings, allowing RNF152 to self-ubiquitinate and sustain degradation at the same levels observed in WT. Finally, combining these two mutants inferred the second strongest stabilization of starting full length populations, given all studied ubiquitin-related functionalities lost activity, while matching the degradation kinetics of the CS mutant or WT. From these data, ubiquitination is showcased as a regulatory step for the degradation of GFP-RNF152. Interestingly, the general effect CHX treatment infers to GFP-RNF152 is generic in regards to if target-specific ubiquitination-related components are functional or not, assuming no affecting preconditions like the possible lack of available of ubiquitin-charged E2 complexes as was inferred for *triple* before treatment. Further studies and repeats would be required to strengthen such postulations, however.

Finally, the actual dependency of ESCRT activity for the degradation of GFP-RNF152 was investigated through combined western blot and microscopy analyzes. Deleting the core ESCRT-II subunit, vps36, caused a complete block of GFP-RNF152 vacuolar degradation (Figure 7C). This was determined through the lack of free GFP accumulation around the 27 kDa region. The full length population of GFP-RNF152 was observed to diminish across the three hour treatment period, however. This is hypothesized to be due to interposed activity of the cytoplasmic proteasome. Despite this, the fundamental takeaway of the necessary functionality of the ESCRT internalization machinery to cause typical vacuolar degradation for GFP-RNF152 persists. This conclusion was confirmed through microscopic assays of ESCRT mutants in which deleting ESCRT machineries blocked the ability of the cell to internalize GFP-RNF152 signal into the vacuole lumen following cycloheximide treatment (Figure 7D). From these results, the

capability of yeast internalization machinery to target an exogenous human protein was confirmed in support of the capabilities of such a system to enact on mammalian targets.

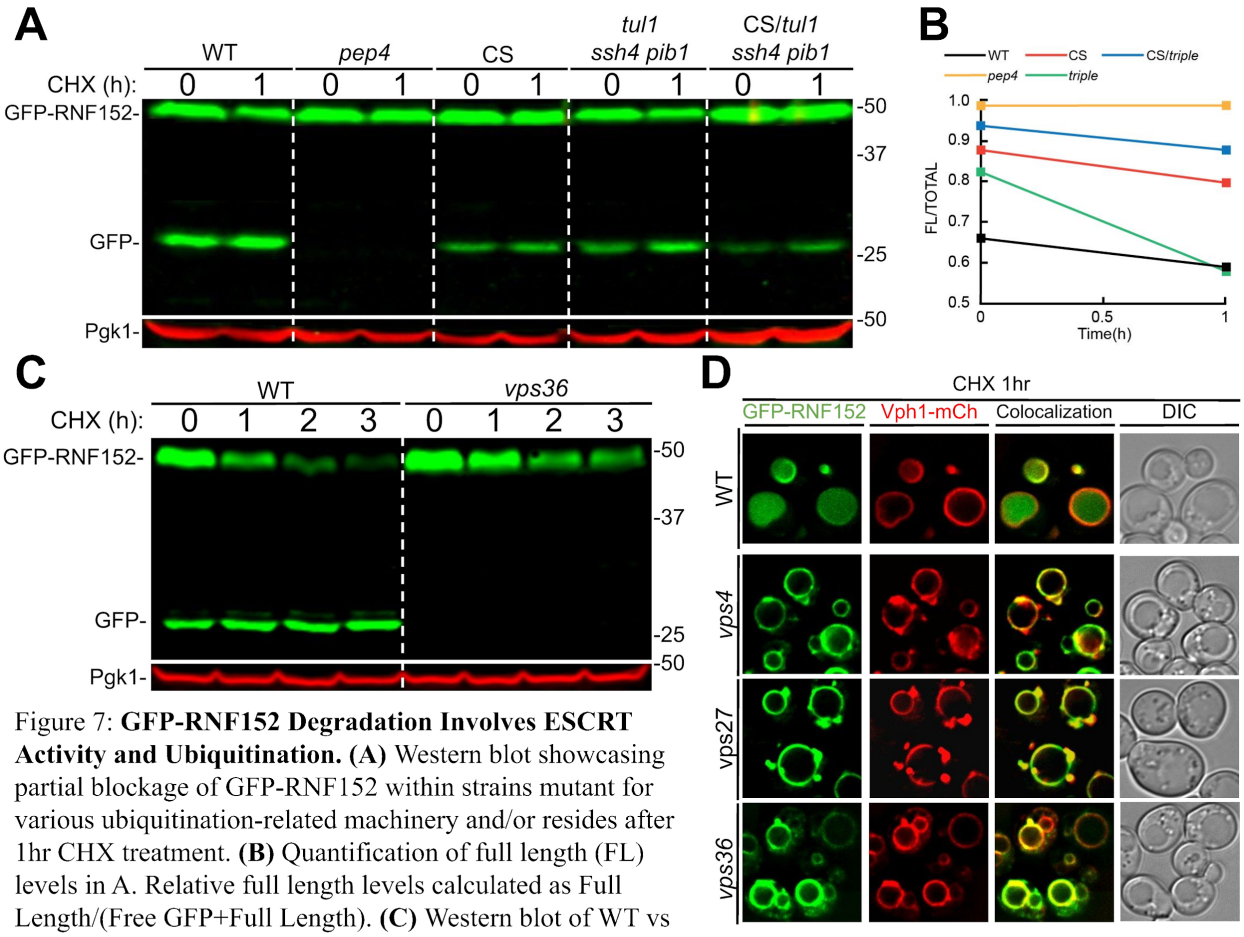

Figure 7: **GFP-RNF152 Degradation Involves ESCRT Activity and Ubiquitination. (A)** Western blot showcasing partial blockage of GFP-RNF152 within strains mutant for various ubiquitination-related machinery and/or resides after 1hr CHX treatment. **(B)** Quantification of full length (FL) levels in A. Relative full length levels calculated as Full Length/(Free GFP+Full Length). **(C)** Western blot of WT vs *vps36* showcasing the necessity for ESCRT activity for Free GFP accumulation of GFP-RNF152 after 1hr CHX treatment. **(D)** Fluorescent microscopy of GFP-RNF152 and Vph1-mCh (vacuole membrane marker) after 1hr CHX treatment.

Discussion

The Possibility of Homologous Internalization Machinery

Under the postulations of transferable intractability, observing the capabilities of a yeast system of vacuolar degradation to degrade ectopically expressed GFP-RNF152 in a regulatable manner supports the possibility of homologous internalization machinery across yeast and mammalian cells. The congruency between the role of ubiquitin and ESCRT machinery for membrane protein degradation stands as a powerful insight into such an evolutionarily conserved mechanism. Extending research decisively lies within the actual identification of internalization machinery within mammalian cells, but this preliminary approach offers invaluable insight into the possible specificities of interactions between machinery and substrate given the yeast system was able to replicate physiologically cellular degradation despite no fundamental adjustment of the mammalian target. This lack of modification possibly implies an even greater evolutionary significance of these mechanisms. The ability for a completely exogenous system to reconstitute regulatable interactions with a mammalian target could imply a high degree of congruence between the homologous systems, which in turn offers profound evolutionary insight to the extent of conservatism. This ultimately could guide the phylogenetic considerations of how such systems evolved throughout time.

Along with this, further basal development of the lysosomal regulation systems offers an increased likelihood of modeling the exact biological mechanisms underlying Lysosomal Storage Diseases. If such opportunities of application arise, the understanding of the mammalian lysosomal machineries and interactomes- and even the fungal counterparts- could drive advancements in medical treatment to a more impactful, scientific methodology of approach.

Chapter 3: Tracing the Vesicular Trafficking of Fth1

Introduction

The study of membrane bound proteins allows for unique considerations of the dynamic nature of biomolecular systems. All cellular structures and their respective biomolecules are arranged in maintained codependencies to provide enough complexity to contribute to biological processes[32]. As previously discussed, a complete understanding of biochemical life must include the entirety of all interactions of all four biomolecules and their immense collection of derivatives. While an encompassing technique of this nature will inentibly utilize elegant exploitations of biophysical principles, cellular biology provides an introductory approach offering cataloging capabilities of modeling interactomes. To formulate the power of this approach, the cellular progression of Fth1p was broadly modeled through the considerations of the necessary pathways Fth1p requires for trafficking. Cellular trafficking recruits diverse bodies of lipid-enclosed vesicles which serve as carriers of cellular machinery and resources. The immense diversity of vesicular trafficking requires a great deal of regulation, thus this critical characteristic of cellular life is flush with interesting, overlapping interactomes. While the extent of this research section does not encompass the entirety of Fth1p's interactome, it stands as an examplative start of the approach scientists can employ to ultimately fully model biochemical life in the full glory of its fluid mosaic nature.

Results

Tracking the Progression of Fth1-GFP Through the Cell

In preliminary research for the degradative profiling of Fth1-GFP, a unique phenomenon was observed in regards to the export of Fth1-GFP from the Endoplasmic Reticulum (ER) following synthesis inhibition. When expressed under the TET-OFF transcription-regulation genetic system, a population of Fth1-GFP molecules were not trafficked out of the ER despite the complete inhibition of nascent transcript production from the addition of the TET-OFF activating drug, doxycycline (Figure 8A). This phenomenon was originally perceived as a complication for the vacuole degradation experiments planned with this strain given populations of the cargo of interest were entrapped in the ER. Extensive testing was thusly performed in an attempt to flush out the retained population of Fth1-GFP. This began through optimization of the transcriptional activity of Fth1-GFP. Fth1-GFP constructs were expressed under a variety of promoters with differing activities. When these considerations proved unsuccessful, optimization of the co-subunit Fet5 was considered under the pretenses of previous Fth1-related research hinting at a quality control system which regulated Fth1 and Fet5's trafficking to the vacuole in a conjoined manner. When Fet5p was overexpressed via the use of an overactive pGPD promoter sequence, Fth1-GFP populations were efficiently trafficked from the ER in a manner reminiscent of continuous trafficking of a limited pool of Fth1p given its transcription was halted from doxycycline addition (Figure 8A). It was inferred that Fth1-GFP required complexation with its Fet5p subunit before the cell allowed its trafficking process to begin, starting with COP-II vesicles for ER-to-Golgi trafficking[33]. This stark effect of an overabundance of its co-subunit causing continuous progression through its trafficking process provided a fundamental

perspective of how the cell regulated its vacuole proteome even through mechanisms housed in the ER.

Transitioning from the ER to the ultimate position of localization generally requires intermediate trafficking through the Golgi apparatus[34]. The Golgi is an amazing organelle in consideration to the simplistic funnelling role it provides to the cell as it acts as a transitive stage of cellular localization. In brief, bountiful cargoes are in-fluxed at the cis-Golgi following which cellular cargoes are sorted and processed through sequential vesicle trafficking events between cisternae in order for related targets to be ultimately out-fluxed together through the trans-Golgi network: a biologically-derived example of the Ship of Theseus. At this point of localization, specificity of cellular process to cargo returns as targets are processed to their final localization through specialized pathways. In regards to Golgi-vacuole specific trafficking pathways, two primary processes exist: the ALP pathway or endosomal trafficking through the VPS CPY pathway[35]. Previous research has hinted at the ability of the cell to traffick Fth1p to the vacuole membrane via the ALP pathway (data not shown) thus the possible role of the VPS pathway was investigated through the considerations of colocalization between Fth1-GFP and VPS pathway related components. The first of these considerations was Sec7, a guanine nucleotide exchanger associated with Golgi-derived vesicles including those involved with both the ALP and VPS pathways. Decisive colocalization between Fth1-GFP and red fluorescent tagged Mars-Sec7 was observable as small trafficking vesicle puncta through microscopy (Figure 8B).

The VPS pathway entails the transitioning of such Golgi vesicles into the macrocellular process of the endocytic pathway in which early endosomal bodies develop into late endosomes. Late endosomes are multivesicular bodies derived from the merging of numerous lipid vesicles such as endoctoic bodies from invagainations of the plasma membrane or Golgi derived VPS

pathway vesicles localizing vacuole membrane targets. The Endocytic pathway can be uniquely visualized through the use of the styryl dye FM4-64, whose staining of the plasma membrane can be chased through the Endocytic pathway, tracking early and late endosomes and the eventual fusion with the vacuole membrane[37]. When Fth1-GFP containing cells were stained with FM4-64, colocalization was significantly observed for trafficking vesicles (Figure 8C). This result confirmed the existence of an endosomal population of Fth1-GFP in support of the involvement of the VPS pathway in Fth1 trafficking. Through these three observations of the necessary processes underlying localization of Fth1-GFP, the progression of this vacuole membrane protein was traced from the ER, to the Golgi, and finally to the vacuole membrane (Figure 8D). Fth1 stands as an interesting candidate for encompassing interaction-cataloging given the involvement of two distinct trafficking pathways in a single target's localization is an abnormal characteristic for an interactome[11].

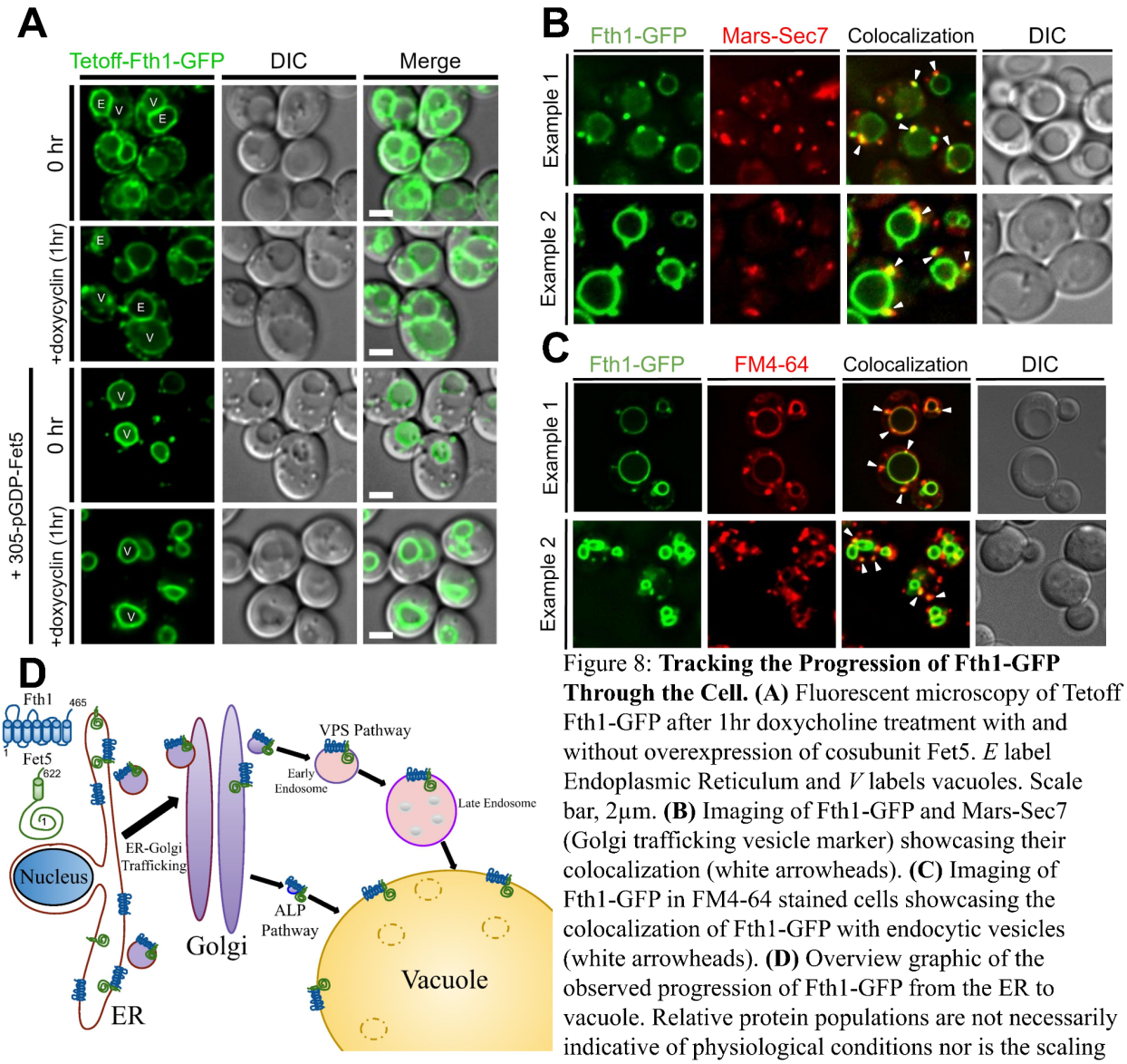

Figure 8: **Tracking the Progression of Fth1-GFP Through the Cell. (A)** Fluorescent microscopy of Tetoff Fth1-GFP after 1hr doxycholine treatment with and without overexpression of cosubunit Fet5. *E* label Endoplasmic Reticulum and *V* labels vacuoles. Scale bar, 2µm. **(B)** Imaging of Fth1-GFP and Mars-Sec7 (Golgi trafficking vesicle marker) showcasing their colocalization (white arrowheads). **(C)** Imaging of Fth1-GFP in FM4-64 stained cells showcasing the colocalization of Fth1-GFP with endocytic vesicles (white arrowheads). **(D)** Overview graphic of the observed progression of Fth1-GFP from the ER to vacuole. Relative protein populations are not necessarily indicative of physiological conditions nor is the scaling of protein and vesicle sizes. The topology of both Fth1p and Fet5p are oriented with their N-terminus lumenal-facing.

Discussion

Through the work accomplished in modeling the trafficking and degradation of Fth1-GFP, a fairly rich perspective of its cellular lifespan is available for continued cataloging. While trafficking has provided a solid foundation to define the Fth1 interactome, continued investigations into assembly, activity, and regulation considerations will be the basis of extenuating research. While an encompassing modeling of cellular life will entail completing such studies for every cellular component, membrane-bound proteins stand as an excellent starting point based on the threshold between protein and lipid interactions such machinery necessitates. Advanced considerations for protein interactions with phosphorylated phosphatidylinositols could be a productive next area of research to study for at least trafficking processes[38]. This collection of modified lipids are essential for the differentiation and regulation of a multitude of lipid vesicles and membranes, whose extent of proteomic interactions still needs to be elucidated. Phosphatidylinositol 3-phosphate is one such derivative whose presence on multivesicular and endosomal bodies is a necessity for the proper functionality of the respective cellular pathways, such as the VPS pathway. These regulatory lipids dynamically interact with protein machinery to contribute to the necessary complexity membranes require to drive biological processes[39]. Fully accounting for these interactions for encompassing biochemical modeling will entail newfound perspectives of how to consider the quasi-ordered distribution of cellular components. Lipids are dynamically intermingled all throughout the cell in a manner dependent on their intractabilities between related cellular structures. One could even argue that subcellular differentiation (and thus activities) could be modeled through topological gradients of a component of interest. Defining cellular localization would entail a more holistic approach through considering the relative probabilities of finding populations of

the biomolecular target across the collection of subcellular organelles and their surrounding areas of definability. This perspective of the dynamic diffusive nature of cellular differentiation could aid in explaining how non-living chemical compounds can project such complicated governance to the total system. It is fathomable to model the entirety of such regulatory relations. The extent of such complexities would require multiple levels of analysis through modern genetic and biochemical approaches or a single biophysical technique capable of identifying and defining the full interactome of a biochemical system. While immense in scale, such work is not outside of the collaborative reach of the scientific community.

Conclusions

Indicative of the complex nature of the vacuole, this research has produced a complexity of conclusions and furthering extensions. Degradative profiling of the key regulatable vacuole membrane proteins provided clarification in regards to the mechanism of internalization. The decisive dependencies for ESCRT activity and ubiquitination provides strong support in favor of the ESCRT-dependent pathway. This clarification arrives in excellent timing given furthering research lies within the modeling of the respective regulatory mechanisms for the mammalian lysosomes. The identification of a possible homologous degradative internalization process through the work done with the ectopic expression of human RNF152 in yeast is of significance in regards to the possible applications lysosomal regulation modeling could provide to the treatment of LSDs. Clearly, this research is only the beginning of these considerations for both lysosomal and encompassing biochemical modeling. The simple identification of mammalian ESCRT homologs persists, along with the general completion of interactome cataloging as was started for Fth1 in regards to its localization path. A great deal of trafficking considerations still remain for even Fth1 given observation of AP-3 vesicles of the ALP pathway have yet to even be directly observed within the cell[11].

While diverse, all of these extending avenues of research ultimately lead to the principal goal of an encapsulating model of biochemical life. This aim is obviously immense, yet under the belief of the discrete nature of cellular components, it is a possible achievement. A conjunction between genetic modeling and a biochemical mindset is a powerful approach to this challenge, in which the interconnected nature of each scientific perspective can provide invaluable insight. The following discussions stand as examples of insights that could potentially

have physiological relevance; offering such extrapolating conclusions not only drives scientific discussion, but offers potential avenues to prioritize in furthering research.

The dynamic nature of Fth1-GFP sequesterment from the ER as observed across varying Fet5 expressions (Chapter 3: Figure 8A) provides insight into possible prerequisites for the trafficking of this protein complex. For example, the assumed requirement of complexation could potentially infer a quaternary binding/interacting domain in which the Fth1-Fet5 complex generates upon formation to allow interaction with budding COPII vesicles or intermittent scaffolding of sorting machinery. This inherent prerequisite of trafficking through complexation also potentially explains the insurance of proper membrane orientation of the Fth1-Fet5 complex as it travels through the Golgi, VPS and ALP pathways, and ultimately localizes to the vacuole membrane. Cellular life would not be possible without such evolutionary conserved advantageous strategies.

The observed stabilization of GFP-RNF152 due to the deletion of the activity of three E3 ligases, of which conversely do not significantly interact with GFP-RNF152 given its degradation dynamics assumed congruent conditions as wildtype following treatment (Chapter 2: Figure 7A-B), could potentially infer a dynamic interaction-based trafficking cascade to regulate the availability of inter-regulated ubiquitin machinery. Lowering the pool of available E3 ligases at the VM surface potentially caused the diminishment of available ubiquitin-charged E2 ubiquitin-conjugating enzymes as explained through the peculiar stabilization of untreated GFP-RNF152. This degree of effect could potentially even extend upstream the interacting cascade of ubiquitin machinery to E1 enzymes or even ubiquitin itself in which the cell's ability to traffick machinery is inherently regulated by relations between the trafficking flux of interacting partners. This mindset is reminiscent of the mosaic lipid composition model

discussed in consideration of the mixed lipid populations between the Golgi, late endosomes, and ultimately all membrane structures (Chapter 3: Figure 8D). This perspective of cellular organization and substructure differentiation resulting from simple descriptions of diffusing biomolecular probability topological gradients infers a chaotic picture of cellular activity. Under the considerations of the interdependency interacting cellular components have upon each other to advantageously localize and infer activity, it is arguable such a mosaically fluid model of biochemical life and explanation of the development of organelle structures is within postulation.

Without question, these hypotheses are beyond the scope of this research, but are potentially within grasp of our scientific community armed with a complete understanding of the basal, inherently objective, physical principles governing the existence of biochemical systems.

Materials and Methodology

Yeast Strains, Plasmids, and Culturing

All yeast strains and plasmid used throughout this research are listed in Table S1. Yeast was grown in either YPD or a selective media YNB culture at 28℃, unless otherwise noted. Stable lines were generated either through the PCR-based genetic modification system as outlined in Longtine et al., 1998, full length homologous recombination, or through plasmid transformation.

Cycloheximide Treatment Assay

Yeast cells containing fluorescently tagged substrates were cultured till the mid-log phase (OD_{600}: 0.5-0.7) before being treated with 100 µM cycloheximide. Imaging or 7OD sample collection for western blotting were completed at each necessary time point.

Rapamycin Treatment Assay

Yeast cells containing fluorescently tagged substrates were cultured till the mid-log phase (OD_{600}: 0.5-0.7) before being treated with 500 ng/ml rapamycin. Imaging or 7OD sample collection for western blotting was completed at each necessary time point.

Glucose Depletion Treatment Assay

Yeast cells containing fluorescently tagged substrates were cultured till the mid-log phase (OD_{600}: 0.5-0.7) before the initial culturing media was replaced with YP, glucose-depleted media following centrifugation of samples. Imaging or 7OD sample collection for western blotting were completed at each necessary time point.

2-Deoxyglucose Treatment Assay

Yeast cells containing fluorescently tagged substrates were cultured till mid-log phase (OD_{600}: 0.5-0.7) before being treated with 0.4 mM 2-deoxyglucose. Imaging or 7OD sample collection for western blotting were completed at each necessary time point.

TET-off Inducible Transcription Inhibition System

Inducible inhibition of transcription through the addition of 2µg/mL doxycycline (a stable analogue of tetracycline) was possible through the use of the yeast TET-off genetic system. Strain construction consisted of replacing the promoter of the GFP fusion construct of interest with a Tet-op promotor which is activated through the binding of the tTA transcription factor. Doxycycline binds to tTA thus competitively inhibiting transcription against the Tet-op promotor. This ultimately halts synthesis of the modified construct.

Microscopy and Image Processing

Microscopy was handled on a Olympus IX71 DeltaVision™ widefield fluorescence microscope with a 100x objective. Image acquisition and processing was performed through the onboard DeltaVision softWoRx software. Light excitations were managed through the included DeltaVision Elite Standard filter sets of FITC (excitation 475/28, emission 525/48) for GFP and nG tags, TRITC (excitation: 542/27, emission: 594/45) for the mCherry tag, and DAPI (excitation: 390/18, emission: 435/48) for the BFP tag. Images were further processed for figure construction using Fiji ((**F**iji **I**s **J**ust) **I**mage J)).

Immunoprecipitation (IP) of Ubiquitinated Substrates

All strains analyzed through IP contained a preliminary genetic deletion of the *doa4* ubiquitin hydrolase to stabilize ubiquitinated cargoes. Samples were cultured in YNB media to OD_{600} 0.25-0.5, and then MYC-tagged ubiquitin was temporally overexpressed from an included

plasmid construct containing a copper inducible promoter (CUP1) through the introduction of 100uM Cu_2SO_4 for 2 hours. Cells were then treated with rapamycin for three hours to induce ubiquitination. Following treatment, approximately 50 OD units of cells were collected by centrifugation at 4°C at 4000 RPM for five minutes.

The cell pellet was resuspended in 500 µl IP buffer (2 mM MgOAc, 1 mM $CaCl_2$, 50 mM HEPES-KOH, pH 6.8, 150 mM KOAc, 15% glycerol, 0.1% digitonin), supplemented with protease inhibitors and 20 mM n-ethylmaleimide (NEM). Whole-cell lysates were collected after samples underwent bead beating at 4°C for ten minutes to disrupt cell membranes. Then, 500 µl of 1.9% digitonin in IP buffer was added to whole-cell lysates to a final concentration of 1%. Membranes were then solubilized by nutating lysates at 4°C for 50 minutes. The sample was pelleted by centrifugation at 140,000 rpm for 20 minutes at 4°C. To capture GFP-tagged cargoes, the lysate was incubated with 25 µl GFP TRAP resin (Chromotek) at 4°C for one hour. The resin was washed four times with 0.1% digitonin in IP buffer by centrifugation at 3,000 g for 30 seconds at 4°C. Proteins bound to the resin were then eluted by incubating with 80 µL sample buffer at 65°C for five minutes. The resulting eluate was analyzed by SDS-PAGE and western blot using antibodies for both MYC and GFP.

Sample Preparation for Western Blotting

After sample collection, one mL of ice cold milliq water and 110 µL of 100% trichloroacetic acid (TCA) was added to cell pellets and then incubated at 4°C for at least 30 minutes. Samples were then pelleted and washed once with 0.1% TCA. Pellets were dissolved in 70 µl 2x boiling buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS) with the addition of 100 µL or glass microbeads, then subjected to bead beating on a vortex mixer at room temperature for five minutes. Samples were then incubated at 65°C for five minutes. 70 µL 2x urea sample buffer

(150 mM Tris, pH 6.8, 6 M urea, 6% SDS, 40% glycerol, 100 mM DTT, bromophenol blue) was then added to mixtures. Samples were again subjected to bead beating at room temperature for five minutes and incubated at 65°C for five minutes. The supernatants were collected from the mixtures following centrifugation and then analyzed through SDS-PAGE running and western blotting.

Western Blot Antibodies

The antibodies used are as follows: G6PDH (1:10,000; A9521, Sigma), Pgk1 (1:5000; 22C5D8, Invitrogen), mouse anti-GFP (1:500; sc-9996, Santa Cruz Biotechnology, Inc.), rabbit anti-GFP (1:3000;TP401, Torrey Pines Biolabs), mouse anti-MYC (1:500; 9E10, Santa Cruz Biotechnology, Inc.), and rabbit anti-MYC (1:2,000; Sigma).

Yeast Strains and Plasmids

Table S1: Yeast strains and plasmids used in this study

S. cerevisiae strains			
stain	**name**	***genotype***	**reference/source**
SEY6210	Wild type	*Matα, leu1-3, 112 ura3-52 his3-200, trp1-901 lys2-801 suc2-D9*	(Robinson et al., 1988)
SEY6210.1	Wild type	*Mata, leu1-3, 112 ura3-52 his3-200, trp1-901 lys2-801 suc2-D9*	(Robinson et al., 1988)
YML108	Fth1-GFP	6210.1, FTH1-GFP::HIS3	(Li et al., 2015a)
YML106	Fet5-GFP	6210.1, FET5-GFP::HIS3	(Li et al., 2015a)
YXY799	Fth1-GFP, *doa4Δ*	6210, FTH1-GFP::TRP1, *doa4Δ::HIS3*	This study
YXY245	Vph1-GFP	6210.1, VPH1-GFP::HIS3	This study
YXY999	Fth1-GFP, *triplemut*	6210.1, FTH1-GFP::KAN, *pib1Δ::HYG, rsp5-1Δ::TRP1, tul1Δ::HIS3*	This study
YML609	Fth1-GFP, *pep4Δ*	6210, FTH1-GFP::TRP1, *pep4Δ::LEU2*	(Li et al., 2015b)
YXY806	Fth1-GFP, *vps4Δ*	6210, FTH1-GFP::HIS3, *vps4Δ::TRP1*	This study
YXY810	Fth1-GFP, *atg1Δ*	6210, FTH1-GFP::HIS3, *atg1Δ::TRP1*	This study

YXY833	Fth1-GFP, *vps23Δ*	6210.1, FTH1-GFP::HIS3, *vps23Δ::TRP1*	This study
YML609	*pep4Δ*	*6210, PEP4Δ::LEU2*	(Li et al., 2015b)
YXY568	Fth1-GFP Mars-Sec7	6210.1, FTH1-GFP::HIS3, MARS-SEC7::TRP1	This study
YXY461	*triplemut*	*6210.1, PIB1Δ::HYG, TUL1Δ::TRP1, SSH4Δ::TRP1*	This study
YXY623	*vps36Δ*	6210.1, *VPS36Δ::HIS3*	This study
YXY914	*vps4Δ*	*6210.1, VPS4Δ::HYG*	This study
YXY595	*vps27Δ*	*6210.1, VPS27Δ::HIS3*	This study
YXY787	Fth1-GFP, 305-pGPD-Fet5	6210.1, 305-pGPD-FET5, pcm189-FTH1-GFP	This study
YXY630	Hxt3-GFP	*6210, ZRC1-BFP::KAN, VPH1-MCHERRY::HYG, HXT3-GFP::TRP1*	This study
YXY631	Hxt3-GFP, *vps4Δ*	*YXY630, VPS4Δ::HIS3*	This study
YXY632	Hxt3-GFP, *vps27Δ*	*YXY630, VPS27Δ::HIS3*	This study
YXY633	Hxt3-GFP, *vps23Δ*	*YXY630, VPS23Δ::HIS3*	This study
YXY634	Hxt3-GFP, *vps36Δ*	*YXY630, VPS36Δ::HIS3*	This study
YXY635	Hxt3-GFP, *snf7Δ*	*YXY630, SNF7Δ::HIS3*	This study
S. cerevisiae plasmids			
vector	**name**	***description***	**reference/study**
pCM189	Fth1-GFP, Tet-off	*Tet-off vector, tet-O7+endogenous promoter C-terminal GFP*	This study
pRS425	pCu-myc-ub	*Copper promotor, Myc-tagged Ubiquitin*	(Li et al., 2015b)
YXP147	GFP-RNF152	*415-pCPY-GFP-RNF152-TADH1*	This study
YXP145	GFP-RNF152 CS	*pBP74A-GFP-RNF152-C12 15 30 34S*	This study

References

1. Stelling, J., Sauer, U., Szallasi, Z., Doyle, F. J., 3rd, & Doyle, J. (2004). Robustness of cellular functions. *Cell*, *118*(6), 675–685. https://doi.org/10.1016/j.cell.2004.09.008
2. Lim, C.-Y. & Zoncu, R. (2016) The lysosome as a command-and-control center for cellular metabolism. J. Cell Biol. 214, 653–664
3. Settembre, C., Fraldi, A., Medina, D. L. & Ballabio, A. (2013) Signals from the lysosome: a control centre for cellular clearance and energy metabolism. Nat. Rev. Mol. Cell Biol. 14, 283–296
4. Budenholzer, L., Cheng, C. L., Li, Y., & Hochstrasser, M. (2017). Proteasome Structure and Assembly. *Journal of molecular biology*, *429*(22), 3500–3524. https://doi.org/10.1016/j.jmb.2017.05.027
5. Li, M., Rong, Y., Chuang, Y.-S., Peng, D. & Emr, S. D. (2015) Ubiquitin-Dependent Lysosomal Membrane Protein Sorting and Degradation. Mol. Cell 57, 467–478
6. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., & Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast (Chichester, England)*, *14*(10), 953–961. https://doi.org/10.1002/(SICI)1097-0061(199807)14:10<953::AID-YEA293>3.0.CO;2-U
7. Hecht, K. A., O'Donnell, A. F. & Brodsky, J. L. (2014) The proteolytic landscape of the yeast vacuole. Cell. Logist. 4, e28023
8. Kucharczyk, R., & Rytka, J. (2001). Saccharomyces cerevisiae--a model organism for the studies on vacuolar transport. *Acta biochimica Polonica*, *48*(4), 1025–1042.
9. Foster, K. G. & Fingar, D. C. (2010) Mammalian Target of Rapamycin (mTOR): Conducting the Cellular Signaling Symphony. J. Biol. Chem. 285, 14071–14077
10. Greiner-Tollersrud, O. K. & Berg, T. (2013) Lysosomal Storage Disorders. Madame Curie Bioscience Database [Internet] (Landes Bioscience)
11. Yang, X. et al. (2020) TORC1 regulates vacuole membrane composition through ubiquitin- and ESCRT-dependentmicroautophagy. J. Cell Biol. 219, e201902127
12. McNally, E. K. & Brett, C. L. (2018) The intralumenal fragment pathway mediates ESCRT-independent surface transporter downregulation. Nat. Commun. 9, 5358
13. McNally, E. K., Karim, M. A. & Brett, C. L. (2017) Selective Lysosomal Transporter Degradation by Organelle Membrane Fusion. Dev. Cell 40, 151–167
14. Karim, M. A., McNally, E. K., Samyn, D. R., Mattie, S. & Brett, C. L. (2018) Rab-Effector-Kinase Interplay Modulates Intralumenal Fragment Formation during Vacuole Fusion. Dev. Cell 47, 80-97.e6
15. Cox, T. M. (2001) Gaucher disease: understanding the molecular pathogenesis of sphingolipidoses. J. Inherit. Metab. Dis. 24, 107–123
16. Vanier, M. T. (2010) Niemann-Pick disease type C. Orphanet J. Rare Dis. 5, 16
17. Bedilu, R. et al. (2002) Variable clinical presentation of lysosomal β-mannosidosis in patients with null mutations. Mol. Genet. Metab. 77, 282–290
18. Bäumner, S. & Weber, L. T. (2018) Nephropathic Cystinosis: Symptoms, Treatment, and Perspectives of a Systemic Disease. Front. Pediatr. 6, 58
19. Elmonem, M. A. et al. (2016) Cystinosis: a review. Orphanet J. Rare Dis. 11, 47
20. Fraldi, A., Klein, A. D., Medina, D. L. & Settembre, C. (2016) Brain Disorders Due to Lysosomal Dysfunction. Annu. Rev. Neurosci. 39, 277–295
21. Rabanal-Ruiz, Y., Korolchuk, V. (2018) mTORC1 and Nutrient Homeostasis: The Central Role of the Lysosome. Int. J. Mol. Sci. 19, 818
22. Grumati, P., & Dikic, I. (2018). Ubiquitin signaling and autophagy. *The Journal of biological chemistry*, *293*(15), 5404–5413. https://doi.org/10.1074/jbc.TM117.000117
23. Song, L., & Luo, Z. Q. (2019). Post-translational regulation of ubiquitin signaling. *The Journal of cell biology*, *218*(6), 1776–1786. https://doi.org/10.1083/jcb.201902074

24. Deshaies, R. J., & Joazeiro, C. A. (2009). RING domain E3 ubiquitin ligases. *Annual review of biochemistry*, *78*, 399–434. https://doi.org/10.1146/annurev.biochem.78.101807.093809
25. Li, M., Koshi, T. & Emr, S. D. (2015) Membrane-anchored ubiquitin ligase complex is required for the turnover of lysosomal membrane proteins. J. Cell Biol. 211, 639–652
26. Migliano, S. M., & Teis, D. (2018). ESCRT and Membrane Protein Ubiquitination. *Progress in molecular and subcellular biology*, *57*, 107–135. https://doi.org/10.1007/978-3-319-96704-2_4
27. Henne WM, Buchkovich NJ, Emr SD. The ESCRT pathway. (2011) Dev Cell. Jul 19;21(1):77-91. doi: 10.1016/j.devcel.2011.05.015. PMID: 21763610.
28. Mizushima N. (2010). The role of the Atg1/ULK1 complex in autophagy regulation. *Current opinion in cell biology*, *22*(2), 132–139. https://doi.org/10.1016/j.ceb.2009.12.004
29. Zhang, W., Yang, X., Chen, L., Liu, Y., Venkatarangan, V., Reist, L., Hanson, P., Xu, H., Wang, Y., & Li, M. (2021) A conserved ubiquitin- and ESCRT- dependent pathway downregulates human lysosomal membrane protein. *Review Commons, [Under Revision]*
30. Mosesso, N., Nagel, M. K., & Isono, E. (2019). Ubiquitin recognition in endocytic trafficking - with or without ESCRT-0. *Journal of cell science*, *132*(16), jcs232868. https://doi.org/10.1242/jcs.232868
31. Okamoto, T., Imaizumi, K., & Kaneko, M. (2020). The Role of Tissue-Specific Ubiquitin Ligases, RNF183, RNF186, RNF182 and RNF152, in Disease and Biological Function. *International journal of molecular sciences*, *21*(11), 3921. https://doi.org/10.3390/ijms21113921
32. Robertson J. L. (2018). The lipid bilayer membrane and its protein constituents. *The Journal of general physiology*, *150*(11), 1472–1483. https://doi.org/10.1085/jgp.201812153
33. Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M., & Schekman, R. (1994). COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell*, *77*(6), 895–907. https://doi.org/10.1016/0092-8674(94)90138-4
34. Boncompain, G., & Perez, F. (2013). The many routes of Golgi-dependent trafficking. *Histochemistry and cell biology*, *140*(3), 251–260. https://doi.org/10.1007/s00418-013-1124-7
35. Feyder, S., De Craene, J. O., Bär, S., Bertazzi, D. L., & Friant, S. (2015). Membrane trafficking in the yeast Saccharomyces cerevisiae model. *International journal of molecular sciences*, *16*(1), 1509–1525. https://doi.org/10.3390/ijms16011509
36. Elkin, S. R., Lakoduk, A. M., & Schmid, S. L. (2016). Endocytic pathways and endosomal trafficking: a primer. *Wiener medizinische Wochenschrift (1946)*, *166*(7-8), 196–204. https://doi.org/10.1007/s10354-016-0432-7
37. Rigal, A., Doyle, S. M., & Robert, S. (2015). Live cell imaging of FM4-64, a tool for tracing the endocytic pathways in Arabidopsis root cells. *Methods in molecular biology (Clifton, N.J.)*, *1242*, 93–103. https://doi.org/10.1007/978-1-4939-1902-4_9
38. Boss, W. F., & Im, Y. J. (2012). Phosphoinositide signaling. *Annual review of plant biology*, *63*, 409–429. https://doi.org/10.1146/annurev-arplant-042110-103840
39. Rautu, S. A., Rowlands, G., & Turner, M. S. (2015). Membrane composition variation and underdamped mechanics near transmembrane proteins and coats. *Physical review letters*, *114*(9), 098101. https://doi.org/10.1103/PhysRevLett.114.098101

Supplementary Materials

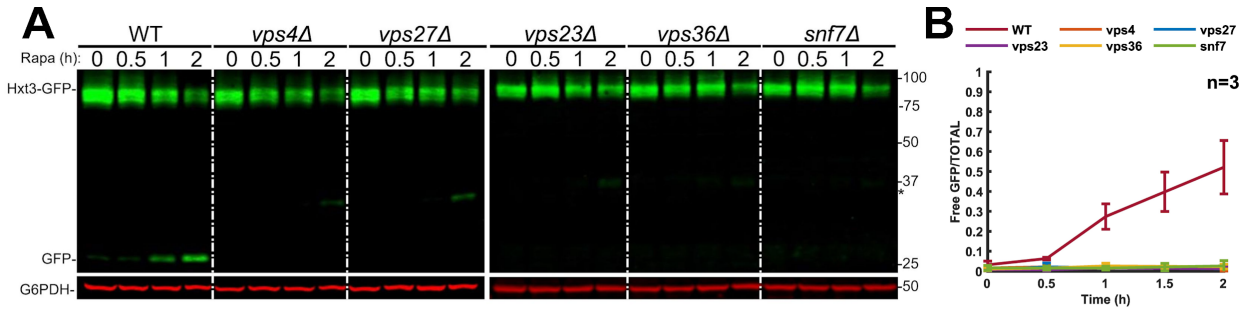

Figure S1: **Hxt3-GFP degradation requires ESCRT (cont.). (A)** Western blot of Hxt3-GFP samples across WT, and five ESCRT mutant strains, *vps4, vps27, vps23, vps36, and snf7* along 2hr rapamycin treatment. ESCRT mutation blocked any observable degradation. * marks an assumed intermediate cleavage product from cytosolic proteasome. **(B)** Quantification of A showcasing any accumulation of free GFP. Relative free GFP levels calculated as Free GFP/(Free GFP+Full Length).

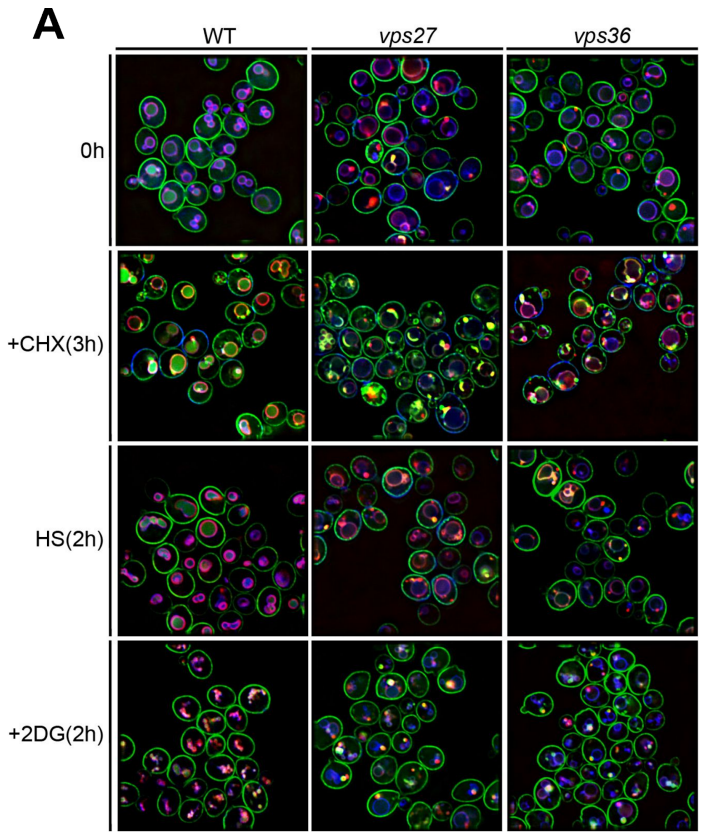

Figure S2: **Tricolor Imaging of Hxt3-GFP across three degradation treatments. (A)** Fluorescent microscopy of Hxt3-GFP, Vph1-mCherry (VM MVB pathway marker), and Zrc1-BFP (VM AP3 pathway marker) across WT, and two ESCRT mutant strains, *vps27* and *vps36*. Three degradation treatments are featured: 3hr CHX, two hour 37℃ heat shock treatment, and 0.4mM 2-deoxyglucose.