Deciphering the Role of p24 Proteins in COPII-mediated Protein Secretion

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ABSTRACT

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In eukaryotic cells, proteins continuously flux through the organelles of the secretory pathway in an essential cellular process called protein secretion. This dynamic process originates at the endoplasmic reticulum (ER), where translating ribosomes push linear peptides into the ER membrane and lumen. ER chaperones assist in folding nascent peptides into three-dimensional conformations and proteins are concentrated into membrane-encapsulated vesicles bound for the Golgi apparatus. ER to Golgi transport is mediated by a set of cytosolic coat proteins called COPII. The COPII coat polymerizes into a lattice on the ER membrane that is able to bend the membrane around secretory cargo and bud off a spherical vesicle.

Protein secretion is subject to rapid changes as a cell responds to its environment and requirements for viability alter. In addition to accommodating short-term demands, such as translational up-regulation, evolved complexity of secretory proteins over time, has also required that secretory components adapt. In both cases changes in secretory demands require that the COPII proteins have an inherent flexibility to navigate these changes without disrupting secretory flux. In this work I have examined a family of quintessential secretory cargo, p24 proteins, that challenge protein secretion. This family of proteins forms a hetero-tetrameric complex that cycles between the ER and the Golgi and mediates transport of glycosylphosphatidylinositol-anchored proteins (GPI-APs). Here I present evidence that

suggests, when present in vesicles, both p24 proteins and their GPI-AP cargo present a challenge to vesicle formation. I posit that three attributes of these proteins present a local barrier to membrane bending: Lumenal asymmetric distribution across the membrane, high cellular abundance and affinity for ceramide rich membranes. I have also elucidated mechanisms that the coat has evolved to accommodate troublesome cargo such as p24 proteins, which enhance structural scaffolding and increase average vesicle size. Finally I present preliminary findings indicating that p24s also contribute to ER homeostasis by preventing aberrant incorporation of proteins into vesicles. Comprehensively, these findings have shed light on the role of p24 proteins in vesicles. Traditionally thought to be canonical ER cargo receptors, these proteins also appear capable of contributing to the composition of the vesicles in which they reside, and impacting trafficking efficiency in two ways: First by directly mediating transport of GPI-APs and second by uniformly packing vesicles to avoid wasteful secretion. My work has contributed to a growing notion in the field that secretory cargo are not inert passengers but active participants in vesicle mediated secretion.

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Chapter 1

Vesicle-mediated Export from the ER: COPII coat function and regulation.

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Abstract

Vesicle trafficking from the endoplasmic reticulum (ER) is a vital cellular process in all eukaryotes responsible for moving secretory cargoes from the ER to the Golgi apparatus. To accomplish this feat, the cell employs a set of conserved cytoplasmic coat proteins – the coat protein II (COPII) complex – that recruits cargo into nascent buds and deforms the ER membrane to drive vesicle formation. While our understanding of COPII coat mechanics has developed substantially since its discovery, we have only recently begun to appreciate the factors that regulate this complex and, in turn, ER-to-Golgi trafficking. Here, we describe these factors and their influences on COPII vesicle formation. Properties intrinsic to the GTP cycle of the coat, as well as coat structure, have critical implications for COPII vesicle trafficking. Extrinsic factors in the cytosol can modulate COPII activity through direct interaction with the coat or with scaffolding components, or by changing composition of the ER membrane. Further, lumenal and membrane-bound cargoes and cargo receptors can influence COPII-mediated trafficking in equally profound ways. Together, these factors work in concert to ensure proper cargo movement in this first step of the secretory pathway.

1-1. Introduction

Eukaryotic protein secretion is an essential cellular process that ensures that proteins destined for the secretory pathway, plasma membrane or extracellular space are delivered with temporal and spatial accuracy. Central to this process is the formation of cargo-bearing vesicles that ferry proteins between compartments of the secretory pathway. Vesicles form from the endoplasmic reticulum (ER) through the concerted action of Coat Protein complex II (COPII) on the ER membrane [1]. Although the basic mechanism of COPII action is relatively well characterized [2, 3], the biophysical details of how the COPII coat deforms the ER membrane remain to be fully understood. In particular, how this process might be

modified to accommodate a wide range of secretory cargo and regulated to meet the dynamic needs of the cell remain unclear. Several recent findings highlight the complexity of vesicle formation from the ER: the potential role that cargo proteins might play in this process is now being explored, as are the various post-translational modifications of the cytosolic COPII machinery. Appreciating that an entire third of the eukaryotic genome is trafficked by vesicular transport from the ER [4], it is not surprising that regulation of this process would be layered, allowing for modification at various points in vesicle formation. In this review we provide a detailed view of how cargo is incorporated into COPII coated vesicles and budded from the ER. In addition we will discuss how recent findings have sent the field in new and exciting directions toward a better understanding of how this pivotal process is intricately regulated.

1-2. Overview of COPII Coat Formation

Transport vesicles are created at the ER via an essential core set of coat proteins that assemble on the cytosolic face of the ER membrane. This core machinery serves multiple functions: it is responsible for inducing curvature in the ER membrane, concentrating cargo into nascent buds, and driving vesicle release. All of these functions are performed by the COPII coat, composed of five proteins: Sar1, Sec23, Sec24, Sec13 and Sec31, which form the minimal machinery required to form vesicles from membranes *in vitro* [1, 2].

Assembly of the COPII coat on the ER membrane occurs in a stepwise fashion, beginning with recruitment of the GTPase Sar1 through GTP loading facilitated by its guanine nucleotide exchange factor (GEF), Sec12 [5]. In the GTP-bound state, Sar1 exposes an N-terminal amphipathic alpha helix that becomes inserted in the ER membrane [6]. Sar1-GTP at the ER subsequently recruits heterodimeric Sec23/24 through the binding of Sec23 to Sar1-GTP, and through interactions of Sec23/24 with the

membrane [2, 7]. Sec24 serves as the principle cargo-binding adaptor for the COPII coat and, once at the ER, associates with cargo molecules to be trafficked to the Golgi apparatus through direct interactions with cargoes or with cargo-bound receptors [8-10]. Protein modules composed of Sar1-Sec23/24-cargoes are referred to as "pre-budding complexes", and represent the basic functional units of the COPII inner coat layer to be incorporated into ER-derived transport vesicles. Following pre-budding complex formation, heterodimers of Sec13/31 are recruited via interaction between Sec23-Sec31 [11]. Sec13/31 forms a cage-like outer coat layer on the nascent vesicle, driving membrane curvature [12-14]. Sec23 is the GTPase activating protein (GAP) for Sar1, with Sec31 also promoting GTP hydrolysis in the completed COPII coat [3]. GTP hydrolysis seems to be required for the ultimate release of vesicles from the donor membrane [15]. After vesicle formation occurs, downstream events lead to uncoating of transport vesicles and recycling of the COPII coat components [1](Figure 1). Although our understanding of COPII-mediated vesicle formation has developed substantially over the past two decades, many details of this process remain unresolved. Here, we discuss recent studies that have contributed to our understanding of the COPII system and its regulatory elements.

1-2.1 Sar1

Sar1 is a small Arf-related GTPase, and is the central player in regulating COPII vesicle formation at the ER. Specific and efficient Sar1 recruitment to the ER membrane is accomplished by its cognate GEF Sec12, which is an ER-resident integral membrane protein [16, 17]. When bound to GTP, Sar1 undergoes a conformational change in its switch I/II regions that exposes an N-terminal amphipathic alpha helix [7, 18]. This helix becomes embedded in the lipid bilayer through interactions of hydrophobic residues with phospholipid groups [6]. The shallow insertion of this amphipathic helix can impart curvature on the membrane [6, 15, 19, 20], although the physiological significance of this curvature remains unclear since

the effect was observed with very high concentrations of Sar1. Perhaps more physiologically important is the apparent requirement for both the helix and GTPase activity in mediating vesicle scission from the membrane [6, 15]. At the membrane, Sar1-GTP is able to recruit Sec23/24 via an extensive binding interface with Sec23 that accounts for ~20% of the surface area of Sar1 [7].

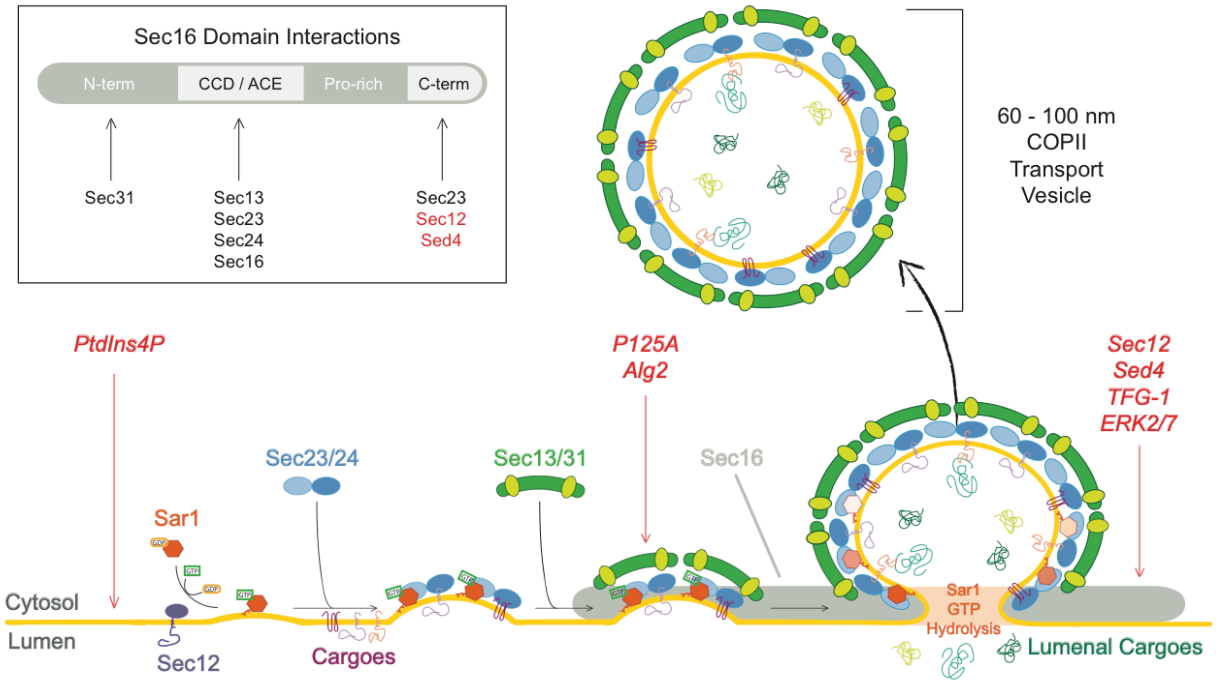

Figure 1. COPII assembly at the ER and regulation by cytosolic factors. COPII coat formation at the ER is initiated by recruitment of Sar to the membrane in the GTP-bound state, enhanced by Sec12 GEF activity. Sar1-GTP recruits Sec23/24 heterodimer through interaction with Sec23. At the ER, Sec24 recruits cargo into pre-budding complexes. Sec13/31 complex is recruited to the inner coat layer through interactions with Sec23. Sec13/31 assembly into the coat drives membrane curvature, facilitating membrane deformation. Lumenal cargoes diffuse into the nascent bud or are recruited through cargo receptor proteins. Vesicle release from the ER is achieved in a manner related to Sar1 GTP hydrolysis. Released COPII vesicles typically fall within a 40 to 100 nanometer range. Sec16 facilitates COPII vesicle formation at the ER, likely through scaffolding COPII components and contributing to ERES structure. Interactions of COPII and COPII-associated components with domains of Sec16 are shown in the box in the upper left. Non-COPII proteins that affect COPII vesicle formation are shown in red italics, with red arrows indicating their proposed targets.

Although the mechanics of Sar1 membrane recruitment and GTPase activity are well understood, its participation in regulating COPII vesicle formation remains somewhat convoluted. Sar1 GTP hydrolysis

appears to control multiple processes, both early and late in COPII vesicle formation. GTP-binding and ER membrane association are essential for the downstream recruitment of Sec23/24 to synthetic liposomes, but cargo-containing membranes may also provide affinity for recruitment of Sec23/24 and in this context Sar1 might instead be more of a regulatory factor than a recruitment anchor. In support of such a model, GTP hydrolysis by Sar1 influences cargo-concentration during pre-budding complex formation [21]. Single-molecule fluorescence studies showed active concentration of cargoes into COPII pre-budding complexes dependent in part on multiple rounds of GTP hydrolysis by Sar1, whereas cargo-free Sar1 and Sec23/24 complexes dissociate more readily from the membrane when GTP is hydrolyzed [21, 22] For large cargo molecules, Sar1-GTP cycling may have an analogous effect on enhancing cargo transport from the ER. Sar1 is important for the efficient packaging of large lipid particles known as chylomicrons [23] and other large cargoes such as procollagen may require Sar1 cycling [24].

Late in COPII vesicle formation, Sar1 GTP hydrolysis likely regulates vesicle scission from the membrane. Release of budded vesicles is inhibited when either the amphipathic helix of Sar1 is deleted [6] or when GTP hydrolysis on Sar1 is prevented [15, 20]. On large unilamellar vesicles, Sar1 forms an ordered scaffold dependent on an omega loop at its C-terminus that tubulates and constricts the underlying bilayer [19, 20]. Scaffolding and constriction of Sar1 in this fashion may facilitate closure of COPII bud necks at the ER, leading to vesicle release. The ability of Sar1 to lower the rigidity of lipid membranes might aid in this constriction process, as was reported with Sar1 complexed with non-hydrolyzable GTP analogs in optical trap studies [25, 26]. After vesicle release from the ER, Sar1 GTP hydrolysis is also thought to contribute to the uncoating of COPII components from formed transport vesicles by destabilizing interactions of the COPII components with the membrane after GTP hydrolysis has occurred [1, 3].

Taken together, these results suggest that Sar1 performs multiple functions during COPII vesicle formation [27]. Whether this results from different Sar1 populations or differential regulation by various factors (i.e. Sec16, Sec12, Sec23, Sec31) that ensure that their specific functions are not undermined by counteracting forces remains to be seen.

1-2.2 Sec23/Sec24

Sar1 recruitment to the ER membrane is followed by the heterodimeric Sec23/Sec24 complex. Sec23/24 forms a bowtie-shaped complex with a concave membrane-apposed surface that may impart curvature on the underlying membrane or simply promote complex binding to already curved membranes [7]. An extensive interaction interface between Sec23 and Sar1 likely drives Sec23/24 recruitment, with additional affinity probably contributed by electrostatic interactions between the basic residues that line the Sec23/24 concave face and acidic phospholipids in the ER [2, 7]. A key part of the Sar1-Sec23 interface is an arginine residue from Sec23 that inserts into the catalytic pocket of Sar1 [7]. This "arginine finger" results in stimulation of Sar1 GTPase activity through stabilization of GTP phosphate groups [7]. Binding of Sec31 to Sec23 and Sar1 through an extended unstructured loop of Sec31 re-orients this arginine residue to further enhance GTPase activity 4- to 10-fold over Sec23/24 alone [3, 11].

In addition to this well-established role of Sec23 in Sar1 GAP activity, recent cryo-EM studies suggest that Sec23 also plays an important role in orienting Sec24 for optimal cargo binding [14]. Rather than aligning directly beneath the outer coat, Sec23 is thought to be able to bind in two distinct locations on Sec31 allowing for alternate conformations of the final three-dimensional geometry [14]. Importantly, these geometries position Sec24 in the open face of the cuboctahedral cage formed by Sec13/31, allowing for binding of cargo with various shapes and sizes [14]. The specific orientation of Sec23/24 is

also thought to influence vesicle size, indicating that placement of this adaptor complex in the coat is potentially important for the transport of larger secretory cargo [14].

Although Sec23p is not known to bind to any cargo directly, it may indirectly impact cargo incorporation via interactions with other coat components influencing aspects such as the Sar1 GTP cycle as well as recruitment of the outer cage. Mammalian cells express A and B isoforms of Sec23, Sar1 and Sec31. Fromme and colleagues identified a missense mutation in Sec23A responsible for cranio-lentinculo-sutural dysplasia (CLSD), which derives from extracellular matrix deficiencies [28]. The mutant Sec23 isoform is unable to recruit Sec13/31 and consequently prevents complete fission of vesicles from the ER. Interestingly, an excess of ER tubules are observed indicating that the pre-budding complex assembles and confers a degree of curvature without the outer cage. The CLSD mutation in Sec23A also decreases the GAP activity of Sec23A. All of these phenotypes are specific for Sar1B; when Sar1A is the sole copy of Sar1 the severity of decreased Sec13/31 recruitment, vesicle budding and GAP activity is diminished [28]. Kim et al describe a second CLSD-associated mutation in Sec23A that also maps to the Sec31A binding interface. Although this mutation also causes increased intracellular retention of collagen, it does not diminish association with Sec31A, and instead activates Sar1B GTPase activity [29]., Vesicle formation in general and traffic of most cargo molecules remain largely unaffected, suggesting that the mutation causes vesicle release from the ER before larger cargo, such as procollagen fibers, can be incorporated. These findings further link the Sar1 GTPase cycle to packaging of large secretory cargo. Additional evidence for regulation of collagen secretion by the TANGO1 cargo receptor via direct interaction with Sec23/24 (discussed below) further substantiates a role for GTPase regulation in collagen trafficking. These distinct mutations in Sec23A illustrate how the same protein-protein interactions within the coat can be differentially modified to impact vesicle trafficking in opposite ways,

suggesting that precise regulation of intra-coat interactions is essential for faithful secretion from the ER.

Post-translational modifications of Sec23 are only beginning to be unmasked, but may have profound impacts on COPII vesicle trafficking. Evidence from Lord and colleagues suggests that phosphorylation and dephosphorylation of specific sites on Sec23 may be able to control the directionality of COPII vesicle transport, with modifications impacting not only budding of COPII vesicles from the ER but also vesicle fusion with the Golgi [30].

Sec24 serves as the principle cargo-binding adaptor for the COPII coat, and is responsible for efficient sorting and recruitment of cargo molecules into pre-budding complexes [8-10]. Cargo molecules associate with Sec24 via interactions between cargo-borne export signals and cargo-binding sites on the surface of Sec24 [10, 31, 32]. Additional isoforms of Sec24 (Iss1/Sfb2 and Lst1/Sfb3 in yeast; Sec24A-D in mammals) may help to broaden the cargo binding capacity for COPII transport vesicles and may also contribute morphological effects by increasing vesicle size [33-35]. Cargo recruitment by Sec24 can be driven by direct cargo-coat interactions or indirect interactions via membrane-spanning receptor proteins that aid in the export of lumenal cargoes from the ER [10, 36]. Binding of cargo to Sec24 results in enhanced stability of pre-budding complexes on membranes even in the context of GTP hydrolysis by Sar1 [21, 37].

Although Sec24 has long been considered a static platform for COPII cargo binding, recent evidence suggests that Sec24 is also critically involved in local and cellular regulatory networks. A novel mutation on the surface of Sec24, termed the m11 mutation, leads to broad defects in COPII vesicle formation [38]. This mutant form of Sec24 displays reduced binding to Sec16 and diminishes the ability of Sec16 to inhibit the GTPase activity of Sar1 *in vitro*, implying a role for Sec24 in regulating COPII vesicle formation

through complex formation with Sec16 [38]. A potential regulatory role for Sec24 is also suggested by new findings that implicate a cellular kinase in post-translational modification of Sec24. One recent study demonstrates that Akt, an important signaling kinase implicated in cancer and diabetes progression, has the ability to phosphorylate mammalian Sec24C; recombinant Akt protein was able to phosphorylate recombinant Sec24C isoform *in vitro* [39]. Phosphorylated Sec24C and Sec24D bind less well to Sec23 [39]. These data imply that Sec24 can also respond to cellular signalling, and perhaps regulate pre-budding complex formation through altered association with Sec23.

1-2.3 Sec13/Sec31

After formation of the pre-budding complex, the Sec13/Sec31 complex binds, forming the outer layer of the COPII coat [40]. The propensity for Sec13/31 to self-assemble into a cage-like spherical structure is suggestive of a role for the outer coat in both collecting the underlying inner coat complexes to organize the vesicle and driving curvature during polymerization [12]. A single unit of the Sec13/31 cage is comprised of a heterotetramer of these proteins. Two Sec31 molecules dimerize tail-to-tail via α-solenoid structures to form a rod. Four rods associate via N-terminal β-propeller domains of Sec31 to form the vertex of the COPII cage. Sec13 lies sandwiched between the α-solenoid and β-propeller domains of Sec31, with Sec31 contributing one β-blade to complement the 6-bladed β-propeller structure of Sec13 [13]. When fully polymerized, the outer cage is believed to optimally form a cuboctahedral geometry, although multiple geometries are possible via this structural model making the outer coat somewhat adaptable to different vesicle shapes [12, 41]. Support for the model that Sec13/31 assembly drives membrane curvature associated with vesicle formation comes from a recent study that explored the role of Sec13 within the COPII coat. Exploiting yeast genetic backgrounds whereby Sec13 becomes dispensable, Copic and colleagues (2012) discovered that reducing the cargo

burden relieves the cellular requirement for Sec13, suggesting the primary role of Sec13 is to provide structural rigidity to the outer coat [42]. Artificially rigidifying Sec31 by deleting a flexible hinge region that encompasses the Sec13 binding site also supported viability absent Sec13. These findings are consistent with cage assembly driven by inter-molecular Sec31 interactions providing the driving force for membrane scaffolding, with Sec13 providing an additional structural role in creating rigid edge for the cage.

Binding of Sec13/31 to the prebudding complex also impacts the catalytic cycle of the coat, increasing the GAP activity of Sec23, and therefore GTP hydrolysis on Sar1, by an order of magnitude [3]. This creates something of a conundrum whereby coat assembly drives maximal catalysis that leads to depolymerization of the coat. How cargo incorporation, vesicle formation and scission all take place before Sar1 GTP hydrolysis stimulates de-polymerization of the coat remains to be fully dissected [3]. The answer to this question is likely to be answered by a multi-faceted regulatory scheme that is continually modified as cargo is incorporated and additional layers of the coat come together on the ER membrane [27].

One type of regulation that has been increasingly explored recently is that associated with secretion of abnormally large cargo from the ER [43]. Since the details of cage geometry have been elucidated, the largest intact cages observed have been 1000 Å in diameter [14]. This is not large enough to accommodate cargoes such as procollagen fibers and chylomicrons, which can be as large as 3,000Å and 10,000Å respectively [23, 43]. This raises the question as to how the composition or geometry of the COPII coat is modified to traffic large cargo. One possibility is that asymmetrical or non-spherical cage geometries provide a mechanism with which to mediate transport of large cargo. Although such arrangements were originally postulated [12], they have recently been experimentally observed by

electron tomography [14]. These authors were subsequently able to show that larger cages of similar geometries can be constructed while maintaining the previously determined angles required for a vertex of four Sec31 molecules [14]. Additional regulation of cage size may come from direct post-translational modification of the outer coat. A recent publication by Jin and colleagues showed that monoubiquitylation of Sec31 allows for the formation of larger COPII vesicles to allow for trafficking of procollagen fibers [44]. Post-translational modifications to the coat could impact how the components interact and possibly even affect GTPase activity of Sar1. This is an especially interesting prospect given the aforementioned finding of the Sec31-Sec23 interface having an impact on the lifetime of the coat and incorporation of large cargo indirectly.

1-3. Cytosolic Regulatory Factors

Although the five COPII coat proteins described above represent the minimal machinery required to bud a transport vesicle, even from naked synthetic liposomes [1, 2], there are clearly additional components that also participate. Indeed, numerous accessory factors are responsible for modulating coat recruitment and COPII vesicle transport, albeit often in poorly-understood ways. Here, we discuss how factors on the cytosolic face of the ER influence COPII coat function.

1-3.1 Sec16

Perhaps the most enigmatic cytosolic factor controlling COPII vesicle formation is Sec16, a large multi-domain protein that associates peripherally with the ER membrane and is essential for ER export *in vivo* [45, 46]. Sec16 localizes to sites of rapid COPII vesicle turnover known as ER exit sites (ERES) and is critical for the maintenance of these structures [46, 47]. Sec16 interacts physically with all of the COPII coat proteins, as well as the ER membrane proteins Sec12 and Sed4, and has a higher lifetime on ER membranes than other COPII components [47-52]. Yet despite its central role in vesicle production, very

little is known about the mechanistic role that Sec16 plays *in vivo*, in large part due to the difficulty associated with working with this 250kDa protein. However, recent structural, biochemical and genetic studies have made significant strides in this area.

One key finding is that Sec16 can impede the GTPase activity of the full COPII coat *in vitro*, implying that this protein can regulate COPII vesicle formation by controlling Sar1 activity [38, 48]. A truncated form of Sec16 that lacks the N-terminal domain, but can still complement a *sec16* deletion, inhibits the Sec31-dependent GTPase activity of Sar1 [38]. This basic finding was confirmed independently by another group that showed the same effect for full-length Sec16 [48]. This Sec16 GTPase inhibitory activity is dependent on a specific site on the surface of Sec24; mutation of this site (*sec24-m11*) diminishes this inhibitory activity *in vitro* and causes accumulation of ER membranes *in vivo* [38]. This observation raises the possibility that COPII vesicle formation and cargo recruitment into nascent vesicles via Sec24 are linked through Sec16 activity, although this model remains speculative.

In addition to a catalytic function for Sec16, it is also implicated more broadly in organization of ERES. Yeast Sec16 can bind directly to membranes *in vitro* and in turn interacts with every COPII coat protein [45, 50, 51, 53]. These observations have lead to the suggestion that Sec16 impacts the formation of ERES by scaffolding the other COPII components during vesicle formation, perhaps further stabilizing the nascent coat by locally reducing Sar1 GTPase activity [38, 47, 48, 53, 54]. In metazoans, another factor also aids Sec16 in its assembly: TRK-fused gene 1 (TFG-1) binds directly to Sec16 and its depletion leads to a reduction in Sec16 and Sec13 at ERES [55]. TFG-1 forms a hexameric complex that helps Sec16 associate properly with the membrane, further implying a scaffolding function either in vesicle formation or in movement of nascent vesicles to an adjacent site of fusion [55].

Sec16 localization to ERES can also be influenced by cellular kinases, in particular ERK2 and ERK7. The MAPK signaling pathway was identified as a potential regulator of ER exit via a siRNA screen performed in HeLa cells [56]. Subsequent investigation revealed that the MAPK member ERK2 was able to phosphorylate Sec16 (confirmed by anti-MAPK substrate immunoblot) and knockdown of ERK2 led to a decrease ERES number [56]. Intriguingly, inclusion of purified ERK2 in an *in vitro* COPII budding assay led to increased vesicle production, suggesting that Sec16 phosphorylation may influence rate of vesicle production at the ER [56]. In a separate study, ERK7 was also found to regulate secretion and Sec16 at ERES. When ERK7 is overexpressed, Sec16 is less abundant at ERES and instead appears dispersed in the cytoplasm [57]. A similar effect is observed during serum and amino acid starvation conditions, where ERK7-mediated ERES disassembly leads to perturbed secretion [57]. These results imply that Sec16 may serve not only as a scaffold for the COPII coat but also a signal integrator, modulating COPII vesicle formation sites in response to cellular conditions. In a similar vein, Sec16 is also responsible for mediating changes in ERES number during increases in cargo load at the ER, indicating that Sec16 may be responsive to other cellular stimuli aside from cell signaling cascades [58].

Through this recent work, it is becoming clear that Sec16 has multiple roles to play at the ER. Future work directed at clarifying how these different roles may be integrated with one another in the context of COPII vesicle formation at the ER will continue to enhance our understanding of this critical secretory protein.

1-3.2 Sec12

Although Sec12 has long been known to aid in the recruitment of Sar1 to the ER membrane through GTP exchange, recent investigations have expanded our understanding of Sec12 catalytic activity and its localization within the ER across various species, both of which may effectively regulate COPII vesicle

biogenesis. The crystal structure of the *S. cerevisiae* Sec12 cytoplasmic domain was recently solved [59], revealing that residues in the Sec12 K-loop bind to a potassium ion. Potassium binding is critical for GEF activity since substitution of potassium for sodium resulted in a 5-fold decrease in Sar1-GTP exchange in an *in vitro* assay [59]. Stimulation of GEF activity through metal binding could potentially have a physiological role in a cellular context, although such a mechanism has not been described.

Restriction of Sec12 to discrete locations within the ER may also affect COPII vesicle formation, but localization of Sec12 seems to vary across species. In *S. cerevisiae*, Sec12 is dispersed throughout the ER, similar to human Sec12 [17, 60]. However, *Pichia pastoris* Sec12 localizes to puncta representing ERES [60, 61]. Recent work has attempted to explain these observations. An investigation by Montegna and colleagues has indicated that *P. pastoris* Sec12 indeed co-localizes with ERES, and that human Sec12 does as well, contrary to previous observations [49]. Further, *P. pastoris* and human Sec12 can both bind directly to the C-termini of their cognate Sec16 homologs, whereas *S. cerevisiae* Sec12 cannot [49, 52]. These results demonstrate that the Sec12-Sec16 interaction may be a conserved method of Sec12 localization across species. Sec12 localized to active sites of COPII vesicle formation via Sec16 would permit the rapid recruitment of Sar1-GTP to the ER in these areas, facilitating high vesicle turnover.

1-3.3 Sed4

A yeast-specific ER-resident protein, Sed4, which has significant sequence homology with Sec12, may in fact play an opposing role in vesicle formation despite this similarity. *SED4* was identified as a multicopy suppressor of an *ERD2* deletion, which encodes the HDEL receptor in yeast [62]. Sed4, like *P. pastoris* and human Sec12, binds to the C-terminus of Sec16 [52, 60]. Despite sharing 45% homology with the cytosolic domain of Sec12, the N-terminal cytoplasmic domain of Sed4 does not display any GEF activity toward Sar1 [63, 64]. On the contrary, recent evidence suggests that Sed4 stimulates Sar1 GTPase

activity in a GAP-like fashion, acting on both Sar1 and Sec23 [63]. Further, Sed4 GAP activity is restricted to cargo-free Sar1 molecules, implying that it may have a cargo concentrating effect on pre-budding complexes entering ERES [63]. Given the opposing activities of Sec12 and Sed4, it remains to be seen (i) how these factors influence one another in *S. cerevisiae*, and (ii) if a factor analogous to Sed4 exists in higher eukaryotes.

1-3.4 Phosphatidylinositol 4-phosphate

Another factor implicated in regulating localization of COPII vesicle production is phophatidylinositol 4-phosphate (PtdIns4P). PtdIns4P plays an important role in Golgi vesicular trafficking, facilitating the recruitment of the GTPase Arf1 to specific lipid subdomains within the Golgi membrane through two pleckstrin homology (PH) domain accessory proteins, Fapp-1 and Fapp-2 [65, 66]. Evidence in mammals suggests that PtdIns4P may have a similar role at the ER. Addition of GST-Fapp1-PH to an *in vitro* COPII budding reaction using microsomal membranes inhibited vesicle formation, suggesting that PtdIns4P is required for COPII budding [67]. Furthermore, Sar1 activation on ER membranes led to enrichment of PtdIns4P, and this phospholipid also accumulated on Sar1-induced tubules by fluorescence microscopy as detected by a Fapp1-PH reporter [67]. These results indicate that PtdIns4P is a critical component of ERES composition, and that this phospholipid may play a regulatory role in modulating COPII recruitment to the ER membrane. These findings raise the possibility for direct ERES regulation through the local activity of PtdIns4P-specific kinases and phosphatases. Indeed, in the same study, incubation of COPII-containing cytosol and ER microsomes in the presence of the cytosolic domain of Sac1 - a yeast ER- and Golgi-localized PtdIns4P phosphatase - led to a reduction in total vesicle production [67]. Cellular kinases that reside at the ER represent candidates for future study on PtdIns4P activity in ERES.

Intriguingly, Sec16 may also be able to respond to changes in lipid composition at the ER; Sec16 localizes to distinct "cup-shaped" structures in metazoans [47, 54]. Evidence from *Drosophila melanogaster* suggests that an arginine-rich domain of dSec16 may bind to the ER membrane at these locations; a deletion construct of Sec16 lacking this region leads to a diffuse localization as detected by fluorescence microscopy [54]. Since arginine-rich domains are known to bind to anionic lipids [68, 69], it remains a distinct possibility that dSec16 may interact with phosphorylated PtdIns to direct ERES localization to specific lipid domains, as a functional link between PtdIns4P and Sec16 has already been established at ERES [58]. Further work will be required to resolve this point, and to see if it is applicable to orthologs of Sec16 in other organisms.

1-3.5 Additional regulators: P125A and ALG2

In mammals, new roles are being found for additional players that seem to act in the biogenesis of COPII vesicles in still poorly understood ways. One such protein, p125A, was initially identified as a Sec23 interacting protein (Sec23IP), binding Sec23 through its N-terminal proline-rich domain in a variety of tissues [70]. This same domain is responsible for recruiting p125A to ERES, and recruitment is enhanced by Sar1-GTP [71]. However, until recently the biological significance of these observations remained clouded. Now, new evidence suggests that p125A also binds Sec13/31: pulldown of Sec31-GST from rat liver cytosol also purifies p125A, dependent on residues 260-600 of p125A [72]. Furthermore, p125A silencing results in Golgi fragmentation and an ER trafficking delay of the model secretory cargo, VSVG [72]. Together, these results suggest that p125A is an important accessory factor that facilitates COPII-mediated trafficking in mammalian cells, perhaps by acting as an adaptor that promotes recruitment of Sec13/31 to Sec23 thereby enhancing the interaction between the inner and outer COPII coat layers.

Alg2 is a penta-EF-hand Ca^{2+}-binding protein that colocalizes at ERES with p125. When Alg2 is depleted, mammalian Sec31A localization at ERES is reduced, implicating Alg2 in ERES stabilization [73]. This protein has also been found to directly bind to mammalian Sec31A in a calcium-dependent manner [74]. For this reason it has been proposed as a possible regulator of the COPII coat. More recent studies have shown that Alg2 may actually inhibit homotypic fusion of vesicles by binding to Sec31 in a Ca^{2+} dependent manner [75]. In a study examining the impact of lumenal Ca^{2+} on early secretory pathway function, Bentley and colleagues found that an increase in lumenal Ca^{2+} promotes binding of Alg2 to Sec31 thereby stabilizing the coat on the membrane and preventing fusion. These findings present an interesting regulatory mechanism in the early secretory pathway reminiscent of the regulated secretion that has been known to exist in the late secretory pathway. As other potential modes of COPII regulation are explored it will be interesting to find if other regulatory mechanisms are in fact conserved throughout the secretory pathway but simply yet to be observed for ER to Golgi transport.

1-4. Lumenal regulation of COPII function: cargo as active participants

A diverse complement of protein cargo as well as membrane-spanning cargo receptors populate each vesicle formed from the ER. A growing body of work has begun to change the way we view secretory cargo. What once appeared to be inert vesicle passengers are now more accurately described as dynamic vesicle occupants, all with specific requirements for vesicle size, cargo composition and even COPII coat composition. This has opened the field up to consider cargo and cargo receptors as lumenal regulators of vesicular trafficking. The following section will discuss several of these cargo and cargo receptors.

1-4.1 Cargo as antagonists to budding: GPI-APs

Glycosylphosphitdylinositol-anchored proteins (GPI-APs) are a highly abundant class of proteins attached to the lumenal leaflet of the ER membrane by lipid-anchors. These proteins have long been known to segregate into distinct ERES that are depleted for more traditional transmembrane cargoes [76]. More recently, these asymmetrically distributed cargoes have been implicated more directly as barriers to vesicle formation due to their unique topology. This model stems from an unusual rescue of *sec13*Δ lethality observed when any of a number of proteins involved in processing or trafficking of GPI-APs is also deleted. The mechanism behind this **b**ypass-of-**s**ec-**t**hirteen (*bst*) phenomenon remains to be fully explored, but it is thought that ERES concentration of asymmetrically oriented cargoes could create local negative curvature that would oppose the curvature of the COPII coat. Sec13 is thus required to confer rigidity to the coat in order to overcome the membrane bending rigidity associated with GPI-AP enriched membranes (Figure 2A). Intriguingly, the yeast Sec24 isoform, Lst1, is also linked to both GPI-AP trafficking and Sec13. Originally defined as a mutant that was **l**ethal-with-**s**ec-**t**hirteen, Lst1 was subsequently appreciated to be a Sec24 paralog required for trafficking of Pma1 from the ER [33]. Lst1 likely functions as a cargo-specific adaptor that recognizes both Pma1 and GPI-APs [9, 33, 77], both of which likely enrich in discrete ceramide-rich domains during ER export [78]. Lst1-containing vesicles are larger than those purely containing Sec23/24 [34] although it remains unclear whether the change in vesicle size is driven by the adaptor or by the underlying cargo. One enticing possibility is that the large Pma1 complex and the asymmetrically distributed GPI-APs create a burden on the ability of the coat to generate sufficient curvature. Traffic of these vesicles is thus more dependent on Sec13 to contribute structural rigidity, which may be augmented by Lst1 to create a larger diameter bud. Such a model is consistent with siRNA experiments in mammalian cells that depleted Sec13 yet caused no defect in bulk secretion, instead creating a specific block in collagen export, which would also require significant bending capacity for the COPII coat [79].

1-4.2 Direct Coat Regulation by Cargo Adaptors: TANGO1

TANGO1 is a transmembrane protein discovered in *Drosophila* that interacts both with collagen in the ER lumen and Sec23/24 in the cytosol. This topology is reminiscent of a canonical cargo receptor that links a lumenal cargo to the coat. However, TANGO1 itself appears to not be packaged into COPII vesicles, suggesting a different mode of action. The current model suggests that TANGO1, in complex with its binding partner CTAGE5, binds both to collagen and Sec23, precluding recruitment of Sec31 and thereby stalling hydrolysis of GTP on Sar1 in order to extend coat polymerization to package bulky collagen fibers (Figure 2B) [80]. Presumably, once the collagen fiber is fully incorporated into the vesicle, TANGO1 dissociates from both the cargo and the coat and Sec13/31 can bind, stimulating Sar1 GTP hydrolysis and vesicle scission. This novel finding is the first direct evidence of an ER cargo receptor playing a regulatory role in coat formation. Given the ever-growing number of cargo receptors being unveiled, especially with the introduction of high throughput screening, it would be surprising to find that the TANGO complex is the only example of this type of regulation.

Cargo may also be able to regulate COPII vesicle formation through direct binding to Sec24, although evidence for such a phenomenon is scant. Cargo load at the ER can indeed cause redistribution of ERES [58], and lifetimes of COPII components at ERES are influenced by cargo amount [81]. Further, examination of the *sec24-m11* allele reveals that Sec24 plays a vital role in regulating the activity of Sar1 through Sec16, as Sec24-m11 abrogates the ability of Sec16 to inhibit Sar1 GTPase activity in an *in vitro* GTPase assay [38]. Due to the proximity of the m11 mutation to the A cargo binding site on yeast Sec24, and given that Sec24-m11 fails to interact normally with Sec16, it is enticing to suggest that cargo and Sec16 may compete or cooperate for interaction with Sec24 at this site to influence COPII vesicle turnover. However, no direct evidence for cargo acting in such a capacity has been described.

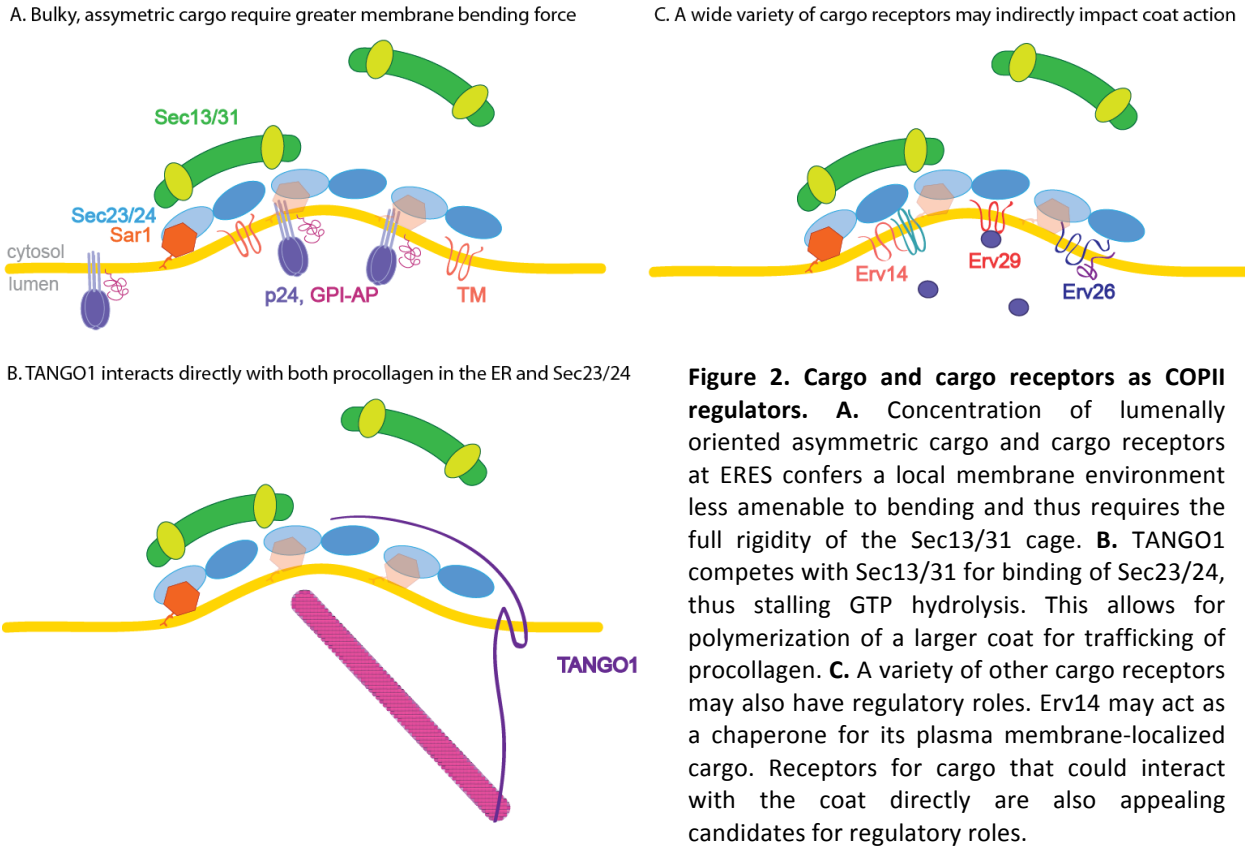

Figure 2. Cargo and cargo receptors as COPII regulators. A. Concentration of lumenally oriented asymmetric cargo and cargo receptors at ERES confers a local membrane environment less amenable to bending and thus requires the full rigidity of the Sec13/31 cage. **B.** TANGO1 competes with Sec13/31 for binding of Sec23/24, thus stalling GTP hydrolysis. This allows for polymerization of a larger coat for trafficking of procollagen. **C.** A variety of other cargo receptors may also have regulatory roles. Erv14 may act as a chaperone for its plasma membrane-localized cargo. Receptors for cargo that could interact with the coat directly are also appealing candidates for regulatory roles.

1-4.3 Additional Cargo Receptors: the ERVs and ERGICs

Canonical cargo receptors function to simply link lumenal cargo molecules to the vesicle coat machinery to ensure efficient capture into nascent vesicles. Examples include yeast Erv29p, which mediates trafficking of multiple soluble cargo proteins [36] (Figure 2C), and mammalian ERGIC53 family members, which facilitate trafficking of glycoproteins [82, 83]. Non-canonical cargo receptors facilitate transport of integral membrane cargoes, which in principle could contain their own ER export motifs. Yeast Erv26p is one such adaptor that mediates ER export of type-II integral membrane proteins. These single pass proteins often have short N-terminal tails with binding signals for trafficking in the late secretory pathway, thus perhaps necessitating the aid of a cargo adaptor for ER exit [84] (Figure 2C). Erv14 is another conserved non-canonical receptor that appears to be required for ER exit of many integral

membrane proteins that ultimately reside in the plasma membrane [85]. A common feature of plasma membrane proteins is that they possess transmembrane domains of greater length than ER or Golgi residents [86]. When such proteins are in transit through the ER, this would create a mismatch between the hydrophobic environment of the lipid bilayer, which is relatively thin in the ER, and the transmembrane domains of the cargoes. One possibility is that Erv14 functions as a chaperone to shield the transmembrane domains of these cargoes and prevent premature degradation that might be triggered by hydrophobic mismatch. Although these cargo receptors may not be representative of canonical, direct regulation, they may be important for modulating coat action indirectly in ways that remain to be explored.

1-5. Conclusions

Our view of COPII coat function is evolving as we appreciate that the basic coat machinery is subject to multiple layers of regulation. Indeed, crosstalk between different regulatory components of the vesicle budding machinery may be essential for proper secretion of a diverse load of cargo at the ER. COPII components themselves are subject to a wide variety of mechanisms that regulate assembly, both temporally and spatially. Beyond the core coat machinery, accessory factors in the cytosol and ER membrane further influence coat dynamics and vesicle formation, through post-translational modification of coat components, direct binding to COPII members, and influencing characteristics of the local membrane environment. Future studies will continue to expand our understanding of how vesicular trafficking from the ER is intricately managed by cellular machinery.

1-6. Addendum to Introduction: Coping with Cargo Diversity

My thesis research has focused on the mechanics of COPII-mediated vesicle formation. I first explored how diverse types of secretory cargo influence the way in which the COPII coat forms a vesicle. Next, I examined how the COPII coat has evolved to facilitate secretion of certain cargo that pose a challenge to sculpting vesicles. Together these findings illustrate how secretory proteins and the COPII coat have co-evolved to maximize efficiency of protein folding and delivery throughout the secretory pathway.

Eukaryotic protein secretion is an essential cellular process conserved from yeast to mammals. Although the complexity of this process varies from single celled organisms to higher eukaryotes, the essential cellular function is the same in all cells: to ensure delivery of proteins throughout the secretory pathway with both temporal and spatial accuracy. The importance of robustly executing a process of this scale is illustrated by the scope of human diseases that result from secretory abnormalities. These disorders occur across tissue types ranging from a congenital form of anemia in the blood to cystic fibrosis in the lung [87]. In single celled organisms, a broad disruption to protein secretion is typically lethal.

A diverse array of proteins are folded and trafficked via the secretory pathway. Various types of protein channels that transport sugars, amino acids or small molecules across the plasma membrane are one example of these diverse cargo. These channels are generally composed of multiple membrane spanning domains that are embedded in the membrane to form a three-dimensional pore [88]. In specialized mammalian cells, large quantities of antibodies constitute the primary secretory cargo. These can be either soluble or membrane bound and depending on the class of antibody can also form ornate structures for optimal antigen recognition [89]. In addition to these manageable secretory clients, over-sized collagen fibrils, that comprise the extracellular matrix of mammalian cells, are also transported via the secretory pathway. Collagen is the most abundant protein in the human body and is trafficked from

the ER as procollagen fibers that can be as large as 300 nm in diameter [90]. With such a heterogenous mix of cargo, the secretory machinery that mediates transport of these cargo must be able to adapt to accommodate the unique requirements of each without disrupting the flow of secretion.

Adaptations to canonical trafficking mechanisms have been explored for cargo, such as collagen, that present a size challenge to vesicular transport. Procollagen fibers and lipoprotein particles called chylomicrons are roughly 3 times the size of a conventional COPII vesicle. However, both types of cargo show dependence on COPII for trafficking [23, 91]. Recent studies on trafficking of procollagen fibers have found that monoubiquitylation of the outer COPII protein Sec31p results in generation of large vesicles and is essential for collagen export [44]. Additionally, binding of a specific adaptor protein called TANGO1 to Sec23p/Sec24p has been identified as a requirement for secretion of collagen VII [24, 80, 92]. These changes to the conventional coat are thought to generate large carriers in two ways. The first is to stall GTP hydrolysis by Sar1p, thus allowing for prolonged polymerization of COPII on the membrane before scission and thus generation of a larger vesicle [93]. There is evidence that membrane scission to release a vesicle is mediated by Sar1p, but the mechanism for this has not yet been determined [6]. The second is by binding adaptor proteins that mediate fusion with ER-Golgi Intermediate Compartments (ERGIC) to create carriers large enough for transport of these difficult cargo [90]. It has also been shown that by altering the geometry of the COPII lattice, vesicle structures can be made that vary in size and flexibility. Altering coat geometry could therefore also contribute to trafficking large cargo [41].

Over-sized proteins are not the only way in which secretory cargo can pose a challenge to trafficking. In yeast many types of multi-spanning membrane proteins are dependent on a cargo adaptor called Erv14p for efficient egress from the ER [94]. Erv14p is a membrane spanning protein that contains a

COPII binding motif as well as domains for binding secretory cargo. Most Erv14p clients are multi-spanning membrane proteins that function in the late secretory pathway [85]. Interestingly, many cargo that are dependent on Erv14p for secretion contain COPII binding signals that allow direct interaction with the coat [95]. Therefore, trafficking of Erv14p clients is mediated by binding of both Erv14p and the client protein to Sec24p. One explanation for this dual binding approach is that multiple signals are required to fully concentrate Sec24p, and generate a robust enough interaction to ensure COPII vesicle incorporation. The multiple-signal requirement may also have evolved to ensure that the coat is only recruited to sites of maximal cargo concentration, to prevent wasting energy on trafficking half-full vesicles. Another explanation is that since these proteins ultimately reside in the plasma membrane, they have longer TMDs than the thickness of the ER membrane. Indeed, it has been shown that the thickness of membranes increases with progression through the secretory pathway and secretory protein TMD lengths reflect this increase [86]. This mismatch of TMD length and membrane thickness could result in exposure of hydrophobic motifs as cargo are folded in the ER. Therefore, in addition to fortifying an interaction with Sec24p, Erv14p could chaperone these exposed hydrophobic regions to ensure such proteins can escape the ER without recognition by the quality control machinery [94, 95]. It is not currently clear if Erv14p functions to enhance Sec24p capture, cargo concentration, cargo chaperoning or a combination of all of these. However, the inability of several Erv14p clients to reach the Golgi in the absence of Erv14p, suggests that multi-spanning membrane cargo present a unique challenge to COPII mediated transport.

Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are another class of secretory cargo with characteristics that require specialized secretory adaptations. GPI-APs are a highly abundant class of proteins that carry out multiple cellular functions primarily at the plasma membrane. In yeast many of

these are involved in cell wall structure and maintenance [96, 97], but in higher eukaryotes GPI-AP family members also include hydrolytic enzymes, receptors and adhesion molecules [98]. Initially translated as single-pass membrane proteins in the ER, the lumenal domain is subsequently transferred to a preassembled glyco-lipid anchor. This is catalyzed by recognition of a c-terminal GPI-attachment sequence by a GPI-transamidase [99]. After covalent attachment to the protein, the GPI anchor undergoes a series of modifications by remodeling enzymes that are essential for ER exit [98].

The GPI anchor inserts the protein into the lumenal leaflet of the ER membrane and consequently GPI-APs are not able to directly bind the cytosolic COPII coat for ER export. Therefore, incorporation of GPI-APs into COPII vesicles is dependent on interaction with a family of ER-Golgi cycling proteins called the p24 proteins [100]. These are single pass type I membrane proteins with small cytosolic tails that bind COPII and have been recently shown to interact with GPI-APs by binding carbohydrate motifs in the GPI anchor [101, 102]. In yeast there are 8 individual p24 family members, Emp24p, Erv25p and Erp1-6, which are thought to form heteromeric complexes by coiled-coiled interactions in membrane proximal lumenal domains [103]. Emp24p and Erv25p are the primary components required for complex stability and function. In yeast, deletion of all 8 p24 genes has illustrated that these proteins are non-essential for viability [104]. However, many GPI-APs are essential and therefore ER egress of these proteins is also essential. Consequently p24 proteins are not required for trafficking of GPI-APs but instead it seems the p24 complex maximizes the efficiency of GPI-AP transport.

Interestingly, depleting p24 proteins results in an array of other phenotypes in addition to delayed trafficking of GPI-APs. The most striking of these is what has been termed the **b**ypass-of-**s**ec-**t**hirteen (*bst*) phenomenon, where depleting p24 proteins allows growth in a strain background containing a normally lethal deletion of *SEC13 [105]*. This suggests that when p24 proteins, and hence GPI-APs, are

depleted from COPII vesicles, the outer COPII protein Sec13p, is no longer essential for vesicle formation. When these proteins are present, vesicles cannot be formed without Sec13p and thus deletion of *SEC13* is lethal. The severity of this phenotype is strongest in the absence of Emp24p and Erv25p due to maximal complex instability, enhanced turnover of remaining p24s and thus near complete p24 depletion from vesicles [106].

A study from our lab sought to determine the full array of gene deletions that allowed growth in the absence of Sec13p, to determine if the phenomenon was specific to p24 and GPI-AP depletion [42]. We performed a high throughput screen in which a strain background containing a *SEC13* deletion was screened against a library of individual strains containing deletions of each of the non-essential genes in *Saccharomyces cerevisiae*. Positive hits were gene deletions that conferred growth when combined with a *SEC13* deletion and thus, like the p24 null strains, were also termed *bsts*. The screen identified a set of seven gene products as core *bsts*. Interestingly all seven are involved in the biogenesis or trafficking of GPI-anchored proteins. Further, we observed that all of the hits from the screen resulted in depletion of proteins from vesicles that have a lumenally asymmetric orientation. GPI-APs are inserted into the lumenal leaflet of the ER and thus the entirety of their mass resides on this side of the ER membrane. The p24 proteins span the membrane but the cytosolic tail is a mere 12 amino acids in length, and therefore the bulk of their mass also resides in the ER lumen. Lumenal asymmetry also explained the one hit from the *bst* screen that was not directly involved in the synthesis or trafficking of GPI-APs: depletion of Erv29p. Erv29p is a cargo receptor for soluble secretory cargo. It is a multi-spanning membrane protein that is able to bind to COPII as well as multiple types of soluble proteins that are secreted from the cell [36]. Erv29p is distributed across the membrane quite evenly, however when bound to varying soluble proteins it also becomes lumenally asymmetric. We proposed that physical

asymmetry across the membrane could create local bending that opposes the direction of vesicle formation. Therefore the presence of these proteins during vesicle formation presents a challenge to the COPII proteins to impose bending and form a vesicle. The transient asymmetry of Erv29p would likely not render the same opposition as the p24 proteins or GPI-APs. In accordance with this reasoning, the growth rescue conferred by *ERV29* deletion in the absence of Sec13p is not as robust as direct p24 and GPI-AP depletion. Therefore, we suggested a model whereby Sec13p is required when difficult cargo are present in vesicles, where it functions to rigidify the coat and facilitate membrane bending and vesicle formation [42].

In further support of a membrane scaffolding function for Sec13p, we showed that fortifying the coat with a mutant form of the Sec13p-binding partner Sec31p also restored viability in the absence of Sec13p [42]. This mutant removes a flexible hinge region of Sec31p, which comprises the binding site for Sec13p, bringing the adjacent domains together. This rigidified mutant, *sec31p-Δhinge*, was viable as the sole copy of Sec31p and importantly was able to confer viability without depletion of potentially burdensome cargo. However, if *sec31p-Δhinge* is grown at a restrictive 37°C its ability to confer viability is decreased. Under these circumstances, weak *sec31p-Δhinge* growth was enhanced by additional depletion of p24 proteins. These data show that vesicle formation is a dynamic interplay between coat composition and cargo composition.

In this thesis I investigate the influence of both cargo and coat composition during COPII vesicle formation. My findings indicate that, amongst ER secretory cargo, p24 proteins pose a unique challenge to forming vesicles and drive the requirement for Sec13p in the outer COPII coat. When the p24 proteins are absent from vesicles, secretion can proceed without Sec13p. This physical burden to vesicle formation is likely exacerbated by the GPI-AP proteins that are escorted into vesicles along with the

p24s. I also explore a potential role for the Sec24p homolog Lst1p in creating vesicles more amenable to accommodating difficult secretory cargo like the p24s. I find that supplementing scaffolding function to the coat can reverse lethality associated with loss of Lst1p function. Next, I present additional evidence of the p24 proteins having a profound influence within vesicles. A curious defect in secretory quality control is observed in the absence of p24 proteins. I show that this defect can be partially reversed by restoring p24s to vesicles and fully reversed by removing Lst1p from the vesicle coat.

The results of my thesis research have led me to conclude that, when efficiently captured in vesicles the highly abundant, lumenally asymmetric p24 proteins carry out multiple functions. The first is to ferry along GPI-APs, increasing the efficiency with which these proteins exit the ER. The second is recruitment of a specialized COPII protein that provides the scaffolding necessary to form a vesicle containing difficult cargo. Finally, by tightly packing vesicles, p24 proteins prevent aberrant secretion of secretory proteins that have not been specifically selected into vesicles. It is becoming increasingly clear that the COPII coat has evolved layers of mechanisms to accommodate changes in secretory cargo, while maintaining faithful secretion. This work highlights a unique secretory cargo that, coordinately with the COPII coat, also adapted over time to enhance the efficiency of protein secretion.

Chapter 2

Traffic of p24 Proteins and COPII Coat Composition Mutually Influence Membrane Scaffolding

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E.L.S contributed the FLIP experiment in figure 2.6 B. C.F.L, A.C, and S.P. contributed yeast strains used. J.C. contributed the spot test in figure 2.7 D and yeast strains. J.G.D contributed all text and remaining experiments.

2-1. Introduction

A recent genome-wide screen performed by Copic et al. found that deletion of *ERV29* was able to rescue lethality of a *SEC13* deletion [42]. This was the one outlier from the rest of the hits from this screen that all directly or indirectly depleted p24 proteins and GPI-APs from vesicles. We posited that, like p24s and GPI-APs, when Erv29p is bound to its soluble secretory cargo the asymmetric, lumenal orientation creates a challenge to local membrane bending. This challenge is met by the rigidity bestowed by Sec13p on the COPII coat [42]. In this study we sought to further explore the impact of secretory cargo on the requirement for Sec13p in COPII vesicle formation. We asked if broad depletion of non-essential cargo could result in Sec13p-bypass viability and identified the p24 proteins as the primary drivers of the *bst* phenotype. This protein family thus presents a unique burden to membrane bending by the COPII coat. We next explored if there was a connection between the challenge p24 proteins present to COPII and their unique affinity for the Sec24p homolog, Lst1p. We propose that, in addition to Sec13p, Lst1p functions to accommodate the burden of p24 proteins in vesicles. Together these COPII proteins bestow properties on the coat to generate vesicles that are better suited to traffic difficult cargo.

2-2. Results

2-2.1 p24 depletion is required for Sec13p bypass

To further probe the hypothesis of bulk lumenal asymmetry resulting in the requirement for Sec13p, we asked if the Sec13p-bypass phenomenon could result from broad cargo depletion from vesicles. To create a greater bulk depletion of cargo from vesicles we tested depletion of double mutants of known and putative ER cargo receptors in a *sec13Δ* null background [82]. In doing so we not only depleted the individual cargo receptors from vesicles but also simultaneously depleted the cargo dependent on that receptor for incorporation into vesicles. We chose to generate the double mutants in query strains

containing deletions of *SEC13* and *ERP1* or *ERV29*. Both query strains exhibit very mild *bst* growth, and therefore we tested for synthetic growth enhancement when combined with a third gene deletion. By assaying for synthetic growth enhancement as opposed to complete rescue we aimed to sensitize the screen and increase the likelihood of novel hits.

Erp1p is a low abundance component of the p24 complex and therefore deletion of this gene only minimally perturbs p24 complex stability. In an *erp1Δ* null background, p24 function remains largely intact [106]. It has been shown that the mild phenotypes observed for Erp1p depletion are exacerbated by additional deletion of *ERP2*, another low-abundance p24 protein, or *ERV29 [42]*. In the query strain deficient for both Sec13p and Erp1p we observed a synthetic growth enhancement with an additional deletion of *ERV14* (Figure 1A, s*ec13Δ erp1Δ erv14Δ*). Erv14p is an ER cargo receptor that facilitates secretion of an array of multi-spanning membrane proteins [94, 95]. We also found that deletion of *ERV14* exacerbated the delay in trafficking of Gas1p, a GPI-AP, in an *erp1Δ* null background (Figure 1B). This assay measures the efficiency of GPI-AP exit from the ER by pulse-chase analysis. Gas1p that reaches the Golgi attains a glycan modification that can be detected as a gel shift to a higher molecular weight when analyzed by SDS-PAGE. Gas1p maturation is measured by the amount of Golgi-modified Gas1p as a percentage of the total at a given time point. In an *EMP24* deletion, where the p24 complex is almost completely destabilized, Gas1p maturation delay is quite severe and less than 60% of pulse-labeled protein is trafficked to the Golgi by 30 minutes. In a WT strain almost all of the pulse-labeled protein is trafficked in this time (Figure 1B). We have previously found that Gas1p trafficking delays correlate strongly with Sec13p-bypass. Because an *ERV14* deletion exacerbated Gas1p maturation delay of an *erp1Δ* null strain, we could not discern if the enhanced *bst* growth, also bestowed by this mutation, was due to Erv14p client depletion or GPI-AP depletion.

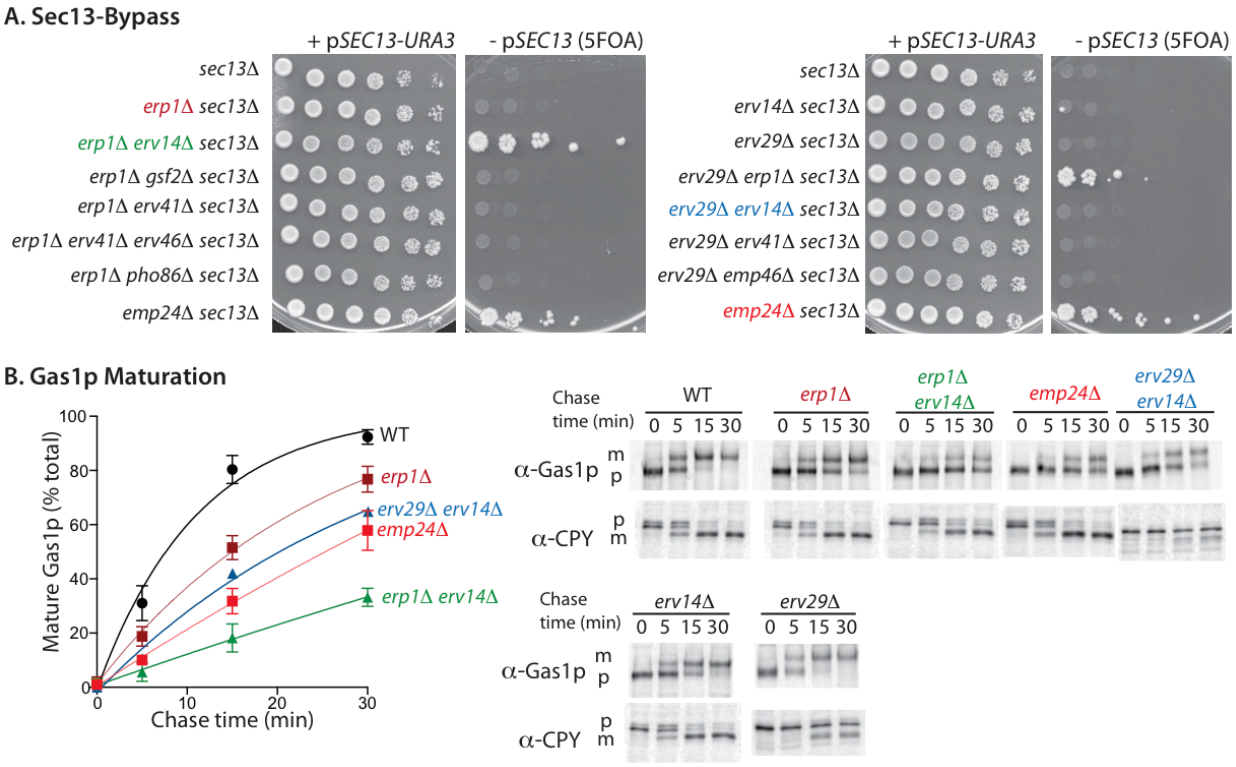

Figure 2.1. p24 depletion is required for Sec13p bypass. (**A**) Strains containing deletions in the cargo receptors indicated in *bst*-sensitized backgrounds - *sec13Δerp1Δ* (left hand side) or *sec13Δerv29Δ* (right hand side) - were spotted as serial dilutions onto media containing 5-FOA to counter-select for the *SEC13-URA3* plasmid and test viability. On standard media (left panels), all strains grew uniformly, whereas growth in the absence of *SEC13* (5FOA, right panels) was sustainable only in the context of an *erp1Δ* background. (**B**) Gas1p trafficking was examined in these strains by pulse-chase analysis. The percent maturation from precursor (p) to Golgi-modified mature (m) forms was quantified by PhosphorImage analysis. Error bars represent SD; n=3-4. Strong Sec13p-independent growth of the *erp1Δ erv14Δ* strain correlated with a robust trafficking delay, compared to an *erp1Δ* single deletant. However, a strong Gas1p trafficking delay did not always correspond to growth in the absence of Sec13p (*erv29Δ erv14Δ*).

The second query strain tested cargo receptor deletions with a strain deficient for Sec13p and Erv29p. Addition of an *ERP1* deletion to this query strain was the only instance in which synthetic enhanced growth was observed (*sec13Δ erv29Δ erp1Δ*). This result had also been reported when the initial screen was published (Figure 1A)[42]. Like Erv14p depletion, the Gas1p trafficking delay of an *ERP1* deletion is exacerbated by *ERV29* deletion and correlated to the strength of *bst* growth (Figure 1B). This suggests that *ERV29* deletion may also disrupt p24 function or GPI-AP trafficking.

Another notable observation from these experiments was that we did not find the reverse correlation between GPI-AP trafficking and *bst* growth to be true. When an *ERV14* deletion was added to the *sec13Δ erv29Δ* background, synthetic enhanced growth was not observed but we found that this strain did display a Gas1p trafficking delay equal to some of the strongest *bsts* (Figure 2B). This also corroborates previous findings of strains with known Gas1p trafficking delays that were not permissive to growth in the absence of Sec13p [42]. These data seem to implicate p24 protein depletion as the primary driver of the Sec13p-bypass phenotype, as we did not observe any growth independent of p24 depletion. Even combined depletion of cargo receptors that both independently exacerbate *erp1Δ* phenotypes did not enhance growth in the absence of Sec13p (e*rv29Δ erv14Δ*).

2-2.2 Local concentration of p24 proteins via COPII interaction drives Sec13p-bypass

If p24 proteins directly present a barrier to vesicle formation, we reasoned that this could be illustrated by altering the local concentration of p24 proteins within the ER membrane. The most abundant p24 proteins, Emp24p and Erv25p, bind to the COPII coat via di-aromatic residues in their short cytoplasmic tails (Figure 2A). Substitution of these motifs with alanines abolishes interaction of p24 cytoplasmic tails with COPII proteins in vitro [102]. We generated these mutants of Emp24p (*emp24-AA*) and Erv25p (*erv25-AA*) and introduced them into strains deficient for Sec13p to examine the impact of locally depleting p24 proteins on the Sec13p-bypass phenotype. Neither mutant could individually confer Sec13p-bypass, but when combined, the double *p24-AA* growth in the absence of Sec13p was as strong as an *EMP24* deletion (Figure 2B). We verified that this was a result of p24 absence from vesicles by visualizing localization of an Emp24p-superfolderGFP fusion protein. This construct inserts sfGFP into the lumenal domain and functions as WT (data not shown). Emp24p-sfGFP localizes to punctate spots throughout the cell in a WT background (Figure 2C, top L). Emp24p-AA-sfGFP is dispersed to the

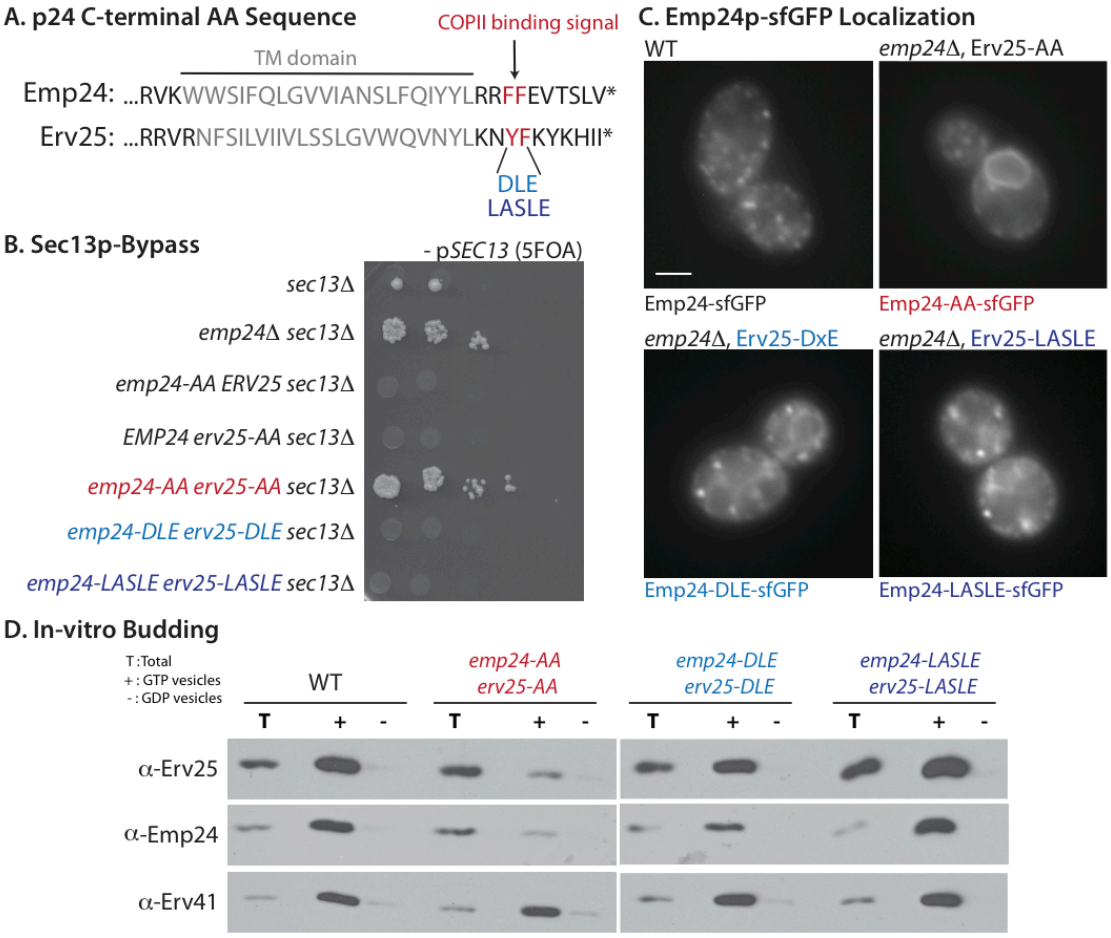

Figure 2.2. Local concentration of p24 proteins via COPII interaction drives Sec13p-bypass. (A) The cytoplasmic tails of Emp24p and Erv25p contain di-aromatic motifs that confer binding to the COPII coat. We substituted these residues with alanine (-AA) or acidic export motifs (-DLE; -LASLE) and examined their effects. (**B**) Individual alanine substitutions in either Emp24p or Erv25p did not confer growth in a *sec13Δ* background, whereas the double mutant strain grew as robustly as an *emp24Δ*. Acidic export motifs reversed this viability. (**C**) Localization of Emp24p fused with superfolder GFP (Emp24-sfGFP) was examined in these backgrounds. In wild-type cells, Emp24p localized to punctate ER exit sites, whereas the alanine double mutant conferred a more diffuse ER pattern; addition of acidic signals restored the punctate localization. Scale bar = 2μm. (**D**) Incorporation of Emp24p and Erv25p into COPII vesicles was tested in vitro. Microsomal membranes were prepared from strains expressing the p24 mutant proteins indicated and used in incubations containing COPII proteins and GTP (+) or GDP (-) that recapitulate vesicle formation. Vesicle fractions were separated from total membranes (T) and all fractions probed with antibodies against the cargo indicated. The alanine-substituted forms of Emp24p and Erv25p were reduced in their capture into COPII vesicles whereas acidic signals restored uptake. Erv41p served as a positive control for vesicle formation.

perinuclear ER suggesting that abrogating COPII binding renders Emp24p unable to concentrate at ERES or leave the ER (Figure 2C, top R). COPII binding efficiency was further probed by an in vitro budding

assay. The double *p24-AA* mutant buds Emp24p and Erv25p less efficiently compared to WT whereas the ER vesicle protein, Erv41p, was captured equivalently in both backgrounds (Figure 2D, L panel). Importantly, both proteins were stably expressed, similar to WT levels (data not shown).

To restore p24 traffic into COPII vesicles we substituted the endogenous COPII binding motif with acidic sorting signals with high Sec24p binding affinity (–DLE, –LASLE) [8]. These mutations reversed Sec13p-bypass growth (Figure 2B), restored punctate localization of Emp24p-sfGFP (Figure 2C, bottom panels), and restored in-vitro budding of Emp24p and Erv25p to WT levels (Figure 2D, R Panel). These data indicate that local capture of p24 proteins into COPII vesicles is central to the Sec13p-bypass phenotype. However we cannot distinguish from these findings whether the effect is specific to p24 proteins or the combined p24 + GPI-AP cargo burden.

2-2.3 p24 mutants with acidic sorting signals partially reverse bst1Δ phenotypes

Delocalization of Emp24p from punctae to diffuse ER has also been observed in a background containing a deletion in *BST1* (Figure 3A, middle panel) [107]. Bst1p is an inositol deacylase required for proper formation of the GPI anchor and was one of the first gene deletions characterized to support growth in the absence of Sec13p [105]. Concentration of p24 proteins at ERES is dependent upon interaction with a fully remodeled GPI-AP. Thus, p24 delocalization in a strain lacking Bst1p likely results from disruption of GPI-AP concentration [107]. Using a Bst1p null strain background to delocalize p24 proteins to the bulk ER, we again tested the local impact of p24 proteins on Sec13p-bypass. When acidic binding signals were attached to Emp24p and Erv25p, deletion of *BST1* no longer caused delocalization of Emp24-sfGFP and punctate localization was restored (Figure 3A, bottom). These signals also partially reversed the Sec13p-bypass growth associated with Bst1p depletion (Figure 3B). In addition to growth in the absence of Sec13p, deletion of *BST1* causes a severe delay in GPI-AP trafficking from the ER as a consequence of

incomplete GPI anchor synthesis. This phenotype was not rescued by the acidic sorting signals (Figure 3C), indicating that the observed reversal of *bst* growth is due solely to restoring p24s and not GPI-APs to vesicles. However, the incomplete reversal suggests that GPI-APs and p24s together likely present the greatest cargo burden with p24s being the primary contributor.

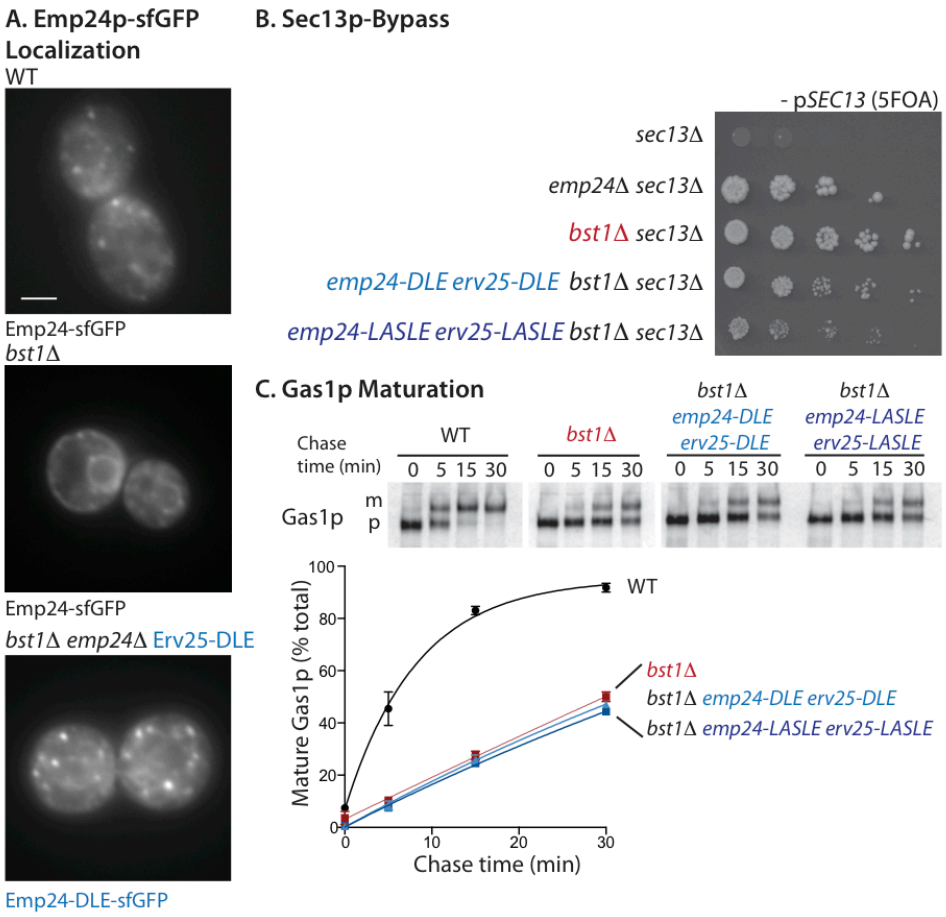

Figure 2.3. p24 mutants with acidic sorting signals partially reverse *bst1Δ* phenotypes. (**A**) Emp24-sfGFP localization was examined in the strains indicated. In a *bst1Δ* background, Emp24-sfGFP redistributed from punctae (WT, upper panel) to disperse bulk ER (*bst1Δ*, middle panel); substituting acidic residues into Emp24p and Erv25p restored punctate localization to a *bst1Δ* strain (*bst1Δ emp24Δ,* lower panel). Scale bar = 2μm. (**B**) The p24 acidic mutants partially reversed the Sec13p-independent growth of a *bst1Δ* strain. (**C**) Gas1p trafficking remained unchanged in all strains containing a *bst1Δ*, strongly supporting a p24 dependence for the Sec13p-bypass phenomenon. Error bars represent SD; n=3.

2-2.4 Altering the Sec24p paralog, Lst1p/Sfb3p, to impair capture of p24 proteins also confers Sec13p-bypass

A subtle but important aspect of adding acidic sorting motifs to p24s is that this drives interaction primarily with Sec24p as opposed to its homolog, Lst1p. The endogenous di-aromatic sorting signals (–FF, –YF) of p24 proteins are able to bind to both coat proteins. The acidic sorting signals –DLE and –LASLE were derived from the Golgi tether Sys1p and the v-SNARE Bet1p respectively. These motifs interact robustly with the same binding site on Sec24p, but do not show evidence of interaction with Lst1p [9]. We sought to determine if biasing binding to a specific COPII cargo adaptor impacts p24 function. If abrogating binding to both Lst1p and Sec24p (*p24-AA*) delocalizes p24s, then deletion of *LST1* might cause partial delocalization since Sec24p interaction remains intact. Instead, in an Lst1p deficient background, Emp24-sfGFP distribution was delocalized, resembling the distribution of the *p24-AA* mutant (Figure 4A). We would therefore also expect deletion of *LST1* to result in robust growth in the absence of Sec13p because p24 proteins appear greatly depleted from vesicles. On the contrary, *LST1* (**L**ethal with **S**ec-**T**hirteen) derives its name from the genome-wide screen in which it was discovered as a gene deletion that enhances lethality of a temperature sensitive *sec13-1* allele [33]. Therefore to query Lst1p involvement in the Sec13p-bypass phenotype we used a mutation in a defined cargo binding site on Lst1p (*lst1-Bsite*) that specifically disrupts binding to p24 proteins but maintains Lst1p integration in the COPII coat [9]. The *lst1-Bsite* mutant also delocalized p24 proteins (Figure 4A, top R) but was not able to grow in a strain bearing a deletion of *SEC13*. However, the *lst1-Bsite* mutant was able to confer Sec13p-bypass in a *sec31-DK* background (Figure 4B). The *sec31-DK* protein contains a point mutation that renders it unable to bind to Sec13p, thereby removing Sec13p from the COPII coat similar to a Sec13p null strain. However, unlike a Sec13p null strain, Sec13p is still expressed in this background and

its other cellular functions such as in the nuclear pore are preserved. Therefore, the *sec31-DK* strain background, while lethal on its own, is healthier overall. In this more robust background, we found that delocalizing p24s via the *lst1-Bsite* mutant, which preserves p24-Sec24p interaction, permitted Sec13p-independent growth. The interpretation of this result is two-fold. First, p24 proteins are partially depleted from vesicles in an *lst1-bsite* mutant background. The second is that the depletion is likely not equal to that of a p24 null background otherwise we would expect the *lst1-Bsite* mutant to be a strong *bst* in a Sec13p null strain. This could be due to interaction with Sec24p, which is able to capture p24 proteins in vesicles as well.

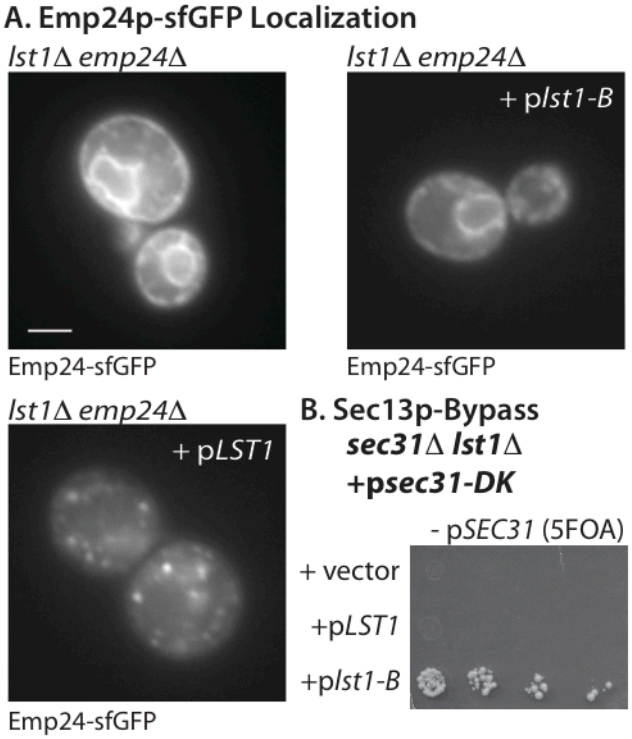

Figure 2.4. Altering the Sec24p paralog, Lst1p/Sfb3p, to impair capture of p24 proteins also confers Sec13p-bypass. (**A**) Emp24-sfGFP localization was examined in the strains indicated. In an *lst1*Δ background, Emp24-sfGFP largely redistributed to the bulk ER. A cargo-binding mutation in the B-site of Lst1p (*lst1-b)* also caused redistribution of Emp24-sfGFP from punctae to bulk ER. Scale bar = 2μm. **(B)** A *sec31Δ lst1Δ* double mutant strain expressing *sec31-DK*, which cannot bind Sec13p, was transformed with the plasmids indicated and growth on 5-FOA was assessed as a measure of Sec13p bypass. The Lst1p-B mutant conferred growth in an otherwise lethal condition.

2-2.5 Lst1p contributes multiple functions to p24 traffic

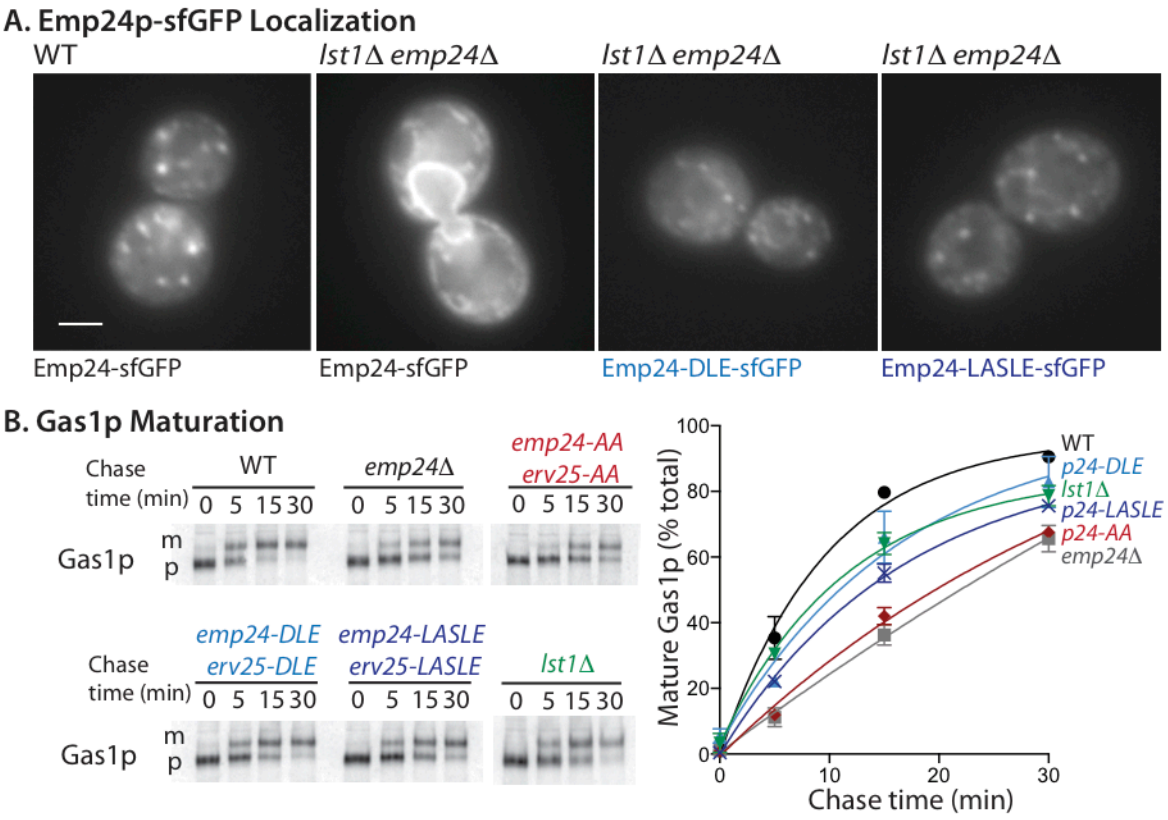

Figure 2.5. Lst1p contributes multiple functions to p24 traffic. (**A**) Emp24-sfGFP localization was examined in the strains indicated. Punctate localization of Emp24-sfGFP in an *lst1Δ* background was rescued by substituting acidic residues into the Emp24p and Erv25p tails. Scale bar = 2μm. **(B)** Gas1p maturation was measured in the strains indicated as described for Figure 1. Alanine substitutions (red) conferred trafficking delays equivalent to that of *emp24*Δ. Acidic signals (blue) partially restored Gas1p trafficking, equivalent to that of an *lst1*Δ (green). Error bars represent SD; n=3-5.

We next tested the ability of Sec24p to substitute for Lst1p in p24 and GPI-AP trafficking. When acidic binding signals were added to Emp24p-sfGFP in an *LST1* deletion strain, localization was restored to punctae, similar to that of a WT strain (Figure 5A, R panels). This confirms that p24–DLE and p24–LASLE predominately associate with Sec24p. A recent publication described a model whereby GPI-AP binding to p24s was required in order for p24s to recruit and bind to Lst1p. This same dependence on GPI-AP binding was not found for p24 interaction with Sec24p. The authors therefore proposed that the p24

proteins do not link GPI-APs to Sec24p and that p24-Lst1p interaction is required for efficient GPI-AP trafficking [101]. Indeed, abolishing interaction with COPII (*p24-AA*) results in a Gas1p trafficking delay equal to that of a p24 null (Figure 5B). However, redirecting p24s to Sec24p (–DLE, –LASLE) results in only a mild delay equivalent to that of a single *LST1* deletion (Figure 5B). This suggests that Sec24p is capable of mediating secretion of GPI-APs via p24 interaction, with a minimal cost to efficiency. If Sec24p is able to traffic GPI-APs, what is the significance of Lst1p in p24 + GPI-AP trafficking?

2-2.6 Sec24p and Lst1p exhibit similar localization and dynamics

One proposed function of Lst1p is to generate large vesicles for trafficking bulky and/or difficult cargo. Visualizing vesicles by electron microscopy that were collected from in vitro budding reactions with Sec23/Sec24, Sec23/Lst1p, or a mixture show that incorporation of Lst1p results in vesicles that are ~10 – 15% larger in diameter [8, 34, 108]. This could explain a partial requirement for Lst1p to traffic p24s and GPI-APs when Sec24p appears capable of trafficking these cargo as well. We have shown that p24 proteins predominantly influence the requirement for Sec13p rigidity in the coat. Perhaps Lst1p provides an additional structural component to the coat to handle difficult cargo. It has been shown that GPI-AP cargo segregate from other types of secretory cargo in vesicles [78, 109]. We posited that if Lst1p creates a unique vesicle population to traffic challenging cargo, which are found in distinct vesicles from other cargo, it might also be present on distinct ERES from Sec24p. We visualized colocalization of Sec24p-mCherry and Lst1p-sfGFP by resolution-limited epifluoresence in WT and *emp24Δ* backgrounds. If the p24 proteins are critical for recruiting Lst1p we imagined its localization might change in the absence of p24s [101]. We found these proteins almost completely colocalized at ERES and this did not change with deletion of Emp24. (Figure 6A). One way in which Lst1p could influence vesicle size is by altering coat dynamics. A larger vesicle requires more COPII proteins and therefore prolonged

association with ERES. We measured turnover of Sec24p-sfGFP and Lst1p-sfGFP in WT and Emp24p null strains by Fluorescence Loss In Photobleaching (FLIP). We did not detect a difference in dynamics between these proteins (Figure 6B) although we did see a decrease in pixel variance of Lst1p-sfGFP from WT to *emp24Δ* backgrounds (Figure 6C). This metric reports the difference between the brightest and darkest spots in an image. Lower pixel variance indicates more Lst1p is diffuse in the cytoplasm in an *EMP24* deletion strain versus tightly bound at ERES. This supports findings that p24 proteins participate in recruitment of Lst1p to ER membranes [101].

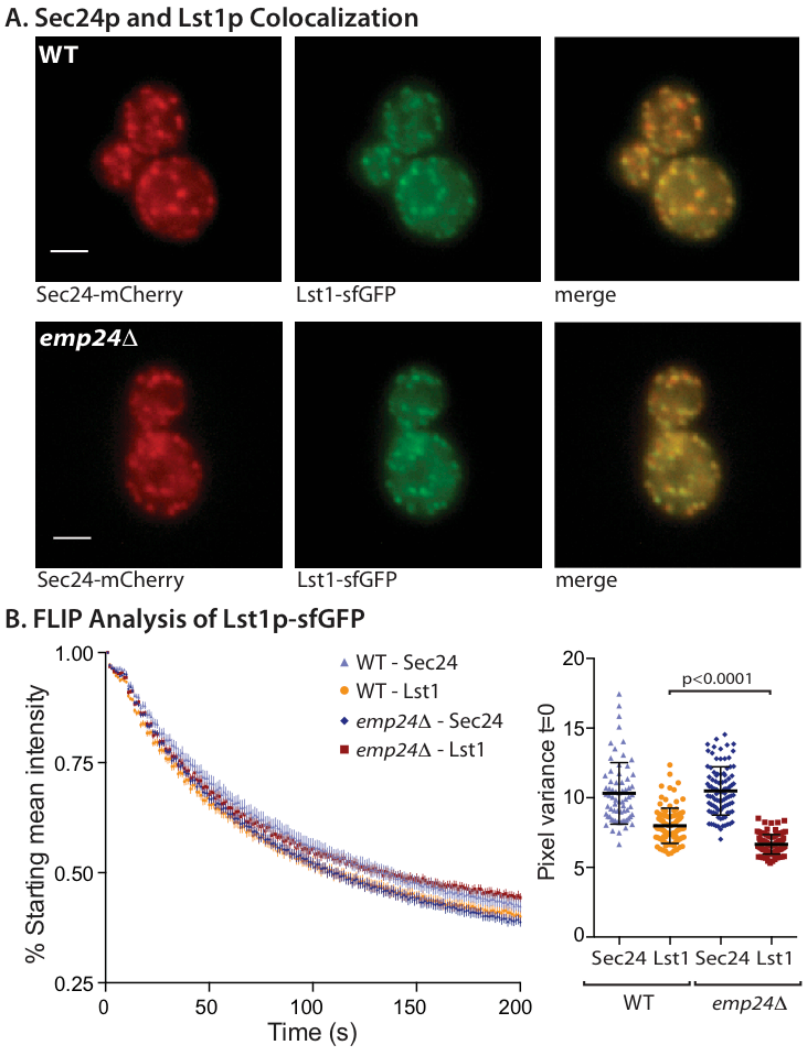

Figure 2.6. Sec24p and Lst1p exhibit similar localization and dynamics. (A) Sec24-mCherry and Lst1-sfGFP appear to largely overlap at ER exit sites in both wild-type (upper panel) and *emp24Δ* (lower panel) cells. Scale bar = 2μm. **(B)** Dynamics of Sec24-sfGFP and Lst1-sfGFP were assessed using Fluorescence Loss in Photobleaching. No significant difference in fluorescence diminution was observed between Sec24p (blue) and Lst1p (red) in either WT or *emp24*Δ backgrounds. Error bars represent SEM; n=12-15. **(C)** Pixel variance, a measurement of the difference between the brightest foci and the dimmest background signal, showed a significant difference between WT and *emp24*Δ cells for Lst1-sfGFP (red) but not for Sec24-sfGFP (blue). Error bars represent SD; n=69-98.

2-2.7 Membrane Sscaffolding by Sec31p can substitute for Lst1p

An additional mechanism for Lst1p function could be that, like Sec13p, Lst1p adds structural rigidity to the coat to accommodate difficult cargo. In this case we would expect that an *lst* phenotype could be rescued by adding a rigidified *sec31p-Δhinge* mutant similar to what we have observed for a *bst* mutation [42]. We first validated our model that *sec31p-Δhinge* rigidifies the coat with a series mutants that add 5, 10 or 20 *GS* – linkers to the excised region of *sec31p-Δhinge* (Figure 7A). These linkers add back flexibility without adding specificity and therefore will not bind Sec13p. The *sec31p-GS* linkers exhibit a gradient in growth defects, with the *sec31p–GS5* being the most robust and *sec31p–GS20* the least robust (Figure 7B). This strongly suggests that restoring flexibility to *sec31Δ-hinge* decreases its ability to compensate for the rigidity deficit of a *SEC13* deletion.

We have previously shown that an exogenous copy of *sec31p-Δhinge*, expressed in addition to the WT chromosomal copy, rescues lethality in a Sec13p null strain [42]. We next tested if an exogenous copy of *sec31-Δhinge* could also rescue lethality in an *lst1Δ sec13Δ* null double mutant or *bst1Δ lst1Δ sec13Δ* null triple mutant background. Deletion of *LST1* reverses the bypass growth of a *BST1* deletion in a Sec13p null background. Therefore *lst*-lethality is dominant to *bst*-rescue, suggesting that whatever Sec13p functions a *bst* mutation alleviates are no longer sufficient for viability when Lst1p is also removed (Figure 7C, left panel). Exogenous *sec31p-Δhinge* did not rescue the double Sec13p, Lst1p null mutant, however viability was restored by addition of a *BST1* deletion to this strain (Figure 7C, right panel). Notably these strains contain chromosomal copies of wild type *SEC31*, which could potentially dilute the rigidifying effect of the plasmid-borne copy of *sec31-Δhinge.* We therefore tested for rescue of Lst1p depletion with *sec31-Δhinge* as the sole copy of Sec31p, which also does not bind Sec13p. We found that in this background the rigidified Sec31p does in fact rescue an *LST1* deletion whereas a non-

rigidified Sec31p that cannot bind Sec13p (–DK) does not (Figure 7D). Altogether we observe that synthetic lethality of depleting Sec13p and Lst1p can be rescued by depletion of troublesome cargo (*bst1Δ*) and/or rigidifying the COPII coat (*sec31-Δhinge*). We propose that these findings point toward a role for Lst1p in coat scaffolding to mediate trafficking of cargo that pose a burden to vesicle formation.

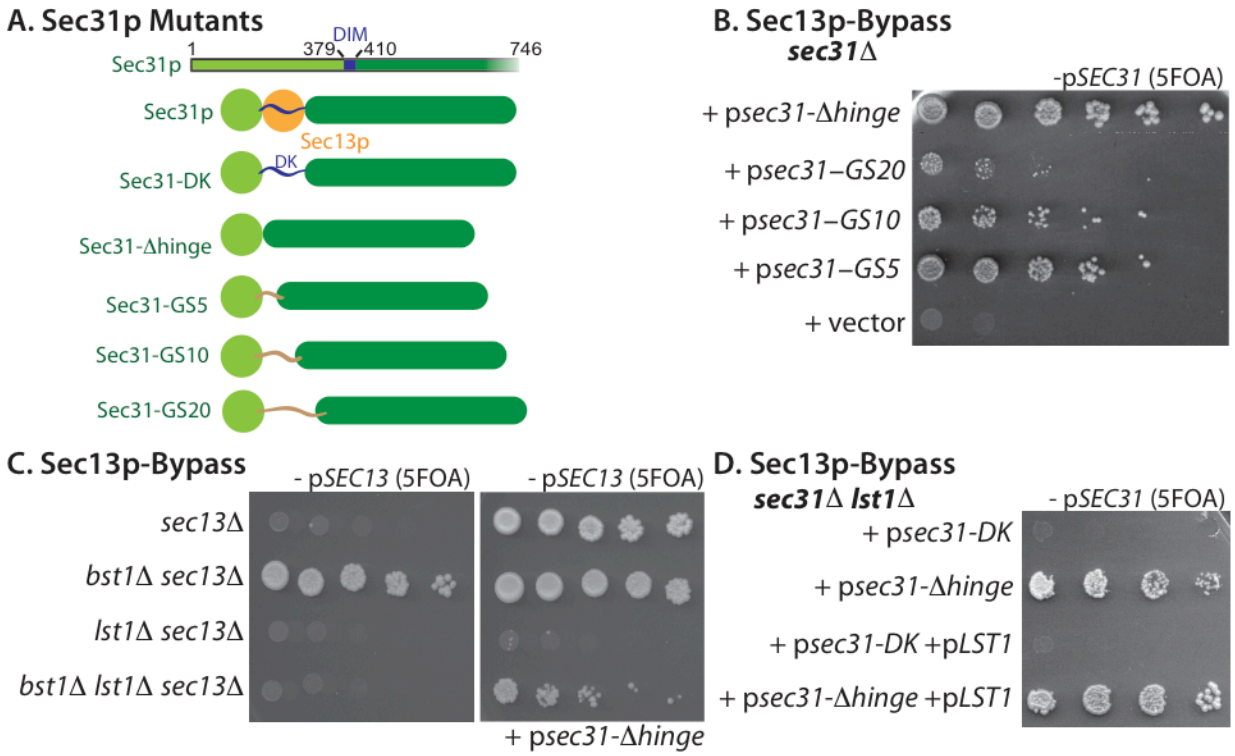

Figure 2.7. Membrane scaffolding by Sec31p can substitute for Lst1p. (**A**) Diagram of the domain structure of Sec31p (top panels), showing the domain insertion motif (DIM) that completes the β-propeller domain of Sec13p; numbers indicate amino acid residues. Sec31-DK contains two substitutions (W386D and A387K) that perturb Sec13p binding. Sec31-Δhinge contains a deletion in the entire DIM plus a flanking flexible domain. Sec31-GS mutants contain 5, 10 or 20 amino acid insertions of glycine-serine repeats. These mutants are also unable to bind Sec13p. **(B)** A *sec31*Δ strain expressing the hinge mutants indicated was tested for growth on 5FOA, revealing a gradient of viability that decreases as the length of the GS linker increases. **(C)** Epistasis analysis of Sec13p-bypass and Sec13p-lethal mutations suggests lethality is dominant: deletion of the Sec24 paralog, *LST1/SFB3*, reversed the viability associated with a *bst1Δ sec13Δ* strain (left panel). A plasmid encoding Sec31-Δhinge was introduced into these strains (right panel) and reversed the dominant lethality of *lst1*Δ in the *bst1Δ lst1Δ sec13Δ* strain. (**D**) In a *sec31Δ lst1Δ* double mutant strain, expression of Sec31-Δhinge conferred growth when both Sec13p and Lst1p were absent from the COPII coat, a normally lethal condition (*sec31-DK*).

2-3. Discussion

ER-derived vesicles sculpted by the COPII coat must be able to accommodate a diverse set of protein cargo. It is easy to imagine that physical properties might influence vesicle formation, when we consider that this process is carried out simply by polymerization of the COPII proteins onto a virtually planar ER membrane. However, the known dependence of some of the most challenging cargo (procollagen, chylomicrons) on COPII for ER exit indicates that the coat must have mechanisms to dynamically adapt to cargo composition without disrupting the flux of protein secretion. We propose that vesicle formation is a stochastic process with inherent flexibility as coat and cargo composition are coordinately optimized for membrane bending and vesicle formation.

One element of this adaptability is illustrated by the Sec13p-bypass phenomenon. We had previously shown that Sec13p becomes non-essential for vesicle formation by depletion of proteins involved in trafficking and synthesis of GPI-APs, as well as a soluble cargo receptor Erv29p. Here we sought to further define this phenotype. GPI-APs are a highly abundant class of proteins that utilize the p24 complex as cargo receptors to pair with COPII and mediate efficient trafficking. We reasoned that several features of these proteins create a local burden to membrane bending and thus explain the requirement for Sec13p rigidity when present: high abundance, predominance in vesicles and largely lumenal orientation. Therefore, an alternative bulk depletion of proteins enriched in ER vesicles may have a similar effect. Indeed Erv29p, a receptor for soluble cargo, also cycles in vesicles between the ER and the Golgi and is mostly lumenal in distribution across the membrane. We tested for Sec13p-bypass by broad depletion of ER cargo receptors, and did not find any gene deletions that rescued lethality independently of p24 depletion. The two deletions we found to enhance weak growth of an *erp1Δ* null strain (*ERV29* and *ERV14*) also exacerbated delay of GPI-AP trafficking. This might lead us to implicate

GPI-AP cargo as the primary cargo burden requiring Sec13p. However, we also observed severe GPI-AP trafficking delays, indicating significant depletion of GPI-APs from vesicles (eg: in *erv29Δ erv14Δ*) that did not confer Sec13p-bypass growth. Given this finding, we sought to test if p24 depletion drives Sec13p-independent growth by altering the local concentration of p24 proteins in vesicles. Utilizing mutations that disrupt COPII binding we found that redistributing p24 proteins from vesicles to the bulk ER was sufficient for robust growth in the absence of Sec13p. Restoring p24 localization with acidic sorting motifs that bind to Sec24p reversed the *bst* rescue. Furthermore, we have shown that while depletion of GPI-APs from vesicles remained constant, restoring p24s with acidic sorting motifs (-DLE, -LASLE) partially reversed Sec13p-bypass viability. This specifuc requirement of Sec13p by p24 proteins suggests that these proteins pose a challenge to the COPII coat for vesicle formation. Notably the reversal of Sec13p-bypass growth was not complete, suggesting thaat perhaps the combined depletion of p24s + GPI-AP cargo allows for total bypass phenotype.

When p24 proteins are restored to vesicles by acidic sorting motifs, they bind only to Sec24p, which could also explain the incomplete reversal of *bst* growth in this background. The endogenous p24-binding motif allows interaction with both Sec24p and Lst1p. The Sec24b-site, where p24 proteins are thought to bind on Sec24p, is also a binding site for many other cargo proteins. These include but are not limited to v-SNAREs, multi-pass membrane channels and cargo receptors ferrying other varieties of secretory cargo into vesicles [9]. Therefore, it is not hard to imagine that when competing for the same binding site on Sec24p, p24s and GPI-APS are not concentrated into vesicles to the same degree as when they are able to utilize both Sec24p and Lst1p for ER exit. In support of this explanation, we find that when p24s cannot bind Lst1p, and therefore traffic by Sec24p alone, growth in the absence of Sec13p is observed (Fig 4B, p*lst1-B*) and Gas1p trafficking operates at a small but significant deficit (Figure 5B,

lst1Δ). Both of these findings are evidence that the p24 proteins are at least partially depleted from vesicles when they bind only to Sec24p compared to when they retain their endogenous binding motif and bind both Sec24p and Lst1p. Therefore, incomplete *bst* reversal of the p24-LASLE/DLE may be a result of partial restoration of p24 proteins to vesicles, as well as combined GPI-AP and p24 burden.

A recent study by Manzano and colleagues has characterized the interaction between p24s, GPI-APs, and Lst1p by crosslinking and immunoprecipitation (IP) experiments. These authors show that when p24s are bound to GPI-APs they have an increased affinity for binding Lst1p compared to when they are not able to bind to GPI-APs [101]. They also observe that p24 binding to Sec24p is not influenced by the presence or absence of GPI-AP binding and that Lst1p, but not Sec24p, IPs with GPI-APs when crosslinked. This suggests that the p24-Lst1p interaction may be optimized to ensure incorporation of GPI-APs into vesicles made with Lst1p. Both COPII proteins are able to IP with p24s, confirming that p24 proteins do interact with both cargo adaptors [101]. From these data Manzano et al. propose that once bound to GPI-APs, p24 proteins recruit Lst1p specifically to ERES to mediate trafficking of GPI-APs.

The p24 proteins are not the only secretory cargo able to bind to Lst1p. Pma1p, a highly abundant plasma membrane ATPase, and Erv29p can also traffic via Lst1p. This raises the question if they also drive Lst1p recruitment [8]. Our observation that Lst1p localization is not dramatically impacted by *EMP24* deletion suggests this is a possibility. Further, if Lst1p is optimal for trafficking p24s and GPI-APs, as Manzano and colleagues suggest, this raises a number of questions about the p24 interaction with Sec24p. Chiefly, why is Gas1p trafficking only mildly delayed in the context of an *LST1* deletion? In our analysis of p24 function we have also found that GPI-AP trafficking is severely delayed in a *sec24p-Bsite* mutant (data not shown). This is the opposite of what we would expect based on the biochemical data from Manzano et al. If Sec24p is capable of trafficking p24s and GPI-APs why traffic by Lst1p at all? A

simple explanation is that *LST1* is a redundant isoform that arose by duplication and alleviated trafficking demands by removing p24 cargo burden from a subset of vesicles. In this scenario Lst1p increased cellular fitness and was thus sustained. Residual Sec24p binding could be vestigial or was maintained because division of labor proved the best way to accommodate the p24 cargo burden. Experiments to measure binding kinetics or other analysis will be necessary to understand if p24 proteins favor one COPII coat adaptor over the other and what implications this has for vesicle formation.

However, there is mounting evidence that suggests the role of Lst1p is more complex than just a redundancy of Sec24p function. First, Lst1p has specificity for a subset of secretory cargo that can be described as difficult cargo. The p24 proteins are highly abundant and have been shown to drive a requirement for Sec13p rigidity. Pma1p is a plasma membrane ATPase that is similarly dependent on Lst1p for export. Pma1p is one of the most abundant secretory proteins in the cell and has also been found to oligomerize into complexes as large as dodecamers prior to exiting the ER [108]. Additionally, Lst1p incorporation into the coat has been shown to create vesicles that are on average ~15% larger than when Sec24p is incorporated alone [34]. It has thus been proposed that Lst1p is a specialized COPII cargo adaptor capable of generating larger or more structured vesicles. Here we present evidence supporting a role for Lst1p in creation of a specialized COPII coat. Removing Sec13p from the coat by preventing binding with Sec31p (*sec31-DK*) is a lethal mutation, similar to deletion of *SEC13*. The rigidified *sec31-Δhinge* rescues this lethality as the sole copy of Sec31p in the cell [42]. Additional deletion of *LST1* in this background, known to exacerbate Sec13p loss of function, is also rescued by the *sec31-Δhinge*. If added as an extra copy of Sec31p, exogenous *sec31-Δhinge* is presumably incorporated into the coat at ~50%. This is sufficient to rescue lethality of Sec13p depletion but cannot rescue

lethality of deleting both *SEC13* and *LST1*. However, in this background addition of a *BST1* deletion, depleting p24 proteins and GPI-APs from vesicles, rescues growth. A *BST1* deletion alone cannot rescue lethality in the absence of Sec13p and Lst1p. Therefore in this background we find that the combined effect of partially rigidifying the coat and cargo depletion can confer growth in the absence of both Sec13p and Lst1p. Finally, we have found that providing extra copies of Sec24p does not rescue in any of these backgrounds (data not shown). This strongly indicates that Lst1p performs a distinct function from that of Sec24p, and more intricate than simply providing an alternative option for cargo binding.

To further explore the function of Lst1p in the COPII coat a deeper understanding of the protein itself and how it interacts with the rest of the coat is necessary. Comparative structural studies of Sec23p/Lst1p and Sec23p/Sec24p could certainly shed light on possible differences between these homologs. Cryo-EM studies of full COPII coats with Lst1p, Sec24p, or both incorporated would be a logical next step in this analysis. Additionally examining the outer cage could be quite insightful as well to determine if the presence of Lst1p somehow alters the geometry of the coat as a whole. Finally studies to further explore cargo segregation and potential segregation of COPII cargo adaptors would elucidate if the effects of Lst1p act on a per vesicle basis or averaged across all vesicles. This may be achievable by microscopy as super resolution techniques continue to improve.

Chapter 3

p24 Function in Vesicle-related Quality Control

Author Contributions:

J.G.D contributed all text and experiments.

3-1. Introduction

Quality control mechanisms in the endoplasmic reticulum (ER) are essential for maintaining homeostasis of protein secretion. There are multiple pathways that comprise ER quality control (ER-QC) and ensure that protein secretion is not incapacitated by errors during protein translation, translocation and folding. Continuous secretion of membrane and proteins is essential for cellular function and growth [110]. Thus quality control mechanisms that ensure the fidelity of this process are of equal importance to viability. The significance of this regulatory mechanism is reflected in the over 100 human disorders that can be attributed to malfunctions in protein folding and/or ER quality control [111].

When proteins fail to attain proper conformation, ER-QC functions primarily in a two-fold approach. The first is ER-associated degradation (ERAD), which integrates with protein folding and trafficking to prevent secretion of aberrant secretory cargo that could potentially wreak havoc on essential cellular processes [112]. Central to ER-QC is accurate recognition of terminally misfolded proteins and exclusion from Golgi bound vesicles. This recognition is tightly regulated, as many of the hallmarks of a misfolded protein, such as exposure of hydrophobic residues, are also characteristic of nascent proteins in the process of folding. To this end it appears that there are multiple mechanisms for ERAD substrate recognition including glycosylation, binding by specific adaptor proteins, signal-mediated adaptor recognition and likely detection of hydrophobic lesions [113]. ERAD also maintains homeostasis of the ER by preventing build-up of misfolded proteins. Misfolded proteins that cannot be corrected are retro-translocated across the ER membrane into the cytosol, ubiquitinated and then degraded by the cytoplasmic proteosome [114, 115].

The second broad arm of ER-QC, the unfolded protein response (UPR), is activated when the build-up of misfolded proteins saturates and overwhelms the ERAD pathway. Accumulation of misfolded proteins

triggers an ER transmembrane sensor called Ire1p to oligomerize. Oligomerization activates Ire1p to cleave an mRNA transcript, *HAC1.* The spliced transcipt codes for a transcription factor, Hac1p, that upregulates targeted gene products to alleviate ER stress. Ire1p recognizes accumulation of misfolded proteins by direct binding of a conserved lumenal domain (cLD) to exposed hydrophobic and basic residues. Binding of hydrophobic lesions to the cLD also promotes oligomerization of Ire1p, augmenting as well as activating the UPR [116]. The UPR results in up-regulation of translocation machinery, ER chaperones, ERAD components, vesicle budding machinery as well as many other targets to ultimately restore ER homeostasis [117].

As the details of protein quality control continue to be revealed it is becoming clear that this is a multi-faceted, highly intricate cellular process. There are many layers of regulation in addition to the canonical ERAD and UPR pathways. For example, it has been shown that upon induction of ER-stress, misfolded GPI-APs that are normally ER retained by chaperones are secreted from the ER. They are delivered to the plasma membrane from where they are internalized and ultimately degraded by the lysosome. This mechanism alleviates the build up of misfolded proteins in the ER allowing for a more rapid return to homeostasis [118]. As eukaryotes evolved, additional mechanisms such as this one arose, affording increased plasticity to accommodate an expanding load of diverse protein substrates. It is therefore quite possible that many important aspects of ER-QC are subtle changes, selected over time for the added fitness conferred by alleviating constraints on secretion.

In yeast, deletion of a single p24 gene results in an array of phenotypes. In chapter 2 I characterized how absence of p24 proteins results in delay of GPI-AP trafficking as well as the bypass-of-sec-thirteen (*bst*) phenotype. In addition to these phenotypes, p24 null strains also exhibit aberrant secretion of misfolded proteins, which are normally ER-retained and degraded, and secretion of the ER resident chaperone

Kar2p. A similar defect has also been observed in *C. elegans* where misfolded GLP-1 and LIN-12 are secreted when p24 activity is decreased [119]. Aberrant secretion is indicative of a breakdown in the quality control mechanisms that are in place to strictly regulate what is incorporated into vesicles.

A previous study in our lab sought to shed light on the QC breakdown associated with secretion of Kar2p. This study performed a high-throughput screen and measured the amount of aberrant Kar2p secreted from strains that each contained a deletion of a non-essential gene in the *Saccharomyces Cerevisiae* genome. The authors found that strains containing deletions in p24 genes had the highest Kar2p secretion index values [120]. These findings indicate that a breakdown in ER-QC occurs when p24 proteins are absent. This could potentially be explained by the finding of Satpute and colleagues, of intentional secretion of misfolded GPI-APs during ER stress. However, it is unclear if deletion of p24 proteins causes ER stress. There have been conflicting reports on depletion of p24s activating the UPR. Deletion of all 8 p24 family members in yeast was not reported induce UPR activation [104]. Whereas another group tested single mutants for UPR activation using the same assay and observed a UPR response [121]. To clear up this discrepancy I measured aberrant secretion in *p24*Δ null strains in a *HAC1* deletion background (data not shown). I found significant secretion remained, independent of a UPR response. This suggests that if p24 proteins indeed play some part in ER-QC, as the quality control breakdown phenotypes indicate, it may be a more passive role that is not intertwined with conventional ER-QC mechanisms.

Here we have generated additional p24 mutants to further probe the role of p24 proteins in vesicular trafficking. We sought to determine the specific p24 functions associated with the diverse phenotypes observed in p24 null strains. We also examine the quality control breakdown phenotypes of p24 mutants in yeast by examining levels of Kar2p secretion in several p24 mutant backgrounds. Our data

support a model for p24 proteins acting as molecular ballasts in vesicles. Due to their unusually high abundance and bulk lumenal orientation, the presence of p24 proteins in vesicles prevents proteins that are not directly or indirectly selected by the COPII coat from being erroneously secreted. Finally, we find that in addition to p24 occupancy in vesicles, the composition of the COPII coat also impacts aberrant secretion. In the absence of Lst1p, aberrant Kar2p secretion is reversed in p24 mutant backgrounds. We propose that, similar to the Sec13p-indepent growth, aberrant secretion is mutually influenced by the presence of p24s in vesicles and Lst1p in the COPII coat.

3-2. Results

3-2.1 Mutating the TM domains of Emp24p disrupts p24 function

The p24 proteins are type I single pass transmembrane (TM) proteins, that function in heteromeric complexes cycling between the ER and the Golgi. They contain small cytoplasmic, C-terminal tails of approximately 12 amino acids that enable interaction with the COPII coat, as discussed in Ch 2. The helical TM domains of p24s have been speculated to serve multiple functions. There are polar residues contained within the membrane domains that were suggested to mediate oligomerization of the p24 complex [103]. Additionally, the p24 complex is known to mediate trafficking of a class of lipid-anchored proteins and hence the transmembrane domains have been speculated to facilitate interaction with the lipid anchor of this cargo. It has since been found that in fact the lumenal domains of p24 proteins mediate both of these functions. Oligomerization of the p24 complex has been shown to occur, at least in part, by coiled-coil interactions of a membrane-proximal region of the lumenal domain. GPI-AP association is mediated by binding of p24 proteins to a carbohydrate group in the GPI anchor that also lies just below the membrane [101, 103].

If the TM domains do not contribute to these key p24 functions, we were interested in further probing how/if the amino acid composition of the TM domain impacts p24 function. We generated three mutants of *EMP24* in which leucine substitutions replaced the entire TM domain (*emp24p-M1*), the N-terminal half (*emp24p-M2*) or the C-terminal half (*emp24p-M3*) (Figure 1A). We introduced these mutants into strains as the sole copy of Emp24p and characterized them by testing protein and complex stability, and assessing the canonical p24 loss-of-function phenotypes: Gas1p trafficking delay; growth in the absence of Sec13p; and aberrant Kar2p secretion. In concert with these mutants we tested the single COPII binding mutant of Emp24p, for direct comparison of the new mutant phenotypes to a mutation we had already largely characterized (*emp24p-AA*).

Belden and Barlow first observed that Emp24p and Erv25p formed a complex by showing that levels of Erv25p were drastically reduced by depletion of Emp24p [122]. Since then it has been shown that Erp1p and Erp2p expression levels are also sensitive to stability of Emp24p, leading to the conclusion that these four proteins are able to form a complex consisting of a dimer of dimers [106]. We therefore performed western blots of both proteins to determine the stability of the Emp24p mutants and Erv25p in the mutant strain backgrounds. We did not find that any of the Emp24p TM mutants were unstable or degraded. Therefore, it is likely that all mutants are effectively transcribed and inserted into the membrane since each maintains the native ER signal sequence. However, when we examined stability of Erv25p in these strains we found that expression of *emp24p-M1* and *M3*, constituting a full TM or C-terminal half substitution, resulted in slightly decreased levels of Erv25p (Figure 1B). This suggests that in these two mutant strain backgrounds, although Emp24p is expressed, it does not efficiently bind to Erv25p. The *emp24p-M1* strain also exhibited strong growth in the absence of Sec13p as well as a strong delay in trafficking of Gas1p (Figure 1C, D; *M1*). The strength of these p24 loss-of-function phenotypes in

this mutant is comparable to an Emp24p null. The *emp24p-M3* strain displayed more mild phenotypes. This strain could not confer growth in the absence of Sec13p, however it did exhibit a defect in Gas1p trafficking, albeit not as severe as *emp24p-M1* (Figure 1C, D; *M3*).

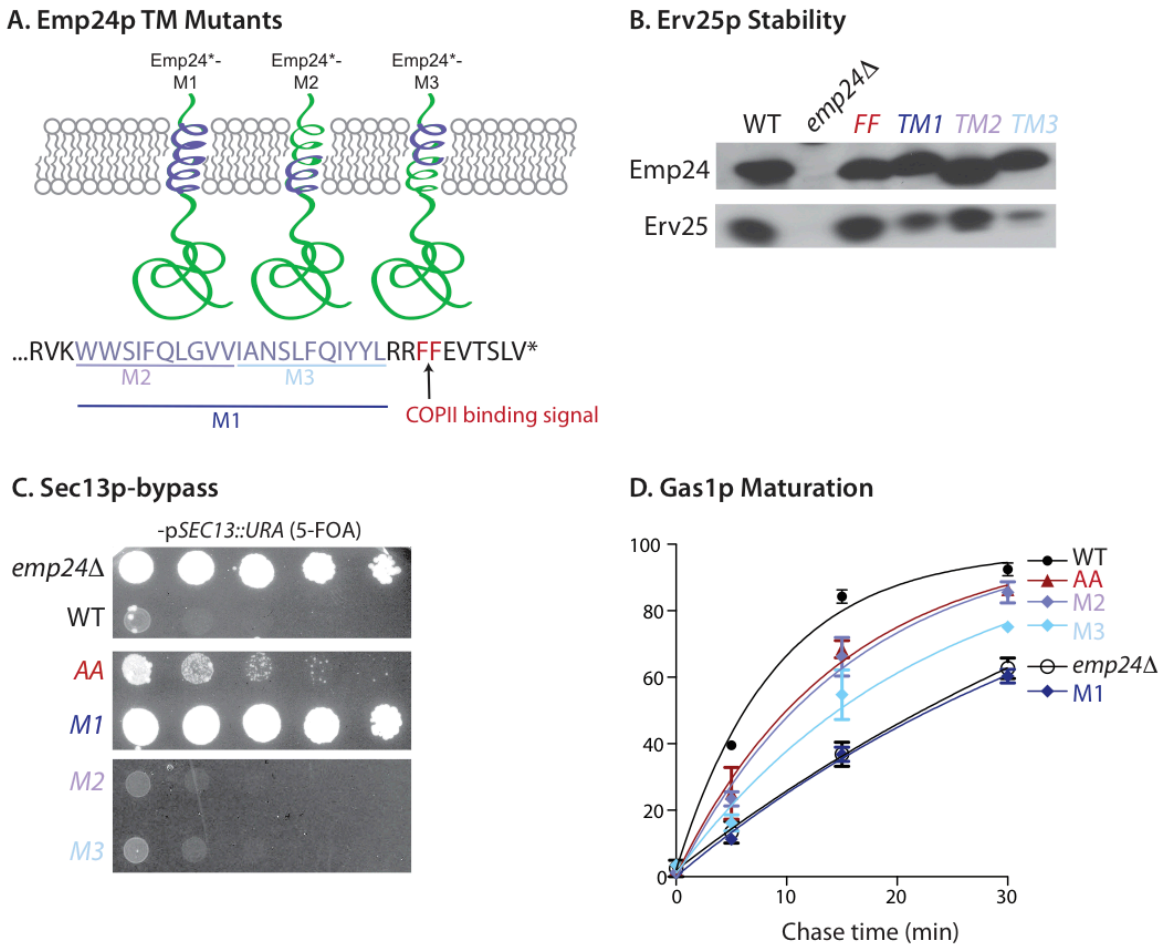

Figure 3.1. Mutating the TM domains of Emp24p disrupts p24 function. (A) Emp24p has a single TM domain of 21 amino acids. TM mutants of Emp24p were made by leucine substitution of the entire TM domain (*M1*), ten amino acids from the N-terminal half of the TM domain (*M2*) or eleven amino acids from the C-terminal half of the TM domain (*M3*). **(B)** Western blots were performed for the strains indicated to examine stable protein expression of Emp24p and Erv25p. Erv25p protein stability was decreased in the *emp24Δ*, *M1* (full TM sub.), and *M3* (C-term TM sub) strain backgrounds. **(C)** Growth was measured in the absence of Sec13p for the strains indicated. The *emp24p-M1* mutant strain conferred robust growth comparable to an *emp24Δ*. The *emp24p-AA* strain conferred mild *bst* growth, whereas the other TM mutants did not confer growth in the absence of Sec13p. **(D)** Gas1p maturation was measured by pulse chase analysis in the strains indicated, as previously described. The *emp24p-M1* strain was again comparable to the *emp24Δ* and exhibited a significant trafficking delay. The N-terminal substitution (*M2*) and the alanine (*AA*) mutants of Emp24p only conferred mild Gas1p trafficking delays. The C-terminal substitution (*M3*) strain exhibited a moderate delay. SD; n=3.

The other two mutants disrupted p24 function to a lesser degree. Erv25p expression was stable in strain backgrounds containing either the N-terminal TM substitution mutant (*emp24p-M2*) or the COPII binding deficient mutant (*emp24-AA*) (Figure 1B). Both of these strains also exhibited a very mild delay in Gas1p trafficking (Figure 1D). However in this particular experiment the *emp24p-AA* strain could support mild growth in the absence of Sec13p whereas the *emp24p-M2* mutant could not confer growth (Figure 1C). The Sec13p-bypass phenotype of the *emp24p-AA* mutant fluctuates between rescue and non-rescue based on small variations in temperature. In Ch 2 we reported that single mutations in the COPII binding motif of Emp24p or Erv25p could not confer growth in the absence of Sec13p. It is perhaps not surprising that the *emp24p-AA* mutant would be on the tipping point of this phenotype since we find the double *p24-AA* mutant exhibits robust Sec13p-bypass growth. The key observation here is that under these experimental conditions an *emp24p-AA* mutant confers weak Sec13p-bypass whereas neither the *emp24p-M2* nor the *emp24p-M3* mutants support any growth.

3-2.2 Depleting p24 proteins drives aberrant Kar2p secretion amongst cargo receptor null strains

To further probe the mechanism behind these findings we tested for a correlation between the p24 phenotypes already examined and Kar2p secretion in these mutants. To measure Kar2p secretion from yeast, a nitrocellulose membrane is applied directly to yeast colonies that were spotted and grown on agarose + rich medium. Kar2p that has escaped the ER is secreted into the extracellular space and binds the membrane, which is subsequently immunoblotted for Kar2p (Figure 2A). The degree of Kar2p secretion of the Emp24p TM mutants and the COPII binding deficient mutant closely correlated with the Gas1p trafficking delay and Sec13p-independent growth of each. The *emp24p-M1* strain secreted Kar2p as robustly as a null strain. The *emp24p-M3* strain showed a moderate secretion phenotype and the emp24p-*M2* and *emp24p-AA* mutants displayed very mild secretion (Figure 2B).

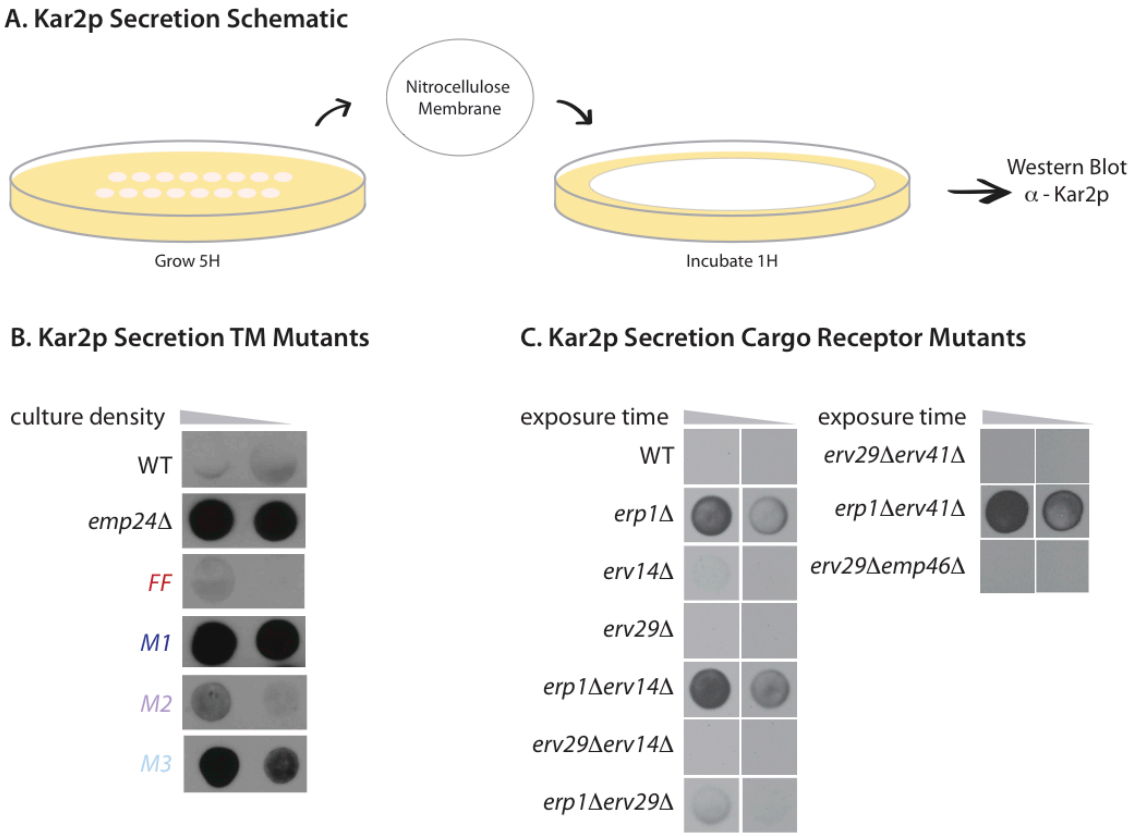

Figure 3.2. Depleting p24 proteins drives aberrant Kar2p secretion amongst cargo receptor null strains. (A) Kar2p secretion from yeast colonies is measured by binding of extracellular secreted Kar2p to a nitrocellulose membrane and subsequent immunoblotting for Kar2p. **(B)** Secretion of Kar2p was examined in strains harboring mutations in the TM and COPII binding domains of Emp24p. The *emp24*Δ and *emp24p-M1* strain backgrounds secreted Kar2p robustly. The *emp24p-M2 and –FF* strains secreted very little Kar2p and the *emp24p-M3* strain exhibited moderately severe secretion. **(C)** Kar2p secretion was also examined in strains with depletions of the ER cargo receptors indicated. Kar2p secretion was only detected in strains with a deletion of *ERP1*, suggesting that p24 depletion is central to this phenotype.

Combining the phenotypes of these mutants, we can conclude that substitution of the full TM domain of Emp24p severely disrupts p24 function. Heteromeric complex formation is hindered, GPI-AP trafficking delayed and based on the Sec13p-bypass phenotype, p24 incorporation into vesicles is likely decreased. The inability to robustly enter vesicles is an interesting finding since this mutant has an intact COPII binding signal. This suggests that complex formation or perhaps association with specific lipid species in the membrane are important for vesicle incorporation as well as direct interaction with COPII.

Substitution of either half of the TM domain with leucines has a lesser impact on p24 function. The C-terminal or cytosolic-proximal half appears more important for complex formation and GPI-AP trafficking whereas substituting the N-terminal half has almost no affect on p24 function.

We next asked if the aberrant Kar2p secretion defect was specific to the absence of p24 proteins. The aforementioned screen from our lab found that p24 deletions had the highest Kar2p secretion index. We wondered if this had something to do with the ER to Golgi cycling of p24 proteins and decided to specifically test ER cargo receptors to see if depletion of these proteins also resulted in secretion of Kar2p. We found that only strains with p24 depletion exhibited substantial Kar2 secretion (Figure 2C). This confirmed that amongst ER cargo receptors, depletion of p24 proteins drives this phenotype much like it drives the Sec13p-bypass growth (Ch 2). Aberrant Kar2p secretion also appears more sensitive to the absence of p24 proteins than Sec13p-bypass growth. A strain with deletions in *ERP1* and *ERV41* secreted Kar2p quite robustly but did not confer growth in the absence of Sec13p. This breaks the correlation between bypass growth and Kar2p secretion phenotypes that we observed for the Emp24p mutants. Another break in the correlation was observed for a strain with depletions of Erp1 and Erv29p. We had previously shown that the additional deletion of *ERV29* in this strain enhanced Sec13p-bypass growth and exacerbated Gas1p trafficking delay (Ch 2, Figure 2). Here we find that *ERV29* deletion rescues the strong Kar2p secretion of a single *ERP1* deletion (Figure 2C, bottom). These data confirm that aberrant Kar2p secretion is primarily sensitive to p24 removal and that as for other p24 null phenotypes, Erv29p may somehow alter the effect.

3-2.3 Erv29p depletion impacts p24 loss-of-function phenotypes

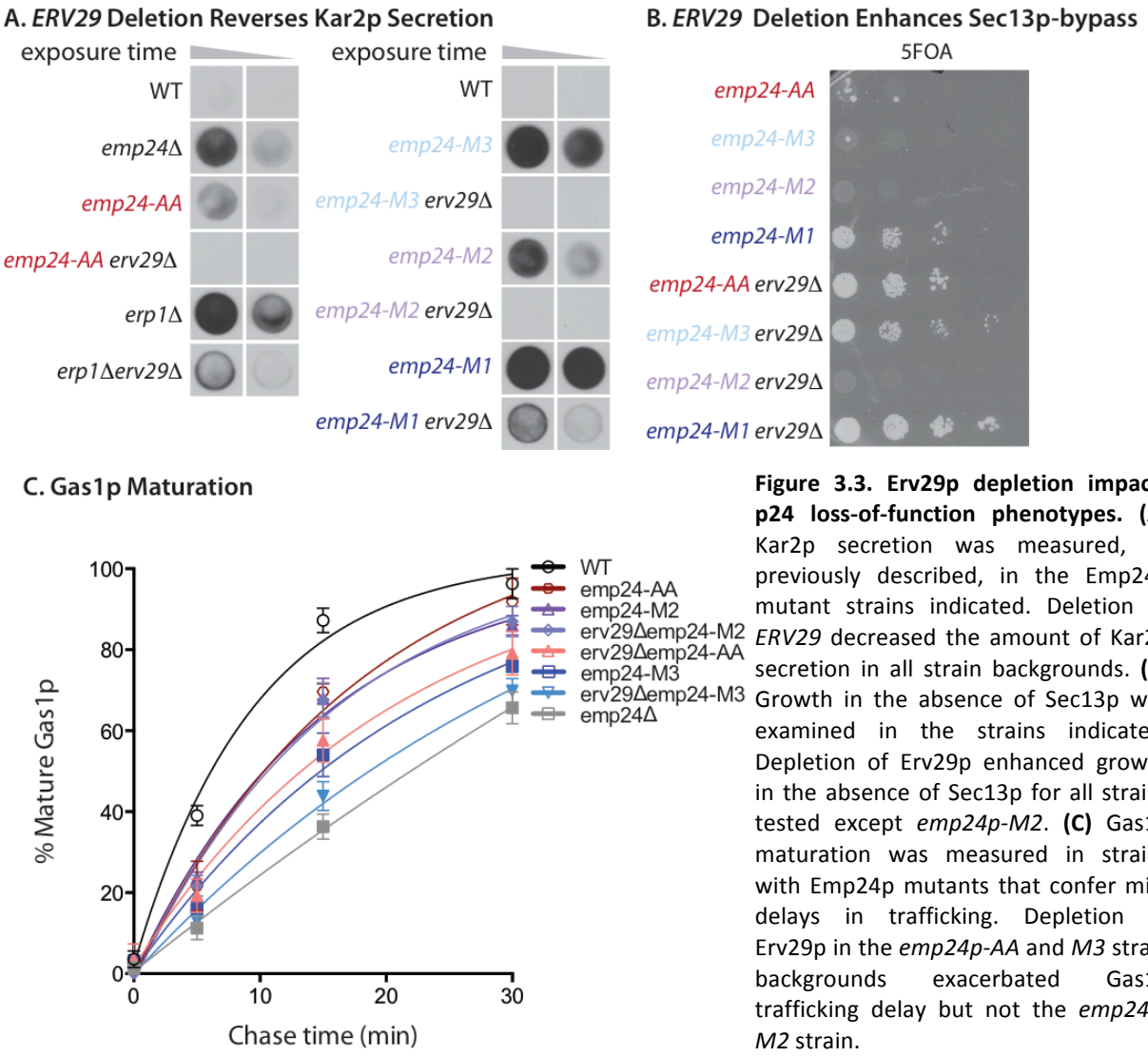

Figure 3.3. Erv29p depletion impacts p24 loss-of-function phenotypes. (A) Kar2p secretion was measured, as previously described, in the Emp24p mutant strains indicated. Deletion of *ERV29* decreased the amount of Kar2p secretion in all strain backgrounds. **(B)** Growth in the absence of Sec13p was examined in the strains indicated. Depletion of Erv29p enhanced growth in the absence of Sec13p for all strains tested except *emp24p-M2*. **(C)** Gas1p maturation was measured in strains with Emp24p mutants that confer mild delays in trafficking. Depletion of Erv29p in the *emp24p-AA* and *M3* strain backgrounds exacerbated Gas1p trafficking delay but not the *emp24p-M2* strain.

Why is Erv29p phenotypically tied to p24 proteins when they do not share any known function? To explore this question we tested the effect of *ERV29* deletion on mutants of Emp24p. We found that Kar2p secretion in all strains bearing mutants of Emp24p was reversed by Erv29p depletion (Figure 3A). This suggests that removal of Erv29p prevents the escape of Kar2p that normally occurs in the absence of p24 proteins. We next examined the effect of depleting Erv29p on Sec13p-bypass growth and Gas1p

trafficking when added to the same strains. We observed that depletion of Erv29p enhanced growth in the absence of Sec13p and exacerbated the Gas1p trafficking delay of all of the Emp24p mutant strains with the exception of the strain expressing *emp24p-M2* (Figure 3B, C). From these data we can conclude that deletion of *ERV29* directly or indirectly affects p24 loss-of-function phenotypes. It further alleviates cargo burden thus enhancing bypass growth, exacerbates the delay in Gas1p trafficking and prevents escape of Kar2p. Interestingly, it appears to only have this effect when p24 function is already perturbed, as evident by the *emp24p-M2* mutant data. The mechanisms behind these observations remain to be determined.

3-2.4 p24 null strains also exhibit aberrant secretion of misfolded CPY which is also rescued by Erv29p depletion*

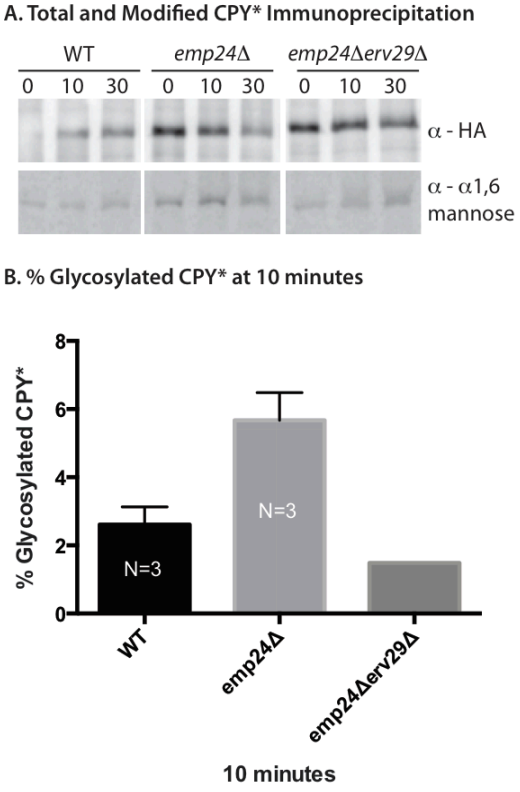

Figure 3.4. p24 null strains also exhibit aberrant secretion of misfolded CPY* which is also rescued by Erv29p depletion. (A) CPY* secretion was measured by pulse-chase analysis and quantification by Phosphorimaging. Secreted CPY* was determined by quantification of the amount of α1,6 mannose modified CPY* (α-α1,6) as a percentage of total CPY*(α-HA). **(B)** An Emp24p null strain exhibited over ~50% more modified CPY* than a WT strain. Depletion of Erv29p decreased CPY* in an *emp24Δ* strain background.

In yeast, p24 null strains also display aberrant secretion of a misfolded variant of the soluble carboxypeptidase Y protein (CPY*). Under WT conditions CPY* is ER-retained and thus does not reach the Golgi whereas the properly folded protein (CPY) is trafficked to the Golgi and acquires an α-1,6 mannose modification. Thus in a WT background we see very low signal when we immuno-precipitate (IP) for α-1,6 modified CPY* (Figure 4A). In an *emp24Δ* null background the same IP yields a significantly stronger signal for α-1,6 modified CPY*. When we quantify the amount of modified CPY* as a percentage of total CPY* we find enhanced Golgi modification of this misfolded variant in the absence of p24 proteins (Figure 4B). In a preliminary experiment, we found that when an *ERV29* deletion was added to this strain, CPY* secretion decreased. This suggests that aberrant secretion of this misfolded protein is also rescued by Erv29 depletion (Figure 4B).

3-2.5 Local concentration of p24 proteins in vesicles and incorporation of Lst1p in the COPII coat influence Kar2p secretion

We have now identified that depletion of p24 proteins results in aberrant protein secretion. Furthermore, both aberrant secretion of Kar2p and CPY* are reversed by removal of Erv29p. We next sought to decipher if p24 vesicle incorporation, like the *bst* phenotype, drives the aberrant secretion of Kar2p. We used the double p24-tail mutants characterized in Ch 2 to test the effect of altering the local concentration of p24s in vesicles on secretion of Kar2p. These mutant proteins contain amino acid substitutions in the cytoplasmic tails that alter binding with the COPII coat. Substitution with alanines (*AA*) abrogates binding altogether, whereas substitution with acidic sorting motifs (*DLE, LASLE*) directs binding to Sec24p. Consistent with our previous data, the double *p24-AA* strain exhibited strong Kar2p secretion, similar to Emp24p null and *emp24p-M1* strains (Figure 5A). This suggests that maintaining p24s in the ER but specifically decreasing their concentration in vesicles permits secretion of Kar2p. The

double *p24-DLE* and *p24-LASLE* exhibited mild Kar2p secretion (Figure 5A). To further probe the impact of restoring p24s to vesicles on Kar2p secretion we expressed the acidic tail mutants in a strain containing a *BST1* deletion. This strain background has been shown to display dispersed localization of Emp24p-sfGFP from punctae to bulk ER (Ch 2, Figure 5A) and secretes Kar2p strongly [120]. We find that expression of the p24 acidic binding mutants again partially rescues Kar2p secretion in a Bst1p null background (Figure 5B). This indicates that restoring p24 proteins to vesicles decreases the amount of Kar2p that is able to escape the ER. This finding also supports the model that the presence or absence of p24 proteins in vesicles drives the aberrant secretion phenotype.

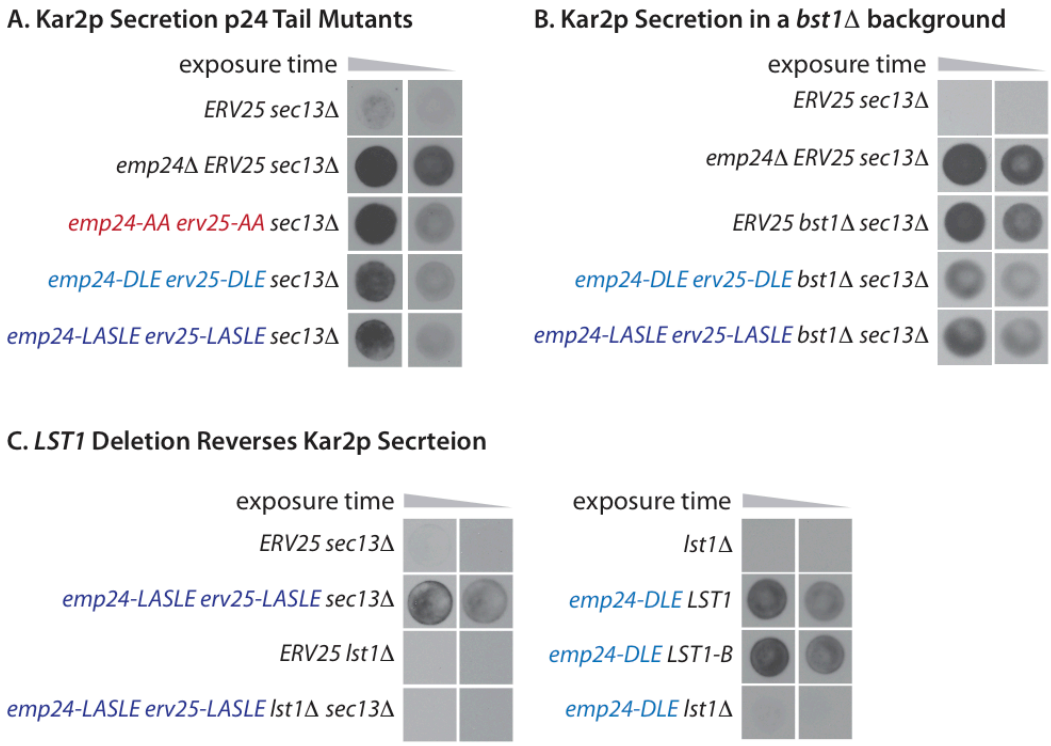

Figure 3.5. Local concentration of p24 proteins in vesicles and incorporation of Lst1p in the COPII coat influence Kar2p secretion. (A) Kar2p secretion was examined in the p24-tail mutant strains indicated (characterized in Ch 2). Alanine substitution of the p24 COPII binding motifs resulted in strong secretion of Kar2p (*emp24p-AA, erv25p-AA)*. Kar2p secretion was decreased when acidic motifs, with affinity for Sec24p, were substituted in the Emp24p and Erv25p tails (*-DxE, -LASLE*). **(B)** Kar2p secretion was measured in a Bst1p null background. The strain with a *BST1* deletion secreted Kar2p as robustly as an Emp24p null strain. Secretion was partially reversed when either *p24-DxE* or *LASLE* was added to a Bst1p null strain background. **(C)** Kar2p secretion was examined in the absence of Lst1p in the COPII coat (*lst1Δ*). Lst1p depletion reversed Kar2p secretion in the p24 acidic tail mutant strains.

Expression of the p24 acidic binding motif mutants also resulted in partial reversal of the Sec13p-bypass phenotype in a Bst1p null background (Ch2, Fig 3). We reasoned this was either a result of GPI-AP depletion decreasing a need for Sec13p rigidity in the COPII coat or incomplete restoration of p24 proteins to vesicles. With the endogenous binding motif, p24 proteins enter vesicles by binding either Sec24p or Lst1p; the acidic sorting motifs bind Sec24p only. We asked if returning p24 proteins to vesicles exclusively via Sec24p was the reason for residual Kar2p secretion. We examined Kar2p secretion in strains expressing p24-DLE and p24-LASLE in the absence of Lst1p. Removing Lst1p ensures p24 acidic sorting mutants exclusively bind to Sec24p. We found that a strain bearing a single *LST1* deletion did not secrete Kar2p. We might have expected the opposite since this may result in partial depletion of p24 proteins from vesicles. However, we also found that when depletion of Lst1p was combined with the acidic sorting mutants, residual Kar2p secretion was rescued (Figure 5C). This was an unexpected finding but can be explained by the fact that removing Lst1p results in exclusive binding to Sec24p but also decreases average vesicle size, as discussed in Ch 2. Therefore, when Lst1p is present and p24 proteins bind only to Sec24p, Kar2p is able to enter the larger vesicles and escape the ER. However in the same scenario, when Lst1p is removed, smaller vesicles are made and Kar2p is no longer able to enter vesicles.

3-3. Discussion

Quality control of protein secretion is critical to cellular homeostasis. Quality control mechanisms in the ER ensure that a continuous, dynamic process is able to maintain fluidity even in the event of malfunction. To facilitate such a feat, specific protein machinery such as ERAD and the UPR work simultaneously to alleviate ER stress. However, one could imagine that as this system has evolved, subtle changes with lower energy requirements could have arisen that alleviated pressure on the

secretory machinery. Higher eukaryotes express multiple isoforms of many COPII components that likely increase efficiency and alleviate constraints [123]. It is possible that over time changes in cargo composition may have also been selected for the benefits conferred on secretory efficiency.

We sought to determine the functions behind the multitude of phenotypes observed in the absence of p24 proteins. We began our analysis with the largely uncharacterized helical membrane domains of p24 proteins. We found that some portion of the TM domain directly or indirectly contributes to p24 complex formation. Therefore the TM domain could either be involved in direct binding with other p24 proteins, or perhaps enhances p24 affinity for specific lipid domains in the ER in which GPI-APs are concentrated. Indeed GPI-APs have been found to require a specific lipid composition in the ER for efficient trafficking [124]. The importance of the TM domain for p24 function was most clearly illustrated by the *emp24p-M1* mutant that harbors a substitution of the entire TM domain with inert leucines. This mutant Emp24 protein is stably expressed but levels of Erv25p are decreased in a strain expressing *emp24p-M1*. This indicates that although the protein is translated and likely inserted into the ER membrane, since its signal sequence remains intact, it does not interact with Erv25p to form a full p24 complex. As a result, in this p24 mutant strain background we observe phenotypes similar to a p24 null. The *emp24p-M1* strain fails to traffic GPI-APs efficiently, confers growth in the absence of Sec13p, and also robustly secretes Kar2p from the ER. This suggests that in addition to not forming a complex with other p24 proteins, this mutant does not efficiently associate with GPI-APs and/or enter COPII vesicles. However, the COPII binding and the GPI-AP binding motifs remain intact. Therefore, it seems that disrupting interactions mediated via the TM domain results in subsequent disruption of other p24 functions. We observed a similar result to a lesser degree for the *emp24p-M3* mutant that contains leucine substitutions in the C-terminal half of the TM domain. Interestingly the *emp24p-M2* mutant,

containing leucine substitution of the N-terminal half of the TM domain, functioned nearly as a WT protein. Therefore, if the TM domain mediates interaction amongst p24 proteins it may primarily employ residues that reside in the C-terminal half of the TM domain.

Mutational analysis of the TM domains also revealed a correlation between the strength of Kar2p secretion with other p24 null phenotypes. We tested the specificity of aberrant secretion to p24 disruption by examining Kar2p secretion in the absence of other ER cargo receptors. We found that, amongst ER cargo receptor depletions, this phenotype was specific to depletion of p24 proteins. We also observed a break in the correlation between p24 null phenotypes. Kar2p secretion appears more sensitive to p24 absence than growth in the absence of Sec13p, since p24 mutations that do not confer bypass growth exhibit significant Kar2p secretion. Additionally, we identified that depletion of Erv29p resulted in a broad reversal of aberrant secretion of both Kar2p and CPY*. Rescue of CPY* secretion is not so unexpected since Erv29p is known to mediate secretion of the WT CPY protein, a soluble secretory cargo. It has also been shown that although CPY* is misfolded, if able to elude the ERAD machinery it can still bind to Erv29p and be secreted [125]. Therefore, aberrant secretion of CPY* in an *emp24Δ* null is still receptor-mediated via Erv29p. However, it is surprising that Kar2p, an ER-resident chaperone, would have a mechanism of receptor-mediated secretion. One explanation is that Kar2p is bound to misfolded proteins that enter vesicles and bind to Erv29p. A second explanation is that Erv29p has a permissive binding motif that allows it to bind pseudo-selectively to soluble cargo. The exact binding motif on Erv29p has not been characterized and Erv29p is known to mediate trafficking of more than one soluble cargo [36]. Therefore, it is possible that the motif is permissive enough to bind various soluble proteins if given access, perhaps even the soluble Kar2p. The question that next arose was how the absence of p24 proteins allows Kar2p and CPY* access to Erv29p.

Depletion of Erv29p also synthetically alters the strength of p24 phenotypes in strains bearing Emp24p mutants. When added to the *emp24p-AA, M1, or M3* strain backgrounds an *ERV29* deletion enhanced the Sec13-bypass growth and exacerbated the delay of Gas1p trafficking. It has also been shown that Erv29p, like p24s, GPI-APs and Pma1p, can bind the COPII cargo adaptor Lst1p [8]. Perhaps the mutual affinity of p24 proteins and Erv29p for this unique COPII component may explain the interconnected phenotypes we observe when both proteins are depleted from vesicles. If this was the case we could speculate models for how Erv29p depletion impacts the three main p24 null phenotypes. *Aberrant secretion:* If both Erv29p and the p24 proteins are concentrated into vesicles sculpted by Lst1p, then specific depletion of p24s could create empty space in these vesicles that would allow CPY* and Kar2p access to Erv29p. *Sec13p-bypass*: If the Sec13-bypass is a result of local depletion of p24 proteins from vesicles, then an additional depletion of Erv29p from the same vesicles could explain the enhanced growth in the absence of Sec13p. *Gas1p trafficking delay*: If Lst1p is required for efficient trafficking of GPI-APs, then depletion of Erv29p and p24s might result in decreased recruitment of Lst1p and thus less efficient capture of GPI-APs. This could be due to a smaller average vesicle size or a lack of flexibility in the COPII scaffold in the absence of Lst1p. Other models are also possible whereby Erv29p has an as yet undiscovered function that is actually tied to p24 function. Additionally, Erv29p depletion could be wreaking havoc in the ER that is resulting in downstream effects that exacerbate the p24 loss of function phenotypes. A closer analysis of Erv29p and also its affinity for Lst1p are required to thoroughly address these questions.

Having identified the p24 null strains as the predominant drivers of Kar2p secretion, we sought to determine what aspect of their absence conferred this phenotype. Altering the direct vesicle incorporation of p24 proteins we find that the aberrant secretion phenotype is in fact due to the

presence or absence of p24 proteins in vesicles. The *p24-AA* mutant data demonstrated that redistributing p24 proteins to dispersed ER enables Kar2p to enter vesicles to a relatively high degree. An important distinction is that p24 proteins are still present throughout the ER in this background, compared to the Emp24p null, but not incorporated into vesicles as efficiently. Therefore, it seems this phenotype, much like the other p24-associated phenotypes, are a consequence of their local concentration in vesicles.

The analysis thus far of the *p24-AA* mutant indicates that the physical absence of p24 proteins in vesicles drives all p24 null phenotypes. However, the reversal of p24 null phenotypes, by addition of acidic binding motifs with affinity for Sec24p, has thus far showed some variability. When p24s are unable to bind to COPII and enter vesicles we observe growth in the absence of Sec13p, delayed Gas1p trafficking and aberrant Kar2p secretion. Therefore, we reasoned that restoring p24 proteins to vesicles should reverse all of the deficiencies observed when they are absent. Instead we find that whereas localization appears restored and Sec13p-bypass reversed (Ch 2, Fig 3), Kar2p secretion and Gas1p trafficking retain mild defects. From these findings we must conclude that populating vesicles with p24 proteins by the endogenous sorting signal that can bind to both Lst1p and Sec24p is not equivalent to populating vesicles with p24s via Sec24p alone. We sought to understand how these two scenarios differ. The primary quantifiable difference between backgrounds in which Lst1p is present or absent is that the presence of Lst1p permits generation of larger vesicle species. In the absence of Lst1p vesicles are comprised only of Sec24p and are ~10-15% smaller in diameter. We found that when we measured Kar2p secretion of the p24 acidic tail mutant strains in the absence of Lst1p, aberrant Kar2p secretion was completely reversed. Therefore, when the p24 proteins are directed to bind Sec24p (-DxE, -LASLE) and Lst1p is present to generate larger vesicles, Kar2p is able to enter vesicles and be secreted.

However, when an *LST1* deletion is added to the same background this larger vesicle population is lost. Kar2p is presumably not able to enter into vesicles when the larger vesicles species is no longer present.

Here we show that the presence and absence of the p24 proteins in COPII-derived vesicles impacts aberrant secretion of proteins. We have found that, amongst ER cargo receptors, the absence of p24 proteins predominantly drives this defect. We have illustrated in Ch 2 that the absence of these proteins has a profound effect on other aspects of secretion. Depletion of p24 proteins from vesicles drives the ability of yeast strains to grow without the essential COPII component Sec13p. The p24 proteins are also able to traffic via a specialized COPII cargo adaptor, Lst1p, thought to accommodate large or troublesome cargo. So it is perhaps not altogether surprising that their absence from vesicles might create a large enough deficit to result in aberrant incorporation of other proteins.

There is much still to be explored to fully understand what role the p24 proteins are playing in ER quality control. Future work would include exploring secretion of other soluble substrates. Most of these experiments were done with Kar2p and need to be repeated with CPY*. It would also be interesting to tease out if the same is observed for integral membrane cargo and/or if the effect is specific to cargo with affinity for Lst1p. Finally, to test the physical ballast model, p24 chimeras and truncations could be generated and expressed in an attempt to alter the physical bulk of p24 proteins in vesicles. Additionally, altering expression levels of p24 proteins would be another way to test the molecular ballast model. The high cellular abundance of these proteins may significantly contribute to their impact on vesicle formation and composition.

Chapter 4

The p24 Proteins Perform a Variety of Key Secretory Functions in COPII Coated Vesicles.

4. Conclusions

The p24 protein family is a unique class of proteins. They are highly abundant and have been implicated in a diverse number of functions throughout the secretory pathway. These include but are not limited to ER cargo receptor, lectin, lipid binding protein, COPI regulator, quality control component and peroxisome proliferation regulator [126]. Higher eukaryotes typically express eight to ten different p24 proteins which, in some tissue types, are essential for viability [127]. In yeast there are eight different p24 proteins which form heteromeric complexes, but the exact composition and specific function of each remains somewhat elusive [128]. Yet, despite their diverse roles in the cell, high abundance and multiple sub families, these proteins are completely non-essential in yeast [104]. In fact deletion of all eight p24 proteins does not show decreased viability beyond the typical p24 null phenotypes of GPI-AP trafficking delay, Kar2p secretion and growth in the absence of Sec13p. Therefore, it is possible that it is through sheer abundance that these proteins seem to impact so many cellular functions. In doing so, they may confer some benefit that their abundance has not been selected against over time. My Ph.D. research has focused on two of these functional nuances of p24 proteins, Sec13p-bypass and aberrant Kar2p secretion. This work has expanded what we know about how eukaryotic protein secretion evolves and adapts to maximize efficiency, minimize waste and accommodate new challenges.

I have found that overall p24 proteins exert the greatest influence at the site of vesicle formation. Altering binding motifs to keep them out of vesicles and trap them in the ER is phenotypically equivalent to removing them entirely by gene deletion. This is most striking with regards to the Sec13p-bypass phenomenon whereby depletion of p24 proteins from vesicles renders a normally essential COPII protein unnecessary. This same p24 depletion also results in the aberrant incorporation of misfolded and ER resident proteins into vesicles. These observations illustrate the physical influence of these

proteins in COPII vesicles. In one scenario they pose a burden to membrane bending and vesicle formation that requires Sec13p to overcome. In the second scenario their abundance appears to prevent wasteful secretion of proteins that would need to be retrieved back to the ER if secreted.

I have also examined the significance of p24 protein association with Lst1p versus Sec24p. Abrogating Lst1p interaction and biasing interaction with Sec24p does not have profound consequences on p24 trafficking of GPI-APs. In fact abrogating interaction with Sec24p (*sec24-Bsite*) appears to be more severe. This calls into question if Lst1p is really required for p24 mediated trafficking of GPI-APs as has been suggested [101]. Furthermore, p24 proteins do not seem to be essential for recruitment or localization of Lst1p, another proposed notion in the field [101]. However, our data show that both the presence of p24 proteins, and the absence of Lst1p from the COPII coat drive a requirement for Sec13p. Removal of p24 proteins permits growth in the absence of Sec13p, whereas a normally non-lethal *LST1* deletion is lethal when Sec13p function is perturbed. The Sec13p requirement in the presence of p24s or absence of Lst1p can be compensated for by incorporation of a rigidified *sec31-Δhinge*. For p24 proteins, this suggests that the presence of p24 proteins in vesicles creates a cargo burden that requires extra rigidity, normally met by Sec13p. For Lst1p, this suggests that the absence of Lst1p from the coat generates a scaffolding deficit that is compensated for by extra rigidity, also normally conferred by Sec13p. Therefore, the p24-Lst1p interaction could have arisen to alleviate the burden posed by p24 proteins by generating a vesicle with an alternate geometry that is more amenable to traffic difficult cargo.

There is evidence for Lst1p forming a specialized coat structure. It has been shown that the coat is capable of polymerizing into alternate geometries, generating vesicles of varying shapes and sizes [41]. We also know that the inner coat makes contacts with the outer Sec13p/Sec31p cage [11]. The

Sec23p/Lst1p dimer may interact differently with the outer cage than Sec23p/Sec24p, and potentially alter overall coat structure. EM studies of purified vesicles have also shown that Lst1p incorporation results in larger average vesicle size. Our finding that depletion of Lst1p rescues nonspecific secretion of Kar2p, may also be evidence that Lst1p forms larger vesicles. If p24 depletion generates vacancies in vesicles for non-specific cargo incorporation, smaller vesicle size could explain how this is reversed by the absence of Lst1p.

From these data, we have developed a model whereby altering the local concentration of p24 proteins in vesicles or Lst1p incorporation in the coat impacts the requirement for Sec13p and creates scenarios where unoccupied space in vesicles allows non-selective secretion of soluble proteins (Figure 1). Under WT conditions, Lst1p promotes generation of larger vesicles in which p24 proteins, GPI-APs, Erv29p and other large cargoes such as Pma1p can concentrate. Smaller vesicles, with little Lst1p incorporated, have a mix of cargoes including p24s, Erv14p and various multi-spanning transmembrane proteins. Misfolded proteins remain in the ER where they are subject to ERAD and Kar2p is ER-retained. Sec13p is required for viability in this background. (Figure 1A). In a p24 null, the absence of these proteins creates significant space inside all vesicles. This is especially apparent in any larger vesicle species that may also contain the soluble cargo receptor, Erv29p. The result is incorporation of misfolded proteins as well as soluble ER resident proteins into vesicles. In the absence of p24 proteins, Sec13p is not required for vesicle formation (Figure 1B). The p24 mutants with acidic sorting signals will no longer be able to bind to Lst1p and are incorporated into vesicles by binding Sec24p. Larger vesicles with a higher Lst1p:Sec24p ratio have less p24s and therefore unoccupied space. Partial Kar2p secretion is exhibited, but less than the *EMP24* deletion because although they are present in vesicles they do not populate larger vesicles to a significant degree. The presence of p24 proteins in vesicles restores the requirement for Sec13p

(Figure 1C). When an *LST1* deletion is added to this background, the larger vesicle species is removed and all proteins are packed into smaller Sec24p-only vesicles with no vacant space for aberrant incorporation of soluble cargo. Here Sec13p is required due to p24 presence as well as Lst1p absence (Figure 1D). In all of these scenarios, removal of Erv29p rescues secretion suggesting that binding to Erv29p is what retains CPY* and Kar2p in vesicles instead of diffusing back out.

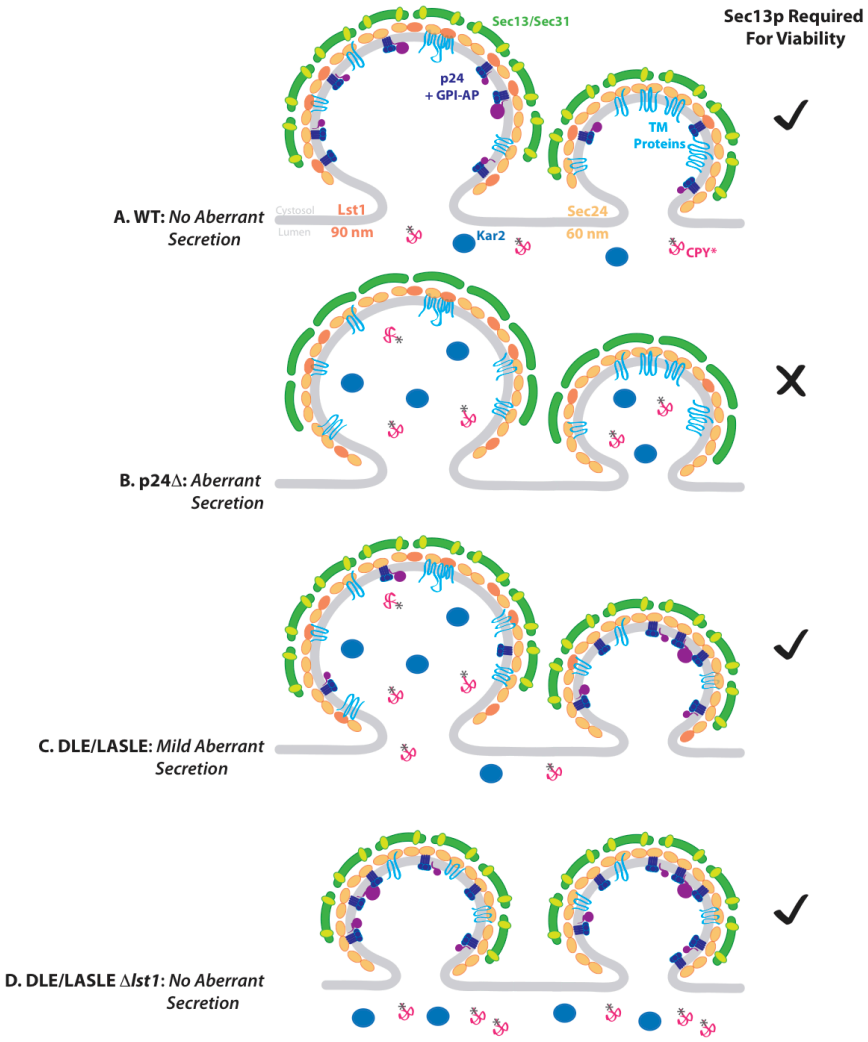

Figure 4.1. p24 proteins impact coat composition and quality control (A) In a WT strain background, p24 proteins populate vesicles and Sec13p is required in the coat to form vesicles. Higher rate of incorporation of Lst1p in the coat creates a larger vesicle species that is primarily occupied by p24s, Pma1p and Erv29p. Kar2p and misfolded proteins such as CPY* are ER retained. **(B)** When p24 proteins are absent from vesicles (*p24Δ*), Sec13p is no longer required for viability. Kar2p and CPY* enter vesicles and bind Erv29p, especially larger vesicles enriched for Erv29p. **(C)** When acidic binding motifs are appended to p24s, p24 proteins enter vesicles via Sec24p only and do not populate larger vesicle species as efficiently as WT. Sec13p is required for viability, but Kar2p and CPY* are able to enter vesicles to a small degree. **(D)** When *LST1* is deleted in the same strain background as (C), p24 proteins enter vesicle via Sec24p only. Sec13p is required for viability and the larger vesicle species is lost. Kar2p and CPY* are not able to enter tightly packed, smaller vesicles.

I was only able to scratch the surface of what role the p24 membrane domains may play in overall p24 function. My preliminary data seem to suggest that this domain is either directly or indirectly involved in promoting p24 complex formation. If the association is direct there may be residues within the TM domain that, in conjunction with the lumenal coiled-coil interactions, promote assembly of the p24 complex. It has been observed that deletion of Erp1p and Erp2p does not disrupt association of Emp24p and Erv25p, and hence the tetrameric p24 complex may form via a dimer of dimers [103]. Perhaps the interaction bringing the Emp24p/Erv25p and Erp1p/Erp2p dimers together is mediated through the TM domains. Faithful complex formation is known to be of principal importance to p24 function, and this is further illustrated by the severe phenotypes displayed by the emp24p-M1 mutant in which the entire TM domain is replaced by leucines.

An alternative explanation for the phenotypes we observe for the TM mutants could be that specific residues in the TM domain are responsible for interacting with lipid species in the ER membrane. In mammalian cells there is evidence for an Emp24p ortholog directly binding to a specific sphingomyelin species via its TM domain, notably the C-terminal half of the TM domain [129]. It has also been shown that GPI-APs have a dependence on ceramides in the ER membrane for efficient egress [124]. Furthermore it has been shown that GPI-APs are able to self-assemble in the ER membrane even in the absence of p24 proteins [107]. If lipid anchored proteins are able to laterally self-assemble within the ER membrane and p24 proteins subsequently associate with GPI-APs to ferry them into vesicles, we might imagine that p24 proteins would similarly segregate within the membrane in order to do so. In addition to GPI-AP proteins, ceramide dependence has also been shown for oligomerization of Pma1p in the ER [108]. Pma1p also shares with GPI-APs and p24s an affinity for the unqiue COPII cargo adaptor, Lst1p. Therefore TM residues of the p24 proteins may mediate association with specific lipid species, to

segregate into ER membrane domains where other Lst1p clients also segregate. This would increase the likelihood of p24 proteins encountering one another thus promoting complex formation as well as their GPI-AP cargo. Additionally this type of concentration of Lst1p clients would increase recruitment of Lst1p and hence vesicle incorporation.

My work with p24 proteins has illuminated the many ways in which this protein family impacts vesicle-mediated protein secretion. They influence COPII coat composition by presenting physical constraints to vesicle formation that drive the requirement for Sec13p rigidity in the outer COPII coat. They can also employ the Sec24p homolog, Lst1p, to generate vesicles that better accommodate difficult cargo proteins. The p24 proteins also impact the ER egress of other secretory cargo. They enhance trafficking efficiency of an abundant class of secretory cargo, GPI-APs. Finally, by populating all vesicle species, p24s prevent wasteful, non-specific secretion of misfolded and ER resident proteins. These multi-faceted functions in COPII vesicles seem to predominantly stem from an unusually high abundance, bulk lumenal orientation across the ER membrane, and their ability to form complexes. It seems that these physical properties of p24 proteins have resulted in their far-reaching influence on so many aspects of secretion. It is intriguing to further speculate that this is no accident, and the many roles of p24 proteins are representative of subtle evolutionary selections over time that enhanced efficiency of protein secretion.

Methods

Yeast Strains

All strains were constructed and grown using standard *S. cerevisiae* methods. Strains (Table 1) were made by crossing, sporulation and dissection of tetrads or by PCR-based integration of auxotrophic or drug resistance markers [130]. Strains containing *EMP24* mutations were made by linearization and integration of PRMP304 to the *TRP*1 locus as described previously [102]. Allelic replacements and integrations of all strains were confirmed by PCR. Strains containing chromosomal fluorescent protein tags were also constructed by PCR based integration to the C-terminus of the gene.

Plasmids

Plasmids used are listed in Table 2. pJD045 was constructed by PCR amplification of the *ERV25* locus (+/-500bp) and ligation into pRS316. The *URA3* marker was replaced with a *HIS3* marker by homologous recombination in yeast. Erv25 mutations in pJD044, pJD055, and pJD066 were generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pJD045. Emp24 mutations in pJD004, pJD006, pJD007, pJD008, pJD058 and pJD065 were made by Quikchange mutagenesis of the PRMP304 integration plasmid [102]. pJD062 was made by digestion of YCplac111-Emp24-CFP to excise *CFP*, followed by GAP repair of the linearized plasmid to incorporate the coding region of superfolder-GFP [131] in the same position. Emp24-sfGFP mutants pJD067, pJD068, pJD069 were generated by Quikchange mutagenesis of pJD062. pJC03 was constructed by PCR amplification of the *LST1* locus (+/-500bp) and ligation into pRS313. pJC04 was made by Quikchange mutagenesis of pJC03. LMB714 was used to tag proteins with sfGFP at the C-terminus. It was constructed from pFA6-GFP-TRP1 [130], which

was digested with BamHI and AscI (NEB) to excise the GFP gene and replace it with a BamHI/AscI flanked PCR fragment containing the sfGFP gene.

*Pulse Chase Analysis-add CPY**

Pulse chase experiments were used to monitor ER-Golgi transport of Gas1p. These experiments were performed as previously described [102]. Strains were grown to mid log phase at 30°C in minimal media lacking uracil or uracil and histidine as required, starved for 15 minutes and labeled with Expre^{35}S^{35}S Protein Labeling Mix (Perkin Elmer) for 5 minutes. The label was chased with excess rich media and samples were taken from 0-30 minutes. Cells were lysed in detergent and Gas1p was immunoprecipitated from cell lysates, separated by SDS-PAGE, and mature and immature bands detected by phosphorimaging using a Typhoon Scanner (GE Healthcare). The protein bands were quantified using ImageQuant software (GE Healthcare) and the percentage of the mature band in each sample plotted.

BST Growth Assay

To observe growth in the absence of Sec13p, strains were grown to saturation at 30°C in media lacking uracil. Serial dilutions of 1:5 were made from the saturated cultures and spotted onto solid media without (control) or with 5-Fluoroorotic acid (5FOA, Gold Biotechnology) to counter-select for pLM246 (*SEC13::URA3*). Strains in figure 2B and 3B were grown on 5FOA lacking histidine to select for the nonessential *ERV25* plasmid. All strains were grown for 5-7 days at 25°C before scanning. Similar experiments tested viability in the *sec31Δ lst1Δ* strain, where the wild-type copy of *SEC31::URA3* was also counter-selected on 5FOA.

In Vitro COPII Budding

In vitro budding was used to examine capture of p24 WT and tail mutant proteins into vesicles and was performed as previously described [8]. Briefly, purified COPII proteins and GTP or GDP were added to microsomal membranes isolated from strains expressing WT or p24 tail mutant proteins (-AA, -DLE, -LASLE). Samples were incubated at 25°C for 30 minutes to permit vesicle formation; donor membranes were removed by centrifugation and vesicles isolated by ultracentrifugation. Samples were analyzed by SDS-PAGE and immunoblot using antibodies against Erv25p, Emp24p and Erv41p.

Fluorescence Microscopy

Imaging of Emp24-sfGFP was performed in cells grown to mid log phase at 30°C in minimal media lacking leucine. Strains used to image Sec24-mCherry and Lst1-sfGFP colocalization were grown in minimal medium lacking tryptophan and supplemented with adenine under the same conditions. Images were taken on a Zeiss AxioImager Z1 upright fluorescent microscope with a Plan-Apochromat 100x/1.4 NA oil immersion objective (Carl Zeiss Ltd.). Images were collected using AxioVision Rel software (Carl Zeiss Ltd.) and processed with Image J (NIH). Fluorescence Loss in Photobleaching (FLIP) was used to measure coat dynamics of Lst1-sfGFP and Sec24-sfGFP as previously described [38]. Briefly, a small region of interest was repeatedly alternatingly photobleached, and then the fluorescence intensity of the whole cell measured for a loss of signal, representing protein that has diffused into the bleaching area. Fluorescence intensity and pixel variance were measured using Image J (NIH) and statistical analysis performed with Prism 5.0 (GraphPad Software).

Western Blot

To western blot for protein stability cultures were grown to mid log phase, lysed and solubilized in 1% SDS + protease inhibitors. Samples were separated by SDS-PAGE and transferred for 2H to PVDF membranes. Membranes were subsequently blocked in 5% milk for 20 minutes and incubated in

primary antibody overnight. The next day membranes were washed, incubated with secondary HRP-conjugated antibody and developed using SuperSignal West reagents. Films were developed using an SRX-101A Medical Film Processor (Konica Minolta).

Kar2 Secretion

To test for Kar2p secretion, strains were grown to saturation at 30°C in selective media based on strain background. Serial dilutions of 1:5 were made from the saturated cultures and both were spotted onto solid media containing rich media plus agarose. Strains were subsequently grown for 5H at 30C to allow for extracellular secretion of Kar2p. A nitrocellulose membrane was then applied directly onto the plate and incubated for an additional 1H at 30C. The nitrocellulose membrane is finally rinsed and immuno-blotted for bound Kar2p, as described above.

Tables

Table 1: Yeast Strains

Strain	Genotype	Source
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
LMY288	BY4741 *sec13Δ*::*HIS3* pLM246 (CEN SEC13-URA3)	[42]
LMY581	LMY288 *emp24Δ*::NATMX	[42]
JDY164	LMY288 *erp1Δ*::NATMX	This study
JDY151	JDY164 *erv14Δ*::KANMX	This study
JDY210	JDY164 *gsf2Δ*::HPHMX	This study
JDY212	JDY164 *pho86Δ*::HPHMX	This study
LMY1008	MATa *his3Δ1 leu2Δ0 met15* lys2* ura3Δ0 sec13Δ*::*HIS3 erp1Δ*::NATMX pLM246	This study
JDY125	LMY1008 *erv41Δ*::KANMX	This study
JDY211	LMY1008 *erv41Δ*::KANMX *erv46Δ*::HPHMX	This study
LMY967	MATa *his3Δ1 leu2Δ0 met15* lys2* ura3Δ0 sec13Δ*::*HIS3 erv29Δ*::KANMX pLM246	[42]
LMY1006	LMY967 *erv41Δ*::KANMX	This study
LMY1007	LMY967 *emp46Δ*::KANMX	This study
JDY150	JDY164 *erv29Δ*::KANMX	This study
JDY149	LMY288 *erv14Δ*::NATMX *erv29Δ*::KANMX	This study
FY833	MATa *his3Δ200 ura3-52 leu2Δ1 lys2* trp1Δ63*	Barlowe Lab
FY834	MATα *his3Δ200 ura3-52 leu2Δ1 lys2* trp1Δ63*	Barlowe Lab
JDY119	FY834 *sec13Δ*::KANMX *erv25Δ*::NATMX pLM246	This study
JDY120	FY833 *sec13Δ*::KANMX *erv25Δ*::NATMX *emp24Δ*::*LEU2* pLM246	This study
JDY136	JDY119 pJD045	This study
JDY137	JDY120 pJD045	This study
JDY133	JDY119 pJD044	This study
JDY138	JDY120 *emp24-AA*::*TRP1* pJD045	This study
JDY135	JDY120 *emp24-AA*::*TRP1* pJD044	This study
JDY175	JDY120 *emp24-DLE*::*TRP1* pJD055	This study
JDY203	JDY120 *emp24-LASLE*::*TRP1* pJD066	This study
JDY186	JDY175 *bst1Δ*::HPHMX	This study
JDY204	JDY203 *bst1Δ*::HPHMX	This study
JDY209	JDY137 *EMP24*::*TRP1 bst1Δ*::HPHMX	This study
JDY192	LMY581 pJD062 (EMP24-SFGFP-LEU)	This study
JDY193	LMY288 *bst1Δ*::NATMX *emp24Δ*::HPHMX pJD062	This study
JDY187	FY833 *sec13Δ*::KANMX *erv25Δ*::NATMX *EMP24*::TRP1 pLM246	This study
JDY194	JDY187 pJD068 pJD044	This study
JDY195	JDY187 pJD069 pJD055	This study
JDY196	JDY195 *bst1Δ*::HPHMX	This study
JDY207	JDY187 pJD067 pJD066	This study
JDY173	MATα *his3Δ1 leu2Δ1 lys2 ura3Δ0 lst1Δ*::NATMX e*mp24Δ*::HPHMX	This study
JDY213	JDY173 pJC03 pJD062	This study

JDY214	JDY173 pJC04 pJD062	This study
JDY215	JDY173 pJD062	This study
JDY216	JDY173 pJD069	This study
JDY217	JDY173 pJD067	This study
JDY218	JDY136 *lst1Δ*::HPHMX	This study
JDY110	MATα *ade2-101c his3Δ200 leu2Δ1 lys2-801am trp1-Δ63 ura3-52 SEC24*-mCherry *LST1*-superfolderGFP	This study
JDY128	JDY110 *emp24Δ*::NATMX	This study
JDY089	LMY288 *SEC24*-superfolderGFP	This study
JDY090	LMY581 *SEC24*-superfolderGFP	This study
JDY166	LMY288 *LST1*-superfolderGFP	This study
JDY168	LMY581 *LST1*-superfolderGFP	This study
LMY342	MATa *his3Δ0 leu2Δ0 met15* lys2* ura3Δ sec13Δ*::*HIS3 bst1Δ*::KANMX pLM246	[42]
LMY760	LMY342 *lst1Δ*::NATMX	This study
LMY1001	MATα *his3Δ200 ura3-52 leu2Δ1 lys2-801am sec13Δ*::KANMX *lst1Δ*::NATMX pLM246	This study
RSY1109	MATa *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 sec31Δ*::*TRP1* pNS3111 (CEN-*SEC31-URA3*)	Schekman Lab
LMY1081	RSY1109 *lst1Δ*::NATMX	This study
JDY009	FY833 *sec13Δ*::KANMX *emp24Δ*::*LEU2 emp24-AA*::*TRP1* pLM246	This study
JDY010	FY833 *sec13Δ*::KANMX *emp24Δ*::*LEU2 emp24-TM11L*::*TRP1* pLM246	This study
JDY011	FY833 *sec13Δ*::KANMX *emp24Δ*::*LEU2 emp24-TM10L*::*TRP1* pLM246	This study
JDY012	FY833 *sec13Δ*::KANMX *emp24Δ*::*LEU2 emp24-TM21L*::*TRP1* pLM246	This study
JDY106	JDY009 *erv29Δ*::*HIS3*	This Study
JDY116	JDY010 *erv29Δ*::*HIS3*	This Study
JDY117	JDY011 *erv29Δ*::*HIS3*	This Study
JDY118	JDY012 *erv29Δ*::*HIS3*	This Study

* This marker was present in one of the parental strains but not checked after tetrad dissection. Possible alleles are *met15Δ0* or *lys2Δ0* depending on the gene.

Table 2: Plasmids

Plasmid	Description	Source
pLM246	pRS316-*SEC13*	[42]
pRMP304	*EMP24*::*TRP1*	Barlowe Lab
pJD004	pRMP304-*emp24-AA* (F196A,F197A)	This Study
pJD045	pRS316-*ERV25-URA*::*HIS*	This Study
pJD044	pJD045-*erv25-AA* (Y204A,F205A)	This Study
pJD058	pRMP304-*emp24-DLE* (F196DLE,F197DLE)	This Study
pJD055	pJD045-*erv25-DLE* (Y204DLE,F205DLE)	This Study
pJD065	pRMP304-*emp24-LASLE* (F196LASLE,F197LASLE)	This Study
pJD066	pJD045-*erv25-LASLE* (Y204LASLE,F205LASLE)	This Study
YCplac111	*EMP24*-CFP	[107]
pJD062	YCplac111-*EMP24*-sfGFP	This Study
pJD067	YCplac111-*emp24-LASLE*-sfGFP	This Study
pJD068	YCplac111-*emp24-AA*-sfGFP	This Study
pJD069	YCplac111-*emp24-DLE*-sfGFP	This Study
pJC03	pRS313-*LST1*	This Study
pJC04	pRS313-*lst1-B* (K543M,R545M)	This Study
LMB714	pFA6-sfGFP-*TRP1* (GFP replaced with sfGFP)	This Study
pBS34	pFA6-mCherry-KANMX	YRC, U. of Washington
pNS3111	pRS316-*SEC31-URA3*	Schekman Lab
pAC20	pNS3111-*URA3*::*LEU2*	[42]
pAC28	pAC20-*sec31-DK* (W386D,A387K)	[42]
pCL23	pAC20-*sec31-Δhinge* (Δaa332-412)	[42]
pJD054	pCL23-*GS5* (5X Gly Ser @ aa332)	This Study
pJD053	pCL23-*GS10* (10X Gly Ser @ aa332)	This Study
pJD050	pCL23-*GS20* (20X Gly Ser @ aa332)	This Study
pJD006	pRMP304-*emp24-M1* (ALL TM::21L)	This Study
pJD007	pRMP304-*emp24-M2* (N-term TM::10L)	This Study
pJD008	pRMP304-*emp24-M2* (C-term TM::11L)	This Study

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