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Traffic of p24 proteins and COPII coat composition mutually influence membrane scaffolding

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Abstract

Eukaryotic protein secretion requires efficient and accurate delivery of diverse secretory and membrane proteins. This process initiates in the endoplasmic reticulum, where vesicles are sculpted by the essential COPII coat. The Sec13p subunit of the COPII coat contributes to membrane scaffolding, which enforces curvature on the nascent vesicle. A requirement for Sec13p can be bypassed when traffic of lumenally oriented membrane proteins is abrogated. Here, we sought to further explore the impact of cargo proteins on vesicle formation. We show that efficient ER export of the p24 family of proteins is a major driver of the requirement for Sec13p. The scaffolding burden presented by the p24 complex is met in part by the cargo adaptor, Lst1p, which binds to a subset of cargo, including the p24 proteins. We propose that the scaffolding function of Lst1p is required to generate vesicles that can accommodate difficult cargo proteins that include large oligomeric assemblies and asymmetrically distributed membrane proteins. Vesicles that contain such cargoes are also more dependent on scaffolding by Sec13p and may serve as a model for large carrier formation in other systems.

Abstract

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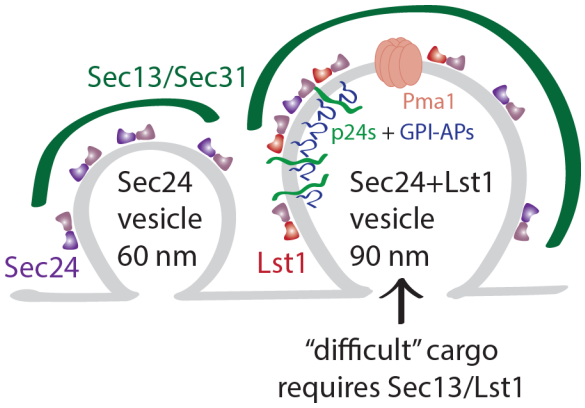

Introduction

Secretory protein export from the endoplasmic reticulum (ER) occurs in transport carriers generated by the COPII coat, a set of five cytoplasmic coat proteins that coordinate to assemble vesicles and populate them with the appropriate cargo [1]. Different components of the COPII coat perform discrete functions. The small GTPase, Sar1p, initiates and regulates coat assembly; the "inner coat", Sec23p/Sec24p, contributes to GTPase activity (via Sec23p) and cargo recruitment (via Sec24p); and the "outer coat", Sec13p/Sec31p, drives membrane curvature. These five COPII coat proteins in isolation suffice to create vesicles from naked liposomes [2]. However, in cells the cargo proteins embedded within the membrane are not inert participants and can contribute to vesicle formation in both positive and negative ways.

Cargo abundance directly influences the number and size of ER exit sites (ERES), which correspond to sites of COPII vesicle formation [3]; acute and chronic induction of cargo protein expression increases the size and number of ERES, respectively [4]. Cargo can also serve as a barrier to vesicle formation: asymmetrically distributed cargo proteins like glycosylphosphorylinositol-anchored proteins (GPI-APs) seem to oppose the positive membrane curvature enforced by the COPII coat thereby driving a requirement for rigidity conferred by Sec13p [5, 6]. Another likely influence of cargo is on the architecture of vesicles themselves. Cargoes like pro-collagen and lipid particles are too large to fit into canonical COPII vesicles and therefore must trigger genesis of non-canonical COPII-dependent structures. Whether these large cargoes directly influence the coat remains unclear, but accessory proteins that interact with various components of the COPII coat may participate in the structural changes that accompany formation of large vesicles [7, 8]. For example, TANGO-1 is proposed to function as a receptor for pro-collagen, linking this lumenal protein to the Sec23/Sec24 cargo adaptor layer [9]. CUL3-KLHL12 is an E3 ligase that ubiquitinates mammalian SEC31, a modification that is required for efficient collagen traffic [10]. Structural studies show that the outer COPII scaffold, formed by Sec13 and Sec31 can adapt to multiple geometries, making both large spheres and elongated tubules under some conditions [11, 12]. Understanding how these geometries are created under different conditions is clearly key to appreciating mechanisms of ER export of diverse cargo.

We previously linked the essential function of the yeast outer coat protein, Sec13p, to traffic of specific classes of asymmetrically oriented cargo, arguing for a unique scaffolding role in the context of such cargoes [5]. A genome-wide screen of the yeast haploid deletion collection identified nine core bypass-of-sec-thirteen (*bst*) mutants [13] that permit viability in the absence of this otherwise essential protein [5]. The majority of *BST* genes encode proteins involved in biogenesis of GPI-APs, including enzymes that remodel the lipid anchor and a family of proteins, the p24 complex [14], that recruits GPI-APs into COPII vesicles [15, 16]. A correlation between the strength of GPI-AP trafficking delays and robustness of growth in the absence of Sec13p lead us to propose that local concentration of these and other asymmetrically distributed cargo molecules, renders cells dependent on Sec13p, which may rigidify the coat scaffold to drive membrane bending [6, 17, 18].

GPI-APs are a unique class of cargo in that they are tethered directly to the ER membrane by a lipid anchor without spanning the bilayer. Yeast GPI-APs are major constituents of the cell wall and are segregated into COPII structures that are distinct from those that contain polytopic plasma membrane proteins [19, 20]. However, GPI-AP depletion from ER exit sites alone does not fully explain the Sec13p bypass phenomenon since many mutants that have delays in GPI-AP maturation are not *BST* mutants [5, 21]. Here, we sought to test whether depletion of cargo more broadly could similarly influence COPII coat requirements using more sensitive and targeted genetic analyses. We find that Sec13p bypass is predominantly associated with defects in p24 family function and GPI-AP traffic, although we confirm our previous conclusion that defects in GPI-AP transport are not sufficient to drive Sec13p bypass. Instead, local concentration of p24 proteins at ER exit sites is central to this phenotype. We further investigate the role of the Sec24p paralog, Lst1p/Sfb3p, in GPI-AP trafficking and Sec13p dependence, finding that Lst1p likely serves two roles: recruitment of p24 proteins (and thus GPI-APs) into COPII vesicles, and structural scaffolding of the membrane around these cargoes. We propose that accumulation of p24 proteins at ER exit sites, driven both by local concentration of GPI-APs and by binding of Lst1p creates a requirement for coat rigidity that can be conferred either by Sec13p or by Lst1p.

Results

Our previous systematic screen for Sec13p-bypass mutants identified one component, Erv29p, that functions as a cargo receptor for soluble lumenal proteins and does not directly participate in traffic of GPI-APs [5]. We therefore asked whether Sec13p bypass could be conferred by depletion of cargo other than GPI-APs from nascent vesicles. We deleted known and putative ER export receptors that might impact local enrichment of entire classes of proteins [22]. Since such components were not identified in our original screens, we tested specific mutants in a sensitized background. Deletion of the p24 protein, Erp1p, yields a mild *bst* phenotype that can be exacerbated by additional mutations [5]. We combined a *sec13*Δ *erp1*Δ strain with deletions in various export receptors and tested for viability on 5-FOA, which counterselects for a *URA3*-containing plasmid carrying wild-type *SEC13*. Deletion of a single cargo receptor, Erv14p, conferred a robust *bst* phenotype in the context of *erp1*Δ (Fig. 1A, left panels). Erv14p is an ER export receptor for many plasma membrane proteins, most of which span the membrane multiple times [19, 23, 24]. Thus, deletion of

Erv14p might broadly exclude large numbers of cargo proteins from vesicles and thus influence the physical properties of the nascent vesicle. However, an *erp1*Δ *erv14*Δ double mutant also showed a severe defect in maturation of Gas1p, an abundant GPI-AP that undergoes Golgi-specific modifications that cause an increase in apparent molecular weight, which can be used as a measure of trafficking efficiency (Fig. 1B). Since Gas1p is not a direct client of Erv14p, the trafficking defect observed in an *erp1*Δ *erv14*Δ strain might indicate secondary effects of deleting Erv14p. A strong defect in GPI-AP maturation in the *erp1*Δ *erv14*Δ strain mirrors the effect of *erp1*Δ *erp2*Δ and *erp1*Δ *erv29*Δ strains, where the individual deletants showed minimal defects in GPI-AP maturation but strong defects when combined [5]. Such GPI-AP delay previously correlated with the strength of the *bst* phenotype, implicating GPI-APs as a cause of Sec13p bypass [5]. We also tested combinations of cargo receptor mutants in an *erv29*Δ background, which we had identified as a weak *bst* mutant that could be exacerbated by additional mutations (Fig. 1A, right panels). Only an *erv29*Δ *erp1*Δ double deletion conferred viability in a *sec13*Δ strain, as previously reported [5]. However, an *erv29*Δ *erv14*Δ strain showed a profound defect in Gas1p maturation (Fig. 1B) but not a *BST* phenotype, confirming that defects in GPI-AP export are not sufficient for Sec13p bypass [5].

Since only two cargo receptor mutants (*erv29*Δ and *erv14*Δ) confer Sec13p bypass, and each required an additional weak p24 mutation to yield robust growth, Sec13p-independent budding appears most strongly linked to conditions where GPI-APs and/or p24 proteins are impaired in their trafficking. Our previous genetic analysis suggested that these effects are local rather than a global feature of ER membrane curvature or composition [5]. This led us to test the effects of p24 protein capture into COPII vesicles. Emp24p and Erv25p both contain di-aromatic signals in their short C-terminal cytoplasmic domains (Fig. 2A) that confer interaction with the COPII coat and are required for p24 complex exit from the ER [25]. We reasoned that mutation of the COPII binding signal should preclude local concentration of the p24 complex into COPII vesicles and might thus confer Sec13p bypass. Indeed, when we mutated both the Emp24p and Erv25p di-aromatic motifs, we observed a strong *BST* phenotype (Fig. 2B), but mutation of either signal on its own remained inviable in a *sec13*Δ strain (Fig. 2B). These mutations caused redistribution of an Emp24p-superfolderGFP fusion protein (Emp24p-sfGFP, with the fluorescent reporter inserted into the lumenal domain) from punctae to bulk ER (Fig. 2C). The punctate localization of the p24 proteins likely corresponds to both ER exit sites (ERES) and cis-Golgi, with the latter localization dependent on ER egress via COPII vesicles. We confirmed this dual localization by co-localizing Emp24p-sfGFP with Sec24p-mCherry, observing that Emp24p was largely in punctae that are distinct from Sec24p-containing spots, although some overlap was detected (Fig. S1A). Mutation of the di-aromatic motifs indeed impaired capture into COPII vesicles generated in vitro; both the Emp24p-AA and Erv25p-AA mutants were stably expressed but failed to enrich in COPII vesicles generated with Sec24p (Fig. 2D; Fig. S2). Replacement of the di-aromatic motifs with acidic motifs (DLE derived from Sys1p and LASLE derived from Bet1p) that interact with the B-site on Sec24p both reversed viability in the context of a *sec13*Δ mutation and returned Emp24p-sfGFP to a punctate localization that corresponded to a mix of ERES and cis-Golgi (Fig. 2B,C; Fig. S1B,C). Consistent with

these phenotypes, Emp24p and Erv25p capture into COPII vesicles generated in vitro was restored when acidic motifs were appended (Fig. 2D; Fig. S2).

Redistribution of Emp24p from punctae to bulk ER has previously been reported in a *bst1*Δ background [26], linking ER export of p24 proteins with lipid anchor remodeling and suggesting that Sec13p-bypass in remodeling mutants might stem in part from altered trafficking of the p24 complex. We therefore tested whether ectopic acidic signals could restore punctate localization to Emp24p-sfGFP in a *bst1*Δ strain. Indeed, these signals were dominant over the *bst1*Δ effect, with Emp24p-DLE-sfGFP reduced in abundance in the bulk ER and enriched in punctae (Fig. 3A). The consequence of this redistribution was reduced viability in the context of a *bst1*Δ *sec13*Δ strain (Fig. 3B). Importantly, restoration of p24 ER export did not rescue the Gas1p maturation defect of a *bst1*Δ strain (Fig. 3C), suggesting GPI-APs still fail to traffic in these strains. We propose that p24 capture into COPII vesicles is a major driver of the requirement for Sec13p function in vivo; altered GPI-AP remodeling (as occurs in the *bst1*Δ mutant) locally depletes both GPI-APs and p24 proteins [26] such that Sec13p is no longer required to scaffold the membrane, By appending strong export signals to the p24 proteins, we can drive these proteins into vesicles independent of GPI-APs, whereupon Sec13p again becomes important. This suggests that local capture of p24 proteins alone can create physical effects that probably stem from their asymmetric distribution and abundance [17, 27].

If local depletion of p24 proteins from vesicles can confer Sec13p bypass, then altering the COPII coat components that confer p24 packaging should similarly yield a *bst* phenotype. The Sec24p paralog, Lst1p/Sfb3p, has been implicated in GPI-AP maturation [26, 28] and direct packaging of p24 proteins [29–31]. We therefore examined the localization of Emp24p-sfGFP in strains lacking this coat adaptor. In an *lst1*Δ strain, Emp24p-sfGFP localized strongly to the bulk ER (Fig. 4A), which we would predict should confer Sec13p bypass. However, *LST1* (for lethal-with-sec-thirteen) disruption is associated with decreased viability of a *sec13-1* strain rather than reversal of lethality [32]. We therefore asked whether a more subtle mutation in Lst1p, corresponding to a known p24-binding site [30], could act as a *BST* mutation and confer viability by virtue of p24 delocalization. When the sole copy of Lst1p contained mutations in the "B-site" that abrogate p24 packaging into COPII vesicles in vitro [30], Emp24p-sfGFP redistributed to the bulk ER (Fig. 4A). Although *lst1-B* was not able to confer viability in a *sec13*Δ background (data not shown), this mutant form of Lst1p restored growth in a strain with mutations in Sec31p (*sec31-DK*; Fig. 4B) that preclude interaction with Sec13p [5], a condition that is normally lethal (Fig. 4B). The *sec31-DK* mutant uncouples Sec13p from the COPII coat but retains other cellular functions of Sec13p (e.g. its role in the nuclear pore in complex with Nup145p), which permits a more robust growth phenotype in the context of *BST* mutations relative to a *sec13*Δ null [5]. Thus, under some permissive conditions, Lst1p B-site mutations can confer Sec13p bypass by preventing efficient local capture of p24 proteins.

We next examined whether ER export of p24 proteins carrying acidic motifs required Lst1p. We observed that p24-DLE and p24-LASLE were punctate even in an *lst1*Δ strain, suggesting that these acidic motifs are recognized predominantly by Sec24p (Fig. 5A). Indeed, the LASLE signal derives from Bet1p, which is unable to bind Lst1p [29]. Thus,

Lst1p appears to be a major driver of ER export of p24 proteins, but Sec24p can substitute if the interaction between Emp24p/Erv25p and Sec24p is strengthened. Moreover, Sec24p can also function in GPI-AP traffic since there is a relatively modest defect in Gas1p maturation in an *lst1*Δ strain (Fig. 5B), suggesting Sec24p compensates. Gas1p maturation was not fully restored in strains expressing p24-DLE and p24-LASLE, and instead more closely resembled that observed in an *lst1*Δ mutant (Fig. 5B). The subtle but significant deficit in GPI-AP trafficking in the p24-DLE and p24-LASLE mutants suggests that even when p24 proteins are restored to ER export by being forced to Sec24p-containing vesicles, GPI-AP trafficking cannot be fully rescued. We propose that GPI-APs require specific functionality associated with Lst1p in order to achieve maximally efficient ER export.

COPII vesicles generated with Lst1p, either alone or in combination with Sec24p, are significantly larger than those generated solely with Sec24p [29, 33]. Perhaps an additional function of Lst1p is to generate larger carriers that can accommodate cargo proteins that favor ceramide-rich membranes such as GPI-APs and the large oligomeric plasma membrane protein, Pma1p, another Lst1p-specific cargo. If Lst1p is required specifically for the generation of larger vesicles capable of accommodating GPI-APs and other cargo proteins with similar requirements, then Lst1p and Sec24p might be physically separated on distinct ER exit sites. We examined the co-localization of Sec24p-mCherry and Lst1p-sfGFP using resolution-limited epifluorescence imaging. Under these conditions, Sec24p was indistinguishable from Lst1p, and deletion of Emp24p had no dramatic effect on distribution of either Sec24p or Lst1p (Fig. 6A). One potential function for Lst1p in generating large COPII carriers could be in altering coat dynamics: larger vesicles require more coat protein to cover the surface area of the vesicle and might therefore require prolonged interaction of the coat with ERES. We tested Sec24p-sfGFP and Lst1p-sfGFP turnover at ERES by fluorescence loss in photobleaching (FLIP) experiments and found no significant differences in the dynamics of the two coat proteins, either in a wild-type background or an *emp24*Δ strain (Fig. 6B). The one difference that we observed was a decrease in the pixel variance for Lst1p-sfGFP in an *emp24*Δ strain relative to wild-type. Pixel variance reports on the cellular distribution of a protein: low variance corresponds to a relatively homogeneous distribution across the cell and high variance corresponds to protein distributed in brighter foci with less background fluorescence. In the case of Lst1p, where pixel variance decreased in an *emp24*Δ strain, the protein most likely became less tightly associated with ERES and more cytoplasmic in distribution, confirming biochemical evidence of reduced Lst1p membrane accumulation in the absence of Emp24p [31]. Finally, we tested whether lack of Lst1p might simply reduce the global abundance of cargo adaptor units such that a critical threshold of inner coat is not attained and larger vesicles are not generated. We overexpressed Sec24p from a multicopy plasmid and tested phenotypes associated with *LST1* deletion. Lethality of a *sec13-1 lst1*Δ strain was not reversed by overexpression of *SEC24*, (Fig. S3A), nor was trafficking of Gas1p restored by overexpression of *SEC24* in a *lst1*Δ null strain (Fig. S3B). In contrast, growth defects associated with trafficking deficits of Pma1p in the absence of Lst1p were rescued by *SEC24* overexpression [32]. This suggests GPI-AP traffic is specifically dependent on Lst1p function independent of cargo adaptor load.

An additional potential function for Lst1p is to confer structural rigidity or altered vesicle geometry to accommodate certain cargoes. Indeed, a structural role for Lst1p could be one basis for the lethality associated with Sec13p dysfunction, since Sec13p likely contributes to membrane bending during vesicle formation [5]. We sought to test this by assessing the ability of Sec31p-Δhinge (Fig. 7A), a rigidified form of Sec31p that cannot bind Sec13p [5], to reverse lethal phenotypes associated with loss of Lst1p. We first further validated our model that Sec31p-Δhinge confers rigidity by adding flexible linker regions of different lengths between the β-propeller and α-solenoid domains of Sec31p. Inserting Gly-Ser repeats of 5, 10 and 20 amino acids conferred a gradient of viability, with the longest flexible linker (Sec31p-GS20) minimally viable (Fig. 7B). Although Sec31p-Δhinge was unable to restore viability to an *lst1*Δ *sec13*Δ strain, a *bst1*Δ *lst1*Δ *sec13*Δ strain was viable when Sec31p-Δhinge was expressed (Fig. 7C). In contrast, overexpression of *SEC24* showed no rescue in any strain (Fig. S3C). Our interpretation of these phenotypes is that rigidity associated with Sec31p-Δhinge cannot compensate for the loss of both Lst1p and Sec13p, but in the context of an additional *bst1*Δ mutation, the combination of reduced membrane bending energy (*bst1*Δ) and enhanced structural rigidity (Sec31p-Δhinge) overcomes a scaffolding deficit, suggesting membrane scaffolding is a partial function of Lst1p. Sec31p-Δhinge also conferred growth in a *sec31*Δ *lst1*Δ double mutant strain where Sec13p cannot interact with Sec31p (Fig. 7D), further suggesting that the combined loss of Lst1p and Sec13p from the COPII coat can be partially overcome by supplementing the coat with additional rigidity.

Discussion

Vesicle formation in vivo is a dynamic process that is capable of adapting to complex physiological conditions. One critical adaptation is responding to changes in cargo abundance and composition such that the appropriate number and size of vesicles are generated to accommodate the specific cargo that transits through the ER. We propose that COPII coat assembly is a stochastic process that possesses intrinsic flexibility to be able to handle diverse cargoes.

In yeast, GPI-APs are abundant components of the cell wall that must be synthesized and trafficked with remarkable efficiency in exponentially growing cells [27]. We previously linked defects in GPI-AP traffic to the bypass-of-sec-thirteen (*BST*) phenotype, whereby the normally essential COPII coat protein, Sec13p, becomes dispensable when GPI-AP traffic is impaired [5]. Here, we attempted to extend this observation to test whether other cargo proteins could similarly influence vesicle budding events. Instead, we find that a requirement for Sec13p is most strongly associated with trafficking of GPI-APs and their receptor complex, the p24 proteins. More specifically, local enrichment of these proteins at sites of vesicle formation (ER exit sites, or ERES) is a primary driver of Sec13p essentiality. Several important properties associated with p24 proteins and their GPI-AP cargo may drive this phenotype. First, both p24 proteins and GPI-APs are asymmetrically distributed across the membrane, which could create local membrane curvature antagonistic to vesicle formation [5, 17, 18]. In addition to physical constraints presented by the proteins themselves, the ceramide-rich membrane domains in which GPI-APs concentrate may also exacerbate a barrier to bending [34]. Finally, both GPI-APs and the p24 proteins are

unusually abundant membrane proteins [27]. Collectively, the predominant p24 complex (Emp24p/Erv25p/Erp1p/Erp2p) in yeast is present at ~130,000 molecules per cell (Saccharomyces Genome Database). This is at least an order of magnitude more abundant than other cargo receptors responsible for ER to Golgi trafficking. The single most abundant GPI-AP, Cwp2, is present at >1.5 million copies per cell, although only a fraction of that mass would be in transit through the ER at any one point in time. Concentration of these abundant, lumenally oriented proteins results in a requirement for Sec13p; when such cargoes are removed from ERES and/or distributed more broadly throughout the ER membrane (ie: *emp24*Δ, *bst1*Δ), vesicle formation can proceed most likely because scaffolding associated with Sec13p is no longer required [5].

The Sec24p paralog, Lst1p, couples GPI-APs and p24 proteins to the COPII coat [30, 31]. *LST1* (for lethal-with-sec-thirteen) was originally isolated in a genetic screen for synthetic lethality with a *sec13-1* temperature sensitive allele [32]. This lethal phenotype is the opposite of the *BST* (Sec13p bypass) phenotype that we would expect for a mutant that causes delocalization of the p24 family. Yet, by specifically abrogating the cargo-binding domain of Lst1p, lethality was transformed into a Sec13p bypass phenotype. That an Lst1p cargo-binding mutant permits Sec13p bypass whereas complete absence of Lst1p exacerbates defects associated with loss of Sec13p suggests that Lst1p performs multiple functions related to vesicle formation. Clearly, one role of Lst1p is to bind p24 proteins to facilitate their enrichment at ER exit sites and capture into COPII vesicles [31]. We propose that Lst1p additionally contributes to the membrane bending energy of the COPII coat, a function that is specifically required for vesicles enriched in "difficult" cargo like p24s and GPI-APs. Indeed, expression of a "rigidified" form of Sec31p, Sec31p-Δhinge, reversed lethal phenotypes associated with *lst1*Δ mutations, suggesting loss of membrane scaffolding is associated with absence of Lst1p. We note that another known substrate for Lst1p is the plasma membrane H+ pump, Pma1p, which is abundant (>1.25 million copies per cell) and found in ceramide-rich membranes [32, 35, 36]. This may be another cargo that has specialized requirements for additional membrane scaffolding that are fulfilled by Lst1p.

Our findings, together with a recent characterization of Emp24p as a lectin that specifically couples GPI-APs to the Lst1p subunit of the COPII coat [31], provide a clear model for how the coat might adapt to these abundant and difficult cargoes. GPI-APs concentrate within the ER upon lipid anchor remodeling, presumably also dependent on the ceramide composition of the ER membrane [19, 26]. Once the glycan portion of the lipid anchor has been remodeled, p24 proteins bind in a manner that triggers some change that permits the specific recruitment of Sec23p/Lst1p to these regions of enrichment [31]. Lst1p functions at least in part to provide additional structural scaffolding to the coat, which is required to overcome the membrane bending energy associated with the concentrated GPI-APs, p24 proteins and perhaps the lipid bilayer itself. Traffic of these "difficult" cargoes is thus uniquely sensitive to loss of Lst1p function and to loss of integrity of the primary coat scaffold, the Sec13p/Sec31p complex. ER export of p24 proteins themselves is more complex, however, since Sec24p can also bind and traffic Emp24p and Erv25p, independent of GPI-AP traffic [30, 31]. Since we do not observe local segregation of Sec24p and Lst1p on distinct ERES, we believe that Lst1p-associated segregation of cargo and altered vesicle architecture likely

operate at the level of individual vesicles, with multiple vesicles generated from each patch of COPII-coated ER. Thus cargo enrichment, receptor binding and coat recruitment correspond to a series of stochastic processes that ultimately result in the local recruitment of the appropriate coat scaffold. As GPI-APs concentrate locally and bind p24 proteins, Lst1p recruitment would be enhanced, thereby facilitating bending of a somewhat rigid membrane and/or altering the diameter of the ensuing vesicle in order to accommodate the specific cargo load.

A long-standing question in ER export is how the canonical COPII coat adapts to handle cargo proteins that cannot be contained in standard 60–80 nm spherical vesicles [7]. We propose that Lst1p-positive vesicles that are enriched in p24 proteins, GPI-APs and large oligomeric membrane proteins like Pma1p [32, 35] are somewhat analogous to carriers that transport collagen and other large structures in mammalian cells [1]. In each case, the physical parameters of the cargo proteins dictate specific requirements, including a larger overall surface area, greater coat rigidity to bend the membrane and delayed scission to promote the creation of larger structures. In the case of GPI-APs, p24 proteins and Pma1p, the "difficult" cargoes link directly to a specialized coat component, Lst1p, that likely influences vesicle architecture. For collagen, an additional layer of communication must exist that signals to the COPII coat that a distinct vesicle geometry is required. Exactly how Lst1p contributes to membrane scaffolding remains to be seen: it could directly influence membrane curvature by virtue of a concave membrane-binding surface [37] or it could alter the geometry of the outer Sec13p/31p scaffold [12] to permit a distinct vesicle architecture [33]. An additional consideration with respect to the size of Lst1p-containing vesicles is the physical nature of the ceramide-rich membranes that Lst1p clients seem to prefer. Although ceramide enrichment may contribute to some requirement for Lst1p, depletion of ceramides still yields large Lst1p vesicles [35], suggesting that vesicle size stems from some intrinsic property of Lst1p. Additional biochemical and morphological dissection of this process is likely to lead to new insight into how cells adapt a canonical coat system to handle non-canonical transport events.

Experimental Procedures

Yeast Strains

All strains were constructed and grown using standard *S. cerevisiae* methods. Strains (Table S1) were made by crossing, sporulation and dissection of tetrads or by PCR-based integration of auxotrophic or drug resistance markers [38]. Strains containing *EMP24* mutations were made by linearization and integration of PRMP304 to the *TRP*1 locus as described previously [25]. 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 S2. 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, pJD058 and pJD065 were made by Quikchange mutagenesis of the PRMP304 integration plasmid [25]. 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 [39] 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 [38], 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

Pulse chase experiments were used to monitor ER-Golgi transport of Gas1p. These experiments were performed as previously described [25]. 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 *sec31*Δ *lst1*Δ 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 [29]. 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 enriched by ultracentrifugation. Samples were analyzed by SDS-PAGE and immunoblot using antibodies against Erv25p, Emp24p and Erv41p followed by detection using chemiluminescence and x-ray film. This method of detection is not

amenable to accurate quantification; budding experiments were performed at least three times and representative images are shown in Figures 2D and S2.

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 [40]. 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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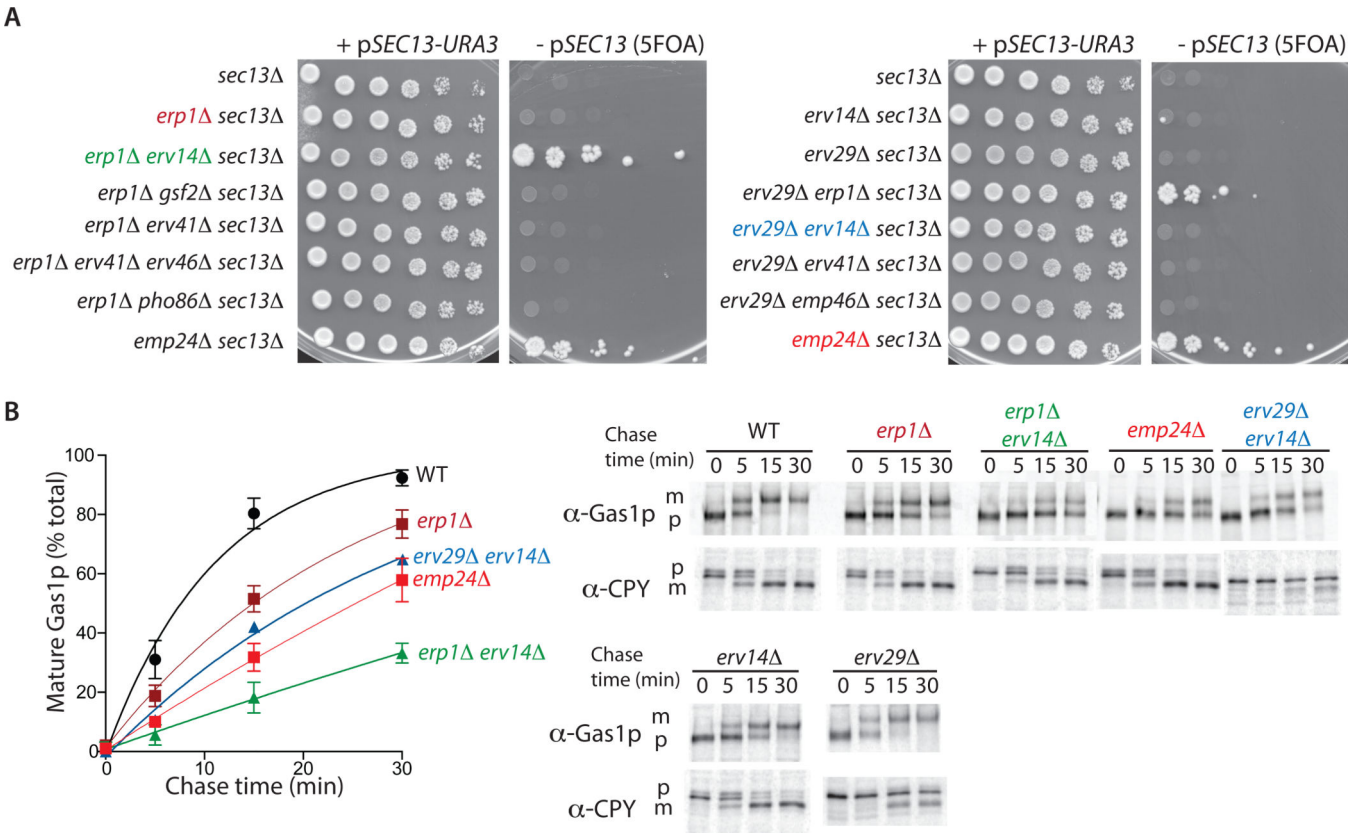

Figure 1. p24 depletion is required for Sec13p bypass

(**A**) Strains containing deletions in the cargo receptors indicated in *bst*-sensitized backgrounds - *sec13*Δ*erp1*Δ (left hand side) or *sec13*Δ*erv29*Δ (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 *erp1*Δ 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 *erp1*Δ *erv14*Δ strain correlated with a robust trafficking delay, compared to an *erp1*Δ single deletant. However, a strong Gas1p trafficking delay did not always correspond to growth in the absence of Sec13p (*erv29*Δ *erv14*Δ).

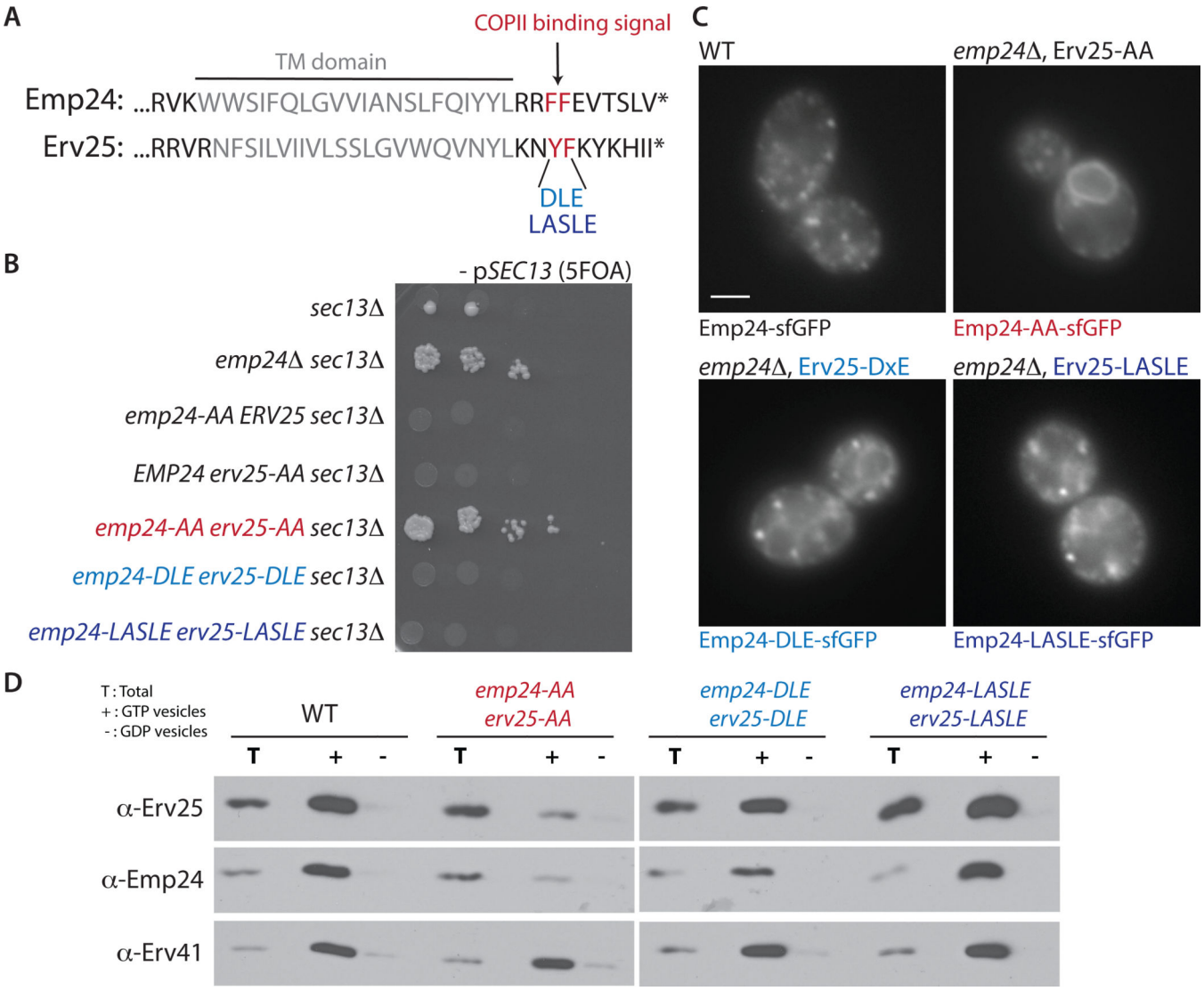

Figure 2. Local concentration of p24 proteins via COPII interaction drives Sec13p-bypass

(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 *sec13*Δ background, whereas the double mutant strain grew as robustly as an *emp24*Δ. 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**) Emp24p and Erv25p capture into COPII vesicles was tested in vitro using microsomal membranes prepared from strains expressing the p24 mutant proteins indicated. Following incubation with COPII proteins and GTP (+) or GDP (−), vesicle fractions were separated from total membranes (T) and cargo proteins detected by immunoblotting 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.

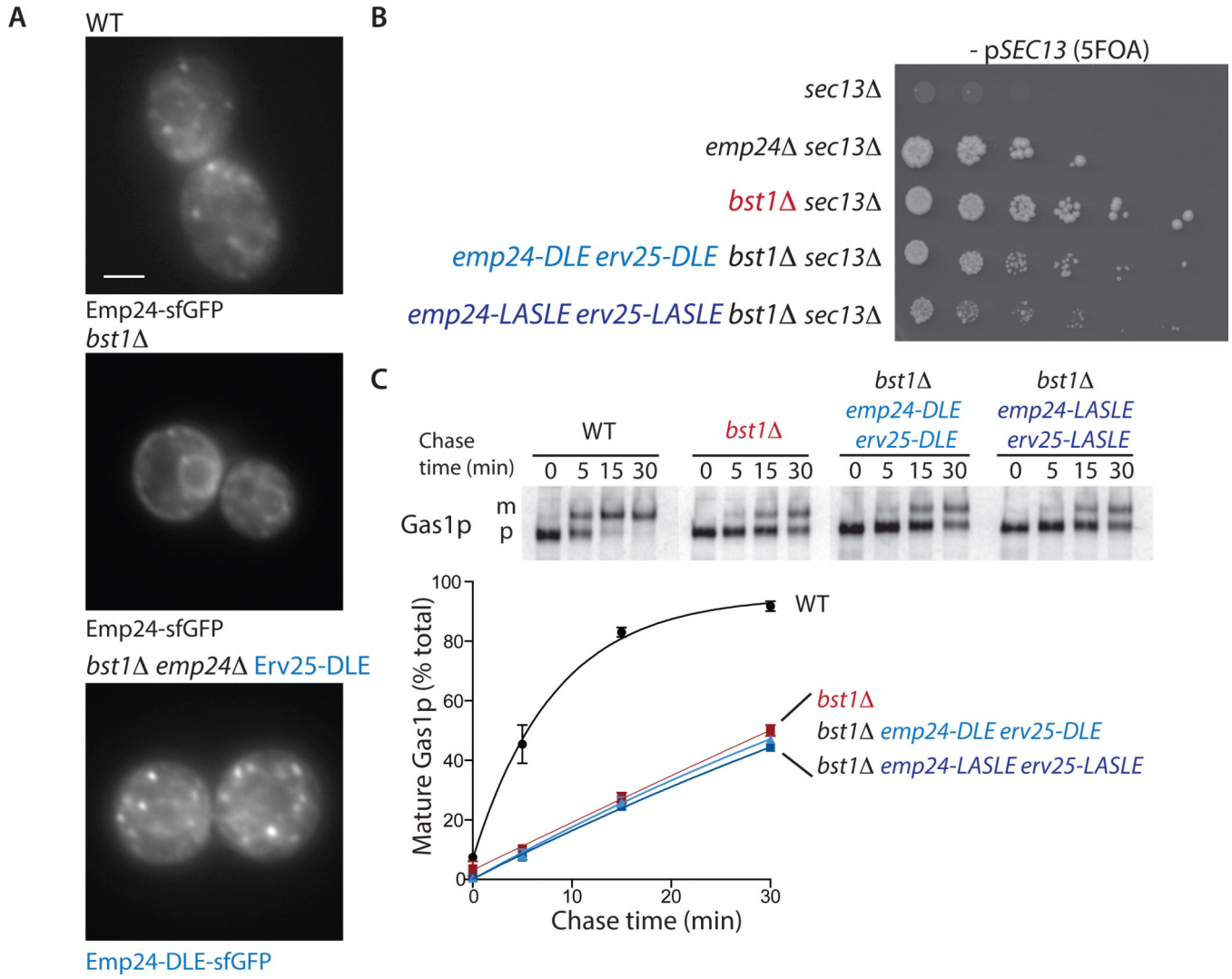

Figure 3. p24 mutants with acidic sorting signals partially reverse *bst1*Δ phenotypes

(**A**) Emp24-sfGFP localization was examined in the strains indicated. In a *bst1*Δ background, Emp24-sfGFP redistributed from punctate ER exit sites (WT, upper panel) to disperse bulk ER (*bst1*Δ, middle panel); substituting acidic residues into Emp24p and Erv25p restored punctate localization to a *bst1*Δ strain (*bst1*Δ *emp24*Δ, lower panel). Scale bar = 2μm. (**B**) The p24 acidic mutants reversed the Sec13p-independent growth of a *bst1*Δ strain. (**C**) Gas1p trafficking remained unchanged in all strains containing a *bst1*Δ, strongly supporting a p24 dependence for the Sec13p-bypass phenomenon. Error bars represent SD; n=3.

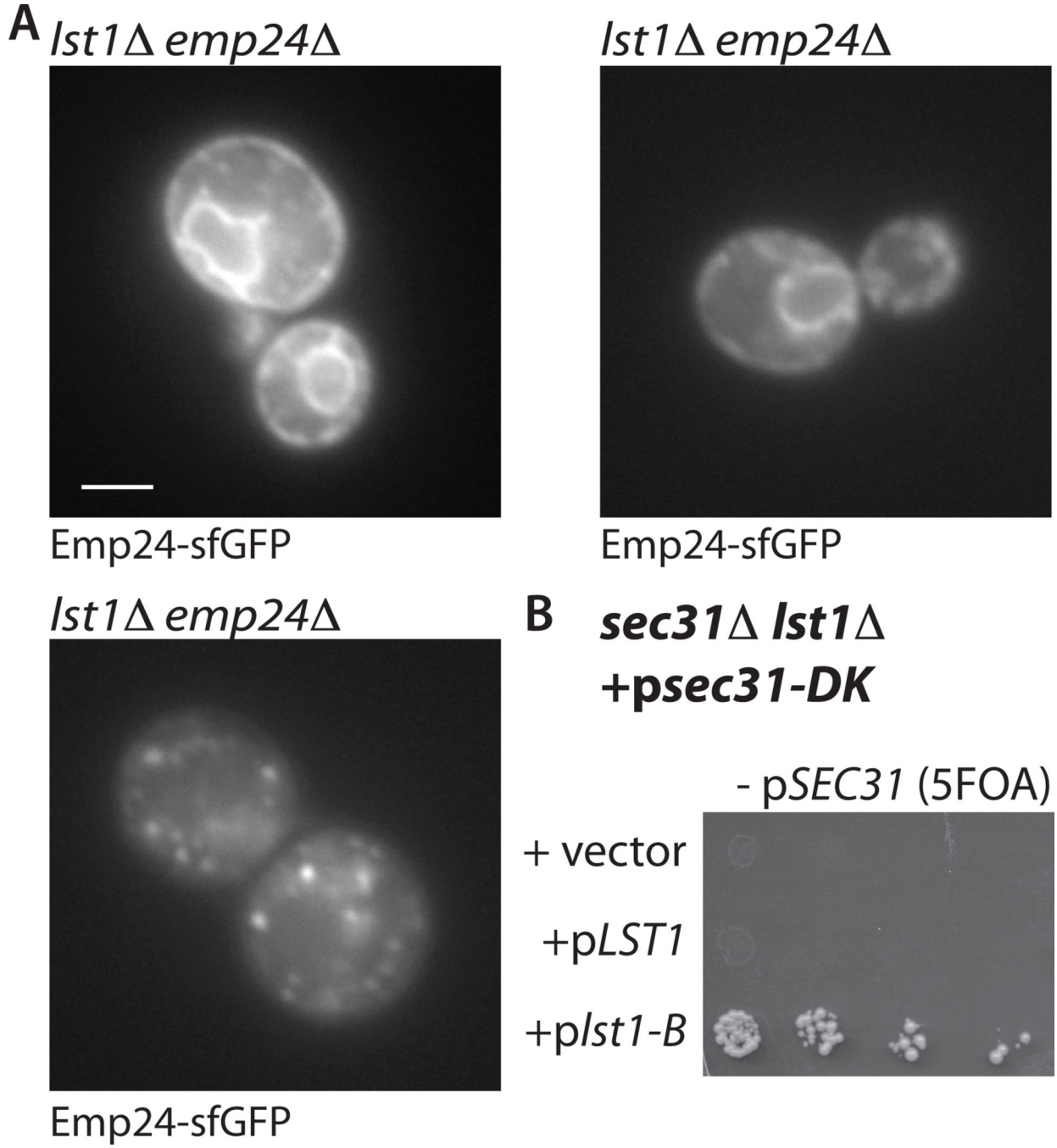

Figure 4. Altering the Sec24p paralog, Lst1p/Sfb3p, to impair capture of p24 proteins also confers Sec13p-bypass

(**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 *sec31*Δ *lst1*Δ 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.

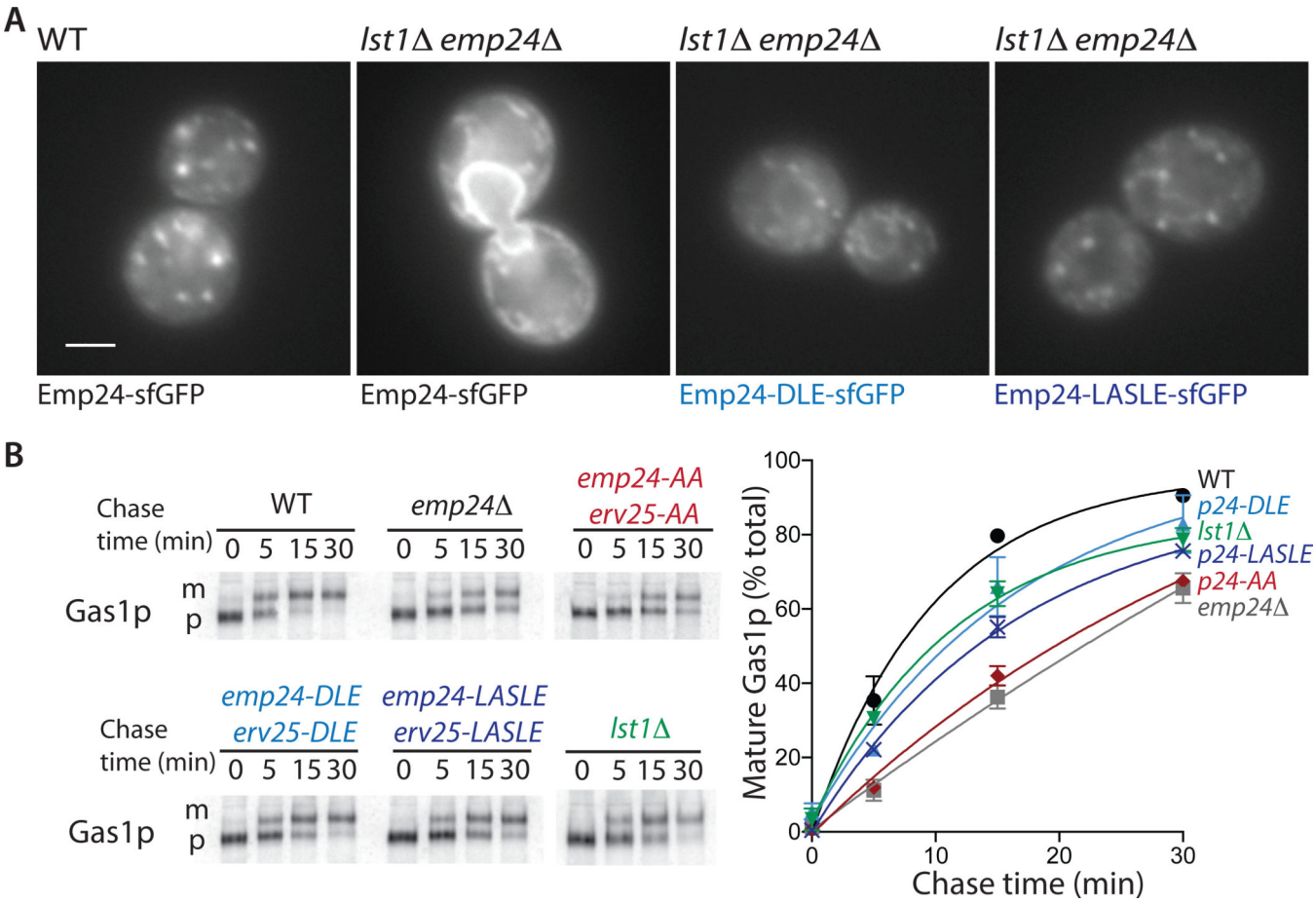

Figure 5. Lst1p contributes multiple functions to p24 traffic

(**A**) Emp24-sfGFP localization was examined in the strains indicated. Punctate localization of Emp24-sfGFP in an *lst1*Δ 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.

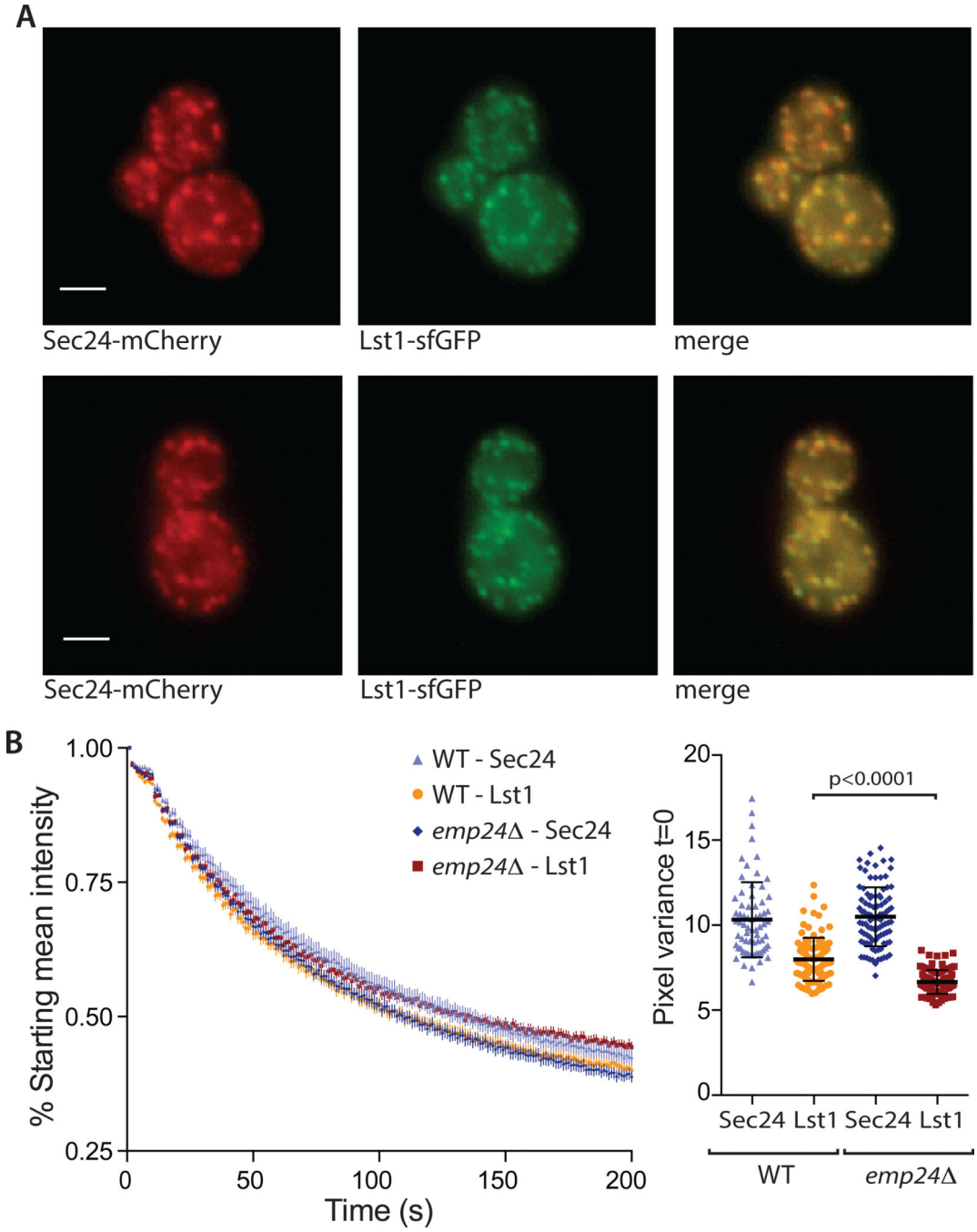

Figure 6. Sec24p and Lst1p exhibit similar localization and dynamics

(A) Sec24-mCherry and Lst1-sfGFP appear to largely overlap at ER exit sites in both wild-type (upper panel) and *emp24*Δ (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.

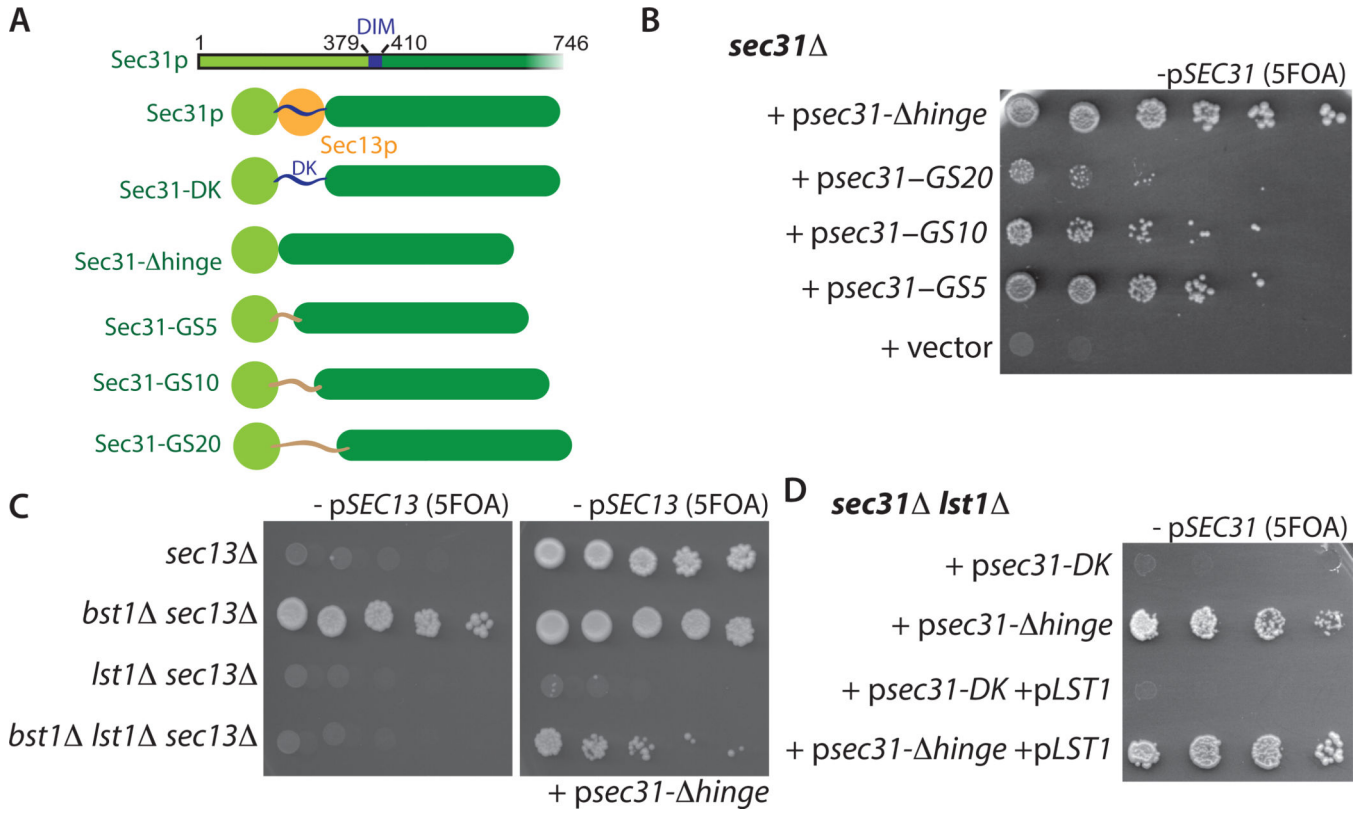

Figure 7. Membrane scaffolding by Sec31p can substitute for Lst1p

(**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 *bst1*Δ *sec13*Δ 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 *bst1*Δ *lst1*Δ *sec13*Δ strain. (**D**) In a *sec31*Δ *lst1*Δ 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*).