Sit4p/PP6 regulates ER-to-Golgi traffic by controlling the dephosphorylation of COPII coat subunits

Deepali Bhandaria, Jinzhong Zhangb, Shekar Menona, Christopher Lorda,c, Shuliang Chenb, Jared R. Helmb, Kevin Thorsenb, Kevin D. Corbettb, Jesse C. Hayb, and Susan Ferro-Novicka

aDepartment of Cellular and Molecular Medicine, Howard Hughes Medical Institute, and bDepartment of Cellular and Molecular Medicine, Ludwig Institute for Cancer Research, University of California at San Diego, La Jolla, CA 92093; cDivision of Biological Sciences, University of Montana, Missoula, MT 59812

ABSTRACT Traffic from the endoplasmic reticulum (ER) to the Golgi complex is initiated when the activated form of the GTPase Sar1p recruits the Sec23p-Sec24p complex to ER membranes. The Sec23p-Sec24p complex, which forms the inner shell of the COPII coat, sorts cargo into ER-derived vesicles. The coat inner shell recruits the Sec13p-Sec31p complex, leading to coat polymerization and vesicle budding. Recent studies revealed that the Sec23p subunit sequentially interacts with three different binding partners to direct a COPII vesicle to the Golgi. One of these binding partners is the serine/threonine kinase Hrr25p. Hrr25p phosphorylates the COPII coat, driving the membrane-bound pool into the cytosol. The phosphorylated coat cannot rebind to the ER to initiate a new round of vesicle budding unless it is dephosphorylated. Here we screen all known protein phosphatases in yeast to identify one whose loss of function alters the cellular distribution of COPII coat subunits. This screen identifies the PP2A-like phosphatase Sit4p as a regulator of COPII coat dephosphorylation. Hyperphosphorylated coat subunits accumulate in the sit4Δ mutant in vivo. In vitro, Sit4p dephosphorylates COPII coat subunits. Consistent with a role in coat recycling, Sit4p and its mammalian orthologue, PP6, regulate traffic from the ER to the Golgi complex.

INTRODUCTION

Proteins destined to traffic through the secretory pathway are sorted into transport vesicles before they tether and fuse to their acceptor membrane (Whyte and Munro, 2002). Tight regulation of these events is required for efficient cargo transport and the maintenance of organelle identity. Genetic and biochemical studies in the yeast Saccharomyces cerevisiae play a major role in identifying the highly conserved components of the secretory apparatus and elucidating the mechanistic details of the first step in the pathway, anterograde transport between endoplasmic reticulum (ER) and Golgi complex (Lord et al., 2013).

The generation of ER-derived transport vesicles begins when the activated form of the GTPhase Sar1p recruits the inner shell of the COPII coat, the Sec23p-Sec24p complex. The coat inner shell sorts cargo into the vesicle before recruiting the outer shell of the coat, the Sec13p-Sec31p complex. These events lead to the hydrolysis of GTP on Sar1p and vesicle budding (Lee et al., 2005; Zanetti et al., 2011). After Sar1p is released from the vesicle, TRAPPI, a multimeric guanine nucleotide exchange factor (GEF), binds to the coat via interaction with Sec23p and recruits the Rab GTPase Ypt1p to the vesicle (Cai et al., 2007; Lord et al., 2011). Subsequently, activated Ypt1p binds to its effector Uso1p, a long coiled-coil tether that links the vesicle to the Golgi (Lord et al., 2011). Once the vesicle tethers to the Golgi, Hrr25p, a serine/threonine kinase, phosphorylates the inner shell of the COPII coat. Eventually, the coat is released and the vesicle fuses with the Golgi. Through sequential interactions with different binding partners (Sar1p-GTP, TRAPPI, and Hrr25p), the coat subunit Sec23p mediates the directionality of these events (Lord et al., 2011).
RESULTS
Identification of a phosphatase that alters the intracellular distribution of COPII coat subunits

We previously showed, by differential fractionation, that phosphorylation of Sec23p drives it from membranes into the cytosol (Lord et al., 2011). In an attempt to identify a phosphatase that dephosphorylates the COPII coat, we screened known phosphatase mutants in the yeast deletion library for a change in the distribution of Sec23p, using differential fractionation as an assay.

The yeast phosphotome contains ∼32 members, which are largely divided into four families: serine/threonine phosphoprotein phosphatases (PPP), magnesium-dependent serine/threonine phosphatases (PPM), protein tyrosine phosphatases (PTP), and dual-specificity phosphatases (DSP), which can dephosphorylate phosphoserine/phosphothreonine, as well as phosphotyrosine residues (Breitkreutz et al., 2010). Only three (Glc7, Cdc14, and Sus72) of these 32 phosphatases are essential for growth in yeast (Figure 1A). None of the temperature-sensitive (ts) mutants in the essential phosphatases, including Glc7p (Supplemental Figure S1A), which was previously implicated in ER-to-Golgi traffic (Bryant and James, 2003), showed a change in the distribution of Sec23p. Of the 29 nonessential phosphatases screened from the library, an increase in the cytosolic pool of Sec23p was observed only in the sit4Δ mutant. This phenotype was confirmed when Sit4p was deleted in our laboratory strain background (Figure 1B, left).

Next we examined the distribution of the other known phosphorylated coat subunits, Sec24p and Sec31p, in the sit4Δ mutant (Salama et al., 1997; Lord et al., 2011). Sec24p, the cargo adaptor of the COPII coat, has two paralogues, Lst1p and Iss1p (Roberg et al., 1999; Shimoni et al., 2000). Available antibodies to Lst1p enabled us to also examine the distribution of this coat subunit. The cytosolic pools of Sec23p and Sec31p were increased in sit4Δ cells (Figure 1B, left, and Supplemental Figure S1B) but not in pph21Δ cells, another phosphatase mutant (Figure 1B, right, and Supplemental Figure S1C). Like Sit4p, Pph21p is a member of the PPP phosphatase family (Figure 1A). Of interest, Lst1p and Sec31p, two highly phosphorylated coat subunits (Stark et al., 2010), appeared to be less stable and migrate more slowly in lysates prepared from the sit4Δ mutant (Figure 1B, compare lanes 1 and 4). When Sit4p was overexpressed, Lst1p (Figure 1C, left) and Sec31p (Figure 1C, right) migrated faster. This increase in mobility was most prominent as the level of Sit4p expression increased (Figure 1C, bottom). Together these findings show that Sit4p, a type 2A serine/threonine phosphatase, regulates the intracellular distribution of COPII coat subunits, as well as the mobility of Sec31p and Lst1p on SDS–polyacrylamide gels.

In vitro transport studies reveal that phosphorylation of the COPII coat is required for vesicle fusion, whereas its dephosphorylation is needed to initiate a new round of vesicle budding (Lord et al., 2011). In an effort to identify the phosphatase that dephosphorylates the COPII coat, we screened mutants in known protein phosphatases for defects in the intracellular distribution of COPII coat subunits. Here we report that Sit4p, a serine/threonine phosphatase, is a key regulator of COPII coat dephosphorylation. In the sit4Δ mutant, COPII coat subunits become hyperphosphorylated and their subcellular distribution is altered. In vitro, Sit4p dephosphorylates coat subunits. Consistent with a role in coat recycling, Sit4p and its mammalian orthologue, PP6, are required for ER-to-Golgi traffic.
Sit4p dephosphorylates Lst1p and Sec31p in vivo and in vitro

To address directly whether the shift in mobility of Lst1p and Sec31p in the sit4Δ mutant is the consequence of hyperphosphorylation of these coat subunits, we treated sit4Δ lysates with calf intestinal alkaline phosphatase (CIP) and analyzed the mobility of these coat subunits on a low-percentage polyacrylamide gel. Both Lst1p and Sec31p migrated faster after CIP treatment (Figure 2A, left, compare lanes 4 and 5). This shift in mobility was not observed when EDTA, a known inhibitor of CIP (Whisnant and Gilman, 2002), was present during the incubation (Figure 2A, left, compare lanes 4–6), or when wild-type lysate was treated with CIP (Figure 2A, left, compare lanes 1–3). We also immunoprecipitated Lst1p and Sec31p from wild-type and sit4Δ-mutant lysates and treated them with CIP (Figure 2A, right, and Supplemental Figure S2A). Interestingly, although we could not easily detect phosphorylation of Lst1p in wild-type lysates, a small but reproducible shift was observed when we treated immunoprecipitated Lst1p with CIP (Figure 2A, right). This shift was more pronounced when Lst1p was immunoprecipitated from the sit4Δ mutant (Figure 2A, right, compare lanes 3 and 4). Together these findings support the proposal that Lst1p and Sec31p are hyperphosphorylated in the sit4Δ mutant.

If Sit4p dephosphorylates Lst1p and Sec31p, it may directly bind to these coat subunits in vitro. As shown in Figure 2B, hexahistidine (His6)-Sit4p bound to glutathione S-transferase (GST) fusions of Lst1p and Sec31p (amino acids [aa] 1–500 and 1115–1273) but not to GST and GST-Sec31p (aa 501–878 and 879–1114). His6-Sit4p also failed to bind to the coat subunit Sec13p (Figure 2B), which is not phosphorylated (Salama et al., 1997). Thus Sit4p binds to coat subunits that are hyperphosphorylated in the sit4Δ mutant. To determine whether Sit4p dephosphorylates Sec31p and Lst1p in vitro, we first phosphorylated GST-Lst1p and GST-Sec31p (aa 1–500) with His6-Hrr25p. Slower-migrating bands were seen only when these fusion proteins were incubated with His6-Hrr25p but not kinase-dead Hrr25p K38R (Figure 2C, lanes 1–3). Both GST-Lst1p and GST-Sec31p (aa 1–500) are substrates for Hrr25p in vitro, as 32P was incorporated into these fusion proteins when they were incubated with the kinase and [γ-32P]ATP in kinase assay buffer (Supplemental Figure S2B). In addition, the more dramatic shift that we observed on SDS-polyacrylamide gels with Lst1p made it possible for us to use the hrr25-5 mutant to demonstrate that this coat subunit is also a substrate of Hrr25p in vivo. These data and the isolation of the hrr25-5 mutant are described in the Supplemental Material (Supplemental Figure S2C and Table S2). Because bacterially expressed Sit4p is not active in vitro (unpublished observations), we immunoprecipitated Sit4p-HA3 from a yeast lysate and used the immunopurified phosphatase to dephosphorylate

![Figure 2](Image)

**Figure 2:** Sit4p dephosphorylates Lst1p and Sec31p in vitro. (A) Left, lysates prepared from wild type (SFNY 1841) and the sit4Δ mutant (SFNY 2045) were incubated at 37°C for 15 min (lanes 1, 4) with CIP (lanes 2, 5) or CIP and EDTA (lanes 3, 6), before they were analyzed on a 6% SDS–polyacrylamide gel by Western blot analysis. Right, same as left, except that the samples were immunoprecipitated before CIP treatment. The asterisk marks the hyperphosphorylated form of Lst1p and Sec31p. (B) Top, equimolar amounts (0.2 μM) of GST, GST-Lst1p, and GST-Sec13p were incubated with Sit4p (0.2 μM), washed, and analyzed by Western blot analysis using anti-His antibody. GST and GST-Sec13p served as negative controls. Bottom, equimolar amounts of GST, GST-Sec31p (aa 1–500, 501–878, 879–1114, 1115–1273), and GST-Sec13p were incubated with Sit4p (0.2 μM) and processed as described. (C) Glutathione–Sepharose beads containing GST-Lst1p and GST-Sec31p (aa 1–500) were incubated without (lane 1) or with His6-Hrr25p K38R (lane 2) or His6-Hrr25p (lanes 3–6) in kinase assay buffer at 30°C for 1 h. The beads were washed and resuspended in phosphatase assay buffer without (lanes 1–4) or with Sit4p-HA3 (lanes 5 and 6) for 1 h at 30°C. EDTA was added (lane 6) to inhibit phosphatase activity. The samples were then analyzed on a 6% SDS–polyacrylamide gel by Western blot analysis.
GST-Sec23p and GST-Sec24p in vitro (Supplemental Figure S2D), suggesting that these coat subunits are also substrates of this phosphatase. This proposal is consistent with the observation that the soluble pool of Sec23p and Sec24p increases in the sit4Δ mutant (Figure 1B, left).

The loss of Sit4p function disrupts COPII vesicle budding in vitro and ER-to-Golgi traffic in vivo

If Sit4p regulates the dephosphorylation of the COPII coat in vivo, the loss of Sit4p function should disrupt secretion. To begin to address this possibility, we compared the trafficking of proteins secreted into the medium in the sit4Δ mutant and wild type. Briefly, wild-type and mutant cells were pulse labeled with S35-ProMix for 6, 8, 10, and 15 min, and proteins secreted into the medium were analyzed on an SDS–polyacrylamide gel. Between 8 and 15 min, several bands were secreted into the medium in wild-type cells. Four of these bands (marked by asterisks) were absent in the sit4Δ mutant at 15 min (Figure 3A, left). Quantitation of one of these bands, p150, is shown in Figure 3A (right). Several other bands were either secreted normally (arrow) or more slowly (arrowheads) in the sit4Δ mutant (Figure 3A, left). Together these findings indicate that secretion of different proteins is delayed in the sit4Δ mutant.

To address whether the defect in secretion is a consequence of disrupting membrane traffic between the ER and Golgi complex, we monitored the trafficking of the vacuolar hydrolase carboxypeptidase Y (CPY). CPY traffics from the ER (p1 CPY) to the Golgi (p2 CPY) before it is proteolytically processed to the mature form (mCPY) in the vacuole (Stevens et al., 1982). When the processing of CPY was monitored in the sit4Δ mutant at 0, 5, 10, and 15 min and compared with wild type, a delay in the conversion of p1 to p2 CPY was observed (Figure 3B, left). Quantitation of this trafficking defect from three separate experiments revealed a significant delay in the conversion of p1 to p2 CPY at 5 min (Figure 3B, right). Whereas p1 CPY was not apparent at 10 and 15 min in the wild type, it lingered in the mutant. Together these findings indicate that membrane traffic between ER and Golgi complex is kinetically delayed in the absence of the phosphatase Sit4p.

It was reported that phosphorylated COPII coat proteins cannot bind to membranes (Dudognon et al., 2004). A prediction of this observation is that COPII vesicle budding should be disrupted in the sit4Δ mutant. To test this prediction, we measured the budding of COPII vesicles from the ER in vitro by monitoring the release of the cargo marker pro-α-factor from the ER (Ruohola et al. 1988; Lian and Ferro-Novick, 1993). Interestingly, sit4Δ-mutant fractions showed a decrease in vesicle budding when compared with wild type.
Sit4p interacts with Hrr25p in yeast and mammalian cells

Phosphatases are frequently found in complexes with kinases that function together in the same process (Breitkreutz et al., 2010). In differential fractionation studies, however, Hrr25p and Sit4p do not cofractionate with each other. Hrr25p largely fractionates with membranes (Figure 4A, left; Lord et al., 2011), whereas Sit4p is largely soluble (Figure 4A, left). These differential fractionation studies were performed in the absence of salt to maintain the association of Hrr25p with membranes. Therefore, to address whether Sit4p and Hrr25p interact with each other under physiological conditions, we immunoprecipitated Sit4p-HA3 from lysates prepared in the presence of 150 mM NaCl. As shown in Figure 4A (right), Hrr25p (lane 3) specifically coimmunoprecipitated with Sit4p-HA. (B) Left, total lysates prepared from HeLa (lanes 1–3) or COS-7 (lanes 4–6) cells were centrifuged at 150,000 × g to generate supernatant (S) and pellet (P) fractions. CK1δ was mostly membrane bound (Figure 4B, left), whereas CK1δ-PP6C, its orthologue, PP6, was largely cytosolic in both HeLa and COS-7 cells, whereas CK1δ was mostly membrane bound (Figure 4B, left, 2011). The defect in membrane fusion may be the consequence of blocking the cycling of the coat on and off membranes. A similar result was obtained when phosphomimetic mutations in Sec23p were analyzed in vitro (Lord et al., 2011).

Components of the secretory apparatus that function in ER-to-Golgi traffic are generally essential for growth (Novick et al., 1980). Although loss of Sit4p leads to a severe growth defect, it is not essential for growth in our laboratory strain background. The growth defect associated with the loss of SIT4 varies in different strain backgrounds depending on which allele of the polymorphic gene SSD1 (Suppressor of SIT4 deletion) is expressed (Sutton et al., 1991). Some alleles of SSD1 allow for the growth of sit4Δ strains, whereas other alleles lead to their inviability. In our strain background, the loss of Sit4p leads to slow growth (Supplemental Figure S3A). When, however, we deleted both SSD1 and SIT4 (ssd1Δsit4Δ) in the same haploid strain, the cells died (Supplemental Figure S3B). This observation suggests that our laboratory strain contains an allele of SSD1 that supports growth and secretion in the sit4Δ mutant.

Sit4p interacts with Hrr25p in yeast and mammalian cells

Previous studies demonstrated that the role of CKIδ, the mammalian orthologue of Hrr25p, is conserved in mammalian cells (Yu and Roth, 2002; Lord et al., 2011). Next we wanted to determine whether the role of Sit4p is also conserved. As was shown for Sit4p, its orthologue, PP6, was largely cytosolic in both HeLa and COS-7 cells, whereas CK1δ was mostly membrane bound (Figure 4B, left,
PP6, the orthologue of Sit4p, is required for ER-to-Golgi traffic in mammalian cells

To address whether PP6 is required for the subcellular distribution of COPII subunits, we used small interfering RNA (siRNA) to deplete cells of PP6 and then examined the distribution of Sec31A. Differential fractionation was performed on COS-7 cells (Figure 5A, middle) transfected with mock or siRNA targeted against PP6 mRNA (Figure 5A, left, and Supplemental Figure S4B), and lysates were separated into supernatant and pellet fractions. As shown in Figure 5A (middle; quantituated on the right), Sec31A distributed between the supernatant and membrane fractions in mock-treated cells (lanes 1–3), whereas in depleted cells it was largely present in the supernatant (lanes 4–6), suggesting that PP6 regulates the recycling of Sec31A on membranes. The loss of PP6 caused a more dispersed punctate localization pattern for the COPII coat (Figure 5B; compare Mock with siPP6-08, described in Materials and Methods; Zeng et al., 2010). This defect was specifically due to the loss of PP6, as it was rescued by the expression of a knockdown-resistant construct of PP6 (Figure 5B; compare siPP6-08 with Rescue). Similar results were obtained with a second siRNA duplex, siPP6-07 (Supplemental Figure S4C).

To determine whether PP6 regulates membrane traffic, we monitored the trafficking of a temperature-sensitive form of vesicular stomatitis virus G (VSV-G) protein (tsO45 VSV-G–green fluorescent protein [GFP]) to the cell surface in mock and PP6 COPII–depleted cells (target sequence, siPP6-08; Supplemental Figure S4D and Figure 6A). To accumulate misfolded tsO45 VSV-G–GFP in the ER, we shifted cells to 40°C and added cycloheximide before shifting the cells to 32°C, a temperature that allows tsO45 VSV-G–GFP to fold into a conformation that is compatible with export from the ER. Trafficking of VSV-G to the cell surface was then monitored by immunofluorescence for 30, 60, and 90 min, using an antibody specific for the ectodomain of VSV-G. As shown in Figure 6A, delivery of VSV-G to the cell surface was delayed in PP6-depleted but not mock-treated cells. Figure 6B shows representative images of surface VSV-G and total VSV-G at 60 min.

To address whether VSV-G traffic was delayed between the ER and Golgi complex in depleted cells, we examined the intracellular pool of VSV-G at 0, 5, and 10 min after shifting the cells to 32°C. At 0 min, VSV-G resided in the ER in mock and depleted cells. By 5 min, some VSV-G reached the Golgi in the mock-treated cells, but delivery was delayed in the depleted cells. By 10 min, most of the VSV-G had reached the Golgi in mock-treated but not mock-treated cells. Figure 6B shows representative images of surface VSV-G and total VSV-G at 60 min.

Consistent with previous studies localizing CK1δ to the Golgi (Milne et al., 2001), CK1δ was found in the perinuclear region of cells and colocalized with the cis-Golgi marker GM130 (Figure 4C, top). Although the trafficking machinery that mediates ER-to-Golgi traffic is highly conserved from yeast to humans, the architecture of the pathway is different. In yeast, COPI vesicles fuse with the Golgi, whereas in mammalian cells, COPII vesicles fuse with a pre-Golgi compartment that matures into the Golgi (Lord et al., 2013). Consistent with the proposal that CK1δ is required for COPII vesicle fusion (Lord et al., 2011), we found it localized to a greater degree with ERGIC-53, which marks the pre-Golgi compartment (Figure 4C, bottom). To determine whether CK1δ and PP6 interact with each other, we prepared lysates in the presence of 150 mM NaCl and precipitated the endogenous copy of PP6 with anti-PP6 antibody from HeLa cell lysates transiently expressing myc-CK1δ. When the precipitates were analyzed by Western blot analysis, PP6 antibody, but not immunoglobulin G (IgG), precipitated CK1δ (Figure 4B, right). The TRAPP II subunit mTrs120 was not present in the precipitate (Figure 4B, right), indicating that CK1δ specifically coprecipitates with PP6.

FIGURE 5: PP6 regulates the intracellular distribution of Sec31. (A) Left, COS-7 cells transfected with mock or PP6 siRNA (siPP6-08) were harvested 72 h posttransfection and immunoblotted with anti-PP6 antibody (top). Actin (bottom) was used as a loading control. Quantitation of PP6 depletion in three separate experiments is shown in Supplemental Figure S4B. Error bars represent SEM. N = 3. Middle, total (T) lysates from either mock (lanes 1–3) or PP6-depleted (lanes 4–6) cells were centrifuged at 150,000 × g to generate supernatant (S) and pellet (P) fractions. Calnexin was used as a fractionation control. Right, quantitation of the supernatant:pellet ratio of Sec31A from three separate experiments. Error bars represent SEM. N = 3. *p < 0.05, Student’s t test. (B) COPII fragments in PP6-depleted cells. COS-7 cells were transfected with mock, PP6 siRNA-08, or PP6 siRNA-08 and pCMV-Myc-hPPP6C-m3-08 (Materials and Methods). The cells were immunostained with anti-COP1 antibody (left) and the number of fragmented structures quantified (right). Error bars represent SEM. N > 100 cells in three independent experiments. ***p < 0.001, Student’s t test. Scale bar, 20 μm.

and Supplemental Figure S4A). Consistent with previous studies localizing CK1δ to the Golgi (Milne et al., 2001), CK1δ was found in the perinuclear region of cells and colocalized with the cis-Golgi marker GM130 (Figure 4C, top). Although the trafficking machinery that mediates ER-to-Golgi traffic is highly conserved from yeast to humans, the architecture of the pathway is different. In yeast, COPII vesicles fuse with the Golgi, whereas in mammalian cells, COPII vesicles fuse with a pre-Golgi compartment that matures into the Golgi (Lord et al., 2013). Consistent with the proposal that CK1δ is required for COPII vesicle fusion (Lord et al., 2011), we found it localized to a greater degree with ERGIC-53, which marks the pre-Golgi compartment (Figure 4C, bottom). To determine whether CK1δ and PP6 interact with each other, we prepared lysates in the presence of 150 mM NaCl and precipitated the endogenous copy of PP6 with anti-PP6 antibody from HeLa cell lysates transiently expressing myc-CK1δ. When the precipitates were analyzed by Western blot analysis, PP6 antibody, but not immunoglobulin G (IgG), precipitated CK1δ (Figure 4B, right). The TRAPP II subunit mTrs120 was not present in the precipitate (Figure 4B, right), indicating that CK1δ specifically coprecipitates with PP6.

PP6, the orthologue of Sit4p, is required for ER-to-Golgi traffic in mammalian cells

To address whether PP6 is required for the subcellular distribution of COPII subunits, we used small interfering RNA (siRNA) to deplete
To determine whether PP6 is required for the transport of endogenous cargoes between the ER and Golgi complex, we examined the recovery of two integral Golgi membrane proteins after washout of brefeldin A (BFA), an assay that recapitulates all steps in ER-to-Golgi transport and Golgi biogenesis. Two Golgi markers were simultaneously monitored: GOS-28, a Golgi-restricted soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) that is present in all cisternae, as well as in intra-Golgi vesicles (Orci et al., 2000); and giantin, a tail-anchored, cis-Golgi membrane protein that interacts with the Golgi matrix but redistributes to the ER in BFA (Miles et al., 2001). Both Golgi markers displayed compact juxtanuclear localization in untreated COS-7 cells and dispersed into punctate and fine cytoplasmic reticular structures subsequent to a 1-h incubation with BFA. These behaviors were indistinguishable in mock and PP6-depleted cells (Supplemental Figure S5, untreated and 0-min washout). On washout of BFA, the pronounced juxtanuclear accumulation returned, with giantin reaccumulating more quickly and completely than GOS-28. Of importance, for both markers, mock-transfected cells reaccumulated juxtanuclear labeling more rapidly than their siPP6-transfected counterparts (Supplemental Figure S5, 75-min washout). This difference was quantifiable for giantin by measuring an objective parameter, the pronounced concentration of Golgi fluorescence (Figure 7B). Because GOS-28 labeling produced lower-contrast juxtanuclear accumulations, objective quantification was more difficult. The BFA results demonstrate that the requirement for PP6 in ER-to-Golgi transport extends to the constitutive export of endogenous cargoes. Together these findings imply that the role of Sit4 in ER-to-Golgi traffic is evolutionarily conserved.

**DISCUSSION**

The serine/threonine kinase Hrr25p was first implicated in membrane traffic when a mutation in hrr25 was identified as a suppressor of the growth and secretion defect of the temperature-sensitive sec12-4 mutant, which blocks the budding of COPII vesicles from the ER (Murakami et al., 1999). This led to the proposal that Hrr25p is a negative regulator of vesicle budding. More recently, we showed that Sec23p and Sec24p are substrates of Hrr25p and phosphorylation of the coat drives the membrane-bound pool into the cytosol (Lord et al., 2011). When Hrr25p activity is disrupted in vitro with the ATP competitive inhibitor IC261, vesicle budding is stimulated, suggesting that an increase in the unphosphorylated pool of the coat enhances budding. Together these findings imply that dephosphorylation of the COPII coat by a phosphatase is needed to recycle the coat for a new round of membrane traffic. To achieve a complete understanding of how phosphorylation and dephosphorylation regulate the membrane association of the COPII coat complex, it is important to identify the phosphatase that dephosphorylates the coat.

To identify this phosphatase, we screened ts and deletion mutants of the known phosphatases in yeast for changes in the distribution of the COPII coat subunit Sec23p. This screen identified one phosphatase, Sit4p, a member of the serine-threonine PPP family. These phosphatase family members contain one catalytic subunit and several regulatory subunits. Sit4p is the catalytic subunit of a multisubunit phosphatase complex that contains four Sit4p-associated proteins, called Sap4p, Sap155p, Sap185p, and Sap190p, which function positively with Sit4p (Luke et al., 1996). We found that the catalytic subunit of the complex, Sit4p, binds directly to COPII coat subunits. Phosphatase activity, however, may depend on its association with the Saps, as we were unable to express active Sit4p in bacteria. To show that Sit4p dephosphorylates the COPII coat, we focused on two of the more heavily phosphorylated COPII coat subunits. Phosphatase activity, however, may depend on its association with the Saps, as we were unable to express active Sit4p in bacteria. To show that Sit4p dephosphorylates the COPII coat, we focused on two of the more heavily phosphorylated COPII coat subunits, Lst1p and Sec31p (PhosphoGRID; Stark et al., 2000). We found that hyperphosphorylated forms of Lst1p and Sec31p accumulate in the sit4A mutant in vivo and demonstrated thatSit4p dephosphorylates Hrr25p-phosphorylated forms of Lst1p and Sec31p in vitro. Sit4p also binds directly to Sec23p and Sec24p, and loss of Sit4p increases the cytosolic pool of these coat subunits, suggesting that Sit4p may dephosphorylate both Sec23p and Sec24p.

COPII coat subunits are present in the cytosol and on ER-derived vesicles. It was postulated that the vesicular pool is selectively phosphorylated by Hrr25p, an orthologue of CKIδ (Lord et al., 2011). How this selectivity is achieved is unknown. The cytosolic phosphatase Sit4p may play a role in ensuring that coat subunits, prematurely phosphorylated in the cytosol, are rapidly dephosphorylated.
FIGURE 7: PP6 is required for ER-to-Golgi traffic in mammalian cells. (A) Left, COS-7 cells transfected with mock or PP6 siRNA (siPP6-08) were transfected with tsO45VSV-G-GFP and incubated at 40°C for 20 h. Cells were then treated with cycloheximide (100 μg/ml final concentration) for 30 min and shifted to 32°C for 0, 5, and 10 min, permeabilized with 0.1% Triton X-100, and immunostained with anti-GM130 antibody (red). Right, data shown on the left quantitated as described in Thayanidhi et al. (2010). Error bars represent the SEM. N = 3. More than 20 cells were examined for each time point in three separate experiments in which ∼89% of the PP6 was depleted. ***p < 0.001, Student’s t test. Scale bar, 20 μm. (B) COS-7 cells were transfected with PP6 siRNA or mock transfected with Lipofectamine reagent alone and grown for 3 d. Cells were then either fixed directly (untreated) or incubated 1 h with BFA, followed by fixation (0-min washout) or recovery in medium without BFA for 75 min before fixation (75-min washout). Quantitation of pronounced giantin Golgi intensity (Materials and Methods) from >125 randomly selected cells for each plotted value. Y-axis value = (Golgi intensity at 75 min washout – Golgi intensity at 0-min washout)/(Golgi intensity of mock cells – Golgi intensity at 0 min) × 100%. Error bars represent SEM. Results are from a single representative experiment. The experiment was performed three times with similar outcomes. **p < 0.01, Student’s t test. Knockdown of PP6 was 90% as assessed by immunoblotting of duplicate coverslips.
Consistent with previously published high-throughput studies (Ho et al., 2000), which suggested a physical interaction between Sit4p and Hrr25p, we showed that Hrr25p and Sit4p directly bind to each other in vitro. Previous studies suggested that Hrr25p mediates phosphorylation of the COPII coat after it tethers to the Golgi, an event that is required to uncoat the vesicle (Lord et al., 2011). Although Hrr25p primarily resides on membranes, a small fraction is also present in the cytosol (Lord et al., 2011; Figure 4A, left) and may access the COPII coat before it can bind to membranes or before COPII-coated vesicles tether to the Golgi. Cytosolic phosphorylation of coat subunits could be counterbalanced by dephosphorylation via Sit4p. Another possibility is that Sit4p may bind to and dephosphorylate Hrr25p, a known phosphoprotein (Li et al., 2007; Holt et al., 2009). Although both hypotheses are possible, additional experiments will be needed to determine the physiological relevance of the Hrr25p/Sit4p interaction.

Consistent with the finding that Sit4p is the phosphatase that recycles the COPII coat, we found that the loss of Sit4p disrupts traffic from the ER to the Golgi complex in vivo and in vitro. In addition, the role of Sit4p in COPII coat recycling appears to be evolutionarily conserved. Depletion of the mammalian orthologue of Sit4p, PP6, by siRNA altered the subcellular distribution of Sec31 and delayed traffic between the ER and Golgi complex. PP6 and CKIδ appear to have the same intracellular distribution as their orthologues, and, like their orthologues, they interact with each other (Figure 4; Lord et al., 2011). Sit4p/PP6 previously was shown to function in the transition from G1 to S phase of the cell cycle (Sutton et al., 1991) and during mitotic spindle formation (Zeng et al., 2010). Therefore our findings uncover a new role for this phosphatase in COPII coat dephosphorylation and the regulation of ER-to-Golgi traffic.

**MATERIALS AND METHODS**

**Strains and growth conditions**

Yeast cells and plasmids used in this study are listed in Table 1 and Supplemental Table S1, respectively. Genomic disruptions and integrations were constructed using the method of Longtine et al. (1998) and confirmed by colony PCR and/or Western blot analysis. Cells were grown in yeast extract peptone, synthetic complete, or minimal medium supplemented with the appropriate amino acids. All media contained 2% glucose as the carbon source unless indicated otherwise.

**Antibodies**

Monoclonal antibodies directed against GM130 and Sec31A were purchased from BD Biosciences (San Jose, CA). Polyclonal anti-PP6 antibody was purchased from Bethyl Laboratories (Montgomery, TX). Polyclonal anti-GST and monoclonal anti-HA and anti-His antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Covance (Berkeley, CA), and Thermo Scientific (Waltham, MA), respectively. Mouse monoclonal anti-GOS-28 antibody was purchased from Stressgen (San Diego, CA). Rabbit polyclonal anti-giantin antibody was purchased from Covance. Monoclonal antibodies directed against COPI (CM1A10) and VSV-G ectodomain were obtained from G. Warren (Max F. Perutz Laboratories, Vienna, Austria). Anti–ERGIC-53 and anti–Li1p antibodies were obtained from H.-P. Hauri (University of Basel, Basel, Switzerland) and Randy Schekman (University of California, Berkeley, CA), respectively. Polyclonal antibody directed against CKIδ was prepared to full-length CKIδ fused to GST and affinity purified on an Affi-Gel column (Bio-Rad, Hercules, CA) preloaded with GST-CKIδ. The flowthrough was discarded and the bound IgG was eluted with 0.2M glycine (pH 2.8) followed by rapid neutralization.

**TABLE 1: Yeast strains used in this study.**

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFNY1308</td>
<td>MATa GAL+ ade2-1 his3-11 ura3-1 can1-100 ssd1-d2 trp1-1 leu2-3 glc7::LEU2 glc7-10::TRP1</td>
<td>Michael Stark, School of Life Sciences, University of Dundee, Dundee, UK</td>
</tr>
<tr>
<td>SFNY1309</td>
<td>MATa GAL+ ade2-1 his3-11 ura3-1 can1-100 ssd1-d2 trp1-1 leu2-3 glc7::LEU2 GLC7::TRP1</td>
<td>Michael Stark</td>
</tr>
<tr>
<td>SFNY1841</td>
<td>MATa GAL+ ura3-52 leu2-3, 112 his3Δ200</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>SFNY1842</td>
<td>MATα GAL+ ura3-52 leu2-3, 112 his3Δ200</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>SFNY2028</td>
<td>MATα ura3-52 leu2-3, 112 his3Δ200</td>
<td>Michael Hampsey, Department of Biochemistry, Robert Wood Johnson Medical School, Piscataway, NJ</td>
</tr>
<tr>
<td>SFNY2029</td>
<td>MATα ura3-52 leu2-3, 112 his3Δ200 ssu72-2</td>
<td>Michael Hampsey</td>
</tr>
<tr>
<td>SFNY2030</td>
<td>MATα GAL+ ade2-1 his3-11 ura3-1 can1-100 trp1-1 leu2-3 bar1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>SFNY2031</td>
<td>MATα GAL+ ade2-1 his3-11 ura3-1 can1-100 trp1-1 leu2-3 bar1::hisGcdc14-1</td>
<td>This study</td>
</tr>
<tr>
<td>SFNY2045</td>
<td>MATα GAL+ ura3-52 leu2-3, 112 his3Δ200 sit4A::His3MX6</td>
<td>This study</td>
</tr>
<tr>
<td>SFNY2046</td>
<td>MATα GAL+ ura3-52 leu2-3, 112 his3Δ200 sit4A::His3MX6</td>
<td>This study</td>
</tr>
<tr>
<td>SFNY2051</td>
<td>MATα ura3Δ0 leu2Δ0 met15Δ0 his3Δ1 hrn25A::KanMX6 pRS315-HRR25(CEN, LEU2+)</td>
<td>This study</td>
</tr>
<tr>
<td>SFNY2070</td>
<td>MATα GAL+ ura3-52 leu2-3, 112 his3Δ200 SIT4-3xHA::His3MX6</td>
<td>This study</td>
</tr>
<tr>
<td>SFNY2179</td>
<td>MATα ura3Δ0 leu2Δ0 met15Δ0 his3Δ1 hrn25A::KanMX6 pRS315-HRR25(CEN, LEU2+) sit4A::His3MX6</td>
<td>This study</td>
</tr>
<tr>
<td>SFNY2219</td>
<td>MATα GAL+ ura3-52 leu2-3, 112 his3Δ200 sit4A::His3MX6 ssd1Δ::KanMX6 pRS316-SIT4(CEN, URA3+)</td>
<td>This study</td>
</tr>
<tr>
<td>SFNY2226</td>
<td>MATα GAL+ ura3-52 leu2-3, 112 his3Δ200 sit4A::His3MX6 GAL1pr-SIT4::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>SFNY2411</td>
<td>MATα GAL+ ura3-52 leu2-3, 112 his3Δ200 pph21Δ::KanMX6</td>
<td>This study</td>
</tr>
</tbody>
</table>
with 1 M potassium phosphate (pH 7.5). The antibody was then buffer exchanged with phosphate-buffered saline (PBS), concentrated, and stored at −80°C in 30% glycerol. Fluorescently labeled secondary antibodies were purchased from Life Technologies (Carlsbad, CA).

**In vitro kinase assay**

Purified GST-fusion proteins (2 μg) immobilized on glutathione–Sepharose beads were incubated with 250 ng of His$_6$-Hrr25p (aa 1–394) or catalytically inactive His$_6$-Hrr25p K38R (aa 1–394) for 1 h at 30°C in kinase buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonylic acid [HEPES], pH 7.4, 2 mM EDTA, 10 mM MgCl$_2$, 1 mM dithiothreitol [DTT], 5 mM cold ATP, 2.5 μCi of [$^{32}$P]ATP, 100 μM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, and protease inhibitors). The beads were washed twice with 1× PBS and eluted in 25 μl of sample buffer by heating to 100°C for 5 min. The samples were analyzed by autoradiography.

**Differential centrifugation**

A total of 100 OD$_{600}$ units of yeast cells was pelleted, resuspended in 2 ml of buffer (1.4 M sorbitol, 100 mM sodium phosphate, pH 7.5, 0.35% 2-mercaptoethanol, and 0.05 μg/ml Zymolyase) and incubated for 30 min at 37°C. The spheroplasts were then centrifuged over a 4-ml sorbitol cushion (1.7 M sorbitol, 500 mM Na$_2$SO$_4$, and 0.5 mg/ml Zymolyase) and incubated for 1 h at 30°C in kinase buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 2 mM EDTA, 10 mM MgCl$_2$, 1 mM dithiothreitol [DTT], 5 mM cold ATP, 2.5 μCi of [$^{32}$P]ATP, 100 μM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, and protease inhibitors). The beads were washed twice with 1× PBS and eluted in 25 μl of sample buffer by heating to 100°C for 5 min. The samples were analyzed by autoradiography.

**CYP pulse chase assay**

Cells were grown overnight at 25°C in minimal medium to early log phase. A total of 16 OD$_{600}$ units was pelleted, resuspended in 3.6 ml of fresh minimal medium, incubated at 37°C for 20 min, and pulse labeled with 400 Ci of [$^{35}$S]methionine for 4 min at 37°C. An aliquot (700 μl) was removed as the 0 min time point, centrifuged, and washed twice with ice-cold 10 mM sodium fluoride/sodium azide. Chase mix (150 μl of 250 mM methionine) was added to the remaining sample. At the desired time points, 700 μl of the cell suspension was removed and processed as described above. The cells were converted to spheroplasts, pelleted at 4000 × g for 3 min, resuspended in 100 μl of 1% SDS, and heated for 5 min at 95°C. The samples were diluted with 900 μl of 1× PBS containing 1% Triton X-100 and centrifuged at 16,000 × g for 15 min, and the supernatant was incubated with 3 μl of anti-CYP antibody for 1 h at 4°C. A 50% slurry of protein A–Sepharose (60 μl) was added, and the samples were incubated for an additional hour. The beads were washed, and protein was solubilized in 1× sample buffer and electrophoresed on an 8% SDS–polyacrylamide gel.

**General secretion assay**

General secretion was measured as described before (Grote et al., 2000). Briefly, cells were grown overnight to early log phase in synthetic complete medium without methionine. The next day, 1.5 OD$_{600}$ units of cells were resuspended in 400 μl of methionine-free medium supplemented with 0.06 mg/ml bovine serum albumin and incubated at 37°C for 20 min. A total of 150 μCi of [$^{35}$S]-ProMix was added to each sample, and aliquots of cells were removed and spun for 5 s at the indicated time points. The medium (300 μl) was transferred to an ice-cold tube containing 30 μl of stop mix (500 mM Na$_2$HPO$_4$, 500 mM NaF), followed by another spin at 14,000 × g for 1 min. The supernatant was then transferred to a fresh tube containing 20 μl of 100% trichloroacetic acid (TCA) with 1 mg/ml sodium deoxycholate and incubated on ice for 1 h. The TCA precipitated proteins were pelleted (14,000 × g for 5 min) and washed twice with ice-cold acetone. The acetone-washed pellets were air dried, resuspended in sample buffer, heated to 100°C for 5 min, and electrophoresed on an 8% SDS–polyacrylamide gel.

**In vitro binding assays with recombinant proteins**

Bacterially expressed and purified His$_6$-Sit4p (0.2 μM) was incubated with equimolar amounts of GST-fusion proteins or GST fusion proteins (2 μg) immobilized on glutathione–Sepharose beads were incubated with 250 ng of His$_6$-Hrr25p (aa 1–394) or catalytically inactive His$_6$-Hrr25p K38R (aa 1–394) for 3 h at 4°C. The immunocomplexes were then bound to 40 μl of protein A beads (50% slurry in IP buffer) for 1 h at 4°C. The beads were washed 3× with IP buffer and eluted in sample buffer by heating to 100°C for 5 min. Immunoprecipitates were then subjected to Western blot analysis.
(0.2 μM) in binding buffer (25 mM HEPES, pH 7.2, 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1 mM EDTA, 1 mM MnCl₂, and protease inhibitors) for 3–4 h at 4°C. The beads were washed 3x with binding buffer and eluted in 25 μl of sample buffer by heating to 100°C for 5 min.

RNA interference
HeLa and COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ incubator. siRNAs targeting human PP6 (siPP6-07, CGCUAGACCUGGACGAAGUA; siPP6-08, GGUUGGAGACCUUCACUUA) were obtained from Dharmacon (Denver, CO). Transfections (cdNA and siRNA) were performed using Lipofectamine 2000 (Life Technologies) as per manufacturer’s instructions.

For the rescue experiments, COS-7 cells transfected with PP6 siRNA-08 were cotransfected with pCMV-Myc-hPPP6C-m3-08, an expression vector that expresses myc-PP6 containing three silent mutations (267 T to C, 268 T to C, and 282 T to C) in the target sequence of si-PP6-08. Cells transfected with PP6 siRNA-07 were cotransfected with pCMV-Myc-hPPP6C-m3-07, an expression vector containing three silent mutations (18 G to A, 21 C to T, and 24 G to A) in the target sequence of si-PP6-07.

Immunofluorescence microscopy
Cells were grown and processed for immunofluorescence as described previously (Yamasaki et al., 2009). The secondary antibody used to localize CK1ε was anti-rabbit IgG conjugated to Alexa 488. Anti-mouse IgG conjugated to Alexa 594 was used to localize ERGIC-53, GM130, COPI, and Sec31A. Images were taken with an LSM 510 confocal microscope using a 100x objective and captured with a digital AxioCam MRm camera (Carl Zeiss Microimaging, Thornwood, NY). For Figure 7B and Supplemental Figure S5, anti-rabbit IgG conjugated to fluorescein isothiocyanate was used to label giantin, and anti-mouse IgG conjugated to Cy3 was used to label GOS-28. Wide-field fluorescence images were captured using a 60x objective on a Nikon E800 microscope (Nikon, Melville, NY) with excitation and emission filter wheels (Chroma Technology, Bellows Falls, VT), a Hamamatsu Orca 2 camera (Hamamatsu, Hamamatsu, Japan), Nikon Z-drive, and OpenLab 5.0 software (Improvision; PerkinElmer, Waltham, MA).

VSV-G trafficking assay
Approximately 24 h after transfecting COS-7 cells with mock/PP6 siRNA at 37°C, we transfected cells with pEGFPdKA206K-N1-VSVG tsO45. The next day, the cells were shifted to 40°C, and after 20 h they were treated with 100 μg/ml cycloheximide. After 30 min, the cells were shifted to 32°C for various times and processed as described previously (Seemann et al., 2000; Yamasaki et al., 2009). For the data in Figure 6, the cells were fixed in 3.7% paraformaldehyde for 15 min and stained with anti-VSV-G ectodomain antibody (primary antibody) and anti-mouse IgG conjugated to Alexa 594 (secondary antibody). Total VSV-G–GFP fluorescence was quantified as before (Seemann et al., 2000). All images were captured at the same setting and exposure. To quantitate the data, the cell area (A) was defined manually and the mean of the fluorescence intensity (I) in that area was measured using ImageJ (National Institutes of Health, Bethesda, MD). The integrated optical density (IOD) was determined by the formula IOD = A × I. Expression levels were normalized by determining the ratio of cell surface to total VSV-G IOD. The data were quantified from three independent experiments, and >30 cells were examined for each time point in every experiment.

BFA recovery assay
The BFA recovery experiments were performed as follows. Three days after transfection with PP6 siRNA or mock transfection with Lipofectamine reagent alone, the medium was replaced with medium containing 2.5 μg/ml BFA. After 1-h incubation at 37°C in BFA, coverslips were dipped several times in PBS and placed into wells of fresh, prewarmed medium containing 10 μg/ml cycloheximide. Coverslips were then fixed at various times and processed for double-label immunofluorescence using anti–GOS-28 and anti-giantin antibodies. Imaging of cells was conducted using a fixed exposure time for each color channel. To quantify giantin Golgi intensity, we used Openlab software. Regions of interest (ROIs) were drawn tightly around each Golgi region and the maximum intensity recorded. A second ROI was drawn abutting outside and following the edge of the first and its mean intensity recorded. Our measure of pronounced “Golgi intensity” (Figure 7B) is defined as the maximum intensity of the first ROI divided by the mean intensity of the second ROI, calculated individually for each cell in every assayed field. For cells without a clear juxtanuclear labeling (e.g., at 0-min washout), the ROI was drawn around the brightest section of the perinuclear area. Image capture, drawing of ROIs, and calculation of intensities were performed doubly blind.

ACKNOWLEDGMENTS
We thank Elizabeth Miller, Seema Mattoo, and Vincent Tagliabracci for advice and M. Stark, D. Morgan, and M. Hampsey for strains. We also thank Y. Jiang, G. Warren, H.-P. Hauri, and R. Schekman for antibodies and Wenyun Zhou for technical support. Salary support for D.B., J.Z, S.M., S.C., and S.F.-N. was provided by the Howard Hughes Medical Institute. S.F.-N. is an Investigator of the Howard Hughes Medical Institute. K.D.C. is supported by the Ludwig Institute for Cancer Research and a grant from the National Institutes of Health (ROI GM104141). Work at the University of Montana was supported by National Institutes of Health Grant GM106323 (to J.C.H.) and by the Center for Structural and Functional Neuroscience.

REFERENCES

Volume 24 | September 1, 2013
Sit4p/PP6 regulates ER-to-Golgi traffic | 2737


