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Article

Keywords: unfolded protein response, phase separation, protein secretion, membrane trafficking

Posted Date: December 21st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1168612/v1>

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Unconventional COPII-mediated secretory protein trafficking continues in the absence of the Sar1 GTPase

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Running title: COPII transport without Sar1

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Abstract

Coat protein complex II (COPII) plays an integral role in the packaging of secretory cargoes within membrane-enclosed transport carriers that leave the endoplasmic reticulum (ER) from discrete membrane subdomains. Lipid bilayer remodeling necessary for this process is driven initially by membrane penetration of the coat subunit Sar1 and further stabilized by assembly of a multi-layer complex of several COPII proteins. However, the relative contributions of these distinct factors to transport carrier formation and protein trafficking remain unclear. Here, we demonstrate that anterograde cargo transport from the ER continues in the absence of Sar1, although the unconventional carriers that form fail to efficiently deliver their contents to subsequent compartments in the secretory pathway. Instead, cargoes accumulate immediately adjacent to the perinuclear Golgi under these conditions, together with components of the COPII coat. Our findings highlight new mechanisms by which COPII promotes transport carrier biogenesis and strongly suggests that the Sar1 GTPase plays a critical role in transport carrier uncoating ahead of membrane fusion and secretory cargo delivery at acceptor compartments.

Keywords: unfolded protein response, phase separation, protein secretion, membrane trafficking

Introduction

In eukaryotic cells, compartmentalization of biochemical processes within membrane-bound organelles improves their efficiency but necessitates the existence of trafficking pathways to move proteins, lipids, and other factors between different subcellular locations (1-5). The oxidative environment of the endoplasmic reticulum (ER) lumen, together with its high concentration of molecular chaperones, facilitates co-translational folding of newly synthesized secretory cargo proteins, which are selectively packaged into coat protein complex II (COPII)-dependent transport carriers that leave the ER from well-organized membrane subdomains (6-10). Loss of COPII function is incompatible with cell survival and development, consistent with its essential role in regulating the movement of thousands of substrates (11-22). However, the mechanisms by which COPII coordinates ER membrane remodeling and subsequent cargo trafficking remain poorly defined, particularly in the case of mammalian cells.

Based on a combination of *in vivo* and *in vitro* data, there currently exist several overlapping models to describe COPII-mediated protein transport (23-25). Perhaps most strikingly, recent cryo-electron microscopy (EM)-based imaging of COPII budding sites has confirmed that biosynthetic cargoes leave the ER via nascent membrane buds, which are linked to an elaborate array of vesicular-tubular membranes that are stably associated with ER subdomains (25). While some literature refers to these tightly juxtaposed organelles collectively as 'ER exit sites', others have distinguished these compartments, based on their unique biochemical environments, dubbing the network of ER-adjacent membranes as ER-Golgi intermediate compartments (ERGIC) (26-29). Super-resolution fluorescence microscopy has defined at least three unique membrane domains that exist in this early portion of the secretory pathway, delineated by the distinct enrichment patterns of several COPII-associated proteins. Specifically, ER subdomains are marked by the Sar1 guanine nucleotide exchange factor (GEF) Sec12, the large scaffolding protein Sec16 that plays a regulatory role in COPII assembly, and cargo receptors in the Tango1/cTAGE5 family (8, 30-32). Components of the COPII complex

accumulate directly adjacent to these regions of the ER, together with TFG, which functions to tether transport intermediates prior to their fusion with one another or neighboring ERGIC membranes (22, 32, 33). ERGIC exhibits elevated concentrations of the lectin ERGIC-53, the Rab1 GTPase, and components of the COPI coat, which play an essential role in the anterograde trafficking of secretory cargoes to the Golgi, as well as retrograde transport to the ER (25, 27, 34-37). The entire interface between the ER and ERGIC is limited to only 300-500 nm in most mammalian cell types, with a high density of membranes present to enable rapid but selective cargo sorting (38).

To promote transport carrier biogenesis and cargo export from the ER, the COPII coat complex must associate directly or indirectly with membranes. Upon GTP loading, Sar1 stably penetrates the outer leaflet of the ER phospholipid bilayer, promoting membrane remodeling and tubulation (39-42). Furthermore, Sec23-Sec24 heterodimers that bind to activated Sar1 and complete the inner layer of the COPII coat also interact with several other ER-associated proteins, including transmembrane secretory cargoes and their receptors, members of the Tango1/cTAGE5 family, and Sec16a (43-47). The assembled inner coat additionally acts as an adaptor layer for nucleation of an outer cage composed of Sec13-Sec31a heterotetramers (9). Together, the multi-layered COPII coat has been suggested to stabilize the high degree of membrane curvature necessary to generate canonical transport intermediates (48, 49). However, it has been difficult to dissect how individual COPII components contribute to this process, as assembly of coat subunits is highly interdependent.

Since multiple inner COPII coat proteins exhibit the ability to associate directly or indirectly with ER subdomains in cells, via protein-lipid or protein-protein interactions, we developed strategies to determine whether COPII-mediated transport continues in the absence of one or the other. Notably, recent studies have suggested that Sar1 activity is dispensable for the trafficking of some cargoes, including potassium ion channels, and in the case of immortalized Caco-2/15 cells, unnecessary for growth and proliferation, although mechanisms

that allow ER export to continue in the absence of Sar1 remain unclear (50, 51). Using a combination of genetic approaches, we demonstrate that the loss of Sar1 results in the formation of unconventional transport carriers that continue to harbor other COPII subunits and mediate the movement of secretory cargoes from the ER. Under these conditions, COPII exhibits behavior similar to that of phase separated liquid droplets, ultimately coalescing around Golgi membranes but failing to fully dissociate to allow efficient fusion of transport carriers and the delivery of cargoes. In contrast, depletion of Sec23-Sec24 heterodimers blocks cargo export from the ER. Taken together, our data highlight new roles for COPII components during membrane remodeling at the ER, potentially in a manner akin to that of early initiators of endocytosis, which undergo liquid-like assembly at plasma membrane subdomains to catalyze membrane deformation (52-54).

Results

Depletion of Sar1 impairs cell growth and activates the unfolded protein response

Current models suggest that the Sar1 GTPase plays an integral role in membrane remodeling necessary for secretory cargo transport from the endoplasmic reticulum (ER) (23, 40). To determine whether additional, Sar1-independent mechanisms exist to support these processes, we first developed an approach to inhibit the functions of both mammalian Sar1 isoforms (Sar1a and Sar1b) by combining CRISPR/Cas9-mediated gene editing and siRNA-mediated protein depletion in human RPE1 cells. A guide RNA directed against the second coding exon of Sar1a was used to independently generate several clonal cell lines, all of which harbored insertions and/or deletions that resulted in frameshifts and introduced early stop codons (**Fig. 1A**). Since each cell line exhibited similar rates of growth, were morphologically indistinguishable, and failed to express Sar1a based on immunoblot analysis (**Fig. 1B and S1A**), we selected one (Clone 3) for further study that contained compound heterozygous mutations within both *SAR1A* alleles (c.158_159insT/c.158_159delT), resulting in disruption of switch 1 residues (amino acids 55-59) and all other domains downstream, which are essential for GTPase activity (55, 56). Attempts to further disrupt Sar1b in this mutant background using CRISPR/Cas9 were unsuccessful, suggesting that RPE1 cells require at least one Sar1 paralog to support growth and proliferation. To circumvent this issue, we used two distinct siRNAs targeting Sar1b in cells lacking Sar1a, which enabled approximately 98% depletion after 72 hours of treatment (**Fig. 1B-D**). Under these conditions, cell proliferation was inhibited and the frequency of cell death was substantially elevated, confirming an essential role for Sar1 function in RPE1 cells (**Fig. S1A, B**).

The phenotypes that result from Sar1 inhibition are consistent with those that occur in response to acute ER stress and activation of the unfolded protein response (UPR), which would be anticipated following a blockade on secretory protein export from the ER (57, 58). To examine whether the UPR is altered in the absence of Sar1, we first examined the expression of

GRP78/BIP, an ER chaperone that binds to unfolded proteins upon ER stress (59). Strikingly, after 48 hours of Sar1 depletion, immunoblot analysis revealed a significant and sustained increase in GRP78/BIP protein levels (**Fig. 1E**). Similarly, quantitative analysis of canonical sensors of the UPR, spliced XBP1 (active form of XBP1) and ATF6 mRNA expression, demonstrated approximately 2-fold increases following siRNA-treatment relative to controls (**Fig. S1C**). Together, these data indicate that Sar1 inhibition leads to activation of multiple branches of the UPR.

Previous studies have suggested that the activation of UPR signaling can modulate the expression of regulatory factors that control COPII-mediated trafficking (60, 61). In particular, several genes encoding COPII subunits and COPII-associated proteins have been shown to be direct transcriptional targets of spliced XBP1 (61). To determine how loss of Sar1 impacts the expression of other early secretory pathway components, we conducted a series of quantitative immunoblot and immunofluorescence experiments. To our surprise, the levels of individual COPII subunits (Sec13 and Sec23) were not significantly altered (**Fig. 1F**). However, several other factors implicated in anterograde secretory protein transport, including Rab1a, p125a/Sec23IP, TFG, and ERGIC-53, were significantly upregulated when Sar1 function was lost (**Fig. 1F**). These data raise the possibility that alternative pathways, which mediate ER membrane remodeling during secretory cargo export, are augmented when the major mechanism to do so is disrupted.

To address this idea, we first examined the distributions of numerous components of the early secretory pathway by immunofluorescence. These studies revealed that the integrity of ER budding sites marked by Sec16a and Tango1 was not disrupted by the loss of Sar1 (**Fig. 2A**). However, the fluorescence intensities of both factors were significantly increased relative to control cells under these conditions (**Fig. 2B**). Similarly, quantitative imaging of Rab1a, TFG, and ERGIC-53 indicated elevated levels of these proteins when Sar1 was inhibited, consistent with immunoblotting studies, but even more striking were their redistributions from peripheral

punctate structures to a perinuclear region that appeared to overlap substantially with the cis-Golgi matrix component GM130 (**Fig. 2C, D**). This was most obvious in the cases of Rab1a and ERGIC-53, which became hyperconcentrated near Golgi membranes under these conditions and were no longer detectable adjacent to ER subdomains harboring Sec16a (**Fig. 2C, D**). These data strongly suggest that conventional ERGIC membranes become destabilized and lose their integrity in absence of Sar1. Consistent with this idea, in cells depleted of Sar1, COPI exhibited a largely cytoplasmic distribution (**Fig. 2E**), suggesting a dependence on Sar1-mediated ER membrane remodeling to subsequently load onto ERGIC membranes and drive anterograde transport of cargoes to the Golgi.

COPII subunits co-assemble into dynamic condensates in the absence of Sar1

Based on prior work demonstrating an integral role for Sar1 in nucleating COPII complexes (40, 62), we initially predicted that its absence would result in COPII subunit dispersal in cells. However, our systematic analysis of the distributions of COPII regulatory factors following Sar1 inactivation demonstrated that the key Sec23-binding protein TFG assembles into several enlarged spherical condensates under these conditions, closely resembling those observed previously upon its overexpression in cells (**Fig. 2D**) (33). We therefore examined whether remaining COPII components exhibited a similar distribution as compared to TFG in the absence of Sar1. These studies identified numerous instances of COPII (inner and outer coat subunits) associating with TFG condensates (**Fig. 2F**). However, the majority of the inner coat, as detected using antibodies directed against Sec24a, accumulated in the perinuclear region, closely juxtaposed to the cis-Golgi following 72 hours of Sar1 depletion (**Fig. 2F, G and S1D**). In contrast, the outer COPII coat (Sec31a) largely failed to localize with Sec24a in this area of the cell, suggesting that perinuclear COPII assemblies had undergone partial uncoating (**Fig. S1D**).

To gain additional insights into the mechanisms underlying redistribution of the COPII inner coat before and after Sar1 depletion, we used CRISPR/Cas9-mediated genome editing to

engineer cells that express Sec23a fused to HaloTag from its endogenous locus (**Fig. 3A**).

Depletion of Sec23b in cells homozygous for HaloTag-Sec23a expression did not impair growth or viability, suggesting that the fusion protein is functional (**Fig. S2A**). Following labeling using a JFX646-conjugated HaloTag ligand, we used live cell imaging to analyze Sec23a dynamics, demonstrating the existence of two populations, which exhibit either long ($>5\ \mu\text{m}$) or short ($<5\ \mu\text{m}$) track displacements over time (**Fig. S2B**). These data indicate that a small fraction of COPII (2.4% on average) moves several microns away from ER subdomains, potentially transiting directly to other organelles, while the vast majority remains at the interface between ER and ERGIC membranes (**Movie S1**). Following the initial phase of Sar1 depletion (48 hours after siRNA treatment), the total number of Sec23a positive structures was reduced by more than 2.5-fold as compared to control cells (**Fig. 3B**). However, in these cells, we found that the number of COPII-decorated spherical condensates larger than $0.8\ \mu\text{m}^3$ in volume increased significantly, many of which subsequently coalesced in the perinuclear region after several hours in a manner reminiscent of vesicle-mediated cargo transport to the Golgi apparatus (**Fig. 3C**). Treatment with nocodazole ($1\ \mu\text{M}$), a microtubule depolymerizing agent, inhibited this redistribution, suggesting that COPII accumulation near Golgi membranes is partially dependent on the microtubule cytoskeleton (**Fig. 3D, E**). Moreover, these data strongly suggest that COPII complexes that assemble in the absence of Sar1 do not remain associated with the ER but instead undergo a combination of slow anisotropic movements and more rapid, albeit brief, directed translocations (**Movie S2**). Consistent with this idea, we found no change in the distribution of ER membranes in cells following Sar1 depletion, again highlighting their distinct behavior as compared to COPII (**Fig. S2C, D**). Notably, in parallel with the redistribution of COPII condensates, the population of smaller COPII-labeled structures less than $0.2\ \mu\text{m}^3$ in volume slowly recovered, indicating an ongoing ability for COPII complex assembly in the absence of Sar1 (**Fig. 3B**).

To better understand how COPII complexes form into discrete structures in the absence of Sar1, we considered the wealth of structural information that exists regarding the interfaces between Sar1 and other COPII subunits (9, 41, 46, 47, 63-65). In particular, previous work indicates that the binding of Sar1 orders a flexible loop within Sec23 known as the 'L-loop', which is important for COPII lattice assembly (9). In the absence of this interaction, the L-loop is predicted to be disordered (9), potentially making Sec23 more likely to undergo liquid-liquid phase separation (LLPS). Consistent with this possibility, lattice light sheet imaging of HaloTag-Sec23a endogenously expressed in cells lacking Sar1 demonstrated it to exhibit liquid-like characteristics, routinely merging and separating from other condensates (followed by spherical relaxation), and also undergoing cycles of partial dissolution and coalescence during their lifespans (**Movies S3 and S4**). This behavior was similar to that seen with the well-characterized stress granule marker G3BP1, which undergoes phase separation following treatment with sodium arsenite (**Fig. 4A and S3A**) (66). Analogous to other liquid droplets, COPII condensates were also sensitive to 1,6-Hexanediol, which is believed to disrupt weak hydrophobic interactions important for LLPS (67), but phase separated structures reformed following washout of the aliphatic alcohol (**Fig. S3B-D**).

Leveraging fluorescence recovery after photobleaching (FRAP) studies, we showed that HaloTag-Sec23a molecules exchange rapidly between condensed droplets and the cytoplasm (**Fig. 4B**). The half-time to recovery varied modestly depending on the size/intensity of structures, with those smaller than $0.2 \mu\text{m}^3$ in volume requiring 3.2 seconds (± 0.3 seconds), while condensates larger than $0.2 \mu\text{m}^3$ in volume required 4.3 seconds (± 0.4 seconds), precisely the same as that reported previously for overexpressed GFP-Sec23a (68). By comparison, HaloTag-Sec23a expressed at endogenous levels in control cells exhibit a half-time to recovery of 2.0 seconds (± 0.5 seconds). These data suggest that the concentration of Sec23 at unique sites may affect its recovery kinetics. Despite these differences, the mobile

fraction of HaloTag-Sec23a was similar under all conditions examined, indicating that the majority of Sec23 is readily exchangeable with the cytoplasmic pool, irrespective of the presence or absence of Sar1 (**Fig. 4B**). This finding is contrasted by the impact of disrupting early secretory pathway function using the fungal metabolite Brefeldin A (BFA), which inhibits COPI assembly independently of Sar1 (69). Specifically, in the presence of BFA (5 μ g/ml), a subset of COPII-associated structures become enlarged, similar to those seen following Sar1 depletion, but their dynamics are significantly altered, exhibiting an increased half-time to recovery (8.2 seconds $\pm$ 1.1 seconds), as well as a reduced mobile fraction (**Fig. 4C**). These data indicate that COPII condensates formed in the absence of Sar1 are unique as compared to structures that assemble as a direct result of COPI inhibition.

To gain a higher resolution view of the impacts resulting from Sar1 depletion, we turned to electron microscopy. In contrast to control cells, which exhibit canonically stacked Golgi cisternae within the perinuclear region, we instead found a dramatic accumulation of spherical membrane-bound compartments in cells lacking both Sar1 isoforms that were highly heterogenous in size (**Fig. 4D and S3E**). Additionally, the lumen of the ER became distended in cells depleted of Sar1, although the morphology of the nuclear envelope remained largely unaffected (**Fig. 4D and S3E**). The distribution of the unusual spherical compartments was similar to that of COPII condensates visualized by fluorescence microscopy, which accumulate in the perinuclear region following Sar1 inhibition. We therefore sought to determine whether the COPII condensates correspond to any known membrane-bound or membraneless organelle. Our findings demonstrated that COPII condensates failed to co-localize with several other proteins known to undergo phase separation, including the P-body component DDX6 and the stress granule marker G3BP1 (70, 71), despite Sar1 inhibition resulting in elevated cellular stress (**Fig. S3F, G**). Similarly, COPII condensates did not associate with autophagosomes, lysosomes, nor did they overlap with the highly disordered protein Sec16a, which has been implicated in the formation of phase separated 'Sec bodies' identified previously in *Drosophila*

S2 cells following nutrient starvation (**Fig. S3H, I**) (72). These data suggest that COPII condensates, which form in the absence of Sar1, represent a new type of liquid droplet that associates with membrane-bound compartments.

COPII directs anterograde cargo transport from the ER in the absence of Sar1

The perinuclear accumulations of COPII and aberrant membrane-bound compartments raises the possibility that Sar1-independent COPII-associated transport carriers continue to form and mediate secretory cargo efflux from the ER. To explore this idea, we leveraged two inducible cargo release systems developed previously in which secreted proteins are initially trapped in the ER but undergo synchronous anterograde trafficking following the addition of a specific chemical agent. In one case, a mutant form of FK506-binding protein (FKBP) fused to dsRED and an ER export signal was targeted to the ER lumen (ss-dsRED), where it assembles into large fluorescent aggregates that can be solubilized by the addition of a synthetic ligand of FKBP (SLF) (73, 74). In the other case, a truncated form of the integral membrane Golgi enzyme Mannosidase II fused to the streptavidin binding peptide and GFP (ManII-SBP-GFP) was co-expressed with a fusion between the human invariant chain of the major histocompatibility complex and streptavidin (Ii-streptavidin), which is retained in the ER. Addition of biotin enables ManII-SBP-GFP to be released from Ii-streptavidin, allowing its entry into the secretory pathway (75). In control cells and cells lacking Sar1a alone, both cargoes accumulated at the Golgi rapidly following release from the ER, based on their increased co-localization with GM130 (**Fig. 5A, B and S4A, B**). To our surprise, elimination of both Sar1 isoforms failed to block the export of either cargo from the ER (**Fig. 5A, B and S4A, B**). In contrast, even partially reduced levels of Sec23 (~75% depletion relative to control cells) fully stalled ER cargo export, despite clear solubilization of ss-dsRED aggregates following addition of SLF (**Fig. 5C and S4C, D**). These data strongly suggest that Sar1, but not Sec23, is at least in part dispensable for COPII-mediated cargo export from the ER.

To directly examine cargo released from the ER relative to COPII in Sar1 depleted cells, we employed super resolution STED microscopy. Specifically, following 60 hours of siRNA treatment targeting Sar1b, cells lacking Sar1a and expressing ss-dsRED were exposed to SLF and processed for immunofluorescence-based imaging using antibodies directed against Sec24a. Under these conditions, we found that released cargo associated directly with COPII condensates, strongly suggesting that at least a subset of these structures represent bona fide transport intermediates (**Fig. 5D**).

COPII condensates accumulate adjacent to Golgi membranes but fail to deliver cargo

The dramatic accumulation of inner COPII coat proteins together with released cargoes in the perinuclear region of cells lacking Sar1 suggests a potential function for the GTPase subsequent to its well-recognized role in ER membrane remodeling. To better define the distribution of COPII relative to the cis-Golgi in the absence of Sar1, we again turned to STED microscopy. Our findings demonstrated that COPII and released cargo localize immediately adjacent to GM130-labeled membranes, strongly suggesting a defect in the fusion of atypical COPII transport carriers that form when Sar1 is depleted (**Fig. 6A, B**). We additionally examined cargo trafficking, specifically from the perinuclear region of control and Sar1-depleted cells. These live cell imaging studies demonstrated that in the absence of Sar1, cargoes that arrive in this region following release from the ER fail to move further through the secretory pathway, in contrast to control cells (**Movies S5 and S6**). These findings are consistent with the idea that COPII coat components must fully disassemble from the surface of transport intermediates to enable membrane fusion.

There are currently conflicting models regarding the mechanisms by which COPII coat disassembly occurs, with some arguing that the presence of Sar1 is necessary, while others suggest that Sar1 dissociates prior to the removal of remaining COPII components (7, 76). To address this question and determine the localization of native Sar1 relative to other components

in the early secretory pathway, we used CRISPR/Cas9-mediated gene editing to place a HaloTag within an internal loop of Sar1a (immediately downstream of alanine 170) that is predicted to be solvent-exposed and far from its switch domains, which mediate GTPase activity (63, 64). We examined its localization using STED microscopy relative to both Sec16a, which marks ER subdomains, and the outer COPII coat component Sec31a. Strikingly, Sar1a exhibited a biased localization and exhibited more overlap with Sec31a as compared to Sec16a (**Fig. 6C, D**). This distribution was similar to that of HaloTag-Sec23a, consistent with the idea that Sar1 continues to associate with COPII transport carriers, even after they are no longer associated with ER subdomains (**Fig. 6C, D**). In contrast, following CRISPR/Cas9 editing to create RPE1 cells that natively express HaloTag-Tango1, we found that its distribution exhibits more overlap with Sec16a as compared to Sec31a, consistent with it remaining at ER subdomains following COPII carrier release (**Fig. 6C, D**). Taken together, these data support a model in which Sar1 normally promotes COPII disassembly from transport intermediates, likely in a GTPase dependent manner (76), to enable fusion with target membrane compartments. In the absence of Sar1, COPII condensates that form to facilitate ER export of cargoes fail to disassemble efficiently, leading to the accumulation of inner COPII coat components and cargoes immediately juxtaposed to Golgi membranes, but unable to undergo fusion (**Fig. 7**).

Discussion

Despite intense study over several decades, the mechanisms by which COPII facilitates the anterograde transport of membrane proteins from the ER remain controversial. Classical paradigms supported by elegant biochemical and reconstitution-based studies suggest that COPII remodels lipid bilayers to form cargo-laden, coated vesicles, while more recent studies raise the possibility that COPII may also promote the formation of highly curved membrane tunnels that concentrate secretory cargoes therein, but fail to coat transport intermediates (4, 23-25). In both cases, COPII is responsible for membrane deformation, a process that has been largely ascribed to the inner coat subunit Sar1, which stably penetrates ER lipid bilayers when bound to GTP, driving membrane tubulation (39-42). Additionally, activated Sar1 has been proposed to be the major recruitment factor for Sec23-Sec24 heterodimers, completing the inner layer of the COPII coat complex (62, 64). However, our findings indicate that Sar1 is surprisingly not required for secretory protein export from the ER, directly challenging the idea that it is indispensable for membrane bending and cargo loading. In contrast, the inner coat component Sec23 is irreplaceable in these processes, supporting a model in which COPII can function independently of Sar1 to remodel ER membranes.

This raises the important question of how Sec23-Sec24 continues to be recruited to ER subdomains following Sar1 inhibition. One possibility is that the scaffolding protein Sec16, which binds directly to Sec23 and Sec24 and localizes independently of COPII to ER budding sites, partially substitutes for Sar1 in this capacity (8, 43, 44). Notably, the levels of Sec16 at ER subdomains are dramatically upregulated in the absence of Sar1, which likely enables enhanced recruitment of remaining COPII components. In an analogous manner, elevated levels of Tango1, which has also been shown to associate directly with Sec23 at ER subdomains, may further promote accumulation of inner COPII coat components irrespective of Sar1 presence, and simultaneous binding of Sec24 to secretory cargoes would additionally aid Sec23-Sec24 membrane association (32, 46, 47). Via these multivalent, but relatively low

affinity interactions, a critical concentration of Sec23-Sec24 heterodimers may be reached to drive their local demixing, creating phase separated condensates. The propensity of COPII components to undergo a phase transition is not unprecedented. During amino acid starvation in *Drosophila* S2 cells, most COPII subunits, with the notable exception of Sar1, co-assemble to form liquid-like stress assemblies that have been dubbed 'Sec bodies', which are believed to function as storage depots to protect early secretory pathway components from degradation until nutrient conditions improve (72). Although COPII condensates observed following Sar1 inhibition appear distinct from 'Sec bodies', both in terms of overall composition and function, the ability of Sec23-Sec24 to undergo phase separation appears to be evolutionarily conserved.

Recent studies using cryo-electron tomography and subtomogram averaging have revealed the overall architecture of the COPII complex assembled on a membrane surface, highlighting a multitude of highly structured interactions between layers of the coat, as well as the association of Sar1 with the lipid bilayer (9, 25, 41). Based on this network of associations, models have emerged to suggest how COPII promotes membrane reorganization to support transport carrier formation and cargo trafficking (9, 25). However, the many low complexity regions found in COPII subunits, particularly those within Sec24 and Sec31 isoforms, remain poorly defined, limiting our understanding of their contributions to this process. Our work demonstrating that COPII continues to facilitate secretory cargo export from the ER in the absence of Sar1 suggests additional roles for the coat in membrane remodeling. In particular, phase separation of COPII while associated with ER membranes may lead to sufficient molecular crowding and steric pressure to promote membrane deformation. This idea is consistent with *in vitro* studies showing that the large hydrodynamic radii of disordered domains from epsin1 or AP180 are adequate to drive membrane tubulation when either is tethered to a bilayer (52, 53). In addition, the liquid-like state of COPII condensates may also facilitate further membrane remodeling events that enable the release of coated transport carriers from the ER, in a manner similar to that shown recently for Eps15-Fcho liquid droplets during endocytosis

(54). Considering our finding that the mobility of COPII subunits within condensates formed following Sar1 depletion is highly similar to that observed in control cells, our data raise the intriguing possibility that COPII phase separation normally contributes to transport carrier biogenesis and secretory efflux from the ER (ie., when Sar1 is present). Further studies will be necessary to fully explore this idea.

Despite ongoing protein export from the ER in the absence of Sar1, cells failed to proliferate under these conditions, suggesting other consequences to endomembrane trafficking. Of particular note, Sar1 inhibition resulted in a lack of discernable ERGIC membranes juxtaposed to ER subdomains, a finding consistent with an absence of canonical COPII transport intermediates, which normally uncoat and fuse to generate these compartments (10). Instead, COPII condensates of various sizes that form in the absence of Sar1 appear to mediate secretory protein trafficking directly to the perinuclear region of cells in a manner dependent on microtubules. This idea is supported by previous work showing that Sec23 interacts with the dynactin complex, enabling movement on microtubules toward centrosomes located near the cell center, together with the Golgi apparatus (77). However, we found that fusion of non-canonical COPII transport carriers with the Golgi was potentially delayed, impeding cargo entry and resulting in a dramatic accumulation of COPII condensates in the perinuclear region over time. These data suggest an important role for Sar1 GTPase activity in fully disassembling the inner COPII coat ahead of membrane fusion, a function that has been speculated for decades, despite a lack of clear evidence in animal cells (76). When taken together, our studies highlight several new functions of COPII, which likely act throughout the lifecycle of ER-derived secretory transport carriers at the ER/ERGIC interface.

Methods

CRISPR/Cas9-mediated genome editing, siRNA-mediated depletion, and cell proliferation studies

To generate human hTERT-immortalized RPE1 cells (CRL-4000 from ATCC) that natively express HaloTag fusion proteins, CRISPR/Cas9-mediated genome editing was used. For HaloTag-Sec23a, a plasmid co-expressing Cas9-GFP and a guide RNA (gRNA; 5'-CTCCACAATGACAACCTATT -3') targeting the amino-terminus of Sec23a was electroporated into cells together with a plasmid-based homology directed repair (HDR) template encoding the HaloTag, a linker, and 800 base pairs of flanking genomic DNA, both upstream and downstream of the Cas9 cut site. Fluorescence activated cell sorting (FACS) was used to identify individual cells that were GFP positive, and homozygous genome edited clones were subsequently validated by fluorescence imaging, immunoblot analysis and Sanger sequencing. For HaloTag-Sar1a and HaloTag-Tango1, synthetic gRNA duplexes comprised of a crRNA (5'-GGGTCACATTCCCCTAAAGG-3' and 5'-AAAAAACCCTGAATTATCTA-3', respectively) and a universal tracrRNA were complexed with recombinant Cas9 and co-transfected with appropriate plasmid-based HDR templates into cells using TransIT-X2 (Mirus Bio). Cells were subsequently labeled with JFX646-HaloTag ligand and subjected to FACS to identify individual genome edited clones. To generate cells expressing EGFP-MAP1LC3B, a synthetic gRNA duplex (5'-TGGTGCAGGGATCTGGGCGG-3') was co-transfected with a HDR template generated previously by the Allen Cell Institute (Addgene #101783). Following FACS, positive clones were identified based on GFP fluorescence. To introduce lesions into the Sar1a coding sequence and disrupt its expression, we targeted exon 2 using a gRNA (5'-GATGTAGTGTTGGAACATGT-3'), which was expressed from a plasmid encoding Cas9-GFP and transfected into cells using FuGENE HD (Promega). Clones isolated by FACS were examined by immunoblot analysis and subjected to Sanger sequencing to confirm bi-allelic editing. Depletion studies were conducted using multiple siRNAs, which were co-transfected into cells using Lipofectamine RNAiMAX

(SAR1B: 5'-GCAUAAACUUGAAUUCAAUA-3' and 5'-CUACCUUCCUGCUAUCAAU-3'; SEC23A: 5'-CAGACUCAUAAUAAUAUGUAU-3' and 5'-AUGACGGUUGUAACUACUAAA-3').

To study cell viability and death, we used a Viability/Cytotoxicity Assay Kit (Biotum). Briefly, the kit contains two markers, Calcein AM and Ethidium homodimer-III (EthD-III), to label live or dead cells, respectively. We measured and compared the average intensity sum of Calcein-AM and Ethidium homodimer-III (EthD-III) in quadruplicates. For quantitative PCR studies, cDNA was produced using the High-Capacity cDNA Reverse Transcription Kit (Thermofisher, 43-688-14) using previously validated primers.

Fluorescence imaging studies and image analysis

Live cell imaging of cargo trafficking from the ER was performed using a Nikon Ti2 spinning disk confocal microscope equipped with a 60x oil immersion objective (1.4 NA) and a Hamamatsu ORCA-Flash4.0 sCMOS camera or a Molecular Devices ImageXpress Micro 4 (IXM4) high content imaging system with a 40x dry objective (0.95 NA) and a sCMOS camera, following transient transfection of plasmids encoding trafficking substrates. For the retention using selective hooks (RUSH) assay (75), streptavidin (100 μ M) was added to the growth media to prevent premature trafficking of cargo. The addition of 200 nM biotin triggered cargo efflux from the ER. Release of ss-DsRED was mediated by the addition of 50 μ M SLF, as described previously (73). For high-speed imaging of HaloTag-Sec23a and GFP-G3BP1 (introduced via lentiviral transduction), a 3i lattice light sheet (LLS) microscope was used, outfitted with a Hamamatsu ORCA-Flash4.0 V3 sCMOS camera, a 25x (1.1 NA) water immersion detection objective, and a 28x (0.71 NA) water immersion illumination objective. Spinning disk confocal microscopy and LLS imaging of fluorescent fusions to G3BP1 and DDX6 were conducted following viral-mediated transduction, as described previously (33), and all HaloTag-fusions

were labeled with dye-conjugated HaloTag ligands (100 nM) overnight prior to washout and imaging.

For diffraction-limited immunofluorescence studies, cells were fixed using 4% paraformaldehyde at 37°C for 15 minutes or 100% methanol at -20°C for 20 minutes, followed by permeabilization and antibody labeling (1 µg/ml) at 4°C overnight (33). After thorough washing, coverslips were incubated with secondary antibodies (AlexaFluor conjugates) for 1 hour and mounted using Vectashield (Vector Laboratories). Imaging datasets were comprised of 25-35 sections separated by 0.3 µm. For stimulated emission depletion microscopy (STED), AbberiorSTAR, Alexa Fluor 555, or Alexa Fluor 594 secondary conjugates were used, prior to mounting using Prolong Diamond Antifade (ThermoFisher Scientific). Coverslips were cured at room temperature in the dark for 24 hours prior to imaging on a Leica TCS SP8 3x superresolution imaging system using the 775 nm depletion laser. Based on the imaging of diffraction-limited fluorescent beads, the full width at half maximum of the STED point-spread function was determined to be 90 nm.

Fluorescence recovery after photobleaching (FRAP) studies were also performed on the Leica SP8 system, using a super continuum white-light laser (65 mW) and an additional 408 nm laser at full power (50 mW). Regions of interest were bleached for 5 frames in a circular pattern, followed by confocal imaging. Data were curated to remove outliers defined as 5 standard deviations from the average intensity at any given timepoint. Analysis was conducted using EasyFRAP. All other image analysis, including co-localization studies and fluorescence intensity measurements, was performed using IMARIS (Bitplane) or FIJI software. For lattice light sheet microscopy studies, beam alignment, dye alignment, and bead alignment were calibrated on the day of each experiment. Point spread functions (PSFs) were generated with numerical apertures of 0.550 and 0.493. Time-lapse studies were conducted in an image format of 1024 x 1024 or 1024 x 512 pixels, stack size of 15-20 µm, and a skewed z-step of 553 nm (deskewed:

300 nm). Each raw time-lapse image series was subsequently deskewed using SlideBook software and processed by constraint iterative deconvolution (3D frequency filter enabled, gaussian noise smoothing at 0.7, and mirrored edge padding (20%) was applied to the x-, y-, and z-planes.

Electron microscopy and immunoblotting studies

For electron microscopy studies, cells grown on sapphire disks and frozen using a Leica EM ICE high-pressure freezer. Samples were freeze-substituted and embedded through a graded series of Epon EMbed 812 (Electron Microscopy Sciences), as described previously (78). Micrographs of 80 nm sections were collected on a Phillips CM120 80 kV transmission electron microscope equipped with an AMT Biosprint 12 series digital camera. Immunoblotting studies were conducted as described (78) using validated antibodies directed against COPB (Santa Cruz), Sec23a (Thermo Scientific), TFG (Novus), Sec22b (from the Schekman lab) Sec13 (Proteintech), Sec12 (Proteintech), pIRE1 (Novus), p125a (Novus Biologicals), Rab1A (Cell Signaling Technology), β -actin (Sigma-Aldrich), ERGIC-53 (Santa Cruz), GM130 (BD Trans), GRP78 (Proteintech), SAR1 (from the Schekman lab), Tango1 (from the Saito lab), and Sec16a (Bethyl).

RNA isolation and quantitative PCR

RNA extraction and quantitative PCR (qPCR) analysis were conducted as described previously (79). Briefly, RNA was extracted from RPE1 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and used to synthesize cDNA with a Superscript III First Strand RT-PCR kit (Invitrogen). RT-qPCR amplifications were performed on the CFX384 Touch Real-time PCR detection system (Bio-Rad). The primers used: *sXBP1* F: CCCTCCAGAACATCTCCCCAT; *sXBP1* R: ACATGACTGGGTCCAAGTTGT; *ATF6* F:

TCCTCGGTCAGTGGACTCTTA, *ATF6* R: CTTGGGCTGAATTGAAGGTTTTG; *GAPDH* F:
AGCCACATCGCTCAGACAC, *GAPDH* R: GCCCAATACGACCAAATCC.

Declaration of Interests

The authors declare no conflicts of interest.

Acknowledgements

This work was supported in part by NIH Grant GM134865 (to A.A.). P.L. received support from T32 GM008688, and H.L. is supported by a UW Stem Cell and Regenerative Medicine Center Graduate Fellowship. F.E. is currently supported by grants from the NIH (DK130919 and DK128136). Cell sorting studies were conducted in the UWCCC Flow Cytometry Laboratory (supported by 1S10RR025483-01), electron microscopy studies were facilitated by the SMPH Electron Microscope Core, and some imaging studies were conducted using instrumentation in the UW Optical Imaging Core. We thank David Ginsburg for the gRNA sequence used to introduce frameshift mutations into the Sar1a locus, Randy Schekman and Kota Saito for antibodies, Kyle Quinney for generating genome edited cells natively expressing HaloTag-Sec23a, Jennifer Peotter for assembling a model figure, and members of the Audhya lab for critically reading this manuscript.

Author Contributions

W.K., P.L., M.G.H., and A.A. designed research; W.K., P.L., M.G.H., K.M., A.T., H.L., R.S., S.B., and A.A. performed research; W.K., P.L., F.E., and A.A. contributed reagents/analytic tools; W.K., P.L., H.L., and A.A. analyzed data; and W.K., P.L., and A.A. wrote the paper.

Data Availability

All reagents generated in this study are available from the corresponding author with a Material Transfer Agreement. All data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request. Source data are provided with this paper. Any additional information required to reanalyze the data reported in this paper is available from the corresponding author upon request.

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Figure Legends

Figure 1. Inhibition of Sar1 differentially impacts multiple components of the early secretory pathway.

(A) Cartoon depicting the human *SAR1A* genomic locus is shown. The position of the gRNA used during CRISPR/Cas9 editing (red line) is highlighted, and the sizes of exons (shown as green boxes) are 1/50th that of introns (shown as black lines). (B and C) Representative immunoblots of extracts generated from control and CRISPR-modified cell lines lacking Sar1a, using antibodies directed against Sar1 and actin are shown. Extracts were generated 72 hours (B) or at the timepoint indicated (C) following siRNA treatment. (D) Quantification of the percentage of Sar1 remaining at different timepoints following Sar1b siRNA treatment (relative to mock siRNA treatment) is provided. Error bars represent mean $\pm$ SEM (n = 4). **p < 0.01, calculated using an ANOVA test. (E) Representative immunoblots of extracts generated from control and CRISPR-modified cell lines lacking Sar1a, using antibodies directed against Grp78 and actin (left) are shown. Quantification of the fold-change in Grp78 levels at various timepoints after siRNA treatment (relative to mock) is displayed. Error bars represent mean $\pm$ SEM (n = 4). **p < 0.01 and *p < 0.05, calculated using an ANOVA test. (F) Representative immunoblots of extracts generated from control and CRISPR-modified cells lacking Sar1a, both in the presence and absence of Sar1b, using antibodies directed against Sec13, Sec23, Rab1, TFG, and actin are shown (left). Quantification of the change in expression of various early secretory pathway components is also provided (right). Error bars represent mean $\pm$ SEM (n = 4). *p < 0.05, calculated using an ANOVA test.

Figure 2. The absence of Sar1 causes a redistribution of COPII. (A, C, D, E, F, and G)

Control cells lacking Sar1a (mock transfected) and cells depleted of Sar1b for 72 hours were immunostained using antibodies directed against Sec16a (A), Tango1 (A), Rab1a; red boxed region is magnified to show relative distributions of Rab1a (red) and GM130 (green) with scale bar, 2 μ m (C), ERGIC-53; arrows highlight juxtaposed distribution relative to Sec16a (D), TFG

(D), COPB1 (E), Sec24a; arrow highlights co-localization of Sec24a and TFG (F), GM130 (G) and GOLGA1 (G). Representative confocal images (maximum intensity projections) are shown. Bars, 5 μ m; inset bars, 2 μ m. (B) Quantification of the fold change in the fluorescence intensities of Sec31a, Sec16a, and Tango1 in the absence of Sar1 (relative to mock siRNA treatment) is shown. Error bars represent mean $\pm$ SEM (n = 15 cells each). **p < 0.01, calculated using an ANOVA test. (E) Representative immunoblots of extracts generated from control and CRISPR-modified cell lines lacking Sar1a, using antibodies directed against COPB and actin are shown (top, right). Extracts were generated 72 hours following siRNA treatment. Quantification of the fold change in COPB levels in the absence of Sar1 (relative to mock siRNA treatment) is provided. Error bar represents mean $\pm$ SEM (n = 4).

Figure 3. Depletion of Sar1 drives Sec23a into enlarged structures that accumulate adjacent to Golgi membranes. (A) Representative immunoblots of extracts generated from control and CRISPR-modified cell lines natively expressing HaloTag-Sec23a, using antibodies directed against Sec23a and actin are shown. (B) Genome edited cells expressing HaloTag-Sec23a and lacking Sar1a (mock transfected) and those depleted of Sar1b for 48 hours were imaged live using spinning disk confocal microscopy following labeling with JFX646-HaloTag ligand (left). Bar, 5 μ m; inset bar, 2 μ m. Quantification of the number of Sec23a-positive sites in cells lacking Sar1a in the presence and absence of Sar1b is shown at various timepoints following Sar1b depletion (right). Error bars represent mean $\pm$ SEM (n = 15 cells each). **p < 0.01 and *p < 0.05, calculated using an ANOVA test. (C) The volume distribution of Sec23a-positive structures in the presence and absence of Sar1 is shown following 48 hours of Sar1b depletion. (D) Control cells lacking Sar1a (mock transfected) and cells depleted of Sar1b for 72 hours, either in the presence or absence of nocodazole (1 μ M), were immunostained using antibodies directed against GM130 to examine its distribution relative to HaloTag-Sec23a labeled with JFX646-HaloTag ligand. Representative confocal images are shown (n = 15 cells,

each condition). Bar, 5 μm ; inset bar, 2 μm . Arrows highlight areas where GM130 and Sec23a are closely juxtaposed. (E) The fluorescence intensity ratios between HaloTag-Sec23a and GM130 are shown in control cells lacking Sar1a and those further depleted of Sar1b at various timepoints after Sar1b depletion in the presence or absence of nocodazole. Error bars represent mean $\pm$ SEM (n = 15 cells each). **p < 0.01 and *p < 0.05, calculated using an ANOVA test.

Figure 4. COPII forms phase separated condensates in the absence of Sar1. (A)

Representative fluorescence intensity measurements of HaloTag-Sec23a condensates (labeled with JFX646-HaloTag ligand) taken over time in cells lacking Sar1 isoforms reveal similar fluctuations as compared to phase separated GFP-G3BP1. (B and C) HaloTag-Sec23a in cells lacking Sar1a, either in the presence or absence of Sar1b, was labeled with JFX646-HaloTag ligand and subjected to photobleaching. Fluorescence recovery is depicted for various sized HaloTag-Sec23a structures, both in the absence (B) and the presence of BFA (5 $\mu\text{g/ml}$) (C). Based on recovery, half times to recovery and percent mobile fractions were calculated (C, right). (D) Representative electron micrographs taken within the perinuclear region of high-pressure frozen cells lacking Sar1a following a mock siRNA treatment or Sar1b depletion. The nuclear envelope (NE) is highlighted in each image. Bar, 500 nm.

Figure 5. COPII-mediated cargo transport continues in the absence of Sar1. (A and C)

Spinning disk confocal microscopy was used to image control RPE1 cells (A), cells lacking Sar1a in the presence and absence of Sar1b (A), and cells lacking Sec23b in the presence and absence of Sec23a (C), each expressing ss-DsRed following treatment with SLF (50 μM) to induce cargo disaggregation and release from the ER. Representative timelapse images are shown (n = 15 cells, each condition). Bars, 5 μm . (B) Quantification of cargo (ManII) accumulation within the perinuclear region (GM130 positive) in the various mutant backgrounds

indicated. Error bars represent mean $\pm$ SEM ($n = 15$ cells each). $**p < 0.01$ and $*p < 0.05$, calculated using an ANOVA test. (D) Cells expressing ss-DsRED and lacking both Sar1 isoforms were imaged using STED microscopy following treatment with SLF ($50 \mu\text{M}$) and immunostained using antibodies directed against Sec24a and GM130. Representative images are shown ($n = 15$ cells). Bar, $2 \mu\text{m}$.

Figure 6. Sar1 is associated with COPII transport intermediates and plays a role in their uncoating. (A) Genome edited cells natively expressing HaloTag-Sec23a (labeled with JFX646-HaloTag ligand) and lacking both Sar1 isoforms were immunostained using antibodies directed against GM130 and imaged using STED microscopy. A representative image is shown ($n = 15$ cells). Bar, $2 \mu\text{m}$. (B) Representative linescan analyses highlighting the relative peak intensities of GM130, HaloTag-Sec23a, and a cargo (ss-DsRed) following its release from the ER ($n = 15$ cells). (C and D) Genome edited cells natively expressing various HaloTag fusion proteins (red) were immunostained using antibodies directed against Sec16a (C, green) or Sec31a (D, green) and imaged using STED microscopy (left). Averaged linescans ($n = 10$) demonstrate the relative distributions of each HaloTag fusion protein as compared to Sec16a (C) and Sec31a (D) (right), with arrows highlighting peak intensities. Bars, $1 \mu\text{m}$.

Figure 7. COPII-mediated cargo transport in the presence and absence of Sar1. A model depicting anterograde protein trafficking pathways from the ER under conditions where Sar1 is present and absent. COPII-mediated trafficking is largely restricted to the ER/ERGIC interface under normal conditions (left), but in the absence of Sar1, ERGIC membranes are disrupted and cargo traffic directly to the perinuclear Golgi (right). Without Sar1, transport intermediates that form are unable to disassemble COPII condensates efficiently from the limiting membrane,

which impedes their ability to fuse with the Golgi and deliver cargo, resulting in an accumulation of inner COPII coat components and cargo immediately adjacent to Golgi membranes.

A **B** **C** **D** **E**

Sar1 Present

Sar1 Absent

Supplementary Files

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