

Regulation of SNARE-mediated fusion at the ER-Golgi interface

Emma Mackey

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Reading Committee:

Alexey J. Merz, Chair

Suzanne Hoppins

Kelly Lee

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Emma Mackey

University of Washington

Abstract

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Emma Mackey

Chair of the Supervisory Committee:

Alexey J. Merz

Department of Biochemistry

SNARE-mediated fusion requires assembly of four SNARE domains (R, Qa, Qb, and Qc) distributed across two membranes, with an SM (Sec1/Munc-18 family) chaperone catalyzing this assembly. We investigated topological requirements for the four SNAREs that mediate ER-Golgi fusion using *in vitro* assays. In the presence of the cognate SM Sly1, we find that a single topology drives efficient fusion: the R SNARE on one membrane versus Qa/Qb/Qc SNAREs on the other. These results prompted us to look upstream, at COPII-SNARE interactions. The assembled COPII coat is known to block fusion. We discovered that of five COPII core subunits, the Sar1 GTPase and Sec23/Sec24 subunits were necessary and sufficient to prevent fusion for prolonged periods, and that Sar1 was dispensable for transient inhibition. When Sec24-interactions with vesicle SNAREs were disrupted, inhibition was relieved, indicating that SNARE sequestration by COPII prevents fusion. Tethers are an essential contact for vesicles before fusion. While many tethers have been proposed, reconstitution has up to now been unsuccessful. I found that of the possible pathways at least one tether combination, Sec17/18 and COGA, were able to promote fusion without additional factors. These observations help to explain how appropriate fusion events are facilitated while inappropriate events are deterred.

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Chapter One: The secretory pathway and its reconstitution

Sequential movement of cargo from the ER to the Golgi was first characterized by Palade. The static organelles that had previously been observed were now able to be visualized as highly dynamic and constantly communicating. Through feeding cells with radiolabeled nutrients, he was able to follow the production and movement of newly synthesized proteins through secretion in pancreatic cells which lent themselves to being a great model of secretion due to their high production rates. This also led to the realization that there is high level organization that maintains differences between organelles, creating organelle identity. Using radioisotope tracers, subcellular fractionation, and electron microscopy, Palade and coworkers had the ability to resolve the production and movement of proteins temporally and spatially (Palade, 1975). This discovery led to a new field, working to understand how intracellular traffic is conducted and controlled. As technology improved so did the field's ability to dissect the complicated processes of vesicular transport.

Vesicular transport can be broken down into a few key steps. First cargo is collected at the budding donor membrane. The vesicle must undergo membrane scission to separate from the donor organelle. The formation of an ER-to-Golgi vesicle requires the coatamer protein complex II (COPII) (Barlowe, et al., 1994). Second, once the vesicle is free, the protein coat used to form the vesicle must dissociate. Third, the vesicle must be recognized via long range protein interactions by the acceptor organelle (Novick, et al., 1981; Zeuschner, et al., 2006). Tethers are the first contact between the two membranes, leading to vesicle capture. The order of these first three steps is not consistent between different organelles and indeed is not agreed upon for the ER to Golgi transport step (Shomron, et al., 2021; Robinson, et al., 2015). Finally, a tether (or tethers) brings the vesicle into close range leading to fusion with the target membrane (Sztul & Lupashin, 2009). The membranes come into close contact, this is the docking step at which the fusion machinery is within range and primed. Finally, four soluble N-ethylmaleimide-sensitive-factor attachment protein receptors (SNAREs) bind tightly to each other across the two membranes. For fusion to occur SNAREs also require a Sec1/Munc18 (SM) family chaperone which guides the fusion reaction (Yu & Shen, 2023). The force generated by this interaction is strong enough to cause membrane fusion (Weber, et al., 1998). The coordination between each of these steps must be tightly controlled to maintain transport directionality.

Following up on this work, Randy Schekman and others turned to *S. cerevisiae* to genetically dissect the genes critical for vesicular transport. Through an initial screen looking for deficiencies of invertase and acid phosphatase activity and exhaustive transmission electron microscopy (TEM), only a single temperature sensitive mutant was identified. This mutant, *sec1-1* had an amazing phenotype of excessive accumulation of secretory vesicles (Novick and Schekman, 1979). An effort to extend this work led to another genetic screen looking specifically for a similar phenotype of vesicle accumulation. However, this temperature sensitive mutant screen once again only led to the discovery of one additional gene (Schekman and Novick, 2004). A breakthrough came with density gradient separation of cells, after the discovery that secretory mutations seemed to impair the growth of the plasma membrane leading cells to be denser than wild-type cells (Henry, et al., 1977). This new approach allowed the Schekman group to find another twenty-two critical secretory genes (Novick et al., 1980, Kaiser & Schekman, 1990). The characterization of these mutants through distinct organelle morphologies gave a jumping off point for future studies of how each protein was involved in secretion. Specifically of interest for my work, Sec17, Sec18, and Sec22 mutants caused large ER tubules, and an accumulation of small vesicles between the ER and the Golgi, this phenotype was distinct from the secretory vesicle phenotype observed for Sec1 mutants, which block later steps in the secretory pathway (Schekman and Novick, 2004).

The function of the Golgi apparatus as the location of protein modification was quickly recognized as a tool for measuring transport. Many secreted proteins are glycosylated. A protein produced by ribosomes at the ER will be initially glycosylated at the ER and not modified until it reaches the proper compartment at the Golgi. This is a measurable output that was captured to measure overall transport levels between these two organelles. Sorting is critical for many cellular and organismal processes such as lysosomal enzyme sorting, neuropeptide secretion, and insulin processing (Kornfeld, 1987; Funkelstein et al., 2010; Steiner and Oyer, 1967). The first reconstitution of traffic measured transport by the increase of glycosylated vesicular stomatitis virus (VSV) G-protein (Balch et al., 1984). The method used by Balch and Rothman pioneered cell transport assays between organelles from different cells. To create a “donor” population of organelle they used a mutant cell line that lacked a glycosylation enzyme and had been fed with radiolabeled carbon. This gave them an input that would have unmodified and radiolabeled proteins allowing separate membrane fractions to be selectively trafficked to and monitored.

Through salt washes and centrifugation the organelles were purified. This fraction of organelles was then combined with an “acceptor” organelle fraction from a wild-type cell line. Combining the two fractions lead to an increase in glycosylated G-proteins not observed with either population alone (Balch et al., 1984). This assay gave the field the discovery of the function of Sec17 in parallel with studies from the Schekman lab showing the importance of this protein complex at several membranes (Kaiser and Schekman, 1990). Clary and Rothman depict the sequential nature of transport as vesicle and acceptor membrane contact, uncoating of the vesicle, and finally fusion. Sec17 and Sec18, known in the mammalian system as alpha-SNAP and NSF respectively, were critical for determining the nature of SNARE mediated fusion (Clary, et al., 1990).

With the understanding of the basic building blocks for fusion, the Rothman lab developed reconstituted proteoliposomes (RPLs) to test the minimal needs for fusion *in vitro* (Weber, et al., 1998). This is the system we have adapted to study fusion in our lab. Weber and Rothman’s initial experiments found that spontaneous SNARE-mediated fusion could occur, and at lower temperatures fusion was stalled at the docking step. Spontaneous fusion is slow and also error prone, and it is well established that *in vivo* an SM chaperone is necessary (Baker & Hughson, 2016; Gillingham & Munro, 2019). The ER-Golgi SM is Sly1, essential for its role in promoting fusion. The SM chaperone works by interacting with the SNAREs and templating the fusion complex (Baker, et al., 2015).

Here I expand on the cell free mechanisms through using a reconstituted system. Proteoliposomes made of a known lipid composition and purified protein serve as mimics for vesicles and the acceptor Golgi membrane (Lupashin, et al., 1996; Schneider, et al., 1999). Through this system I dissect the intrinsic topological restrictions of SNAREs and their chaperone for fusion, how coat protein interactions disrupt fusion, and begin efforts of reconstituting tethering.

Chapter Two: SNARE topology and restriction by COPII

This chapter is adapted in full from the publication:

Mackey, E. J., Takenaka, T., Miller, E. A., & Merz, A. J. (2026). *Sequential restriction of SNARE-mediated fusion by the COPII inner coat and SNARE chaperones* (p. 2026.02.02.703378). bioRxiv. <https://doi.org/10.64898/2026.02.02.703378>

Introduction

Vesicular traffic within the endomembrane system controls subcellular protein distribution, organelle biogenesis and turnover, and secretion. The final step of vesicular transport, fusion of two compartments, is a nexus of specificity and regulation. Fusion events are executed by the SNARE (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein family (Bombardier and Munson, 2015; Jahn et al., 2023). Fusion requires assembly of four distinct SNAREs into a parallel α -helical bundle that spans the donor and acceptor membranes — the pre-fusion *trans* SNARE complex (Hanson et al., 1995; Poirier et al., 1998; Sutton et al., 1998; Weber et al., 1998). Following fusion, the hyperstable *cis* SNARE complex is disassembled by an ATPase complex composed of Sec17 and Sec18 (α -SNAP and NSF, in mammals). This recycles and reenergizes SNAREs so that they can catalyze subsequent rounds of fusion (Hanson et al., 1995; Khan et al., 2025; Mayer et al., 1996).

Each class of membrane fusion event employs a unique set of SNAREs, though some SNARE proteins operate in more than one pathway (Grissom et al., 2020). Each SNARE bundle contains four parallel helices in a stereotypical arrangement (Fasshauer et al., 1998; Sutton et al., 1998). Based on sequence homology and position within the bundle, the four helices are named Qa, Qb, Qc, and R. In this nomenclature, Q and R denote the presence of either glutamine (Q) or arginine (R) residues that contribute side chains to a central “ionic” packing layer near the middle of the bundle. At the presynaptic membranes of neurons, only two SNAREs are anchored by C-terminal transmembrane helices: VAMP2/Synaptobrevin (R) resides on vesicles, whereas Syntaxin1a (Qa) is on the plasma membrane. A third SNARE, SNAP-25, contains both Qb and Qc helices, separated by an unstructured linker. SNAP-25 associates with the target membrane through palmitoyl modifications to the linker (Rizo and Xu, 2015). Most fusion events, however, employ four separate SNARE polypeptides, each bearing its own C-terminal membrane anchor.

Defining which SNAREs must reside on donor versus acceptor membranes is the SNARE topology problem.

SNAREs bearing C-terminal transmembrane domains are inserted into membranes at the ER through the tail-anchor pathway (Mateja and Keenan, 2018). SNAREs are then exported from the ER as cargo in COPII vesicles (Miller et al., 2002). They then traffic to steady-state locations in the secretory and endolysosomal systems. These locations are dynamically maintained through cycles of anterograde and retrograde traffic. During these processes, there are numerous opportunities for off-target fusion to occur. Consequently, mechanisms that restrict the range of allowed SNARE fusion topologies are thought to be critical determinants of directionality and specificity — and ultimately, of organelle identity (Koike and Jahn, 2022). The sets of SNARE topologies that can assemble into productive fusion machines *in vivo* are constrained by: (i) the intrinsic assembly properties of SNAREs themselves (McNew et al., 2000a); (ii) chaperones that catalyze *trans*-SNARE complex assembly and proofread assembled complexes (Choi et al., 2018; Dubuke and Munson, 2016; Schwartz et al., 2017; Zhang and Hughson, 2021); and (iii) factors such as vesicle coats that control which SNARE proteins are present on which membranes (Miller et al., 2002; Mossessova et al., 2003).

Among the SNARE chaperones, Sec1/mammalian Unc-18 (SM) proteins are central. SMs are thought to be essential for all SNARE-mediated fusion in cells (Rizo and Sudhof, 2012). After many years of controversy, breakthrough structures of the endolysosomal SM Vps33 suggested a unifying model: SMs are enzymes that catalyze assembly of a half-zipped *trans* “template complex” between the Qa and R SNAREs (Baker et al., 2015; Jahn et al., 2023; Rodkey et al., 2008). This model makes a key prediction about SNARE topology: Qa and R SNAREs must always function *in trans* to one another. How SM proteins interact with Qb and Qc SNAREs, and whether SMs impose additional topological restrictions on Qb or Qc SNARE function, are unresolved questions.

We study the first trafficking event in the secretory pathway: fusion of ER-derived COPII carriers at the *cis*-Golgi or the precursor ER-Golgi intermediate compartment (ERGIC). The COPII coat comprises five subunits that sequentially assemble at the ER exit site (Antonny et al., 2001; Sato and Nakano, 2005). Upon GTP binding, the Sar1 GTPase associates with the membrane (Van der Verren and Zanetti, 2023). Sar1 then recruits the Sec23/24 complex (or variant complexes containing Sec24 paralogs). Sec24 binds the cytoplasmic tails of

transmembrane cargo molecules to capture and concentrate them for ER export. Finally, the Sec13/31 complex associates with and stabilizes the nascent coat lattice. Although the spatiotemporal dynamics of COPII uncoating and the features of COPII carriers are under intense discussion, uncoating cannot occur until Sar1-bound GTP is hydrolyzed. Before GTP hydrolysis, COPII blocks fusion of the carrier with target membranes (Barlowe et al., 1994; Cai et al., 2007b; Oka and Nakano, 1994). Though the mechanism through which COPII blocks fusion has not been fully dissected, COPII contains SNARE binding sites that might prevent SNAREs from entering pre-fusion complexes (Mossessova et al., 2003; Miller et al., 2003). *In vivo*, COPII may uncoat partially, or in stages. COPII subcomplexes may contribute to vesicle tethering at Golgi precursor membranes (Angers and Merz, 2011; Cai et al., 2007a).

There are seven possible 1:3 or 2:2 SNARE topologies. In yeast, COPII-mediated anterograde traffic requires four SNAREs: Sed5 (Qa), Bos1 (Qb), Bet1 (Qc), and Sec22 (R) (Banfield et al., 1994; Newman and Ferro-Novick, 1987; Novick et al., 1980; Shim et al., 1991; Sogaard et al., 1994). At least three different — and incompatible — topologies have been proposed to catalyze fusion in the COPII pathway (Fig. 1.1E, 1.1C or 1.1F, and 1.1I). In studies with chemically defined reconstituted proteoliposomes (RPLs), Rothman's lab assigned Bet1 (Qc) to the COPII vesicle, opposite Sed5 (Qa), Bos1 (Qb), and Sec22 (R) on the target membrane (McNew et al., 2000a; Parlati et al., 2000; Parlati et al., 2002). However, no SM chaperone was present in these fusion experiments. Work using complex yeast cell extracts and temperature-sensitive SNARE mutants challenged the Rothman lab's interpretation (Cao and Barlowe, 2000; Liu and Barlowe, 2002; Miller et al., 2005). These experiments suggested that Sec22 (R), Bos1 (Qb) and Bet1 (Qc) function on the COPII vesicle, opposite Sed5 (Qa) on the Golgi target membrane. Experiments using chemically defined RPLs, but now in the presence of the SM Sly1, yielded yet another result: robust fusion was observed with the R SNARE opposite Qa, Qb, and Qc (Duan et al., 2024a; Duan et al., 2024b; Furukawa and Mima, 2014; Jun and Wickner, 2019). These studies, however, did not explore alternative SNARE topologies.

Here, we use chemically defined reconstitutions to explore all possible ER-Golgi SNARE topologies, in the presence and absence of the essential SNARE chaperones Sly1 (SM), Sec17, and Sec18. Lipid compositions mimic Golgi membrane composition. As there is occasionally heterotypic fusion, we chose to mimic this lipid environment so that both populations would have identical composition, making SNARE topology the only variable. In the absence of Sly1

there is little or no fusion, consistent with the lethal phenotype of *sly1* Δ null alleles. In the presence of Sly1, one of seven possible topologies catalyzes efficient fusion: R opposite Qa, Qb, and Qc. Building on these results, we explore how the COPII coat prevents fusion until the coat depolymerizes. Surprisingly, although COPII packages all four SNARE proteins into ER-derived carriers, we find that COPII-SNARE binding, through a specific site on Sec24, is necessary to block fusion. We propose a working model in which both COPII suppression of fusion, and Sly1 facilitation of fusion, are consistent with a unique functional SNARE topology. This arrangement may not only facilitate anterograde traffic, but also provide a mechanism for suppressing inappropriate fusion events across alternative topologies that might disrupt organelle identity and maturation.

Results

Sly1 stimulates fusion with strict SNARE topological restrictions

Crystal structures of SNAREs complexed with the lysosomal SM Vps33 suggest that R and Qa SNAREs must function *in trans* (Baker et al., 2015). This is consistent with the arrangement of neuronal exocytic SNAREs, which have only two rather than four transmembrane anchors. Elegant force spectroscopy studies corroborated this model, demonstrating that an SM, a Qa-SNARE, and an R-SNARE can form a partially-zipped intermediate called the template complex (Baker et al., 2015) of SM proteins (Sly1, Sec1, and Vps45) with client SNARE complexes implies that the requirement for Qa and R-SNAREs to act on opposite membranes is generalizable (Fig. 1.1A; Fig. S1; Fig. S2)(Baker et al., 2015). We therefore hypothesized that *any* arrangement that places the R and Qa SNAREs *in trans* should drive fusion in the presence of a cognate SM. Accordingly, Qb and Qc SNAREs should be able to drive fusion when anchored in either membrane.

To test this model, we prepared RPLs bearing all possible topologies: four sets with one SNARE versus the remaining three (Fig. 1.1B-E), three sets with pairs of two SNAREs in opposition (Fig. 1.1F-H), and one set with all four SNAREs present on both membranes, recapitulating homotypic vesicle fusion (Fig. 1.1I). The SNAREs and chaperones were present at near-physiological concentrations (Supplementary Table S1). Of the seven unique topologies, four place the R and Qa SNAREs *in trans*, while three place the R and Qa *in cis*. We measured fusion between RPL populations using two orthogonal FRET pairs, one reading out lipid mixing and the other, content mixing. Our analyses focused on content mixing, the fusion reaction endpoint. We set up reactions with paired RPL populations, took baseline fluorescence readings, and then added Sly1 to initiate fusion. To our surprise, Sly1 initiated rapid and efficient fusion with only one of the seven unique topologies: the R SNARE versus the Qa/Qb/Qc SNAREs (Fig. 1.2A-B). With this topology rapid fusion immediately followed Sly1 addition.

A second topology, R/Qc vs. Qa/Qb, drove substantial fusion but with slower kinetics. Ten minutes into the reaction, about half as much fusion occurred as with the R vs. Qa/Qb/Qc topology (Fig. 1.2B). Thus, there appears to be some plasticity for Qc SNARE topology; however, the lower efficiency of the R/Qc opposite Qa/Qb topology suggests that it is not preferred. In extensive mutational studies of Sly1 and the Qa-SNARE Sed5, even a ~50% decrease in fusion *in vitro* correlated with conditional lethality *in vivo* (Duan et al., 2024a; Duan

et al., 2024b). Among the seven unique topologies only these two drove substantial fusion, with the remaining five virtually inactive.

These findings are in tension with previous work suggesting that fusion is catalyzed solely by the Qc vs. R/Qa/Qb topology (McNew et al., 2000a; Parlati et al., 2000; Parlati et al., 2002). That conclusion was based on lipid mixing experiments in a chemically defined system, where Sly1 was not present. The apparent discrepancy results, at least in part, from our use of content mixing as a primary endpoint. When we examined lipid rather than content mixing, very slow but reproducible lipid mixing was detected with the the Qc vs. R/Qa/Qb topology. This signal was independent of Sly1 addition. In comparison, the R vs. Qa/Qb/Qc topology catalyzed essentially no lipid mixing unless Sly1 was present, at which point the signal quickly outpaced the Qc vs R/Qa/Qb topology (Fig. S3). Lipid mixing without content mixing could indicate accumulation of a stalled or hemifused state. Thus, our experimental findings are at least partially consistent with the Rothman group's. Nevertheless, as content mixing is minimal and unchanged in the presence of Sly1, and given that loss of Sly1 is lethal *in vivo* (Duan et al., 2024b; Ossig et al., 1991), we suggest that the Qc vs. R/Qa/Qb and the R/Qc vs Qa/Qb topologies do not catalyze physiologically relevant fusion *in vivo*.

We also tested RPLs with all four SNAREs on both membranes, a configuration that mimics homotypic fusion, as reported to occur with mammalian COPII carriers in a cell-free system (Xu and Hay, 2004). These events require the mammalian Sec17/Sec18 homologs α -SNAP/NSF, ATP, and mammalian Sly1 (SCFD1). As expected from these studies and experiments with RPLs (Duan et al., 2024a; Duan et al., 2024b; Jun and Wickner, 2019), in the absence of Sec17/18:ATP we observed little or no homotypic fusion of 4-SNARE RPLs (Fig. 1.2A-B). Thus, we next asked whether Sec17/18:ATP can promote Sly1-dependent fusion across a broader range of SNARE topologies.

Sec17/18 and SNARE topology

Sec17/18:ATP disassemble post-fusion *cis*-SNARE complexes, releasing SNAREs to catalyze subsequent rounds of fusion (Hanson et al., 1997; Mayer et al., 1996). These enzymes also disassemble unproductive *cis*- and *trans*-SNARE complexes that assemble aberrantly (Choi et al., 2018; White et al., 2025), and they can in some cases help otherwise non-fusogenic SNARE complexes to function. We hypothesized that cycles of SNARE assembly and

disassembly might allow alternative SNARE topologies multiple chances to form productive *trans* complexes, leading to fusion. Alternatively, Sec17 and Sec18 might directly stimulate SM-dependent fusion (Ma et al., 2016; Orr and Wickner, 2022; Schwartz et al., 2017; Schwartz and Merz, 2009; Song et al., 2021; Zick et al., 2015).

In the presence of Sec17/18:ATP and Sly1, again at near-physiological concentrations, there was rapid fusion with the 4 SNARE vs 4 SNARE (homotypic) configuration (Fig. 1.3H), consistent with previous work. No fusion occurred when R and Qa SNAREs opposed Qb and Qc SNAREs (Fig. 1.3E). Some fusion did occur when the R/Qc pair opposed Qa/Qb (Fig. 1.3G), but this fusion was slower than with the R vs. Qa/Qb/Qc topology, and Sec17/18 and ATP did not increase the rate or total amount of fusion for this topology.

There was a delayed fusion signal with the R/Qb/Qc vs. Qa topology. Over the first 10 minutes virtually no fusion was detected; however, by 30 minutes there was a noticeable signal (Fig. 1.3B). It is important to note that heterotypic fusion generates post-fusion RPL populations that display all four SNAREs. Sec17/18:ATP may allow the products of slow or inefficient first-round fusion events to undergo additional rounds of more efficient fusion that do not reflect the activity of the starting SNARE topology. A delayed signal may suggest that inefficient fusion gradually creates a third population of RPLs bearing all four SNAREs, which can then fuse efficiently with at least one of the starting populations. We infer that this occurs with the R/Qb/Qc vs. Qa topology.

Sly1 binds the Qa SNARE Sed5 with such high affinity ($K_D < 1$ nM) that under physiological conditions the complex probably does not dissociate, even during Sec18-mediated complex disassembly. (Demircioglu et al., 2014; Grabowski and Gallwitz, 1997; Khan et al., 2025). Indeed, a chimeric Sly1-Sed5 fusion protein can restore viability to a *sed5Δ* null mutant, demonstrating that Sed5 and Sly1 can function without dissociation (Gao and Banfield, 2020). We therefore asked whether pre-assembly of Sed5 and Sly1 prior to docking and Sec17/18 activity might alter the topological specificity of fusion. When this order of addition was tested for the R vs Qa/Qb/Qc topology, there was a small but reproducible increase in the initial rate of fusion (Duan et al., 2024a). To test whether the order of Sly1 addition alters topological requirements, we tested four additional topologies by adding Sly1 to Qa-bearing RPLs before priming by Sec17/18 or tethering (Fig. S4A). Early Sly1 addition did not stimulate fusion in the Qc vs R/ Qa/Qb topology (Fig. S4C). For both the R/Qc vs Qa/Qb and the R/Qb/Qc vs Qa

topologies there was a decrease in the lag before fusion and a mild increase of total fusion stimulated (Fig. S4D-E). While there was a decrease in the lag before onset of fusion, there was no rapid fusion upon initiation of the reaction, as seen with the homotypic topology (Fig S4F). Early Sly1 addition does not appear to modify the topological constraints on fusion.

Qb and Qc SNAREs transmembrane anchors are needed for efficient fusion

The yeast vacuole and presynaptic SNARE systems include one (Qc) or two (Qb and Qc) SNARE domains that lack a C-terminal transmembrane anchor. In chemically defined reconstitutions of yeast vacuole fusion (which included the HOPS tethering complex), fusion could be driven by soluble Qb and Qc SNAREs. We tested the limits of early secretory SNARE topology in reactions with Qb and Qc SNAREs that lack transmembrane anchors. The R and Qa SNAREs were always *in trans*, and when present, full length Qb or Qc SNAREs were *in cis* to the Qa. Soluble Qb or Qc SNARE cytoplasmic domains lacking transmembrane helices (Qb 1-222 and Qc 1-123), were added to reactions that contained the other full-length SNAREs, Sec17/18, ATP, and Sly1 (Fig. 1.4A). Even when soluble SNAREs were present at high concentrations (up to 4 μ M), only modest fusion occurred, far below the level of fusion for the R vs Qa/Qb/Qc topology (Fig. 1.4B). Soluble Qb drove slightly more efficient fusion than the other configurations, and this mild stimulation was observed only in the presence of Sly1 and Sec17/18/ATP (Fig. 1.4C). These results provide further evidence that early secretory Qb and Qc SNAREs drive efficient, Sly1-dependent fusion when anchored in the same membrane as the Qa SNARE.

Sar1 and Sec23/24 are necessary and sufficient for COPII inhibition

With a better understanding of SNARE topological constraints in hand, we turned our attention to the machinery that forms the ER-Golgi carrier organelle. In cell extract experiments, when the full COPII coat (Sar1:GPPNP, Sec23/24, and Sec13/31) was locked onto the vesicle, fusion was blocked. This indicated that Sar1 GTP hydrolysis and at least partial COPII disassembly are prerequisites for fusion (Barlowe et al., 1994; Oka and Nakano, 1994). Because these early studies required the full COPII coat to form carriers, it was not possible to rigorously define the minimal set of COPII components that prevents fusion. Moreover, it was unclear whether additional factors might also be needed for COPII to block fusion. Although Sar1:GDP

dissociation occurs rapidly, it is unclear whether the rest of the COPII complex dissociates at the same time, or if some subunits remain associated with the carrier (Antonny et al., 2001; Barlowe et al., 1994; Cai et al., 2007b; Lord et al., 2011; Sato and Nakano, 2005). It was therefore of interest to determine which subunits directly inhibit fusion.

To reactions containing RPLs bearing the R vs Qa/Qb/Qc SNAREs, we added purified COPII components to test how each component contributes to fusion inhibition (Fig. 1.5A-B). The Sec23/24 and Sec13/31 complexes were added to reactions at or slightly below their *in vivo* concentrations, while Sar1 was added to about twice its *in vivo* concentration (see Supplementary Table S1). This was done to increase Sar1:GTP levels, since the Sar1 nucleotide exchange factor Sec12 was not present in the reactions (Barlowe and Schekman, 1993).

Fusion was tested under both hydrolyzing (GTP) and non-hydrolyzing (GPPNP) conditions. As expected, in reactions with any combination of COPII protein and GTP we saw high levels of fusion both early (10 min) and near completion of the reaction (30 min). Sar1 GTP hydrolysis allows COPII to dissociate from the membrane for fusion to proceed. The initial rate of fusion, even in the presence of Sar1 alone, was lower than in reactions lacking COPII at 10 minutes ($p=0.04$) and 30 minutes ($p=0.05$). This decrease is observed across all three conditions. We suspect that membrane insertion of the Sar1 N-terminal helix partially opposes fusion (see Discussion).

In the presence of GPPNP, fusion was strongly impaired with all five COPII proteins present (Fig. 1.5C). These results recapitulate the original yeast extract studies and reveal that COPII by itself inhibits fusion, without any additional factors. We next tested combinations of the COPII components for inhibition of fusion. In reactions containing Sar1:GPPNP and Sec23/24, but lacking Sec13/31, fusion was inhibited as completely as in the presence of all five COPII components. Thus, the inner coat complex (Sar1:GPPNP and Sec23/24) is sufficient to block fusion. The Sec13/31 outer shell does not contribute to inhibition (Fig. 1.5C).

In the yeast extract system, higher concentrations of COPII, Sec23/24 complex, or recombinant GST fusions to Sec23 or Sec24 inhibit COPII carrier tethering and fusion, even under GTP-hydrolyzing conditions (Barlowe, 1997; Cai et al., 2007b). We therefore tested whether Sec23/24 could inhibit fusion when Sar1 was not present. There was substantial inhibition early in the reaction, but this inhibition diminished over time (Fig. 1.5D). Sec23/24 binds membranes through its affinities for Sar1:GTP, the membrane itself, and cargo proteins

including SNAREs. The results suggest that, even without Sar1, Sec23/24 heterodimer can partially inhibit fusion. This is consistent with experiments where Sar1 is released rapidly during carrier formation but the remaining COPII subunits dissociate more slowly (Barlowe, 1997; Cai et al., 2007b). Perhaps unexpectedly, Sec23/24 alone appears to inhibit fusion almost as completely as Sar1:GPPNP and Sec23/24 for the initial 10 minutes of the reaction, but by 30 minutes behaves more like Sar1:GTP and Sec23/24 (Fig. 1.5D). We conclude that Sec23/24 is primarily responsible for fusion inhibition, while Sar1:GPPNP causes stable Sec23/24 association and prolonged inhibition of fusion. The early inhibition observed here may indicate that, *in vivo*, active mechanisms promote fusion by stripping Sec23/24 from the carrier. Candidate factors for such an activity include the tethering factor Grh1, casein kinase I, and, in multicellular organisms, TFG (Behnia et al., 2007; Hanna et al., 2017; Humphreys et al., 2021; Lord et al., 2011).

Sec23/24 binding interactions with SNAREs are necessary for inhibition

Does COPII act as a relatively nonspecific “steric mesh” that prevents the membranes from coming into close contact, or does inhibition depend mainly on specific regulatory coat-SNARE interactions? Stereospecific interactions between Sec23/24 and SNAREs package SNAREs at ER exit sites, ensuring that COPII carriers are competent to fuse with target membranes. However, COPII-SNARE interactions might directly regulate the ability of SNAREs to mediate fusion. There are three distinct cargo binding sites on Sec24, each recruiting different SNAREs.

Missense mutations in the Sec24 cargo binding sites disrupt interactions with SNAREs and other cargo. Two specific point mutants, L616W and W897A, are of special interest. Like wild type Sec24, these mutants assemble into stable lattices and do not impair COPII carrier formation. These mutants were originally identified through defects in their ability to package SNAREs into vesicles. In our experiments, the SNAREs are already present on the RPLs, so the cargo binding mutants can be used to test whether Sec24-SNARE binding interactions are important for COPII inhibition at the fusion stage. Sec24 L616W disrupts the B-site, impairing its interaction with the Qc and R SNAREs, and *in vivo*, cannot support cell viability (Miller et al., 2003). Sec24 W897A disrupts the A-site, which mediates Qa SNARE binding. *In vivo*, the W897A mutation is lethal if the partially redundant Sec24 paralog Sfb2 is deleted (Miller et al.,

2005). In fusion assays with GPPNP, the B-site mutant Sec23/Sec24 (Sec24 L616W) inhibited fusion less strongly than the wild type, and similarly to Sar1-only conditions (Fig. 1.6C-D). In marked contrast, mutation of the Qa-only A-site pocket (Sec24 W897A) did not relieve inhibition in the presence of GPPNP. Moreover, in reactions with GTP and the A-site mutant, fusion was somewhat slower than with wild type Sec23/24. (Fig. 1.6A-D). As we had found above that wild-type Sec23/24 was capable of transiently inhibiting fusion without Sar1, we tested whether this inhibition required interactions with SNAREs. We found that Sec23/24 containing either SNARE binding mutation had little or no inhibitory effect. (Fig. 1.6E-F). We conclude that SNARE sequestration within the Sec24 B-site is the primary mechanism through which COPII inhibits fusion.

Discussion

Our results argue that ER-Golgi fusion requires the R SNARE oppose the Qa and Qb SNAREs. The Qc SNARE may act on either membrane, but is most efficient *in cis* to the Qa and Qb SNAREs. As discussed below, strong evidence indicates that the Qa SNARE Sed5 functions on the Golgi acceptor membrane. Thus, the R SNARE Sec22 is the primary fusogenic SNARE on the COPII carrier. Consistent with these findings, we find that Sec24 binding to R and Qc SNAREs is the primary mechanism through which COPII inhibits fusion. This paints a satisfying picture: COPII first ensures that the R (and perhaps Qc) SNAREs required for fusion are packaged into the carrier. It then holds them ready but inactive until the coat's inner shell has been shed.

Little or no basal fusion occurs in the absence of Sly1 with any SNARE topology, consistent with the lethality of *sly1* Δ mutants. In the presence of Sec17/18 and ATP, fusion is strongly stimulated when all four SNAREs are present on both membranes, since disassembly of *cis*-SNARE bundles is required for fusion in the homotypic configuration. Even when all SNAREs are present on both membranes, it is reasonable to assume that productive fusion reactions employ the R SNARE in *trans* to Qa/Qb/Qc. Thus, the intrinsic properties of SNAREs and the SM act together to enforce topological constraints on fusion. The requirement for R and Qa SNAREs to operate *in trans* is consistent with crystallographic, biophysical, and modeling studies. In contrast, it is not yet possible to explain the requirement for Qb and Qc SNAREs to operate *in cis* to the Qa.

Previously, Rothman's group examined basal ER-Golgi fusion in the absence of the SM Sly1, and identified a unique functional topology: Qc opposite Qa, Qb, and R (McNew et al., 2000a; Parlati et al., 2000; Parlati et al., 2002). This discrepancy between our experiments results from the presence or absence of Sly1, the fusion readouts used, and perhaps other differences in methodology. We primarily used content mixing signals while the Rothman experiments relied solely on lipid mixing. Importantly, we observed very slow but reproducible lipid mixing with the Qc vs. Qa/Qb/R topology, but there was no stimulation by Sly1 with this topology (Fig. S3). Additionally, our studies used different lipid-to-protein ratios, different lipid compositions, and different lipid mixing reporters. *In vivo*, all SNARE-mediated fusion requires an SM (Sudhof and Rothman, 2009).

Experiments with cell extracts demonstrated that Sed5 (Qa) and Sly1 must act at the target membrane, and are not needed on the COPII carrier (Cao and Barlowe, 2000; Miller et al., 2005). Cao and Barlowe further suggested that Bos1 (Qb) and Bet1 (Qc) function only on the COPII carrier, along with Sec22 (R) (Liu and Barlowe, 2002). In contrast, our experiments suggest that fusion is most efficient when all three Q SNAREs are *in cis*. Fusion with the R/Qb/Qc vs Qa topology was slow and exhibited a long lag after initiation. A stronger fusion signal was observed when Sly1 was preincubated with the Qa SNARE RPLs, but preincubation did not overcome the long lag (Fig. S4E). We conclude that the R/Qb/Qc vs Qa topology does not efficiently catalyze fusion. The basis for the discrepancy between our results and those of Cao and Barlowe is unclear. One possibility is that additional factors present in the cell extract system could influence SNARE topology requirements. For example, the use of temperature-sensitive SNARE mutants may perturb interactions with COPII or tethering factors upstream of *trans* SNARE complex assembly. Interactions between the Uso1/p115 tether and R, Qb and Qc SNAREs were recently established (Bravo-Plaza, et. al., 2023). Thus, it is possible that these SNAREs have functions in tethering distinct from their roles in direct catalysis of fusion.

Previous chemically defined reconstitutions of fusion yielded divergent results with respect to the specificity and selectivity of SNARE topological restriction. These studies were mostly done in the absence of SMs (Fukuda et al., 2000; Furukawa and Mima, 2014; McNew et al., 2000a; Parlati et al., 2000; Parlati et al., 2002; Zwilling et al., 2007). In the yeast vacuole system, the HOPS complex contains the SM Vps33, but HOPS contains five other subunits, some of which bind SNAREs (Dulubova et al., 2001; Lobingier and Merz, 2012; Song and Wickner, 2017; Wickner et al., 2023). In the vacuole system the Qc SNARE is soluble. Surprisingly, no published experiments in this highly characterized system have directly compared fusion with native (non-mutant) SNAREs in the three possible arrangements of R, Qa, and Qb. To our knowledge, in fact, only one previous study, from Shen's laboratory, has systematically explored SNARE topology in the presence of an SM and without the potentially confounding presence of other tethering factors (Rathore et al., 2011). In their experiments, chimeric neuronal SNAREs bearing Qb and Qc SNARE domains were fused to C-terminal transmembrane domains. Fusion was totally dependent on Munc18-1, and efficient only with the R vs. Qa/Qb/Qc topology. Although the Shen group's system is synthetic, their results suggest

that underlying mechanisms that enforce topology in the early secretory pathway may be conserved across SM-SNARE systems.

Yeast cells lacking the R-SNARE Sec22 are thermosensitive and grow slowly, but are viable (Dascher et al., 1991; Liu and Barlowe, 2002; Ossig et al., 1991). In these cells the R-SNARE Ykt6 substitutes for Sec22 (Liu and Barlowe, 2002). Ykt6 is promiscuous, operating in other Golgi fusion events as well as vesicle and autophagosome fusion with lysosomes (Bas et al., 2018; Gao et al., 2018; Kweon et al., 2003; Matsui et al., 2018; McNew et al., 1997; Takáts et al., 2018; Tateishi et al., 2025; Watanabe et al., 2024). Ykt6 lacks a transmembrane anchor, instead associating with membranes through farnesyl and geranylgeranyl moieties appended Cys-Cys motif at its C-terminus (McNew et al., 1997; Sakata et al., 2021; Shirakawa et al., 2020; Tateishi et al., 2025). If Ykt6, like Sec22, acts *in trans* to Qa, Qb, and Qc SNAREs, then it must drive fusion with no α -helical transmembrane anchor on one membrane. However, when either the Qa or R transmembrane segment of a neuronal SNARE complex was replaced with prenyl anchors in a minimal reaction system, anchors longer than the geranylgeranyl group were required to initiate lipid mixing (McNew et al., 2000b). It will be illuminating to examine the mechanisms of Ykt6-mediated fusion in chemically defined systems that incorporate SMs and other cofactors.

COPII is a paradigm for understanding how coats couple carrier formation to downstream docking and fusion events. In crude extract systems the complete COPII complex prevents fusion (Barlowe et al., 1994; Oka and Nakano, 1994). Our experiments identify Sar1 and Sec23/24 as both necessary and sufficient for this inhibition (Fig. 1.5C). Sec23/24 partially inhibited fusion without Sar1. Binding between the SNAREs and Sec24 is critical for SNARE packaging into vesicles with disruption of the SNARE-COPII interaction leading to absence of SNAREs in COPII vesicles. Distinct sites bind different SNAREs (Miller et al., 2003, Miller et al. 2005, Mossessova et al., 2003). The Sec24 B mutant employed here, L616W, most strongly impairs packaging of Sec22 (R) and Bet1 (Qc). We found that L616W allows fusion to occur in the presence of GPPNP-locked Sar1. Thus, the Sec24 B site, in addition to cargo packaging, specifically prevents fusion by sequestering SNAREs. Consistent with this model, in cell-free extracts recombinant Sec23 inhibited tethering and subsequent fusion, while excess recombinant Sec24 inhibited fusion without disrupting tethering (Barlowe, 1997; Cai et al., 2007b). The assembled COPII mesh may also prevent fusion by preventing membrane approach, but it is not

entirely clear how complete, patchy, or dynamic the COPII coat is *in vivo*, particularly in the presence of GTP rather than its non-hydrolyzable analogs.

Unexpectedly, Sar1 partially inhibited fusion in the absence of other COPII subunits. Penetration of the membrane cytoplasmic leaflet by Sar1's amphipathic N-terminal helix generates positive membrane curvature. This curvature is hypothesized to promote COPII carrier formation and carrier scission from the ER exit site (Kozlov and Taraska, 2023; Lee et al., 2005; Paul et al., 2023; Van der Verren and Zanetti, 2023). In contrast, lysolipids added to the cytoplasmic leaflet promote positive curvature and fission, and potentially inhibit fusion (Chernomordik et al., 1993; Günther-Ausborn et al., 1995; Melia et al., 2006; Reese and Mayer, 2005; Schwartz and Merz, 2009; Vogel et al., 1993). COPII carriers budded from native ER membranes are enriched in lysolipids (Melero et al., 2018). As COPII carriers form in the presence of Sar1, GTP hydrolysis and Sar1 dissociation may relieve compression within the outer leaflet, thereby relaxing positive membrane curvature and allowing fusogenic membrane structures to form. In an alternative model, Sar1:GTP directly engages SNARE proteins and places them in an inactive state. The interplay between Sar1 and SNARE-mediated fusion will require additional scrutiny.

The directionality of vesicle transport is regulated by sequential interactions of SNAREs with coat proteins, other SNAREs, and chaperones. We have shown that a specific interaction between Sec24 and SNAREs prevents fusion, ensuring that the vesicle cannot back fuse with the ER, and maintaining functional separation from other SNARE-containing membranes. Topological restrictions further confer fusion specificity to membranes based on the presence or absence of specific SNAREs and SM proteins. The requirements for fusion we have identified support a model of strict sequential and topological regulation throughout the transport process, starting with SNARE packaging and ending with the fusion event itself.

Materials and Methods

Protein purification

Full-length SNAREs and Sly1 were purified as described (Duan et al., 2024b). Soluble SNAREs were purified in the same manner as Sly1. Sar1 was purified as described (Shimoni and Schekman, 2002). Sec23/24 complexes and Sec13/31 complexes were purified as described (Stancheva et al., 2020). Plasmids used for protein expression and purification are listed in Table S2.

Preparation of RPLs

Reconstituted proteoliposomes were made as previously described (Duan et al., 2024b) with minor modifications. Lipid compositions were adjusted such that each population was identical, with the exception of the fluorescent reporter lipid. Table S3 describes the liposome compositions and vendor sources.

Fusion assays

Fusion was assayed and calibrated as described (Duan et al., 2024b). For reactions including RPLs, unlabeled streptavidin and soluble SNAREs were pre-mixed before the addition to a final concentration of 3% PEG, Sec17/18 (100 nM, 50 nM, or omitted), ATP regenerating mixture to 1 mM, and additional buffer. For reactions containing COPII proteins, Sar1 was added at 2611 nM, Sec23/24 and mutant variants were added at 314 nM, Sec13/31 was added at 407 nM. These reactions were immediately transferred to the plate reader and baseline fluorescence readings were acquired. Fusion was initiated by adding Sly1 (100 nM). Content mixing calibration was carried out for each RPL pair tested. 100% was determined by the total possible content mixing signal when both liposome populations were lysed with detergent in the absence of unlabeled competing streptavidin. Zero percent fusion was calibrated by taking baseline fluorescence measurements of reactions containing RPLs but not soluble cofactors.

AlphaFold3 modeling

Models of SNARE and SM interactions were modeled using the Google AlphaFold3 server. SNAREs were truncated from the N-terminus to their SNARE domain. R and Qa SNAREs

maintained the remainder of the protein which Qb and Qc SNAREs were truncated at their –3 layer to show a partially fused complex. Modeling parameters were set to defaults.

Estimation of *in vivo* protein concentrations

In vivo protein copy numbers and concentrations (Supplementary Table S1) were estimated as before (Duan et al., 2024b). From <https://www.yeastgenome.org>, curated Protein Abundance data for each protein were filtered to include only estimates for cells grown in YEPD media, and with no other specified treatments (drugs, *etc.*). Geometric means of the filtered copy number data are reported, to decrease sensitivity to high-count outlier measurements. Our estimates are in good agreement with comparable estimates by others (Ho et al., 2018). The volume of a haploid yeast cell is assumed to be ~67 fL, based on light microscopy and X-ray tomography measurements (Chan et al., 2016; Chan and Marshall, 2014; Uchida et al., 2011). The volume of accessible cytosol (cytoplasm minus major organelles including nucleus, ER, vacuole, mitochondria etc.) is taken as 75% of this volume, or 50 fL, similar to the fraction estimated by Uchida et al.

Figures

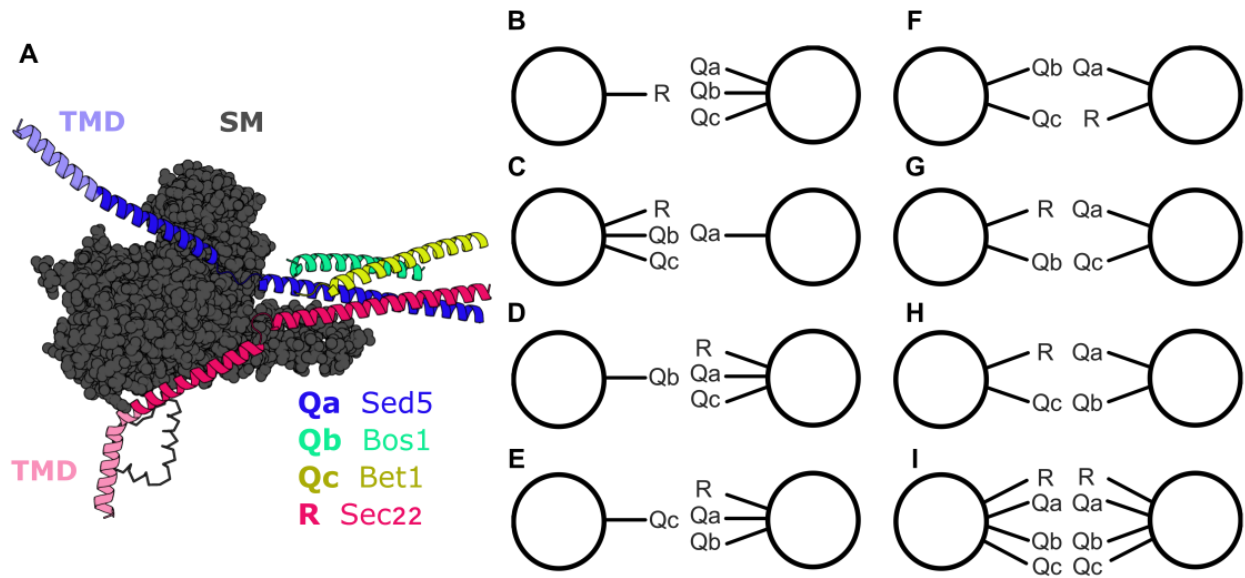


Figure 1.1. Possible SNARE topologies

(A) AlphaFold 3 model of the ER-Golgi trans-SNARE bundle in a template complex with Sly1. Qa and R SNAREs are modeled including only their SNARE domains through their transmembrane helices (Qa: 235-340, R: 111-214). The transmembrane domains are depicted in a lighter shade, as noted. Qb (residues 149-182) and Qc (residues 36-78) SNARE domains are modeled from α -helical packing layers -8 to -3 within the SNARE bundle (Fasshauer et al., 1998; Sutton et al., 1998). (B-I) Schematics represent the RPL pairs prepared and tested. (B-H) The seven minimal, unique SNARE topology pairs are shown as well as, (I) the eighth topology tested, which mimics homotypic fusion.

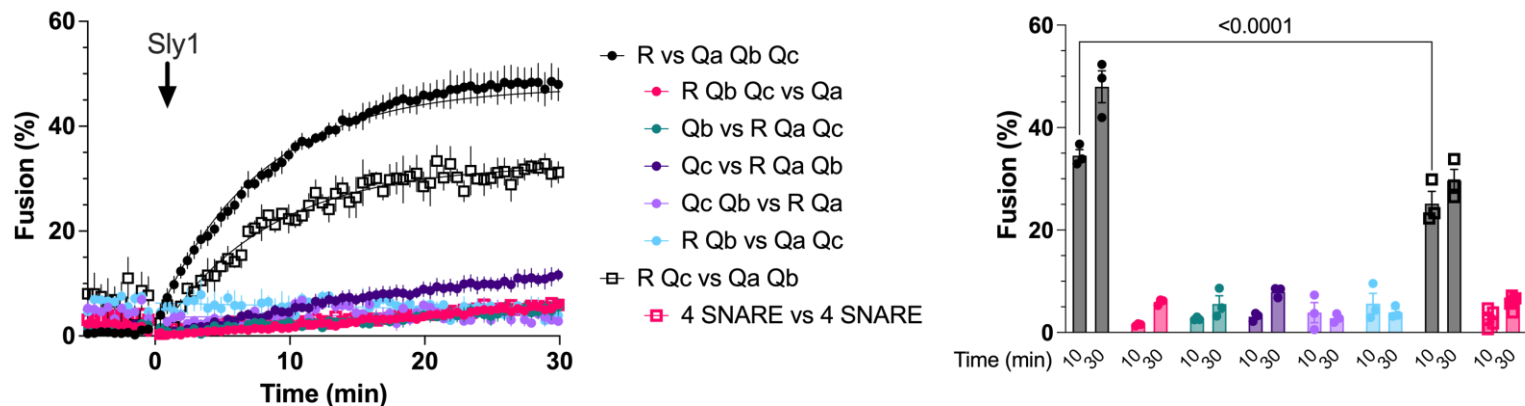


Figure 1.2. Rapid, efficient fusion in the presence of Sly1 occurs with select topologies(A) Fusion assays were carried out for each pair of RPLs. Fusion reactions ran for thirty minutes after initiation. RPL populations were mixed together for a baseline reading. Reactions were initiated by the addition of 100 nM Sly1. Lines show fits of a 2-parameter kinetic model. 100% fusion was determined by determining the total content mixing of two RPL populations by bursting with detergent. **(B)** Fusion for each configuration at 10 and 30 min. is shown, from the datasets plotted in (A). Both plots show means from 3 separate experiments, with error bars spanning $\pm$ S.E.M.). The P value indicated by the horizontal bar in (B) is from 2-way ANOVA with correction for multiple comparisons.

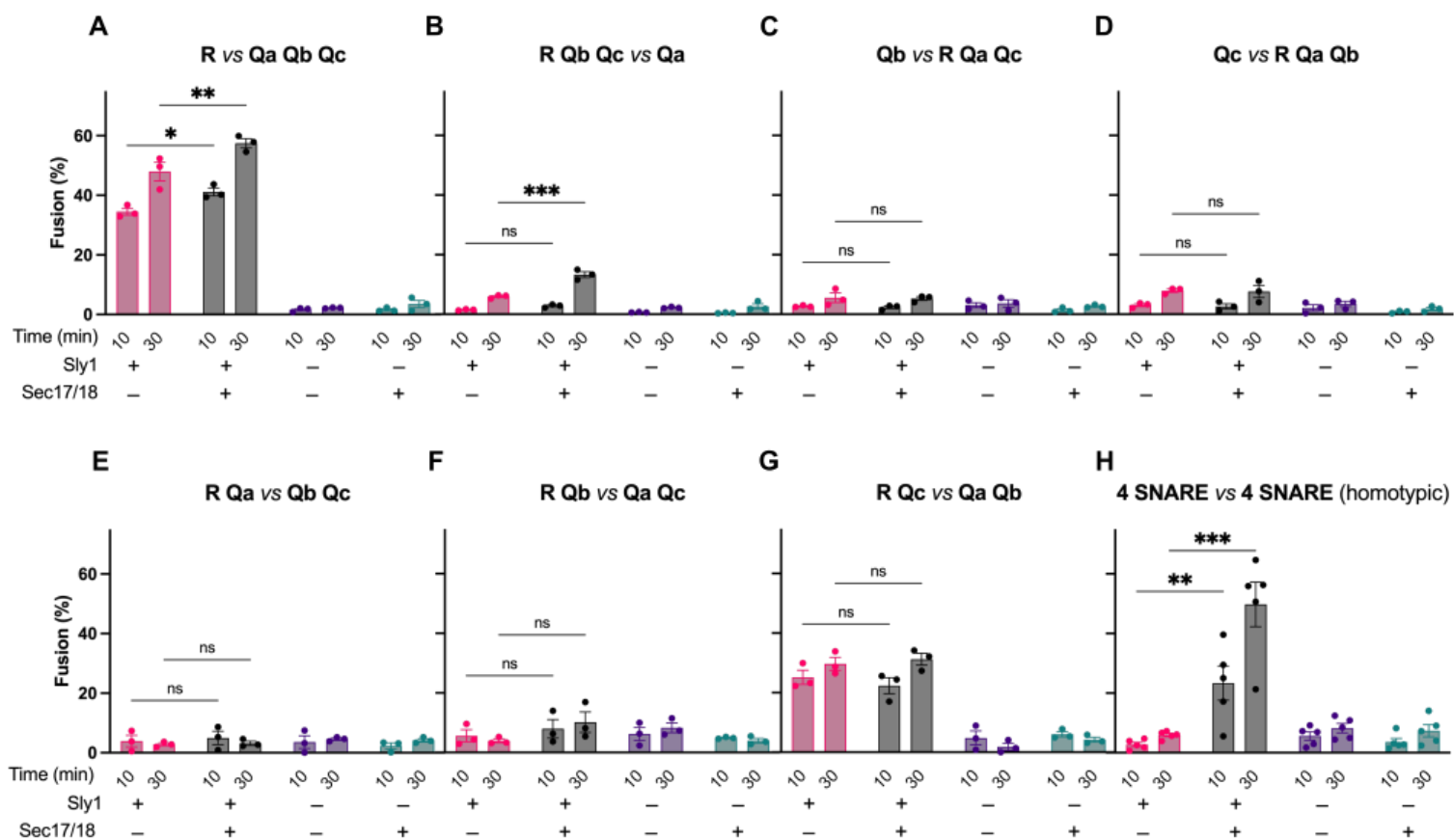


Figure 1.3. Sec17/18 and topological restriction of fusion

Fusion reactions were assembled as in Fig. 2. Where indicated, Sec17/18 (100 nM and 50 nM respectively) and ATP regenerating system (1 mM), were added to the RPLs 5 min. before the 5 min. baseline reading. Fusion was then initiated by adding 100 nM Sly1 at time= 0, then monitored for 30 min. Individual time points at 10 and 30 min. were taken for each reaction. Vertical bars show means $\pm$ S.E.M. for 3 independent experiments. P-values for selected 2-way ANOVA comparisons are indicated by horizontal bars (ns= $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P < 0.001$).

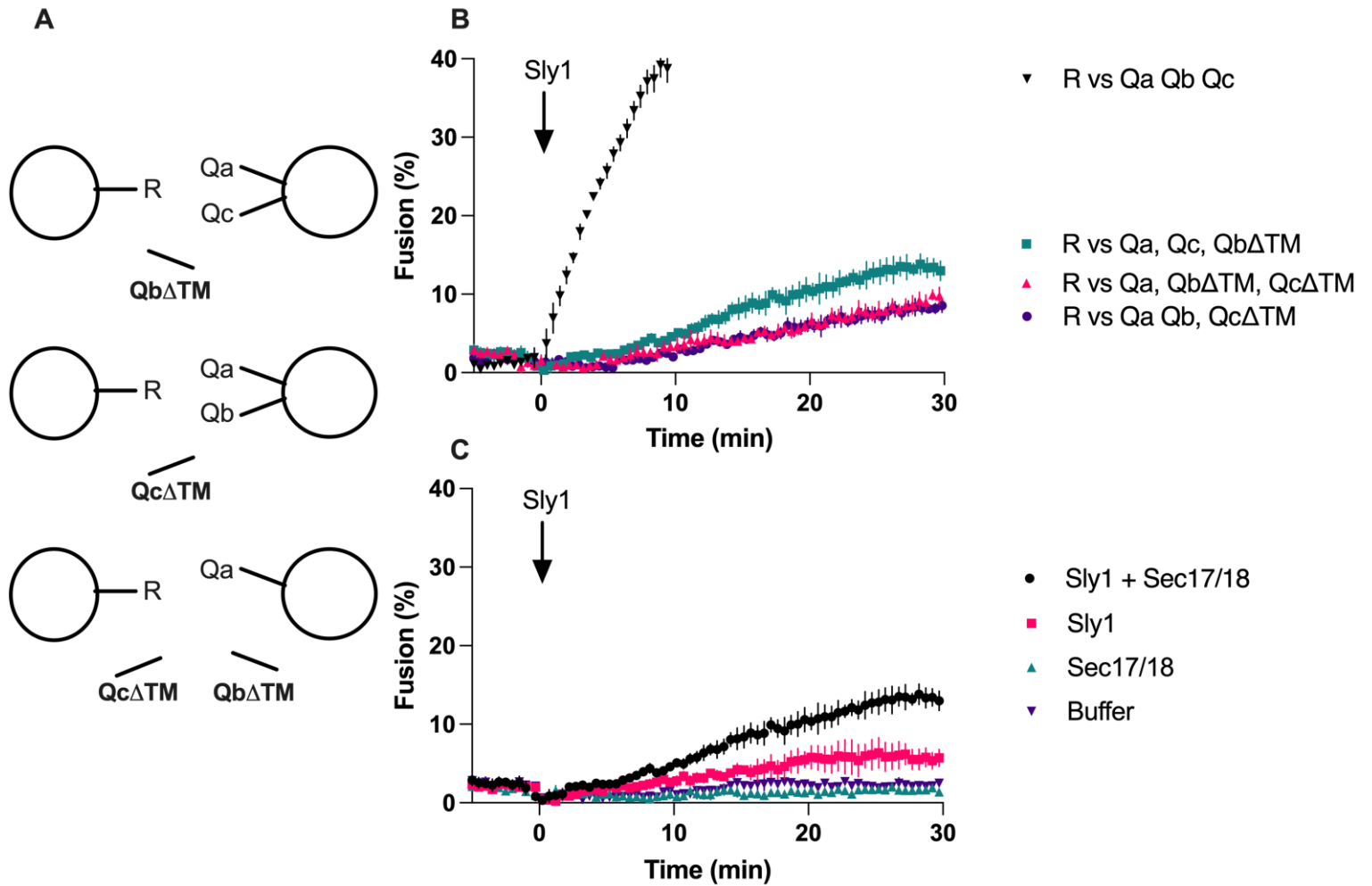


Figure 1.4. Qb and Qc SNAREs lacking membrane anchors do not drive efficient fusion

(A) Schematics show topologies used in soluble SNARE configurations. (B, C) Fusion reactions were set up and calibrated as described above with Sec17/18 (100 nM and 50 nM respectively) and ATP regenerating system (1 mM), and soluble SNAREs (4 μ M each) were added prior to the 5 min baseline reading. Fusion was initiated by adding 100 nM Sly1 at time =0. (C) Fusion time course showing fusion conditions tested for R vs Qa in the presence of QbΔTM and QcΔTM (4 μ M each). Points show means $\pm$ S.E.M. from 3 independent experiments.

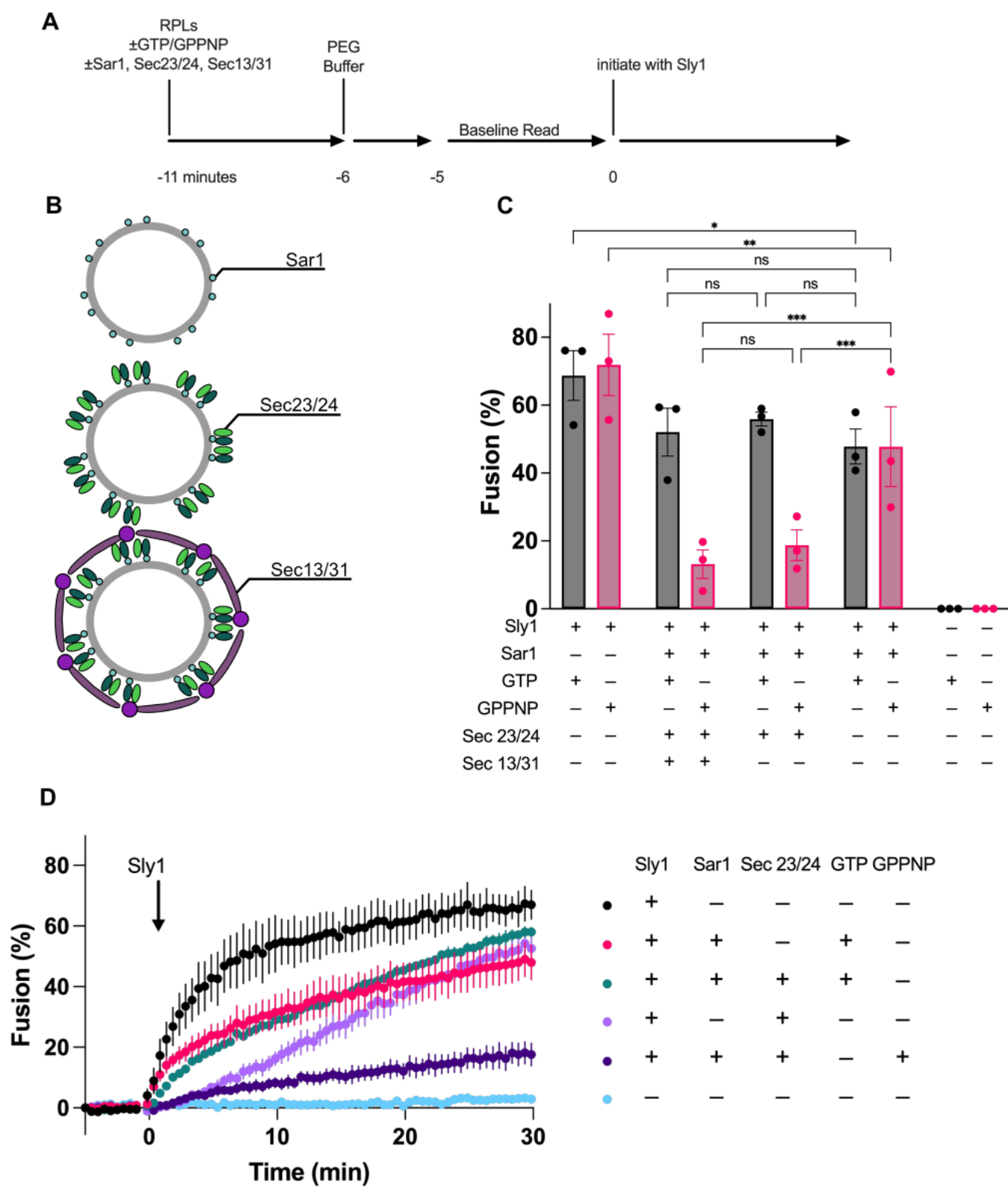


Figure 1.5. Sar1 and Sec23/24 are sufficient to inhibit fusion

Fusion assays were performed and calibrated as described above. Different combinations of COPII subunits were added to determine the minimal requirements for fusion. Sar1 was added to 2611 nM, Sec23/24 and mutant variants were added to 314 nM, and Sec13/31 was added to 407 nM. **(A)** Timeline showing the addition of each component to COPII fusion assays. **(B)** Schematic representation of the COPII layers and the tested conditions. **(C)** Fusion was measured after the addition of Sly1 at time=0 for 30 min. The last five minutes ($t = 25$ to 30 min) were averaged for each assay. Bars show means $\pm$ S.E.M. for 3 separate experiments. Grey bars show GTP conditions for each noted COPII protein combination; magenta bars show GPPNP conditions. P-values for selected 2-way ANOVA comparisons are indicated by horizontal bars (ns= $P > .05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P < 0.001$). **(D)** Fusion reactions with Sec23/24 and different nucleotide and GTPase conditions. The plot shows means $\pm$ S.E.M from 3 independent experiments.

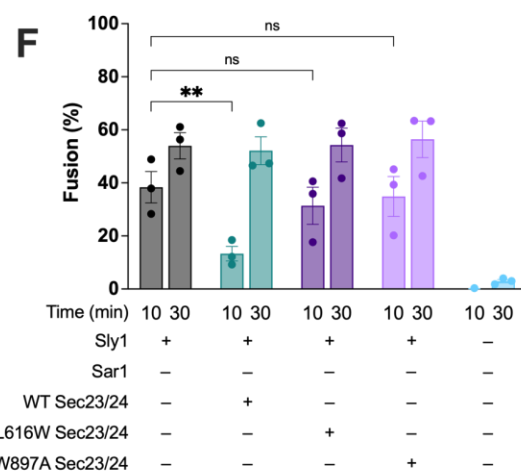
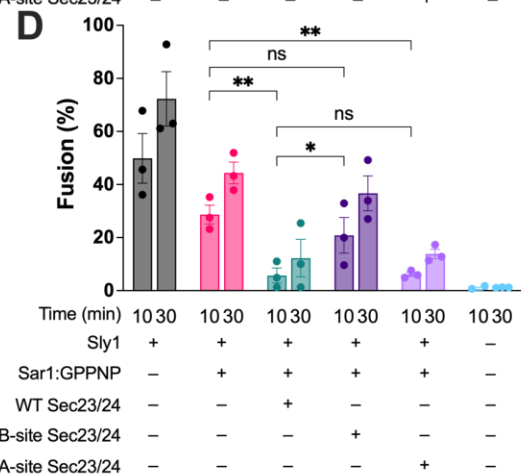
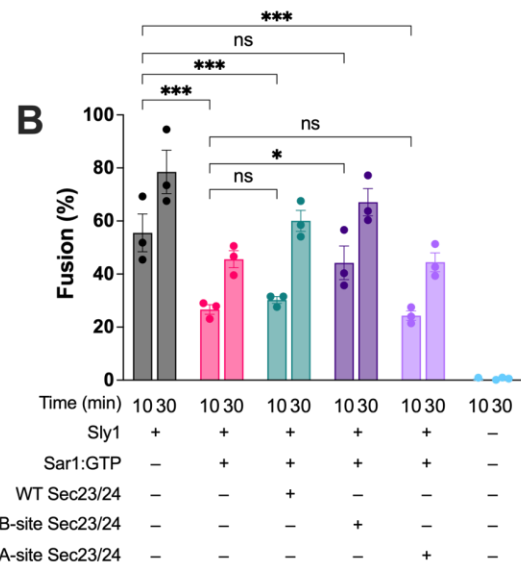
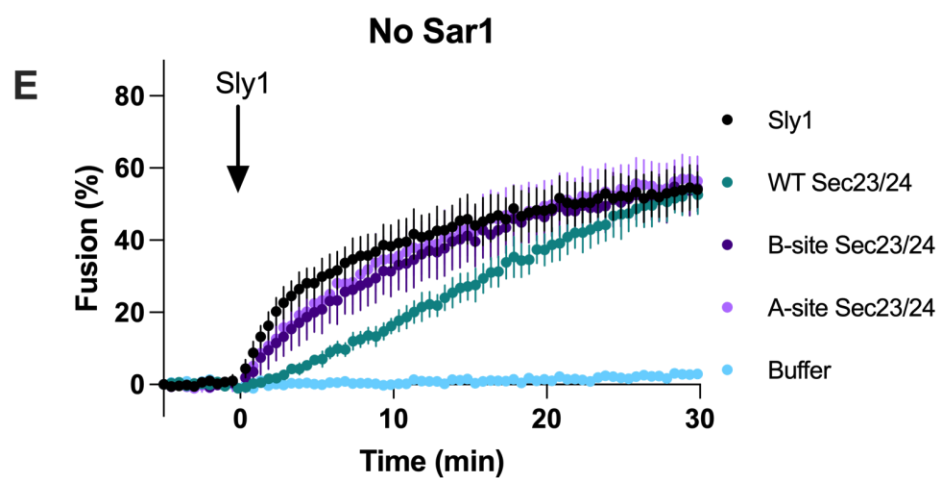
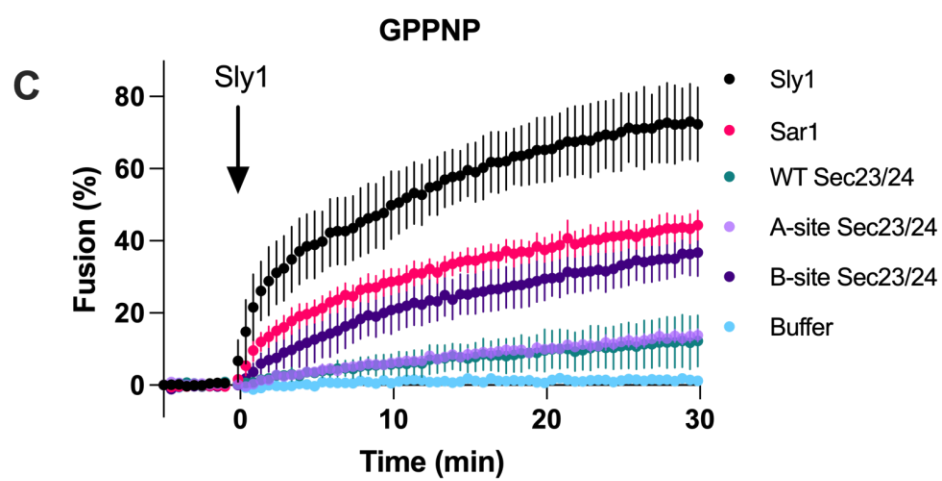
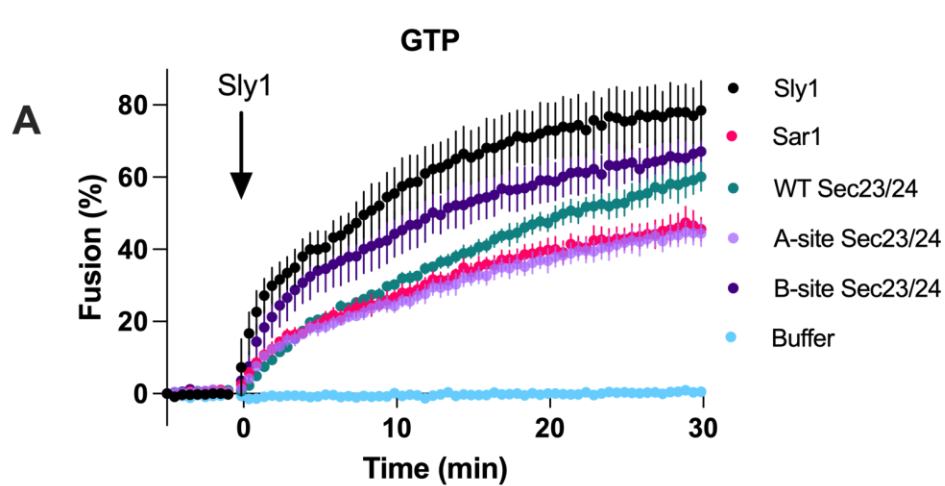
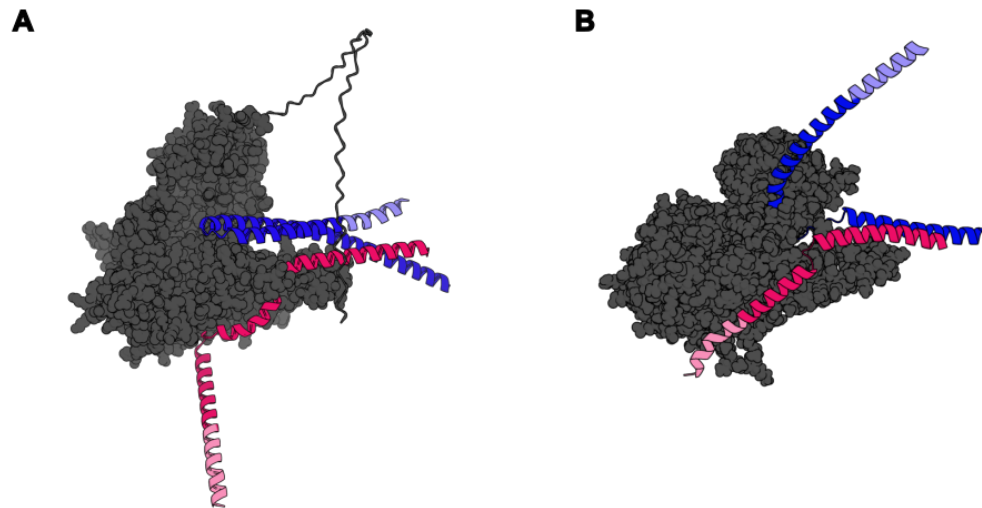


Figure 1.6. Sec24-SNARE interactions regulate fusion

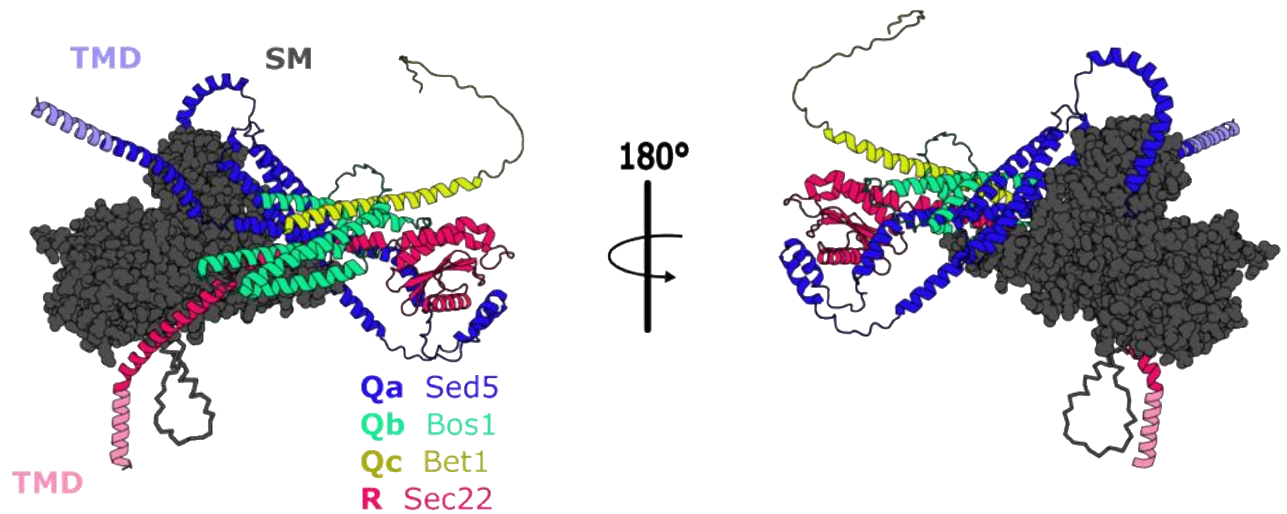
Fusion assays were performed as previously described. Reactions included permutations of nucleotides, Sar1, and Sec23/24 wild-type, or Sec23 A-site (W897A) or B-site (L616W) mutant protein, added to the same concentrations as in Fig. 5. Fusion was measured after addition of Sly1 at time = 0. Sar1 was added to 2611 nM, Sec23/24 and mutant variants were added to 314 nM, and Sec13/31 was added to 407 nM. **(A & B)** Reactions contained GTP. **(C & D)** Reactions contained GPPNP. **(E & F)** Reactions contained no nucleotide and no Sar1. **(A, C, & E)** Fusion assays initiated with 100 nM Sly1 at time=0. Each point represents the means of 3 independent reactions $\pm$ S.E.M. **(B, D, & F)** Measurements at 10 and 30 min (± 0.5 min) were taken from the time courses shown in panels A, B, or E respectively. Bars represent means with error bars representing $\pm$ S.E.M. P-values for selected 2way ANOVA comparisons are indicated by horizontal bars (ns= $P > .05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P < 0.001$).

Supplementary Figures



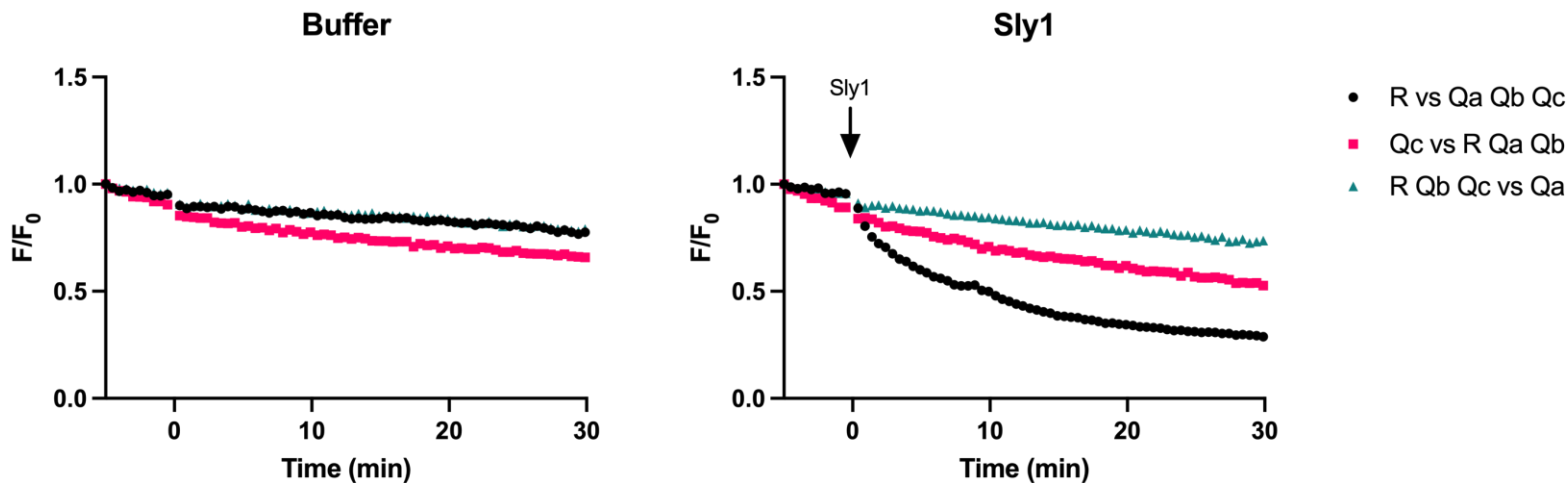
Supplementary Figure 1.

AlphaFold3 modeling of SMs and cognate Qa and R SNAREs. **(A)** Sec1 (SM) with Sso1 (Qa) and Snc2 (R). Sec1 is shown in grey with residues 670-724 shown as ribbon for clarity. Sso1 (residues 183-289), is colored blue with the TMD in light blue (274-289). Snc2 (residues 20-115), is colored red with the TMD in pink (98-115). **(B)** Vps45 (SM) with Tlg2 (Qa) and Ykt6 (R) AlphaFold 2 model. Vps45 is shown in grey. Tlg2 (residues 243-336) is colored blue, with its TMD shown in light blue (318-336). Ykt6 (residues 138-199) is colored red with its membrane-proximal region (186-199) in pink. The C-terminal prenyl lipids that anchor Ykt6 to the membrane are not modeled.



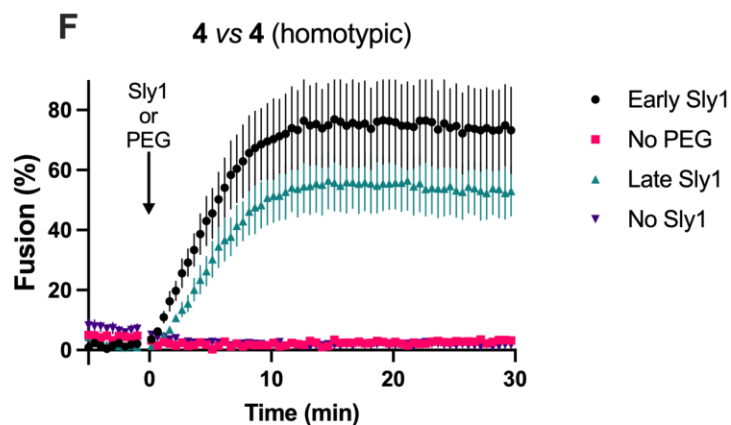
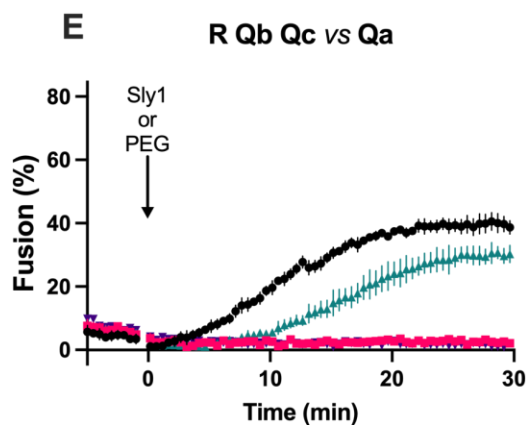
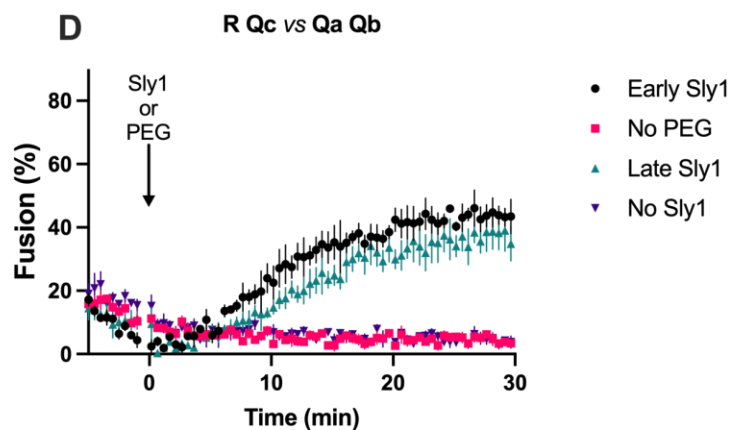
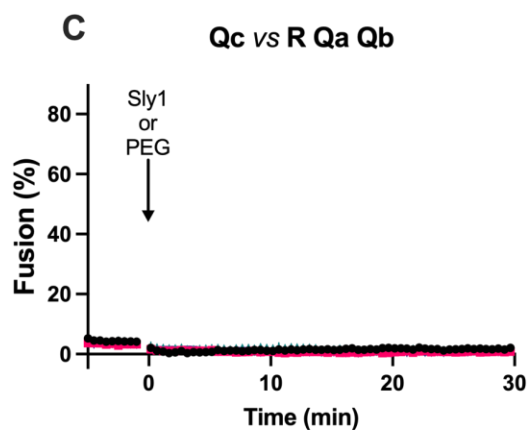
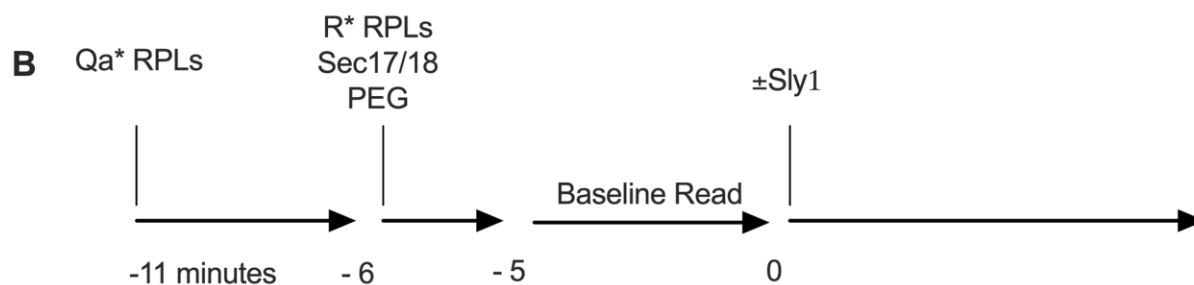
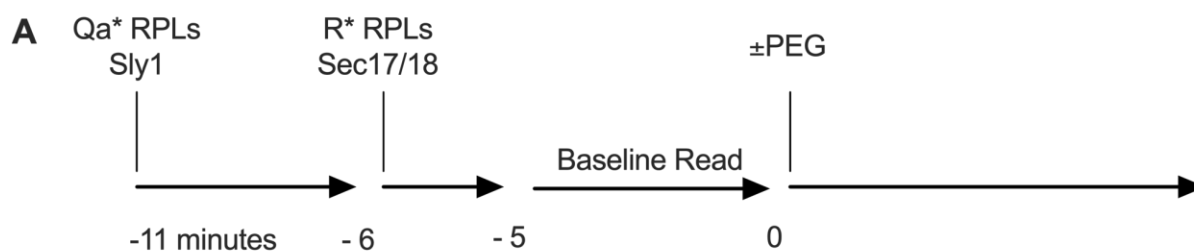
Supplementary Figure 2. The N-terminal regulatory domains of SNAREs are not predicted to modify their SNARE templating interactions with Sly1

AlphaFold3 models of Sly1 with full length R (Sec22) and Qa (Sed5) as well as the Qb (Bos1) (1-182), and Qc (Bet1) (1-78) to their -3 layer. Modeling includes the N-terminal domains depicting additional interactions between the SNAREs. The absence or presence of the Sec22 N-terminal longin domain does not dramatically alter the positions of the Qa or R SNARE domains on Sly1 domain 3a, or the binding of Sec22 Phe186 in conserved pocket on Sly1 (also seen in published and deposited but unpublished Vps33-Nyv1 crystal structures: PDB 5BV0, 9ZCS, 9BZE, 9ZCT).



Supplementary Figure 3. Lipid mixing with representative topologies

Representative lipid mixing signals in the absence (A) and presence (B) of Sly1. Raw fluorescence was normalized to the starting signal for each population. Note that in panel A, the traces for R vs. Qa Qb Qc, and R Qb Qc vs. Qa overlap so closely that they are not distinguishable.



Supplementary Figure 4. Effects of Sly1 order-of-addition

Order of addition experiments to determine if Sly1 and Qa interactions can additionally stimulate fusion before the addition of the other RPL population. (**A** and **B**) Schematic representations of the order of addition of each component to the fusion assays in this figure. The top panel (**A**) shows early Sly1 addition, while the bottom panel shows late sly1 addition. Qa* represents the RPL population bearing the Qa SNARE (with or without other SNAREs, depending on the topology tested). For the homotypic RPL set only one population was added at this time point. Sec17/18 and ATP were added in all experiments. Experiments with Sly1 added early to the Qa* population (**A**) were initiated with PEG at time=0. This order of addition was used for the black (circle) and pink (square) traces in the below graphs. Experiments initiated with Sly1 added late (**B**) are similar to the order-of-addition used all other topology experiments in this study. This order of addition was used for the teal (triangle) and purple (inverted triangle) traces. (**C-F**) Time course traces, each for a different SNARE topology, showing the kinetic effects of adding Sly1 to the Qa* population.

Supplementary Tables

Supplementary Table 1. Comparison of estimated <i>in vivo</i> protein concentrations versus concentrations used in this study's fusion reconstitution experiments					
	Cytosol volume, μm^3 (fL)	Copy number, molecules/cell	Concentration in or exposed to cytosol, nM	Concentration in RPL fusion assays in this study, nM	Notes
Sec17	50	11,364	378	100	SNARE adapter for Sec18
Sec18	50	8,115	270	50	SNARE disassembly ATPase
Sly1	50	6,380	212	100	SM family SNARE chaperone
Sed5	50	2,728	91	≤ 200	Golgi Qa-SNARE; Sly1 client
Bos1	50	2,870	95	≤ 200	Golgi Qb-SNARE
Bet1	50	3,772	125	≤ 200	Golgi Qc-SNARE
Sec22	50	3,288	109	≤ 200	Golgi/ER R-SNARE
Sar1	50	22,568	750	1600	COPII small GTPase
Sec23	50	14,568	484	300	COPII inner shell
Sec24	50	8,873	295	300	COPII inner shell
Sfb2	50	3,615	120	-	COPII inner shell (Sec24 paralog)
Sfb3	50	7,835	260	-	COPII inner shell (Sec24 paralog)
Sec13	50	19,617	652	400	COPII outer shell & nuclear pore
Sec31	50	14,493	481	400	COPII outer shell

Supplementary Table 2. Plasmids used in this study

plasmid ID	Description	Source/reference
<i>E. coli</i> SNARE expression plasmids		
AMP1792	pET-30::His ₆ -(3C)-Sed5	(Furukawa and Mima, 2014)
AMP2020	pET-30:: His ₆ -(3C)-Bos1(Q153D)	(Furukawa and Mima, 2014), but modified per (Duan et al., 2024)
AMP1794	pET-30:: His ₆ -(3C)-Bet1 (with corrected missense mutation)	(Furukawa and Mima, 2014) but corrected as (Duan et al., 2024)
AMP1795	pET-41::GST- His ₆ -(3C)-Sec22	(Furukawa and Mima, 2014)
AMP1977	pET-30:: His ₆ -(3C)-Bos1(Q153D)(1-222)	This Study
AMP1976	pET-30:: His ₆ -(3C)-Bet1(1-123)	This Study
<i>E. coli</i> SNARE chaperone expression plasmids		
AMP1547	pTYB12::intein-CBD-Sec17	(Schwartz and Merz, 2009)
AMP77	pQE9:: His ₆ -SEC18	(Haas and Wickner 1996)
<i>E. coli</i> Sly1 expression plasmids		
AMP1649 (pBL51)	pHIS-Parallel1:: His ₆ -(TEV)-Sly1	(Lobingier and Merz, 2014)
AMP1651	pHIS-Parallel1:: His ₆ -(TEV)-Sly1-20	(Duan et al., 2024)
<i>E. coli</i> miscellaneous expression plasmids		
AMP1881	pET-49::GST- His ₆ -(3C)	(Duan et al., 2024)
AMP2019	pET-30:: His ₈ -HRV3C(Protease)	(Duan et al., 2024)
AMP2016	pET-49::GST- His ₆ -(Thrombin)-HRV3C(Protease)	(Duan et al., 2024)

Supplementary Table 3. SNARE RPL lipid compositions used in this study

ER mimetic RPLs				Golgi mimetic RPLs		
Lipid	Vendor	mol%		Lipid	Vendor	mol%
18:1 (Δ 9-Cis) PC	Avanti 850375	35.0%		18:1 (Δ 9-Cis) PC	Avanti 850375	35.0%
16:0-18:1 PE	Avanti 850757	16.0%		16:0-18:1 PE	Avanti 850757	14.0%
Soy PI	Avanti 840044	6.3%		Soy PI	Avanti 840044	6.3%
16:0-18:1 PS	Avanti 840034	5.6%		16:0-18:1 PS	Avanti 840034	5.6%
16:0-18:1 PA	Avanti 840857	3.5%		16:0-18:1 PA	Avanti 840857	3.5%
18:1 DG	Avanti 800811	1.4%		18:1 DG	Avanti 800811	1.4%
Brain PI(4)P	Avanti 840045	2.0%		Brain PI(4)P	Avanti 840045	2.0%
Ergosterol	Sigma 45480	30.0%		Ergosterol	Sigma 45480	30.0%
16:0 Marina Blue™ DHPE	Invitrogen M12652	0.2%		16:0 NBD-PE	Invitrogen N360	2.2%

Chapter three: Efforts towards dissecting tethering at the cis-Golgi

Introduction

First contact between a vesicle and the target membrane is through long-range tethers. There are two families of vesicle tethers: the CATCHR family protein complexes and Golgins, coiled-coil proteins (Chau, et al., 2016; Witkos and Lowe, 2016). The CATCHR family are multi-subunit-complexes which interact with membranes, Rabs, SNAREs, and often contain the SM protein. For Golgi to ER retrograde transport, this is conducted by the Dsl1 complex (Meiringer, et al., 2011; DAmino, et al., 2024). Dsl1, is the most evolutionarily divergent and does not interact with a Rab (Santana-Molina, et al., 2021). For the vacuole system, HOPS carries out this process. For ER-Golgi anterograde transport, the COG complex and Transport particle protein (TRAPP(I, II and III)) have been suggested to be the canonical tether (Aragon-Ramirez, et al., 2026; Cai, et al., 2011; Muschalik and Munro, 2018). Additional proposed tethers include coiled-coil proteins which are not as structurally rigid. Consisting of a homodimer coiled-coil region and sometimes globular head domains, these proteins interact with the vesicle on one end and the target membrane, or additional linking proteins on the other (Muschalik and Munro, 2018). In mammals many of these proteins have been implicated in ER-Golgi anterograde transport, such as Giantin, GM130, and p115. In yeast the p115 homolog, Uso1, is highly associated with ER-Golgi transport and has been suggested to be the essential tether (Cao and Barlowe, 1998). While there is disagreement in the field over how vesicles reach the target membrane there are likely several possible tethers. One commonality between almost all tethers is the reliance on Rab proteins.

Rab proteins are often referred to as the “zip codes” of vesicular transport (Koike & Jahn, 2022). Rabs are small GTPase proteins that associate with membranes when they are in their GTP-bound active state. Rabs are lipid-modified (*bis*-geranylgeranylated) at their C-terminus, to be able to insert into a membrane (Pylypenko, et al., 2006). This marks the membrane for proper transport. Rabs undergo cycles of activation and inactivation. In their GDP-bound, inactive state they interact with GDI (GDP dissociation inhibitor). When bound to GDI the lipid anchor is tucked into a hydrophobic pocket of GDI. This allows the inactive Rab:GDI complex to be freely soluble in the cytoplasm. GDI interacts with all Rabs. The Rab then will become GTP activated through interactions with the GEF. Rabs will insert into the membrane with GDI and will either

undergo GTP-exchange or be re-extracted. The spontaneous hydrolysis rate of GTP by a Rab varies between proteins but is very low across the family. In the membrane-anchored, GTP activated state, the Rab can now interact with its effectors. Effectors include tethers, SMs, SNAREs and many other fusion participants (Grosshans, et al., 2006). Rab effectors also include a variety of other proteins including lipid modifying enzymes and cytoskeletal motors. The Rab becomes inactive when GTP is hydrolyzed to GDP, either spontaneously or with the help of a GAP. The sequential interaction with the GAP and then GDI extracts the Rab from the membrane to start the cycle over again. The Rab of interest in the ER-Golgi pathway is Ypt1, or Rab1 in mammals. This creates a large technical block for reconstitution into our system. Only through natively purifying from *S. cerevisiae* or through geranylgeranyl modification *in vitro* can the correctly modified protein be procured, or by reconstitution of the lipidation process. Additionally, Ypt1 is difficult to fluorescently label for live cell imaging, with any size linker causing mis-localization and/or activity issues in a similar Rab, Rab10 (Hata, et al., 2025). The difficulties of studying Ypt1 have caused a large gap of knowledge surrounding how this Rab interacts with tethers and where it is crucially located for membrane transport.

The Ypt1 effector Uso1 exists as a dimer, the extensive ~1000 residues making up the coiled-coil region being responsible for this oligomerization. The mammalian Uso1, p115, has been shown to not only dimerize in the coiled-coil region but also in the N-terminal globular head domain (GHD) (Sapperstein, et al., 1995). It appears that this dimerization is transient, though there is a consistent fraction of the population that exists in this state *in vitro* (An et al., 2009). Such dimerization without the coiled coil region of Uso1 has not been found, the head domain appears to only exist as a monomer. Structural studies of p115 suggest that there is a conformational change of the GHD that allows for this state switching (Heo, et al., 2020). Comparisons of the “open” and “closed” conformations of bovine p115 find one region at the 200th residue and one region at the 600th residue that clash in the closed form. These structures inform us that the dimerizing interface has distinct structural states as a monomer or as a dimer. The functional relevance of dimerization of the GHD has not been extensively studied, especially as it did not seem to be conserved across species.

The Uso1 GHD interacts with SNARE proteins (Bravo-plaza, et al., 2023). Computational and biochemical results suggest at least three of the ER- Golgi SNAREs interact with the GHD. These interactions span the outer face of Uso1 that would be exposed if the Uso1

GHD did interact like p115. Our models suggest Uso1 could dimerize and interact with SNAREs simultaneously. Interestingly, the GHD interacts with SNAREs that exist on both the vesicle and the Golgi membrane. As the GHD is large and structured it makes it an attractive target for testing interactions with effectors of the transport pathway.

Despite the interest in Uso1 as a lead candidate for controlling tethering there are several other candidates of interest. The conserved oligomeric Golgi complex (COG), is a highly conserved octameric complex made up of two lobes, A and B, each consisting of 4 subunits. COG exists in cells as an octameric complex as well as both of its tetrameric subcomplexes (Willett, et al., 2016). Loss of COG function leads to defects in glycosylation, trafficking defects, and organelle morphology alterations. COG interacts with GTP-bound Ypt1. Cells are completely viable with the loss of any or all lobe B subunits, but any deficiency in lobe A leads to severe growth defects or lethality (Lees, et al., 2010; Ram, et al., 2002). At least two COG subunits, Cog2 and Cog3, are implicated in ER-Golgi transport (Willett, et al., 2016).

The aim of dissecting the tethering pathways is to understand the multiple complex ways in which a vesicle can be recognized by the Golgi. What determines if a vesicle is targeted for the Golgi? The Rab family is highly implicated in marking vesicles for their target compartment. With the multiple effectors of Ypt1 it would stand to reason that there are multiple modes of recognition. How this redundancy is utilized in the cell remains unknown. Here we expand on the interactions between Uso1 and Ypt1 through genetic modification of predicted binding sites, and initial efforts to reconstitute tethering in our fusion assay system.

Results

Modeling of Ypt1-Uso1 binding sites

Mammalian Rab1 and yeast Ypt1 are known to interact with p115 and Uso1, respectively. However, the site of Ypt1/Rab1 interaction on Uso1, as well as the specific activity conferred, is less clear. I used AlphaFold3 to predict the binding site interactions between two Uso1 head domains and two Ypt1 proteins (Fig. 2.1). This predicted binding site disagrees with several published predictions of Ypt1-Uso1 binding sites, placing Ypt1 at the extreme N terminus, however, in recent studies the AF3 model has been published, but only one of each protein was modeled (Lorente-Rodriguez & Barlowe, 2011; Bravo-Plaza, et al., 2023). Interestingly in our prediction, Ypt1 sits at the homodimer interface between Uso1 head domains. This suggests that in *S. cerevisiae*, dimerization may occur — and be regulated by Ypt1 binding. Importantly, the binding interface appears to be at the Ypt1 switch I domain, a region known to undergo conformational changes dependent on the nucleotide state. The predicted binding site on the Uso1 side is at a location that is predicted to undergo conformational change with Uso1 dimerization (Heo, et al., 2020). This further supports that Ypt1 modification of this region could be regulatory.

In contrast to earlier modeling by other groups, which had placed the Rab very close to the GHD N-terminus, my models suggest that Ypt1 interacts with Uso1 at different sites, across the homodimer interface between the two GHDs (Lorente-Rodriguez & Barlowe, 2011). A dimer of the purified Uso1 GHDs has not been found despite extensive biochemical experiments (Bravo-Plaza, et al., 2023). Our predicted structure, along with published data on oligomeric states of Uso1 and p115, suggests the hypothesis that the GHDs dimerize conditionally, in the presence of Ypt1:GTP (Fig. 2.1). Most of the predicted interactions between Ypt1 and the Uso1 homodimer are near the 200th residue of the GHD. However we see some predicted interactions across the Uso1 dimer pair, near the 650th residue. Additionally, I compared the predicted structure for Ypt1:GTP:Mg²⁺ with that of Ypt1 lacking the nucleotide and metal ion. Ypt1 switch I domain (residues 37-45) undergoes a conformational change when GTP is bound (Fig. 2.1A). This region has been highlighted as interacting with effectors only with the activation of Ypt1 (Sclafani, et al., 2010). Comparing this region between AF3 predictions, we see that there is a change in the unstructured loop between the two predicted states (Fig. 2.1B). The movement of

this loop specifically increases the interactions predicted between Ypt1 and the second Uso1 head. This model strongly suggests a specific binding site for Ypt1-activated Uso1 dimerization, potentially a critical step for vesicle tethering.

Based on our AlphaFold3 predictions, I designed amino acid substitutions to the Uso1 GHD to test the impact of the binding site on cell viability. I designed mutations on four helices of the putative Uso1 binding sites. Each mutant consists of alanine substitutions at amino acids predicted to interact or are within 4 Å of the modeled Ypt1. As Ypt1 is predicted to bind at the dimer interface, three of the helices are around the residues 150-200 and one lies on the other Uso1 interface around residue 650. I introduced alanine substitutions at four unique sites, three on one face of the Uso1 dimer interface, and one on the other (Fig. 2.2). Based on the predicted relative strengths, the first three sites were prioritized.

Cell viability of Uso1 mutants

The mutation sites were tested for their impact on tethering through growth assays. Site one S188A T189A, site two H218A K221A L222A F225A, site three L268A E271A, and site four E647A K651A T652A were inserted individually into a LEU marked Uso1 plasmid for yeast expression. The background strain had an *uso1Δ* deletion. As *USO1* and *YPT1* are essential genes, their deletion leads to a lethal phenotype. If my mutations lead to a functional disruption of either protein there will be a decrease in cell viability. To test the relative viability of these mutants I used a 5-FOA (5-Fluoroorotic acid) growth assay. This growth assay allows us to assess lethality of essential genes by initially having a wildtype copy of the essential gene that can be ejected from the cell by a counter-selectable marker.

Of the three sites tested, we found that one mutant had a consistent growth defect at 37°C when it was the only copy of Uso1 provided (Fig. 2.3 bottom right). This mutant (H218A K221A L222A F225A; from here on called mutant two) was the only mutant tested with a substantial growth defect. The growth defect was only observed at an elevated temperature, but was partially dominant, with slower growth at 37°C when a wildtype copy of *USO1* was present (Fig. 2.4 bottom left). This dominant phenotype agrees with the argument that the Uso1 head domain functions in the context of an Uso1 homodimer. Assuming an even mixture of wildtype and mutant proteins some mutants will dimerize with wildtype potentially inactivating the wildtype copy from functioning properly.

Rescue of Uso1 mutant with Sly1-20

The growth defect from the site two mutant suggests there is a critical impact at this predicted site between Uso1 and Ypt1, although it is not a completely lethal mutation. This highlights there may be additional impacts with the dimerizing binding site between Ypt1 and Uso1. As both *USO1* and *YPT1* deletions can be rescued by *SLY1-20*, a hyperactive suppressor mutant, we tested the addition of Sly1-20 to the growth assay. Sly1-20 mutant is in a constitutively active state, with an open amphipathic helix known to aid it close range tethering (Duan et al., 2024). We found that *SLY1-20* was able to fully rescue the growth defect caused by the site two mutant (Figure 2.4). This suggests that the mutation made to Uso1 is specifically interfering with this trafficking pathway.

Reconstitution of tethering

Our reconstituted fusion assays use a crowding agent, polyethylene glycol (PEG), or an engineered protein, GST-P4M-KA, to overcome the lack of tethering factors (Duan, et al., 2024). Several tethering factors have been implicated as critical for vesicle capture at the Golgi. It is likely that there is redundancy in the system *in vivo*, with redundant systems that tether vesicles for ER to Golgi transport. However, we have not yet discovered even a minimal set of factors that can complete this function. Of the possible tethers Uso1 and COG are our lead candidates given their necessity for viability. Ypt1 is also a critical factor. In order to test if these components could augment suboptimal amounts of PEG in our assays, I performed a PEG dilution series with the addition of all the tethering factors available.

The tethering factors added in this experiment were COG-A (Cog1,2,3,4), Uso1-Bonsai, and Sec17/18. COG-A was used because COG-B, the other half of the COG hetero-octamer, can be fully deleted without causing a defect in cell growth or viability (Lees, et al., 2010). Some studies have also suggested that COG-A and COG-B function separately (Willett, et al., 2013). Uso1-Bonsai is a variant of Uso1 that removes a long segment of the coiled-coil region present in the *S. cerevisiae* protein (amino acids 1124-1691) but absent from almost all other eukaryotes. Uso1-Bonsai is close to the length of the human ortholog, and fully complements an *uso1Δ* null mutation *in vivo*. COG-A and Uso1-Bonsai were chosen for this study because they can be expressed and purified from *E. coli*. Tethering *in vivo* and in cell extract assays depends on Ypt1

activity and its interactions with its effectors, and thus was added. Before the assay, GDP-bound Ypt1 was exchanged to GPPNP.

To our surprise, there was increased fusion with the addition of all the tethers without Ypt1 (Fig. 2.5A). Unexpectedly, the presence of Ypt1 inhibited fusion at all PEG concentrations (Fig. 2.5). As we decreased PEG concentration to zero we still found that the tethering factors stimulated fusion, and the addition of Ypt1 impaired fusion (Fig. 2.5D). The fusion signal was extremely rapid after the addition of Sly1, consistent with our expectations for tethering, which is thought to be a rate-limiting step. In these experiments, Ypt1 was added to 5 μ M making it around 10 times more concentrated than the expected cellular concentration ($\sim$ 500 nM, Duan, unpublished). It is possible that the delayed fusion signal with addition of Ypt1 in all PEG concentrations is due to Ypt1 membrane insertion altering the lipid packing in the membrane cytoplasmic leaflet, which may be unfavorable for formation of a widely hypothesized “hemifusion stalk” intermediate on the fusion pathway. Future experiments will need to determine the appropriate concentration of Ypt1 and how the addition of this protein can specifically stimulate fusion. To further dissect the tethering results we did find, we determined which of the added tethering factors impacted the fusion signal.

COG-A and Sec17/18 are sufficient to partially reconstitute Ypt1-independent tethering

Our first experiment added all of the tethering factors that we have purified to date, in an effort to stimulate fusion in the absence of a synthetic factor (PEG or GST-P4M-KA). I aimed to determine which factors were minimally required for this stimulation. Each tether was added individually to fusion reactions without PEG (Fig. 2.6 A). No individual component stimulated fusion to the same extent as the mixture of Sec17/18, COG-A, and Uso1-Bonsai in combination. Uso1-Bonsai alone did not stimulate fusion, as might be expected without addition of Ypt1. Both COG-A and Sec17/18 gave mild stimulation above baseline, but again, did not reach the levels found with the complete set of factors. Next I tested pairwise sets of factors (Fig. 2.6 B). In this experiment I found that Sec17/18 and COG-A in combination stimulates fusion in the absence of PEG at a similar level to all the complete set of factors. This suggests in this experimental set up without the functional addition of Ypt1, COG-A and Sec17/18 are capable of tethering vesicles for fusion. As each of these components was individually able to minimally increase fusion it remains unclear if the fusion signal is a result of COG-A and Sec17/18 working in combination

to tether vesicles or if the signal is a result of the combinations of each component individually. Up to this point, tethering between the ER and Golgi had not been reconstituted. The mechanisms of the observed tethering remain unknown, and how Ypt1 may be added to stimulate fusion are both open questions that need to be solved.

Discussion

Tethering at the *cis*-Golgi is complex, presenting a major question for decades in this field. Strong genetic interactions suggest a redundant web of functionally associated tethers. It is very likely that there is more than one pathway for vesicles from the ER to be accepted at the *cis*-Golgi. Our goal has been and will continue to be to reconstitute these pathways until we understand how the tethers function with each other and other fusion factors. In this work we used mutational analysis to locate the Uso1-Ypt1 binding interface and have found one way to reconstitute some tethering function, albeit Ypt1-independent.

The Uso1-Ypt1 binding interface leaves us with several questions. It will be critical to biochemically assess if Ypt1 is able to dimerize the Uso1 GHD. A conformational change of the Uso1 head domain could help in the tethering function, and how it conveys that dimerization would also be insightful for understanding the Golgin class of tethers. Identifying the binding site also raises questions about how the SNAREs interacting with the GHD interact with the Ypt1 activation cycle. We also have immediate plans to test the growth defects caused by the fourth site mutation; preliminary results suggest it has a strong growth defect.

The reconstitution of tethering in our fusion system is a preliminary result using only one subset of possible tethering factors. Additional factors we would like to test, include the complete COG heterooctamer, Grh1, Bug1, and a previously uncharacterized factor identified in our genetic screens. Additionally in this experiment we only used one SNARE topology, we recognize it is likely that tether-SNARE interactions could be critical for this and other tethering pathways, potentially requiring different topological orientations. We are excited at the prospect that COG-A and Sec17/18 appear to reconstitute some tethering function. How this combination can tether vesicles without the presence of Ypt1 leads to several questions. Importantly, some structural predictions of COG-A and Sly1 suggest a possible interaction. More studies will be needed to determine if this interaction is critical for the observed tethering function.

Acknowledgements: We would like to thank Dr. Christopher Fromme for the Ypt1:GDI protein. Dr. Fred Hughson for the COG-A purification protocol, Tom Duan for purifying COG-A and making several constructs for tether purification, and Ariel Lin for purifying Uso1-Bonsai.

Figures

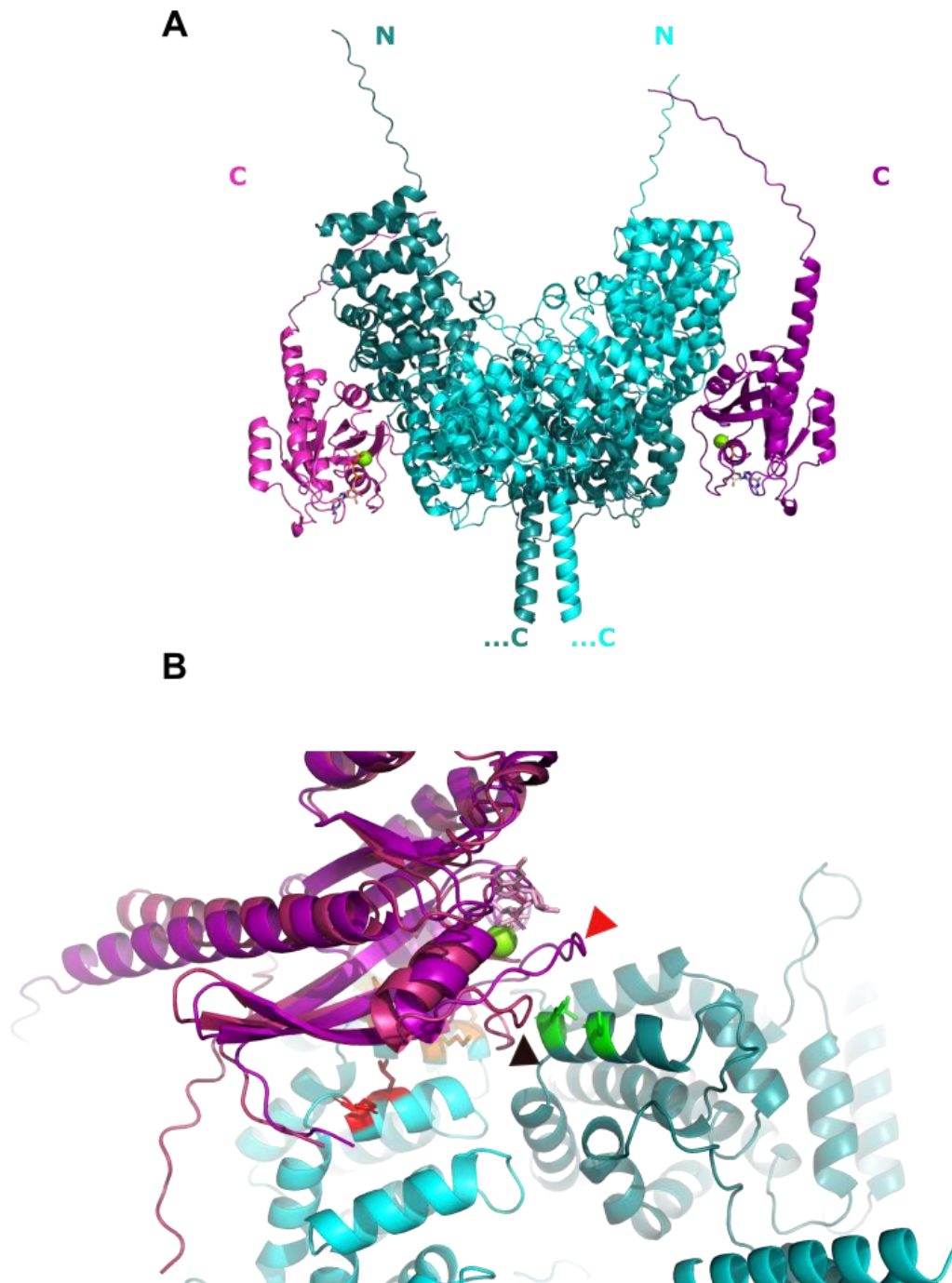


Figure 2.1 Predicted Uso1 and Ypt1 interactions in the presence of GTP

AlphaFold3 predictions of Ypt1 and Uso1 interacting. Two copies of Ypt1 were modeled and shown in magenta and dark pink. Mg²⁺ is shown as a green circle, and GTP as sticks. Two copies of the Uso1 GHD (1-750) are shown in cyan and teal. (A) Ypt1 is predicted to interact with an Uso1 homodimer interface. This interface is consistent with mammalian Uso1/p115 but not experimentally demonstrated to occur in *S. cerevisiae*. The C-terminus of Ypt1 is predicted to point in the same direction as the N-terminus of Uso1 as labeled. As a truncated version of Uso1 is modeled the C-terminus of the modeled region is labeled. (B) Overlay of Ypt1 without nucleotide and Ypt1 with GTP. The switch 1 loop (33-45) shows a difference in relation to the second Uso1 interface. The GTP bound model loop is labeled with the red arrow, the no nucleotide model loop is labeled with the black arrow.

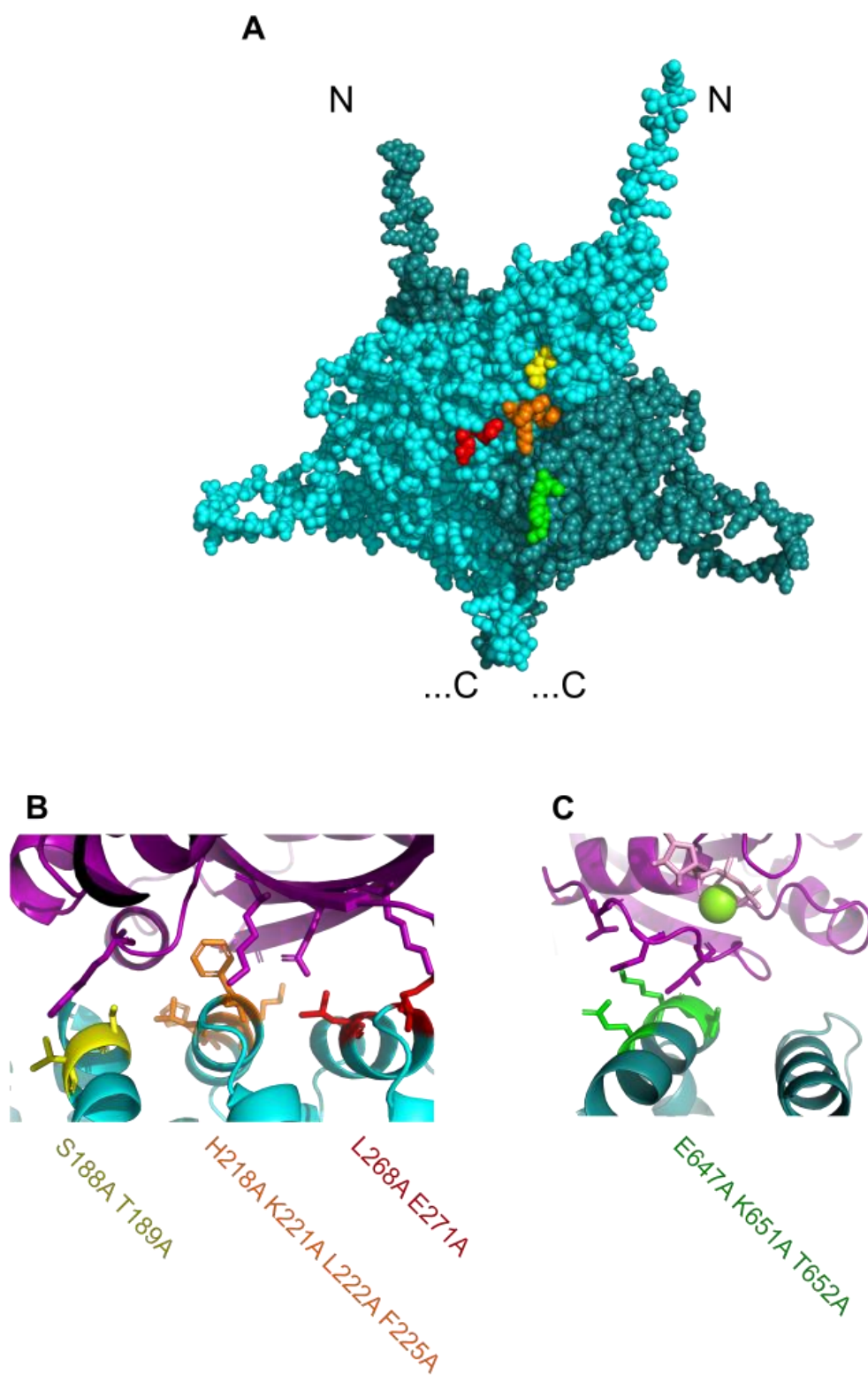


Figure 2.2 Predicted Uso1-Ypt1 binding interface

AlphaFold3 interactions between Uso1 (1-750) and Ypt1:GTP:Mg²⁺ predicted several interactions within 4 angstroms. Mutants were chosen based on predicted interactions and distance from Ypt1. (A) The Uso1 dimer as colored in Figure 2.1 without Ypt1 shown. Additional the four tested mutation sites are colored in red(1), orange(2), yellow(3), and green(4). The N- terminus is labeled for each Uso1 protein, The C-terminus of the modeled region is also labeled. (B) The first three tested sites are on one Uso1 interface. (C) the fourth site is on the dimerizing Uso1. The mutations made are listed below in the corresponding color.

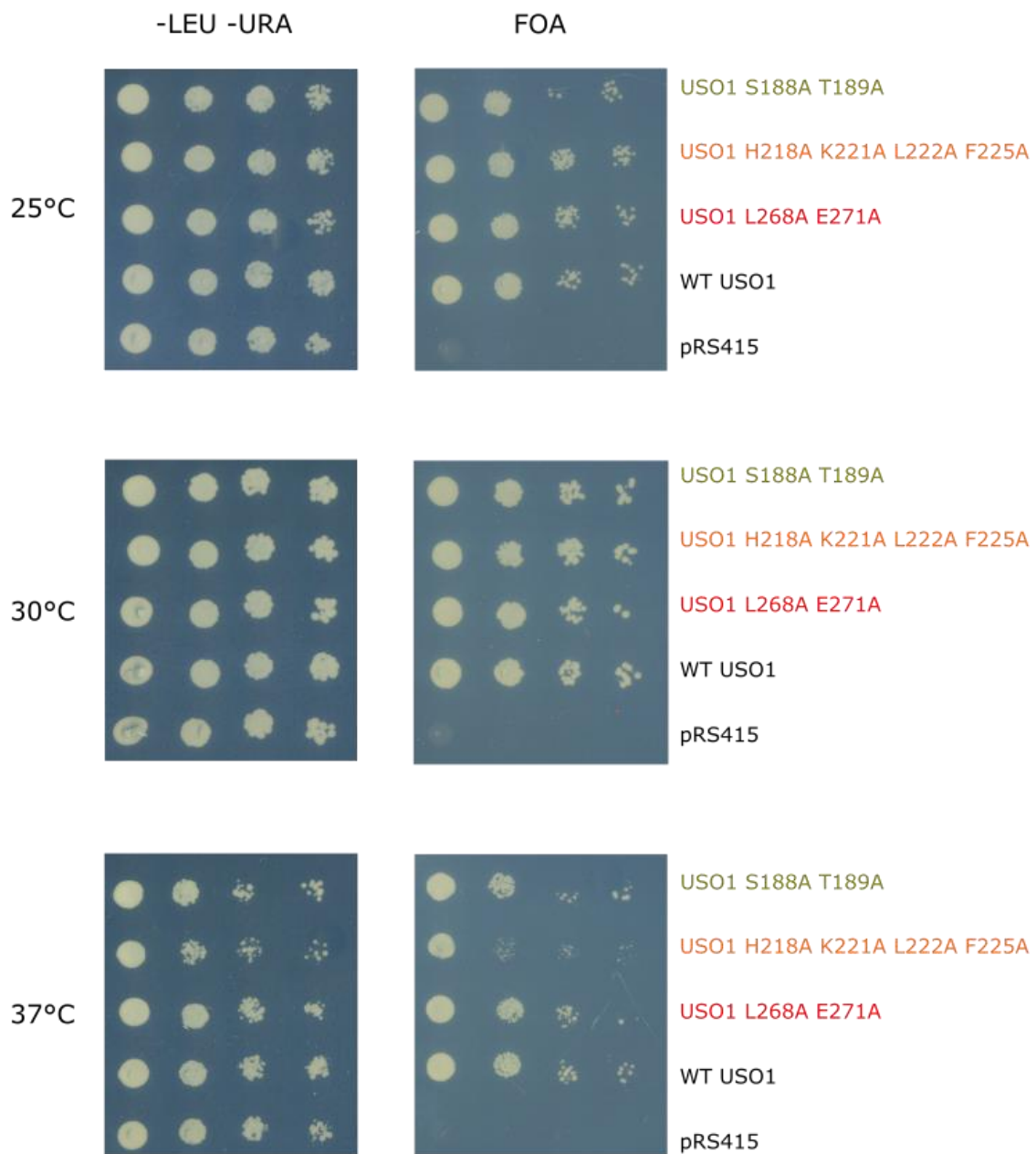


Figure 2.3 Growth Assays of *Uso1* mutants

The three mutants were each transformed into *S. cerevisiae* Δ USO1 pRS416::USO1. Cells were grown and diluted to an OD of .5, then diluted 1:20 three times to create the dilution series. Cells were spotted onto FOA plates and –LEU –URA plates and grown at 25°C, 30°C, and 37°C for four days. Mutation labels are color coded to correspond with Figure 2.2.

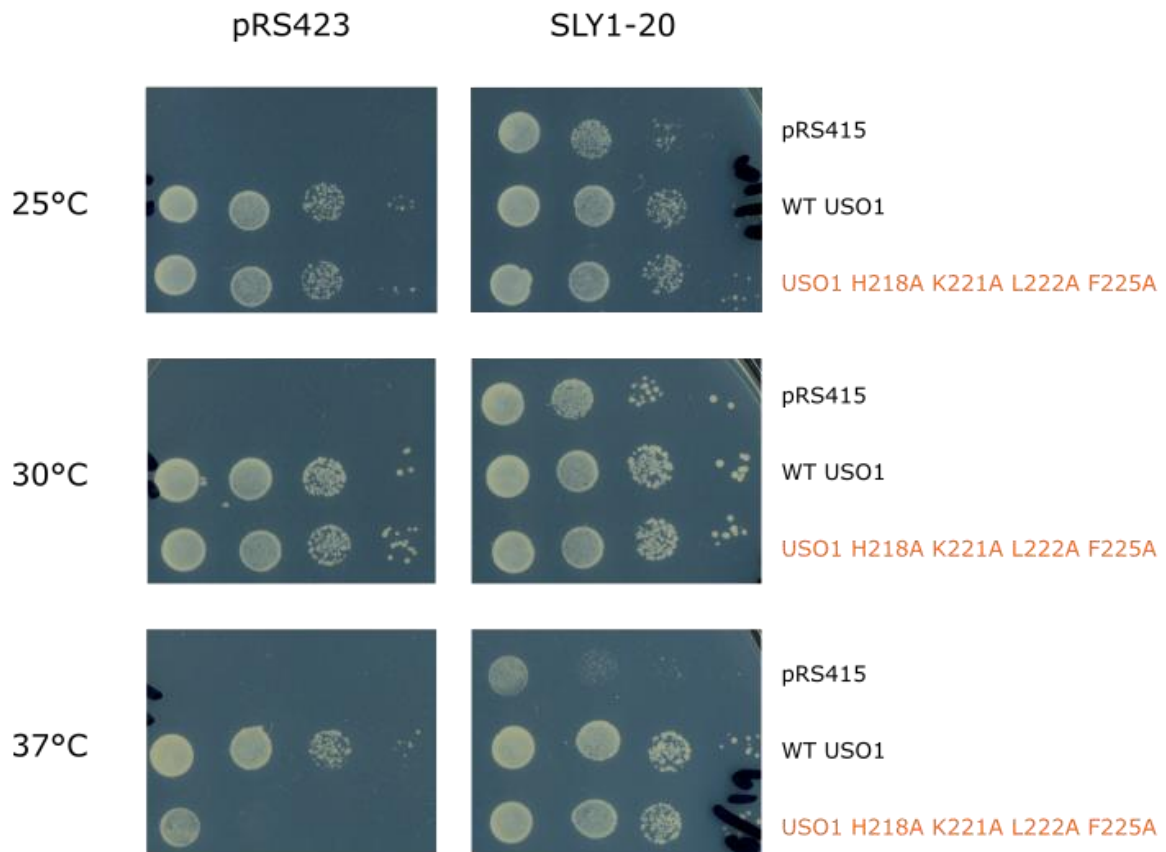


Figure 2.4 Growth rescue of *Uso1* mutant with overexpression of *Sly1-20*

The site two mutant, wildtype, and an empty control cell lines previously indicated were transformed with pRS423::SLY1-20. Cells were grown on FOA plates to remove the balancing USO1::URA plasmid then replica plated and grown on -LEU -HIS plates to test the rescue of this phenotype. Cells were grown and diluted as previously described. Liquid cultures were plated and grown for four days at the temperatures indicated.

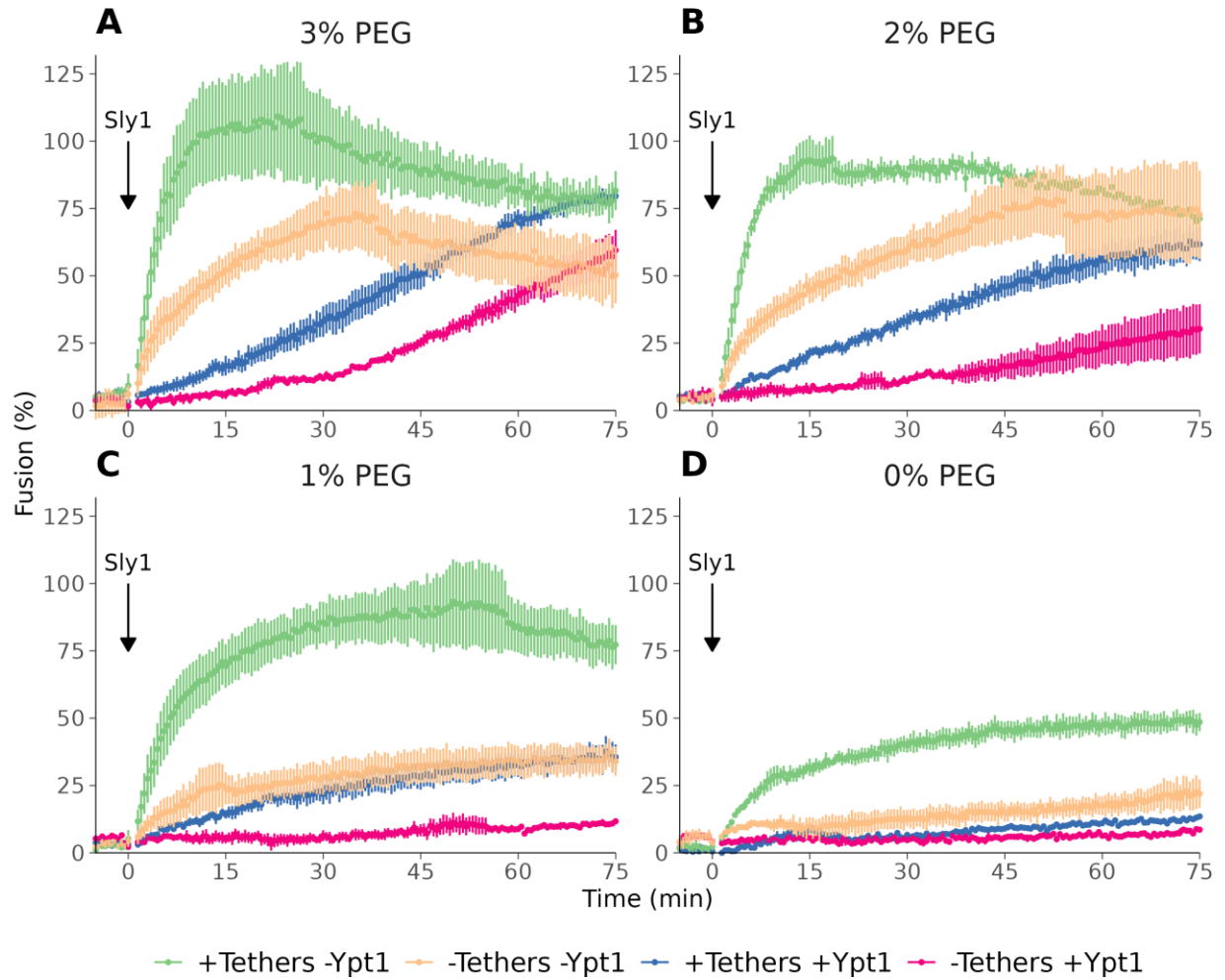


Figure 2.5 Fusion with tethering factors and decreasing PEG concentrations

Fusion reactions carried out as described above in variable concentrations of PEG. For conditions including tethers, 100 nM Uso1-bonsai, 333 nM COGA, 50 nM Sec18 and 100 nM Sec17 were added to the reaction. Reactions containing Ypt1 had 5 μ M Ypt1:GDI complex added. Ypt1 was nucleotide exchanged with EDTA and excess magnesium added after the addition of GTP. The nucleotide exchange protocol was carried out for all conditions. When PEG was added it was done so before the baseline reading. Fusion was initiated after a five minute baseline reading to initiate fusion. (A) 3% PEG was added to the reaction, this is the standard condition for fusion assays without tethering factors. (B) 2% PEG was added. (C) 1% PEG was added. (D) No PEG was added.

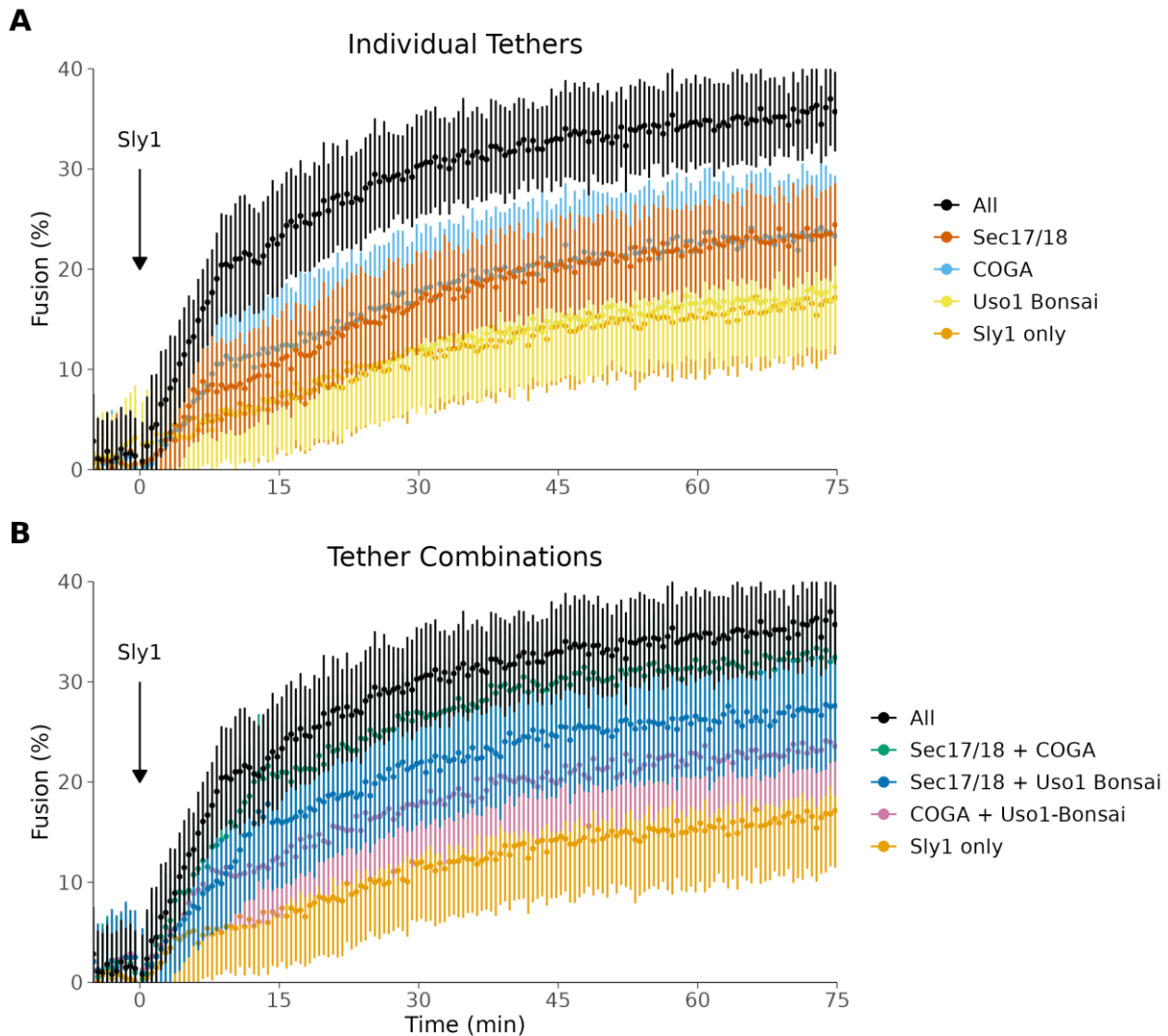


Figure 2.6 Minimal tethering components needed for reconstitution of tethering
 Fusion assays were set up as previously described. Tethers were added at 100 nM each except Sec18 which was added at 50 nM. Mock nucleotide exchange was carried out on all vesicle populations despite no addition of Ypt1 to these experiments. (A) only one of each tethering factor was added to each reaction to determine the impacts of each component individually. (B) Combinations of tethering factors were added before the initiation of fusion reaction.

Chapter four: Extended RPL preparation and floatation methods

This chapter is adapted in full from a submission to Methods in Enzymology

Making Reconstituted Proteoliposomes

Lipid and detergent stocks dissolved in chloroform or methanol or a combination of both are used. Lipid solutions purchased from avanti can be stored at -80 or -20 in clean vials (rinsed with the appropriate solvent) after being topped with argon and caps sealed with teflon and parafilm. Ergosterol was dissolved in chloroform to 15mg/ml and octa-beta glucoside was dissolved in methanol to 146.2 mg/ml. Prepare 1.25L of FB160M1 for each 1mL RPL prep.

Lipid solutions are mixed via Hamilton Gastight syringes into .5ml glass containers under a fume hood. Syringes are flushed immediately prior to use with the correct solution, with PI4P being the only lipid stored in chloroform/methanol/water mixture; this syringe is marked for this use only. After fluorescent lipids are added the tubes are covered in foil and remaining steps are conducted in the dark. Once all lipids and detergents are combined, the glass containers are placed under a nitrogen stream to dry for around 10 minutes. At this point there should be a fine lipid film on the bottom of the glass with minimal liquid remaining. To completely dry the lipids the mixtures are then placed in a speed-vac attached to a desiccator to capture the remaining chloroform. Subject to medium speed for 30 minutes is sufficient to remove the remaining liquid and condense the film to the bottom of the tube. If a speed-vac is not available the lipid mixture may alternatively be placed in a pressurized desiccator for 3 hours to remove the remaining chloroform.

Prepare dialysis cassettes during nitrogen stream drying. Add 2 grams of biobeads and 1L of FB160M1 to a beaker. Stir vigorously at 4°C for 5-10 minutes. Add 3 mL 20K MWCO dialysis cassette. For several RPL preps they may be added to the same beaker with additional biobeads. Do not exceed 2 mL total per 1 L of FB160M1. Continue to stir at a high speed until use, this allows the biobeads to degas as well as equilibrate the dialysis cassettes.

Once lipid films are formed and dried the material may be topped with argon gas and capped and sealed if teflon and parafilm and left at -80 overnight but may also be used immediately. Once dehydrated, the lipid mixture may now be rehydrated in the desired buffer. For a 1mL prep we begin by adding 210 μ L of 37°C FB160M1, top with argon gas and seal the tubes with teflon and

parafilm. Using a warm buffer is critical to fully resuspend the lipid mixture. The mixture is then bath sonicated for 5 minutes then nutated for 20 minutes at room temperature. This is repeated for three cycles, between cycles the tubes may be vortexed this can help especially before the initial cycle. Tubes should remain upright for the entire process to prevent loss of liquid.

During the second cycle prepare SNARE proteins for addition to lipid solution. On ice, prepare one epindorf tube for each desired RPL population. Add the appropriate amount of protein, and protease for tag cleavage. Once the lipids are resuspended add 210 μL of lipid mixture to each protein mixture. At this point keep everything on ice or at 4°C to prevent protein degradation. Add content mixing probes and additional buffers. Nutate lipid-protein mixture at 4°C for 30 minutes to allow for full tag cleavage.

There should be around 4-5 μL of lipid mixture remaining. Take 3 μL of each lipid mixture and add to separate PCR tubes, this will be used to quantify the lipid concentration of the final RPLs. Add 45 μL of FB160M1 to the lipid mixture and cover with foil and place in -20°C for lipid quantification the following day.

Using 3 mL syringes and 18 gauge needles, add the lipid-protein micelle mixture to the dialysis cassettes, do not touch the dialysis membrane. After addition of the mixture remove any air in the cassette, this may require removing the syringe and and purging. Remove as much air as possible. Replace the dialysis cassettes in the biobead FM160M1 beaker. Dialyze for 16-18 hours.

Rescue the dialysis contents. Add air to the cassette before removing the liquid. Place the post dialysis RPL mixture into a 5 mL tube and measure the amount of volume rescued. Mix an equal volume of 70% Histodenz mixture with the post dialysis RPLs, vortex well but gently. With a syringe and 18 gauge needle add to a clear ultra centrifuge tube on ice. Using a clean syringe and needle, slowly layer at least 1 mL of 25% Histodenz mixture over the RPL Histodenz mixture. Using a clean syringe and needle, slowly layer at least 200 μL of FB160M1 mixture over the Histodenz until the liquid is at the brand mark of the ultracentrifuge tube. Completely filling the tube is critical to prevent the tube from splitting and losing all of your sample.

In an ultracentrifuge spin in a SW60ti rotor, at 55K RPM, for 1.5hours, at 4°C. Remove tubes from the rotor and place directly on ice. Cut a quarter of the way up a p200 pipette tip off, to create a wider opening to the tip, this minimizes the disruption of RPLs for rescue. All RPLs should be in the top layer of the gradient. Gently move the pipette tip around the edge of the tube

to release the vesicles from the tube and concentrate them into the center of the tube. Typically, populations with more SNAREs tend to clump together and will be easy to remove all at once. Keep track of how much volume was removed to calculate the yield. You may pipette off more volume if you think it will increase your yield. Generally, avoid pipetting up any of the histodenz layers and try to minimize the total volume. The rescued volume will look clumpy. Vortex gently to resuspend until the mixture looks homogenous.

To determine the concentration of RPLs use the sample from the previous day to create a standard curve. The original diluted sample is at 1.25 mM lipid concentration, serial dilute 1:2 three times with FB160M1. Using a fluorometer, measure the Marina Blue populations with excitation at 370 nm, cutoff at 420 nm, and emission at 465 nm; and NBD with excitation at 460 nm, cutoff at 515 nm, and emission at 538 nm. We use 384 well plates, specifically black low-volume round bottom plates. Low-volume wells are critical to accurately determine fusion. This curve will allow you to correctly determine the amount of RPLs to use for each fusion reaction. RPLs should be diluted 1:10, 1:20 and 1:40 to accurately determine the concentration. The ideal usable concentration is around 2-3 mM. RPLs can be further diluted to this ideal concentration or left at a higher concentration. Aliquot each population into 10 μ L aliquots in strip tubes and flash freeze with liquid nitrogen and store at -80°C. Frozen RPLs can be stored for at least 3 years without decreased functionality.

Fusion assays

Using a fluorometer for time series readings of 30 minutes to 1 hour, we simultaneously read lipid mixing and content mixing signals every 30 seconds, at medium intensity for 6 seconds, shaking for 5 seconds between each read at 27°C. Lipid mixing is measured with excitation at 370 nm, cutoff at 420 nm, and emission at 465. Content Mixing is measured with excitation at 565 nm, cutoff at 630 nm, and emission at 670 nm.

The following basic additions to a fusion assay can be modified to test the addition of soluble proteins, or other desired factors. Unlabeled streptavidin is first mixed with the population which contains the biotin conjugated probe. This allows for quenching of any free probe or burst RPLs in this population. To the same tube the other RPL population is added. In a separate tube a final concentration of 3% PEG and buffer are combined. If the only conditions being tested are the later addition of other soluble factors such as an SM protein these two tubes can be combined

after the PEG has been diluted with a buffer, then 17.5 μ L of this mixture added to each well. Alternatively the RPL mixture can be added to each well and then the PEG and buffer mixture after. In this later set up additional soluble proteins such as tethering factors or additional chaperones can be added between the addition of the mixed RPLs and the PEG buffer mixture. This allows the proteins of interest to interact with the RPLs before they are in a highly crowded environment. Once the initial set up is completed the plate is placed in the fluorometer. Start a new reading and allow for a baseline reading for 5 minutes. Interrupt the read and add 2.5 μ L of either the SM protein or buffer for a negative control. Amend the current run to continue to read for the rest of the experiment. In a highly fusogenic population a dip in lipid mixing signal and an increase in content mixing signal should be immediately obvious.

Floated protein association

After the conclusion of a fusion assay, the end result materials can be floated against a gradient to determine protein association with the RPLs. This can also be conducted with a single RPL population before a fusion assay to specifically associate a protein with only one population. Similar to the float step in the making of RPLs, the desired RPL/protein mixture is combined with equal volume 70% Histodenz. Add this layer to a TLA 100.1 compatible thick walled tube. This should amount to less than 50 μ L of volume total. Overlay with 100 μ L of 25% Histodenz with a syringe and needle. It is critical to not have the layers mix or drop onto the first layer. At an angle add the layer slowly to the tube. Overlay with 30-50 μ L of FB160M1 buffer. The use of a TLA100 rotor allows for much smaller volumes, however because it is a fixed angle rotor the volume must leave space at the top of the tube to prevent spilling. The angle also makes the material stick to the top wall rather than gather in the center of the tube. Because of the content mixing probes the resulting clump of resired RPLs and proteins will be easy to visualize. Spin in a tabletop ultracentrifuge for 20 minutes at 80,000 RPM at 24°C. Remove tubes from the rotor and place directly on ice. Cut a quarter of the way up a p200 pipette tip off, to create a wider opening to the tip, this minimizes the disruption of RPLs for rescue. All RPLs should be in the top layer of the gradient. Gently move the pipette tip around the edge of the tube to release the vesicles from the tube and concentrate them into the center of the tube. A typical rescue volume is about 10 μ L. Any proteins that are bound to the membranes will remain associated and be able to be qualified with an SDS-PAGE gel visualized with coomassie, silver stain, or western blot.

-Buffers

FB160M1 : 20 mM HEPES KOH pH 7.0, 160 mM CH₃COOK, 10% (w/v) glycerol, 1 mM (CH₃COO)₂Mg

70% iso-osmolar histodenz: 70% histodenz (w/v) in FB160M containing only 2% glycerol.

25% iso-osmolar histodenz: dilute 70% iso-osm histodenz with FB160M.

Chapter five: Concluding remarks

Here I have shown the strong preference for a single SNARE topology for vesicle traffic from the ER to the Golgi. This pathway uniquely has a SM protein that is not involved in a larger complex, allowing us to specifically determine the preference for the R SNARE opposing the three Q SNAREs in a minimal system. It was equally interesting that the Qc SNARE has some topological flexibility. The R/Qc opposing the Qa/Qb can also produce fusion, although less than the preferred topology. Determining the topological needs of the fusion machinery of this pathway is not possible in vivo. Prior studies using temperature sensitive mutants suggested that the Qb and Qc SNAREs were required on the vesicle (Barlowe, 1994). This results demonstrates the requirements for the minimal fusion machinery. Sly1 physically interacts with the Qa and R SNAREs making any implication of topological requirements of the Qb and Qc SNAREs a question of how the regulation is conferred. We do note that over long periods of time and with the additional chaperones Sec17/18 we see increased stimulation for the flexibility of the Qb SNARE when the Qc SNARE is on the R SNARE membrane. My leading questions concluding this study are two fold, how do the Q SNARE interactions on one membrane promote fusion that they are unable to do in trans, and how can the addition of tethering factors impact the topological requirements.

The preference conferred for the R vs Qa/Qb/Qc topology could be caused by the Q SNAREs forming a pre fusion cluster, the N-terminal domains of the Q SNAREs could aid in template complex formation, or membrane dynamics could be critical. While our experiments have been unable to resolve the possible explanations for the topological requirements for Sly1 mediated fusion I suggest that the R SNARE requires flexibility while initiating interactions with Sly1 which aren't possible when interacting with a cis SNARE. As our structural predictions suggest that the R SNARE slots into Sly1 in the pre fusion state, this interaction could be difficult to achieve in the SNARE domain of the R SNARE is already engaged and is necessarily engaged for all SNAREs to interact in fusion.

Another critical question is how tethers interact with SNAREs. It has been demonstrated that there is a need for the Qc and Qb SNAREs on the vesicle (Cao, et al., 2000). Additional work shows potential interactions between the Qb and Qc SNAREs with the GHD of Uso1 (Bravo-Plaza, 2023). Taken together this poses an interesting question of how SNAREs not

involved in fusion may be necessary on either membrane solely for tethering. If tethers interact with SNAREs as a means of recognition it would not be beneficial for the end fusion product to recognize SNARE on their incorrect membrane. Perhaps tethers recognize the SNAREs on their proper membrane and their binding serves as an intermediate step coordinating SNAREs between tethering and fusion. As my work has touched on both of these subjects I am eager to see how these questions may resolve.

My experiments on COPII resolve how the coat inhibits fusion. A SNARE binding pocket of Sec24 interacts with either the R or Qc SNAREs sequestering it from interacting in fusion reactions. In combination with my topology results this would be a logical interaction for regulating fusion as vesicles depart the ER. The surprising data from this experiment go further to suggest that Sar1 is not critical for this inhibition. This suggests that the SNARE-Sec24 interaction is strong enough to transiently interact without membrane recruitment of the Sec23/24 dimer. It is likely that Sar1 dissociates upon GTP hydrolysis, potentially leaving Sec23/24 bound to the membrane through an abundance of cargo interactions. Sec23/24 have also been found to interact with several Golgi located proteins. Once again it comes down to what the role may be between Sec23/24 and tethering factors. The most attractive model would be a direct handoff of the R SNARE from Sec24 to either a tether or directly to Sly1.

Understanding the steps of vesicle initiation through docking on two membranes is the next necessary area of study for this field. Several tethers have been identified but their functions are less well understood. We have identified the binding site between Uso1 and Ypt1. Ypt1 sits at the interface of a suspected Uso1 dimer interface. While I was unable to finish the biochemical experiments needed to confirm the presence of a dimer I believe the location of the binding site poses an intriguing hypothesis; Does Ypt1-Uso1 binding cause a dimerization conformational change of the Uso1 GHD? The answer to this question could potentially offer an explanation of how Golgins receive and confer Rab signals.

My additional results of reconstitution of tethering through the addition of COG-A and Sec17/18 perhaps provide more questions than answers. Most interestingly, this interaction was not Ypt1 dependent. COG-A does have SNARE and Sly1 binding sites and Sec17/18 could elevate the number of available SNAREs for COG-A to interact with. More biochemical experimentation is needed to dissect how these factors create functional tethering. Despite the questions that arise, tethering between the ER and Golgi had not been reconstituted in any way

prior to these experiments. With many unanswered questions, my results are exciting and suggest that reconstitution of tethering of multiple ER to Golgi pathways is close.

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