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Review

The inner workings of intracellular heterotypic and homotypic membrane fusion mechanisms

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Intracellular trafficking is a field that has been intensively studied for years and yet there remains much to be learned. Part of the reason that there is so much obscurity remaining in this field is due to all the pathways and the stages that define cellular trafficking. One of the major steps in cellular trafficking is fusion. Fusion is defined as the terminal step that occurs when a cargo-laden vesicle arrives at the proper destination. There are two types of fusion within a cell: homotypic and heterotypic fusion. Homotypic fusion occurs when the two membranes merging together are of the same type such as vacuole to vacuole fusion. Heterotypic fusion occurs when the two membranes at play are of different types such as when an endosomal membrane fuses with a Golgi membrane. In this review, we will focus on all the protein components – Rabs, Golgins, Multisubunit tethers, GTPases, protein phosphatases and SNAREs – that have been known to function in both of these types of fusion. We hope to develop a model of how all of these constituents function together to achieve membrane fusion. Membrane fusion is a biological process absolutely necessary for proper intracellular trafficking. Due to the degree of importance multiple proteins are required for it to be properly carried through. Whether we are talking about heterotypic or homotypic fusion, any defects in the fusion machinery can result in disease states such as Parkinson's and Alzheimer's disease. Although much research has significantly expanded our knowledge of fusion, there is still much more to be learned.

Keywords. Golgi; Rabs; SNAREs; tethers; vacuole

Abbreviations: COG, Conserved oligomeric Golgi complex. A multi tethering complex composed of eight subunits known to be localized to the Golgi membrane; GARP, Golgi-associated retrograde protein. A multi tethering complex composed of four subunits known to be localized to the Golgi membrane; Golgin, Coiled-coil protein known to localized primarily to the Golgi membrane; MTC, Multi-tethering complex. Name given to tethers known to be made up by many protein subunits; Rab, Small GTPase that is part of the Ras superfamily; SNARE, Soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Vps1, Vacuolar protein sorting-associated protein 1. Yeast homologue to dynamin. GTPase protein; LMA1, Vacuolar binding protein that aids in Vam3 retention prior to fusion; Sec17, Alpha-soluble NSF attachment protein. Protein that binds to *cis*-SNAREs; Sec18, ATPase involved in SNARE disassembly along with Sec17; HOPS, Multi tethering complex that functions in vacuolar fusion; YcK3p, Kinase that phosphorylates Vps41 of the HOPS complex; Mon1–Ccz1, Guanine nucleotide exchange factor of Ypt7

1. Introduction

For years, lipid membranes have been characterized as the gatekeepers of the cell; they function to ensure that only necessary molecules enter the cell while keeping harmful substances out. These lipid membranes are composed of one or two leaflets of long fatty acid chains with each fatty acid containing a phospholipid head, otherwise known as a lipid bilayer. In 1972, Singer and Nicolson proposed the, now widely accepted, fluid mosaic model of the lipid membrane (Singer and Nicolson 1972). Within this model lies the idea that proteins associate with the lipid membrane to form a mosaic. Proteins can either be peripheral, lying on the margin of the membrane, or integral, inserted within the lipids (Goni 2014). Membranes owe much of their capabilities to these proteins. Among these abilities is membrane fusion mediated by proteins. Membrane fusion is an important biological process that occurs between two different cell types, called intercellular membrane fusion as well as between membranes within a single cell, termed intracellular membrane fusion. Intracellular membrane fusion is an essential process for a cell to remain viable since it is a terminal step in intracellular membrane trafficking. Although this process may sound simplistic, there is much

more at work than two membranes contacting each other in order for fusion to occur. Fusion is a complex process that involves N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), the most widely known fusion proteins, working alongside other components (Han *et al.* 2017). The types of proteins necessary for fusion can be grouped into four different categories: Rabs, SNAREs, tethering proteins, as well as a few other accessory proteins. Each of these components interact at different time points of the fusion process, and malfunctions in anyone of these groups of proteins can lead not just to fusion defects but a variety of medical malignancies. For example, defects in certain SNARE proteins have been associated with congenital myasthenic syndromes, which are genetic disorders that lead to impaired neuromuscular transmission (Rodriguez Cruz *et al.* 2018). Disorders caused from malfunction in Rab proteins vary from neurological disorders to cancer as well as diabetes (Li and Marlin 2015). Dysregulation in tethers such as the conserved oligomeric Golgi (COG) complex can lead to congenital disorders of glycosylation (CDG)-type II. Additionally, neurodegenerative diseases such as Parkinson's and Alzheimer's have been associated with a dysregulation of COG and its Rab and SNARE binding partners (Climer *et al.* 2015).

2. Components of proper membrane fusion

2.1 *Rab GTPases*

Throughout the fusion process, various proteins step in at different time intervals. The previous protein passes on the baton to the next one. Although this is a common concept in fusion, Rab proteins are unique in the sense that they are scattered all throughout the fusion steps. They can be found at the start of fusion, during protein recruitment and all the way up to the fusion step interacting with other fusion proteins (Hutagalung and Novick 2011).

Specifically, Rabs are a group of GTPase proteins that are ubiquitously found within a cell. They have been critically known to function in fusion events of different membrane compartments. In humans, there have been over 70 different Rabs identified (Seabra *et al.* 2002; Tzeng and Wang 2016). Together they form the largest subset of the Ras superfamily (Pereira-Leal and Seabra 2001). The function of Rab proteins is attributed to cycling of GTP/GDP binding, which correlates with membrane association and dissociation steps (Schwartz *et al.* 2007). Active Rab found at organelle membranes includes the GTP-bound form, while inactive Rab is composed of the GDP-bound form located at the cytoplasm (Martinez and Goud 1998). At the cytoplasm, 11 prenylation where a prenyl moiety is added to their C-terminal. This increases their hydrophobicity and thus induces them to associate with the membrane (Schwartz *et al.* 2007). At the membrane, the inactive GDP form of the Rab protein undergoes activation when a guanine nucleotide exchange factor (GEF) adds a nucleotide to convert it into the GTP/active form (Hutagalung and Novick 2011). To convert back to the inactive form, a GTPase-activating protein (GAP) aids in GTP hydrolysis, which allows for a GDP dissociation inhibitor to bind in order to maintain the Rab protein in its inactive cytosolic form (Schwartz *et al.* 2007). GDP and GTP are able to bind to the Rab protein via a G-domain that is universally conserved across all Rabs, with varying amino acids within this domain being responsible for GDP and GTP binding within different Rabs. This means that different Rabs can be activated by different mechanisms. As well, the same Rab can be activated by varying mechanisms (Langemeyer *et al.* 2014). When active, Rabs are membrane bound. They are charged with the task of recruiting different effector proteins to perform membrane fusion. Before fusion can initiate, the target membrane and the vesicular membrane have to come into close proximity to each other. Rab proteins are able to help in this step by recruiting effector proteins that act as tethers to bring the membranes together. Rabs can bind both coil–coil tethers known as the Golgins as well as multisubunit tethers such as the Golgi-associated retrograde protein (GARP) and the COG complex (Hutagalung and Novick 2011). Recent studies have indicated that the GRIP domain of Golgins such as in golgin-97, GCC88 and GCC185 is important for them to interact with different Rab proteins and anchor them down to the membrane (Cheung and Pfeffer 2016). More of how Rab to Golgin interactions facilitate fusion will be illustrated in the later heterotypic section.

2.2 *Tethering proteins: GARP*

Although there are a variety of tethers that function at different sites within the cell, the GARP (Vps fifty-three (VFT)) complex is of particular importance for heterotypic fusion which will be explained further in the heterotypic section. In particular, it plays a role in the transport of cargo from the endosome to the Golgi membrane, otherwise known as retrograde trafficking. The GARP complex is a multisubunit complex composed of Ang2, Vps52, Vps53 and Vps54 in mammalian cells, while yeast cells contain Vps51, Vps52, Vps53 and Vps54 (Bonifacino and Hierro 2011).

2.3 *Tethering proteins: COG*

Another important tethering complex for heterotypic fusion is the COG complex. It is also a multi-subunit tether that forms part of the CATCHR family. This is namely important in intra-Golgi trafficking, and vast research has documented its interaction with various fusion proteins as well as molecular motors. Since it is most important in tethering vesicles that carry glycosylation enzymes,

mutations in this complex can lead to a group of disorders known as CDG (Zeevaert *et al.* 2008). Given that COG is a multi-subunit tether, it is composed of peptides, COG 1–8. It is further divided into two lobes: Lobe A and Lobe B. Lobe A is comprised of COG subunits 1–4, while Lobe B is made up of subunits 5–8 (Willett *et al.* 2013). Since the COG complex interacts with a variety of proteins such as Rabs, SNAREs, SM proteins and coil–coil tethers, there are various models that propose how COG maintains all of these interactions in order to promote Golgi trafficking. The first model proposes that COG acts to initiate the formation of vesicle docking stations. In this way, it acts to recruit proteins necessary for the proper fusion proteins to arrive at the target site in order for the donor vesicle to dock and initiate fusion. The second model proposes that COG acts as a tether to connect the arriving vesicle to the target membrane. The third model suggests that COG acts to stabilize the SNAREs on the target membrane just prior to fusion. As a final model, it has been proposed that COG acts to perform all of these functions (initiate docking stations, act as a tether, stabilize SNAREs) in the same fusion reaction (Willett *et al.* 2013). This review will focus on the role of COG as a tether at the *trans*-Golgi network (TGN).

2.4 *Tethering proteins: Golgins*

Lining the Golgi membrane are Golgins, also known as coil–coil proteins. This name is given to them due to most of their structure being made up of coils or a collection of two or more alpha helices that extend into a rod-like structure (Gillingham and Munro 2003). Although these proteins are well conserved through evolution, much of their coil regions are not conserved giving the idea that these regions are just spacers. Additionally, their structure includes unstructured regions that could help in hinging and Golgin flexibility, a mechanism which would be useful when bringing captured vesicles closer to the target destination (Oas and Endow 1994). When lining the Golgi membrane, they are anchored either by their C-terminus or the help of a small GTPase such as Arl1 or Rab proteins. Golgins using the Arl1 protein as a Golgi membrane binding mechanism contain a specific C-terminally located domain called the GRIP domain. In this class of Golgins golgin-97, golgin-245, GCC88 and GCC185 are included (Panic *et al.* 2003; Wu *et al.* 2004). In addition to having GTPase binding sites at their C-terminus, Golgins have been shown to contain multiple Rab binding sites along the length of their coil–coil region, which could help in capturing incoming vesicles and guiding them to the correct region at the Golgi surface (Sinka *et al.* 2008). In their cytosolic N-terminal domain, Golgins contain mechanisms that help to capture different types of carrier vesicles, which will be explained further in the following heterotypic fusion section (Wong *et al.* 2017).

2.5 *Tethering proteins: homotypic fusion and protein sorting (HOPS)*

A major tether in homotypic vacuolar fusion is the HOPS complex, which is made up of six different vacuolar protein sorting (Vps) subunits: Vps11, Vps16, Vps18, Vps33, Vps39 and Vps41. Of these, Vps11, Vps16, Vps18 and Vps33 form the core complex known as the Vps core. The other two subunits then associate to form what is known as the HOPS complex (Seals *et al.* 2000; Wurmser *et al.* 2000). In vacuolar fusion, HOPS plays a well-characterized role by interacting with the small GTPase Rab7, which aids in its tethering function that will be explained in more detail in the later homotypic section (Lin *et al.* 2014).

2.6 *SNAREs*

To complete fusion, SNARE proteins are required. Currently more than 60 members have been associated with this protein superfamily, all of which share a 60–70 amino acid stretch known as the SNARE motif. In addition to the SNARE motif, these proteins also contain a linker region as well as a transmembrane domain (Han *et al.* 2017). The transmembrane domain allows for the SNARE to anchor on to lipids of their target membranes (Malsam and Sollner 2011). However, there are seven SNAREs that are known to not contain a transmembrane domain. These SNAREs anchor on to the target membrane via lipid modifications, Vam7 is one such SNARE that will be discussed in the homotypic fusion section (Hong and Lev 2014). The SNARE motif is a key domain in the SNAREs' ability to function in fusion. This motif is located at the cytosolic end of the SNARE and generally spans for about 60–70 amino acids in length, which contains heptad repeats that have the ability to form coil–coil structures (Malsam and Sollner 2011).

There are two types of SNARE proteins: *t*-SNAREs and *v*-SNAREs, which are needed for proper fusion to occur (Han *et al.* 2017). With regard to trafficking, *v*-SNAREs are found within the vesicle membrane, while *t*-SNAREs are localized to the target membrane (Schiavo *et al.* 1997). As a way to further differentiate between *t*- and *v*-SNAREs, it can be noted that most *v*-SNAREs have an arginine residue located at the center of the SNARE domain for which they can also be called R-SNAREs. On the other hand, *t*-SNAREs tend to contain a glutamine or aspartate residue leading researchers to call them Q-SNAREs (Ungermann and Langosch 2005). Further, *t*-SNAREs can be subcategorized into Q_a, Q_b and Q_c SNAREs. This is based on the amino acid located at the central layer in addition to structural features (Malsam and Sollner 2011).

For fusion to occur, it has been shown that the cytosolic SNARE domains of these SNAREs interact to form a *trans*-complex (Han *et al.* 2015). This complex is also termed the

trans-v/-t-SNARE complex or SNAREpins (Malsam and Sollner 2011). The *trans*-complex is a composite of a four-helix SNARE bundle, which aids in bringing the two membranes of interest into close proximity. Further, this interaction aids the two membranes' transition from a hemifusion state to a full-fusion state (Wu *et al.* 2016). For fusion to occur, SNARE 'zippering' must ensue from the N-terminal end of the SNAREs to the C-terminal end. As the SNAREs zipper together, they generate a force that can pull the two opposing membranes together, which allows fusion of the membranes and content mixing to occur (Sudhof and Rothman 2009).

In the following sections, this review will discuss how all these protein components work in concert in order for heterotypic and homotypic fusion to occur. Proteins implicated in heterotypic and homotypic fusion are listed in table 1.

3. Heterotypic fusion

Heterotypic fusion is primarily defined as occurring between two different types of membranes. For example a Golgi to vacuole fusion would be heterotypic as the Golgi and vacuole are different types of membranes. Although there are different types of homotypic fusion reactions that can be discussed, this review will focus on endosome to Golgi fusion. This fusion reaction is the result of retrograde transport and therefore, many proteins that recycle through this transport are reliant on endosome to Golgi heterotypic fusion. Shiga toxin and cation independent mannose-6-phosphate receptor are some of the proteins known to use this type of transport (McKenzie *et al.* 2012). Although priming, tethering and fusion are the three main steps in a fusion reaction, for heterotypic fusion, focus will be placed on the Rab proteins involved as well and the different tethering mechanisms and finally the final fusion step.

3.1 *Rabs*

Active, GTP bound, Rab9 binds to the late-endosomal membrane as well as the TGN and has been shown to act in the recycling of cation-independent mannose-6-phosphate receptor (CI-MPR) towards the TGN membrane (Shapiro *et al.* 1993). In contrast, more recent research found that constitutively active Rab9 leads to a trafficking of CI-MPR to the late endosome as the final destination, suggesting that Rab9 functions in anterograde trafficking of CI-MPR towards the endosome (Kucera *et al.* 2016). Since the latter is a novel additive function of Rab9, further exploration may be required to resolve the controversy. Another GTPase that has undoubtedly been shown to play a role in retrograde trafficking is Rab7b (Progida *et al.* 2010). Specifically, Rab7b has been implicated in the regulation of CI-MPR, TGN46, Shiga toxin and toll-like receptors 4 and 9 (Bucci *et al.* 2010). Rab7-dependent trafficking is initiated by recruiting Rab7 to its target membrane by a Rab escort protein (Kucera *et al.* 2016). At the membrane, Rabs are activated by Mon1–Ccz1, a GEF that adds a GTP in order to aid in membrane insertion of Rab7, as seen in figure 1 (Seabra and Wasmeier 2004; Nordmann *et al.* 2010). Once anchored to the membrane, steady-state distribution of Rabs, including Rab7, is enforced by the interaction of the Rab effector (Rab interacting lysosomal protein (RILP) with the hypervariable C-terminal domain (HVD) on Rab proteins. This idea was proposed based on the observation that RILP knockdowns or HVD replacements lead to a mislocalization of Rab7. Specifically, the N-terminal residues (174–183) of the Rab7 HVD are important for Rab binding to RILP and thus late endosome localization (Li *et al.* 2014). Once at the membrane, studies indicate that Rab7 is able to recruit the retromer complex, which allows for the transport of the vesicle derived from the late endosome to the TGN. The recruitment of the retromer is demonstrated by the interaction of Rab7 with both Vps35 and Vps26 (figure 1), components of the retromer complex (Priya *et al.* 2015). Further, it is the palmitoylated form of Rab7 that has been shown to aid in this interaction, but palmitoylated Rab7 is not necessary for Rab7 interaction with RILP indicating that palmitoylation is specific for the proper trafficking of endosome-derived vesicles to the TGN. Palmitoylation of Rab7 occurs at the cysteine residues 83 and 84 (Modica *et al.* 2017).

Table 1. Protein function in heterotypic and homotypic fusion

Proteins involved in heterotypic fusion	Proteins involved in homotypic fusion
Rabs: Rab7, Rab9, Rab6/Ypt6	Sec18p/NSF
Mon1–Ccz1	Sec17/alphaSnap
Retromer complex	LMA1
Golgins	Pah1
TBC1D23	HOPS
WASH complex	Ypt7
COG complex	Mon1–Ccz1
GARP	Yck3p
SM proteins/Vps45	Vps1
SNAREs: syntaxin 6/Tlg1, Syntaxin 16/Tlg2, Vti1, VAMP4	SNAREs: Vam7, Nyv1, Vam3, Vti1

3.2 *Tethering*

At the Golgi membrane lie the TGN Golgins, which are anchored via their GRIP domain to the Golgi membrane. Once at the TGN, Golgins act to capture vesicles at a distance and bring them in closer to the target membrane surface. Although there are a few different models that propose how this is done, this area of research is still under investigation. However, recent research has proposed that N-terminal of Golgins contains certain vesicle binding motifs (Wong *et al.* 2017). Of the four TGN Golgins that capture endosomal-derived vesicles, GCC88 has an N-terminally located 60 amino acid residue stretch that was shown to be necessary for proper capture of endosomal vesicles.

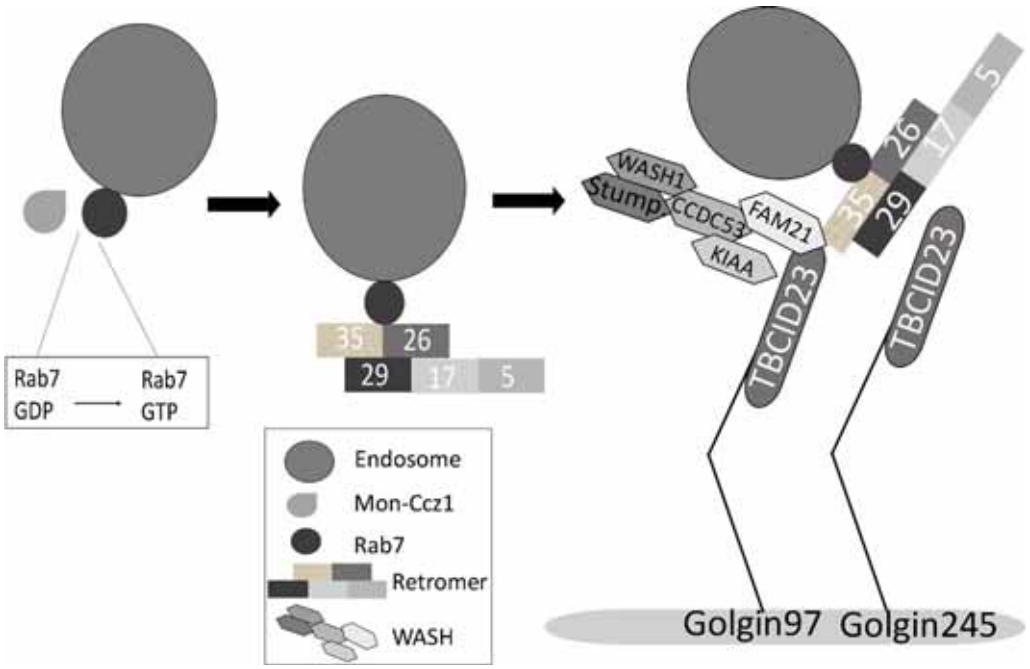

Figure 1. Endosomal to Golgin interaction at the endosomal membrane, Rab7 is first recruited, where it is then able to go from its inactive GDP bound form to its, GTP bound form with the help of the GEF, Mon-Ccz1 (Seabra and Wasmeier 2004; Nordmann *et al.* 2010). Once bound to the endosome, Rab7 can interact with Vps35 and Vps26 of the retromer complex (Priya *et al.* 2015). Simultaneously, Vps35 interacts with the FAM21 subunit of the WASH complex (Seaman 2012). This interaction is important because as the vesicle approaches, the FAM21 subunit of WASH is able to interact with TBCID23, a protein that interacts and attaches to the N-terminal domain of golgin-97 and golgin-245. Via TBCID23, golgin97 is able to tether the vesicle into the Golgi membrane (Shin *et al.* 2017).

Furthermore, the results indicated that the first 21 amino acids of the N-terminus, of golgin-97 and golgin-245, were necessary for proper vesicle binding. Further, when golgin-97 and golgin-245 were compared, it was found that they shared phenylalanine at the second position of the vesicle capturing motif. When this phenylalanine was mutated, they found that all tethering activity was lost in both of these Golgins, concluding that these two types of Golgins bind similar vesicles that differ from the vesicles that GCC88 recognizes, since GCC88 did not share this phenylalanine. This means that Golgins may be able to discriminate between different vesicles that target the Golgi membrane (Wong *et al.* 2017). Although this research pinpoints the N-terminus as necessary for vesicle binding, there is still ongoing research to pinpoint where and how Golgins bind the opposing membrane.

One area of research indicates that instead of binding to vesicle membranes directly, Golgins appear to bind to bridging factors that mark the endosomal membrane. TBC1D23 (figure 1), a member of the TBC (tre-2/bub2/Cdc16) family of Rab GAP, is one such bridging factor. Research showed that mislocalization of either golgin-97 or golgin-245 to the mitochondria lead to TBC1D23 mislocalization to the mitochondrial membrane as well, leading to the conclusion that the Golgins need to be first localized to the Golgi membrane before TBC1D23 can be recruited. At the TGN, TBC1D23 is able to bind to the Golgin's N-terminus where it acts as a bridging factor between the Golgins and the endosome-derived vesicle. Specifically, the carrier-binding domain of TBC1D23 binds to the FAM21 subunit of the Wiskott-Aldrich syndrome protein and Scar homologue (WASH) complex on the approaching vesicles (figure 1) (Shin *et al.* 2017). The WASH complex is known to function alongside the retromer in endosomal protein recycling. The retromer to WASH complex association is facilitated by the interaction of the Vps35 subunit of the retromer and the Fam21 subunit of the WASH complex (Seaman 2012) (figure 1). Additionally, it was shown that TBCID23 was necessary for golgin-97 and golgin-245 to capture CI-MPR laden vesicles but not necessary for GCC88 to capture CI-MPR containing vesicles, further reinforcing the idea that each of these Golgins have their own unique ways to discriminate between varying cargo carrying vesicles (Shin *et al.* 2017). The Golgin, GCC185 also supports this idea, as it has been shown to interact with the clathrin adaptor AP-1 (figure 2, panel A). Instead of requiring a bridging protein for this interaction, GCC185 is able to bind to AP-1 via its specific amino acid residues (939–1031) located at the N-terminus (Brown *et al.* 2011). Once the vesicle is bound to the N-terminal arms of GCC185, the collapsible model, proposed by Cheung and colleagues, has GCC185 being flexible enough to collapse into the TGN membrane and therefore bringing the bound vesicle close enough to the TGN membrane (Cheung *et al.* 2015)

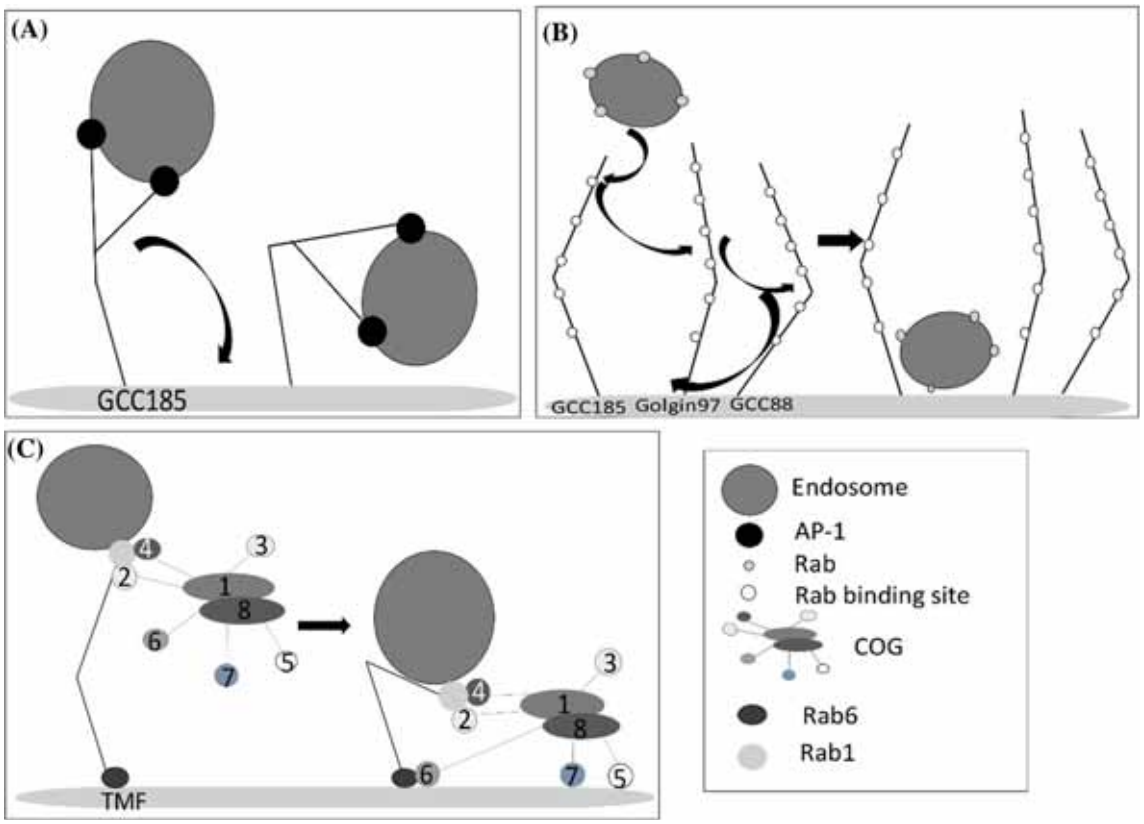

Figure 2. Tethering varieties between endosome and Golgi membrane. There are a variety of ways that Golgins can tether in incoming vesicles. Panels (**A–C**) highlight some of the proposed mechanisms of Golgin tethering. (**A**) GCC185 is able to recognize the adaptor protein, AP-1, decorating incoming vesicles, via its N-terminally located splayed ends (Brown *et al.* 2011). Additionally, it is known to contain a hinge region that allows it to collapse on to the Golgi membrane along with its captured vesicle (Cheung *et al.* 2015). (**B**) Several Golgins have been shown to contain a variety of Rab binding sites along their lengths (Hayes *et al.* 2009). Via the tentacular model, it is proposed that Rab proteins decorating the incoming endosome can transiently interact with these Rab binding sites until the endosome has made it to its target destination along the Golgi membrane (Sinka *et al.* 2008). (**C**) Aside from acting as lone tethers, Golgins have been shown to work along with the MTC. One such example is between the Golgin, TMF and the MTC, COG. In this example, TMF is initially anchored on to the Golgi membrane via a Rab6 interaction. Its cytosolic, N-terminal domain interacts with Rab1 on the incoming endosome. Simultaneously, COG4 of the COG complex is able to interact with Rab1 while COG2 interacts with the TMF head. This aids in bringing the vesicle into closer proximity to the Golgi membrane. Once close enough to the Golgi membrane, COG6 of the COG complex is able to interact with Rab6 and complete the tethering activity of the incoming endosome (Miller *et al.* 2013).

(figure 2A). However this model has only been proposed for GCC185 based on the structural analysis of this protein. More studies have to be carried out on the rest of the Golgins to determine how it is that they get the vesicle close enough to the target membrane once it is bound. One theory that has arisen is the tentacular model. In this model, Golgins surround the Golgi membrane forming a meshwork containing various vesicular binding sites (figure 2B). Since different Golgins share similar binding partners such as Rabs and other vesicular coat proteins, the incoming vesicle can make its way towards the Golgi membrane through its promiscuous, transient protein–protein interactions with Golgin-localized proteins (Munro 2011) (figure 2B). For example, GCC185 was shown to contain five binding sites along its length for 14 different Rab proteins including Rab6 and Rab9 (Hayes *et al.* 2009). Furthermore, the TGN-localized golgin-97 was shown to contain multiple binding sites for Rab6, Rab19 and Rab30, while the GCC88 Golgin was shown to have several binding sites for Rab6, Rab19 and Rab30 upstream of its GRIP domain. This redundancy in Rab binding sites supports the tentacular model. It also explains why lethal phenotypes are rare when specific Rabs or Golgins are depleted from cell lines (Sinka *et al.* 2008). However more research has to be carried out to confirm this model.

Aside from models that have Golgins acting as lone tethers, it is suggested that Golgins can interact with multisubunit tethers such as COG. For example, TATA element Modulatory factor (TMF) is another Golgin lining the Golgi membrane and is proposed to interact with the COG complex. This Golgin is anchored to the Golgi membrane via Rab6 interaction that connects the C-terminal coil to the Golgi membrane (figure 2C). In this model, the N-terminal TMF head interacts with incoming vesicles via Rab1 on the

vesicle. To reinforce the tethering activity COG4 also binds Rab1 while Cog 2 simultaneously binds the TMF head. The TMF Golgin then uses its centrally located hinge region to bring the vesicle closer to the target TGN. Once close enough to the TGN membrane, COG6 can bind to Rab6 GTPase to fully tether the vesicle (Miller *et al.* 2013). This research highlights some important concepts. First, it reiterates the idea that different Golgins discriminate between different cargo vesicles via distinct Rab and coat protein interactions. Second, it proposes the idea that coil–coil tether and multi tethering complexes (MTCs) can function together in tethering events. This could signify that the two types of tethers could also be working together in other fusion events not yet identified. Lastly, this research proposes that Rab1 could be serving in retrograde traffic when previous research classified Rab1 as an anterograde protein (Miller *et al.* 2013), indicating that Rab1 could be functioning in multiple roles that need to be further studied. Taking this pioneering work as a platform, research studies can aim at figuring out if the norm is for MTCs and Golgins to work together or if that is an anomaly seen only between TMF and COG. Also, is it just Rabs that drive the type of Golgin interactions that vesicles have or is it also cargo dependent?

Moving on, once the vesicle is tethered and close enough to the TGN membrane, SNARE to SNARE interaction is the next step that needs to occur in order for fusion to be completed. Once at this interface, another tether may be aiding in the SNARE to SNARE interaction. GARP has been shown to bridge Syntaxin6, a *t*-SNARE on the TGN and Rab6 on transport vesicles (figure 3). The human homologue to Vps51 of the GARP complex, Ang2, was shown to interact with Syntaxin6, which is the human homologue to yeast Tlg1. This interaction is specifically between the $_{37}$LxxYY$_{41}$ motif of the N-terminal part of Ang2 and the N-terminal Habc domain of Syntaxin6 (Abascal-Palacios *et al.* 2013). Additionally, a similar interaction was observed between Vps51 and Tlg1 in yeast. It was found that N-terminally located amino acid residues 18–30 on Vps51 interact with the N-terminal domain of Tlg1 forming a 3 helix bundle (Fridmann-Sirkis *et al.* 2006). On the other end of the GARP complex is Vps52, which binds to Ypt6, the yeast homologue of Rab6, on incoming endosome-derived vesicles (Saimani *et al.* 2017). Much can be speculated about these GARP interactions, but one hypothesis is that GARP is the bridging factor that brings SNARE into close enough proximity to form the four helical bundles necessary for fusion. This is based on a pull-down analysis that demonstrated that Ang2 also interacts with Vti1a. Since Vti1a does not contain a Habc domain to interact with Ang2, this pull-down could be due to indirect interactions caused by the SNARE assembly, enforcing the idea that GARP serves as a bridge to bring the SNAREs in close proximity to each other (Abascal-Palacios *et al.* 2013). Worth noting is the finding that

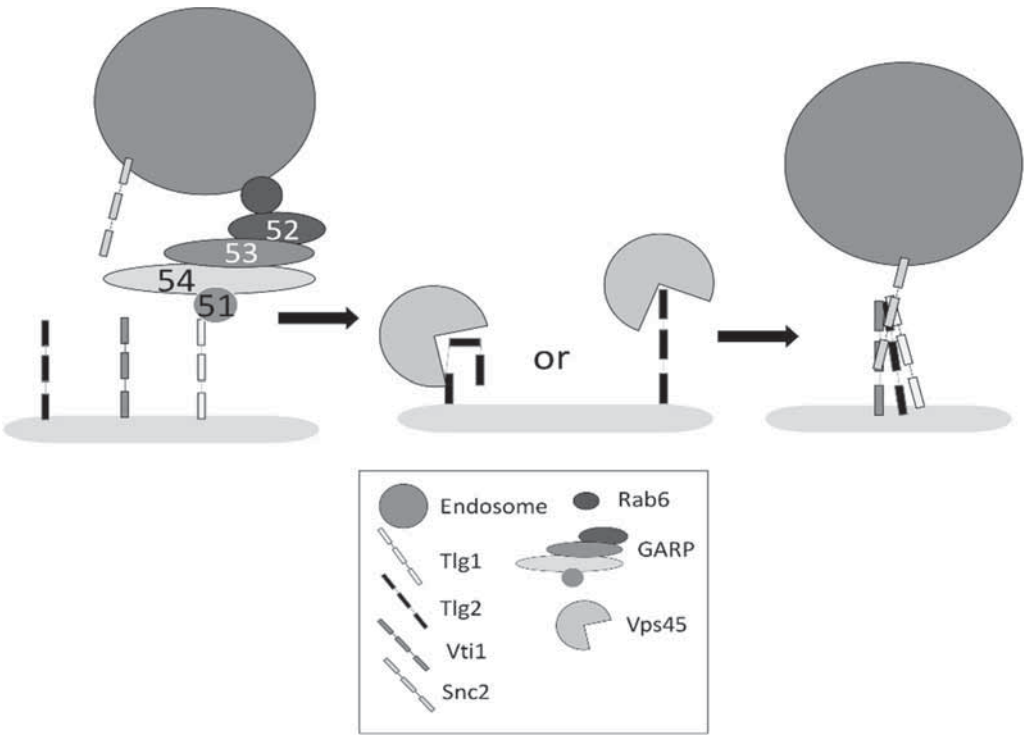

Figure 3. SNARE interactions necessary for fusion another tethering mechanism for fusion to proceed involves the GARP complex. Here the Vps51 subunit is able to bind to Tlg1 while the Vps52 subunit is able to bind to Ypt7 (Fridmann-Sirkis *et al.* 2006; Saimani *et al.* 2017). This interaction brings the endosome closer to the Golgi membrane. Additionally, the SNARE, Tlg2 must be activated for fusion (Struthers *et al.* 2009). There are two proposed mechanisms that this can occur. In one scenario, Vps45 interacts with the C-terminal region on the closed conformation of Tlg2 to activate it. In a second scenario, Vps45 interacts with the N-peptide of Tlg2 (Furgason *et al.* 2009). Lastly fusion is able to occur via SNARE bundling and the formation of the *trans*-SNARE complex.

Vps53 and Vps54 GARP subunits also interacted with Syntaxin6, Syntaxin16 and VAMP4 (Perez-Victoria *et al.* 2010).

Another multisubunit tether that has been implicated in endosome to TGN fusion is the COG complex. This complex is thought to serve a similar role to the GARP complex, bridging the incoming vesicle SNARE with the TGN target SNAREs to form the four helical bundles. It was found that COG6, of the COG complex, directly interacted with Syntaxin6. This interaction is specific to the N-terminal coil–coil domain of COG6 and the SNARE domain of Stx6. Additionally, cells depleted in COG6 showed failure of Stx6 to localize to the TGN and had a reduction in the Stx6–Stx16–Vtia–VAMP4 complex. These results lead to the speculation that Cog6 works to stabilize Stx6 interactions with the other three SNAREs (Laufman *et al.* 2011). With all these tethers playing a role, a question that comes to mind is whether specific cargos prefer specific tethers or is specificity not as important as long as the desired destination is still reached.

3.3 *Fusion*

Once tethering is complete, the next step in fusion is the formation of the four helical bundles. However, before this bundle can be formed, one critical SNARE, Tlg2 in yeast or Syntaxin16 in mammalian cells interacts with the SM protein Vps45. This interaction is thought to activate Tlg2/Syntaxin16 for SNARE binding (Struthers *et al.* 2009). As shown in figure 3, Vps45 binds to a C-terminal region on Tlg2 when Tlg2 is in a closed conformation. Additionally, Vps45 is able to bind to the N-peptide of Tlg2 when Tlg2 is in an open conformation. Both of these conformations of Tlg2 compete for binding of Vps45, indicating that this could serve as a potential regulatory mechanism for fusion, but further exploration needs to be performed to confirm this. (Furgason *et al.* 2009). In a similar manner, Vps45 is able to bind to Syntaxin16 (Burkhardt *et al.* 2008). This type of SM protein to Syntaxin interaction has been proposed to facilitate binding between the Syntaxin SNARE and the other three SNAREs; however, exactly how this interaction helps to form the SNARE bundle is still unknown (Carpp *et al.* 2006). Once the three *t*-SNAREs on the TGN and the one *v*-SNARE on the incoming vesicle come in contact they form the *trans*-SNARE complex or SNARE pin and fusion of the two vesicles can proceed (figure 3).

4. Homotypic fusion

Homotypic fusion involves the joining of two of the same type of membranes. There are various models that can be thought of, endosome to endosome fusion, Golgi to Golgi fusion, vacuole to vacuole fusion and so on. In here, I will focus on vacuole to vacuole fusion, one of the most widely studied types of homotypic fusion. In order for vacuolar fusion to occur, three main steps need to be achieved first: priming, docking and fusion. Each step is governed by specific sets of proteins (Seeley *et al.* 2002).

4.1 *Priming*

In priming (figure 4), Sec18p/NSF and Sec17/αSNAP work together to dissociate the *cis*-SNARE complex that was formed during the previous fusion reaction. After the fusion step, the SNAREs are held together in a *cis*-conformation by Sec17. Sec18, one of the most important players of priming, detaches itself from the vacuolar membrane and transfers itself on to Sec17 where Sec18 works to sequester Sec17 from the SNARE bundle, thereby releasing the SNAREs for the next fusion activity (Ungermann *et al.* 1998; Starr *et al.* 2016).

Taking a closer look, evidence suggests that ATP bound Sec18 resides on the vacuolar membrane by attaching itself to the phosphomonoester head group of phosphatidic acid (PA) where it also serves as a membrane receptor for LMA1 (Xu *et al.* 1998; Starr *et al.* 2016). Meanwhile, Sec17 resides on the *cis*-SNARE complex. The mammalian Sec17 α-SNAP is known to utilize its N-terminal 63 and C-terminal 37 residues for proper SNARE binding (Hohl *et al.* 1998). Next, in the budding yeast it was shown that the PA phosphatase, Pah1, helps in the conversion of PA to diacylglycerol (figure 4). When this conversion occurs, Sec18 is released from the vacuolar membrane and is able to indirectly bind the *cis*-SNARE complex by using its N-terminal domain to bind Sec17/α-SNAP (Hohl *et al.* 1998; Starr *et al.* 2016). In mammalian cells, this interaction is mediated via N-(1–160) and C-(160–295) amino acid residues on α-SNAP that interact with NSF. However, it is the C-terminus of α-SNAP that has been shown to be important for triggering ATPase activity of NSF required for the release of α-SNAP from the SNAREs and the subsequent disassembly of Vam7 and Nyv1 (Barnard *et al.* 1997; Boeddinghaus *et al.* 2002). Interestingly, Vam7 is released to the cytoplasm since it has been shown that Vam7 shuttles between the cytoplasm, when it is not needed for fusion and the vacuolar membrane (figure 4). The distinctive Vam7's mobility is attributed to a lack of a transmembrane domain, which is replaced by a PX domain that requires PtdIns for proper vacuolar binding. Unlike Vam7 and Nyv1, Vam3 and Vti1 release have not been shown to be dependent on Sec18/17 ATP hydrolysis (Boeddinghaus *et al.* 2002). Additionally, LMA1 is released from Sec18 and is transferred to the free Vam3 (figure 4). It is believed that LMA1 binding to Vam3 helps in stabilizing Vam3 and the vacuolar membrane until the next fusion cycle (Xu *et al.* 1998).

4.2 *Docking/tethering*

Once the *cis*-SNARE complex has been dissolved, the individual SNAREs are ready for another round of fusion.

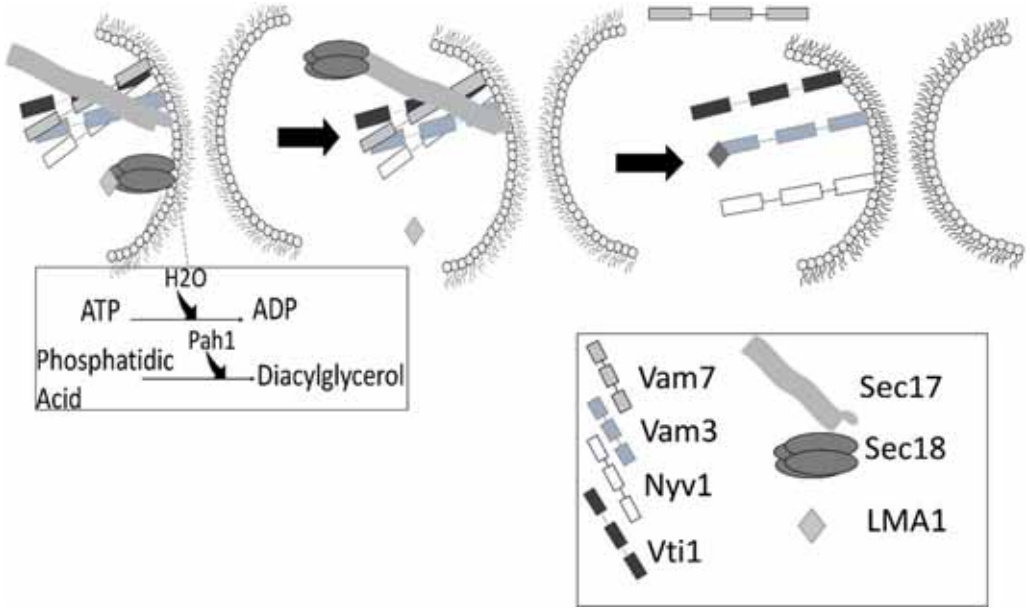

Figure 4. Vacuolar priming is the first step in fusion. At this point, the SNAREs are in the *cis*-conformation meaning that they are bundled up on one single membrane. They need to be separated in order to undergo the next fusion cycle (Ungermann *et al.* 1998; Starr *et al.* 2016). For this to occur, Sec18, located on the vacuolar membrane is dissociated when there is a change in lipid composition. That is PA is converted to diacylglycerol via the help of the Pah1 protein (Hohl *et al.* 1998; Starr *et al.* 2016). As Sec18 is released, it is able to attach itself on to Sec17 and LMA1 is released (Hohl *et al.* 1998; Xu *et al.* 1998; Starr *et al.* 2016). By way of ATP hydrolysis, Sec17 is able to dissociate itself from the SNARE bundle and the SNAREs are able to separate. As this separation occurs, Vam7 is released on to the cytoplasm for later recruitment and LMA1 protein is able to attach itself on to Vam3 in order to stabilize it (Xu *et al.* 1998; Cheever *et al.* 2001; Kramer and Ungermann 2011).

This next fusion cycle first requires docking via small GTPases and the MTC, HOPS, along with the help of certain vacuole lipids (Hickey and Wickner 2010). Not only is HOPS the main tether during vacuolar fusion, but it also functions to aid in fusion by binding to SNAREs and by inhibiting *trans*-SNARE complex disassembly mediated by Sec17 and Sec18 (Xu *et al.* 2010).

HOPS can perform its tethering activity by interacting with Ypt7, a yeast Rab protein, and by binding to vacuolar lipids (Hickey and Wickner 2010). When interacting with Ypt7, HOPS is able to bring together opposing vacuolar membranes. Taking a closer look, HOPS acts as a sort of bridge where it uses its Vps39 subunit to activate and bind Ypt7 on donor membranes while the Vps41 subunit, located on the opposite end of the complex, interacts with Ypt7 on target membranes (figure 5) (Stroupe *et al.* 2006; Auffarth *et al.* 2014; Ho and Stroupe 2016). In this scenario, the GEF, Mon1–Ccz1, is the most upstream factor driving docking and then fusion. It works by converting Ypt7 from its GDP bound form to its active GTP form (Langemeyer *et al.* 2018). Also, the HOPS complex is phosphorylated on its Vps41 subunit by the vacuolar membrane-localized kinase, Yck3p. Once phosphorylated, the Vps41 subunit of HOPS can bind to the GTP bound form of Ypt7 (figure 5) (Cabrera *et al.* 2010; Zick and Wickner 2012; Ho and Stroupe 2016). Additionally, HOPS has been shown to have an affinity to binding to phosphorylated phosphoinositides such as PI4P, PI-3,5-bisphosphate ($PI(3,5)P_2$) and PI-4,5-bisphosphate ($PI(4,5)P_2$) but does not show a strong interaction with nonphosphorylated phospholipids such as phosphatidylinositol (PI), phosphatidylserine or PA and acidic lipids. Further, it was the fully intact HOPS complex that displayed this interaction rather than individual HOPS subunits (Stroupe *et al.* 2006). Taken together, HOPSs' affinity for Ypt7 and vacuolar lipids, may help in promoting the enrichment of HOPS to the site of fusion allowing for multiple HOPS subunits to aid in the tethering activity (Stroupe *et al.* 2006) (figure 5).

4.3 *Fusion*

In addition to being necessary for docking, the HOPS complex is also capable of binding to SNAREs, proving to be a linking factor between the intermediate docking stage and the final fusion of SNAREs. Evidence suggests that HOPS can drive fusion via its three SNARE binding subunits: Vps18, Vps16 and Vps33. Specifically, Vps16 and Vps18 interact with the PX domain of Vam7, while Vps33 shows a positive interaction with the assembled SNARE complex (Kramer and Ungermann 2011). More recent research found that Vps33 of the HOPS complex was able to bind to the SNARE motif of Vam3 while simultaneously binding the SNARE motif of Nyv1 at a site outside the clef

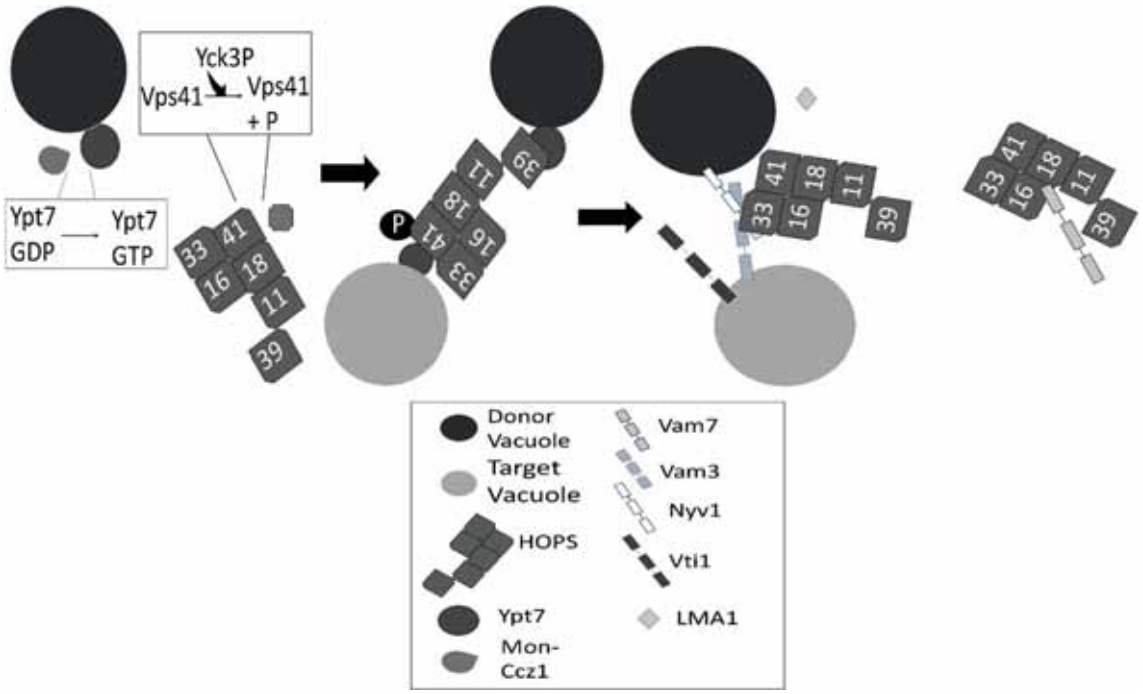

Figure 5. Vacuolar tethering for tethering, a few steps need to proceed. Ypt7 needs to be activated at the vacuolar membrane via the GEF, Mon-Ccz1 (Langemeyer *et al.* 2018). Also, Vps41 of the HOPS complex must be phosphorylated by the kinase Yck3p. This phosphorylation reaction allows Vps41 to interact with Ypt7 on the target vacuole while Vps39 of the hops complex is able to interact with Ypt7 on the approaching vacuole (Stroupe *et al.* 2006; Auffarth *et al.* 2014; Ho and Stroupe 2016). Additionally, HOPS is charged with the task of recruiting Vam7 from the cytoplasm to the site of fusion. This is done by Vam7 interacting with Vps16 and Vps18 of the HOPS complex (Cheever *et al.* 2001; Kramer and Ungermann 2011). Also, Vps33 of the HOPS complex is able to bind to Nyv1 and Vam3 to form what appears to be a half zippered SNARE bundle (Baker *et al.* 2015). When Vam3 interacts with Vps33, LMA1 is able to be dissociated from Vam3 (Ungermann *et al.* 1999; Muller *et al.* 2002).

of Vps33 (figure 5). This interaction leads to the formation of what looks like a half zippered SNARE (Baker *et al.* 2015). Upon this SNARE zippering, LMA1 is released from Vam3 via the help of Vtc (vacuolar transport protein). However, the precise mechanism is still obscure (Ungermann *et al.* 1999; Muller *et al.* 2002).

Further aiding in the idea that HOPS may be necessary to drive fusion, a new model is proposed where HOPS helps in recruiting Vam7 from the cytoplasm to the vacuolar fusion site by interacting with the PX domain of Vam7 (figure 5). Once Vam7 is at the vacuolar membrane, the PX domain of Vam7 can interact with PI3P (Cheever *et al.* 2001; Kramer and Ungermann 2011). However, this interaction is disrupted by Vam7's centrally located coil–coil domain known as the polybasic region (PBR) made up of Arg-164, Arg-168, Lys-172, Lys-175, Arg-179 and Lys-185. Experimental evidence shows that as the PX domain of Vam7 sits on vacuolar lipids preferentially binding to PI3P lipids, two lone acidic patches on this domain interact with the positively charged PBR domain of Vam7, suggesting a conformational change of Vam7 that needs to be further assessed. This interaction competes against the interaction between the PX domain and vacuolar lipids allowing Vam7 to free itself for the vacuolar membrane and interact with the acidic surface of the SNARE bundle in order to stabilize the complex. When the amino acids of the PBR domain were mutated to alanine, there was an increased Vam7 binding to the vacuolar membrane and a decrease in binding to its cognate SNARES (Miner *et al.* 2016). It has been shown that the N-terminal domain of Vam7 is also necessary to associate with the other two Q-SNAREs, Vam3 and Vti1, to aid in the formation in the 3Q-SNARE subcomplex as well as allow for interaction of the R-SNARE, Nyv1 to form the *trans*-SNARE complex (Xu and Wickner 2012).

Once the SNARE complex is in its *trans* form, Vps33 of HOPS aids in protecting it from premature Sec17 and Sec18-dependent disassembly via its 3a distal domain. It is proposed that this occurs only when Vps33 is able to bind to SNAREs as well as opposed vacuolar membranes. This is a way to discriminate between the *cis*- and *trans*-conformation of SNAREs and prevent the protection of the *cis* form (Xu *et al.* 2010; Baker *et al.* 2015) (figure 6). At the trans-conformation, zippering occurs and SNARE complexes are able to facilitate the transmission of energy to the vacuolar membrane for fusion (Zorman *et al.* 2014; Miner *et al.* 2016). For optimal membrane fusion, it has been shown that Sec17 may play on last role other than disassembly. By binding its N-terminally located apolar loop into the lipid membrane, Sec17 can

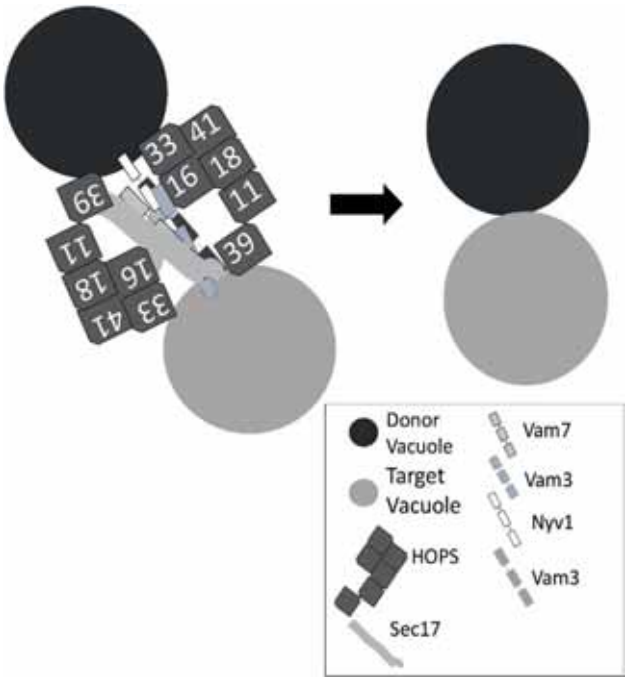

Figure 6. Vacuolar fusion just prior to fusion, as the SNAREs are interacting in a SNARE bundle, HOPS plays a role by maintaining the SNARE formation so that Sec17 and Sec18 do not act prematurely to dissociate the forming *trans*-SNARE complex (Xu *et al.* 2010; Baker *et al.* 2015). Once the SNARE complex has been formed, Sec17 has been shown to interact with the SNAREs as well as promote lipid rearrangement in order to aid in fusion (Song *et al.* 2017).

produce lipid rearrangement for fusion to fully occur as well as bind to the zippered *trans*-SNARE complex in order to stabilize it (Song *et al.* 2017) (figure 6).

In a similar manner to LMA1, another protein has been known to interact with Vam3 in vacuole to vacuole fusion, which is Vps1, the yeast homologue to mammalian dynamin, which has long been regarded as a fission protein. However, recent scientific research has pointed that Vps1 could be acting as a fusion protein as well. This is a confounding concept since it is hard to understand how one protein can do two completely contradictory jobs. In 2014, researchers found that Vps1 had the capability to control vacuolar membrane fusion by generating a tethering complex between the different SNAREs. Vps1 is able to do this by facilitating the interaction of Vam3, a Qa SNARE, with the HOPS complex. Specifically, Vps1 interacts with the SNARE domain of Vam3. However, there seems to be a threshold of how much Vps1 is helpful in forming the SNARE complex. It was shown that too much Vps1 decreases the amount of SNARE complex formation. This leads to the speculation that increasing quantities of Vps1 could sequester Vam3 from the SNARE complex by keeping it attached to the HOPS complex. This hints at the proposed mechanism of Vps1 functioning as a fusion protein: first Vps1 binds to the SNARE domain of Vam3 and sequesters it to the HOPS complex, then, in order to get Vam3 to form part of the SNARE complex, Vps1 must disassociate. This poses the idea that Vps1 could be a primer for SNARE complex formation (Alpadi *et al.* 2013).

Not only is Vps1 a prerequisite for SNARE complex formation, but also it is crucial for fusion to completely proceed. This indicates that Vps1 is necessary for the transition that takes vesicular and target SNAREs from the hemi fusion state all the way up to content mixing of two individual vacuoles. Furthermore, it is the polymerized form of Vps1 that is needed for content mixing to occur. This polymerized form of Vps1 is able to recruit Vam3 to the fusion site and provide the high local concentrations of t-SNAREs (Peters *et al.* 2004; Kulkarni *et al.* 2014). This recruitment of Vam3 is necessary to coordinate the *trans*-SNARE complex. When Vps1 was mutated at the K642, I649 and Y628 amino acid residues, its polymerization was inhibited. Although these mutations still allowed some *trans*-SNARE formation, it still resulted in a drastic reduction in lipid and content mixing. Efforts to salvage the phenotype were useless: no amount of Vam3 upregulation rescued fusion. Further, when research studies attempted to bypass Vps1 requirement, by artificially stimulating fusion through the addition of Vam7 and chlorpromazine, their efforts proved futile. These results demonstrate the high degree for which Vps1 polymerization is necessary for proper fusion (Kulkarni *et al.* 2014). Not only is Vps1 vital for vacuole fusion, but also other findings have implicated dynamins in the fusion steps throughout other parts of the cell. For example, the human homologue of Vps1, dynamin2, was shown to play a role in the fusion of lytic granules with the plasma membrane. It has also been suggested that the mechanochemical properties of dynamin and dynamin-related proteins play a role in exocytosis in order to form a fusion pore. This observation was seen in natural killer cells, which are a part of the innate immune system in humans. Specifically, dynamin2 mediates the ending steps of the fusion of these lytic granules with the plasma membrane (Arneson *et al.* 2008).

In addition to functioning alongside SNAREs to coordinate fusion, Vps1 has also been shown to interact with other fusion components. A recent study revealed that Vps1 also interacts with the GARP tethering complex, which works in endosome to Golgi heterotypic fusion. The interaction between Vps1 and GARP was further narrowed down to be between the E127 and Y129 amino acid residues of Vps51 of the GARP complex with Vps1 (Saimani *et al.* 2017). These findings that Vps1 is found to function with tethers and SNAREs tell us that Vps1 could be doing more for the cell than just fission, as previously thought. More work needs to be carried out in order to completely classify Vps1 as a fission and fusion protein.

5. Concluding remarks

Although scientists have come a long way in understanding the inner workings of a cell, much remains to be explored and learned. A prime example being the current

understanding of fusion. What was once thought to be a job solely completed by SNAREs has now been described as much more complex. It is now recognized that fusion requires tethers and other accessory proteins in order to aid the SNARE proteins to carry through the full fusion process (D'Agostino *et al.* 2017). Nevertheless, there are still a few holes to be filled when it comes to understanding the exact mechanism of action. So far, there is a vague idea of how all these proteins work together, and with this, I have developed a basic skeletal framework of how I hypothesize these proteins to interact in heterotypic and homotypic fusion scenarios. As research continues, these models can be further developed to become more accurate. Particular interest should be put on understanding how different tethers work together. As explained in the heterotypic section, the TMF Golgin and COG complex have been shown to be cooperating to tether incoming vesicles. In a similar manner, other tethers could be working together, but this needs to be further explored. With regard to the Golgins, it was seen that at least one of them, GCC185, had a hinge that aided in its flexibility. Is it that other Golgins also contain this flexibility, and further, do other multi-complex tethers also contain a similar elasticity? It seems that such a capacity would certainly aid in their ability to tether incoming vesicles. Also, pertaining to the Golgins, more research should be done on the Rab binding sites located along their lengths. Do different cargo laden vesicles compete for binding along these binding sites, and does this aid in their promiscuity? If so, is promiscuity a rule of thumb when it comes to Golgins, or are their certain Golgins or certain scenarios where Golgins are faithful to a certain type of vesicle/cargo? Additionally, when talking about cargo, an emphasis should be placed on how different cargos dictate the route and mode of fusion. As scientists focus more on these questions, it will not be surprising to find further proteins aiding in fusion; perhaps serving as linking factors between the already known protein interactions. Almost anything is possible in this complex world of cell trafficking and fusion that remains to be seen.

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