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## Multifaceted Mechanism of Vps1 Mediated Endosome-to-Golgi Fusion in Vitro

Ehsan Suez

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**MULTIFACETED MECHANISM OF VPS1 MEDIATED ENDOSOME-TO-GOLGI  
FUSION *IN VITRO***

A Master's Thesis

Presented to

The Graduate College of  
Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Ehsan Suez

August 2020

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# **MULTIFACETED MECHANISM OF VPS1 MEDIATED ENDOSOME-TO-GOLGI FUSION *IN VITRO***

Biology

Missouri State University, August 2020

Master of Science

Ehsan Suez

## **ABSTRACT**

To maintain cell homeostasis, protein recycling through intracellular membrane fusion is an important cellular process. Both Vps1 and Ypt6 have been implicated in protein recycling from the endosome to the *trans*-Golgi Network (TGN). SNARE proteins are thought to be the key regulator in this membrane fusion mediated protein recycling mechanism. I studied membrane fusion events by incorporating purified proteins into liposomes. A series of data suggest that high concentration of SNARE proteins inhibits fusion unlike the opposite popular notion. Also, the data suggests that Vps1 acts on membrane fusion dynamics in a manner that lower concentrations of Vps1 enhance fusion in compared to its higher concentration counterpart. Moreover, it was found that Vps1's GTPase activity seems to be inadequate to enhance fusion. Finally, the data suggests that Vps1 and Ypt6 do not act in a synergistic mode despite both of them possessing GTPase activity.

**KEYWORDS:** membrane fusion, Vps1, Ypt6, SNARE, reconstitution assay, GTPase activity

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A Master's Thesis  
Submitted to the Graduate College  
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August 2020

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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I dedicate this thesis to my mother.

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## INTRODUCTION

Membrane fusion is a biophysical reaction that is of fundamental importance in biological systems for diverse processes such as organelle biogenesis, neurotransmission, and hormone secretion, as well as various post-endocytic and exocytic pathways. There are different types of membrane fusion, and these processes result from a series of coordinated cellular activities. Improper membrane fusion can lead to human diseases such as Alzheimer's or neutropenia. A previous study showed that defects in the intracellular trafficking from the endosome to *trans*-Golgi Network (TGN) have a positive correlation with Alzheimer's disease. Biallelic mutations in certain fusogenic protein cause defective endosomal intracellular protein trafficking, which leads to congenital neutrophil defect syndrome <sup>1</sup>. Together, these have made it important to study the underlying mechanism of membrane fusion and its regulators.

### **Regulators of SNAREs and SNARE Mediated Fusion**

Fusion of membranous organelles play a key role in eukaryotes life <sup>2</sup>. Protein factors that regulate vesicular/membrane trafficking pathways can be grouped into four major categories: SNAREs ("SNAP REceptor"), NSF (N-ethylmaleimide sensitive factor) and SNAP (Soluble NSF attachment protein)AP, Rab GTPase and tethering complex, and SM proteins <sup>3</sup>. SNARE (Soluble NSF Attachment protein Receptor) are the central component to the fusion of endomembranes <sup>4-6</sup>. SNARE proteins are membrane associated proteins containing a SNARE motif that stretches ~ 60 amino acids in length with heptad repeats. SNAREs are thought to be fundamental fusion factors for two reasons: their subcellular localization at donor (or vesicle) and target membranes and their ability to form extremely stable, SDS-resistant complexes that

bring adjacent membranes in close apposition to promote fusion of them <sup>7</sup>. SNAREs are either vesicle localized ( $\nu$ -SNARE) or target-membrane bound ( $t$ -SNARE) <sup>8</sup>. To promote membrane fusion, these  $\nu$ - and  $t$ -SNAREs form a four-helix bundle referred to as “trans-SNARE complex” or “SNAREpins” <sup>9,10</sup>.

### **SNARE Interaction with Other Fusion Factors**

Several studies suggested that SNAREs are sufficient to be the minimal membrane fusion machinery <sup>9,11</sup>, whereas recent studies suggested some additional factors, including SM proteins, synaptotagmins, and complexins that influence the minimal fusion machinery <sup>2</sup>. SM proteins harbor a conserved ~600 amino acid sequence folding into an arch-shaped “clasp” structure <sup>12</sup>. As such, SM proteins work as a clasp to bind both  $\nu$ -SNARE and  $t$ -SNARE components of zipper SNARE complexes spatially and temporally <sup>2,3</sup>. One of the best known SM proteins for membrane fusion is Vps45p functioning for membrane fusion at Golgi <sup>13</sup>. Synaptotagmins act as a  $\text{Ca}^{2+}$  sensor in the fusion process <sup>14</sup>. Reduced fusion activity was observed in mice having expressed synaptotagmin whose  $\text{Ca}^{2+}$  binding affinity was reduced <sup>15,16</sup>. Complexins are proteins that can bind to SNARE strongly <sup>17,18</sup> and activate SNARE complex to pave the way for the synaptotagmin action, which then triggers fusion <sup>2</sup>.

As stated above, the formation of SNARE complex is influenced by their interaction with SM proteins like Vps45p <sup>19</sup>. Myelofibrosis, thrombasthenia, and neutropenia are well-known diseases linked with mutation of Vps45p <sup>20</sup>. Vps45p also regulates cellular levels of SNAREs, including Tlg2p and Snc2p, and thus maintains membrane trafficking through endosomal system. A study has shown that the C-terminal half of the Snc2p motif (53-88) is the key player in mediating the interaction with Vps45p <sup>21</sup>. Tlg2  $t$ -SNARE is 396 amino acids long, exhibiting

a domain structure typical of syntaxins <sup>22</sup>, and it mediates membrane fusion at the Golgi together with its partners, including 2 *t*-SNAREs, Tlg1 and Vti1, and Snc2 *v*-SNARE to form a functional SNARE complex <sup>20,23–25</sup>. All these 4 SNAREs are implicated in mediating fusion of endosome-derived vesicles with late Golgi <sup>24,26,27</sup>. Among these, the above-stated three *t*-SNAREs, Tlg1, Tlg2, and Vti1, are associated with the late Golgi, and the fourth SNARE Snc2, a *v*-SNARE, is localized on the donor membrane or endosome-derived vesicle. <sup>21</sup>.

### **Summary of Vps1 Function in The Endosome to Golgi Traffic**

While Dynamins play a central role in endocytic process in mammalian cells, Dynamin Like Proteins (DLP) play a marginal endocytic role in the budding yeast, *Saccharomyces cerevisiae* <sup>28</sup>. Rather, these yeast DLPs, including Vps1, are mainly implicated in the post-endocytic targeting to the vacuole and the late endosome-to-Golgi trafficking <sup>29</sup>. In addition, Vps1 functions for transportation of Carboxypeptidase Y (CPY) from the Golgi toward the endosome: loss of Vps1 leads to missorting at the Golgi and mistargeting of CPY to outside of the cell <sup>30</sup>. The exact mode of action of Vps1 at the Golgi for supporting CPY traffic has been elusive, but it has been proposed that Vps1 might be playing a role in the scission process to release Golgi-derived vesicles carrying CPY. Furthermore, it was shown that loss of Vps1 results in mislocalization of essential protein factors, including clathrin, for the Golgi-to-endosome traffic of CPY, manifesting an addition role of Vps1 in recruitment of clathrin to the exit site of late Golgi (Goud Gadila et al., 2017). However, Goud Gadila et al (2017) found that the loss of Vps1 has no impacts on Golgi recruitment of the adaptor GGA1, one of two distinct types of clathrin adaptors along with the clathrin adaptor AP-1 <sup>33</sup>. Two more essential factors required for the Golgi-to-endosome delivery of CPY are Vps34, a Golgi-associated lipid kinase, and Vps15, a

serine/threonine protein kinase. In particular, Vps34-Vps15 heterodimers have been proposed to directly bind to Vps10-CPY complex to facilitate CPY sorting into the clathrin-coated vesicle at the late Golgi <sup>34</sup>.

Studies discovered that CPY binds to its receptor Vps10 at the Golgi, and the Golgi-derived vesicle carrying the CPY-Vps10 complex travels towards and unloads the cargo at the late endosome/prevacuolar compartment <sup>35,36</sup>. In the late endosome, CPY and Vps10 are separated from each other, and then Vps10 is recycled back to the Golgi. CPY-laden late endosomes fuse with the vacuole to unload CPY in the lumen of the vacuole <sup>34-36</sup>. In cells lacking Vps1, accumulation of Vps10 occurs both at the late endosome and vacuolar membrane due to a retrieval defect from both organelles <sup>37</sup>. This reflects a similar scission role of Vps1 at the exiting site of these organelles, indicating Vps1 being a bona fide molecular scissors for the endosome-to-Golgi traffic <sup>37</sup>. This finding is consistent with the notion that classical dynamin is implicated in function for the retrograde traffic from the late endosome to the TGN <sup>38,39</sup>.

It is also believed that Vps1 helps recycling Snc1, a SNARE protein, from early endosomes to the late Golgi by exerting its scission activity at the endosome. To support this notion, a recent study using Vps1 mutants lacking their intrinsic GTPase function found that cells expressing these mutant variants of Vps1 displayed enlarged cytoplasmic GFP-Snc1 puncta instead of being localized at the growing bud of yeast cells <sup>40,41</sup>, manifesting a defect of Snc1 retrieval from or a defect of scission of vesicles carrying the cargo Snc1 at the endosome. Along with the suggested scission role, Vps1 appears to play a role in recruitment of molecular components for proper fusion at the Golgi. In particular, two key proteins required for the fusion event, including Vps51 and Tlg1, were found to be mislocalized in the absence of Vps1. The

former is a component of the GARP complex essential for early step of the fusion and the latter is a *t*-SNARE at the Golgi <sup>40</sup>.

### **Structural Significances Between Vps1 and Human Dynamin**

Dynamin primarily concentrates at the endosomal compartment and also has a widely accepted function to tighten the elongated vesicle neck, leading to vesicle fission by constricting the collar created by dynamin around the neck of the vesicle <sup>42</sup>. It is proposed that dynamin's endosomal function depends on both its intrinsic ability of self-assembly and GTP hydrolysis <sup>43</sup>. For the latter, dynamin GTPase domain locates to its N-terminus, and by hydrolyzing GTP into GDP, it helps driving membrane remodeling <sup>44</sup>. This domain is connected to a stalk domain that is involved in dynamin self-assembly, which leads to form a dynamin dimer that is targeted to the neck of the emerging vesicle <sup>45</sup>. The precise function of recruited dynamin is a subject of controversy. While the two-stage model proposes that dynamin assembly itself triggers membrane constriction, the constrictase/ratchet model proposes that not every dynamin monomer contributes equally to membrane constriction <sup>46</sup>. Nevertheless, both models support the notion that a constriction of the neck of an emerging vesicle is caused by the GTPase activity of dynamin <sup>47,48</sup>.

Vps1 is a dynamin-related protein (DRP) and shows sequence homology with human dynamin 1 by containing a catalytic GTPase domain, a three-helix bundle termed the bundle signaling element (BSE), and a helical stalk formed by three helices from the Middle domain and one from the GTPase Effector Domain <sup>49</sup>. While Vps1 lacks both a PH and a PRD (Proline Rich Domain) compared to human dynamin, Vps1 and other fungal DRPs carry two distinctive domains absent in dynamin: Insert A and Insert B. Another difference between Vps1 and

dynamamin 1 is the robust stimulation of dynamamin GTPase activity observed in the presence of liposomes, whereas stimulation of Vps1's GTPase activity by liposomes is minimal. It was proposed that the dynamamin PH domain is critical for regulation and facilitation of lipid stimulated GTP hydrolysis<sup>43</sup>. Vps1 lacks the PH domain, which may account for the basal level of GTPase activity. Yet, Vps1 is capable of binding phospholipid bilayers via its Insert B domain.

A pseudoatomic modeling identified a novel, biologically significant interface named “ $\alpha$ B interface” that forms during Vps1 helical self-assembly. The formation and stability of this interface may be affected by close approach of nearby Insert A from adjacent GTPase domain. Thus, GTPase domain interactions of Vps1 was also shown to be significantly important for Vps1's endosomal function<sup>43</sup>.

### **Small GTPase Mediated Membrane Trafficking**

Rab GTPases are associated with most organelles in the cell and serve as binding platforms for the unique localization of protein machinery for membrane trafficking<sup>50-54</sup>. Rabs are a subfamily of the Ras superfamily of small GTPases, and they are highly conserved throughout the eukaryotic kingdom. Rabs can switch between an active GTP-bound state (Rab-GTP) and an inactive GDP-bound form (Rab-GDP). Guanine nucleotide exchange factor (GEF) is required to transform Rabs into an active form, while GTPase activating protein (GAP) is required to transform back to an inactivated form<sup>55</sup>. Specially, Rab GTPases like Rab5 and Rab7 work as organelle markers and have functions in endosome and lysosome biogenesis<sup>56</sup>. Rab6 was suggested to be the main regulator of the Endosome-to-Golgi trafficking<sup>57</sup>. A previous study showed that depletion of GTP-bound Ypt6, the yeast homolog of the mammalian Rab6, leads to a reduction of the GARP complex recruitment to the TGN. It was also proposed that the

interaction between Ypt6 and Vps52 might facilitate a triggering effect on the assembly of GARP complex to the tethering site at TGN<sup>58</sup>

### **Principles of Lipid Mixing and Content Mixing**

Membrane fusion can either be hemi-fused or completely fused. In hemi-fusion state, two membranes mix lipids but don't mix their luminal contents, while complete fusion involves both inner and outer leaflets of membranes to form a pore that will allow luminal contents to be mixed. *In vitro* membrane fusion reconstitution assays are very helpful for studying this fusion process and can be either done by ensemble approach or single-particle approach<sup>59</sup>, both of which are FRET based methods.

Förster resonance energy transfer (FRET) is a process by which an initially electronically excited fluorophore (the Donor) transfers its excitation energy to another fluorophore (the Acceptor). By using Struck method<sup>60</sup> that used this FRET mechanism, Rothman showed that neuronal SNARE proteins are fusogenic<sup>9</sup>. He used two different class of vesicles: one vesicle with *v*-SNAREs (synaptobrevin/VAMP) containing quenched mixture of NBD-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-7-nitro-2-1,3-benzoxadiazol-4-yl) and Liss Rhod-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-lissamine rhodamine B sulfonyl) while other vesicle with *t*-SNAREs (syntaxin and SNAP-25) alone. When both fused, they exchanged lipid, leading to dequenching and increased reading of NBD-PE fluorescence<sup>9</sup>. The drawback of this method is its inability to differentiate between hemi-fusion and complete fusion since SNARE-mediated lipid mixing can occur without sufficient content mixing<sup>61,62</sup>.

Ensemble based content mixing assays have shown different problems including potential leakiness of proteoliposomes, aggregation, and vesicle rupture that may plague ensemble



experiments<sup>62</sup>. To cope with this downside of the FRET-based ensemble mixing assays, a single-particle assay was developed using both lipid mixing and content mixing indicators, which helps define the precise stage of fusion between docking, hemi-fusion and complete fusion<sup>62–64</sup>. In this simultaneous single-particle assay, vesicles containing *t*-SNAREs (syntaxin and SNAP-25) are immobilized and subsequently tethered to surface through neutravidin-biotin linkage. Vesicles containing *v*-SNAREs (synaptobrevin and synaptotagmin) contain Sulforhodamine B as a content marker and lipid dye DiD as a lipid marker. Incubation can be started with the condition that lacks  $\text{Ca}^{2+}$ , then followed by injection of  $\text{Ca}^{2+}$  to measure effect of  $\text{Ca}^{2+}$  in mixing. Fluorescence reading (red for lipid mix and green for content mix) allowed simultaneous reading of both lipid mixing and content mixing<sup>59</sup>. Further improvements have recently been made to use content proteins that are linked to fluorophores such as Cy5 and PhycoE via streptavidin or biotin. An increase of FRET between Cy5 and PhycoE is measured to quantify the degree of content mixing. The simultaneous assay can relate lipid and content mixing rates on top of quantifying the percent of monitored fusion whether it is true or not (leaky fusion). This simultaneous mixing assay has become more popular for analyzing the multiple stages of the fusion reaction and interpreting those results more accurately compared to either lipid mixing or content mixing alone. But due to numerous advantages, lipid mixing assays are still considered the standard and more frequently used.

### **Problem Statement and Hypothesis**

Vps1 has traditionally been proposed as a membrane fission protein that works as a scission factor, but its role in homotypic fusion of vacuolar membranes has only been minimally discussed<sup>65–67</sup>. Mammalian Rab6 homologue Ypt6 is shown to regulate the endosome-to-Golgi

trafficking, in particular at the stage of endosome fusion at the TGN <sup>68</sup>. Interestingly, a previous study from our lab has presented evidence of physical interaction between Vps1 and Ypt6 <sup>46</sup>.

Given their interaction and their proposed function at the stage of membrane fusion, addressing the possibility that Vps1 functions synergistically with Ypt6 for membrane fusion would provide significant insights into the molecular mechanism behind the endosome-to-TGN trafficking.

To answer this, my thesis project involved using an *in vitro* lipid mixing assay in which reconstituted v-SNARE carrying proteoliposomes that mimics endosome-derived vesicles and t-SNARE proteoliposomes that mimics TGN are mixed in the presence or absence Vps1 to measure the extent of fusion. This *in vitro* membrane fusion reconstitution experiment will help define the potential collaborative nature of the dynamin-related protein Vps1 with Ypt6 the main regulation of cargo trafficking for the endosome-to-TGN protein. To this end, my study will focus on answering the following major scientific questions:

1. Does Vps1 play a role in fusion in a dose-dependent manner?
2. Do highly concentrated SNARE proteins drastically enhance membrane fusion?
3. Does the presence of GTP and Vps1 increase the rate of membrane fusion?
4. Does the interaction between Vps1 and Ypt6 affect fusion?

## MATERIALS AND METHODS

### Transformation, Expression and Purification of GST Tagged Protein

PCR-amplified *TLG2* sequence was inserted between the *Bam*H1 and the *Xho*1 site of pGEX-5X-2 (KKD 376). Restriction digestion of the plasmid with *Bam*H1 and *Xho*1 was conducted to confirm the insertion of *TLG2* gene. *TLG2* -containing pGEX-5x-2 plasmid (KKD 376) was then introduced into BL21 Rosetta (DE3) pLysS competent cells (Novagen), and positive transformants were selected from on LB + Ampicillin + Chloramphenicol plates. The resulting positive colonies with the cloned vector were inoculated into 3 mL starter cultures of Luria-Bertani medium (LB) with 4  $\mu$ L of 100 mg/mL Ampicillin and 6  $\mu$ L of 35 mg/mL Chloramphenicol at 37°C overnight with shaking (250 rpm). The overnight samples were diluted in a fresh 4 mL culture of LB medium (with Ampicillin and Chloramphenicol) the following morning and allowed to grow to an OD of ~0.1 measured at 600 nm. The culture was then incubated at 37°C and allowed to grow until it reaches OD of ~0.5 measured at 600 nm. Then the 4 mL cultures were then split into a 1 mL uninduced (UI) sample and a 3 mL induced (I) sample. The induced samples were inoculated with 1 mM Isopropyl  $\beta$ -D-1- thiogalactopyranoside (IPTG), and both UI and I samples were incubated on ice for 15 minutes with vigorous shaking every few minutes. After the ice incubation, both UI and I samples were tested for protein expression at varying temperatures of 22°C, 30°C, and 37°C (for 8 hrs, 6 hrs, and 3 hrs, respectively) shaking at 270 rpms. All samples were centrifuged at 3000 g at 4°C for 20-25 minutes, using a table-top centrifuge (Beckman J2-HS). The supernatant was removed by decanting, and the pellet was instantly flash frozen using liquid nitrogen and was stored immediately at -80°C.

Pellets from both UI and I samples were resuspended with 500  $\mu$ L 1X PBS (Phosphate Buffered Saline, pH 7.4). Then, 100  $\mu$ L of 5X SDS (Sodium Dodecyl Sulfate) lysis buffer was applied to both UI and I samples and vortexed until no visible clump remained. Branson 250 sonicator was then used for sonicating the sample for 30 seconds with output controls set to 2-3. The samples were then spun at 4°C for 10 minutes at 13,000 g using a microcentrifuge. Then, samples were boiled at 95°C for 5 minutes and were spun at maximum speed at 4°C for 1 minute. Twelve  $\mu$ L of the supernatant was collected after the centrifugation was done and applied to an SDS-PAGE. The gel apparatus was set at 100 volt and ran for an hour. The induced and uninduced samples were applied on adjacent wells of the gel for visual comparison of the existence of proteins. After an hour, SDS-PAGE gels were stained with Coomassie blue for 3-5 minutes and later, were de-stained overnight on a rocking machine at room temperature (Figure 1).

After the small-scale trials, it was found that, a 6 hours of induction at 30°C was optimal for expression of GST-Tlg2. With the same condition stated above, protein expression was induced GST-Tlg2 in a 500 mL culture at 30°C. After the induction period, the cell suspension was centrifuged at 3000 g (4500 rpm) in a JA-14 rotor for 30 minutes at 4°C. The pellet was then flash frozen and stored at -80°C as described earlier.

The bacterial pellet was resuspended with ice cold 30 mL of 1X PBS for 250 mL culture. Then, 150 U of 50 U/ $\mu$ L DNaseI was used per 1 gram of bacterial pellet. Fresh 200  $\mu$ L lysozyme was added per 1 gram of pellet. While vortexing gently on ice, 30  $\mu$ L of 1M PMSF (phenylmethylsulfonyl fluoride), 1.8  $\mu$ L of 1 mg/mL pepstatin A, and 1  $\mu$ L of 10  $\mu$ g/mL leupeptin were added in order. The lysed sample was transferred to 50 mL Beckman centrifuge tube for a sonication on ice for maximum of 60 seconds with output control set at 2-3. The

sonicated suspension was centrifuged in a Beckman J2-HS centrifuge. Rotor JA-20 was used at 15000 g for 25-30 minutes at 4°C. The supernatant was filtered through a 0.45 µm filter.

A GSTrap column (GE Healthcare) was used for a purification process. The column was equilibrated with 25-30 mL of binding buffer (1x PBS) prior to protein mixture application. The column was then washed with 25-30 mL of binding buffer (1x PBS). An elution buffer (30 mL of 50 mM Tris-HCl and 15 mM Glutathione, pH 8) was prepared and applied through the column. Eluted protein fractions were collected, and their protein content was checked with SDS gel electrophoresis. Fractions containing the protein of interest was subject to an overnight dialysis. The dialysis buffer was made of 10 mM Tris and 100 mM NaCl (pH 7.4). The next morning, 30 K MWCO Pierce protein concentrator was used to concentrate the protein. The purified, concentrated protein was saved in 20% glycerol.

### **Lipid Mixing Proteoliposome Reconstitutions**

The syringes were cleaned with 10 passes of acetone followed by 10 passes of chloroform before and after handling of phospholipids. For reconstitution of *t*-SNARE containing proteoliposomes, 200 µL of 15 mM premixed lipid solution in chloroform in a 10 x 75 mm glass tube was dried by a gentle stream of nitrogen and later, trace amount of chloroform was removed using vacuum for 30 minutes. The premixed lipid solution used in this step consists of DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and DOPS ((1,2-dioleoyl-sn-glycero-3-phospho-L-serine, sodium salt) in an 85:15 molar ratio. The dried lipid film was then dissolved by gentle agitation in room temperature for 15 minutes in 1 mL of reconstitution buffer (25 mM HEPES-KOH, 400 mM KCl, 10% Glycerol, 1 mM DTT, pH 7.4) containing 40 nM *t*-SNARE complex and 1% OG (octyl β-D-glucopyranoside). After that, additional 2 mL of reconstitution

buffer was added while vortexing vigorously to dilute the OG detergent. OG was then removed by dialysis in 4 liters of reconstitution buffer at room temperature containing 4g of SM2 beads (Bio-Rad) overnight in 4°C. Proteoliposomes were then recovered and concentrated by flotation in a Nycodenz rate zonal gradient. Total 3 mL dialysates were divided in half. Each 1.5 mL of dialysate was mixed with 1.5 mL of 80% (w/v) Nycodenz dissolved in reconstitution buffer and then was overlaid with a 750  $\mu$ L of 30% Nycodenz (w/v) in reconstitution buffer, followed by 250  $\mu$ L reconstitution buffer lacking glycerol. The samples were then centrifuged in a SW60 Beckman rotor at 55,000 rpm for 3 hours and 40 minutes at 4°C. Gradients used to prepare *t*-SNARE vesicles resulted in two closely spaced bands (opaque bands against a dark background). Proteoliposome vesicles were harvested from the from the top 250  $\mu$ L layer. Harvested proteoliposome vesicles were put together in a volume of total 450-500  $\mu$ L total.

For reconstitution of *v*-SNARE containing proteoliposomes, 200  $\mu$ L of 6 mM premixed lipid solution in chloroform in a 10 x 75 mm glass tube was dried by a gentle stream of nitrogen and later, trace amount of chloroform was removed using vacuum for 30 minutes. The premixed lipid solution used in this step contained DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DOPS ((1,2-dioleoyl-sn-glycero-3-phospho-L-serine, sodium salt), NBD-DPPE ((1,2-dioleoyl-snglycero-3-phosphoethanolamine-N-7-nitro-2-1,3-benzoxadiazol-4-yl)), and Lis-Rhodamine-DPPE (1,2- dioleoyl-sn-glycero-3-phosphoethanolamine -N-lissamine rhodamine B sulfonyl) in an 82:15:1.5:1.5 molar ratio. The resulting thin lipid film was dissolved in 400  $\mu$ L of reconstitution buffer (25 mM HEPES-KOH, 400 mM KCl, 10% Glycerol, 1 mM DTT, pH 7.4) containing 40 nM *v*-SNARE (Snc2p) and 1% OG. After that, 800  $\mu$ L of reconstitution buffer was added to the sample while vortexing vigorously to dilute the OG detergent. OG was then removed by dialysis in 1.5 liters of room temperature reconstitution buffer containing 1.5g of

SM2 beads (Bio-Rad) overnight in 4°C. Proteoliposomes were then recovered and concentrated by flotation in a Nycodenz rate zonal gradient. Total 1.2 mL dialysates were mixed with 1.2 mL of 80% (w/v) Nycodenz dissolved in reconstitution buffer and then was overlaid with a 750 µL of 30% Nycodenz (w/v) in reconstitution buffer, followed by 250 µL reconstitution buffer lacking glycerol. The samples were then centrifuged in a SW60 Beckman rotor at 48,000 rpm for 4 hours at 4°C. Gradients used to prepare *v*-SNARE vesicles results in two closely spaced bands (opaque bands against a dark background). Proteoliposome vesicles were harvested from the top 250 uL layer. A total of 100-150 µL of harvested proteoliposome vesicles was enough for the experiment.

### **Lipid Mixing Fusion Assay**

Black Costar 96-well FlouroNunc plates (Thermo Scientific) were used for the Lipid mixing fusion assay. Five µL of *v*-SNARE containing donor vesicles were applied in each well and were mixed with 45 µL of *t*-SNARE containing acceptor vesicles by gentle pipetting. Lipid concentrations and SNARE proteins concentrations are provided in table 1 and table 2. The 96-well plate containing the mixed liposomes was then preincubated at 4°C protected from light for 2 hours with gentle agitation. After incubation, 100 µM of GTP was added in designated well if required. 6xHN-Vps1 was added last at a concentration of either 1 nM, 10 nM, 100 nM, 500 nM, or 1000 nM. The samples were then incubated at 37°C with gentle agitation for 15 minutes and placed in the fluorescent plate reader. We used 460 nm as excitation wavelength and 538 nm as emission wavelength with 515 nm wavelength as cutoff filter. As more fusion occurs, an increase of NBD fluorescence was observed which was monitored by reading the plate every 2 minutes for 2.5 hours. A consistent temperature of 37°C and intermittent shaking in every 5 seconds were

maintained throughout the measurement. After that, the plate was removed from the reader. Ten  $\mu\text{L}$  of 1% Triton X-100 was applied in all of the wells at the same time using a multichannel pipette. The endpoint reading was taken. The resulting data was then normalized. The change of NBD signal over time was calculated and presented as the quantification of fusion. Based on that data, bar chart and line graph were prepared from each experiment.

### **Statistical Analysis**

Four experiments were done to test my four hypotheses and they were referred as like experiment 1 (for hypothesis one), experiment 2 (for hypothesis two) and so on. Experiment 1 and 3 were done in repeats of three. Then, the mean and standard deviation of the three-data sets were determined using Excel. Student's T-test (2 tails, two sample unequal variance) was performed using Excel and results were reported as p-values. The  $P\text{-value} \leq 0.05$  is represented with one asterisk (\*). One-way ANOVA was done for experiment 1 using Excel and results were reported as p-value. To get the % increasement of fusion rate in experiment one, normalized value at min three and minute thirty was obtained for each group and the rate changes were calculated, and average values were determined alongside respective standard deviation for each group.



## RESULTS

### Vps1 Affects Membrane Lipid Mixing

Tlg2, Tlg1, Vti1, and Snc2 are essential SNARE proteins to stimulate the retrograde membrane fusion of endosome-derived vesicles with the *trans*-Golgi (TGN). Purified recombinant SNARE proteins were quantitated with Bradford assay and validated with SDS-PAGE gel electrophoresis. To test if Vps1 augments SNARE-mediated proteoliposome fusion, a varying-concentrations of Vps1, ranging from 1 nM to 1000 nM, were added to the mixture of V-SNARE proteoliposome (Donor) and t-SNARE proteoliposome (Acceptor). While varying concentration of Vps1 didn't seem to stimulate lipid mixing overall, fusion rate change dropped when using Vps1 of 100 nM (16.24%) or 500 nM (13%) or 1000 nM (7.99%) compared to Vps1 of 1 nM (21.44%) or Vps1 of 10 nM (21.84%) (Figure 2). All experiments were performed in triplicate and repeated two or three times. Together, the results indicate that concentrations of Vps1 at 10 nM or lower stimulates SNARE-mediated fusion rates as opposed to lower level of fusion when treated with higher concentrations of Vps1.

### Too Much SNARE Doesn't Stimulate Fusion; Rather Inhibits

A previous study revealed that higher concentration of SNAREs further augmented *in vitro* membrane fusion<sup>2</sup>, and therefore, I aimed to test my hypothesis that a fusion assay with 2  $\mu$ M of *t*-SNAREs, 50 times higher concentration than that of experiment 1, and 6.6 nM of *v*-SNAREs, 3 times higher concentration than that of experiment 1, would significantly elevate lipid mixing. However, my experimental results showed the exact opposite result. Instead of drastic stimulation, the highly concentrated SNAREs in proteoliposomes have indeed inhibited fusion (Figure 3). This finding from my preliminary experiments suggest this inhibits fusion, but

more work is needed to confirm this. Furthermore, addition of 1  $\mu$ M Vps1 inhibited lipid mixing drastically, consistent with Figure 1 results.

### **Assessment if GTP Helps Vps1 to Enhance Lipid Mixing**

Vps1 binds to and hydrolyzes GTP to facilitate membrane remodeling activities such as membrane fission. In light of the finding that Vps1 is capable of stimulating SNARE-mediated membrane fusion rate (Figure 2), I hypothesized that Vps1 in the presence of GTP will induce increased levels of membrane fusion. Recently, Varlakhanova et al (2018) determined the  $K_m$  value of *C. thermophilum* Vps1 of 60  $\mu$ M. Therefore, 100  $\mu$ M of GTP was added to the mixture to test my hypothesis. However, the addition of GTP in a mixture with 10 nM Vps1 resulted in no noticeable increase in lipid mixing between the donor and acceptor membrane (Figure 4). On the other hand, the same amount of GTP with 500 nM Vps1 induced slightly elevated lipid mixing without statistical significance (Figure 4). This finding suggests that the observed effect of Vps1 on assisting membrane fusion does not require GTP hydrolysis at the dose used.

### **Combined Treatment of Vps1 and Ypt6 Doesn't Enhance SNARE-Mediated Lipid Mixing**

The yeast homologue of Rab6 is Ypt6, which was previously shown to interact with Vps1<sup>46</sup>. The present study assessed the hypothesis that the degree of lipid mixing will increase with the presence of both Vps1 and Ypt6. While, 1  $\mu$ M Vps1 alone seemed to stimulate the fusion the most, 1  $\mu$ M of Ypt6 alone didn't seem to largely enhance the fusion rate. Vps1 and Ypt6 were together added with 100  $\mu$ M of GTP and that showed even less fusion than Vps1 alone did. This study was done without replication and more experiments can be done to address the underlying reason behind that (Figure 5).

## DISCUSSION

Although SNARE proteins play a key role in regulating membrane fusion, their exact role in fusion and mode of action have been extensively debated. “Minimal machinery” hypothesis proposed by Weber <sup>9</sup> has been proven inadequate to explain the fusion mechanism. More recent models of membrane fusion have been using other proteins (such as SMs, tethers, Sec 17/ $\alpha$ -SNAP and/or synaptotagmin) and lipids that hold critical functions in the fusion reaction while incorporating basic SNARE pairing and zipper mechanism. Because these other proteins are thought to be just as essential as SNAREs to reconstitute true fusion mechanisms <sup>69</sup>, one of my research goals was to see if sufficient fusion can occur only by using highly concentrated SNARE proteins without using any of the helper proteins. Despite using highly concentrated SNARE proteins, fusion tends to decrease instead of increasing. It may be that a certain threshold of SNARE protein is needed for sufficient fusion events, and more than that level doesn't help fusion at all.

Another key component of intracellular trafficking is Vps1 that has shown both fission and fusion activities. What determines which of these modes will be activated is a question that needs to be addressed. One of my thesis project goals was to see if the concentration of Vps1 works as a regulating factor for that. We used different concentrations of Vps1 and found out that lower concentrations helped to stimulate fusion, but higher concentrations doesn't. One of the possible explanations for the observation is that a certain level of Vps1 is needed to gather enough SNARE proteins to cause sufficient fusion.

Vps1 is a known GTPase and is believed to break GTP and use that energy to create more fusion. So, addition of 100  $\mu$ M of GTP in a reconstitution assay is supposed to enhance fusion.

While there was no visible enhancement of fusion when used beside 10 nM of Vps1, a slight elevation was observed when used beside 500 nM of Vps1. So, apparently GTP doesn't seem to help fusion here. We speculate that incorporation of other helper proteins may help Vps1 to use GTP in a way that will enhance fusion to a greater extent.

Previous studies have shown that Vps1 locates at the TGN and its interaction with Ypt6 can affect membrane fusion dynamics *in vivo* <sup>46</sup>. We wanted to see if addition of Ypt6 alongside Vps1 in my reconstitution assay can significantly affect fusion or not. We found out that Vps1 and Ypt6 together don't help to enhance fusion *in vitro*. This supports the prior finding that Vps1 and Ypt6 don't work in a sequential pathway.

### **Concentration of Vps1 Affects the Efficiency of Fusion**

Previous studies have shown that both oligomerization and GTPase activity of Vps1 are crucial for binding the SNARE domain of Vam3 to increase the number of available *trans*-SNAREs for fusion and subsequent transition from hemifusion to content mixing in homotypic vacuolar fusion <sup>66,67</sup>. While a previous study from the Kim lab <sup>70</sup> shows that addition of GTP may stimulate fusion, we can't deny the possibility that other key factors like HOPS or GARP complex is necessary for a full fusion and content mixing. While mixing the proteoliposomes and Vps1 and/or GTP before reading the fluorescence, the final volume was kept constant unlike previous experiments in our lab that didn't keep the final volume constant. This led us to the conclusion that more fusion was observed when the Vps1 was lower. This raised the question whether the concentration of Vps1 is playing a key role here to determine the rate of the fusion between the liposomes. To address this question, we decided to use different concentration of Vps1 I found that, 10 nM of Vps1 has shown optimum fusion between the liposomes while

higher concentration of Vps1 shows decreasing fusion efficiency. This supports the very notion that concentration indeed plays a very crucial role to dictate fusion efficiency. I speculate that too much Vps1 may block the binding site for SNARE domains to interact. This concept is backed up by the finding that Vps1 is not only an essential part of the fission machinery, but also controls membrane fusion by regulating trans-SNARE formation <sup>66</sup>. According to this study, wild type Vps1 in its native polymeric state binds Vam3 and brings together multiple SNARE complexes satisfying or exceeding the local threshold of SNAREs necessary to promote trans-SNARE formation and hence becomes sufficient for complete fusion. In contrast, Vps1 mutants that can't assemble properly are not able to attain this threshold SNARE abundance for complete fusion. Vps1 in these polymerization-defective mutants is unable to gather sufficient Vam3 molecules for complete fusion and the process is stalled at the hemifused state (Kulkarni 2014). One of the hypotheses behind that is Vps1 polymers might organize hotspots of fusion containing multiple *t*-SNAREs. Release of Vam3 (*t*-SNARE) from Vps1 might provide *t*-SNAREs at high local concentration, stimulating efficient multiple *trans*-SNARE pairing and enhancing fusion. The other hypothesis is, displacement of Vps1 from Vam3 might coincide with a conformational change on Vam3 facilitating downstream interactions of Vam3-Vam3 and subsequent fusion. The interaction with GED domain of Vps1 with Vam3 would place Vam3 in an ideal position to regulate Vps1 depolymerization, which depends on the GED. This depolymerization of Vps1 should activate Vam3 and enhance fusion <sup>65</sup>. Another study has revealed that Vps1 binds to the SNARE domain of Vam3 and promote vacuolar fusion unlike mutated Vps1. To compensate for that, adding rVps1 actually didn't help. While rVps1 with a concentration of up to 0.6  $\mu$ M was added, 80% of the fusion activity was rescued. But when more concentrated rVps1 was used, then less fusion occurred. In fact, 6  $\mu$ M of rVps1 almost

completely inhibited the fusion process. Here, concentrated rVps1 inhibited trans-SNARE formation and subsequently inhibited the SNARE complex formation <sup>66</sup>. This is consistent with my experiment- derived results as my experiments also showed that after a certain level, increasing amounts of Vps1 inhibit fusion rather than promoting it.

### **Too Much SNAREs Inhibit Fusion Instead of Enhancing**

Several vacuolar fusion model studies used the lipid mixing assay and have shown significant lipid dequenching with SNAREs in relatively high concentrations. But very little fusion was detected when SNAREs alone were used without the addition of the tether HOPS for vacuolar fusion <sup>71</sup>. One of the studies has also shown that substantial lysis accompanies high SNARE densities. So, when we try to measure full content mixing, we might get fusion reading that occurs from lipid dequenching by lysis and reannealing <sup>72</sup>. This study also used high concentration SNARE proteins only without the addition of HOPS. This study showed that the use of only high concentration SNARE proteins doesn't enhance fusion and actually inhibits the fusion process. Perhaps, SNARE proteins need to reach a certain threshold level for an efficient fusion event. Also, addition of HOPS can help if too much SNARE proteins are involved.

### **Addition of GTP Doesn't Necessarily Affects Fusion Depending on Vps1 Concentration**

The catalytic GTPase domain of Dynamin is believed to hydrolyze GTP and uses the energy produced by the hydrolysis event to drive membrane remodeling <sup>44</sup>. Previous study from Kim lab has suggested that Vps1 might enhance fusion when GTP is added but the evidences were not unequivocal <sup>70</sup>. While one experiment showed that addition of GTP results in highest fusion efficiency, another experiment showed addition of non-hydrolyzable variant GTyP

provides highest fusion efficiency. This raised the question if GTP hydrolysis capability of Vps1 can regulate fusion efficiency or not. In this study, I used the same concentration of GTP as used in previous studies, but varying Vps1 concentration was used. No matter if the Vps1 concentration was lower or higher, significant fusion enhancement was not observed. This observation is consistent with the finding that stimulation of Vps1's GTPase activity by liposomes is minimal compared to robust stimulation of Dynamin<sup>43</sup>. The lack of a pH domain in Vps1, unlike its human counterpart dynamin, may be the reason why Vps1 can't make the most of the GTP to enhance fusion to a greater extent.

### **Functional Relationship Between Vps1 and Ypt6 on SNARE Mediated Endosome-to-TGN Lipid Mixing**

A previous study<sup>46</sup> has suggested that the interaction of Ypt6 and Vps1 does not depend on whether the Ypt6 is GTP-bound or GDP-bound, and that both Vps1 and Ypt6 act for Snc1 recycling as a GTPase. The loss of Vps1 or Ypt6 leads to a retrograde GFP-Snc1 trafficking defect toward the Golgi, and whether Vps1 and Ypt6 act in a sequential pathway or not was the question that needed to be answered. To answer the question whether Vps1 and Ypt6 function cooperatively with each other or not, Kim lab previously carried out experiments and tried to find out which one of these two proteins functions as an upstream or downstream factor for the other. Results from those experiments have indicated that both of these proteins function as a GTPase for Snc1 trafficking to the TGN and the GTPase activity of Vps1 may function redundantly with that of Ypt6. The next question was finding out the possible common downstream effectors of Vps1 and Ypt6 that might function in endosome-derived vesicle tethering/fusion at the TGN. The data from Makaraci<sup>46</sup> supports the notion that both Vps1 and

Ypt6 act redundantly on the GARP, but their specific binding patterns are different. Moreover, these experiments strengthened the notion that Vps1 functions upstream of the tethering and fusion or facilitates the downstream steps. Whether Vps1 and Ypt6 both simultaneously exerts their GTPase function in the presence of GTP was my major question. In my experiment, though both of the proteins together have shown to enhance fusion in the presence of GTP, it was less than the fusion mediated by Vps1 alone. This correlates with the previous findings from Kim lab that, Vps1 and Ypt6 don't work in a sequential pathway.

## **Limitations**

One of the most significant limitations of this study is that I have followed only a lipid mixing protocol, meaning my data might have only been resulted from hemifusion rather than full fusion. To get the actual data on full fusion, the use of a simultaneous lipid and content mixing protocol is necessary to distinguish the fusion efficiency from hemifusion and full fusion. Also, unlike membrane fusion assays developed by researchers<sup>66,67</sup>, the assays in this work did not incorporate tethering factors like HOPS that facilitates both lipid and content mixing. Also, addition of tethering factors such as GARP or PEG may help to facilitate lipid and/or content mixing.

## **Future Direction**

A simultaneous lipid and content mixing assay has to be done while incorporating Vps1. While some of my studies were done in replication, others were not done in replication. So, these future studies should be done (n=3) with three technical replicates for each sample condition being studied. As GARP complex has been shown to be a key regulator in the fusion process, more



research has to be done by incorporating Vps51. SM protein has also been known to play a role in membrane fusion dynamics. Therefore, preincubating the liposomes with purified Vps45 might provide new findings in the future.

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Table 1: Lipid concentrations used for lipid mixing assay

<b>Premixed lipids for Lipid Mixing Assay (6 mM)</b>				
<b>Lipid Stocks</b>	<b>Mol%</b>	<b><i>v</i>-SNARE lipids</b>	<b>Mol%</b>	<b><i>t</i>-SNARE lipids</b>
DOPC 18:1(25mg/ml)	82	30.94 $\mu$ L	85	80.18 $\mu$ L
DOPS 18:1 (10mg/ml)	15	14.58 $\mu$ L	15	36.45 $\mu$ L
NBD-PE 18:1 (1mg/ml)	1.5	16.63 $\mu$ L	-	-
Liss Rhod-PE (1mg/ml).	1.5	23.43 $\mu$ L	-	-
Chloroform (200 $\mu$ L total)	-	114.41 $\mu$ L	-	83.37 $\mu$ L

Table 2: SNARE protein concentrations for lipid mixing assay

<b><i>v</i>-SNARE Liposomes</b>				
<b><i>v</i>-SNAREs</b>	<b>Molar</b>	<b>Volumes</b>	<b>Molar</b>	<b>Volumes (for experiment 2 only)</b>
Snc2 (84 $\mu$ M)	2.2 nM	10 $\mu$ L	6.6 nM	30 $\mu$ L
Reconstitution Buffer w/ 1% $\beta$ OG	-	390 $\mu$ L	-	370 $\mu$ L
<b><i>t</i>-SNARE Liposomes</b>				
<b><i>t</i>-SNAREs</b>	<b>Molar</b>	<b>Volumes</b>	<b>Molar</b>	<b>Volumes (for experiment 2 only)</b>
Tlg2p (28.17 $\mu$ M)	40nM	1.5 $\mu$ L.	2 $\mu$ M	46 $\mu$ L
Tlg1p (16.98 $\mu$ M)	40nM	2.36 $\mu$ L	2 $\mu$ M	115 $\mu$ L
Vti1p (13.23 $\mu$ M)	40nM	3.02 $\mu$ L	2 $\mu$ M	146 $\mu$ L
Reconstitution Buffer w/ 1% $\beta$ OG	-	993 $\mu$ L		307 $\mu$ L

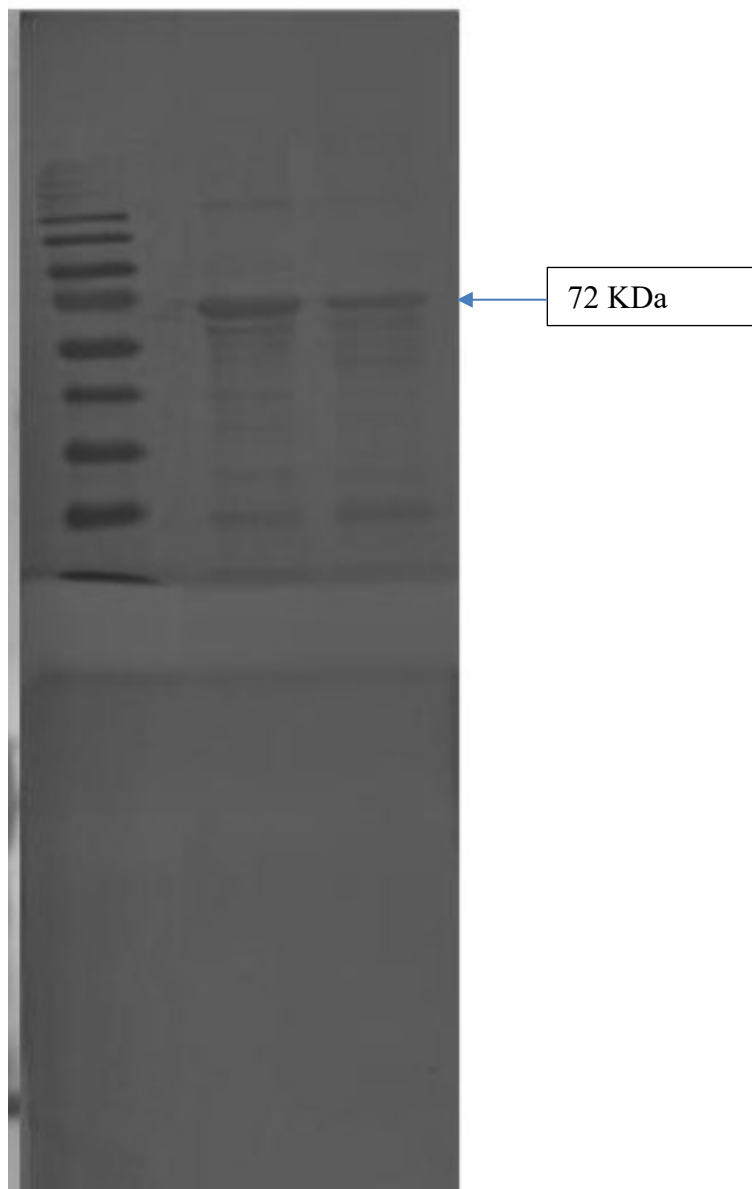


Figure 1: Yeast endo-to-Golgi fusion protein used in this study. Proteins were visualized after Coomassie blue staining using Azure C-series advanced imaging system; GST-Tlg2p (72 kDa).



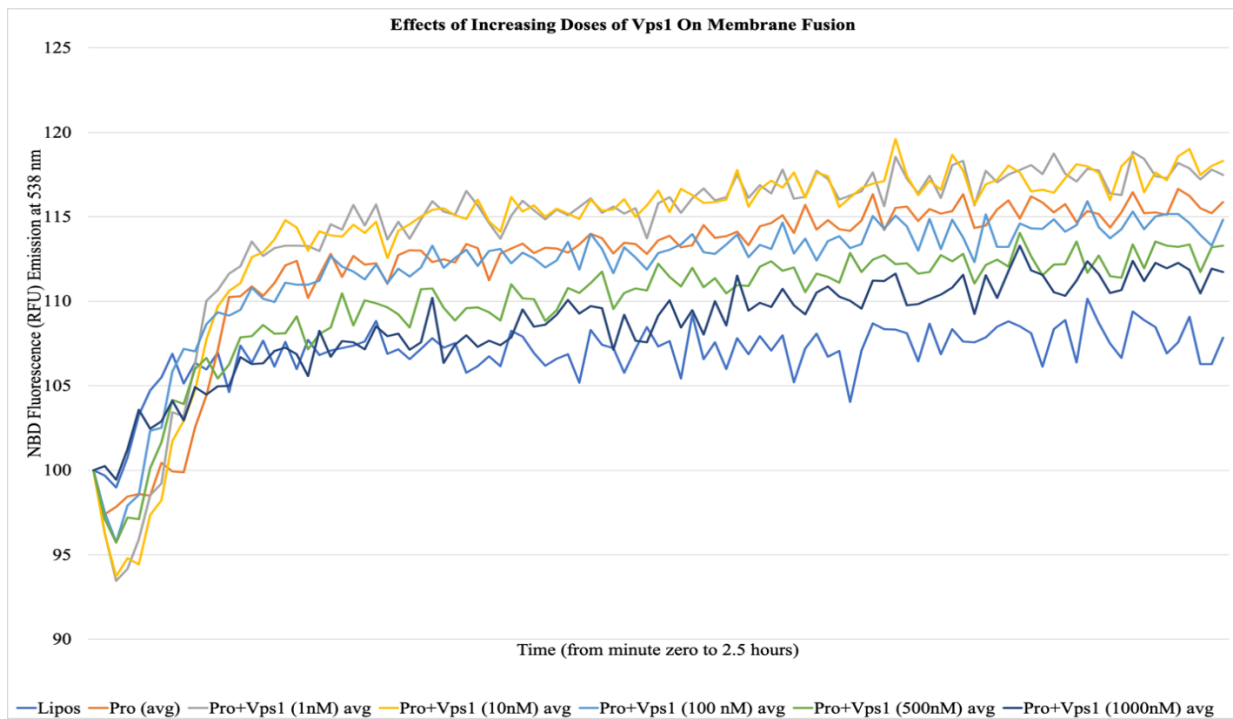


Figure 2A

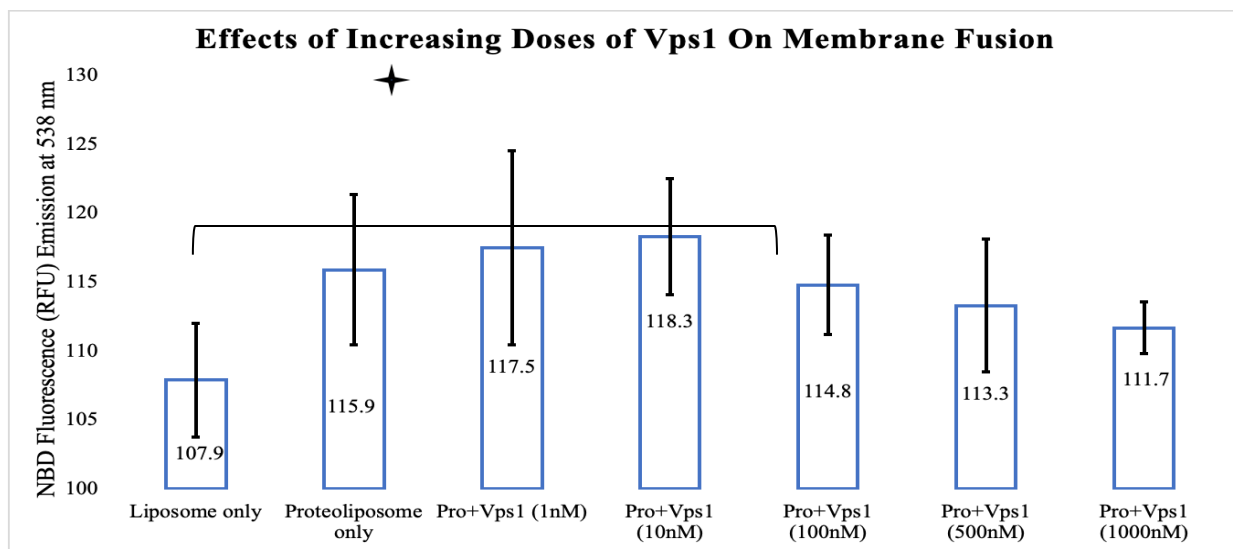


Figure 2B

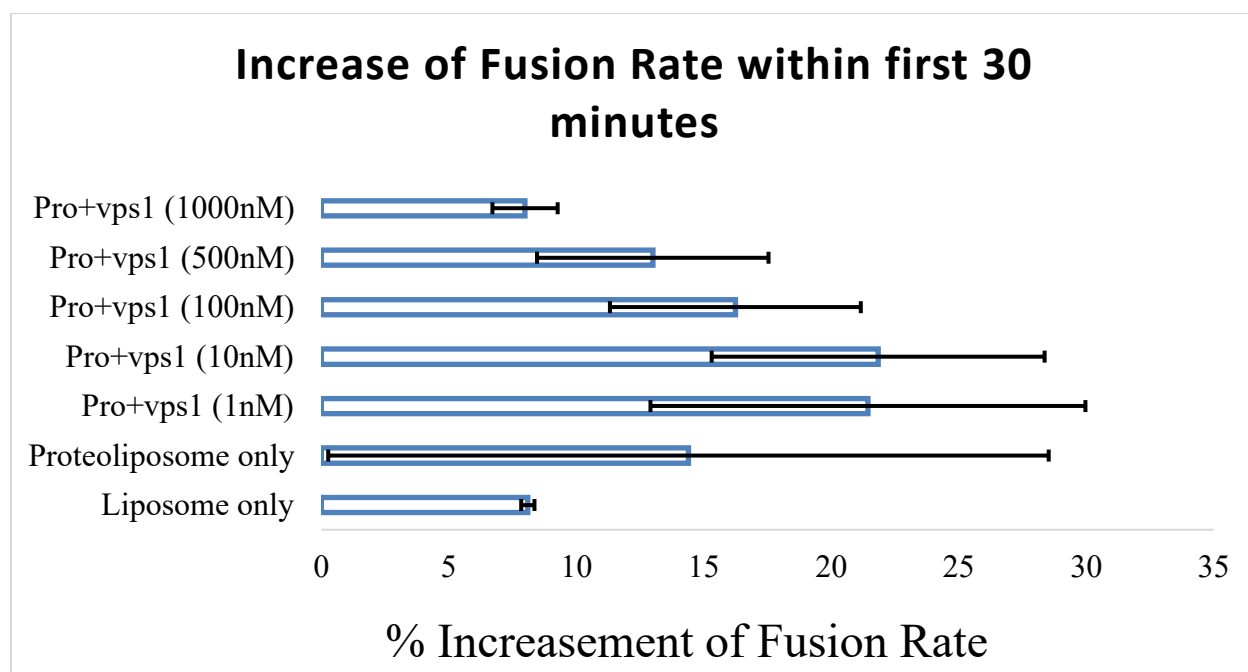


Figure 2C

Figure 2: Effects of increasing doses of Vps1 on membrane fusion. Liposomes and proteoliposomes for the lipid mixing assay were prepared as described in the methods. Control wells were prepared with 5  $\mu$ L of fluorescent  $\nu$ -liposomes and reconstitution buffer lacking the prepared  $t$ -liposomes (liposome only). All other wells contained  $\nu$ -liposomes,  $t$ -liposomes, and differing amounts of Vps1 as indicated in the figure and Table 2. Lipid mixing assays were monitored by recording the increase of NBD-PE fluorescence (excitation 460 nm/emission 538 nm) as it departs from nearby Liss Rhod-PE molecules during lipid mixing for 2.5 hours. For each group, the initial reading was normalized to 100 and the corresponding final reading was obtained as compared to that initial value. All groups were performed in duplicate, and the average value was obtained. (A) Raw kinetic data showing increasing NBD fluorescence over time, signifying elevated fusion. (B) Averaged final reading values and standard deviation for each group were determined. Student's  $t$ -test was performed using Microsoft Excel program. One asterisk indicates p-value being smaller than 0.05. One-way ANOVA was performed using Microsoft Excel program. P-value was 0.238 and F crit value was 3.87. (C) After the initial dip was observed at minute 3, stimulated fusion was observed until minute 30. Enhancement of fusion rate between this time was calculated and presented here.

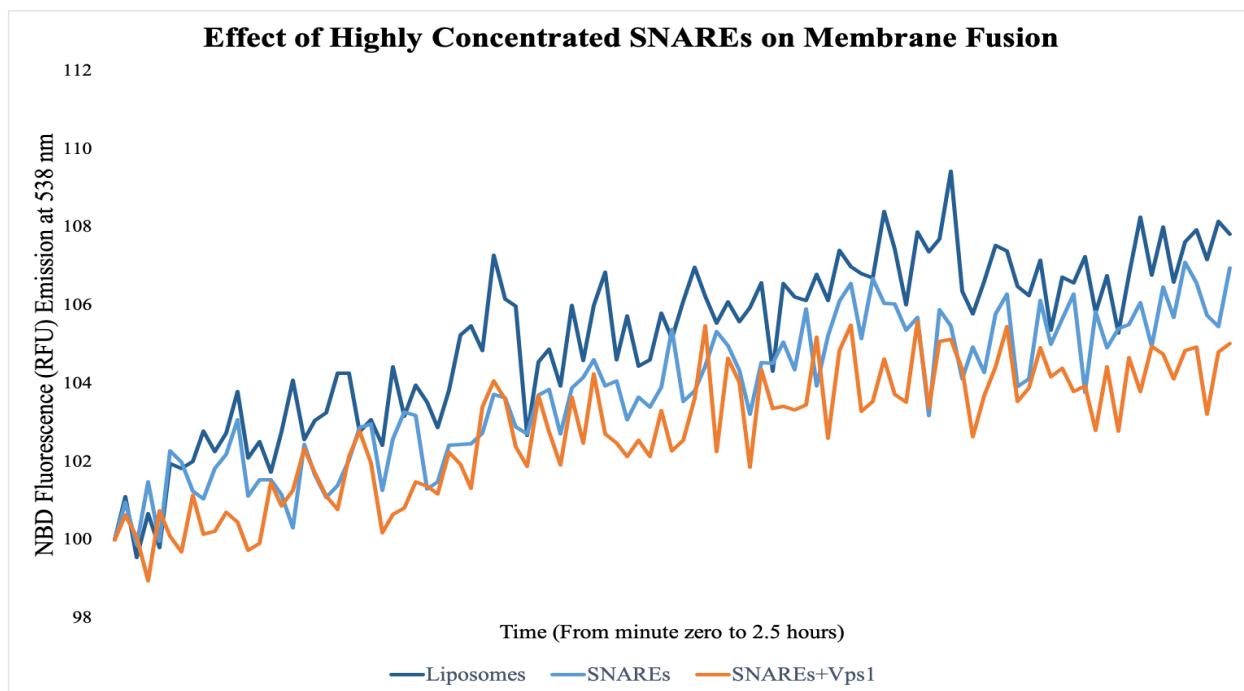


Figure 3A

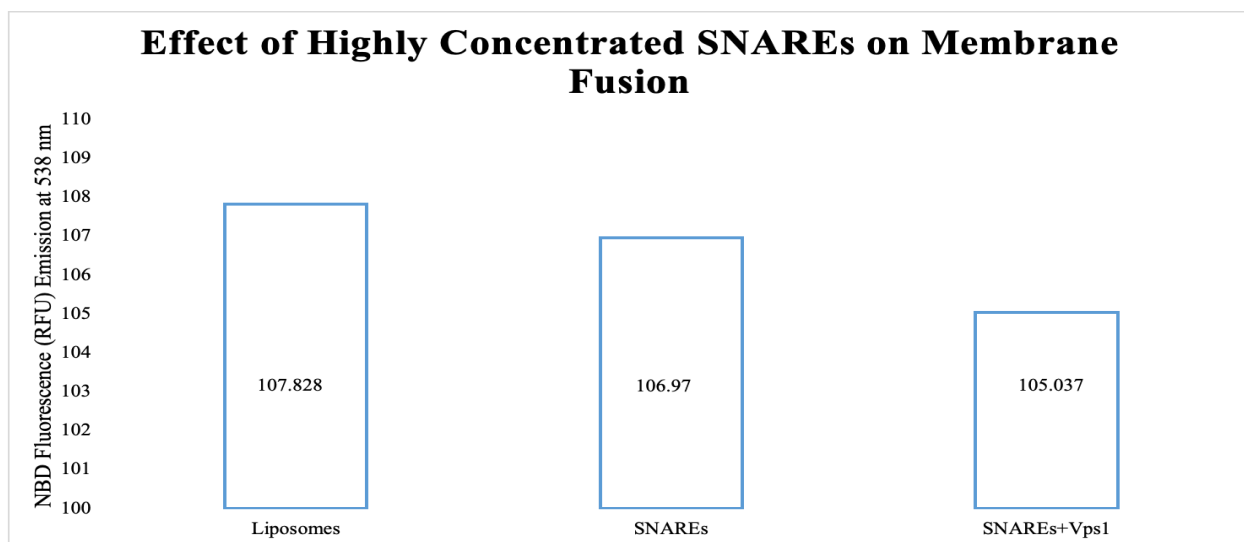


Figure 3B

Figure 3: Effects of highly concentrated SNAREs on membrane fusion. Liposomes and proteoliposomes for the lipid mixing assay were prepared as described in the methods. Control wells were prepared with 5  $\mu$ L of fluorescent *v*-liposomes and reconstitution buffer lacking the prepared *t*-liposomes (liposome only). All other wells contained *v*-liposomes, *t*-liposomes as

indicated in the figure and Table 2 and 1  $\mu$ M of Vps1 (4.6  $\mu$ L). Lipid mixing assays were monitored by recording the increase of NBD-PE fluorescence (excitation 460 nm/emission 538 nm) as it departs from nearby Liss Rhod-PE molecules during lipid mixing for 2.5 hours. For each group, the initial reading was normalized to 100 and the corresponding final reading was obtained as compared to that initial value. (A) Raw kinetic data showing increasing NBD fluorescence over time, signifying elevated fusion. (B) Averaged final reading values for each group were determined. This experiment was done without replication.

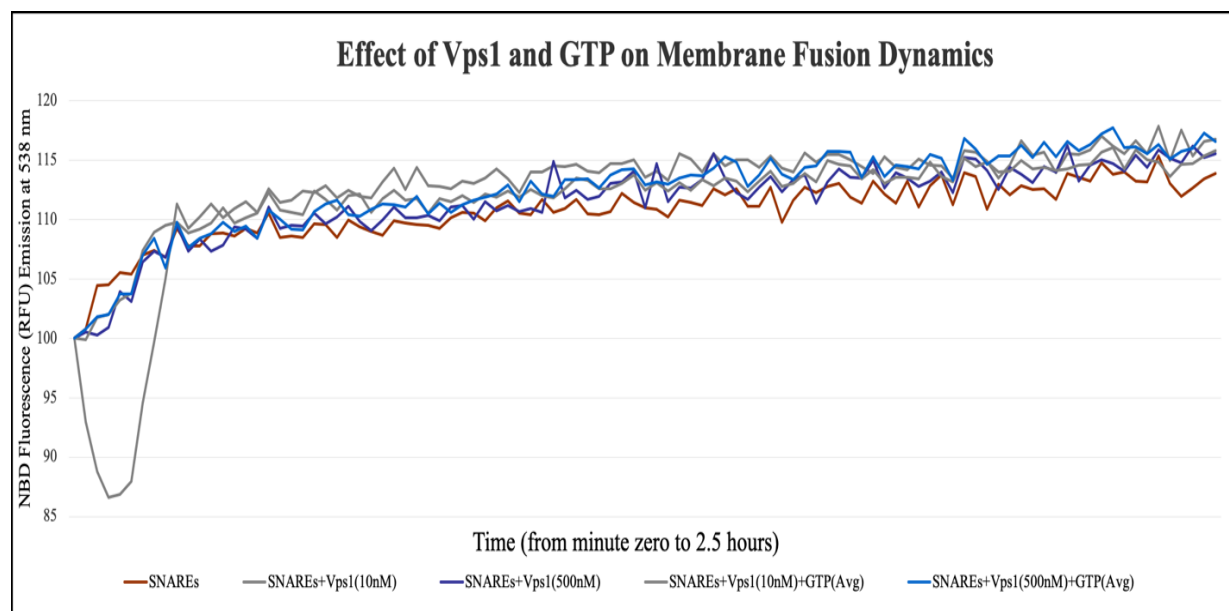


Figure 4A

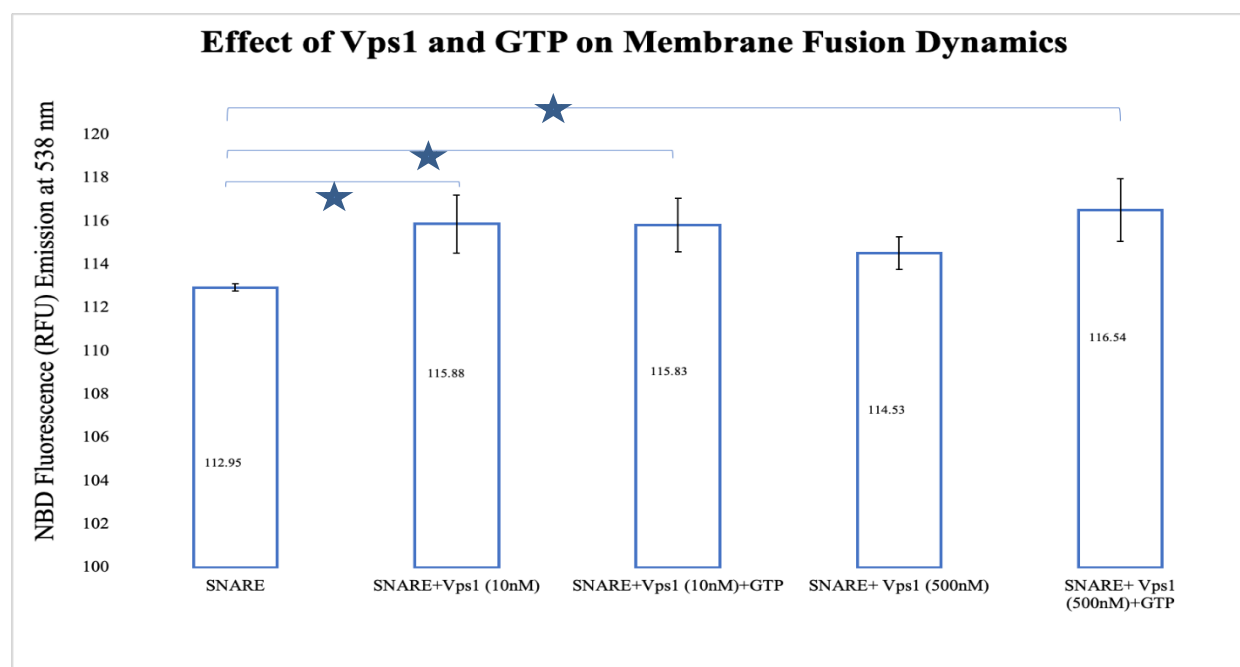


Figure 4B

Figure 4: Effect of Vps1 and GTP on membrane fusion dynamics. Proteoliposomes for lipid mixing were prepared as described in the method section. Control wells (SNARE) were prepared by including 5  $\mu$ L *v*-liposomes and 45  $\mu$ L *t*-liposome without Vps1. Experimental sample wells contained differing amounts of Vps1 in the presence or absence of 100  $\mu$ M GTP as indicated in the figure and Table 2. Lipid mixing assays were monitored by recording the increase of NBD-PE

fluorescence (excitation 460 nm/emission 538 nm) as it departs from nearby Liss Rhod-PE molecules during lipid mixing for 2.5 hours. For each group, the initial reading was normalized to 100 and the corresponding final reading was obtained as compared to that initial value. All groups were performed in duplicate, and the average value was obtained. (A) Raw kinetic data showing increasing NBD fluorescence over time, signifying elevated fusion. (B) Average final reading and standard deviation for each group were determined. Student's *t*-test was performed using Microsoft Excel program. One asterisk indicates p-value being smaller than 0.05.

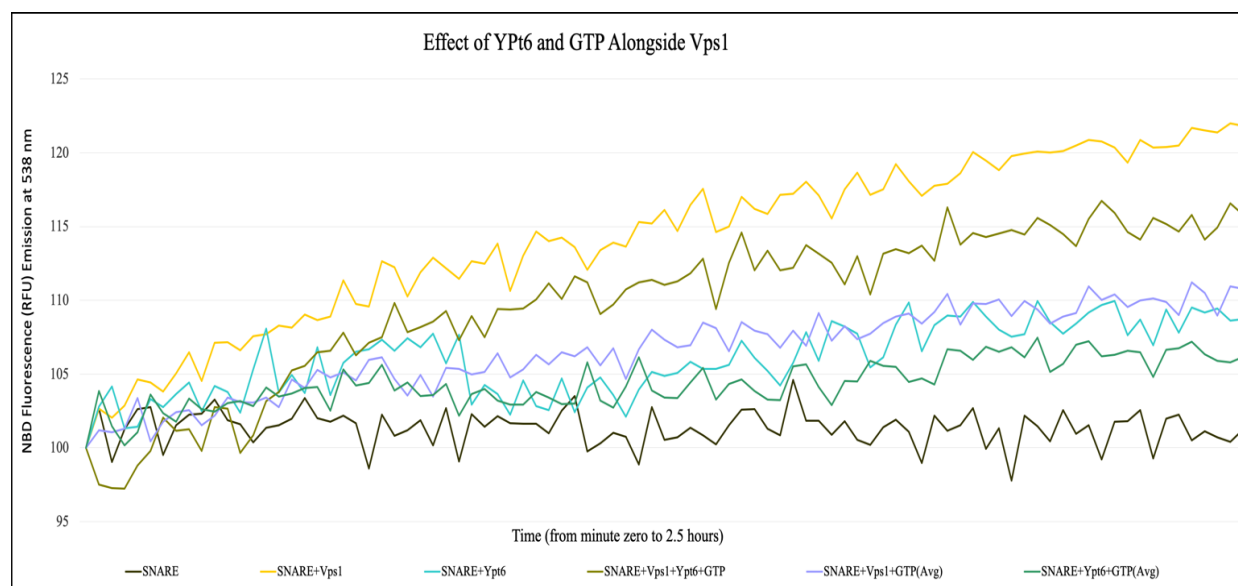


Figure 5A

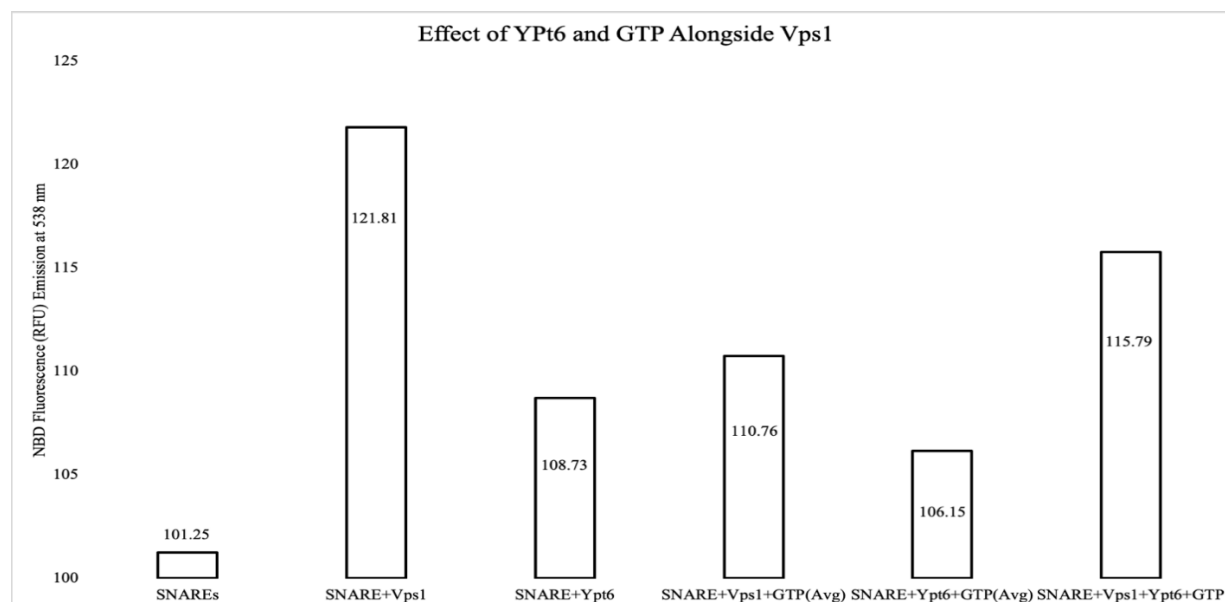


Figure 5B

Figure 5: Effect of Ypt6 and GTP alongside Vps1 on membrane fusion dynamics. Proteoliposomes for lipid mixing were prepared as described in the methods. Control wells were prepared with 5  $\mu$ L *v*-liposomes and 45  $\mu$ L *t*-liposome as in SNAREs only and 1  $\mu$ M of Vps1 and 1  $\mu$ M of Ypt6 were added to test samples as shown in the figure legend and Table 2. Lipid mixing assays were monitored by recording the increase of NBD-PE fluorescence (excitation 460 nm/emission 538 nm) as it departs from nearby Liss Rhod-PE molecules during lipid mixing for 2.5 hours. For each

group, the initial reading was normalized to 100 and the corresponding final reading was obtained compared to that initial. (A) Raw kinetic data showing increasing NBD fluorescence over time, signifying elevated fusion. (B) Average final reading for each group were determined. This experiment was done without replication.