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Functional Connection Between Yeast Dynamin and Retromer at the Endosome

Christopher Robert Trousdale

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FUNCTIONAL CONNECTION BETWEEN YEAST DYNAMIN AND
RETROMER AT THE ENDOsome

A Masters Thesis
Presented to
The Graduate College of
Missouri State University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science, Biology

By
Christopher R. Trousdale
July 20, 2015
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FUNCTIONAL CONNECTION BETWEEN YEAST DYNAMIN AND RETROMER AT THE ENDOSONME

Biology

Missouri State University, July 20 2015

Master of Science

Christopher R. Trousdale

ABSTRACT

Intracellular trafficking from the late endosome to Golgi in cells is termed retrograde transport, essential for recycling of important macromolecules including cell membrane receptors. Retrograde transport is regulated by a family of proteins known as the “Retromer” composed of 5 VPS proteins (Vps5, Vps17, Vps26, Vps29, and Vps35). Retromer acts as the coat proteins for vesicles emerging from late endosomes. Loss of Retromer function has been previously implicated in both Parkinson’s and Alzheimer’s disease. Vps1, a yeast dynamin-like protein, plays a role in intracellular trafficking. Vps1 has been shown to localize at the endocytic sites to promote pinching off of endocytic vesicles. The goal of this study was to further investigate the relationship between the Retromer and Vps1. My data show colocalization between Vps1 and the Retromer, and that Vps1 knockout cells show a decrease in Retromer targeting to endosomes, a phenotype reminiscent of human Alzheimer’s disease. In order to evaluate the functional relationship of the Retromer and Vps1, colocalization and interaction studies, both genetic and physical, were conducted. The data suggest that various Retromer proteins interact with Vps1 on both the genetic and physical levels. I explore this possible relationship, further expanding Vps1’s role as an intracellular trafficking mediator.

KEYWORDS: retromer, retrograde transport, yeast, trafficking, cargo, vps1

This abstract is approved as to form and content

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Chairperson, Advisory Committee
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INTRODUCTION

Vesicular Trafficking and the Retromer

Eukaryotic cells, being more intricately organized than prokaryotic cells, require a multitude of cellular pathways in order to survive, grow, and perform the given functions unique to each cell. Of these many biological processes, a wide range of trafficking pathways are vital in regards to the homeostasis of eukaryotic cells (Munn, 2000; Wendland et al., 1998). Eukaryotic cells contain many different organelles, all of which serve a different function. One of the ways these organelles traffic proteins, nutrients, and other materials to and from each other is through vesicles, known as vesicular trafficking. The most basic form of vesicular trafficking is endocytosis (Fig. 1A). Endocytosis comprises the invagination of the plasma membrane, resulting in the internalization of extracellular molecules. Endocytic cargo can range from nutrients to signaling molecules and even some toxins (Arlt et al., 2015; Shaw et al., 2001). These cargoes are delivered to endosomal compartments (Fig. 1A), where cargo sorting into cargo-specific transport carriers occurs for transport to their next destinations (Munn, 2000; Wendland et al., 1998).

Intracellular trafficking as a whole is conserved among all eukaryotes, especially between yeast and mammalian systems. Minute differences do exist, as the yeast (S. cerevisiae) Golgi are free floating and not stacked as they are in the mammalian system (Fig. 1B) (Feyder et al., 2015). The cargo traffic from endosomes to the lysosome/vacuole (Fig. 1A, B) is named degradation traffic since these cargoes are being degraded at the lysosome/vacuole, while some cargoes pass through the trans-Golgi
network (TGN) to be recycled to the plasma membrane (Fig. 1A, B) (Arlt et al., 2015; Conibear and Stevens, 1998). The latter is the prominent form of the two types of endosomal recycling traffic in yeast, which involves the process of retrograde transport (Fig. 1A, B). Retrograde transport encompasses all traffic of molecules from the endosome to the Golgi (Bonifacino and Hurley, 2008; Burd and Cullen, 2014). The other type of recycling occurs when proteins are trafficked from the early endosome immediately back to the plasma membrane, and is known as the rapid recycling pathway (Fig. 1B) (McDermott and Kim, 2015). The mechanism behind this traffic is not currently known.

Molecules that are recycled to the TGN via the retrograde transport pathway include a number of proteins, the most common examples being membrane receptors such as the carboxypeptidase Y (CPY) receptor Vps10 in yeast and Mannose-6-Phosphate receptor in mammalian cells (Burd and Cullen, 2014; McGough and Cullen, 2011; Nothwehr et al., 1999; Seaman et al., 1997). Vps10 is a transmembrane receptor protein, containing an intraluminal domain, a transmembrane domain, and a cytosolic tail domain (Seaman et al., 1997). In the Golgi, the luminal domain of Vps10 binds pro-CPY, and the receptor-cargo complex is trafficked to the endosome. Once at the endosome, pro-CPY is released from Vps10, and Vps10 is recycled back to the Golgi (Marcusson et al., 1994). This mechanism of endosome-to-Golgi recycling is mirrored by Mannose-6 Phosphate Receptor (M6PR) in mammalian cells. The function of retrograde transport is the correct and efficient retrieval of Vps10 and related receptors such as M6PR. A 5 protein complex known as the Retromer, first characterized in yeast by Seaman et al in
acts as the selector molecule that correctly recognizes and binds the cytosolic tail of Vps10 for retrograde recycling (Seaman, 2005).

**Retromer Structure in Yeast**

In yeast (*S. cerevisiae*), the Retromer complex consists of 5 Vps (Vacuolar Protein Sorting) family proteins (Seaman et al., 1998), Vps5, Vps17, Vps26, Vps29, and Vps35 (Fig. 2 A&B). Vps5 and Vps17, also known as sorting nexins (SNXs), are heterodimerized to form a "tubulation complex," which is responsible almost exclusively for remodeling endosomal membranes in order to form a tubule-shaped extension to which Vps10 and other cargo molecules are selected for recycling (Fig. 2B). These proteins contain 2 important domains: a phox homology (PX) domain (Song et al., 2001) and a BAR (Bin–Amphiphysin–Rvs) domain (Bonifacino and Hurley, 2008; van Weering et al., 2012). Phox domains contain a sequence that binds phosphatidylinositol 3-phosphate (PtdIns3P or PI3P), the main phosphorylated phosphatidyl inositol in endosomal membranes (Gillooly et al., 2000). The ability to bind to PI3P on endosomal membranes is essential to the function of these sorting nexins, as N-terminal truncations of either Vps5 or Vps17 show defects in CPY trafficking (Seaman and Williams, 2002; Song et al., 2001). Furthermore, the PX domain binding activity to PI3P acts as a membrane “anchor” from which the curved dimer can induce curvature to the membrane to which it is bound (Griffin et al., 2005; Seaman and Williams, 2002). The BAR domain serves two concurrent purposes in the complex: dimerization and membrane remodeling (Bonifacino and Hurley, 2008; van Weering et al., 2012). C-terminal BAR domains of both SNX proteins show the ability to bind the opposing SNX C-terminal BAR domains, resulting in dimerization (van Weering et al., 2012). These two SNX proteins create what
is referred to as a SNX-BAR dimer (Horazdovsky et al., 1997). As consequence of this interaction coupled with the PI3P binding of the PX domain, global curvature is induced in the bound membrane (Griffin et al., 2005; Seaman and Williams, 2002). As multiple SNX-BAR dimers bind to the forming vesicular membrane, the vesicle begins to adopt a tubular shape (Fig. 2B). This culminates in a tubule shaped vesicle bud, from which individual vesicles are pinched off (Fig. 2B) and trafficked towards the TGN.

The second, larger subunit of the Retromer is known as the “Cargo Recognition Complex” (CRC), and is made up of 3 Vps proteins in both yeast and mammalian cells: Vps26, Vps29, and Vps35 (McGough and Cullen, 2011; Seaman et al., 1998; Seaman, 2012)(Fig. 2). Vps35 shows the highest homology between yeast and mammalian (human) forms, displaying 61% similarity in nucleotide sequence, and as high as 70% sequence homology in the N-terminal tail section (Edgar and Polak, 2000). Vps29 contains less homology between the two major kingdoms, showing approximately 50% nucleotide sequence similarity, though notably the yeast form of Vps29 is considerably longer than the mammalian one (Edgar and Polak, 2000; Haft et al., 2000). Vps26 in yeast are also longer than in mammalian cells, and show approximately 33% amino acid sequence homology to their mammalian counterparts (Haft et al., 2000). This trimeric complex is directly responsible for recognizing cargo molecules and binding to them. The 3 component proteins bind together, each utilizing separate domains to form the trimeric complex. For example, Vps29 and Vps35 form a complex, bound together when Vps29’s metallophosphoesterase fold, which can bind metal ions, interacts with the C-terminal end of Vps35’s Alpha-solenoid structure (Bonifacino and Hurley, 2008; Swarbrick et al., 2011; Wang et al., 2005). Within this interaction, Vps35’s Alpha-solenoid structure
actually wraps around Vps29 to form the two protein complex (Hierro et al., 2007). Vps26 contains an N-terminal arrestin domain that binds to the N-terminal end of Vps35’s Alpha-solenoid domain (Gokool et al., 2007; Reddy and Seaman, 2001; Shi et al., 2006). Though Retromer cargoes encompass a large assortment of different proteins, they do show similar amino acid sequences, notably high amounts of hydrophobic and aromatic residues (Bonifacino and Hurley, 2008). For example, CIMPR (Cation Independent Mannose-6 Phosphate Receptor) and Sortilin have been shown to contain specific motifs, WLM and FLV, respectively, while Vps10 contains the sequence FYVFSN (Nothwehr et al., 2000; Seaman et al., 1997). The presence of these residues is important, as Vps35 shows the ability to inherently recognize and bind these residues, facilitating their cargo selection (Bonifacino and Hurley, 2008; Seaman, 2007, 2012). In addition to selectively binding its cargo, both ends of the Vps26-Vps35-Vps29 trimer are able to bind to the N-terminal sequence of SNX-BAR proteins. Outside of its interaction with the SNX-BAR dimer, the CRC complex shows a minor ability to influence membrane shape (Bonifacino and Hurley, 2008; Burd and Cullen, 2014; Seaman et al., 1998). These characteristics culminate in the ideal that the Retromer acts as a “coat” complex, covering all sides of the forming tubule (Hierro et al., 2007). Even though much is known in regards to the yeast Retromer, very little has been discovered as to how these tubules pinch off of the endosomal membrane. Recently, the yeast trafficking protein Vps1 and the sorting protein Mvp1 have been implicated as being an integral part of how Retromer coated vesicles are pinched off from the endosome (Chi et al., 2014). However, the implication of Vps1 as a pinchase has yet to be confirmed with direct evidence. Further research is required in order to confirm this hypothesis.
**Mammalian Retromer Structure**

The mammalian Retromer shows slight variability in composition when compared to yeast, mainly in the SNX-BAR dimer and Vps26. Mammalian cells contain several SNX proteins, wherein the Retromer uses four of these (SNX1, SNX2, SNX5, and SNX6) (Bonifacino and Hurley, 2008; Farias et al., 2014; Griffin et al., 2005; McGough and Cullen, 2011). Within these four proteins, however, mammalian cells show an overlap in function, as SNX1 and SNX2 combine to form a heterodimer with one of either SNX 5 or SNX6. Thus, the SNX-BAR dimer is a collaborative complex involving four proteins in recurring pairs: SNX1/5, SN1/6, SNX2/5 and SNX2/6 (Farias et al., 2014; van Weering et al., 2012; Wassmer et al., 2007). In the case of Vps26, mammalian cells contain two paralogues of the protein, Vps26A and Vps26B (Bugarcic et al., 2011; Kerr et al., 2005). Though these two proteins are similar in size, sequence (~80% homology), and functionality, they do exhibit different specificity, binding to distinct Retromer cargo molecules in an independent manner (Bugarcic et al., 2011; Kerr et al., 2005). For example, CRCs containing Vps26B show the capability to bind Golgi phosphoprotein 3 (GOLPH3) and TBC1 Domain Family Member 5 (TBC1D5) proteins, but not CI-M6PR, a well-documented Retromer cargo molecule. Vps26A, however, exhibits normal CI-M6PR binding (Bugarcic et al., 2011). The inability of Vps26B to bind CI-M6PR can be explained by its distinct C-terminal tail section that is different from that of Vps26A (Bugarcic et al., 2011). It could be surmised that the mammalian Retromer shows this variability to accommodate several different cell types (Griffin et al., 2005; Schwarz et al., 2002), cargo molecules, or even cellular changes during the stages of development that would not be necessary in lower eukaryotes (Bujny et al.,
Thus, CRCs are formed using either Vps26A or Vps26B, though both combinations occur simultaneously in cells. Furthermore, independent deletion of either Vps26 paralogue shows differing effects (Bugarcic et al., 2011). For example, deletion of Vps26A leads to decrease in Vps29/Vps35 expression, a Golgi phenotype that shows heavy fragmentation, and most importantly embryonic cell death (Seaman, 2004). Conversely, cells containing a knockout of Vps26B show no detrimental phenotypes, appearing to be completely normal. This leads to the assumption that the two paralogues do not exhibit functional redundancy (Kim et al., 2010). Overall, it would appear that Vps26A is the more important molecule based on its greater range of binding partners, as well as the greater severity of defects seen in cells without Vps26A. This begs the question of how Vps35 selectively interacts with both paralogues of Vps26, and whether mammalian cells have a compensatory mechanism if Vps26A is downregulated. Is Vps26B overexpressed in such a situation? Or is there a different protein that can substitute for Vps26A?

**Retromer Recruitment to the Endosome**

Given that the yeast Retromer exists in the cytosol as a stable heteropentamer (Bonifacino and Hurley, 2008), its recruitment is different from that of the mammalian Retromer. In modern Retromer research there are two emerging hypotheses as to the recruitment of the Retromer to the endosome in mammalian cells. One hypothesis proposes the CRC is the first subcomplex of the Retromer to be recruited to endosomes (Harbour et al., 2010), while a second hypothesis supposes that the SNX-BAR dimer binds endosomal membranes before CRCs arrive (van Weering et al., 2012). Studies have shown that the CRC is recruited to endosomal membranes via interaction between Vps35
and the late endosome membrane protein Rab7. Furthermore, CRC units lacking Vps26 seem to lose the ability to bind to Rab7 via Vps35 (Priya et al., 2015). It is possible this inhibition serves as a method of preventing association of incompetent Retromer CRCs with late endosomal membranes. This leads to the idea that assembly of the trimeric structure must be completed before the CRC can be recruited to endosomes.

Interestingly, biochemical protein binding profiles of Vps35 concluded the cytosolic existence of several versions, both complete and incomplete, of the CRC (Norwood et al., 2011). Vps35 has shown the ability to bind Vps26 and Vps29 independently, creating both Vps26-Vps35 and Vps35-Vps29 binding groups (Norwood et al., 2011). Further research into the specifics of how distinctly different and complete CRCs are formed in the cytosol would aid in understanding of the hypothesis that CRCs are first recruited to endosomes. In regards to the second hypothesis, very little has been expanded upon the idea of SNX-BAR dimers pre-existing on endosomal membranes prior to formation of the full complex of the Retromer. One piece of suggestive evidence towards this hypothesis is that SNX-BAR dimers display the ability to bind endosomal membranes independently of the CRC. This leads to the suggestion that SNX-BAR dimers can be recruited to the membrane before the process of tubulation occurs (van Weering et al., 2012). Another idea for the hypothesis is that CRCs are recruited by the small GTPase Rab7, as it has been shown to bind the Retromer CRC (Priya et al., 2015; Rojas et al., 2008; Seaman et al., 2009). Therefore Rab7 would seem a likely protein to recruit the CRC to SNX-BAR coated tubules. Additionally, SNX3 has been implicated to be involved in the recruitment of the Retromer to the endosome, as SNX3 also binds the CRC (Harrison et al., 2014) and aids Rab7 in CRC recruitment. Studies looking at time
based-localization of Retromer components to fluorescently marked endosomes could be one future direction to pursue. Mutational studies of Retromer-Retromer binding domains, looking at the time-lapse recruitment to marked endosomes could be another avenue of research. Regardless of the experiment, more work must be done in the area of recruitment, as great insight as to how the Retromer is recruited is paramount for the furthered understanding of the endosomal protein sorting system.

**Retromer Cargo**

In mammalian cells, the Retromer recognizes several different cargo proteins (Table 1), including M6PR (Mannose-6 Phosphate Receptor) (Arighi et al., 2004; Seaman, 2004), a mammalian functional homolog of Vps10 (Seaman et al., 1997), and SORLA (Sortilin-Related Receptor L, also known as SORL1) (Harbour et al., 2010; Lane et al., 2010). In mammalian cells and *Drosophila* the signaling pathway Wnt has been found to utilize the Retromer in recycling the Wnt signaling factor Wls from endosomes back to the Golgi (Belenkaya et al., 2008; Coudreuse et al., 2006; de Groot et al., 2013). Also, membrane iron transporter DMT1 (Divalent Metal Transporter 1) shows evidence of dependency on Retromer-mediated sorting and recycling from endosomes to the Golgi (Tabuchi et al., 2010). The Retromer is extensively studied in mammalian neural dendritic cells and has been implicated as a major sorting complex for the recycling of β2ARs (Beta-2 adrenergic receptors) (Choy et al., 2014; Temkin et al., 2011). Several studies also conclude that receptors for APP (Amyloid Precursor Protein) are recycled within neural cells in a manner consistent with other Retromer cargo (Choy et al., 2012; Lane et al., 2010; Nielsen et al., 2007; Sullivan et al., 2011; Vieira et al., 2010). Further within the realm of neural development and function, BACE1 (β-secretase-1), an
important protease in the development of neurons, has also been shown to be a Retromer-targeted cargo (Muhammad et al., 2008; Wang et al., 2012). Clearly, the mammalian Retromer is involved in a large number of different pathways that utilize the Retromer as a specific protein sorting complex for efficient protein recycling. Even with this list of cargoes, it is very possible there are several more to be discovered. The investigation into such possibilities would do well to further scientific understanding of just how widely used the Retromer is within cells.

**Retromer and Intracellular Pathogens**

**Viral Pathogens.** As the Retromer has been shown to recycle a myriad of different molecules within mammalian cells, it has also been shown that several pathogens utilize the Retromer in their intracellular pathology. Human Immunodeficiency Virus (HIV-1) has been shown to use Retromer-based recycling in the process of virion construction (Fig. 3A), specifically involving the two component envelope (Env) proteins (gp120 and gp41) (Blot et al., 2003; Groppelli et al., 2014). Not only do Retromer proteins show intracellular colocalization with HIV-1 particles *in vivo*, but Retromer proteins Vps26 and Vps35 have been shown to physically interact directly with the cytoplasmic tail of the Env protein (Fig. 3A). These results, in combination with a host of Retromer knockout studies in HIV-1 infected cells, suggest that the Retromer plays a critical role in the retrieval of Env proteins once they are endocytosed (Groppelli et al., 2014).

Similarly to HIV-1, the Retromer has also been shown to modulate intracellular movement of Human Papillomavirus (HPV), specifically HPV serotype 16, which is
most notable for its well documented role in cervical cancer. HPV is a non-enveloped double-stranded DNA virus containing two major capsid proteins (L1 and L2) that function in cooperative fashion to mediate viral entry into host cells (Fig. 3B) (Lipovsky et al., 2013; Sapp, 2013). The virus is taken up into the cell through a process known as "micropinocytosis" (Schelhaas et al., 2012). Recent studies showed that capsid proteins L1 and L2 colocalize with Retromer CRC components in mammalian cells (Popa et al., 2015). L1 and L2 have also been shown to physically interact with all 3 proteins of the CRC. As expected, cells in which Retromer proteins are knocked down by siRNA show defects in HPV's ability to infect cells. This infection inhibition result is consistent for across several HPV serotypes (Lipovsky et al., 2013). This Retromer knockdown result reflects that the Retromer is necessary for the virus to escape the early endosome, and describes how the virus appears to become trapped in the endosome when the Retromer CRC is inactivated (Lipovsky et al., 2013). Sequence analysis and mutational studies have confirmed that the L2 protein contains a carboxy-terminal domain that specifically mediates L2 binding with Retromer CRC proteins (Popa et al., 2015). The logical analysis of these results leads to the theory that L2 proteins directly interact with Retromer CRCs, likely by penetrating the endosomal membrane, though it is also possible that L2 interacts with a receptor that is then bound by the Retromer (Fig. 3B) (Popa et al., 2015; Sapp, 2013). Both HIV-1 and HPV appear to hijack the Retromer complex and its recycling pathway for the ultimate gain of the virus.

In addition to HPV and HIV-1, the Retromer has been shown to be vital to Herpesvirus Saimiri (HVS) ability to fully infect cells. However, in contrast to the previous examples, HVS does not utilize the Retromer to increase its virulence, but rather
negatively affects the Retromer and the virus benefits (Fig. 3C). In this instance, HVS tyrosine kinase-interacting protein (Tip) shows interaction with Retromer protein Vps35 (Kingston et al., 2011). Looking further into this interaction, Tip shows binding ability with two distinct regions of Vps35; from amino acid 1-120 and from 500-796. HVS infected cells show mislocalization of Tip and Vps35 proteins to the lysosome (Fig. 3C). These proteins appear to aggregate with each other within the lysosome, suggesting that Tip actually deregulates the Retromer by directly interrupting the function of the CRC; however, Tip does not affect the expression levels of CRC subunit proteins (Kingston et al., 2011). The interaction between Tip and Vps35 is specific to HVS survival, resulting in downregulation of CD4 on the cell surface (Fig. 3C). CD4 is an integral cell surface receptor that acts to activate antigen-presenting cells (Kingston et al., 2011). The resulting lack of CD4 receptors at the cell surface is essential to HVS’s ability to avoid detection and destruction. Additionally, Tip-Vps35 interaction has been implicated to have a large role in the ability of HSV to immortalize T-cells (Kingston et al., 2011).

**Bacterial Pathogens.** In bacteria-caused infectious disease, the bacterium *Shigella dysenteriae* secretes a toxin known as Shiga toxin (Stx) as a major component of its virulence. Enterohemorrhagic *Escherichia coli* also produce Shiga toxins, termed Shiga-like toxins or verotoxins (Bryan et al., 2015). Stx has been shown to utilize Retromer function once bound to toxin receptors on the cell membrane and endocytosed (Fig. 4) (Bujny et al., 2007; Popoff et al., 2007). Stx shows cellular colocalization with SNX1 coated vesicles following endocytosis (Popoff et al., 2009), which suggests Stx is trafficked by way of endosomes (Fig. 4). Cells in which SNX1 was knocked down via RNAi (RNA interference) display disruption of Stx trafficking, such that Stx is retained
in the peripheral cellular structure as opposed to normal localization in the perinuclear area (Bujny et al., 2007). Similar experiments describe that the loss of Vps26 results in Stx being localized to endosomal vesicles, essentially becoming stuck and unable to locate to the vicinity of the nucleus (Popoff et al., 2007). These data are suggestive that the Retromer is required for proper Stx function within the cell (Fig. 4), another example of the utilization of the Retromer in infective disease pathology.

**Disease via Retromer Dysfunction**

The Retromer however, is not only relevant in the area of microbial pathogens, but also has been implicated to play a role in the disease pathology of several degenerative diseases. The two most prominent examples are Alzheimer's and Parkinson’s disease. Both are fairly widespread and well known in our society, yet the specifics of why each disease occurs and how they progress are still not clear. However, pathways and processes within the cell, such as Retromer activity, have been identified as contributors to the disease, expanding the role of the Retromer in human disease pathology.

**Alzheimer’s disease.** Alzheimer’s disease (AD) is a neurodegenerative disease characterized by loss of cell function in the brain. A hallmark of AD is Amyloid Beta (Aβ) plaques, aggregates of Aβ protein in patient brain tissue (Fig. 5A). Amyloid Beta proteins are created by improper cleavage of the cellular Amyloid Precursor Protein (APP). First, APP, a plasma membrane protein, is made in ER and targeted to the plasma membrane via the secretory pathway, and then is endocytosed for its recycling. In normal cells, APP is first cleaved in the Endosome by α-secretase (Ishiura, 1991), then
trafficked via Retrograde Transport to the Golgi (Vieira et al., 2010), destined for the plasma membrane (Fig. 5A). At the membrane, APP is further cleaved by γ-secretase. BACE1, a known Retromer cargo (Wang et al., 2012), is recycled between the endosome and the Golgi via the Retromer, allowing BACE1 to improperly cleave APP (Fig. 5A). From this point the improperly cleaved APP is then recycled to the plasma membrane, where γ-secretase recognizes and cleaves it (Fig. 5A). The upshot of this is that the combination of BACE1 and γ-secretase cleavage results in the release of Aβ (Choy et al., 2012). APP trafficking by retrograde transport is dependent on the Vps10 homolog receptor SORLA (Muhammad et al., 2008) and the Retromer coat complex (Vieira et al., 2010), as APP has been shown to physically bind to both (Small et al., 2005; Vieira et al., 2010). Additionally, both APP and SORLA colocalize with Retromer protein Vps35 in vivo, suggesting both are Retromer cargo (Small et al., 2005; Vieira et al., 2010).

Reduced expression of Vps35 has long been implicated in the pathology of AD, as tissue samples from patients with AD contain lowered levels of Vps35 and Vps26 (Small et al., 2005). Several knockdown studies have described that the severity of Vps35 is positively correlated with Aβ production (Choy et al., 2012; Muhammad et al., 2008; Small and Petsko, 2015; Sullivan et al., 2011; Vieira et al., 2010). Moving forward, finding ways of detecting reduced levels of Vps35 in humans could prove advantageous to the diagnosis of AD. Also, it may be possible to reverse this underexpression using microRNA and other genetic technologies. A combination of earlier diagnosis and better treatments could go a long way to successfully fighting the battle against Alzheimer’s disease.

**Parkinson’s disease.** Parkinson’ disease (PD), is another neurodegenerative disease with strong links to Retromer dysfunction in neurons, and is the second most
common neurodegenerative disease in the US behind only AD (Deng et al., 2013). Unlike AD, the specific underlying mechanisms are not quite as well understood in PD, as there are many knowledge gaps yet to be filled. The Retromer however has been identified as a possible contributor to PD in a few different ways. First, a mutation of the VPS35 gene (p.D620N) has been linked to familial inherited and idiopathic forms of PD, though these examples account for only 0.1 to 1% of all documented cases (Follett et al., 2014). The p.D620N mutation of Vps35 does, however, exhibit some very interesting effects on retrograde trafficking. Studies on the mutated form of Vps35 suggest that cells have completely lost function of the Retromer altogether (Small and Petsko, 2015), however other studies conclude that the mutation results only in the loss of retrograde transport to the Golgi (McGough et al., 2014), or defective autophagosome formation (Zavodszy et al., 2014). First, cells expressing mutated Vps35 also exhibit large defects, such as enlarged endosomes that are mistargeted to the area around the nucleus (Fig. 5B), a result that has been confirmed using patient tissue samples (Follett et al., 2014). Second, cells with dysfunctional Retromer have been shown to mistarget DMTII and Wls proteins to the lysosome (Fig. 5B). DMTII deficiencies have been linked to iron accumulation in PD patient samples (Deng et al., 2013), and Wls, a signaling molecule in the WNT/β-catenin signaling pathway, is necessary for a multitude of neuronal signaling functions, including development and cell-cell communication (Belenkaya et al., 2008; Coudreuse et al., 2006). Deficiencies in either of these pathways contribute to impaired cell function. Thirdly, dysfunction of the Retromer is involved in the accumulation of Lewy Bodies, a hallmark of PD (Follett et al., 2014). Lewy Bodies are formed when excess cellular α-synuclein oligomerizes in cells and is excreted to the ECM (Fig. 5B). α-synuclein is
normally degraded by the enzyme Cathepsin D, a known cargo of the CIMPR receptor (Follett et al., 2014). As described earlier, CIMPR is a target protein for retrograde transport, and thus a cargo of Retromer. Cathepsin D is synthesized in the ER then trafficked to the Golgi, where it binds to CIMPR to be transported to the endosome (Fig. 5B). Once at the endosome, Cathepsin D releases from CIMPR, destined for the lysosome where it becomes active, and CIMPR is recycled to back to the Golgi via the Retromer (Follett et al., 2014). However, if this traffic is inefficient or nonfunctional, Cathepsin D cannot reach the lysosome, and α-synuclein is not properly degraded. This excess accumulation of α-synuclein then leads to the production of Lewy Bodies (Fig. 5B) which contribute to neurodegeneration of neurons and the onset of Parkinson’s disease. It may be possible in the future to look to the Retromer in the early diagnosis and possible treatment of PD, though extensive research would be required to reach that point. Regardless, the more that is known of the mechanisms behind Parkinson’s disease the better scientists and researchers can strategize about treatments and detection.

**Diabetes Mellitus.** While the bulk of known diseases linked to Retromer dysfunction are neurodegenerative, there are other non-neural examples. Diabetes Mellitus (DM) is one example which, while having no direct evidence linking the Retromer to its causation, has been linked to DM via a few different proteins. The Vps10-family receptor SorCS1 has been linked to both Type 2 and Type 1 DM via Genome Wide Association Studies (GWAS) (Goodarzi et al., 2007; Liang et al., 2009; Paterson et
al., 2010). Vps26a has also been linked to Type 2 DM by GWAS (Kooner et al., 2011).
Even though these findings do not draw a direct link between DM and the Retromer, they
shed light into a possible relationship between the Retromer and DM, an increasingly
common disease among humans.

**Prospective Retromer Research Trends**

As various studies and experiments have shown, the Retromer complex is a vital
part of cellular trafficking machinery in cells. Despite the Retromer’s specified action in
recycling, it has been shown to be heavily involved in extracellular virulence of several
pathogens, as well as dysfunctional neurodegenerative diseases. While the yeast
Retromer and the mammalian Retromer show conserved action, they are markedly
different in structure and composition, though this divergence is easily explained with the
evolutionary mechanics required for multicellular organisms when compared to single-
celled eukaryotes like *S cerevisiae*. The future of Retromer research is certainly a bright
one, considering the wealth of unknown variables in the system, such as specific
recruitment, further inter-connectivity to other protein sorting, and the intricacy of
Retromer action in human neural cells as it relates to disease. There are several valid and
interesting questions to be investigated in connection with the Retromer. One can hope
the future brings answers to many of these questions: How does Vps35 selectively bind to
the different paralogues of Vps26? How is Retromer binding into subcomplexes
regulated? Are there any changes in the Retromer through different disease states? How
are Retromer coated tubules pinched off in yeast or mammalian systems? Is the yeast
Retromer recruited differently than the mammalian Retromer? How are Retromer
proteins individually recruited to form these intricate complexes? Outside of these
questions of Retromer function and regulation, the possibility of Retromer-developed
treatments and detection methods for diseases like Alzheimer’s and Parkinson’s or even
HIV and HPV provide a whole new avenue for Retromer research. Overall, retrograde
transport is but one small subcategory of cellular trafficking, and yet its importance
cannot be overstated.

**Vps1**

**A Dynamin-Like Protein and Implication as Scission Protein.** Vps1 is a yeast homolog of the mammalian protein Dynamin, which hydrolyzes GTP to accomplish its function (Ekena et al., 1993; Vater et al., 1992). Dynamin forms an oligomer of a chain-like spiral around the neck of the budding vesicle, at which point GTP binds to the dynamin chain and is hydrolyzed. This induces a conformational change, resulting in the scission of vesicles budding from plasma membranes during endocytosis (Ekena et al., 1993; Ferguson and De Camilli, 2012). Vps1, showing 45% total homology with Dynamin, contains the potential to accomplish this same function in yeast (Vater et al., 1992). Vps1 is comprised of 3 domains, a GTPase domain, a GED domain, and a Middle domain. Vps1 has been linked to several different intracellular trafficking pathways in yeast including endocytosis, endosomal, anterograde, and retrograde traffic (Burda et al., 2002; Chi et al., 2014; Ekena et al., 1993; Hayden et al., 2013; Lukehart et al., 2013; Vater et al., 1992; Wang et al., 2011). In these pathways Vps1 is theorized to aid in vesicle budding and scission of the budding vesicles, similar to the function of Dynamin (Ferguson and De Camilli, 2012). Additionally, cells in which Vps1 is knocked out show severe recycling defects (Hayden et al., 2013; Lukehart et al., 2013; Wang et al., 2011),
and these recycling defects in the absence of Vps1 similarly resemble those of Retromer-deficient cells in both AD and PD pathology (Small and Petsko, 2015).

**Previous Lab Findings and Proposed Function in Retrograde Pathway.** Our lab has recently provided evidence for Vps1’s involvement in several pathways. First, our lab recently found that Vps1 colocalizes with the well-known Golgi marker Sec7 (Lukehart et al., 2013). Second, our lab published data showing Vps1 colocalization with the endosomal marker PI3P (Hayden et al., 2013), which occurs abundantly at the endosome in yeast. Third, Dr. Kim’s research group at MSU produced data showing that Vps1 and clathrin interact and proposed that Vps1 acts as the scission protein in anterograde traffic, pinching off clathrin coated vesicles from the Golgi bound for the endosome (personal communication with Shiva Kumar Goud Gadila/Michelle Williams). Fourth, our lab produced data implicating Vps1 is involved in recycling traffic via physical interaction with Trans-Golgi Network (TGN) recycling factor Ypt6, strengthening the implication of Vps1’s action in vesicle fusion at the Golgi (personal communication with Pelin Makaraci). Fifth, data was also produced suggesting Vps1 interacts with as several proteins involved in protein sorting at the endosome (personal communication with Bryan Banh). Three proteins of the Endosomal Sorting Complexes Required for Transport, known as ESCRTs, were found to physically interact with Vps1; ESCRTII proteins Vps22 and Vps36, and ESCRT III protein Vps24 (personal communication with Aria McDermott). Recently, a group from Yale published data implicating that Vps1 aids in the formation of retrograde vesicles at the endosome (Chi et al., 2014). This finding supports the notion that Vps1 may act as a scission protein at the endosome (Fig. 6).
HYPOTHESIS AND GOALS

In this study, I investigated the functional relationship between Vps1 and the coat protein complex known as the Retromer in the Retrograde trafficking pathway from the endosome to the Golgi. As described previously, Vps1 and the Retromer both localize at the endosome. Therefore I hypothesize that the Retromer and Vps1 will colocalize within the cells at the endosome. I further hypothesize that Vps1 and the Retromer are interdependent on one another. Furthermore, I hypothesize that the proteins of the Retromer and Vps1 are functionally related and will interact on the genetic level. Finally, I hypothesize that the proteins of the Retromer will interact with Vps1 on the physical level.

Here I provide evidence that Vps1 colocalizes with the 5 proteins of the Retromer complex (Vps5, 17, 26, 29, and 35) in vivo, as well as evidence that Retromer proteins are able to be correctly recruited to the endosome with or without the presence of Vps1. I provide evidence that the amount of Retromer recruitment is in fact diminished in cells that lack Vps1 versus that of wild type cells. Finally, genetic interaction studies reveal that only Vps35 interacts with Vps1; however yeast-two-hybrid analysis reveals that all five Retromer proteins show positive physical interaction with Vps1.
MATERIALS AND METHODS

Media

All media were prepared using DI water in small autoclave-safe bottles. Standard YPD (yeast-peptone), 2X YPD, and selective (ex: -His) media were prepared in 90ml batches, then autoclaved before use. For a standard 3ml yeast culture, 2.7ml of media was added, followed by 0.3ml of a prepared 20% Dextrose solution. LB media was made, autoclaved, and used for bacterial strains. 3ml of this media was added plus either 8µl of Kanamycin (50mg/ml) or 9µl of Ampicillin (100mg/ml) to make a standard 3ml bacteria culture. For cultures utilizing dropout media, such as DDO (Double DropOut), TDO (Triple DropOut), or QDO (Quadruple DropOut), 3ml of media was used for each culture.

Yeast Strain Construction

Yeast strains used in this study are listed in Table 1. Green and Red fluorescent tagged fusion proteins (GFP and RFP, respectively) were constructed using either a GFP or RFP construct integrated at the 3’ end of the gene coding region through homologous recombination as previously described (Kim et al., 2006; Longtine et al., 1998; Nannapaneni et al., 2010). Strains containing gene deletions were constructed by replacing the complete gene reading frame in wild type cells with either a KanMX6, HIS, or TRP cassette, as described previously (Longtine et al., 1998). Transformants were plated on selective media, based on the inserted cassette (YPD+Kan, SD/-HIS, or SD/-TRP), and confirmed using both colony PCR and fluorescent microscopy. Resulting positive colonies were grown in liquid medium lacking in nutrients required to maintain
selectivity for positive colonies. The plasmid encoding DsRed-FYVE was introduced into yeast strains using a one-step transformation protocol as previously described (Chen et al., 1992), following which cells were plated on selective media lacking leucine (SD/-LEU). Positive colonies were confirmed using fluorescent microscopy.

**Colony PCR**

All transformed cells were confirmed using colony PCR. DNA was either extracted using MasterPure™ Yeast DNA Purification Kit (Epicentre MPY80200) or using a “Quick and Dirty” method. Extracted DNA was used as template and was added into PCR mix [19µl Sterile H2O, 2.5µl 10x Ammonium Buffer, 1µl MgCl2, 0.5µl 10x dNTP mix, 0.5µl 10uM Forward primer, 0.5µl 10µM Reverse Primer, 0.3µl Bull Taq, and 1µl Template]. PCR protocol was then run on a BioRad C1000 Thermal Cycler. Amplified PCR samples were run on a 1% Ethidium Bromide agarose gel with 5µl of 6X Sample Loading Buffer for confirmation against 1X 1Kb DNA Ladder (New England BioLabs, N3232L).

**Fluorescence Microscopy**

GFP/RFP tagged cells were also partially confirmed via fluorescent microscopy. For this process cells were grown in their respective selective media overnight, and their OD was measured using a Thermo Scientific Biomate 3 Spectrophotometer. Cells at an OD between 0.6 and 0.8 are spun down (1ml of culture, 1500 rpm for 3 min) and visualized using a spinning disk confocal system that includes an inverted Olympus IX81 microscope, a Yokogawa CSUX1 spinning disk head, a 100× numerical aperture (NA) 1.4 PlanApo oil objective, and an Electron Amplified CCD (ImagEM, Hamamatsu) or a conventional fluorescence microscope (ORCA camera). The image was focused at an
equatorial plane of the cells under oil immersion at 100x magnification. Exposure for all cases was set to 200ms. Simultaneous two-color imaging was done using an image splitter to separate red and green emission signals. All images were taken focused on the center plane of the cell.

**Quantitative Analysis of Retromer Colocalization**

Yeast strains containing GFP-tagged copies of all five Retromer proteins (Vps5, Vps17, Vps26, Vps29, and Vps35) with wild type Vps1 or *vps1∆* carrying the DsRed-FYVE plasmid were grown in their respective selective medium overnight at 30˚C until reaching an OD between 0.6-0.8. Cells were then imaged using a spinning-confocal microscope (ImagEM). Once a sufficient number (n = 30) of these cells were captured for each sample, the pictures were analyzed. The number of GFP and DsRed puncta were counted separately, and then compared to give a colocalization percentage. This percentage represents the approximate amount of colocalization between the GFP and DsRed proteins *in vivo*. Yeast strains containing GFP-tagged Retromer proteins and RFP-tagged Vps1 were also analyzed in the same manner as DsRed (described above) with the exception of Vps5-GFP mRFP-Vps1. Due to loss of Vps5-GFP puncta (most likely due to mutation in the tagged protein sequence), this strain was constructed by one-step transforming Vps5-GFP yeast cells with an mRFP-Vps1-URA vector. Once all cells were counted for all 3 sets, statistical analysis was run on the data, computing the average amount of colocalization in each strain, as well as standard deviation and corresponding error bars (Microsoft Excel).
Quantitative Analysis of GFP-tagged Retromer Puncta

Yeast strains containing GFP-tagged copies of all five Retromer component proteins (Vps5, Vps17, Vps26, Vps29, and Vps35) in either wild type Vps1 or vps1Δ were grown in their respective selective medium overnight at 30°C until reaching an OD between 0.6-0.8. Cells were then imaged using fluorescent microscope (ORCA). Once a sufficient number (n = at least 30) of cells were captured for each sample, the pictures were analyzed, counting the number of GFP puncta in each cell. The number of GFP puncta represents the ability of the Retromer complex to be correctly targeted within its functional pathway. Three independent experiments were compiled, and statistical analysis was performed on the data, computing the average number of puncta in each cell type, as well as standard deviation and corresponding error bars (Microsoft Excel).

Genetic Interaction/Synthetic Lethality Assay

Tetrad Dissection. Two strains of S. cerevisiae of two different haploid sex types (1 MATa and 1 MATα), each containing a different gene deletion (e.g.: vps1Δ and vps5Δ) were applied to a YPD plate in parallel lines, and incubated overnight at 30°C. The next day the plate was replica plated twice onto a new YPD plate, this time forming a pound sign by crossing the lines (#). This allows the haploid cells to mate. The next day the (#) YPD plate was replica plated onto a plate of media lacking methionine and lysine (SD/-MET/-LYS), and incubated overnight at 30°C. This plate was used to ensure growth of only diploid cells, signifying a successful mating procedure. The next day cells from the previous plate were replated for isolation, and incubated for 2 nights at 30°C until clearly defined colonies of good size (~2-3mm) were produced. A single colony from this
isolation streak was used to create two spots (~25mm each) on a 2X YPD plate, which was incubated for 12 hours at 30˚C, and then the 25mm spots were replated onto a new 2X YPD plate and incubated for 12 hours at 30˚C. From the second 2X YPD plate, cells were spread onto an MSPO (Minimal SPOrulation) plate in the same 25mm spot fashion.

MSPO is a media used to “starve” cells, which triggers a sporulation process in diploid cells, creating haploid spores. These haploid spores are found as tetrads, very small quadruplets of cells that spawn from a single mother cell. During meiosis, homologous combination causes crossing over of genetic material, hopefully creating a double mutant. The MSPO plate was incubated at room temperature for 2-3 days, at which point a slide was prepared from the MSPO colonies to check for the presence of tetrads. Once tetrads are confirmed on the slide using a light microscope, cells were prepared for dissection.

For tetrad dissection, a small amount of cells from the MSPO plate are suspended in a 1% zymolase solution, which removes the cell wall. This solution was incubated at room temperature for 20 minutes. During this incubation, a YPD plate was cut with two parallel lines in the media using a sterile scalpel in order to form an island. To the zymolase solution of cells Sterile PCR water (100µl) was added to optimize cell density. Of this solution 30µl was applied to one end of the island and allowed to flow down the island to the other side of the plate.

The dissection was performed on a dissection microscope (Nikon 50i). The YPD plate was placed upside down in the scope stage and the scope was focused of the cells covering island. Individual tetrads were picked up off the plate using a glass micro-needle (Singer) and transferred to a section of the plate below the island, where each tetrad was
broken into 4 individual cells, forming a grid. Once a sufficient number of tetrads (6-8) were dissected, the YPD plate was incubated for 2 days at 30°C.

**Genotyping and Synthetic Lethality Growth Assay.** Each cell from the dissection plate was plated onto a new YPD plate in a short (0.5cm) line following the grid pattern from the dissection plate, and incubated overnight at 30°C. This plate was then replica plated onto four different plates (YPD+Kanamycin, SD/-MET, SD/-LYS, and SD/-TRP), each either lacking an amino acid or containing an antibiotic. These plates allow the genotype of the cell to be determined. From these plates, cells containing the genotype of interest (double mutants) were grown in YPD liquid.

Double mutant cells, their respective single mutant parental cells, and wild type control cells were all grown in YPD overnight at 30°C to be used for the Synthetic Lethality Growth Assay. The next day the OD\textsubscript{600} of all cultures was taken, and cultures were diluted to an OD\textsubscript{600} of 1. Serial dilutions were carried out on a 96 well plate using a factor of 5, then plated onto two YPD plates and incubated for 2 days, one at 30°C and one at 37°C. The rationale for that assay is that if the two genes knocked out in the double mutant are functionally related/contained in the same pathway, the cells will show synthetic lethality (death) or synthetic sickness (severely inhibited growth) when grown under stress at 37°C.

**Yeast-Two-Hybrid Vector Construction and Mating Assay**

**Vector construction.** Yeast-Two-Hybrid strains were constructed using the In-Fusion \textregistered HD Cloning Kit User Manual (Clontech, Mountain View, CA). Plasmids were constructed containing 1 bait protein (Vps1) and 5 prey proteins (Vps5, 17, 26, 29, and
The generation of the bait vector, pGBK77-Vps1 utilized the following primers: Forward primer CATGGAGGCCAATTCATGGATAGCATTTATTTTCTAC and Reverse primer of GCAGGTCGACGGATCCAACAG AGGAGACGATTGACTAG.

The generation of the 5 prey vectors (plasmid pGADT7) used the primers as listed in the Primer Table. To clone Vps1 gene into pGBK77 (Clontech), the vector was linearized using BamHI and EcoRI, and the Vps1 gene was amplified using Purified Genomic DNA. Amplified PCR product was then ligated into the linearized pGBK77 vector at the corresponding cloning sites, and the ligated vector plasmid was transformed in *E coli* using the Stellar Competent Cells Protocol PT5055-2 (Clontech), and plated onto Luria broth agar plates containing 25µg/ml kanamycin (LB+KAN). Colony PCR and EcoRI/BamHI restriction digest was used to confirm positive transformants that contain the pGBK77-Vps1 vector. Plasmid vectors of pGADT7 were constructed using the same steps, with the Luria broth plates containing 50µg/ml ampicillin (LB+AMP).

**Mating Assay.** pGBK77-Vps1 plasmid vectors were purified from *E coli* using PureYieldTM Plasmid Miniprep System (A1223, Promega). The purified bait vector pGBK77-Vps1 was transformed into strain Y2H Gold yeast cells (Clontech; MATa) and prey vectors were transformed into strain Y187 yeast cells (Clontech; MATα) using the polyethylene glycol/lithium acetate protocol outlined in Matchmaker® Gold Yeast Two-Hybrid System User Manual (Clontech). Cells were plated on media lacking tryptophan (SD/-TRP) or leucine (SD/-LEU) for BD and AD vectors, respectively. These vectors contain reporter gene for Histidine Synthesis, Adenine Synthesis, Production of α-Galactosidase, and Aureobasidin resistance. Plates were incubated at 30°C for 2-3 day, and positive transformants were verified using colony PCR.
Positive bait and prey colonies were liquid mated using the protocol contained in Matchmaker Gold Yeast Two-Hybrid System User Manual (Clontech). Mated cultures were plated onto stringent media lacking both tryptophan and leucine (SD/-TRP/-LEU), referred to as Double Dropout (DDO), and incubated for 3-4 days at 30°C. Once colonies had grown on DDO plates, they were replica plated onto more stringent media lacking tryptophan, leucine, and histidine (SD/-TRP/-LEU/-HIS), and media lacking tryptophan, leucine, histidine, and adenine (SD/-TRP/-LEU/-HIS/-ADE), designated as Triple Dropout (TDO) and Quadruple Dropout (QDO) respectively. These plates were then incubated for 4-5 days at 30°C.

In order to determine the strength of protein-protein interactions between the bait and prey proteins, spotting assays were performed for each mated set of cells (e.g.: Vps1/Vps5, Vps1/Vps17, and so on). The strength of the interactions is indicated by the number of reporter genes that are activated. Thus, the stronger the interaction, the more stringent media the cells will survive and grow on. To set up the spotting assay, cells were grown in 3 ml DDO liquid media cultures for 2 days at 30°C, at which point the OD$_{600}$ of each culture was measured, and adjusted to an OD of 1.5 via dilution. Serial dilutions were carried out on a 96 well plate using a factor of 5, then plated onto DDO, TDO, and QDO plates, which were incubated at 30°C for 4-5 days.

The relative binding affinities were measured and analyzed, using a growth spotting assay. The mated strains, containing prey vectors with each of the five Retromer proteins and Vps1 as bait, were spotted on three QDO agar plates, following the method described above. After 4 days of incubation at 30°C, the intensity of the spotted colonies
was measured, the background intensity was subtracted, and the adjusted intensities of
the positive control and negative control were normalized to 100% and 0%, respectively.

**Statistical Analysis**

All statistical tests were performed using Student’s T-Test (2 tails, Two-Sample
unequal variance) and results were reported as $p$-values (Microsoft Excel). Statistical
significance was defined as having a $p$-value < 0.05. Standard Deviation is shown on
graphs using error bars.
RESULTS

Vps1 and Retromer Colocalize

Previously, Dr. Kim’s research group at MSU published data that demonstrated Vps1, the yeast Dynamin-like protein, localizes to the Golgi (Lukehart et al., 2013) and to endosomes in yeast cells (Hayden et al., 2013). As both organelles are part of the Retrograde Transport pathway, I hypothesized that Vps1 would colocalize with Retromer as well. During this study, Chi et al 2014 published data that show Vps17 and Vps1 colocalization in vivo. As this is only 1 protein of the Retromer, I strived to test all 5 proteins with Vps1. To investigate this, I constructed yeast strains in which Vps1 was C-terminally tagged with red fluorescent protein (Vps1-RFP) and then C-terminal tagged each individual Retromer protein with a green fluorescent protein (ex: Vps26-GFP), creating 5 distinct strains. I used confocal fluorescent microscopy to evaluate the possible colocalization, which was partially seen in all 5 strains (Fig. 7A). The partial colocalization percentages of the Retromer with Vps1 are as follows: 30.603% ± 8.422 for Vps5, 31.316% ± 8.695 for Vps17, 27.270% ± 6.448 for Vps26, 34.330% ± 7.653 for Vps29, and 33.086% ± 6.205 for Vps35. These results indicate that Vps1 and the complete Retromer do partially colocalize in vivo in yeast cells.

Retromer Proteins Targeted Correctly In the Absence of Vps1

As Vps1 is an important cellular trafficking protein, the loss of Vps1 function has been linked to many severe trafficking defects (Chi et al., 2014; Wang et al., 2011), including those reminiscent of Alzheimer’s and Parkinson’s disease (Small and Petsko, 2015). Here I evaluate the potential effects of the loss of Vps1 on the cellular recruitment
of the Retromer to the endosome, where retrograde transport cargo selection takes place. Two sets of strains were used for this experiment: one set of 5 strains of wild type yeast (WT) containing Retromer proteins C-terminally tagged with GFP (1 stain for each Retromer protein), and a second set containing the identical Retromer-GFP proteins using a \(VPS1\) null mutant cell (\(vps1\Delta\)). Then all 10 strains were transformed with a plasmid that contains the gene for the endosomal marker-recognition module DsRed-FYVE. FYVE is an amino acid motif that binds to PI3P in endosomal membranes (Gillooly et al., 2000). When conjugated with DsRed, FYVE acts as a red fluorescent marker for endosomes. The first set of strains fulfilled two purposes, as both a confirmation that the Retromer resides at the endosome, and as a control for comparison with \(vps1\Delta\) strains. I evaluated the localization of the GFP and RFP proteins using confocal fluorescence microscopy, concluding that there is very little difference in colocalization between the WT and \(vps1\Delta\) strains (Fig. 8A, 9A). Yeast strains containing WT Vps1 showed Retromer-endosome partial colocalization percentages of 23.260% ± 8.369 for Vps5, 26.113% ± 8.173 for Vps17, 25.629% ± 10.013 for Vps26, 23.738% ± 8.861 for Vps29, and 25.742% ± 10.105 for Vps35 (Fig. 8B). Conversely, \(vps1\Delta\) strains showed Retromer-endosome partial colocalization percentages of 28.044% ± 8.742 for Vps5, 22.309% ± 11.069 for Vps17, 24.919% ± 7.711 for Vps26, 24.552% ± 8.683 for Vps29, and 26.928% ± 9.363 for Vps35 (Fig. 9B). When \(vps1\Delta\) strains were compared statistically to the partial colocalization percentages of WT cells, four of the Retromer proteins proved to be not statistically different (Vps17, 26, 29, 35; \(p\)-values in Fig. 9B), while the difference between colocalization of Vps5 in WT and \(vps1\Delta\) strains was statistically significant with a \(p\)-value of \(p = 0.034\). The results of this experiment show that while
Vps1 and Retromer proteins do indeed partially colocalize, Vps1 is not required for the correct targeting of Retromer to the endosome. Additionally, these results suggest the lack of Vps1 does not drastically affect the Retromer-endosome colocalization ratio (Fig. 8B, 9B).

**Retromer Recruitment Is Diminished In the Absence of Vps1**

While I have shown data suggesting Vps1 is not necessary for correct Retromer targeting to endosomes, if Vps1 functions within the retrograde pathway then it is certainly plausible that the loss of Vps1 would affect the efficiency of Retromer function. The way I have chosen to test this hypothesis is by evaluating the amount of Retromer puncta that occurs in cells of both WT (Vps1 containing) and Mutant (vps1Δ) yeast strains. For this experiment, cells that contained GFP-tagged Retromer fusion proteins of both WT and vps1Δ genotypes (constructed for the previous experiment) were grown overnight and their Retromer-GFP localization was evaluated using conventional fluorescence microscopy (n = 30). Four Retromer proteins (Vps5, 26, 29, 35) displayed a marked decrease in Retromer puncta number (Fig. 10A) that was statistically significant (Fig. 10B; p-values listed in legend). In WT cells, the average number of puncta per cell was 7.893 ± 2.052 for Vps5-GFP, 7.702 ± 1.896 for Vps17-GFP, 8.399 ± 2.098 for Vps26-GFP, 8.924 ± 2.297 for Vps29-GFP, and 9.321 ± 2.103 for Vps35-GFP. In vps1Δ cells, the average number of puncta per cell was 6.041 ± 1.957 for Vps5-GFP, 7.608 ± 2.199 for Vps17-GFP, 5.309 ± 1.442 for Vps26-GFP, 6.634 ± 2.074 for Vps29-GFP, and 7.616 ± 2.239 for Vps35-GFP (Fig. 10B). Overall, the data suggests that Retromer recruitment is only minimally affected by the loss of Vps1, indicating that while vps1Δ
show small decreases in Retromer puncta, the differences are not significant enough to imply any sort of real defect in Retromer efficiency to target endosomes.

**Vps35 Genetically Interacts with Vps1**

When two proteins are involved in the same pathway or cellular process, they are said to be genetically interacting. Therefore, I sought to determine if Vps1 showed evidence of genetic interaction with any of the 5 Retromer proteins. To test this, I used what is called a Synthetic Lethality Assay (Fig. 11). I constructed double null mutants of each Retromer protein with Vps1 \((vps1\Delta vps5\Delta, vps1\Delta vps17\Delta, vps1\Delta vps26\Delta, vps1\Delta vps29\Delta, \text{and} vps1\Delta vps35\Delta)\) and evaluated them for Synthetic Lethality. The only Retromer protein that showed Synthetic Lethality was that of Vps35 (Fig. 11), though it can be argued that the other 4 Retromer proteins showed slight Synthetic Sickness (abnormal growth) as opposed to death. This evidence suggests that Vps35 and Vps1 do indeed interact on the genetic level, and thus are required for yeast to survive in stressed conditions.

**Vps1 Physical Interacts with Several Retromer Proteins**

While Genetic Interaction assays can determine whether or not two proteins function within the same or parallel pathway(s), it does not have the ability to detect whether two proteins physically bind with each other *in vivo*. Proteins that are functionally related in a pathway often physically bind to one another as a part of their function in the pathway. Given that Vps35 showed evidence of genetic interaction, the logical next step was to test for physical interaction. In order to investigate whether Vps1 and each of the Retromer proteins bind to each other inside yeast cells, I performed a Yeast-Two-Hybrid Physical Interaction Assay. Of the 5 Retromer proteins, 4 showed
evidence of physical interaction with Vps1. Cells containing BD-Vps1 and AD-Vps5, AD-Vps26, AD-Vps29, or Vps-35 showed growth on QDO (Quadruple DropOut, SD/-Ade/-His/-Leu/-Trp) medium (Fig. 12A), indicated the activation of both the HIS and ADE reporter genes. The HIS reporter gene that codes for the production of histidine, an amino acid which QDO medium lacks, while the ADE reporter gene codes for the production of adenine, a nucleobase that QDO medium also lacks. Growth of QDO medium is evidence for a strong physical interaction between two proteins. The positive control, consisting of known binding partners SV40 Large T-Antigen and p53 also showed growth on QDO plates (Fig. 12A). In order to quantitate the binding affinities between Vps1 and Vps5, 26, 29, and 35, the mean integrated density (IntDen) value of the positive control colonies from 3 experiments was normalized to 100%, and the relative cell density of the experimental colonies were compared to that of the positive control. The relative cell densities of four Retromer proteins compared to the positive control are as follows (Fig. 12B): Vps5 showed a relative cell density of 30.40% ± 23.8%, while Vps26 showed a cell density of 12.99% ± 23.1%. Vps29 showed a cell density of 54.7 ± 12.8% and Vps35 showed a cell density of 41.8 ± 25.3%. The higher the relative cell density, the stronger the interaction, which causes more cell growth on stringent media. Using this evaluation method, the four Retromer proteins, in order of strength of physical interaction with Vps1 is as follows: Vps29, Vps35, Vps5, Vps26. Therefore, Vps29 and Vps35 show the strongest binding affinity with Vps1 while Vps26 shows the weakest of those that grew on QDO. Vps17 did not show any quantifiable growth on QDO plates and very weak growth on TDO plates (Fig. 12), leading to the assumption that Vps17 and Vps1 have a transient, weak interaction.
DISCUSSION

Throughout the entirety of this study, our lab has been on the leading edge of research in the yeast Retromer field. Vps1 is well established trafficking protein in yeast, yet its possible relationship with the Retromer has yet to be fully investigated. Therefore the Vps1-Retromer topic was a novel area that I have now provided novel insight into this relationship. While there are a lot of questions my data brings into light, my findings raise new questions previously unasked in the realm of yeast research. By investigating this possible relationship, I have opened a new realm of research directions for not only our own lab, but for the yeast Retromer field. This study stands as a large achievement within the scientific community here at our university.

Vps1 has been previously shown to localize to the endosome (Hayden, Williams et al. 2013), as well as colocalize with Retromer protein Vps17 (Chi, Liu et al. 2014). Whereas the aforementioned study only showcased colocalization between Vps1 and Vps17, here I have expanded the investigation of this relationship to include all 5 proteins of the Retromer complex in yeast. While it is possible that the colocalization of Vps1 and Vps17 supports the foundation for the argument that Vps1 is involved in the Retromer pathway, without confirmation that Vps1 colocalizes with both major subcomplexes of the Retromer, this argument is incomplete. Thus, I have provided new data, confirming that all five Retromer proteins partially colocalize with Vps1 inside the yeast system. The two proteins only partially colocalize for a few reasons. First, while Vps1 and the Retromer do colocalize, they don't function completely dependently of one another within the cell. That means that Vps1 isn't only present at the endosome, as described
previously. Second, Vps1 has been shown to localize with most biological membranes in yeast, most recently with the ER protein Ste24 (personal communication with Bryan Banh). This localization is likely due to an intrinsic membrane binding domain. While no distinct binding domain exists in Vps1, there is a sequence that lies between the GED and Middle domains that may exhibit this ability. If this sequence does indeed bind to membranes, this domain would likely have a role in targeting Vps1, a yet to be determined aspect of the protein. This possible recruitment could help further explain how Vps1 is recruited to the endosome. Furthermore, the presence of Vps1 and the Retromer colocalized puncta hints at a possible functional relationship between Vps1 and the Retromer complex that has yet to be fully investigated. This colocalization may be a result of interdependency between Vps1 and the Retromer within retrograde transport, which logically involves recruitment to the endosome.

The recruitment of the Retromer complex to the endosome is a highly debated issue that is vital to the understanding of how the complex correctly identifies and sorts through several known cargoes (Harbour et al., 2010; van Weering et al., 2012). Therefore, if Vps1 is implicated as an important player in Retromer function (Chi et al., 2014), it is entirely possible that Vps1 may play a role in the recruitment of the Retromer. This was an area of the Vps1-Retromer relationship that was largely unstudied. Based on colocalization data with Vps1, I investigated the possible role of Vps1 in Retromer recruitment. Several previous studies have reported the cellular localization of the Retromer components to the endosome (Belenkaya et al., 2008; Chi et al., 2014; Hayden et al., 2013; Seaman, 2004, 2007), so colocalization between the Retromer proteins and the endosomal marker DsRed-FYVE was an expected result. However, in cells lacking
Vps1 (\textit{vps1}\Delta), it is largely unknown if Retromer localization will be affected. Given the loss of Vps1 causes a host of trafficking defects (Hayden et al., 2013; Lukehart et al., 2013; Wang et al., 2011), so it is a likely assumption that Retromer recruitment could be affected. However, in my study, all five components of the yeast Retromer showed an unaffected ability to correctly target late endosomes in \textit{vps1}\Delta cells. While this is too surprising of a finding, it does help to further categorize the defects in \textit{vps1}\Delta cells. Also, it suggests that Retromer recruitment to the endosome and Vps1 recruitment to the endosome are completely independent. This means that while the two both end up at the endosome, Vps1 is not required for the Retromer to be recruited. Interestingly, the potential role of Vps1 in the function of Retrograde Transport clearly must not occur before the recruitment of either Vps1 or the Retromer to the endosome. If this is true, then by what mechanism might Vps1 use to recognize and associate with the Retromer?

The Retromer Tubulation Complex, (Vps5/17) contains both PX and BAR domains, which intrinsically bind membranes. This allows Vps5/17 to be targeted to PI3P in endosomal membranes. The Retromer CRC (Vps26/29/35) contains domains that target the CRC to cargo proteins present at the endosome. The combined action of all these domains correctly targets the Retromer to the endosome. Here I have shown that this process is unaffected in the absence of Vps1, which leads to the question of how exactly Vps1 is recruited and targeted to the endosome. It is possible that Vps1 is recruited by the Retromer, though I have not fully explored this possibility. Notably, I have only investigated one half of the question here. It remains to be seen whether the Retromer is required for successful Vps1 recruitment to the endosome. Also, if the Retromer will assemble at the endosome without Vps1, and if Vps1 is acting as the scission molecule
for Retromer coated vesicles, what happens to the Retromer when Vps1 is not present to pinch off tubules? Does the Retromer simply stay attached to the cargo on the tubule membrane? Or does the Retromer recognize the lack of Vps1 and dissociate from the membrane?

Despite the non-requirement of Vps1 in the recruitment of the Retromer, this does not rule out a further relationship between the Retromer and Vps1. In fact, data from a previous study by Chi et al., 2014 provided evidence that vps1Δ cells show a marked increase in Retromer puncta number. In this study, puncta count, and thus the targeting ability of Vps17 and Vps26, showed large increases in number when Vps1 was knocked out. However, in my experiment using all five Retromer proteins, I observed slightly contradictory data to the previous study. While the decreases I observed are not of large magnitude, they are found to be statistically relevant in 4 of 5 cases. It should be noted that my experiment focused on a single plane of view within the cell, at approximately the center of each cell. This is important because my puncta numbers in general are lower than in the 2014 Chi study where they counted total puncta throughout entire cells. So while my view of focus was not as expansive, the center of the cell is most often a good indicator of complete cell expression levels; a decrease in the center plane of focus correlates to a decrease in number throughout the cell. If this is the true case, what is causing the loss of puncta number? I hypothesize that this decrease in puncta number in vps1Δ cells versus WT cells is a sign of mild downregulation of the Retromer proteins themselves, and indicative of a possible overall downregulation of traffic within the retrograde transport system. The most logical explanation would be that vps1Δ cells show lowered expression levels of the Retromer proteins when compared to WT, an
experiment yet to be completed. If lowered Retromer protein expression is the outcome of this experiment that would tend to strengthen the tie of the Retromer to neurodegenerative diseases such as Alzheimer’s and Parkinson’s, which both display lowered expression of Retromer (Deng et al., 2013; Muhammad et al., 2008; Small, 2008; Small and Petsko, 2015). These results are contradictory to the experiment of Chi et al., 2014 in two major ways. First, I did not see as large a magnitude of difference in the puncta number in either WT or vps1Δ cells. Though my data shows statistical significance, the WT cells are still only a few puncta higher than the vps1Δ cells on average, whereas in their study the difference was very large. Second, the difference I did see in puncta number was the reverse of the Chi et al., 2014 experiment. Over the whole Retromer, WT cells showed a larger number of puncta per cell when compared to the vps1Δ cells. This is divisively different from what was reported in the 2014 study. Therefore it is my observation that vps1Δ cells do show a lower average number of puncta than WT cells, a discrepancy I attribute to a possible downregulation in Retromer expression. Alternatively, it could be theorized that the decrease of Retromer puncta could be the upshot of inefficient scission of Retromer-coated vesicles at the endosome. Vps1 has been previously implicated to act as a scission protein in many intracellular trafficking pathways (Ferguson and De Camilli, 2012). If Vps1 does in fact act as the scission protein for retrograde transport, one could surmise that the loss of this function would cause inefficient release of budded Retromer-coated vesicles, leading to the decrease in Retromer puncta. This dysfunction would likely lead to an accumulation of Retromer cargo proteins in the late endosome, an avenue for future yeast Retromer research.
While the localization of Vps1 and the Retromer has been previously identified, the possibility of these proteins being functionally related has yet to be fully explored. In effort to fully explore this possible relationship, the genetic relationship of the Retromer and Vps1 was tested. For two genes to be “Genetically Interacting,” they must be related in function by way of their respective proteins, meaning that the proteins made from the two genes have some kind of functional relationship in a cellular pathway. The concept of synthetic sickness implicates a functional relationship in one of three pathways: A Linear Pathway, a Multiprotein Complex, or a Parallel Pathway. Option 1 involves proteins being involved in the very same pathway, with one either serving as down or upstream regulator of the other, whereas option 2 involves the two proteins binding into a multiprotein complex in the pathway. With option 2 it is possible the two proteins could be overlapping in function within the complex, or they could serve completely separate functions in the complex. Option 3 involves the two proteins being the primary regulators in parallel pathways, which means they can possibly compensate for a lack of the other. While my evidence only implicates one Retromer protein (Vps35) to be genetically interacting with Vps1, it also shows what may be synthetic sickness in Vps17, Vps26, and Vps29, though the weakness of the double knockout cells is very small. While the sickness may be present, the most relevant data is the synthetic lethality displayed between Vps1 and Vps35. My hypothesis is that options 1 and 2 are the most likely explanation for the functional relationship between Vps1 and Vps35. However, it is not possible to differentiate between the 2 options with genetic interaction alone. In following with the concept of the synthetic lethality assay, a cell lacking both Vps1 and Vps35 would be expected to show severe defects in intracellular trafficking. As explained
above, \textit{vps1}\textDelta cells show large trafficking defects (Hayden et al., 2013; Lukehart et al., 2013), and both AD and PD pathologies involve a deficiency of Vps35 (Deng et al., 2013; Small and Petsko, 2015). Therefore, if both proteins were knocked out, the cell would be under severe stress. Vps1 is implicated to be involved almost universally in trafficking and Vps35’s role as the cargo binding protein in the Retromer highlights it as the most important Retromer protein to the complex as a whole. Basically cells with neither protein would be improperly trafficking important molecules, would have extremely limited recycling ability, and normal proteins recycled by retrograde transport would possibly accumulate in the endosomes and vacuole. The lack of efficient recycling would affect the cells ability to function optimally. This activity alone would be taxing on the cell, and could explain the cells inability to survive in stressed conditions.

Considering both of these possible mechanisms, it makes sense that Vps1 and Vps35 must have some kind of functional relationship within the pathway of retrograde traffic.

Genetic interaction can be an indicator of physical interaction between proteins. The rationale is that if two proteins are located in the same area of the cell (colocalized) and are functionally related genetically, it is a likely possibility that those two proteins are also interacting on the physical level, most likely by binding to each other. My data demonstrate that not just Vps35, but also Vps5, Vps26, and Vps29 show evidence of physical interaction with Vps1. The positive control, containing known binding partners Large T Antigen and p53 shows a very strong interaction, while the negative control, cells containing Large T Antigen and Lamin show absolutely zero interaction. Interestingly, Vps17 only shows a possible weak, transient interaction, despite its close functionality and similar amino acid sequence to Vps5. This poses a few puzzling
questions, largely due to the functional overlap of Vps5 and Vps17, as well as both proteins being fairly homologous to one another (Horazdovsky et al., 1997; Seaman and Williams, 2002). However, the data does give legitimacy to the possibility of the CRC (Vps26-Vps35-Vps29) directly binding Vps1. This opens the possibility of the CRC and Vps1 being able to recognize each other, which could lead to a possible recruitment mechanism, a point of contention described earlier. If it is possible for Vps1 to bind the CRC, which could further explain the relationship between the Retromer and Vps1, as well as give solid support to the argument that Vps1 may act as the scission protein for the retrograde transport pathway. Certainly if the Retromer exhibits the ability to bind to Vps1 \textit{in vivo}, this provided evidence for the scission protein hypothesis, as binding the Retromer could be the mechanism by which Vps1 targets the endosomal tubule in order to perform the scission function. Perhaps Vps1 recognizes the CRC and binds it, then assembles around the tubule in order to pinch of vesicles. This hypothesis would mean that the Retromer is recruited before Vps1.

**Conclusions**

Taken together, my data provides further evidence towards the possibility of Vps1 to act as a scission protein in Retrograde Transport, as I have presented a basis for a relationship between the Retromer and Vps1. Further research must be done into the physical interaction relationship, as well as mechanistic studies that may be able to identify a more concrete recruitment profile for the Retromer. Nevertheless, the data shown here gives an extensive look into the localization and functional relevance of Vps1 to the Retromer. In addition to recruitment, my data lends evidence that may strengthen the Retromer-Alzheimer/Parkinson’s link, and hopefully can provide further insight into
the pathology of these diseases. In the future, I am hopeful that the entirely of the Retromer/retrograde transport system can be discovered and characterized, as it would be incredibly helpful to neurodegenerative and other human disease. Though it cannot be said with certainty, I present that these findings deliver a pivotal step in the clarification of the Retromer system, with specific insight into the relationship between the Retromer and Vps1.

**Future Directions**

Moving forward from these results, there are several future experiments that would help solidify the data I collected. The Yeast-Two-Hybrid physical interactions results reported here need to be confirmed using an alternative protein-protein binding assay, such as a GST Pulldown Assay. The recruitment question needs to be completed, determining whether or not Vps1 is able to colocalize to the endosome in the absence of the Retromer. The decrease in Retromer puncta must be explored, and qPCR can be used to determine what change, if any, occurs in the expression of the Retromer in WT versus \( vps1\Delta \) cells. Also, a localization experiment of the Retromer cargo Vps10 to see where Vps10 accumulates in WT versus \( vps1\Delta \) cells would further shed light on the possible role of Vps1 as the scission protein for retrograde transport. These experiments are just a few possible examples our lab may be able to investigate in the field of yeast Retromer.
REFERENCES


association study identifies a novel major locus for glycemic control in type 1 diabetes, as measured by both A1C and glucose. Diabetes 59, 539-549.


Table 1. Mammalian Retromer Cargo. Examples of Mammalian Retromer Cargo proteins. Known mammalian cargoes listed by name, common notation, function, and reference.

<table>
<thead>
<tr>
<th>Cargo Name</th>
<th>Notation</th>
<th>Function</th>
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<td>Cation-Independent Mannose-6 Phosphate Receptor</td>
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<td>Mannose-6 Phosphate receptor</td>
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<td>Wntless (mammalian homolog GPR177)</td>
<td>Wls</td>
<td>Wnt recycling factor</td>
<td>(de Groot et al., 2013)</td>
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<td>Divalent Metal Transporter 1</td>
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<td>(Tabuchi et al., 2010)</td>
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<td>Beta-2 adrenergic receptors</td>
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<td>(Priller et al., 2006)</td>
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Table 2. Yeast Strains Used In This Study

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Figure 1: Intracellular Trafficking Pathways in Mammalian and Yeast (S. cerevisiae) (Trousdale and Kim, 2015). A: Endocytosed cargo may consist of several different types of proteins, from receptors to nutrients to signal molecules. Upon completion of Endocytosis (pink), cargo can be delivered to several different final places within the cell. Post-endocytosed vesicles are targeted to early endosome (EE) that eventually matures into late endosomes (LE). From LE, cargo can either be trafficked to the lysosome for degradation (yellow arrows), or trafficked to the Golgi via Retrograde Transport (blue arrows). Retrograde transport is a major recycling pathway for endocytosed proteins. In addition, secretory proteins that are manufactured in the ER are trafficked to the Golgi, then to the plasma membrane. This pathway is referred to as the Secretory pathway (green arrows). B: Fates of endocytosed molecules in Saccharomyces cerevisiae. Endocytosed cargo can immediately travel back to the plasma membrane (red). Through Retrograde Recycling (blue), where proteins are sorted at the endosome and trafficked to the Golgi, cargo can be redistributed to the plasma membrane via secretory vesicles (SV). Notably, the Golgi in yeast is not stacked like it is in the mammalian system, instead existing as independent Cis, Medial, and Trans versions. Some cargoes selected at LE are trafficked to the vacuole in which they are degraded by enzymes.
Figure 2: Retromer Complex-Mediated Tubule Formation at the Late Endosome (Trousdale and Kim, 2015). A: the Cargo Recognition Complex (CRC) is recruited by SNX-BAR dimers bound to the endosomal membrane. Vps35 mediates the cargo selection by directly binding to the CPY receptor Vps10. Vps10 is the Retromer target cargo being transported to the Golgi. B: Tubules being formed off the membrane of the Late Endosome, coated with the 2 subcomplexes of the Retromer. After vesicles are successfully pinched off from the tubule, the Retromer begins the process of uncoating, likely heading back to the endosome to bind more cargo.
Figure 3: Pathways by which HIV-1, HPV, and HVS exploit the Retromer during infection of cells (Trousdale and Kim, 2015). **A:** Human Immunodeficiency Virus (HIV-1) infects the cell and integrates its genome into the host cells genome by way of its Reverse Transcriptase/DNA Integrase activity. From this point the viral genome is transcribed and viral proteins are synthesized from the resulting mRNA. Viral components, such as Envelope proteins (Env) are trafficked to the plasma membrane where new virions are assembled as they bud from the host cell. Proteins that are not integrated into virions are endocytosed, recycled from the late endosome to the Golgi using the Retromer, and then redistributed back to the plasma membrane for incorporation into virions. **B:** Human Papillomavirus (HPV) binds to host cells and is endocytosed. Once the virus inside the endosome reaches the late endosome stage, major and minor capsid proteins, L1 and L2, respectively, bind to Retromer CRCs, allowing the virus to disassemble the capsid, releasing the viral genome into the cytoplasm while effectively avoiding acidification of the endosome as it fuses with a lysosome. **C:** Herpesvirus Saimiri (HVS) produces a protein called Tip that physically binds to Vps35 at the endosome, blocking its activity, rendering the cells ability to recycle receptor proteins nonfunctional. This results in the downregulation of CD4 on the cell surface.
Figure 4: Pathway in which Shiga Toxin (Stx) utilizes the Retromer in its pathogenic cycle (Trousdale and Kim, 2015). Bacterial pathogen *Shigella dysenteriae* secretes a toxin known as Shiga-Toxin (Stx), composed of 1 α-subunit (red) and 4 β-subunits (blue). Stx binds to toxin receptors on the cell surface, and is endocytosed by gut epithelial cells and then trafficked to the *Trans*-Golgi Network (TGN) by way of Retromer-mediated retrograde transport. After moving from the TGN to the *cis*-Golgi, the toxin is transported to the Endoplasmic Reticulum (ER), where it blocks protein synthesis. This inhibition eventually leads to cell death, which in turn causes major symptoms of Shigellosis, a form of dysentery.
Figure 5: Retromer Dysfunction and Disease (Trousdale and Kim, 2015). A: Alzheimer’s disease (AD) is thought to be caused by improper cleavage of APP. In the non-amyloidogenic pathway, APP is endocytosed, and is cleaved by α-secretase in the endosome, and trafficked via SORLA through retrograde recycling to the plasma membrane, where it is cleaved by γ-secretase. In the amyloidogenic pathway known Retromer cargo protein BACE1 is recycled between the Endosome and Golgi via the Retromer, resulting in possible APP cleavage via a β-secretase called BACE1 (β-site APP cleaving enzyme) in the endosome. When improperly cleaved APP reaches the plasma membrane, γ-secretase cleaves the protein, resulting in the release of Amyloid Beta protein (Aβ), which aggregates into Amyloid Plaques, a hallmark feature of AD. B: Parkinson’s disease (PD) is linked to the Retromer in three main ways: 1. Mutation of the Vps35 protein, specifically the p.D620N mutation present in about 1% of familial autosomal inherited PD. Proteins showing this mutation have been characterized by Retromer coated endosomes being mislocalized to the nuclear area of the cell. 2. PD tissue samples have shown increased levels of iron in cells, likely due to mislocalization of Divalent Metal Transporter II (DMTII) to the lysosome in cells with Retromer deficiency. In addition to DNTII, WNT Signaling molecule Wls has also been shown to be mislocalized to the lysosome. 3. Cathepsin D. When Retromer function is impaired, CIMPR is inefficiently trafficked to the endosome; therefore Cathepsin D does not reach the lysosome. This causes a buildup of α-synuclein, which is excreted and aggregates to form Lewy Bodies, a hallmark feature of PD.
Figure 6: Vps1’s Proposed Action at the Late Endosome. A vesicle coated in with the Retromer complex containing Retromer cargo protein Vps10 forms a tubule as it buds off of the late endosome. Dynamin-like Protein Vps1 is proposed to come in and bind to the membrane near the base of the tubule, at which point GTP binds the spiral-chain-like oligomer of Vps1 and is hydrolyzed by Vps1’s GTPase domain. This triggers a conformational change in the Vps1 proteins, extending the spiral upwards along the tubule, effectively pinching off the vesicle for transport to the Golgi. **Inset:** A newly pinched off Retromer coated vesicle begins to uncoat as it traffics towards the Golgi.
Figure 7: Retromer Proteins Colocalize with Vps1. A: Representative images of wild type strains expressing N-terminal GFP tagged Retromer proteins and N-terminal RFP tagged Vps1. Arrowheads indicate colocalized puncta. All 5 Retromer proteins show colocalization with Vps1 in vivo. B: Quantification of colocalization between Retromer-GFP and Vps1-RFP puncta. The average colocalization percentage shown is out of 100% with error bars showing Standard Deviation.
Figure 8: Retromer Proteins Localize to the Endosome in WT Cells. 

A: Retromer proteins colocalize with the Endosome. Representative images of wild type strains expressing N-terminal GFP tagged Retromer proteins and the Endosomal marker DsRed-FYVE. Arrowheads indicate colocalized puncta. All 5 Retromer proteins show colocalization with DsRed-FYVE. 

B: Quantification of Retromer-GFP colocalization with DsRed-FYVE. The average percentage of colocalization in each cell shown is out of 100% with error bars showing Standard Deviation.
Figure 9: Retromer Proteins Localize to the Endosome in vps1Δ Cells

A: Retromer proteins colocalize with the Endosome. Representative images of vps1Δ strains expressing N-terminal GFP tagged Retromer proteins and the Endosomal marker DsRed-FYVE. Arrowheads indicate colocalized puncta. All 5 Retromer proteins show colocalization with DsRed-FYVE.

B: Quantification of Retromer-GFP colocalization with DsRed-FYVE. The average percentage of colocalization in each cell shown is out of 100% with error bars showing Standard Deviation. P-values for statistical comparison of WT (Fig. 8) and vps1Δ (Fig. 9) partial colocalization: Vps5 p = 0.034, Vps17 p = 0.136, Vps26 p = 0.760, Vps29 p = 0.72, Vps35 p = 0.639.
Figure 10: Retromer Targeting is Decreased in Cells Lacking Vps1. A: Representative images of wild type and vps1Δ strains expressing N-terminal GFP tagged Retromer proteins. B: Quantification of Retromer targeting in WT and vps1Δ strains with error bars showing Standard Deviation. Cells were grown in selective medium, imaged, and the number of puncta were counted in each cell (n = 75). Data analyzed by two-tailed, unpaired student’s T-test, giving p-values for each Retromer proteins: Vps5 p = 6.83E-11, Vps17 p = 0.181, Vps26 p = 5.14E-31, Vps29 p = 7.86E-12, and Vps35 p = 1.56E-09.
Figure 11: Vps1 and Vps35 genetically interact. Dilutions of haploid double mutants (ex: \textit{vps1}\Delta\textit{vps5}\Delta) were grown on YPD plates for 2 days at 30°C. Abnormal or Inhibited growth indicate Synthetic Sickness/Lethality, respectively. The double mutant \textit{vps1}\Delta\textit{vps35}\Delta shows no growth (Synthetic Lethality) at 37°C, suggesting a functional relationship between Vps1 and Vps35 \textit{in vivo}. 
Figure 12: Vps1 Physically Interacts with Several Retromer proteins. A: A dilution series of cell strains containing both AD and BD plasmids. Growth on stringent media (TDO/QDO) indicates strength of interaction based on activation of reporter genes (ADE/HIS). Vps5, Vps26, Vps29, and Vps35 growth on QDO suggest Vps1 physically binds with these Retromer proteins in vivo. B: Quantification of Retromer-Vps1 Yeast-Two-Hybrid physical interaction assay. Relative cell densities on QDO media were used to calculate representative binding affinities for all strains tested, with error bars showing Standard Deviation.