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The Functional Relationship between the Retromer and Yeast Dynamin at the Endosome

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Abstract:
Membrane trafficking from the late endosome to the 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). The 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 a range of intracellular trafficking pathways necessary for endocytic, secretory, and recycling pathways. Vps1 acts as a scission protein in yeast endocytosis by binding to actin. The retromer and Vps1 are hypothesized to work in concert in the retrograde pathway, with Vps1 acting in a similar manner to its role in endocytosis. We found that Vps1 and retromer colocalize in cells, and both localize to the endosome in yeast. Functionally, Vps1 and retromer component protein Vps35 exhibit evidence of a genetic relationship. Though Vps1 displays no ability to bind to individual retromer components in vivo,double deletion of Vps1 and retromer componentsresults in more severe defects in Vps10 receptor trafficking when compared with cells lacking Vps1 or retromer components.

Keywords: *retromer, dynamin, Vps1, relationship, Vps10, Vps1, and endosome.*

1. Introduction

The vesicular traffic from the endosome towards the Golgi is known as retrograde transport (Bonifacino & Hurley, 2008). Retrograde transport is a vital trafficking process in regards to cellular homeostasis, as several proteins depend on this recycling in order to function. Vps10, the most prominent example in *Saccharomyces cerevisiae*, requires recycling between the endosome and the Golgi for proper transport of its cargo, Carboxypeptidase Y (CPY) (M. N. Seaman, Marcusson, Cereghino, & Emr, 1997; M. N. J. Seaman, Michael McCaffery, & Emr, 1998). CPY is made in the ER as its precursor protein pro-CPY, trafficked to the Golgi, then transported via Vps10 to the endosome, and from the endosome to the vacuole, where it is processed into functional CPY (Marcusson, Horazdovsky, Cereghino, Gharakhanian, & Emr, 1994). A few commonly known mammalian proteins that depend on retrograde trafficking include: the mammalian CPY homolog Mannose 6-Phosphate Receptor (M6PR) (Arighi, Hartnell, Aguilar, Haft, & Bonifacino, 2004; M. N. Seaman, 2004), Divalent Metal Transporter II (DMTII) (Tabuchi et al., 2010), and Amyloid Precursor Protein (APP) (Vieira et al., 2010).

Retrograde transport is mediated by a protein coat complex known as the Retromer, which is responsible for binding cargo proteins (e.g. Vps10) and binding endosomal membrane to induce membrane curvature (Bonifacino & Hurley, 2008). In yeast, the Retromer is composed of five Vps (Vacuolar Sorting Protein) proteins: Vps5, Vps17, Vps26, Vps29, and Vps35(M. N. J. Seaman et al., 1998). The Retromer exists as two subcomplexes that assemble into a stable heteropentamer (Bonifacino & Hurley, 2008). The first subcomplex, known as the tubulation complex (TC), is composed of Vps5 and Vps17 heterodimer, alternatively known as the SNX-BAR dimer, as both Vps5 and Vps17 are homologs of the mammalian SNX (Sorting Nexin) proteins, SNX1/2 and SNX5/6, respectively (Bonifacino & Hurley, 2008; Griffin, Trejo, & Magnuson, 2005). These mammalian SNX proteins contain a BAR domain, through which they heterodimerize (Horazdovsky et al., 1997; van Weering, Verkade, & Cullen, 2012), and a Phox (PX) domain through which they are able to bind endosomal membranes (M. N. Seaman & Williams, 2002; Song et al., 2001). The second

subcomplex is known as the cargo recognition complex (CRC), and is a trimer of Vps26,Vps35, and Vps29, in both yeast and mammalian versions (Haft et al., 2000). The CRC is responsible for binding cargo for trafficking via retrograde transport. In addition to this, the CRC binds to the SNX-BAR dimer, thus forming the heteropentamer in yeast (Bonifacino & Hurley, 2008).

While retrograde transport is an extensively studied mechanism in both yeast and mammalian systems, there is a largely unknown component to its function. As with most vesicular traffic derived from membranes, a scission protein is often present with the explicit function of pinching off vesicles as they bud off a parent membrane. While this process can be aided by both actin filaments(Palmer et al., 2015)and a motor proteins (Granger, McNee, Allan, & Woodman, 2014), the action of a scission protein greatly enhances the efficiency of the pinching off of these vesicles. In mammalian endocytosis, a protein known as dynamin fills this role. Dynamin uses a GTPase domain that hydrolyzes GTP to accomplish its function (Ekena, Vater, Raymond, & Stevens, 1993; Vater, Raymond, Ekena, Howald-Stevenson, & Stevens, 1992). Dynamin forms an oligomer in 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 of the dynamin polymer, which causes the scission of vesicles budding from plasma membranes during endocytosis (Ekena et al., 1993; Ferguson & 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 Middle domain, and the GED domain. Vps1 has been linked to several different intracellular trafficking pathways in yeast including endocytosis, endosomal, anterograde, and retrograde pathways (Burda, Padilla, Sarkar, & Emr, 2002; Ekena et al., 1993; Goud Gadila et al., 2017; Hayden et al., 2013; Lukehart, Highfill, & Kim, 2013; Saimani, Smothers, McDermott, Makaraci, & Kim, 2017; Wang, Sletto, Tenay, & Kim, 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 & De Camilli, 2012). Additionally, cells in which Vps1 is knocked out show severe recycling defects (Goud Gadila et al., 2017; Saimani et al., 2017),which resembles those of retromer-deficient cells in both Alzheimer's and Parkinson's disease pathology(Small & Petsko, 2015).

Vps1 has been shown to be present at the endocytic site, most likely to act as a tubulation/scission factor for invaginating vesicles, and has a capacity to bind actin at the neck of such vesicles (Palmer et al., 2015). In addition, Vps1 has previously shown to be located at the endosome (Banh et al., 2017), and thus it is possible Vps1 is performing a similar function to its role in endocytosis. Indeed, two recent studies revealed that Vps1 is implicated in aiding in the scission of retromer-coated vesicles emerging from the endosome and the vacuole (Arlt, Reggiori, & Ungermann, 2015; R. J. Chi et al., 2014), initiating an exploration of the potential functional relationship between the retromer and Vps1. In these efforts, they only reported that Vps1 colocalizes with Vps17, a retromer protein. We sought to further characterize the spatial relationship between Vps1 and the retromer in this study, and we present here that all five retromer components display colocalization with Vps1. However, it appears that the recruitment of retromer components to the endosome does not depend on the presence of Vps1. Finally, we provide evidence for a functional relationship between retromer and Vps1, as manifested by a synthetic lethal effect in a double mutant stain lacking Vps1 and Vps35.Here, the possible relationship between retromer and Vps1 is explored in depth, furthering expanded the implication that Vps1 may function in scission of retromer coated vesicles as a part of retrograde transport.

2. Materials and Methods

2.1. Strain Construction

Yeast strains used in this study and their genotypes are listed in Table 1. Green and Red Fluorescent Protein tagged fusion proteins (GFP and RFP, respectively) were constructed using either a GFP or RFP sequence integrated at the 3' end of the gene coding region through homologous recombination as previously described (Bartlett et al., 2015; Longtine et al., 1998). 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+Kanamycin, SD/-Histidine, or SD/-Tryptophan), and positive transformants were confirmed using colony PCR. Resulting positive colonies were grown in liquid medium lacking in nutrients required to maintain selectivity for positive colonies. Plasmids encoding DsRed-FYVE(Odorizzi, Babst, & Emr, 1998)and Vps10-GFP(gift from Dr. Gerst, Weizmann Institute of Science) were introduced into yeast strains using a one-step transformation protocol as previously described (Chen, Yang, & Kuo, 1992), following which cells were plated on selective media lacking leucine (SD/-Leucine). Positive colonies were confirmed using fluorescent microscopy.

2.2. Fluorescence Microscopy

GFP and 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 were 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). Colocalization images were 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 (DV2, Photometrics) to separate red and green emission signals.

2.3. Tetrad Dissection for Genetic Interaction

Single null mutant strains (haploid) were constructed, including a *vps1*Δ strain of sex type *MATα* and individual retromer knockouts (e.g. *vps5*Δ) of sex type *MATa*. These were mated using a plate method, and grown on minimal media to induce tetrad formation. Tetrads were dissected using a dissection microscope (Nikon 50i) and grown for 2 days. Resulting colonies were genotyped to confirm double null mutant genotype. WT, single, and double null mutant strains (e.g. *vps1*Δ*vps5*Δ) were grown at 30˚C and 37˚C as part of a spotting Assay.

2.4. Physical Interaction

Yeast-Two-Hybrid strains were constructed as described in the In-Fusion ® HD Cloning Kit User Manual (Clontech Laboratories, Mountain View, CA). To clone *VPS1* gene into pGBKT7 (Clontech), the vector was linearized using *BamH*I and *EcoR*I, and the *VPS1* gene was amplified using a purified genomic DNA template. The amplified PCR product was then ligated into the linearized pGBKT7 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 Vps1 in the pGBKT7. Each retromer gene was cloned into pGADT7 using the same steps described above, with the Luria broth plates containing 50µg/ml ampicillin (LB+AMP).

pGBKT7-Vps1 plasmid vectors were purified from *E coli* using Pure Yield™ Plasmid Miniprep System (A1223, Promega, Madison, WI). The purified bait vector pGBKT7-Vps1 was transformed into strain Y2H Gold yeast cells (Clontech; *MATa*) and prey vectors harboring retromer genes 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. Plates were incubated at 30˚C for 2-3 day, and positive transformants were verified using colony PCR.

Positive bait and prey colonies were mated using the protocol contained in Matchmaker Gold Yeast Two-Hybrid System User Manual (Clontech). The matedcells were spotted onto stringent media lacking both tryptophan, leucine, and histidine (SD/-TRP/-LEU/-HIS), referred to as Triple Dropout (TDO), and incubated for 3-4 days at 30˚C.

2.5. Spotting Assays

For Genetic Interaction: WT, single, and double null mutants were spotted on YPD plates after being diluted by a factor of 5 and grown for 2 days at 30°C and 37°C in order to test for genetic interaction

For Yeast-Two-Hybrid: AD and BD positive mated colonies, along with positive/negative controls were spotted on TDO plates at a dilution factor of 5 and grown for 3-4 days.

3. Results

3.1. Vps1 Colocalizes with All Retromer Subunits.

An important aspect of two proteins having a functional relationship is whether or not the proteins reside in the same regions of the cell. Therefore, the first step in this study was to establish the cellular localization of Vps1 and the retromer by looking for colocalization between the two. In order to test this, yeast strains were engineered to express C-terminally RFP-tagged Vps1 (Vps1-RFP) and a C-terminally GFP-tagged retromer subunit (ex: Vps35-GFP), creating 5 distinct strains. Confocal fluorescent microscopy was used to evaluate the possible colocalization, which was partially seen in all 5 strains (Fig. 1A). 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 (Fig. 1B). These results indicate that Vps1 and the individual Retromer subunits do partially colocalize *in vivo* experiments with yeast cells.

3.2. Retromer Subunits Targeted Correctly in the Absence of Vps1

We evaluated 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 a retromer protein that is C-terminally tagged with GFP (1 strain for each retromer protein), and a second set containing the identical retromer-GFP proteins in a *vps*1 null mutant cell (*vps1*Δ). 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: a confirmation that the retromer resides at the endosome, and as a control for comparison with *vps1*Δ strains. We evaluated the localization of the GFP- and DsRed-tagged proteins using confocal fluorescence microscopy, concluding that there is very little difference in colocalization between the retromer and the endosome in WT versus *vps1*Δ strains (Fig. 2 A, C). Yeast strains containing endogenous 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. 2B). Conversely, *vps1*Δ 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. 2D).Our statistical analysis showed that the extent of colocalization of each GFP-tagged retromer protein to the endosome in *vps1*Δis not

statistically highly different from that of WT cells (*p*-values listed in Fig. 2 legend). This result suggests that while Vps1 and retromer proteins do indeed partially colocalize, Vps1 is not required for the correct targeting of retromer to the endosome.

3.3. The Number of Vps26- and Vps35-GFP Puncta Increased in the Absence of Vps1

If Vps1 functions with the retromer within the retrograde pathway, then it is certainly plausible that loss of Vps1 would affect the efficiency of retromer function. To test the possibility, we examined the number of retromer-GFP puncta in cells of both WT and *vps1*Δ cells. Two retromer proteins (Vps26 and Vps35) in *vps1*Δ cells displayed a marked increase in retromer puncta number (Fig. 3A), when compared with WT cells ($p<0.01$).In WT cells, the average number of retromer-GFP puncta per cell was 4.12 ± 1.14 for Vps5-GFP, 4.3 ± 0.7 for Vps17-GFP, 3.13 ± 0.45 for Vps26-GFP, 4.27 ± 0.58 for Vps29-GFP, and 3.96 ± 0.23 for Vps35-GFP (Fig. 3B). In *vps1*Δ cells, the average number of retromer-GFP puncta per cell was 4.27 ± 0.93 for Vps5-GFP, 4.53 ± 0.92 for Vps17-GFP, 5.33 ± 0.89 for Vps26-GFP, 4.35 ± 0.45 for Vps29-GFP, and 6.62 ± 1.05 for Vps35-GFP (Fig. 3B).Overall, small increase in Vps26- and Vps35-GFP puncta in *vps1*Δ strains suggests at least one of three things: that the expression of these retromer subunits may be increased, the recruitment of them to the endosome increased, or the dissociation of them from the endosome decreasedin *vps1*Δ strains.

3.4. Vps35 Genetically Interacts with Vps1

If combined mutations of two genes implicated in an essential cellular process lead to cell death, it is expected that the two genes have a functional relationship in regard to the biological pathway. To investigate if Vps1 showed evidence of genetic interaction with any of the 5 Retromer proteins, we constructed double null mutants of each retromer protein with Vps1 (*vps1*Δ*vps5*Δ, *vps1*Δ*vps17*Δ, *vps1*Δ*vps26*Δ, *vps1*Δ*vps29*Δ, and *vps1*Δ*vps35*Δ) and evaluated them for synthetic lethality. The only retromer protein that showed synthetic lethality was that of Vps35 (Fig. 4), though it can be argued that the other 4 retromer proteins showed no noticeable synthetic growth sickness. This result suggests that Vps35 and Vps1 do indeed interact on the genetic level, most likely for efficient retrograde trafficking.

3.5. Vps1 Does Not Physical Interact with Retromer Proteins

While synthetic lethality assays can determine whether or not two gene products function within the same or parallel pathway, it does not have the ability to detect whether two proteins physically bind with each other. 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 with *VPS1*, 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, a Yeast-Two-Hybrid assay was used. Of the 5 Retromer proteins, none showed evidence of physical interaction with Vps1 since cells co expressing BD-Vps1 with AD-Vps5, AD-Vps26, AD-Vps29, or AD-Vps35 did not grow on TDO plates (Fig. 5). However, the positive control, consisting of known binding partners, SV40 Large T-Antigen and p53, showed growth on TDO plates.

3.6. Vps1 and Retromer Double Deletion Mutants Display More Severe Vsp10 Retrieval Defects

Vps1 is implicated in Vps10, a transmembrane receptor, retrieval from endosomes and vacuole.Loss of Vps1 results in accumulation of Vps10 at the vicinity of the rim of the vacuole forming ring-like structures(Arlt et al., 2015; Richard J Chi et al., 2014) (Fig 6A). Likewise, Vps10 recycling from vacuoles depends on the retromer (Fig 6A), consistent with a previous report(Arlt et al., 2015). Therefore, we hypothesized that Vps1 and retromer double deletion mutants would display more severe Vps10 retrieval defects than Vps1 or retromer single mutant cells. Interestingly, in two double mutant strains (*vps1*Δ*vps5*Δand *vps1*Δ*vps17*Δ) the mean percent of cells displaying normal Vps10-GFP distribution (punctated structure in the cytoplasm instead of ring-like structures) was 9.5% and 4.85%, when compared to single mutant cells of *vps1*Δ (22.3%), *vps5*Δ (14.8%), and *vps17*Δ (8.6%). However, the rest of the double mutant cells (*vps1*Δ*vps26*Δ*, vps1*Δ*vps29*Δ, and *vps1*Δ*vps35*Δ) showed no significant differences from corresponding single mutant cells (Fig. 6A and B).

4. Discussion

Vps1 has been previously shown to localize to the endosome (Banh et al., 2017; Williams & Kim, 2014), as well as colocalize with Vps17, a retromer subunit (R. J. Chi et al., 2014). Whereas the aforementioned study only showcased colocalization between Vps1 and Vps17, here we 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, we have provided new data, confirming that all five Retromer proteins partially colocalize with Vps1 inside the yeast system.

The fact that Vps1 and the Retromer do partially colocalize is consistent with the findings that report the location of Vps1 at several biological membranes in yeast, including the Golgi, peroxisome, and vacuole (Goud Gadila et al., 2017; Saimani et al., 2017; Williams & Kim, 2014). The molecular mechanisms underlying Vps1 targeting to these various organelles remain unknown. However, a recent report provided a hint toward the mechanism by showing that Vps1 interacts with PI3P, PI4P, PI5P, and PS (phosphatidylserine) (Smaczynska-de et al., 2015). In particular, the authors demonstrated that the point mutationof serine 599 into aspartate abolish Vps1's interaction with PI4P and PI5P, but not affecting its interaction with PI3P and PE. However, the question of whether the Ser 599 is required for Vps1's targeting to aforementioned organelles is yet to be investigated.

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 in Retromer function (R. J. Chi et al., 2014), it is entirely possible that Vps1 may play a role in the recruitment of the Retromer. Based on colocalization data with Vps1, The possible role of Vps1 in Retromer recruitment was investigated. Several previous studies have reported the cellular localization of the Retromer components to the endosome (Belenkaya et al., 2008; R. J. Chi et al., 2014; M. N. Seaman, 2004, 2007), so colocalization between the Retromer proteins and the endosomal marker DsRed-FYVE was an expected result. However, in cells lacking Vps1 (*vps1*Δ), it was largely unknown if Retromer localization would be affected. Given the loss of Vps1 causes a host of trafficking defects (Banh et al., 2017; Goud Gadila et al., 2017; Saimani et al., 2017), it is a likely assumption that Retromer recruitment could be affected. However, in this study, all five components of the yeast Retromer showed an unaffected ability to correctly target late endosomes in *vps1*Δ cells. While this is too surprising of a finding, it does help to further categorize trafficking defects in *vps1*Δ cells. Also, it suggests that Retromer recruitment to the endosome and Vps1 recruitment to the endosome are completely independent. It is well known that the dimeric retromer tubulation subcomplex consists of Vps5 and Vps17 that contain both PX and BAR domains, which intrinsically bind membranes (Horazdovsky et al., 1997; M. N. Seaman & Williams, 2002; Song et al., 2001; van Weering et al., 2012). This allows Vps5 and Vps17 to be targeted to PI3P in endosomal membranes. The trimeric retromer cargo recognition subcomplex (Vps26, 29, and 35) contains domains that target the subcomplex to cargo proteins present at the endosome (Bonifacino & Hurley, 2008). By tying our retromer localization data in *vps1*Δ cells up with these known information regarding retromer recruitment to the endosome, one can conclude that retromer targeting is controlled by their own intrinsic membrane or cargo binding domains, not by Vps1. Given that vesicle coating by the retromer proceeds the scission of the vesicle by Vps1, it is rather possible that Vps1 is recruited by the Retromer, though this possibility has not been fully explored. Notably, we 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.

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 *vps1*Δ cells show a marked increase in Retromer puncta number. Their study was only focusing on counting the number of Vps17- and Vps26-GFP puncta in cells lacking Vps1. However, in our experiment we examined all five Retromer proteins, providing slightly contradictory data to the previous study by Chi et al. (2014). Vps26-GFP puncta in *vps1*Δ cells increased in number in our experiment (Fig. 3), which is in good agreement with the precious study, whereas Vps17-GFP puncta number in the same mutant cells did not change appreciably. Noteworthy, Vps35-GFP dots increased significantly in number in the same mutant cells. What is causing the upturn of Vps26- and Vps35-GFP puncta number in the mutant? One possible hypothesis is that this increase in puncta number in *vps1*Δ cells versus WT cells is a sign of mild upregulation of the Retromer proteins themselves. Alternatively, the increase may be due toan overall down regulation of traffic within the retrograde transport system. In order words, loss of Vps1 may cause a delay of departing vesicles from the endosome, thus down regulating the traffic toward the Golgi. The rationale behind this idea is that Vps1 has been previously implicated to act as a scission protein in many intracellular trafficking pathways (Ferguson and De Camilli, 2012;Palmer et al., 2015). 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 increase in retromer puncta.

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 an 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. A linear pathway involves proteins being involved in the very same pathway, with one either serving as down or upstream regulator of the other, whereas a multiprotein complex involves the two proteins binding into a multiprotein complex in the pathway. With a multiprotein complex, it is possible the two proteins could be overlapping in function within the complex, or they could serve completely separate functions in the complex. A parallel pathway involves the two proteins being the primary regulators in similar pathways, which means they can possibly compensate for a lack of the other. While our evidence only implicates one retromer protein (Vps35) to be genetically interacting with Vps1 (Fig. 4), 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. Therefore, our hypothesis was that a cell lacking both Vps1 and Vps35 would be expected to show severe defects in intracellular trafficking. However, *vps1*Δ*vps35*Δ cells displayed no noticeable additive traffic defects of Vps10-GFP when compared with *vps1*Δ or *vps35*Δ cells (Fig. 6). Considering the quantitated defect of Vps10-GFP retrieval in cells lacking only Vps35 was severe enough (>95% of cells displayed Vps10-GFP traffic defects), it appears that the effect of the additional loss of Vps1 on this traffic was negligible. Surprisingly, two double mutant strains, *vps1*Δ*vps5*Δ and *vps1*Δ*vps17*Δ, displayed more severe defects in Vps10-GFP retrieval than each of their corresponding single KO mutant strains did. One likely explanation for this phenomena is that the scissor Vps1 has a functional connection with the coat retromer at the endosome. Given the notion that vesicle coating precedes the scission of the vesicle, deficiency of scission activity due to the loss of Vps1 could be further aggravated with a loss of retromer such as Vps5 and Vps17. Though through our genetic interaction analysis did not clearly present the functional relationship between Vps1 with these two retromer components, our Vps10-GFP retrieval assay results solidify the possibility of a functional connection with Vps1 along with Vps5 and Vps17 in the context of Vps10 retrieval process. Nevertheless, none of the functionally implicated retromer components including Vps5, Vps17, and Vps35 physically interacts with

Vps1 based on our yeast two hybrid assay (Fig. 5). Therefore, it may be that Vps1 and the retromer are situated in close proximity to each other at the endosome, but their interaction is too feeble and transient to detect with the current protein interaction assays.

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Annexure

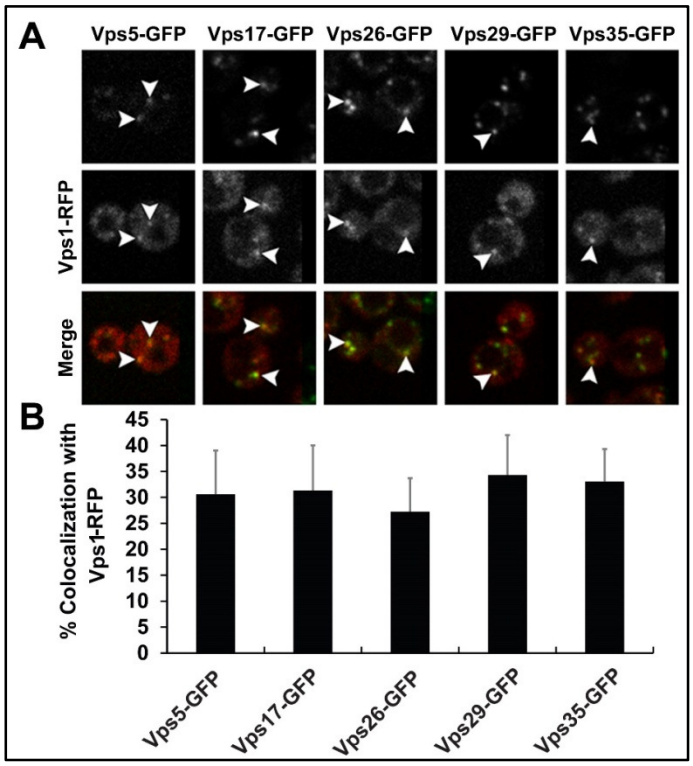

Figure 1: Retromer proteins colocalize with Vps1. A: Representative images of wild type strains expressingC-terminal GFP tagged Retromer proteins and C-terminal RFP tagged Vps1. Arrowheads indicate colocalized puncta. All 5 Retromer proteins show partial colocalization with Vps1 in vivo. B: Quantification of colocalization between Retromer-GFP and Vps1-RFP puncta. The average colocalization percentage shown is out of 100%.

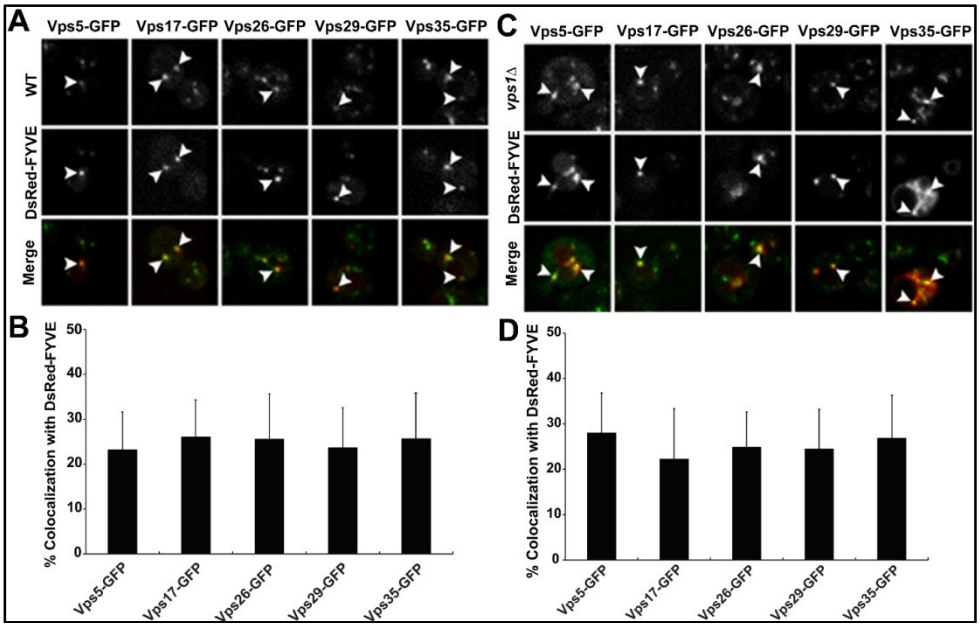

Figure 2: Retromer proteins localize to the endosome in WT and vps1Δ cells. A: Representative images of wild type strains expressingC-terminal GFP tagged Retromer proteins and the endosomal marker DsRed-FYVE. Arrowheads indicate colocalized puncta. All 5 Retromer proteins show colocalization with DsRed-FYVE. B: Quantification of Retromer-GFP colocalization with DsRed-FYVE in WT cells. The average percentage of colocalization in each cell shown is out of 100%. C: Representative images of vps1Δ strains expressingC-terminal GFP tagged Retromer proteins and the endosomal marker DsRed-FYVE. Arrowheads indicate colocalized puncta. D: Quantification of Retromer-GFP colocalization with DsRed-FYVE in vps1Δ cells. The average percentage of colocalization in each cell shown is out of 100%. p-values for statistical comparison of WT (B) and vps1Δ (D) partial colocalization: p (Vps5) = 0.034, p (Vps17) = 0.136, p (Vps26) = 0.760, p (Vps29) = 0.72, p (Vps35) = 0.639.

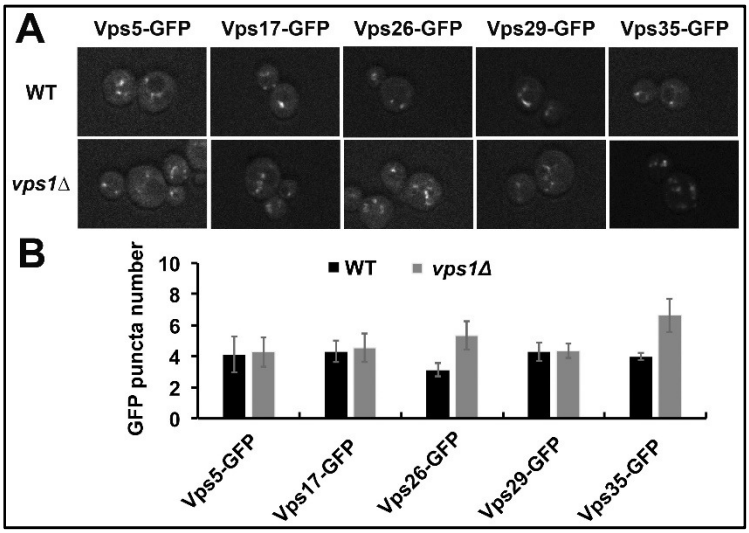

Figure 3: The mean number of Vps26- and Vps35-GFP carrying puncta increased in cells lacking Vps1. A: Representative images of WT and vps1Δ strains expressingC-terminal GFP tagged Retromer proteins. B: Quantification of Retromer puncta number in WT and vps1Δ strains. Cells were grown in selective medium, imaged, and the number of puncta were counted in each cell. Data analyzed by two-tailed, unpaired student's T-test, giving p-values for each Retromer proteins: p (Vps5) = 0.86, p (Vps17) = 0.75, p (Vps26) = 0.019, p (Vps29) = 0.85, and p (Vps35) = 0.013.

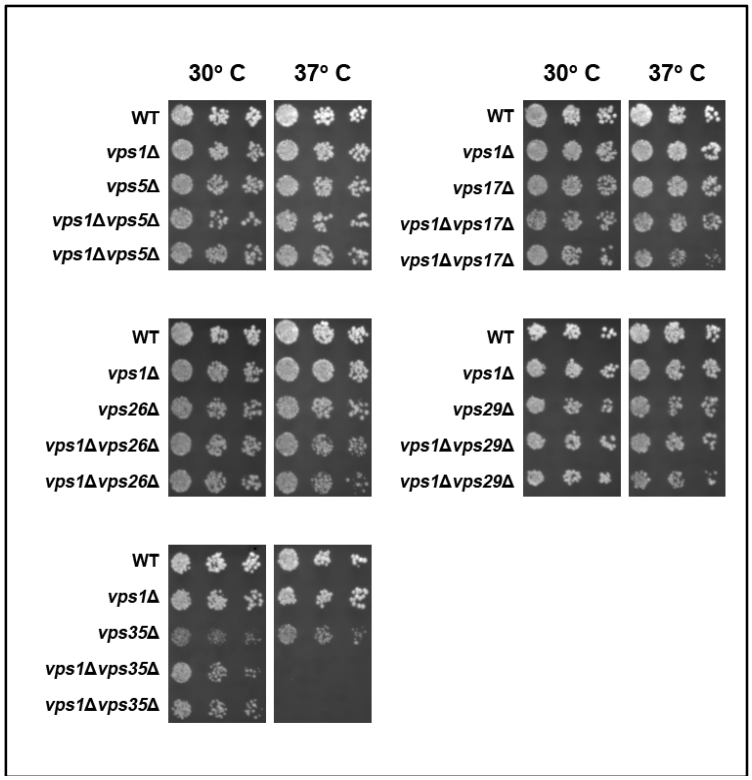

Figure 4: VPS1 and VPS35 genetically interact. Haploid double mutants (ex: vps1Δvps5Δ) were grown to logarithmic phase, and serial dilutions were spotted onto YPD plates for 2 days at 30˚C or at 37˚C. Abnormal or inhibited growth indicates synthetic sickness or lethality, respectively. The double mutant vps1Δvps35Δ shows no growth (synthetic lethality) at 37˚C, suggesting a functional relationship between Vps1 and Vps35 within the retrograde trafficking pathway.

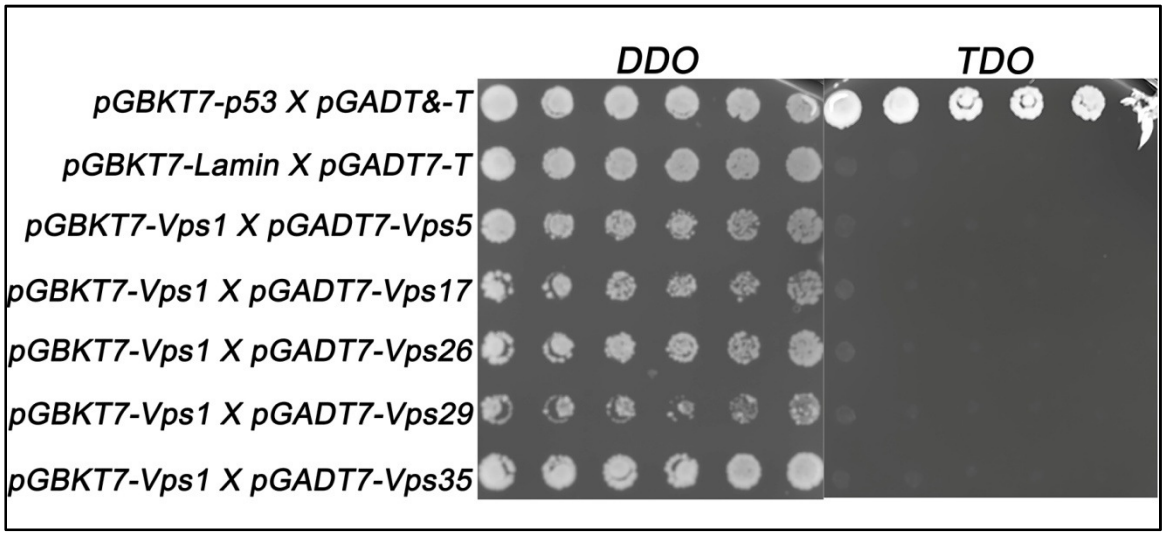

Figure 5: Vps1 does not interact with the retromer proteins. A dilution series of cell strains containing both an AD-retromer subunit and BD-Vps1 plasmids. Growth on selective media (TDO) indicates activation of reporter gene (HIS), which allows cells to produce histidine and to grow. Only positive control cells expressing p53 and SV40 T antigen grew on TDO, but all tested cells coexpressing Vps1 with each of the retromer proteins did not grow on TDO, suggesting Vps1 does not bind with these retromer proteins in vivo.

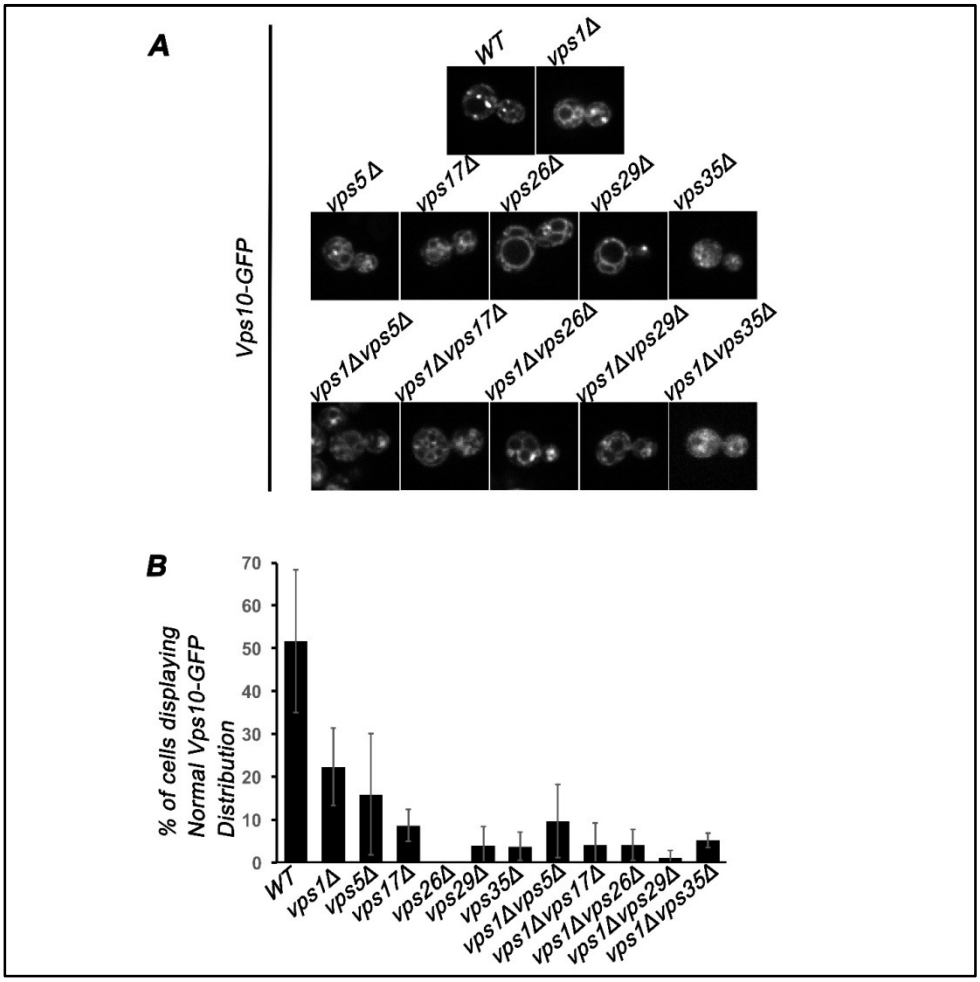

Figure 6: Vps10-GFP retrieval is more severely compromised in double deletion mutant strains (vps1Δvps5Δ and vps1Δvps17Δ) lacking Vps1 and a retromer component. A: Representative images of Vps10-GFP in strains of WT, vps1Δ, retromer single KO mutants, and Vps1 and retromer double deletion mutants. The vast majority of WT cells displayed punctated Vps10-GFP without a ring-like structure, whereas retromer single KO, vps1Δ, and all double deletion mutant strains contained multiple ring-like structures labeled with Vps10-GFP. B: Quantification of percent of cells displaying normal Vps10-GFP distribution. Cells that do contain Vps10-GFP puncta but do not contain ring-like structures were considered as normal.

Strain Table 1. All yeast strains used for this study.

Strain Number	Strain Name	Source	Genotype
KKY 0002	Wild Type (BY4741)	Invitrogen	*MAT a his3Δ1 leu2D met15Δ ura3Δ*
KKY 0343	Wild Type	John Cooper	*MAT α his3ΔuraΔleuΔtrpΔlysΔ*
KKY 0344	Wild Type	John Cooper	*MAT a his3ΔuraΔleuΔtrpΔlysΔ*
KKY 0352	*vps1Δ*	This Study	KKY0343 *VPS1::KanMx6*
KKY 0925	Vps1-RFP	This Study	KKY0343 *VPS1-RFP-KanMx*
KKY 1215	Vps26-GFP Vps1-RFP	This Study	KKY0925 *VPS26-GFP-HIS*
KKY 1216	Vps29-GFP Vps1-RFP	This Study	KKY0925 *VPS29-GFP-HIS*
KKY 1514	Vps5-GFP mRFP-Vps1	This Study	KKY0343 *VPS5-GFP-HIS*, [*mRFP-VPS1-URA*]
KKY 1283	Vps17-GFP Vps1-RFP	This Study	KKY0925 *VPS17-GFP-HIS*
KKY 1265	Vps35-GFP Vps1-RFP	This Study	KKY0925 *VPS35-GFP-HIS*
KKY 1134	Vps17-GFP	This Study	KKY0343 *VPS17-GFP-HIS*
KKY 1136	Vps5-GFP	This Study	KKY0343 *VPS5-GFP-HIS*
KKY 1145	Vps35-GFP	This Study	KKY0343 *VPS35-GFP-HIS*
KKY 1153	Vps26-GFP	This Study	KKY0343 *VPS26-GFP-HIS*
KKY 1177	Vps29-GFP	This Study	KKY0343 *VPS29-GFP-HIS*
KKY 1155	*vps1Δ* Vps17-GFP	This Study	KKY0352 *VPS17-GFP-HIS*
KKY 1157	*vps1Δ* Vps35-GFP	This Study	KKY0352 *VPS35-GFP-HIS*
KKY 1160	*vps1Δ* Vps26-GFP	This Study	KKY0352 *VPS26-GFP-HIS*
KKY 1178	*vps1Δ* Vps29-GFP	This Study	KKY0352 *VPS29-GFP-HIS*
KKY 1267	*vps1Δ* Vps5-GFP	This Study	KKY0352 *VPS5-GFP-HIS*
KKY 1189	Vps29-GFP DsRed-FYVE	This Study	KKY1177 [*DsRed-FYVE-LEU*]
KKY 1183	Vps26-GFP DsRed-FYVE	This Study	KKY1153 [*DsRed-FYVE-LEU*]
KKY 1217	Vps17-GFP DsRed-FYVE	This Study	KKY1134 [*DsRed-FYVE-LEU*]
KKY 1218	Vps5-GFP DsRed-FYVE	This Study	KKY1136 [*DsRed-FYVE-LEU*]
KKY 1271	Vps35-GFP DsRed-FYVE	This Study	KKY1145 [*DsRed-FYVE-LEU*]
KKY 1219	*vps1Δ* Vps5-GFP DsRed-FYVE	This Study	KKY1267 [*DsRed-FYVE-LEU*]
KKY 1220	*vps1Δ* Vps17-GFP DsRed-FYVE	This Study	KKY1155 [*DsRed-FYVE-LEU*]
KKY 1197	*vps1Δ* Vps35-GFP DsRed-FYVE	This Study	KKY1157 [*DsRed-FYVE-LEU*]
KKY 1193	*vps1Δ* Vps29-GFP DsRed-FYVE	This Study	KKY1177 [*DsRed-FYVE-LEU*]
KKY 1186	*vps1Δ* Vps26-GFP DsRed-FYVE	This Study	KKY1160 [*DsRed-FYVE-LEU*]
KKY 1292	*vps5Δ*	This Study	KKY0002 *VPS5::HIS*
KKY 1288	*vps26Δ*	This Study	KKY0002 *VPS26::HIS*
KKY 1224	*vps17Δ*	This Study	KKY0002 *VPS17::HIS*
KKY 1321	*vps35Δ*	This Study	KKY0002 *VPS35::HIS*
KKY 1397	*vps29Δ*	This Study	KKY0002 *VPS29::HIS*
KKY 1328	*vps1Δvps5Δ*	This Study	*MAT α his3ΔuraΔleuΔlysΔ VPS1::KanMx6 VPS5::HIS*
KKY 1330	*vps1Δvps17Δ*	This Study	*MAT α his3ΔuraΔleuΔmetΔ VPS1::KanMx6 VPS17::HIS*
KKY 1332	*vps1Δvps26Δ*	This Study	*MAT a his3ΔuraΔleuΔtrp VPS1::KanMx6 VPS26::HIS*
KKY 1413	*vps1Δvps35Δ*	This Study	*MAT a his3ΔuraΔleuΔlysΔ VPS1::KanMx6 VPS35::HIS*
KKY 1425	*vps1Δvps29Δ*	This Study	*MAT a his3ΔuraΔleuΔ VPS1::KanMx6 VPS29::HIS*
KKY 1254	Y2HGold	Clontech	*MAT a, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$–Gal1$_{TATA}$–His3, GAL2$_{UAS}$–Gal2$_{TATA}$–Ade2 URA3:: MEL1$_{UAS}$–Mel1$_{TATA}$ AUR1-C MEL1*
KKY 1255	Y187	Clontech	*MAT α, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met–, URA3:: GAL1$_{UAS}$–Gal1$_{TATA}$–LacZ, MEL1*
KKY 1272	pGBKT7-LAM	This Study	KKY1254[*pGBKT7-LAM*]
KKY 1273	pGADT7-T	This Study	KKY1255[*pGADT7-T*]
KKY 1274	pGBKT7-53	This Study	KKY1254[*pGBKT7-53*]
KKY 1275	pGBKT7-Vps1	This Study	KKY1254 [*pGBKT7-VPS1*]
KKY 1399	pGADT7-Vps17	This Study	KKY1255 [*pGADT7-VPS17*]
KKY 1408	pGADT7-Vps5	This Study	KKY1255 [*pGADT7-VPS5*]
KKY 1409	pGADT7-Vps26	This Study	KKY1255 [*pGADT7-VPS26*]
KKY 1410	pGADT7-Vps29	This Study	KKY1255 [*pGADT7-VPS29*]

KKY 1411	pGADT7-Vps35	This Study	KKY1255 [*pGADT7-VPS35*]
KKY 1412	pGBKT7-Vps1 pGADT7-Vps17	This Study	KKY1275/KKY1399 (diploid)
KKY 1462	pGBKT7-Vps1 pGADT7-Vps5	This Study	KKY1275/KKY1408 (diploid)
KKY 1463	pGBKT7-Vps1 pGADT7-Vps26	This Study	KKY1275/KKY1409 (diploid)
KKY 1464	pGBKT7-Vps1 pGADT7-Vps29	This Study	KKY1275/KKY1410 (diploid)
KKY 1465	pGBKT7-Vps1 pGADT7-Vps35	This Study	KKY1275/KKY1411 (diploid)
KKY 1886	Wild type Vps10-GFP	This Study	KKY002 [*pAD54-VPS10-GFP*]
KKY 1887	*vps1*Δ Vps10-GFP	This Study	KKY352 [*pAD54-VPS10-GFP*]
KKY 1888	*vps5*Δ Vps10-GFP	This Study	KKY1292 [*pAD54-VPS10-GFP*]
KKY 1889	*vps17*Δ Vps10-GFP	This Study	KKY1224 [*pAD54-VPS10-GFP*]
KKY 1890	*vps26*Δ Vps10-GFP	This Study	KKY1288 [*pAD54-VPS10-GFP*]
KKY 1891	*vps29*Δ Vps10-GFP	This Study	KKY1397 [*pAD54-VPS10-GFP*]
KKY 1892	*vps35*Δ Vps10-GFP	This Study	KKY1321 [*pAD54-VPS10-GFP*]
KKY 1893	*vps1*Δ*vps5*Δ Vps10-GFP	This Study	KKY1328 [*pAD54-VPS10-GFP*]
KKY 1894	*vps1*Δ*vps17*Δ Vps10-GFP	This Study	KKY1330 [*pAD54-VPS10-GFP*]
KKY 1895	*vps1*Δ*vps26*Δ Vps10-GFP	This Study	KKY1332 [*pAD54-VPS10-GFP*]
KKY 1896	*vps1*Δ*vps29*Δ Vps10-GFP	This Study	KKY1425 [*pAD54-VPS10-GFP*]
KKY 1897	*vps1*Δ*vps35*Δ Vps10-GFP	This Study	KKY1413 [*pAD54-VPS10-GFP*]