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Identification of a conserved motif required for Vps35p/Vps26p interaction and assembly of the retromer complex

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Short title: An essential conserved motif required for retromer assembly.

Abbreviations: vps - vacuolar protein sorting; CIMPR – cation independent mannose 6-phosphate receptor; GFP – green fluorescent protein; Snx – sorting nexin; IP – immunoprecipitate; CPY – carboxypeptidase Y; 3-AT – 3-amino-1,2,4-triazole.

Synopsis

The retromer complex is a conserved cytoplasmic coat complex that mediates the endosome-to-Golgi retrieval of vacuole/lysosome hydrolase receptors in yeast and mammals. The recognition of cargo proteins by retromer is performed by the Vps35p/VPS35 component which together with Vps26p/VPS26 and Vps29p/VPS29 forms the cargo-selective subcomplex. In this report we have identified a highly conserved region of Vps35p/VPS35 that is essential for the interaction with Vps26p/VPS26 and for assembly of the retromer complex. Mutation of residues within the conserved region results in mutants of Vps35p/VPS35 which cannot bind to Vps26p/VPS26 and are not efficiently targeted to the endosomal membrane. These data implicate Vps26p/VPS26 in regulating Vps35p/VPS35 membrane association and therefore suggest a role for Vps26p/VPS26 in cargo recognition.

Introduction

In eukaryotic cells the sorting and transport of membrane proteins in the endocytic and secretory pathways is mediated by cytoplasmic coat proteins that can both recognise cargo through intrinsic sorting motifs and can assemble to deform the membrane into a vesicle or tubule. Different coat proteins function at different sites in the cell and thereby help to generate and maintain the exquisite specificity of protein targeting to the organelles that comprise the endocytic and secretory pathways. A common feature of the various coat proteins is that they are multimeric protein complexes comprising several subunits which assemble into a single functional unit which can perform the tasks of cargo recognition and membrane deformation (reviewed in [1-3]).

A good example of a multimeric coat complex is the retromer complex. Retromer is a conserved cytoplasmic coat that functions in endosome-to-Golgi retrieval and was first characterised in yeast *Saccharomyces cerevisiae* [4,5]. Yeast retromer comprises

five subunits each encoded by a vacuole protein sorting (*VPS*) gene. Loss of retromer function results in a vacuole protein sorting defect and secretion of the vacuolar hydrolase, carboxypeptidase Y (CPY), due to the failure to retrieve the CPY sorting receptor, Vps10p [4,6]. Although yeast retromer is a stable heteropentameric complex, retromer can be phenotypically and biochemically dissected into two subcomplexes; Vps35p, Vps29p and Vps26p comprise the cargo-selective component of the retromer complex whilst the sorting nexins Vps5p and Vps17p dimerize to drive vesicle/tubule formation through their Bin/Aphiphysin/Rvs (BAR) domains and intrinsic self-assembly activity [reviewed in 7]. In mammalian cells, VPS35, VPS29 and VPS26 form a complex which can bind cargo such as the cation-independent mannose-6-phosphate receptor (CIMPR) [8]. Sorting nexin-1 (Snx1) along with sorting nexins-2, -5 and -6 function as the mammalian orthologs of the yeast Vps5p/Vps17p dimer to drive tubulation of endosomal membranes [9, 10]. Unlike yeast, mammalian retromer does not form a stable heteropentameric complex as the association of the VPS35/29/26 complex with the sorting nexin complex is dynamic and transient [11].

The importance of retromer in endosomal membrane protein sorting and transport has been revealed recently through a variety of studies that have implicated retromer in the processing of the amyloid precursor protein [12], transcytosis of the polymeric IgA receptor [13], Wnt gradient formation [14] and trafficking of the Ftr1p protein required for iron homeostasis in yeast [15]. Some progress has been made in understanding how retromer assembles and the function of the individual subunits. For instance, Vps35p and its mammalian ortholog can bind to cargo proteins such as Vps10p and the CIMPR respectively [6, 8]. VPS29 is a phosphoesterase which can dephosphorylate the serine preceding the acidic dileucine sorting motif in the tail of the CIMPR [16]. The structure of VPS26 was recently solved revealing an unexpected structural similarity to the arrestin family of proteins hinting that VPS26 may also have a role to play in binding cargo proteins [17]. The association of Vps29p with Vps35p has been shown to depend upon a conserved hydrophobic patch on the surface of Vps29p. Mutation of valine at position 109 in Vps29p or position 90 in mammalian VPS29 blocks assembly of Vps29p with Vps35p and prevents VPS29 recruitment to the membrane in mammalian cells [18]. A separate hydrophobic patch on Vps29p centered on a leucine residue at position 252 is

essential for the association of the Vps35p/Vps29p/Vps26p subcomplex with the Vps5p/Vps17p members of yeast retromer complex [18].

Other studies in yeast have shown that Vps26p plays a vital role in mediating the interactions between Vps35p and other retromer components, Vps5p/Vps17p and also in regulating the membrane association of Vps35p [19]. As Vps35p/VPS35 must be membrane-associated to recognise cargo proteins such as Vps10p and the CIMPR the regulatory role that Vps26p plays in Vps35p membrane association has clear implications for the ability of Vps35p to sort cargo proteins. In mammalian cells binding of VPS26 to VPS35 requires a mobile loop in the C-terminal domain of VPS26 [17]. It is currently unknown however, how Vps35p associates with Vps26p. In this study, we have identified a critical region of Vps35p that is required for the interaction with Vps26p. This region comprises a highly conserved N-terminal motif, P-R-L-Y-L, that is predicted to be part of a β -sheet. Mutation of the arginine or adjacent leucine results in a *vps35* mutant that cannot bind to Vps26p and therefore fails to retrieve Vps10p from endosomes to the Golgi. As the P-R-L-Y-L motif is conserved in mammalian VPS35, we have determined what role this motif has in assembly of mammalian retromer and found that it is essential for binding of VPS26 and is required for the membrane association of VPS35.

Experimental

Reagents and media

Most reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK) with the following exceptions: restriction enzymes were purchased from New England Biolabs (Hitchin, Herts, UK). Effectene was obtained from Qiagen (Crawley, West Sussex, UK), Fugene6 from Roche (Lewes, East Sussex, U.K.), protein-A sepharose and ^{125}I -protein-A from Amersham Biosciences (St. Albans, Herts, U.K.). Fluorescently labelled secondary antibodies were obtained from Molecular Probes (Paisley, Scotland, UK). Antibodies against mammalian VPS35 and VPS26 are described in [20]. Yeast strains used are described in table 1. Standard yeast media was used for culturing the yeast and selecting transformants.

Production of the vps35 mutant alleles.

Generation of the *vps35-27* dominant negative allele (previously named *vps35dn*) has been described previously [4]. After sequencing to identify the mutations present, the mutations were separated using standard molecular biology techniques by cutting the mutant and wildtype DNA and splicing together to separate the mutations and generate the *vps35-27a-e* alleles. The Y55H mutation was separated from other mutations by a unique *KpnI* site. Digestion of wildtype *VPS35* and *vps35-27* with *KpnI* followed by gel purifying the vector and insert fragments allowed construction of *vps35-27b* and *c*. The Y55H, L99P and F112L mutations were separated from the I247T, S275L and M457T mutations by a unique *SpeI* site. Digestion of wildtype *VPS35* and *vps35-27* with *SpeI* followed by gel purifying the vector and insert fragments allowed construction of *vps35-27a* and *d*. The *vps35-27e* allele was generated by digestion of *vps35-27b* with *SpeI* followed by ligation of the *SpeI* fragment into wildtype *VPS35*. To produce the *vps35L99P*, *vps35F112L*, *VPS35R107A* and *VPS35L108P* mutants, site directed mutagenesis was performed using the QuickChange kit from Stratagene (Cambridge, UK) following manufacturers instructions. All mutants and alleles were sequenced to confirm the presence of the required mutation(s).

Yeast metabolic labelling experiments.

CPY sorting experiments were performed as described in [19] and are outlined briefly here. To determine how much CPY is secreted, cells grown to an OD_{600nm} of 0.8 were collected by centrifugation and resuspended in 0.5ml of selective media so that the final concentration was 5 ODs per ml. The cells were labelled with ³⁵S-methionine for 10 minutes followed by a 30 minutes chase. The cells were then transferred to a tube on ice containing 0.5 ml spheroplasting buffer (50 mM Tris-HCl pH 8.0, 2 M sorbitol, 40 mM NaAzide, 20 mM dithiothreitol and 20 µg/ml α2 macroglobulin) for 10 minutes. After addition of zymolyase, the cells were incubated for 15 minutes at 30°C to allow spheroplasting to occur. The cells were then centrifuged and the supernatant (extracellular; E) and cell pellet (intracellular; I) fractions treated with 10% trichloroacetic acid (TCA) to precipitate the proteins. After centrifugation and washes with acetone, the pellets were dried and resuspended into 100 µl cracking buffer (6M urea, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS, 1% β-mercaptoethanol) to

solubilise the proteins. The samples were diluted with the addition of 1 ml immunoprecipitation (IP) buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA), cleared by centrifugation and transferred to a fresh tube. CPY was recovered from the intracellular and extracellular fractions by immunoprecipitation. In the whole cell CPY sorting assay, 5 ODs of cells were labelled with ^{35}S methionine for 10 minutes and chased for 30 minutes before the whole culture was transferred to ice and precipitated with 10% TCA. After centrifugation, the pellets were washed with acetone, dried, resuspended into cracking buffer and then lysed by vortexing with glass beads and heating to 70°C. The samples were diluted with IP buffer, cleared by centrifugation and then transferred to a fresh tube. CPY was recovered by immunoprecipitation.

Cell fractionation experiments to determine the membrane localisation/association of Vps10p, Vps35p and Vps29p were performed as described in [21, 4]. Native immunoprecipitation experiments to evaluate the assembly of the yeast retromer complex *in vivo* were performed as described in [19, 22].

Yeast two hybrid analysis.

VPS35 was cloned into the two hybrid 'bait' vector pGBT9 by PCR of a full length mouse VPS35 which introduced a *Bam*HI site at the 5' end. The PCR product was first cloned using the pCRblunt vector (Invitrogen, Paisley, Scotland, UK). VPS35 was then subcloned into pGBT9 by excision from pCRblunt using *Bam*HI followed by ligation into *Bam*HI cut pGBT9. Mouse VPS26 was obtained as a full length EST from the IMAGE consortium and subcloned from pCMVSPORT using *Sal*I and *Nsi*I to excise VPS26 and ligated into the prey vector pGAD424 which had been cut with *Sal*I and *Pst*I. Mouse VPS29 was obtained as a full length EST from the IMAGE consortium and subcloned into pGAD424 using *Eco*RI and *Not*I to cut at the 5' and 3' ends respectively. The *Not*I site was filled with T4 polymerase and the fragment was ligated into pGBT9 which had been cut with *Eco*RI and *Sma*I

Native immunoprecipitations from mammalian cells.

Transient transfections were performed using Effectene (Qiagen) or Fugene6 (Roche) following manufacturers instructions. Native immunoprecipitations of GFP-

tagged VPS35 constructs transiently expressed in HeLa cells were performed as described in [18]. VPS26 immunoprecipitations were performed in an identical fashion using our anti-VPS26 antisera [20]. SDS-PAGE and Western blotting were performed as described in [19]

Immunofluorescence microscopy.

Fluorescence microscopy was performed as described in [20].

Results

In previous studies the generation of a dominant negative *vps35* mutant (*vps35dn*) has been described [4]. This allele of *vps35* is designated *vps35-27* and was produced using random PCR mutagenesis and gapped-plasmid repair. This procedure very often introduces several mutations. Therefore to identify which mutation (or combination of mutations) is required for the dominant phenotype, the *vps35-27* allele was sequenced and six different mutations were found, all of them in the N-terminal half of the protein (see table 2). Some of the mutations result in changes to highly conserved amino acids such as the Y55H, L99P and I247T mutations. In figure 1a, the sites of the various mutations are shown on an alignment of yeast Vps35p with homologues from plant, chicken and humans. Secondary structure prediction shows that some of the mutations (i.e. Y55H, L99P and I247T) fall in regions that are predicted to form α -helical or β -sheet secondary structure.

To pin-point which mutation(s) is conferring the dominant negative phenotype, the *vps35-27* allele was subjected to restriction digestion using enzymes that cut at unique sites in between the various mutations. The mutant DNA was then ligated into wildtype *VPS35* to generate a new allele carrying one or more of the original six mutations (see table 2). The resulting alleles were transformed into wildtype yeast and carboxypeptidase Y (CPY) sorting was evaluated by pulse/chase analysis. The dominant negative phenotype clearly is caused by either the L99P or F112L mutation (or the combination of both) (see figure 1b). Site directed mutagenesis was therefore used to generate alleles carrying only the L99P mutation or the F112L mutation. Analysis of these alleles conclusively demonstrate that the dominant negative phenotype of the *vps35-27* allele is

the result of the L99P mutation which falls in a highly conserved region predicted to form a β -sheet.

The L99P mutation is able to confer a dominant negative phenotype. To further examine the importance of this region of Vps35p to its function the amino acids residues (P97, R98, Y100 and L101) surrounding and adjacent to L99 were mutated individually to alanine. The resulting mutants were ligated into centromeric plasmids for wildtype expression levels and transformed into *vps35 Δ* yeast. A whole cell CPY sorting experiment was performed to evaluate the ability of the alanine scanning mutants to complement the CPY sorting defect in *vps35 Δ* yeast. In figure 2a (upper panel), the CPY sorting experiment shows that the L99P mutant and the R98A mutant are both unable to restore normal CPY sorting in the *vps35 Δ* strain. The inability of the L99P and R98A mutants to rescue CPY sorting in the *vps35 Δ* strain was not due to a lack of expression of the mutant Vps35 proteins as all the mutants were expressed and detectable in lysates (figure 2a, lower panel).

It has previously been demonstrated that Vps35p is required as part of the retromer complex to mediate retrieval of Vps10p from endosomes to the Golgi [4, 5]. Logically, as the L99P mutant is unable to rescue CPY sorting in a *vps35 Δ* strain, one would expect that Vps10p should be mislocalised in *vps35 Δ* yeast expressing the L99P mutant. In figure 2b the results of a cell fractionation experiment are shown. In wildtype cells, Vps10p is predominately localised to the P100 fraction which contains endosomal and Golgi membranes along with small vesicles. However, when *VPS29*, *VPS26* or *VPS35* are deleted, Vps10p becomes mislocalised to the P13 fraction which contains vacuolar membranes [4,21]. Expression of wildtype *VPS35* in the *vps35 Δ* strain rescues this phenotype, but expression of the L99P mutant results in Vps10p mislocalisation to the vacuolar membrane fraction identical to that observed in the *vps35 Δ* strain with empty vector (see figure 2b).

Additionally, when the effect of the L99P mutation upon the localisation of Vps29p or the Vps35 protein itself is investigated it becomes apparent that the L99P mutation confers a phenotype more similar to the effect observed when Vps26p is missing. Loss of Vps35p results in the displacement of Vps29p from the P100 fraction ([4]; and figure 2b - compare wildtype to *vps35 Δ* cells). In yeast expressing the L99P

mutant, Vps29p remains associated with the P100 so the L99P mutant does not behave like a *vps35Δ* strain with respect to Vps29p localisation. The L99P mutation does however result in a quantifiable shift of the Vps35 protein from the P100 to S100 fraction. This is very similar to the effect upon Vps35p observed in a *vps26Δ* mutant and hints that perhaps the L99P mutation is causing the loss of interaction between Vps35p and Vps26p. The localisation of Vps26p to the P100 fraction was moderately affected by deletion of either *VPS35* or *VPS29* (see figure 2b, lower panel) and therefore the localisation of Vps26p does not inform as to whether the L99P mutation in Vps35p affects the interactions with other subunits such as Vps26p. Deletion of both *VPS35* and *VPS29* did not have an additive effect on the P100 localisation of Vps26p which remained associated with the P100 fraction suggesting that Vps26p can bind membranes independently of Vps35p or Vps29p.

The effect of the L99P mutation upon the localisation of Vps10p was also investigated in wildtype cells expressing high copy plasmids (2 μ) carrying either wildtype *VPS35* or the *vps35L99P* mutant allele. In figure 2c, there is little apparent shift of Vps10p from the Golgi (P100) fraction to the vacuolar (P13) fraction and no effect was observed on the localisation of Vps26p. This is consistent with the observation that expression of the mutant *vps35* alleles in a wildtype background results in a more modest CPY sorting defect than was observed in *vps35Δ* cells expressing the *vps35* mutants from low copy centromeric plasmids.

To assess the effect of the L99P mutation on the assembly of the retromer complex in yeast, a native immunoprecipitation experiment was performed. Unfortunately the Vps35p antisera does not recognise Vps35p under native conditions, therefore we used the antisera against Vps5p or Vps26p which have both been used previously for native immunoprecipitations of retromer [19, 22]. When a lysate from wildtype cells is incubated with either anti-Vps5p or anti-Vps26p, it is possible to immunoprecipitate (IP) the retromer complex as a heteropentamer, each of the subunits being detectable in secondary immunoprecipitates (see figure 3a). However, when Vps35p is deleted, it is no longer possible to co-IP all five subunits. In lysates from *vps35Δ* cells, Vps5p remains associated with Vps17p but no longer assembles with the other members of the complex. Vps26p can only IP itself in lysates from *vps35Δ* cells.

This is consistent with previous data which has shown that Vps35p acts as a binding platform necessary for retromer assembly as a heteropentamer [4, 19 and 22]. When lysates from cells expressing the L99P mutant are evaluated, it is observed that the L99P mutation has a similar effect on retromer assembly as is seen in *vps35Δ* cells, i.e. it is impossible to co-IP the retromer complex as a heteropentamer (figure 3a).

The L99 residue is very highly conserved among orthologs of Vps35p. Therefore to better understand the role that this region of Vps35p plays in retromer function we next investigated the interactions of mammalian VPS35 using the yeast two-hybrid system. Mouse VPS35 was cloned into the 'bait' vector (pGBT9) of the "Matchmaker" two hybrid system and mouse VPS29 and VPS26 were cloned into the 'prey' vector (pGAD424). These constructs were transformed into the two hybrid reporter strain (HF7c) and the interaction between VPS35 and VPS26 and VPS29 was determined by growth on plates lacking histidine. No growth was observed when either VPS35 was expressed with empty 'prey' vector or VPS26 or VPS29 was co-transformed with empty 'bait' vector (data not shown) so none of the bait or prey constructs was able to autoactivate.

Wildtype VPS35 can interact with both VPS26 and VPS29. Using the inhibitor of the *HIS3* reporter gene, 3-amino-1,2,4-triazole (3-AT) it is possible to gauge the strength of the interaction between VPS35 and VPS26 or VPS29. Strong interactions will be resistant to inhibition with 3-AT whilst weak interactions will result in poor expression of the *HIS3* gene and yeast growth will be inhibited by 3-AT. The interaction with VPS26 is robust and can allow growth in the presence of 2 mM 3-AT. The interaction between VPS35 and VPS29 is somewhat less strong and is susceptible to inhibition in the presence of 2 mM 3-AT. The VPS35 mutants R107A and L108P (equivalent to R98A and L99P in yeast) are compromised in their ability to interact with VPS26 and virtually no growth is seen on plates containing 2 mM 3-AT. The VPS35L108P mutant behaves like wildtype VPS35 with respect to its interaction with VPS29, the VPS35R107A mutant however appears able to interact with VPS35 even in the presence of 2 mM 3-AT suggesting that the VPS35R107A mutant can more strongly interact with VPS29 than wildtype VPS35.

We next examined the ability of the mammalian VPS35 mutants to interact with VPS26 *in vivo* using native immunoprecipitations. Cells were transfected with GFP-tagged VPS35 constructs and then lysed and the lysates treated with antibodies against either GFP or VPS26. Additional controls are provided in the form of the GFP-tagged VPS29 constructs. Wildtype VPS29-GFP can assemble with VPS35 and VPS26 but the VPS29V90D-GFP mutant cannot [18]. These constructs therefore act as positive and negative controls respectively. In figure 4a, GFP-wildtypeVPS35 is able to co-IP VPS26, but the R107A and L108P mutants fail to interact with VPS26 although the R107A mutant was relatively poorly expressed compared to wildtype GFP-VPS35 or the L108P mutant. Similarly, when antibodies against VPS26 are used to IP the lysates, anti-VPS26 can IP wildtype GFP-VPS35 (along with endogenous VPS35) but the R107A and L108P mutants were not detected in these immunoprecipitates (figure 4b).

The interaction between Vps35p and Vps26p has been shown to be important for the membrane association of Vps35p ([19]; this study figure 2b). To determine if the localisation of VPS35 to endosomal membranes requires the interaction with VPS26, we have investigated the localisation of the GFP-tagged VPS35 constructs by fluorescence microscopy. In figure 5a, GFP-wildtypeVPS35 can target to membranes and colocalise with VPS26 but the R107A and L108P mutants are detected only in the cytoplasm. The mutation of the R107 or L108 residues in mammalian VPS35 therefore blocks recruitment of VPS35 to the endosomal membrane.

Discussion

In this study we have identified a conserved region of Vps35p and its mammalian ortholog (VPS35) that is required for the interaction with Vps26p/VPS26. The mutation that confers the dominant negative phenotype observed in cells expressing the *vps35-27* allele was determined to be a L-P substitution at residue 99 which is in the middle of a conserved motif P-R-L/M-Y-L. It is worth noting that of all Vps35p orthologs present in the database, only the *Arabidopsis* ortholog has the methionine residue instead of the first leucine. All other Vps35p orthologs conform to the P-R-L-Y-L consensus. The *vps35-27* allele was first described in [4] but the site of the mutation(s) that conferred the phenotype was not known then. The *vps35-27* allele was found to be suppressed by

overexpression of *VPS29* [4]. In this study we have found that the L99P mutation results in a Vps35 protein that can no longer bind to Vps26p. Therefore the cause of the dominant effect of the *vps35-27* allele is likely to be the titration of Vps29p into inactive complexes, hence overexpression of *VPS29* is able to restore CPY sorting in cells expressing the *vps35-27* allele.

When the L99P *vps35* mutant is expressed at wildtype levels in a *vps35Δ* background, the CPY sorting phenotype is identical to a null mutant. Alanine scanning mutagenesis of the P-R-L-Y-L motif revealed that the arginine is also critical for Vps35p function as mutation to alanine also results in a null phenotype. Interestingly the other alanine scanning mutants were all able to complement the CPY sorting defect in the *vps35Δ* strain indicating that some residues of the P-R-L-Y-L motif are more important than others and that the L99P mutation which confers a null phenotype may be due to local instability in that region of the Vps35p due to the introduction of a proline instead of the leucine. All of the *vps35* alleles were readily detectable so none of the mutations resulted in a protein that was intrinsically unstable and rapidly degraded.

Loss of retromer function has been shown to result in the mislocalisation of the vacuolar hydrolase receptor, Vps10p, to the vacuole [4, 5 and 21]. This is also true for the Vps35L99P mutant. This experiment also provides the first clue as to the underlying defect in the Vps35L99P mutant. When the localisation of Vps29p in cells expressing the L99P mutant was examined it was found to be similar to those cells expressing wildtype Vps35p and not like the *vps35Δ* cells in which Vps29p becomes cytosolic. When the distribution of Vps35p was investigated, it was found that the Vps35L99P protein was present in the cytosolic (S100) fraction to a similar degree as was observed in a *vps26Δ* strain. This hints at a defect in the Vps35p-Vps26p interaction.

The loss of the interaction between Vps35p and Vps26p, which was suggested by the data from figure 2b, was confirmed by native immunoprecipitation from yeast lysates and by two hybrid analysis of the interaction between mammalian VPS35 and VPS26. In yeast the L99P mutant did not assemble into the retromer complex which was immunoprecipitated with antibodies against either Vps5p or Vps26p. Our antisera against Vps35p does not recognise the protein under native conditions so it was not possible to IP Vps35p directly to assess the interactions of the L99P mutant. It has been

previously demonstrated that Vps26p facilitates the proper assembly of Vps35p/Vps29p with Vps5p/Vps17p [19]. Therefore, the inability of Vps5p to co-IP Vps35p/Vps29p is consistent with a loss of interaction between Vps35p and Vps26p due to the L99P mutation. Similarly, the inability of Vps26p to co-IP any of the other retromer subunits from cells expressing the L99P mutant is in-line with the hypothesis that the L99P mutant cannot bind to Vps26p.

The data from the two hybrid analysis of the interactions between mammalian VPS35 and mammalian VPS26, VPS29 strongly agrees with the data obtained from the studies in yeast. Wildtype VPS35 and VPS26 interact strongly, the strength of the interaction is resistant to inhibition with 2 mM 3-AT. The L108P and R107A VPS35 mutants were only able to weakly interact with VPS26 and growth of the yeast containing these combinations of bait and prey was inhibited by 2 mM 3-AT. Curiously, the interaction between VPS35 and VPS29 was actually increased due to the R107A mutation. This observation hints that perhaps VPS29 can interact with a region of VPS35 close to where VPS26 interacts.

Native immunoprecipitation experiments of GFP tagged-VPS35 constructs transiently transfected into mammalian cells confirmed that the R107A and L108P mutants do not interact with VPS26 *in vivo* although the R107A mutant did not efficiently transfect resulting in lower expression of this mutant than was observed for the L108P mutant. The reason for the relatively poor expression of the R107A mutant is currently unknown but was observed on repeated occasions. One possibility is that the ability of the VPS35R107A mutant to interact with VPS29 (see figure 3b) resulted in this construct being dominant negative and therefore toxic to cells expressing it.

Loss of the interaction between VPS35 and VPS26 results in VPS35 being unable to localise to endosomal membranes. Wildtype GFP-VPS35 colocalised with VPS26 whilst the R107A and L108P mutants were present only in the cytoplasm.

In summary, we have identified a critical conserved region of Vps35p/VPS35 that is necessary for the interaction with VPS26. Loss of interaction with Vps26p results in a null phenotype and mislocalisation of Vps10p to the vacuole. Mutation of R107 or L108 in mammalian VPS35 abolishes the interaction with mammalian VPS26 and results in VPS35 being unable to target to the membrane. We have previously shown that a

conserved valine on Vps29p/VPS29 is essential for the interaction with Vps35p/VPS35. Mutation of V109 or V90 respectively results in the mutant Vps29p/VPS29 being unable to bind Vps35p/VPS35 and unable to target to endosomal membranes [18]. With this data in mind we can propose a model of recruitment of retromer to the membrane that is controlled by Vps26p/VPS26. Through unknown mechanisms (that may require SNX1 and SNX2 in mammalian cells [11]), Vps26p/VPS26 is recruited to endosomal membranes. Vps35p/VPS35 follows and binds Vps26p/VPS26 through the mobile loop in Vps26p/VPS26 [17] and the P-R-L-Y-L motif in Vps35p/VPS35. Vps29p/VPS29 binds last and this is mediated through the C-terminus of Vps35p/VPS35 [23] and the V109/V90 residues in Vps29p/VPS29 [18]. It is also possible that Vps35p can interact with Vps29p independently of Vps26p which has been demonstrated through the use of crosslinking in *vps26Δ* cells [4]. Vps29p is required for the subsequent assembly of the cargo selective Vps35/29/26p subcomplex with Vps5/17p to create the pentameric retromer complex. In yeast, this involves the L252 residue of Vps29p which is necessary for this interaction [18]. In this model the assembly of retromer on the endosomal membrane is stepwise and ordered and facilitates retromer in its function of membrane protein sorting for endosome-to-Golgi retrieval.

An alternative model is also possible in which the cargo-selective subcomplex of Vps35/29/26p assembles as a unit prior being recruited onto the membrane. The Vps35p/29/26p subcomplex may then interact with the membrane via multiple weak interactions occurring through the Vps35p and Vps26p subunits both of which appear able to associate with the membrane (at least partially) independently of the other subunits. Hence loss of the interaction between Vps35p and Vps26p (due to the L99P mutation) does not (in yeast) result in a complete loss of membrane association. The interactions that govern assembly of the retromer complex are summarized in figure 6.

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Strain	Genotype	source or ref.
SEY 6210	<i>MATα leu2-3,112 ura3-52 his3Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	[24]
MSY2600	SEY 6210 <i>vps26Δ::LEU2</i>	[4]
PSY1-29	SEY 6210 <i>vps29Δ::HIS3</i>	[21]
EMY18	SEY 6210 <i>vps35Δ::HIS3</i>	[21]
MSY10-21	SEY 6210 <i>VPS10-6myc</i>	[4]
HF7c	<i>MATα ura3-52 his3Δ200 trp1-Δ901 lys2-801 ade2-101 leu2-3,112 gal4-542, gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4 17mers)₃-CYC1-LacZ.</i>	Clontech (Paisley, Scotland, UK)

Table I. Yeast strains used in this study.

Allele	Mutations					
<i>vps35-27</i>	Y55H	L99P	F112L	I247T	S275L	M457T
<i>vps35-27a</i>				I247T	S275L	M457T
<i>vps35-27b</i>		L99P	F112L	I247T	S275L	M457T
<i>vps35-27c</i>	Y55H					
<i>vps35-27d</i>	Y55H	L99P	F112L			
<i>vps35-27e</i>		L99P	F112L			

Table II. Mutations in the *vps35* alleles.

Figure legends.

Figure 1. Identification of the mutation that confers the dominant negative phenotype of the *vps35-27* allele. A. Protein sequence alignment of yeast (*Saccharomyces cerevisiae*, Sc) Vps35p N-terminal half with orthologs from plant (*Arabidopsis thaliana*, At), Chicken (*Gallus gallus*, Gg) and human (*Homo sapiens*, Hs). The position of the various mutations present in the *vps35-27* allele are indicated. The yeast Vps35p sequence was analysed by the jpred secondary structure prediction program

(<http://www.compbio.dundee.ac.uk/~www-jpred/>). Regions of α -helical predicted structure are shown as cylinders, arrows depict regions predicted to form β -sheet structure. B. CPY sorting assay of wildtype cells (SEY6210) expressing the *vps35-27* allele and derivatives a-e. Cells were pulse labelled with ^{35}S -methionine for 10 mins and chased for 30 mins before the intracellular (I) and extracellular (E) fractions were separated from each other and CPY recovered by immunoprecipitation. Expression of the *vps35-27* allele at high copy number results in secretion of the Golgi-modified (p2) form of CPY. The alleles derived from *vps35-27* that have the L99P mutation secrete p2CPY whilst those without do not.

Figure 2. Alanine-scanning mutagenesis of the P-R-L-Y-L motif and cell fractionation to localise Vps10p, Vps29p and Vps35p. A. CPY sorting of *vps35 Δ* cells expressing the P-R-L-Y-L alanine-scanning mutants. *vps35 Δ* cells were transformed with centromeric plasmids (low copy number) to express wildtype *VPS35*, the L99P mutant or the P-R-L-Y-L motif mutants. Cells were pulse labelled with ^{35}S -methionine for 10 mins and chased for 30 mins before the culture was transferred to ice and precipitated with 10% trichloroacetic acid. After lysis, total CPY was recovered by immunoprecipitation. The L99P and R98A mutants both fail to complement the CPY sorting defect in *vps35 Δ* yeast. In the lower panel, Vps35p was recovered by immunoprecipitation showing that all the mutants are equally expressed. B. Cells were labelled with ^{35}S -methionine before being fractionated into P13 (vacuolar membrane), S100 (cytosolic) and P100 (Golgi, endosomes and small vesicles) fractions to determine the distribution of Vps10p, Vps29p and Vps35p. Loss of retromer function results in Vps10p being mislocalised to the vacuolar membrane (P13) fraction and a similar phenotype is produced in cells

expressing the L99P mutant. When the levels of Vps35p in each fraction is quantitated, it is apparent that the behaviour of Vps35L99P protein is similar to Vps35p in *vps26Δ* cells. C. Wildtype cells (MSY10-21) expressing either wildtype *VPS35* or the *vps35L99P* mutant were fractionated as above and Vps10p and Vps26p were recovered by immunoprecipitation. In a wildtype background there is little discernable shift of Vps10p to the vacuolar (P13) fraction due to the L99P mutation.

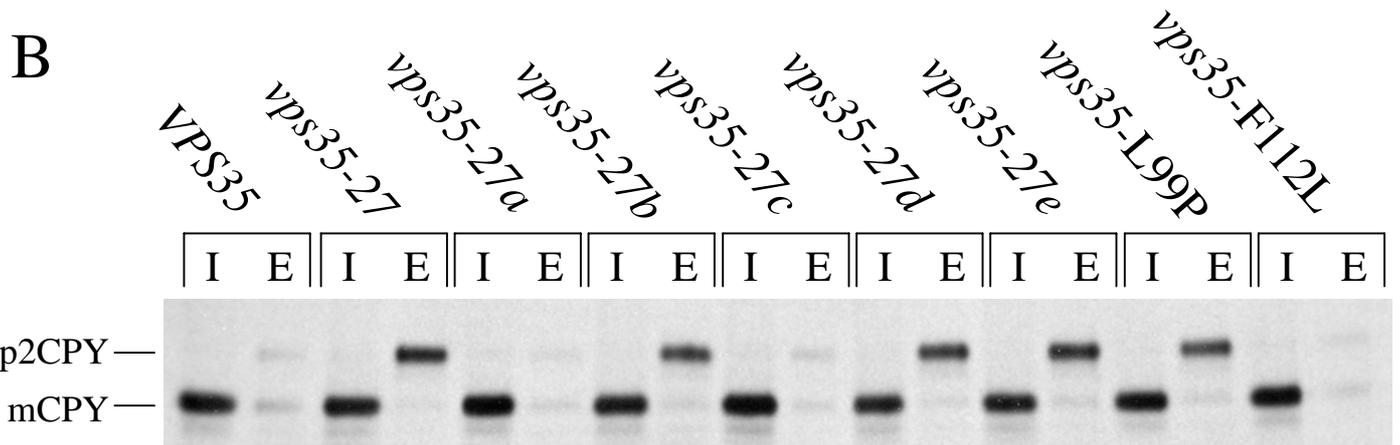
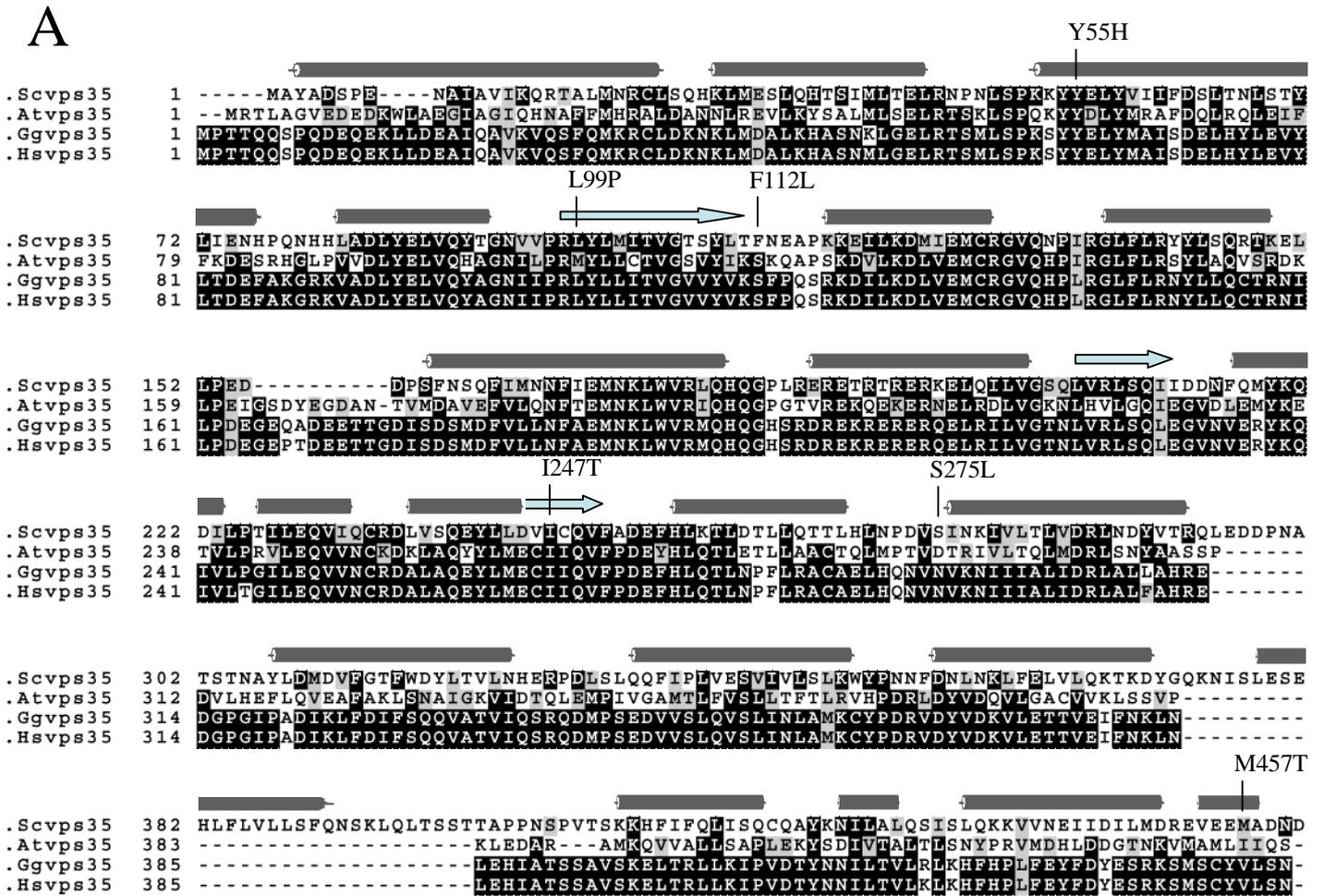
Figure 3. The L99P/L108P mutation in yeast Vps35p or mammalian VPS35 abolishes the interaction with Vps26p/VPS26. A. Yeast cells were labelled with ³⁵S-methionine before lysis. The retromer complex was immunoprecipitated under native conditions using antisera against either Vps5p or Vps26p. After the primary IP, the samples were re-solubilised and the individual retromer proteins were immunoprecipitated under non-native conditions. The Vps35pL99P mutant does not assemble into the retromer complex. B. Yeast two hybrid analysis of the interaction between mammalian VPS35 and VPS26 and VPS29. VPS35 wildtype, R107A and L108P mutants in the two hybrid ‘bait’ vector were transformed into the two hybrid reporter strain (HF7c) along with either VPS26 or VPS29 in the ‘prey’ vector. Transformants were grown in liquid culture before being spotted onto YNB plates lacking histidine (but with either 0, 1 or 2 mM 3-AT added) in a serial dilution. Growth on the –His plates is indicative of an interaction between bait and prey, the strength of the interaction can be gauged by the ability of the yeast to grow in the presence of 3-AT. The R107A and L108P mutations in VPS35 abolishes the interaction with VPS26.

Figure 4. The R107A and L108P mutations abolish the interaction with mammalian VPS35 in vivo. A. HeLa cells were transiently transfected with GFP-tagged wildtype VPS35 or the R107A or L108P mutants. Additionally, cells stably expressing either VPS29-GFP or VPS29V90D-GFP constructs were used as positive and negative controls. After lysis, the lysates were incubated with anti-GFP antisera. Wildtype GFP-VPS35 binds VPS26 but the R107A and L108P mutants do not interact with VPS26. The VPS29-GFP protein was able to co-IP with VPS35 and VPS26 but the V90D mutant does not. B. Similarly when anti-VPS26 antisera is used to IP the lysates, wildtype GFP-

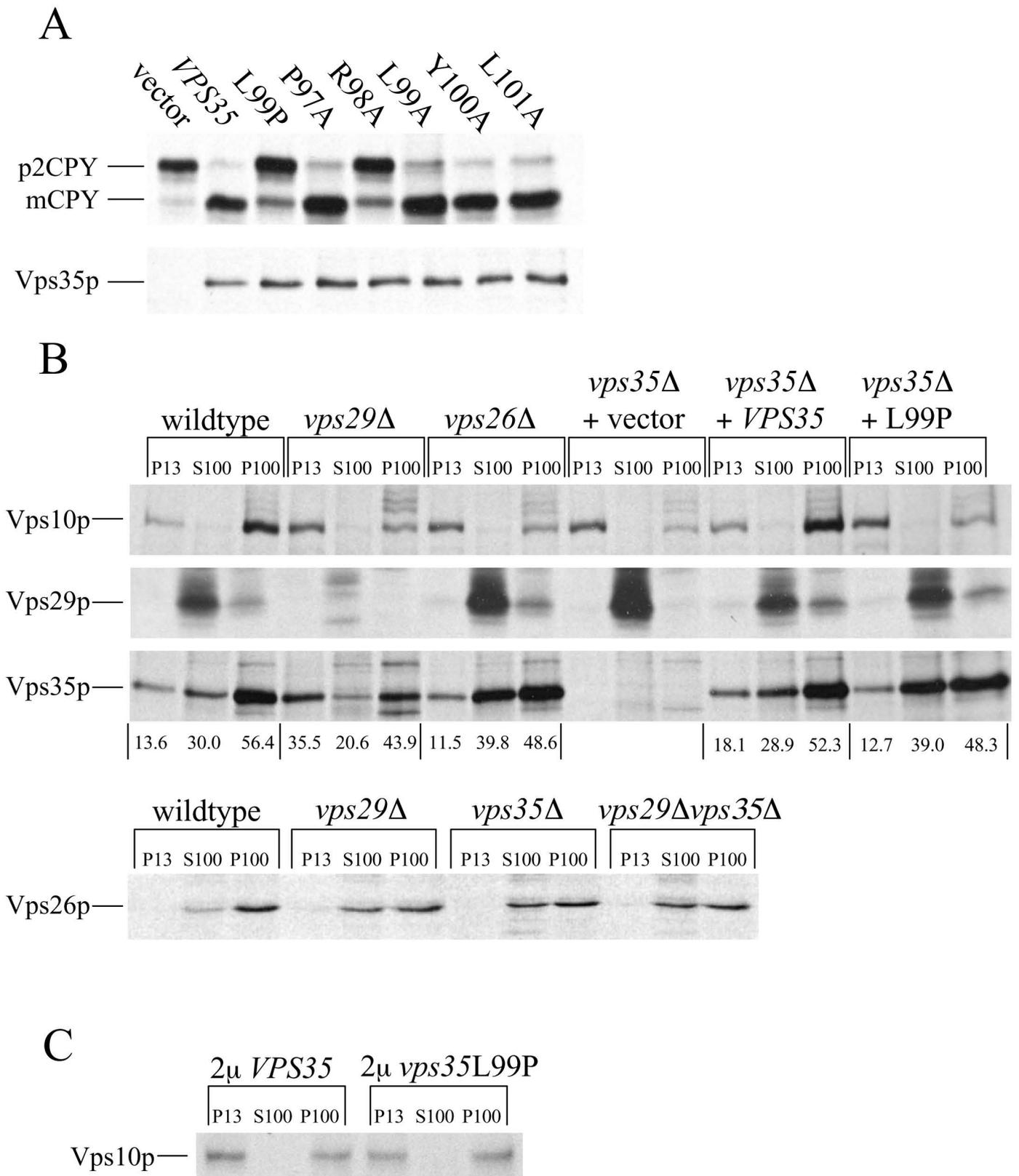
VPS35 can co-IP with VPS26 but the R107A and L108P mutants both fail to interact with VPS26.

Figure 5. The R107A and L108P mutations in VPS35 prevent recruitment to the endosomal membrane. HeLa cells were transiently transfected with GFP-tagged VPS35 constructs. After fixation, the cells were labelled with antibodies against VPS26. Wildtype VPS35 can be recruited to the membrane to colocalise with VPS26 but the R107A and L108P mutants are only visible in the cytoplasm. Scale bar = 20 μ m.

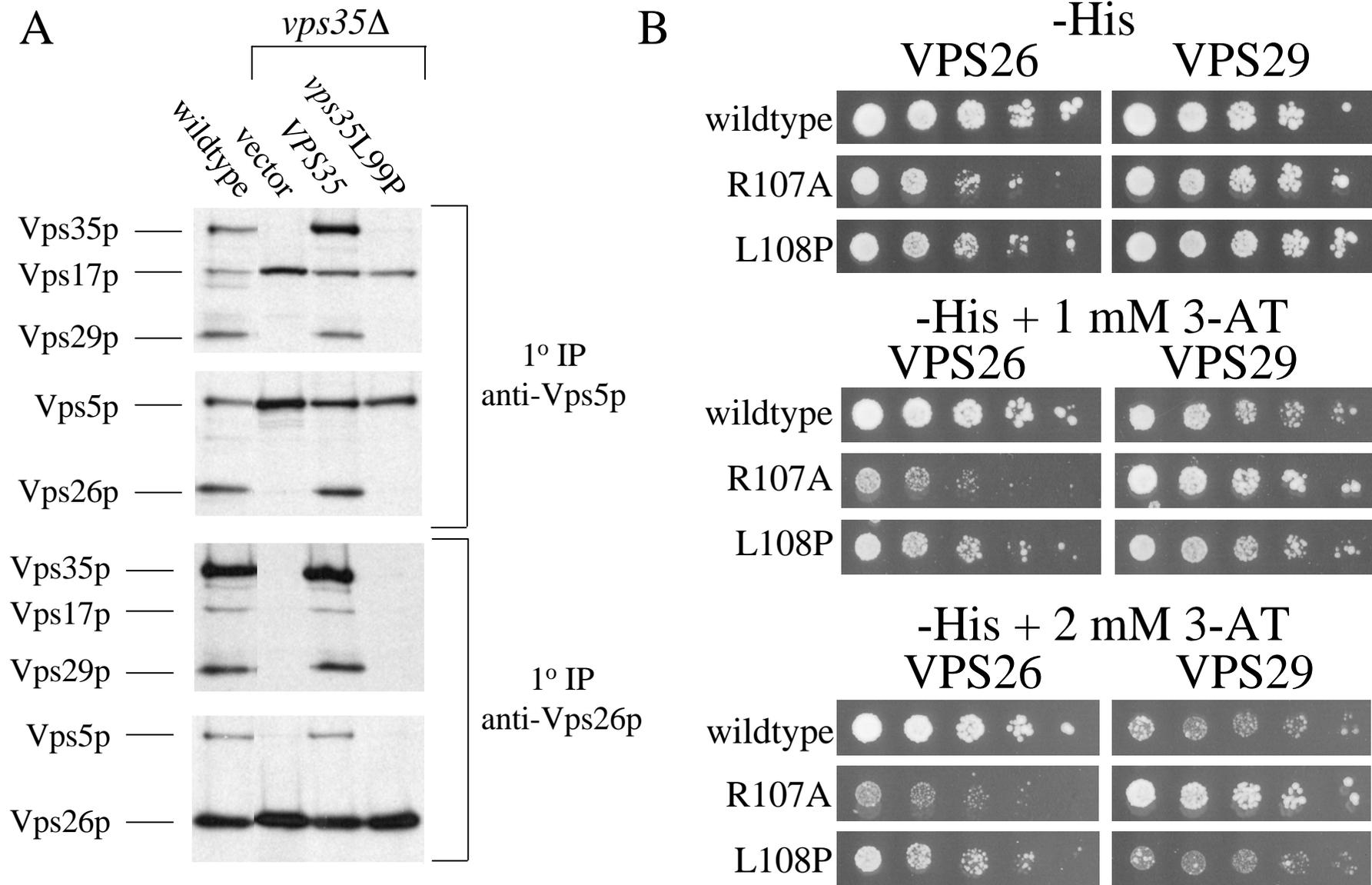
Figure 6. A schematic model depicting key features of the assembly of the retromer complex. Vps35p/VPS35 binds to Vps26p/VPS26 via the P-R-L-Y-L motif, the R and adjacent L residues being critical for this binding. Vps29p/VPS29 binds at the C-terminus of Vps35p/VPS35 through a conserved valine (V109 or V90) on Vps29p/VPS29 [23,18]. Vps29p is necessary for the assembly of the pentameric retromer complex containing Vps5p and Vps17p and the L252 residue of Vps29p is essential for this interaction. The assembly of retromer could occur on the endosomal membrane (as shown in figure 6) or the cargo-selective subcomplex (Vps35/29/26p) could assemble in the cytoplasm prior to the recruitment onto the membrane.



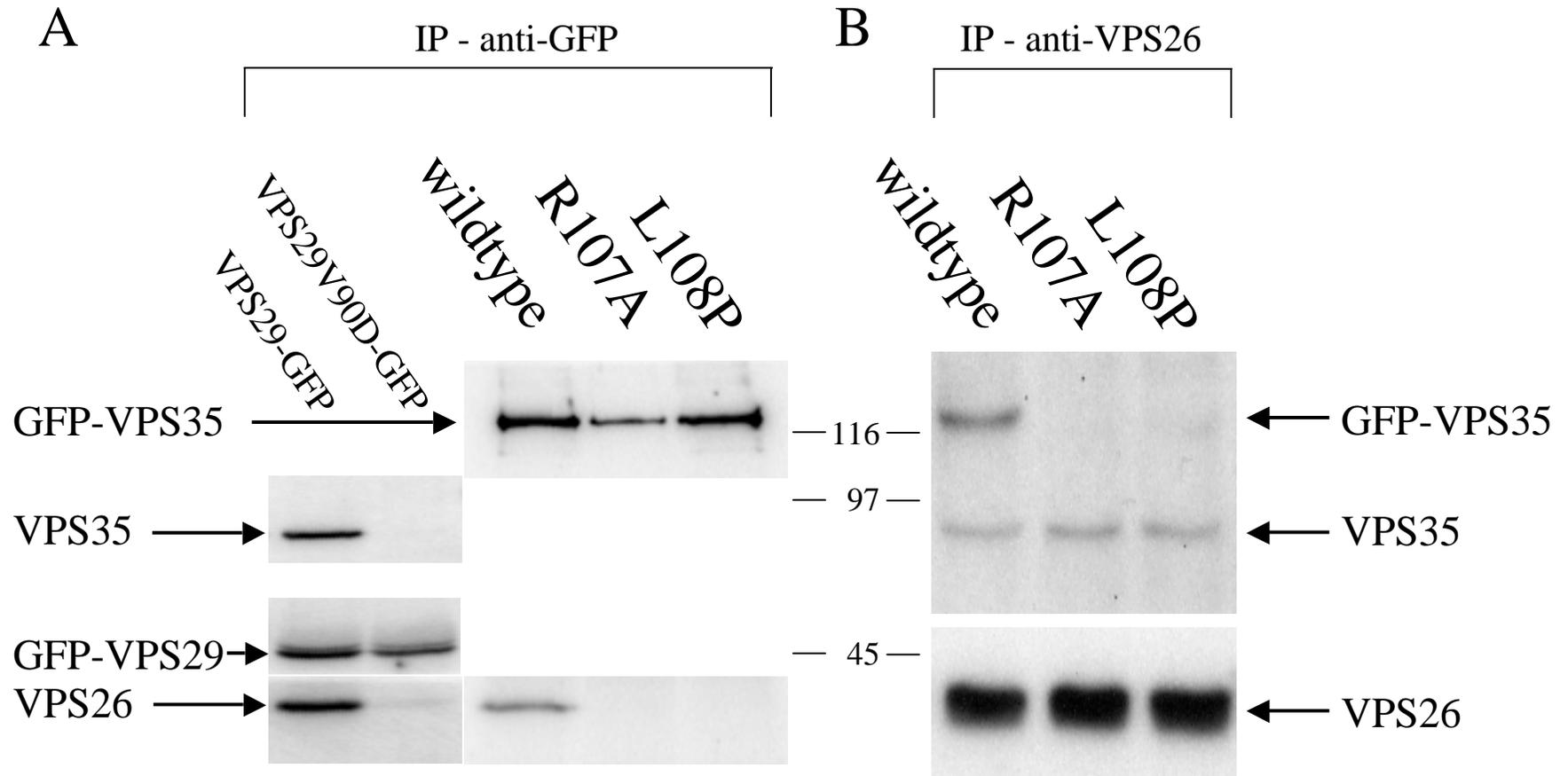
Gokool *et al.*, Figure 1.



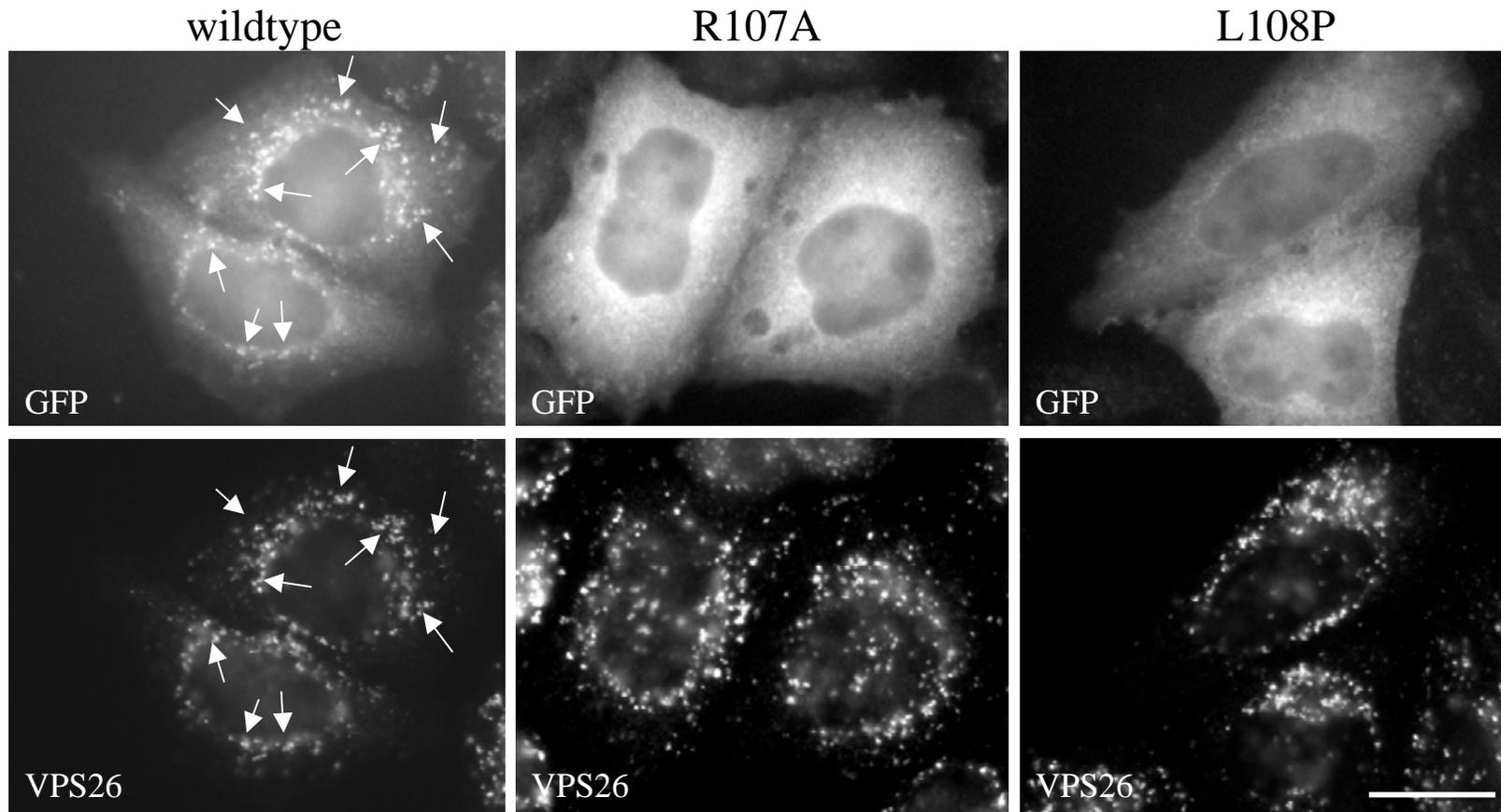
Gokool *et al.*, Figure 2.



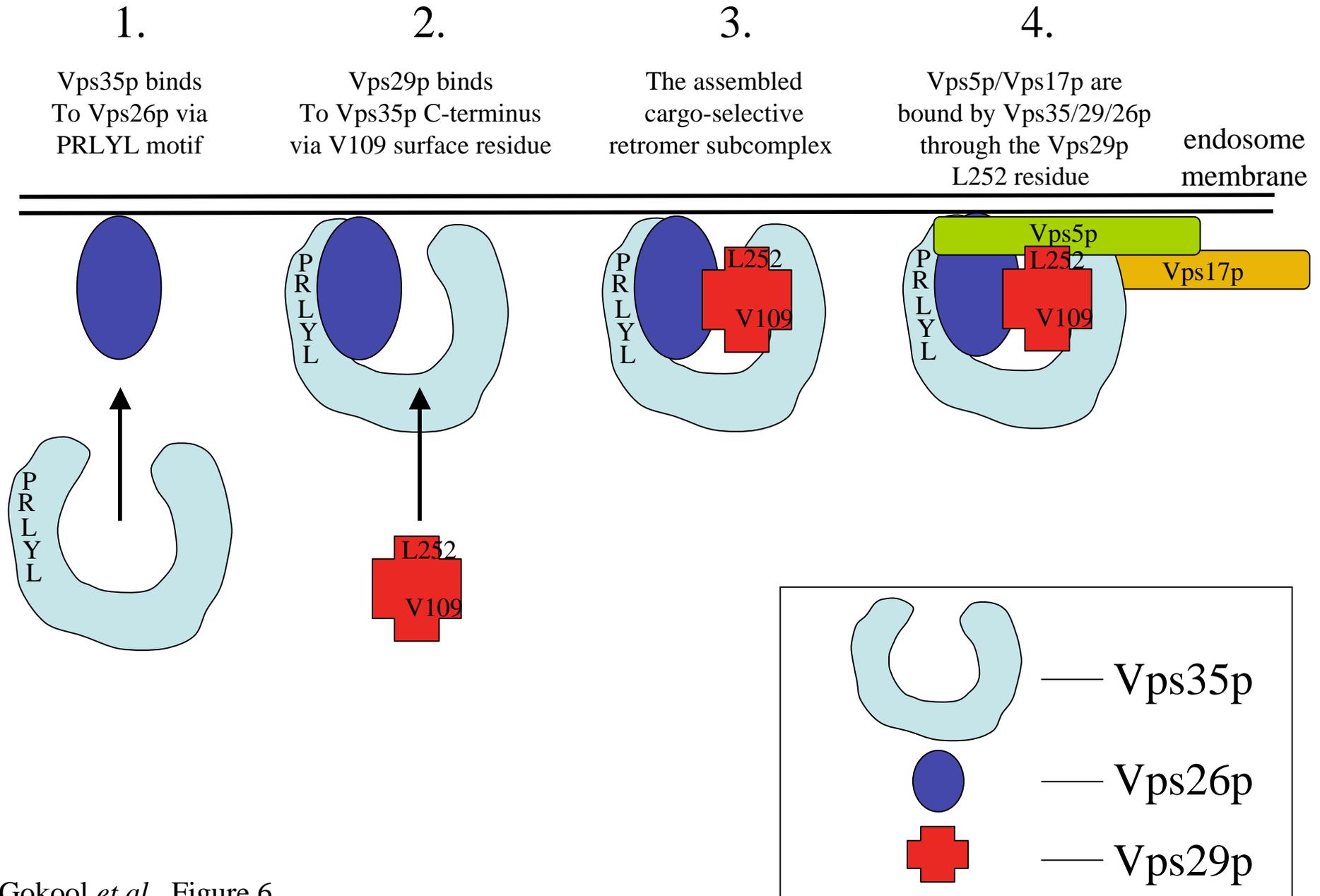
Gokool *et al.*, Figure 3.



Gokool *et al.*, Figure 4.



Gokool *et al.*, Figure 5.



Gokool *et al.*, Figure 6.