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Atypical Parkinsonism-associated retromer mutant alters endosomal sorting of specific cargo proteins

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eTOC Summary

Mutations in the retromer complex, which is involved in sorting integral membrane proteins from endosomes to cellular compartments, are associated with atypical Parkinsonism, but how these mutations affect retromer function remains unclear. Through a quantitative proteomic analysis of the retromer interactome, McMillan et al. reveal a new mechanism for perturbed endosomal sorting in Parkinsonism.

Abstract

The retromer complex acts as a scaffold for endosomal protein complexes that sort integral membrane proteins to various cellular destinations. The retromer complex is a heterotrimer of VPS29, VPS35 and VPS26. Two of these paralogues, VPS26A and VPS26B, are expressed in humans. Retromer dysfunction is associated with neurodegenerative disease, and recently three VPS26A mutations (p.K93E; p.M112V and p.K297X) were discovered to be associated with atypical Parkinsonism. Here, we applied quantitative proteomics to provide a detailed description of the retromer interactome. By establishing a comparative proteomic methodology, we identify how this interactome is perturbed in atypical Parkinsonism-associated VPS26A mutants. In particular, we describe a selective defect in the association of VPS26A(p.K297X) with the SNX27 cargo adaptor. By showing how a retromer mutant leads to altered endosomal sorting of specific PDZ ligand-containing cargo proteins, we reveal a new mechanism for perturbed endosomal cargo sorting in atypical Parkinsonism.

Introduction

Retromer is a highly conserved heterotrimer of VPS29, VPS35 and VPS26, of which two paralogues, VPS26A and VPS26B, are expressed in humans (Seaman, 2012; Burd and Cullen, 2014). Retromer is associated with the cytosolic face of endosomes where it scaffolds a multi-protein complex that orchestrates the sorting of integral membrane proteins (termed cargos) into transport carriers destined for the plasma membrane, the *trans*-Golgi network (TGN) and specialised organelles (Seaman et al., 1998; Cullen and Korswagen, 2011; Burd and Cullen, 2014). Defects in retromer are associated with neurological disease. Retromer expression is lowered in brains of patients with Alzheimer's disease and Parkinson's disease, and retromer mutations are observed in familial and sporadic forms of these diseases (Small et al., 2005; Muhammad et al., 2008; Vilariño-Guell et al., 2011; Zimprich et al., 2011; Vardarajan et al., 2012; MacLeod et al., 2013; Shannon et al., 2014; Vilariño-Guell et al., 2014; Rovelet-Lecrux et al., 2015). For example, the autosomal dominant Parkinson disease-linked VPS35(p.D620N) mutation leads to perturb retromer function by disrupting the association with the actin-nucleating Wiskott-Aldrich syndrome and SCAR homolog (WASH) complex (McGough et al., 2014a, Zavodszky et al., 2014). Further mutations in the VPS26A sub-unit, VPS26A(p.K93E); VPS26A(p.M112V) and VPS26A(p.K297X), have also been linked to atypical Parkinsonism (Gustavsson et al., 2015). How these mutations perturb retromer function remains unclear. Alterations in retromer accessory proteins are observed in neurological disease, including in Down's syndrome and infantile myoclonic epilepsy the retromer cargo adaptor sorting nexin-27 (SNX27) (Wang et al., 2013; Damseh et al., 2015). Identifying retromer accessory proteins and how they assemble to form a functional complex is essential in defining the molecular details of retromer activity and in providing insight into the pathoetiology of retromer-associated disease.

Here, we have applied proteomics to provide the first detailed, quantitative description of the retromer interactome. By establishing a comparative proteomic methodology, we identify how this interactome is perturbed in VPS26A mutants in patients with atypical Parkinsonism (Gustavsson et al., 2015). In particular we describe the identification of a selective defect in the association of VPS26A(p.K297X) with SNX27. Through establishing that this leads to perturbed endosomal sorting of specific cargo proteins we reveal a new mechanism for perturbed endosomal trafficking in Parkinsonism.

Results and Discussion.

Quantitative identification of interacting proteins for individual retromer subunits.

Retromer's role in cargo sorting is mediated, in part, through an ability to recruit accessory proteins (Harbour et al., 2010). Limited information is available regarding the range of retromer interacting proteins. We previously identified VPS35 interacting proteins using SILAC proteomics (McGough et al., 2014a and 2014b). Here, we extended this procedure using VPS29, VPS26A and VPS26B in order to: validate known interactions; determine whether VPS26A and VPS26B display distinct interactions that may distinguish their functions; and, to increase the detection power of our overall analysis. Previously, proteins not strongly enriched in the VPS35 interactome were excluded (McGough et al., 2014b). Such proteins may be relevant to retromer function, with their low enrichment due to weak interaction or through association with a subunit other than VPS35. Comparison of the VPS35 interactome with those for VPS29, VPS26A and VPS26B would highlight these proteins, despite them being weakly enriched in an individual interactome.

To achieve this we lentivirally transduced human retinal pigment epithelial-1 (RPE-1) cells to generate cell populations expressing GFP-tagged VPS26A, VPS26B or VPS29 (Supp. Fig. 1A-F). For the VPS29 interactome we grew GFP-VPS29 expressing cells in amino acids of "medium" mass (R6K4), alongside cells expressing GFP grown in unlabelled, "light" amino acids (R0K0). We subjected these cells to GFP-trap immunoprecipitation, resolved the combined co-immunoprecipitates by SDS-PAGE and identified proteins using LC-MS/MS. From duplicate experiments, a single list of VPS29 interacting proteins was generated by excluding proteins quantified from a single unique peptide and any that were <4-fold enriched in the GFP-VPS29 immunoprecipitate (Fig. 1A, Table S1). The two filtered lists were combined by excluding proteins not present in both lists leading to 53 proteins being identified as the VPS29 interactome (Fig. 1A, 1B, Table S1).

For VPS26A and VPS26B interactomes we used triple SILAC, growing GFP, GFP-VPS26A and GFP-VPS26B expressing cells in R0K0, R6K4 and "heavy" (R10K8) labelled medium respectively. From duplicate experiments, we again generated a single list of proteins that interacted with each of the VPS26 paralogues (Fig. 1A, Table S1), with the difference that we considered both the R6K4/R0K0 ratio and count (for VPS26A) and the R10K8/R0K0 ratio and count (for VPS26B) when filtering. If a protein met the required criteria for inclusion in the filtered list for one VPS26 paralogue but not the other, we included that protein to avoid removing proteins that interact with only one paralogue. The identified 141 proteins defined the VPS26 interactome (Fig. 1A, 1B, Table S1).

To gain insight into potential functional differences between VPS26 paralogues we sort to identify paralogue-specific interactomes. Here we examined the mean R10K8/R6K4 ratio. For the VPS26B-specific interactome we excluded proteins with a mean R10K8/R6K4 ratio below 10, while for the VPS26A-specific interactome we excluded proteins with a mean R10K8/R6K4 ratio above 0.1. Using these criteria, all VPS26 interactors were common to VPS26A and VPS26B, the only exception being VPS26B's association with a heat shock protein, HSPH1 (Fig. 1B, Table S1).

Identification of the detailed retromer interactome.

In the same parental RPE-1 cells, we previously generated a VPS35 interactome in duplicate (McGough et al., 2014a and 2014b), so we combined these data into a single VPS35 interactome (Fig. 1A, Table S1). To identify proteins consistently immunoprecipitated by retromer, we identified proteins common to the VPS35, VPS29, and VPS26A and VPS26B interactomes. To avoid excluding proteins that may have been filtered out of the individual interactomes we cross-referenced each protein in the retromer interactome with the unfiltered lists for each retromer subunit. If a protein was found in an unfiltered list at an enrichment ≥ 2-fold, we considered that protein to be present in the interactome of that retromer subunit for our comparison (Fig. 1C). Gene ontology analysis (PANTHER Classification System – $p<0.05$) revealed the majority of interactors had roles in endosomal transport (Fig. 1D). Network analysis using esyN (Fig. 1E) (Bean et al., 2014), revealed known retromer interactors, the WASH complex and its accessory proteins (Gomez and Billadeau, 2009; Harbour et al., 2010; Harbour et al., 2012), including the CCC complex (Phillips-Krawczak et al., 2015), and various other proteins including SNX27 (Temkin et al., 2011; Steinberg et al., 2013), ANKRD27 (VARP) (Hesketh et al., 2014; McGough et al., 2014a; Bean et al., 2015), ANKRD50 and SDCCAG3 (McGough et al., 2014a), TBC1D5 (Seaman et al., 2009), and DNAJC13 (Shi et al., 2009; Popoff et al., 2007; Freeman et al., 2014). Most of these 'core' retromer accessory proteins were located within the central region of the Venn diagram (Fig. 1C). This region contained two proteins not previously shown to interact with retromer, the Rab10 guanine-nucleotide exchange factor DENND4C (Yoshimura et al., 2010), and the polycystin family member PKD2 (Chapin and Caplan, 2010). GFP-nanotrap immunoisolation and quantitative western analysis confirmed association of retromer with several interactors including SNX27, DENND4C and PKD2 (Fig. 1F). DENND4C and PKD2 proteins co-localised with retromer decorated endosomes (Supp. Fig. 2A, 2B) and the functional relevance of these interactions will be described elsewhere.

Missense VPS26A mutants do not perturb heterotrimeric retromer assembly or its endosome association.

We next turned our attention to how the atypical Parkinsonism associated VPS26A mutants affect assembly of the retromer interactome. Using lentiviruses to transduce RPE-1 cells with constructs encoding for GFP-VPS26A, GFP-VPS26A(p.K93E), GFP-VPS26A(p.M112V), or GFP-VPS26A(p.K297X), we titrated the viral load to produce cell lines where expression of the GFP-tagged transgene approached endogenous levels and was observed to lower the expression of endogenous VPS26A, presumably as a result of competition for inclusion of the GFP-tagged transgene into the stable heterotrimeric complex and resultant destabilisation of the non-complexed endogenous VPS26A (Fig. 2A). Note, truncation of the last 31 amino acids of VPS26A leads to the molecular weight of the GFP-tagged protein being smaller (Fig. 2A). GFP-nanotrap immunoisolation and quantitative western analysis revealed that each VPS26A mutant retained the ability to form a heterotrimeric complex (Fig. 2B, 2C). Quantitative confocal imaging established each mutant retained association with endosomes labelled for retromer (VPS35 positive) and the WASH complex (FAM21 positive) (Fig. 2D, 2E).

VPS26A(p.K297X) displays a loss-of-association with the cargo adaptor SNX27 and an enhanced association with PKD2 and DENND4C.

To identify loss-of and gain-of-function phenotypes associated with each VPS26A mutant, we developed an unbiased, quantitative and directly comparative proteomic methodology. RPE-1 cells lentiviral transduced with encoding constructs for wild-type GFP-VPS26A or GFP-VPS26A mutants were grown in light and medium heavy SILAC media. Cells were subjected to GFP-trap, the immunoprecipitates combined and the proteins resolved by SDS-PAGE and identified by LC-MS/MS. Data were expressed as a quantified enrichment ratio of the VPS26A mutant over wild-type VPS26A. A ratio for the interacting protein approaching 1.0 was indicative of unperturbed association, while a ratio approaching 0.1 or 10.0 were indicative of a reduced or enhanced association to the VPS26A mutant respectively (Fig. 3A).

Consistent with the data in Figure 2B and 2C, all VPS26A mutants retained an unperturbed enrichment with VPS29 and VPS35 as well as the WASH complex (Fig. 3A). For the VPS26A(p.K93E) and VPS26A(p.M112V) mutants no major alterations were observed with any of the core retromer accessory proteins (enrichment ratios between 0.6 and 2.8). In contrast, the VPS26A(p.K297X) mutant showed a dramatic reduction in SNX27 association (ratio of 0.01). An increase in the enrichment ratio of VPS26A(p.K297X) for PKD2 (ratio 17.172), and DENND4C (ratio 6.555) was also observed, which we confirmed through

quantitative western analysis (Fig. 3B-D). Thus truncation of the last 31 amino acids of VPS26A, as observed in VPS26A(p.K297X), leads to a pronounced loss-of and enhancement-of VPS26A's ability to associate with specific retromer accessory proteins.

VPS26A(p.K297X) fails to associate with SNX27 in direct binding assays.

The PDZ domain of SNX27 binds to VPS26 (Gallon et al., 2014). Using purified proteins, binding of VPS26A to GST-SNX27 was readily observed by Coomassie staining and western blotting (Fig. 4A). While the same was true for VPS26A(p.K93E) and VPS26A(p.M112V), binding to VPS26A(p.K297X) was undetectable. Binding of VPS26A to SNX27 enhances SNX27's affinity for PDZ ligands (Gallon et al., 2014; Chan et al., 2016). In agreement with the pull-down experiments, VPS26A(p.K93E) and VPS26A(p.M112V) enhanced binding of SNX27 to a peptide corresponding to the PDZ ligand of Kir3.3 by more than 10-fold, while VPS26A(p.K297X) displayed no ability to enhance this binding affinity (Fig. 4B, 4C).

Structurally, the β-hairpin in the SNX27 PDZ domain binds to a groove in the arrestin-like structure of VPS26A (Gallon et al., 2014). Within this structure, Lys93 and Met112 of VPS26A reside in close proximity to the SNX27 interacting surface, but they do not form direct contacts (Fig. 4D). Lys297 also does not directly contact SNX27. However this residue and adjacent C-terminal sequences are important for stabilizing the linker (residues 152-164) between the N and C-terminal domains. Therefore the deletion of these C-terminal sequences likely prevents SNX27 interaction by destabilizing the linker region and preventing its binding to the SNX27 β-hairpin.

Loss of VPS26A(p.K297X) association to SNX27 leads to defective sorting of SNX27 cargo.

Over 100 cell surface integral proteins require SNX27 to prevent their lysosomal degradation and maintain their cell surface levels (Steinberg et al., 2013). Essential to this sorting is the interaction of VPS26 with SNX27 (Gallon et al., 2014). One cargo is the glucose transporter GLUT1. Suppression of retromer or SNX27 leads to re-routing of endocytosed GLUT1 to LAMP1-labelled late endosomes/lysosomes (Steinberg et al., 2013). To establish whether the VPS26A mutations rescue the GLUT1 sorting defect observed upon RNAi-mediated suppression of VPS26 expression (Fig. 5A – see also Gallon et al., 2014), siRNA-resistant GFP-tagged VPS26A or mutant VPS26A was expressed in VPS26A and VPS26B suppressed RPE-1 cells. Expression of siRNA resistant GFP-VPS26A, GFP-VPS26A(p.K93E) or GFP-VPS26A(p.M112V) fully rescued the knockdown phenotype (Fig. 5B, 5C). In contrast, GFP-VPS26A(p.K297X) failed to rescue the sorting defect (Fig. 5B, 5C). This mirrors the GLUT1

sorting defect upon uncoupling of SNX27's association with retromer (Steinberg et al., 2013), and is consistent with the loss of SNX27 binding to VPS26A(p.K297X).

Under these conditions immunofluorescence staining established that steady-state distribution of the cation-independent mannose 6-phosphate receptor (CI-MPR) was unaltered (Supp. Fig. 2C). Perturbed retromer function is characterised by an alteration in CI-MPR distribution from a normal localisation at the TGN to peripherally dispersed endosomes (Arighi et al., 2004; Seaman, 2004). Also unaltered by expression of the VPS26A mutants was the steady-state distribution of $\alpha_5\beta_1$-integrin, a cargo that undergoes endosome sorting to the plasma membrane through a retromer-independent pathway (Steinberg et al., 2012) (Supp. Fig. 2D). Finally, as each VPS26A mutant was able to assemble into the retromer heterotrimer at the expense of endogenous VPS26A (Fig. 2A), we considered their potential as function blocking dominant negatives. In RPE-1 cells GLUT1 steady-state distribution was significantly perturbed upon over-expression of GFP-VPS26A(p.K297X); a similar phenotype was not observed with wild-type GFP-VPS26A or GFP-VPS26A(p.K93E) or GFP-VPS26A(p.M112V) (Fig. 5D). Overall, these functional data are consistent with the unperturbed interactomes of VPS26A(p.K93E) and VPS26A(p.M112V), and establish that the inability of VPS26A(p.K297X) to associate with SNX27 manifests as a clear and specific defect in endosomal sorting of SNX27-retromer cargo.

Cargoes for SNX27 mediated sorting include neuronal receptors such as β2-adrenergic receptor, G protein–activated inward rectifying potassium type 2 receptor, serotonin receptor, the parathyroid hormone receptor, as well as NMDA and AMPA receptors (Joubert et al., 2004; Lauffer et al., 2010; Balana et al., 2011; Cai et al., 2011; Temkin et al., 2011; Wang et al., 2013; Chan et al., 2016). Loss of VPS26A(p.K297X) association to SNX27 therefore provides further evidence of the importance of the SNX27-retromer-WASH sorting pathway for neuronal health and provides mechanistic insight into how retromer function is perturbed in this form of atypical Parkinsonism. In developing an unbiased and comparative proteomic methodology we provide a powerful tool for defining disease-associated loss-of and gain-of-function phenotypes as typified by the enhancement of VPS26A(p.K297X) binding to two newly identified retromer interacting proteins: PKD2 and DENND4C. While the role of these proteins in retromer-mediated sorting are unclear, our study highlights a novel regulatory feature of the carboxy-terminal tail of VPS26A in the scaffolding function of retromer.

Materials and Methods

Antibodies

Antibodies used in this study were: rabbit monoclonal α5 integrin (Abcam, ab150361), mouse monoclonal β–actin (Sigma-Aldrich, A1978), rabbit monoclonal CI-MPR (Abcam, ab124767), rabbit polyclonal DENND4C (Sigma-Aldrich, HPA014917), rabbit monoclonal EEA1 (Cell Signalling, C45B10), mouse monoclonal GFP (green fluorescent protein, Roche, 11814460001), rabbit monoclonal GLUT1 (Abcam, 115730), mouse monoclonal LAMP1 (H4A3, DSHB), rabbit polyclonal PKD2 (Santa Cruz, sc-25749), mouse monoclonal SNX27 (Abcam, ab77799), rabbit polyclonal Strumpellin (Santa Cruz, 87442), rabbit polyclonal VPS35 (Abcam, 97545), rabbit monoclonal VPS35 (Abcam, 157220), rabbit polyclonal VPS26A (Abcam, ab137447), rabbit polyclonal VPS29 (Abcam, ab98929), Rabbit polyclonal WASH1 and FAM21 (gifts from D.D. Billadeau (Mayo Clinic, Rochester, MN)).

Generation of GFP-VS26A and GFP-VPS26A mutant lentiviral vectors

VPS26A was sub-cloned from an original plasmid which was a kind gift from Dr Rohan Teasdale (University of Queensland, Kerr et al., 2005) into the lentiviral vector pXLG3. To produce siRNA-resistant VPS26A six silent base mismatches (C187T; A189G; A192G; C195T; A198G; A201G) were introduced into the ORF, generating resistance to VPS26A siRNA oligonucleotide 1 from the ON-TARGET plus human SMART pool (Dharmacon). To produce siRNA-resistant VPS26B seven silent base mismatches (G795A; C798T; T799A; C800G; G804A; C805A; C807G) were introduced into the ORF, generating resistance to VPS26B siRNA oligonucleotide 2 from the ON-TARGET plus human SMART pool (Dharmacon) (Gallon et al., 2014). The VPS26A(p.K93E), VPS26A(p.M112V) and VPS26A(p.K297X) mutations were generated using site-directed mutagenesis using the following primers:

VPS26A(p.K93E): 5’-gttctccaggtaaggctagttcctccactaggtttacaaattcatga-3’
VPS26A(p.M112V): 5’-gactcagagcagaagttatgattttgaatttgtgcaagttgaaaagcc-3’
VPS26A(p.K297X): 5’-gaagtccggagttcactaagcatattagaagcatgttttag-3’

Cell culture and generation of lentiviral vector expressing stable RPE1 cell lines

Lentiviral particles were produced in HEK293T cells before being added to RPE-1 cells at near endogenous levels to produce stably expressing cell lines. RPE-1 and HEK293T cells were maintained in DMEM (Sigma, D5796) supplemented with 10% (v/v) foetal bovine serum (Sigma, F7524) and penicillin/streptomycin (Gibco, 15140122) under standard conditions.

Immunoprecipitation and western blot analysis

RPE-1 cell lines stably transduced with the desired GFP-tagged constructs were lysed in Tris-based immunoprecipitation buffer (50 mM Tris–HCl, 0.5% NP40 and Roche Protease inhibitor cocktail) and the GFP immuno-precipitated with GFP-trap beads (Chromotek). Immuno-blotting was performed using standard procedures. Detection was carried out on a Licor Odyssey Infrared scanning system using fluorescently labelled secondary antibodies.

SILAC

RPE-1 cell lines stably transduced with the desired GFP-tagged constructs were grown in SILAC (stable isotope labelling with amino acids in culture) DMEM (ThermoScientific, 89985) supplemented with 10% (v/v) dialysed FBS (Sigma-Aldrich, F0392). Cells were grown in light (R0K0), medium (R6K4) or heavy labelling (R10K8) for at least 6 passages to achieve full labelling (amino acids were obtained from Sigma apart from K4 which was from ThermoScientific). Cells were lysed in immunoprecipitation buffer (50 mM Tris–HCl, 0.5% NP40, Roche Protease Inhibitor Cocktail) and GFP was immuno-precipitated with GFP-trap beads (Chromotek) for 1 hour. Samples were pooled and separated on Nupage 4-12% pre-cast gels (Invitrogen) and subjected to LC–MS/MS (liquid chromatography-tandem mass spectrometry) analysis on an Orbitrap Velos mass spectrometer (ThermoScientific).

Isothermal titration calorimetry (ITC)

The rat SNX27 PDZ domain and human VPS26A proteins were purified as described previously (Gallon et al., 2014; Chan et al., 2016). Briefly, SNX27 PDZ domain was purified as a GST fusion protein followed by thrombin cleavage to remove the GST tag, and VPS26A was purified via an N-terminal His-tag. Proteins were then gel filtered into ITC buffer (50 mM Tris (pH 8), 100 mM NaCl) using a Superose 200 column. The synthetic Kir3.3 peptide was purchased from Genscript. ITC experiments were performed on a Microcal iTC200 instrument in ITC buffer. Peptides were titrated into 40 μM SNX27 PDZ domain solutions at 25°C (supplemented with 40 μM hVPS26A proteins when required). Data were processed using ORIGIN to extract the thermodynamic parameters ΔH, K_a ($1/K_d$) and the stoichiometry n. ΔG and ΔS were derived from the relationship: $\Delta G = -RT\ln K_a$ and $\Delta G = \Delta H - T\Delta S$.

GST pull-down experiments

GST-tagged SNX27 PDZ domain (1 nmol) was mixed with VPS26A proteins (1 nmol) in 500 μl 25 mM Tris (pH 8), 300 mM NaCl, 1 mM DTT and bound to 25 μl glutathione sepharose resin. After 2 h incubation at 4°C, the resin was washed four times with pull-down buffer and bound proteins eluted in SDS-PAGE sample buffer. Western blot analysis was done using a nitrocellulose membrane and the iBlot semi-dry transfer system (Life Technologies). His-

tagged proteins were detected by ECL on photographic film, using a primary mouse anti-penta-His antibody (Qiagen, 34660) and goat anti-mouse HRP-coupled secondary antibody (ThermoFisher, A16072).

RNAi-mediated suppression of endogenous VPS26A and VPS26A

RPE-1 cells stably expressing GFP, wild type or mutant VPS26A were transfected either with a ON-TARGET plus non-targeting control pool (Dharmacon) [sequences 5'-UGGUUUACAUGUCGACUAA-3', 5'-UGGUUUACAUGUUGUGUGA-3', 5'-UGGUUUACAUGUUUUCUGA-3', 5'-UGGUUUACAUGUUUUCCUA-3'] or with the VPS26A suppression oligonucleotide 1 [sequence 5′-GCUAGAACACCAAGGAAUU(dTdT)-3′] and VPS26B suppression oligonucleotide 2 [sequence 5′-GAAGUUCUCUGUGCGCUAU(dTdT)-3′] of the ON-TARGET plus human SMART pool (Dharmacon) to suppress endogenous VPS26A and VPS26B. Cells were reverse-transfected using DharmaFECT (Dharmacon), then transfected again 12 hours later according to manufacturer's instructions. 48 hours after the second transfection cells were fixed and stained.

Immunofluorescence staining

Cells were fixed in 4% (v/v) paraformaldehyde in PBS for 20 minutes before being permeabilized with either 0.1% (v/v) Triton X-100 or 0.1% (w/v) saponin for 5 minutes. Cells were incubated with 1% (w/v) BSA for 10 minutes followed by incubation for 1 hour in the indicated primary antibody. The cells were then incubated with the appropriate Alexa Fluor secondary antibody (405, 488, 568, Invitrogen) for 1 hour with DAPI being used as a nuclear stain before being mounted on coverslips with Fluoromount-G (eBioscience, 00-4958-02).

Image acquisition

Microscopy images were recorded at room temperature on a confocal laser-scanning microscope (Leica SP5 AOBS) attached to an inverted epifluorescence microscope (DMI6000). A 63x, NA 1.4, oil immersion objective (Plan Apochromat BL) and the standard SP5 system acquisition software and detector were used. Images were analysed with the Volocity software package (Perkin Elmer). To filter noise, thresholds were applied uniformly across conditions. The colocalization tool on the Volocity software was used to calculate the Pearson's correlation and colocalization coefficient. Analysis is based on the quantification of over 100 cells from three independent experiments.

Statistical analysis

All quantified western blot and confocal colocalization data are the mean of at least three independent experiments. The raw data from the immuno-precipitation western blotting were first normalized to the GFP to account for differences in amount of protein and then to the VPS26A control. Results are expressed as a percentage of the VPS26A control. Mean and standard error were calculated, followed by a one way ANOVA and posthoc Dunnett test to determine statistical significance. Colocalization data was averaged across individual experiments and the mean and standard error calculated across the three experiments. A one way ANOVA followed by a posthoc Dunnett test was then used to analyze statistical significance. For all statistical tests, a P = value<0.05 was considered significant and is indicated by an asterisk.

Plasmids

VPS29 was sub-cloned from an original plasmid which was a kind gift from Dr Rohan Teasdale (University of Queensland). VPS26B and DENND4C was cloned from Hela cDNA into a pEGFPC1 vector (Clontech) and then subcloned into the XLG lentiviral vector. Full length mouse PKD2 was a kind gift from Professor Jing Zhou (Havard Medical School). The 1-703 PKD2 truncation was cloned from Hela cDNA into a pEGFPN1 vector (Clontech).

Online Supplemental material

Fig. S1 shows the protein expression levels of GFP-tagged VPS26A, VPS26B and VPS29 in RPE-1 cells as well as GFP-trap immunoprecipitation and immunofluorescence showing that the GFP-tagged retromer components can still associate into the retromer heterotrimer. Fig. S2 shows that PKD2 and DENND4C can associate with retromer on endosomes by immunofluorescence and that the missense VPS26A mutants do not perturb trafficking of the CI-MPR or α5 integrin. Table. S1 contains the SILAC quantified interactomes of the different retromer subunits in RPE-1 cells.

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Figure Legends

Figure 1 | Identification of the detailed retromer interactome.

A | Schematic describing the filtering of retromer subunit interactomes. B | Venn diagram showing the proteins that interact with VPS29 (green), VPS35 (grey), VPS26A (blue) and VPS26B (orange). C | Venn diagram showing retromer accessory proteins. Color code as in B. D | Proteins identified in C were subjected to gene ontology analysis using the PANTHER classification system. E | Proteins identified in C were subjected to esyN analysis generating a network of protein interactions. Each node represents a protein and each connecting line represents an interaction indicated by experimental evidence. F | RPE-1 cells expressing GFP-tagged VPS26A, VPS26B, VPS29 and VPS35 were subjected to GFP-nanotrap immunoisolation followed by quantitative western analysis for validation of DENND4C, PKD2 and SNX27 association. Data is from a single experiment representative of three independent experiments.

Figure 2 | Missense VPS26A mutants do not perturb assembly of the retromer heterotrimer or its endosome association.

A | Fluorescence based western blot showing the expression levels of GFP-VPS26A and GFP-VPS26A mutants compared to endogenous VPS26A. Expression of GFP-VPS26A(p.K297X) cannot be seen due to the antibody recognizing the C terminus of VPS26A. B | Fluorescence based western analysis after GFP-trap immunoprecipitation of GFP-VPS26A and GFP-VPS26A mutants with endogenous VPS35 and VPS29. C | Quantification of B from 3 (VPS35), and 4 (VPS29) independent experiments. Data expressed as a percentage of the GFP-VPS26A control. No statistically significant difference was observed. D | Immunofluorescence staining of VPS35, EEA1, LAMP1, FAM21 in RPE-1 cells expressing GFP-VPS26A or the GFP-VPS26A mutants. Scale bar: 20 μm. E | Colocalization of GFP-VPS26A or GFP-VPS26A mutants with VPS35, EEA1, LAMP1 or FAM21 from three independent experiments. No statistically significant difference was found.

Figure 3 | VPS26A(p.K297X) leads to a loss-of and enhancement-of VPS26A's association with specific components of its interactome.

A | Logarithmic graph showing the interactors identified from comparative SILAC proteomics of GFP-VPS26A versus GFP-VPS26A(p.K93E), GFP-VPS26A(p.M112V) or GFP-VPS26A(p.K297X). The SILAC ratio is the fold-enrichment of proteins in GFP-VPS26A mutant over GFP-VPS26A. Red circles indicate either a pronounced enhancement or loss of association with GFP-VPS26A(K297X). Data average of n = 2-3 independent experiments. B and C | Fluorescence based western analysis after GFP-trap immunoprecipitation of GFP-VPS26A, GFP-VPS26A(p.K93E), GFP-VPS26A(p.M112V) and GFP-VPS26A(p.K297X). D | Quantification of data from three independent experiments. Data are expressed as a percentage of the GFP-VPS26A control and analyzed by a one way ANOVA followed by a Dunnett posthoc test (*, $p<0.05$, **, $p<0.01$)

Figure 4 | VPS26A(pK297X) mutation directly impairs SNX27 interaction.

A | Binding of GST-SNX27 PDZ domain to purified His-tagged VPS26A mutants as detected by Coomassie staining (top) or Western blotting with anti-His antibody (bottom). B | Binding of the Kir3.3 peptide to the SNX27 PDZ domain was measured by ITC either to SNX27 alone (black circles) or in the presence of wild-type VPS26A (blue circles) or the VPS26A mutants (green through to red circles). Top shows raw data and bottom shows integrated and normalized data. C | Binding affinities of the Kir3.3 peptide (PPESESKV) to the SNX27 PDZ domain. D | Structure of VPS26A (gold ribbon) in complex with SNX27 (blue ribbon and surface) (Gallon et al., 2014), indicating the sites of VPS26A mutants (coloured spheres). The

site of Kir3.3 interaction is modelled based on the SNX27-Kir3.3 structure (Balana et al., 2011) (yellow sticks).

Figure 5 | Loss of VPS26A(p.K297X) association with SNX27 leads to the mis-trafficking of SNX27 dependent cargo.

A | Immunofluorescence co-staining of GLUT1 with LAMP1 after RNAi-mediated suppression of VPS26 expression. A non-targeting RNAi was used as a control. B | Immunofluorescence co-staining of GLUT1 with LAMP1 in RPE-1 cells stably expressing GFP-VPS26, or GFP-VPS26A mutants after RNAi-mediated suppression of VPS26 expression. C | Quantification of colocalization of GLUT1, CI-MPR and α5-integrin with LAMP1 from three independent experiments. Data were analyzed by a one-way ANOVA followed by a Dunnett posthoc test compared to the GFP-VPS26A control (*, $p<0.05$, **, $p<0.01$). D | Immunofluorescence co-staining of GLUT1 and LAMP1 in RPE-1 cells overexpressing GFP-VPS26A, or the GFP-VPS26A mutants. Scale bar for A, B and E: 10 μm.

Supplementary Figure Legends

Supplementary Figure 1 | Expression of GFP-tagged retromer components in lentivirally transduced RPE-1 cells.

A-C | Fluorescence based western blots showing the protein expression levels of GFP-tagged VPS26A, VPS26B and VPS29. β-actin as loading control. D-E | Fluorescence based western blots after GFP-trap immunoprecipitation of GFP-tagged retromer components with the indicated antibodies. The GFP-tag does not disrupt incorporation into the retromer heterotrimer. F | Immunofluorescence staining of VPS35 in RPE-1 cells expressing GFP-tagged retromer components. The GFP-tag does not affect association to VPS35 decorated endosomes. DAPI was used as a nuclear stain. Scale bar: 20 μm. Zoomed in image: 10 μm.

Supplementary Figure 2 | PKD2 and DENND4C are associated with retromer-decorated endosomes and missense VPS26A mutants do not perturb trafficking of the CI-MPR or α5 integrin.

A | Immunofluorescence staining of endogenous VPS35 in RPE-1 cells transiently transfect with constructs encoding for GFP-tagged full length PKD2 or the 1-703 PKD2 truncation mutant. PKD2 is transported from the endoplasmic reticulum (ER) to the somatic plasma membrane from where it can enter the endocytic network (Hoffmeister et al., 2011; Kim et al., 2014). The steady-state distribution of PKD2 is primarily in the ER but removal of the

carboxy-terminal ER retention signal, as in the 1-703 truncation mutant, releases PKD2 to the plasma membrane and the endocytic network. Here it shows extensive co-localisation with retromer decorated endosomes. Scale bar: 10 μm. B | Consistent with published work (Borner et al., 2012), immunofluorescence staining of endogenous VPS35 in HeLa cells transfected with GFP-DENND4C revealed a predominant cytoplasmic localisation with some association of DENND4C on VPS35 positive endosomes. Scale bar: 10 μm. Zoomed in image: 5 μm C | Immunofluorescence co-staining of CI-MPR with LAMP1 in RPE-1 cells stably expressing GFP-VPS26A, or GFP-VPS26A mutants after RNAi-mediated suppression of VPS26 expression. Scale bar: 10 μm. D | Immunofluorescence co-staining of α5 integrin with LAMP1 in RPE-1 cells stably expressing GFP-VPS26A, or GFP-VPS26A mutants after RNAi-mediated suppression of VPS26 expression. Scale bar: 10 μm.

Supplementary Table 1 | SILAC quantified interactomes of the different retromer subunits in RPE-1 cells.

A | VPS29 interactome raw data. B | Filtered and combined VPS29 interactome. C | VPS26 interactome raw data. D | Filtered and combined VPS26 interactome. E | VPS26 paralogue-specific interactomes. F | Filtered and combined VPS35 interactome.

Supplementary References

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