Parkinson’s disease linked Vps35 R524W mutation impairs the endosomal association of retromer and induces α-synuclein aggregation.

Jordan Follett¹, Andrea Bugarcic¹, Zhe Yang¹, Nicholas Ariotti¹, Suzanne J. Norwood¹, Brett M. Collins¹, Robert G. Parton¹,² & Rohan D. Teasdale¹,*

¹ Institute for Molecular Bioscience, The University of Queensland, St Lucia, Queensland, 4072, Australia

² University of Queensland, Centre for Microscopy and Microanalysis, Brisbane, Queensland 4072, Australia

*Corresponding author: Rohan Teasdale, +61-3202-9143, r.teasdale@uq.edu.au

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Abstract

Endosomal sorting is a highly orchestrated cellular process. Retromer is a heterotrimeric complex which associates with endosomal membranes and facilitates the retrograde sorting of multiple receptors, including the cation-independent mannose-6-phosphate receptor for lysosomal enzymes. The cycling of retromer on and off the endosomal membrane is regulated by a network of retromer-interacting proteins. Here, we find that Parkinson’s disease-associated Vps35 variant, R524W, but not P316S, is a loss-of-function mutation as marked by a reduced association with this regulatory network and dysregulation of endosomal receptor sorting. Expression of Vps35 R524W containing retromer results in the accumulation of intracellular α-synuclein positive aggregates, a hallmark of Parkinson’s disease (PD). Overall the Vps35 R524W containing retromer has a decreased endosomal association which can be partially rescued by R55, a small molecule previously shown to stabilize the retromer complex, supporting the potential for future targeting of the retromer complex in the treatment of Parkinson’s disease.
Introduction

Parkinson’s disease (PD) primarily affects the dopaminergic neurons of the substantia nigra pars compacta; a small anatomical region located within the midbrain that regulates movement. On a cellular level, PD is a complex disease characterized by the subcellular formation of large, perinuclear accumulations of aggregated proteins, called Lewy Bodies (LBs). The most abundant protein found in LBs is the pre-synaptic plasma membrane-associated protein α-synuclein, but they may also contain other proteins and lipids (1-3). The formation of LBs, which individual neurons are unable to clear, has been directly linked to activation of apoptotic pathways in the dopaminergic neurons (2).

Rare familial mutations in a range of proteins have been identified and have provided significant insight into the molecular pathways involved in the manifestation of PD (reviewed in (4)). Recently, a number of point mutations (D620N, P316S, R524W, L774M) within the retromer subunit Vps35 were associated with late-onset PD and analysis of 144 individuals confirmed that overall levels of Vps35 mRNA in the substantia nigra were significantly decreased in PD-affected patients (5-7). Retromer is a heterotrimeric complex composed of Vps29. Vps35 and one of the two Vps26 subunits, Vps26A or Vps26B (8-10). Retromer has a central role in the sorting of receptor cargo within endosomal membranes, which is essential to coordinate the specific spatio-temporal localisation of individual receptors enabling them to perform their varied functions (11,12). One such cargo is the cation-independent mannose-6-phosphate receptor (CI-M6PR) that controls the sorting of lysosomal enzymes such as cathepsin D which is required for protein turnover (11-13). Retromer serves as a multi-functional scaffold forming an interaction hub for a wide array of endosome-associated proteins, collectively termed the retromer interactome. These interactions aid in the formation of the cargo-containing tubulovesicular membrane carriers destined for other compartments such as the Golgi apparatus and plasma membrane. The diverse proteins that associate with retromer include regulatory molecules, proteins required for membrane recruitment, and protein complexes that control membrane tubulation and scission (reviewed in (14,15)). It is the spatial and temporal coordination of the interactions between the retromer and retromer interactome that enables it to coordinate multiple endosome-derived trafficking pathways (16).

Characterisation of the PD-causing Vps35 D620N mutation demonstrated that Vps35 D620N-retromer is unable to correctly traffic cargoes such as CI-M6PR and post synaptic AMPA receptors, the former of which results in improper processing and trafficking of cathepsin D (17,18). Moreover, previous reports suggest that the aforementioned sorting defect may arise from perturbed interactions with the WASH complex, a protein complex implicated in endosome-to-Golgi receptor sorting, and consequently, may lead to a down regulation of autophagy (19-21). Together, these studies support the importance of the late endocytic network in the degradation of cellular proteins and highlight the importance of a specific protease, cathepsin D, in overall lysosomal function and the degradation of luminal α-synuclein.

Here, we report the cellular characterisation of two familial PD-linked Vps35 variants, Vps35 P316S and Vps35 R524W. While the P316S variant appears to have little impact on retromer assembly or function, Vps35 R524W is poorly recruited to endosomes and impairs the recruitment of retromer-dependent interacting proteins and the trafficking of CI-M6PR. Similar to that observed for Vps35 D620N, expression of Vps35 R524W induced higher levels of α-synuclein positive aggregates, when compared to that of Vps35 WT or Vps35 P316S. These findings provide insight into the underlying molecular mechanism of PD-linked Vps35 R524W, and retromer’s involvement in the molecular pathways associated with PD.
Results

Vps35 P316S and Vps35 R524W are incorporated into retromer complexes

To determine if the formation of the heterotrimeric retromer complex in the presence of Vps35 variants P316S and R524W is altered, in vitro isothermal titration calorimetry (ITC) and in vivo co-immunoprecipitation were employed using full-length recombinant Vps35 proteins and GFP fusion constructs, respectively. The arginine 524 residue is present in an exposed loop between α-helices of the reiterating pairs of HEAT-like α-helical repeats, and contributes indirectly to the VPS35 interface with VPS29 as shown in the co-crystal structure (22). The proline 316 residue is predicted to be within an intervening loop, so its substitution is unlikely to disrupt the overall Vps35 structure. As demonstrated by ITC, Vps35 P316S retained interaction with retromer subunits Vps26A and Vps29 ($K_d = 1.5\text{ nM}$ and $170\text{ nM}$, respectively) at thermodynamic profiles similar to that of wild-type Vps35 ($K_d = 1.1\text{ nM}$ and $250\text{ nM}$, respectively). The Vps35 R524W mutant also demonstrated a similar binding affinity to that of the wild-type Vps35 for Vps29 ($K_d = 303\text{ nM}$), and Vps26A ($K_d = 1.4\text{ nM}$) (Fig 1A). In support of these in vitro experiments, co-immunoprecipitation from HeLa cells transiently expressing GFP fusion constructs demonstrated that Vps35 P316S and Vps35 R524W interact with retromer subunits Vps26A and Vps29, in whole cell lysates or the cytosolic fraction (Fig 1B, C). The incorporation of the PD associated variants of Vps35 into Vps26A-retromer complexes was confirmed by co-immunoprecipitation of endogenous Vps26A (Fig 1D). Collectively, this data demonstrates that both PD-associated mutations can form a stable retromer trimer and their expression does not alter steady state protein levels of endogenous retromer subunits. However, comparison of the relative amounts of the retromer subunits co-precipitated indicates that a portion of Vps35 R524W does not appear to be complexed to Vps26A and Vps29.

Vps35 R524W-containing retromer has diminished endosome recruitment

The subcellular localization of Vps35 P316S and Vps35 R524W was determined in HeLa cells transiently expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP. Consistent with the sub-cellular localization of Vps35 WT-GFP, Vps35 P316S-GFP demonstrated a high level of endosomal recruitment and displayed a high level of co-localization with Vps26A on these endosomes (Fig 2A). In contrast to this, while Vps35 R524W-GFP showed partial endosomal recruitment where it co-localised with Vps26A, a significant fraction of the protein also showed a diffuse localisation to the cytosol (Fig 2A).

Membrane fractionation was employed to determine the relative proportion of retromer associated with membranes. HeLa cells transiently transfected with GFP fusion constructs were lysed in sucrose-containing buffer, and cytosolic and microsome fractions were isolated. Loading controls and purity of the fractions were monitored using late endosome marker LAMP1 (microsome) and β-tubulin (cytoplasmic). Consistent with the previously described data, wild-type retromer proteins, including Vps35 WT-GFP, were observed associated with membranes and within the cytosol. Vps35 P316S-GFP showed a similar distribution to Vps35 WT-GFP whereas Vps35 R524W-GFP was predominantly distributed in the cytosol with a proportional decrease in the protein associated with membranes. Further, Western blotting of endogenous Vps26A, Vps35 and Vps29 demonstrate no significant shift in total levels of retromer from the microsomal fraction following expression of the GFP fusion constructs (Fig 2B). The biochemical and immunofluorescence findings together suggest impaired endosome association of Vps35 R524W.

We employed confocal microscopy to further examine the endosome morphology and retromer localization in the presence of Vps35 P316S and Vps35 R524W (Fig 2C). HeLa cells transiently expressing GFP fusion constructs were immunolabeled with antibodies against early endosome marker, EEA1 or late endosomal marker,
LAMP1. Consistent with the expression of Vps35 WT-GFP, confocal microscopy revealed recruitment of Vps35 P316S-GFP to EEA1 positive endosomes (R_w = 0.2130, R_p316s = 0.2013, Fig 2D), while cells expressing Vps35 R524W-GFP demonstrated reduced overlap with EEA1 positive compartments (R_r524w = 0.1551, Fig 2D). However, no differences in the morphology of EEA1 positive endosomes, when compared to neighbouring untransfected cells, was observed in transfected cells expressing any of the Vps35 proteins (Fig 2C). Immunolabelling of HeLa cells with antibodies against LAMP1 revealed no gross morphological changes of late endosome compartments in cells expressing Vps35 WT-GFP or Vps35 P316S-GFP. In contrast, expression of Vps35 R524W induced some swelling of the late endosome, but did not influence the overall localization of LAMP1 positive compartments when compared to neighbouring untransfected cells (Fig 2C). Further, consistent with previous observations, LAMP1 displayed minor overlap with Vps35 WT-GFP, Vps35 P316S-GFP and Vps35 R524W-GFP (R_w = 0.0330, R_p316s = 0.0518, R_r524w = 0.0428 respectively, Fig 2D).

**Sub-cellular localization of Vps35 mutants using APEX-GBP**

To resolve the redistribution of Vps35 generated by the various point mutations at a higher resolution, we utilized a modified ascorbate peroxidase (APEX) tag for characterizing subcellular protein distribution with electron microscopy. Recently, a genetically encoded plasmid for modular detection of any GFP-tagged protein of interest has been developed that employs co-transfection of APEX directly conjugated to a GFP-binding peptide (GBP) (23). When APEX-GBP is expressed it binds to the GFP-tagged protein resulting in the formation of specific electron density at the site of the protein after the diaminobenzoic acid (DAB) reaction in the presence of H_2O_2 and post-fixation with osmium tetroxide (23). Control expression of APEX-GBP alone and APEX-GBP + GFP resulted in significant electron density in the cytoplasm of transfected cells but no enriched electron density at endosomes/multivesicular bodies (Fig. 3A & B). However, the expression of Vps35 WT-GFP with APEX-GBP resulted in specific and enriched electron density at the site of morphologically identifiable endosomes (Fig. 3C). Vps35 P316S-GFP was enriched at endosomes and present on the external leaflet of endosomes, analogous to the WT (Fig. 3D). Vps35 R524W-GFP when co-transfected with APEX-GBP had reduced enrichment at the cytosolic face of endosomes and an increase in the abundance of soluble reaction product (Fig. 3E), resembling the expression of APEX-GBP alone and GFP + APEX-GBP. As an additional positive control we analyzed the distribution of Vps35 D620N-GFP, a point mutant that we have previously demonstrated generates an enlarged and clustered endosomal network. Strikingly, this mutant resulted in specific reaction product at enlarged and morphologically distinct endosomes and a reduction of the soluble pool of the protein (Fig. 3F).

**The association of Vps35 R524W with regulators of the retromer complex is impaired**

Retromer does not bind directly to membranes and its recruitment to endosomes is controlled by its capacity to coordinate interactions with a number of membrane-associated proteins. Association of the retromer complex with the endosomal membrane is positively regulated by the small GTPase Rab7a, and negatively regulated by the RabGAP TBC1D5 (24). Following recruitment to the endosome, additional retromer-dependent sorting machinery such as the Arp2/3 activating complex, WASH, is recruited to aid in cargo sorting (25-27). We examined the sub-cellular localization of the WASH complex subunits FAM21, Rab7a and TBC1D5 and their ability to interact with the retromer complex via co-immunoprecipitation following expression of Vps35 P316S-GFP and Vps35 R524W-GFP. HeLa cells transiently expressing GFP fusion constructs were fixed and immunolabeled with antibodies against endogenous FAM21 (Fig 4A), Rab7a (Fig 4D) and TBC1D5 (Fig 4G). Consistent with previous findings, Vps35 WT-GFP or Vps35 P316S-GFP demonstrated high level of co-localization (R_w = 0.8900; R_p316s = 0.9035 (Fig 4B)) in contrast to cells expressing Vps35 R524W-GFP where a significant decrease in co-localization with FAM21 (R_r524w = 0.8011, Fig 4B) was observed. Similar to this, HeLa
cells expressing Vps35 WT-GFP or Vps35 P316S-GFP immunolabeled with antibodies against Rab7a or TBC1D5 displayed a moderate, but consistent, amount of co-localization (Rab7a: \( R_W = 0.2828; \) \( R_{P316S} = 0.2856; \) TBC1D5: \( R_W = 0.2967; \) \( R_{P316S} = 0.2820, \) Fig 4E and Fig 4H, respectively). In contrast to these observations, expression of Vps35 R524W revealed a marked decrease in overlap with both Rab7a and TBC1D5 (\( R_{R524W} = 0.2166 \) and \( R_{R524W} = 0.2056 , \) Fig 4E and Fig 4H, respectively). Further, consistent with the previously described LAMP1 immunolabelling (Fig 2C), Rab7a-positive endosomes demonstrated swelling following expression of Vps35 R524W, but not Vps35 P316S or Vps35 WT, whereas endosomes positive for FAM21 or TBC1D5 displayed no evidence of gross morphological changes or differences in subcellular localization when compared to Vps35 WT-GFP or Vps35 P316S-GFP expression.

Co-immunoprecipitation was employed to investigate the ability for Vps35 P316S and Vps35 R524W-containing retromer to interact with, FAM21, Rab7a and TBC1D5 (Fig 4). HeLa cells expressing GFP fusion constructs were lysed and protein complexes were immunoprecipitated using GFP NanoTrap, resolved by SDS-PAGE and identified using Western blotting technique. Using antibodies against GFP and endogenous FAM21, Rab7a and TBC1D5, no differences in the ability for Vps35 P316S-GFP-containing retromer to interact with FAM21, Rab7a or TBC1D5 was observed, as determined by comparison to the levels of co-precipitated protein using Vps35 WT-GFP (Fig 4C, 4F and 4I, respectively). In contrast, Vps35 R524W-GFP demonstrated a clear reduction in its capacity to co-precipitate FAM21, Rab7a and TBC1D5, despite the total levels of all proteins being similar. Overall, Vps35 R524W consistently shows a decreased level of recruitment to endosomes, which is reflected in a lower level of interaction with proteins known to function in its recruitment to membranes. To determine if the expression of Vps35 R524W was interfering with recruitment of these retromer interacting proteins we examined the level of co-localisation between endogenous Vps26A and TBC1D5. A non-significant change was observed between control cells and those expressing the Vps35-GFP proteins (data not shown) consistent with these PD-associated Vps35 variants not interfering with endogenous retromer recruitment.

**Vps35 R524W expression induces α-synuclein aggregation**

Next, we employed the recently described retromer stabilising agent (28), R55, in an attempt to rescue the diminished membrane recruitment of Vps35 R524W-GFP containing retromer. For this, we employed both immunofluorescence and biochemical methodologies described above. HeLa cells transiently expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP were incubated with 5 µM R55 or DMSO control for 48 hours, fixed and immuno-labelled with the early endosomal marker, EEA1. As previously observed (Fig 2C), in cells incubated with DMSO vehicle control co-localisation analysis between GFP and EEA1 showed a decrease in Vps35 R524W-GFP at the early endosome (\( R_W = 0.214, \) \( R_{P316S} = 0.213, \) \( R_{R524W} = 0.158; \) Fig 5A & 5B). Interestingly, treatment of cells transfected with Vps35 R524W-GFP and treated with R55 agent demonstrated an increased endosomal localization (Fig 5A & 5B). However, the presence of R55 did not completely rescue the loss of Vps35 R524W-GFP in the microsome fraction to a level equivalent to that observed for Vps35 WT-GFP or Vps35 P316S-GFP (Fig 5C).

α-synuclein is the major component of LBs, a prominent phenotype in PD pathogenesis. Although the underlying cause of LB formation is not fully understood it appears to be caused by the perturbation of several distinct cellular homeostasis processes, including defects in endosomal degradation pathways (29,30). To address if retromer plays a role in the accumulation of aggregated α-synuclein, we used the SH-SY5Y neuroblastoma cell model which endogenously expresses α-synuclein, possesses machinery needed for dopamine uptake and metabolism, and responds
to external stimuli, including depolarisation using KCl (31). To confirm the suitability of this assay, the induced α-synuclein aggregates were confirmed to contain phosphorylated α-synuclein (32), autophagy component p62 (32,33), and Lysosome marker, Lamp1 (34) (Fig 6A). These aggregates are also thioflavin – S positive indicating that some of the aggregated α-synuclein is in a fibrillar form (Fig 6A) Furthermore, the ability of this method to induce higher molecular weight aggregates of GFP-α-synuclein was confirmed biochemically (Fig 6B). All of these properties have been associated with α-synuclein aggregates formed in other Parkinson disease models and/or Lewy bodies.

The reduced recruitment of Vps35 R524W to endosomes would impair retromer’s function and therefore the expression of this Vps35 PD-associated mutant may reduce α-synuclein clearance and promote aggregation. Untransfected SH-SY5Y cells and cells expressing GFP, Vps35 WT-GFP, Vps35 D620N-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP were incubated with 50 mM KCl for 60 min and allowed to recover in complete growth medium for 48 hours, immunolabeled for endogenous α-synuclein and transfected cells were scored as positive or negative for aggregates. α-synuclein positive aggregates were observed in 7.2% of untransfected SH-SY5Y cells, 13% of SH-SY5Y cells expressing GFP and 20% in those expressing Vps35 WT-GFP. In comparison to these control cells 29% of Vps35 P316S-GFP expressing cells, 43% of Vps35 R524W-GFP expressing cells and 56% of Vps35 D620N-GFP expressing cells were observed to have α-synuclein aggregates (Fig 6C & D). Concurrent treatment of untransfected SH-SY5Y cells 5μM R55 for 48 resulted in a consistent reduction in the total percentage of aggregates observed (4%) (Fig 6D). Likewise, a reduction in the total number of aggregates was witnessed in R55 treated SH-SH5Y cells expressing GFP or Vps35 WT-GFP (9 and 15%, respectively). This trend was also observed for SH-SY5Y cells expressing either Vps35 P316S-GFP (21%) or Vps35 R524W-GFP (33%), however this was not seen in cells expressing Vps35 D620N-GFP (50.2%, Fig 6D), potentially owing to its nature as a likely dominant negative protein. Therefore, the expression of these PD-associated Vps35 mutants can directly influence the formation of large α-synuclein positive aggregates, a hallmark of PD.

Retrograde sorting is delayed in the presence of Vps35 R524W

To test the functional impact of Vps35 R524W expression on the retrograde endosomal cargo sorting, we investigated the recycling of the well-characterised retromer cargo, CI-M6PR. Previous reports mapped the interaction between retromer and the cytosolic tail of the CI-M6PR to amino acids 500-693 of the Vps35 subunit (11). Given the location of the point mutations on the Vps35 in relation to the Vps29 and the cargo-interacting domain within Vps35, the interaction of retromer with CI-M6PR, modulation of soluble lysosomal enzyme delivery, relative co-localisation and kinetics of retrograde trafficking were analysed as previously described (10,11,17). Vps35 P316S containing retromer was found to co-precipitate the M6PR at levels identical to that of the wild-type retromer whereas Vps35 R524W displayed decreased levels of M6PR (ranging from 15-60% less precipitant than Vps35 WT levels) (Fig 7A). Expression of Vps35 P316S-GFP, like Vps35 WT-GFP, did not disrupt the lysosomal delivery of cathepsin D, which is dependent on mannosylation and binding to the CI-M6PR for the transport of the Golgi-processed precursor into the endosomal system. In contrast, expression of Vps35 R524W-GFP clearly disrupted the trafficking itinerary of the CI-M6PR as secretion of pro-cathepsin D into the extracellular media was observed after protein synthesis was inhibited for 7 hours (Fig 7B). Next, the sub-cellular distribution of the endogenous CI-M6PR relative to retromer was examined. The CI-M6PR was localised to punctate and perinuclear organelles in cells transfected with Vps35 WT-GFP and Vps35 P316S-GFP and demonstrated a small amount of overlap with retromer (R_{WT} = 0.210 and R_{P316S} = 0.204; Fig 7C, D). In comparison, expression of Vps35 R524W-GFP was found to alter the sub-cellular distribution of the CI-M6PR from predominantly perinuclear staining to dispersed puncta. These dispersed CI-M6PR-positive puncta demonstrated increased co-localisation with the Vps35 R524W-GFP endosomal retromer (R_{R524W} = 0.261) relative to controls. Given, the observed deficit in retrograde
trafficking, we investigated the Golgi morphology in HeLa cells transfected with all GFP constructs using antibodies against trans-Golgi protein, p230. HeLa cells expressing the described constructs demonstrated no marked differences in overall Golgi morphology or distribution (data not shown).

HeLa cells were co-transfected with CD8-CIM6PR to monitor the retrograde delivery of internalised antibodies to the TGN using a well-established antibody uptake assay (10). Cells were incubated on ice with anti-CD8 antibody and chased at 37°C for up to 30 min. In cells expressing CD8-CIM6PR and GFP only, strong CD8 immunostaining was observed on membranes positive for p230, demonstrating efficient delivery of the reporter to the TGN network following retromer-mediated transport through the endosome. Consistent with this, strong CD8 immunostaining was observed to overlap with p230-positive membranes in cells expressing either Vps35 WT-GFP or Vps35 P316S-GFP at 30 min post-chase (Fig 7E). However, in cells expressing Vps35 R524W-GFP, the majority of the p230-positive membranes did not display evidence of CD8 staining at 30 min post-chase and showed strong CD8-CIM6PR localization to dispersed punctate structures, reminiscent of endosomes, indicative of significant delays in the retrograde sorting pathway (Fig 7E). Based on these multiple assays, the expression of the PD-associated Vps35 R524W mutant retromer protein impacts significantly on the retrograde trafficking pathway and accumulates CI-M6PR in endosomes due to an inefficiency to transport proteins from endosomes to the TGN.

**SNX27-retromer dependent recycling of GLUT1 is unaffected in the presence of Vps35 R524W**

In addition to retrograde trafficking of cargo, retromer also functions to recycle proteins from endosomes to the plasma membrane by forming a complex with SNX27 (35,36). To determine if Vps35 P316S-GFP or Vps35 R524W-GFP expression impacts the retromer-mediated recycling pathway, we investigated the sub-cellular localization of endogenous SNX27 in transfected HeLa cells (Fig 8A, B). Co-localization analysis revealed a moderate overlap between both Vps35 WT-GFP and SNX27 (R=0.3562) and Vps35 P316S-GFP with SNX27 (R = 0.3851), whereas analysis of Vps35 R524W-GFP with SNX27 demonstrated a marked reduction in co-localization (R=0.2659; Fig 8B). Membrane fractionation showed unchanged levels of SNX27 recruited to membranes (data not shown) and the sub-cellular localization relative to endogenous Vps26A (Fig 8C) and overall morphology of SNX27-positive endosomes in the presence of Vps35 R524W-GFP was not altered (Fig 8A), consistent with the notion that SNX27 is recruited to the endosome independent of the retomer complex. Relative to Vps35 WT-GFP, less SNX27 was co-immunoprecipitated with Vps35 R524W-GFP (Fig 8D). To determine if this decrease in SNX27 binding to Vps35 R524W retromer had a dominant negative effect on the recycling pathway as it does for the retrograde pathway we analysed GLUT1 as a representative and well-established SNX27-retromer dependent cargo (36,37). HeLa cells expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP were immunolabeled with antibodies raised against endogenous GLUT1 and LAMP1 (Fig 8E). Confocal microscopy revealed localization of GLUT1 to both the cell surface and punctate structures in cells expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP, reminiscent of the neighbouring untransfected cells staining. Additionally, intracellular localization of GLUT1 demonstrated minimal overlap with late endosome marker, LAMP1, supporting uninterrupted recycling in the presence of Vps35 P316S-GFP or Vps35 R524W-GFP (Fig 8E). To further confirm these results, we labelled the plasma membrane of transfected cells with biotin and used streptavidin-based precipitation to assess the cell surface levels of GLUT1 and TfnR. Biotinylation revealed similar cell surface levels of retromer dependent cargo, GLUT1 and retromer independent receptor, TfnR, in cells expressing GFP, Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP. We also find Vps35 D620N expression does not impact recycling of GLUT1 (Fig 8F), consistent with previous reports (19). In support of this finding, HeLa cells expressing all GFP fusion constructs demonstrated identical levels of glucose uptake compared to controls and was consistent with GLUT1 biotinylation observations (Fig 8G). Therefore, the reduced
levels of Vps35 R524W-GFP from SNX27-positive endosomes does not appear to negatively impact the recycling function of retromer.

Discussion

While the PD linked point mutation Vps35 P316S does not destabilize the retromer’s high affinity trimer structure, Vps35 R524W displayed some evidence of impaired trimer formation, modulation of retromer’s cellular functions and induction of α-synuclein aggregation, which is an underlying hallmark of PD. Despite genetic evidence linking this mutation to late onset PD, expression of Vps35 P316S did not display any distinct cellular phenotypes when compared to expression of Vps35 WT. Vps35 R524W demonstrated poor recruitment to the endosome and its incorporation negatively impacted the interaction of retromer with several well-established retromer interacting proteins. Further, this mislocalization of Vps35 R524W-containing retromer resulted in reduced endosome-to-Golgi but not endosome-to-plasma membrane receptor sorting. This was demonstrated by several CI-M6PR trafficking assays where we show the redistribution of endogenous CI-M6PR from the perinuclear space to dispersed puncta, late endosome swelling and detectable levels of pre-cathepsin D in the culture medium which was mirrored by the decrease in cathepsin D processing in the cells. Finally, we demonstrate that expression of the R524W mutation leads to increased levels of α-synuclein positive aggregation.

Previous studies demonstrate the recruitment of additional endosomal machinery such as the WASH complex, TBC1D5 and members of the SNX-BAR to the endosome assist retromer in mediating sorting of its cargo to the TGN (20,38,39). However, it is the combination of the small GTPase Rab7a and PX-containing protein SNX3 that is thought to initiate this cascade by recruiting retromer to the membrane (24,40). The current view of retromer mediated transport is that the Vps35-Vps26-Vps29 proteins form a stable core trimer that is a hub for associating with regulatory and cargo proteins (25). Our data shows that although the core complex remains unaffected by the P316S. The R524W mutation partially disrupts this formation and also negatively impacts on the association with regulatory molecules needed for cargo sorting. Previously, Vps35-Rab7a-SNX3 interaction studies demonstrated that binding of Rab7a and SNX3 is within the first 300 amino acids of Vps35 and immediately adjacent to the Vps26-Vps35 interface (41). Despite this, we showed that R524W but not the P316S mutation within Vps35 severely impacts the retromer-Rab7a interaction and Vps35 R524W-retromer membrane association, coupled with reduced interactions with the WASH complex subunit FAM21, the RabGAP TBC1D5 and PDZ motif cargo adaptor SNX27. While Vps35 R524W-retromer displayed impaired binding to multiple retromer-associated proteins, we suggest this is a consequence of reduced endosome recruitment rather than a direct impact on the binding surfaces of all these molecules. Interestingly, the use of R55, a recently described retromer stabilising agent (28), showed a rescue phenotype in membrane association of the Vps35 R524W-retromer, possibly by stabilising a conformational change needed for retromer-Rab7a interaction required for membrane recruitment. Therefore, these findings suggest that the observed defect in Vps35 R524W expressing cells arises from its diminished interaction with machinery needed to recruit retromer to the membrane, second to a partial loss of stable incorporation into the retromer trimer.

Given the described role of CI-M6PR in trafficking of cathepsin D, a protease needed for clearance of α-synuclein (42,43), and the importance of retromer in correct localization of the CI-M6PR and sorting of lysosomal cathepsin D (11,13,44) it is not unexpected that expression of Vps35 mutants that perturb trafficking demonstrate increased frequency of forming intracellular aggregates. Additionally, a complete knockout of cathepsin D leads to extensive levels of high molecular weight α-synuclein species and increase in Lewy Body numbers in brains of knockout animals (30). Together, these findings support the concept that regulation of the lysosomal pathway and its luminal content plays a fundamental role in turnover of proteins, a pathway that is already highly susceptible to errors during ageing (45). In order to examine the role of retromer in α-synuclein
aggregation and clearance, we used a non-pharmacological based stimuli (KCl) rather than other reagents (Rotenone, Bafilomycin A1) which target mitochondrial and lysosomal function, respectively, and may indirectly impact retromer’s cellular function (46,47). Using this assay, our findings indicate that the expression of Vps35 R524W leads to CI-M6PR and cathepsin D mistrafficking, suggesting a direct correlation between the loss of retromer, the Vps35 R524W point mutation and the production of α-synuclein aggregates in a cellular system. Consistent with these results is the previously described PD linked Vps35 mutation (Vps35 D620N) was also reported to impair the degradation of α-synuclein, a product of diminished endo-lysosomal functionality, further supporting a relationship between receptor trafficking and α-synuclein aggregate production (21). Several lines of evidence also demonstrate the requirement of both Rab7 and TBC1D5 in maintaining constant autophagic flux, retromer-dependent trafficking and optimal function of the lysosomal compartment (30,38,48-50). Although these studies do not conclusively demonstrate a direct link between the retromer complex, autophagic flux itself and formation of α-synuclein aggregates, they do support the emerging notion that retromer-dependent machinery is required for functionality of the endolysosomal-autophagy clearance pathways.

The interaction between the SNX27 PDZ domain and the Vps26 subunit of the retromer complex has been shown to be important in regulation of the recycling pathway (37), but their recruitment to the endosomal membrane appears to be independent of each other (36). Here we confirm the membrane association of SNX27 is not impaired in the presence of Vps35 R524W expression and also demonstrate recycling of GLUT1, a known PDZ motif-containing cargo, was identical to that observed in the presence of Vps35 WT-GFP expression. This may be due to the interaction of the endogenous SNX27 with the more abundant, endogenous retromer over the poorly recruited Vps35 R524W-retromer leading to the uninterrupted recycling of SNX27-dependent cargo observed here. Therefore, the PD-linked R524W mutation does not influence the recycling trafficking pathway in our experimental conditions, like the PD-linked Vps35 D620N mutation which appears to be similarly functional in GLUT1 recycling.

Overall, we have demonstrated that Vps35 R524W containing retromer poorly interacts with known endosomal machinery and consequently impacts the retrograde cargo sorting properties of the retromer complex and not its role in recycling SNX27-dependent cargo. Expression of Vps35 R524W is similar to the sorting defect witnessed for the Vps35 D620N mutation linked to PD in which, the CI-M6PR is not efficiently delivered to the TGN (7,17,19). However, Vps35 D620N-containing retromer reportedly interacts with accessory proteins TBC1D5 and SNX27 identical to that observed for wild-type retromer but has minor reductions in its ability to interact with the WASH complex (19,21), whereas Vps35 R524W has reduced interactions with TBC1D5, SNX27 and the WASH complex. Vps35 D620N broadly modifies endosome morphology (17), whereas Vps35 R524W expression alters late endosome morphology with minimal enrichment at the endosome, supporting very distinct changes to the molecular and cellular properties of the retromer sorting pathways in the presence of the two mutations. Despite these described differences between the D620N and R524W Vps35 PD linked mutations, it is clear that the retrograde sorting properties of retromer, and not its role in endosome to plasma membrane trafficking, is indeed defective in PD. Although this does not rule out Vps35 P316S as being causative in PD, it infers that the primary defect leading to disease onset may be through a currently undefined retromer-dependent mechanism.

Experimental procedures:
DNA constructs
Vps35 WT-GFP has been described previously (17) and was used to generate P316S and R524W point mutations. Quikchange mutagenesis (Stratagene) was employed using the following PCR mutagenic primer pairs:

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GCTCACCGTGAAGATGGATCCGGAATCCC
AGCGGAT and 3′-ATCCGCTGGGATTCCAGCCATCTTCACG
GTGAGC or 5′-GCTGGTGGAAATCAGTGGATTCGCTTCACA
CTG and 3′-CAGTGTGAAGCGAATCCACTGATTTCCACC
AGC, respectively.

The pCMU-CD8/C1-M6PR and Vps35 D620N-GFP constructs were described previously (17).

Antibodies

The following primary antibodies were purchased as indicated: Mouse monoclonal anti-CIM6PR, anti α-synuclein, anti SNX27 (C16), rabbit polyclonal anti-GLUT1, anti-SQSTM1 / p62, rabbit polyclonal anti-GLUT1, anti-SQSTM1 / p62, anti-Vps26A and rabbit monoclonal anti alpha Synuclein (phospho S129) (Abcam); Mouse monoclonal anti-GFP and rabbit polyclonal anti-GFP were from Life technologies and Roche applied science, respectively; Goat polyclonal raised against Vps35 (IMGENEX); Mouse monoclonal anti-human EEA1, p230 and LAMP1 (BD Transduction Laboratories); Rabbit monoclonal anti Rab7a (Cell signaling); Mouse monoclonal Anti-CD8 (eBioscience); Mouse monoclonal anti-β-tubulin (Sigma Aldrich); Sheep polyclonal anti TGN-46 (AbD Serotec); Goat polyclonal anti-TBC1D5 (C-14) (Santa Cruz Biotechnology); Rabbit polyclonal anti-cathepsin D and anti-FAM21C (Merck Millipore). Rabbit polyclonal against Vps29 was generated in house and used previously (10). Secondary Goat anti-mouse IgG Alexa Fluor568, Donkey anti-mouse IgG Alexa Fluor546 and Donkey anti-goat IgG Alexa Fluor647 (Life Technologies); Horse-radish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Dako); IRdye 680 and IRdye 800 conjugated fluorescence secondary antibodies (LI-COR Biosciences).

Cell culture and Transfection

HeLa cells were grown in a humidified 37°C incubator with 5% CO2 and maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco) and 2mM L-glutamine (Life Technologies). Mamalian constructs were transfected into cells using LipofectAMINE 2000 (Life Technologies) according to manufacturer’s instructions. This routinely achieved 50-70% transfection efficiency for HeLa cells and 25-40% for SH-SY5Y cells.

Protein purification and isothermal titration calorimetry (ITC)

Constructs of mouse VPS35, VPS29 and VPS26A for expression in Escherichia coli were described previously (17) and all VPS35 point mutations were engineered using the Quikchange mutagenesis kit (Stratagene). Recombinant proteins used for the ITC experiments were prepared and analysed as described (51). Experiments were performed in 20 mM Tris (pH 8.0), 200 mM NaCl, 1 mM DTT at 283 K using a MicroCal iTC200 (GE Healthcare).

Immunoprecipitations

Transfected HeLa cell monolayers were washed in ice-cold phosphate buffered saline (PBS) and lysed in TK lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10 mM Na4P2O7, 30 mM NaF, 2 mM Na3VO4, 10 mM EDTA, 0.5 mM AEBSF and Complete Mini protease inhibitor cocktail (Roche)) for 10 min on ice. Whole cell lysates were centrifuged at 17,000 x g and supernatant collected. These supernatants were incubated with GFP-NanoTrap beads for 1-2 h or anti-Vps26A antibodies overnight at 4°C under constant rotation. Vps26A antibodies were complexed by addition of Protein G sepharose beads. GFP immunoprecipitation performed from isolated cytosol was conducted in HES buffer described below. Complexes were dissociated from the beads by boiling after three consecutive washes with TK lysis buffer.

Western immunoblotting

Cell lysate samples were subjected to bichorionic acid (BCA) assay (Thermo Scientific) to determine protein concentration. Equivalent amounts of protein per sample (10-50 µg) were resolved by SDS-PAGE, transferred onto PVDF membrane (Immobilon-P and Immobilon-FL; Millipore) according to the manufacturer’s instructions.
Immunoblotting using ECL and Odyssey infrared imaging system (LI-COR Biosciences) was performed as per manufacturer’s instructions.

**Electron Microscopy**

EM was performed as described previously (23). Briefly, HeLa cells were seeded onto 3cm dishes and transfected with a DNA ratio of 1:1 GFP-tagged protein of interest to APEX-GBP using Lipofectamine 2000 (Invitorgen) as per the manufacturer’s instruction. Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at room temperature and washed repeatedly in 0.1 M sodium cacodylate buffer. The DAB reaction was performed for 30 min at room temperature in the presence of H2O2. Cells were then post-fixed in 1% Osmium Tetroxide for 2 min then washed again in 0.1 M sodium cacodylate. Cells were serially dehydrated in increasing percentages of ethanol and subsequently serially infiltrated with increasing percentages of LX112 resin and polymerized at 60°C overnight. Ultrathin sections (60nm) were cut on an Ultracut UC6 ultramicrotome (Leica) and on a JEOL 1011 electron microscope at 80 kV fitted with a Morada Soft Imaging camera (Olympus) at 2-fold binning.

**Indirect Immunofluorescence, confocal microscopy and co-localization Analysis**

HeLa cells grown on glass coverslips were washed with room temperature PBS twice prior to fixation with 4% PFA for 20 min. Cells were permeabilized using 0.1% Triton X100/PBS for 10 min at room temperature and subsequently blocked using 2% BSA/PBS for 45 min. Cells were labelled with primary and secondary antibodies and counterstained with DAPI nuclear stain. Coverslips were mounted using Fluorescent Mounting Medium (Dako) and imaged using a Zeiss LSM710 Upright Scanning Laser confocal fluorescent microscope equipped with a 63× Plan-Apochromatic objective and Argon, HeNe1, HeNe3 lasers. All images were analyzed using Zeiss LSM 5.0 and Adobe Photoshop software. Pearson’s correlation coefficients were calculated using the Image J plugin ‘Colocalization finder’ from the NIH. Fields of view containing both transfected and non-transfected cells were segregated by generating regions of interest around transfected cells, cropping selected regions, splitting into respective grey-scale channels and applying threshold settings. Co-localization analysis was conducted on 3 independent experiments with 10 images per group containing 5-10 transfected cells per field of view. Co-localization values were exported to GraphPad Prism 5 software and tabulated accordingly.

**Microsomal Fractionation**

HeLa cells grown in 15 cm² culture dishes were washed twice on ice in PBS, collected and homogenized with 20 passages through a 22G ¾ needle in buffer containing 20 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM EDTA, 2 mM Na3VO4, 10 mM EDTA and 0.5 mM AEBSF. Lysates were spun at 500 x g for 5 min, and a portion of the resulting supernatant was collected and stored as the whole cell lysate. The resulting supernatant was further centrifuged at 17,200xg for 20 min to isolate the crude plasma membrane fraction followed by 175,000xg for 75 min to isolate the microsome fraction from the remaining cytosolic fraction. Pelleted microsomes were re-suspended in homogenization buffer (20 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM EDTA, 2 mM Na3VO4, 10 mM EDTA and 0.5 mM AEBSF).

**α-synuclein aggregation**

SH-SY5Y cells grown on glass coverslips were incubated with 50mM KCl (Sigma Aldrich) for 60 min at 37°C. Following the treatment, cells were either fixed (0 h time point) or allowed to recover in complete growth medium for up to 48 h, when they were fixed and immunofluorescence microscopy performed using anti-α-synuclein antibody. For detection of α-synuclein aggregates by Western blotting, SH-SY5Y cells stably expressing GFP-α-synuclein (52) were collected for analysis 48hrs after KCl treatment as described (53) and resolved on a 6% SDS-PAGE to separate HMW α-synuclein. Thioflavin-S (Sigma Aldrich) staining was employed following washes with ethanol and performed as described (54).

**Cathepsin D Secretion assay**

HeLa Cells plated in 6-well dishes 48 h prior to use were washed in 37°C PBS, incubated with serum free DMEM, supplemented with 2 mM L-Glutamine and Cyclohexamide (final concentration 100 µg/mL). Medium and cell lysate samples were
collected at 0, 3 and 7 h post-chase, centrifuged at 17,000 x g for 10 min at 4 °C and supernatants were transferred to fresh tubes. Collected medium was precipitated using Trichloroacetic acid (TCA) for 10 min on ice, boiled and subjected to western blotting.

**Antibody uptake assay**

HeLa cells grown on glass coverslips were co-transfected with the previously described GFP/CD8-Cl-M6PR fusion constructs for 16 h. Transfected cells were serum starved in DMEM supplemented with 2 mM L-glutamine for 4 h prior to being chased at 37°C with anti-CD8 antibody. Post chase coverslips were fixed in 4% PFA and subjected to indirect immunofluorescence.

**Cell surface biotinylation**

16-18 hrs post transfection, transfected HeLa cells in 15 cm² cell culture plates were washed 3 times with ice-cold PBS, followed by incubation with 0.25 mg/ml of EZ-link Sulfo-NHS-LC-LC Biotin (Thermo Fisher Scientific) for 10 min on ice, when reactions were quenched by 50 mM Glycine for 10 min. Cells were harvested in TK lysis buffer and protein concentrations were determined by BCA protein quantitation assay. Equal amounts of protein lysates were incubated with Neutroavidine Agarose beads (Thermo Fisher Scientific) for 1 hour at 4 °C. After washing the beads 3 times with TK lysis buffer, beads were boiled in 2 x SDS sample loading dye, and pull-down samples were subjected to SDS-PAGE/immunoblotting.

**Glucose Uptake Assay**

Transfected HeLa cells in a 12-well plated were washed with warmed Krebs-Ringer’s Phosphate (KRP) buffer containing 10 mM HEPES, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 1.25 mM MgSO₄, 0.6 mM Na₂HPO₄, 0.4 mM NaH₂PO₄. Cell monolayers were incubated for 10 min in the presence of 50 µM 2-deoxy-D-glucose and 1 µCi/ml 2-deoxy-H³-glucose. The amount of glucose transported into the cells was determined by harvesting the cells in 1% Triton X-100 and the associated radioactivity quantified using a MicroBeta liquid scintillation counter (Perkin Elmer Biosciences). The counts per min (cpm) were normalized to total protein concentrations.

**Statistics**

All statistical analyses were complete using GraphPad Prism software 6 and described in the appropriate figure legends.
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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article

Author Contributions: J.F. performed the majority of experiments with N.A and R.G.P contributing the EM data presented in Fig 3; S.J.N and B.M.C contributing the ITC data presented in Fig 1A; A.B contributed the trafficking assay presented in Fig 7E and Z.Y contributed the uptake assays presented in Fig 8F and 8G. RDT conceived and coordinated the study and wrote the paper with J.F. All authors reviewed the results and approved the final version of the manuscript.
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References:

D620N Mutation Linked to Parkinson's Disease Disrupts the Cargo Sorting Function of Retromer.
*Traffic* **15**, 230-244

neurotransmitter receptor trafficking to synapses is altered by the Parkinson's disease VPS35

19. McGough, Ian J., Steinberg, F., Jia, D., Barbuti, Peter A., McMillan, Kirsty J., Heesom, Kate J.,
Whone, Alan L., Caldwell, Maeve A., Billadeau, Daniel D., Rosen, Michael K., and Cullen, Peter J.
(2014) Retromer Binding to FAM21 and the WASH Complex Is Perturbed by the Parkinson Disease-
Linked VPS35(D620N) Mutation. *Current Biology* **24**, 1670-1676


WASH complex association and inhibits autophagy. *Nat Commun* **5**


23. Ariotti, N., Hall, Thomas E., Rae, J., Ferguson, C., McMahon, K.-A., Martel, N., Webb, Robyn E.,

recruitment of the cargo-selective retromer subcomplex is catalysed by the small GTPase Rab7 and
inhibited by the Rab-GAP TBC1D5. *Journal of Cell Science* **122**, 2371-2382

The cargo-selective retromer complex is a recruiting hub for protein complexes that regulate

complex is mediated by the extended ‘tail’ of Fam21 binding to the retromer protein Vps35. *Biochem J* **442**, 209-220

FAM21 tail link the WASH actin regulatory complex to the retromer. *Mol Biol Cell* **23**, 2352-2361


30. Cullen, V., Lindfors, M., Ng, J., Paetau, A., Swinton, E., Kolodziej, P., Boston, H., Saftig, P., Woulfe,

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Figure Legends:

**Figure 1. Parkinson’s disease linked Vps35 mutants P316S and R524W do not disrupt trimer formation**

A) Isothermal Titration Calorimetry of Vps35 point mutations with retromer subunits, Vps29 (left panel) and Vps26A (right panel). GFP-NanoTrap immunoprecipitations from HeLa cells B) whole cell lysates or C) cytosolic subcellular fraction of transiently expressing GFP, Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP followed by immunoblotting analysis of precipitated complexes with anti-GFP, anti-Vps26A and anti-Vps29 antibodies. D) Endogenous Vps26A was immunoprecipitated from HeLa cells transiently expressing GFP, Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP followed by immunoblotting analysis of precipitated complexes with anti-Vps35, anti-Vps26A and anti-Vps29 antibodies. Within the control lane the same experimental procedure was applied in the absence of the primary anti-Vps26A antibody.

**Figure 2. Vps35 R524W disrupts recruitment of retromer to the endosomal membrane**

A) Confocal analysis of HeLa cells transiently expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP immunolabeled with anti Vps26A (n=3, 10 images per group). Scale bar: 5µm. B) Representative immunoblotting (n=4) of HeLa cells transiently expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W subjected to fractionation, SDS-PAGE and immuno-labelling with antibodies against the listed proteins. C) Representative immunofluorescence images of HeLa cells transiently expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP labelled with EEAI or LAMP1 followed by counterstaining with DAPI. Scale bar: 5µm. D) Analysis of co-localization from (c) represented by Pearson’s correlation co-efficient. Graphs representative of three independent experiments with 10 images per group with 5-7 transfected cells per field of view (Error bars represent +/- SEM; *p<0.05; ANOVA with Tukey’s multiple comparisons test.)

**Figure 3. Ultrastructural resolution of Vps35-GFP**

A) HeLa cells transfected with APEX-GBP alone demonstrate strong electron dense reaction product in the cytoplasm. Insets demonstrate no enriched electron density to control endosomes. B) EGFP co-transfected with APEX-GBP results in exclusively cytoplasmic electron density. C) Vps35 WT-GFP + APEX-GBP demonstrates significant electron density at the endosomes of transfected cells. Arrows demonstrate endosomes with increased electron density compared to the cytoplasm. D) Vps35 P316S-GFP closely resembles the WT construct. Arrows demonstrate endosomes with increased electron density when compared to the cytoplasm. E) Vps35 R524W-GFP demonstrated a reduction in endosome association and an increase in soluble reaction product. Arrows show endosomes with enriched electron density over the cytoplasm, arrowheads demonstrate endosomes without electron density. F) Vps35 D620N-GFP caused enlarged endosomes with strong reaction product in transfected cells. * denotes untransfected adjacent cells. Scale Bars, 1 µm.

**Figure 4. Vps35 R524W has diminished association with regulators of retromer**

A) Immunofluorescence staining of FAM21 in HeLa cells transiently expressing Vps35 WT-GFP, Vps35 P316S-GFP and Vps35 R524W-GFP followed by DAPI counterstaining. Scale bar: 5µm. B) Analysis of co-localization from (A) represented by Pearson’s co-efficient. Graphs representative of three independent experiments with 10 images per group with 5-7 transfected cells per field of view ( Error bars represent +/- SEM, *p <0.05; ANOVA with Tukey’s multiple comparisons test ). C) Representative co-immunoprecipitation of GFP, Vps35 WT-GFP, Vps35 P316S-GFP and Vps35 R524W-GFP with endogenous FAM21 from HeLa cells. D) Representative immunofluorescence images of HeLa cells transiently expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP labelled with late endosome marker, Rab7. Scale bar: 5µm. E) Analysis of co-localization from (D) represented by Pearson’s
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c-o-efficient. Graphs representative of three independent experiments with 10 images per group with 5-7 transfected cells per field of view (Error bars represent +/- SEM, *p<0.05; ANOVA with Tukey's multiple comparisons test). F) Representative co-immunoprecipitation of GFP, Vps35 WT-GFP, Vps35 P316S-GFP and Vps35 R524W-GFP with endogenous Rab7a from HeLa cells. G) Localization of TBC1D5 in HeLa cells expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP counterstained with DAPI. Scale bar: 5µm. H) Analysis of co-localization from (G) represented by Pearson’s co-efficient. Graphs representative of three independent experiments with 10 images per group with 5-7 transfected cells per field of view (Error bars represent +/- SEM, *p<0.05; ANOVA with Tukey's multiple comparisons test). I) Representative western blots of TBC1D5 co-immunoprecipitation with Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP containing retromer from HeLa cells.

Figure 5. Pharmacological stabilisation of retromer increases its total levels and membrane recruitment
A) Confocal images of HeLa cells transiently expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP incubated with DMSO or 5 µM R55 immunolabeled with EE1 and counterstained with DAPI. Scale bar: 5µm. B) Graphical representation of co-localization observed in (A) from two independent experiments with 10 images per construct and time point. Error bars displayed as +/- SEM; *p<0.05 by ANOVA followed with Tukey’s multiple comparisons test. C) Representative western blots of HeLa cells transiently expressing GFP, Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP treated with 5 µM R55 or DMSO for 48hrs (n=3). Membranes were also analysed using anti-LAMP1 for loading control.

Figure 6. Parkinson’s disease linked Vps35 point mutations increase the production of α-synuclein positive aggregates
A) SH-SY5Y cells were untreated or induced by KCl treatment to form α-synuclein aggregates (arrows) and immunofluorescence was performed with anti-α-synuclein in combination with antibodies to phosphorylated α-synuclein (phospho S129), autophagy marker p62, lysosome marker LAMP1 and protein aggregation marker, Thioflavin S. Scale bar: 2µm. B) SH-SY5Y cells stably expressing GFP alone or GFP-α-synuclein were depolarized with 50mM KCl of 60 mins or sham treated and left to recover for 48hrs. Soluble and insoluble fractions were generated and analyzed by Western blotting as described in the experimental procedures. 40 µg of protein was loaded in each lane. A higher exposure (High Ex.) of the GFP-α-synuclein is shown while the lower exposure (Low Ex.) is equivalent to the soluble fraction. C) Confocal immunofluorescence images of SH-SY5Y cells expressing GFP fusion constructs, immunolabeled with anti-α-synuclein and counterstained with DAPI following treatment with KCl. Scale bar: 5µm. D) Graph representing number of transfected SH-SY5Y cells bearing α-synuclein aggregates following KCl treatment prior to incubation with DMSO or 5 µM R55 for 48 hrs. Graph represents the proportion of cells with α-synuclein aggregated from 20-30 random images from 3 independent experiments. Error bars displayed as +/- SEM; *p<0.05 by ANOVA followed Tukey's multiple comparisons test.

Figure 7. Expression of Vps35 R524W perturbs localization and sorting of Cl-M6PR
A) Representative co-immunoprecipitation of GFP fusion constructs with CD8-M6PR reporter resolved by SDS-PAGE. Membranes were immunolabelled with anti-CD8. B) Representative immunoblots of culture medium and cell lysates from HeLa cells transiently transfected with Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP and incubated with 100µg/mL of cyclohexamide for up to 7hrs (n=3). Membranes were probed with antibodies raised against β-tubulin and Cathepsin D. C) Confocal analysis of HeLa cells transiently expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W immunolabeled with antibodies against endogenous Cl-M6PR and counterstained with DAPI. Scale bar: 5µm. D) Graphical representation of co-localization observed in (C). Graph represents three independent experiments with 10 images per group with 5-7 transfected cells per field of view (n=3, Error bars represent +/- SEM, *p<0.05;
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ANOV A followed with Tukey's multiple comparisons test). E) Representative images of HeLa cells expressing CD8-CIM6PR and GFP fusion constructs at 30 min post chase with anti-CD8 and immunolabeled with anti-p230 (red) and anti-CD8 (green) antibodies. Scale bar: 5µm.

Figure 8. Vps35 R524W does not disrupt SNX27-dependent cargo recycling
A) Immunofluorescence of HeLa cells transiently expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP immunolabelled with anti-SNX27. Scale bar: 5µm. B) Graphical representation of colocalization observed in A. Graphs representative of three independent experiments with 10 images per group with 5-7 transfected cells per field of view (error bars represent +/- SEM, *p <0.05; by ANOVA followed with Tukey's multiple comparisons test.). C) Pearson’s co-efficient of SNX27 with Vps26A in HeLa cells expressing Vps35 WT-GFP, Vps35 P316S or Vps35 R524W-GFP (error bars represent +/- SEM, *p <0.05; ANOVA followed with Tukey's multiple comparisons test). D) Co-immunoprecipitation using GFP NanoTrap of GFP, Vps35 WT-GFP,Vps35 P316S-GFP and Vps35 R524W-GFP with endogenous SNX27. E) Representative immunofluorescence images of HeLa cells expressing Vps35 WT-GFP, Vps35 P316S-GFP or Vps35 R524W-GFP followed by co-immunolabelling with anti-GLUT1 and anti-LAMP1. Scale bar: 5µm. F) Representative western blots (n=3) of HeLa cells expressing GFP fusion constructs showing biotinylation of cell surface and total GLUT1 levels. G) Graphical demonstration of glucose uptake in HeLa cells transiently transfected with GFP fusion constructs. Data representative of 3 independent experiments conducted in triplicate. Error bars represent +/- SEM.
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Parkinson's disease linked Vps35 R524W mutation impairs the endosomal association of retromer and induces α-synuclein aggregation
Jordan Follett, Andrea Bugarcic, Zhe Yang, Nicholas Ariotti, Suzanne J. Norwood, Brett M. Collins, Robert G. Parton and Rohan D. Teasdale

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