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Identification of Alzheimer disease associated variants in genes that regulate retromer function

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Abstract

The proteolytic processing of amyloid precursor protein (APP) to generate the neurotoxic Aβ peptide is central to the pathogenesis of Alzheimer disease (AD). The endocytic system mediates the processing of APP by controlling its access to secretases that cleave APP. A key mediator of APP localization is SorL1 – a membrane protein that has been genetically linked to AD. The retromer complex is a conserved protein complex required for endosome-to-Golgi retrieval of a number of physiologically important membrane proteins including SorL1. Based on the prior suggestion that endocytosis and retromer sorting pathways might be involved, we hypothesized that variants in other genes in this pathway might also modulate AD risk. Genetic association of AD with 451 polymorphisms in 15 genes encoding retromer or retromer-associated proteins was tested in a Caucasian sample of 8,309 AD cases and 7,366 cognitively normal elders using individual SNP and gene-based tests. We obtained significant evidence of association with *KIAA1033* (Paris p = 0.025), *SNX1* (Paris p =0.035), *SNX3* (p = 0.0057) and *RAB7A* (Paris p = 0.018). Ten *KIAA1033* SNPs were also significantly associated with AD in a group of African Americans (513 AD cases, 504 controls). Findings with four significant *SNX3* SNPs in the discovery sample were replicated in a community-based sample of Israeli-Arabs (124 AD cases, 142 controls). We show that Snx3 and Rab7A proteins interact with the cargo-selective retromer complex through independent mechanisms to regulate the membrane association of retromer and thereby are key mediators of retromer function. These data implicate additional AD risk genes in

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the retromer pathway and formally demonstrate a direct link between the activity of the retromer complex and the pathogenesis of AD.

1. Introduction

The localization of membrane proteins to discrete and specific compartments within eukaryotic cells is governed by a complex interplay of protein-protein interactions in which a sorting motif(s) in the cytoplasmic tail of a membrane protein is recognized by membrane-associated 'coat' proteins to direct the respective membrane proteins into a tubule or vesicle for transport to another compartment. A failure in the fidelity of sorting processes can lead to a range of pathologies. Sometimes the failure occurs when a sorting motif is mutated – a notable example being the mutation of the NPXY motif identified as causal in familial hypercholesterolemia by Brown and Goldstein [3]. Alternatively the molecular machinery that recognizes sorting motifs is at fault, for example, patients with deficient AP-3 function in Hermansky-Pudlack syndrome [10].

There has been a growing appreciation recently of the importance of correct protein sorting in regulating the processing of amyloid precursor protein (APP) and therefore the proteins that function in mediating localization to the post-Golgi endocytic system have been of great interest to studies of the underlying causes of late-onset Alzheimer disease (AD). Recently the retromer complex, an endosomally-localized protein complex, has been implicated in regulating APP processing (Figure 1) [8,61].

The retromer complex is a conserved endosome-associated protein complex that was first identified in yeast as essential for the endosome-to-Golgi retrieval of the CPY-sorting receptor, Vps10p. The studies first conducted in yeast revealed that retromer comprises five proteins, (encoded by vacuole protein sorting – VPS - genes) that are arranged into two functionally distinct subcomplexes; a cargo-selective trimer of Vps35p, Vps29p and Vps26p and a structural complex proposed to drive vesicle or tubule formation made of a dimer of the yeast sorting nexin proteins, Vps5p and Vps17p [53]. The retromer complex is conserved across all eukaryotes underscoring its vital role in mediating endosomal protein sorting [24].

Since retromer was first identified in yeast, studies in a variety of systems have identified cargo proteins that require retromer for their localization, and accessory proteins that function with retromer in endosomal protein sorting. For example, the small GTPase Rab7A associates with the cargo-selective retromer complex to mediate it's localization to endosomes [47]. Other retromer-associated proteins include TBC1D5, a rab GTPase activating protein, Eps15-homology domain containing protein-1 (EHD1) and the WASH complex [15,17,52].

Membrane proteins that depend on retromer for their proper localization, and therefore are considered to be retromer cargo proteins, now include; the cation-independent mannose 6-phosphate receptor (CIMPR), Wntless, a protein required for the secretion of the Wnt morphogen, DMT-1, a divalent cation transporter and the Vps10-family members sortilin and SorL1 (also known as SorLA) [4,9,13,41,51,52,62]. Since SorL1 has been shown to associate with APP and regulate the processing of APP [27], it has been of interest to studies directed at understanding the pathology of AD. The pathophysiological importance of the physical association of SorL1 and its four type I membrane homologs (sortilin, sorCS1, sorCS2 and sorCS3) with APP has been elevated by the identification of AD-linked single nucleotide polymorphisms (SNPs) in the genes encoded by these loci [29,43–46].

Retromer is required to mediate the localization of SorL1 and loss of retromer results in increased production of the pro-aggregatory neurotoxic Aβ peptide. Indeed a reduction in the levels of retromer has been detected in neuronal tissue of AD-patients suggesting a causal link between retromer activity and AD [26,38,41,57] and carriers of some AD-associated *SORL1* haplotypes have lower transcription of *SORL1* [46]. Therefore genes encoding retromer subunits or retromer-associated proteins and genes that function in mediating endosomal protein sorting are prime candidates for analyses directed towards identifying AD-linked SNPs.

In this study, we analyzed the association of AD with SNPs in genes that have putative retromer function or interact with the retromer-complex to mediate endosomal protein sorting. Four out of 15 retromer or retromer-associated genes (*SNX3, RAB7A, KIAA1033* and *SNX1*) showed significant evidence of association with AD in SNP-based and/or gene-based analyses. Intriguingly, loss of Snx3 or Rab7A function results in a reduction in endosome-associated retromer although the data presented herein suggests that Snx3 and Rab7A regulate retromer membrane association through distinct mechanisms.

2. Methods

2.1 Datasets

Summarized information from tests of genetic association of AD with SNPs located in the candidate gene regions was culled from a recent large genome-wide association study (GWAS) conducted by the Alzheimer Disease Genetics Consortium (ADGC) [39]. Naj et al. computed results for SNPs throughout the genome in their discovery sample composed of 8,309 AD cases and 7,366 cognitively normal elders from ten independent Caucasian data sets (Table 2). Details of the quality control and statistical analysis including genotype imputation procedures and genetic models has been published elsewhere [39]. GWAS datasets containing African Americans (513 AD cases, 504 controls) [35] and Israeli-Arabs (124 AD cases, 142 controls) from a consanguineous community [55] were used to replicate top-ranked findings in the Caucasian datasets. Although these samples are modest are modest in size, evidence of replication in populations which are genetically distinct from the discovery sample would make the findings more robust and generalizable [35, 55].

2.2 Gene selection

We tested the genetic association of AD with eight genes encoding the subunits of the mammalian retromer-complex [16,66] and with seven other retromer-associated genes (Table 1). The GTPase Rab7A mediates the localization of the retromer to the endosome [47]. FAM21C, KIAA1033, KIAA0196 form subunits of the human WASH-complex and are involved in retromer-dependent endosomal protein sorting [17]. The Eps15 homology domain-containing protein-1 (EHD1) interacts with the retromer to facilitate endosome-to-Golgi retrieval [15]. FK506 binding protein 15(FKBP15) has shown evidence of being involved in regulation of early endocytic transport [64]. The sorting nexin 3 (Snx3) has been shown to interact with the cargo-selective subcomplex of the retromer to sort Wntless (Wnt-binding protein) [19].

2.3 Study heterogeneity and gene-based multiple testing correction

Heterogeneity of odds ratios for each SNP among datasets was assessed using the Cochran's Q and I^2 statistics [20]. We corrected for testing multiple SNPs in a gene after accounting for correlation between SNP genotypes due to linkage disequilibrium. Each gene tested was treated as an independent hypothesis and the effective number of tests per gene was obtained by a method described by Li and Ji [32]. The Versatile Gene-based Association Study (VEGAS) approach [34] was used to summarize the strength of association of a gene with

AD based on the number of SNPs tested in the gene and size of the gene. This method computes a gene-based test statistic based on the SNP p-values within the gene, and then uses simulation to calculate an empirical gene-based p-value taking into account all genes in the genome.

2.4 Antibodies, biochemicals and other reagents

Unless otherwise stated, general biochemical reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK). Polyclonal antibodies against Vps26, Vps35, GFP and Snx1 have been described previously [51,52]. Polyclonal anti-actin, anti-Snx3 and monoclonal anti-Rab7a were purchased from Sigma-Aldrich (Poole, Dorset, UK). Monoclonal antibodies against Snx1 and EEA1 were purchased from BD Bioscience (Oxford, UK). The monoclonal anti-GFP was purchased from Invitrogen, (Paisley, Scotland, UK). The monoclonal anti-CD8 antibody was produced from a hydridoma cell line as described previously [50]. Fluorescently labeled secondary antibodies for immunofluorescence were purchased from Molecular Probes (Paisley, Scotland, UK). Small interfering (si) RNA for RNAi experiments was purchased from Dharmacon (Lafayette, CO, USA). ^{125}I-protein A for western blotting was purchased from Perkin Elmer (Waltham, MA, USA). Effectene used in transfections was purchased from Qiagen (Crawley, Sussex, UK).

2.5 Generation of GFP-construct and cell lines

GFP-tagged Snx3 was produced by amplifying an EST encoding human Snx3 incorporating *BamH*I and *Sal*I sites at the 5' and 3' ends respectively. The PCR product was cloned initially into pCR Blunt (Invitrogen – Paisley, Scotland, UK) sequenced and then subcloned into pEGFP C1 at the *Bgl*II and *Sal*I sites. The GFP-Snx3 construct was then subcloned into pIRESneo2 following excision with *Nhe*I-*BamH*I and cloned into *Nhe*I-*BamH*I digested vector. Similar methodology was applied to generate the GFP-Syntaxin 6 cell line. The GFP-RhoB construct was generously provided by Professor H. Mellor (University of Bristol, UK) and was cloned into pIRESneo2 similarly to GFP-Snx3. The other GFP-tagged cell lines have been reported previously in [17,52].

2.6 Yeast two-hybrid analysis

The yeast two hybrid system (and most of the constructs) have been described previously [17]. The pGAD424-Snx3 construct was generated by subcloning Snx3 from pCR Blunt into a modified pGAD424 in which the *BamH*I and *Sal*I sites in the multi-cloning site of pGAD424 were in the same reading frame as pEGFP C1.

2.7 Native immunoprecipitations

The immunoprecipitation of GFP-tagged proteins from HeLa cell lysates was performed as described previously [52] using the following lysis buffer; 100 mM Mes-NaOH, pH 6.5, 200 μM sodium orthovanadate; 0.5 mM EGTA, 1% digitonin and protease inhibitors using a polyclonal anti-GFP antisera described previously [52]. The CD8-CIMPR and CD8-SorL1 expressing cell lines have been reported elsewhere [14,50]. The immunoprecipitation of the CD8-reporter proteins was performed as described previously [14,50]. SDS-PAGE and western blotting was performed as described previously [51].

2.8 RNAi-mediated knockdowns

The silencing of Snx3, Rab7A etc expression was achieved by transfection of siRNA into the HeLa cells used as described previously [51]. For large scale siRNA knockdown experiments (e.g. in the CD8-reporter expressing cell lines) a single transfection of 500 pmol of siRNA was performed for cells grown on 140 mm tissue culture dishes. 48 hours post-

transfection, each dish was trypsinised and the cells were seeded into two new 140 mm dishes. The cells were used for the respective experiment 24 hours later.

2.9 Automated microscopy

Following siRNA-mediated knockdown of target genes cells were seeded on 96-well plates and allowed to attach overnight. Cells were then fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes and blocked with 3% BSA in PBS at 4°C overnight. Next, cells were labelled with anti-VPS26 and anti-Snx1 or anti-EEA1 antibodies for 1 hr at room temperature followed by Alexa Fluor 488-labeled secondary antibody. Finally, cells were also treated with a Whole Cell Stain Blue (Dharmacon, Lafayette, CO, USA) for 1 hr at room temperature before imaging on a Cellomics Arrayscan automated microscope, using a 2-channel Spot Detector protocol. The whole cell stain was used for focusing and for the definition of the cell outlines; in the 2nd channel endosomes were counted with the Spot Detector Algorithm using an identical intensity threshold for all conditions. To measure the overlap coefficient for the CD8-reporters and VPS26, cells treated with siRNA were seeded into four wells of a 24-well plate and allowed to adhere overnight. Following fixation and antibody staining using polyclonal anti-VPS26 and monoclonal anti-CD8, the cells were further stained with Hoecsht to visualize the nucleus. Using the nucleus for focusing, the cells were imaged on a Cellomics Arrayscan automated microscope and the intensity of the VPS26 staining that was coincident with the CD8-reporter protein was measured using the Colocalization Algorithm.

3. Results

3.1 AD is Associated With KIAA1033, RAB7A, SNX1, and SNX3

Nominally significant evidence of AD association was observed with at least one SNP in seven of the 15 retromer-related genes (Table 3 and Supplementary Table 1). In four of these seven genes (*KIAA1033, RAB7A, SNX1* and *SNX3*), approximately 50% or more of the SNPs were significantly associated with AD at p=0.05. Two SNPs in *SNX3* were significant after multiple testing correction (best p=0.0056 for rs12524840) and two SNPs in *RAB7A* nearly met the gene-based significance threshold (rs9831813, p=0.0058 rs7631994, p=0.0059). Analysis of the individual SNP results using the VEGAS approach revealed that AD was significantly associated with *RAB7A, KIAA1033* and *SNX1* at the gene level (Table 3). This gene-based analysis also showed suggestive evidence of association with *SNX3* (p=0.06). This result may have been tempered by heterogeneity of odds ratios among studies for five *SNX3* SNPs ($p<0.05$).

Examination of results for SNPs in these four genes in the replication datasets revealed no evidence of association in *RAB7A* or *SNX1*. However, in the Israeli-Arab dataset nearly significant results ($p\leq0.08$) were obtained with four *SNX3* SNPs all of which were nominally significant ($p\leq0.03$) with the same pattern of effect in the Caucasians (Supplementary Table 2). Ten SNPs distributed across the proximal half of *KIAA1033* were significantly associated with AD in AAs (Supplementary Table 2). Remarkably, these SNPs intercalate the 28 SNPs significantly associated with AD in Caucasians. Considering results from both single SNP and gene-based association analyses, *SNX3* and *RAB7A* provided the most compelling evidence of association with AD. Consequently, these genes were studied further for genetic interactions with *SORL1* and each other and for effects on retromer function.

All possible two-way interactions of SNPs from *SNX3* (28 SNPs), *RAB7A* (32 SNPs) and *SORL1* (152 SNPs) were tested in models including terms for the SNPs and their interaction. The most significant evidence of interaction was found involving rs2276346 in

SORL1 and 14 *RAB7A* SNPs ($0.0019 \leq p \leq 0.0037$), however these results did not survive multiple testing correction (threshold p-value for 280 independent tests = 0.00018). Nominally significant interactions were also observed with *SORL1* SNP rs11512475 and five *SNX3* SNPs ($0.0064 \leq p \leq 0.0080$) which also did not remain significant after multiple test correction (threshold p-value for 196 independent tests = 0.00026). None of the interactions in models including *RAB7A* and *SNX3* SNPs were significant at $p < 0.01$.

3.2 Interaction of Snx3 and Rab7A with retromer

We investigated the interaction between mammalian retromer and Snx3 initially by using the yeast two-hybrid (Y2H) system. The Snx3 gene was expressed in the yeast HF7c strain from the pGAD424 ('prey') vector and thus tested for an interaction with retromer components and retromer-associated proteins (Figure 2A). Growth of the yeast on media lacking histidine is indicative of an interaction and was only observed for the combination of Snx3 and Vps35.

We next generated a HeLa cell line stably transfected with a GFP-Snx3 construct. The GFP-Snx3 fusion colocalizes with Vps26 on punctate endosomal structures (Figure 2B). The Y2H interaction of Snx3 and Vps35, and the colocalization of GFP-Snx3 with Vps26 suggest an in-vivo interaction is likely. Therefore we performed native immunoprecipitations (IPs) on a panel of HeLa cell lines stably expressing various endosomally localized GFP-tagged proteins. We observed that GFP-Snx3 co-IPs the Vps26 and Vps35 proteins consistent with an association with retromer (Figure 2C). In line with our previously published data [48], GFP-Rab7A co-IPs retromer as does Vps29-GFP that serves as a positive control for these experiments. Other GFP-fusion proteins (e.g. Rab5, Rab9) do not co-IP retromer demonstrating the specificity of the assay. In addition to analyzing the native IPs by western blotting we also subjected the samples to SDS-PAGE and silver staining (see figure 2D). The bands corresponding to Vps26 and Vps35, although faint, are visible in the GFP-Snx3 and GFP-Rab7A lanes.

We next investigated the functional significance of the Snx3-retromer interaction by performing siRNA-mediated knockdowns of Snx3 and other retromer-associated proteins. Figure 3A and 3B shows cells that have been treated with siRNA to knockdown (KD) Snx1, Snx3, Rab7A, Vps26 and KIAA1033 and then fixed and labeled with antibodies to stain Vps26 and the endosomal protein EEA1. The Snx3 and Rab7A KDs both reduced the intensity of the Vps26 staining but did not markedly affect the EEA1 staining indicating that endosomal structures persist after Snx3 or Rab7A KD. To quantify the effect of the KDs on the endosomal localization of retromer, the cells were imaged using an automated microscope to determine the number of Vps26-positive endosomes. This experiment revealed that the loss of Rab7A and Snx3 both result in a pronounced reduction in the number of Vps26-positive endosomes (Figure 3C). The effect of the loss of Snx3 or Rab7A on Snx1-positive endosomes was much less (Figure 3D) and no effect on the number of EEA1-positive endosomes was observed for any knockdown performed (Figure 3E).

The reduction in the Vps26 staining observed after KD of Snx3 (or Rab7A) could be due to loss of membrane association or loss of the protein – the immunofluorescence based assays cannot distinguish these two possibilities. Therefore lysates from the various KDs were analyzed by western blotting. As shown in Figure 3F, there was no loss of the Vps26 protein following the Snx3 (or Rab7A) KD although Vps26 KD does result in loss of the Vps26 protein. The efficacy of the siRNA targeting Snx3 or Rab7A was confirmed by western blotting lysates of cells stably transfected with GFP-Snx3 or GFP-Rab7A that had been treated with siRNA to silence Snx3 or Rab7A and is shown in Supplemental Figure S1.

Thus we conclude that the reduction in Vps26 labeling observed in Figure 3A–3C is not caused by loss of the protein and is likely to be due to loss of membrane association. To test this hypothesis we performed a simple fractionation assay to separate membrane-associated (pelletable) and cytosolic (soluble) retromer. In Figure 3G, the pellet (P) and soluble (S) fractions from control, Snx3 and Rab7A KD cells have been analyzed by SDS-PAGE and western blotting. The Snx3 and Rab7A knockdowns both result in a shift of Vps26 and Vps35 from the pellet fraction to the soluble fraction. Data from three blots was quantified and is shown in Figure 3H. Loss of either Snx3 or Rab7A resulted in a pronounced change in the membrane association of retromer with both Vps26 and Vps35 becoming predominately soluble consistent with Snx3 and Rab7A regulating the membrane association of retromer. Simultaneous KD of both Snx3 and Rab7A did not cause further loss of retromer membrane association (data not shown) suggesting that there are additional factors responsible for a basal level of membrane association of the cargo-selective retromer complex.

If loss of Snx3 results in reduced membrane association of the cargo-selective retromer complex, does increased Snx3 promote retromer membrane association? To address this question, cells expressing Vps29-GFP or GFP-Vps35 where the elevated expression results in increased cytoplasmic localization were transfected with red fluorescent protein-tagged Snx3. We observed that in cells transiently transfected with RFP-Snx3 the localization of Vps29-GFP and GFP-Vps35 appears more punctate and endosomal (Supplementary Figure 2).

The data presented so far shows that Snx3 is required for the membrane association of retromer – similar to Rab7A. Therefore we wondered if Rab7A and Snx3 interact with each other either directly or through a third-party protein. Thus large scale anti-GFP native IPs were performed on cells stably expressing GFP-Snx3, GFP-Rab9 (a negative control), GFP-Rab7A and Vps29-GFP (a positive control). The native IPs were analyzed by mass spectrometry (see Supplementary Table 3). Other than retromer proteins, we did not identify any proteins that co-IPed with both GFP-Snx3 and GFP-Rab7 and therefore Snx3 and Rab7A are unlikely to interact with each other directly or indirectly unless via retromer.

3.3 Snx3 and Rab7A act independently to regulate retromer membrane association

We next investigated whether Snx3 and Rab7 act together or independently to regulate retromer association. Initially, cells stably expressing GFP-Snx3 were treated with siRNA to KD either Snx3 or Rab7A. In Figure 4A, the Snx3 KD abolished expression of GFP-Snx3 and leads to a reduction in the Vps26 staining. Loss of Rab7A expression also caused a reduction in Vps26 staining but there was an apparent increase in the brightness of the GFP-Snx3 labeling in Rab7A knockdown cells. When the same KDs were performed in cells stably expressing GFP-Rab7A we noticed that loss of Snx3 resulted in reduced punctate GFP-Rab7A labeling along with diffuse Vps26 labeling. The Rab7A KD did not affect expression of the GFP-Rab7A as this construct utilizes murine Rab7A that is resistant to the siRNA that silences endogenous Rab7A expression in the HeLa cells and hence Vps26 labeling appears normal.

Lysates from the KDs shown in Figure 4A and 4B were analyzed by SDS-PAGE and western blotting. We observed that the Rab7A KD in the GFP-Snx3 cells did result in an increase of GFP-Snx3 levels (Figure 4C) that is consistent with the increased fluorescence (Figure 4A). To test whether loss of Snx3 expression could be compensated by increased expression of Rab7A, Snx3 KDs were performed on a panel of cells expressing GFP-fusion proteins that localize to endosomes including the Rab7A-Q67L mutant that is constitutively active. The number of Vps26-positive endosomes was determined using an automated microscope (Figure 4D). In control cells there were ~40 Vps26 endosomes per 1000 μm^2

(similar to untransfected HeLa cells) although increased Rab5 or Rab7 expression can respectively decrease or increase the number of Vps26 positive endosomes. In the Snx3 knockdown cells, the number of Vps26 positive endosomes was reduced to ~10 per 1000 μm^2 although in cells expressing GFP-Rab7A it is ~15 per 1000 μm^2, this number is no greater than was observed for untransfected HeLa cells. We conclude therefore that elevated expression of Rab7A (even constitutively active Rab7A-Q67L) cannot compensate for loss of Snx3. Furthermore, when native immunoprecipitation of GFP-Snx3 or GFP-Rab7A was performed on cells treated with siRNA to abolish Snx3 or Rab7A expression, we observed that knockdown of Snx3 abolishes the interaction of retromer with the GFP-Rab7A protein whereas some association of retromer with GFP-Snx3 was retained after loss of Rab7A expression (Figure 4E).

The inability of the cargo-selective retromer complex to associate with endosomal membranes following siRNA KD of Snx3 or Rab7A would be predicted to significantly impact the association of the cargo-selective complex with cargo proteins such as the CIMPR or SorL1. We tested this hypothesis by immunoprecipitating CD8-reporter proteins that contain the cytoplasmic tails of the CIMPR or SorL1 after siRNA KD of Snx3 or Rab7A. Figure 5A shows that loss of Snx3 or Rab7A greatly reduces the amount of retromer proteins (Vps35 and Vps26) that co-IP with the respective CD8-reporter protein. The KD of Snx3 or Rab7A does not however, alter the amount of CD8-reporter protein that IPs or levels of Vps35 or Vps26 proteins (Figure 5B) Additionally in Figure 5C, we also measured the intensity of the immunofluorescence signal of Vps26 protein that was coincident with the respective CD8-reporter. The KD of Snx3 or Rab7A markedly reduces the intensity of the Vps26 staining consistent with the loss of membrane association shown in Figure 3. This experiment supports the hypothesis that Snx3 and Rab7A mediate the association of retromer with cargo proteins such as SorL1.

4. Discussion

We identified significant association of AD with specific SNPs in or at the gene level for four of the 15 retromer and retromer-associated genes tested in a large group of Caucasian AD cases and controls. *RAB7A, KIAA1033* and *SNX1* attained gene-level significance and *SNX3* contained SNPs significant after correction for multiple testing. Significant findings for several *SNX3* SNPs were replicated in a sample of Israel-Arabs. We also obtained evidence of replication for *KIAA1033* in African Americans, although with a completely different set of SNPs. These non-overlapping findings may be due to distinct population linkage disequilibrium patterns among SNPs in the *KIAA1033* gene region reflecting association with the same or different causal variants.

No retromer or retromer-related genes including *SORL1* contained a signal approaching genome-wide significance in the largest GWAS published to date [38]. However, failure to meet this threshold, due to small effect size or allelic heterogeneity, does not mean that a gene or variant is not associated as demonstrated by genetic and functional evidence for *SORL1* [28–30,41,42,44–46,57,58]. Our use of GWAS data to address a specific hypothesis with far fewer tests requires much lower thresholds for significance.

Association of Rab7A protein with retromer has been observed in enteric amoeba, human tissue culture cells and yeast [5,40,47,52]. The Snx3 protein is a member of the sorting nexin family and contains a PX domain that binds to phosphotidylinositol 3-phosphate (PtdIns3-P). The yeast homolog of Snx3 is called Grd19p and interacts with the retromer complex to mediate endosomal protein sorting of specific cargo proteins [59,60]. The interaction of retromer with either Rab7A or Snx3 therefore appears to be conserved across species and through evolution suggesting that these interactions are of profound functional importance.

Indeed, while this manuscript was in preparation, Harterink et al. [19] reported that Snx3 functions in retromer-mediated sorting of Wntless, a membrane protein required for Wnt secretion, although that study overlooked the evidence that Snx3 also affects Rab7A localization [36] thereby complicating the interpretation of the role of Snx3 in regulating retromer membrane association.

It is intriguing that both *SNX3* and *RAB7A* contained AD-linked SNPs as herein we show that loss of either Snx3 or Rab7A expression leads to very similar phenotypes, specifically a reduction in the membrane association of the cargo-selective retromer complex. We have also previously reported that both Snx3 and Rab7A knockdowns result in a defect in endosome-to-Golgi retrieval [17,52]. Interestingly, data presented herein and in a previous study [7] indicate that loss of Snx3 expression also alters the localization of Rab7A hinting that the mechanism by which Snx3 affects the membrane association of retromer could be through altered Rab7A localization and necessitating a more detailed examination of the relationship between Snx3 and Rab7A.

We found no evidence for a direct association of Snx3 with Rab7A, therefore it is unclear why the loss of Snx3 should affect Rab7A localization. Attempts to compensate for the loss of Snx3 by increasing the expression of Rab7A, including the constitutively active Q67L mutant, were also unsuccessful. Given that loss of Rab7A results in a quantitative increase in the level of GFP-Snx3 but no rescue of the Vps26 localization we conclude that Rab7A and Snx3 regulate the membrane association of the cargo-selective retromer complex through distinct mechanisms. This conclusion is consistent with lack of evidence for genetic interaction of *RAB7A* with *SNX3* on risk of AD.

Studies undertaken in yeast and nematode have implicated Snx3 in directing specific cargo proteins into a retromer-mediated retrieval pathway [24,29]. It is possible therefore that Snx3 aids in stabilizing the membrane association of the cargo-selective retromer complex by concentrating membrane proteins into discrete patches thereby elevating the local concentration of cytoplasmic tails for retromer to interact with. Additionally the Y2H data we present here shows that Snx3 can interact directly with Vps35 and therefore this interaction may also contribute to facilitating the stable association of retromer with endosomal membranes thereby promoting the association with cargo proteins such as CIMPR and SorL1 (see Figure 6). It is noteworthy however that, unlike Rab7A, Snx3 is not conserved across all eukaryotes being absent in plants. Therefore, while Snx3 is clearly important for retromer function in mammalian cells, it is dispensable in some other eukaryotes.

Trafficking of the amyloid precursor protein (APP) from the cell surface via the endocytic pathways plays a key role in the generation of amyloid β-peptide (Aβ), the aberrant accumulation of which is postulated to be central to the pathogenesis of AD [36]. Thus, pathways modulating APP-sorting through the membrane or altering APP cleavage by the secretases are key to understating the pathophsyiology of AD. Multiple studies have shown that retromer deficiency elevates levels of endogenous Aβ in the brain and causes hippocampal dysfunction and neurodegeneration [38,56]. SorL1 is involved in trafficking of APP from the cell surface to the Golgi-complex and reduced expression of *SORL1* leads to elevated Aβ levels and an increased risk of AD [1,2,12,42,49,58]. Several candidate-gene and targeted studies across different ethnic groups have shown the strong association of *SORL1* with AD [6,22,23,27,28,30,33,46,63]. Recently, the other members of the Vps10p-domain sorting receptor family (*SORCS1, SORCS2, SORCS3* and *SORT1*) have been demonstrated to be genetically and functionally associated with AD [26,43,45]. Because loss of Snx3 or Rab7A expression results in displacement of the cargo-selective retromer complex from the endosome membrane leading to a marked reduction in the ability of

retromer to recognize cargo proteins (see Figure 6), the most likely explanation for the association of Snx3 and Rab7A with AD is through defective protein sorting of retromer cargoes such as SorL1. Connections of *SORL1* with *RAB7A* and *SNX3* are suggested by our genetic interaction analyses. Precise and more significant statistical evidence for interactions among these and other retromer-associated genes will likely emerge from studies of additional large datasets assembled by several international AD genetics consortia. A direct influence of the novel AD-associated genes reported herein (*RAB7A, SNX1, SNX3*, and *KIAA1033*) on APP processing or Aβ has not been reported, and thus future studies should explore these relationships. However, it has been reported from multiple studies that loss of retromer function or loss of retromer-association with SorL1 result in increased processing of APP to Aβ [14,26,37,45,55] and, therefore, it is expected that APP processing will be altered in Snx3 or Rab7A knockdowns.

Although none of the retromer or retromer-associated genes have yet emerged as strong candidates in agnostic approaches such as GWAS [25,39], perhaps due to their modest effect on genetic risk [43,45,46] or intragenic heterogeneity [45,46], large-scale GWAS have identified genome-wide significant association of AD with nine novel loci [17,21,25,54], three of which --- *PICALM, BIN1* and *CD2AP* --- lend additional support to perturbation of membrane trafficking as an important mechanism in AD pathogenesis. Genetic studies in budding yeast indicate an essential role of the Bin1 homolog in endocytosis, however, this role appears to be non-essential for homologs in fission yeast, fruit flies, and mice [31]. *CD2AP* is involved in membrane trafficking occurring during receptor endocytosis and cytokinesis [37]. Although the role of *PICALM* in AD pathogenesis is poorly understood, it is believed to be involved in APP metabolism via the endocytic pathway [48].

A hypothesis-driven pathway approach allowed us to apply gene association methods with a much lower statistical significance threshold than required for a GWAS. Evidence for association of AD with the retromer-associated genes *RAB7A* and *SNX3*, as well as interactions of these genes with *SORL1* on AD risk, present a compelling argument for further studies of protein-trafficking and APP sorting pathways in AD etiology. However, lack of association of the retromer-complex genes *VPS35, VPS26* and *VPS29* underscores the limitation of even hypothesis-driven genetic studies that typically evaluate common variants because they are known and, at best, are powered to detect association with them. The advent of high-throughput gene sequencing technologies has demonstrated that much of the genetic risk for common diseases is likely due to rare variants [11]. Recent studies have shown that rare mutations in *VPS35* cause Parkinson disease [65,67]. It is also possible that these vacuolar protein sorting genes exert an effect on AD risk through interaction with other genes. This possibility can be studied statistically and by functional studies *in vitro*.

Supplementary Material

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Acknowledgments

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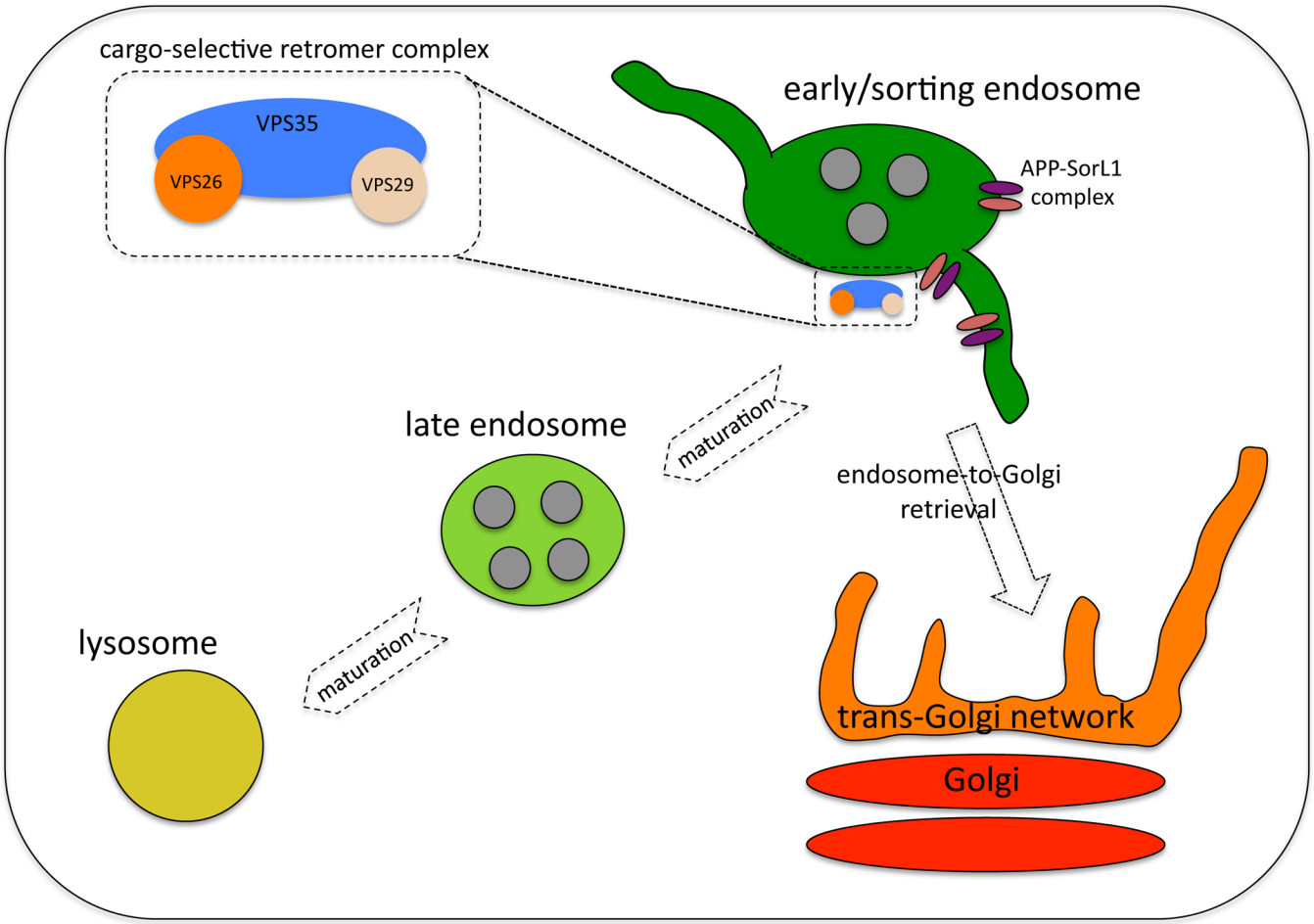


Figure 1.
Schematic diagram of the endocytic pathway and the role of retromer in sorting APP and SorL1. The SorL1 protein associates with APP. The cargo-selective retromer complex interacts with SorL1 to direct the APP-SorL1 complex into an endosome-to-Golgi retrieval pathway. Aberrant APP localization to late endosomal compartments increases processing to the neurotoxic Aβ peptide.

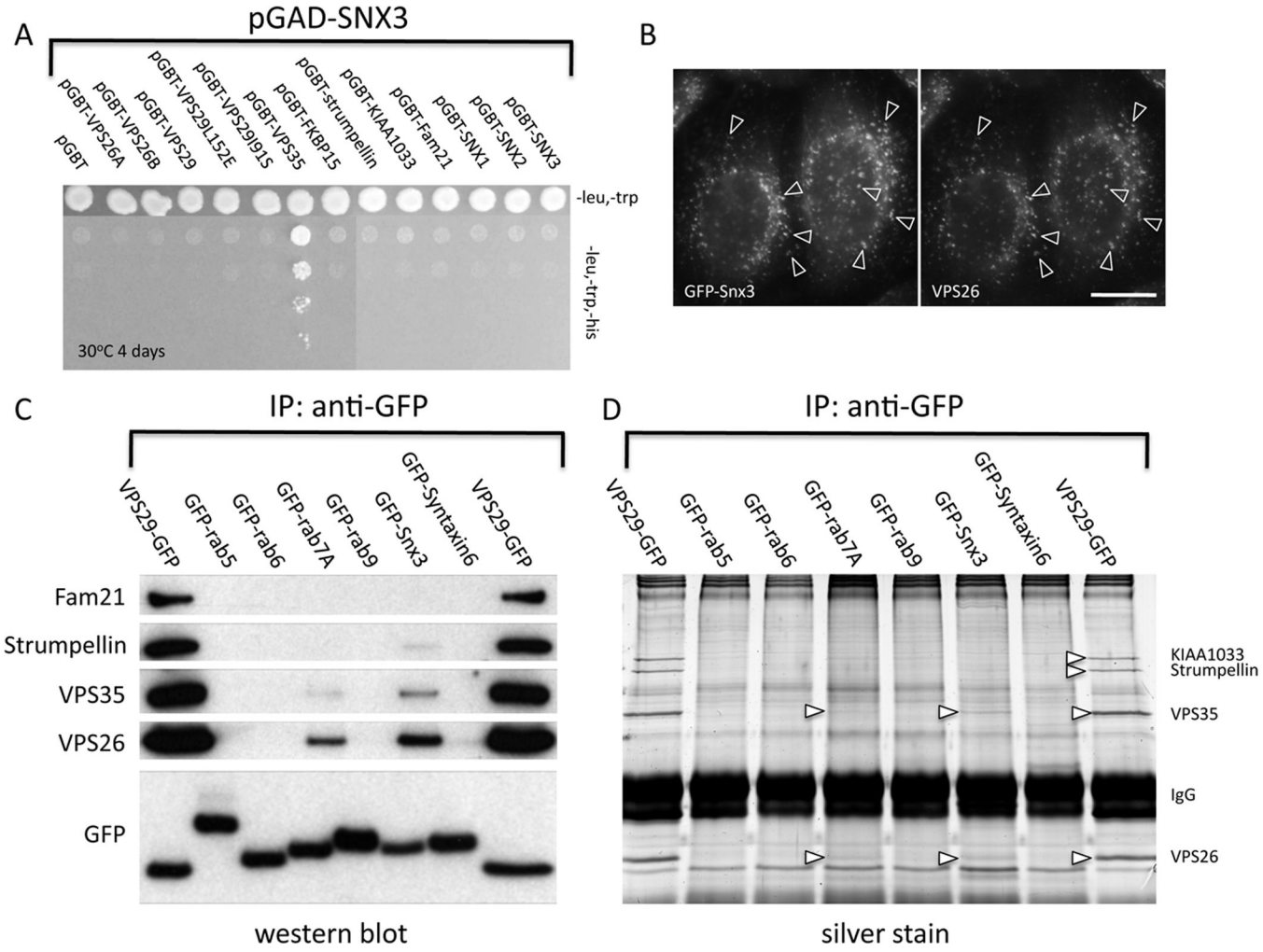
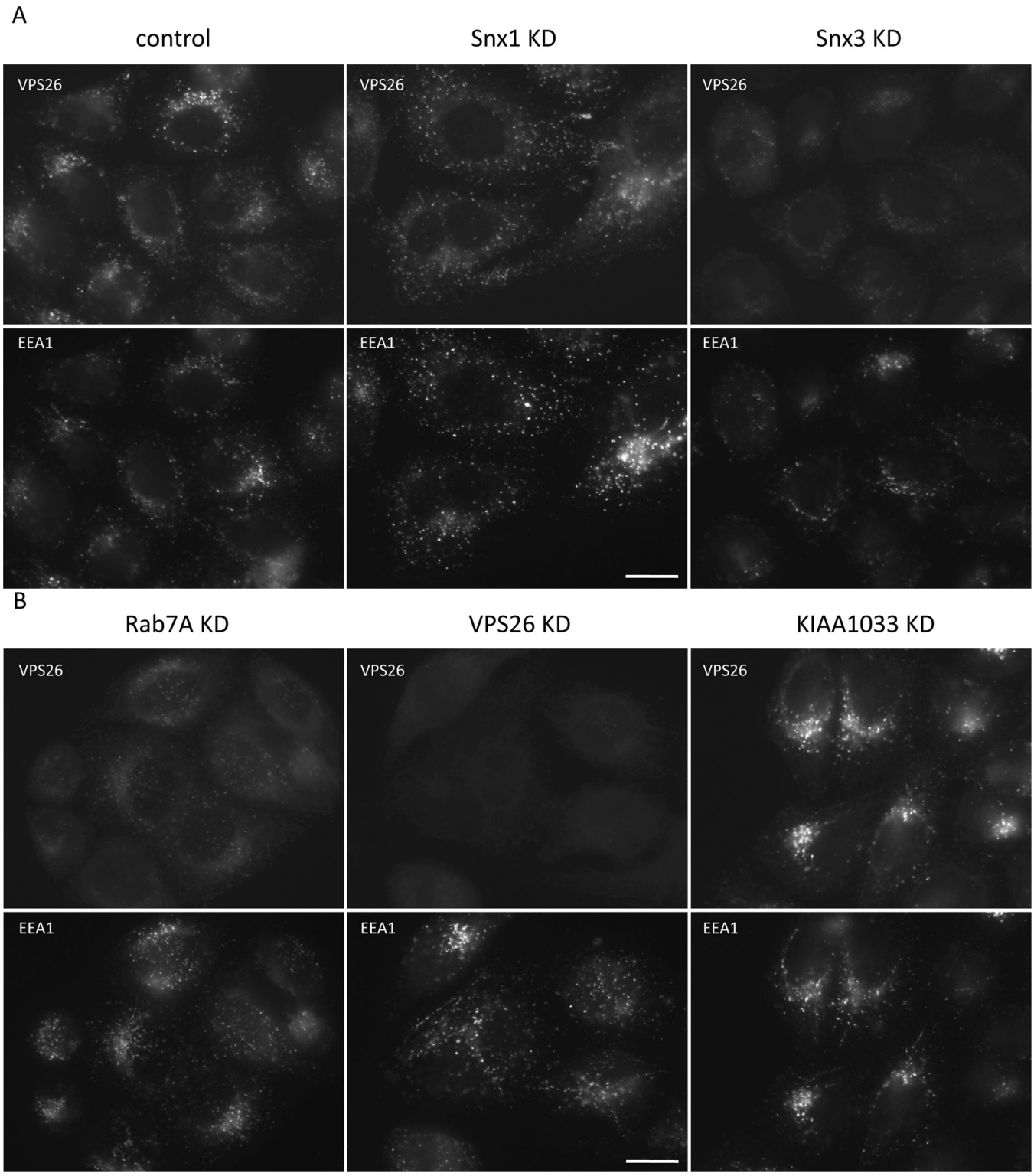


Figure 2.

Snx3 interacts with the cargo-selective retromer complex through VPS35. **A**. Yeast two-hybrid analysis of Snx3 interactions with retromer and retromer-associated proteins. The yeast HF7c strain was transformed with the pGAD-Snx3 plasmid along with pGBT9 plasmids expressing the various retromer proteins. Transformants were spotted out onto –leu,-trp media or onto –leu,-trp,-his media as a dilution series. Only yeast expressing both Snx3 and VPS35 grow on the triple dropout media indicating an interaction between Snx3 and VPS35. **B**. HeLa cells stably transfected with GFP-Snx3 were fixed and labeled with anti-GFP and anti-VPS26. The GFP-Snx3 protein colocalizes with VPS26 (indicated with arrow-heads) Bar = 20μm. **C**. HeLa cells stably expressing various GFP-tagged proteins were lysed and treated with anti-GFP to IP the respective proteins. GFP-Snx3 co-IPs the VPS26 and VPS35 proteins as does GFP-Rab7A (as reported previously). The VPS29-GFP construct is a positive control in this experiment and co-IPs significantly more retromer proteins. **D**. Identical samples to C were subjected to SDS-PAGE and silver staining. The VPS35 and VPS26 bands are visible in the GFP-Rab7A and GFP-Snx3 lanes (indicated with arrow-heads).



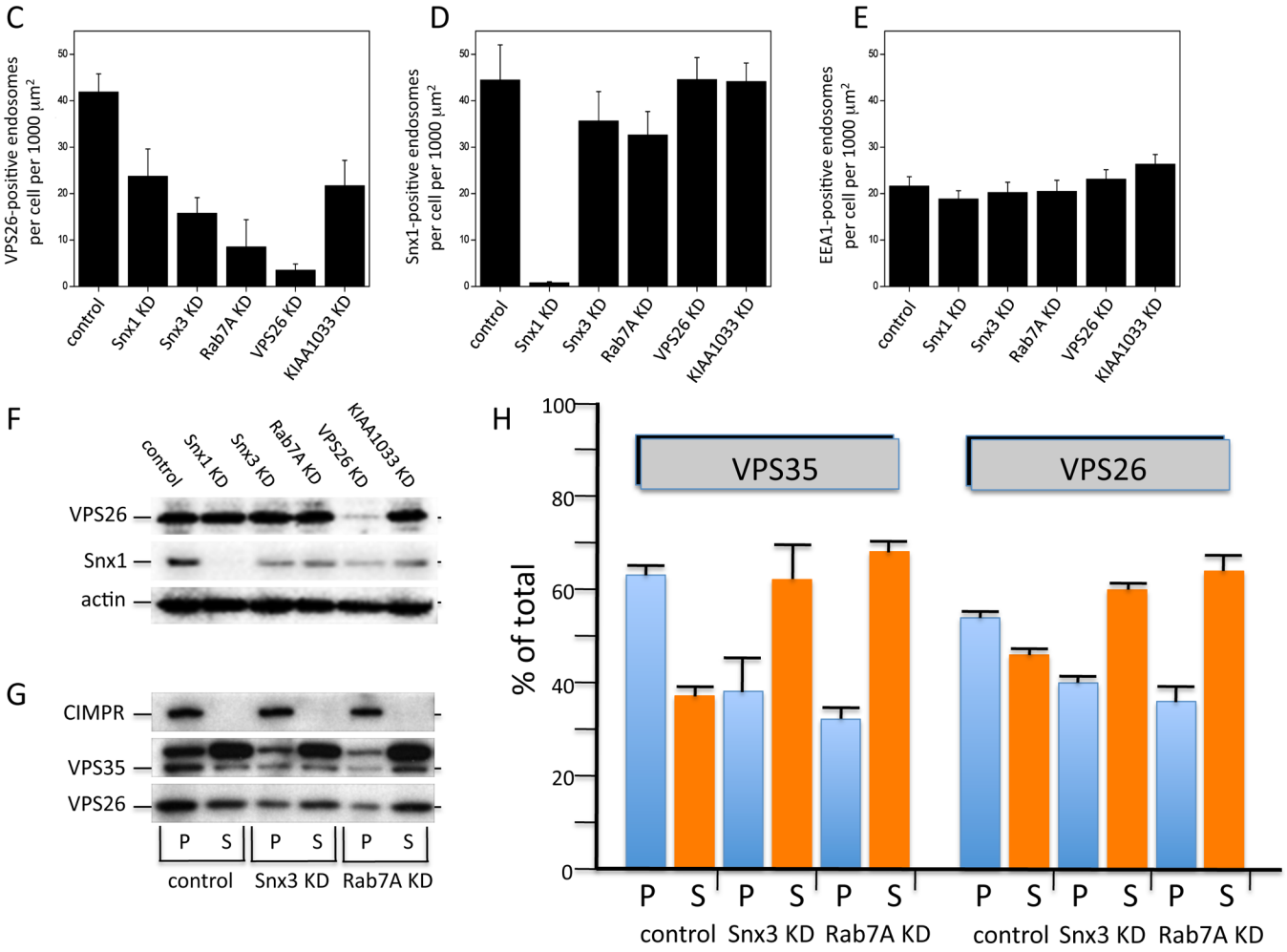
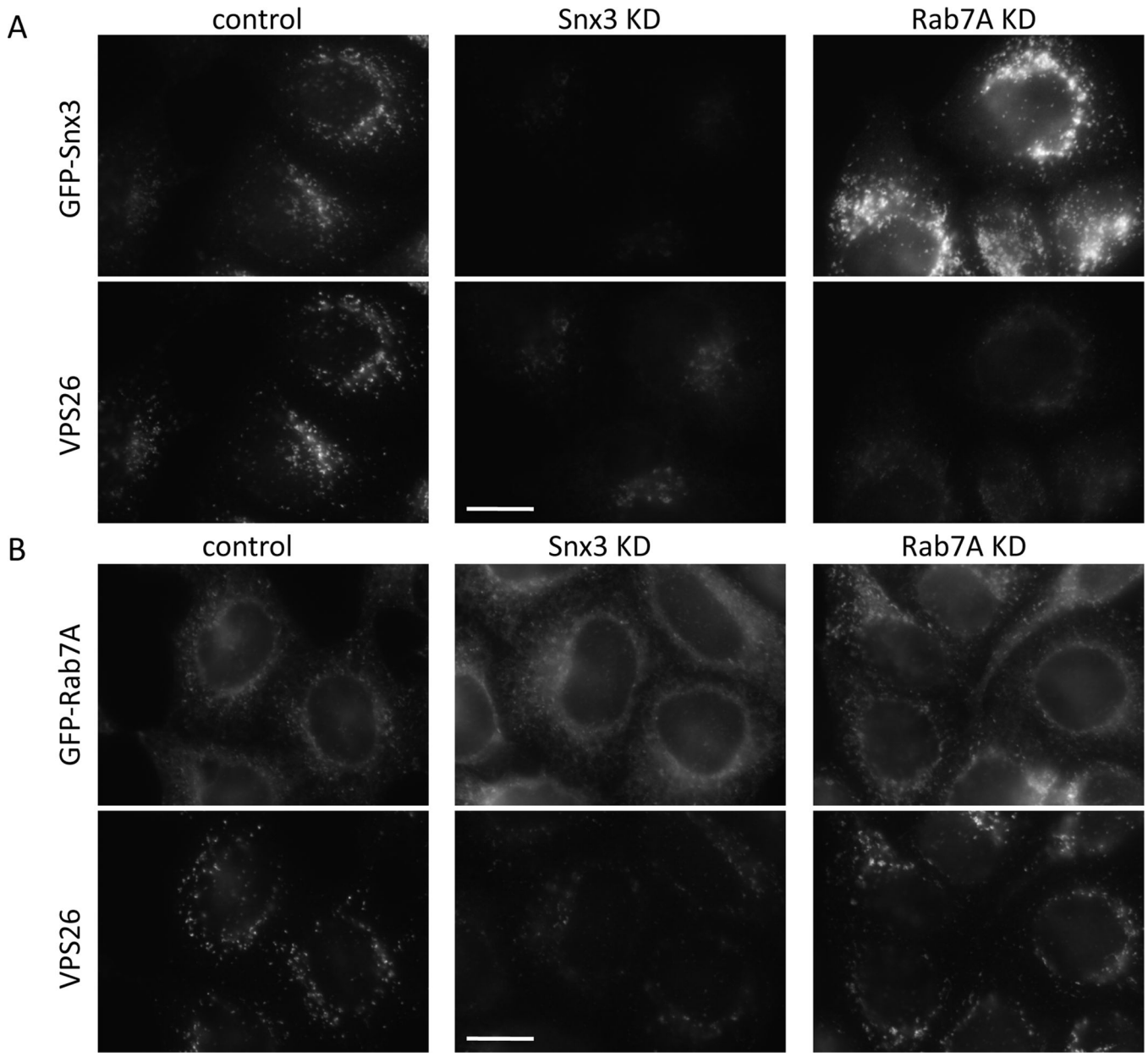


Figure 3.

Snx3 is required for the membrane association of the cargo-selective retromer complex. **A** & **B**. HeLa cells were treated with siRNA to abolish the expression of Snx1, Snx3, Rab7A, VPS26 or the WASH complex protein, KIAA1033. After fixation the cells were labeled with antibodies to VPS26 and EEA1. Loss of Snx3 expression results in a reduction of the VPS26 labeling comparable to loss of Rab7A. Bar = 20μm. **C, D** & **E**. Cells were treated with siRNA as in A and B. The number of VPS26-positive (C) endosomes was determined using a Cellomics ArrayScan Quantitative microscope along with the number of Snx1-positive (D) or EEA1-postive (E) endosomes. Loss of Snx3 or Rab7A results in a marked reduction in the number of VPS26-positive endosomes whilst little effect was observed for Snx1 or EEA1. **F**. Samples from the cells treated with the various siRNA were western blotted for VPS26 and Snx1. The reduction in the VPS26 signal observed in A, B and C is not due to the loss of the protein. **G**. HeLa cells were treated with siRNA to abolish Snx3 or Rab7A expression. The cells were then fractionated to separate membranes and membrane-bound proteins (P) from soluble cytosolic proteins (S). The samples were analyzed by SDS-PAGE and western blotting. The VPS26 and VPS35 proteins redistribute into the soluble (cytosolic) fraction after loss of Snx3 or Rab7A expression. **H**. The experiment performed in G was repeated a further two times and the data from the three experiments was averaged and is shown as a graph.



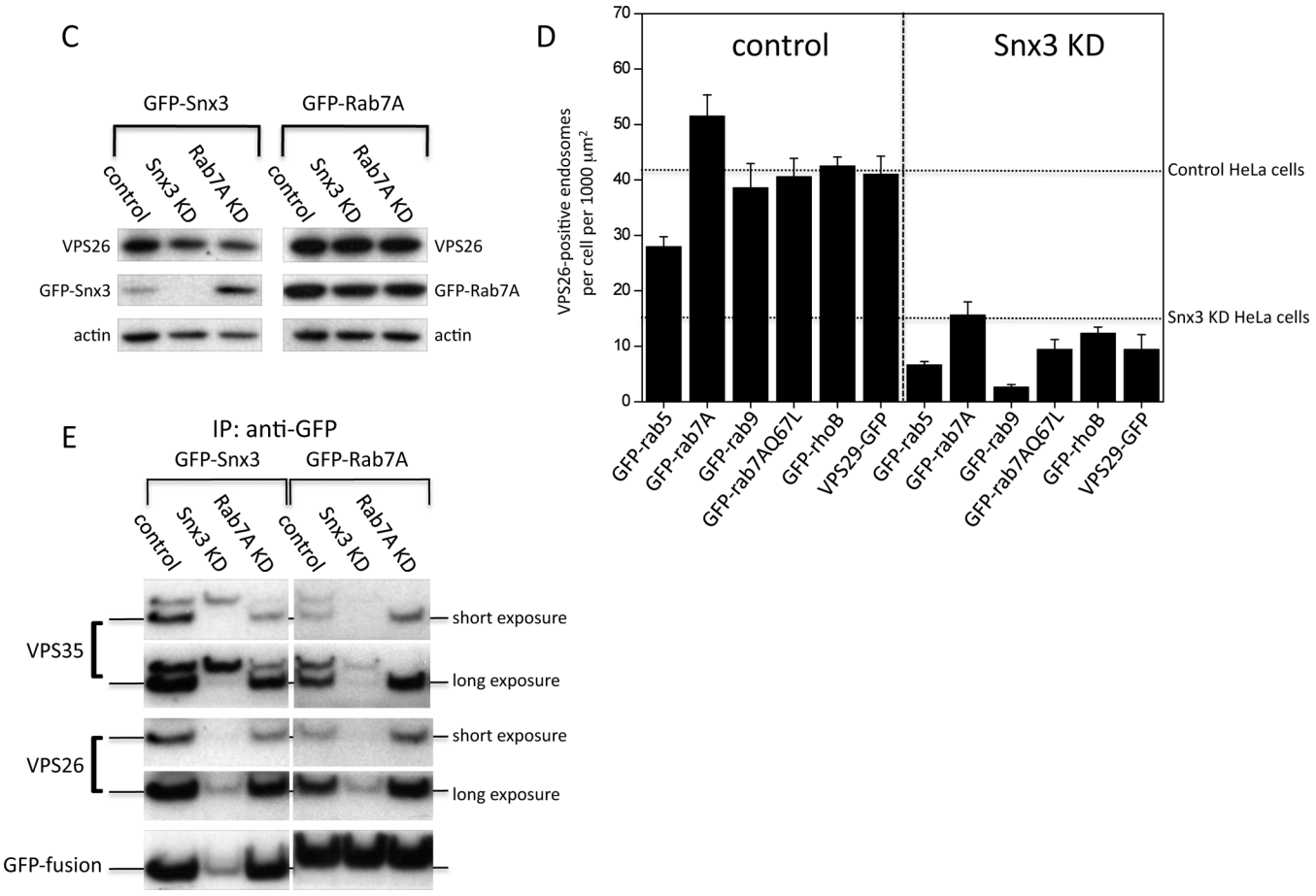


Figure 4.

Loss of Snx3 and Rab7A both affect retromer-association but through different mechanisms. **A**. HeLa cells stably expressing GFP-Snx3 were treated with siRNA to abolish Snx3 or Rab7A expression. Loss of Snx3 or Rab7A result in a similar reduction in VPS26 labeling but loss of Rab7 appears to increase the intensity of the GFP-Snx3 labeling. Bar = 20μm. **B**. HeLa cells stably expressing GFP-Rab7A were treated as in A. In this experiment, loss of Snx3 expression reduces the punctate labeling of the GFP-Rab7A and also results in a loss of VPS26 labeling. The GFP-Rab7A construct is of murine origin and is therefore resistant to the siRNA targeting human Rab7A and hence VPS26 labeling is similar to control cells. Bar = 20μm. **C**. Lysates from cells shown in A and B were subjected to SDS-PAGE and analyzed by western blotting. The Rab7A knockdown increases the GFP-Snx3 levels consistent with the increased intensity of GFP-Snx3 signal observed in A. **D**. Cells stably expressing various GFP-tagged endosomal proteins were treated with siRNA to abolish Snx3 expression. VPS26-positive endosomes were then quantified using the Cellomics ArrayScan microscope. In all cases, loss of Snx3 expression results in a marked reduction in the number of VPS26-positive endosomes indicating that increased expression of Rab7A or the constitutively active Rab7A Q67L mutant is unable to rescue the loss of Snx3. For comparison, the number of VPS26-positive endosomes determined for control and Snx3 KD cells in Figure 2C is shown by the dotted lines. **E**. Cells stably expressing GFP-Snx3 or GFP-Rab7A were treated with siRNA to KD Snx3 or Rab7A expression. Lysates were immunoprecipitated using anti-GFP and following SDS-PAGE, VPS35 and VPS26 were detected by western blotting. The KD of Snx3 abolishes the association of retromer with

GFP-Rab7A, loss of Rab7A expression reduces but does not completely abolish the interaction between GFP-Snx3 and retromer.

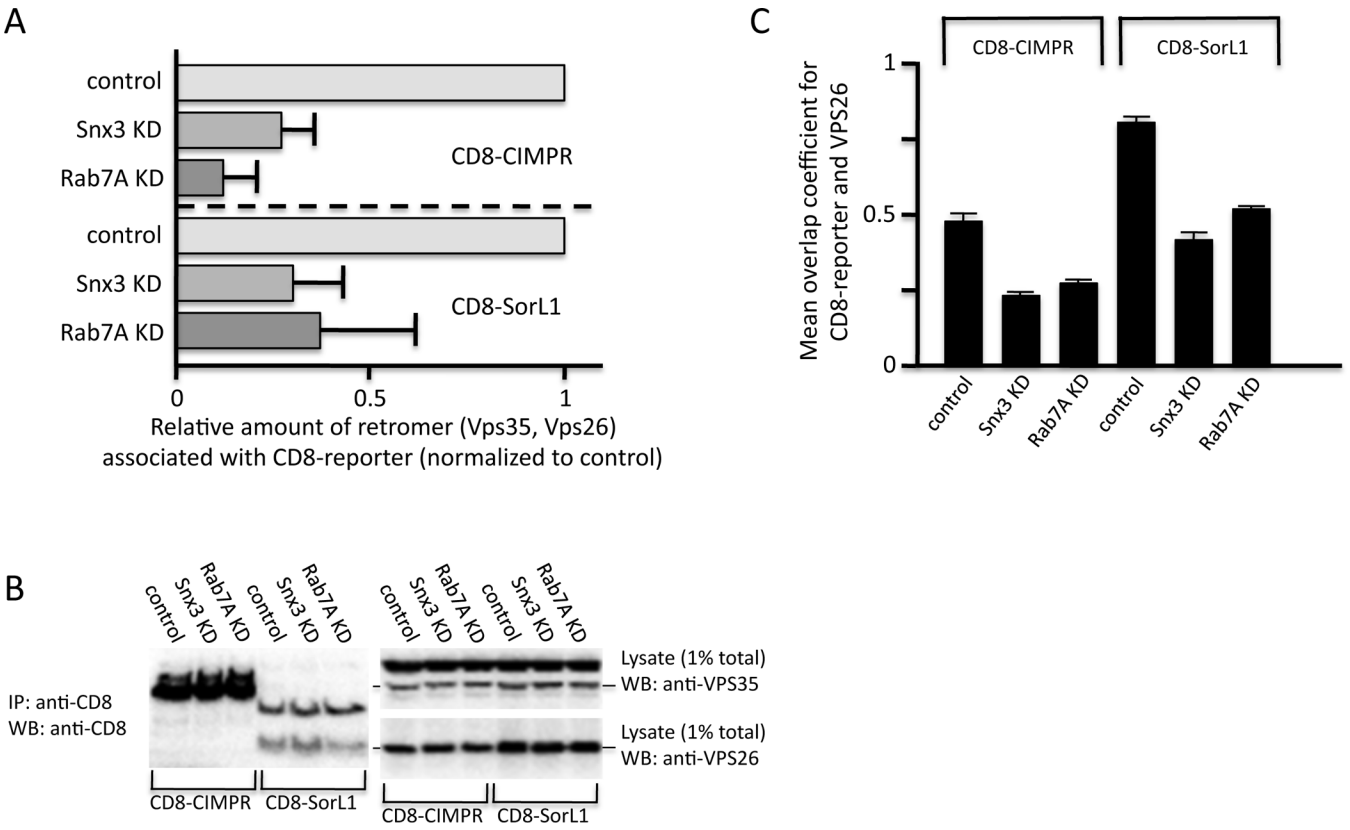


Figure 5.

Snx3 and Rab7A are required for retromer association with cargo proteins. **A**. Cells stably expressing CD8-CIMPR or CD8-SorL1 were treated with siRNA to knock down Snx3 or Rab7A expression. Following lysis, the CD8-reporter proteins were immunoprecipitated with anti-CD8 and subjected to SDS-PAGE. Vps35 and Vps26 were detected by western blotting. Signals from three independent blots were quantified and normalized to the control, errors bars are mean deviation. **B**. The level of CD8-reporter protein immunoprecipitated is not affected by the Snx3 or Rab7A KDs and neither are levels of Vps35 or Vps26 in the lysates. **C**. The intensity of the Vps26 immunofluorescence signal that is coincident with the respective CD8-reporter protein was measured using an automated microscope. 250 cells per well and at least three wells for each condition were imaged. Error bars are standard deviation. These data show that the KD of Snx3 or Rab7A markedly reduces the association of retromer with cargo proteins.

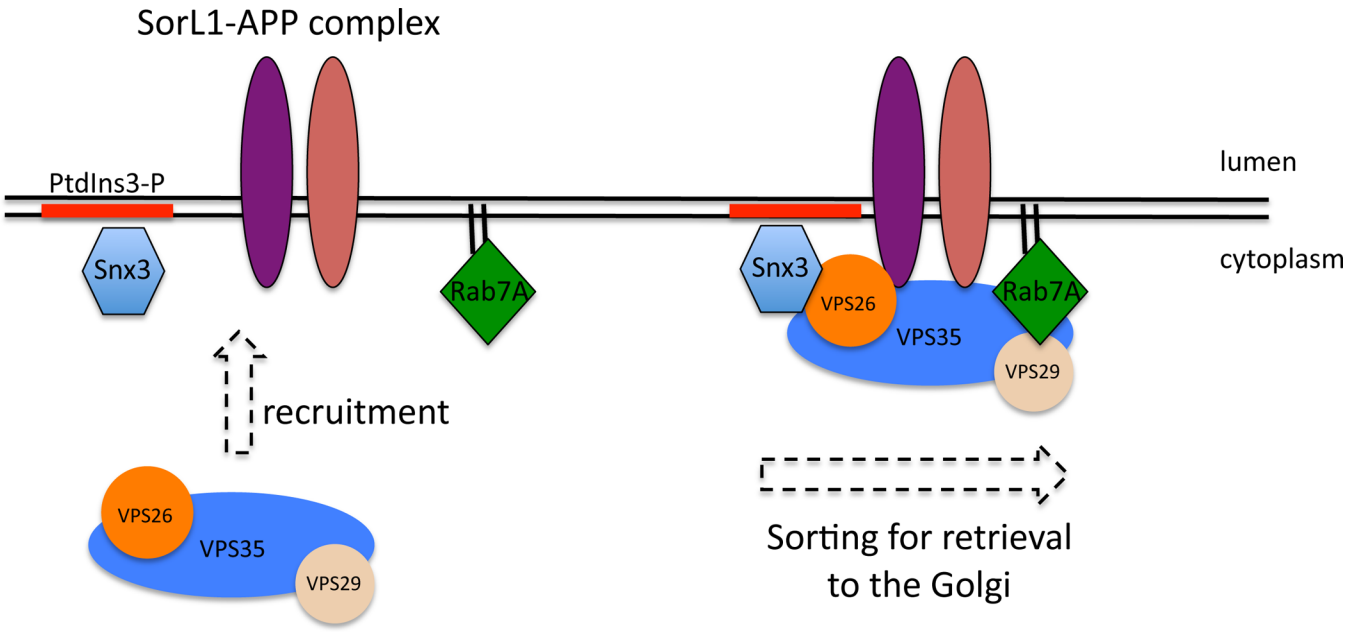


Figure 6.
Schematic diagram of the role of Snx3 and Rab7A in promoting the recruitment of the cargo-selective retromer complex to the endosomal membrane thereby facilitating the association of retromer with cargo proteins such as SorL1. This mechanism may therefore contribute to the proper trafficking of APP and regulate access of APP to the β-secretase.

Table 1

Retromer-complex and retromer-associated genes tested for genetic association with AD

RETROMER COMPLEX	RETROMER ASSOCIATED
SNX1	EHD1
SNX2	FAM21C
VPS26A	FKBP15
VPS26B	KIAA0196
VPS29	KIAA1033
VPS35	RAB7A
SNX5	SNX3
SNX6	

Table 2

Characteristics of the GWAS datasets

	Cases					Controls			
	Cases (N)	**Female (N, %)**	**Age at onset (mean ± SD)**	**Age at exam (mean ± SD)**	***APOE ε4* (allele %)**	**Controls (N)**	**Female (N, %)**	**Age at exam (mean ± SD)**	***APOE ε4* (allele %)**
ACT	566	357(63%)	83.90 (4.8)	84.72 (4.9)	0.26	1696	947 (56%)	81.08 (6.0)	0.11
ADC1	1566	850 (54%)	72.47 (7.1)	81.61 (7.0)	0.42	515	305 (59%)	75.00 (8.0)	0.16
ADC2	738	377 (51%)	73.19 (7.1)	80.06 (7.2)	0.39	160	110 (69%)	75.68 (7.9)	0.16
ADNI	268	113 (42%)	75.30 (7.2)	77.96 (6.5)	0.42	173	70 (40%)	78.6 (5.5)	0.14
GenADA	669	380 (57%)	74.59 (6.2)	80.36 (6.2)	0.38	713	456 (64%)	74.21 (7.0)	0.13
UM/VU/MSSM	1186	764 (64%)	74.06 (7.8)	77.48 (6.9)	0.36	1135	696 (61%)	74.00 (8.3)	0.12
MIRAGE	509	324 (64%)	71.16 (6.5)	75.97 (6.6)	0.36	753	440 (58%)	72.04 (7.2)	0.23
NIA-LOAD	1811	1176 (65%)	73.57 (6.7)	82.49 (7.1)	0.46	1575	947 (60%)	73.99 (8.5)	0.20
OHSU	132	81 (61%)	86.10 (5.5)	90.40 (5.2)	0.23	153	84 (55%)	83.86 (7.6)	0.08
TGEN2	864	633 (73%)	74.91 (7.2)	82.00 (7.6)	0.40	493	186 (38%)	80.19 (8.7)	0.11
TOTAL	**8309**	**5055 (61%)**	--	--	--	**7366**	**4241 (58%)**	--	--

Table 3

SNP-based and gene-based association test results

GENE	#SNPs	#SNPs SIGNIFICANT AT 0.05	#SNPs SIGNIFICANT AFTER MULT TESTING	BEST P-VALUE	MULTIPLE TESTING THRESHOLD	PARIS P-VALUE
EHD1	18	3	0	0.033	0.010	0.13
FAM21C	2	0	0	0.31	0.025	0.33
FKBP15	95	0	0	0.11	0.0033	0.44
KIAA0196	40	3	0	0.044	0.0056	0.18
KIAA1033	56	27	0	0.0080	0.0045	0.025
RAB7A	32	17	0	0.0058	0.0050	0.018
SNX1	17	16	0	0.035	0.017	0.035
SNX2	43	0	0	0.15	0.0056	0.56
SNX3	28	13	2	0.0057	0.0071	0.059
SNX5	40	0	0	0.18	0.0071	0.32
SNX6	24	0	0	0.14	0.0071	0.44
VPS26A	28	0	0	0.066	0.0062	0.21
VPS26B	12	1	0	0.034	0.0062	0.25
VPS29	3	0	0	0.26	0.017	0.59
VPS35	4	0	0	0.82	0.025	0.90

suggestive evidence

significant at p<0.05

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