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Figure 1

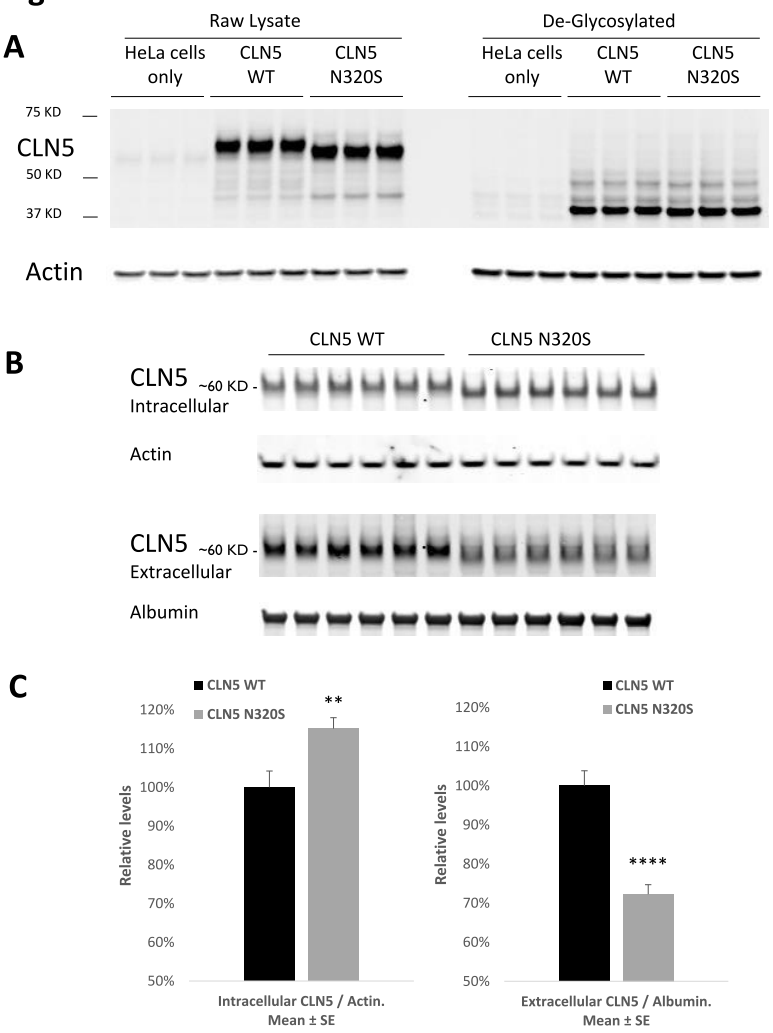


Figure 2

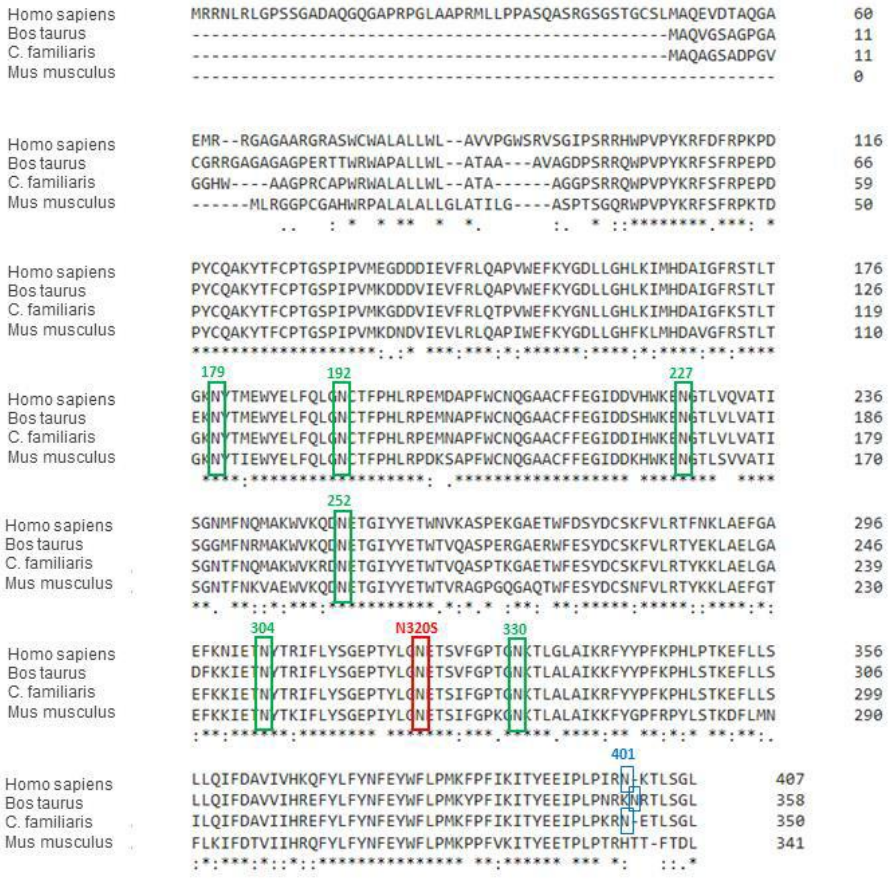


Figure 3

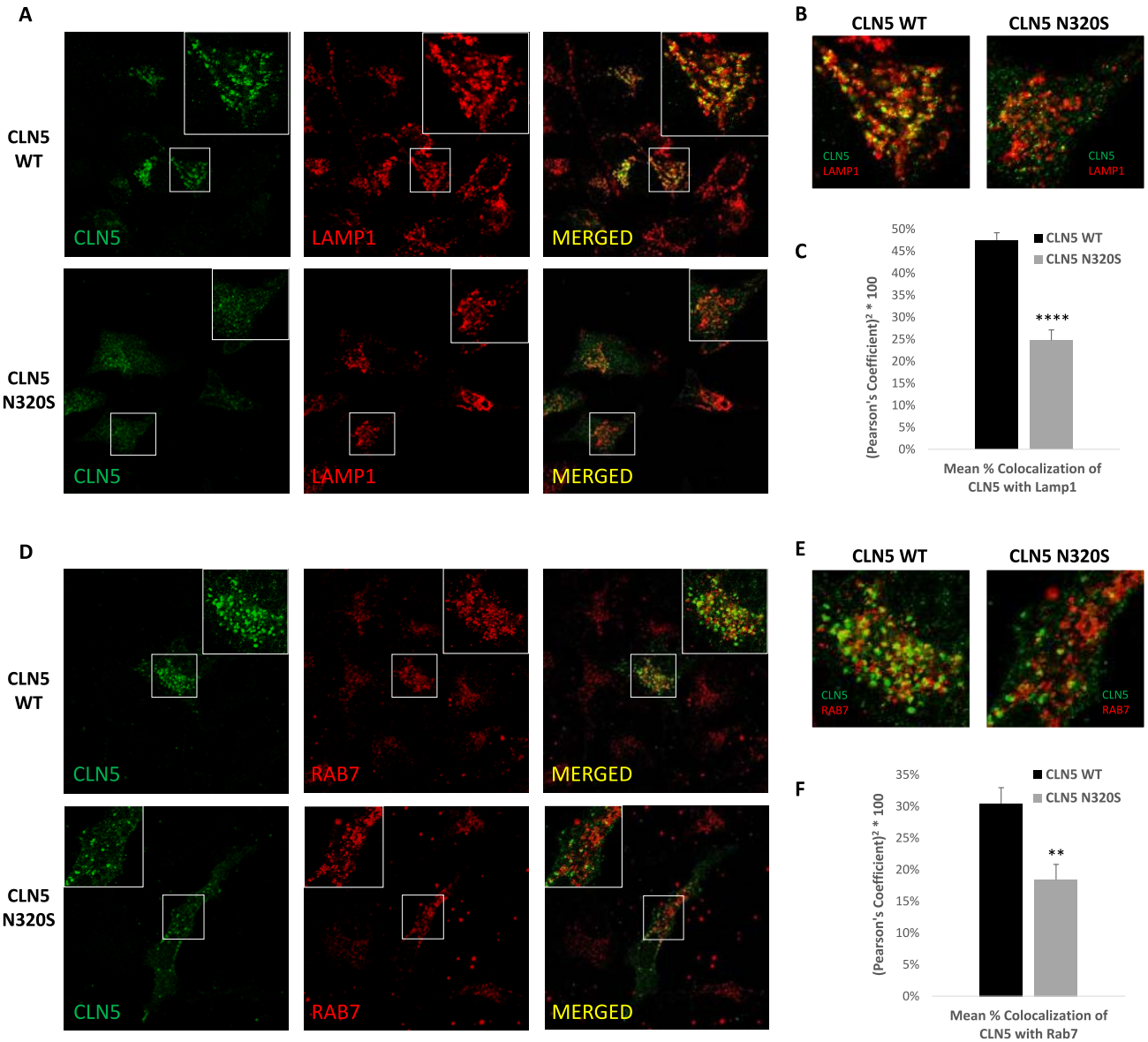


Figure 4

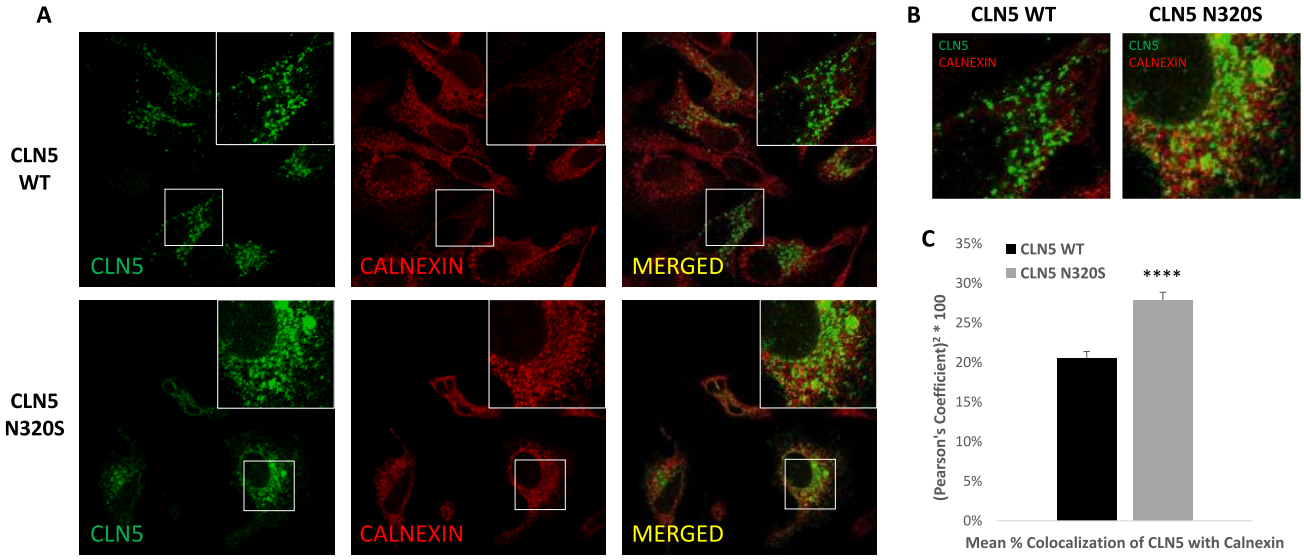


Figure 5

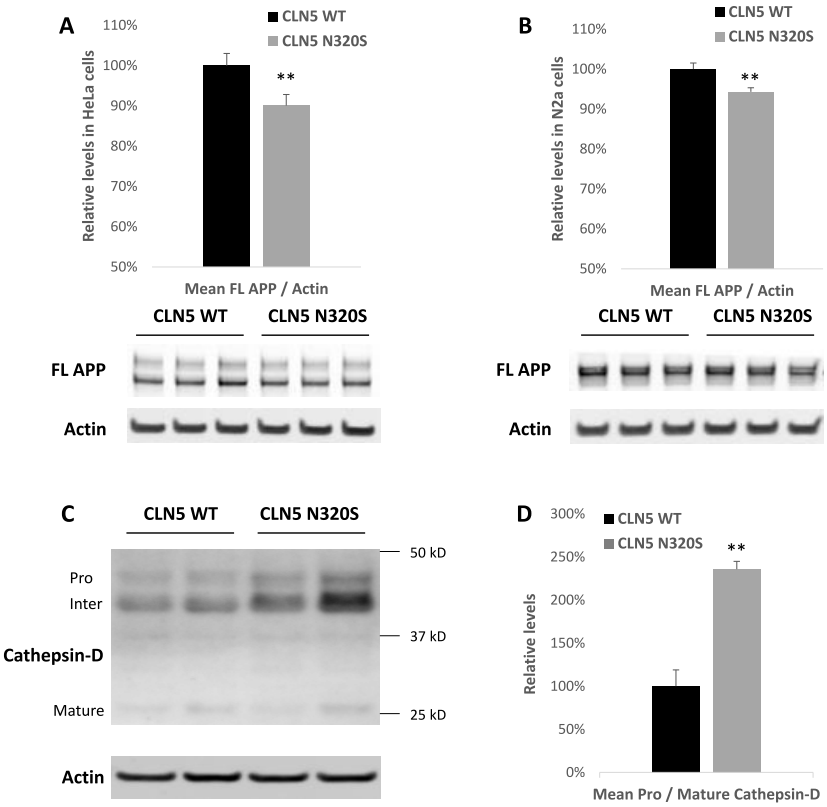
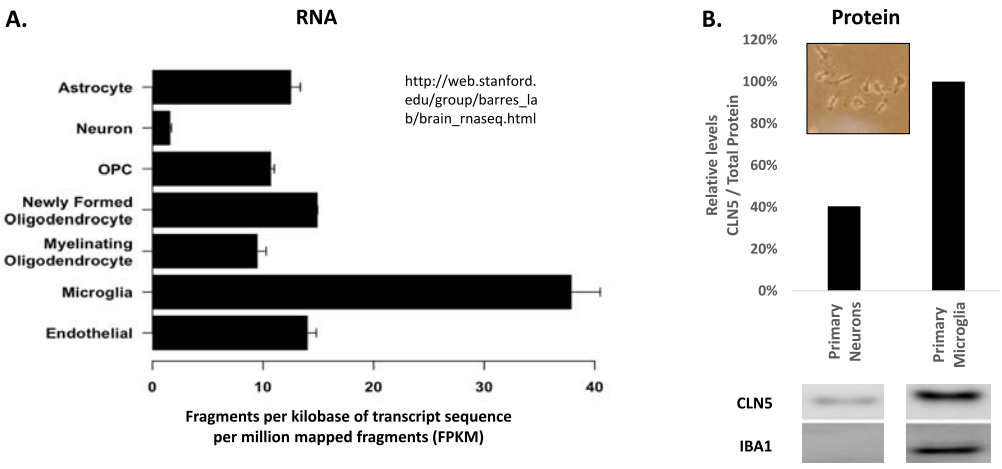


Figure 6



An Alzheimer's linked loss-of-function CLN5 variant impairs Cathepsin D maturation consistent with a retromer trafficking defect

Running head: A *CLN5* variant in Alzheimer's disease

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ABSTRACT

In a whole exome sequencing study of multiplex Alzheimer's disease (AD) families we investigated three neuronal ceroid lipofuscinosis genes that have been linked to retromer, an intracellular trafficking pathway associated with AD-- Ceroid lipofuscinosis 3 (CLN3), Ceroid lipofuscinosis 5 (CLN5) and cathepsin D (CTSD). We identified a missense variant in CLN5 c.A959G (p.Asn320Ser) that segregated with AD. We find that this variant causes glycosylation defects in the expressed protein, which causes it to be retained in the endoplasmic reticulum with reduced delivery to the endolysosomal compartment, CLN5's normal cellular location. The AD-associated CLN5 variant is shown here to reduce the normal processing of Cathepsin D and to decrease levels of full-length APP, suggestive of a defect in retromer-dependent trafficking.

INTRODUCTION

Lysosomal storage diseases (LSDs) are a group of inherited disorders that typically cause neurodegeneration early in life(1). Recent observations have established that genetic variants that cause one type of LSD, Gaucher's disease, can act as a risk factor for developing a late-onset neurodegenerative disease, Parkinson's disease(2). This observation suggests that genes that cause other LSDs might act as risk factors for other late-onset neurodegenerative disorders, notably Alzheimer's disease (AD). While over the past decade over 25 genes increasing risk of AD have been identified, a large part of the genetic contribution to AD remains to be clarified (3).

With this question in mind, we focused on a select group of genes that cause another LSD, Neuronal ceroid lipofuscinosis (NCL), because they have been directly or indirectly associated with retromer trafficking(4, 5). Retromer is a multi-modular protein assembly that has been linked to the pathogenesis of late-onset AD(6, 7), and is now considered the 'master conductor' of endosomal sorting and trafficking(8). Among the group of NCL genes we focused on these three: Ceroid Lipofuscinosis 3 (*CLN3*) whose expressed protein functions in trafficking the mannose-6-phosphate receptor (M6PR), a key cargo of retromer(9); *CLN5* whose expressed protein is located at endosomal membranes and has been shown to function in the recruitment of retromer to endosomal membranes(4); and CTSD, whose expressed protein, Cathepsin D, requires the normal retromer-dependent trafficking of M6PR to deliver pro-Cathepsin D to the endosome, during which it is processed to its mature form Cathepsin D.

To explore whether genetic variation in any of these three genes increase risk of AD, we capitalized on data from a whole exome sequencing study of multiplex Alzheimer's disease (AD) families. To validate variant(s) identified in these analyses, we then turned to cell culture to determine whether they have deleterious effects on normal

function. Finally, because all three genes converge on Cathepsin D, we tested whether the abnormal function caused by identified variant(s) would affect the normal processing of this protein.

MATERIALS AND METHODS

DNA isolation and sequencing. High molecular weight DNA was isolated from either fresh or frozen blood stored at -80^{o}C using the Gentra Puregene and FlexiGene kits (Qiagen). When high quality DNA from blood was unavailable (13 probands), DNA was isolated from lymphocyte cell lines. TruSeq DNA Preparation and Exome Enrichment kits (Illumina, San Diego, CA) were used to prepare indexed genomic DNA (gDNA) libraries and isolate exonic regions for high throughput sequencing. Multiplexed DNA samples were sequenced in batches of up to 12 samples on Illumina's Genome Analyzer IIx, HiSeq 2000, and MiSeq platforms (http://www.illumina.com). Paired-end reads were performed over 82-307 sequencing cycles, yielding high coverage at an average depth of >60x per sample and interval region captured.

Downstream bioinformatics analysis of sequence data. Using the Burrows Wheeler Aligner (10) the reads obtained from the pooled sequencing were aligned to the human reference genome build 37 (http://bio-bwa.sourceforge.net/). Quality control of the sequencing data was done using established pipelines, including base alignment quality calibration and refinement of local alignment around putative indels using the Genome Analysis Toolkit (GATK)(11). Variants were called and recalibrated using multi-sample calling with GATK's UnifiedGenotyper and VariantRecalibrator modules. Reliably called variants were annotated by ANNOVAR(12) including *in-silico* functional prediction using POLYPHEN(13) and extent of cross-species conservation using PHYLOP(14).

Statistical analyses of sequence data. We tested segregation and AD association of individual single nucleotide variants (SNVs) in the sequenced samples. To validate SNVs prioritized from these analyses, we genotyped them in all family members of the families in which they were discovered, and a set of 438 unrelated, unaffected population controls of similar ancestry (68.1% women, mean age at examination = 82.0 ± 7.3, *APOE* ε4 allele frequency = 9.8%) using the Sequenom MassARRAY platform. We compared their allele frequencies in affected individuals with unaffected samples from this follow-up genotyping using Fisher's exact test. The 438 unaffected, unrelated controls were determined to be of the same ethnic background as the familial cases using methods described previously(15). We also compared the allele frequencies in affected individuals with the publicly available ExAC data. Because of the lack of an optimal ethnically matched control data set for Caribbean Hispanics, we used the ExAC Latino cohort for an estimate of the population allele frequencies of identified variants.

Design and preparation of the CLN5 constructs. Plasmids expressing the WT and c.A959G (N320S) CLN5 proteins (human) tagged with flag at c-terminal were designed using Thermofisher's GeneArt portal. A flexible poly-glycine linker (6xG) was inserted between the protein and the tag. mRNA sequence for CLN5 was acquired from the National Center for Biotechnology Information (NCBI). The constructs were then subcloned into pcDNA 3.1 (+) Hygro vectors with CMV promotor.

Cell culture. HeLa cells were cultured using DMEM + 10% FBS and glutamax, with penicillin, streptomycin and amphotericin B to prevent microbial contamination. Mouse neuroblastoma (N2a) cells were cultured in 50% DMEM (high glucose) & 50% Opti-MEM + 10% FBS and Glutamine (2mM) with penicillin and streptomycin to prevent microbial contamination. Primary mouse cortical neuron cultures were performed as described previously (16). Primary microglia were cultured as described previously with slight modifications (17). Briefly brain homogenate from day 1 pups were plated onto poly d

ornithine coated flasks in DMEM/F12 + 10% FBS and glutamax containing macrophage colony stimulating factor (mCSF). At day 7 microglia were dislodged from the bottom of the flask by tapping the flask gently. Careful tapping results in floating of microglia prior to other cells / debris. Medium containing microglia was passed through cell strainer to remove remaining debris. Suspension was spun down at 500g and the pellet was re-suspended in fresh medium and plated in 6 well plates and allowed to grow and stabilize for 7 days prior to harvesting.

Transfection of cells with CLN5 WT and N320S variant and biochemistry. WT and N320S CLN5 expressing plasmids were transfected into HeLa and N2a cells using lipofectamine. Cells were harvested 24 hours after transfection, culture medium was also collected at the same time. Lysates from the samples were run on NuPAGE® Bis-Tris 4-12% gels, transferred onto nitrocellulose membranes using iblot and were probed with antibodies against CLN5 (Abcam ab170899 1:500), actin (Novus NB600-535 1:2000), albumin (abcam ab3781 1:2000), Iba1 (Novus NB100-1028 1:500) Amyloid Precursor Protein (Abcam ab32136 1:10,000) and cathepsin D (Abcam ab75852 1:500). For the deglycosylation experiments Endo H kit from New England Biolabs (P0702L) was used with the recommended protocol (https://www.neb.com/protocols/2012/10/18/endo-hf-protocol), briefly lysates were denatured in glycoprotein denaturing buffer at 100°C for 10 min, then incubated in glycobuffer3 and Endo H at 37°C for 1 hour. To generate stable cell lines some wells of the HeLa cells expressing the CLN5 WT and N320S variant plasmids were selected with hygromycin B for ~14 days. The surviving stably transfected cells were plated in 6 well plates, and were harvested after 48 hours for biochemistry.

Immunocytochemistry. HeLa cells were transfected with CLN5 plasmids in a 24 well plate (with coverslips) using lipofectamine 2000. Twenty-four hours after transfection cells were fixed using 4% paraformaldehyde for 10 min and permeabilized using digitonin (0.01%) for 10 min. To block nonspecific staining cells were incubated in 5% donkey

serum overnight. Cells were probed for CLN5 (Abcam ab170899 1:250, Sigma SAB1412697 1:200), LAMP1 (R&D AF4800 1:500), Rab7 (SC-376362 1:50), and calnexin (Genescript A01240 1:500) primary antibodies prepared in 1% donkey serum. Secondary antibodies (Life technologies) conjugated with alexa fluor dyes were used. Images were taken using Zeiss LSM 700 META confocal microscope equipped with a 63× Plan-Apochromat objective andHeNE1, HeNe2 and argon lasers. Colocalization analysis was performed using ImageJ's JACoB plugin.

RESULTS

Whole exome sequencing of multiplex AD families

Whole exome sequencing was performed in 31 Caribbean Hispanic families (98 affected and 12 unaffected relatives) from the Estudio Familiar de Influencia Genetica en Alzheimer (EFIGA). Families selected for sequencing had at least four affected individuals meeting NINCDS-ADRDA(18) standard criteria for AD and were free of known mutations in *APP*, *PSEN1*, *PSEN2*, *GRN* and *MAPT*. Analysis of the sequence data of the *CLN5* gene identified a rare missense variant (rs199609750; c.A959G, population frequency: 7.418e-05) that segregated with AD status in one multiplex family, present in two affected but no unaffected individuals (frequency in affected: 2.0%, frequency in unaffected: 0%). This variant was also significantly associated with AD when genotyped in all family members and compared to 438 internally genotyped controls of similar ancestry in which the mutant allele was entirely absent ($p<0.0001$), or when compared to population Latino controls in the ExaC database (allele frequency=0.0006; $p<0.0001$). No variants in *CLN3* or *CTSD* were segregating with AD status in these families.

Altered post-translational processing in the CLN5 c.A959G (N320S) variant

To validate possible biological effects of the CLN5 c.A959G (N320S, rs199609750) AD variant, we began by expressing WT and c.A959G (N320S) CLN5 in HeLa cells. The WT

CLN5 displayed an expected molecular weight of 60kD, however we observed a slight shift of ~2.5kD in the c.A959G (N320S) variant (**Fig. 1A**). This shift indicates the possibility of an aberrant post-translational modification, as c.A959G is a missense mutation and should not result in any form of premature truncation of the protein.

CLN5 has 8 asparagine (N) glycosylation sites and all of these sites are glycosylated in WT human CLN5(19). The c.A959G missense mutation results in the replacement of the amino acid at position 320, an asparagine with serine (N320S) where asparagine 320 is one of the glycosylation sites (**Fig. 2**). Moharir et al. (2013) also indicated that lack of N-glycosylation on certain sites in CLN5, including N320, impairs CLN5 trafficking and function(19). Based on site-directed mutagenesis of individual asparagine residues to glutamine on each of the N-glycosylation consensus sites followed by colocalization studies, they categorized the mutants into three groups: 1. Folding of the protein without which CLN5 is retained in the ER (N179Q, N252Q, N304Q, N320Q), 2. glycolysation involved in endosome/lysosome trafficking without which CLN5 is accumulated in the Golgi (N401Q) and 3.) glycolysation involved in lysosomal function (N192Q, N227Q)), suggesting that there are functional differences in various N-glycosylation sites of CLN5 which differentially affect folding, trafficking, and lysosomal function of CLN5. We hypothesized therefore that this novel amino acid substitution (N320S) should also interfere with N-glycosylation at this particular site. To confirm this glycosylation deficit, sugar moieties were stripped away from these proteins before subjecting them to gel electrophoresis. After the deglycosylation reaction both proteins, the WT and the N320S mutant, resolved at a location close to 37kD and the difference in molecular weights between them disappeared. We also noticed a 15% increase in the levels of intracellular CLN5 N320S variant (**Fig. 1B,C**). This increase in levels can be explained by the glycosylation defect, as it can lead to abnormal folding of CLN5 resulting

in endoplasmic reticulum (ER) entrapment (19, 20). If so, and since CLN5 can be secreted into the extracellular space (19, 21) , ER retention of the CLN5 N320S variant should be associated with its diminished secretion. Accordingly, we analyzed CLN5 levels in cell culture medium, and confirming the hypothesis we find a significant decrease in the CLN5 N320S variant released to the media compared to WT CLN5 (**Fig. 1C)**. These experiments establish that the novel AD-linked CLN5 variant is aberrantly glycosylated and provide biochemical evidence that suggests that the variant might be trapped in the ER.

Impaired cellular localization and retromer-mediated trafficking in the CLN5 N320S variant

We then turned to confocal microscopy to further confirm this interpretation by comparing the intracellular localization of WT CLN5 to the AD-associated N320S variant. WT CLN5 is proteolytically cleaved and glycosylated prior to its transport to the endosomal-lysosomal compartments(21-23). Confirming the hypothesis, compared to WT CLN5, the AD-associated N320S variant showed increased co-localization with ER markers, but decreased co-localization with markers of the endosomes and lysosomes (**Fig. 3 and 4; Supplemental Material**).

Upon further biochemical analysis of HeLa cells, transiently expressing the WT and the N320S CLN5, we observed a small but consistent decrease in intracellular full-length APP (~10% decrease, p=0.01), when N320S CLN5 was expressed. To confirm this finding we repeated these experiments in mouse N2a cells and found a similar decrease in full-length APP in these neuroblastoma cells (**Fig. 5A-B**).

The glycosylation deficiency and ER retention of CLN5 N320S variant suggests that it is a loss-of-function mutation. One function assigned to CLN5 is its role in retromer trafficking—a pathway firmly linked to AD etiology by animal, cell biology and genetic

studies—based on a study that found that CLN5 depleted cells show evidence of retromer dysfunction(4). The study relied on previous observations establishing that a reliable cellular readout of retromer dysfunction is transport defects of Cathepsin D to the endosomal-lysosomal compartments, a secondary consequence of the dependence of retromer for mannose-6-phosphate receptor recycling. During the transport to the endosomal-lysosomal compartments pro-cathepsin D is processed into its mature form(24), and retromer dysfunction is therefore associated with a relative increase in pro-cathepsin D compared to mature cathepsin D(25). We relied on this cellular readout to determine whether the AD-associated CLN5 N320S mutant causes a partial loss of CLN5 function. We measured pro-cathepsin D and mature cathepsin D in HeLa cell lines stably expressing WT and N320S CLN5 constructs. Compared to cell lines expressing WT CLN5, we observed a significant increase in pro-cathepsin D levels in the cell lines expressing CLN5 N320S mutant (**Fig. 5C-D**).

DISCUSSION

Numerous studies focusing on Neuronal Ceroid Lipofuscinosis (NCL) have identified many NCL related mutations(26). Our findings identify, we believe for the first time, a variant in one of these genes that is linked to late-onset AD. While CLN5's function is not fully understood, this transmembrane protein is normally located at endosomal membranes(19), and one study suggested that it plays a role in retromer trafficking(4). Retromer is a multi-modular protein assembly that is now considered a 'master conductor'(8) of endosomal sorting and trafficking. Each retromer module is made up of a group of proteins that serves a dedicated role, and the modules work together in support of retromer trafficking function(6). One key module is the 'membrane-recruiting' module, which functions in recruiting retromer's 'cargo recognition' module to the membrane of endosomes. The complete list of proteins that are part of the membrane-recruiting module remains unknown, but a previous study has provided strong evidence that CLN5,

which normally resides in the endosome, functions in this module(4). This observation prompted us to include *CLN5* in our genetic analysis.

In a whole exome sequencing study we identified the missense N320S (p.Asn320Ser) variant in *CLN5* to segregate with AD status in families multiply affected by the disease. This variant was also significantly associated with AD when genotyped in all family members and compared to 438 internally genotyped controls of similar ancestry in which the mutant allele was entirely absent, or when compared to population Latino controls in the ExaC database. Using a combination of molecular biology, biochemistry, and immunofluorescence experiments we further validated this variant, functionally demonstrating that it is glycosylation deficient, which causes the expressed protein to be partially trapped in the ER and reduces its normal delivery to the endolysosomal system. Guided by a previous study(4) that showed that CLN5 deficiency affects retromer's function, we demonstrate that an effective deficiency in endosomal CLN5 caused by the missense variant results in a shift in the relative levels of pro-cathepsin D, an established phenotype of retromer dysfunction (24, 25, 27, 28), and a reduction in full-length APP. Genomic and cell biological findings have linked retromer dysfunction to AD pathogenesis(6). More than simply identifying a CLN5 variant genetically linked to AD and validating that it causes a loss-of-function, our results suggest that it converges onto established pathophysiological mechanisms of disease.

We postulate that the identified N320S missense mutant might mediate its AD-associated toxicity by affecting retromer function in microglia. CLN5 is highly expressed in the brain and within the brain CLN5 is heavily enriched in microglia (**Fig. 6**) (29, 30), a cell type linked to AD. Microglia are activated upon tissue damage and are critical for brain homeostasis through clearance of cellular debris. The identification of CLN5 as a microglial gene associated with AD is in line with the implication of the microglial gene TREM2 (encoding a phagocytic receptor) as an AD susceptibility gene(31). Notably,

retromer deficiency has been found in microglia of AD brains(32), but the mechanisms underlying this deficiency are still unclear. Thus, besides providing additional evidence for the role and molecular mechanisms of retromer dysfunction in AD, and while the missense N320S (p.Asn320Ser) variant should be further validated in additional independent AD datasets and the CLN5 gene should be further scrutinized in various ethnic groups for additional potential disease-associated variants, our findings provide critical support for the link between retromer, microglia and AD. Future studies relying on genetically engineered mice expressing the CLN5 mutation are required to better establish the functional consequence of this mutation on the brain and its contribution to various AD-related pathologies, including the potential effect on APP processing suggested by this study.

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FIGURE LEGENDS

Fig 1. Glycosylation deficits in AD variant CLN5 N320S. CLN5 WT and N320S AD variant plasmids were transfected into HeLa cells. After 24 hours the cells were lysed and probed for CLN5. (A) CLN5 migrates to ~60 KD with a difference of approximately 2.5 KD between WT and AD variants, however, when sugar moieties were removed using the enzyme endoglycosidase H, CLN5 resolved to a lower location on the gel at ~37 KD and the molecular weight difference disappeared. (B) The relative level of CLN5 inside the cell was compared to its level in the medium after 24 hours of transfection. WT CLN5 band was observed at ~60KD in both intra & extracellular compartments. (C) Quantification showed a significant increase and decrease in intracellular and extracellular CLN5(N320S) variant respectively. Here and in figures below, asterisks denote * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, and **** = $p<0.0001$.

Fig 2. Sequence alignment of CLN5 (performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/)). The red box indicates the N-glycosylation site corresponding to human N320 which is conserved among different species and at which the identified variant rs199609750 (c.A959G) exerts its effect. The green boxes indicate the six additional N-glycosylation sites conserved among different species. The blue boxes indicate the N-glycosylation site corresponding to human N401, which is not conserved in rodents. Sequences used in this alignment: H. sapiens (NP_006484.1), Bos Taurus (ABD83352.1), Canis lupus familiaris (NP_001011556.1), Mus musculus (NP_001028414.1).

Fig 3. The AD variant CLN5 N320S is reduced in the endolysosomal system. HeLa cells grown on coverslips were transfected with CLN5 WT and N320S variant plasmids. Cells were fixed with PFA, and stained for CLN5 and LAMP1. (A and B) Co-localization of WT and N320S CLN5 with LAMP1. WT CLN5 staining is more punctate compared to N320S and has a higher colocalization with the lysosomal marker LAMP1. (C) Analysis from 3 independent immunofluorescence experiments showing reduced co-localization of N320S CLN5 with LAMP1 (total no of cells analyzed = 75). (D, E and F) CLN5 co-localization with late endosomal marker RAB7 also followed a pattern similar to LAMP1 colocalization (total no of cells analyzed = 27). For each experiment, the image/cell showing the Pearson correlation value closest to the mean was selected as representative image. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, and **** = $p<0.0001$.

Fig 4. Impaired intracellular trafficking in CLN5 N320S cells. (A and B) Co-localization of WT and N320S CLN5 with ER marker calnexin. (C) Analysis from 3 independent immunofluorescence experiments showing increased co-localization of N320S CLN5 with ER. (total no of cells analyzed = 189). The image/cell showing the Pearson correlation value closest to the mean was selected as representative image. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, and **** = $p<0.0001$.

Fig. 5. Reduction of full-length APP and effect on Cathepsin D processing. HeLa and N2a cells were transfected with CLN5 WT and N320S variant plasmids. After 24 hours, the cells were lysed and probed for full-length APP. (A & B) Full-length APP levels were significantly reduced in CLN5 N320S expressing HeLa and N2a cells. (C) Some wells of the HeLa cells expressing the CLN5 WT and N320S variant plasmids were selected with hygromycin to generate stable cell lines. Stable cell lines were plated in 6 well plates, and after 48 hours the cells were lysed and probed for cathepsin D. (D) Quantification revealed a significant increase in the ratio of pro versus mature cathepsin D. ** = $p<0.01$.

Fig 6. CLN5 Expression in different brain cells. (A) CLN5 RNA expression measured in Fragments per kilobase of transcript sequence per million mapped fragments (FPKM), reproduced from Ben Barres / Jia Wu database (30) (B) Lysates from primary mouse neurons and primary mouse microglia were probed with anti-CLN5 antibody and anti-IBA1 antibody. Protein levels were normalized to total protein by ponceau stain. (B, inset) Primary microglia at day 7 of culture.