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C9orf72 Intermediate Repeats are Associated with Corticobasal Degeneration, Increased *C9orf72* Expression and Disruption of Autophagy

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Author Contributions

E.B.L. and S.-C.L conceived and designed this study. C.P.C performed RNA and protein expression experiments in patient brain and iPSCs. M.P. performed repeat size and risk allele genotyping. Y.K.T., W.Y.H. and S.-C.L performed C9orf72 overexpression autophagy experiments. F.H., W.T., C.M.M., W.W.S., B.L.M., C.G., J.P.G.V., C.L.W., F.M.B., S.R., H.K., J.C.T., C.T., M.D., B.G., V.M.V.D., V.M.Y.L., J.Q.T., K.Y.M., H.L., D.W.D, and G.D.S. performed neuropathology analysis and provided DNA samples. C.P.C and E.B.L wrote the manuscript and all authors approved the final manuscript.

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Abstract

Microsatellite repeat expansion disease loci can exhibit pleiotropic clinical and biological effects depending on repeat length. Large expansions in *C9orf72* (100s-1000s of units) are the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal degeneration (FTD). However, whether intermediate expansions also contribute to neurodegenerative disease is not well understood. Several studies have identified intermediate repeats in Parkinson's disease patients, but the association was not found in autopsy confirmed cases. We hypothesized that intermediate *C9orf72* repeats are a genetic risk factor for corticobasal degeneration (CBD), a neurodegenerative disease that can be clinically similar to Parkinson's but has distinct tau protein pathology. Indeed, intermediate *C9orf72* repeats were significantly enriched in autopsy-proven CBD (n=354 cases, odds ratio=3.59, p-value=0.00024). While large *C9orf72* repeat expansions are known to decrease *C9orf72* expression, intermediate *C9orf72* repeats result in increased *C9orf72* expression in human brain tissue and CRISPR/cas9 knockin iPSC derived neural progenitor cells. In contrast to cases of FTD/ALS with large *C9orf72* expansions, CBD with intermediate *C9orf72* repeats was not associated with pathologic RNA foci or dipeptide repeat protein aggregates. Knock-in cells with intermediate repeats exhibit numerous changes in gene expression pathways relating to vesicle trafficking and autophagy. Additionally, overexpression of *C9orf72* without the repeat expansion leads to defects in autophagy under nutrient starvation conditions. These results raise the possibility that therapeutic strategies to reduce *C9orf72* expression may be beneficial for the treatment of CBD.

Keywords

Neurodegeneration; Corticobasal degeneration; C9orf72 repeat expansion; Parkinsonism; Autophagy

Introduction

Large hexanucleotide repeat expansions (G_4C_2) in *C9orf72* are the most common genetic cause of the neurodegenerative diseases amyotrophic lateral sclerosis (ALS) and frontotemporal degeneration (FTD)[19, 49, 60]. ALS/FTD is characterized neuropathologically by the presence of TDP-43 inclusions which are tightly linked to neurodegeneration[35]. While the pathophysiologic mechanisms that lead to TDP-43 aggregation and neurodegeneration in the setting of large *C9orf72* repeat expansions are still unclear, both a loss of normal *C9orf72* function and a toxic gain of function attributed to repetitive RNA and protein aggregates have been proposed[19, 36, 60, 63, 67]. At the protein level, C9orf72 regulates several vesicle trafficking pathways [4, 26, 79] and is necessary for proper myeloid cell function in mice, but knockout of the protein does not cause neurodegeneration[12, 58]. On the other hand, the presence of the repeat expansion is sufficient to induce neurodegeneration in several mouse models[15, 42]. There is also uncertainty in the repeat size threshold necessary to cause disease. Repeat expansions that cause ALS/FTD are typically hundreds to thousands of units long and can differ substantially between tissues[10, 70], making it difficult to accurately determine the pathogenic repeat size threshold. There have been reports of pathogenic repeats as small as 55–100 units in patient blood[23, 28]. In contrast, non-diseased individuals typically harbor repeats of 2–8 units[20]. Several studies have explored whether intermediate *C9orf72* expansions (e.g. those larger than controls but not large enough to cause ALS/FTD) are associated with risk for other neurodegenerative diseases, and these have often yielded conflicting results[1, 20, 30, 32, 37, 56, 80, 84, 86]. For instance, a significant association between intermediate expansion carriers and Parkinson's Disease (PD) was reported in clinically diagnosed PD[56] and confirmed in a large meta-analysis[73], but these associations were not found in an autopsy confirmed PD cohort[57]. Other studies with very small numbers of cases have also suggested that intermediate *C9orf72* repeats are associated with atypical Parkinsonian syndromes[13, 64]. Thus, there is some evidence that intermediate expansions in *C9orf72* may confer risk for neurodegenerative diseases other than ALS/FTD, but more exploration is needed to better understand the molecular pathways and exact neurodegenerative diseases that are affected.

Here, we sought to determine whether intermediate expansions in *C9orf72* are a risk factor for corticobasal degeneration (CBD), a rare neurodegenerative disease that shares some similar clinical features with PD such as limb rigidity, bradykinesia, tremor and apraxia[5]. Patients that present with these neurologic features are often diagnosed with corticobasal syndrome (CBS) because these symptoms can also be caused by several other neurodegenerative diseases; definitive diagnosis of CBD requires neuropathology autopsy examination[33]. CBD is characterized by hyper-phosphorylated tau aggregates in neurons and astrocytes that are morphologically distinct from those found in Alzheimer's disease and

other tauopathies[21]. Only ~25–50% of patients diagnosed with CBS are confirmed as CBD at autopsy[11, 39, 40], making clinically defined cohorts difficult to use for genetic studies of CBD due to underlying neuropathological heterogeneity. In this study, we determine whether *C9orf72* intermediate expansions may be a risk factor for CBD by measuring repeat size in the largest cohort of autopsy confirmed cases ever tested. In addition, we use post mortem CBD brain tissue to confirm the genetic findings, use CRISPR/cas9 to modify the repeat number in human iPS cells at the endogenous locus, and determine the effects of *C9orf72* expression on autophagy. In doing so, we identify a novel mechanism for how intermediate repeat expansions may affect CBD pathogenesis that is distinct from large expansions that cause ALS/FTD.

Materials and Methods

Case Samples

Genomic DNA for repeat expansion size screening was obtained from 354 autopsy confirmed CBD cases from 17 institutions. Institutions contributing the vast majority of cases included Mayo Clinc, University College London and the University of Pennsylvania. CBD patient cerebellum tissue for RNA expression studies was obtained from the Center for Neurodegenerative Disease Research brain bank at the University of Pennsylvania and from the Mayo Clinic brain bank. Brain autopsy material is not considered human subjects research. However, legal consent for autopsy was obtained in all instances. As a control cohort, we utilized a previously published global cohort of healthy controls for which *C9orf72* repeat sizing was available[73].

Assessment of Repeat Expansion Size and SNP Genotyping

The CBD cohort was screened for intermediate C9orf72 repeat expansions using fluorescent labeled touchdown PCR followed by fragment-length analysis on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). PCR used 50ng genomic DNA in a final volume of 20ul containing 0.5U of Amplitaq Gold (Applied Biosystems) and a final concentration of 1x Amplitaq Buffer I, 5uM reverse primer, 5uM 6FAM-fluorescent labeled forward primer, 1M Betaine, 5% DMSO, 0.25mM 7-deaza-dGTP, and 0.25mM of each dNTP. For detection of full expansions using repeat-primed PCR, the Roche FastStart (Roche, Basel, Switzerland) polymerase system was used to amplify 1ug of genomic DNA in a reaction with 0.9 mM $MgCl_2$, 200nM dNTP mix, 180nM 7-deaza-dGTP, 1.4uM flanking primers, 0.175uM repeat primer, 7% DMSO, .93M Betaine and 0.125 U polymerase. For both intermediate and full expansion detection, a touchdown PCR cycling program was used where the annealing temperature was gradually lowered from 70°C to 56°C in 2°C increments with a 3-minute elongation time at 72°C for each cycle. SNP genotyping was carried out using Taqman probes as previously described[19]. All primer sequences are listed in Supplemental Table 1.

Detection of RNA foci

RNA foci detection was performed on paraffin embedded cerebellum sections as follows: Slides were deparaffinized with Xylene, followed by Xylene/Ethanol (1:1) and then rehydrated with an ethanol series (100%, 90%, 80%,70% for 3 mins each). Slides were

incubated for 5 minutes in water, then digested with proteinase K (20ug/mL) for 10 minutes at 37 degrees in a buffer containing 10mM Tris-HCL pH 8 and 0.5% SDS in DEPC-treated water. Slides were then washed twice with water then dehydrated with increasing ethanol series and allowed to air dry. Slides were then prehybridized for 1 hour at 66 degrees in hybridization buffer containing 50% Foramide, 2X SSC, 50mM $NaHPO_4$, and 10% Dextran sulfate in DEPC-treated water. Slides were hybridized with 40nM probe (LNA 5TYE563C4G2; Exiqon (Denmark) # 300500, batch # 621440) at 66 degrees overnight. Slides were then washed once with 2X SSC + 0.1% Tween for 5 mins at room temperature, then 3 times with 0.1X SSC for 10 minutes at 65 degrees. Slides were then rinsed with water, stained with DAPI and mounted with coverslips using Prolong glass antifade mountant (Invitrogen, Carlsbad, CA). Cases for RNA foci detection included 12 non-expanded CBD, 9 intermediate CBD, 4 non-expanded control cases (negative control) and 5 full expansion FTD/ALS cases (positive control).

Detection of Dipeptide Repeat proteins and Measurement of DNA methylation

Paraffin embedded cerebellum sections were deparaffinized and stained with antibodies against p62, GA (1:7500), GP (1:7500) or GR (1:100) as previously described[48]. 116 non-expanded CBD, 9 intermediate CBD, 4 non-expanded control cases (negative control) and 5 full expansion FTD/ALS cases (positive control) were stained. For measurement of DNA methylation in CBD patient genomic DNA, 100ng DNA was digested using the restriction enzymes HhaI and HaeIII (New England Biolabs, Ipswich, MA) followed by qPCR as previously described[41].

RNA Expression Analysis from Postmortem Brain and repeat edited NPCs

RNA was extracted from approximately 100mg of cerebellum tissue using Trizol reagent (Life Technologies, Carlsbad, CA). RNA integrity was tested on an Agilent 2100 bioanalyzer using the RNA Nanochip 6000 kit (Agilent, Santa Clara, CA). Only RNA samples with RIN values > 6 were included in this study. For repeat edited NPCs, cells were pelleted at day 12 post induction and RNA was extracted using the RNeasy Kit (Qiagen). cDNA was prepared using random hexamers and Superscript III (Invitrogen) according to manufacturer's protocol. RT-qPCR was performed using SYBR Green reagents (Roche) on a StepOne Plus Real-Time PCR Machine (Life Technologies). RNA levels were normalized to the geometric mean of two housekeeping genes (ACTB, GPS1) using the ΔΔCt method. All primer sequences are listed in Supplemental Table 1.

CRISPR Editing of Repeat Size

CRISPR gRNAs were designed using the MIT CRISPR design tool (http://crispr.mit.edu/) and cloned into the Cas9-GFP PX458 vector (Addgene, Cambridge, MA) as previously described[59]. Repair templates were generated via PCR cloning using genomic DNA from intermediate repeat carriers. PCR was carried out using the FastStart Polymerase (Roche) with additives for GC rich templates (360nM 7-deaza-dGTP, 7% DMSO, 930mM Betaine). PCR products containing 2 or 28 repeats were cloned into PGEM Easy-T vector (Promega, Madison, WI) and verified by sequencing. A 2 bp substitution was made at the PAM site in the repair template using QuickChange site directed mutagenesis (Aligent) in order to prevent Cas9 cutting of the repair template and to incorporate a restriction enzyme site (See

Supplemental Figure 3). PX458 and repair template vectors were co-transfected into WT iPSCs[46] using Viafect reagent (Promega). On day 2 post transfection, GFP+ cells were sorted via FACS (BD FACS Aria II) to 96 well plates for clonal isolation. DNA was extracted from 24 well plates of confluent clones using 0.2% SDS lysis buffer followed by phenol-chloroform extraction. DNA from clones was PCR amplified and screened for homology directed repair by restriction enzyme digest. Repeat size was measured as outlined above on positive clones, and all CRISPR edited cell lines were Sanger sequenced via PCR cloning (10 clones/cell line).

Differentiation of iPSCs to Neural Progenitor Cells

IPSCs were differentiated via dual SMAD inhibition as previously described[66]. Single cells were plated at high density ($2\times10^5/cm^2$) in mTeSR1 (StemCell Technologies, Vancouver, Canada) + 10uM ROCK inhibitor Y27632 (ATCC) on Matrigel (Corning, Corning, NY) coated 6 well plates. The following day, media was changed to neural induction media (see Shi et al.[66] for complete formulation) containing 10uM SB431542 (Tocris, Bristol, United Kingdom) and 1uM dorsomorphin (Sigma, St. Louis, MO). Neural induction media was changed each day. At day 6, cells were split 1:2 into neural induction media + ROCK inhibitor and media changes were continued until day 12. Day 12 NPCs were characterized for expression of NPC markers by immunofluorescence and RT-qPCR. For western blot analysis, NPCs were passaged again at day 12 and collected at day 16 post induction.

Western Blots and Antibodies

For detection of C9orf72, day 16 NPCs were pelleted, lysed (50mM Tris-HCl, 750mM NaCl, 5mM EDTA, 2% SDS) and sonicated using a probe sonicator (Sonics Vibra-cell), then spun at 21,000 g for 30 mins. BCA analysis (Pierce, Thermo Fisher) was performed on cleared lysate to quantify protein expression. 50ug of protein was run on an 8% Tris-Glycine gel, transferred using the Transblot Turbo system (Biorad, Hercules, CA) to a nitrocellulose membrane and blocked for 1 hour at room temperature with Odyssey Licor Blocking buffer (Licor, Lincoln, NE). Membranes were blotted with the following antibodies overnight at 4 degrees: anti-C9orf72 1:1000 (Millipore, Burlington, MA, ABN1645), anti-GAPDH 1:1000 (Cell signaling Technology, Danvers, MA 2118S). IR dye secondary antibodies (Licor) were used to detect protein on a Licor Odyssey. Protein was quantified using Image Studio software (Licor).

For C9orf72 expression and autophagy experiments, cell lysates were prepared in ice-cold radio immunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris (pH8.0) supplemented with protease and phosphatase inhibitors (PhosphoSTOP, Roche). Cells were lysed for 20 min and centrifuged for 10 min at 13,500 rpm. Protein concentration of the soluble fractions were determined using BCA reagent (Thermo Fisher Scientific). 20–70ug of total protein was resolved using 10% or 12% denaturing polyacrylamide gel electrophoresis and transferred to PVDF membrane (Immobilon-P, PVDF). Proteins on PVDF membranes were blocked using 5% low-fat milk in TBST containing 0.1% Tween-20 and incubated with primary antibody in SuperBlock (TBS, Thermo Fisher Scientific) overnight at 4°C. The primary antibodies

used were: LC3 (ProteinTech, 14600–1-AP, 1:1000), and GAPDH (ProteinTech, 60004–1-Ig, 1:5000). The membranes were washed in TBST. Anti-rabbit or anti-mouse horse-radish peroxidase (HRP) conjugated secondary antibodies (1:3000, Thermo Fisher Scientific) in 5% milk in TBST was incubated with the membranes for 1 hour at room temperature. The membranes were incubated with WestPico or WestFemto chemiluminescent substrate (Thermo Fisher Scientific) and exposed to Amersham Hyperfilm ECL films (GE Healthcare Life Sciences). The films were developed using Konica Minolta film developer. Protein band intensity was quantified using LI-COR Image Studio Lite.

RNA sequencing in knockin intermediate repeat cells

50 base pair paired end RNA sequencing was carried out on day 12 post induction isogenic NPC lines (See Supplemental Table 2) with 3 biological replicates per group and 2 technical replicates per cell line. Libraries were prepared using the TrueSeq Stranded mRNA and Ribo-zero rRNA removal kits (illumina, San Diego, CA). Sequencing was carried out on a Hiseq 2500 (illumina) and at least 100 million reads were generated per biological replicate. Adapter sequences were removed using Cutadapt[50]. Reads were aligned to the genome (GRCh38) using STAR (v2.6) [22] with the default mapping parameters and the read counts per gene were counted using the quantMode function and genecode v29 annotation. Differentially expressed genes between the short repeat group and intermediate repeat group were called using the DEseq2 program (v1.22)[45] with default parameters. Over enrichment analysis of biological pathways was carried out using WebGestalt (2019) on genes that were either upregulated or downregulated (adjusted p-value < 0.05) in the intermediate repeat group. For comparison of genes dysregulated in full expansion carriers, differentially expressed gene lists (adjusted $p > 0.05$ and >2 fold change) from two studies[24, 63] that compared gene expression in iPSC derived neurons with full expansions vs. low repeat controls were compared against the differentially expressed genes in this study (intermediate repeat vs low repeat).

Generation of stable C9orf72 expressing cell lines, transfection and drug treatments

The generation of an inducible single-copy C9orf72 transgene using Flp-In TRex-HeLa cells was as described previously[31]. All cell lines used in this study were maintained in DMEM (GE Healthcare HyClone, SH30243.01) with 10% FBS (GE Healthcare HyClone, SV30160.03) and 1% Pen-Strep (Thermo Fisher Scientific, 15070063) at 37°C and 5% CO_2. Tetracycline (Sigma-Aldrich, T7660) was added (100 ug/ml) for 18 h to induce the expression of C9orf72 transgene. Parental HeLa cells treated with tetracycline were used as controls in the experiments shown in Figure 6b and 7b. All plasmid transfections in cell lines were performed using Lipofectamine 2000 (Thermo Fisher Scientific, 11668019) or TurboFect (Thermo Fisher Scientific, R0531) as per the manufacturer's instruction. For autophagy experiments, Chloroquine (CQ, Sigma-Aldrich) was dissolved in water (20 mM) and used at 40μM in the media. Autophagy induction using Earle's Balanced Salt Solution (EBSS, Gibco) was incubated on cells following two successive PBS rinses. Incubation time for each condition is stated in the respective figure legends.

Results

Screening of CBD patient DNA for *C9orf72* expansions

To test the hypothesis that intermediate size expansions in *C9orf72* are a risk factor for CBD, genomic DNA from 354 autopsy confirmed CBD cases was screened using a PCR-based sizing assay (Figure 1a). We compared CBD patients to controls from a previously published global meta-analysis of *C9orf72* repeat size [73]. We found that 1.84% of alleles from CBD cases (corresponding to 3.7%, or 13 cases) harbored intermediate length repeat expansions compared to 0.52% of controls (Figure 1b–c, OR = 3.59, p = 0.00024). This analysis was based on a minimum size cutoff of 17 which was determined a priori and based on prior independent studies[7, 73]. The increase in *C9orf72* intermediate repeat allele frequency was not due to an increase in the frequency of the *C9orf72* repeat expansion haplotype between CBD patients verses non-diseased controls or general global populations (Supplemental Table 3), and we did not detect any expansions larger than 29 repeats (Supplemental Table 4). We conducted a post-hoc analysis on our CBD cohort for each repeat length cut off below 18 repeats which revealed that repeats as low as 10 units was associated with CBD (p = 0.026, OR = 1.36), where 61 out of 354 CBD cases had *C9orf72* repeat alleles that were at least 10 units. This included 5 cases which had two alleles at 10 or greater. Although this post-hoc analysis is considered exploratory[9], there appeared to be a somewhat graded increase in CBD risk with increasing repeat size (Figure 1d, Supplemental Table 5). Overall these results indicate that intermediate expansions in *C9orf72* may be a genetic risk factor for CBD, and the risk may be additive with higher repeat sizes conferring increased CBD risk.

C9orf72 expression from intermediate expansion carriers

Next, we determined how intermediate repeat expansions affect expression of the *C9orf72* gene. Previous studies have shown that large expansions in *C9orf72*, which cause ALS/FTD, reduces mRNA from the expanded allele[8, 19, 77]. This reduction in expression is often associated with hypermethylation of the *C9orf72* promoter region[41, 52, 61, 81]. To test whether intermediate expansions alter *C9orf72* DNA methylation and mRNA levels, we analyzed post mortem cerebellum samples from a subset of 47 CBD cases for which frozen tissue was readily available. We measured repeat expansion size, DNA methylation and *C9orf72* mRNA levels of the two major isoforms[76] (Figure 2a, V2 and V3 isoforms). In this subgroup analysis, we again observed high frequencies of intermediate repeat expansions between 17 and 29 units (Supplemental Figure 1). Intermediate size expansions did not correlate with hypermethylation of the promoter region, as is observed in full expansion cases (Supplemental Figure 2). Surprisingly, total *C9orf72* mRNA levels were positively correlated with total *C9orf72* repeat size (Figure 2b left, $R^2 = 0.276$, p = 0.0034).This positive correlation was not observed for the major isoform, V2, which has a transcription start site downstream from the repeat expansion (Figure 2c left, $R^2 = 0.00496$, p = 0.717). In contrast, V3 has a transcription start site upstream from the repeat expansion and shows a robust positive correlation between repeat expansion size and mRNA levels (Figure 2d left, $R^2 = 0.667$, $p < 0.0001$). Cases with >17 total repeats exhibited ~2.5 to 5 fold increase in variant 3 mRNA compared to cases with shorter repeats. Finally, analysis of the variant 3 to variant 2 ratio also reveals a striking positive correlation with larger repeat

sizes (Figure 2e left, $R^2 = 0.849$, $p < 0.0001$). Similar results were obtained when using the largest repeat allele as opposed to the total *C9orf72* repeat length (Figure 2b–d, right). However, total repeat number produced a stronger positive correlation to *C9orf72* mRNA levels, supporting an additive effect of repeat size on expression.

To confirm the positive relationship between *C9orf72* repeat size and mRNA expression, we analyzed publicly available SNP eQTL data from the Genotype-Tissue Expression (GTEx) Project[44]. Large *C9orf72* repeat expansion mutations invariably occur within a 140kb haplotype block with a defined series of SNPs[49, 53]. The SNP rs3849942 is a commonly used marker for this haplotype block in which the G allele is associated with small non-expanded *C9orf72* alleles, and the A allele is associated with both larger intermediate to full repeat expansions[19]. We observe the same trend in our CBD genomic DNA cohort where the rs3849942 A risk allele was associated with larger *C9orf72* repeat sizes (Figure 2f). Using rs3849942 as a marker for longer/intermediate repeats (as full expansions are rare in unselected populations), we used GTEx data to determine if this SNP is associated with altered *C9orf72* expression. We found that the risk allele is significantly associated with increased *C9orf72* expression across numerous tissue types, with the largest effect size occurring in neural tissues (Figure 2g). Expanding the analysis to 20 haplotype defining SNPs[53], we found that 12 of the SNPs on the risk allele are associated with increased *C9orf72* expression in both the cortex and basal ganglia (Figure 2h). In contrast, there were no significant eQTL SNPs that led to a decrease in expression. These results provide additional evidence that intermediate expansions lead to increased *C9orf72* expression.

CRISPR Editing of *C9orf72* expansion size in iPSCs

In order to confirm the previous findings experimentally and to determine whether the risk haplotype versus the intermediate *C9orf72* repeats themselves are driving increased *C9orf72* gene expression, CRISPR/cas9 was used to modify the repeat size in previously characterized induced pluripotent stem cells (iPSCs) from a non-diseased individual[46]. A repair template containing either 2 or 28 repeats was used to alter the size of the repeat expansion in iPSCs that normally have alleles of either 2 or 6 repeats (Figure 3a–b; Supplemental Figure 3). Clonal lines were derived by FACS and were screened for changes in repeat size and for the presence of the knocked-in allele by PCR followed by restriction enzyme digest (Figure 3b). Genotype, including repeat size, was confirmed by repeat primed PCR (Figure 3c) and Sanger sequencing (Supplemental Figure 4). Despite using a repair template with 28 repeats, several different iPSC clones with repeat sizes ranging from 23 to 28 were obtained. Additional isogenic iPSC clones were also obtained with two or six *C9orf72* repeats.

Given that both neurons and glial cells are affected in CBD[5], we used the dual SMAD inhibition method[14, 66] to differentiate edited iPSCs with intermediate *C9orf72* expansions and isogenic controls with small repeats into neural progenitor cells (NPCs) as determined by uniform expression of the NPC marker Pax6 (Supplemental Figure 5). RT-qPCR of these edited NPC lines showed a remarkably consistent pattern of higher *C9orf72* mRNA expression in clones with intermediate *C9orf72* repeats compared to isogenic controls with small repeats (Figure 3d). Consistent with the data from CBD post mortem

brain tissue, we again saw no change in the amount of variant 2 mRNA (Figure 3e) but a robust increase in variant 3 mRNA levels in all intermediate *C9orf72* repeat cell lines (Figure 3f–g). This effect was not an artifact of neuronal differentiation, as undifferentiated iPSCs also exhibited increased *C9orf72* mRNA levels (Supplemental Figure 6). Thus, these experiments demonstrate a causal relationship between intermediate *C9orf72* expansions and increased *C9orf72* mRNA levels.

Intermediate repeat carriers do not have RNA foci or DPR pathology

Full expansion carriers have characteristic neuropathology in post mortem brain and spinal cord tissue that includes nuclear RNA foci of G_4C_2 transcripts[19] and aggregates of dipeptide repeat proteins (DPRs) that co-stain with p62[6]. Staining for DPRs using p62 antibody or DPR specific antibodies (GA, GP and GR) was carried out on 125 CBD cases in hippocampus and/or cerebellum, including 9 cases with intermediate repeats (repeat sizes 17–29, Figure 4a). Interestingly, all of these cases were negative for the aggregates typically seen in full expansion cases (Figure 4a). Additionally, RNA foci were not detected in CBD cerebellum tissues with intermediate repeats, nor were they detected in cases with low repeat numbers (Figure 4b). In comparison, full expansion FTD/ALS cases had abundant DPR aggregates together with RNA foci in ~18% of nuclei. Thus, the major pathologic hallmarks of full expansion carriers are not present in CBD cases, leading us to hypothesize that the underlying mechanisms of intermediate expansions and CBD risk may be distinct from the large repeat expansions that cause ALS/FTD.

Elevated C9orf72 protein and global gene expression changes in intermediate repeat cell lines

To test whether C9orf72 protein expression is higher in cells with intermediate repeats, protein expression was measured in isogenic repeat edited NPCs. NPC lines with intermediate repeats exhibited a consistent ~40% increase in C9orf72 protein levels when compared to isogenic low repeat number controls (Figure 5a–b). Thus, intermediate size repeat expansions increase both *C9orf72* mRNA and protein levels.

To determine whether overexpression of C9orf72 in the context of intermediate repeats alters expression of other genes, RNA sequencing was performed on 3 edited intermediate NPC lines and 3 low repeat isogenic control lines (Supplemental table 2, Supplmental file 1). We found 1,307 genes significantly downregulated (adjusted p-value < 0.05 and fold change > 2) in intermediate repeat cell lines and 959 genes upregulated (Figure 5c). Next, overrepresentation analysis was performed on gene ontology pathways in the differentially expressed genes. Genes down regulated in intermediate repeat cell lines (Figure 5d) are highly enriched for metabolic processes, especially those involving nutrient catabolism. Genes upregulated in intermediate repeat cell lines (Figure 5e) are enriched in vesicle trafficking and protein degradation pathways including golgi vesicle transport, response to ER stress and autophagy. We then looked in more detail at specific genes within the most highly dysregulated biological processes. When categorizing vesicle trafficking genes according to their function[69], there is modest increase in expression of numerous endosomal/lysomal genes, including *C9orf72* (Figure 5f). Interestingly, there is a consistent and sizable decrease in expression of genes involved in secretory pathways in intermediate

repeat cell lines, indicating a possible defect in vesicle secretion (Figure 5f). Additionally, key genes involved in autophagy are modestly increased in intermediates (Figure 5g). However, several genes that have been shown to regulate autophagy in response to nutrient starvation[3, 71, 85] and ER stress[78] such as *TMEM74* and *TMEM74B* are markedly downregulated in intermediate repeat cells (Figure 5g), suggesting a defect in autophagy under stress conditions. Finally, we compared the differentially expressed genes in our dataset to previously published[24, 63] gene expression studies between control (low repeat) and full expansion cells derived from ALS/FTD patients. Interestingly, we see a distinct group of dysregulated genes between control and intermediate repeat cells than between control and full expansion cells (Figure 5h), indicating that different processes are affected by intermediate expansions versus full expansions.

Over-expression of C9orf72 impairs starvation-induced autophagy

Next, a cell culture model was used to further validate the observed changes in biological pathways in the RNA sequencing data and determine if these were in fact caused by *C9orf72* over expression. Since *C9orf72* is known to regulate autophagy[16, 18, 65, 74] and this pathway was altered in the RNA sequencing data, we generated an inducible isogenic HeLa cell line expressing a single-copy of human C9orf72 long isoform without the repeat expansion tagged with GFP and 6xHis-FLAG tag. The transgene expression level is comparable, i.e., 1:1, to that of endogenous C9orf72 and is under the tight control of tetracycline (Supplmental Figure 7 and [31]). Importantly, the transgene also associates with the autophagy initiation complex, thereby confirming that the GFP-tagged C9orf72 recapitulates the known endogenous gene function[31]. As a readout of autophagy, we utilized an LC3 (microtubule-associated protein light chain 3)-based assay (Figure 6a). During autophagosome assembly, LC3-I undergoes lipidation via conjugation with a phosphatidylethanolamine group to become LC3-II [54, 82]. LC3-II is normally rapidly degraded during autophagy via fusion with the lysosome, but blocking lysosome fusion allows for accumulation of LC3-II to serve as a marker of autophagic activity[72]. Immunoblotting in C9orf72 overexpressing cells revealed that basal LC3-II levels are ~20% higher in C9orf72 overexpressing cells compared to parental HeLa cells (lane NGM, normal growth medium, Figure 6b–c), indicating that either autophagosome production is increased or lysomal degradation of LC3-II is reduced under normal conditions. When autophagosome-lysosome fusion was blocked with chloroquine (CQ)[51], LC3-II accumulates at a higher level in CQ-treated condition when compared to their untreated counterparts, and at a similar level between the parental HeLa and C9orf72 overexpressing cells (Lane NGM+CQ, Figure 6b–c). Interestingly, when autophagy was induced using nutrient-deficient medium, EBSS (Earle’s balance salt solution), the accumulation of LC3-II was reduced by ~40% in the C9orf72 overexpressing cells when compared with parental HeLa cells (Lane EBSS+CQ, Figure 6b–c), suggesting that C9orf72 overexpression blocks starvation-induced autophagy. To further corroborate the biochemical findings, a GFP-LC3 stable HeLa cell line[87] was transfected with C9orf72 construct linked via P2A peptide to mCherry to better visualize the transfected cells. Consistent with the immunoblotting results above, ~50% more LC3-II puncta were observed in the C9orf72 overexpressing conditions in normal growth media. (NGM panel, Figure 6d–e). However, there was ~30% reduction of

LC3-II puncta in C9orf72 overexpressing cells under starvation (EBSS+CQ panel, Figure 6d–e), again indicating that there is a failure of autophagy initiation in starvation conditions.

To more accurately measure the actual autophagic flux or rate, a time course without and with chloroquine treatment was performed (Figure 7a). With the lysosomes blocked, LC3-II accumulated at a similar rate in C9orf72 overexpressing cells under normal conditions as the parental HeLA cells (Figure 7b–c, top blot). Under starvation conditions, however, the rate of accumulation was markedly reduced (Figure 7b–c, bottom blot) compared to the parental HeLa cells. Taken together, these results suggest that C9orf72 overexpression promotes autophagy under nutrient rich conditions but limits the cell's ability to induce autophagy during times of nutrient stress.

Discussion

In this study, we demonstrate that intermediate repeat expansions in *C9orf72* may be a genetic risk factor for CBD, thus broadening the spectrum of neurodegenerative diseases implicated by this gene. This study highlights the importance of using autopsy confirmed cases to better understand the genetics of neurodegenerative diseases that have similar or overlapping clinical manifestations. Although intermediate repeats are found in only a small percentage of CBD patients, the magnitude of risk conferred by intermediate repeats is comparable or greater than other known genetic risk factors for CBD[34]. This study assembled the most CBD cases yet tested, and due to the rarity of CBD, a replication cohort is not yet available for follow-up analysis. While we established an a priori hypothesis based on the previously established but arbitrary cutoff of 17 repeat units or larger to define intermediate expansion carriers, our findings suggest that both CBD risk and *C9orf72* expression increases in an additive manner with increasing repeat length where perhaps C9orf72 intermediate expansions could be defined as at least 10 units. Interestingly, initial reports of *C9orf72* repeat mutation cohorts, defined at that time as at least 30 repeats, included one case of autopsy-confirmed CBD[68]. We did not detect any expansions larger than 29 repeats in our CBD cohort, and intermediate expansion carriers did not have the characteristic RNA foci and dipeptide repeat pathology seen in full expansions. Future studies will need to further define the size threshold that determines whether carriers are more likely to develop risk for CBD versus ALS/FTD.

C9orf72 joins a list of other repeat expansion containing genes in which the length of the repeat confers a pleiotropic effect on the disease phenotype. For instance, intermediate length CGG repeats in the gene *FMR1* result in the neurodegenerative disease Fragile X-associated tremor/ataxia syndrome (FXTAS) linked to toxicity of repeat RNA[29], whereas full expansions lead to near complete silencing of *FMR1* and the neurodevelopmental disorder Fragile X Syndrome[75]. Similarly, intermediate CAG expansions in *Ataxin-2* are a risk factor for ALS[25], whereas full expansions cause spinocerebellar ataxia type 2[62]. For *C9orf72*, large expansions cause ALS/FTD[19, 60], whereas we demonstrate that intermediate expansions confer ~3.5 fold higher risk for CBD. Full expansions mutations in *C9orf72* have been associated with reduction of *C9orf72* gene expression and a gain of repeat associated pathology including nuclear repeat RNA accumulation and dipeptide repeat protein production. [19, 27, 67, 76] In contrast, we show that intermediate expansions

cause an increase in *C9orf72* mRNA levels in both CBD post mortem cerebellum and knock-in isogenic neural cell lines. This mechanism is also supported by publicly available SNP eQTL data, which shows that numerous SNPs on the expansion haplotype are associated with increased *C9orf72* expression. Thus, it appears that maintaining proper levels of C9orf72 may be necessary for proper neuronal function, with increased expression observed in CBD patients and decreased expression observed in ALS/FTD patients.

C9orf72 is thought to function as Rab guanine exchange factor[38], which participates in a host of vesicle trafficking pathways[55]. C9orf72 has been shown to regulate endocytosis[26], vesicle secretion[4], intracellular trafficking[4, 26, 58, 67] and autophagy[16–18, 74, 79, 83]. Indeed, we observe numerous gene expression changes in all of these vesicle trafficking pathways in knock-in NPCs with intermediate expansions. The large number of significant gene expression changes detected in this study is likely due to the depth of sequencing (~100 million reads/sample) and the use of isogenic CRISPR edited controls, which removes variability due to genetic background. Particularly striking changes were observed in genes involved in vesicle secretion and autophagy. The involvement of autophagy pathways in numerous neurodegenerative diseases has been well documented [55], and increased levels of C9orf72 may enhance CBD risk by disrupting autophagy pathways necessary for responding to stress. *C9orf72* has been shown to regulate autophagy initiation through the *ULK1* complex[31, 65, 79], and we observe increased expression of autophagy initiation genes in the intermediate repeat knockin NPCs. Interestingly, over expression of C9orf72 seems to increase autophagy initiation under normal conditions, but inhibits autophagy induction under nutrient stress. Several other studies have implicated *C9orf72* as regulator of the cellular stress response[2, 16, 43, 47], and we observe dysregulation of genes involved in the ER stress response and nutrient biosynthesis pathways in intermediate repeat knockin NPCs. In particular, the gene *TMEM74* and its paralog *TMEM74B* were high downregulated in *C9orf72* NPCs with intermediate repeats. *TMEM74* has been shown to be a potent activator of autophagy induced via nutrient starvation[85] and is thought to function via a unique pathway that does not require initiation via the canonical *ULK1* autophagy initiation complex[71]. This alternative initiation pathway may be affected by increased C9orf72 levels and could explain why we observe an autophagy defect in C9orf72 over expressing cells under nutrient starvation. Future studies will need to further define the molecular mechanisms by which *C9orf72* overexpression alters specific autophagy pathways.

Overall, the findings presented here illustrate the complexity of *C9orf72* repeat expansion mutations, with the length of expansion conferring risk for different neurodegenerative diseases via opposing mechanisms. Notably, the proportion of CBD cases with intermediate C9orf72 repeats is still relatively low (~4%) indicating that the overall genetic risk associated with intermediate repeats is modest. Likewise, given that CBD is a rare disease, an otherwise normal individual harboring an intermediate repeat is unlikely to develop CBD. Finally, these results indicate that autophagy pathways are dysregulated by elevated C9orf72 levels and suggest a link between *C9orf72* expression and neurodegenerative disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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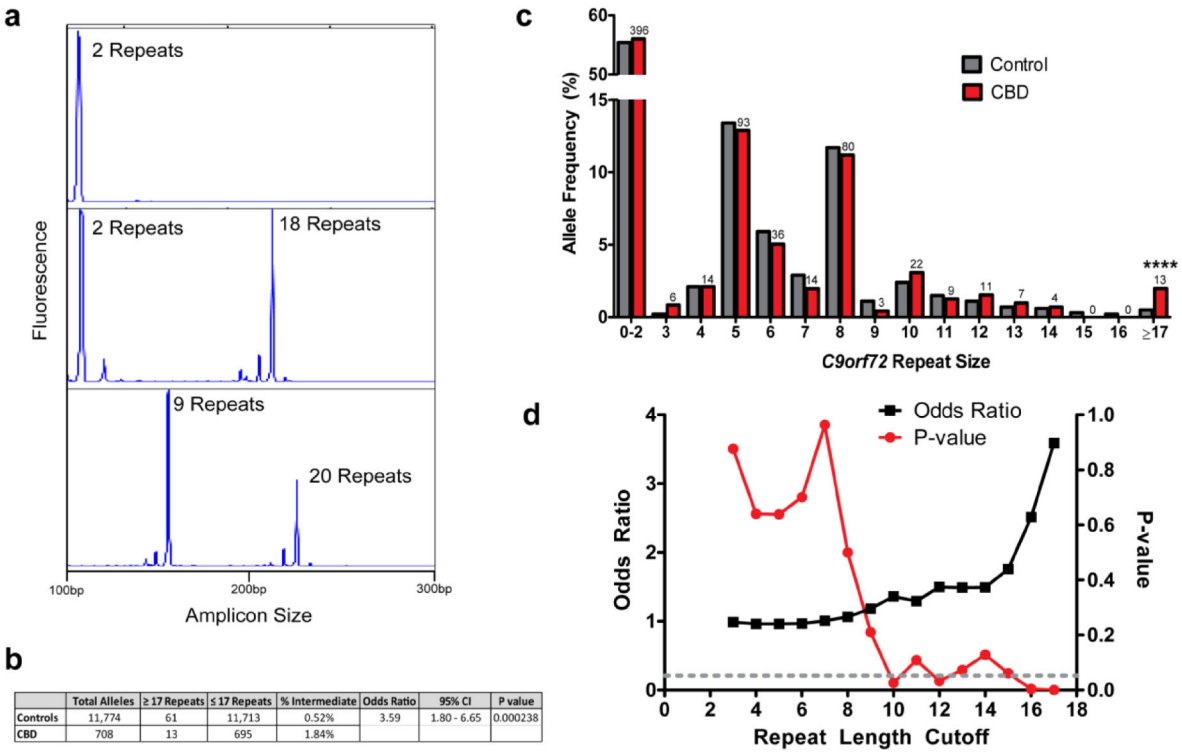

b

	Total Alleles	≥ 17 Repeats	≤ 17 Repeats	% Intermediate	Odds Ratio	95% CI	P value
Controls	11,774	61	11,713	0.52%	3.59	1.80 - 6.65	0.000238
CBD	708	13	695	1.84%			

Figure 1: *C9orf72* repeat sizing of CBD.

a) Example capillary electrophoresis traces of CBD cases measured from genomic DNA using a fluorescent PCR assay with genotypes of 2,2 (top), 2,18 (middle) or 9,20 (bottom). **b)** Summary statistics for repeat size screening of CBD cases (n = 708 alleles) and previously published controls (n = 11,774 alleles)[73]. Fisher's exact test: OR = 3.59, 95% CI = 1.80 – 6.65, p = 0.000238. **c)** Allele frequencies of repeat sizes in CBD cases and controls. The number of alleles in the CBD cohort for each size is depicted above the bars. **d)** Results of Fisher's exact test between CBD cases and controls at each allele size cutoff. Dotted gray line represents $p < 0.05$ significance level.

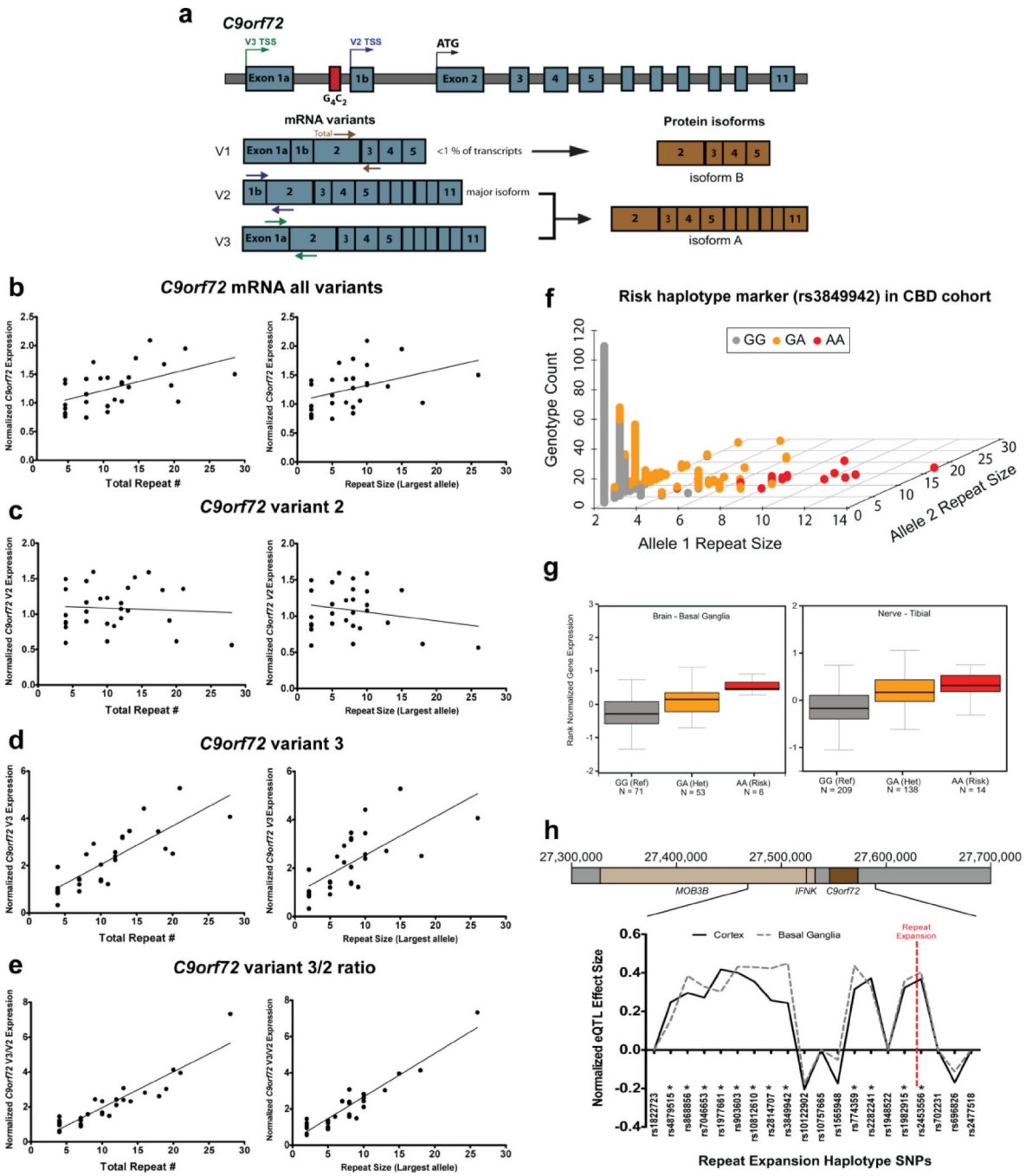

Figure 2: Effect of intermediate repeats on *C9orf72* expression.

a) Diagram of the *C9orf72* locus with mRNA and protein isoforms. Transcription start sites (TSS) are labeled for each mRNA variant on the genomic DNA. Colored arrows on the mRNA depict primers used for RT-qPCR. **b)** Correlation between total *C9orf72* repeat size (left) or largest allele (right) and *C9orf72* mRNA levels from all variants measured by RT-qPCR using RNA from CBD patient cerebellum (Total repeats: n = 29, R^2 = 0.276, p = 0.0034; Largest allele: n = 29, R^2 = 0.1613, p = 0.0308). Data is normalized to samples with 4 total repeats. **c)** Correlation between total *C9orf72* repeat size (left) or largest allele (right)

and variant 2 mRNA levels (Total repeats: n = 29, R^2 = 0.00496, p = 0.717; Largest allele: n = 29, R^2 = 0.04432, p = 0.2730). **d)** Correlation between total *C9orf72* repeat size (left) or largest allele (right) and variant 3 mRNA levels (Total repeats: n = 29, R^2 = 0.667, p < 0.0001; Largest allele: n = 29, R^2 = 0.4874, p < 0.0001). **e)** Correlation between total *C9orf72* repeat size (left) or largest allele (right) and the variant 3 mRNA to variant 2 mRNA ratio (Total repeats: n = 29, R^2 = 0.849, p < 0.0001; Largest allele: n = 29, R^2 = 0.8869, p < 0.0001). **f)** Genotyping results for rs3849942 in the CBD patient genomic DNA cohort (n = 708 alleles). The A allele is associated with intermediate and full length repeat expansions. **g)** SNP eQTL data from the Gtex project showing the effect of rs3849942 on *C9orf72* expression in basal ganglia (left, p = 9.00E-07) and tibial nerve (right, p = 2.80E-16). For box plots, center line indicates median, boxes are upper and lower quartiles, error bars are standard error of the mean. Numbers of samples are indicated below each group. **h)** Normalized GTEx eQTL effect size from cortex and basal ganglia on *C9orf72* expression for all 20 SNPs in the consensus repeat expansion risk haplotype (from Mok et al. 2012[53]). * indicates SNPs with a significant effect on *C9orf72* expression. Red dotted line indicates the location of the *C9orf72* repeat expansion.

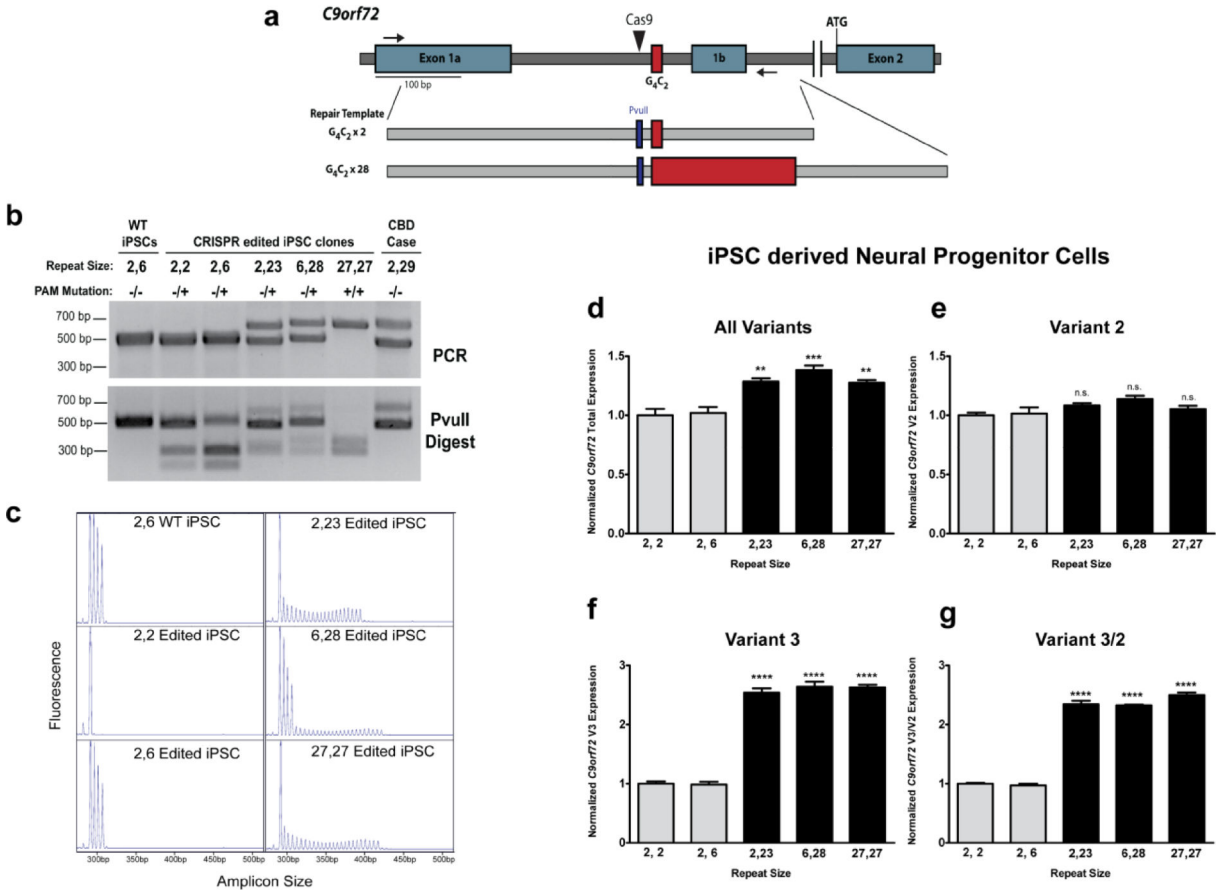

Figure 3: CRISPR/cas9 knock-in of intermediate repeats into iPSCs.

a) Diagram of *C9orf72* genomic locus and double stranded repair templates used for knocking in intermediate repeats. Black triangle represents the CRISPR/cas9 cut site. Black arrows indicate the PCR primers used in (b). Red boxes indicate the G_4C_2 repeat. Blue boxes indicate the 2 bp substitution included in the repair templates to mutate the Cas9 PAM site and generate a PvuII restriction enzyme site. **b)** PCR (top) and PvuII digest (bottom) of CRISPR edited iPS cell lines using PCR primers spanning the CRISPR cas9 cut site and repeat expansion. Digestion of the PCR product indicates that HDR occurred. **c)** Capillary electrophoresis traces of repeat primed PCR from CRISPR edited iPSCs. **d-g)** RT-qPCR from the indicated variants of *C9orf72* from neural progenitor cells differentiated from CRISPR edited iPSCs. Light bars correspond to low repeat lines while black bars correspond to intermediate repeat lines. N = 3 biological replicates per cell line; ANOVA (All variants: $F = 18.3$, $p = 0.0001$; Variant 2: $F = 2.9$, $p = 0.08$; Variant 3: $F = 216.0$, $p < 0.0001$; V2/V3: $F = 460.0$, $p < 0.0001$) followed by Bonferroni post hoc test between low and intermediate repeat cell lines. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Error bars are standard error of the mean.

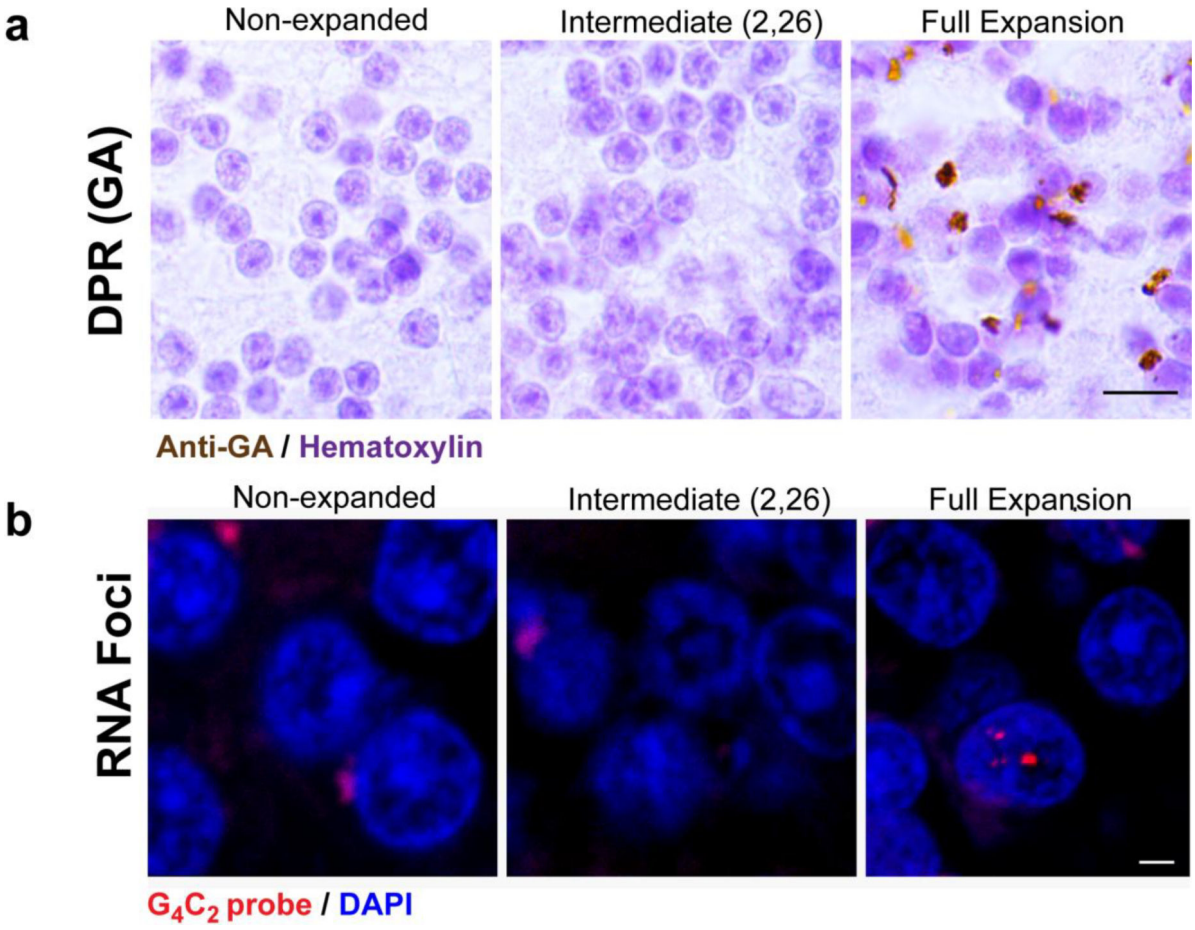

Figure 4: Absence of repeat pathology in intermediate expansion cases.

a) Representative Immunohistochemistry images of non-expanded control (n=4 cases) or non-expanded CBD (n = 116 cases), intermediate expansion CBD (n = 9 cases), and full expansion FTD/ALS (n = 5 cases) post mortem cerebellar samples using anti-GA or p62 antibodies. Intermediate expansion carriers were also negative for GP/GR aggregates. Scale bar 10μm. **b)** Representative fluorescence in situ hybridization images of non-expanded control (n=4 cases) or non-expanded CBD (n = 12 cases), intermediate expansion CBD (n = 5 cases), and full expansion FTD/ALS (n = 5 cases) post mortem cerebellar samples using a probe complimentary to the G_4C_2 repeat. Scale bar 5μm.

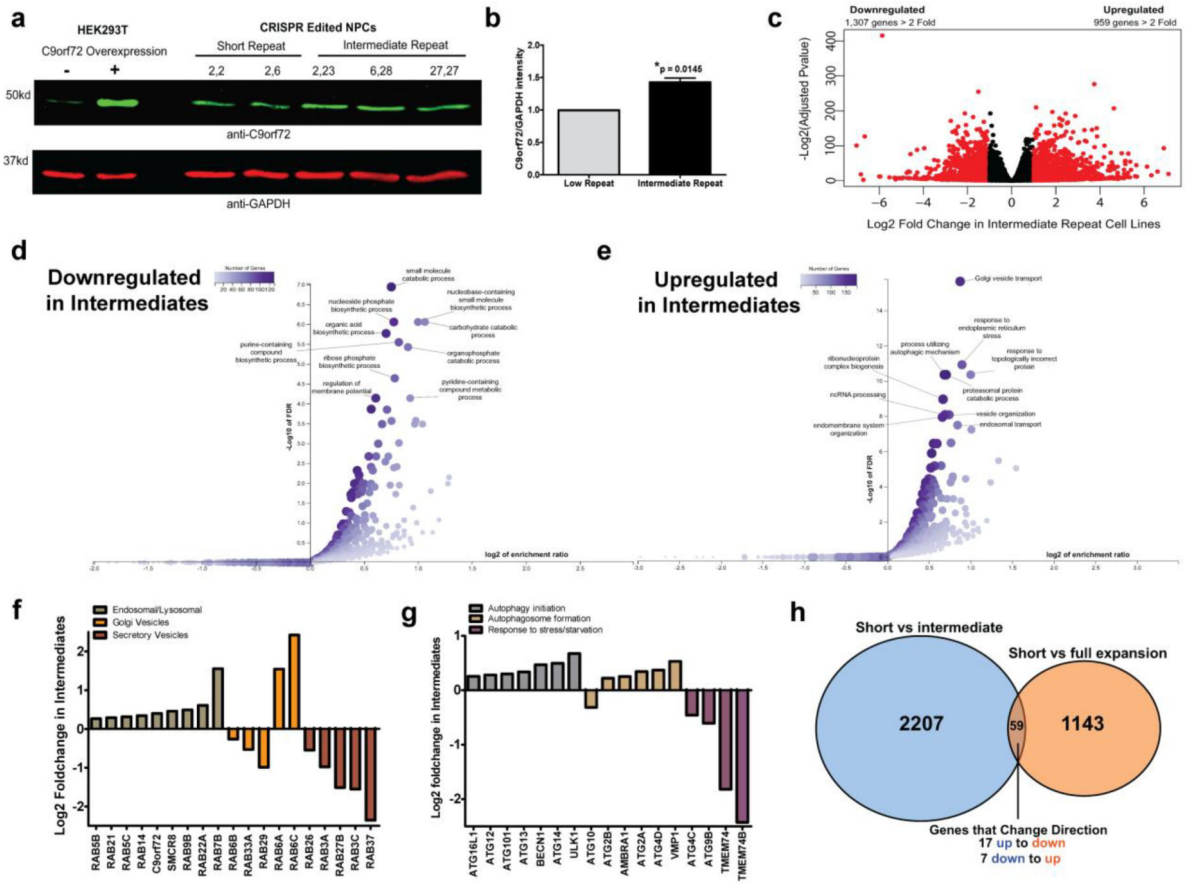

Figure 5: Elevated C9orf72 protein levels and gene expression changes in intermediate repeat NPCs

a) Western blot of protein expression from CRISPR edited NPCs. N = 2 low repeat and 3 intermediate repeat number cell lines. C9orf72 antibody was validated in HEK293T cells transfected with C9orf72 overexpression plasmid (Lanes 1–2). Representative blot of 2 technical replicates is shown. **b)** Quantification of C9orf72 protein levels from repeat edited NPCs. Protein expression was normalized to GAPDH as a loading control and the average of 2 technical replicates is reported. N = 2 low repeat and 3 intermediate repeat number cell lines. Two tailed t-test, p = 0.0145. Error bars are standard error of the mean. **c)** RNA sequencing analysis of 3 low repeat NPC lines (max repeat size 2 or 6) and 3 intermediate repeat (max repeat size 23, 27 or 28) NPC lines. Differentially expressed genes with an adjusted p-value < 0.05 and greater than 2 fold change are depicted as red dots. **d-e)** Overrepresentation analysis of biological processes on differentially downregulated genes (d) or upregulated genes (e) in NPCs with intermediate repeats. Labels highlight the top 10 most significant biological processes. **f)** Changes in gene expression of genes involved in vesicular trafficking. All genes shown are significantly differentially expressed (adjusted p < 0.05) between low repeat and intermediate repeat NPCs. **g)** Changes in gene expression of genes involved in autophagy. All genes shown are significantly differentially expressed (adjusted p < 0.05) between low repeat and intermediate repeat NPCs. **h)** Comparison of overlap in differentially expressed genes between low repeat NPCs versus intermediate repeat NPCs (this study) or low repeat vs full expansion neurons from patient derived iPSCs

from two previous studies [24, 63]. Only genes with adjusted $p < 0.05$ and fold change > 2 were used in this comparison.

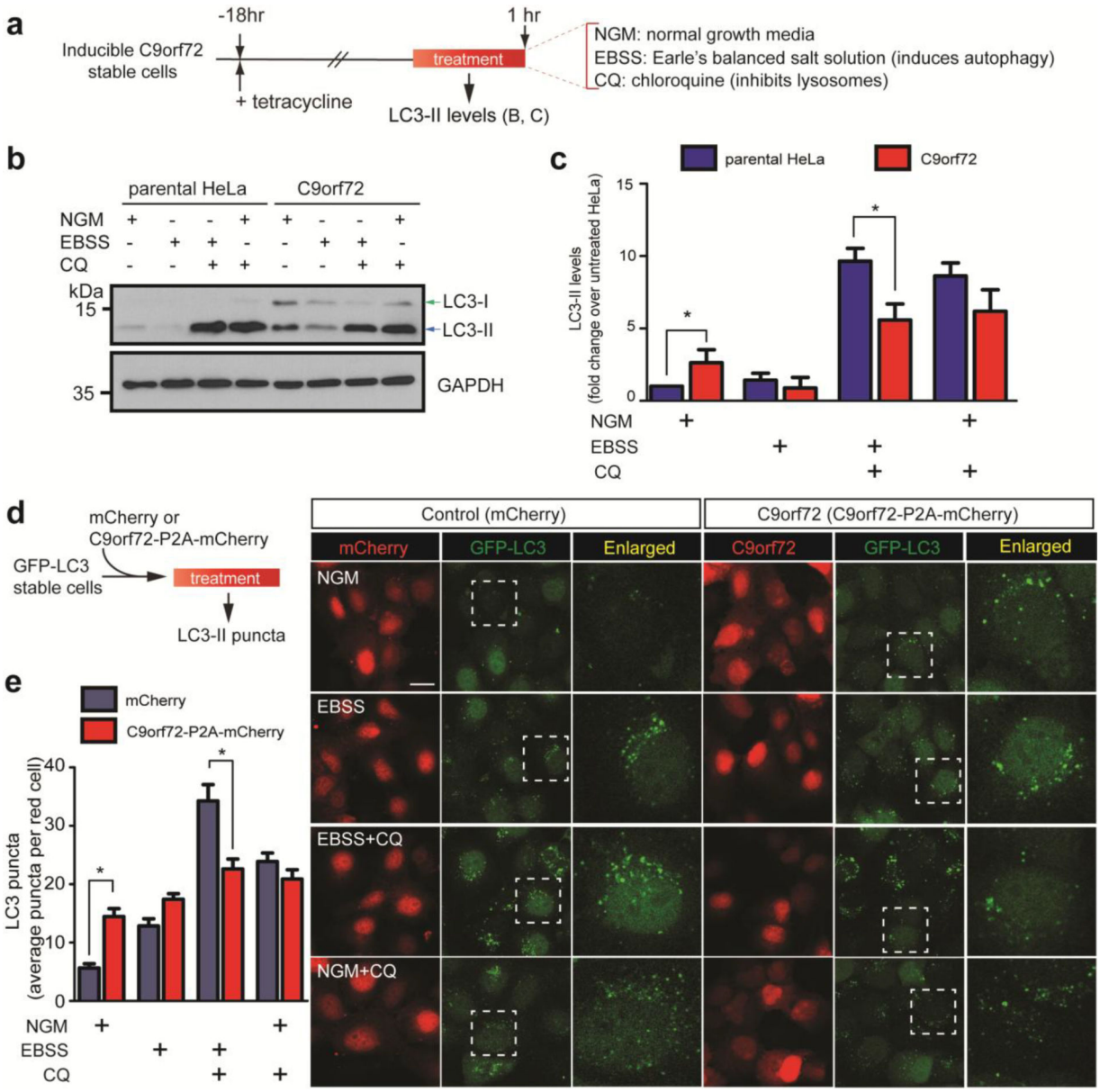

Figure 6: Autophagy defects in inducible C9orf72 over-expressing cells

a) Schematic of experimental procedure for LC3-II protein detection in C9orf72 HeLa stable cells. **b)** Representative western blot showing LC3-II expression levels of parental HeLa cells and C9orf72 cells first treated with treated with tetracycline to induce C9orf72 transgene expression then with EBSS and/or CQ for 1 hour. **c)** Bar chart corresponding to (a) showing pooled data of LC3-II protein levels expressed as fold change over parental HeLa cells in normal growth media without CQ treatment. Data represents three independent experiments with * $p<0.05$, Student's t-test. **d)** Schematic of experimental procedure for the detection of LC3-II puncta using fluorescence microscopy in parental HeLa cells overexpressing GFP-LC3 and mCherry (control) or C9orf72-P2A-mCherry plasmids. The cells were treated with NGM, EBSS, EBSS+CQ or NGM+CQ for 2 hours and green puncta associated with GFP-LC3 in mCherry-overexpressing cells were analyzed

using ImageJ particle count. At least 25 cells were analyzed per treatment group. Scale bar = 20 μm. **e)** Bar chart corresponds to the average LC3 puncta count in (d) with * $p < 0.05$, Student's t-test. Error bars represent standard error of the mean (S.E.M).

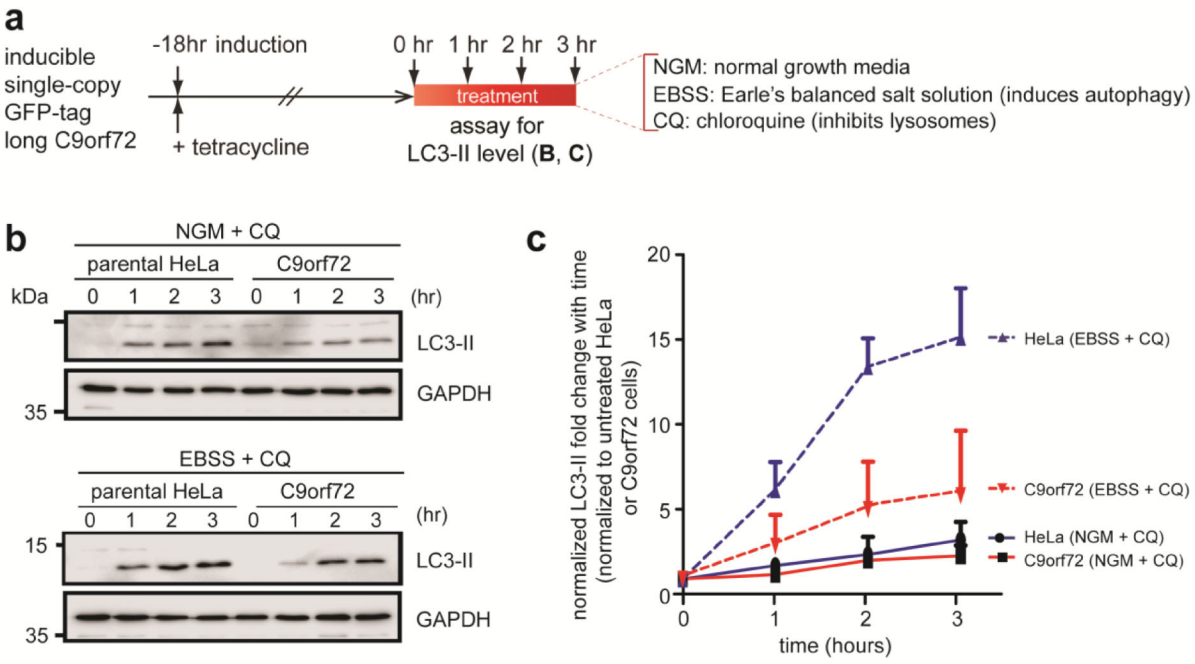

Figure 7. Kinetics of starvation induced autophagy in C9orf72 over-expressing cells

a) Schematic of time-course experiments on autophagy in C9orf72 HeLa stable cells. **b)** Representative western blot showing LC3-II expression levels of parental HeLa cells and C9orf72 cells first treated with tetracycline to induce C9orf72 transgene expression then with either normal growth media (NGM)+CQ or EBSS+CQ for 0, 1, 2, and 3 hours. **c)** Time course chart corresponding to (b) showing pooled data of LC3-II protein levels expressed as fold change over respective controls, i.e. parental HeLa cells or C9orf72 cells grown in normal growth media without CQ treatment. Data represents three independent experiments with error bars representing standard error of the mean (S.E.M).