Regulation of human cerebral cortical development by *EXOC7* and *EXOC8*, components of the exocyst complex, and roles in neural progenitor cell proliferation and survival

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Abstract

Purpose—The exocyst complex is a conserved protein complex that mediates fusion of intracellular vesicles to the plasma membrane and is implicated in processes including cell polarity, cell migration, ciliogenesis, cytokinesis, autophagy, and fusion of secretory vesicles. The essential role of these genes in human genetic disorders however, is unknown.

Methods—We performed homozygosity mapping and whole exome sequencing of consanguineous families with recessively inherited brain development disorders. We modeled an *EXOC7* splice variant *in vitro* and examined *EXOC7* mRNA expression in developing mouse and human cortex. We modeled *exoc7* loss-of-function in a zebrafish knockout.

Results—We report variants in exocyst complex members, *EXOC7* and *EXOC8*, in a novel disorder of cerebral cortex development. In *EXOC7*, we identified four independent partial loss-of-function (LOF) variants in a recessively inherited disorder characterized by brain atrophy, seizures, and developmental delay, and in severe cases, microcephaly and infantile death. In *EXOC8*, we found a homozygous truncating variant in a family with a similar clinical disorder. We modeled *exoc7* deficiency in zebrafish and found the absence of *exoc7* causes microcephaly.

Conclusion—Our results highlight the essential role of the exocyst pathway in normal cortical development and how its perturbation causes complex brain disorders.

Keywords

exocyst; EXOC7; EXOC8; microcephaly; developmental delay

INTRODUCTION

Eight genes in the human genome, *EXOC1 – EXOC8*, encode the exocyst complex, a multimeric, evolutionarily-conserved complex that traffics vesicles within the cell to the plasma membrane for fusion. The exocyst complex has been shown to play a role in several cellular processes, including cell polarity, cell migration, ciliogenesis, cytokinesis, autophagy, and fusion of secretory vesicles[1], but human disorders associated with definitive

loss-of-function mutations in any of these components have not yet been reported. Although a missense mutation in *EXOC8*[2] was reported in a single case of Joubert syndrome (MIM 213300), and a missense mutation in *EXOC4*[3] was reported in a case of Meckel-Gruber syndrome (MIM 249000), the pathogenicity of these two mutations has not yet been confirmed. As such, the essential role of individual proteins of the exocyst complex remains unclear.

Several of the reported functions of the exocyst complex, cell polarity and migration, cytokinesis, and ciliogenesis[1,4–6] are integral processes during cerebral cortical development and so motivated us to test the hypothesis that mutations in exocyst encoding genes cause brain development disorders. First, establishing cell polarity and cell migration are essential for cortical development. Mutations in radial glial cell progenitors (RGC) polarity genes, such as *Pals1* and *Par3*, disrupt cortical development through massive cell death or premature cell cycle exit, respectively[7,8]. In addition, mutations in genes required for neuronal migration can cause one of several cortical malformations, including lissencephaly (MIM 607432, *PAFAH1B1/LIS1*[9]), double cortex syndrome (MIM 300067, *DCX*[10, 11]), and cortical dysplasia (MIM 610031, *TUBB3*[12], *TUBB5*[13], *KIF5C*[14], *KIF2A*[14]). Second, robust and rapid cell division of cortical progenitors is essential for cortical development and several genetic causes of microcephaly (MIM 251200) exhibit disrupted cytokinesis as a result of supernumerary (*CDK5RAP2*[15, 16], *KATNB1*[17,18]), or missing spindle poles (*ASPM*[19], *WDR62*[20]) stemming from dysfunctional centrosomes[21]. Finally, defects in cilia formation lead to ciliopathy syndromes (MIM 209900), complex syndromes with disrupted brain development (*INPP5E*[22], *C2CD3*[23], *BBS1*[24]).

Here we provide a systematic analysis of variants in two exocyst components, by defining several variants in *EXOC7* and *EXOC8*. We identify four independent, partial loss-of-function variants in *EXOC7*, associated with developmental brain disorders of variable severity characterized by developmental delay, seizures, brain atrophy, microcephaly, and infantile death. We also describe one loss-of-function variant in *EXOC8* similarly associated with severe developmental delay, seizures, brain atrophy, microcephaly, and premature death. We further provide a zebrafish genetic model of *EXOC7* loss-of-function and offer genetic evidence that *EXOC7* is required for neuron survival.

MATERIALS AND METHODS

Human subjects

This study was conducted with the approval of institutional review boards and according to the ethical standards of the participating institutions: Boston Children's Hospital, University of California San Diego, the Faculty of Medicine, United Arab Emirates University, and AP-HP Sorbonne Université. Informed consent was received from all participants. Permission was received to publish patient photographs.

IACUC approval of zebrafish housing and experiments

A complete description of the husbandry and environmental conditions in housing for the fish used in these experiments is available as a collection in protocols.io dx.doi.org/

10.17504/protocols.io.mrjc54n. All animals were cared for humanly and all experiments were approved by Boston Children's Hospital IACUC.

Whole exome sequencing

DNA was extracted from whole blood and whole exome sequencing was performed (See Supplement). We filtered out variants with allele frequency >10% in Middle Eastern population[25].

Sanger sequencing

Primers surrounding the reported variant in each family were used for PCR and subsequent Sanger sequencing to confirm genotype from whole exome sequencing and determine segregation within the family.

Minigene cloning and expression

A ~5kb section of human *EXOC7* locus was amplified with primers (F: AAGGACTGAAGGAGCATTTC, R: CAGGGAGTCGAAGGTCTTCT) from a BAC and cloned into pCAG expression vector. The splice acceptor variant from family I was introduced with site-directed mutagenesis. WT or splice variant containing vector was transfected into mouse N2A cells and after 48 hours RNA was isolated and retrotranscribed into cDNA. N2A cells were cultured at 37 °C and 5% CO_2 in high-glucose DMEM (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

HAP1 mutant cell line

HAP1 human cell line was cultured at 37 °C and 5% CO_2 in high-glucose DMEM (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The splice acceptor variant from Family I was introduced into a HAP1 cell line as a hemizygous variant using CRISPR/Cas9 mutagenesis (Supplemental Methods) [26] (Horizon Discovery). Immunoblot performed using human EXOC7 antibody (Abcam, ab118792).

Exoc7 alternative splicing in developing cortex

Alternative splicing analysis of *EXOC7* in developing human (GW13–16) and mouse (E14.5) cortex was performed as described previously[27]. Aligned BAM files from RNA sequencing datasets were analyzed with the MISO pipeline (version 0.4.6) to determine the inclusion frequency of alternatively spliced exons.

Generation of *exoc7* mutant zebrafish lines

Exoc7 mutant zebrafish were generated by CRISPR/Cas9 mutagenesis. Cas9 mRNA (250 ng/µl) and *exoc7* targeting guide RNA (target: CCGTCCTCATCCTGGACGCC, 80 ng/µl) were injected into 1-cell embryos. Embryos developed to adulthood and then Sanger sequencing was used to identify potential heterozygous *exoc7* mutant carriers in F1 progeny. A 1-bp frameshift deletion in exon 5 was identified and this fish was backcrossed to WT to generate heterozygous carriers. This allele is *mh111*.

Toluidine blue staining of zebrafish

5 dpf embryos were fixed in 4% PFA overnight at 4 °C and then embedded in JB-4 resin according to manufacturer's protocol (Polysciences Inc). Fish were sectioned at 2 μm, and then matching sections were stained with toluidine blue and imaged with a bright-field microscope.

Immunostaining of zebrafish progenitor cells

5 dpf embryos were fixed in 4% PFA overnight at 4°C, embedded in OCT, and sectioned coronally at 20 μm on a cryostat. Matched sections were stained with a primary antibody against Sox-2 (Abcam, ab97959). Tissue was permeabilized and blocked in 3% BSA, 0.3% Triton X-100, 0.3% sodium azide in PBS. Primary antibodies were incubated overnight at 4°C. Sections were stained with Alexa secondary antibodies and Hoechst. Imaging was done on Zeiss 510 confocal microscope. Sox-2 positive nuclei in telencephalon were counted.

TUNEL staining in developing zebrafish

5 dpf embryos were fixed in 4% PFA overnight at 4°C, embedded in OCT, and cryo-sectioned coronally. Apoptotic cells in matched sections were labeled with TUNEL staining using the Apoptag kit (Millipore) according to the manufacturer's instructions. Imaging was done on Zeiss 510 confocal microscope. TUNEL positive cells in telencephalon were counted.

RNAscope

RNAscope on human fetal brain tissue was performed according to manufacturer's protocol (ACDBio). Tissue was fixed in 4% PFA, frozen, and sectioned at 20 μm on a cryostat.

Quantification and Statistical Analysis

In all analyses, mean values are presented for pooled data and errors bars are SEM. For all quantifications, statistical significance was determined using a two-tailed, unpaired t-test (GraphPad Prism).

RESULTS

EXOC7 and *EXOC8* mutations in recessive developmental disorders

In mapping developmental disorders affecting the cerebral cortex, we identified variants in *EXOC7* and *EXOC8* associated with recessive brain development syndromes with a range of symptom severity including developmental delay, seizures, brain atrophy, microcephaly, and infantile death (Table 1).

Family I is a consanguineous family with the most severely affected children, having infantile lethality with neonatal micrencephaly, seizures, and arthrogryposis (Figures 1A, S1A). The family had two daughters who were born with myoclonic seizures and arthrogryposis multiplex. One had documented microcephaly (−2.7 SD), and both died within the first months of life. Imaging of both siblings showed a cerebrum smaller than the skull cavity with a thin cortex and extremely simplified gyri, enlarged ventricles, reduced

white matter, and a very small cerebellum and brainstem (Table S1). These findings are consistent with global cerebral cortical maldevelopment, and likely brain atrophy, reflecting neuronal loss (Figure 1A). Homozygosity mapping in this family identified a region on chromosome 17 linked to disease with a statistically suggestive maximum LOD score of 1.93 (Figure S2A). Whole exome sequencing identified a homozygous splice variant in *EXOC7* within this region (exon 7 splice acceptor, NC_000017.10:g.74087318T>C (hg19), c.809–2A>G, Figure 1A); this variant is heterozygous in 2/251,414 alleles from normal controls (frequency = 7.96×10^{-6}) and never homozygous (gnomAD database[28]). This variant mutates a highly conserved base, disrupts the canonical splice acceptor for exon 7 (ag|G > gg|G), and segregates perfectly with disease in this family. One additional rare, homozygous variant was found in the same linkage region that caused a missense mutation in *CYB5D2* (NC_000017.10:g.4057982C>T (hg19), p.Arg136Trp), a gene that encodes a heme binding protein[29] with low expression in the developing cerebral cortex (Figure S4). In the gnomAD database, this *CYB5D2* variant is heterozygous in 3/276,426 alleles from normal controls (frequency = 1.22×10^{-5}, 0 homozygous alleles), and there are 3 additional homozygous missense and 1 homozygous stop-gain variants in *CYB5D2* in gnomAD. Although little is known about *CYB5D2* function, its expression is much lower than *EXOC7* in developing cortex and the greater relative severity of the *EXOC7* splice acceptor variant favor it as causative in this family.

Family II has one affected male child, of consanguineous parents, with severe developmental delay, seizures, and mild microcephaly (−2.6 SD) (Figure 1B). Brain imaging showed central and cortical atrophy prominent in the temporal lobes (Table S1). Whole exome sequencing identified a homozygous 3-base pair deletion in exon 3 of *EXOC7* that removes a serine at position 48 (NC_000017.10:g.74097928_74097930delGAT (hg19), Ser48del, Figure 1B). This variant is heterozygous in 2/276,426 alleles from normal controls (frequency = 7.24×10^{-6}) and never homozygous (gnomAD), and segregates perfectly with disease. Recent work presents two important roles for EXOC7's N-terminal domain, (1) binding to EXOC8 to promote exocyst complex assembly[30] and (2) binding to Wave regulatory complex for cell migration[31]. As Ser48 is located in this region, its deletion could disrupt these functions, consistent with it being a pathogenic variant.

Family III is non-consanguineous with two affected fetuses with mild microcephaly and cerebellar hypoplasia, confirmed by autopsy following termination of pregnancy (Figures 1C, S1F–H, Table S1). Whole exome and Sanger sequencing identified two compound heterozygous variants in *EXOC7* in the affected fetuses that segregated with disease. One variant is the same exon 7 splice variant found in Family I (NC_000017.10:g.74087318T>C (hg19), c.809–2A>G, Figure 1C). The second variant is an in-frame deletion of 15 bp that removes five amino acids in exon 10 (NM_001145297.2: c.1212_1226delTGGGCTGATGCTTGA, Figures 1C, S3). This variant is absent from normal controls (gnomAD).

Family IV is consanguineous with three siblings affected with recessively inherited seizures, intellectual disability, and developmental delay (Figure 1D). Brain imaging showed atrophy in two of the three affected siblings (Table S1). Homozygosity mapping identified a single linkage region on chromosome 17 with the maximum possible LOD score of 2.9 (Figure

S2B). Whole exome sequencing identified a homozygous missense variant in exon 15 of *EXOC7* within the linkage region (NC_000017.10:g.74081807C>T (hg19), Ala523Thr, Figure 1D). This variant is heterozygous in 2/277,066 alleles from normal controls (frequency = 7.22×10^{-6}) and never homozygous (gnomAD), and segregates perfectly with disease. Alanine 523 is a highly conserved amino acid that is conserved from humans to zebrafish.

Family V exhibits recessively inherited global developmental delay with regression, seizures, and microcephaly in three daughters of consanguineous parents (Figures 1E, S1B–E). Brain imaging showed atrophy in all three affected individuals (Table S1). Homozygosity mapping identified multiple linkage regions with a maximum possible LOD score of 2.5, including a large region on chromosome 1 (Figure S2C). Whole exome sequencing identified a homozygous 2-base pair deletion in exon 1 of *EXOC8* in the linkage region (NC_000001.10:g.231471676_231471677delCT (hg19), Asp607Ter, Figure 1E), which is absent from normal controls (gnomAD). This frameshifting deletion creates a premature stop codon at amino acid 607, short of the full-length protein (725 aa), and segregates perfectly with disease in this family. One additional homozygous variant was detected in the family (stop gain in *RP1L1*). In gnomAD, this variant is present as a homozygote in one individual and *RP1L1* is associated with adult-onset retinitis pigmentosum (OMIM 613587), so this variant is unlikely to contribute to microcephaly in this family.

In total, we report four novel, missense and splice site variants in *EXOC7* and one novel truncating variant in *EXOC8* in families with a recessive syndrome of brain atrophy, seizures, and developmental delay, and in more severe cases microcephaly and infantile death (Table 1). The presence of cerebral atrophy in all families indicates neurodegeneration and suggests *EXOC7* and *EXOC8* are required for neuronal survival. Loss-of-function variants in *EXOC7* have not been previously linked to human disease, and we have recently reported one homozygous missense variant in *EXOC8* in a single case of Joubert syndrome[2].

EXOC7 splice variant disrupts mRNA spicing patterns and reduces protein expression

EXOC7 is an alternatively spliced gene with 5 verified transcripts[32]; three of which include exon 7. We generated a minigene assay to model the exon 7 splice acceptor variant found in both Families I and III and found this variant disrupts splicing and decreases EXOC7 protein level. The minigene was constructed using 5 kb of genomic DNA from the human *EXOC7* locus spanning exon 6 to exon 9 (Figure 2A). RT-PCR of mRNA transcribed from the minigene plasmid expressed in mouse N2A cells revealed three splicing disruptions caused by the human variant (Figure 2A). First, cDNA encoding a high-abundance transcript (including exons 6, 7, and 9) was isolated from wild-type minigene but completely absent from the variant (Figure 2A). A low-abundance larger product that could not be sub-cloned for sequencing was found in wild-type and likely encodes a transcript including exons 6, 7, 8, and 9 (*, Figure 2A). Second, two novel out-of-frame splice forms that are predicted to encode early truncations were found exclusively in variant minigene cDNA (Figure 2A). Form A splices-in the last 37 bases of intron 6 causing a frameshift which is predicted to

create a premature stop codon at amino acid 286, well short of the full-length protein of 684 amino acids. Form B splices-out the first 14 bases of exon 7 causing a frameshift, predicted to create a premature stop codon at amino acid 290. An isoform that skips exon 7 and includes exons 6 and 9 was observed in both wild-type and variant minigenes. These minigene results show that the variant disrupts *EXOC7* mRNA splicing. In order to assess the variant's effect on EXOC7 protein we mutated a HAP1 cell line to encode the patient variant (hemizygous). Immunoblot of EXOC7 protein showed reduced expression in variant HAP1 cells (Figures 2B, 2C). We detected two isoforms, a larger isoform (1) was significantly reduced by 50% (two-tailed t-test, p = 0.045) and a smaller isoform (2) showed a trend towards reduction (two-tailed t-test, p = 0.088). Together, these results support the pathogenicity of this variant.

EXOC7 is highly expressed in developing cerebral cortex

EXOC7 is highly expressed in the developing human cortex, consistent with the affected individuals' phenotypes. *In situ* hybridization using RNAscope in human fetal cortex showed abundant *EXOC7* expression in the ventricular zone (adjacent to the ventricle and co-expressing *VIM*), the outer subventricular zone (co-expressing *TBR2*) and in the cortical plate (adjacent to the pial surface) (Figures 3A, 3B). Similarly, *Exoc7* is expressed in developing mouse cortex (MGI). RNA sequencing analysis of human fetal cortex[33] confirms this expression pattern with expression in the ventricular zone and the inner and outer subventricular zones at a similar level as *ASPM*, a canonical microcephaly gene, and in the cortical plate (Figure 3C). *EXOC7* expression in both progenitors and postmitotic neurons suggests it has important roles in both cell types during cortical development. Notably, exon 7 of *EXOC7*, which contains a splice acceptor variant in Families I and III, is differentially spliced in cortical progenitors compared to postmitotic cortical neurons. Using a previously published method[27], we identified differentially spliced exons in the developing cortex with RNA sequencing of separated cortical progenitors and postmitotic neurons isolated from both developing mouse and human brain tissue. During fetal human cortical development (GW15), exon 7 is included in 93% of transcripts from the cortical plate and 47% of transcripts from ventricular zone (Figure 3D). Similarly, during cortical development in mice (E14.5), exon 7 is included in 87% of transcripts isolated from the cortical plate and 35% of transcripts from the ventricular zone (Figure 3D). The role of this differential splicing in the regulation of *EXOC7* function in development is unknown, but our evidence that the splicing variant identified in Families I and III alters this differential expression by eliminating expression of the exon 7 included isoform (Figure 2) suggests it is pathogenic by disrupting cortical development.

Exoc7 is essential for vertebrate embryonic development

EXOC7 protein is highly conserved among vertebrates (Figure 4A). To further characterize the function of *EXOC7* in brain development, we examined *Exoc7* loss in both zebrafish and mouse. Mice deficient for *Exoc7* have been created by Lexicon Pharmaceuticals and are reported to be homozygous lethal (personal communication). This is consistent with the phenotype for loss-of-function mutations in other exocyst components also reported to be early embryonic lethal[34,35], and suggests that the three human alleles identified retain some function and are partial loss-of-function.

To facilitate analysis of *Exoc7* function during development, we took advantage of the ease of genome editing in the zebrafish. We created an *exoc7* mutant zebrafish with predicted loss-of-function of the encoded protein that allowed us to examine the function of *exoc7* in development. We used CRISPR/Cas9 mutagenesis to create a 1-bp deletion in exon 5 of *exoc7* that generates a predicted frameshift and subsequent nonsense mutation. This mutation is predicted to lead to a prematurely truncated protein 185 amino acids long, well short of the full-length peptide at 735 (Figure 4B).

We found that *exoc7* is essential for zebrafish development. At 5 days post fertilization (dpf), approximately 25% of progeny from a heterozygous incross showed head edema and small eyes, consistent with Mendelian inheritance (Figures 4C, D, G). Genotyping confirmed that these phenotypes were associated with loss of *exoc7* and demonstrated that the phenotype was highly penetrant (Figure S5A). The mutant fish from the incross (edema and small eys) die shortly after day 5, showing *exoc7* is essential for early zebrafish survival.

Quantification of the small eye phenotype revealed that the eye area was reduced by 26% in mutant fish (two-tailed t-test, 3.6×10^{-24}, Figure S5B). Broader characterization of the phenotype in *exoc7* mutant fish revealed general defects in head size although this was partially masked with the present edema. To account for the changes caused by the edema, we measured the distance from the outside of one eye to the other and then subtracted the regions of edema (Figure 4C). Even with this conservative measure, head diameter was 4% smaller in *exoc7* null fish compared to clutch controls (two-tailed t-test, p = 6.8×10^{-5}, Figure 4E). We detected no difference in body length in mutant fish, suggesting that these defects are specifically caused by the loss of *exoc7* and not simply by developmental delay or allometric changes (two-tailed t-test, p = 0.21, Figure 4F).

Cellular defects in *exoc7* null developing telencephalon

To identify cellular mechanisms underlying microphthalmia and microcephaly in *exoc7* mutant zebrafish (Figure 4G), we measured apoptosis and counted progenitor cells in the developing telencephalon. At 5 dpf, we found a 3-fold increase in the number of apoptotic cells (TUNEL stain) in the telencephalon of *exoc7* null zebrafish (Control n=5, *exoc7* KO n=5, two-tailed t-test, p = 0.0023, Figure 4H). At the same age, we also found a 53% decrease in the number of Sox2-positive telencephalon progenitor cells (Control n=4, *exoc7* KO n=5, two-tailed t-test, p = 0.0019, Figure 4I). The number of Sox2-positive neuroprogenitors was also decreased in the retina of *exoc7* null fish, with a 76% decrease compared to controls (Control n=11, *exoc7* KO n=10, two-tailed t-test, p = 3.0×10^{-6}, Figures S5C). We examined Hoechst-stained mitotic figures in developing telencephalon and did not find a detectable increase in abnormal mitoses in exoc7 null fish, suggesting normal cytokinesis (Figure S5E). Together, these results highlight specific cellular defects that drive microcephaly in zebrafish in the absence of *exoc7*, and further suggest that the atrophy and microcephaly observed in humans with *EXOC7* mutations reflect loss of proliferating progenitor cells and postmitotic neurons.

DISCUSSION

We identified four independent presumably hypomorphic variants in *EXOC7* and one predicted loss of function variant in *EXOC8* causing a recessive human brain development disorder characterized by brain atrophy, seizures, and developmental delay and in more severe cases, microcephaly and infantile death. We show that *EXOC7*, a member of the mammalian exocyst complex, is highly expressed in developing human cortex. In addition, a zebrafish model of *Exoc7* deficiency recapitulates the human disorder with increased apoptosis and decreased progenitor cells during telencephalon development, suggesting that the brain atrophy in human cases reflects neuronal degeneration. These findings provide key inroads into understanding the role of the exocyst complex in normal cortical development and complex neurodegenerative disorders.

Exocyst variants cause a range of brain development disorders

Our work represents the first systematic genetic analysis of the role of exocyst components in human genetic disease. The four distinct alleles that we describe in *EXOC7* show a range of severity consistent with the degree to which they likely damage the protein, with the splicing variant being most severe, a five amino acid deletion having similar severity, a one amino acid deletion being less severe, and an amino acid substitution mutation being the mildest. However, all families share CNS disease and specifically cortical atrophy.

We also report a null variant in *EXOC8* associated with severe phenotypes within this spectrum. Interestingly, we previously reported that a single affected individual with Joubert syndrome had a homozygous missense mutation (E265G) in *EXOC8*[2]. This variant occurred at a highly conserved amino acid and was predicted to be damaging to protein function. Careful clinical review of the affected individuals here confirms that they do not have classic signs of Joubert syndrome. It is possible that these two alleles, E265G and Asp607Ter, lead to different clinical syndromes based on differing mutation severity, where a hypomorphic missense mutation causes Joubert syndrome and a null mutation causes cortical atrophy and microcephaly. Consistent with this idea, homozygous null *Exoc8* mice are reported to have early embryonic lethality (MGI, Mouse Phenotyping Consortium). Here we report that loss-of-functions variants in either *EXOC7* or *EXOC8* lead to highly overlapping clinical features, suggesting perhaps that disruption of the exocyst complex broadly impairs normal cortical development. This is supported by previous work reporting an individual with Meckel-Gruber Syndrome and microcephaly had a homozygous missense variant (Gln578Arg) in another exocyst component, *EXOC4*[3]. The exact mechanism for exocyst dysfunction causing microcephaly and cortical atrophy is unknown, but previous work and our current results suggest the exocyst may be essential for multiple molecular processes during cortical development. Joubert syndrome and Meckel-Gruber syndrome both have features of ciliopathies, and the exocyst is reported to localize to the primary cilium where some members are required for normal ciliogenesis (*EXOC5*[36]). In developing zebrafish, a ciliopathy phenotype of abdominal or cardiac edema, upward tail curvature and small eyes has been observed with loss of *exoc5*[37], and knockdown of Joubert syndrome gene *arl13b*[38] (OMIM 612291). We find that *exoc7* mutant zebrafish have a phenotype with some ciliopathy features including small eyes with edema but missing other features such as

abdominal edema or upward tail curvature (Figure S5D). Further investigation will be required to determine if loss of *exoc7* causes mild cilia dysfunction. Interestingly, the patients we report with *EXOC7* hypomorphic variants do not have classic ciliopathy features. We find that loss of *EXOC7* leads to apoptosis, cell loss, and atrophy in the developing brain and further studies will determine which cellular processes (or combination thereof) are disrupted including RGC polarity and cilia function.

Exocyst complex in cortical development

Our results agree with previous studies of *Exoc7* function in neurons and add new details for its role in brain development. Previous work reported that expression of dominant negative *Exoc7* in developing mouse cortex impaired neuron migration[39] and *in vitro Exoc7* knockdown in cultured neurons disrupted polarization and prevented process outgrowth[4,5]. A conditional mouse knockout of *Arp2/3*, an actin binding protein that interacts with *Exoc7*, in neuroprogenitors showed cortical disorganization characterized by radial glia process truncation, impaired neuron migration, and abundant apoptosis[40]. Here we find *exoc7* deficiency in the zebrafish developing telencephalon is also associated with abundant apoptosis.

We report the first identification of human variants in an exocyst member, *EXOC7*, and show these variants and a null variant in another exocyst member, *EXOC8*, cause a neurodevelopmental syndrome of brain atrophy, seizures, and development delay with microcephaly and infantile death. This study exposes key, shared properties of two exocyst components in brain development. Our *exoc7* loss-of-function zebrafish model provides a new tool that can shed light on the mechanisms of the exocyst complex in the developing brain supporting the role of *EXOC7* as the cause of this complex neurological disorder.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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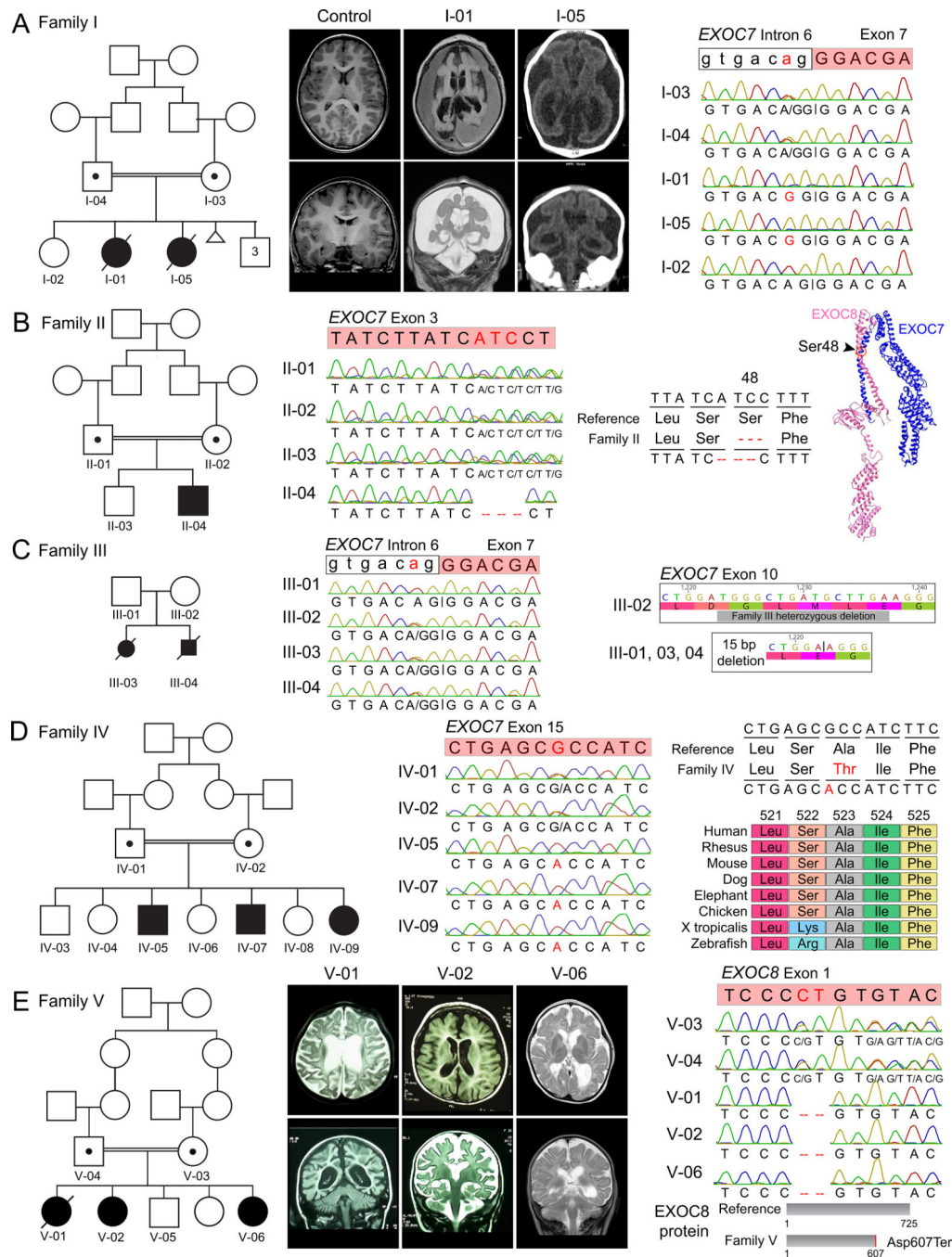
Figure 1. *EXOC7* variants cause a recessive brain development disorder

(A) Left, pedigree of Family I showing consanguineous parents and recessive inheritance of lethal microcephaly in I-01 and I-05. Middle, coronal and axial brain MRI (I-01) or CT (I-05) show extremely simplified gyral pattern, small cortex, and fluid accumulation with age-matched normal MRI for comparison. Right, Sanger sequencing of *EXOC7* intron 6 / exon 7 boundary shows intronic A>G variant that mutates the canonical splice acceptor (ag| G to gg|G). This variant is homozygous in affected individuals and segregates with disease. (B) Left, pedigree of Family II showing consanguineous parents and recessive inheritance of

brain atrophy, microcephaly, and seizures in II-04. Middle, Sanger sequencing of *EXOC7* exon 3 reveals a homozygous 3-base pair ATC deletion in the affected individual that segregates with disease. Right, this deletion removes amino acid Serine 48, which is located in the EXOC7 N-terminal region responsible for binding to EXOC8[30]. (C) Left, pedigree of Family III showing recessive inheritance of fetal microcephaly and cerebellar hypoplasia in III-03, III-04. Middle, Sanger sequencing showing *EXOC7* exon 7 heterozygous splice acceptor variant. Right, diagram of 15 bp heterozygous deletion in *EXOC7* exon 10. (D) Left, pedigree of Family IV showing consanguineous parents and recessive inheritance of brain atrophy and seizures in IV-05, IV-07, and IV-09. Middle, Sanger sequencing of *EXOC7* exon 15 reveals a homozygous G>A variant in affected individuals that segregates with disease. Right, this variant changes amino acid 523, a highly conserved amino acid from humans to zebrafish, from alanine to threonine. (E) Family V has recessive inheritance of a syndrome of developmental regression and delay, seizures, brain atrophy, and early death. Homozygosity mapping and exome sequencing reveals a homozygous 2 base-pair deletion in *EXOC8* that causes early protein truncation. aa: amino acid.
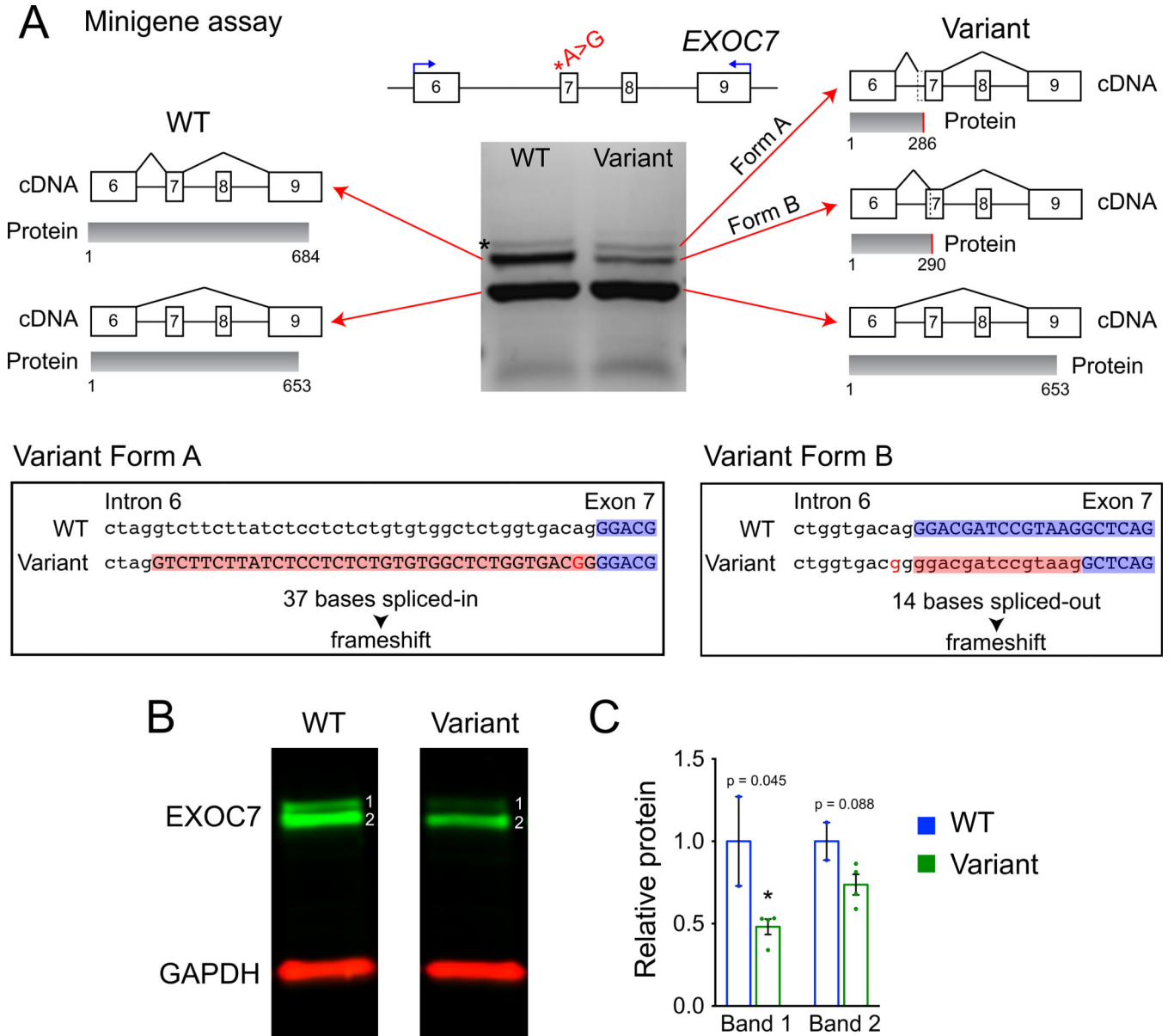
Figure 2. Exon 7 splice acceptor variant disrupts splicing

(A) Top, diagram of *EXOC7* human minigene construct with splice acceptor variant. Blue arrows mark RT-PCR primers used to generate cDNA products shown in gel image. Arrows from each band in gel point to splicing diagram determined by Sanger sequencing of the cDNA. WT minigene generated two in-frame isoforms (left), whereas variant minigene generated one in-frame isoform and two novel out-of-frame isoforms (right). Two variant isoforms encode novel stop codons that lead to premature protein truncation. *: low-abundance product that could not be sub-cloned for sequencing. (B) Immunoblot of EXOC7 protein in WT and variant HAP1 cells showing reduction of two EXOC7 isoforms. GAPDH is a protein loading control. (C) Quantification of (B) showed significant 50% reduction of larger EXOC7 band (Band 1, $p = 0.045$), while the lower weight band (Band 2) was not significantly reduced. P-values calculated by two-tailed t-test. Error bars represent SEM.
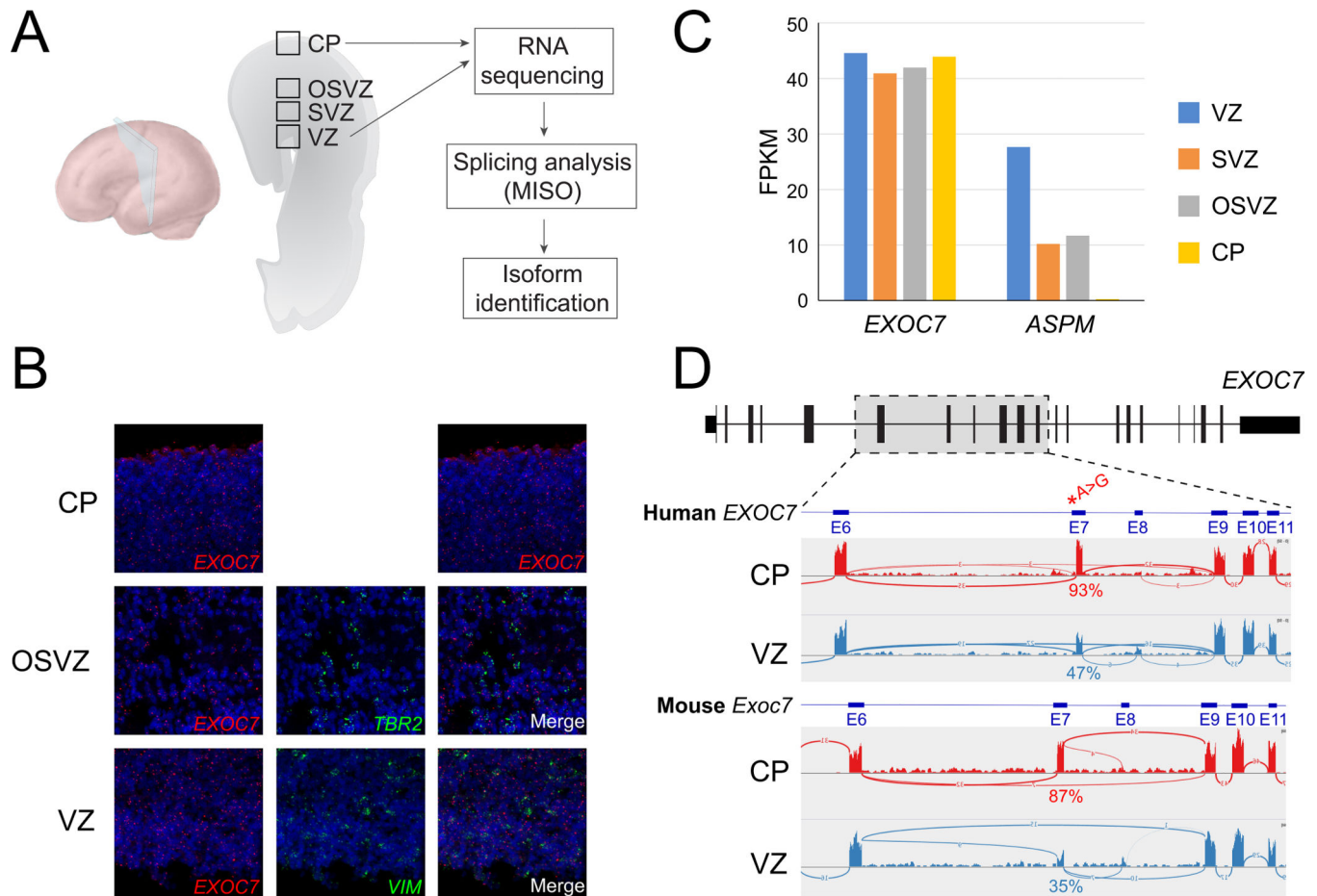
Figure 3. *EXOC7* is highly expressed in developing cortex

(A) Diagram of cortical section of human fetal cortex indicating locations of RNAscope imaging in (B) and RNA sequencing in (C). (B) RNAscope imaging of fetal human cortex shows *EXOC7* expression in VZ and OSVZ, two progenitor zones and in CP, the location of post-mitotic neurons. (C) *EXOC7* is highly expressed in developing human cortex and shown in comparison to *ASPM*. Expression levels measured based on RNA-sequencing[33]. (D) RNA sequencing data from developing human fetal cortex (GW15) and mouse cortex (E14.5) showing differential inclusion of exon 7 in CP vs VZ. FPKM: Fragments Per Kilobase of transcript per Million mapped reads.
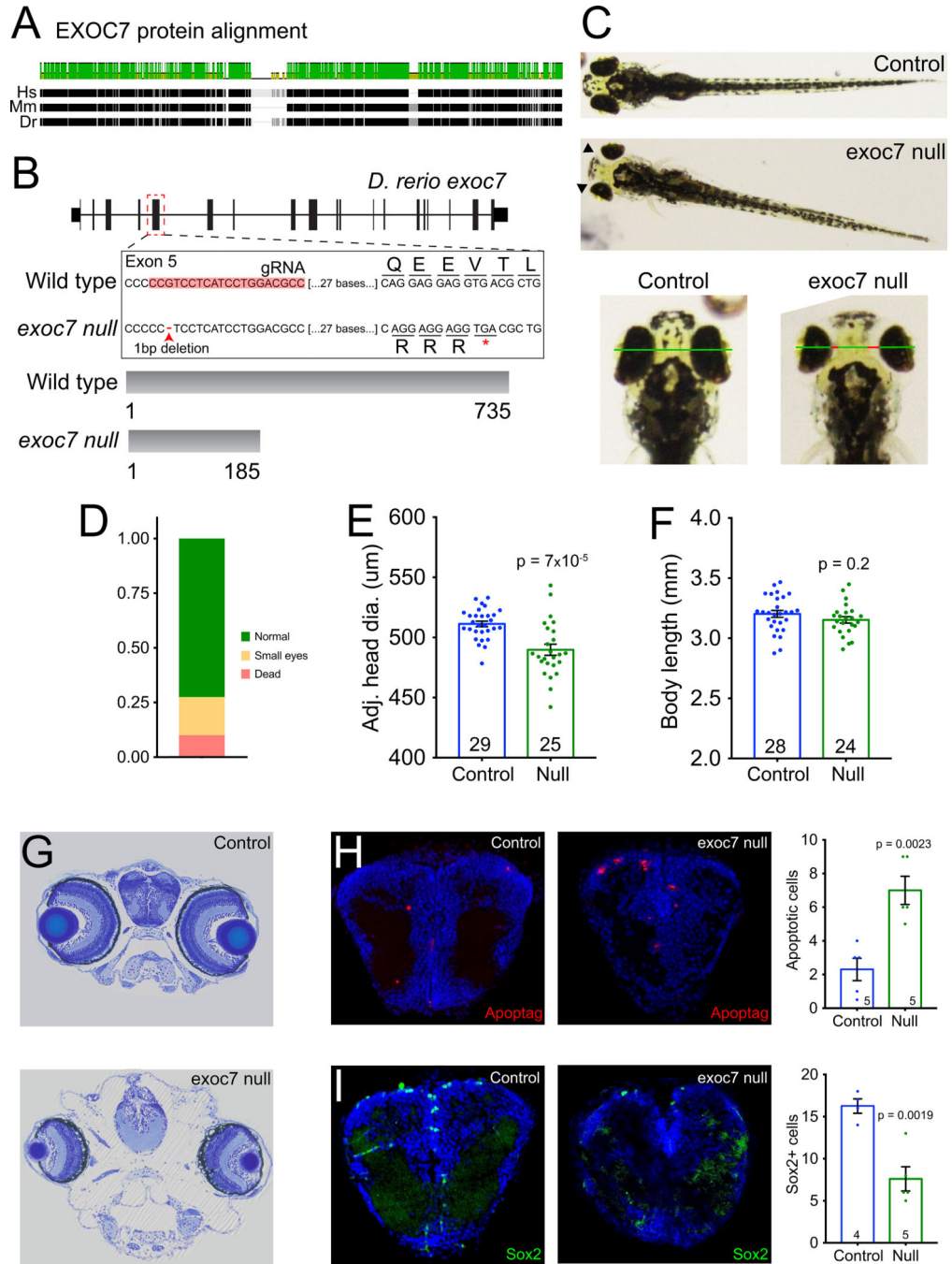
Figure 4. *Exoc7* is essential for zebrafish telencephalon development

(A) Exoc7 amino acid sequence is highly conserved between human, mice, and zebrafish. (B) *exoc7* 1bp frameshift deletion mutation in exon 5 is confirmed by DNA sequencing and predicted to cause a frame shift and subsequently a protein truncation through a premature STOP codon at amino acid 186. (C) *exoc7* homozygous mutant fish have gross developmental abnormalities by 5 dpf notably small eyes and head edema. Green line shows measurement of adjusted head diameter calculated by subtracting edema (red lines). (D) Heterozygous *exoc7* zebrafish crosses generated mutant fish (small eye/edema or dead) at

expected Mendelian ratio. Genotyping confirmed that phenotypically mutant larvae were homozygous for the *exoc7* mutation. (E) Quantification of adjusted head diameter, which is significantly reduced in homozygous mutant fish. (F) Body length is not significantly changed in homozygous mutant fish. (G) Toluidine blue stain of 5 dpf wild-type and *exoc7* mutant zebrafish. (H) Apoptag staining shows a significant increase of apoptotic cells in the *exoc7* mutant telencephalon. (I) Immunohistochemical staining of neuronal progenitors using Sox2. The number of Sox2+ progenitors is significantly decreased in the *exoc7* mutant telencephalon. P-values calculated with two-tailed t-test. Error bars represent SEM.

Table 1.

Variant summary for each family.

	Family I	Family II	Family III	Family IV	Family V
Maximum LOD score	1.93	Singleton	Non-consanguineous	2.9	2.5
Gene	*EXOC7*	*EXOC7*	*EXOC7*	*EXOC7*	*EXOC8*
Variant type	Splice variant	In-frame deletion	Splice variant & in-frame deletion	Missense	Frameshift deletion
Variant	Exon 7 splice acceptor (c.809-2A>G)	Ser48del (GGAT>G)	Exon 7 splice acceptor (c.809-2A>G) & Exon 10: c.1212_1226 delTGGGCTGATGCTTGA	Ala523Thr (C>T)	Asp607Ter (CCT>C)
Segregates in family	Yes	Yes	Yes	Yes	Yes
gnomAD frequency	2/251,414 alleles, heterozygous	2/276,426 alleles, heterozygous	Splice: 2/251,414 alleles, heterozygous & Deletion: absent	2/277,066 alleles, heterozygous	Absent