Biallelic variants in the small optic lobe calpain *CAPN15* are associated with congenital eye anomalies, deafness and other neurodevelopmental deficits.

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Abstract

Microphthalmia, coloboma and cataract are part of a spectrum of developmental eye disorders in humans affecting ~12 per 100,000 live births. Currently, variants in over 100 genes are known to underlie these conditions. However, at least 40% of affected individuals remain without a clinical genetic diagnosis, suggesting variants in additional genes may be responsible. Calpain 15 (CAPN15) is an intracellular cysteine protease belonging to the non-classical Small Optic Lobe (SOL) family of calpains, an important class of developmental proteins, as yet uncharacterised in vertebrates. We identified five individuals with microphthalmia and/or coloboma from four independent families carrying homozygous or compound heterozygous predicted damaging variants in *CAPN15*. Several individuals had additional phenotypes including growth deficits, developmental delay and hearing loss. We generated *Capn15* knockout mice that exhibited similar severe developmental eye defects, including anophthalmia, microphthalmia, and cataract, and diminished growth. We demonstrate widespread *Capn15* expression throughout the brain and central nervous system,

strongest during early development, and decreasing postnatally. Together, these findings demonstrate a critical role of *CAPN15* in vertebrate developmental eye disorders, and may signify a new developmental pathway.

Introduction

Developmental eye anomalies, including anophthalmia (absent eye), microphthalmia (small eye) and coloboma (disruption of the optic fissure closure), collectively known as AMC, are a genetically heterogeneous group of disorders affecting between 11.9 and 30 per 100,000 live births(1). Single gene alterations can underlie these conditions, the most frequent being in *SOX2*(2). To date, ~100 genes have been consistently associated with these phenotypes and are included in standard structural eye disorders diagnostic panels (https://panelapp.genomicsengland.co.uk/panels/509/). Data for congenital cataracts are similar, with an estimated prevalence of 1.91 to 4.24 per 10,000 children (3) and nearly 100 genes considered diagnostic (https://panelapp.genomicsengland.co.uk/panels/230/). There is a degree of genetic overlap in the underlying causes of AMC and cataract, as seen with variants in *GJA8* (4). However, at least 40% of individuals with AMC (depending on phenotype) remain without a genetic diagnosis (2, 5), indicating that additional unidentified genetic factors contribute to these conditions.

Calpains are intracellular cysteine proteases(6) with important roles in development. There are four conserved families: Classical, PalB, Transformer (Tra), and Small Optic Lobe (SOL), with 'Classical' being the best characterized. All calpain isoforms have a conserved catalytic domain and each family is characterized by unique domains: a C-terminal penta-EF

hand domain in Classical calpains; a C-terminal C2 domain in Tra calpains; an N-terminal MIT domain and an additional C-terminal C2-like domain in PalB calpains, and an N-terminal zinc finger domain that binds polyubiquitin(7) and a C-terminal SOL homology domain (SOLH) in SOL calpains(8, 9).

Embryonic mice with a homozygous disruption of the murine Classical calpain small subunit gene *Capn4* (causing disruption of the activities of Classical calpains Capn1 and Capn2) die midgestation, displaying defects in the cardiovascular system, hemorrhage, and accumulation of erythroid progenitors(10). Furthermore, pre-implantation embryonic lethality between the morula and blastocyst stage is observed in mice with a homozygous *Capn2* deletion (11). Tra-3 (Transformer 3) calpain was first discovered in the nematode *C. elegans*, as a sex determination gene(12). Later, studies suggested that Tra-3 cleaves Tra-2, which then interacts with transcription factor Tra-1 both directly and indirectly to promote female development(13, 14). In addition, *C. elegans* with a single nucleotide polymorphism in Tra-3 displayed a smaller body size when animals were grown at low temperature(15). In humans, variants in the orthologue of the Tra calpain (*CAPN5*) that hyperactivate its protease activity are associated with inflammatory vitreoretinopathy, hearing loss and developmental delay (16).

An atypical calpain, *CAPN15* (also known as *SOLH or SOL calpain*) is located on chromosome 16p13.3(17), a region previously implicated in inherited cataracts with microphthalmia(18). It was first characterized in the fruit fly *Drosophila*(19, 20), where loss of SOL calpain leads to a 50% reduction in the volume of the optic lobes due to degeneration and absence of certain classes of columnar neurons(20). In *Aplysia*, the orthologue of

CAPN15 is required for the induction of long-term plasticity implicated in non-associative memory, but not the long-term plasticity implicated in associative memory (21, 22).

Using whole exome sequencing (WES) we identified five individuals from four independent families with likely pathogenic homozygous or compound heterozygous variants in *CAPN15*, displaying microphthalmia and/or coloboma plus additional phenotypes including growth delay. To characterize the role of CAPN15 in vertebrates, we generated a *Capn15* knockout mouse. *Capn15*$^{(-/-)}$ mice displayed developmental eye anomalies including anophthalmia, microphthalmia and cataract, as well as reduced body weight similar to our affected individuals. Our data shows that *CAPN15* plays a critical role in eye development and growth in vertebrates. It is the first intracellular protease linked to AMC, indicating a new pathway and is an important gene to include on diagnostic gene panels for developmental eye disorders and cataract.

Results

Identification of five individuals with CAPN15 mutations and eye anomalies

WES of 55 individuals with developmental eye disorders from a UK cohort (Supplemental Methods) identified one individual with unilateral microphthalmia and bilateral coloboma with a previously unreported homozygous missense variant in *CAPN15* exon 13 (NM_005632.2:c.2905G>A; NP_005623.1:p.(Gly969Ser); chr16:602863 [hg19]) (Individual 1) (Figure 1A). Subsequently, four additional individuals with AMC spectrum disorders and biallelic *CAPN15* variants from three independent families were identified by WES. Individual 2, recruited to the Deciphering Developmental Disorders (DDD) project, was identified with compound heterozygous *CAPN15* variants. The first variant was a paternally inherited missense variant in exon 8 (NM_005632:c.2159C>T; NP_005623.1:p.(Ser720Phe);

chr16:601394 [hg19]), absent from the gnomAD database (http://gnomad.broadinstitute.org/)(23). The second variant was a rare maternally inherited missense variant in exon 10 (rs762523863; NM_005632:c.2398C>T; NP_005623.1:p.(Arg800Trp); gnomAD minor allele frequency = 0.00001986) (chr16:602103 [hg19]) (Figure 1B). Individual 3 carries a homozygous missense variant in *CAPN15* exon 13 (NM_005632.3:c.3083G>A; p.(Arg1028Lys); chr16: 603041 [hg19]) absent from the gnomAD database(23) (Figure 1C). Finally, individuals 4 and 5, siblings and offspring of distantly related parents, were ascertained through a Canadian cohort of 51 patients presenting with anomalies, with or without neurodevelopmental disorders (24). They harbour a homozygous missense variant in *CAPN15* exon 6 (NM_005632.3:c.1838C>T; NP_005623.1:p.(Ser613Leu); chr16:599467 [hg19]) (Figure 1D). This variant is extremely rare, reported in only 3 individuals on the gnomAD database (minor allele frequency 0.00001421), with no individuals with homozygosity for this variant observed. All five variants are predicted damaging by SIFT(25) and PolyPhen-2(26) and were validated by Sanger sequencing. In each case, the identified variants were inherited from asymptomatic heterozygous parents with no family history of eye anomalies. The siblings of Individual 1 were found to be asymptomatic heterozygous carriers. No genotyping data is available for the unaffected sibling of Individuals 4 and 5. The segregation of disease with biallelic *CAPN15* variants in these five families confirms a recessive pattern of inheritance. No other likely pathogenic variants in known AMC genes were identified in these individuals by WES.

Detailed phenotypic descriptions of each individual are provided in Table 1, Figure 2 and the Supplemental Results. All five affected individuals presented with AMC spectrum disorders; two individuals (1 and 2) had microphthalmia, four had coloboma (1, 3, 4, and 5), and Individual 1 also had a unilateral lens opacity (cataract). Interestingly, some individuals

showed additional delay in growth and development. Three individuals showed delayed growth, including short stature (1, 2, and 4), low weight (1 and 2), reduced head circumference (1 and 2) and clinical microcephaly affecting Individual 4. Furthermore, Individuals 2, 4 and 5 presented with developmental delay, in addition to cognitive delay (2) and autism (2 and 5). Finally, three individuals (2, 4 and 5) were diagnosed with hearing loss and both Individuals 2 and 4 had simple external ear features. It is also interesting to note the presence of multiple miscarriages in Family 1.

As the c.2905G>A (Individual 1) and c.3083G>A (Individual 3) variants affect the first and last nucleotides of *CAPN15* exon 13, respectively, we investigated their impact on splicing. For c.2905G>A we generated cDNA from blood samples from Individual 1 and their parents. However, PCR and Sanger sequencing across exons 11-14 revealed no alteration in splicing (data available on request). Similarly, for Individual 3 we observed no impact on splicing after performing RTPCR on RNA extracted from LCLs derived from this patient (data available on request).

Within *CAPN15*, all five variants are located within the C-terminal portion of the protein and affect amino acids conserved across multiple vertebrate species (Figure 3). However, only p.(Ser613Leu) (Individuals 4 and 5) and p.(Ser720Phe) (Individual 2) are located within an annotated region of the protein, both lying within the Calpain catalytic domain. As no 3D structure for CAPN15 is available, we used I-TASSER (27-29) to generate a predicted structure. Site Directed Mutator (SDM; http://marid.bioc.cam.ac.uk/sdm2/prediction) was applied to this model to predict the impact of the variants on CAPN15 stability (Table 2). This indicated that p.(Gly969Ser) and p.(Arg1028Lys) may decrease protein stability ($\Delta\Delta G$ = -3.27 and -1.15, respectively), whereas p.(Ser613Leu) is predicted to increase stability ($\Delta\Delta G$

= 1.85). Interestingly, both of the mutations predicted to decrease CAPN15 stability are in the highly conserved SOLH domain that is present only in SOL calpains and whose structure and function is unknown.

Capn15$^{(-/-)}$ mice have anophthalmia, microphthalmia and cataract

The protein encoded by the human CAPN15 gene (NP_005623.1) has 87.4% identity/90.9% similarity to the mouse orthologue NP_001334263 using EMBOSS Needle (30). Mouse embryonic stem cells with a *lacZ*-Neo cassette inserted into the *Capn15* locus were obtained from the International Mouse Phenotyping Consortium (IMPC)(31) and used to generate *Capn15* knockout mice (Supplemental Figure 1A). Disruption of *Capn15* was confirmed by Western blotting of brain homogenates using an antibody raised against the C-terminal of the protein (Supplemental Figure 1B).

In a cross of *Capn15* heterozygous mice, the incidence of *Capn15$^{(-/-)}$* pups was 12%, significantly lower than the expected 25% (12%, p=1.28e^{-5}, Chi Square test; WT n=52, *Capn15$^{+/-}$* n=155, *Capn15$^{-/-}$* n=29). This indicates that homozygous loss of the gene results in decreased viability, and is of further interest given the presence of multiple miscarriages in Family 1. Furthermore, *Capn15$^{(-/-)}$* mice that were successfully weaned weighed 11% (+/-3% SEM) less than their littermate WT or heterozygous mice (p=0.003 and p=0.0006, respectively. Student's t-test; WT n=45, *Capn15$^{+/-}$* n=71, *Capn15$^{-/-}$* n=18), recapitulating the delayed growth and development in several of our affected individuals (Figure 4A). Most significantly, homozygous *Capn15* KO mice displayed a range of developmental eye disorders overlapping those observed in the 5 affected individuals with biallelic *CAPN15* variants. These included anophthalmia (18%), microphthalmia (26%) and cataract (31%) (Figure 4B-E). Less than 6% of heterozygous mice overall displayed developmental eye

disorders (anophthalmia 1%, microphthalmia 2%, and cataract in 2%). No significant difference was observed between the left and right eyes of adult *Capn15* KO mice (>12 weeks of age) (p=0.15) and there was no preference for bilateral or unilateral presentation. Therefore, after pooling left and right eyes, the presence of these phenotypes was significantly increased in *Capn15* KO mice compared to heterozygous or WT mice (***p<0.0001; WT n=30, $Capn15^{+/-}$ n=160, $Capn15^{-/-}$ n=90 including offspring from parents who were both $Capn15^{-/-}$; Figure 4F). This difference in eye phenotype was present in mice at 6 and 12 weeks of age, suggesting that these anomalies were the result of aberrant development.

Capn15 is highly expressed in the brain and eye

To investigate the role of *Capn15* in the brain during development, *lacZ* expression was examined in $Capn15^{(lacZ\text{-}Neo)}$ heterozygous mice. X-gal staining of sagittal sections of E12 embryos showed that *Capn15* was primarily expressed in the outer layer of the central nervous system, in particular the mantle zone of the pallium and subpallium (forebrain), and the mantle zone of the rhombomere (hindbrain) (Figure 5A). At E18 *Capn15* was expressed widely in the nervous system with high levels in the subventricular zone, immediately next to the ventricular zone. Expression was strongest in the deep cortical layer of the cerebral cortex, and neuroepithelium layer in the hippocampus, but not the pyramidal layer. By P3, *Capn15* was ubiquitously expressed in the brain from the olfactory bulb to the cerebellum and also within the retina, particularly in the ganglion cell layer (Figure 5C-D). To further explore the pattern of expression over time, *Capn15* protein expression in the rat brain was examined over time using immunoblotting. Here, expression was found to be highest during embryonic development (E14-E18 brains), decreasing significantly after birth (P0-adult) (Figure 5E, F).

Discussion

Our data demonstrate the importance of the protease *CAPN15* in mammalian eye and brain development. We identified likely pathogenic recessively inherited variants in five individuals with AMC and/or cataract and additional phenotypes including delayed growth and development. These phenotypes were recapitulated in a mouse model, with further data supporting expression of the gene during brain development.

Our cases showed microphthalmia and/or coloboma, while 18% of knockout mice had the more severe anophthalmia. The milder phenotypic range displayed in the humans may be explained by all five variants being missense. These variants could impact protein function by destabilising protein structure, affecting the calpain catalytic activity, or its regulation by the SOLH domain. This is likely to be less damaging to overall function than the total loss of the gene in the mouse knockout. This may also account for the presence of AMC phenotypes at low frequency in heterozygous mice, which were not detected in humans heterozygous for the missense variants. Alternatively, it may reflect the small sample group of humans or interspecies variation. However, given the lack of a functional assay for CAPN15, it is difficult to determine whether the variability between mouse and human phenotypes and/or amongst the individuals carrying distinct missense changes is due to different effects of these variants on the protein. The penetrance of the eye phenotype in the mouse was also variable, even with a complete knockout, suggesting a complex relationship between the loss of CAPN15 and the eye phenotype. Therefore, screening of additional individuals with AMC would help to determine both the full phenotypic range associated with variants in this gene, as well as potential genotype-phenotype correlations. Furthermore, only Individual 1 had cataract, while 31% of mice had this phenotype. This may reflect ascertainment bias, as none

of the participants or cohorts included in this study was recruited on the basis of this phenotype. However, recent reports show that variants in *GJA8*, a gene associated with isolated cataract, are also a significant cause of microphthalmia, accounting for over 1% of cases of AMC in large cohorts (4).

Developmental delay and hearing loss were present in three individuals with *CAPN15* variants. Our data shows strong embryonic expression of *Capn15* in the brain, suggesting that it is important in neurodevelopment. Therefore, the *CAPN15* variants may underlie these phenotypes as well as eye anomalies. Similarly, growth delay was observed in three individuals and the *Capn15*$^{-/-}$ mice also exhibited a significant growth deficit.

There is little known about the function of the SOL calpain family, of which CAPN15 is the sole human member. This family diverged from other calpain families before the origin of metazoans and is conserved in all metazoans, suggesting a fundamental role for the protein (9). Two of the identified variants are in conserved residues in the calpain catalytic domain present in all members of the calpain family, while a third variant lies adjacent to it. However, while the N-terminal of the SOL calpain has been shown to bind to poly-ubiquitin(7), there have been no functional studies on the calpain or *SOLH* domain of this family of genes. In *Drosophila*, the large loss of neurons in the optic lobe was thought to be due to degeneration(20), but a role for Capn15 in early differentiation during development was not excluded. In *Aplysia*, SOL calpain is important for the induction of plasticity associated with non-associative learning(21), perhaps due to cleavage of Protein Kinase Cs (PKCs) into persistently active Protein Kinase Ms (PKMs)(7, 32). Further investigation of how Capn15 performs these functions may also shed light on its role during eye development. Furthermore, as other calpains function to regulate transcription through

regulated cleavage of transcription factors (33), and since many genes underlying ocular anomalies are transcription factors(2), investigation of the role CAPN15 plays in early developmental transcriptional pathways may also prove a valuable avenue of research.

Together, our data meet criteria for inclusion of *CAPN15* in clinical genetic diagnostic testing for AMC according to both the Clinical Genome Resource (ClinGen) (www.clinicalgenome.org)(34, 35) and Genomics England PanelApp (https://panelapp.genomicsengland.co.uk/#!Guidelines). Therefore, these data will contribute to improved care for AMC patients and their families.

Conclusions

We demonstrate for the first time the importance of *CAPN15* in mammalian eye development, identifying likely pathogenic variants in five individuals with AMC from four families, supported by a mouse KO model and embryonic expression studies. These findings translate to clinical benefit by adding a new gene to clinical diagnostic testing, and potentially a new pathway in the increasingly complex network involved in human eye development.

Materials and Methods

Ethics approval and consent to participate

The UK cohort of 316 individuals with ocular anomalies was recruited as part of a national 'Genetics of Eye and Brain anomalies' study (REC 04/Q0104/129). Informed consent was obtained according to the tenets of the Declaration of Helsinki. Individual 2 was recruited into the Deciphering Developmental Disorders (DDD) Study, which has UK Research Ethics Committee approval (10/H0305/83, granted by the Cambridge South REC, and GEN/284/12 granted by the Republic of Ireland REC). For individual 3, informed written consent was obtained from all participants under an IRB approved protocol (KFSHRC RAC# 2070023). Individuals 4 and 5 are members of a Canadian study approved by the institutional ethics review board of Université de Sherbrooke (project 12–167). All participants or their legal guardians provided written consent. All studies on mice were approved by the Montreal Neurological Institute/McGill University Animal care and use committee protocol 2009–5784

Capn15 antibody

Polyclonal antibodies were raised in rabbits against the carboxy terminal of Capn15 using the following epitope: CDVAGLHGPRPL. A cysteine residue was added to the N-terminal of the peptide to enhance coupling. Peptides were coupled to KLH-maleimide and SulfoLink coupling resin (Thermo Fisher Scientific) according to manufacturer's instruction. After conjugation to KLH-maleimide, rabbits were injected and after four boosts the final serum was affinity purified on SulfoLink columns as previously described(36).

Generation of a Capn15 KO mouse

We obtained embryonic stem cells with a *lacZ*-Neo cassette inserted into the mouse *Capn15* locus from the International Mouse Phenotyping Consortium (IMPC)(31). The strategy used by the consortium was to insert a cassette that provides a strong exon entry site coupled with a stop codon, with *lacZ* produced from internal ribosome entry site translation (31, 37). To remove this cassette, these mice were bred with mice that contain flippase (FLP) recombinase that recognizes a pair of FLP recombinase target (FRT) sequences that flank the genomic region containing this cassette leaving lox sites surrounding the calpain catalytic domain exons 4-6 (based on NCBI reference sequence NM_001347334.1). These mice were then bred against mice expressing germline Cre recombinase to remove the floxed exons and generate a KO line (Supplemental Figure 1).

Dissections

Adult mice were transcardially perfused with ice-cold phosphate-buffered saline (PBS) followed by 4% (wt/vol) ice-cold paraformaldehyde (PFA) in PBS. Brains were post-fixed in 4% PFA for 45min at 4^{o}C, rinsed in PBS, and cryoprotected in 30% sucrose/PBS overnight at 4^{o}C. The following day, brains were embedded in Tissue-Plus^{TM} O.C.T. compound (Fisher Healthcare) and flash frozen in 2-methylbutane chilled in dry ice. The brains were kept at −80°C until further use.

X-gal staining

Sections of 20µm were incubated overnight at 37°C in solution containing 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 2 mM $MgSO_4$, 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 0.2% NP-40, 0.1% sodium deoxycholate, and 1.5 mg/ml X-gal. Sections were rinsed in PBS, washed in ethanol (50% for 1min, 70% for 1min, 95% for 1min and 100% for 2X1min), cleared in xylene, and mounted with Permount (Thermo Fisher Scientific).

Western blotting

Embryonic brains were homogenized manually in lysis buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 6 mM $MgCl_2$, 2 mM EDTA, 1.25% NP-40, 0.125% SDS, 25 mM NaF, 2 mM $Na_4P_2O_7$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mg/ml leupeptin, and 4 mg/ml aprotinin. Before loading, 5X sample buffer was added to the lysate and samples were incubated at 95^{o}C for 5min. Proteins were resolved by SDS-PAGE on Bis-Tris gel and transferred to nitrocellulose membrane (Bio-Rad). The blots were blocked in TBST (TBS + 0.1% Tween) containing 4% skim milk for 30min at room temperature and then incubated with primary antibodies overnight at 4^{o}C. After washing 3 times with TBST, the blots were incubated with HRP-conjugated secondary antibodies for 1 hour at RT, and washed again 3 times in TBST. The Western Lightning Plus-ECL kit (NEL103001EA; PerkinElmer LLC Waltham, MA USA) was used as per manufacturer's instructions to detect protein bands. The primary antibody used was homemade rabbit anti-Capn15 antibody (1:1000) raised against the C-terminus of Capn15. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000). Antibodies were diluted in Tris buffered saline with Tween containing 4% skim milk powder.

Eye phenotype quantification

Mice eyes were examined and grouped as follows: seems normal, obvious cataract, small eye and no eye. This categorisation was performed for both eyes of each mouse. The analysis was performed without the knowledge of the genotype of the mice.

Quantification of immunoblotting

Immunoblots were scanned and imaged using the public domain Image J program developed at the U.S. National Institute of Health (https://imagej.nih.gov/ij/). We calibrated our data with the uncalibrated optical density feature of NIH image, which transforms the data using the formula $\log_{10}[\frac{x}{254}]$, where x is the pixel value (0–254). We used the Ponceau image for each gel to normalize the amount of SOL calpain in brains at different ages to the amount of SOL calpain in E12-E14 brains run on the same gel.

Whole Exome Sequencing

A UK cohort of 316 individuals with ocular anomalies, principally AMC, was recruited as part of a national 'Genetics of Eye and Brain anomalies' study (REC 04/Q0104/129). Informed consent was obtained according to the tenets of the Declaration of Helsinki. Patients were screened by indication in known AMC genes. Fifty-five individuals from the cohort were screened by whole exome sequencing (WES) as described previously(38, 39). Individual 2 was recruited into the Deciphering Developmental Disorders (DDD) Study, which has UK Research Ethics Committee approval (10/H0305/83, granted by the Cambridge South REC, and GEN/284/12 granted by the Republic of Ireland REC).

For individual 3, WES was performed as previously described (40). Sanger sequencing and segregation analysis were completed. Informed written consent was obtained from all participants under an IRB approved protocol (KFSHRC RAC# 2070023).

Individuals 4 and 5 are siblings who were identified through whole-exome sequencing of a Canadian cohort of 51 patients presenting dysmorphisms with or without neurodevelopmental disorders (24).

The following primers were used for sequencing: For Individual 1: Forward Primer CCATCATCCTGCTCACCGA and Reverse Primer GGCACGCTATCCTGGGTAC; For Individual 2 Exon 8: Forward Primer GCAACATGAAGGTGGACGAT and Reverse Primer AGCTGCCGTTCCAGGAGAAA; For Individual 2 Exon 10: Forward Primer GGAGGGCTTCCTATTATAGG and Reverse Primer AACACCAGGATGCACAGGTC and For Individual 3: Forward Primer GCAGGGGTCCCGAGA and Reverse Primer CAGACCGGCGACCTCT.

cDNA and splicing studies

Blood samples were collected from Individual 1 and her parents, RNA was extracted using a RNeasy Mini kit (Qiagen) and cDNA generated using a high capacity reverse transcriptase kit (Applied Biosystems). To examine the impact of the variant on splicing, forward and reverse PCR primers were designed mapping to exons 11 and 14, respectively (Forward: GTCAAGAAGTTCGTCAGCTG, Reverse: CTGTCCAGTCACTGAGGAAG).

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or http://www.ddduk.org/access.html (http://www.ddduk.org/access.html) for full acknowledgement.

Conflicts of Interest

The authors state no conflicts of interest in this study.

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Figure Legends

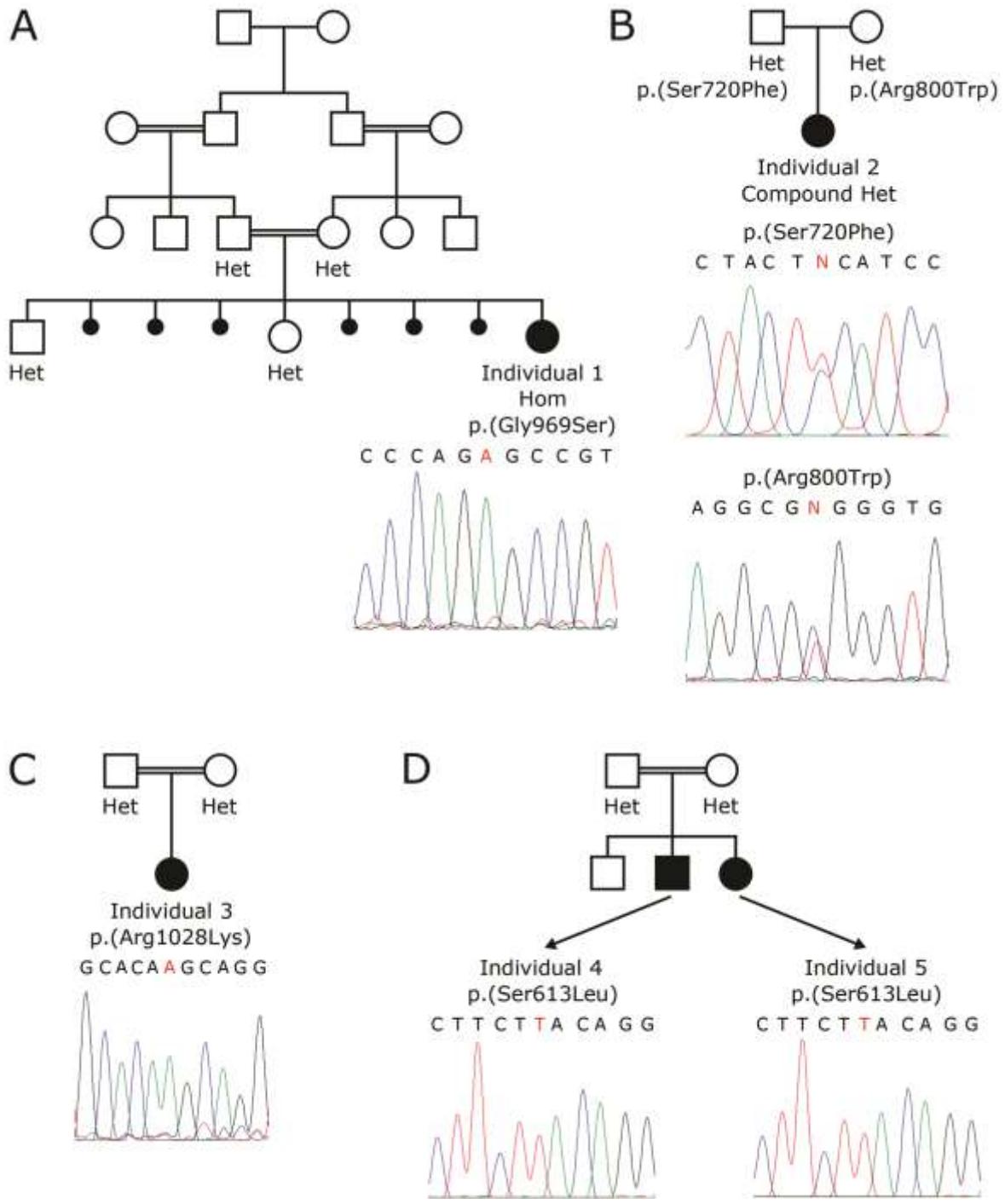
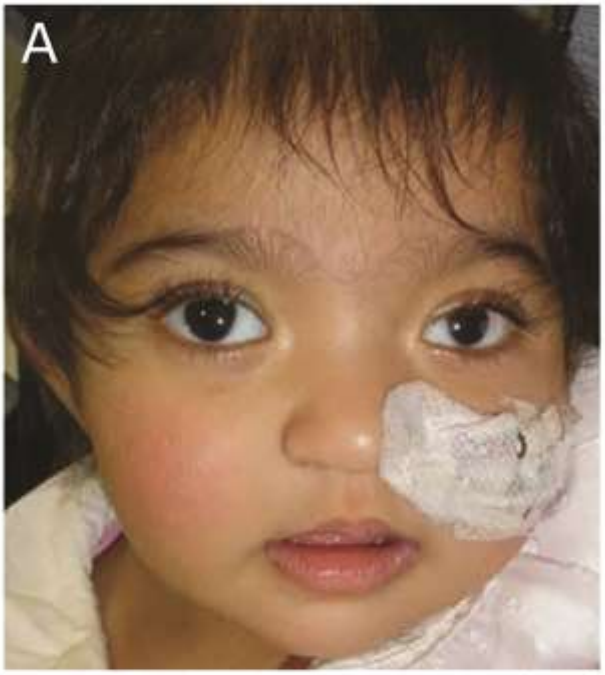


Figure 1. Pedigrees of the five Individuals with *CAPN15* variants. Chromatograms of Sanger sequencing validation of the variants in affected individuals shown. Position of variant indicated in red. Note that sequencing for Individual 3 was performed from cDNA. **(A)** Individual 1 **(B)** Individual 2 **(C)** Individual 3 **(D)** Individuals 4 and 5.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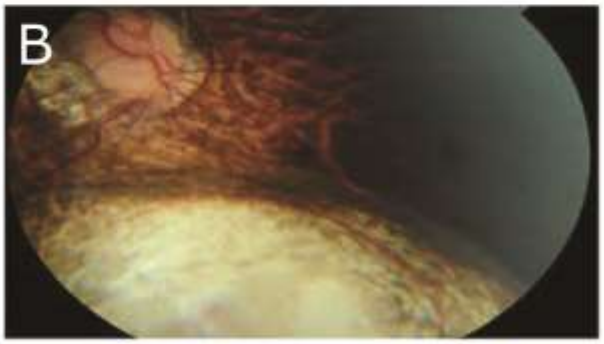
B


C


D

Figure 2. Phenotypes of Individuals 1, 3 and 4. (A) Right iris and chorioretinal coloboma, and left mild microphthalmia with an iris and chorioretinal coloboma involving the disc in Individual 1. **(B, C)** Inferonasal chorioretinal coloboma (B) and iris coloboma of the right eye (C) in Individual 3. **(D)** Bilateral iris and chorioretinal coloboma (more prominent in the left eye), and dysmorphic features including left ptosis, left simple ear, and prominent columella in Individual 4.

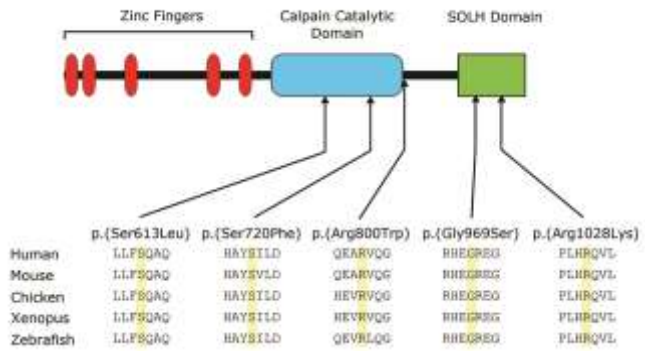

Figure 3. Schematic of CAPN15 showing the location of the variants identified in Individuals 1-5. Zinc fingers shown in red, the calpain catalytic domain in blue and the SOLH domain in green. The conservation of the affected amino acids in human, mouse, chicken, *Xenopus* and zebrafish is indicated.

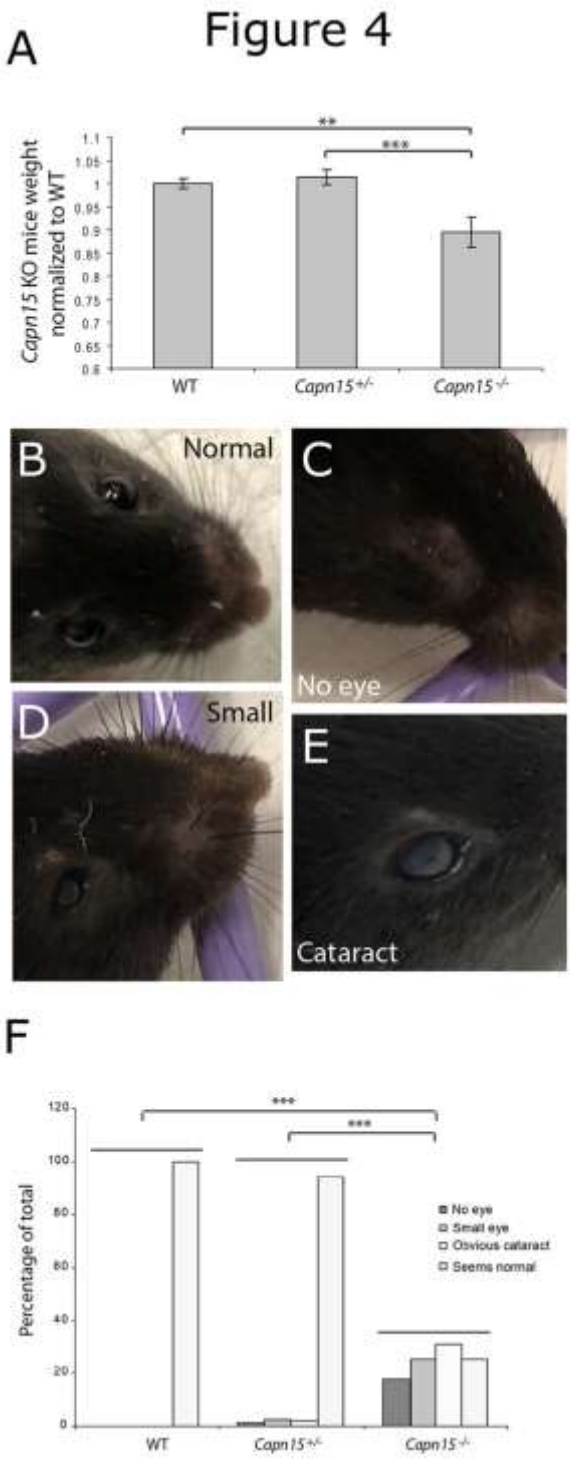

Figure 4. *Capn15* KO mice display growth and eye anomalies (A) Mean weights of *Capn15* KO and *Capn15* heterozygous mice after weaning normalized to the mean weight of WT mice. Error bars represent SEM. **(B-E)** Representative photographs of the eye phenotypes of *Capn15* KO mice; normal (B), anophthalmia (C), microphthalmia (D) and cataract (E). **(F)** Comparison of the percentage of eye anomalies in WT, *Capn15*

heterozygous and *Capn15* KO mice at more than 12 weeks of age. ** = p=0.003, *** = p=0.0006.

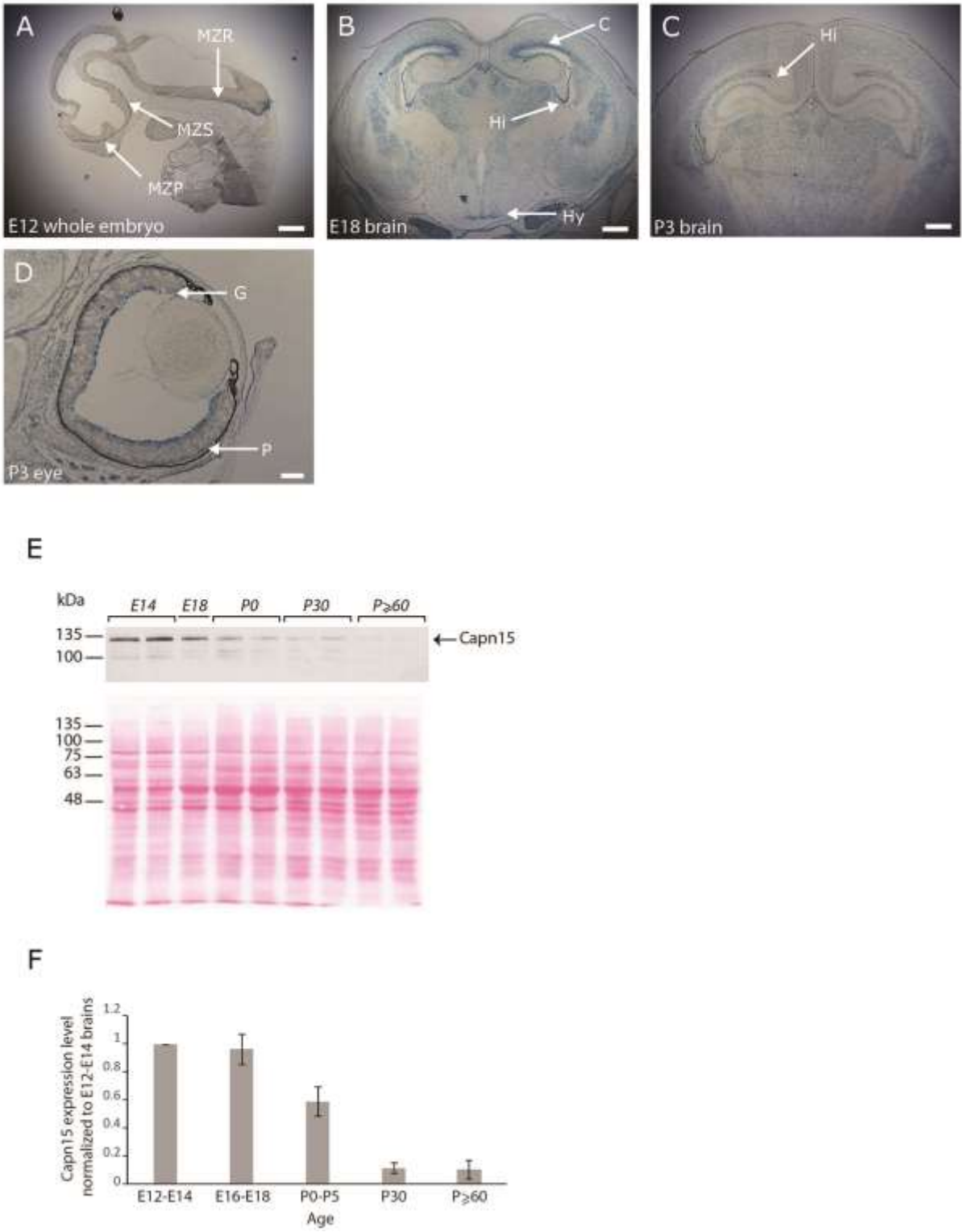

Figure 5. *Capn15* is enriched in rodent brain and eyes during early development. (A-D) X-gal staining of sections from E12, E18 and P3 *Capn15*$^{(lacZ\text{-}Neo)}$ heterozygous mice. Scale

bar is 200µm. (A) Sagittal section of whole embryo at E12 showing *Capn15* expression in the mantle zones of the pallium, subpallium, and rhombomere. (B) Coronal section of the brain at E18 showing expression in the cerebral cortex, hippocampus. (C) Coronal section of the brain at P3 showing generalised expression, including the hippocampus. (D) Sagittal section of the eye at P3, highlighting expression in the ganglion cells of the retina. (E) Western blots using protein extracts from rat brains at E14, E18, P0, P30 and P≥60 showing decreasing Capn15 expression with increasing age. Experiment was performed in triplicate. **(**F) Quantification of *Capn15* expression in rat brain. Capn15 expression was normalized to E12-E14 brains; E12-E14 n=3, E16-E18 n=4, P0-P5 n=4, P30 n=2, and P≥60 n=3. Error bars represent SEM. Abbreviations: C (cortex), G (ganglion cell layer), Hi (hippocampus), Hy (hypothalamus), MZP (mantle zone of pallium), MZR (mantle zone of rhombomere), MZS (mantle zone of subpallium), P (photoreceptor cell layer).

Table 1: Phenotypes of the five human individuals identified carrying homozygous or compound heterozygous *CAPN15* variants. Individuals 4 and 5 are siblings. y (years).

Individual	**1**	**2**	**3**	**4**	**5**
Variant	p.(Gly969Ser)	p.(Ser720Phe) p.(Arg800Trp)	p.(Arg1028Lys)	p.(Ser613Leu)	p.(Ser613Leu)
Eye	Unilateral microphthalmia, bilateral coloboma, unilateral lens opacity	Bilateral microphthalmia, iridio-corneal adhesions	Bilateral myopia, unilateral coloboma	Bilateral coloboma, unilateral ptosis	Unilateral coloboma
Growth	Poor weight gain	Delayed	Normal	Short stature	Normal
Developmental delay	Normal	Cognitive delay, autism	Normal	Global delay	Developmental delay, autism spectrum disorder

Head circumference	19th % (3.5y)	5th % (14y)	Normal	Microcephaly	Normal
Hearing	Normal	Hearing loss	Normal	Deaf	Deaf
Other	Laryngeal cleft, dysphonia, hirsute, sacral dimple	Bicuspid aortic valve, horseshoe kidney, simple ears, unusually shaped small pituitary	-	Facial dysmorphism, imperforate anus, unilateral simple ear, prominent columella	Imperforate anus with vaginal fistula, hemivertebrae

Table 2: Site Directed Mutator (SDM; http://marid.bioc.cam.ac.uk/sdm2/prediction) analysis of the impact of CAPN15 variants on protein stability.

Variant	ΔΔG	Stability
p.S613L	1.85	Increased
p.S720F	0.52	Increased
p.R800W	0.24	Increased
p.G969S	-3.27	Reduced
p.R1028K	-1.15	Reduced