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Identification of a Novel *GJA8* (Cx50) Point Mutation Causes Human Dominant Congenital Cataracts

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Hereditary cataracts are clinically and genetically heterogeneous lens diseases that cause a significant proportion of visual impairment and blindness in children. Human cataracts have been linked with mutations in two genes, *GJA3* and *GJA8*, respectively. To identify the causative mutation in a family with hereditary cataracts, family members were screened for mutations by PCR for both genes. Sequencing the coding regions of *GJA8*, coding for connexin 50, revealed a C > A transversion at nucleotide 264, which caused p.P88T mutation. To dissect the molecular consequences of this mutation, plasmids carrying wild-type and mutant mouse ORFs of *Gja8* were generated and ectopically expressed in HEK293 cells and human lens epithelial cells, respectively. The recombinant proteins were assessed by confocal microscopy and Western blotting. The results demonstrate that the molecular consequences of the p.P88T mutation in *GJA8* include changes in connexin 50 protein localization patterns, accumulation of mutant protein, and increased cell growth.

Cataracts are clouding of the lens inside the eye which leads to a decrease in vision. Types of cataracts can be defined by the age at onset: congenital, juvenile, presenile and senile or age-related cataracts. Congenital cataracts are responsible for 10–30% of childhood blindness[1–3]. They can be subdivided according to their anatomical location within the lens, their appearance, and most commonly by a combination of these two parameters[1,4]. Congenital cataracts are a genetically heterogeneous lens disease. Currently, there are about 39 genetic loci identified as causing isolated or primary cataracts have been mapped and 18 distinct genes responsible for nonsyndromic hereditary cataracts have been identified including *BFSP1, BFSP2, CHMP4B, CRYAA, CRYAB, CRYBA1/A3, CRYBA4, CRYBB1, CRYBB2, CRYBB3, CRYGC, CRYGD, CRYGS, GJA3, GJA8, HSF4, LIM2, MAF, MIP, PITX3* and *LIM2*[5–8].

In the present study, we sought to identify the genetic defect in a four-generation family in which congenital cataracts are present in an autosomal dominant pattern. Gap junctional intercellular communication (GJIC) plays an important role in the maintenance of tissue independence and homeostasis in multicellular organisms. Gap junctions are composed of connexin (Cx) molecules[9]. Because cataracts are associated with mutations in two genes,*GJA3* and *GJA8*, coding for connexin 50 (Cx50) and connexin 46 (Cx46), respectively[10], mutations were directly screened in recruited family members by PCR of *GJA3* and *GJA8*.

To dissect the molecular consequence of the mutation identified here, the ORFs of wild-type and mutant mouse *Gja8* gene coding for Cx50 were inserted into eukaryotic gene expression vectors and ectopic expression of the recombinant proteins and cellular localization of corresponding proteins were assessed by confocal microscopy and Western blotting.

Results

Clinical data. The family comprised 4 affected individuals from a four generation pedigree(Figure 1A).The proband is a 17-year-old male (IV: 3). His phenotype is bilateral complete opacification of the fetal, infantile, and adult nucleus and the cortex, and its phenotype is of total cataracts (Figure 1B), with low vision (20/200). Other family members have similar cataracts patterns. There was no family history of other ocular or systemic abnormalities. Hospital records indicated that the opacity either was present at birth or developed during the first few months of life usually, but did not progress with age.

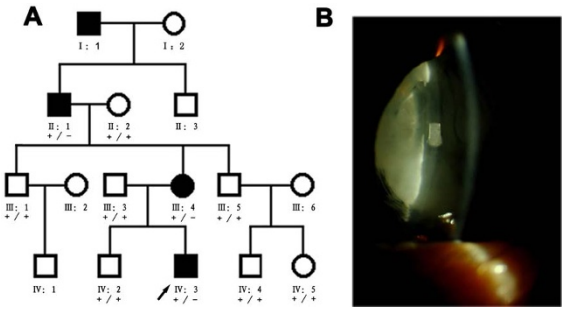

Figure 1 | **Cataracts pedigree and phenotype.** (A). Cataracts pedigree. Squares and circles symbolize males and females, respectively. Black and white lines denote affected status and unaffected status, respectively. Arrow indicates proband. Individuals underlined in blue represent those enrolled in the study. + represents wild-type *GJA8* allele, − represents allele with mutation. (B). Photographs of affected individuals of this family. The phenotype of the proband (IV: 3) is bilateral complete opacification of the fetal nucleus and the cortex; its phenotype is total cataract.

p.P88T Mutation in *GJA8* identification and analysis. Direct cycle sequencing of the amplified fragments of the *GJA8* gene in one affected individual (IV: 3) identified a C > A transversion in exon 2 at nucleotide 264 (Figure 2A) with no other changes in the coding sequence of GJA8. This transversion caused a substitution of Threonine (T) for Proline (P) at codon 88 (p.P88T). Results of additional sequencing demonstrated that this mutation co-segregated with all affected individuals in the family, and was not observed in any of the unaffected family members. Denaturing HPLC analysis confirmed this mutation, which cosegregated with all affected individuals in the family, and this mutation was not observed in any of the unaffected family members or 100 normal controls. The remainder of the coding sequence, including that of *GJA3*, did not show any sequence change. The p.P88T mutation was not present in multiple databases, including the dbSNPs (v130), HapMap, 1000 Genome, and 702 in-house exome data.

Multiple-sequence alignments of Cx50 in various species were obtained (Figure 2B). We found that codon 88, where mutation (p.P88T) occurred, is located within a highly conserved region. To date, 33 mutations have been identified in human Cx50; we summarize all reported mutations in Figure 3. The prevalence of changes in proline 88 indicates that this residue is a mutation hot spot. The p.P88T substitution changes a nonpolar residue to a polar residue at this key site in the protein. The online bioinformatics software SIFT (http://sift.jcvi.org/) predicts a deleterious effect of this substitution. Taken together, these data indicate that the p.P88T substitution is a causative disease mutation rather than a simple polymorphism.

Different expression pattern and high expression level of mutant protein. To dissect the molecular consequences of the mutation identified in this family, we generated the patients-associated Flag-tagged and GFP-tagged Cx50 mutants. Specifically, genes coding for wild-type and mutant Cx50 were inserted into two eukaryotic gene expression vectors (pSIN and pEGFPc1), respectively. Meanwhile, Flag, which is a robust and commonly used epitope, was also added to the pSIN construct.

Confocal immunocytochemical analysis showed that cells transfected with wild-type (WT) Cx50 had characteristic punctate plasma membrane staining. Limited staining was also detected intracellularly in 293 cells (Figure 4A). In contrast, the localization of proteins affected by the p.P88T connexin mutation was different, with lower level of plasma membrane staining and evidence of accumulations of the protein in the cytoplasm and plasma membrane. A similar gene expression pattern has been observed with a different cell line (human lens epithelial cells, HLE cells; Figure 4B) and a different tag (EGFP; Figure 4C, 4D).

We further took advantage of a membrane-tagged expression of RFP, encoded by the pmTmG plasmid (original from Addgene http://www.addgene.org/17787/), and used it as control for labeling the plasma membrane, which confirms that the p.P88T Cx50 mutant protein has a distinct expression pattern of connexins (see Supplementary Fig. S1online), compared with that of wild-type. This finding is consistent with a previous study that demonstrated ectopic expression of P88S and P88Q Cx50 mutant protein in an expression pattern that is distinct from wild-type[4,11].

Western blotting analysis of extracts from the cells expressing connexins clearly showed a much higher expression of mutated connexins (Figure 5). Furthermore, several different lengths of Cx50 (about 60 kd, 50 kd, 43 kd) have been detected, possibly as a result of cleavage by caspase-3-like protease during expression. In addition, protein post-translational modification may exist[12].

Mutated Cx50 has a positive effect on cell growth. Current evidence suggests that Cx50 is likely to play a role in cell growth during lens development. We therefore investigated the effects of the mutation on this process by observing the cell colony size in puromycin selected cells with stable expressing Flag-tagged Cx50 proteins. We found that stable ectopic expression of mutated Cx50 led to a much larger colony size when compared with wild-type control (Figure 6). Furthermore, cell growth curves (see Supplementary Fig. S2 online) also showed higher rates of cell growth for lines tranfected with mutated Cx50 protein. These data indicate that at least, mutated Cx50 has a positive effect on cell growth *in vitro*.

Discussion

More than 90% of the soluble proteins in the lens are crystallins. In our previous studies, we identified several mutations in different

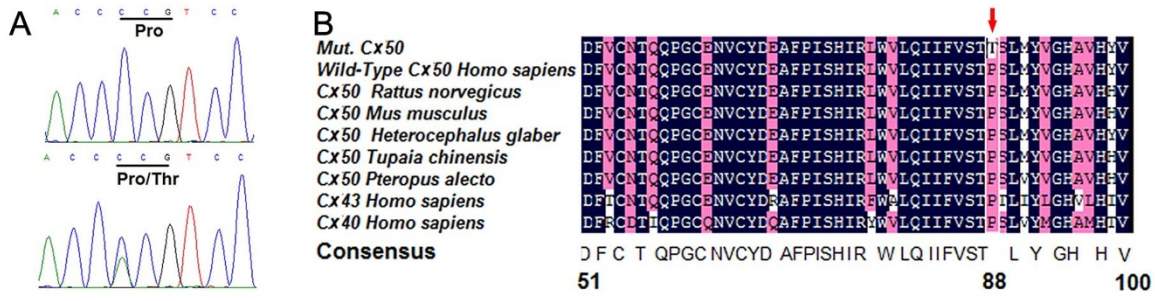

Figure 2 | **Mutation analysis.** (A). DNA sequence chromatogram analysis. DNA sequence chromatograms of the unaffected members (top) and affected members (bottom) in a family with autosomal dominant total cataracts. A single base alteration of C > A transversion in exon 2 causes a conservative substitution of Pro to Thr at codon 88 (p.P88T). (B). Multiple protein sequence alignments. Multiple-sequence alignment of Cx50 from different species and Cx family members (Cx43, Cx40) from human revealed that codon 88, where the mutation (p.P88T) occurred, was located within a highly conserved region. The "mut." sequence indicates the sequence with the mutation detected in this family.

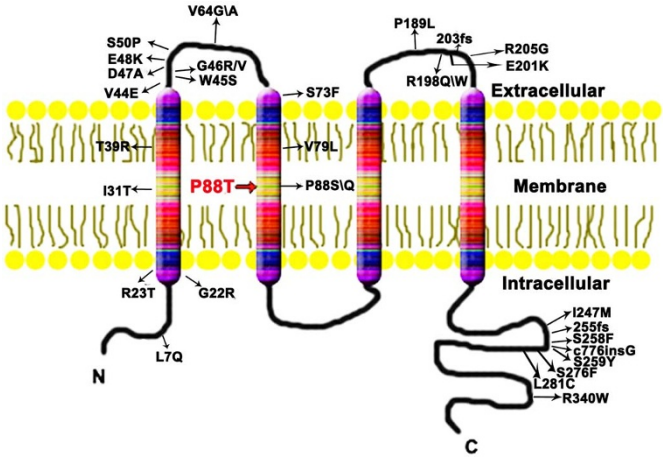

Figure 3 | Schematic diagram of Cx50 reported mutations. Schematic diagram of Cx50 protein containing all reported human mutations associated with cataracts. The identified mutation in this study is marked with red arrow.

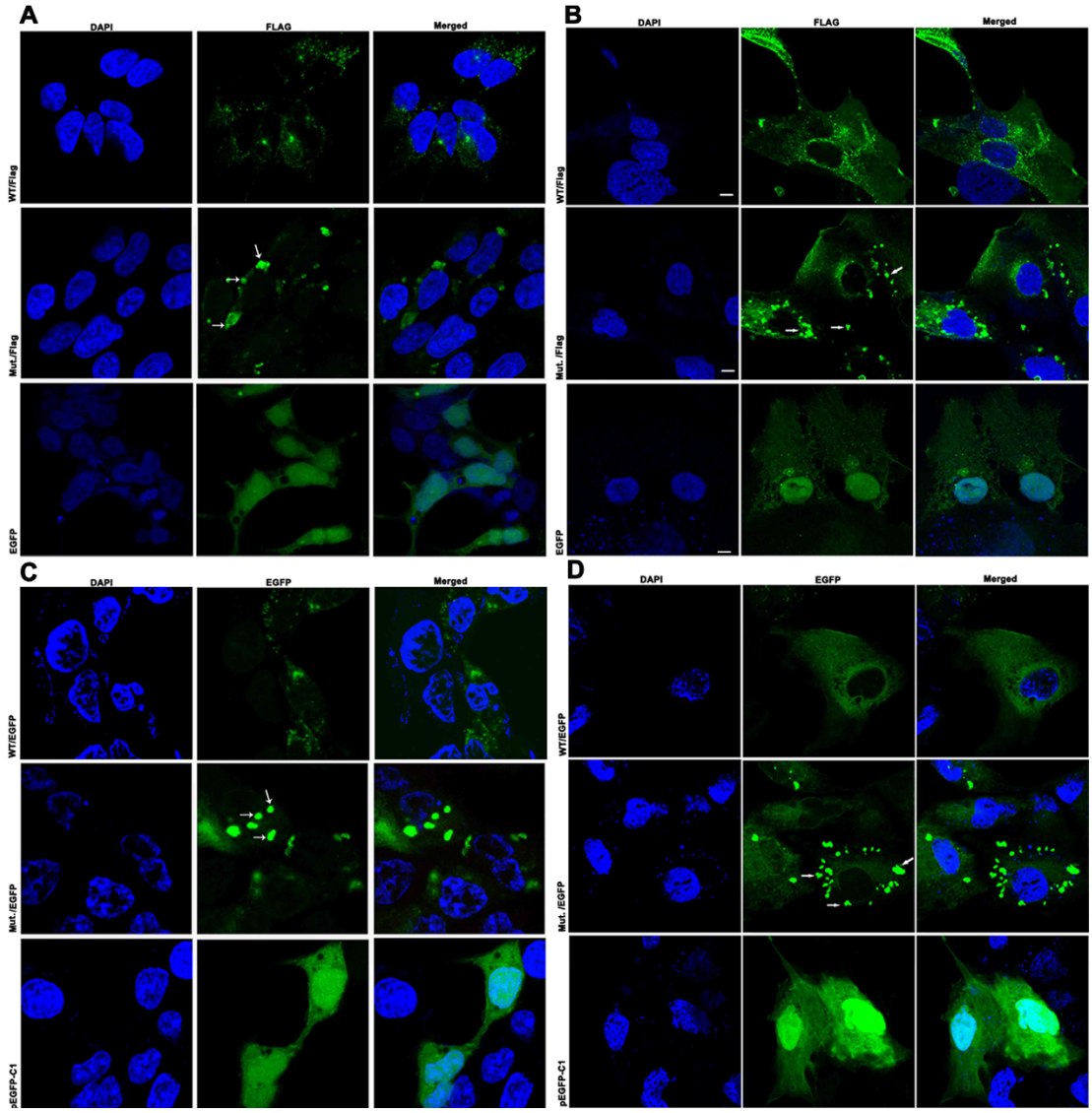

Figure 4 | Distinct protein localization patterns of p.P88T mutation. (A, B). Immunofluorescent imaging of Cx50-Flag and EGFP (control) showing transiently transfected 293 cells (A) and HLE cells (B) with Cx50-WT, p.P88T mutant, and pSin EGFP backbone plasmids (control). Cells were immunostained with anti-Flag monoclonal antibody. DAPI shows nuclear DNA staining. Arrows mark the accumulation of the mutant protein in the cytoplasm and plasma membrane. (C, D). Immunofluorescent imaging of EGFP fusion proteins showing transiently transfected 293 cells (C) and HLE cells (D) with Cx50-WT p.P88T mutant, and pEGFPc1 backbone plasmids (control). DAPI shows nuclear DNA staining.

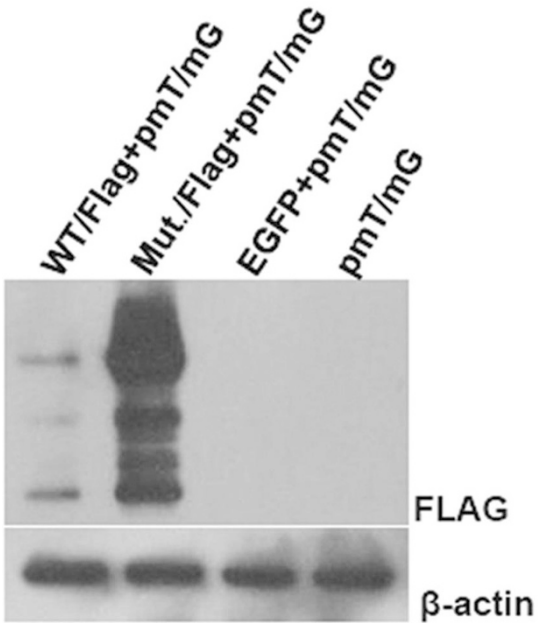

Figure 5 | Much higher expression level of p.P88T mutant protein. Western blotting for Cx50 and EGFP (control). The blots were probed with the anti-Flag antibody. Only two groups of cells produced a protein band, which includes about 60 kDa, 50 kDa, 43 kDa. No band was detected in the EGFP control.

genes responsible for inherited cataracts[13–16]. In order to maintain solubility properties of crystallins and to ensure transparency, the lens must have sophisticated mechanisms to control ionic balance and water content. Hence, an extensive network of gap junctions provides low-resistance pathways for both electrical and metabolic coupling of lens epithelial–epithelial, epithelial–fiber and, and fiber–fiber cells[17]. Three connexins (Cx43, Cx46 and Cx50) have been identified in human lens[4,18]. Because mutations in *GJA1*, coding for Cx43, cause human occulodentodigital dysplasia (ODDD), which is a distinct disease from cataracts, we excluded it as a candidate gene responsible for cataracts in the present family. Cx46 and Cx50 are the two most abundant connexins in lens fiber cells and lens epithelial cells, and mutations in these two genes have been reported to cause cataracts. To date, there are 33 identified mutations in human Cx50, summarized in Figure 3. p.P88S (CCG to TCG, mutation nucleotide underscored, with phenotype Zonular pulverulent cataracts), p.P88Q (CCG to CAG, with Lamellar pulverulent and balloon-like with Y-sutural cataracts) but not p.P88T (CCG to ACG, with total cataracts, this work) were previously reported[4,19].

The mutation identified here highlights that the mutational hot spot residue 88 of Cx50 is critical for its function, and provides additional evidence to show that cataracts is a genetically heterogeneous disease, especially since different mutations at the same residue render different phenotypes.

Proline can introduce bends in transmembrane helices, and because of its rigidity can act as a conformational 'switch', allowing parts of proteins to adopt alternative conformations, such as domain-swapped dimmers[20]. Mutations of proline residues in connexins may disrupt the protein structure and further effect the translocation and subcellular localization of the final protein. Our initial hypothesis is that the mutation may just disrupt the location of the protein, causing the mutant protein to predominantly localize in the cytoplasm, compared to localization of the wild-type protein in the cellular membrane. But both wild-type and mutant recombinant proteins are localized to the cytoplasm and cell membrane (Figure 4; supplementary Figure S1). The distinctive recombinant proteins expression level of the same construct was observed in different cell lines (Figure 4).

We observed a much higher expression level of p.P88T mutant Cx50 protein (Figure 5), compared with the wild-type, which is consistent with the results of the P88S mutant protein[21]. The altered degradation of the P88S mutant protein observed in that study was proposed to lead to the higher expression level of P88S. A similar mechanism may allow the p.P88T mutant to achieve a much higher protein expression level.

There are reports that connexin may control cell growth[22], while whether p.P88T mutation have the effects on the cells growth or not is still an open question. We were surprised to find that stable ectopic expression of mutated Cx50 led to a much larger colony when compared with wild-type control (Figure 6), and higher growth rates were confirmed by cell growth curve assay (see Supplementary Fig. S2 online). These data indicate that mutated Cx50 has a positive effect on cell growth *in vitro*. Xia et al. showed that mice heterozygous for a Gja8 point mutation (R205G) have cataracts and smaller lenses, while severe cataracts are seen in homozygous mice[23]. While further studies are needed to determine whether lens cells from the patients have a growth advantage or not, results from this *in vitro* cell growth assay clearly show a striking difference between the wild-type and mutant protein.

As to the accurate gene diagnosis of cataracts, so far, it is still an ongoing challenge, because the relatively large numbers (18) of disease genes make it one of the most complicated single-gene genetic disorders. Although Capture Next Generation Sequencing offers alternative mutation screening techniques for heterogeneous inherited diseases[24], the cost and bioinformatics analysis represent the limiting factors for gene diagnosis. Here, we showed an example of using a functional cloning approach

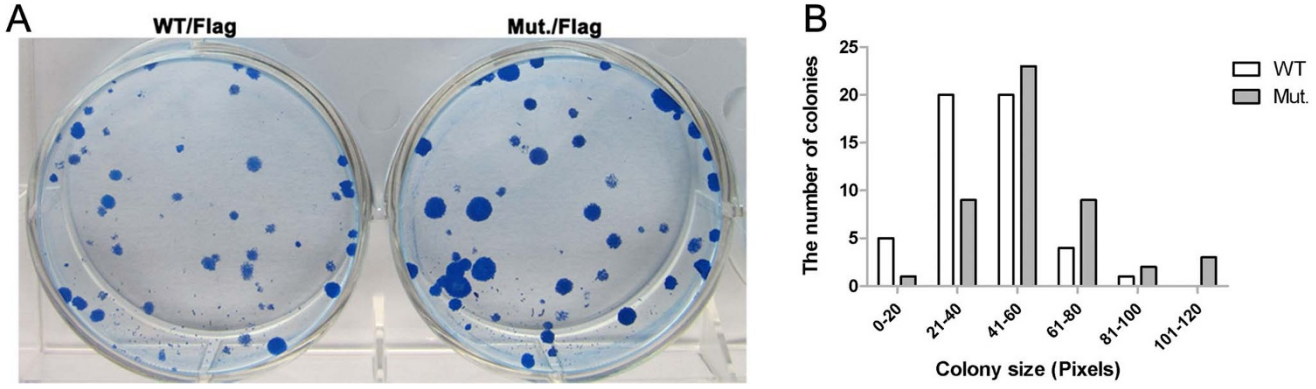

Figure 6 | A positive effect of p.P88T mutant protein on cell growth. (A, B). Colony size of 293 cells with stable ectopic overexpression of Cx50. Stable ectopic overexpression of p.P88T mutated Cx50 led to a much larger cell colony when compared with wild-type control.

to identify the responsible gene for hereditary cataracts. It will be valuable for genetic counseling of the family members and prenatal diagnosis. This study also highlights that it may be meaningful to directly screen these connexin coding genes for mutations in families with human hereditary cataracts, especially families with total cataracts.

We conclude that a novel mutation in Cx50, p.P88T, has been identified as the causative mutation in a family with hereditary dominant congenital cataracts. We found a distinct localization pattern for mutant protein, an increased accumulation of mutant protein, and a positive effect on cell growth, which may account for the molecular mechanisms underlying cataracts in this family.

Methods

Patients. This study conformed to the tenets of the Declaration of Helsinki (sixth revision, 2008) and was approved by the Ethics Committee of the Eye hospital, Wenzhou Medical University. The family members in this study have given written informed consent to publication of their case details. Ten members from the family were recruited, including three affected and seven unaffected members (Figure 1). Clinical and ophthalmological examinations were performed by ophthalmologists. A 5 ml venous blood sample was drawn into an ethylenediamine tetraacetic acid (EDTA) sample tube from every subject. Genomic DNA was extracted from peripheral blood leukocytes using the Wizard Genomic DNA Purification Kit (Promega, Beijing, China) according to the manufacturer's instructions.

Mutation screening and analysis. We designed a set of eight primer pairs (see Supplementary Table S1 online) to amplify the exons and flanking intron sequences of GJA3 (NM_021954) and GJA8 (NM_005267). Oligonucleotide primers and PCR information for amplification are shown in Supplementary Table S1. PCR products were sequenced on an ABI PRISM 3730 DNA Sequencer. Denaturing high-performance liquid chromatography (DHPLC, Wave DHPLC; Transgenomic, San Jose, CA) was used to confirm the identified mutation in the *GJA8* gene using a commercial system on the patients, family members, and 100 normal controls as previously described[14]. Briefly, gene specific PCR primers (see Supplementary Table S1 online, Exon2a) were used to amplify the fragment that harbored the point mutation. After the amplification, about 100 ng of the PCR product was denatured for 3 min at 96°C and then gradually reannealed by decreasing sample temperature from 95 to 55°C over a period of 30 min. PCR products were then separated with a DHPLC machine as follows: initial concentration at 44% of buffer A (0.1 M triethylammonium acetate, TEAA; Transgenomic) and 56% of buffer B (0.1 M TEAA containing 25% acetonitrile; Transgenomic) at 62.0°C.

Using the websites of NCBI and UCSC, we obtained the Cx50 sequence from Homo sapiens, Rattus norvegicus, Mus musculus, Heterocephalus glaber, Tupaia chinensis, Pteropus alecto and sequences of Cx40 and Cx43 from Homo sapiens.

Using DNAMAN biosoftware, multiple-sequence alignment of Cx50 sequences from these species was obtained. Furthermore, we used the online bioinformatics software SIFT (http://sift.jcvi.org/) to predict whether the amino acid substitution p.P88T in Cx50 could have a phenotypic effect.

Cloning and expression of proteins. Recombinant mouse wild-type and mutant p.P88T Cx50 coding fragments were prepared as previously described[14]. Flag sequence was added into the coding fragments to get a C-terminal Flag tag. Genes coding for Cx50 wild-type, Cx50 mutant, and EGFP proteins were inserted into eukaryotic expression Vectors pSin or pEGFPc1 to obtain pSin-WT, pSin-Mut., pSin–EGFP, pEGFPc1-WT, and pEGFPc1-mut., respectively. DNA sequencing confirmed the desired specific sequence in each construct.

The recombinant plasmids were expressed in human embryonic kidney (293) cells and human lens epithelial cells (HLE). Briefly, cells were seeded at day 0 and transfected on day 1. For transient gene expression, cells were fixed with 4% paraformaldehyde at day 3 and then incubated with corresponding antibodies. To label plasma membrane, membrane-tagged expressions of RFP, coding from pmTmG plasmid, were used as a control. Stable cell lines of the transfection (pSin vectors) were obtained by selecting with puromycin for an additional 10 days. To measure colony size, the puromycin-resistant colonies from 293 cells were obtained by puromycin selection and then trypsinized & re-seeded at the same cell numbers. After another twelve days, the dishes were stained with Commassie Blue. Cell growth curve was performed according to manufacturer's protocol (Roche, cat No.05015944001).

Immunofluorescence and Western blotting. The cells were permeabilized with 0.1% Triton X-100 and blocked in solution, then incubated with anti-Flag antibody. It was recognized with secondary antibodies and DAPI was used to label the cellular nuclei. The cells were observed under laser confocal microscope.

The expression of the wild-type and mutant proteins was analyzed on SDS-PAGE under reducing conditions. The proteins were recognized by anti-Flag antibodies.

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Author contributions

F.G. formulated the idea of the paper and supervised the research. X.L.G., Y.L.Z., X.L., Y.M.W., J.N.L., J.H.Y., Z.B.J. and J.Q. collected the samples and performed the experiments. X.L.G. and F.G. performed data analyses, F.G. wrote the manuscript. All authors have read and approved the final manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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