Mutations in PIKFYVE cause autosomal dominant congenital cataract

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Abstract

Congenital cataract, an ocular disease predominantly occurring within the first decade of life, is one of the leading causes of blindness in children. Through whole exome sequencing of a
Chinese family with congenital cataract, we identified a disease-causing mutation (p.G1943E) in PIKFYVE, which affecting the PIP kinase domain of the PIKfyve protein. We demonstrated that heterozygous/homozygous disruption of PIKfyve kinase domain, instead of overexpression of PIKFYVE<sup>G1943E</sup> in zebrafish mimicked the cataract defect in human patients, suggesting that haploinsufficiency, rather than dominant-negative inhibition of PIKfyve activity caused the disease. Phenotypical analysis of pikfyve zebrafish mutants revealed that loss of Pikfyve caused aberrant vacuolation (accumulation of Rab7<sup>+</sup>Lc3<sup>+</sup> amphisomes) in lens cells, which was significantly alleviated by treatment with the V-ATPase inhibitor bafilomycin A1 (Baf-A1). Collectively, we identified PIKFYVE as a novel causative gene for congenital cataract and demonstrated the potential application of Baf-A1 in treatment of congenital cataract.

**Introduction**

Congenital cataract is partial or complete opacification of the lens that occurs at birth or during the first decade of life. It is a common ocular abnormality that causes visual impairment and blindness during infancy (Khokhar et al., 2017), accounting for 5%-20% blindness in children worldwide (Sheeladevi, Lawrenson, Fielder, & Suttle, 2016). According to the location and shape of the lens opacities, congenital cataract can be divided into seven clinical types: nuclear cataract, polar cataract, lamellar cataract, nuclear with cortical cataract, cortical cataract, sutural cataract, and total cataract (Zhai et al., 2017). Different types of cataracts cause different levels
of visual impairment in patients. A variety of factors, including gene mutations that affect lens metabolism are closely associated with cataract (J. Li, Chen, Yan, & Yao, 2020), and autosomal dominant congenital cataract is the most common mode of inheritance (Berry, Georgiou, et al., 2020). To date, mutations in at least 50 genes involved in lens structure and development have been linked to isolated congenital cataract (Berry, Ionides, et al., 2020; Shiels & Hejtmancik, 2017), including crystallin genes (CRYAA, CRYAB, CRYBB1, CRYBB2, CRYBB3, CRYBA1/A3, CRYBA2, CRYBA4, CRYGC, CRYGD and CRYGS) (Bhat, 2003; Zhuang et al., 2019), membrane protein genes (GJA3, GJA8, MIP and LIM2) (Berry, Francis, Kaushal, Moore, & Bhattacharya, 2000; Beyer, Ebihara, & Berthoud, 2013; Pei et al., 2020), growth and transcription factor genes (PITX3, MAF and HSF4) (Anand, Agrawal, Slavotinek, & Lachke, 2018), beaded filament structural protein genes (BFSP1 and BFSP2) (Song et al., 2009) and other genes (CHMP4B and EPHA2) (Dave et al., 2016; Shiels et al., 2007). Although these findings have made tremendous contributions to our understanding of the genetic etiology of congenital cataract, new disease causative genes as well as the underlying molecular mechanisms remain to be discovered (Berry, Georgiou, et al., 2020).

In this study, we identified a missense mutation (p.G1943E) in the phosphatidylinositol phosphate kinase (PIPK) domain of PIKfyve from a Chinese cataract family. The human PIKFYVE gene encodes a large protein consisting of 2,098 amino acids, which contains six evolutionarily conserved domains (Kawasaki et al., 2012), including zinc finger phosphoinositide kinase (FYVE) domain, plextrin homology domain, β-sheet winged helix
DNA/RNA-binding motif, cytosolic chaperone CCTγ apical domain-like motif, spectrin repeats (SPEC), and C-terminal fragment PIPK domain (S. Li et al., 2005; Shisheva, 2008). The N-terminal FYVE domain can bind to PtdIns3P on the membrane, while the C-terminal is the kinase domain which has the catalytic function for the production of PtdIns(3,5)P2 (Shisheva, Sbrissa, & Ikonomov, 1999). The in-vivo function of PIKfyve in the development of lens and its association with congenital cataract have not been documented. Different from the previously reported PIKfyve mutations affecting the chaperonin-like domain that cause fleck corneal dystrophy (CFD), a disease characterized by tiny white flecks scattered in corneal stroma (Gee et al., 2015; Kawasaki et al., 2012; S. Li et al., 2005), the p.G1943E mutation was revealed for the first time in the kinase domain of PIKfyve to cause congenital cataract in human patients.

Using zebrafish models, we demonstrated that disruption of the PIPK domain in Pikfyve caused early-onset cataract defect and treating the pikfyve-deficient embryos with V-ATPase inhibitor bafilomycin A1 (Baf-A1) significantly alleviated the vacuolation defect in the lens of zebrafish mutants.

Results

Cataract family and clinical characteristics

We recruited a large Chinese Korean family affected with congenital cataract consisting of 31 family members including 12 affected individuals across 4 generations (Figure 1A). All the
family members were Chinese Korean except one married-in individual (III-8) who was Han Chinese. There was no consanguineous marriage in this family. Bilateral cataract was observed in every generation of the pedigree and affected parents transmitted the disease to both males and females, suggesting an autosomal dominant inheritance. No other ocular and systemic abnormalities were found in these patients. Clinical information was obtained from all patients except two deceased individuals (Table 1). 7 of the 10 living patients were diagnosed as cataract in their first decade of life, with the smallest age of diagnosis at 2 years old. The best corrected visual acuity (BCVA) of these patients ranged from 20/125 to 20/20. The proband (III-9) showed nuclear pulverulent cataract in both eyes (Figure 1B) with vision loss (OD: 20/40, OS: 20/66) since childhood. The mother (II-4) of the proband also had nuclear pulverulent cataract in both eyes, while his father (II-3) had no cataract in either eye. The son (IV-5) of the proband had Y-sutural cataract. Among the other affected members of this family, individuals II-8, III-5, III-6 and IV-3 presented nuclear pulverulent opacity in both eyes, whereas individual III-1 and his son (IV-1) showed peripheral cortical punctate opacity in both eyes. All patients of this family did not show any abnormalities in fundus photography and optical coherence tomography (OCT) examinations.

Identification of a PIKFYVE missense mutation in the cataract family
To identify the causative gene mutation responsible for congenital cataract in this family, we performed whole exome sequencing (WES) for four selected family members including three affected members (II-1, III-9 and IV-5) and one unaffected member (II-5; Figure 1A). The average sequencing depth was 184× and 97% of the targeted regions were covered with a read depth of at least 20×. Overall, 101,852–102,401 sequence variants were identified in each individual. Nonsynonymous or splice site variants that were shared among all three affected members but were absent from the unaffected member were filtered to retain those with minor allele frequencies < 1% in the genome aggregate database (gnomAD; https://gnomad.broadinstitute.org/). We first looked at variants in known cataract genes but did not find any disease causative mutations in this family. Further filtering on in silico prediction of pathogenicity refined the candidate list to three genes (PIKFYVE, NPHS1 and FPR1; Table 2). We then verified and evaluated these candidate variants in the whole family using Sanger sequencing. Only the p.G1943E missense mutation in the PIKFYVE gene completely segregated with the disease in the family (Figure 1A).

As shown in Figure 2A, in contrast to the healthy control, the PIKFYVE gene in the cataract patients carried a G to A heterozygous mutation in the 42nd exon, which leads to the substitution of a highly conserved amino acid glycine (G) by glutamate (E) at position 1943 in the PIPK domain (Figures 2B and 2C). To interpret how this mutation altered the function of PIKfyve, we first determined the protein stability of PIKfyveWT and PIKfyveG1943E by western blot analysis of pCS2(+) -CMV-PIKfyveWT and pCS2(+) -CMV-PIKfyveG1943E transfected HEK293T
cells at 10 and 20 hours post transfection. As shown in Figure 2D, the expression level of PIKfyve\textsuperscript{G1943E} was comparable with that of PIKfyve\textsuperscript{WT}, suggesting that the p.G1943E mutation has little or no effect on the protein stability of PIKfyve. Recently, a middle-to-low resolution structure of PIKfyve was resolved by cryo-EM (PDB ID:7K2V) (Lees, Li, Kumar, Weisman, & Reinisch, 2020). Using this structure as template, we predicted the structure of the p.G1943E mutant form of PIKfyve PIPK domain. As shown in Figures 2E and 2F, the p.G1943E mutation sits in a linker connecting the N-lobe (gold) and the C-lobe (cyan) of PIPK domain. This loop (red) acts as a hinge between N-lobe and C-lobe (Figure 2F) and forms a highly restricted conformation. However, the p.G1943E mutation introduces a negatively charged residue to the loop. This negatively charged residue not only changes the surface electrostatic potential of PIPK domain (Figure 2G), but also generates charge repulsions between the loop and N-lobe (D1872 and D1871) (Figure 2F), thereby potentially affecting the kinase activity of PIKfyve.

**Disruption of PIPK domain of PIKfyve caused early-onset cataract in zebrafish**

Bioinformatics analyses suggest that the p.G1943E mutation introduces a negative charged residue in the loop, which could potentially affect the kinase activity of PIKfyve. To directly demonstrate that heterozygosity of the p.G1943E mutation caused haploinsufficiency rather than dominant-negative inhibition of PIKfyve activity in human patients, we generated a pikfyve-deficient zebrafish mutant model using the CRISPR/Cas9-directed gene editing technology.
To maximally mimic the p.G1943E mutation in human patients, we designed single-guide RNA (sgRNA) in exon 40 to target the C terminal PIPK domain and screened out the pikfyveΔ8 allele, which harbored an 8-bp deletion and a 112-bp insertion in exon 40. As shown in Figure 3A, this pikfyveΔ8 mutation introduced a premature stop codon immediately downstream of the insertion site, thereby presumably generating a truncated Pikfyve protein that lacks the kinase activity. Phenotypical analysis revealed that pikfyveΔ8 homozygous mutants developed normally before 5 days post-fertilization (dpf), but later showed severe developmental defects and all died between 7 dpf and 9 dpf (Figure 3-figure supplement 1 A and B). Under stereomicroscope, we noticed that compared to siblings (sib), lens of pikfyveΔ8 mutants were less transparent at 5 dpf (Figure 3B). To characterize this phenotype in more details, we followed up the development of lens in all genotypes at different stages under differential interference contrast (DIC) microscope. Our results showed that bubble-like vacuoles first appeared in the developing lens of pikfyveΔ8 homozygous mutants at 3 dpf (Figures 3C and 3D). Strikingly, the number and size of vacuoles both increased drastically by 5 dpf and almost dominated the lens of mutants. Interestingly, compared to the wild-type (WT) control, pikfyveΔ8 heterozygote mutants also manifested significantly higher number of vacuoles in their lens (Figures 3C and 3D), suggesting that normal development of lens highly depends on the activity of Pikfyve and heterozygous disruption of Pikfyve is sufficient to cause cataract phenotype. In addition to loss of function study of Pikfyve, we also utilized a ubiquitously expressed ubiquitin (ubi) promoter to generate
transgenic lines $Tg(ubi:PIKfyve^{WT})$ and $Tg(ubi:PIKfyve^{G1943E})$ to overexpress $PIKfyve^{WT}$ and $PIKfyve^{G1943E}$ in zebrafish respectively. As expected, both of these two zebrafish lines showed normal development of the lens during early stages ($Figure$ $3$-figure supplement 1 C). Collectively, these findings strongly supported the hypothesis that haploinsufficiency, rather than dominant-negative inhibition of $PIKfyve$ activity by the p.G1943E mutation caused cataract in human patients. Furthermore, we noted that the vacuolation phenotype was partially rescued in $pikfyve^{Δ8};Tg(ubi:PIKfyve^{WT})$ zebrafish ($Figure$ $3$-figure supplement 1 D and E). These findings, together with the observations that pharmacological inhibition of $PIKfyve$ activity by inhibitor YM201636 also induced aberrant vacuolation in human lens epithelial cells HLEB3 ($Figure$ $3E$), suggested that function of $Pikfyve$ in the regulation of cataract formation is highly conserved across species and our zebrafish cataract model would potentially be an excellent system for further preclinical study.

**Detailed characterization of cataract phenotypes in $pikfyve^{Δ8}$ mutants**

To delineate the details of cataract phenotype in $pikfyve^{Δ8}$ mutants, we crossed $pikfyve^{Δ8}$ mutant with the reporter transgenic line $Tg(cryaa:DsRed)$, in which expression of $DsRed$ was controlled by promoter of the crystalline gene $cryaa$, the major constitutive components of lens fibers. Intriguingly, in comparison with the evenly distributed $DsRed$ signals in the lens of siblings, vacuoles in $pikfyve^{Δ8}$ mutants almost occupied the surface of the lens and all of them were $DsRed$
negative (Figure 4A), indicating that these vacuoles did not contain lens fibers. Meanwhile, hematoxylin-eosin (HE) staining, together with ZL-1 antibody and DAPI co-staining revealed that while disruption of Pikfyve seemingly had no effects on the enucleation process of lens, lens fibers in pikfyveΔ8 mutants were less organized than those in siblings (Figures 4B and 4C). To further characterize the vacuoles and lens structure in high resolution, we utilized transmission electron microscope (TEM) to visualize the ultrastructure of lens in both siblings and pikfyveΔ8 mutants. We could detect large vacuoles in the mutant lens at 3 dpf and 5 dpf, while only several tiny vacuoles in the sibling lens (Figure 4D). Lens fiber cells of WT embryos were mildly edema, and their nuclei were oval with uniform chromatin. The lens fibers (white arrows) were arranged neatly and tightly. Furthermore, mitochondria (black triangles) of WT were slightly swollen without vacuoles. In contrast, lens fiber cells of the pikfyveΔ8 mutants, were obviously edematous; and the nuclei were irregularly shaped. Besides, the arrangement of lens fibers was loose and deformed. Also, lipid droplets were formed in pikfyveΔ8 mutants. Compared to the WT, mitochondria (black triangles) of pikfyve mutants were obviously swollen and enlarged. Mitochondrial crests were reduced, and most of them were aberrantly vacuolated. In addition, autophagic lysosomes appeared swollen and vacuolated.

Vacuoles in pikfyveΔ8 mutants were amphisomes
To define the nature of vacuoles in the lens of pikfyveΔ8 mutants, we conducted time-lapse imaging to monitor their behaviors during zebrafish development. Our results showed that vacuoles in pikfyveΔ8 mutants were highly dynamic and small vacuoles were frequently found to be fused with each other to form larger vacuoles (Figure 5A, white arrows). This feature, together with previous findings showing that PIKfyve was an essential regulator of endomembrane homeostasis (Hasegawa, Strunk, & Weisman, 2017), prompted us to further investigate whether vacuoles in pikfyveΔ8 mutants were actually endocytic vesicles. To probe this issue, we generated fusion mRNAs encoding GFP and the markers of endocytic vesicles (i.e., the small GTPase Rab5c, Rab7 and Rab11a), which were injected into zebrafish embryos to specifically label early, late and recycling endosomes in pikfyveΔ8 mutants. Our results showed that in pikfyveΔ8 mutants, almost all vacuoles were positive for the late endosome marker Rab7-GFP (Figure 5C, white arrows). By contrast, the early endosome marker Rab5c-GFP and recycling marker Rab11a-GFP showed no co-localization with vacuoles (Figures 5B-5D). As autophagy was also closely-related to the organelle membrane system and had been shown to be regulated by PIKfyve (Vicinanza et al., 2015), we therefore checked the status of autophagosomes in pikfyveΔ8 mutants by injecting lc3b-mcherry fusion mRNA. As shown in Figure 5E, a proportion of vacuoles in pikfyveΔ8 mutants were also positive for the autophagosome marker Lc3b. Taken together, these data implied that vacuoles in pikfyveΔ8 mutants were amphisomes formed by the fusion of autophagosome and late endosome.
Baf-A1 partially rescued the vacuole defect in the lens of *pikfyve*Δ8 mutant zebrafish

Baf-A1 is a specific inhibitor of V-ATPase. Previous work has shown that the vacuole phenotype induced by PIKfyve deficiency in COS-7 cells could be rescued by Baf-A1 ([Compton, Ikonomov, Sbrissa, Garg, & Shisheva, 2016](https://doi.org/10.1101/2021.06.25.449865)). We thus investigated whether the vacuolation defect in the lens of *pikfyve*Δ8 mutant zebrafish could also be rescued by Baf-A1. Indeed, we found that vacuole number in the lens of *pikfyve*Δ8 mutants treated with 1 µM Baf-A1 was significantly lower than that in the control group treated with dimethyl sulfoxide (DMSO; Figures 6A and 6B). To further validate that Baf-A1 could directly alleviate cataract defect, rather than just delay the phenotype, we imaged the lens of the same mutant zebrafish before and after Baf-A1 treatment. As shown in Figures 6C and 6D, while vacuole numbers of all the mutant embryos showed a slight increase after DMSO treatment or without treatment, we observed significant decrease in vacuole number in all mutants with mild or severe phenotype.

On the other hand, a previous study also showed overexpression of transient receptor potential mucolipin 1 (TRPML1) could partially rescue the vacuole phenotype in *PIKfyve*-deficient macrophages ([Krishna et al., 2016](https://doi.org/10.1101/2021.06.25.449865)). However, in our system, ectopic expression of *Trpml1* by mRNA injection failed to rescue vacuolation phenotype in *pikfyve* mutant lens (Figure 6 – figure supplement 1). Thus, V-ATPase rather than Trpml1 might be a key component for the formation or maintenance of the large vacuoles in the lens of *pikfyve* mutant zebrafish, and inhibition of V-ATPase by Baf-A1 could directly alleviate the cataract phenotype.
Discussion

In this study, we identified a missense mutation (p.G1943E) in PIKFYVE responsible for congenital cataract in a Chinese Korean family (Figures 1 and 2). This mutation is very rare in the general population. In the gnomAD database, only 4 individuals (including 3 East Asians and 1 Latino/Admixed American) carry the p.G1943E mutation, representing an extremely low allele frequency of 0.00002 (Table 2). The degree and morphology of lens opacity in the cataract family were phenotypically heterogeneous (Table 1), which is consistent with previous findings (Berry, Ionides, et al., 2020). Most of the patients in this family developed cataract and vision loss in their childhood. Meanwhile, the majority of patients had nuclear pulverulent cataract, while the others had nuclear Y-sutural cataract or peripheral cortical punctate cataract (Table 1). The heterogeneity in clinical manifestations might be ascribed to interactions between genetic and environmental factors during lens development (Berry, Ionides, et al., 2020).

Mutations in PIKFYVE have been reported to be associated with CFD (Gee et al., 2015; Kawasaki et al., 2012; Kotoulas et al., 2011; S. Li et al., 2005). However, none of the PIKFYVE mutations in these studies caused congenital cataract in addition to CFD, except two of the CFD patients showed cataract formation in 66 and 58 years old respectively (Kotoulas et al., 2011). Interestingly, we also did not observe any corneal defect in patients with congenital cataract in this study. It is noted that all CFD related mutations in PIKFYVE, either homozygous or heterozygous, are distributed in two regions (i.e., amino acids 667-843 and 1490-1538),
corresponding to cytosolic chaperone CCTγ apical domain-like motif and SPEC domains respectively. By contrast, the p.G1943E mutation identified in this study is located in the C-terminal PIPK domain. Based on these findings, we hypothesized that different domains of PIKfyve might exert different functions by binding with different partners. This hypothesis is further supported by other evidence. First, the predicted protein structure suggested that, instead of gross disruption of PIKfyve protein structure, the p.G1943E mutation only mildly altered the 3-D conformation of PIPK domain by changing its surface electrostatic potential and generating charge repulsions between the loop and N-lobe (Figures 2G and 2F). Therefore, it is reasonable to argue that this mutation in PIPK domain may specifically alter its kinase activity or change the binding surface with other proteins, while keep the N-terminal cytosolic chaperone CCTγ apical domain-like motif and SPEC domains functional intact. Second, in our pikfyveΔ8 zebrafish mutants, while the C-terminal PIPK domain was truncated in PikfyveΔ8 protein, the N-terminal cytosolic chaperone CCTγ apical domain-like motif and SPEC domains were not affected. Accordingly, we did not observe any cornea defect in the zebrafish mutants either.

Inhibition of PIKfyve in COS-7 cells had been shown to induce large vacuoles through promoting the enlargement of both early and late endosomes (Ikonomov, Sbrissa, & Shisheva, 2006; Rutherford et al., 2006). Further studies revealed that the Ca²⁺ releasing channel, endolysosome-localized mucolipin TRPML1 acts downstream of PIKfyve to trigger membrane fusion/fission process (Dong et al., 2010) or promote lysosome/phagosome maturation (Dayam, Saric, Shilliday, & Botelho, 2015; Kim, Dayam, Prashar, Terebiznik, & Botelho, 2014).
Interestingly, while overexpression of TRPML1 partially alleviated the vacuole phenotype in PIKfyve-deficient macrophages (Krishna et al., 2016), the rescue effect was not observed in the lens of pikfyveΔ8 zebrafish mutants (Figure 6 – figure supplement 1), suggesting that PIKfyve might function in a context dependent manner. Consistent with this idea, we also noted that vacuoles in macrophages and lens cells of pikfyveΔ8 mutants were differently stained by lysosome marker (Figure 5 – figure supplement 1). On the other hand, vacuole formation in PIKfyve-deficient COS-7 cells and macrophages could also be inhibited by a drug called Baf-A1 (Compton et al., 2016; Isobe et al., 2019). In this study, we found that Baf-A1 could partially rescue the vacuole defect in the lens of pikfyveΔ8 mutant zebrafish (Figures 6A-6D). Baf-A1 is a macrolide antibiotic that inhibits V-ATPase, the ATP-dependent proton pump located on the membrane of organelles. The cellular acidification process mediated by V-ATPase may affect many basic biological processes, including membrane trafficking (in particular endosome maturation and fusion between autophagosomes and lysosomes (Hammond et al., 1998; Yamamoto et al., 1998), protein degradation and autophagy (Bowman, Siebers, & Altendorf, 1988; Yamamoto et al., 1998; Yoshimori, Yamamoto, Moriyama, Futai, & Tashiro, 1991). Interestingly, several works in yeast also identified mutations in V-ATPase that did not affect proton pump function, but indeed caused defects in vacuole fusion (Strasser, Iwaszkiewicz, Michielin, & Mayer, 2011). Moreover, another study in drosophila demonstrated that inhibition of vesicle fusion by Baf-A1 did not depend on V-ATPases, but relied on Ca²⁺ sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) pump, the secondary target.
of Baf-A1 (Mauvezin, Nagy, Juhasz, & Neufeld, 2015). Therefore, the target of Baf-A1 and
underlying mechanisms still need to be further investigated. These mechanisms will finally
contribute to the potential clinical application of the drug in the treatment of congenital cataract.
### Materials and methods

#### Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|------------------------------------|-------------|---------------------|-------------|------------------------|
| Cell line (*Homo sapiens*)         | HEK293T     | Laboratory cell bank of Shenzhen PKU-HKUST Medical Center |             |                        |
| Cell line (*Homo sapiens*)         | HLEB3       | ATCC                | ATCC® CRL-11421™ |                        |
| Strain, strain background (*Danio rerio*) | *pikfyve*Δ8 mutant | This paper          |             |                        |
| Strain, strain background (*Danio rerio*) | *Tg(ubi:PIKFYVE*WT) | This paper          |             |                        |
| Strain, strain background (*Danio rerio*) | *Tg(ubi:PIKFYVE*G1943E) | This paper          |             |                        |
| Strain, strain background (*Danio rerio*) | *Tg(cryaa:DsRed;i*l-1b:GFP-F) | DOI: 10.1242/dmm.014498 |             |                        |
| Chemical compound, drug            | YM201636    | Selleck, China      | S1219       | 800 nM for 4 hours     |
| Chemical compound, drug            | bafilomycin A1 | MedChemExpress, NJ, USA | HY-100558 | 1 μM for 4.5 hours   |
| Antibody | Anti-PIKfyve antibody | Abcam, UK | ab62467 |
|----------|----------------------|-----------|---------|
| Antibody | Anti-Lens Fiber Cell Marker antibody [ZL-1] | Abcam, UK | ab185979 |
| Sequence-based reagent (Homo sapiens) | Human PIKfyve genotyping fwd | This paper | PCR primer |
| Sequence-based reagent (Homo sapiens) | Human PIKfyve genotyping rev | This paper | PCR primer |
| Sequence-based reagent (Homo sapiens) | Human PIKfyve cDNA cloning fwd | This paper | PCR primer |
| Sequence-based reagent (Homo sapiens) | Human PIKfyve cDNA cloning rev | This paper | PCR primer |
| Sequence-based reagent (Danio rerio) | Zebrafish pikfyve genotyping fwd | This paper | PCR primer |
| Sequence-based reagent (Danio rerio) | Zebrafish pikfyve genotyping rev | This paper | PCR primer |

**Pedigree and patients**

A 4-generation pedigree consisting of 31 family members (*Figure 1A*) was recruited from the eye clinic of Shenzhen Eye Hospital, Shenzhen, Guangdong, China. All the living family
members underwent a complete ocular examination. Twelve individuals in this family were
diagnosed with congenital cataract (Figure 1B, Table 1), while the other 19 individuals were
unaffected. The diagnosis of congenital cataract was based on 1) lens opacity detected at birth or
during the first decade of life; and 2) no known other cause (e.g., trauma, iatrogenic, or
inflammatory disease) (Berry, Georgiou, et al., 2020). The study protocol was approved by the
Independent Ethics Committee of Shenzhen Eye Hospital, in accordance with the tenets of the
Declaration of Helsinki. Written informed consent was obtained from all study participants.

Whole exome sequencing and Sanger sequencing

DNA was extracted from blood or hair samples of 21 family members (Figure 1A). WES was
performed on 4 selected family members (II-1, II-5, III-9 and IV-5). Exome capture was
performed using a SureSelect Human All Exon V6 capture kit (Agilent Technologies, Santa
Clara, CA). Samples were sequenced on a HiSeq 4000 next-generation sequencing system
(Illumina, San Diego, CA). Sequence reads were aligned to the human reference genome (hg19)
using the Burrows-Wheeler Aligner (BWA) (H. Li & Durbin, 2009). Sequence variants were
called using the Genome Analysis Toolkit (GATK) with the Best Practices for SNP and Indel
discovery in germline DNA (McKenna et al., 2010). The variants were annotated using
Annotate Variation (ANNOVAR) (Wang, Li, & Hakonarson, 2010) and then filtered based on
the following criteria: 1) non-synonymous SNPs or indels in the exon region or the splice site
region; 2) novel or minor allele frequency < 1% in gnomAD; 3) PolyPhen score > 0.2; 4)
genomic evolutionary rate profiling (GERP) score > 2.5; and 5) combined annotation dependent
depletion score (CADD) > 10. Polymerase chain reaction (PCR) and Sanger sequencing were
used to verify the identified mutations from WES and screen the other family members for the
mutations. The primer sequences used in PCR and Sanger sequencing were: 1) forward primer:
5'-TTTTGACCTTCTCTTGATTAGAGG-3' and 2) reverse primer: 5'-
AAATATGGCCTAGTAACCAAAGTTAAA-3'.

Cell culture and YM201636 treatment

The HLEB3 cells (ATCC® CRL-11421™) were obtained from ATCC and the HEK293T cells
were obtained from laboratory cell bank of Shenzhen PKU-HKUST Medical Center. Cell culture
was performed according to the instructions (Andley, Rhim, Chylack, & Fleming, 1994). The
HLEB3 cells were plated into 6-well plates and grown for 4 days. The cells were then treated
with YM201636 (Selleck, China) at a working concentration of 800 nM for 4 hours before
imaging. Cells only treated with DMSO were used as control.

Protein detection by western blot

Transfection of the HEK293T cells was performed according to manufacturer’s instructions of
Lipofectamine RNAiMAX Reagent. Cells were transfected with 2 µg pCS2(+)‐CMV‐
PIKFYVEWT or pCS2(+)‐CMV‐PIKFYVEG1943E and harvested after 48 hours for western blot
analysis. Expression of the house keeping gene GAPDH was used as the loading control. The
ImageJ software (National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/) was used to measure the intensity of the bands and quantify the expression of PIKfyve protein. The anti-PIKfyve (ab62467, Abcam, Cambridge, UK), anti-GAPDH (ab9485, Abcam) primary antibodies and goat anti-rabbit (ab205718, Abcam) secondary antibodies were used in this study.

Zebrafish husbandry

The AB strain zebrafish were raised and maintained according to the standard protocols (Westerfield, 1993). The wild type and transgenic lines including pikfyveΔ8, Tg(cryaa:DsRed;il-1b:GFP-F) (Nguyen-Chi et al., 2014), Tg(ubi:PIKFYVEWT) and Tg(ubi:PIKFYVEG1943E) were used in this study.

Generation of zebrafish pikfyve mutant

The sgRNA was designed with the online tool Crisprscan (Vejnar, Moreno-Mateos, Cifuentes, Bazzini, & Giraldez, 2016). The sgRNA was synthesized by in vitro transcription with the MEGAshortscript™ T7 Transcription Kit (AM1354, Ambion, Austin, TX, USA) and purified by the MEGAclear™ Kit (AM1908, Ambion). The sgRNA, together with the Cas9 protein (EnGen® Cas9 NLS, S. pyogenes, M0646M, NEB, Ipswich, MA, USA) was injected into zebrafish embryos at one-cell stage. The final concentration was 100 ng/μL for each sgRNA
and 800 ng/μL for Cas9 protein. Stable mutant line was screened by the T7 endonuclease 1 (T7E1) digestion and Sanger sequencing.

**Generation of transgenic zebrafish lines**

The coding sequence of wild type and G1943E mutant form of human PIKFYVE was placed downstream of *ubi* promoter (*Mosimann et al., 2011*), which was then cloned to a modified PBSK vector containing Tol2 element (*Kawakami, Shima, & Kawakami, 2000*). The plasmid and transposon mRNA were then injected into embryos at one-cell stage. Injected embryos were raised into adulthood and screened for germline transmission by PCR and whole-mount in situ hybridization (WISH) of F1 embryos.

**Histology**

Zebrafish embryos were fixed in 4% PFA at 5 dpf and then dehydrated in 30% sucrose. The whole embryos were mounted in optimal cutting temperature compound and frozen in -80°C. Cryo-section was performed at 10 μM intervals in transverse planes from the head. HE staining and antibody staining were conducted according to the standard protocols. ZL-1 antibody (ab185979, Abcam) was used in this study. TEM analysis of the lens was completed by Servicebio Technology (Wuhan, China).

**Imaging of zebrafish lens**
Images were taken under ZEISS LSM980 Confocal Laser Scanning Microscope (Carl Zeiss Microscopy, NY, USA). The 63× objective (Plan-Apochromat 63×/1.40 Oil DIC M27) was used in this study. For time-lapse imaging experiment, 4-dpf zebrafish was mounted in 1.0% low-melting agarose with 0.02% tricaine. The lens was directly imaged under ZEISS CellDiscoverer 7 microscope (Carl Zeiss Microscopy). A 50× water lens (Plan-Apochromat 50×/1.2) was used and images were taken every 3 minutes. Images were analyzed by ImageJ (National Institutes of Health).

**Detection of endosomes and autophagosomes in zebrafish lens**

Coding sequences of *rab5c*, *rab7* or *rab11a* were fused with GFP sequence and cloned into the pCS2+ vector. mRNAs were synthesized *in vitro* using the mMESSAGE mMACHINE SP6 kit (AM1908, Ambion). 1-2 nL mRNA (100 ng/μL) was injected into zebrafish embryos at one-cell stage. At 3.5 dpf, embryos were mounted in 1% low-melting agarose and imaged under the confocal microscope.

**Baf-A1 treatment**

Baf-A1 powder (HY-100558, MedChemExpress, NJ, USA) was dissolved to the concentration of 1 mM in DMSO for stock, and diluted to 1 μM in egg water before use. Zebrafish embryos were treated with either 1‰ DMSO or 1 μM Baf-A1 solution at 28.5°C for 4.5 hours and washed out by egg water before confocal imaging.
Statistical analysis

All statistical analyses were performed using the SPSS statistics software v24.0 (IBM SPSS Statistics, NY, USA). Data was represented as mean±SD. Two-tailed Student’s t-tests were used for comparisons between two groups. Statistical significance was shown as n.s., \( p > 0.05 \); \(*p < 0.05\); \(***p < 0.001\); and \(****p < 0.0001\). Statistical plots were generated using the ggplot2 package (Wickham, 2009) in R (R Core Team, 2017).

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Competing interests

The authors declare no competing interests.
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Figure 1. Pedigree structure and ocular manifestations of the cataract family

(A) Pedigree of the family with congenital cataract. Squares denote males and circles denote females; Symbols crossed by a line indicate deceased individuals. Filled symbols indicate affected individuals, while open symbols indicate unaffected individuals. All affected family members had bilateral congenital cataract. The arrow denotes the proband. The individuals marked with an asterisk (*) are analyzed by whole exome sequencing. Genotypes of the \textit{PIKFYVE} mutation (p.G1943E) are indicated below each symbol (+, wildtype allele; -, mutant allele). (B) Slit-lamp photographs showing the transparent lens of an unaffected individual (IV-6) and the nuclear pulverulent cataract in the left eye of the proband (III-9).
Figure 2. The PIKFYVE mutation identified from the cataract family

(A) Sanger sequencing chromatogram showing the cDNA sequences from a healthy control and a cataract patient. The heterozygous c.5828G>A missense mutation in the patient is indicated by red arrow. (B) A schematic diagram showing the human PIKfyve domains. The p.G1943E mutation in the PIPK domain is indicated by the red arrow. (C) Protein sequence alignment of PIKfyve orthologs in vertebrates. The black triangle denotes the conserved glycine at position 1943. (D) Western blot analysis of PIKfyveWT and PIKfyveG1943E expression in HEK293T cells that were transiently transfected with either pCS2(+)-CMV-PIKfyveWT or pCS2(+)-CMV-PIKfyveG1943E. The protein levels were normalized by GAPDH expression. Experiments were repeated three times. (E) Predicted structure model of the p.G1943E mutant form of PIKfyve PIPK domain generated by the PHYPRE2 server (http://www.sbg.bio.ic.ac.uk/~phyre2/html/). N-lobe, C-lobe and the hinge linker are shown in gold, cyan and red respectively. The mutation residue E1943 is shown in sticks and labeled with green. The negatively charged residue D1872 close to E1943 side chain is also shown in sticks. (F) A schematic demonstrating the organization
of PIKfyve PIPK domain. N-lobe, C-lobe and the hinge linker are shown in gold, cyan and red respectively. The position of the p.G1943E mutation is labeled with a yellow star. (G) Surface electrostatic potential comparison of the PIPK domain of PIKfyve between wild type (WT) and p.G1943E mutant. The electrostatic potentials are presented as heat maps from red to blue, and the electrostatic potential scales are shown in the lower panel. See Figure 2-source data for details.

Figure 3. Disruption of the PIPK domain of PIKfyve in zebrafish caused early-onset cataract

(A) A schematic diagram showing the generated pikfyve<sup>Δ8</sup> mutant allele. The underlined base pairs are the sgRNA target. The deleted base pairs are shown in dark blue while inserted ones are...
shown in red. The stop codon introduced in the mutant form is shown in the grey box. (B) Representative images showing the lens of sibling and pikfyveΔ8 mutants at 5 dpf. (C) Representative differential interference contrast (DIC) images showing the lens of pikfyve+/+, pikfyve+/Δ8 and pikfyveΔ8/Δ8 embryos at 3 dpf and 5 dpf. The scale bars represent 10 μm in (B) and (C). (D) Quantification of vacuole number in the lens of pikfyve+/+, pikfyve+/Δ8 and pikfyveΔ8/Δ8 embryos at 3 dpf (n=7 for pikfyve+/+, n=10 for pikfyve+/Δ8; n=7 for pikfyveΔ8/Δ8) and 5 dpf (n=7 for pikfyve+/+, n=11 for pikfyve+/Δ8; n=6 for pikfyveΔ8/Δ8). (E) Representative images of HLEB3 cells treated with DMSO or PIKfyve inhibitor YM201636 for 4 hours. All experiments were repeated three times. The scale bars represent 25 μm. See Figure 3-source data for details.

**Figure 3 – figure supplement 1. Characterization of pikfyve-deficient zebrafish mutants**

(A) Gross morphology of 5-dpf and 7-dpf siblings and pikfyveΔ8 mutants. The scale bars represent 500 μm. (B) Survival rate of pikfyveΔ8 mutants (n=48) and siblings (n=50). (C)
Representative images showing the lens of 5-dpf wild type (WT), Tg(ubi:PIKfyve\(^{WT}\)) and Tg(ubi:PIKfyve\(^{G1943E}\)) zebrafish embryos. (D) Representative images showing the lens of 5-dpf pikfyve\(^{Δ8}\) and pikfyve\(^{Δ8};\) Tg(ubi:PIKfyve\(^{WT}\)) zebrafish. The scale bars represent 20 μm in (C) and (D). (E) Quantification of the vacuole number in the lens of 5-dpf pikfyve\(^{Δ8}\) (n=4) and pikfyve\(^{Δ8};\) Tg(ubi:PIKfyve\(^{WT}\)) (n=7) zebrafish embryos. *, \(p < 0.05\), Student’s t-test. All experiments were repeated three times. See Figure 3 supplement 1-source data for details.

Figure 4. Detailed characterization of cataract phenotypes in pikfyve\(^{Δ8}\) mutants
(A) Confocal imaging of the lens of 5-dpf sibling and *pikfyveΔ8* mutants in Tg(cryaa:DsRed) transgenic background. (B) Hematoxylin-eosin (HE) staining of 5-dpf sibling and *pikfyveΔ8* mutant zebrafish lens after cryostat section. (C) ZL-1 antibody staining of 5-dpf sibling and *pikfyveΔ8* mutant zebrafish lens. (D) Transmission electron microscope (TEM) images of the lens of siblings and *pikfyveΔ8* mutants at 3 dpf and 5 dpf. LD: lipid droplet; N: nucleus; ASS: autophagy lysosome. All results were confirmed in three different individuals. All the scale bars represent 10 μm.

**Figure 5.** Characterization of vacuoles in *pikfyveΔ8* mutants
(A) Time-lapse imaging indicating the dynamic changes of vacuole formation in the lens of 4-dpf pikfyveΔ8 mutants. White arrows indicate the fusion process of two small vacuoles. (B) Representative images showing the lens of 3.5-dpf sibling and pikfyveΔ8 mutants injected with gfp-rab5c mRNA. (C) Representative images showing lens of 3.5-dpf sibling and pikfyveΔ8 mutants injected with gfp-rab7 mRNA. (D) Representative images showing lens of 3.5-dpf sibling and pikfyveΔ8 mutants injected with gfp-rab11a mRNA. (E) Representative images showing lens of 3.5-dpf sibling and pikfyveΔ8 mutants injected with mcherry-lc3b mRNA. All experiments were repeated three times. All the scale bars represent 10 μm.

Figure 5 – figure supplement 1. Lysosomes of pikfyveΔ8 mutant zebrafish

Confocal images of the lens and macrophages of 4-dpf pikfyveΔ8 mutants in Tg(mpeg1:dsredx) background after LysoSensor staining. The results were confirmed in three different individuals. The scale bars represent 20 μm.
Figure 6. Baf-A1 partially rescued the vacuole defect in the lens of *pikfyve*Δ8 mutant zebrafish

(A) Representative confocal images of the lens of 4-dpf *pikfyve*Δ8 mutants treated with DMSO or Baf-A1 for 4.5 hours. (B) Quantification of the vacuole number in the lens of 4-dpf siblings and *pikfyve*Δ8 mutant embryos treated with DMSO or Baf-A1 (n=10 for sibling groups; n=7 for mutant groups). (C) Confocal images of the lens of 4-dpf *pikfyve*Δ8 mutants with no treatment or after 4.5 hours treatment with DMSO or Baf-A1. (D) Quantification of the vacuole number in C (n=6 for each group). All experiments were repeated three times. All the scale bars represent 20 μm. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001, Student’s t-test. See Figure 6-source data for details.
Figure 6 – figure supplement 1. Overexpression of *trpml1* failed to rescue the lens defects in *pikfyve* mutants.

(A) Confocal images of the lens of 4-dpf *pikfyve*Δ8 mutants and *pikfyve*Δ8 mutants injected with *trpml1* mRNA. The scale bars represent 20 μm. (B) Quantification of the vacuole number in the lens of 4-dpf *pikfyve*Δ8 mutants (n=4) and *pikfyve*Δ8 mutants injected with *trpml1* mRNA (n=6). n.s., not significant, *p* > 0.05, Student’s t-test. See Figure 6 supplement 1-source data for details.
### Table 1. Clinical characteristics of living patients in the cataract family

| Patient | Gender | Age at diagnosis (years) | BCVA before surgery (OD, OS) | Cataract type (OU) | Surgery (Y/N) | BCVA after surgery (OD, OS) |
|---------|--------|--------------------------|-----------------------------|-------------------|-------------|---------------------------|
| II-1    | F      | 9                        | —                           | —                 | Y           | 20/160, 20/125            |
| II-4    | F      | 12                       | 20/40, 20/32                | nuclear pulverulent | N           | NA                        |
| II-8    | M      | 4                        | 20/100, LP                  | nuclear pulverulent | Y           | 20/40, FC                 |
| III-1   | M      | 47                       | 20/25, 20/20                | peripheral cortical punctate | N           | NA                        |
| III-5   | M      | 6                        | 20/40, 20/40                | nuclear pulverulent | Y           | 20/20, 20/20              |
| III-6   | M      | 7                        | 20/40, 20/50                | nuclear pulverulent | Y           | 20/16, 20/16              |
| III-9   | M      | 6                        | 20/40, 20/66                | nuclear pulverulent | Y           | 20/28, 20/25              |
| IV-1    | M      | 22                       | 20/20, 20/20                | peripheral cortical punctate | N           | NA                        |
| IV-3    | M      | 3                        | 20/30, 20/25                | nuclear pulverulent | N           | NA                        |
| IV-5    | M      | 2                        | 20/125, 20/125              | nuclear and Y-sutural | N           | NA                        |

BCVA: best corrected visual acuity; OD: eye, oculus dexter; OS: eye, oculus sinister; OU: eye, oculus uterque; Y: yes; N: no; F: female; M: male; LP: light perception; FC: finger counting; —: unknown; NA: not applicable.
| Gene    | SNP ID     | Chromosome position (bp; hg19) | cDNA change | Amino acid change | MAF (gnomAD) | PolyPhen | GERP | CADD |
|---------|------------|-------------------------------|-------------|-------------------|--------------|-----------|------|------|
| PIKFYVE | rs771244880| chr2:209217490                | c.5828G>A   | p.G1943E          | 0.00002      | 0.999     | 5.09 | 26.00|
| NPHS1   | rs114849139| chr19:36330456                | c.2869C>G   | p.V957L           | 0.001        | 0.987     | 4.27 | 14.34|
| FPR1    | rs78488639 | chr19:52249959                | c.289G>T    | p.L97M            | 0.008        | 0.795     | 2.55 | 10.70|

MAF: minor allele frequency; gnomAD: genome aggregation database; PolyPhen: polymorphism phenotyping; GERP: evidence of evolutionary conservation; CADD: combined annotation dependent depletion.

**Source data**

- Figure 2-source data 1. Raw data for intensity of bands in Figure 2D.
- Figure 2-source data 2. Raw data for the full raw unedited blots in Figure 2D.
- Figure 3-source data 1. Raw data for quantification in Figure 3D.
- Figure 3 supplement 1-source data 1. Raw data for quantification in Figure 3 supplement 1B and 1E.
- Figure 6-source data 1. Raw data for quantification in Figure 6B and 6D.
- Figure 6 supplement 1-source data 1. Raw data for quantification in Figure 6B.