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Latent TGF-β binding protein-2 is essential for the development of ciliary zonule microfibrils

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Latent TGF-β-binding protein-2 (LTBP-2) is an extracellular matrix protein associated with microfibrils. Homozygous mutations in *LTBP2* have been found in humans with genetic eye diseases such as congenital glaucoma and microspherophakia, indicating a critical role of the protein in eye development, although the function of LTBP-2 *in vivo* has not been well understood. In this study, we explore the *in vivo* function of LTBP-2 by generating *Ltbp2*$^{-/-}$ mice. *Ltbp2*$^{-/-}$ mice survived to adulthood but developed lens luxation caused by compromised ciliary zonule formation without a typical phenotype related to glaucoma, suggesting that LTBP-2 deficiency primarily causes lens dislocation but not glaucoma. The suppression of *LTBP2* expression in cultured human ciliary epithelial cells by siRNA disrupted the formation of the microfibril meshwork by the cells. Supplementation of recombinant LTBP-2 in culture medium not only rescued the microfibril meshwork formation in *LTBP2*-suppressed ciliary epithelial cells but also restored unfragmented and bundled ciliary zonules in *Ltbp2*$^{-/-}$ mouse eyes under organ culture. Although several reported human mutant LTBP-2 proteins retain normal domain structure and keep the fibrillin-1-binding site intact, none of these mutant proteins were secreted from their producing cells, suggesting secretion arrest occurred to the LTBP-2 mutants owing to conformational alteration. The findings of this study suggest that LTBP-2 is an essential component for the formation of microfibril bundles in ciliary zonules.

INTRODUCTION

Primary congenital glaucoma (PGC) is an inherited disease commonly found in children as early as their first year of life, often resulting in blindness. Recently, homozygous mutations in *LTBP2* were reported in patients with PCG who develop high intraocular pressure (IOP), megalocornea, buphthalmos and ectopia lentis (1–3). On the other hand, other groups reported on homozygous *LTBP2* mutations in patients with an autosomal recessive ocular disease who presented with megalocornea, microspherophakia and lens dislocation with secondary glaucoma later in life or without glaucoma (4–6). These results clearly identify causative *LTBP2* mutations that lead to genetic eye diseases including glaucoma, although it remains unclear whether glaucoma is the primary phenotype or a secondary result of other primary defect such as lens dislocation.

LTBP2 encodes a matrix protein, latent transforming growth factor beta (TGF-β)-binding protein-2 (LTBP-2), which is one of four LTBP family proteins (LTBP-1–4) (7,8). LTBPs are multidomain glycoproteins containing epidermal growth

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factor (EGF)-like domains and eight-cysteine domains, both of which are also found in other matrix proteins, namely fibrillin-1, -2 and -3. As the name indicates, LTBP-1, -3 and -4 bind to TGF-β molecules and function to keep the signaling molecule inactive in the extracellular matrix of tissues until further induction releases active TGF-β from the complex (7). Despite the homology with and a domain structure similar to that of other LTBPs, LTBP-2 does not bind to TGF-β molecules and seems to have a function distinct from direct involvement in TGF-β signaling (9). Several reports, including ours, indicated an association between LTBP-2 and microfibrils, which mainly comprise fibrillin-1 and -2, and suggested a role for LTBP-2 in the elastic fiber assembly process (10–13). Shipley *et al.* created *Ltbp2*-deficient mice and reported that the homozygous mutant mice died at an early embryonic stage, suggesting an essential role of LTBP-2 in early embryogenesis of mice (14). However, this gene-knockout study of mice was inconsistent with human studies because no birth defects and/or early developmental abnormalities other than eye diseases were reported in human *LTBP2* mutations. Because of this discrepancy, it has been understood that LTBP-2 has different functions among species *in vivo*.

In this study, we independently created conditional *Ltbp2*-mutant mice with minimum alterations in the mouse genome and created systemic homozygous null mutant mice to confirm their phenotype as previously reported (14). Surprisingly, the homozygous null mutant mice survived to adulthood without any gross abnormalities. We analyzed the phenotypes of the mutant mice and concluded that *Ltbp2* null mutant mice developed lens dislocations owing to the disorganized formation of ciliary zonules, which comprise bundles of microfibrils. We further analyzed the function of LTBP-2 and found that the protein plays an essential role in the assembly of large microfibril bundles in both cultured cells and organ-cultured mouse eyes. We also investigated the pathogenic effects of human *LTBP2* mutations and found that secretion arrest occurs in all reported LTBP-2 mutants that conserve the fibrillin-1-binding domain.

RESULTS

Ltbp2-mutant mice survived to adulthood

To investigate the biological function of LTBP-2 *in vivo*, we created conditional knockout mice that could delete exon 1 of *Ltbp2* in the presence of Cre-recombinase (Fig. 1A). The neomycin cassette (pgk-Neo) was removed from the floxed allele by *FRT*/Flp-mediated recombination. We tested the Cre-recombinase-dependent elimination of *Ltbp2* expression *in vivo* by mating heterozygous floxed mice with transgenic mice expressing Cre-recombinase in the germline to produce systemic heterozygotes ($Ltbp2^{+/-}$). The $Ltbp2^{+/-}$ mice were intercrossed to produce systemic *Ltbp2* homozygous mutant mice ($Ltbp2^{-/-}$). Using reverse transcription–polymerase chain reaction (RT–PCR), western blot and immunohistochemical analyses, we observed no detectable *Ltbp2* transcripts or LTBP-2 proteins in $Ltbp2^{-/-}$ tissues (Fig. 1B–D), indicating that the expression of *Ltbp2* was completely lost in the homozygous mutant mice. Contrary to a previous report (14), $Ltbp2^{-/-}$ mice survived to adulthood without showing gross developmental defects; furthermore, the homozygous mutant mice were fertile, indicating no reproductive abnormalities. By intercrossing $Ltbp2^{+/-}$ mice, we obtained homozygous mutant mice at the expected Mendelian ratio (Supplementary Material, Table S1), also indicating no embryonic lethality of the mutant mice. From these results, we concluded that lack of *Ltbp2* expression caused no severe effects on development and reproduction of mice *in vivo*.

Lack of LTBP-2 had no obvious contribution to tissue elasticity

LTBP-2 has been found to associate with fibulin-5, a protein necessary for elastogenesis (15–17), and was suggested to regulate elastic fiber assembly *in vitro* (8,10,11). Down-regulation of *LTBP2* by siRNA in human dermal fibroblasts allows fibrillin-1-independent deposition of fibulin-5 and elastin in the matrix (10). Therefore, we investigated whether there were any detectable abnormalities in the elastic fibers in *Ltbp2*-mutant mice. Histological examination did not reveal any morphological changes such as a sign of emphysema in $Ltbp2^{-/-}$ lung tissues (Supplementary Material, Fig. S1A). Electron microscopic observation also demonstrated normal elastic fiber formation in the extracellular matrices of the lungs and normal elastic lamellae in the aortic media of the mutant mice at different developmental stages (Supplementary Material, Fig. S1B). We also analyzed elastic features of the aortae by measuring arterial diameters under continuous increases in intra-aortic pressure and found no significant difference in aortic stiffness between the wild-type and $Ltbp2^{-/-}$ mice (Supplementary Material, Fig. S1C). On the basis of these results, we concluded that the lack of LTBP-2 does not lead to major abnormalities of elastic fibers in elastin-rich tissues in mice.

Null mutations in *Ltbp2* does not result in congenital glaucoma in mice

Although *LTBP2* mutations have been reported to cause congenital ocular diseases including glaucoma in humans (1–6,18), it remains unclear whether glaucoma is the primary phenotype or a secondary symptom owing to other congenital diseases such as ectopia lentis. These reports prompted us to investigate whether *Ltbp2*-mutant mice had any phenotypes related to glaucoma. Anterior chamber angle closure is one of the morphological abnormalities linked to high IOP, which leads to the optic nerve damage found in glaucoma. By histological examination, we found that the angles of $Ltbp2^{-/-}$ mouse eyes were not closed compared with those of wild-type eyes (Supplementary Material, Fig. S2A). Transmission electron microscopy showed no evident structural abnormalities in the trabecular meshwork and Schlemm's canal in $Ltbp2^{-/-}$ eyes (Supplementary Material, Fig. S2B). We measured the IOP of wild-type and $Ltbp2^{-/-}$ mice by placing a glass needle into the eyes under anesthesia *in vivo* and found no significant increase of IOP in $Ltbp2^{-/-}$ mouse eyes at both young and old ages (Supplementary Material, Fig. S2C). These results suggested that $Ltbp2^{-/-}$ mice developed none of the typical phenotypes of glaucoma that involved an increase in IOP. It is often found that human patients develop normal-tension glaucoma, wherein optic nerves are damaged even though the IOP is within normal range. Therefore, we tested whether there were any neuronal

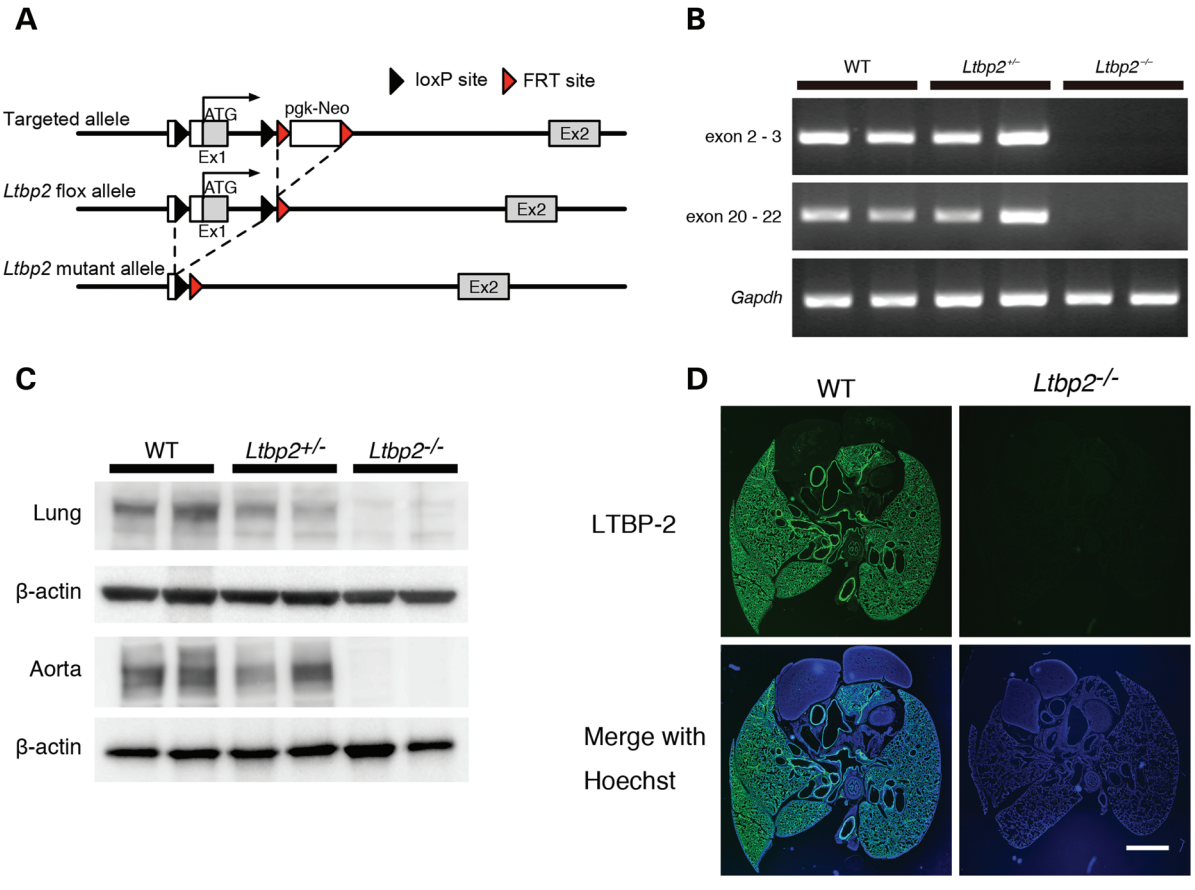

Figure 1. Generation of *Ltbp2* null mice. (**A**) Schematic representation of the strategy to target the *Ltbp2* gene. The pgk-Neo cassette flanked by FRT sites was removed by crossing with flippase-expressing mice. Subsequently, the exon 1 flanked by loxP sites was removed by crossing with mice expressing Cre-recombinase in the germline. (**B**) RT–PCR analysis showing complete loss of *Ltbp2* mRNA in $Ltbp2^{-/-}$ mice. Total RNA from lungs of 10-week-old mice was extracted and reverse-transcribed to cDNA, followed by PCR. Although only exon 1 of the *Ltbp2* gene was deleted, primers designed to amplify exon 2–3 or exon 20–22 did not produce PCR product in $Ltbp2^{-/-}$ samples, indicating that the *Ltbp2* mRNA was disrupted by nonsense-mediated decay. The expression level of *Gapdh* was used as an internal control (Bottom). (**C**) Western blot analysis of lung and aorta extract from 10-week-old wild-type and $Ltbp2^{-/-}$ mice, using an anti-LTBP-2 antibody. β-actin was used as an internal control. (**D**) Immunofluorescent staining of thoracic tissues of P5 wild-type and $Ltbp2^{-/-}$ mice using an anti-LTBP-2 antibody. LTBP-2 was specifically expressed in lungs and vessels of wild-type mice. Lower panels are superimposition of upper panels with Hoechst 33258 staining. Scale bar, 1 mm.

damages in $Ltbp2^{-/-}$ mouse eyes by measuring the density of the optic nerve and retinal ganglion cells; however, we found no difference on comparing with wild-type mouse eyes (Supplementary Material, Fig. S2D and E). Finally, we examined the visual function of the $Ltbp2^{-/-}$ mice by observing the retinotopic map in the visual cortex of the mice using optical imaging of intrinsic signals (Supplementary Material, Figs S3 and S4). The retinotopic maps in the wild-type and $Ltbp2^{-/-}$ mice appeared to cover a wide range of the visual field ($-30°$–$20°$ in azimuth), and we found no noticeable difference between them (Supplementary Material, Fig. S3B). Quantitative analysis of the cortical representation of the visual field also showed no significant difference between the wild-type and $Ltbp2^{-/-}$ mice (Supplementary Material, Fig. S3C). Therefore, we concluded that $Ltbp2^{-/-}$ mice do not have a visual field deficit in the horizontal plane. We also analyzed the visual acuity of $Ltbp2^{-/-}$ mice and found no significant difference compared with the acuity of wild-type mice (Supplementary Material, Fig. S4). These results indicated that $Ltbp2^{-/-}$ mice have no deficit in detecting projected images on the retina and in transmitting the information to the visual cortex of the brain.

Ltbp2 mutations cause ectopia lentis because of ciliary zonule fragmentation

LTBP2 mutations have also been reported in humans with ectopia lentis (3,4,6,18). Therefore, we next analyzed phenotypes related to lens dislocation in $Ltbp2^{-/-}$ mice. Indeed, the mutant mice developed lens dislocation into the posterior chamber of the eyes (Fig. 2A and B). Ciliary zonules act as ligaments anchoring the lens to the ciliary process and are made of bundles of fibrillin microfibrils (3,19). Immunofluorescent staining, immunotransmission electron microscopy (immuno-TEM) and western blot analyses revealed the presence of LTBP-2 in ciliary bodies, including the ciliary epithelial cells and ciliary zonules, in wild-type mouse eyes (Fig. 2C, Supplementary Material, Figs S5 and S6A and C). Immuno-TEM of negatively stained ciliary zonules revealed that LTBP-2 is localized to the junction of microfibrils and connecting filaments, a structure previously reported in ciliary zonules (20) (Supplementary Material, Fig. S6D and E). The fibrous structure of ciliary zonules, which is visualized by immunohistochemical examination using

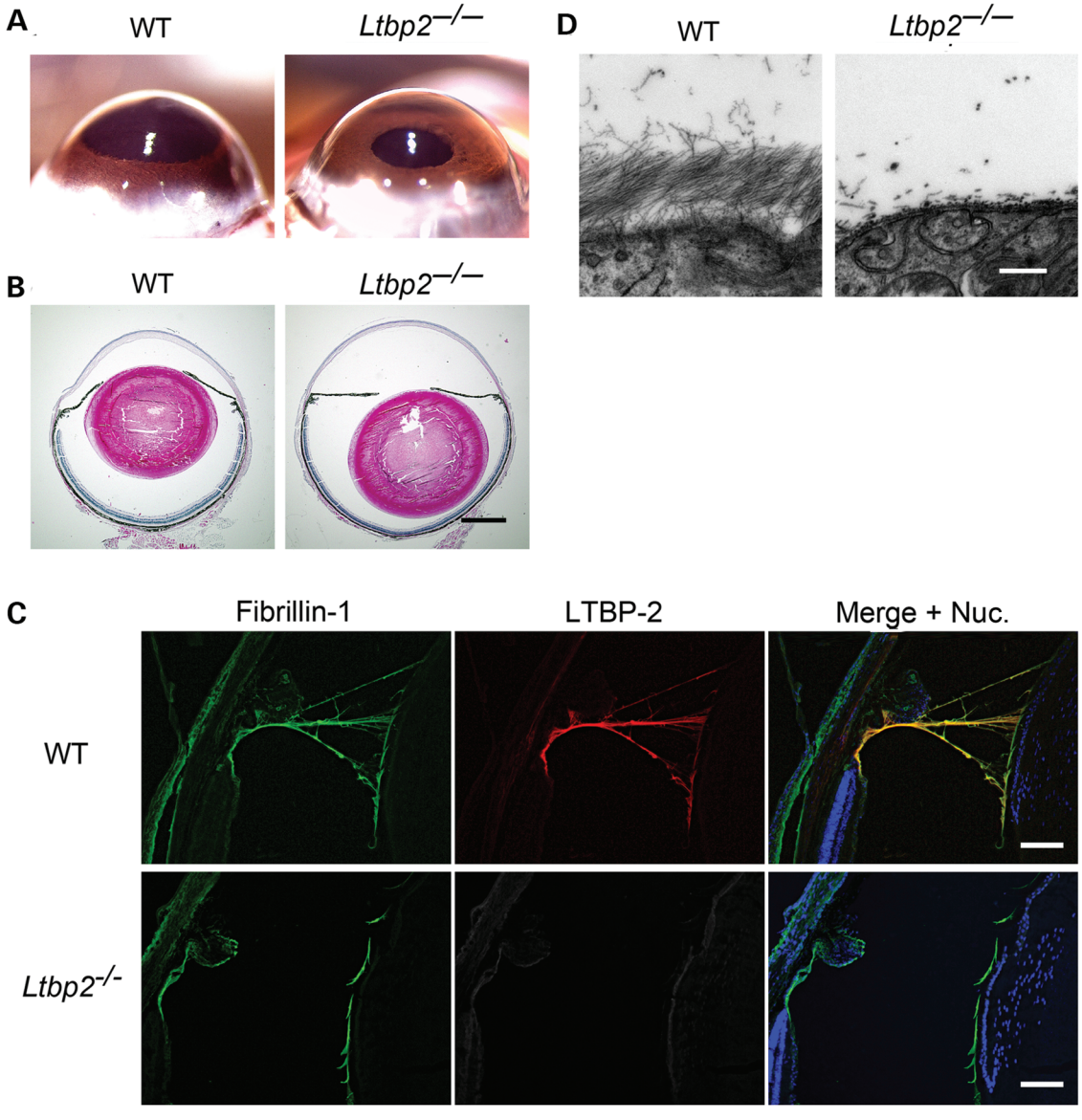

Figure 2. *Ltbp2* deficiency causes ciliary zonule fragmentation. (**A**) Pictures of eyes of wild-type and *Ltbp2*$^{-/-}$ mouse at 12 months. The irises of wild-type mouse eyes were convex as they were pushed by lenses, whereas the irises of *Ltbp2*$^{-/-}$ mouse eyes appeared flat. (**B**) HE-stained histological sections of mouse eyes. The lens of *Ltbp2*$^{-/-}$ mouse eye was dislocated posteriorly. Scale bar, 1 mm. (**C**) Immunofluorescent staining of 8-week-old *Ltbp2*$^{-/-}$ and wild-type mouse eyes using anti-fibrillin-1 and anti-LTBP-2 antibodies. The right images were produced by superimposition of the left and middle images, together with Hoechst 33258 nuclear staining. Scale bars, 100 μm. Nuc., nuclei. (**D**) TEM of ciliary zonules of 10-week-old wild-type and *Ltbp2*$^{-/-}$ mouse eyes. Ciliary zonules of wild-type eye were composed of thick bundles of microfibrils, but those of *Ltbp2*$^{-/-}$ eye were fragmented. Scale bar, 500 nm.

anti-fibrillin-1 antibody, was present in wild-type mouse eyes, whereas the structure was disorganized in *Ltbp2*-mutant mouse eyes at early stages after birth (Supplementary Material, Fig. S6B) and largely missing in later stages (Fig. 2C), even though the expression levels of fibrillin-1 were similar in the eyes of both genotypes (Supplementary Material, Fig. S5A). Electron microscopy also detected microfibrils as thick bundled structures on the surface of the ciliary epithelial cells of wild-type mouse eyes, whereas no bundled structures of microfibrils were observed on the ciliary epithelial cells of *Ltbp2*$^{-/-}$ mouse eyes, leaving only fragmented remnants of microfibrils (Fig. 2D). These results indicated that LTBP-2 has an essential role in forming the thick bundled structure of microfibrils in ciliary zonules. mRNAs for *Ltbp1* and *3* were expressed in the ciliary body, suggesting that these could not compensate for the absence of LTBP-2 (Supplementary Material, Fig. S5D).

LTBP-2 is required for microfibril assembly on ciliary epithelial cells

From these results, we expected that LTBP-2 functioned to construct the microfibrils of ciliary zonules. To test this hypothesis, we cultured human non-pigmented ciliary epithelial cells and analyzed microfibril formation in the presence or absence of LTBP-2. The primary cultured ciliary epithelial cells produced

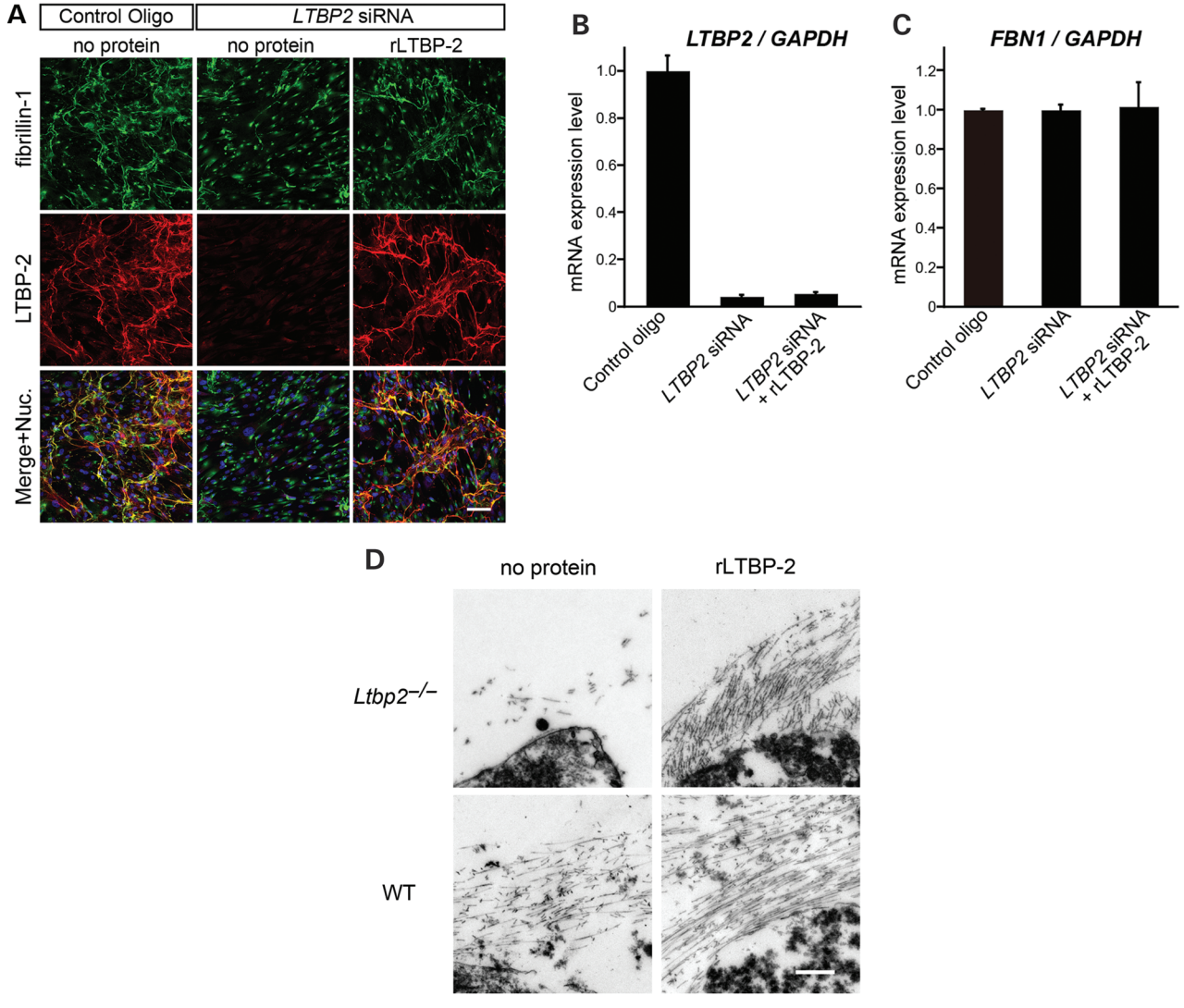

Figure 3. LTBP-2 induced microfibril assembly in cell culture (**A**–**C**) and in organ culture (**D**). (A) Human non-pigmented ciliary epithelial cells (HNPCEC) were transfected with siRNA as indicated and cultured in 4% serum containing medium with or without recombinant LTBP-2 (rLTBP-2), 15 μg/ml for 7 days. Cells were stained with anti-fibrillin-1 (upper panels) and anti-LTBP-2 antibodies (middle panels). Superimpositions of the upper and middle panels together with Hoechst nuclear staining (lower panels) showed co-localization of LTBP-2 and fibrillin-1. *LTBP2* knockdown abrogated fibrillin microfibril meshwork formation, and supplementation of rLTBP-2 rescued the knockdown effect in HNPCEC. Scale bar, 100 μm. (B) *LTBP2* knockdown efficiency in HNPCEC. Total RNA from siRNA-transfected HNPCEC was extracted 7 days after transfection. *LTBP2* and *GAPDH* mRNA levels were measured with quantitative real-time PCR. (C) *LTBP2* knockdown or rLTBP-2 supplementation did not affect the expression of fibrillin-1. *FBN1* mRNA levels were measured as in (B), using *GAPDH* mRNA levels as internal controls. Data are presented as mean ± SEM of five independent experiments, each performed in triplicate. (D) Eye explants of P5 wild-type and $Ltbp2^{-/-}$ mice were cultured with or without rLTBP-2. After 7 days culture, ciliary zonules were evaluated by TEM. $Ltbp2^{-/-}$ mouse eye cultured without rLTBP-2 (upper-left), with rLTBP-2, 15 μg/ml (upper-right), wild-type eye cultured without rLTBP-2 (lower-left), with rLTBP-2, 15 μg/ml (lower-right). rLTBP-2 not only rescued the formation of ciliary zonules in $Ltbp2^{-/-}$ mouse eyes but also increased microfibril bundles in the ciliary zonules of wild-type mouse eyes. Scale bar, 500 nm.

microfibrils that were visualized by the anti-fibrillin-1 antibody on the surface of the cells (Fig. 3A). We found that LTBP-2 co-localized to fibrillin-1, showing a fibrous pattern on the surface of the cells. When we suppressed the expression of *LTBP2* mRNA by siRNA, we found that fibrillin-1-positive microfibrils were markedly decreased on the *LTBP2* knockdown cells (Fig. 3A), although the siRNA suppressed the expression of LTBP-2 but not that of fibrillin-1 (Fig. 3B and C). We then supplemented the culture medium of ciliary epithelial cells transfected with *LTBP2* siRNA with recombinant LTBP-2 protein and observed the restoration of microfibril formation on the cells; this excluded the possibility that the decrease in microfibrils by *LTBP2* siRNA was an off-target effect (Fig. 3A). The added LTBP-2 protein co-localized with fibrillin-1 on the microfibril meshwork. The requirement of LTBP-2 in microfibril formation seems to be cell type-specific, because suppression of *LTBP2* mRNA by siRNA in human dermal fibroblast did not affect the development of microfibril meshwork (Supplementary Material, Fig. S7). Through an organ culture experiment *ex vivo*, we further tested whether supplementation of recombinant LTBP-2 was effective in restoring ciliary zonules in *Ltbp2*-mutant mouse eyes. We cultured the anterior cup of eye

from P5 animals in culture medium for 7 days with or without recombinant LTBP-2 supplementation and observed microfibril formation in the ciliary body by electron microscopy. Only fragmented microfibrils were detected on the ciliary epithelial cells of *Ltbp2*-mutant mouse eyes without LTBP-2 supplementation; however, the production of bundled unfragmented microfibrils was observed in mutant eyes that were cultured in the presence of LTBP-2 (Fig. 3D). These results suggested that LTBP-2 promotes the assembly of mature microfibrils in ciliary zonules.

Pathological *LTBP2* mutations cause loss of secretion and lack of fibrillin-1 binding activity

It has been previously reported that LTBP-2 directly binds to fibrillin-1, one of the main components of microfibrils (11). Several groups have determined that missense, nonsense and frame-shift mutations in *LTBP2* in humans are responsible for familial eye diseases such as glaucoma and microspherophakia (Supplementary Material, Table S2) (1–6). We expected that these mutations would cause a loss of function of *LTBP2*, resulting in malformation of microfibrils in the eyes; therefore, we tested the impact of mutations in LTBP-2 for secretion and binding to fibrillin-1. We first narrowed down the critical domain for fibrillin-1 binding on LTBP-2. Because intact LTBP-2 binds to the N-terminal domain of fibrillin-1 (11), we expressed the recombinant N-terminal domain of fibrillin-1 (rF23) (21) with a myc-tag in HEK293T cells and recovered the protein in cultured medium to use for LTBP-2 binding. For LTBP-2, we separated the protein into five domains and expressed each domain with a FLAG tag into the culture medium of HEK293T cells (Fig. 4A). We then mixed both the culture media containing myc-tagged fibrillin-1 fragment and FLAG-tagged LTBP-2 fragments and immunoprecipitated LTBP-2 fragments by anti-FLAG antibody beads to find out which LTBP-2 domain binds to fibrillin-1. We found that LTBP-2 binds to fibrillin-1 at the latter half of the calcium-binding EGF (cbEGF)-like repeat domain (LTBP-2-D; Fig. 4B). When we looked at the reported human *LTBP2* mutations, we found that most of the LTBP-2 mutants did not have this fibrillin-1-binding domain because of premature termination (Supplementary Material, Table S2); however, we noticed three frame-shift mutants and one missense mutant containing the fibrillin-1-binding domain. To test whether these mutants had the ability to bind to fibrillin-1, we prepared expression vectors for these mutants by site-directed mutagenesis. As a control, we also prepared expression vectors for LTBP-2 variants that contained single amino acid replacements caused by single-nucleotide polymorphisms (SNPs) deposited at Genbank (Supplementary Material, Table S3). Prepared expression vectors were individually transfected into HEK293T cells, and the culture medium from these transfected cells was collected for immunoprecipitation with fibrillin-1 fragments. However, when we tested for the presence of recombinant proteins in the cultured medium, we found that none of the LTBP-2 mutants were efficiently secreted in the medium, although the proteins were detected in cell lysates (Fig. 5A). Normal and polymorphic LTBP-2 variants were all detected in both the cell lysate and cultured medium, indicating that the failure of secretion is specific to mutant proteins. Because all the LTBP-2 mutants were produced in the cells, we prepared cell lysates and analyzed the fibrillin-1 binding activity of the mutant proteins. Among the four LTBP-2 mutants, three mutants had no binding activity to fibrillin-1, whereas one missense mutant LTBP-2 (p.C1438Y) showed fibrillin-1 binding activity equivalent to that of normal and polymorphic LTBP-2 variants (Fig. 5B). These data suggest that pathogenic mutations cause structural alterations in the proteins, leading to secretion failure, inactivation of fibrillin-1 binding activity or both.

DISCUSSION

LTBP-2 is the only protein that does not bind to TGF-β in the LTBP family; therefore, it is suspected to have a biological function other than TGF-β signal transduction. Shipley *et al*. reported on the early embryonic lethality of systemic homozygous *Ltbp2* knockout mice (14), suggesting an essential function of the protein at the embryonic stage in mice, although the reason of the lethality was not identified. On the other hand, it has been reported by several groups that human homozygous mutations of *LTBP2* cause ocular diseases including primary or secondary glaucoma, megalocornea, microspherophakia and ectopia lentis but no developmental defects in organs other than eyes (1–6). These findings indicate a sharp contrast of phenotypes between mice and humans, because many of the human *LTBP2* mutations were nonsense mutations at the N-terminal region and considered equivalent to null mutations. In the process of generating conditional *Ltbp2* knockout mice, we generated systemic *Ltbp2* homozygous mutant mice by deleting the first exon containing the starting ATG codon. Unexpectedly, the homozygous mutants showed no outcomes such as embryonic lethality, gross developmental abnormalities or impaired fertility. Because we deleted the pgk-Neo cassette (Fig. 1A), the artificial effect of the exogenous sequences was minimal in our study compared with that in the original *Ltbp2*-mutant mice analysis. We assume that the *Ltbp2*-mutant mice created in the previous report may have had an unexpected disruption of a critical gene(s) in addition to *Ltbp2* or that the remaining artificial DNA sequences, including the pgk-Neo cassette, may have affected the expression of the neighboring genes that were required for embryonic development.

We extensively analyzed any detectable ocular abnormalities and visual function in the *Ltbp2*-mutant mice and found that the mutant mice exhibited severely fragmented ciliary zonules in the eye without any sign of glaucoma. Homozygous mutations in *LTBP2* have been originally reported to cause glaucoma in humans (1,2); however, some of the following reports have also described *LTBP2* mutations for other eye diseases such as megalocornea, microspherophakia and Weill–Marchesani syndrome (3–6,18). Currently, researchers disagree as to whether glaucoma is a primary symptom or a secondary effect of another abnormality caused by *LTBP2* mutations. Interestingly, the reported symptoms for all patients with *LTBP2* mutations include zonule weakness and lens dislocation, suggesting a malformation of ciliary zonules as the primary defect caused by *LTBP2* mutations. Indeed, lens dislocation is often associated with secondary glaucoma, as observed in Marfan syndrome and Weill–Marchesani syndrome (22). Increasing number of evidences suggest association of microfibril deficiency with glaucoma (23). One

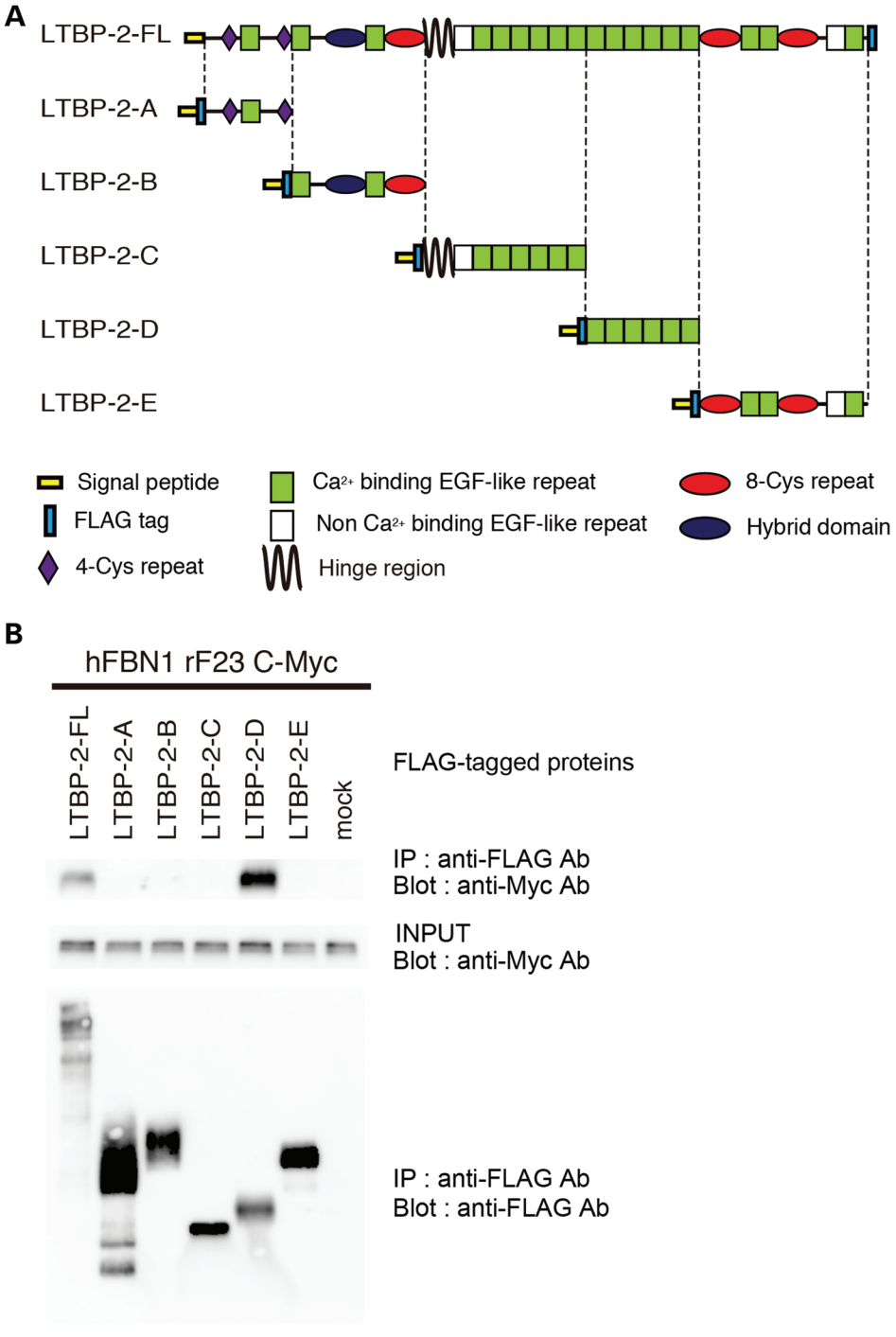

Figure 4. *In vitro* binding assay showing interaction of LTBP-2 with fibrillin-1. (**A**) Domain structure of the human full-length LTBP-2 and the LTBP-2 fragments. The full-length LTBP2 was expressed as a C-terminal FLAG-tagged protein, and LTBP-2 fragments were expressed as N-terminal FLAG-tagged proteins flanked by the preprotrypsin signal sequence in HEK293T cells. (**B**) Specific interaction of fibrillin-1 with LTBP-2-D fragment. An N-terminal fragment of human fibrillin-1 (rF23) was independently expressed as a C-terminal Myc-tagged protein. Mixture of conditioned media and cell lysates from cells transfected with the LTBP-2 constructs and with the fibrillin-1 construct were incubated and immunoprecipitated with anti-FLAG antibody, followed by western blot analysis with anti-Myc antibody and anti-FLAG antibody.

possible explanation is that moving lens causes adhesion of iridocorneal angle by pushing the iris forward. Another possibility is that fragmented zonule microfibrils occlude the trabecular meshwork or Schlemm's canal, resulting in high IOP. We therefore assume that the *LTBP2* mutations do not cause PCG; however, some patients with the mutations develop symptoms of glaucoma at an early stage of life as a secondary effect of lens dislocation and/or fragmentation of ciliary zonules. Screening of gene mutations in the United States, United Kingdom, India and Saudi families and sporadic cases

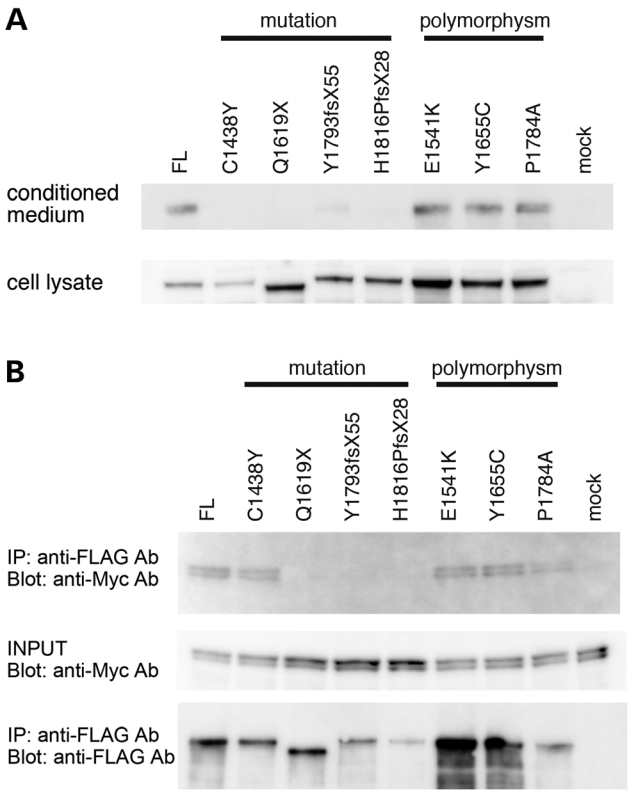

Figure 5. Human *LTBP2* mutations cause loss of secretion and lack of fibrillin-1 binding activity. (**A**) HEK293T cells were transiently transfected with FLAG-tagged human LTBP-2 cDNAs with point mutations reported in ocular diseases or with known polymorphisms. Transfected cells were cultured in serum-free medium for 48 h, and the cell lysates and the conditioned media were harvested and analyzed by western blot with anti-FLAG antibody. (**B**) *In vitro* binding assay showing interaction of LTBP-2 mutants and variants with fibrillin-1. Mixtures of the media and cell lysates of HEK293T cells transfected with FLAG-tagged LTBP-2 mutants or variants were incubated with independently expressed Myc-tagged N-terminal fragment of fibrillin-1. The reactants were subjected to immunoprecipitation with anti-FLAG antibody, followed by western blot analysis with anti-Myc antibody or anti-FLAG antibody.

of PGC did not find *LTBP2* mutations (24–27), possibly because these PCG subjects were all primary and had no other ocular manifestations, including lens dislocation. Our mice data are in accordance with these clinical investigations.

We observed that *Ltbp2*-mutant mice developed lens luxation because of abnormal ciliary zonule formation (Fig. 2, Supplementary Material, Figs S6A and S7). Through electron microscopic observation, thick and continuous microfibrils were barely detected in the mutant mice, indicating that LTBP-2 is involved in the formation of mature ciliary zonules made of bundled microfibrils (Fig. 2D). Because LTBP-2 binds to fibrillin-1, which is the major component of microfibrils, LTBP-2 may have a role in the development of mature bundles of microfibrils so that ciliary zonules obtain mechanical strength. Microfibrils consist of fibrillins, particularly fibrillin-1 in case of ciliary zonule microfibrils, with the parallel head-to-tail alignment (28); this occurs without a major structural contribution of other extracellular matrix proteins. LTBP-2 may not be directly involved in the formation of the fibrillin polymer; instead, the protein may be associated with the fibrillin polymer to form larger bundles of fibers. By proteomics analysis, Cain *et al*. reported that extensively purified zonular microfibrils consist of fibrillin-1 as the largest component, with a small contribution from microfibril-associated glycoprotein-1 (MAGP-1) as the matrix proteins (29). Although they did not detect LTBP-2 by their proteomics analysis, we detected the presence of LTBP-2 in ciliary zonules in intact eye tissue (Fig. 2C, Supplementary Material, Figs S5 and S6), indicating LTBP-2 is present but may not covalently or strongly interact with fibrillin-1 in ciliary zonules. Davis *et al*. reported that ciliary zonule microfibrils were laterally connected by small filaments (20). Our electron microscopic observation detected a lateral association of LTBP-2 to a microfibril (Supplementary Material, Fig. S6E), suggesting that LTBP-2 may contribute to the formation of thick microfibrils by tying fibrillin microfibrils into bundles. Further investigation is necessary to determine whether LTBP-2 constitutes the small connecting filaments.

LTBP-2 binds to fibrillin-1 at the C-terminal half of the long cbEGF-like repeat domain (Fig. 4A and B) that corresponds to exon 23 to 28 in the *LTBP2* genomic structure. Most of the *LTBP2* mutations found in humans are located on earlier exons of *LTBP2*, resulting in premature termination of the gene product. However, some of the pathogenic *LTBP2* mutations are localized to the C-terminal region in the primary structure of the protein; therefore, the mutant proteins should contain the fibrillin-1-binding domain. To uncover the pathogenic mechanism of *LTBP2* mutations, we produced recombinant LTBP-2 mutant proteins in cultured HEK293T cells to perform immunoprecipitation with a recombinant fibrillin-1 fragment and found that none of the mutants were efficiently secreted from the producing cells (Fig. 5A). All the LTBP-2 variant proteins that contained nonpathogenic amino acid substitutions because of SNPs were as efficiently secreted as the original LTBP-2 protein; therefore, only pathogenic mutations affected the secretion of the matrix protein, which could be caused by conformational alteration. It is interesting that although the mutations are downstream of the fibrillin-1-binding domain, all but one missense LTBP-2 mutants had no binding to fibrillin-1 by immunoprecipitation (Fig. 5B). This finding suggested that these mutations may cause such conformational changes that result in not only secretion arrest but also loss of fibrillin-1 binding activity.

The addition of recombinant LTBP-2 in a culture medium of human ciliary epithelial cells transfected with *LTBP2* siRNA restored the microfibril meshwork formation in the extracellular matrix of the cells (Fig. 3A). The recombinant protein also increased the production of stable microfibrils in organ-cultured eye tissues from both wild-type and *Ltbp2*-mutant mice (Fig. 3D), suggesting an essential function of LTBP-2 in the construction of ciliary zonules. *Ltbp2*-deficient mice, however, did not show an apparent abnormality in the matrix of other tissues such as the lung, aorta, and skin (Supplementary Material, Fig. S1), although *Ltbp2* mRNA was abundantly expressed in these tissues in wild-type mice (14). We assume that stable microfibril formation in ciliary zonules critically depends on LTBP-2; however, other matrix proteins may compensate for the lack of LTBP-2 in the other tissues. There are 3 other LTBP family members both in mice and human. Among these, *Ltbp1* and *3* were expressed in the ciliary body (Supplementary Material, Fig. S5D), suggesting that LTBP-1 and 3 are not sufficient to compensate for the lack of LTBP-2. Mutant mice lacking an isoform of LTBP-4 develop pulmonary emphysema as well as cardiomyopathy associated

with disrupted elastic fiber formation (30). Further studies using double-mutant mice deficient in *Ltbp2* and *Ltbp4* are required to address the question whether LTBP-2 has a cryptic function in tissues other than ciliary zonules.

In summary, we demonstrated the essential function of LTBP-2 in the formation of ciliary zonules, which consist of microfibrils. Homozygous mutant mice lacking LTBP-2 exhibited lens dislocation because of the disruption of normal ciliary zonule formation, thus resembling some symptoms of human patients with *LTBP2* homozygous mutations. Further studies including *Ltbp2*-mutant mice and gene mutations related to ectopia lentis in humans will lead to understanding of the microfibril organization as well as the pathology of glaucoma.

MATERIALS AND METHODS

Mice

All mice used in this study were maintained on normal lab diet. All procedures were conducted according to the Guideline for Animal experimentation at Kansai Medical University and Tottori University.

Human samples

The human cadaveric eye was obtained from San Diego Eye Bank. A written consent was acquired from the donor regarding eye donation for research purpose. Tissue procurement was adherent with the Declaration of Helsinki. The globe was initially cut into three parts and homogenized in 8 M urea using a Polytron homogenizer (PT10−35, Kinematica AG). After centrifugation, the supernatants were subjected to western blotting.

Generation of *Ltbp2* knockout mice

The coding sequence in the exon 1 of mouse *Ltbp2* was flanked by two loxP sequences inserted in 5′ untranslated region of the exon 1 and intron 1. The pgk-Neo cassette flanked by FRT sites was removed by crossing with flippase-expressing mice (31). To generate *Ltbp2* null allele, $Ltbp2^{flox/+}$ mice were crossed with Ayu-1 Cre knock-in mice, which express Cre in multiple tissues including germ line (32). See Supplementary Material, Methods, for construction of the targeting vector.

Antibodies and immunodetection

Primary antibodies used were as follows: rabbit anti-LTBP-2 polyclonal (made by immunizing rabbits with recombinant mouse LTBP-2, 1:1000, Supplementary Material, Fig. S2C, Fig. 5A and B; 1:100, Fig. 1D, Supplementary Material, Fig. S6A; 1:50, Supplementary Material, Fig. S6C and E), chicken anti-LTBP-2 polyclonal (made by immunizing chickens with recombinant mouse LTBP-2, 1:100, Fig. 2C), mouse anti-LTBP-2 monoclonal 8F1 (made by immunizing mice with recombinant human LTBP-2, 1:100, Fig. 3A; 1:1000, Supplementary Material, Fig. S5C), rabbit anti-fibrillin-1 polyclonal PR217 (Elastin Products Company, 1:100, Fig. 3A), rabbit anti-mouse fibrillin-1 polyclonal (made by immunizing rabbits with recombinant mouse fibrillin-1 fragment, 1:100, Fig. 2C and Supplementary Material, Fig. S6B) and rabbit anti-γ-Synuclein polyclonal (Abcam, 1:100, Supplementary Material, Fig. S2E) antibodies. See Supplementary Material, Methods, for details.

Cell culture

HEK293T cells and human non-pigment ciliary epithelial cells (HNPCEC) purchased from Sciencell were maintained in DMEM (Invitrogen) supplemented with 2 mM glutamine, 100 units/100 mg/ml penicillin/streptomycin and 10% FBS at 37°C in 5% CO_2. To develop fibrillin microfibrils, confluent HNPCECs were cultured on coverslips in DMEM/F12 (Invitrogen) supplemented with 2 mM glutamine, 100 units/100 mg/ml penicillin/streptomycin and 10% FBS with or without 15 μg/ml of recombinant LTBP-2 at 37°C in 5% CO_2 for 7 days without changing the medium.

Plasmid construction

Human full-length LTBP-2 cDNA was kindly provided by J Keski-Oja (University of Helsinki). pEF6/ssFLAG, which contains a preprotrypsin signal sequence followed by a FLAG tag and 6×His tag before multiple cloning sites, was used to add an N-terminal FLAG tag (33). pEF6/FLAG or pEF6/Myc were used to add a C-terminal FLAG tag or a C-terminal Myc tag (16). FLAG-tagged human LTBP-2 fragments LTBP-2A, B, C, D and E were previously described (10). The following four mutant cDNAs and three polymorphism cDNAs of human LTBP-2 were generated by site-directed mutagenesis and subcloned into pEF6/ssFLAG: c.4700G>A (p.C1438Y), c.5242C>T (p.Q1619X), c.5763delC (p.Y1793fsX55), c.5833dupC (p.H1816PfsX28), c.5008G>A (p.E1541K), c.5351A>G (p.Y1655C), c.5737C>G (p.P1784A). All constructs were confirmed by sequencing (ABI Prism 3100). An N-terminal fragment of human fibrillin-1 rF23 cDNA was kindly provided by Lynn Sakai (Shriners Hospital for Children) and subcloned into pEF6/Myc and pEF6/ssFLAG.

Protein purification

Recombinant human and mouse LTBP-2 with a FLAG tag and a 6×His tag was purified using TALON affinity resin (Takara) from serum-free conditioned medium of HEK293T cells stably transfected with pEF6/ssFLAG-human LTBP-2 or pEF6/FLAG-mouse LTBP-2.

Transfection, *in vitro* binding assay and western blotting

HEK293T cells were transfected using LipofectAMINE PLUS (Invitrogen) or FuGENE HD Transfection Reagent (Promega). After transfection, they were cultured in serum-free DMEM/F12 (Invitrogen). The conditioned media were subjected to immunoprecipitation with anti-FLAG M2 affinity gel followed by western blotting as described previously (33).

RNAi

Duplex RNA oligonucleotides (Stealth Select RNAi) and control oligonucleotides (Stealth RNAi™ siRNA negative control) were purchased from Invitrogen. Mixed siRNA duplexes (at final concentration 10 nM) were reverse-transfected into HNPCECs using Lipofectamine RNAi MAX (Invitrogen). The oligonucleotides

sequences of LTBP2 siRNAs are provided in Supplementary Material, Table S6.

AUTHORS' CONTRIBUTIONS

T.I., T.O., T.O.A. and T.N. designed the study; T.O., Y.F., H.Y., K.N., M.H., K.K., Y.H. and T.N. performed the experiments; T.I., T.O., Y.F. and T.N. collected and analyzed the data; K.T. and K.K. provided the reagents; T.I., T.O.A. and T.N. wrote the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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