Spata7 is required for maintenance of the retinal connecting cilium

Jiaxiong Lu1, Kaitlyn Xiong2, Xinye Qian1, Jongsu Choi1, Yoon-Kyung Shim3, Jacob Burnett3, Graeme Mardon3 & Rui Chen1,2

SPATA7, an early onset LCA3 retinal disease gene, encodes a putative scaffold protein that is essential for the proper assembly of the connecting cilium (CC) complex in photoreceptors. Previous studies have shown that SPATA7 interacts with other photoreceptor-specific ciliary proteins, such as RPGR and RPGRIP1, and maintains the integrity of CC integrity. However, although it is known that Spata7 is required for early formation of the CC, it is unclear if Spata7 is also required for the maintenance of the CC. To investigate Spata7 function in the retina at the adult stage, loss of function was induced in the adult retina upon tamoxifen induction of an inducible Spata7 knockout allele (Spata7*; UbcCreERT2/+). The phenotype of mutant retina was characterized by a combination of histology, immunobiochemistry, and electroretinography (ERG). Our results demonstrated that Spata7 is also essential for maintaining the integrity of the mature retinal CC. Loss of Spata7 in adults caused phenotypes similar to those seen in germline mutant mice, including photoreceptor cell degeneration and defective ERG responses. Close examination of the CC revealed significantly shortened NPHP1 length as a result of Spata7 deletion. Furthermore, mislocalization of rhodopsin, leading to ER stress-mediated apoptosis, was observed in the retinal layers. Our results indicate that Spata7 is required not only for the establishment but also for the maintenance of the CC of photoreceptors.

Retinal photoreceptor cells contain a primary cilium known as the connecting cilium (CC)1. The CC is a slender structure that connects the outer (OS) and inner segments (IS) of photoreceptors and mediates a critical protein trafficking function2,3. Mutations in several photoreceptor-specific and common cilia genes can lead to deficient morphogenesis and/or dysfunction of the CC and result in retinal ciliopathies, a group of inherited retinal degenerative diseases including Retinitis pigmentosa (RP)4 and Leber congenital amaurosis (LCA)5.

Proper formation and maintenance of the CC is essential for proper function of photoreceptor cells. Many genes that are important for CC development are also essential for CC maintenance. For example, both early and late loss of function of intracellular transport factors KIF3A or IFT88 result in CC defects and photoreceptor degeneration6. In contrast, it has been observed that although NPHP1 is crucial during OS development, once the CC is established, a low level of NPHP1 is sufficient to maintain the proper function of the CC6.

We previously identified SPATA7 (spermatogenesis-associated protein 7) as a disease gene in the LCA3 locus7,8. Mechanistic studies indicate that Spata7 is critical for the integrity of microtubule filaments of the CC and physically interacts with multiple members of the RPGR and NPHP complexes8. Loss-of-function mutations in SPATA7 cause rapid photoreceptor degeneration and lead to LCA in humans9,10,11. Similarly, severe early-onset photoreceptor degeneration is observed in Spata7 germine knockout (Spata7*+) mice and lead to rapid retinal degeneration defects in adults causing rapid photoreceptor degeneration in the adult retina5. In addition, consistent with observations in germline Spata7*+ mice, photoreceptor cells of tamoxifen-induced Spata7ikO mice exhibit rhodopsin mislocalization in the IS and outer nuclear layer (ONL) and ER stress activation. Taken together, our results indicate that Spata7 is required for both the initial establishment and continued maintenance of the retinal CC.

1Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX, USA. 2Department of Molecular and Human Genetics, Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX, USA. 3Department of Pathology, Baylor College of Medicine, Houston, TX, USA. *email: ruichen@bcm.edu
Figure 1. Loss of SPATA7 expression in retinas of Spata7<sup>flox/−</sup>; UbcCreERT2/+ mice. (A) Schematic of the Spata7 <sup>flox</sup> allele and breeding scheme for generating Spata7<sup>flox/−</sup>; UbcCreERT2 mice. Numbered rectangles represent Spata7 exons. (B–D) Anti-SPATA7 staining (pink) of retinal cryosections showing absence of Spata7 protein in 4-month-old Spata7<sup>flox/−</sup>; UbcCreERT2/+ mice with tamoxifen (injected at 3 months of age) (C) compared to non-injected Spata7<sup>flox/−</sup>; UbcCreERT2/+ mice (B) and tamoxifen-injected Spata7<sup>flox/−</sup> mice (D). Nuclei are stained with DAPI (blue). INL, inner nuclear layer; IS, inner segment; ONL, outer nuclear layer; OS, outer segment; CC, connecting cilium. (E) PCR amplification of genomic DNA shows complete deletion of the floxed portion of Spata7 following tamoxifen induction in Spata7<sup>flox/−</sup>; UbcCreERT2 mice. (1) Non-injected Spata7<sup>flox/−</sup>; UbcCreERT2 and (2) tamoxifen injected Spata7<sup>flox/−</sup>; UbcCreERT2. Scale bar = 20 µm.
Results

UbcCreERT2 successfully deletes Spata7 in adult mouse retinas. Previous studies have focused on investigating the role of SPATA7 in the developing retina using germline Spata7 knockout mice\(^9,12\). To determine the role of SPATA7 in maintaining the photoreceptor CC in the mature mammalian retina, we generated an inducible Spata7 knockout mouse (Spata7\(^{flox/-}\); UbcCreERT2\(^+/+\)) by cross-breeding conditional Spata7 knockout mice (Spata7\(^{flox/flox}\); UbcCreERT2\(^+/+\)) with germline Spata7 knockout mice harboring the UbcCreERT2 transgene\(^15,16\) (Spata7\(^{-/-}\); UbcCreERT2\(^+/+\)) (Fig. 1A). Cre expression was induced by injecting tamoxifen at 3 months of age, when mouse retinas have fully matured\(^17\). Previous studies have shown that Spata7 localizes to the CC, which connects the IS and the OS\(^12\). UbcCreERT2-mediated Spata7 deletion was evaluated by staining for Spata7 protein in the CC of Spata7\(^{flox/-}\); UbcCreERT2\(^+/+\) mice one month after tamoxifen induction. Immunostaining showed the absence of Spata7 in the CC of tamoxifen-injected Spata7\(^{flox/-}\); UbcCreERT2\(^+/+\) mice, whereas Spata7 protein was readily detected in control mice, including Spata7\(^{flox/-}\); UbcCreERT2\(^+/+\) mice without tamoxifen induction and Spata7\(^{flox/-}\) mice without the UbcCreERT2 allele (Fig. 1B–D). We performed PCR on genomic DNA extracted from isolated mouse retinas using primers flanking the Spata7 allele\(^14\), and no band corresponding to was detected for tamoxifen-injected Spata7\(^{flox/-}\); UbcCreERT2\(^+/+\) mice compared with control mice (Fig. 1E). These data indicate that the Spata7 conditional allele was completely deleted in adult mouse retinal cells upon tamoxifen induction, making the Spata7\(^{flox/-}\); UbcCreERT2\(^+/+\) mouse a suitable model for studying Spata7 function in adult retina.

Figure 2. Significant photoreceptor degeneration due to loss of Spata7 in adult mouse retina. (A) Hematoxylin and eosin staining of paraffin-embedded retinal sections shows reduced ONL thickness in 6-month-old Spata7\(^{flox/-}\); UbcCreERT2 mice following tamoxifen induction at 3 months of age. Tamoxifen non-injected Spata7\(^{flox/-}\); UbcCreERT2 mice and 3-month-old wild-type and Spata7\(^{-/-}\) mice act as controls. ONL outer nuclear layer, INL inner nuclear layer, GCL ganglion cell layer. (B) Spider plot showing retinal morphometry reveals reduced ONL thickness resulting from loss of Spata7 either in the germline or in adults. The thickness of the ONL was measured at 20 equally spaced positions along the vertical meridian of the retina. Position 0 corresponds to the optic nerve head. Each point represents the mean ± SEM obtained for each group (n ≥ 3 mouse retinas). (C) 6-month-old Spata7\(^{flox/-}\); UbcCreERT2 mice with tamoxifen induction at 3 months of age exhibit reduced ERG scotopic a-wave and b-wave and photopic b-wave amplitudes compared to non-injected controls. Statistical analysis was performed using the Mann–Whitney test (**p < 0.001). N = 10 and N = 8 for Spata7\(^{flox/-}\); UbcCreERT2 mice without or with tamoxifen induction, respectively.
Spata7flox/− compared to controls (Fig. 2C). These data indicate that the loss of both cone and rod photoreceptors in the adult mouse retina leads to photoreceptor cell degeneration. In the amplitudes of both scotopic and photopic waves in tamoxifen-induced Spata7flox/−; UbcCreERT2/+ mice compared to wildtype and non-injected Spata7+/− mice (Fig. 2B). Consistent with the morphological changes described above, electroretinograms (ERGs) performed on tamoxifen-injected Spata7flox/−; UbcCreERT2/+ mice 3 months after induction showed a significant reduction in the amplitudes of both scotopic and photopic waves in tamoxifen-injected Spata7flox/−; UbcCreERT2/+ mice compared to controls (Fig. 2C). These data indicate that the loss of both cone and rod photoreceptors in the induced adult Spata7 knockout mice causes impaired photoresponsiveness. Collectively, these results demonstrate that, similar to what has been observed in germline mutant retinas, loss of Spata7 in adult mouse retinas leads to photoreceptor cell degeneration.

Protein mis-localization and ER stress activation in Spata7 adult knockout mouse retina. Previous studies have indicated that the integrity of the CC is lost in germline Spata7 mutants, resulting in defective phototransporting, ectopic accumulation of protein in the IS of photoreceptor cells, and ER stress-mediated apoptosis2. To test if a similar phenotype is observed in adult Spata7 knockout mice, immunofluorescence staining was performed to assess the levels of rhodopsin mis-localization and ER stress activation. Substantial mislocalization of rhodopsin to the IS and ONL was observed in the induced adult Spata7 knockout mice across the entire retina was measured by retinal morphometry. Similar to Spata7−/− mice, tamoxifen-injected Spata7flox/−; UbcCreERT2/+ mice also presented with severely reduced ONL thickness (70% in tamoxifen-injected Spata7flox/−; UbcCreERT2/+ mice and 65% in Spata7−/− mice) compared to wildtype and non-injected Spata7flox/−; UbcCreERT2/+ mice (Fig. 2B). Consistent with the morphological changes described above, immunofluorescence staining was performed on retinal sections of tamoxifen injected Spata7flox/−; UbcCreERT2/+ mice three months after induction. H&E results indicated that the induced conditional knockout mice showed approximately 70% reduction in ONL thickness compared to control mice (Fig. 2A). Photoreceptor degeneration across the entire retina was measured by retinal morphometry. Similar to Spata7−/− mice, tamoxifen-injected Spata7flox/−; UbcCreERT2/+ mice presented with severely reduced ONL thickness (70% in tamoxifen-injected Spata7flox/−; UbcCreERT2/+ mice and 65% in Spata7−/− mice) compared to wildtype and non-injected Spata7flox/−; UbcCreERT2/+ mice (Fig. 2B).

Loss of Spata7 leads to microtubule destabilization in the DCC. To further assess if loss of Spata7 in the adult mouse retina leads to disruption of the CC, we performed immunostaining against acetylated α-tubulin and NPHP1. The retinal CC can be partitioned into two regions: the proximal CC (PCC), which is homologous to the transition zone (TZ) of primary cilia, and the distal CC (DCC), a photoreceptor-specific extension of the ciliary TZ2. Acetylated α-tubulin localizes to the tubulin-based cytoskeletal core along the entire CC23,27. NPHP1, a member of the NPHP–MKS complex24 that normally localizes along the entire CC, only localizes to the PCC in germline Spata7 mutants25,26. As shown in Fig. 4A, consistent with the phenotype of germline mutants, NPHP1 signal was significantly reduced in the DCC in induced Spata7flox/−; UbcCreERT2/+ mice compared to controls (Fig. 4A). Both the germline knockout and adult-specific knockout mouse models showed more than a 60% reduction in NPHP1 signal length (Fig. 4B). Interestingly, significantly shortened acetylated α-tubulin signal was observed in tamoxifen-injected Spata7flox/−; UbcCreERT2/+ retinas (Fig. 4C). These results indicate that adult-specific knockout of Spata7 leads to axoneme microtubule destabilization in the DCC.

Discussion

In this study, we showed that inducible conditional knockout of Spata7 in adult mouse retinas results in a rapid reduction in ONL thickness and decreased response to light, indicating that Spata7 is essential for maintaining adult mouse retinal integrity and function. Combined with our previous reports, this study indicates that Spata7 is required for both establishment and maintenance of the CC. Our findings suggest that Spata7 is continuously required during retinal development and post-maturation. This study provides a more detailed understanding of the CC and helps clarify the overlooked roles of Spata7 in matured retina.

In our previous report, we generated tissue-specific Spata7 knockout mouse models by crossing Spata7flox/lox mice with photoreceptor-specific Crx-Cre mice or retinal pigment epithelium (RPE)-specific Bst1-Cre mice, demonstrating sufficient and pathogenic roles of Spata7 during photoreceptor development14. However, the roles of Spata7 in matured photoreceptor cells remained to be elucidated. We applied the UbcCreERT2/+ mouse...
Figure 4. Conditional loss of Spata7 in adult mouse retinas leads to collapse of the distal region of the CC. (A) Retinal cryosections of 6-month-old Spata7flx/−; UbcCreERT2 mice with or without tamoxifen induction and 3-month-old wildtype and Spata7−/− mice were costained for NPHP1 (red) and acetylated α-tubulin (green). A single-cilium image of NPHP1 localized to the distal CC is shown on the right. Bars for single-cilium images are 1 µm. (B and C) Histogram displaying NPHP1 (B) and acetylated α-tubulin (C) signal length from 6-month-old Spata7flx/−; UbcCreERT2 mice with or without tamoxifen induction and 3-month-old wild-type and Spata7−/− mice. Statistical analysis was performed using two-way ANOVA analysis (***p < 0.001). N = 5 mice per genotype, 10 CC per animal.
SPATA7 is necessary to replenish the protein pool. That SPATA7 is continuously required for the proper structure and function of the CC, and newly synthesized C). The biological molecular behind this remains unknown and is worth future investigations. These data suggest that Spata7 and activation of ER stress-mediated cell death. Additionally, given that the loss of in the adult retina during retina development, including disruption of CC integrity and protein trafficking along microtubule tracks as a ciliary gate that controls access of both membrane and soluble proteins to the photoreceptor outer segment26. Protein–protein interactions are thought to play critical functions to maintain the integrity of microtubule filaments of the CC. The absence of SPATA7 leads to mislocalization of DCC proteins and destabilized axonomal microtubules6. Shortened DCC length as inferred from NPHP1 expression is observed in both Spata7+/− and tamoxifen-injected Spata7floxs/+; UbcCreERT2/+ mice. Similarly, the lengths of expression of other DCC proteins are predicted to be reduced in both germline knockout mice and adult-specific knockout mice. However/Interestingly, tamoxifen-injected Spata7floxs/+; UbcCreERT2/+ mice and Spata7floxs/− mice showed different microtubule patterns. In contrast to the thin and elongated axonomal microtubules observed in Spata7−/− mice, axonomal microtubules in the adult Spata7 conditional knockout mouse model were almost completely collapsed (Fig. 4A, C). The biological molecular behind this remains unknown and is worth future investigations. These data suggest that Spata7 is continuously required for the proper structure and function of the CC, and newly synthesized SPATA7 is necessary to replenish the protein pool.

In summary, we propose that Spata7 is also required for maintenance of the retinal CC post-maturation. Loss of Spata7 in mature retinas results in a degeneration phenotype similar to what is observed due to loss of Spata7 during retina development, including disruption of CC integrity and protein trafficking along microtubule tracks and activation of ER stress-mediated cell death. Additionally, given that the loss of Spata7 in the adult retina results in more widespread ER stress in inner retina layers and more severe axonemal microtubule collapse, Spata7 may have a larger number of requirements in mature retinas.

Methods
Mouse strain generation, breeding, and genotyping. This study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org). All animals were handled in accordance with the policies on the treatment of laboratory animals’ protocol (AN-4175) approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Mice were housed on a standard diet and on a 6 am–6 pm light cycle, 20 ± 2°C with 50 ± 5% relative humidity. UbcCreERT2/+ mice were purchased from the Jackson Laboratory (Stock No: 007001). Spata7 iKO (Spata7floxs/+; UbcCreERT2/+ ) mice were generated by crossing Spata7floxs/+ mice with Spata7−/−; UbcCreERT2/+ mice. Spata7 iKO mice were obtained at the expected frequency (50%), and UbcCreERT2 mice are homozygous embryonic lethal9. The genotyping protocols for Spata7+/− and Spata7floxs/+ alleles were described previously10,14. We used a genomic PCR assay to detect the Spata7 allele using the following primers: P1 (5′-CACATTCCATCCCGATCTTTTA-3) and P2 (5′-CTGACTAGGAGGAGTAGAAAAG-3′). Spata7 deletion was assessed by the presence of a 500 bp band after running an agarose gel.

Tamoxifen induction. Tamoxifen (Sigma-Aldrich, CAS#10540-29-1) dissolved in corn oil at a concentration of 25 mg/ml was intraperitoneally injected into adult mice (3-month-old) for 5 consecutive days. Mice were then maintained for another 3 months before retinal assays were conducted.

Immunohistochemistry and immunostaining. Immunofluorescent staining with paraffin sections were conducted as previously described (Reference). Briefly, eyes were enucleated and fixed in modified Davidson’s fixative overnight for paraffin embedding. Eye sections (7 µm) were deparaffinized, and antigen retrieval was performed by boiling sections in 0.01 M Tris, EDTA buffer (pH 9.0) or 10 mM sodium citrate buffer (pH 6.0) for 30 min. Cooled slides were then washed in PBS, incubated for 1 h in hybridization buffer (10% normal goat serum, 0.1% Triton X-100, in PBS), and incubated with primary antibody overnight. Primary antibodies used were anti-Spata7 (1:100, custom antibody made by Bethyl Laboratories), anti-Rhodopsin (1D4) (1:200, Santa Cruz Biotechnology sc-57432), anti-CHOP (1:200, Santa Cruz Biotechnology sc-575), anti-ATF6 (1:200, Novus Biologicals NB1P-40256), anti-P-PERK (Thr980) (1:100, Invitrogen MA5-15033), anti-NPHP1 (aa 394–687) (1:100, from Greg Pazour lab)26, and anti-acetylated-alpha-tubulin (1:200, Santa Cruz Biotechnology sc-23950). The next day, slides were washed in PBS, incubated with secondary antibody for 2 h, incubated with DAPI at room temperature, then mounted with anti-fade medium (Prolong; Invitrogen) and coverslipped. Fluorescent images were captured with a Zeiss Axio Imager.Z1 microscope (Zeiss Axio Imager). Relative fluorescent intensities were measured by ImageJ. All immunostaining experiments were performed independently using five biological replicates.

ERGs. Mice were dark-adapted overnight and anesthetized with Rodent-III (22 mg/kg ketamine, 4.4 mg/kg xylazine, and 0.37 mg/kg acepromazine; intraperitoneal injection). Both pupils were dilated using tropicamide (1.0%) and phenylephrine (2.5%) and corneas were anesthetized with proparacaine (1.0%). After removing excess fluid, a drop of Goniosoft (2.5%) was placed on each cornea to keep the eye moistened and improve contact between the cornea and the ERG electrode (N1530NNC). For the scotopic recordings, luminescence at 10−1,
10^3, 10^2 scot cd*s/m² was used to record scotopic ERG a- and b-waves on a UTAS Visual Diagnostic System and EMWIN software (LKC Technologies, Gaithersburg, MD, USA). After scotopic ERG recordings, mice were light-adapted (30 cd*s/m²; white light) for 5 min, and then photopic ERGs were recorded with photopic luminance of 5, 50, and 500 scot cd*s/m² as previously described. A minimum of 10 mice were used for each genotype for ERG analyses. Mann–Whitney tests were performed for statistical analysis (**p < 0.001).

Quantification and data analysis. Statistical analysis of immunostaining was performed using two-way ANOVA analysis (**p < 0.001). N = 5 mice per genotype, 3 staining slides per animal. CHOP + cells were counted in the 200um region adjacent to the optic nerve. Three retinal sections were counted per animal. N = 5 mice per genotype. Error bar is SEM.

Data availability
The datasets generated and/or analyzed during the current study are available in the [Jiaxiong] https://drive.google.com/drive/u/0/folders/12gzP931uPv6BIDgnBt5FL3TzXwLQ9K_d.

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J.L., J.B., G.M., and R.C. built the experimental construct. J.L., K.X., and Y.S. performed all the assays. J.L., K.X.,
G.M., and R.C. conceived the study, interpreted the data, and wrote the manuscript. X.Q. and C.J. processed
the data and assisted with their interpretation. All co-authors discussed the results and commented on the
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Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to R.C.

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