Arf-like protein 13b (ARL13b) is a small GTPase that functions as a guanosine nucleotide exchange factor (GEF) for ARL3-GDP. ARL13b is located exclusively in photoreceptor outer segments (OS) presumably anchored to discs by palmitoylation, whereas ARL3 is an inner segment cytoplasmic protein. Hypomorphic mutations affecting the ARL13b G-domain inactivate GEF activity and lead to Joubert syndrome (JS) in humans. However, the molecular mechanisms in ARL13b mutation–induced Joubert syndrome, particularly the function of primary cilia, are still incompletely understood. Because Arl13b germline knockout mice are lethal, we generated retina-specific deletions of ARL13b in which ARL3-GTP formation is impaired. In mouse retinal Arl13b heterozygote central retina at postnatal day 6 (P6) and older, outer segments were absent, thereby preventing trafficking of outer segment proteins to their destination. Ultrastructure of postnatal day 10 (P10) central retinal Arl13b–/− photoreceptors revealed docking of basal bodies to cell membranes, but mature transition zones and disc structures were absent. Deletion of ARL13b in adult mice via tamoxifen-induced Cre/loxP recombination indicated that axonemes gradually shorten and outer segments progressively degenerate. IFT88, essential for antero–grade intraflagellar transport (IFT), was significantly reduced at tam Arl13b−/− basal bodies, suggesting impairment of intraflagellar transport. AAV2/8 vector-mediated ARL13b expression in the ret Arl13b−/− retina rescued ciliogenesis.

Primary cilia are hairlike protrusions involved in mechanotransduction (kidney epithelial cells), smell (olfaction), or vision (retinal photoreceptors). Ciliogenesis and maintenance of cilia depend on intraflagellar transport (IFT) and many other factors, including Arf-like GTPases (ARL proteins) of the RAS superfamily (reviewed in Ref. 1). ARL13b was discovered in Caenorhabditis elegans and zebrafish cilia during a search for genes causative of Bardet-Biedl syndrome and cystic kidney disease (2, 3). In mammals, ARL13b was expressed in cilia of all organs examined, including cerebellum, distal renal collecting ducts, olfactory epithelial cells, and photoreceptors (4–7). A splice-acceptor site mutation in exon 2 of mouse Arl13b (hennin mutation, hnn) was associated with defects in neural tube patterning, limbs and eyes attributed to defects in the sonic hedgehog (shh) signaling pathway, and homozygous mutants did not survive beyond E14.5 (8). A kidney-specific deletion of Arl13b led to kidney fibrosis and decreased ciliogenesis (5, 9).

ARL13b mutations R79Q and R200C in the G-domain of the human ARL13b gene caused Joubert syndrome (JS), a syndromic ciliopathy affecting multiple tissues (10, 11). Both mutant proteins disable ARL13b GTPase-activity and localize normally to cilia, but are unable to rescue ciliogenesis defects (12). ARL13b was shown to be the guanine nucleotide exchange factor (GEF) of ARL3 (13) and each, ARL13b-R79Q and ARL13b-R200C, impedes the GEF activity for ARL3. GEF activity was specific for ARL3; ARL2 was unaffected (13). The crystal structure derived from Chlamydomonas reinhardtii ARL13b revealed that the GEF activity of ARL13b is mediated by its G-domain and a coiled-coil C-terminal region (13) (PDB 5DI3).

We investigated the relationship among ARL3, its GEF, ARL13b (13), and its GAP, RP2 (14) in photoreceptor ciliogenesis as a means to understand disease etiology (Fig. 1). The photoreceptor outer segment is a modified primary cilium containing a stack of ~800 discs dedicated to reception of light and phototransduction (15). Unique among ciliated cells, the entire structure is replaced every 10 days by phagocytosis of the distal tip balanced by nascent disc assembly at the proximal outer segment, a process that requires massive protein synthesis in the inner segment and efficient trafficking pathways through the transition zone (TZ) (reviewed in Refs. 15–17). ARL3 was shown to be required for ciliogenesis as a retina-specific deletion of Arl3 prevented formation of the photoreceptor transi-
tion zone (18). Depletion of ARL3 in mature rods revealed that, additionally, ARL3 acts as a cargo displacement factor (CDF) disrupting the complex of lipiddated cargo with the solubilization factors, PDE6D and UNC119, which prevents GTP hydrolysis and prolongs the life-time of ARL3-GTP, associated with X-linked retinitis pigmentosa (XLRP) (19, 20). Abundance of ARL3-GTP in Rp2h+/− retina interfered with trafficking of lipiddated proteins of the phototransduction cascade (PDE6 and GRK1) (21). Correspondingly, transgenic expression of dominantly active ARL3-Q71L leads to mistrafficking of lipiddated proteins and retina degeneration (22).

Here, a conditional knockout of Arl13b (i.e. Arl13b−/−) was generated by expressing Cre recombinase in retina during embryonic development beginning at ~embryonic day 9 (E9). The results show that i.e. Arl13b−/− photoreceptors degenerate rapidly after failing to form mature transition zones and outer segments. Depletion of ARL13b in the adult by tamoxifen-inducible Cre/loxP recombination (tamArl13b−/−) destabilized axonemes and transition zones, leading to progressive photoreceptor degeneration. Significant reduction of IFT88 at tamArl13b−/− basal bodies suggested impairment of IFT upon deletion of Arl13b. scARL13b-AAV8 vector, injected into the i.e. Arl13b−/− subretinal space at eye opening, partially compensated and rescued photoreceptor degeneration.

Results

Generation of conditional Arl13b knock-out mice

ARL13b contains a G-domain characteristic of Arf-like proteins, a proline-rich domain, several coiled-coil motifs for protein–protein interactions, and a VXPX-like ciliary targeting signal, RVEP (Fig. 2A) (23, 24). To generate a conditional knockout, we used Arl13b+/− mice in which exon 2 is flanked by loxP sites (Fig. 2B) (25). Cre/loxP-recombination was initiated at E9 by breeding Arl13b+/− mice with transgenic Six3-Cre (18, 26) to yield Arl13b+/−Six3-Cre mice, abbreviated i.e. Arl13b−/− to indicate retina-specific knockout. LoxP-directed recombination is predicted to delete exon 2 resulting in a frameshift mutation and truncation of ARL13b at codon 2 of exon 2 (Fig. 2C). Genotyping of the floxed gene was performed using primers P1 and P2 flanking loxP in intron 2 with tail DNA (Fig. 2D), and primers P3 and P4 with retina DNA as template (Fig. 2E). Deletion of exon 2 in retina genomic DNA of P15 i.e. Arl13b−/− offspring was confirmed using primers P1 and P4 flanking the loxP sites of introns 1 and 2 (Fig. 2F).

Polyclonal anti–ARL13b antibody (Proteintech Group Inc.) recognized two polypeptides (48 kDa and 60 kDa) by immunoblotting (Fig. 2G); the 48 kDa species corresponds to unmodified ARL13b (calculated M, 48,000), while the slower-moving polypeptide may be posttranslationally modified by N-terminal palmitoylation (8, 27, 28), or represent an unknown splice variant. Both polypeptides are detectable in P17 retina knock-out lysates, as far peripheral retina still elaborates outer segments (Fig. 2H, g–i). Immunohistochemistry using i.e. Arl13b−/− frozen sections and monoclonal anti–ARL13b antibody (NeuroMab) showed that ARL13b is an outer segment protein expressed as early as P6 (Fig. 2H, a–c). At P6, ARL13b localized in nascent OS in close proximity to the basal body (Fig. 2Ha, enlargement in lower panel). At P10 and P15 maturing outer segments contained ARL13b (Fig. 2H, b and c, and lower panel enlargements). Arl13b was also detectable in the inner segment (Fig. 2H, b and c, arrows). By contrast, ARL13b was undetectable in i.e. Arl13b−/− central retina at P6, P10, and P15 (Fig. 2H, d–f; enlargements show the presence of centriolcos, identified by transgenic expression of CETN2 fused to EGFP (EGFP-CETN2), but absence of fully developed transition zones. While Six3-Cre–mediated recombination starts at E9 in the central retina (26), recombination is delayed in the peripheral retina because of delayed expression of Cre recombinase (18, 29). Therefore, in the far peripheral retina, ARL13b was detectable and rudimentary outer segments still formed between P10 and P15 (Fig. 2H, g–i).

Impaired i.e. Arl13b−/− photoreceptor transition zone formation leads to OS protein mislocalization and rapid photoreceptor degeneration

Deletion of Arl13b is expected to prevent formation of ARL3-GTP, a protein with two known functions: as a cargo displacement factor (30, 31) and as a factor enabling TZ formation (18). Employing transgenic EGFP-CETN2 as a centriole/TZ marker (29, 32), we monitored the rate of photoreceptor degeneration by immunohistochemistry (Fig. 3, A–C). Rhodopsin, detectable at P6 when nascent i.e. Arl13b−/− outer segments form (Fig. 3Aa), normally localizes distal to basal bodies (Fig. 3Aa, right panel) but mislocalized to inner segments (IS) and outer nuclear layer (ONL) of the i.e. Arl13b−/− photoreceptor (Fig. 3A, d–f). In P10 and P15 heterozygous control retinas, rod outer segments developed rapidly, expressing rhodopsin (Fig. 3Aa, a–c); PDE6 (Fig. 3B, a and b); and IQCB1/NPHP5, a transition zone marker (33, 34) (Fig. 3C, a and b) (red); basal body, daughter centrioles, and transition zones can be distinguished (white arrowheads in Fig. 3Aa, a–c, right panels). Based on EGFP-CETN2 expression, centriolcos persist as green puncta although TZ extensions are not detectable in central i.e. Arl13b−/− photoreceptors from P6 to P15 (Fig. 3Aa, d–f, right panels); rhodopsin and PDE6, normally destined for the OS, accumulated in the IS and ONL (Fig. 3, Ae and B, c and d). Rhodopsin was nearly completely absent in the P15 central i.e. Arl13b−/− retina, presumably degraded by endoplasmic reticulum–associated protein degradation (ERAD) (Fig. 3Af). IQCB1/NPHP5, labeling the transition zone and proximal
axoneme, was absent, consistent with failure to form a functional connecting cilium and outer segment (Fig. 3C, c and d). A whole retina immunoblot (P17) revealed significant traces of rhodopsin and GRK1 originating from far peripheral retina; rod transducin (Tα) and PDE6 expressions persisted more strongly (Fig. 3D). ARL3-GDP levels appear unaffected by ARL13b
Absence of outer segments in retAr13b−/− cones

Cone pigment trafficking in heterozygous control and retAr13b−/− retinas expressing EGFP-CETN2 was also explored (Fig. 4A). Cone outer segments were identified by anti-ML-opsin (Fig. 4A, a and b) and anti-S-opsin (Fig. 4B, a and b) immunoreactivity in P10 and P15 control retinas. Control cone OS are shown connected to transition zones (yellow arrows) and basal bodies (white arrowheads) (Fig. 4, A and B, a and b, right panels). Although opsins distributed throughout mutant cones, intense accumulation was observed in bloated and deformed inner segments (Fig. 4, A and B, c and d, right panels). Peripheral proteins (GRK1, cone transducin-γ, and cone PDE6) were not detectable (not shown). Photopic b-waves, using flash intensities of −1.6 log cd s−2 and higher, were highly suppressed at P15 (Fig. 4C). Optokineti c tracking (OKT) responses at 2 months of age were extinguished in retina-specific knock-out mice (p < 0.0001), indicating that the mice were unable to respond to light because of retAr13b−/− rod and cones being completely degenerated (Fig. 4D). By contrast, OKT responses of heterozygous knock-out mice were indistinguishable from wild-type (WT) littermates, suggesting haplosufficiency.

retAr13b−/− rods degenerate faster than retAr3−/− rods

ARL3-GDP cannot be activated by GDP/GTP exchange in the retAr13b−/− retina, whereas in the retAr3−/− retina, both ARL3-GDP and ARL3-GTP are absent. ARL3-GDP levels appear stable in the P17 retAr13b−/− retina (Fig. 3D). To explore differences in the degeneration rate, we compared the rate of retAr13b−/− retina degeneration with that of retAr3−/− retina (Fig. 5). Plastic sections of the three genotypes (WT, retAr13b−/−, and retAr3−/−) (Fig. 5A) showed severely reduced retAr13b−/− and retAr3−/− OS/IS morphology indicating the retAr13b−/− and retAr3−/− retinas degenerated as early as P15. retAr13b−/− ONL thickness decreased significantly faster at P15 and P30 than observed in retAr3−/− (Fig. 5B). At 1 month of age only one nuclear row remained in retAr13b−/− retina, whereas retAr3−/− photoreceptors retained three rows of nuclei (Fig. 5B); the different degeneration rates suggest that ARL13b may have an additional function.

retAr13b−/− ultrastructure

Ultrastructural analysis at postnatal days P10 and P15 revealed normal rod photoreceptor ciliogenesis in control animals. In P10 WT retina, basal bodies dock to the photoreceptor cell membrane, transition zones extend, disc membranes assemble, and OS are elaborated (Fig. 6A, a and b). In P10 retAr13b−/− photoreceptors, basal bodies docked to the cell membrane (Fig. 6A, c and e), rudimentary transition zones formed (yellow arrow) but ended in membranous bags (white asterisk) without recognizable disc structure (Fig. 6A, d and f). At P15, WT photoreceptor structure appears advanced with well-aligned basal bodies, transition zones and disc membrane in the OS (Fig. 6B, a and b). By contrast, P15 retAr13b−/− basal bodies are either undocked to membrane or docked to membranes with distal and subdistal appendages (Fig. 6B, c-f). Docked basal bodies (Fig. 6D) developed stunted TZs connected to membranous bags. Stunted TZs, revealed by electron microscopy, were not identified by confocal microscopy despite presence of the EGFP-CETN2 transgene. This may perhaps be attributed to loss of CETN2 docking sites in the knockout, thereby eliminating EGFP fluorescence. The electron microscopy results indicate a requirement of ARL13b GEF activity and ARL3-GTP for normal photoreceptor TZ structure.

ARL13b depletion in the adult mouse

To explore TZ stability as a function of ARL3-GTP, we deleted ARL13b progressively at 1 month of age using a tamoxifen-inducible Cre/loxP recombination system, CAG-CreER (35). Nuclear translocation of CreER was induced by intraperitoneal injection of tamoxifen on five consecutive days, and the degeneration rate was assessed in retina cryosections of eyes harvested 2, 3, and 6 weeks later (Fig. 7). At 2 weeks post tamoxifen induction (2WPI), ARL13b was present in treated WT outer segments, but undetectable in tamAr13b−/− retina (Fig. 7A, compare a and b). Although tamAr13b−/− outer segments appeared of normal length (Fig. 7A, d-f), rhodopsin, PDE6, and Tα displayed retention in the tamAr13b−/− inner segments (Fig. 7A, d-f, lower panels). Tamoxifen treatment of tamAr13b+/+ mice had no effect on rhodopsin (Fig. 7Ac), PDE6, or Tα trafficking (results not shown). At 3WPI, rhodopsin, PDE6, and Tα (Fig. 7B, a-d) were retained much more strongly in the tamAr13b−/− inner segments suggesting outer segment shortening/degeneration. At 6WPI tamAr13b−/− outer and inner segments were absent with only one row of nuclei surviving (Fig. 7, C, b-d, and G). Scotopic a-wave amplitudes were significantly reduced at 2WPI and 3WPI (Fig. 7D and E) and extinguished at 6WPI (Fig. 7F).

Convergent outer segments, identified by anti-ML-opsin antibody, appeared normal and were attached to their TZ (yellow arrows)
in tamoxifen-treated WT animals at 2WPI (Fig. 8Aa) and 3WPI (Fig. 8Ac). At 3WPI in tamAr13b−/− retina, ML-opsin started to mistraffic and accumulated in the inner segment (Fig. 8Ad); cone outer segments started to disintegrate accompanied by appearance of EGFP-CETN2 in the mutant inner segments (Fig. 8Ad, lower panel enlargement), as also seen for rods (Fig. 7Bd). The tamAr13b−/− photopic b-wave (Fig. 8B) and OKT (Fig. 8C) responses at 2WPI were ~50% reduced; photopic ERG was strongly attenuated at 3WPI (Fig. 8D). At 6WPI, the photopic b-wave was completely extinguished (Fig. 8E).
Impairment of intraflagellar transport in \textit{\textsuperscript{tam}Arl13b\textasciitilde} retinas

Anterograde IFT along microtubules depends on association of IFT particles with kinesin-2 molecular motors and IFT particles (36). IFT88, an IFT-B particle required for anterograde IFT, is present at the transitional zone (yellow arrow) and centrioles (white arrowhead) are indicated. Mutant retina fails to form OS and cone opsin accumulate throughout the cell. Enlargements are shown, right. Left side of each panel shows DAPI (blue). Scale bar, 20 µm; for enlargements, 5 µm. C, amplitudes of photopic ERG b-wave and a-wave of 8 WT and \textit{\textsuperscript{tam}Arl13b\textasciitilde} mice as a function of flash intensity; diminished photopic ERG response is because of functional peripheral cones. D, OptoMotry (n = 4) of \textit{\textsuperscript{tam}Arl13b\textasciitilde} mice compared to WT controls. OKTs of heterozygous knockouts were comparable with WT controls and extinguished in \textit{\textsuperscript{tam}Arl13b\textasciitilde} mice with statistical significance (***, p < 0.0001; ns, not significant; error bars indicate S.D., n = 4).

**Viral rescue of \textit{\textsuperscript{tam}Arl13b\textasciitilde} photoreceptors**

A self-complementary AAV2/8 vector expressing ARL13b (scAAV-Arl13b) was generated to attempt the "rescue" of...
mutant photoreceptors. Virus was injected subretinally at P12–P15, and retinas were harvested 6 weeks later. Significant increase in ARL13b expression in WT mice injected with scAAV-Arl13b (Fig 10, upper), compared with uninjected controls (Fig. 10, lower) confirmed expression and correct localization. ONL thickness and OS protein trafficking in both central and peripheral retina signaled partial rescue (Fig. 11, A and B) and specifically, expression of the microtubule-associated protein, RP1, in treated rerlArl13b retinas. Both mutants show rapid loss of OS and reduced nuclear tiers of the outer nuclear layer (ONL). Photoreceptor OS in rerlArl13b and rerlArl3 sections are absent at P10 and the ONL is reduced to 6–7 rows of nuclei in rerlArl13b and 7–8 rows of nuclei in rerlArl3 (d–f). At 1 month of age, rerlArl13b has only one nuclear row remaining in the ONL and rerlArl3 has 2–3 rows of nuclei left (g–i). Scale bar, 20 μm. B, nuclei numbers at P10, P15, and 1 month of age in WT, rerlArl13b, and rerlArl3 retinas. Both knockouts are predegenerate at P10 and degenerate rapidly, whereas rerlArl3 retina degenerates faster than rerlArl3 retina (n = 3; **, p < 0.05; ***, p < 0.001; ****, p < 0.0001). At 1 month of age, one ONL layer remains in rerlArl13b and 2–3 layers in rerlArl3.

Discussion

Mouse photoreceptor outer segments are modified primary cilia that form during early postnatal development (38–42). Maturation of the mother centriole into the basal body, docking of the basal body to the cortex of the cell, generation of the transition zone and extension of the axoneme occur in several unsynchronized steps, employing numerous proteins, some of which harbor unknown function. At P0, mother (MC) and daughter centrioles (DC) are present in the rod cell cytoplasm, and the distal end of the MC is covered by a vesicle. Around P3–P4, the MC has matured and docks to the cell cortex, assuming its function as basal body and microtubule organization center (MTOC). The basal body is a highly conserved, barrel-shaped, microtubule-based structure that remains associated with the daughter centriole through filamentous bundles. The MC is decorated with distal and subdistal appendages that enable microtubule nucleation and membrane anchoring; ultrastructural cross-sections reveal a symmetrical array of nine microtubules in a triplet arrangement (9(3)+0). Tubulin subunits are incorporated into the emerging TZ, a doublet microtubule structure (9(2)+0) anchored to the basal body. Around P6–P7, a singlet microtubule (9(1)+0) emerges forming the axoneme, and the first stacks of discs are synthesized (disc morphogenesis). Outer segment formation is complete at P21 (38).

In this communication, we explored the consequences of retina-specific deletion of ARL13b, the GEF for ARL3 (13) on basal body docking and TZ maturation. We monitored the formation of TZ by two independent methods, using immunohistochemistry with EGFP-CETN2 as a centriole and TZ marker (Figs. 2–4) and high-resolution electron microscopy (Fig. 6). We found that photoreceptor axonemes and outer segment discs did not form when ARL13b was absent before onset of ciliogenesis. rerlArl13b basal bodies docked to the cell membrane and formed distal and subdistal appendages, but the TZ never formed correctly, and membranous bags attached to stunted Tzs never contained OS disc structures (Fig. 6, A, c–f and B, c–f). These results align with our earlier result that, in the absence of ARL3 during early postnatal development, TZ for-
mation is impaired and photoreceptor outer segments do not form, establishing ARL3-GTP, the downstream effector of ARL13b, as a factor in ciliogenesis and TZ formation (18).

**Figure 6.** *retArl13b* photoreceptor ultrastructure. A, electron micrographs of P10 WT (a and b) and mutant (c–e) distal inner segments. The mother centriole, or basal body (BB), typically contacts a ciliary vesicle and extends nine microtubule doublets to form the proximal axoneme, which itself becomes surrounded by a sheath constituting the periciliary membrane. As the sheath fuses with the plasma membrane, the basal body docks to the photoreceptor cortex, stabilized by fibers, and extends a transition zone. Well-developed connecting cilia with outer segment discs (a and b) contrast with aborted or stunted transition zones of mutant photoreceptors (c–f). Because Sx3Cre recombinase is driven in a central-to-peripheral sequence, central photoreceptors of the mutant are affected severely. BB docking occurs in P10 *retArl13b* rods (c and e), but axoneme extension is aborted (d, yellow arrow) with elaboration of undefined membranous bags (asterisk) in slightly more peripheral retina (d and f). B, mature TZs (CC) and outer segments with stacks of disc membrane form in P15 WT photoreceptors (a and b). BB are shown docked to the plasma membrane (yellow arrows) of littermate mutant photoreceptors (c–f), decorated with distal and subdistal appendages, and forming unspecified membranous bags (f). BB with appendages are shown in tangential section (e and f).

The results in mouse photoreceptors are in contrast to *C. elegans*, zebrafish, and hTert-RPE1 cells where absence of ARL13b did not prevent formation of cilia. In *C. elegans*, studies in *Arl-13* mutants (worm Arl-13 is the homolog of mammalian ARL13b) showed that truncated cilia were formed with various structural deformities (43, 44). In zebrafish, knockdown of *Arl-13* (*sco* mutant) led to multiple cilia-associated phenotypes,
including left-right asymmetry, kidney pronephric cysts, and body curvature (45). In sco mutants, cilia are disorganized but their ultrastructure (9(2)/H11001, nine microtubules doublets and one doublet in the center) appears to be intact. In a zebrafish arl13b null mutant, cilia and photoreceptor outer segments are shortened (46), and retina degeneration progressed very slowly over weeks. In hTert-RPE1 cell lines in which ARL13b was deleted, cilia were present but significantly shorter (24). In Arl13b null mice (hnn), cilia are formed but are short with specific defects in the ciliary axoneme structure, accompanied by defects in sonic hedgehog (shh) signaling resulting in embryonic lethality (8, 10, 47). In kidney-specific deletions of mouse, ductal cilia were absent, a defect that led to rapid cyst formation and renal failure, and mutant mice died at P60 (5). Taken together, strong interspecies differences can be observed upon depletion of ARL13b. The phenotype of ARL13b deletion is strongest in mouse kidneys and photoreceptors where transition zones are stunted and axonemes are absent.

Figure 7. Tamoxifen-induced ARL13b depletion in the adult affects rods. A, at 2 weeks post injection (2WPI), ARL13b was (a) present in tamAr13b+/+ outer segments, but (b) absent in tamAr13b−/− outer segments. c. OS developed normally in tamoxifen-injected WT controls at 2 to 6WPI. In tamAr13b−/− retina at 2WPI, rhodopsin (d), PDE6 (e), and rod transducin (f) accumulated in the inner segments. Areas of interest are enlarged (lower panels). B, in tamAr13b+/+ retina at 3WPI, rhodopsin localizes normally to the outer segments (a). In tamAr13b+/− retina at 3WPI, rhodopsin (b), PDE6 (c), and transducin (d) mislocalized in the IS as OS degenerate. C, in tamAr13b+/− retina at 6WPI, rhodopsin still localizes normally to the outer segments (a). In tamAr13b−/− retina at 6WPI, rod photoreceptors are completely degenerated (b–d). D–F, scotopic a-wave amplitudes of tamoxifen-induced Arl13b knockouts at 2WPI (D), 3WPI (E), and 6WPI (F). Error bars indicate S.D., n = 5. G, ONL thickness (μm) was measured at 2WPI, 3WPI, and 6WPI in tamAr13b+/+ and tamAr13b−/− retinas; only one nuclear row survived in tamAr13b−/− retina at 6WPI. **, p < 0.05; ****, p < 0.0001.
In retina-specific deletion of ARL13b (this paper) and ARL3 (18), basal bodies appear to dock to the cell cortex, but mature TZ/OS are never formed. The consequence is accumulation of outer segment proteins in the inner segment, nuclear and synaptic regions, but the precise mechanism leading to abortion of ciliogenesis and rapid photoreceptor degeneration is unknown.
A possible trigger to cause degeneration may be the participation of ARL13b and ARL3-GTP in intraflagellar transport. IFT requires IFT-A and IFT-B particles, in addition to heterotrimeric kinesin-2, to transport cargo into cilia or flagella (48–50). In C. elegans Arl-13 mutants, IFT-A and IFT-B particles required for IFT were disrupted suggesting that ARL-13 and, based on its GEF activity also ARL-3, regulate IFT particle integrity thus linking ARL-13 and ARL-3 to IFT (1, 27, 43). In hTert-RPE1 cells, ARL13b interacts with IFT-B particles, but its ciliary localization is independent of this interaction (24). In ARL13b knock-out RPE1 cells, which have shortened cilia, retrograde IFT is disabled, leading to accumulation of IFT-B and IFT-A particles at the ciliary tips (24).

IFT in photoreceptors is important for maintenance of the axoneme (29, 51) that extends into the outer segment. Retina-specific deletion of KIF3a, the obligatory subunit of heterotrimeric kinesin-2, prevented formation of photoreceptor TZ and axonemes, exactly as observed in retina-specific deletions of ARL3 and ARL13b. Tamoxifen-induced deletion of ARL13b showed increased solubilization of EGFP-CETN2 in rods and cones (Figs. 7 and 8). Apparently, EGFP-CETN2, normally tightly associated with TZ and centrioles, lost its ability to interact with centrioles/TZ, became increasingly soluble, and accumulated in the inner segment (Fig. 9, A and B). Furthermore, labeling axonemes with RP1 (Fig. 9A) and AcTub (Fig. 9B) in ARL13b knockouts indicated that axonemes shortened at 2WPI and became stunted at 3WPI, suggesting that failure to maintain the basal body–axoneme cytoskeleton may be a key event leading to photoreceptor degeneration. Gradual shortening of the axoneme at 2WPI and 3WPI was obtained when KIF3a was deleted in the adult mouse using tamoxifen induction (29), and even the degeneration rates with tamoxifen-induced deletions of KIF3a, ARL3, and ARL13b are nearly identical.

Figure 9. Axoneme and basal body/TZ cytoskeleton destabilization. A. TamAr13b/− and controls probed with anti-RP1 as in Fig. 7. Hatched boxes are enlarged as lower panels. RP1 is a MAP (microtubule-associated protein) and localizes to the proximal axoneme (red) which is attached to the TZ (green) (a and b). In tamAr13b/− retina at 2WPI (d) and 3WPI (e), the axoneme area interacting with RP1 is reduced and CETN2 interaction with centrioles/TZ is impaired (d and e, lower panels). B. anti-AcTub identifies acetylated α-tubulin which stabilizes the proximal microtubule axoneme (a–c). In tamAr13b/− retina at 2WPI (d) and 3WPI (e), AcTub labeling of the axoneme is reduced and EGFP-CETN2 solubilizes forming a long flare into the ONL (d and e, lower panels). At 6WPI, the photoreceptors have degenerated (Af and Bf) compared with controls (Ac and Bc). Scale bar = 20 μm; in enlargements, scale bar = 20 μm. Please note: Ac and Af and Bc and Bf are not on the Egfp-Cetn2 background. C. Egfp-Cetn2−/− tamAr13b/− (a and b) and Egfp-Cetn2−/− tamAr13b/− sections were probed with anti–giantin antibody (red). CETN2 mislocalizes to the IS/Golgi of mutant retina (c and d). Scale bar, 5 μm. D. reduced lengths (μm) of RP1-responsive axonemes in tamAr13b/− photoreceptors at 2WPI, 3WPI, and 6WPI (****, p < 0.0001).
ARL13b and the mouse photoreceptor transition zone

We determined the localization of IFT88, an obligatory IFT-B particle essential for anterograde IFT in photoreceptors (36, 52). The results show that IFT88 is nearly absent in ret^−/− basal bodies where cargo for IFT is assembled, thus strongly suggesting impairment of anterograde IFT upon deletion of ARL13b. Interference with transport of tubulin subunits and axoneme building blocks could explain fully the progressive OS shortening and eventual photoreceptor degeneration in tamoxifen-induced ARL13b deletions.

Experimental procedures

Animals

Procedures were approved by the University of Utah Institutional Animal Care and Use Committee and were conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Floxed Arl13b mice (Arl13b^f/f) were provided by Dr. Tamara Caspary (Emory) and maintained in a 12:12 h dark-light cycle. A transgenic mouse expressing EGFP-CETN2 fusion protein (The Jackson Laboratory, stock number 008234) was used to identify centrioles with fluorescence microscopy (32).

Generation of Arl13b gene knock-out mouse

Arl13b^f/f mice were mated with Six3-Cre transgenic mice to delete exon 2 generating retina-specific Arl13b knockouts (ret^Arl13b) (Fig. 2) (26, 35, 53). The intron 2 loxP site was genotyped using primer pair Arl13b-CondF3 5′-GGATAGACCTTGCCCTTCTG (P1) and Arl13b-Cond949R 5′-AACTGGGACACCCAAATGAG (P2) with genomic tail DNA as a template (Fig. 2D). The intron 1 loxP site was identified with primers Arl13b-CondF 5′-AGGACGGTTAGAAACACTG (P3) and Arl13b-CondR 5′-AAGGCCAGCTGGTGTTATTT (P4) using tail DNA (Fig. 2E). Deletion of exon 2 in Arl13b^f/f;Six3 (ret^Arl13b^−/−) mouse retina was verified with primers P3 and P4 (Fig. 2F) and retina DNA (Fig. 2F). Six3-Cre mice were genotyped with Cre-specific primer set Six3Cre159 5′-TCGATGCGAAGGAATGAG and Six3Cre160 5′-TTCCGGCTATACGTAACAGG. The Egfp-Cetn2^+ transgene was identified with the primer set, Egfp-Cetn2^+/−F 5′-TGAACGAAATCTTCCCAGTTTCA and Egfp-Cetn2^+−R 5′-ACTTCAAGATCCC-GCCACACAT. Absence of the rd8 mutation was confirmed by PCR as described previously (54).

Confocal immunohistochemistry

Animals were dark-adapted overnight and sacrificed under dim red light. The eyecups and retina cryosections for confocal microscopy were prepared as described (55). Briefly, sections were incubated with the following polyclonal primary antibodies: VPP (anti-rhodopsin, 1:500) (56); rod Tα (anti–transducin-α, 1:500, Santa Cruz Biotechnology); anti–M/L-opsin (1:500, Chemicon); anti–cone PDE6 (1:500, CytoSignal); anti-INPP5E (1:200, Promega); anti–S-opsin (1:500, Chemicon); MOE (anti–GRK1, 1:500, Santa Cruz Biotechnology), and anti–transducin-γ (1:500). Monoclonal antibodies included G8 (anti–GRK1, 1:500, Santa Cruz Biotechnology) and anti-Arl13b (1:200, NeuroMab). Anti–IQCBI/NPHP5-KK antibody was a gift of Dr. Edgar Otto (University of Michigan) (57). The anti–IFT88 antibody was a gift of Dr. Gregory Pazour (University of Massachusetts Medical School). Alexa Fluor 555 conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were diluted 1:1000 in blocking solution (2% BSÂ, 0.1% Triton X-100, 0.1 M phosphate buffer, pH 7.4). Images were acquired using a Zeiss LSM800 confocal microscope.

Immunoblotting

Retinas were isolated and lysed in 100 µl of 50 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM EDTA, 0.2% Triton X-100 with 2 µl of 100 mM PMSF and 2 µl protease inhibitor. Material was sonicated for 2 × 20 pulses at 30% intensity and spun at 15,000 rpm for 10 min. Protein concentration was determined by Bradford assay; retina lysate proteins were then separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane processed as described (55). Primary antibodies were diluted 1:300 for anti-ARL13b (Proteintech), 1:300 for anti-ARL3 (18), 1:500 for anti-rhodopsin (1D4), 1:500 for anti–rod Tα (Santa Cruz Biotechnology), 1:500 for anti-PDE6 (MOE, CytoSignal), 1:200 for anti–GRK1 (G8, Santa Cruz Biotechnology), and 1:1000 for anti–β-actin (Sigma-Aldrich). Secondary antibodies (Odyssey) were iR680 goat anti-mouse (1:5000) and iR800 goat anti-rabbit (1:3000). Images were acquired using an Odyssey scanner.
Electroretinography (ERG)

Scotopic and photopic electroretinogram responses were recorded from P15 WT, \( \text{ret}^{Arl13b^{+/+}} \), and \( \text{ret}^{Arl13b^{-/-}} \) mice using a UTAS BigShot Ganzfeld system (LKC Technologies, Gaithersburg, MD). ERGs were measured as described previously (18, 21).

Optomotry

Optomotor reflex-based tests were performed using an OptoMotry system (CerebralMechanics Inc.). Mice 2 months of age were adapted to room light (150–250 lux), and optokinetic tracking was performed under light conditions to test cone-mediated vision. Briefly, rotation speed (12 degrees/sec-
ond) and contrast were kept constant as described (58). Visual acuities of mice were quantified by increasing the spatial frequency of the rotating grating (0.03–0.39 cycles/degree) until the maximum frequency threshold was tracked.

**Transmission electron microscopy**

Isolated mouse eyecups were fixed by immersion in 2% glutaraldehyde–1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4 °C overnight (32, 46). The eyecups were postfixed with 1% osmium tetroxide in 0.1 M cacodylate for 1 h, buffer-rinsed, stained en bloc with uranyl acetate, and subsequently dehydrated in an ascending series of methanol solutions. Eyecups were embedded in Epon resin (Ted Pella, Inc., Redding, CA) for sectioning. 1-µm plastic sections were cut to face and orient photoreceptors near the optic nerve. Retina ultrathin (60 nm) sections were cut onto slot grids with carbon-coated Formvar film (Electron Microscopy Sciences, Hatfield, PA) and poststained with uranyl acetate followed by lead citrate. Transmission electron microscopy was performed at 75 kV using a JOEL electron microscope.

**Generation of the AAV2/8 shuttle vector and virus**

Mouse Arl13b expression cassette under the control of the G protein–dependent receptor kinase (GRK1) promoter was placed in between the two inverted terminal repeats of an AAV2/8 shuttle plasmid (33). To generate a self-complementary vector, one inverted terminal repeats of the shuttle plasmid was mutated. The vector was packaged into AAV8 capsid. Production and purification of the vector were performed following a protocol described previously (59). Vector quantification was conducted by real time PCR using linearized plasmid standards and primers against the GRK promoter.

**Statistics**

SigmaPlot12 was used for statistical analysis using Student’s t test and the level of statistical significance was set p = 0.05.

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