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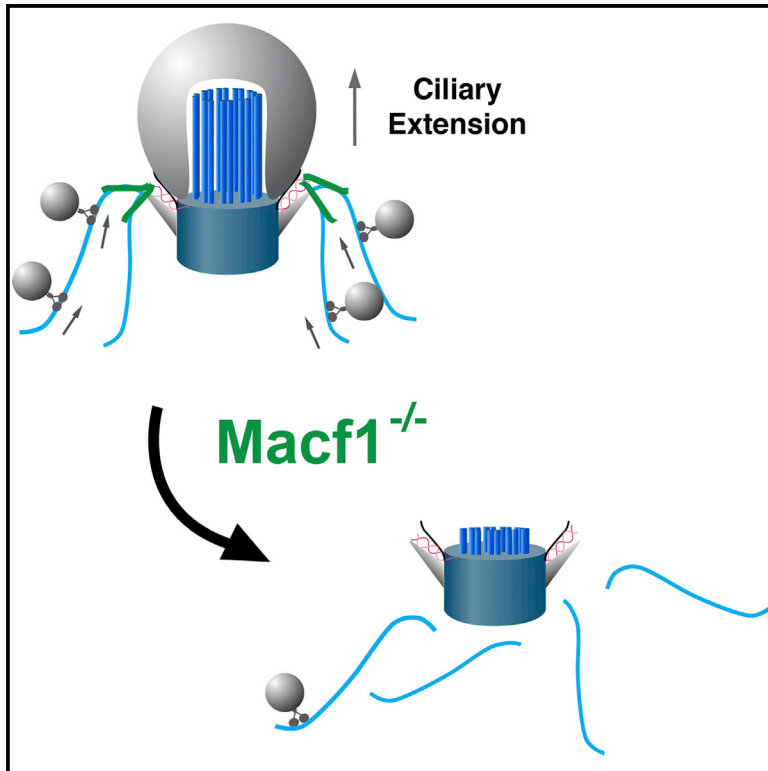
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Loss of MACF1 Abolishes Ciliogenesis and Disrupts Apicobasal Polarity Establishment in the Retina

Graphical Abstract



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In Brief

May-Simera et al. find that MACF1, a giant protein that mediates microtubule and actin interactions, is essential for ciliogenesis and maintenance. In the developing retina, MACF1 is required for establishing apicobasal polarity in photoreceptors, thus highlighting the importance of cilia in providing the correct positional cues.

Highlights

- MACF1 is required for apical basal body docking and ciliogenesis
- MACF1 is critical for maintenance of the photoreceptor sensory cilium
- Primary cilia may provide positional cues for apicobasal polarization of photoreceptors

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Loss of MACF1 Abolishes Ciliogenesis and Disrupts Apicobasal Polarity Establishment in the Retina

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SUMMARY

Microtubule actin crosslinking factor 1 (MACF1) plays a role in the coordination of microtubules and actin in multiple cellular processes. Here, we show that MACF1 is also critical for ciliogenesis in multiple cell types. Ablation of *Macf1* in the developing retina abolishes ciliogenesis, and basal bodies fail to dock to ciliary vesicles or migrate apically. Photoreceptor polarity is randomized, while inner retinal cells laminate correctly, suggesting that photoreceptor maturation is guided by polarity cues provided by cilia. Deletion of MACF1 in adult photoreceptors causes reversal of basal body docking and loss of outer segments, reflecting a continuous requirement for MACF1 function. MACF1 also interacts with the ciliary proteins MKKS and TALPID3. We propose that a disruption of trafficking across microtubules to actin filaments underlies the ciliogenesis defect in cells lacking MACF1 and that MKKS and TALPID3 are involved in the coordination of microtubule and actin interactions.

INTRODUCTION

While the primary cilium was first identified over a century ago (Zimmermann, 1989), its significant roles in regulating developmental pathways and tissue homeostasis were only recognized in the past decade (Eggenchwiler and Anderson, 2007; Gerdes et al., 2009; Singla and Reiter, 2006). A genetically heterogeneous group of human diseases, termed ciliopathies, many involving retinal dysfunction, are now known to be associated with primary cilia dysfunction (Rachel et al., 2012). The mammalian retina develops from a single layer of neuroectoderm into a light-sensing multilayered neuroepithelial structure required for

visual function. The retinal neuroepithelium is composed of multipotent progenitors that undergo differentiation, migration, and patterning to develop into a fully laminated mature retina comprising polarized photoreceptors and five other neural cell types (Reese, 2011). Understanding the development and homeostasis of the retina is important, because it both serves as a paradigm for understanding CNS development and provides a basis for developing stem cell transplantation as a therapy for retinal degeneration, a common cause of blindness. Of particular importance is the development of photoreceptors, the light-sensing neurons required for visual perception. Upon exiting the cell cycle, maturation of photoreceptors requires cell polarization coupled with highly organized intercellular connections, prior to extension of the connecting cilia and outer segments. These characteristics make the mammalian retina an excellent model in which to study both ciliogenesis and polarization during tissue morphogenesis.

A key cellular event during retinal development is the establishment and maintenance of cell polarity, aided by a highly dynamic cytoskeletal infrastructure composed of microtubules and actin filaments. The migration and anchoring of centrosomes to the apical domain is a hallmark of apicobasal polarity establishment. Centrosomal anchoring is also required for the extension of a microtubule-based ciliary axoneme during ciliogenesis. Ciliogenesis is initiated when the mother centriole docks with a primary ciliary vesicle within the cytoplasm (Garcia-Gonzalo and Reiter, 2012; Sorokin, 1962). The ciliary vesicle grows in size by the fusion of additional secondary vesicles, and the centriole begins to extend microtubule structures to initiate the formation of the ciliary axoneme. Two different modes of ciliogenesis may be used by different cell types: an “intracellular” pathway, where vesicles dock to the centriole cytoplasmically prior to axoneme extension and fusion with the plasma membrane; and an “extracellular” pathway, in which centrioles migrate and dock at the plasma membrane directly, followed by axoneme extension (Ghossoub et al., 2011; Molla-Herman et al., 2010). Once docked, the centriole becomes the basal

body and anchors the membrane-encapsulated cilium. Several studies have highlighted a primary role for the actin cytoskeleton in addition to microtubules in these processes. (Boisvieux-Ulrich et al., 1987, 1990; Hirota et al., 2010; Klotz et al., 1986; Lemullois et al., 1987, 1988; Molla-Herman et al., 2010). Specifically, docking of the basal body at the cell membrane requires active actin cytoskeletal remodeling, mediated via apical activation of the GTPase RhoA (Pan et al., 2007). Therefore, proteins that mediate interactions between actin and microtubules may be important during ciliogenesis.

Microtubule actin crosslinking factor 1 (MACF1/ACF7), one of two spectraplakins found in mammals, is a giant protein with multiple functional motifs. It interacts with F-actin, microtubules, and intermediate filaments to organize cytoskeletal networks and participate in intracellular trafficking (Sonnenberg and Liem, 2007; Suozzi et al., 2012). MACF1 concentrates at distal ends (plus ends) of microtubules and polarized cell borders, where it couples the microtubule network to membrane-associated junctions, facilitating actin-microtubule interactions at the cell periphery and enabling rapid turnover of focal adhesion molecules (Wu et al., 2008). The *Drosophila* MACF1 homolog *shot* localizes to the apical side of stable microtubule extensions in the rhabdomere terminal web of developing photoreceptors (Mui et al., 2011), structures that coordinate membrane-cytoskeleton trafficking (Chang and Ready, 2000). Genetic interaction studies also suggest that *shot* modulates the Crumbs (Crb) membrane domain, a component required for establishing apicobasal cell polarity during rhabdomere elongation (Mui et al., 2011). Interestingly, MACF1 is one of the most abundant proteins in the photoreceptor ciliary proteome (Liu et al., 2007) and interacts with ciliopathy protein MKKS/BBS6 (May-Simera et al., 2009). These observations suggest that MACF1-mediated interaction between microtubule and actin networks may play a role in ciliogenesis and photoreceptor differentiation. To test these hypotheses, we ablated *Macf1* in select tissues and show that *Macf1* is essential for cilia biogenesis and maintenance, which, in turn, play critical roles in retinal lamination during development and homeostasis of mature photoreceptors.

RESULTS

MACF1 Is Expressed in the Developing Postnatal Retina

Two MACF1 isoforms have been reported in mouse (Lin et al., 2005). RNA sequencing (RNA-seq) analysis found that the developing retina expressed predominantly the *Macf1a* RNA transcript (Figure 1A). *Macf1a* RNA peaked in rod photoreceptors around postnatal day (P)3. Because germline loss of *Macf1* is early embryonic lethal (Chen et al., 2006; Kodama et al., 2003), we carried out conditional ablation by crossing *Macf1*-floxed mice (*Macf1^{fl/fl}*) (Wu et al., 2008) with mice expressing Six3p-Cre to excise *Macf1* in retinal progenitors from embryonic day (E)9 onward (*Macf1^{fl/fl};Six3-Cre*). To assay for the MACF1 protein, we generated a polyclonal antibody against the N-terminal domain of MACF1 (Figure 1A). MACF1 was absent in the conditional *Macf1^{fl/fl};Six3-Cre* retinas (Figures 1B and 1C). In control retina, MACF1 concentrated at the apical domain of the neuroblast layer (NBL; Figure 1C, white arrow) and in the inner plexiform layer (IPL) during early postnatal development. As the retina

matured, MACF1 localization was enriched in the plexiform layers and at the apical edge of the outer (photoreceptor) nuclear layer (ONL) (Figure 1C). In the adult retina (P28), MACF1 protein colocalized with the basal body beneath the photoreceptor connecting cilium. This labeling was corroborated by immuno-electron microscopy (immuno-EM), which revealed MACF1 at basal body appendages (Figure 1D; Figure S4).

Ablation of *Macf1* Disrupts Retinal Lamination and Arrests Photoreceptor Maturation

To evaluate retinal function, electroretinograms (ERGs) were performed at 6 weeks of age. ERG a- and b-waves were absent in both dark- and light-adapted *Macf1^{fl/fl};Six3-Cre* mutants, indicating loss of both rod and cone function (Figure 2A). Mutant mice were non-responsive to a 10-Hz flicker test, which isolates cone function, further confirming cone functional deficit. Histological analysis revealed severe retinal dysplasia in the MACF1 null retina, predominantly affecting the photoreceptor layer (ONL) (Figure 2B). At P0, *Macf1* mutant retina was indistinguishable from control, but by P5, the disruption of outer retina lamination was evident. In control retinas, the inner retinal layer (INL) and ONL began to emerge as distinct layers, yet separation of the layers in the mutant was severely disrupted. By P10, the ONL in the mutant appeared split and fragmented. At maturity, mutant photoreceptor inner and outer segments were absent, and no separation between the INL and ONL was evident. Disruption of *Macf1* using Rxp-Cre (*Macf1^{fl/fl};Rxp-Cre*) resulted in a similar but slightly milder retinal phenotype (Figure S1).

Despite severe disruption of the outer retina, retinal cell fate appeared to progress normally, as indicated by the appearance of cell-specific markers. Rhodopsin and cone opsins were expressed in *Macf1^{fl/fl};Six3-Cre* mutant photoreceptors, though not polarized at the apical domain by P5 (Figure S2A) or localized to any structure that resembles outer segments by P21 (Figure 3A). Markers for amacrine, horizontal, and ganglion cells (choline acetyltransferase [ChAT], calbindin, and calretinin) in the inner retina were comparable to controls (Figure 3B; ChAT not shown). Bipolar cell disorganization was evident at P10, with some cell bodies interspersed with photoreceptors, and this became more severe at P21 (Figure 3C). Because bipolar cells make direct synaptic contact with photoreceptors, bipolar cell disorganization is likely secondary to photoreceptor lamination defects. Inner retina neurons (ganglion and amacrine cells) were spared, indicating that the loss of *Macf1* in the retina primarily disrupts photoreceptor cell development.

Staining for OTX2, a marker for undifferentiated neuroblasts in the NBL, was similar between *Macf1* mutant and control at P0 (Figure S2B), as was staining for early-born retinal neurons, including amacrine, horizontal, and ganglion cells (Figure S2C). Dividing cells, marked by phosphohistone-H3, localized entirely to the apical margin of the neuroepithelium in controls at P0. In contrast, dividing cells frequently located ectopically in the mid-NBL of *Macf1* mutants (Figures S2D and S2E). These results suggest that MACF1 function is dispensable in early-born retinal neurons but necessary during differentiation of later-born photoreceptor/retinal neurons (Reese, 2011).

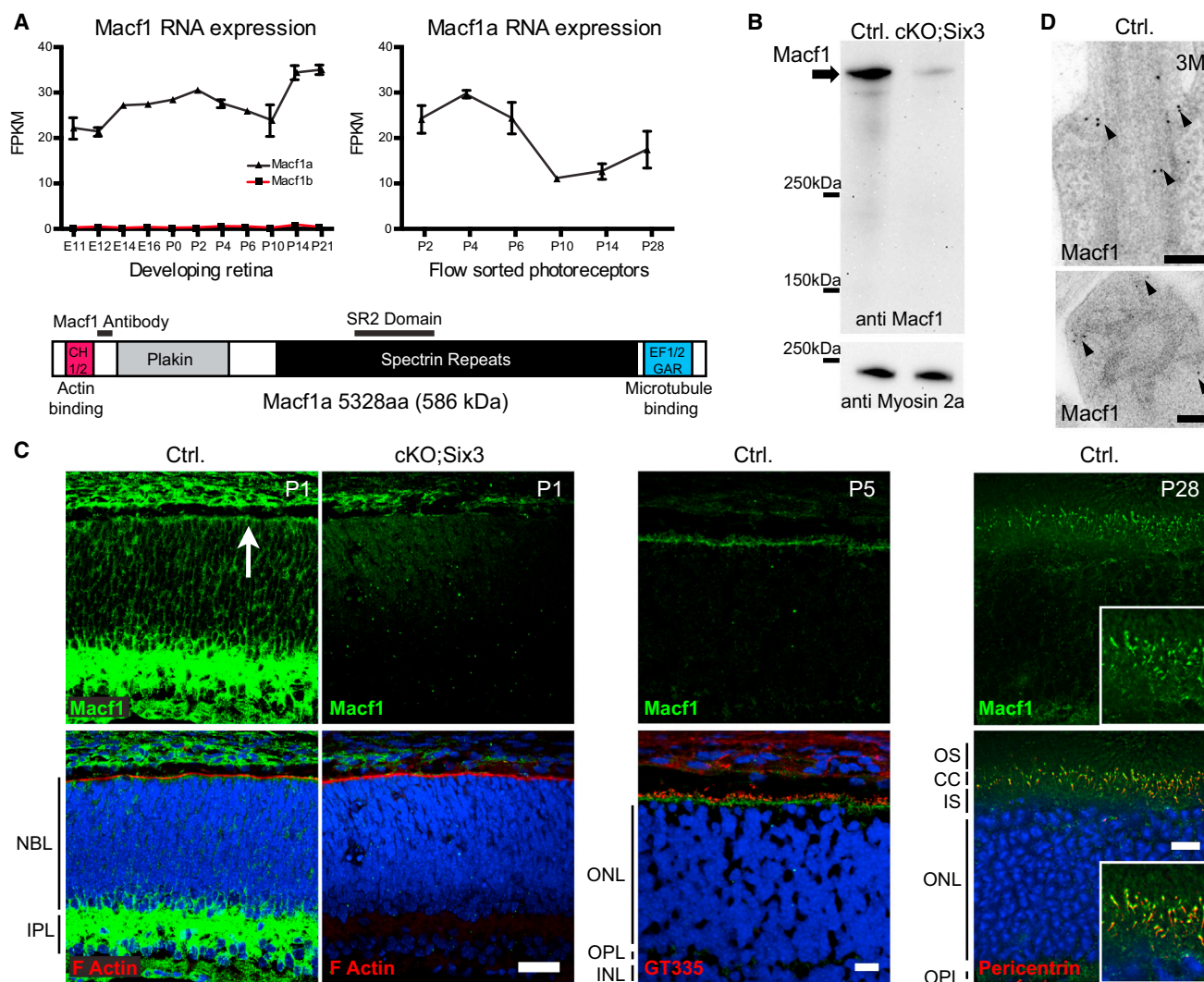


Figure 1. Macf1 Is Expressed throughout Vertebrate Retina Development

(A) RNA-sequencing data from developing mouse retina and flow-sorted photoreceptors. FPKM (fragments per kilobase of transcript per million fragments mapped) indicates the level of transcription. Schematic of MACF1a protein domains and localization of antibody epitope is shown.

(B) Immunoblots of control (Ctrl.) and *Macf1*-excised (cKO;Six3) mouse retinal lysates probed with MACF1 antibody show a reduction in expression in the mutant.

(C) Immunohistochemistry of retinal sections using antibodies against MACF1 in control and *Macf1*-excised sections. To visualize relevant structures in each time point, F-actin was stained with phalloidin, the emerging connecting cilium was stained with GT335, and the base of the cilium was stained with pericentrin. OPL, outer plexiform layer; OS, inner segment; IS, inner segment; CC, connecting cilia.

(D) Immuno-EM using an antibody against MACF1. Immuno-gold particles were concentrated near the basal body in the photoreceptor inner segment (3 months; 3M).

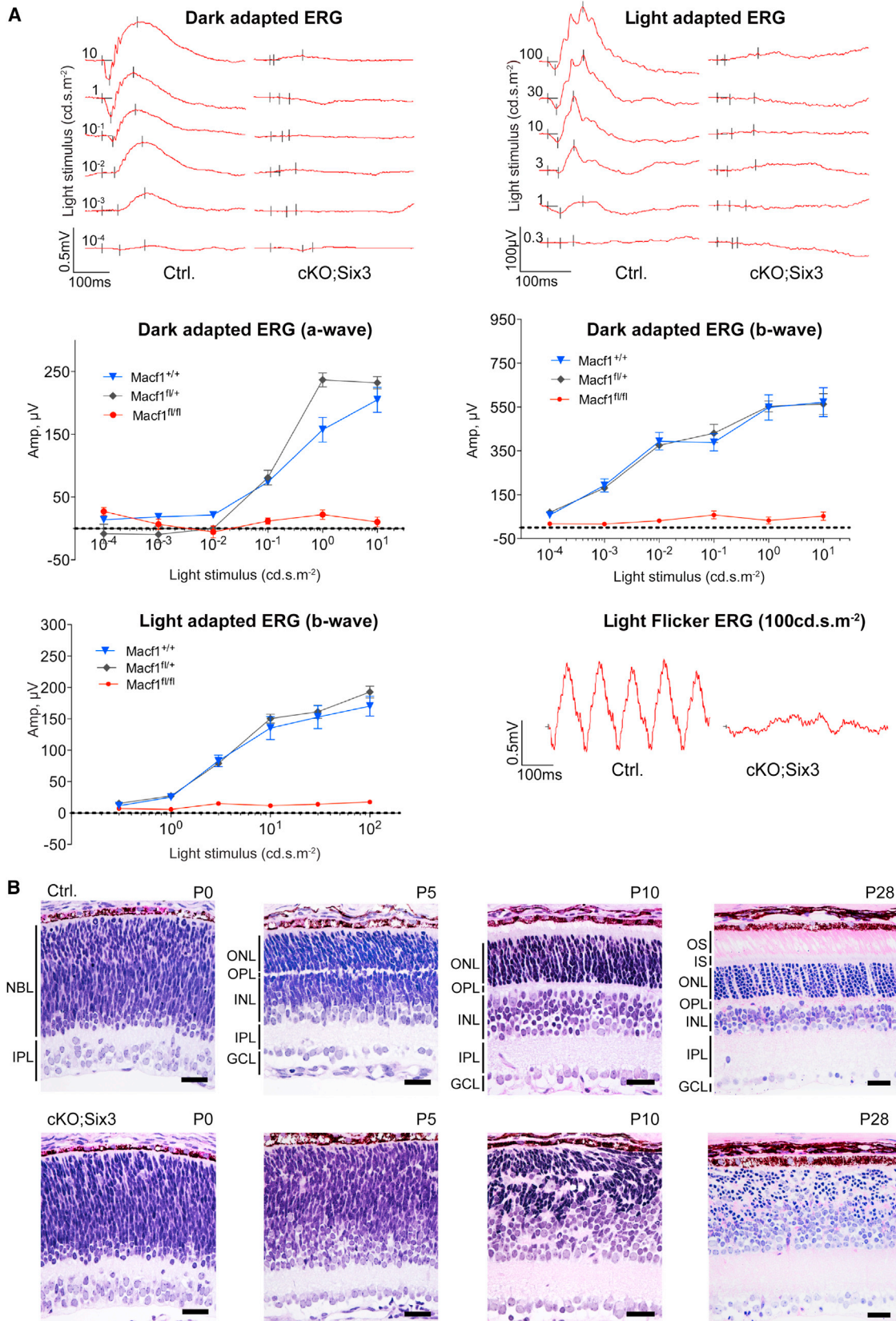
Scale bars represent 50 μ m in (C) and 100 nm in (D).

See also Figure S4.

Polarity of Developing Photoreceptors Is Disrupted in *Macf1* Null Retina

By P10, photoreceptors are well polarized, as indicated by the apical alignment of ciliary rootlets, and begin to elaborate outer segments (Figure 4A; Figure S2F). In *Macf1* mutants, however, rootlets fail to align along the apical margin of the retina (Figure 4A). Localization of the Crumbs protein complex is an early determinant of apical junctions and marker of apicobasal polarity. Discontinuous CRB1 localization along the apical edge of

mutant neuroepithelia at P5 (Figure 4B) and P0 (Figure 4C) indicates a defect in polarity establishment. Other polarity markers, including PALS1 and PAR3, also failed to align at the apical margin of the NBL in P0 mutant retina (Figure 4C). Electron-dense junctional complexes were observed by transmission electron microscopy (TEM) in P1 and P5 mutant retinas, suggesting that junctions could still form where neighboring cells are aligned (Figures S3A–S3C). These data suggest that *Macf1* function is essential for the establishment of photoreceptor polarity.



Failed Ciliogenesis Underlies Loss of Apicobasal Polarity of Developing Photoreceptors

At P5, ciliary rootlets were aligned to the apical edge of the ONL. Co-labeling with glutamylated tubulin (GT335), a ciliary transition zone marker, showed emergence of the connecting cilium (Figure 4D). In contrast, ciliary rootlets were scattered throughout all retina layers of *Macf1* mutants, and the connecting cilium was absent. As early as P0, displaced basal body profiles could be observed throughout the developing NBL of mutant retinas (Figure 4E). The early stages of ciliary biogenesis were observed in electron micrographs taken at the apical edge of the NBL in P1 retina. A ciliary vesicle docks onto the distal end of the mother centriole/basal body (Figure 4F, left panel), begins to nucleate the microtubule-based ciliary axoneme (middle panel), and eventually fuses with the plasma membrane (right panel). Basal bodies identified in multiple TEM images were scored on the basis of having a ciliary vesicle or an emerging cilium, and the results were quantified (Figure 4G). Mislocalized basal bodies throughout the mutant retina were difficult to observe; therefore, only basal bodies near the apical membrane could be readily identified and quantified, and thus, the percentage of abnormal basal body profiles is likely an underestimate. In *Macf1* mutants, the majority of basal bodies lacked a docked ciliary vesicle, which suggests that they are unable to fuse with the plasma membrane, and thus, ciliogenesis is inhibited. Therefore, in the retina, failed ciliogenesis in the *Macf1* mutant precedes or coincides with the beginning stage of retinal lamination. We propose that cilia biogenesis and positioning are a determinant of the neuroepithelial lamination, similar to what has been reported in brain development (Wilsch-Bräuninger et al., 2012).

Macf1 Is Required for Maintaining the Photoreceptor Outer Segments in Adult Retina

To determine whether MACF1 also plays a role in fully differentiated photoreceptors, we disrupted the gene in 3-month-old *Macf1*-floxed (*Macf1^{fl/fl}*) mice by subretinal injection of AAV8-Cre recombinase under the control of a rhodopsin kinase promoter (AAV8-RKp-Cre). AAV8-RKp-EGFP was co-injected to visualize transduced cells. Wild-type (non-floxed) mice were also injected and served as negative controls. Six weeks after injection, AAV transduction coverage was greater than 70% and restricted to photoreceptors, as indicated by GFP (Figure 5A). Compared to the controls, ERG a- and b-waves were significantly decreased in injected *Macf1*-floxed mice under dark-adapted conditions (Figure 5B). Additionally, ONL thinning, upregulation of GFAP (glial fibrillary acidic protein, an indicator of photoreceptor degeneration), and mislocalized rhodopsin were observed readily in injected floxed mice (Figures 5C and 5D).

TUNEL staining revealed apoptotic photoreceptor cells (Figures 5E and 5F). TEM images of inner segments showed a loss of basal body anchoring at the connecting cilium in injected mutants (Figure 5G). Basal bodies were “undocked” from the apical inner segments, similar to the observed loss of basal body docking in developing photoreceptors. Numerous detached basal bodies could be found in injected floxed mice (Figure S4A), but none were found in controls. The average distance between a basal body and the base of the outer segment was 1.83 μm in mutant compared to 1.29 μm in control (Figure 5H). Quantification was performed using TEM images. These data suggest that MACF1 is also required for actively maintaining basal body positioning in mature photoreceptors.

Macf1 Is Required for Ciliogenesis in Multiple Tissues and Cell Types

Next, we sought to determine whether MACF1 was broadly required for ciliogenesis. Ependymal epithelial cells line the ventricular walls of adult brain and are covered with multiple motile cilia. Development of ventricular neuroepithelia requires polarization of a primary cilium, which precedes the emergence of multiple motile cilia on mature ependymal cells (Mirzadeh et al., 2010; Tissir et al., 2010). Embryos in which MACF1 was excised using *Foxg1p-Cre* (expressed in the developing telencephalon) did not survive past E18.5, a time when primary cilia are well developed. Scanning electron microscopy of E18.5 mutants showed a loss of primary cilia on ependymal epithelia (Figure 6A, red arrows). Additionally, a failure of the lateral ventricles to expand dorsally resulted in smaller ventricles (Figures 6A and S5A, red arrows). Using a different Cre driver, *Six3p-Cre* (expressed in the ventral forebrain), mutants survived to adulthood but exhibited slightly enlarged ventricles (Figure S5B), a hallmark of cilia mutant mouse models and human ciliopathy patients (Rachel et al., 2015). Altered shapes of lateral ventricles were also found in mutant mice in which MACF1 was conditionally ablated in the brain (Goryunov et al., 2010).

An additional ciliated tissue targeted by *Foxg1p-Cre* is the cochlea sensory epithelium, which contains polarized actin-based stereociliary bundles that sit atop rows of mechanosensory hair cells. Closely attached to each stereociliary bundle is a microtubule-based kinocilium, which is required for correct bundle development. Stereocilia bundle abnormalities have been reported in numerous cilia mutants (Jones et al., 2008; May-Simera et al., 2015; Ross et al., 2005). *Macf1* mutant cochlea epithelia exhibited shortened actin bundles (Figure 6B) and shorter kinocilia (Figures 6C and 6F) compared to controls. The stereocilia and kinocilia defects are consistent with enriched distribution of MACF1 at the cuticular plate and at the kinocilium basal body (Antonellis et al., 2014).

Figure 2. Loss of *Macf1* Impairs Retinal Function and Disrupts Retinal Lamination

(A) Representative ERG traces from control (*Macf1^{+/+};Six3-Cre/Macf1^{fl/fl};Six3-Cre*) (Ctrl.) and mutant (*Macf1^{fl/fl};Six3-Cre*) (cKO;Six3) mice at 6 weeks of age ($n = 5-6$ mice). Mean a- and b-wave amplitudes at six different light intensities are plotted, with error bars indicating \pm SEM. Both rod and cone functions were reduced in *Macf1* homozygous mutants. Amp, amplitude.

(B) H&E staining of developing retina showed severe retinal dysplasia in *Macf1* mutant mice (*Macf1^{fl/fl};Six3-Cre*), predominantly affecting the outer retina by P5. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars represent 50 μm .

See also Figure S1.

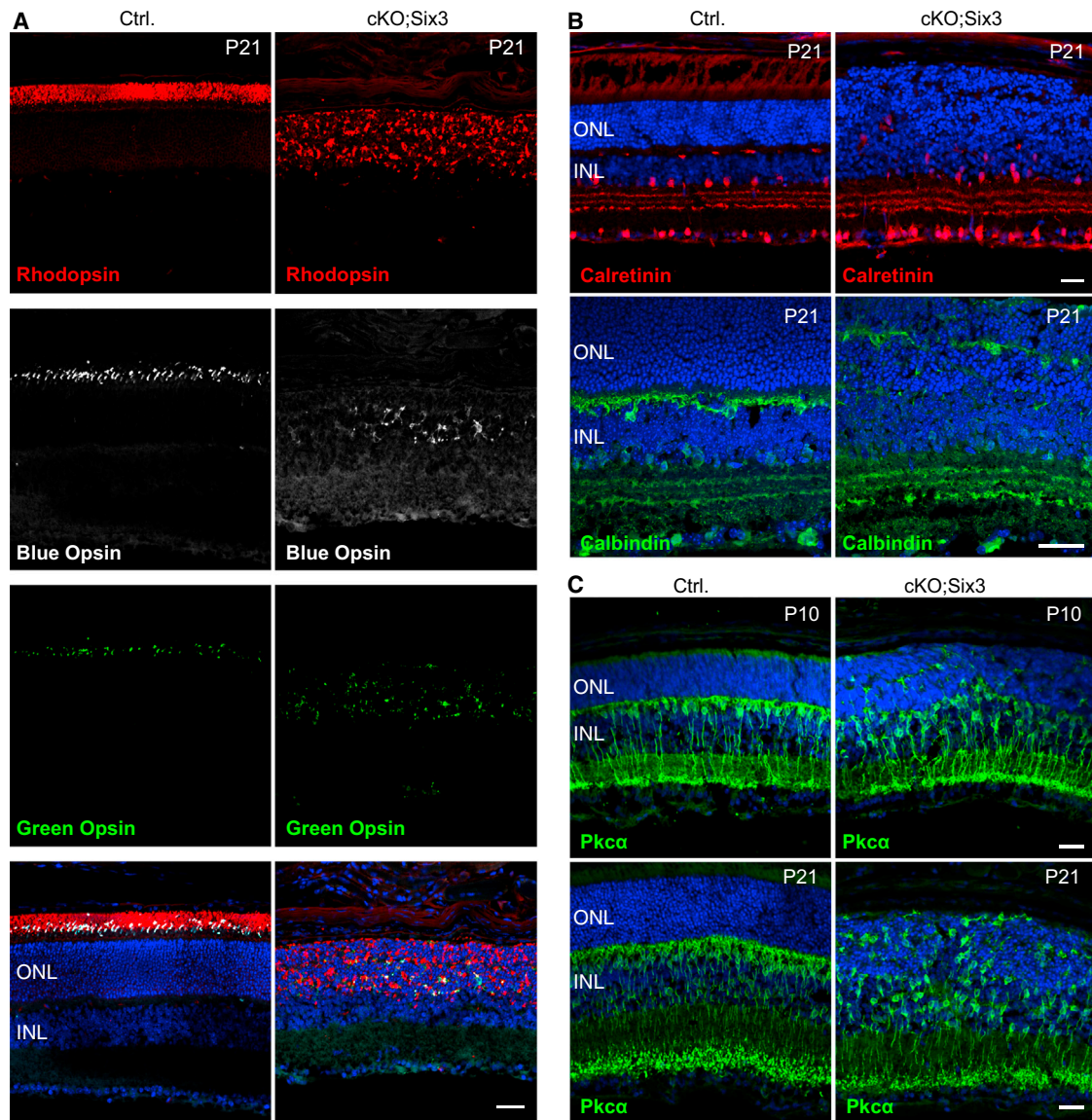


Figure 3. Photoreceptors Differentiate and Inner Retinal Neuronal Morphologies Are Preserved in *Macf1* Mutant Retina

(A) Immunohistochemistry on retina sections (P21) stained with antibodies against rhodopsin, blue opsin, and green opsin, showing normal photoreceptor differentiation in *Macf1*-null retina (cKO;Six3-Cre). Ctrl., control.

(B) Staining for calretinin and calbindin showed that amacrine, horizontal, and ganglion cells were relatively unaffected in *Macf1*-null retina.

(C) Staining for PKC α to label bipolar cells (PKC α) showed increased disruption with age (P10 versus P21) and predominantly affected the scleral side of the retina. ONL, outer nuclear layer; INL, inner nuclear layer. Scale bars represent 25 μ m.

See also Figure S2.

Deletion of MACF1 in immortalized mouse embryonic fibroblasts (MEFs) abolished ciliogenesis upon serum starvation (Figures 6D and 6G). Ciliary axoneme markers Arl13b and acetylated tubulin confirmed the loss of ciliary extension. Retention of GT335 at the basal body in mutant cells, in comparison to GT335 expansion into the proximal cilium in control, corroborated these findings (Figure 6E). Structurally, basal bodies were present, and high-resolution fluorescence microscopy showed that markers for basal body distal (CEP164) and subdistal appendages (NINEIN, EB1) remained unchanged in the mutant

(Figures S6A–S6C). These data indicate that MACF1 is broadly required for ciliogenesis.

Macf1 Interacts with Ciliary and Basal Body Proteins and Promotes Anchoring of Microtubules to the Mother Centriole

We previously found a direct interaction between MACF1 and basal body protein MKKS (BBS6) (May-Simera et al., 2009). To confirm these findings, we performed co-immunoprecipitation pull-down assays using both tagged constructs and endogenous

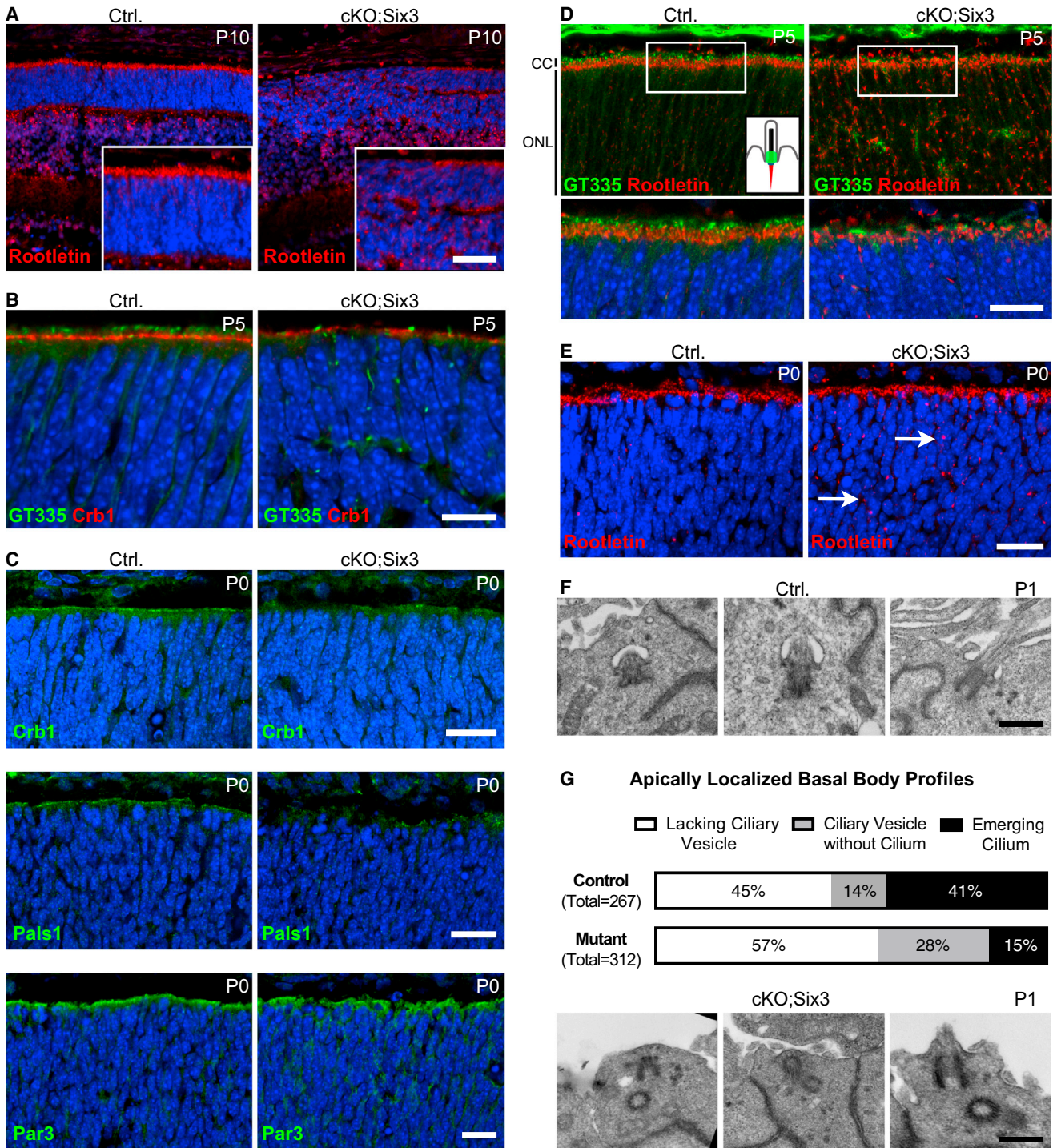


Figure 4. Polarity, Basal Body Docking, and Cilia Extension Are Disrupted in *Macf1* Mutant Retina

Immunohistochemistry on control (*Macf1^{fl/+};Six3-Cre*) (Ctrl.) and *Macf1* mutant (*Macf1^{fl/fl};Six3-Cre*) (cKO;Six3) retina sections.

(A) Ciliary rootlets (rootletin) were aligned along the apical edge of the neuroepithelium in control retina but abnormally distributed in *Macf1* mutants.

(B) Compared to control, *Macf1* mutant retina showed discontinuous localization of polarity marker Crb1 and failed alignment of the ciliary connecting cilium (GT335).

(C) As early as P0, Crb1 and additional polarity markers Pals1 and Par3 were abnormally distributed in *Macf1* mutant retina.

(D and E) Mislocalization of basal bodies (rootletin) was observed in mutant retina at (D) P5 and (E) P0, and the emergence of the transition zone (GT335) was only observed in controls.

(legend continued on next page)

protein. After co-transfecting HEK293 cells with MACF1-SR2-HA (hemagglutinin) and full-length MKKS-myc constructs, and using an anti-HA antibody for pull down, we were able to identify MKKS-myc (Figure S6E). We were also able to pull down transfected MKKS with cell-endogenous MACF1, using our custom made antibody (Figure S6F). Because the same MACF1 domain (SR2) was also identified as a binding partner with basal body protein TALPID3 (yeast two-hybrid screen), we similarly performed a co-immunoprecipitation pull-down assay. GFP-tagged TALPID3 was able to pull down MACF1-SR2-HA (Figure S6G). These findings suggest that MACF1-dependent functions during ciliogenesis may be mediated, in part, through interactions with TALPID3 and BBS6.

Closer examination revealed a loss of microtubule anchoring in mutant MEFs (Figure 7A) and cochlea hair cells (Figure 7B). Examination of microtubule growth after nocodazole treatment revealed normal nucleation but perturbed microtubule anchoring (Figure 7C). Loss of MACF1 was accompanied with an increase in actin stress fibers (Figure S6D). PCM1, a component of the pericentriolar matrix (PCM) required for ciliary trafficking (Stowe et al., 2012), was condensed around centrioles upon loss of MACF1, rather than dispersed as seen in control MEFs (Figures 7D and 7E). Altered PCM formation and aberrant microtubule anchoring, could, in turn, disrupt trafficking to the centrosome (Dammermann and Merdes, 2002). Intriguingly, localization of DNAH5 (dynein heavy chain 5) was lost from mutant cells (Figure 7F). Although traditionally a motile ciliary protein, DNAH5 labeled the basal body in control cells yet was completely absent in the mutant. This suggests the presence of axonemal dyneins at the base of the cilium, similar to Leishmania (Wheeler et al., 2015), where they may be assisting cargo loading.

Changes in the Cellular Transcriptome and Proteome upon Loss of *Macf1*

To explore the downstream molecular and cellular events brought on by the loss of MACF1, we analyzed the transcriptome and proteome of homozygous and heterozygous MEF cells by RNA-seq and mass spectrometry analyses. Mass spectrometry on whole-cell lysates from *Macf1* mutant and control MEFs identified 90 proteins that were significantly differentially expressed with a fold change of 1.5 or higher (Figures S7A–S7C; Table S1). We analyzed identified proteins using a Gene Ontology tool (<http://www.geneontology.org>). We found hits for focal adhesions and/or adherens junctions, as well as proteins related to vesicles and extracellular vesicles (Figure S7C). The role of MACF1 in focal adhesions has been well documented, yet the association with vesicles was surprising and further suggests a role in vesicle trafficking upstream of ciliogenesis. Strikingly, RNA-seq analysis (Figures S6, S7D, and S7E) revealed primarily downregulation of biological processes involved in cell migration and movement of

cellular components, as well as changes in focal adhesion and intercellular junctional components (Figure S7E). These findings are consistent with the known role of MACF1 in cell migration and focal adhesion dynamics.

DISCUSSION

Because MACF1 was identified as a potential interacting partner with basal body protein MKKS (BBS6) (May-Simera et al., 2009) and is highly enriched in the photoreceptor ciliary proteome (Liu et al., 2007), we hypothesized that *Macf1* is important for ciliary function and retinal development. In this study, we found defects caused by *Macf1* deletion in multiple cell types that were attributable to its ubiquitous requirement during the early stages of ciliogenesis. MACF1 was also shown to be actively required in differentiated tissues to maintain basal body positioning and for localization of key polarity proteins. Moreover, lack of ciliogenesis in *Macf1*-null retina perturbed the apicobasal polarity of photoreceptors, causing severe disruption of retinal lamination, primarily affecting photoreceptors, concomitant with loss of visual function. In the developing retina, loss of MACF1 abolished docking of the basal body and subsequent extension of the ciliary axoneme at the apical domain of the neuroepithelia. As a result, the photoreceptor connecting cilium and outer segments failed to develop. Absence of MACF1 in ependymal epithelia lining the brain ventricles also impaired ciliogenesis and resulted in ventricle malformation. Although the kinocilium in the developing cochlea emerged, its length was significantly shortened. It is unclear why the kinocilium in the cochlea was partially retained, but functional redundancy (there are two spectraplakins homologs in the mammalian genome), the diversity of *Macf1* splice variants, and the incomplete action of the Cre driver in the cochlea are possible explanations. In MEF cells in which MACF1 loss was complete, ciliogenesis was entirely abolished. Thus, our study identifies a previously unrecognized function of MACF1 in ciliogenesis and apicobasal polarity establishment in the neuroepithelia.

Although MACF1 is known for its coordination of microtubules and actin at focal adhesions (Karakesiosoglou et al., 2000), the ciliogenic defects suggest impaired cytoskeletal interactions at the basal body. We observed enriched MACF1 labeling around the base of the connecting cilium, a finding consistent with the photoreceptor ciliary proteome data (Liu et al., 2007) and what has been found at the kinocilium of cochlear hair cells (Antonellis et al., 2014). In cells lacking MACF1, the anchoring of microtubules around the basal body was disrupted. PCM1, a protein required for radial organization of microtubules at the basal body (Dammermann and Merdes, 2002; Farina et al., 2016), was also less dispersed in the absence of MACF1. Two other known direct binding partners of MACF1, EB1 and EB3, promote ciliogenesis via microtubule anchoring at the subdistal appendages, similarly to the proposed mechanism for *Macf1* (Schröder

(F) Electron micrographs of basal bodies at the apical edge of the neuroblast layer in P1 retina. Images from control retina show a docked ciliary vesicle (left), initiation of ciliary axoneme extension (middle), and fusion with the apical membrane (right).

(G) These events were quantified and were less readily observed in *Macf1* mutant retina. Lower panel shows examples of basal body profiles found in mutant. ONL, outer nuclear layer; CC, connecting cilia. Scale bars represent 50 μ m in (A), 25 μ m in (B)–(D), 10 μ m in (E), and 500 nm in (F) and (G).

See also Figures S2 and S3.

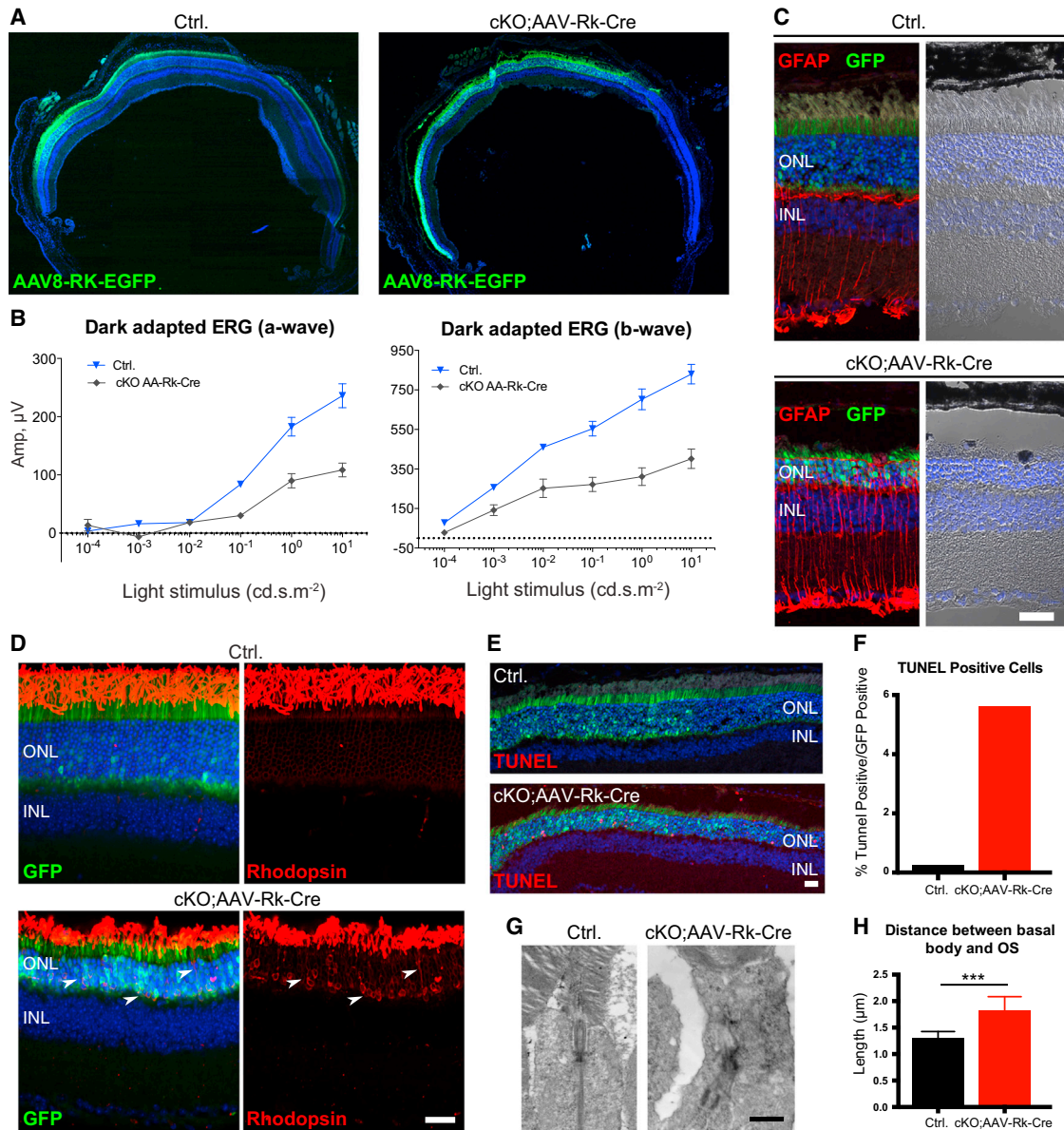


Figure 5. Macf1 Is Required for Photoreceptor Homeostasis in Mature Retina

(A) Whole-retina cross-sections of *Macf1*^{fl/+} (Ctrl.) and *Macf1*^{fl/fl} (cKO;AAV-Rk-Cre) mice 4–6 weeks post co-injection of AAV-Rk-Cre and AAV8-RKp-EGFP. Co-transduced regions are visualized by GFP expression.

(B) Mean ERG a- and b-wave amplitudes (Amp) (n = 4 mice) are plotted, with error bars indicating \pm SEM.

(C) Confocal and corresponding differential interference contrast (DIC) images show elevated GFAP expression extending into a thinning outer nuclear layer (ONL) in the mutant. INL, inner nuclear layer.

(D) Vibratome sections show rhodopsin mislocalization in the ONL of mutant retina.

(E and F) TUNEL-positive nuclei (red) were readily observed in mutant, but not control, retinas.

(G) Electron micrographs of the basal body at the photoreceptor transition zone.

(H) Quantification of “dropped” basal bodies in control and mutant photoreceptors, measured as the distance between the basal body profile and start of the outer segment (n > 10 basal bodies per treatment group).

Scale bars represent 50 μ m in (C)–(E) and 500 nm in (G).

See also Figure S4.

et al., 2011). We also found that MACF1 interacts with MKKS and TALPID3, basal body proteins that, when lost, result in ciliopathy phenotypes (Ross et al., 2005; Stephen et al., 2015). Interest-

ingly, the behavior of MACF1-null centrosomes, basal body misorientation, and disrupted docking, is strikingly similar to that caused by loss of TALPID3 (Stephen et al., 2015). This

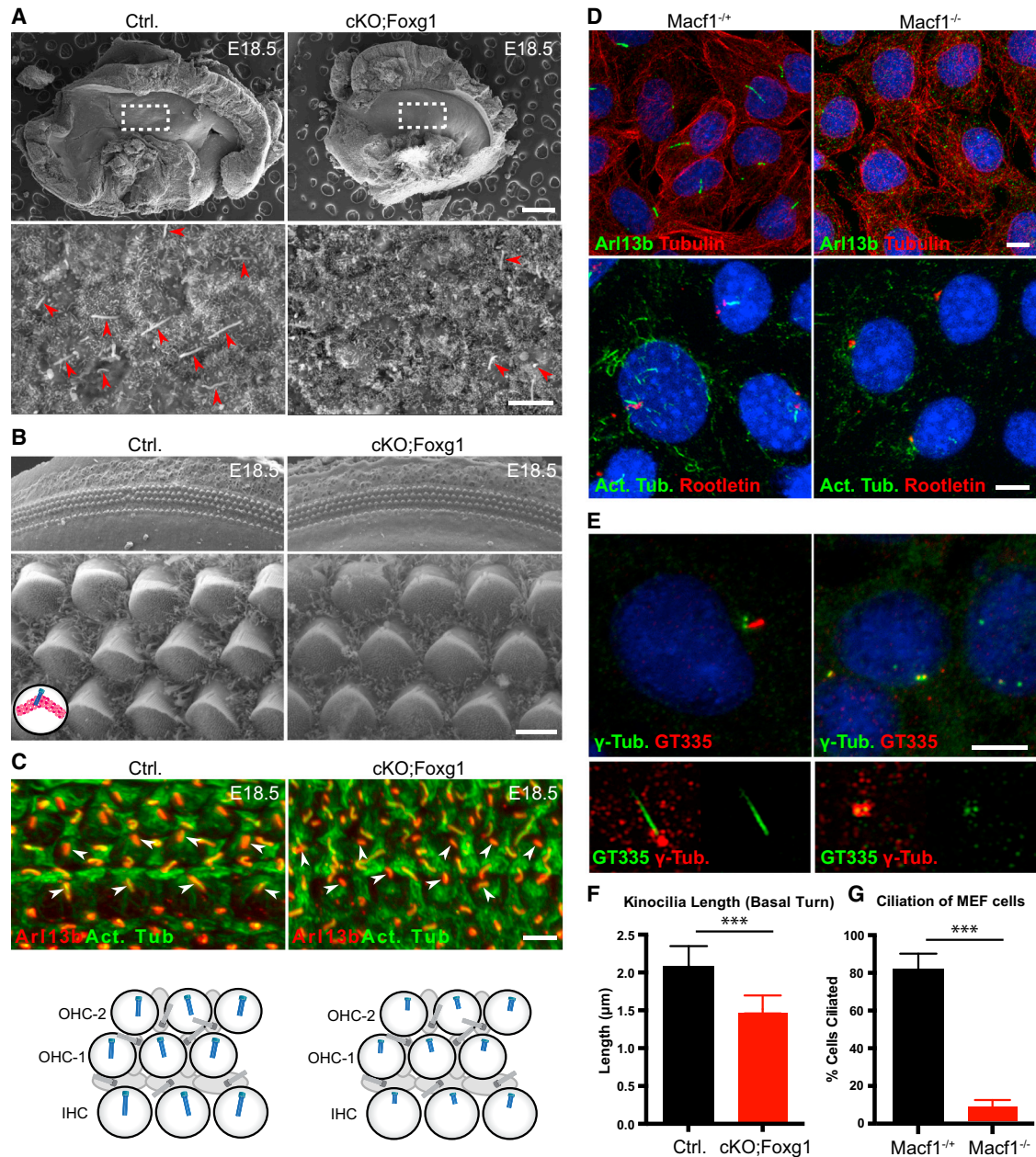


Figure 6. Cilia Fail to Extend in Multiple *Macf1*-null Cell Types

(A) Scanning electron micrographs (SEMs) of exposed lateral ventricle in *Macf1*^{fl/fl};*Foxg1*-Cre (Ctrl) and *Macf1*^{fl/fl};*Foxg1*-Cre (cKO;Foxg1) brain show diminished ventricle size (upper panels) in the mutant. Ventricle epithelial cells have a primary cilium extending into the ventricle (lower panels, red arrows), which were scarce and stumpy in the mutant compared to control.

(B and C) SEM (B) and immunohistochemistry (C) of the cochlea basal turn. One row of inner hair cells (IHC) is separated from three rows of outer hair cells (OHC) in both control and mutant (B, upper panel). Higher magnification images revealed shorter outer hair cells in the mutant (lower panel). The microtubule-based kinocilium at the vertex of each stereocilia bundle was also slightly shorter (inset cartoon; pink represents actin bundles, and blue represents kinocilium). A schematic representation of hair cells (round circles, blue kinocilia) and support cells (gray ovals, gray primary cilia) is shown. Hair cell kinocilia were shortened in *Macf1* mutants, while support cell cilia were unaffected.

(D) Staining of confluent serum-starved MEFs with ciliary markers Arl13b and acetylated tubulin showed failure of ciliary axoneme extension in *Macf1*-null MEFs. Tubulin was increasingly dispersed in the mutant, and ciliary rootlets (rootletin) were still present in mutant cells.

(E) In heterozygous cells, the ciliary transition zone (GT335) extended past the basal body, unlike in mutant, where it was confined to the basal body (top panels, confocal; lower panels, super-resolution).

(legend continued on next page)

phenotype has been previously associated with abnormal actin organization ultimately leading to ciliogenesis failure (Dawe et al., 2007a; Dawe et al., 2007b) as is seen in TALPID3 null cells (Yin et al., 2009). Accumulation of actin stress fibers were also observed in our mutant MEFs, consistent with slowed actin filament dynamics, as previously reported (Wu et al., 2008).

Disruption of the cytoskeleton around the basal body likely impairs ciliary trafficking, and because maturation of the cilium relies on robust trafficking of cilia components, this suggests that *Macf1* may be required for cilia-targeted vesicle exchange at this actin/microtubule interface. Cilia-targeted vesicles arrive at the basal body via microtubules and are subsequently transferred to the mother centriole via an actin-mediated process (Benmerah, 2013; Molla-Herman et al., 2010). Interestingly, localization of ciliopathy proteins have been observed in cellular regions where high rates of microtubule-to-actin trafficking occur (Hernandez-Hernandez et al., 2013; Sedmak and Wolf- rum, 2010). This may explain the interactions that we demonstrated between MKKS and TALPID3. Both MKKS and TALPID3 bind to the same central domain of the extremely large MACF1 protein, far from the distinct actin- and microtubule-binding sites located at opposite ends of the polypeptide. Associations with these proteins may affect the functional conformation of MACF1, thus serving to modulate interactions with microtubules and actin filaments. Both *Macf1* and *Talpid3* null cells are able to initiate microtubule nucleation but have delayed microtubule growth (Yin et al., 2009). Loss of DNAH5 from the basal body in *Macf1* null cells may also suggest a role for axonemal dyneins in facilitating cargo loading similar to what has been shown in Leishmania (Wheeler et al., 2015). Support for the role of MACF1 in vesicle trafficking also comes from an earlier report showing that MACF1 was required for protein transport from the Golgi to the cell periphery (Kakinuma et al., 2004). Alternatively, recent publications point to the successive remodeling of actin filament architecture and disruption of branched actin networks as having a stimulatory role in ciliogenesis (Antoniades et al., 2014; Reiter et al., 2012), such as in the movement of basal bodies apically and the accumulation of ciliary vesicles, although MACF1 and TALPID3 null cells also abnormally accumulate pericentriolar satellites, as shown by condensed PCM1 localization (Stephen et al., 2015). Previous studies showed that MACF1 appears to accelerate branched-actin-filament remodeling at focal adhesions (Wu et al., 2008). Therefore, loss of *Macf1* may cause failure of ciliogenesis due to loss of actin remodeling at the basal body, similar to its suggested role at focal adhesions. These two scenarios need not be mutually exclusive and could both operate in executing MACF1-mediated basal body movement and apical docking.

It is quite remarkable that our RNA-seq data analysis identified marked downregulation of biological processes involved in cell migration and movement of cellular components, as well as changes in focal adhesion and cellular junctional components.

Defective cell migration, due in part to an inability to remodel focal adhesions, is a well-documented cellular defect upon loss of *Macf1*. Thus, the RNA-seq data reveal a downstream effect on the mutant cell transcriptome that could have further contributed to the observed defects in cell migration and focal adhesion dynamics. Mass spectrometry analysis revealed overlapping findings and additional changes in proteins involved in vesicular trafficking. These findings are consistent with the known role of MACF1 in cell migration and focal adhesion dynamics and with our hypothesis that MACF1 plays a role in promoting vesicle trafficking at the base of the cilium. The identification of genes up- or downregulated in response to *Macf1* loss should prove very useful for understanding the cell biological mechanism of MACF1 function and for future studies.

Ciliogenesis is a critical determinant of neuroepithelial lamination (Paridaen et al., 2013; Wilsch-Bräuninger et al., 2012). Cellular machinery involved in the formation of epithelial polarity is also required in the formation and function of primary cilia (Delous et al., 2009; Fan et al., 2004; Rodriguez-Boulan and Macara, 2014; Sfakianos et al., 2007). A recent study showed that cilia are required for cell adhesion (Schouteden et al., 2015), which is a prerequisite of establishing polarity (Rodriguez-Boulan and Macara, 2014). Ablation of *Macf1* in the developing retina resulted in a severe defect in photoreceptor polarity and outer segment formation. Inner retinal morphology was relatively well preserved, and subtle disruptions seemed to have occurred as a secondary phenomenon. The promoters that are used to drive Cre-mediated excision of *Macf1* are expressed early and in multiple retinal neuron progenitors (Furuta et al., 2000; Swindell et al., 2006); therefore, the specific defect in photoreceptors may result from their unique reliance on polarity-mediated developmental cues. In contrast to inner retinal neurons, photoreceptor development and maturation are critically dependent on neuroepithelial configuration and apicobasal polarization (Reese, 2011). Once inner retinal neurons detach from the apical junctions, they no longer manifest apicobasal polarity. In contrast, when photoreceptors fail to develop cilia, they lose their position cues along the apicobasal axis. As observed in *Macf1* mutant retinas, many photoreceptors orient their apical domain toward the inner retina (split retina). Once photoreceptors assume this orientation, they are unable to form junctional complexes with neighboring cells due to misaligned adherens junctions. Thus, our data showed that photoreceptor terminal differentiation is guided by polarity cues provided by primary cilia.

In addition to a critical function during retinal development, we also found that MACF1 is required for ongoing basal body positioning. When *Macf1* was deleted in adult photoreceptors, the basal body detached from its apical inner segment location and “sank” into the interior of the inner segment, a phenotype not previously observed in other ciliary mutants. These data

(F) Kinocilium length was quantified using Arl13b and acetylated tubulin-stained IHC images.

(G) Quantification of ciliation in *Macf1* heterozygous and null MEFs cells ($n > 400$ cells counted in four separate experiments).

Scale bars represent 0.5 mm in (A) and 5 μ m in (B)–(E).

See also Figure S5.

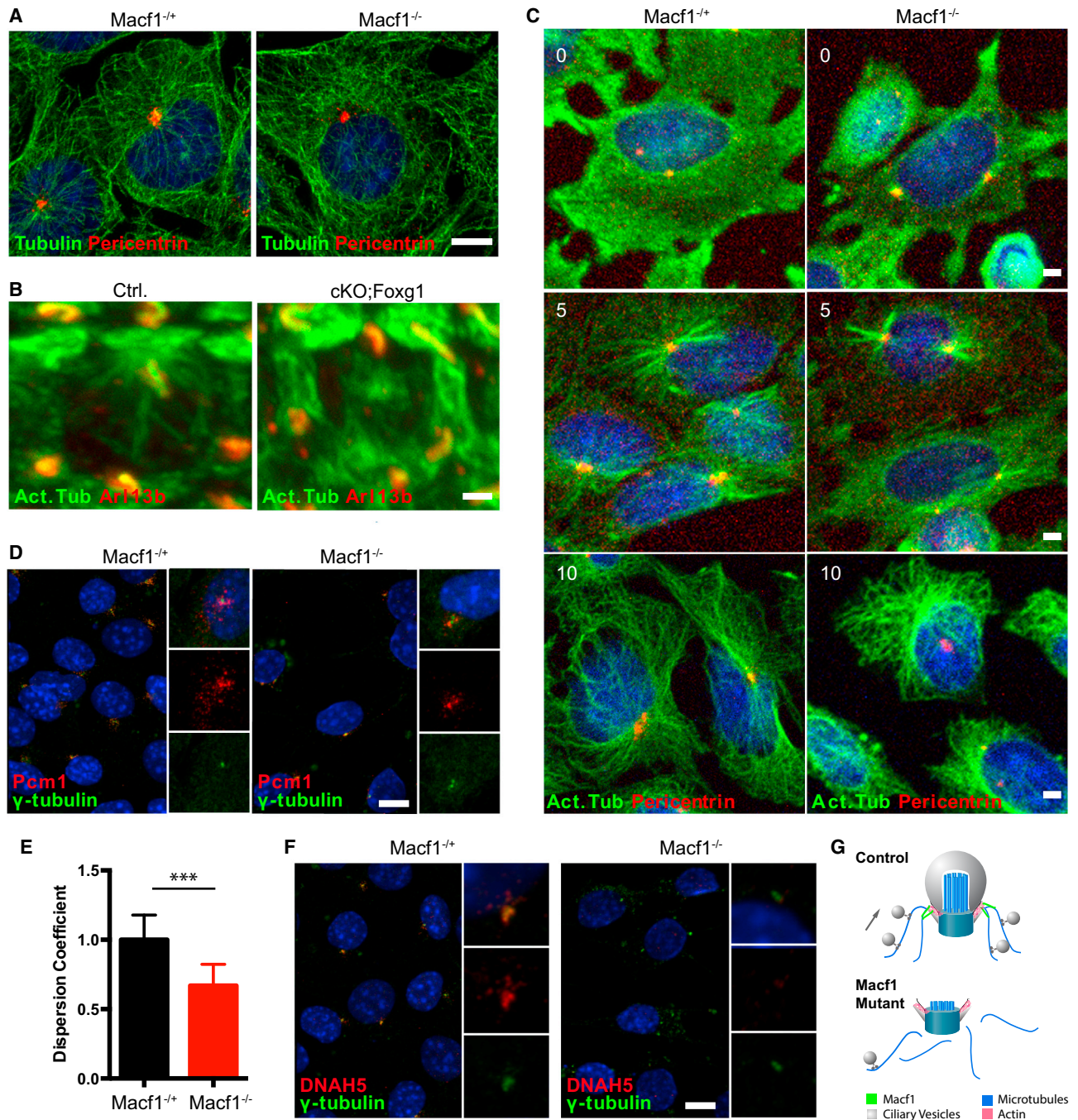


Figure 7. Loss of MACF1 Disrupts Microtubule Anchoring to Subdistal Appendages

(A) In control MEFs, microtubules (tubulin) extended radially from an anchored centrosome (pericentrin), which was lost in mutant.
 (B) Outer hair cells of control cochlea showed microtubules (acetylated tubulin) emanating from the base of the kinocilium (Arl13b) and extending across the cell. In the mutant, microtubules were disconnected from the kinocilium and abnormally bundled in the cell center.
 (C) Immunohistochemistry of stable microtubules (acetylated tubulin) and the centrosome (pericentrin) in MEFs at 0, 5, and 10 min post-nocodazole treatment. Although microtubules began to nucleate from the centrosome in both control and mutant cells, within 10 min, microtubules were no longer anchored to the centrosome in mutant cells.
 (D and E) Pericentriolar material (PCM1) was more dispersed around the centrosome in control cells compared to mutant.
 (F) DNAH5 (red) localized to the basal body (green) in control cells but was missing from the mutant.
 (G) Schematic representation of the loss of microtubule anchoring and vesicle trafficking at the basal body, resulting in the lack of ciliary extension in the mutant. Scale bars represent 5 μ m in (A), 2 μ m in (B) and (C), and 10 μ m in (D) and (F). See also Figure S7.

suggest that the photoreceptor basal body requires an active MACF1-mediated process to remain at its location similarly to basal body anchoring at the apical membrane, which also requires microtubule association with cortical actin lying just beneath the plasma membrane (Antoniades et al., 2014; Reiter et al., 2012). This could also explain why the establishment of cilia goes hand in hand with establishing polarity (Delous et al., 2009; Fan et al., 2004; Rodriguez-Boulan and Macara, 2014; Sfikianos et al., 2007), which is an active process that needs to be maintained throughout life.

The early embryonic lethality (at gastrulation) of germline-ablated *Macf1* mice precludes a detailed phenotypic analysis and suggests that a complete absence of *Macf1* is incompatible with life in mammalian species, including humans. This is not surprising, given the fundamental roles of MACF1 in multiple cell types. We postulate that hypomorphic alleles of *Macf1*, or mutations that ablate select isoforms of MACF1, could lead to human syndromes characterized by loss or dysfunction of cilia. These may include retinal dystrophy, brain defects, cystic kidney, situs inversus, and other manifestations of ciliopathies. Future genetic investigations should uncover what human disease entities may be associated with gene mutations in *Macf1*.

EXPERIMENTAL PROCEDURES

Mice

Macf1^{flox/flox} mice were crossed with *Six^{Cre}*, *Rx^{Cre}*, or *Foxg1^{Cre}* animals. Heterozygous and homozygous conditional knockout mice were identified with respect to the targeted allele by PCR. For postnatal staging, up to 24 hr after birth was considered P0. *Macf1^{flox/+};Cre* mice were used as controls, unless otherwise stated. All experiments with animals were approved by the Animal Care and Use Committee at the National Eye Institute.

Electroretinography

ERG responses were recorded with an Espion E2 system (Diagnosys). For dark-adapted responses, stimulus flash intensity varied from -4.0 to $1 \log \text{sc cd.s/m}^2$ (scotopic candela – second/meter²), with inter-stimulus intervals of 10 to 60 s. Light-adapted ERG responses were obtained with flash intensities from 0.3 to 100sc cd.s/m^2 , and responses elicited at each intensity were averaged 20 times.

Light and Electron Microscopy, Immunofluorescence, Immuno-EM, and Immunoblotting

These procedures were carried out using standard protocols and described in greater detail in the Supplemental Experimental Procedures.

Deletion of *Macf1* in Adult Photoreceptors Using AAV8-RK-Cre

Using a Hamilton syringe, subretinal injections of $1 \mu\text{L}$ sterile PBS containing AAV8-RK-Cre at $1 \times 10^9/\mu\text{L}$, AAV8-RK-EGFP at $5 \times 10^8/\mu\text{L}$, and $1 \times$ fluorescein were performed on adult *Macf1^{flox/flox}* and *Macf1^{flox/+}* mice. Animals were anesthetized, and pupils were dilated as for ERGs. Eyes were harvested 3–5 weeks post-injection.

Statistics

Unpaired Student's *t* tests were conducted to compare measured values between control and mutant samples. A *p* value of less than 0.05 was considered significant, and a *p* value less than 0.01 was considered highly significant. Error bars indicate SD unless otherwise noted. ***, *p* < 0.001.

ACCESSION NUMBERS

The accession number for the data reported in this paper is GEO: GSE86997.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.09.089>.

AUTHOR CONTRIBUTIONS

H.L.M.-S. and T.L. were responsible for conception and experimental design. H.L.M.-S., J.D.G., C.G., M.C., F.K., K.B., T.B., S.M.C., K.D.K., R.S.P., N.P., and M.G.D. performed experiments. M.W.K. and M.G.D. provided reagents. H.L.M.-S. and J.D.G. generated figures. H.L.M.-S. and T.L. co-wrote the manuscript.

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