Nf2 fine-tunes proliferation and tissue alignment during closure of the optic fissure in the embryonic mouse eye

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Abstract

Uveal coloboma represents one of the most common congenital ocular malformations accounting for up to 10% of childhood blindness (1~ in 5,000 live birth). Coloboma originates from defective fusion of the optic fissure (OF), a transient gap that forms during eye morphogenesis by asymmetric, ventral invagination. Genetic heterogeneity combined with the activity of developmentally regulated genes suggest multiple mechanisms regulating OF closure. The tumor suppressor and FERM domain protein neurofibromin 2 (NF2) controls diverse processes in cancer, development and regeneration, via Hippo pathway and cytoskeleton regulation. In humans, *NF2* mutations can cause ocular abnormalities, including coloboma, however, its actual role in OF closure is unknown. Using conditional inactivation in the embryonic mouse eye, our data indicates that loss of *Nf2* function results in a novel underlying cause for coloboma. In particular, mutant eyes show substantially increased RPE proliferation in the fissure region with concomitant acquisition of RPE cell fate. Cells lining the OF margin can maintain RPE fate ectopically and fail to transition from neuroepithelial to cuboidal shape. In the dorsal RPE of the optic cup, *Nf2* inactivation leads to a robust increase in cell number, with local disorganization of the cytoskeleton components F-actin and pMLC2. We propose that RPE hyperproliferation is the primary cause for the observed defects causing insufficient alignment of the OF margins in *Nf2* mutants and failure to fuse properly, resulting in persistent coloboma. Our findings indicate that limiting proliferation particularly in the RPE layer is a critical mechanism during optic fissure closure.

Introduction

Congenital ocular malformations such as anophthalmia, microphthalmia and coloboma (MAC) are prevalent in ~1 in 3-4,000 individuals and are the cause for over 25% of childhood blindness worldwide (1-3). Uveal coloboma alone may account for up to 10% of childhood blindness, 1~ in 5,000 live birth (4, 5) for reviews, see (6-8); it is most commonly observed as missing tissue in the ventro-nasal region of the eye and originates from a failure in apposition and fusion of the margins of the optic fissure (OF). The OF forms as a ventral groove by asymmetric invagination, extending from the vitreal side to the proximal junction with the forebrain that allows mesenchymal cells to migrate in and form the hyaloid vessel (9-11). Closure of the OF occurs when mesenchymal cells end migration; the laterally growing edges of the retinal pigmented epithelium (RPE) and retina cells lining the OF margins appose to fuse and form a continuous optic cup. Fusion starts at the midpoint of the OF and moves in both directions distally and proximally (9, 12). An opening remains near the center of the optic cup (optic disc) for ganglion cell axons and blood vessels. Coloboma can affect vision minimally or result in congenital blindness, depending on the severity, timing, involvement of tissue and location in the optic cup (for reviews, see (6-8, 13)). The etiology of coloboma is complex, and defects result from disturbance of heterogenous genetic and/or environmental factors suggesting multiple mechanisms in regulating the closure process, including cell autonomous and cell non-autonomous tissue interactions. In humans, a subpopulation of colobomata is a result of mutations in developmentally important genes, for example *CHD7, SOX2, BMP4, GDF6, SHH, STRA6, PAX2, PAX6, PORCN, VSX2, TFAP2A, MITF* and *SIX3*; however, the origins for many OF closure defects are unknown (for reviews, see (3, 7, 8, 13, 14)). Additional genes have been

identified in animal models, providing important insight into the biological function in regulating OF closure. More recently, substantial progress has been made in understanding critical processes mediating OF closure; including growth and patterning of the ventral optic cup and optic nerve head (15-19), cell-cell contact and signaling (20-30), crosstalk with migrating hyaloid precursors and extracellular matrix components (31-35), cytoskeleton dynamics (36, 37), epigenetics (38), degradation of ECM and cellular proteins (39-41), programmed cell death, survival and cell proliferation (42, 43) (for reviews, see (7, 8, 13)). Elegant in vivo imaging studies in zebrafish and excellent anatomical analyses in chick have characterized important morphogenetic and cellular behavior during OF closure (20, 27, 34, 44-48). In addition, comprehensive gene expression analyses have identified novel candidate genes potentially relevant for regulating OF closure (27, 46, 49-51).

Gene mutations of Hippo signaling pathway components represent novel associations with MAC, particularly coloboma (7, 23, 52, 53). Mutations in the Neurofibromin 2 gene (*NF2*) result in the autosomal dominant disorder Neurofibromatosis Type 2 (NF2, OMIM # 101000), a relatively rare syndrome characteristic of neoplastic lesions. Human *NF2* mutations associate frequently with ocular abnormalities (52, 54-56). Particularly, impaired vision can occur due to lens opacities, cataract, optic nerve meningioma, optic nerve hamartoma, cataract, epiretinal membranes, combined retinal and RPE hamartoma, and paralytic strabismus. Importantly, some patients with loss of *NF2* heterozygosity also exhibit coloboma, consistent with *Nf2* being identified as a coloboma gene in mouse (52, 57, 58) (this study).

The *Nf2* gene encodes the FERM domain protein MERLIN, affiliated with Ezrin, Radixin, and Moesin (ERM) proteins. It exerts multiple functions in tumor suppression, development, and regeneration in diverse organs and tissues, for example by restricting proliferation, controlling apoptosis, and promoting differentiation, apicobasal polarity and junctional complex formation (59-61). NF2 localizes primarily to the cell cortex and links the cortical actomyosin cytoskeleton to the plasma membrane. It associates with α-catenin to promote establishment of intercellular junctions and polarity complexes (62). In addition, NF2 can localize to the nucleus and act as a contact-dependent tumor suppressor by inhibiting CRL4DCAF1 ubiquitin ligase (60). Importantly, among other regulatory inputs, NF2 also controls the Hippo pathway to balance organ size, proliferation and behavior of stem cells; it binds to and activates several Hippo pathway components, including the Hippo kinases MST1/2 and LATS1/2 (60, 61). Loss of Hippo signaling leads to nuclear translocation of the transcriptional co-activators YAP/TAZ, often associated with abnormal proliferation in cancer and during development. Human mutations of *YAP* and its transcriptional effector *TEAD* cause ocular abnormalities; heterozygous loss of *YAP* can result in coloboma, and the autosomal dominant ocular disorder Sveinsson's chorioretinal atrophy (SCRA, OMIM # 108985) that is characterized by atrophic regions in choroid and retina (53, 63, 64). Recent studies in Drosophila, zebrafish and mouse elucidated functions of *Yap/Taz* during ocular development, with some aspects of evolutionary conservation (58, 65-70) (for reviews, see (71-73)).

Our study focuses on the role of *Nf2* during OF closure in mouse. NF2 protein is expressed in developing ocular tissues and is required for development of the lens and the optic cup,

particularly of the ciliary margin into ciliary body and iris (57, 58, 74-76). Coloboma occurs upon tissue-specific *Nf2* deletion (57, 58), however, it is unclear how loss of *Nf2* function affects the closure process. Our data demonstrates that loss of *Nf2* function results in ectopic proliferation in the RPE layer in the OF region with concomitant maintenance of RPE cell fate, without affecting Wnt/β signaling activity, apicobasal polarity or cell death. In *Nf2* conditional mutants, RPE cells in the temporal OF margin maintain columnar organization instead of transitioning to cuboidal shape. In the dorsal RPE of the optic cup, *Nf2* loss of function leads to a substantial increase in cell number, accompanied with local disorganization of cytoskeleton components F-actin and pMLC2. We propose that these abnormalities in the *Nf2* mutant optic cup cause insufficient alignment of the OF margins, resulting in a failure to attach and fuse properly, and, thus, leading to a persistent coloboma. Our analysis provides novel insights into the underlying mechanisms during OF closure as well as into the specific function of *Nf2* in this process.

Results

Loss of *Nf2* function leads to a narrow coloboma in the ventral optic cup

Germline disruption of the *Nf2* gene causes early embryonic lethality before eye development commences, due to a lack of extraembryonic ectoderm and mesoderm (around E6.5) (77). To investigate the role of *Nf2* during closure of the optic fissure (OF), we conditionally disrupted the *Nf2* gene using *Rx3-Cre* that is constitutively active in the early optic vesicle starting at E8.75 (76, 78, 79). In the optic cup, *Rx3-Cre* is active in the domains of the presumptive neural retina, retinal pigment epithelium (RPE) and lens vesicle (78, 79). In mouse, OF closure starts at E10.5 and is completed by E12.5, evident by continuous pigmentation in the ventral optic cup at

E13.5 (Fig.1 A, C). *Rx3-Cre*-mediated, conditional ablation of *Nf2* (hereafter *Nf2*$^{lox/lox}$*;Rx3-Cre*) results in failure of OF fusion in both eyes of each embryo (E13.5-E17.5; Fig.1B, D; n=22; 11 independent litters). In *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups, the OF margins are tightly apposed, suggesting a defect late, during the final process of closing (Fig.1D). Expression of laminin in the central ventral optic cup is persistent, confirming failed degradation of the basement membrane between the OF margins (Fig.1F; see Suppl. Fig.1A for sagittal section scheme). Laminin persistence shows 100% penetrance in the distal optic cup, close to the ciliary margin, while some *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos can exhibit laminin degradation proximally at E13.5 (33%, 3/9 eyes from n = 7 embryos; 5 independent litters). We did not observe any ocular or extraocular defects, including coloboma and persistent laminin expression in *Nf2*$^{lox/+}$*;Rx3-Cre* eyes (not shown; n = 3; 3 independent litters). Thus, coloboma in *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes is less likely to be a delay in timing since failed closure in the distal and frequently in the proximal region of the optic cup is persistent at later stages. Compared to controls, the RPE layer in *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes appears slightly darker (Fig.1D, H; Suppl. Fig.1C) and increased in height (Fig.1G, H; brackets). The pigmented OF margins extend into the open OF (Fig.1H; arrowhead), not detectable at earlier stages (not shown). In addition, *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos display additional, fully penetrant tissue fusion defects in closure of eye lids, lip and palate, as previously observed (not shown) (57).

A 2.4kB human *Nf2* promoter element was recently identified to generate a *NF2-lacZ* reporter mouse, showing robust activation specifically in the RPE between E10.5 and E12.5 (80). During mouse eye development, NF2 protein is transiently expressed in the retina and continuously

present in RPE and ciliary margin (58, 74). *Nf2* reporter activation and NF2 protein expression in the eye matches expression of the Hippo pathway target YAP. YAP1 protein normally shows cytoplasmic and nuclear localization in ocular tissues, in the mouse optic vesicle and optic cup, particularly the RPE, as well as in the adjacent periocular mesenchyme (53, 58, 67, 71, 81, 82). YAP1 expression in the developing RPE is dynamic; in the optic vesicle it is preferentially nuclear while late embryonically and in the adult it is localized in both the nucleus and cytoplasm (not shown)(53, 58, 67, 81, 82). Consistently, we observed YAP1 expression in the cytoplasm and nucleus in retina and RPE during OF closure (Fig.1I; Suppl. Fig.1D). In *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups, nuclear localization of Yap is increased in RPE, and also in retina and lens (arrowheads in Fig.1K; Suppl. Fig.1E; E11.0-E11.5; n=5; 4 independent litters). Thus, our data indicate that *Nf2* function is required for retention of (phosphorylated) YAP in the cytoplasm during OF closure. The *NF2-lacZ* reporter, therefore, may not contain the required elements to reveal activity in other embryonic tissues such as the retina (80).

<u>In the ventral optic cup of *Nf2*$^{lox/lox}$*;Rx3-Cre*, RPE markers persist in the OF</u>

Loss of *Nf2* results in increased nuclear YAP localization (see above), and YAP is required and sufficient to control RPE cell fate in zebrafish and mouse (66, 67). Therefore, we analyzed retina and RPE markers in the OF region between E11.5 and E13.5, during and after OF alignment and fusion, respectively (Fig.2). The expression patterns of the pan-ocular paired PAX6 transcription factor (n=3; 3 independent litters) and retina-specific VSX2 homeodomain transcription factor (n=4; 4 independent litters) are unchanged in the ventral optic cup of *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos at E11.5 (Fig.2B, D). Dorsoventral patterning is required for establishing the retinotectal

projection of retinal ganglion cells, and ventral optic cup specialization is particularly important for OF closure (83). Dorsal localization of the T-box transcription factor TBX5 is unaltered in the *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup (Fig.2F; arrow; n=4, E11.5-E13.5; 4 independent litters; refs). Similarly, ventral expression of the paired homeodomain transcription factor PAX2, essential for OF closure and ventral patterning of the optic cup, is robust in the OF margins in *Nf2*$^{lox/lox}$*;Rx3-Cre* OF margins, indicating no major defect (Fig.2H). Therefore, general tissue patterning of retina and dorsoventral regionalization is not affected by loss of *Nf2* function.

The bicoid transcription factor Otx2 is required for RPE cell fate and RPE differentiation at the advanced optic vesicle stage (84, 85). We observed that expression of OTX2 is ectopically maintained in the OF in several E11.5 *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos (Fig.2J; arrowheads; 57%; n=7; 4 independent litters). At E13.5 all analyzed eyes showed persistent OTX2 expression in the OF (Fig.2L; arrowheads; 100%; n=4; 4 independent litters), consistent with extended pigmentation into the OF (Fig.1D, H; Suppl Fig.1B). We confirmed maintenance of RPE markers in the OF in E11.5 *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos by analyzing expression of the bHLH transcription factor MITF, a critical key regulatory factor for RPE cell fate and OF closure (18, 86, 87). Normally, MITF is present in the approaching margins at E11.0 (Suppl Fig.2A; arrowheads) and becomes progressively excluded from the ventral OF margins at E11.5 during OF closure (Fig. 2M). In *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups at E11.0, MITF protein shows normal localization in the OF (Suppl Fig.2B; arrowheads; n=4; 2 independent litters). At E11.5, MITF-labeled cells extend into the lower (ventral) OF in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups (Fig. 2N; arrows; 100%; n=4; 2 independent litters), suggesting persistent presence of RPE precursors in the OF. Abnormally upregulated

RPE fate in the OF due to FGF pathway deficiency leads to failed OF fusion and YAP can cooperate with Pax transcription factors to promote Mitf expression in neural crest progenitors (88-90). Therefore, to test whether excessive *Mitf* dosage in the OF margins is preventing OF closure, we removed one functional *Mitf* allele by crossing the $Mitf^{Mi}$ allele into $Nf2^{lox/lox}$*;Rx3-Cre* (87). Normally, heterozygous $Mitf^{Mi/+}$ optic cups are less pigmented (Suppl. Fig.2D). In haplo-insufficient $Nf2^{lox/lox}$*;Rx3-Cre;*$Mitf^{Mi/+}$ optic cups, pigmentation is strongly reduced at E13.5, however, coloboma is still apparent (Suppl Fig.2F, H; 4 eyes from n=2 embryos; 2 independent litters). Thus, loss of *Nf2* function can result in maintenance of RPE cell fate in the OF margins, however, it may not be the primary cause for defective OF closure in $Nf2^{lox/lox}$*;Rx3-Cre* eyes.

Others and we have shown that Wnt/β-catenin signaling is required and sufficient for MITF and OTX2 expression in the mouse optic cup (91-94). RPE-specific β-catenin gene disruption causes coloboma (94). However, gain of function of the pathway by disruption of the Wnt/β-catenin inhibitor *Axin2* can also result in coloboma with persistent expression of MITF and OTX2 (19), similarly to $Nf2^{lox/lox}$*;Rx3-Cre*. Importantly, several studies have shown that NF2 can associate with LRP6 and blocks its phosphorylation resulting in inhibition of Wnt/β-catenin pathway activation (95). To determine whether Wnt/β-catenin pathway activity is hyperactivated upon loss of *Nf2* function, we utilized a knock-in $Axin2^{LacZ}$ reporter (96). We did not detect obvious changes in $Axin2^{LacZ}$ (β-GAL) expression in $Nf2^{lox/lox}$*;Rx3-Cre* heterozygous for $Axin2^{LacZ}$ (Fig. 2P; arrows; n = 4; 3 independent litters), indicating that Wnt/β-catenin pathway activity is not restrained by *Nf2* function.

Apicobasal polarity in the ventral optic cup is unaffected in the absence of NF2

NF2 participates in assembly of plasma membrane protein complexes and coupling them with the cortical actin cytoskeleton (57, 59, 60, 62). In the mouse epidermis, *Nf2* disruption can result in disorganization of F-Actin, misplacement of polarity complex proteins and aberrant, diffuse localization of tight junction proteins; thus, a general role for NF2 in cell-cell attachment during tissue fusion has been proposed during neural tube closure (57, 62). In the developing ciliary margin, *Nf2* deletion causes dysregulation of apical junction proteins affecting apico-basal polarity (58). In the ventral optic cup of *Nf2*$^{lox/lox}$*;Rx3-Cre*, F-Actin localization appears normal (Fig.3B; E11.0-E11.5; n=7; 4 independent litters). In medaka, reduction of YAP results in decreased tension of the actomyosin network in embryonic tissues (97). Therefore, we reasoned that increased nuclear YAP may promote actomyosin activity in *Nf2*$^{lox/lox}$*;Rx3-Cre* ventral optic cups. Critical for actomyosin contractility is phosphorylation of myosin light chain II (pMLC2) that is normally localized to the apical RPE surface in the optic cup (Fig.2C) (93, 98). In the ventral RPE of *Nf2*$^{lox/lox}$*;Rx3-Cre*, adjacent to the OF, we observed no obvious changes in apical localization of pMLC2 protein (Fig.3D; arrow; E11.5; n=3; 2 independent litters), suggesting that actomyosin activity is not affected by loss of *Nf2*. Similarly, localization of ZO-1, indicative for intercellular apical junction formation between retina and RPE, is not altered in *Nf2*$^{lox/lox}$*;Rx3-Cre* (Fig.3F; E11.0-11.5; n=5; 5 independent litters). Furthermore, NF2 regulates assembly of adherens junctions (AJ) in the mouse epidermis, through direct association with α-catenin, linking it to polarity protein complexes (62). However, we observed no difference in distribution of the AJ core protein α-catenin in the OF and apical surfaces between control and

Nf2$^{lox/lox}$*;Rx3-Cre* optic cups (Fig.3G, H; arrows; E11.5; n = 3; 2 independent litters). Thus, our data suggest that loss of *Nf2* in the optic vesicle does not interfere with apicobasal polarity, formation of adherens/tight junctions and actomyosin organization in the ventral optic cup during closure of the OF.

During OF closure, ventral RPE proliferation is abnormally increased in *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes

Depending on the species, OF closure is tightly regulated by a balance of programmed cell death (apoptosis) and proliferation. In zebrafish, apoptosis is not abundant in the ventral optic cup, and cell death or proliferation are not required for OF closure (27, 34, 45, 99, 100). In chick and in mammals, retinal apoptosis is prevalent and retinal proliferation is decreased in the vicinity of the OF margins during closure (9, 10, 27, 101-103). Accordingly, abnormal changes in cell number by altered cell death or cell divisions in the optic cup can result in OF closure defects and colobomata (43, 104). Since *Nf2* disruption in mouse causes altered apoptosis during neural tube closure (57), we first analyzed programmed cell death using TUNEL labeling. In the ventral optic cup of E11.5 *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos, apoptosis was not significantly altered in the ventral quadrant harboring the OF (V2; Suppl. Fig.3A-D). Nuclear localization of YAP can stimulate cell division, and a significant increase in RPE marker-labeled cells and BrdU incorporation in the RPE layer is observed in conditional *Nf2* mutants using Nestin-Cre (57, 58). However, spatial and temporal information critical for the process of OF closure is lacking; these studies were performed after completion of OF closure, and tissue analysis was not regionally correlated with the OF. Thus, we performed a detailed proliferation analysis in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups during OF closure. Phospho-histone H3-labeling to detect

proliferating cells in G2/M of the cell cycle revealed an upward trend in the ventral *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup that was not significant (Fig.4A-C; E11.5; n = 3; 2 independent litters). We observed a small but significant increase of phospho-histone H3-labeled cells in the ventral quadrant that includes the OF region (Fig.4D). Since the number of G2/M cells, particularly in the RPE, was generally very low in the optic cup, we analyzed EdU incorporation during S-phase of the cell cycle as an additional proliferation marker (Fig.4E-J). We determined changes in proliferation specifically during the initial alignment of the OF margins, a critical stage for the subsequent attachment and fusion processes during OF closure (E11.0; n = 4; 2 independent litters). The percentage of EdU-labeled cells is significantly increased by 46% in the RPE in *Nf2*$^{lox/lox}$*;Rx3-Cre* (Fig. 4F, G; control: 19.91% ± 1.74 s.d.; *Nf2*$^{lox/lox}$*;Rx3-Cre*: 29.07% ± 4.60 s.d.). In contrast, no significant change is detectable in the retina (Fig.4G, H). The RPE-specific increase in EdU-labeled cell number is specific for the ventral portion in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups, by 79% (Fig. 4F, I; V1-V3; see Suppl. Fig. 3C for subdivision scheme; control: 27.82% ± 5.66 s.d.; *Nf2*$^{lox/lox}$*;Rx3-Cre*: 49.86% ± 7.10 s.d.), while the dorsal RPE shows no significant change at this stage (Fig.4F, I). Significantly increased numbers of EdU-labeled RPE cells are observed in all subdivisions of the ventral optic cup (Fig.4J), including the optic fissure region (V2; Fig. 4J). Consistent with increased proliferation, the total number of cells in the ventral optic cup is significantly expanded in *Nf2*$^{lox/lox}$*;Rx3-Cre* (Suppl. Fig.3E). In summary, our data indicates that *Nf2* disruption in the optic vesicle results in abnormally increased RPE proliferation specifically in the ventral optic cup early in the process of OF closure.

Ectopic RPE cell number in the dorsal optic cup of *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos

As described above, we detected no significant increase in the percentage of EdU incorporation in the dorsal *Nf2*$^{lox/lox}$*;Rx3-Cre* RPE (Fig. 4I). However, we observed supernumerary cells and frequent cellular crowding in the MITF-labeled RPE layer of the dorsal optic cup (Fig. 5B; arrowheads; n = 4; 2 independent litters). Quantification of total cell number revealed a robust significant increase by 47% specifically in the dorsal *Nf2*$^{lox/lox}$*;Rx3-Cre* RPE layer (Fig. 5C; n = 4; 2 independent litters; control: 183.75% ± 15.13 s.d.; *Nf2*$^{lox/lox}$*;Rx3-Cre*: 271.00% ± 10.42 s.d.). In contrast, total cell number in the dorsal retina did not change in *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos (Fig.5D). Furthermore, in the dorsal RPE layer of *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups, we observed regions with irregular F-actin organization and mislocalization of pMLC2 (Fig.5F, H; arrowheads). Abnormally increased number of RPE cells with disorganization of the RPE cell layer was maintained later at E13.5, using OTX2 as a marker for differentiating RPE cells (Fig.5K; arrowheads). Collectively, our data indicates that ocular *Nf2* disruption leads to a significant increase of cell number in the dorsal and ventral RPE layer of the optic cup. This occurs with concomitant acquisition of RPE cell fate and mis-localization of F-Actin and pMLC2 in the dorsal RPE layer. However, size of the *Nf2*$^{lox/lox}$*;Rx3-Cre* eye is unchanged, since measurement of the ocular diameter in embryos did not reveal a significant difference (Suppl. Fig. 3F; at least 6 eyes per genotype from n = 6 embryos; 5 independent litters).

Alignment and cellular height is altered in the OF margins of the *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup

Early in the process of OF closure in control embryos (E11.0), the fissure margins become tightly aligned, shaped with a convex surface toward the basal space (Fig.6A; arrowheads). In the

Nf2$^{lox/lox}$*;Rx3-Cre* OF, the shape of the basal surface of the OF margins is convex, similar to controls, however, alignment of the margins is less advanced (Fig.6B, arrowheads). At E11.5, margins in control embryos are well aligned along most of the OF with a flattened basal surface (Fig.6C; arrowheads). In E11.5 *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos, we noticed that the shape of the approaching OF margins is consistently altered; the margins maintain a convex shape and the actual extent of contact area between both OF margins is often reduced in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups (Fig.6D; arrowheads). We quantified this by measuring the extent by which the margins become tightly apposed and determined that the OF in *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes shows a significant reduction (Fig. 6E; 9 eyes; n = 7 embryos; 3 independent litters).

In *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups, we observed a tighter cellular packing and crowding with occasional formation of a double layer in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups (Fig.4F; arrowheads). This is most likely caused by increased proliferation particularly in the ventral RPE layer that is continuous with the ventral OF margins (V2; Fig.4J). In addition, we found that the ventral marginal OF layers appear increased in height along the apicobasal axis and columnar in shape, in comparison to the rather cuboidal organization in controls (Fig.6C, D; brackets). We measured the apicobasal height of cells lining each temporal and nasal margin in the ventral OF (Suppl. Fig.3G) and observed a significant increase in cellular height particularly in the temporal OF in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups (Fig. 6F; at least 6 eyes from 5 embryos per genotype, from a minimum of 3 independent litters). In the nasal OF in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups, cell height measurement showed an upward trend that is not significant (Suppl. Fig. 3H). These results suggest that, in the absence of *Nf2* function, convex shape of the OF margins is maintained,

combined with a columnar organization, resulting in insufficient alignment and subsequent failure to fuse.

Discussion

In summary, our results demonstrate that conditional, ocular disruption of the *Nf2* gene at the optic vesicle stage results in a fully penetrant, distally persistent late-stage defect in OF closure in the mouse embryo (see Fig. 6G for a proposed summary). *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes show increased nuclear localization of YAP protein in ocular tissues, without apparent defects in formation of junctional complexes or apicobasal polarity in the ventral optic cup. Here we identify expansion specifically of the RPE layer as a unique underlying cause for coloboma. In *Nf2*$^{lox/lox}$*;Rx3-Cre* mutant eyes, more cells in the RPE layer in the ventral optic cup, including in the OF margins, proliferate and acquire RPE fate. In the OF margins, cells extend in height or double up as a second layer, and the RPE layer maintains columnar organization instead of transitioning into cells of cuboidal shape. In addition, in the RPE of the dorsal optic cup, *Nf2* loss of function leads to a robust increase in cell number, with local disorganization of the cytoskeleton components F-actin and pMLC2. We propose that this specific effect on proliferation in the RPE layer in the *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup may result in changes in mechanodynamics within the growing optic cup, causing the OF margins to align insufficiently.

<u>Nf2 is required for balancing RPE cell number during OF closure</u>

During optic cup morphogenesis, the RPE is required for growth and OF closure; extreme, broad removal of RPE by diphtheria toxin in the early optic cup causes microphthalmia and coloboma

(29, 105). Growth of the RPE during eye development is dynamic; proliferation is high in the entire optic vesicle (50-60%) and decreases in the RPE substantially soon after invagination (12-25%) (106-109). During normal OF closure, cell number in the mammalian optic cup is tightly balanced by cell proliferation and programmed cell death (13, 43, 104). Particularly close to the approaching OF margins, proliferation is lower compared to other regions of the optic cup, and deregulated proliferation leads to coloboma (43). This is assumed to avoid cellular crowding in the fissure to allow cellular reorganization. However, it is unclear from these studies whether cell number specifically in the RPE layer is critical for OF closure (43, 89, 104). *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes exhibit a substantial increase in proliferation in the ventral RPE when the OF margins are approaching (by 79%; Fig.4I), as well as a robust increase of overall cell number in the dorsal RPE. Furthermore, RPE cell expansion is only observed when *Nf2* loss occurs in the optic vesicle and early optic cup, but not at later time points (58)(SR and SF; unpublished observations). This is consistent with the RPE's inability to renew itself, once differentiation and further maturation commences (110). Few factors have been identified that can promote proliferation and expansion of RPE; these include transgenic hyperactivation of Notch signaling, resulting in microphthalmia and coloboma (29, 111), or loss of factors that normally limit proliferation in the developing RPE, such as the cyclin-dependent kinase inhibitor p27Kip1 or DAPL1 (112-114). Collectively, our analysis reveals a unique role for *Nf2* in regulating closure of the optic fissure by restricting RPE proliferation in the optic cup.

Ectopic cells in the outer layer of the *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup acquire RPE fate

In the growing optic cup, the presumptive RPE slowly proliferates to maintain a single layer and differentiates into pigmented cells in a central to peripheral and dorsal to ventral gradient, which is tightly coordinated with growth of the neural retina (86, 108, 109). Abnormal increases in proliferation in the presumptive RPE in the early optic cup is usually induced by loss of RPE key regulatory gene function or by direct misexpression of factors stimulating proliferation, for example, by modulation of extracellular matrix molecules, extracellular signaling pathways (e.g. FGF, Wnt, Shh), overexpression of oncogenes and loss of key RPE determination genes (110, 115). Since the presumptive RPE is bipotential at the early optic cup stage, these manipulations result in transdifferentiation of RPE into retina, with concomitant acquisition of neural retina-specific cell fate and formation of an ectopic retina (for review, see (110)). Our findings are unusual; in *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes, proliferation of the presumptive RPE is strongly upregulated without inducing transdifferentiation into retina, and more RPE cells are produced. This effect is associated with an increase in nuclear YAP localization, consistent with YAP/TAZ's role in regulating RPE cell fate and differentiation in zebrafish and mouse (58, 65-67). In addition, YAP mutant eyes develop coloboma in zebrafish. Thus, YAP/TAZ likely exerts a dual role in promoting proliferation and differentiation in the presumptive RPE layer in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups. In premigratory neural crest progenitors, YAP cooperates with Pax3 to transcactivate Mitf, thus, YAP/TAZ may directly regulate RPE-specific gene expression (90).To further confirm that YAP/TAZ are required for RPE expansion in *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos, we attempted to block YAP/TEAD interaction by administering Verteporfin; however, this was not successful, possibly due to solubility issues. A recent analysis in mouse revealed that NF2

coordinates later proliferation and differentiation of peripheral retina, ciliary body/iris and RPE by limiting nuclear YAP/TAZ activation (58). Thus, it is very likely that RPE expansion in *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes is mediated via nuclear translocation of YAP/TAZ and activation of TEAD.

Coloboma may be due to persistent buckling of the OF margins

In *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups apposition of both OF margins is significantly decreased; the margins show a higher degree of bending and do not flatten as observed in controls during OF alignment (Fig.6). Although, some optic cups can exbibit fusion at varying degrees proximally close to the optic disc, the OF closure defect in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups is always persistent in the distal optic cup at later stages, excluding a simple delay. While we cannot rule out other potential mechanisms (e.g. constraints provided by the extracellular matrix), our results suggest that over-proliferation in *Nf2*$^{lox/lox}$*;Rx3-Cre* RPE is likely to be the primary defect. Expanded proliferation may exert a critical mechanical force for promoting tissue buckling, thereby possibly resulting in prolonged convex bending of the OF margins. In a potentially similar scenario, experimentally induced, localized increases in proliferation due to constitutively activated β-catenin can exert mechanical forces to stimulate cortical folding in the embryonic mouse telencephalon (116). Thus, it is possible that similar effects occur on mechanodynamics in the *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup.

Our observations suggest that shape and organization of the RPE layer may be important for OF closure. RPE morphogenesis during formation of the optic cup is dynamic; RPE cells transition from a pseudostratified columnar epithelium into a single layer of cuboidal cells (for reviews,

see (85, 86)). Interestingly, in zebrafish, RPE cells flatten further and expand around the retina in the optic cup, independent of cell proliferation and possibly by recruitment of additional cells into the layer (45, 117) (for review, see(118)). Electron microscopic studies in mouse and hamster suggest that marginal OF cells in the immediate vicinity of the first fusion contact re-orient their polarity during degradation of the basement membrane (9, 10). This is supported by live imaging studies in zebrafish, revealing extensive movements and incorporation of OF marginal cells into retina as well as RPE during closure (34, 48). In *Nf2*$^{lox/lox}$*;Rx3-Cre* RPE, cells in the temporal OF margin do not properly transition to a cuboidal monolayer at the time of closure (Fig.6F). It is possible that the expansion of *Nf2*$^{lox/lox}$*;Rx3-Cre* RPE results in cellular packing/crowding, preventing cells from re-orienting properly in the OF. This re-arrangement may be necessary to prepare for fusion at the right time. However, we cannot fully exclude that the cells lining the OF harbor a primary intrinsic defect that ultimately prevents proper dissolution of the basement membrane.

Role of Hippo signaling in OF closure in humans

Hippo pathway components have been recently identified as genes with clinical relevance for coloboma (23, 52, 53, 63). Heterozygous loss of function mutations in *YAP1* were identified in 2 unrelated families exhibiting coloboma (3). According to previous studies using animal models, *YAP1* may be important for regulating RPE cell fate and RPE proliferation during OF closure in humans (66, 67). However, YAP1 is also present in the periocular mesenchyme (Fig.1; (53)), which could be of importance for studies addressing the syndromic occurrence of coloboma. Furthermore, a mutation in the *TEAD1* gene causes SCRA, preventing it from interacting with

YAP1 (63, 119). SCA manifests as bilateral stripes of atrophic retina and choroid; however, the contribution of RPE defects in disease pathology is not well understood. The protocadherin FAT1 represents a potential upstream regulator of the Hippo pathway, and it is expressed in the RPE (23). A new clinical syndrome with frequent ocular manifestations, in particular coloboma, is reportedly caused by frameshift mutations in the FAT1 gene that may restrict nuclear localization of YAP. While FAT1 may be critical for cell-cell contact during OF closure, a direct effect on YAP localization was not observed (23). Furthermore, *NF2* mutations cause a variety of ocular defects particularly in the RPE; for example, increased pigmentation and combined retinal and RPE hamartomas (52, 120). Importantly, coloboma was observed in NF2 patients with germline nonsense mutations (52). Thus, it is possible that hyperproliferation of the RPE represents an underlying mechanistic cause for some of the observed ocular abnormalities, including coloboma, in individuals with inherited *NF2* mutations.

Material and Methods

Mice

Animal procedures were approved by the Vanderbilt University Medical Center Institutional and Animal Care and Use Committee. *Nf2*$^{lox/lox}$ mice (76) were crossed with *Rx3-Cre* (79), *Axin2*^{lacZ} (Jax strain #009120); (96)) and *Mitf*^{Mi} (Jax strain #001573; (121, 122)). Mouse lines were maintained in a mixed genetic C57BL/6 and CD-1 background. No ocular or extraocular defects were detected in *Nf2*$^{lox/+}$*;Rx3-Cre* eyes, and littermates without *Cre* allele were used as controls. Some samples were heterozygous for the *Axin2*^{lacZ} reporter allele, which did not reveal any difference to samples harboring the *Axin2*^{wt} allele. Some control samples were

heterozygous for the *Rd8* mutation, and no difference to *Rd8* wildtype controls was observed. The *Rd8* mutation results from a mutation in the gene *Crb1* (123, 124), however, only ocular abnormalities at postnatal ages such as retinal disorganization and photoreceptor degeneration have been observed (125). For timed pregnancies, counting started at day 0.5 when the vaginal plug was detected. Embryonic tissues were genotyped using the following primer combinations (19, 76, 78). The *Nf2* primers CTT CCC AGA CAA GCA GGG TTC (forward, P4) and GAA GGC AGC TTC CTT AAG TC (reverse, P5) generate amplicons for wt (305 bp) and *lox* (442 bp); *Rx3-Cre* primers: GTT GGG AGA ATG CTC CGT AA (forward) and GTA TCC CAC AAT TCC TTG CG (reverse) produce a 362 bp amplicon; and the *Axin2*^{lacZ} primers: AAG CTG CGTCGG ATA CTT GAG A (Cs), AGT CCA TCT TCA TTC CGC CTA GC (Cwt), TGG TAA TGC TGC AGT GGC TTG (ClacZ) generate the *Axin* wt (493 bp) and *Axin*^{lacZ} amplicons (400 bp). For genotyping of the *Mitf*^{Mi} allele, tissue samples were processed and genotyped by Transnetyx (Cordova, TN) using Taqman with custom-designed primers: CCTTTCCCATGCTCTTTTCTTGAAG (forward), CTAGCTCCTTAATGCGGTCGTTTAT (reverse), and the following reporter probes to detect the 3 nucleotide mutation: ACGAAGAAGAAGATTTAAC (wildtype allele) , AACGAAGAAGATTTAAC (*Mitf*^{Mi} allele). To detect proliferating cells in S-phase of the cell cycle, pregnant dams received intraperitoneal EdU injections of 30 µg/g body weight 2 hours before sacrificing (Thermofisher/Invitrogen, Rockford, IL; #E10187).

Immunohistochemistry

Embryos at the indicated ages were processed as previously described (Bankhead15). Briefly, embryonic heads were fixed in 4% paraformaldehyde (PFA) for 1 hour, cryoprotected in

sucrose, sectioned on a Leica CM1950 cryostat at 12 µm and stored at -80C until further use. For immunohistochemical analysis, sections were processed using antigen retrieval with triton X-100 (0.1%, 1%) and hot citrate buffer pH 6.0. The following primary antibodies or markers were used: Alpha-catenin (1:2,000; Sigma, St. Louis, MO; #C2081), β-galactosidase (1:5,000; Cappel; MP Biomedicals, Aurora, OH; #55976), phospho-histone H3 (1:1,000; EMD Millipore, Billerica, MA; #06-570), laminin (1:2,000; Abcam, Cambridge MA, USA; #ab30320), MITF (1:800; Exalpha, Shirley, MA; # X1405M), OTX2 (1:1,500; EMD Millipore, Temecula; #AB9566), OTX2 (1:700; R&D Systems, Minneapolis, MN; #AF1979), PAX2 (1:800; BioLegend, San Diego, CA; #901001), PAX6 (1:500; BioLegend, San Diego, CA; #901301), Phalloidin (1:50; Invitrogen, Carlsbad, CA; #A12379), phospho-Myosin Light Chain 2 (1:80; Cell Signaling, Danvers, MA; #3674), PHH3 (Upstate Cell Signaling Solutions; Lake Placid, NY; #06-570), Tbx5 (1:200; Novus Biologicals; Centennial, CO; NBP183237), Vsx2 (!:800; Exalpha; Shirley, MA; #X1180P), YAP1 (1:100; Cell Signaling, Danvers, MA; #14074), ZO1 (1:100; Thermofisher, Rockford, IL, #40-2200). The following secondary antibodies were applied: goat anti-mouse, conjugated with Alexa Fluor647 (1:800; ThermoFisher/Invitrogen; #A32728), donkey anti-mouse Alexa Fluor647 (1:800; Thermofisher/Invitrogen; #A32728), donkey anti-mouse Alexa Fluor®594 (1:1,000; Jackson ImmunoResearch; West Grove, PA; #715-585-150), goat anti-rabbit Alexa Fluor568 (1:1,000; ThermoFisher/Invitrogen; #A11036), donkey anti-rabbit Alexa Fluor®488 (1:1,000; Jackson ImmunoResearch; West Grove, PA; #711-545-152), donkey anti-rabbit Alexa Fluor®594 (1:1,000; Jackson ImmunoResearch; West Grove, PA; #711-585-152), goat anti-mouse Alexa Fluor647 (1:800; ThermoFisher/Invitrogen; #A32728), donkey anti-goat Alexa Fluor 568 (1:800; ThermoFisher/Invitrogen; #A110357), donkey anti-goat TRITC (1:800; Jackson

ImmunoResearch; West Grove, PA; #705-025-147), donkey anti-sheep Cy™3 (1:800; Jackson ImmunoResearch; West Grove, PA; #713-165-003). For detection of apoptotic cells, ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (EMD Millipore, #S7110) was used according to the manufacturer's instructions. EdU detection was performed using the Click-iT® EdU Imaging Kit (Thermofisher/Invitrogen, Rockford, IL; #C10637). Sections were counter-labeled with DAPI (Thermofisher/Invitrogen, Rockford, IL; #D3571) and mounted in Prolong Gold Antifade. A minimum of 3 eyes from 3 different embryos from at least 2 individual litters were analyzed per genotype, time point and marker.

Embryo and whole eye images were captured using an Olympus SZX12 stereomicroscope, equipped with the Olympus MicroFire digital microscope camera U-CMAD3. Epifluorescence images were captured using an upright Olympus BX51 microscope (Tokyo, Japan), mounted with an Olympus XM10 camera. For confocal imaging, the ZEISS LSM 880 system was used. Images were processed using Fiji (NIH) and Adobe Photoshop CS6 software.

Quantification analyses

For quantification of TUNEL, PHH3-labeling, and EdU incorporation, labeled cells were counted in cryostat sections midway through the optic cup (see Suppl. Fig. 1A for sagittal section scheme). The optic cup was sub-divided into 6 regions of interest (see Suppl. Fig. 3C) allowing comparison of dorsal and ventral individual subdivisions (D1, D2, D3 and V1, V2, V3) as well as combined data subdivisions to generate values for dorsal (D1-D3) and ventral optic cup domains (V1-V3). The percentage of cells incorporating EdU was calculated by determining the

number of total cells using DAPI-labeled nuclei. Cell diameter measurements were performed on whole eye images (examples are shown in Fig. 1C, D) by drawing a horizonal line across the center of the eye globe, measured using Fiji software (NIH). To calculate the extent of attachment between OF margins, sagittal cryostat sections labeled with Phalloidin were analyzed. Attachment was defined for tightly apposed OF margins enclosing an area with phalloidin labeling apparent mostly as a single border between the OF margins (for examples, see Fig. 3A; Fig. 6C, D; Suppl. Fig. 3F). No attachment (set as "0") was defined as a distinguishable gap between the two apposing phalloidin-labeled OF margins, with detectable mesenchymal cells present in the OF (for example, see Fig. 3B). Attachment of OF margins was measured by drawing a straight vertical line, analyzed using Fiji software. Cellular height was calculated using Phalloidin-labeled images and Fiji software. Specifically, a straight line was applied along the apicobasal extent of each cell (Suppl. Fig. 3F), and 2-4 cells for each temporal or nasal margin of the OF was measured for each sample.

Statistical analysis was performed using Prism version 8 (Graphpad, San Diego, CA) for 2-Way ANOVA with Sidak's multiple comparison or Student's T-test. Differences were only considered significant when $p < 0.05$.

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Figure Legends

Figure 1

Nf2 disruption in ocular tissues causes coloboma in the embryonic mouse eye. Sagittal view with nasal orientation to the left. A: Control embryonic head at E13.5. B: *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos exhibit cleft lip and palate (arrowhead). C: higher magnification of E13.5 control eye. D: In *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos at E13.5, the ventral eye shows an OF closure defect with a narrow gap (arrowhead) and the pigment appears slightly darker. E: Immunolabeling for the basement marker laminin in the E13.5 ventral optic cup of controls confirms absence of laminin and successful fusion of the OF region (arrowhead). F: Laminin is persistent in the ventral optic cup in E13.5 *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos indicating failed OF fusion (arrowhead). G: Brightfield image of the ventral optic cup in control showing pigmentation of the RPE in the fused OF

(arrowhead). H: In the ventral optic cup of E13.5 *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos, pigmentation extends into the unfused OF (arrowhead) and the RPE layer appears slightly increased in height (bracket, compare to bracket in G). I: During OF closure at E11.5, YAP1 protein expression is predominantly cytoplasmic in the lens and retina (arrowhead), and in the RPE YAP1 is localized both to the cytoplasm and nucleus. K: In *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes, YAP1 expression is predominantly nuclear in lens, retina and RPE (arrowheads).

Scale bars: 100 µm in E; 50 µm in I,K.

Figure 2

Analysis of tissue patterning in the *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup reveals persistence of RPE markers in the open optic fissure. A-L, O, P: E11.5; M, N: E13.5. A-B: Expression of the pan-ocular protein PAX6 is unaltered in *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes (B). Asterisks label overlying OF. C-D: The retina-specific homeodomain protein VSX2 is normally expressed in *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes (D). E-F: Analysis of TBX5 expression shows normal patterning of the dorsal optic cup in *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos (F; arrow). G-H: In the ventral *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup, the key transcription factor PAX2 is normally localized (H). I-J: Expression of the RPE determination transcription factor OTX2 can be maintained in the open fissure of *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes (J, arrows). K-L: At E13.5, coloboma is persistent in *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos and the margins of the unclosed OF are lined with OTX2-positive cells (L, arrows). M-N: The bHLH transcription factor MITF is present in the RPE layer of the optic cup (M). In *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos, MITF localization extends into the OF (N; arrows). O-P: Axin2 reporter expression, a readout for

Wnt/β-catenin pathway activation, is not altered in the ventral RPE in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups (P, arrows). Sagittal view with nasal orientation to the left.

Scale bars: 100 μm in A, E, G.

Figure 3

Apicobasal polarity and intercellular junction assembly are maintained during OF closure in the ventral optic cup in *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos. A-D, G, H: E11.5; E-F: E11.0. A-B: Phalloidin labeling shows apical localization of F-Actin on retina and RPE surfaces in control ventral optic cup (A, arrow). Asterisk marks the overlying OF. The apical localization of F-actin is unaltered in *Nf2*$^{lox/lox}$*;Rx3-Cre* ventral optic cups (B; arrow). C-D: Localization of pMLC2 on the apical surface of the RPE layer appears normal in *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos (D; arrow). E-F: Apical distribution of the tight junction component ZO-1 is not affected in the *Nf2*$^{lox/lox}$*;Rx3-Cre* ventral optic cup (F; arrow). The cadherin associated protein α-catenin shows normal localization in the OF and apical space between retina and RPE in *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes (H, arrows). Sagittal view with nasal orientation to the left.

Scale bars: 20 μm in G, H.

Figure 4

Proliferation is upregulated in the RPE layer in the *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup. A-D: E11.5; E-J: E11.0. A-B: Phospho-histone H3 (pHH3) labeling of control (A) and *Nf2*$^{lox/lox}$*;Rx3-Cre* (B) detects ocular cells in G2/M phases of the cell cycle (red label). Dapi is used as a nuclear label (blue). Quantification shows that the number of phospho-histone H3-labeled cells is not changed in

the ventral optic cup in *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes, however (C), there is a small significant increase in the ventral-most area that harbors the OF (V2; D). E-J: EdU labeling and quantification. EdU-labeled cells in control (E; green label), with nuclear Dapi co-labeling (blue). In the RPE of the *Nf2*$^{lox/lox}$*;Rx3-Cre* ventral optic cup, the number of EdU-labeled cells is increased (F; arrowheads), in comparison to controls (E, arrowheads). Quantitative analysis of EdU incorporation reveals that the number of cells in S-phase is significantly upregulated specifically in the RPE but not in the retina (G, H). The increase in EdU-labeled cell number is significant for the ventral RPE, while the dorsal RPE shows no change (I). Significant stimulation of proliferation in the ventral RPE applies to all regions in the ventral optic cup, including the V2 region harboring the OF (J). Data are shown as means ± s.d., and p values are indicated above the horizontal lines in each graph (significant < 0.05). Statistical analysis was performed using unpaired, two-tailed Student T-test or 2-Way ANOVA with Sidak's multiple comparison. Images show sagittal view with nasal orientation to the left.

Scale bar: 100 µm in A.

Figure 5

Cell number in the RPE layer is increased in the *Nf2*$^{lox/lox}$*;Rx3-Cre* dorsal optic cup. A-B: MITF labeling reveals increased RPE layer thickness in *Nf2*$^{lox/lox}$*;Rx3-Cre* (B; arrowheads point to ectopic RPE cells located apically). C-D: Quantification indicates a significant increase in total cell number in the dorsal RPE in *Nf2*$^{lox/lox}$*;Rx3-Cre* (C; see Suppl. Fig.3C for subdivisions), while cell number is unchanged in the dorsal retina (D). Data are means ± s.d., Student's T-test was applied for statistical analysis, and p values are indicated on the horizontal lines in each graph.

F: Phalloidin-labeling reveals irregularities in localization in the dorsal RPE layer in *Nf2*$^{lox/lox}$*;Rx3-Cre* (arrowheads). H: In addition, mislocalization of pMLC2 is detectable in the dorsal RPE layer in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups (arrowheads). I-K: OTX2 labeling shows that supernumerary cells persist in the dorsal optic cup at E13.5 (K; arrowheads).

Scale bars: 50 μm in A, C.

Figure 6

Rx3-Cre-mediated disruption of *Nf2* results in defective alignment of OF margins during the closure process. A-D: F-actin localization in the OF at E11.0 (A, B) and at E11.5 (C, D) in control (A, C) and *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups (B, D). A: In E11.0 control embryos, the OF margins become tightly aligned, shaped with a convex surface toward the basal space (arrowheads). B: In the *Nf2*$^{lox/lox}$*;Rx3-Cre* OF, shape of the basal surface of the OF margins is convex, similar to controls, however, alignment of the margins is less advanced (B, arrowheads). At E11.5, the OF margins in controls are well aligned along most of the OF with a flattened basal surfaces (C, arrowheads). The RPE layer consists of cuboidal cells (bracket). D: In *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes, the OF margins align only partially (arrowheads), exhibit convex shape, and the cells in the RPE layer show increased height in the apicobasal axis, thereby appearing columnar (bracket). E: Quantification of aligned OF margins. Extent of attached margins was measured in the OF, as outlined in representative examples shown in C, D for aligned OF between arrowheads. In *Nf2*$^{lox/lox}$*;Rx3-Cre*, few margins show close alignment, which is often much reduced, compared to controls (lack of alignment defined = 0). Data are means ± s.d. and Student's T-test was applied for statistical analysis. F: Analysis of cellular height along the apicobasal axis of cells

lining the ventral OF margins (see brackets in C, D; Suppl. Fig. 3F) reveals a significantly increased cellular height in the temporal OF margin. Statistical analysis was performed using nested T-test, data are means (with minimal and maximal points), and p values are indicated on the horizontal lines in each graph. G: Proposed model summarizing our observations, for explanation, see text (left: control optic cup; right: *Nf2*$^{lox/lox}$*;Rx3-Cre*). The arrows point to potential effects of RPE hyperproliferation on the mechanodynamics of optic cup growth and apposition of OF margins. Sagittal view with nasal orientation to the left.

Scale bars: 50 µm in A, C.

Supplemental Figure 1

A) Cartoon of scheme for sagittal sections. B-C: Low magnification images of E13.5 bright field of control eye (B) and *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup (C). In *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups, pigmentation extends deep into the unfused OF (C; arrowhead) and the RPE appears darker (C; arrow; compare with B). D-E: Lower magnification of YAP1 immunolabeling in E11.5 control and in *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos. YAP1 expression shows increased nuclear localization in *Nf2*$^{lox/lox}$*;Rx3-Cre* lens, retina and RPE (E; arrowheads). Asterisks label the OF in D, E. Sagittal view with nasal orientation to the left.

Scale bars: 100 µm in B, C; 50 µm in D, E.

Supplemental Figure 2

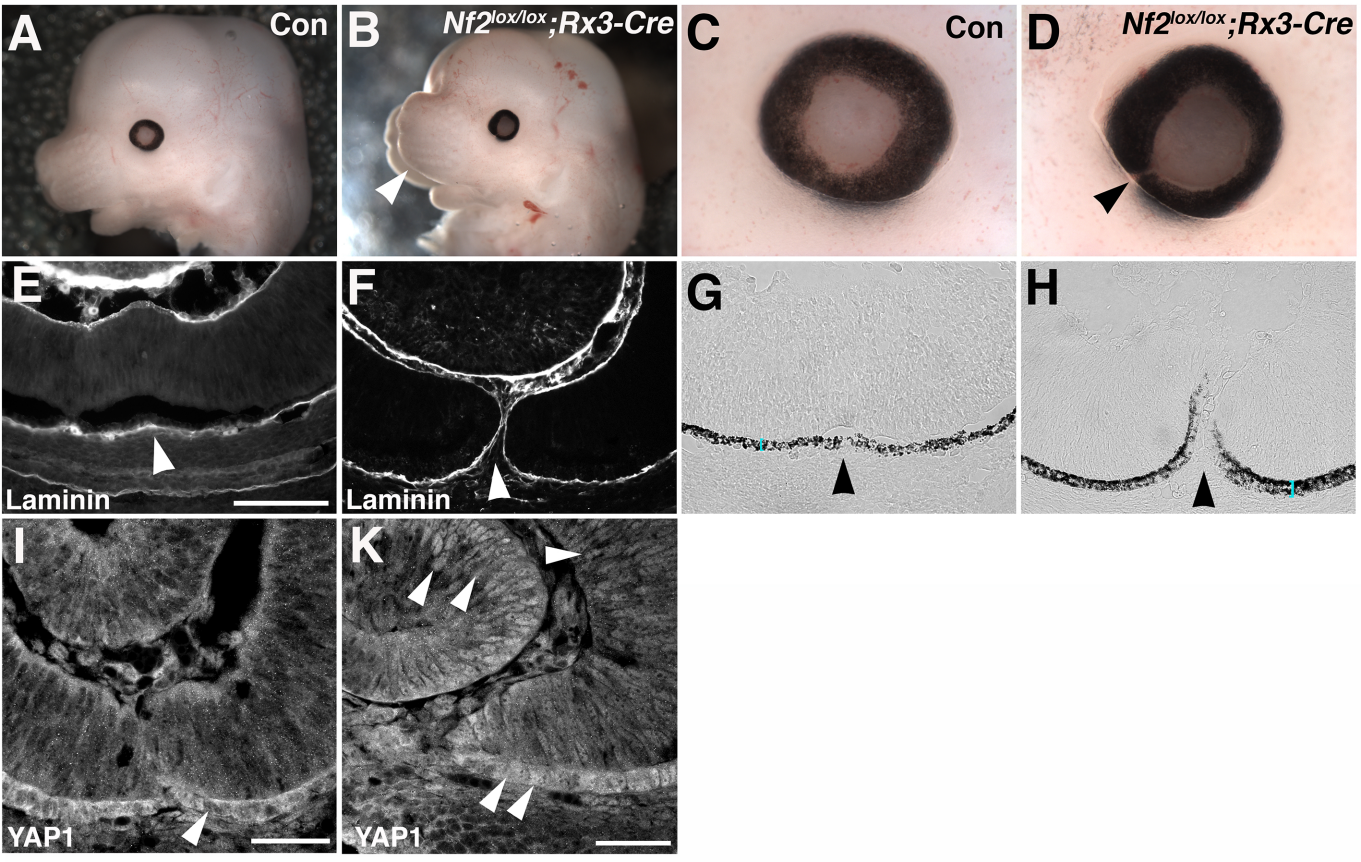
Investigation of MITF protein localization and *Mitf* function in *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes. A-B: At E11.0, MITF expression appears similar in the closing OF in control (A) and *Nf2*$^{lox/lox}$*;Rx3-Cre* eyes

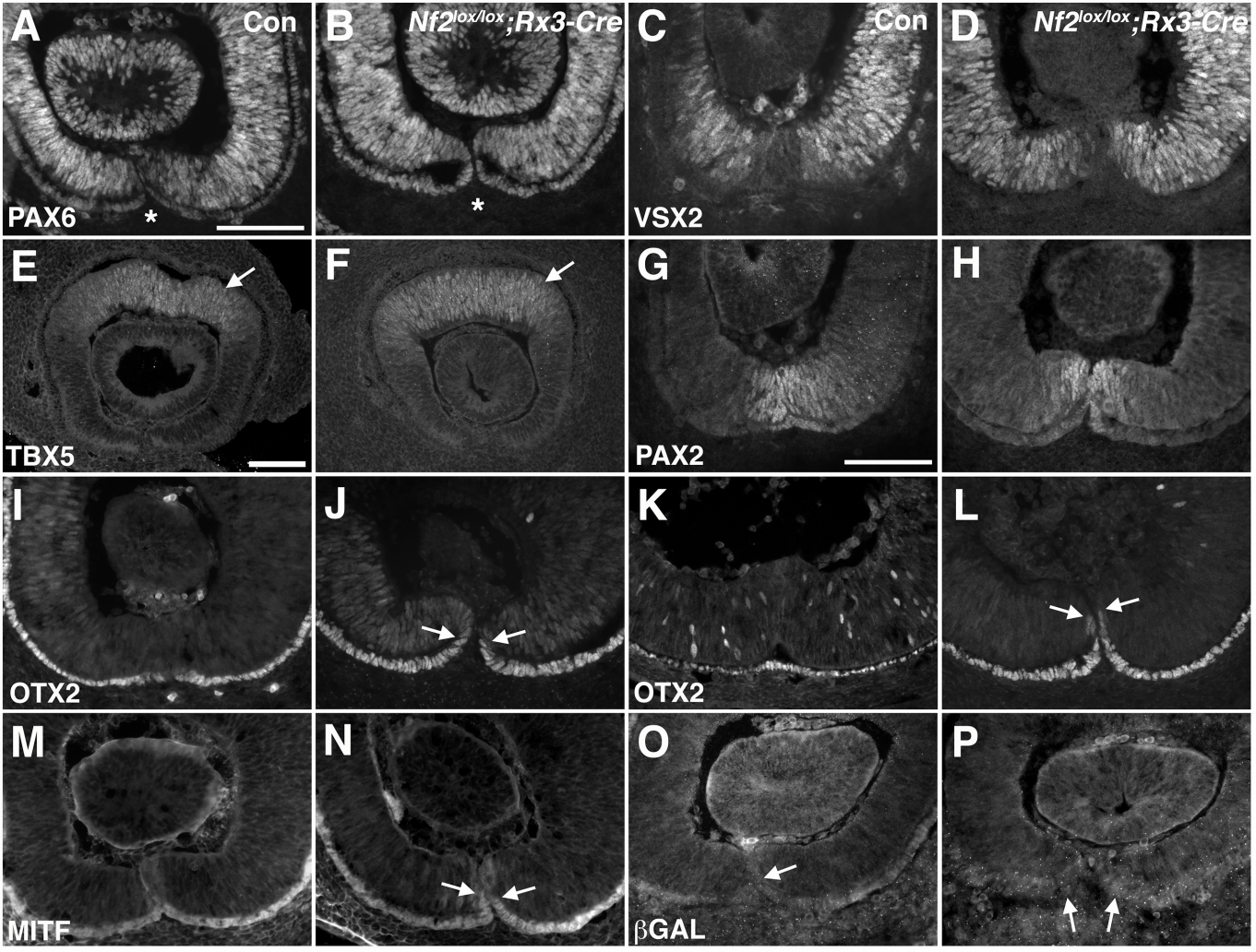
(B; arrowheads). Arrow in B points to MITF expression in the periphery. C-H: *Mitf* haploinsufficiency does not rescue coloboma in the *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup. C-D: In control eyes, removal of one *Mitf* allele results in reduction of pigmentation (D). G: Sagittal view of *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cup showing persistent laminin expression ventrally (also shown in Fig. 1F at higher magnification). In *Nf2*$^{lox/lox}$*;Rx3-Cre;Mitf*$^{Mi+/-}$ optic cups, OF closure is not prevented, confirmed by persistence of laminin expression in the ventral optic cup (H). Arrowheads (A, B, E, F) and asterisks (A, B, G, H) label the OF. Sagittal view with nasal orientation to the left. Scale bars: 50 µm in A, 200 µm in C, G.

Supplemental Figure 3

Analysis of apoptosis, eye diameter and height measurements of the RPE in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups. A-D: TUNEL-labeled cells in the OF in controls (A; arrows) and in *Nf2*$^{lox/lox}$*;Rx3-Cre* (B; arrows). Asterisks mark the overlying optic fissure. C: Scheme of subdivisions used for quantification in the optic cup. D: Quantification of TUNEL-labeled cells shows no significant change in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups. E: The total number in ventral regions was quantified using Dapi labeling (see scheme in C). V1+3 contains counts of the two ventrolateral compartments and shows a significant increase in total cell number in *Nf2*$^{lox/lox}$*;Rx3-Cre*. V1-3 shows cell number counts for all three ventral regions combined. The region harboring the OF (V2) can exhibit a clear gap in *Nf2*$^{lox/lox}$*;Rx3-Cre* optic cups, therefore, the cell number can be underestimated. However, the total cell number shows still a significant increase, when V2 is included. F: No significant difference of the eye diameter is detectable between controls and *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos. G: Example for measuring the apicobasal height of RPE cells in the

nasal and temporal OF margins during alignment. Measurements were performed using ImageJ. Asterisks mark the overlying optic fissure. H: Nested T-test analysis reveals no significant change in cellular height along in the apicobasal axis in cells lining the nasal OF margins in *Nf2*$^{lox/lox}$*;Rx3-Cre* embryos. Data are means (with minimal and maximal points). Statistical analysis for data shown in D and F was performed using Student T-test and in E using 2-Way ANOVA with Sidak's multiple comparison. Data are means ± s.d., and p values are indicated above the horizontal lines in each graph. Sagittal view with nasal orientation to the left. Scale bars: 100 µm in B, C.


A
Con
B
Nf2lox/lox;Rx3-Cre
C
Con
D
Nf2lox/lox;Rx3-Cre
E
Laminin
F
Laminin
G
H
I
YAP1
K
YAP1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Con
PAX6
*
B
Nf2lox/lox;Rx3-Cre
*
C
Con
VSX2
D
Nf2lox/lox;Rx3-Cre
E
TBX5
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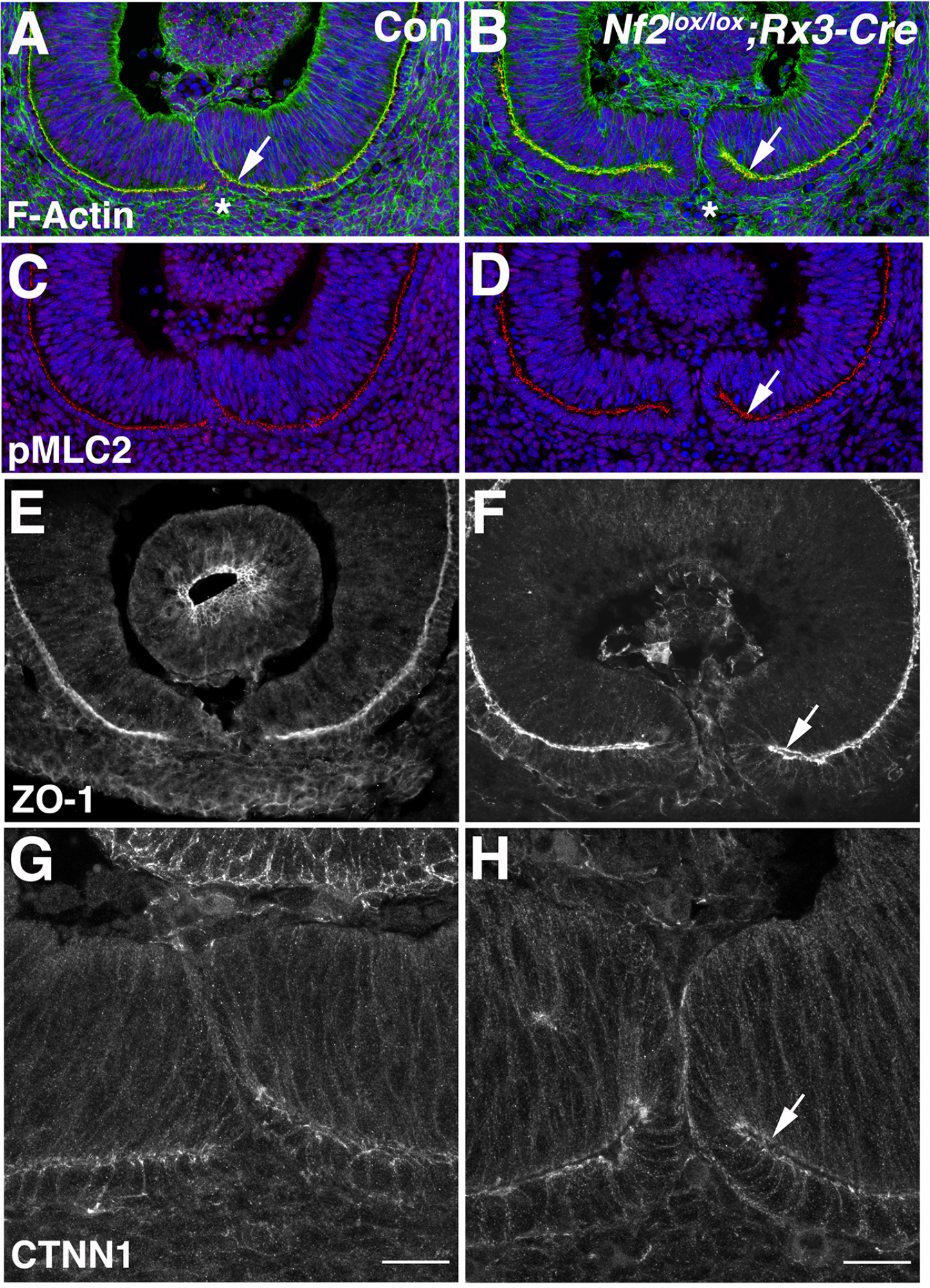
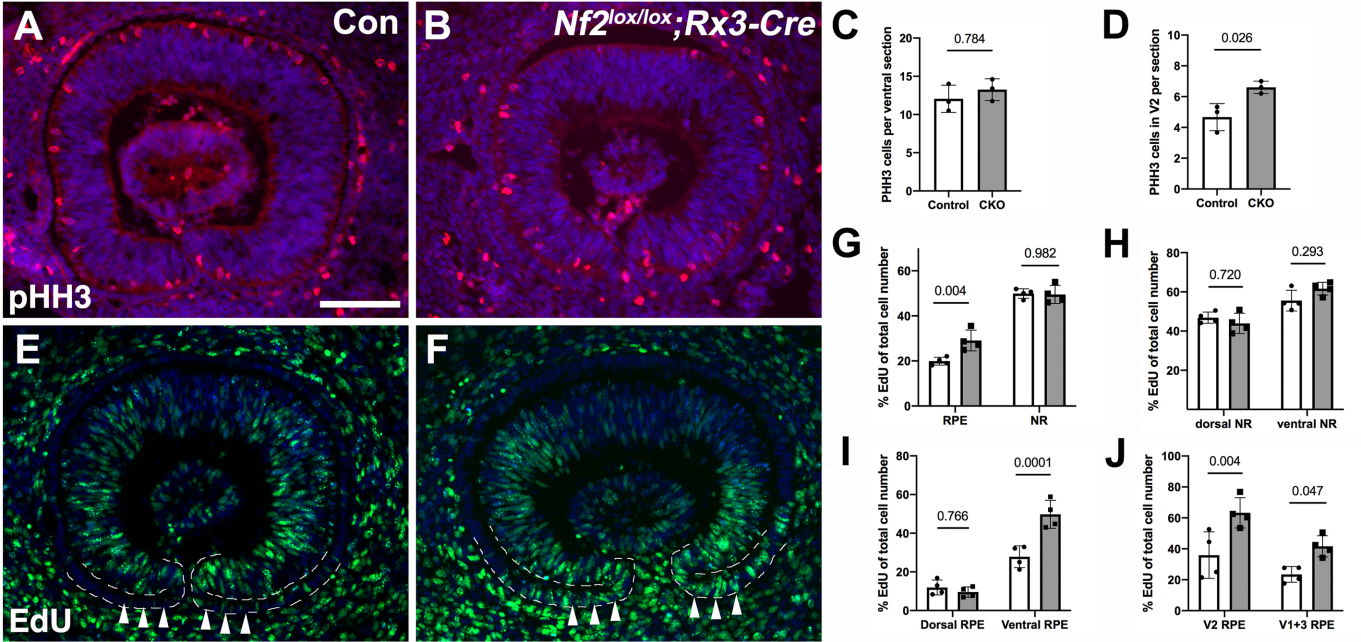
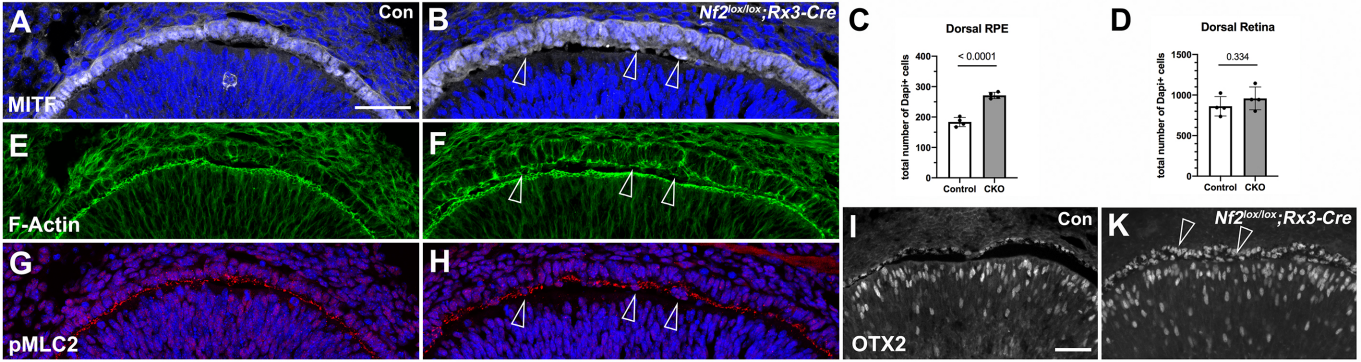
N
O
βGAL
P


A
Con
F-Actin
*
B
Nf2lox/lox;Rx3-Cre
*
C
pMLC2
D
E
ZO-1
F
G
CTNN1
H


A
Con
pHH3
B
Nf2lox/lox;Rx3-Cre
C
PHH3 cells per ventral section
0.784
Control
CKO
D
PHH3 cells in V2 per section
0.026
Control
CKO
E
EdU
F
G
% EdU of total cell number
0.004
0.982
RPE
NR
H
% EdU of total cell number
0.720
0.293
dorsal NR
ventral NR
I
% EdU of total cell number
0.766
0.0001
Dorsal RPE
Ventral RPE
J
% EdU of total cell number
0.004
0.047
V2 RPE
V1+3 RPE


A
Con
MITF
B
Nf2lox/lox;Rx3-Cre
C
Dorsal RPE
< 0.0001
total number of Dapi+ cells
400
300
200
100
0
Control
CKO
D
Dorsal Retina
0.334
total number of Dapi+ cells
1500
1000
500
0
Control
CKO
E
F-Actin
F
G
pMLC2
H
I
Con
OTX2
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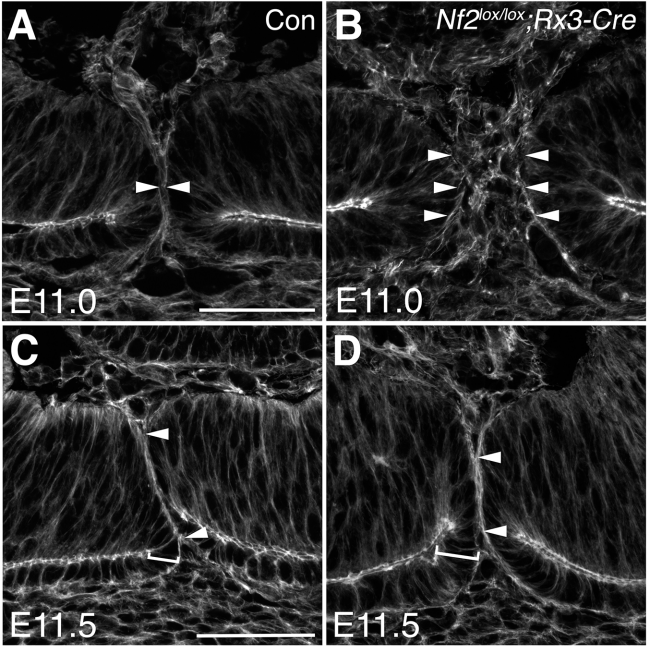
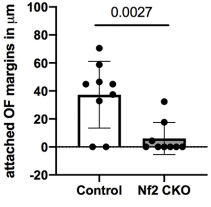
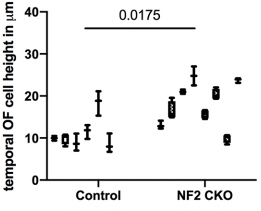
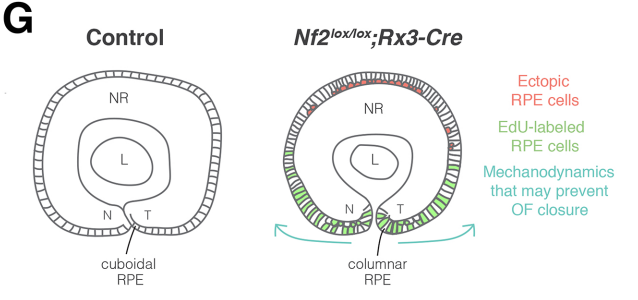
Nf2lox/lox;Rx3-Cre


A
Con
E11.0
B
Nf2lox/lox;Rx3-Cre
E11.0
C
E11.5
D
E11.5


E
0.0027
attached OF margins in µm
100
80
60
40
20
0
-20
Control
Nf2 CKO


F
0.0175
temporal OF cell height in µm
40
30
20
10
0
Control
NF2 CKO


G
Control
Nf2lox/lox;Rx3-Cre
NR
L
N
T
cuboidal RPE
NR
L
N
T
columnar RPE
Ectopic RPE cells
EdU-labeled RPE cells
Mechanodynamics that may prevent OF closure