Current Biology, Vol. 14, 711–717, April 20, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.cub.2004.04.006

Zebrafish Mosaic Eyes Is a Novel FERM Protein Required for Retinal Lamination and Retinal Pigmented Epithelial Tight Junction Formation

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Summary

Polarization is a common feature of many types of cells, and we are beginning to understand how cells become polarized. The role of cell polarity in development and tissue morphogenesis, however, is much less well understood. Our previous analysis of the *mosaic eyes* (*moe*) mutations revealed that *moe* is required for retinal lamination and also suggested that zebrafish *moe* function is required in the retinal pigmented epithelium (RPE) for the proper localization of adjacent retinal cell divisions at the apical neuroepithelial surface. To understand the function of *moe* in the RPE, we cloned the *moe* locus and show that it encodes a novel FERM (for 4.1 protein, ezrin, radixin, moesin) domain-containing protein. Expression of *moe* in the eye, kidney, and brain reflects phenotypes found in *moe*$^{-}$ mutants, including RPE and retinal lamination defects, edema, and small or absent brain ventricles. We show that *moe* function is required for tight junction formation in the RPE. We suggest that *moe* may be a necessary component of the *crumbs* pathway that regulates apical cell polarity and also may play a role in photoreceptor morphogenesis.

Results and Discussion

Previously, we showed that the *moe* gene is required for retinal lamination and RPE integrity; our experiments also suggested that *moe* function is required in the RPE for the correct apical localization of adjacent retinal cell divisions [1]. To understand the molecular basis of retinal and RPE defects in *moe*$^{-}$ mutants, we cloned the *moe* locus.

The *moe* locus consists of 25 coding exons (see Supplemental Data at http://www.current-biology.com/content/supplemental) that encode a novel FERM domain-containing protein. The predicted protein is 776 amino acids and approximately 87 kDa with the FERM domain at the amino terminus (Figure 1A). We designed primers for RT-PCR and PCR to examine this gene in wild-type (wt), *moe*^{b781}, and *moe*^{b882} embryos. The *moe*^{b781} allele contains a C to T transition (Figure 1B) that changes Gln142 to a stop codon, truncating the protein in the FERM domain (Figure 1A). RT-PCR fails to amplify product from *moe*^{b882}. Genomic sequence of *moe*^{b882} contains a T to A transition in the splice donor site of exon 5 (Figure 1B); this change predicts a transcript that retains intron 5 with a stop codon 84 base pairs into the intron, truncating the protein in the FERM domain with 27 missense amino acids at its carboxy terminus (Figure 1A; data not shown). The phenotype of the *moe*^{b781} allele is indistinguishable from the *moe*^{b476} deficiency. The *moe*^{b882} allele may be slightly less severe, the brain ventricles are not as reduced as in the *moe*^{b781} allele and the *moe*^{b476} deficiency, although the retina appears just as disorganized. It is possible that the *moe*^{b882} allele is hypomorphic or that it is a null and the phenotype is modified by the Tübingen strain background.

Injection of translation-blocking morpholino into one-to-two-cell stage wt embryos phenocopies *moe* mutations (Figure 1C). Nearly all injected embryos (68/71) display this phenotype, including tail curvature, small or absent brain ventricles, distended heart, pericardial edema, and patchy RPE (the remaining three embryos appeared wild-type, suggesting injection error).

C. elegans, *Drosophila*, and human genomes contain highly conserved *moe* homologs, whose functions are largely unknown. The human protein EPB41L5 on chromosome 2q14 has 61% overall identity with zebrafish Moe and 87% identity in the N′-terminal half. A second human protein, EPB41L4B, on 9q22 also shares strong identity in the N′-terminal half, as does *Drosophila*, CG9764 (flybase entry: Yurt [2]), and *C. elegans*, U80955. We provide alignment of the FERM domains of these proteins in the Supplemental Data (available on *Current Biology*'s website).

In zebrafish, the *moe* gene is adjacent to the *ptpn4* gene, which contains a FERM domain that is homologous to the FERM domain in Moe (see Supplemental Figure S1 on *Current Biology*'s website). In humans, this genomic arrangement is conserved, the *moe* homolog (EPB41l5 on 2q14) is next to *PTPN4* and in addition, the other *moe*-like gene (EPB41L4B on 9q31) is next to *PTPN3*. In *C. elegans*, both the *moe* homolog (U80955) and *ptp-1* are on chromosome III (III-5 and III-3, respectively), and in *D. melanogaster*, both the *moe* homolog (CG9764) and *ptpmeg* are on chromosome 3 (3R-87E11 and 3L-61B3, respectively). We performed phylogenic analysis of the FERM domains of these proteins along with the FERM domains of *D. melanogaster* and human MOESIN. The resulting phylogenic tree groups the Moe-like proteins together and the PTP-like proteins together, with the exception of *D. melanogaster* Ptpmeg (Figure 1D). The topology suggests that the *ptp*-like and *moe*-like genes derived from tandem duplication of a common ancestral gene and that chromosomal duplication events in the vertebrate lineage generated the additional copies of the *moe* and *ptpn* genes.

Zebrafish *moe* is expressed ubiquitously at early developmental stages (Figure 2A) but later expression is restricted and found in central nervous system (Figures 2B and 2H), ear (Figure 2E), kidney podocytes (Figure 2F), and eye (Figures 2C and 2G). Several cell types in the eye express *moe*, including photoreceptors, inner nuclear layer cells, retinal precursors in the marginal zone, and cells in the anterior chamber (Figure 2G). In 5 day postfertilization brain, cells in the white matter

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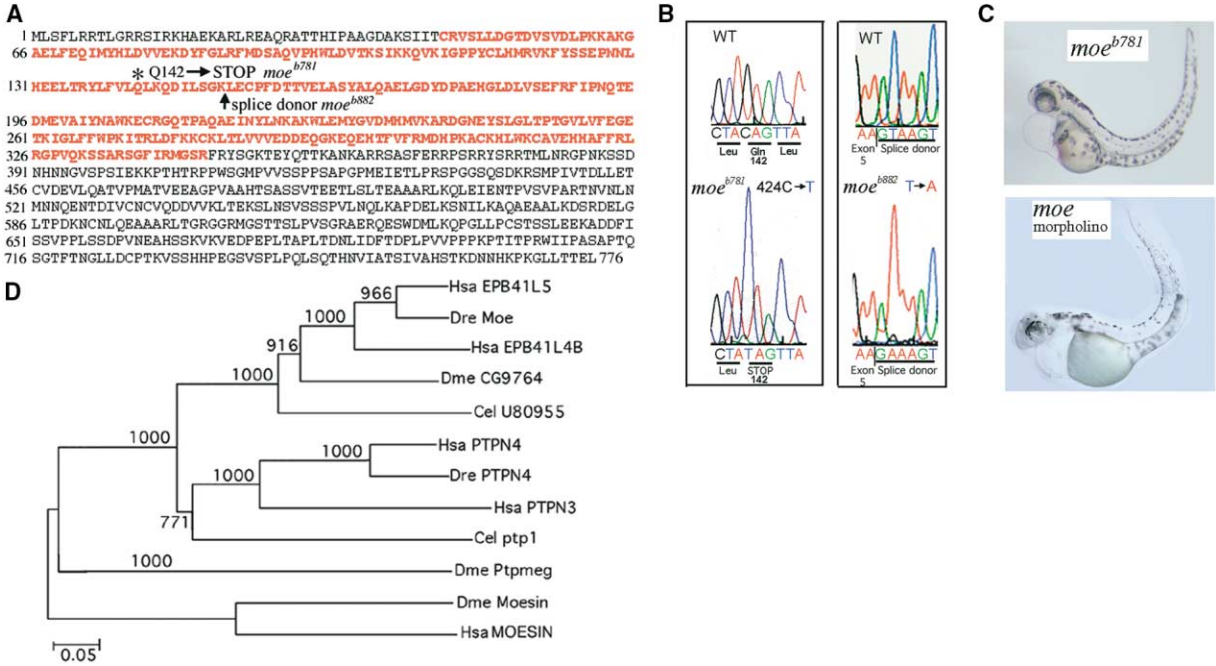

Figure 1. The *moe* Locus Encodes a FERM Domain-Containing Protein

(A) Moe contains a FERM domain (indicated in red) in the N′ terminus, mutation positions are indicated, moe^{b781} (*) and moe^{b882} (arrow).

(B) Sequence traces of point mutations. The moe^{b781} mutation is a C→T transition that changes glutamine142 (Q142) to a stop codon. The moe^{b882} mutation is a T→A transition in the splice donor site of exon 5.

(C) Injection of 1 ng of translation-blocking antisense morpholino oligonucleotides into one to two cell wt embryos phenocopies the *moe* mutations.

(D) Phylogeny of Moe-like and PTPN-like proteins in humans (Hsa), zebrafish (Dre), *D. melanogaster* (Dme), and *C. elegans* (Cel). The tree was calculated using the neighbor joining method in ClustalX software. Numbers at branch points are bootstrap values. The tree was constructed with 1000 replications giving bootstrap values representing the probability of each node in the tree. Branch lengths are proportional to distances between sequences. Human MOESIN and *Drosophila* moesin were used as outgroup proteins to root the tree.

and surrounding ventricles express *moe*; these may be ependymal, glial, and neural stem cells (Figure 2H).

Mutations in *moe* cause an apparent phenotype like that caused by mutations in the *nagie oko* (*nok*) gene [3]; both mutations cause curvature of the tail (in most moe^{-} mutants the tail turns up, in nok^{-} mutants the tail turns down), edema of the pericardium, patchy retinal pigmented epithelium, and brain ventricle defects (all ventricles are absent in nok^{m520} mutants, only the hindbrain ventricle is present, albeit very small, in moe^{-} mutants). We made double mutants of nok^{m520} and moe^{b476} (nok^{-};moe^{-}). We examined a clutch of 145 embryos resulting from paired crosses of $nok^{m520/+}$;$moe^{b476/+}$ individuals. We genotyped mutants at 48 hr postfertilization (hr) and identified 9 nok^{-};moe^{-} mutants, 23 moe^{-} mutants, and 34 nok^{-} putative mutants (see Supplemental Experimental Procedures on *Current Biology*'s website). The doubles are like single mutants: they exhibit edema of the pericardium and loss of brain ventricles; eight out of nine nok^{-};moe^{-} mutants had downturned tails and the other had a straight tail (data not shown). These results suggest *nok* and *moe* act in the same genetic pathway.

Nok is a zebrafish homolog of *Drosophila* Stardust [4, 5] and mammalian PALS1 [6], both of which interact physically with Crumbs proteins [4, 5, 7]. Crumbs proteins contain a FERM-protein binding motif necessary for Crumbs function [8] that is functionally conserved in vertebrates [9]. Moe is a good candidate for this FERM-protein that binds Crumbs. Because *stardust* [4, 5] and *crumbs* [10–12] are necessary for epithelial polarity and PALS1 and CRB3 localize to tight junctions and may function in tight junction assembly [13, 14], we examined whether *moe* is also necessary for epithelial polarity by characterizing tight junctions, a feature of established epithelial polarity [15].

We used antibodies to examine several components of tight junctions. In wt eyes, antibodies against Zonula Occludens-1 [16] (ZO-1; Figure 3A), cingulin [17] (Figure 3C), and occludin [18] (Figure 3E) label the apical surface of RPE at 3 days. In contrast, in moe^{-} RPE we observe little or no labeling with these antibodies (Figures 3B, 3D, and 3F), suggesting that tight junctions are absent.

We examined younger animals to learn whether moe^{-} mutants fail to form tight junctions or whether tight junctions form but then disappear. In wt RPE of 24 hr embryos, we observe little or no anti-cingulin labeling (data not shown); at 30 hr, labeling is apparent (Figure 4A) and increases later (Figures 4C and 4E). Even at early stages in moe^{-} RPE, we observe no significant labeling (Figures 4B, 4D, and 4F), although abnormal plaques of anti-cingulin in moe^{-} retinas are present (Figures 4D and 4F, arrows). In wt eyes, anti-cingulin labeling is concentrated at the apical RPE surface (Figure 4G) and in moe^{-}

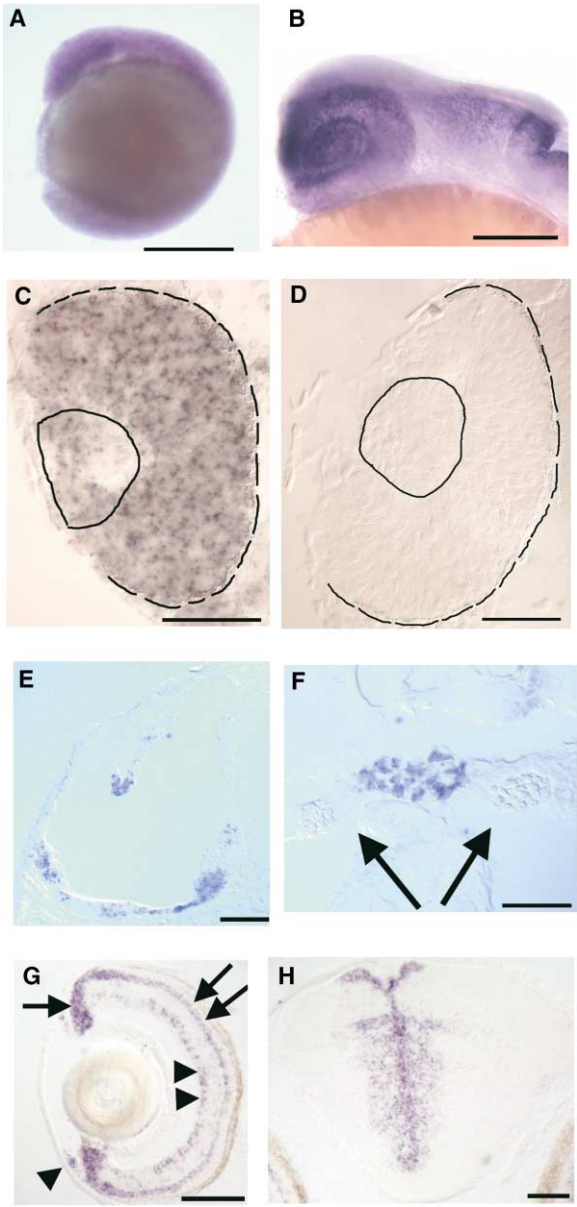

Figure 2. Expression of *moe* Accounts for Mutant Phenotypes

(A) At 13 somite stage, expression is ubiquitous. (B) At 24 hr, expression becomes restricted mainly to the CNS. (C) Section through wt eye at 30 hr showing expression in retinal epithelial cells, RPE, and lens. (D) Section through *moe*^{b476} mutant sibling at 30 hr (included on the same slide for in situ hybridization as [C]). (E) Ear expression at 5 days includes the cristae, maculae, and septum. (F) Kidney (podocyte) expression at 5 days; arrows indicate the pronephric tubules. (G) Several cell types in the eye express *moe* at 11 days, including photoreceptors (double arrows), inner nuclear layer cells (double arrowheads), progenitor cells in the marginal zone (arrow), and cells in the anterior chamber (arrowhead). (H) Expression in the white matter and ventricular area of the brain at 11 days. Scale bars, (A), 300 μm; (B), (G), and (H), 100 μm; (C)–(F), 50 μm. (C) and (D), lens is circled and the basal extent of the RPE is indicated by black dashed line. (A) and (B), side views, anterior to the left, dorsal up; (C)–(H) cross sections, dorsal up.

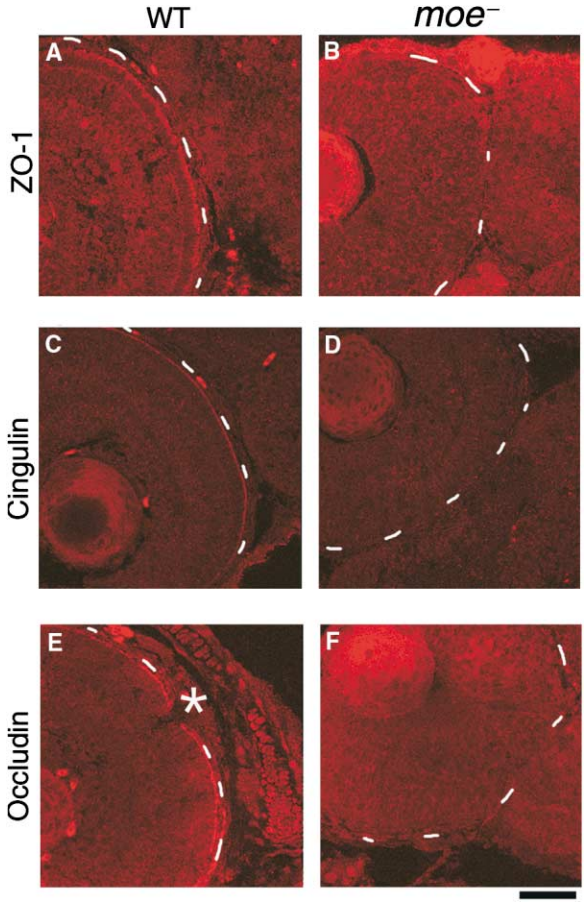

Figure 3. Tight Junctions Are Absent at 3 Days in *moe*$^{-}$ RPE

At 3 days in wt RPE, antibodies against ZO-1 (A), cingulin (C), and occludin (E) label the apical surface. In *moe*$^{-}$ (*moe*^{b476}) RPE, antibodies against ZO-1 (B), cingulin (D), and occludin (F) fail to label the apical surface. Confocal images of cryostat cross sections; dashed lines indicate the basal RPE, and "*" indicates optic nerve head. Scale bar, 50 μm.

mutants no such concentration of labeling is apparent (Figure 4H), although disorganized plaques of anti-cingulin labeling are present in other areas (Figure 4H, arrows).

We examined whether anti-cingulin labeling is also absent in *nok*$^{-}$ and *nok*$^{-}$;*moe*$^{-}$ double mutant RPE. No anti-cingulin labeling is apparent in *nok*$^{-}$ (Figure 5B) and *nok*$^{-}$;*moe*$^{-}$ RPE (Figure 5C), suggesting that *nok* function is also required for tight junction formation in RPE.

These results suggest that Moe is required for tight junction formation, either directly for assembly of the tight junction or upstream in apical cell polarity formation. Anti-cingulin, anti-occludin, and anti-ZO-1 antibodies presumably label RPE cells, although we cannot rule out the possibility that they also label the most apical region of retinal cells that adjoin the RPE. This seems unlikely for several reasons: (1) tight junction structures have not been reported in retinal cells, (2) our mosaic analysis suggests that *moe* function is required in the RPE [1], and (3) the timing of antibody labeling is difficult

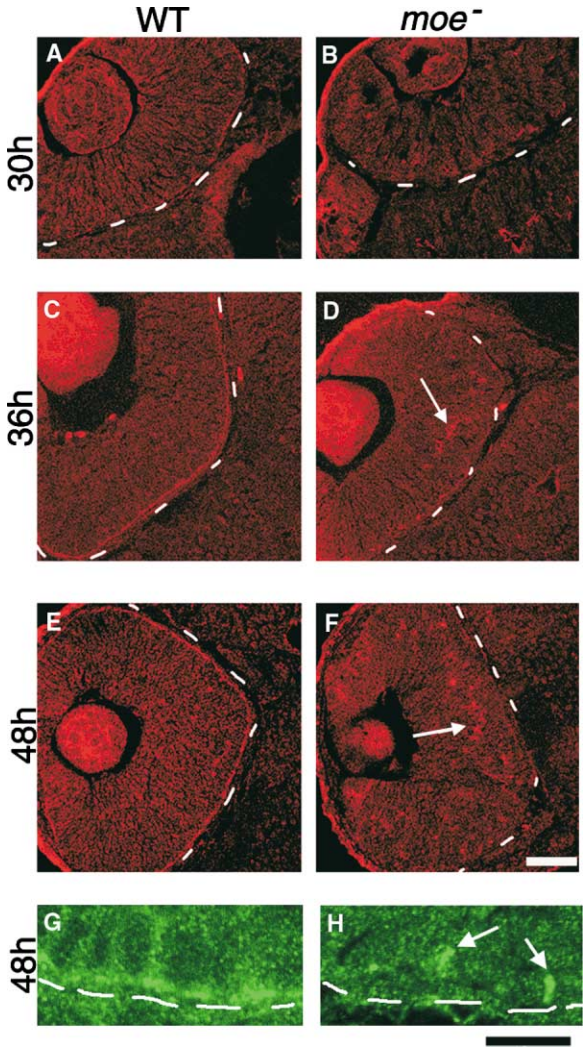

Figure 4. Tight Junctions Fail to Form in *moe*$^{-}$ RPE

Time course of tight junction development in RPE indicated by anti-cingulin antibody. In wt eyes, anti-cingulin becomes visible at around 30 hr (A), more uniform at 36 hr (C), and more intense at 48 hr (E). At all stages examined, 30 hr (B), 36 hr (D), and 48 hr (F), little or no anti-cingulin labeling is visible in *moe*$^{-}$ (*moe*^{b476}) RPE. Higher magnification views of the RPE region in wt (G) and *moe*^{b781} mutant (H) eyes. Ectopic plagues of anti-cingulin labeling are apparent in the *moe*$^{-}$ retina at 36 hr ([D], arrow) and 48 hr ([F] and [H], arrow). Confocal images of cryostat cross sections; dashed lines indicate the basal RPE. Scale bar: (A)–(F), 50 μm; (G) and (H), 10 μm.

to reconcile with the timing of cell differentiation occurring in the retina.

To examine further the possibility that *moe* acts in the same pathway as *nok*, we assayed the localization of Nok protein in *moe*$^{-}$ mutants. Anti-Nok antibody labels the ventricular surface of the brain and apical RPE in wt embryos at 48 hr (Figures 5G and 5I). In *moe*$^{-}$ mutants, we find labeling in the ventricular region in the brain, although it is less organized (Figure 5H) and Nok is less concentrated or absent in the apical RPE (Figures 5H and 5J). Ectopic Nok plaques are evident in the retina (Figure 5H, arrows), similar to the cingulin plaques observed in *moe*$^{-}$ mutants (Figure 4). These results suggest that proper localization of Nok requires *moe* function.

The predominant defects in *moe*$^{-}$ mutants are consistent with *moe* expression and potential function in tight junction formation. Edema suggests kidney dysfunction, small or absent brain ventricles suggest defects in ependyma, and early expression in RPE is consistent with its role in tight junction formation. Interestingly, *moe* expression in RPE around 24 hr corresponds well with our observation that between 24 and 30 hr Moe function becomes critical for tight junction formation (Figure 4) and for proper localization of adjacent retinal cell divisions [1].

We propose two models that are not mutually exclusive to explain how *moe* may function in the RPE to regulate cell divisions of adjacent retinal epithelial cells at the apical surface. (1) *moe* is required for tight junction formation. Loss of tight junctions in *moe* mutants leads to loss of RPE barrier function. This could result, for example, in diffusion of factors into the retina from the overlying mesenchyme that disrupt retinal cell divisions. (2) *moe* is required for RPE polarity, upstream of tight junction formation. Loss of RPE polarity in *moe* mutants could result in the mislocalization of factors normally targeted to the apical surface of the RPE that influence retinal cell divisions. Recent work has shown that the anterior rim of the retina and RPE have retinal organizing activity [19]. The anterior rim activity but not the RPE activity can be blocked by soluble Frizzled 5 receptor. The anterior rim expresses Wnt2b, and exogeneously applied Wnt2b has retinal organizing activity. Wnt1 and Wnt10b localize to the apical surface of the RPE [20] and, thus, are candidates for the RPE retinal organizing activity.

Moe is a new member of the family of FERM domain proteins, some members of which serve as linkers between cytoskeleton and transmembrane proteins [21]. We hypothesize that *moe* may be a key component of the *crumbs* pathway that contributes to apical cell polarity and that Moe may link the cytoskeleton with the transmembrane protein Crumbs. An alternative hypothesis is that *moe* and *crumbs* (with *nok*) act in parallel pathways necessary for tight junction formation. Mutations in human *CRB1* are associated with retinal disorders, retinitis pigmentosa 12 (RP12) [22] and Leber's congenital amaurosis (LCA1) [23–25]. Expression of *moe* in retina is similar to mammalian *Crb1* [26]; both are expressed in photoreceptors and inner nuclear layer cells. In *Drosophila*, Crumbs plays an important role in photoreceptor morphogenesis [27, 28]. The early onset of RP12 and LCA associated with CRB1 mutations suggests that it may also play a role in photoreceptor morphogenesis, and it is possible that Moe may cooperate with CRB1 in this process.

Little is known about the function of the *moe* homolog *yurt* in *Drosophila* and whether it interacts in the *crumbs* pathway. The phenotype of *yurt* mutants has been described, and mutants fail to complete germ band retraction, dorsal closure, and head involution [2]. Weak *crb* alleles also fail to complete germ band retraction and dorsal closure [29], and the first defects observed in strong *crb* alleles are irregularities in the arrangement of adherens junction material in the amnioserosa [30]. It is becoming increasingly clear that forces from the amnioserosa initially drive dorsal closure [31]. Yurt pro-

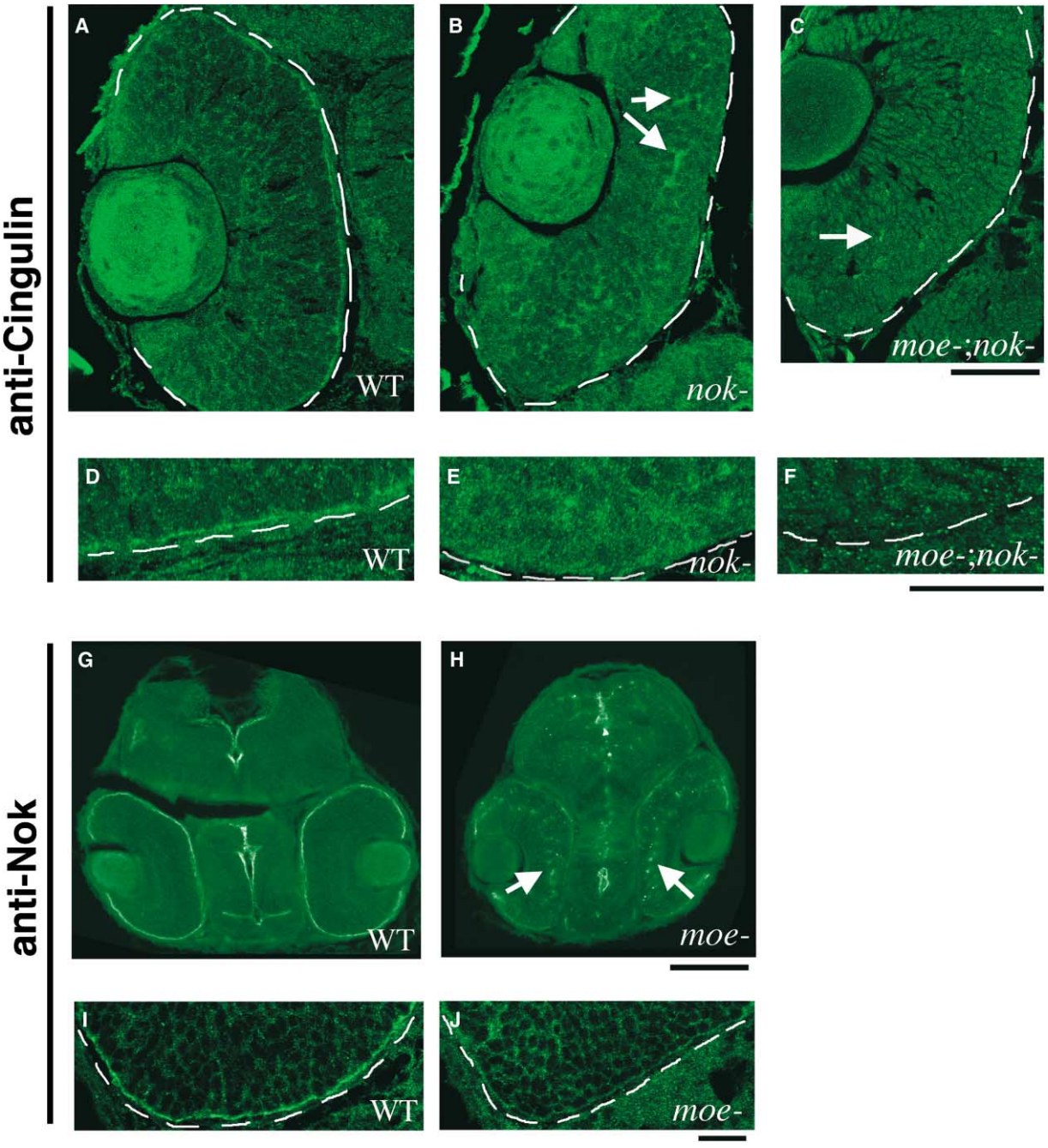

Figure 5. Cingulin Is Absent in Apical RPE of *nok*$^{-}$ Mutants and *moe*$^{-}$;*nok*$^{-}$ Double Mutants

Cingulin labeling in wt (A and D), *nok*$^{-}$ mutant (B and E), and *moe*^{b476};*nok*$^{-}$ double mutants (C and F) eyes. (A and D) In wt eyes, anti-cingulin labels the apical RPE. In *nok*$^{-}$ mutant (B and E) and *moe*^{b476};*nok*$^{-}$ double mutant (C and F) eyes, no anti-cingulin labeling is found in the RPE region. At 48 hr in wt embryos Nok localizes to the brain ventricular surface (G) and the RPE (I). In *moe*$^{-}$ (*moe*^{b781}) mutants (H) Nok appears to localize to the brain ventricular surface, although it is more punctate, localization to the RPE is reduced, and ectopic plaques (arrows) form in the retina (H). Nok localizes to the apical RPE in wt eyes (I), and this apical localization is largely lost in *moe*$^{-}$ mutants (J). Confocal images of cryostat cross sections (A–F, I, and J); dashed lines indicate the basal RPE. Scale bars: (A)–(F), (I), and (J), 50 μm; (G) and (H), 100 μm.

tein overlaps with adherens junctions in the amniose-rosa and is localized to the apical and lateral domains of the epithelial plasma membrane [2]. Crumbs also localizes to the apicolateral and apical domains of the epithelial plasma membrane [12], but it is still unknown whether Yurt and Crb colocalize.

Recently, the first glimpse into the retina of patients with *CRB1* mutations and LCA1 has revealed lamination defects [32] that seem remarkably similar to the retinal phenotypes observed in *moe* [1] and *nok*$^{-}$ [3] zebrafish mutants. Other abnormalities associated with *moe* mutations [1] are not found in humans with *CRB1* [22–25]

mutations. This difference may indicate that Moe interacts with additional members of the Crumbs family. Another possibility is that Moe interacts with Discs large (Dlg) proteins that also contain binding motifs for unknown FERM proteins [33] and contribute to a genetic hierarchy that interacts with *crumbs* in *Drosophila* [34, 35].

The identification of the *moe* locus is added to the growing list of genes that are required for retinal lamination in zebrafish, including *heart and soul*, which encodes an atypical PKC [36]; *parachute* and *glass onion*, which encode N-cadherin [37–39]; and *nok* [3]. It will be interesting to determine whether these genes act in the same or parellel pathways required for normal retinal development.

Supplemental Data

Data including Experimental Procedures and two figures that describe the positional cloning of the *moe* locus and alignments of the N′-terminal regions of human, *Drosophila*, *C. elegans*, and zebrafish Moe homologous proteins are available at http://www.current-biology.com/content/supplemental.

Acknowledgments

We thank Kiyoshi Kikuchi for isolating the moe^{b882} allele; John Willoughby for help with SSCP analysis; Jeremy Wagner for technical help; Jocelyn McAuley and the University of Oregon fish facility staff for fish care; Sandra Citi for tight junction antibodies; Xiangyun Wei for anti-Nok antibody; Wolfgang Driever for the nok^{m520} fish; Chris Amemiya for PAC library; Angel Amores for assistance in PAC screening; the Sanger Institute for PAC sequencing; and Ulli Tepass, John Postlethwait, Judith Eisen, and Chris Doe for helpful comments on the manuscript. We are grateful to John Postlethwait for assistance with phylogenic analysis. Supported by a NRSA fellowship (EY06794-01) to A.M.J., NIH (HD22486, DC04186), and the W.M. Keck and M.J. Murdoch Foundations.

Received: September 9, 2003
Revised: February 27, 2004
Accepted: March 1, 2004
Published: April 20, 2004

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Accession Numbers

Accession numbers for PACs are AL672097 (PAC P37o5), AL713996 (PAC P201c3), and AL672129 (PAC P104f18); the accession number for *moe* is AY590884 and for *ptpn4* is AY590885.