Atypical Cadherin Fat1 Is Required for Lens Epithelial Cell Polarity and Proliferation but Not for Fiber Differentiation

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Purpose. The Fat family of atypical cadherins, originally identified in *Drosophila*, play diverse roles during embryogenesis and adult tissue maintenance. Among four mammalian members, Fat1 is essential for kidney and muscle organization, and is also essential for eye development; Fat1 knockout causes partial penetrant microphthalmia or anophthalmia. To account for the partial penetrance of the Fat1 phenotype, involvement of Fat4 in eye development was assessed. Lens phenotypes in Fat1 and 4 knockouts were also examined.

Methods. Fat1 and Fat4 mRNA expression was examined by in situ hybridization. Knockout phenotypes of Fat1 and Fat4 were analyzed by hematoxylin and eosin (H&E) and immunofluorescent staining.

Results. We found Fat4 knockout did not affect eye induction or enhance severity of Fat1 eye defects. Although Fat1 and Fat4 mRNAs are similarly expressed in the lens epithelial cells, only Fat1 knockout caused a fully penetrant lens epithelial cell defect, which was apparent at embryonic day 14.5 (E14.5). The columnar structure of the lens epithelial cells was disrupted and in some regions cell aggregates were formed. In these multilayered regions, apical cell junctions were fragmented and the apical-basal polarity was lost. EdU incorporation assay also showed enhanced proliferation in the lens epithelial cells. Interestingly, these defects were found mainly in the central zone of the epithelial layer. The lens epithelial cells of the germinative zone maintained their normal morphology and fiber differentiation occurred normally at the equator.

Conclusions. These observations indicate that Fat1 is essential for lens epithelial cell polarity and proliferation but not for terminal differentiation.

Keywords: lens, Fat1, Fat4

The lens, as part of the eye's dioptric apparatus, plays an important role in transmitting and focusing light onto the photosensitive cells of the retina. It is comprised of an epithelial layer that covers the anterior surface of a regularly packed, spheroidal mass of transparent fiber cells. While the thin epithelial layer does not contribute much to the optical function of the lens, it is essential for lens growth and maintenance.[1] The epithelial layer provides the progenitor cells for the generation of new lens fibers. Epithelial cells located at the lens equator exit the cell cycle and those cells that shift below the equator differentiate into highly elongated fiber cells that are progressively added to the fiber mass throughout life. The epithelial cells are also important for the homeostasis of the avascular lens fibers and associate intimately with the fiber cell apical surface, thus facilitating communication and interaction between the two forms of lens cells.[2,3] A disturbance in the integrity of the epithelial sheet usually leads to cataract; for example, conditions that induce an epithelial-mesenchymal transition result in the formation of opaque fibrotic plaques and subcapsular cataract.[1]

The Fat family of proteins are atypical cadherins that first came to prominence because of their role in suppressing growth in *Drosophila*.[4] Later studies in *Drosophila* also showed that Fat, together with the cadherin Dachsous, has a key role in regulating planar cell polarity (PCP[5,6]). In vertebrates there are four *Fat* genes, *Fat1–4*, that appear to have some tissue specific and redundant roles in development.[7] Analysis of Fat4 knockout (KO) mice has identified a key role for this Fat family member in regulating vertebrate PCP.[8] Initial analysis of Fat1 KO mice showed that homozygous mutants died within 48 hours of birth with kidney formation failure and later studies identified an essential role for Fat1 in muscle shape organization.[9,10] Although in the initial study no defects associated with tumor suppressive function were observed, several mutations in the *Fat1* gene have been identified in human cancers and a role in proliferation control has been reported recently.[11]

Fat1 KO also causes microphthalmia and anophthalmia in mice (small or no eye formation defects, respectively) and based on the presence of large numbers of apoptotic cells in the abnormal eyes it was concluded that degeneration or apoptosis may cause the abnormal eye phenotype.[9] The eye phenotype was partially penetrant, suggesting compensation of the defect by other member(s) of the Fat family. Fat2 KO mice appear to have no apparent ocular defects and the eyes of Fat3 KO mice appear mostly normal except for some changes in the morphology of amacrine cells in the retina.[12,13] The Fat4 KO mouse dies at birth and exhibits various developmental defects associated with PCP, but so far an eye phenotype has been not described. Given this background on the Fat family, and

because we had previously shown PCP to operate in the lens, we set out to determine if Fat1 and/or Fat4 have a role in lens development.

Materials and Methods

Mice

The use of animals in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Generation of Fat1 and Fat4 KO mice were described previously.[7,8] Double KO embryos were generated by crosses between Fat1;Fat4 double heterozygous parents.[7] Wild-type C57BL6 mice were used for in situ hybridization.

In Situ Hybridization

Embryos aged between embryonic day 9.5 (E9.5) and E18.5 were obtained by setting up timed matings. Dissected embryos were processed for in situ hybridization as described previously.[14] Polymerase chain reaction primers used to generate DNA templates for riboprobe synthesis are shown in Supplementary Table S1. Two probes that were generated from different regions of the gene showed similar staining patterns, confirming specificity of the probes to target genes.

Measurement of Eye Area

Serial sagittal sections of lenses were hematoxylin and eosin (H&E)–stained and the eye diameter measured. The sections with the maximum diameter were selected as representing the central region of the eyes. The surface of the eye cavity and underlying inner surface of the cornea was outlined manually to measure the eye area with the Analyze tool of ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Immunofluorescent Microscopy

Paraffin sections were used for immunofluorescent staining as described before.[15] The following primary antibodies were used in this study: rabbit antibodies against β-catenin (H102, sc7199; Santa Cruz Biotechnology, Dallas, TX, USA), Aquaporin 0/MIP26 (#AQP01-A; Alpha Diagnostic, San Antonio, TX, USA), β-crystallin,[16] p57/Kip2 (H-91, sc-8298; Santa Cruz Biotechnology), ZO-1 (33-9100; Zymed, San Francisco, CA, USA), Fat1 (35B; gift from Thorne Lab[17]); mouse antibodies against β-catenin (clone 14, 610154; Transduction Laboratories, Franklin Lakes, NJ, USA), E-cadherin (clone 36, 610182; Transduction Laboratories), α-smooth muscle actin (α-SMA; M085129; DAKO, Glostrup, Copenhagen, Denmark); and goat antibodies against nuclear Lamin B (M20, sc-6217; Santa Cruz Biotechnology) and Scribble (C20, sc-11049; Santa Cruz Biotechnology). Incorporated EdU was detected by Click-iT EdU Imaging Kits (C10337; Molecular Probes, Eugene, OR, USA).

Results

Fat1 and Fat4 Are Similarly Expressed in Lens Epithelial Cells During Morphogenesis

We first examined the transcriptional patterns of Fat1 and Fat4 during eye development (Fig. 1). No distinct expression of either *Fat* gene was detected in the lens placode or optic cup at E9.5 (Figs. 1A, 1B). The first signal for Fat4 was detected in lens pit cells at E10.5 (Fig. 1D, arrow) while Fat1 was not detected at this stage (Fig. 1C). The lens vesicle has formed by E11.5 and a transient signal in the posterior cells of the vesicle was detected with Fat1 probes (Fig. 1E, arrow) but the expression of Fat4 was confined to the anterior cells of the vesicle (Fig. 1F, arrow). By E12.5, cells in the posterior part of the vesicle have begun to elongate into primary fibers and both Fat1 and Fat4 were excluded from the primary fibers but localized to the anterior cells of the vesicle that differentiate into the lens epithelium (Figs. 1G, 1H). From this stage on, little or no Fat1 or Fat4 expression was detected in the lens fibers and prominent expression was restricted to the lens epithelial cells (Figs. 1I–L). Fat1/4 expression was also detected to varying degrees in mesenchymal cells in the vicinity of the developing eye (Figs. 1E–H, asterisks). Expression of both *Fat* genes was similarly detected in the optic cup at E12.5 (Figs. 1G, 1H, arrowheads). At later stages the expression of both Fat1 and Fat4 was detected at the distal margin of the optic cup that forms the ciliary body and iris (Figs. 1I–L, large arrowheads). In neural retina Fat1 was detected in the outer layers (Figs. 1I, 1K', small arrowheads), while Fat4 expression was detected in the inner layers (Fig. 1J, small arrowheads). By E18.5 Fat4 expression formed a distinct lamina in the inner layer (Fig. 1L', arrowheads).

Fat1, But Not Fat4, Has a Major Effect on Eye Formation

Similar to previous reports,[9] gross morphologic examination of heads of Fat mice consistently showed that eyes were small in Fat1 KOs, whereas the eyes appeared similar to those in wild types if one allele of Fat1 was present (Figs. 2A–C). Note that the small eye defect was observed on a Fat4 wild-type background (Fig. 2C). Expression of Fat4 mRNA from early stages of eye morphogenesis was suggestive of a role for Fat4 during this process; however, we found that Fat4 KO had minimal, if any, influence on eye formation. The size of eyes in Fat4 KO embryos was equivalent to that of controls (Figs. 2D, 2E). We also looked for synergetic effects between Fat1 and Fat4 on eye formation; depletion of one allele of Fat1 on Fat4 KO background did not result in small eye formation (Fig. 2F). Occasionally Fat1 KO embryos had no eyes in the absence of one allele of Fat4 (Fig. 2G). This observation indicated that an enhancement of the Fat1 phenotype could be expected in the absence of both Fat4 alleles (i.e., in the Fat1;Fat4 double KO [DKO]). However, these DKO embryos did form eyes (Fig. 2H), suggesting the anophthalmia observed in $Fat1^{-/-}$;$Fat4^{+/-}$; embryos was not a result of synergetic effect of Fat4 depletion. Rather it appears the small/no eye defect was primarily associated with the loss of Fat1 and the variation of the defect was a reflection of partial penetrance of the Fat1 phenotype.

Eye size was quantified by measuring the eye area in sections through the center of eyes from each group (Figs. 2, 3). Based on the observation that heterozygous depletion of Fat1 and Fat4 as well as homozygous depletion of Fat4 did not affect eye formation, combinations of Fat1 and Fat4 genotypes were divided into groups as follows: (1) genotypes of $Fat1^{+/+}$; $Fat4^{+/+}$, $Fat1^{+/+}$;$Fat4^{+/-}$, $Fat1^{+/-}$;$Fat4^{+/+}$, and $Fat1^{+/-}$; $Fat4^{+/-}$ were grouped as controls, (2) $Fat1^{+/+}$;$Fat4^{-/-}$ and $Fat1^{+/-}$;$Fat4^{-/-}$ were placed in the Fat4 KO group, and (3) $Fat1^{-/-}$;$Fat4^{+/+}$, $Fat1^{-/-}$;$Fat4^{+/-}$, and $Fat1^{-/-}$;$Fat4^{-/-}$ were placed in Fat1 KO group (Fig. 3). The quantification data shows that although the eye sizes of Fat4 KO embryos tended to be marginally smaller than the sizes of controls (in fact, in E13.5 litter 1 [Fig. 3A], they were statistically smaller than controls; control $n = 8$, Fat4 KO $n = 10$, $P < 0.05$), this slight difference did not reach statistical significance in E13.5 litter 2 and E14.5 samples (Figs. 3B, 3C; E13.5 litter 2 control $n = 6$,

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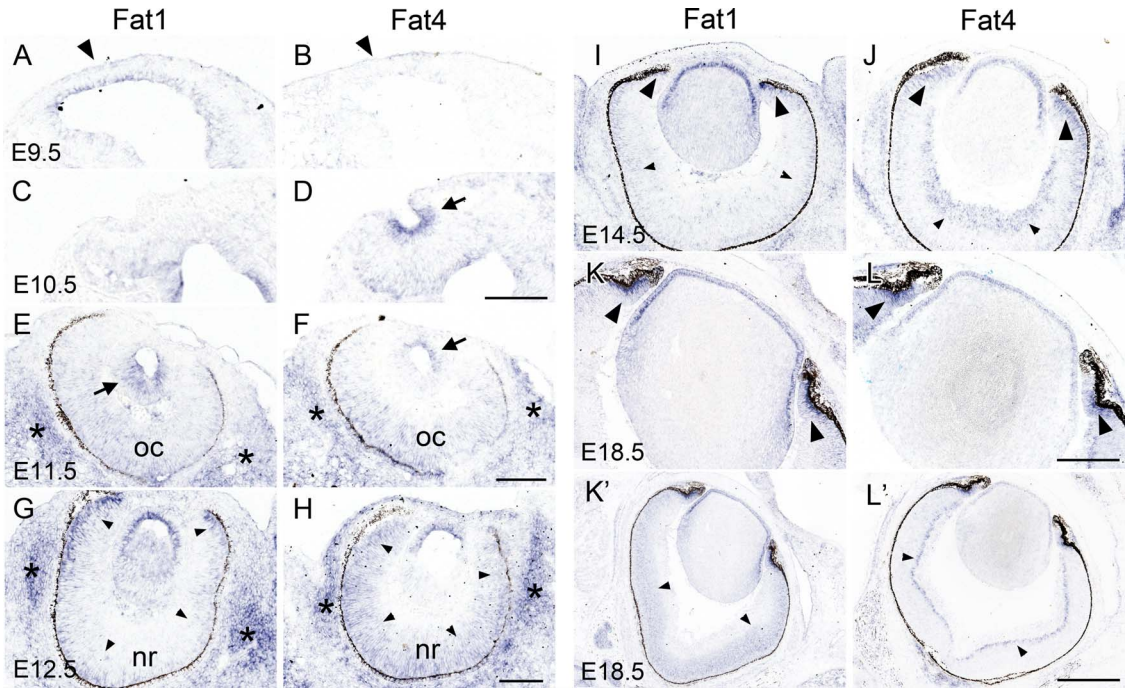

Figure 1. Expression of Fat1 and Fat4 during lens development. Cryosections of mouse embryos were processed for in situ hybridization with probes for Fat1 (**A**, **C**, **E**, **G**, **I**, **K**, **K'**) and Fat4 (**B**, **D**, **F**, **H**, **J**, **L**, **L'**) at E9.5 (**A**, **B**), E10.5 (**C**, **D**), E11.5 (**E**, **F**), E12.5 (**G**, **H**), E14.5 (**I**, **J**), and E18.5 (**K**, **K'**, **L**, **L'**). Both Fat1 and Fat4 were undetectable in the surface ectoderm of the presumptive lens region (*arrowheads*) at E9.5 (**A**, **B**). The first signal for Fat4 was detected in lens pit cells at E10.5 (*arrow*, [**D**]) while Fat1 was not detected at this stage (**C**). Fat1 was first detected in the posterior cells of the lens vesicle at E11.5 (*arrow*, [**E**]), while Fat4 signal was found in the anterior cells of the lens vesicle at this stage (*arrow*, [**F**]). By E12.5, Fat1 and Fat4 showed a similar expression pattern in the anterior cells of the lens vesicle that differentiate into the lens epithelial layer (**G**–**L**). Mesenchymal cells surrounding the optic cup (oc) also showed strong expression of Fat1 and Fat4 at E11.5 and E12.5 (*asterisks*, [**E**–**H**]). In the oc at E12.5, both Fat1 and Fat4 were detected in the outer layers of the developing neural retina (nr, *arrowheads*, [**G**, **H**]). After this stage Fat1 was restricted to the outer layers (*small arrowheads*, [**I**, **K'**]), whereas Fat4 was concentrated to the inner layer (*small arrowheads*, [**J**]). Fat4 formed a distinct lamina of expression on the inner side of the developing neural retina at E18.5 (*arrowheads*, [**L'**]). Both Fat1 and Fat4 were detected at the periphery of the retinal cup where the ciliary body and iris differentiate (*large arrowheads*, [**I**–**L**]). *Scale bars*: (**A**–**D**) 100 μm, (**E**, **F**) 100 μm, (**G**, **H**) 100 μm, (**I**–**L**) 200 μm, (**K'**, **L'**) 400 μm.

Fat4 KO $n = 8$, E14.5 control $n = 12$, Fat4 KO $n = 4$) by standard *t*-test analysis ($P > 0.05$). In contrast, the sizes of Fat1 KO eyes were significantly smaller than the eye sizes of controls in E13.5 litter 1 and E14.5 samples (Figs. 3A, 3C; E13.5 litter 1 control $n = 8$, Fat1 KO $n = 5$ [sixth sample showed anophthalmia], E14.5 control $n = 12$, Fat1 KO $n = 3$ [fourth sample showed anophthalmia]). Statistical analysis was not applicable for E13.5 litter 2 (Fig. 3B) because of the small number of Fat1 KO samples ($n = 2$, although they also appeared smaller than controls). These results indicate that although the mRNA expression pattern is similar for Fat1 and Fat4, only Fat1 is essential for eye formation and Fat4 has only a minimal influence on eye size regulation.

Fat1 Depletion Disrupts Columnar Morphology of Lens Epithelial Cells but Does Not Affect Fiber Differentiation

We also found Fat4 depletion did not affect lens formation (data not shown), while Fat1 knockouts consistently exhibited morphologic defects in the lens epithelium. Localization of β-catenin serves to delineate the cell margins and clearly showed that, instead of the regular packing characteristic of columnar epithelial cells in wild-type lenses (Figs. 4A, 4A'), the epithelial layer in Fat1 KO ($Fat1^{-/-}$;$Fat4^{+/+}$, $Fat1^{-/-}$;$Fat4^{+/-}$, and $Fat1^{-/-}$;$Fat4^{-/-}$) lenses was consistently thinner and the cells did not develop or maintain a columnar morphology and consequently packed irregularly (Figs. 4B, 4B'). This defect became obvious at E14.5 and all five lenses with the Fat1 KO genotype showed multilayering of the lens epithelial cells, but this defect was not seen in any of the four Fat4 KO or the other 20 control lenses (Fig. 4B, arrows, Fig. 5). We noticed that the morphologic defect in the epithelial cells was not apparent in the germinative zone (i.e., the region directly above the lens equator; Figs. 4A, 4B) but rather tended to be more centrally located. Furthermore, the epithelial cells at the lens equator in Fat1 KOs appeared to maintain normal patterns of fiber differentiation, similar to those in controls; for example, expression of an epithelial marker, E-cadherin (Figs. 4C, 4D), disappeared at the equator while the fiber cell markers Aquaporin 0 (AQP0; Figs. 4E, 4F) and β-crystallin (Figs. 4G, 4H) were induced in the transition zone and readily detected in the fiber mass of Fat1 KO lenses as observed in the controls. Normal fiber differentiation depends on cell cycle exit and this was also evident in Fat1 KO lenses as induction of a postmitotic marker, Kip2/p57, was detected just anterior to the lens equator in the similar pattern to the controls (Figs. 4I, 4J). No ectopic EdU-positive cells were found in the fiber cell compartment, again confirming cell cycle exit upon fiber differentiation occurred normally in Fat1 KO lenses (see below, Fig. 6A).

Localization of the apical cell junction marker, ZO-1, showed that in the multilayered region of the Fat1 KO lens, the contiguous apical cell junction region was lost (Fig. 5A). In the Fat1 KO lenses ZO-1 localization was patchy and discontinuous (Fig. 5A, arrows), compared with the reactivity for ZO-1 that extended along the apical junctions of control lenses (Fig. 5A, arrowheads). This indicated loss of cell polarity

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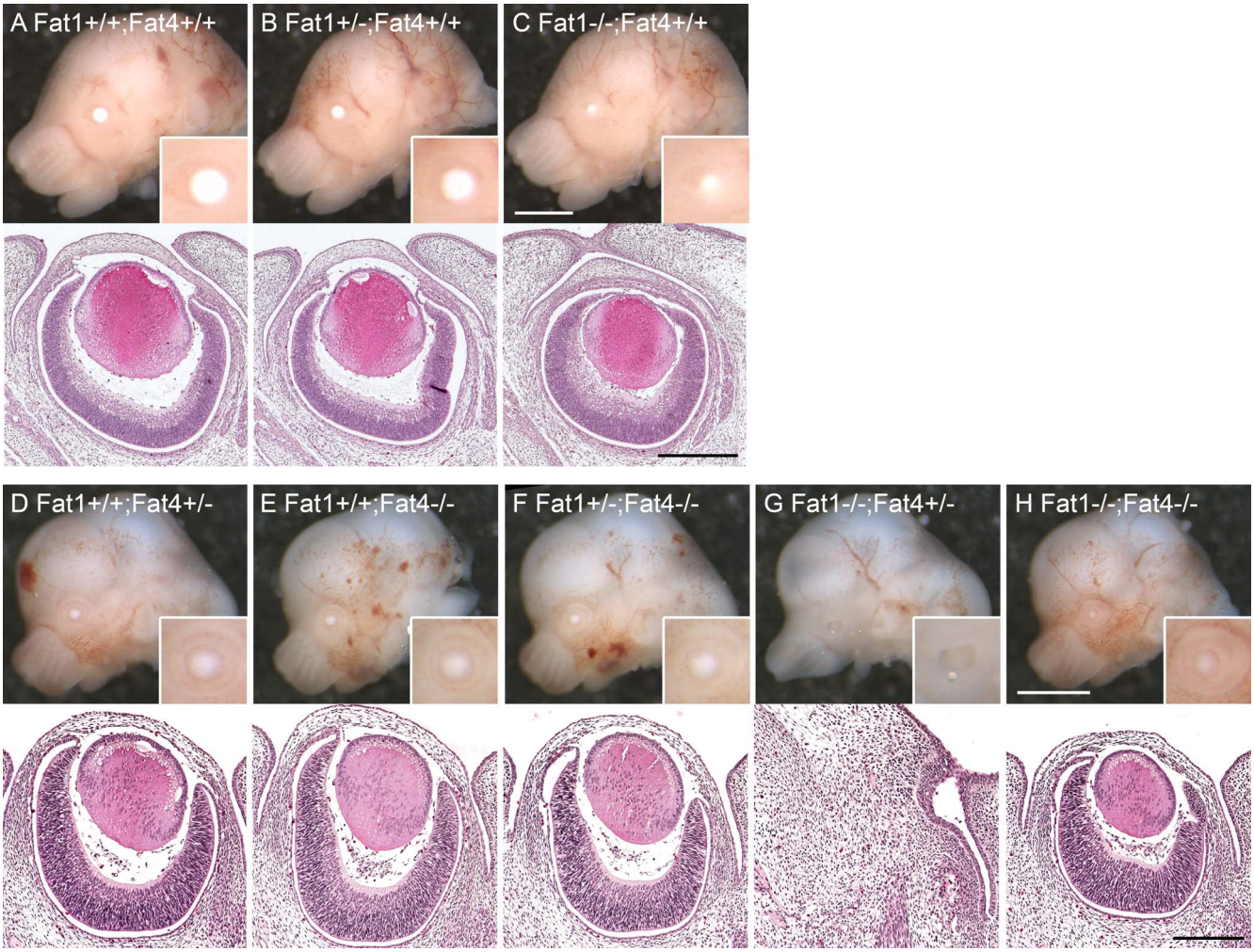

Figure 2. Eyes were small or absent in Fat1 KO mice. Surface eye appearance (*top rows*) and H&E staining of sectioned eye tissues (*lower rows*) from littermates at E14.5 (**A–C**) and E13.5 (**D–H**). At E14.5, Fat1 heterozygous embryos (**B**) have healthy eyes like wild-type embryos (**A**), but Fat1 KO embryos (**C**) have small eyes. At E13.5, Fat4 heterozygous (**D**), and KO (**E**) embryos have healthy eyes and depletion of one allele of Fat1 from Fat4 KO embryos does not induce obvious eye defects (**F**). Some Fat1 KOs lack eye development (**G**) while others exhibit small eyes (**H**). However, the absence of eyes does not seem to be influenced by Fat4 removal (Fat4 is heterozygous in [**G**]) since the Fat1;Fat4 DKO shown in (**H**), does not have a more severe phenotype than that shown in (**G**), rather it appears more normal exhibiting only a small eye phenotype. Thus, the small eyes that form in DKO embryos appear to be associated with Fat1 KOs but not with Fat4 depletion. The embryo shown in (**G**) also has facial malformations. *Scale bars*: (**A–C**) *top row* 2 mm, H&E images 400 μm, (**D–H**) *top rows* 2 mm, H&E images 200 μm.

and indeed in this region the nuclei exhibited more rounded shapes compared with the elongated nuclei that tended to follow the columnar shape of the cells in controls (Fig. 5A, see white nuclear lamin B [NLB] staining). Some of these cells in the multilayers also showed immunoreactivity for α-SMA, a marker for epithelial mesenchymal transition (EMT; Fig. 5B, arrow); a process that does not normally occur in the lens but is a feature of some subcapsular cataracts.[18] In summary, these observations indicate that Fat1 is required to maintain the normal morphology of lens epithelial cells, especially those in the central zone, but is not essential for epithelial to fiber differentiation.

Increase of DNA Synthesis in the Lens Epithelium of Fat1 Knockout Mice

Because Fat proteins are known as tumor suppressors and their role in cell cycle control has been suggested, we examined cell proliferation in E14.5 embryos by EdU incorporation assay (Fig. 6). After 2-hour incubation, the number of nuclei labeled with EdU appeared more abundant in the lenses of Fat1 KO mice compared with lenses of controls and Fat4 KO mice (Fig. 6A). Quantitative analysis showed that EdU incorporation rates of the control ($Fat1^{+/+}$; $Fat4^{+/+}$ and $Fat1^{+/-}$;$Fat4^{+/+}$), double heterozygous control ($Fat1^{+/-}$;$Fat4^{+/-}$; because of the large number of samples they were split into two controls) and Fat4 KO ($Fat1^{+/-}$;$Fat4^{-/-}$) were almost the same; the mean rates in the germinative zone were 20.5%, 22.7%, and 21.0% (21.6% altogether) and in the central zone the rates were slightly less at 15.2%, 15.8%, and 14.2% (15.1% altogether) for control, double heterozygous control, and Fat4 KO, respectively. EdU incorporation rates were significantly increased in Fat1 KO lens epithelial cells in both zones (against the total means of controls, *t*-test, $P < 0.05$); the rates were 26.4% in the germinative zone and 27.7% in the central zone. Note the central zone showed similar or even higher incorporation rate than that of the germinative zone in Fat1 KO lenses, whereas in the controls and Fat4 KOs the central zones consistently showed lower rates. This higher incorporation rate in the central zone indicates that this zone

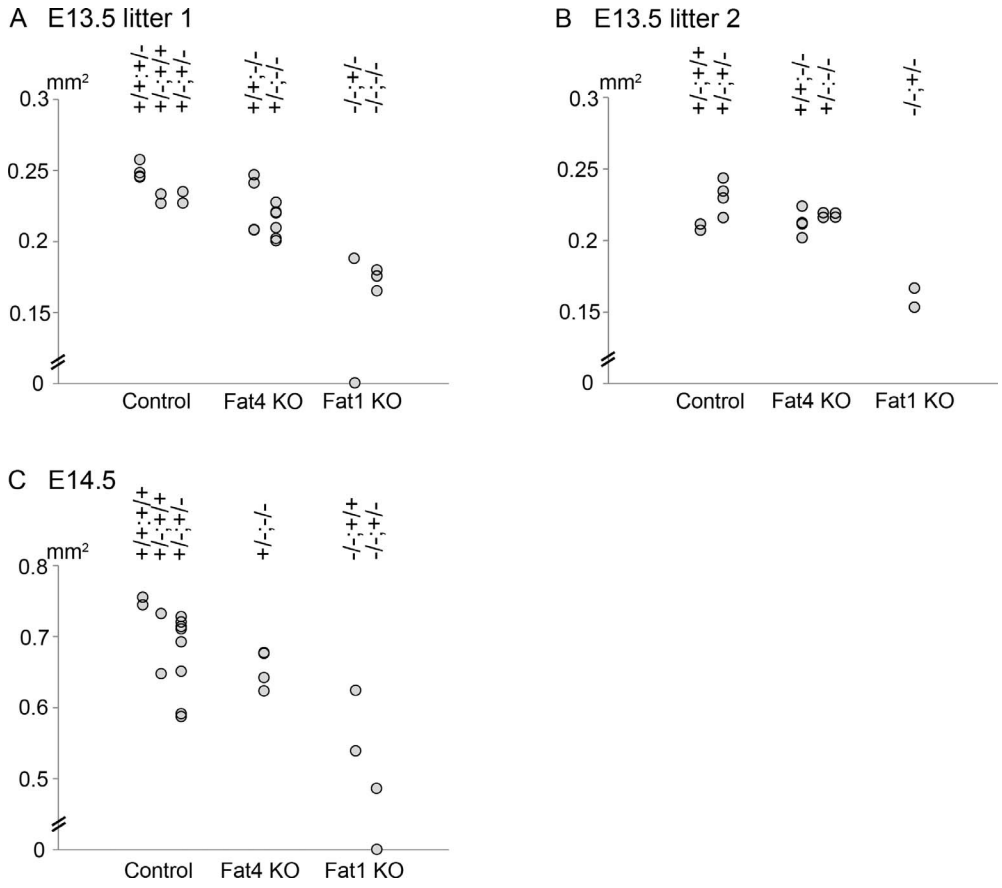

Figure 3. Fat4 depletion has only minimal effect on eye size. Paraffin sections were prepared from the Fat1;Fat4 embryos at E13.5 (**A**, **B**) and E14.5 (**C**) and the central sections were selected to measure eye cavity area (mm^2). Genotypes are indicated at the top of plots (+/+;+/– shows Fat1[+/+];Fat4[+/–], etc.). According to the size similarity the samples were grouped into control (+/+;+/+, +/–;+/+, +/+;+/–, and +/–;+/–), Fat4 KO (+/+;–/– and +/–;–/–) and Fat1 KO (–/–;+/+, –/–;+/–, and –/–;–/–). Two embryos with the Fat1(–/–);Fat4(+/–) genotype showed no eye formation; therefore, eye areas were marked as zero (**A**, **C**).

is more sensitive to the effects of Fat1 depletion. This observation indicates that Fat1 is essential for cell cycle control in the lens epithelial cells and that this requirement appears to be most prominent in the central zone.

Fat1 Localizes to Cell Membrane Region of Lens Epithelial Cells

We examined the cellular localization of Fat1 in the lens with a specific antibody raised against a region of the human Fat1 cytoplasmic tail, which has high homology between human and rodent sequences.[17] Consistent with the in situ hybridization pattern, Fat1 protein was detected in the lens epithelial cells but not in the fibers (Fig. 7A). Fat1 protein was localized at the apical cell junctions and weakly on the cell membranes of the lens epithelial cells at E14.5 (Figs. 7A, 7B); control rabbit IgG did not detect these signals (Supplementary Fig. S1). Because the knockout of Scribble[19] also disrupted the columnar structure of lens epithelial cells similar to that seen when Fat1 is depleted, we compared Fat1 localization with Scribble localization. Similar to previous reports,[20] Scribble localized to the apical cell junctions of the lens epithelial cells and in this region was similar to Fat1 and β-catenin localization (Fig. 7B). In postnatal lenses, Fat1 was similarly detected at the apical cell junctions and the cell membranes in both the central and the germinative zone of the epithelial cells (Figs. 7C, 7D).

Discussion

Functional Divergence of Fat1 and Fat4 During Eye Formation

A major finding in this study is that while there is prominent expression of Fat1 and Fat4 in the lens, it is only the absence of Fat1 that results in a lens phenotype. Both Fat1 and Fat 4 are similarly expressed in the lens pit and in the anterior cells of the lens vesicle before becoming prominently expressed in the epithelium; neither Fat was detected in fibers. Although they have similar expression patterns, it is only Fat1 KO mice that have an eye/lens phenotype; lenses of Fat4 KOs do not appear to have any significant differences from lenses of wild types. Taken together with the observation that Fat4 depletion did not significantly enhance the Fat1 KO phenotype, this indicates that Fat1 and Fat4 have distinct roles during eye development with little or no functional redundancy, at least during the embryonic stages examined in this study. Because Fat4 did not show compensative activity it is still unclear why Fat1 shows partial penetrance on eye phenotypes. The fact that the members of Fat family show complex patterns of functional redundancy, as well as the observation that their phenotypes are varied depending on mouse backgrounds, makes this a difficult issue to resolve.[7]

For some functions at least, Fat4 is known to interact with another atypical cadherin, Dachsous (Dchs1). For example, Fat4 and Dchs1, have been shown to work together in the PCP

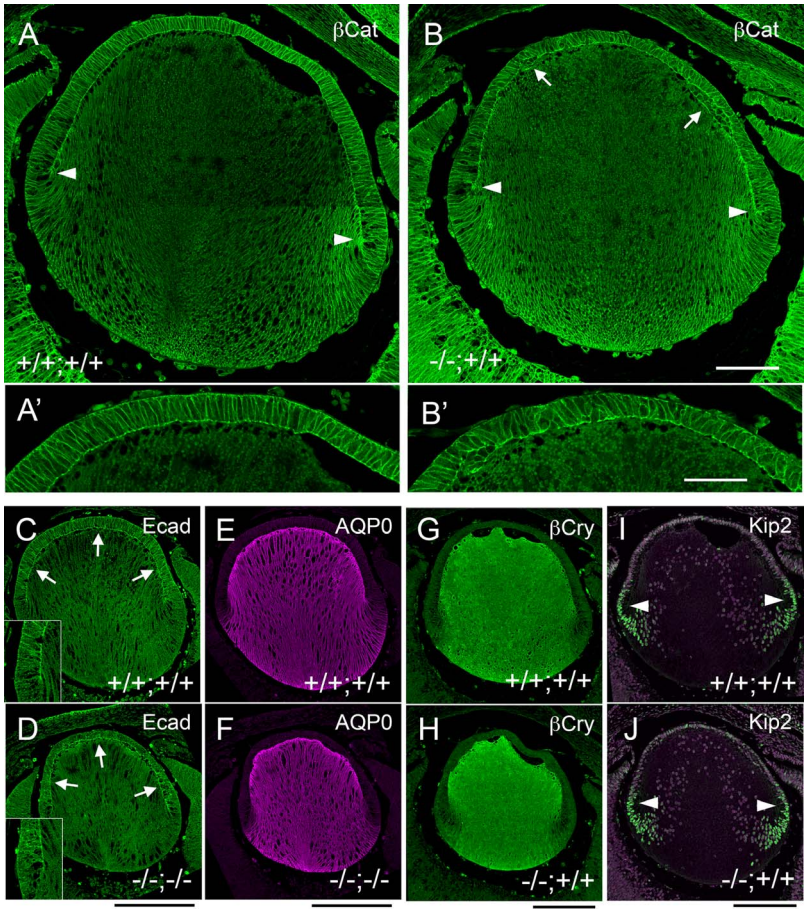

Figure 4. Lens epithelial cells lose their columnar structure in the central region but show normal terminal differentiation at the transition zone in Fat1 KO mice (E14.5). (**A**, **B**) Lens epithelial cells tend to be columnar and are regularly aligned in wild-type lenses (**A**, **A'**); in contrast, the epithelial cells in Fat1 KO lenses, particularly those anteriorly situated (usually referred to as the central region of the epithelium), exhibit more irregular shapes (**B**, **B'**). Some multilayering regions are also evident ([**B**], *arrows*). However, the morphology and arrangement of lens epithelial cells in the germinative/transition zone of Fat1 KO lenses appears to be more or less normal, as does the fulcrum structure at the lens equator (*arrowheads*, [**A**, **B**]). (**C**–**J**) In both wild-type and Fat1 KO lenses, the epithelial cell marker E-cadherin ([**C**, **D**], *arrows*) and the fiber-specific proteins AQP0 (**E**, **F**) and β-crystallin (**G**, **H**) are induced normally. Note the cytoplasmic signal in the lens fibers in the E-cadherin images is nonspecific background. A postmitotic marker, Kip2/p57 (*green*), is also induced normally in the germinative/transition zone in both wild-type and Fat1 KO lenses (*arrowheads*, [**I**, **J**], nuclei are labeled with nuclear lamin B, *purple*). *Scale bars*: (**A**, **B**) 200 μm, (**A'**, **B'**) 100 μm, (**C**–**F**) 200 μm, and (**G**–**J**) 200 μm.

pathway and Dchs1 KO causes similar defects throughout the body that are observed in Fat4 KOs.[21] The lack of expression of Dchs1 in the lens (Supplementary Figs. S2A–G), is also consistent with the absence of a functional role for Fat4 in the PCP pathway at these embryonic stages. Interestingly, we did detect a temporally and spatially regulated expression of Four-jointed (Fjx1), a golgi kinase that mediates Fat4 and Dchs1 interaction, in the lens (Supplementary Figs. S2H–N). Four-jointed may work independently from Fat4 and Dchs1 in the lens, but it also leaves open the possibility that the Fat4/Dchs1/Fjx1 interaction might have a role during stages of lens development not examined here.

Fat1 Is Required to Maintain the Lens Epithelial Phenotype and Regulate Its Cell Cycle Activity

Although the lens phenotype of Fat1 KO mice varies in severity, a consistent characteristic is loss of distinct apical-basal polarity and multilayering in some regions. Some of the cells in small multilayered plaques fluoresce for α-SMA, an indicator of an epithelial mesenchymal transition. This epithelial function of Fat1 may be mediated by VASP/Ena proteins, the actin regulators that modulate cell migration and cell–cell contact formation because their direct binding to Fat1 has been shown.[22,23] Consistent with this is our observation that Fat1 was detected at the apical cell junctions of the lens epithelial cells and that VASP has also been detected at the apical cell junctions of the lens epithelial cells at the lens pit stage.[24] In this case, the variability of the eye/lens phenotype of Fat1 knockout mice may indicate involvement of other, as yet unknown, background factors that influence this cell-cell contact function of Fat.

We also noticed that the prominent disruption of the columnar structure of lens epithelial cells in Fat1 KO mice closely resembled the phenotype induced in Scribble-depleted lenses.[19] Scribble is a PDZ domain-containing adaptor protein which is essential for apical cell junction formation and also for cell proliferation. Interestingly its physical and functional interaction with Fat1 has been indicated in Zebrafish and *Drosophila*.[25,26] We showed colocalization of Fat1 and Scribble at the apical cell junctions. The columnar structure and the apical cell junctions are also disrupted in aPKC conditional KO mouse lenses.[27] However, in aPKC mutants the prominent defect is first seen at the equator where the apical tips of the elongating lens fibers are not retained at the fulcrum and are dislocated to the posterior side. Thus, Fat1 may function with

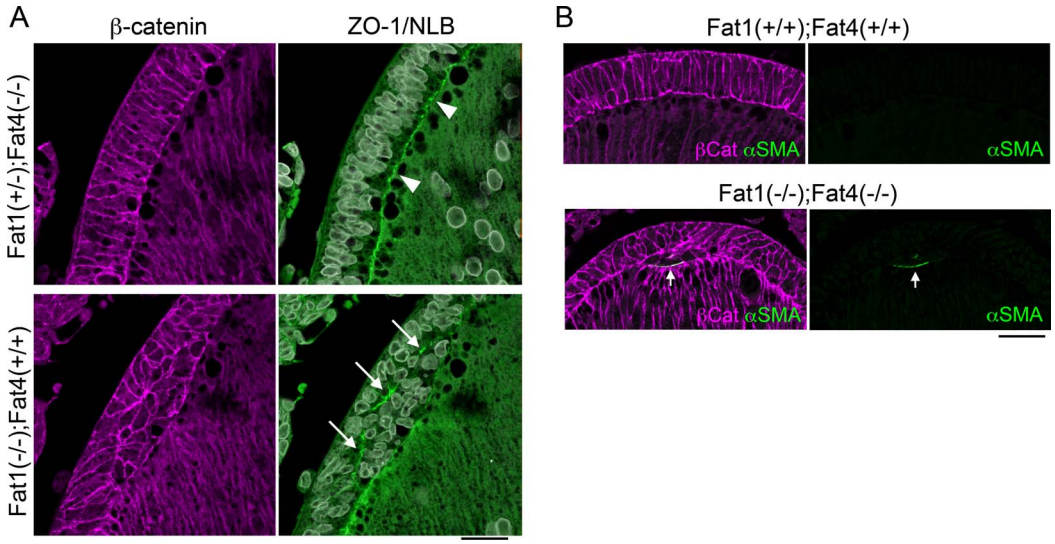

Figure 5. Apical-basal polarity is deficient in the lens epithelium of Fat1 KO mouse embryos at E14.5. (**A**) β-catenin reactivity (*purple*) delineates cell borders and shows multilayered epithelial cells in Fat1 KO lenses. ZO-1 reactivity (*green*) localizes the apical cell junctions of the cells that collectively form a contiguous barrier at the apical surface of lens epithelial cells in control lenses (*arrowheads*). In some Fat1 KO lens epithelial cells, especially in the multilayered region, apical localization of ZO-1 is lost and this causes discontinuity in apical cell junction formation (*arrows*). Nuclear lamin B (NLB) staining (*white*) shows nuclei in multilayered cells tend to have more rounded rather than the elongated shapes characteristic of controls. (**B**) Patches of reactivity for α-SMA (*green*) in regions of multilayering (*arrow*) indicate that some cells of Fat1 KO have undergone an epithelial mesenchymal transition (EMT), a process that does not normally occur in wild-type lenses. *Scale bars*: (**A**) 25 μm, and (**B**) 25 μm.

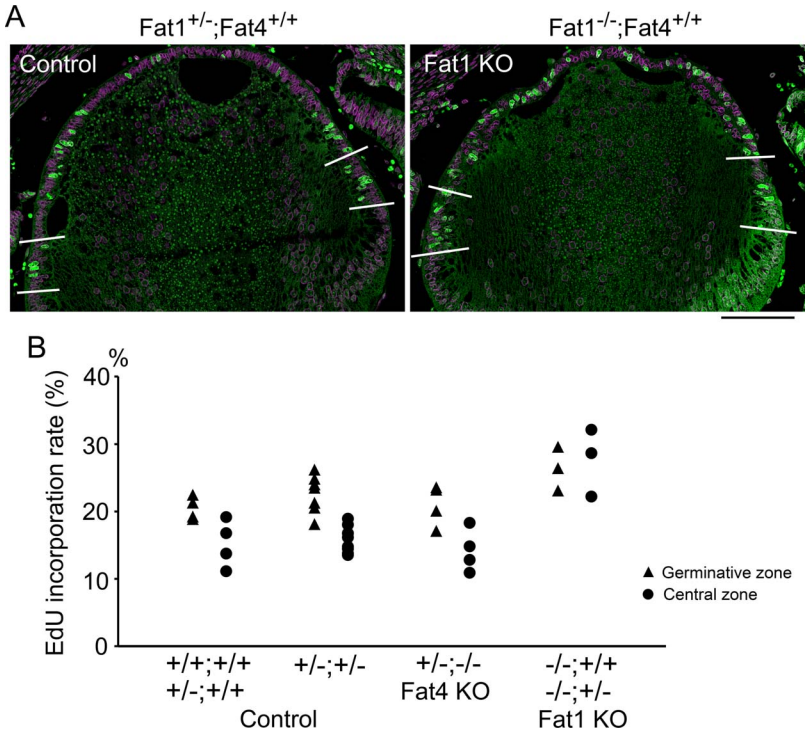

Figure 6. DNA synthesis is enhanced in the lens epithelium of Fat1 KO mice at E14.5. EdU was administrated to pregnant mice and embryos were collected after 2-hours incubation and processed for paraffin sectioning. (**A**) Confocal microscope images showing EdU-positive cells (*green*) and counter staining of nuclear lamin B (*purple*). The *white bars* delineate the boundaries of the germinative zone epithelial cells (the greater part of the epithelium, the central zone, lies anterior to this). *Scale bar*: 100 μm. (**B**) The number of EdU-positive cells of the germinative (*triangles*) and central (*circles*) zones were counted from three nonsequential lens sections to examine EdU incorporation rates. Lenses were categorized into four groups according to Fat1;Fat4 genotype (wild-type or one allele of Fat1 is missing, Fat1 and Fat4 double heterozygote, Fat4 KO, and Fat1 KO). Fat1 KO lenses showed a significantly higher EdU incorporation rate compared with the control groups, whereas no significant difference was detected between Fat4 KO and control lenses.

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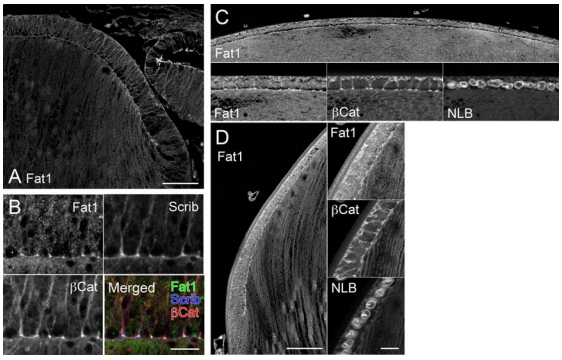

Figure 7. Cellular localization of Fat1 in the lens at E14.5 (**A**, **B**) and P12 (**C**, **D**). (**A**) Immunoreactivity of Fat1 antibody was detected in the lens epithelial cells at the apical cell junctions and weakly on the cell membranes. (**B**) Fat1 at the apical cell junctions colocalized with Scribble and β-catenin (in the *merged panel* the apical junctions are labeled in *white* because of colocalization of three proteins). (**C**, **D**) In postnatal lenses, Fat1 was similarly detected at the apical cell junctions and on the cellular membrane of the central (**C**) and equator (**D**) epithelial cells. *Scale bars*: (**A**) 50 μm, (**B**) 10 μm, and (**C**, **D**) 50 μm (high magnification panels 12.5 μm).

the Scribble-Dlg complex rather than the aPKC-Par polarity proteins.

In addition to the loss of apical-basal polarity, the Fat1 KO lenses showed an increased level of DNA synthetic activity in the epithelium of Fat1 but not Fat4 KO mice. In *Drosophila*, the tumor suppressive activity of Fat has been linked to the Hippo pathway and it has been shown to have a functional interaction with some components of this pathway. A role for the Hippo pathway in the lens has been identified in Merlin/NF2 and YAP KO mice. In these mutants the lens epithelial cells showed premature[28] or delayed[29] cell cycle exit, suggesting the Hippo pathway is essential for regulating the transition from proliferation to fiber differentiation in the lens. In contrast, the current study showed that transition from proliferation to differentiation compartments occurred normally in Fat1 KO lenses at the equator as in control lenses. Thus, Fat1 regulation of the Hippo pathway may not be a feature of lens cells.

Fat1 regulation of the cell cycle has also been linked to canonical Wnt/β-catenin pathway. Recently, Fat1 was shown as a responsible gene for tumors linked to aberrant Wnt activation.[11] They showed binding between Fat1 and β-catenin was associated with activation of Topflash reporter. In the lens, Wnt/β-catenin signaling is required to promote the formation of an intact epithelial layer between E12.5 and E14.5[30] and its disruption causes premature fiber differentiation, whereas forced activation causes increased progression of epithelial cells through the cell cycle and a delay of differentiation.[31,32] Obviously, further examination is required but this raises the possibility that Fat1 may influence lens cell proliferation/differentiation through its involvement with several signaling pathways.

It is interesting that depletion of Fat1 mainly affects the lens epithelial cells of the central zone but has less effect on the cells of the germinative zone. Because Fat1 is expressed in both zones, its activity may be restricted to the central zone by other regulatory factors; for example, transcriptional activity of β-catenin in the canonical Wnt/Fz pathway has been detected in the central lens epithelial cells while its regulator, sfrp2, mRNA shows complimentary expression and is restricted to the germinative zone.[14] Also the Notch signaling targets, Hes5 and Herp2, are expressed only in the germinative zone.[33,34] These observations indicate differences in the two zones at the molecular level. A precise mechanism that generates different characters in each zone has not yet been elucidated. However, it has been suggested that the spatial correlation to the surrounding ocular tissues and aqueous compartments specifies these zones.[16] In this study, it was noted that the germinative zone is bathed by posterior chamber aqueous, whereas the central zone is bathed by anterior chamber aqueous. In line with this, it is possible that the differences in responses to Fat1 depletion between central and germinative zones may reflect such differences.

Intriguingly, a recent study in *Drosophila* identified another role for Fat that is independent of Hippo and PCP regulatory functions. The McNeill laboratory[35] showed that when Fat gets cleaved, a cytoplasmic fragment, Ft^{mito}, is imported into mitochondria. Loss of Fat led to a switch in the metabolism of larvae from oxidative phosphorylation to glycolysis. Essentially the study identified a key role for Ft^{mito} in electron transport in the mitochondria and loss of Fat resulted in increases in reactive oxygen species (ROS). In this context, it is interesting to note that other studies have shown a switch to glycolysis in tumor cells supports their growth and that ROS production can stimulate stem cell proliferation. If a similar role in mitochondrial function for Fat operates in vertebrates as in *Drosophila*, this may underlie the enhanced proliferation seen in our Fat1 KO mice. Also, in the event of a reduction in efficiency of the Fat/mitochondrial interaction in the lens epithelium and a concomitant increase in ROS production, even if the effect is slight, such a loss of efficiency over time could contribute to cataract progression in older individuals. Indeed, ROS production itself has been shown to impair the function of mitochondria and promote the generation of more ROS with further damaging effects.[36] Consequently, when considering the role of Fat in the lens, or in any other system, we need to look beyond the well-known roles this important family of atypical cadherins has in cell–cell contact as well as PCP and Hippo pathway signaling.

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