Prox1 function is crucial for mouse lens-fibre elongation

Jeffrey T. Wigle[1], Kamal Chowdhury[2], Peter Gruss[2] & Guillermo Oliver[1]

Although insights have emerged regarding genes controlling the early stages of eye formation, little is known about lens-fibre differentiation and elongation. The expression pattern of the *Prox1* homeobox gene suggests it has a role in a variety of embryonic tissues, including lens[1]. To analyse the requirement for *Prox1* during mammalian development, we inactivated the locus in mice. Homozygous *Prox1*-null mice die at mid-gestation from multiple developmental defects; here we describe the specific effect on lens development. *Prox1* inactivation causes abnormal cellular proliferation, downregulated expression of the cell-cycle inhibitors *Cdkn1b* (also known as *p27^{KIP1}*) and *Cdkn1c* (also known as *p57^{KIP2}*), misexpression of E-cadherin and inappropriate apoptosis. Consequently, mutant lens cells fail to polarize and elongate properly, resulting in a hollow lens. Our data provide evidence that the progression of terminal fibre differentiation and elongation is dependent on *Prox1* activity during lens development.

We inactivated mouse *Prox1* by inserting the gene encoding β-galactosidase (LacZ), in frame, at amino acid 224 of *Prox1* (Fig. 1*a*,*b*). The resulting LacZ activity allowed analysis of *Prox1* expression throughout development. In the developing eye, *Prox1* was first expressed at approximately embryonic day (E) 9.5 over the lens placode (Fig. 1*c*); at E10.0 in the whole lens vesicle (Fig. 1*d*); and at E12.5 in the anterior proliferating epithelium and fibre cells (Fig. 1*e*). During normal mouse lens development after the formation of the lens vesicle (Fig. 2*a*), the mitotically active epithelial cells from the anterior pole (Fig. 2*c*) are induced to differentiate into fibre cells in the equatorial region encircling the lens (Fig. 2*c*). The fibre cells elongate anteriorly to fill the hollow lens vesicle, eventually producing a lens consisting of postmitotic differentiated fibre cells expressing crystallin proteins and covered anteriorly by a monolayer of proliferating epithelial cells (Fig. 2*c*,*e*).

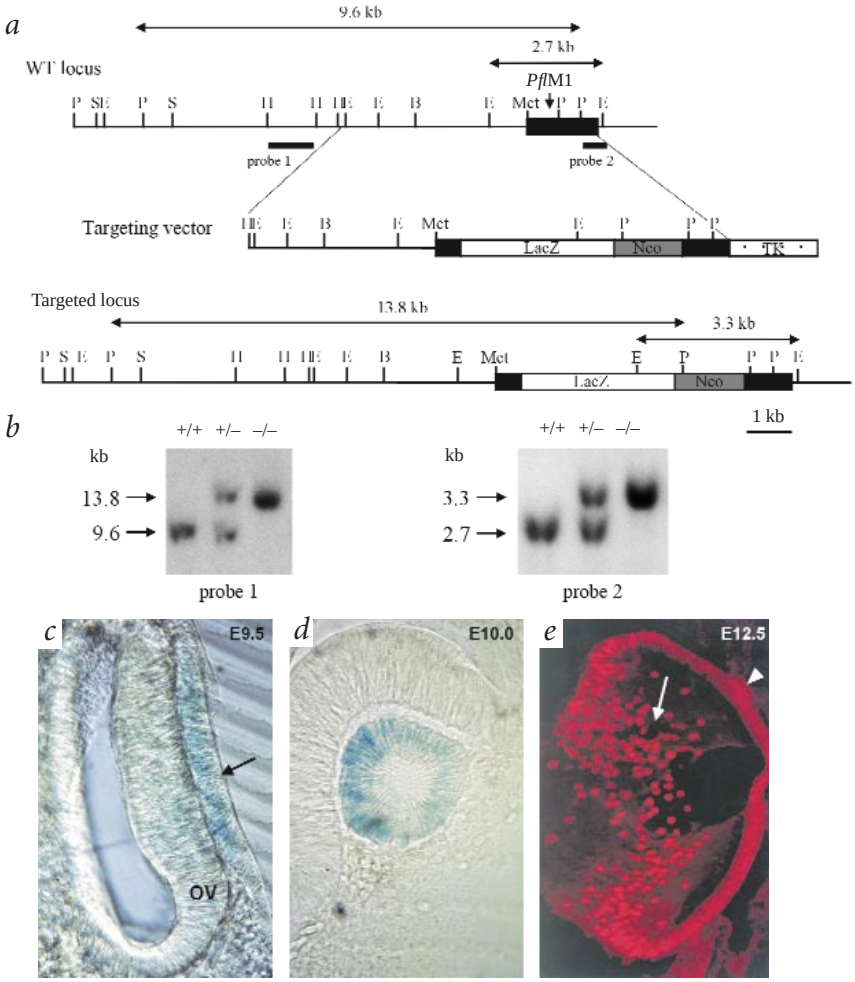


Fig. 1 Targeted disruption of *Prox1*. ***a***, Structure of wild-type and targeted *Prox1* loci. *Prox1* was inactivated by the in-frame insertion of a β-galactosidase-neomycin resistance cassette in the *Pfl*MI site 224 aa downstream of the initiation methionine (Met). ***b***, Mice and embryos were genotyped by Southern-blot analysis using genomic DNA isolated from tails or yolk sacs, digested with *Pst*I and hybridized with a 1.2-kb external genomic fragment (probe 1), or digested with *Eco*RI and hybridized with a 0.3-kb internal fragment (probe 2). For *Pst*I, the 9.6-kb fragment is indicative of the wild-type allele and the 13.8-kb fragment originates from the targeted allele. For *Eco*RI, the 2.7-kb band represents the wild-type locus and the 3.3-kb fragment represents the targeted allele. ***c***, β-galactosidase detection of normal *Prox1* expression at E9.5 in the lens placode (arrow) in contact with the optic vesicle (OV). ***d***, At E10.0, *Prox1* normal expression (LacZ) is detected in the entire lens vesicle. ***e***, At E12.5, Prox1 protein is observed in the anterior dividing epithelium (arrowhead) and lens fibres (arrow). In contrast to the *Drosophila melanogaster prospero* gene, Prox1 protein does not exhibit unequal protein distribution in lens cells.

[1]*Department of Genetics, St. Jude Children's Research Hospital, 332 North Lauderdale St, Memphis, Tennessee 38105, USA.* [2]*Max Planck Institute for Biophysical Chemistry, Am Fassberg, 37077 Goettingen, Germany. Correspondence should be addressed to G.O. (e-mail: guillermo.oliver@stjude.org).*

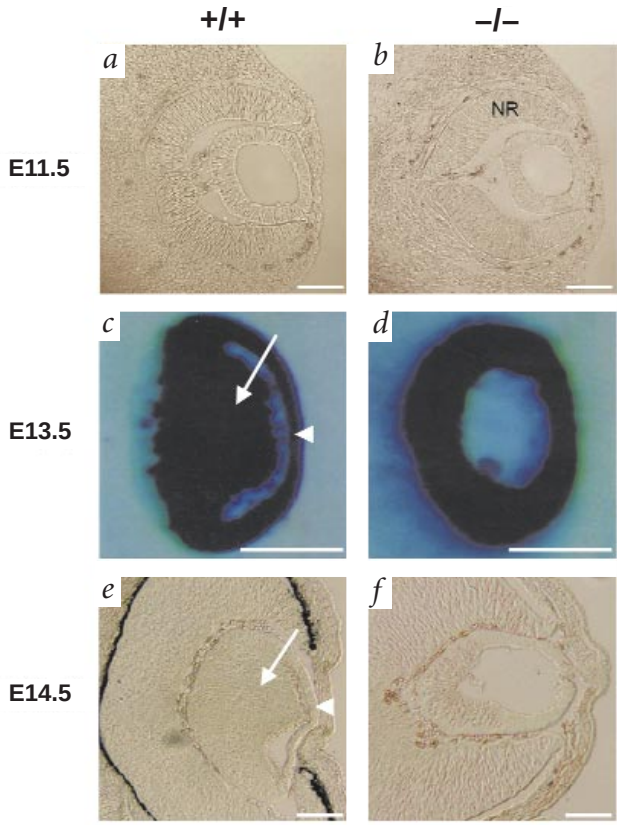


Fig. 2 Lens-fibre elongation is affected in *Prox1*$^{-/-}$ mice. Normal lens-fibre development in wild-type E.11.5–14.5 embryos is revealed by Nomarski imaging (***a***,***e***) or LacZ detection (***c***). Lens-fibre elongation (arrow) towards the anterior epithelia (arrowhead) is observed in (*c*) and (*e*). *Prox1*$^{-/-}$ lens is shown in (*b*), (*d*) and (*f*). The initial formation of the lens vesicle is not affected in mutant lens (***b***). At E13.5, lack of lens-fibre elongation is noticeable by the hollowed appearance of the lens (***d***), which becomes more obvious by E14.5 (***f***). NR, neuroretina; scale bar, 100 μm.

Histological analysis of *Prox1*$^{-/-}$ eye at different stages of development revealed abnormal lens development (Fig. 2). Although the initial induction of the lens vesicle (Fig. 2*b*) is unaffected, there is minimal elongation of the lens fibres towards the anterior epithelium, so the cystic lumen persists and a hollow lens forms (Fig. 2*d*,*f*). Beginning at E12.5, a small reduction of the overall size of the eye was also observed.

During normal lens development, a precise transition from actively proliferating epithelial cells to terminally differentiating non-proliferating lens fibre occurs. BrdU (5-bromodeoxyuridine)-incorporation assays indicated equivalent proliferation in wild-type and mutant lens until approximately E11.5 (Fig. 3*a*,*b*). We saw no differences in wild-type and mutant anterior germinal epithelium from E12.5–14.5 lenses (Fig. 3*c*–*f*), but in E12.5–14.5 lenses we found many BrdU-positive cells posteriorly (Fig. 3*d*,*f*). Thus, *Prox1* activity is necessary for the temporal withdrawal of these cells from the cell cycle. To address this possibility directly, we examined the expression of two cell-cycle regulators, *Cdkn1b* and *Cdkn1c*, in wild-type and mutant lens. These cell-division kinase inhibitors are normally expressed during the differentiation and elongation of lens fibres[2,3]. Lack of *Cdkn1c* leads to lens vacuolization, inappropriate entry of lens fibre cells into S-phase and increased apoptosis[2], whereas *Cdkn1b*-deficient mice do not show any developmental defects in the eye[3]. Analysis of *Cdkn1b*$^{-/-}$*Cdkn1c*$^{-/-}$ double-mutant mice show that the two genes function cooperatively to regulate cell-cycle exit and differentiation of lens-fibre cells[3]. Starting at E13.5, mice lacking these two gene products show vacuolated lens, failed lens-fibre elongation and suppressed crystallin expression[3].

Immunohistochemical analysis of E12.5–14.5 wild-type lens reveals Cdkn1b expression in postmitotic lens-fibre cells located in the equatorial region, with sporadic expression in the anterior dividing epithelium (Fig. 4*a*,*c*). At the same stage of development, Cdkn1c expression is localized in the equatorial region and in cells at the posterior region of the lens (Fig. 4*e*,*g*). Expression of both proteins was reduced or absent in *Prox1*$^{-/-}$ lens (Fig. 4). In some cases, a very low level of diffuse and non-nuclear staining for Cdkn1b was detected at E12.5 (Fig. 4*b*) and E14.5 (Fig. 4*d*). We detected no *Cdkn1c* expression at E12.5 (Fig. 4*f*), whereas we saw a very low number of positive cells at E14.5 over the posterior region of the mutant lens (Fig. 4*h*). A possible explanation for this is that the young fibre cells normally expressing these two cell-cycle regulators are missing in mutant lens. *Sox1* (ref. 4), however, which is induced in young fibre cells, is still expressed in mutant lens (Fig. 6*f*). This indicates that the observed downregulation in *Cdkn1b*/*Cdkn1c* expression is not due to the specific lack of the expressing tissue in mutant lens, but instead is a direct consequence of the absence of *Prox1* activity.

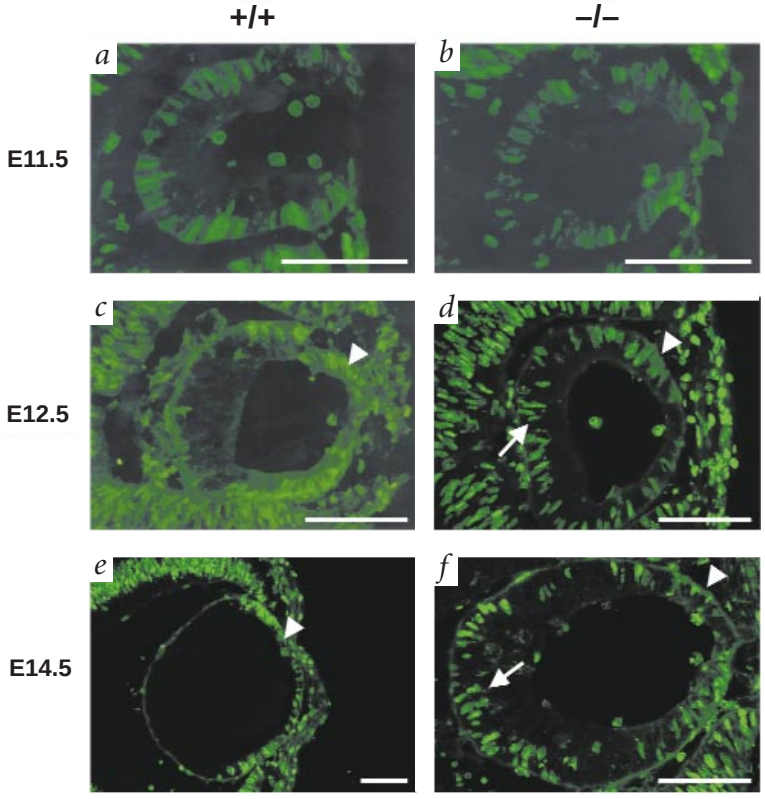


Fig. 3 Altered cellular proliferation in *Prox1*$^{-/-}$ lens. At the lens vesicle stage, BrdU incorporation shows no difference between wild-type and mutant eyes (***a***,***b***). Later in development, proliferating cells are normally located in the anterior epithelium of wild-type lens (***c***,***e***, arrowhead) and are absent from the posterior region. In contrast, BrdU-positive cells are abnormally found in the posterior lens-fibre cells (arrow) in mutant lens (***d***,***f***). Scale bar, 100 μm.

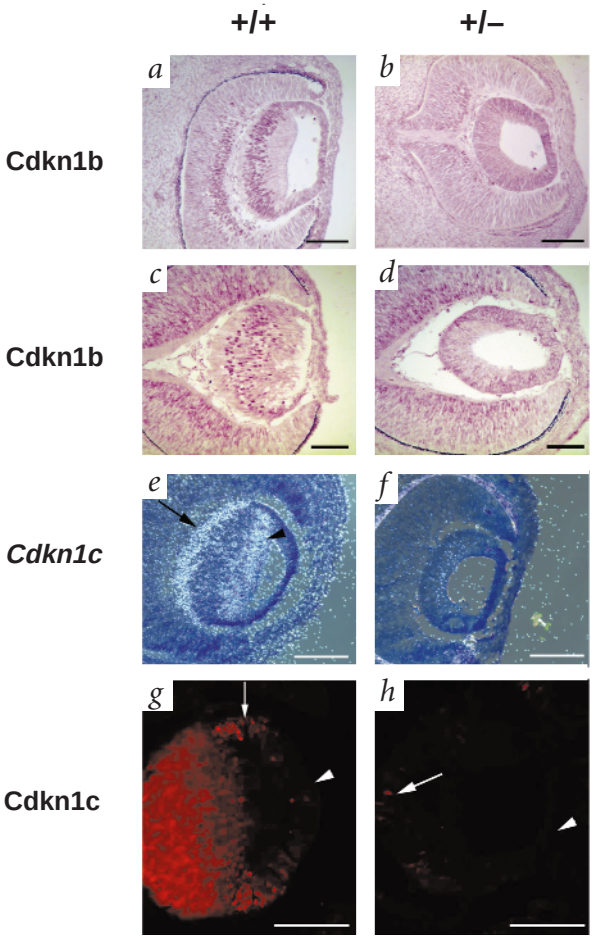


Fig. 4 *Prox1* regulates *Cdkn1b* and *Cdkn1c* expression in the lens. Immunohistochemical analysis of Cdkn1b protein shows expression in the posterior part of the wild-type lens at E12.5 (***a***) and in the lens fibres at E14.5 (***c***). Some expression is also observed in the neuroretina. In mutant littermates, a diffuse and non-nuclear staining is observed at E12.5 (***b***) and E14.5 (***d***). *In situ* hybridization at E12.5 revealed *Cdkn1c* mRNA in cells located in the equatorial (arrowhead) and posterior (arrow) regions of wild-type lens (***e***). No expression is observed in mutant littermates (***f***). At the protein level, Cdkn1c is found in equatorial (arrow) and posterior cells of E14.5 wild-type mice (***g***), whereas in mutant littermates a few barely detected stained cells are observed in the posterior region (***h***, arrow). Arrowheads indicate anterior epithelia. Scale bar, 100 μm.

To determine whether the abnormal cellular proliferation in *Prox1*$^{-/-}$ lens affected lens-fibre differentiation, we compared expression of genes encoding crystallins in wild-type and mutant eye by RT-PCR (α-, β- and γ-crystallins) and *in situ* hybridization (α-A-crystallin). Precisely regulated temporal and spatial accumulation of crystallins is critical for normal lens-fibre development. Expression of crystallin genes begins at approximately E12.0 in mice[5]. In *Prox1*$^{-/-}$ lens, the expression of most crystallin genes is slightly downregulated, but still detectable at E14.5 (Fig. 5). Expression of *Crygb* and *Crygd* (encoding γ-B- and γ-D-crystallin, respectively), however, was not detected in mutant lens (Fig. 5), which may also contribute to the lens-fibre phenotype. For example, inactivation of *Sox1* produces a lens phenotype similar to that of *Prox1* mutants[4] and it is associated with the downregulation or lack of all γ-crystallin transcripts. In addition, an effect on fibre-cell elongation was also reported for the *Cat2*^{Elo} mouse, which harbours a mutated *Cryge* gene (encoding γ-E-crystallin; ref. 6).

The expression of most genes encoding α-, β- and γ-crystallins (except γ-B- and γ-D-crystallin) suggests that cell-fibre differentiation takes place in *Prox1*$^{-/-}$ lens. To confirm this, we examined the expression of the intermediate filament markers CP49 and filensin, late-stage markers of lens-fibre differentiation[7]. Both markers were expressed in E14.5 mutant lens (data not shown). This indicates that despite the loss of *Cdkn1b* and *Cdkn1c*, portions of the fibre-cell differentiation process are still initiated, but are then arrested in the absence of *Prox1*.

Our results support the notion that *Prox1* regulates a subset of changes in gene expression that occur during fibre-cell differentiation and elongation. In normal lens, epithelial cells begin to express lens-fibre markers on withdrawal from the cell cycle, and then elongate anteriorly. The normal pattern of differentiation implies that anterior epithelial cells remain proliferative, whereas non-proliferating posterior cells withdraw from the cell cycle, enter terminal differentiation and give rise to elongating fibre cells. The abnormal presence of dividing cells in the posterior of *Prox1*$^{-/-}$ lens would therefore be predicted to affect this process. To test this hypothesis, we analysed expression of E-cadherin, a marker usually associated with proliferating epithelial cells in a developing embryo[8]. Although we saw similar levels of E-cadherin expression throughout the entire lens vesicle of E11.5 wild-type and mutant embryos (data not shown), they differed after initiation of fibre-cell elongation. Starting at approximately E12.5, E-cadherin expression in wild-type lens is restricted to the

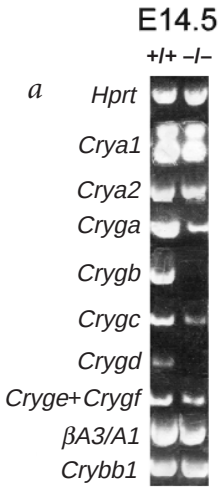
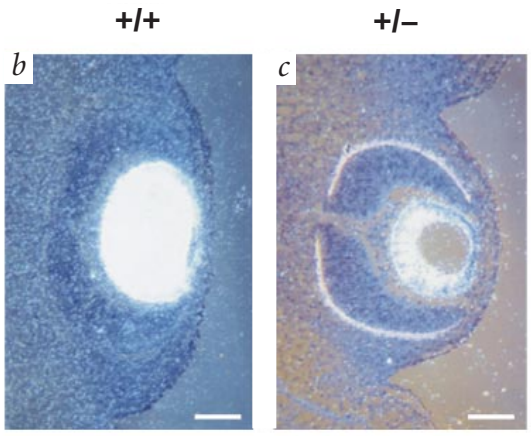


Fig. 5 Analysis of crystallin gene expression. ***a***, RT-PCR analysis of E14.5 wild-type and mutant lens. Similar amounts of cDNA were used in the amplification reactions as seen for *Hprt*. No significant differences are observed in the expression of the genes encoding α- and β-crystallins between wild-type and mutant lens. Slight downregulation is detected in mutant lens for *Cryga*, *Crygc*, *Cryge* and *Cyrgf*, whereas no *Crygb* and *Crygd* expression is observed. ***b***,***c*** *In situ* hybridization analysis of *Crya1* expression in wild-type and mutant E14.5 lens shows no major differences. Scale bar, 100 μm.



Crya1

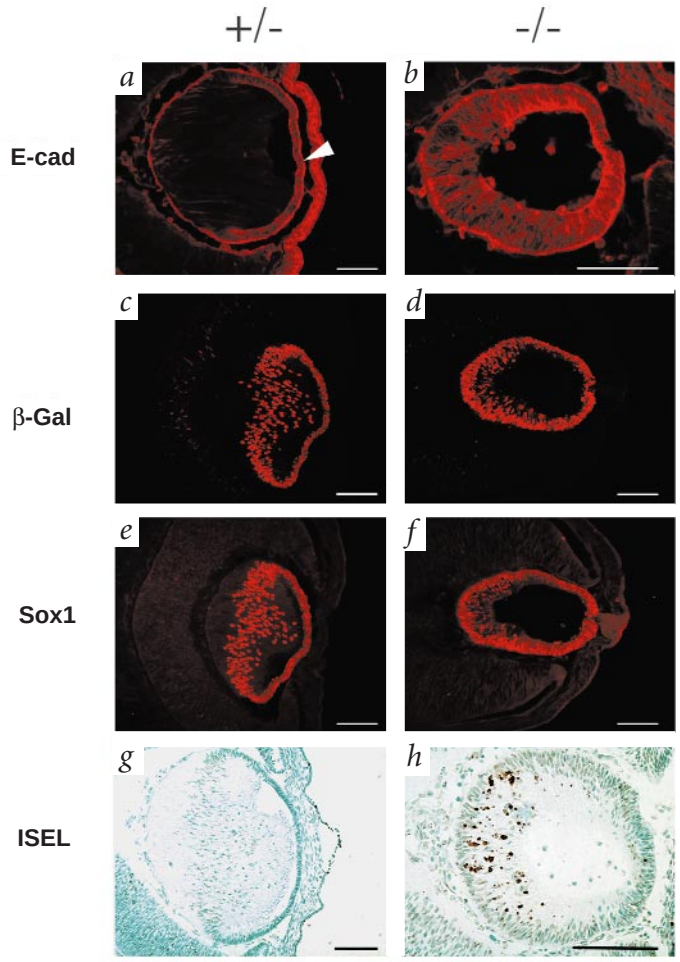


Fig. 6 E-cadherin is uniformly expressed in *Prox1* mutant lenses. ***a***, E-cadherin is normally expressed in the proliferating anterior epithelium of E14.5 lens (arrowhead), whereas in the mutant lens (***b***) E-cadherin is expressed uniformly throughout the entire mutant lens. ***c***, Prox1 (detected with an anti-β-galactosidase antibody) and Sox1 (***e***) proteins are normally located in the anterior proliferating epithelium and differentiating fibre cells. The expression of both gene products is also found over the posterior lens fibres of mutant lens (***d***,***f***). ***g***, Apoptosis is not observed in E14.5 wild-type lens, whereas a large number of ISEL-positive cells (brown nuclei) are seen in the posterior part of *Prox1*$^{-/-}$ lens (***h***). Scale bar, 100 μm.

anterior proliferative epithelium (Fig. 6*a*). In contrast, at similar stages of *Prox1*$^{-/-}$ lens development we saw a uniform, antero-posteriorly graded expression of E-cadherin throughout the entire lens (Fig. 6*b*). This result indicates that the proliferating cells found in the posterior lens compartment retained epithelial characteristics. Thus, the abnormal presence of proliferating epithelial cells in the posterior *Prox1*$^{-/-}$ lens may disturb the normal regionalization of the lens, and therefore the process of lens-fibre elongation. Inappropriate expression of Prox1 and Sox1, which normally exhibit regionalized expression during lens development, supports this concept (Fig. 6*c*–*f*). The detection of increased programmed cell death in the posterior region of *Prox1*$^{-/-}$ lens (Fig. 6*g*,*h*) suggests that the problems in patterning, failure to exit the cell cycle and failure to elongate leads to the apoptosis of fibre cells.

We have shown here that *Prox1* function is required for the temporal expression of the cell-cycle inhibitors *Cdkn1b* and *Cdkn1c* in differentiating lens fibres. It is possible that downregulation of the expression of these genes results in the abnormal presence of dividing cells in the posterior region of *Prox1*$^{-/-}$ lens. These posterior cells behave similarly to the normal proliferating epithelial cells from the anterior lens and therefore also express E-cadherin (and possibly other cadherin species). In *Prox1*$^{-/-}$ mice, steps leading to hepatocyte terminal differentiation and migration are also affected (B. Sosa-Pineda *et al.*, manuscript in preparation). In this mutant tissue, high expression of E-cadherin (and some extracellular matrix proteins) is observed surrounding the hepatocytes, which are unable to bud off from the liver diverticulum (data not shown). One interpretation of the eye phenotype may be that in *Prox*$^{-/-}$ lens, the uniform expression of E-cadherin along the entire mutant lens affects terminal differentiation and elongation of lens fibres. Alternatively, the lack of *Prox1* activity may arrest fibre terminal differentiation, and as a consequence, E-cadherin expression persists in the posterior compartment of the mutant lens. Our results show that the progression of terminal fibre differentiation and elongation in the lens is dependent on *Prox1* activity. For lens-fibre differentiation and elongation, a direct signal from the surrounding neural retina is required[9]. It may be that *Prox1*$^{-/-}$ lens is defective in reception and/or transduction of some of these signals allowing lens-fibre differentiation to initiate but not progress.

Methods

Generation of *Prox1*$^{-/-}$ mice. A β-galactosidase-neomycin resistance cassette was fused in frame to the N-terminal part of *Prox1*. A 5.1-kb *Sma*I-*Xho*I fragment containing LacZpA-pGKNeopA sequences was blunt-ended and ligated into the *Pfl*MI-linearized and blunt-ended *Prox1* construct. An F9 polyoma early promoter-derived HSV TK gene was added downstream of the 3′ homology for negative selection. R1 embryonic stem (ES) cells were electroporated and selected following standard procedures. Positive clones were used to generate chimaeras by morula aggregation. We performed tail and yolk-sac DNA isolation and PCR amplification of genomic DNA to identify the mutated allele. *Prox1*$^{+/-}$ embryos appeared normal, and the distribution of β-galactosidase expression agreed with that observed by *in situ* hybridization analysis[1]. We found that 100% of the newborn heterozygous mice died within approximately 3 d after birth, suggesting a haplo-insufficient effect of *Prox1*. To overcome this, the original chimaeras were crossed to four different genetic backgrounds. In three of these backgrounds the mortality remained unchanged, whereas the crosses to the NMRI background produced 1 of 30 surviving heterozygous newborn mice, suggesting that a genetic modifier for *Prox1* is present in this strain. From the crosses of these surviving *Prox1*$^{+/-}$ animals, no newborn *Prox1*$^{-/-}$ mice were found, indicating that the absence of *Prox1* is lethal *in utero*. Subsequent analysis revealed that *Prox1*$^{-/-}$ embryos die at approximately E14.5.

Detection of LacZ activity in embryos and tissues. We performed X-gal staining of mouse embryos with a post-fixation in 4% paraformaldehyde at 4 °C overnight. Vibratome sections were performed as described[10].

Immunohistochemistry. Embryos were fixed overnight in 4% paraformaldehyde, cryoprotected with 30% sucrose in PBS overnight, embedded in tissue freezing medium and cryostat sectioned (10 μM).

Antibodies. Primary antibodies were: rabbit anti-β-galactosidase (Cappel), rabbit anti-Sox1, rabbit anti-Prox1 (G.O., unpublished data), rat anti-uvmorulin/E-cadherin (Sigma), rabbit anti-Cdkn1b (Santa Cruz Biotechnology), goat anti-Cdkn1c (Santa Cruz Biotechnology), rabbit anti-CP49, rabbit anti-filensin and mouse anti-BrdU (Becton-Dickinson). For fluorescence staining, the following secondary antibodies were used: Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), Alexa 488 conjugated goat anti-mouse IgG (Molecular Probes), Cy3-conjugated goat anti-rat IgG (Jackson ImmunoResearch) and Cy3-conjugated donkey anti-goat IgG (Jackson ImmunoResearch). For horseradish peroxidase stainings of

Cdkn1b, biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch) was used followed by the Vectastain Elite ABC kit and Vectastain VIP substrate kit (Vector Laboratories).

***In situ* hybridization.** T3 or T7 RNA polymerase *in vitro* transcribed sense or antisense 35S-labelled RNA probes were generated from Bluescript KSII subclones containing different coding regions of *Crya1* and *Cdkn1c*, as described[11].

BrdU labelling of embryos. For BrdU-incorporation assays, pregnant mice were injected with 5-bromo-2′-deoxyuridine (0.2 ml of 15 mg/ml; Boehringer) in 0.86% saline. After 1 h, embryos were removed by Caesarean section and processed for cryosectioning. Cyrosections were denatured for 15 min at RT in HCl (2 N), neutralized with sodium borate (0.1 M, pH 8.5) for 15 min and incubated overnight with mouse anti-BrdU monoclonal antibody.

ISEL staining. Cryosections (10 μm) were ISEL (ref. 12) stained with the FragEL kit (Oncogene) according to the manufacturer's protocol. ISEL-stained sections were counterstained with methyl green (Oncogene).

RT-PCR amplification. Eyes were dissected from E14.5 embryos and RNA was isolated using TRIZOL reagent (Life Technologies) following the manufacturer's instructions. After genotyping of the embryos by PCR, the total RNA isolated from four eyes for each genotype was used for cDNA synthesis using a cDNA synthesis kit (Pharmacia) and random hexamer primers. One-tenth of this reaction was used as a template for PCR amplification using the same primers and conditions reported previously for genes encoding α-, β- and γ-crystallins, as well as for the *Hprt* used as control[4]. For *Crybb1* amplification, the following primers were used: 5′–GATACAGCACCAGGA–3′ (exon 1) and 5′–CTTCTCCAGGA-CAAA–3′ (exon 2). PCR amplification was done at 90 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min for 30 cycles. *Cryge* and *Crygf* were distinguished after amplification by digestion with *Bgl*II, which only cuts *Cryge* PCR products. Slight downregulation of both gene products was observed in mutant lens (data not shown).

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