A forkhead gene, *FoxE3*, is essential for lens epithelial proliferation and closure of the lens vesicle

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In the mouse mutant *dysgenetic lens* (*dyl*) the lens vesicle fails to separate from the ectoderm, causing a fusion between the lens and the cornea. Lack of a proliferating anterior lens epithelium leads to absence of secondary lens fibers and a dysplastic, cataractic lens. We report the cloning of a gene, *FoxE3*, encoding a forkhead/winged helix transcription factor, which is expressed in the developing lens from the start of lens placode induction and becomes restricted to the anterior proliferating cells when lens fiber differentiation begins. We show that *FoxE3* is colocalized with *dyl* in the mouse genome, that *dyl* mice have mutations in the part of *FoxE3* encoding the DNA-binding domain, and that these mutations cosegregate with the *dyl* phenotype. During embryonic development, the primordial lens epithelium is formed in an apparently normal way in *dyl* mutants. However, instead of the proliferation characteristic of a normal lens epithelium, the posterior of these cells fail to divide and show signs of premature differentiation, whereas the most anterior cells are eliminated by apoptosis. This implies that *FoxE3* is essential for closure of the lens vesicle and is a factor that promotes survival and proliferation, while preventing differentiation, in the lens epithelium.

[*Key Words:* forkhead; lens epithelium; *dysgenetic lens; FoxE3;* cataract]

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Lens formation during vertebrate eye development is a classic example of induction. (Spemann 1901) The optic vesicle—an outgrowth from the ventral forebrain that later forms the retina—induces the surface ectoderm to invaginate and bud off the lens vesicle. A polarity soon develops in the primitive lens; posterior cells differentiate into elongated, crystallin-expressing, primary lens fibers that gradually fill the vesicle, whereas the anterior surface retains a layer of undifferentiated, proliferating cells. These cells of the anterior lens epithelium continue to divide throughout life and as they migrate posteriorly into the equatorial region of the lens, they start to differentiate into secondary lens fibers (McAvoy 1980; Piatigorsky 1981).

Several transcription factors are essential for lens development. The homeodomain proteins Lhx2, Rx, and Six3 are involved in formation of and induction by the optic vesicle (Oliver et al. 1995; Mathers et al. 1997; Porter et al. 1997; Loosli et al. 1999). Lens-forming competence of the head ectoderm depends on the paired homeodomain protein Pax6 (Fujiwara et al. 1994; Gehring 1996; Furuta and Hogan 1998) and heterozygous loss of function of *PAX6* is responsible for aniridia in humans (Jordan et al. 1992; Glaser et al. 1994). Necessary components of lens fiber differentiation and maturation include growth arrest and crystallin accumulation. The basic leucine zipper (bZIP) protein L-Maf (Ogino and Yasuda 1998) and Sox high mobility group (HMG) domain proteins (Kamachi et al. 1998; Nishiguchi et al. 1998) activate α- and γ-crystallin gene expression, respectively. Cell cycle arrest requires the retinoblastoma protein (Morgenbesser et al. 1994) and the cyclin-dependent kinase (*Cdk*) inhibitors *Cdkn1b* ($p27^{Kip1}$) and *Cdkn1c* ($p57^{Kip2}$) (Zhang et al. 1998), which depend on the homeodomain protein Prox1 for their expression (Wigle et al. 1999). The cues that induce lens polarity, epithelial proliferation, and fiber differentiation are differences in growth factor content between the aqueous and vitreous humor (Hyatt and Beebe 1993; Schulz et al. 1993; Klok et al. 1998; Potts et al. 1998).

Here we report the cloning of a forkhead transcription factor gene, *FoxE3*, which is expressed in the lens epithelium. We show that *FoxE3* is mutated in a classic mouse model with defect lens development.

Results

Cloning of FoxE3

In a screen for forkhead genes from a human genomic library (Pierrou et al. 1994), previously we isolated a par-

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tial clone containing a novel forkhead motif that we named *FREAC8* (*FOXE3* according to the latest nomenclature) (Larsson et al. 1995). To locate the spatial and temporal distribution of expression, we isolated genomic clones of the mouse homolog to perform in situ hybridization. Following mouse gene nomenclature recommendations, we call this gene *FoxE3*. Sequencing of 7 kb around the forkhead motif of *FoxE3* revealed a single open reading frame of 864 nucleotides, corresponding to a 288-amino-acid protein (Fig. 1). Comparison with the sequence of human *FOXE3* cDNA clones (Å Blixt, M. Ormestad, M. Sparacio, and P. Carlsson, unpubl.) confirmed that *FoxE3* lacks introns and verified the predicted borders of the coding sequence. The amino acid sequence of FoxE3 has no obvious similarity to other proteins in the databases, outside the forkhead domain.

FoxE3 *is expressed in the lens*

Whole-mount in situ hybridization of mouse embryos from E8.5 to E10.75 and sections of embryos and adult tissues showed that *FoxE3* is expressed in the lens of the developing eye (Fig. 2). Expression is first detected as a small dot on the surface ectoderm around E9.5 (Fig. 2a) and then rapidly increases as the lens placode is formed (Fig. 2b,g). *FoxE3* expression becomes confined to the lens vesicle as this structure detaches from the surface ectoderm and initially it is evenly distributed throughout the vesicle (Fig. 2f). In situ hybridization of sections through the developing lens showed that *FoxE3* expression is switched off in posterior cells of the lens vesicle as these cells start to differentiate to lens fibers, but remains high in the anterior cells (Fig. 2h). This pattern of

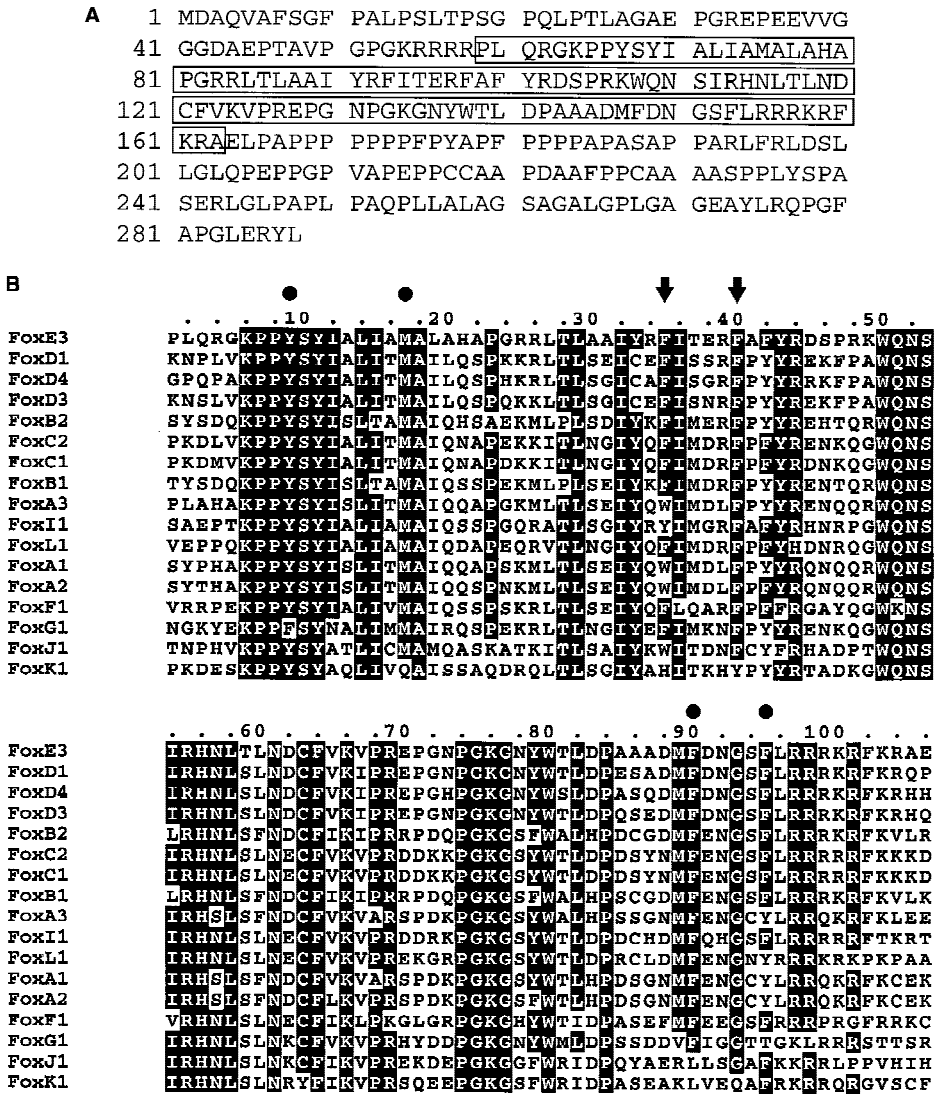

Figure 1. (*a*) Amino acid sequence of *FoxE3* predicted from DNA sequence. The forkhead domain is boxed. (*b*) The forkhead domain of *FoxE3* aligned with other mouse forkhead proteins in the SwissProt database. Phe93 and Phe98 of *FoxE3* are indicated with arrows and other amino acids in the methionine-aromatic rosette with solid circles. For conversion between the Fox nomenclature and old names, see http://www.biology.pomona.edu/fox.html.

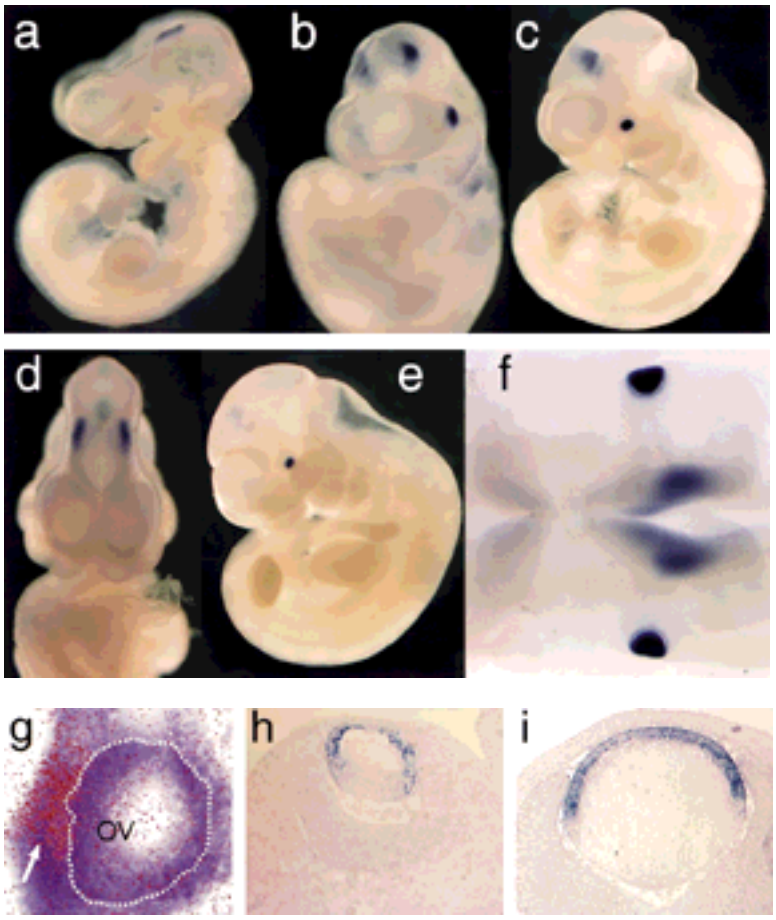

Figure 2. *FoxE3* expression in embryonic development. (*a*) Whole mount in situ hybridization of 23-somite mouse embryo showing the first signs of *FoxE3* expression on the lateral head ectoderm and on the cephalic neural folds. (*b*) 25-somite stage embryo (E9.5) with expression in lens placode and caudal forebrain. (*c*) Side view and (*d*) frontal view of 30-somite embryo (E10.25) with high expression in lens vesicle and weaker expression in caudal, dorsolateral diencephalon. (*e*) 35-somite embryo (E10.5) with high expression in lens vesicle and fading brain expression. (*f*) Dorsal view of clarified 30-somite embryo showing *FoxE3* expression in lens vesicles and diencephalon (out of focus). (*g*) Radioactive in situ hybridization showing lens placode (arrow) expression of *FoxE3* (red) in section of E9.5 embryo. Dotted line indicates the border of the optic vesicle (OV). (*h*) Section through the eye of E12.5 embryo showing *FoxE3* expression in the anterior part of the lens vesicle, but turned off in the differentiating primary lens fibers in the posterior part. (*i*) Section through the eye of E14.5 embryo demonstrating *FoxE3* expression confined to the anterior epithelium of the lens.

FoxE3 expression, limited to the undifferentiated cells covering the anterior surface of the lens (Fig. 2i), is retained throughout embryogenesis and into adulthood. Apart from the lens, *FoxE3* expression is seen in only one location during a brief period of development. It first appears on the neural folds in the cephalic region at about the same time as expression in the lateral head ectoderm (Fig. 2a). After closure of the anterior neuropore, expression is found in the most caudal, dorsolateral parts of the diencephalon (Fig. 2b,c,d). The expression level peaks at the 25- to 30-somite stage (E9.5–E10) and then rapidly vanishes. From E11.5, in situ hybridization failed to detect *FoxE3* expression at any developmental stage in any tissue, except for in the anterior lens epithelium.

FoxE3 *is colocalized with* dysgenetic lens

Given its early onset and restricted expression pattern, combined with the well-documented role of forkhead genes in embryogenesis (for review, see Kaufmann and Knöchel 1996), *FoxE3* was an obvious candidate gene for developmental lens defects. Previously, we have localized the human homolog *FOXE3* to 1p32 (Larsson et al. 1995) and although no human lens disorder has been linked to this chromosomal region, the mouse mutation *dysgenetic lens* (*dyl*) (Sanyal and Hawkins 1979) has been mapped to an area of chromosome 4 syntenic with human 1p32 (Sanyal et al. 1986; Jänne et al. 1995). *dyl* arose spontaneously in Balb/c mice and segregates as an autosomal recessive trait. The most striking phenotype in this mutant is a persistent connection between the lens and the corneal epithelium (Fig. 3f), which results from failure of the lens vesicle to close and separate from the ectoderm (Fig. 3b; Sanyal and Hawkins 1979). Reduced size, irregular shape, and disorganized structure with large vacuoles are other characteristics of the lenses of *dyl* mice (Fig. 3; Sanyal and Hawkins 1979). Lens fiber elongation and expression of crystallins are initiated in an apparently normal way (Brahma and Sanyal 1984, 1987), but the number of fibers is drastically reduced, resulting in a diminutive, irregular, and cataractic lens where lens fiber material is sometimes expelled to the exterior through the persistent ectodermal connection (Fig. 3b). In wild-type lens, a well-defined epithelial layer is seen on the anterior surface (Fig. 3e), which is replaced by elongated cells posterior of the equatorial zone (Fig. 3g). In *dyl* lens, the epithelial layer is formed during lens vesicle polarization, but then gradually disappears and is absent by the time of birth, leaving a cataractic lens where cell morphology is similar on either side of the lens equator (Fig. 3h). Apart from the eye defects, *dyl* mice appear to be normal.

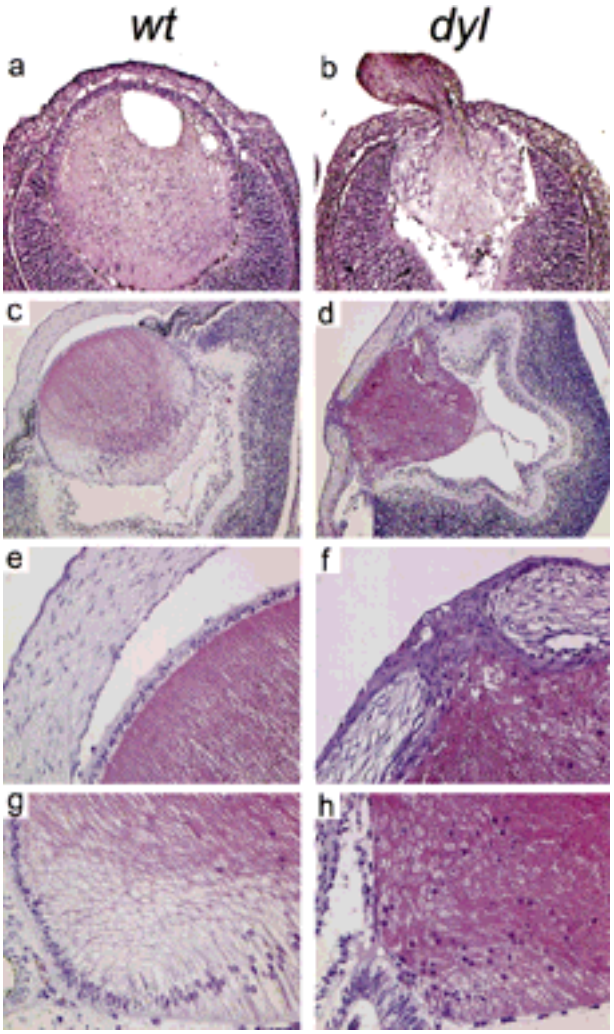

Figure 3. Hematoxylin and eosin-stained sections through the eye of wild-type (*a*,*c*,*e*,*g*) and *dyl* (*b*,*d*,*f*,*h*) mice. (*a*,*b*) Eyes from E14.5 embryos. The lens vesicle fails to close in *dyl* mutants, which in some cases leads to lens material being ejected to the exterior (*b*). (*c–h*) Eyes from newborn mice. Note the small, irregularly shaped lens (*d*) and the persistent connection between lens and cornea (*d*,*f*) in *dyl* mutants. In the equatorial zone of the lens from wild-type animals (*g*) there is a transition from the well-developed anterior epithelium to posterior elongated lens fibers. In *dyl* mutants (*h*) no clear difference is seen between anterior and posterior side and a defined epithelium is missing.

To establish whether *FoxE3* is located in the chromosomal region to which *dyl* has been mapped, we localized *FoxE3* with fluorescent in situ hybridization (FISH). As shown in Figure 4, the cytogenetic position of *FoxE3* is 4C7, which is in perfect agreement with the genetic mapping of *dyl* to linkage position 49.6 cM of chromosome 4 (Sanyal et al. 1986; Jänne et al. 1995).

dyl *mutants have mutations in* FoxE3

The entire coding region plus 600 bp of flanking sequences of *FoxE3* were determined from a homozygous *dyl* mouse. Comparison with the previously determined wild-type sequence revealed five single nucleotide substitutions, three of which alter the predicted amino acid sequence of FoxE3. One missense mutation substitutes a glutamic acid for glutamine at position 253 in the carboxy-terminal part of the protein and the other two occur close to each other within the forkhead box. Both the latter are T → C transitions that result in phenylalanines being replaced by other amino acids: Phe-93-Leu and Phe-98-Ser (Fig. 4c). The *dyl* mutant is maintained on a Balb/c genetic background, whereas our wild-type sequence was derived from 129/Sv DNA. To determine whether the observed mutations are restricted to the *dyl* mutant, or reflects differences between the two inbred strains, we also sequenced *FoxE3* from a normal Balb/c mouse. The two translationally silent mutations and the Gln-253-Glu substitution turned out to be differences between Balb/c and 129/Sv, whereas the two mutations in the forkhead box of *FoxE3* are specific for the *dyl* mutant.

The dyl *phenotype cosegregates with* FoxE3 *mutations*

The progeny from sibling matings of *dyl*/+ mice, obtained from *dyl*/*dyl* × Balb/c crossings, were scored for the *dyl* phenotype by visual inspection of the lens under stereomicroscope (2 weeks postpartum or later) or by microscopic examination of histological sections (embryos or newborn pups). Each individual was also genotyped with regard to the T277C mutation (corresponding to the Phe-93-Leu substitution) in the forkhead box of *FoxE3*. As shown in Table 1, only $FoxE3^{T277C}/FoxE3^{T277C}$ homozygotes exhibited the lens defects characteristic of *dyl* mutants.

The lens epithelium fails to proliferate in dyl *mutants*

In situ hybridization of sections through developing eyes from *dyl* embryos showed that *FoxE3* is expressed in an apparently normal way in this mutant during early lens development. At later stages the morphological differences between wild-type and mutant lens make a comparison difficult; hardly anything remains of the lens epithelium in *dyl* embryos at time points later than E15.5. At E14.5, however, the epithelium has become well defined and the general morphology of *dyl* and wild-type lenses remain similar enough to allow a direct comparison. At this stage *FoxE3* expression is confined to the anterior lens epithelium of both wild-type and mutant (Fig. 5a,b).

The lens morphology of *dyl* mice suggests that the primary defect, leading to a drastic reduction in the number of secondary lens fibers, is failure of the lens epithelium to proliferate. To investigate this, we assayed DNA synthesis in the developing eye of E14.5 and E15.5 embryos by BrdU incorporation. In the anterior epithelium of wild-type lenses from both time points, a high percentage of the cells had gone through S-phase and stained BrdU positive (Fig. 5c,e). In lenses from E14.5 *dyl* em-

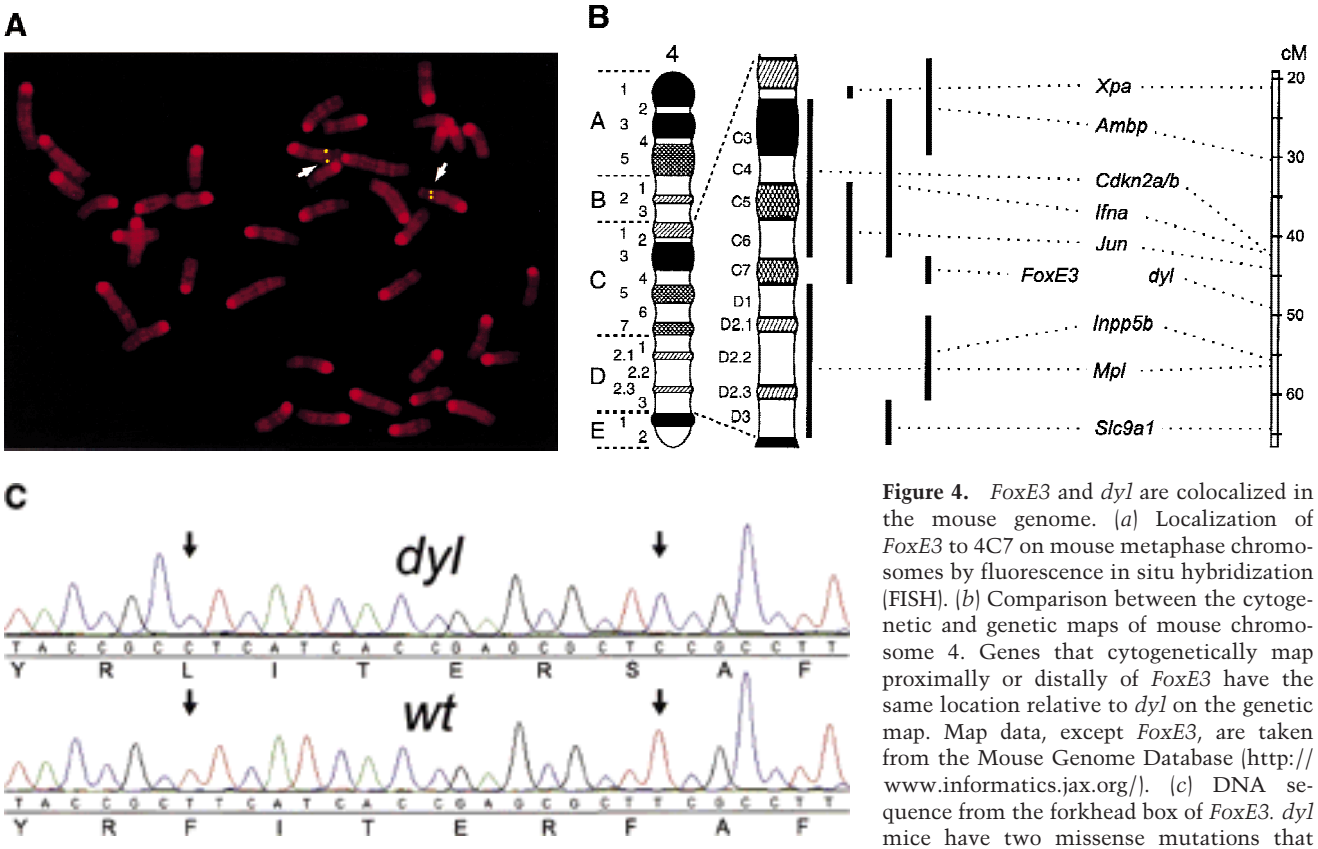

Figure 4. *FoxE3* and *dyl* are colocalized in the mouse genome. (*a*) Localization of *FoxE3* to 4C7 on mouse metaphase chromosomes by fluorescence in situ hybridization (FISH). (*b*) Comparison between the cytogenetic and genetic maps of mouse chromosome 4. Genes that cytogenetically map proximally or distally of *FoxE3* have the same location relative to *dyl* on the genetic map. Map data, except *FoxE3*, are taken from the Mouse Genome Database (http://www.informatics.jax.org/). (*c*) DNA sequence from the forkhead box of *FoxE3*. *dyl* mice have two missense mutations that substitute leucine and serine for Phe-93 and Phe-98, respectively.

bryos a morphologically distinguishable epithelium is present (for example, see Fig. 5b), but only a few cells had incorporated BrdU (Fig. 5d). At E15.5 no BrdU-positive cells could be detected in the lens (Fig. 5f). Consistent with this, the expression of proliferation markers also differ between *dyl* and wild-type. The lens epithelium from wild-type embryos contains mRNA for *Mki67* (Fig. 5g) and *Pcna* (Fig. 5i,k), encoding proliferation associated antigens, with the highest expression levels found in the germinative zone next to the neural retina. In *dyl* mutants, expression of these genes is barely detectable and the residual expression has a more anterior distribution (Fig. 5h,j,l).

dyl *lens epithelial cells are eliminated through premature differentiation and apoptosis*

In a normal developing lens, a distinct border separates the dividing cells of the epithelium from the posterior, differentiating cells and this border coincides with the limit for *FoxE3* expression. A possible role for *FoxE3*, compatible with the lack of epithelial growth in the *dyl* mutant, could be to maintain this border by preventing the expression in epithelial cells of genes involved in growth inhibition and differentiation. *Cdkn1c* (= $p57^{KIP2}$) encodes an inhibitor of Cdks and is highly expressed in the equatorial zone of the lens, where cell cycle exit occurs (Fig. 6a; Zhang et al. 1998). In wild-type E14.5 lens there is no overlap in expression between *FoxE3* and *Cdkn1c* (Figs. 5a and 6a,c), whereas in the *dyl* mutant the expression of *Cdkn1c* extends anteriorly into the epithelium (Fig. 6b,d). In addition, the expression level of *Cdkn1c* is much lower in *dyl* lens, approximately equal to that near the tip of the neural retina (Fig. 6b,d), whereas the wild-type expression is comparable to that in the periocular chondrogenic mesenchyme (Fig. 6a,c). The homeodomain protein Prox1 is essential for *Cdkn1c* expression in lens, as shown by the absence of *Cdkn1c* mRNA in lenses of *Prox1* null mice (Wigle et al. 1999). *Prox1* is expressed in the entire lens, but only

Table 1. *Cosegregation of the* $FoxE3^{T277C}$ *mutation and the* dyl *phenotype*

	+/+	$FoxE3^{T277C}$/+	$FoxE3^{T277C}/FoxE3^{T277C}$
Connection between lens and cornea. Small cataractic lens.	0	0	24
No connection between lens and cornea. Lens not cataractic.	23	52	0

Progeny from *dyl*/+ × *dyl*/+ crossings were scored for the *dyl* phenotype and were genotyped with regard to the $FoxE3^{T277C}$ mutation. The number of individuals in each category is indicated.

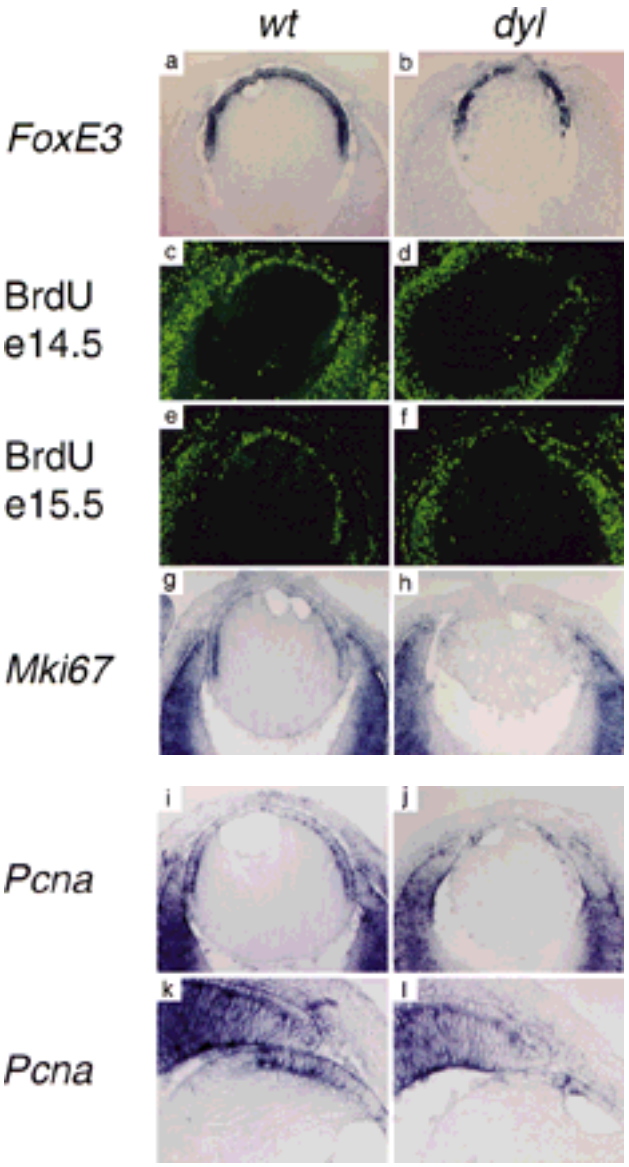

Figure 5. The lens epithelium fails to proliferate in *dyl* mutants. In situ hybridization show similar patterns of *FoxE3* expression in wild-type (*a*) and *dyl* (*b*) E14.5 lens. BrdU incorporation reveals intense proliferation of epithelial cells in wild-type lens (*c*,*e*), but a sparse distribution of replicating cells in E14.5 *dyl* lens (*d*). At E15.5 the difference is even more pronounced with only a few BrdU-positive cells in the mutant (*f*). The expression of genes encoding proliferation associated antigens *Mki67* (*g*,*h*) and *Pcna* (*i*–*l*) is down-regulated and displaced anteriorly in *dyl* mutants. All sections except *e* and *f* are from stage E14.5.

weakly in the epithelium and with the highest mRNA levels found in the equatorial zone, coinciding with the site of *Cdkn1c* expression (Fig. 6e). In the *dyl* mutant lens, the high level of *Prox1* expression extends anteriorly into the epithelium and, like *Cdkn1c*, overlaps with the expression of *FoxE3* (Fig. 6f).

Differentiation is closely linked to cell cycle arrest in lens development. The *Crya1* gene, encoding α1-crystallin, is expressed at a low level in the epithelial cells, but is induced transcriptionally to very high levels in the equatorial zone and stays high in the differentiating lens fibers (Fig. 6g). In the *dyl* mutant, epithelial cells express *Crya1* at about the same level as the rest of the lens and no induction is seen in the equatorial zone (Fig. 6h). Only in the most anterior epithelial cells is the *Crya1* expression lower. E-cadherin is expressed throughout the lens epithelium and this expression is retained until the cells reach the equatorial zone and start to differentiate (Fig. 6i). The presence of E-cadherin-expressing cells on the anterior surface of the lens from E14.5 *dyl* mice verify the presence at this stage of an epithelial layer, despite the lack of proliferation (Fig. 6j). In the mutant, expression of E-cadherin does not extend as far posteriorly as in the wild-type lens, which, together with the altered expression pattern of *Crya1*, show that the border between differentiated and undifferentiated cells is moved forward.

Platelet-derived growth factor (PDGF) A is secreted by the iris and the ciliary body, next to the germinative zone of the lens (Orr-Urtreger and Lonai 1992; Reneker and Overbeek 1996) and the *Pdgfra* gene, encoding the PDGFα receptor, is expressed in the lens epithelium (Fig. 6k,m; Morrison-Graham et al. 1992; Orr-Urtreger and Lonai 1992). In the *dyl* mutant, *Pdgfra* expression is diminished compared to wild-type and the residual expression is restricted to the most anterior part of the epithelium (Fig. 6l,n). The lack of proliferation and the down-regulation of *Pdgfra* expression suggest that lens epithelial cells in the *dyl* mutant may be experiencing a decreased growth factor receptor signaling. Because many cell types are protected from apoptosis by growth factor stimulation, we investigated whether the curtailed growth of the lens epithelium in *dyl* mutants was accompanied by programmed cell death. No signs of apoptosis could be detected in wild-type E14.5 lens, whereas the *dyl* mutant lens had many apoptotoic cells in the anterior part of the epithelium (Fig. 6o,p).

Discussion

Of 70 forkhead proteins in the SwissProt database described from metazoans and *Saccharomyces*, 67 have an aromatic amino acid in the position that corresponds to Phe-93 in FoxE3. The other site of mutation in *dyl* mice, Phe-98, is even more conserved. Here, 62 of 70 proteins have phenylalanine and 5 additional proteins have other aromatic amino acids. In the cocrystal structure of the forkhead protein FoxA3 (HNF3γ) bound to DNA (Clark et al. 1993), the aromatic residues corresponding to Phe-93 and Phe-98 of *FoxE3* are located in helix 2 of the forkhead helix–turn–helix motif and in the T′ loop connecting helix 2 and helix 3. Together with conserved aromatic residues from the amino-terminal end of the forkhead domain and from the carboxy-terminal second wing (W2), these amino acids generate a rosette-like structure in which a conserved methionine is surrounded by five aromatic side chains (Fig. 1b; Clark et al. 1993). A few proteins distantly related to the forkhead

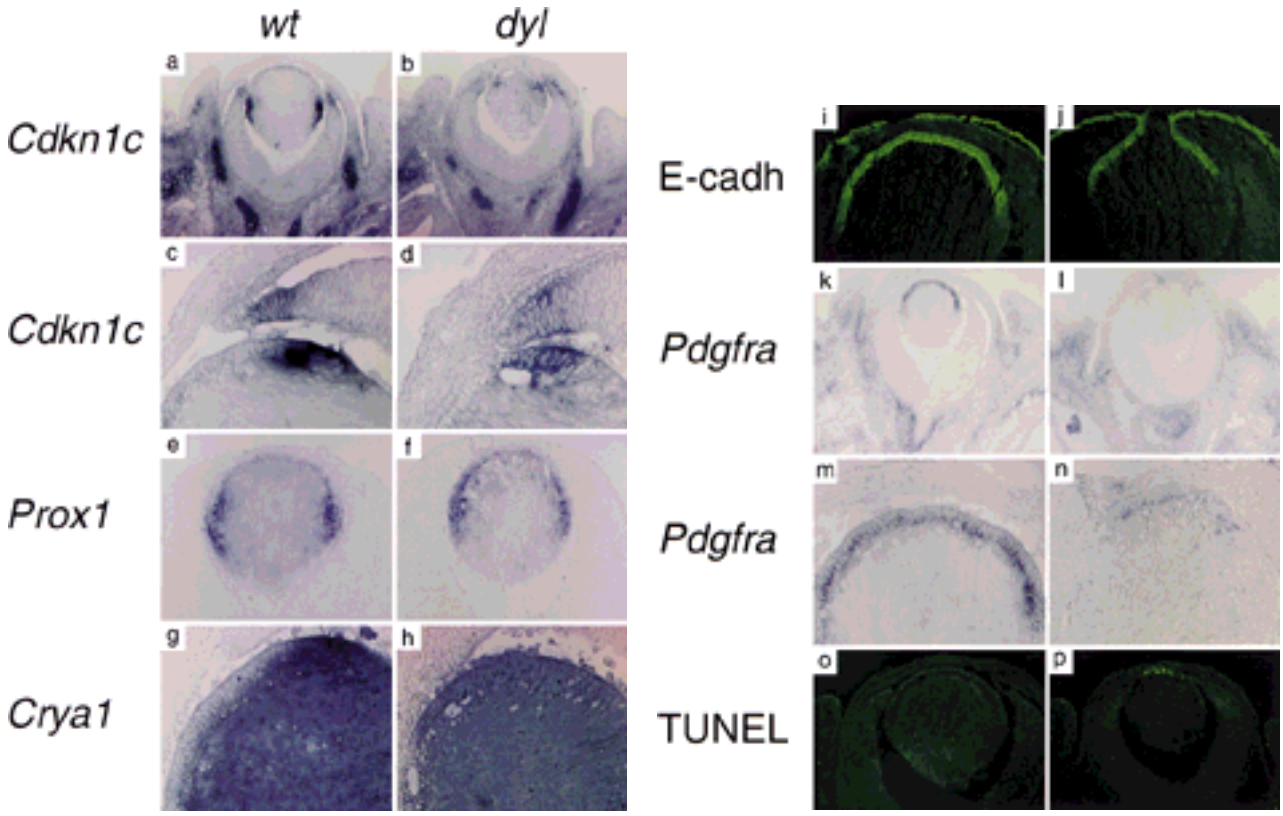

Figure 6. The lens epithelial cells in *dyl* mutants are eliminated by premature differentiation and apoptosis. In situ hybridization (*a*,*b*,*g*,*h*,*k*,*l*,*m*,*n*), immunohistochemistry (*i*,*j*), and TUNEL assay (*o*,*p*). The *Cdk* inhibitor *Cdkn1c* is expressed in the equatorial cells of the lens (*a*,*c*). In the *dyl* mutant the expression level of *Cdkn1c* is reduced and the expression is displaced anteriorly, overlapping with that of *FoxE3* (*b*,*d*). *Prox1* is mainly expressed in the equatorial, nonepithelial cells (*e*), but in the *dyl* mutant the highest expression level is seen in the posterior part of the epithelium (*f*). *Crya1* expression is low in the epithelium and highest in the equatorial zone (*g*). In *dyl*, *Crya1* is evenly expressed throughout the lens, except in the most anterior cells (*h*). E-cadherin is expressed in the epithelium of wild-type (*i*) and dyl (*j*) lens reaching further to the posterior in wild-type. *Pdgfra* is expressed in the lens epithelium (*k*,*m*), but is down-regulated and restricted to the most anterior cells in *dyl* (*l*,*n*). The anterior cells in the lens epithelium undergo apoptosis in *dyl* mutants (*p*), whereas no signs of cell death can be detected in wild-type lens (*o*). All sections are from E14.5 embryos.

main group have nonaromatic amino acids in either of the two positions corresponding to Phe-93 and Phe-98. These proteins invariably deviate from consensus in several other positions of the aromatic rosette and thus appear to rely on different intramolecular interactions. Deletion of, or mutations in, the human forkhead gene *FOXC1* (previously known as *FREAC3* or *FKHL7*) cause malformations in the anterior segment of the eye (Mears et al. 1998; Nishimura et al. 1998). In one extended pedigree the *FOXC1* mutation that cosegregates with the eye defects is a single nucleotide transition that substitutes a serine for a phenylalanine in the forkhead domain (Phe-112-Ser) (Nishimura et al. 1998). Phe-112 of FOXC1 corresponds to one of the residues in *FoxE3* (Phe-98) that is mutated in *dyl* mice and in both cases is the phenylalanine replaced by a serine. The symptoms of patients carrying the Phe-112-Ser mutation in *FOXC1* are as severe as in families where the gene is deleted, which indicates that Phe-112-Ser is a null allele. By analogy, we predict that Phe-98-Ser is a null allele of *FoxE3*. In conclusion, each of the two substitutions in the forkhead domain of *FoxE3* found in *dyl* mice would be predicted to obliterate DNA binding. The presence of two such mutations, together with the colocalization of *dyl* and *FoxE3* in the mouse genome, cosegregation of the mutations with the *dyl* phenotype and defects in *dyl* mice being confined to the tissue where *FoxE3* is expressed, strongly suggests that mutations in *FoxE3* are responsible for the *dyl* phenotype.

During normal development, the lens vesicle closes and separates from the ectoderm, a process that fails in *dyl* mice (Fig. 3; Sanyal and Hawkins 1979) and implies *FoxE3* as an essential regulator of this event. In later stages of lens development, *FoxE3* appears to be necessary for the growth and survival of the lens epithelium. In the absence of functional FoxE3 (i.e., in *dyl* mutants), initial anteroposterior polarization of the lens vesicle proceeds normally; by E13.5–14.5 a defined anterior epithelium is morphologically distinguishable and can also be identified by the presence of *FoxE3* mRNA and E-cadherin. This polarity is thought to depend on differences in growth factor content between the aqueous and

vitreous humor that bathe the anterior and posterior surfaces of the lens, respectively (Hyatt and Beebe 1993; Schulz et al. 1993; Klok et al. 1998; Potts et al. 1998). Lens fiber differentiation also appears to be independent of *FoxE3*, as judged by the expression of crystallins (Brahma and Sanyal 1984, 1987), and primary fibers fill the lumen of lens vesicles in wild-type and mutant alike. However, although the cells of a normal lens epithelium enter a phase of intense cell division, which provides the progenitors of the secondary lens fibers, the epithelial cells of a *dyl* mutant fail to proliferate and eventually disappear. One reason for this appears to be a changed expression pattern of genes known to promote cell cycle arrest. The posterior part of the lens epithelium, the germinative zone, is normally the area in which the most rapid growth occurs, but in the absence of FoxE3 activity, *Cdkn1c* and *Prox1* are expressed here. Cdkn1c blocks cell cycle progression directly by inhibiting Cdks and Prox1 indirectly by activating *Cdkn1c*. The cells in what should have been the germinative zone respond to lack of FoxE3 activity by growth arrest, as illustrated by loss of expression of *Pcna* and *Mki67* and lack of BrdU incorporation. Judged by the elevated level of *Crya1* expression and the reduced range of E-cadherin expression, the fate of the most posterior of the epithelial cells instead appears to be premature differentiation.

Cells in the equatorial zone of the lens, immediately adjacent to the epithelium, show a dramatic reduction in *Cdkn1c* expression in the *dyl* mutant. The link between FoxE3 activity in the epithelial cells and *Cdkn1c* expression in the neighboring cells can only be speculated on, but may involve a paracrine factor produced by the epithelium, the synthesis of which depends on *FoxE3*.

Although the posterior part of the lens epithelium in *dyl* mutants show signs of cell cycle arrest and premature differentiation, the most anterior cells express E-cadherin, but not *Cdkn1c* or high levels of *Crya1*. However, the lack of BrdU incorporation show that, in spite of this, no proliferation occurs. Instead, these cells appear to be eliminated by programmed cell death. *Pdgfra*, encoding the PDGFα receptor, is highly expressed in the lens epithelium and this expression appears to depend in part on FoxE3, as seen by the diminished expression level in *dyl* lens. The ligand, PDGF A, is secreted into the aqueous humor by the ciliary body, the iris, and the corneal endothelium and stimulates growth of the lens epithelium (Brewitt and Clark 1988; Potts et al. 1994; Reneker and Overbeek 1996). That *Pdgfra* is important for the response of the epithelium is shown by the developmental lens defects with reduced number of secondary lens fibers seen in *Patch/Patch* mouse embryos, homozygous for a null mutation in *Pdgfra* (Morrison-Graham et al. 1992). Therefore, it seems likely that the reduced *Pdgfra* expression contributes to the failure of *dyl* lens epithelium to proliferate, but the more severe phenotype of *dyl* compared to *Patch* mutants suggests that other types of growth factor signaling may also depend on *FoxE3*. The aqueous humor contains survival factors for the epithelial cells of the lens (Hyatt and Beebe 1993; Ishizaki et al. 1993; Renaud et al. 1994; Chow et al. 1995) and the apoptotic fate of these cells in the *dyl* mutant implies *FoxE3* as a component in the generation or reception of such signals.

Classic transplantation experiments in chick embryos demonstrated the importance of the lens epithelium for cornea development (Coulombre and Coulombre 1964; Genis-Galvez 1966; Genis-Galvez et al. 1967) and recent work on mouse embryos with null mutations in the forkhead gene *FoxC1* (*Mf1*) shows that formation of the anterior chamber depends on proper differentiation of the posterior corneal endothelium (Kidson et al. 1999). The cornea in *dyl* mutants appears poorly differentiated and lacks the dense, highly structured appearance of a normal cornea (Fig. 3c–f). The anterior chamber is missing (Fig. 3c,d) and the posterior surface of the cornea adheres to the lens, presumably due to a poorly developed corneal endothelium (Fig. 3e,f). The nature of the signal produced by the lens epithelium that induces differentiation of the cornea remains unknown, but it is likely to depend, at least partially, on *FoxE3*, either directly or indirectly through maintenance of the lens epithelium.

The eye defects of *dyl* mice resemble clinical findings in certain cases of anterior segment dysgenesis, in particular Peters' anomaly (Peters 1906; Stone et al. 1976), which is characterized by keratolenticular adhesion, central leucoma, microphthalmia, and adhesion of the iris. Occasionally, Peters' anomaly is caused by mutations in *PAX6* (Hanson et al. 1994), but in the majority of cases this gene can be excluded and the cause of the defects remains unknown (Churchill et al. 1998). The human homolog of *FoxE3*, *FOXE3*, located at 1p32 (Larsson et al. 1995), must be considered a candidate gene for this disorder.

Materials and methods

Cloning of FoxE3

A genomic 129/Sv λ library (Stratagene) was screened with a probe from the human *FOXE3* gene (Larsson et al. 1995) and overlapping *FoxE3* clones were isolated. A 7-kb *Bgl*II fragment centered around the *FoxE3* forkhead box was subcloned and sequenced on a Beckman CEQ2000 using GPS-1 transposon insertions (New England Biolabs).

PCR amplification and sequencing

DNA was prepared from Balb/c (Charles River) and *dyl/dyl* mice (Jackson Laboratory, Maine) and the *FoxE3* gene was amplified in two overlapping pieces with the primers (GGGATGGGGCCCAGAGACTGACTC and CGCAGGAAGCTACCGTTGTCGAAC; GCCCTACTCATACATCGCGCTCAT and TGGAGGAGGGCAGGGAAGGCTTAG). PCR products from *dyl* and wild-type mice were sequenced directly with the same primers used for amplification.

Genotyping of FoxE3^{T277C}

A 270-nucleotide fragment from the forkhead box of *FoxE3* was amplified from tail biopsies with the primers GCCCTACTCATACATCGCGCTCAT and CGCAGGAAGCTACCGTTGTCGAAC. Digestion of the product with *Mnl*I generated three fragments from the wild-type allele (150, 114, and 6 nucleotides)

and four fragments from the $FoxE3^{T277C}$ allele (114, 82, 68, and 6 nucleotides), which were separated on 3% MetaPhor agarose gels.

Chromosome localization

Fluorescence in situ hybridization of mouse metaphase chromosomes (Helou et al. 1998) was performed with a 9-kb digoxigenin-labeled probe containing *FoxE3*. Hybridization signals were visualized with FITC-anti digoxigenin and banding patterns by DAPI counterstaining.

Histological sections

Paraffin sections (3 µm) of heads from newborn (3 days) wild-type or *dyl* mice and cryostat sections (8 µm) from embryos were stained with eosin and hematoxylin.

In situ hybridization and immunohistochemistry

In situ hybridizations of whole mount mouse embryos (Rosen and Beddington 1994) and cryosections (Henrique et al. 1995) were performed with digoxigenin-labeled antisense RNA probes. The *FoxE3* probe consists of a 1-kb fragment from the 3′ end of the gene. Plasmids used to generate probes for *Pdgfra* were kindly provided by Dr. C. Betsholtz (Göteborg University, Sweden), for *Mki67* by Dr. H. Igarashi (Kumamoto University, Japan), and for *Crya1* by Dr. J. Piatigorsky (National Eye Institute, Bethesda, Maryland). Probes for *Pcna* were generated from IMAGE cDNA clone No. 605791. Unique parts of *Cdkn1c* (pos 200–931 in the sequence with GenBank accession no. U22399) and *Prox1* (pos 132–869 in the sequence with GenBank accession no. AF061576) were amplified by PCR from genomic mouse DNA and riboprobes were transcribed from promoters appended to the primers. The authenticity of PCR products and cDNA clones were verified by DNA sequencing. No signals were observed when control sense probes were used. Radioactive in situ hybridizations of cryosections were performed as described (Mahlapuu et al. 1998) with two ^{33}P-labeled antisense oligonucleotides from *FoxE3* (CAGGGAAGGCTTAGCCCAAGCAAGGCTCGGGGACCCAGCGAATTG and CTCAGGCTGCAAGCCCAACAGGCTGTCCAGGCGGAAGAG). E-cadherin was detected with a rat monoclonal antibody (kindly provided by Dr. H. Semb) and visualized with a FITC-conjugated secondary antibody (DAKO).

BrdU and TUNEL

Kits from Roche Biochemicals and fluorescence microscopy were used to identify BrdU incorporation and apoptotic cells by the TUNEL assay in cryosections. In vivo BrdU labeling of embryos was achieved by intraperitoneal injection of 0.1 µmole of BrdU/g body weight, 4 hr before the pregnant females were sacrificed.

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Note

The GenBank accession no. for the *FoxE3* locus (7kb) is AF142647.

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