Six3 activation of *Pax6* expression is essential for mammalian lens induction and specification

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The homeobox gene *Six3* regulates forebrain development. Here we show that *Six3* is also crucial for lens formation. Conditional deletion of mouse *Six3* in the presumptive lens ectoderm (PLE) disrupted lens formation. In the most severe cases, lens induction and specification were defective, and the lens placode and lens were absent. In *Six3*-mutant embryos, *Pax6* was downregulated, and *Sox2* was absent in the lens preplacodal ectoderm. Using ChIP, electrophoretic mobility shift assay, and luciferase reporter assays, we determined that *Six3* activates *Pax6* and *Sox2* expression. Misexpression of mouse *Six3* into chick embryos promoted the ectopic expansion of the ectodermal *Pax6* expression domain. Our results position *Six3* at the top of the regulatory pathway leading to lens formation. We conclude that *Six3* directly activates *Pax6* and probably also *Sox2* in the PLE and regulates cell autonomously the earliest stages of mammalian lens induction.

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Introduction

In vertebrates, the lens originates from the presumptive lens ectoderm (PLE). The lens placode, a thickened region of the head surface ectoderm (SE), is the first morphologic sign of lens development. Interactions between the lens placode and the optic vesicle lead to the formation of the lens pits and neuroretina. Lens induction begins in the head SE before contact between the PLE and optic vesicle is established (Grainger *et al*, 1992, 1997; Sullivan *et al*, 2004).

The homeobox gene *Pax6* is an evolutionarily conserved key regulator of metazoan eye development (Hill *et al*, 1991; Jordan *et al*, 1992; Glaser *et al*, 1992; Quiring *et al*, 1994; Ashery-Padan *et al*, 2000), and *Pax6* heterozygosity results in ocular abnormalities such as aniridia in humans (Glaser *et al*, 1994) and small eye in rodents (Hogan *et al*, 1986; Hill *et al*,

1991; Matsuo *et al*, 1993). Furthermore, in *Pax6*^{-/-} mice, the lens and other eye structures do not form (Hill *et al*, 1991; Grindley *et al*, 1995). Conditional deletion of *Pax6* in the mouse lens prevents lens placode formation (Ashery-Padan *et al*, 2000).

Lang (2004) has proposed that the preplacodal phase of *Pax6* expression regulates the later placodal phase of expression. In this context, BMP and FGF signaling act upstream of the *Pax6* pathway controlling lens placode formation (Lang, 2004). Approximately 40 conserved, non-coding sequences potentially involved in *Pax6* regulation have been identified in the *Pax6* locus (see trafac.cchmc.org), and two *Pax6* enhancers, *ectoderm enhancer* (EE) (Williams *et al*, 1998; Kammandel *et al*, 1999; Xu *et al*, 1999; Dimanlig *et al*, 2001) and *SIMO* enhancer (Kleinjan *et al*, 2001), are important for lens lineage expression. However, the upstream regulators of the preplacodal phase of *Pax6* have remained elusive. Meis family proteins regulate *Pax6* activity in the PLE (Zhang *et al*, 2002); however, Meis1-deficient embryos have partially duplicated retinae and small lenses, which suggests that either Meis2 compensates for Meis1 or other unidentified factors regulate *Pax6* in the PLE (Hisa *et al*, 2004).

Heterozygous mutations in the transcription factor *Sox2* that produce hypomorphic conditions occur in about 10% of humans with anophthalmia or severe microphthalmia (Fantes *et al*, 2003; Hagstrom *et al*, 2005; Ragge *et al*, 2005; Zenteno *et al*, 2005). *Sox2* is known to regulate δ -crystallin expression in the chick in a complex with *Pax6* (Kamachi *et al*, 2001). *Sox2*-null mice die at preimplantation (Avilion *et al*, 2003), thereby preventing analysis of this gene's role(s) in eye development. *Sox2* expression is absent in mutant mouse strains with severe lens defects such as *Bmp4*, *Pax6*^{Sey/Sey} and *Bmp7* (Furuta and Hogan, 1998; Wawersik *et al*, 1999).

Sox2 expression precedes lens placode formation (Furuta and Hogan, 1998) and its upregulation in the PLE is one of the earliest indicators of lens specification and is dependent on *Pax6* (Furuta and Hogan, 1998; Zygari *et al*, 1998). Upregulation of *Sox2* was not detected in the SE of *Pax6*^{Sey-1Neu}-homozygous embryos in which lens induction is defective (Furuta and Hogan, 1998). However, the early unaffected phase of *Pax6* expression in the SE of *Pax6*-Le conditional mutant embryos was sufficient to upregulate *Sox2* expression in this tissue (Ashery-Padan *et al*, 2000), a finding that indicates that upregulation of *Sox2* in the PLE is dependent on early, but not late *Pax6* activity.

Six3 protein expression was reported to start at around E9.5 in the vertebrate lens placode (Oliver *et al*, 1995), and forced *Six3* expression in fish and mouse embryos promotes ectopic lens (Oliver *et al*, 1996) and neural retina formation (Loosli *et al*, 1999; Lagutin *et al*, 2001). Until recently, investigating the *in vivo* role of *Six3* in eye development was not possible, because the prosencephalon, the brain region from which eyes form, is severely truncated in

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Six3^{-/-} mice (Lagutin *et al*, 2003). However, by using a *Cre/loxP* approach to inactivate *Six3* in the developing eye, we can now address this question.

Results

Cre/loxP-mediated removal of Six3 activity from the PLE

To address the *in vivo* role of *Six3* during lens morphogenesis, we generated the floxed *Six3* mouse line *Six3*^{f/w} (nomenclature and details in Supplementary Figure S1). During the initial crosses of *Six3*^{f/w} mice with the *Le-Cre* mouse strain (Ashery-Padan *et al*, 2000), the transgenic *Cre* strain was occasionally germline active. This allowed us to generate the *Six3* Δ allele in which the floxed sequence encoding the Six domain and homeodomain of *Six3* was permanently deleted (Supplementary Figure S1). The generation of the *Six3* ^{Δ/Δ} mice confirmed that the *Six3* Δ allele was a true null allele; the prosencephalon truncation in *Six3* ^{Δ/Δ} mice was indistinguishable from that previously reported in *Six3*^{-/-} mice (Lagutin *et al*, 2003). Furthermore, antibody staining of the generated *Six3*-mutant lens failed to detect any signal (see below) when we used an anti-*Six3* antibody (our unpublished data) that recognizes a *Six3* epitope downstream of the floxed DNA sequence.

Six3 ^{Δ/w} ; *Le-Cre* mice were generated by crossing *Six3* ^{Δ/w} mice with *Le-Cre* mice in which *Cre* activity in the PLE was reported to start at around E9.0 (Kammandel *et al*, 1999; Ashery-Padan *et al*, 2000). *Six3*^{f/f} mice were generated by intercrossing *Six3*^{f/w} mice. Finally, *Six3*^{f/ Δ} ; *Le-Cre* mice were obtained by crossing *Six3*^{f/f} females with *Six3* ^{Δ/w} ; *Le-Cre* males. *Six3*^{f/w} embryos, which were indistinguishable from wild-type embryos, were used as controls.

Six3 mediates lens morphogenesis

Gross morphological analysis of the generated *Six3*^{f/ Δ} ; *Le-Cre* mice revealed several lens phenotypes, including drastically reduced lens size, cataracts, or absence of the lens (Figure 1A–D, A'–D'). These defects were even more obvious in the analysis of lens sections. Three major phenotypes were observed at any particular stage: mild (type I), moderate (type II), and severe (type III). A summary of this analysis is included in Supplementary Table 1.

Defects in lens development were evident as early as E10.5. Reduction in the size of the invaginating lens pit was observed in mildly (not shown) and moderately affected mutant embryos (Figure 1F and G). Embryos with the severe (type III) lens phenotype showed minimal thickening and no invagination of the PLE (Figure 1H). The shape of the optic vesicle was also defective (Figure 1H), although *Six3* was not removed from it (Figure 3B' and E'). Conditional deletion of *Pax6* from the PLE also resulted in defective invagination of the optic vesicle (Ashery-Padan *et al*, 2000). Variability in the severity of lens defects was also observed at later stages. At E12.5, the lens vesicle was small but relatively normal in mildly affected mutant embryos (Figure 1J), smaller and abnormal in others (Figure 1K), and totally absent in type III embryos (neuroretina was also deformed; Figure 1L). At E14.5, some embryos had an abnormally persistent lens stalk and disorganized lens fibers (Figure 1N), and others had a very small and abnormal lens (Figure 1O and P). Thus, removal of *Six3* from the PLE during lens induction disrupts lens formation.

The time and extent of Le-Cre-mediated Six3 excision from the PLE is variable

As mentioned before, *Le-Cre* activity in the PLE was reported to start around E9.0. (Kammandel *et al*, 1999; Ashery-Padan *et al*, 2000). To determine whether the variability in the severity of the lens phenotype reflects differences in the timing and extent of *Six3* excision from the PLE, we characterized the temporal and spatial activity of *Le-Cre* by using the *R26R* strain (Soriano, 1999). We found that the time and extent of *Le-Cre* activity was variable (Supplementary Figure S2). At around the 22-somite stage, the most common and rather specific *Cre* activity was detected in the lens SE (Supplementary Figure S2A and B). In addition, a less common and more variable phase of *Le-Cre* activity was also identified in the putative lens SE as early as the 7- to 11-somite stage (Supplementary Figure S2C–I); variations in the time and extent of *Le-Cre* were observed between the left and right eyes of individual embryos (Supplementary Figure S2C and D). X-gal⁺ cells were also found in mesenchymal and neural cells (Supplementary Figure S2G–I), and in a few cases, X-gal-expressing cells were ubiquitously distributed along most of the embryo (Supplementary Figure S2F). Consistent with these results, we also found variability in the time and extent of *Cre* protein expression in the PLE; extensive *Cre* expression was seen in the PLE of a 16-somite-stage embryo (Supplementary Figure S2J); in contrast, fewer positive cells were seen in the same region of a somite-matched littermate (Supplementary Figure S2K).

According to these results, the variability in the time and extent of *Six3* removal from the PLE (compare Figure 3B' with Supplementary Figure S3D and G) and, therefore, the severity of the *Six3* lens phenotype are probably direct consequences of the variable onset of *Le-Cre* activity. In some E9.5 *Six3*-mutant embryos, high levels of *Six3* remained in the PLE (Supplementary Figure S3D and G); therefore, the levels of *Pax6* and *Sox2* expressed in the PLE were normal or only mildly reduced (Supplementary Figure S3E and F). These differences could account for the milder (types I and II) phenotypes observed in some *Six3*-mutant embryos at later stages.

In this experimental setting, *Six3* deletion from the PLE probably ranged from about the 7-somite stage to E9.5; from E9.0 to E9.5 for most of the characterized less severe lens mutant embryos; and starting from the 7-somite stage onward for the reduced percentage of embryos that exhibited the most severe lens phenotype.

Lens differentiation is not affected in mildly or moderately affected Six3-mutant lenses

Six3 mutants with mild lens phenotypes (types I and II) have lens pits and lens vesicles, indicating that lens placode formation was not severely affected. This conclusion was supported by the normal expression of the early lens placodal markers *Pax6*, *Sox2*, *Mab211l*, *sFrp2*, and *FoxE3* at E10.5 (data not shown), and *FoxE3* (Figure 2A') and *Prox1* (Figure 2B') at E12.5. Lens differentiation also appeared unaffected, as shown by the normal expression of β -crystallin (Figure 2C'). However, an increase in cell death was observed in the lens pit at E10.5 (see Figure 2D'), and although the percentage of BrdU⁺ cells was unaffected at this stage (Figure 2E'), at E12.5 proliferating cells were abnormally located in the posterior

Figure 1 Conditional deletion of *Six3* in the PLE causes severe lens phenotypes. Compared to age-matched control lenses (A–D), *Six3*^{fl/a};*Le-Cre* mutant eyes showed a range of lens defects at representative stages. (A') At E12.5, the mutant eye appeared smaller, and the shape was abnormal. The shape of the retinal-pigmented epithelium (arrowhead) was defective. (B') At E14.5, defects in the shape of the mutant eyes were consistently identified, and the lens (arrowhead) was extremely reduced. (C') Three-month-old isolated mutant eyes exhibited obvious retinal defects such as irregular shape (arrow). The isolated lens was very small and degenerated and appeared to have cataracts (arrowhead). (D') At 6 months, the eye lids of *Six3*^{fl/a};*Le-Cre* mutant mice were closed (arrowhead), suggestive of anophthalmia or microphthalmia. (E–P) H & E-stained sections of control and *Six3*^{fl/a};*Le-Cre* mutant lenses. At E10.5, some mutants exhibited a smaller abnormal lens pit (arrows in F, G). (H) No lens pit (arrow) was present in those with the most severe phenotype, and the retina appeared to have been duplicated (arrowhead). At E12.5, some type I mutants exhibited slightly reduced lens vesicle (arrow in J), and some type II mutants showed greatly reduced ones (K). No lens vesicle was present in type III mutants (arrow in L). Similar phenotypic variations were seen at E14.5. The lens was smaller (N) or very reduced and abnormal (O, higher magnification in P). The lens stalk was frequently observed (arrow in N).

lens compartment (Figure 2F'). Therefore, in moderately affected *Six3*-mutant lenses, cells in the posterior margin do not exit the cell cycle, and lens polarity is probably not established, as also indicated by the ectopic expression of *FoxE3* in that region (Figure 2A').

Lens induction/specification is defective in the severely affected *Six3*-mutant lens

Our studies focused on the severe lens phenotype (type III), which most likely resulted from early (7-somite stage onwards), extensive removal of *Six3* activity from the PLE.

Le-Cre activity was already abundant in the PLE of *Six3*-mutant embryos at E9.5 (Figure 3A) and started decreasing at E10.5 (Figure 3A'). Lens specification normally occurs at around E9.0–E9.5. In severely affected embryos, *Six3* expression was already removed from the mutant PLE at E9.5 (Figure 3B'); *Six3* expression in the neural retina remained unaffected. *Pax6* expression was drastically reduced (Figure 3C'), and *Sox2* expression was absent in the mutant PLE (Figure 3D'). The few *Pax6*-expressing cells remaining in some mutant PLE were most likely remnants of *Pax6* that was expressed before Cre-mediated deletion of *Six3*.

Figure 2 Lens specification and differentiation is not altered in the moderately affected *Six3*^{flb}/*Le-Cre* mutants. Expression of *FoxE3* (A'), *Prox1* (B'), and β -crystallin (C') in the moderately affected E12.5 lenses indicated that placode formation and lens differentiation had occurred in some *Six3*^{flb}/*Le-Cre* embryos. Nevertheless, the polarity of the lens vesicle was affected; persistent abnormal *FoxE3* expression was detected in the posterior part of the lens vesicle (A', arrow). (D') Increased apoptosis was detected in E10.5 *Six3*-mutant lens pit (arrowhead in rectangle; $3 \pm 1\%$ for the control, $25 \pm 5\%$ for the mutant, $n = 3$). (E-E') The percentage of BrdU⁺ cells was normal in E10.5 *Six3*-mutant lens pit (arrowhead in rectangle; $42 \pm 0.6\%$ for the control, $41 \pm 1\%$ for the mutant, $n = 3$); however, ectopic BrdU⁺ cells were found in the posterior part of the lens vesicle at E12.5 (arrowheads in F').

Consistent with these results, *Six3* expression was not detected in the mutant PLE at E10.5 (Figure 3E') but was normal in the neural retina, and no lens-like structures were present, as indicated by the absence of thickening and invagination of the SE. In adjacent sections, *Pax6* expression was barely detectable (Figure 3F') in the mutant SE, and *Sox2* was absent (Figure 3G'). Consequently, expression of downstream markers of the invaginating lens pit such as *Prox1* (Figure 3H'), *sFrp2* (Figure 3I'), and *FoxE3* (Figure 3J') was also undetected in the *Six3*-mutant lens. Expression of *Meis1* (Figure 3K') and *Meis2* (data not shown), which directly regulate *Pax6* expression in the lens placode (Zhang *et al*, 2002), appeared unaffected.

Six3 is a direct transcriptional regulator of *Wnt1* in the developing mouse forebrain (Lagutin *et al*, 2003), and the Wnt/ β -catenin signaling pathway mediates mouse lens development (Smith *et al*, 2005). To determine whether alterations in the Wnt/ β -catenin pathway influenced lens formation in *Six3*-mutant embryos, we used the available *Bat-Gal* reporter mouse that expresses *lacZ* in response to activation of the canonical Wnt pathway (Maretto *et al*, 2003). Control and *Six3*-mutant E9.5 and E10.5 PLE isolated from crosses of *Bat-Gal* and *Six3*^{flb}/*Le-Cre* mutant mice were both negative for X-gal staining (data not shown). In addition, no nuclear localization of β -catenin was observed in the SE of mutant embryos (data not shown).

These results indicate that the lens phenotypes resulting from the specific removal of *Six3* are probably not related to defects in the Wnt/ β -catenin pathway. However, the drastically reduced expression of *Pax6* and the absence of

Sox2 in the type III *Six3*-mutant PLE indicate that lens induction/specification are defective in these severely affected embryos. Thus, *Six3* activity in the PLE is essential during lens induction.

The time of *Six3* removal determines the severity of the lens phenotype

To remove *Six3* activity at different developmental stages as efficiently as possible but without compromising its role in forebrain formation (Lagutin *et al*, 2003), we used the *CAGG-CRE-ER* mouse strain in which ubiquitous Cre activity is induced by tamoxifen (Hayashi and McMahon, 2002). Floxed *Six3* (*Six3*^{flb}) females were bred with *Six3*^{flb}/*CAGG-CRE-ER* males, and two injections of tamoxifen were given to the pregnant dams at approximately E7.8 and early E8.5 for early *Six3* deletion, and at E9.5 and E10.0 for late deletion; embryos were then isolated at E10.5. Both protocols deleted *Six3* activity efficiently (Figure 4A' and G'), although defective forebrain patterning was observed in some of the early-injected embryos, which were then no longer considered for the analysis of the lens.

No thickening or invagination of the PLE was seen at least in one side of six of the seven E10.5 mutant embryos in which tamoxifen was administered early (E7.8 and E8.5; Figure 4A' and B'). *Pax6* and *Sox2* expression were not detected in the PLE but were observed at lower levels in the optic cup (Figure 4C' and D'). The expression of *FoxE3*, *sFrp2*, and *Prox1* was not detected in the mutant PLE (data not shown), but that of *Meis1* (Figure 4E') and *Meis2* (data not shown) was unaffected. Thus, lens specification was defective, a phenotype

Figure 3 Lens induction and specification are defective in the severely affected *Six3^{f/fΔ};Le-Cre*-mutant lens. Cre activity was restricted to the PLE in E9.5 (A) and E10.5 (A') *Six3^{f/fΔ};Le-Cre*-mutant embryos. (B') *Six3* expression was deleted from the mutant PLE (arrow) but was unaffected in the developing optic vesicle (arrowhead). (C') Pax6 expression in the PLE was drastically reduced (arrow), and that of Sox2 was absent (arrow in D'); however, Pax6 and Sox2 expression in the optic vesicle remained normal (arrowheads in C', D'). (E'–K') Lens induction and specification was arrested, as indicated by the lack of thickening or invagination of the PLE and the absence of Sox2 expression. In E10.5 *Six3^{f/fΔ};Le-Cre* mutant embryos, *Six3* expression in the PLE was deleted (arrows in E') but not that in the neural retina lineage. In this embryo, the optic vesicle appeared to be duplicated. (F') Pax6 expression was downregulated in the PLE (area between arrowheads), and that of Sox2 (G', region between the two arrows) was absent. Expression of lens differentiation markers Prox1 (H'), *sFrp2* (I'), and *FoxE3* (J') was not detected in the mutant tissue, where the lens pit should have formed. (K') Expression of *Meis1* remained unchanged.

similar to that of the severely affected type III embryos described above when using *Le-Cre*. Interestingly, although the shape of the developing optic cup was abnormal, typical retinal progenitor markers (*Pax6*, *Sox2*, *Chx10*) were still detected (Figure 4C', D' and F').

Lens specification was not obviously affected (i.e., the lens pit was normal or slightly smaller) in E10.5 mutant embryos ($n = 7/7$) in which tamoxifen was administered later (E9.5

and E10.0; Figure 4G'). *Pax6* expression appeared normal in the mutant lens pit (Figure 4H'), and that of *Sox2* was normal or slightly reduced (Figure 4I'), characteristics reminiscent of those described for the types I and II *Six3*-mutant lenses when using *Le-Cre*. A graphic representation of these phenotypes is presented in Figure 4J.

To complement these studies, we also examined early deleted (tamoxifen injection at approximately E7.8 and

Figure 4 The timing of *Six3* deletion is crucial for the lens phenotype. Ubiquitous, early deletion of *Six3* via tamoxifen injection of pregnant *Six3^{f/fΔ};CAGG-Cre-ER* dams at E7.8 and E8.5 (A'–F') and late deletion via injection at E9.5 and E10.0 (G'–I') were performed. Mutant embryos were analyzed at E10.5. Only early deletion of *Six3* caused a failure in lens specification. (A') *Six3* expression was not detected in the PLE (arrow) or optic vesicle (arrowhead). (A', B') The PLE did not thicken and the lens placode did not form. (C', D') Defective lens induction and specification was confirmed by the absence of Pax6 and Sox2 from the PLE of the mutant embryos (arrows). (E') Expression of Meis1 remained in the SE. Although *Six3* was deleted ubiquitously and the shape of the optic vesicle was abnormal, neural retina expression of Pax6 (arrowhead in C'), and Chx10 (arrow in F') remained. (G'–I') Embryos subjected to late *Six3* deletion exhibited normal or slightly small lens pits. Pax6 expression was not affected (arrow in H'), and Sox2 was either not affected or was slightly reduced (I'). (J) Graphic representation of the number of analyzed mutant embryos with early or late *Six3* deletion per the observed lens phenotype (normal, small, or no lens pit (LP)).

early E8.5) *Six3*-mutant embryos at around E9.0 (18-somite stage, prior to the upregulation of Sox2 in the PLE). In two independent *Six3*-mutant embryos (Figure 5A' and D'), only a few *Six3*-expressing cells remained in the PLE, and *Six3* expression was efficiently deleted in the evaginating optic cup (bilateral differences in the rate of *Six3* deletion and severity of the lens phenotype were occasionally observed).

In adjacent sections, Pax6 expression was consistently reduced in the mutant PLE (Figure 5B' and E'), although at different levels; it was almost undetectable in some embryos (Figure 5E') and reduced in others (Figure 5B'). Normally, low levels of Sox2 expression are detected in the PLE at this early stage (Figure 5C and F); however, Sox2 expression was further reduced in the PLE of one of the analyzed mutant

Figure 5 Early deletion of *Six3* downregulates *Pax6* preplacodal expression. Ubiquitous, early deletion of *Six3* by injection of tamoxifen at E7.8 and E8.5 in *Six3^{fl/Δ};CAGG-Cre-ER* mutant embryos analyzed at E9.0 (18-somite stage). (**A'**, **D'**) *Six3* activity was almost completely removed from the PLE (arrowheads) and optic vesicle. *Pax6* (**B'**, **E'**) and *Sox2* (**C'**, **F'**) expression was reduced in the PLE, although with varying efficiency.

littermates (Figure 5C') and not detected in the PLE of the second one (Figure 5F'). Our results suggest that the downregulation of *Pax6* and consequent lack of upregulation of *Sox2* in the PLE results from the removal of *Six3* at early stages.

Together, these results argue that the time of *Six3* deletion is critical for the lens phenotype, and early *Six3* deficiency is responsible for the failure in lens specification. Early *Six3* deletion drastically downregulates *Pax6* and *Sox2* expression in the PLE, and late *Six3* deletion minimally affects their expression.

Six3* expression in the PLE precedes that of *Pax6

We have now demonstrated that early conditional removal of *Six3* from the PLE downregulates *Pax6* preplacodal expression and arrests lens formation; the resulting lens phenotype is similar to that of *Pax6*^{-/-} embryos (Grindley *et al*, 1995). Therefore, the *Six3*-promoted lens phenotype may be caused by *Six3*'s direct regulation of preplacodal *Pax6* in the head SE. To support this proposal, *Six3* expression in the presumptive lens SE must start as early as (if not before) that of *Pax6*. To test this, we characterized the expression profiles of the two proteins starting at around the 5-somite stage (E8.0). Besides strong expression in the anterior neuroectoderm, *Six3* was also detected in the head SE that will become lens ectoderm (Figure 6A); barely detectable levels of *Pax6* were seen in the adjacent sections (Figure 6A'). Around the 7- to 8-somite

stage, *Pax6* become visible in the PLE (Figure 6B'); at around E8.5–9.5, *Six3* and *Pax6* were both localized in the PLE (Figure 6C and C'), lens placode, and optic vesicle (Figure 6D and D').

Six3 PLE expression at around early E9.5 is initially independent of *Pax6*; later, it becomes *Pax6*-dependent (Purcell *et al*, 2005). The finding that *Six3* expression in the head SE precedes that of *Pax6* suggests that *Six3*-mediated upregulation of *Pax6* is a necessary early step in the commitment toward a lens fate. Supporting this argument, *Six3* expression in the PLE was not overtly affected in 6- or 8-somite-stage *Pax6*^{Sey-1Neu/Sey-1Neu}-mutant embryos (Figure 6E' and F'), but was downregulated at later stages (Figure 6G') and was absent at around E9.5–E10.5 (Figure 6H'). In contrast, *Six3* expression in the neural retina was unaffected.

We conclude that *Six3* activity in the SE precedes that of *Pax6*, and that *Pax6* activity, although not necessary to induce *Six3* expression, is later required for *Six3* maintenance and upregulation in the PLE.

***Six3* binds to *Pax6* and *Sox2* lens enhancers**

Pax6 deficiency causes a lens phenotype (Grindley *et al*, 1995) similar to that of the severe type III *Six3* mutants. This similarity led us to hypothesize that *Six3* directly activates *Pax6* expression during early lens morphogenesis (i.e., during lens induction). In addition, it has been suggested that *Sox2* is downstream of the *Pax6* preplacodal phase (Furuta

Figure 6 Six3 expression in the PLE precedes that of Pax6 and is initially unaffected in *small eye*-mutant embryos. (A) Six3 is expressed in the PLE (arrowhead) of a wild-type 5-somite-stage embryo. (A') Pax6 expression is not yet detected in that region. At later stages, Six3 (B–D) and Pax6 (B'–D') are detected in the PLE (arrowheads) and optic vesicles. Six3 expression in the PLE was examined in *small eye* (*Pax6^{Sey-1Neu/Sey-1Neu}*)-mutant embryos. Pax6 was not required for the initial expression of Six3 in the PLE (arrowheads in E', F') but was required for its maintenance in the PLE at later stages (arrowheads in G', H').

and Hogan, 1998). Sox2 expression was absent from the PLE of type III *Six3*-mutant lenses. In this context, Six3 could also directly or indirectly activate Sox2 expression in the PLE.

During lens formation, *Pax6* is regulated by the *EE* (Williams *et al*, 1998; Kammandel *et al*, 1999; Xu *et al*, 1999) and *SIMO* enhancers (Kleinjan *et al*, 2001). In the case of *Sox2*, three lens enhancers (*N3*, *N4*, *L*) have been identified in the chick (Uchikawa *et al*, 2003); however, only *N3* and *N4* function during lens specification. We have identified a consensus core ATTA motif as the typical Six3 DNA-binding motif (Zhu *et al*, 2002). Visual inspection of the available DNA sequences of *EE*, *SIMO*, *N3* and *N4* enhancer regions identified multiple ATTA motifs (Supplementary Figure S4B–E).

To determine whether Six3 binds *Pax6* and *Sox2* lens enhancers *in vitro*, we performed an electrophoretic mobility shift assay (EMSA) using DNA fragments representing *EE*, *SIMO*, *N3*, and *N4*. Full-length Six3 protein specifically retarded the four different enhancer elements (Figure 7A). The binding specificity was reflected by the reduced amount of complex that formed when nonradioactive probes were added, and a super-shift of the GST-Six3 probe complex was seen when using an anti-Six3 antibody (Figure 7A).

The presence of more than one retarded band for each enhancer suggests that more than one Six3-binding site is present in each element.

To determine whether Six3 directly binds to *Pax6* and *Sox2* regulatory elements *in vivo*, we initially performed ChIP assays on the prospective head (Six3-expressing) and trunk (Six3-free) regions of E8.5 (11- to 17-somite stage) wild-type mice. TaqMan-based real-time PCR was used for quantification (details in Supplementary data). For *EE*, *SIMO*, and *N4*, the ratio for the head was significantly higher (> 5) than the ratio for the trunk (1.20–2.97), indicating that Six3 protein was bound to these enhancers when using chromatin derived from the Six3-expressing head region (Figure 7B). For the *N3* enhancer, there were no considerable differences in the ratio between heads and trunks, indicating that Six3 did not bind to it. This result could be due to the fact that in the chick, *N3* is normally active around the lens placode stage (equivalent to approximately 25-somite stage in mice) (Uchikawa *et al*, 2003), and the chromatin sample used for the ChIP assay was from 11- to 17-somite mouse embryos.

Mouse Six3 is a transcriptional repressor (Kobayashi *et al*, 2001; Zhu *et al*, 2002; Lopez-Rios *et al*, 2003); however, transcription factors often behave as activators or repressors,

Figure 7 Six3 is a direct activator of *Pax6* and most likely of *Sox2* lens lineage expression. **(A)** Bacterially expressed Six3 protein (full-length) and [32 P]-labeled *Pax6* EE, *Pax6* SIMO, *Sox2* N3, and *Sox2* N4 enhancer fragments were subjected to EMSA. Six3-bound DNA fragments are indicated by solid arrowheads and super-shifted bands (following addition of anti-Six3 antibody), by open arrowheads. The binding specificity was determined by the ability of the complex to be competed by 400 $\times$ excess of nonradioactive probes. Position of free probe is indicated by the solid arrows. **(B)** Six3 protein was enriched in selected regions of the *Pax6* EE, *Pax6* SIMO, and *Sox2* N4 enhancers *in vivo*. Chromatin prepared from 11- to 17-somite stage heads and trunks was used for the ChIP assays and real-time PCR analysis. For *Pax6* EE, *Pax6* SIMO, and *Sox2* N4 enhancers, the calculated ratio when using the heads as a DNA source was significantly higher (>5) than the ratio obtained when using the trunks (1.2–2.97), a result indicating that Six3 protein was bound to those regions. For the *Sox2* N3 enhancer, the ratio for the head (1.52) was relatively similar to that for the trunk (1.2–2.97), suggesting that Six3 protein was not bound to that region, at least at that particular stage. **(C)** Luciferase transcriptional reporter assays showed that Six3 activates *Pax6* lens lineage expression in the context of the *Pax6* EE. The *pCAB-Six3* (blue bars) plasmid activated the *Pax6* lens lineage reporter plasmid (*Pax6-EE-luc*); *pCAB-Six3VP16* (green bars) enhanced this activation, and *pCAB-Six3EnR* (red bars) attenuated it. Each bar represents six replicates ($\pm$ s.d.). **(D)** Luciferase reporter assay also showed that *Pax6* EE regulates *Pax6* lens lineage expression by Six3. *Pax6-ΔEE-luc* (red bars) attenuated the activation of luciferase activity (blue bars). Each bar represents six replicates ($\pm$ s.d.). **(E)** Six3 might also regulate *Sox2* lens lineage expression. The *pCAB-Six3* slightly increased RL-TK-N4 reporter activity (green bars), and slightly affect that of RL-TK-N3 (red bars), as compared to the activity of the control RL-TK reporter (blue bars). Each bar represents four replicates ($\pm$ s.d.). **(F)** Electroporation of *pCAB-Six3-IRES-GFP* into the SE of chick embryos at HH8 stage (4- to 5-somite stage) specifically expanded the *Pax6* expression domain only on the electroporated side (arrow). **(G)** Proposed gene-regulation network for lens cell fate specification. Solid arrows indicate direct regulation, and dashed arrows indicate potential direct regulation that has not yet been proven.

depending on the context. To determine whether Six3 functions as a transcriptional activator in the PLE, we generated a *Pax6* lens lineage luciferase reporter by inserting a luciferase-coding fragment under the control of *Pax6 EE*. Cotransfection with a *pCAB-Six3* expression plasmid activated the *Pax6* lens lineage reporter (*Pax6-EE-luc*) (Figure 7C); *pCAB-Six3VP16* expression plasmid enhanced the activation more than two-fold, and the repressor form of Six3 (*pCAB-Six3EnR*) attenuated it. Furthermore, reporter activation was attenuated when the *Pax6 EE* (*Pax6EE*) was missing (Figure 7D). Similar experiments using DNA fragments from the *SIMO* enhancer did not produce consistent results (data not shown). Therefore, at least in the context of *Pax6 EE*, Six3 activates *Pax6* lens lineage expression.

A similar approach was used with *N3* and *N4* enhancer elements, except that the *pRL-TK* reporter plasmid was selected, as carried out in the original Sox2 enhancer study (Uchikawa *et al*, 2003). Cotransfection with the *pCAB-Six3* expression plasmid slightly increased *N4* lens lineage reporter and slightly decreased *N3*. The *pCAB-Six3VP16* expression plasmid slightly enhanced the activation of *N4*, and *pCAB-Six3EnR* did not have any obvious effect (data not shown). These results suggest that Six3 activates Sox2 expression, although the two lens enhancers could respond differently.

Electroporation of mouse Six3 promotes ectopic Pax6 expression in the chick SE

Our data suggest that Six3 specifically activates preplacodal *Pax6* expression in the PLE during the early-somite stage. To test this hypothesis further, we electroporated a full-length *Six3* expression construct (*pCAB-Six3-IRES-GFP*) or GFP (*pCAB-IRES-GFP*) into one side of the ectoderm, posterior and lateral to the PLE of Hamburger–Hamilton (HH) stage 8 chick embryos (4- to 5-somite stage). After overnight culture, we assessed the expression of *Pax6* and *Sox2* and performed GFP immunostaining to visualize the electroporated cells. Misexpression of Six3, but not that of GFP, led to an enlarged *Pax6* domain on the electroporated side of the SE ($n = 7/10$ embryos; arrow in Figure 7F). Ectopic *Pax6* expression was not induced in the trunk, and ectopic expression of *Sox2* was never observed ($n = 0/10$; data not shown). Similar expansion of *Pax6* expression and no change in *Sox2* expression were observed when an activated form of Six3 was electroporated (*pCAB-Six3VP16*) (data not shown, $n = 10/20$). Misexpression of *Six3-EnR* did not overtly affect *Pax6* expression (data not shown, $n = 21$).

Six3-mediated activation of *Pax6* lens lineage expression was highly temporally specific. When HH9–HH10 embryos were electroporated and analyzed 24 h later (when the lens vesicle had formed), we never observed ectopic *Pax6* expression in the Six3-electroporated cells of the SE. Thus, Six3 directly activated *Pax6* preplacodal expression in the PLE *in vivo* most likely only during a very brief period of early lens development.

Discussion

In this paper, we identified Six3 as a critical early regulator of *Pax6* and *Sox2* expression during lens induction and specification. Conditional removal of Six3 activity in the PLE promoted a range of eye defects; in the most severe cases, no lenses were formed. We also showed that Six3 expression

in the head SE precedes that of *Pax6* and that Six3 expression is initially not affected in the SE of *Pax6*^{−/−} *small eye*-mutant embryos.

Six3 activity in PLE is essential for lens specification

A key consequence of removing Six3 from the PLE at the early somite stage was the lack of thickening and invagination of the PLE, the efficient downregulation of *Pax6* expression, and the complete removal of Sox2 expression from the PLE of the most severely affected embryos. This finding suggests that, similar to the *Pax6 small eye*^{−/−} phenotype, the identified defective upregulation of *Pax6* and Sox2 in the PLE of the *Six3*-mutant embryos affects the acquisition of the critical expression threshold of genes necessary to regulate the onset of lens placode formation. Ultimately, this defective regulation results in defective lens induction/specification (i.e., type III lenses).

As mentioned above, *Pax6* maintains its own expression in the PLE (Grindley *et al*, 1995), and *Pax6* activity is necessary to upregulate that of Sox2. In the generated *Six3*-mutant embryos, the reduction in the levels of Sox2 expression was more sensitive to the removal of Six3 activity than that of *Pax6*. This result suggests that in addition to the *Pax6*-dependent regulation of Sox2, upregulation of Sox2 expression in the PLE also requires activation by Six3 in a *Pax6*-independent manner.

Phenotypic variability in Six3-mutant lenses

The generated *Six3*-mutant embryos display a range of lens phenotypes: lens specification failed in the most severely affected mutants, but was normal in the milder ones. This variability most likely reflects differences in the time and extent of *Six3* deletion, which ultimately affects the rate and time of *Pax6* and Sox2 downregulation in the PLE: early deletion of *Six3* causes the failure in lens specification, but deletion at later stages results in milder lens phenotypes. In support of this argument, we report that in the genetic background used for our analysis, there is variability in the timing and extent of *Le-Cre* activity and in the rate and efficiency of *Six3* deletion (as a consequence also in the extent of *Pax6* and Sox2 downregulation).

Spatial and temporal variation in the extent of Sox2 downregulation is seen when comparing standard *Pax6*-null mice (Grindley *et al*, 1995) with conditional *Pax6*-null animals (Ashery-Padan *et al*, 2000); however, no variability in the lens phenotype has been reported in the generated *Pax6*-conditional mutants in which *Le-Cre* was also used (Ashery-Padan *et al*, 2000). It could be argued that as indicated by the authors, the reported phenotype (no lens placode formation but normal Sox2 expression; Ashery-Padan *et al*, 2000) is the result of late *Pax6* deletion by *Le-Cre* (E9.0–E9.5). However, based on our analysis of the *Le-Cre* activity profile using the *R26R* reporter strain, early removal of *Pax6* activity and therefore complete downregulation of Sox2 expression could have also taken place in a reduced but undetected percentage of the generated *Pax6*^{fl/fl}; *Le-Cre* embryos (assuming their genetic backgrounds were similar to the ones we used for these studies).

A requirement of a timely critical threshold of *Pax6* expression for lens placode formation has already been suggested (van Raamsdonk and Tilghman, 2000; Davis-Silberman *et al*, 2005), and specific conditional deletion of

a single copy of *Pax6* from the developing lens results in a smaller lens (Davis-Silberman *et al*, 2005). This dosage sensitivity may be more relevant during early stages of lens placode formation, because the proposed *Pax6* autoregulatory loop maintains *Pax6* expression starting at this stage (Grindley *et al*, 1995). Therefore, the fewer the number of *Pax6*-expressing cells that remain at the preplacodal stage, the more severe the resulting lens phenotype will be.

According to this, the mildly affected *Six3* mutant lens (type I and type II) are most likely the consequence of less extensive and/or later deletion of *Six3* in the PLE, which in turn lead to milder downregulation of *Pax6* (similar to that of *Pax6* *small eye*^{+/-} mice that have smaller lenses) and *Sox2* levels. It should be mentioned that we did not find any lens phenotype in the *Six3*-heterozygous lenses (data not shown), a result indicating that one functional allele of *Six3* is sufficient for normal lens specification and that more than 50% of *Six3* should be depleted to promote *Pax6* or *Sox2* downregulation.

***Six3* is a direct activator of *Pax6* expression**

Results from the analysis of the generated *Six3*-mutant embryos suggested that *Six3* directly regulates *Pax6* and *Sox2* expression in the PLE. Support for this proposal was provided by a combination of *in vitro* and *in vivo* experimental assays.

Pax6 *EE* is located about 3.9-kb upstream of the *Pax6* *P0* promoter (Kammandel *et al*, 1999) and can direct reporter expression to the PLE from E9.0 onward (Kammandel *et al*, 1999; Ashery-Padan *et al*, 2000). However, lens development is not completely blocked in *EE*-deficient mice (Dimanlig *et al*, 2001); this finding supports the proposal that *Pax6* expression in the lens is mediated by multiple regulatory elements. *Pax6* expression in the lens placode of *EE*-deficient embryos is reduced, and these embryos exhibit a relatively mild lens phenotype (i.e., reduced placodal cell proliferation and a small lens pit and lens vesicle) (Dimanlig *et al*, 2001). The *Pax6* *SIMO* enhancer was identified because patients with aniridia have mutations in this regulatory element (Kleinjan *et al*, 2001). The *SIMO* enhancer also directs reporter expression in the lens lineage starting at around E9.5 (Kleinjan *et al*, 2001). However, given that *Pax6* expression in the PLE can be detected as early as the 7- to 8-somite stage (E8.0), additional regulatory elements, by themselves or in cooperation with the *EE* or *SIMO* enhancers, are probably responsible for the early preplacodal phase of *Pax6* expression.

The *Sox2* *N4*, *N3*, and *L* enhancers were identified by extensive sequence analyses and reporter assays in chick embryos (Uchikawa *et al*, 2003).

We have now demonstrated *in vitro* that *Six3* directly binds *Pax6* *EE* and *SIMO* enhancers and *Sox2* *N3* and *N4* enhancers. Furthermore, we demonstrated *in vivo* that *Six3* binds the *EE*, *SIMO* and *N4* enhancers during early lens specification. The lack of enrichment of *Six3* in *Sox2* *N3* could be due to the fact that the embryonic stage used for the ChIP analysis was earlier than the stage at which *Sox2* *N3* was reported to be active in the chick (Uchikawa *et al*, 2003). Those data demonstrate that *Six3* directly regulates *Pax6* and *Sox2* *in vivo*, although activation of *Sox2* by *Six3* might be in cooperation with additional factors.

Six3 can act as a transcriptional repressor (Kobayashi *et al*, 1998; Zhu *et al*, 2002; Lopez-Rios *et al*, 2003). Yet, injection of

Six3 RNA causes the ectopic expansion of *Pax6* expression into the prospective midbrain and cerebellum of medaka fish (Loosli *et al*, 1999), which suggests that *Six3* also activates *Pax6* expression in some contexts. Results from our luciferase reporter assay have now determined that *Six3* activates the *Pax6* lens lineage expression. Furthermore, our chick electroporation studies demonstrated that the *Pax6* expression domain expanded in the electroporated SE of HH8 chick embryos; no expansion was seen when HH9–HH10 embryos were used. Together, these findings support the proposal that *Six3* directly regulates the expression of *Pax6* (and most likely also of *Sox2*) during a brief, crucial period of early lens development, that is, during the preplacodal stage. Later on, *Pax6* expression appears to be independent of *Six3*.

A working model of lens induction

Six3 expression in the PLE was first detected at around the 5-somite stage. We propose that during lens development two phases of *Six3* activity occur, and it is the initial phase of *Six3* expression in the PLE that is crucial for the regulation of lens induction and specification. Based on our results, *Six3* can act as either an activator or repressor, depending on the cellular context and its specific protein partners; the early phase of *Six3* activity that precedes that of *Pax6* is necessary to activate *Pax6* and *Sox2* expression in the PLE (Figure 7G).

In addition, there are evidences to suggest that these three genes regulate each other in a complex regulatory network (Loosli *et al*, 1999; Goudreau *et al*, 2002; Aota *et al*, 2003). Our analysis of *Pax6*^{*Sey-1^{Neu}/Sey-1^{Neu}*} mutant embryos (Figure 6) provides further support to this proposal. In this case, although initial *Six3* expression in the PLE is unaffected, the lack of *Pax6* activity in the PLE fails to maintain *Six3* expression in that structure during later stages (it is not yet known whether maintenance of *Six3* expression requires direct activation by *Pax6*). This results in the absence of the lens placode and failure in lens formation.

In summary, we propose that as lens development progresses, the initial downregulation of preplacodal *Pax6* expression promoted by the lack of *Six3* activity leads to the eventual failure of *Pax6* own activation and its maintenance in the PLE. Absence of *Six3* and *Pax6* activity in the mutant PLE results in the defective upregulation of *Sox2* expression in this tissue. Altogether, these defects lead to the failure in lens induction and specification observed in the most severe *Six3*-mutant embryos.

Materials and methods

Mouse lines

Descriptions of the generation of the *Six3*-targeting construct and mouse strains used are presented as Supplementary data.

Immunohistochemistry and *in situ* hybridization

Standard procedures were used. The list of used primary antibodies and *in situ* probes is provided in the Supplementary data.

BrdU and TUNEL assays

Time-mated female mice were injected with BrdU (0.1 mg/g body weight, intraperitoneally), and embryos were harvested 1.5 h later. Anti-BrdU monoclonal antibody (1:10; BD Biosciences) was used. The TUNEL assay was performed using the ApopTag Kit (Chemicon), according to the manufacturer's instructions. For counting of BrdU⁺ cells and TUNEL⁺ cells, sections through the middle of the lens pits were used. The results represent the average percentage of three embryos.

ChIP assay

The ChIP Kit (Upstate) protocol was followed, except that the nuclear fraction was used. Briefly, 13 E8.5 wild-type embryos were harvested. Heads (Six3-expressing) and trunks (Six3-negative) were dissected, and nuclear fractions were subjected to separate ChIP assays. Half of the chromatin was immunoprecipitated with the Six3 antibody (4 μ l). Chromatin was purified by QIAquick PCR Purification Kit (Qiagen) and suspended in 50 μ l EB buffer (Qiagen). A 2- μ l sample of DNA from each of the four samples (head and trunk, with and without Six3 immunoprecipitation) was used for TaqMan real-time PCR (ABI7900HT) analysis (Supplementary data).

DNA sequence analyses

Pax6 and *Sox2* genomic DNA sequences were downloaded from Ensembl database (www.ensembl.org). A comparison of the two sequences was performed using Vista Tools (genome.lbl.gov/vista).

Plasmids

To generate the EMSA templates, PCR-amplified DNA fragments containing selected regions from *Pax6 EE*, *Pax6 SIMO*, *Sox2 N3*, and *Sox2 N4* were first cloned into the pGEM-T easy plasmid (Promega) and verified by DNA sequencing. DNA sequences of the primers used are provided in the Supplementary data and in Supplementary Figure S4.

For the luciferase assay, the mouse *Pax6* lens lineage reporter plasmids *Pax6-EE-luc* and *Pax6- Δ EE-luc* were generated by replacing the *lacZ*-coding sequence from the *P0-3.9-lacZ* parental plasmid (Williams *et al*, 1998) with the luciferase reporter gene. For *Sox2*, a conserved 1380-bp DNA region that included *Sox2 N3* enhancer was generated by PCR using the following primers:

5'-AGATCTTTTCTCAAACTGAAATAGCAAACA-3' and

5'-AGATCTCTGAGCTTACCCCTGACTC-3' and cloned into the *Bgl*III site of the pRL-TK (Promega) reporter plasmid. For *Sox2 N4*, the same amplified DNA fragment (502 bp) used for the EMSA assay was cloned into the *Bgl*III site of pRL-TK.

Effectors for the luciferase assay were made as follows: the plasmids *pCAB-Six3-IRES-GFP*, *pCAB-Six3VP16-IRES-GFP*, and *pCAB-Six3EnR-IRES-GFP* were obtained by inserting *Six3 cDNA*, *Six3VP16*, and *Six3EnR*, respectively, into blunted *Bam*HI-*Cla*I sites of the *pCAB-IRES-GFP* vector (McLarren *et al*, 2003).

EMSA

*Not*I digestion was used to release the four DNA templates from their parental plasmids. Klenow enzyme was used to end-label these double-stranded DNA fragments with [α -³²P]dCTP. The EMSA was then performed as described in the Supplementary data.

Luciferase assay

Cos7 cells were maintained in culture at 37°C in DMEM with 10% FBS, 50 μ g/ml penicillin-streptomycin, and 2 mM glutamine. Cos7 cells were seeded into 24-well plates and transfected with the proper reporter/effector mixtures using Fugene 6 (Roche) according to the manufacture's instructions. Details of the *Pax6* and *Sox2* reporter luciferase assays are presented in the Supplementary data.

Chick electroporation

Fertilized chicken eggs (Henry Stewart, UK) were incubated at 38°C in a humidified incubator for 26–36 h, until they reached HH8–HH10 stage. Overexpression of *pCAB-Six3-IRES-GFP* in HH8 embryos was performed in modified New culture as described previously (Stern and Ireland, 1981) using 1–2 mg/ml DNA and four 50-ms pulses of 7 V (McLarren *et al*, 2003). Embryos were grown for 12–14 h, and the expression of *Pax6* and *Sox2* was analyzed by whole-mount *in situ* hybridization followed by immunostaining with polyclonal anti-GFP antibodies (Molecular Probes). HH9–HH10 embryos were electroporated *in ovo* as described previously, with the exception that the electrical stimulus was increased to 20 V (Bhattacharyya *et al*, 2004). After 24 h, the expression of β -crystallin and *Pax6* was analyzed using the same methods described for the younger chick embryos.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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