Mutually regulated expression of *Pax6* and *Six3* and its implications for the *Pax6* haploinsufficient lens phenotype

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Pax6* is a key regulator of eye development in vertebrates and invertebrates, and heterozygous loss-of-function mutations of the mouse *Pax6* gene result in the *Small eye* phenotype, in which a small lens is a constant feature. To provide an understanding of the mechanisms underlying this haploinsufficient phenotype, we evaluated in *Pax6* heterozygous mice the effects of reduced *Pax6* gene dosage on the activity of other transcription factors regulating eye formation. We found that *Six3* expression was specifically reduced in lenses of *Pax6* heterozygous mouse embryos. Interactions between orthologous genes from the *Pax* and *Six* families have been identified in *Drosophila* and vertebrate species, and we examined the control of *Pax6* and *Six3* gene expression in the developing mouse lens. Using *in vitro* and transgenic approaches, we found that either transcription factor binds regulatory sequences from the counterpart gene and that both genes mutually activate their expression. These studies define a functional relationship in the lens in which *Six3* expression is dosage-dependent on *Pax6* and where, conversely, *Six3* activates *Pax6*. Accordingly, we show a rescue of the *Pax6* haploinsufficient lens phenotype after lens-specific expression of *Six3* in transgenic mice. This phenotypic rescue was accompanied by cell proliferation and activation of the platelet-derived growth factor α-R/cyclin D1 signaling pathway. Our findings thus provide a mechanism implicating gene regulatory interactions between *Pax6* and *Six3* in the tissue-specific defects found in *Pax6* heterozygous mice.

homeodomain proteins | transcription factors | eye development

The *Pax* gene family encodes a group of transcription factors containing a paired DNA-binding domain and, in some cases, a paired-type homeodomain (1). In vertebrates, *Pax* genes include nine members that hold essential developmental roles, as exemplified by the phenotypes found in homozygous null mutants (2). An interesting feature of *Pax* genes, and one that is shared with a restricted group of gene families (3), is that dominant phenotypes frequently accompany heterozygous loss-of-function mutations. Typically, the phenotype only partly reflects the expression pattern, and the affected organ is reduced in size in the heterozygote and is absent in the homozygote. A well-studied example of this dosage-dependent effect is found with the *Pax6* gene (4, 5). Mice that are homozygous for *Pax6* null mutations are anophthalmic, whereas a 50% reduction of *Pax6* gene dosage causes a microphthalmic phenotype (4). Overexpression of *Pax6* in eye structures of transgenic mice also causes a microphthalmic phenotype (6), indicating that stringent controls must be set on *Pax6* expression levels for normal eye development to occur.

An understanding of the mechanisms by which single allele mutations of certain genes, but not of others, lead to an haploinsufficient syndrome remains an unresolved issue (3). It has been proposed that *Pax6* exerts a dosage effect (7) by which certain effector genes may not respond predictably to Pax6 protein levels that are below a threshold value. Eye development in vertebrates and invertebrates is regulated by a conserved network of transcription factors and nuclear proteins (8), and we tested the hypothesis that reduced *Pax6* gene dosage may lead to abnormal gene expression within this network. We were particularly interested in studying the effects of reduced *Pax6* gene dosage on the activity of the *Six3* gene, because both genes are expressed in an overlapping fashion during mouse eye development (9), and regulatory networks involving genes orthologous to *Pax6* and *Six3* have been demonstrated in eyes of vertebrates and *Drosophila* (10, 11). Moreover, disruption of *Pax6* function in the prospective lens ectoderm of the mouse abolishes *Six3* expression, suggesting a dependence of *Six3* on *Pax6* function in this structure (12).

Here, we describe the regulatory interactions between *Pax6* and *Six3* in the developing mouse lens. Our results reveal a gene relationship in which *Six3* expression is dosage-dependent on *Pax6* function and where, conversely, *Six3* activates *Pax6* expression. We found this gene relationship to be tissue-specific. Furthermore, we show that the *Pax6* haploinsufficient lens phenotype is rescued in transgenic mice expressing *Six3* under the regulation of a lens-specific gene promoter. Our results thus provide a mechanism explaining how heterozygous loss-of-function mutations of the mouse *Pax6* gene may lead to the tissue-specific defects observed in the *Small eye* mutant. Moreover, these findings suggest that similar regulatory interactions may underlie haploinsufficient syndromes resulting from single-allele mutations of other transcription factor-encoding genes.

Materials and Methods

Transgene Constructs and Generation of Transgenic Mice. The *αASix3* transgene was generated by inserting a 1.4-kb *Six3* cDNA fragment into polylinker restriction sites located between the *αA-crystallin* promoter and simian virus 40 (SV40) intron and polyadenylation sequences of plasmid pACP3 (13) (see Fig. 2*A*). The transgene was linearized with *Sac*II and readied for microinjection. A similar approach was used to construct a modified *αA-crystallin* promoter/*Pax6* cDNA transgene (*αAmPax6*), except that two additional Pax6 binding sites were inserted in the promoter region (L.W.R., unpublished work). Transgenic mice were generated (14) and bred in the FVB/N background. Two *αASix3* transgenic lines (nos. 40 and 53) were studied. Both displayed the same phenotype based on morphology and molecular analysis. Eight *αAmPax6* lines were generated; analysis was done on line OVE1078. NMRI mice harboring a targeted mutation of the *Pax6* gene (*Pax6*^{lacZ}) (15) were backcrossed for at least four generations in the FVB/N background before mating with *αASix3* transgenic mice. For staging of embryos,

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Abbreviations: EMSA, electrophoretic mobility-shift assay; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; PDGF, platelet-derived growth factor; E*n*, embryonic day *n*; SV40, simian virus 40.

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noon of the day of vaginal plug formation was considered as embryonic day 0.5 (E0.5). Genotyping was done by PCR (13) or Southern blotting (15) with genomic DNA extracted from mouse tails.

Histology and *in Situ* Hybridization. Embryos were harvested, fixed in 4% paraformaldehyde, and processed in Paraplast. Transverse sections of the eyes were done at 8-μ thickness as described (16). Eyes from 3-week-old mice were dissected, fixed in Carnoy's solution, processed in JB-4 plastic (Polysciences), and sectioned, according to a previously published protocol (17). *In situ* hybridization experiments using ^{35}S-labeled riboprobes were performed as described (16). A transgene-specific probe (probe B, see Fig. 2*A*) consisted of a 220-bp *Bgl*II–*Bam*HI fragment, containing SV40 polyadenylation sequences. The *Six3* (9), *FoxE3* (18), *Prox1* (19), and *Eya1* and *2* (provided by R. Maas, Harvard University, Cambridge, MA; ref. 20) riboprobes have been described.

Image Analysis. Bright- and dark-field images were captured with an Olympus BX60 microscope fitted with a charge-coupled device, and analyzed with the imaging software ANALYSIS (Soft-Image System, Münster, Germany). For gray-scale measurements, dark-field images were captured at identical light intensities and exposure times and saved as 8-bit black and white images. Selected ocular regions were outlined, the remaining image was masked, and gray-scale values were measured on individual pixels. Results were expressed as percentages, with values of 0 and 100 being given to black and white, respectively.

Immunohistochemistry. The following antibodies were used: anti-β- and anti-γ-crystallins, anti-αA-crystallin, anti-MIP26, anti-filensin, and anti-CP49 [kindly provided, respectively, by J. S. Zigler (National Eye Institute, Bethesda), K. Kato (Institute for Developmental Research, Aichi, Japan), J. Horwitz (Jules Stein Eye Institute, Los Angeles), and R. A. Quinlan (University of Dundee, Dundee, U.K.)]. Anti-Cdc2, Cdk4, Cdkn1b, Cdkn1c, platelet-derived growth factor (PDGF)α-R, fibroblast growth factor-R types 1 and 2 antibodies (Santa Cruz Biotechnology), anti-insulin-like growth factor I-R (GroPep, Adelaide, Australia), anti-cyclin D1 (Zymed), anti-Pax6 (American Type Culture Collection), anti-Six3 (kindly provided by G. Oliver, St. Jude Children's Research Hospital, Memphis, TN; ref. 21), anti-Sox1 (obtained from R. Lovell-Badge, National Institute for Medical Research, London; ref. 22), and anti-phospho p44/42 mitogen-activated protein kinase (Cell Signaling Technology, Beverly, MA). In all cases, appropriate Alexa-488-conjugated secondary antibodies (anti-mouse, -rabbit, or -goat) were used and cell nuclei were counterstained with propidium iodide (all from Molecular Probes). Fluorescence was recorded with a SLM410 Zeiss confocal microscope.

BrdUrd Labeling and Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick-End Labeling (TUNEL). For BrdUrd labeling, gravid females were injected i.p. with a BrdUrd/fluorodeoxyuridine mix (10 μm/1 μm, Amersham Pharmacia) at 0.01 ml/g of body weight, and embryos were harvested 3 h afterward. After processing in Paraplast, sections were reacted with a mouse monoclonal anti-BrdUrd antibody (Roche Molecular Biochemicals) using conditions suggested by the manufacturer. TUNEL staining was done by using ApopTag Plus Fluorescein Apoptosis Detection reagents (Intergen, Purchase, NY) as specified.

Electrophoretic Mobility-Shift Assays (EMSAs) and Cell Transfections. EMSAs were done according to a previously published protocol (23). Expression vectors used for cell transfections were cloned in the cytomegalovirus promoter-driven pCS2+ vector. The *Pax6*-luciferase reporter contained six tandem copies of a 20-bp

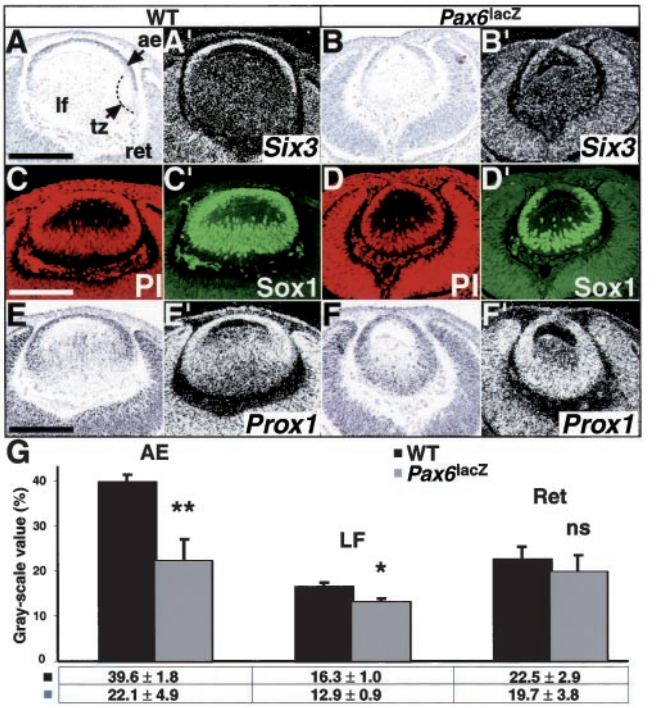

Fig. 1. Dosage-dependent reduction of *Six3* expression in lenses of *Pax6*^{lacZ} embryos. Reduced *Six3* expression is found in lenses of E12.5 *Pax6*^{lacZ} embryos (*B*′), compared with controls (*A*′). *Six3* expression in the retina (ret) is unchanged. Expression of Sox1 (*C*–*D*′) and *Prox1* (*E*–*F*′) is unchanged in wild-type (WT) and *Pax6*^{lacZ} embryos. (*A*, *B*, *E*, and *F*) Bright field, hematoxylin. (*A*′, *B*′, *E*′, and *F*′) Dark field. (*C*–*D*′) Fluorescence microscopy. ae, anterior epithelial cells; lf, lens fiber cells; ret, retina; tz, transition zone; PI, propidium iodide. (Bar = 100 μm.) (*G*) Gray-scale measurement of *Six3* expression. For each embryo, measurements were done in the lens (AE and LF) and retina (Ret) as described in *Materials and Methods*. Values were calculated in E12.5 *Pax6*^{lacZ} (gray) and wild-type (black) embryos. *Six3* expression was reduced in lenses of *Pax6*^{lacZ} embryos, whereas expression in the retina was unchanged. Results are the average ± SE of measurements from four separate embryos. Statistical difference: **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

sequence (5′-CTCATTGCCCATTCAAATAC-3′) derived from a *Pax6* lens enhancer (24). The *Six3*-luciferase reporter contains four tandem copies of a 45-bp sequence containing an inverted Pax6-binding site (5′-CCGCCCAGAGCCT**TGCAGTTGAGCGGAAAAG**CACTCCTGGTGCAG-3′), located approximately 2 kbp upstream of exon 1 of mouse *Six3*. Both reporter constructs were cloned in pGL3-promoter (Promega). Cultures and transfections of COS7 cells were prepared as described (23). Transfection efficiency was estimated by including a constant amount (0.3 μg) of cytomegalovirus promoter-LacZ plasmid while the total amount of transfected DNA was kept constant (2 μg) in all assays. Forty hours after transfection, cells were harvested, and luciferase assay was carried out by using a Berthold (Nashua, NH) LB9501 luminometer. β-Galactosidase activity was measured with the Galacto-Light kit (Tropix, Bedford, MA). For all experiments three samples were transfected and processed independently. Each experiment was performed at least twice.

Results

***Six3* Expression in the Lens Requires *Pax6* Function in a Dosage-Dependent Manner.** We examined the regulatory hierarchy of genes involved in lens development by comparing the expression of eye-specific transcription or nuclear factors in the eyes of E12.5 wild-type and *Pax6* heterozygous embryos. Reduced *Six3* expression was found in lenses of *Pax6*^{lacZ} embryos (Fig. 1*A*′, *B*′,

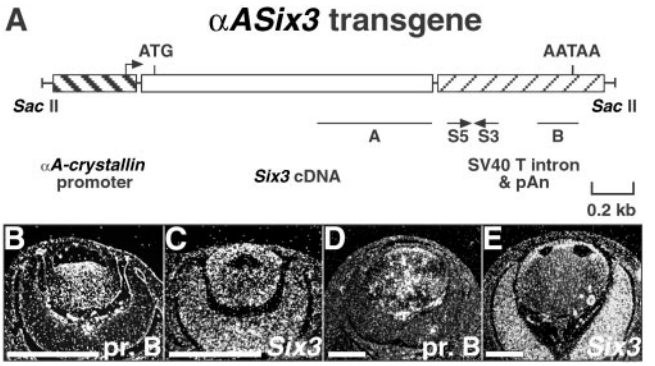

Fig. 2. Structure and expression of the *αASix3* transgene. (*A*) Diagram of the DNA construct used for the generation of *αASix3* transgenic mice. The *αASix3* transgene was generated by inserting the full-length coding sequences of the murine *Six3* cDNA into polylinker restriction sites of plasmid pACP3. The arrow represents the site of transcription initiation. A 0.6-kbp fragment derived from the *Six3* cDNA was used as an *in situ* hybridization probe (probe A). A transgene-specific riboprobe (probe B) was derived from the SV40 polyadenylation sequences. The location of primers used for PCR genotyping (S5 and S3) is shown. AATAA, SV40 polyadenylation (pAn) site; ATG: translation initiation site. (*B–E*) Expression of the transgene. Using riboprobe B, transgene expression was detected in *αASix3* transgenic embryos from E12.0 (*B*) and was restricted to lens fiber cells at all stages examined (*D*, E13.5). Expression of the transgene is compared with *Six3* expression in age-matched controls (*C* and *E*). (Dark field; bar = 100 μm.)

and *G*), whereas expression levels of *Eya1* and *2*, *FoxE3*, *Prox1*, and Sox1 (Fig. 1 *C–F′* and data not shown) were the same in wild-type and *Pax6*^{lacZ} embryos. In contrast, *Six3* expression in the retina of *Pax6*^{lacZ} embryos was unchanged (Fig. 1 *A′*, *B′*, and *G*). The lens-specific reduction of *Six3* expression in *Pax6*^{lacZ} embryos indicates a dosage dependence of *Six3* expression on *Pax6* levels in the lens, whereas *Pax6* function is not essential for *Six3* expression in the retina (23).

Lens-Specific Expression of *Six3* Rescues the *Pax6* Haploinsufficient Lens Phenotype. Because of its specificity, we speculated that this dosage-dependent reduction of *Six3* expression might contribute to the *Pax6* haploinsufficient lens phenotype. We therefore attempted to rescue this lens defect by increasing *Six3* gene dosage, using a transgene (*αASix3*, Fig. 2*A*) linking a 0.4-kbp murine *αA-crystallin* gene promoter to a 1.4-kbp mouse *Six3* cDNA (9). With a transgene-specific riboprobe, embryonic expression was initially detected at E12.0 (Fig. 2*B*). Expression of the transgene in lens fiber cells overlapped that of the endogenous *Six3* gene from E12.0 to E13.5 and was maintained in the differentiated fiber cells (Fig. 2 *B–E*). We found that transgene expression levels were lower in *αASix3/Pax6*^{lacZ} embryos than in *αASix3/Pax6*$^{+/+}$ embryos (not shown), this difference being likely caused by the presence of a Pax6-responsive site in the *αA-crystallin* promoter sequences used in the transgene construct (25).

Lenses from E14.5 *αASix3/Pax6*^{lacZ} embryos were larger than those of age-matched *Pax6*^{lacZ} embryos, but remained smaller than wild-type controls (Fig. 3 *A–C*). This phenotypic rescue was found in *Pax6*^{lacZ} mice hemizygous for the *αASix3* transgene and was fully penetrant in the FVB/N background. The increased lens size was maintained in 3 week-old *αASix3/Pax6*^{lacZ} mice (Fig. 3 *I–K*), although at this later stage lens morphology was abnormal and included the presence of vacuoles and ballooned fiber cells (Fig. 3 *K* and *O*). To explain this phenotypic rescue, we first examined the effects of *Six3* expression on lens fiber cell proliferation, differentiation, and death. We studied cell proliferation in embryos by using the BrdUrd incorporation assay, which detects cells entering the S phase. BrdUrd-incorporating

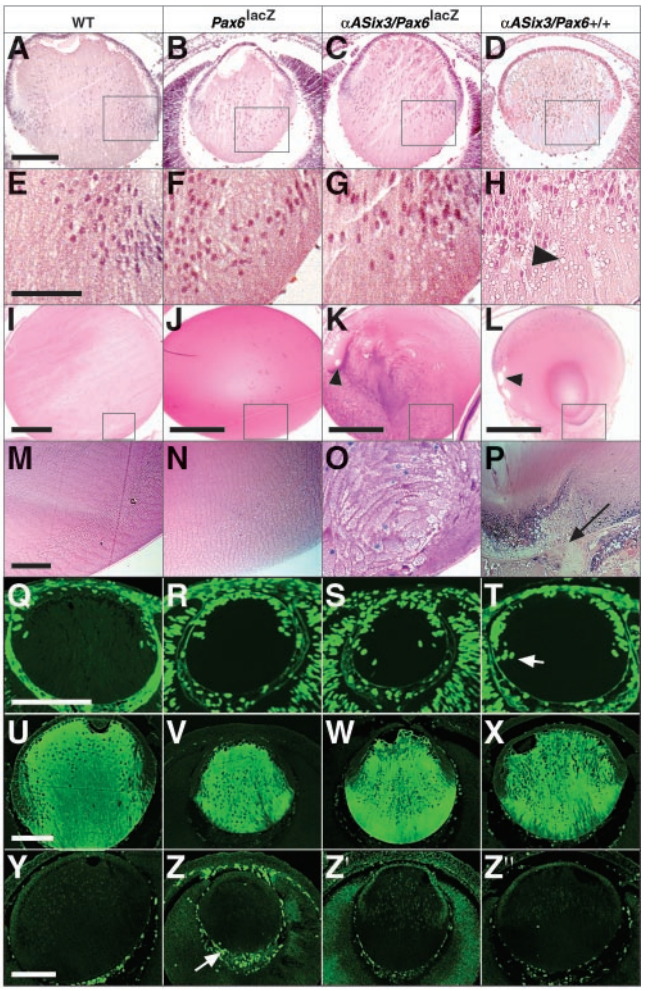

Fig. 3. Rescue of the *Pax6* haploinsufficient lens phenotype in *αASix3/Pax6*^{lacZ} transgenic mice. (*A–P*) Effects of *Six3* expression on lens morphology. Transverse sections from eyes of E14.5 (A--*H*) and 3-week-old (*I–P*) mice. Note the increased lens size in *αASix3/Pax6*^{lacZ} mice (*C* and *K*) compared with age-matched *Pax6*^{lacZ} mice (*B* and *J*), and the microphakia in *αASix3/Pax6*$^{+/+}$ transgenic mice (*D* and *L*). The boxed areas in *A–D* and *I–L* are enlarged in *E–H* and *M–P*, respectively. Note the presence of vacuoles (arrowhead, *H*) in fiber cells of E14.5 *αASix3/Pax6*$^{+/+}$ transgenic embryos. Large vacuoles (arrowheads, *K* and *L*) and disorganized fiber cell arrangement are present in lenses of 3-week-old *αASix3/Pax6*^{lacZ} and *αASix3/Pax6*$^{+/+}$ transgenic mice (*O* and *P*). Posterior rupture of the lens capsule is seen in the latter (arrow, *P*). (*Q–T*) Lens fiber cell proliferation in embryos harboring an *αASix3* transgene. BrdUrd immunochemistry in E12.5 embryos demonstrating the presence of proliferating cells in the lens fiber cell compartment and transition zone (arrow) of *αASix3/Pax6*^{lacZ} (*S*) and *αASix3/Pax6*$^{+/+}$ transgenic embryos (*T*), but not in controls (*Q* and *R*). (*U–X*) Normal lens maturation in *αASix3/Pax6*^{lacZ} transgenic embryos. The differentiation marker β-crystallin is normally expressed in fiber cells of E14.5 *αASix3/Pax6*^{lacZ} transgenic embryos (*W*) [compare with wild-type (*U*) and *Pax6*^{lacZ} (*V*) controls]. The same marker is expressed heterogeneously in fiber cells of a E14.5 *αASix3/Pax6*$^{+/+}$ transgenic embryo (*X*). (*Y–Z″*) TUNEL assay. The lens fiber cell compartment of E13.5 *αASix3/Pax6*$^{+/+}$ transgenic embryos contains rare nuclei that were positive for the TUNEL assay (*Z″*). At the same stage, numerous TUNEL-positive nuclei are found in the degenerating hyaloid vessels of transgenic and control embryos (arrow). [Bar = 100 μm, except *E–H* (50 μm) and *I–L* (500 μm).]

lens fiber cells were present in E12.5 (Fig. 3*S*) and E13.5 *αASix3/Pax6*^{lacZ} embryos, but not in the lens fiber cell compartment of controls (Fig. 3 *Q* and *R*). We next examined the effects of *Six3* expression on lens differentiation and cell death. At stage E14.5, the expression of the fiber cell differentiation

markers αA-, β- and γ-crystallins, MIP-26, filensin, and CP-49 (Fig. 3 *U–W* and data not shown), as well as the number of TUNEL assay-positive fiber cells (Fig. 3 *Y–Z′*), were the same in fiber cells of *αASix3/Pax6*^{lacZ} and control embryos. Altogether, these results indicate that the improved lens size in *αASix3/Pax6*^{lacZ} embryos resulted, in part, from fiber cell proliferation. Importantly, differentiation and survival of fiber cells in *αASix3/Pax6*^{lacZ} embryos were normal, leading to overall lens growth.

***Six3* Overexpression Causes Defective Lens Maturation and Fiber Cell Death.** In contrast to the improved lens size in *αASix3/Pax6*^{lacZ} transgenic mice, *αASix3/Pax6*$^{+/+}$ transgenic mice were microphakic (Fig. 3 *D* and *L*). The severity of the microphakia was greater in embryos homozygous for the αASix3 transgene (not shown). The latter effect was found in either the *Pax6*$^{+/+}$ or *Pax6*^{lacZ} background, indicating that *Six3* gene dosage was the primary factor affecting the lens phenotype. *αASix3/Pax6*^{lacZ} transgenic embryos were microphakic from stage E14.5 onward (Fig. 3*D*). In sections done on adult eyes, the lenses had a disorganized fiber cell arrangement accompanied by posterior rupture of their content into the vitreous chamber (Fig. 3 *L* and *P*).

BrdUrd incorporation assay revealed lens fiber cell proliferation in *αASix3/Pax6*$^{+/+}$ transgenic embryos, an effect similar to that observed in *αASix3/Pax6*^{lacZ} embryos (Fig. 3 *S* and *T*). We examined the effect of increased *Six3* expression on lens fiber cell maturation and survival. From E14.5 onward, a heterogeneous distribution of lens differentiation markers was observed in lenses of *αASix3/Pax6*$^{+/+}$ embryos, when compared with controls (Fig. 3 *U* and *X*). Lastly, from stage E14.5 onward, vacuoles were present in fiber cells of *αASix3/Pax6*$^{+/+}$ embryos, but not in age-matched controls (Fig. 3 *E* and *H*), indicating a process of cell death. However, fiber cells were rarely stained with TUNEL (Fig. 3*Z′′*). Cell death thus resulted either from necrosis or from a process differing from the common forms of apoptosis. These results thus suggest that, in addition to defective maturation, *Six3* overexpression causes fiber cell death.

Mutually Regulated Expression of *Pax6* and *Six3* in the Lens. Members of the vertebrate *Pax* and *Six* gene families participate in gene feedback loops regulating eye, kidney, ear, and muscle formation (reviewed in ref. 26). To further address the effects of *Six3* expression in the developing lens, we examined the regulatory relationship between *Pax6* and *Six3*. In lenses of transgenic embryos, expression of *Six3* up-regulated that of Pax6 (Fig. 4 *A–F*). Reciprocally, up-regulated Six3 expression was found in fiber cells of *αA-crystallin* (modified)*/Pax6* (*αAmPax6*) transgenic embryos (Fig. 4 *G–L*). Expression of other eye-specific factors (*Eya1, Eya2*, *FoxE3*, *Prox1*, and Sox1) was unchanged in lenses of transgenic embryos and controls (not shown).

A 107-bp *Bgl*II–*Acc*I DNA fragment, located 5 kbp upstream of the *Pax6* (P0) transcription start site, has previously been shown to direct reporter gene activity in lenses of transgenic embryos (24). This fragment was examined by EMSA for the presence of Six3-binding sites. We observed the shift of electromobility of a 20-bp sequence (5′-CTCATTGCCCATTCAAATAC-3′) derived from the 5′ part of the enhancer (not shown). We examined whether this sequence has Six3-dependent transcriptional activity. In cell transfection assays, luciferase activity was increased after cotransfecting a reporter construct containing a multimer of this sequence with a Six3 expression vector (Fig. 4*M*). Additionally, overlapping phage clones containing 27 kbp of the *Six3* gene were sequenced and evaluated for the presence of sequences related to the Pax6-paired domain DNA-binding sites (27). An oligonucleotide derived from one potential inverted site (5′-TGCAGTTGAGCGGAAAAG-3′), located 2 kbp upstream of the first coding exon, shifted the Pax6 protein in EMSA (not shown). Moreover, a luciferase reporter construct containing a multimerized oligonucleotide was activated after cotransfection with a Pax6 expression vector (Fig. 4*N*). The results of EMSAs and cell transfection assays, combined with those in transgenic embryos, clearly indicate that each gene constitutes a direct target for the counterpart transcription factor, and that *Pax6* and *Six3* expression is mutually activated during mouse lens development.

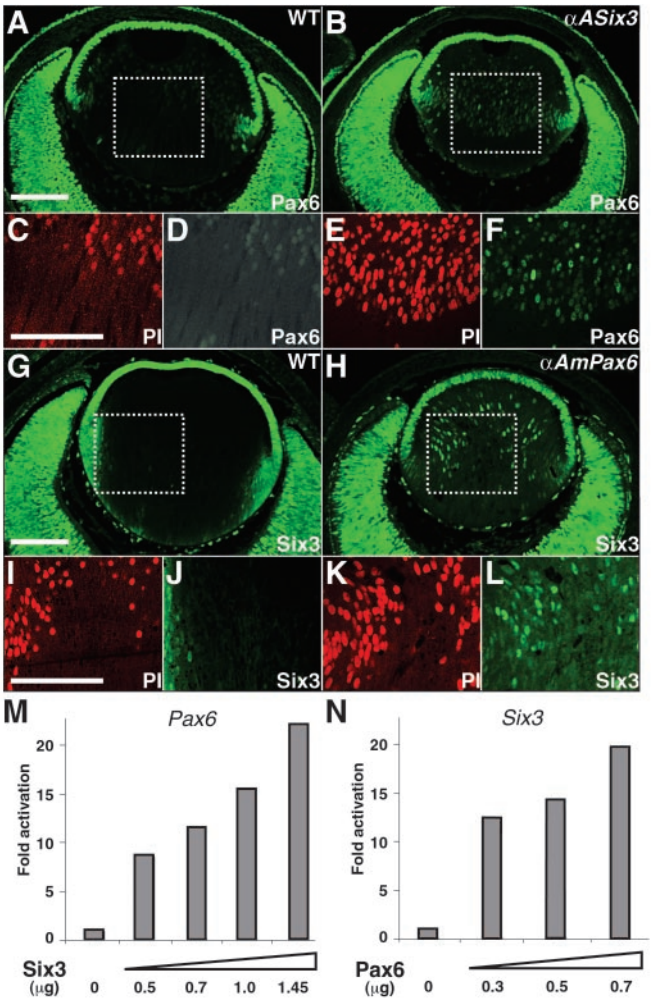

Fig. 4. Mutually regulated expression of *Pax6* and *Six3* in the developing lens. (*A–L*) Mutual activation of *Six3* and *Pax6* expression in transgenic embryos. Fiber cell nuclei from E14.5 *αASix3* transgenic embryos are immunoreactive for the Pax6 protein (*B*, *E*, and *F*) whereas only faint staining is detected in the fiber cell compartment of wild-type controls (*A*, *C*, and *D*). Reciprocally, Six3-immunoreactive fiber cells are present in E14.5 *αAmPax6* transgenic embryos (*H*, *K*, and *L*), but not in controls (*G*, *I*, and *J*). [Bar = 100 μm (*A*, *B*, *G*, and *H*).] (*M* and *N*) Cell transfection assays. COS7 cells were transiently transfected with a *Pax6*-luciferase reporter and increasing amounts of a Six3 expression vector (*M*), or with a *Six3*-luciferase reporter and a Pax6 expression vector (*N*). In both *M* and *N*, a dosage-dependent activation of the reporter is seen after transfection of increasing amounts of the expression construct. Values are expressed as the fold increase in luciferase activity in the presence of the expression plasmid, compared with the level of activity with reporter plasmid alone.

Up-Regulation of PDGFα-R and Cyclin D1 After Lens-Specific Expression of *Six3* or *Pax6* in Transgenic Mice. We further evaluated the cell proliferation in *αASix3/Pax6*^{lacZ} and *αASix3/Pax6*$^{+/+}$ transgenic embryos by studying the expression of cell cycle regulatory proteins in fiber cells. We found increased levels of cyclin D1

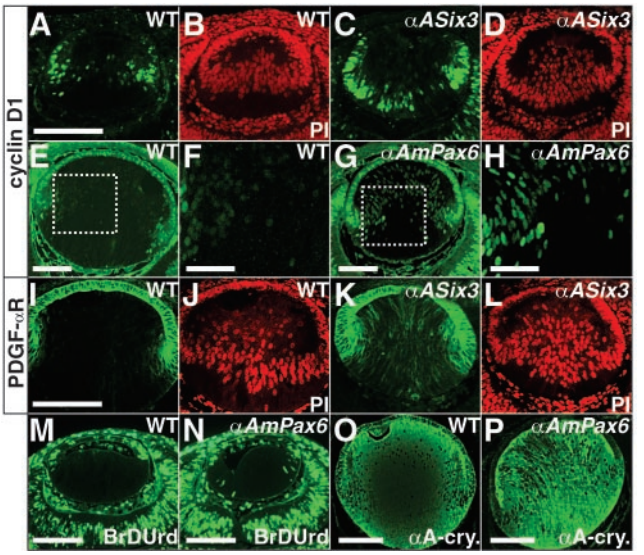

Fig. 5. PDGFα-R and cyclin D1 up-regulation in transgenic embryos expressing *Six3* or *Pax6* in the lens. (*A–H*) Cyclin D1 immunochemistry. Increased numbers of cyclin D1-reactive nuclei are detected in the fiber cell compartment of E12.5 α*ASix3* (*C* and *D*) and E14.5 α*AmPax6* (*G* and *H*) transgenic embryos, compared with controls (*A*, *B*, *E*, and *F*). The boxed areas in *E* and *G* are enlarged in *F* and *H*. (*I–L*) Up-regulation of PDGFα-R expression in fiber cells of α*ASix3* transgenic embryos. PDGFα-R activity is up-regulated in the lens fiber cell compartment of E12.5 α*ASix3* (*K* and *L*) transgenic embryos. PDGFα-R immunoreactivity is present in anterior epithelial cells, but not in fiber cells of controls (*I* and *J*). (*M–P*) Lens fiber cell proliferation and defective maturation in α*AmPax6* transgenic embryos. Lens fiber cell proliferation is observed in E12.0 α*AmPax6* embryos (*N*), whereas the same changes are absent in age-matched controls (*M*). A heterogeneous distribution of αA-crystallin is found in the lens of a E14.5 α*AmPax6* transgenic embryo (*P*), compared with a control (*O*). WT, wild type. [Bar = 100 μm, except *F* and *H* (50 μm).]

(Fig. 5 *A–D*), whereas expression levels of Cdkn1c, Cdkn1b, Cdc2, and Cdk4 were unchanged (not shown). The up-regulated cyclin D1 activity was accompanied by an increase in the activated form of p44/42 mitogen-activated protein kinase (ERK), indicating a fiber cell response to an extracellular mitogenic signal (not shown). We further evaluated this signaling pathway by studying the lens expression levels of receptor tyrosine kinases that act upstream of ERK. Expression levels of PDGFα-R were up-regulated in fiber cells of αASix3 transgenic embryos, whereas expression levels of insulin-like growth factor I-R, and fibroblast growth factor types 1 and 2 were unchanged (Fig. 5 *I–L* and data not shown). Moreover, fiber cell proliferation, ERK activation, and up-regulated expression of PDGFα-R and cyclin D1 were found in lenses of α*AmPax6* transgenic embryos (Fig. 5 *E–H* and *M–N*, and data not shown). Defective fiber cell maturation was also demonstrated in α*AmPax6* transgenic embryos (Fig. 5 *O* and *P*), an effect similar to that observed after *Six3* overexpression (see Fig. 3 *U* and *X*). Therefore, in gain-of-function studies, the effects of either transcription factor in the lens were similar, as a likely consequence of the cross-regulation between both genes. These results also argue that both transcription factors participate, along with *FoxE3*, *Prox1*, and *Cdkn1c* (18, 19, 28), in a gene network controlling cell proliferation in the lens. In this context, the mutual activation of *Pax6* and *Six3* may serve to limit the fluctuations of expression levels of either transcription factor (3). Stable expression of Pax6 and Six3 may be required for the coordinate expression of various components of the cyclin D1/PDGFα-R signaling pathway, thus facilitating the progression between different phases of the cell cycle.

Discussion

In this study, we described the mechanisms regulating the expression of *Pax6* and *Six3* in the developing mouse lens. Using both *in vitro* and transgenic approaches, each transcription factor was shown to directly interact with the counterpart gene, and mutual activation of gene expression was demonstrated. Importantly, *Six3* expression was specifically reduced in lenses of *Pax6* heterozygous embryos. Hence, our findings invoke a model of gene regulation in the lens in which *Six3* expression is dosage-dependent on *Pax6*, and where *Six3* activates *Pax6* transcription. This model indicates a pivotal role for the Six3 transcription factor in this regulatory pathway. Moreover, it provides an explanation for the tissue-specific defects observed in haploinsufficient syndromes. Accordingly, we have tested this regulatory model and shown, by means of a transgenic experiment, that *Six3* expression in lenses of *Pax6* heterozygous embryos restores this structure to a near-normal size.

Previous studies have highlighted some of the mechanisms underlying haploinsufficient syndromes (3, 7, 29). The *Pax6* haploinsufficient lens phenotype has been related to a delay in lens formation, possibly because of an initial failure of the Pax6 protein to reach a threshold level (7). Although the mechanisms controlling the expression of *Pax6* and *Six3* were not directly evaluated at early stages of lens formation, prior findings show that both genes interact at these developmental stages, and therefore likely contribute to the early lens defect observed in *Pax6* heterozygous embryos. Indeed, experiments involving the conditional disruption of the *Pax6* gene in the prospective lens ectoderm (12) have shown that *Pax6* function is required for the initiation of Six3 expression in this structure. However, it remains unclear whether *Six3* regulates *Pax6* function at early stages of lens formation, because the consequences of *Six3* disruption in the mouse have not been reported. Indirect evidence for such an interaction however comes from the targeted deletion of a 340-bp lens-specific *Pax6* enhancer (30), which includes the 107-bp fragment analyzed in our study. This study confirms that this 340-bp enhancer controls early (E9.0–12.0) lens-specific *Pax6* expression, implying a role for the Six3-binding site it contains in the regulation of early *Pax6* expression. This study also indicates the existence of lens-specific enhancer(s) controlling *Pax6* expression at later (>E12.0) developmental stages. The increased *Pax6* expression resulting from the activation of the additional lens-specific enhancer(s) leads to a rescue of the early microphakic phenotype observed in homozygous and heterozygous mutant embryos. These results are therefore analogous to the phenotypic rescue observed in our study and provide independent proof that genetic manipulations at later stages of lens formation can correct early developmental defects.

Prior studies have indicated distinct roles for *Pax6* during eye formation (31, 32). The function of *Six3*, however, remains unclear. Previous studies have indicated roles for the closely related *XSix6/Optx2* gene (33) and the *Drosophila* ortholog *sine oculis* (34) in proliferative control. We have shown that, in lenses of transgenic mouse embryos, *Six3* expression induces cell proliferation. Similarly, lens fiber cell proliferation was observed in α*AmPax6* transgenic embryos, this common effect likely reflecting the cross-regulation between both genes. Our results show that a signaling pathway involving PDGFα-R, ERK-1, and cyclin D1 mediates the effects of *Six3* or *Pax6* on cell proliferation. Expression levels of these factors were increased in transgenic mice, whereas levels of other receptor tyrosine kinases (insulin-like growth factor I-R, fibroblast growth factor-R types 1 and 2) or factors known to regulate lens fiber cell proliferation (Cdkn1c, *Prox1*, and *FoxE3*) (18, 19, 28) were unchanged in transgenic embryos and controls. Although our results would predict critical functions for both PDGFα-R and

D-type cyclins in lens development, disruption of PDGFα-R (35) or cyclin D1 (36) in the mouse have not resulted in any lens defects. However, inactivation of individual PDGF receptors or cyclins in mice appears to be functionally compensated (36, 37), and further analysis would be warranted to determine their exact function during lens development.

Our results indicate that the mutual activation of *Pax6* and *Six3* expression results from direct interactions between each transcription factor and the counterpart gene. These findings agree with studies suggesting a general role for Pax6 as an activator of transcription (38), but appear in conflict with recent studies indicating that Six3 acts as a transcriptional repressor (39, 40). Six3-mediated repression seems dependent on its interaction with vertebrate members of the Groucho-related family of corepressors (39). Transcription factors that interact with Groucho-related proteins are subdivided into two main subclasses (41). A first class consists of constitutive repressors that bind Groucho proteins in a stable fashion. In contrast, conditional repressors are converted from activators to repressors of transcription depending on the presence or absence of a coactivator protein, or whether the context of the DNA-binding site facilitates the recruitment of Groucho-related proteins. Our results suggest that Six3 functions as a conditional repressor. Similar to the *Drosophila* Dorsal (42) and the mammalian Pax5 (43) proteins, the activity of Six3 appears context-dependent, because it can function as a transcriptional activator after binding to native DNA sequences. These results indicate an added complexity in the regulation of target genes by Six3. Moreover, members from all three subgroups of the Six family have been shown to interact with Groucho-related proteins (39). Our findings thus suggest that some, if not all, of these factors may exert dual effects on transcription.

Altogether, our results indicate a conservation of the regulatory mechanisms regulating the expression of *Pax6* and *Six3* orthologs in the eyes of mouse and *Drosophila* (10, 34). During early mouse lens development, *Six3* acts as a downstream target of *Pax6* function (12). Subsequently, we show that both genes directly interact to positively regulate their gene expression levels. A similar regulatory strategy has been described in the developing *Drosophila* eye between *Pax6/eyeless* and *sine oculis*, and the Eya coregulatory protein (10, 34). On the basis of nucleic acid comparisons, *Six3* and the related *Six6/Optx2* share close homologies to the *Drosophila Optix* gene (44), whose activity is independent of that of *Pax6/eyeless* (45), and are more distantly related to *sine oculis*. A possible interpretation is that *Sine oculis* and *Six3* have retained from a common ancestral gene their regulatory relationship to *Pax6/eyeless*, whereas this same strategy has been lost for *Optix* and *Six6/Optx2*. This common gene remains to be identified, although the search for such an ancestral gene in a basal organism (46) should provide novel perspectives on the evolution of both *Pax* and *Six* genes.

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