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The LIM homeobox transcription factor Lhx2 is required to specify the retina field and synergistically cooperates with Pax6 for Six6 trans-activation

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ABSTRACT

In mammals, a limited set of homeobox-containing transcription factors are expressed in the presumptive eye field and required to initiate eye development. How these factors interact together at the genetic and molecular level to coordinate this developmental process is poorly understood. We found that the Lhx2 and Pax6 transcription factors operate in a concerted manner during retinal development to promote transcriptional activation of the *Six6* homeobox-gene in primitive and mature retinal progenitors. *Lhx2* demarcates the presumptive retina field at the neural plate stage and *Lhx2* inactivation delays initiation of *Rx*, *Six3* and *Pax6* expression in this domain. The later expressed *Six6* is properly activated in the pituitary/hypothalamic axis of *Lhx2*$^{-/-}$ embryos, but expression fails to be initiated in the optic vesicle. Lhx2 and Pax6 associate with the chromatin at several regions of *Six6 in vivo* and cooperate for trans-activation of *Six6* regulatory elements *in vitro*. In retinal progenitor/stem cells, both *Lhx2* and *Pax6* are genetically required for proper *Six6* expression and forced co-expression of Lhx2 and Pax6 can synergistically trans-activate the *Six6* locus. Our work reveals how two master regulators of eye development coordinate their action to sequentially promote tissue-specific transcriptional initiation and full activation of a retinal determinant gene.

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Introduction

The optic vesicle of vertebrates originates from the neural tube and is first detected between embryonic stages (e) 8.25 and e8.5 as a bilateral evagination of the anterior neural plate, named the optic sulcus (Marquardt, 2003; Oliver and Gruss, 1997). Around e9.5, the intimate contact between the optic vesicle and the eye surface ectoderm induces the transformation of the ectoderm into a lens placode, which is revealed by the proliferation and thickening of the ectoderm (Oliver and Gruss, 1997). In turn, the lens placode induces the evagination of the optic vesicle into an optic cup around e10.0. The optic cup ultimately gives rise to the fully mature neural retina. How retinal specification is established at the neural plate stage and how definitive retinal identity is promoted and maintained in retinal progenitors remain important issues in developmental biology. The homeobox-containing transcription factors Pax6, Rx, Six3 and Lhx2 are genetically required for eye formation and over-expression of Pax6, Rx or Six3/Six6 can induce ectopic retinal tissues in frog and fish embryos (Bernier et al., 2000; Carl et al., 2002; Chow et al., 1999; Hill et al., 1991; Loosli et al., 1999; Mathers et al., 1997; Porter et al., 1997; Zuber et al., 1999). Although the genetic function of these factors has been highly studied, the molecular mechanisms by which they coordinate retinal development and establish definitive retinal identity remain poorly understood.

Pax6 is a member of the paired-box and homeobox-containing gene family (PAX) of transcription factors and has been used as a prototype to study eye development in several model organisms (Gehring and Kazuho, 1999; Gehring, 2002). In mice, *Pax6* is expressed starting at e8.0 in the eye surface ectoderm, and in the eye neural ectoderm, which gives rise to the optic vesicle (Walther and Gruss, 1991). Despite being anophthalmic at later stage of development, *Pax6*-null embryos form an optic vesicle that arrests in development prior to the optic cup stage (Grindley et al., 1995; Hogan et al., 1986). In the optic vesicle of *Pax6* mutants, neuroepithelial (NE) progenitors over-proliferate and display an abnormal cell cycle kinetic, possibly owing to downregulation of cyclin-dependent kinase inhibitors (Duparc et al., 2007). Conditional mutagenesis of *Pax6* in the e11.0 distal retina revealed that *Pax6* is required at the time of retinogenesis to maintain progenitor cells proliferation and generate retinal cell types diversity, in part through transcriptional activation of pro-neural genes (Marquardt et al., 2001). Although *Pax6* is being considered to operate at the apex of the genetic cascade governing eye and retinal development, expression of *Lhx2*, *Rx*, *Otx2*, *Six3* and *Six6* in the optic vesicle of *Pax6* mutants is unaffected, revealing that early retinal specification does occur in the absence of *Pax6* (Bernier et al., 2001; Jean et al., 1999).

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Lhx2 is a transcription factor that plays an essential role in mammalian's eye development and that is conserved in lower vertebrate species (Porter et al., 1997; Zuber et al., 2003). *Lhx2* encodes a member of the LIM homeobox-containing transcription factors family (*LHX*). LHX proteins can activate or repress gene transcription by direct DNA binding and association with co-activators or co-repressors through their LIM domain (Agulnick et al., 1996). In mice, *Lhx2* is required for the development of numerous organs, including the eye, the telencephalon and blood system (Porter et al., 1997). While little is known about how *Lhx2* operates, experiments have showed that red blood cell defects in *Lhx2*-null mice are non-cell autonomous and mediated by abnormalities that lie within the liver (Porter et al., 1997). In contrast, most abnormalities present in the CNS appear to be cell autonomous, as revealed by chimera aggregation studies (Porter et al., 1997). *Lhx2* is also expressed by immature B and T lymphocytes, but not by hematopoietic stem cells (Wu et al., 1996). Notably, some chromosomal translocations involved in human leukemia's appear to include the *LHX2* locus, and Lhx2 over-expression can immortalized human hematopoietic stem cells (Wu et al., 1996). Recent genetic studies have also revealed that *Lhx2* is required for the self-renewal of epithelial stem cells, but the underlying molecular mechanism remains elusive (Rhee et al., 2006).

Herein we report on the characterization of *Lhx2* function during the earliest steps of retinal development. We found that *Lhx2* represents the first retinal determinant gene with Rx within the presumptive retina field, and that *Lhx2* mutation results in delayed induction of *Six3*, *Rx* and *Pax6* expression in this domain. Later on, *Lhx2* is also required for *Six6* expression initiation in the optic vesicle. In retinal tissue, Lhx2 and Pax6 proteins are bound to the chromatin at the *Six6* locus, and can cooperate to trans-activate Six6 regulatory elements *in vitro*. In retinal progenitor/stem cells, Lhx2 and Pax6 are genetically required for *Six6* expression and can cooperate to synergistically trans-activate the *Six6* gene. Our work reveals that *Lhx2* is required to establish primitive retinal identity at the neural plate stage by allowing initiation of retinal-determinant genes expression, and how later *Lhx2* cooperates with *Pax6* to establish definitive retinal identity and promote cellular proliferation.

Materials and methods

Animals

Adult mice from the albinos CD1 or 129sv strains were purchased from Charles River (St-Constant, Qc., Canada). *Pax6* mutant mice are a gift from Peter Gruss (Max-Planck Institute, Goettingen) and *Lhx2* mutant mice from Heiner Westphal (National Institute of Health, Bethesda). Embryos stage was determined according to the time of vaginal plug.

In situ hybridization

Embryos were dissected in PBS, fixed overnight in 4% paraformaldehyde at 4 °C and embedded in Paraplast (Monoject Scientific). Sections (10 μm) were cut and dried onto super-frost glass slides (Fisher Scientific). ^{35}S-labelled RNA probes using SP6, T3 or T7 RNA polymerase were done with Boehringer enzyme according to the directive of the company. Exposure time for the radioactive RNA *in situ* hybridization was 15 days. For *in situ* hybridizations on whole embryos, preparations were hybridized with digoxigenin-labelled RNA probes and visualized with alkaline phosphatase-coupled anti-digoxigenin antibody (1/2000) (Boehringer) and NBT/BCIP substrate (Boehringer) at pH 9.5. For cryosection, embryos were cryoprotected in 30% sucrose/PBS overnight at 4 °C, embedded in cryomatrix solution and snap-frozen in liquid nitrogen. Specimens were cut using a cryostat (Leica) at 10 μm and used for non-radioactive *in situ* hybridization.

RT-PCR and quantitative real-time PCR

All primers were designed to flank individual exons and tested by PCR in RT+ and RT− control extracts. Total RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcription (RT) was performed using 1 μg of total RNA and the MML-V reverse transcriptase (Invitrogen). PCR amplification was performed using the HotStar TAQ polymerase (Invitrogen). PCR was run as follow; 94 °C for 10 min, followed by 30 cycles of denaturing at 94 °C, annealing at 57 °C and extension at 72 °C in an Applied Biosystems thermal cycler. Real-time PCR was performed using the Platinum SYBRGreen SuperMix (Invitrogen) and a Real-Time PCR apparatus (BioRad).

Chromatin immunoprecipitation

ChIP was performed using the ChIP Assay kit (Upstate) according to the manufacturer's instructions. Briefly, 0.5×10^6 cells were sonicated to shear the chromatin. Chromatin-associated proteins were precipitated using goat anti-Lhx2 (Santa Cruz), rabbit anti-Pax6 (US Biological) or rabbit anti-IgG (Upstate) antibodies. Samples were heated to reverse the protein–DNA crosslinks and the DNA recovered by phenol/chloroform/isoamyl alcohol extraction. Genomic DNA was used as template for PCR amplification using primers to the *Six6* loci.

Luciferase assay

Four distinct genomic DNA fragments from the *Six6* locus (−3964 to −2465, −2480 to −1263, −1284 to −75 and +638 to +1703) were cloned into the pGL3 Luciferase Reporter Vector (Promega). *Pax6*, *Lhx2*, *Six3* and *Otx2* cDNAs were cloned into the expression vector pCS2+. Reporter vectors were transfected with either one expression vector or a combination using Lipofectamine 2000 (Invitrogen). 48 h post-transfection, cells were lysed using Passive Lysis Buffer (Promega). Cells lysates were analyzed using the Dual Glow Luciferase Assay System (Promega).

Cell culture

Optic vesicles of e9.5 embryos were dissected-out with tungsten needles in HBSS, as described (Duparc et al., 2007). Optic vesicles were directly triturated in HBSS using needles (20G-10x; 22G-5x), in order to obtain a suspension of single cells. After centrifugation, cells were placed in neural stem cell (NSC) media: DMEM/F12 (Invitrogen) containing 0.25% glucose, B27 supplement, Heparin (2 μg/ml; SIGMA), Gentamycin (25 μg/ml; Invitrogen) and human recombinant FGF2 (10 ng/ml; Preprotech). Cells were cultured in 6 well plates (Sarstedt) for 3 to 10 days at 37 °C in 5% CO_2 atmosphere. When applicable, BrdU (SIGMA) was added to the culture media at 10 μg/ml. For passage, single retinal spheres were dissociated with an enzyme-free solution (CHEMICON). After trituration, the single cell suspension was harvested at 300 g for 5 min and washed twice with HBSS. Cells were plated at 2000 cells/ml in neural stem cell media. Cell viability was evaluated using a hemacytometer and trypan blue exclusion staining.

Immunofluorescence and immunohistochemistry

For BrdU-labeling experiments, retinal spheres were directly frozen in liquid nitrogen and post-fixed after sectioning using 100% ETOH for 30 min and 4% PFA/PBS for 10 min. Sections or cells were treated with DNase I/0.05% HCl for 30 min in order to reveal BrdU epitopes. Samples were blocked in 1% BSA (Vector laboratories)/0.1% Tween 20/PBS solution and incubated with the primary antibodies overnight at 4 °C. After washes with PBS, samples were incubated with appropriate secondary antibodies for 1 h at RT. Antibodies

used: anti-BrdU (SIGMA), anti-P-H3 (Upstate), and anti-Ki67 (Abcam). For immunohistochemistry labeling, 4% PFA/PBS fixed retinal spheres were equilibrated in sucrose and embed in OCT compound. Frozen sections were analyzed by using the Vectastain® ABC kit (Vector) according to the manufacturer instructions. Peroxidase substrates used is the Vector® DAB (Sigma). Observations were made under microscope (Leica DMRE, Leica Microsystems) and images were captured with a digital camera (Retiga EX; QIMAGING; with OpenLab, ver.3.1.1 software; Open-Lab, Canada). Primary antibody used is the rabbit anti-cleaved caspase-3 (Cell signaling).

DNA micro-array

Total RNA was prepared using TRIzol reagent (Invitrogen) and purified by the RNeasy MiniElute Cleanup kit (Qiagen) from 6 WT and 6 *Lhx2*$^{-/-}$ e9.0 embryos or 3 WT and 3 *Pax6*$^{-/-}$ retinal stem cell cultures at passage 2. Microarray analysis using BeadChip Mouse Genome (Illumina) or GeneChip Mouse Expression Set 430 array, which contains ~39,000 transcripts (Affimetrix) was performed at the Centre d'innovation at Genome Quebec (McGill university, Montreal, PQ). Data were analyzed using the FlexArray software.

DNA electroporation

Mouse *Pax6* and *Lhx2* cDNAs were cloned into the EF1α-CMV/GFP lentiviral vector (L. Cheng, Johns Hopkins University). Retinal stem cells were nucleofected with plasmids DNA using the Mouse Neural Stem Cell Nucleofector Kit according to manufacturer's instructions (Amaxa Biosystems), plated on matrigel (BD Bioscience) in NSC media for 36 h and sorted for GFP expression by fluorescence activated cell sorting.

Virus

Mouse *Six6* cDNA (Jean et al., 1999) was cloned into EF.V.CMV.GFP (L. Cheng, Johns Hopkins University) and transfected in 293T cells with helper vectors (F. Boudreau, Sherbrooke University) using Lipofectamine 2000 (Invitrogen). Viral supernatants were ultra-centrifuged and exposed to single cell suspensions O/N. Aggregates were dissociated to single cells and plated at 2000 cells/ml in NSC media for 1 week. GFP positive retinal spheres were visualized using a fluorescence-mounted inverted microscope (LEICA).

Statistical analysis

Statistical differences were analyzed using Student's *t*-test for unpaired samples. An analysis of variance (ANOVA) followed by the Dunnett test was used for multiple comparisons with one control group. In all cases, the criterion for significance (*P* value) was set as mentioned in the respective figures.

Results

Lhx2 demarcates the presumptive retina field

To characterize *Lhx2* expression pattern during mouse eye development, we performed *in situ* hybridizations on whole embryos (WISH). *Lhx2* expression was first detected in the anterior neural plate at the 2 somites stage in the region corresponding to the prospective retina field (Fig. 1A) (Furukawa et al., 1997; Mathers et al., 1997; Zuber et al., 2003). From e9.5 to e12.5, robust *Lhx2* expression was observed in the optic vesicle and eye, prospective telencephalon, and limb bud (Figs. 1B and C). On embryo sections, *Lhx2* expression was detected at e10.5 in all components of the eye neuroectoderm i.e. optic stalk, optic cup and retinal-pigment epithelium (RPE), and in the ventral diencephalon (Fig. 1D). In the e12.5 and e17.5 retina, *Lhx2* expression is most intense at the retinal ciliary margin and distal RPE, with strong expression also in the outer neural retina and RPE (Figs. 1E and F). Notably, comparative expression analysis at e8.25 revealed that *Lhx2* is co-expressed with *Rx* in the anterior neural plate and that *Lhx2* overlap-with and is contained within *Rx* expression domain (Fig. 1G). In contrast, *Six3* expression is predominant in the prospective pituitary/hypothalamic axis and ventral forebrain, and nearly absent from the retina field (Fig. 1G) (Oliver et al., 1995). *Pax6* has a broader and more diffuse expression throughout the presumptive eye domain, consistent with its later expression in both epithelial and neuroepithelial derivatives (Fig. 1G) (Walther and Gruss, 1991).

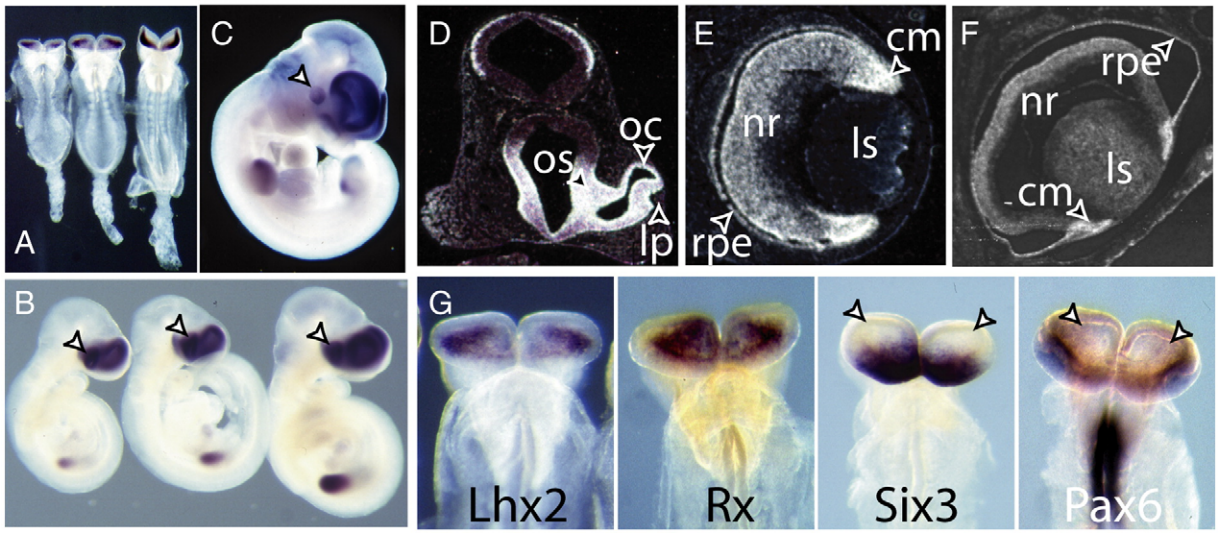


Fig. 1. *Lhx2* is expressed in the visual system and demarcates the retina field (A–G). (A–C) Whole-mount *in situ* hybridization shows that *Lhx2* expression is detectable in the anterior neural plate of mouse embryos at the 2 somites stage (A), in the optic vesicle (arrowheads), telencephalic vesicle and limb from e9.0 (B), and in the eye (arrowheads), neocortex and limb bud at e12.5 (C). (D–F) On embryos sections, robust *Lhx2* expression was detected in the optic cup (oc) and optic stalk (os) at e10.5, in the retinal-pigment epithelium (rpe), central neural retina (nr) and ciliary marginal zone of the retina (cm) at e13.5 and e17.5. No expression was detectable in ectodermal derivatives such as the lens placode (lp) and lens (ls). Non-specific background signal is observed in the ocular mesenchyme in panel D. (G) In 2 somites stage embryos, *Lhx2* and *Rx* expression demarcates a specific region of the anterior neural plate corresponding to the presumptive retina field, where *Six3* and *Pax6* are not yet expressed (arrowheads).

Lhx2 is required to initiate Six3, Rx and Pax6 expression within the retina field

Eye development in *Lhx2* mutant embryos arrest at the optic vesicle stage around e9.0, allowing gene expression analysis at this stage and earlier. To better understand *Lhx2* function in eye development, we performed comparative DNA micro-array analysis on e9.0 WT and *Lhx2*$^{-/-}$ forebrains. These experiments revealed that several genes involved in eye/retinal development are downregulated in *Lhx2*$^{-/-}$ forebrains, including Mitf, Chx10, Vax2, Rx (Rax), Tbx5, Fzd5 and Six6 (Table 1). In sharp contrast, the *Pax6* mutation is not associated with a downregulation of *Lhx2*, *Rx*, *Six3*, *Chx10*, *Otx2* and *Six6* expression in the forebrain and optic vesicle (Bernier et al., 2001; Duparc et al., 2006; Jean et al., 1999).

Table 1
Comparative gene expression analysis between WT and *Lhx2*$^{-/-}$ embryos

Accession number	Gene	Description	Fold change
NM_011445.1	*Sox6*	SRY-box containing gene 6	−0,9082
NM_011384.2	***Six6***	**Sine oculis-related homeobox 6 homolog**	**−0,9108**
NM_001085495	*Arfgef2*	ADP-ribosylation factor guanine nucleotide-exchange	−0,9412
NM_178192.1	*Hist1h4a*	Histone 1, H4a	−0,9438
AK084437	*D230046H12Rik*	Unknown (Riken)	−0,9641
NM_013603.1	*Mt3*	Metallothionein	−0,9667
NM_029768.2	*Use1*	Unconventional SNARE in the ER 1 homolog	−0,9724
AK018697	*Ldlr*	Low density lipoprotein receptor	−0,9785
NM_011921	*Aldh1a7*	Aldehyde dehydrogenase family 1, subfamily A7	−0,9854
AK013968	*3110001N23Rik*	Unknown (Riken)	−0,9872
	9530086O07Rik	Unknown (Riken)	−1,0055
NM_176841.2	*Ccdc88a*	Coiled coil domain containing 88A	−1,0313
NM_001033193.1	***Fzd5***	**Frizzled homolog 5**	**−1,0448**
NM_007740.2	*Col9a1*	Procollagen, type IX, alpha 1	−1,0536
NM_175657	*Hist1h4m*	Histone 1, H4m	−1,0674
NM_199065	*Slitrk1*	SLIT and NTRK-like family, member 1	−1,0994
AK050619	*P38ip*-pending	Transcription factor (p38 interacting protein)	−1,1068
	B830012L14Rik	Unknown (Riken)	−1,1287
AK084485	*D330006D04Rik*	Unknown (Riken)	−1,1665
NM_009719.4	*Neurog3*	Neurogenin 3	−1,2109
AK032066	*Osp94*	Osmotic stress protein 94 kDa	−1,2355
NM_024226	*Rtn4*	Reticulon 4, transcript variant 5	−1,2406
AK054516	*E330035H20Rik*	Unknown (Riken)	−1,2491
NM_153551.1	*Dennd1c*	DENN/MADD domain containing 1C	−1,3124
NM_011537	***Tbx5***	**T-box 5**	**−1,3659**
AK084692	*Stk18*	Serine/threonine kinase 18	−1,3736
NM_008469.1	*Krt1-15*	Keratin complex 1, acidic, gene 15	−1,4316
AK013410	*Rab14*	RAB14, member RAS oncogene family	−1,4464
NM_145463	*Shisa2*	Shisa homolog 2	−1,4861
	C230053D17Rik	Unknown (Riken)	−1,4912
AK054453	*E330027G05Rik*	Unknown (Riken)	−1,5453
AK084661	*D330026I07Rik*	Unknown (Riken)	−1,5460
NM_013833	***Rax***	**Retina and anterior neural fold homeobox**	**−1,6921**
NM_011912.1	***Vax2***	**Ventral anterior homeobox containing gene 2**	**−1,7093**
NM_001083587	*Tsn3*	Tensin 3	−1,7541
AK042261	*Sh3pxd2b*	SH3 and PX domains 2B	−1,9207
NM_013467	*Aldh1a1*	Aldehyde dehydrogenase family 1, subfamily A1	−1,9287
NM_007701.2	***Chx10***	**C. elegans ceh-10 homolog**	**−1,9390**
NM_010024.1	*Dct*	Dopachrome tautomerase	−2,0174
NM_001009950	*Slc38a8*	Solute carrier family 38, member 8	−2,0912
NM_007799	*Ctse*	Cathepsin E	−2,1450
NM_008601	***Mitf***	**Microphthalmia-associated transcription factor**	**−2,2651**
XM_122498.1	*Tm7sf1*	Transmembrane 7 superfamily member 1	−2,6245
NM_010024	***Dct***	**Dopachrome tautomerase**	**−3,2626**

Six forebrains from each genotypes at e9.0 were analyzed using Illumina BeadArray™ technology. Genes in bold have been involved in eye development and are downregulated in *Lhx2*$^{-/-}$ embryos.

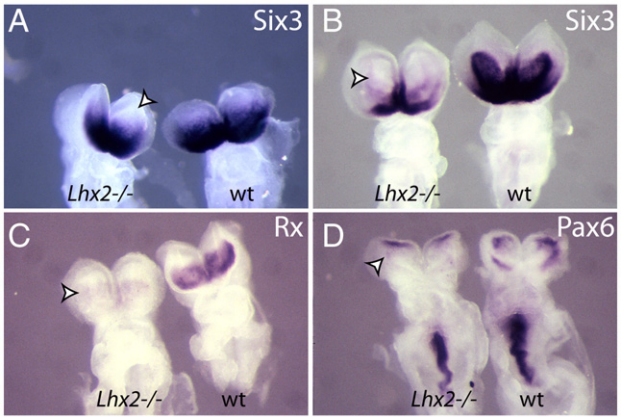


Fig. 2. *Lhx2* is required for *Six3*, *Rx* and *Pax6* expression in the retina field, as revealed by *in situ* hybridization (A–D). (A and B) In *Lhx2*$^{-/-}$ embryos, *Six3* expression is detected in the pituitary/hypothalamic axis and ventral forebrain, but not in the retina field (arrowheads) at neural plate (A) and neural fold (B) stages. (C and D) In *Lhx2*$^{-/-}$ embryos, *Rx* and *Pax6* expression is absent or highly reduced in the retina field (arrowheads).

Based on the above result, we hypothesized that *Lhx2* may control the expression of eye genes at the earliest steps of retinal specification. To test this, we performed WISH on e8.25/e8.5 WT and *Lhx2*$^{-/-}$ embryos, thus prior to any visible morphological abnormalities in *Lhx2* mutants. In e8.25 WT embryos, *Six3* expression was robust in the presumptive pituitary/hypothalamic axis and ventral forebrain region, but expression in the retina field was only initiated. At e8.5, *Six3* expression in the optic sulcus was well established (Figs. 2A and B). In contrast, although *Six3* expression was present in the presumptive pituitary/hypothalamic axis and ventral forebrain of *Lhx2* mutants, expression failed to be initiated in the retina field and optic sulcus (Figs. 2A and B). Similarly, *Pax6* and *Rx* expression was absent or reduced in the anterior neural plate of *Lhx2* mutants at e8.25 (Figs. 2C and D). These results show that *Lhx2* is required at the earliest stage of retina specification to initiate *Six3*, *Rx* and *Pax6* expression within the retina field.

Lhx2 is required for Six6 expression in the optic vesicle

Although gene expression fails to be initiated at the neural plate stage, *Six3* expression was present (but reduced) at e9.0 in the optic vesicle of *Lhx2* mutants (Fig. 3B and E). *Six3* expression was also reduced in the prospective telencephalic vesicle of *Lhx2* mutants (Fig. 3B). In contrast to *Six3*, expression of the *Six3*-related homeobox gene *Six6* is only initiated at 3–4 somites stage in the prospective pituitary/hypothalamic axis, and later spreads to the presumptive ventral optic stalk and optic vesicle at e9.0 (Jean et al., 1999). In *Lhx2* mutants, we observed normal *Six6* expression pattern in the pituitary/hypothalamic axis at e9.0, but expression in the optic vesicle was not detected (Figs. 3C and D). To further confirm this observation, we performed WISH using *Rx* as control for expression in the optic vesicle. *Rx* expression was reduced but present in the optic vesicle of *Lhx2*$^{-/-}$ embryos (Fig. 3A). To quantify these observations, we performed gene expression analyses on forebrain extracts from e9.0 WT and *Lhx2* mutant embryos by Real-time PCR. Because the optic vesicle of *Lhx2* mutants is smaller even at this stage, we normalized for eye genes expression using Rx as standard. Even after normalization, the expression of *Six3* and *Six6* was still reduced by 75% and 85% in *Lhx2* mutant forebrains, respectively (Fig. 3E). These results suggest that *Lhx2* is required to initiate *Six6* and also possibly *Six3* expression in the optic vesicle.

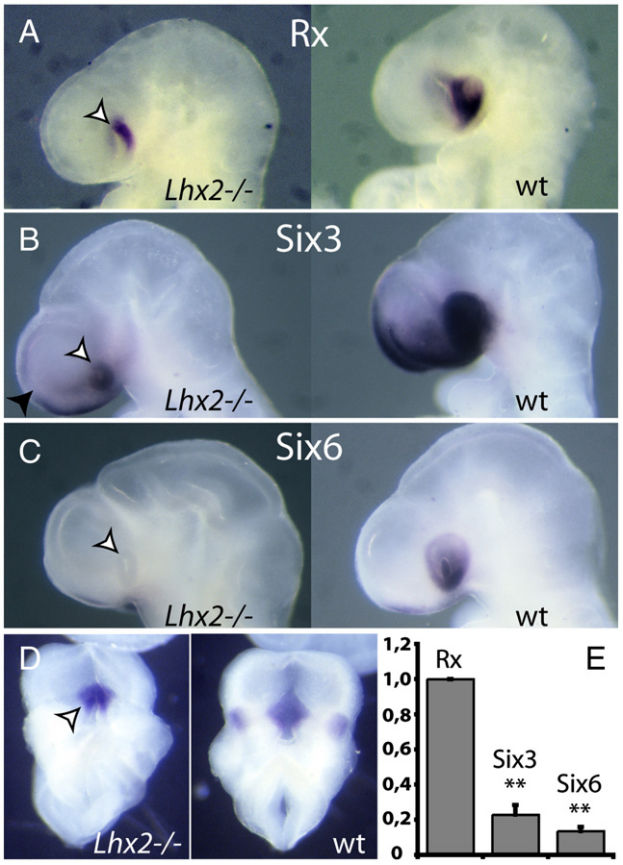


Fig. 3. *Lhx2* is required for *Six6* expression in the optic vesicle. (A–D) Whole-mount *in situ* hybridization shows that in e9.0 *Lhx2*$^{-/-}$ embryos, *Rx* (A) and *Six3* (B) expression is present but reduced in the optic vesicle (arrowheads). *Six3* expression is also reduced in the telencephalic vesicle of *Lhx2*$^{-/-}$ embryos (black arrowhead in panel B). In *Lhx2*$^{-/-}$ embryos, *Six6* expression is detected in the pituitary/hypothalamic axis (arrowhead in D) but not in the optic vesicle (C). Real-time PCR analysis of e9.0 forebrains revealed that when compared to WT littermates ($n=4$), *Six3* and *Six6* expression is highly reduced in *Lhx2* mutants ($n=3$), even when normalized to *Rx* expression level (E), which was set to 1. $^{**}P<0.01$.

Lhx2 and Pax6 can bind to the chromatin at the Six6 loci in vivo and trans-activate Six6 regulatory elements in vitro

Our results suggest that Lhx2 might bind to *Six6* promoter regions for expression in the developing retina. To test this, we characterized the *Six6* gene for putative Lhx2 DNA binding sites using the TRANSFACT algorithm. Several putative sites were identified in the 5′ promoter region and intron I of *Six6* (Fig. 4B). Several putative DNA binding sites were also identified for Pax6 (Fig. 4B). To test for potential association of Lhx2 or Pax6 with the chromatin at these regions *in vivo*, we performed chromatin immunoprecipitation (ChIP) experiments on e12.5 mouse retinas (Duparc et al., 2007). Based on the location of the putative DNA binding sites identified, we scanned 18 chromatin domains each covering ~300 base pairs of genomic DNA. Lhx2 association with the chromatin was found in genomic DNA regions corresponding to binding sites −3795, −1650 and −290 of *Six6* (Fig. 4A). Pax6 association with the chromatin was found in genomic DNA regions corresponding to binding sites −1650, −290, +685 and +4180 of *Six6*. Common and robust association of Lhx2 and Pax6 with the chromatin was found at sites −1650 and −290. Control ChIP with an anti-IgG antibody or amplification of the β-*globin* promoter suggests that this association is specific (Fig. 4A-inset, and data not shown).

Based on the bioinformatics and ChIP results, we hypothesized that Lhx2 and Pax6 directly regulate *Six6* transcription. To test this, we performed Luciferase assay in 293T cells using 4 distinct genomic DNA fragments covering the 5′ promoter region (position −3964 to −75) and intron I (position +638 to +1703) of *Six6* (Fig. 4C). We found that Lhx2 alone was not sufficient to induce significant trans-activation of these DNA fragments. Pax6 alone could trans-activate fragment 1 (position −3964 to −2465) and 3 (position −1284 to −75) just above the baseline level, which was established at 3 (Fig. 4C). Notably, adding Lhx2 and Pax6 together resulted in relatively strong trans-activation of fragments 1 and 2 (position −2480 to −1263) (Fig. 4C). Robust activation of fragment 2 using both factors correlated with co-association of Lhx2 and Pax6 with the chromatin at position −1650, which is contained within fragment 2. We also tested if adding additional factors, such as Six3 and Otx2, could result in a more pronounced trans-activation of *Six6* regulatory elements (Zuber et al., 2003). Adding all factors together did not enhanced Pax6 and Lhx2 activity on *Six6* DNA fragments. In contrast, it apparently interfered with the previously observed activity on fragment 1 and 2 (Fig. 4C). These results suggest that Lhx2 and Pax6 are associated with the chromatin at the *Six6* locus *in vivo* and can trans-activate *Six6* regulatory elements *in vitro*.

Lhx2 is required for Six6 expression in retinal progenitor/stem cells

We previously reported on the isolation and characterization of NE retinal progenitors present in the mouse optic vesicle (Duparc et al., 2007). These NE progenitors display all the characteristics of retinal stem cells. We performed dissociated cultures of WT and *Lhx2*-mutant optic vesicles at e9.0 in serum-free media to isolate NE progenitors in a neurosphere assay (Duparc et al., 2007). Retinal colonies from *Lhx2* mutants were smaller and less abundant then from WT littermates (Fig. 5A). Considering the reduce size of the optic vesicle in *Lhx2* mutants, we performed secondary colony formation assay (self-renewal assay) from single spheres, allowing normalization of the number of cells plated/well. Single spheres were dissociated to single cell suspensions and 2000 cells/ml were re-plated in the same media. Under these conditions, retinal colonies were smaller and less abundant in the absence of *Lhx2* (Fig. 5B and data not shown). To identify the underlying cellular defect, we first measured the mitotic index (PH3^{+}/DAPI^{+} cells) in WT and *Lhx2*$^{-/-}$ colonies. No significant differences were found between both genotypes (data not shown). The Ki67 antigen is expressed at all phases of the cell cycle, but not in G0, and is used as a marker for progenitors (Scholzen and Gerdes, 2000; Endl et al., 2001). To analyze the cell cycle, spheres from both genotypes were pulsed with Bromodeoxy-Uridine (BrdU) for 60 min and analyzed on sections with antibodies against BrdU and Ki67 (Chenn and Walsh, 2002; Klezovitch et al., 2004). BrdU incorporation assays revealed a marked reduction in the number of BrdU^{+}/DAPI^{+} cells (Fig. 5C) and BrdU^{+}/Ki67^{+} progenitor cells (Fig. 5D) in *Lhx2*$^{-/-}$ colonies, suggesting that *Lhx2*$^{-/-}$ progenitors are partially arrested at the G1 phase of the cell cycle. To further test this, we evaluated the frequency of progenitors not having entered the S phase (i.e. Ki67^{+}/BdrU^{-} cells) after chronic exposure to BrdU for 12 h. We found that the frequency of unlabeled progenitors was significantly higher in *Lhx2*$^{-/-}$ colonies, again suggesting that *Lhx2*$^{-/-}$ progenitors have a tendency to be arrested in G1 (Fig. 5E). We also measured the frequency of apoptotic cells (activated caspase-3^{+}/DAPI^{+} cells) and found a significant increase in the number of apoptotic cells in *Lhx2*$^{-/-}$ retinal spheres (Fig. 5H).

To test if this system was relevant to study gene regulation, we performed Real-time PCR analysis on WT and *Lhx2*$^{-/-}$ retinal colonies. We found that *Six3* and *Six6* expression was reduced by 50% and 70% in *Lhx2*$^{-/-}$ colonies, respectively, thus similarly as in the optic vesicle (Fig. 5F). Work performed in medaka (*Oryzias latipes*) revealed that Six3 and Six6 proteins could promote cell cycle entry and DNA replication in part by preventing the physical interaction of

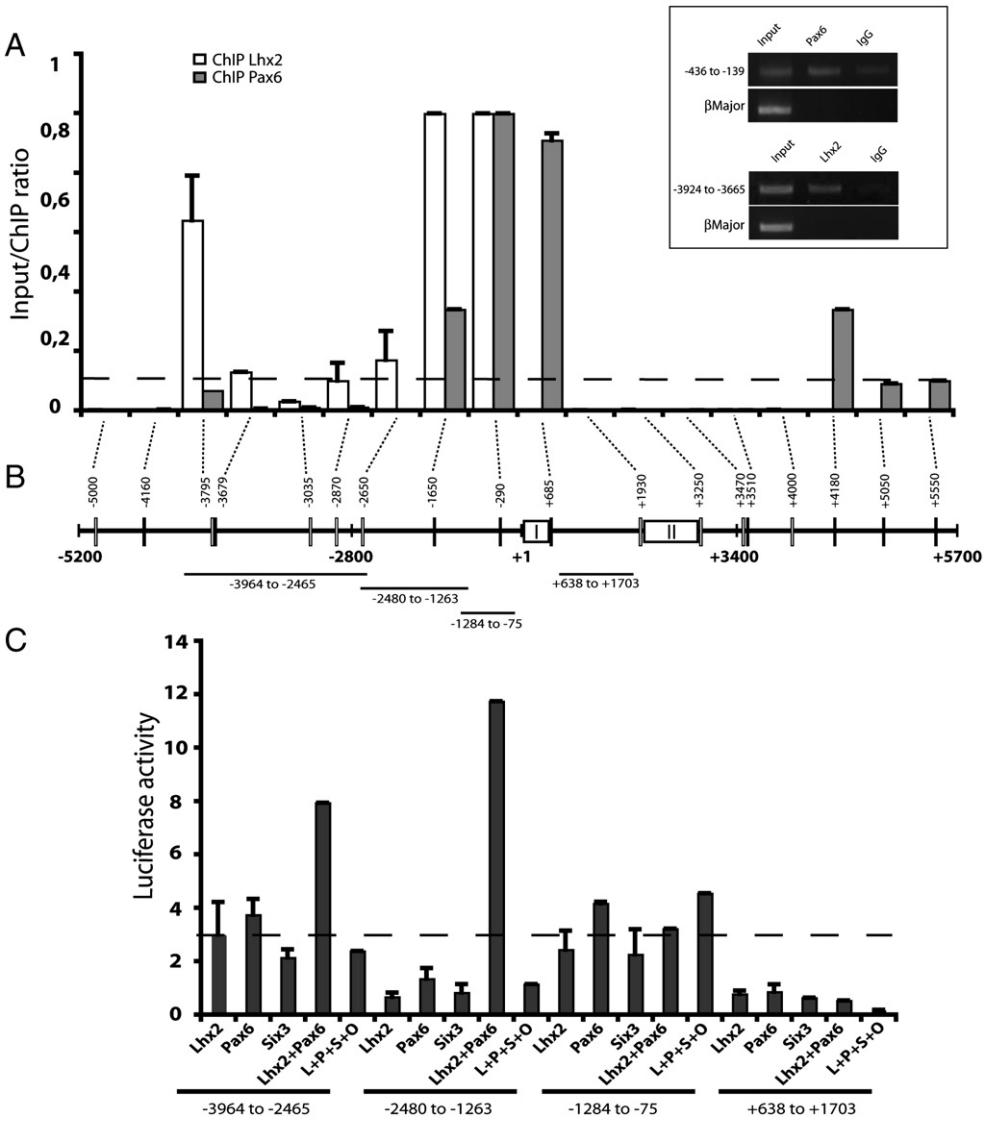


Fig. 4. Lhx2 and Pax6 can bind to the chromatin at the *Six6* loci *in vivo* and trans-activate *Six6* regulatory elements *in vitro*. (A) ChIP scanning experiment at the *Six6* loci on e12.5 mouse retinas using anti-Lhx2, anti-Pax6 and anti-IgG antibodies ($n=2$). (A and B) Based on the location of several putative Lhx2 or Pax6 DNA binding sites covering about 10 k base pairs of genomic DNA at the *Six6* loci (B), PCR primers were designed to amplified 18 DNA fragments containing these sites. Quantitative analysis of the input/ChIP ratio for each fragment reveals an enrichment of Lhx2 or Pax6 proteins on the chromatin at different regions of *Six6* (A). ChIP enrichment was found significant when above the established arbitrary baseline level of 0.1. Control anti-IgG antibody and amplification of a fragment of the β-globin (βMajor) promoter suggest that the observed associations are specific (inset). (B) Physical map of the *Six6* loci showing the 4 DNA fragments used in Luciferase assays. The white boxes correspond to exon I and II of *Six6*. (C) Luciferase assays were performed in 293T cells using 4 genomic DNA fragments ($n=3$), and Luciferase activity was found significant when above the established arbitrary baseline level of 3.

Geminin with Cdt1, the main component for the assembly of the pre-replication complex (Del Bene et al., 2004). Six3 and Six6 may also promote cell proliferation by repressing the transcription of cyclin-dependent kinase inhibitors (Gestri et al., 2005; Li et al., 2002). We compared cyclin-dependent kinase inhibitors (i.e. $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) expression in WT and $Lhx2^{-/-}$ retinal colonies, micro-dissected e9.25 forebrains, and micro-dissected e9.0 optic vesicles by Real-time PCR. We found that the expression of all 3 cyclin-dependent kinase inhibitors was increased in $Lhx2^{-/-}$ samples *in vitro* and *in vivo*, with the most dramatic and consistent up-regulation observed for $p27^{Kip}$ (Figs. 5F and G).

Over-expression of Six3 or Six6 in Medaka or Xenopus embryos resulted in increased retinal progenitor cells proliferation and ectopic retinal tissue formation (Bernier et al., 2000; Loosli et al., 1999; Zuber et al., 1999). We rationalized that reduced expression of *Six6* and *Six3* in $Lhx2^{-/-}$ retinal colonies may explain the proliferation defect. To test this, we over-expressed Six6/GFP or GFP alone in WT and $Lhx2^{-/-}$ retinal colonies using lentiviruses. We observed that cells infected with multiple Six6/GFP viral copies entered into apoptosis within 48 h. Similar results were obtained with a Six3/GFP lentivirus. However, using lower viral concentrations, we could achieve conditions where the Six6/GFP virus was non-toxic. In clonal dissociation assays, we found that $Lhx2^{-/-}$ retinal colonies infected with the Six6/GFP virus were larger than those infected with the GFP virus after 1 or 2 passages (GFP virus: 194±14 μm; Six6/GFP virus: 230±24 μm, $P=0.02$). These results suggest that restoring *Six6* expression in $Lhx2^{-/-}$ retinal colonies can partially rescue the growth defect, and reveal that Six3/Six6 gene-dosage is extremely sensitive in this particular cellular context. Collectively, our data show that $Lhx2^{-/-}$ retinal progenitor/stem cells generate smaller colonies due to a proliferation defect and elevated apoptosis, and suggest that the reduced proliferation phenotype is link to *Six6* and *Six3* down-regulation and increased cyclin-dependent kinase inhibitors activity.

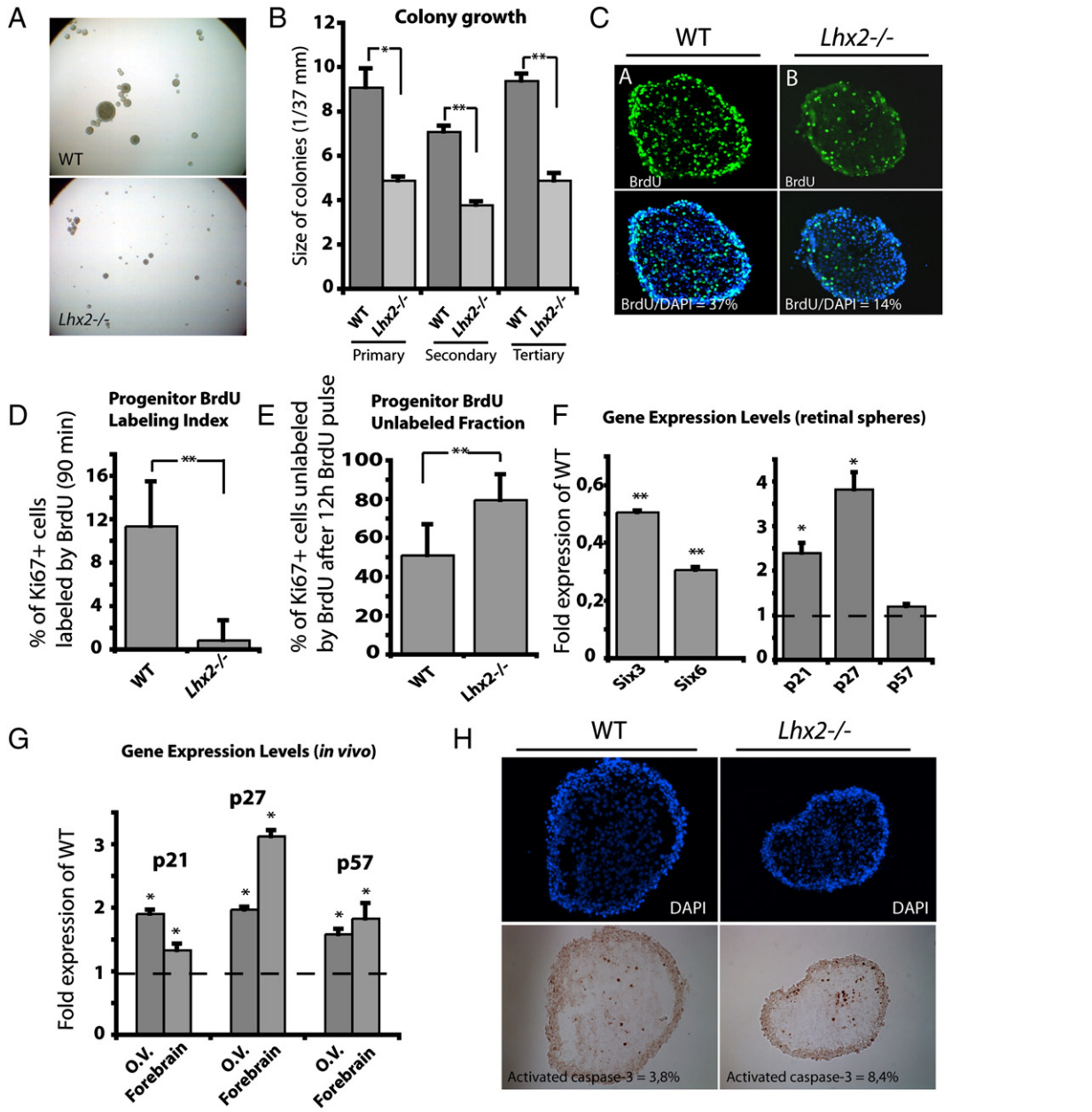


Fig. 5. *Lhx2* is required for *Six6* expression in retinal progenitor/stem cells. (A and B) Retinal spheres from *Lhx2*$^{-/-}$ embryos are smaller than WT littermates in primary cultures and after serial passages of single spheres (Primary culture $n=5$, Secondary $n=5$, Tertiary $n=3$). (C and D) Immunofluorescence on sections of retinal spheres exposed to BrdU for 90 min revealed a marked reduction in BrdU incorporation in *Lhx2*$^{-/-}$ spheres ($n=3$) compared to WT ($n=3$). BrdU saturation experiment reveals that most Ki67^{+} *Lhx2*$^{-/-}$ progenitors have not yet incorporate BrdU after12 h of BrdU exposition (E). Real-time PCR analysis of *Six3* and *Six6* (F), and *p21*^{Cip1}, *p27*^{Kip1} and *p57*^{Kip2} expression levels in *Lhx2*$^{-/-}$ ($n=5$) and WT ($n=5$) retinal spheres (F), *Lhx2*$^{-/-}$ ($n=2$) and WT ($n=2$) e9.0 optic vesicles (O.V.), and *Lhx2*$^{-/-}$ ($n=2$) and WT ($n=2$) e9.25 forebrains ($n=2$) (G). Immunohistochemistry on *Lhx2*$^{-/-}$ and WT retinal sphere sections using an anti-activated caspase-3 antibody. Sections were mounted with DAPI to calculate the number of caspase-3^{+} cells over the total number of DAPI^{+} cells/section (H). *$P<0.05$; **$P<0.01$.

Pax6 is required for Six6 expression in retinal progenitor/stem cells

Pax6 is not required for *Six6* expression in the optic vesicle (Jean et al., 1999). However, based on the ChIP and Luciferase assay results, we hypothesized that *Pax6* is required for *Six6* expression maintenance. We performed global gene expression analysis using DNA micro-arrays to compare WT and *Pax6*-null retinal colonies isolated from e9.5 optic vesicle (Duparc et al., 2007). These experiments revealed that in *Pax6*$^{-/-}$ retinal colonies, *Six6* is the most down-regulated gene from the entire array (Fig. 6A). To confirm this, we performed gene expression analyses on individual WT and *Pax6*$^{-/-}$ retinal spheres by RT-PCR. We found that in contrast with *Six3*, *Lhx2* and *Otx2*, which expression is apparently normal, expression of *Six6* is undetectable in *Pax6*$^{-/-}$ samples (Fig. 6B). These results reveal that *Pax6* is genetically required for *Six6* expression in retinal progenitor/stem cells.

Lhx2 and Pax6 can synergistically activate Six6 expression in retinal stem cells

To further explore the relationship between Lhx2, Pax6 and Six6 gene activation, we forced Lhx2 or Pax6 expression in cultured retinal stem cells. Retinal colonies were dissociated to single cell suspensions and electroporated with the Lhx2/GFP, Pax6/GFP or GFP-only DNA constructs (Fig. 7). At the time of optimal GFP expression (i.e. 36 h post-electroporation), cells were sorted by fluorescence activated cell

A

Experiment1	Experiment2		
Fold change	Fold change	Accession	Gene
-57,656848	-13,678414	AF050130	**Six6**
-27,617735	-12,198791	AV271189	Unknown
-25,55683	-10,410566	BB039510	Enpp3
-21,622613	-21,794276	AF237702	Shmt1
-20,255741	-12,582181	BC011272	**Pax6**
-14,869398	-38,752774	C86550	Dcpp1
-10,029585	-7,7758217	BM240873	Btla
-9,3993656	-4,961376	NM_009387	Tk1
-7,829564	-4,2806747	BG070087	Unknown
-7,7373348	-5,6176802	D89669	Cyp24a1
-7,677381	-4,7866593	NM_009369	Tgfbi
-7,1835815	-13,666596	NM_023245	Palmd
-7,0349684	-8,4578295	L04961	Xist
-6,9133153	-5,5826691	BB533460	Tgfbi

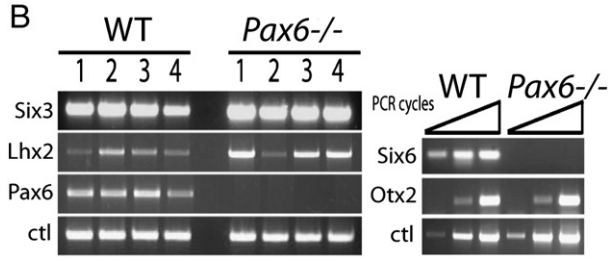


Fig. 6. *Pax6* is required for *Six6* expression in retinal progenitor/stem cells. Comparative DNA micro-array (Affimetrix Chip) analysis of *Pax6*$^{-/-}$ and WT retinal spheres reveals that *Six6* is the most under-expressed gene in *Pax6*$^{-/-}$ cells (A). RT-PCR analysis on 4 individual retinal spheres from *Pax6*$^{-/-}$ and WT genotypes reveals that *Six6* expression is not detectable in *Pax6*$^{-/-}$ cells, in contrast to *Six3*, *Lhx2* and *Otx2*. *Gapdh* was used as the internal control (ctl) for normalization (B). *$P<0.05$; **$P<0.01$.

sorting for GFP expression and analyzed by Real-time PCR. When compared together, GFP-positive and GFP-negative cells electroporated with the control plasmid displayed near identical *Six6* expression levels. However, GFP-positive cells electroporated with the Lhx2/GFP construct had 9–10 fold higher *Six6* expression levels and 2–3 fold higher *Six3* expression levels (Fig. 7). Pax6/GFP over-expression alone had no detectable effect on *Six3* or *Six6* expression. To test the possibility of cooperative activation, we electroporated a DNA mixture containing Lhx2 and Pax6 constructs. Notably, Lhx2 and Pax6 constructs could induce together a ~40 folds activation of *Six6* transcription (Fig. 7). The impact on *Six3* expression was comparable to that of Lhx2/GFP over-expression alone. These results suggest that Lhx2 and Pax6 can synergistically activate *Six6* in retinal stem cells.

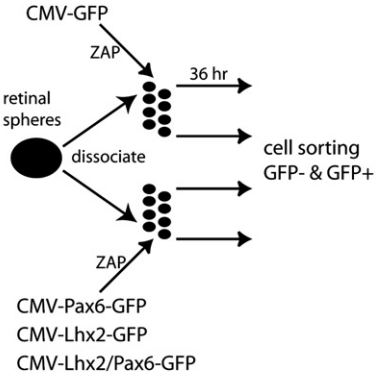
Discussion

Our data indicate that *Lhx2* demarcates the presumptive retina field with *Rx* in the anterior neural plate, and that *Lhx2* is required to establish primitive retinal identity at this stage by either directly inducing or allowing initiation of retinal genes expression. Although *Lhx2* mutants develop optic vesicles, similarly as *Pax6*-null mouse or *Six3*-morphan medaka embryos, the impact of Lhx2 loss-of-function on gene expression is unique. In both *Pax6* and *Six3* mutants, initiation of retinal gene expression (i.e. Rx, Pax6, Lhx2, Otx2 and Six3) is normal at the neural plate and early optic vesicle stages, indicating that these factors are not required for gene expression initiation (Bernier et al., 2001; Carl et al., 2002). Similarly, *Six6* expression is apparently normal in the optic vesicle of *Pax6*-mutants (Jean et al., 1999). In contrast, expression of *Pax6*, *Rx*, *Six3* and *Six6* is absent or delayed in the retina field and highly reduced in the optic vesicle of *Lhx2* mutants. *Six3* expression is also reduced in the telencephalic vesicles of *Lhx2* mutants (see Fig. 3B). In this context, it is noteworthy that an inverse genetic relationship has been proposed in the zebrafish forebrain, where *Six3* apparently regulates *Lhx2* expression and where *Lhx2* over-expression could overcome *Six3* knockdown (Ando et al. 2005). However, this genetic relationship is most unlikely in the mouse visual system. Hence, while *Six3* is expressed earlier than *Lhx2* in the anterior neural plate (Oliver et al. 1995), its expression in the presumptive retina field occurs 6–12 h after that of *Lhx2* and *Rx*.

At first glance, our results suggest that Lhx2 may activate directly *Six3*, *Rx* and *Pax6* transcription in the retina field, and thus operates at the upper most position in the genetic hierarchy governing retina formation. However, over-expression of Lhx2 alone in retinal stem cells, while inducing *Six6* expression, had little impact on *Six3* or *Pax6* expression. Furthermore, there is no report of eye-inducing activities of Lhx2 in other model organisms. Thus, Lhx2 may not be sufficient to activate the expression of "upstream regulators" by its own in gain-of-function experiments, but is clearly required for gene expression initiation in the retina field.

One possible alternative interpretation of these observations is that *Lhx2* may be involved in "potentiating" the expression of early retinal determinant genes prior to the establishment of retinal identity. Gene potentiation or priming as been proposed to be an important mechanism for the developmental expression of lineage-specific transcription factors prior to the establishment of definitive

A



B

	Relative expression levels				
	Lhx2	Pax6	Six3	Six6	Hprt
CMV-GFP-	1.0	1.0	1.0	1.0	1.0
CMV-GFP+	1.0	1.0	1.0	1.0	1.0
CMV-Pax6-GFP-	0.5	1.3	4.0	0.8	1.0
			II	II	
CMV-Pax6-GFP+	0.7	9.3	3.4	**0.9**	1.0
CMV-Lhx2-GFP-	1.04	1.4	1.6	1.6	1.0
			^	^^^	
CMV-Lhx2-GFP+	9.28	0.9	3.1	**9.9**	1.0
CMV-Lhx2/Pax6-GFP-	0.95	1.1	0.8	0.4	1.0
			^	^^^	
CMV-Lhx2/Pax6-GFP+	1.71	4.3	2.3	**41.3**	1.0

Fig. 7. Lhx2 and Pax6 can synergistically activate *Six6* expression in retinal progenitor/stem cells. An experimental system was designed to electroporate plasmid DNA into cell suspensions of dissociated retinal spheres (A). After 36 h of culture on matrigel, cells were sorted for GFP expression by fluorescence activated cell sorting (FACS) and RNA was isolated from the GFP− and GFP+ cell populations ($n=2$). Quantification of *Lhx2*, *Pax6*, *Six3* and *Six6* expression in FACS purified cells using Real-time PCR. Hprt was used as internal control for normalization of samples concentrations. Cells electroporated with the GFP− only vector and sorted as GFP+ were used as standard reference (set at 1) for the Pax6/GFP+ and Lhx2/GFP+ cell populations (B). Cells sorted as GFP− were used as standard reference (set at 1) for the Pax6/GFP− and Lhx2/GFP− cell populations. These experiments reveal that *Six6* can be activated by Lhx2 alone, but not by Pax6 alone, or by the combination of Lhx2 and Pax6 (numbers in bold).

identity in multi-potent hematopoietic stem cells (Bottardi et al., 2007). Gene potentiation involves the maintenance of an accessible chromatin conformation in multi-potent stem cells, counterbalancing possible epigenetic silencing at specific loci (Bottardi et al., 2007; Szutorisz et al., 2005). Whether Lhx2 has a function in modulating chromatin organization or accessibility for other transcription factors remains to be evaluated.

Once primitive retinal identity is established in the anterior neural plate and optic sulcus, it is predicted that "master regulators of eye development" such as Rx, Pax6, Six3, Lhx2 and Otx2 would cooperate to establish definitive retinal identity by inducing high expression levels of retinal determinant genes. The underlying mechanisms to induce high gene expression levels could involve synergistic activation and positive autoregulatory feedback loops. We found here that the concerted action of Lhx2 and Pax6 results in the synergistic activation of *Six6* transcription. Notably, this correlates with association of both Pax6 and Lhx2 with the chromatin at the *Six6* loci, sometime on the same chromatin region, suggesting the possible formation of a yet uncharacterized molecular complex. We also found that *Lhx2* is required for *Six6* expression initiation in the optic vesicle, and that *Pax6* is required for *Six6* expression maintenance in cultured retinal stem cell isolated from the optic vesicle. Interestingly, our results are in accordance with studies performed in medaka and *Xenopus* embryos. In *Xenopus*, combined over-expression of *Pax6*, *Six3* and *Otx2* results in more robust ectopic eye induction than over-expression of *Pax6* alone, suggesting either synergistic activation of retinal gene expression and/or more efficient re-programming of retinal competence in non-retinal tissues (Zuber et al., 2003). In medaka, inactivation of *Pax6* or *Six3* is associated with normal retinal gene expression at early optic vesicle stages, but gene expression is lost later on, showing that *Pax6* and *Six3* are individually required for gene expression maintenance (Carl et al., 2002).

One of the most striking phenotype of *Lhx2* mutants is the reduced optic vesicle and forebrain size owing to reduced cell proliferation (Porter et al. 1997). In *Lhx2*-null retinal progenitor/stem cell cultures, we found that proliferation was highly reduced and that progenitors were stalled at the G1 phase of the cell cycle at higher frequencies than normal. Apoptosis was also elevated compare to WT controls. The observed cell cycle phenotype correlated with reduced *Six3* and *Six6* expression and increased expression of cyclin-dependent kinase inhibitors in retinal progenitor/stem colonies, e9.25 forebrains and e9.0 optic vesicles of *Lhx2* mutants. These observations indirectly support the hypothesis that Six3 and Six6 can repress cyclin-dependent kinase inhibitors expression (Gestri et al., 2005; Li et al., 2002). They also highlight the unanticipated opposite function of Pax6 in repressing retinal progenitor/stem cells proliferation through activation of cyclin-dependent kinase inhibitors expression (Duparc et al. 2007). The complexity of the system is further revealed by the simultaneous requirement for *Pax6* in the maintenance of *Six6* expression in retinal colonies (this study). The biological significance of the apparent genetic antagonism between *Lhx2* and *Pax6* in optic vesicle growth is unclear at the moment but could allow the establishment of equilibrium in NE progenitors cell cycle kinetics.

In conclusion, our results suggest a model for early retinal specification where *Lhx2* delineates the retina field with *Rx* and allows induction of eye gene expression in this domain, possibly by "potentiating" the chromatin. Once primitive retinal identity is established, Lhx2 cooperates with Pax6 for robust trans-activation of downstream target genes such as *Six6* in order to establish and maintain definitive retinal identity. The herein proposed model likely also involves Six3, Rx and Otx2 in a much more complex molecular network, and could represent a common strategy to establish and maintain cell type identity in different regions of the developing CNS.

Acknowledgments

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