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Proper differentiation of photoreceptors and amacrine cells depends on a regulatory loop between NeuroD and Six6

Ivan Conte*,†, Raquel Marco-Ferreres†, Leonardo Beccari, Elsa Cisneros, José María Ruiz, Noemí Tabanera and Paola Bovolenta‡

SUMMARY

Timely generation of distinct neural cell types in appropriate numbers is fundamental for the generation of a functional retina. In vertebrates, the transcription factor *Six6* is initially expressed in multipotent retina progenitors and then becomes restricted to differentiated retinal ganglion and amacrine cells. How *Six6* expression in the retina is controlled and what are its precise functions are still unclear. To address this issue, we used bioinformatic searches and transgenic approaches in medaka fish (*Oryzias latipes*) to characterise highly conserved regulatory enhancers responsible for *Six6* expression. One of the enhancers drove gene expression in the differentiating and adult retina. A search for transcription factor binding sites, together with luciferase, ChIP assays and gain-of-function studies, indicated that NeuroD, a bHLH transcription factor, directly binds an 'E-box' sequence present in this enhancer and specifically regulates *Six6* expression in the retina. NeuroD-induced *Six6* overexpression in medaka embryos promoted unorganized retinal progenitor proliferation and, most notably, impaired photoreceptor differentiation, with no apparent changes in other retinal cell types. Conversely, *Six6* gain- and loss-of-function changed *NeuroD* expression levels and altered the expression of the photoreceptor differentiation marker *Rhodopsin*. In addition, knockdown of *Six6* interfered with amacrine cell generation. Together, these results indicate that Six6 and NeuroD control the expression of each other and their functions coordinate amacrine cell generation and photoreceptor terminal differentiation.

KEY WORDS: Neurogenesis, Retinal development, Transcription regulation, Medaka

INTRODUCTION

The vertebrate retina is a layered structure composed of six neuronal and one glial cell type, which are organised in three cellular layers: the ganglion cell layer, comprising retinal ganglion (RGC) and displaced amacrine cells, the inner nuclear layer (INL), which contains bipolar, horizontal and amacrine interneurons and Müller glial cells, and the outer nuclear layer (ONL), where rod and cone photoreceptors are located (Rodieck, 1998).

These cell types differentiate from common multipotent retinal progenitors in a loosely conserved temporal order, where RGCs are always the first to be generated. Although secreted factors, such as Fgfs and Shh, are crucial for the onset and propagation of retinal differentiation (Esteve and Bovolenta, 2006; Martinez-Morales et al., 2005), combinations of proneural basic helix-loop-helix (bHLH) and homeodomain (HD)-type transcription factors (TFs) determine the intrinsic properties of retinal precursors and regulate their differentiation into specific cell types (Hatakeyama and Kageyama, 2004; Wang and Harris, 2005). For example, Ath5, a bHLH-TF, renders postmitotic retinal precursors competent to generate RGCs and activates the expression of HD-TFs such as Brn3 and Islet 1, which are required for RGC differentiation (Hatakeyama and Kageyama, 2004). Similarly, the bHLH TF NeuroD is expressed in postmitotic retinal cells that originate amacrine and photoreceptor cells, although with notable variations among vertebrate species (Liu et al., 2008; Moore et al., 2002; Ochocinska and Hitchcock, 2007; Ochocinska and Hitchcock, 2009; Yan and Wang, 1998). Co-expression of the HD-TF Crx drives NeuroD-positive progenitors toward the photoreceptor fate, activating rod- or cone-specific determinants (Hennig et al., 2008). Although similar TF combinations have also been determined for the remaining retinal neurons (Hatakeyama and Kageyama, 2004; Wang and Harris, 2005), the precise transcriptional network required for the full differentiation of each retinal cell type is still poorly understood and probably involves additional components.

Six3 and *Six6* are two highly related members of the *Six*/*sine oculis* family of HD-TFs. Both genes act as transcriptional repressors by interacting with members of the *Groucho* family of transcriptional co-repressors (Beccari et al., 2009; Marco-Ferreres et al., 2009) but there is also evidence for their role as transcriptional activators (Jeong et al., 2008; Liu et al., 2006; Reichman et al., 2010). In most vertebrate species, both genes are strongly expressed starting at the gastrula stages in the anterior neural plate, where they are required for early forebrain specification. Overexpression of either gene induces ectopic retinal tissues and increases retinal neuroblast proliferation (Del Bene et al., 2004; Loosli et al., 1999; Lopez-Rios et al., 2003; Zuber et al., 1999). In vertebrates, genetic inactivation of *Six3* causes loss of brain structures anterior to the midbrain, including the eyes (Lagutin et al., 2003). *Six6*-null mice instead present strong pituitary defects and retinal hypoplasia, often associated with the absence of the optic chiasm and nerves (Li et al., 2002). Possibly owing to these early defects, the function of *Six3* and *Six6* during retinal neurogenesis has been barely explored. Both TFs are initially expressed in the entire retinal neuroepithelium, *Six6* with

Instituto Cajal, CSIC and CIBER de Enfermedades Raras (CIBERER), Avda Doctor Arce 37, 28002 Madrid, Spain.

*Present address: Telethon Institute of Genetics and Medicine (TIGEM) and Institute of Genetics and Biophysics 'A. Buzzati-Traverso', CNR, Via Pietro Castellino, 80131 Naples, Italy
†These authors contributed equally to this work
‡Author for correspondence (bovolenta@cajal.csic.es)

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a ventroanterior^{high} to dorsoposterior^{low} gradient. Thereafter, *Six3* become mostly localised to the amacrine, horizontal and RGC cells, whereas *Six6* withdraws from most differentiating precursors but is retained in RGC, amacrine and progenitor cells of the ciliary margin (Bovolenta et al., 1998; Conte and Bovolenta, 2007; Kawakami et al., 1996; Li et al., 2002; Manavathi et al., 2007). Although *Six3* alone has no effect, its co-expression with *NeuroD* or the related *Math3* increases the number of amacrine cells (Inoue et al., 2002). *Six3* might also be involved in photoreceptor differentiation as its HD interacts with specific DNA elements in the *Rhodopsin* promoter and stimulates its transcription, although this activity is normally repressed by Metastasis-associated protein 1 (Manavathi et al., 2007). Whether *Six6* has similar or different functions in retinal cell type specification is unknown.

Probably owing to teleost genome duplication and subfunctionalisation of the *Six3/6* paralogue genes (Conte and Bovolenta, 2007), the expression of *Six6* in medaka fish starts later in development than in other vertebrate species (Conte and Bovolenta, 2007; Lopez-Rios et al., 2003), making the medaka fish an ideal model to study the possible function of *Six6* during retinogenesis. Here, we took advantage of this model and investigated *Six6* function in the retina, looking, as a starting point, for the elements that regulate *Six6* expression. Combining phylogenetic footprinting with bioinformatic prediction of DNA binding sites, we identified and characterized a highly conserved *Six6* retinal enhancer that is functionally recognised by NeuroD. NeuroD-mediated activation of *Six6* in vivo promotes unorganized retinal progenitor proliferation and unexpectedly impairs photoreceptor differentiation, with no apparent changes in other retinal cell types. Notably, knockdown of Six6 decreases NeuroD expression levels and alters markers of photoreceptor and amacrine cell differentiation. Therefore, we propose that Six6 and NeuroD control each other's expression and function to coordinate the terminal differentiation of photoreceptors and the genesis of amacrine cells.

MATERIALS AND METHODS

Sequence analysis

The available vertebrate *Six6* and *NeuroD* genomic sequences were retrieved from public databases (http://genome.ucsc.edu/; http://genome.jgi-psf.org/) and aligned to identify putative regulatory modules on the basis of sequence conservation (Conte and Bovolenta, 2007). The TRANSFAC, Jaspar and rVISTA tools were used to predict putative binding sites for known TFs (Bryne et al., 2008; Matys et al., 2003).

DNA constructs

A 7 kb genomic fragment upstream of the coding sequence of the medaka fish (*Oryzias latipes*) *Six6*, including the first 9 coding nucleotides, was isolated from genomic DNA using specific primers (see Table S1 in the supplementary material) and cloned in frame with a nuclear *eGFP* reporter gene into the pSKII-I*Sce*I-eGFP vector to create the cI construct. Four deleted constructs, pSKII-I*Sce*I-*Six6*-7kbΔ*Eco*RV (cIII), pSKII-I*Sce*I-*Six6*-7kbΔ*Xho*I-*Nsi*I (cIV), pSKII-I*Sce*I-*Six6*-7kbΔ*Xho*I-*Sph*I (cV) and pSKII-I*Sce*I-*Six6*-7kbΔ*Xho*I-*Hin*dIII (cVI), were obtained by digestion with the indicated enzymes. The sequence containing only the retinal enhancer b (RE-b) was isolated by PCR amplification from the medaka and mouse genomes with specific primers (see Table S1 in the supplementary material) and cloned upstream of the tyrosine kinase promoter in the pSKII-I*Sce*I-Tk-eGFP vector (cVII-cVIII and cXIV-cXV constructs). One kilobase of the 3′UTR of *Six6* (named D) was amplified by PCR and cloned downstream of *eGFP* into an *Hpa*I site of the cI construct to generate cII. For Luciferase assays, 1.7 kb of the c*Six6* genomic sequence containing the B and C clusters and including 9 nucleotides of the coding region was cloned in frame with the *Luciferase* reporter into the pGL3 basic vector (cIX). The B cluster, isolated by restriction enzyme digestion (*Eco*RV or *Sma*I), and the RE-b, obtained by PCR amplification, were inserted in sense and antisense orientation into the polylinker of pSKII-I*Sce*I-Tk-eGFP (cX and cXI) and pGL3 promoter vector (cXII and cXIII). Mutations in the *NeuroD* binding site of the constructs were generated by PCR and cloned in a similar manner (cXVI; see Table S1 in the supplementary material). Constructs were verified by automated sequencing (Secugen, S.L, Madrid).

Establishment of transgenic lines

The Cab inbred medaka strain was used throughout the study. Stages were determined according to Iwamatsu (Iwamatsu, 2004). Transgenesis and monitoring of *eGFP* expression in the living lines was performed as described (Conte and Bovolenta, 2007). Three independent stable transgenic lines were generated for all tested constructs.

Isolation of *NeuroD* cDNA

The *NeuroD* cDNA was obtained by RT-PCR from total RNA of embryos collected at different developmental stages using specific primers.

mRNAs and morpholino injections

In vitro synthesis of the mouse or medaka *NeuroD* and *Six6* mRNAs was performed as described (Esteve et al., 2003). *NeuroD* mRNAs were injected at 10-100 ng/μl, which induced a dose-dependent phenotype. Selected working concentrations were 50-75 ng/μl for *NeuroD* and 75 ng/μl for *Six6*. Control embryos were injected with 15 ng/μl of *eGFP* mRNA. A morpholino (MO; Gene Tools LLC, Oregon, USA) was designed against the 5′UTR of *Six6*: 5′-GGCTTCTCCAGTGTTT-CCTTCACCC-3′. A control MO carrying five mismatches was used as a control. The specificity and inhibitory efficiency of MO-*Six6* was determined by co-injecting the MO with a synthetic 5′UTR-*Six6*+*eGFP* mRNA. eGFP intensity was quantified with ImageJ, as previously reported (Esteve et al., 2004; Ruiz et al., 2009). MO-*Six6* at 90 μM fully abrogated eGFP fluorescence. MO was injected into one blastomere at the two-cell stage. At least three independent experiments were performed for each marker and condition.

Wholemount in situ hybridization

Wholemount in situ hybridizations were performed, photographed and sectioned as described (Conte and Bovolenta, 2007). Antisense and sense riboprobes for the medaka *Six6*, *Otx2*, *Crx*, *Rhodopsin*, *Pax6*, *NeuroD*, *CycD1* (*Ccnd1*) and *Meis2.2* were used. The mRNA localization was revealed using the NBT/BCIP (purple precipitate), NBT (light blue precipitate) or Fast Red (red fluorescent precipitate) substrates.

Immunohistochemistry

Embryos were processed for immunocytochemistry as described (Esteve et al., 2004; Ruiz et al., 2009). The following primary antibodies were used: anti-phospho-Histone-H3 (1/1000; Roche Diagnostics), rabbit anti-Pax6 (1/1000, PRB-278P Covance), rabbit anti-Six3 (raised against the mouse Six3 RLQHQAIGPSGMRSLAEPG C-terminal sequence) and a rabbit polyclonal anti-Otx2 (1/300, Abcam, ab21990) raised against the 250-289 human amino acid sequence. This peptide shows 90% and 68% identity with Otx2 and Crx, respectively, suggesting that the antiserum might recognize both proteins. The secondary antibodies were from Molecular Probes (used at 1/1000 dilution).

Transient transfection and Luciferase assays

Dissociated cell cultures were prepared from chick retinas as described (Lopez-Rios et al., 2008). In each experiment, cultured cells were cotransfected with the cIX, cXII, cXIII and cXVI constructs (100 ng), the expression vectors (100 ng; pcDNA3/Ath3, pcDNA3/Ath5, pcDNA3/NeuroD, pcDNA3/E47, or pcDNA3 alone) and the RL-TK plasmid with *Renilla Luciferase* (10 ng) as a transfection efficiency control. Cells were harvested 48 hours after transfection. Reporter activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Each assay was performed in duplicate. Results are shown as mean ± s.d. for at least three independent assays.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were performed as described (Martinez-Morales et al., 2003). Recombinant mouse *NeuroD-myc* was synthesized in vitro using a TNT kit (Promega). In competition assays, 1-500 fold excess of unlabelled mutated double-stranded oligonucleotide was used. In supershift assays, reactions included 2.5ng/μl of rabbit anti-myc polyclonal antibody or 2.5ng/μl of rabbit anti-HA polyclonal antibody (Sigma-Aldrich). The oligonucleotide primers used for the EMSA are shown in Table S1 in the supplementary material.

Chromatin immunoprecipitation

ChIP assays were performed with a commercial kit (Millipore) following the manufacturer's instructions. P19 cell line was transfected with the chick *Six6* promoter alone or with a myc-tagged mouse *NeuroD*. Chromatin was immunoprecipitated with 2 μg of either rabbit α-myc antibody (Sigma) or a goat IgG (Sigma). DNA was analysed by PCR (Roche) to amplify regions containing the putative NeuroD binding sites on chick or mouse *Six6* promoters. Fold enrichment is expressed as the ratio of myc to control IgG signal.

RESULTS

Six6 hypothalamic and retinal expression is controlled by different HCNE

Phylogenetic footprinting based on the alignment of orthologous genomic sequences from related teleost species has been successfully used to identify the regulatory code of the *Six3.2* gene (Conte and Bovolenta, 2007). We applied a similar strategy to identify cis-regulatory elements controlling *Six6* expression, focusing particularly on the retina. The sequence of the medaka *Six6* loci was retrieved from public databases using the *Six6* coding sequence (AM353044) as a query. Alignment of about 20 kb flanking the *Six6* gene with the corresponding regions from fugu, tetraodon and stickleback (Fig. 1A,B) identified four clusters (named A-D; Fig. 1B,C) of highly conserved non-coding elements (HCNE). Three of them (A-C) lie in the first 7 kb upstream of the *Six6* coding region, whereas an additional cluster (D; Fig. 1B,C) was identified in the 3 kb downstream region (Fig. 1A). To address their regulatory potential, the 7 kb genomic fragment was amplified from medaka genomic DNA and fused with a nuclear *eGFP* reporter to generate construct I (cI) (Fig. 1C). cI was then assayed for possible enhancer activity in medaka embryos, as previously reported (Conte and Bovolenta, 2007). Three stable cI transgenic lines showed comparable and robust eGFP expression only in the retina. The expression began at stage 24 and was thereafter observed in this region with a pattern that matched endogenous *Six6* expression (Fig. 1D-F; see Fig. S2A in the supplementary material). eGFP fluorescence was observed with progressively increasing intensity, first in the retinal neuroepithelium (Fig. 3A-B) and then in the RGC and amacrine cells (Fig. 1C; Fig. 3C,F,H). Because cI did not recapitulate *Six6* hypothalamic expression (Fig. 1D), we asked whether the cluster of HCNE contained in the 3 kb fragment downstream of *Six6* was needed to drive expression in this region. Fusion of this amplified fragment downstream of *eGFP* in construct cI (cII; Fig. 1C) was sufficient to observe reporter expression in the hypothalamus, although a weak signal was

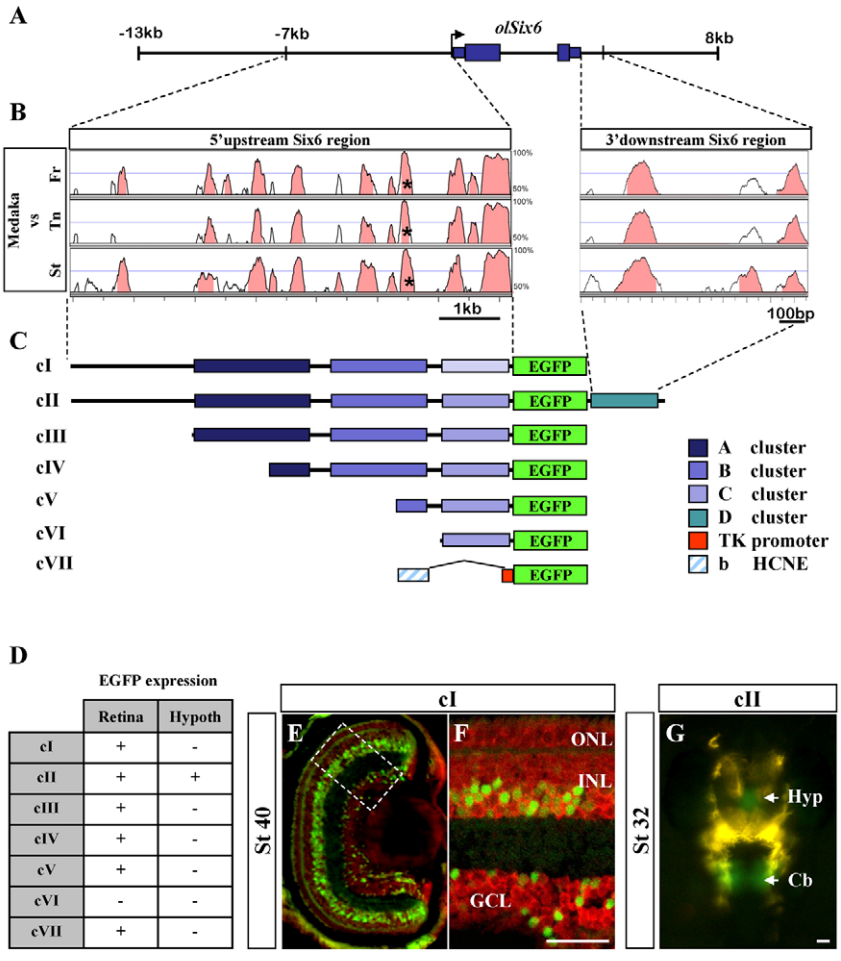

Fig. 1. Characterization of the medaka *Six6* regulatory region. (**A**) Schematic drawing of the genomic region containing the medaka *Six6* gene. The blue boxes represent the *Six6* exons. (**B**) Vista comparison of teleost *Six6* genomic loci. Blocks of conserved sequences (75% identity over 100 bp) are indicated in pink. Asterisks indicate the conserved block corresponding to the retinal enhancer b. (**C**) Schematic representations of the constructs (cI to cVII) used for transgenesis analysis. (**D**) Summary of the eGFP expression pattern observed in transgenic embryos. (**E**,**F**) eGFP expression in the retina of cI transgenic embryos recapitulates endogenous *Six6* retinal expression. F is a magnified view of the boxed region in E. (**G**) Dorsal view of a cII transgenic embryo shows eGFP expression in the hypothalamus (Hyp) and ectopically in the cerebellum (Cb). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar: 50 μm.

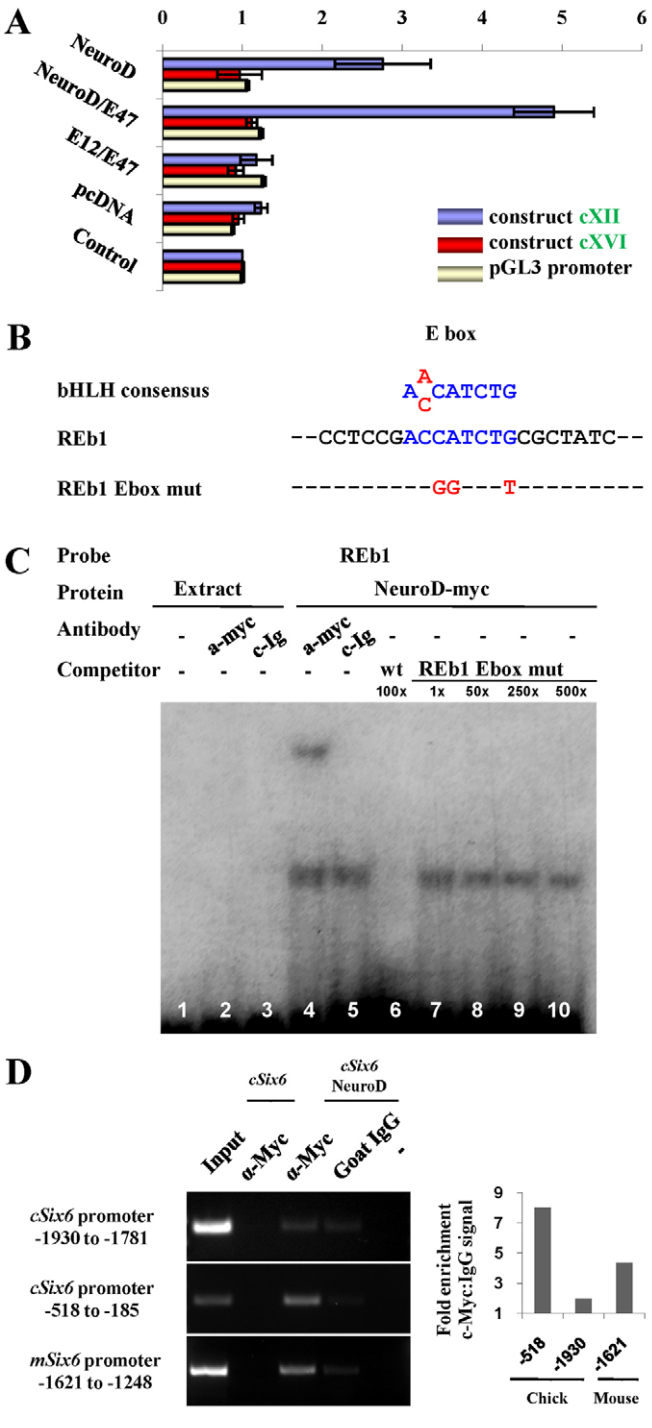

Fig. 2. NeuroD binds and transactivates the conserved E-Box in the RE-b. (**A**) NeuroD activates *Luciferase* expression under the control of the RE-b (construct cXII) in transient transfection assays using chick retinal cells. Maximal activation is observed when the cofactor E47 is cotransfected with NeuroD. Mutations in the conserved E-box (construct cXVI) abrogate NeuroD-mediated reporter activation. (**B**) E-Box consensus sequence and oligonucleotides used for in vitro assays. REb1, oligonucleotide covering part of the medaka RE-b sequence and containing the highly conserved E-box (highlighted in blue); REb1 E-Box mut, oligonucleotide containing a mutated E-box (changes are indicated in red). (**C**) EMSA assay performed with a ^{32}P-labelled bRE1 probe and NeuroD-myc translated in vitro. NeuroD-myc forms a complex with the labelled probe that is specifically supershifted by anti-myc but not by control antibodies (lanes 4 and 5, respectively). This binding is competed by an excess of the same unlabelled oligonucleotide (lane 6) but not by increasing concentrations of the E-box mutant oligonucleotide (lanes 7-10). (**D**) NeuroD specifically immunoprecipitates the mouse and chick *Six6* enhancer region containing the NeuroD binding site in ChIP assays. No ChIP was detected in cells transfected with a control vector (pCMV) or when the chromatin was immunoprecipitated with unspecific goat IgGs.

detected also in the cerebellum (Fig. 1G), suggesting that additional repressor elements might refine *Six6* spatial expression, as reported for *Six3.2* (Conte and Bovolenta, 2007).

To identify which, if any, of the HCNE contained in the cI construct was responsible for *Six6* expression in the retina, we generated a series of constructs (cIII-cVI) where HCNE were progressively deleted (Fig. 1C). Embryos carrying constructs cIII-cV showed the same pattern of reporter expression observed with cI, whereas no eGFP fluorescence was observed in those carrying cVI (Fig. 1D), restricting the region responsible for expression to a stretch of about 180 base-pairs (bp) corresponding to the first HCNE of cluster B (Fig. 1B-D). This region appeared necessary and sufficient to drive expression in the retina as stably transfected embryos carrying the cVII construct, where HCNE-b was combined with the minimal tyrosine kinase promoter, efficiently expressed eGFP with the expected retinal pattern. The HCNE-b was therefore named retinal enhancer b (RE-b).

If RE-b had an important evolutionarily conserved role in regulating *Six6* expression in the retina, it should be present in the *Six6* loci of vertebrates other than teleosts. mVista- and Multialign-based alignment of the characterised medaka *Six6* regulatory region with the human, mouse, chicken and *Xenopus tropicalis* orthologues demonstrated extensive sequence conservation of RE-b among all vertebrate phyla (see Fig. S1 in the supplementary material). Supporting the relevance of this conservation, substitution of the medaka RE-b in cVII with those derived from mouse or chick resulted in three independent stable transgenic lines with an expression pattern identical to that observed with the medaka elements (compare Fig. S1B,C in the supplementary material with Fig. 1F).

NeuroD specifically binds and activates the *Six6* RE-b

To determine potential trans-acting factors, we next analysed RE-b from different vertebrates with TRANSFAC and Jaspar softwares (Bryne et al., 2008; Cartharius et al., 2005). This analysis identified a highly conserved consensus E-box binding site within the RE-b (Fig. 2A,B). E-boxes bind TFs of the bHLH family, including the proneural genes *Ath3*, *Ath5* and *NeuroD*, which have been implicated in the specification of RGC, amacrine and photoreceptor precursors (Hatakeyama and Kageyama, 2004), where *Six6* is also expressed (Conte and Bovolenta, 2007) (see Fig. S2A-C,E in the supplementary material). We thus asked whether these factors could activate the *Six6* retinal enhancer in *Luciferase* reporter assays in cultures of dissociated cells from embryonic day 5 (E5) chicken retinas. Cells were transiently transfected with the reporter construct containing the chick RE-b coupled to the *Luciferase* gene (cXII) together with different combinations of mouse Ath3, Ath5, NeuroD and E47, a ubiquitously expressed bHLH protein that forms heterodimers with other family members, enhancing their

activity (Longo et al., 2008; Naya et al., 1995). Notably, in all the tested combinations, only the presence of *NeuroD* led to a significant activation of reporter expression (see Fig. S1 in the supplementary material). This activation was particularly evident when *NeuroD* was cotransfected with *E47* (Fig. 2A; see Fig. S1 in the supplementary material), in line with the notion that these two factors form particularly stable heterodimers (Longo et al., 2008). This activation was no longer observed when transfections were repeated with a reporter construct carrying three point-mutations in the E-box of the RE-b (cXVI; Fig. 2A,B).

The specificity of NeuroD binding was confirmed by EMSA and ChIP assays. NeuroD-myc protein formed a complex with a P^{32}-labelled oligonucleotide derived from the RE-b and containing the E-box binding site (Fig. 2B,C). The migration of the complex was specifically retarded by anti-myc antibodies and binding to the probe was competed by an excess amount of the same unlabelled oligonucleotide, but not by increasing concentrations of its mutated version (Fig. 2C). In line with this finding, anti-myc antibodies, but not a control IgG, immunoprecipitated chick RE-b in P19 cells transfected with NeuroD-myc and the chick *Six6* promoter (Fig. 2D). NeuroD-myc immunoprecipitated the mouse RE-b from P19 chromatin (Fig. 2D) with the same specificity, further proving that *Six6* RE-b is a direct target of NeuroD.

NeuroD overexpression expands the domain of *Six6* expression causing an increase in cell proliferation

Together, these studies identified *NeuroD* as a strong candidate to regulate *Six6* expression in the retina. Consistent with this possibility, the distribution of *NeuroD* and *Six6* mRNAs partially overlapped in the embryonic wild-type retinas (see Fig. S2 in the supplementary material). As shown in different vertebrate species (Conte and Bovolenta, 2007; Lopez-Rios et al., 1999; Toy and Sundin, 1999), medaka *Six6* mRNA is localised to the entire retinal neuroepithelium at the beginning of neurogenesis, albeit its levels are slightly lower in the region where *NeuroD*-positive cells begin to accumulate (see Fig. S2A-D in the supplementary material). These results were also confirmed by the presence of nuclear *eGFP* reporter expression in cells of cI transgenic retinas (*Six6* cI>eGFP) where *NeuroD* mRNA was localised in the cytoplasm (Fig. 3A,B). The photoreceptor progenitor nature of these *NeuroD*- and *Six6*-positive cells was further confirmed by the expression of *Crx*/*Otx2* (see Materials and methods), established photoreceptor markers (Nishida et al., 2003) (Fig. 3D,E). However, *Six6* expression was progressively downregulated in photoreceptors (Fig. 3B; see Fig. S2C,E in the supplementary material), whereas a strong *Six6* and

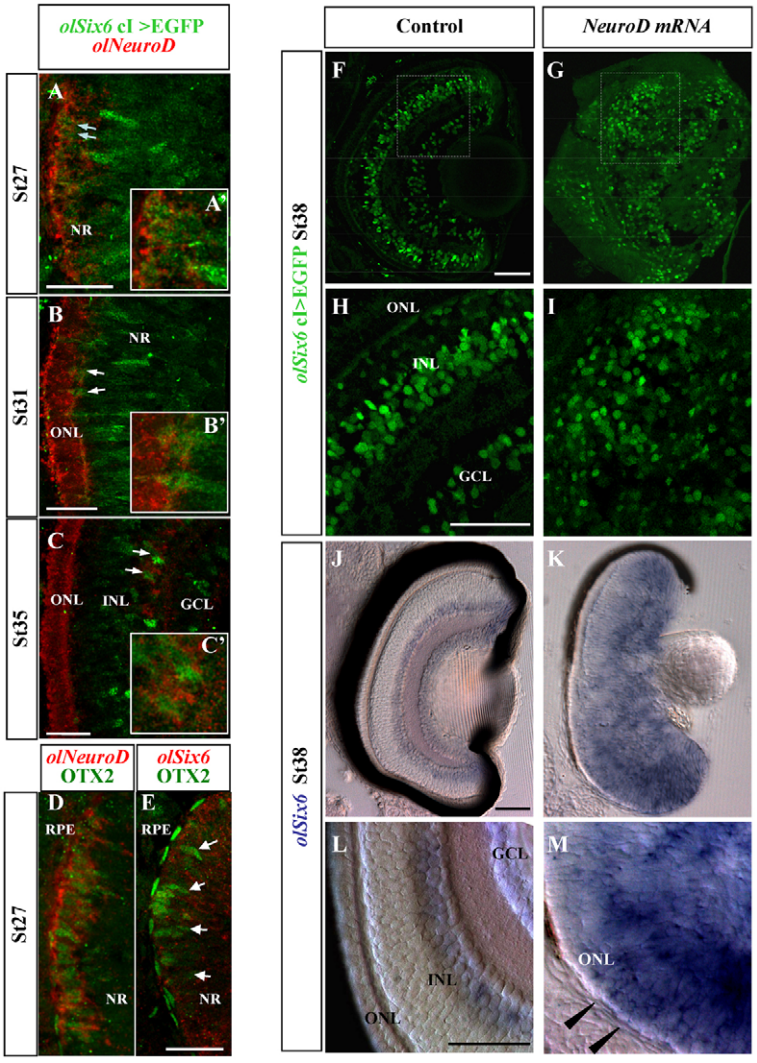

Fig. 3. *NeuroD* overexpression expands the domain of *Six6* expression. (**A-C'**) Retinal sections from embryos of the *Six6 cI>eGFP* transgenic line (stages 27, 31, 35) hybridized with a *NeuroD* antisense probe (red) and immunostained for eGFP. White arrows indicate the cells shown in the higher magnification insets (A'-C'). (A) At stage 27, the cytoplasmic *NeuroD* mRNA staining colocalises with nuclear eGFP expressed under the control of *Six6* in developing photoreceptor precursors. (B) eGFP expression decreases in the photoreceptor layer as *NeuroD* expression increases. (C) Both genes are co-expressed in a subclass of amacrine cells. (**D**,**E**) Cryostat sections from stage 27 wild-type retinas were hybridized in toto with *NeuroD* (D) or *Six6* (D) antisense probes (red) and immunostained with anti-Otx2 (green). White arrows in the neural retina (NR) in E indicate colocalisation (double-labelled cells). Otx2 colocalises with both *NeuroD* and *Six6* in emerging photoreceptor precursors. (**F-I**) Frontal sections from stage 38 control or *NeuroD*-injected *Six6 cI>eGFP* embryos. *NeuroD* mRNA activates eGFP expression in most of the retinal neuroepithelium (G,I). H and I are higher magnification views of the boxed regions in F and G, respectively. (**J-M**) Frontal sections from stage 38 control (J,L) and *NeuroD*-injected (K,M) embryos hybridized for *Six6*. Black arrowheads in M indicate absence of ventral pigmented epithelium. Am, amacrine cells; GCL, ganglion cell layer; INL, inner nuclear layer; NR, neural retina; ONL, outer nuclear layer; RPE, retinal pigmented epithelium. Scale bars: 25 μm in A-E; 50 μm in F-M.

NeuroD colocalisation was maintained in an amacrine cell subpopulation (Fig. 3C; see Fig. S2E,F in the supplementary material).

To understand if *NeuroD*-directed regulation of *Six6* occurs in vivo, we tested whether *NeuroD* overexpression could expand the *Six6* expression domain in both wild-type and transgenic embryos. In contrast to the restricted expression observed in control *eGFP*-injected embryos (Fig. 3F,H), injections of *NeuroD* mRNA (50-75 ng/μl) in *Six6* cI>eGFP transgenic embryos activated eGFP expression in most of the retinal neuroepithelium (stage 38; Fig. 3G,I). Likewise, *NeuroD* overexpression in wild-type embryos expanded *Six6* mRNA distribution, which, in the most affected embryos, was also observed in layers where it is normally absent (Fig. 3K,M). No ectopic *Six6* expression was observed in regions other than the retina, supporting a tissue-specific *NeuroD*-mediated activation of *Six6*.

Notably, *NeuroD* mRNA overexpression caused abnormal eye development in most embryos (75±5%; *n*=1750). In the majority of the affected embryos (54±3%), the eyes were slightly larger and shifted ventroanteriorly with absent or reduced ventral retinal pigmented epithelium (Fig. 3G,K,M) and optic stalks, which acquired neural retina characteristics. As an extreme phenotype, 20±3% (*n*=1750) of the affected embryos developed a giant cyclopic eye positioned in the anterior-most of the embryo (see Fig. S3B,D,F in the supplementary material). In these cyclopic eyes, the retina often appeared duplicated with fused inner nuclear layer (INL) and ganglion cell layer (GCL) easily recognised by immunocytochemical markers, whereas the photoreceptor layer was apparent only in the periphery of the retina (see Figs S3 and S4 in the supplementary material). Cyclopia characterised all the embryos (*n*=250) injected with high *NeuroD* concentrations (~100 ng/μl). No central nervous system (CNS) regions other than the eyes were affected in *NeuroD*-injected embryos, at least on the basis of morphological inspection.

Gain of *Six6* (also known as *Optx2*) function in *Xenopus* leads to an increase in cell proliferation (Zuber et al., 1999), explained by the finding that Six6 directly represses the expression of cyclin inhibitors like *p27Kip1* (Li et al., 2002). If the *NeuroD* phenotype is mostly mediated by direct *Six6* activation, we should expect a similar increase in cell proliferation and this increase should be abrogated by the injection of a *Six6*-specific morpholino (*olSix6*-MO). In line with this idea, the number of retinal cells immunostained with anti-PH3 antibodies, a marker for mitotic cells, was significantly increased in *NeuroD*-treated embryos (Fig. 4A,B,J). Notably, PH3-positive cells were not confined to the ventricular surface of the neuroepithelium or the ciliary marginal zone (CMZ), as observed in controls (Fig. 4A), but were dispersed within the thickness of the retina (Fig. 4B). Similarly, although *CycD1* mRNA, an additional proliferation marker, localised only to the CMZ in stage 38 control retinas, expression was still observed in the ventral retina of *NeuroD*-injected embryos at the same stage (Fig. 4D-G). The co-injection of *Six6*-MO (90 μM) together with *NeuroD* mRNA was sufficient to bring both proliferation and *CycD1* expression back to the level observed in controls (Fig. 4C,H,I,J). Notably, *Six6*-MO did not counterbalance the tendency to cyclopia induced by a high concentration of *NeuroD* (data not shown) nor the optic stalk defects observed even with lower *NeuroD* doses (Fig. 4C,H, arrows).

Together, these data indicate that *NeuroD*-mediated activation of *Six6* controls retinal cell proliferation, whereas ectopic activation of other *NeuroD* target genes are responsible for optic stalk defects.

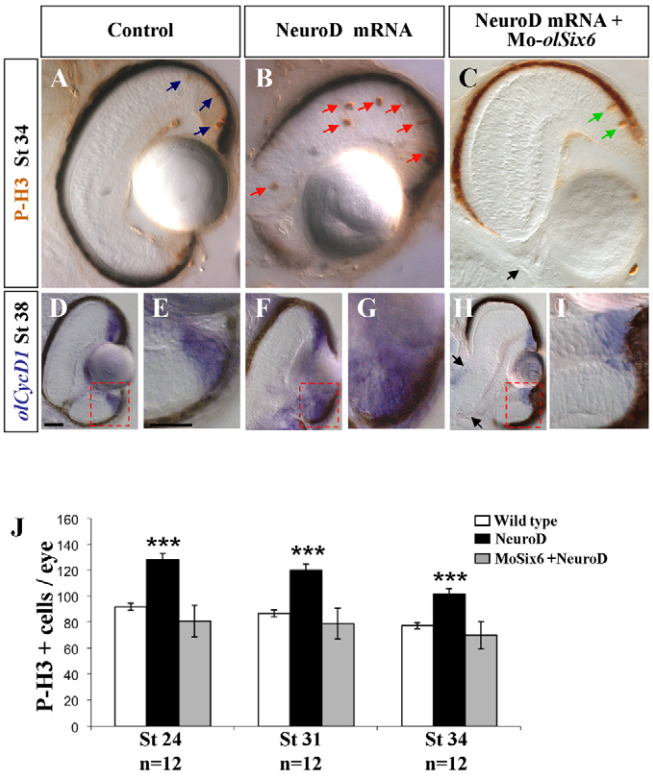

Fig. 4. *NeuroD*-mediated *Six6* expansion increases retinal cell proliferation. (**A-C**) Frontal sections from stage 34 control (A), *NeuroD* mRNA (B) or *NeuroD* mRNA plus MO-*Six6* (C)-injected embryos immunostained with anti-PH3. In contrast to controls, PH3-positive cells are increased and dispersed throughout the retina of *NeuroD*-injected embryos. This phenotype is rescued by MO-*Six6* co-injection. Arrows in A-C indicate PH3-positive cells. (**D-I**) Frontal sections from stage 38 control (D,E), *NeuroD* (F,G) or *NeuroD* plus MO-*Six6* (H,I)-injected embryos hybridized for *CyclinD1*. *CycD1* expression is expanded in the ventral retina of *NeuroD*-injected embryos. Arrows in H indicate optic stalk defects. E, G and I are higher magnification views of the boxed areas in D, F and H, respectively. (**J**) Quantification of PH3-positive cells per eye in control, *NeuroD* and *NeuroD* plus MO-*Six6* injected embryos. PH3-positive cells were counted from eye sections of six embryos for each stage. Results are shown as mean ± s.d. of PH3-positive cells counted for each eye and validated by χ^2-tests for statistical significance. ***, $P<0.0001$.

NeuroD-mediated *Six6* activation interferes with photoreceptor differentiation

NeuroD participates in the specification and differentiation of vertebrate amacrine and photoreceptor cells, although with differences among species (Kanekar et al., 1997; Moore et al., 2002; Morrow et al., 1999). For example, in *Xenopus*, *NeuroD* promotes the generation of amacrine cells (Kanekar et al., 1997; Moore et al., 2002), whereas in zebrafish, it initiates photoreceptor progenitor withdrawal from the cell cycle and, when ectopically expressed, favours photoreceptor generation at the expenses of Müller glial cells (Ochocinska and Hitchcock, 2007; Ochocinska and Hitchcock, 2009). In medaka fish, *NeuroD* and *Six6* are co-expressed in differentiating amacrine cells as well as in photoreceptor progenitors, although in the latter, co-expression is only transient (Fig. 3A-C; see Fig. S2 in the supplementary material). We therefore asked whether *NeuroD* overexpression in the medaka retina had similar consequences as those described in

zebrafish or *Xenopus* and which of the putative *NeuroD* activities could be specifically counterbalanced by *Six6*-MO, thus confirming a possible physiological role of *Six6* as a downstream target of *NeuroD*.

NeuroD-mediated overexpression of *Six6* led to a disorganised growth of the eye where, in the most severe cases, specific cell layers were indistinguishable (Fig. 3G,K; see Figs S3 and S4 in the supplementary material). To overcome this problem, we selected for analysis only those *NeuroD*-injected embryos where retinal layers could still be recognised (54±3%). Analysis of the expression of *Pax6*, *Meis2.2*, *Otx2* and *Six3*, markers for RGC, amacrine, bipolar and horizontal cells, respectively, did not show any statistically significant defects in *NeuroD* overexpressing embryos when compared with age-matched controls (Fig. 5A-B′,D-E′,G-H′; see Fig. S4A-D,G in the supplementary material). By contrast, *Rhodopsin* expression, a marker for differentiated photoreceptors, was markedly reduced or totally absent in *NeuroD*-overexpressing retinas (40%; *n*=30; Fig. 5J-K′). This reduction did not reflect a complete loss of the photoreceptor lineage, as Crx-positive postmitotic photoreceptor precursors (Garelli et al., 2006) were still observed (see Fig. S4E,F in the supplementary material). *Rhodopsin* expression was completely rescued when *Six6*-MO was co-injected with *NeuroD* (Fig. 5L,L′), indicating that these alterations involve *Six6* activity. As observed before, *Six*6-MO did not counterbalance the tendency to cyclopia induced by *NeuroD* nor the apparent extension of the retina into the optic stalk, frequently observed in both *NeuroD* and *Six6*-MO plus *NeuroD*-injected embryos (Fig. 5C,F,I,L).

Six6 and *NeuroD* act in a regulatory loop to control photoreceptor differentiation and amacrine cell specification

The data reported above suggest that high *Six6* levels interfere with *Rhodopsin* expression and thus with photoreceptor maturation. If this was the case, interference with *Six6* expression alone should force photoreceptor differentiation ahead of time. Surprisingly, analysis of *Six6*-MO-injected embryos at stages 30-34, when photoreceptor differentiation is still ongoing, revealed the opposite effect. Approximately 67% of the morphants (*n*=40) expressed a very low level of or no *Rhodopsin* at stage 34, whereas a milder reduction was observed only in 35% of the misMO-injected control embryos (Fig. 6A,B). At stage 38, 31% of the morphant embryos showed a *Rhodopsin* decrease, suggesting that reduced *Six6* levels interfere with the onset of photoreceptor terminal differentiation but not their specification because, as observed in *NeuroD*-injected retina, *Crx* expression appeared unaltered (Fig. 6C,D).

To explain this apparently contradictory result, we hypothesized that *Six6* and *NeuroD* might act in a regulatory loop, where *Six6* would initiate and/or maintain *NeuroD* expression and both genes would be required, directly or indirectly, to regulate *Rhodopsin* levels. In line with this hypothesis, the appearance of the first NeuroD-positive cells around stage 27 was delayed (see Fig. S5A in the supplementary material) and the overall *NeuroD* expression levels were decreased in the retina of *Six6*-MO-injected embryos (Fig. 6E,F; 40%, *n*=60) as compared with misMO-injected controls. At later developmental stages, the decrease was also evident in both the amacrine and photoreceptor layers, but whereas Crx-positive photoreceptor precursors were normally generated, Pax6 and Six3-positive amacrine precursors were significantly diminished (see Fig. S4H-L in the supplementary material). Conversely, *Six6* overexpression increased *NeuroD* mRNA levels in the retina (60%; *n*=30; Fig. 6G,H; see Fig. S5 in the supplementary material), which was ectopically observed in the optic stalk (Fig. 6H) and throughout the thickness of the retinal neuroepithelium in embryos with the strongest phenotype (see Fig. S5B in the supplementary material). In a lower proportion of embryos (25%), *Six6* mRNA induced ectopic patches of pigmented cells in the brain with an associated ectopic expression of *NeuroD* (Fig. 6H,J,L), suggesting that *Six6* can initiate *NeuroD* expression in tissues other than the retina. In agreement with what was observed after *NeuroD*-mediated Six6 overactivation, *Six6* mRNA

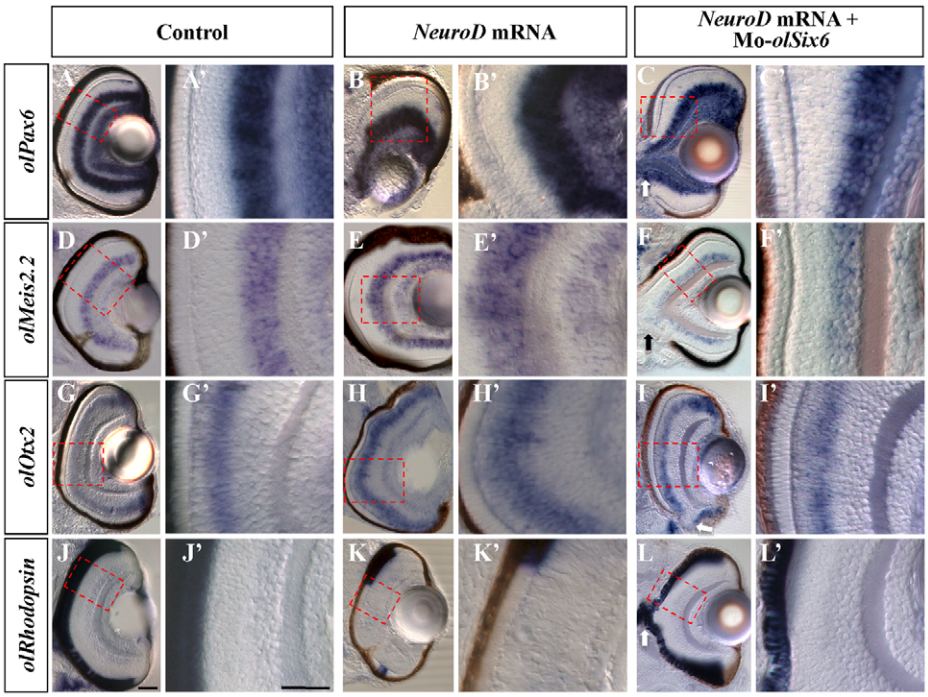

Fig. 5. NeuroD-induced activation of *Six6* specifically reduces photoreceptor differentiation. (**A-L′**) Frontal sections from stage 38 control, *NeuroD* and *NeuroD* plus MO-*Six6*-injected embryos. Embryos were hybridized with the following probes: *Pax6* and *Meis2.2* (amacrine and ganglion cells; A-F′); *Otx2* (bipolar cells; G-I′); *Rhodopsin* (differentiated photoreceptors; J-L′). *NeuroD*-injected embryos show a strong reduction of *Rhodopsin* expression, which is counteracted by MO-*Six6* co-injection. No major alterations were observed in the distribution of other cell type markers. Arrows in C,F,I,L indicate optic stalk defects. A′-L′ are magnified views of the boxed areas in A-L. Scale bars: 50 μm.

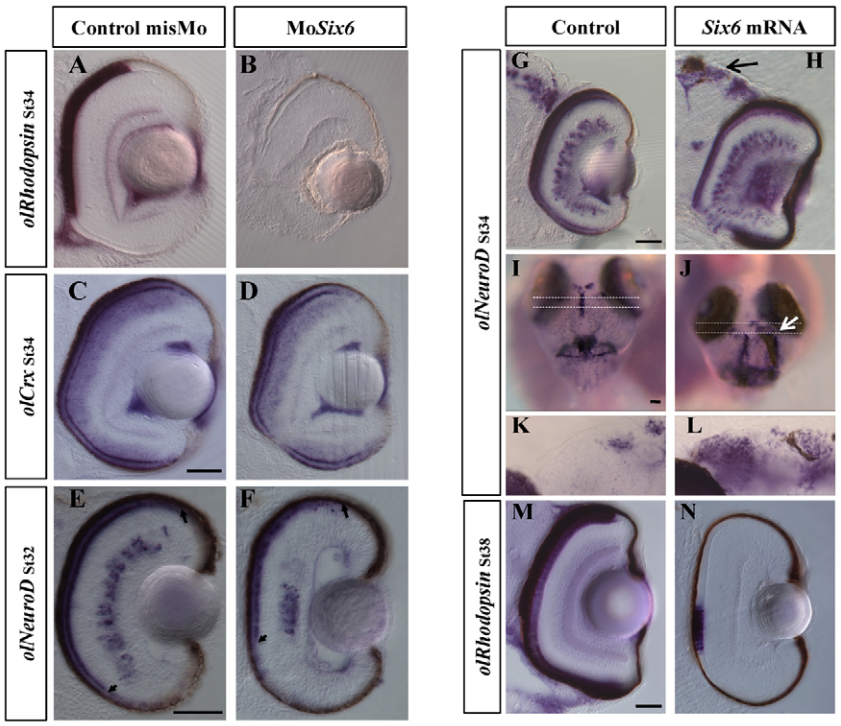

Fig. 6. Alterations of *Six6* expression modifies *NeuroD* expression, amacrine cell specification and photoreceptor differentiation. (**A-F**) Frontal sections from stages 34 (A-D) and 32 (E,F) misMO control or MO-*Six6*-injected embryos hybridized with *Rhodopsin*-, *Crx*- and *NeuroD*-specific probes. *Rhodopsin* (A,B) and *NeuroD* (E,F) mRNAs are downregulated in *Six6* morphants, whereas *Crx*-positive photoreceptor progenitors appear unaffected (C,D). (**G-N**) Control *eGFP* and *Six6* mRNA-injected embryos at stages 34 (G-L) and 38 (M,N) were hybridized with *NeuroD*- and *Rhodopsin*-specific probes. *Six6* gain-of-function increases retinal expression of *NeuroD* (H,J,L), which is also expressed in ectopic patches in the brain (L and arrows in H,J). Dashed lines in I,J indicate the level of the transverse sections shown in G,H,K,L. *Rhodopsin* expression is largely absent in *Six6*-overexpressing embryos (M,N). Scale bars: 50 μm.

injection did not affect the generation of amacrine cells while strongly reducing *Rhodopsin* but not *Crx* expression (Fig. 6M,N; data not shown). Together, these results suggest that *Six6*, together with *NeuroD*, is necessary but, by itself, is not sufficient to specify amacrine cells. Furthermore, the mutual regulation of *Six6* and *NeuroD* contributes to photoreceptor differentiation.

DISCUSSION

Generation of neuronal diversity is largely initiated by the cooperation between TFs of proneural bHLH and HD types. Proneural genes act as general determinants driving proliferating precursors towards a neuronal phenotype in broad domains of the CNS (Guillemot, 2007; Powell and Jarman, 2008) but their activity is made context specific by the interaction with locally expressed HD-TFs. According to this general principle, we have shown that *NeuroD*, a widely expressed proneural gene, regulates *Six6* expression exclusively in the retina. *Six6*, in turn, is sufficient to activate *NeuroD* expression, even ectopically. Physiologically, this regulatory loop appears to control retinal photoreceptor maturation and amacrine cell specification. These conclusions are based on genomic and functional experiments in the medaka fish where the late onset of *Six6* expression provided a unique opportunity to study time-specific functions of *Six6* in the retina.

Six6 is strongly expressed in the retina and hypothalamus of all vertebrate species so far analysed (Aijaz et al., 2005; Conte and Bovolenta, 2007; Conte et al., 2005; Gallardo et al., 1999; Jean et al., 1999; Lopez-Rios et al., 1999). We previously used a two-step genome comparison strategy, followed by a highly reproducible transgenic analysis to dissect the regulatory code of *Six3.2* (Conte and Bovolenta, 2007). Using the same approach, we have demonstrated that only two clusters of HCNE (B and D) are sufficient to reproduce the entire expression domain of *Six6* in the medaka fish. The B element is responsible for the retinal expression, whereas the D cluster, alone or in combination with the 5′ regulatory region, is responsible of the hypothalamic expression. The pattern of the eGFP reporter was highly reproducible in all injected embryos (roughly 97%, albeit with intensity differences, thus excluding chromosome position effects) and verified by the establishment of three independent stable transgenic lines for each of the tested constructs, supporting that clusters B and D contain functionally important cis-regulatory sequences. Despite their conservation, clusters A and C did not activate *eGFP* reporter expression, although subtle regulatory activities below the resolution of our analysis cannot be excluded. Alternatively, their conservation might reflect other important roles in the control of gene transcription, including the regulation of chromatin structure (Glazko et al., 2003; Gomez-Skarmeta et al., 2006) or, in the particular case of module C, minimal promoter functions. Proper *Six6* expression might additionally require a yet unidentified repressor, similar to that described for *Six3.2* (Conte and Bovolenta, 2007), which could normally silence the ectopic eGFP signal observed in the cerebellum upon injection of the downstream hypothalamic enhancer.

The strong sequence conservation indicates an evolutionary conserved role for both the B and D regions. However, we focused our attention on and demonstrated the functional relevance only of the RE-b, a 200 bp region contained in the upstream B cluster. This enhancer, independently of its species origin, was sufficient to recapitulate endogenous *Six6* expression in the retina of medaka fish embryos and in chick retinal cultures, strongly supporting its functional relevance from teleost to mammals. Although binding sites for other TFs are likely to be present in this region, we identified only a strongly conserved consensus E-box. A recent study showed that Lhx2 and Pax6, two TFs involved in the specification of the eye anlage (Porter et al., 1997; Zuber et al., 2003), act synergistically to activate the early expression of *Six6* in the mouse optic vesicles (Tetreault et al., 2009). Consistent with the late expression of *Six6* in medaka (Conte and Bovolenta, 2007; Lopez-Rios et al., 2003), binding sites for these two TFs were not found in the *Six6* promoter.

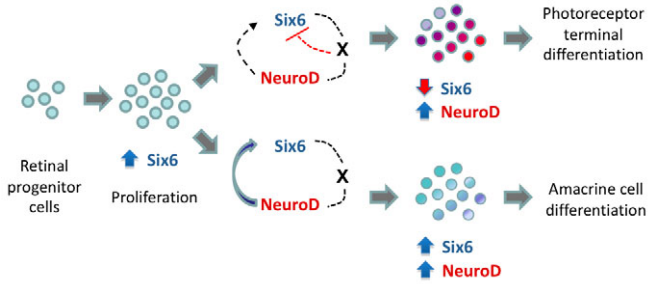

Fig. 7. Model of Six6 and NeuroD cross-regulation in retina neurogenesis. Different combinatorial levels of Six6 (blue) and NeuroD (red) might regulate retinal progenitor proliferation, amacrine cell specification and photoreceptor differentiation (see text for further details). Solid lines indicate direct regulations, whereas broken lines indicate probable indirect regulations that might also involve the activity of uncharacterized factors (X). The red dashed line indicates the activity of a putative repressor that might negatively regulate Six6 in differentiating photoreceptors.

Different subfamilies of bHLH proteins can form heterodimers and recognize the same canonical E-box sequence (Hernandez et al., 2007; Powell and Jarman, 2008). We cannot exclude that a number of bHLH might compete to occupy the conserved *Six6* E-box and control different phases of *Six6* expression, as reported for *Ath5* regulation in RGCs (Hernandez et al., 2007). Nevertheless, our results support a highly specific role of the NeuroD-E47 heterodimer in the binding and transactivation of the E-box in the *Six6* enhancer. Indeed, E47 alone or the related Ath5 and Ath3 had no effects, although their expression also partially overlaps with that of *Six6* in the retina (Brown et al., 1998; Brown et al., 2001; Inoue et al., 2002; Kanekar et al., 1997). This specificity is further supported by our EMSA, ChIP and in vivo studies. *NeuroD* mRNA injections lead to an increased and ectopic expression of *Six6* in the entire retina but in no other CNS regions, strongly arguing for a temporal and tissue-specific NeuroD-mediated regulation of *Six6*. This observation is also consistent with the possible existence of repressors that antagonise NeuroD-mediated expansion of *Six6* outside the retina or, alternatively, with the existence of a retinal-specific positive cofactor. On the contrary, *Six6* overexpression results in the activation of *NeuroD* expression, even ectopically. Notably, forced expression of Six6 alone or in combination with Groucho co-repressors generates ectopic retina-like tissue in the forebrain (Lopez-Rios et al., 2003), similar to that observed here. Together, these results suggest that *Six6* might indirectly regulate *NeuroD*, for example, by normally repressing a *NeuroD* negative regulator. Alternatively, *Six6* might work as transcriptional activator as recently suggested by the observation that the protein can activate the promoter of the gene encoding the RdCVF (rod-derived cone viability factor), a trophic factor expressed in rods (Reichman et al., 2010). These possibilities are not mutually exclusive and might be context-dependent, involving differential transcriptional networks in amacrine and photoreceptor cells, which might be refined by the availability of other co-factors. For example, E47, although ubiquitously expressed, might be particularly abundant in amacrine cells, whereas other bHLH cofactors, such as Hes6 (Bae et al., 2000), might be abundant in photoreceptors.

Six3 and *Six6* loci might have arisen from the duplication of a common ancestor (Gallardo et al., 1999), which implies the possible existence of similar regulatory elements. Co-expression of *Six3* and *NeuroD* increases amacrine cell numbers (Inoue et al., 2002), and protein-protein interaction between Six3 and NeuroD has been reported (Tessmar et al., 2002). Despite some parallels with *Six6*, we could not find sequence conservation by comparing the regulatory elements that drive late retinal expression of *Six3.2* (Conte and Bovolenta, 2007) with that identified for *Six6*. Accordingly, no difference in *Six3* expression was observed in the medaka fish retinas upon *NeuroD* overexpression, further confirming the specificity of NeuroD-Six6 regulation.

NeuroD promotes neuronal cell fate acquisition and links cell cycle withdrawal with terminal differentiation in different neural cell populations (Lee et al., 1995; Farah et al., 2000; Chae et al., 2004). In the retina, its function has been linked with the generation of amacrine and photoreceptor cells (Inoue et al., 2002; Kanekar et al., 1997; Moore et al., 2002; Morrow et al., 1999; Yan et al., 2005; Yan and Wang, 2004). Six6 activity, instead, has been mostly associated with undifferentiated proliferating precursors (Li et al., 2002; Zuber et al., 1999). It was therefore somewhat surprising to find that NeuroD directly activates *Six6* expression. *NeuroD* overexpression combined with MO-mediated inhibition of *Six6* confirmed this relationship and proved that the phenotypic alterations of *NeuroD* overexpression in part resembled those reported for *Six6* gain-of-function in other vertebrates (Bernier et al., 2000; Zuber et al., 1999), as expected for a direct regulation of *NeuroD* over *Six6*. Our overexpression studies uncovered additional and previously unnoticed defects caused by *NeuroD* gain-of-function, which were not antagonised by *Six6*-MO and thus probably mediated by additional NeuroD targets. These defects included the ventral displacement of the eye, the loss of the ventral retinal pigmented epithelium and, most notably, the transformation of the optic stalk in tissue with neural retina characteristics. These defects culminated with cyclopia and retinal hyperplasia in the most-affected embryos. This is in striking contrast with the results of NeuroD overexpression in *Xenopus*, where the eyes were reduced in size owing to precocious neural differentiation (Kanekar et al., 1997; Lee et al., 1995). Defects somewhat similar to those we observed have been instead described as a consequence of the overexpression of the related *Xenopus Ath3* (Takebayashi et al., 1997). Given the redundant function of *NeuroD* and *Ath3* (Inoue et al., 2002), it is possible that, in medaka fish, *NeuroD* has acquired functions exerted by *Ath3* in other species.

Although we initially observed a regulation of Six6 by NeuroD, the overall picture that emerged from our studies suggests that NeuroD and Six6 undergo a mutual regulation. Alignment of the *NeuroD* loci from different teleost species identified a number of conserved putative Six6 binding sites (Marco-Ferreres et al., 2009) in the putative regulatory region of *NeuroD* (see Fig. S6 in the supplementary material). However, ChIP and Luciferase assays failed to validate the functional relevance of these putative binding sites (see Fig. S6 in the supplementary material), suggesting, although not definitively proving, that Six6 regulates *NeuroD* expression in the retina indirectly. An indirect regulatory mechanism might be particularly appropriate for photoreceptor precursors where the two genes are only transiently co-expressed. Otx2 and Crx, which colocalise with both Six6 and NeuroD, are possible candidates, especially because Otx2 function has been already associated with *NeuroD* regulation (Hennig et al., 2008).

Taken together, our data suggest the following plausible model (Fig. 7). *Six6* expressed in multipotent progenitors activates the expression of *NeuroD* in a cell subpopulation, which become committed to generate photoreceptors and a subset of amacrine cells (Masland, 2001). *Six6* is necessary but not sufficient for

amacrine cell generation. Its knockdown significantly decreases amacrine cell number, whereas its direct or indirect (*NeuroD*-mediated) overexpression does not significantly modify it, suggesting that additional factors, for example the related *Six3* (Inoue et al., 2002), are required for amacrine cell generation. In committed photoreceptors, *NeuroD* and *Six6* are both required to initiate the differentiation program, which seems particularly sensitive to both low and high Six6 levels. Although in adult human retinas SIX6 protein is abundantly localized to the ONL (Aijaz et al., 2005), the *Six6* mRNA (which does not necessarily reflect protein concentration) felt below detectable levels as photoreceptors began to differentiate, indicating that a significant amount of Six6 is required, directly or indirectly, to initiate *Rhodopsin* expression. This might be then maintained by the cooperative activity of NeuroD with other factors, including Otx2 or possibly Six3 (Manavathi et al., 2007). Indeed, an ordered sequence of E-boxes and putative Six3 and Six6 binding sites are highly conserved in the *Rhodopsin* promoter, although our preliminary observations indicate that knockdown of *Six6* has only a modest effect on *eGFP* reporter expression when a *Xenopus Rhodopsin* promoter *eGFP* construct was co-injected in medaka fish embryos (Fadool, 2003) (data not shown). This suggests that Six6-mediated regulation of *Rhodopsin* expression is probably indirect. Why sustained high levels of Six6 might have a repressive effect on *Rhodopsin* expression is unclear but it might involve repression and/or sequestration of other transcription factors.

In zebrafish, *NeuroD* conditionally overexpressed at late stages of differentiation increases photoreceptor generation, whereas its downregulation does not affect their specification (Ochocinska and Hitchcock, 2009). In line with the latter observation, the reduced levels of *NeuroD* observed in *Six6* morphants did not interfere with photoreceptor precursor generation but, contrary to what was observed in zebrafish, early medaka *NeuroD* or *Six6* overexpression did not increase photoreceptor number. This indicates that additional and NeuroD-independent mechanisms must specify this fate. These are probably operative only at late stages of neurogenesis when *NeuroD* is conditionally overexpressed in zebrafish.

Previous works had shown that Six6 plays an important role in retinal proliferation (Li et al., 2002; Zuber et al., 1999); however, the possible functions of this HD-TF during retinal cell specification remained unknown. Our study uncovered a novel role of *Six6* in amacrine and photoreceptor differentiation in cooperation with *NeuroD*. This cooperation, together with the observation that Lhx2 and Pax6 regulate *Six6* expression in mammals (Tetreault et al., 2009), constitutes a starting point towards the full identification of the transcriptional network controlling mammalian Six6 function. This, in turn, might help define the molecular causes of human inborn eye defects such as anophthalmia, microphthalmia and coloboma, which have been associated with genetic alterations of the *SIX6* locus (Ahmad et al., 2003; Gallardo et al., 1999; Gallardo et al., 2004).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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Table S1. Oligonucleotides used in this study

Primer name	Sequence (5′-3′)
pSKII-I*Sce*I-*olSix6*-7kb F	AATGGCATTTGACATAAGCC
pSKII-I*Sce*I-*olSix6*-7kb R	CTTAACAAGTGCCTGGTTCTC
olSix6 RE-b F	ATGAACAGCTTCGGGTACCGAAGG
olSix6 RE-b C	GAAACACTCGAGGGAAGAAGTGAG
mSix6 RE-b F	CCGCGAACTGTGAAGATCTG
mSix6 RE-b R	GATTCCCAATTGACATCCAC
*ol*Six6 3′UTR F	GACAGTGAATGTGACATCTG
*ol*Six6 3′UTR R	GATTACCGCTGACCAGTTTG
cSix6 RE-b F	TAATATCAGGGTTTTCCGTC
cSix6 RE-b R	TCTCTGTAGTTCCCCGTGTGG
cSix6 RE-b NeuroD mut F	GAGTGGCCCTGGGATCCCCGGAAGCGAGAC
cSix6 RE-b NeuroD mut R	CCGCCG ATATCCTGCGGGAGAGGCTGATTCG
olNeuroD cDNA F	GAATACCCGCATCAGGTCAC
olNeuroD cDNA R	TTTGGAATTATCAGCTGAGC
olSix6 5′UTR *Eco*RI F	TCAGGGAATTCGCTCGATAAGTTTG
olSix6 5′UTR *Nco*I R	CAAGATGGGCAACTGGACCATGGAG
RE-b E-box EMSA F	GGGACACCTCCGACCATCTGCGCTTC
RE-b E-box EMSA R	GGGATAGCGCAGATGGTCGGAGGTGTC
RE-b E-box mut EMSA F	GGGACACCTCCGACGGTCTTCGCTATC
RE-b E-box mut EMSA F	GGGATAGCGAAGACCGTCGGAGGTGTC
cSix6 Nd BS (ChIP) –1930 to –1781 F	AGGGGTTTCCGTCAGAATGAGCTTA
cSix6 Nd BS (ChIP) –1930 to –1781 R	CCTTTCCTTTAGTATCAAGTACAAT
cSix6 Nd BS (ChIP) –518 to –185 F	GAGTGGCACTGGGATCCCCGGAAGCGAGAC
cSix6 Nd BS (ChIP) –518 to –185 R	CCGCCGTGCTGCTGCGGGAGAGGCTGATTCG
mSix6 Nd BS (ChIP) –1621 to –1248 F	CCGCGAACTGTGAAGATCTG
mSix6 Nd BS (ChIP) –1621 to –1248 R	GATTCCCAATTGACATCCAC

ol, *Oryzias latipes*; m, mouse; c, chick.