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Six3 inactivation reveals its essential role for the formation and patterning of the vertebrate eye

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SUMMARY

The establishment of retinal identity and the subsequent patterning of the optic vesicle are the key steps in early vertebrate eye development. To date little is known about the nature and interaction of the genes controlling these steps. So far few genes have been identified that, when over-expressed, can initiate ectopic eye formation. Of note is *Six3*, which is expressed exclusively in the anterior neural plate. However, 'loss of function' analysis has not been reported. Using medaka fish, we show that vertebrate *Six3* is necessary for patterning of the anterior neuroectoderm including the retina anlage. Inactivation of *Six3* function by morpholino knock-down results in the lack of forebrain and eyes. Corroborated by gain-of-function experiments, graded interference reveals an additional role of *Six3* in the proximodistal patterning of the optic vesicle. During both processes of vertebrate eye formation, *Six3* cooperates with *Pax6*.

Key words: Eye development, Six3, Proximodistal patterning, Medaka, Morpholino

INTRODUCTION

Vertebrate eye development is initiated at the end of gastrulation with the determination of the retina anlage in the anterior neuroectoderm. Subsequently, the initially single eye field splits into two symmetric retinal primordia that evaginate laterally from the forebrain and become proximodistally patterned into optic stalk and retina (Chow and Lang, 2001; Jean et al., 1998).

Members of the evolutionarily conserved Six and Pax families of transcription factors (Bernier et al., 2000; Cheyette et al., 1994; Loosli et al., 1998; Oliver et al., 1995; Quiring et al., 1994; Toy et al., 1998; Zuber et al., 1999) share a temporally and spatially overlapping expression in the developing retina. Loss- and gain-of-function data indicate a key role for *Pax6* in eye development (reviewed by Gehring and Ikeo, 1999). Both *Six3* and *Pax6*, when overexpressed, mutually activate each others expression resulting in the formation of ectopic eye structures, suggesting also for *Six3* a role in the establishment of retinal identity (Chow et al., 1999; Kobayashi et al., 1998; Loosli et al., 1999).

The transcription factor *Six3* contains a homeodomain and a Six domain (Oliver et al., 1995; Toy et al., 1998), and is specifically expressed in the anterior neuroectoderm including the eye field and specific parts of the abutting surface ectoderm in all vertebrates analyzed so far (Bovolenta et al., 1998; Granadino et al., 1999; Loosli et al., 1998; Oliver et al., 1995; Seo et al., 1998; Zhou et al., 2000). In addition, at subsequent stages of development *Six3* is expressed in the developing retina. Overexpression of *Six3* results in expanded and ectopic retinal primordia in medaka (Loosli et al., 1999) and *Xenopus* (Bernier et al., 2000), and in an enlarged forebrain in zebrafish (Kobayashi et al., 1998). However, it remains elusive how and to what extend *Six3* contributes to this process as experimental loss-of-function data for *Six3* have not been reported so far.

We used a morpholino oligo-based gene knock-down approach (Nasevicius and Ekker, 2000) and gain-of-function analysis in the teleost medaka (*Oryzias latipes*) to determine the role of *Six3* in the initial establishment of the retinal primordia. Inactivation of *Six3* leads to the complete absence of forebrain and eyes, indicating the key role of *Six3* in patterning of the anterior neural plate and establishment of retinal identity.

We further established hypomorphic conditions, under which retinal precursor cells still form, to address the later function of *Six3*, identifying a role of *Six3* in proximodistal patterning of the optic vesicle. Our epistasis analysis establishes a genetic network and shows that *Six3* function in proximodistal patterning is mediated by the regional specification gene *Vax1*. Strikingly, the hypomorphic phenotypes closely resemble humans heterozygous for mutations in *Six3*, who suffer from holoprosencephaly, microcephaly and microphthalmia.

MATERIALS AND METHODS

Medaka stocks

Wild-type *Oryzias latipes* from a closed stock at EMBL-Heidelberg were kept as described (Köster et al., 1997).

Morpholino injections and control experiments

Morpholinos (Gene Tools, LLC, OR) targeted against *Six3*, *Pax6* and *GFP* were dissolved in 1×Yamamoto ringer (Yamamoto, 1975). Morpholino dilutions were injected into one cell of medaka embryos at the two-cell stage resulting in an even distribution. Morpholinos targeted against GFP (MoGFP: 5′-CAGCTCCTCGCCCTTGCTCACCATG-3′), which did not affect embryonic development, were injected at 0.06 mM. Binding specificity of MoSix3 was shown using an RNA containing the respective morpholino target sequence fused to the GFP-coding region (MoSix3t-GFP: 5′-GTCCGCCGCCTGCCTTCCCACACGC-GFP-3′). Co-injection of MoSix3t-GFP RNA (130 ng/μl at one-cell stage) and MoSix3 (0.05 mM at the two cell stage together with the red fluorescent lineage tracer dye rhodamine dextran (RD) (to follow its distribution) completely blocks GFP expression. However, GFP expression was not affected when five base pairs of the target sequence were exchanged (MoSix3ct-GFP: 5′-GaCCGCCGaCTGCCTaCCCtCACcC-GFP-3′). To test the Pax6 morpholino (0.06 mM) specificity, MoPax6t-GFP (5′-ATGATGCAG-AACAGTCACAGTGGCG-GFP-3′) and MoPax6ct-GFP (5′-ATa-ATGCAaAACAGTaACAaTGGaG-GFP-3′) constructs were used. Co-injection of MoPax6 with MoPax6t-GFP RNA blocks GFP expression, whereas co-injection with MoPax6ct-GFP does not affect GFP expression.

RNA injections

In vitro transcription of mouse *Six3* RNA and injection at 100 ng/μl into medaka embryos was performed as described (Loosli et al., 1999).

Whole-mount in situ hybridization and vibratome sectioning

Whole-mount in situ hybridization was performed using digoxigenin and fluorescein labeled RNA riboprobes as described (Loosli et al., 1998). Vibratome sections were cut following standard procedures (Loosli et al., 1999).

RESULTS AND DISCUSSION

Specific interference with *Six3* and *Pax6* function

To determine the role of *Six3* in patterning of anterior neuroectoderm, we interfered with *Six3* function by injecting a morpholino oligonucleotide (Nasevicius and Ekker, 2000) directed against *Six3* mRNA (MoSix3) that specifically blocks translation. Control experiments show that MoSix3 specifically and stably inhibits its target in a concentration-dependent manner (Fig. 1 and Materials and Methods). Sequence specificity of MoSix3 was shown using RNA containing the respective morpholino target sequence fused to the GFP-coding region (MoSix3t-GFP). Co-injection of MoSix3t-GFP RNA and MoSix3 completely blocks GFP expression (*n*=22/24; Fig. 1A-C). Conversely, GFP expression was not affected when five base pairs of the target sequence were exchanged (MoSix3ct-GFP) (*n*=36/36; data not shown) indicating the specificity of MoSix3. The morpholino induced phenotype can be specifically titrated by co-injection of RNA containing the morpholino target site: co-injection of MoSix3 and its target MoSix3t-GFP did not affect the embryonic morphology, whereas co-injection of MoSix3 and MoSix3ct-GFP resulted in the Six3 morphant phenotype (data not shown). Finally, the Six3 morphant phenotype can be rescued by co-injection of mouse *Six3* RNA (data not shown).

Analogous control experiments have been performed for the Pax6 morpholinos. Co-injection of MoPax6 with MoPax6t-GFP RNA blocks GFP expression (*n*=19/23; Fig. 1D-F), whereas co-injection with MoPax6ct-GFP does not affect GFP expression (*n*=25/25).

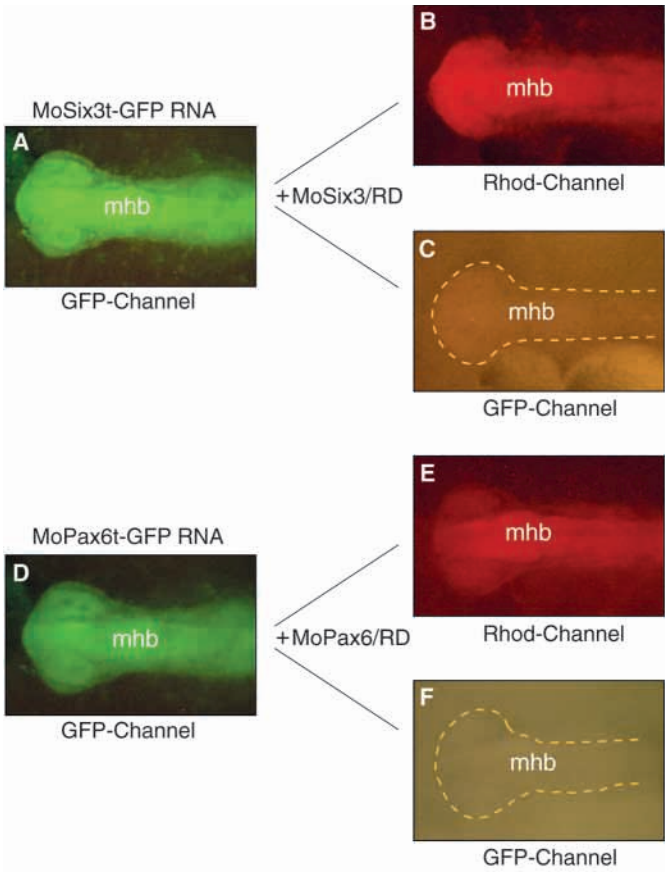

Fig. 1. Specific interference with gene translation using Six3 (MoSix3) and Pax6 (MoPax6) morpholinos. (A-F) Dorsal views of embryos at early somitogenesis stage (21); anterior is towards the left. (A,D) Embryos injected with control RNA at the one-cell stage show overall GFP expression. (B,C,E,F) At the two-cell stage, embryos were co-injected with the respective morpholinos and rhodamine dextran (RD) and analyzed using the GFP and rhodamine (Rhod) channel. mhb, mid-hindbrain boundary.

These control experiments show that both the Six3 and Pax6 morpholinos interfere specifically with their targets in a concentration-dependent manner and thus allow us to study the role of these genes in eye development in the loss-of-function situation, using either hypomorphic or amorphic conditions.

Graded inactivation of *Six3* function

Inactivation of *Six3* by injection of high amounts of MoSix3 (Six3 morphants) causes an eyeless phenotype and the deletion of the forebrain. In these embryos already at late neurula/early somitogenesis stages morphologically abnormal cells were detected in the presumptive forebrain, indicating ectopic cell death (Fig. 2A,B). TUNEL labeling (Winkler et al., 2000) in Six3 morphants at the late gastrula stage revealed a large number of apoptotic cells in a region overlapping with the *Six3* expression domain in the anterior neuroectoderm (Fig. 2C-E). Thus, in the absence of *Six3* function, these cells undergo ectopic cell death resulting in the absence of forebrain and eye structures. This shows that *Six3* function is essential for the

establishment and maintenance of the anterior neuroectodermal identity including the presumptive forebrain and the retina anlage, consistent with the early expression pattern at the late gastrula/early neurula stage.

Six3 functions in the formation of the proximodistal axis of the optic cup

Six3 continues to be expressed also in the developing retina and forebrain, suggesting additional later functions. In line with this, *Six3* overexpression induces the formation of patterned optic cups at ectopic locations in the brain (Loosli et al., 1999). Using molecular markers specific for the proximal region [optic stalk, *Vax1* (Winkler et al., 2000)] and distal region [neuroretina, *Rx2* (Loosli et al., 1999)] of the developing optic cup, we found that *Six3* overexpression by mRNA injection results in stalk cells positive for *Vax1* in the midbrain abutting the *Rx2* expressing ectopic neuroretina (Fig. 2F-H). The formation of a complete proximodistally patterned ectopic optic primordium hints at an important role of *Six3* in later aspects of eye patterning. In severe Six3 morphants, however, this later role cannot be examined, due to the complete absence of these structures.

We circumvented this problem by using lower MoSix3 concentrations that generate hypomorphic phenotypes in a concentration-dependent manner (Table 1 and Fig. 2I-K). Mildly affected Six3 morphants exhibit mild cyclopia, small eyes and a reduction of proximal regions of the pigmented retinal epithelium (PRE). The intermediate phenotype is characterized by severe cyclopia and the loss of forebrain structures. TUNEL analysis of embryos at the late gastrula stage revealed apoptotic cells scattered over the entire anterior neuroectoderm. When compared with embryos injected with higher amounts of MoSix3, the abundance of apoptotic cells was lower. We observed a close correlation between the abundance of ectopic cell death at the late gastrula stage (intermediate, 32%; mild, 41%; *n*=79) and the phenotypic severity at subsequent stages of development (intermediate, 33%; mild, 40%; *n*=159; compare with Table 1). Mutations in the human *SIX3* gene result in holoprosencephaly (Granadino et al., 1999; Wallis et al., 1999). In these heterozygous individuals, this dominant disorder causes craniofacial malformations that include cyclopia, microphthalmia and midfacial defects. Thus, the medaka Six3 morphant faithfully phenocopies reduced *Six3* activity in humans, indicating that this human holoprosencephaly disorder can be studied using the morpholino-based medaka model.

Differential requirement for *Six3* along the proximodistal axis of the eye

We analyzed mildly affected morphants at early somitogenesis stages using specific molecular markers. The homeobox gene *Vax1* is expressed in the developing ventral forebrain and optic stalk (Hallonet et al., 1998; Winkler et al., 2000) and loss of function analysis in mouse show its essential role for the normal development of these proximal eye structures (Hallonet et al., 1999). In mildly affected Six3 morphants, *Vax1* expression was completely lost. This highlights a severe reduction of proximal eye structures (*n*=14/16; Fig. 3A,B) and an essential regulatory input of *Six3* on *Vax1* expression. On the other hand, the two expression domains of the distal retina marker *Rx2* were fused anteriorly and reduced in size (*n*=15/25; Fig. 3C,D) consistent with the observed microphthalmia (Fig. 2J). The finding that the proximal region of the retinal primordium is more sensitive to reduced *Six3* levels than the distal region indicates a role of *Six3* in proximodistal patterning, in part mediated by the transcriptional regulation of *Vax1*.

In these Six3 morphants the telencephalic *Emx1* expression domain is significantly smaller and non-overlapping with the *Rx2* expression domain (Fig. 3C,D), consistent with the loss of

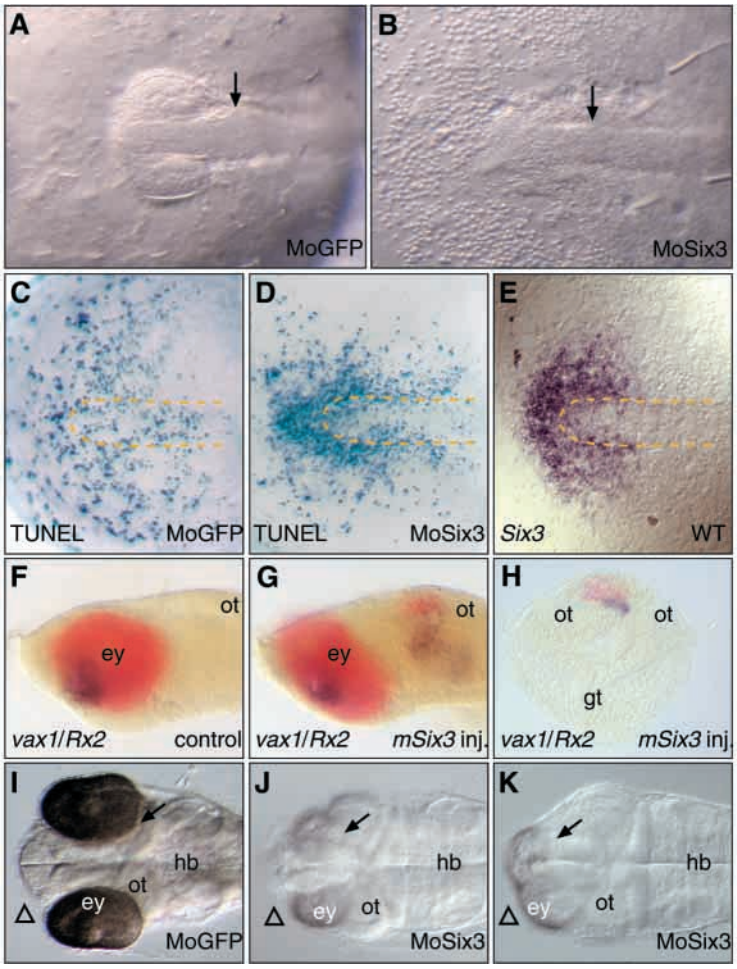

Fig. 2. *Six3* is required for retina and forebrain development. (A-E) Dorsal views of embryos (A,B) at six-somite stage (21) and (C-E) late gastrula stage (16), anterior is towards the left. (B) Injection of high MoSix3 concentrations results in loss of anterior structures (severe) compared with MoGFP control injected embryos (A). Arrow indicates region of forming mid-hindbrain boundary. (C,D) Ectopic cell death (TUNEL) in response to MoSix3 overlapping with (E) *Six3* expression. Broken yellow line outlines forming embryonic axis. (F,G) Lateral views and (H) *Rx2* (red) and *Vax1* (purple) in situ analysis of (F) GFP control and (G,H) mouse *Six3*-injected embryos at somitogenesis stage (23); (F-H) transverse section (dorsal is upwards) at the midbrain level of another embryo. *Six3* overexpression results in ectopic formation of proximal (*Vax1*) and distal (*Rx2*) eye structures in the midbrain (22%; *n*=37). (I-K) Dorsal views of organogenesis stage embryos (30). (J,K) MoSix3-injected embryos exhibit a small eye (mild) and a cyclopic (intermediate) phenotype. Arrow indicates proximal retinal structures lost, arrowhead indicates forebrain structures lost (K). ey, eye; gt, gut; hb, hindbrain; inj., injected; ot, optic tectum.

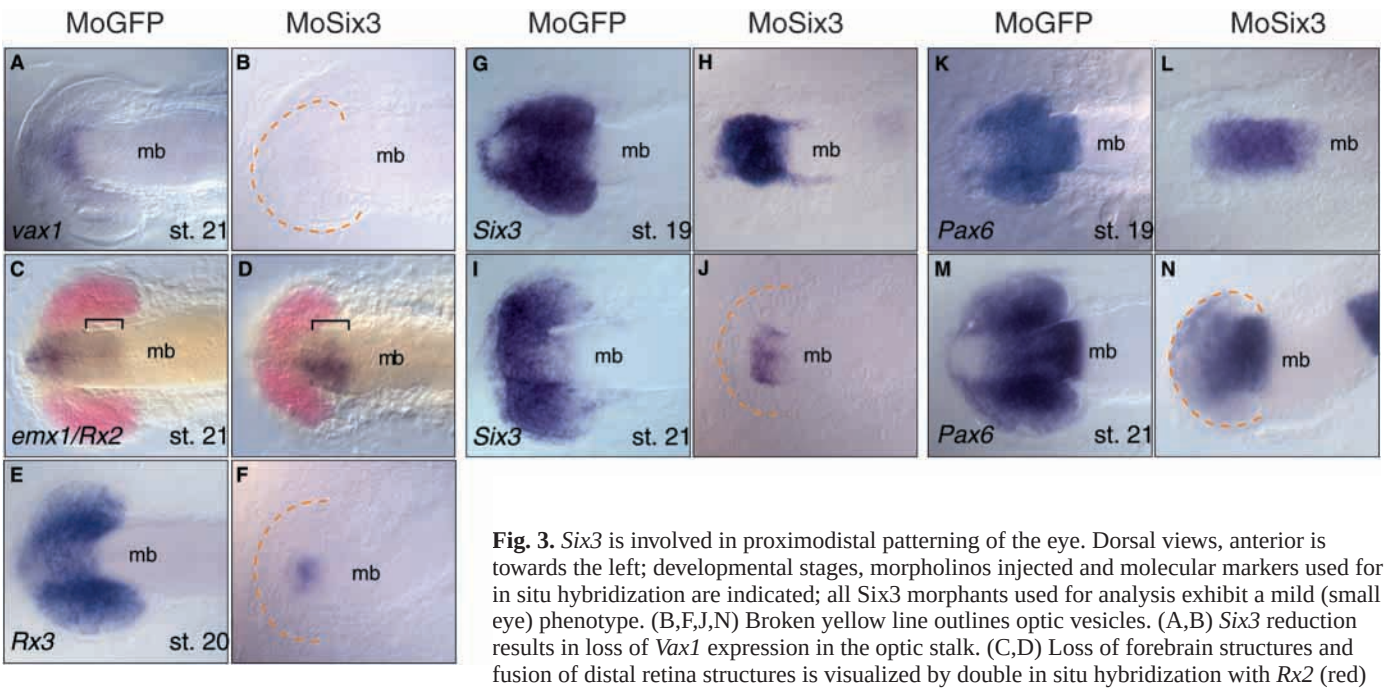

Fig. 3. *Six3* is involved in proximodistal patterning of the eye. Dorsal views, anterior is towards the left; developmental stages, morpholinos injected and molecular markers used for in situ hybridization are indicated; all Six3 morphants used for analysis exhibit a mild (small eye) phenotype. (B,F,J,N) Broken yellow line outlines optic vesicles. (A,B) *Six3* reduction results in loss of *Vax1* expression in the optic stalk. (C,D) Loss of forebrain structures and fusion of distal retina structures is visualized by double in situ hybridization with *Rx2* (red) and *Emx1* (purple). Bar indicates region of remaining *Emx1*-expressing tissue. (E,F) Retinal *Rx3* expression is lost in Six3 morphants. (G-N) In Six3 morphants *Six3* is lost and *Pax6* is reduced in the retina at six-somite stage (21). mb, midbrain.

forebrain structures at later stages of development (Fig. 2K) and the microcephaly observed in individuals with *SIX3* mutations (Wallis et al., 1999).

The expression of *Rx3*, *Pax6* and *Six3* itself depends on *Six3* function

As retinal precursor cells are still present in mildly affected morphants, as visualized by *Rx2* expression, we were able to study the role of *Six3* in the genetic network that underlies early eye development. Consistent with the demonstrated post-transcriptional effect of morpholinos on gene expression (Nasevicius and Ekker, 2000), initial *Six3* transcript levels and distribution are not affected in Six3 morphants (Fig. 3G,H). It has been suggested that *Six3* acts in a regulatory feedback loop (Loosli et al., 1999). We demonstrate this at later stages (six-somite stage), when expression of *Six3* was not detectable in the retinal precursor cells of Six3 morphants, whereas weak expression was observed in the region of the developing hypothalamus (Fig. 3I,J). This shows that *Six3* expression in retinal precursor cells crucially depends on *Six3* activity, confirming the suggested role of *Six3* in maintenance of its own expression. However, for its hypothalamic expression *Six3* receives additional independent input at this stage.

Table 1. Concentration-dependent effects upon injection of morpholinos targeted against *Six3* (MoSix3)

MoSix3 concentration (mM)	Severe phenotype	Intermediate phenotype	Mild phenotype	Wild type	Dead or not gastrulated	*n*
0.013	0%	0%	14%	78%	8%	50
0.025	0%	2%	36%	56%	6%	50
0.04	2%	22%	59%	11%	6%	66
0.05	9%	33%	40%	12%	6%	159
0.1	69%	0%	7%	12%	12%	26

Injection of MoSix3 results in the formation of either small (mild phenotype), cyclopic (intermediate phenotype) or no (severe phenotype) eyes.

Proliferation and morphogenesis of the retinal primordium requires the activity of the homeobox gene *Rx3* (Loosli et al., 2001). Genetic epistasis analysis in medaka indicated a regulatory input of *Six3* to *Rx3* expression. To evaluate the contribution of *Six3*, we analyzed *Rx3* expression in Six3 morphants. Notably, while retinal *Rx3* expression initially appears unaffected, reduced *Six3* function results in the loss of *Rx3* expression at the four-somite stage (Fig. 3E,F). Thus, *Rx3* receives an initial input that is at least partially independent of *Six3* and in a second phase depends entirely on *Six3* function in the developing optic vesicle. *Rx3* remained expressed only in the region of the forming hypothalamus, where *Six3* and *Rx3* expression domains do not overlap. The observed microphthalmia can in part be explained by the loss of *Rx3*, resulting in a reduced proliferation of retinal progenitor cells.

Overexpression of the evolutionarily conserved transcription factor *Pax6* results in ectopic *Six3* expression and eye formation (Chow et al., 1999). On the other hand, ectopic retina formation in response to *Six3* overexpression in fish is preceded by ectopic *Pax6* expression (Loosli et al., 1999), indicating that *Six3* and *Pax6* can crossregulate each other during eye formation. Similar to *Six3*, the expression of *Pax6* is not eye specific, but their respective domains overlap in the retina anlage at the late gastrula stage (Loosli et al., 1998; Oliver et al., 1995). It has been argued that the region of overlapping expression defines the retina anlage and its formation requires their crossregulatory interaction (Loosli et al., 2001). In Six3 morphants, up to the onset of somitogenesis, *Pax6* transcript levels were unaltered (Fig. 3K,L and data not shown). At the six-somite stage, however, the *Pax6* expression level in the retinal precursor cells was significantly reduced, but unaffected

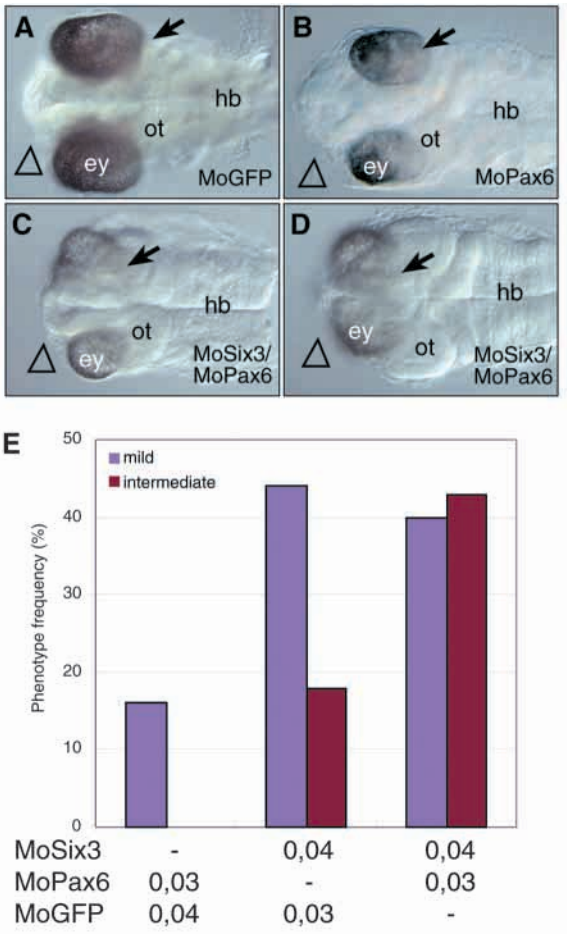

Fig. 4. Cooperation of *Pax6* and *Six3* in eye development. (A-D) Dorsal views of embryos at organogenesis stage, anterior is towards the left; injected morpholinos are indicated. (B) MoPax6 results in development of small eyes compared with control embryos (A). (C,D) Co-interference with *Six3* and *Pax6* function results in formation of either small (mild) or cyclopic eyes (intermediate). Arrow in D indicates proximal retinal structures lost; arrowhead indicates forebrain lost. (E) MoPax6/MoSix3 co-injection (concentrations in mM) (n=473) shifts the mild (blue) towards the intermediate phenotype (red) when compared with MoPax6/MoGFP (n=50) or MoSix3/MoGFP-injected embryos (n=50). ey, eye; hb, hindbrain; ot, optic tectum.

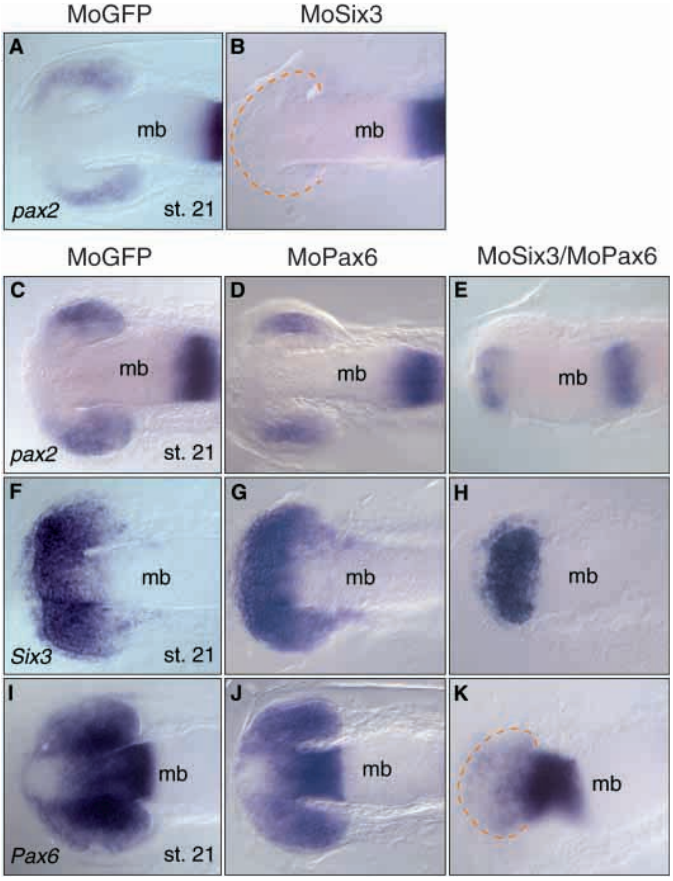

Fig. 5. Differential effects of MoSix3, MoPax6 or MoSix3/MoPax6 co-injected embryos. Dorsal views of embryos at the six-somite stage, anterior is towards the left; injected morpholinos and molecular markers for in situ hybridization are indicated. (A,B) MoSix3 results in the loss of *Pax2* expression. (C,E) Retinal *Pax2* is still expressed even in more severely affected MoSix3/MoPax6 double morphants. (F,H,I,K) In MoSix3/MoPax6 double morphants retinal *Pax6* but not *Six3* expression is reduced at early somitogenesis. (D,G,J) *Pax2*, *Six3* and *Pax6* expression are not affected in Pax6 morphants. broken yellow line outlines optic vesicle in B,K. mb, midbrain.

Table 2. Concentration-dependent effects upon injection of morpholinos targeted against *Pax6* (MoPax6)

MoPax6 concentration (mM)	Small eyes	Wild type	Dead or not gastrulated	*n*
0.015	9%	80%	11%	55
0.03	12%	78%	10%	50
0.06	38%	50%	12%	101
0.09	39%	49%	12%	57

Injection of MoPax6 results in the formation of small eyes.

in the presumptive dorsal forebrain where *Six3* is not expressed (Fig. 3M,N). The finding that *Pax6* expression in retinal precursor cells is sensitive to *Six3* activity corroborates their regulatory interaction (Bernier et al., 2000; Chow et al., 1999; Loosli et al., 1999). Although at early stages both genes appear to be independent of *Six3* function, the strength of this regulatory interaction increases during the development of the retinal primordium.

Interaction of *Six3* and *Pax6* during eye development

We used the morpholino-based gene knockdown approach to interfere with *Pax6* function. The specific and stable inhibitory effect of MoPax6 on its target was verified in control experiments (see above, Fig. 1 and data not shown). Pax6 morphants exhibit a ‘small eye’ phenotype in a concentration-dependent manner (Table 2 and Fig. 4A,B). In situ hybridization analysis of Pax6 morphants injected with either 0.03 mM (Fig. 5D,G,J) or 0.06 mM MoPax6 (data not shown) revealed that, at the early stages of development, *Pax6* interference does not affect the expression of all molecular markers, for example, *Pax2*, *Six3*, *Pax6* or *Rx* genes (Fig.

5D,G,J and data not shown). This is in good agreement with the analysis of the respective murine homologs in small eye *Pax6*$^{-/-}$ mutant mice (Bernier et al., 2001).

To address the cooperation of *Six3* and *Pax6* in optic vesicle formation, we co-injected MoSix3 and MoPax6. A MoPax6 concentration was used that, when injected alone, results in a very low frequency of the small eye phenotype (Fig. 4E). This was combined with MoSix3 at a concentration that causes predominantly a weak hypomorphic phenotype when injected alone (Fig. 4E). The co-injection results in a significant change of the ratios of the two observed phenotypes. In MoSix3-injected embryos, less than one third of the affected embryos exhibit the intermediate phenotype, whereas co-injection results in a more than twofold increase (Fig. 4E). The co-injection of MoSix3 and MoPax6 leads to phenotypes that are morphologically indistinguishable from morphant phenotypes caused by higher concentrations of MoSix3 alone (compare Fig. 2J with Fig. 4C and Fig. 2K with Fig. 4D and data not shown). Although MoPax6 alone only mildly affects eye formation, it potentiates the MoSix3 interference. This indicates that the genetic network underlying early retina formation and the subsequent patterning of the eye depends on the combined regulatory input of *Six3* and *Pax6*.

Different effects on gene expression in Six3/Pax6 double morphants

To examine this interaction in more detail, we analyzed the expression of molecular markers upon co-interference. Whereas the expression of *Rx2*, *Rx3*, *Vax1* and *Emx1* were similarly reduced (data not shown), we observed a differential response of the transcription factor *Pax2* in morphologically indistinguishable single and double morphants. *Pax2* expression in the retina was not detectable in single injected Six3 morphants (Fig. 5A,B). In MoSix3/MoPax6 co-injected morphants however, *Pax2* is still expressed (Fig. 5C,E). Even in the more severely affected double morphants, *Pax2* expression levels were not affected in the remaining retinal tissue. In addition, in Pax6 morphants *Pax2* expression remained unchanged (Fig. 5C,D). Thus *Pax2* expression is sensitive only to *Six3* interference but not to *Pax6* interference at early developmental stages. This differential molecular response indicates that *Six3* and *Pax6* have partially non-overlapping sets of target genes, consistent with the different phenotypes observed in the single Six3 and Pax6 morphants.

In addition, the expression of *Six3* and *Pax6* was differentially affected comparing single Six3 and Six3/Pax6 double morphants. Although *Six3* expression at the six-somite stage is strongly reduced in single MoSix3-injected embryos (Fig. 3I,J), its expression level remains unaffected in Pax6 or double morphants (Fig. 5F-H). However, retinal *Pax6* expression at this stage was more severely reduced in double morphants than in Six3 morphants (Fig. 3M,N, Fig. 5I,K). *Pax6* knockdown alone had no effect on its own expression (Fig. 5I,J). Thus, *Pax6* expression in retinal precursor cells depends on the combined regulatory input of *Pax6* and *Six3*. Conversely, *Six3* expression is less sensitive to this combined input indicating a mainly *Six3*-dependent regulation. Consistent with our results, it has been reported that *Six3* is still expressed in the retinal primordium of *Pax6*$^{-/-}$ mouse embryos (Bernier et al., 2001).

Conclusions

Our experiments demonstrate that *Six3* is essential for the formation of a discrete domain within the anterior neuroectoderm. In the absence of *Six3* function, cells in the *Six3* expression domain undergo apoptosis resulting in the absence of forebrain and eye. Conversely, overexpression of *Six3* results in retinal hyperplasia, indicating that one function of *Six3* is the control of proliferation in the presumptive retinal cells. In addition, *Six3* functions in the determination of the naive anterior neuroectoderm as loss of function results in the absence of the respective structures, while ectopic *Six3* expression leads to their ectopic formation.

Interference with gene function on the translational level using the gene knockdown technique offers advantages compared with other loss-of-function approaches. They allow to generate hypomorphic conditions, under which feedback loops can be uncovered. Our analysis provides direct evidence that *Six3* acts in a regulatory feedback loop on its own expression in vertebrates. Initially, *Six3* expression in the retina is unaffected but subsequently becomes crucially dependent on *Six3* function. However, *Pax6* has no influence on *Six3* expression at the early stages of eye development. This finding is consistent with *Pax6*$^{-/-}$ mutant mice where *Six3* is still expressed. Interestingly, the genetic network underlying gene regulation in the forming hypothalamus appears to be different from what we observed in the retina. Here, *Six3* and *Rx3* expression patterns do not overlap at early somitogenesis stages. The expression of both genes is neither affected in Six3 single nor Six3/Pax6 double morphants. It will be interesting to determine how the early expression of these genes is regulated and to what extend these genes are involved in the development of the hypothalamus.

Our gene knockdown data show that *Six3* and *Pax6* interact genetically at early stages of eye development. However, the morphological and molecular consequences of the loss of *Six3* function are more severe. *Pax6* acts on gene expression including its own only in cooperation with *Six3*. As seen for Pax6 morphants, small eye *Pax6*$^{-/-}$ mouse embryos initially form optic vesicles (Hill et al., 1991; Matsuo et al., 1993; Quinn et al., 1996; Ton et al., 1991), indicating that also in mouse *Pax6* is not required for the formation of the retina anlage in the neuroectoderm.

TUNEL-labeling experiments at the late gastrula stage revealed that injection of lower amounts of either MoSix3 or MoSix3/MoPax6 results in a reduced and variable degree of apoptosis as compared with high amounts of MoSix3 (data not shown). In both, single- and double-injected morphants, cells that undergo ectopic cell death were distributed over the entire anterior neural plate under all different hypomorphic conditions analyzed, suggesting a similar mechanism acting in both, single and double morphants.

In both, single- and double-injected morphants, the distribution of apoptotic cells at the late gastrula stage shows no regionalization, suggesting that proximodistal patterning is a later function of *Six3*. The close correlation of the abundance of cell death at the late gastrula stage and phenotypic severity at subsequent stages suggests that the early role of *Six3* (determination) and its later function (patterning) are not completely independent. Future experiments will address to what extent the mechanisms for determination and patterning are coupled.

Following its role in the determination and formation of the retina anlage, *Six3* functions in proximodistal patterning of the optic vesicle. *Six3* activity regulates the expression of the regional specification gene *Vax1*, which is required for the formation of ventral forebrain and proximal eye structures. Graded loss of *Six3* function shows that the distally expressed genes *Rx2* and *Pax6* are less sensitive than *Vax1* underscoring the proximodistal patterning activity of *Six3*. In addition, at this stage *Six3* controls proliferation and morphogenesis by regulating the expression of *Rx3*, which is essential for these processes in the developing optic vesicle (Loosli et al., 2001). Future experiments will aim to identify factors mediating the differential activity of *Six3* along the proximodistal axis.

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REFERENCES

Bernier, G., Panitz, F., Zhou, X., Hollemann, T., Gruss, P. and Pieler, T. (2000). Expanded retina territory by midbrain transformation upon overexpression of Six6 (Optx2) in Xenopus embryos. *Mech. Dev.* **93**, 59-69.

Bernier, G., Vukovich, W., Neidhardt, L., Herrmann, B. G. and Gruss, P. (2001). Isolation and characterization of a downstream target of Pax6 in the mammalian retinal primordium. *Development* **128**, 3987-3994.

Bovolenta, P., Mallamaci, A., Puelles, L. and Boncinelli, E. (1998). Expression pattern of *cSix3*, a member of the Six/sine oculis family of transcription factors. *Mech. Dev.* **70**, 201-203.

Cheyette, B. N. R., Green, P. J., Martin, K., Garren, H., Hartenstein, V. and Zipursky, S. L. (1994). The Drosophila sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. *Neuron* **12**, 977-996.

Chow, R. L. and Lang, R. A. (2001). Early eye development in vertebrates. *Annu. Rev. Cell Dev. Biol.* **17**, 255-296.

Chow, R. L., Altmann, C. R., Lang, R. A. and Hemmati-Brivanlou, A. (1999). Pax6 induces ectopic eyes in a vertebrate. *Development* **126**, 4213-4222.

Gehring, W. J. and Ikeo, K. (1999). Pax 6: mastering eye morphogenesis and eye evolution. *Trends Genet.* **15**, 371-377.

Granadino, B., Gallardo, M. E., Lopez-Rios, J., Sanz, R., Ramos, C., Ayuso, C., Bovolenta, P. and Rodriguez de Cordoba, S. (1999). Genomic cloning, structure, expression pattern, and chromosomal location of the human SIX3 gene. *Genomics* **55**, 100-105.

Hallonet, M., Hollemann, T., Wehr, R., Jenkins, N. A., Copeland, N. G., Pieler, T. and Gruss, P. (1998). Vax1 is a novel homeobox-containing gene expressed in the developing anterior ventral forebrain. *Development* **125**, 2599-2610.

Hallonet, M., Hollemann, T., Pieler, T. and Gruss, P. (1999). Vax1, a novel homeobox-containing gene, directs development of the basal forebrain and visual system. *Genes Dev.* **13**, 3106-3114.

Hill, R. E., Favor, J., Hogan, B. L. M., Ton, C. C. T., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. and van Heyningen, V. (1991). Mouse small eye results from mutations in a paired-like homeobox containing gene. *Nature* **354**, 522-525.

Jean, D., Ewan, K. and Gruss, P. (1998). Molecular regulators involved in vertebrate eye development. *Mech. Dev.* **76**, 3-18.

Kobayashi, M., Toyama, R., Takeda, H., Dawid, I. B. and Kawakami, K. (1998). Overexpression of the forebrain-specific homeobox gene six3 induces rostral forebrain enlargement in zebrafish. *Development* **125**, 2973-2982.

Köster, R., Stick, R., Loosli, F. and Wittbrodt, J. (1997). Medaka spalt acts as a target gene of hedgehog signaling. *Development* **124**, 3147-3156.

Loosli, F., Köster, R. W., Carl, M., Krone, A. and Wittbrodt, J. (1998). Six3, a medaka homologue of the *Drosophila* homeobox gene sine oculis is expressed in the anterior embryonic shield and the developing eye. *Mech. Dev.* **74**, 159-164.

Loosli, F., Winkler, S. and Wittbrodt, J. (1999). Six3 overexpression initiates the formation of ectopic retina. *Genes Dev.* **13**, 649-654.

Loosli, F., Winkler, S., Burgtorf, C., Wurmbach, E., Ansorge, W., Henrich, T., Grabher, C., Arendt, D., Carl, M., Krone, A. et al. (2001). Medaka eyeless is the key factor linking retinal determination and eye growth. *Development* **128**, 4035-4044.

Matsuo, T., Osumi-Yamashita, N., Noji, S., Ohuchi, H., Koyama, E., Myokai, F., Matsuo, N., Toniguchi, S., Dari, H., Jseki, S. et al. (1993). A mutation at the *Pax-6* gene in rat small eye is associated with impaired migration of midbrain crest cells. *Nat. Genet.* **3**, 299-304.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-220.

Oliver, G., Mailhos, A., Wehr, R., Copeland, N. G., Jenkins, N. A. and Gruss, P. (1995). Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. *Development* **121**, 4045-4055.

Quinn, J. C., West, J. D. and Hill, R. E. (1996). Multiple functions for Pax6 in mouse eye and nasal development. *Genes Dev.* **10**, 435-446.

Quiring, R., Walldorf, U., Kloter, U. and Gehring, W. J. (1994). Homology of the eyeless gene of drosophila to the small eye gene in mice and aniridia in humans. *Science* **265**, 785-789.

Seo, H. C., Drivenes, O., Ellingsen, S. and Fjose, A. (1998). Expression of two zebrafish homologues of the murine Six3 gene demarcates the initial eye primordia. *Mech. Dev.* **73**, 45-57.

Ton, C. C. T., Hirvonen, H., Miwa, H., Weil, M. M., Monaghan, P., Jordan, T., van Heyningen, V., Hastie, N. D., Meijers-Heijboer, H. and Drechsler, M. (1991). Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. *Cell* **67**, 1059-1074.

Toy, J., Yang, J.-M., Leppert, G. and Sundin, O. H. (1998). The Optx2 homeobox gene is expressed in early precursors of the eye and activates retina-specific genes. *Proc. Natl. Acad. Sci. USA* **95**, 10643-10648.

Wallis, D. E., Roessler, E., Hehr, U., Nanni, L., Wiltshire, T., Richieri-Costa, A., Gillessen-Kaesbach, G., Zackai, E. H., Rommens, J. and Muenke, M. (1999). Mutations in the homeodomain of the human SIX3 gene cause holoprosencephaly. *Nat. Genet.* **22**, 196-198.

Winkler, S., Loosli, F., Henrich, T., Wakamatsu, Y. and Wittbrodt, J. (2000). The conditional medaka mutation eyeless uncouples patterning and morphogenesis of the eye. *Development* **127**, 1911-1919.

Yamamoto, T. (1975). *Medaka (killifish), Biology and Strains*. Tokyo: Keigaku Publishing Company.

Zhou, X., Hollemann, T., Pieler, T. and Gruss, P. (2000). Cloning and expression of *xSix3*, the *Xenopus* homologue of murine *Six3*. *Mech. Dev.* **91**, 327-330.

Zuber, M. E., Perron, M., Philpott, A., Bang, A. and Harris, W. A. (1999). Giant eyes in Xenopus laevis by overexpression of XOptx2. *Cell* **98**, 341-352.