Specification of the vertebrate eye by a network of eye field transcription factors

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Summary

Several eye-field transcription factors (EFTFs) are expressed in the anterior region of the vertebrate neural plate and are essential for eye formation. The *Xenopus* EFTFs *ET, Rx1, Pax6, Six3, Lhx2, tll* and *Optx2* are expressed in a dynamic, overlapping pattern in the presumptive eye field. Expression of an EFTF cocktail with *Otx2* is sufficient to induce ectopic eyes outside the nervous system at high frequency. Using both cocktail subsets and functional (inductive) analysis of individual EFTFs, we have revealed a genetic network regulating vertebrate eye field specification. Our results support a model of progressive tissue specification in which neural induction then *Otx2*-driven neural patterning primes the anterior neural plate for eye field formation. Next, the EFTFs form a self-regulating feedback network that specifies the vertebrate eye field. We find striking similarities and differences to the network of homologous *Drosophila* genes that specify the eye imaginal disc, a finding that is consistent with the idea of a partial evolutionary conservation of eye formation.

Key words: Neural patterning, Eye field specification, Ectopic eye formation, Genetic network, Noggin, Otx2, ET, Rx1, Pax6, Six3, Lhx2, Tll, Optx2, *Xenopus laevis*, Transcription factor cocktails

Introduction

The first morphological evidence of eye formation in vertebrates is a bilateral expansion of tissue from the early forebrain to form the optic vesicles. It has been known for nearly 70 years, however, that presumptive eye tissue (eye field) exists prior to optic vesicle formation. In the salamander, a small piece of anterior neural plate can be isolated 6 hours prior to optic vesicle formation, and remarkably, after another 24 hours in vitro this tissue will transform into a single small but histologically normal eye (Lopashov and Stroeva, 1964). Modern molecular evidence shows that the eye anlagen is specified at the neural plate stage when a group of eye field transcription factors, EFTFs, are expressed in the anterior neural plate. The EFTFs include *ET*, *Rx1*, *Pax6*, *Six3*, *Lhx2*, *tll* and *Optx2* (also known as *Six6*). Genetic evidence clearly demonstrates the importance of these EFTFs in vertebrate eye formation. Mutations of *PAX6*, *SIX3* and *OPTX2* in human result in malformations affecting the eyes (Wawersik and Maas, 2000). The targeted, or spontaneous mutation of *Pax6*, *Rx* (*Rax* – Mouse Genome Informatics) *Lhx2*, *Tll*, *Six3* and *Six6* in mouse, results in animals with abnormal or no eyes (Hill et al., 1991; Lagutin et al., 2003; Li et al., 2002; Mathers et al., 1997; Porter et al., 1997; Tucker et al., 2001; Yu et al., 2000). Similar phenotypes have been observed when homologues of *Six3*, *Pax6*, *tll*, *Rx1* and *Optx2* genes have been functionally inactivated in other vertebrate species (Carl et al., 2002; Chow et al., 1999; Hollemann et al., 1998; Isaacs et al., 1999; Loosli et al., 2001; Zuber et al., 1999). Not only are these EFTFs necessary for eye formation, but in some contexts they are also sufficient. Overexpression of *Pax6*, *Six3*, *Rx* and *Optx2* homologues can expand or induce eye tissues in the nervous system of vertebrates (Andreazzoli et al., 1999; Bernier et al., 2000; Chow et al., 1999; Chuang and Raymond, 2001; Loosli et al., 1999; Mathers et al., 1997; Oliver et al., 1996; Zuber et al., 1999).

Many of these EFTFs were originally identified as homologs of genes required for eye formation in *Drosophila melanogaster*. For example, *Pax6* is a homologue of *Drosophila eyeless* and *twin of eyeless* (Quiring et al., 1994), and *Six3* and *Optx2* are homologues of *Drosophila sine oculis* (Oliver et al., 1995). The *Drosophila* genes, *twin of eyeless* (*toy*), *eyeless* (*ey*), *eyes absent* (*eya*), *sine oculis* (*so*), *dachshund* (*dac*), *eye gone* (*eyg*) and *optix* either induce ectopic eyes or are required for normal eye formation (Hanson, 2001; Heberlein and Treisman, 2000; Kumar, 2001; Wawersik and Maas, 2000). The expression patterns of *toy*, *ey*, *so*, *eya*, *dac* and *eyg* overlap in the *Drosophila* eye field during its specification (Kumar and Moses, 2001a). It has been proposed that the overlapping expression patterns of these genes drives eye specification and is regulated by the Notch and EGFR signaling systems (Kumar and Moses, 2001a). Dominant-negative Notch receptor blocks compound eye formation, while constitutively activate Notch induces *ey* and *toy* expression and ectopic fly eyes (Kurata et al., 2000). A role for Notch signaling in vertebrate eye formation is

Table 1. Primer sets used for PCR analysis

Target gene	Upstream primer (5′ to 3′)	Downstream primer (5′ to 3′)	Cycle number	Number of bp	Accession number/reference
ET	CCT ATC CTT GAC TTG CTA CA	GTT TTG GGG AAG GAG GGT AT	24	255	AF173940
Pax6	GCA ACC TGG CGA GCG ATA AGC	CCT GCC GTC TCT GGT TCC GTA GTT	28	450	U76386
Six3	TTG TCT GTC TGT CTC TTG TT	TTC TGT GTT TGG TTT ATC TC	30	369	AF183571
Rx1	CCC CAA CAG GAG CAT TTA GAA GAC	AGG GCA CTC ATG GCA GAA GGT T	28	416	AF017273
tll	ACT TGC CTC TCG TGC TGC TCT ACT G	ATC CGG TCG GGT TGC TCA TCT T	30	351	U67886
Lhx2	ACC CTC CTC CCC CAT TAC TCA C	AGG GCA TAT CTG GGC ATC TTC A	30	461	AY141037
Optx2	ACA GAG CAG CGG CGG CAA AGA	GAG CGC TCC CTG GTA CTG TGA CTG A	30	296	AF081352
NCAM	CAC AGT TCC ACC AAA TGC	GGA ATC AAG CGG TAC AGA	30	343	Xenbase
Otx2	GGA TGG ATT TGT TAC ATC CGT C	CAC TCT CCG AGC TCA CTT CCC	25	315	U19813
XAG	GAC TGG TGC TGT TCA ACC TTG	CAT TGG GAA ATA ACT GGG ACC	25	349	U76752
H4	CGG GAT AAC ATT CAG GGT ATC ACT	ATC CAT GGC GGT AAC TGT CTT CCT	24	189	Hollemann et al., 1998

Primer sets were designed from the indicated GenBank sequence or from the indicated source (see Materials and methods for the details of the PCR reaction conditions). Xenbase primer sequences can be found at http://www.xenbase.org/

suggested by similar experiments. Mice homozygous for a hypomorphic Notch2 mutation have bilateral microphthalmia (McCright et al., 2001), while activation of Notch signaling induces the expression of *Pax6*, *Six3* and *Rx* and causes eye duplications and ectopic eye tissue formation (Onuma et al., 2002).

In *Drosophila*, it has been possible using genetics to show that these genes act as a network with hierarchical components and multiple steps of feedback regulation including functional protein interactions (Chen et al., 1997; Pignoni et al., 1997). More recently, overexpression and inactivation studies have begun to shed light on the transcriptional network of EFTFs involved in vertebrate eye formation. Overexpression of *Pax6*, *Six3*, *Optx2* and *Rx* upregulate each other's expression, while inactivation of each can reduce the expression of the others (Andreazzoli et al., 1999; Bernier et al., 2000; Chow et al., 1999; Chuang and Raymond, 2001; Goudreau et al., 2002; Lagutin et al., 2001; Lagutin et al., 2003; Loosli et al., 1999; Zuber et al., 1999). For example, *Pax6* and *Six3* crossregulate each other's expression in both medakafish and mouse (Carl et al., 2002; Goudreau et al., 2002). As in *Drosophila*, functional interactions among the vertebrate EFTFs involve protein-protein complexes and multiple levels of regulation (Li et al., 2002; Mikkola et al., 2001; Stenman et al., 2003), implying that a complex network must exist.

As in the salamander, *Xenopus laevis* neural plate explants form eye tissue in vitro. When *Xenopus* anterior neural plate explants are isolated with underlying prechordal mesoderm at stage 12.5, two retinas form, demonstrating that the eye field is specified as early as stage 12.5 (Li et al., 1997). The frog EFTFs, *ET*, *Pax6*, *Six3*, *Rx1*, *Lhx2*, *tll* and *Optx2*, are expressed together in the *Xenopus* anterior neural plate prior to stage 15 (Bachy et al., 2001; Casarosa et al., 1997; Hirsch and Harris, 1997; Hollemann et al., 1998; Li et al., 1997; Mathers et al., 1997; Zhou et al., 2000; Zuber et al., 1999). In this paper, we test the idea proposed by Kumar and Moses for *Drosophila* eye field specification, in order to determine if the coordinated expression of EFTFs can also specify the vertebrate eye field. We find that EFTF cocktails not only induce ectopic eye fields in *Xenopus*, but generate ectopic eyes at high frequency outside the nervous system. In addition we provide an initial characterisation of the functional interactions among the EFTFs involved in vertebrate eye field specification.

Materials and methods

Animals

Fertilised eggs were obtained from pigmented *Xenopus* injected with 500 U of human chorionic gonadotropin (Sigma-Aldrich Company, UK) to induce egg laying. Embryos were dejellied with 3.3 mM DTT in 200 mM TrisHCL (pH 8.8) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

RNA microinjection

Capped RNA was synthesised in vitro from pCS2.Xnoggin, pCS2.XOtx2, pCS2R.XET, pCS2R.XPax6, pCS2R.XSix3, pCS2+.XRx1, pCS2R.XLhx2 (3), pCS2+mt.X-tll, pCS2.XOptx2, pCS2.nucβgal or pCS2GFP template DNA using the Message Machine kit (Ambion, Austin, TX). X-Gal staining was performed on embryos injected with 200 ng βgal as previously described (Turner and Weintraub, 1994). *GFP* was sometimes used (500 ng per embryo) in place of βgal to label injected embryos, when there was a concern that βgal staining would obstruct in situ staining.

RT-PCR analysis

For animal cap assays, embryos were injected at the two-cell stage with the indicated RNA(s). Ectodermal explants (animal caps) were isolated from stage 8.5 embryos using the Gastromaster (XENOTEK Engineering, Belleville, IL). Total RNA was isolated from embryos or pools of ten stage 21 animal caps by extraction with RNAzol B reagent (Tel-Test, Friendswood, TX, USA). After treatment with RQ-1 DNAse (Promega, Poole, UK) to remove contaminating genomic DNA, first-strand cDNA synthesis was performed by reverse transcription with random hexamers in a volume of 20 μl. Histone H4 PCR was performed using 1 μl of template in a final reaction volume of 12.5 μl to determine the relative amount of cDNA in each sample. Subsequent PCR was performed using normalised amounts of template. Cycling conditions were: 92°C, 2 minutes then 92°C, 45 seconds; 56 or 65°C, 45 seconds; 72°C, 45 seconds, for 24-30 cycles and ended with a single extension step of 72°C for 10 minutes. An annealing temperature of 65°C was used for the Optx2 primer set; all other primer sets were annealed at 56°C. The primers used are shown in Table 1. Radiolabelled PCR products were separated on 7% polyacrylamide gels, expression levels were determined using a Storm 860 Phosphoimager with ImageQuant ver. 4.1 software (Molecular Dynamics, Sunnyvale, CA) and normalised to H4 as a loading control.

For multistage analysis, RNA was isolated from three embryos per stage and a total of four sets of RNAs from staged embryos were tested yielding similar results. For animal cap assays, each experiment was performed between three and five times to ensure reproducibility. Control experiments (not shown) with cloned templates demonstrated that the amplification efficiencies did not vary between primer sets

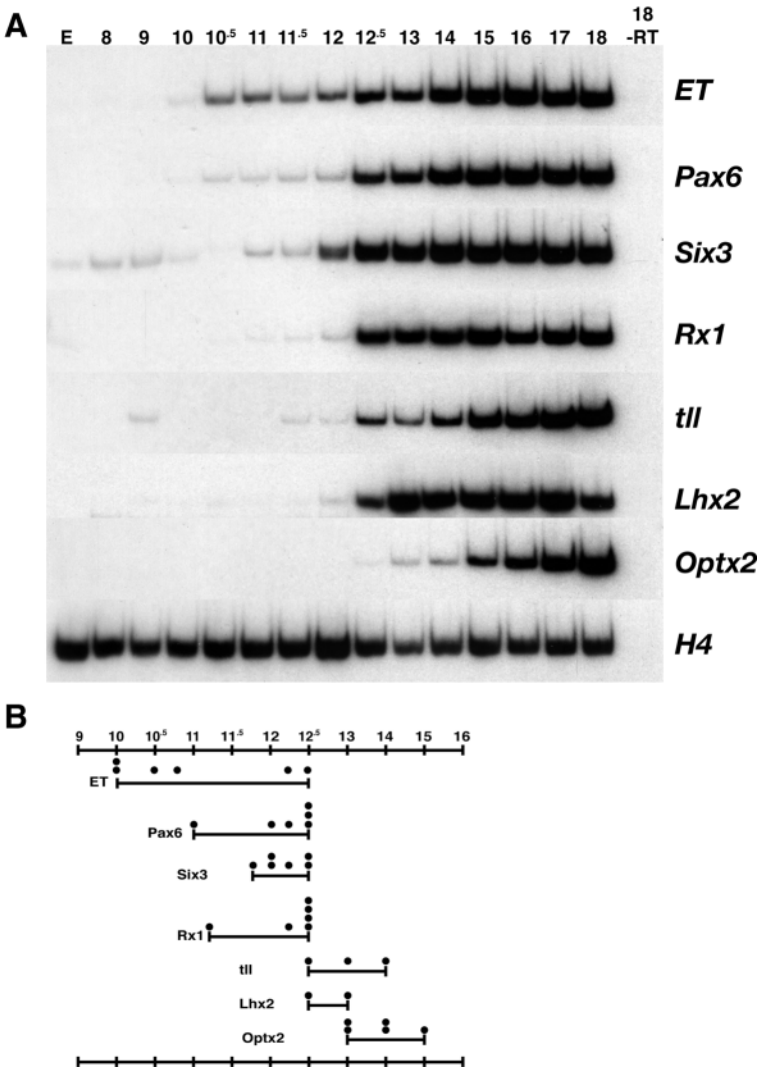


Fig. 1. Relative timing of EFTF expression. RT-PCR was used to detect the expression of *ET*, *Pax6*, *Six3*, *Rx1*, *tll*, *Lhx2* and *Optx2* in the unfertilised embryo (E) and until stage 18 of development. The transient expression of *Six3* and *tll* prior to stage 10.5 was detected in four independent experiments. PCR amplification of Histone H4 demonstrates that approximately equivalent amounts of cDNA templates were used. A duplicate set of reactions from stage 18 embryo RNA were run without reverse transcriptase to test for contaminating plasmid and genomic DNA (18 –RT). The PCR products were subcloned and sequenced to confirm their identities. (B) Schematic showing the results of multiple experiments. Each dot represents the developmental stage at which strong induction was observed.

using these conditions. A no reverse transcription control was included in each reaction to check for the presence of contaminating genomic and plasmid DNA. Subcloning and sequencing confirmed the identities of the amplified products.

cDNA identification and sequence analysis

XSix3 was isolated by screening a stage 42 head cDNA library (a gift from P. A. Krieg, University of Texas, Austin, TX) with an *XSix3* PCR-amplified fragment that was obtained as previously described (Andreazzoli et al., 1999). Plating, hybridisation and washing conditions have been described previously (Franco et al., 1991). The *XSix3* predicted amino acid sequence is identical to that described by Zhou and colleagues (Zhou et al., 2000). A full-length cDNA was cloned into the *Eco*RI/*Xho*I site of pBS(SK-) vector. A complete description of the cloning and sequence of the *Xenopus Lhx2* will be given elsewhere (M.E.Z., unpublished). *Xenopus Lhx2* sequence has been submitted to GenBank under Accession Number AY141037.

In situ hybridisation

Whole-mount single and double in situ hybridisation on *Xenopus* embryos was performed as previously described (Andreazzoli et al., 1999; Harland, 1991). Bleaching of pigmented embryos was carried out following color reaction as described by Mayor et al. (Mayor et al., 1995). To determine the change in eye field diameter, the injected side of embryos was first determined by staining for βgal expression or using a fluorescent dissecting microscope to detect GFP. The diameter of the *Rx1* expression domain in the rostrocaudal dimension on the injected side was then compared with that of the uninjected side.

Results

Vertebrate EFTF expression is coordinated and suggests a genetic hierarchy

To determine the relative timing of vertebrate EFTF expression, we used RT-PCR to establish the developmental stage at which each is first and strongly expressed. Only *Six3* is expressed at detectable levels in the egg (Fig. 1A). Early *Six3* expression is transient and lost by stage 10.5. *ET*, *Pax6*, *Rx1*, *tll*, *Lhx2* and *Optx2* were first detected at stages 10, 10.5, 11, 11.5, 12 and 12.5, respectively. In contrast to the first detectable expression, strong expression of *Pax6*, *Six3*, *Rx1*, *tll* and *Lhx2* is nearly simultaneous and starts between stages 12 and 12.5, while strong induction of *ET* and *Optx2* occurs by stages 10.5 and 14/15, respectively. Some variation in the expression of these genes was observed from experiment to experiment (Fig. 1B). However, the relative timing of expression was consistent in each experiment. These results demonstrate a tightly coordinated, strong expression of five EFTFs within a 30 minute time span. In addition, these results suggest that: (1) *ET* expression does not require the expression of *Pax6*, *Six3*, *Rx1*, *tll*, *Lhx2* or *Optx2*; and (2) *Optx2* is not required for the initial expression of *ET*, *Pax6*, *Six3*, *Rx1*, *tll* or *Lhx2*.

The EFTFs are expressed in overlapping patterns during vertebrate eye field formation

Interactions suggested by the synchronised timing of EFTF expression could only operate if these factors were colocalised. Therefore, we used double whole-mount in situ hybridisation to determine the relative expression patterns of the eye field transcription factors.

We first compared the expression domains of these genes with *Otx2*, which is required for the establishment of presumptive forebrain and midbrain territories (Kablar et al., 1996; Pannese et al., 1995). Because the eye field originates within the forebrain, mice deficient in *Otx2* lack eyes (Acampora et al., 1995; Matsuo et al., 1995). At gastrula stages, *Otx2* is expressed in the entire presumptive anterior neuroectoderm (Fig. 2A), but between the end of gastrulation and the beginning of neurulation (stage 12.5/13) it is

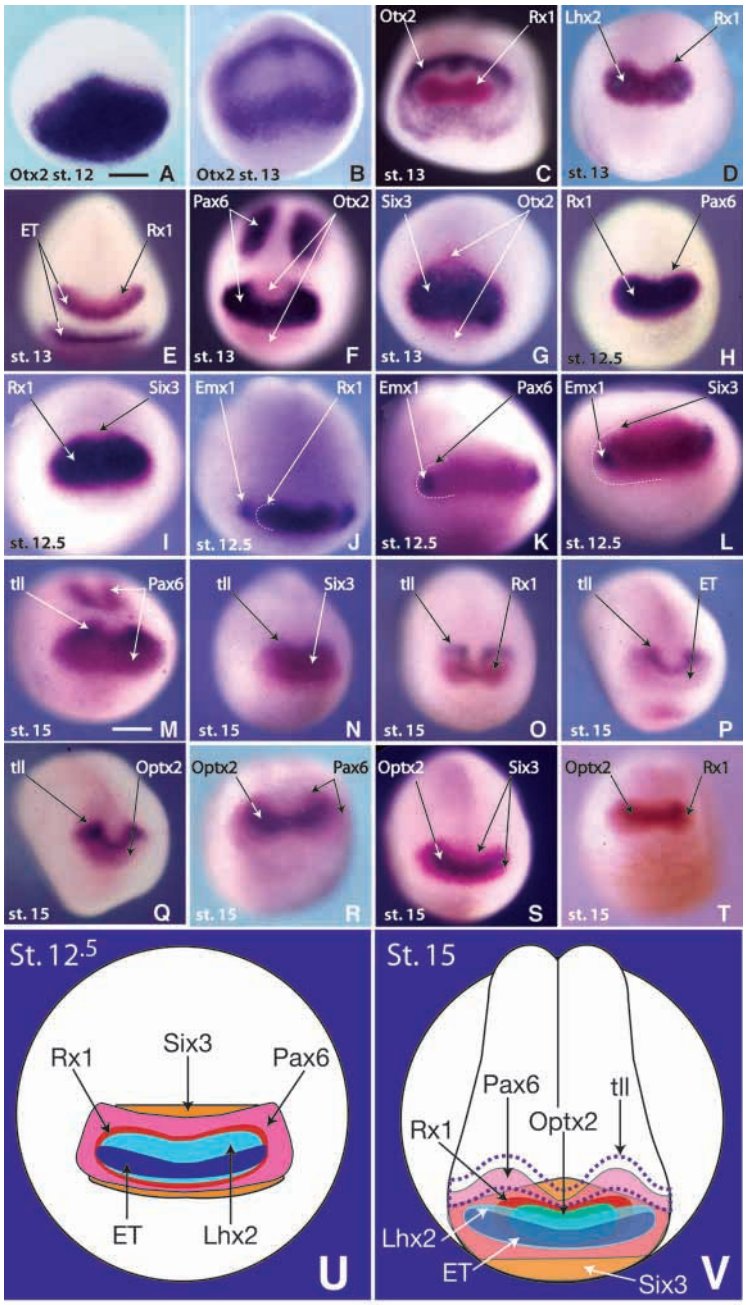


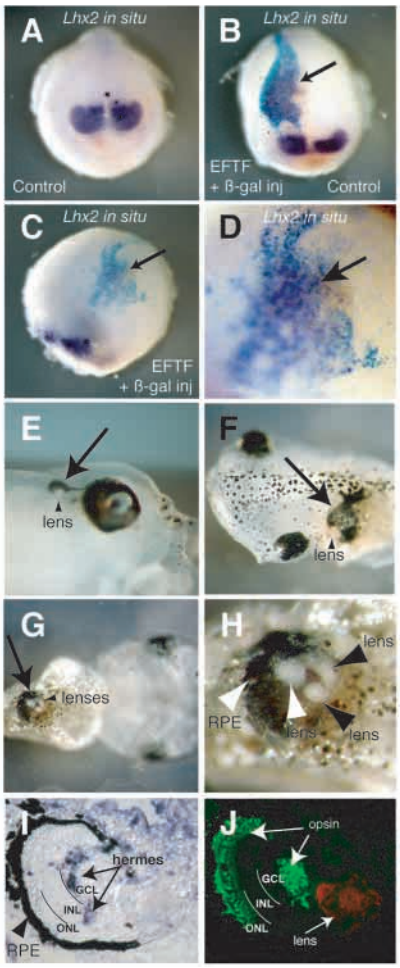
Fig. 2. Comparison of EFTF expression patterns by double whole-mount in situ hybridisation. *Otx2* expression at stage 12 (A) and 13 (B). In C-I and K-T, the dark blue stain is the expression pattern of the gene named on the left, while the magenta stain is the expression pattern of the gene named on the right, at the stages shown. For example, in C, *Otx2* is dark blue and *Rx1* is magenta. (J) Both *Emx1* and *Rx1* stain dark blue. (J-L) The *Rx1* (J), *Pax6* (K) and *Six3* (L) expression borders are indicated by a broken line. A schematic summary of the overlapping expression patterns of the eye field transcription factors at stage 12.5/13 (U) and 15 (V) is shown. Scale bars: in A, 300 μm for A-L; in M, 300 μm for M-T.

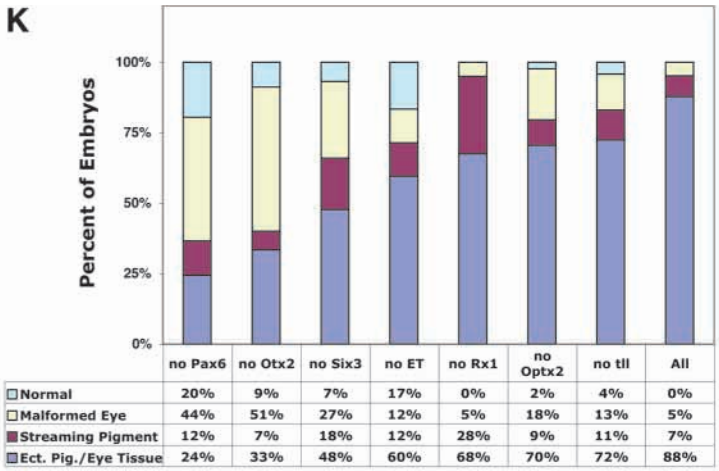
downregulated in the medial region of its expression domain (Fig. 2B). This 'hole' in the *Otx2* expression domain, is the approximate location of the eye field.

ET, *Pax6*, *Six3*, *Rx1* and *Lhx2* are all first detectable in the presumptive eye field before the completion of gastrulation and the beginning of neurulation (stage 12) (not shown). *Rx1* is expressed neatly within the inner limits of the 'hole' in the *Otx2* expression domain (Fig. 2C), and within the *Rx1* domain are the even smaller expression domains of *Lhx2* and *ET* (Fig. 2D,E). Both *Pax6* and *Six3* expression domains are slightly larger that the *Rx1* domain and overlap that of *Otx2* (Fig. 2F-I). To define the anterior and lateral expression boundaries of these genes more clearly, we used the homeodomain-containing transcription factor *Emx1* as a positional marker. *Emx1* is expressed in the rostral neural plate in the telencephalic primordium at early neural stages (Pannese et al., 1998). The expression domains of *Rx1* and *Emx1* do not overlap, although both *Pax6* and *Six3* overlie *Emx1* expression confirming that the lateral expression of *Rx1* (and therefore *Lhx2* and *ET*) lies within both the *Pax6* and *Six3* expression domains (Fig. 2J-L). Although the *Six3* expression domain clearly extends beyond the anterior limit of *Emx1* (Fig. 2L), the most anterior limit of *Pax6* expression is coincident with *Emx1* (Fig. 2K). *ET*, *Pax6*, *Six3*, *Rx1* and *Lhx2* thus have overlapping, but not identical, expression domains in the eye field region. The *ET* expression domain is the most restricted of these genes within the presumptive eye field and the *Six3* domain is the broadest. One can think of concentric rings of expression in domains of decreasing size – *Six3* > *Pax6* > *Rx1* > *Lhx2* > *ET* (Fig. 2U).

By midneurula stages (stage 14/15), *tll* and *Optx2* expression can be detected by WISH. *tll* is first observed in a narrow stripe of cells in the prechordal region of the neural plate. As described by Holleman et al. (Hollemann et al., 1998), the expression domain of *tll* overlaps the posterior and lateral *Pax6* expression domain (Fig. 2M), distinct from the eye field. By contrast, *Six3* expression overlaps *tll* expression medially (Fig. 2N). The expression domains of *Rx1*, *ET* and *Optx2* closely border, but do not significantly overlap the expression domain of *tll* (Fig. 2O-Q). These results suggest that *tll* is unlikely to be required for eye field specification as it is expressed after the eye field forms and only partially overlaps the eye field region. *Optx2* transcripts are detected within the *Pax6*, *Six3*, *Rx1* and *Lhx2* expression domains (Fig. 2R-T and not shown).

Clearly, some of the EFTFs are expressed outside the definitive eye field, consistent with the roles of genes like *Pax6* and *Six3* in the development of other nearby structures, such as the olfactory epithelium and the hypothalamus (Lagutin et al., 2003; Oliver et al., 1995; Van Heyningen and Williamson, 2002). Within the eye field – the expression patterns of the EFTFs are dynamic and follow the morphogenesis of the neural plate, including the lateral migration of the eye field as it begins to separate. This is illustrated by comparing their expression patterns at stage 12.5/13 and stage 15 only 3 hours later (Fig. 2U,V). These results demonstrate that the anterior neural plate is subdivided into molecularly distinct domains that express specific subsets of the EFTFs.





	no Pax6	no Otx2	no Six3	no ET	no Rx1	no Optx2	no tll	All
Normal	20%	9%	7%	17%	0%	2%	4%	0%
Malformed Eye	44%	51%	27%	12%	5%	18%	13%	5%
Streaming Pigment	12%	7%	18%	12%	28%	9%	11%	7%
Ect. Pig./Eye Tissue	24%	33%	48%	60%	68%	70%	72%	88%

Fig. 3. Coordinated expression of EFTFs induces ectopic *Lhx2* expression and ectopic eye-like structures outside the nervous system. (A-D) In situ hybridisation for *Lhx2* expression (violet) in stage 20 embryos. (A) Uninjected embryo shows the normal expression pattern of *Lhx2*. (B-D) *Otx2*, *ET*, *Pax6*, *Six3*, *Rx1*, *tll*, *Optx2* and β-gal RNAs were injected into one cell of two-cell stage embryos. β-gal staining (light blue) shows the injected side. Arrow indicates to ectopic *Lhx2* expression (violet). (E-H) Embryos injected with *Otx2*, *ET*, *Pax6*, *Six3*, *Rx1*, *tll* and *Optx2* RNAs, and grown to stage 45. Arrows indicate ectopic eyes and arrowheads point to lens. (I,J) Sections through ectopic eyes reveal the layering of ganglion (GCL), inner nuclear (INL) and outer nuclear (ONL) cell layers. (I) The retinal ganglion cells are detected using the marker, *hermes* (violet). Rod photoreceptors are identified in the outer nuclear layer, by the detection of opsin (green, J). Opsin also stains a rosette of cells between the GCL and the lens. Lens was detected using anti-crystalline antibodies and stains red in J. (K) Cocktail subsets reveal the relative importance of EFTFs for eye tissue induction. Animals were scored according to severity of phenotype – from ectopic pigment/eye tissue (most severe) to normal animals. When all the factors were present, most embryos developed ectopic pigment or eye tissue (Ect. Pig./Eye Tissue). When *Pax6* was left out of the cocktail, for example, the frequency of ectopic pigment or eye tissue was greatly reduced and 20% of the embryos were unaffected (Normal).

The coordinated overexpression of EFTFs is sufficient to generate secondary eye fields and ectopic eyes outside the nervous system

To determine if the coordinated expression of EFTF genes is sufficient to generate eye fields and eyes in vertebrates, we expressed a cocktail of seven of the EFTFs in developing *Xenopus* embryos. We injected *Otx2*, *ET*, *Pax6*, *Six3*, *Rx1*, *tll* and *Optx2* RNAs simultaneously into one blastomere at the two-cell stage with βgal to identify the injected side of the embryo. *Lhx2* was intentionally left out of the cocktail, as we needed an early marker to identify the presence of ectopic eye field. Preliminary experiments demonstrated that the absence of *Lhx2* from the cocktail had little effect on the observed phenotypes. Coordinated expression of the EFTF cocktail induced ectopic expression of *Lhx2* in 100% of injected embryos. Ectopic *Lhx2* was detected both within and outside the nervous system (Fig. 3B-D), whereas its normal expression domain is limited to the anterior neural plate (Fig. 3A). When the injected embryos were grown to stage 45, we found ~90% of these embryos expressed ectopic retinal pigment epithelium (RPE) on the injected side. Sections taken through this ectopic tissue and immunostained for opsin, revealed that photoreceptors were often associated with the ectopic pigment. Approximately 20% of injected embryos clearly developed quite large ectopic eyes, the most striking aspect of which was their location. Ectopic eyes were detected near the CNS, but were also often found at locations far from the CNS, e.g. in the belly region and even at the anus (Fig. 3E-H). These tissues expressed markers for differentiated retinal ganglion, rod and cone photoreceptor cells, RPE and lens (Fig. 3I-J and not shown), indicating that they were indeed eyes as defined by the cell types detected as well as their morphology.

Cocktail subsets reveal crucial circuit components

The high efficiency with which cocktails of EFTFs generate ectopic eye tissue enabled us to determine those most crucial for eye formation. To do this, we systematically injected cocktail subsets lacking one of the EFTFs and determined their efficiency at inducing ectopic eye tissues. The most dramatic reductions in ectopic eye tissue were observed when *Pax6* was

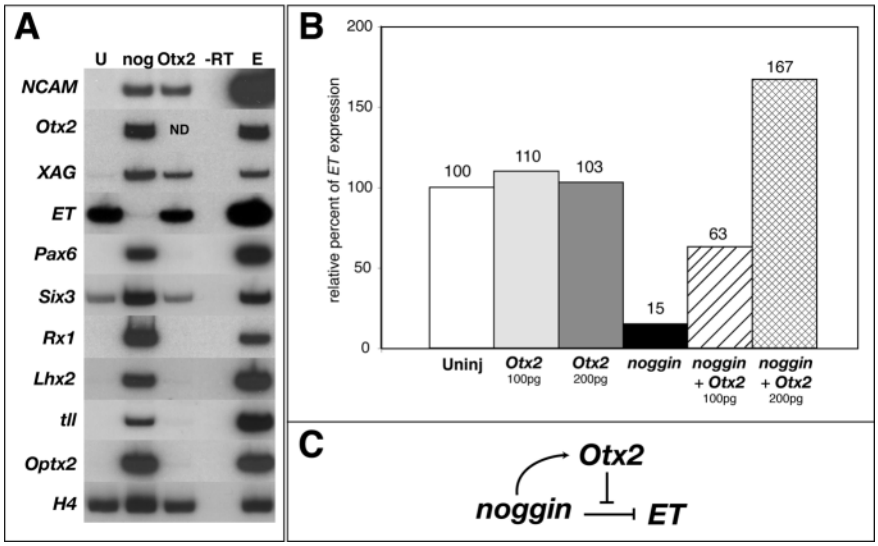
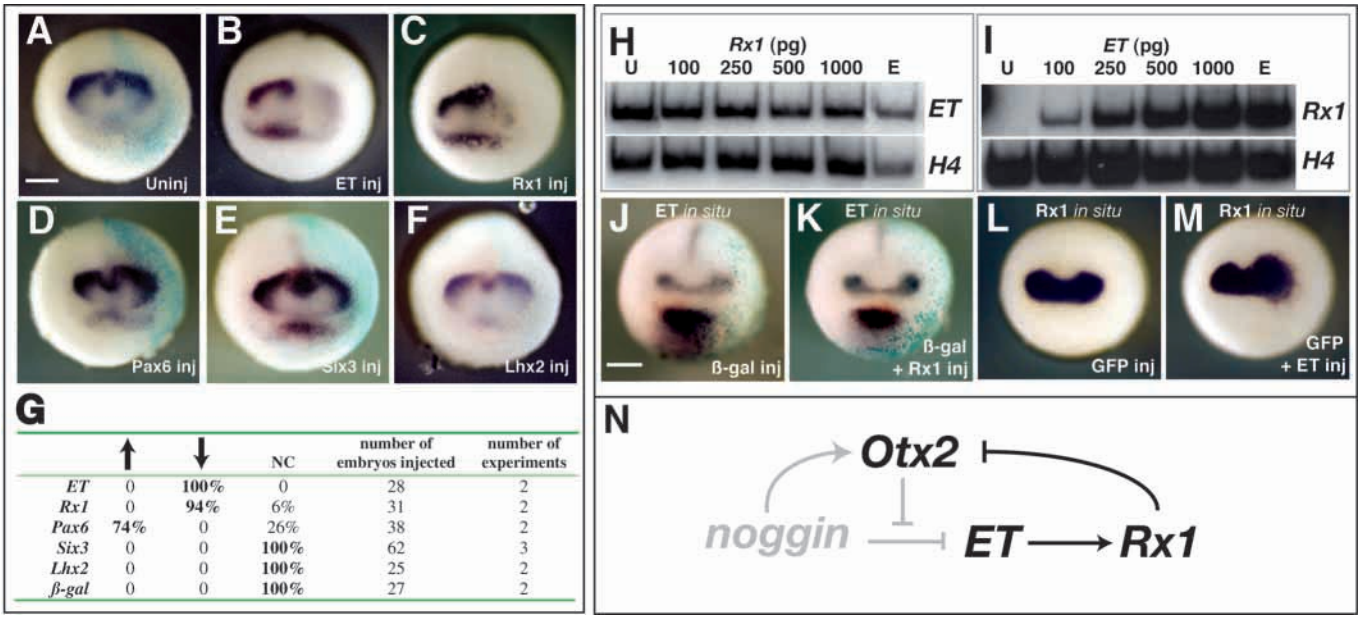


Fig. 4. *Noggin* but not *Otx2* regulates eye field transcription factor expression while *Otx2* blocks the repression of *ET* by *noggin*. (A) RT-PCR was used to detect changes in the expression of the EFTFs in response to *noggin* (10 pg) and *Otx2* (200 pg). The effect of *Otx2* on its own expression was not determined (ND). The presence of *ET* and *Six3* in uninjected animal caps (U) was not a result of DNA contamination as neither transcript was detected when duplicate samples were amplified in the absence of reverse transcriptase (–RT). Uninjected sibling embryos 'E' were used as a positive control for PCR. Histone H4 was used as a loading control. (B) RT-PCR was used to determine the relative expression of *ET* in ectodermal explants from embryos injected with *Otx2*, *noggin* or both. The percent of *ET* expression relative to uninjected controls is shown above each bar of the graph. (C) Interpretation of the combined results from A and B.

removed (Fig. 3K), followed by *Otx2*, *Six3* and *ET*. Removal of *Rx1*, *Optx2* or *tll* from the EFTF cocktails affected ectopic eye tissue induction to a lesser extent (Fig. 3K). The strong effect of removing *Pax6*, *Otx2* and *Six3* from the cocktails may have been predicted, as numerous studies have demonstrated these genes are required for eye formation. However, a crucial role for *ET* in early eye formation has not been reported. Conversely, the relatively small effects of removing *Rx1*, *Optx2*, *tll* and *Lhx2* from the cocktails is intriguing because each of these genes has been shown to be required for normal eye formation. Remembering that this is non-mutant tissue, a possible explanation is that *Rx1*, *Optx2*, *tll* and *Lhx2* can be induced to sufficient levels by the remaining EFTFs – compensating for their removal. The strong ectopic expression of *Lhx2* seen in embryos injected with EFTF cocktails (Fig. 3B-D) certainly supports this hypothesis. The genetic hierarchy



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	↑	↓	NC	number of embryos injected	number of experiments
ET	0	**100%**	0	28	2
Rx1	0	**94%**	6%	31	2
Pax6	**74%**	0	26%	38	2
Six3	0	0	**100%**	62	3
Lhx2	0	0	**100%**	25	2
ß-gal	0	0	**100%**	27	2

Fig. 5. *ET*, *Rx1* and *Pax6* regulate *Otx2* expression. Embryos were injected into one blastomere at the two-cell stage with RNA of the indicated gene. Whole-mount in situ hybridisation was used to detect *Otx2* expression in embryos injected with 100 pg *ET* (B), 400 pg *Rx1* (C), 200 pg *Pax6* (D), 200 pg *Six3* (E) or 500 pg *Lhx2* (F) RNA. Embryos in A,D-F were co-injected with βgal RNA to identify the injected side. In B and C, the embryos were not stained for βgal expression so that the repression of *Otx2* could be more easily visualised. Scale bar: 300 μm. (G) Quantitation of the effect of EFTFs on *Otx2* expression. Percent of embryos with an increase (↑), decrease (↓) or no change (NC) in *Otx2* expression. *ET* induces *Rx1* expression. (H,I) *Rx1* injection did not effect *ET* expression, while *ET* induced *Rx1* expression in *Xenopus* animal caps in a dose-dependent manner. Histone H4 was used as a loading control; U, uninjected; E, parallel, uninjected embryo. (J-M) Whole-mount in situ hybridisation was used to detect *ET* (J-K) and *Rx1* (L-M) expression in stage 13 *Xenopus* embryos injected with 200 pg *Rx1* (K) or *ET* (M) RNA. In (J,K), embryos were injected with βgal RNA. In L,M, GFP RNA was used to detect the injected side of the embryo. The right side is the injected side in J-M. Scale bar: 300 μm. (N) Interpretation of the results of Figs 4, 5.

suggested by the timing of EFTF expression (Fig. 1) is also consistent with this idea as the four EFTFs that are deemed most crucial by the cocktail subset method are expressed earlier than, and may therefore induce the expression of, *Rx1*, *Optx2*, *tll* or *Lhx2*.

EFTFs are induced by the combined action of *noggin* and *Otx2*

The above results suggest that eye field formation might result from a series of progressive inductions. Extending this hypothesis prior to eye field specification, the ectoderm is converted into the neural plate in response to neural inducers. Next, presumptive forebrain is specified by the regulated expression of *Otx2*. Finally, the eye field forms within the presumptive forebrain. If this model were correct, one would expect that both *noggin* and *Otx2* are upstream of the EFTF genes, and may activate them either directly or indirectly. We therefore used the animal cap assay to test the effect of *noggin* and *Otx2* on the expression of the EFTFs.

In untreated animal caps, only *ET* and *Six3* were detected (U, Fig. 4A), consistent with their early expression in the embryo (Fig. 1). The neural inducer, *noggin*, dramatically increased the expression of many of the eye field transcription factors including *Pax6*, *Six3*, *Rx1*, *Lhx2*, *tll* and *Optx2*. Interestingly, *noggin* strongly repressed *ET* expression. Similar results were found with another neural inducer, *chordin* (data not shown). Both *noggin* and *Otx2* induced the neural marker *NCAM* and the cement gland marker *XAG*. Unlike *noggin*, however, *Otx2* did not alter the expression of *ET*, *Pax6*, *Six3*, *Rx1*, *Lhx2*, *tll* or *Optx2*. The inability of *Otx2* to induce any EFTFs suggests that their regulation is *Otx2* independent.

The strong repression of *ET* by *noggin* and *chordin*, however, raised the question of how the initial expression of *ET* is turned on in the eye field. We therefore considered the possibility that *Otx2* inhibits the repression of *ET* by *noggin*. To test this idea, *noggin* mRNA was injected with and without *Otx2* mRNA, and the effect on *ET* expression was determined in animal caps. *Otx2* alone had no effect on *ET*, while *noggin* repressed *ET* expression to 15% of control levels (Fig. 4B). However, co-expression of *Otx2* with *noggin* not only rescued *ET* expression, but increased *ET* levels beyond that of controls in a dose-dependent manner. These results indicate a potentially crucial role for *Otx2* in eye field formation. Neural induction alone results in a neural plate resistant to *ET* expression; however, subsequent expression of *Otx2* in the anterior neural plate permits *ET* expression and perhaps subsequent eye field formation (Fig. 4C).

ET, *Rx1* and *Pax6* regulate *Otx2* expression in the anterior neural plate and presumptive eye field

The loss of *Otx2* expression between stages 12 and 13 is synchronised with the induction of several EFTFs in the eye field (Fig. 2), suggesting that they may repress *Otx2* expression in the anterior neural plate during normal embryonic development. It was, in fact, previously shown that overexpression of *Rx1* represses *Otx2* expression in the neural plate (Andreazzoli et al., 1999). To test if other EFTFs are also capable of regulating *Otx2* expression, we injected the EFTFs into one cell of two-cell stage embryos and determined their effect on *Otx2* expression using whole-mount in situ hybridisation.

Otx2 expression normally extends both rostral to the neural plate, in a region corresponding to the cement gland anlagen, and posterior to the eye field, in the region fated to be the primordium of the mesencephalon (Eagleson et al., 1995), Fig. 2B, Fig. 5A). We found that both *ET* and *Rx1* repressed *Otx2* expression throughout the entire anterior neural plate (Fig. 5B,C). *Otx2* expression was repressed in 100% of embryos injected with *ET* RNA and 94% of embryos injected with *Rx1* RNA (Fig. 5G). In 74% of embryos injected with *Pax6*, there was an expansion of the *Otx2* expression domain (Fig. 5D,G). Interestingly, *Otx2* expression was expanded laterally and caudally but not into the eye field by *Pax6* (Fig. 5D). Neither *Six3* nor *Lhx2* altered *Otx2* expression (Fig. 5E,F). These results demonstrate that both *ET* and *Rx1* are able to repress the expression of *Otx2* in the eye field region.

As *ET* is expressed before *Rx1*, it is possible that the repression of *Otx2* by *ET* is indirect – mediated through *Rx1*. To test this possibility, we first used the animal cap assay to determine the effect of *ET* and *Rx1* on each other's expression. *Rx1* neither induced nor repressed *ET* expression in animal caps at concentrations as high as 1000 pg (Fig. 5H). However, *ET* strongly induced the expression of *Rx1* in a dose-dependent manner (Fig. 5I).

To test this pathway in vivo, we injected *ET* or *Rx1* RNA and assayed for changes in *Rx1* or *ET* expression in stage 13 embryos. To target the eye field, we injected *ET* or *Rx1* RNA into dorsal blastomeres at the four-cell stage. Consistent with the animal cap assays, *Rx1* had no effect on *ET* expression at stage 13 (compare Fig. 5J-K). As predicted, *ET* strongly induced the expression of *Rx1* in 93% of injected embryos (n=29). Interestingly, *Rx1* induction was only observed in the anterior neural plate in the presumptive eye field region (Fig. 5M). When *ET* was injected ventrally, *Rx1* induction was not observed (not shown). When *ET* was injected at the two-cell stage (resulting in the expression of *ET* throughout an entire half of the embryo), *Rx1* induction was again only detected in the anterior neural plate, including the presumptive eye field region (not shown).

The downregulation of *Otx2* in the eye field can thus be explained by the fact that *ET* induces the expression of *Rx1*, which then represses *Otx2* (Fig. 5N). However, these results, do not rule out the possibility that *ET* also represses *Otx2* independently of *Rx1*. In fact, there is some indication that this pathway may also be operative as induction of *Rx1* by *ET* was detected in most but not all embryos while *ET* repressed *Otx2* expression in all embryos tested. In addition, we found that in the presumptive cement gland region, *ET* represses *Otx2* (Fig. 5B) without inducing *Rx1* (Fig. 5M), implicating an *Rx1*-independent mechanism in this tissue.

Both *Otx2* and *noggin* potentiate functional interactions between the EFTFs.

The inducing effect of *ET* on *Rx1* is limited to the anterior forebrain, suggesting that *Otx2*, although not an inducer of EFTFs itself, may provide an environment that primes the anterior neuroectoderm for eye field formation (Fig. 4). If so, co-injection of *Otx2* might potentiate the effects of *ET* on the activation of downstream EFTFs. *ET* is an obvious candidate for co-injection experiments as it is expressed earlier than all other EFTFs and has the most restricted expression domain.

As predicted by the above model, *Otx2* strongly potentiates the induction of *Rx1* by *ET* in the animal cap assay (Fig. 6A).

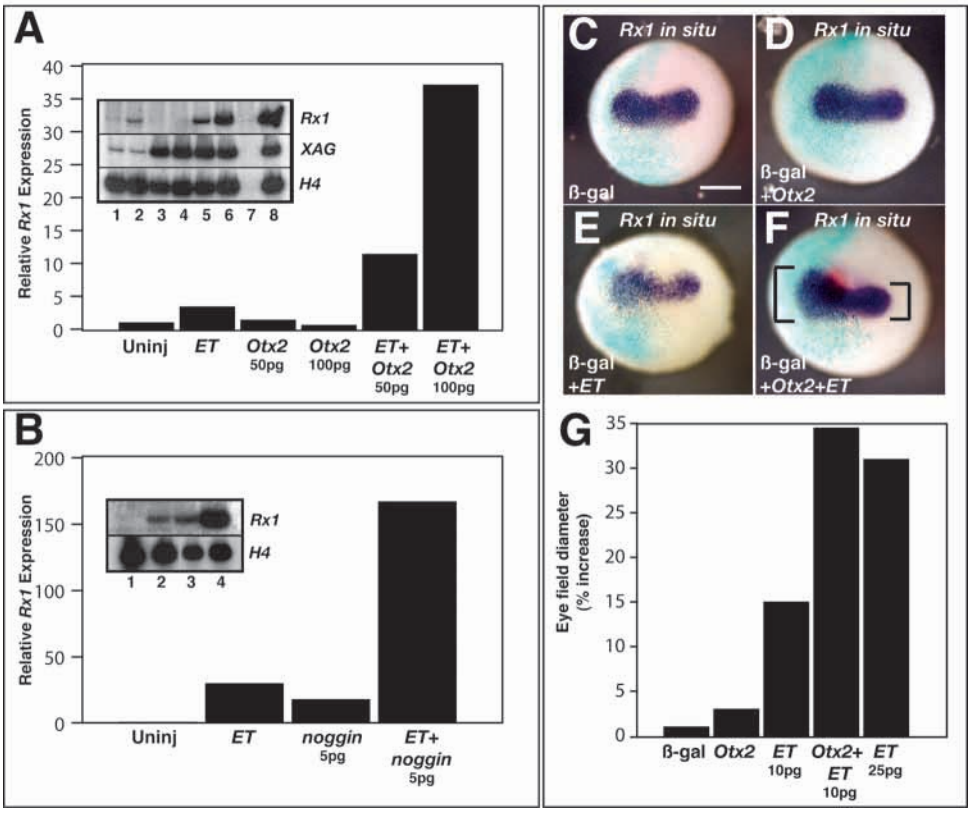

Fig. 6. *Otx2* and *noggin* potentiate the induction of *Rx1* by *ET*. (A,B) RT-PCR was used to detect changes in *Rx1* and *XAG* expression in ectodermal explants from *Xenopus* embryos injected with *noggin*, *Otx2* and *ET*. *ET* (100 pg) was injected alone, with 50 or 100 pg of *Otx2* (A), or 5 pg *noggin* (B). (A) Lane 1, uninjected; lane 2, *ET* (100 pg); lane 3, *Otx2* (50 pg); lane 4, *Otx2* (100 pg); lane 5, *ET* (100 pg) + *Otx2* (50 pg); lane 6, *ET* (100 pg) + *Otx2* (100 pg); lane 7, embryo, no reverse transcription; lane 8, embryo, *XAG* induction was used as a positive control for *Otx2* activity. (B) Lane 1, uninjected; lane 2, *ET* (100 pg); lane 3, *noggin* (5 pg); lane 4, *ET* (100 pg) + *noggin* (5 pg). (C-G) *Rx1* expression was normalised to Histone H4 then set relative to uninjected controls. *Otx2* potentiates the *ET* induced expansion of *Rx1* expression in the anterior neural plate. Whole-mount in situ hybridisation was used to detect *Rx1* expression at stage 13 in embryos injected with βgal alone (C), or in combination with 25 pg *Otx2* (D), 10 pg *ET* (E) or both *Otx2* and *ET* (F). (G) The rostrocaudal diameter of the *Rx1* expression domain on the injected side (βgal-positive) was measured and compared with the uninjected (βgal-negative) side of the embryo (see F for an example).

This is in spite of the fact that *Otx2* alone does not induce *Rx1* expression in vitro. The effect is dose dependent as co-injection of 50 and 100 pg of *Otx2* with *ET* induced a three- and eleven-fold increase in *Rx1* expression. *Noggin* also potentiates the *Rx1* induction by *ET*. Weak *Rx1* expression was detected in animal caps from embryos injected with suboptimal amounts of *ET* or *noggin*. However, co-injection of *noggin* and *ET* RNAs at these same concentrations induced a greater than five-fold increase in *Rx1* expression (Fig. 6B).

We next examined the effect of *Otx2*:*ET* co-injection on *Rx1* expression in vivo. *Otx2* (25 pg) only slightly increased *Rx1* expression in stage 13 embryos (Fig. 6D,G), while *ET* (10 pg) expanded the average domain of *Rx1* expression by 15% (Fig. 6E,G). However, co-injection of *Otx2* with *ET* increased the average eye field diameter by nearly 35% (Fig. 6F,G), approximately equivalent to the effect of 25 pg of *ET* alone (Fig. 6G). These results are consistent with the model of progressive tissue specification described above.

The circuitry of the EFTF network revealed by systematic overexpresssion studies in animal caps

The relative timing, spatial expression patterns, cocktail subset experiments and directed overexpression studies suggest that early genes such as *ET* may be required for the expression of later expressed genes in the eye field and rule out the possibility that later genes such as *Optx2* are required for the initial induction of earlier EFTFs. Nevertheless, dominant-negative *Optx2* and *tll* constructs and *Optx2* knockouts retard eye field growth and thus lead to reduced levels of other EFTFs, including *ET* and *Pax6* (Hollemann et al., 1998; Li et al., 2002; Zhu et al., 2002; Zuber et al., 1999). Because of the possibility of such indirect feedback effects on EFTF expression, it is difficult to unravel the EFTF network using a loss-of-function approach alone, especially when the initial expression of these genes in the eye field is so closely synchronised. To overcome this problem, we used a systematic approach, injecting embryos with one EFTF at a time, and screening the injected caps using RT-PCR to detect changes in the expression of the remaining EFTFs.

A representative experiment and a summary of our results are shown in Fig. 7A,B. This data was then assembled into a circuit using Occam's razor (Fig. 7C) that shows the most parsimonious set of necessary interactions needed to explain the results. These results confirm many of the predictions made

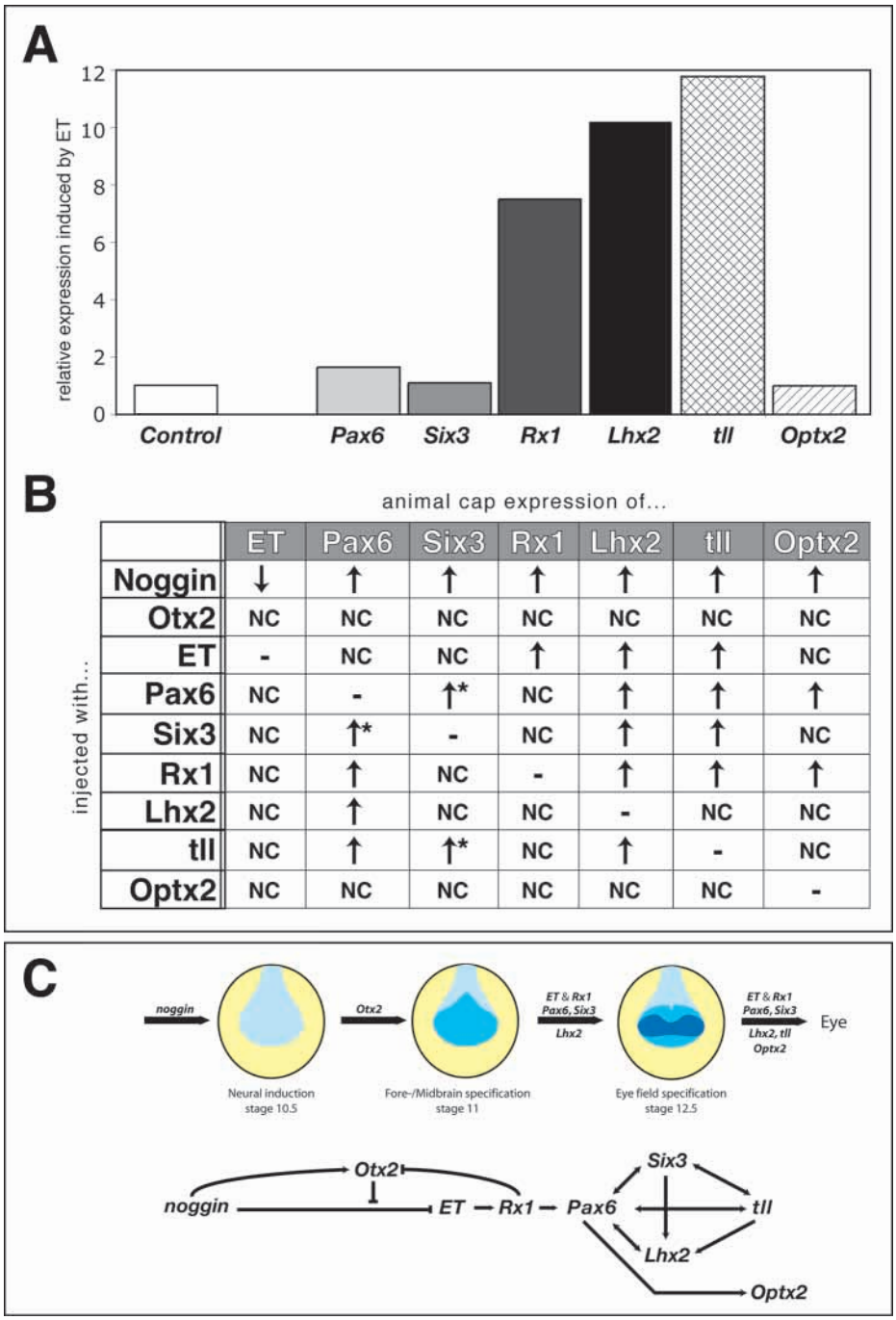

Fig. 7. Epigenetic interactions among the eye field transcription factors define a genetic network during eye field formation. (A) *ET* induces the expression of a subset of EFTFs. RT-PCR was used to detect changes in EFTF expression in ectodermal explants isolated from embryos injected with 200 pg of *ET*. The fold induction represents the relative expression of the EFTFs when compared with uninjected controls. (B) Epigenetic interactions between *noggin*, *Otx2* and the EFTFs. ↑, induction of target gene; ↓, repression of target gene; NC, no change in target gene expression; *some variability in these inductions was observed. (C) Summary model of eye field induction in the anterior neural plate. Light blue indicates the neural plate, blue shows the area of *Otx2* expression and dark blue represents the eye field.

from the expression studies (Figs 1, 2) and incomplete cocktail experiments (Fig. 3). *ET*, positioned at the front of the circuit induces the expression of *Rx1*, *Lhx2* and *tll*, which in turn induce the expression of *Pax6*:*Lhx2*:*tll*:*Optx2*, *Pax6* and *Pax6*:*Six3*:*Lhx2*, respectively. However, *ET* is unique in that none of the EFTFs studied here can induce its expression in the animal cap assay. Conversely, *Optx2*, the last of these genes to be expressed during eye formation, is induced by both *Pax6* and *Rx1*, yet is unable to induce any of the earlier expressed EFTFs. The four EFTFs expressed earliest and deemed most crucial by the incomplete cocktail method are not only situated towards the front end of the circuit, but are also factors like *Pax6*, *Six3* and *Otx2* that previous studies have demonstrated are central to eye formation. *Pax6* and *Six3* induce each other's expression as well as that of *Lhx2* and *tll*. *Pax6* also induces *Optx2*. *Lhx2* and *tll* were induced by five and four of the six EFTFs, respectively, confirming the hypothesis that a sufficient amount of these genes could be induced by the remaining EFTFs to compensate for their removal from the eye inducing cocktails. In summary, *ET* at the front of the circuit induces *Rx1*, which activates a crossregulatory network, including *Pax6*, *Six3*, *Lhx2* and *tll*, followed by *Optx2* induced by *Pax6*.

Additional interactions may also exist and are not ruled out by the present data set. The working model illustrated in Fig.

7C forms a framework that further experiments may build on to define more precisely the genetic interactions required for vertebrate eye formation.

Discussion

Eye specification in flies and vertebrates

Kumar and Moses proposed a two-stage mechanism for *Drosophila* eye specification. First the eye-antennal imaginal disk complex is subdivided into two distinct presumptive organ fields. Next, specific organ identities (eye versus antenna) are defined. In the embryo prior to eye specification, *toy*, *ey*, *so*, *eya*, *dac* and *eyg* have only partially overlapping expression patterns. However, at the second larval stage their expression patterns are coordinated and the eye becomes specified (Kumar and Moses, 2001a). In the vertebrate, we have shown that inductive and patterning events prepare the anterior neural plate for formation of the vertebrate eye field. However, it is the coordinated expression of the EFTFs that is required for the specification of the eye field. We find this to be a remarkable example of mechanism conservation, given the large evolutionary distance between these species and the differences in the development and morphology of fly and vertebrate eyes.

In the fly, *toy*, *ey*, *so*, *eya*, *dac* and *eyg* are co-expressed in the second larval stage and the elimination of any of them reduces the probability of eye formation (Kumar and Moses, 2001a). In *Xenopus*, *ET*, *Rx1*, *Pax6* and *Six3* are co-expressed in the anterior neural plate and the elimination of any of them from a cocktail of EFTFs injected into the *Xenopus* embryo reduces the frequency of ectopic eye tissue formation.

These remarkable similarities in general developmental design are perhaps logically predicated based on the functional and structural homologies between the *Drosophila* eye genes and the vertebrate EFTFs (Hanson, 2001; Wawersik and Maas, 2000). *orthodenticle* (*otd*) the *Drosophila* homolog of Otx genes is required for development of the eye, antenna and anterior brain, and is normally expressed in a wide domain that spans the dorsal midline and encompasses the entire dorsal head ectoderm (Finkelstein and Boncinelli, 1994). Its expression is turned off in the head midline during development and in the part of the visual primordium that forms the posterior optic lobe and the larval eye (Royet and Finkelstein, 1996). This is strikingly similar to the changes we see in the *Xenopus Otx2* expression pattern. The *optomotor-blind* (*omb*) gene is a member of the *Tbx2* T-box subfamily. *ET* shares more sequence homology with *omb* than any other gene in the fly genome (not shown). *omb* expression is first detected in the optic lob anlagen, later expanding to a larger part of the developing larval brain (Poeck et al., 1993). In the eye imaginal disc, *omb* is detected in glial precursors, posterior to the morphogenetic furrow and in the optic stalk. Null *omb* mutants die in pupal stage and show severe optic lobe defects (Pflugfelder et al., 1992). The *Drosophila Rx* homolog is not expressed in the larval eye imaginal discs nor the embryonic eye primordia (Eggert et al., 1998; Mathers et al., 1997). However, it is expressed prior to *ey* in the procephalic region from which the eye primordia originates, suggesting a role for *Drosophila Rx* prior to *ey* during eye formation in the fly (Eggert et al., 1998; Mathers et al., 1997). It has therefore been suggested that *Drosophila Rx* may only be required for early brain development (Eggert et al., 1998). Finally, our results showing *Pax6* as the most critical component of the *Xenopus* EFTF cocktail with respect to the induction of ectopic eyes meshes well with the general prominence given to *Pax6* and its *Drosophila* homologues *ey* and *toy* as transcription factors centrally involved in early eye development (Wawersik and Maas, 2000).

The functional interactions among the genes required for *Drosophila* eye formation have been extensively investigated (Heberlein and Treisman, 2000; Kumar and Moses, 2001b). Using the ectodermal explant assay, we identified functional epistatic interactions among the vertebrate EFTFs. There are some striking similarities with the functional interactions among the fly EFTFs. For example, we see induction of *Six3* and *Optx2* by *Pax6* and induction of *Pax6* by *Six3* in ectodermal explants (Fig. 7B). In *Drosophila*, *ey* can induce ectopic *so* and *optix* expression and ectopic eye formation induced by co-expression of *so* with *eya* results in the activation of the *ey* gene (Halder et al., 1998; Niimi et al., 1999; Pignoni et al., 1997; Seimiya and Gehring, 2000).

Some differences between fly and vertebrate eye formation are also evident. We found that *tll* was able to induce the expression of *Pax6*, *Six3* and *Lhx2*, and that *Pax6* and *Six3* induce *tll* expression. *Drosophila tll* does not require *ey* or *so* in the embryonic visual system (Daniel et al., 1999; Rudolph et al., 1997). We found *Lhx2* to be induced by all the EFTFs investigated in this report with the exception of *Optx2* (Fig. 7A,B). The gene *apterous* (*ap*) is the most homologous *Drosophila* gene to *Lhx2*; however, *apterous* loss-of-function mutants have no reported defect in eye formation (Bourgouin et al., 1992; Cohen et al., 1992; Lundgren et al., 1995).

Vertebrate EFTFs and their functions

It is interesting to examine the results of this paper in light of studies, particularly knockout studies, on specific EFTFs in other vertebrates. *Otx2*$^{-/-}$ mice lack forebrain and midbrain (Acampora et al., 1995; Matsuo et al., 1995). In *Xenopus*, anterior structures are also lost when *Otx2* fused to the engrailed transcriptional repressor is expressed in embryos (Isaacs et al., 1999). An early requirement for *Otx2* in vertebrate eye formation is implied from studies in which *Pax6*, *Six3*, *Optx2* or *Rx* overexpression result in the formation of ectopic eye tissues, because as Chuang and Raymond observed, ectopic eye tissue is only generated in the head region defined by *Otx2* expression (Chuang and Raymond, 2002). Using EFTF cocktails, we were able to generate ectopic eyes outside of the nervous system (Fig. 3). *Otx2* clearly potentiates the functional interaction among the EFTFs and is a crucial component of the mix (Figs 3, 6). However, a more detailed analysis will be required to determine if ectopic eye formation outside the nervous system is a result of including *Otx2* in the cocktail.

Our results suggest a role for *ET* as an initiator of eye field specification. Originally identified as a T-box family member expressed very early in the eye field, (Li et al., 1997), subsequent overexpression studies showed that *ET* is involved in the dorsoventral patterning of the eye (Wong et al., 2002). The more than 50 T-box family members identified have been classed into five subfamilies (Papaioannou and Silver, 1998; Wilson and Conlon, 2002). *ET* is a member of the *Tbx2* subfamily that includes the *Tbx2*, *Tbx3*, *Tbx4* and *Tbx5* genes,

and is most similar to *Tbx3*. The mouse and chicken orthologues of *Tbx2*, *Tbx3* and *Tbx5* are all expressed in overlapping domains within the dorsal neural retina of the embryonic optic cup (Chapman et al., 1996; Gibson-Brown et al., 1998; Sowden et al., 2001) – very similar to the expression pattern of *Xenopus ET* (Li et al., 1997; Takabatake et al., 2000). Evidence of a role for *Tbx3* in mammalian eye formation is limited. Mouse *Tbx3* has been detected in preimplantation embryos as early as 3.5 days post coitum (Bollag et al., 1994; Chapman et al., 1996) and in the retinal primordia (Takabatake et al., 2000), but no *Tbx3* null mutants have been reported. Hypomorphic mutations in human *Tbx3* cause Ulnar-mammary syndrome (UMS), an autosomal dominant disorder affecting limb, apocrine-gland, tooth, hair and genital development with no apparent effect on the eye (Bamshad et al., 1997). However, the mouse *Tbx3* is clearly expressed in some embryonic tissues that are unaffected in the human syndrome (Bamshad et al., 1997; Chapman et al., 1996). It may be that the human mutations responsible for UMS do not affect its role in these other tissues, or that other T-box family members compensate for a defective form of *Tbx3*. Interestingly, the putative orthologue of zebrafish *Tbx2*, *tbxc*, is expressed in the single eye field at the end of gastrulation (~10 hours post fertilisation, hpf), while the putative zebrafish *Tbx3* orthologue is not expressed prior to 24 hpf and is not reported to be expressed in the retina (Dheen et al., 1999; Ruvinsky et al., 2000; Yonei-Tamura et al., 1999). Thus, it may be that the zebrafish *tbx2*, not zebrafish *tbx3* is the functional homologue of *Xenopus ET*.

Overexpresssion studies are very useful in characterising genetic networks, but clearly do not rule out the existence of parallel pathways and additional intermediates. For example, the fact that *noggin* can induce *Rx1* while repressing *ET* means that a parallel pathway for *Rx1* induction must exist and that *ET* expression is not essential for *Rx1* induction. Whether *ET* is required in vivo for *Rx1* induction is not known. However, the question of requirement and the normal pathway of activation are different issues. The observation that *ET* is expressed prior to *Rx1* and induces *Rx1* expression, and that this activity is enhanced in neuralised tissue suggests very strongly that this pathway is active in the embryo.

The role of *Rx* in vertebrate eye formation has been investigated in more detail than *ET*. *Rx* homologues have been identified in humans, rodents (mouse and rat), chicken, fish (zebrafish, medakafish and cavefish), as well as frog (Casarosa et al., 1997; Loosli et al., 2001; Mathers et al., 1997; Ohuchi et al., 1999; Strickler et al., 2002; Tucker et al., 2001). Mice lacking functional *Rx* homologues do not develop eyes (Mathers et al., 1997; Tucker et al., 2001). In $Rx^{-/-}$ mice, neither *Pax6* nor *Six3* are upregulated in the presumptive optic area as early as E9.0 (Zhang et al., 2000). These results are consistent with our own, indicating that *Rx* has an early role in eye formation and is upstream of *Pax6* and *Six3*. The medakafish mutant eyeless (*el*), is the result of an intronic insertion into the *Rx3* locus (Loosli et al., 2001). *Rx3* is required for evagination and proliferation of the optic vesicle. In medaka *Rx3* mutants, both *Tbx2* and *Tbx3* expression in the retina is lost, suggesting that *Rx3* is genetically upstream of these genes or that Tbx2/3 are expressed in tissues lost or re-patterned in *Rx3* mutants (Loosli et al., 2001). This is in contrast to our results, which show *Rx1* is downstream of *ET*. Two *Rx* homologues have been reported in *Xenopus* (*Rx1* and *Rx2*) and *medakafish* (*Rx2* and *Rx3*), while three have been identified in zebrafish (*rx1*, *rx2* and *rx3*) (Casarosa et al., 1997; Chuang et al., 1999; Loosli et al., 2001; Mathers et al., 1997; Winkler et al., 2000). Medakafish *Rx3* shares greater sequence homology with *Xenopus XRx2* than *XRx1* (not shown). In medaka *Rx3* mutants, *Rx2* expression is unaffected and morphogenetic movements are normal until optic vesicle evagination, *Rx2*-positive retinal tissue forms and the separation of the single retinal field into the two eye primordial is unaffected (Winkler et al., 2000). Medakafish *Rx2* is exclusively expressed in presumptive and differentiated retinal tissue during and after gastrulation (Loosli et al., 2001; Mathers et al., 1997). These results suggest that medakafish *Rx2*, or an as yet unidentified medaka *Rx* homolog is acting as the *Rx1* functional homologue in *Xenopus*.

$Rx^{-/-}$, $Pax6^{-/-}$, $Lhx2^{-/-}$ and $Six3^{-/-}$ mice all lack eyes (Grindley et al., 1995; Mathers et al., 1997; Porter et al., 1997; Tucker et al., 2001), but the morphological defects seen in these embryos also give clues to the order in which they are required during eye formation. $Rx^{-/-}$ embryos do not develop optic sulci, vesicles or cups, which normally form between stages E8.5 and E9.5 (Zhang et al., 2000). $Pax6^{-/-}$ (*Sey*) and $Lhx2^{-/-}$ mice, however, do develop optic vesicles, which form optic stalks and rudimentary optic cups (Grindley et al., 1995; Porter et al., 1997). In $Pax6^{-/-}$ animals, *Rx1*, *Six3* and *Lhx2* expression is unaffected as late as E10.5 (Bernier et al., 2001; Zhang et al., 2000). Recently, Lagutin and colleagues demonstrated a requirement for *Six3* during forebrain development (Lagutin et al., 2003). $Six3^{-/-}$ mice die at birth, and lack head structures anterior to the midbrain, including the eyes. Mouse *Six3* expression is first detected at E7.0 to E7.5 in the anterior neuroectoderm and the first morphological abnormalities in $Six3^{-/-}$ mice are seen at E8.0. *Rx1* expression, although significantly reduced, is still detected at E8.5 in the anterior neural plate of *Six3*-null animals, demonstrating that early *Rx1* expression does not require *Six3*. By contrast, neither *Rx1* nor *Pax6* is detectable at optic vesicle stages, as these structures do not develop. Interestingly, we also detect *Six3* expression prior to eye field formation in the frog. *Xenopus Six3* is detected weakly until stage 9, is lost, and then increases dramatically during eye field specification (Fig. 1). Perhaps there results point to a twofold role for *Six3* in eye formation – an early neural patterning function then as a component of the self-regulating network responsible for eye field specification. Our animal cap analysis indicates that *Optx2* does not regulate the expression of any of the EFTFs, consistent with the observation that *Pax6*, *Six3* and *Rx* expression are normal in the small eyed $Six6^{-/-}$ mouse (Li et al., 2002). Dominant-negative *tll* constructs inhibit the growth of the optic vesicle in *Xenopus* (Hollemann et al., 1998), and $tll^{-/-}$ mice show signs of retinal degeneration 3 weeks after birth that eventually result in visual defects (Yu et al., 2000). Our results similarly argue that *tll* and *Optx2* are not involved in the earliest steps of eye field formation, but that *ET*, *Rx1*, *Pax6*, *Six3* and *Lhx2* are part of a self-regulating network of nuclear factors in vertebrates that helps specify the eye field.

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