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Six3 and Six6 activity is modulated by members of the groucho family

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SUMMARY

***Six3* and *Six6* are two genes required for the specification and proliferation of the eye field in vertebrate embryos, suggesting that they might be the functional counterparts of the *Drosophila* gene *sine oculis (so)*. Phylogenetic and functional analysis have however challenged this idea, raising the possibility that the molecular network in which Six3 and Six6 act may be different from that described for SO. To address this, we have performed yeast two-hybrid screens, using either *Six3* or *Six6* as a bait. In this paper, we report the results of the latter screen that led to the identification of TLE1 (a transcriptional repressor of the *groucho* family) and AES (a potential dominant negative form of TLE proteins) as cofactors for both SIX6 and SIX3. Biochemical and mutational analysis shows that the Six domain of both SIX3 and SIX6 strongly interact with the QD domain of TLE1 and AES, but that SIX3 also interacts with TLE proteins via the WDR domain. *Tle1* and *Aes* are expressed in the developing eye of medaka fish (*Oryzias latipes*) embryos, overlapping with the distribution of both *Six3* and *Six6*. Gain-of-function studies in medaka show a clear synergistic activity between *SIX3*/*SIX6* and *TLE1*, which, on its own, can expand the eye field. Conversely, *AES* alone decreases the eye size and abrogates the phenotypic consequences of *SIX3/6* over-expression. These data indicate that both *Tle1* and *Aes* participate in the molecular network that control eye development and are consistent with the view that both Six3 and Six6 act in combination with either Tle1 and/or Aes.**

Key words: Eye, Aes, Tle1, Transcriptional repression, medaka

INTRODUCTION

Eye development is a multi-step process controlled by genes highly conserved throughout evolution. *Six3* and *Six6*, two members of the Six/sine oculis family, are highly conserved genes required for the initiation of eye development in vertebrates. *Six* genes code for transcriptions factors characterised by an homeo (HD) and a Six domain (SD) (Gallardo et al., 1999; Kawakami et al., 2000; Rodriguez de Cordoba et al., 2001). *Six3* and *Six6* are expressed in the anterior neural plate in an overlapping domain, more restricted and delayed for *Six6*, that comprises the prospective eye field and diencephalic ventral derivatives, where their expression is maintained at later stages (Bovolenta et al., 1998; Loosli et al., 1998; Lopez-Rios et al., 1999; Zuber et al., 1999).

The evolutionarily conserved importance of *Six* genes in eye development is illustrated by gain- and loss-of-function analysis in different species (Pignoni et al., 1997; Pineda et al., 2000; Seimiya and Gehring, 2000). In vertebrates, *Six3* over-expression induces the enlargement of the eye and the ectopic appearance of retina primordia in medaka fish (Loosli et al., 1999) and *Xenopus* (Bernier et al., 2000) embryos, as well as forebrain expansion in zebrafish (Kobayashi et al., 1998). In a similar way, *Six6* over-expression increases the eye size in *Xenopus* (Bernier et al., 2000; Zuber et al., 1999), controlling retinal neuroblast proliferation (Zuber et al., 1999) and induces trans-differentiation of dissociated pigment epithelium cells into neural retina phenotypes (Toy et al., 1998). In human, loss-of-function mutations in *SIX3* cause holoprosencephaly type II (Pasquier et al., 2000; Wallis et al., 1999), whereas *SIX6* has been associated with anophthalmia and pituitary defects (Gallardo et al., 1999). The relevance of *Six3* in head bilateralisation is also demonstrated by loss-of-function experiments in medaka that implicates *Six3* in proximodistal patterning of the eye (Carl et al., 2002). Therefore, while gain-of-function studies point to the capability of both genes to control eye field growth, loss-of-function analysis and their specific expression pattern suggest that their function may have diversified.

Comparison between the molecular networks that control *Drosophila* and vertebrate eye development and the observation that mutations in the *so* gene disrupt the development of the entire fly visual system, had originally led to the proposal that *Six3* may be the functional counterpart of the *Drosophila sine oculis* (*so*) gene (Oliver et al., 1995). However, isolation of two additional *Drosophila Six* genes, *optix* and *Dsix4* (Seo et al., 1999), and phylogenetic analysis of the *Six* family members has shown that *Six3* and *Six6* are more closely related to *optix* than to *so*, which is instead closely related to *Six1* and *Six2* (Gallardo et al., 1999). To initiate eye development SO requires the interaction with the product of the *eyes absent* gene (*eya*), which in turn binds to the

Dachshund protein (Chen et al., 1997; Pignoni et al., 1997). This complex acts downstream of *eyeless* (*ey*) and regulates *ey* expression with a positive feed-back loop. Functional conservation of this interaction has been demonstrated in vertebrates in the development of the somites, where Pax3, Dach2, Eya2 and Six1 act synergistically to induce muscle formation (Heanue et al., 1999). Whereas Six1, Six2, Six4 and Six5 interact with different Eya proteins, inducing their translocation to the nucleus, Six3 does not appear to interact with vertebrate Eya proteins (Ohto et al., 1999). *Optix,* the *Drosophila Six3* ortholog, is expressed in the eye imaginal disk and does not interact with *eya*, but on its own induces ectopic eye formation upon over-expression, with a mechanism that is independent from that of *so* (Seimiya and Gehring, 2000).

These data altogether suggest that the genetic network in which *Six3*/*Six6* (and possibly *optix*) operate may include cofactors other than those described for the fly SO and the vertebrate Six1 products. To search for these possible components and to compare SIX3 and SIX6 interactions, we have performed a two-hybrid screen using either *Six3* (Tessmar et al., 2002) or *Six6* as a bait. Here, we report the results of the latter screening, that has identified TLE1, a transcriptional repressor of the *groucho* family and AES, a truncated form of TLE proteins (Chen and Courey, 2000), as potential cofactors for both SIX6 and SIX3. The functional significance of these interactions is supported by biochemical analysis and by the overlapping distribution of both *Tle1* and *Aes* with those of *Six3* and *Six6* within the prospective eye regions. Furthermore, gain-of-function studies in medaka embryos show a clear synergic activity between *SIX3*/*SIX6* and *TLE1*, which, on its own, can expand the eye field. Conversely, *AES* alone decreases the eye size and abrogates the phenotypic consequences of *SIX3*/*6* over-expression.

MATERIALS AND METHODS

Yeast two-hybrid analysis

A *Xenopus* oocyte cDNA library [generated and kindly provided by Drs S. Pierce, D. Kimelman, M. Chen and J. A. Cooper (Yost et al., 1998)] cloned in pVP16f1 was screened using the pJ694a yeast reporter strain and c*Six6*, cloned in pGBDUC3, as bait. Primary positive colonies were isolated for their ability to grow in SD-Leu-Ura-Ade plates, and re-screened for their ability to recuperate specific interaction after bait plasmid loss in 5-FOA medium. The resulting clones were grouped by sequencing, restriction analysis and dot-blot hybridisation criteria. The adopted screening procedure is described elsewhere (Agatep et al., 1999; Parchaliuk et al., 1999).

The full-length or partial coding sequences of human *SIX1* (h*SIX1*), h*SIX3*, h*SIX6*, mouse *Six2* (m*Six2*) and m*Six4* were cloned in pGBDUC3, while the full-length or partial coding sequences of h*TLE1*, h*TLE3* and h*AES* were cloned in pVP16f1, using specific primers. The resulting constructs were used to analyse protein interaction in the two-hybrid assay, as follows. pGBDUC3 and pVP16f1 plasmids were transformed into the pJ694α and pJ694a strain, respectively, and the resulting clones were mated to generate diploid strains that were tested for their ability to grow on SD-Leu-Ura, SD-Leu-Ura-Ade and SD-Leu-Ura-Ade-His + 3AT. Full-length *TLE1* and *TLE3* plasmids were a generous gift from Dr S. Stifani. The entire h*AES* coding sequence was amplified by RT-PCR from human adult muscle mRNA. Point mutantions of F87E and V95P, L99P in the Six domain of the human SIX3, and F9E and V17P, L21P in the Six domain of human SIX6 were generated by in vitro mutagenesis (Quickchange site-directed mutagenesis kit; Stratagene) using specific primers and the respective wild-type plasmids as template. Deletion of amino acids 87-103 of human SIX3 and amino acids 9-25 of human SIX6 were obtained by PCR amplification using the forward primers SIX3Δ87-103Fw: ATGTTCCAGCTGCCCACCCTCAACGACATC-GAGCGGCTG and SIX6Δ9-25Fw: ACCATGTTCCAGCTGCCCA-TCTTGAATGATGTGGAGCGCCTG. The amino-terminal deletions of both SIX3 and SIX6 were obtained by PCR amplification and subsequent cloning.

GST pull-down assays

pGEX-*TLE1*$_{1-135}$ (QD) and pGEX-*TLE3*$_{490-772}$ (WDRD) were a generous gift from Dr S. Stifani. Full-length h*AES* was cloned into pGEX-A expression vector to generate a GST-AES fusion protein. Recombinant proteins were purified from induced cultures and bound to a glutathione resin (AP Biotech). All proteins were quantified by SDS-PAGE and Coomassie staining, and equivalent amounts (5 μg) of protein were used in each assay. Full-length h*SIX3*, h*SIX6* and h*SIX1* were cloned into pCDNA3-Flag using specific primers. These plamids were used to generate full-length proteins using the TnT T7 Coupled Rabbit Reticulocyte Lysate System (Promega). Proteins were analysed by SDS-PAGE and western blotting using a specific monoclonal anti-Flag antibody (Sigma) prior to interaction assays. In vitro synthesised Flag-tagged SIX3, SIX6 and SIX1 proteins were incubated with GST fusion proteins bound to 30 μl of glutathione resin in binding buffer (PBS, 0,1% NP-40, 100 μM PMSF, 1 μg/ml leupeptine and 2 μg/ml aprotinine), overnight at 4°C. Pelleted resins were extensively washed in binding buffer and PBS, boiled in Laemmli loading buffer and examined by SDS-PAGE. Gels were transferred to nitrocellulose membranes that were sequentially incubated with anti-Flag antibody (1:6000), HRP-labelled goat anti-mouse secondary antibody (1:10000) and ECL chemiluminescent system (AP Biotech). Blots were exposed on ECL Hyperfilm (AP Biotech).

Cloning of medaka *Tle1*, *Tle3* and *Tle4* probes

First strand cDNA was generated by oligo (dT) reverse transcription using total mRNA from stage 23 medaka embryos. The degenerate primers used for specific PCR amplification of the different members of the *Groucho* family are the followings: *Tle1*, 5′-AAYATHGARATGCAYAARCARGC-3′ and 5′-RAACCAYTTNCC-RCARTGNGCRA-3′; *Tle3*, 5′-AARGGNTNYGTNAARATHT-GGGA-3′ and 5′-CCNGTIACDATRTAYTTRTCRTC-3′; *Tle4*, 5′-AARGGNTGYGTNAARGTITGGGA-3′ and 5′-RAACCAYTT-NCCRCARTGNGCRA-3′. The TD-PCR conditions used are as follows: 95°C for 30 sec, 60°C 30 sec (–1°C per cycle), 72°C 2 minutes, for 20 cycles, followed by an additional 20 cycles with a constant annealing temperature of 60°C. *Aes* probe corresponded to the medaka EST sequence Olc21.06f (Medaka EST project, University of Tokyo). The amplified products were cloned into pGEM-T Easy vector (Promega) and sequenced. The sequences were aligned with those of their orthologues and paralogues to confirm unequivocally their identity as the *Tle1*, *Tle3* and *Tle4* medaka genes. All sequences have been deposited in the databases with accession numbers AY158892, AY158893 and AY158894.

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation was performed as described previously using DIG-labelled probes (Loosli et al., 1998). *Six3*, *Pax6*, *Otx2* (Loosli et al., 1998) and *Rx2* (Loosli et al., 1999) probes have been described previously.

mRNA injections

Full-length *TLE1*, *AES*, *SIX3* and *SIX6* were cloned into pCS2+ vector using specific primers. The plasmids were linearised and in vitro transcribed using the SP6 Message mMachine kit (Ambion). The synthesised mRNA was purified using Quiaquick RNeasy columns

(Quiagen), precipitated, quantified and injected in 1× Yamamoto Ringer (Yamamoto, 1975) into one blastomere in the two to four cell stage of medaka embryos. All the injection solutions included 30 ng/ml of h*GFP* mRNA as a lineage tracer. Both *TLE1* and *AES* mRNA were injected at different concentrations (50-250 ng/µl). The induced phenotypes were dose dependent. Selected working concentrations were 100 ng/µl for *TLE1* mRNA and 200 ng/µl for *AES*. The corresponding SIX3 and SIX6 plasmids were used as templates for in vitro mutagenesis, as described above.

RESULTS

Six6 two-hybrid screen

A yeast two-hybrid library generated from *Xenopus* oocytes was screened with the entire coding sequence of the chick *Six6* gene to identify possible evolutionarily conserved interacting partners. The initial 484 true-positive clones were analysed by rounds of random sequencing and grouping by dot-blot hybridisation with a final classification in five different groups as follows. (1) *Esg-1*/*Tle1* isoform A (181 clones); (2) *Esg-1*/*Tle1* isoform B (203 clones); (3) *Esg-1*/*Tle1* isoform C (25 clones); (4) *Aes* (22 clones); (5) not in frame, could not be amplified, etc (55 clones). Thus, the vast majority of the clones corresponded to the *Esg-1*/*Tle1* and the *Aes* genes, two members of the Groucho family of co-repressors (Chen and Courey, 2000). In groups 1-4 all the clones analysed included as a minimal region the coding sequences of the highly conserved glutamine-rich domain (QD). Interactions of *Esg-1*/*Tle1* and *Aes,* though the QD domain were also identified in a parallel screen performed with the medaka *Six3* gene as a bait (Tessmar et al., 2002). Interaction of the Six3.2 protein with another member of the Groucho family (Grg3) has also been described in zebrafish, though interaction was tested for the WD-40 repeats (WDR) domain of the molecule (Kobayashi et al., 2001).

Drosophila Groucho and its vertebrate homologues, known also as TLE [transducin-like enhancer of split, according to nomenclature in humans (Stifani et al., 1992)], are long-range co-repressor proteins that do not bind directly to DNA but are recruited to the template through protein-protein interaction with specific sets of DNA-binding transcription factors (reviewed by Chen and Courey, 2000; Fisher and Caudy, 1998). In vertebrates, there are four different TLE proteins: TLE1, TLE2, TLE3 and TLE4 (Koop et al., 1996; Miyasaka et al., 1993; Schmidt and Sladek, 1993; Stifani et al., 1992). As schematised in Fig. 1A, for human, Groucho/TLE proteins are characterised by the highly conserved N-terminal Gln-rich (QD) and C-terminal WD-40 repeats (WDR) domains. Interactions with DNA-binding proteins have been frequently mapped to the WDR domain, but there are several examples of interactions through the QD and multiple contact points have been reported for a number of proteins, including Pax5, BF1, NK3 and UTY (Choi et al., 1999; Grbavec et al., 1999; Eberhard et al., 2000; Yao et al., 2001). The QD domain is in addition responsible for oligomerization between members of the family, a prerequisite for efficient transcriptional repression. In addition to Groucho/TLE proteins, both invertebrate and vertebrate genomes code for a truncated family member, known as AES, composed only of the QD and GP domains (Fig. 1A). Because AES lacks most of the domains present in TLE proteins, but is able to associate with itself and TLE proteins through the QD domain, it has been proposed that AES behaves as a negative regulator of the repression mediated by TLE, possibly diminishing the local concentration of repressor units (Chen and Courey, 2000; Fisher and Caudy, 1998; Muhr et al., 2001; Roose et al., 1998). Evidence however exists that AES, when fused to a DNA binding domain, can also behave as a repressor (Ren et al., 1999) and that in some cases fails to compete with the repressor activity of TLE proteins (Eberhard et al., 2000).

Differential interaction of SIX3 and SIX6 with AES and TLE1

Six genes code for proteins with two highly conserved domains: the homeo domain (HD), responsible for DNA binding and the Six domain (SD), involved in both DNA and protein binding (Kawakami et al., 1996). These two domains are nearly identical in SIX3 and SIX6. The N-terminal portion is longer in SIX3 and includes a Gly-rich region of unknown function, absent in SIX6. The C terminus is the most divergent domain with the important exception of the last nearly identical 15 amino acids (Rodriguez de Cordoba et al., 2001).

Taking advantage of the strong conservation of both the Six and Groucho families of proteins, we have used the human genes to map the interactions between these two classes of molecules. On the basis of the structural and functional domains described above, we generated a series of constructs containing the full-length or specific domains of *SIX* and *groucho*/*TLE* human genes (Fig. 1A). These constructs were used in a yeast two-hybrid analysis, which shows that both full-length SIX3 and SIX6 interact strongly with the entire TLE1 and AES proteins, as judged by growth in highly selective media (Fig. 1B). This interaction is mediated by the QD domain of Gro/TLE proteins and the N-terminal region of SIX proteins, which includes the Six domain (SD). The latter is probably responsible of the interaction, since the N-terminal region of SIX6, which is composed almost exclusively by the SD, behaves similarly to that of SIX3. Comparable results were also obtained with SIX1 and with the mouse Six2 but not with mouse Six4, which, under stringent conditions, interacted only with the isolated QD of TLE1 (Fig. 1B). Interestingly, *Drosophila* Optix showed similar interactions with Groucho as well as with TLE1 and AES (Fig. 1B).

Interaction between the Six domain of Six3.2 and the isolated WDR domain of Ggr3, the orthologue of human *TLE3*, has been described in zebrafish (Kobayashi et al., 2001). In our analysis, a weak interaction between the full-length or $SIX3_{1-205}$ and TLE1 or TLE3 WDR domain was observed but only under low stringency conditions (Fig. 1C). A similar weak interaction was detected with mSix4 but, most interestingly, not with SIX6.

The interactions of SIX3, SIX6 and SIX1 (for comparison) with TLE/AES were further validated with GST pull-down assays, using in vitro synthesised Flag-tagged proteins. Western blot analysis confirmed that the three SIX proteins specifically co-precipitated with AES as well as with the TLE1 QD (Fig. 1D). In agreement with our two hybrid analysis, a lower amount of SIX3, but not of SIX6 or SIX1, co-precipitated with the WDR domain of TLE1 (Fig. 1D).

In conclusion, these data indicate that there is a comparable interaction of SIX3 and SIX6 with AES through the QD domain. However, the interaction of SIX3 with TLE1 is

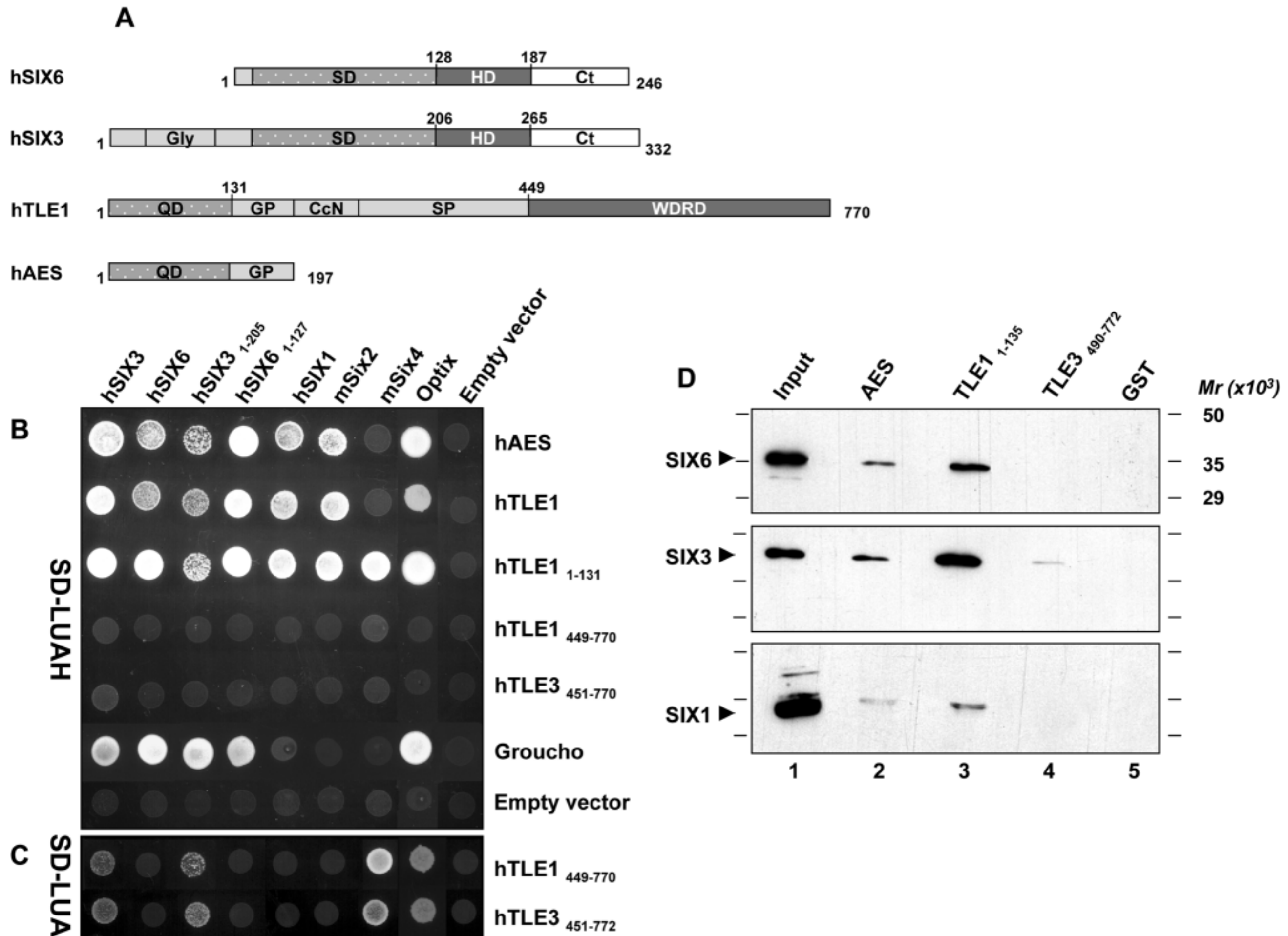

Fig. 1. SIX6 and SIX3 interact with TLE1 and AES. (A) Schematic diagram of the domain organisation of human SIX6, SIX3, TLE1 and AES. All vertebrate TLE proteins have the same organisation, as illustrated for TLE1. Besides the QD and WDR domains, these proteins have GP, CcN and SP domains, which have been shown to be involved in transcriptional repression, nuclear localisation and protein interactions. (B) High stringency two-hybrid analysis of the interactions between SIX3, SIX6, SIX1, mSix2, mSix4 and Optix with AES, TLE and Groucho proteins. Constructs containing the full-length or specific domains of SIX and Groucho/TLE human genes were used to map the interactions between these two classes of molecules. (C) Low stringency two-hybrid analysis of the interactions of SIX proteins with the WDR domain of TLE1 and TLE3. (D) Western blot analysis of pull-down experiments using GST::AES (lane 2) and GST::TLE1 proteins (lanes 3, 4) and in vitro synthesised Flag-tagged SIX proteins. Lane 1 shows the respective SIX proteins translated by TnT (input). Lane 5 shows control pull-downs with GST alone. SD, Six domain; HD, Homeo-domain; Ct, C-terminal domain; Gly, glycine-rich region; QD, glutamine-rich domain; GP, glycine-proline rich region; CcN, casein kinase II/cdc2 kinase site/nuclear localisation domain region; SP, serine-proline rich region; WDRD, WD-40 repeats domain.

expected to be stronger than that of SIX6 because of the ability of SIX3 to interact with TLE proteins via two different domains.

Medaka *Tle1* and *Aes* genes are expressed since early stages of eye development

To assess the possible in vivo relevance of these interactions during eye development, we investigated whether in medaka embryos the expression of *groucho/Tle* genes, in particular *Tle1* and *Aes*, overlaps with that of *Six3* and *Six6* at different stages of eye development. To this end we generated probes to the medaka *Aes*, *Tle1* and the closely related *Tle4* (Choudhury et al., 1997), as well as for the *Tle3* gene.

The results of whole-mount in situ hybridisation analysis are shown in Fig. 2. Medaka *Tle1* transcripts are first detected during early neurula stage, in the most anterior part of the embryonic body (data not shown). At late neurula stage, *Tle1* but not *Tle3* shows a prominent expression in the anterior brain, including the evaginating optic vesicles (Fig. 2D,G), overlapping with the expression domain of *Six3* (Fig. 2A-C) (Loosli et al., 1998) and of *Six6* at later stages of development (Fig. 2P-R). Like *Six3* and *Six6*, *Tle1* expression was detected at high levels in the eye domain as well as in the ventral diencephalon through optic cup and eye differentiation stages (Fig. 2B-C,E-F,Q-R). In contrast, *Tle3* mRNA was detected in the lens but not in other eye structures (Fig. 2H,I). Both *Tle1* and *Tle3* showed additional sites of expression in the CNS including, for *Tle1*, the hindbrain and the fore-, mid- and hindbrain for *Tle3* (Fig. 2D-I). In comparison to *Tle1*, *Tle4* has a later onset and a weaker expression but this is confined to the

eye, particularly the neural retina and the optic stalk (Fig. 2K), and to the ventral diencephalic region, including the optic chiasm (Fig. 2L).

Aes expression was detected in the anterior neural tube, localised to the evaginating optic vesicle and the prospective midbrain region (Fig. 2M). At later stages, *Aes* mRNA became more widely distributed throughout the embryo with clear levels in the eye and in the ventral diencephalon (Fig. 2N,O), overlapping with *Six3* and *Six6* expressions.

In conclusion, the spatiotemporal expression of both Tle1 and Aes are compatible with their associations with Six3 and/or Six6 during retina specification and morphogenesis. Tle4 is an additional candidate but only at later stages of development. These ideas are further supported by the observation that similar overlapping distributions are conserved in chick embryos (data not shown). In the medaka eye, the possible interaction between Tle3 and Six3 may be limited to lens tissue, the only site where the expression of the two genes overlaps.

TLE1 over-expression induces an enlargement of the eye field and reinforces *SIX3*/*SIX6* capability of initiating retina formation

Biochemical and expression analysis are consistent with the idea that Tle1 and Aes participate in the molecular network that controls eye development, as potential cofactors for Six3 and Six6. To test the functional significance of these interactions we over-expressed *TLE1* or *AES* alone or in combinations with *SIX3* or *SIX6* in medaka embryos.

The morphological and molecular consequences of *TLE1* RNA injections into a single blastomere of embryos at the two- to four-cell stage are shown in Fig. 3. The most prominent phenotypic feature of the injected embryos is an enlargement of the optic vesicles, which is maintained in more developed eyes and it is often accompanied by bulging of the midbrain (Fig. 3A). These morphological changes were observed in 39% of the injected embryos (91/232) and are similar to those observed with injections of low doses of *Six3* RNA (not shown) (Loosli et al., 1999). In the affected embryos, endogenous *Six3* expression domain was generally enlarged to a variable degree into the midbrain (Fig. 3B). Similarly, the expression of both *Pax6* (Fig. 3F) and that of *Rx2* (Fig. 3D), a retina marker, was also consistently expanded as compared to controls (Fig. 3C,E). In addition, while *TLE1* over-expression was not found to induce the appearance of ectopic *Rx2* transcripts in the midbrain, ectopic isolated patches of *Pax6* expression were observed in the midbrain (Fig. 3F). These alterations were detected also at later stages of development and were restricted to the fore- and mid-brain. Thus, in spite of the bulging, the midbrain was normally specified, as judged by *En2* and *Pax2* expression (not shown). Furthermore, the posterior limit of *Otx2* expression at the isthmus was located normally, though somewhat tilted due to midbrain alterations (Fig. 3H). No patterning defects were ever observed in more posterior regions of the embryos. Injections of similar concentrations of *TLE2* was not followed by enlargement of the eye field or by other obvious morphological alterations (not shown).

Injections of *Six3* RNA in medaka embryos leads to a concentration-dependent expansion of the eye and other brain structures, which is accompanied, at higher doses, by the appearance of additional ectopic *Rx2*-positive retina

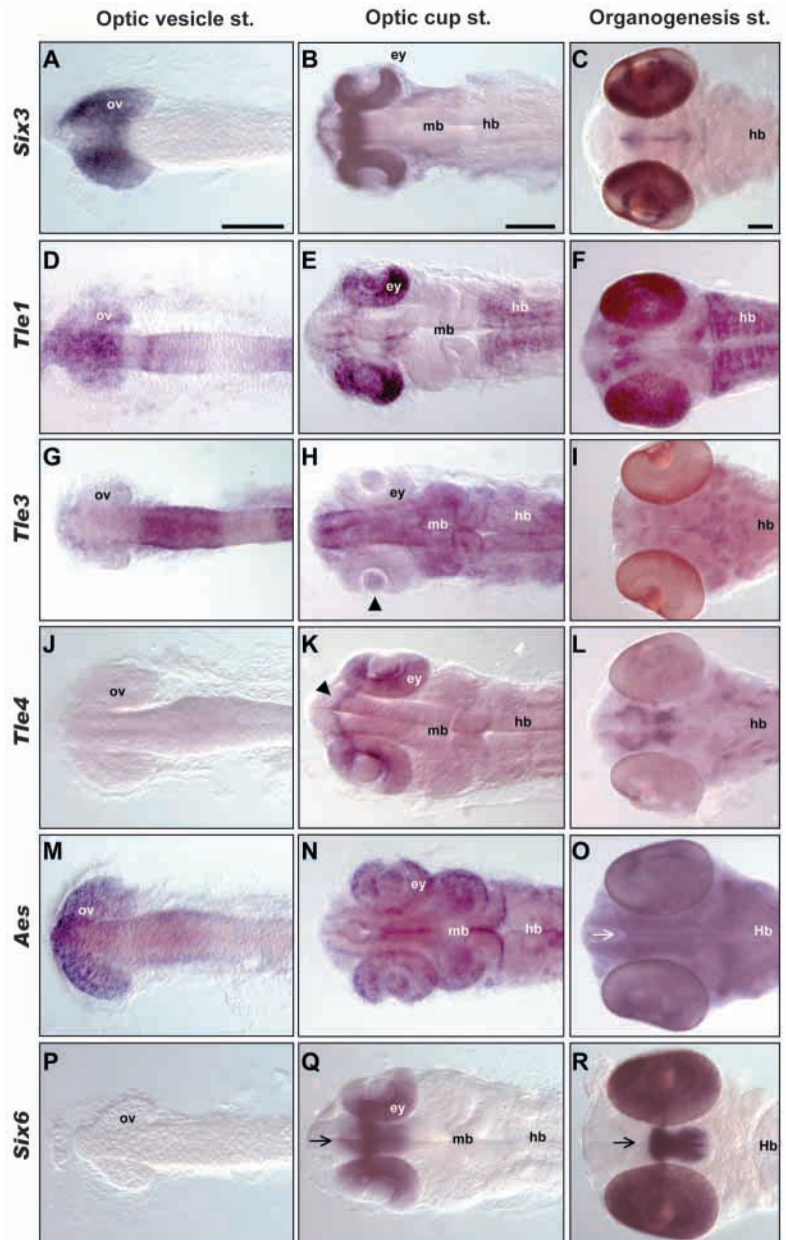

Fig. 2. Comparison of the expression domains of *Six3*, *Six6* and *Gro/Tle* genes in medaka embryos. Whole-mount in situ hybridisations at different developmental stages as indicated at the top of each colum. All embryos are dorsalview, anterior to the left. Embryos were hybridised with probes to *Six3* (A-C), *Tle1* (D-F), *Tle3* (G-I), *Tle4* (J-L), *Aes* (M-O) and *Six6* (P-R). Note how *Tle1* and *Aes* are expressed in the eye field from early stages. Arrowhead in H indicates the lens vesicle; arrowhead in K, the optic stalk; arrows in O, Q and R, the ventral diencephalon. ov, optic vesicle; ey, eye; mb, midbrain; hb, hindbrain. Scale bars 0.1 mm.

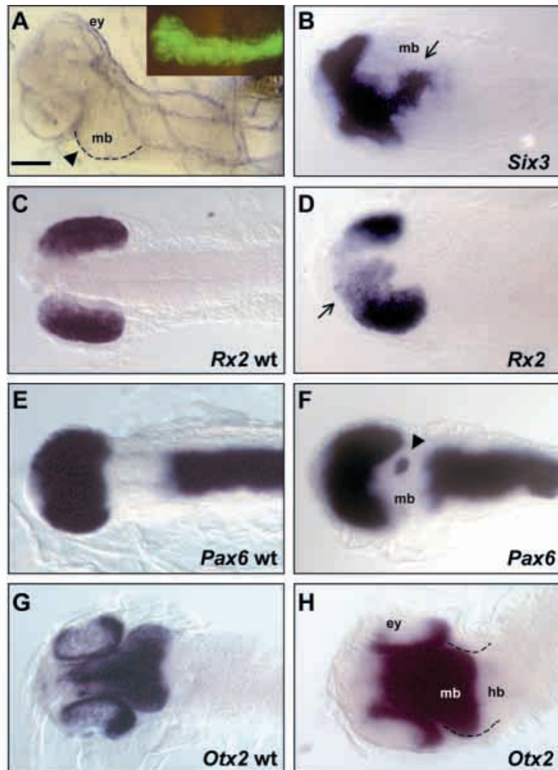

Fig. 3. *TLE1* over-expression enlarges the eye field in medaka embryos. Dorsal (A-D,G,H) and ventral (E,F) views (anterior to the left) of *TLE1*-injected embryos. (A) Over-expression of *TLE1* (inset shows expression of the co-injected *GFP* mRNA) causes a visible enlargement of the optic vesicles and bulging of the midbrain (arrowhead). Whole-mount in situ hybridisations demonstrate that expression of *Six3* (B) and *Rx2* (D) are expanded (arrows) compared to control embryos (C, and Fig. 2B). Note that *TLE1* injections lead to the expansion of the posterior domain of *Pax6* and to the appearance of ectopic *Pax6* expression (arrowhead in F), as compared to controls (E). *Otx2* expression was similar to that of controls (G,H). ey, eye; mb, midbrain; hb, hindbrain; wt, wild type. Scale bar: 0.1 mm.

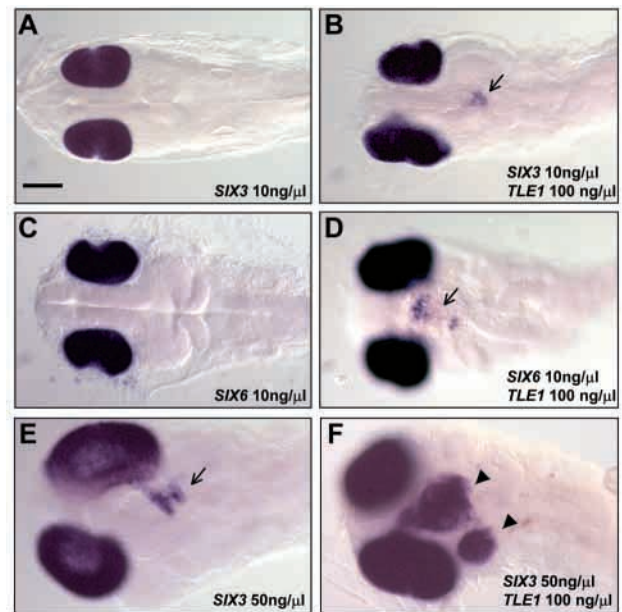

Fig. 4. *TLE1* synergizes with *SIX3* and *SIX6* in over-expression assays. Dorsal views of stage 24 embryos injected with either *SIX3* (A,B,E,F) or *SIX6* (C,D) mRNA alone (A,C,E) or in combination with *TLE1* (B,D,F). Concentrations are indicated in the panels. Embryos were hybridised to detect *Rx2* expression. Note that 10 ng/µl of *SIX3* or *SIX6* are not effective in inducing ectopic *Rx2* expression (A,C). Co-injection with *TLE1*, clearly boosts *SIX3* and *SIX6* activity and induces ectopic *Rx2*-positive tissue in the midbrain (arrows in B and D). The phenotype induced by higher doses of *SIX3* (E, ectopic midbrain expression. arrow) was also enhanced by *TLE1* co-injections (F), leading to the striking appearance of additional separate patches of ectopic *Rx2* expression (arrowheads in F). Scale bar: 0.1 mm.

tissue in the dorsal midbrain (Loosli et al., 1999). *Six6* over-expressed in *Xenopus* embryos induces similar enlargements of the eye field (Bernier et al., 2000; Zuber et al., 1999), which, in medaka, are also followed by the formation of ectopic *Rx2*-positive retina tissue, though with less efficiency than with *Six3* (F. L., J. W., unpublished observations and Fig. 6C, Table 1). If Tle1 acts as a cofactor for either Six3 or Six6, it should be expected that co-injections of *TLE1* with sub-optimal concentrations of either *SIX3* or *SIX6* can mimic the phenotypic consequences of injecting higher doses of *SIX3*/*SIX6* RNA (i.e. the appearance of ectopic *Rx2*-positive tissue). As shown in Table 1, *SIX3* or *SIX6* RNA concentrations below 20 ng/µl were ineffective in inducing ectopic *Rx2* expression. About 50 ng/µl of mRNA were generally required to induce this phenotype in roughly half of the injected embryos (Fig. 4E). However, when clearly sub-optimal concentrations (10 ng/µl; Fig. 4A,C) of either *SIX3* or *SIX6* were co-injected with *TLE1* (100 ng/µl), a significant number of embryos (Table 1) presented ectopic expression of *Rx2*, besides an enlargement of the eye (Fig. 4B,D). This synergic activity was also observed with higher doses of *SIX3*/*SIX6*, resulting in the striking appearance of several independent ectopic *Rx2*-positive sites (Fig. 4F). In all the cases analysed, these patches were confined to the midbrain, as in the *SIX3*/*SIX6* over-expression.

Table 1. Percentage of embryos showing ectopic expression of *Rx2*

	SIX3 10 ng/µl	*SIX3* 20 ng/µl	*SIX3* 50 ng/µl	*SIX3* 10 ng/µl+ *TLE1* 100 ng/µl	*SIX3* 50 ng/µl+ *AES* 200 ng/µl	*SIX6* 10 ng/µl	SIX6 20 ng/µl	*SIX6* 50 ng/µl	*SIX6* 10 ng/µl+ *TLE1* 100 ng/µl	*SIX6* 50 ng/µl+ *AES* 200 ng/µl
% of ectopic *Rx2* expression in the midbrain	0% (0/32)	6% (2/32)	47% (15/32)	18% (12/68)	7% (4/54)	0% (0/30)	0% (0/38)	38% (20/52)	7% (4/56)	3% (2/58)

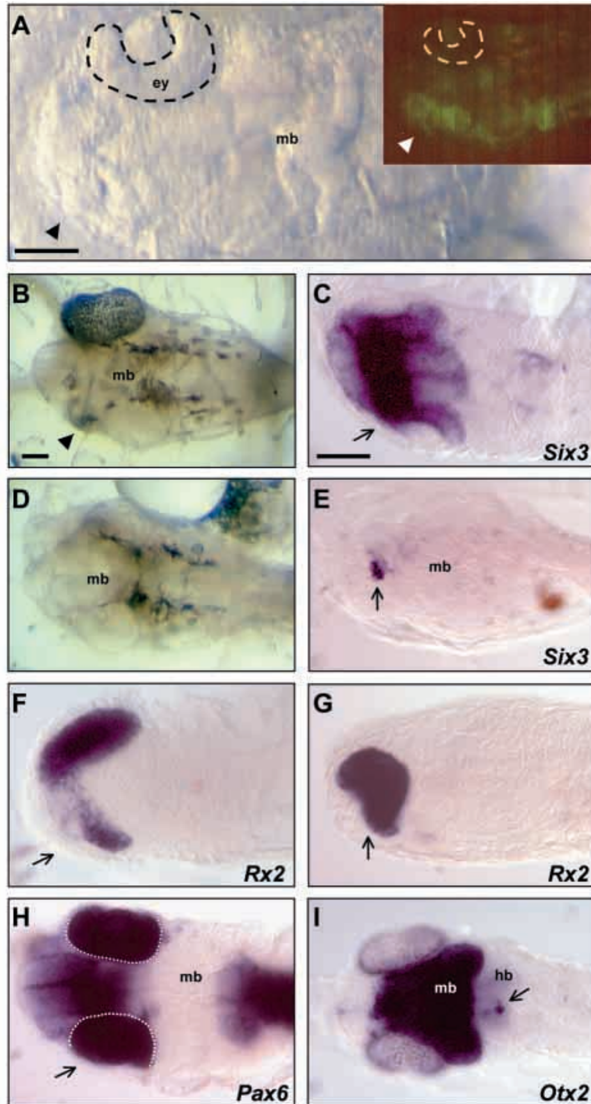

Fig. 5. *AES* over-expression reduces the eye size in medaka embryos. Dorsal (except E, ventral) views of embryos at stage 24 (A,C,G,H,I), stage 32 (B,D,E) and stage 20 (F) injected with *AES.* Anterior is to the left. Dotted white lines indicate the extent of the eye domains. Embryos show a unilateral (A) or bilateral (D) loss of the eye(s). (A inset) Expression of the co-injected *GFP* mRNA. (B) The same embryos as in A but at a later stage of development, showing that the failure of eye formation (arrowhead) is permanent. Whole-mount in situ hybridisations demonstrate that the reduction or absence of the eye(s) is accompanied by a decrease of the expression domain of *Six3* (arrows in C,E) and *Rx2* (arrows in F,G). Apart from the reduced domain of the affected eye (arrow in H), *Pax6* (H) and *Otx2* (I) expression domains were similar to those of wild-type embryos. Occasionally, ectopic expression of *Otx2* was observed in the hindbrain (arrow in I). mb, midbrain. Scale bars: 0.1 mm.

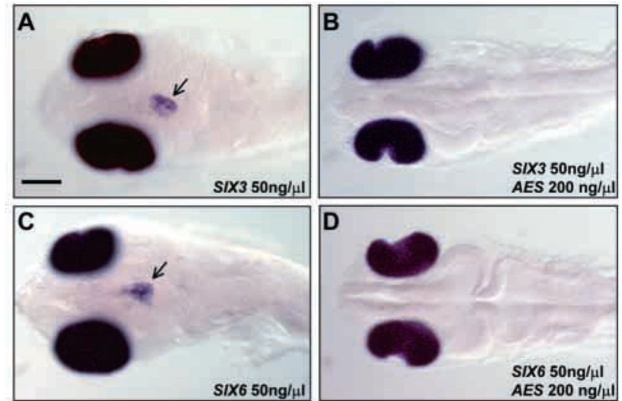

Fig. 6. *AES* abrogates *SIX3*- and *SIX6*-induced phenotypes. Dorsal views of stage 24 injected embryos hybridised with *Rx2* probe. Embryos injected with 50 ng/µl of either *SIX3* (A) or *SIX6* (C) show ectopic *Rx2* expression in the midbrain (open arrows). This phenotype is inhibited by co-injections of *AES* mRNA (B,D). Scale bar: 0.1 mm.

AES over-expression leads to eye hypoplasia and counteracts *SIX3/SIX6* gain of function phenotype

The data described above indicate that TLE1 per se can enlarge the eye field and its interaction with SIX3/6 boosts the capability of these factors to initiate ectopic retina tissue formation. In agreement with the idea that AES may function as a dominant negative form of TLE protein, *AES* mRNA injections generated a visible reduction of the eye size in 50/182 (27%) of the injected embryos (Fig. 5A). This was not due to a delayed development of the eye since it was observed also at later developmental stages (Fig. 5B). Consistent with this phenotype, *Six3* and *Rx2* expression was reduced in all the affected embryos analysed (Fig. 5C,F). In a smaller proportion of the embryos, the effect of *AES* over-expression was more dramatic, leading to the presence of a single eye field (Fig. 5G) or to the loss of both eyes (Fig. 5D). In the latter case, the expression of *Six3* was restricted to the midline of the ventral diencephalon, possibly corresponding to prospective hypothalamic and pituitary region (Fig. 5E). Mildly affected embryos with a moderate reduction of the eye size presented no other obvious brain malformations, as judged by normal *Pax6* expression (Fig. 5H). *Otx2*-positive midbrain tissue appeared morphologically normal, even though ectopic *Otx2* expression into the hindbrain was occasionally observed (Fig. 5I).

Furthermore, *AES* over-expression abrogated significantly the ectopic formation of *Rx2*-positive tissue in the midbrain, when co-injected with amounts of either *SIX3* or *SIX6* mRNA (50 ng/µl) capable of inducing ectopic retina-like tissue (Fig. 6). Thus, in the presence of *AES*, the frequency of appearance of *Rx2*-positive tissue in the tectum decreased from 47% to 7% for *SIX3* and from 38% to 3% for *SIX6* (Table 1). This was an ‘all-or-none’ effect and no intermediate levels of *Rx2* expression were observed in the co-injections.

Altogether these data show that TLE1 and AES have opposing effects on SIX3 and SIX6 protein activities and thus uncover how Six3/Six6 act as repressors and function in the determination and maintenance of retinal identity.

Mutant SIX proteins that do not interact with TLE1/AES are unable to initiate ectopic retina formation

To test whether the overexpression phenotype of SIX3/SIX6 relies on the recruitment of endogenous Groucho proteins, we generated mutant forms of both SIX3 and SIX6 in which these interactions were disrupted. Secondary structure analysis of the

Table 2. Biochemical and functional properties of mutant SIX3 and SIX6 proteins

Form injected	Interaction with TLE1	Interaction with AES	Percentage of ectopic *Rx2*-expressing embryos: 50 ng/μl	10 ng/μl+ TLE1 100 ng/μl	50 ng/μl+ AES 200 ng/μl
SIX3*	++	++	47% (15/32)	18% (12/68)	7% (4/54)
SIX6*	++	++	38% (20/52)	7% (4/56)	3% (2/58)
SIX3Δ87-103	−	−	0% (0/28)	0% (0/32)	0% (0/43)
SIX6Δ9-25	−	−	0% (0/43)	0% (0/40)	0% (0/35)
SIX3-V95P, L99P	−	−	0% (0/35)	0% (0/31)†	0% (0/34)
SIX6-V17P, L21P	−	−	0% (0/37)	0% (0/41)†	0% (0/22)
SIX3-F87E	−	+	0% (0/34)	0% (0/39)	0% (0/32)
SIX6-F9E	−	−	0% (0/40)	0% (0/25)	0% (0/20)

*These data corresponds to those detailed in Table 1 and are included for comparison.
†SIX mutant proteins at 50 ng/μl were coinjected with TLE1 at 100 ng/μl.

Six domain (http://cubic.bioc.columbia.edu/predictprotein) reveals its potential folding in four α-helix stretches. Therefore, we generated a series of N-terminal deletions in both SIX3 and SIX6, carrying sequential deletions of each of these helical regions and assayed their interactions by two-hybrid analysis.

SIX3Δ1-86 and SIX6Δ1-8 were still able to interact strongly with both full-length AES and TLE1, as expected given that these constructs include the entire Six domain. However, the inclusion of the first predicted α-helix in the deletion (SIX3Δ1-103 and SIX6Δ1-25), clearly impaired the interaction of both SIX proteins with TLE1 and AES (not shown). To further analyse the importance of these region for the interaction, we specifically deleted only the first helical region in the Six domains of SIX3 and SIX6 (SIX3Δ87-103 and SIX6Δ9-25) and generated four different point mutations in the same stretch of amino acids: SIX3-V95P, L99P; SIX6-V17P, L21; SIX3F87E and SIX6F9E. The first two double point mutations affect highly conserved residues and are predicted to lead to the disruption of the helical structure. The other two point mutations have been described very recently as being necessary for Six3 interaction with Groucho proteins (Zhu et al., 2002). As shown in Table 2, all six mutations lead to a loss of interaction with TLE1 and AES, with the exception of SIX3F87E, which still shows a weak interaction with AES. To assay the functional relevance of these mutations, we over-expressed them in medaka embryos. Table 2 shows that all of them are complete loss-of-function mutations, unable to affect eye development and induce ectopic *Rx2*-expressing retinal structures in the midbrain. Moreover, when co-injected with TLE1, no functional synergism is observed, not even when the amount of mutant RNA is raised to 50 ng/μl. When co-injected with AES, none of the mutant forms of SIX showed any functional interaction with AES, in spite of the weak biochemical interaction shown by SIX3F87E, as mentioned above.

These data strongly support the hypothesis that the specific interaction between TLE and Six3/Six6 is crucial for normal eye development and the cause of the over-expression phenotype observed in our studies.

DISCUSSION

Transcriptional repression is emerging as one of the fundamental mechanisms underlying the progressive specification of the neural plate. Thus, dorsoventral and rostrocaudal patterning of the neural tube is achieved through cross-repressive events between different classes of transcription factors expressed in abutting domains (Jessell, 2000; Nakamura, 2001; Simeone, 2000). Many of these molecules recruit TLE proteins for their activity. This is the case for instance of Nkx proteins, Pax6 and Dbx2 in the ventral and dorsal domains of the spinal cord (Muhr et al., 2001), or of En1, En2 and Pax5 in the midbrain (Eberhard et al., 2000). We have shown here that Gro/TLE transcriptional cofactors also participate in the network of genes that control eye specification in vertebrates, interacting with SIX3 and SIX6. Four lines of evidence support this idea. First, Gro/TLE proteins bind in vitro to both SIX3 and SIX6. Second, *Tle1* and *Aes* are expressed in the eye field, overlapping with the expression domains of *Six3* and *Six6*. Third, *TLE1* synergizes with *SIX3* and *SIX6* in inducing ectopic retina tissue, a function that is inhibited by *AES*, a dominant negative regulator of Gro/TLE activity. Finally, mutations in the Six domain of SIX3 and SIX6 that disrupt interaction with TLE1 and AES, prevent the phenotypic consequences observed after *SIX3*/*SIX6*-TLE1/*AES* co-injections.

Six3 and Six6 have different biochemical interactions

The Gro/TLE is a family of conserved transcriptional co-repressors required for many developmental processes in both invertebrates and vertebrates. Gro/TLE proteins are capable of interacting with a variety of DNA-binding transcription factors and, once recruited to DNA, mediate transcriptional repression through a series of mechanisms. These include multimerization of TLE proteins along the DNA template and interaction with histones and histone deacetylases, capable of altering the local chromatin structure (reviewed by Chen and Courey, 2000; Courey and Jia, 2001). The repression activity of Gro/TLE proteins is inhibited in many cases by AES, a shorter version of these proteins, composed essentially of the QD domain that mediates AES function (Muhr et al., 2001; Ren et al., 1999; Roose et al., 1998). Therefore, Gro/TLE proteins might be considered as multipurpose modulators of transcription. Our two-hybrid analysis has identified both Tle1 and Aes as co-factors of Six6. A screen of the same library, performed in similar conditions, showed that Six3 has the capability of

interacting with the same two Gro/Tle proteins. Interestingly, however, while no other candidates emerged from the Six6 screen, several additional proteins were isolated as Six3-interacting factors. These did not include any Eya proteins, even though PCR analysis confirmed their presence in the yeast two-hybrid library (Tessmar et al., 2002). These results further support the idea that the conserved SO/Six1 interaction with Eya proteins is not a feature of the Optix/Six3/Six6 branch of the family (Heanue et al., 1999; Ohto et al., 1999; Seimiya and Gehring, 2000).

Mapping of the SIX/TLE interaction domains using the human proteins identified additional differences between SIX3 and SIX6. Both proteins interact, through the Six domain, with the QD domains of AES and TLE1. The main but not exclusive function of the QD domain is mediating homo- and hetero-oligomerization among Gro/Tle proteins (Pinto and Lobe, 1996). Our results showing a specific interaction between the QD domain of TLE and the first putative alpha helix of the Six domain of SIX3/SIX6 are consistent with data reported for other transcription factors binding TLE proteins through the QD domain (McLarren et al., 2000; Ren et al., 1999). In addition, SIX3, but not SIX6, shows an additional interaction with the WDRD. Therefore, in spite of their strong homology, SIX3 and SIX6 behave differently in their interaction with other proteins. In particular, in the case of Gro/TLE interaction, the SIX3/TLE1 complex might be favoured and more effective than that formed by SIX6/TLE1, since simultaneous interactions through different domains may be necessary for a more efficient recruitment of TLE to DNA tethered factors (Eberhard et al., 2000).

The nature of Six3 and Six6 as transcriptional repressors has been previously proposed on the basis of over-expression studies in *Xenopus* and zebrafish, where fusions of Six3 or Six6 with the *engrailed* repression domain could mimic *Six3* or *Six6* over-expression phenotypes (Kobayashi et al., 2001; Zuber et al., 1999). In zebrafish, this assumption was further validated showing that in a yeast two-hybrid assay the Six domain of *Six3.2* could interact with the WDR domain of Tle/Grg3 (Kobayashi et al., 2001). Our results confirm and extend these observations demonstrating, as a result of two-hybrid screens, that both Six3 and Six6 interact with Groucho/Tle proteins through the conserved QD domain. Furthermore, the identification of a novel interaction between Six3/Six6 and Aes suggests alternative mechanisms of Six3/Six6 activity, including Six3/Aes- and/or Six6/Aes-mediated transcriptional derepression strategies.

Tle1 and Aes have opposing activity in retina development

The amino acid sequences of Gro/TLE family members are highly conserved. For instance, the WDR and QD domains of TLE1 or TLE3 share 93% and 84% of identity, respectively. Thus, it is not surprising that in vitro both molecules interact with SIX3 and SIX6. However, expression analysis and functional data point to Tle1 and Aes as the most likely partners of Six3 and Six6 activities in the early patterning of the eye in medaka. Thus, *TLE2* over-expression does not perturb eye development, and *Tle3* expression in the eye is limited to the lens vesicle. In contrast, *Tle1* and *Aes* are expressed from early stages in the eye field, overlapping with the distribution of *Six3* and later with that of *Six6*. *Tle4*, the expression of which was first detected at optic cup stages, restricted almost exclusively to the eye, is an additional candidate for Six3/6 functions during retina differentiation. *TLE1* over-expression enlarges the retina field, expanding the expression of both *Six3* and *Rx2*, without major modifications in the expression of other anterior markers such as *Otx2*. Although the precise function of *AES* is still controversial (Eberhard et al., 2000), *AES* over-expression considerably reduces the eye size and the expression of *Six3* and *Rx2*, supporting the idea that in the eye, as in dorso-ventral patterning of the neural tube and in *Xenopus* axis formation (Muhr et al., 2001; Roose et al., 1998), Aes might act as an inhibitor of Tle function. A priori, we cannot exclude that these effects might be mediated, at least in part, by the interaction with transcription factors expressed in the eye field other than Six3 and Six6. For example, the sequence of Rx proteins includes an engrailed homology (eh1) related motif, known to mediate Tle recruitment in other proteins (Eberhard et al., 2000; Muhr et al., 2001). In addition, the interaction of Tle1 with En1, En2, Pax2 or Pax5, all of which are involved in midbrain patterning (Araki and Nakamura, 1999; Eberhard et al., 2000), may explain the alteration of this structure that we observed in several gain-of-function embryos. However, co-injection experiments of wild-type and mutated SIX proteins with Gro/TLE family members support the idea that *TLE1* and *AES* overexpression phenotypes are the result of the modulation of endogenous Six3/Six6 activity by TLE1/AES. Critical concentrations of either *SIX3* or *SIX6* induces the ectopic formation of retina tissue in the anterior brain. The number and size of these ectopic structures is increased when *TLE1* is co-injected. Furthermore, *TLE1* allows the formation of ectopic structures even at suboptimal concentrations of *SIX3*/*SIX6*, an effect that is not observed with the injections of SIX proteins carrying mutations that abolish the interaction with TLE1. *AES* efficiently abrogates this phenotype, substantiating further the model that *TLE1*/*AES* are modulating *SIX3*/*SIX6* function. In agreement with a specific involvement of *TLE1*/*AES* in eye development, we never observed any malformations in the posterior regions of the embryos. Furthermore, the reported phenotypes caused by overexpression of other *Gro*/*Tle* are quite distinct from those we observed. mRNA injection of X*Grg4*/*Tle4* in *Xenopus* oocytes inhibits *Tcf*-dependent axis formation, an event that is instead enhanced by X*Grg5*/*Aes* (Roose et al., 1998). In ovo electroporation of the *Grg4*/*Tle4* chick homologue inhibited *En2* and *Pax5* expression, altering mesencephalic borders (Sugiyama et al., 2000). Complementing our observations, while this paper was under revision, Zhu et al. (Zhu et al., 2002) reported that Six3 interaction with Groucho proteins is also relevant for other steps of vertebrate eye development, namely lens morphogenesis in the chick and photoreceptors differentiation in the rat retina.

Although we have not addressed this issue, it is likely that Gro/Tle proteins cooperate with Six3/Six6 in the development of other structures where these genes are strongly co-expressed. This might be the case for the pituitary gland, the development of which may require Six3/6 functions (Gallardo et al., 1999). Interestingly, Tle1 is expressed during mouse pituitary organogenesis, where it has been shown to interact at least with Hesx1 to prevent the activity of Prop1, a paired-like transcriptional activator related to Hesx1 (Dasen et al., 2001).

Possible models for Tle/Aes modulation of Six3/Six6 transcriptional activities

The results of our gain-of-function studies are consistent with a simple model, in which both Six3 and Six6 can act in combination with either Tle1 and/or Aes. Six3 and Six6 may bind to distinct DNA binding sites. Their interaction with either Tle1 or Aes will lead to transcriptional repression or activation, respectively. In a more elaborated possibility, both Six3 and Six6 could be the DNA binding elements of a larger transcriptional repressor complex, the repressosome (Courey and Jia, 2001), formed by Tle proteins and additional factors recruited through interaction with Six3 or Tle1. In agreement with this idea, Six3 is able to directly contact other nuclear factors including SWI/SNF proteins (Tessmar et al., 2002), involved in the chromatin remodelling required during transcription repression (Sudarsanam and Winston, 2000). Aes recruitment into the complex would provide a mechanism of derepression. Alternatively, other factors could compete with Tle1 for binding to Six3 (Tessmar et al., 2002), thus modulating Six3/Tle activity in a way similar to that described for TLE1, Cbfa1 and HES1 (McLarren et al., 2000). These two models imply that both Six proteins interact with Gro/TLE with a similar affinity. However, yeast two-hybrid and biochemical analyses suggest that the SIX3/TLE1 interaction might be stronger than that of SIX6/TLE1, because it is mediated by an additional binding site. Furthermore, if the homeodomain on its own confers DNA binding specificity, Six3 and Six6 could compete for the same DNA binding sites, as only a single amino acid substitution differentiates their HD (Gallardo et al., 1999). Therefore, as a third possibility, the Six3/Tle1 complex could act as a transcriptional repressor unit, the activity of which could be regulated by a dominant negative complex formed by Six6/Aes. This model provides a specific function for both Six3 and Six6 and is compatible with the available expression, gain- and loss-of-function data on the two molecules. The Six6 expression pattern is more restricted than that of Six3 and, in general, occurs later in development (Gallardo et al., 1999; Lopez-Rios et al., 1999). Thus, Six3 patterning activities in the anterior neural plate could be alleviated by subsequent expression of Six6 in this tissue, allowing the Six6-Aes complex to displace Six3-Tle1 from their binding sites and releasing the repression state of the regulated loci. This would be in agreement with the observations that in humans, impairment of either *SIX3* or *SIX6* function is associated with different phenotypes (Gallardo et al., 1999; Wallis et al., 1999). When over-expressed, however, larger amounts of either SIX3 and SIX6 are readily available to interact indistinctly with either Tle1 and Aes. This results in a comparable behaviour where both SIX3 and SIX6 increase neuroblasts proliferation and impose retinal identity to 'competent' neural tissue (Bernier et al., 2000; Loosli et al., 1999; Toy et al., 1998; Zuber et al., 1999) (this report).

This repression-derepression strategy based on the differential interaction of closely related Six family members with Gro/Tle proteins could be extended conceptually to other *Six* genes. Indeed, Six1, Six2 and Six4 interact with both Tle1 and Aes in vitro.

In conclusion, Gro/Tle proteins participate in the genetic network that controls eye patterning in vertebrates. We propose that in vivo Tle1 and Aes do have differential interactions with Six3 and Six6, contributing to diversify the function of these two closely related *Six* genes. Whether the complex of Six3/6 with Tle/Aes is needed for eye specification throughout evolution, remains to be established. However, as shown here, Optix interacts with Groucho in a similar fashion and an *Aes* orthologue is present in the *Drosophila* genome (Chen and Courey, 2000), suggesting that *optix* activity in eye development may also require these cofactors.

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