

FoxG1 and TLE2 act cooperatively to regulate ventral telencephalon formation

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SUMMARY

FoxG1 is a conserved transcriptional repressor that plays a key role in the specification, proliferation and differentiation of the telencephalon, and is expressed from the earliest stages of telencephalic development through to the adult. How the interaction with co-factors might influence the multiplicity and diversity of FoxG1 function is not known. Here, we show that interaction of FoxG1 with TLE2, a *Xenopus tropicalis* co-repressor of the Groucho/TLE family, is crucial for regulating the early activity of FoxG1. We show that TLE2 is co-expressed with FoxG1 in the ventral telencephalon from the early neural plate stage and functionally cooperates with FoxG1 in an ectopic neurogenesis assay. FoxG1 has two potential TLE binding sites: an N-terminal eh1 motif and a C-terminal YWPMSPF motif. Although direct binding seems to be mediated by the N-terminal motif, both motifs appear important for functional synergism. In the neurogenesis assay, mutation of either motif abolishes functional cooperation of TLE2 with FoxG1, whereas in the forebrain deletion of both motifs renders FoxG1 unable to induce the ventral telencephalic marker *Nkx2.1*. Knocking down either FoxG1 or TLE2 disrupts the development of the ventral telencephalon, supporting the idea that endogenous TLE2 and FoxG1 work together to specify the ventral telencephalon.

KEY WORDS: FoxG1, TLE, *Xenopus*, Telencephalon

INTRODUCTION

The vertebrate forebrain is a complex neural network consisting of a wide variety of highly specified neurons and glia. The correct localisation and timed differentiation of a variety of neuron subtypes requires the coordinated action of a large number of signalling cascades and transcriptional regulators (Hebert and Fishell, 2008). One factor involved in the timing of neuronal differentiation and in the specification of subdomain identity is the transcriptional repressor FoxG1, a member of the Fox/Forkhead family of winged-helix transcription factors [previously known as BF-1 and renamed Foxg1 for mouse, FoxG1 for other chordates and FOXG1 for human (Kaestner et al., 2000)]. FoxG1 expression is conserved throughout vertebrates in dividing progenitors of the ventricular zone and early postmitotic neurons in the telencephalic neuroepithelium (Bourguignon et al., 1998; Murphy et al., 1994; Tao and Lai, 1992; Zhao et al., 2009). Loss- and gain-of-function experiments in different species have established FoxG1 as a maintenance factor for neural progenitors. Knockout of *Foxg1* leads to premature differentiation in the dorsal telencephalon and to a drastic increase in early-fate CR neurons accompanied by a loss of late progenitors (Hanashima et al., 2004; Xuan et al., 1995). Conversely, in gain-of-function experiments in frogs, a high dose of FoxG1 expands the progenitor population (Bourguignon et al., 1998; Hardcastle and Papalopulu, 2000), whereas in chicken it causes overgrowth of the

neural tube (Ahlgren et al., 2003). siRNA knockdown experiments have demonstrated a role for the level of FoxG1 in controlling the timing of cortical layer differentiation (Shen et al., 2006), suggesting a concentration-dependent activity.

A second important role of FoxG1 is during the specification of the ventral telencephalon (subpallium) and its delineation against the dorsal telencephalon (pallium) and basal diencephalon (hypothalamus). In the mouse *Foxg1* knockout, all markers for the ventral telencephalon are lost from an early stage, suggesting that the ventral telencephalon is never specified, while dorsal markers spread ventrally (Martynoga et al., 2005). In fish, morpholino (MO) knockdown experiments demonstrated a similar loss of the subpallium, accompanied by a ventral expansion of dorsal telencephalon markers and a ‘slippage’ of ventral telencephalic cells into the hypothalamic territory of the diencephalon (Danesin et al., 2009). Recently, it has been shown that Foxg1 is required cell-autonomously in the acquisition of ventral (subpallial) telencephalic identity in the mouse (Manuel et al., 2010).

How does FoxG1 exert these functions? At the molecular level, FoxG1 acts mainly as a transcriptional repressor by direct and indirect mechanisms (Bourguignon et al., 1998; Dou et al., 2000; Li et al., 1995; Seoane et al., 2004; Yao et al., 2001). FoxG1 shows direct repression on reporter constructs (Li et al., 1995; Yao et al., 2001), and in vivo it directly represses *p27Xic* and *wnt8b* (Hardcastle and Papalopulu, 2000; Danesin et al., 2009). However, it can also negatively regulate TGFβ signalling by binding to Smad and FoxO transcription factors (Dou et al., 2000; Seoane et al., 2004). The proliferation-promoting effect of mouse Foxg1 is independent of its DNA-binding ability (Dou et al., 2000; Hanashima et al., 2002). Transcriptional repression by FoxG1 is partly mediated by recruiting transcriptional co-repressors of the Groucho/Transducin-like enhancer of split (TLE) and AT-rich interaction domain (ARID) families (Sonderegger and Vogt, 2003; Tan et al., 2003; Yao et al., 2001). These co-repressors, in turn,

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recruit chromatin-modifying enzymes such as histone deacetylases to the transcription factor complex (Chen et al., 1999) or possess demethylation activity themselves (Yamane et al., 2007).

The Groucho/TLE family of transcriptional co-repressors is utilised by a large number of transcription factors to confer repressor activity. TLEs can enhance the activity of active repressors, turn inactive transcription factors into repressors, or even convert transcriptional activators into repressors. Groucho/TLEs are involved in the regulation of a variety of signalling pathways, including Notch, Wnt, TGF β superfamily and EGF signalling, and show partially overlapping expression in various developing tissues (Buscarlet and Stifani, 2007; Chen and Courey, 2000; Gasperowicz and Otto, 2005; Hasson and Paroush, 2006; Zamparini et al., 2006). Expression of TLE1 and TLE3 has been reported in the ventricular zone of the murine telencephalon (Dehni et al., 1995; Leon and Lobe, 1997) and overexpression of TLE1 causes a delay or inhibition of neurogenesis in vivo and in cultured cortical neurons (Nuthall et al., 2004; Yao et al., 2000). Even though the full mechanism of TLE inhibition of neurogenesis in the developing forebrain still has to be resolved, TLE1 has been shown to bind a group of transcription factors involved in telencephalon patterning and differentiation, including Hes1, FoxG1 and members of the Six family (Kobayashi et al., 2001; Nuthall et al., 2002; Yao et al., 2001). Transfected FoxG1 acts as a repressor of cortical neurogenesis and this effect can be enhanced by TLE1 or reversed by the distant family member TLE6 (Marcal et al., 2005). Since FoxG1 is co-expressed with members of the Groucho/TLE family (Yao et al., 2001), we hypothesised that there might be a specific requirement for individual TLEs for the activity of FoxG1 in forebrain development.

Here, we describe the cloning and characterisation of *Xenopus tropicalis* TLE2, about which little was known. We show that a conserved N-terminal eh1 motif is necessary for the physical interaction of FoxG1 with TLE2, whereas the C-terminal domain, which has previously been suggested to contain a TLE binding motif, is unnecessary. Nevertheless, we find that the C-terminal domain is necessary for the functional synergism of FoxG1 with TLE2, either alone or in combination with the N-terminal domain. We also show that FoxG1 is co-expressed with TLE2 in the ventral telencephalon (subpallium), and either FoxG1 or TLE2 knockdown abolishes or reduces the development of this region. Our findings suggest that a spatially restricted member of the Groucho/TLE family, TLE2, interacts with FoxG1 to specify the ventral telencephalon.

MATERIALS AND METHODS

Sequences and constructs

BLAST searches with human TLE sequences were performed in an EST database (<http://informatics.gurdon.cam.ac.uk/online/xt-fl-db.html>) and EST clusters were retrieved.

Clusters Xt7.1-ANBT192.5.5, Xt7.1-TEgg056g07.3 and Xt7.1-TGas107e13.3 contain the homologue of *TLE1*, *TLE2* and *TLE4*, respectively; Xt7.1-CABD14417.5 contains the homologue of the short form *AES*. *X. tropicalis* sequence IDs: TLE1 (GU014558), TLE2 (GU014559), TLE4 (GU014560) and AES (GU014561). Human sequence IDs: TLE1 (NP_005068.2), TLE2 (AAH17364), TLE3 (AAH43247) and TLE4 (NP_008936). Additional BLAST search with the JGI *X. tropicalis* genome server (<http://genome.jgi-psf.org/Xentr4/Xentr4.home.html>) confirmed these results and did not identify any other sequences. Sequence alignments were performed by CLUSTALW (Larkin et al., 2007) on <http://align.genome.jp/> using standard settings.

To generate antisense RNA probes, we used the following single EST clones: TGas001p14 for *TLE1*, TEgg002e09 for *TLE2*, TGas107e13 for *TLE4* and TGas097a10 for *AES*.

Full-length constructs and Flag-tagged constructs were cloned from EST sequences by PCR and inserted into pCS2 using the following restriction sites: *TLE1* was cloned via *Xba*I, *TLE2* via *Xba*I, *TLE4* via *Eco*RI/*Xba*I and *AES* via *Eco*RI/*Xba*I. Since there was no full-length clone available for *TLE4*, the sequence was first amplified from *X. tropicalis* stage (st.) 19 cDNA using 5'-GCATTAGGCTTACTATTAACACAAGGAGTC-3' and 5'-TACAAGTATAATACAAATTCAGAGAATCACAA-3'. Flag epitopes (DYKDDDDK) were introduced by linker-PCR at the N-terminus directly after the start methionine. *X. tropicalis* FoxG1 was not found in the EST database and was PCR cloned from genomic sequence in pSC2. *X. tropicalis* FoxG1 has no introns in the coding sequence.

Xenopus laevis FoxG1-HA has been described elsewhere (Regad et al., 2007) and was used as a template for all mutants. FoxG1-N3A-HA was generated by mutating I20, L23 and V24 into alanines using the Stratagene QuikChange II Site-Directed Mutagenesis Kit and the QuikChange Primer Design Program (www.stratagene.com) to design oligos. Similarly, FoxG1-C4A-HA was generated by mutating L309, V311, L314 and V315 into alanines. FoxG1-HA constructs to mutate F233, F246, W255 and F260 into alanines were generated the same way. For FoxG1- Δ C-HA, sequences encoding amino acids 1-253 and 261-436 were amplified by recombinant PCR using 5'-TGGTGCAAGGAGAGCAGGGAGCCGCTCTATC-CATAAAGGT-3' and 5'-GCGGGCTCCCTGCTCTCCTTGACACCAC-CCAAGGGCCAGC-3' as inner primers and SP6 and T7 as outer primers. The final fragment was ligated, following *Eco*RI/*Xba*I digestion, into pCS2.

mRNA injections and embryo processing

Xenopus embryos were injected in one cell at the two-cell stage and processed as described (Bourguignon et al., 1998). *lacZ* mRNA (200 pg) was co-injected as a lineage tracer. All mRNA injections were performed in *X. laevis*, using *X. laevis* FoxG1 and *X. tropicalis* TLE2 mRNA. Antisense probes for *X. laevis* *N-tubulin* and *X. tropicalis* *elrC* have been described previously (Bourguignon et al., 1998; Carruthers et al., 2003). For other *X. tropicalis* antisense probes we used the following ESTs: for *Dlx5*, clone TNeu071c08; for *TLE1*, TGas001p14; for *TLE2*, TEgg002e09; for *AES*, TGas097a10; and for *emx1*, TNeu056k18. *emx1* was cloned by PCR into pSC2.

X-Gal staining and in situ hybridisation were carried out as described (Bourguignon et al., 1998).

MO design and injection

All MO experiments were performed in *X. tropicalis* with MOs designed against the *X. tropicalis* genes. MOs were designed and produced by Gene Tools. All MOs used were FITC labelled. The Control MO is the standard Gene Tools control 25-mer (5'-CCTCTTACCTCAGTTACAATTTATA-3'). The upstream/ATG FoxG1 MO is 5'-TCACAAGGATTGGAGCCG-GACACTC-3' (-26 to -2); the TLE2 exon 1-intron 1 splice MO is 5'-ACGTCACACAACACACTTACCGGAG-3'. To detect the efficiency of the TLE2 exon 1-intron 1 splice MO, we performed semi-quantitative RT-PCR using primers that anneal in exon 1 (5'-CAAACCGAGACAGTCC-CACT-3') and exon 3 (5'-TTTCTGTTGCCAGCTTTTCA-3') of *TLE2*. The predicted product size is 220 bp. Interfering with the splicing event would lead to larger products or to premature degradation of the mRNA.

Cell culture transfections

HEK 293T cells from ATCC (30-2002) were maintained in DMEM supplemented with 10% FBS (DE14-801FH, Bio-Whittaker) and penicillin/streptomycin. Individual plasmid DNA constructs were transfected into cells plated in 6-well plates with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The following amounts were used: GFP, 1 μ g; FoxG1-HA and FoxG1 mutants, 0.5 μ g; TLE1-Flag and TLE2-Flag, 1.5 μ g. Each transfection was supplemented with pCS2 to a total of 2 μ g DNA.

Co-immunoprecipitation and western blotting

Co-immunoprecipitation (co-IP) and western blotting of HEK 293T cells were performed by standard methods. Forty-eight hours post-transfection, PBS-washed HEK 293T cells were scraped using 0.5 ml IP-lysis buffer (50

mM Tris-acetate pH 7.5, 300 mM NaCl, 1 mg/ml BSA, 2% IGEPAL or NP40, protease inhibitors) and rotated for 20 minutes at 4°C followed by 10 minutes centrifugation at 16,000 g.

Supernatant was collected as cell lysate and 10 µl kept for expression analysis. Cell lysates were supplemented with another 0.5 ml IP-lysis buffer and 1.5 µg anti-Flag antibody (F1804, Sigma) and rotated for 3 hours at 4°C. Following the addition of 30 µl preincubated Protein A/G Sepharose (sc-2003, Santa Cruz), samples were rotated overnight at 4°C. After washing three times with IP-washing buffer (50 mM Tris-acetate pH 7.5, 300 mM NaCl, 0.5% IGEPAL, 0.1% SDS), Sepharose beads were incubated with 30 µl SDS-loading buffer and, after heating for 10 minutes at 95°C and centrifugation for 3 minutes at 16,000 g, the supernatant was loaded on a 10% SDS-PAGE gel.

Western blotting was performed in a semi-dry system according to the manufacturer's instructions (Hoefer). PVDF membrane (Millipore) was blocked with 5% dry milk powder in PBS containing 0.1% Tween 20 for 30 minutes followed by incubation with anti-Flag-HRP (1:1000; A8592, Sigma) or anti-HA-HRP (1:1000; 12013819001, Roche) antibodies in blocking buffer overnight. Following three washing steps in PBS containing 0.1% Tween 20, blots were developed using ECL reagents (GE Healthcare).

Cryosectioning and immunohistochemistry

For *X. tropicalis* FoxG1 immunohistochemistry, embryos were fixed for 24 hours at 4°C in Dent's Fixative (80% methanol, 20% DMSO), washed three times for 10 minutes in PBS, transferred into 15% fish gelatine/15% sucrose for 16 hours, sectioned on a cryostat and stained following standard protocols (Regad et al., 2007).

For tissue culture immunohistochemistry, HeLa cells grown on coverslips were fixed with PBS containing 4% paraformaldehyde for 20 minutes and processed as described (Regad et al., 2007).

The following primary antibodies were used: rabbit anti-FoxG1 (1:50) (Regad et al., 2007), mouse anti-phospho-histone H3 (anti-pH3; 1:500, Upstate) and rat anti-HA (1:200, Roche).

For sectioning after whole-mount in situ hybridisation, embryos were processed as above and dried sections (2 hours at room temperature) were treated for 2 minutes with acetone followed by 2 minutes in PBS containing 0.1% Triton X-100 and then embedded in 90% glycerol.

TUNEL staining

X. tropicalis embryos were injected in one cell at the two-cell stage with 7.5 ng of the respective FITC-conjugated MO (Control, TLE2 or FoxG1 MO). At st. 28, embryos were fixed and sectioned at 12 µm using a Leica CM3050 S cryostat. TUNEL staining was performed using the TMR Red In Situ Cell Death Detection Kit according to the manufacturer's instructions (Roche). The injected side was identified using mouse anti-FITC primary (1:250, Roche) and Alexa Fluor 488 goat anti-mouse secondary (1:500, Molecular Probes) antibodies.

Electroporation

X. tropicalis embryos were injected at the one-cell stage with 15 ng *X. tropicalis* FoxG1 MO. At st. 25, embryos were injected in the brain ventricle with 30 µl of a mixture of 1 µg/µl CMV-β-gal and 100 ng/µl CMV-FoxG1 DNA (*X. laevis*) or mutated versions thereof (note that the *X. tropicalis* MO does not recognise *X. laevis* FoxG1 as it is targeted to an upstream region that is not present in the *X. laevis* construct). Immediately after injection, embryos were electroporated using home-made platinum electrodes and an SD9 stimulator (Grass Technologies) at the following settings: 20V, 50-millisecond pulse duration, 1-second interpulse space, six pulses. Embryos were fixed and analysed for β-gal and Nkx2.1 expression at st. 31-32.

RESULTS

Isolation of *X. tropicalis* TLEs

We scanned the largest available *X. tropicalis* EST collection (<http://informatics.gurdon.cam.ac.uk/online/xt-fl-db.html>) for homologues of the six human members of the Groucho/TLE family (Gasperowicz and Otto, 2005). Three of the resulting four hits could easily be assigned as the homologues of human *TLE1*, *TLE4* and *AES* based on sequence homology. The last sequence had only 64%

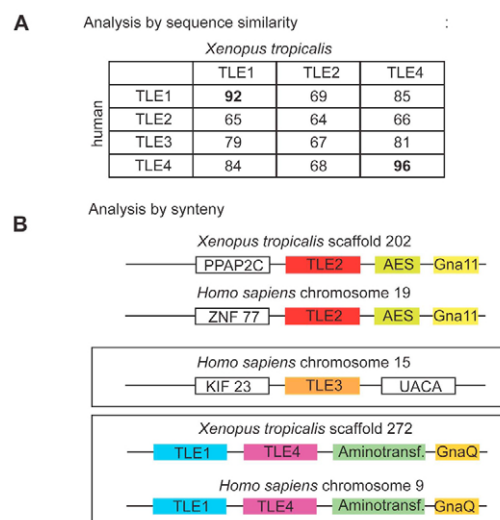


Fig. 1. Assignment of *X. tropicalis* TLE homologues by sequence homology and synteny. (A) *X. tropicalis* and human TLE1 and TLE4 are clearly homologous but TLE2 is difficult to assign to a human TLE based on sequence similarity. (B) However, synteny analysis reveals that *X. tropicalis* and human TLE2 share the same chromosomal neighbours, including AES. Similarly, TLE1 and TLE4 show the same inverted tandem chromosomal arrangement in both species.

and 67% identity to human TLE2 and TLE3, respectively. However, synteny analysis revealed that the sequence has the same neighbouring chromosomal genes as human and mouse *TLE2/Tle2* (Fig. 1). We therefore decided to name this gene *X. tropicalis* TLE2, a decision supported by phylogenetic tree analysis (see Fig. S1 in the supplementary material). The adjacent gene to TLE2 in the human, murine and *Xenopus* genome is *AES/Grg5*, a truncated member of the TLE family that has been generated by tandem gene duplication (Bajoghli, 2007) and shows dominant-negative activity (Roose et al., 1998). Also, TLE1 and TLE4 are arranged adjacent to each other in both the human and *Xenopus* genome. *X. tropicalis* TLE1, TLE2, TLE4 and AES were fully sequenced and TLE1, TLE2 and TLE4 were shown to have the typical Groucho family domain structure (Fig. 2). *Xenopus laevis* TLE2, TLE4 and AES have been cloned before and named *ESG1*, *Xgrg-4* and *AES/Xgrg-5*, respectively (Choudhury et al., 1997; Roose et al., 1998).

Expression of TLEs and FoxG1 in *X. tropicalis*

At the neural plate stage (st. 16), all three TLEs (*TLE1*, *TLE2* and *AES*) were expressed throughout the anterior neural plate (ANP). Expression of *TLE1* appeared to be stronger at the anterior-most neural fold, similar to *FoxG1* expression in the presumptive telencephalic area (Fig. 3, left column). For *TLE2* and *AES*, the strongest expression was in a small horseshoe-shaped area in a more medial part of the ANP that is fated to give rise to the ventral diencephalon (Eagleson and Harris, 1990). At late neurula stage (st. 22-23) *TLE1*, *TLE2* and *AES* were partially co-expressed, showing areas of stronger and weaker expression along the neural tube as well as in the eye primordium. In their anterior-most expression they all extended into the ventral forebrain region and possibly overlapped with *FoxG1* (Fig. 3, middle column). At early tadpole stage (st. 28), the expression of the individual TLEs became more specific (Fig. 3, right column). *TLE1* showed strong expression in the telencephalon, the eye, mid- and hindbrain and the branchial

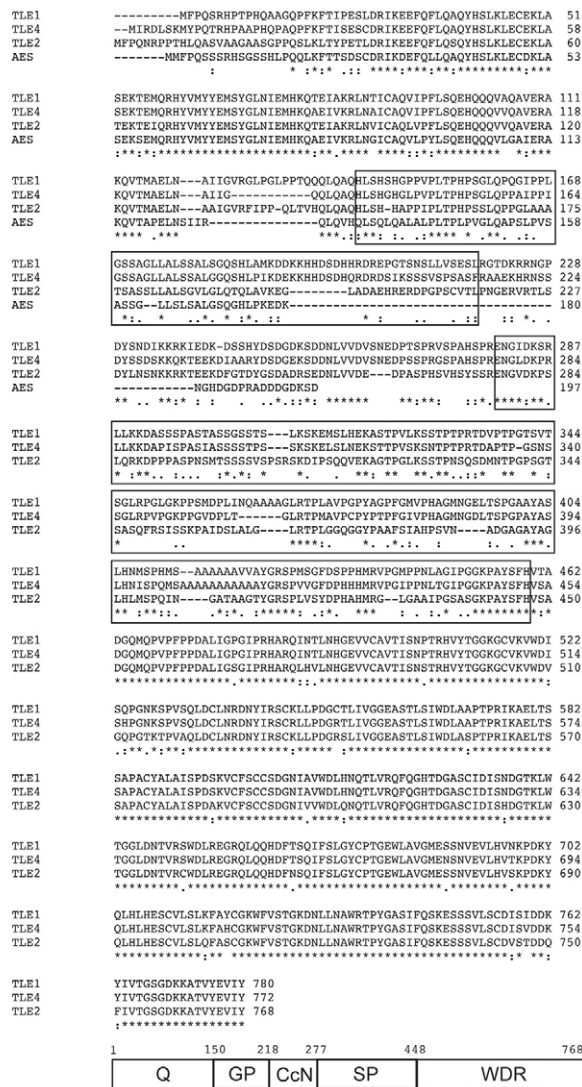


Fig. 2. Amino acid sequence alignment of *X. tropicalis* TLE1, TLE2, TLE4 and AES. The domain structure of a typical Groucho protein is shown diagrammatically at the bottom (reviewed by Jennings and Ish-Horowicz, 2008). The numbers on the diagram correspond to the amino acid sequence of *X. tropicalis* TLE2. The Q domain is responsible for homo- and heterodimerisation with other TLEs (Grbavec et al., 1998), the adjacent GP (glycine/proline-rich) domain recruits histone deacetylases (HDACs), the CcN domain contains phosphorylation sites and a nuclear localisation signal and SP is a serine/proline-rich domain. The WDR (WD Repeat) domain is highly conserved and most transcription factors interact with TLEs via this domain. To demarcate the different domains, the GP and SP domains are boxed in the sequence alignment. Definitions of conservation features: asterisk, positions that have a single, fully conserved residue; colon, one of the following ‘strong’ groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW; full-stop, one of the following ‘weaker’ groups is fully conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY.

arches and weaker expression along the neural tube. *TLE2* appeared to be expressed in two stripes in the midbrain/diencephalon area and in the eye, weakly along the neural tube and diffusely in the branchial arch region. However, in whole-mounts, *TLE2* appeared to be absent from the telencephalon (Fig. 3, red arrowhead). *AES*

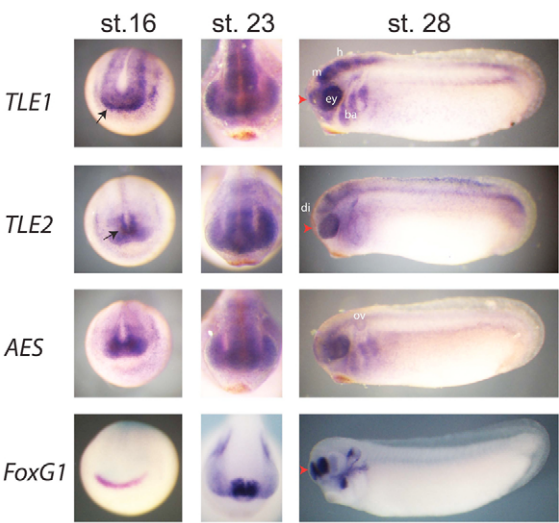


Fig. 3. Expression of *X. tropicalis* TLEs. Expression patterns of *TLE1*, *TLE2*, *AES* and *FoxG1* as shown by whole-mount in situ hybridisation of *X. tropicalis* embryos at neural plate stage (st. 16; frontal view), early tailbud (st. 22–23; frontal view), and late tailbud (st. 28; lateral view). *TLE1* and *TLE2* show expression throughout the anterior neural plate (st. 16), but *TLE1* is strongest in the anterior neural ridge (black arrow), whereas *TLE2* is strongest in a more medial ridge (black arrow). Note the expression of *TLE1*, but not *TLE2*, in the forebrain of st. 28 embryos, which largely overlaps with the telencephalic expression of *FoxG1* (telencephalon indicated by red arrowheads). Ey, eye; m, midbrain; h, hindbrain; ba, branchial arches; di, diencephalon; ov, otic vesicle.

showed expression in the forebrain, eye, branchial arches, otic vesicle and along the neural tube, similar to the expression of *AES* in *X. laevis* (Molenaar et al., 2000). The expression of *TLE4* was low and diffuse (data not shown), unlike *X. laevis* *TLE4* (*Xgrg-4*), which is widely expressed but is higher in neural tissues (Molenaar et al., 2000). *FoxG1* was expressed throughout the telencephalon, the olfactory placodes and the cranial nerves as well as in the anterior retina. This expression pattern is identical to that of *X. laevis* *FoxG1* and the two gene products are 96% identical at the amino acid level (see Fig. S2 in the supplementary material).

In cleared or sectioned whole-mount embryos at st. 22 (Fig. 4D), *X. tropicalis* *TLE1* and *TLE2* were expressed in the forebrain and also in more posterior parts of the CNS. Within the telencephalon, *TLE1* showed a substantial overlap with *FoxG1* (Fig. 4B,G), whereas *TLE2* overlapped with *FoxG1* only in the ventral-most telencephalon (Fig. 4B,K,E). *FoxG1* and *TLE2*, but not *TLE1*, were also co-expressed in the anterior optic chiasm, which is the ventral boundary of the telencephalon with the diencephalon (Fig. 4C,H,L) (Bachy et al., 2002).

FoxG1 synergises with TLE2

FoxG1 affects neuronal differentiation in a dose-dependent manner when it is ectopically overexpressed in *Xenopus* embryos (Bourguignon et al., 1998). A low dose of *FoxG1* cell-autonomously enhances neurogenesis, whereas a higher dose cell-autonomously suppresses endogenous neurogenesis. Thus, *FoxG1* misexpression forms a convenient assay to test for synergism of a factor with *FoxG1*. To examine whether *FoxG1* and *TLE2* work together, we injected *TLE2* mRNA together with a low dose of *FoxG1*. Low-dose injection of *FoxG1* mRNA alone

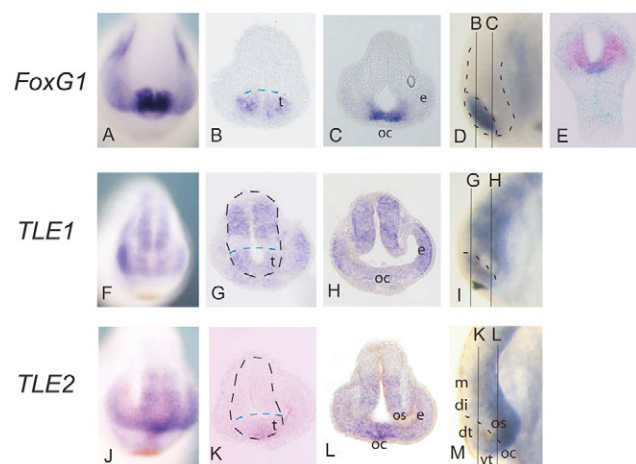


Fig. 4. Region-specific expression of *TLE1* and *TLE2* in the developing telencephalon. (A–M) Whole-mount in situ hybridisation for *FoxG1* (A–E), *TLE1* (F–I) and *TLE2* (J–M) in *X. tropicalis*. All images except E (st. 28) represent st. 22 embryos. (B,C,G,H,K,L) Transverse sections (20 μ m) of whole-mount stained embryos. The neural tissue is outlined by black dashed lines, and the dorsal border of the telencephalon is indicated by blue dashed lines. The plane of the sections is shown in cleared embryos (D,I,M, anterior to the left, eyes removed). Black dashed lines in G and K indicate the telencephalon and the whole brain tissue. (E) A double in situ stained for *FoxG1* (purple) and *TLE2* (blue) at st. 28; the plane of section is similar to C. di, diencephalon; dt, dorsal telencephalon; e, eye; oc, optic chiasm; os, optic stalk; m, mesencephalon; t, telencephalon; vt, ventral telencephalon. Annotation based on Bachy et al. (Bachy et al., 2002).

caused ectopic neurogenesis (97%, $n=39$) on the injected side (Fig. 5A,G), whereas high-dose injection resulted in suppression of the lateral stripe of primary neurogenesis and in ectopic neurons adjacent to the injected region (90%, $n=39$) (Fig. 5B,C,G), as previously described (Bourguignon et al., 1998). Injection of 200 pg *TLE2* mRNA had no effect (82%, $n=40$) (Fig. 5D,G) or resulted in minor ectopic neurogenesis (18%, $n=40$), but co-injection with 50 pg *FoxG1* resulted in a predominant high-dose phenotype (58%, $n=38$) (Fig. 5E–G). This shows that the presence of the co-repressor *TLE2* can change the activity of *FoxG1*. We were not able to test *TLE1* in this assay as it was very poorly expressed in the embryo from the injected mRNA (data not shown).

TLE2* and *FoxG1* physically interact via an N-terminal eh1 domain in *FoxG1

We next asked whether TLEs physically interact with *FoxG1*. Co-immunoprecipitation (co-IP) and western blotting of transfected HEK 293T cells showed that both *TLE2* and *TLE1* interact with *FoxG1*. The interaction with *TLE1* might be weaker, as consistently less *FoxG1* was immunoprecipitated with *TLE1* than with *TLE2* (Fig. 5H).

The binding of TLEs to their target transcription factors is, in most cases, mediated via their WDR domain. This domain recognises two conserved motifs in the proteins that TLEs interact with: a WRPW motif and an engrailed homology 1 (eh1) domain, FXIXXIL (reviewed by Buscarlet and Stifani, 2007; Gasperowicz and Otto, 2005), which is present in eight classes of the homeodomain protein superfamily and in more than 50% of all members of the Fox family (Buscarlet and Stifani, 2007; Gasperowicz and Otto, 2005;

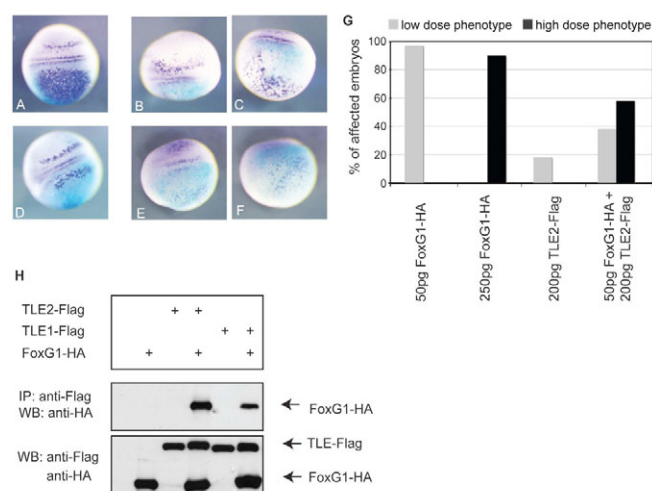


Fig. 5. *FoxG1* interacts physically and cooperates functionally with *TLE2*. (A–F) *X. laevis* embryos were injected at the two-cell stage and processed for X-Gal staining (light blue) and whole-mount in situ hybridisation for *N-tubulin* (purple) at st. 16. Low-dose injection of *X. laevis FoxG1* (50 pg) gives rise to ectopic neurogenesis throughout the lateral ectoderm (A), whereas high-dose injection (250 pg) causes a complete suppression of endogenous neurogenesis on the injected side and ectopic neurogenesis outside the X-Gal staining area (B,C). Injection of *X. tropicalis FoxG1* mRNA gave rise to similar phenotypes (not shown). Co-expression of 200 pg *X. tropicalis TLE2* with 50 pg *X. laevis FoxG1* (E,F) gives rise to a phenotype that is similar to high-dose injections (250 pg) of *FoxG1* alone (B,C). *TLE2* alone has no effect (D). (A,B,D,E) Dorsal views; (C,F) lateral views. In all panels, anterior is to the left. (G) Percentage of low-dose and high-dose phenotypes resulting from each injection. (H) Co-immunoprecipitation of *X. laevis FoxG1*-HA and *X. tropicalis TLE*-Flag with anti-Flag antibody followed by western blotting with anti-HA. The bottom panel shows loading of equal amounts of lysates followed by western blotting with anti-Flag and anti-HA. *TLE2*-Flag strongly interacts with *FoxG1*-HA, whereas *TLE1*-Flag expressed at similar levels shows slightly weaker binding.

Yaklichkin et al., 2007b). An interaction between human *TLE1* and murine or chicken *Foxg1* has been mapped to a C-terminal domain of *Foxg1* (Sonderegger and Vogt, 2003; Yao et al., 2001), comprising amino acids 276–336 of mouse *Foxg1* (which corresponds to amino acids 229–292 in *Xenopus FoxG1*). This domain contains a peptide that shows structural similarity to WRPW motifs and is conserved in *Xenopus FoxG1* [Y254WPMSFSLSH (Yao et al., 2001)].

To test the potential of each motif to contribute to the interaction between *FoxG1* and *TLE2*, we changed either the three conserved hydrophobic amino acid residues of the N-terminal eh1 to alanines (*FoxG1*-N3A-HA) or deleted the C-terminal motif (amino acids 254 to 260, *FoxG1*- Δ C-HA; see Fig. 6A,B). In transfected HEK 293T cells, the C-terminal mutation did not have an effect on *TLE2* binding, whereas *FoxG1*-N3A-HA lost the interaction with *TLE2* (Fig. 6C). A double mutant, *FoxG1*-N3A- Δ C-HA, also did not interact with *TLE2* (Fig. 6C). *FoxG1* contains a double CK1-phosphorylation motif near the N-terminus that is required for its nuclear localisation (Regad et al., 2007). This phosphorylation site overlaps with the N-terminal eh1 motif and the lack of interaction between *FoxG1*-N3A-HA and *TLE2* could be due to a failure of *FoxG1* to localise to the nucleus. However, in transfected HEK 293T cells, both wild-type and mutant *FoxG1* were nuclear (Fig. 6E, right panel).

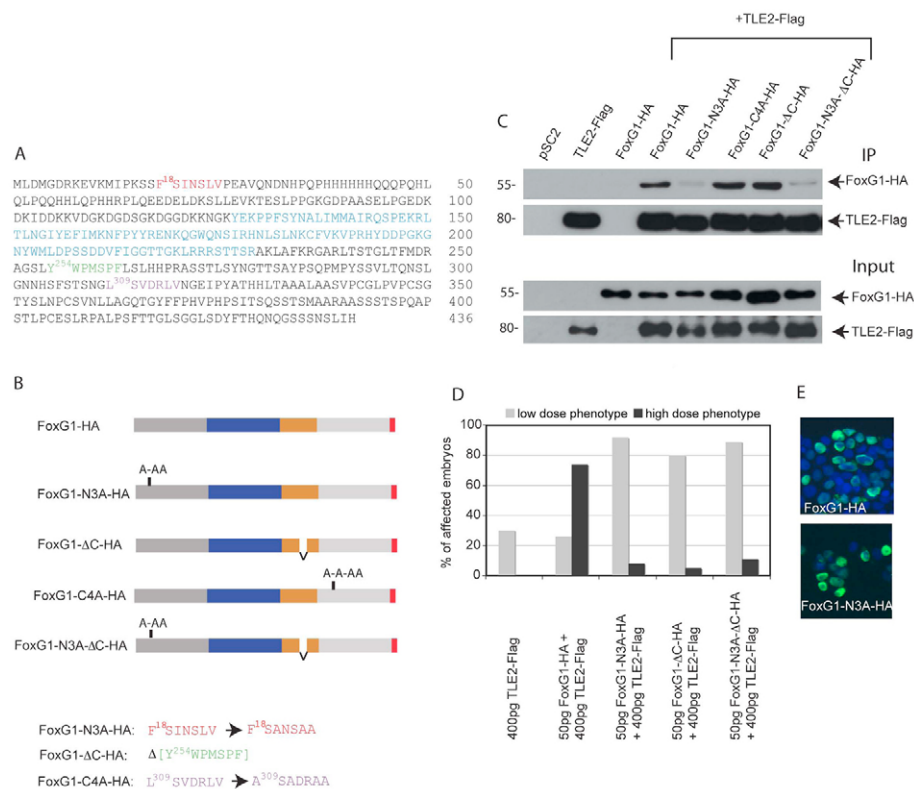


Fig. 6. Motifs in FoxG1 that are responsible for the binding and functional cooperativity of FoxG1 and TLE2. (A) Amino acid sequence of *X. laevis* FoxG1 and potential TLE binding motifs. The DNA-binding domain is shown in blue, the N-terminal eh1 motif is highlighted in red, the C-terminal WRPW-like motif in green and the cryptic eh1 motif in purple. (B) Schematic representation of the FoxG1 mutants. The N-terminal domain is shown in dark grey, the DNA-binding domain in blue, the potential TLE1 interaction domain in orange and the HA epitope in red. Beneath is shown the sequence of mutated motifs (same colour coding as in A). (C) Immunoprecipitation (IP) of HEK 293T cells transfected with FoxG1-HA or FoxG1 mutants together with TLE2-Flag, showing that mutation of the N-terminal eh1 motif (FoxG1-N3A-HA), either alone or simultaneously with the C-terminal motif (FoxG1-N3A-ΔC-HA), leads to loss of binding to TLE2. The top two panels show IP with anti-Flag followed by western blotting with anti-Flag and anti-HA. Lower panels show the input (pre-IP lysates) analysed by western blotting with anti-Flag and anti-HA. (D) The phenotypes of embryos injected with the indicated amounts of *X. tropicalis* TLE2 and *X. laevis* FoxG1 or FoxG1 mutant mRNA were scored as low-dose or high-dose as in Fig. 5. TLE2 does not cooperate with any of the three mutants to convert a low-dose FoxG1 injection into a high-dose phenotype. (E) HEK 293T cells transfected with either FoxG1-HA or FoxG1-N3A-HA. Both proteins are nuclear.

A computational analysis of eh1 motifs within the Fox family of transcription factors revealed that in addition to the fully conserved eh1 motif at the N-terminus (F18SINSLV), there is a 'remnant' eh1 motif in the C-terminal region of FoxG1 that lacks the highly conserved phenylalanine [L309SVDRLV (Yaklichkin et al., 2007b)]. Mutation of the four hydrophobic amino acid residues of the remnant C-terminal eh1 motif to alanines (FoxG1-C4A-HA) did not affect TLE binding (Fig. 6C) and was not investigated further. In parallel, we tested the ability of all FoxG1 mutants to bind TLE1, and the results were very similar to those obtained with TLE2 (data not shown).

Both the N-terminal and C-terminal domains are responsible for functional cooperation with TLE2

To address the functional activity of the FoxG1 mutants, we assessed the potential of the mutants to convert a low-dose FoxG1 mRNA injection into a high-dose phenotype. Injection of 400 pg TLE2 mRNA alone had only a minor effect on neurogenesis, showing a mild low-dose phenotype (30%, $n=61$) (Fig. 6D). Injection of wild-type FoxG1 together with TLE2 showed a strong conversion to a high-dose phenotype (74%, $n=46$); however, the FoxG1-N3A-HA and the double mutant FoxG1-N3A-ΔC-HA produced a very low

percentage of the high-dose phenotype when co-injected with TLE2 (8%, $n=50$ and 11%, $n=45$, respectively). Surprisingly, the deletion mutant FoxG1-ΔC-HA was unable to efficiently convert a low-dose injection into a high-dose phenotype in the presence of TLE2 (5%, $n=66$) (Fig. 6D). All three FoxG1 mutants were able to induce a low-dose or high-dose phenotype when overexpressed alone, although the double mutant showed a reduced low-dose phenotype, in the sense that the number of ectopic neurons observed was lower (data not shown). This suggests that all mutants are expressed and are functionally active and, in addition, that the double mutant is more severe than the single mutants. Taken together, these results suggest that the N-terminal eh1 motif within FoxG1 plays a crucial role in the physical and functional interaction with TLEs. The C-terminal motif does not appear necessary for interaction with TLE2, but it is required for functional synergism, which could be indirect.

Knockdown of FoxG1 or TLE2 leads to reduction of the ventral telencephalon

If FoxG1 and TLE2 interact in vivo in the developing forebrain we would expect common aspects in their respective knockdown phenotypes. FoxG1 is encoded by an intronless gene and was knocked down by an ATG/upstream MO, whereas TLE2 was

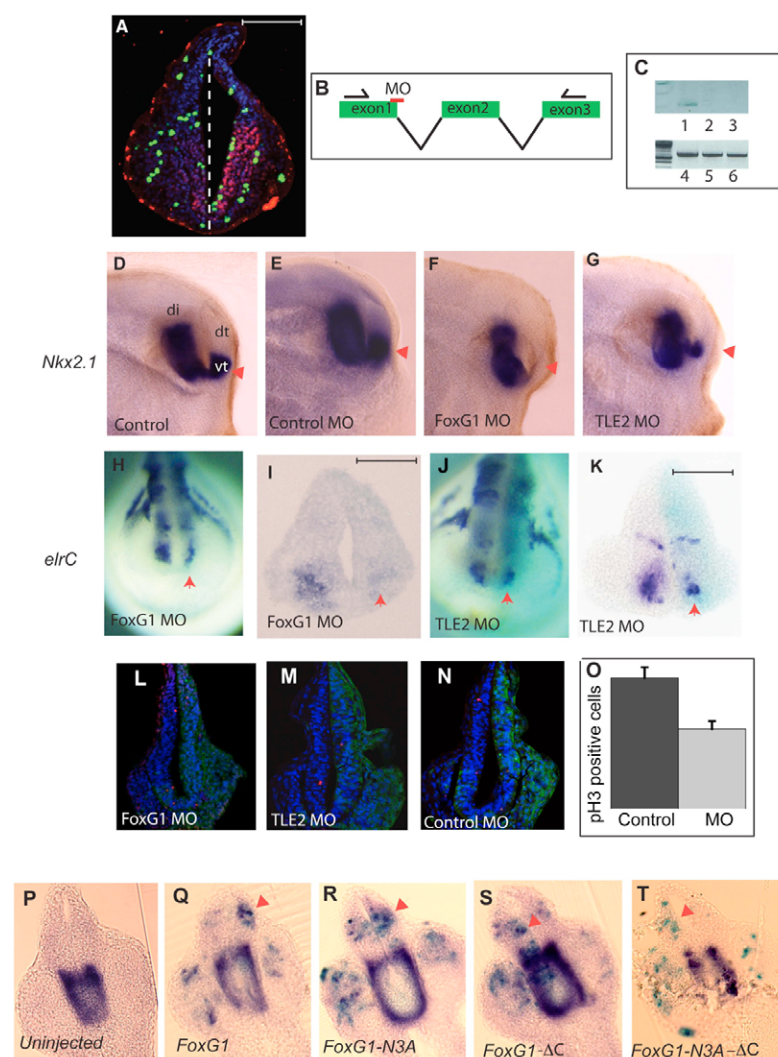


Fig. 7. The effect of FoxG1 and TLE2 MO on forebrain development. (A) Transverse sections of forebrain of FoxG1 MO-injected (7.5 ng) embryos (st. 28) stained with DAPI (blue), anti-pH3 (green) and anti-FoxG1 (red). Note the reduction of FoxG1 staining and in pH3-positive cells on the MO-injected (left) side in the presumptive FoxG1 expression region. Dashed white line indicates the midline. (B) Schematic representation of the TLE2 splice MO (red) and location of PCR primers in relation to the exon-intron arrangement of TLE2. (C) Semi-quantitative PCR from embryos injected with 10 ng Control MO (lanes 1, 4), 10 ng TLE2 MO (lanes 2, 5) or 20 ng TLE2 MO (lanes 3, 6). Primers used were *ornithine decarboxylase* (*ODC*) as control (lanes 4-6) and TLE2 exon 1 and TLE2 exon 3 (lanes 1-3) (see B). (D-G) *Nkx2.1* expression in the forebrain of st. 34 control embryos and embryos injected at the one-cell stage with 10 ng Control MO (E), 15 ng FoxG1 MO (F) and 15 ng TLE2 MO (G). The expression in the ventral telencephalon (red arrowhead) is reduced or missing in TLE2 and FoxG1 morphant embryos (F,G). di, diencephalon, dt, dorsal telencephalon, vt, ventral telencephalon. (H-N) Embryos injected in one cell at the two-cell stage with FoxG1 MO (7.5 ng) and TLE2 MO (7.5 ng) processed for in situ hybridisation for *elrC* (H-K) and TUNEL staining (red nuclei; L-N). Injected side is to the right, as marked by X-Gal staining (H-K) or FITC (L-N). (H,J) Frontal views; (I,K-N) transverse sections through the forebrain of st. 19 (H-K) or st. 32 (L-N) embryos. Reduced forebrain *elrC* expression on the MO-injected side is indicated by red arrows. There was no statistically significant increase in TUNEL staining. (O) In FoxG1 MO-injected forebrains there was a 40% decrease ($P=0.003$, paired *t*-test, $n=12$ embryos) in pH3-positive cells. (P-T) Transverse sections through the forebrain of FoxG1 MO-injected embryos, subsequently electroporated with wild-type FoxG1 and various FoxG1 mutated versions, as indicated, together with *lacZ* DNA and analysed for *Nkx2.1* expression. Light blue (X-Gal staining) indicates cells that have taken up the electroporated DNA (red arrowheads indicate examples of electroporated cells); dark purple indicates cells expressing *Nkx2.1*. The experiment was repeated four times with similar results. See Materials and methods for details. Scale bars: 100 μ m.

knocked down by injection of a splice MO targeted to the exon 1-intron 1 boundary. The knockdown of FoxG1 protein was determined by immunohistochemistry with anti-FoxG1 antibody (Fig. 7A), and the effectiveness of the TLE2 splice MO was assessed by aberrant splicing as detected by PCR (Fig. 7B,C).

To assess possible similarities in the loss-of-function phenotype we injected Control, FoxG1 or TLE2 MO into *X. tropicalis* embryos and performed whole-mount in situ hybridisation for the ventral telencephalon marker *Nkx2.1*. *Nkx2.1* is also expressed in the diencephalon, but within the telencephalon it is good marker for the ventral-most part of the subpallium: the pallidum or medial ganglionic eminence (Bachy et al., 2002). In FoxG1 MO-injected embryos, the subpallial expression of *Nkx2.1* was entirely missing (100%, $n=11$), consistent with previous data from mouse and fish (Martynoga et al., 2005; Manuel et al., 2010; Danesin et al., 2009), and in TLE2 MO embryos it was greatly reduced (70%, $n=10$) (Fig. 7G).

To assess whether FoxG1 knockdown also affects neurogenesis in the *Xenopus* forebrain, we studied the effect of FoxG1 and TLE2 MO knockdown on *elrC* expression, which is one of the earliest markers of neuronal differentiation, being expressed in cells undergoing the transition from proliferation to differentiation (Carruthers et al., 2003; Good, 1995; Perron et al., 1999). We observed a reduction in the expression domain of *elrC* in the

forebrain of st. 19 embryos after either FoxG1 or TLE2 MO injection (Fig. 7H-K). Loss of *elrC* expression could indicate a failure of early neural specification, increased cell death or a failure to exit the progenitor compartment. We did not observe a statistically significant increase in cell death with either MO (Fig. 7L,M,N; see Fig. S3E in the supplementary material). Failure to exit the progenitor compartment should result in an increase in proliferation. However, we found a significant reduction (by 40%) in pH3-positive cells in the FoxG1 MO-injected side as compared with the control side in both st. 19 and st. 28 embryos ($n=12$, $P=0.003$) (Fig. 7O; see also anti-pH3-stained green nuclei in Fig. 7A), suggesting that the loss of *elrC* is due to a loss of neural specification in the ventral forebrain, rather than to any prolonged maintenance of cells in the progenitor state.

To address whether the loss of ventral telencephalic fate is accompanied by an increase in dorsal markers, we assessed the expression of *emx1*, a dorsal telencephalic (pallial) marker (Bachy et al., 2002). *emx1* expression was expanded ventrally in the FoxG1 MO embryos, but not in the TLE2 MO embryos (see Fig. S3 in the supplementary material), suggesting that FoxG1 knockdown, but not TLE2 knockdown, causes a widespread respecification.

Finally, to test for functional synergism of FoxG1 with TLE2 in vivo, we tested the ability of wild-type and mutated FoxG1 to induce *Nkx2.1* expression in a FoxG1 MO background (Fig. 7P-T). We

found that wild-type FoxG1 and the N-terminal and C-terminal mutants (FoxG1-N3A and FoxG1-ΔC) were able to induce *Nkx2.1*, suggesting that in the forebrain each motif is able to compensate for the absence of the other. However, the double mutant, in which both N-terminal and C-terminal motifs are affected (FoxG1-N3A-ΔC), was unable to induce *Nkx2.1*. This finding supports the notion that cooperation between the TLE2-binding eh1 motif and the C-terminal WRPW-like motif is necessary for ventral telencephalon specification.

DISCUSSION

Cloning and expression of *X. tropicalis* TLEs

In this study, we have cloned and characterised the expression of four *X. tropicalis* TLEs: *TLE1*, *TLE2*, *TLE4* and *AES*. We have shown that TLE1, TLE2 and AES are highly expressed in the nervous system; however, they did not show identical expression patterns. We focused on the expression of TLE1 and TLE2 in the developing forebrain, specifically the telencephalon. Expression of *TLE2* has previously been described only by northern blotting (Choudhury et al., 1997).

We have shown that these genes are co-expressed at the neural plate stage, but show a bias in the strength of expression towards different future subdomains of the forebrain. Later on, these differences are amplified, as TLE1 is expressed throughout the telencephalon whereas TLE2 is restricted to the ventral-most part, i.e. the subpallium and the optic chiasm, which forms the border between telencephalon and diencephalon and where TLE1 is not expressed. TLE1 and TLE2 are expressed in more-posterior regions in distinct patterns, which we have not detailed here. The mouse homologue of TLE1 is expressed in the embryonic telencephalon (Dehni et al., 1995; Xuan et al., 1995; Yao et al., 1998). TLE2 exhibits very limited expression in the mouse forebrain (Grbavec et al., 1998) and is also expressed in the developing mammalian pancreas and in chicken feather buds (Hoffman et al., 2008; Houghton et al., 2003).

Whether TLEs have distinct or overlapping (redundant) functions is an open question and there are data to support either possibility (Gasperowicz and Otto, 2005; Yao et al., 1998). Our expression analysis of *Xenopus* TLE1 and TLE2 supports the view that these transcriptional co-factors have distinct functions; their neural expression profiles only partially overlap, providing the potential to interact with different transcription factors.

The N-terminal eh1 motif is important for physical association of TLEs with FoxG1

Previous studies in chicken and mouse that analysed the interaction between TLE1 and Foxg1 used large deletions to identify potential binding sites (Sonderegger and Vogt, 2003; Yao et al., 2001). Both groups identified a polypeptide of ~65-70 amino acids C-terminal of the Foxg1 DNA-binding domain as crucial for the interaction with TLE1 in in-vitro assays. Within the binding region, the short peptide motif YWPMSPLSLH was reported to show similarity to WRPW motifs (Yao et al., 2001). Another study has shown that the anti-neurogenic activity of TLE1 is due to binding of TLE1 to Hes1 and other WRPW motif-containing proteins, rather than to eh1 motif-containing binding partners, such as FoxG1 (Buscarlet et al., 2008). Therefore, it was unclear whether the co-repressor TLE1, or the eh1 motif of Foxg1, is important for the activity of Foxg1 in the developing telencephalon.

However, in co-IP assays performed in cell culture between FoxG1 and TLE2, we could not detect any effect on TLE2 binding to FoxG1 when the C-terminal motif is deleted. The mutation of

single aromatic amino acids (crucial components of both known motifs) in this region also had no effect on binding (data not shown). By contrast, we identified an N-terminal eh1 motif in FoxG1 as the main binding site for TLE2. Although the co-IP assay does not allow us to conclude with certainty that the interaction between FoxG1 and Groucho/TLE is direct, the eh1 motif shows the strongest conservation with known TLE binding sites (Yaklichkin et al., 2007a; Yaklichkin et al., 2007b).

How can the differences to previous reports be explained? One possibility is that FoxG1 interacts with TLE1 and TLE2 via different domains. However, this is not supported by our data, as we have not found any differences between TLE1 and TLE2 in co-IP assays (except for a lower amount of immunoprecipitated FoxG1 with TLE1). Another possibility is that the differences are due to the assays used. For example, the work on TLE1 used in vitro assays with bacterially derived proteins, which might lack important modifications necessary for binding to the eh1 motif. Indeed, a previous study has shown that the phosphorylation state of S/T residues next to the initial phenylalanine in an eh1 motif has an influence on the interaction with Groucho (Goldstein et al., 2005) and we have previously described a double casein kinase I phosphorylation site that overlaps with the N-terminal eh1 motif (Regad et al., 2007). TLEs can also be post-translationally modified, for example by hyperphosphorylation, which is also induced by the presence of FoxG1 (Buscarlet et al., 2008; Nuthall et al., 2002; Nuthall et al., 2004), or by sumoylation (Ahn et al., 2009). Our analysis bears the advantage of having co-factors and protein modifications present and, under these conditions, which better resemble those in vivo, we detect the N-terminal eh1 motif of FoxG1 as the main binding motif for TLE2.

Functional cooperation of TLE2 with FoxG1 in vivo

We have used a *Xenopus* neurogenesis assay (Bourguignon et al., 1998) to assess the cooperation of FoxG1 with TLE2 in the embryo. Since the expression of FoxG1 in the posterior neural plate and lateral ectoderm is ectopic, this assay can only be used to show functional synergism, rather than to draw any conclusions on the specific activity of FoxG1 in the developing forebrain. With this assay, we have shown that TLE2 modulates the activity of FoxG1. When expressed together with TLE2, a low dose of FoxG1 suppresses endogenous neurogenesis, which is otherwise only observed when FoxG1 is overexpressed at a high dose. We interpret this finding to mean that the presence of TLE2 allows FoxG1 to function more efficiently as a transcriptional repressor. This is analogous to the observation that co-expression of TLE1 enhances the repressor activity of FoxG1 on a FoxG1-sensitive reporter construct (Yao et al., 2001).

Mutating the N-terminal eh1 motif (FoxG1-N3A-HA) leads to a loss of functional cooperation with TLE2, consistent with the loss of TLE2 binding by this mutant in co-IP experiments. The FoxG1-ΔC-HA mutant, which represents a deletion of the C-terminal YWPMSPL motif, also shows a lack of functional cooperation with TLE2 in the neurogenesis assay. This is intriguing because deletion of this site does not abolish interaction with TLE2 in co-IP assays. Therefore, we suggest that even though this site is not necessary for TLE2 binding, it is important for the functional cooperation of FoxG1 with TLE2. One explanation is that a third factor exists, which binds FoxG1 at this motif and has an influence on FoxG1/TLE activity. As the motif YWPMSPL also contains a MAP kinase consensus site (P-X-S/T-P), it could serve as a possible protein-protein interaction site that is dependent on phosphorylation.

Our own work has also uncovered differences that depend on the assay used. In the neurogenesis assay, mutation of either the N-terminal or C-terminal motif is sufficient to interfere with the functional cooperation of exogenous FoxG1 and TLE2. However, within the forebrain, each motif seems to be able to compensate for the absence of the other. In both assays, simultaneous interference with both motifs renders FoxG1 inactive. One possible explanation is that both mutants are compromised in recruiting co-repressors, but different target genes have different threshold requirements for transcriptional repression. The presence of different additional co-factors or FoxG1/TLE modifications in the different assays might also play a role. Although we do not yet fully understand the details of FoxG1-TLE interaction, the common denominator in all the functional assays used in this work is that the double mutant is more severely affected than any single mutant. These findings suggest that both the N-terminal eh1 motif and the C-terminal WRPW-like motif contribute cooperatively to FoxG1 activity, either by interaction with TLE alone or in combination with other co-factors.

FoxG1 and TLE2 knockdown affect ventral forebrain specification

If FoxG1 and TLE2 interact *in vivo* one would expect that the phenotype of knocking down FoxG1 would be similar to that of knocking down TLE2. Indeed, both FoxG1 and TLE2 knockdown affected Nkx2.1 expression in the ventral telencephalon. The expression of Nkx2.1 in this area was eliminated with the FoxG1 MO and reduced with the TLE2 MO. This is consistent with the expression profile of FoxG1 and TLE2 and, particularly, with their co-expression in the ventral telencephalon (subpallium). The fact that FoxG1 knockdown has more severe effects than TLE2 knockdown in the telencephalon can be explained by the observation that FoxG1 is expressed throughout the entire telencephalon, whereas TLE2 is restricted to the ventral-most part.

The requirement for FoxG1 in the specification of the ventral telencephalon is consistent with previous findings in mouse and zebrafish (Danesin et al., 2009; Martynoga et al., 2005). Here, we suggest that this event is mediated by the specific interaction of FoxG1 with the transcriptional co-repressor TLE2, which might provide a mechanism for the cell-autonomous role of FoxG1 in this region (Manuel et al., 2010). Although we have focused on TLE2, we cannot exclude a contribution by TLE1 and finer assays will be needed to distinguish the contribution of each factor.

One of the hallmarks of the FoxG1 knockout and knockdown phenotype in mice and zebrafish is premature neuronal differentiation, particularly in the dorsal forebrain (pallium) (Danesin et al., 2009; Xuan et al., 1995), which transiently results in a greater number of neurons. In *Xenopus*, we have not seen this increase but instead observed a reduction in newly postmitotic neurons in the telencephalon of st. 19 tadpoles, marked by the expression of *elrC*. This is unlikely to reflect a delay in neurogenesis accompanied by increased proliferation, as we detected this reduction over a long period of time (st. 19 to st. 25) and we observed a reduction in mitotic cells. Therefore, we suggest that the reduction of *elrC* expression signifies a failure in the specification of telencephalic tissue that might precede the specification of neurogenesis. Indeed, this would be consistent with our earlier observation that FoxG1 is sufficient to induce the neuroepithelial marker Sox3 in isolated naïve ectoderm (animal caps) (Hardcastle and Papalopulu, 2000). The difference between the *Xenopus* phenotype and that seen in mouse and zebrafish, in which an increased number of neurons is transiently detected, could be due to phylogenetic differences in the development of the forebrain. Alternatively, partial compensation by other genes,

as originally suggested for the mouse (Xuan et al., 1995), might result in different phenotypes. In this respect, it might be relevant that in zebrafish there are three Foxg1 genes: *foxg1a* is the main Foxg1 paralogue expressed in the telencephalon, but *foxg1b*, which would not be targeted by the *foxg1a* MO, is transiently expressed in the ventral telencephalon (Zhao et al., 2009). At present, we cannot exclude the possibility that the reduction in *elrC* is due to premature neurogenesis that depletes the overall number of progenitors and hence reduces the overall number of neurons generated.

In conclusion, based on the specific overlap of expression between FoxG1 and TLE2 in the ventral telencephalon, their physical and functional interaction as shown by co-IP and a neurogenesis assay, the induction of Nkx2.1 in the forebrain and the similar phenotypes resulting from MO knockdown, we suggest that FoxG1 and TLE2 synergise to specify the ventral telencephalon. It remains to be seen whether other regionally expressed co-factors, such as JARID1B (PLU-1; KDM5B) (Tan et al., 2003), mediate some of the other activities of FoxG1.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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