En2, Pax2/5 and Tcf-4 transcription factors cooperate in patterning the Xenopus brain

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Among Xenopus Lef/Tcf, XTcf-4 has an outstanding role. In early development it is located exclusively in the midbrain where it is essential for midbrain and isthmus development. In order to identify transcription factors responsible for the restriction of XTcf-4 expression we isolated a 3.8 kb fragment of the XTcf-4 promoter. We found that this promoter fragment is sufficient to mimic endogenous XTcf-4 expression in the midbrain. Characterization of putative binding sites for en2 and pax2/5 revealed that en2, but not pax2/5 directly represses XTcf-4 promoter activity. Gain-of-function experiments in Xenopus embryos confirmed this en2-mediated repression. Loss-of-function experiments demonstrate that both en2 and pax2/5 are essential for endogenous XTcf-4 expression. The primary effect of pax2/5 depletion thereby appears to be a reduced en2 expression at neurula stages. Because en2 can compensate for the depletion of pax2/5, we assume a hierarchical regulation of gene expression in the midbrain/isthmus region with pax2/5 acting upstream of en2. Furthermore, since the XTcf-4 expression domain does not overlap with the expression domains of the isthmus marker genes en2 and pax2/5, we conclude that the knock-down of en2 and pax2/5 results in a downregulation of a paracrine growth factor regulating XTcf-4 expression. We found that the growth factor for this non-cell-autonomous effect of en2 and pax2/5 is wnt-1 acting on the — 1437 Lef/Tcf binding site on the XTcf-4 promoter. We provide evidence that the main nuclear wnt transducer for the autoregulation of XTcf-4 is XTcf-1.

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Introduction

After a transient activation step in the blastula and a stabilizing step in the gastrula, posterior identity of neural tissue is established by transforming signals (Stern, 2001). Neural induction largely depends on inhibition of BMP signalling, although recent studies revealed increasing complexity by identifying additional signalling molecules including FGF and Wnt involved in this process (Stern, 2001). Anterior-posterior patterning of the neural tissue is established by four secondary organizing centres, as sources of secreted signalling molecules: the anterior neural ridge, the zonula limitans intrathalmatica, the midbrain/hindbrain boundary (isthmus) and rhombomere 4. Organization of the midbrain and the anterior hindbrain is regulated by the FGF-8 and Wnt secreting midbrain/hindbrain boundary (Liu and Joyner, 2001; Wurst and Bally-Cuif, 2001). This organizing centre is established already during gastrulation by a Wnt gradient, which controls the expression of gbx2 and otx2 in a dose dependent manner. The border of otx and gbx expressing cells gives rise to a tissue stripe expressing the homeobox transcription factor engrailed-2 (en2), which demarcates the midbrain/hindbrain boundary (Hidalgo-Sanchez et al., 2005). In addition to its role as transforming signal for posteriorizing the CNS (Kiecker and Niehrs, 2001), recent findings indicate a function of wnt/β-catenin signalling in establishing the blastula chordin noggin expression centre and in stabilizing neural identity (Heeg-Truesdell and LaBonne, 2006; van Venrooy et al., 2008).

Key transcription factors which define midbrain identity belong to the super-family of homeobox transcription factors: pax2 and pax5 to the paired class, en2 to the Antennapedia class (Holland et al., 2007). Pax2 and pax5 form together with pax8 a closely related subfamily of pax genes, which evolved in early vertebrate evolution by gene duplication (Bassham et al., 2008). Zebrafish embryos homozygous for a pax2.1 mutant allele, no isthmus, fail to develop a midbrain and cerebellum (Brand et al., 1996). Pax5, a closely related family member can compensate for the loss of pax2 in mice (Bouchard et al., 2000) and double knock-out mice of pax2/5 revealed some redundant function (Schwarz et al., 1997). Interestingly, pax5 seems to regulate the expression of pax5, since it directly binds to and activates the pax5 enhancer (Pfeffer et al., 2000). In chicken, gain-of-function experiments revealed that pax5 and pax2 induce trans-differentiation from diencephalon to tectum (Funahashi et al., 1999; Okafuji et al., 1999), whereby pax5 additionally induces isthmus specific marker genes, including en2 and fgf8 (Funahashi et al., 1999).

Mice mutant for en2 showed a 30% reduction in the size of the cerebellum (Joyner et al. 1991). The weakness of the effect might be due to functional redundancy of en2 and its closest homolog en1.
Indeed, mice heterozygous null for en1 and homozygous null for en2 showed progressive degeneration of dopaminergic neurons in the substantia nigra (Sgado et al., 2006). In *Xenopus*, a gradient of en2 protein repels growth cones of Xenopus axons originating from the temporal retina and attracts nasal axons, an effect that was blocked by inhibitors of protein synthesis (Brunet et al., 2005).

Among the Lef/Tcf transcription factors, only Tcf-4 has been shown to be involved in antero-posterior patterning of the CNS. Although expressed more anterior in the midbrain, Xtcf-4 is essential for en2 expression (Kunz et al., 2004). Interestingly, Xtcf-4 expression is controlled by an autoregulatory loop via a Lef/Tcf site on the Xtcf-4 promoter (Koenig et al., 2008).

The outstanding role of Xtcf-4 in brain-patterning prompted us to analyze the molecular mechanisms underlying its expression. Therefore, we isolated a 3.8 kb fragment of the Xtcf-4 promoter and found that this fragment is sufficient to mimic endogenous Xtcf-4 expression. We identified putative binding sites for en2 and pax2/5 and showed that en2 represses the Xtcf-4 promoter. Loss-of-function experiments revealed that in the absence of en2 or pax2/5 endogenous Xtcf-4 is robustly downregulated. Interestingly, en2 can substitute for the loss of pax2/5. Since Xtcf-4 and en2 are expressed in a non-overlapping manner in adjacent tissues, a paracrine factor secreted from the isthmus seems to regulate Xtcf-4. We provide evidence that this paracrine factor is Wnt-1, acting downstream of en2 and activating the Xtcf-4 promoter via the −1437 Lef/Tcf binding site. We assume that in addition to an autoregulatory Wnt-Xtcf-4 loop controlling Xtcf-4 expression (Kunz et al., 2004; Koenig et al., 2008), a direct repression by en2 excludes Xtcf-4 from a Lef/Tcf site on the Xtcf-4 promoter (Koenig et al., 2008).

### Material and methods

### Plasmids and constructs

Xtcf-4 promoter fragments were amplified by genome walking as previously described (Koenig et al., 2008). Genomic DNA was amplified with Plushion Polymerase (New England Biolabs) and the following primers: 5′-AACCTCGAGATGTCAGCCT CTTGCTCTGTTG-3′ and pGL3 reporter plasmids and veri

mRNA was synthesized in vitro using the mRNA message machine kit (Ambion). 100 pg of en2, pax5 and pax2 mRNA was coinjected with 4 pg dextran-FITC as lineage tracer into the animal hemisphere of one blastomere of *Xenopus* 2-cell stage embryos. Although diffusing in the embryo, dextran-FITC staining allowed us to identify the injected site at neurula stages and to sort left hand injected embryos from right hand injected ones. Embryos were kept as previously described (Kunz et al., 2004). Embryos were staged according to Nieuwkoop and Faber (1967) and fixed at stages 26–28 (unless otherwise noted) in MEMFA (0.1 M MOPS pH 7.2, 2 mM EGTA, 1 mM MgSO4, and 3.7% formaldehyde). Whole-mount in situ hybridization was performed according to previously described procedures (Gawantka et al., 1995). Localization of mRNA was visualized using anti-digoxigenin antibodies conjugated to alkaline phosphatase, followed by incubation with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Images were captured on a Leica MZFLIII microscope using a digital camera (Qimaging) and Improvision software (Openlab).

### Promoter analysis in Xenopus

500 pg DNA of the different Xtcf-4 promoter constructs was injected into one blastomere of two-cell stage embryos and stained for fluorescence of the promoter driven EGFP protein or analyzed by whole-mount in situ hybridization for the localization of EGFP mRNA. Transgenes were released from the vector backbone by NotI digestion and integrated by sperm nuclei transplantsations (REMI) as described elsewhere (Sekkali et al., 2008).

For quantification of promoter activity by luciferase assays, 500 pg of the Xtcf-4 promoter constructs was coinjected with 100 pg CMV-β-galactosidase and the indicated morpholino. cDNA and mRNA into both blastomeres of 2 cell stage embryos, 5 embryos were pooled and analyzed in reporter gene assays (at least in triplicates) at the indicated stages.

### Transfection, reporter gene assays

XTC cells were transfected with TransPass (New England Biolabs) according to manufacturer's description. Forty-eight hours after transfection cells were harvested. Reporter gene assays were performed as described (Gradl et al., 1999).

### Results

We cloned 3.8 kb of the Xtcf-4 gene upstream of the transcription start site (Fig. 1A, Acc. No. EU085381) and fused several fragments of this promoter to the reporter EGFP. While a −1755 bp promoter fragment was almost ubiquitously active (not shown), the activity of a −2720 bp promoter fragment injected into one blastomere of two-cell stage embryos was restricted to the belly region (Fig. 1B). Finally, EGFP driven by the −385 bp promoter fragment was expressed exclusively in the midbrain (Figs. 1C and D), similar to endogenous Xtcf-4 (Figs. 1C and D). The overall frequency of EGFP positive embryos was only 2%. However, all of these seven embryos showed an EGFP expression restricted exclusively to the midbrain.

To increase the frequency of EGFP positive embryos we decided to generate transgenic frogs. Indeed, the percentage of EGFP positive embryos largely increased (15.2% n = 112), still showing a restricted promoter activity in the brain. Compared to the promoter-injected embryos, the EGFP-signal in the transgenic embryos is shifted more posterior to the midbrain/hindbrain boundary (Fig. 1E) as it is typical for the tadvole stage (see ISH Fig. 2).

Injection of a −385 bp fragment driving luciferase in principle yielded in the same result. Only few embryos showed a very faint
signal in the midbrain region (not shown). Consistent with this low
frequency and weakness of the signal, luciferase activity of the
injected −3.8 kb promoter construct was also much weaker than
shorter promoter fragments and only five fold more active than the
promoter-less pGL3 vector (Fig. 1F). A similar reduction of promoter
activity was also observed in transfected XTC cells (Supplementary
Fig. 1A), indicating that repressing elements are located between
−2.7 kb and −3.8 kb of the XTcf-4 promoter.
We conclude from these observations that regulatory repressing elements restricting XTcf-4 expression to the midbrain are located within 1.1 kb spanning from $-2720$ to $-3855$. Sequence analysis by Transcription Element Search Software (TESS, http://www.cbil.upenn.edu/cgi-bin/tess/tess) revealed putative binding sites for pax2/5, en2 and p53. To verify whether these elements, indeed,

Fig. 2. A) Superposition of en2, pax2 and XTcf-4 expression at different stages in schematic embryos. The superposition is based on the in situ hybridization depicted. B) Expression patterns of XTcf-4, pax2 and en2 as revealed by whole-mount in situ hybridization. C) Double staining of en2 (red) and XTcf-4 (blue) revealed that the expression fields of these transcription factors do not overlap. The bar indicates the cutting plane for the sections shown in D).

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regulate XTcf-4 promoter activity, we mutated them and tested the promoter responsiveness in luciferase reporter gene assays. While co-transfected fgf8, p53 and an oncogenous p53 mutant did not change the activity of a −3773 bp XTcf-4 promoter (not shown), co-transfection of en2 repressed the promoter in XTC cells (Fig. 1G) and co-transfection of pax5, but not pax2 activated it (Supplementary Fig. 1B). In either case, the regulation of the XTcf-4 promoter activity was dependent on the integrity of the corresponding binding site: mutation of the pax2/5 site abolished pax5 mediated activation, mutation of the en2 site abolished en2 mediated repression. En2 mediated repression of the XTcf-4 promoter was also found in Xenopus embryos when we injected our promoter constructs together with en2 mRNA (Fig. 1H). We could not observe a pax2 and pax5 dependent regulation in the embryo (data not shown). Thus, a direct regulation of the XTcf-4 promoter by pax2/5 remains doubtful. Hence, the spatial restriction of XTcf-4 expression appears to be directly regulated by en2. Considering that en2 expression in the isthmus depends on XTcf-4 (Kunz et al., 2004) and that en2 is a direct wnt target gene (McGrew et al., 1999), one might speculate upon a cross-regulation of XTcf-4 and en2 and by ISH from neurula to tadpole stages a direct regulation of XTcf-4 and en2 in the early posterior midbrain.

A direct regulation would require a co-expression of the corresponding transcription factors. Therefore we monitored the expression profile of XTcf-4 and en2 and by ISH from neurula to tadpole stage. Because we found a pax2/5 responsive element on our XTcf-4 promoter which is functional in the cell culture system we also included pax2 in this analysis. While en2 and pax2 expression overlapped at the isthmus in neurula and tadpole stages XTcf-4 transcripts were found clearly separated from them in the midbrain up to tailbud stage, while in the tadpole stage XTcf-4 expression field has expanded posteriorly forming an interface with the en2 expression domain (Fig. 2). Since en2 and XTcf-4 are not co-localized until tadpole stages a direct regulation of XTcf-4 by en2 was unexpected. However, we have previously shown by antisense morpholino injections that the expression of isthmus specific marker genes including en2, fgf-8 and wnt-1 depend on the presence of XTcf-4 (Kunz et al., 2004). To analyze the cross-regulation of XTcf-4, en2 and pax2/5 in more detail we performed gain-of-function and loss-of-function experiments.

Consistent with its role as repressor we found a downregulation of XTcf-4 expression upon en2 overexpression (Figs. 3A and B, Supplementary Fig. 2). 76% of the en2 injected embryos showed a reduced expression of XTcf-4 at the injected side. This effect appears to be specific for the midbrain marker gene XTcf-4, since neither pax2 nor pax5 expression was altered following en2 mRNA injection (not shown). Overexpression of pax2 had no effect on XTcf-4 expression (Figs. 3A and B), Pax5 mRNA injection occasionally increased XTcf-4 expression (17.1%, n = 70). Interestingly, pax5 and pax2 overexpression did not alter the expression of en2 (Supplementary Fig. 2 and data not shown).

Although overexpressed en2 inhibits XTcf-4 expression and XTcf-4 promoter activity, endogenous en2 is required for proper XTcf-4 expression. Antisense morpholino oligonucleotide mediated knockdown of en2 reduced XTcf-4 expression in 54% of the injected embryos (n = 61, Figs. 4A and B, Supplementary Fig. 2). Single injections of either pax2 or pax5 morpholino did not alter the expression of XTcf-4 (not shown) although each of them specifically suppressed pax2 or pax5 translation in the TNT system (Supplementary Fig. 3). However, simultaneous injection of pax5 and pax2 specific morpholinos led to reduced XTcf-4 levels in a significant portion of the injected embryos (Figs. 4A and B). The effects of the antisense morpholino oligonucleotides were specific, since coinjection of en2 mRNA and pax2 mRNA, respectively, restored normal XTcf-4 expression (Figs. 4A and B).

Given that the expression of pax2 was reduced following en2 depletion (Figs. 4C and D) and en2 was also reduced following pax2/5 depletion, these transcription factors seem to function as key
regulators of midbrain and isthmus development. This was further confirmed by a reduced expression of Xwnt-1 at the isthmus. Both, knock-down of en2 and knock-down of pax2/5 resulted in a severe reduction of Xwnt-1 expression (Fig. 4E). This reduction of Xwnt-1 was not seen before the onset of zygotic XTcf-4 expression at late neurula stages (Fig. 4F).
Since XTcf-4 is not co-expressed with pax2/en2 until tailbud stages, but depends on the presence of these transcription factors, we hypothesized that a growth factor expressed under the control of en2 and pax2/5 is secreted by the isthmus and activates XTcf-4 expression in the adjacent midbrain. The observed reduction of Xwnt-1 expression in en2 and pax2/5-depleted embryos prompted us to unravel whether, indeed, canonical Wnts are the secreted growth factors downstream of en2 regulating XTcf-4 expression in the midbrain. Therefore, we tried to restore XTcf-4 expression by coinjection of 50 pg wnt cDNA together with the en2 specific morpholino. Indeed, coinjected wnt-8 and wnt-1, but not wnt-5A partially restored XTcf-4 expression in en2 depleted embryos (Figs. 5A and B).

Furthermore, we found that the activity of the −3773 XTcf-4 promoter construct was drastically reduced (32% remaining activity) following en2 depletion (Fig. 5C). Considering that this promoter fragment is only five fold as active as the empty pGL3 vector (Fig. 1F), the −3.8 kb promoter activity drops in the presence of the en2 morpholino almost to basal levels. This effect of the en2 morpholino was completely reverted by coinjected wnt-1 or wnt-8 cDNA (Fig. 5C).

The −3773 XTcf-4 promoter construct with mutated en2 binding site was still sensitive (66% remaining activity) to en2 depletion (Fig. 5D), indicating that XTcf-4 promoter restoration by en2 is at least partially indirect. Since the activity of both, wild type (Fig. 5C) and mutated (Fig. 5D) promoter constructs was completely restored by coinjected wnt-1 and wnt-8, we assume that this indirect regulation is mediated by canonical wnt signalling. A shorter −1775/+501 promoter fragment was even strongly repressed (7% remaining activity) by en2 depletion (Fig. 5E), although this fragment does not contain a consensus en2 binding site. Most likely, this strong repression reflects, that the basal activity of the shorter promoter fragment is much higher compared to the −3773/+501 construct (Fig. 1F). Importantly, also the activity of the short −1775/+501 promoter fragment was partially restored by coinjected wnt-1 and wnt-8, but not by coinjected wnt-5A (Fig. 5E). After mutation of the −1437 Lef/Tcf binding site on the −1775/+501 promoter fragment the promoter did no longer respond on wnt injections (Fig. 5F), confirming that the regulation of the XTcf-4 promoter by en2 is at least partially mediated by wnt/β-catenin signalling driven activation of the Lef/Tcf family members Xllef-1 (Molenaar et al., 1998) and XTcf-1 (Roel et al., 2003) remain as predominant Lef/Tcf in the isthmus. But if the direct effect of en2 on the XTcf-4 expression were to exclude it from the isthmus, then why would en2 be essential for XTcf-4 expression? A probable explanation is, that target genes other than XTcf-4 are regulated by en2 or synergize with en2 in regulating XTcf-4 in the midbrain. In zebrafish, such a synergism between en2 and fgf-8 is necessary to position the border between the diencephalon and the midbrain (Scholpp et al., 2003). However, in transient transfection experiments fgf-8 had minor influence on the 3.8 kb XTcf-4 promoter fragment (not shown), indicating that a similar synergism for regulating XTcf-4 expression is not relevant. Wnt/β-catenin signalling acts as positive regulator of XTcf-4 expression (Kunz et al., 2004; Koenig et al., 2008) and is controlled by en2 in two ways: On one hand, the expression of Xwnt-1 at the isthmus depends on the presence of engrailed (this study), on the other hand, engrailed regulates the wnt/β-catenin pathway activity by destabilizing β-catenin (Bachar-Dahan et al., 2006). Lacking en2, in either case, results in an indirect regulation of XTcf-4 expression via the Lef/Tcf binding site. Thus, a balanced en2 level restricted to the isthmus guarantees the correct XTcf-4 expression by excluding it from the en2 expressing cells. In parallel, en2 allows XTcf-4 expression in adjacent cells via the action of the paracrine factor wnt-1. With time, the positive action of wnt signalling seems to gain the upper hand, since in later stages, XTcf-4 is also expressed at the isthmus adjacent region in the posterior midbrain (Fig. 2, and Kunz et al., 2004).

In order to analyze which Lef/Tcf is responsible for the auto-regulation of XTcf-4, we depleted XTcf-1, XTcf-3 and Xllef-1, which are all present in the brain (Molenaar et al., 1998; Roel et al., 2003 and Supplementary Fig. 4) by injecting the corresponding antisense morpholinos. Depletion of XTcf-3 (n = 30) and Xllef-1 (n = 36) had only minor effects on XTcf-4 (Fig. 6A). However, following depletion of XTcf-1 by antisense morpholino injection (Supplementary Fig. 3B) we observed a severe reduction of XTcf-4 expression (Figs. 6A and B) in more than 75% of the injected embryos. This reduction was dose dependent. Interestingly, also en2 expression was reduced upon XTcf-1 depletion, while XTcf-3 and Xllef-1 morpholino injections showed no effects on en2 expression (Figs. 6C and D). Compared to the reduction of XTcf-4 (in more than 75% of the injected embryos) and compared to the en2 reduction following XTcf-4 depletion (63% of the injected embryos, Kunz et al., 2004) a reduced en2 expression following XTcf-1 depletion was only observed in 27% of the injected embryos.

Remarkably, the mutual regulation of en2 and pax2/5 observed in tailbud stages (Fig. 4C) does not exist at neurula stages. As seen in Fig. 4F depletion of pax2/5 resulted already in a reduced en2 expression at the onset of brain compartmentation, while en2 depletion did not suppress pax2 expression at this time point. This prompted us to ask whether pax2/5, en2 and XTcf-4 expression might be regulated in a hierarchical manner also reflecting the relatively late expression of XTcf-4. In order to unravel such a hierarchy in regulation we tried to restore XTcf-4 expression in en2 depleted embryos by coinjection of pax5 mRNA. We observed that pax5 did not restore XTcf-4 expression in en2 depleted embryos (Fig. 7). However, overexpressed en2 restored XTcf-4 expression in pax2/5 depleted embryos (Fig. 7), indicating that XTcf-4 is not directly regulated by pax, but instead mainly regulated by the pax2/5 target gene en2. Consistently, XTcf-4 could restore neither en2 expression in pax2/5 depleted embryos nor pax2 expression in en2 depleted embryos (Fig. 7A and data not shown).

Taken together, our data revealed three major mechanisms regulating XTcf-4 expression and its restriction to the midbrain (Fig. 8): (1) Autoregulation via canonical wnt signalling and XTcf-1 is necessary for XTcf-4 expression. (2) Direct repression by en2 excludes XTcf-4 from the isthmus. (3) Indirect and non-cell autonomous activation via pax2/5 → en2 → wnt-1 allows XTcf-4 expression in the midbrain.

Discussion

En2, pax2/5 and Tcf-4 are key transcription factors in the developing CNS and essential for brain-patterning by the isthmus organizer (Wurst and Bally-Cuif, 2001; Kunz et al., 2004). However, little is known about a cross-regulation of these transcription factors in a regulatory network. Here we demonstrate that the key transcription factors of the isthmus en2, pax2 and pax5 are essential for the expression of the midbrain specific marker gene XTcf-4. Both, en2 and pax2/5 are essential for the expression of Xwnt-1, which then activates XTcf-4 expression via the −1743 Lef/Tcf binding site on the promoter. Surprisingly, en2 regulates the promoter also directly. We can show that overexpression of en2 represses XTcf-4 promoter activity and endogenous XTcf-4 expression. Thus, en2 appears to exclude XTcf-4 from the isthmus. The also more posteriorly expressed Lef/Tcf family members Xllef-1 (Molenaar et al., 1998) and XTcf-1 (Roel et al., 2003) remain as predominant Lef/Tcf in the isthmus. But if the direct effect of en2 on the XTcf-4 expression were to exclude it from the isthmus, then why would en2 be essential for XTcf-4 expression? A probable explanation is, that target genes other than XTcf-4 are regulated by en2 or synergize with en2 in regulating XTcf-4 in the midbrain. In zebrafish, such a synergism between en2 and fgf-8 is necessary to position the border between the diencephalon and the midbrain (Scholpp et al., 2003). However, in transient transfection experiments fgf-8 had minor influence on the 3.8 kb XTcf-4 promoter fragment (not shown), indicating that a similar synergism for regulating XTcf-4 expression is not relevant. Wnt/β-catenin signalling acts as positive regulator of XTcf-4 expression (Kunz et al., 2004; Koenig et al., 2008) and is controlled by en2 in two ways: On one hand, the expression of Xwnt-1 at the isthmus depends on the presence of engrailed (this study), on the other hand, engrailed regulates the wnt/β-catenin pathway activity by destabilizing β-catenin (Bachar-Dahan et al., 2006). Lacking en2, in either case, results in an indirect regulation of XTcf-4 expression via the Lef/Tcf binding site. Thus, a balanced en2 level restricted to the isthmus guarantees the correct XTcf-4 expression by excluding it from the en2 expressing cells. In parallel, en2 allows XTcf-4 expression in adjacent cells via the action of the paracrine factor wnt-1. With time, the positive action of wnt signalling seems to gain the upper hand, since in later stages, XTcf-4 is also expressed at the isthmus adjacent region in the posterior midbrain (Fig. 2, and Kunz et al., 2004). However, in early stages, there is a clear gap between the expression domains of en2 and XTcf-4. This gap might reflect that additional factors regulate proper localization of early XTcf-4 expression. Alternatively, in the gap region the amount of en2 is too low to be detected by ISH. Anyway, canonical wnt signalling seems to be a dominating mechanism to regulate the expression of the wnt effector XTcf-4. Among the Lef/Tcfs, XTcf-1 (but not Xllef-1 and XTcf-3) appears indispensable for XTcf-4 activation, and wnt ligands secreted by an intact isthmus are required to activate the −1473 Lef/Tcf site on the XTcf-4 promoter.
The role of the paired box transcription factors pax2 and 5 on the expression of XTcf-4 appears to be more general. Although we could identify a pax responsive element on the XTcf-4 promoter within the 1.1 kb responsible for correct localization, endogenous XTcf-4 seems to be regulated by pax2/5 in an indirect manner. We show that a depletion of pax2/5 results not only in a reduced XTcf-4 expression, but also in a reduced en2 expression. This is consistent with pax2/5 knock-out mice where en2 is completely absent and where the

![Diagram A](image1.png)

**Fig. 5.** A) Coinjection of 50 pg cDNA encoding for wnt-1 (enMo+wnt1) or wnt-8 (enMo+wnt8) restored XTcf-4 expression in en2 depleted embryos (enMo). Coinjected wnt-5A cDNA (enMo+wnt5A) had no effect on XTcf-4 expression. The asterisk marks the injected side. B) Quantification of the reduced XTcf-4 expression. n: number of analyzed embryos. C) Depletion of en2 by injection of 4 pmol of the en2 morpholino (enMo) results in reduced activity of the 3.8 kb promoter fragment and weakens the 3.8 kb promoter fragment with mutated en2 site (D). In either case the promoter activity was restored by coinjection of 50 pg wnt-1 (enMo+wnt1) and 50 pg wnt-8 (enMo+wnt8) cDNA. E) The activity of the 1.8 kb promoter fragment was drastically reduced in en2 depleted embryos. Coinjected wnt-1 and wnt-8, but not wnt-5A significantly increased the promoter activity. F) The 1.8 kb promoter fragment with mutated Lef/Tcf binding site was less reduced than the corresponding unmutated promoter and not activated by coinjected wnts. Given are mean values and standard errors of n=4, each consisting of five embryos. P values of student t-test are indicated, n.s.: not significant.
**Fig. 6.** Depletion of XTcf-1 (Tcf1Mo), XTcf-3 (Tcf3Mo) and XLef-1 (Lef1Mo) have different effects on XTcf-4 expression (A) and en2 expression (C). 4 pmol of the indicated morpholino antisense oligonucleotides was injected into one blastomere of two-cell stage embryos and stained for the expression of XTcf-4 (A) and en2 (C). The asterisk marks the injected site. B and D show the quantification of reduced marker gene expression for different amounts of XTcf-1 morpholino injections. n: number of analyzed embryos.

**Fig. 7.** En2 restores XTcf-4 expression in pax2/5 depleted embryos. A) 4 pmol of the indicated morpholino antisense oligonucleotides was coinjected with 200 pg of the indicated mRNA into one blastomere of two-cell stage embryos and analyzed for the expression of XTcf-4 and pax2. The asterisk marks the injected side. B) Quantification of XTcf-4 reduction in en2 and pax2/5 depleted embryos and in cross-rescue experiments. While pax5 is unable to rescue the en2 phenotype, en2 restores the XTcf-4 expression after the pax2/5 knockdown. n: number of analyzed embryos.
mesencephalon/metencephalon primordium is lost (Schwarz et al., 1999). It seems to be likely that similar to the murine en2 promoter (Song et al., 1996) in Xenopus en2 is directly regulated by pax2/5. This is also confirmed by our finding that at neurula stages depletion of pax2/5 already resulted in decreased en2 expression, while wnt-1 was not yet reduced. The absence of a pax2/5 binding site on a 2.8 kb fragment of the en2 promoter construct (Acc. No. AF152960) rather reflects that these promoter fragments lack important regulatory elements.

In contrast to multimerized Lef/Tcf consensus binding sites, which restrict GFP expression to regions of active wnt/Lef/Tcf-catenin signalling in Xenopus (Geng et al., 2003; Denayer et al., 2006) or zebrafish (Dorsky et al., 2002), the single functional Lef/Tcf site at position −1437 on the Xtcf-4 promoter is not sufficient to restrict reporter gene expression to the brain. Instead, GFP driven by Xtcf-4 promoter fragments without the localization sequence (−2720 to −3855) is excluded from the brain and enriched in the endoderm, supporting the idea of Barolo (2006) that for proper expression of wnt/Tcf target genes Lef is not all that is needed. Instead the additional activation of transcription factors needs to be integrated to define the expression pattern of spatially restricted genes such as Xtcf-4. For Xtcf-4, repressing elements rather than activating elements appear to be essential for the spatial restriction.

Coinjection experiments revealed, that en2 can substitute for pax2/5 in regulating Xtcf-4 expression. Thus, the 1.6-fold activation of the Xtcf-4 promoter by co-transfected pax5 (not pax2) seems to be of minor relevance for regulating Xtcf-4 expression. Instead, our results suggest a hierarchic gene expression with pax2/5 regulating en2 and en2 regulating Xtcf-4 in two ways: directly via the en2 binding site on the Xtcf-4 promoter to keep Xtcf-4 excluded from the isthmus, and indirectly via Wnt-1, to allow its expression in the midbrain. Hereby, Xtcf-1 seems to be the key transcription factor of the Lef/Tcf family mediating Xwnt-1 signalling.

Although en2 is a direct wnt target gene (McGrew et al., 1999) and Xtcf-4 is necessary for en2 expression (Kunz et al., 2004), Xtcf-4 is not sufficient to replace en2 for pax2/5 expression. Again, one might suggest a direct regulation of en2 by pax2/5 similar to the mouse and similar to the direct regulation of gbx2 by pax2 in zebrafish (Islam et al., 2006).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.02.011.

References


