

Noncanonical Wnt-4 signaling and EAF2 are required for eye development in *Xenopus laevis*

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Wnt-4 is expressed in developing neural and renal tissue and is required for renal tubulogenesis in mouse and *Xenopus*. The function of Wnt-4 in neural differentiation is unknown so far. Here we demonstrate that Wnt-4 is required for eye development in *Xenopus laevis*. This effect of Wnt-4 depends on the activation of a β -catenin-independent, noncanonical Wnt signaling pathway. Furthermore, we report the identification of EAF2, a component of the ELL-mediated RNA polymerase II elongation factor complex, as a target gene of Wnt-4 signaling. EAF2 is specifically expressed in the eye and EAF2 expression was dependent on Wnt-4 function. Loss of EAF2 function results in loss of eyes and loss of Wnt-4 function could be rescued by EAF2. In neuralized animal caps, EAF2 has properties characteristic for an RNA polymerase II elongation factor regulating the expression of the eye-specific transcription factor Rx. These data add a new layer of complexity to our understanding of eye development and give further evidence for the importance of noncanonical Wnt pathways in organ development.

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Introduction

Specification of cells during development becomes evident by differential gene expression and is regulated by extracellular growth factors. Wnt proteins form a family of secreted glycoproteins which can activate different intracellular signaling pathways. The canonical Wnt/ β -catenin pathway involves stabilization of cytoplasmic β -catenin, which can act as a transcriptional coactivator (Logan and Nusse, 2004). Noncanonical Wnt pathways are per definition independent of β -catenin signaling (Kühl *et al*, 2000a; Veeman *et al*, 2003). These pathways involve activation of calcium-sensitive enzymes like protein kinase C and calcium calmodulin-depen-

dent kinase II or activation of Jun-N-terminal kinase (JNK) through members of the rho family of small GTPases. This latter pathway is also known as the planar cell polarity pathway, which is involved in *Drosophila* epithelial cell polarity as well as *Drosophila* eye development (Mlodzik, 1999). In vertebrates, noncanonical Wnt signaling is activated by Wnt-5A, Wnt-11, and Wnt-4 (Du *et al*, 1995) and regulates dorso-ventral patterning of the embryo, cell migration, and heart development (Veeman *et al*, 2003). In *Xenopus*, mouse, and chicken embryos, Wnt-4 is expressed in distinct expression domains in neural tissues as well as the developing excretory system (McGrew *et al*, 1992; Parr *et al*, 1993; Hollyday *et al*, 1995; Saulnier *et al*, 2002). Loss-of-function studies have shown that Wnt-4 is required for kidney organogenesis in mouse and *Xenopus*. Wnt-4 has also been linked to axonal pathfinding in rat embryos (Lyuksyutova *et al*, 2003). The role for Wnt-4 in regulating gene expression during neural differentiation, however, is poorly defined so far.

The eye derives from the anterior neural tissue, the fore-brain. The early eye field is characterized by expression of several marker genes including the homeobox transcription factor Rx and the paired box transcription factor Pax-6. During neurulation this initially continuous eye field is divided, resulting in two lateral expression domains of Rx and Pax-6 by a midline-derived inhibitory signal. Pax-6, Rx, and other transcription factors expressed in the developing eye are thought to constitute a positive autoregulatory feedback loop (Zuber *et al*, 2003). However, upstream signaling pathways and growth factors that contribute to this feedback loop are not known. In a recent report, the Wnt receptor *Xenopus* Frizzled-3 (Fz-3) has been implicated in eye development, suggesting that Wnt signaling might play a role during early eye development (Rasmussen *et al*, 2001). The Wnt ligand activating Fz-3 during eye development, however, is unknown so far.

As the neural functions of Wnt-4 have been poorly investigated to date, we aimed to (i) uncover the potential roles for Wnt-4 in neural patterning and (ii) to identify the downstream factors by which Wnt-4 can mediate its effects. Here, we show that Wnt-4 and its downstream factor EAF2 are required for eye development in *Xenopus laevis*. Furthermore, our data implicate noncanonical Wnt signaling in early eye development.

Results

Wnt-4 is required for eye development in *Xenopus*

During *Xenopus* embryogenesis, Wnt-4 expression starts at the onset of neurogenesis at stage 12.5–13 with two characteristic spots in the anterior brain region that persist until later stages (McGrew *et al*, 1992; Figure 1A–C). Whole-mount *in situ* analyses using marker genes specific for the early eye field (Pax-6, Rx) or the midbrain marker Pax-2 indicate that Wnt-4 is expressed immediately adjacent to the early eye field

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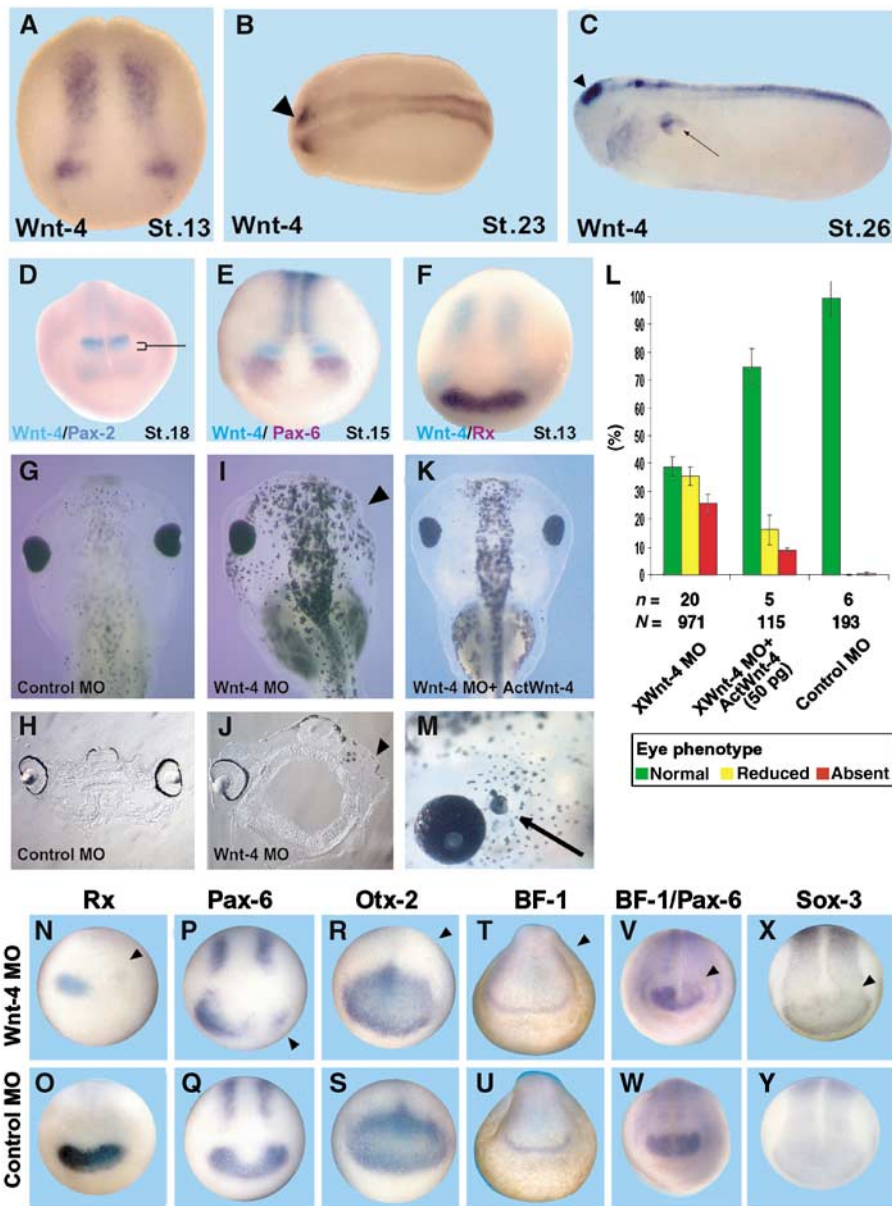


Figure 1 Inhibition of Wnt-4 function by an antisense MO leads to loss of eye structures. (A–C) Wnt-4 is expressed in neural tissue as monitored by whole-mount *in situ* hybridization of *X. laevis* embryos of different stages as indicated. Expression of Wnt-4 in neural tissue starts at stage 13. Two characteristic spots in the anterior neural plate persist until later stages (arrowhead). Arrow in (C): pronephros expression. (D–F) Mapping of the anterior-most neural expression domain of Wnt-4. (D) Double *in situ* hybridization with the midbrain marker Pax-2, (E) the early eye field marker genes Pax-6 or (F) Rx, indicate that Wnt-4 is expressed at the forebrain–midbrain boundary immediately caudally to the eye field. The bracket in (D) indicates the expression of Wnt-4 in the forebrain region that does not overlap with Pax-2. (G, I) Injection of a Wnt-4 MO, but not a control MO, interferes with eye development as judged by a loss of RPE. (H, J) Sections of those embryos indicate the complete loss of eyes. (K) Co-injection of Wnt-4 MO and an activin–Wnt-4 fusion is able to revert the phenotype in a significant manner. (L) Statistical evaluation of experiments. *n* = number of independent experiments, *N* = number of embryos scored. Error bars indicate standard error means. (M) Overexpression of Wnt-4 leads to development of ectopic eye structures in rare cases (Arrow). (N–Y) Wnt-4 MO or control MO unilaterally injected embryos were analyzed by whole-mount *in situ* hybridization against Rx (N, O), Pax-6 (P, Q), Otx-2 (R, S), BF-1 (T, U), BF-1/Pax-6 simultaneously (V, W) or Sox-3 (X, Y). Loss of Wnt-4 function specifically leads to a loss of Rx and Pax-6 in the early eye field, but not of Otx-2, BF-1, or Sox-3. Arrowheads in (I, J, N, P, R, T, V, X) indicate the injected side of the embryo.

(Figure 1D–F) and marks the forebrain/midbrain boundary. Additionally, Wnt-4 is expressed in the pronephros (Figure 1C) as well as in the floor plate. To uncover a potential role of Wnt-4 during neural development, we pursued two independent strategies: We first performed neural specific loss-of-function analyses for Wnt-4 and, second, we aimed to identify genes that are transcriptionally upregu-

lated by Wnt-4 in neuralized animal caps of *Xenopus* embryos.

In order to analyze the function of Wnt-4 in neural tissues, we used a characterized Wnt-4 antisense morpholino oligo (Wnt-4 MO) that has previously been shown to interfere with translation of the endogenous Wnt-4 protein (Saulnier *et al*, 2002) and has been used to study the function of Wnt-4

during pronephros development. The Wnt-4 MO or a control MO was injected unilaterally into one dorsal-animal blastomere at the eight-cell stage to target the presumptive anterior neural region. In all, 61% of Wnt-4 MO- but not control MO-injected embryos showed strongly reduced (35%) or absent eyes (26%) at stage 42 of development (Figure 1G, I, and L), as judged by the size of the retinal pigment epithelium (RPE). This result was confirmed by histological sections (Figure 1H and J). Whereas normally developed eyes have a multi-layered structure, this is missing in Wnt-4 MO-treated embryos on the injected side. As a complete loss of RPE is a simple and reliable way to monitor developmental eye defects, we used this phenotype to analyze the outcome of further experiments described below. To rescue the Wnt-4 MO-induced phenotype, we used an Activin-Wnt-4 fusion construct which consists of the activin-derived propeptide and the mature Wnt-4 protein. The Wnt-4 MO does not target RNA coding for this fusion protein and after translation cleavage of the propeptide releases a mature and functional Wnt-4 protein (Saulnier *et al*, 2002). Coinjecting 50 pg of this RNA with Wnt-4 MO significantly reduced the number of embryos with eye defects on the injected side (Figure 1K and L). In addition, we overexpressed Wnt-4 RNA in dorsal-animal blastomeres of eight-cell stage embryos to analyze Wnt-4 gain-of-function embryos. Most of these embryos displayed defects in gastrulation movements as described earlier by others (Du *et al*, 1995). However, in rare cases (1–2%) embryos were obtained with ectopic eye structures (Figure 1M) similar to those injected with the Wnt receptor Fz-3 (Rasmussen *et al*, 2001) or Pax-6 (Chow *et al*, 1999).

We next characterized the loss-of-function defects in eye development at the molecular level. Unilateral injection of Wnt-4 MO into dorsal-animal blastomeres resulted in a loss of Rx and Pax-6 expression at stages 13/14 on the injected side (Figure 1N–Q). This effect on gene expression was specific for Pax-6 and Rx expression as the fore- and midbrain marker gene *Otx-2* or the forebrain marker BF-1 was not affected (Figure 1R–U). Note that *Otx-2* is not considered to be an eye marker gene at this stage of development (see Supplementary Figure 1). Pax-6 expression in the spinal cord (Figure 1P and Q) and the expression of the pan-neural marker Sox-3 were not affected (Figure 1X and Y). These data indicate that Wnt-4 is specifically required for eye-specific marker gene expression. At later stages, both the olfactory and the otic placode were established normally (data not shown). In addition, double *in situ* staining for Pax-6 and BF-1 revealed that a knockdown of Wnt-4 function interfered solely with Pax-6 expression (Figure 1V and W). As a consequence, the possibility that the loss of eyes is due to defects in gastrulation movements can be excluded. Our findings thus clearly establish a specific requirement for Wnt-4 in early eye development of *Xenopus*.

Wnt-4 exerts its function through a noncanonical Wnt pathway

We next addressed the question of which intracellular signaling pathway is triggered by Wnt-4 during eye development. Wnt-4 has recently been suggested to activate β -catenin-independent, noncanonical Wnt signaling pathways, which can be activated by a deletion mutant of dishevelled (*dsh*), *dsh* Δ DIX (Du *et al*, 1995; Boutros *et al*, 1998; Veeman *et al*, 2003). Coinjection of *dsh* Δ DIX RNA and Wnt-4 MO resulted

in a significant reversal of the Wnt-4 MO phenotype (Figure 2A, C, and E). Injection of GFP RNA had no effect. In contrast, overexpression of β -catenin RNA can mimic signaling via the canonical Wnt/ β -catenin pathway. Neural specific overexpression of β -catenin RNA was unable to rescue the Wnt-4 MO phenotype (Figure 2A, B, and E). As an early overexpression of β -catenin might interfere with aspects of early axial and neural patterning (Kiecker and Niehrs, 2001), we also overexpressed β -catenin later in development by means of plasmid injections. CMV-driven β -catenin overexpression after onset of zygotic transcription (Stage 8) did

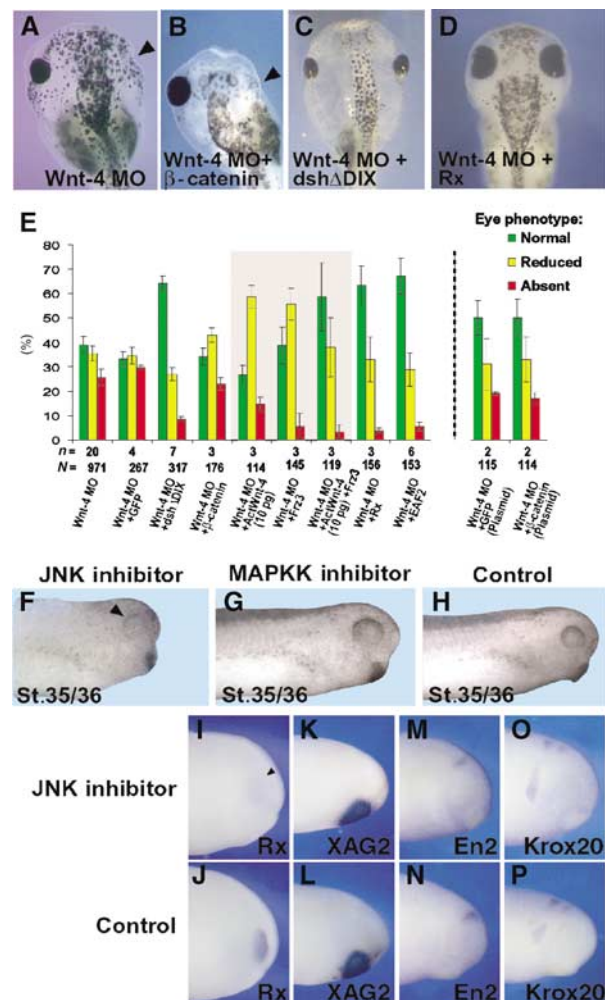


Figure 2 The effect of Wnt-4 is mediated through a β -catenin-independent noncanonical Wnt pathway. (A–C) Injection of *dsh* Δ DIX (C), but not β -catenin RNA (B), rescues the eye phenotype of Wnt-4 MO (A)-injected embryos. (D) Injection of Rx RNA rescues the eye phenotype of Wnt-4 MO. Arrowheads indicate missing eyes. (E) To quantify the rescue experiments, embryos were characterized according to the phenotype observed as normal, reduced eye or absent eye. Mean values of *n* experiments are given. *N* = number of embryos scored. Error bars indicate standard error means. For the β -catenin DNA rescue experiments, an independent set of Wnt-4 MO embryos is given for comparison. (F–H) Treatment of embryos with the JNK inhibitor SP600125 from stage 11 to 22 results in strongly reduced RPE (arrowhead) at stages 35/36. (G, H) Treatment with the MAPKK inhibitor PD98059 does not affect eye development. (I–P) Marker gene analyses of JNK inhibitor-treated embryos by *in situ* hybridization (stage 24) using different marker genes as indicated.

not rescue the Wnt-4 MO phenotype (Figure 2E). We therefore conclude that Wnt-4 signals through β -catenin-independent signaling pathways.

Recently, Wnt-4 has been shown to activate JNK (Cai *et al*, 2002) as does dshADIX (Boutros *et al*, 1998). In support of a role for JNK activity in Wnt-4 induced noncanonical signaling, treatment of *Xenopus* embryos with the JNK inhibitor SP600125, but not an inhibitor against MAPKK, during stages of early eye development (St.11–22) resulted in a small eye phenotype at stage 30 (Figure 2F–H). A molecular analysis of this phenotype revealed a downregulation of Rx expression at stage 24, whereas XAG2, a cement gland marker, En-2, a marker for the midbrain–hindbrain border, as well as Krox-20, a hindbrain marker, remained unchanged (Figure 2I–P). Thus, these data indicate that Wnt-4 regulates eye development in *Xenopus* through a noncanonical Wnt pathway and implicate JNK to be involved in this pathway.

As Fz-3 has been implicated in eye development and in noncanonical Wnt signaling (Rasmussen *et al*, 2001), we tested whether Fz-3 RNA is able to rescue the Wnt-4 MO phenotype. Indeed, a low amount of Fz-3 RNA (10 pg) reduced the number of eyeless embryos but did not reduce the overall occurrence of an eye phenotype. Interestingly, injection of a low dose of Act-Wnt-4 RNA (10 pg) had also only a modest rescuing activity. However, coinjection of Fz-3 and low dose of Act-Wnt-4 RNA (10 pg) synergistically rescued the Wnt-4 MO phenotype (Figure 2E). Taken together, these data implicate Fz-3 in Wnt-4-mediated signaling.

Finally, we tested the ability of Rx as a potential downstream gene to rescue the Wnt-4 MO phenotype. Therefore, we injected Rx RNA together with Wnt-4 MO and monitored eye development. Interestingly, injection of Rx RNA was able to significantly revert the Wnt-4 MO phenotype in *Xenopus* (Figure 2A, D, and E).

Identification of Wnt-4 downstream genes

We performed a PCR-mediated subtractive cDNA screen (Figure 3) using the animal cap assay to elucidate in an alternative approach the role of Wnt-4 in neural patterning and to identify potential *Wnt-4* downstream genes in neural tissue. Animal caps were neuralized by injecting noggin RNA into two-cell stage *Xenopus* embryos. FGF was subsequently added to neuralized animal cap cultures, resulting in caps expressing anterior as well as posterior neural marker genes. These noggin/FGF-treated caps define the default state for the screen and we asked which genes are upregulated by over-expressing Wnt-4 by RNA coinjection into two-cell stage embryos. A subtractive cDNA library was constructed from treated animal caps when controls reached stage 22. This procedure results in a cDNA library consisting of cDNA clones likely to be enriched in the noggin/FGF/Wnt-4 pool versus the noggin/FGF pool. To eliminate false-positive clones, inserts of 600 clones were PCR amplified, spotted onto nylon membranes and subsequently hybridized with radioactively labeled cDNA probes derived from both RNA pools. Using this experimental approach, we identified 44 genes that were more highly expressed after treatment with Wnt-4. To narrow down the number of genes of potential interest, these clones were sequenced and their spatial expression patterns were determined by *in situ* hybridization techniques using *Xenopus* embryos at different developmental stages. A list of genes upregulated by Wnt-4 treatment in

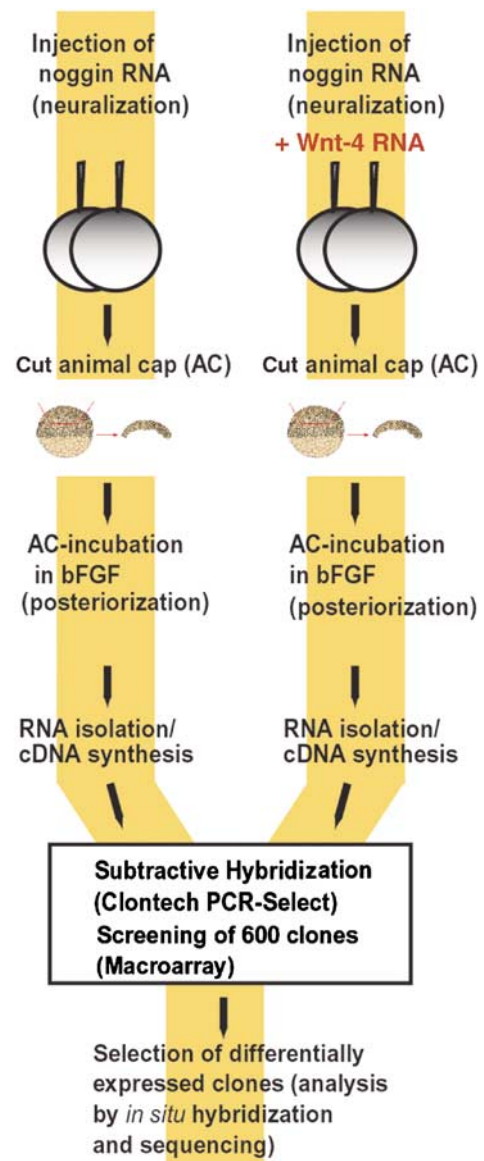


Figure 3 Identification of *Wnt-4* target genes by subtractive cDNA cloning. Schematic drawing of the subtractive cDNA screen. Animal caps were treated either with noggin and FGF (left branch) or with noggin, FGF, and Wnt-4 (right branch) as indicated. RNA was isolated from these caps and enriched for those RNA species to be upregulated by Wnt-4 following the Clontech protocol. Of those clones obtained, 600 were spotted as a macroarray and rehybridized with radioactively labeled cDNA pools of both samples. Verified clones were sequenced and the spatio-temporal expression was determined by whole-mount *in situ* hybridization. See Supplementary Table for a list of identified genes.

FGF/noggin-treated animal caps is given in Supplementary Table 1.

EAF2 is a Wnt-4 downstream factor

One of the potential downstream genes identified in the screen is the *Xenopus* homolog of EAF2 (Figure 4A), which is an ELL-associated factor and part of the ELL-mediated RNA polymerase II elongation factor complex (Li *et al*, 2003; Simone *et al*, 2003). In *Xenopus*, EAF2 is expressed maternally in a ubiquitous manner (data not shown). Tissue-specific, zygotic expression of EAF2 starts at stage 18.5 in

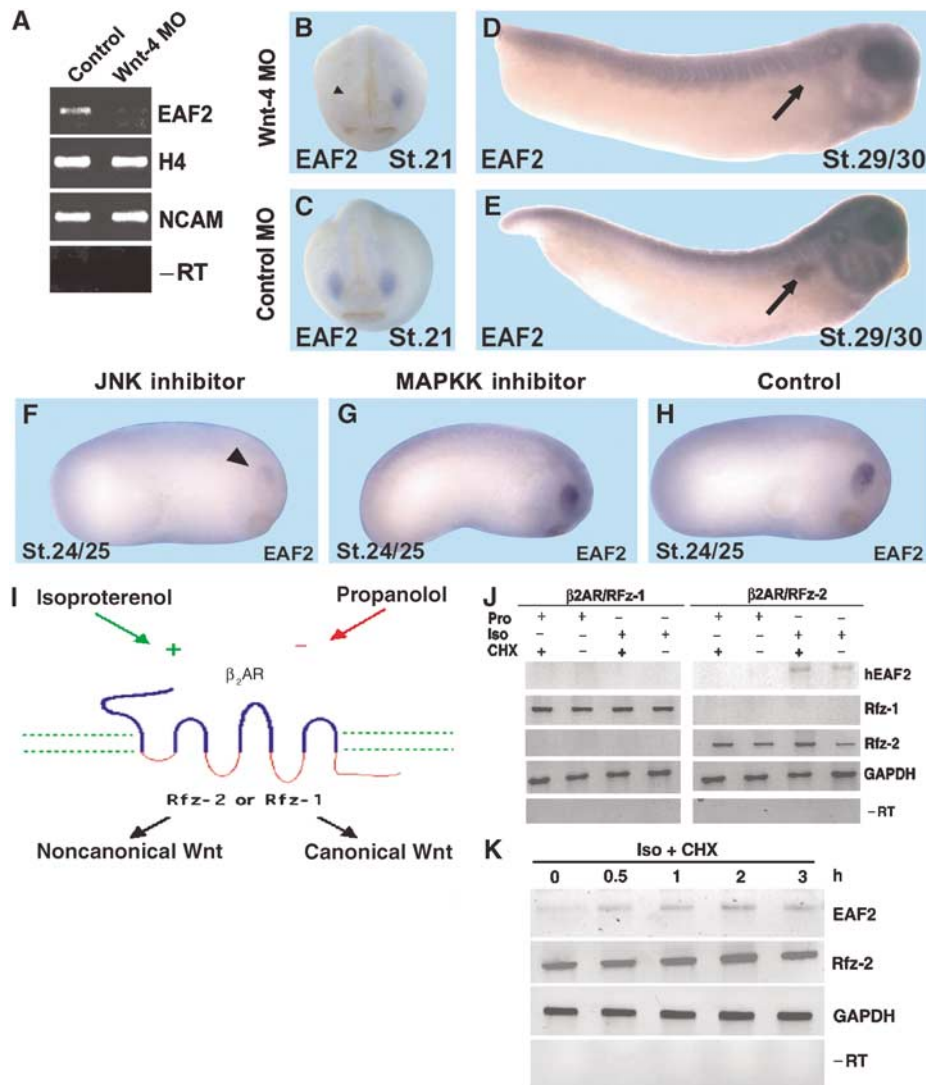


Figure 5 EAF2 is a Wnt-4 downstream factor. (A) RT-PCR analysis demonstrate that *EAF2* gene expression is downregulated in Wnt-4 loss-of-function embryos, confirming the results obtained in the Wnt-4 gain-of-function screen. (B–E) Targeted injection of the Wnt-4 MO (B, D), but not a control MO (C, E), into blastomeres that will give rise to anterior neural tissue (B, C) or the pronephric kidneys (D, E), respectively, indicate that expression of EAF2 in these tissues depends on *Wnt-4* gene function at stage 21 or stage 29/30, respectively. Arrowhead in (B) indicates missing expression of EAF2 in the eye anlage after Wnt-4 MO injection. Arrows in (D) and (E) highlight the pronephric anlage. (F–H) Treatment of embryos with the JNK inhibitor, but not the MAPKK inhibitor, from stage 11 to 22 leads to a downregulation of EAF2 expression in the eye at stage 24/25. (I) Schematic drawing of inducible Frizzled receptors and their signaling specificity as previously reported. (J) HEK293 cells were stably transfected with inducible β₂AR/Rfz-1 or β₂AR/Rfz-2 receptors, as indicated and verified by RT-PCR for Rfz-1 or Rfz-2 intracellular loops. Activation of β₂AR/Rfz-2, but not β₂AR/Rfz-1, by the β₂-adrenergic agonist isoproterenol (Iso), but not the antagonist propranolol (Pro), leads to activation of *EAF2* gene expression, as monitored by RT-PCR within 4 h of treatment. Activation is resistant to treatment with cycloheximide (CHX). GAPDH served as loading control. –RT: negative control. (K) Time course of EAF2 activation after stimulation as indicated.

pronephros, indicate that expression of EAF2 in both organs depends on Wnt-4 (Figure 5B–E). In addition, inhibiting JNK function by SP600125 treatment from stage 11 to 22 resulted in downregulation of EAF2 expression (Figure 5F–H). We subsequently addressed the question whether EAF2 might be a direct target gene of noncanonical Wnt signaling. Due to the lack of purified Wnt-4, we made use of characterized inducible Frizzled receptors encompassing extracellular and transmembrane domains of the β₂ adrenergic receptor (β₂AR) and intracellular loops of Frizzled receptors (Figure 5I). These receptors can be stimulated or inhibited by β-adrenergic agonist (isoproterenol) or antagonists (propranolol), respectively. The β₂AR/Rfz-1 chimeric receptor

couples to the Wnt/β-catenin pathway, whereas the β₂AR/Rfz-2 chimeric receptor couples to noncanonical Wnt signaling (Liu *et al*, 1999, 2001; Kühl *et al*, 2000b). These receptors were stably transfected into human embryonic kidney cells (HEK293), challenged with isoproterenol or propranolol, and the expression of human EAF2 was monitored by RT-PCR. Interestingly, hEAF2 was induced by the activated β₂AR/Rfz-2, but not by the β₂AR/Rfz-1 chimeric receptor (Figure 5J). Upregulation of EAF2 transcription was observed as early as 30 min after stimulation of the β₂AR/Rfz-2 receptor (Figure 5K). This activation was resistant to treatment with cycloheximide, an inhibitor of eukaryotic translation. This observation is consistent with the idea that EAF2

upregulation might be a direct response in the chosen cell line upon cell stimulation. In summary, we have identified *EAF2* as a gene regulated by Wnt-4 activity in explant cultures and during embryonic organogenesis.

EAF2 is required for eye development in *X. laevis*

Recently, it has been demonstrated that RNA polymerase II elongation factors like ELL, Foggy/Spt5, or Pandora/Spt6 play important roles during development (Guo *et al*, 2000; Eissenberg *et al*, 2002; Keegan *et al*, 2002). We therefore asked whether *EAF2* is required for eye development. We first tested whether an *EAF2* MO inhibits translation of *EAF2* RNA (5'UTR + *EAF2*) in an *in vitro* transcription and translation assay (Figure 6A). The *EAF2* MO clearly blocked translation of *EAF2* RNA, whereas a control MO had no effect. An *EAF2* control RNA construct lacking the 5'-UTR (Δ 5'UTR + *EAF2*) was not targeted by the *EAF2* MO and was therefore used for rescue experiments. We next injected the *EAF2* MO into those blastomeres that will give rise to the eye region. Unilateral

injection of *EAF2* MO results in 50% of embryos with reduced or absent eyes on the injected side. Loss of complete eye structures was observed in 24% of the embryos (Figure 6B–D) as judged by loss of RPE. The loss-of-eye phenotype could be rescued by coinjection of an *EAF2* construct (Δ 5'UTR + *EAF2*) that is not a target for the *EAF2* MO, whereas injection of RNA coding for GFP had no effect (Figure 6D). Molecular analysis indicated that *EAF2* loss-of-function embryos display a loss of Rx, Pax-6, and Otx-2 expression in the eye region consistent with the morphological phenotype (Figure 6E–J). As *EAF2* expression in the eye starts at stage 18.5, we also analyzed expression of marker genes at earlier stages of development (St.13/14). As expected, we did not observe an effect on marker gene expression at this stage, implicating that *EAF2* is involved in maintenance of eye-specific marker gene expression. Interestingly, overexpression of *EAF2* does not result in enlarged or ectopic eye structures (data not shown), implicating a permissive rather than an instructive function of *EAF2* during eye development. Taken together, our data clearly indicate that *EAF2* function is required for eye development and highlight an essential function of *EAF2* during embryonic development. As *EAF2* expression in the eye depends on Wnt-4 function and both, Wnt-4 MO and *EAF2* MO injections result in the same phenotype, we next analyzed whether *EAF2* RNA injection is sufficient to rescue the Wnt4 MO phenotype. Coinjection experiments clearly indicate that *EAF2* can revert the effect elicited by Wnt-4 MO (Figure 2E), indicating that both might act through a common downstream target.

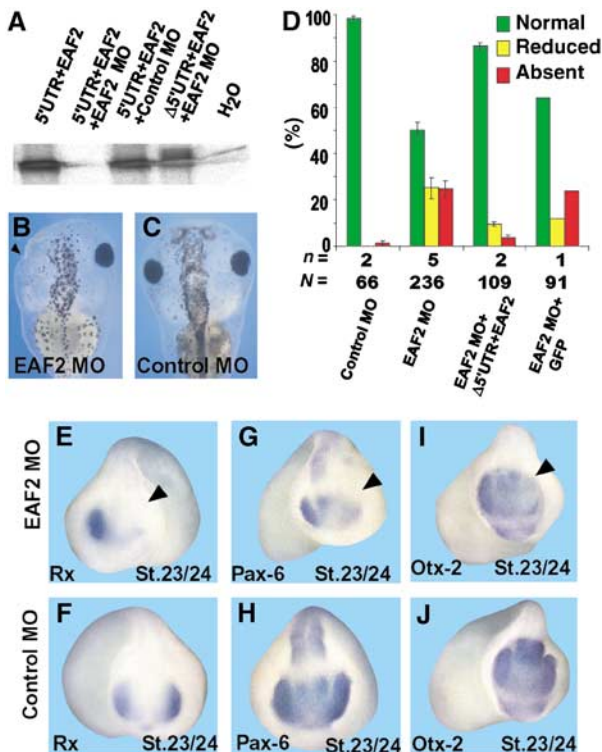


Figure 6 *EAF2* is required for eye development. (A) The *EAF2* MO used inhibits translation of *EAF2* RNA (5'UTR + *EAF2*), whereas the control MO does not. An *EAF2* construct lacking the 5'UTR (Δ 5'UTR + *EAF2*) is not targeted by the MO. Data of transcription and translation assays, resulting in production of ³⁵S-labeled proteins, are shown. (B, C) Unilateral injection of *EAF2* MO, but not control MO, into dorsal-animal blastomeres of eight-cell stage embryos results in loss of eyes, as indicated by a loss of RPE (arrow head). (D) Statistical evaluation of phenotypes observed. n = number of independent experiments, N = number of embryos scored. Error bars indicate standard error means. (E–J) Marker gene analysis for Rx, Pax-6 or Otx-2 expression, as indicated by whole-mount *in situ* hybridization of unilaterally *EAF2* MO-injected embryos (E, G, I) or control MO-injected embryos (F, H, J). All the three marker genes are downregulated in the developing eye. The arrowheads in panels (E, G, I) indicate the injected sides of the embryos.

EAF2 functions as an RNA polymerase II elongation factor

We next explored whether *EAF2* indeed functions as a component of an RNA polymerase II elongation complex. To address this point, we adopted an established RNA polymerase II elongation assay to the *Xenopus* animal cap system (Guo *et al*, 2000). We focused these experiments on the regulation of Rx transcription as Rx was able to rescue Wnt-4 MO-injected embryos and Rx expression depends on Wnt-4, *EAF2*, and JNK. Due to RNA polymerase II pausing and template escape during transcription, a population of RNA transcripts is generated from the *Rx* gene locus that differs in RNA length in noggin-neutralized animal caps. This can be shown by RT-PCR using primers specific for different regions of the generated RNAs (Figure 7A), that is, the signal for a primer pair proximal to the transcription start site is stronger than the signal for more distal primer pairs although all of them show comparable signals on a DNA template. Overexpression of *EAF2* in noggin-neutralized caps led to an increase in signal strength for the distal primer, Rx(d) (Figure 7B). The signal for the proximal primer pair Rx(p) remained unchanged, indicating that *EAF2* indeed increases the number of Rx full-length transcripts and thus likely functions as an RNA polymerase II elongation factor. This effect on Rx transcription was not observed when GFP RNA was overexpressed instead of *EAF2* RNA. We also did not observe an effect of *EAF2* on Pax-6 transcription (Figure 7B). This effect of *EAF2* on Rx expression thus might explain why *EAF2* is able to rescue the Wnt-4 MO phenotype.

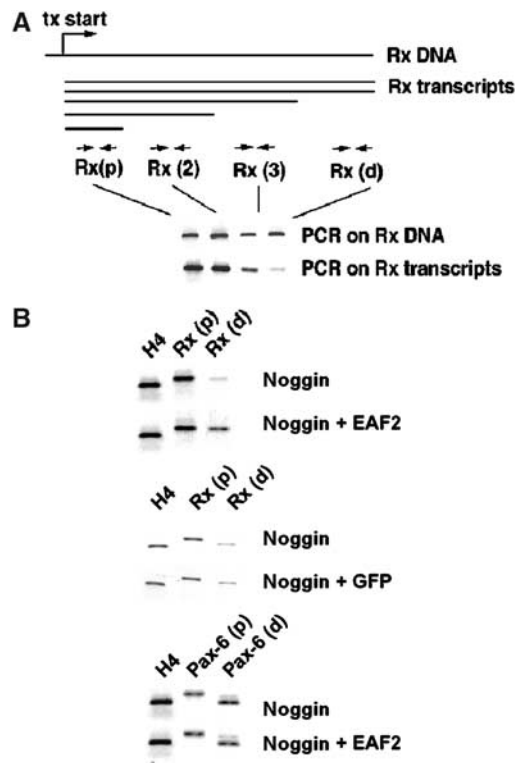


Figure 7 EAF2 has properties of an RNA polymerase II elongation factor. (A) During transcription, RNA transcripts of different lengths are generated due to pausing and template escape, as indicated in the schematic drawing. Relative occurrence of Rx transcripts of different lengths can be monitored by RT-PCR in noggin-neutralized animal caps using different RT-PCR primer sets (Rx (p), Rx (2), Rx(3), Rx(d)). Tx: transcription start site. (B) Overexpression of EAF2, but not GFP RNA, in noggin-neutralized animal caps leads to an increase in full-length transcripts as monitored by the distal Rx primer, Rx(d). H4 served as loading control. Transcription of Pax-6 was not affected by EAF2 overexpression.

Discussion

Detailed studies of transcription factors that are expressed at early stages of eye specification and morphogenesis of the eye have given us great insight into the molecular steps required for eye development (Chow and Lang, 2001). However, the molecular nature of growth factors involved in early eye development has remained largely unknown so far. Here, we report that Wnt-4 is required for early steps of eye development in *X. laevis*. This finding is based on loss-of-function experiments with subsequent analyses of marker gene expression. Furthermore, our gain-of-function studies led to the identification of a novel Wnt-4 downstream factor, EAF2, which is specifically expressed in the developing eye and which is required for eye development. In this context, Wnt-4 acts through a noncanonical Wnt pathway that is independent of β -catenin but involves activation of JNK.

***XWnt-4* is required for *Xenopus* eye development**

Several transcription factors are known to pattern the anterior neural plate of vertebrate embryos and function in eye development (Zuber *et al*, 2003). These transcription factors regulate each other's expression and form a network controlling specification of eye fate. Expression of these genes occurs

after neural induction, but growth factors that initiate their expression or contribute to maintenance of expression are presently unknown. A similar network of transcription factors is involved in specification of eye fate in *Drosophila*, where signaling by EGFR, Notch, Hedgehog, and Wingless has been implicated in eye specification (Royet and Finkelstein, 1997). We have shown here that Wnt-4, a vertebrate member of the Wnt/wingless family of secreted glycoproteins, is required for eye development in a vertebrate embryo, *X. laevis*.

Based on several observations, our experiments suggest that Wnt-4 is required for maintenance rather than initial induction of eye-specific marker genes. First, expression of Pax-6 or Rx in the anterior neural plate starts during gastrulation at stage 11–12 and is strongly upregulated at stage 12.5 (Zuber *et al*, 2003). However, neural Wnt-4 expression is not detectable until stage 12.5–13. Thus, the initial expression of eye marker genes precedes the expression of Wnt-4. Second, overexpression of noggin in animal caps was sufficient to induce Pax-6 and Rx in the absence of Wnt-4 expression, further supporting the notion that Wnt-4 is not required for Pax-6 or Rx induction (data not shown). However, we observed a loss of Pax-6 and Rx expression in the prospective eye field as early as stage 13 in Wnt-4 MO-injected embryos, clearly indicating that Wnt-4 function is required at this stage and thus for maintenance of marker gene expression. In rescue experiments, we were able to show that Rx is able to rescue the Wnt-4 MO phenotype, whereas initial experiments suggest that Pax-6 is unable to revert the Wnt-4 MO phenotype. Indeed, previous publications placed Rx upstream of Pax-6 in eye development (Mathers *et al*, 1997; Zhang *et al*, 2000; Zuber *et al*, 2003). Rx^{-/-} mice do not develop an optic vesicle and lack expression of Pax-6 and Six-3, whereas Pax-6^{-/-} mice develop an optic vesicle, which is, however, abnormally small and malformed (small eye phenotype). Our data implicate Rx as a target for Wnt-4 action, which will require analyses of the *Xenopus* Rx promoter. Experiments aimed at analyzing the *Xenopus* Rx promoter are currently underway.

The effect of Wnt-4 on eye development might be mediated by binding to the Wnt receptor Fz-3. Fz-3 is expressed in the diencephalon and loss-of-function experiments reveal the requirement of Fz-3 for eye development and eye-specific marker gene expression (Rasmussen *et al*, 2001). Just recently, Fz-3 has been shown to be functionally coupled to Wnt-4 during axonal pathfinding in the rat neural tube (Lyuksytova *et al*, 2003). In addition, it has been suggested that Fz-3 is able to activate a noncanonical Wnt pathway (Kühl *et al*, 2000a,b; Rasmussen *et al*, 2001). This is consistent with our observation described above that Wnt-4 signals through noncanonical Wnt pathways during eye development and that low doses of Wnt-4 and Fz-3 synergistically rescue the Wnt-4 MO phenotype.

Strikingly, Wnt-4 knockout mice were reported to lack any obvious eye phenotype (Stark *et al*, 1994). It needs to be noted though that other Wnt members are coexpressed with Wnt-4 in the posterior diencephalon in mice (Parr *et al*, 1993), but not *Xenopus* (Wolda and Moon, 1992), including Wnt-7B. This Wnt member was reported not to fall into the class of Wnt proteins that act through β -catenin (Naylor *et al*, 2000). Accordingly, Wnt-7B was mentioned to activate a noncanonical Wnt signaling pathway (Rosso *et al*, 2005).

Therefore, Wnt-7B may compensate for the loss of Wnt-4 in Wnt-4^{-/-} mice.

Wnt-4 signals through a noncanonical Wnt pathway

Wnt-4 has recently been shown to act through activation of JNK (Cai *et al*, 2002) and thus through the Wnt/JNK pathway. Here we provide further evidence for the existence of a Wnt-4-triggered Wnt/JNK signaling pathway as a deletion mutant of *dsh*, *dsh* Δ DIX, that has been shown to activate noncanonical Wnt, and JNK signaling is sufficient to rescue the Wnt-4 MO phenotype. In addition, treatment of embryos with the JNK inhibitor SP600125 during stages of early eye development resulted in eye defects. Thus, a decrease in JNK signaling activity can phenocopy a Wnt-4 loss-of-function situation and activation of JNK signaling can revert the Wnt-4 MO phenotype. Taken together, these findings strongly indicate that Wnt-4 signals through JNK during eye development. This observation is further supported by the finding that JNK1^{-/-}, JNK2^{+/-} mutant mice also display an eye phenotype with reduced Pax-6 expression although during later stages of eye development (Weston *et al*, 2003). In parallel, we were able to exclude a role for Wnt/ β -catenin signaling during these early stages of eye development, as coinjection of Wnt-4 MO and β -catenin RNA or DNA did not rescue the eye-specific phenotype. Further supporting our conclusion, Wnt-4 did not posteriorize neuralized animal caps, whereas Wnt-3A and β -catenin did (data not shown). This, however, does not exclude the possibility that Wnt/ β -catenin signaling might act at later stages of eye development, that is, lens development, as recently suggested (Stump *et al*, 2003).

Noncanonical Wnt signaling has been shown to regulate cell migration processes, that is, during gastrulation or migration of neuronal precursor cells (Veeman *et al*, 2003). These processes involve the transmembrane proteins strabismus or prickle to be part of a noncanonical Wnt signaling pathway resembling the *Drosophila* planar cell polarity pathway. However, when we injected a strabismus MO (Darken *et al*, 2002) into animal neural blastomeres, we never observed the loss of eye phenotype which is characteristic for the Wnt-4 MO (data not shown). In addition, our marker gene analyses also indicate that Wnt-4 MO injection into dorsal-animal blastomeres does not interfere with cell movements. In summary, we conclude that the effect of neural Wnt-4 MO injections is on differentiation rather than cell migration.

Wnt-4 as a long-range signaling molecule

Wnt-4 is expressed at the forebrain/midbrain boundary and its expression does not overlap with the primary eye field, raising the question how Wnt-4 mediates its effect on eye development. As Wnts are lipid modified and as they strongly interact with proteoglycans (Logan and Nusse, 2004), it is widely assumed that they act as short-range signaling molecules. However, recent findings also argue for a long-range signaling activity of Wnts. Recently, it has been shown that Wnt-3A can induce β -catenin signaling with properties consistent as a long-range signaling molecule (Kiecker and Niehrs, 2001) during anterior–posterior patterning of the neural tube. During inner ear development, Wnt-7A is also able to signal over a long distance (Dabdoub *et al*, 2003). Patterning of the somites occurs by Wnt proteins derived

from the adjacent dorsal neural tube as well as the ectoderm at least in part through noncanonical Wnt signaling (Chen *et al*, 2004). Wnt-4 has also been implicated as a long-range signaling molecule during anterior–posterior guidance of commissural axons in rat embryos (Lyuksyutova *et al*, 2003). Consistent with this hypothesis, the *Drosophila* homolog wingless also can signal over a long distance (Zecca *et al*, 1996; Neumann and Cohen, 1997). Our findings that EAF2 can be regulated by Wnt-4 signaling and that the expression domains of both genes in neural tissue are just adjacent to each other support the idea of Wnt-4 to signal as a long-range molecule.

A potential function of RNA polymerase II elongation for eye development

Using a subtractive cloning strategy, we identified EAF2 as a potential downstream factor in neural Wnt-4 signaling, which is known to interact in mammals with the RNA polymerase II elongation factor ELL. Within eukaryotes, several elongation factors exist that regulate the activity of RNA polymerase II. These elongation factors are regulated by interacting proteins and show some specificity with respect to the genes they regulate (Shilatfard *et al*, 2003). Interfering with RNA polymerase II elongation by specifically targeting individual components of elongation complexes thus might result in specific phenotypes during development. In *Xenopus*, EAF2 is highly enriched in the developing eye with lower expression in the pronephros, the epiphysis, and the somites. This expression pattern is conserved between species as the mouse EAF2 homolog is expressed in the eye, the developing kidney, and the somites as well (Li *et al*, 2003). Whether this reflects a conserved function of EAF 2 is currently unknown as knock-out mice for EAF2 are not available. On a functional level, our data demonstrate that EAF2 is essential for normal eye development as EAF2 regulates Rx expression.

How is the function of EAF2 coupled to the function of Wnt-4 during eye development? Whereas we observed a downregulation of eye-specific marker genes after knock-down of Wnt-4 function as early as stage 13, eye-specific expression of EAF2 starts not earlier than stage 18. Nevertheless, EAF2 can rescue the phenotype observed after Wnt-4 MO treatment, suggesting an identical molecular target. Indeed, we were able to demonstrate that Rx can rescue the Wnt-4 MO phenotype, whereas Rx itself is regulated by EAF2. This suggests that Wnt-4 is required for early aspects of eye development in the absence of EAF2 expression. Later, Wnt-4 induces EAF2, which itself is involved in maintaining the expression of eye-specific marker genes in a stable feedback loop.

We also analysed a potential function of EAF2 during pronephros development. Knockdown of pronephric EAF2 gene function resulted in abnormal pronephric development affecting, however, not only the EAF2-expressing tubules but also the pronephric duct (data not shown). Pronephric defects occurred with high frequency in conjunction with abnormal somitogenesis, as revealed by marker gene analysis. As the paraxial mesoderm, which gives rise to the somites, is an important source of signals inducing pronephric development, it is likely that the large-scale nature of the pronephric defects is a consequence of disrupting an earlier, EAF2-dependent step essential for paraxial mesoderm

differentiation. This precludes at present a detailed analysis of EAF2 function in pronephric development.

In summary, our data provide a first example for a growth factor regulating the expression of an RNA polymerase II elongation factor component. Thereby we also add a new layer of complexity on the regulation of gene expression by extracellular factors. Having established a link between non-canonical Wnt-4 signaling and EAF2 expression and function, our data also provide a new molecular mechanism as to how biological effects of noncanonical Wnt signaling might be mediated.

Materials and methods

Embryos

Xenopus embryos were obtained using standard procedures and staged according to Nieuwkoop and Faber (1967).

RNA transcription, microinjections, and explant cultures

Capped RNAs were transcribed *in vitro* using the SP6 or T7 Message Machine Kit (Ambion). For the animal cap assay, embryos were injected at the two-cell stage and caps were dissected at stage 8. The explants were cultured in LCMR (43 mM NaCl, 0.85 mM KCl, 0.37 mM CaCl₂, 0.19 mM MgCl₂, 5 mM HEPES, pH 7.2) including 0.5% BSA and penicillin/streptomycin (Invitrogen, final concentrations: penicillin 20 U/ml, streptomycin 20 µg/ml) until uninjected control embryos reached stage 22. RNA amounts injected were: noggin 400 pg; XWnt-4 1000 pg. For FGF treatment, bFGF (Promega) was added at a concentration of 200 ng/ml. The JNK inhibitor SP600125 (Alexis) was used at a concentration of 12.5 µM, consistent with the inhibitory function of this compound against JNK in different cell-based assays (Bennett *et al*, 2001). Optimal concentration and timing of inhibitor treatment were evaluated experimentally (see Supplementary Figure 2). The MAPKK inhibitor PD98059 (Calbiochem) was used at 2 µM.

RNA isolation and RT-PCR analyses

Total RNA was isolated from explanted tissues using Purescript RNA Isolation Kit (Biozym) and subsequently analyzed by RT-PCR. First-strand cDNA was synthesized according to the Invitrogen protocol using Superscript II reverse transcriptase. The PCR reaction mixtures were prepared using the Master Amp PCR Core Kit (Epicentre Technologies) and PCR was performed under standard conditions at 55°C annealing temperature. Primer sequences are given in Supplementary data.

Whole-mount *in situ* hybridization and histology

For the analyses of marker gene expression by whole-mount *in situ* hybridization, embryos were injected unilaterally into one dorsal-animal blastomere at the eight-cell stage and cultured until indicated stages. Embryos were fixed in MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO₄, and 4% formaldehyde) for 1.5–2 h at room temperature, washed in 1 × MEM solution and dehydrated in EtOH. Whole-mount *in situ* hybridization was performed according to a standard protocol. For histology, embryos were refixed in MEMFA for 1–2 h and embedded in gelatine/BSA. Vibratome sections were cut at 30 µm thickness and coverglass mounted with 90% glycerol, 10% 0.1 M Tris-Cl (pH 7.4).

Wnt-4 and EAF2 antisense MO

The Wnt-4 MO was used as published (Saulnier *et al*, 2002). A standard MO with the base composition 5'-CCTCTT ACCTCAGTTACAATTTATA-3' or a Wnt-4 4-base mismatch MO (Saulnier *et al*, 2002) was used as a control. The EAF2 MO used

was a 25-mer MO with the base composition 5'-AGCTGCTCCATT CATCTGCCGCGCC-3'. The functionality of the EAF2 MO was tested using the transcription and translation assay TNT-Kit (Promega). The 5'UTR + EAF2 construct consists of the whole open reading frame and an additional 10 bp of the 5'UTR. These 10 bp are missing in the Δ5'UTR + EAF2 construct. Oligos were resuspended in sterile water and injected unilaterally into a single blastomere at the eight-cell stage in doses of 5 ng per embryo. One dorsal-animal blastomere was selected to target anterior neural tissue, while a ventral vegetal blastomere was selected to target the developing pronephric kidney. For rescue experiments, 5 ng of Wnt-4 MO was injected together with different RNAs or DNA as indicated. Amounts of RNA were: dshΔDIX: 50 pg, β-catenin RNA: 150 pg, β-catenin DNA: 100 pg, EAF2: 1 ng, Activin-Wnt-4: 10 or 50 pg, Fz-3: 10 pg, Rx: 200 pg, Pax-6: 200 pg. The β-catenin RNA induced secondary axes when overexpressed on the ventral side of four-cell stage embryos, indicating the functionality of the RNA used.

Subtractive cDNA screen

In order to identify *Wnt-4* downstream genes activated in neuralized animal caps, we followed the protocols of the following Clontech kits: SMART PCR cDNA synthesis kit, PCR-Select cDNA subtraction kit and PCR-Select differential screening kit. The original identified EAF2 clone contained 950 bp of the 3'UTR and we completed the EAF2 sequence by a RACE procedure (GeneRacer Kit, Invitrogen). The 5' RACE primer used was: 5'-GAT CTG CCA GGC ACT GAG GGA ATT T-3' using an annealing temperature of 65°C, resulting in a 1800 bp fragment with the 5'UTR and the open reading frame. The full sequence of EAF2 was deposited under the accession number AJ579576. A related EST clone was found in the database with the accession number CA988261, which likely represents an allelic variation.

RNA polymerase II elongation assay

For the RNA polymerase II elongation assay, animal caps were neuralized by injection of 400 pg of noggin RNA at two-cell stage. Caps were cut at stage 8 and cultured until stage 20 in 1 × MBSh (10 mM HEPES (pH 7.5), 88 mM NaCl, 2.5 mM KCl, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 2.4 mM NaHCO₃). The length of RNA transcripts was monitored by RT-PCR. Primer sequences are given in Supplementary data.

Cell culture

Human embryonic kidney cells (HEK293) were stably transfected with expression plasmids coding for β₂AR/Rfz-1 or β₂AR/Rfz-2. Correct expression of these constructs was verified by RT-PCR using primers specific for Rfz-1 or Rfz-2 intracellular loops. To activate or inactivate these receptors, cells were treated with the β₂-adrenergic agonist isoproterenol (100 µM) or the β₂-adrenergic antagonist propranolol (30 µM) and gene expression was analyzed by RT-PCR. Primers are given in Supplementary data. Cycloheximide was used at 500 ng/ml simultaneously to agonist/antagonist treatment. Functionality of the cycloheximide used was tested in a TNT assay.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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