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Direct control of *Hoxd1* and *Irx3* expression by Wnt/β-catenin signaling during anteroposterior patterning of the neural axis in *Xenopus*

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ABSTRACT During and after gastrulation, the neural axis in vertebrates is patterned along the antero-posterior axis by the combined activity of signaling factors secreted in the neural ectoderm and the underlying mesoderm. These signals divide the neural axis into four major divisions: the forebrain, midbrain, hindbrain and spinal chord. Among the signals that pattern the neural axis, Wnts play a prominent role and many patterning genes have been found to be direct Wnt/β-catenin target genes, including several homeobox domain-containing transcription factors. Here we show that *HoxD1* and *Irx3* are transcriptionally induced by the Wnt pathway during neurulation. Using induction in the presence of the translation blocking drug cycloheximide and chromatin immunoprecipitation assays, we confirm that *HoxD1* and *Irx3* are both direct Wnt target genes. In addition, we identified *Crabp2* (cellular retinoic acid binding protein 2) as an indirect target that potentially links the activities of Wnt and retinoic acid during antero-posterior patterning.

KEY WORDS: *Wnt signaling, anteroposterior axis, Hox genes, Xenopus, patterning*

Introduction

During early embryonic development in vertebrates the neural tube is patterned along the anteroposterior (AP) axis. A combined activity of signals divide the neural tube into four major parts: forebrain, midbrain, hindbrain, and spinal cord, and these different regions can be detected soon after the formation of the neural plate. These signals are thought to be initially transferred from the mesoderm that underlies the future neural plate during gastrulation and afterwards also become expressed in the neural plate proper. Extensive evidence has been obtained to implicate the FGF, Wnt, and retinoic acid (RA) signaling pathways in the caudalization of the neural axis (Durston *et al.*, 1997, Gamse and Sive, 2000, Gavalas and Krumlauf, 2000, Gomez-Skarmeta *et al.*, 2003). In function of the combined activity of these factors boundaries of Hox gene expression are established along the AP axis, which in the neural plate have been suggested to be involved in establishing different identities in the hindbrain and spinal cord (Deschamps *et al.*, 1999, Iimura and Pourquie, 2007, McGinnis and Krumlauf, 1992). Also other homeodomain containing transcription factors are induced in specific domains along the AP axis, including *Cdx* genes and transcription factors that pattern the tissue rostral from the most anterior expressed *Hox* genes (Deschamps *et al.*, 1999).

Among the signals that pattern the neural axis in *Xenopus*, Wnts have recently taken centre stage. Several Wnts are expressed along the dorsal AP axis and a morphogen gradient by Wnt/β-catenin signaling is shown to exist in the neural plate at the end of gastrulation with the highest activity posterior, which gradually decreases anteriorly, and is completely absent in the most rostral structures (Kiecker and Niehrs, 2001). This gradient is most likely established by the activities of a posterior-to-anterior

Abbreviations used in this paper: AP, anteroposterior; ChIP, chromatin immunoprecipitation; CHX: cycloheximide; Dex, dexamethasone; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; RA, retinoic acid; WISH, whole mount *in situ* hybrization.

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gradient of Wnt ligands and an opposing anterior-to-posterior gradient of Wnt antagonists. Consequently, several patterning genes are proposed to be expressed in function of specific thresholds of Wnt/β-catenin signaling activity in combination with other competence factors (Ille and Sommer, 2005, Yamaguchi, 2001). In this process Wnt signaling is expected to cooperate with other factors that have been shown to be implicated in the patterning of the neural axis, such as FGF, RA, Nodal, Sonic Hedgehog and BMP.

In order to identify patterning genes that are under direct control of Wnt/β-catenin signaling, we deployed transgenic *Xenopus laevis* embryos that carry binary inducible constructs that can either inhibit or mimic Wnt/β-catenin signaling (Denayer *et al.*, 2008a, Deroo *et al.*, 2004). We identified several homeodomain-containing transcription factors that are induced by Wnt signaling in the neurula stage embryo. Of these, *Cdx1*, *Cdx4*, *Msx1*, *Msx2* and *Otx2* have all been described before as direct Wnt target genes (Fujimura *et al.*, 2009, Hussein *et al.*, 2003, Lickert *et al.*, 2000, Miller *et al.*, 2007, Pilon *et al.*, 2006, Pilon *et al.*, 2007, Westenskow *et al.*, 2009). We here identify two other homeodomain-containing genes, *Hoxd1* and *Irx3*, as direct Wnt/β-catenin target genes. In addition, we identified an indirect target *Crabp2* that may link Wnt and RA signaling in the hindbrain.

Results

Identification of novel Wnt/β-catenin targets using transgenic Xenopus *embryos*

In order to search for new target genes of the canonical Wnt/β-catenin pathway, we generated *Xenopus laevis* embryos that are transgenic for an optimized hormone-inducible, multicomponent Wnt pathway activating or repressing system (Denayer *et al.*, 2008a, Deroo *et al.*, 2004). These embryos constitute an ideal source for differential gene expression analysis since the integrated Wnt pathway interfering systems specifically intersect at the nuclear endpoint of the pathway. The Wnt pathway activating and repressing system, and the corresponding transgenic embryos are further referred to as pVP16 and pEnR, respectively (Fig. 1A). To control for possible aspecific back-

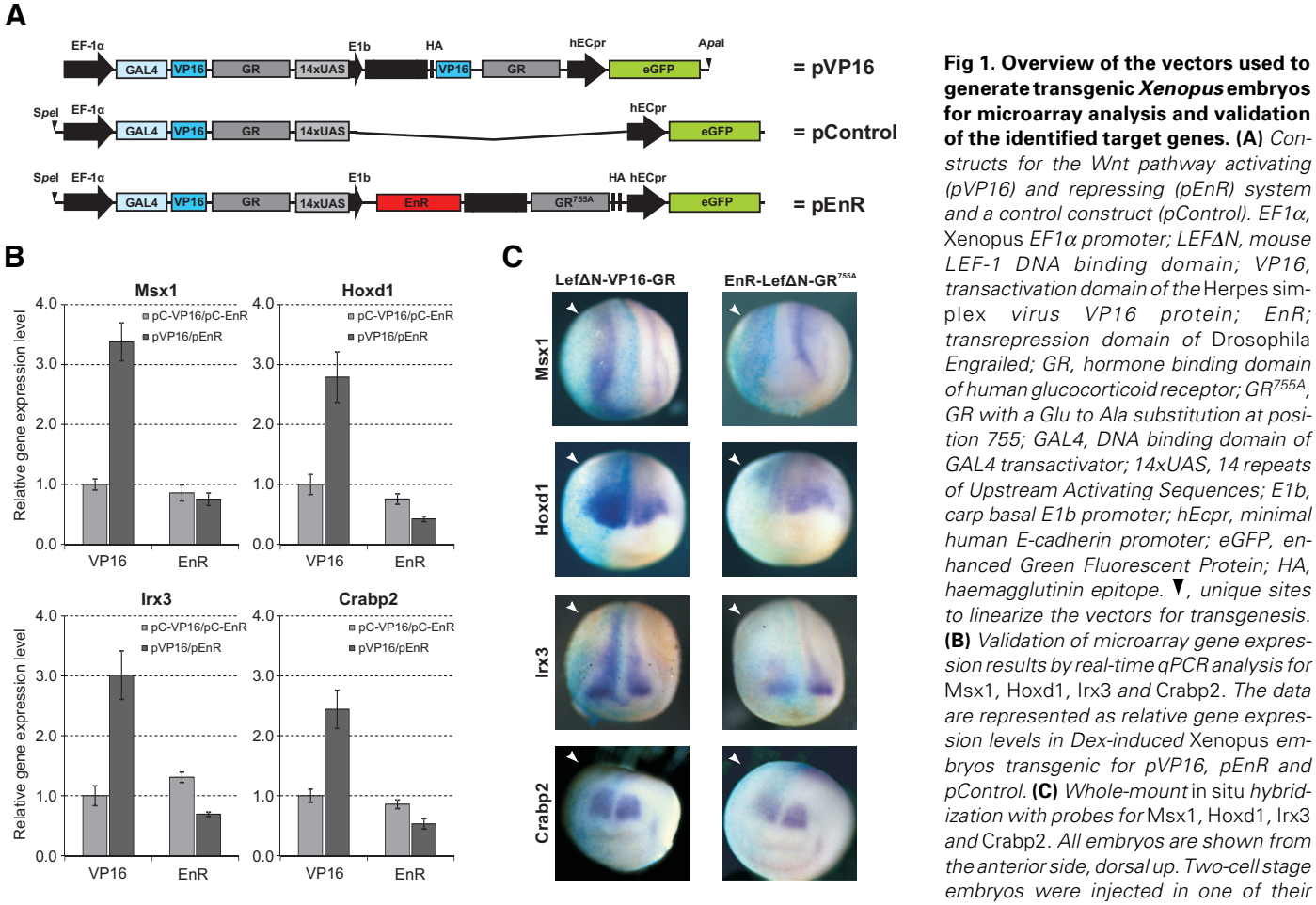

Fig 1. Overview of the vectors used to generate transgenic *Xenopus* embryos for microarray analysis and validation of the identified target genes. (A) *Constructs for the Wnt pathway activating (pVP16) and repressing (pEnR) system and a control construct (pControl). EF1α,* Xenopus *EF1α promoter; LEFΔN, mouse LEF-1 DNA binding domain; VP16, transactivation domain of the* Herpes simplex *virus VP16 protein; EnR; transrepression domain of* Drosophila *Engrailed; GR, hormone binding domain of human glucocorticoid receptor; GR^{755A}, GR with a Glu to Ala substitution at position 755; GAL4, DNA binding domain of GAL4 transactivator; 14xUAS, 14 repeats of Upstream Activating Sequences; E1b, carp basal E1b promoter; hEcpr, minimal human E-cadherin promoter; eGFP, enhanced Green Fluorescent Protein; HA, haemagglutinin epitope.* ▼*, unique sites to linearize the vectors for transgenesis.* **(B)** *Validation of microarray gene expression results by real-time qPCR analysis for* Msx1, Hoxd1, Irx3 *and* Crabp2. *The data are represented as relative gene expression levels in Dex-induced* Xenopus *embryos transgenic for pVP16, pEnR and pControl.* **(C)** *Whole-mount* in situ *hybridization with probes for* Msx1, Hoxd1, Irx3 *and* Crabp2. *All embryos are shown from the anterior side, dorsal up. Two-cell stage embryos were injected in one of their blastomeres with 15 pg RNA for the Dex inducible Wnt activating construct (LefΔN-VP16-GR) and 300 pg for the Dex inducible Wnt repressing construct (EnR-LefΔN-GR^{755A}) together with 50 pg of β-galactosidase RNA as a tracer. The injected side is indicated by an arrowhead. All embryos were treated with Dex at late gastrula (st12.5) until end-neurula (st17). Upregulation in LefΔN-VP16-GR injected embryos is seen for* Msx1 *(71%, n=14),* Irx3 *(67%, n=12) and* Hoxd1 *(76%, n=21). An expanded expression domain was frequently observed for* Crabp *(33%, n=15). Reduced expression is seen in EnR-LefΔN-GR^{755A} injected embryos for* Msx1 *(87%, n=24),* Irx3 *(79%, n=14),* Hoxd1 *(92%, n=25) and* Crabp *(47%, n=15).*

ground signals of the induction regimen, embryos with a transgenically integrated control vector (pControl), which lacks the Wnt pathway interfering fusion but still possesses the common remaining elements of the multicomponent systems, were generated (Fig. 1A).

During gastrulation, normally developing F_0 transgenic embryos were singled out on the basis of their non-mosaic genuine eGFP expression, which is included as a selection marker in all three transgenesis vectors. This selection aimed at embryos with more or less comparable eGFP intensity, as this could be used as a rough and indirect reference for an approximately equal copy number of the integrated transgene. The transgenic binary Wnt interfering constructs were induced by the addition of dexamethasone (Dex) to the cultivation buffer. In order to enrich for primary Wnt target genes, we wanted to keep the time frame of Dex treatment as short as possible. To accommodate this, a kinetic experiment was previously performed by doing semi-quantitative RT-PCR for the known direct Wnt target gene *En2* (McGrew *et al.*, 1999), on transgenic pEnR embryos incubated with Dex from end-gastrula stage onwards for different time periods. This experiment showed that a >3 fold repression of *En2* occurred as soon as 7 hours post Dex treatment (data not shown). Therefore, in our Wnt target screen, the pEnR transgenic embryos were incubated with Dex from stage 12.5 (end-gastrula stage) onwards till 7 hours later, when they approximately reached end-neurula stage (stage 17). In parallel, embryos transgenic for the pControl vector underwent the same Dex regimen. While for efficient repressive activity a minimal time for the decay of pre-made Wnt target RNA needs to be considered, this is not the case for the Wnt pathway activating system. The latter is believed to require a shorter Dex treatment to become appropriately functional. Therefore, pVP16 transgenic embryos were subjected to 4 hours of Dex treatment. To synchronize the endpoint, the transgenic pVP16 embryos were only induced by Dex at stage 13.5-14 for 4 hours. Embryos transgenic for the pControl vector underwent the same treatment in parallel. Following the appropriate Dex treatment, total RNA was extracted from those 4 different pools of transgenic embryos. Each RNA sample was then used for hybridization against a *Xenopus laevis* Affymetrix array.

We sought for genes which were activated in the pVP16 transgenic embryos and/or repressed in the pEnR transgenics. Only a small number of genes (~ 100) were actually affected by our Wnt-interfering constructs. In total, 86 genes were annotated and 21% (= 18) of these were already described in the literature to be Wnt target genes (e.g. *Axin2*, *Dkk1*, *Sp5*, Wnt 1-responsive Cdc42 homologue). Interestingly, many of the genes are expressed in regions where Wnt ligands are known to be expressed (data to be reported elsewhere), including genes encoding homeodomain-containing transcription factors involved in AP patterning of the neural axis.

Identification and characterization of AP patterning genes

We further focused our research on those potential Wnt/β-catenin target genes that have been clearly documented to play a role in AP patterning of the neural axis. In addition to the 5 genes that have already been identified as direct Wnt/β-catenin target genes, i.e. *Cdx1*, *Cdx4*, *Otx2*, *Msx1* and *Msx2* (Fujimura *et al.*, 2009, Hussein *et al.*, 2003, Lickert *et al.*, 2000, Miller *et al.*, 2007, Pilon *et al.*, 2006, Pilon *et al.*, 2007, Westenskow *et al.*, 2009), we identified the homeobox-containing genes *Hoxd1*

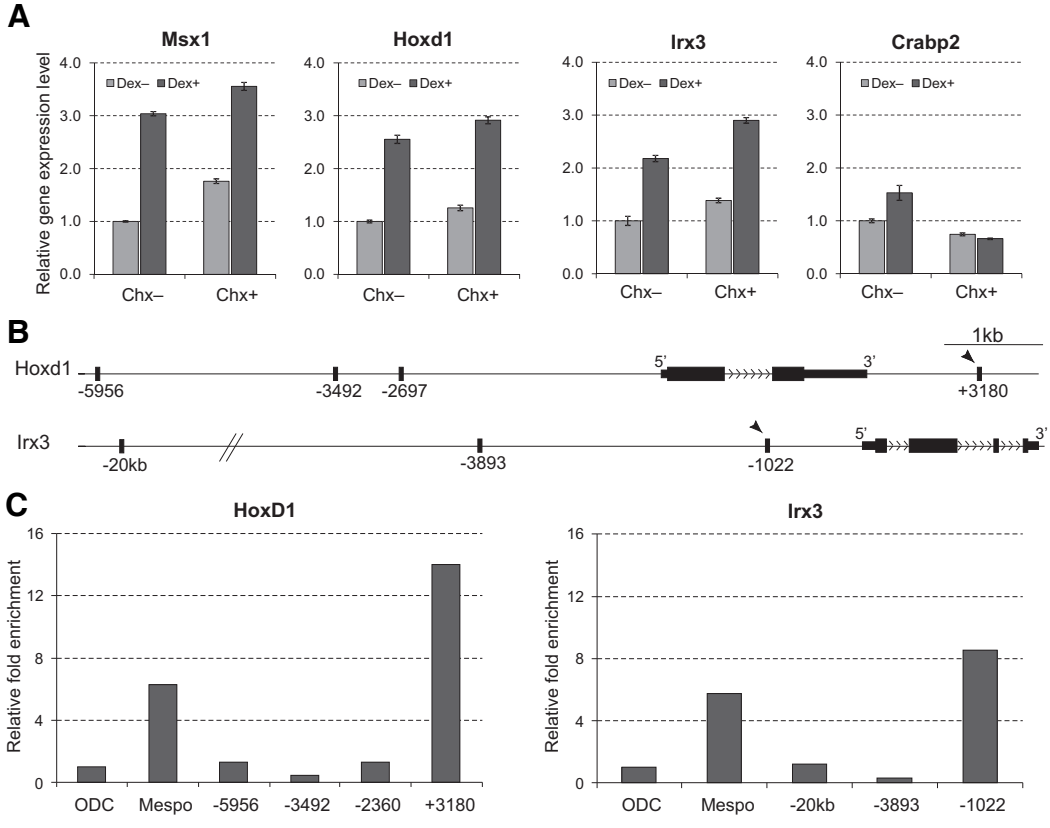

Fig 2. Identification of *Hoxd1* and *Irx3* as direct Wnt target genes by qRT-PCR analysis after cyclohexamide (CHX) treatment and chromatin immunoprecipitation (ChIP) experiments. (A) *Influence of the translation inhibitor CHX on Wnt-induced expression of* Msx1, Hoxd1, Irx3 *and* Crabp2. *Embryos were injected at four-cell stage in each blastomere with 25 pg Dex inducible Wnt-activating RNA (LefΔN-VP16-GR) and treated with Dex and/or CHX from mid-neurula (st15) until end-neurula (st17).* **(B)** *Schematic overview of predicted LEF1/TCF consensus sites at the* Hoxd1 *and* Irx3 *gene loci. ChIP positive sites are indicated by arrows. The numbers refer to the distance (in bases) from the start codon.* **(C)** *ChIP assay on WT* Xenopus tropicalis *embryos detecting binding of the β-catenin/LEF1/TCF complex to the* Hoxd1 *and* Irx3 *gene loci.* Mespo *was included as a positive control and a region in the* ODC *gene served as a negative control. Data are shown as fold enrichment relative to the negative control.*

and *Irx3* as potential novel direct target genes. In addition, we focused our attention on the *Crabp2* gene since it encodes a protein that potentially feeds into the RA signaling pathway.

We confirmed the data from the micro-array using quantitative reverse transcriptase PCR (qRT-PCR) analysis for *Hoxd1*, *Irx3* and *Crabp2*. As a positive control we included the *Msx1* gene. Significant induction of all four genes was found in Dex-induced pVP16 transgenic animals compared to the pControl (4 hour). Similarly, all genes were suppressed in the Wnt-inhibiting pEnR transgenics (Fig. 1B). The relative upregulation following Wnt pathway activation was much stronger than the relative downregulation seen in pEnR versus pControl. This could be anticipated, considering the relatively strong induction capacity of the VP16 transactivation domain in the integrated chimeric transgene. One could also imagine that the capacity to appropriately elevate the expression of a specific gene is stronger than the capacity to repress it, taking into account the additional time needed to decay pre-made target RNA in the latter case.

In order to analyze which expression domains of the potential novel target genes were under control of Wnt/β-catenin signaling, we performed whole mount *in situ* hybridization (WISH) analysis on embryos injected unilaterally at the 2-cell stage with synthetic RNA encoding either the Wnt-mimicking LefΔN-VP16-GR or the Wnt-inhibiting EnR-LefΔN-GR^{755A}, both previously characterized (Denayer *et al.*, 2008a, Deroo *et al.*, 2004). RNA encoding β-galactosidase was coinjected as a tracer. At stage 12.5 the chimeric proteins were activated by the addition of Dex to the culture buffer and embryos were further grown in the presence of Dex for 7 hours up until stage 17. Embryos were fixed and processed for WISH with probes for *Msx1* (positive control), *Hoxd1*, *Irx3* and *Crabp2*. Expression of all genes was clearly upregulated or expanded *(Crabp2)* by the LEFΔN-VP16-GR RNA and repressed in embryos injected with EnR-LefΔN-GR^{755A} RNA (Fig. 1C).

Characterization of Hoxd1 *and* Irx3 *as direct Wnt/β-catenin target genes*

In order to investigate whether the *Hoxd1*, *Irx3* and *Crabp2* genes are direct targets of the Wnt/β-catenin pathway, we performed gene induction assays in the presence of the translation-blocking drug cycloheximide (CHX). Embryos were injected with LEFΔN-VP16-GR RNA and cultivated until stage 15, when Dex and/or CHX were added to the culture buffer. Two hours later embryos were lysed and RNA was extracted and used for qRT-PCR analysis. The prerequisite of a direct Wnt target gene is that it is activated by LEFΔN-VP16-GR (+ Dex) even in the presence of CHX. If the gene is indirectly induced by an intermediate transcription factor (that is Wnt-induced) it should not be upregulated in the presence of CHX. As is clear from Fig. 2A, *Msx1* (positive control), *Hoxd1* and *Irx3* are upregulated by LEFΔN-VP16-GR in the presence of CHX, confirming that they are direct target genes. In contrast, the gene *Crabp2* was not induced by Dex in the presence of CHX, indicating that it is an indirect target gene.

To further characterize the *Hoxd1* and *Irx3* genes as novel direct Wnt/β-catenin target genes, we performed chromatin immunoprecipitation (ChIP) assays. Since no genomic information is available for *Xenopus laevis*, we performed these assays in the highly related organism *Xenopus tropicalis* for which the genome is sequenced. We first performed *in silico* screening of the *Hoxd1* and *Irx3* gene loci for the presence of potential LEF1/TCF binding sites. This identified 4 regions in the *Hoxd1* locus, 3 in the upstream region at positions -5956, -

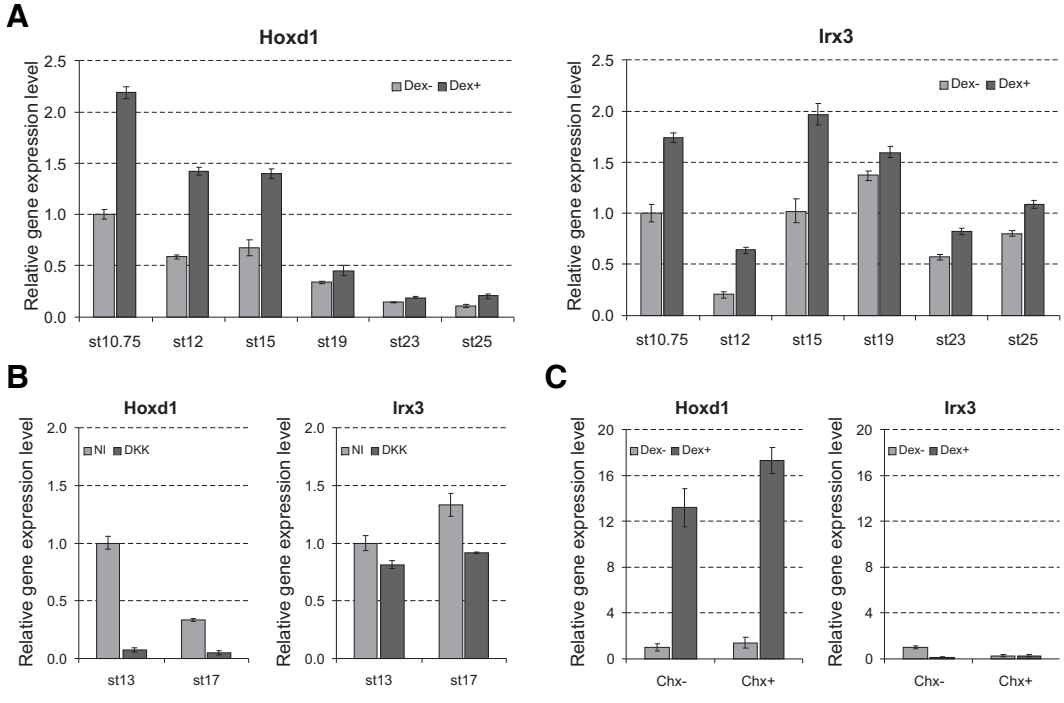

Fig. 3. Spatio-temporal restricted Wnt-responsiveness of *Irx3* and *Hoxd1* and confirmation of *Irx3* and *Hoxd1* targets of endogenous Wnt signaling. (A) *Evaluation of Wnt induced expression of* Irx3 *and* Hoxd1 *at different developmental time points. Embryos were injected at the four-cell stage in each blastomere with 25 pg Wnt activating RNA (LefΔN-VP16-GR) and treated with Dex for 2 hours at stages 10.75, 12, 15, 19, 23 and 25, followed by qRT-PCR analysis.* **(B)** Irx3 *and* HoxD1 *are down-regulated by the Wnt inhibitor Dkk1. Embryos were injected at the four-cell stage in each blastomere with 25 pg Dkk1 RNA. Expression of* Irx3 *and* HoxD1 *was analyzed by qRT-PCR at stage 13 and stage 17 compared to non-injected embryos.* **(C)** *Response of* Irx3 *and* HoxD1 *to the Wnt activating construct in animal caps. Embryos were injected at the four-cell stage in each blastomere with 25 pg Dex-inducible Wnt activating RNA (LefΔN-VP16-GR). Animal caps were dissected at stage 8, treated at stage 15 with Dex and/or CHX for 2 hours and used for qRT-PCR analysis.*

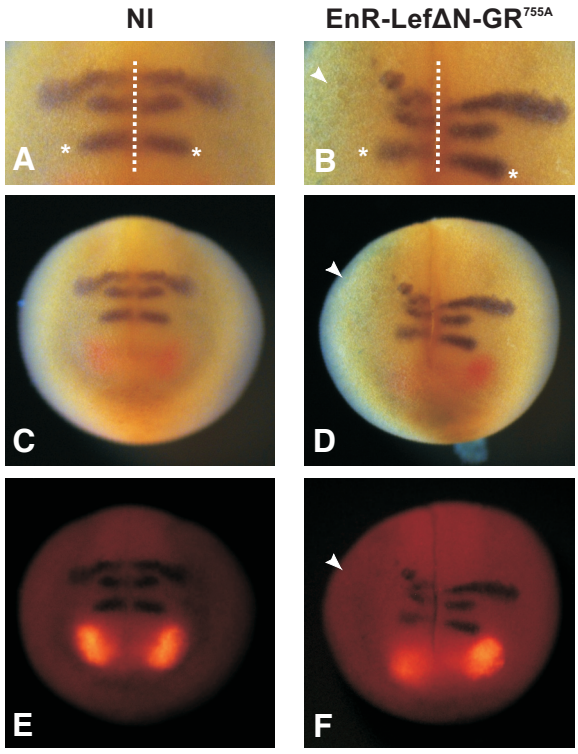

Fig. 4. Wnt repression during neurulation can induce a posterior shift in *Krox20* and *Engrailed-2* expression. *Embryos were injected unilaterally with 300 pg Dex-inducible Wnt-repressing RNA (EnR-LefΔN-GR755A) at the four-cell stage in two blastomeres* **(B,D,F)**. *As a tracer, 50 pg of β-gal RNA was co-injected. Non-injected (NI) embryos were included as a control* **(A,C,E)**. *Both injected and control embryos were treated with Dex from late gastrula (st12.5) until st20. The most anterior stripe (*) represents* En2 *expression, the other 2 show expression of* Krox20 *in rhombomeres 3 and 5. Expression of* Krox20 *and* Engrailed-2 *was frequently shifted to a more posterior position. In contrast, expression of* Rx2a *(shown in Fast Red staining) was not shifted. Bright field* **(A-D)** *and red fluorescent images* **(E, F)** *are shown. The injected side is indicated by an arrowhead. The dotted line indicates the dorsal midline.*

3492 and -2360 from the start codon. In addition, a potential binding site is present in the 3' region at position +3180 (Fig. 2B). For the *Irx3* gene, we found potential LEF1/TCF binding sites in positions -1022 and -3893 and more upstream at -20 kb. Stage 17 embryos were cross-linked and lysed, and DNA was immunoprecipitated. Since we do not have specific antibodies that recognize all Lef1/Tcf proteins in *Xenopus*, we performed the ChIP assay with a polyclonal antibody against β-catenin, which is the obligate nuclear interaction partner for all LEF1/TCF factors that transmit Wnt/β-catenin signaling. Immunoprecipitated DNA was then used for qPCR with primers that span the potential LEF1/TCF sites. The known Wnt target gene *Mespo* served as a positive control (Wang *et al.*, 2007) and normalization was done against DNA immunoprecipitated with rabbit IgG. For the *Irx3* gene, only the DNA fragment spanning the potential LEF1/TCF binding site in the proximal promoter at position -1022 was enriched (Fig. 2C). None of the potential binding sites in the promoter region of the *Hoxd1* was found to be immunoprecipitated by β-catenin antibodies. However, the LEF1/TCF binding site at position +3180, i.e. downstream of the coding sequence, was significantly enriched in the ChIP experiment (Fig. 2C).

Together, these data strongly indicate that both *Hoxd1* and *Irx3* are direct target genes of the canonical Wnt/β-catenin pathway involved in AP patterning of the neural axis. The *Crabp2* gene, however, has the characteristics of an indirect target.

Spatio-temporal analysis of the Hoxd1 *and* Irx3 *Wnt target genes*

To determine the developmental stages when the *Hoxd1* and *Irx3* genes are under control of the Wnt/β-catenin signaling, we injected embryos with the inducible LEFΔN-pVP16-GR Wnt mimicking RNA and added Dex at different developmental time points. Embryos were lysed 2 hours after induction and RNA was isolated and used for qRT-PCR analysis. Induction of both *Hoxd1* and *Irx3* was observed at early and late gastrula stages (stage 10.75 and 12) and in neurulas (stage 15). Both genes were less responsive to Wnt signaling at later stages (Fig. 3A). To further prove the specificity of our Wnt-interfering constructs, we analyzed the expression of *Hoxd1* and *Irx3* in embryos ectopically expressing the secreted Wnt inhibitor Dkk1. Injection of Dkk1 RNA reduced the expression of both *Hoxd1* and *Irx3* at the gastrula and neurula stages (Fig. 3B).

The restricted expression patterns of *Hoxd1* and *Irx3* despite the ubiquitous presence of the Dex-activated pVP16 construct in whole embryos (Fig. 1C) suggests that the induction of both genes is very context dependent. To further address this issue, we investigated the response to the Wnt-mimicking pVP16 construct in animal caps, which consist of ectoderm destined to form epidermis. When the sibling embryos reached stage 15, the animal caps were activated for 2 hours with Dex in the absence or presence of CHX. Interestingly, while the *Hoxd1* gene was strongly induced in the animal caps, this was not the case for *Irx3* (Fig. 3C). This indicates that the expression of the latter is strictly context dependent.

Together, these experiments show that the induction of both Wnt target genes is under strict spatio-temporal control.

Neurula stage Wnt inhibition parallels the effects of Hoxd1 and Irx3 depletion on AP marker gene expression

In order to further characterize the effect of Wnt inhibition on AP marker gene expression, we unilaterally injected 4-cell stage embryos into the 2 left blastomeres with EnR-LefΔN-GR755A and β-galactosidase RNA, the latter as a tracer. Embryos were induced at stage 12.5 and fixed at stage 20. Embryos were then processed for WISH to detect the expression of *En2*, which is a marker of the midbrain-hindbrain boundary whose expression domain overlaps at the neurula stage with the anterior transversal stripe of *Irx3* expression (Bellefroid *et al.*, 1998). In addition, we analyzed the expression of *Krox20*, which labels the rhombomeres 3 and 5 in the hindbrain, and is within the expression domain of *Hoxd1* (McNulty *et al.*, 2005). We observed reduced expression for both *Krox20* (82% of the embryos analyzed, n=33) and *En2* (73%, n=33) in the injected site. In addition, residual marker gene expression was frequently shifted towards a more posterior position, relative to the expression of *Rx2a*, a marker for

the eye field (Fig. 4). This was especially the case for *Krox20* (64%) but less eminent for *En2* (18%). Repression of *Krox20* and *En2* has previously been observed in embryos depleted for *Irx* genes (Rodriguez-Seguel *et al.*, 2009). A posteriorly shifted expression of *Krox20* has been reported for embryos injected with Hoxd1 morpholino (McNulty *et al.*, 2005) and in embryos with grafted tissue expressing the Wnt antagonist Dkk1 in the neural plate (Kiecker and Niehrs, 2001). These data suggest partially overlapping phenotypes of Wnt inhibition and Irx3 and Hoxd1 depletion.

Discussion

We screened for genes that are induced by the Wnt/β-catenin pathway in neurula stage *Xenopus* embryos and identified two homeodomain-containing genes involved in AP patterning that are under direct transcriptional control of the Wnt/β-catenin pathway, i.e. the *Hoxd1* and the *Irx3* gene. Interestingly, *Hoxd1* and its paralogues *Hoxa1* and *Hoxb1* were very recently also identified as direct Wnt targets in gastrula stage embryos (In der Rieden *et al.,* 2010).

The *Hoxd1* homeobox gene belongs to the highly conserved family of *Hox* genes that are expressed in colinear fashion and establish a complex developmental regulatory system that provides cells with specific positional identities on the AP axis (Deschamps *et al.*, 1999, Duboule, 1998, Iimura and Pourquie, 2007, McGinnis and Krumlauf, 1992). In *Xenopus*, *Hoxd1* is first expressed in the ventrolateral mesoderm and later in the posterior ectoderm, presumptive hindbrain and its associated neural crest (McNulty *et al.*, 2005). Knockdown of *Hoxd1* generates defects in hindbrain and neural crest derivatives (McNulty *et al.*, 2005). We here report that inhibition of the Wnt/β-catenin pathway after gastrulation induces similar patterning defects, potentially via the repression of *Hoxd1*, although we cannot exclude the involvement of other Wnt target genes.

Irx3, formerly also known as *Xiro3* in *Xenopus,* is an iroquois-related transcription factor that contains one homeobox DNA binding domain. The *Xenopus* and mouse orthologs are expressed early in the prospective neural plate in a subset of neural precursor cells (Bellefroid *et al.*, 1998). In the developing forebrain of the chicken, *Irx3* expression is restricted to the posterior region. Consistent with the role of Wnts as posteriorizing agents in neural tissue, Wnt signaling was shown to be sufficient to induce *Irx3* in forebrain explants (Braun *et al.*, 2003). Knockdown of *Irx3* in *Xenopus* was shown to induce a caudally shifted forebrain, reduced midbrain and smaller and caudally shifted midbrain-hindbrain boundary (Rodriguez-Seguel *et al.*, 2009). The latter phenotype, reflected by a caudel shift in *En2* expression, was also eminent in our Wnt-inhibited embryos.

Using ChIP experiments, we identified the potential Wnt-responsive Lef1/TCF binding sequences in the *Hoxd1* and *Irx3* gene loci. For the *Irx3* gene we identified a binding site at position -1022. We could not identify evolutionary conservation for this site. However, the *Irx3* promoter region is on the whole poorly conserved between mammals and *Xenopus*. Conversely, two highly conserved potential LEF1/TCF sites are present around position – 3 kb of most mammalian genomes, but these sites are absent in *Xenopus* (data not shown). In contrast to the non-conserved sites in *Irx3*, the LEF1/TCF binding site at position +3180 in the 3' region of the *Hoxd1* gene, as well as the surrounding sequences, shows high evolutionary conservation. The LEF/TCF binding site is positioned at the start of the *Hoxd* gene cluster. *Hox* genes show a linear arrangement within four clusters in the genome and they are expressed in a temporally and spatially defined pattern. Several mechanisms have been proposed to mediate colinear expression of the *Hox* genes (Durston *et al.*, 2010, Kmita and Duboule, 2003). Interestingly, the *Hoxa-1* and *Hoxb-1* genes possess evolutionarily conserved 3' enhancers that control responsiveness to endogenous retinoids (Langston *et al.*, 1997). Our results suggest the existence of a Wnt-responsive enhancer at a similar position in the *Hoxd1* locus. A profound functional analysis of this region is currently in progress.

Evidently, the expression of the induced *Hoxd1* and *Irx3* is not under exclusive control of Wnt signaling. Similar to the other Wnt target genes *Cdx1*, *Cdx2*, *Otx2*, *Msx1* and *Msx2*, they most likely are also subject to regulation by other secreted factors in the neural plate, such as FGF and RA. For *Hoxd1* it has been documented that it is directly induced by RA signaling (Kolm and Sive, 1995). Similar cooperative activity may explain the observed spatially restricted expression of *Irx3* and the inability of our Wnt-mimicking construct to activate *Irx3* expression in animal caps. Besides spatial control of gene induction, we also observed that *Irx3* and *Hoxd1* were only poorly induced by the Wnt-mimicking construct in post-neurula stages.

In conclusion, our results add two more homeodomain containing transcription factors to the growing list of direct Wnt/β-catenin genes that pattern the AP axis in the neural axis in vertebrates.

Material and Methods

Plasmid construction

The construction of the pVP16, pC-VP16, pControl, pCMV-LEFΔNVP16GR and pCMV-EnRLEFΔNVP16GR plasmids has been described previously (Denayer *et al.*, 2008a, Deroo *et al.*, 2004).

In vitro *transcription and RNA injection*

Constructs were linearized with *Not* I, and *in vitro* synthesis of capped RNA was performed using SP6 RNA polymerase as described previously (Vleminckx *et al.*, 1997). Eggs were collected from female *Xenopus laevis* frogs, fertilized and dejellied. Embryos were injected at the 2- or 4-cell stage. During injection embryos were incubated in 1 X MMR (0.1 M NaCl; 1.8 mM KCL; 2 mM $CaCl_2$; 1 mM $MgCl_2$; 5 mM Hepes pH7.6), 6% Ficoll (Amersham Pharmacia), and afterwards transferred and grown in 0.1x MMR. For animal cap assay, animal pole regions were dissected at stage 8 in 1 x MMR and afterwards transferred and grown in 0.5 x MMR.

Trangenesis procedure

Generation of transgenic embryos was carried out essentially as described (Deroo *et al.*, 2004).

Dexamethasone and cycloheximide treatment

Both dexamethasone (Dex) and cycloheximide (CHX) were used at 10 μM. Embryos were treated as indicated.

Xenopus laevis *microarray analysis*

Total RNA was isolated using Trizol reagent (Life Technologies), followed by an additional lithium chloride precipitation step. For each of the 4 RNA samples, at least 10 transgenic embryos were pooled. RNA was quantified by absorbance at 260 nm, and the RNA quality was verified using 2100 Bioanalyzer (Agilent). For the microarray analysis, commercial Affymetrix Genechip *Xenopus laevis* Genome Arrays were used.

These gene chips contain 15500 probe sets, each consisting of multiple 25-mer oligonucleotide probes, representing approximately 13600 gene clusters and 14400 gene transcripts (http://www.affymetrix.com/products/arrays/specific/*Xenopus*.affx). Linear RNA amplification, probe labeling, hybridization, scanning and image analysis were carried out at the MicroArray Facility (MAF) of Flanders Institute for Biotechnology (VIB).

The Affymetrix array data were normalized using the GeneChip Robust Multi-Array (GCRMA) method implemented in the BioConductor package "gcrma" (http://www.bioconductor.org) (Wu and Irizarry, 2004). The GCRMA method takes into account PerfectMatch and MisMatch probes as well as probe sequence composition. After normalization, genes with a low mean intensity over all samples were filtered out since they represent noise. Based on the overall intensity distribution of our data, a $\log_2$ mean intensity of 3.5 was chosen as filtering threshold. Genes were considered differentially expressed when they showed a 2-fold induction ratio for the Wnt-mimicking construct divided by the Wnt-interfering construct and substracted by the ratio of the control constructs (i.e. to compensate for potential effects of the Dexamethasone).

Whole mount* in situ *hybridization (WISH)

Antisense RNA probes for *HoxD1*, *Irx3*, *Crabp2*, *Msx1*, *Engrailed-2, Rx2a* and *Krox20* were synthesized from cDNA using digoxygenin or fluorescein as a label (Roche Molecular Biochemicals). Embryos were prepared, hybridized and stained as described (Harland, 1991). For detection, BM purple, NBT/BCIP or Fast Red (Roche Molecular Biochemicals) was used as a substrate for alkaline phosphatase. β-Gal staining was performed as previously described (Denayer *et al*., 2008b). Images were captured on a Zeiss Lumar V12 stereomicroscope with an AxioCam camera and assembled via Axiovision software.

Real-time Quantitative RT-PCR analysis

Total RNA was isolated using the Aurum Total RNA fatty and fibrous tissue kit (Biorad). For each RNA sample at least 10 embryos were pooled. cDNA was prepared with oligo (dT) and random hexamer primers using the iScript cDNA Synthesis Kit (Biorad) according to the manufacturer's instructions.

Real-time qPCR analysis was performed using the SYBR green PCR master mix (Applied Biosystems) on a LightCycler® 480 Real-Time PCR System (Roche). Primers for measuring gene expression levels (as well as for ChIP experiments) were designed using Primer express 1.0 software (Perkin-Elmer applied Biosystems) and sequences are listed in Table S1. For all primer pairs, a "no-template control" was included. For measuring gene expression, a "no-amplification control" was also included and all values were normalized to the level of the housekeeping gene ornithine decarboxylase (ODC).

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed according to the method described before (Blythe *et al*., 2009), with minor modifications: we used *Xenopus tropicalis* embryos at stage 17 and cross-linking time was diminished to 30 min. After homogenization, embryos were sonicated with a Brandson cell disruptor to obtain DNA fragments of less than 1000 bp. Immunoprecipitation was performed with rabbit anti-β-catenin polyclonal antiserum (kind gift from Dr. Barry Gumbiner). Purfied normal rabbit IgG was purchased from Santa Cruz (SC-2027). Instead of sepharose beads, protein G Dynabeads (Invitrogen) were used.

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