Hox in frogs: xenopus reveals novel functions for vertebrate Hox genes

Bardine, N.

Chapter 3

Two *Hoxc6* transcripts are differentially expressed and regulate primary neurogenesis in *Xenopus laevis*

Nabila Bardine[1], Cornelia Donow[1], Brigitte Korte[1], Antony J. Durston[2], Walter Knöchel[1], Stephan A. Wacker[1]

[1] Institute of Biochemistry, University of Ulm, 89081 Ulm, Germany

[2] Institute of Biology, University of Leiden, 2333 AL Leiden, The Netherlands

Abstract

Hox genes are key players in defining positional information along the main body axis of vertebrate embryos. In *Xenopus laevis, Hoxc6* was the first homeobox gene isolated. It encodes two isoforms. We analyzed in detail their spatial and temporal expression pattern during early development. One major expression domain of both isoforms is the spinal cord portion of the neural tube. Within the spinal cord and its populations of primary neurons *Hox* genes have been found to play a crucial role for defining positional information. Here we report that a loss-of-function of either one of the *Hoxc6* products does not affect neural induction, the expression of general neural markers is not modified. However, Hoxc6 does widely affect the formation of primary neurons within the developing neural tissue. Manipulations of *Hoxc6* expression severely change the expression of the neuronal markers *N-tubulin* and *Islet-1*. Formation of primary neurons and formation of cranial nerves are affected. Hence, Hoxc6 functions are not restricted to the expected role in anterior-posterior pattern formation, but they are crucial for the initial formation and differentiation of primary neurons in the nervous system of *Xenopus laevis* as well.

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Introduction

Hox genes encode a family of transcription factors related to the homeotic factors of *Drosophila* (Akam 1989; Lemons and McGinnis 2006), where they were initially discovered (Lewis 1978). *Hox* genes are crucial for patterning and organizing structures along the main body axis (Krumlauf 1994). These genes are organized in clusters in a wide range of animals. Vertebrates possess multiple copies of *Hox* gene clusters, as a result of successive genome duplications (McGinnis and Krumlauf 1992; Lemons and McGinnis 2006; Duboule 2007; Deschamps 2007). During development, *Hox* genes are activated sequentially according to their position within the cluster, leading to unique combinations of *Hox* genes expressed at different axial levels, referred to as Hox code (Duboule and Morata 1994; Kessel 1994; Kessel and Gruss 1991). When ectopically expressed or mutated, *Hox* genes lead to so called homeotic transformations, i.e. one part of a body is transformed into another.

Hox gene functions have been extensively studied by generation of gain-of-function and loss-of-function mutants. This confirmed the role of *Hox* genes in defining axial identities (McGinnis and Krumlauf 1992). Furthermore, *Hox* genes were also connected to the patterning of the neural tube and its subtypes of neurons (Dasen et al. 2003; Song and Pfaff 2005; Nordström et al. 2006; di Sanguinetto et al. 2008). *Hox* gene expression has been correlated to the identities of motor neurons along the spinal cord (Shah et al. 2004). Indeed, in the developing spinal cord, motor neurons occupy discrete columns with different identities and projections (Tanabe and Jessell 1996). This columnar organization has been proven to be under the control of sequential phases of different Hox-C protein activities, in response to some gradient molecules, like the fibroblast growth factors (Dasen et al. 2003; Liu et al. 2001; Bel-Vialar et al. 2002; Guthrie 2004).

Hoxc6, an *Antennapedia* homologue, was the first *Hox* gene cloned in the vertebrate *Xenopus* (Carrasco et al. 1984). Two different proteins were described, a long and a short protein, due to the existence of two distinct promoters (PRI and PRII respectively, (Cho et al. 1988)). The two proteins share an identical DNA binding sequence, the homeodomain. The localization of these Hoxc6 proteins has been reported in previous studies.

The generation of a polyclonal anti-serum against Hoxc6 (formerly called XlHbox1) proteins enabled immuno-localization studies in *Xenopus*, mouse and zebrafish on the basis of the sequence conservation between the different species (Oliver et al. 1988; Wright et al.

1989; Molven et al. 1990). These studies focused on late stages of development. Hoxc6 protein localization was analyzed in stage 12 and 13 mouse embryos, and in a stage 45 *Xenopus* tadpole (Oliver et al. 1988). Hoxc6 proteins were found in the nervous system. In particular they were found in some neuronal populations as sensory neurons and interneurons in zebrafish, and in dorsal root ganglia in *Xenopus laevis* (Cho et al. 1988; Molven et al. 1990). Another study was based upon Northern-blot data on a stage 41 tadpole, and showed *in situ* hybridization on sections of a few additional stages using a probe recognizing both forms (at that time called *Xeb-1* (Carrasco and Malacinski 1987)). However, the analysis of expression of *Hoxc6* transcripts in early development has been quite incomplete, especially in relation to their potential functions with respect to primary neuron formation and patterning.

Here we show a detailed expression analysis of the two *Hoxc6* transcripts in *Xenopus laevis* during early development. We mainly focused on gastrulation and neurulation, using whole mount *in situ* hybridization for both transcripts. They exhibit different spatial patterns of expression, whereas their temporal patterns look identical. The two transcripts are predominantly co-expressed in the spinal cord during development of *Xenopus laevis* in accordance with the previous immuno-localization data.

We analyzed the functions of the two transcripts by loss-of-function and gain-of-function experiments. Specific depletion of either one of the two transcripts failed to affect pan-neural markers, whereas, after the double knockdown, the expression of *N-CAM* was reduced. However, the formation of primary neurons was affected by a single knockdown of either one of the isoforms. This neuronal phenotype is reversed by ectopic expression of *Hoxc6*. The results of our experiments point towards a novel basic function of *Hoxc6*, not in anterior-posterior patterning, but more generally in the formation and differentiation of primary neurons *in Xenopus laevis*.

Results and Discussion

Temporal and spatial expression patterns of two *Hoxc6* transcripts

The *Xenopus Hox* gene *Hoxc6* is under the control of two promoters, PRI and PRII (Cho et al. 1988). The organization of the two transcripts in *Xenopus laevis* is depicted in Figure 1

A. The distal PRI promoter is a common 5‘ regulatory unit for the *Hoxc4*, *Hoxc5,* and *Hoxc6* genes, whereas the proximal promoter PRII exclusively regulates *Hoxc6* (Simeone et al. 1988; Boncinelli et al. 1991; Coletta et al. 1991). The PRI promoter leads to the production of a transcript of 2.2 Kb. This long transcript encodes the shorter protein of 152 amino acids (hereafter called short form, SF). The PRII promoter leads to a smaller transcript of 1.8 Kb and encodes a longer protein of 234 amino acids (hereafter called long form, LF). The two proteins differ only in 82 amino acids at their N-terminus, but share an identical C-terminus including the homeodomain (Cho et al. 1988; Chariot and Gielen 1998; Sharpe et al. 1988; Shimeld et al. 1993).

The different localizations of these Hoxc6 proteins from the aforementioned immunohistochemistry in *Xenopus*, mouse and zebrafish, lead to the conclusion that the *Hoxc6* gene might be under tissue specific regulation (Oliver et al. 1988; Wright et al. 1989; Molven et al. 1990). The localization of transcripts in the whole embryo during the early development of mouse, frog or fish has only partially been documented. Here we have analyzed the temporal and spatial expression patterns of the *Hoxc6* isoforms in *Xenopus laevis* by RT-PCR and whole mount *in situ* hybridization, from the one cell stage until tadpole stages. In Figure 1 A, the positions of the PCR primers are indicated. These were also used to generate the probes for the *in situ* hybridization.

Both, the *Hoxc6* LF and SF transcripts are maternally expressed, although the level of LF is very low. Their maternal expression decreases rapidly (Figure 1 B). Zygotic expression appears during mid-gastrulation (after stage 11). Their expression persists during gastrulation and neurulation. Both transcripts have constant levels of expression at least until stage 27 (Figure 1 B).

The spatial expression patterns of both *Hoxc6* transcripts were analyzed by whole mount *in situ* hybridization. The probe recognizing the SF transcript is specific for this transcript. The probe for the LF transcript is located in the ORF and spans the first 246 nucleotides specific to the LF and 210 nucleotides common to both isoforms.

In accordance with the temporal expression patterns, neither transcript was detected by *in situ* hybridization at early gastrulation (Figure 1, C and L). Expression of both isoforms was found from midgastrula stages until tadpole stages. The expression increases during gastrulation. At the end of gastrulation, both transcripts were found in the posterior axial and paraxial regions (Figure 1 D, E and M, N). As neurulation proceeds, both isoforms were prominently expressed in the neural tube region, in the paraxial mesoderm and in the tailbud (Figure 1, F-H and O-Q). At tadpole stages, the expression of both transcripts persists mainly in the neural tube (Figure 1 I-K and R-T). The LF transcript shows a sharp

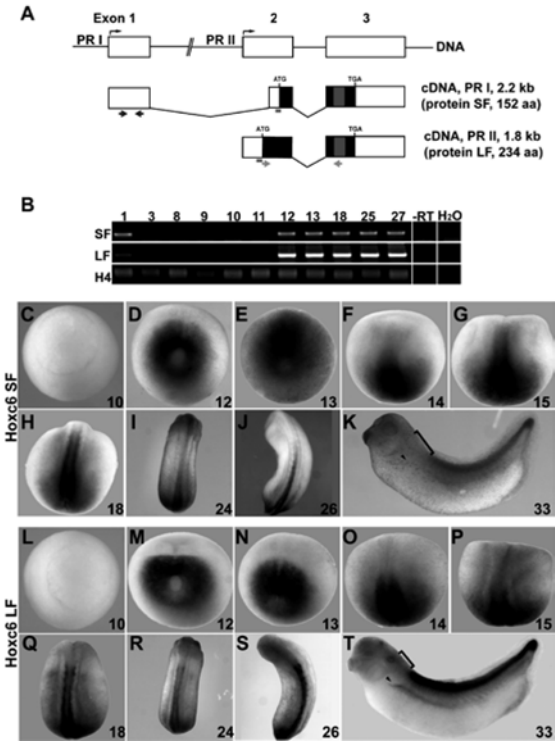


Figure 1. Temporal and spatial expression pattern of the *Hoxc6* isoforms

A Structure of *Hoxc6* isoforms in *Xenopus laevis*. *Hoxc6* isoforms derive from 2 different promoters. The distal promoter (PR I) leads to a 2.2 Kb precursor transcript, coding for the short form (SF) protein of 152 aa. The proximal promoter encodes a 1.8 Kb transcript leading to the long form (LF) protein of 234 aa. Black boxes indicate the open reading frame, grey boxes are the homeodomain. Red bars indicate the binding sites of the morpholino-oligonucleotides. The blue and green arrows show the primer sites that served for the RT-PCR and to generate the probes for *in situ* hybridization.**B** Temporal expression pattern of *Hoxc6* isoforms. RT-PCR was carried out using embryos at the indicated stages from 1 to 27. *H4* is used as a loading control, –RT is shown for stage 12 RNA.**C to K** show SF expression patterns from beginning of gastrulation until tadpole stages. Stages are indicated by the numbers **L to T** show the LF pattern from beginning of gastrulation until tadpole stages. The arrowheads in **K** and **T** indicate the location of the pronephric anlage. The squared brackets indicate the different distances between the anteriormost border of expression of each isoform and the otic vesicle area.

anterior boundary of expression in the neural tube. The transcript encoding the SF was detected with a more caudally located anterior boundary in the neural tube (using the otic vesicle as a landmark). Its pattern is more diffuse than that of the LF transcript (Fig.1, J-K and S-T, squared brackets). Moreover, at tadpole stages, expression of the LF transcript was found in the region of the pronephric anlage, while SF transcripts were not detected in this region (Fig.1, K, T, arrowheads). Our data show that the two *Hoxc6* transcripts do not have identical expression patterns. The anterior expression border of the SF appears to be more posterior. In addition the SF is not expressed in the pronephros or pronephric duct. To confirm the tissue localization of *Hoxc6* transcripts, we performed transversal sections of tadpoles. The *Hoxc6* expression was compared to those of known tissue specific markers. Thus, comparable sections were stained for *MyoD*, a somite specific marker (Hopwood et al. 1989, Figure 2 E), for *Xlim-1*, labeling the intermediate mesoderm of the pronephric anlage and the neural tube (Chan et al. 2000, Figure 2 F), and for *FoxF1* as a marker for lateral plate mesoderm (Köster et al. 1999, Figure 2 G).

Strongest expression for both isoforms of *Hoxc6* was found in the neural tube with exception of the floor plate (Figure 2 A-D). Comparing the expression of the *Hoxc6* isoforms to the other markers confirmed that both *Hoxc6* transcripts are expressed in somitic tissue. An additional expression domain of the LF was found in the pronephric anlage. The schemes in Figure 2 H indicate the position of the sections within the embryo, and the positions of the different tissues within the sections. In addition sections were analyzed originating from regions in front of the expression domain, as detected by whole mount *in situ* hybridization. Interestingly expression of both, the LF and the SF, were detected in the neural tube at the level of branchial arches (Figure 2 I, J).

These observations highlight the difference in tissue expression between the two *Hoxc6* transcripts. Both forms are expressed within the neural tube, and in the somitic mesoderm. However, staining in the cross sections also demonstrates a specific high level of expression of the LF transcript in the pronephric anlage. These spatial data suggest that the expression of *Hoxc6* transcripts is differentially regulated in different tissues.

Knockdown of either one of the isoforms of *Hoxc6* failed to affect *N-CAM* and *Notch* expression in the neural plate

To investigate *a* possible function of *Hoxc6* during *Xenopus* neurulation, we performed loss-of-function studies using antisense morpholino-oligonucleotides against both *Hoxc6* isoforms (MO; their positions are indicated as red bars in Figure 1 A). First, the function of *Hoxc6* MOs was tested *in vitro* in a transcription-translation assay to verify that *Hoxc6* transcripts are inhibited efficiently (Figure 3 A). MO2 preferentially blocks translation of the SF, and MO3 exclusively inhibits the translation of the LF. This nomenclature for the MOs will be used in the description below. The addition of the standard control MO (ctMO) does not affect the efficiency of translation in the reticulocyte lysate of either of the wild type *Hoxc6* forms. A construct coding for a LF transcript lacking the MO3 binding site (Figure 3 A, LF(insens.)) was tested in this *in vitro* assay as well. MO2 does recognize an internal sequence within the LF transcript. To exclude strong cross reactions we tested the potential interference of MO2 with the translation of the LF(insens.) construct. We found that addition of either MO2 or both, MO2 and MO3, failed to block the translation of this insensitive transcript of the LF (Figure 3 A). This confirms that MO2 does not efficiently disturb the translation of the LF transcript, and therefore proves its specificity to knockdown the SF.

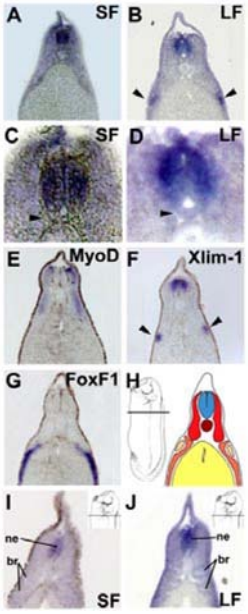


Figure 2. Tissue localisation of *Hoxc6* isoforms in cross sections

A SF expression in the somitic tissue and neural tube. **B** Expression of LF in somitic tissue and neural tube; in addition LF is expressed in the pronephric anlage (arrowheads). **C, D** Close-ups of the neural tubes shown in **A** and **B**. Expression of the *Hoxc6* isoforms is detected in all cells of the neural tube with exception of the floorplate. **E** *MyoD* expression in the somites. **F** *Xlim-1* expression in the neural tube and in the pronephric anlage (arrowheads). **G** *FoxF1* expression in the lateral plate mesodem. **H** Position of the shown sections within stage 27 embryos, and localization of different tissues in the sections. Neural tube (blue); somite mesoderm (red); notochord (brown), endoderm (yellow), lateral plate mesoderm (dark orange), the pronephric anlage (light orange). **I** Cross section at the level of the branchial arches (br) as indicated in the inlay. Expression of SF is found in the neural tube (ne), even though it was not visible in whole mount *in situ* hybridization. **J** Expression of LF in the neural tube at the same level.

To analyze the endogenous function of *Hoxc6* transcripts for neural induction as the first step of neurogenesis, MO2 or MO3 were injected at the 2-cell stage into both cells at the animal pole of embryos. We monitored the expression of the neural marker *Sox-2*, which labels the neural plate in the *Xenopus* neurula (Mizuseki et al. 1998). Neither injection of MO2, knocking down the SF (Figure 3 D), nor injection of MO3, knocking down LF (Figure 3E), nor the combined injection of both MOs (Figure 3 F) showed differences compared to uninjected or ctMO injected embryos (Figure 3 B, C). Identical results were obtained for another two neural markers (*Nrp-1*, *Xenopus Notch*, shown in the Supplemental Figure 1 A-J). It has been reported that Hoxc6 proteins bind to the promoter of the general neural marker *N-CAM* and activate its transcription in NIH 3T3 cells. This activation is stronger, when both isoforms bind the promoter simultaneously (Jones et al. 1993).

Thus, we asked whether depletions of *Hoxc6* transcripts have any effect on *N-CAM* expression *in vivo*. *N-CAM* expression was not affected by ctMo injection compared to uninjected controls (Figure 3 G, H). After depletion of either one or the other transcript, *N-CAM* expression was not significantly affected (Figure 3 I, J). Nevertheless, combined injection of the two MOs led to a dramatic down-regulation of *N-CAM* expression (Figure 3 K). An identical effect was observed on the epidermal ectoderm marker *XK81A1* (Supplemental figure 1 K-O).

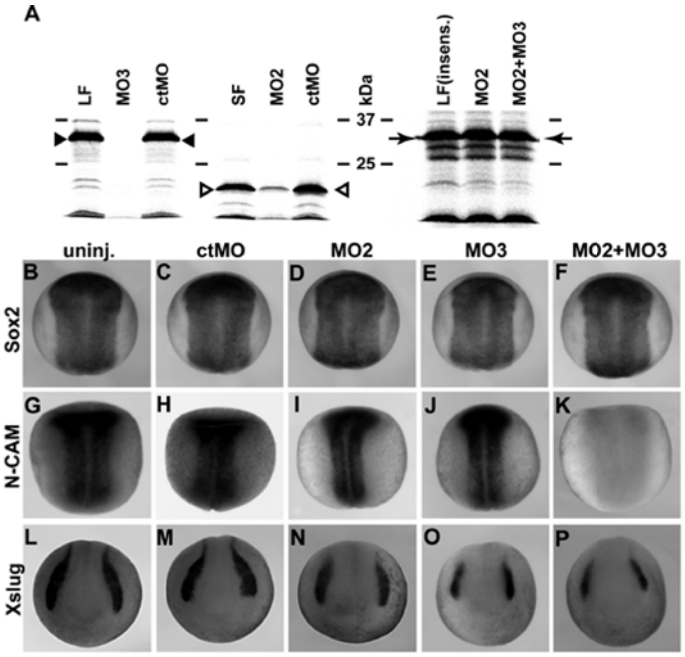
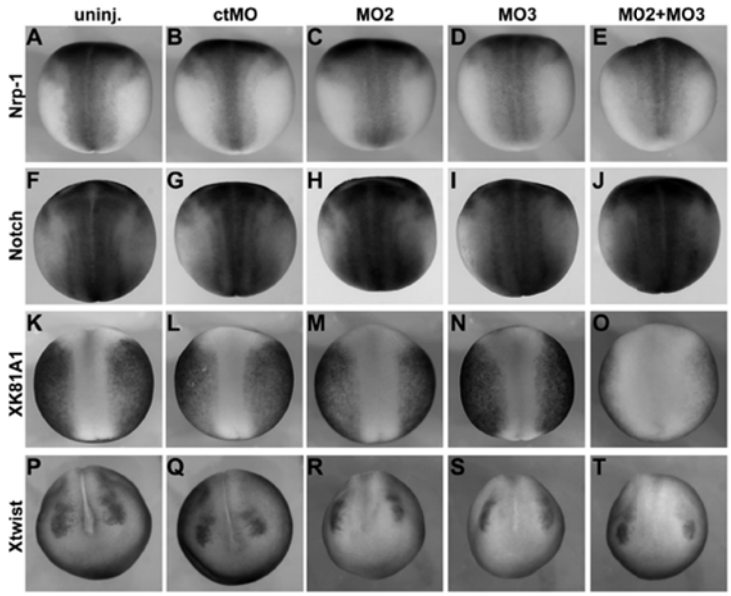


Figure 3. The effect of *Hoxc6* knockdowns on neural markers

A Specificity of MO nucleotides was tested *in vitro* using a coupled transcription-translation assay. *In vitro* synthesis of LF was inhibited by MO3, but not by the ctMO (black arrowheads). *In vitro* synthesis of the SF was reduced by MO2, but not by ctMO (open arrowheads). Translation of a LF construct lacking the MO3 binding site (LF insensitive) is neither inhibited by MO2 nor by MO2 combined with MO3 (arrows). This demonstrates that the SF specific MO2 does not affect synthesis of the LF, even though its target sequence is present in the LF mRNA.**B to F** The neural marker *Sox2*

The analysis of neural crest markers revealed a significant effect of the knockdown of the LF (separate or combined with the knockdown of SF), but weak or no effects of the knockdown of the SF (*Xslug* in Figure 3 L-P, *Xtwist* in Supplemental Figure 1 P-T). The effects of a knockdown of *Hoxc6* on the epidermal and neural crest ectoderm are unexpected, since no expression of Hoxc6 was detected in these tissues at the analyzed stages. We do not have an explanation yet. One can speculate that is effect must be indirect, acting via the inhibition of Hoxc6 in the neuroectoderm or the mesoderm.

Altogether, our data indicate that depletion of a single *Hoxc6* isoform in *Xenopus* does not affect general neural induction or the initial neural tissue formation. We also show that *N-CAM* expression in the *Xenopus* embryo is under a synergetic control of both *Hoxc6* products as has previously been reported for a cell culture (Jones et al. 1993).



Supplemental Figure 1. The effect of *Hoxc6* knockdowns on different markers

Expression patterns of different markers in uninjected embryos, and after the injection of ctMO, MO2, MO3, and a combination of MO2 and MO3 as indicated above the photographs. **A to E** Expression of the neural marker *Nrp-1*. **F to J** Expression of the neural marker *Notch*. **K to O** Expression of the epidermal marker *XK81A1*. **P to T** Expression of the neural crest marker *Xtwist*.

Knockdown of either one of the isoforms of *Hoxc6* leads to defects in formation of primary neurons

Neural development starts with neural induction during gastrulation. A few hours later the first neurons begin to differentiate in the *Xenopus* neural plate, located in three bilateral longitudinal stripes, so called columns. This pattern reflects the functional subdivision of the early nervous system. Thus, motor neurons, interneurons and sensory neurons derive from the medial, intermediate and lateral column respectively (Hartenstein 1993; Diez del Corral and Storey 2001). It has been shown that Hox-C proteins can influence the identity of motor neurons within the spinal cord columns of chicken (Dasen et al. 2003; Dasen et al. 2005).

Furthermore, *Hox* mutants have been shown to exhibit defects in motor axon projections, consistent with an alteration in their identity (Wahba et al. 2001; Tiret et al. 1998; Wu et al. 2008). We therefore asked whether a *Hoxc6* knockdown has any influence on the formation and identity of primary neurons in *Xenopus*. We monitored the expression of *N-tubulin* upon MO injections. *N-tubulin* is a pan-neuronal marker expressed in neurons undergoing the differentiation process (Figure 4 A). Its expression is not affected by the injection of the ctMO (Figure 4 B). Injection of MO2 resulted in, at most, weak effects (Figure 4 C). When the MO3 was injected, *N-tubulin* expression was extensively down-regulated (Figure 4 D), indicating an impaired formation of primary neurons. Half-sided injections of the different MOs (Supplemental Figure 2 A-D) and the comparison with ctMO injections show that this down-regulation does not result from a delay in development. The knockdown of the LF by injection of MO3 was counteracted by co-injection of the insensitive LF-mRNA, which in addition resulted in ectopic *N-tubulin* expression (Figure 4 E). It is worthwhile noticing that the loss of the LF transcript leads to a stronger effect than the depletion of the SF transcript. This could indicate a potential difference in the functions of the corresponding Hoxc6 proteins. Next we investigated the effect of Hoxc6 depletion on some subpopulations of neurons. The LIM homeodomain protein Xisl-1 is expressed in the medial and lateral parts of the spinal cord. Amongst others it controls the trajectory of the motor axons along the dorso-ventral axis of the limb (Kania and Jessell 2003).

In an uninjected or a ctMO injected neurula stage embryo, *Xisl-1* positive neurons form a discrete set of columns reminiscent of *N-tubulin* expression pattern, but with fewer cells (Figure 4 F, G). The depletion of either the SF transcript or the LF transcript causes a decrease in number of *Xisl-1* positive cells (Figure 4 H, I, Supplemental Figure 2 E-G). Again, the loss of the LF transcript gives a severe phenotype and the depletion of the SF a weak effect. Next we investigated the effect of Hoxc6 depletion on some subpopulations of neurons. The LIM homeodomain protein Xisl-1 is expressed in the medial and lateral parts of the spinal cord. Amongst others it controls the trajectory of the motor axons along the dorso-ventral axis of the limb (Kania and Jessell 2003). In an uninjected or a ctMO injected neurula stage embryo, *Xisl-1* positive neurons form a discrete set of columns reminiscent of *N-tubulin* expression pattern, but with fewer cells (Figure 4 F, G). The depletion of either the SF transcript or the LF transcript causes a decrease in number of *Xisl-1* positive cells (Figure 4 H, I, Supplemental Figure 2 E-G). Again, the loss of the LF transcript gives a severe phenotype and the depletion of the SF a weak effect. Later on, *Xisl-1* is expressed in cranial nerves, in the neural crest of the branchial arches, and along the spinal cord as described ((Brade et al. 2007), and Figure 4 K, L).

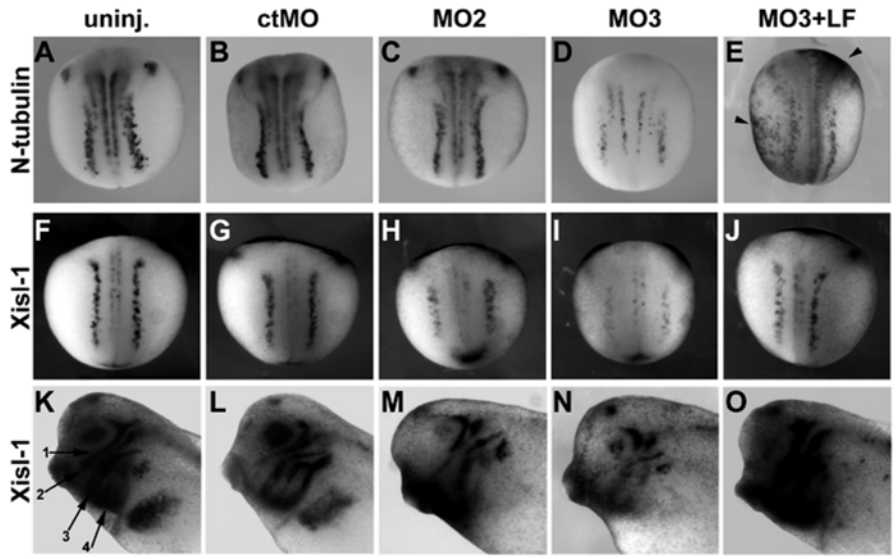
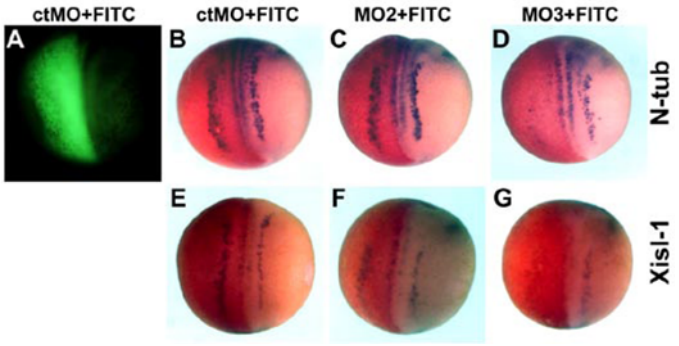


Figure 4. The effect of *Hoxc6* knockdowns on formation of primary neurons and cranial nerves

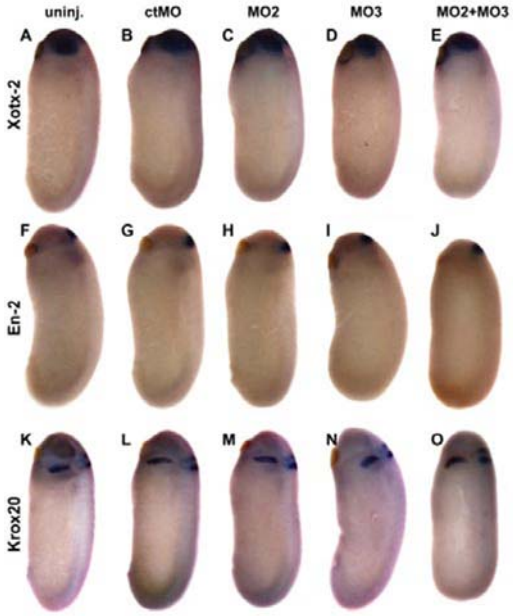
Markers specific for primary neurons (*N-tubulin*, *Xisl-1*) and for cranial nerves (*Xisl-1*) were analyzed at a neurula stage and a tadpole stage by *in situ* hybridization upon *Hoxc6* depletion. Shown are uninjected (uninj.) embryos, and embryos injected with ctMO, MO2, MO3, and a combination of MO3 with an MO-insensitive mRNA for LF, as indicated above the photographs.**A to E** Analysis of the neuronal marker *N-tubulin*. Arrowheads indicate ectopic N-tubulin expression.**F to J** Expression of *Xisl-1* at a neurula stage for the same set of treatments. No ectopic expression was found.**K to O** Expression of *Xisl-1* in the head of a tadpole. Numbers in **K** indicate the different branchial arches and the corresponding cranial nerves.

Especially the *Hoxc6* LF hypomorph shows a severe decrease in *Xisl-1* expression in the corresponding structures (Figure 4 M, N). These results are in accordance with previous studies. With *in vivo* injections of a functional antibody it has been shown that inhibition of the function of the LF protein leads to severe defects at the level of hindbrain and anterior spinal cord (Wright et al. 1989). Moreover, in a rescue experiment we find that co-injection of MO3 with an MO- insensitive LF transcript restores the loss of *Xisl-1* positives neurons (Figure 4 J) and of *Xisl-1* expression in the region of the cranial nerves (Figure 4 O). However, in contrast to the *N-tubulin* expression no significant ectopic activation was observed for Xisl-1. This rescue experiment highlights a predominant role of *Hoxc6* (at least the LF protein) in regulating *Xisl-1* positive neurons in *Xenopus*, as it does in chicken (Dasen et al. 2005).



Supplemental figure 2 Half-sided knockdowns of the *Hoxc6* isoforms confirm an *Hoxc6*-specific effect on neuronal markers

Embryos were injected in one out of two blastomeres with a mix of FITC-dextran and the different MOs. The different combinations are indicated above the photographs.**A** In vivo photograph of an half-sided injected embryo. **B to D** *N-tubulin* expression after half-sided injections. The Fast-Red staining of FITC indicates the injected side. **E to G** Expression of *Xisl-1* after half-sided injections. Again the Fast-Red staining of FITC indicates the injected side.



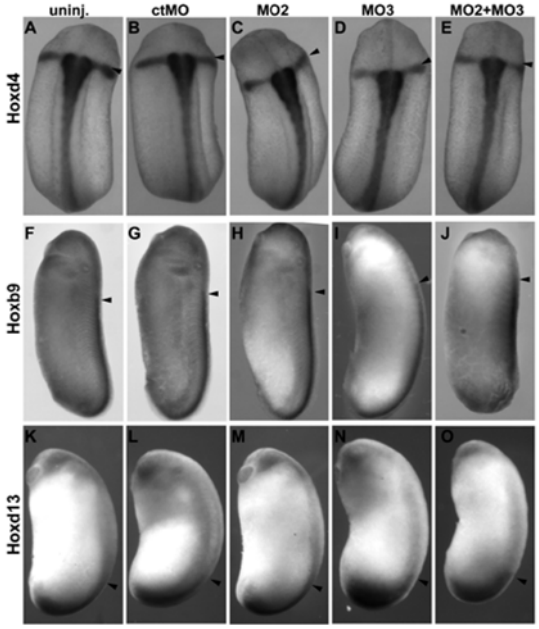
Supplemental figure 3. The effect of *Hoxc6* knockdowns on anterior-posterior marker genes of the head region

A to E Expression of the anterior marker *Xotx-2* in uninjected embryos, and in embryos injected with ctMO, MO2, MO3, or with the combination of MO2 and MO3, as indicated above the photographs. **F to J** Expression of the midbrain-hindbrain boundary marker *En-2* after the same set of treatments. **K to O** Expression of the hindbrain marker *Krox-20* after the same set of treatments.

Interestingly, at least parts of this effect must be indirect, since it affects cells (i.e. the cranial nerves), which do not express *Hoxc6* at the analyzed stages. We could not confirm the idea that the effect may result from a homeotic transformation, since the analysis of a series of anterior-posterior marker genes did not reveal significant changes in the anterior-posterior patterning (Supplemental Figure 3 and 4). The observed (and probably indirect) effect on early neural crest formation may be part of the explanation (see above).

Together, our results show that the depletion of Hoxc6 proteins leads to a general loss in primary neuron formation as shown by a down-regulation of *N-tubulin*, and especially a loss of *Xisl-1* positive neurons.

As indicated above, this effect is neither due to a reduction of neural precursors, nor the result of changes in anterior-posterior patterning. Rather, the quantity or quality of differentiation of primary neurons from these cells is affected.



Supplemental figure 4. The effect of *Hoxc6* knockdowns on anterior-posterior marker genes of the trunk region

A to E Expression of *Hoxd4* in uninjected embryos, and in embryos injected with ctMO, MO2, MO3, or with the combination of MO2 and MO3, as indicated above the photographs. **F to J** Expression of *Hoxb9* after the same set of treatments. **K to O** Expression of *Hoxd13* after the same set of treatments. Arrowheads in all photographs indicate the anterior border of expression seen in whole mount *in situ* hybridization.

This decrease in the number of *Xisl-1* positive neurons was reversed by co-injection of the MO3 (knocking down the *Hoxc6* LF) with the mRNA encoding a MO-insensitive form of *Hoxc6* LF. These results highlight a major function of *Hoxc6* in neurogenesis in *Xenopus* laevis. In contrast to neural induction, formation and differentiation of primary neurons is strongly affected by the depletion of the *Hoxc6* LF product indicating a crucial role of *Hoxc6* in the formation and differentiation of primary neurons. This places Hoxc6, on the pathway of neurogenesis, between neural induction and the specification of primary neurons.

Injection of mRNAs encoding either one of the isoforms of *Hoxc6* leads to ectopic expression of the neuronal marker *N-tubulin*

In the rescue experiment shown in Figure 4 we observed ectopic expression of the neuronal marker *N-tubulin*. We therefore asked, if the injection of mRNA encoding either one of the *Hoxc6* isoforms ectopically activates neural and neuronal markers. In comparison to uninjected embryos the expression of the general neural marker *N- CAM* was not significantly affected after injections of LF or SF mRNA (Figure 5 A-C). The neuronal marker N-tubulin is ectopically activated in both, embryos injected with LF mRNA and embryos injected with SF mRNA (Figure 5 D-F). Surprisingly no ectopic expression of *Xisl-1* was found in corresponding embryos of the same experiments. The explanation may be that the Hoxc6 ectopically induces primary neurons different from the *Xisl-1*-positive motor neurons. Further experiments confirm a close relation of *Hoxc6* to *N-tubulin*. A comparison of the temporal and spatial expression patterns of *N-tubulin* with *Hoxc6* patterns shows extensive overlaps. Expression of *N-tubulin* was detected from midgastrula stages on, overlapping with *Hoxc6* in time (Figure 6 A, Figure 1 B) and space (Figure 6 C-F, spatial Hoxc6 patterns in Figure 1). We also analyzed the effect of ectopic expression of the *Hoxc6* isoforms on animalcaps at a neurula stage. RT-PCR data show that injection of either SF mRNA or LF mRNA resulted in activation of *N-tubulin* expression (Figure 6 B). In accordance with our gain-of-function experiments, *N-CAM* was not activated in these caps. As a positive control injection of the mRNA for the neural inducer Noggin was used. In these *noggin* injected caps both, *N-CAM* and *N-tubulin,* are activated. The activation of *N-tubulin* by *Hoxc6* injection appears already during gastrulation, as it is shown by the half-sided injection of the LF mRNA (Figure 6 G). Rescue with mRNAs for both, LF and SF, on MO2 or MO3 injected embryos confirm this role of *Hoxc6* for the regulation of

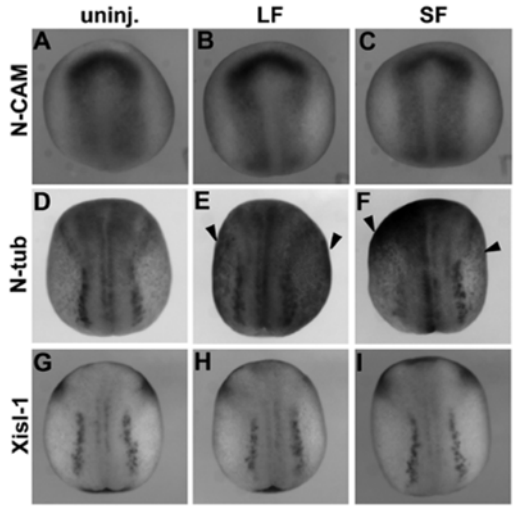


Figure 5 Effect of overexpression of the LF and the SF on neural and neuronal markers.

A to C Expresssion of the neural marker *N-CAM* in uninjected embryos (uninj.), and in embryos injected with mRNA encoding either the LF or the SF as indicated above the photographs.**D to F** Expression of the neuronal marker *N-tubulin* after the same set of treatments. Arrowheads indicate ectopic activation of *N-tubulin* expression.**G to I** Expression of the neuronal marker *Xisl-1* after the same set of treatments.

N-tubulin. Consistent with the experiments shown in figure 4, MO2 has a weak downregulating effect and MO3 has a strong downregulating effect on *N-tubulin* (Figure 7 A, F). Coinjection with different doses of LF mRNA did not result in a rescue after loss of *N-tubulin* with MO2 (Figure 7 B, C), whereas the MO3 effects were clearly rescued (Figure 7 G, H). In addition ectopic activation of *N-tubulin* was observed very often. Coinjections of the MOs with different doses of the SF mRNA rescued the weak MO2 effects (Figure 7 D, E). In MO3 injected embryos, we never observed restauration of *N-tubulin* even at such high doses of SF mRNA, that gastrulation is affected (Figure 7 I, J). Also ectopic *N-tubulin* activation is only infrequently found, in the shown cases presumably due to the malformations. So, in correspondence with the loss-of-function experiments, the gain-of-function experiments and the rescue experiments especially with *Hoxc6* LF show effects on the primary neuron formation, but not on the general neural marker *N-CAM*. This again places *Hoxc6* as a necessary component on the pathway of neurogenesis, in a position somewhere between the early steps of neural induction and the specification and differentiation of primary neurons.

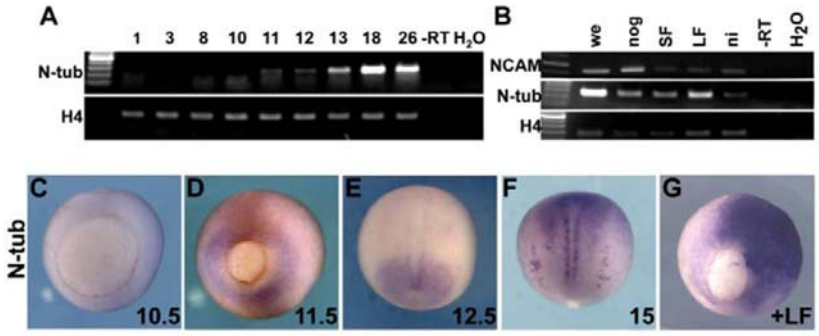


Figure 6 The early temporal and spatial pattern of *N-tubulin* and its activation by *Hoxc6* isoforms.

A A time course of *N-tubulin* expression analyzed by RT-PCR. Numbers indicate the stages. *H4* is used as loading control. **B** Activation of *N-tubulin* in animal caps by *Noggin* (nog), SF and LF at early neurulation. As additional controls uninjected animal caps (ni) and whole embryos (we) are shown. *H4* is used as loading control. **C to F** Spatial expression pattern of *N-tubulin* at gastrula and early neurula stages as indicated by the numbers. **G** Ectopic activation of *N-tubulin* during gastrulation after half-sided injection of LF mRNA.

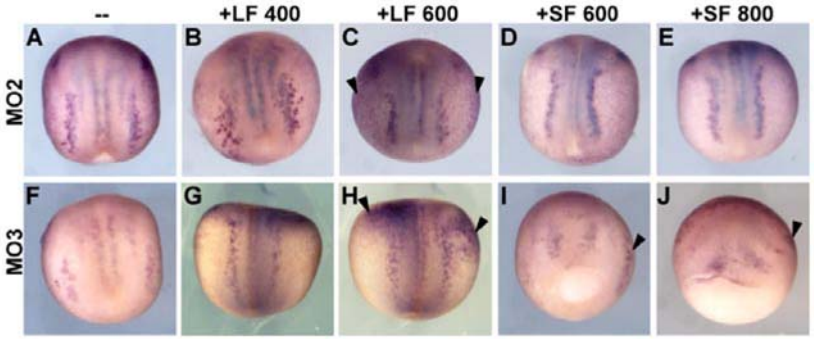


Figure 7 Effects of LF and SF mRNA on embryos either injected with MO2 or with MO3.

A to E Expression of *N-tubulin* is shown in embryos after injection with MO2 alone, and in combination with different doses of either LF or SF mRNA, as indicated above the photographs. The numbers describe the amount of mRNA in pg per embryo. Arrowheads indicate ectopic activation of *N-tubulin* expression. **F to J** Expression of *N-tubulin* in embryos after injection with MO3 alone and in combination with different doses of either LF or SF mRNA. Arrowheads in **G** indicate ectopic activation of *N-tubulin* expression. Expression labeled with arrowheads in **I** and **J** may be ectopic or may result from the defect of gastrulation.

Experimental Procedures

Cloning of the *Hoxc6* expression construct

The complete open reading frame of *Hoxc6* (BC084319) was amplified using primers including *BamHI* and *XhoI* restriction sites (F: 5'-**GGATCC**ATGAATTCCTATTTCACTAACCCTT-3'; R: 5'-**CTCGAG**GGGTGTCTCTCCATTCACTCTTT-3'). The short form *Hoxc6* was amplified using the following primers with *BamHI* and *EcoRI* restriction sites: (F: 5'-CG**GGATCC**ATGCTCACTAGCTGCAGGCAGA-3'; R: 5'-G**GAATTC**TCACTCTTTGCCTTGTCCCTCT-3'). After ligation of the PCR product into pGEM-T Easy vector (Promega), *Hoxc6* long form (LF, previously called PRII) was excised by *BamHI*/*XhoI* digestion and ligated into CS2+ vector (Turner and Weintraub 1994). The *Hoxc6* short form (SF, previously called PRI) was excised by EcoRI/BamHI and ligated to CS2+. These constructs were checked by sequencing. Since the LF and the SF constructs do not encode the 3' sequences that are recognized by the MOs (see below), they are insensitive to the MO knockdown.

Injection of morpholinos and mRNA

Embryos were staged according to (Nieuwkoop and Faber 1956). *In vitro* fertilization, embryo culture, and mRNA injection, were carried out as previously described (Wacker et al. 2000; Winklbauer 1990). Morpholino injections were done as mRNA injections, except that 5 nl of MO solution per cell were injected in both blastomeres at stage 2. The concentrations are stated below.

Two anti-sense morpholino oligonucleotides (MOs, Gene-Tools Inc.) were designed for the *Hoxc6* transcripts, which encode the two different isoforms. Each MO was designed to be effective against one of the isoforms (compare Figure 1 A). The sequences of the MOs are as follows: *Hoxc6*, MO2-5'-TCTATTACAACACAAACCGGAGGTCG-3', MO3-5'-ATTCATATCTTCTCCTTTACCTGCC-3'. MO3 targets the LF transcript, while MO2 is effective against the SF transcript. SF and LF are designating the size of the protein in the present report. Thus LF transcript refers to the formerly called PRII product. Respectively, SF refers to PRI. Morpholinos and mRNAs were diluted in Gurdon's buffer (15 mM Tris pH 7.5, 88 mM NaCl, 1 mM KCl) and injected at two cell stage in the animal hemisphere. The amounts injected are 15 ng or 20 ng per embryo of *Hoxc6* MOs and of the standard control MO. For some experiments MOs were co-injected with FITC dextran (M=10000, Molecular probes) in one cell out of two cell stage embryo. In all experiments, control MO (standard control, Gene-Tools Inc.) injections were also carried out and embryos processed for marker analysis. Control MO (ctMO) embryos never gave different results from the

uninjected controls. The function and specificity of the MOs were tested *in vitro* using the Sp6 coupled transcription and translation assay (Promega). For gain-of-function and rescue experiments 600 pg of *Hoxc6* MO-insensitive mRNA were used, if not differently stated. Up to 800pg of *Hoxc6* SF mRNA were injected alone or in combination with MOs.

Detection of gene expression by *in situ* hybridization

Whole mount *in situ* hybridization was performed as previously described (Harland 1991; Wacker et al. 2004), except that the RNAse step was omitted. Antisense digoxigenin-labeled probes were: *FoxF1* (*XFD13*, (Köster et al. 1999)), *Xlim1* (Chan et al. 2000), *N-tubulin* (Chitnis and Kintner 1995; Marcus et al. 1998), *Xisl-1* (Brade et al. 2007), *Sox-2* and *Hoxd4* (Jansen et al. 2007), *Hoxb9* and *Hoxd13* (Wacker et al. 2004), *MyoD* and *Xtwist* (Hopwood et al. 1989), *Krox-20* (Bradley et al. 1993), *En-2* (Hemmati-Brivanlou et al. 1991), *Xotx2* (Blitz and Cho 1995), epidermal keratin *XK81A1* (Jonas et al. 1989), *Nrp-1* (Richter et al. 1990), *Xenopus Notch* (Coffman et al. 1990), *Xslug* (Mayor et al. 1995), *N-CAM* (Kintner and Melton 1987). The SF probe was generated by cloning a part of the precursor messenger (Figure 1 A). The primers used are as follows: F 5'-GGAATTCGGAAAAGGCATTTGCACACGCCA-3'; R: 5'-CCGCTCGAGTGCTACAGCCCGGAACTCTGG-3'. The LF probe was cloned by amplifying a fragment from the start codon until the homeodomain from the plasmid coding for the long form protein. The primers used are: F 5'-ATGAATTCCTATTTCACTAACCCT-3'; R: 5'-TTTGGTAACGGGAATAGATCT-3'. The fragment was ligated to the *pDRIVE* vector. For embryos injected with MOs and FITC, A antiFITC-antibody and Fast-Red were used to reveal the FITC at first. The DIG labeled probes for *N-tubulin* or *Xisl-1* and their detection followed using the standard methods as mentioned above. In some cases embryos were bleached with hydrogen peroxide after the *in situ* hybridization. For sections embryos were embedded in gelatin blocks after *in situ* hybridization, and dissected at 50 μm intervals.

Animal cap assay

Embryos were injected at two cell stage in both cells with *noggin* (500 pg per embryo), *Hoxc6 LF* (600 pg per embryo), *Hoxc6 SF* (600 pg per embryo) mRNAs. Caps were dissected at stage 8.5 and cultured until control siblings reached stage 15. Samples were processed to RNA extraction and RT-PCR.

RNA isolation and RT-PCR analysis

Total RNA was extracted from different stages embryos or caps by Trizol (Invitrogen) and phenol chloroform extraction. The extract was cleaned using the RNAeasy columns kit (Qiagen). The first strand cDNA synthesis (Fermentas) was carried out according to the manufacturer instructions. The following primers were used:

H4 (F: 5'-CGGGATAACATTCAGGGTA-3'; R: 5'-TCCATGGCGGTAACTGTC-3'); SF (F: 5'-GGAATTCGGAAAAGGCATTTGCACACGCCA-3'; R: 5'-CCGCTCGAGTGCTACAGCCCGGAACTCTGG-3'; LF (F: 5'-ATGAATTCCTATTTCACTAACCCT-3'; R: 5'-TTTGGTAACGGGAATAGATCT-3'); N-tubulin (F: 5'-ACACGGCATTGATCCTACAG-3'; R: 5'-AGCTCCTTCGGTGTAATGAC-3'); N-CAM (F: 5'-GCCCCTCTTGTGGATCTTAGTGA-3'; R: 5'-ACAGCGGCAGGAGTAGCAGTTC-3').

The positions of the primers for LF and SF are indicated in figure 1 A.

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