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

Bardine, N.

Chapter 5

HoxC6 is required for somitogenesis in *Xenopus*

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Abstract

Hox genes are involved in the regionalization of the vertebrate anteroposterior body axis In mouse, such a role has been shown for the *HoxC6* gene in the patterning of the axial skeleton. We investigated the developmental role of *Hoxc6* in *Xenopus laevis* and unexpectedly find a requirement for somitogenesis through regulation of the *Xenopus X-Delta-2*. This role contrasts sharply with the known functions of *Hox* genes in vertebrates and indicates a putatively ancestral, function for *Hoxc6* in *Xenopus*. Our observations challenge the current perception of the universality of *Hox* gene functioning in tetrapods and suggest that evolutionary changes in the amniote body plan have resulted in a different implementation of the *Hox* patterning system.

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Introduction

In vertebrates, the axial skeleton derives from metameric structures, called somites, appearing during body elongation on each side of the neural tube (Cinquin, 2007). These
somites are generated in an anterior to posterior sequence, at the anterior end of the unsegmented presomitic mesoderm (PSM) (Pourquié, 2002). Although somites are morphologically uniform, they will give rise to the different vertebrae specific for the axial level they are positioned.

Hox transcription factors are major players in conferring positional information along the anteroposterior axis in vertebrates (Krumlauf, 1994; Burke *et al.,* 1995; Lemons and McGinnis, 2006). The developmental roles of some *Hox* genes in vertebrates have been characterized by knockout and transgenic analysis in the mouse and by morpholino knockdowns in *Xenopus* and zebrafish. Single *Hox* knockouts do generally not lead to dramatic phenotypes (due to the high functional redundancy within the paralogous groups) but double, triple and quadruple knockouts have been very informative about the roles of *Hox* genes in development (McNulty *et al*., 2005; Wellik and Capecchi, 2003; McClintock *et al.,* 2002; van den Akker *et al*., 2001; Horan *et al*., 1995a, 1995b). In the mesoderm, patterning by the *Hox* genes has been shown to underlie the regionalization of the axial skeleton and *Hox* genes are believed to specify the cervical, thoracic, lumbar, sacral and caudal anatomical regions (Kessel and Gruss, 1991). *Hox* phenotypes are in general interpreted as "homeotic transformations", in which metameres adopt an identity normally associated with a different anteroposterior position. In the mouse, no *Hox* mutations are known to affect the basal underlying segmental organization of the trunk. However, a direct link between any *Hox* somitic domain of expression and any mutant phenotype has not yet been made. Nonetheless, it has been reported that *Hox* gene function is relevant within the presomitic mesoderm (McIntyre *et al.,* 2007). Thus, *Hox10* group function is required within the time window during which the thoracic/lumbar transition somites are being generated (Carapuço *et al*., 2005).

A link between body patterning and segmentation was first proposed in mouse. *Hoxd1* expression and *Hoxd3* expression were down-regulated in the somitomeres of the RBPjk-null mutant, suggesting that Notch signaling is somehow required for the correct expression of *Hox* genes during mouse somitogenesis (Zákány *et al.,* 2001). It has been shown that Notch mutants exhibit homeotic transformations and changes in vertebral identity in mouse and man (Cordes et *al.,* 2004; Turnpenny *et al*., 2007). In addition, in *Xenopus*, loss of function of the paralogous group 1 (PG1) leads to somitogenesis defects and a loss of

X-Delta-2 expression which already starts within the PSM before somite formation (Peres *et al*., 2006; Peres and Durston, 2006). Conversely, loss of function of *X-Delta-2* impairs early expression of *Hox* genes during gastrulation. This data shed light on a tight link between patterning and segmentation in *Xenopus*. Loss of PG1 function also leads to the downregulation of moreposterior *Hox* genes (McNulty *et al.,* 2005). Interestingly, none of the PG1 genes is expressed in the trunk somites at tadpole stages (except *Hoxa1* which is expressed in the most anterior somites) while more posterior *Hox* genes such as *Hoxb4* are expressed in those structures. It has been reported that *Hoxb4* ectopic expression affects somite formation in *Xenopus* without affecting other mesodermal derivatives (Harvey and Melton, 1988). Here, we investigate the potential link between the down-regulated *Hox* genes in the PG1 loss of function (as *Hoxc6*) and the somitogenesis process in *Xenopus*. We used a morpholino knockdown approach to disturb expression of several *Hox* gene*s* in *Xenopus.* Our results show a requirement for *Hoxc6* function for a proper somitogenesis in *Xenopus*.

Results

Knockdown of *Hoxc6* leads to loss of segmentation

To investigate the role of *Hox* genes in a more primitive, anamniote tetrapod we performed loss of function by injecting morpholino antisense oligonucleotides into early blastomeres in *Xenopus laevis*. We injected morpholinos against *Hoxd1*, *Hoxb4* (unpublished data), *Hoxc6*, *Hoxb9, Hoxa7*, and *Hoxb7* (Peres personal communication). Injection with the anti *Hoxc6* morpholino, but not with any other morpholino against any one of the other *Hox* genes tested resulted in a clear and unexpected segmentation phenotype (Fig. 1 and 2). *Hoxc6* encodes two different proteins, a long form (LF) and a short form (SF) (Cho *et al.,* 1988). Our results show that the loss of the LF leads to a severe segmentation loss while the loss of the SF does not seem to affect somites formation (Fig. C and D). Segmentation of the frog PSM has been shown to be mediated by a Nocth ligand, *X-Delta-2* (Jen *et al*., 1997). *X-Delta-2* is expressed in the anterior half of a somitomere. Moreover, *X-Delta-2* was down-regulated upon PG1 genes knockdown in *Xenopus* (Peres *et al.,* 2006).

Thus, embryos were analyzed for expression for *X-Delta-2* and they showed loss of anterior somitic boundaries (Fig. 3). In addition, embryos were analyzed for *PAPC* (Kim et *al.,* 2000) and *Thylacine1* (Moreno and Kintner,2004) expression, both genes are expressed in the anterior part of the prospective somite (Fig. 4). These data confirmed the loss of anterior somite character. Analysis of a somite posterior marker, like *Uncx4.1* (Bussen *et al*., 2004), show a similar severe downregulation as seen for anterior markers as *X-Delta-2* (Fig. 3 and data not shown).

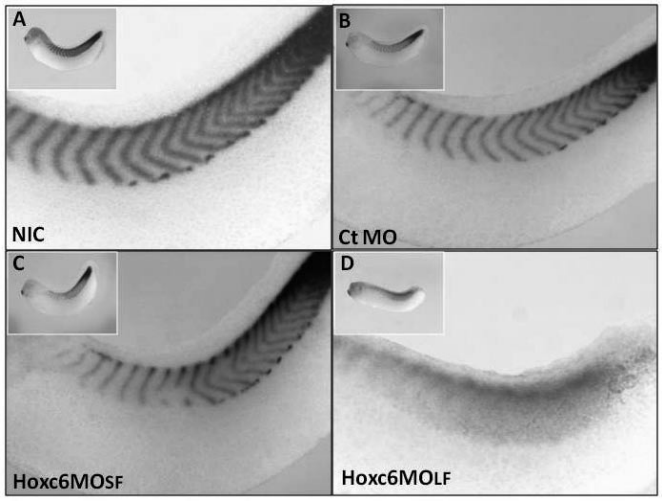

Figure1: *Hoxc6* knockdown leads to severe segmentation phenotype in *Xenopus*. Shown are uninjected control embryo (A), Control morpholino (CtMO) injected embryo (B), MO targeting the short form *Hoxc6* (C), and MO targeting the long form Hoxc6 (D) stained by whole mount in situ hybridization for the expression of *MyoD*. In D, note that the typical segmented pattern of *MyoD* is lost when the long form HoxC6 protein is lost.

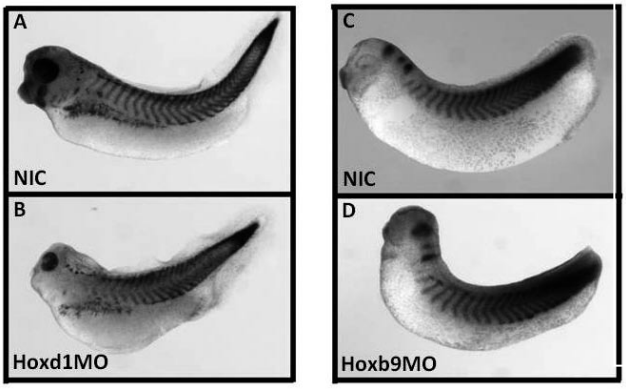

Figure 2: *Hoxd1* or *Hoxb9* knockdown does not affect trunk segmentation in *Xenopus*. Shown are uninjected control, *Hoxd1* MO injected, and *Hoxb9* MO injected stained by whole-mount in situ hybridization for *MyoD*, and *Engrailed2/Krox-20/MyoD*. Note that segmentation is unaffected in both cases.

Since it has been reported that *Mespo* (Joseph and Cassetta, 1999) is activated in the anterior PSM by *Tbx6* (Li *et al.,* 2006), we further analyzed *Hoxc6* LF morphans for the expression of these two markers. Their expression seems to be unchanged in the PSM (data not shown). Altogether, our results show that the loss of HOXC6 LF protein in *Xenopus* severely disturbs the polarity of the forming somites while the unsegmented PSM does not seem to be affected.

The loss of somites is specific for *HOXC6* long protein isoform

Our group previously reported the loss of segmentation upon the loss of PG1 *Hox* genes (Peres *et al*., 2006). This effect was through the loss of *X-Delta-2* expression during early development of *Xenopus*. Surprisingly, a single member of the PG1 genes, m*Hoxb1*, rescued *X-Delta-2* expression, but this was insufficient to restore a normal segmented phenotype. Here, we decided to investigate if other *Hox* genes (anterior and posterior to *Hoxc6*) were able to rescue the segmentation phenotype obtained by *Hoxc6* LF knockdown.

Figure 3: *X-Delta-2* expression is downregulated in the PSM after knocking down Hoxc6 long protein. *X-Delta-2* pattern in an uninjected control embryo (A) or control morpholino injected embryo (B) is dramatically lost after injection of *Hoxc6* MOLF. Note in C that the stripes are lost showing a loss of anterior half identity of the prospective

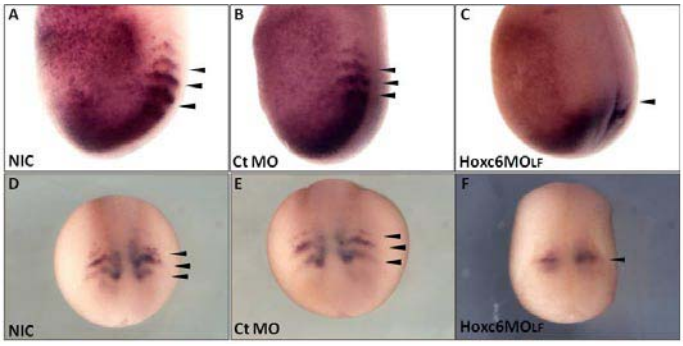

Figure 4: *PAPC* and *Thylacine 1* expression is downregulated in the PSM after knocking down Hoxc6 long protein. *PAPC* (A) and *Thylacine 1*(D) expression pattern in an uninjected control embryo or control morpholino injected embryo (B and E respectively) is dramatically lost after injection of *Hoxc6* MOLF (*PAPC* in C and *Thylacine1* in F). Note that in C and F the stripes are lost showing a loss of anterior half identity of the prospective somites after depletion of *Hoxc6*.

Surprisingly, members from the same paralogs 6, *Hoxa6* and *Hoxb6,* did not rescue the loss of segmentation (data not shown). The *Hoxc6* short form did not restore a proper

segmentation pattern either (Fig.5 D). Segmentation was not restored by neither *Hoxd1*, *Hoxa7* nor *Hoxb9* (Fig.5 C, E, F). However, *Hoxc6* coding for the long form protein rescued the segmented pattern. These results point towards a role of *Hoxc6* LF in segmentation.

The Drosophila homologue of *HoxC6* , *Antennapedia*, rescues the segmentation phenotype.

Hoxc6 was the first vertebrate gene cloned on the basis of its homology to the Drosophila gene, *Antennapedia* (*Antp*, (Carasco *et al.,* 1984)). We investigated whether Drosophila *Antp* could rescue the segmentation phenotype in *Xenopus* after depletion of Hoxc6 LF. Surprisingly, *Antp* mRNA seems to behave like *Hoxc6* LF mRNA and restores a segmented pattern (Fig. 6) while a segmentation gene, *Fushi taratzu* does not show the same behaviour (data not shown). These data suggest that a conserved function of *Hoxc6* mediates segmentation in the frog.

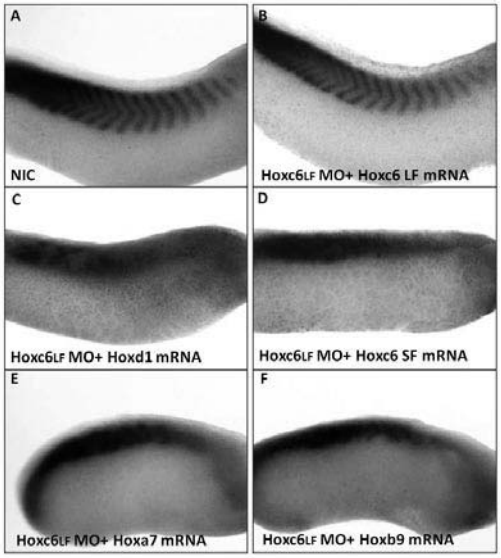

Figure 5: *Hoxc6* LF rescues segmentation defects while *Hoxd1*, *Hoxc6 SF*, *Hoxa7* and *Hoxb9* fail to rescue this phenotype. Is shown MyoD expression in an uninjected control tadpole (A), injected with MO targeting Hoxc6 LF +*Hoxd1* (C), +*Hoxc6* LF (B), +, *Hoxc6 SF*, + *Hoxa7* (E), + *Hoxb9* (F). Only *Hoxc6* mRNA encoding the long form Hoxc6 protein is able to rescue the segmentation loss.

HoxC6* targets *X-Delta-2

It has been previously shown that the PG1 *Hox* genes play a role during segmentation in *Xenopus* via *X-Delta-2* (Peres *et al*., 2006). PG1 genes knockdown reduces *X-Delta-2* expression at neurula stages. However, *X-Delta-2* expression was restored by

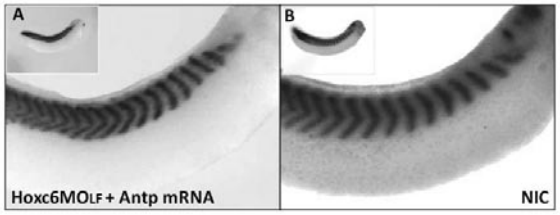

Figure 6: The *Drosophila* homolog of *Hoxc6*, *Antennapedia* rescues the segmentation phenotype induced by *Hoxc6* LF knockdown. MyoD expression is shown in uninjected control (B), and in MO+ *Antp* mRNA (A).

injection of the murine *Hoxb1*. This rescue was not sufficient to restore a segmented pattern at tadpole stages. In addition, we observed a severe downregulation of some posterior *Hox* genes such as *Hoxc6* upon PG1 genes loss of function. Here we investigated the potential link between *Hoxc6* and *X-Delta-2*. *Hoxc6* LF knockdown specifically downregulates *X-Delta-2* expression at early stages in *Xenopus* as mentioned above (Fig. 3) without affecting other mesodermal markers (data not shown). Conversely, ectopic expression of *Hoxc6* LF triggers upregulation of *X-Delta-2* expression within the PSM in agreement with microarray data (Michaut *et al*., manuscript in preparation). Moreover, *Hoxc6* LF mRNA as well as its Drosophila homologue, *Antp,* restore the typical pattern of *X-Delta-2* in the *Hoxc6* LF hypomorph (Fig. 7 and data not shown). These data suggest that *Hoxc6* could directly regulate the expression of *X-Delta-2* in the PSM in *Xenopus*, and that this function seems to be a conserved feature during evolution.

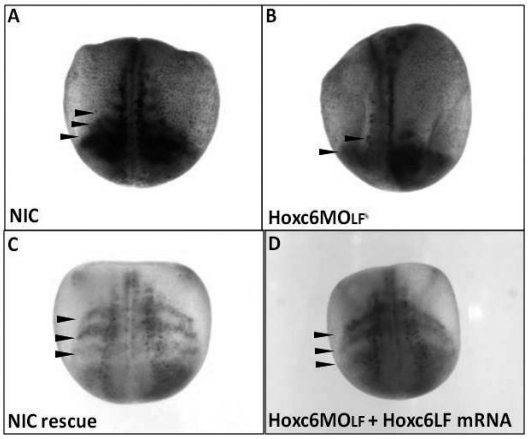

Figure 7: *Hoxc6* LF restores *X-Delta-2* expression pattern within the PSM. *X-Delta-2* expression with the stripes at neurula stage in an uninjected embryo (A, C), and in an embryo injected with Hoxc6MOLF (B), and in an embryo co-injected with MO targeting the LF *Hoxc6* and the mRNA insensitive to this morpholino (D). Upon *Hoxc6 LF* loss of function, *X-Delta-2* expression is downregulated as seen by the loss of the Delta stripes. The typical expression pattern is rescued by coinjection of Hoxc6 LF mRNA insensitive to the morpholino (D).

Discussion

In vertebrates, somitogenesis.results in the formation of metameric structures called somites. Despite their similarity, different somites will give rise to diverse vertebrae in the axial skeleton. *Hox* genes are among the major players in the specification of the morphological identity of the vertebrae (Krumlauf, 1994). The paraxial mesoderm starts to be patterned during gastrulation, prior to somite formation. The patterning process

continues later during somitogenesis. Moreover, a tight link between patterning and somitogenesis has been shown in *Xenopus* (Peres *et al.,* 2006) and mouse (Zákány *et al.,* 2001). In the frog, knockdown of the PG1 *Hox* genes leads to segmentation defects, as well as to downregulation of posterior *Hox* genes, like *Hoxc6*. The latter has been shown to be expressed within the somitic mesoderm (Oliver *et al.,* 1988). So, we investigated a potential link of *Hoxc6* and segmentation in *Xenopus*.

In this study, we show that *Hoxc6* long form (LF) function is required for *X-Delta-2* expression during *Xenopus* development. Our group has previously shown that loss of function of *X-Delta-2* in *Xenopus* leads to downregulation of *Hoxc6* at gastrula stages (Peres *et al*., 2006). This downregulation was rescued by *X-Delta-2* expression. These data and our results suggest a feedback loop between *X-Delta-2* and *Hoxc6*. In addition, these results show a requirement for Notch signaling for proper *Hox* expression in *Xenopus*. Indeed, *Hox* genes expression, *Hoxd1* and *Hoxd3*, was down-regulated in a Notch signaling mutant (Zákány *et al*., 2001). It was also suggested that the expression of *Hox* genes in the mouse PSM is crucial for specifying vertebral identity (Carapuço *et al.,* 2005), thus suggesting a tight regulation of *Hox* gene expression before somites formation.

Here in *Xenopus*, loss of function of *Hoxc6* LF severely disrupts segmentation, resulting in loss of somite identity. Indeed, anterior and posterior somite markers are lost resulting in the loss of boundaries between somites, and a proper segmentation pattern (Fig. 1). This phenotype is specific to *Hoxc6* LF because no other *Hox* genes (*Hoxd1*, *Hoxa7* and *Hoxb9*) could rescue the segmentation defects. Members from the paralogous 6 group did not rescue the segmentation loss. *Hoxc6* LF only could rescue the segmentation defects in *Xenopus.* These results suggest a difference in function between the two *Hoxc6* genes. It had already been shown that the two HOXC6 proteins show a differential expression pattern, strongly suggesting a difference in function (Oliver *et al.,* 1988).

Whether *Hox* genes are upstream of somitogenesis and expression of *Delta-like* genes in higher vertebrates remains to be investigated. Moreover, the requirement of a single *Hox* gene for proper segmentation has not been reported up to date. Our study also showed that *X-Delta-2* is a putative direct target of *Hoxc6* LF. Indeed downregulation of *X-Delta-2* is restored upon *Hoxc6* injection in *Hoxc6LF* hypomorph. This function of *Hoxc6* is shared with its Drosophila homologue, *Antennapedia* suggesting an ancestral requirement of *Hoxc6* during segmentation in *Xenopus*. If *Xenopus* represents the ancestral condition of vertebrates, this could mean that there is either a complete dissociation of the pathway inducing or alternatively that the genetic pathway has been consolidated and that now multiple paralogous groups are directly upstream of *X-Delta-2*.

In PG1 loss of function, expression of posterior *Hox* genes has been shifted posteriorly or down-regulated. The latter is the case of *Hoxc6*. We speculate that loss of segmentation on PG1 could be through downregulation of *Hoxc6*, and subsequently *X-Delta-2*. *Hoxd1* and *mHoxb1* fail to rescue the segmentation defects in PG1 genes loss of function (Peres *et al.,* 2006). A similar phenotype is fully rescued by *Hoxc6* LF as we have shown. A rescue of PG1 segmentation phenotype by *Hoxc6* LF mRNA is likely to occur (currently under investigation). This would emphasize a predominant role of *Hoxc6* in *Xenopus* segmentation.

Experimental procedures

Embryos

Xenopus laevis embryos were stages according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). Culture of embryos and buffers were as described (McNulty *et al*., 2005).

Detection of gene expression by in situ hybridization

The whole mount in situ hybridization protocol used was a modified protocol from previously described (Harland, 1991). After in situ hybridization, embryos were bleached when necessary as previously reported (Song and Slack, 1994). Digoxigenin-labelled probes were made from the following plasmids: Krox-20 (Bradley *et al*., 1993); *Engrailed-2* (Hemmati-
Brivanlou *et al*., 1991); *MyoD* (Hopwood *et al*., 1989); X-Delta-2 (Jen *et al.,* 1997). *XTbx6*, *Mespo* and *Uncx4.1* were obtained from the NIBB Xenopus cDNA resource group.

Injection of morpholinos and mRNA

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. Morpholinos are as follows: Hoxd1 MO (McNulty *et al.,* 2005); Hoxc6LF MO: 5'-ATTCATATCTTCTCCTTTACCTGCC-3' ; Hoxc6 SF MO: 5'-TCTATTACAACACAAACCGGAGGTCG-3' ; Hoxb9 MO: 5'-TGACCGACCCACCAGCTTCTCCCCA -3' ; and the standard control morpholino (ctMO) from Genes-Tools Inc. (Gene-Tools Inc.). Hoxc6 MOs were used at concentrations

of 15 to 17,5ng per embryo; Hoxd1 MO as reported previously; Hoxb9 MO 20 ng; and ctMO up to 40ng.
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 construct does not encode the 3' sequences that are recognized by MO LF.

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