Chapter 3

Hoxc6 loss of function truncates the main body axis in Xenopus

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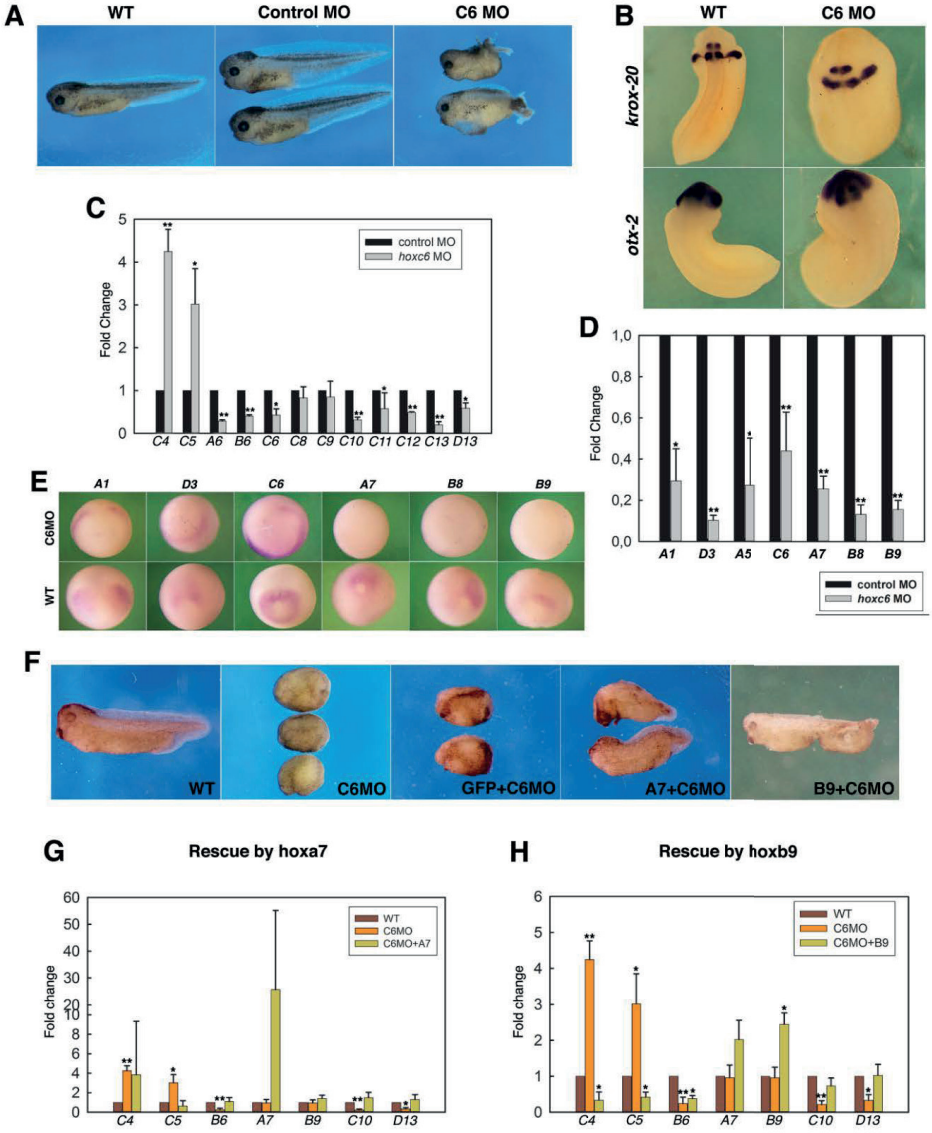
During animal development, the formation of the anterior-posterior (A-P) axis is of fundamental importance, because it sets the stage for subsequent tissue differentiation and organogenesis. Patterning along the A-P axis requires the expression of Hox genes to specify positional information (Alexander et al., 2009). In mammals, these genes are organized into four chromosomal (HOXA-D) clusters containing 39 members. Equivalent genes within each cluster are divided into 13 paralogous groups (HOX1-13) (Burke et al., 1995; Carroll, 1995). During A-P patterning, Hox genes are expressed in a temporal sequence that matches their 3' to 5' arrangement on the chromosome (temporal collinearity) (Dolle and Duboule, 1989; Duboule, 2007; Izpisua-Belmonte et al., 1991). This precisely controlled timing in Hox gene expression potentially provides a timing mechanism for the formation of A-P structures, that are laid down from head to tail progressively during early development (Eyal-Giladi, 1954; Gamse and Sive, 2000; Gamse and Sive, 2001; Nieuwkoop, 1952; Stern et al., 2006). Actually, Hox temporal collinearity is prerequisite for establishing the correct spatial pattern of Hox gene expression (Durston and Zhu, 2015; Wacker et al., 2004a), which also reflects their 3' to 5' order on the chromosome (spatial collinearity) (Duboule and Dolle, 1989; Graham et al., 1989; Lewis, 1978). Therefore, not only is temporal collinearity an interesting feature of Hox genes, it is also likely to play a crucial role in A-P patterning. Nonetheless, the nature of Hox temporal collinearity has yet to be fully elucidated.

In an attempt to understand what drives the Hox timer (temporal collinearity), we did Hoxc6 loss of function (LOF) in the Xenopus by injection of a *hoxc6* morpholino (MO) (5'-ATTCATATCTTCTCCTTTACCTGCC-3') at 2-cell or 4-cell stage. The effectiveness and specificity of this MO have been demonstrated previously (Bardine et al., 2009). Remarkably, injection of 60ng *hoxc6* MO cut off the posterior part of the axis completely, whereas injection of the same amount of control MO did not affect axis formation (Fig. 1A). Consistent with the phenotype, we observed that the expression domains of *krox-20* (Bradley et al., 1993), a rhombomere marker, and *otx-2* (Li et al., 1994; Mori et al., 1994), a midbrain marker, expanded posteriorly (Fig. 1B).

We then asked: why did Hoxc6 LOF give such a severe developmental consequence? To answer this question, we examined the expression of all the 9 genes in the HOXC cluster (*hoxc4, 5, 6, 8, 9, 10, 11, 12* and*13*) and all the 3 genes in the hox6 paralogous group (*hoxa6, b6* and *c6*) at st.26 (Fig. 1C). In Hoxc6 LOF embryos, *hoxc6* was downregulated by 50%, confirming the morpholino worked. The downregulation of *hoxc6* was accompanied by upregulation of *hoxc4* and *c5* and by downregulation of most Hox genes that are posterior to *hoxc6* in the HOXC cluster. These results indicate two previously identified types of Hox interactions within the HOXC cluster: posterior prevalence (Harding et al., 1985; Schneuwly et al., 1987; Zhu et al., 2017) (the repression or antagonism of anterior Hox genes by posterior ones, e.g. the repression of *hoxc4* and *c5* by Hoxc6), and posterior induction (Hooiveld et al., 1999; Zhu et al., 2017) (the induction of posterior Hox genes by anterior ones, e.g. the induction of *hoxc8,9,* and *10-13* by Hoxc6) (reviewed in Durston and Zhu, 2015). Interestingly, *hoxa6* and *b6* were also downregulated by Hoxc6 LOF, suggesting interactions also exist among Hox clusters. This explains why Hox genes from other clusters (e.g. *hoxd3, a5,* and *d13*) behaved similarly as those from the HOXC cluster (Fig. 1C and data not shown). Together, these results show that Hoxc6 LOF cuts off the axis at an extremely specific position: the front end of the Hoxc6 expression zone: the neck-thorax boundary. This massive effect is due to the fact that Hoxc6 LOF affects the expression of Hox genes from all the four clusters.

Since Hox genes show temporal collinearity and begin their A-P patterning activity during gastrulation (Deschamps et al., 1999; Forlani et al., 2003; Gaunt and Strachan, 1994; Iimura and Pourquie, 2006; Wacker et al., 2004b), we then examined the expression of different Hox genes at the gastrula stage (st.12.5) (Fig. 1D and 1E). In Hoxc6 LOF gastrulas, the expression levels of *hoxc6, a7, b8* and *b9* were significantly lower than those in the control. Interestingly, we also noted downregulation of *hoxa1, d3* and *a5* in these embryos. While at first sight this may seem contradictory to posterior prevalence, it is compatible with and explainable by our recent result that posterior prevalence starts to exert its influence from st.15 in paraxial mesoderm (Zhu et al., 2017). What is important is that results in Fig. 1D and 1E suggest Hoxc6 LOF cuts off the temporal sequence of Hox expression and stops the Hox timer.

If Hox interactions do occur, posterior Hox genes should be able to partially or fully rescue the truncated Hox sequence in Hoxc6 LOF embryos. To test this hypothesis, we ectopically expressed *hoxa7* and *hoxb9* in these embryos. Ectopic expression of both genes rescued the Hoxc6 LOF phenotype (Fig. 1F). These rescues were specific as GFP was unable to rescue the axis. Interestingly, *hoxb9* ectopic expression in these embryos restored the tail part of the axis, showing a gap in the axis between the *hoxc6* expression position and the *hoxb9* domain. In *hoxa7* rescued embryos, *hoxa7, c10* and *d13* were upregulated, while *hoxc5* was downregulated. Compared with its expression in the Hoxc6 LOF group, *hoxc4* expression was only slightly affected (Fig. 1G). In hoxb9 rescued embryos, the expression of *hoxb9*, *c10, c12* and *d13* were restored, while *hoxd3*, *c*4 and *c5* were downregulated (Fig. 1H and data not shown). All these results can be explained by posterior prevalence and posterior induction. The upregulation of *hoxb6* in hoxa7 rescued embryos and upregulation of *hoxa7* in hoxb9 rescued embryos does, however, complicate the picture. Nevertheless, it is still reasonable to propose that Hox interactions play a crucial role in Hox temporal collinearity, especially when considering that temporal collinearity requires synchronization among different clusters and cells.


A
WT
Control MO
C6 MO
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C6 MO
krox-20
otx-2
C
Fold Change
control MO
hoxc6 MO
C4 C5 A6 B6 C6 C8 C9 C10 C11 C12 C13 D13
D
Fold Change
A1 D3 A5 C6 A7 B8 B9
control MO
hoxc6 MO
E
A1 D3 C6 A7 B8 B9
C6MO
WT
F
WT
C6MO
GFP+C6MO
A7+C6MO
B9+C6MO
G
Rescue by hoxa7
Fold change
WT
C6MO
C6MO+A7
C4 C5 B6 A7 B9 C10 D13
H
Rescue by hoxb9
Fold change
WT
C6MO
C6MO+B9
C4 C5 B6 A7 B9 C10 D13

Figure 1 *Hoxc6* knockdown in Xenopus embryos has a massive effect on A-P axis formation and Hox gene expression. (A) Injection of 60ng Hoxc6 MO at the two-cell or four-cell stage leads to a significant truncation of the posterior axis, whereas injection of control MO has no observable effect on axis formation. (B) The expression of *krox-20*, a rhombomere marker, and *otx-2*, a midbrain marker, are expanded posteriorly by depletion of Hoxc6. (C) Quantitative RT–PCRs showing the expression of all the 9 genes in the HoxC cluster (*hoxc4, 5, 6, 8, 9, 10, 11, 12* and *13*), all the 3 genes in the Hox6 paralogue group (*hoxa6, b6* and *c6*) and *hoxd13* at stage 26. *Hoxc4* and *c5* are upregulated by *hoxc6* knockdown, while most of the other Hox genes are downregulated. Data are presented as fold change relative to control and indicate mean + SD from 3-6 biological replicates. The measurements were normalized to *ODC*. (*p < 0.05, **p < 0.01). (D) Quantitative RT-PCRs showing the expression of *hoxa1, d3, a5, c6, a7, b8*, and *b9* at st.12.5. (E) Whole mount in situ hybridization (WISH) for the expression of *hoxa1, d3, c6, a7, b8* and *b9* at the gastrula stage (st.12.5). Note that *hoxc6* and Hox genes that are posterior to it are downregulated. (F-H) The rescue of posterior axis and Hox gene expression in Hoxc6-depleted embryos by *hoxa7* and *hoxb9* overexpression. Co-injection of *hoxa7* or *hoxb9* mRNA (~1ng in total) can partially rescue the Hoxc6 MO phenotype (E). The rescue of phenotype is accompanied by rescue of Hox gene expression. In *hoxa7* mRNA and Hoxc6 MO co-injected embryos, the expression of *hoxa7, b9, c10* and *d13* are upregulated, while hoxc5 is downregulated (F). *Hoxb9* co-injection rescues *hoxb9, c10* and *d13*, but not *hoxc4* and *c5* (G).

The LOF phenotype for Hoxc6 in our study is striking. It is much more extreme than the mouse LOF phenotype for the same gene (Garcia-Gasca and Spyropoulos, 2000) or even than the mouse LOF phenotype for the whole Hox6 paralogue group (McIntyre et al., 2007). There are clearly differences between the amniote mouse and the anamniote Xenopus. In part this difference may reflect the fact that, in Xenopus but possibly not in mouse, expression of the other later *hox6* paralogues depends on the earlier Hoxc6. The most important difference however is likely to be that, while the entire axis is specified early in anamniotes like Xenopus (Durston and Zhu, 2015), the mouse A-P axis is specified over a much longer period and is dependent on axial growth (Steventon et al., 2016; Young et al., 2009). The striking Hoxc6 LOF phenotype in Xenopus emphasises the importance of collinear Hox-Hox interactions in Hox collinearity. While further studies are needed to elucidate the molecular mechanisms that mediate Hox interactions, we believe these interactions drive Hox collinearity. This study also gives a new clue to the mechanism of vertebrate axial patterning.

We present evidence above that Hox interactions regulate the sequence of Hox genes expressed during temporal collinearity. However, there is no reason to expect that this also entirely determines exact timing of this process or its evident synchronisation with segmentation. In this context, it is very interesting that recent articles propose an interaction between Hox temporal collinearity and the somitogenesis clock (Durston and

Zhu, 2015; Gouveia et al., 2015). It is likely that this authentic biological clock adds precision to an important developmental mechanism.

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