Regulatory role for a conserved motif adjacent to the homeodomain of Hox10 proteins

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SUMMARY

Development of the vertebrate axial skeleton requires the concerted activity of several Hox genes. Among them, Hox genes belonging to the paralog group 10 are essential for the formation of the lumbar region of the vertebral column, owing to their capacity to block rib formation. In this work, we explored the basis for the rib-repressing activity of Hox10 proteins. Because genetic experiments in mice demonstrated that Hox10 proteins are strongly redundant in this function, we first searched for common motifs among the group members. We identified the presence of two small sequences flanking the homeodomain that are phylogenetically conserved among Hox10 proteins and that seem to be specific for this group. We show here that one of these motifs is required but not sufficient for the rib-repressing activity of Hox10 proteins. This motif includes two potential phosphorylation sites, which are essential for protein activity as their mutation to alanines resulted in a total loss of rib-repressing properties. Our data indicates that this motif has a significant regulatory function, modulating interactions with more N-terminal parts of the Hox protein, eventually triggering the rib-repressing program. In addition, this motif might also regulate protein activity by alteration of the protein’s DNA-binding affinity through changes in the phosphorylation state of two conserved tyrosine residues within the homeodomain.

KEY WORDS: Hox genes, Axial patterning, Transgenics, Vertebrate development, Mouse

INTRODUCTION

Hox genes are key regulators of embryonic development (Krumlauf, 1994; Pearson et al., 2005). Genes of this family are typically organized in genomic clusters, although some exceptions to this rule have been described recently (Duboule, 2007). Whereas invertebrates contain a single Hox cluster, the Hox genes of vertebrates are distributed in several clusters, which are thought to have arisen by sequential duplication of a single ancestral cluster (Duboule, 2007). As a consequence, individual Hox genes in a given cluster have close relatives in one or more of the others. The homologs in the different clusters, defined according to their relative position and sequence similarities, constitute the so-called paralog groups. There are 13 paralog groups in most vertebrates (Duboule, 2007). Members of the same paralog group are often redundant for particular functions (Mallo et al., 2010). This is most evident for genes in paralog groups 9, 10 and 11 as their roles in patterning the axial skeleton became evident only after simultaneous inactivation of all members of the group (Wellik and Capecechi, 2003; McIntyre et al., 2007). In addition, some Hox gene functions are paralog specific and not shared by members of other groups. This is illustrated by the distinct phenotypes obtained with members of different paralog groups when assayed under the same experimental conditions. For instance, when precociously expressed in the presomitic mesoderm of transgenic embryos, Hoxb6 induces ectopic ribs, Hoxa10 blocks rib formation, Hoxa11 produces fusions between adjacent ribs and vertebrae, and Hoxc13 truncates the axial skeleton (Carapuço et al., 2005; Vinagre et al., 2010; Young et al., 2009). Interestingly, these phenotypes are mostly consistent with functions assigned to them through other experimental approaches (Wellik and Capecechi, 2003; McIntyre et al., 2007; Mallo et al., 2010; Young et al., 2009). This indicates that group-specific functions must derive from sequence or structural characteristics intrinsic to the Hox proteins.

The specificity of Hox gene activity has been a subject of intense research but it is still far from being understood. The activity of Hox proteins depends to a large extent on their ability to control gene expression as DNA-binding proteins (Pearson et al., 2005). Hox proteins bind DNA through the homeodomain (HD), which is the motif that defines these proteins as a family (Pearson et al., 2005). Domain-swapping experiments have shown that, in some cases, the HD plays a central role in dictating the functional specificity of the protein (Chan and Mann, 1993). How the HD could provide this specificity has been difficult to explain on the basis of pure DNA-binding properties, as different Hox proteins bind very similar target sequences (Ekker et al., 1994; Noyes et al., 2008). In flies, the presence of co-factors can account for at least part of this specificity (Mann et al., 2009). The most prominent of those co-factors are Exd and Hth. Exd is able to bind both a hexapeptide located N-terminal to the homeodomain in many Hox proteins (Chan and Mann, 1996) and the Ubda motif, C-terminal to the homeodomain of Ubx and AbdA (Merabet et al., 2007). Exd (alone or together with Hth) has been shown to refine the DNA-binding properties of Hox proteins (Joshi et al., 2007; Slattery et al., 2011), thus helping to explain functional specificities for some Drosophila Hox proteins. Although the vertebrate homologs of Exd and Htd (Pbx, Meis and Prep proteins) are also able to bind...
vertebrate Hox proteins (Phelan et al., 1995), it is not clear from genetic experiments to what extent they contribute to Hox gene function in general or specificity in particular (Capellini et al., 2006; Capellini et al., 2008; Moens and Selleri, 2006).

A number of reports indicate that not only does functional specificity reside outside the HD for some Hox proteins, but different functions for a given Hox protein might also depend on different areas of the protein. One example of this is provided by the Drosophila and Artemia Ubx proteins; although they are both capable of inducing abdominal characteristics in thoracic segments, only the Drosophila protein was able to inhibit limb formation (Ronshaugen et al., 2002). This latter function was mapped to sequences C-terminal to the homeodomain and does not depend on differential DNA-binding properties between the two Ubx molecules (Galant and Carroll, 2002; Ronshaugen et al., 2002). In vertebrates, when the Hoxa11 HD was replaced with that of Hoxa13, Hoxa10 or Hoxa4, the resulting chimeric alleles were still able to fulfill Hoxa11 patterning activity in the skeleton, but had different effects in the Hoxa11-dependent development of other structures (Zhao and Potter, 2001; Zhao and Potter, 2002). For instance, whereas Hoxa11 containing either the Hoxa13 or Hoxa10 HD was able to fulfill Hoxa11 functions in the male reproductive tract, Hoxa11 with the Hoxa13, Hoxa10 or Hoxa4 homeodomains failed to promote normal development of the limbs and female reproductive tract (Zhao and Potter, 2001; Zhao and Potter, 2002). Similarly, the differential regulation of Six2 by Hoxa2 and Hox11 proteins has been shown to depend not on the identity of their HDs but on N- and C-terminal regions flanking this domain (Yallowitz et al., 2009)

Genes of the paralog group 10 are unique among vertebrate Hox genes in their ability to block rib formation, a characteristic that plays a central role in the evolution of the vertebrate axial skeleton (Wellik and Capecchi, 2003; Mallo et al., 2010). Genetic experiments showed that this property is shared by all group 10 members (Wellik and Capecchi, 2003), thus providing the grounds for comparisons to identify the molecular determinants for this activity. In addition, transgenic Hoxa10 expression in the presomitic mesoderm produces totally rib-less embryos (Carapuço et al., 2009), which provides a convenient functional test for the gene’s activity. In this work, we show that a conserved sequence located adjacent to the N-terminal end of the homeodomain of Hox10 proteins is required for their rib-repressing activity in mice. This motif includes two potential phosphorylation sites, which are essential for Hoxa10 activity, as their mutation into alanines resulted in a total loss of rib-repressing properties. The activity of this motif seems to require interactions with more N-terminal parts of the Hox protein. In addition, it might also modulate protein activity by alteration of the protein’s DNA-binding affinity through changes in the phosphorylation state of two conserved tyrosine residues within the HD.

MATERIALS AND METHODS

Sequence comparisons

Sequence alignments were performed with the ClustalW2 program using Hox10 protein sequences from different species obtained from public databases.

Production of mutant constructs and transgenic mice

All proteins used in this study contained a FLAG-tag at their N-terminal end. The tags were first introduced into the Hoxa10 and Hoxb9 proteins by cloning oligonucleotides encoding for a methionine followed by the FLAG sequence into the S’ end of their corresponding cDNAs, so that the FLAG-tag was immediately downstream of the start codon and linked to the second amino acid of the tagged protein. The cDNAs corresponding to the different mutant and chimeric proteins were then produced on these basic cDNAs using an overlapping PCR mutagenesis strategy. To construct Hoxa10amt, the Hoxa10 cDNA was cut with SalI and HindIII, blunted by filling in with the Klenow polymerase and re-ligated. The sequence of all constructs was verified by direct sequencing. To express the cDNAs in cultured cells, they were cloned into the pSport1.1 expression plasmid. To express the cDNAs in transgenic embryos, they were cloned downstream of the msd enhancer of the Dll1 gene (Beckers et al., 2000) and upstream of the SV40 polyadenylation signal. The constructs used in this work are summarized schematically in Fig. 1. All cloning procedures were performed according to standard cloning techniques (Sambrook et al., 1989).

To produce transgenic mice, the relevant constructs were liberated from bacterial sequences and gel purified with the QiAquick gel extraction kit (Qiagen). Transgenic animals were then produced by pronuclear microinjection and oviduct transfer according to standard procedures (Hogan et al., 1994). Transgenic fetuses were recovered by cesarean section at embryonic day (E) 18.5 and the skeletal phenotypes analyzed by Alcan Blue/Alizarin Red staining as previously described (Mallo and Brändlin, 1997).

The significance of the absence of rib-repressing activity of the different constructs considering the total number of transgenics analyzed was estimated using a one-tailed two-proportion z-test (http://in-silico.net/statistics/ztest/two-proportion), with significance at $P<0.05$.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using nuclear extracts of HEK293T cells transfected with expression constructs encoding the relevant wild-type or mutant Hox proteins, using Lipofectamine 2000 (Invitrogen), according to manufacturer’s specifications. Nuclear extracts were prepared as described in Wadman et al. (Wadman et al., 1997). The amount of Hox proteins obtained in these extracts was estimated by western blot using the anti-
FLAG M2 antibody (Sigma). The EMSA probe consisted of the sequence AAAGGCATGACTAATTGCATGGTAACTGGAGAAATGCTTTCTCTCTCTCTGGGGTGAAGCCTGCATG, corresponding to the H1 enhancer of Myf5 (Buchberger et al., 2007), purified by PCR, end labeled with 32P using polynucleotide kinase and purified with the QIAquick PCR Purification Kit (Qiagen). EMSA reactions (20 μl) contained 10 mM Hepes (pH 7.9), 75 mM KCl, 2.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 3% Ficoll, 2 mg/ml BSA, 0.2 mg/ml polyd/dc, 2 μl of protein (corresponding to 1:50 of the nuclear extract obtained from a confluent 35 mm plate) and 2 μl of probe. Binding reactions were incubated at room temperature for 30 minutes and separated on a 5% polyacrylamide gel in 0.5X TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA).

RESULTS

Hox10 proteins contain group-specific protein motifs

The strong redundancy observed among Hox10 genes in their ability to block lumbar rib formation (Wellik and Capecchi, 2003) suggests that the rib-repressing properties of the corresponding proteins must reside in parts shared by the three group members. Alignment of the mouse Hoxa10, Hoxc10 and Hoxd10 protein sequences revealed the presence of a few conserved areas outside the HD (Fig. 2A). These included two small sequences, NWLTAKS and RENRIRELT, located immediately adjacent to the N- and C-terminal ends of the HD, respectively (for simplicity, we will refer to these sequences as M1 and M2 motifs, respectively). Extension of these analyses to Hox10 proteins of other vertebrate species revealed a strong phylogenetic conservation of these motifs (Fig. 2B). We did not find the M2 motif or a similar sequence in any other vertebrate or invertebrate Hox protein. However, we found a sequence with similarity to M1 in Hox paralog group 9 proteins (NWLHARS), which has been considered to be a modified hexapeptide/linker region (LaRonde-LeBlanc and Wolberger, 2003). Despite their resemblance, the motifs in Hox9 and Hox10 proteins differ in two out of the seven residues and we could not find a Hox10-type M1 motif in any Hox9 protein or vice versa, indicating that they are paralog group specific. One of the differences between the motifs observed in Hox9 and Hox10 proteins is the presence of a histidine or a threonine in position –4 (relative to the homeodomain), respectively. The crystal structure of HOXA9 suggests an essential role for His –4 in the three dimensional structure of this motif (LaRonde-LeBlanc and Wolberger, 2003). Therefore, the presence of a Thr residue in this position might have significant influence on the structural and/or functional properties of the Hox10 proteins. Taken together, these analyses indicate that the M1 and M2 motifs are hallmarks of Hox10 proteins and suggest that they might be involved in activities common to all of them, among which rib repression is the best documented (Wellik and Capecchi, 2003).

The M1 motif is necessary but not sufficient for Hoxa10 rib-repressing activity

To test whether the M1 and M2 motifs are required for Hoxa10 rib-repressing activity, we first generated deletion mutants and tested their activity by expressing them in transgenic mice. Hoxa10 proteins lacking the M2 motif (Hoxa10ΔM2) retained the ability to block rib formation (Fig. 3C and Table 1). This indicates that, despite its strong conservation among Hox10 proteins, M2 seems not to be involved in this redundant Hox10 function. Analysis of M1 deletion mutants (Hoxa10ΔM1) gave a different result, as we could not find anyDll1-Hoxa10ΔM1 transgenic embryo with the typical rib-less phenotype (Table 1). In two out of the 16 transgenics analyzed we observed significant rib defects, but these were much weaker than those typically observed in Dll1-Hoxa10 embryos (Fig. 3D and Table 1). These results indicate that Hoxa10 lacking M1 is almost unable to repress rib formation, thus demonstrating the requirement of the M1 motif for this function.

For some Drosophila Hox proteins, the HD together with the conserved sequences adjacent to its N-terminal end have been shown to be sufficient to produce a functional protein (Papadopoulos et al., 2010). To test whether this was also the case...
It seems that it is able to link the protein to the networks controlling rib development. It further suggests that the outcome of such interaction might depend on other areas of the Hox protein. Interestingly, the activity of Hoxb9M1 was different to that of a similar molecule consisting of the N-terminal region of Hoxb9 linked to M1, HD and M2 of Hoxa10 (Hoxb9NT/a10CT). In particular, Hoxb9NT/a10CT also lacked rib-repressing properties, but we could not identify any sign of cervical rib formation in any Dll1-Hoxb9NT/a10CT fetus (Table 1). Altogether, our results suggest that the rib-repressing activity of Hoxa10 might require interaction between M1 and the N-terminal part of the Hoxa10 protein. In addition, although the HD and/or M2 of Hoxa10, together with M1, are not enough to promote inhibition of rib formation, they might still participate in the interactions between M1 and other parts of the protein that control rib development.

**M1 activity requires its conserved phosphorylatable residues**

One of the fingerprints of the M1 motif of Hox10 proteins is the presence of a Thr at –4 position. Threonine is a target for phosphorylation, a modification that often modulates protein function, including that of Hox proteins (Berry and Gehring, 2000; Jaffe et al., 1997; Taghli-Lamallem et al., 2008). To understand whether such a modification could play a role in Hoxa10 rib-repressing activity, we replaced the Thr –4 for an alanine (Hoxa10T>A) and tested its activity in transgenic mice. Hoxa10T>A retained at least a large part of its rib-repressing activity, as revealed by the presence of transgenic fetuses with strong rib phenotypes (Fig. 4D,E and Table 1). One of these transgenics was totally rib-less (Fig. 4D), resembling the phenotype typically observed in Dll-Hoxa10 transgenics. In addition, we had two additional Dll1-Hoxa10T>A specimens with strong rib deficiencies, although they were clearly far from complete (Fig. 4E and Table 1). The presence of these intermediate rib phenotypes, which, as discussed above, we never observed expression of the wild-type Hoxa10, indicates that although Hoxa10T>A is competent to promote full rib repression, its activity might not be as strong as that of the wild-type Hoxa10. However, despite this, our results indicate that phosphorylation of Thr –4 is not essential to confer full repressing activity to Hoxa10. The M1 motif also contains a serine at –1 position, raising the possibility that phosphorylation at this residue could compensate for an eventual need of this modification at Thr –4. To test this, we changed Ser –1 to Ala both alone (Hoxa10S>A) or combined with a similar change in Thr –4 (Hoxa10ST>AA) and evaluated the activity of these proteins in transgenic mice. Hoxa10S>A seemed to retain full rib-repressing activity, as the rib phenotypes found in Dll1-Hoxa10S>A transgenic fetuses were either a complete absence of ribs or very minor defects in the ribs at the borders of the rib cage (Fig. 4C and Table 1), thus resembling typical phenotypes of Dll1-Hoxa10 transgenics. However, we were unable to detect skeletal abnormalities in any of the Dll1-Hoxa10ST>AA fetuses that we generated (Fig. 4F and Table 1). Together, these results indicate that the presence of at least one hydroxyl group in M1 is required to confer rib-repressing activity to Hoxa10, which is consistent with the involvement of phosphorylation within M1 in the regulation of Hoxa10 rib-repressing activity.

**DNA-binding properties of the Hoxa10 mutant versions**

The most common mechanism of Hox activity requires HD-mediated binding to DNA (Pearson et al., 2005). As the M1 motif is located adjacent to this DNA-binding domain, it is possible that...
it could affect the binding properties of the protein, similar to what has been described for other regions outside the HD (Liu et al., 2008). Therefore, we tested whether the loss of rib-repressing activity of the Hoxa10ST>AA and the Hoxa10ST>AA mutant proteins could be related to changes in their DNA-binding properties. For this, we used the Hox target site in the H1 enhancer of Myp5 (Buchberger et al., 2007), which is involved in the Hox10-mediated rib-repression process (Vinagre et al., 2010). Hoxa10ST>AA bound its target within H1 with an affinity comparable to that of the wild-type Hoxa10 protein (Fig. 5). The restoration of DNA-binding affinities of Hoxa10ST>AA by mutating the two conserved tyrosine residues to phenylalanines is consistent with the likelihood that modifications in the conserved tyrosine residues within the HD could represent the origin of the reduced binding properties of Hoxa10ST>AA.

Increased DNA-binding capacity of Hoxa10ST>AA/YY>FF relative to Hoxa10ST>AA was associated with a slight increase in rib-repressing activity because, contrary to Dll1-Hoxa10ST>AA transgenics, some Dll1-Hoxa10ST>AA/YY>FF specimens presented mild rib phenotypes (Fig. 6 and Table 1). However, the activity of Hoxa10ST>AA/YY>FF was clearly far from complete as none of the rib-less phenotype was not detected, the P value for a significant absence of a rib-less phenotype on the basis of the number of transgenics analyzed is indicated (one-tailed two-proportion z-test, significant at P<0.05).

### Table 1. Summary of the transgenics analyzed and the phenotypes observed for the different constructs with a brief description of the intermediate rib phenotypes observed for some of the constructs

<table>
<thead>
<tr>
<th>Construct*</th>
<th>Total analyzed</th>
<th>Rib-less</th>
<th>Intermediate phenotypes</th>
<th>Minimal‡</th>
<th>Full activity§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoxa10</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>yes</td>
</tr>
<tr>
<td>Hoxa10M1</td>
<td>16</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>(P=0.010)</td>
</tr>
<tr>
<td>Hoxa10M2</td>
<td>11</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>yes</td>
</tr>
<tr>
<td>Hoxa10N1</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>(P=0.036)</td>
</tr>
<tr>
<td>Hoxa10S1A</td>
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<td>4</td>
<td>0</td>
<td>3</td>
<td>yes</td>
</tr>
<tr>
<td>Hoxa10S1A</td>
<td>11</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>yes</td>
</tr>
<tr>
<td>Hoxa10ST&gt;AA</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(P=0.036)</td>
</tr>
<tr>
<td>Hoxa10ST&gt;AA/YY&gt;FF</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>(P=0.030)</td>
</tr>
<tr>
<td>Hoxb9MT</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(P=0.012)</td>
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<tr>
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<td>11</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>(P=0.036)</td>
</tr>
<tr>
<td>Hoxa10ST&gt;AA/YY&gt;FF</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>(P=0.044)</td>
</tr>
</tbody>
</table>

*The indicated cDNAs were expressed under the Dll1-msd enhancer.

‡Minimal rib phenotypes defined as unilateral or bilateral absence of no more than two ribs.

§Full activity defined as presence of rib-less phenotypes. When rib-less phenotype was not detected, the P value for a significant absence of a rib-less phenotype on the number of transgenics analyzed is indicated (one-tailed two-proportion z-test, significant at P<0.05).

*These embryos had small ectopic ribs in the cervical area.

Table 1. Summary of the transgenics analyzed and the phenotypes observed for the different constructs with a brief description of the intermediate rib phenotypes observed for some of the constructs. The indicated cDNAs were expressed under the Dll1-msd enhancer. Minimal rib phenotypes defined as unilateral or bilateral absence of no more than two ribs. Full activity defined as presence of rib-less phenotypes. When rib-less phenotype was not detected, the P value for a significant absence of a rib-less phenotype on the basis of the number of transgenics analyzed is indicated (one-tailed two-proportion z-test, significant at P<0.05).
development of the paralog group 10 are able to block rib formation and this function is essential for patterning of the lumbar area (Wellik and Capecchi, 2003; Carapuço et al., 2005). Genetic studies revealed that the three members of this paralog group are redundant in this function (Wellik and Capecchi, 2003), suggesting that group 10 proteins should share some sequence or structural property relevant for setting up the program leading to the block of rib formation. Sequence comparison revealed that, besides the HD, Hox10 proteins share just a few conserved regions. Among them, we identified two small motifs flanking the HD. These motifs show strong phylogenetic conservation and seem to be group 10 specific, suggesting that they might play a role in Hox10 functions. Our analysis of the relevance of these motifs to rib development revealed that one of them, here named M1, is involved in this process. The motif located at the C-terminal end of Hox10 proteins (M2) seems to be dispensable for the rib-repressing activity of Hoxa10, although a fine regulatory role in this process cannot be completely ruled out. Gene inactivation studies revealed that Hox10 genes are also redundant in limb patterning, most particularly the proximal segment (stylopod) of the hindlimb (Wellik and Capecchi, 2003). It remains to be determined whether M2 is involved in this process.

Our studies indicate that M1 is required for the rib-repressing activity of Hox10 proteins because removal of this motif rendered Hoxa10 virtually inactive in our transgenic assay. However, our data also indicates that M1 is not sufficient to confer rib-repressing properties to Hox proteins, but requires the presence of other parts of the Hox10 protein. The lack of abnormal rib phenotypes in Dll1–Hoxb9NT/a10CT and Dll1–Hoxa10ST>AA transgenic fetuses indicates both that the presence of the Hoxa10 HD is insufficient to bring M1 active in rib-repression, and that the N-terminal part of Hoxb9 cannot replace the equivalent area of Hoxa10, suggesting that the existence of Hox group-10-specific element(s) is required to activate the rib-repressing program upon interaction with M1. Sequence comparisons of Hox10 proteins revealed the presence of a few small conserved regions scattered through the first half of their N-terminal region, which are obvious candidates to be involved in the rib-repressing function of Hox10 proteins. Experiments are currently under way in our laboratory to test whether or not they are relevant to this common Hox10 function in the axial skeleton.

It is not clear what drives the functional connection between M1 and other areas of Hoxa10 to promote rib repression. As discussed above, our data suggests that this should include direct or indirect interactions between M1 and the N-terminal region of the protein. One possibility is that M1 modulates an activity in the N-terminal region that is eventually responsible for triggering the program that blocks rib development. Specific modulation of N-terminal activity by sequences adjacent to the homeodomain has been described for...
other Hox proteins (Merabet et al., 2003; Joshi et al., 2010). This modulation could be mediated through specific M1-dependent post-translational modifications in the N-terminal region that would eventually trigger the rib-repressing program. Our comparison of the in vivo and in vitro behavior of Hoxa10ST- AA and Hoxa10ST- AAA- FF indicates that M1 could indeed be involved in the phosphorylation of tyrosine residues in the HD. However, understanding whether M1 activates rib-repressing properties in the N-terminal region of Hoxa10 through specific post-translational modifications will require a direct analysis of proteins with active and inactive M1 motifs. Another possibility is that M1 is necessary to recruit co-factors that interact with the N-terminal part of the protein to repress rib formation. The first obvious candidates for such factors are Pbx proteins, because of their ability to bind most Hox proteins (Moens and Selleri, 2006). Indeed, the M1 equivalent in HOXA9 has been shown to interact with Pbx proteins (LaRonde-LeBlanc and Wolberger, 2003), suggesting that the M1 motif of Hox10 proteins could also interact with Pbx. However, even if such interaction occurs, it is not very probable that Pbx proteins mediate interactions between M1 and N-terminal region in the rib-repressing process. Genetic data supports this conclusion as inactivating mutations in Pbx genes are associated with deficient rib development (Capellini et al., 2008), which is the opposite to what would be expected for a Hox10 co-factor. Our analysis of Hoxa10ST- AA also argues against an involvement of Pbx proteins in Hox10 rib-repressing activity. In particular, the mutant M1 motif in Hoxa10ST- AA contains the cryptophan residue thought to be critical for Pbx-Hox interactions (LaRonde-LeBlanc and Wolberger, 2003) and it is thus expected that the mutant M1 is still able to bind Pbx proteins. However, Hoxa10ST- AA (or Hoxa10ST- AAA- FF) lacks rib-repressing properties, thus suggesting that this activity is independent of Pbx. In addition, experiments performed in our laboratory, aimed at identifying Hoxa10-Pbx1 interactions in the context of the H1 enhancer, did not provide any evidence for such interactions (supplementary material Fig. S1). In conclusion, although we cannot formally rule out an involvement of Pbx proteins in the Hoxa10-mediated rib-repressing process, all evidence seems to go against Pbx proteins as Hox10 co-factors in this process. Considering that M1 can be somehow regarded as a functional equivalent of the hexapeptide present in Hox1-8 proteins, other factors that could mediate M1 activity are proteins related to Bip2, as this protein, a member of the TFIID complex involved in transcription initiation, has been shown to bind the hexapeptide of Antp (Prince et al., 2008). Whether Bip2 or related proteins are indeed involved in the functional interactions of M1 remains to be determined. Clearly, identification of bona fide M1-binding partners will require a systematic experimental approach.

Our data is compatible with phosphorylation within M1 being essential to promote and/or regulate its function in the rib-repression process. M1 contains conserved Thr and Ser residues, which are essential for its function as their mutation to Ala completely removes rib-repressing activity from the Hoxa10 protein. According to our data, the presence of either the Thr or Ser is enough to keep Hoxa10 activity in rib development, as individual Hoxa10ST- AA and Hoxa10ST- A mutants conserved rib-repressing properties. Although both residues seem to be sufficient to promote Hoxa10 rib-repressing activity, the small phenotypic differences between Dll1-Hoxa10ST- AA and Dll1-Hoxa10ST- AAA transgenics suggest that, physiologically, the relative weight of the Thr (actually the most Hox10 specific) in this process is slightly higher than that of the Ser.

Comparison of the phenotypes of Dll1-Hoxa10ST- AA, Dll1- Hoxa10ST- AA and Dll1-Hoxa10ST- AAA- FF transgenic fetuses, suggests that modification of the Thr or Ser is required for productive interactions between M1 and the N-terminal region, leading to activation of the rib-repressing program. Interestingly, our data indicates that M1 might also be implicated in an additional type of regulatory process involving interactions between M1 and the HD, ultimately controlling the phosphorylation state of two conserved tyrosine residues within the HD (Y332 and Y349), which influences the DNA-binding properties of the molecule. Our data is compatible with phosphorylation of Y332 and Y349 being the origin of the small differences in activity that we detected for Hoxa10ST- AA compared with that of Hoxa10ST- AA and Hoxa10ST- AAA- FF, suggesting that this mechanism might also be part of the physiological control of Hox10 activity during rib development. Such a mechanism resembles that described to modulate Hox10 activity during γ-interferon-induced differentiation of myeloid cells (Eklund et al., 2000; Eklund et al., 2002). In myeloid cells, it has been shown that this regulation requires the activity of Shp1 (Ptpn6 – Mouse Genome Informatics) (Eklund et al., 2002). Involvement of this factor in the control of rib development is not probable, however, as SHP1 has been described to be hematopoietic-specific (Plutzky et al., 1992) and Shp1 null mutant mice appear to have no rib problems (Kozlowski et al., 1993). It remains to be determined whether the factors that mediate M1 interactions with the N-terminal region and HD are the same or different. It is clear that identification of molecules interacting with M1 and analysis of how modifications in M1 affect those interactions and/or their functional output will be essential to understand how Hox10 proteins control rib development.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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