**Controlling Hox gene expression and activity to build the vertebrate axial skeleton**

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**ABSTRACT**

It has long been known that Hox genes are central players in patterning the vertebrate axial skeleton. Extensive genetic studies in the mouse have revealed that the combinatorial activity of Hox genes along the AP body axis specifies different vertebral identities. In addition, Hox genes were instrumental for the evolutionary diversification of the vertebrate body plan. In this review, we focus on fundamental questions regarding the intricate mechanisms controlling Hox gene activity. In particular, we discuss the functional relevance of the precise timing of Hox gene activation in the embryo. Moreover, we provide insight into the epigenetic regulatory mechanisms that are likely to control this process and are responsible for the maintenance of spatially restricted Hox expression domains throughout embryonic development. We also analyze how specific features of each Hox protein may contribute to the functional diversity of Hox family. Altogether, the work reviewed here further supports the notion that the Hox programme is far more complex than initially assumed. Exciting new findings will surely emerge in the years ahead.

## INTRODUCTION

The observation of intriguing phenotypes in the fruit fly in which a specific part of the body is transformed into another was the first step towards the identification of Hox genes (Bateson, 1894). These genes were then mapped through extensive genetic analyses, which further confirmed their essential role in conferring segmental identities along the anterior-posterior (AP) axis of the *Drosophila* embryo (Lewis, 1978). It was later discovered that they encode transcription factors that share a DNA-binding domain of 60 aminoacids, known as the homeodomain (HD) (McGinnis et al., 1984). Mammals have 39 Hox genes distributed in four linear clusters, thought to result from a double duplication event (Krumlauf, 1994). These genes can be classified in 13 paralog groups (PGs) according to sequence homologies and their position within the cluster (Krumlauf, 1994). The number of PG members in each cluster varies from 9 to 11, and each PG is represented in at least two clusters.

In vertebrates, one of the developmental processes under Hox gene control is the formation of the axial skeleton. The axial skeleton derives from the somites, mesodermal segmental structures symmetrically organized on both sides of the developing spinal cord (reviewed in Dubrulle and Pourquié, 2004). All somites look morphologically similar but produce skeletal elements (vertebrae) with individual morphological characteristics that vary depending on their position along the AP embryonic axis (reviewed in Brent and Tabin, 2002). From a gross anatomical perspective, the vertebral column can be divided in 5 different regions: cervical, thoracic, lumbar, sacral and caudal. The total vertebral number in the animal's spine and their distribution among these regions define the axial formula, which vary widely among vertebrate species. Hox genes are major regulators of the patterns of somitic differentiation that produce the appropriate axial formula (Krumlauf, 1994). In particular, it is thought that specific combinations of Hox genes along the AP axis are responsible for the generation of vertebrae with distinct anatomical properties (reviewed in Wellik, 2007).

A large number of studies, mainly using the mouse as a model, have highlighted the complexity of the Hox patterning activities leading to the production of a properly organized axial skeleton (reviewed in Mallo et al., 2010). Regional patterning functions of Hox genes result from a combination of both unique functional features associated to each Hox protein and the appropriate control of their spatial and temporal expression.

The enormous variations in the axial formula among vertebrates might thus derive to a large extent from a diversification of the mechanisms regulating Hox gene expression and activity during embryonic development. In this review we will first discuss some of the mechanisms controlling Hox gene expression and their relevance to build a properly organized axial skeleton. In addition, we will analyze the features that confer different Hox proteins the ability to regulate specific patterning processes during development of the paraxial mesoderm.

## DEVELOPMENTAL REGULATION OF HOX GENE ACTIVATION

In vertebrates, Hox gene expression is initiated in cells of the posterior primitive streak that contribute to extraembryonic mesoderm and then expands anteriorly into prospective cells of the embryo proper (initiation phase; reviewed in Deschamps et al., 1999). Sequential activation of Hox genes directly reflects their position within the cluster, with expression of more posterior (5') genes being progressively initiated at later developmental time points (a property commonly known as temporal collinearity; Izpisúa-Belmonte et al., 1991). The initial Hox expression domains are transmitted to the nascent paraxial mesoderm during gastrulation, creating the first Hox expression patterns in this mesodermal tissue (establishment phase; reviewed in Deschamps et al., 1999). During this time, gradients of retinoic acid (RA), Fgf and Wnt signalling have been shown to modulate Hox gene expression, possibly through the concerted action of the transcriptional regulators Cdx (reviewed in Mallo et al, 2010). Once the anterior limits of Hox expression are established, the Polycomb group (PcG) and the Trithorax group (trxG) proteins have an essential role in maintaining them throughout embryonic development (maintenance phase; reviewed in Deschamps et al., 1999).

Hox gene activation occurs concurrent with embryonic growth in the vertebrate embryo and, consequently, the progressive temporal activation of these genes acquires a spatial component, with 3' Hox genes being expressed at more anterior embryonic levels than those at the 5' end of the cluster. In agreement with this, Hox gene expression in the frog embryo is initiated in a temporally collinear sequence in the non-organiser mesoderm at the gastrula stage and this coincides with the establishment of AP identities in this tissue (Wacker et al., 1994). These mesodermal cells are no longer competent to express additional Hox genes once they involute at the blastopore lip during gastrulation and leave the "Hox induction field", and therefore their AP identity is fixed at this early embryonic stage. Further to this, the collinear activation of Hox

genes was shown to control the time of cell ingression during gastrulation in chicken embryos and thus the time at which these cells reach the embryonic axis (Iimura and Pourquie, 2006). Overall, these results strongly support the notion that the time of Hox gene activation in the mesoderm during gastrulation largely defines the AP Hox expression domains and controls vertebral identity.

**Timing of Hox gene activation and alterations in the axial skeleton**

A variety of studies showed that in different vertebrates specific Hox genes are expressed at AP levels corresponding to particular characteristics of the axial skeleton. For instance, early comparative analyses focusing on the cervical to thoracic transition revealed that this anatomical feature was typically associated with the activation of genes corresponding to the *HoxPG6* (Gaunt, 1994; Burke et al., 1995). This suggested a role for *HoxPG6* genes in setting the start of the rib-containing areas of the axial skeleton. Support for this hypothesis was provided by transgenic experiments in mice showing the ability of the *Hoxb6* gene to induce ectopic ribs (Vinagre et al., 2010). Therefore, the mechanisms regulating the precise timing of *HoxPG6* activation in the different vertebrate species will have a direct impact on the number of vertebral segments in their necks.

More recently, a similar correlation between Hox gene expression and vertebral patterns was made at the caudal end of the trunk. In particular, it was observed that activation of *HoxPG10* to *HoxPG13* genes is strongly delayed in snake and caecilian embryos, roughly corresponding to the trunk to tail transition and with the end of the elongating axis (Woltering et al., 2009; Di-Poï et al., 2010). These results indicated that the remarkably long dorsal (rib-containing) regions characteristic of these animals might have resulted from changes in the timing of activation of those posterior Hox genes.

The functional connection between the timing of Hox gene activation and axial patterning was further demonstrated experimentally in the mouse. Some of these experiments involved premature expression of several Hox genes using transgenic approaches (Kessel et al., 1990; Lufkin et al., 1992; Carapuço et al., 2005; Vinagre et al., 2010). These transgenic mice displayed different types of homeotic transformations in their axial skeleton that correlate with an anterior expansion of the activity of the genes expressed from the transgene. Although these experiments could be interpreted in terms of altered spatial expression, the tight connection between developmental time

and AP levels indicate that they could also be the consequence of earlier than normal activation of the specific Hox gene activity. Consistent with this hypothesis, premature activation of *Hoxd4* produced phenotypes reminiscent to those resulting from over-expression of this gene using an enhancer of a more anterior Hox gene (Lufkin et al., 1992; Bel-Vialar et al., 2000).

A different set of studies tested the functional consequences of modifying known regulatory regions within the Hox loci. Deletion of an essential *Hoxc8* enhancer in the mouse largely reproduced the phenotypic characteristics observed upon *Hoxc8* gene inactivation (Juan and Ruddle, 2003). Interestingly, removal of this enhancer delayed *Hoxc8* activation but seemed to have no effects on its expression at later developmental stages. Moreover, the chicken counterpart of the *Hoxc8* enhancer drives reporter gene expression to more posterior axial levels in the paraxial mesoderm of transgenic mouse embryos (Belting et al., 1998). This is consistent with a reduced number of thoracic vertebrae in the chicken compared to the mouse. Similar conclusions derive from *in vivo* studies in the *Hoxd* cluster. In particular, removal of a regulatory region within the posterior area of this cluster resulted in delayed activation of *Hoxd11* and produced a posterior transposition of the sacrum similar to that observed in *Hoxd11* mutant mice (Zákány et al., 1997). When in a complementary set of experiments this enhancer was replaced for the corresponding zebrafish regulatory element, *Hoxd11* activation was detected at earlier developmental times and resulted in the transposition of the sacrum in the opposite direction, reducing the lumbar region by one segment (Gérard et al., 1997). These experiments confirm that the precise temporal control of the initial Hox gene activation is essential for patterning processes in the paraxial mesoderm. In addition, they show that evolution of discrete regulatory regions might have played an essential role in setting species-specific activation times for Hox gene expression and could therefore have been instrumental in setting the different axial formulas characteristic of the various vertebrate species.

**Epigenetic regulation of the Hox clock in axial progenitors**

The association between temporal aspects of Hox gene activation and mesodermal patterning implies that understanding the mechanistic and molecular basis of temporal Hox gene activation is pivotal to further our knowledge on the morphogenesis and evolution of the vertebrate axial skeleton. A number of elegant genetic studies in the mouse, performed more than a decade ago, suggested the existence of repressive

environments within the Hox clusters. Introduction of *Hoxd9/lacZ* and *Hoxd11/lacZ* transgenes into an ectopic posterior genomic position in the *Hoxd* cluster (between *Hoxd13* and *Evx2*) resulted in delayed reporter gene expression that specifically correlated with the site of integration, although the restricted spatial distribution of the transgenes remained largely unaffected (van der Hoeven et al., 1996). Furthermore, successive insertions of the *Hoxd9/lacZ* transgene upstream of the *Hoxd* cluster revealed the genomic location of a regulatory element imparting this repressive environment (Kondo and Duboule, 1999). Deletion of this element results in premature Hox gene expression during the initial phase of activation and leads to homeotic transformations of the axial skeleton (Kondo and Duboule, 1999). Correct axial Hox gene expression patterns are nevertheless largely resumed later in development. Altogether, these studies were consistent with a higher order regulatory mechanism that establishes a repressive environment at the 5' end of the cluster and specifically controls the sequential initiation of Hox gene expression. A lower order regulatory mechanism, involving the transcriptional activation by gene-specific short-range regulatory elements, would later regulate Hox expression during the establishment phase, generating spatially restricted expression domains.

It was later proposed that a particular chromatin structure is responsible for this repressive environment in the cluster and that progressive opening of the chromatin would result in a sequential 3' to 5' release of Hox genes from repression and into transcriptional activation (Kmita and Duboule, 2003). Support for this hypothesis was provided by more recent reports showing that the timing of Hox gene activation strongly correlates with structural modifications of the chromatin of the cluster in a 3' to 5' direction (Chambeyron and Bickmore, 2004; Chambeyron et al., 2005; Morey et al., 2007; Soshnikova and Duboule, 2009; Noordermeer et al., 2011). In particular, sequential activation of *Hoxb* genes in embryonic stem (ES) cells upon addition of RA correlated with the progressive acquisition of active histone modifications (di-methylation of lysine 4 at histone H3 (H3K4me2) and acetylation of lysine 9 at histone H3 (H3K9ac)) at the promoters of *Hoxb1* and *Hoxb9* (Chambeyron and Bickmore, 2004). Moreover, a systematic analysis of the distribution of two histone modifications (tri-methylation of lysine 27 at histone H3 (H3K27me3) and tri-methylation of lysine 4 at histone H3 (H3K4me3)) during *Hoxd* cluster activation in the primary body axis of the mouse embryo revealed that such a mechanism also occurs in vivo (Soshnikova and Duboule, 2009; Noordermeer et al., 2011). Temporal collinear expression of *Hoxd*

genes was shown to correlate with a progressive collinear decrease in the distribution of the repressive mark H3K27me3 and an increase in the active mark H3K4me3 (Fig. 1). These dynamic changes in the chromatin precede the initiation of *Hoxd* expression and are not determined by a spreading mechanism as initially envisaged, as the distribution of the histone marks is largely correct upon disruption of cluster integrity (Soshnikova and Duboule, 2009). Interestingly, in addition to these epigenetic modifications, chromatin decondensation and nuclear repositioning of active chromatin domains have been consistently observed, concurrent with gene activation, both in ES cells and in the AP embryonic axis (Chambeyron et al., 2005; Morey et al., 2007; Noordermeer et al., 2011).

Although the strong correlation observed between specific histone modifications and Hox expression in early embryos suggests that they should have an important biological role, their functional relevance remains elusive. The histone modifications associated to the active and inactive chromatin states during Hox cluster activation suggest involvement of PcG and trxG proteins in this process. PcG and trxG genes were initially identified in the fruit fly (Lewis, 1978), where they were shown to be involved in the maintenance of appropriate Hox gene expression domains after their initial activation (Simon et al., 1992). PcG proteins seem to function in two large multimeric complexes known as Polycomb Repressive Complexes (PRC). The histone methyltransferase activity of PRC2 deposits H3K27me3 to histones in the target chromatin. This histone modification is then recognized by PRC1, whose activity promotes chromatin condensation and silencing of the associated loci (Cao et al., 2005; Eskeland et al., 2010), possibly through monoubiquitination of H2AK119 (Cao et al., 2005). trxG activity is also associated to a multimeric complex that introduces the active H3K4me3 modification to target nucleosomes, thus inducing local chromatin remodelling and gene activation (Milne et al., 2002). The basic principles of PcG and trxG activity seem to be similar in vertebrate systems, although the existence of a larger number of components adds another level of complexity to the analysis of their function (reviewed in Schuettengruber et al, 2007).

Genetic studies in mice revealed that PcG and trxG genes are essential for proper AP patterning of the axial skeleton (van der Lugt et al., 1994; Akasaka et al., 1996; van der Lugt et al., 1996; Coré et al., 1997; Yu et al., 1998; del Mar Lorente et al., 2000; Terranova et al., 2006). In particular, mice deficient for the PRC1 proteins Bmi1 (van der Lugt et al., 1994), M33 (Coré et al., 1997), Mel18 (Akasaka et al., 1996) and

Ring1A (del Mar Lorente et al., 2000) display posterior transformations of the axial skeleton, which correlate with anteriorization of Hox gene expression in the paraxial mesoderm. The skeletal phenotypes observed in all these mutant embryos are typically mild and only the expression of a specific subset of Hox genes seemed to be affected. This may reflect a functional redundancy between different components of the PRC1. In agreement with this, simultaneous inactivation of *Bmi1* and *Mel18* (Akasaka et al., 2001) and of *Bmi1* and *M33* (Bel et al., 1998) resulted in more severe skeletal phenotypes. Alternatively, the mild phenotypes may indicate that multimeric complexes are assembled in a time and tissue-specific manner, each being involved in a specific subset of regulatory processes.

In contrast, inactivation of the PRC2 members *Eed*, *Ezh2* and *Suz12* all had drastic effects in mouse embryonic development as mutant embryos died during gastrulation with severe AP patterning defects (Shumacher et al., 1996; O'Carroll et al., 2001; Pasini et al., 2004). Early embryonic lethality of PRC2 mutant embryos most probably reflects the broad biological role of these proteins in the regulation of gene expression and precludes the analysis of the axial skeleton at late developmental stages. However, *Eed* hypomorphic mutants display posterior homeotic transformations of the axial skeleton reminiscent of those found in PRC1 mutants (Shumacher et al., 1996), suggesting the involvement of both PRCs in Hox gene regulation. It is possible that the stronger phenotypes observed in PRC2 mutant embryos is the consequence of less redundant activities among components of this complex. Conditional deletion of members of the PRC2 complex will be required to further analyze their role in Hox gene regulation and axial patterning, in particular during the initial activation of Hox gene expression.

Expression analyses indicated that inactivation of PRC1 components produced anterior shifts in the somitic expression of specific Hox genes. However, the initial timing of Hox gene activation seemed largely not affected. Therefore, while the requirement for PRC1 to maintain Hox expression domains seems supported by these data, the contribution of this complex to the initial steps of Hox gene activation is not clear. However, studies carried out on mutant mice for the PRC1 protein M33 provided some evidence for a functional link between PcG activity and the initial activation of Hox gene expression (Bel-Vialar et al., 2000). In particular, M33 mutant embryos have facilitated access to RA responsive elements within the *Hoxd* cluster, because low RA doses produce premature Hox gene activation and homeotic transformations in the axial

skeleton of these mutants. This is consistent with a more labile repressed state of the Hox clusters in the absence of M33 and suggests that, in addition to their well-known function in epigenetic maintenance of positional identity (reviewed in Soshnikova 2011), PcG proteins may also play an essential role in the early repression of Hox genes. Clear evidence for this is still lacking and awaits further investigation.

Inactivation of *Mll*, an essential component of the trxG complex, revealed that trxG proteins are also involved in Hox gene regulation in the mouse (Yu et al., 1995; Yu et al. 1998). Expression analysis showed that Hox activation is properly initiated in *Mll*-deficient embryos. However, as development progresses, the expression domains of specific Hox genes become progressively lost in the paraxial mesoderm, resulting in bidirectional homeotic transformations of the axial skeleton (Yu et al., 1995; Yu et al. 1998). Therefore, similar to PcG, trxG genes clearly play a role in the maintenance phase of Hox gene expression, but their involvement in initial Hox gene activation processes is less clear. Further work will be required to identify the mechanism of this activation.

## HOX GENE REGULATION BEYOND TRANSCRIPTION

Although most of our knowledge regarding Hox gene regulation focuses on mechanisms influencing gene transcription, recent data suggest the existence of functionally relevant post-transcriptional regulatory processes influencing Hox protein production during embryonic development.

One of those mechanisms involves activity of miRNAs, a class of small non-coding RNAs that regulates gene expression by controlling the stability and/or translation efficiency of specific target mRNAs (reviewed in Filipowicz et al., 2008). Three highly conserved miRNAs families, miR-10, miR-196 and miR-615, are produced within the Hox clusters (Griffiths-Jones et al., 2008) and have been shown to target specific Hox transcripts (Mansfield et al., 2004; Yekta et al., 2004; Hornstein et al., 2005; Woltering and Durston, 2008). Interestingly, experimental modification of miR-10 and miR-196 expression levels produced phenotypes that are consistent with abnormal Hox gene expression (Woltering and Durston, 2008; McGlinn et al., 2009; He et al., 2011). For instance, when miR-196 was knocked-down in chicken embryos a cervical vertebra acquired thoracic characteristics (McGlinn et al., 2009). Moreover up- and down-regulation of this miRNA during zebrafish development produced transformations in the axial skeleton affecting the cervical and thoracic areas (He et al.,

2011). Therefore, miR-196 seems to be involved in patterning processes during development of the paraxial mesoderm. However, whether miR-196 regulation of skeletal development is mediated by modulation of Hox protein production needs to be directly demonstrated. Interestingly, Hox cluster-encoded miRNAs seem to target preferentially anterior Hox genes. On the basis of this observation, it has been proposed (Yekta et al., 2008) that miRNA activity might help to explain the functional dominance that posterior Hox genes often exert over those encoded from more 5' areas of the cluster (reviewed in Wellik et al., 2007).

In addition to miRNAs, it has been recently shown that the cellular levels of ribosomal proteins might also be instrumental in the control of Hox protein production. In particular, mice heterozygous for an inactivating mutation in the ribosomal protein L28 (RPL28) presented homeotic transformations in their axial skeletons resembling Hox mutant phenotypes (Kondrashov et al., 2011). Interestingly, reduced levels of Rpl38 had negative effects on the translational efficiency of a specific subset of Hox transcripts, without a general impact on the cell's translational competence. So far, it is not clear what makes the affected transcripts particularly sensitive to Rpl38 deficiencies. Also, it is not known whether this represents an exceptional mechanism of Hox gene regulation or if it is the first example of a more general mechanism to fine tune protein levels at the translational level.

In conclusion, it is possible that spatial and temporal regulation of Hox protein production could involve multiple layers of regulation to ensure appropriate distribution of Hox activity during embryonic development.

## THE INTRINSIC FUNCTIONAL PROPERTIES OF HOX PROTEINS

The functional specificity of vertebrate Hox genes cannot be solely explained on the basis of their differential patterns of expression. A number of reports showed that different Hox genes produce dissimilar morphological alterations when assayed under the same experimental conditions. For instance, transgenic expression of *Hoxb6*, *Hoxb9*, *Hoxa10* or *Hoxa11* using the same enhancer all lead to different alterations in the embryo's axial skeleton (Carapuço et al., 2005; Vinagre et al., 2010; Guerreiro et al., 2012). Remarkably, genetic studies revealed a high degree of functional conservation among members of the same PG (Horan et al., 1995; Manley and Capecchi, 1997; Wellik and Capecchi, 2003; McIntyre et al., 2007). This was clearly demonstrated by

the analyses of compound mutants for the *HoxPGs 9*, *10* and *11*. The full blown null phenotype was only observed after complete removal of all PG members, being the presence of a single allele of any of the paralog members enough to produce a significant rescue the mutant phenotype (Wellik and Capecchi, 2003; McIntyre et al., 2007). Although this functional specificity could depend on the temporal and spatial activation of each of the relevant genes, the coincident phenotypes resulting from inactivation and premature activation of the different paralog groups (Wellik and Capecchi, 2003; Carapuço et al., 2005) suggest the existence of functional signatures in the Hox proteins. Importantly, functional redundancy among paralog members might be different depending on the paralog group. *HoxPG8* genes could exemplify this, as the skeletal phenotypes of the compound paralog mutants cannot be explained by their functional redundancy (van den Akker et al., 2001) and distinct phenotypes were obtained upon transgenic activation of *Hoxb8* or *Hoxc8* in the same embryonic territory (Pollock et al., 1995).

The molecular signatures providing functional specificity to the vertebrate Hox proteins are still largely unknown but several reports have started to shed light on this issue. Hox genes encode transcription factors that selectively regulate transcription of downstream genes. Therefore, clarification of the mechanisms controlling differential selection of the relevant promoter/enhancer elements, as well as those regulating activation or repression of those elements, is essential to understand how Hox gene activity is translated into specific patterning and morphogenetic processes. As we will discuss next, extensive molecular analyses have allowed the identification of several domains and motifs within the Hox proteins playing relevant roles in their function.

## Lessons from Hox domains and motifs

### *The Homeodomain*

The HD is the region of the Hox proteins that had attracted most attention. Structurally, it is folded in three α-helices preceded by a flexible N-terminal arm. The third helix (recognition helix) and the N-terminal arm are involved in specific contacts with DNA (Kissinger et al., 1990; Billeter et al., 1993). Systematic analyses of the DNA binding properties of different HDs revealed that residues at the third helix provide DNA binding specificity and serve to sub-classify the HDs present in different protein families (Noyes et al., 2008). The HDs of Hox proteins largely fall into the same subgroup of HDs and, therefore, the DNA binding properties of most Hox proteins are

very similar, making it conceptually unclear how the HD could provide functional specificity. Nevertheless, early functional studies in *Drosophila* showed that the HD may indeed confer distinct *in vivo* functional properties to individual Hox proteins (Lin and McGinnis, 1992; Zeng et al., 1993).

Zhao and Potter (2001; 2002) investigated whether the HD was able to specify Hox functions in vertebrates. The Hoxa11 HD was replaced with that of Hoxa4, Hoxa10 or Hoxd13 using a knock-in strategy. Extensive analyses of mice carrying HD-swapped alleles showed a variety of phenotypic alterations, overall indicating that the HD is indeed relevant for Hox functional specificity in vertebrates. However, this specificity seemed to be restricted to certain tissues and, remarkably, no defects were observed in the axial skeleton of those HD-swapped mutants, suggesting that the axial patterning properties of Hox proteins might reside outside the HD. A similar conclusion was obtained from transgenic experiments using chimeric Hox proteins (Sreenath et al., 1996). Hoxa4 and Hoxc8 demonstrated distinct functional properties, as they suppress or induce rib growth in the cervical region of transgenic mice when both are expressed under the control of the *Hoxa4* promoter. When the activity of a chimeric Hoxa4 protein containing the Hoxc8 HD was analyzed, the corresponding transgenic mice exhibited a Hoxa4 phenotype, although with slightly weaker expressivity (Sreenath et al., 1996). Therefore, although the available data is still limited, it seems that Hox functional specificity in patterning of the axial skeleton resides mostly outside the HD.

*The Hexapeptide*

Genetic and molecular studies in *Drosophila* led to the identification of an additional conserved motif playing fundamental roles in some aspects of both Hox gene activity and functional specificity. This motif, termed the hexapeptide (HX), is localized N-terminally to the HD, from which it is separated by a sequence known as the linker region (Fig. 2A). Interestingly, the length of the linker region is a paralog-specific feature of Hox proteins, which led to the suggestion that it may also play a role in Hox specificity (reviewed in der Rieden et al., 2004). However, so far there is no experimental evidence showing that the linker region contributes to the functional specificity of vertebrate Hox proteins and therefore, we will not further discuss it in this review.

The HX contains a core sequence (YPWM) centered on a tryptophan residue that is critical for its function. In *Drosophila*, the activity of the HX has been mostly

linked to its interactions with Exd, which increase specificity and binding affinity of Hox proteins to their target DNA sequences (reviewed in Laurent et al., 2008; Mann et al., 2009).This motif is invariably present in vertebrate Hox proteins from PGs 1 to 8, suggesting that it might have important roles in vertebrate Hox protein function. Similarly to what has been described in *Drosophila*, the HX of vertebrate Hox proteins seems to provide an interaction surface for the vertebrate Exd homologs, the Pbx proteins (reviewed in Moens and Selleri, 2006). Biochemical studies on vertebrate Hox/Pbx interactions indicate that they follow the same rules as those characterized for their *Drosophila* counterparts, including the requirement for the conserved tryptophan in the Hox HX (Neuteboom et al., 1995). Therefore, dimerization with Pbx proteins could be an important determinant of vertebrate Hox protein functional specificity (Fig. 2B).

Analysis of *Pbx* mutant mice suggest that these proteins could indeed play a role in Hox gene activity. *Pbx1* mutant mice show alterations in the axial skeleton that could be interpreted as combinations of Hox mutant phenotypes (Selleri et al., 2001). Further analyses, including compound inactivation of members of the *Pbx* family, indicated the presence of significant functional redundancy among family members in different tissues. Interestingly, *Pbx1*/*Pbx2* double mutants contained strong defects in the axial skeleton, including reduced rib size, suggesting that Pbx proteins could be required for the rib-promoting properties of Hox proteins (Capellini et al., 2008), although this possibility has so far not been directly explored. It should be noted, however, that in vertebrate embryos the relationship between *Pbx* and Hox genes might not be restricted to direct protein interactions as in the limb buds *Pbx* genes seem to be required for activation of Hox gene expression (Capellini et al., 2006).

Although some of the malformations observed in *Pbx* mutants could be explained by alterations in Hox gene activity, the complexity of the *Pbx* mutant phenotypes does not allow to clearly differentiate between Hox-dependent and Hox-independent functions of *Pbx* genes. To solve these problems mutant mice were generated to produce specific Hox proteins containing a non-functional HX. Mice carrying mutations in the Hoxb8 HX showed homeotic transformations affecting the rib cage (Medina-Martinez and Ramírez-Solis, 2003). These defects resembled those observed in the upper thoracic rib cage of *Hoxb8* null mice (van den Akker et al., 1999) suggesting that Hoxb8 requires binding to Pbx cofactors for its normal function. However, these mice also presented phenotypic characteristics in their axial skeletons

resembling some characteristic features of loss-of-function mutations in *Hoxa7*, *Hoxb7* and *Hoxb9*, without any obvious effects on their expression (Medina-Martinez and Ramírez-Solis, 2003). This suggests that interaction with Pbx might be required to fine tune Hoxb8 binding properties, thus promoting regulation of its physiological targets and preventing interactions with the DNA target sites of other closely related Hox proteins. The validity of this hypothesis *in vivo* remains to be directly tested. A similar genetic approach revealed that normal Hoxa1 activity in the hindbrain and neural crest requires an intact HX (Remacle et al., 2004). Considering the phenotypes described for Hoxb8, it will be important to understand whether the HX-less Hoxa1 protein also acquires dominant negative functions on the activity of other Hox proteins.

Genetic and molecular studies in *Drosophila* revealed that the Exd/Hox complex is often bound by an additional partner, Hth, to form a functional trimeric complex on specific DNA targets (Ryoo et al., 1999; Ebner et al., 2005). Vertebrates have several Hth homologs, the Meis and Prep proteins, that are also able to form heterotrimeric complexes with Hox and Pbx proteins (Berthelsen et al., 1998; Jacobs et al., 1999; Schnabel et al., 2000), suggesting a potential role for the Meis/Prep cofactors in the modulation of vertebrate Hox protein function. Recently it was also shown that Meis proteins can promote the HX-independent interactions between a large number of fly and mouse Hox proteins and Exd/Pbx (Hudry et al., 2012). This study thus suggests that in the presence of Meis Hox/Pbx interactions might not always follow the classical mechanisms and further reinforce the idea that Meis cofactors may contribute to the specificity of functions. Whether this is indeed the case is still a matter of debate mostly because mutant phenotypes for *Meis1* or *Prep1* do not share many phenotypic traits with Hox mutant mice (Hisa et al., 2004; Ferretti et al., 2006). Despite this, it is still possible that the functions of Meis1/Prep1 are covered by redundant activities provided by other members of the family. Evaluation of this possibility must await the production and analysis of the relevant mutants.

*A divergent hexapeptide*

Hox proteins of PGs 9 to 13 do not contain a canonical HX motif. However, HoxPG9 and HoxPG10 proteins have a paralog group-conserved motif adjacent to the HD with some structural and functional similarities with the HX (Fig. 2A). In particular, these motifs contain a conserved tryptophan located six residues N-terminally to the HD, which, according to the HoxA9-Pbx1 crystal structure, is involved in Hox protein

interactions with Pbx1 (LaRonde-LeBlanc and Wolberger, 2003). Biochemical analyses have shown that mouse Hoxb9 and Hoxa10 proteins bind DNA cooperatively with Pbx1 following patterns resembling those described for anterior Hox proteins (Shen et al., 1997). Intriguingly, structural analyses indicate that HoxA9 DNA binding properties are not affected by the presence of Pbx1 as a cofactor (LaRonde-LeBlanc and Wolberger, 2003), thus questioning to which extent could Pbx1 be involved in functional fine tuning of HoxPG9 (and maybe HoxPG10) protein binding to their DNA targets, as suggested for HX-containing Hox proteins.

Functional analysis in transgenic embryos revealed that the divergent HX (NWLTAKS) of Hoxa10 is necessary, but not sufficient, for the rib-repressing activity of Hoxa10 (Guerreiro et al., 2012). However, it is not clear to which extent NWLTAKS activity requires interaction with Pbx proteins. In particular, a physiological DNA target of Hoxa10 involved in its rib-repression function (Vinagre et al., 2010) does not contain a Pbx1 binding motif and Hoxa10/Pbx1 failed to interact in vitro when assayed on this DNA target (Guerreiro et al., 2012).

Regardless of the possible involvement of Pbx1, it appears that the activity of NWLTAKS requires the presence of its conserved threonine and serine because when these were replaced by alanine residues (NWLAAKA), the resulting Hoxa10 protein lost rib-repressing activity in transgenic mice (Guerreiro et al., 2012). This result suggests a role for phosphorylation in this process. The control of Hox gene activity by phosphorylation has been previously reported in other biological contexts. In vertebrates, HoxA9 and HoxA10 activity in the hematopoietic system has been shown to be regulated by phosphorylation (Eklund et al., 2000; 2002; Vijapurkar et al. 2004). In both cases phosphorylation occurred in aminoacids within the HD and results in a drastic reduction of the protein's capacity to bind DNA. Interestingly, the Hoxa10 protein carrying the mutant NWLAAKA motif also has reduced DNA binding properties, which depend on phosphorylation of the same residues of the HD that control Hoxa10 activity in myeloid cells (Guerreiro et al., 2012). This suggests that the activity of NWLTAKS is in part mediated through interactions with the protein's HD that modulate the state of phosphorylation of specific residues within the HD. However, the role of NWLTAKS for the Hoxa10 rib repressing properties is not restricted to this interaction, because blocking phosphorylation in the HD results in very moderate rescue of the rib-suppressing properties of the NWLAAKA-containing Hoxa10 protein (Fig. 2C) (Guerreiro et al., 2012).

Phosphorylation has also been shown to influence Hox activity in *Drosophila* embryos (Jaffe et al., 1997; Berry and Gehring, 2000; Taghli-Lamallem et al., 2008), indicating that this modification might be a more general regulatory mechanism of Hox protein activity. In the fly, the best studied case is that of a casein kinase II phosphorylation site at the C-terminal end of Antp, which has been shown to be required for a subset of the protein's functions during *Drosophila* development (Jaffe et al., 1997). Interestingly, this motif is phylogenetically conserved in the ortolog vertebrate Hox proteins (HoxPG6) suggesting that it might also be functionally relevant for HoxPG6 protein function. This possibility has so far not been experimentally explored.

*Other relevant regions and motifs of Hox proteins*

In addition to the conserved domains and motifs, Hox proteins contain additional sequences of variable sizes both N-terminal and C-terminal to the HD. These are the less conserved regions of Hox proteins, making them likely candidates to contribute significantly to the protein's functional specificity. Several studies performed using *in vitro* systems described the existence of activation and repression motifs outside the HD able to regulate transcriptional activity on reporter systems (Rambaldi et al., 1994; Zappavigna et al., 1994; Schnabel and Abate-Shen, 1996; Zhao et al. 1996; Viganò et al., 1998). However, the functional relevance of these and other protein regions to the regulation of physiological processes has rarely been addressed. Nevertheless, it has been shown that the N- and C-terminal regions of Hox proteins are key determinants of the functional outcome of the protein's interaction with a given regulatory element in the embryo. For instance, in branchial arch and facial mesenchyme the C-terminal region of HoxPG2 proteins is required to repress *Six2* expression while in the nephrogenic mesenchyme HoxPG11 proteins activate *Six2* from the same enhancer, requiring the combined activity of its N- and C-terminal regions (Yallowitz et al., 2009). An equivalent dichotomy was observed for Hoxb6 and Hoxa10 activity on the regulation of rib development. The activity of both Hox proteins seems to include interactions with the same enhancer in the hypaxial regulatory region of the *Myf5* gene (Vinagre et al., 2010). However, the functional outcome of both interactions is the opposite, with Hoxb6 activating and Hoxa10 silencing the enhancer's activity. The finding that the N-terminal part of Hoxa10 is essential for its function in rib-repression (Guerreiro et al., 2012) indicates that this region of the protein must contain the

functional signatures driving the Hoxa10-specific effects upon the protein's interaction with the enhancer. Whether the same is true for Hoxb6 awaits direct experimental evaluation.

The manifestation of disorders caused by mutations in the N-terminal region of Hox proteins also argues for their importance in regulating Hox function. Specifically, limb and genital malformations result from the expansion of a poly-alanine motif localized in the N-terminal regions of HoxA13 and HoxD13 (Goodman et al., 1997; Utsch et al., 2002).

The identification of functionally relevant areas within the N- and C-terminal regions of Hox proteins is complicated by the typical lack of recognizable protein motifs and the virtual lack of structural information on these areas, most notably the N-terminal regions. In some cases, nature has helped to identify relevant domains when two species contain functionally different versions of the same Hox protein. A paradigmatic case was the identification of a leg-repressing activity in the C-terminal end of the *Drosophila* Ubx, which is lacking in the protein from *Artemia* (Galant and Carroll, 2002; Ronshaugen et al., 2002). However, these examples are rather rare. An alternative approach is to take advantage of the strong redundancy among PG members and search for group-specific signatures in the sequence. This approach has been taken for the HoxPG10 proteins and led to identify the NWLTAKS motif as a functional component of the Hoxa10 rib-repressing activity (Guerreiro et al., 2012). Other regions have also been reported to be shared by all HoxPG10 proteins, which are thus candidates to be involved in the common activities of this paralog group (Guerreiro et al., 2012), although direct evaluation of the functional weight of most of these regions is still missing.

**More pieces to the Hox puzzle: other potential Hox cofactors**

The wide diversity of functions performed by Hox proteins indicates that their spectrum of interactions is likely to be much wider than the classical Hox/Pbx/Meis complexes. It is possible that current genome wide efforts to identify natural targets for Hox proteins will provide a set of physiological Hox-regulated enhancers whose sequence can give clues about the identity of additional Hox cofactors.

As mentioned above Hoxa10 and Hoxb6 regulate rib development by controlling the activity of a distal enhancer of the *Myf5* gene (Vinagre et al., 2010). This enhancer, known as H1 (Buchberger et al., 2007), is also regulated by Pax3 and Six1/4 (Bajard et

al., 2006; Giordani et al., 2007). The Pax and Hox binding sites overlap within this enhancer, suggesting a possible functional interaction between Pax3 and Hox proteins. Recent biochemical analyses have shown that Hoxb6, but not Hoxa10, indeed binds cooperatively with Pax3 to the H1 enhancer *in vitro* (Guerreiro et al., 2013), indicating that Pax3 can be an important cofactor to confer functional specificity to HoxPG6 proteins (Guerreiro et al., 2013). Interestingly, interactions between Hox and Pax proteins have also been described for other members of these families in different biological contexts, both in mouse and *Drosophila* (Gong et al., 2007; Plaza et al., 2008), indicating that Pax proteins might be a more general Hox protein cofactor than currently recognized. Quite remarkably, Hoxb6 rib-promoting activity appears to be independent of its capacity to bind DNA (Guerreiro et al., 2013) and could thus be mediated through its interactions with Pax3 (Fig. 2D). A similar functional independence from DNA binding has been already described for other vertebrate Hox proteins (Zappavigna et al., 1994; Williams et al., 2005a; 2005b). For instance, studies in cultured cells showed that mutant HoxPG13 proteins unable to bind DNA keep the ability to modulate transcription through interactions with cofactors (Williams et al., 2005a; 2005b). Additional work is needed to further explore this unconventional Hox mode of action and to determine whether it may constitute an additional mechanism to confer functional specificity to Hox proteins.

Analysis of another functional Hox-regulated enhancer led to the identification of Deformed Epidermal Autoregulatory Factor-1 (Deaf-1) as a potential cofactor of the *Drosophila* Hox protein Dfd in the control of Dfd-responsive elements (Gross and McGinnis, 1996). Interestingly, the phenotypic abnormalities displayed by *Deaf-1* mutant mice include transformations of cervical segments and rib cage abnormalities, which could result from abnormal Hox gene activity (Hahm et al., 2004). It is thus possible that Deaf-1 acts as a Hox protein partner, although it is still unclear whether these proteins physically interact with each other. It is possible that the eventual Hox/Deaf-1 interaction requires the input of additional factors. In particular, Deaf-1 has been identified as a partner of Lmo4 (Sugihara et al., 1998). LIM-only (LMO) proteins are transcription factors that seem to be involved in the assembly of multiprotein complexes by conferring docking sites for other factors (reviewed in Rabbitts, 1998). The finding that the targeted deletion of *Lmo4* in mice results in skeletal defects in the rib cage and cervical vertebrae similar to those observed in *Deaf-1* mice suggests that Lmo4 and Deaf-1 could both be part of a multiprotein complex regulating Hox protein

activity during patterning of the axial skeleton. However, as attractive as this hypothesis can be, key aspects of this potential interaction, like identification of direct protein interactions or their presence within the same protein complex have never been reported.

## CONCLUDING REMARKS

Over the last decades, significant advances have been made in our understanding of how the activity of the different Hox genes generate precise species-specific patterns in the axial skeleton. However, a number of important questions remain unanswered. In particular, the mechanistic and molecular control of the precise timing of Hox gene activation at early embryonic stages is only starting to be understood. In this review, we have discussed experimental evidence that support the notion that this control is essential for the establishment of AP positional information and subsequent specification of different vertebral identities. In addition, several studies suggest that changes in the timing of Hox activation may be a key driving force in the evolutionary diversification of the vertebrate body plan, which further reinforces the functional relevance of such a control and the need to understand it.

It has been suggested that the temporal collinear activation of Hox genes could have been a major evolutionary constraint maintaining the clustered organization of Hox genes in the genome of organisms that develop through progressive addition of embryonic tissue to caudal regions (Monteiro and Ferrier, 2006). In fact, taking into account its synchronization with morphogenetic movements during gastrulation and the somitogenesis clock in these organisms, temporal control of Hox gene activation could prevent posterior Hox genes from being expressed too early in the embryo, in cells fated to produce more anterior vertebral structures, which would be crucial for correct embryonic development and AP patterning.

In recent years, a potential functional role for the chromatin in the regulation of the temporal activation of Hox genes in epiblast cells has been uncovered. In particular, several studies have revealed a significant correlation between specific histone modifications, nuclear organization and timing of Hox gene activation. At present, this evidence is exciting but merely correlative, and future experiments should evaluate and further dissect the functional contribution of these experimental observations for the Hox clock and ultimately to the patterning of the axial skeleton.

As for the Hox proteins, very little is known about the details of their activity as transcription factors controlling specific regulation programs. The Hox paradox, illustrated by the high *in vivo* functional specificity of Hox proteins, compared to their very similar DNA binding affinities *in vitro,* is only partially resolved. It is commonly thought that interactions with cofactors contribute to Hox specificity of action. So far, the known Hox cofactors fall short to provide a comprehensive explanation for the diversity of patterns produced by the different Hox proteins, often in the same tissues. Clearly, future investigations aiming at identifying additional Hox partners and analyzing the way they interact with and modulate Hox function will lead to a better understanding of the crucial roles played by Hox genes in the development and evolution of the axial skeleton. In addition, considering the high functional redundancy observed among PG members, it is possible that the identification and characterization of HoxPG-specific motifs will help to understand the mechanisms that provide functional specificity to Hox proteins. These signatures are likely to be involved in interactions with other molecules, including those controlling specific post-translational modifications potentially contributing to the control of Hox specific functions.

In the past years, we have witnessed substantial increase in the ways gene activity can be regulated. In this review, we have discussed the impact that miRNAs or quantitative parameters in the synthesis of ribosomal proteins have on fine tuning the production of Hox gene products. Many other non classical forms of gene regulation will surely be found to also play relevant roles in Hox-mediated patterning processes. It is possible that as we gain deeper knowledge about the full complexity of the mechanisms controlling Hox gene expression and activity, many of the still non answered questions and open debates about how the combined activity of the different Hox genes is translated into precisely built morphological structures will be finally solved.

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FIGURE LEGENDS

**Figure 1.** Epigenetic regulation of the Hox clock during gastrulation controls vertebral identity. Progressive 3' to 5' chromatin opening at the Hox clusters correlates with a colinear increase in active histone marks (H3K4me3; blue circle) and a decrease in repressive marks (H3K27me3; green circle), and precedes the sequential activation of Hox gene expression (from light to dark grey arrows) in the gastrulating mouse embryo. Spatially restricted Hox gene expression patterns are then established along the primary body axis of the mouse embryo. Genes located at the 3' end of the Hox cluster are expressed at ealier time points and thus at more anterior axial levels than those at the 5' end. The combinatorial activity of Hox genes in the somitic mesoderm provides positional identity along the AP axis and subsequently leads to the specification of the different vertebrae – cervical (C, in orange), thoracic (T, in blue), lumbar (L, in green), sacral (S, in yellow) and caudal (C, in pink). Experimental evidence suggests that changes in the timing of Hox gene activation impairs correct axial patterning and produces homeotic transformations of the axial skeleton. Moreover, it strongly correlates with the different skeletal morphologies observed in several vertebrate species and is therefore likely to contribute to the diversity of the vertebral formula.

**Figure 2.** Hox motifs are important for Hox activity. **A:** General structure of HoxPG1-8 and HoxPG10 proteins. The conserved motifs and domains are represented and include the homeodomain (blue), the linker region (LR, grey), the hexapeptide (HX, orange) and the NWLTAKS motif (pink). Additional hypothetical functional motifs are represented as light blue rectangles. **B:** Schematic representation of regulation of a Hox target gene by Hox and Pbx proteins. A Hox responsive element containing Pbx (P) and Hox (H) binding sites is represented. Hox interact with Pbx through the hexapeptide motif (orange rectangle) and the resulting complex binds the Hox responsive element and modulates transcription of a target gene. **C:** Schematic representation of the regulatory function of NWLTAKS motif (pink rectangle) at HoxPG10 through interactions with other regions of the protein. Once phosphorylated (represented as yellow circles) at the NWLTAKS motif, HoxPG10 proteins are able to bind the Hox binding site (H), through the HD, and to participate in interactions with the N-terminal

part of the protein triggering the rib-repressing program (left side of the figure). In the absence of phosphorylation NWLTAKS, HoxPG10 proteins are less efficient binding to DNA, which is related with phosphorylation of specific residues within the HD, and fail to interact with the N-terminal region resulting in a protein without rib-repressing properties. (right side of the figure). **D:** Schematic representation of regulation of *Myf5* expression by Hox and Pax proteins. The H1 enhancer containing Pax (X) and Hox (H) binding sites is represented. HoxPG6 interacts with Pax3 and the resulting complex binds the H1enhancer through Pax HD (purple). Transcription of *Myf5* is activated without need of the direct binding of HoxPG6 to the H1 enhancer.

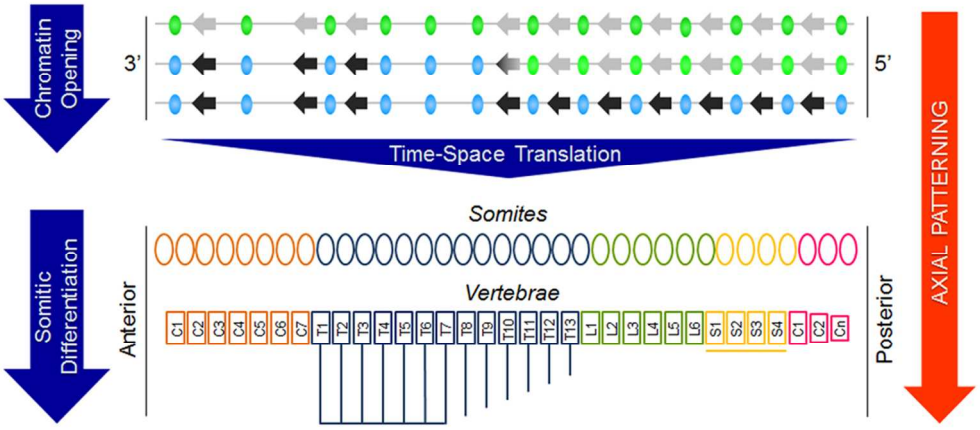


Figure 1
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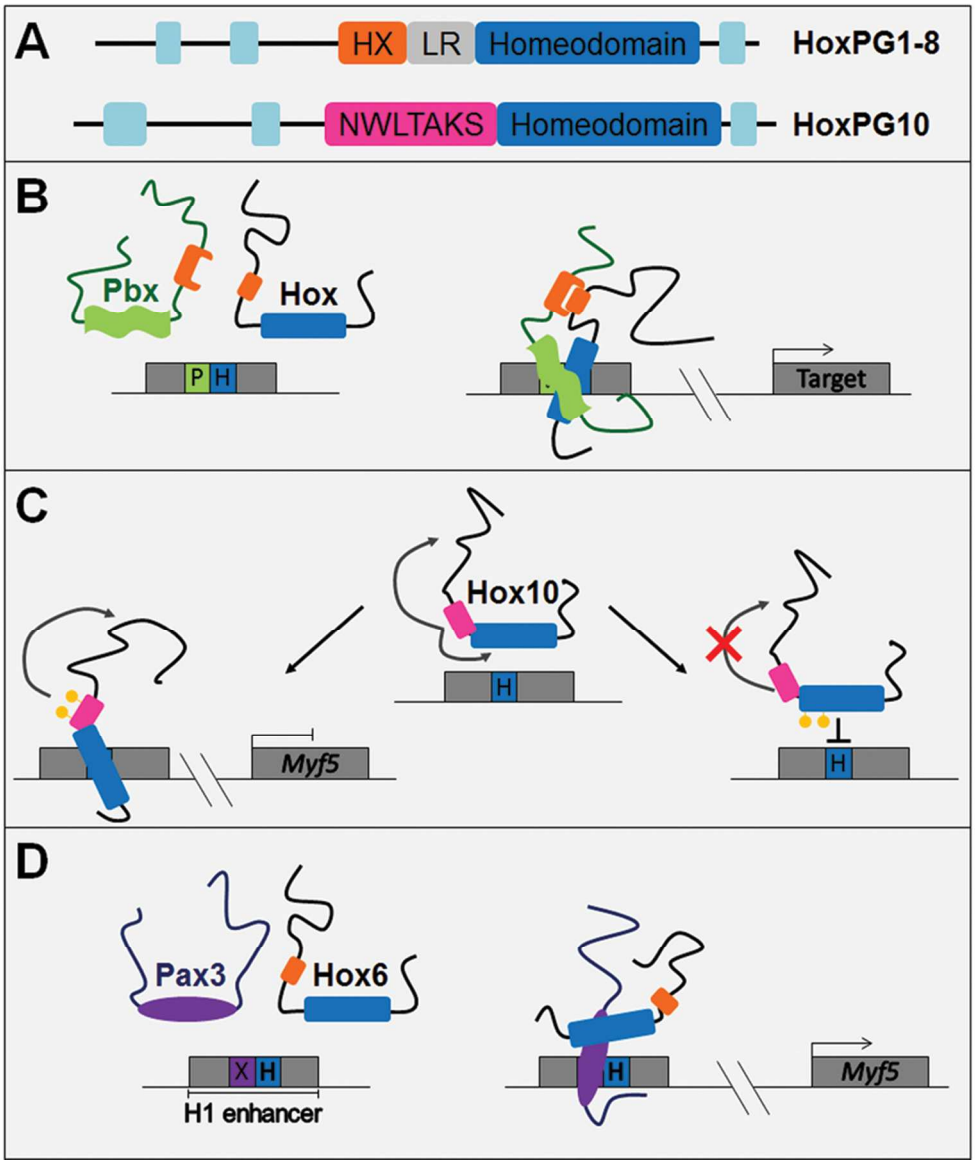


Figure 2
114x133mm (300 x 300 DPI)