HOX GENES CONTROL THE SPECIFICATION OF GLOBAL VERTEBRAL DOMAINS

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To Scott, the best of friends.

"Science may set limits to knowledge, but should not set limits to imagination."

– **Bertrand Russel**

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ABSTRACT

The development of an animal from embryo to adult is an actively regulated process, largely controlled through differential gene expression. Hox genes are key modulators of embryonic development. Among other functions, they are essential for patterning the body plan by conferring identity to segments along the anterior-posterior axis. In vertebrates, Hox genes can specify the identity of both individual vertebrae and global vertebral domains in the axial skeleton. Hox group 10 is responsible for the layout of the lumbar region by inhibiting rib formation, while Hox group 11 defines the sacral domain of the skeleton. It was previously thought that ribs were set out by default. Hence, it was suggested that another Hox gene would have to inhibit rib formation in the cervical domain, similarly to Hox group 10 in the caudal part of the skeleton. We produced mice bearing ribs in every vertebrae by over-expressing *Hoxb6* in the PSM under the control of the *Dll1* promoter, showing that the thoracic area is formed through the activity of Hox group 6 genes that specifically induce rib formation, and that the cervical domain is defined as the area that precedes Hox group 6 expression. In this study, we used our two Hox over-expression mouse models with complementary rib phenotypes to study the molecular mechanisms of rib development. Our previously described *Dll1-Hoxa10* transgenics are completely rib-less, while the *Dll1-Hoxb6* embryos form ectopic ribs throughout the whole length of their skeletons. Interestingly, our findings indicate that the rib patterning cues provided by these two Hox genes are first interpreted in the muscle-forming myotomal compartment through the regional-specific hypaxial expression of *Myf5/Myf6* genes. We believe this is a non-myogenic *Myf5/Myf6*-specific function that is translated to the bone-forming sclerotome through the activity of FGF and PDGF growth factors. Our data support a functional model for the concerted development and evolution of the vertebral musculoskeletal system.

In addition, we also studied which unique protein features of particular Hox paralogous groups confer them their functional specificity. We focused on the rib-inhibiting properties of Hoxa10 and used them as a measure of Hox group 10's function. We identified three Hox group 10-specific conserved peptide motifs and investigated whether they could account for the rib-repressing function of this Hox group. Our results indicate that at least one of these motifs is necessary, but not sufficient for Hoxa10's function, and suggest a potential role for the other two. Interestingly, by generating chimeric proteins between Hoxa10 and Hoxa11, a non-rib-inhibitory protein, we were able to produce intermediate phenotypes that are reminiscent of both *Hoxa10* and *Hoxa11* over-expression phenotypes. Our results point to a critical role of specific peptide domains located outside the homeodomain for conferring functional specificity to Hox proteins.

SUMÁRIO

O desenvolvimento de um animal, desde embrião até adulto, é um processo activamente regulado e controlado, em grande parte, através da expressão diferencial de genes. Os genes Hox são moduladores do desenvolvimento embrionário por excelência. Para além de outras funções, são essenciais para a padronização do plano axial, uma vez que conferem identidade aos segmentos ao longo do eixo anterior-posterior. Em vertebrados, os genes Hox especificam não só vértebras individuais, mas também grupos vertebrais completos do esqueleto axial. O grupo Hox 10 é responsável pela planificação da região lombar ao inibir a formação de costelas, ao passo que o grupo Hox 11 define o domínio sacral do esqueleto. Anteriormente pensava-se que as costelas eram determinadas por defeito, tendo sido sugerido que a zona cervical seria formada pela actividade repressora de costelas, conferida por um gene Hox com uma função idêntica à do grupo Hox 10 na parte caudal do esqueleto. Neste trabalho, produzimos murganhos que possuem costelas em todas as vértebras, através da sobre-expressão de *Hoxb6* na mesoderme pré-somítica, sob o controlo do promotor *Dll1*. Os nossos dados demonstram que a região torácica é determinada pela acção de genes do grupo Hox 6 que induzem a formação de costelas, e que a zona cervical é definida como a área que precede a actividade do grupo 6. Utilizámos dois modelos de murganho com fenótipos complementares nas costelas para estudar os mecanismos moleculares do desenvolvimento das mesmas. Os nossos transgénicos *Dll1-Hoxa10*, anteriormente descritos, não desenvolvem costela alguma, enquanto que os embriões *Dll1-Hoxb6* formam costelas ectópicas ao longo de todo o comprimento do seu esqueleto. Curiosamente, os nossos dados indicam que a informação providenciada por estes dois genes Hox é inicialmente interpretada pelo compartimento gerador de músculo, o miótomo, através da activação hipaxial de *Myf5/Myf6* que é depois traduzida para o esclerótomo (gerador de osso) pela

actividade de factores de crescimento FGF e PDGF. Os nossos resultados apoiam um modelo funcional para o desenvolvimento e evolução do sistema musculo-esquelético dos vertebrados.

Adicionalmente, também investigámos que características proteicas exclusivas de grupos Hox particulares lhes conferem a sua especificidade funcional. Focámo-nos na capacidade de inibir costelas do grupo Hox 10 e usámo-la como uma medida da função do mesmo. Identificámos três domínios peptídicos conservados e exclusivos do grupo Hox 10 e investigámos se estes poderiam ser responsáveis pelas capacidades repressoras de costelas deste grupo Hox. Os nossos resultados indicam que pelo menos um destes domínios é necessário mas não suficiente para a função da proteína Hoxa10 e ainda sugerem um potencial papel para os outros dois domínios peptídicos. Curiosamente, através da produção de proteínas quiméricas entre Hoxa10 e Hoxa11 (uma proteína que não tem propriedades repressoras de costelas), obtivemos fenótipos intermédios, reminiscentes daqueles produzidos pela sobre-expressão individual de cada uma das proteínas referidas. Os nossos resultados apontam para um papel crucial de domínios peptídicos específicos, localizados fora do homeodomínio, na atribuição da especificidade funcional às proteínas Hox.

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GLOSSARY

A-P Anterior-posterior

BMP Bone Morphogenetic Protein

BIP2 Bric-à-brac interacting protein 2

Cdx Caudal type homeobox

Disco disconnected

Disco-r disco-related

Dll Delta Like

Exd Extradenticle

FGF Fibroblast Growth Factor

FGFR Fibroblast Growth Factor Receptor

Gdf11 Growth differentiation factor 11

HOM Homeotic

HOM-C Homeotic Complex

Hth Homothorax

Hox Homeobox genes

Lfng Lunatic fringe

Meox Mesenchyme homeobox

Mng Myogenin

MRF Myogenic Regulatory Factors

Pax Paired box

Pbx Pre B cell leukemia transcription factor

PcG Polycomb Group

Pdgf	Platelet derived growth factor
PdgfR	Platelet derived growth factor Receptor
PSM	Presomitic Mesoderm
RA	Retinoic Acid
RBP	Recombining Binding Protein
Scx	Scleraxix
Shh	Sonic Hedgehog
TALE	Three Amino acid Loop Extension
Tbx	T box
TGFβ	Transforming Growth Factor β
TrxG	Trithorax Group
Tsh	tea-shirt
WNT	Wingless-wint

CHAPTER I – INTRODUCTION

"In the beginning, there was nothing. Then God said, "Let there be light".
And there was still nothing, but now you could see it."

– **Groucho Marx**

The development of a well-organized, fully functional animal from a single cell has amazed scientists throughout history. Most of the early embryological studies can be traced back to ancient Greek philosophers who studied reproduction, differentiation, growth and traits' inheritance as part of a field known as *generation*. In the fourth century B.C., Aristotle (384-322 B.C.) published the first acknowledged study of comparative anatomical embryology, and he was also the first to use the chicken egg as a model to study development (Aristotle 350 B.C.).

For the longest time, from the sixteen hundreds to mid nineteen century, two opposing theories on the origin of life were intensely debated all over the world. The theory of *preformation* defended that since all creatures were originated at the same time as a result of God's work of creation, each generation had to be present in a fully formed miniature version within its progenitors' egg or sperm. The opposing theory of *epigenesis*, originally conjectured by Aristotle, favored that each embryo was formed *de novo* through the gradual development of parts from an undifferentiated mass (Speybroeck *et al*. 2002; Pinto-Correia 1997; Gilbert 2003).

In the twentieth century, modern genetics was born and advanced with the discovery of the DNA as the physical substrate of genes. This had a tremendous impact in the way scientists study the development of organisms and today, developmental biology is thriving fast growing field that integrates many different disciplines ranging from molecular biology to Evo Devo.

I.I How to make an embryo

"Let the Sperm of a man by itself be putrefied in a gourd glass, sealed up, with the highest degree of putrefaction in Horse dung, for the space of forty days, or so long until it begin to be alive, move, and stir, which may easily be seen. After this time it will be something like a Man, yet transparent, and without a body. Now after this, if it be every day warily, and prudently nourished and fed with the Arcanum of Mans blood, and be for the space of forty weeks kept in a constant, equal heat of Horse dung, it will become a true, and living infant, having all the members of an infant, which is born of a woman, but it will be far less. This we call Homunculus".

- **Paracelsus, *Of the Nature of Things.***

There is one way of going from genotype to phenotype, and that is through development. Tomas Morgan realized this in a time when embryology and genetics were one and the same (Morgan 1926). Mechanistically speaking, the way we go from genotype to phenotype is through differential gene expression, the ultimate masterpiece of development.
When asking the question of how molecular changes in development lead to concrete morphological differences, one particular family of genes, the Hox genes, often stands out. The study of particular functions of this family of genes in the development of the mouse embryo is main subject of this thesis work.

I.II Hox genes

"Here, there is one central field. Development. How the egg turns into the organism. But development ultimately includes all of biology: and it will have to be put on a molecular basis."

– **Sydney Brenner**

Hox genes have captured the imagination of scientists like few others, due to their power to shape animal morphology and their implications in both developmental and evolutionary processes.

The concept of *homeosis* was first contemplated by William Bateson, who described it as "'something that has been changed into the likeness of something else'' (Bateson 1894). Two of the most paradigmatic homeotic transformations are observed in the *Bithorax* and *Antennapedia* mutants (Fig. 1). The genes in the origin of those remarkable phenotypes were informatively named Homeotic (HOM) genes, and extensive genetic and molecular analyses indicated they are part of a gene complex that controls segment identity in *Drosophila* (Lewis 1978; McGinnis and Krumlauf 1992). The *Drosophila* Homeotic Complex (HOM-C) is composed of eight genes organized in two loci (or complexes) in *Drosophila*'s chromosome three (Lewis 1978). The *Antennapedia* complex includes the *Labial* (*Lab*), *Proboscipedia* (*Pb*), *Deformed (Dfd), Sex combs reduced* (*Scr)* and *Antennapedia (Antp)* genes responsible for the specification of the head and first thoracic segments (Wakimoto *et al*. 1984). The Bithorax complex contains the *Ultrabithorax (Ubx) gene*, which specifies the third thoracic segment, and the *Abdominal A* (*Abd-A*) and *Abdominal B* (*Abd-B*) genes, which are responsible for the identities of the abdominal segments (Casanova and White 1987; Sánchez-Herrero *et al*. 1985).

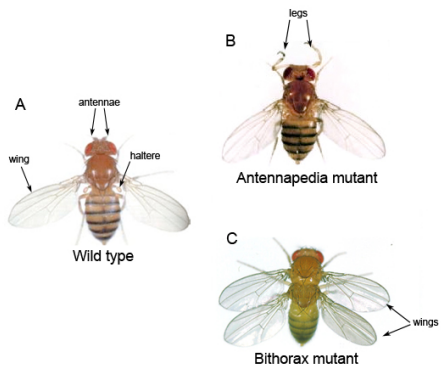

Figure 1: Classic examples of homeotic mutations. **A.** A wild type fruit fly, *Drosophila melanosgaster,* showing one pair of antennae in the head, one pair of wings in the second

thoracic segment and one pair of halteres in the third thoracic segment. **B.** In the *Antennapedia* mutant the antennae are converted into leg structures by a mutation in the regulatory region of the *Antennapedia* gene, which causes it to be expressed in the head. C. The *Bithorax* mutant is a four-winged fruit fly in which the third thoracic segment is transformed into another second thoracic segment (i.e., transformation of halteres into wings). This mutant results from three mutations in *cis* regulatory regions of the *Ultrabithorax* gene (adapted from various sources).

Molecular analysis of the homeotic genes led to the discovery of the homeobox, a conserved 180 base pair long sequence that encodes a 60 amino-acid DNA binding domain called the homeodomain (McGinnis *et al*. 1984; McGinnis *et al*. 1984; Gehring *et al*. 1990; Gehring 1987). Although transcription-independent functions of Hox genes have been described (Brunet *et al*. 2005; Nédélec *et al*. 2004; Topisirovic *et al*. 2005; Prochiantz and Joliot 2003), the DNA-binding properties of their homeodomains conferred them the title of transcription factors (Gehring *et al*. 1990).

Over the past 20 years, Hox homologs have been found in virtually every billaterian animal (Garcia-Fernàndez 2005), including chordates, arthropods, platyhelminths, nematodes, and nemertines, where they consistently control axial identities and the formation of the body plan (Kmita-Cunisse *et al*. 1998; Salser and Kenyon 1994; Bayascas *et al*. 1998; Carroll 1995; Garcia-Fernàndez and Holland 1994; Krumlauf 1994; McGinnis and Krumlauf 1992).

Whereas ancestral Hox genes were organized in a single cluster, genome duplication events gave rise to four distinct clusters (A-D) in most vertebrates, which in mammals contain 39 genes (Duboule 2007) (Fig. 2). Worthy of note is that teleost fishes seem to have undergone an additional round of genome duplication that produced seven or eight clusters, depending on the species (Prince *et al*. 1998; Amores 1998). Vertebrate Hox genes can be classified in 13 paralogous groups according to sequence homology and position within the cluster. Probably due to secondary gene losses, not every Hox paralog is represented in each cluster (Prince 2002) (Fig.2).

Hox gene activation within the cluster follows a temporal and spatial sequence that correlates with their physical order in the chromosome, a phenomenon known as colinearity (reviewed in Kmita and Duboule 2003; Krumlauf 1994). In particular, the “temporal colinearity” manifested in mammals and short germ-band insects, represents the sequential activation of Hox genes, with 3’-located genes being expressed earlier than more 5’-located genes (reviewed in Kmita and Duboule 2003).

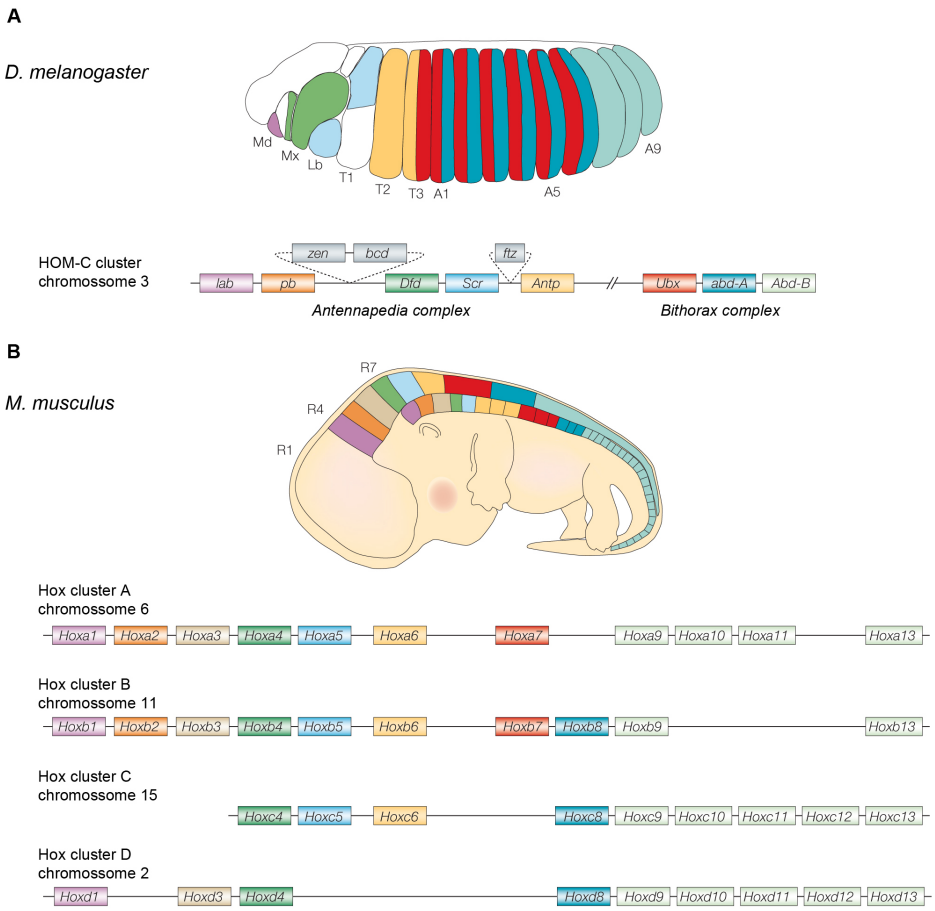

Figure 2: Schematic representation of the fruit fly *Drosophila melanogaster*, (A) and mouse, *Mus musculus*, (B) Hox clusters. In both panels (A and B) embryos are represented and the colors stand for the relative expression domains of corresponding Hox genes (top), which are represented according to their distribution in the chromosome (bellow). The *Antennapedia* and *Bithorax* complexes are indicated (adapted from Pearson *et al*. 2005).

From an evolutionary perspective, the relationship between colinear Hox activation and morphogenesis has been suggested to work as a constraint to maintain the clustering of Hox genes (Ferrier and Minguillon 2003; Kmita and Duboule 2003). This view, however, has been recently challenged after the cloning and analysis of Hox genes in new model organisms (like urochordates), in which some level of coordination of Hox gene expression is achieved even in the absence of clustering (Lemons and McGinnis 2006; Monteiro and Ferrier 2006; Galliot 2005). This suggests that the precise mechanism regulating the expression of these genes is irrelevant, provided that the correct spatiotemporal pattern of expression is met (Kmita and Duboule 2003)

HOX EXPRESSION AND REGULATION

Hox genes are activated in the primitive streak when this structure is almost fully extended, and then in its caudal-most part, in a domain that contributes mostly to extra-embryonic mesoderm (Gaunt and Strachan 1994; Deschamps and Wijgerde 1993; Deschamps *et al*. 1999). The initial expression domains subsequently stretch anteriorly until they reach their definitive distribution in the three germ layers (Deschamps *et al*. 1999). Interestingly, there is evidence of a priming mechanism in the posterior section of the primitive streak that sets the conditions for Hox transcriptional activation 12 hours before it actually begins (Forlani 2003).

Recent work in chicken suggested that activation of Hox gene expression in the early epiblast is responsible for controlling the timing of ingression through the primitive streak (Limura and Pourquié 2006).

The progressive activation of Hox genes following their chromosomal position (temporal colinearity), generates anterior limits of expression for the different Hox paralog genes progressively located in more posterior embryonic areas, which also reflect the gene's position within the cluster (spatial colinearity). While activation of Hox gene transcription typically

produces sharp anterior expression limits, this expression often gradually decrease towards the posterior end of the embryo. Overall, this expression scheme results in overlapping patterns of expression of different Hox genes along the AP axis (Kessel and Gruss 1990; 1991).

The initial activation of Hox genes in the primitive streak is critical for regional identity because it is the main determinant of the final Hox expression domains. WNT and FGF signals, which are involved in the formation and proper functioning of the primitive streak, likely regulate initial Hox expression in this region (Forlani 2003). Later, these expression domains seem to be further modulated and refined in the presomitic mesoderm (PSM) by FGF, WNT and RA signals (Aulehla *et al*. 2003; Dubrulle *et al*. 2001) that control axial elongation and the rate of somite formation (Dubrulle and Pourquié 2004a) (discussed in more detail below). Additionally, genes involved in the segmentation program also have an effect on the regulation of Hox gene expression, as illustrated by the analysis of loss- or gain-of-function mutations in genes of the Notch signaling pathway (Cordes *et al*. 2004). The Gdf11 signaling pathway might also have an important role in regulating Hox expression. *Gdf11* is a member of the TGFβ family that is expressed in the primitive streak and in the tail bud (McPherron *et al*. 1999). Deletion mutants of this gene or its receptors display anterior homeotic transformations in their axial skeletons that correlate with altered expression of particular Hox genes (Andersson *et al*. 2006; Oh and Li 1997; Oh *et al*. 2002; Liu *et al*. 2001; McPherron *et al*. 1999). Another important group of Hox regulators is the *Cdx* family of genes. *Cdx* genes directly regulate expression of Hox genes in a dose-dependent fashion (Subramanian *et al*. 1995; Gaunt *et al*. 2004; Charité *et al*. 1998; Pownall *et al*. 1996) and can also indirectly mediate the effects of FGFs and WNTs in Hox expression (Isaacs *et al*. 1998; Deschamps and van Ness 2005).

Long-term maintenance of Hox transcriptional states seems to be achieved through epigenetic marks. The trithorax group (trxG) is involved in the epigenetic maintenance of active Hox genes in the correct segments, whereas the polycomb group (PcG) is required for blocking their activation in the wrong spatial domains (Ringrose and Paro 2004). It has been suggested that the combined action of PcG and trxG proteins ensures that Hox gene expression is properly restricted to specific embryonic domains (Mahmoudi and Verrijzer 2001).

HOX FUNCTION AND SPECIFICITY

Hox genes are involved in a wide variety of developmental and cellular functions, including cell proliferation, cell death, cell adhesion, cell cycle regulation and cell migration (Pearson *et al*. 2005). At a higher morphological level, Hox genes help building organs either by modulating the positional identity of a given structure or by producing novel structures. In either case, in order to achieve their function, Hox genes must both control signaling pathways and establish complex regulatory networks (Hombríaa and Lovegrove 2003).

The extraordinary functional specificity of Hox genes *in vivo* contrasts with their indiscriminate binding specificity *in vitro*, which is due, to some extent, to the high degree of similarity and conservation of their homeodomains (Hoey and Levine 1988).

The structure of the homeodomain has been resolved by nuclear magnetic resonance spectroscopy and x-ray crystallography (Kissinger *et al*. 1990; Otting *et al*. 1990), and consists of three α-helixes and a flexible N-terminal arm next to the first helix. The third helix is the recognition helix that contacts the DNA major grove at a six base-pair sequence containing a four base pair recognition core, T(A/T)AT (Ekker *et al*. 1991; Pearson *et al*. 2005). This “TNAT” sequence is extremely common in the genome, which

raises questions about how Hox proteins identify their correct target sites from the multitude of potential binding sites.

Hox specificity has been extensively connected to the interaction with other proteins, usually DNA-binding proteins themselves (Mann and Chan 1996; Mann and Affolter 1998; Mann 1995). The most common Hox cofactors comprise the PBC and MEIS classes of TALE proteins. The first class includes Exd in *Drosophila* and the Pbx proteins in vertebrates, whereas the MEIS class contains Hth in the fly and the vertebrate Meis and Prep homeodomain containing proteins (Moens and Selleri 2006). Similarly to Hox genes themselves, Hox partners were initially identified because their mutation led to morphological patterning defects in *Drosophila*. The first of these was Exd, which was indentified as a Hox cofactor because its loss-of-function produced homeotic transformations in specific segments without affecting Hox gene expression (Rauskolb *et al*. 1995; Rauskolb *et al*. 1993; Peifer and Wieschaus 1990). Pbx1 was independently identified in vertebrates through a chromosomal translocation that caused human preB cell leukemia (Nourse *et al*. 1990; Kamps *et al*. 1990; Rauskolb *et al*. 1993). Pbx/Exd primarly interact with Hox proteins through the three amino acid loop in their homeodomain, which binds a tryptophan-containing hexapeptide motif (NY/FP/DWMK/R), located N-terminal to the homeodomain and present in Hox proteins from paralog groups 1 to 8 (Piper *et al*. 1999; Chang *et al*. 1995; Knoepfler and Kamps 1995; Neuteboom *et al*. 1995; Passner *et al*. 1999; Phelan *et al*. 1995).

The second class of TALE cofactors, which includes Meis and Prep proteins, regulates Hox activity in several ways: by binding DNA together with Hox, by forming part of ternary Hox-PBC-MEIS DNA-binding complexes, and by modulating Pbx/Exd activity independently of DNA (Mann and Morata 2000; Moskow *et al*. 1995; Knoepfler *et al*. 1997; Chang *et al*. 1997; Berthelsen *et al*. 1998a; Berthelsen *et al*. 1998b).

In addition to PBC and MEIS, other proteins have been reported to work as Hox partners in *Drosophila*. These include disco, disco-r and tsh zinc finger proteins, and more recently BIP2 (Prince *et al*. 2008). However, whether or not these proteins are also Hox cofactors in vertebrates remains to be discovered (Moens and Selleri 2006).

Despite extensive evidence indicating that cofactor cooperative binding helps improve Hox binding specificity, the *in vivo* effects on Hox function are still controversial, most particularly in vertebrates. Hox/PBC complexes can act either as transcriptional activators or repressors, depending on the target and the developmental context. This led to the suggestion that the Hox-mediated transcriptional outcome at a particular target gene is determined not by the presence of cofactors but rather by their ability to recruit co-activators or co-repressors to specific regulatory sequences (Saleh *et al*. 2000; Kobayashi 2003; Gebelein *et al*. 2004). Conversely, others have reported that some targets usually repressed by Hox genes, become activated when the hexapeptide is mutated (Merabet *et al*. 2003; Galant *et al*. 2002), suggesting that PBC binding alone could stipulate the transcriptional status of Hox proteins, at least in some situations. Considering the available information, it seems that Hox proteins obey very few rules when it comes to their functional activity, and even for any given Hox protein, specificity seems to be achieved differently depending on the particular set of downstream targets in question (Pearson *et al*. 2005; Merabet *et al*. 2009; Foronda *et al*. 2009). It is therefore critical that search for these targets is taken as a priority.

A reasonable number of genes have been demonstrated to be directly or indirectly regulated by Hox genes in a variety of organisms (Bobola *et al*. 2003; Kutejova *et al*. 2005; Pearson *et al*. 2005). This number has increased enormously in the last years with the application of systematic approaches in different model organisms using microarray technology (Cobb and Duboule 2005; Hersh *et al*. 2007; Hueber *et al*. 2007; Leemans

et al. 2001; Rohrschneider *et al*. 2007; Zhao and Potter 2001). The results of these studies suggest, as expected, that the expression of many genes involved in cellular functions is Hox-regulated. However, another interesting insight that stands out is that 63-69% of targets are unique for a specific Hox protein (Lu *et al*. 2003; Saleh *et al*. 2000; Shen *et al*. 2001), which is at odds with previous predictions that suggested a higher number of common targets. Moreover, Hox proteins can bind to chromatin-modifying proteins, such as histone deacetylases and acetyltransferases, possibly regulating downstream targets through epigenetic mechanisms (Lu *et al*. 2003; Saleh *et al*. 2000; Shen *et al*. 2001).

The paradox of functional specificity has yet another side to it, that has to do with Hox protein structure. Sequence analysis of Hox proteins, led to the identification of key residues outside of the homeodomain that are critical for Hox function. The best characterized is the above-mentioned hexapeptide, which has also been called PBC interaction domain (PID) (Phelan *et al*. 1995; Morgan *et al*. 2000; Merabet *et al*. 2009; Johnson *et al*. 1995; Chang *et al*. 1995). Interestingly, the area between this domain and the homeodomain, called linker region, was shown to be relevant for Hox activity, particularly its size and amino-acid composition (Gebelein *et al*. 2002; In Der Rieden *et al*. 2004; Merabet *et al*. 2003). In addition to these, other protein domains seem to have a role in conferring functional specificity to Hox proteins, by interacting with other cofactors, by mediating specific DNA contacts or by promoting proper folding of the protein into its correct three-dimensional structure. These residues are usually located around the homeodomain, either N- or C-terminally (Lin and McGinnis 1992; Gibson *et al*. 1990; Furukubo-Tokunaga *et al*. 1993; Dessain *et al*. 1992; Berry and Gehring 2000; Passner *et al*. 1999; Zeng *et al*. 1993). Importantly, some of these motifs are paralog-specific signatures, which have been proven to be essential for the specific functional activity of Hox proteins *in vivo* (Merabet *et al*. 2009). In summary, the study of these protein motifs can enhance our

understanding of the mechanisms by which Hox activity regulates specific developmental processes.

I.III A Body Plan: The vertebrate axial skeleton

"Nature does nothing uselessly."
– **Aristotle**

The axial skeleton composed of vertebrae is the ultimate hallmark of vertebrates. It provides support, allows moment and offers protection to vital organs. The skeleton also plays a role in maintaining the body's homeostasis, as it is involved in endocrine regulation, serves as storage of minerals and is the site of blood cell production.
The axial skeleton displays an overt segmented pattern with repeats of individual vertebrae, which are usually classified into 5 distinct groups: cervical (C), thoracic (T), lumbar (L), sacral (S), and caudal (Cd), according to anatomical features. The quantitative distribution of vertebrae among each of these anatomical regions is often called the "axial formula". This varies dramatically among different classes of vertebrates but is remarkably conserved within species, and is though to have been an important evolutionary phenomenon in the establishment and radiation of different vertebrate groups (Carroll 1988; Gadow 1933). Take the neck region as an example of this variation: ducks have 16 cervical vertebrae, while swans have as many as 25 and some snakes only 1. Whereas this variability is often related with function and adaptation to the environment, there are also developmental and evolutionary constraints at work, as seems to be the case of the cervical vertebrae in mammals. Ranging from long-necked giraffes to humans, virtually all mammals have 7 cervical vertebrae, and this conservation has been suggested to be due to developmental constraints,

as changes in this formula are often associated with increased incidence of cancer and neural problems (Galis 1999).

Both the cause and consequences of the diversity of axial formulas in the animal kingdom have provoked speculation from morphologists for centuries, and will be discussed later. For now, I will focus on the embryonic origin of the skeleton, the somites.

SOMITOGENESIS

One of the most noticeable illustrations of segmentation in vertebrates occurs during early embryonic development in the process of somite formation. Somites are paired mesodermal structures that form periodically from the unsegmented presomitic mesoderm, and represent the embryological origin of the skeletal muscle of the trunk and limbs, the axial skeleton and the dermis of the back (Brand-Saberi and Christ 2000).

Somites are part of the paraxial mesoderm (Jouve *et al*. 2002; Freund *et al*. 1996), and as such, are located symmetrically on both sides of the neural tube, and flanked laterally by the intermediate and lateral plate mesoderm. This position within the embryo is important for the establishment of the tissue interactions that control somite differentiation.

The process of somite formation is controlled at least at two different stages of development. It first relies on the emergence of paraxial mesodermal cells from the epiblast and later on the oscillatory activity of a variety of signaling processes in the PSM (Pourquié 2001). The formation of the paraxial mesoderm is closely associated with the morphogenetic movements of gastrulation. During the regression of the primitive streak, somitogenic stem cells are deposited and become a resident population first in the primitive streak and then in the tail bud. It is thought that the somitic mesoderm mostly derives form these progenitors (Stern 1992; Schoenwolf *et al*. 1992; Selleck and Stern 1991; Hatada and Stern 1994; Psychoyos and Stern 1996).

It has been suggested that production of somitic mesoderm occurs in two consecutive phases. According to this hypothesis, anterior somites would derive from the paraxial mesoderm produced from the primitive streak, and posterior somites would originate from the tail bud, once it becomes the source of paraxial mesoderm after the closure of the neuropore (Catala *et al*. 1995; Tam 1984; Pourquié 2001). However, some authors consider the distinction between the two phases somewhat artificial because some lines of evidence suggest that paraxial mesoderm production, and consequently, somite formation is a continuous process (Pourquié 2001).

The generation of paraxial mesoderm requires that proliferation, specification and migration events take place in an orderly fashion. The molecular control of these processes is mostly provided by the coordinate activity of at least three main signaling cascades: the WNT, FGF and RA pathways. Disrupting any of these pathways has severe consequences to axis formation, typically producing axial truncations resulting from impaired paraxial mesoderm formation (Pourquié 2001). In particular, mutations in the *Wnt3a* gene, the *FGFR1*α isoforms or the RA-producing enzyme Raldh2, resulted in disrupted somitogenesis after the formation of the first few somites (Greco *et al*. 1996; Takada *et al*. 1994; Yamaguchi *et al*. 1994; Niederreither *et al*. 1999; Ciruna and Rossant 2001). Mutations in the T-box genes *Brachyury* or *Tbx6*, which are regarded as mediators of the WNT signaling pathway, produced similar phenotypes, further documenting the requirement of these signals to produce mesodermal precursors (Yamaguchi *et al*. 1999; Chapman *et al*. 1996). Additionally, in some of these mutants, the posterior somitic tissue was replaced by ectopic neural tubes, indicating that these signaling pathways are involved in the specification of the paraxial mesoderm vs. neuroectoderm fate in the tail bud (Chapman and Papaioannou 1998; Yamaguchi *et al*. 1999; Yoshikawa *et al*. 1997).

Somitogenesis is a sequential, directional and synchronous process, where somites are formed at the anterior border of the PSM, resulting in the progressive addition of new somites posterior to older ones (Yamaguchi 1997; Pourquié 2001; Dubrulle and Pourquié 2004a). The pace of somite formation is a species-specific characteristic. In the mouse, each new somite sprouts from the PSM every 120 minutes, whereas this process takes 90 minutes in chick and only 30 minutes in zebrafish (Dequéant and Pourquié 2008). Histologically, the vertebrate PSM gives the impression of being a loose mesenchyme with no morphological organization. However, during somite formation, the PSM cells change their adhesion properties and become progressively more epithelized (Duband *et al*. 1987), culminating with the establishment of a new somitic border.

This process of spatial and temporal segment specification is tightly regulated, and thought to depend on an intrinsic molecular clock that sets the pace of somite formation, associated with a maturation front that determines the location of the new inter-somitic border (Baker *et al*. 2006a; Schnell *et al*. 2002; Baker *et al*. 2006b). The first experimental evidence of such a molecular oscillator came with the documentation of the dynamic expression of the gene *Hairy1* in the PSM of chick embryos (Palmeirim *et al*. 1997). This gene is expressed as a wave that sweeps the PSM in a posterior to anterior direction once every 90 minutes (the time that takes to form a new somite in chick), to finally merge in a band that correspond to the anterior part of the next somite to be formed. Hence, the last site of *Hairy1* expression in each cycle labels the position of the next inter-somitic boundary.

Since the discovery of the first oscillating gene, many other genes were found to have similar dynamic expression in the PSM. Most of these are components of the Notch and WNT pathways (Dubrulle and Pourquié 2004a). The Notch signaling pathway is involved in the correct synchronization of segmentation of the paraxial mesoderm. Mice bearing

mutations in genes related to this pathway, namely, *Notch1*, *Delta1*, *Delta3*, *RBPjk*, *Mesp2* and *Lfng*, typically display impaired formation of caudal somite due to incorrect segmentation of the PSM (Saga *et al*. 1997; Wong *et al*. 1997; Oka *et al*. 1995; Zhang and Gridley 1998; Evrard *et al*. 1998; Conlon *et al*. 1995). In addition, recent data from thorough microarray studies suggest that the clock might be more complex than initially imagined and involve at least three signaling pathways (Goldbetera and Pourquié 2008; Dequéant *et al*. 2006; Mallo 2007).

Apart from the segmentation clock, it is generally accepted in the field that the sign of competence for segmentation in PSM cells is given by two opposing morphogenetic gradients: a caudal-to-rostral gradient of FGF/WNT signaling and a rostral-to-caudal gradient of RA that would block and promote segmentation, respectively. The area of interception of the activities provided by the two gradients is known as the determination front and marks each new somitic border (Diez del Corral *et al*. 2003; Moreno and Kintner 2004; Dubrulle *et al*. 2001; Aulehla *et al*. 2003). According to this model, the maturation state of PSM cells is determined by a threshold of FGF activity, in which high levels of FGF would maintain cells in an immature state (Dubrulle *et al*. 2001). The FGF gradient is determined by the transcription of *Fgf8* messenger RNA exclusively in the growing posterior tip of the embryo, which gets progressively degraded in the recently formed tissues, resulting in an mRNA gradient in the posterior part of the embryo that is translated into gradient of Fgf8 protein, and FGF signaling activity (Dubrulle and Pourquié 2004b). Interestingly, by altering the FGF levels using beads soaked in Fgf8, it is possible to manipulate somite size in a dose dependent manner, with somites becoming progressively smaller with higher Fgf8 levels, a result that has been interpreted as a slowing down of the progression of the determination front (Dubrulle *et al*. 2001; Dubrulle and Pourquié 2004b). RA signaling is the second component of the determination front. The RA gradient results from

the activity of two enzymes, Raldh2 and Cyp26, involved in the synthesis and degradation of RA, respectively. While RA is synthesized by *Raldh2*, which is expressed at high levels in the recently formed somites and at low levels in the anterior PSM, it is degraded by Cyp26, which is strongly expressed at the tip of the tail bud, thus leading to a rostral-to-caudal gradient of RA in the PSM (Sakai *et al*. 2001; Niederreither *et al*. 2003; Blentic *et al*. 2003). The RA gradient controls the progression of the determination front by antagonizing the instructions provided by the FGF signaling. Interesting and consistently, lack of RA results in smaller somites, whereas too much RA leads to enlarged somites (Maden *et al*. 2000; Dubrulle and Pourquié 2004a; Diez del Corral *et al*. 2003). The last component known to be present in the PSM and influence the positioning of segmental boundaries is the WNT pathway (Aulehla *et al*. 2003). Over-expressing *Wnt3a* in the caudal end of chick embryos leads to the formation of smaller somites (Aulehla *et al*. 2003). In the mouse, *Wnt3a* is strongly expressed in the growth zone of the primitive streak and then in the tail bud, and it was suggested that secreted Wnt3a would diffuse to form a caudal-to-rostral gradient in the PSM (Aulehla *et al*. 2003). In agreement with this idea, it was shown that *Axin2*, a direct target of the WNT signalling cascade, has a cyclic expression in the PSM, oscillating at the same frequency but out of phase with the Notch pathway, suggesting a possible negative regulation of Notch activity by the WNT signaling via Axin2 (Aulehla *et al*. 2003). Therefore, according to this model, while Notch signaling plays a role in somitic boundary formation by being linked to the oscillator, the WNT pathway puts together the clock and the morphogen gradients in a comprehensive mechanism controlling the segmentation process (Aulehla and Herrmann 2004). The temporal information provided by the segmentation clock has to be translated into a spatial pattern in the PSM that defines each new segmental border. The Mesp2 transcription factor is key in this process. Inactivation of this gene results in the absence of border

formation and in the failure to establish an anterior-posterior pattern within each somite (Morimoto *et al*. 2005; Takahashi *et al*. 2000). In the anterior PSM, Mesp2 modulates an on/off state of Notch signaling that corresponds to the future somitic boundary through the activation of *Lfng* transcription (Morimoto *et al*. 2005; Oginuma *et al*. 2008; Oginuma *et al*. 2010).

SOMITE DERIVATIVES

Once formed, the epithelial somites progressively differentiate into distinct compartments as a response to signals from surrounding tissues, giving rise to different cell lineages. The ventro-medial part of the epithelial somite undergoes an epithelial-to-mesenchymal transition to form the sclerotome, which is the source of the axial skeleton (Fig. 3). The dorso-lateral part, the dermomyotome retains its epithelial character and with further maturation of the somite, cells delaminate from its edges and migrate underneath to form the myotome that will originate the skeletal muscles of the trunk. Once the myotome is formed, the remaining part of the dermomyotome is called dermotome and will form the dermis of the back (Borycki and Emerson 2000; Brand-Saberi and Christ 2000) (Fig. 3). More recently, a fourth somitic compartment was indentified, the syndetome, that is generated from the dorsolateral border of the early sclerotome and gives rise to the axial tendons (Brent *et al*. 2003) (Fig. 3).

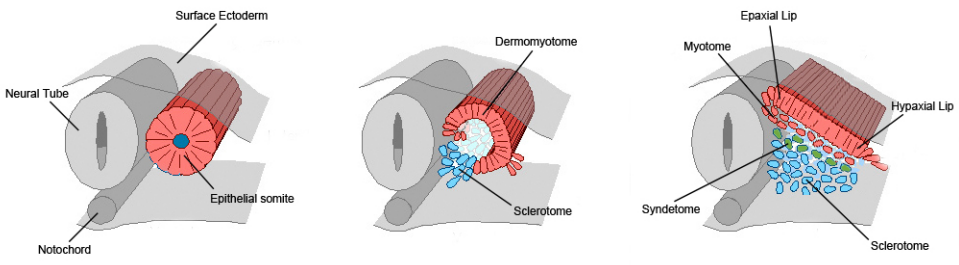

Figure 3: Schematic representation of somite differentiation into four compartments. The epithelial somite in response to signals from the surrounding tissues differentiates into dermomyotome and sclerotome. Cells from the dermomyotome then delaminate and move

underneath to form the myotome. A fourth somitic compartment, the syndetome, is generated within the sclerotome (adapted from various sources).

THE SCLEROTOME

During differentiation, the sclerotome is further compartmentalized into rostral and caudal halves (Stern and Keynes 1987), and also medial and lateral domains (Freitas *et al*. 2001; Brent and Tabin 2002). These sub-domains, which can be identified by the expression of different molecular markers, originate distinct structures. The rostro-caudal compartmentalization of the sclerotome, underlies the phenomenon of resegmentation, which is crucial for the development of the vertebral column and has strong implications in the metamerization of the peripheral nervous system (Christ and Wilting 1992; Rickmann *et al*. 1985; Christ *et al*. 1979). Resegmentation of the sclerotome results in each vertebra being formed by the posterior half of one somite and the anterior half of the next (Aoyama and Asamoto 2000; Christ *et al*. 1998; Bagnall *et al*. 1988). The dorsomedial sclerotome generates the spinous process and contributes to the distal ribs; the ventromedial sclerotome originates the vertebral bodies, neural arches, intervertebral discs and proximal ribs; and the ventrolateral scleromome also contributes to the distal part of the ribs (Olivera-Martinez *et al*. 2000; Freitas *et al*. 2001; Huang *et al*. 2000).

Pax1, Nkx3.1 and *Nkx3*.2 are the first molecular markers activated when the presumptive sclerotome is induced (Schneider *et al*. 2000; Kos *et al*. 1998; Tribioli and Lufkin 1997; Rodrigo *et al*. 2003; Peters *et al*. 1999; Müller *et al*. 1996; Ebensperger *et al*. 1995). In early somite development, activation of *Pax1* in the ventro-medial part of the epithelial somite labels the emergence of the sclerotome. *Pax1* is expressed even before these cells undergo the epithelial-to-mesenchimal transition that characterizes the formation of the sclerotome, and remains a good ventromedial sclerotomal marker during vertebral morphogenesis (Monsoro-Burq 2005; Christ *et al*. 2000). Pax9 is activated slightly later and acts redundantly with Pax1 (Peters *et al*. 1999;

Müller *et al*. 1996). In the *Pax1/Pax9* double mutant mice, the development of the ventral part of the vertebra, but not of the neural arches, is defective (Peters *et al*. 1999). *Pax1* is genetically upstream of *Nkx3.2* because expression of this gene cannot be detected in *Pax1/Pax9* double mutants (Rodrigo *et al*. 2003; Tribioli and Lufkin 1997). However, *Pax1* is expressed normally in the somites of *Nkx3.2* mutant embryos, although later steps of vertebral differentiation are severely defective in these animals (Tribioli and Lufkin 1997). *Nkx3.1* expression is activated in newly formed somites but mice carrying null mutations in this gene do not have any skeletal defects (Schneider *et al*. 2000; Kos *et al*. 1998). Altogether, these results suggest that the activation of *Pax1* is the key event that triggers sclerotome formation during development (Monsoro-Burq 2005).

It is well established that sclerotome formation requires signals from axial midline tissues. Shh and Noggin, secreted by the notochord, are important to induce and maintain *Pax1* expression (Marcelle *et al*. 1999; Chiang *et al*. 1996; Hammerschmidt and McMahon 1998). Loss-of-function *Shh* mice fail to form the entire vertebral column (Chiang *et al*. 1996). However, in these mutants, the sclerotome is formed, although significantly smaller in size, and *Pax1* is activated, but its expression is not maintained, suggesting that Shh is involved in the maintenance of the sclerotomal program rather than in its activation. In *Noggin* knockout mutants, *Pax1* expression is delayed, indicating that this molecule is involved in sclerotomal specification (Dockter 2000; McMahon *et al*. 1998). It has been suggested that, in this process, Noggin is mostly required to inhibit the dorsalizing activity of BMP4, provided by the lateral plate mesoderm (Dockter 2000; McMahon *et al*. 1998; Christ *et al*. 2004). However, it has been shown that Noggin can also induce low levels of *Pax1* on its own (Liem *et al*. 2000; McMahon *et al*. 1998). The ectoderm, on the other hand, produces WNT molecules that prevent Pax1 activation (Capdevila *et al*. 1998). Therefore, proper

sclerotomal formation clearly depends on the correct equilibrium between dorsalyzing and ventralizing signals.

THE DERMOMYOTOME

The dermomyotome generates myotomal muscle progenitor cells and the dermis of the back. It is composed of a central epithelial sheet that is surrounded by contiguous lips (Huang and Christ 2000; Ordahl and Le Douarin 1992). The dermomyotome is marked by the expression of *Pax3*, contrasting with the activation of *Pax1* in the sclerotome (Birchmeier and Brohmann 2000). This transcription factor starts to be expressed in the entire somite, but with further differentiation, *Pax3* expression becomes restricted to the dermomyotome and, finally, it is up-regulated in the lateral edge of the dermomyotome (Williams and Ordahl 1994; Goulding *et al*. 1994; Bober *et al*. 1994). *Pax3* is required for the proper formation of both migratory and non-migratory muscle precursor cells. Indeed, in mice *Pax3* mutant mice, migratory myogenic precursors cells fail to delaminate and, consequently, these mutants lack muscles in the limbs and diaphragm (Epstein *et al*. 1995; Tremblay *et al*. 1998; Daston *et al*. 1996).

While the formation of the sclerotome is dependent on the notochord, dermomyotome development is mostly controlled by signals from the dorsal neural tube and surface ectoderm. WNT signals seem to have a prominent role in this process (Fan *et al*. 1997; Fan and Tessier-Lavigne 1994; Dietrich *et al*. 1997). However, notochord produced, long range acting *Shh* is thought to be necessary for both survival and proliferation of myogenic precursors, in particular those in the epaxial compartment (Krüger *et al*. 2001; Duprez *et al*. 1998; Teillet *et al*. 1998).

During dermomyotome differentiation, cells at the dorso-medial lip (DML) delaminate and migrate underneath to form the epaxial myotome that will originate the back muscles The central dermomyotome looses its epithelial morphology and forms the dermatome, which is the origin of the dorsal

dermis (Olivera-Martinez *et al*. 2000). The ventro-lateral dermomyotome behaves differently at different axial levels. At limb bud levels, cells delaminate and migrate to invade the lateral plate mesoderm, and differentiate into limb muscles (Chevallier *et al*. 1977). At the interlimb level, cells from the ventro-lateral lip (VLL) of the dermomyotome move underneath to produce the hypaxial myotome. During this process, the ventrolateral dermomyotome and hypaxial myotome invade the lateral plate mesoderm together as a somitic bud, which will contribute to the formation of the body wall muscles (Brent and Tabin 2002; Brand-Saberi and Christ 2000) (Fig. 4).

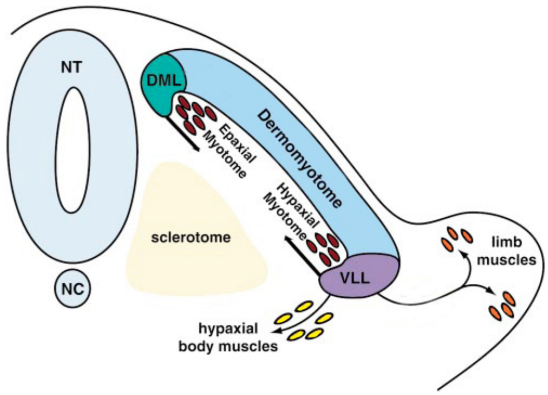

Figure 4: Somitic sources of muscle progenitors for epaxial, hypaxial, and limb muscles. Myogenic progenitors originate in the dorsal-medial and ventral-lateral lips of the dermomyotome. Cells of the dorsal-medial lip (DML) migrate ventrolaterally, differentiate, and give rise to epaxial back muscles. The ventral-lateral lip (VLL) cells migrate underneath to form the ventral hypaxial body wall muscles at interlimb levels (*yellow*); and migrate to form the limb musculature at limb levels (*orange*) (adapted from Pownall *et al*. 2002).

Limb and hypaxial progenitors activate a particular expression profile in order to coordinate their migration with the activation of the myogenic program, which should only occur upon progenitors' arrival to their final sites of differentiation (Birchmeier and Brohmann 2000). These genes include: *Pax3* and *Msx1* transcription factors, which are required for progenitor cell migration (Bober *et al*. 1994; Epstein *et al*. 1995; Goulding *et al*. 1994; Houzelstein *et al*. 1999); c-*met*, a *Pax3* target, and its ligand SF/HGF

(scatter factor/hepatocyte growth factor), which are essential for delamination of migratory precursor cells from the epithelial dermomyotome (Maina *et al*. 1996; Bladt *et al*. 1995; Birchmeier and Gherardi 1998; Dietrich *et al*. 1999); and *Lbx1*, another *Pax3* target, that is only expressed in migratory precursor cells and is critical for the migration of muscle precursors into the limb bud (Gross *et al*. 2000; Mennerich *et al*. 1998; Brohmann *et al*. 2000; Jagla *et al*. 1995; Dietrich *et al*. 1998).

Muscle development from the myotome is under the control of myogenic regulatory factors (MRFs): *MyoD*, *Myf5*, *Myogenin*, and *Myf6/MRF4*. These are a family of conserved basic helix-loop-helix (bHLH) transcription factors (Weintraub *et al*. 1991), characterized by their ability to activate the skeletal muscle program in many non-muscle cell types. This property has bestowed them the title of master regulators of muscle progenitor cell specification and differentiation (Davis *et al*. 1987; Choi *et al*. 1990). In the mouse, *Myf5* becomes active slightly before *MyoD* and despite some differences in their expression, these genes seem to compensate for each other because, while both individual mutants develop normal musculature, double mutants completely fail to form skeletal muscles (Pownall *et al*. 2002; Braun *et al*. 1992; Tajbakhsh *et al*. 1996). The accumulation of studies on single and compound loss-of-function mutants of the different MRF factors led to the current view that *Myf5* and *Myf6* are at the top of the myogenic cascade. MyoD seems to operate downstream of *Myf5/Myf6* in some precursors and in parallel with them in others, and *myogenin* operates as the final effector in the pathway, controlling terminal differentiation (Carvajal and Rigby 2010; Kassar-Duchossoy *et al*. 2004).

In addition to its role in the migration of muscle progenitors, *Pax3* has a broader role in myogenesis that is evident in *Pax3* and *Myf5* double mutants that have no muscle in the trunk (Tajbakhsh *et al*. 1997; Bajard *et al*. 2006). Indeed, Pax3 is responsible for the activation of *MyoD* and, thus, the myogenic program in *Myf5*-deficient mice. The Six homeoproteins and their

cofactors, Eya and Dach, are also important regulators of myogenesis. They cooperate with Pax proteins and MRFs and form a complex regulatory network of muscle development (Grifone *et al*. 2005).

Cis Regulation of Muscle Development

The different functions of MRFs are reflected in how the transcription of each of these genes is regulated. Genes situated higher in the myogenic hierarchy, such as *Myf5* and *Myf6*, respond to more signaling pathways than those acting at the bottom of the myogenic cascade, such as *myogenin* (Carvajal and Rigby 2010). Relevant to this work is the remarkably complex arrangement of distinct enhancers that are necessary to drive particular aspects of *Myf5* and/or *Myf6* expression (Carvajal *et al*. 2008). However, while *Myf6* transcriptional regulation seems to require at least four enhancer elements to specifically drive expression in the limbs, somitic bud, central thoracic myotome, ventral and dorsal myotome (Fomin *et al*. 2004; Carvajal *et al* 2001; Pin *et al*. 1997), *Myf5* regulation is much more complex. *Myf5* expression is controlled by at least five elements in different compartments in the somites (Hadchouel 2003; Buchberger *et al*. 2003; Teboul *et al*. 2002; Teboul *et al*. 2003; Summerbell *et al*. 2000), at least three in the limbs (Buchberger *et al*. 2003; Hadchouel *et al*. 2000; Hadchouel 2003; Bajard *et al*. 2006), two in the developing central nervous system (CNS) (Summerbell *et al*. 2000; Daubas *et al*. 2009; Daubas *et al*. 2000), and at least five in the branchial arches (Summerbell *et al*. 2000; Carvajal *et al* 2001; Patapoutian *et al*. 1993). Interestingly, while most regulatory regions of the *Myf5/6* locus specifically activate discrete fractions of either *Myf5* or *Myf6* expression, some data is consistent with the existence of at least one shared regulatory element between the two genes (Carvajal *et al* 2001). The elucidation of how these two linked genes are regulated so that their expression patterns are sometimes distinct and other times overlapping is of foremost interest

for understanding the evolution of distal elements that control gene expression.

THE SYNDETOME

In order to function properly, the musculoskeletal system depends on the coordinated development of not only bone and muscle, but also tendons. However, whereas the formation of the first two somite derivatives has been extensively studied, tendon development has been overlooked until fairly recently.

The bHLH transcription factor *scleraxis* (*Scx*) is expressed both in mature tendons of the limbs and trunk, and in their progenitors in the developing somite (Schweitzer *et al*. 2001) (Fig. 3). It has been recently suggested that these tendon progenitors define a fourth somitic compartment, the syndetome. The syndetome originates from a domain at the dorsolateral edge of the early sclerotome that has the potential to activate either the tendon or cartilage programs (Brent *et al*. 2003). It has been shown that a myotomal signal, possibly involving FGF signaling, induces Scx expression in sclerotomal cells localized next to the myotome. Moreover, the analysis of *Sox5/Sox6* double mutants, in which the chondroprogenitors cannot differentiate into cartilage, indicated that in order to form cartilage it is necessary to actively repress tendon development in the dorsolateral sclerotome (Brent *et al*. 2005).

MAKING SENSE OF SEGMENTS: HOX GENES AT WORK

The road to the axial formula is slippery and involves many complex interactions between different tissues and regulatory factors, ranging from transcription factors to signaling molecules (Mallo *et al*. 2009; Wellik 2007). The AP patterning of the body plan is largely controlled by the activity of Hox genes, which eventually dictate the differentiation plan for the different segments.

One of the early challenges in the Hox field was to establish a correlation between Hox gene expression and function in the patterning of the axial skeleton. The expression of Hox genes is typically characterized by sharp anterior borders that have been correlated with skeletal phenotypes of several single Hox loss-of-function mutants and over-expression transgenics. These correlations led to the proposal of the “Hox code” model (Kessel *et al*. 1991; Burke *et al*. 1995; Burke 2000). This model states that the segment-specific morphological identities would result from combinatory “code” of Hox expression, which is distinctive in each segment along the AP axis (Kessel *et al*. 1991; 1990). An alternative model for Hox patterning activity was proposed on the basis of the phenomenon of “phenotypic suppression” described in *Drosophila* (Struhl 1983; Morata 1993) to explain the observation that individual Hox loss-of-function mutants often displayed phenotypes only in the most anterior regions of their expression domains, despite their broad expression patterns (Duboule and Morata 1994). This model, called “posterior prevalence”, postulates that posteriorly expressed Hox genes are functionally dominant over anterior genes. (Duboule and Morata 1994; Kmita and Duboule 2003). Recently, this subject was revisited and new data suggested that miRNAs could work as a mechanism to enforce the prevalence of posterior Hox genes by repressing anterior Hox instructions (Yekta *et al*. 2008). Nevertheless, neither the “Hox code” or “the posterior prevalence” models can entirely explain the phenotypes produced in several gain-of-function and loss-of-function studies of either individual Hox genes or whole paralogous groups (Mallo *et al*. 2010).

Comparative analysis of the anterior limits of expression of Hox genes in the paraxial mesoderm of different vertebrates revealed a correlation between the anterior boundary of expression of different Hox genes and the physical transition between morphologically distinct areas of the axial skeleton (Wellik 2007; Burke 2000; Burke *et al*. 1995) (Fig. 5). A striking example of this is the somitic anterior expression border of *Hox6* genes, which lies at

the prospective boundary between cervical and thoracic vertebrae in vertebrates with different numbers of cervical vertebrae. This transition is mapped to somites 18-19 in the goose, 16-17 in chick, 9-12 in the mouse, 2-3 in xenopus and 1-2 in zebrafish (Molven *et al*. 1990; Burke *et al*. 1995; Burke 2000). Interestingly, the early activation of *Hox6* genes was later implicated as the cause of the reduction of the cervical region in pythons (Cohn and Tickle 1999), further suggesting that the Hox paralog group 6 is critical for the cervical-to-thoracic transition in vertebrates.

Loss-of-function studies of single Hox genes often changed the identity of individual vertebral elements, which became most perceptible when the phenotype affected anatomical boundaries (Boulet and Capecchi 1996; Carpenter *et al*. 1997; Chisaka and Capecchi 1991; Condie and Capecchi 1993; Davis and Capecchi 1994; Jeannotte *et al*. 1993; Kostic and Capecchi 1994; Le Mouellic *et al*. 1992; Small and Potter 1993; Suemori *et al*. 1995; Horan *et al*. 1994). Compound mutants, in particular those affecting the same paralog group, often resulted in progressively more severe phenotypes (Horan *et al*. 1995b; McIntyre *et al*. 2007; Wellik and Capecchi 2003), which is consistent with some degree of redundancy between paralogs that had already been suggested based on overlapping expression patterns (Kessel *et al*. 1990; Horan *et al*. 1995a; Gruss and Kessel 1991; Burke *et al*. 1995; Burke 2000). More recently, analysis of compound mutants that included all members of the paralog group unveiled yet another level of Hox function in controlling the specification of global vertebral domains, which will be discussed in the next section.

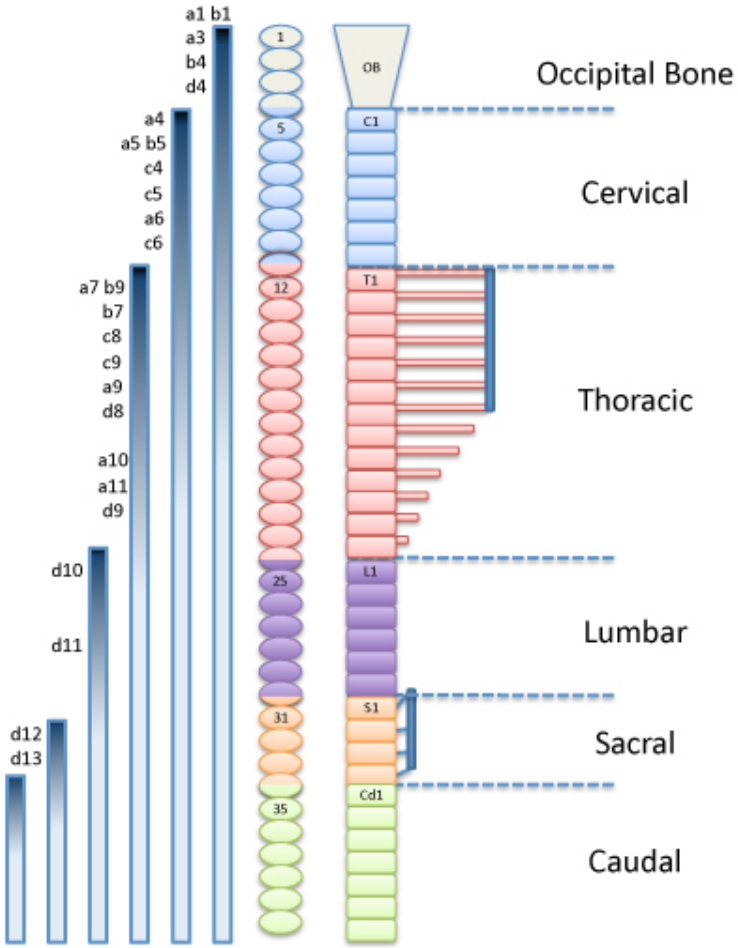

Figure 5: Schematic representation of vertebral domains of the axial skeleton, aligned with somite levels of the mouse and expression of the Hox genes. The anterior limit of expression of specific *Hox* genes is indicated as the top of the gradient, which represents the progressive decrease in expression anterior to posterior. OB: occipital bone; C: cervical; T: thoracic; L: lumbar; S: sacral; Cd: caudal (adapted from Burke *et al*. 1995; Favier and Dollé 1997).

Specification of Global Vertebral Domains

The first experimental evidence that Hox genes could also control whole anatomical vertebral regions came form the simultaneous inactivation of all three paralog genes from Hox group 10 (Wellik and Capecchi 2003). In these compound mutants the prospective lumbo-sacral region exhibited ectopic ribs, thus acquiring thoracic-like features (Fig. 6 A). More recently, the role of Hox group 10 in the patterning of the lumbar domains was further

demonstrated by the precocious over-expression of *Hoxa10* in the PSM of developing mouse embryos, which resulted in the overall inhibition of rib formation and, consequently, in completely rib-less mice (Carapuço *et al*. 2005) (Fig. 6 B). Together, these reports indicate that Hox group 10 is essential for the formation of the lumbar region by specifically blocking rib formation. Interestingly, the same studies showed that Hox group 11 is responsible for the development of the sacral domain. Whereas the loss-of-function of paralog group 11 results in the absense of the sacrum (Wellik and Capecchi 2003), over-expression of *Hoxa11* in the PSM entails a "sacralization" of the lumbar region by promoting sacral-like fusions in adjacent vertebrae (Carapuço *et al*. 2005).

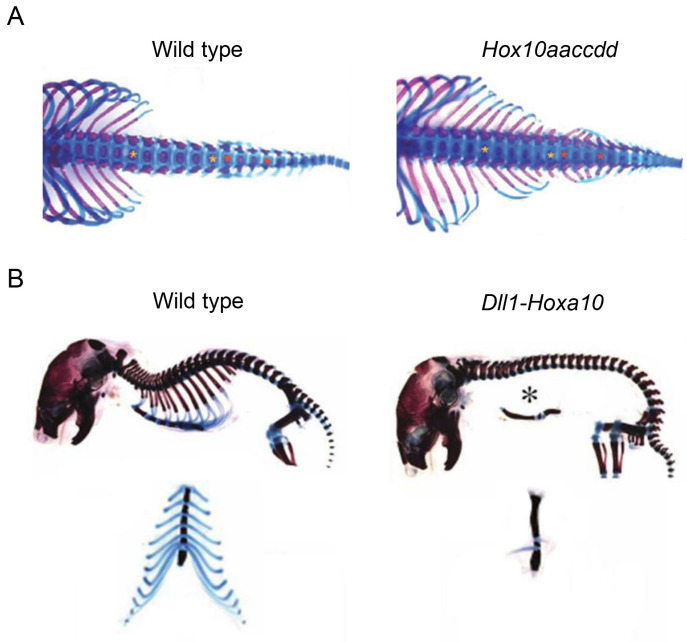

Figure 6: The role of Hox paralog group 10 in the patterning of the axial skeleton. The inactivation of all six alleles of Hox group 10 results in ectopic rib formation in the presumptive lumbar and sacral regions of the skeleton (A). Over-expression of a member of Hox group 10 in the PSM of mouse embryos driven by the *Dll1* promoter results in complete blocking of rib development (B). Adapted from Wellik and Capecchi 2003 (A) and Carapuço *et al*. 2005 (B).

It is noteworthy that in the patterning of the ribcage, the generation of sternal versus floating ribs seems to be controlled by Hox group 9 (McIntyre *et al*. 2007). These genes are specifically important to produce floating ribs, since the global inactivation of this paralog group resulted in an increased number or sternal ribs (13-14 instead of the normal 7) (McIntyre *et al*. 2007). Much less is known about the patterning mechanisms of the neck region. Inactivation of members of the paralogous Hox groups 3-5 demonstrated that these genes have some role in specifying particular morphologies in the cervical vertebrae (Horan *et al*. 1995b; McIntyre *et al*. 2007; Condie and Capecchi 1994). However, a global transformation of the cervical region into the identity of another vertebral domain has not been achieved so far. It has been proposed that ribs are set out by default and, consequently, the cervical domain would be specified by a particular Hox gene or group that would inhibit rib formation in the neck similarly to Hox group 10 in the lumbo-sacral domain (Wellik and Capecchi 2003). However, this gene has not been identified yet and this hypothesis is somewhat at odds with the previously mentioned analysis of the anterior limits of expression of Hox group 6 in different vertebrates (Burke *et al*. 1995).

The work presented in this thesis is integrated in these global patterning functions of Hox genes. We were interested in understanding the mechanisms by which Hox genes control the specification of global vertebral domains, in particular those involving regional specification of rib formation.

I.IV Muscle and Bone: There is no "I" in Team

"Science... never solves a problem without creating ten more."
– **George Bernard Shaw**

A functional musculoskeletal system requires the coordinated development of muscle, bone and tendon. Increasing amount of evidence indicates that this coordination is, to a large extent, the consequence of a dynamic dialog between different somitic compartments.
Inactivation of *Myf5* results in a fairly normal muscle development, but in strikingly severe rib defects (Tajbakhsh *et al*. 1996; Braun *et al*. 1992). Initially, the skeletal phenotype in *Myf5* mutants was suggested to result from impaired production of myotomal inductive signals and/or from the disruption of patterning interactions necessary for rib formation (Braun and Arnold 1995; Braun *et al*. 1992). This idea was further supported by the generation of three different loss-of-function alleles of the *Myf6* gene, located 8 Kb upstream of *Myf5* on the mouse chromosome 10 (Patapoutian *et al*. 1995; Zhang *et al*. 1995; Braun and Arnold 1995), which produced variable defects in rib formation. However, the role of *Myf5/Myf6* in rib development was later revisited and questioned when new *Myf5* alleles were produced that showed no distinguishable rib defects (Kaul *et al*. 2000). This led to the suggestion that the rib abnormalities in Myf mutant mice resulted from some interference with the activity of another gene in the same genomic area as a consequence of the production of the mutant alleles. However, unexpected skeletal defects associated with genes involved in myogenic development are not exclusive of *Myf5/Myf6*. In addition to well-characterized skeletal muscle defects, *myogenin* mutant embryos present abnormal sternal formation (Nabeshima *et al*. 1993; Hasty *et al*. 1993; Vivian *et al*. 2000). The *splotch* mouse mutants, which carry a mutation in the *Pax3* gene, display, among other phenotypes, abnormal

intercostal muscles and several rib deficiencies (Dickman *et al*. 1999; Henderson *et al*. 1999). Another interesting example is the loss-of-function *Six1* mutants. Mice lacking *Six1* die at birth due to severe rib malformations and show extensive muscle hypoplasia specially in particular hypaxial muscles (Laclef 2003). *Six1/Six4* double mutants show an aggravation of the *Six1*-null phenotype (Grifone *et al*. 2005), suggesting that these genes act redundantly in this function.

The above-mentioned studies demonstrate that alterations in the activity of several myogenic genes are often accompanied by impaired rib formation. There are a number of potential inductive signals are expressed in myotome that could potentially mediate these myotome-sclerotome interactions. Among these, FGFs and PDGFA seem to be prime candidates for such activity (Grass *et al*. 1996; Tallquist *et al*. 2000). PDGFRα starts to be expressed in the PSM and ephithelial somite, but later becomes restricted to the sclerotome and dermatome (Schatteman *et al*. 1992; Orr-Urtreger *et al*. 1992; Orr-Urtreger and Lonai 1992). PDGFRα loss-of-function mutants have a normal initial sclerotomal patterning, but later exhibit rib, sternum and vertebrae abnormalities (Soriano 1997). These mutants have perturbed myotomal patterning, which again suggests the skeletal defects could be due to a disruption of myotome-to-sclerotome signaling. Interestingly, PDGFA, a PDGFRα ligand normally expressed in the myotome, is absent in ribless *Myf5* null mutants, and knocking-in PDGFA into the *Myf5* locus resulted in a partial rescue of rib development (Tallquist *et al*. 2000). Similarly, *Fgf4* and *Fgf6* expression was shown to be down-regulated in *Myf5* mutant mice (Grass *et al*. 1996). So far, there is no solid genetic data to validate or deny involvement of these FGFs in myotomal-sclerotomal interactions. However, a number of experiments performed in chicken embryos seem to support a role for FGF signaling in this process. In particular, expression of myotomal *Fgf8* has been suggested to influence rib development (Huang *et al*., 2003). In chicken embryos, ectopic Fgf8 protein

increases sclerotomal proliferation and enhances rib development, whereas inhibition of FGF signalling by SU5402 causes deletions in developing ribs (Huang *et al*. 2003).

Interestingly, chick-quail chimeras have shown the production of tendon progenitors form the dorsolateral sclerotome in response to FGFs secreted from the adjacent myotome (Brent *et al*. 2003). Thus, it seems that FGFs have a pivotal role in the communication between the somitic muscle and cartilage cell lineages involved both in the formation of tendons and ribs.

All in all, these results demonstrate the importance of a correct communication between the different somite compartments, and that this dialog is likely achieved through the use of signaling molecules, such as FGFs and PDGFs growth factors.

I.V Objectives

The overall goal of this work was to contribute to the general understanding of the function of Hox genes in the patterning of global domains of the vertebrate axial skeleton. Here we look at Hox function from two perspectives:

1. Which physiological mechanisms and pathways underlie Hox-mediated patterning of rib formation.
2. Which intrinsic features of Hox proteins confer them their functional specificity.

CHAPTER II – HOX SPECIFICATION OF GLOBAL VERTEBRAL DOMAINS INVOLVES INTERACTIONS WITH MYOGENIC-RELATED FACTORS *

*__Adapted from Vinagre T__, Moncaut N, Carapuço M, Nóvoa A, Bom J, Mallo M. *Evidence for a myotomal Hox/Myf cascade governing non-autonomous control of rib specification within global vertebral domains.*
Dev Cell. 2010 Apr 20; 18(4):655-61. PMID: 20412779

II.I Summary

Hox genes are essential for the patterning of the axial skeleton. Hox group 10 has been shown to specify the lumbar domain by setting a rib-inhibiting program in the presomitic mesoderm (PSM). We have now produced mice with ribs in every vertebra by ectopically expressing Hox group 6 in the PSM, indicating that Hox genes are also able to specify the thoracic domain. We show that the information provided by Hox genes to specify rib-containing and rib-less areas is first interpreted in the myotome through the regional specific control of *Myf5* and *Myf6* expression. This information is then transmitted to the sclerotome by a system that includes FGF and PDGF signaling to produce vertebrae with or without ribs at different axial levels. Our findings offer a new perspective on how Hox genes produce global patterns in the axial skeleton and support a redundant non-myogenic role of *Myf5* and *Myf6* in rib formation.

II.II Background

Hox genes have been classically described to be involved in the production of vertebrae with individual characteristics (R Krumlauf 1994; Mallo *et al*. 2009; Wellik 2007). More recently, it was discovered that Hox genes also play essential roles in defining global vertebral domains (Wellik and Capecchi 2003). In particular, it was shown that Hox group 10 is responsible for the layout of the rib-less lumbar region by diverting it from a rib-containing thoracic identity (Wellik and Capecchi 2003; Carapuço *et al*. 2005). In addition, Hox group 11 was demonstrated to be required for the formation of the sacrum (Wellik and Capecchi 2003). However, it remains unclear whether or not Hox genes are involved in the global specification of the thoracic and cervical domains. Moreover, the mechanism by which Hox genes control these processes is completely unknown.

Wellik and Capecchi (2003) proposed that ribs are set out by default and that the rib-less cervical domain would result from the rib-blocking activity of

other Hox genes acting similarly to Hox group 10 in the lumbar region (Wellik and Capecchi 2003). However, this hypothesis is difficult to reconcile with published expression patterns for Hox genes (Burke *et al*. 1995), which instead suggest an alternative hypothesis. In particular, the anterior limit of expression of members of the Hox group 6 correlates with the cervical to thoracic transition in a variety of vertebrates bearing a different number of cervical vertebrae (Burke *et al*. 1995), indicating that this Hox group might have a role in promoting rib formation. Here we present evidence supporting this hypothesis, showing that Hox control of rib formation is mediated by regulation of *Myf5* and *Myf6* expression in the hypaxial myotome through the interaction with a relevant enhancer. Moreover, our transgenic analyses indicate that myotomal *Myf5/Myf6* activation triggers a non-autonomous effect mediated by PDGF and FGF signaling, promoting rib formation in the adjacent sclerotome. Our data support a redundant non-myogenic role of *Myf5* and *Myf6* in the processes leading to rib formation.

II.III Results

OVER-EXPRESSION OF HOX GROUP 6 INDUCES ECTOPIC RIB FORMATION

In order to test whether Hox group 6 activity could induce rib formation, we employed a transgenic approach to over-express *Hoxb6* either in the presomitic mesoderm (PSM) or in the somites of mouse embryos. Whilst somite-exclusive expression gave mild phenotypes (Fig. 7, compare A with C), the extended expression of *Hoxb6* in PSM cells resulted in the formation of ectopic ribs throughout the whole length of the axial skeleton (Fig. 7, compare A with B), without affecting the total number of vertebrae.

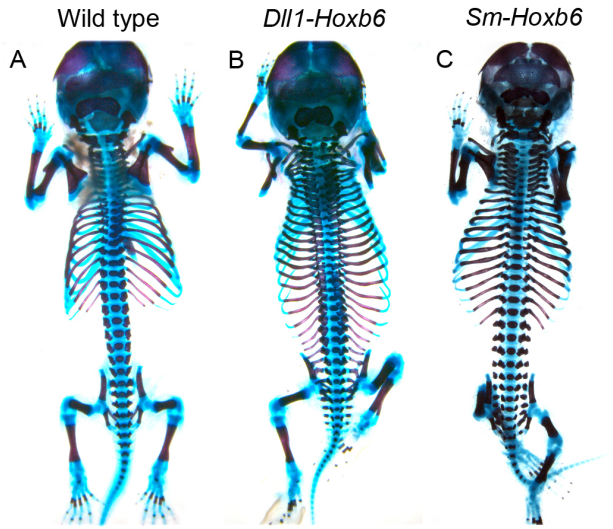

Figure 7: *Hoxb6* over-expression in the PSM induces ectopic rib formation. Hoxb6 expression in the PSM driven by the Dll1 promoter induces ectopic rib formation through the whole A-P length of the skeleton (A), whereas Hoxb6 expression in the somites driven by the Sm promoter gives mild skeletal phenotypes (B). Skeletal staining of wild type (A), *Dll1-Hoxb6* (B) and Sm-Hoxb6 (C) E18.5 fetuses. Equivalent phenotypes were observed in 4 out of 9 *Dll1-Hoxb6* transgenics.

In these transgenics, the prospective cervical area contained ribs fused laterally to form an apparent articular surface for the forelimbs, which were slightly displaced rostrally. The prospective lumbar area also displayed ectopic ribs, progressively decreasing in size in a caudal direction, presumably following the physiological decrease in size of the lower thoracic ribs. In the presumptive sacral area, the vertebrae lost their characteristic morphology and assumed rib-like features, while keeping the lateral fusions typical of the sacral region.

The normal expression of Hox group 10 genes seen in *Dll1-Hoxb6* transgenics (Fig. 8 A-C) indicates that the rib phenotype of *Dll1-Hoxb6* embryos does not result from down-regulation of Hox group 10 genes, despite the similarities in the phenotypes of these transgenics compared to the global group 10 deletion mutants (Wellik and Capecchi 2003).

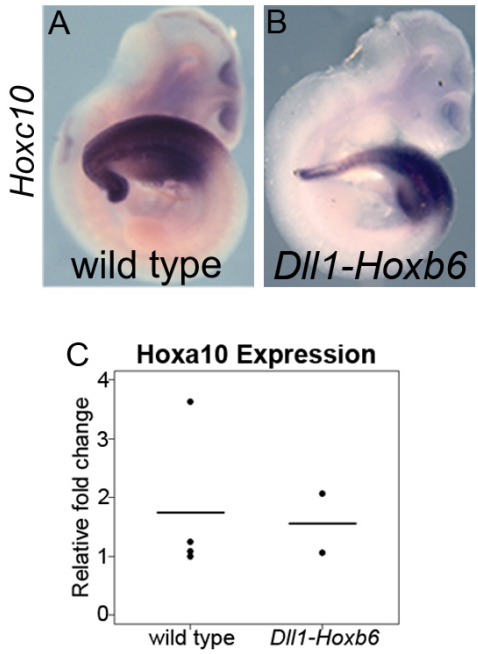

Figure 8: Normal Hox group 10 expression in Dll1-Hoxb6 transgenics. Whole mount *in situ* hybridization of E10.5 wild type (A) and Dll1-Hoxb6 (B) embryos; *Hoxc10* expression is unchanged in *Dll1-Hoxb6* embryos. Quantitative RT-PCR analysis shows normal expression levels of *Hoxa10* in PSM and first somites of E10.5 Dll1-Hoxb6 (n=2) compared with wild type embryos (n=4) (C). GAPDH was used as the endogenous control. Each measurement is the average of duplicate PCR of individual samples. The bar shows the average value for each class.

In sum, Hox paralog groups 6 and 10 seem to modulate the processes leading to rib formation in antagonistic ways. Hence, the "snake-like" (*Dll1-Hoxb6*) transgenics together with our previously described rib-less (*Dll1-Hoxa10*) embryos (Carapuço *et al*. 2005) provide an ideal complementary system to study how Hox genes control rib formation.

HOX GROUPS 6 AND 10 CONTROL REGIONAL HYPAXIAL EXPRESSION OF GENES IN THE *MYF5/MYF6* PATHWAY

Because ribs derive from the sclerotome (Huang *et al*. 2000), we expected this somitic compartment to be affected in our transgenics. However, we

found no significant differences in the expression patterns of sclerotomal markers such as *Pax1*, *Pax9* and *Meox2* in the Hox transgenics (Fig. 9 A-I).

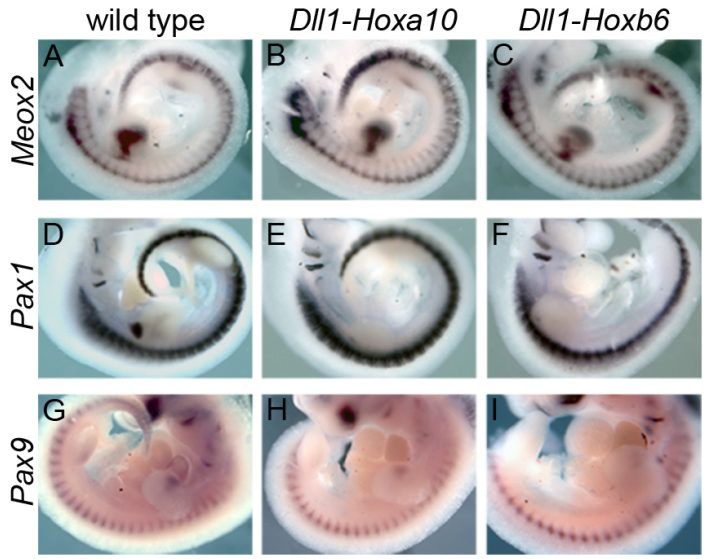

Figure 9: Normal sclerotomal patterning in Hox transgenics. Unchanged expression pattern of several sclerotomal markers in *Dll1-Hoxa10* and *Dll1-Hoxb6* transgenics. Whole mount *in situ* hybridization of E10.5 (A-F) and E11.0 (G-I) wild type (A, D, G), *Dll1-Hoxa10* (B, E, H) and *Dll1-Hoxb6* (C, F, I) embryos: *Meox2* (A-C), *Pax1* (D-F) and *Pax9* (G-I).

Given the normal patterning of the sclerotome in our Hox transgenics, we decided to analyze the expression of genes that have been associated with rib deficiencies in genetic studies. Several mutations of the myogenic factor *Myf5* have been produced and, whereas myogenesis remains relatively normal, some mutants displayed strong rib defects that resembled the phenotypes observed in our *Dll1-Hoxa10* transgenics (Carapuço *et al*. 2005; Braun *et al*. 1992; Tajbakhsh *et al*. 1996).

In wild-type embryos, *Myf5* expression follows specific regional patterns. While it is expressed in the dorso-medial (epaxial) myotome of somites at all rostro-caudal levels, it is only detected in the ventrolateral (hypaxial) myotome of somites located between the limb buds, which are those producing rib-bearing vertebrae (Fig. 10 A, A'), and never at the limb level

somites (Fig. 10 C, C' and I, I'). In both *Dll1-Hoxa10* and *Dll1-Hoxb6* transgenic embryos the distribution of *Myf5* transcripts was clearly affected. Interestingly, the changes in *Myf5* expression were region-specific, correlating with the relative changes seen in rib development.

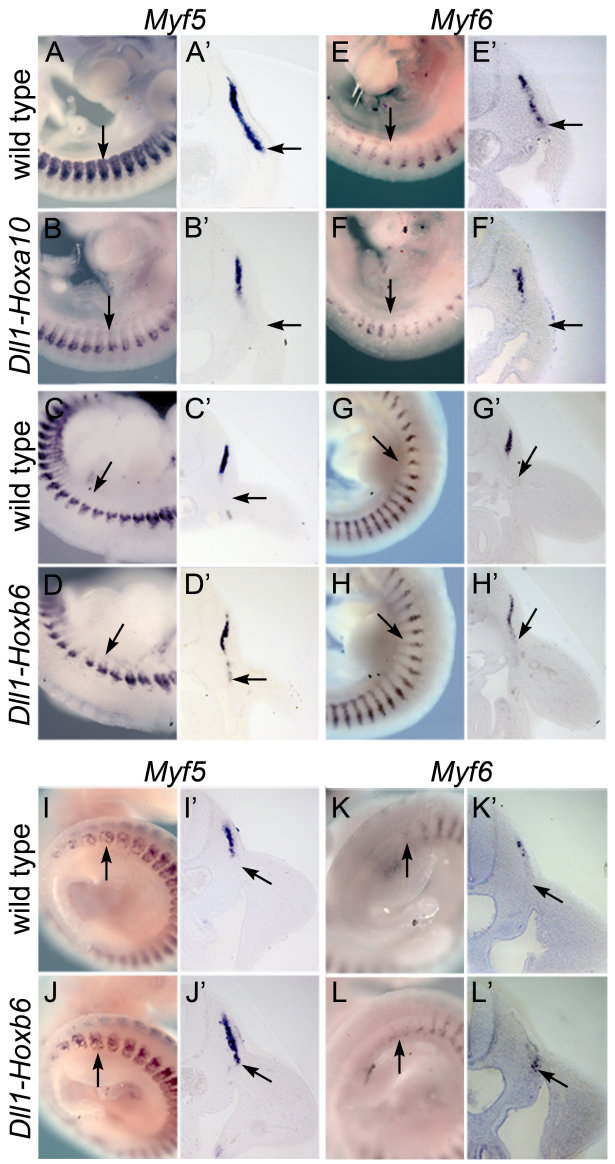

Figure 10: Hox groups 6 and 10 modulate regional expression *Myf5* and *Myf6*. Whole-mount *in situ* hybridization of wild type (A, A'; C, C'; E, E'; G, G'; I, I' and K, K') *Dll1-Hoxa10* (B, B'; F, F') and *Dll1-Hoxb6* (D, D'; H, H'; J, J' and L, L') mouse embryos with *Myf5* (A–D' and I-J') and *Myf6* (E-H' and K-L') probes. Pictures focus on interlimb somites of *Dll1-Hoxa10* embryos and their controls (compare A, A' with B, B' and E, E' with F, F'), forelimb somites of

Dll1-Hoxb6 embryos and their controls (compare C, C' with D, D' and G, G' with H, H') and hindlimb level somites of *Dll1-Hoxb6* embryos and their controls (compare I, I' with J, J' and K, K' with L, L'). Arrows indicate the area of differential expression. Vibratome sections are shown at the arrow level for each marker. Images correspond to embryos at E10.0 (~28-31 somites), except for panels E and F, which are E9.5 (~24 somites).

In *Dll1-Hoxa10* transgenics *Myf5* was down-regulated specifically in the hypaxial myotome of interlimb somites (prospective thoracic region) (Fig. 10 B, B'), and in *Dll1-Hoxb6* transgenic embryos *Myf5* was ectopically activated in the ventrolateral domain of somites at limb (both fore limb and hind-limb) and neck levels (prospective rib-less regions) (Fig. 10 D, D' and J, J'). Thus, we observe a strong positive correlation between rib development and hypaxial *Myf5* expression.

Mutations in *Myf6* have also been associated with severe rib deficiencies resembling those seen in *Dll1-Hoxa10* transgenics (Braun and Arnold 1995), indicating that this gene could also be a target of Hox gene activity. Expression analysis showed patterns similar to those observed for *Myf5*. In *Dll1-Hoxa10* transgenic embryos, *Myf6* was severely down-regulated, most prominently in the hypaxial myotome of the interlimb area (Fig. 10 compare E, E' with F, F'). Conversely, we found ectopic *Myf6* activation in the hypaxial myotomal domain of somites at limb and neck levels in *Dll1-Hoxb6* transgenics (Fig. 10 compare G, G' with H, H' and K, K' with L, L'). Interestingly, hypaxial expression at the hindlimb level of *Dll1-Hoxb6* transgenics preceded that of the epaxial domain (Fig. 10 L, L'), thus mimicking the temporal pattern that has been described for the interlimb region in wild-type embryos (Summerbell *et al*. 2002). Together, these results indicate that Hox groups 6 and 10 are able to control *Myf5* and *Myf6* regional specific expression in the hypaxial myotome in a pattern that closely correlates with rib development. Interestingly, *in situ* analysis of *Myf5* mutants with normal ribcages (Kaul *et al*. 2000; we will refer to these mutants as $Myf5^{loxp/loxp}$) revealed that while *Myf6* expression was down-regulated in the epaxial myotome, expression of *Myf6* in the hypaxial myotome of interlimb somites was clearly conserved (Fig. 11 A, A', B, B'),

displaying a pattern complementary to that found in *Dll1-Hoxa10* transgenics. Altogether, these results indicate that Hox genes are able to control the expression of *Myf5* and *Myf6* in the domain that is relevant for rib formation and are consistent with a redundant role for these genes in rib induction.

To further evaluate *Myf5/Myf6* activity in the Hox transgenics, we tested the expression of suggested downstream effectors. We first assayed *Pdgfa* and *Fgf4*, which were shown to be down-regulated in *Myf5* mutant embryos (Grass *et al*. 1996; Tallquist *et al*. 2000). Expression of these genes followed patterns similar to those described for *Myf5* and *Myf6*. In *Dll1-Hoxa10* embryos these growth factors failed to be activated in the hypaxial domain of interlimb somites, while the remaining expression domains appeared largely unaffected (Fig. 11 compare G, G' with H, H' and I with J). Conversely, *Dll1-Hoxb6* embryos presented ectopic *Pdgfa* and *Fgf4* expression in the hypaxial domain of limb and neck somites (Fig. 11 compare K, K' with L, L' and M with N).

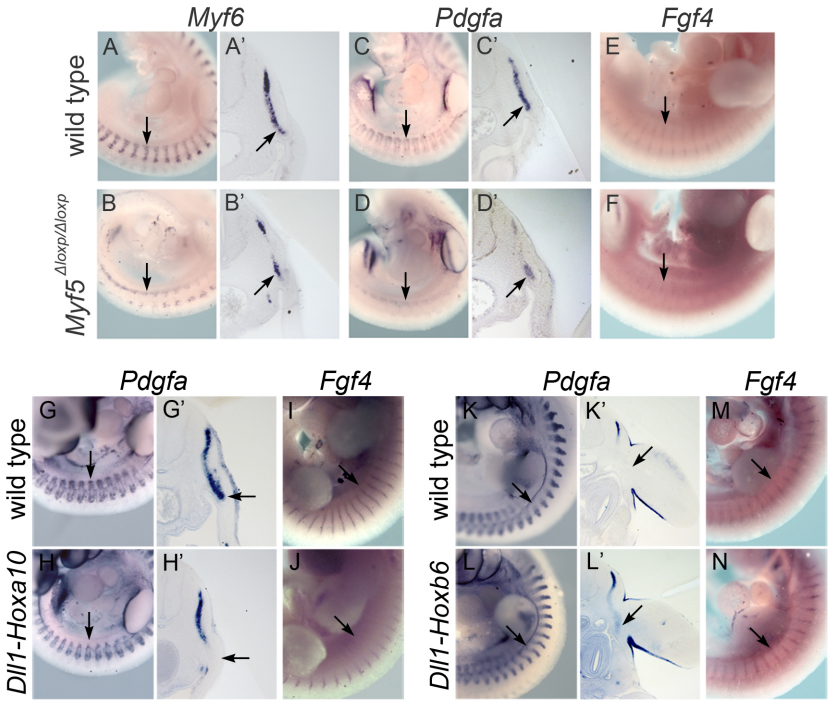

Figure 11: Hox groups 6 and 10 modulate regional expression of genes in the Myf5/6 pathway. Whole-mount *in situ* hybridization of wild-type (A, A'; C, C'; E and G, G'; I; K, K'; M), *Myf5*$^{\Delta loxp/\Delta loxp}$ (B, B'; D, D'; F), *Dll1-Hoxa10* (H, H'; J) and *Dll1-Hoxb6* (L, L'; N) mouse embryos with *Myf6* (A-B'), *Pdgfa* (C-D'; G-H'; K-L') and *Fgf4* (E; F; I; J; M; N) probes. Pictures focus on interlimb somites of *Dll1-Hoxa10* embryos and their controls and forelimb somites of *Dll1-Hoxb6* embryos and their controls. Arrows in A-F indicate the area of conserved expression and in G-N the area of differential expression. Vibratome sections are shown at the arrow level for each marker. Images correspond to embryos at E10.0 (~28-31 somites) except for panels E, F, I, J, M, N, which are E11.0 (~40 somites).

We also observed that *Pdfga* and *Fgf4* expression was conserved almost exclusively in the hypaxial myotome of interlimb somites of *Myf5*$^{\Delta loxp/\Delta loxp}$ embryos, although the levels were lower than in control specimens (Fig. 11 C-F).

Interestingly, not all *Myf5* targets were similarly affected in the Hox transgenic embryos. Myogenin (*Mgn*), a *Myf5* target gene in the myogenic cascade (Pownall *et al*. 2002), was up-regulated in the ventrolateral myotome of limb and neck somites of *Dll1-Hoxb6* transgenics but its expression was not affected in *Dll1-Hoxa10* embryos (Fig. 12 A-D). While the *Mgn* pattern observed in *Dll1-Hoxb6* embryos could result from activation by *Myf5/Myf6*, the fact that *Mgn* is not down-regulated in *Dll1-Hoxa10* interlimb hypaxial somites can be attributed to normal *MyoD* expression (Fig. 12 E, F), which is also upstream of *Mgn* (Pownall *et al*. 2002). The persistent hypaxial expression of myogenic genes like *MyoD* and *Mgn* in *Dll1-Hoxa10* transgenics is consistent with the presence of muscles in the whole circumference of the prospective thoracic area of these transgenics (Fig. 12 G). In addition, induction of an interlimb-like *Mgn* expression pattern in the hypaxial myotome at forelimb and neck levels of *Dll1-Hoxb6* transgenics is in agreement with the presence of intercostal muscles associated with the ribs in the neck of these transgenics (Fig. 12 H, I). Further analysis of the *Dll1-Hoxa10* embryos with additional myotomal markers, such as *Six1* or *Pax3*, also revealed no significant differences when compared to wild-type littermates (Fig. 12 J,K and L,M).

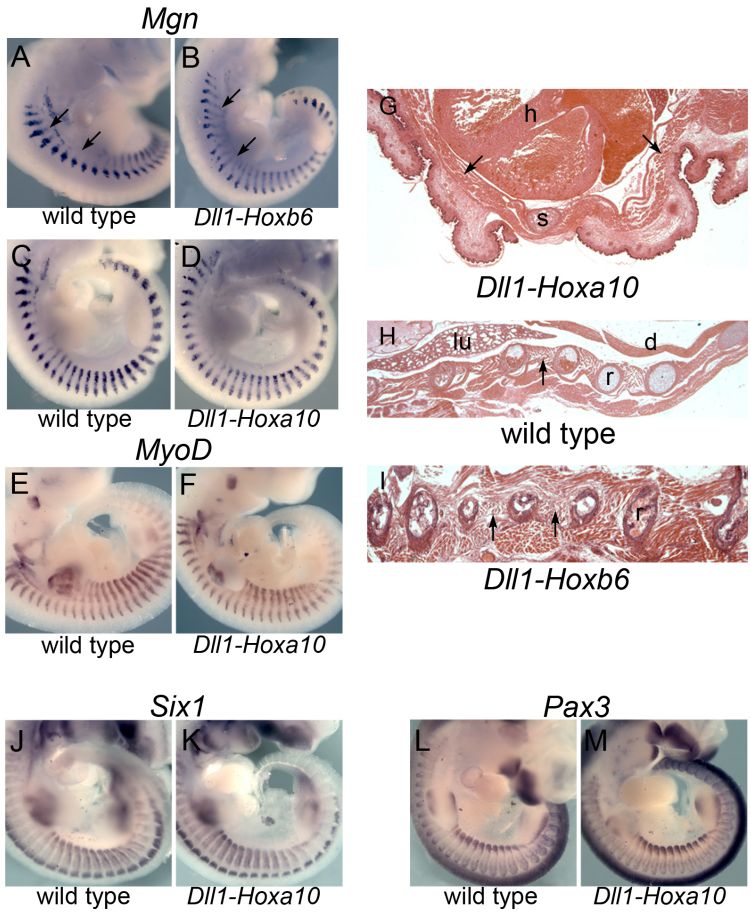

Figure 12: Myotomal and muscle analysis of Hox transgenics. Whole-mount *in situ* hybridization of E10.5 wild type (A, C, E, J, L), *Dll1-Hoxb6* (B) and *Dll1-Hoxa10* (D, F, K, M) embryos with *Mgn* (A-D), *MyoD* (E, F), *Six1* (J, K), *Pax3* (L, M). Transverse section through the thorax of a E18.5 *Dll1-Hoxa10* (G) embryo, showing muscle tissue (arrows) that reaches the most ventral part of the embryo and attaches to the sternum (s). The picture is oriented with the ventral part of the specimen at the bottom. Frontal section through the ribcage of a wild type (H) or the neck of a *Dll1-Hoxb6* transgenic embryo (I), at E18.5 showing intercostal muscles (arrows) connecting adjacent ribs (r). Pictures are oriented with rostral to the left and medial to the top. d, diaphragm; h, heart; lu, lung.

These results suggest that the effect of Hox groups 6 and 10 is quite specific for *Myf5/Myf6* and not a result of a general effect on the myotome. Furthermore, the expression of *Fgf4* and *Pdgfa* in *Dll1-Hoxa10* and *Myf5*$^{\Delta loxp/\Delta loxp}$ embryos suggests their involvement in a *Myf5/Myf6*-specific pathway associated with rib development.

HYPAXIAL *MYF6* EXPRESSION RESCUES THE RIB-LESS *DLL1-HOXA10* PHENOTYPE

To determine if the Hox-modulated expression of *Myf5*/*Myf6* is key to rib development, we tested whether *Myf6* could rescue the rib-less *Dll1-Hoxa10* phenotype when expressed in the hypaxial somite. As *Pax3* expression seems to be unaffected by *Hoxa10* (Fig. 12 L, M), we used an enhancer of this gene that promotes expression in the hypaxial somite (Brown *et al*. 2005). *Pax3Pr-Myf6* transgenic embryos showed no apparent skeletal phenotype, which was expected since the hypaxial *Pax3* enhancer reproduces the normal expression of this gene in the hypaxial somite at the different axial levels (Brown *et al*. 2005) (Fig. 13 A'-C'). For the rescue experiment we produced *Pax3Pr-Myf6*::*Dll1-Hoxa10* double transgenics. Three of the seven double transgenics generated had recognizable rib phenotypes, which were much less severe than those observed in *Dll1-Hoxa10* transgenics, with the remaining four double transgenics having a wild type-like phenotype.

While *Dll1-Hoxa10* transgenics showed strong rib phenotypes, typically a complete absence of ribs in 65% of the cases (Fig. 13 B; Table 1; Carapuço *et al*. 2005), *Pax3Pr-Myf6*::*Dll1-Hoxa10* double transgenics showed a mild alteration in the total number of ribs combined with the presence of variable abnormal patterns such as rib fusions, proximal gaps and distorted rib insertions in the sternum (Fig. 13 C, Tables 1 and 2).

These results indicates that *Myf6* expression in the hypaxial somite is sufficient to rescue the *Hoxa10*-induced rib phenotype, thus, providing further evidence of a direct contribution of this myogenic factor to the rib phenotypes obtained in the Hox transgenics and its involvement in rib development.

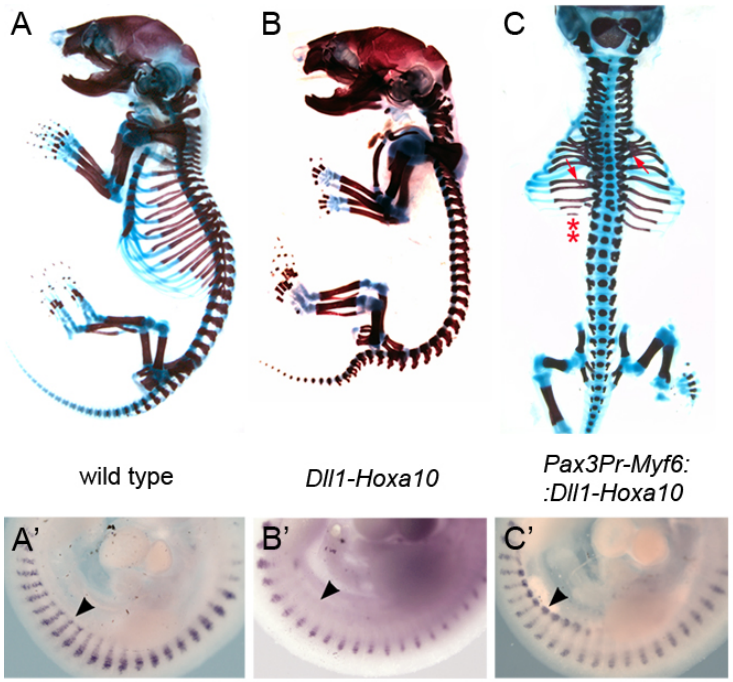

Figure 13: Rescue of the *Dll1-Hoxa10* phenotype with hypaxial *Myf6*. Skeletal staining of wild type (A), *Dll1-Hoxa10* (B) and *Dll1-Hoxa10::Pax3Pr-Myf6* (C) E18.5 fetuses. A', B' and C' show *Myf6* expression in the corresponding transgenics at E10.0.

Table 1: Comparison of the skeletal phenotype of *Pax3pr-Myf6*, *Dll1-Hoxa10* and *Pax3pr-Myf6::Dll1-Hoxa10* fetuses. Data is represented both as the number embryos showing a particular phenotype/total number embryos analyzed, and as percentages.

	Pax3pr-Myf6	***Dll1-Hoxa10***	***Pax3pr-Myf6: :Dll1-Hoxa10***
Wild type FVB/N phenotype[1]	**7/7 (100%)**	**2/14 (14.29%)**	**4/7 (57.14%)**
Thoracic rib defects	**0/7 (0%)**	**3/14 (21.43%)[2]**	**3/7 (42.86%)[3]**
Complete rib-less phenotype	**0/7 (0%)**	**9/14 (64.29%)**	**0/7 (0%)**

[1] **60% of our FVB/N derived fetuses contain a small rib in L1.**

[2] **Variable rib defects in T1, T2 and T13.**

[3] **See Table 2 for details.**

Table 2: Skeletal phenotype of *Pax3Pr-Myf6::Dll1-Hoxa10* fetuses. Description of the skeletal abnormalities of the three affected *Pax3Pr-Myf6::Dll1-Hoxa10* out of a total of seven individuals.

	Number of Ribs	Sternal insertion	Proximal rib fusions	Proximal Gaps
#1	12 right side 10 left side	Distorted	Several fusions at different levels	----------
#2	14 right side 12/13 (fused) left side	Distorted	The most caudal ribs are fused together	Proximal gap in T2/T3
#3	12 right side 11 left side	Normal	----------	Proximal gap in T2 /T12

BINDING OF HOX GROUPS 6 AND 10 PROTEINS TO AN ENHANCER THAT DRIVES HYPAXIAL EXPRESSION OF *MYF5*

Among the different control regions that have been described for *Myf5*, an enhancer was identified that drives expression in the somitic domain affected in our Hox transgenics (Buchberger *et al*. 2007; Bajard *et al*. 2006; Giordani *et al*. 2007). The homology element 1 (H1) of this enhancer (Buchberger *et al*. 2007), also known as 147 bp enhancer (Bajard *et al*. 2006), contains the sequence CTAATTG, which fits with predicted target sequences for Hoxb6 and Hoxa10 (Noyes *et al*. 2008). This potential Hox-binding site seems to be important for enhancer activity according to transgenic reporter assays (Buchberger *et al*. 2007).

To test if our candidate Hox proteins bind this enhancer *in vivo*, we performed Chromatin Immunoprecipitation (ChIP) experiments on PSM isolated from mouse embryos. We could consistently immunoprecipitate the H1 enhancer element but not other genomic areas using specific antibodies for both Hox group 6 and 10 proteins (Fig. 14 A). This result indicates a physiological positioning of these Hox proteins at a genomic region that drives *Myf5* expression in the hypaxial myotomal domain and is consistent with a Hox-mediated regulation of *Myf5* in this embryonic region.

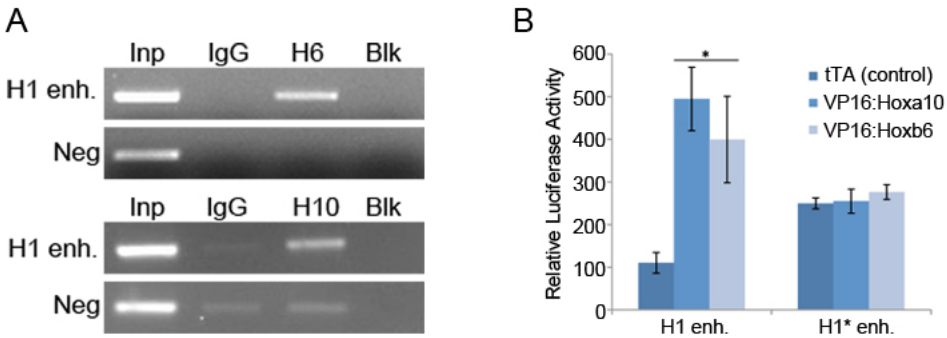

Figure 14: *Myf5/Myf6* as functional targets of Hox groups 6 and 10 genes. A. ChIP from PSM of E9.5 wild-type mouse embryos using antibodies against Hoxc6 (H6), Hoxa10 (H10) or an unspecific control IgG (IgG), and PCR amplification of the Homology 1 enhancer element (H1) and negative control region (Neg). Inp, input; Blk, blank. These results are representative of three independent experiments. B. Luciferase activity from wild type and mutated H1 enhancer (H1enh. and H1*enh., respectively), driven by VP16:Hoxa10, VP16:Hoxb6 or the tetracycline transactivator (tTA), as a control. The activation from the H1 enhancer is statistically significant (VP16:Hoxa10 $p<0.01$ and VP16:Hoxb6 $p<0.04$). The values are presented as the mean and standard error of the triplicates from a representative experiment.

When tested using a luciferase reporter assay in cultured cells, both Hoxa10 and Hoxb6 fused to VP16 activated transcription from the wild type H1 enhancer, but not from a mutant version of this element lacking the Hox-binding site (Fig 14 B), further validating the capability of Hox proteins to bind to the CTAATTG sequence of the H1 enhancer. The mutant version of H1 used in these experiments still contained intact the Pax3 and Six1-binding sites also present in this enhancer, indicating that the CTAATTG site is the main target sequence for Hox proteins in this regulatory element.

IMPAIRED MYF5 HYPAXIAL EXPRESSION IN H1-MUTATED BAC REPORTER LINE

The study of the different regulatory elements that contribute to specific features of the full expression pattern of a particular gene has typically involved analyzing the expression driven by each individual element separately. However, this approach neglects the importance of the genomic

context in which these elements are integrated, as well as potential interactions between different regulatory elements.

We were interested in understanding the relative importance of specific binding sites to the activity of hypaxial enhancers in the physiological context of the *Myf5/6 locus*. Moreover, we wanted to develop the tools to study how each of these enhancers behaves individually and in association with other regulatory elements. To do this, we used a BAC reporter (B195-APZ), which includes the complete regulatory sequences of the *Myf5/6* locus and incorporates the LacZ reporter into the *Myf5* gene and the AP reporter into the *Myf6* gene (Carvajal *et al* 2001), as a template to modify specific sites by homologous linear recombination. We started by inserting a 22-mer oligonucleotide upstream the H1 element (erB195-APZ) to allow differentiation from the endogenous sequence. This new BAC template was used to mutate the CTAATTG Hox-binding site in the H1 element. In addition, we mutated another similar putative Hox binding site, located 95Kb upstream the *Myf5* transcriptional start site (from here on named P95 enhancer), within a genomic region that has been suggested to include early *Myf5* hypaxial enhancers (Carvajal *et al* 2001, J. Carvajal – personal communication). BACs carring mutations in both Hox binding sites were also constructed (Fig. 15).

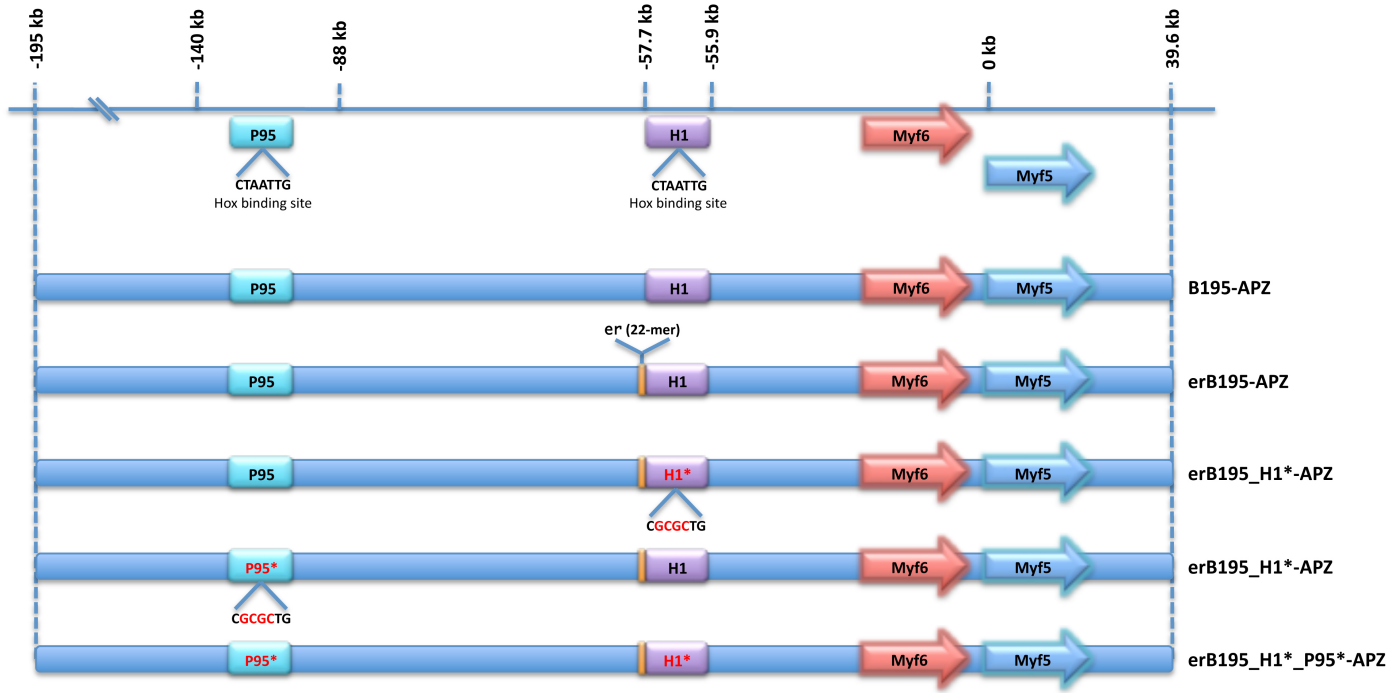

Figure 15: Schematic representation of *Myf5/6* BAC reporters. Physical map of the genomic region included in each reporter. Distances upstream (negative) and downstream (positive) of the transcriptional start site of *Myf5* are indicated. *Myf5* gene is represented in blue alluding to the LacZ reported inserted into its' sequence and Myf6 is represented in red as a reference to the AP reporter inserted into its' genetic sequence. The P95 prospective enhancer is represented in light blue, whereas the H1 enhancer is represented in purple. Modifications from the original B195-APZ are indicated as follows: in the erB195-APZ was inserted a 22-mer sequence; the erB195_H1*-APZ has a mutant Hox binding site in the H1 enhancer, erB195_P95*-APZ has a mutant Hox binding site in the P95 enhancer, and erB195_H1*_P95*-APZ which includes mutations in the Hox binding sites of both enhancers.

To this date, we have been able to establish a reasonably good BAC reporter line that has a mutated Hox-binding site in the H1 enhancer (*erB195_H1*-APZ*). We used *B195-APZ L20* embryos (kindly given by J. Carvajal) as preliminary controls to evaluate the β-galactosidase activity in this transgenic line. However, these are not ideal controls because they lack some aspects of *Myf5* expression. Nevertheless, focusing on the somitic expression, which is the most relevant for this work, we observed that in our *erB195_H1*-APZ* embryos, *Myf5* expression – as analyzed by β-galactosidase staining – was specifically down-regulated in the hypaxial myotome of interlimb-level somites. In these embryos, with the exception of the limb domain, which also requires the intact Hox binding site (Buchberger *et al*. 2007), the remaining aspects of *Myf5* expression remained mostly unaltered. In particular, the expression in the epaxial somite, branchial arches, and brain was maintained (Fig. 16, Table 3). These results confirm that the Hox binding site within the H1 enhancer is crucial to drive a correct *Myf5* expression in the hypaxial myotome (Fig. 16), even in the context of the context of the *Myf5* genomic environment provided by the *B195-APZ* BAC. Currently, efforts are being made towards establishing the remaining lines (Table 3).

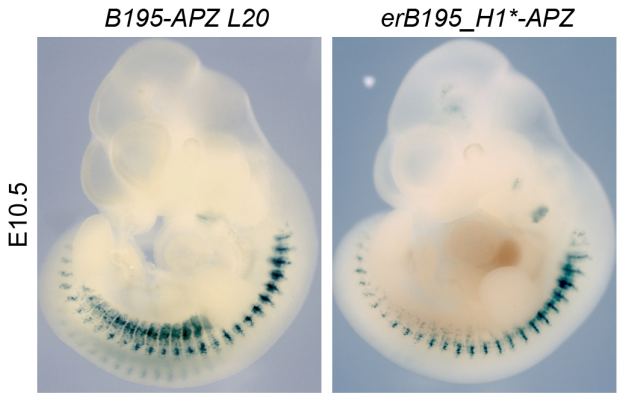

Figure 16: β-galactosidase staining of *B195-APZ L20* (control) and *B195_H1*-APZ* E10.5 embryos. *Myf5* expression in the hypaxial myotome of er*B195_H1*-APZ L20* embryos is severely reduced compared to controls, whereas expression in the epaxial myotome remains normal.

Table 3: Summary of the expression profile for all BAC reporter lines.

Constructs	Founders	Expression of Transgenes					
		Epaxial Myotome	Hypaxial Myotome	Branchial Arches	Brain	Limbs	Comments
erB195_H1-APZ*	#137[m]	-	-	-	-	-	
	#1[f]	N.A.	N.A.	N.A.	N.A.	N.A.	Currently analyzing F1's
	#173[f]	-	-	-	-	-	
	#326[f]	-	-	-	-	-	
erB195_H1-APZ*	#1[m]	+	-	+	faint	-	Mosaic founder. Currently indentifying consistent F1's
	#11[f]	N.A.	N.A.	N.A.	N.A.	N.A.	Currently analyzing F1's
erB195_P95-APZ*	#11[f]	N.A.	N.A.	N.A.	N.A.	N.A.	Currently analyzing F1's
*erB195_H1*_P95*-APZ*			N.A.				Soon to be injected

[m] **Male**

[f] **Female**

ALTERED HYPAXIAL EXPRESSION OF *LBX1* IN *DLL1-HOXA10/B6* TRANSGENICS

Ladybird homeobox gene (*Lbx1*) is a homeobox transcription factor that has been implicated in several biological functions, including epithelial-to-mesenchimal transition, and the migration of muscle precursor cells (MPCs) to the developing limb (Brohmann *et al*. 2000; Gross *et al*. 2000; Yu *et al*. 2009). In *Lbx1*-deficient mice migration of the lateral, but not ventral, hypaxial muscle precursors to the limbs is compromised, which resulted in a strong deficiency in the development of limb muscles (Brohmann *et al*. 2000; Gross *et al*. 2000).

At E10.5, *Lbx1* is normally expressed in the hypaxial dermomyotomal lip of forelimb and hind-limb level somites, while it is completely absent from interlimb level somites (Fig. 17 A, C). This regional distribution of *Lbx1* expression is somehow complementary to the expression domains of *Myf5/Myf6* that we have shown to be under Hox regulation. Therefore, we decided to analyze whether the expression of this gene was also affected in our Dll1-Hox transgenics.

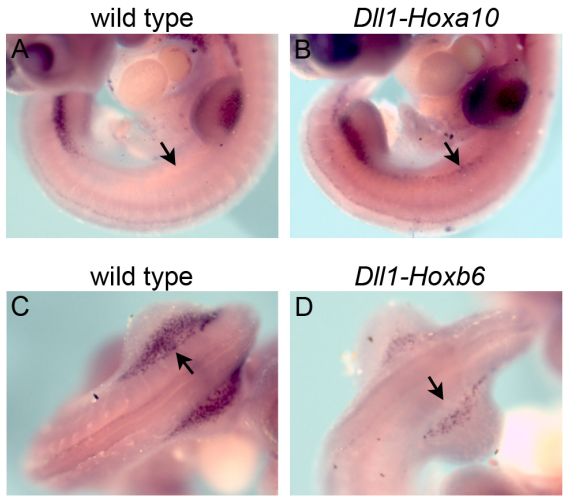

Figure 17: *Lbx1* regional expression is altered in Dll1-Hox transgenics. *Lbx1* is normally expressed in the hypaxial lip of neck and limb-level somites (A, C, arrows). In *Dll1-Hoxa10* embryos, *Lbx1* is up-regulated specifically in interlimb-level somites (B, arrow). Conversely in *Dll1-Hoxb6* transgenics, *Lbx1* is severely down-regulated in limb-level somites (D, arrow).

In *Dll1-Hoxa10* transgenics we observed ectopic expression of *Lbx1* in the ventral-most hypaxial lip of interlimb somites (Fig. 17 B), whereas in *Dll1-Hoxb6* embryos we saw a severe down-regulation of *Lbx1* in the limbs and limb-associated somites (Fig. 17 D). This indicates that *Lbx1* expression is also regulated by Hox genes, although with a pattern complementary to that of *Myf5/6*, and could indicate the involvement of *Lbx1* in the processes leading to rib formation.

We have shown that *Lbx1* is antagonistically regulated by Hoxa10 and Hoxb6, which raises the hypothesis of a potential functional role of this gene in the processes regulating rib formation. However, given the primary myotomal function of this gene, the question of whether this is a crucial or a rather secondary role remains to be determined. We decided to investigate this question by expressing *Lbx1* under the control of the previously mentioned *Pax3* hypaxial promoter. If *Lbx1* is a key regulator of rib development, similarly to *Myf5/6*, then the prediction would be that by over-expressing it in the hypaxial interlimb somites we could recapitulate at least some characteristics of the *Dll-Hoxa10* ribless phenotype. Skeletal analysis of *Pax3Pr-Lbx1* transgenics showed that both the number of ribs and the general axial formula were correct (Fig. 18 A-C), indicating that *Lbx1* up-regulation in the hypaxial dermomyotomal lip, at least on its own, seems not to be sufficient to block rib formation. However, we could see significant alterations in rib morphology in some of these embryos, mostly including distal fusions and misshaped ribs.

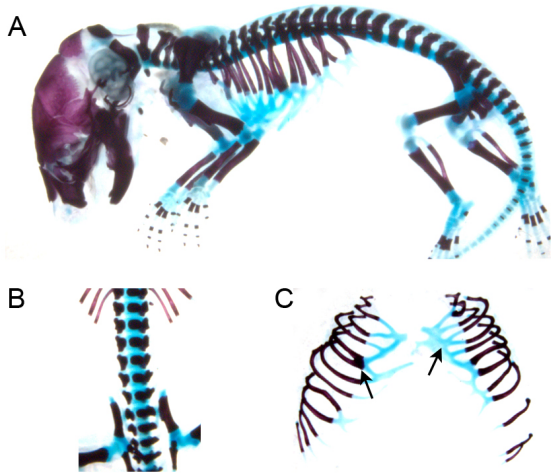

Figure 18: *Lbx1* expression in hypaxial interlimb somites results in mild rib defects. Skeletal staining of E18.5 *Pax3Pr-Lbx1* (A-C). A. Whole skeleton view. B. Lumbar detail showing normal number of lumbar vertebrae. C. Thoracic detail showing different types of distal rib fusions (arrows) and absence of the sternum.

Interestingly, in the most strongly affected *Pax3Pr-Lbx1* transgenic embryo we observed a complete absence of the sternum, a phenotype that together with the rib malformations resembles the *Six1*-null skeletal phenotype (Laclef 2003), another example of a mutation on a myogenic gene that results in rib defects, resuscitating old questions about how independent are early myotome and sclerotome differentiation. These results indicate that, while hypaxial up-regulation of *Lbx1* in interlimb somites is not enough to explain the *Dll1-Hoxa10* rib phenotype, it is possible that this gene is part of the regulatory network controlling rib development downstream of Hox genes.

II.IV Discussion

In this study we show that specification of global vertebral domains in the vertebrate axial skeleton is controlled by the balanced activity of different Hox genes. It had been previously shown that Hox groups 10 and 11 play essential roles in the patterning of the lumbar and sacral regions, respectively (Wellik and Capecchi, 2003; Carapuço *et al*. 2005). Our results

now indicate that Hox genes of the paralog group 6 are able to provide the instructions to generate the thoracic area. According to our data, the presence of ribs is not a default state (Wellik and Capecchi, 2003) but rather the result of a positive activity of Hox genes that triggers processes leading to rib induction. In caudal areas Hox group 10 proteins override this activity to generate the rib-less areas of the skeleton. In our model, the cervical domain is passively determined as the region anterior to the start of the rib-determining Hox activity (Fig. 19). Surprisingly, we found that the primary target of the rib-forming/rib-blocking activities of Hox genes does not seem to be the sclerotome, but rather specific genes expressed in the myotomal compartment. In particular, we show that the primary targets of Hox genes are *Myf5* and *Myf6* specifically in the hypaxial myotome. This implies a non-myogenic function of *Myf5*/*Myf6* that controls rib development. The role of *Myf5* in rib formation has been a matter of controversy. Initial studies pointed to *Myf5* as a central player in the processes leading to rib development (Braun *et al*. 1992). However, when other *Myf5* mutants were produced that exhibited no rib defects (Kaul *et al*. 2000), it was suggested that the rib determining factor was not *Myf5* itself but another gene somehow linked to it. A decade later, such a gene has not been identified and recent new data once more associated *Myf5* with rib development (Haldar *et al*. 2008; Gensch *et al*. 2008). Among the genes located close to *Myf5* in the genome only *Myf6* stands out as a candidate to be involved in rib development since rib phenotypes have been described in some mutants for this gene (Braun and Arnold 1995; Patapoutian *et al*. 1995; Zhang *et al*. 1995).

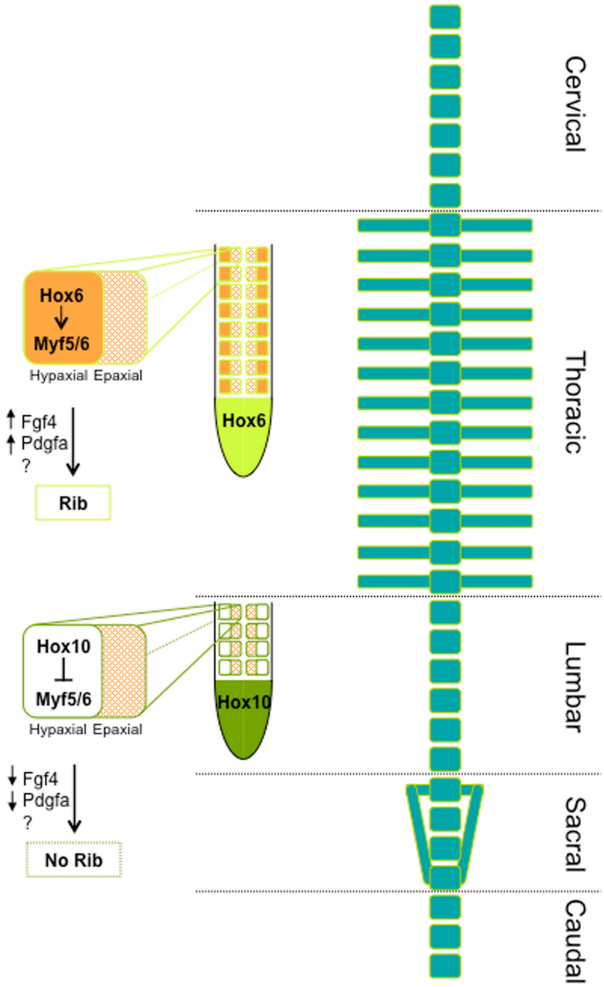

Figure 19: Hox groups 6 and 10 specify global vertebral domains. Schematic representation of Hox-mediated specification of the different vertebral domains of the axial skeleton. On the right panel, the adult cervical, thoracic, lumbar, sacral and caudal vertebral regions of the skeleton are displayed. The left panel shows a representation of the forming somites at different levels. In the prospective thoracic somites, Hox group 6 is activated (light green), thereby up regulating *Myf5* and its downstream effectors *Pdgfa* and *Fgf4* in the hypaxial fraction of the myotome (orange), ultimately leading to rib formation. In the prospective lumbar-caudal somites, Hox group 10 is activated (green), resulting in the down regulation of *Myf5*, *Pdgfa* and *Fgf4* in the hypaxial myotome (white), leading to inhibition of rib formation in those vertebrae.

Interestingly, rib deficiencies have been observed only when inactivation of either *Myf5* or *Myf6* also affected expression of the other gene (Braun and Arnold 1995; Patapoutian *et al*. 1995; Zhang *et al*. 1995; Braun *et al*. 1992; Tajbakhsh *et al*. 1996; Yoon *et al*. 1997; Kassar-Duchossoy *et al*. 2004).

This suggests that *Myf5* and *Myf6* have redundant functions in rib formation and that it is the double inactivation of both genes that causes the rib phenotypes in particular *Myf5* and *Myf6* mutants, rather than the effects on an additional rib-determining gene in the *Myf* genomic area. Our results with both *Dll1-Hoxa10* transgenics and $Myf5^{loxp/loxp}_{\Delta}$ mutants are fully consistent with this hypothesis. In addition, the involvement of the Myf factors in rib development is also supported by the ability of *Myf6* to rescue the rib-less *Dll1-Hoxa10* phenotype, when expressed in the hypaxial somite. Our observations that Hox-driven information seems to be first interpreted by a specific population of myotomal *Myf5*/*Myf6*-expressing cells could indicate that these cells can directly contribute to the ribs. However, while cell tracing experiments have shown contribution of *Myf5*-expressing cells to the ribs (Gensch *et al*. 2008; Haldar *et al*. 2008), they seem to represent a rather small fraction of the rib chondrocytes to fully explain *Myf5* contribution to rib development. In addtion, *Myf6*-expressing cells were not found in the sclerotomal compartment using a similar cell tracing strategy (Haldar *et al* 2008). Therefore, it seems more likely that the *Myf5*/*Myf6*-expressing cells convey their rib-forming information to the sclerotome through a cell non-autonomous mechanism. Our results suggest that members of the FGF and PDGF signaling pathways are involved in this mechanism, an idea that is also supported by genetic studies consistent with the participation of FGFs and PDGFs in rib formation. In particular, inactivation of *Pdgf*α receptor resulted in severe rib anomalies (Soriano 1997) and insertion of a *Pdgfa* cDNA in the *Myf5* locus significantly rescued the *Myf5* rib phenotype (Tallquist *et al*. 2000). The involvement of *Fgf4* in rib formation has not been genetically addressed but a variety of experiments performed in chicken embryos suggest that FGF signaling is important for rib formation (Huang *et al*. 2003). Altogether, these results strongly suggest that FGF and PDGF signaling are important components of the mechanism that transmits patterning information from *Myf5*/*Myf6* to the sclerotome.

Regulation of *Myf5*/*Myf*6 by Hox genes may be a complex process. While the activity of *Hoxa10* and *Hoxb6* seems to be required before somites are formed, their effect is only detected at a later developmental stage in a specific somitic domain. This observation seems to be at odds with a simple transcriptional activation (Hoxb6) or repression (Hoxa10) mechanism, as is the normal expression of *Myf5* and *Myf6* in the tail tip of *Dll1-Hoxb6* transgenics (data not shown). Therefore, Hox proteins must functionally interact with other factors to modulate spatial and temporally specific activity of the *Myf5*/*Myf6* regulatory region. Pax3 and Six1/4 are likely candidates to be involved in this process, as they also interact functionally with the H1 enhancer through binding sites located at both sides of the Hox site (Giordani *et al*. 2007; Bajard *et al*. 2006). Interestingly, expression of a dominant negative version of Pax3 from the *Pax3* locus down-regulated *Myf5* and *Myf6* expression in the hypaxial myotome of interlimb somites without affecting other myogenic factors like *MyoD* or *Mgn* (Bajard *et al*. 2006), which resembles our observations in *Dll1-Hoxa10* transgenics. This suggests that *Hoxa10* activity could involve functional inactivation of *Pax3*. If this is the case, it cannot occur at the transcriptional level, as *Pax3* expression seemed normal in *Dll1-Hoxa10* transgenics. Direct competition for binding to the enhancer is also unlikely because *Hoxa10* activity is observed when this gene is expressed in the PSM and not in the somites (Carapuço *et al*., 2005), and *Pax3* is only expressed in the somites. A similar spatial-temporal gap is observed between *Pax3* expression and *Hoxb6* activity in the transgenics. This suggests a sequential activity of Hox proteins and Pax3 (and probably Six1/4) to activate *Myf5*/*Myf6* expression in the hypaxial myotome. A possible scenario is that Hox proteins provide a label to the *Myf5*/*6* hypaxial enhancer, which would promote (Hoxb6) or block (Hoxa10) binding and/or activation by Pax3 later in the differentiating somite, eventually regulating *Myf5*/*Myf6* expression. Interestingly, a “label based” mechanism to modulate of cell type-specific recruitment of

transcription factors to distal enhancers has been recently reported (Lupien *et al*. 2008). Experiments are currently in progress to test if Hox/Pax3 interactions are also mediated through an equivalent mechanism. Of note, interactions between Hox and Pax proteins with differential functional outcomes have also been described for other members of the Hox and Pax families (Yallowitz *et al*. 2009). Therefore, Hox-Pax functional interactions could be a general theme in vertebrate development.

It has been suggested that regulation of hypaxial *Myf5* expression by *Pax3* might require, in addition to the H1 enhancer, other still not identified earlier acting elements (Bajard *et al*. 2006). Similarly, it is possible that Hox-mediated modulation of *Myf5*/*Myf6* expression in the hypaxial myotome could involve additional components, which is consistent with the complex regulation of the *Myf5/Myf6* locus (Carvajal *et al*. 2008). A probable location for such elements is the genomic region between 88 and 140 kb upstream of the *Myf5* gene, which has been reported to contain early hypaxial enhancers (Carvajal *et al*. 2001). Consistent with this, the Rigby laboratory has recently identified a potential hypaxial enhancer located around 95kb upstream of the *Myf5* transcriptional start site, here named P95 enhancer, which also contains a Hox binding site (J. Carvajal, personal communication). We are currently analyzing the effect of both individual and collective mutations in the H1 and P95 enhancers on *Myf5* expression using BAC reporter transgenics. The preliminary data we collected on the BAC line bearing a mutation in the Hox binding site of the H1 enhancer, suggests that this binding site is necessary for the expression of *Myf5* in the hypaxial myotome and in the limbs. Although β-galactosidase activity in this BAC reporter line is not as strong as in others reported elsewhere (Carvajal *et al* 2001), it shows all the correct elements of *Myf5* expression, except for those affected by the H1 mutation, and validates previous observations made using regular transgenic reporter lines (Buchberger *et al*. 2007). We expect that the analysis of the BAC transgenic lines produced here will

provide insights into the interactions between the different enhancers that regulate the complex expression elements of the *Myf5/6* locus, and will help understand how transcription factors like Hox6/10, Pax3 or Six1/4 (among others) modulate the activity of these enhancers and their interactions.

The paradoxical involvement of myogenic-related factors in rib formation is far from being resolved. Here we provide additional evidence that *Myf5/6* are important in rib development. Mutations in other myogenic factors, including *myogenin*, *Six1* and *Pax3* have also been reported to give rib phenotypes (Dickman *et al*. 1999; Laclef 2003; Hasty *et al*. 1993; Henderson *et al*. 1999; Nabeshima *et al*. 1993; Vivian *et al*. 2000), indicating that they might also somehow affect rib formation. Additionally, we show here that another myogenic gene, *Lbx1*, could also play a role in the control of rib development, since its expression is altered in our Hox transgenics. It is still unclear whether the Hox-mediated regulation of *Lbx1* is direct or indirect, and very little is know about this gene's regulation. However, we searched for phylogenetically conserved potential Hox binding sites in the *Lbx1* promoter and found one that seems promising. We preformed band-shift assays to determine whether Hoxb6 and Hoxa10 could bind this site of the *Lbx1* regulatory region *in vitro* and obtained very preliminary results suggesting that they can (data not shown). If these results are confirmed, they could point to a direct Hox/Lbx1 interaction, which would be interesting to explore because it would mean that Hox6 and Hox10 proteins could have opposite activities in two different enhancers/promoters, i.e., Hox6 would promote the activation of *Myf5/6* and the repression of *Lbx1* in limb-level somites, while Hox10 would repress *Myf5/6* and activate *Lbx1* in interlimb-level somites. Nevertheless, an indirect relationship between Hox genes and *Lbx1* is also a possibility. A study in *Xenopus* has determined a correlation between *Lbx1* expression and *Myf5* activation in epaxial muscle formation (Martin and Harland 2006). It would be interesting to investigate how *Myf5/6* and *Lbx1* interact in the processes leading to rib development.

Another noteworthy aspect is that the finding that *Lbx1* expression is specifically modulated in our Hox transgenics, builds up the argument that Hox genes seem to be involved in a regional-specific control of expression/function of specific genes in the hypaxial myotome (limb vs. interlimb), which is critical for rib formation.

The complete answer to this fascinating predicament could require a better understanding the embryonic origins of both muscle and bone. Classical studies pointed to a complete independence between myotome-derived and sclerotome-derived structures. However, while some reports suggest that ribs exclusively develop from the sclerotome (Huang *et al* 2000), others indicate that the distal-most sternal part of ribs originate from the ventrolateral dermomyotome (Kato and Aoyama, 1998), suggesting potential interactions between the developing ribs and intercostal muscles during their migration and differentiation (Brent and Tabin 2002, Sudo *et al* 2001). This work provides additional evidence of the requirement of these interactions to the proper development of ribs.

The Hox-mediated patterning process we describe in this manuscript serves as a mechanism for the establishment of global vertebral domains (i.e., cervical, thoracic, lumbar) through the specification of rib-containing and rib-less areas of the skeleton. Whether Hox genes use a similar mechanism to specify the individual features that characterize the different vertebrae, or this is elicited by direct control of sclerotomal development, remains to be determined. However, the primary involvement of myotomal components in the specification of global vertebral domains provides an evolutionarily efficient mechanism that ensures the concomitant evolution of the ribs and their associated muscles, to produce animals with properly organized axial musculoskeletal systems. Curiously, rib development in turtles follows a plan that differs from that typically observed in other amniotes, resulting in the formation of the carapace. This specific rib development is associated with turtle-specific *Myf5* hypaxial expression in the trunk (Ohya *et al*. 2006)

and development of specific muscle attachments (Nagashima *et al*. 2009), further suggesting the importance of the *Myf5*-rib connection in the evolution of the body plan.

II.V Materials and methods

Plasmid-based Constructs

The *Dll1-Hoxa10* construct was previously described (Carapuço *et al*. 2005). The *Sm-Hoxb6* and *Dll1-Hoxb6* constructs were generated by insertion of the human *Hoxb6* cDNA (IMAGE: 4548382) downstream of the *Sm* (Carapuço *et al*. 2005) and *Dll1* (Beckers *et al*. 2000) enhancers, respectively, and upstream of the SV40 polyadenylation signal. The *Pax3Pr-Myf6* and *Pax3Pr-Lbx1* constructs were generated by cloning the *Myf6* (IMAGE: 8733960) and *Lbx1* cDNAs (IMAGE: 8733926) downstream the hypaxial enhancer of the *Pax3* gene (Brown *et al*. 2005) and upstream of the SV40 polyadenylation signal.

BAC constructs

BAC modifications were done by homologous linear recombination. Briefly, linear recombination cassettes containing the mutated region flanked by two arms with homology to the region of interest of the original BAC B195-APZ were produced using a two-stage PCR-based mutagenesis approach (Fig. 20). In this strategy, two DNA fragments with overlapping ends (the 3' end of one fragment overlaps with the 5' end of the other) containing the desired mutation were amplified separately in a first PCR round. These fragments were then used to generate a single extended fragment in an initial pre-amplification round, followed by a second PCR amplification reaction using external primers to produce the final DNA fragment containing the targeted mutation. Recombination cassettes were denatured, ethanol-precipitated, and resuspended in 20 μL of ice-cold H_2O.

The BAC recombineering was preformed as described elsewhere (Carvajal *et al* 2001). Briefly, an overnight pre-culture of DY380 cells containing the BAC clone to be modified and the temperature-inducible λ-recombinase system were incubated overnight at 30ºC. 200µL of these cultures were used to inoculate 10mL of selective medium (LB with 12.5µg/µL Chloramphenicol) and incubated at 32°C with vigorous shaking (>300 rpm) until OD600 was 0.6 ± 0.05. Cultures were induced to express the recombination function in a 42°C waterbath for 15 minutes. Cultures then incubated on ice for 20 minutes, made electrocompetent by standard methods, and resuspended in a final volume of 40 µL.

The denatured cassettes (250-700ng) mixed with the 30 µL of electrocompetent DY380 cells carrying the BAC clone to be modified were electroporated using the Bio-Rad GenePulser™ system (1.75 kV, 200 ohms & 25 µF). Electroporated cells were diluted 1:10^6 into a final volume of 50 mL of LB and aliquoted into a single 96-well plate (500 µL per well), resulting in an average of 10–50 cells per well, and incubated overnight at 32°C. Pools were screened using the appropriate primers (Table 4). Two to four positive pools were plated to obtain single colonies, screened again to identify single positive clones. Clones were sequenced using one internal primer for the mutation and one primer external to the recombination cassette.

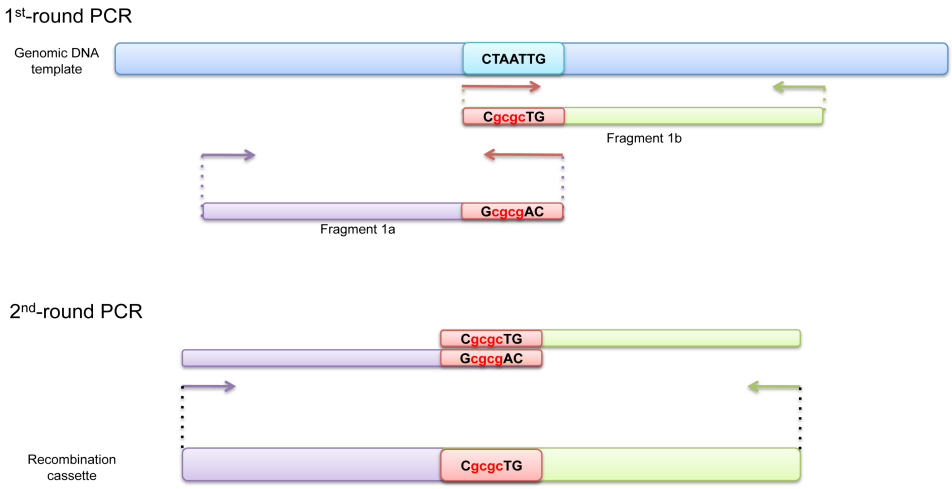

Figure 20: Schematic representation of the two-stage PCR strategy used to produce the BAC recombination cassettes.

Transgenic and mutant mice

Transgenic embryos were produced by pronuclear injection according to standard methods. All transgenic mice used in this work have a FVB/N genetic background. Of note, normal fetuses derived from our FVB/N colony present a slight deviation from the typical axial formula, as they contain a small rib in L1 with a penetrance of about 60%, which is also observed in non-affected transgenics with this genetic background.

The *Myf5*$^{loxp/loxp}_{\Delta}$ mutants have been previously described (Kaul *et al*. 2000).

Genotyping

Embryos (E9.5-E11.5) were genotyped using yolk sac's DNA. The embryos were dissected, the yolk sacs collected and incubated overnight in 50µL of lysis buffer (50mM KCl, 10mM Tris-HCl pH8.3, 2mM $MgCl_2$, 0.45% Tween-20, 0.45% NP40) with 200µg/mL of proteinase K at 50°C. The proteinase K was inactivated by heating the lysates at 95°C for 30 min and 2µL of the lysate was used directly for genotyping by PCR (see primers' details in Table 4).

Fetuses at E18.5 and pups at 3 weeks post-partum were genotyped using DNA obtained from a either a portion of the intestines or the tail tip, respectively, which was treated with 0.5mL of Laird's buffer (100mM Tris-Hcl pH8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl) containing 100μg/mL of proteinase K, at 50°C, overnight. The DNA was precipitated with 0.5 mL of isopropanol and dissolved in 500μL of TE buffer pH 8.0 at 37°C for 3 hours, and 2μL of the lysate was used for the PCR reaction (see primers' details in Table 4).

Table 4: Details of primers used to genotype plasmid-based and BAC transgenics

	Primer Sequence	**Annealing temperature**
Dll1-Hoxa10	Hoxa10_F 5' AGCGAGTCCTAGACTCCACGC 3' Hoxa10_R 5' GTCCGTGAGGTGGACGCTACG 3'	65ºC
Sm/Dll1-Hoxb6	Hoxb6_F 5' TAGGTCGGAGCACTGTCGTCC 3' Hoxb6_R 5' CGAACACGCTCTTGTCCTGCG 3'	65ºC
Pax3Pr-Myf6	Myf6-genot-F AGACTGCCCAAGGTGGAGAT Myf6-genot-R AATGTTCCAAATGCTGGCTG	55ºC
Pax3Pr-Lbx1	Lbx1-gen-F2 GCACTCCAACAAGCCGCTGACGCC Lbx1-Gen-R GAGCATTGGTGAGGCCCAGCTGC	65ºC
erB195-APZ	H1-Rec-check-F GCCTGCCTTTAACGCAGTGTC X-rev CGATGCAGTTCGTCCTAGATG	60ºC
erB195_H1*-APZ	X-for CATCTAGGACGAACTGCATCG H1-mut-genot-R CGCTCCAGTTACCATGCAGCGCG	60ºC
erB195_P95*-APZ	P95-mut-genot-F CACCCGAACACAAAGCGCGCTG P95R CAATTGCTCCCTCTACTCTAACATCTGT	59ºC

β-galactosidase Staining

Embryos at E10.5 were dissected and fixed in Mirky's Fixative overnight at 4ºC, washed 3 times for 10 minutes in Wash Buffer (0.02% Tween-20 in PBS) and stained with Staining solution (5mM K3Fe(CN)6; 5mM K4Fe(CN)6.3H2O; 2mM MgCl2; 0.4mg/mL X-gal; 0.02% Tween-20/NP40) at 37ºC protected from light from 5 hours to overnight, depending on the embryonic stage. The stained embryos were post-fix in Mirky's overnight at 4ºC and stored indefinitely at 4ºC.

Skeletal Staining

Skeletal preparations were made using the alcian blue/alizarin red staining method (Mallo and Brändlin 1997). Briefly, fetuses were dissected at E18.5, eviscerated, skinned and fixed in absolute ethanol. The cartilaginous skeleton was stained for 12-24 hours in an alcian blue solution and fixed again in absolute ethanol overnight. Soft tissues were digested in a 2% Potassium Hydroxide solution for 6 hours after which the bony skeleton was stained in an alizarin red solution. The embryos were cleared 2% Potassium Hydroxide solution for 6 hours and stored in 25% glycerol.

In situ Hybridization

Whole mount *in situ* hybridization (ISH) was performed as described elsewhere (Kanzler *et al*. 1998) All *in situ* probes used in this work were DIG-labeled, antisense RNA probes (see Table 5 for probe information**)**. ISH-stained embryos were embedded in gelatin/albumin and sectioned with a vibratome.

Table 5: Details of the RNA probes used for *in situ* hybridization.

cDNA	Source	Backbone	Linearized	RNA Polymerase	Size
Hoxc10	RT-PCR	pKS	EcoRI	T3	1091
Myf5	Kindly given by J. Carvajal and P. Rigby	pKS	XbaI	T7	350
Myf6	IMAGE: 8733960	pCR4-TOPO	NotI	T3	840
Pdgfa	IMAGE: 3495629	pCMV-Sport6	EcoRI	T7	5424
Fgf4	Kindly given by G. Martin	pKS	BamHI	T3	620
Lbx1	Sub-cloned from IMAGE: 8733926	pKS	EcoRi	T7	1000
Pax1	RT-PCR	pKS	HindIII	T7	300
Pax3	Kindly given by P. Gruss	N.A.	NotI	T3	N.A.
Pax9	Kindly given by B. Herrmann	pKS	SalI	T7	900
Meox2	IMAGE: 3591924	pCMV-Sport6	EcoRI	T7	2229
Six1	Kindly given by N. Bobola	pKS	ClaI	T7	623
MyoD	Kindly given by E. M. Fuechtbauer	pKS	SpeI	T7	1700
Myogenin	Kindly given by A. Kispert	pKSII	NotI	T7	1000

Chromatin Immunoprecipitation

The ChIP assays were preformed according to a protocol described elsewhere (Kutejova *et al*. 2008) with some modifications. Briefly, PSM from E9.5 mouse embryos were dissected in PBS and fixed in 1% formaldehyde for 23 minutes at 4°C. The embryonic tissues were disintegrated with a 25-gauge needle and the sonicated cross-linked material was centrifuged at 4500rpm at 4ºC for 5 minutes, and the resulting pellet ressuspended in 500µL of PIPES buffer (5mM PIPES, 85mM KCl, 0.5% NP40, protease inhibitors) and incubated 10 minutes at 4ºC, rotating. The samples were centrifuged at 5000 rpm at 8ºC for 5 minutes and the resulting pellet

ressuspended in 200µL of Lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1, protease inhibitors) and incubated 15-20 minutes at 4ºC, rotating. The cross-linked samples were sonicated to 200-1000 bp fragments, centrifuged at 14000 rpm at 8ºC for 10 minutes and the supernatant recovered. The sonicated lysate was diluted 1/10 in ChIP Dilution Buffer (1% Triton, 2mM EDTA, 150mM NaCl, 20mM Tris-HCl pH 8.1). At this point, 100µL of sample were collected to use as Input sample. Samples were immunoprecipitated using Hoxc6 antibody (Abcam ab41587), Hoxa10 antibody (kindly provided by J. Dasen) or control rabbit IgG (Abcam ab27478), pre-bound to Dynabeads Protein A (Invitrogen) in ChIP Dilution Buffer, overnight at 4ºC. The chromatin bound to the Dynabeads was washed 6 times for 10 minutes at 4ºC with RIPA buffer (50mM Hepes, 1mM EDTA, 0.7% Sodium-Deoxicholate, 1% NP40, 0.5% LiCl) and 2 additional times with 1x TE buffer pH8.0, and the beads collected with a magnetic concentrator between washes. The DNA was eluted from the beads by adding 100µL of Elution Buffer (1% SDS, 100mM $NaHCO_3$) and incubated at 65ºC for 6-16 hours. The immunoprecipitated DNA was purified using Qiaquick PCR kit (Qiagen) and PCR-amplified using primers for the H1 enhancer and for a negative control region (Table 6).

Table 6: Details of the primers used in ChIP experiments for the amplification of the H1 enhancer and a negative control region.

	Primer Sequence	**Annealing temperature**
H1	H1-ChIP-F 5' GCCATCTACTCTCACACACCATAC 3' H1-ChIP-R 5' CCACGCTAAAATACAGACATGCAG 3'	60ºC
Negative control	Myf5-ChIP-NegF 5' CTGGCGTGTCTCCCTCTCTGCTGAA 3' Myf5-ChIP-NegR 5' GCTCCGAAGGCTGCTACTCTTGGCT 3'	60ºC

Luciferase Assays

For the luciferase assays, reporter plasmids were made by cloning the wild type or a mutant version of the H1 enhancer in which the CTAATTG was replaced for CGCGCTG upstream of the minimal promoter of the pGL3-Promoter Vector plasmid. Transfections were performed on HEK-293T cells using reporter plasmids together with plasmids expressing either *VP16:Hoxa10*, *VP16:Hoxb6* or, as a control, the tetracycline transactivator (tTA) (Gossen and Bujard 1992) using Lipofectamine 2000. The pCMV-b plasmid was included in all electroporations for normalization. Luciferase activity was measured on cell extracts 24 hours after transfection and normalized to β-galactosidase activity. Significance was evaluated using Student's t-test.

Quantitative RT-PCR

To quantify transcript levels, total RNA was extracted with Trizol (Sigma) according to the manufacturer's protocol. cDNAs were synthesized by random priming using Superscript II reverse transcriptase (Invitrogen) and the mRNA levels were determined by qPCR using QuantifastTM FYBR® Green PCR Kit (Qiagen), and primers specific to the *Hoxa10* cDNA (Table 7).

Table 7: Details of the primers used to quantify the *Hoxa10* cDNA

	Primer Sequence	**Annealing temperature**
Hoxa10	Hoxa10F 5' AGCGAGTCCTAGACTCC 3' Hoxa10R 5' GTCCGTGAGGTGGACGCTACG 3'	65ºC

II.VI Acknowledgments

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CHAPTER III – MECHANISMS OF HOX FUNCTIONAL SPECIFICITY: THE ROLE OF HOX10-SPECIFIC MOTIFS

III.I Summary

Hox genes are key regulators of embryonic development. Among other functions, they are essential for vertebral identity. Despite their binding to identical DNA sequences *in vitro*, Hox proteins display exquisitely specific functions, suggesting their functional activity may reside outside of the homeodomain and involve cooperation with other proteins. Hox group10 has been shown to be responsible for the formation of the lumbar region by inhibiting rib formation, whereas Hox group 11 controls the layout of the sacral region. We have indentified two small protein motifs of Hoxa10 that are necessary for its rib-inhibiting function, and show that the homeodomain of Hoxa10 is not sufficient to confer Hoxa10-like function in a non-rib-inhibiting protein, such as Hoxa11.

III.II Background

One of the most frequently asked questions in the transcriptional regulation field is how transcription factors specifically select and properly regulate their target genes. This presumably straightforward question includes at least two aspects: how do transcription factors explicitly select their correct DNA target sequences among the myriad of potential binding sites throughout the genome (Rohs *et al*. 2010; Garvie and Wolberger 2001), and how do they determine whether to activate or repress a specific target gene (Joshi *et al*. 2010). A paradigmatic illustration of this problem is provided by the Hox family of transcription factors. These are phylogenetically well-conserved proteins that belong to the widespread homeodomain-containing superfamily of transcription factors and have fundamental roles in conferring AP axis determination and segment identities (R Krumlauf 1994). Hox proteins share a highly conserved 60 amino acids domain, called the homeodomain, which is known to mediate DNA binding (Gehring *et al*., 1990), through the recognition of a consensus core motif, T(A/T)AT (Pearson *et al*. 2005).

The apparent relaxed *in vitro* binding of Hox proteins to a common core sequence contrasts with their remarkably specific *in vivo* functional activities (Mann *et al*. 2010). This seems to imply that the DNA-binding specificities of Hox proteins might not be restricted to the homeodomain alone (Ekker *et al*. 1994; Desplan *et al*. 1988; Affolter *et al*. 1990; Hoey and Levine 1988; Ekker *et al*. 1991). In fact, a number of studies, have shown that specificity also requires non-homeodomain residues, typically in the N- or C-terminal vicinity of the homeodomain (Zhao and Potter 2002; 2001; Zeng *et al*. 1993; J M Passner *et al* 1999; Dessain *et al*. 1992; Furukubo-Tokunaga *et al*. 1993; Gibson *et al*. 1990; Lin and McGinnis 1992; Joshi *et al*. 2007; Mann and Hogness 1990). These non-homeodomain motifs seem to behave as unique protein signatures, often showing paralog specificity, suggesting their possible implication in Hox paralog function and redundancy (Merabet *et al*. 2009).

A classical example of a more generically displayed Hox protein signature is the “hexapeptide”, also known as PID, located shortly upstream of the homeodomain, which has been extensively implicated in Hox protein interaction with PBC cofactors (comprising the fly Exd and the vertebrate Pbx) (Morgan *et al*. 2000; Chang *et al*. 1995; Phelan *et al*. 1995; Johnson *et al*. 1995; Knoepfler and Kamps 1995). In vertebrates, the hexapeptide has been described in Hox paralog groups 1 to 8, but it is not present in more posterior Hox paralog proteins. In addition, biochemical analyses indicate that some members of more posterior paralog groups do not bind DNA as a complex with Pbx (Shen *et al*. 2001). Moreover, Hox genes also have PBC-independent functions (Pederson *et al*. 2000; Peifer and Wieschaus 1990; Percival-Smith and Hayden 1998; Prince *et al*. 2008), and the hexapeptide has been reported not only to be present in non-Hox proteins (Knoepfler *et al*. 1999; Löhr *et al*. 2001; Brown *et al*. 1994; In Der Rieden *et al*. 2004), but also associated with proteins other than PBC (Prince *et al*. 2008), further

suggesting that other peptide motifs and cofactors could be involved in conferring specificity to posterior Hox paralog proteins.

Here we present a functional approach to study Hox specificity *in vivo*. We indentified three Hox paralog-group-10-specific peptide motifs as potential candidates to mediate the rib-inhibiting capacity of Hoxa10. We show that at least one of these domains is necessary for Hoxa10's function and present preliminary data suggesting a critical role for the other two, which seems to be homeodomain-independent.

III.III Results

SPECIFIC PEPTIDE MOTIFS ARE IMPORTANT FOR HOX GROUP 10'S FUNCTION

As mentioned above, Hox group 10 is responsible for the formation of the lumbar region of the skeleton by inhibiting rib formation (Wellik and Capecchi 2003, Carapuço *et al* 2005). This functional property is specific to Hox group 10 and, to our knowledge, not shared by any other Hox protein (Carapuço *et al* 2005). We were interested in indentifying particular protein signatures that could account for Hox group 10's function.

A simple sequence alignment of Hox group 10 proteins of a variety of vertebrates showed these proteins have at least three conserved motifs outside the homeodomain, which are remarkably conserved among vertebrates (Fig. 21). Importantly, these are not present in other Hox paralogs, suggesting that they may play a role in the specific function of Hox group 10.

To address the functional requirement of these domains, we first deleted d2 from the Hoxa10 protein (Fig. 22 A) and evaluated the rib-inhibiting capacity of these mutants when expressed in the PSM under the control of the *Dll1*Δ promoter.

Figure 21: Sequences of several Hox group 10 proteins of different vertebrates, from Xenopus leavis to Homo sapiens. Three conserved motifs among these proteins are highlighted in green (d1), red (d2) and orange (d3).

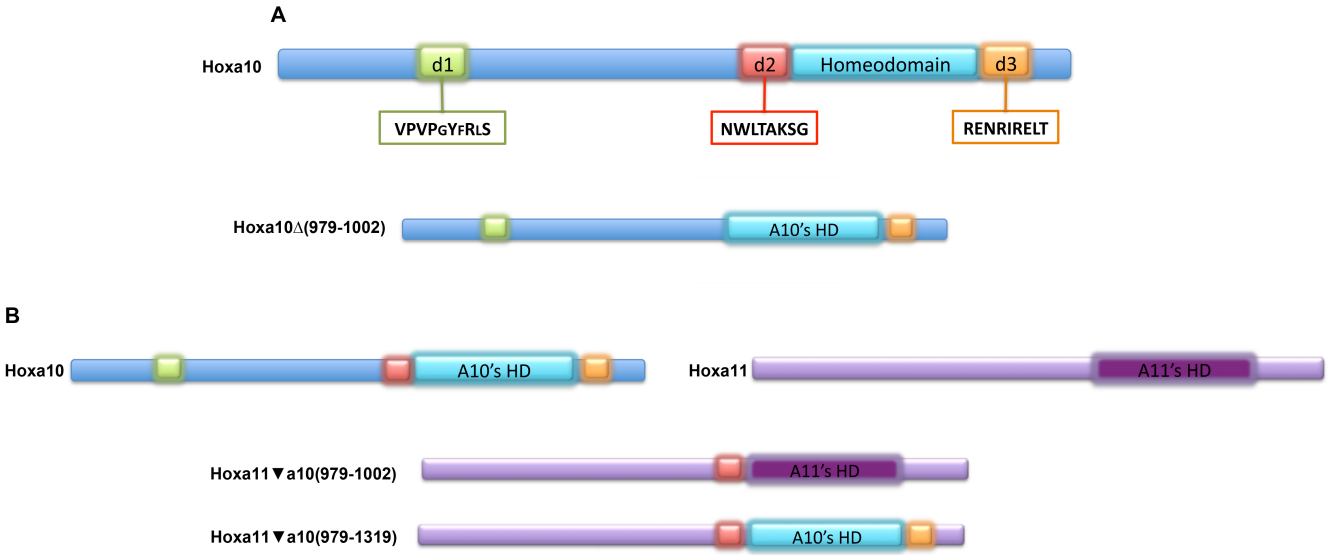

Figure 22: Schematic representation of the Hoxa10 deletion mutants (A) and the chimeric proteins between Hoxa10 and Hoxa11 (B). A. The d2 domain was removed form Hoxa10 to produce the Hoxa10Δ(979-1002) mutant protein. B. Chimeric proteins between Hoxa10 and Hoxa11. The d1 domain from Hoxa10 was inserted in the Hoxa11 sequence producing the Hoxa11▼a10(979-1002), while the Hoxa11▼a10(979-1319) is composed of the N-terminal fraction of Hoxa11, and the d2 and homeodomain of Hoxa10.

Analysis of transgenic embryos expressing the d2 deletion mutant of Hoxa10 (*Dll1-Hoxa10∆(979-1002)*) did not reveal any skeletal phenotype in their axial skeletons, indicating that removal of the d2 motif impaired the rib-inhibiting capacity of Hoxa10 (Fig. 23 A-C).

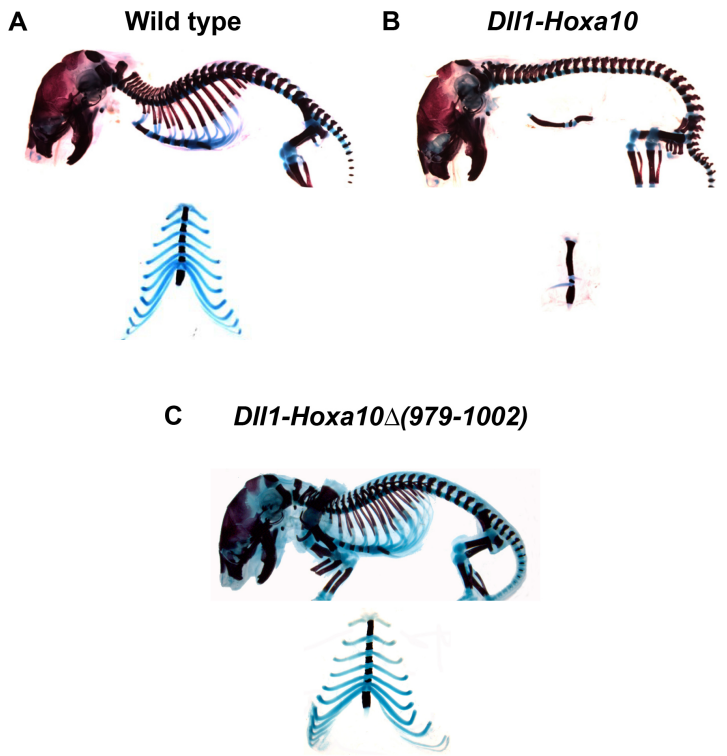

Figure 23: Phenotypic analysis of the axial skeleton of two Hoxa10 deletion mutants. Skeletal staining of wilde type (A), *Dll1-Hoxa10* (B), and *Dll.Hoxa10∆(979-1002)* (C) E18.5 fetuses. PSM over-expression of *Hoxa10* results in ribless embryos (B). The deletion of the d2 domain of the Hoxa10 protein results in the recovery of a wild type-like phenotype (C).

These results suggest that the conserved d2 domain is necessary for Hoxa10 function. Similar deletion analyses of the d1 and d3 domains are currently in progress in the Mallo laboratory.

To further understand the functional competence of these domains, we decided to address if they were sufficient to confer Hoxa10 function to a different Hox paralog protein that does not block rib formation when

ectopically expressed in the PSM. For this we produced chimeric proteins between relevant domains of Hoxa10 and Hoxa11. Hoxa11 is involved in the formation of the sacrum (Wellik and Capecchi, 2003) and when over-expressed in the PSM originates mild rib-fusions as well as some fusions between lumbar vertebrae reminiscent of the characteristic sacral-level fusions, without affecting the total number of ribs (Carapuço *et al*, 2005).

We produced two different chimeric mutants. In one of them we introduced the domain d2 into the Hoxa11 sequence (*Dll1-Hoxa11▼a10(979-1002)*), and in the other we replaced the Hoxa11's homeodomain and C-terminal region with a fragment that included d2, the Hoxa10's homeodomain, the d3 domain, and the respective C-terminal region (*Dll1-Hoxa11▼a10(979-1319))* (Fig. 22 B).

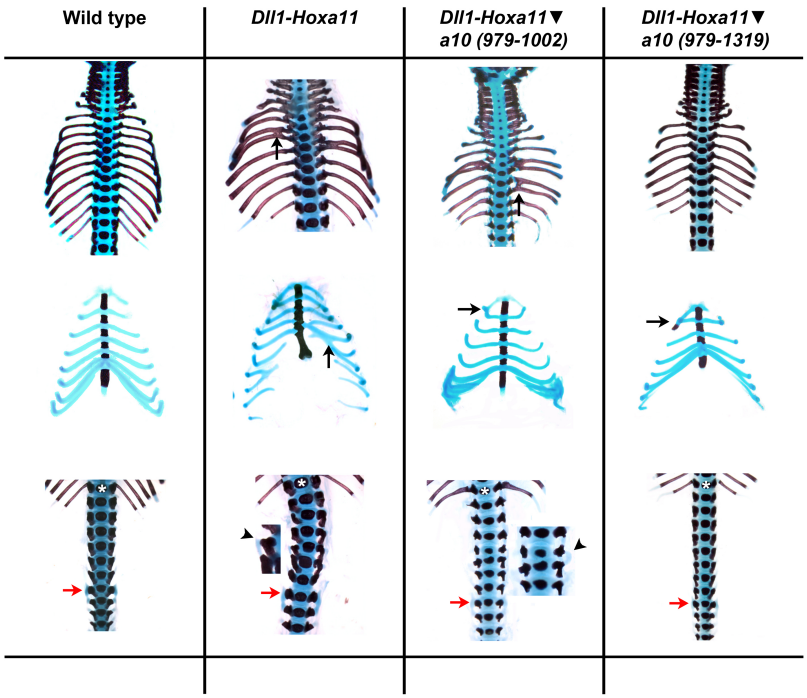

Figure 24: Phenotypic analysis of the axial skeleton of two transgenics of chimeric proteins. Overexpression of *Hoxa11* in the PSM results in several rib and vertebral fusions, whereas the two chimeric mutants, *Dll1-Hoxa11▼a10(979-1002)* and *Dll1-Hoxa11▼a10(979-1319)* result in an intermediate phenotype with Hoxa11-like fusions and also a diminution of the number of ribs. Black arrows indicate different rib fusions, black arrowheads show sacral-like fusions in lumbar vertebrae, asterisks mark the last thoracic vertebra and red arrows indicate the first sacral vertebra.

These transgenics show an intermediate phenotype between *Dll1-Hoxa10* and *Dll1-Hoxa11*, the phenotype of the *Dll1-Hoxa11▼a10(979-1319)* being closer to a typical *Dll1-Hoxa10* phenotype than that of *Hoxa11▼a10(979-1002)* transgenics. They display rib and sacral-like fusions reminiscent of Hoxa11 transgenics and a reduction in the number of ribs (varying from 12-10 ribs) resembling Hoxa10 activity (Fig. 24). Despite the more or less severe reduction in the overall number of ribs, the sacrum seems to be correctly positioned, even though in *Dll1-Hoxa11* it is sometimes displaced anteriorly by 1-3 vertebrae. The rib fusions in *Dll1-Hoxa11▼a10(979-1002)* are both proximal and distal, similarly to what was found *Dll1-Hoxa11* animals, whereas in *Dll1-Hoxa11▼a10(979-1319)* they are mostly proximal and usually in the first sternal ribs. Also, we could only observe sacral-like fusions between lumbar vertebrae in the *Dll1-Hoxa11▼a10(979-1002)* transgenics (Fig. 24).

These results show that the larger the fragment of Hoxa10 inserted into the Hoxa11 protein, the more Hoxa10-like the mutant phenotype is. Nevertheless, despite the apparent requirement of the conserved motifs, neither d2 alone or together with Hoxa10's homeodomain and the d3 domain are sufficient to confer a rib-inhibiting capacity to the Hoxa11 protein, suggesting the possible requirement of additional protein domains.

III.IV Discussion

It is well established that the homeodomain is critical to Hox DNA-binding both *in vitro* and *in vivo*. Yet, this does not account for how the exquisite *in vivo* functional specificity of Hox genes is achieved. Among the possible explanations to this paradox, the most popular is that particular cofactors confer local specificity (Mann and Chan 1996). However, given the widespread expression of most Hox cofactors and the fact that they seem to be able to bind the majority of Hox proteins, a proper explanation for the

mechanisms underlying target regulation by these genes is still lagging behind (Mann and Affolter 1998).

A growing amount of studies are leading to new perspectives on how binding specificity is achieved. Recent data from *Drosophila* suggested that Hox DNA-binding might be comprised of two steps: one that involves contacts between the recognition helix of the homeodomain and AT-rich sequences in the DNA major grove, and another that helps discriminate the correct binding sites from the myriad of generic AT-rich sequences. In the second step, the specificity would be achieved from contacts between the DNA minor grove and residues in the N-terminal arm and linker region (Joshi *et al*. 2007). Other studies that focused on this linker region between the PID and the homeodomain show a strong conservation in linker size within Hox paralogs, and suggest that both the sequence of the linker and its size are important for Hox function (In Der Rieden *et al*. 2004; Gebelein *et al*. 2002; Merabet *et al*. 2003). It has even been suggested that a bigger linker would reflect a less stringent binding, whereas a shorter linker would favor a more stable DNA-binding. Given that linkers seem to be smaller in more posterior Hox paralogs, this could represent a molecular-based justification for the posterior prevalence phenomenon (In Der Rieden *et al*. 2004; Laronde-leblanc and Wolberger 2003).

All things considered, it is not surprising that peptide motifs outside the homeodomain might have an important role in establishing specific Hox protein functions. We indentified three conserved protein motifs (d1, d2 and d3), present in Hox paralog group 10 proteins of all vertebrates represented in current databases and which are exclusive of this Hox paralog group (Fig. 21). The strong conservation of these motifs in the only group of Hox genes with rib-inhibiting properties suggests a possible role in rib repression. We developed a functional assay to address Hox specificity *in vivo*. Using the transgenic *Dll1-Hoxa10* ribless mouse model (Carapuço *et al* 2005) as readout of Hoxa10's function, we began by specifically deleting d2 and

found that this motif is necessary for the rib-inhibiting function of Hoxa10 (Fig. 23). Additionally, to analyze if this and the other conserved domains were sufficient to induce a Hoxa10'-like phenotype, we produced chimeric Hox proteins and showed that particular pieces of Hoxa10 can mimic its rib-repressing function to some extent when inserted into the non-rib-inhibitory backbone of Hoxa11 (Fig. 24). However, the resulting embryos have a Hoxa10/Hoxa11 intermediate phenotype, with typical Hoxa11 fusions associated with a moderate reduction in the number of ribs, meaning that these domains are not sufficient to induce a full force Hoxa10-like rib-less phenotype. Interestingly, the phenotype of the *Dll1-Hoxa11▼a10(979-1319)* chimeras, where both d2 and d3 are inserted into the Hoxa11 protein, is closer to the complete rib-less phenotype than that of the *Dll1-Hoxa11▼a10(979-1002)*, in which only the d2 domain was introduced into the Hoxa11 backbone. It should be considered that the first chimeric protein also contain the Hoxa10's homeodomain is also present, which could also be functionally meaningful. However, given the high similarity between the homeodomains of these two closely related proteins, it is more likely that the moderate rib-inhibiting capacity of these transgenics mostly dependes on the d2 and d3 motifs. Nevertheless, the rather mild phenotypes of both these chimeric protein transgenics indicates that there must be some other characteristic in the Hoxa10 protein that confers the proper conformational configuration or provide an additional interacting interface to allow a complete rib-inhibiting function. The d1 domain is a strong potential candidate to exert this role, and experiments aimed to explore this domain are in progress. The ideal goal would be to identify the correct combination of Hox group 10 peptide motifs that confers comprehensive rib-inhibiting properties to a Hox protein with no intrinsic rib-inhibiting properties. This would allow us to understand which Hox protein components are relevant for binding specificity on the one hand, and for functional specificity on the other.

It would be important to determine the structural properties of the Hox10 motifs within the protein's 3D structure and how it relates to the target DNA sequence. Unfortunately, the cristal structure of Hox10 proteins has not been resolved yet. However, some ideas can be extrapolated from the crystal structure of the Hoxa9/Pbx/DNA complex. Hoxa9 is a closely related protein to Hoxa10 that contains a motif that is very similar to d2 (NWLTAKS), but differs in a threonine-to-histidine and a lysine-to-arginine transformation (NWLHARS). The tryptophan (W) residue in the NWLHARS sequence seems to be essential for Hox-Pbx interaction in Hox paralogs 1-8 (Chang *et al*. 1996; Shen *et al*. 1997; Laronde-leblanc and Wolberger 2003). This tryptophan residue is also located within the Hoxa10's d2 motif, which could indicate that this motif helps provide specificity through the interaction with Pbx. Experiments to test the functional relevance of this tryptophan by evaluating the rib-inhibiting properties of a Hox10 protein with the tryptophan replaced by an alanine, are currently underway in the Mallo laboratory. However, it should also be noted that so far there is no evidence of Hox10 interactions with Pbx proteins, and the Hox target site within the H1 enhancer does not fit to a Hox/Pbx binding site, but rather to a Hox binding site. In addition, the presence of Pbx1 seems not to modify the DNA binding properties of Hoxa9 (Laronde-leblanc and Wolberger 2003), suggesting that this interaction might not be important to provide binding specificities to this Hox protein.

Recent data from our laboratory suggests a possible role of phosphorylation in the rib-repressing function of Hoxa10. The NWLTAKS motif contains two potential phosphorylation sites, and the threonine is a Hox10-specific signature. Indeed, mutation of these serine and threonine within d2 to alanine, resulted in the loss o rib-inhibiting properties of the mutant Hox10 protein (I. Guerreiro and M. Mallo, unpublished observations). This would be an interesting twist to this story given that protein phosphorylation is a key mechanism of many developmental functions (Brinkworth *et al*. 2003), and it

has been reported that Hox genes can be functionally regulated through phosphorylation (Jaffe *et al*. 1997). Curiously, this threonine is the one that is substituted by a histidine in the Hoxa9 protein, suggesting this could in fact be a key residue to confer rib-repressing activity to Hoxa10.

Interestingly, a similar function of serine and threonine phosphorylation sites was described in inhibiting the leg-repressing function of *Ubx* in *Drosophila*. In the fruit fly, *Ubx* is largely expressed in abdominal segments where it has a leg-inhibiting function. However, the homologous gene in a multi-limbed arthropod, the brine shrimp *Artemia*, does not have the same limb-repressing activity. This inability to repress leg formation was shown to be associated with serine and threonine phosphorylation sites in the C-terminal region of the *Artemia* Ubx, which are absent from the *Drosophila* homolog (Ronshaugen *et al*. 2002).

The search for the mechanisms of Hox function and target regulation has raised more questions than it has answered. Overall, what is clear is that Hox genes resort to a multitude of molecular strategies, and rarely obey to standardized systems while carrying out their functions.

III.V Materials and methods

Generation of transgenic constructs

Hox group 10 sequences were obtained from NCBI database and vertebrates' sequences aligned using the multiple sequence alignment tool CLUSTALW.

The *Dll1-Hoxa10* and *Dll1-Hoxa11* constructs were previously described (Carapuço *et al*. 2005). To produce the deletion and chimeric proteins, the relevant regions of the *Hoxa10* and *Hoxa11* cDNAs were PCR amplified and the sequences cloned to produce the mutant cDNA versions. All the sequences were confirmed by direct DNA sequencing and the final mutant cCNA versions were inserted downstream the *Dll1* (Beckers *et al*. 2000) enhancer, and upstream of the SV40 polyadenylation signal.

Transgenic constructs were released from the bacterial plasmid backbone, purified using a gel-extraction kit (QiAquick gel-extraction kit, Quiagen) and embryos were produced by pronuclear injection according to standard methods. All transgenic mice used in this work have a FVB/N genetic background. The functional activity of the constructs was evaluated by analyzing the effects on rib formation. Fetuses were dissected at E18.5, a portion of the intestine used to extract DNA for genotyping by PCR (see primers' details in Table 8), and skeletal preparations made using the alcian blue/alizarin red staining method (Mallo and Brändlin 1997).

Table 8: Sequence of primers used to genotype the different transgenics generated.

Tansgenics	Primer Sequence	Annealing Temperature
Dll1-Hoxa10	Hoxa10_F 5' AGCGAGTCCTAGACTCCACGC 3' Hoxa10_R 5' GTCCGTGAGGTGGACGCTACG 3'	65ºC
Dll1-Hoxa11	Hoxa11_F 5' AACTTCAAGTTCGGACAGCGG 3' Hoxa11_R 5' TCAGTGAGGTTGAGCATGCGG 3'	65ºC
Dll.Hoxa10Δ(620-649)	Hoxa10_F 5' AGCGAGTCCTAGACTCCACGC 3' Hoxa10_R 5' GTCCGTGAGGTGGACGCTACG 3'	65ºC
Dll.Hoxa10Δ(979-1002)	Hoxa10_F 5' AGCGAGTCCTAGACTCCACGC 3' Hoxa10_R 5' GTCCGTGAGGTGGACGCTACG 3'	65ºC
Dll1-Hoxa11▼a10(979-1002)	Oct F 5' AATTCGGCCGGCAACTGGCTCA CAGCAAAGAGT 3' A11-3'-2 5' CAGTCGACTCCATGCATCCCTC TCTTGCAC 3'	65ºC
Dll1-Hoxa11▼a10(979-1319)	A11-5'-1 5' CAGAATTCAAAGAGGCAGCT GCAGTGGAG 3' Hoxa10_R 5' GTCCGTGAGGTGGACGCTACG 3'	65ºC

III.VI Acknowledgements

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CHAPTER IV – FINAL CONSIDERATIONS ON HOW TO MAKE A SKELETON

"Every science begins as philosophy and ends as art."

- **Will Durant**

In this thesis work, we addressed two different levels of complexity of Hox function: 1) how are the Hox-provided instructions interpreted and translated into region-specific vertebral morphologies, and 2) which intrinsic protein characteristics confer functional specificity to particular Hox paralog proteins.

SPECIFICATION OF GLOBAL VERTEBRAL DOMAINS

The separation of the axial skeleton into distinct vertebral domains has several functional and evolutionary implications. Hox genes were originally thought to be responsible for providing specificity to individual segments (Pederson *et al*. 2000; Peifer and Wieschaus 1990; Percival-Smith and Hayden 1998; Prince *et al*. 2008). More recently, Wellik and Capecchi suggested that, in vertebrates, Hox genes also determine whole skeletal areas with similar vertebral morphologies. The work presented in this thesis clearly reinforces the idea of a Hox role in establishing global patterns in the axial skeleton. It had been previously shown that Hox genes play a role in this process by defining the lumbar and sacral areas of the skeleton. Hox paralog group 10 specifies the lumbar domain by inhibiting rib formation and thus diverting it from a rib containing identity (Wellik and Capecchi 2003; Carapuço *et al*. 2005). Additionally, Hox group 11 is essential for the formation of the sacrum (Wellik and Capecchi 2003). Hox group 9 also seems to have a regional patterning function (McIntyre *et al*. 2007). Inactivation of the whole paralogous group resulted in ribcages with increased numbers of sternal ribs (13-14 instead of the normal 7), which suggested these genes are important to generate floating ribs (McIntyre *et al*. 2007). Interestingly, Hox group 9 rib patterning activity is likely to be required in the lateral plate mesoderm and not in the somites, as determined by expression analysis of members of this Hox group (McIntyre *et al*. 2007). The sternum derives from the lateral plate mesoderm (Brent and Tabin 2002; Wellik 2007; Monsoro-Burq 2005), and it has been shown

that the development of distal ribs requires migration of somitic mesoderm into the somatopleura (Sudo *et al*. 2001), and might involve interactions of rib and sternal precursors. It is possible then, that Hox group 9 modulates the response of the lateral plate mesoderm to these migration signals in order to control the production of sternal versus floating ribs.

We expanded the concept of Hox genes defining global vertebral domains by showing that the rib-containing area of the skeleton is not set out by default, as previously suggested (Wellik and Capecchi, 2003), but is rather the result of the activity of Hox group 6 proteins that specifically activate the rib forming program to produce the thoracic region (Vinagre *et al*. 2010). Accordingly, the rib-less cervical domain is the vertebral area between the skull and the area of Hox group 6 induction. This hypothesis is consistent with the expression patterns described for members of the Hox group 6 in vertebrates with a different number of cervical vertebrae. In particular, it has been shown that the anterior limits of expression of *Hoxc6* correlate with the cervical-to thoracic transition in different vertebrates with necks containing different numbers of cervical vertebrae (Burke 2000; Burke *et al*. 1995; Cohn and Tickle 1999; Molven *et al*. 1990). A striking example of this phenomenon is provided by the analysis of *Hoxc6* expression in pythons (Cohn and Tickle 1999). These reptiles, as well as other snakes, have an expansion of the thoracic region, with ribs in every pre-cloaca vertebrae (except for the atlas, the first cervical vertebra); the *Hoxc6* anterior expression boundary in these animals correlates with this thoracic expansion (Cohn and Tickle 1999 *et al*. 1999), further suggesting that Hox group 6 is important for rib formation. Remarkably, by overexpressing *Hoxb6* in the PSM of mouse embryos we were able to emulate the peculiar axial regionalization of snakes in a different vertebrate, which provides experimental evidence to support the hypothesis that changes in the expression of Hox genes can account for the evolution of morphological transitions in the axial skeleton. It would be interesting to determine whether

the same effect could be reproduced in other vertebrates with a different regionalization of their axial skeleton.

The contrasting effects that Hox groups 6 and 10 have on rib formation suggested that they could modulate the same set of targets in opposite manners. Interestingly, we show in this thesis that the primary targets of Hox activity in rib formation seem to be genes expressed in the myotome. This was a surprising finding because, given the embryological origin of ribs, we expected to find these targets among sclerotomal genes. However, we found that Hox genes modulate rib formation by regulating the hypaxial expression of *Myf5* and *Myf6*. This gives these two genes a new function in addition to their role in myogenesis, which might have important evolutionary implications, namely the coordinated development of muscles and bones as part of the same biological system. It has been previously shown that the precursor cells of the ribs and intercostal muscles are closely connected within the somite, and that this relationship could be essential for proper rib morphogenesis (Evans 2003). Our data support this idea and suggest that the Hox patterning information, thus interpreted in the hypaxial myotome, is likely transmitted to the adjacent sclerotome by PDGF and FGF signals that promote skeletogenesis. This cell non-autonomous mechanism is supported by the correlated myotomal expression of *Pdgfa* and *Fgf4* with that of the *Myf5/Myf6* genes in our Hox transgenic embryos as well as in the *Myf5*$^{\Delta loxp/\Delta loxp}$ mutants (Grass *et al*. 1996; Vinagre *et al*. 2010), and is consistent with data from others showing the involvement of FGF and PDGF signaling in rib formation (Grass *et al*. 1996; Tallquist *et al*. 2000; Soriano 1997; Huang *et al*. 2003).

The separation between myotome and sclerotome as the sources of muscle and bone, originally shown by fate-mapping experiments, denotes the existence of separate somitic lineages but does not exclude that cooperation between them is required to form a proper musculoskeletal system. An excellent example that illustrates the importance of interactions

between the muscle and cartilage precursors is the establishment of the tendon lineage (Brent *et al*. 2005). Examination of muscle-deficient *Myf5/MyoD* double mutants revealed the absence of tendon formation, and suggested a role for the specified myotome in axial tendon development. Furthermore, analysis of the cartilage-deficient *Sox5/Sox6* compound mutants indicated a common origin of the tendon and cartilage lineages, and indicated that the two cell types are alternative differentiation fates of sclerotomal progenitors (Brent *et al*. 2005). Preliminary data from our lab also brings Hox genes to this specific patterning process. In particular, we found that the main syndetomal marker, *scleraxis*, is specifically down-regulated in the hypaxial myotome of the rib-less *Dll1-Hoxa10* embryos (N. Moncaut and M. Mallo, unpublished data), which further emphasizes the pivotal role of Hox genes in the coordinated development of the musculo-skeletal system.

Initial patterning of the somite into different compartments requires both signals form the surrounding tissues and the activation of specific internal genetic cascades. The ventral midline *Shh* has been shown to regulate several of these fates (Brent and Tabin 2002). During sclerotome formation, *Shh* is important for the expression of several genes involved in axial skeleton development, including *Pax1*, *Pax9*, *Sox9* and *Nkx3.2* (Zeng *et al*. 2002; Murtaugh *et al*. 2001; Murtaugh *et al*. 1999; Buttitta *et al*. 2003). It has been proposed that different levels of *Shh* are required for the patterning of particular domains within the sclerotome. Thus, the proper balance of *Shh* activity would help specify the ventromedial cartilage fate or the dorsolateral tendon lineage from this compartment (Brent and Tabin 2002; Brown *et al*. 2005). In addition, *Shh* has been shown to induce and maintain myogenic factors such as *Myf5*, as well as to regulate FGF expression in the myotome (Fraidenraich *et al*. 2000; Gustafsson *et al*. 2002; Krüger *et al*. 2001; Teboul *et al*. 2003). Another interesting piece of the puzzle that needs clarification is how the Hox patterning instructions given in the PSM, exert their effects

on *Myf5/Myf6* transcription in the hypaxial myotome of differentiated somites. As previously discussed, the bridging of this spatio-temporal gap likely involves Pax3 and Six1/Six4 proteins, acting cooperatively with Hox genes, because they are also required for the activity of the hypaxial *Myf5* enhancer bound by Hox 6 and 10 proteins (Giordani *et al*. 2007; Bajard *et al*. 2006), and abnormal rib development has been described in mice bearing mutations for the *Pax3* and *Six1/Six4* genes (Grifone *et al*. 2005; Henderson *et al*. 1999).

The control of rib development through a Hox-mediated regulation of *Myf5/Myf6* expression presents a possible model for the coordinated evolution of the vertebrate body plan and the formation of a functional musculoskeletal system. The road to fully understand how Hox genes specify axial skeleton patterning might still be long, but this work and the establishment of the Hox-Myf link is surely a step forward.

Hox Functional Specificity

The paradoxical contrast between the significant Hox functional specificity *in vivo* and their relaxed DNA binding properties *in vitro* has been the subject of extensive debate in the Hox field. The homeodomain is the prime DNA binding domain of Hox proteins, as well as of other transcription factors also containing this motif. However, while some amino acids within the homeodomain have been shown to play important roles in Hox DNA binding specificity (Berger *et al*. 2008; Noyes *et al*. 2008; Chan *et al*. 1993), the overt conservation of its sequence in Hox proteins with clearly different biological functions argues against the homeodomain being the main determinant of Hox specificity *in vivo*.

Following earlier studies in *Drosophila* that indicated that the homeodomain and neighboring C- and N-terminal residues were responsible for most functional specificities of Hox proteins (Zeng *et al*. 1993; Dessain *et al*. 1992; Mann and Hogness 1990; Kuziora and McGinnis 1989;1990; Gibson

et al. 1990; Furukubo-Tokunaga *et al*. 1993; Lin and McGinnis 1992), homeodomain swapping experiments in mice have shown that, whereas some Hox functions seem to correlate with the identity of the homeodomain, the axial skeleton of mice bearing chimeric Hox alleles is not significantly affected by the identity of the homeodomain (Zhao and Potter 2002; 2001), which further indicates that the homeodomain on its own cannot account for Hox functional specificity in axial patterning.

It has been described that Hox proteins contain several levels of amino acid signatures outside their homeodomain (Merabet *et al*. 2009). Relevant for this work is the conservation of specific peptide motifs within paralogs that are thought to be key in conferring functional specificity to members of the same paralog group. These paralog-specific signatures have been proposed to be the basis of the functional redundancy observed within members of the same paralog group (Merabet *et al*. 2009). We identified three highly conserved, and apparently Hox group 10-specific protein motifs and investigated whether they could be required for the rib-blocking properties of these proteins. We observed that at least one of these motifs, the d2 motif, is critical for Hoxa10's function in the patterning of the lumbar region of the skeleton. However, our experiments show that this and another motif (d3), either alone or in combination with the homeodomain are not sufficient to confer a completely Hoxa10-like function to a different Hox protein that does not posses rib-inhibiting properties. These results could either be a consequence of the requirement of additional protein motifs for Hox group 10 rib-inhibiting function or, alternatively, the remaining protein sequence of the recipient Hoxa11 protein can obstruct the activity of these foreign motifs.

Some of the most popular explanations for the *in vivo* functional specificity of Hox proteins implicate the participation of partners or cofactors. The d2 motif that we found to be necessary for the function of Hoxa10 comprises a conserved tryptophan (Trp) residue that has been shown to be important for

the interaction with PBC proteins (Chang *et al*. 1996; Shen *et al*. 1997), thus suggesting that interaction with Pbx might be important for Hox group 10 rib-inhibiting function. However, some studies showed that members of the Pbx family are only capable of increasing the DNA-binding specificity of anterior Hox proteins but not of Abd-B-related proteins (Laronde-leblanc and Wolberger 2003), which is the group in which Hox group 10 has been classified. In addition, it has been described that the DNA binding properties of Hoxa9, which also contains a Trp-containing motif different but closely related to that in Hox10 proteins, are not modified by the presence of Pbx1 in the complex (Laronde-leblanc and Wolberger 2003). In addition, the DNA binding site that we have identified within the H1 Myf5 enhancer does not match a consensus Pbx/Hox binding site, but rather a Hox binding site. Hence, the mechanism underlying Hox group 10-specific rib-repressing activity is most likely Pbx-independent, and possibly involves alternative cofactors, post-translational modifications or the recruitment of chromatin-modifying proteins. In fact, several studies in different biological contexts have associated Hoxa10 with the recruitment of histone modifying enzymes (Bei *et al*. 2007; Shen *et al*. 2001; Lu *et al*. 2003; Hassan *et al*. 2007; Gordon *et al*. 2010), and a similar situation could take place in the control of rib development. Overall, it would be interesting to determine the mechanism by which these conserved protein motifs influence Hox function. Preliminary data from the Mallo laboratory suggest that protein phosphorylation of specific residues within the d2 domain might be important for Hoxa10's functional specificity (Guerreiro *et al*, unpublished results). A deeper analysis of mutant and chimeric proteins, as well as the identification of functional partners is likely going provide interesting clues for understanding how Hox group 10 achieves its specific function in rib formation.

Our understanding of the processes that underlie the molecular basis of evolution of the vertebrate body plan is steadily progressing. Hox genes

have long been identified as crucial factors in conferring segment identity along the AP axis, both in vertebrates and in invertebrates. Our data demonstrates that the regional patterning of the axial skeleton relies not only on the correct spatio-temporal control of the expression of Hox genes and their specific targets, but also on unique features of each particular Hox paralogous group. This work provides new insights into the molecular mechanisms of Hox function in the patterning of global vertebral domains.

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APPENDIX I

Mallo, M., Vinagre, T., and Carapuço, M. (2009). The road to the vertebral formula. The International journal of developmental biology 53, 1469-81.

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The road to the vertebral formula

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ABSTRACT In vertebrates, the paraxial mesoderm differentiates into several structures, including the axial skeleton. The genetic mechanisms that control positional information in the paraxial mesoderm along the anterior-posterior axis are responsible for the development of a skeleton with the appropriate vertebral formula, i.e. a specific number of cervical, thoracic, lumbar, sacral and caudal vertebrae. These control mechanisms are complex and involve molecules of different kinds, including transcription factors, like those encoded by the *Hox* genes, and signalling molecules, like those involved in Gdf11, FGF, retinoic acid or WNT signalling. Recent experiments indicate that most of the positional information for the paraxial mesoderm is encoded during the initial steps of its development in the presomitic mesoderm, although it is only decoded later during differentiation of the somites. The genesis of positional identity may be linked to the process of somitogenesis, which also occurs in the presomitic mesoderm as a result of complex interactions involving oscillatory activity of components of the Notch and WNT signalling pathways and antagonistic gradients of FGF/WNT and retinoic acid. The possible connections between *Hox* genes and all these signalling processes to generate a properly patterned axial skeleton are discussed in this review.

KEY WORDS: *Hox gene, signalling, somitogenesis, patterning, skeleton*

A quick look at a book of comparative anatomy is enough for one to realize that the axial skeleton of all vertebrates is composed of repeated units. We call these "vertebrae", and they come in an endless variety of sizes and shapes. In a second look we see that, despite their enormous diversity, we can still classify them in discrete groups according to general anatomical considerations: cervical (C), in the neck; thoracic (T), those with ribs; lumbar (L), spanning the abdomen; sacral (S), supporting the hindlimbs; and caudal (C), in the tail. The distribution of the vertebrae among the various groups is what we know as the vertebral formula, which represents one of the distinctive features of the different vertebrates. For instance, if we just focus on the neck, we see that snakes have just one cervical vertebra, mammals 7, chickens 14, and swans 25. The vertebral formula of the mouse, which is the focus of this review, consists of 7 cervical, 13 thoracic, 6 lumbar, 4 sacral and 30 caudal.

Embryologically, the axial skeleton derives from the somites, paired segmental structures located at both sides of the neural tube (Dubrulle and Pourquié, 2004). The somites are formed sequentially in an anterior to posterior sequence by chopping off fragments from the anterior end of the presomitic mesoderm (PSM) with a size and at a pace characteristic of each species (Dubrulle and Pourquié, 2004). The PSM represents the most posterior portion of the paraxial mesoderm, which is morphologically not segmented. The process of somitogenesis is closely linked to the posterior growth of the embryo. Indeed an equilibrium is maintained between formation of somites at the anterior end of the PSM and deposition of new mesenchymal cells at its posterior extremity, provided first by the primitive streak and later by the tail tip (Dubrulle and Pourquié, 2004).

After formation, somites differentiate progressively, eventually leading to the formation of the axial skeleton, the musculature of the body and limbs, and the dermis of the back (Brent and Tabin, 2002). Somite differentiation starts with the formation of two compartments, the sclerotome and the dermomyotome, in the ventro-medial and dorso-lateral parts of the epithelial somite,

Abbreviations used in this paper: AbdB, abdominalB; Acvr, activin receptor; A-P, anterior-posterior; BMP, bone morphogenetic protein; C, cervical vertebra; Dll, delta-like; FGF, fibroblast growth factor; Gdf, growth differentiation factor; HOM-C, homeotic complex; hsp, heat shock protein; L, lumbar vertebra; PcG, polycomb group; PDGF, platelet-derived growth factor; PSM, presomitic mesoderm; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid responsive element; S, sacral vertebra; T, thoracic vertebra; TGF, transforming growth factor; TrxG, trithorax group.

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respectively. The dermomyotome will then produce the myotome, which is the origin of the muscle cells, and the dermatome, which produces the dermis. The axial skeleton originates from the sclerotomal cells that delaminate from the epithelial somite and migrate to surround the neural tube and the notocord. Formation of the vertebrae does not follow a simple one somite-one vertebra rule. Instead, each individual vertebra is formed by the posterior and anterior halves of adjacent somites, a process known as resegmentation (Bagnall *et al.*, 1988). In addition, the boundary between anterior and posterior sclerotomal compartments of a somite (intrasomitic border) becomes the intervertebral disc in the fully developed vertebral column.

The differentiation of the somites is controlled at two different levels. One level includes the mechanisms responsible for the formation and differentiation of the somitic compartments, which are mostly conserved throughout the length of the axial skeleton (Marcelle *et al.*, 2002). The second level of control provides positional information in the anterior-posterior (A-P) axis, thus accounting for the genesis of morphologically distinct structures from somites located at different axial levels. These latter mechanisms are the subject of this review.

The genetic control of segmental identity

Genetic experiments have identified a variety of molecules that play essential roles in the control of segmental identity in the axial skeleton. In general, and perhaps rather artificially, they can be subdivided in transcription factors and signalling molecules. A schematic representation of selected phenotypes derived from mutations in several of these factors is shown in Fig. 1.

Transcription factors

The most classical regulators of segmental identity in many organisms and tissues are the members of the Hox gene family. The initial idea for such a role stems from the analysis of the genetic basis of the homeotic phenotypes that had been described for the first time in *Drosophila* mutants more than 100 years ago (Bateson, 1894; Lewis, 1978). Mammals contain 39 Hox genes distributed in four genomic clusters, with each cluster sharing structural similarities with the *Drosophila* HOM-C complex (Krumlauf, 1994). The different members of the vertebrate clusters are classified in 13 groups (refered to as paralogs) according to sequence homologies and their position within the cluster.

The involvement of Hox genes both in the control of axial identities and in the evolution of the vertebral axis is suggested by comparison of Hox gene expression profiles in vertebrate species with a different axial formula (Gaunt, 1994; Burke *et al.*, 1995). In those studies it was shown that the anterior expression boundaries of equivalent Hox genes in different species do not maintain the same absolute somite number but are transposed in register with specific anatomical landmarks. In addition, relative shifts in *Hox* gene expression observed in different areas along the axis reflected the relative expansion and contraction of morphological regions (Gaunt, 1994; Burke *et al.*, 1995; Cohn and Tickle, 1999). Therefore, it is very probable that the Hox genes had a fundamental role in the evolution of the vertebrate axial skeleton.

While these comparative studies suggest the involvement of the Hox genes in the specification of vertebral identities, the demonstration of this idea was provided by extensive genetic experiments in the mouse (Chisaka and Capecchi, 1991; Le Mouellic H *et al.*, 1992; Condie and Capecchi, 1993; Dolle *et al.*, 1993; Jeannotte *et al.*, 1993; Ramirez-Solis *et al.*, 1993; Condie and Capecchi, 1994; Horan *et al.*, 1994; Kostic and Capecchi, 1994; Horan el al., 1995; Rancourt *et al.*, 1995; Fromental-Ramain *et al.*, 1996; Chen and Capecchi, 1997; Manley and Capecchi, 1997; Chen *et al.*, 1998; Godwin and Capecchi, 1998; van den Akker *et al.*, 2001; Economides *et al.*, 2003; Wellik and Capecchi, 2003). Both ectopic expression and inactivation of many Hox genes resulted in skeletal phenotypes scored as identity transformations, which varied depending on the specific Hox gene or genes involved in the experiment (Krumlauf, 1994). Those experiments showed that, in general, Hox genes located at the 3' end of the clusters (also called "anterior" Hox genes on the basis of their expression domains) are involved in the specification of anterior structures and those located towards the 5' end of the cluster (also known as "posterior" Hox genes) are responsible for the control of posterior vertebral identities. However, despite many years of intensive research, we still do not understand the mechanism by which Hox genes control vertebral identities, nor do we have a satisfactory explanation for the interactions among the different Hox genes.

One of the first hypotheses to explain how Hox genes generate regional identity in the paraxial mesoderm stemmed from analyses in mouse of the consequences of the ectopic expression of particular Hox genes on the developing axial skeleton and from the correlation of alterations in Hox gene expression with homeotic transformations in embryos that had been exposed to retinoic acid (RA) at different gestation times (Kessel and Gruss, 1991; Kessel, 1992). According to this hypothesis, the combination of various Hox gene products co-expressed in a given somite or "Hox codes" would specify the final morphology of the resulting vertebra. However, this simple combinatorial model failed to explain the skeletal phenotypes of the growing list of Hox mutant mice. Among the characteristics that seemed apparent from the initial gene inactivation experiments was that the domain of activity of the Hox genes was reduced to their most anterior expression domain. These results, together with the functional hierarchy existing among HOM-C gene products in *Drosophila*, suggested the "posterior prevalence" model for the patterning activity of Hox genes (Bachiller *et al.*, 1994; Duboule and Morata, 1994). According to this model the function of "posterior" (5') Hox genes is prevalent over that of their more "anterior" (3') relatives. Accordingly, the most "posterior" Hox gene expressed at a given A-P level would dictate the morphogenetic programme. However, this model also fails to explain many of the Hox mutant phenotypes. Currently, the activity of Hox genes in the control of vertebral identity is usually explained as a combinatorial code that considers that both the functional weight and outcome of the activity of Hox genes depends on their specific Hox partners at each particular axial level. While this model is flexible enough to explain almost any possible phenotype, it is also too vague to provide useful predictions or explanations.

Despite the clear difficulties to provide a unified view of Hox gene function, several lessons can be learned from the analysis of the large palette of Hox mutant phenotypes already available. Functional redundancy/synergistic activity among members of paralog groups is a very common feature of Hox genes. A

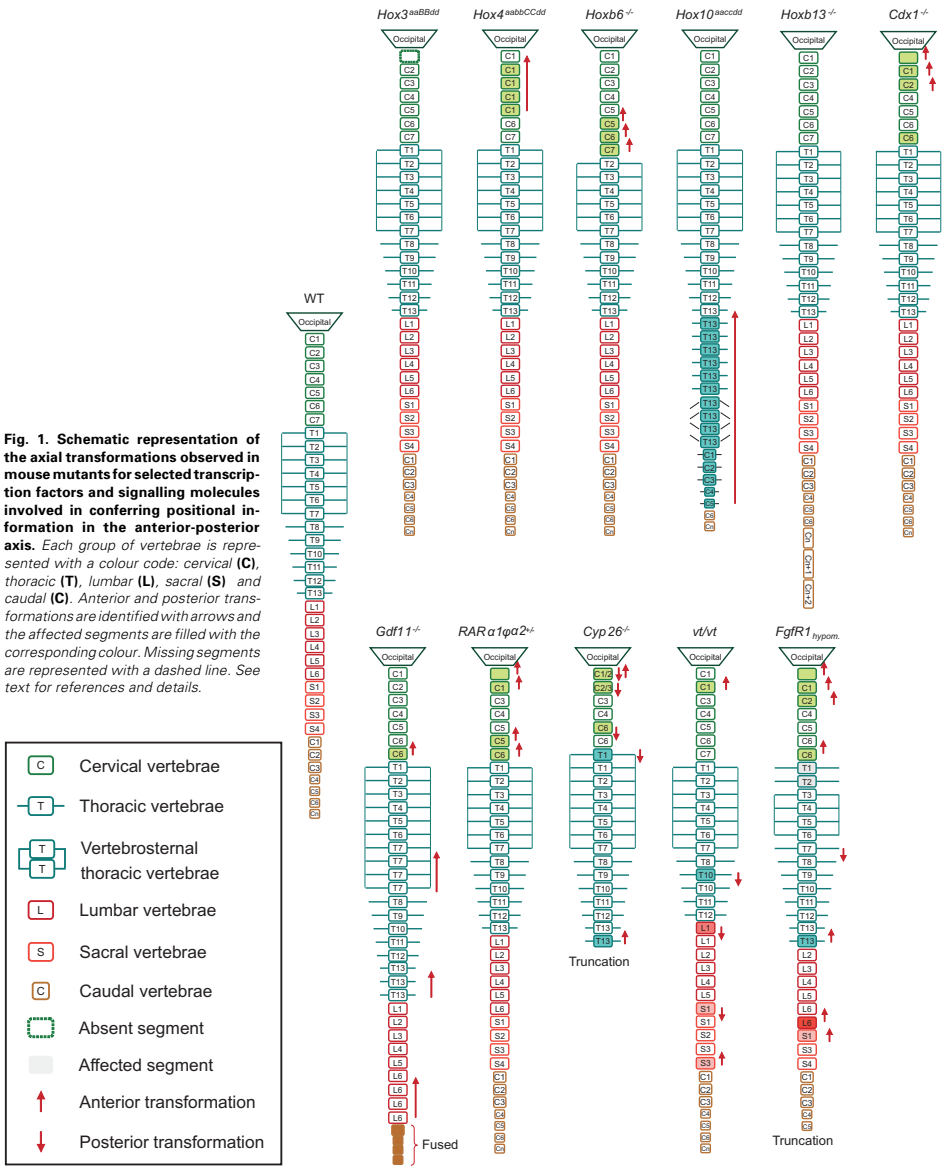

Fig. 1. Schematic representation of the axial transformations observed in mouse mutants for selected transcription factors and signalling molecules involved in conferring positional information in the anterior-posterior axis. *Each group of vertebrae is represented with a colour code: cervical* **(C)**, *thoracic* **(T)**, *lumbar* **(L)**, *sacral* **(S)** *and caudal* **(C)**. *Anterior and posterior transformations are identified with arrows and the affected segments are filled with the corresponding colour. Missing segments are represented with a dashed line. See text for references and details.*

paradigmatic example for functional redundancy is provided by the Hox paralog groups 10 and 11. Both groups contain 3 members and therefore a diploid total of 6 alleles. It has been shown that these genes have strong patterning effects in the lumbar and sacral areas, respectively, which were only revealed when all 6 alleles of the paralog group were inactivated (Wellik and Capecchi, 2003). The identity of the specific paralog member seems not to be as important as the total number of functional alleles expressed in the embryo, as a single allele of any of the paralog genes seems to be enough to rescue most of the phenotype (Wellik and Capecchi, 2003). For other paralog groups, the threshold levels of activity required for normal development are higher. As a consequence, the phenotypes observed in the compound mutants show a dose-dependent increase in the transformations (Condie and Capecchi, 1994; Horan el al., 1995; Fromental-Ramain *et al.*, 1996; Chen and Capecchi, 1997; Manley and Capecchi, 1997; Chen *et al.*, 1998). For instance, mutants for the paralog group 4 show anterior transformations of the cervical vertebrae from quite mild in the single mutants to quite extensive in the triple mutant for the paralog 4 genes of the a, b and d clusters, in which several cervical vertebrae are transformed into a C1 identity (Horan el al., 1995). In addition, and contrary to what seems to happen with the Hox groups 10 and 11, the functional weight for each of the paralog members appears to be slightly different as revealed by the specific single and compound mutants. At the moment, the level of redundancy of many other paralog groups is not clear because the lethality of some Hox mutations complicates the genesis of global paralog mutants. It should be noted, however, that for some Hox paralogs, in particular group 8, mutations in specific members of the group seem to rescue the phenotype derived from inactivating mutations in another member of the group, as revealed by the analysis of compound mutant mice (van den Akker *et al.*, 2001). This finding indicates that redundancy is also not a universal principle of Hox gene activity.

Also important in this discussion is the finding that genes of the same paralog group often have not only redundant functions but also unique activities. An example for this is the Hox paralog group 9. While both *Hoxa9* and *Hoxd9* seem to be required at the lumbar level, apparently only *Hoxa9* has an influence on the lower thoracic/thoraco-lumbar transition (Fromental-Ramain *et al.*, 1996).

Another very interesting characteristic of Hox gene activity is that paralog Hox groups usually have specific functional characteristics that differentiate them from other paralog Hox groups. Typical examples are the adjacent paralog groups 3 and 4, both involved in the patterning of the cervical region. While the absence of members of group 3 leads to the loss of a vertebral segment (Condie and Capecchi, 1994; Manley and Capecchi, 1997), mutations in group 4 result in identity changes in the cervical area (Horan *et al.*, 1995). Another good example is provided by the already mentioned groups 10 and 11, both belonging to the AbdB class of Hox genes. While the Hox group 10 genes specify lumbar identities, the activity of group 11 genes is required for the genesis of sacral vertebrae (Wellik and Capecchi, 2003). However, this rule of one paralog-one function is also not universal in the Hox world, as synergistic interactions among members of different paralog groups have also been reported. Among other examples we could mention the defects affecting the cervical-thoracic transition of trans-heterozygotes between *Hoxb9* and *Hoxb8* or *Hoxb7* (Chen and Capecchi, 1997) or the apparent non-allelic complementation of the *Hoxb5-Hoxb6* genes in axial patterning (Rancourt *et al.*, 1995).

A recurrent subject in the Hox-dependent vertebral phenotypes is also that the areas of the axial skeleton most typically affected in the Hox mutants are the transitions between vertebral domains. Thus, alterations in the first cervical vertebrae, the cervico-thoracic and thoraco-lumbar transitions, or the number of vertebro-sternal ribs (those attached to the sternum) are frequently reported associated to mutant mice for a variety of Hox genes. On the contrary, alterations in vertebrae in the middle of vertebral domains (like T4 or L3) are seldom reported. It is possible that some of this imbalance is explained by the easier identification of modifications in the first groups as compared to those in the second. However, it may also imply that these vertebral transitions represent fundamental changes in somite differentiation, which are more sensitive to disruption than the mechanisms involved in refining the global programmes to produce individual structures. Interestingly, an experimental proof for the existence of some kind of Hox-dependent global mechanisms responsible for the development of specific vertebral domains was provided in a recent report by Wellik and Capecchi (2003). In this study inactivation of all six Hox group 10 alleles resulted in animals with ribs in the prospective lumbar vertebrae, indicating a global requirement for Hox group 10 activity to pattern the lumbar area, mostly by blocking development of ribs from the corresponding segments. Likewise, complete inactivation of the Hox group 11 resulted in the transformation of prospective sacral and caudal vertebrae into a lumbar-like identity, indicating the requirement of a positive Hox group 11 activity to produce sacral and caudal identities (Wellik and Capecchi, 2003). Whether this principle of global determination of vertebral domains extends to other areas of the axial skeleton awaits experimental evaluation.

Finally, it should be noted that, while the general principle, mostly derived from the *Drosophila* field, considers that Hox genes are involved in providing identity to segments and not in the segmentation process itself, particular Hox mutant phenotypes are associated with the loss or gain of vertebral segments. *Hoxb13* mutant mice have extra caudal vertebrae, indicating that these mice produce extra segments in the paraxial mesoderm (Economides *et al.*, 2003). Conversely, in compound *Hoxa3;Hoxd3* and *Hoxb3;Hoxd3* mutants the first cervical vertebra, the atlas, fails to form (Condie and Capecchi, 1994; Manley and Capecchi, 1997). It is not clear whether the loss of the atlas is a consequence of the absence of the corresponding somitic domain, its inability to differentiate and subsequent loss, or the "skipping" of one whole segment in the differentiation programme. Interestingly, the *Hoxd3* gene was found to be expressed with a cyclic behaviour in the PSM, which could indicate a link between this gene and the segmentation process (Zakany *et al.*, 2001) (see below).

Another homeobox gene that has been shown to be involved in identity processes in the paraxial mesoderm is *Gbx2* (Carapuço *et al.*, 2005). Mice mutant for this gene show an axial phenotype closely resembling that produced by mutations in the *Hoxc8* gene (van den Akker *et al.*, 2001). It is not clear how this gene controls vertebral identities because it is not expressed in the somites and its inactivation does not affect Hox gene expression (Carapuço *et al.*, 2005). Actually, the phenotypes of these mutants provide a good argument in favour of the requirement of homeodomain-

containing activity in the PSM to control segmental identity in the paraxial mesoderm (see below).

The Cdx genes are another family of transcription factors also required for the proper control of vertebral identities. In mammals, this family is composed of three genes, *Cdx1*, *Cdx2* and *Cdx4*, with homology to the *Drosophila* gene *caudal* (Pollard and Holland, 2000). Mice mutant for the Cdx genes show homeotic transformations in their axial skeletons, although usually not as extensive as those observed in the Hox mutants. *Cdx1* null mutants display anterior transformations that affect the cervical and upper thoracic regions (Subramanian *et al.*, 1995). Lack of *Cdx2* leads to preimplantation embryonic lethality, thus hampering the analysis of their skeletons (Chawengsaksophak *et al.*, 1997). However, *Cdx2*$^{+/-}$ embryos present an anterior homeotic shift in the axial skeleton, albeit seemingly subtle and restricted to the cervico-thoracic transition, indicating that the *Cdx2* gene also plays a role in the control of vertebral identities (van den Akker *et al.*, 2002; Chawengsaksophak *et al.*, 1997; 2004). Inactivation of *Cdx4* did not affect development of the axial skeleton (van Nes *et al.*, 2006). However, inactivation of this gene did increase the transformations caused by mutations in the *Cdx1* and *Cdx2* genes, indicating both that *Cdx4* also plays a role in the specification of axial identities and that the Cdx genes have redundant functions (van Nes *et al.*, 2006). Redundancy was also observed between *Cdx1* and *Cdx2*, as the combination of mutant alleles for both genes gave rise to more severe skeletal defects than the single mutants (van den Akker *et al.*, 2002).

With the exception of *Cdx1*, which is also expressed in the anterior paraxial mesoderm, expression of the Cdx gene family is mostly localized to posterior embryonic areas (Meyer and Gruss, 1993; Gamer and Wright, 1993; Beck *et al.*, 1995), suggesting that their activity on the control of vertebral identities might be mediated by other factors. The similarity of the axial phenotypes of the Cdx mutant mice with those of the mutants in several Hox genes suggested functional interactions between the two gene families. The expression domains of particular Hox genes suffered posterior shifts in the Cdx mutants, which were more accentuated when the Cdx mutations were combined (Subramanian *et al.*, 1995; van den Akker *et al.*, 2002), lending support to this hypothesis and placing the Hox genes downstream of the Cdx. In agreement with this idea, consensus response elements for the Cdx proteins were identified in the promoter DNA sequences of a number of Hox loci (Subramanian *et al.*, 1995; Knittel *et al.*, 1995; Pownall *et al.*, 1996; Charité *et al.*, 1998; Isaacs *et al.*, 1998; Gaunt *et al.*, 2004; Tabariès *et al.*, 2005). Interestingly, some of these consensus Cdx response elements have been shown to be able to transduce positional information to regulate Hox gene expression in the mesoderm and neurectoderm in a dose-dependent manner. And, while Cdx genes are mainly thought to be positive regulators of Hox gene expression, it has recently been shown that these genes may also be involved in blocking Hox gene expression. In particular, a Cdx-responsive enhancer was identified in the *Hoxa5* locus that is required for the proper positioning of the caudal limit of expression of this gene by a repressive mechanism (Tabariès *et al.*, 2005). All together, these data indicate that Cdx genes might control vertebral identities indirectly through their effects on Hox gene expression. It should be noted, however, that the alterations of Hox gene expression in *Cdx1* mutant embryos are quite mild, indicating that Cdx genes could also have a Hox-independent role in skeletal patterning.

Another group of genes that is also important for the proper control of vertebral identities in mammals are the homologs of the *Drosophila* Trithorax (TrxG) and Polycomb (PcG) groups. It is generally believed that the members of these large groups of genes are involved in epigenetic processes to stabilize the transcriptional state of different developmentally relevant genes (Pirrotta 1998), including Hox genes, although recent reports suggest that these proteins could have a more direct role in transcriptional processes (Breiling *et al.*, 2001; Saurin *et al.*, 2001; Milne *et al.*, 2002; de Graff *et al.*, 2003; Wang *et al.*, 2004; de Napoles *et al.*, 2004). While the TrxG genes are thought to maintain Hox gene activity in the appropriate domains, the PcG genes seem to be involved in keeping them repressed in the complementary regions (Pirrotta, 1998). Accordingly, the role of these genes in the control of regional identities in the paraxial mesoderm is thought to be indirect, mediated by their effect on Hox gene expression. Consistent with this idea, inactivation of the *Trx* homolog *Mll* resulted in homeotic transformations in the axial skeleton associated with the down-regulation of specific Hox genes after their seemingly normal induction (Hanson *et al.*, 1999; Yu *et al.*, 1998). Conversely, mice bearing mutations in elements of the PcG genes, like *Mel18*, *Bmi1*, *M33*, *Mph1*, *Ring1A* or *Eed*, showed derepression of some Hox genes outside their normal domain associated with homeotic transformations of the axial skeleton (Akasaka *et al.*, 1996; van der Lugt *et al.*, 1994; Schumacher *et al.*, 1996; Core *et al.*, 1997; Takihara *et al.*, 1997; del Mar Lorente *et al.*, 2000; Wang *et al.*, 2002). PcG genes seem to synergize in their activity since compound mutations for some of these genes have been shown to enhance the phenotypes associated with the individual genes (Bel *et al.*, 1998; Akasaka *et al.*, 2001). However, it should be noted that a constant characteristic of these mutant mice is that their homeotic phenotypes are quite mild and do not always correlate with the expected type of transformation. For instance, the *Ring1A* mutants show a combination of posterior (e.g., T12 to L1) with anterior (e.g., C2 to C1) type transformations, which is contrary to the posterior type only transformations to be expected for this kind of gene (del Mar Lorente *et al.*, 2000).

Interestingly, the TrxG and PcG genes seem to function antagonistically as the vertebral transformations and altered Hox expression patterns of *Mll*-deficient and *Bmi1*-deficient mice were normalized when both *Mll* and *Bmi1* were deleted (Bel *et al.*, 1998; Akasaka *et al.*, 2001).

Recent work from several laboratories has provided the basis to begin to understand the molecular mechanisms of the activity of the TrxG and PcG genes. A thorough description of such mechanisms is beyond the scope of this review and the interested reader is referred to recent reviews on the subject (e.g., Cernilogar and Orlando, 2005; Grimaud *et al.*, 2006)

Signalling molecules

In addition to the "classical" determinants of positional identity in the axial skeleton discussed above, genetic and teratogenic studies uncovered the requirement of molecules belonging to different signalling systems for the development of a normal axial formula.

Gdf11 (also called Bmp11), a member of the TGFβ family of

signalling molecules (Nakashima *et al.*, 1999), has been shown to play an important role in the patterning of the axial skeleton. Inactivation of this gene in mice produced strong deviations from the normal vertebral formula (McPherron *et al.*, 1999). *Gdf11* mutant mice have several extra thoracic and lumbar segments at the expense of caudal vertebrae. Interestingly, the *Gdf11*$^{-/-}$ mutants apparently have a normal number of somites, which suggests that *Gdf11* is not affecting the rate of somite formation but rather their positional identity (McPherron *et al.*, 1999). Overall, the mutant phenotype was interpreted as a general anterior homeotic transformation of posterior segments. Molecular analyses of these mutants indicated abnormal expression of selected Hox genes, including a posterior expansion of *Hoxc6* and *Hoxc8* in the developing vertebrae by 2 or 3 segments and a posterior shift in the rostral limit of the *Hoxc11* and *Hoxc10* expression domains, the latter following the caudal displacement of the hindlimb also observed in these mutant embryos.

A series of genetic experiments have also identified the receptors that apparently mediate Gdf11 activity in the control of segmental identities in the axial skeleton. *Activin receptor IIB* (*AcvrIIB*) mutant mice show multiple patterning defects, including vertebral transformations that resemble the *Gdf11*$^{-/-}$ phenotype, although less severe (Oh *et al.*, 1997). The milder phenotypes of *AcvrIIB*$^{-/-}$ mice relative to the *Gdf11* mutants suggested that other type II receptor(s) for the Tgfβ family must be compensating for the lack of *AcvrIIB*. Specifically, *Activin receptor IIA* (*AcvrIIA*) seems to be involved. While disruption of *AcvrIIA* do not produce phenotypes in the axial skeleton (Matzuk, *et al.*, 1995; Song *et al.*, 1999), reducing the *AcvrIIA* dose in the context of an *AcvrIIB* mutant background increased the severity of the axial phenotypes, indicating that these two receptors cooperatively mediate the activity of Gdf11 in the context of vertebral specification (Oh *et al.*, 2002). Biochemical studies showing binding of Gdf11 to these receptors further support this conclusion (Oh *et al.*, 2002). Recently, the type I Tgfβ receptor that could be functionally interacting with the type II receptors and Gdf11 was identified as ALK5 (Andersson *et al.*, 2006). This type I receptor was shown biochemically to interact with Gdf11 in an AcvrIIB-dependent manner. In addition, the severity of the *AcvrIIB*$^{-/-}$ phenotype was increased when one allele of *ALK5* was inactivated, suggesting a functional role for *ALK5* in anterior-posterior skeletal patterning via Gdf11 signalling *in vivo* (Andersson *et al.*, 2006).

Other signalling pathways, including those of FGFs, WNTs and retinoic acid (RA), have also been implicated in the control of axial identity in the paraxial mesoderm. As these factors also play essential roles at earlier stages of mesodermal development, it is sometimes difficult to evaluate the extent of their involvement in regional specification processes. However, for all these signalling pathways there is enough data available to say with confidence that they also play a role in the control of segmental identity in the paraxial mesoderm.

It has long been known that an imbalance of vitamin A can have severe teratogenic effects (Weston *et al.*, 2003). RA is the main active metabolite of vitamin A and was among the first signalling molecules to be experimentally associated with A-P patterning processes in the paraxial mesoderm. As already discussed above, it has been shown that administration of high RA doses results in a number of skeletal defects, including homeotic transformations in the axial skeleton (Kessel and Gruss, 1991; Kessel, 1992). These include both anterior and posterior types of transformation and affect vertebrae at all axial levels, the specific type of transformation being stage- and dose-dependent (Kessel and Gruss, 1991; Kessel, 1992). RA is not only a teratogen affecting the axial skeleton; it also plays a physiological role in the determination of regional identities in the paraxial mesoderm, as revealed by the phenotypes of compound mutants for the retinoic acid receptors (RAR) (Lohnes *et al.*, 1994). Mice carrying specific combinations of mutant alleles for RARs had abnormal skeletal phenotypes which also included vertebral malformations scored as homeotic transformations. Interestingly, these transformations affected almost exclusively the cervical area, indicating either that RA is not involved in the physiological control of vertebral identities in more caudal areas or that these effects are mediated by a different set of receptors. Expression of a dominant negative form of a RAR in chondrogenic cells also produced alterations in the cervical vertebrae (Yamaguchi *et al.*, 1998). These data were interpreted as suggesting that the activity of RA in skeletal development is required in the differentiating mesenchyme.

Manipulation of RA metabolism in the embryo provided further support for the requirement of proper spatial control of RA activity during embryonic development in general and during axial patterning in particular. Inhibition of RA production through the inactivation of *Raldh2* confirmed the need of this signalling pathway for mesodermal development (Niederreither *et al.*, 1999). However, these mutants were not informative regarding the involvement of RA in the control of spatial identities in the axial skeleton because the embryos died at midgestation stages. Conversely, inactivation of *Cyp26*, an enzyme involved in the catabolism of RA, produced vertebral phenotypes very similar to those resulting from exogenous administration of RA (Sakai *et al.*, 2001; Abu-Abed *et al.*, 2001). Interestingly, the analysis of RA activity in these mutants using a RA reporter transgene, *RARE-hsp-lacZ*, revealed that Cyp26-mediated inactivation of RA signalling occurs locally in the PSM, indicating that the effects of excessive RA signalling on the axial skeleton derive from its activity in this unsegmented area of the paraxial mesoderm (Sakai *et al.*, 2001). The possible relevance of this finding will be discussed later.

The similarity of the skeletal phenotypes of compound RAR mutants, *Cyp26*$^{-/-}$ animals and RA-exposed embryos with those of *Cdx1* and *Wnt3a* mutants (see later) suggests an interaction among these factors. Indeed, molecular analyses indicate that expression of *Wnt3a* is negatively modulated by increased RA signalling (Sakai *et al.*, 2001; Abu-Abed *et al.*, 2001), and the *Cdx1* promoter contains RA responsive elements (RARE) (Houle *et al.*, 2000). In addition, it has been known since the early days of research on Hox genes that RA signalling has a strong influence on Hox gene expression (Simeone *et al.*, 1990), and RAREs have been found within the Hox complexes (Lanston *et al.*, 1997; Zhang *et al.*, 1997). Consistent with this, abnormal Hox gene expression was found in embryos with altered RA signalling (altered both by genetic or pharmacological procedures) and, given the role of Hox genes in the control of segmental identity in the paraxial mesoderm, it has been suggested that it is the abnormal expression of these genes that

determines the axial phenotypes derived from misregulated RA signalling. However, clear experimental evidence supporting this hypothesis is still lacking.

Fgfr1-mediated FGF signalling is also involved in the control of positional information in the paraxial mesoderm. Inactivation of *Fgfr1* leads to severe gastrulation abnormalities and early embryonic lethality (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994) which complicates the analysis of the role Fgfr1 may play in skeletal patterning. Nevertheless, the genetic analysis of a series of hypomorphic and activated *Fgfr1* alleles showed that Fgfr1-dependent FGF signalling is required for proper A-P patterning of the paraxial mesoderm (Partanen *et al.,* 1998). In this study, it was shown that mice carrying hypomorphic alleles of the *Fgfr1* gene had homeotic transformations in their axial skeleton, predominantly with anterior characteristics. These skeletal malformations were associated with caudal truncations of variable severity depending on the specific *Fgfr1* genotype. In contrast, an activating mutation that converted the tyrosine autophosphorylation site (Y766) into a phenylalanine led exclusively to posterior transformations (Partanen *et al.,* 1998). Associated with these skeletal alterations, the authors found subtle alterations in Hox gene expression. This led them to suggest that the role of FGF signalling in the control of positional information in the paraxial mesoderm is to establish appropriate Hox gene expression. Thus, FGF signalling would determine vertebral identities indirectly through the activity of Hox genes (Partanen *et al.,* 1998). The involvement of FGFs in mesodermal patterning was also suggested by studies in *Xenopus* that correlated FGF overexpression with up-regulation of Hox genes, although the impact on vertebral identities was never evaluated directly (Pownall *et al.*, 1996).

Genetic analyses of *Wnt3a* also suggested its involvement in the establishment of segmental identities in the paraxial mesoderm. Complete inactivation of this gene leads to severe truncation of the body axis posterior to the forelimb level (Takada *et al.*, 1994; Ikeya and Takada, 2001). However, even in these strongly truncated embryos, analysis of the cervical skeleton revealed the presence of anterior vertebral transformations (Ikeya and Takada, 2001). The analysis of *Wnt3a* heterozygous mice and of *vestigial tail* (*vt*) mutants, which carry a hypomorphic mutation for the *Wnt3a* gene (Greco *et al.*, 1996), confirmed the involvement of this factor in A-P patterning processes of the axial skeleton, extending all along the anterior-posterior axis (Ikeya and Takada, 2001). The effects of Wnt3a in patterning the paraxial mesoderm seem to be at least partially mediated by control of the *Cdx1* gene, which was found to be strongly downregulated in response to total or partial reductions in *Wnt3a* activity (Ikeya and Takada, 2001). In agreement with this, the skeletal phenotypes of the *Wnt3a* and *Cdx1* mutants are very similar. In addition, studies on the *Cdx1* promoter revealed the presence of functional β-catenin responsive elements (Lickert *et al.*, 2000). However, as the *Cdx1* mutant phenotype does not include transformations posterior to the cervical/upper thoracic region (Subramanian *et al.*, 1995), the activity of Wnt3a in these more posterior areas must be mediated by a Cdx1-independent mechanism. Hox gene expression was also analysed in the *Wnt3a* mutants, and very subtle or no alterations were found when compared to wild type littermates (Ikeya and Takada, 2001), arguing against an extensive role of these genes downstream of *Wnt3a* in the A-P patterning of the paraxial mesoderm.

Where is segmental identity determined?

Grafting experiments performed more than 30 years ago indicated that somites already contain their positional information while they are still being formed in the anterior presomitic mesoderm (Kieny *et al.*, 1972). In those experiments, presomitic mesoderm from a given stage grafted to an equivalent position of an embryo at an earlier developmental stage diffentiated according to the prospective somitic position of the donor tissue. However, several years later, as the genetic determinants of segmental axial identities started to be evaluated and Hox genes took centre stage, their specific and remarkable expression patterns in the somites led to the assumption that it is their somitic expression that is relevant to their function in the control of segmental identities in the paraxial mesoderm. This is actually one of the basic assumptions of the “Hox code” and “posterior prevalence” models. Likewise, the grafting experiments by Kieny *et al.* (1972) were later explained by the expression of specific Hox genes in the graft-derived somites, which corresponded to the patterns appropriate for the donor tissue (Nowicki and Burke, 2000).

Nonetheless, until recently, the functional relevance of Hox gene activity in the somites was not directly tested, and there are descriptions of Hox-associated vertebral phenotypes which are clearly inconsistent with Hox gene activity being required in the somites. For instance, axial phenotypes were observed in embryos that recovered appropriate somitic Hox gene expression domains after retarded activation in the presomitic mesoderm (Zakany *et al.*, 1997). Similarly, specific genetic manipulations in the Hox complexes resulted in transient precocious expression of some *Hox* genes in the paraxial mesoderm, which did not affect their final somitic expression, but nonetheless produced abnormal phenotypes in the axial skeleton (Kondo and Duboule, 1999). While these results clearly highlight the importance of timing for the function of Hox genes, they also indicate that proper somitic Hox gene expression is not sufficient for normal patterning of the axial skeleton. The phenotypes of several Hox mutant mice are also inconsistent with Hox gene activity being required in the differentiating somites. For instance, malformations in the upper thoracic vertebrae observed in *Hoxb9* mutant embryos were exacerbated when one or both alleles of the *Hoxa9* gene, which is not expressed at the corresponding somitic level, were also inactivated (Fromental-Ramain *et al.*, 1996). Even more striking is the case of the *Hox10* paralog group. As mentioned earlier, genetic data clearly showed that the genes of this paralog group are essential for the patterning of the lumbar area and that the three members of this group have equivalent functions in this process (Wellik and Capecchi, 2003). However, expression of these genes not only fails to reach the proper somitic level but also shows strong variations among the group members (Carapuço *et al.*, 2005).

A direct evaluation of the spatial requirements for Hox gene activity showed that at least in some cases Hox genes are able to imprint specific segmental identity to somites when they act during their formation in the PSM, and that somitic Hox gene expression alone is not sufficient (Carapuço *et al.*, 2005). This seems to be the case for the Hox10 group, as *Hoxa10* can extend

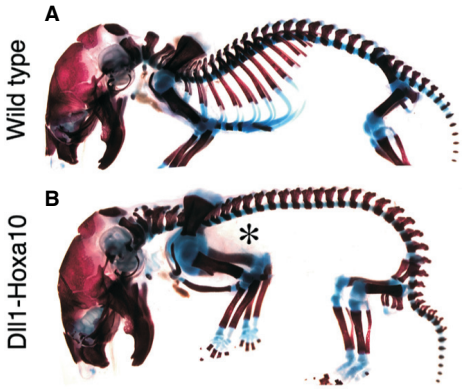

Fig. 2. Effect of expression of *Hoxa10* in the presomitic mesoderm of transgenic embryos. (A) *Skeleton of a newborn wild type mouse.* **(B)** *Skeleton of a newborn transgenic mouse in which the* Hoxa10 *gene was expressed in the presomitic mesoderm using an enhancer of the the* Dll1 *promoter. Note the complete absence of ribs (asterisk). The skeletons were stained using the alcian blue-alizarin red method.*

its dominant activity to block formation of ribs anterior to the lumbar area when ectopically expressed in the prospective thoracic PSM (Fig. 2) but not in the corresponding somites (Carapuço *et al.*, 2005). In other cases Hox gene expression in the PSM or in the somites seems to be required for alternative functional activities. This was clear for the Hox11 group, which, as discussed above, is required for the production of both sacral and caudal type vertebrae (Wellik and Capecchi, 2003). In this case expression in the PSM is required for the formation of the sacrum and expression in the somites is responsible for giving a caudal signature to the vertebrae (Carapuço *et al.*, 2005). The extent to which the function of other Hox genes is required in the somites, in the PSM, or even in earlier stages of development of the paraxial mesoderm remains to be determined. Interestingly, it was recently reported that Hox gene expression in the epiblast acts dominantly to determine specific cellular behaviours during gastrulation and possibly at later stages in the differentiation of the paraxial mesoderm (Iimura and Pourquié, 2006).

A challenging aspect of addressing the above discussed ideas is to find a mechanism that complies to the principle of being set in the PSM and only translated later during differentiation of the somite. Among the possible approaches to this problem is the identification of the system that translates the patterning information into a morphogenetic programme. Although our knowledge about these systems is in general limited, some information is available regarding rib formation, which would impact on the understanding of the global mechanisms for the formation of the thoracic and lumbar areas, the latter because it seems to require rib inhibition (Wellik and Capecchi, 2003).

Ever increasing evidence supports the idea that signals provided by the myotome are crucial for the proper development of ribs. Mutations in myogenic regulatory factors, like *Myf-5* or *myogenin*, lead to the absence of the major distal part of the ribs (Braun *et al.*, 1992; Wang *et al.*, 1996). The expression of these genes is confined to the myotome without apparent sclerotomal contribution (Bober *et al.*, 1991; Hopwood 1991; Ott *et al.*, 1991; Hinterberger *et al.*, 1991; Pownall *et al.*, 1992), indicating the need for interactions between somitic compartments in the development of ribs. Among the strongest candidates to mediate these interactions are Fgf4, Fgf6 and Pdgfα. These factors are expressed in the myotome (Goldfarb, 1990; Orr-Urtreger and Lonai, 1992; deLapeyriere *et al.*, 1993) and are downregulated in the somites of *Myf5*-deficient mice (Grass *et al.*, 1996; Tallquist *et al.*, 2000). In addition, while the requirement of Fgf4 and Fgf6 for rib formation has still not been tested genetically, disruption of signalling through PDGFs leads also to malformations in vertebrae, ribs and sternum (Soriano, 1997). In addition, functional Myf5-binding sites have been found in the *Pdgfα* promoter in mice and humans and knocking-in *Pdgfα* into the *Myf5* locus partially rescues rib formation defects typical of *Myf5*-deficient mice, indicating that *Pdgfα* is a *bona fide* downstream effector of Myf5 (Tallquist *et al.*, 2000).

On the basis of this information, it is tempting to speculate that the patterning activities provided in the presomitic mesoderm (Kieny *et al.*, 1972; Carapuço *et al.*, 2005), Hox-dependent or not, at least regarding rib development, could be effectively translated at a later stage in the modulation of myotomal-sclerotomal interactions, either by controlling the production of myotomal signals or by modulating the sclerotomal responses to those signals. However, irrespective of the mechanism, it is somehow specifically encoded in the PSM and not in the already formed somites, suggesting that the encoding system may be linked to specific features of the PSM. We will attempt to address this issue in the next sections.

Are *Hox* genes functionally connected to the signalling systems operating in the patterning of the paraxial mesoderm?

As discussed earlier in this review, it is clear that both signalling molecules and homeodomain-type transcription factors are involved in the control of segmental identities in the paraxial mesoderm. However, the functional connection between these two groups of molecules, if any, is not so clear. As is implicit in the above discussion, the classical view is that the various signalling pathways modulate expression of the Hox genes, which are then responsible for specifying the identity of the different vertebral segments. This would reconcile the apparent discrepancy observed in the mutants for several of these signalling systems, in which phenotypes are typically associated to somitic differentiation but expression or activity are mostly restricted to the most caudal parts of the paraxial mesoderm (Takada *et al.*, 1994; McPherron *et al.*, 1999; Sakai *et al.*, 2001; Corson *et al.*, 2003). Accordingly, efforts were always made to find modifications in Hox gene expression in any mutant with an altered axial skeleton. In some cases the observed alterations in Hox expression patterns are clear and somewhat extensive (e.g., in *Gdf11* mutants), but in others they are so subtle that it is hard to imagine that these modifications could be causally connected to the observed phenotypes (e.g., in *Wnt3a* mutants).

An alternative hypothesis, which is not necessarily mutually

exclusive with that outlined in the previous paragraph, is based on the finding that the activity of the Hox genes is required in the PSM (Carapuço *et al.*, 2005). According to this hypothesis, Hox genes would modulate the activity of the signalling processes that are involved in the establishment of axial identities in the paraxial mesoderm. These could include those signalling pathways discussed above (i.e. RA, FGF, WNT) and others that we still have not considered in this review, e.g. involving members of the Notch superfamily. Although to our knowledge no data are available so far that directly prove or disprove this hypothesis, there is evidence indicating that Hox genes can indeed modify the activity of signalling pathways. For instance, we have shown that *Hoxa2* modulates the response of mesenchymal cells of the second branchial arch to Fgf8 (Bobola *et al.*, 2003). And components of several signalling pathways have been reported as downstream targets of Hox genes in several biological contexts (Mallo and Magli, 2006). Also provocative is the recent finding that Hox gene expression in the epiblast modulates gastrulation movements of the targetted cells (limura and Pourquié, 2006), because a role in this process was also described for FGF and BMP signalling (Miura *et al.*, 2006). Thus, it is conceivable that Hox genes could modulate the activity of these signalling pathways. In biological systems, gene expression and morphogenetic mechanisms are often maintained by feedback loops after their initial induction. If this principle also applies to Hox genes and signalling processes, altered signalling could also leave its signature in abnormal Hox gene expression, which is what is found in many of the signalling mutants.

Are segmentation and segmental identity functionally connected?

During recent years considerable effort was made towards understanding the molecular basis of somitogenesis. The leading model to explain this process is that known as "clock and wavefront", initially proposed by Cooke and Zeeman (1976). This model proposes the existence of an oscillating signal in the PSM (the clock) that sets the pace for somite formation, and of a "determination front" which sets the position along the AP axis where cells respond to the oscillatory signal to create a segmentation domain. A lot of evidence has now accumulated supporting this model, which also provided the key information to understand the process of somitogenesis in molecular terms. Many recent reviews cover the different aspects of this process (Aulehla and Herrmann, 2004; Dubrulle and Pourquié, 2004; Giudicelli and Lewis, 2004; Gridley, 2006), so we will only describe it very briefly to help understand the possible connection between segmentation and positional information in the paraxial mesoderm.

The first experimental evidence for a cyclic molecular activity in the PSM was the dynamic expression of the chicken *Hairy1* gene (Palmeirim *et al.*, 1997). Expression of this gene was found as a wave running through the PSM in a posterior to anterior direction with a periodicity that matched the pace of somite formation. Since then, many other genes were found to have an equivalent oscillatory expression in mice, chicken, zebrafish and *Xenopus*, indicating that this mechanism is conserved among vertebrates (reviewed in Aulehla and Herrmann, 2004; Dubrulle and Pourquié, 2004; Giudicelli and Lewis, 2004; Gridley, 2006). In general, these cycling genes are components of the Notch and WNT signalling pathways. Interestingly, all Notch pathway members cycle mostly in phase, suggesting that they are functionally linked. Conversely, the oscillation of these genes is largely out of phase with the cycles of the WNT pathway members. In addition, the cycling activities of both pathways seem to be functionally connected, WNT being apparently upstream of the Notch (Aulehla *et al.*, 2003). The molecular nature of the wavefront seems to include opposing gradients within the PSM: Fgf8/Wnt3a in a posterior to anterior direction and RA in an anterior to posterior direction. Fgf8 is thought to keep PSM cells in an undifferentiated state. As mesodermal cells move anteriorly through the PSM, they will be exposed to progressively lower Fgf8 levels until they reach a level of FGF signalling low enough to allow activation of the segmentation programme. This area would be the "determination front". The anterior-posterior RA gradient seems to be functionally antagonistic to that of FGFs and it has been proposed that it functions by opposing FGF activity and/or by directly activating genes involved in the segmentation process (Diez del Corral and Storey, 2004). The WNT signalling pathway, acting through Wnt3a, was proposed to integrate clock and gradients in a global mechanism controlling the segmentation process (Aulehla and Herrmann 2004).

The connection, if any, between the formation of the somites and the specification of the vertebral type they will produce is not clear. Experiments in which the Fgf8 gradient in the PSM of chicken embryos was artificially altered resulted in abnormal activation of Hox gene expression (Dubrulle *et al.*, 2001). In particular, exogenous application of Fgf8 in the posterior PSM resulted in smaller somites anterior to the bead, compensated by larger somites posteriorly. Associated with this effect, activation of *HoxB9* and *HoxA10* seemed to be shifted anteriorly in the operated side. The authors interpreted this premature activation of Hox gene expression as resulting from cells in the somites anterior to the Fgf8 bead being exposed to an extra oscillation cycle, thus suggesting a connection between the segmentation clock and specification of axial identity, as determined by Hox gene expression (Dubrulle *et al.*, 2001). In these experiments, however, it was not analysed whether Hox gene activation was a direct effect of Fgf8 [as it has been shown to be in other biological contexts (Johnson *et al.*, 1994; Pownall *et al.*, 1998; Bel-Vialar *et al.*, 2002)] and if the altered Hox gene expression actually resulted in identity transformations in the axial skeleton.

A link between the segmentation clock and Hox gene activation was also suggested by the finding that some Hox genes, including *Hoxd1* and *Hoxd3*, show a dynamic expression profile in the PSM of mouse embryos somewhat resembling the expression of genes ascribed to the segmentation clock (Zakany *et al.*, 2001). Expression of *Hoxd1* in this area was shown to be dependent on Notch signalling, one of the main components of the segmentation clock, further reinforcing the connection between segmentation and positional information. Inactivation of *Hoxd1* resulted in fusions of the first two cervical vertebrae, but it was not possible to assess if this phenotype results from the lack of activation of this gene in the PSM by the Notch signalling because of the early lethality associated to the global inactivation of this signalling pathway (Oka *et al.*, 1995). A partial answer to this question was provided by an independent report using transgenic approaches to modulate Notch signalling in the PSM. Expression of a dominant negative form of the Notch ligand Dll1 using two different promot-

ers resulted in alterations in the axial skeleton that were scored as identity changes (Cordes *et al.*, 2004). These anatomical phenotypes were associated with subtle changes in the expression of some Hox genes, but a causal relationship between the morphological and molecular phenotypes remains to be determined. Homeotic transformations were also reported for other mutants in members of the Notch signalling pathway (Cordes *et al.*, 2004), although the proper characterization of the identity changes and the evaluation of their extent were complicated by the strong segmentation phenotypes also observed in these mice (Zhang and Gridley, 1998; Evrard *et al.*, 1998).

Another indirect indication of a possible functional connection between segmentation and segmental identity processes in the paraxial mesoderm is provided by the interesting association of vertebral transformations scored as homeotic transformations with alterations of the signalling pathways that create the gradients in the PSM (FGF, RA, WNT). While, as discussed above, various explanations were hypothesized for these phenotypes, it is also possible that they are related to deviations from the proper functioning of the segmentation clock. If this is indeed the case, it would favour a thus far hypothetical link between somitogenesis and positional information in the paraxial mesoderm. Nonetheless, such a hypothesis awaits direct experimental evaluation.

Concluding remarks

Years of intense research have resulted in the identification of many of the genetic determinants of positional information in the paraxial mesoderm. However, suprisingly little is known about how these genes work to produce a properly patterned axial skeleton. Very recent data suggest, although in part quite indirectly, that the patterning of the axial skeleton is programmed by interactions between Hox genes and several signalling systems. It is even possible that the generation of the patterning information is linked to the processes leading to formation of somites, a potential connection worth exploring using direct experimental approaches.

The potential connection between Hox and signalling systems also suggests how Hox genes could be modulating specific morphogenetic processes. Earlier in this review we have discussed that the Hox-mediated modulation of rib formation might be mediated through influencing signalling processes between the myotome and sclerotome. This influence of Hox gene activity on signalling could be part of a mechanism by which they control development of vertebral structures other than the ribs. Accordingly, we speculate that Hox genes might provide positional information by modulating different signalling pathways in specific ways and that it is the global outcome of these signalling activities which dictates the specific morphogenetic programmes. Different combinations of Hox genes would determine different profiles of signalling activities, thus generating different structures. In this context, it is worth noting that most of the known signalling pathways have an effect on skeletogenesis and thus modulation of signalling processes could eventually mean modulation of skeletogenic processes. Obviously, there are still too many unanswered questions regarding how positional information is encoded and decoded in the paraxial mesoderm, which surely will keep us busy for years to come.

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APPENDIX II

Vinagre, T., Moncaut, N., Carapuço, M., Bom, J., Novoa, A., and Mallo, M. (2010). Evidence for a Myotomal Hox / Myf Cascade Governing Nonautonomous Control of Rib Specification within Global Vertebral Domains. Developmental Cell 18, 655-661.

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Short Article

Evidence for a Myotomal Hox/Myf Cascade Governing Nonautonomous Control of Rib Specification within Global Vertebral Domains

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SUMMARY

Hox genes are essential for the patterning of the axial skeleton. Hox group 10 has been shown to specify the lumbar domain by setting a rib-inhibiting program in the presomitic mesoderm (PSM). We have now produced mice with ribs in every vertebra by ectopically expressing Hox group 6 in the PSM, indicating that Hox genes are also able to specify the thoracic domain. We show that the information provided by Hox genes to specify rib-containing and rib-less areas is first interpreted in the myotome through the regional-specific control of *Myf5* and *Myf6* expression. This information is then transmitted to the sclerotome by a system that includes FGF and PDGF signaling to produce vertebrae with or without ribs at different axial levels. Our findings offer a new perspective of how Hox genes produce global patterns in the axial skeleton and support a redundant nonmyogenic role of *Myf5* and *Myf6* in rib formation.

INTRODUCTION

Hox genes have been classically described to be involved in the production of vertebrae with individual characteristics (Krumlauf 1994; Wellik 2007; Mallo et al., 2009). More recently, it was discovered that Hox genes also play essential roles in defining global vertebral domains (Wellik and Capecchi, 2003). In particular, it was shown that Hox group 10 is responsible for the layout of the rib-less lumbar region by diverting it from a rib-containing thoracic identity (Wellik and Capecchi, 2003; Carapuço et al., 2005). In addition, Hox group 11 was demonstrated to be required for the formation of the sacrum (Wellik and Capecchi, 2003). However, it remains unclear whether or not Hox genes are involved in the global specification of the thoracic and cervical domains. Moreover, the mechanism by which Hox genes control these processes is completely unknown.

Wellik and Capecchi, (2003) proposed that ribs are set out by default and that the rib-less cervical domain would result from the rib-blocking activity of other Hox genes acting similarly to Hox group 10 in the lumbar region. However, this hypothesis is difficult to reconcile with published expression patterns for Hox genes (Burke et al., 1995), which instead suggest an alternative hypothesis. In particular, the anterior limit of expression of members of the Hox group 6 correlates with the cervical-to-thoracic transition in a variety of vertebrates bearing a different number of cervical vertebrae (Burke et al., 1995), indicating that this Hox group might have a role in promoting rib formation. Here we present evidence supporting this hypothesis, showing that Hox control of rib formation is mediated by regulation of *Myf5* and *Myf6* expression in the hypaxial myotome through interaction with a relevant enhancer. Moreover, our transgenic analyses indicate that myotomal *Myf5*/*Myf6* activation triggers a nonautonomous effect mediated by PDGF and FGF signaling, promoting rib formation in the adjacent sclerotome. Our data support a redundant nonmyogenic role of *Myf5* and *Myf6* in the processes leading to rib formation.

RESULTS

Overexpression of Hox Group 6 Induces Ectopic Rib Formation

In order to test whether Hox group 6 activity could induce rib formation, we employed a transgenic approach to overexpress *Hoxb6* either in the presomitic mesoderm (PSM) or in the somites of mouse embryos. While somite-exclusive expression gave mild phenotypes (see Figures S1A and S1B available online), the extended expression of *Hoxb6* in PSM cells resulted in the formation of ectopic ribs throughout the whole length of the axial skeleton (Figures 1A and 1B), without affecting the total number of vertebrae. In these transgenics, the prospective cervical area contained ribs fused laterally to form an apparent articular surface for the forelimbs, which were slightly displaced rostrally. The prospective lumbar area also displayed ectopic ribs, progressively decreasing in size in a caudal direction, presumably following the physiological decrease in size of the lower thoracic ribs. In the presumptive sacral area, the vertebrae lost their characteristic morphology and assumed rib-like features, while keeping the lateral fusions typical of the sacral region.

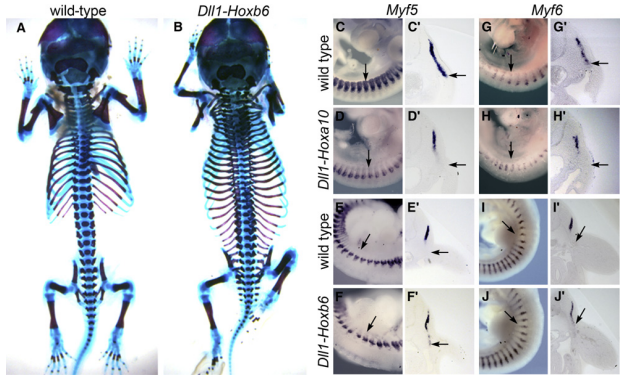

Figure 1. Control of Rib Formation and Myf5/Myf6 Expression by Hox Genes

(A and B) *Hoxb6* overexpression in the PSM induces ectopic rib formation. Skeletal staining of wild-type (A) and *Dll1-Hoxb6* (B) E18.5 fetuses. Equivalent phenotypes were observed in 4 out of 9 transgenics.

(C–J) Hox groups 6 and 10 modulate regional expression *Myf5* and Myf6. Whole-mount in situ hybridization of wild-type (C, C′, E, E′G, G′, I, and I′), *Dll1-Hoxa10* (D, D′, H, and H′), and *Dll1-Hoxb6* (F, F′, J, and J′) mouse embryos with *Myf5* (C–F′) and *Myf6* (G–J′) probes. Pictures focus on interlimb somites of *Dll1-Hoxa10* embryos and their controls and forelimb somites of *Dll1-Hoxb6* embryos and their controls. Arrows indicate the area of differential expression. Vibratome sections are shown at the arrow level for each marker. Images correspond to embryos at E10.0 (~28–31 somites), except for (G) and (H), which are E9.5 (~24 somites). See also Figure S1.

The normal expression of Hox group 10 genes seen in *Dll1-Hoxb6* transgenics (Figures S1C–S1E) indicates that the rib phenotype of *Dll1-Hoxb6* embryos does not result from downregulation of Hox group 10 genes, despite the similarities in the phenotypes of these transgenics compared to the global group 10 deletion mutants (Wellik and Capecchi, 2003). Therefore, Hox paralog groups 6 and 10 seem to modulate the processes leading to rib formation in antagonistic ways. Hence, the ''snake-like'' (*Dll1-Hoxb6*) transgenics together with our previously described rib-less (*Dll1-Hoxa10*) embryos (Carapuço et al., 2005) provide a complementary system to study how Hox genes control rib formation.

Hox Groups 6 and 10 Control Regional Hypaxial Expression of Genes in the *Myf5/Myf6* Pathway

Because ribs derive from the sclerotome (Huang et al., 2000), we expected this somitic compartment to be affected in our transgenics. However, we found no significant differences in the expression patterns of sclerotomal markers such as *Pax1*, *Pax9*, and *Meox2* in the Hox transgenics (Figures S1F–S1N). Hence, we decided to analyze the expression of genes that have been associated with rib deficiencies in genetic studies. Several mutations of the myogenic factor *Myf5* have been produced, and, whereas myogenesis remains relatively normal, some mutants displayed strong rib defects that resembled the phenotypes observed in our *Dll1-Hoxa10* transgenics (Braun et al., 1992; Tajbakhsh et al., 1996; Carapuço et al., 2005). In wild-type embryos, *Myf5* expression follows specific regional patterns. While it is expressed in the dorso-medial (epaxial) myotome of somites at all rostro-caudal levels, it is only detected in the ventrolateral (hypaxial) myotome of somites located between the limb buds, which are those producing rib-bearing vertebrae (Figures 1C, 1C′, 1E, and 1E′; Figures S1O and S1O′). In both *Dll1-Hoxa10* and *Dll1-Hoxb6* transgenic embryos, the distribution of *Myf5* transcripts was clearly affected. Interestingly, the changes in *Myf5* expression were region specific, correlating with the relative changes seen in rib development. In *Dll1-Hoxa10* transgenics *Myf5* was downregulated specifically in the hypaxial myotome of interlimb somites (prospective thoracic region) (Figures 1D and 1D′), and in *Dll1-Hoxb6* transgenic embryos *Myf5* was ectopically activated in the ventrolateral domain of somites at limb and neck levels (prospective rib-less regions) (Figures 1F and 1F′; Figures S1P and S1P′). Thus, we observe a strong positive correlation for rib development and hypaxial *Myf5* expression.

Mutations in *Myf6* have also been associated with severe rib deficiencies resembling those seen in *Dll1-Hoxa10* transgenics (Braun and Arnold, 1995), indicating that this gene could also be a target of Hox gene activity. Expression analysis showed patterns similar to those observed for *Myf5*. In *Dll1-Hoxa10* transgenic embryos, *Myf6* was severely downregulated, most prominently in the hypaxial myotome of the interlimb area (Figures 1G–1H′; see also Figures 3A′ and 3B′). Conversely, we found ectopic *Myf6* activation in the hypaxial myotomal domain of somites at limb and neck levels in *Dll1-Hoxb6* transgenics (Figures 1I–J′; Figures S1Q–S1R′). Interestingly, hypaxial expression at the hindlimb level of *Dll1-Hoxb6* transgenics

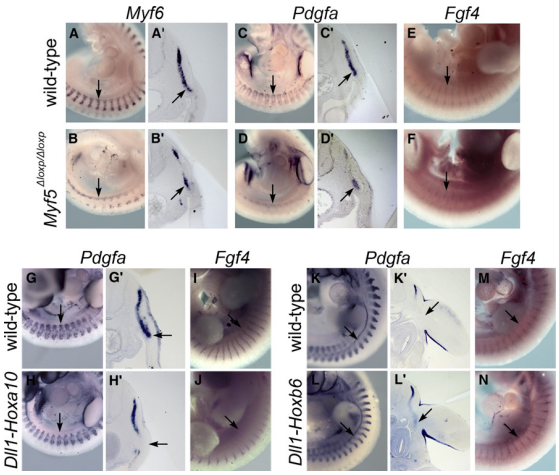

Figure 2. Hox Groups 6 and 10 Modulate Regional Expression of Genes in the Myf5/6 Pathway

Whole-mount in situ hybridization of wild-type (A, A′, C, C′, E, G–I, and K–M), *Myf5*$^{\Delta loxp/\Delta loxp}$ (B, B′, D, D′, and F), *Dll1-Hoxa10* (H–J), and *Dll1-Hoxb6* (L–N) mouse embryos with *Myf6* (A–B′), *Pdgfa* (C–D′, G–H′, and K–L′), and *Fgf4* (E, F, I, J, M, and N) probes. Pictures focus on interlimb somites of *Dll1-Hoxa10* embryos and their controls and forelimb somites of *Dll1-Hoxb6* embryos and their controls. Arrows in (A)–(F) indicate the area of conserved expression and in (G)–(N) the area of differential expression. Vibratome sections are shown at the arrow level for each marker. Images correspond to embryos at E10.0 (~28–31 somites) except for (E), (F), (I), (J), (M), and (N), which are E11.0 (~40 somites). See also Figure S2.

preceded that of the epaxial domain (Figures S1R and S1R′), thus mimicking the temporal pattern that has been described for the interlimb region in wild-type embryos (Summerbell et al., 2002). Together, these results indicate that Hox groups 6 and 10 are able to control *Myf5* and *Myf6* regional specific expression in the hypaxial myotome in a pattern that closely correlates with rib development. Interestingly, in situ analysis of *Myf5* mutants with normal ribcages (Kaul et al., 2000; we will refer to these mutants as *Myf5*$^{\Delta loxp/\Delta loxp}$) revealed that while *Myf6* expression was downregulated in the epaxial myotome, expression of *Myf6* in the hypaxial myotome of interlimb somites was clearly conserved (Figures 2A–2B′), displaying a pattern complementary to that found in *Dlll1-Hoxa10* transgenics. Altogether, these results indicate that Hox genes are able to control the expression of *Myf5* and *Myf6* in the domain that is relevant for rib formation and are consistent with a redundant role for these genes in rib induction.

To further evaluate *Myf5*/*Myf6* activity in the Hox transgenics, we tested the expression of suggested downstream effectors. We first assayed *Pdgfa* and *Fgf4*, which were shown to be downregulated in *Myf5* mutant embryos (Grass et al., 1996; Tallquist et al., 2000). Expression of these genes followed patterns similar to those described for *Myf5* and *Myf6*. In *Dll1-Hoxa10* embryos these growth factors failed to be activated in the hypaxial domain of interlimb somites, while the remaining expression domains appeared largely unaffected (Figures 2G–2J). Conversely, *Dll1-Hoxb6* embryos presented ectopic *Pdgfa* and *Fgf4* expression in the hypaxial domain of limb and neck somites (Figures 2K–2N; Figures S2A–S2B′). We also observed that *Pdfga* and *Fgf4* expression was conserved almost exclusively on the hypaxial myotome of interlimb somites of *Myf5*$^{\Delta loxp/\Delta loxp}$ embryos, although the levels were lower than in control specimens (Figures 2C–2F).

Interestingly, not all *Myf5* targets were similarly affected in the Hox transgenic embryos. Myogenin (*Mgn*), a *Myf5* target gene in the myogenic cascade (Pownall et al., 2002), was upregulated in the ventrolateral myotome of limb and neck somites of *Dll1-Hoxb6* transgenics, but its expression was not affected in *Dll1-Hoxa10* embryos (Figures S2C–S2F). While the *Mgn* pattern observed in *Dll1-Hoxb6* embryos could result from activation by *Myf5*/*Myf6*, the fact that *Mgn* is not downregulated in *Dll1-Hoxa10* interlimb hypaxial somites can be attributed to normal *MyoD* expression (Figures S2G and S2H), which is also upstream of *Mgn* (Pownall et al., 2002). The persistent hypaxial expression of myogenic genes like *MyoD* and *Mgn* in *Dll1-Hoxa10* transgenics is consistent with the presence of muscles in the whole circumference of the prospective thoracic area of these transgenics (Figure S2M). In addition, induction of an interlimb-like *Mgn* expression pattern in the hypaxial myotome at forelimb and neck levels of *Dll1-Hoxb6* transgenics is in agreement with the presence of intercostal muscles associated with the ribs in the neck of these transgenics (Figures S2N and S2O). Further analysis of the *Dll1-Hoxa10* embryos with additional myotomal markers, such as *Six1* or *Pax3*, also revealed no significant differences when compared to wild-type littermates (Figures S2I–S2L).

These results suggest that the effect of Hox groups 6 and 10 is quite specific for *Myf5*/*Myf6* and not a result of a general effect on the myotome. Furthermore, the expression of *Fgf4* and *Pdgfa* in *Dll1-Hoxa10* and *Myf5*$^{\Delta loxp/\Delta loxp}$ embryos suggests their involvement in a *Myf5*/*Myf6*-specific pathway associated with rib development.

Hypaxial *Myf6* Expression Rescues the Rib-less *Dll1-Hoxa10* Phenotype

To determine if the Hox-modulated expression of *Myf5*/*Myf6* is key to rib development, we tested whether *Myf6* could rescue the rib-less *Dll1-Hoxa10* phenotype when expressed in the hypaxial somite. As *Pax3* expression seems to be unaffected by *Hoxa10* (Figures S2K and S2L), we used an enhancer of this gene that promotes expression in the hypaxial somite (Brown

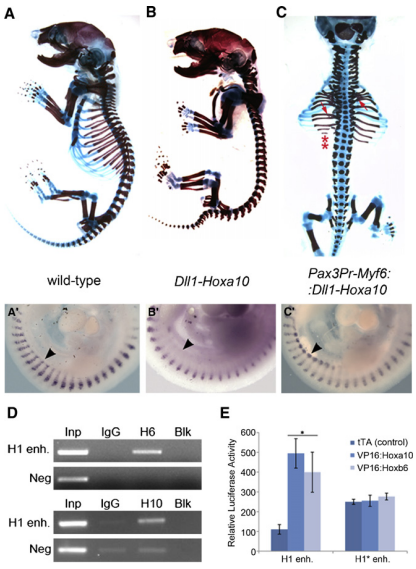

Figure 3. *Myf5*/*Myf6* as Functional Targets of Hox Groups 6 and 10 Genes

(A–C) Rescue of the *Dll1-Hoxa10* phenotype with hypaxial *Myf6*. Skeletal staining of wild-type (A), *Dll1-Hoxa10* (B) and *Dll1-Hoxa10::Pax3Pr-Myf6* (C) E18.5 fetuses. (A′), (B′) and (C′) show *Myf6* expression in the corresponding transgenics at E10.0.

(D) Chromatin immunoprecipitation from PSM of E9.5 wild-type mouse embryos using antibodies against Hoxc6 (H6), Hoxa10 (H10), or an unspecific control IgG (IgG), and PCR amplification of the Homology 1 enhancer element (H1) and negative control region (Neg). Inp, input; Blk, blank. These results are representative of three independent experiments.

(E) Luciferase activity from wild-type and mutated H1 enhancer (H1enh. and H1*enh., respectively), driven by VP16:Hoxa10, VP16:Hoxb6 or the tetracycline transactivator (tTA) as a control. The activation from the H1 enhancer is statistically significant (VP16:Hoxa10 $p < 0.01$ and VP16:Hoxb6 $p < 0.04$). The values are presented as the mean and standard error of the triplicates from a representative experiment.

et al., 2005). *Pax3Pr-Myf6* transgenic embryos showed no apparent skeletal phenotype, which was expected since the hypaxial *Pax3* enhancer reproduces the normal expression of this gene in the hypaxial somite at the different axial levels (Brown et al., 2005) (Figures 3A′–3C′). For the rescue experiment we produced *Pax3Pr-Myf6*::*Dll1-Hoxa10* double transgenics. Three of the seven double transgenics generated had recognizable rib phenotypes, which were much less severe than those observed in *Dll1-Hoxa10* transgenics (Figures 3A–3C). In particular, while *Dll1-Hoxa10* transgenics showed strong rib phenotypes, typically a complete absence of ribs in 65% of the cases (Figure 3B; Table 1) (Carapuço et al., 2005), *Pax3Pr-Myf6*::*Dll1-Hoxa10* double transgenics showed a mild alteration in the total number of ribs combined with the presence of variable abnormal patterns such as rib fusions, proximal gaps, and distorted rib insertions in the sternum (Figure 3C; Table 1; Table S1). This result indicates that *Myf6* expression in the hypaxial somite is sufficient to rescue the *Hoxa10*-induced rib phenotype, thus providing further evidence of a direct contribution of this myogenic factor to the rib phenotypes obtained in the Hox transgenics and its involvement in rib development.

Binding of Hox Group 6 and 10 Proteins to an Enhancer that Drives Hypaxial Expression of *Myf5*

Among the different control regions that have been described for *Myf5*, an enhancer was identified that drives expression in the somitic domain affected in our Hox transgenics (Bajard et al., 2006; Buchberger et al., 2007; Giordani et al., 2007). The homology element 1 (H1) of this enhancer (Buchberger et al., 2007), also known as 147 bp enhancer (Bajard et al., 2006), contains the sequence CTAATTG, which fits with predicted target sequences for Hoxb6 and Hoxa10 (Noyes et al., 2008). This potential Hox-binding site seems to be required for enhancer activity according to transgenic reporter assays (Buchberger et al., 2007). To test if our candidate Hox proteins bind this enhancer in vivo, we performed chromatin immunoprecipitation experiments on PSM isolated from mouse embryos. We could consistently immunoprecipitate the H1 enhancer element but not other genomic areas using specific antibodies for both Hox group 6 and 10 proteins (Figure 3D). This result suggests a physiological positioning of these Hox proteins at a genomic region that drives *Myf5* expression in the hypaxial myotomal domain and is consistent with a Hox-mediated regulation of *Myf5* in this embryonic region.

When tested using a luciferase reporter assay in cultured cells, both Hoxa10 and Hoxb6 fused to VP16 activated transcription from the wild-type H1 enhancer, but not from a mutant version of this element lacking the Hox-binding site (Figure 3E), further validating the capability of Hox proteins to bind to the CTAATTG sequence of the H1 enhancer. The mutant version of H1 used in these experiments still contained intact the Pax3 and Six1-binding sites also present in this enhancer, indicating that the CTAATTG site is the main target sequence for Hox proteins in this regulatory element.

DISCUSSION

In this study we show that specification of global vertebral domains in the vertebrate axial skeleton is controlled by the balanced activity of different Hox genes. It had been previously shown that Hox groups 10 and 11 play essential roles in the patterning of the lumbar and sacral regions, respectively (Wellik and Capecchi, 2003; Carapuço et al., 2005). Our results now indicate that Hox genes of the paralog group 6 are able to provide the instructions to generate the thoracic area. According to our data, the presence of ribs is not a default state (Wellik and Capecchi, 2003) but rather the result of a positive activity of Hox genes that triggers processes leading to rib induction. In caudal areas Hox group 10 proteins override this activity to generate the rib-less areas of the skeleton. In our model, the

Table 1. Comparison of the Skeletal Phenotype of *Pax3pr-Myf6*, *Dll1-Hoxa10*, and *Pax3pr-Myf6::Dll1-Hoxa10* Fetuses

	Pax3pr-Myf6	*Dll1-Hoxa10*	*Pax3pr-Myf6::Dll1-Hoxa10*
Wild-type FVB/N phenotype[a]	7/7 (100%)	2/14 (14.29%)	4/7 (57.14%)
Thoracic rib defects	0/7 (0%)	3/14 (21.43%)[b]	3/7 (42.86%)[c]
Complete rib-less phenotype	0/7 (0%)	9/14 (64.29%)	0/7 (0%)

Data are represented both as the number embryos showing a particular phenotype/total number embryos analyzed, and as percentages.
[a] 60% of our FVB/N-derived fetuses contain a small rib in L1.
[b] Variable rib defects in T1, T2, and T13.
[c] See Table S2 for details.

cervical domain is passively determined as the region anterior to the start of the rib-determining Hox activity (Figure S3).

Surprisingly, we found that the primary target of the rib-forming/rib-blocking activities of Hox genes does not seem to be the sclerotome, but rather specific genes expressed in the myotomal compartment. In particular, we show that the primary targets of Hox genes are *Myf5* and *Myf6* specifically in the hypaxial myotome. This implies a nonmyogenic function of *Myf5*/*Myf6* that controls rib development. The role of *Myf5* in rib formation has been a matter of controversy. Initial studies pointed to *Myf5* as a central player in the processes leading to rib development (Braun et al., 1992). However, when other *Myf5* mutants were produced that exhibited no rib defects (Kaul et al., 2000), it was suggested that the rib determining factor was not *Myf5* itself but another gene somehow linked to it. A decade later, such a gene has not been identified, and recent new data once more associated *Myf5* with rib development (Gensch et al., 2008; Haldar et al., 2008). Among the genes located close to *Myf5* in the genome, only *Myf6* stands out as a candidate to be involved in rib development, because rib phenotypes have been described in some mutants for this gene (Braun and Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995). Interestingly, rib deficiencies have been observed only when inactivation of either *Myf5* or *Myf6* also affected expression of the other gene (Braun et al., 1992; Braun and Arnold, 1995; Patapoutian et al., 1995; Tajbakhsh et al., 1996; Zhang et al., 1995; Yoon et al., 1997; Kassar-Duchossoy et al., 2004; this work). This suggests that *Myf5* and *Myf6* have redundant functions in rib formation and that it is the double inactivation of both genes that causes the rib phenotypes in particular *Myf5* and *Myf6* mutants, rather than the effects on an additional rib-determining gene in the *Myf* genomic area. Our results with both *Dll1-Hoxa10* transgenics and $Myf5^{\Delta loxp/\Delta loxp}$ mutants are fully consistent with this hypothesis. In addition, the involvement of the Myf factors in rib development is also supported by the ability of *Myf6* to rescue the rib-less *Dll1-Hoxa10* phenotype, when expressed in the hypaxial somite.

Our observations that Hox-driven information seems to be first interpreted by a specific population of myotomal *Myf5*/*Myf6*-expressing cells could indicate that these cells can directly contribute to the ribs. However, while cell-tracing experiments have shown a contribution of *Myf5*-expressing cells to the ribs (Gensch et al., 2008; Haldar et al., 2008), they seem to represent too small a fraction of the rib chondrocytes to fully explain *Myf5* contribution to rib development. In addition, *Myf6*-expressing cells were not found in the sclerotomal compartment using a similar cell tracing strategy (Haldar et al., 2008). Therefore, it seems more likely that the *Myf5*/*Myf6*-expressing cells convey their rib-forming information to the sclerotome through a cell nonautonomous mechanism. Our results suggest that members of the FGF and PDGF signaling pathways are involved in this mechanism, an idea that is also supported by genetic studies consistent with the participation of FGFs and PDGFs in rib formation. In particular, inactivation of *Pdgf-alpha* receptor resulted in severe rib anomalies (Soriano, 1997), and insertion of a *Pdgfa* cDNA in the *Myf5* locus significantly rescued the *Myf5* rib phenotype (Tallquist et al., 2000). The involvement of *Fgf4* in rib formation has not been genetically addressed, but a variety of experiments performed in chicken embryos suggest that FGF signaling is important for rib formation (Huang et al., 2003). Altogether, these results strongly suggest that FGF and PDGF signaling are important components of the mechanism that transmits patterning information from *Myf5*/*Myf6* to the sclerotome.

Regulation of *Myf5*/*Myf6* by Hox genes may be a complex process. While the activity of *Hoxa10* and *Hoxb6* seems to be required before somites are formed, their effect is only detected at a later developmental stage in a specific somitic domain. This observation seems to be at odds with a simple transcriptional activation (Hoxb6) or repression (Hoxa10) mechanism, as it is the normal expression of *Myf5* and *Myf6* in the tail tip of *Dll1-Hoxb6* transgenics (Figure S1E). Therefore, Hox proteins must functionally interact with other factors to modulate spatial and temporally specific activity of the *Myf5*/*Myf6* regulatory region. Pax3 and Six1/4 are likely candidates to be involved in this process, as they also interact functionally with the H1 enhancer through binding sites located at both sides of the Hox site (Bajard et al., 2006; Giordani et al., 2007). Interestingly, expression of a dominant-negative version of Pax3 from the *Pax3* locus downregulated *Myf5* and *Myf6* expression in the hypaxial myotome of interlimb somites without affecting other myogenic factors like *MyoD* or *Mgn* (Bajard et al., 2006), which resembles our observations in *Dll1-Hoxa10* transgenics. This suggests that *Hoxa10* activity could involve functional inactivation of *Pax3*. If this is the case, it cannot occur at the transcriptional level, as *Pax3* expression seemed normal in *Dll1-Hoxa10* transgenics. Direct competition for binding to the enhancer is also unlikely, because *Hoxa10* activity is observed when this gene is expressed in the PSM and not in the somites (Carapuço et al., 2005), and *Pax3* is only expressed in the somites. A similar spatial-temporal gap is observed between *Pax3* expression and *Hoxb6* activity in the transgenics. This suggests a sequential activity of Hox proteins and Pax3 (and probably Six1/4) to activate *Myf5*/*Myf6* expression in the hypaxial myotome. A possible

scenario is that Hox proteins provide a label to the *Myf5/6* hypaxial enhancer, which would promote (Hoxb6) or block (Hoxa10) binding and/or activation by Pax3 later in the differentiating somite, eventually regulating *Myf5*/*Myf6* expression. Interestingly, a "label-based" mechanism to modulate cell type-specific recruitment of transcription factors to distal enhancers has been recently reported (Lupien et al., 2008). Experiments are currently in progress to test if Hox/Pax3 interactions are also mediated through an equivalent mechanism. Of note, interactions between Hox and Pax proteins with differential functional outcomes have also been described for other members of the Hox and Pax families (Yallowitz et al., 2009). Therefore, Hox-Pax functional interactions could be a general theme in vertebrate development.

It has been suggested that regulation of hypaxial *Myf5* expression by *Pax3* might require, in addition to the H1 enhancer, other still not identified earlier acting elements (Bajard et al., 2006). Similarly, it is possible that Hox-mediated modulation of *Myf5*/*Myf6* expression in the hypaxial myotome could involve additional components, which is consistent with the complex regulation of the *Myf5*/*Myf6* locus (Carvajal et al., 2008). A probable location for such elements is the genomic region between 88 and 140 kb upstream of the *Myf5* gene, which has been reported to contain early hypaxial enhancers (Carvajal et al., 2001).

The Hox-mediated patterning process we describe in this manuscript serves as a mechanism for the establishment of global vertebral domains (i.e., cervical, thoracic, and lumbar) through the specification of rib-containing and rib-less areas of the skeleton. Whether Hox genes use a similar mechanism to specify the individual features that characterize the different vertebrae, or this is elicited by direct control of sclerotomal development, remains to be determined. However, the primary involvement of myotomal components in the specification of global vertebral domains provides an evolutionarily efficient mechanism that ensures the concomitant evolution of the ribs and their associated muscles, to produce animals with properly organized axial musculoskeletal systems. Curiously, rib development in turtles follows a plan that differs from that typically observed in other amniotes, resulting in the formation of the carapace. This specific rib development is associated with turtle-specific *Myf5* hypaxial expression in the trunk (Ohya et al., 2006) and development of specific muscle attachments (Nagashima et al., 2009), further suggesting the importance of the *Myf5*-rib connection in the evolution of the body plan.

EXPERIMENTAL PROCEDURES

The *Dll1-Hoxa10* construct was previously described (Carapuço et al., 2005). The *Sm-Hoxb6* and *Dll1-Hoxb6* constructs were generated by insertion of the human *Hoxb6* cDNA (IMAGE: 4548382) downstream of the *Sm* (Carapuço et al., 2005) and *Dll1* (Beckers et al., 2000) enhancers, respectively, and upstream of the SV40 polyadenylation signal. The *Pax3Pr-Myf6* construct was generated by cloning the *Myf6* cDNA (IMAGE: 8733960) downstream of the hypaxial enhancer of the *Pax3* gene (Brown et al., 2005) and upstream of the SV40 polyadenylation signal. Transgenic embryos were produced by pronuclear injection according to standard methods. All transgenic mice used in this work have a FVB/N genetic background. Of note, normal fetuses derived from our FVB/N colony present a slight deviation from the typical axial formula, as they contain a small rib in L1 with a penetrance of about 60%, which is also observed in nonaffected transgenics with this genetic background. The $Myf5^{\Delta loxp/\Delta loxp}$ mutants have been previously described (Kaul et al., 2000). Fetuses were dissected at E18.5 and skeletal preparations made using the alcian blue/alizarin red staining method (Mallo and Brändlin, 1997). Whole-mount in situ hybridization (ISH) was performed as described elsewhere (Kanzler et al., 1998). ISH-stained embryos were embedded in gelatin/albumin and sectioned with a vibratome.

Chromatin immunoprecipitation assays were performed using PSM from E9.5 mouse embryos. Briefly, PSM were dissected in PBS and fixed in 1% formaldehyde. After tissue homogenization, samples were sonicated and immunoprecipitated using Hoxc6 antibody (Abcam ab41587), Hoxa10 antibody (kindly provided by J. Dasen), or control rabbit IgG (Abcam ab27478), prebound to Dynabeads Protein A (Invitrogen). The immunoprecipitated DNA was PCR-amplified using primers for the H1 enhancer: GCCATC TACTCTCACACACCATAC and CCACGCTAAAATACAGACATGCAG; and for a negative control region: CTGGCGTGTCTCCCTCTCTGCTGAA and GCTCCGAAGGCTGCTACTCTTGGCT.

For the luciferase assays, reporter plasmids were made by cloning the wild-type or a mutant version of the H1 enhancer in which the CAATTA was replaced for CGCGCTG upstream of the minimal promoter of the pGL3-Promoter Vector plasmid. Transfections were performed on 293T cells using reporter plasmids together with plasmids expressing either VP16:Hoxa10, VP16:Hoxb6 or, as a control, the tetracycline transactivator (tTA) (Gossen and Bujard, 1992) using Lipofectamine 2000. The pCMV-β plasmid was included in all electroporations for normalization. Luciferase activity was measured on cell extracts 24 hr after transfection and normalized to β-galactosidase activity. Significance was evaluated using Student's t test.

To quantify transcript levels, total RNA was extracted with TriReagent (Sigma) according to the manufacturer's protocol. cDNAs were synthesized by random priming using Superscript II reverse transcriptase (Invitrogen), and the mRNA levels were determined by qPCR using QuantifastTM FYBR Green PCR Kit (QIAGEN). The primers used were *Hoxa10*F: AGCGAGTCCTA GACTCC and *Hoxa10*R: GTCCGTGAGGTGGACGCTACG; *Hoxa11*F: AACTT CAAGTTCGGACAGCGG and *Hoxa11*R: TCAGTGAGGTTGAGCATGCGG; *Myf5*F: TCCTCAGGAATGCCATCCGC and *Myf5*R: GACAGTAGATGCTGT CAAAG; and *Myf6*F: AGACTGCCCAAGGTGGAGAT and *Myf6*R: AATGTTC CAAATGCTGGCTG.

SUPPLEMENTAL INFORMATION

The Supplemental Information includes three figures and one table and can be found with this article online at doi:10.1016/j.devcel.2010.02.011.

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Develpomental Cell 18

Supplementary Information

Evidence for a Myotomal Hox/Myf Cascade Governing Nonautonomous Control of Rib Specification within Global Vertebral Domains

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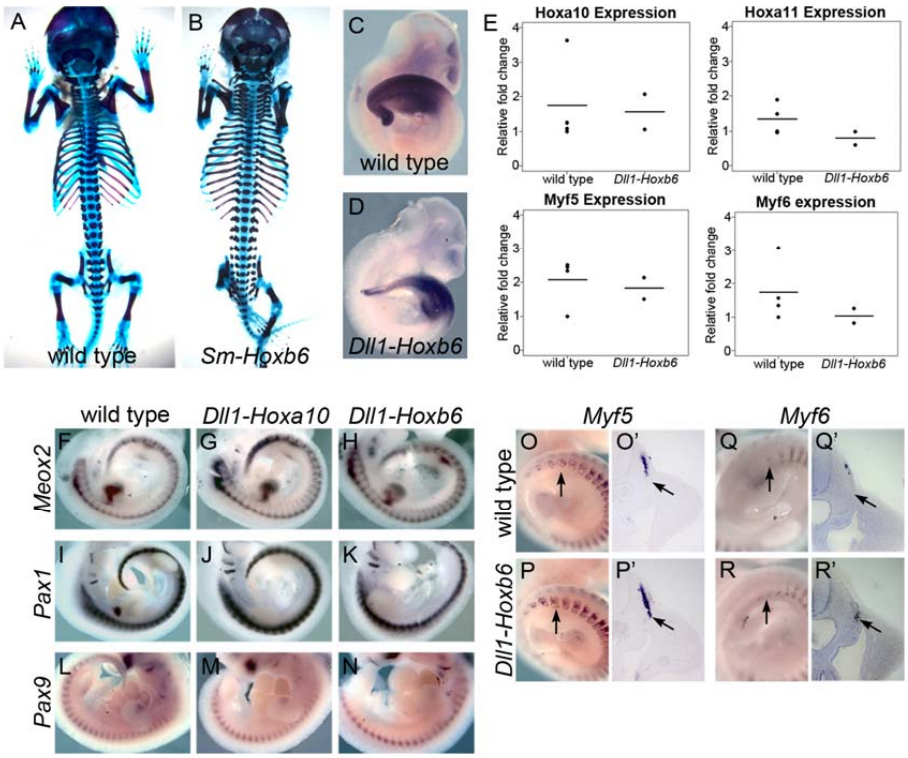

Figure S1. A,B. Mild skeletal phenotype in *Sm-Hoxb6* embryos. Skeletal staining of wild type (A) and *Sm-Hoxb6* (B) E18.5 fetuses. **C-E.** Normal Hox group 10 expression in *Dll-Hoxb6* transgenics. Whole mount *in situ* hybridization of E10.5 wild type (C) and *Dll1-Hoxb6* embryos (D). *Hoxc10* expression is unchanged in *Dll1-Hoxb6* embryos. Quantitative RT-PCR analysis shows normal expression levels of *Hoxa10*, *Hoxa11*, *Myf5* and *Myf6* in PSM and first somites of E10.5 *Dll1-Hoxb6* (n=2) compared with wild type embryos (n=4). GAPDH was used as the endogenous control. Each measurement is the average of duplicate PCR of individual samples. The bar shows the average value for each class. **F-N.** Unchanged expression pattern

of several sclerotomal markers in *Dll1-Hoxa10* and *Dll1-Hoxb6* transgenics . Whole mount *in situ* hybridization of E10.5 (F-K) and E11.0 (L-N) wild type (F, I, L), *Dll1-Hoxa10* (G, J, M) and *Dll1-Hoxb6* (H, K, N) embryos: *Meox2* (F-H), *Pax1* (I-K) and *Pax9* (L-N). **O-R.** Hind-limb level expression of *Myf5* and *Myf6*. Whole-mount *in situ* hybridization of E10.0 wild type (O, Q) and *Dll1-Hoxb6* embryos (P, R) with *Myf5* (O-P) or *Myf6* (Q, R). The arrows indicate the areas of differential expression. Vibratome sections (O'-R') were done at the arrow level.

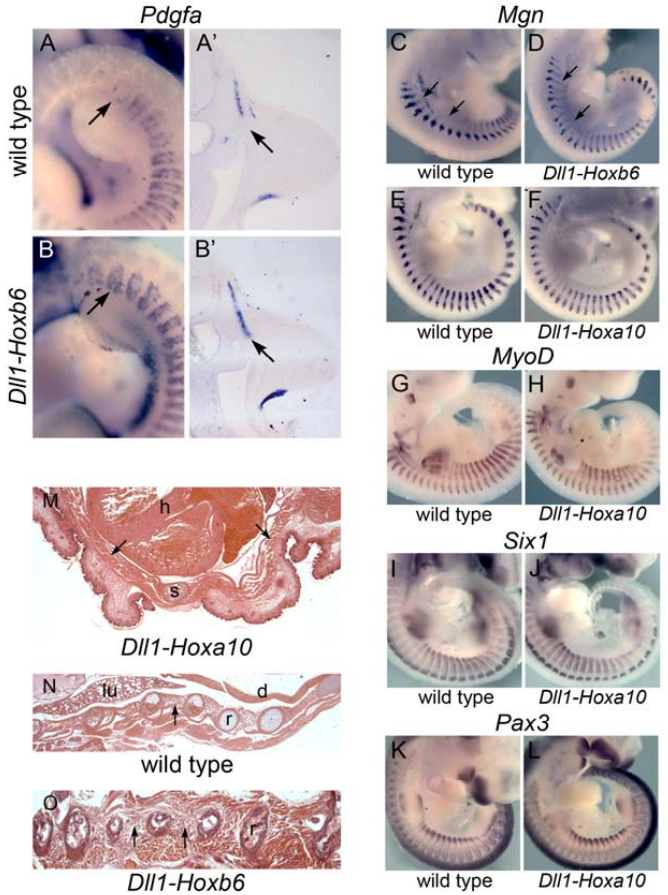

Figure S2. Myotomal and muscle analysis of Hox transgenics. A, B. Hind-limb level expression of *Pdgfa*. Whole-mount *in situ* hybridization of E10.0 wild type (A) and *Dll1-Hoxb6* embryos (B). The arrows indicate the areas of differential expression. Vibratome sections (A', B') were done at the arrow level. **C-L.** Myotomal markers in Hox transgenics. Whole-mount *in situ* hybridization of E10.5 wild type (C, E, G, I, K), *Dll1-Hoxb6* (D) and *Dll1-Hoxa10* (F, H, J, L) embryos with *Mgn* (C-F), *MyoD* (G, H), *Six1* (I, J), *Pax3* (K, L). **M.** Transverse section through the thorax of a E18.5 *Dll1-Hoxa10* (A) embryo, showing muscle tissue (arrows) that reaches the most ventral part of the embryo and attaches to the sternum (s). The picture is oriented with the ventral part of the specimen at the bottom. **N, O.** Frontal section through the ribcage of a wild type (N) or the neck of a *Dll1-Hoxb6* transgenic (O) embryo at E18.5 showing intercostal muscles (arrows) connecting adjacent ribs (r). Pictures are oriented with rostral to the left and medial to the top. d, diaphragm; h, heart; lu, lung.

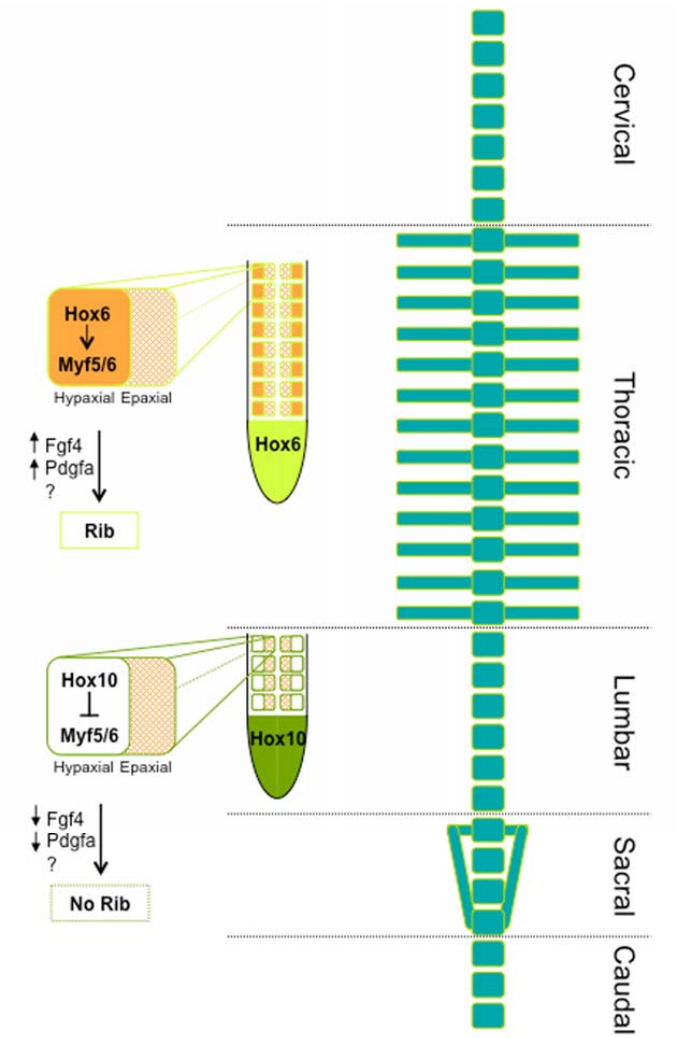

Figure S3. Hox groups 6 and 10 specify global vertebral domains. Schematic representation of Hox-mediated specification of the different vertebral domains of the axial skeleton. On the right panel, the adult cervical, thoracic, lumbar, sacral and caudal vertebral regions of the skeleton are displayed. The left panel shows a representation of the forming somites at different levels. In the prospective thoracic somites, Hox group 6 is activated (light green), thereby up regulating *Myf5* and its downstream effectors *Pdgfa* and *Fgf4* in the hypaxial fraction of the myotome (orange), ultimately leading to rib formation. In the prospective lumbar-caudal somites, Hox group 10 is activated (green), resulting in the down regulation of *Myf5*, *Pdgfa* and *Fgf4* in the hypaxial myotome (white), leading to inhibition of rib formation in those vertebrae.

Table S1. Skeletal phenotype of *Pax3Pr-Myf6::Dll1-Hoxa10* fetuses. Description of the skeletal abnormalities of the three affected *Pax3Pr-Myf6::Dll1-Hoxa10* out of a total of seven individuals.

	Number of Ribs	**Sternal insertion**	**Proximal rib fusions**	**Proximal Gaps**
#1	12 right side 10 left side	Distorted	Several fusions at different levels	----------
#2	14 right side 12/13 (fused) left side	Distorted	The most caudal ribs are fused together	Proximal gap in T2/T3
#3	12 right side 11 left side	Normal	----------	Proximal gap in T2 /T12