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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

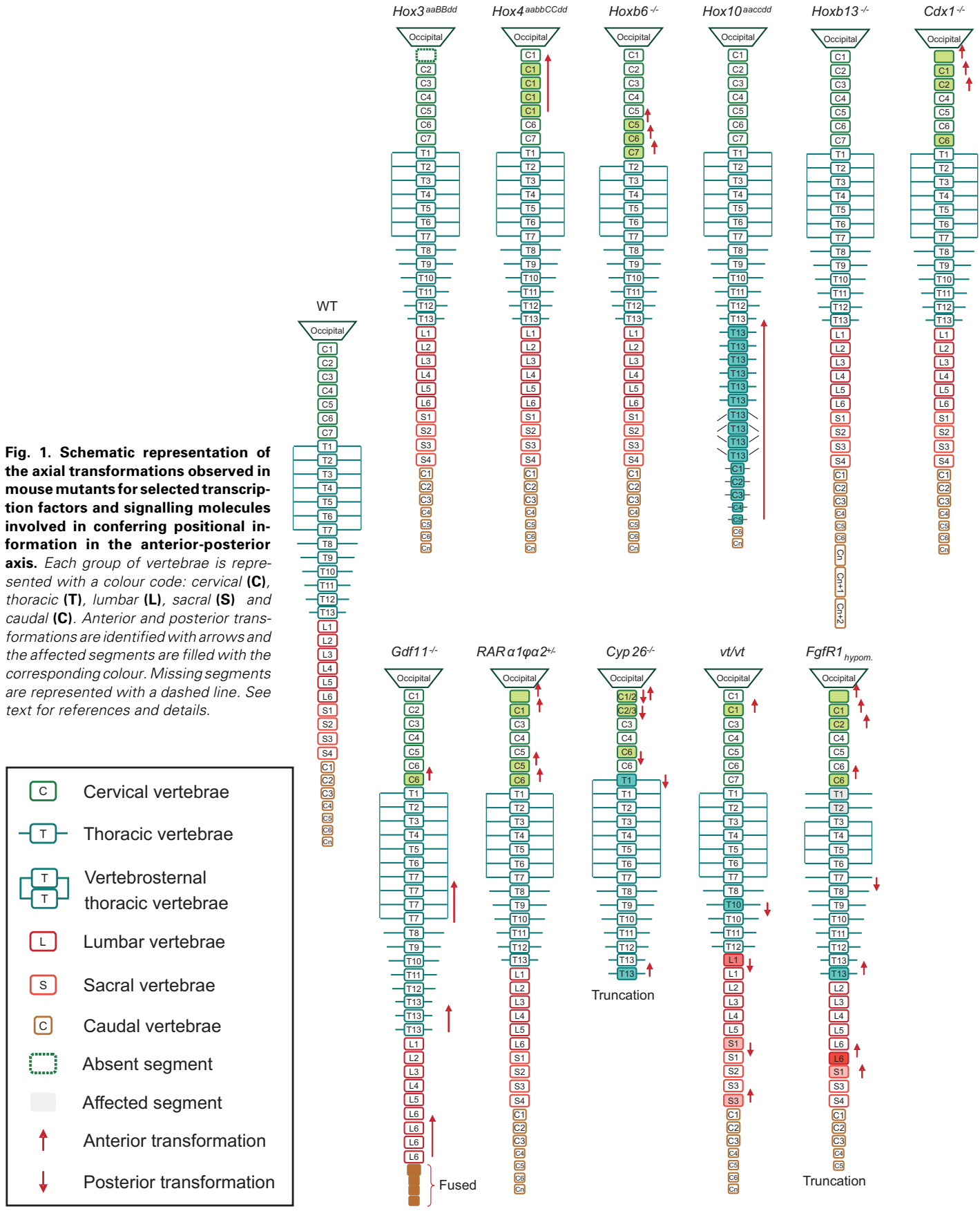

Fig. 1. Schematic representation of the axial transformations observed in mouse mutants for selected transcription factors and signalling molecules involved in conferring positional information in the anterior-posterior axis. *Each group of vertebrae is represented with a colour code: cervical* **(C)**, *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

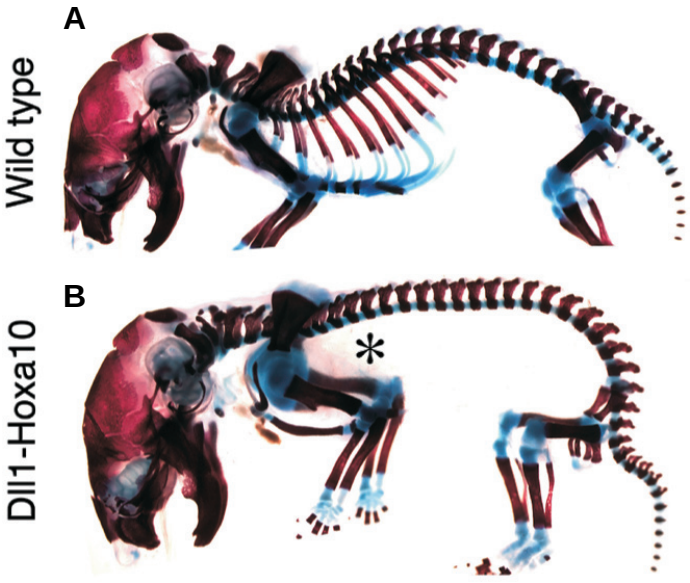

Fig. 2. Effect of expression of *Hoxa10* in the presomitic mesoderm of transgenic embryos. (A) *Skeleton of a newborn wild type mouse.* **(B)** *Skeleton of a newborn transgenic mouse in which the* Hoxa10 *gene was expressed in the presomitic mesoderm using an enhancer of the the* Dll1 *promoter. Note 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 (Iimura 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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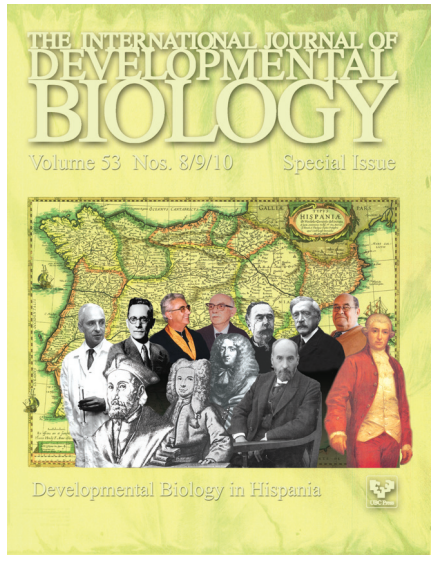

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