Hox Patterning of the Vertebrate Axial Skeleton

Deneen M. Wellik*

The axial skeleton in all vertebrates is composed of similar components that extend from anterior to posterior along the body axis: the occipital skull bones and cervical, thoracic, lumbar, sacral, and caudal vertebrae. Despite significant changes in the number and size of these elements during evolution, the basic character of these anatomical elements, as well as the order in which they appear in vertebrate skeletons, have remained remarkably similar. Through extensive expression analyses, classic morphological perturbation experiments in chicken and targeted loss-of-function analyses in mice, *Hox* genes have proven to be critical regulators in the establishment of axial skeleton morphology. The convergence of these studies to date allows an emerging understanding of *Hox* gene function in patterning the vertebrate axial skeleton. This review summarizes genetic and embryologic findings regarding the role of *Hox* genes in establishing axial morphology and how these combined results impact our current understanding of the vertebrate *Hox* code. *Developmental Dynamics 236:2454–2463, 2007.* © 2007 Wiley-Liss, Inc.

INTRODUCTION

Hox genes are homeodomain-containing transcription factors that were first described in *Drosophila* for their ability to cause segmental homeotic transformations of the body plan (Lewis, 1963, 1978). While flies have eight *Hox*, or *HomC*, genes located in a single cluster, mammals have 39 *Hox* genes arranged in four clusters (Fig. 1). Tandem duplications within the ancestral *Hox* cluster followed by genome duplication events have resulted in a relatively fixed arrangement in mammals with four clusters, composed of 2 to 4 members of 13 paralogous groups. Paralogous *Hox*

genes have been established based on sequence similarity and position within the cluster (each paralogous group is shown color coded in Fig. 1). Upon the discovery that *Hox* genes have been conserved throughout vertebrate evolution, it was proposed that they might be important in patterning the vertebrate body plan. The most obvious read-out of anterior–posterior (AP) patterning in vertebrates is the axial skeleton.

Most of the axial skeleton is derived from the somites, serially homologous structures that form on each side of the neural tube during the establishment and growth of the AP axis. The

sclerotome of the first 4.5 somites migrate and fuse to form the occipital skull (Goodrich, 1930). In the rest of the axial skeleton, the somitic sclerotome differentiates and undergoes resegmentation, in which the posterior half of one somite and the anterior half of the next most caudal somite give rise to a single axial vertebral element. The thoracic skeleton is not derived solely from somitic mesoderm, however. In addition to the vertebral elements and the ribs, which are derived from somitic mesoderm, the thoracic skeleton also has an abaxial component—the sternum, which arises from two bands in the lateral

plate mesoderm that fuse at the midline. Thus, an understanding of the development of the rib cage requires the consideration of the two distinct tissues from which it derives and is patterned.

Many studies over the past 2 decades have characterized the details of *Hox* expression in vertebrate embryos, the response of *Hox* gene expression to embryologic and genetic manipulation, as well as the effects of targeted loss of *Hox* function on axial patterning. In this review, *Hox* expression from its onset at gastrulation through the establishment of anterior boundaries at mid-gestation will be reviewed. Embryological and genetic attempts to understand *Hox* gene function during axial patterning will also be addressed. How the accumulated results impact our understanding of the "*Hox* code" will be discussed, along with future experiments that might further clarify our understanding of *Hox* function in axial patterning.

HOX EXPRESSION

Early Expression and Establishment of Anterior Limits

Upon the discovery of vertebrate *Hox* genes, expression patterns were analyzed in some detail. Early studies established that *Hox* gene expression was initiated during gastrulation stages with a temporal pattern in which more 3′ genes in the complex (i.e., *Hoxa1*, *Hoxb1*, *Hoxa2*, *Hoxb2*) turn on during early gastrulation stages and more 5′ genes are expressed later in the tail bud, after anterior somites have formed. Comparisons between *Hox* gene expression patterns consistently demonstrated that the temporal delay of 5′ *Hox* gene expression, along with progressive generation and growth along the AP axis, translates into spatial differences in the anterior expression limits of these genes, phenomena later referred to as temporal and spatial colinearity. Overall, *Hox* expression levels increase through approximately embryonic day (E) 12.5 in mice, at which time, expression in the neural tube and somitic mesoderm are fixed, with an anterior limit specific for each gene (Holland and Hogan, 1988; Gaunt et al., 1990; Izpisua-Belmonte et al., 1991; Kessel and Gruss, 1991).

In cases where the anterior limits of expression have been detailed before E12.5 in the mouse, it has been shown that many early *Hox* expression boundaries are not identical to their boundaries at E12.5 (Deschamps and Wijgerder, 1993; Gaunt and Strachan, 1996; Chen and Capecchi, 1997; Deschamps et al., 1999). *Hox* expression appears to spread anteriorly after ingression through the primitive streak, and early *Hox* expression boundaries in the somites from E9.5 through E12.5 demonstrate both anterior and posterior changes before fixed boundaries are established (Deschamps and Wijgerder, 1993; Chen and Capecchi, 1997; Deschamps et al., 1999; Iimura and Pourquié, 2006; McIntyre, et al., 2007). Expression boundary changes are not due to migration of cells already expressing a particular *Hox* gene; rather, *Hox* expression is gained or lost from adjacent cells, resulting in changes in the anterior limit of expression. Forlani et al., have shown that acquisition of *Hox* expression limits in the neuroectoderm and mesoderm are independent and that the apparent "spreading" of *Hox* expression during these early stages require cell–cell contact between more posterior cells that express *Hox* genes in early stages and more anterior cells that gain *Hox* expression at later, postgastrulation stages (Forlani et al., 2003).

Recent work suggests that it may be the *Hox* genes themselves that are responsible for the timing of ingression of chicken epiblast tissue through the primitive streak (Iimura and Pourquié, 2006). In this study, Iimura and Pourquié present evidence that the temporal acquisition of colinear *Hox* gene expression in the early epiblast controls ingression through the primitive streak. By expressing a posterior *Hox* gene, such as *Hoxb9*, in the epiblast adjacent to the primitive streak, earlier and more anteriorly than it is normally expressed, they show that the ectopic *Hoxb9*-expressing epiblast cells, which would normally ingress at earlier time points and end up in more anterior somites, are held in the epiblast until later stages when endogenous *Hoxb9*-expressing cells ingress. Thus, the *Hoxb9*-overexpressing cells end up more posteriorly than their normal fate. This work provides evidence that *Hox*

genes themselves direct the translation of temporal colinearity into the spatial colinearity that is initially established at the primitive streak.

Anterior Limits

By approximately E12.5, the *Hox* genes demonstrate stable anterior limits of expression in the mesoderm, and it is this boundary that has generally been correlated with phenotypes in the axial skeleton in single mutant mice. Much published literature has examined expression at these later time points. Detailed accounts and discussions of these results can be found in previous reports and reviews (Kessel and Gruss, 1991; Burke et al., 1995; Burke, 2000; and references therein).

Several prescient correlations between *Hox* expression and axial morphology have been noted in these studies. Kessel and Gruss noted that a combined examination of the expression literature suggested that changes in vertebral morphology correlated with the fixed expression of different sets of *Hox* genes. For example, the third through fifth cervical vertebrae (C3, C4, C5), which have nearly identical morphologies, have a single unique combination of *Hox* expression, while C6 and C7, which each have distinct morphologies, also have unique additions or combinations of *Hox* genes expressed in that AP region (Kessel and Gruss, 1991).

By comparing the anterior expression limits of many paralogous group *Hox* genes in both chickens and mice, a correlation was demonstrated between these expression limits and the boundaries of morphologically distinct regions of the axial skeleton (Burke et al., 1995; Burke, 2000). For example, the anterior boundaries of the *Hox6* genes lie close to the boundary between cervical and thoracic vertebrae in both organisms, even though this transition occurs between the 10th and 11th somite in mice and between the 18th and 19th somite in chicken (mice have 7 cervical vertebrae compared with 14 in chicken). The anterior limit of *Hox10* gene expression lies at the boundary between thoracic and lumbar vertebrae and the anterior expression boundary of *Hox11* genes lie close to the lumbar–sacral transition in both animals as well. Together, the

fixed colinear patterns of *Hox* gene expression suggest that these genes are likely to be very important regulators of the vertebrate body plan.

Expression Limits in Somitic Vs. Lateral Plate Mesoderm

As noted above, the axial skeleton is not derived solely from somitic mesoderm (Nowicki and Burke, 2000; Burke and Nowicki, 2003; Nowicki et al., 2003). The sternum of the thoracic skeleton, or rib cage, forms from the lateral plate mesoderm, which is also the source of the connective tissue surrounding the distal ribs. Musculoskeletal patterning is controlled by the connective tissue lineage, and Burke and colleagues have suggested that the patterning of primaxial and abaxial domains is independent (Nowicki and Burke, 2000; Burke and Nowicki, 2003; Nowicki et al., 2003). A manifestation of this could be that *Hox* encoded patterning information in the somite is distinct from that in the lateral plate mesoderm. Unfortunately, less information has been reported regarding *Hox* expression in lateral plate mesoderm. Recently, we have examined the expression of the *Hox5*, *Hox6*, and *Hox9* group genes and have found that anterior limits in the somitic mesoderm and lateral plate mesoderm at several developmental stages are offset considerably (Fig. 2, and McIntyre et al., 2007), much as the neural and somitic anterior limits are also distinct for many *Hox* genes. Thus, the differences in *Hox* expression in the two tissues that contribute to the thoracic skeleton support the possibility that *Hox* patterning of these two tissues in the axial skeleton occur independently.

HOX FUNCTION

Axial Fate Determination: Classic Embryology

Embryologic experiments performed in chick have shown conclusively that commitment to axial fate occurs at very early stages, even before segmentation of the presegmented mesoderm (PSM). Kieny et al. showed that donor PSM, when transplanted heterotopically into a host embryo, results in the formation of the axial pattern associ-ated with the donor AP location (Kieny et al., 1972). Nowicki and Burke later expanded on this work and demonstrated that, in the hetero-topic grafts, *Hox* expression from the donor tissue is maintained in the somitic mesoderm after transplantation, consistent with the postulate that it is the *Hox* regulated activity that is responsible for somite-derived axial patterning (Nowicki and Burke, 2000). Of interest, their work also showed this is not true for donor tissue that contributes to the abaxial domain. *Hox* expression in donor somite cells that migrate into the host lateral plate mesoderm, adopt the *Hox* expression pattern of the host. This work provides further evidence that *Hox* patterning in the primaxial and abaxial components of the skeleton are independent and that special consideration must be given to the patterning of the rib cage.

Genetics of *Hox* Function

Targeted mutations have been generated in every *Hox* gene, and individual mutations in genes in the *Hox3* group through the *Hox11* group have been reported to have defects in the axial skeleton (Chisaka and Capecchi, 1991; LeMouellic et al., 1992; Condie and Capecchi, 1993; Jeannotte et al., 1993; Small and Potter, 1993; Davis and Capecchi, 1994; Horan et al., 1994, 1995a,b; Kostic and Capecchi, 1994; Davis et al., 1995; Rancourt et al., 1995; Suemori et al., 1995; Boulet and Capecchi, 1996; Fromental-Ramain et al., 1996a; Carpenter et al., 1997; Chen and Capecchi, 1997, 1999; Manley and Capecchi, 1997; Chen et al., 1998; van den Akker et al., 1999, 2001; Garcia-Gasca and Spyropoulos, 2000; van den Akker et al., 2001; Wahba et al., 2001; Wellik and Capecchi, 2003, McIntyre et al., 2007). As expected from their expression patterns, more 3′ *Hox* genes exhibit phenotypes in anterior regions of the axial vertebrae and more 5′ *Hox* genes display phenotypes in more posterior regions of the axial skeleton, consistent with their colinear expression. For instance, loss of *Hoxd3* function results in defects of the first and second cervical vertebrae, C1 and C2, while loss of *Hoxd11* function causes changes in sacral patterning (Condie and Capec-chi, 1993; Davis and Capecchi, 1994). However, colinearity has not been uniformly demonstrated, most notably in the thoracic region. Phenotypes from single and multiple mutants in the *Hox5*, *Hox6*, *Hox7*, *Hox8*, and *Hox9* group genes have been reported to include defects in the first rib (T1). The reason for the sensitivity of this portion of the rib cage to loss of *Hox* function and the apparent lack of colinearity in this region of the axial skeleton has not been clear.

The accumulated genetic results from single *Hox* mutations do not present strong correlations with phenotypes from *HomC* loss-of-function in *Drosophila*. Loss of *HomC/Hox* function in *Drosophila* consistently results in anterior homeotic transformations of the body plan, wherein the identity of parasegments along the AP axis normally expressing a specific *Hox* gene are transformed to the identity of parasegments immediately anterior to the loss-of-function region (Lewis, 1963, 1978). The reciprocal gain-of-function experiments in *Drosophila*, in which more posteriorly expressed *Hox* genes are ectopically expressed in anterior regions resulted in posterior homeotic transformations. Together, these experiments provided strong evidence that the *HomC* genes are critical AP patterning cues in the development of the body plan in *Drosophila*. In single *Hox* mutant mice, however, some defects have been characterized as anterior homeotic transformations—the kinds of transformations that would be expected from *Drosophila* genetics (examples include transformations of C3 to C2 in *Hoxa4* mutants [Horan et al., 1994], T1 to C7 transformation in *Hoxa7/b7* double mutants [Chen et al., 1998] and a 14th rib on the first lumbar vertebrae in *Hoxb9* mutants [Chen and Capecchi, 1997]). Other single loss-of-function mutants have resulted in posterior homeotic transformations (such as ectopic rib formation on the seventh cervical vertebra (C7) in *Hoxa5* and *Hoxa6* mutants [Jeannotte et al., 1993; Kostic and Capecchi, 1994] and conversion of T13 into a lumbar phenotype in *Hoxa11* mutants [Davis et al., 1995], reviewed in [Krumlauf, 1993, 1994]). Still other phenotypes, such as loss of sternabrae, or fusion of ribs in several *Hox5* through *Hox9* mutants, are not easy to classify as homeotic changes at all. Taken to-

gether, these data have not provided a coherent genetic interpretation of *Hox* patterning of the vertebrate axial skeleton.

Paralogous *Hox* Patterning

Upon the discovery that multiple clusters of *Hox* genes are present in vertebrates, it was recognized that the duplications of genes may also provide redundant genetic material and that quite sophisticated mutations might be required to understand the functions of vertebrate *Hox* genes (Kessel and Gruss, 1990; Maconochie et al., 1996). This has, indeed, turned out to be the case. In fact, in every case where combinations of more than one member of a *Hox* paralogous group has been mutated, synergistic phenotypes have resulted (Condie and Capecchi, 1994; Davis et al., 1995; Horan et al., 1995b; Fromental-Ramain et al., 1996a,b; Chen and Capecchi, 1997, 1999; Manley and Capecchi, 1997; Chen et al., 1998; Studer et al., 1998; Gavalas et al., 2001; van den Akker et al., 2001; Wahba et al., 2001; Wellik et al., 2002; Wellik and Capecchi, 2003, McIntyre et al., 2007). This finding has proven to be particularly important in axial patterning. In the earliest reports looking at the redundant function of paralogous *Hox* genes, Condie and Capecchi showed that loss of function of *Hoxd3* resulted in a remodeling of the craniocervical joint and a partial fusion of C1, the atlas, to the occipital bone (Condie and Capecchi, 1993). Single mutants for *Hoxa3* do not display defects in the craniocervical joint. However, when *Hoxa3/Hoxd3* double mutant embryos were generated, the atlas is no longer present at newborn stages (Condie and Capecchi, 1994). This finding is likely to represent an anterior homeotic transformation wherein the first prevertebral skeletal element that normally gives rise to the atlas becomes part of the base of the skull, following the fate of the first four and a half somites (Goodrich, 1930). Horan et al., demonstrated a similar redundant function for the *Hox4* paralogous genes. While single mutants from this paralogous group result in incompletely penetrant defects only in C2 or C3, removal of three of the four paralogous *Hox4* genes (*Hoxa4*,

Hoxb4, and *Hoxd4*) result in fully penetrant anterior homeotic transformations of C2 through C5 (Horan et al., 1995b). These early studies demonstrate the existence of functional redundancy among *Hox* paralogous genes and the importance of removing this redundancy to understand the genetic function in vertebrates.

Since these studies, complete paralogous mutants have been generated for the *Hox5*, *Hox6*, *Hox7*, *Hox8*, *Hox9*, *Hox10*, and *Hox11* genes (Chen et al., 1998; van den Akker et al., 2001; Wellik and Capecchi, 2003; McIntyre et al., 2007). In all cases, more severe axial phenotypes are produced in paralogous mutants than in single or double mutant combinations. In several cases, the complete axial skeletons from these animals have been analyzed in detail, and a more coherent genetic understanding of *Hox* patterning of the axial skeleton has been gained from these studies. Paralogous mutants consistently demonstrate anterior homeotic transformations along extended regions of the axial skeleton (schematized in Fig. 3, aqua-shaded elements). *Hox5* triple mutants demonstrate anterior homeotic transformations of C3 through the first thoracic vertebra (T1) to a C2, or axis-like phenotype, while *Hox6* paralogous mutants show anteriorizing transformations in C6 through T6 (McIntyre et al., 2007). *Hox9* quadruple mutants show dramatic transformations of the posterior thoracic vertebrae, with 13 or 14 ribbed vertebrae attached to the sternum instead of the usual complement of 7 (McIntyre et al., 2007). *Hox10* triple mutants evidence a transformation of lumbar and sacral vertebrae to posterior thoracic fate, with transformed vertebrae showing small rib projections. In the *Hox11* paralogous mutants, the sacral vertebrae and early caudal vertebrae are anteriorly transformed to a lumbar-like fate (Wellik and Capecchi, 2003).

Between adjacent paralogous mutant groups, however, there is significant overlap in the AP regions that exhibit phenotypes (Fig. 3, orange-shading). Within these overlapping regions, the effect of loss of *Hox* paralogous function is distinct. For instance, in both *Hox5* and *Hox6* paralogous mutants, the first thoracic verte-

brae display anterior homeotic transformations. In the case of the *Hox5* mutants, however, the first thoracic vertebrae show transformations of the dorsal neural arch toward a C2 fate (Fig. 4, compare *Hox5* to wild-type T1). Ribs initiate, but do not extend from this vertebrae. In *Hox6* mutants, T1 is converted to the phenotype of the next most anterior vertebra, C7, with no neural arch morphology and no rib formation (Fig. 4). Similar examples are shown for "L1" of the *Hox9* and *Hox10* paralogous mutants and for "S1" of the *Hox10* and *Hox11* paralogous mutants (Fig. 4). Thus, it appears that each set of paralogous *Hox* genes directs specific morphologies in colinear AP-restricted regions of the somite-derived skeleton. While these functions are redundant between paralogous genes, the function of each paralogous group appears to be distinct, with each group imparting specific morphologies to regions of the axial skeleton.

Hox genes also appear to play a very important role in patterning the abaxial portion of the thoracic skeleton. The combined genetic results indicate that sternal patterning is coded differently from that of the somite-derived vertebral elements. In *Hox5*, *Hox6*, and *Hox9* paralogous mutants, the sternum is mispatterned along its entire AP length (McIntyre et al., 2007, Fig. 3, purple-shaded areas). The changes in pattern do not exhibit clear colinearity. Furthermore, the nature of the sternal defects cannot be classified as homeotic transformations. Together, these data suggest independent *Hox* patterning mechanisms exist in the lateral plate mesoderm and must be considered in the development of the thoracic skeleton.

Hox Cluster Deletion

The phenotypes of the paralogous mutants demonstrate that there is redundancy among *Hox* paralogous genes in axial patterning, therefore, it might be anticipated that loss of *Hox* gene function in *cis* (*Hox* genes from the same cluster) would be less likely to cause severe consequences in axial patterning. This appears to be true. Deletion of the *HoxC* cluster was reported to cause only mild
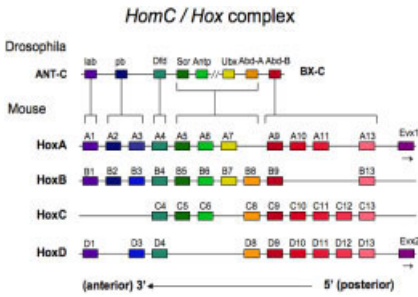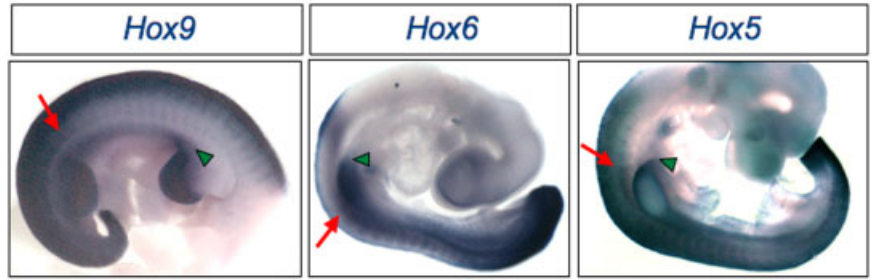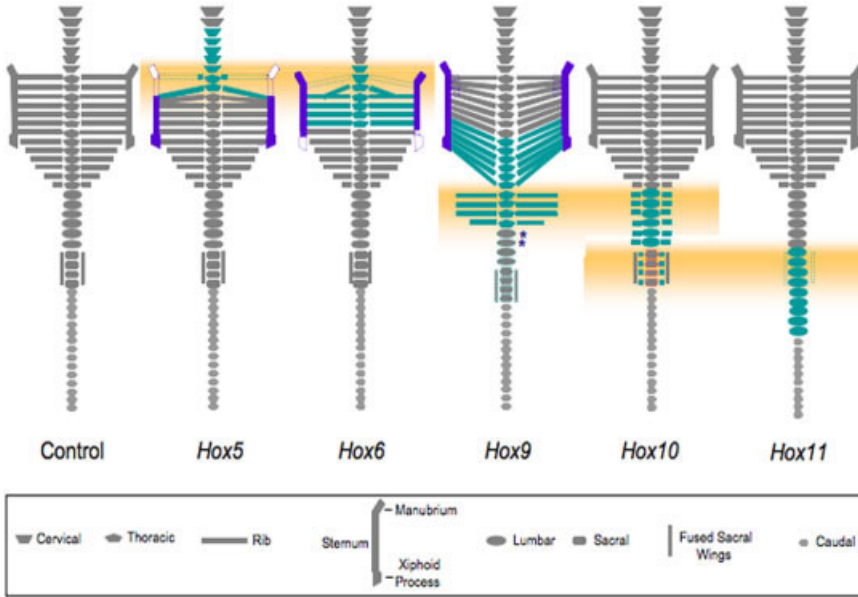
Fig. 1.

Fig. 2.

Fig. 3.

Fig. 5. Schematic of overlaps in and differences between the somite-derived primaxial phenotypes and the lateral plate-derived, abaxial phenotypes of *Hox* paralogous mutants. The regions for both primaxial and abaxial defects are shown as color-coded bars adjacent to the segments affected in paralogous mutants. Note the differences in AP position as well as the overlap differences in the primaxial versus the abaxial phenotypes.
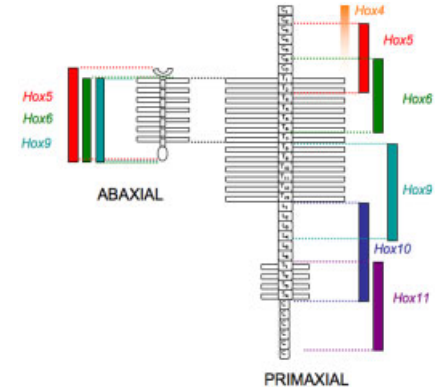

Fig. 1. Schematic of relationship between *Drosophila* and mouse *Hox* genes. *Hox* genes are shown as colored boxes in their order on the chromosome. Orthologous genes between *Drosophila* and mouse, and paralogous mouse genes are shown color-coded.

Fig. 2. Representative *Hox* expression patterns during embryogenesis. In these embryos, a combination of probes demonstrates the expression of *Hox5*, *Hox6*, and *Hox9* paralogous genes. *Hox5* and *Hox6* expression is shown at E9.5; *Hox9* expression is shown at E10.5. For each group, the somite anterior expression boundary (red arrow) is offset from the lateral plate expression boundary (green arrowhead).

Fig. 3. Schematic representation of regions of reported phenotypes in *Hox* paralogous mutants. Different vertebral elements are denoted by unique shapes, shown in the bottom panel. Aqua-shaded areas demonstrate the regions of anterior homeotic transformations of the somite-derived primaxial phenotypes. Purple-shaded areas show the lateral plate-derived, abaxial phenotypes for each group. The orange background highlights the regions of phenotypic overlap between adjacent paralogous mutants.
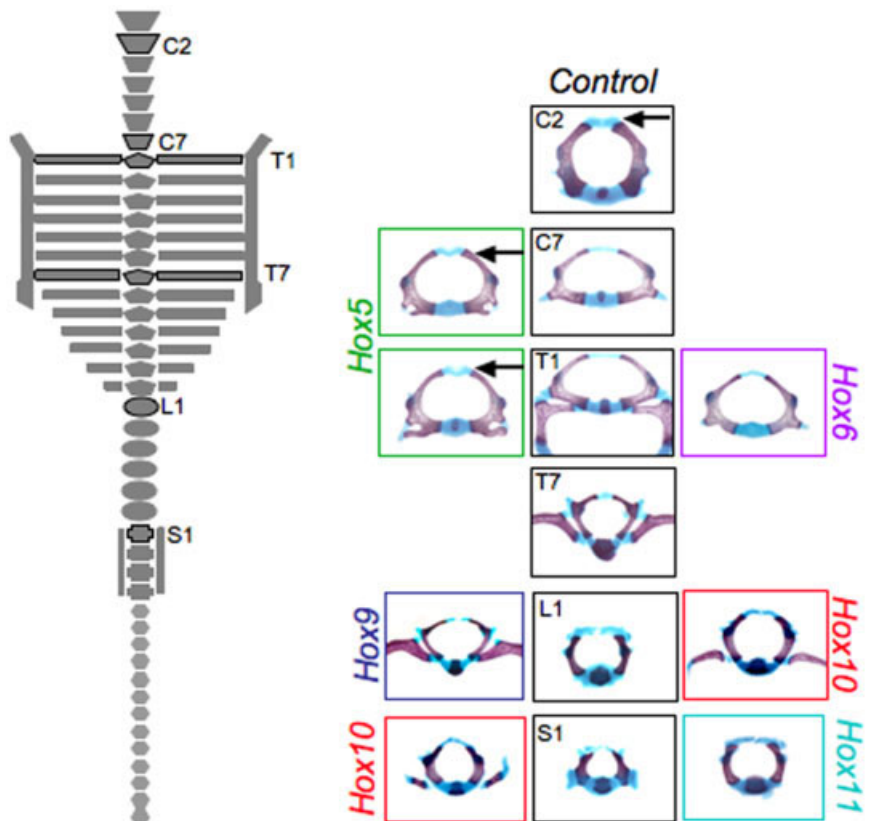
Fig. 4.

transformations of the axial skeleton (Suemori and Noguchi, 2000). Deletions in the *HoxA* and *HoxD* cluster have also been generated (Zakany and Duboule, 1999; Spitz et al., 2001; Kmita et al., 2005), and while the axial phenotypes have not been described in detail, the authors state that the phenotypes caused by the *HoxD* deletions are largely a combination of single mutant phenotypes (Spitz et al., 2001). The most severe phenotypes have been reported for mice carrying a deletion of the *HoxB* cluster. The phenotypes in the somite-derived, primaxial skeletons are relatively minor. However, the sternal phenotype is quite severe, with a failure of closure of the sternal bands and severely shortened and mispatterned sternum (Medina-Martinez et al., 2000). Interestingly, *Hox5* through *Hox9* genes have all been shown to play roles in patterning the sternum, and while *HoxA*, *HoxC*, and *HoxD* complexes possess only two to four members of the *Hox5* through *Hox9* genes, the *HoxB* cluster contains all five members. As the paralogous mutant phenotypes discussed previously indicate that *Hox* contribution to sternal phenotype appears not to be colinear and significant overlap of expression and function occurs throughout the lateral plate-derived sternum, it is possible that the more severe phenotype in the *HoxB* cluster deletion is caused by the loss of a greater number of *Hox5* through *Hox9* genes that contribute to sternal morphology. Severe phenotypes would not be anticipated (and have not been reported) in the somite-derived primaxial skeleton because fewer *Hox* genes within a single complex are functionally active in each region.

Fig. 4. Changes in specific vertebral elements for the *Hox5*, *Hox6*, *Hox9*, *Hox10*, and *Hox11* paralogous mutants. On the left side of the panel, a diagram of the axial skeleton is shown, with specific vertebral elements shown in the right panel marked (C, cervical; T, thoracic; L, lumbar, S, sacral). Wild-type, control elements from specific vertebral positions are denoted by letter and number. The analogous segment from the paralogous mutants are shown on the right and left, with colored boxes for each paralogous mutant group.-

CONCLUSION: THE NATURE OF THE "HOX CODE"

The advent of the ability to generate targeted loss-of-function mutations in mice has allowed a detailed genetic examination of the function of *Hox* genes in mammalian development. Combined with critical experiments in chick that highlight the determination and derivation of axial structures (Kieny et al., 1972; Nowicki and Burke, 2000; Nowicki et al., 2003, Christ et al., this issue, pages 2382–2396), our knowledge of *Hox* function in vertebrates has greatly increased. As predicted shortly after the discovery of the duplications of the *Hox* clusters in mice (Kessel and Gruss, 1990, 1991), this analysis has been grueling and has required complicated combination of mutants generated by numerous investigators but has resulted in a dramatic increase in the knowledge of how the "*Hox* code" operates in vertebrates.

An important conclusion to be drawn from these studies is that the genetic function of vertebrate *Hox* genes are indeed similar to the function originally ascribed to the *Hox/HomC* genes of *Drosophila* by Ed Lewis (Lewis, 1978). Loss of paralogous *Hox* function consistently results in anterior homeotic transformations of colinear regions of the somite-derived axial skeleton. Full support for this conclusion has been demonstrated only many years after the first loss-of-function mutations were reported for this gene family (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992; LeMouellic et al., 1992).

The biggest hurdle has been due to the substantial functional redundancy that exists among paralogous *Hox* genes that contribute to axial morphology. In all paralogous groups that contribute to axial morphology (*Hox3* through *Hox11*), the functions of these genes are redundant and loss-of-function of multiple paralogous genes, without exception, has resulted in more dramatic phenotypes in the axial skeleton (Condie and Capecchi, 1994; Horan et al., 1995b; Manley and Capecchi, 1997; Chen et al., 1998; van den Akker et al., 2001; Wellik and Capecchi, 2003; McIntyre et al., 2007).

Reasons for the high degree of redundancy among the *Hox* genes is beyond the scope of this review, but are likely due to shared regulatory mechanisms within the *Hox* clusters necessary for the establishment of colinear expression, which is inextricably linked to their physical arrangement on the chromosome (reviewed in Duboule and Morata, 1994; Duboule, 1998; Kmita and Duboule, 2003; Duboule and Deschamps, 2004).

The combined genetic analyses have also led to less anticipated conclusions regarding the function of *Hox* genes in axial patterning. The overlap in phenotypes between adjacent paralogous mutant groups was not predicted by early mammalian loss-of-function studies or by previous work on *Drosophila*. This is a unique feature of vertebrate *Hox* patterning. In all cases where adjacent paralogous groups have been examined carefully, the contribution by each paralogous group to phenotype in overlapping regions is distinct. Thus, vertebral morphology is the result of the combination of characteristics imparted by the *Hox* paralogous genes functioning in that AP region. This analysis is not complete, as all paralogous group mutants have not been examined at the level of individual vertebrae, but current work suggests at least two paralogous groups contribute significantly to the patterning of each vertebral element.

These findings reflect on our current understanding of the *Hox* patterning of the axial skeleton. Kessel and Gruss proposed the existence of a "*Hox* code," in which "the identity of a vertebral segment is specified by a combination of functionally active *Hox* genes" (Kessel and Gruss, 1991). This model has since been interpreted to suggest that it is a combination of ALL *Hox* proteins that are ever expressed in a specific AP region that will contribute to phenotype. This latter notion is not supported by early results on single *Hox* mutants, which generally demonstrate defects only in one or two vertebrae at the anterior limit of expression. Current results on paralogous mutants also do not support the latter interpretation of the *Hox* code. However, the original assertion that each vertebral element is patterned according to the combina-

tion of Hox proteins active in each AP region is clearly supported by the genetic results (Fig. 5). Combinations of the activity of at least two paralogous group genes would allow for the continuum of morphologies that exist in the vertebrate axial skeleton. At the anterior limits of some paralogous genes, the new *Hox* activities provide rather dramatic changes to phenotype, such as the changes in the morphology of the first cervical vertebrae, the atlas, which provides the craniocervical joint, to repeating vertebrae by *Hoxa3/d3* (Condie and Capecchi, 1994), cessation of rib formation, coded by the *Hox10* paralogous genes (Wellik and Capecchi, 2003; Carapuco et al., 2005), or the formation of sacral vertebrae at the caudal end of the skeleton by *Hox11* paralogous genes (Wellik and Capecchi, 2003). In other regions of the axial skeleton, these genes provide less dramatic morphological changes, such as broadening of the rib angles in the thoracic skeleton by the *Hox6* genes and curtailing of rib growth around the circumference of the animal and attachment to the sternum by *Hox9* genes (McIntyre et al., 2007). Together, reported *Hox* activities account for most of the modifications of axial structure that occur along the AP axis.

The accumulated genetic results also raise important questions regarding the model of posterior prevalence. This model was originally proposed to address the response of *Hox* gene expression and proposed activity to ectopic placement of retinoic acid in the limb, and is defined as "the property of a given *Hox* gene to realize its functional potential only in the domains in which it is the most posteriorly expressed *Hox* gene" (Duboule, 1991). With respect to AP patterning, this model holds that "the patterning information at one particular body level primarily relies on one HOX protein (or a group of paralogs), rather than on a combination of proteins" (Kmita and Duboule, 2003). Recent genetic results suggest that there is a continuum of *Hox* activity from at least two paralogous groups that are functioning in any AP location and that the next most posterior set of paralogous genes do not prevent the function of the adjacent, anterior group. Thus, the genetic results do not support the

simplest interpretation of posterior prevalence. However, it should be noted that the axial skeleton regains normal patterning posterior to the anterior homeotic transformations. Thus, the activity of any *Hox* paralogous group does not extend to the caudal end of the embryo, as does the expression of *Hox* mRNA in the early embryo. Whether Hox protein expression is similar to *Hox* mRNA expression, and what limits the function of each paralogous group posteriorly is not currently understood. Until information regarding in which cells each of the Hox proteins are expressed, and at what stage morphological patterning information is imparted in the developing axial skeleton, it will be impossible to clearly resolve mechanistic postulates regarding *Hox*-mediated patterning events.

The recognition that lateral plate mesoderm contributes to the formation of the thoracic skeleton is critical to understanding *Hox*-mediated axial patterning. The combined genetic results allow the conclusion that somite-derived *Hox* patterning and lateral plate-derived *Hox* patterning of the rib cage occur with a high degree of independence. While the somite-derived primaxial skeleton demonstrates colinearity of expression and function in the axial skeleton, this is not true of the lateral plate-derived abaxial skeleton. The *Hox5* through *Hox9* paralogous groups affect the morphology of the entire sternum and do not demonstrate colinearity in this region (Fig. 5). Of interest, each of these paralogous group mutants affect the formation of the first sternabra and first rib attachment, suggesting that this region is a node for establishing a coordinated pattern between the primaxial and abaxial skeleton.

While the number of vertebrae in different vertebrate organisms varies considerably, no combinations of *Hox* mutations studied to date have resulted in a change in the total number of vertebrae in mice. Thus, *Hox* gene function does not appear to regulate the number of somites or vertebrae formed, but only the morphology achieved by these structures. Based on the recent study demonstrating the importance of *Hox* gene function during gastrulation (Iimura and Pourquié, 2006), it would be interesting to

know how gastrulation would be affected by loss of all *Hox* function. One might expect very drastic consequences in the ability of epiblast cells to gastrulate if all *Hox* function was removed. With respect to *Hox* function postgastrulation, however, combined evidence suggests that *Hox* genes do not regulate somite number in an organism.

In all mutant combinations generated and examined to date, interestingly, the only type of vertebrae that have not been shown to change dramatically with loss of *Hox* paralogous function are the mid-thoracic vertebrae. Mid-thoracic vertebrae look very similar to one another, and these vertebrae appear largely unaffected by *Hox* mutations. Additionally, no set of *Hox* loss-of-function mutants results in significant loss in the number of ribbed vertebrae. The rib angles and number of ribs attaching to the sternum can be modified, but no paralogous mutant group demonstrates loss of more than 1 of the 13 thoracic ribs. The combined results suggest that the more anterior *Hox* genes (*Hox3* through *Hox5*) have been co-opted to allow more anterior somites to form vertebrae instead of becoming part of the skull, and *Hox6* through *Hox8* genes modify existing thoracic structure. *Hox9* genes repress the ability of posterior ribs to extend and attach to the sternum and *Hox10* genes repress rib formation posterior to the thoracic vertebrae. *Hox11* genes actively promote sacral morphology. These data are consistent with ribbed thoracic vertebrae that extend laterally around much of the body wall being the only axial phenotype that is not *Hox* dependent and, therefore, representing a developmental, and perhaps evolutionary, "ground state." However, the argument that more sophisticated combinations of *Hox* mutations are required to see transformations of this region of the thoracic vertebrae is at least one alternative explanation for this observation. Loss-of-function of all four complexes would have to be generated, conditionally and only in the somites, to address this question completely. The early function of *Hox* genes in the gastrulating embryo (Iimura and Pourquié, 2006) and the lateral plate contribution to thoracic patterning (McIntyre et al., 2007) would otherwise prevent or complicate the interpretation.

PERSPECTIVE

Genetic analyses that have been performed on *Hox* genes in mice over the past 15 years has contributed substantially to our knowledge regarding their function in patterning the axial skeleton. The molecular mechanisms involved in these events, however, are almost completely lacking. To begin to address molecular explanations for *Hox*-mediated patterning events in the axial skeleton, two main questions must be answered: WHEN and WHERE?

Perhaps the most critical lack of information involves the timing when *Hox*-coded axial patterning events occur. While careful embryological experiments in chicken show definitively that *Hox* expression is determined in the presomitic mesoderm, this does not provide information regarding when these genes are functionally important. With respect to patterning the axial skeleton, it is only several days after the onset of expression that sclerotomal cells begin to acquire morphologies specific to their AP location. When the regulation of these patterning events occur is not clear.

Carapuco et al. reported very interesting results demonstrating that ectopic expression of *Hoxa10* anterior to its normal expression in the lumbar vertebrae results in the repression of all ribs in the thoracic region. By comparing the expression from two transgenic enhancers, they demonstrate that rib repression correlates with earlier expression in the PSM (Carapuco et al., 2005). However, characterization of expression from the two transgenic constructs used in this study was not extended to later stages, so it is difficult to conclude that the only difference between these two enhancers is the onset of expression. It is possible that the duration and amplitude of expression from these two constructs, and the spatial domains activated in these transgenic lines at later stages, also differ substantially and contributes to the difference in the ability of these two constructs to repress rib outgrowth. Evidence that anterior limits of *Hox* expression changes after exit from the PSM, in some cases to substantially more anterior regions, along with evidence that the more anterior boundaries correlate with genetic phenotype, supports the possibility that the functional *Hox* activity in the somite-derived axial skeleton occurs at later time points.

The second piece of critical information in distinguishing between different potential mechanisms of *Hox* function in axial patterning is the establishment of the spatial distribution of Hox proteins at the time when Hox proteins are actively regulating vertebral morphology. The majority of *Hox* expression literature has focused on mRNA expression, not protein expression. In cases where protein and mRNA expression have been compared directly, significant differences have been noted (Nelson et al., 1996; Brend et al., 2003). Our current understanding of the "*Hox* code" suggests that at least two paralogous groups are functioning at any AP location to achieve wild-type vertebral morphology. No information exists to suggest whether the two paralogous groups are expressed in the same cells and perform presumably different functions or if, at the time points when morphology is being established, the active paralogous group proteins are expressed in distinct subsets of cells. As a further extension, the current genetic results demonstrate that posterior prevalence is not observed between adjacent paralogous groups of *Hox* genes, but if adjacent groups of genes are not expressed in the same cells, it is possible that some yet more posterior groups of *Hox* proteins are expressed in the same cells and repress anterior *Hox* gene function. Without a clear understanding of the cellular distribution of Hox protein expression—at time points when pattern is actually established—it is not possible to provide clear support or refutation of existing models.

It is only by careful temporal analyses of *Hox* gene function in the somitic mesoderm that we will understand when functionally important events occur in axial patterning. Temporal conditional mutants, such as those controlled by tamoxifen-Cre or doxycycline-inducible Cre may allow the establishment of WHEN *Hox* genes function in axial patterning. The generation of good antibodies to Hox proteins, such as those described by Dasen et al. (2005), or studies using live fluorescent reporters of Hox protein expression, such as the ones described previously (Godwin et al., 1998; Arenkiel et al., 2004), will allow the further dissection of WHERE Hox proteins are expressed during these critical patterning events. Only after these important parameters are delineated are we likely to be able to propose and test mechanistic hypotheses regarding *Hox* function in axial patterning.

REFERENCES

Arenkiel BR, Trvdyk P, Gaufo GO, Capecchi MR. 2004. Hoxb1 functions in both motoneurons and in tissues of the periphery to establish and maintain the proper neuronal circuitry. Genes Dev 18: 1539–1552.

Boulet AM, Capecchi MR. 1996. Targeted disruption of Hoxc-4 causes esophageal defects and vertebral transformations. Dev Biol 177:232–249.

Brend T, Gilthorpe J, Summerbell D, Rigby PWJ. 2003. Multiple levels of transcriptional and post-transcriptional regulation are required to define the domain of *Hoxb4* expression. Development 130: 2717–2718.

Burke AC. 2000. Hox genes and the global patterning of the somitic mesoderm. Cur Top Dev Biol 47:155–181.

Burke AC, Nowicki JL. 2003. A new view of patterning domains in the vertebrate mesoderm. Dev Cell 4:159–165.

Burke AC, Nelson CE, Morgan BA, Tabin C. 1995. Hox genes and the evolution of vertebrate axial morphology. Development 121:333–346.

Carapuco M, Novoa A, Bobola N, Mallo M. 2005. *Hox* genes specify vertebral types in the presomitic mesoderm. Genes Dev 19:2116–2121.

Carpenter EM, Goddard JM, Davis AP, Nguyen TP, Capecchi MR. 1997. Targeted disruption of Hoxd-10 affects mouse hindlimb development. Development 124:4505–4514.

Chen F, Capecchi MR. 1997. Targeted mutations in hoxa-9 and hoxb-9 reveal synergistic interactions. Dev Biol 181:186–196.

Chen F, Capecchi MR. 1999. Paralogous mouse Hox genes, Hoxa9, Hoxb9 and Hoxd9, function together to control development of the mammary gland in response to pregnancy. Proc Natl Acad Sci U S A 96:541–546.

Chen F, Greer J, Capecchi MR. 1998. Analysis of Hoxa7/Hoxb7 mutants suggests

periodicity in the generation of different sets of vertebrae. Mech Dev 77:49–57.

Chisaka O, Capecchi MR. 1991. Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene hox-1.5. Nature 350:473–479.

Chisaka O, Musci TS, Capecchi MR. 1992. Developmental defects of the ear, cranial nerves and hindbrain resulting from targeted disruption of the mouse homeobox gene Hox-1.6. Nature 355:516–520.

Condie BG, Capecchi MR. 1993. Mice homozygous for a targeted disruption of Hoxd-3 (Hox-4.1) exhibit anterior transformations of the first and second cervical vertebrae, the atlas and the axis. Development 119:579–595.

Condie BG, Capecchi MR. 1994. Mice with targeted disruptions in the paralogous genes hoxa-3 and hoxd-3 reveal synergistic interactions. Science 370:304–307.

Dasen JS, Tice BC, Brenner-Morton S, Jessell TM. 2005. A Hox regulatory network establishes motor neuron pool identity and target muscle connectivity. Cell 123: 477–491.

Davis AP, Capecchi MR. 1994. Axial homeosis and appendicular skeleton defects in mice with a targeted disruption of hoxd-11. Development 120:2187–2198.

Davis AP, Witte DP, Hsieh-Li HM, Potter SS, Capecchi MR. 1995. Absence of radius and ulna in mice lacking hoxa-11 and hoxd-11. Nature 375:791–795.

Deschamps J, Wijgerder M. 1993. Two phases in the establishment of HOX expression domains. Dev Biol 156:473–480.

Deschamps J, van den Akker E, Forlani S, deGraaf W, Oosterveen T, Roelen B, Roelfsema J. 1999. Initiation, establishment and maintenance of Hox gene expression patterns in the mouse. International Journal of Developmental Biology 43:635–650.

Duboule D. 1991. Patterning in the vertebrate limb. Curr Opin Genet Dev 1:211–216.

Duboule D. 1998. Vertebrate Hox gene regulation: clustering and/or colinearity. Curr Opin Genet Dev 8:514–518.

Duboule D, Deschamps J. 2004. Colinearity loops out. Dev Cell 6:738–740.

Duboule D, Morata G. 1994. Colinearity and functional hierarchy among genes of the homeotic complexes. TIG 10:358–364.

Forlani S, Lawson KA, Deschamps J. 2003. Acquisition of Hox codes during gastrulation and axial elongation in the mouse embryo. Development 130:3807–3819.

Fromental-Ramain C, Warot X, Lakkaraju S, Favier B, Haack H, Birling C, Kierich A, Dolle P, Chambon P. 1996a. Specific and redundant functions of the paralogous Hoxa-9 and Hoxd-9 genes in forelimb and axial skeleton patterning. Development 122:461–472.

Fromental-Ramain C, Warot X, Messadecq N, LeMeur M, Dolle P, Chambon P. 1996b. Hoxa-13 and Hoxd-13 play a crucial role in the patterning of the limb autopod. Development 122:2997–3011.

Garcia-Gasca A, Spyropoulos DD. 2000. Differential mammary morphogenesis along the anteroposterior axis in Hoxc6 gene targeted mice. Dev Dyn 219:261–276.

Gaunt SJ, Strachan L. 1996. Temporal colinearity in expression of anterior Hox genes in developing chick embryos. Dev Dyn 207:270–280.

Gaunt SJ, Coletta PL, Pravtcheva D, Sharpe PT. 1990. Mouse Hox-3.4: homeobox sequence and embryonic expression patterns compared with other members of the Hox gene network. Development 109:329–339.

Gavalas A, Trainor P, Ariza-McNaughton L, Krumlauf R. 2001. Synergy between Hoxa1 and Hoxb1: the relationship between arch patterning and the generation of cranial neural crest. Development 128:3017–3027.

Godwin AR, Stadler HS, Nakamura K, Capecchi MR. 1998. Detection of targeted GFP-Hox gene fusions during mouse embryogenesis. Proc Natl Acad Sci U S A 95:13042–13047.

Goodrich ES. 1930. Studies on the structure and development of vertebrates. London: Macmillan.

Holland PWH, Hogan BLM. 1988. Expression of homeo box genes during mouse development: a review. Genes Dev 2:773–782.

Horan GS, Wu K, Wolgemuth DJ, Behringer RR. 1994. Homeotic transformation of cervical vertebrae in Hoxa-4 mutant mice. Proc Natl Acad Sci U S A 91:12644–12648.

Horan GS, Kovacs E, Behringer RR, Featherstone MS. 1995a. Mutation in paralogous Hox genes result in overlapping homeotic transformations: evidence for unique and redundant function. Dev Biol 169:359–372.

Horan GS, Ramirez-Solis R, Featherstone MS, Wolgemuth DJ, Bradley A, Behringer RR. 1995b. Compound mutants for the paralogous *hoxa-4*, *hoxb-4*, and *hoxd-4* genes show more complete homeotic transformations and a dose-dependent increase in the number of vertebrae transformed. Genes Dev 9:1667–1677.

Iimura T, Pourquié O. 2006. Collinear activation of Hoxb genes during gastrulation is linked to mesoderm cell ingression. Nature 442:568–571.

Izpisua-Belmonte JC, Falkenstein H, Dolle P, Renucci A, Duboule D. 1991. Murine genes related to the Drosophila AbdB homeotic genes are sequentially expressed during development of the posterior part of the body. EMBO J 10:2279–2289.

Jeannotte L, Lemieux M, Charron J, Poirier F, Robertson EJ. 1993. Specification of axial identity in the mouse: role of the Hoxa-5 (Hox1.3) gene. Genes Dev 7: 2085–2096.

Kessel M, Gruss P. 1990. Murine developmental control genes. Science 249:374–379.

Kessel M, Gruss P. 1991. Homeotic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. Cell 67:89–104.

Kieny M, Mauger A, Sengel P. 1972. Early regionalization of somitic mesoderm as studied by the development of the axial skeleton of the chick embryo. Dev Biol 28:142–161.

Kmita M, Duboule D. 2003. Organizing axes in time and space; 25 years of colinear tinkering. Science 301:331–333.

Kmita M, Tarchini B, Zakany J, Logan M, Tabin C, Duboule D. 2005. Early developmental arrest of mammalian limbs lacking HoxA/HoxD function. Nature 435: 1113–1116.

Kostic D, Capecchi MR. 1994. Targeted disruptions of the murine Hoxa-4 and Hoxa-6 genes result in homeotic transformations of components of the vertebral column. Mech Dev 46:231–247.

Krumlauf R. 1993. Mouse Hox genetic functions. Curr Opin Genet Dev 3:621–625.

Krumlauf R. 1994. Hox genes in vertebrate development. Cell 78:191–201.

LeMouellic H, Lallemand Y, Brulet P. 1992. Homeosis in the mouse induced by a null mutation in the Hox-3.1 gene. Cell 69:251–264.

Lewis EB. 1963. Genes and developmental pathways. Am Zool 3:33–56.

Lewis EB. 1978. A gene complex controlling segmentation in *Drosophila*. Nature 276:565–570.

Lufkin T, Dierich A, LeMeur M, Mark M, Chambon P. 1991. Disruption of the Hox-1.6 homeobox gene results in defects in a region corresponding to its rostral domain of expression. Cell 66:1105–1119.

Maconochie M, Nonchev S, Morrison A, Krumlauf R. 1996. Paralogous Hox genes: function and regulation. Annu Rev Genet 30:529–556.

Manley NR, Capecchi MR. 1997. Hox group 3 paralogous genes act synergistically in the formation of somitic and neural crest-derived structures. Dev Biol 192: 274–288.

McIntyre DM, Rakshit S, Yallowitz AR, Loken L, Jeannotte L, Capecchi MR, Wellik DM. 2007. *Hox* Patterning of the vertebrate rib cage. Development 134:2981–2989.

Medina-Martinez O, Bradley A, Ramirez-Solis R. 2000. A large targeted deletion of Hoxb1-Hoxb9 produces a series of single-segment anterior homeotic transformations. Dev Biol 222:71–83.

Nelson CE, Morgan BA, Burke AC, Laufer E, DiMambro E, Murtaugh LC, Gonzales E, Tessarallo L, Parada L, Tabin C. 1996. Analysis of *Hox* gene expression in the chick limb bud. Development 122: 1449–1466.

Nowicki JL, Burke AC. 2000. *Hox* genes and morphological identity: axial patterning versus lateral patterning in the vertebrate mesoderm. Development 127: 4265–4275.

Nowicki JL, Takimoto R, Burke AC. 2003. The lateral somitic frontier: dorso-ventral aspects of anterio-posterior regionalization in avian embryos. Mech Dev 120: 227–240.

Rancourt DE, Tsuzuki T, Capecchi MR. 1995. Genetic interaction between hoxb-5

and hoxb-6 is revealed by nonallelic non-complementation. Genes Dev 9:108–122.

Small KM, Potter SS. 1993. Homeotic transformations and limb defects in Hoxa-11 mutant mice. Genes Dev 7:2318–2328.

Spitz F, Gonzalez F, Peichel C, Vogt TF, Duboule D, Zakany J. 2001. Large scale transgenic and cluster deletion analysis of the HoxD complex separate an ancestral regulatory module from evolutionary innovations. Genes Dev 15:2209–2214.

Studer M, Gavalas A, Marshall H, Ariza-McNaughton L, Rijli FM, Chambon P, Krumlauf R. 1998. Genetic interactions between Hoxa1 and Hoxb1 reveal new roles in regulation of early hindbrain patterning. Development 125:1025–1036.

Suemori H, Noguchi S. 2000. Hox C cluster genes are dispensable for overall body plan of mouse embryonic development. Dev Biol 220:333–342.

Suemori H, Takahashi N, Noguchi S. 1995. Hoxc-9 mutant mice show anterior transformation of the vertebrae and malformation of the sternum and ribs. Mech Dev 51:265–273.

van den Akker E, Reijnen M, Korving J, Brouwer A, Meijlink F, Deschamps J. 1999. Targeted inactivation of Hoxb8 affects survival of a spinal ganglion and causes aberrant limb reflexes. Mech Dev 89:103–114.

van den Akker E, Fromental-Ramain C, deGraaf W, LeMouellic H, Brulet P, Chambon P, Deschamps J. 2001. Axial skeletal patterning in mice lacking all paralogous group 8 Hox genes. Development 128:1911–1921.

Wahba GM, Hostikka SL, Carpenter EM. 2001. The paralogous Hox genes Hoxa10 and Hoxd10 interact to pattern the mouse hindlimb peripheral nervous system and skeleton. Dev Biol 231:87–102.

Wellik DM, Capecchi MR. 2003. Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton. Science 301:363–366.

Wellik DM, Hawkes PJ, Capecchi MR. 2002. Hox11 paralogous genes are essential for metanephric kidney induction. Genes Dev 16:1423–1432.

Zakany J, Duboule D. 1999. Hox genes and the making of sphincters. Nature 401: 761.