Hox Genes and Segmentation of the Hindbrain and Axial Skeleton

Tara Alexander, Christof Nolte, and Robb Krumlauf

Stowers Institute for Medical Research, Kansas City, Missouri 64110;
email: tba@stowers.org, cdn@stowers.org, rek@stowers.org

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Key Words

rhombomeres, somites, vertebrae, neural crest, global patterning, homeotic transformation, fibroblast growth factor (Fgf), retinoic acid (RA), caudal homeobox gene (*Cdx*), variant hepatocyte nuclear factor 1 gene (*vhnf1*), mesoderm, colinearity

Abstract

Segmentation is an important process that is frequently used during development to segregate groups of cells with distinct features. Segmental compartments provide a mechanism for generating and organizing regional properties along an embryonic axis and within tissues. In vertebrates the development of two major systems, the hindbrain and the paraxial mesoderm, displays overt signs of compartmentalization and depends on the process of segmentation for their functional organization. The hindbrain plays a key role in regulating head development, and it is a complex coordination center for motor activity, breathing rhythms, and many unconscious functions. The paraxial mesoderm generates somites, which give rise to the axial skeleton. The cellular processes of segmentation in these two systems depend on ordered patterns of *Hox* gene expression as a mechanism for generating a combinatorial code that specifies unique identities of the segments and their derivatives. In this review, we compare and contrast the signaling inputs and transcriptional mechanisms by which *Hox* gene regulatory networks are established during segmentation in these two different systems.

Contents

INTRODUCTION

Hox genes are a large family of related genes that encode helix-turn-helix transcription factors. In many animal species, this gene family plays an important role in regulating the specification of positional identities of tissues along the anterior-posterior (A-P) axis during development (Carroll 1995, Krumlauf 1994, McGinnis & Krumlauf 1992). In most vertebrates, excluding fish, there are 39 *Hox* genes organized into four separate chromosomal clusters (**Figure 1**). Within each cluster, all genes have the same orientation with respect to transcription. These clusters arose by duplication and divergence from a common ancestral complex; and on the basis of similarities, in both the sequence and position of genes in the complexes, it is possible to identify 13 paralogous groups (PG) (**Figure 1*c***). A hallmark of clustered *Hox* genes is the direct correlation between their linear arrangement along the chromosome, and the timing and A-P boundaries of their expression during early development (Duboule & Dollé 1989, Graham et al. 1989, Lewis 1978). This property is termed colinearity and results in the establishment of ordered domains of expression that provide a combinatorial *Hox* code for

***Hox* genes:** a highly conserved family of homeodomain transcription factors that regulate axial information

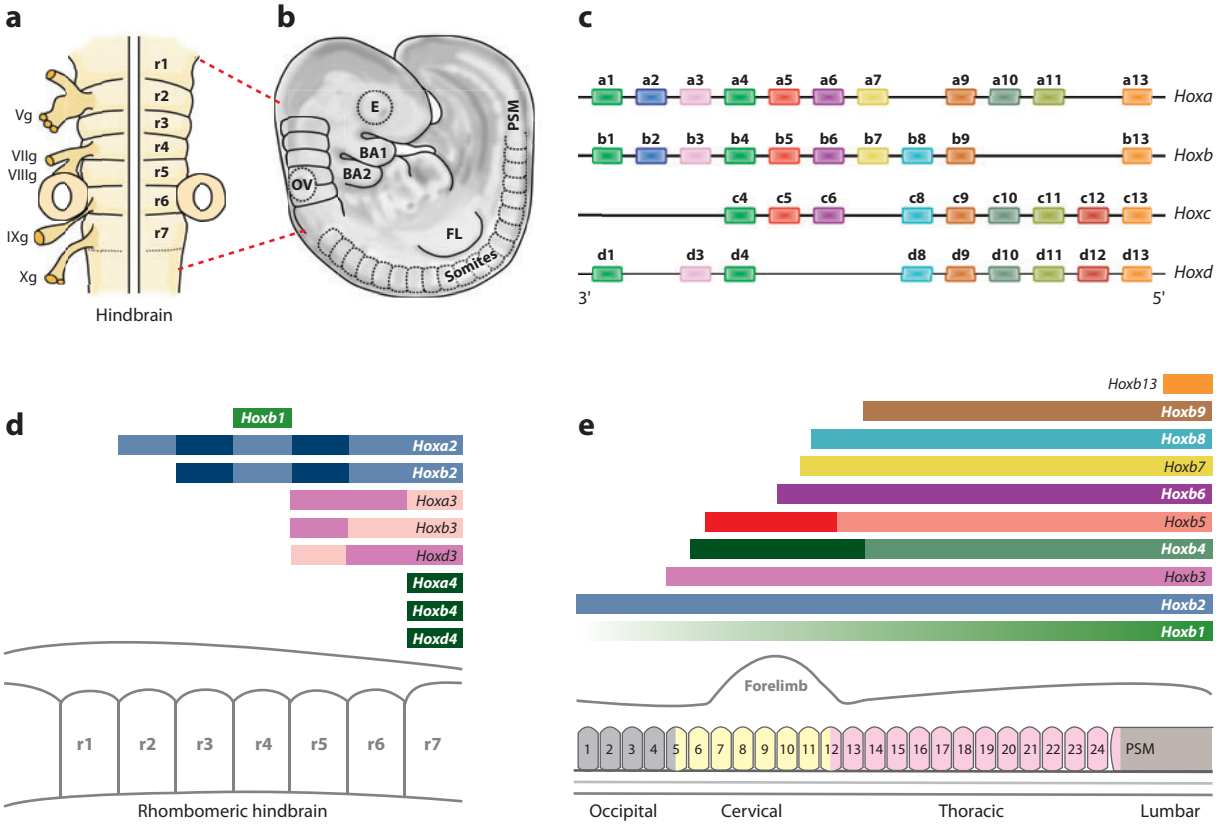

Figure 1

The mammalian *Hox* cluster. (*a*) Depiction of a segmented vertebrate hindbrain displaying the rhombomeres and their associated cranial motor nerves. For clarity the cranial ganglia are displayed on only one side of the segmented hindbrain. Shown are the five most obvious ganglia (Vg-Xg) through which the motor and sensory nerves pass. (*b*) A 9.5 dpc mouse embryo illustrating the hindbrain and somites. Other features include the developing eye (E), otic vesicle (OV), branchial arches (BA1, BA2), forelimb (FL) and presomitic mesoderm (PSM). (*c*) *Hox* genes in the mammal are organized into four clusters (*Hoxa*, *Hoxb*, *Hoxc*, and *Hoxd*) that are arrayed on separate chromosomes. Within each cluster, the *Hox* genes are arranged in a linear order that reflects their initiation and placement of their anterior border of expression. Thus, members of the first paralogous group (*Hoxa1*, *Hoxb1*, *and Hoxd1*) are generally expressed first and have the most anterior border of expression, whereas members of the thirteenth paralogous group (*Hoxa13*, *Hoxb13*, *Hoxc13*, *and Hoxd13*) are expressed last and have the anterior borders of expression in the most posterior regions. (*d*) *Hox* gene expression in the 9.5 dpc mouse hindbrain. The borders of expression domains colocalize with rhombomeric boundaries. Higher domains of expression are indicated by darker shading domains, and members within a paralogous group are displayed in the same color. (*e*) *Hox* gene expression in the developing somitic column of the vertebrate embryo. For illustrative purposes, only *Hox* genes from the *Hoxb* complex are shown. For some *Hoxb* members, their mRNA distribution along the A-P axis varies and is shown as a gradient. As within the developing hindbrain, the staggered arrangement of their anterior borders within somites is a property of their physical ordering along the chromosome; this phenomenon is known as colinearity.

specifying distinct regional properties along embryonic axes (Kmita & Duboule 2003). Within vertebrate species alone, the products of the *Hox* genes are used to impart A-P positional identity within the paraxial mesoderm, lateral plate mesoderm, neuroectoderm, neural crest, and endoderm. Major signaling pathways, such as fibroblast growth factor (Fgf), retinoic acid (RA), and Wnt, play important roles in establishing the *Hox* codes in these different developmental contexts. Subsequently, the *Hox* code is redeployed to provide patterning information to the developing limbs and urogenital system.

Hindbrain: region of the brain that coordinates motor activity, breathing rhythms, and many unconscious functions

Rhombomeres: segmental compartments in the hindbrain (rhombencephalon) that control neural organization and architecture

Somites: epithelial blocks of mesodermal cells that give rise to vertebrae and ribs, dermis of the skin, and skeletal muscles of the body wall and limbs

Hindbrain rhombomeres and trunk somites are transient, serially homologous structures that are critical for organizing many later developmental processes. The segments of the paraxial mesoderm (somites) are formed in a reiterative progression, concomitant with the laying down of the body axis, whereas the rhombomeres form within the pre-existing neural tube. These repeating units are then modified by segment-specific Hox activity. Patterning of both systems involves coordination between morphological segmentation and the generation of unique profiles of *Hox* gene expression within each segment (Galis 1999, Kessel & Gruss 1991, Lumsden 2004, Lumsden & Krumlauf 1996). In turn, differential Hox function between segments underlies the institution of segment-specific developmental programs that regulate the identity of these segments and their derivatives. Thus, differential Hox activity creates regional diversity from repeated units.

Whereas *Hox* expression in both the hindbrain and the paraxial mesoderm must be coordinated with morphological segmentation, the genesis of the segments in each system is quite distinct. It is postulated that segmentation has arisen independently many times during evolution, suggesting that there are probably important differences in the molecular and cellular pathways that govern segmentation in different contexts. For example, Fgf, RA, and Wnt signaling pathways play important roles in these segmental processes but, due to differences in timing and levels of expression, these signaling cascades generate distinct outcomes. Despite these differences, one common theme appears to be that ordered expression of *Hox* genes is coupled to specification of segmental identity. Therefore, building a picture of the regulatory networks that establish and maintain the coordinated patterns of *Hox* expression and function provides an opportunity to compare and contrast patterning and morphogenesis in the hindbrain and axial skeleton. This review provides an overview of how segments form, and what is known about the upstream factors and signals that govern the establishment of the *Hox* cascade in the vertebrate hindbrain and paraxial mesoderm. In light of the conserved nature of these processes, extrapolation of data from a variety of vertebrate systems has been used to generate a working model of events.

HOX GENES AND HINDBRAIN SEGMENTATION

Hindbrain Segmentation

Segmentation in the hindbrain (rhombencephalon) is a progressive process that occurs over a fairly short period of time to divide the future hindbrain territory into discrete units. Initially, the hindbrain appears as a smooth, featureless sheet of cells, which then undergoes a period of transient compartmentation into seven segments known as rhombomeres (r) (Lumsden 2004, Lumsden & Krumlauf 1996, Moens & Prince 2002). Rhombomeres represent lineage-restricted cellular compartments formed by cell segregation. Cell sorting between rhombomeres is regulated by the Eph/ephrin bidirectional signaling pathway (Mellitzer et al. 2000). The *Eph* receptors and their membrane-bound ligands, the *ephrins*, are expressed in complementary rhombomeres; receptors are expressed in r3 and r5, whereas their ligands are expressed in r2, r4, and r6. This establishes alternating differences in adhesion and repulsion that repeat with a two-segment periodicity and leads to sorting between adjacent cell populations (Mellitzer et al. 1999, Xu et al. 1999). This differential sorting mechanism creates segregated groups of cells that respond to local signals and adopt distinct characteristics. Understanding the molecular basis for establishing both the two-segment periodicity and alternating pattern of cell properties, behaviors, and patterns of gene expression in hindbrain segmentation is critically important for building an accurate picture of regulatory networks that control segmentation in this context.

Hindbrain Segmentation and Head Development

Although the physical separation of the hindbrain into seven rhombomeric segments is transitory, this early organization plays a fundamental role in head development and in maintaining the neural architecture in postsegmental and adult stages of development (Pasqualetti et al. 2007, Wingate & Lumsden 1996). A major component of the bone and connective tissue that contributes to craniofacial development is derived from cranial neural crest cells, which migrate from the hindbrain rhombomeres (Koentges & Matsuoka 2002, Le Douarin & Kalcheim 1999). Correlating with the two-segment periodicity, relatively little neural crest is derived from r3 and r5, and this arises due to interactions between rhombomeres, and between rhombomeres and their surrounding environment. The hindbrain, cranial neural crest, and ectodermal placodes combine to form the cranial nerves of the adult medulla oblongata and pons, whose physical patterning can be traced back to their rhombomeres of origin (**Figure 1*a***). Hence, rhombomere segmentation governs the formation and organization of nerve nuclei, ganglion root positioning, patterns of neurogenesis, and neural circuitry, which underpins its conserved role as a complex neural coordination center.

The A-P boundaries and patterns of *Hox* gene expression are tightly linked to rhombomeric segments (Keynes & Krumlauf 1994, Lumsden & Krumlauf 1996, Maconochie et al. 1996). In accord with the property of colinearity, most of the genes from PG 1–4 display ordered and nested domains of expression, which have anterior boundaries that map to the junction between rhombomeres (Hunt et al. 1991, Wilkinson et al. 1989). With few exceptions, genes within a cluster have an A-P boundary of expression that varies with a two-segment periodicity from the adjacent genes. The A-P boundary of *Hoxb2* maps to the r2/r3 junction, whereas *Hoxb3* marks the r4/r5 boundary, and *Hoxb4* maps to the r6/r7 junction (**Figure 2*b***). *Hox* genes within a given PG also generally have the same boundaries of gene expression. Thus, members from *Hox* groups 2, 3, and 4 have anterior boundaries that map to the r2/r3, r4/r5, and r6/r7 boundaries, respectively (**Figure 2*b***).

Exceptions to the two-segment periodicity in boundaries of *Hox* expression are *Hoxa2*, *Hoxa1*, and *Hoxb1*. *Hoxa2* expression extends up the r1/r2 boundary and is the only *Hox* gene expressed in r2. In the mouse, at 8.0 days post coitum (dpc), both *Hoxa1* and *Hoxb1* are expressed up to the presumptive r3/r4 boundary. However, by 9.5 dpc *Hoxa1* is rapidly downregulated in the hindbrain, whereas the expression of *Hoxb1* becomes restricted to r4. This illustrates that domains of *Hox* expression are dynamic during the period of segmentation, and there can also be segment specific variations in the levels of expression (**Figure 2**). Of the 12 *Hox* genes in PG 1–4, only *Hoxd1* and *Hoxc4* are not expressed in the hindbrain.

The nested domains of *Hox* expression, which are also observed in non-neural ectoderm, and cranial neural crest cells and their derivatives form a *Hox* code that regulates patterning in the branchial region of the head (Trainor & Krumlauf 2000, 2001). The expression of *Hox* genes in the hindbrain and cranial neural crest is regulated independently (Maconochie et al. 1999). Therefore, patterns established in the rhombomeres are not passively translated into the arches when neural crest cells migrate, although regulatory events within the hindbrain impact the establishment of *Hox* expression in neural crest cells.

During neural development, *Hox* genes begin to display differential expression that correlates with the onset of neurogenesis. Within the hindbrain, columns of different classes of neurons begin to form, and many of these are correlated with rhombomeres and rhombomere boundaries. These columns correspond to the expression domains of known factors in neurogenesis, and functional studies have begun to demonstrate that *Hox* genes play later roles in regulating patterns of neurogenesis and regional identity (Kiecker & Lumsden 2005).

Cranial neural crest cells: multipotent cells that delaminate from the midbrain and hindbrain to generate most bone and connective tissues of the head

Function of *Hox* Genes in Rhombomere Identity

Functional support for the role of *Hox* genes in regulating the segmental identity of rhombomeres has come through analyses of phenotypes arising from loss- and gain-of-function experiments in several species (Lumsden 2004, Maconochie et al. 1996, Rijli et al. 1998, Moens & Prince 2002). The requirement for Hox proteins in many different tissues often results in complex defects in *Hox* mutants. Moreover, functional compensation between genes can mask regulatory activity. Despite these difficulties, genetic studies have provided insight into the role of *Hox* genes in control of segmental patterning in the hindbrain.

The products of the PG1 genes, *Hoxa1* and *Hoxb1*, play multiple roles in the mouse hindbrain, which, in part reflect their regulatory relationship, because *Hoxa1* helps to activate *Hoxb1* in r4. In *Hoxa1* mutants, r5 is lost, and there is a fusion between r4 and r6 (Carpenter et al. 1993, Mark et al. 1993). In *Hoxb1* mutants, there is a failure to maintain the identity of r4, and it adopts an r2-like character (Studer et al. 1996). Conversely, ectopic

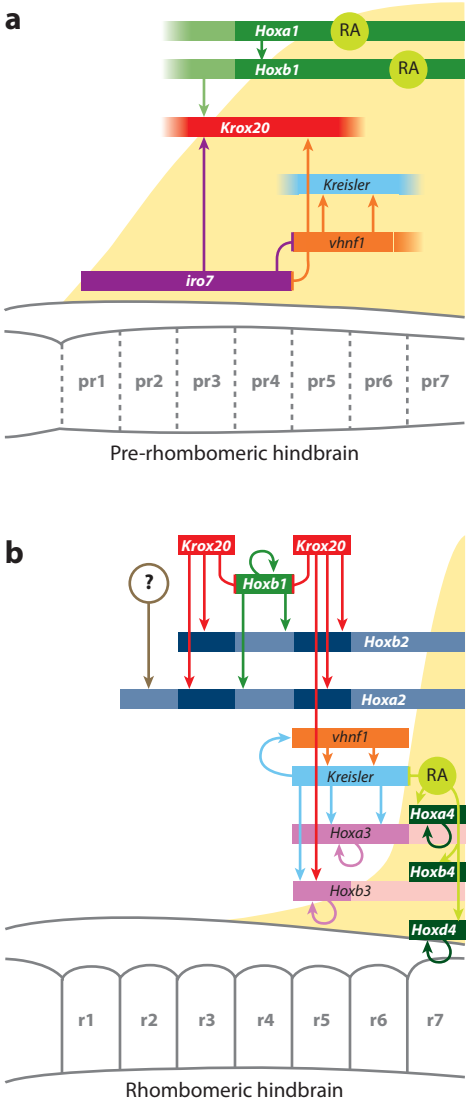

Figure 2

Gene patterning network of the vertebrate hindbrain. (*a*) Network prior to the appearance of the rhombomeres. The yellow background represents the retinoic acid (RA) gradient produced by the Raldh2 (Aldh1a2) enzyme that is located in the somites flanking the caudal hindbrain. In response to RA, the expression of *Hoxa1* and *Hoxb1* (both in *green*) is initiated in the neural tube by retinoic acid response elements (RAREs) located in their 3′ flanking sequences. Hoxb1 directly activates expression of *Krox20* (*red*) in the presumptive r3 territory. In the zebrafish, reciprocal interactions between iro7 (*purple*) and vhnf1 (*orange*) partition the hindbrain into two parts. These domains are further subdivided by the actions of group 1 paralogs, Krox20 and kreisler (*light blue*). (*b*) Network at the appearance of the rhombomeres. The borders of genes that are expressed in the hindbrain coincide with its segmentation. The earlier expression patterns of *Krox20* and *Hoxb1* become localized to specific rhombomeres, and at this time *Hoxa1* expression is no longer detected in the hindbrain. The expression of other *Hox* genes is mediated by crossregulatory [i.e., *Hoxb1* regulating group 2 paralogs (*dark blue*) expression in r4], upstream regulators such as Krox20 and kreisler and in response to RA [i.e., group 4 paralogs (*dark green*)]. Through the opposing action of members of the Cyp26 family (not shown), the availability of RA (*yellow background*) domain has been posteriorized to the caudal end of the hindbrain. Several *Hox* genes display higher levels of expression in different rhombomeres, as indicated by the darker blue shading for *Hoxb2* and *Hoxa2* in r3 and r5. Many of the *Hox* genes autoregulate (*circular arrows*). The expression of *Hoxd3* is not shown.

expression of *Hoxa1* or *Hoxb1* leads to a transformation of r2 into an r4 character (Zhang et al. 1994). In *Hoxa1/Hoxb1* compound mutants, groups of cells form in the position of future r4, but they fail to adopt a segmental identity, and no neural crest cells migrate from this segment (Gavalas et al. 2001, Rossel & Capecchi 1999, Studer et al. 1998). Hence, in the absence of these genes, future r4 is locked in a ground state and unable to enter hindbrain patterning. In zebrafish, the role of *hoxb1a* and *hoxb1b* in patterning the hindbrain appears to be conserved (Moens & Prince 2002). Therefore, *Hoxa1* and *Hoxb1* work together to specify r4 identity, and *Hoxa1* has an additional role in forming r5.

Hoxa2 is the only PG2 member expressed in r2, and it is required to maintain segmental properties of r2. In *Hoxa2* mutants, there is a reduction of r2 and an expansion of r1, resulting in an enlargement of the cerebellum at the expense of the pons (Gavalas et al. 1997). Major defects are observed in cranial neural-crest cell derivatives of the second branchial arch, and supporting evidence from other species indicates that *Hoxa2* plays a conserved role in regulating the differentiation of bone and connective tissue in craniofacial development (Trainor & Krumlauf 2001, Rijli et al. 1998). *Hoxa2* and *Hoxb2* are both expressed in r3-r7 (**Figure 2*b***). In mouse, analyses of single and compound mutants for these genes show that *Hoxa2* influences the size of r3, and *Hoxb2* contributes to the maintenance of r4 identity (Davenne et al. 1999, Gavalas et al. 2003). In double mutants, the segmentation of the r3-r5 region is relatively normal, but the inter-rhombomeric boundaries between r1 and r4 are missing. Therefore, input from *Hoxa2* and *Hoxb2* is needed to generate the correct r2/r3 boundary.

For PG3 genes, embryos with single and double mutant combinations of *Hoxa3*, *Hoxb3*, or *Hoxd3* display vertebral defects and many other abnormalities. However, hindbrain patterning appears to be normal, although there are defects in the formation of the IX cranial nerve (Manley & Capecchi 1997). The loss of all three PG3 genes results in altered motor neuron development in r5 and r6 and the ectopic activation of *Hoxb1* in r6 (Gaufo et al. 2003). This demonstrates that the PG3 proteins work in concert to regulate the identity of r5 and r6 in part through repression of *Hoxb1*. The PG4 genes *Hoxa4*, *Hoxb4*, and *Hoxd4* are expressed up to the r6/r7 border in the hindbrain, and mutants display severe skeletal abnormalities. However, no hindbrain or neurological defects have been reported even in compound mutants in which all three of these PG4 genes are deleted (Horan et al. 1995).

Although this is beyond the scope of this review, phenotypes in *Hox* mutants reveal coordinated defects in derivatives of the rhombomeres: the neurons and cranial neural crest, and in cranial ganglia. These defects underscore how early segmental organization and *Hox* expression impact later processes of craniofacial development and neural architecture, and indicate that *Hox* genes perform multiple roles in elaborating the segmental plan of head development.

Retinoic acid (RA): a vitamin A derivative that functions as a morphogen to instruct developmental pathways

THE INDUCTIVE PHASE OF *HOX* EXPRESSION IN THE HINDBRAIN

Retinoids and Initiation of *Hox* Expression

Evidence from in vitro and in vivo studies supports a role for retinoic acid (RA) in initiating early *Hox* gene expression and patterning the hindbrain (Gavalas 2002, Gavalas & Krumlauf 2000, Maden 2002). In several vertebrate model systems, adding RA during early embryogenesis results in an expansion of the posterior hindbrain at the expense of the anterior hindbrain. Conversely, reducing the amount of available RA, or inhibiting retinoid signaling, results in a posterior expansion of anterior hindbrain characteristics at the expense of the posterior hindbrain. These RA-dependent changes in the segmentation program of the hindbrain directly correlate with changes in *Hox* gene expression (Gavalas 2002).

Regulatory studies indicate that RA directly activates some *Hox* genes. The *Hoxa1*,

Fibroblast growth factor (Fgf): a large family of secreted ligands that sends signals to regulate growth, survival, migration, and patterning processes in development

Hoxb1, *Hoxa4*, *Hoxb4*, and *Hoxd4* genes all contain retinoic acid response elements (RAREs) that control aspects of their neural expression (Gould et al. 1998, Marshall et al. 1994, Packer et al. 1998, Studer et al. 1998, Zhang et al. 2000). Typically, these RAREs are bound by a heterodimer composed of a retinoid X receptor (RXR) and a RA receptor (RAR). In the absence of ligands, they recruit corepressors, but when RA is present, they undergo an allosteric change and recruit coactivators. The RAREs that are located downstream of the coding exons of *Hoxa1* and *Hoxb1* genes are required to initiate their expression up to the r3/r4 boundary in the hindbrain (Dupé et al. 1997, Studer et al. 1998) (**Figure 2*a***).

Several models have been proposed to account for the experimental evidence on the roles of RA in regulating hindbrain segmentation. However, by integrating information on the synthesis and degradation of RA, a common picture begins to emerge (Niederreither & Dollé 2008). Levels of RA vary along the A-P axis of the hindbrain. At the caudal end of the hindbrain, RA is present at its highest concentration, and the concentration progressively decreases in rostral directions. In the early hindbrain region, RA is initially generated by a metabolic enzyme, Retinaldehyde dehydrogenase 2 (Raldh2), expressed in the somites that flank the caudal hindbrain, which converts retinaldehyde into RA (Niederreither et al. 1997). RA from the somites diffuses into the neural tube to influence hindbrain patterning including the regulation of *Hox* expression (Niederreither & Dollé 2008). As development proceeds and more somites are formed, the source of RA synthesis regresses in a posterior direction. To counterbalance synthesis, members of the Cyp26 family degrade retinoids, and they are dynamically expressed in the anterior hindbrain during embryonic development, where they are required for normal hindbrain patterning (Abu-Abed et al. 2001, Sakai et al. 2001).

These findings suggest a model in which domains and boundaries of RA in the hindbrain shift over time in concert with changes in the source of RA and its degradation. The activation of *Hox* genes is also progressive, and there are distinct periods when a *Hox* gene is competent to respond to an inducing signal. Therefore, the relative levels of RA available and the windows of competence will determine if and when any given *Hox* gene is capable of being induced in the hindbrain (Hernandez et al. 2007, Sirbu et al. 2005).

Fgf Signaling and Initiation of *Hox* Expression

Fibroblast growth factor (Fgf) signaling is important in initiating *Hox* gene expression in the hindbrain. In the zebrafish, *Fgf3* and *Fgf8* are expressed before hindbrain segmentation and become localized to the prospective r4 territory (Maves et al. 2002, Walshe et al. 2002). Reducing the expression of *Fgf3* and *Fgf8* alters the expression of many key genes associated with segmentation, as evidenced by the loss of *Hoxa2* expression in r2-r5, *Krox20* expression in r5, and *valentino/kreisler* expression in r5. Hence, Fgf signaling participates in regulating the identity of r5 and r6 in the zebrafish hindbrain.

The patterns of expression of *Fgf* orthologs vary between species, suggesting that different members of the family may participate in inductive events. In the chick embryo, activating Fgf signaling in the presumptive r7-r8 territory induces *Krox20* and *kreisler* expression, whereas inhibition abolishes the expression of these same genes (Marin & Charnay 2000). This supports a conserved role for Fgf signaling in regulating the identity of the r5-r6 region through the activation of *Krox20* and *kreisler*. Hence, the induction of *Hox* genes by Fgfs may be indirect.

vhnf1 and Induction of *Hox* Genes

In the zebrafish, there is evidence that Fgf and RA signaling regulates the expression of *variant hepatocyte nuclear factor* (*vhnf1*) in the future r5 and r6 territories (Hernandez et al. 2004). *vhnf1* encodes a homeodomain transcription factor that is transiently expressed in the presumptive

r5 and r6 (Aragón et al. 2005). The loss of *vhnf1* results in abnormal gene expression in the r4-r6 region. The r4 expression domain of *Hoxb1* expands posteriorly, whereas *Krox20* expression is reduced in r5, and the expression of *kreisler* (*valentino*) is abolished in r5 and r6. Conversely, ectopic expression of *vhnf1* results in an anterior expansion of *kreisler* expression. These studies demonstrate that *vhnf1* functions to specify early r5/r6 identity by repressing early r4 genes (*Hoxb1*) and by activating r5 and r5/r6 specific genes, such as *Krox20* and *kreisler*, which in turn directly regulate rhombomere-specific expression of *Hox* genes (Sun & Hopkins 2001, Wiellette & Sive 2003) (**Figure 2**). In support of this model, analysis in the mouse has shown that vhnf1 binds to the *kreisler* gene and directly regulates expression in r5 and r6 (Kim et al. 2005). RA signaling directly regulates neural expression of the *vhnf1* gene through a RARE located in its fourth intron (Pouilhe et al. 2007). Furthermore, *vhnf1* expression in r5 and r6 is dependent on the presence of two binding sites for *kreisler*, suggesting that *kreisler* and *vhnf1* form a direct positive feedback loop to maintain expression in r5 and r6.

Iroquois Genes and Induction of *Hox* Genes

In zebrafish, *vhnf1* expression anterior to r5 is repressed by the product of an *Iroquois* (*Irx/Iro*) gene. The *Iroquois* (*Irx/Iro*) gene complex was first characterized in *Drosophila*, and it encodes homeodomain transcription factors of the three amino acid extension (TALE) subclass. The TALE superfamily also includes members of the *Pbx*, *Meis*, and *Prep* gene families, which function as cofactors for Hox activity (Burglin 1997). In *Drosophila*, members of Iro-C activate proneural genes, and their function seems to have been conserved in vertebrates. In zebrafish, both *iro1* and *iro7* genes are expressed in the hindbrain and the posterior expression of *iro7* corresponds to the future r4/r5 boundary (Lecaudey et al. 2004). Both iro7 and vhnf1 are involved in a repressive loop, whereby iro7 represses *vhnf1* expression in r4 and vhnf1 blocks *iro7* expression in r5 (**Figure 2*a***).

ESTABLISHMENT OF HOX EXPRESSION IN RHOMBOMERES

Before morphological segmentation of the hindbrain, the inductive events described above activate *Hoxa1*, *Hoxb1*, *kreisler*, *Krox20*, *vhnf1*, and *Irx* expression in the developing hindbrain with distinct domains that ultimately mark the future rhombomeric segments. As cells segregate, the borders of these expression domains sharpen and visible segments appear. *Hox* expression becomes further refined by direct regulation through upstream factors, such as Krox20 and kreisler, and through cross- and autoregulatory interactions between the *Hox* genes themselves. These inputs begin to define a gene regulatory network for establishing segmentally-restricted domains of *Hox* expression in the hindbrain.

Krox20 and Activation of *Hox* Genes

Krox20 is a zinc finger transcription factor that is expressed in prospective r3 and r5 of the hindbrain. In the absence of *Krox20*, r3 and r5 cells form initially, but they are lost at later stages (Schneider-Maunoury et al. 1997, Voiculescu et al. 2001). Fate mapping suggests that r3 and r5 either switch their adhesive properties or acquire the identity of an adjacent even-numbered segment, which leads them to intermingle with neighboring segments. The regulation of *Krox20* expression is complex and involves the input of the Wnt, RA, and Fgf signaling pathways. Three *cis*-regulatory modules contribute to control of *Krox20*, and these integrate inputs from Krox20 itself (autoregulation), vhnf1 in r5, and Hox/Pbx in r3 (Chomette et al. 2006, Wassef et al. 2008). In the zebrafish, both *Iro7* and *Meis1.1* control expression of *Krox20* in r3 (Stedman et al. 2009).

Krox20 exerts a role in segmental identity by directly activating the transcription of *Hoxa2*, *Hoxb2*, and *EphA4* in r3 and r5

(Maconochie et al. 1996, Nonchev et al. 1996). In combination with kreisler, Krox20 activates *Hoxb3* expression in r5 (Manzanares et al. 2002). Conversely, Krox20 represses the expression of *Hoxb1* in r3 and r5 by binding with PIASxβ, which is required for *Hoxb1* expression in r4 (Garcia-Dominguez et al. 2006). Hoxb1 or Hoxb2 may feedback through a Hox/Pbx motif in a regulatory module of *Krox20* to help initiate or maintain its expression. There is evidence that early expression of *Hoxa1* synergizes with *Krox20* to specify r3 identity (Helmbacher et al. 1998). These studies demonstrate a critical role for Krox20 in activating the *Hox* genes essential for regulating the identity of r3 and r5 (**Figure 2*b***).

kreisler (*kr*) and Activation of *Hox3* Genes

The *kreisler* (*kr*) mutation is an X-ray induced chromosomal inversion of a gene, which encodes for a basic domain-leucine zipper (bZIP) transcription factor of the Maf family that is expressed in r5 and r6 (Cordes & Barsh 1994). In *Kr* mutant mice, r5 and r6 fail to acquire a proper segmental identity, and adopt an r4 character instead (Giudicelli et al. 2003, Manzanares et al. 1999b, McKay et al. 1994). In zebrafish, *valentino*, the ortholog of *kreisler*, has a conserved role in regulating the identity of r5 and r6 (Moens et al. 1996, 1998). As described above, kreisler and vhnf1 are involved in a direct positive feedback loop in r5 and r6, triggered by RA and Fgf signaling. With respect to *Hox* genes, regulatory analyses have demonstrated that kreisler directly binds to *cis*-modules upstream of *Hoxa3* and *Hoxb3* to activate their expression in r5 and r6 (Manzanares et al. 1997, 1999a).

Auto- and Cross-regulation Among *Hox* Genes

Once segmental *Hox* gene expression has been initiated by the signaling pathways and upstream factors that function in early hindbrain patterning, auto- and crossregulation among the *Hox* genes play an important role in maintaining rhombomeric expression. In the developing hindbrain, Hox/Pbx-dependent autoregulatory elements (AREs) have been found in regulatory modules of *Hoxb1*, *Hoxa3*, *Hoxb3*, and *Hoxb4* genes. Transient RA signaling activates *Hoxb1* and *Hoxb4*, and they positively regulate their own expression in r4 and r7 by autoregulation (Gould et al. 1997, Pöpperl et al. 1995). The *Hoxa3* and *Hoxb3* genes are transiently activated by kreisler, and in turn autoregulation reinforces this expression in r5 (Manzanares et al. 2001) (**Figure 2*b***).

These AREs also function as *Hox*-response elements that are capable of mediating cross-regulatory inputs by other *Hox* genes. For example, Hoxa1 and Hoxb2 modulate r4-restricted expression of *Hoxb1* through interaction with its AREs. Similar cross-regulatory interactions are observed by Hox3 members on the *Hoxa3* ARE (Manzanares et al. 2001) and Hox4 members on the *Hoxb4* ARE (Gould et al. 1997, Serpente et al. 2005). Further evidence for cross-regulation in the hindbrain is illustrated by the pivotal role played by Hoxb1 in regulating r4 identity. *Hoxa2* and *Hoxb2* are also expressed in r4, and the regulatory basis of their expression is mediated through direct activation by Hoxb1 (Maconochie et al. 1997, Tümpel et al. 2007). This establishes a regulatory cascade in r4, in which RA induces *Hoxa1* and *Hoxb1*, which in turn stimulate *Hoxb1* via its ARE. Hoxb1 then activates *Hoxa2* and *Hoxb2* in r4, and they feed back into the *Hoxb1* ARE to reinforce expression at later stages in r4. Analyses of single and compound mutants for members of this network provide functional support for the relevance of these regulatory relationships (Davenne et al. 1999, Gavalas et al. 2003, Studer et al. 1998).

The Hindbrain Regulatory Network

The model that emerges for segmental regulation of *Hox* genes indicates that key signals and upstream factors have multiple inputs at many

levels (**Figure 2**). There is not a strict hierarchy of functions. *Krox20* can regulate segmental identity via modulation of *Hox* genes, but it can also contribute to cell sorting by regulation of *EphA4*. *Hox* genes themselves can regulate the formation of a segment (*Hoxa1* in r5), segmental identity, and cell sorting. Regulatory analyses reveal many examples of feedforward and feedback loops that reinforce or restrict expression in rhombomeres. This presents a challenge in characterizing detailed gene regulatory networks of hindbrain segmentation, complete with *Hox* target genes. However, multiple inputs that reinforce expression may help to explain why this is such a robust and conserved regulatory cascade in vertebrate development.

SEGMENTATION OF PARAXIAL MESODERM

Formation of the Paraxial Mesoderm

In this section, we briefly discuss aspects of paraxial mesoderm development that are relevant to understanding how global A-P patterning is established within this tissue. Cells that will contribute to the future paraxial mesoderm are located in the epiblast adjacent to and within the primitive streak. These cells will ingress primarily through the rostral primitive streak to form the definitive paraxial mesoderm (Kinder et al. 1999). There are two populations of paraxial mesoderm progenitors. One group of cells ingresses through the primitive streak from the adjacent epiblast to contribute to short stretches of the paraxial mesoderm that form the lateral somite. Another, stem cell-like population resides within the streak over time and generates descendants that give rise to the medial somite at all axial levels (Iimura et al. 2007, Psychoyos & Stern 1996, Wilson & Beddington 1996). This implies that there are mechanisms for synchronizing the axial identity between these two populations of somitic precursors.

The temporal coordination of these cell behaviors is tightly regulated. Recruitment of nascent paraxial mesodermal cells to the primitive streak depends on BMP signaling (Miura et al. 2006), whereas Fgf signaling is required for the migration of these cells away from the primitive streak (Ciruna et al. 1997, Sun et al. 1999). Fgfs are also required upstream of *Tbx6* for the specification of paraxial mesoderm identity (Chapman et al. 2003, Ciruna & Rossant 2001), and Wnt3a plays a role in regulating paraxial mesoderm versus neural cell fate decisions (Yoshikawa et al. 1997). It is tempting to speculate that many of these processes, directly or indirectly, impact A-P patterning of the paraxial mesoderm by Hox proteins. Consistent with this, mutant alleles of *Fgfr1* lead to vertebral homeotic defects correlated with changes in *Hox* expression (Partanen et al. 1998). Mutation of the murine *type IIB activin receptor* or *Wnt-3a* also disrupts A-P patterning associated with shifts in the anterior boundaries of *Hox* gene expression (Oh & Li 1997).

Following neuropore closure in the mouse at 10.5 dpc, ingression of mesodermal progenitors from the epiblast ceases (Wilson & Beddington 1996) and subsequently, cells of the paraxial mesoderm are generated by the tailbud (Cambray & Wilson 2007). Many of the same signaling molecules that are present during gastrulation continue to be expressed in the tailbud with a spatial arrangement analogous to that found in the primitive streak and node, although levels can vary with the maturation of the tailbud. Consequently, *Hox* genes and other regulators of A-P patterning are likely to be exposed to changing regulatory inputs as the formation of paraxial mesoderm proceeds during development.

Somitogenesis

The newly formed paraxial mesoderm begins to expand, and the population between the cells emerging from the primitive streak and the most newly formed somite is referred to as the presomitic mesoderm (PSM) (**Figure 4**). Bilateral pairs of somites that flank the neural tube form sequentially with anterior somites being

Epiblast: the primordial outer layer of the blastula that gives rise to the ectoderm and contains cells capable of forming the endoderm and mesoderm

Primitive streak: the thickened posterior area of the epiblast composed of cells that proliferate and migrate to form the mesoderm and help to establish the future longitudinal axis of the early embryo

Node: a thickening at the anterior end of the primitive streak which acts a signaling center

Mesenchyme: groups of loosely organized undifferentiated cells mostly derived from mesoderm, in contrast to epithelial cells which are tightly interconnected

Sclerotome: the mesenchymal compartment formed from the ventral somite after differentiation that will give rise to vertebrae

older than posterior somites. An oscillatory mechanism (a clock) coupled with a morphogen gradient (a wavefront) underlies both the periodicity of somite formation and the proper partitioning of paraxial mesoderm cells into somites of the appropriate size (Dequeant & Pourquié 2008).

The oscillator is characterized by cyclic transcriptional activity manifesting as waves of expression progressing through the PSM in a posterior to anterior direction. Components of both the Fgf and Notch signaling cascades have been shown to cycle in-phase with one another, whereas Wnt target genes have been shown to oscillate in the converse phase (Aulehla et al. 2003, Dequeant et al. 2006, Palmeirim et al. 1997). Overlaying this dynamic pattern of gene expression are P-A gradients of FGF and Wnt (Diez del Corral & Storey 2004, Dubrulle et al. 2001, Dubrulle & Pourquié 2004). High levels of Fgf signaling are believed to keep the posterior two-thirds of the PSM in an undetermined state with respect to the segmentation program. Along the P-A Fgf gradient, there is a threshold (the determination front) in which cells alter their response and begin the process of segmentation and somite formation (Dequeant & Pourquié 2008). RA signaling, mediated by *Raldh2*-dependent synthesis of RA in anterior somites, may antagonize Fgf signaling to help set the determination front during the initiation of somitogenesis (Diez del Corral & Storey 2004, Sirbu & Duester 2006). The determination front is maintained at a fixed relative position in the PSM over time as a result of a balance between the rates that cells are added and leave the PSM to form somites.

The past decade has seen remarkable progress in unraveling the molecular series of events underlying somitogenesis. As briefly outlined above, Wnt, Fgf, RA, and Notch are key players in the complex and dynamic processes that occur within the PSM. It is intriguing that genetic studies have implicated each of these pathways as upstream regulators of *Hox* gene expression within the paraxial mesoderm, coupling somitogenesis and the establishment of vertebral positional identity.

Differentiation of the Somites

The newly formed somite consists of a mesenchymal core surrounded by a block of epithelial cells. During development, somite differentiation proceeds progressively in an anterior to posterior direction. The somite undergoes a number of morphological changes in response to signals arising from the neighboring tissues such as the notochord, neural tube, ectoderm and lateral plate mesoderm (Christ et al. 2004) (**Figure 3*a***). Somites are polarized along their A-P, D-V, and mediolateral axes. The ventral half of the somite undergoes an epithelial-to-mesenchymal transition to form the sclerotome, which generates the vertebrae of the axial skeleton. The dorsal half of the somite retains its epithelial character and forms the dermomyotome, giving rise to the dorsal dermis and muscles of the back and limbs.

Although they respond in stereotyped ways to inductive cues, the sclerotomes differentiate into vertebrae that are clearly different from each other according to their position along the A-P axis. This is illustrated by the different anatomical types of vertebrae, i.e., cervical, upper thoracic, lower thoracic, lumbar, sacral, and caudal vertebrae. However, defining morphological characteristics can be seen between vertebrae within an anatomical unit (**Figure 3*b–d***) and along the entire vertebral column. Newly formed somites already possess the A-P information needed to generate their ultimate vertebral identity, because somites moved from one A-P level to another will generate a vertebral structure characteristic of its origin and not its new location (Fomenou et al. 2005, Nowicki & Burke 2000). This A-P information is thought to be governed by early differences in the developmental programs regulated by the *Hox* code.

Hox Expression in Nascent and Presomitic Mesoderm

Hox genes are expressed in nested domains along the A-P axis of paraxial mesoderm throughout the progressive process that leads to the generation of the vertebral column. *Hox*

expression patterns are initiated in a temporally colinear manner within the caudal epiblast and primitive streak cells (Deschamps & Wijgerde 1993, Gaunt & Strachan 1996, Iimura & Pourquié 2006). Once initiated, the expression of any given gene then spreads towards the mid-to-anterior primitive streak, where the paraxial mesoderm progenitors reside (**Figure 4*a*,*b***). A subset of expression then expands beyond the node into the posterior neural plate. This pattern begins with the PG 1 *Hox* genes, and it is reiterated for each successive *Hox* gene along the *Hox* clusters in a progression from the 3′ end of each complex to the 5′ end. The temporal window that separates the first appearance of expression of PG1 to PG9 in the caudal primitive streak is very narrow due to the rapid elaboration of this region of the embryo. However, there is a clear, colinear temporal order in the onset of *Hox* expression at and anterior to the node (**Figure 4**). The *Hox* code is not passively carried through the primitive streak, however, because the Hox proteins themselves play a role in timing the ingression of nascent paraxial mesodermal cells (Iimura & Pourquié 2006). By regulating the sequential ingression of cells into the paraxial mesoderm, temporal colinearity becomes translated into spatial colinearity.

Hox expression boundaries are not fully determined at the time of gastrulation. Expression boundaries continue to be subject to regulatory influences as cells move through the PSM and become incorporated in somites and also throughout later stages in somite development. Whereas the temporally colinear onset of *Hox* gene expression in the anterior primitive streak presages later spatial colinearity within

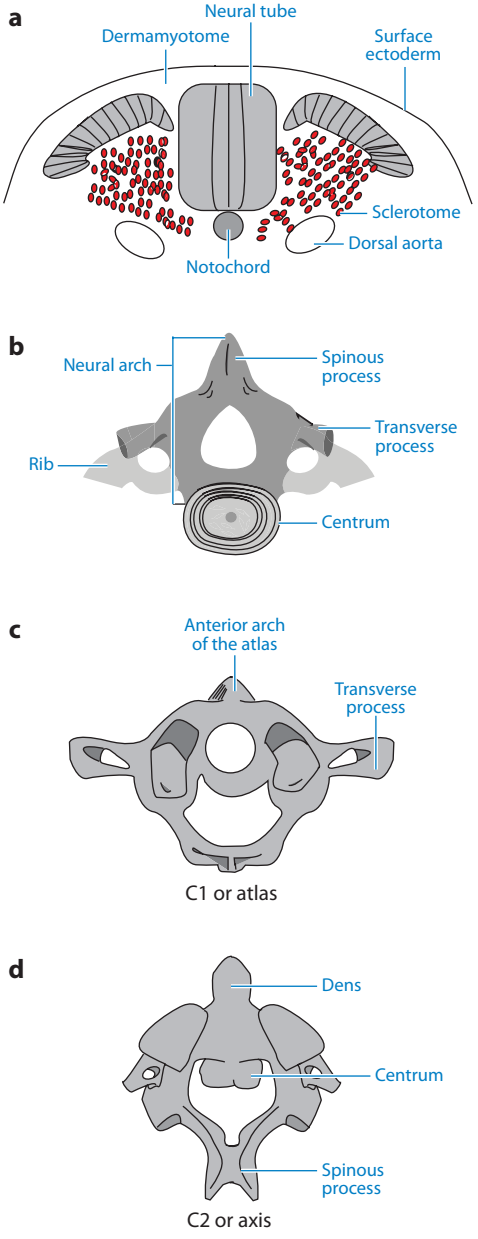

Figure 3

Somite differentiation and vertebral morphology. (*a*) The somite differentiates into an epithelial dermamyotome dorsally and a mesenchymal sclerotome (*red*) ventrally in response to inductive signals from neighboring structures such as the surface ectoderm, neural tube, notochord, and dorsal aorta. The sclerotome will give rise to the vertebrae. (*b*) Represented is a prototypical thoracic vertebra demonstrating characteristic vertebral features such as the centrum, neural arch, and spinous and transverse processes. Variations in the morphology of the neural arch and processes between many of the vertebrae make the vertebral column an ideal system for assessing the role of *Hox* genes in assigning segmental identity. For example, clear differences in the morphology of (*c*) the atlas and (*d*) the axis provide visual landmarks for determining the presence of partial or complete homeotic transformations of these vertebrae.

the somites, the anterior boundaries of *Hox* gene expression are not solely dependent on lineage transmission. Lineage tracing studies have shown that the expression of most *Hox* genes in the anterior primitive streak is initiated in cells that will form somites anterior to the definitive expression boundary of the respective gene (Forlani et al. 2003). This suggests that the anterior boundaries of *Hox* expression in the paraxial mesoderm are established by activation in a broad domain that becomes refined through repressive mechanisms that occur in the PSM and/or in the somites. There is evidence that Fgf signaling plays a role in refining the early *Hox* expression boundaries that are associated with specific somites (Dubrulle et al. 2001).

Transplantation of the caudal PSM to more anterior levels has shown that axial identity has already been established (Fomenou et al. 2005). Because the caudal PSM is composed of cells that have traversed the primitive streak and become committed to the paraxial mesoderm most recently, this places the establishment of axial identity early in the formation of the paraxial mesoderm. Hence, the later modulation of *Hox* expression in the PSM and somites may only be important for the refinement of the spatial pattern that is generated during the process of gastrulation, perhaps to coordinate *Hox* boundaries with precise somites.

Hox Expression and the Segmentation Clock

A number of experiments suggest that the clock and wavefront mechanism that drives the periodic segmentation of PSM tissue into somites also regulates *Hox* gene expression. The expression of at least four genes is temporally dynamic within the rostral PSM with a periodicity that corresponds to that of the known cyclic genes (Zakany et al. 2001). It remains possible that other *Hox* genes also cycle in the PSM. Functional support for this link comes from evidence that the expression of at least two *Hox* genes is dramatically reduced, if not absent, specifically in the paraxial mesoderm of mice mutant for the Notch effector RBPJk (Zakany et al. 2001).

Hox Expression in the Somites

Following activation, most *Hox* genes are dynamically expressed in the somites and their derivatives (pre-vertebrae). The main exceptions are the PG1 *Hox* genes, whose expression extends into the unsegmented paraxial mesoderm that is anterior of the first somite and then is rapidly lost. During somite development and differentiation, the domains of *Hox* expression that are established at earlier stages can shift in anterior and posterior directions. There can also be sharp posterior and anterior boundaries. The pattern of these changes varies considerably from gene to gene, suggesting additional regulatory inputs are selectively altering the initial colinear domains of nested *Hox* expression. This might be part of the process of redeploying *Hox* genes for later functions in somite development, in addition to their early role in regulating segmental identity.

The regulatory basis and relevance of these later patterns of *Hox* expression have not been examined in great detail. However, the targeted deletion of enhancers required for precise temporal activation of *Hoxc8*, *Hoxd10*, and *Hoxd11* in paraxial mesoderm shows that correct early *Hox* gene activation is critical for the control of vertebral identity and that later domains of somite expression are not dependent on the early regulatory regions (Juan & Ruddle 2003, Zakany et al. 1997). Hence, *Hox* expression patterns in paraxial mesoderm, from induction to formation of the axial skeleton, are refined by multiple regulatory inputs within the PSM, somites, and surrounding tissues following their initial activation during gastrulation (**Figure 4*c***). This is probably mediated by independent *cis*-regulatory modules that direct the dynamic patterns of *Hox* expression in paraxial mesoderm and its derivatives.

Function of *Hox* Genes in Somitic Identity

Analyses on patterning of the axial skeleton have provided some of the strongest evidence in vertebrates that *Hox* genes exert their functions

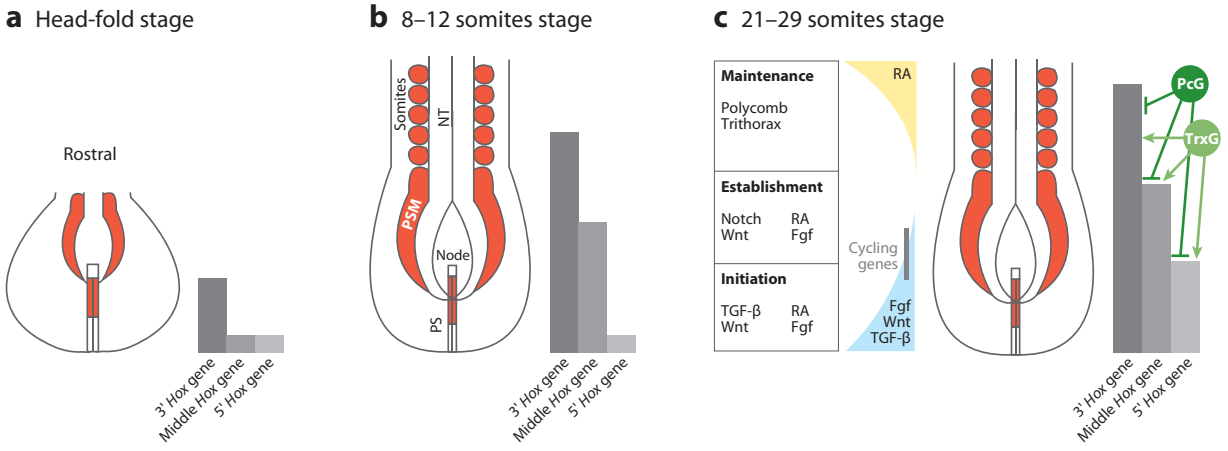

Figure 4

Temporal and spatial colinearity of *Hox* expression in the developing paraxial mesoderm. Depicted are schematics of the posterior region of the developing mouse embryo. The nascent and definitive paraxial mesoderm is shown in (*red*). (*a*) The colinear appearance of *Hox* expression in the posterior-most embryo occurs in rapid succession for most *Hox* genes during the head-fold stage. (*b*) The onset of *Hox* expression in the nascent paraxial mesoderm during the expansion phase displays clear temporal colinearity as seen in the progressively more 5′ *Hox* genes expressed in this region over time. Thus, temporal colinearity during this phase directly contributes to spatial colinearity within the developing paraxial mesoderm. (*c*) *Hox* expression in the paraxial mesoderm is characterized by multiple phases that correspond to the initiation of expression in progenitor cells in the primitive streak, the establishment of defined A-P boundaries in developing somites and the maintenance of these boundaries. Disruption of Wnt, TGF-β, Fgf, and RA signaling leads to the disruption of axial identity and corresponding changes in *Hox* gene expression. Similarly, disruption of the function of PcG and TrxG proteins leads to changes in the maintenance phase of *Hox* gene expression. NT, neural tube; PS, primitive streak; PSM, presomitic mesoderm.

as selector genes by regulating regional identity. The distinct features of individual structures along the A-P axis of the vertebral column have facilitated phenotypic analyses in *Hox* loss- and gain-of-function mutations in mice. The defects include malformed vertebrae, vertebral fusions, rib fusions, and vertebral homeotic transformations. Homeotic transformations in the context of the vertebral column describe a class of phenotypes in which a vertebra acquires the characteristics of its immediate anterior or posterior neighbor, whereas the total number of vertebrae remains constant. A number of patterns have emerged from these studies that clarify the function of *Hox* genes in global patterning of the paraxial mesoderm.

Strengthening the argument that *Hox* genes function in the PSM to assign axial identity to the somites, it has been demonstrated that overexpression of *Hoxa10* in the PSM and newly formed somites results in homeotic transformations within the vertebral column (Carapuco et al. 2005). Overexpression of this gene specifically within the somites leads to vertebral dysmorphogenesis rather than homeotic transformations, illustrating later roles for the Hox proteins as well. Deletion of an enhancer responsible for the early activation of *Hoxc8* delays the anterior expansion from 8 dpc until 8.5 dpc and is sufficient to induce homeotic transformations in the cervical and upper thoracic regions similar to a null allele of the gene (Juan & Ruddle 2003). This further supports the idea that the global patterning of somites occurs prior to somite formation.

Based on loss-of-function mutations, most members of the Hox PG 3–13 play roles in specifying the identity of the postcranial axial skeleton. Vertebral homeotic transformations caused by *Hox* gene mutations do not always involve the transformation of an entire vertebra to that of another along the rostrocaudal axis but instead may lead only to the transformation of specific vertebral features (Horan et al. 1995).

In other cases, the mutation of a *Hox* gene leads to more complete homeotic transformations of one or many vertebrae. Although there is a general trend for the phenotypes resulting from single *Hox* gene mutations to reflect the spatial colinearity of *Hox* gene expression, there are numerous exceptions (**Figure 5*a***). In contrast, a comparative study of mice with mutations in an entire PG demonstrated colinearity of Hox function along the vertebral column and evidence for functional compensation between groups (**Figure 5*b***) (McIntyre et al. 2007). However, there is also evidence that PGs perform distinct roles in vertebral patterning, even when the same vertebrae are affected.

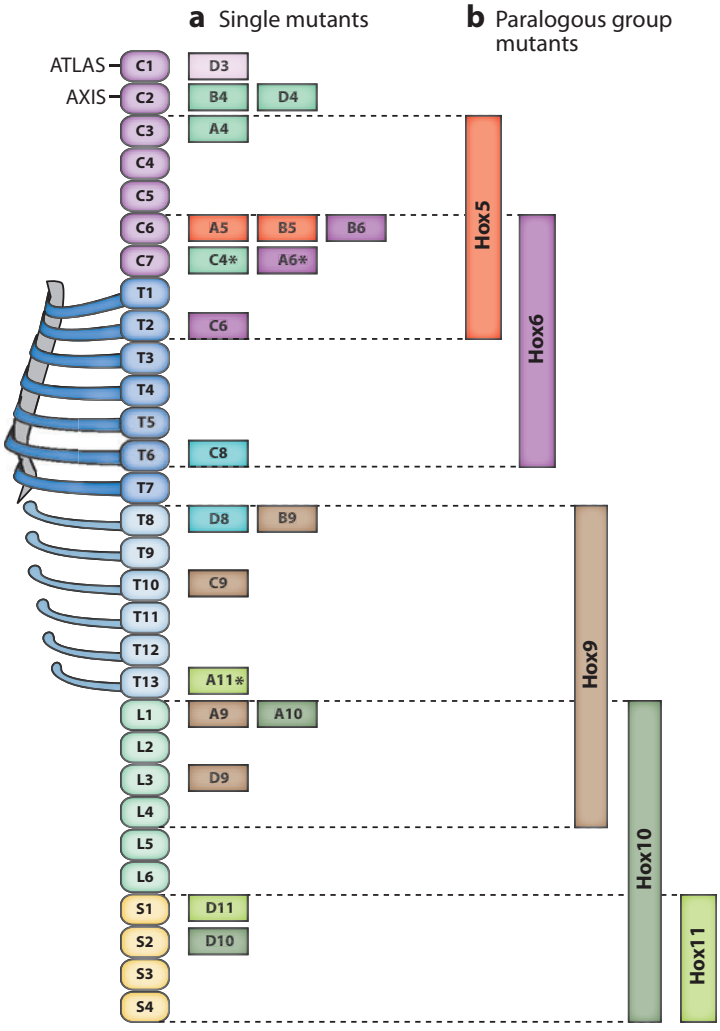

Figure 5

Patterns of homeotic transformations in *Hox* mutant mice. (*a*) The most anterior vertebra that shows either a partial or complete homeotic transformation as the result of a given *Hox* gene mutation is indicated. Members of the same PG are color-coded equivalently. (*b*) The range of phenotypes in mice with mutations in entire PGs is depicted. Two emergent patterns are the reflection of spatial colinearity in the order of *Hox* phenotypes along the A-P axis and the functional overlap, in some cases, of different *Hox* genes in patterning the same vertebrae. Asterisks indicate the few examples of posterior homeotic transformations that are observed in some single *Hox* gene mutants.

Based on the phenomenon of phenotypic suppression, which was first characterized in *Drosophila*, a posterior prevalence model has been postulated to account for how the function of posterior Hox proteins overrides the function of coexpressed anterior Hox proteins (Gonzalez-Reyes & Morata 1990). According to this model, segmental identity is imparted by the most 5′ Hox PG that is expressed at a given axial level (Duboule & Morata 1994). In many cases, loss-of-function *Hox* alleles lead to defects in much broader domains than expected from a strict interpretation of the posterior prevalence model, i.e., the defects extend into regions where more 5′ *Hox* genes are expressed. There is also evidence that levels of expression can influence function in more posterior territories. Therefore, although this accounts for many of the observed *Hox* mutant phenotypes in the axial skeleton, there are numerous exceptions to the posterior prevalence-based models.

The combinatorial model posits that a somite acquires its segmental identity from the specific complement of *Hox* genes it expresses (Kessel & Gruss 1991). A corollary of this model is that distinct Hox proteins have unique functions. However, many studies have shown that *Hox* genes within the same PG and between different PG may be functionally equivalent (Zhao & Potter 2001). For instance, the coding sequences of *Hoxa3* and *Hoxd3* were shown to be functionally interchangeable (Greer et al. 2000). By comparing compound mutants, there is further evidence that dosages or levels of gene expression play an important role in determining which genes may share functions in patterning a region. Despite the difficulties in

generating a unified model to account for function, these models provide important clues into the nature and readout of the vertebral *Hox* code and, cumulatively, illustrate that multiple mechanisms are likely to be employed in a context-dependent manner.

ACTIVATION OF HOX EXPRESSION IN THE FUTURE PARAXIAL MESODERM

Despite the wealth of information on the functional roles of Hox proteins in regulating segmental identity in the paraxial mesoderm compared with hindbrain, relatively little is known about the signaling pathways, upstream transcription factors, and *cis*-regulatory modules that directly control *Hox* expression in this context. Such regulatory information is challenging to obtain because of the progressive nature of the segmentation process itself. The timing of events, from the emergence of cells from the primitive streak to somite formation, is relatively rapid, and they are associated with extensive cell migration, tissue movements, cell proliferation, and oscillating signals. In the absence of a series of well characterized *cis*-regulatory modules that mediate the diverse aspects of initiation and maintenance of dynamic *Hox* expression in paraxial mesoderm, regulatory relationships have been inferred largely by assaying for changes in *Hox* expression following experimental and genetic perturbations to the system.

Retinoids in *Hox* Gene Regulation

RA plays a key role in vertebral patterning, in part, via regulation of *Hox* expression. Exogenous RA treatment or disruption of RA activity leads to distinct homeotic transformations and to alterations in *Hox* expression (Kessel & Gruss 1991, Niederreither & Dollé 2008). Studies that detail the patterns of RA activity in the primitive streak and paraxial mesoderm are beginning to clarify when and where RA may act (Molotkova et al. 2005, Sirbu & Duester 2006). Based on the expression of RA-synthesizing enzymes (*Raldh2* and *Cyp1b1*) and RA-responsive reporter gene activity, RA is present during the time that precedes and coincides with the onset of expression of most *Hox* genes in the posterior primitive streak and epiblast. However, RA activity regresses anteriorly into the PSM by the time that expression of all but the most 3′ *Hox* genes are successively activated in anterior regions of the primitive streak (Niederreither et al. 1999, Sirbu & Duester 2006).

These findings imply that RA exerts different regulatory inputs into *Hox* activation in the primitive streak in a stage-dependent manner. RA may differentially act on 3′ versus 5′ *Hox* genes in mesoderm similar to the regulation of *Hox* expression by RA in the chick neural tube (Bel-Vialar et al. 2002). RA may be involved in the initial induction of almost all of the *Hox* genes in the posterior primitive streak, whereas other factors modulate subsets of *Hox* genes in the more anterior territories. In support of complex inputs by RA, ectopic treatment of embryos with RA can alter vertebral patterning in distinct early and late time periods, but there is a refractory period during which no defects are observed. Furthermore, RA plays a role in maintaining the bilateral symmetry of somite formation.

TGF-β Family Members in *Hox* Gene Regulation

BMP signaling also contributes to *Hox* gene expression (McPherron et al. 1999, Oh & Li 1997, Oh et al. 2002). Homeotic transformations are observed in *type IIB activin receptor* mutants, and the mutation of *Gdf11* (*Bmp11*) leads to widespread anterior homeotic transformations throughout the axial skeleton, extending from the cervical through lumbar vertebrae (McPherron et al. 1999). The mutation of *Gdf11* results in complex shifts in boundaries of *Hoxc6*, *Hoxc8*, *Hoxc10*, and *Hoxc11* expression. The mesodermal component of *Gdf11* expression is localized to the primitive streak during gastrulation and to the tailbud during secondary body development (McPherron et al. 1999). Thus, *Gdf11* expression includes domains that contain the progenitors of the paraxial

mesoderm and newly ingressed presomitic cells and supports models in which *Hox*-determined segmental identity is set up early in the genesis of the somites.

Wnts in *Hox* Gene Regulation

In embryonic stem cell models, Wnt signaling is required for the development of mesoderm, and BMP and Wnt specify mesodermal fates by the activation of *Hox*- and *Cdx*-dependent pathways (Lengerke et al. 2008, Lindsley et al. 2006). In an analysis of 12 Wnt family members, only *Wnt-3a* was found to be expressed in domains of the primitive streak fated to contribute to the paraxial and lateral plate mesoderm (Takada et al. 1994). Two Wnt mutants have been analyzed for skeletal mutations: *vestigial tail* (*Wnt-3a*$^{vt/vt}$), which is a *Wnt* hypomorph, and the loss-of-function mutant, *Wnt-3a*$^{neo/neo}$ (Ikeya & Takada 2001). In the *Wnt-3a*$^{vt/vt}$ mutants, posterior transformations are observed from the midthoracic to lumbar regions, and anterior homeotic transformations are observed in the sacral regions and in C2. The *Wnt-3a*$^{neo/neo}$ mutants also display a partial C2 to C1 transformation. These mice do not form somites caudal to somite 9, and therefore effects on more posterior vertebrae could not be scored (Takada et al. 1994). Both mutants display shifts or loss of *Hoxd3* and *Hoxb4* expression. Thus, Wnt-3a signaling appears to differentially regulate distinct subsets of *Hox* genes.

Cdx Transcription Factors in *Hox* Gene Regulation

There are three murine *Cdx* family members, *Cdx1*, *Cdx2*, and *Cdx4*, which display discrete and overlapping spatial and temporal expression patterns (Beck et al. 1995, Gamer & Wright 1993, Meyer & Gruss 1993). In the mesoderm, expression of *Cdx2* and *Cdx4* only extends as far rostrally as the PSM, whereas the expression of *Cdx1* extends into the somites. *Cdx1* expression begins at 7.5 dpc in the primitive streak, which coincides with the initiation of expression of the 3′ *Hox* genes. At 8.5 dpc, Cdx protein is present in all of the somites, but as more somites begin to form and differentiate, levels begin to regress posteriorly. A single mutation of these genes reflects their staggered expression pattern in that *Cdx1* results in anterior homeotic transformations in the upper cervical through thoracic regions, whereas *Cdx2* affects the axial identities that begin with lower cervical vertebrae and extend through thoracic vertebrae (Chawengsaksophak et al. 1997, Subramanian et al. 1995, van den Akker et al. 2002). However, compound mutants have uncovered roles for *Cdx2* in the upper cervical region, and functions for all three genes extended as far caudally as the lumbosacral transition. The anterior homeotic transformations in *Cdx* mutant mice corresponded to posterior shifts in the rostral expression domain of each of the *Hox* genes analyzed, which indicates that they are Cdx targets. There is evidence that Cdx proteins act as direct regulators of *Hox* gene expression. Consistent with this idea, the *cis*-regulatory regions of *Hoxa7*, *Hoxb8*, and *Hoxc8* have been found to contain Cdx binding motifs that are important for regulatory activity (Charité et al. 1998, Subramanian et al. 1995, Taylor et al. 1997).

The Fgf, RA, and Wnt signaling pathways converge upon regulation of the *Cdx* genes (Allan et al. 2001; Bel-Vialar et al. 2002; Houle et al. 2000, 2003; Pilon et al. 2007). RA and Wnt response elements have also been identified in the regulatory regions of *Cdx* genes. In the *Wnt3a* hypomorph mutant *vestigial tail* (*vt*), the caudal domain of *Cdx1* expression was reduced, whereas *Cdx2* and *Cdx4* expression was unaffected. Hence, morphogen gradients active within the PSM and somites might be translated into a gradient of Cdx transcription factor activity that regulates *Hox* genes.

THE MAINTENANCE OF *HOX* GENE EXPRESSION PATTERNS

There are a variety of mechanisms that could be employed to ensure the proper propagation of early patterns of *Hox* expression in paraxial mesoderm through later development and into adulthood. In the hindbrain, auto- and

cross-regulatory interactions between the *Hox* genes are critical for maintaining segmental expression in rhombomeres. Epigenetic mechanisms are also fundamentally important for ensuring the continuation of appropriate expression. In *Drosophila*, the maintenance of *Hox* expression is regulated by members of the Polycomb (PcG) and trithorax (TrxG) groups of proteins, and these proteins play key roles in regulating *Hox* expression in paraxial mesoderm. The canonical model is that PcG proteins are involved in perpetuating the appropriate repressed state of a *Hox* gene, whereas the TrxG proteins are essential for maintaining active *Hox* expression domains. In mouse mutants, both TrxG-mediated activation and PcG-mediated repression are established between 8.5 and 9.5 dpc. Thus, they are generally not involved in the initial development of *Hox* expression domains in mesoderm (Akasaka et al. 2001, Yu et al. 1998). However, recent evidence suggests that the PcG proteins may play roles within regions in which *Hox* genes are actively expressed and also during the earliest phases of *Hox* gene expression (Boyer et al. 2006). By modulating factors and pathways in early development that contribute to the control of *Hox* gene expression, PcG and TrxG proteins might indirectly regulate early *Hox* expression in paraxial mesoderm. Regardless of these early roles, PcG and TrxG protein complexes are major regulators that control vertebral identity in later stages of development.

CONCLUSION

Hox proteins are able to transform uniform segments into remarkably elaborate structures. We have seen tremendous progress in clarifying how segment-specific Hox activity is set up in the rhombomeres. Although many of the same upstream activators of the hindbrain *Hox* code function in the paraxial mesoderm, it has been more difficult to resolve a coherent upstream regulatory network in this context. This is due, in part, to the complexity and rapidity of development in the posterior embryo as the *Hox* genes are first beginning to be expressed. In the hindbrain, *Hox* expression and function are intimately tied to the formation of the rhombomeres. In contrast, Hox proteins establish segmental identity within the paraxial mesoderm prior to the formation of overt segments, although early patterning must later be coordinated with specific somites. Thus, Hox patterning of the rhombomeres and somites shares fundamental features, such as colinearity and a combinatorial *Hox* code, and possesses unique attributes that speak to the versatility of Hox-based patterning systems.

SUMMARY POINTS

1. The Hox family of transcription factors is expressed in an ordered pattern in segments of the hindbrain and paraxial mesoderm, which forms a molecular code for regulating regional diversity from similar repeated units.
2. Misexpression or mutation of *Hox* genes results in homeotic transformation, the conversion of one structure into another.
3. The hindbrain is formed by dividing a region of the neural tube into seven segmental compartments (rhombomeres) that control major aspects of the formation and functional organization of neuronal, bone and connective tissue elements in head development.
4. Compartmentalization of the vertebral column occurs by the periodic addition of a bilateral pair of somites to the posterior end of the elongating A-P body axis, which becomes the foundation for the segmental organization of many features of the trunk such as the vertebrae, nerves, and muscles.

5. Early expression of *Hox* genes is initiated by the retinoic acid (RA) and fibroblast growth factor (Fgf) signaling pathways. Subsequent expression is modulated by auto- and cross-regulatory interactions among the *Hox* genes themselves. Epigenetic programs are then locked in by members of the Polycomb and trithorax groups.

FUTURE ISSUES

1. Identify regulatory elements that mediate the basis of the diverse phases of *Hox* expression in paraxial mesoderm and somites.
2. Distinguish between direct and indirect inputs of signaling events and epigenetic mechanisms in the control of *Hox* expression.
3. Further characterize how early segmental organization is translated into a full elaboration of the body plan and understand the later functional roles for Hox proteins in these processes.
4. Although segmentation of the hindbrain and paraxial mesoderm is a highly conserved process in vertebrates, we need to understand how this may vary between species.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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