

# Hox Genes and Limb Musculoskeletal Development

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**Abstract** In the musculoskeletal system, muscle, tendon, and bone tissues develop in a spatially and temporally coordinated manner, and integrate into a cohesive functional unit by forming specific connections unique to each region of the musculoskeletal system. The mechanisms of these patterning and integration events are an area of great interest in musculoskeletal biology. *Hox* genes are a family of important developmental regulators and play critical roles in skeletal patterning throughout the axial and appendicular skeleton. Unexpectedly, *Hox* genes are not expressed in the differentiated cartilage or other skeletal cells, but rather are highly expressed in the tightly associated stromal connective tissues as well as regionally expressed in tendons and muscle connective tissue. Recent work has revealed a previously unappreciated role for *Hox* in patterning all the musculoskeletal tissues of the limb. These observations suggest that integration of the musculoskeletal system is regulated, at least in part, by *Hox* function in the stromal connective tissue. This review will outline our current understanding of *Hox* function in patterning and integrating the musculoskeletal tissues.

**Keywords** Hox genes · Musculoskeletal tissue integration · Musculoskeletal development · Limb patterning · Connective tissue · Development and patterning

## Introduction

How tissues are patterned and then integrated with one another during development is an important and highly complex

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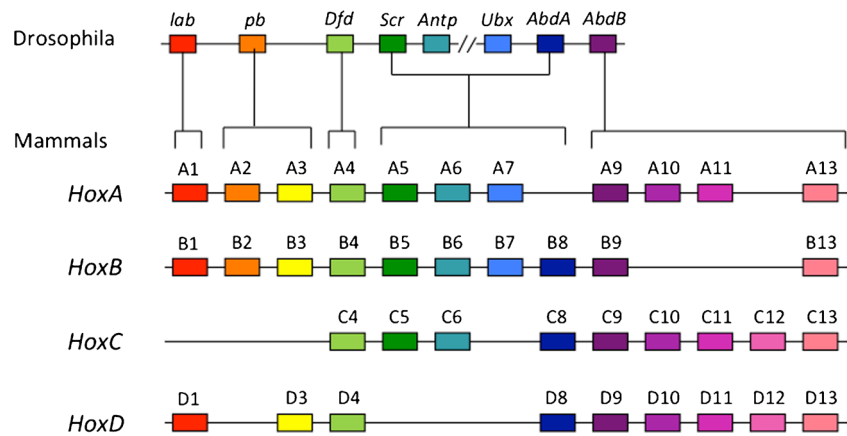
question in developmental biology. The interaction between cells of different embryonic origins and between different tissues within the organism is essential for proper development and function. The musculoskeletal system is an example of a highly complex set of structures that integrate cell types from several distinct embryonic origins. The musculoskeletal system is composed of three basic components: muscle, tendon, and bone, which undergo substantial changes to achieve their final form. One family of highly conserved developmental regulators that have been shown to be important for the integration and patterning of the musculoskeletal tissues is the *Hox* genes. This review will provide an overview of the role of *Hox* genes in musculoskeletal development with an emphasis on how *Hox* regulates musculoskeletal development and patterning in the limb.

## Hox Function and Skeletal Patterning

*Hox* genes are a family of highly conserved homeodomain-containing transcription factors that were first described in the fruit fly, *Drosophila*. These genes instruct what has been referred to as positional identity along the anterior to posterior (AP) body axis. The collinear arrangement of these genes along the chromosome reflects their spatial and temporal expression within the organism (Fig. 1) [1–6].

The linked *Hox* cluster underwent duplications during vertebrate evolution. This resulted in a total of 39 *Hox* genes in all mammalian species, arranged on four chromosomal clusters (Fig. 1) [2, 7]. The collinear arrangement of the *Hox* genes has been maintained from flies to mammals. The genes in each cluster are further subdivided into 13 paralogous groups based on sequence similarity and position within the cluster [8]. Members of each paralogous group share similar expression domains, and significant functional redundancy has been retained among paralogs. In many cases, assessing *Hox* gene function has required combining loss-of-function mutations in multiple members within the paralogous groups [6, 9–23].

**Fig. 1** Schematic of *Drosophila* and mammalian *Hox* genes clusters. Colored boxes represent linear arrangement (3'–5') along the chromosome. Color-coding depicts orthologous relationships between *Drosophila* and mammalian *Hox* genes, and paralogous relationships within the mammalian *Hox* clusters



Along the AP axis of the body, *Hox* genes are expressed in collinear, but overlapping domains within the somites and input from multiple *Hox* paralogous groups are responsible for establishing correct positional identity of the vertebrae (reviewed in detail [24, 25]). This combinatorial code of *Hox* expression along the AP axis results in vertebrae morphology being determined by patterning information from multiple *Hox* genes, usually two or more paralogous groups. When loss of an entire paralogous group occurs, the region that normally receives this input is patterned by the remaining *Hox* genes in the region. In the majority of cases this leads to anterior homeotic transformations in which the vertebrae assume a more anterior morphology [6, 13–15].

In addition to the role of *Hox* genes in patterning the axial skeleton, the posterior most *Hox* paralogs (*Hox9-13*) pattern the limb skeleton along the proximodistal (PD) axis. The vertebrate limb can be divided into three segments: the proximal stylopod (humerus/femur), the medial zeugopod (radius and ulna/tibia and fibula), and the distal autopod (hand/foot bones). In contrast to the anterior homeotic transformations observed in the axial skeleton, loss of *Hox* paralogous groups in the limb results in a complete loss of patterning information within a specific limb segment. For example, loss of *Hox10* paralogous genes results in severe stylopod mispatterning, loss of *Hox11* paralogous genes results in severe zeugopod mispatterning, and loss of *Hox13* paralogous genes results in a complete loss of autopod skeletal elements [6, 10–12, 26]. The difference between axial and limb patterning is because of the nonoverlapping function of *Hox* paralogous groups within the limb. The posterior *HoxA* and *HoxD* clusters are expressed in both the forelimb and the hindlimb, while the *HoxC* cluster is only expressed in the hindlimb [6, 10, 27]. With loss of function for all posterior *HoxA* and *HoxD* genes in the limb, there are severely truncated skeletal elements [28]. Additionally, posterior *Shh* expression is not initiated or maintained demonstrating a combined requirement for *Hox* function in early limb AP patterning as well as PD patterning [28].

Roles for two other paralogous groups, *Hox5* and *Hox9*, have recently been described in patterning the AP axis of the

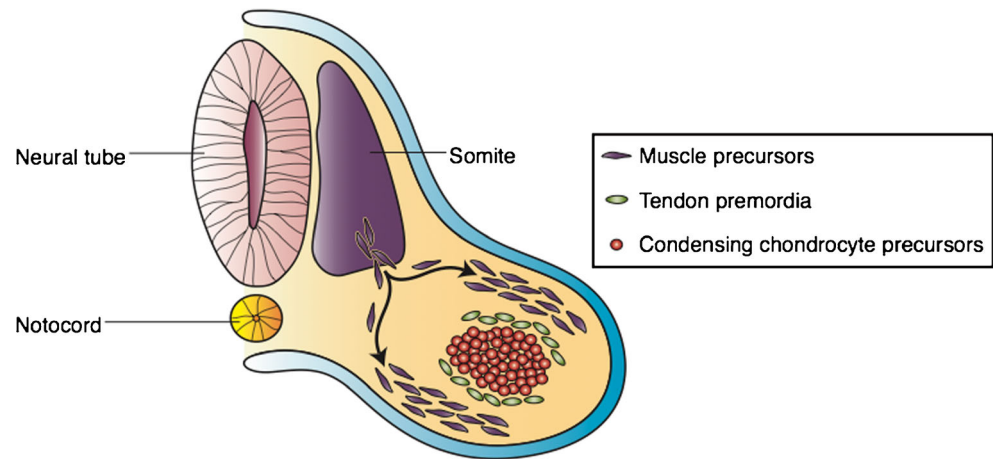
forelimb. With complete loss of *Hox9* genes (*Hoxa9*  $-/-$ ; *Hoxb9*  $-/-$ , *Hoxc9*  $-/-$ , *Hoxd9*  $-/-$ ), *Shh* expression is not initiated, disrupting AP patterning within the developing limb bud [29]. *Hox9* promotes posterior *Hand2* expression resulting in the inhibition of the hedgehog pathway inhibitor *Gli3*, allowing for induction of *Shh* expression in the posterior limb bud. *Gli3* expression is not repressed when *Hand2* is lost in the posterior limb bud. The overexpression of *Gli3* results in a complete loss of *Shh* expression and no AP axis is established. This results in only one skeletal element in each limb segment, identical to the phenotype resulting from loss of *Shh* function [29]. Loss-of-function mutations in the *Hox5* paralogous group (*Hoxa5*  $-/-$ , *Hoxb5*  $-/-$ , *Hoxc5*  $-/-$ ) results in loss of repression of anterior *Shh* expression in the limb bud leading to anterior patterning defects in the limb [30]. In this case, *Hox5* has been reported to interact with *Plzf* and functions to restrict *Shh* to the posterior limb bud [30].

### Vertebrate Limb Musculoskeletal Development

Musculoskeletal development is a complex process whereby bone, tendon, and muscle tissues must be appropriately patterned and precisely connected for physiologically relevant movement. An excellent model for studying musculoskeletal system development is the vertebrate limb. During limb development, bone, tendon, and muscle precursors differentiate and are coordinately patterned and integrated into a functional unit. There is significant knowledge on the developmental regulation of bone, tendon, and muscle tissue individually (reviewed in detail [31–33]) however, our understanding on how these tissues integrate into a cohesive unit is less clear. Work in recent years has begun to tease apart the mechanisms behind these coordinated patterning events, and it is clear that there is substantial communication between these tissues as limb pattern is established.

The limb musculoskeletal system is composed of mesodermal tissue that is derived from two distinct embryonic compartments (Fig. 2). The lateral plate mesoderm gives rise to the limb bud itself and the cartilage and tendon precursors arise

**Fig. 2** The limb musculoskeletal tissues are derived from two distinct mesodermal compartments. Tendon (*green*) and chondrocyte (*red*) precursors are derived from the lateral plate mesoderm within the limb bud, and tendon primordia arise adjacent to the centrally localized chondrocyte condensations. Muscle precursors (*purple*) delaminate from the ventral somite adjacent to the limb and migrate into the limb bud as dorsal and ventral muscle masses



within the lateral plate mesoderm of the limb bud [34, 35]. In contrast, muscle precursors differentiate from the dermomyotomal compartment of the axial somites adjacent to the limb field and migrate into the limb bud to form the limb musculature [36–39].

In the mouse, the forelimb bud emerges at embryonic day 9 (E9) from the lateral plate mesoderm on each side of the embryo. The skeletal pattern of the limb arises as *Sox9*-positive cartilage precursors condense centrally within the limb bud mesenchyme from proximal (stylopod) to distal (autopod) beginning around E11.5 [40]. Concurrently, muscle precursor cells delaminate from the ventrolateral dermomyotome of the somites adjacent to the limb bud and migrate into the limb as dorsal and ventral masses, where they continue to proliferate, aggregate, and differentiate into muscle tissue [36]. Finally, tendon primordia arise in the dorsal and ventral limb mesenchyme directly from the lateral plate mesoderm then align between the muscle masses and the skeletal elements (Fig. 2) [41, 42]. The dorsal and ventral muscle bundles segregate into individual anatomic groups as muscle connective tissue cells align along future sites of splitting and initiate cell death [41, 43, 44]. This process generally proceeds in a proximal to distal and dorsal to ventral fashion along the limb axis. Bone, tendon, and muscle precursors undergo concurrent differentiation and patterning in a highly coordinated fashion, however, there is a significant lack of understanding on the mechanisms behind these highly coordinated patterning events.

#### Early Patterning Events are Tissue Autonomous

Muscle precursors themselves have no intrinsic or predetermined patterning information. Classical chick-quail graft experiments, where somites outside the normal limb region are grafted into the axial region adjacent to the limb, demonstrated that muscles from any somite are capable of migrating into the limb and forming normal limb musculature [36, 37]. More complex single-cell lineage analysis has demonstrated that transplanted somitic muscle precursors are not

biased to form specific anatomic muscles [45]. If tendon primordia are surgically removed, muscle progenitors still migrate into the limb bud mesenchyme and are able to undergo differentiation demonstrating that the differentiation of muscle tissue is autonomous and does not require signals from the tendon [41]. Markers of early tendon progenitors have been described relatively recently, but it is not clear if they mark the earliest tendon progenitors [42]. Therefore, there has been no genetic ablation of all limb tendon progenitors. The generation of a tendon-less limb mouse will be necessary to test whether early muscle patterning events occur in the complete absence of tendon progenitors.

Various muscle-less limb models have demonstrated that early patterning of the connective tissue and skeletal elements do not require the presence of muscle. Initiation of tendon and muscle connective tissue pattern occurs normally in muscle-less limb models in both mouse and chick [41, 42, 46, 47]. The *Sox9*<sup>+</sup> skeletal elements are also normally patterned in the absence of muscle.

#### Muscle and Tendon/Muscle Connective Tissue Interactions are Required for Later Patterning Events

After the initial prepatterning events of the tendon and the muscle connective tissue occur, integration of the muscle, bone, and tendon requires interaction between tissues. While early specification and patterning of tendon and muscle connective tissue is normal in muscle-less limbs, tendon progenitors are progressively lost in a proximal to distal fashion [41]. After migrating into the limb, patterning of the dorsal and ventral muscle masses requires interactions with the tendon. If tendon primordia are surgically removed from the limb, proper muscle patterning is disrupted and aberrant muscles form [41]. The formation of aberrant muscle in the absence of tendon demonstrates a role for tendon in negatively regulating muscle formation in specific regions of the limb [41].

There is growing evidence that the connective tissue elements of the limb, tendons, and muscle connective tissue,

have critical roles in patterning the muscle. The T-box transcription factors *Tbx4* and *Tbx5* are broadly expressed in the limb bud mesenchyme including the muscle connective tissue and tendon precursors [48]. Loss of *Tbx5* gene function results in ectopic splitting of the dorsal and ventral limb muscle masses and disruption of the muscle connective tissue organization. Additionally, levels of  $\beta$ -Catenin and N-cadherin were reduced. These data suggest that the organization of connective tissue and catenin/cadherin mediated cell adhesion are required for connective tissue-mediated muscle patterning.

The transcription factor *Tcf4*, a member of the Tcf/Lef family which acts downstream of *Wnt* signaling, has been identified as a marker of muscle connective tissue cells [49]. Overexpression of *Tcf4* results in the formation of ectopic muscle and loss of *Tcf4* disrupts muscle fiber type switching, suggesting that the muscle connective tissue directs differentiation of muscle precursors [49, 50]. There is also evidence that *Fgf4* signals from muscle at sites of tendon attachment function to maintain expression of tendon specific genes [47]. Together these data demonstrate a critical requirement for muscle-tendon interactions for maintenance and patterning of both tissues.

#### Muscle and Tendon Interactions with Bone are Necessary for Patterning Bone Ridges

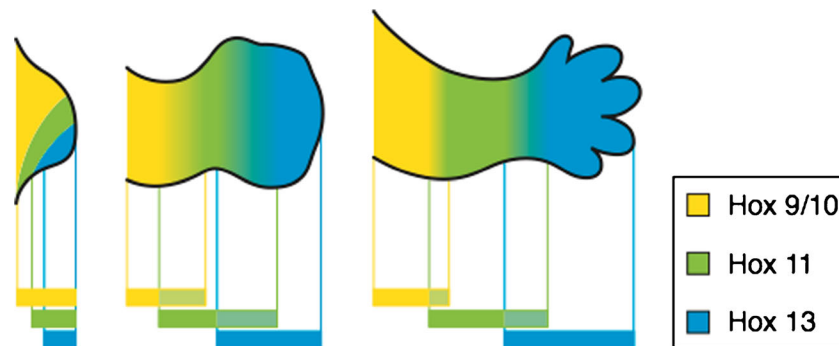
Attachment of muscle to the bone through tendon is important in patterning the skeletal elements at sites of tendon attachment. It has long been appreciated that mechanical forces generated in muscle and transmitted to the bone through tendon attachments influence the final skeletal morphology [51–53]. In *Scx* null mutants, tendon condensation and differentiation is disrupted and consequently the deltoid tuberosity does not form on the humerus demonstrating a requirement for proper tendon formation in the development of this bone process [54]. Recent data provide evidence that bone ridge cells originate from a pool of *Scx*<sup>+</sup>; *Sox9*<sup>+</sup> progenitor cells [55–57]. Bone ridge patterning appears to be a biphasic process of initiation and growth stages, and signals from the

tendon are critical to initiate formation of these skeletal structures [58]. Additionally, there is also a well-described role for muscle loading in regulating proper tendon structure [57, 59, 60]. These studies highlight the critical requirement for muscle, tendon and bone interactions in regulating bone morphology. What determines the sites of tendon-bone attachment is still a largely unanswered question in the field.

#### Establishment of Regional Patterning

Despite significant data on the requirement for crosstalk between muscle, tendon, and bone during musculoskeletal patterning, the mechanisms responsible for these processes are still under active exploration. A critical component in patterning the musculoskeletal system is the incorporation of regional differences in patterning along the AP axis of the vertebrae and the PD axis of the limb. The same tissue components, muscle, tendon, and bone, are present throughout the limb, but substantial morphologic differences and unique connections occur within these tissues along the axial and limb axes. While there is significant information on the development of muscle, tendon, and bone, how these tissues form precise connections and become patterned and integrated into a functional unit is largely unknown. Work published recently from our laboratory suggests that this regional patterning information is provided, at least in part, by the master developmental regulators, the *Hox* genes.

*Hox* genes expression is initiated very early in limb bud development. The posterior *Hox* genes are expressed in a nested and highly overlapping pattern within the limb bud. As the limb develops and grows in length, the *Hox* paralogous groups become progressively restricted to their appropriate functional domains such that *Hox9* and *Hox10* are expressed in the stylopod, *Hox11* is expressed in the zeugopod, and *Hox13* is expressed in the autopod (Fig. 3) [6, 10–12, 26, 61]. This restricted expression pattern is then maintained throughout embryonic development [61].



**Fig. 3** Posterior *Hox* paralogous genes are progressively restricted to specific limb regions during development. In the early limb bud, *Hox 9–13* paralogs are expressed in an overlapping and nested pattern. As the limb develops, *Hox* paralogs are progressively restricted resulting in

*Hox9/10* (yellow) expressed proximally (stylopod), *Hox11* (green) expressed medially (zeugopod), and *Hox13* (blue) expressed distally (autopod)

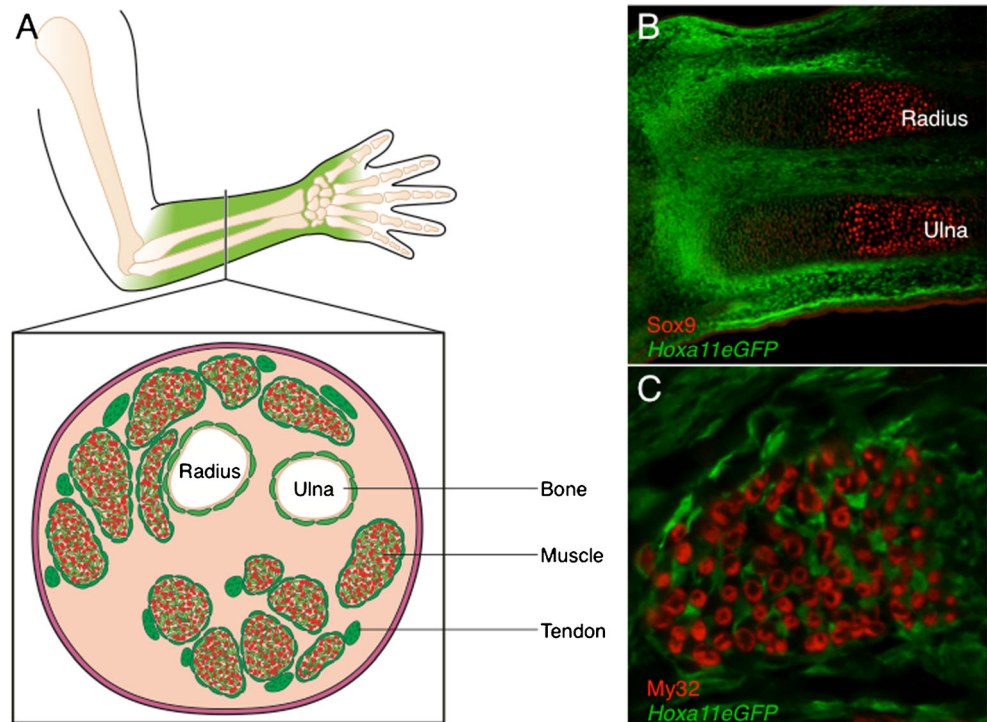
The regionalized expression and function of *Hox* genes along the limb axis makes them an attractive candidate for directing regionalized patterning and integration of the musculoskeletal tissues. A *Hox11eGFP* knock-in mouse model was utilized to examine *Hox11* expression in detail within the developing limb. Characterization of *Hox11eGFP* expression in whole mount and in longitudinal limb sections shows broad expression throughout the distal limb bud mesenchyme early in development. This expression rapidly becomes restricted to the presumptive zeugopod [61•]. Interestingly, *Hox11eGFP*<sup>+</sup> cells are largely excluded from the *Sox9*<sup>+</sup> chondrogenic precursor population at all stages of development [61•]. Careful examination of *Hox11eGFP* expression shows *Hox11eGFP*<sup>+</sup> cells in the outer perichondrial layer surrounding the presumptive zeugopod condensations (Fig. 4A and B). Analysis with markers for muscle and tendon revealed *Hox11eGFP* expression within the muscle connective tissue and all cells of the tendon (Fig. 4A and C). Throughout limb development, *Hox11eGFP*<sup>+</sup> cells are always excluded from muscle and bone and remain expressed in the stromal connective tissues (Fig. 4) [61•]. *Hox13GFP* expression has also been reported to be expressed in the perichondrium and excluded from condensing cartilaginous elements, however, detailed co-expression analysis with muscle and tendon markers has not yet been examined [62].

The cartilage elements destined to become the limb skeletal elements still condense in *Hox* loss-of-function mutants but they are not elaborated to form the appropriate anatomic bones [26]. The observation that *Hox* is not expressed in the skeletal elements but in the perichondrial stromal layer surrounding

the elements significantly changes how the skeletal phenotypes observed in these mutants are interpreted. These data strongly suggest that signals from the perichondrial stromal cells have an important role in determining the morphology and patterning of the skeletal elements.

*Hox11eGFP* expression in the connective tissues of the limb was a surprising finding and prompted analysis of the muscle and tendon phenotypes in *Hox11* mutants. A potential role for *Hox* in mediating muscle or tendon development had not been previously investigated in detail. In addition to the well-described regional skeletal phenotypes observed in *Hox* paralogous loss-of-function mutants, severe mispatterning of the zeugopod muscles and tendons is observed in *Hox11* loss-of-function mutants [61•]. Early in development, tendon progenitors are specified in *Hox11* mutants and appear similar to controls, suggesting that *Hox* functions in patterning and integration but does not function in specification of connective tissue. In *Hox11* mutants, the migration of muscle precursors into the limb occurs normally and dorsal and ventral muscle masses are present. However, the subsequent splitting of the masses into individual muscle bundles is abnormal and loss or fusion of various muscle groups is observed [61•]. Muscle differentiation, however, still occurs as mature muscle cells are observed in these mutants. The muscle-specific phenotypes in *Hox11* mutants suggest a critical role for *Hox* in connective tissue-mediated patterning of the muscle but not in directing muscle migration or differentiation. These data provide strong evidence that *Hox* function in the connective tissue stromal cells is critical for regionally patterning all components of the musculoskeletal system.

**Fig. 4** *Hox11* is expressed in the connective tissue (stromal) compartment of the forelimb. **A**, *Hox11eGFP* expression is restricted to the zeugopod and excluded from bone and muscle cells. *Hox11* is expressed in the outer perichondrium surrounding the bones, in the muscle connective tissue, and in the tendons. **B**, Longitudinal section of E14.5 forelimb showing expression of *Hox11eGFP* (green) surrounding the *Sox9*<sup>+</sup> (red) skeletal elements. **C**, Transverse view of an individual forelimb muscle bundle at E14.5 showing expression of *Hox11eGFP* (green) in the muscle connective tissue surrounding individual muscle (red) cells. *My32* myosin 32



Development and patterning of the skeletal elements remains normal in *Hox11* compound mutants where one of the four functional alleles of forelimb *Hox11* (either *Hoxa11* or *Hoxd11*) is still expressed [6, 10, 26, 61•]. *Hox11* compound mutants allow us to address the question of whether the muscle and tendon phenotypes observed in *Hox11* double mutant animals represent independent roles for *Hox11* in patterning these tissues, or whether the phenotypes are a consequence of the severely disrupted skeletal patterning. In *Hox11* compound mutants, skeletal patterning is preserved but regional mispatterning of the muscles and tendons is still observed, demonstrating that the muscle and tendon phenotype is independent of skeletal pattern [61•]. These analyses also demonstrate that skeletal patterning and muscle/tendon patterning can be uncoupled. Analysis of limb muscle and tendon phenotypes in other posterior *Hox* gene paralogous mutants has not been investigated in detail, but we hypothesize that similar muscle and tendon phenotypes will be observed.

### Perspectives and Future Directions

These observations raise very important questions of how *Hox* genes function in the musculoskeletal system and how they contribute to integrating the patterning of muscles, tendons, and bones. Many studies highlight the importance of the connective tissues in patterning the limb, and the expression of *Hoxa11eGFP* in the stromal connective tissues of the limb is consistent with an important role for *Hox* in these stromal cells in directing the pattern of musculoskeletal tissues [41, 48–50•, 58•, 61•]. The continuum of *Hoxa11eGFP* expression from the muscle connective tissue, into the tendon, and then the perichondrium surrounding the bone suggests of a role for *Hox11* in integrating patterning information between these tissues. The connective tissue extracellular matrix is made up of largely the same components throughout the limb and identification of region and tissue specific markers is important for understanding how these tissues direct regional patterning. Expression of *Hoxa11eGFP* provides a unique tool to specifically identify these connective tissue elements for analysis within the zeugopod. Generation of fluorescent alleles and conditional loss of function experiments for other *Hox* genes will be beneficial in addressing these questions. Our recent work strongly suggests that connective tissue cells possess regional identity information and play a critical role in establishing proper region-specific pattern of the musculoskeletal tissues [61•].

Musculoskeletal injury and disease can have a devastating impact on a patient's quality of life and there is a great need for novel and improved treatments and therapies. As master developmental regulators, understanding and exploiting the *Hox* signaling program could be invaluable for the development of

usable stem cell therapies designed to treat musculoskeletal injury and disease. Our observations suggest that different *Hox* programs may need to be activated to direct appropriate stem cell activity for use in repair at specific axial or proximal/distal levels.

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### Compliance with Ethics Guidelines

**Conflict of Interest** K. M. Pineault and D. M. Wellik declare that they have no conflicts of interest.

**Human and Animal Rights and Informed Consent** All studies by K. M. Pineault and D. M. Wellik involving animal research were performed after approval by the appropriate institutional review boards.

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