PERSPECTIVE

Rethinking the proximodistal axis of the vertebrate limb in the molecular era

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The development of the vertebrate limb has long served as a paradigm for understanding the fundamental processes by which an undifferentiated field of cells gains spatial pattern and undergoes coordinated differentiation to produce the exquisitely complex structures that characterize our functional anatomy. The limb bud emerges from the flank as a mound of seemingly homogeneous mesenchymal cells within an ectodermal jacket. Yet within days this mass of cells gives rise to skeletal elements, muscles, and connective tissues organized in the form of a mature limb. Looking at one's own arm or leg, this organization can immediately be appreciated along three different axes: The back of the hand (dorsal) is different from the palm (ventral), the thumb (anterior) is distinct from the little finger (posterior), and the upper arm (proximal) is different from the lower arm and the hand (distal). The process by which differences along the proximodistal axis are established has been particularly controversial, with competing models proposed to explain the outcome of both classical and genetic manipulations. However, if one examines the two major models that have been previously proposed, neither of them is tenable in the context of our current knowledge of gene activity in the developing limb. Proximodistal patterning therefore needs to be placed into a new framework based directly on the molecular data.

The first key insight into the process of limb patterning came almost 60 yr ago when John Saunders discovered that the apical ectodermal ridge (AER), a thickened ridge of ectodermal cells that runs along the anterior–posterior axis of the distal limb bud (equivalent to a ridge running along the distal edge of the hand from which the finger tips will eventually form) is necessary for the successful outgrowth of the limb along the proximodistal axis (Saunders 1948). To explain how different structures arise at different proximodistal levels as the limb bud grows out, a model was subsequently proposed based on progressive specification of increasingly distal cell fates in a domain ~300 μ m deep directly below the AER (Summerbell et al. 1973). According to this view, under the

influence of the AER, cells are maintained in a so-called progress zone. These cells continuously acquire ever more distal positional information through the influence of an internal clock that is kept active as long as the cells receive signaling from the overlying AER. This process gives the cells a distal fate proportional to the length of time the cells remain in the progress zone. When cells move out of the range of AER signaling—i.e., out of the progress zone—the clock stops and their proximodistal fate therefore becomes fixed. This happens continuously, as all the cells in the progress zone—indeed, all of the cells of the limb bud—are dividing. As more cells are produced, only the members of the population closest to the distal end remain within the 300- μ m signaling range of the AER. Thus the first cells to be forced outside the progress zone, through the growth of the limb, become the upper arm while the last become the digits (Fig. 1A). (Note that the 300- μ m depth of the progress zone is only an approximation, as are the sizes of several subsequently discussed regions such as the 200- μ m domain of cell death following AER extirpation. The numbers quoted are based on published estimates and give a rough indication of where in the limb bud certain events occur, but should not be considered to be precise measurements.)

This model can explain, in an elegant manner, several important classical experiments. First, the earlier in limb development the AER is removed, the more severe the truncation that results (Summerbell 1974; Rowe and Fallon 1982). Following early AER extirpation only proximal parts of the limb develop, while at later stages more distal structures additionally form. This can be interpreted as the consequence of prematurely stopping the clock within the cells of the progress zone upon AER removal. Cells lying within the progress zone would still contribute to limb structures but could only form them to the proximodistal level specified at the time of the surgery.

Second, when a subset of cells in the early progress zone are killed, for example, by X irradiation, distal structures form while proximal ones are reduced or absent (Goff 1962; Pinot 1970; Summerbell 1978; Wolpert et al. 1979). This is explained by the requirement that the depleted cell population in the progress zone needs to undergo additional proliferation before achieving a size

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Figure 1. (A) The Progress Zone Model. Initially, the entire limb mesenchyme has a proximal identity. Left on its own it would develop into proximal, stylopod structures (red). However, the mesenchyme at the tip of the limb bud, the progress zone (PZ), is exposed to Fgf and becomes respecified at a slightly more distal, zeugopod (yellow) fate. As limb development proceeds, the progress zone cells divide, and as a result of this growth, not all of the cells remain within range of the Fgf signal. Those too far from the tip maintain their already specified fate, whereas those still in close proximity to the tip are once again respecified to a still more distal, stylopod (orange) fate. (B) The Early Specification Model. All of the proximodistal segments—stylopod (red), zeugopod (yellow), and autopod (orange)—are specified early in limb development. These segment-specific progenitor pools expand as the limb bud grows.

sufficient to force some cells out of reach of the AER. Until this happens, all the cells in the progress zone become distalized without leaving any outside the progress zone with a memory of more proximal fate specification.

Swapping AERs between leg and wing buds (Zwilling 1959) and between early and late stage limb buds (Rubin and Saunders 1972) has no effect on skeletal pattern. These results establish that the AER, while required for proximodistal outgrowth, does not instruct different proximodistal cell fates, and further indicate that the necessary factor(s) produced by the AER are the same for the wing and the leg and do not change with time. Subsequently, it was discovered that distal skeletal elements were rescued following AER extirpation by proteins of the fibroblast growth factor (Fgf) family (Niswander et al. 1993; Fallon et al. 1994) and at least four family members—Fgf4, Fgf8, Fgf9, and Fgf17—are indeed specifically expressed in the mouse AER (Martin 1998; Sun et al. 2000). Thus the progress zone could in principle be redefined as the set of cells within the distal limb bud that are within range of Fgf signaling from the AER. According to this view, Fgfs are the permissive factors that allow the progress zone cells to maintain the activity of their internal clock and hence permit their continued, progressive acquisition of ever more distal fates.

More recently, an alternative view that can be termed the “Early Specification Model” was developed to ex-

plain the distal truncation seen following AER removal (Dudley et al. 2000). Extensive apoptosis is seen in the distal-most 200 μ m following AER extirpation (Rowe et al. 1982; Dudley et al. 2002). As the limb grows, the 200- μ m domain of cell death becomes proportionally a smaller portion of the total limb bud, leading to progressively less distal truncations in skeletal pattern. Indeed, fate mapping indicates that marked distal cells are not incorporated into skeletal structures following AER removal (Dudley et al. 2002). Thus, if the early specification model were correct, the skeletal truncations following AER extirpation would reflect the apoptotic loss of already specified distal segments (rather than indicating the extent of distal specification at the time of the experiment, as the Progress Zone Model would propose).

Both the Progress Zone Model and the Early Specification Model can explain the classical AER extirpation and X-irradiation experiments (for review, see Mariani and Martin 2003). Nonetheless, when critically assessed in light of more recent molecular data, neither of these models is completely satisfactory. The phenotypes resulting from conditional removal of Fgf gene activity in the AER (Lewandoski et al. 2000; Sun et al. 2002) are difficult to reconcile with the Progress Zone Model without some additional less-than-straightforward assumptions. Moreover, there is currently no convincing evidence for a cell-autonomous clock capable of affecting

limb patterning, which is at the heart of the Progress Zone Model. It is worth noting, in this regard, that very intriguing cycles of expression of the gene *hairy2* have recently been identified during distal limb outgrowth (Pascoal et al. 2007). However as *hairy2* is believed to function to transduce Notch signaling, and genetic removal of all canonical or noncanonical Notch activity in the limb fails to produce a segmentation phenotype (Pan et al. 2005), the relevance of this observation remains unclear. Thus, while it is certainly an exciting finding worthy of additional study, for the moment at least a concrete molecular mechanism for a progress zone clock is still lacking. On the other hand, the Early Specification Model does not provide an explanation for how the distinct proximodistal progenitor pools are established, nor is it clear whether there is even space in the earliest limb bud to accommodate distinct zones of cells corresponding to each of the later limb segments (Wolpert 2002). In our view, however, the biggest problem with both of these models is the rather striking lack of support for either one from molecular expression studies.

The Progress Zone Model makes a strong prediction that the entire population of progress zone cells, 300 μ m wide, would coordinately undergo successive changes in the expression of a series of distinct specification genes. Moreover, according to that model, the changes in proximodistal specification gene expression would be based entirely on an internal clock and would not be dependent on inductive signaling. The Early Specification Model makes the equally strong prediction that the expression of specification genes would form small stripes across the early limb bud that would subsequently expand into wider stripes as the respective progenitor pools proliferate. It has been 17 years since the nested patterns of Hox genes provided the first molecular insights into limb patterning (Dolle et al. 1989), and literally hundreds of limb expression patterns have since been described, including markers for each of the developing proximodistal limb segments. Yet no patterns have emerged such as those predicted by either of the two major models of proximodistal patterning. There are no genes known to arise progressively in the distal mesenchyme that are independent of instructive inductive cues, and there are no genes known to be expressed in stripes reflective of the future limb segments in the earliest limb bud. Indeed, the best markers for the individual limb segments, such as *Hoxa13* for the autopod, are expressed in patterns that directly contradict both models (see below).

One can always argue that true proximodistal specification genes, with expression patterns as predicted by one of the two models, remain undiscovered. However, eventually such an excuse for the lack of evidence begins to ring hollow, especially as several large-scale expression screens by *in situ* hybridization and microarray have not revealed genes with patterns matching the predictions of either of the two models. A parallel can be drawn to the state of paleontology in the late 1970s when the failure to observe gradual changes in the fossil records could no longer satisfactorily be excused on the basis of an incomplete fossil record, and punctuated

equilibrium was finally appreciated as a major mode of evolutionary change (Gould and Eldredge 1972). We have, therefore, re-examined the available molecular information on gene expression along the proximodistal limb axis to see if the existing data itself could form the basis for a new way of conceptualizing proximodistal specification.

Toward a modern view of proximodistal specification

A gene activity-based framework for understanding the specification of the most proximal portion of the limb (the stylopod) has already been provided by Mercader et al. (2000). They describe the critical role of two signaling centers in establishing this aspect of proximodistal patterning, one in the flank (producing retinoic acid) and one at the distal tip of the limb bud (producing members of the Fgf family). We expand on this model to additionally address the specification of more distal limb segments by considering the dynamic changes in gene expression in the distal limb and the signaling centers that coordinate them. In addition, we consider another key level of complexity in the process of defining the limb segments. Even as the progenitors of the eventual proximodistal segments are being specified, the progenitor pools that will ultimately contribute to each limb segment are rapidly expanding through continued proliferation. We suggest that the length of time cells proliferate before committing to differentiation will significantly influence the size of the progenitor pools at the time differentiation is initiated, which consequently will have a major impact on the ultimate size of the skeletal elements produced. Thus, although the humerus and phalanges are normally of similar size when they first condense, if the humerus progenitors proliferate for a shorter period of time before commencing differentiation, the humerus condensation and hence the element itself will be shorter than normal.

The cells at the distal tip of the limb bud are maintained in an apparently undifferentiated state due to the influence of signals from the AER (Globus and Vethamany-Globus 1976). To put this observation in modern terms, we suggest that during limb bud outgrowth, commitment to differentiation and chondrogenic condensation occurs as cells exit the domain influenced by Fgf signaling from the AER. We will refer to this Fgf-responding domain that roughly corresponds to the distal region previously identified as the progress zone, as the “undifferentiated zone.” We will refer to the proximal border of this domain as the “differentiation front.” Initially the undifferentiated zone encompasses the entire limb bud, but as the mass of mesenchyme becomes large enough, the most proximal cells are not longer within the range of Fgf signaling and, therefore, are able to enter various differentiation pathways. This process continues with the resultant well-known wave of proximal-to-distal differentiation (Fig. 2A). As this is occurring, dynamic patterns of gene expression are unfolding. Without reference, for now, to the way proximodistal gene expression is regulated, we postulate that a cell’s proximodistal

Figure 2. Establishment of distinct molecular domains along the proximodistal limb axis. (A) Approximate map of the undifferentiated zone in the distal 250 μ m of the limb bud (tan) at various stages of limb development. (B) Comparison of the domains of expression of Meis1 (a stylopod marker; blue) and AP2 (an undifferentiated zone marker; tan) at different stages of limb development. (C) Comparison of the domains of expression of Meis1 (a stylopod marker; blue) and Hoxa11 (a zeugopod marker; red) at different stages of limb development. (D) Comparison of the domains of expression of Hoxa11 (a zeugopod marker; red) and Hoxa13 (an autopod marker; green) at different stages of limb development. Hoxa11 and Hoxa13 expression overlap at early stages of their expression (brown).

specification is based on the segment-specific genes it expresses at the time it exits the undifferentiated zone.

While the mechanisms that pattern the distinct limb segments remain controversial, we can follow the evolving domains of segment-specific gene expression as a surrogate providing an indication, in relative terms, of when the limb segments are specified and gain insight from the way those genes are regulated. On such a basis, the first segment of the limb to be specified in the model we are proposing is the stylopod, under the influence of the proximal and distal signaling centers as suggested by

Mercader et al. (2000) (Fig. 2B). Because these signaling centers are fairly close to one another in early, tiny limb buds, the expression domains of genes positively regulated by each of these signaling centers initially overlap. As the limb bud grows and the distance between the proximal and distal signaling centers increases and the expression domains of the genes they regulate become distinct (Fig. 2B).

Thus, as the early limb bud starts to expand, the most proximal cells are the first to find themselves out of range of the AER. Because they are beyond the influence

of AER signals, but are close to the source of flank-derived signals, the moment they cross the differentiation front they cease expressing distal markers and only maintain stylopod-specific gene expression. At the same time, as they are pushed out of range of the AER signals, they commit to enter cellular differentiation pathways. This process continues as the limb bud grows, with more progenitors escaping the influence of the AER and joining the stylopod until the limb bud is large enough that cells forced by cell expansion out of range of the AER are also out of range of flank signals. At that point, we suggest that the size of the stylopod progenitor pool has been fixed, and subsequent cells pushed across the differentiation front will form more distal limb segments.

In our aquatic ancestors, this might have been all that was required for proximodistal specification, as the autopod (wrist/ankle and digits) is believed to be a “neomorphic” evolutionary innovation of the tetrapod lineage (Sordino et al. 1995) and the ancestral appendage was in essence limited to a stylopod and a zeugopod. In that setting, the proximal and distal signaling centers would be sufficient to establish two proximodistal domains, one established within the range of the flank signals and one out of range of the flank signals.

In tetrapods, where a series of distal limb segments needs to be specified and expanded, the process becomes more complex. As the entire limb bud distal to the stylopod is out of range of flank signaling, this proximal signaling center ceases to be important for more distal specification. However, a third signaling center, the zone of polarizing activity (ZPA) acts in concert with the AER to initiate dynamic patterns of expression that evolve through cross-regulation within the distal cells themselves as well as under the continuing influence of the AER and ZPA.

Of course, the ZPA is traditionally thought of as a signaling center organizing the anteroposterior axis. However, the reality is that limb patterning is achieved as an integrated whole, with critical cross-interactions between the signaling centers patterning the several limb axes as well as integration at the level of the regulation of key target genes, such as the Hox genes (see below). In particular for our current discussion, we will argue that the activity of the ZPA is also important to take into account in considering the regulation of genes specifically expressed in the zeugopod and autopod.

How we view signals from the AER and ZPA influencing the proximodistal patterning of the zeugopod and autopod can be more easily explained in the context of the gene expression patterns described below. As in the specification of the stylopod, it will be seen that there is a coordination between the dynamic changes in distal expression domains and the onset of differentiation, as the effective range of AER signaling demarcates both the subset of cells in the distal limb bud where proximodistal gene expression patterns can be modified by continued AER signaling and the depth of the undifferentiated zone.

For simplicity, in the discussion that follows we will focus exclusively on markers for the major subdivisions

of the limb, the stylopod, zeugopod, and autopod. However, to fully understand proximodistal patterning, ultimately additional segments must also be considered. The wrist/ankle are distinct from digits within the autopod. Moreover, at least in principle, each phalanx within a digit could also be viewed as a distinct segment. In our discussion (below) of the molecular data in support of our model, we will continue to focus on markers for each of the three major limb subdivisions; for the sake of simplicity, however, it will be important in the future to expand this analysis, taking into account additional proximodistal domains. For example, specification of the wrist could be followed utilizing the difference in late expression patterns of *Hoxa13* (entire autopod) and *Hoxd13* (autopod exclusive of wrist).

Markers for proximodistal limb segments

The best markers available for the stylopod segment are *Meis1* and *Meis2*, which encode related TALE-homeodomain proteins. The Meis proteins regulate the activities of the transcription factor Pbx and Hox genes (for review, see Moens and Selleri 2006). The Meis genes are more than just markers for the stylopod, as they may themselves play a role in proximal specification; since ectopic expression of these genes can reprogram proximodistal fate at least to some extent (Capdevila et al. 1999; Meracader 2000). However, it is important to note that gene inactivation studies have indicated that neither of these genes is essential, at least individually, for normal limb development (Azcoitia et al. 2005).

Arguably the best markers discovered thus far for the distal limb segments are the 5' members of the *Hoxa* and *Hoxd* clusters, at least at later stages of development. Early in limb development, these genes have dynamic patterns of expression (see below); however, by the time cells in the chondrogenic condensations start to differentiate into cartilage, *Hoxa11* is an excellent and very precise marker for the entire zeugopod, while *Hoxa13* is an equally good marker for the entire autopod (Yokouchi et al. 1991). It is important, however, to bear in mind that these genes are themselves not actually involved in segment specification. For example, when all *Hoxa11* activity is removed from the limb bud (*Hoxa11* and *Hoxd11* in the forelimb [Davis et al. 1995], and *Hoxa11*, *Hoxc11*, and *Hoxd11* in the hindlimb [Wellik and Capecchi 2003]), the zeugopod condensations (the cartilaginous anlagen of the radius and ulna or tibia and fibula) form relatively normally. In the absence of the influence of these Hox genes, however, there is a failure to form normal growth plates at the proximal and distal ends of the zeugopod bones and the zeugopod is extremely reduced (Boulet and Capecchi 2004). Thus *Hox11* gene activity is indeed critical, quite specifically for normal zeugopod development, but is not required to specify the zeugopod itself.

Although the *Hoxa* genes do not appear to play a role in segment specification per se, their final expression domains are exactly congruent with the distal limb segments. Thus, their regulation must reflect a mechanism

for bringing their domains into precise register with the segment boundaries. Hence, either they are regulated by the true specification genes, or are coregulated by the same processes that control the expression of the specification genes. In either case, since no genes directly involved in distal specification have been described, these Hox genes can be used as surrogates for understanding the formation of the distinct zeugopod and autopod segments.

In addition to the genes marking the future limb segments, there are also genes whose expression reflects the activity of the signaling centers influencing the proximodistal axis. Thus the Retinoic Acid Receptor β (RAR β) is expressed in the proximal-most limb reflecting retinoid signaling from the flank (Dolle et al. 1990; Smith and Eichele 1991). Similarly, a large number of genes expressed distally throughout limb development are thought to be directly regulated by Fgfs produced within the AER. It is worth noting, however, that for most of these genes the assumption that they are regulated by FGF signaling from the AER is based on AER removal experiments, in which the subridge cells die within a few hours of ridge removal. Thus the observed loss of expression of these genes and rescue of their expression following ridge removal by the application of FGF beads might be due to cell death and prevention of cell death, respectively, rather than any direct effect of FGF signaling on the genes themselves. Nevertheless, several of these genes are expressed in multiple places in the developing embryo in regions of Fgf signaling, supporting the idea that they could indeed be Fgf dependent in the distal limb as well. These genes—including, for example, AP2 (Chazaud et al. 1996; Shen et al. 1997), Fgf10 (Ohuchi et al. 1997), Sprouty1 and Sprouty2 (Chambers and Mason 1999; de Maxim et al. 1999; Minowada et al. 1999), and the distal mesenchymal domain of Msx1 (Ros et al. 1992)—are expressed to slightly differing distances below the AER, presumably reflecting distinct levels of response to a distal-to-proximal Fgf protein gradient. The most broadly expressed of these extends roughly 250 μ m from the distal tip. The size of the expression domain of any one of these genes is relatively constant through the patterning phase of limb development. In this respect, they are similar to the relatively constant-sized distal zone where the mesenchyme is kept in an apparent undifferentiated state by signaling from the AER (Globus and Methamany-Globus 1976). Thus, we consider expression of genes such as AP2 as markers for the distal region where cells are held in an undifferentiated state, rather than reflecting a particular state of proximodistal specification. We do not, however, mean to imply that any of these specific distal markers are themselves necessarily involved in regulating commitment to differentiation.

Dynamic patterns of expression in the context of proximodistal specification

For simplicity, we will focus on four genes as markers for this discussion: Meis1 for the stylopod, Hoxa11 for the zeugopod, Hoxa13 for the autopod, and AP2 for the un-

differentiated zone actively responding to AER/Fgf signaling. When the limb bud first becomes evident (stage 17 in the chick forelimb), Meis1 is expressed throughout the limb bud (Capdevila et al. 1999; Mercader et al. 2000) presumably in response to signaling from the flank (Fig. 2B; Mercader et al. 2000). At the same time, the entire limb bud also expresses AP2 under the presumed influence of AER signals (Chazaud 1996; Shen 1997). As first discussed by Mercader et al. (2000), the complete overlap of these domains can fit with a model based on proximal and distal signaling centers, since at early stages, every cell in the limb bud should be within range of both flank and AER signaling, although direct evidence for the necessity of a proximal signaling center in limb development is lacking. As the limb bud grows, three different zones emerge, a proximal domain that continues to express Meis1, a distal domain expressing only AP2, and a middle region expression both genes. These observations also fit the model, since it predicts that as the limb bud enlarges, cells in the proximal region will only be within range of signals from the flank, cells in the distal region will only be within range of signals from the AER, and cells in the middle region will be within range of both signals. At still later stages, the Meis1-expressing domain proximally and AP2-expressing domain distally are nonoverlapping, and a domain of increasing size is generated between them where neither of these markers is expressed. Again, this fits the model, which predicts that at later stages there will be a domain in which cells are out of range of both flank and AER signals.

Against the backdrop of the expansion and separation of the Meis1 and AP2 expression domains, Hoxa11 and Hoxa13 are activated, and their expression patterns evolve to ultimately correspond to the zeugopod and autopod, respectively. Hoxa11 expression is initiated at a time when Meis1 and AP2 still overlap significantly (stage 19 in the chick forelimb) (Nelson et al. 1996; Vargesson et al. 2001), in a posteriorly based distal stripe overlapping both Meis1 and AP2 (Fig. 2C). Over time, however, the proximal domain of Hoxa11 expression becomes restricted such that it attains a mutually exclusive domain relative to Meis1 (Capdevila et al. 1999; Mercader et al. 2000). Thus, once Hoxa11 expression has been initiated in the distal limb overlapping the early domain of Meis1, like Mercader et al. (2000), we propose that the subsequent mutually exclusive pattern of expression of Meis1 and Hoxa11 is achieved through a combination of flank signals promoting Meis1 expression, distal signals antagonizing Meis1 expression, and mutual negative regulation between Meis1 and Hoxa11 enhancing the transition (see below). Note, however, that to the extent that flank signals play a role in establishing proximodistal limb boundaries, they must do so relatively early, as a mid-stage limb bud can be grafted to the dorsal surface of a second, host limb bud and a normal proximodistal skeletal pattern will result, in the absence of any continued interaction with flank signals. Thus from mid-bud stages onward the distal signals combined with mutual Meis1–Hoxa11 antagonism must suffice to refine their mutually exclusive domains.

Since, in this model, the range of Fgf signaling defines both the territory where *Meis1* will be repressed and the extent of the distal undifferentiated zone, it assures that the *Meis1* and *Hoxa11* zones finally become mutually exclusive precisely at the time the last of the *Meis1*-expressing cells are pushed across the differentiation front by the expansion of the limb bud. Subsequent cells to cross the differentiation front thus express *Hoxa11* but not *Meis1*. Hence, in the early limb bud, proximal cells start differentiation while expressing only *Meis1*, and when subsequently the mutually exclusive domains of *Meis1* and *Hoxa11* are established, cells start differentially expressing only *Hoxa11*. In other words, while cells transiently express both these markers in the early limb bud, the coordination of their dynamic expression with the differentiation front means that at the time differentiation actually takes place cells express either the stylopod or zeugopod marker, but not both.

Shortly after *Hoxa11* expression is dissociated from *Meis1* expression at the proximal limit of the *Hoxa11* domain, *Hoxa11* expression is down-regulated in the distal handplate such that it also has a mutually exclusive border with the late distal expression domain of *Hoxa13*. *Hoxa13* expression is initiated after *Hoxa11* (stage 21 in the chick forelimb) (Nelson et al. 1996; Vargesson et al. 2001), in a very small domain at the posterior-distal margin, initially overlapping with both *Meis1* and *Hoxa11* (Fig. 2D). *Hoxa13* expression then expands to form a narrow distal stripe overlapping with the distal expression of *Hoxa11* but no longer overlapping the proximal expression domains of *Meis1* (stage 23 in the chick forelimb). Finally, *Hoxa13* expression broadens to encompass the distal 200–300 μ m as the handplate extends (stage 25 in the chick), at the same time as *Hoxa11* is down-regulated distally (Yokouchi et al. 1991; Nelson et al. 1996). Once again, we suggest that the process of distal *Hoxa13* expansion and *Hoxa11* down-regulation is coordinated with the time at which cells leave this undifferentiated zone such that cells do not cross the differentiation front while expressing both *Hoxa11* and *Hoxa13*. At the same time as the *Hoxa11* is completely down-regulated distally and the *Hoxa13* expression domain reaches its full 200- to 300- μ m width, the last of the *Hoxa11*-expressing cells moves across the differentiation front. Subsequently, when differentiation is initiated in more distal cells, they express only *Hoxa13*. At this point, the three cardinal limb segments, the stylopod, zeugopod, and autopod, are each distinctly defined by the expression of *Meis1*, *Hoxa11*, and *Hoxa13*, respectively.

Regulation of gene expression along the proximodistal axis

Of these markers, it is easiest to understand the regulation of AP2, which is expressed in the distal undifferentiated zone mesenchyme at every stage of limb bud development. In all likelihood, AP2 (like other markers for this domain) is strictly dependent on Fgf signaling from the AER. Following AER extirpation, AP2 expression is

completely lost within 4–8 h, and this down-regulation can be rescued by ectopic Fgf (Shen et al. 1997). Other distal makers have similarly been shown to be exquisitely sensitive to loss of the AER. For example, the mesenchymal expression of *Msx1* subjacent to the AER is down-regulated as quickly as 2 h after AER removal and completely absent after 4 h (Ros et al. 1992). Like AP2, distal *Msx1* expression can be maintained by ectopic application of Fgf-soaked beads (Fallon et al. 1994). Moreover, other distally expressed genes such as the markers of the Sprouty family are not only expressed in the domain of the distal Fgf signaling in the limb bud, but also in many other areas of Fgf signaling in the embryo. Thus, although in principle the expression of these genes in the distal limb bud could be independent of Fgf signaling, in which case (as discussed above), the down-regulation of AP2, *Msx1*, Sprouty, and other genes following AER removal could be attributed to the rapid wave of distal cell death that follows AER extirpation (Rowe et al. 1982; Dudley et al. 2002), the most parsimonious explanation is that they do depend on active Fgf signals in the limb. Moreover, even if those specific genes are not themselves regulated by Fgf, it is clear from the 200- to 250- μ m region of distal cell death following AER removal that their expression domains are approximately coincident with the range of Fgf signaling and can therefore be used as surrogates for following the cells responding to Fgf as the limb bud expands along the proximodistal axis.

Using *Meis1* as a marker for the proximal limb segment, the entire early limb bud transiently carries this molecular signature of the stylopod. It has been postulated that the expression of *Meis* is based on signaling by retinoic acid (RA) from the flank, as ectopic RA expands *Meis1* expression distally at later stages while RA antagonist prevents proximal *Meis1* expression (Mercader et al. 2000). Interestingly, *Meis1* expression also appears to be negatively regulated by Fgf signaling, as the endogenous *Meis1* expression is down-regulated by ectopic Fgf protein (Mercader et al. 2000). Thus one can hypothesize that, at the earliest stages when the entire limb bud presumably sees high levels of both RA and Fgf, RA in effect “wins,” and as a consequence *Meis1* is expressed. As the limb bud grows, gradients of both molecules are presumably set up such that toward the middle of the limb bud, cells see lower RA concentrations, under which conditions Fgf is able to act as an inhibitor of *Meis1*, and in this way could contribute to the formation of the sharp boundary of *Meis1* expression.

The regulation of *Hoxa11*, the marker of the zeugopod segment, is less clear. As discussed above, *Hoxa11* expression is initiated in a broad distal domain, under the AER at a time when most of the limb bud also expresses *Meis1*. There are reports that, like the *Hoxd* genes (Laufer et al. 1994; Koyama et al. 1995; Vogel et al. 1995), *Hoxa11* requires Fgf signaling from the AER (Hashimoto et al. 1999). However, this is only observed at early stages (stage 20 in the chick forelimb), whereas *Hoxa11* expression is stably expressed and independent of AER activity at later stages (stage 25 in the chick forelimb) (Hashimoto et al. 1999; Vargesson et al. 2001). This

could represent a transition in *Hoxa11* regulation, but in light of the current appreciation of distal cell death following AER removal (Rowe et al. 1982; Dudley et al. 2000), an alternative explanation would be that *Hoxa11* expression is never directly dependent on AER signaling. Rather, at early stages the relatively small and distally restricted domain of *Hoxa11* expression is lost by apoptosis following AER removal. At later stages AER removal results in loss of distal cell populations, but the *Hoxa11* domain is larger and extends more proximally than the wave of cell death and hence is largely unaffected by it. Consistent with this explanation for the loss of *Hoxa11* in early limb buds, an Fgf bead can rescue *Hoxa11* expression if applied immediately upon AER extirpation, but not 12 h later (Hashimoto et al. 1999), when the wave of apoptosis has already taken place (Dudley et al. 2000).

In contrast, *Hoxa13* does seem to be directly regulated by Fgf signaling (Hashimoto et al. 1999; Vargesson et al. 2001). AER removal results in a very rapid loss of *Hoxa13* expression, which is rescued by application of exogenous Fgf (Vargesson et al. 2001). However, ectopic Fgf is not capable of expanding the *Hoxa13* domain proximally, suggesting that additional signals must also be involved in establishing the limits of its domain of expression (Vargesson et al. 2001). One such factor that could act to restrict *Hoxa13* to its normal distal domain is RA, since ectopic application of RA results in a down-regulation of *Hoxa13* (Mercader et al. 2000). Interestingly, when ectopic RA blocks distal *Hoxa13* expression, there is a concomitant distal expansion of *Hoxa11* expression. Thus the effect of RA on *Hoxa11* appears to be position dependent. At the proximal border of its normal domain, RA signaling inhibits *Hoxa11* expression, whereas at its distal border, RA induces *Hoxa11*. This can be interpreted in terms of reciprocal and mutually antagonistic proximodistal gradients of RA and Fgf (Mercader et al. 2000).

Not surprisingly, there are also regulatory relationships between these various transcription factors themselves. Consistent with their mutually exclusive domains of expression, *Hoxa13* misexpression represses *Meis1* while *Meis1* misexpression down-regulates *Hoxa13* (Capdevila et al. 1999). Test for cross-interactions between *Meis1* and *Hoxa11* have not been reported, but it seems likely that these genes also have a reciprocal negative regulatory relationship. Perhaps surprisingly, however, *Hoxa11* and *Hoxa13*, which share a very sharp boundary in the late limb bud, do not appear to directly regulate one another, and *Hoxa13* misexpression has no effect on *Hoxa11* expression (Yokouchi et al. 1995). This suggests that perhaps their adjacent, nonoverlapping expression domains are established by reciprocal response to a common signal.

One possible candidate for a common signal affecting both *Hoxa11* and *Hoxa13* expression is Sonic hedgehog (Shh). While Shh acts mainly along the anterior-posterior axis, its activity is integrated at multiple levels with signals primarily affecting other axes. For example, Shh is known to be required in addition to Fgf signals for *Hoxd13* expression (Koyama 1993; Laufer 1994). *Hoxa13*,

described above, is initiated in a tiny posterior-distal domain that correlates with the location of highest Shh signaling in the ZPA. Moreover, recent studies of *Hoxa13* and *Hoxa11* expression in Shh-deficient mouse embryos shows that *Hoxa13* expression is lost in the absence of Shh, and at least in preliminary analyses, *Hoxa11* fails to be down-regulated in the distal limb bud (T. Toyooki and A. McMahon, pers. comm.).

In short, these collective studies reveal a complex set of regulatory interactions controlling the temporally dynamic expression patterns of *AP2*, *Meis1*, *Hoxa11*, and *Hoxa13*. Ultimately these patterns resolve in final expression domains that uniquely define each of the future limb segments. We have suggested that establishing these domains of gene expression depends on both inductive signaling and cross-regulation between the genes themselves. The coordination of these events with the expansion of cells across the differentiation front assures that cells only express genes characteristic of a single limb segment at the time they start differentiation. We would further propose that when a discontinuity between progenitor pools is reached—for example, when *Hoxa13*-positive cells follow *Hoxa11*-positive cells across the differentiation front—they will have distinct cellular properties and respond differently to extracellular cues, leading to the initiation of a new limb segment and distinct skeletal elements.

Through expression analysis of *Meis1*, *Hoxa11*, and *Hoxa13* at progressive stages of limb development, it becomes apparent that they do not support either the Early Specification or Progress Zone Models of proximodistal specification. The expression domains of these markers are very dynamic. At early stages they are not found in a simple prepattern of the limb segments that simply expands, as would be suggested by the Early Specification Model. Thus, the molecular data suggest that the Early Specification Model simply cannot be right. Similarly, the activation of *Hoxa13* in a tiny posterior-distal domain that expands first into a narrow distal stripe at the distal tip (much thinner than the postulated progress zone) and then spreads into a 250- μ m-wide handplate does not match the coordinated promotion of all the cells within a 250- μ m progress zone to an autopod fate, as is hypothesized by the Progress Zone Model. Moreover, the regulation of the genes considered here depends on extracellular induction of gene expression rather than being established by an internal, autonomous clock, the mechanism that is at the heart of the Progress Zone Model. While one can, nonetheless, hypothesize that eventually additional markers will be identified whose expression patterns and modes of regulation will support either the Early Specification or the Progress Zone Model, we suggest that until such genes are indeed found, it will be most productive to base thinking about the proximodistal limb axis on the data that do exist.

In summary, neither the Progress Zone Model nor the Early Specification Model fit the large amount of molecular expression data generated in the last decade. As an alternative, we have utilized these data to provide a new framework for viewing proximodistal limb pattern-

ing. The emerging picture is one of dynamic expression domains of specification genes in the context of the growth of the limb bud and a distally maintained differentiation front. The result is the progressive generation and expansion of progenitor pools specific for each limb segment. This is not so much a new model as a dynamic description of molecular events responsible for defining proximodistal limb segments.

A major weakness in the foregoing discussion is that the genes we use as markers for proximodistal specification in our analysis are, with the possible exception of *Meis1*, not actually involved in determining segment identity. They are, however, the best markers currently available, and (as discussed above) are brought into quite precise register with the segment primordial prior to the overt differentiation of the proximodistal elements. Even so, it remains possible that the true proximodistal specification genes are regulated quite differently. Nonetheless, we have undertaken this survey of currently known gene regulation out of the belief that the current molecular data demand a shift in the way we think about proximodistal patterning, and the importance of this analysis, if any, lies in a way of viewing the problem rather than in the details. It is important that specification of the proximodistal axis be conceptualized on a molecular basis and that future molecular and genetic studies be focused in that direction rather than toward proving or disproving the classical models. At the same time, it will be extremely important to revisit the classical experiments, such as AER extirpation and X-irradiation studies, with modern molecular tools to gain a deeper understanding of proximodistal limb patterning and how it is established.

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Rethinking the proximodistal axis of the vertebrate limb in the molecular era

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