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Review

Integration of the transcriptional networks regulating limb morphogenesis

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ABSTRACT

The developing limb is one of the best described vertebrate systems for understanding how coordinated gene expression during embryogenesis leads to the structures present in the mature organism. This knowledge, derived from decades of research, is largely based upon gain- and loss-of-function experiments. These studies have provided limited information about how the key signaling pathways interact with each other and the downstream effectors of these pathways. We summarize our current understanding of known genetic interactions in the context of three temporally defined gene regulatory networks. These networks crystallize our current knowledge, depicting a dynamic process involving multiple feedback loops between the ectoderm and mesoderm. At the same time, they highlight the fact that many essential processes are still largely undescribed. Much of the dynamic transcriptional activity occurring during development is regulated by distal cis-regulatory elements. Modern genomic tools have provided new approaches for studying the function of cis-regulatory elements and we discuss the results of these studies in regard to understanding limb development. Ultimately, these genomic techniques will allow scientists to understand how multiple signaling pathways are integrated in space and time to drive gene expression and regulate the formation of the limb.

Introduction

The early developing limb, formed by budding out from the lateral plate mesoderm, is a bundle of mesenchymal cells surrounded by an ectodermal jacket. This superficially simple structure will grow and differentiate to form the highly conserved structures of the tetrapod limb including a single long skeletal element in the proximal limb (the stylopod), two long skeletal elements in the medial limb (the zeugopod), and a wrist/ankle connected to a variable number of digits in the distal limb (the autopod) (Fig. 1). The stereotypical structure of the vertebrate limb provides an excellent model system to understand the genetic regulation of pattern formation. The structure itself is not critical for embryonic development meaning that genetic manipulations need not necessarily affect the survival of the embryo. Furthermore, the external location of the limb on the embryo enables a wide range of embryonic manipulations.

Genetic and embryological studies have identified many of the key signals controlling different temporal stages of limb development. Most of these signals, such as the WNT, HOX, BMP, Hedgehog and FGF proteins, play equally essential roles in many other aspects of embryonic development as well as homeostasis in adult tissues. Disruption of these developmental signals have also been linked to congenital limb defects in humans (Bruneau et al., 2001; Kang et al., 1997; Lehmann et al., 2003; Muenke et al., 1994; Utsch et al., 2002; Vortkamp et al., 1991). However, the transcriptional targets of these signals and how these key signals interact with each other in the developing limb are largely unknown. In this review we highlight current knowledge of the genetic interactions controlling limb development and highlight modern genomic techniques that can be used to reveal key unknown processes.

The dynamic transcriptional events occurring during development are largely regulated by cis-regulatory elements that are present outside the proximal promoter (Chandler et al., 2007; Jeong et al., 2006; Michos et al., 2004; Montavon et al., 2011). Much of the vertebrate genome is under the influence of such developmentally active cis-regulatory elements (Ruf et al., 2011). Extensive work has been performed to identify such cis-regulatory elements in cultured cells, especially those involved in the regulatory networks controlling pluripotency in ES cells (Boyer et al., 2005; Creyghton et al., 2010; Loh et al., 2006; Rada-Iglesias et al., 2011). These techniques have begun to be utilized to study limb development and we believe they will rapidly improve our understanding of the transcriptional events regulating the formation of the limb. Specifically, they will aid in the identification of cis-regulatory elements, the transcription factors to which they are bound and the genes that they regulate.

Genetic networks regulating limb development

In this portion of the review we will discuss the transcriptional events regulating three distinct processes in limb development;

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(1) Initiation of limb development, (2) establishment of developmental axes, (3) limb outgrowth and patterning. We have generated detailed gene regulatory networks for these three processes (Figs. 2–4). We depict the transcriptional processes of limb development using BioTapestry, a well-established, freely available, visualization tool that has been used to map multiple developmental processes (Longabaugh et al., 2005; Longabaugh et al., 2009). The network diagrams shown in this review are for the forelimb, but the largely similar hindlimb networks are available as supplementary material (Figs. S1–S3). The forelimb and hindlimb network diagrams are also available as a downloadable and modifiable resource. These networks can function as a powerful resource to help scientists contextualize novel data within previously published interactions. The primary references (PubMed identifiers) underlying each link can be obtained within the BioTapestry software by right-clicking the genetic link and selecting experimental data.

There are a number of challenges to visualizing dynamic gene regulatory networks during development. In constructing this gene regulatory network, we have made a number of assumptions and simplifications. First, we have only included genes and interactions with well-established roles; we have deliberately left out important genes such as *Prdm1/Blimp1* and *Lmx* members whose function in limb development is not well understood (Robertson et al., 2007; Tzchori et al., 2009). Processes depending on multiple, redundant members of gene families (e.g., 5′ *Hox* and *Bmp* genes) are shown as the collective effort of the gene family. Additional assumptions are pointed out in the context of individual figures. Most of the experimental data was generated through loss-of-function studies that do not precisely pinpoint the time-window for gene activity. We have in some cases had to use our best guess when determining when a particular process begins or ends. We use data obtained from mouse and chick experiments interchangeably. However, when different we have used mouse nomenclature (e.g., *Wnt3* instead of *Wnt3a*). Finally, our non-dynamic models do not depict quantitative variations in gene expression.

The experimental data underlying the network is discussed within the body of the review. However, a number of general features are readily apparent. For example, many genes and signaling pathways, such as the BMP genes, are used reiteratively in many different aspects of limb development and even perform conflicting functions. There is also a paucity of information regarding the activators or downstream targets of many of the signals regulating limb patterning (e.g., genes defining dorsal identity). We highlight unknown inputs or outputs as boxes. In addition, there are multiple levels of reciprocal signaling between the ectoderm and the mesoderm that occur at all stages of limb outgrowth. While the directionality of these signaling pathways is well understood, the genes and transcription factors mediating these signals are not. Finally, for most nodes in the network it is unknown whether the interactions are direct or indirect. We have indicated experimentally validated direct interactions by placing magenta diamonds under the relevant links in Figs. 2–4 and Figs. S1–S3. Unless explicitly stated otherwise, the genetic interactions discussed below are indirect. Well-characterized networks containing experimentally defined cis-regulatory inputs have been generated in invertebrate systems (reviewed in Davidson, 2010). There are currently no vertebrate developmental systems characterized to this level of detail, and as this information becomes available in future studies, it will allow for greater resolution of the network.

Initiation of limb bud development

This section of the review discusses the genetic interactions regulating the initiation of limb bud development. Fig. 2 and Fig. S1 display visual representations of these interactions for the forelimb and hindlimb, respectively. In the chicken, *Wnt2b* and *Wnt8c* are expressed in the lateral plate mesoderm in the future forelimb and hindlimb domains, respectively. Ectopic application of WNT2B or WNT8C to the flank of chicken embryos results in the formation of ectopic limbs (Kawakami et al., 2001). WNT2B and WNT8C promote limb development through their ability to stabilize β-catenin which can also stimulate ectopic limb development in chickens (Kawakami et al., 2001). *Wnt2b* is not expressed in the lateral plate mesoderm of mice and its deletion does not result in any overt limb phenotype (Ng et al., 2002; Witte et al., 2009). Nonetheless, early conditional deletion of *β-catenin* in the future hindlimb forming mesoderm

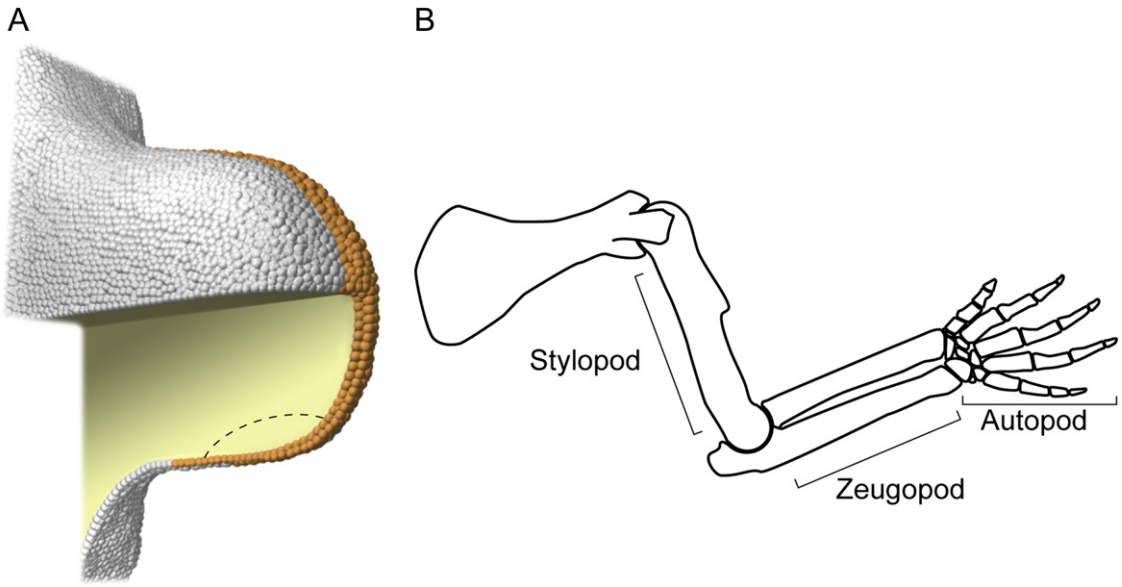

Fig. 1. Key features of limb development. (A) Schematic of a limb bud in the 'outgrowth' phase. The apical ectodermal ridge (AER) is highlighted in orange. The cutout show the interior mesenchyme (yellow) with the approximate boundaries of the zone of polarizing activity (ZPA) indicated by a dashed line. (B) The stereotypical structure of the vertebrate limb containing stylopod, zeugopod and autopod elements. The schematic shows a mouse limb; a chick limb has morphological differences but the same 3 proximal–distal domains. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

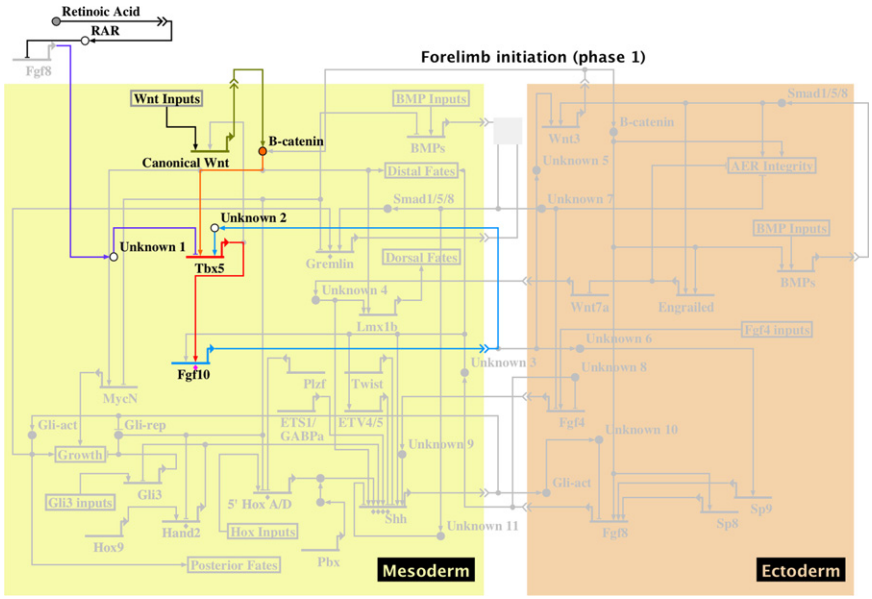

Fig. 2. Genetic interactions underlying forelimb induction. This figure summarizes the signals known to initiate the formation of the limb bud. Retinoic acid signaling from the mesoderm restricts expression of *Fgf8* in proximity to the future limb field. This prevents FGF8-mediated repression of *Tbx5*. The links between *Fgf8* and *Tbx5* are colored to add emphasis even though they are inactive in the *wildtype* limb. The induction of *Fgf10* is the key event in initiating limb bud formation. Probable direct interactions have a magenta diamond underneath the link. Other interactions may be direct or indirect. Colored circles indicate proteins that mediate the relevant transcriptional processes. Unknown transcriptional effectors of a secreted signaling molecule are represented by white circle labeled 'Unknown'. Unknown transcriptional inputs or outputs are highlighted within a box. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

causes a complete failure of hindlimb development with a similar requirement for β-catenin also proposed for the forelimb. Additional data shows that *Islet1* is also essential for hindlimb development and helps stabilize β-catenin. It is likely that the stabilization of β-catenin by *Islet1* occurs through the induction of an uncharacterized WNT ligand (Kawakami et al., 2011).

The TBX proteins, TBX5 and TBX4, are essential factors for forelimb and hindlimb development, respectively. Loss of *Tbx5* causes the failure of forelimb initiation while loss of *Tbx4* causes limb bud formation to halt prematurely (Agarwal et al., 2003; Naiche and Papaioannou, 2003; Naiche and Papaioannou, 2007; Rallis et al., 2003). Although *Tbx4* and *Tbx5* are expressed for extended periods during limb development, conditional deletion studies suggest that their functional role is limited to limb bud formation (Gibson-Brown et al., 1996; Hasson et al., 2007; Naiche and Papaioannou, 2007). The TBX proteins regulate the expression of *Fgf10*, which is essential for limb bud initiation (Sekine et al., 1999). In *Tbx5* null mice there is a complete loss of *Fgf10* expression in the forelimb, while in *Tbx4* null mice *Fgf10* expression is initiated in the hindlimb but not maintained (Naiche and Papaioannou, 2003; Naiche and Papaioannou, 2007; Rallis et al., 2003).

In the chicken, *Tbx5* is downstream of *Wnt2b* but whether *Tbx4* is downstream of *Wnt8c* is unknown (Ng et al., 2002; Rallis et al., 2003). WNT mediated induction of *Tbx5* is sufficient to explain the subsequent induction of *Fgf10* and the initiation of forelimb development. In the hindlimb however, *Fgf10* expression must be regulated by other factors in addition to *Tbx4*. Conditional deletion of either *β-catenin* or *Islet1* in mice results in a complete loss of *Fgf10* expression in the hindlimb but the epistatic relationship between these two inducers is currently unclear. Additional work in mice and chicken embryos has identified a positive feedback loop between *Fgf10* and the *Tbx* genes as well as feedback between the *Tbx* genes and *Wnt2b*/*8c* in chickens (see Fig. 2 and Fig. S1) (Agarwal et al., 2003; Ng et al., 2002; Sekine et al., 1999; Takeuchi et al., 2003). Although the *Tbx* genes continue to be expressed in the limb mesenchyme at later stages of limb development their only essential function is during limb bud initiation (Gibson-Brown et al., 1996; Hasson et al., 2007; Naiche and Papaioannou, 2007).

Retinoic acid also provides key signals in the initiation of forelimb bud development (compare Fig. 2 to Fig. S1). Genetic disruption of retinoic acid synthesis causes a loss of *Tbx5* expression and a loss of forelimb development (Mic et al., 2004; Niederreither et al., 2002; Sandell et al., 2007). Duester and colleagues propose that retinoic acid generates a permissive environment for forelimb development by repressing FGF8 signaling in the intermediate mesoderm alongside the forelimb forming domain (Zhao et al., 2009). A lack of *Fgf8* expression in proximity to the hindlimb forming domain might explain why retinoic acid signaling is not required for hindlimb development (Tzchori et al., 2009).

Dorsal–ventral axis formation

The following sections of the review discuss the transcriptional interactions establishing the developmental axes. Fig. 3 and Fig. S2 display visual representations of these interactions for the forelimb and hindlimb, respectively. The dorsal–ventral axis is established by unknown signals emanating from the somites that pattern the prelimb mesenchyme (Michaud et al., 1997). The regulation of dorsal–ventral patterning is then transmitted onto the nascent limb ectoderm. The ectoderm establishes dorsal–ventral polarity through the expression of *En1* and *Wnt7a* in the ventral and dorsal ectoderm, respectively. *En1* functions to restrict *Wnt7a* to the dorsal ectoderm. Loss of *En1* results in *Wnt7a* expression in the dorsal and ventral ectoderm and the loss of ventral structures such as foot pads (Loomis et al., 1996; Wurst et al., 1994). *Wnt7a* promotes dorsal cell fate by positively regulating *Lmx1b* (mouse) or *Lmx1* (chicken) expression in the sub-ectodermal mesenchyme (Chen et al., 1998; Dreyer et al., 1998; Riddle et al., 1995; Vogel et al., 1995). Loss of either *Wnt7a* or *Lmx1b* results in the ventralization of the dorsal limb such that paw pads are now present on the dorsal limb surface (Chen et al., 1998; Dreyer et al., 1998; Parr and McMahon, 1995). While WNT7A is initially thought to maintain *Lmx1b* expression through non-canonical (β-catenin independent) WNT signaling

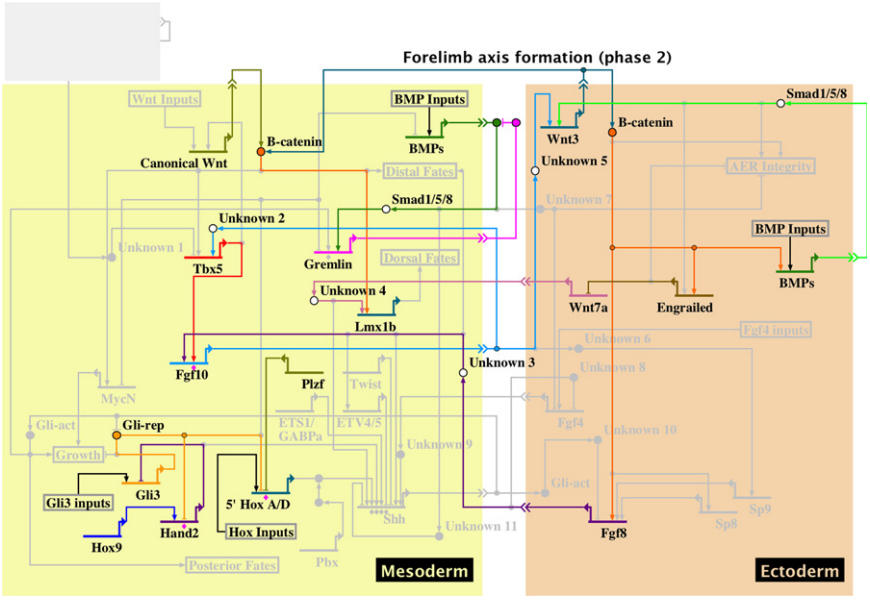

Fig. 3. Gene regulatory networks defining the developmental axes of the forelimb. The developmental axes that are essential for the patterning of the limb are established at the very earliest stages of forelimb formation. Mesenchymal *Fgf10* and epithelial *Fgf8* form a positive transcriptional feedback loop that is critical for limb development. How FGF8 and FGF10 regulate transcription is unknown. BMP signaling in the mesenchyme can negatively affect the formation of the future AER but this effect is countered by BMP mediated induction of the BMP antagonist *Gremlin*. Probable direct interactions have a magenta diamond underneath the link. Other interactions may be direct or indirect. Colored circles indicate proteins that mediate the relevant transcriptional processes. Unknown transcriptional inputs or outputs are highlighted within a box. Unknown transcriptional effectors of a secreted signaling molecule are represented by white circle labeled 'Unknown'. Smad 1/5/8 is also indicated with a white circle because it has not been definitively linked to the BMP-responsive processes depicted on the diagram. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

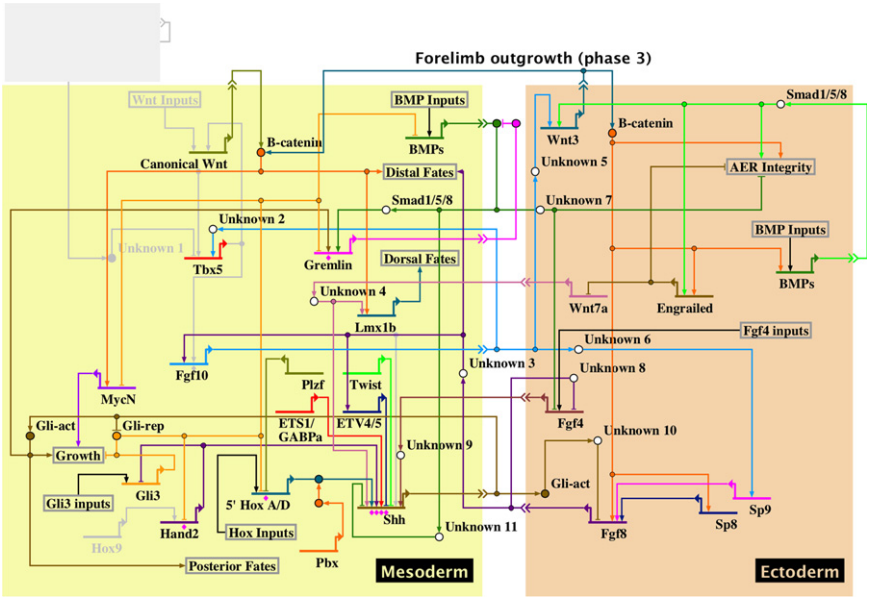

Fig. 4. Gene regulatory networks driving forelimb outgrowth. This figure summarizes the transcriptional events that drive the outgrowth of the forelimb and ultimately control the patterning of the forming limb. The domain labeled 'Ectoderm' now includes a morphologically distinct AER. The identity of factors controlling AER induction and maintenance are undefined. Initiation and maintenance functions have therefore been grouped together in the node labeled 'AER Integrity'. An additional epithelial-mesenchymal feedback between *Fgf4* and *Shh*, present at this time, is essential for regulating *Shh* expression and the subsequent formation of the posterior limb. Although *Plzf* is predominantly active in the hindlimb it does play a minor role in forelimb development. Probable direct interactions have a magenta diamond underneath the link. Other interactions may be direct or indirect. Colored circles indicate proteins that mediate the relevant transcriptional processes. Unknown transcriptional inputs or outputs are highlighted within a box. Unknown transcriptional effectors of a secreted signaling molecule are represented by white circle labeled 'Unknown'. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

there is evidence that a canonical (β-catenin dependent) signal also controls *Lmx1b* transcription (DeLaurier et al., 2006; Kengaku et al., 1998). Additional evidence suggests that WNT7A can also signal through the canonical WNT pathway in the limb and other tissues (Adamska et al., 2005; Adamska et al., 2004; Stenman et al., 2008). It is therefore currently unclear whether WNT7A, or some of the many additional WNT ligands expressed in the limb, are responsible for β-catenin dependent *Lmx1b* expression (Hill et al., 2006; Witte et al., 2009).

As shown in Fig. 3 and Fig. S2, ectodermal WNT and BMP signaling regulate ventral limb identity by positively regulating *En1* expression. Conditional deletion of *BmpR1a*, *Wnt3* or *β-catenin*, in the developing

mouse limb ectoderm, results in a loss of *En1* expression and a subsequent dorsalization of the ventral limb (Ahn et al., 2001; Barrow et al., 2003). Likewise, ectopic activation of WNT or BMP signaling in the dorsal ectoderm of developing chick limbs results in the expression of markers of the ventral ectoderm within the tissue (Barrow et al., 2003; Pizette et al., 2001).

Proximal–distal axis formation

The apical ectodermal ridge (AER) is a portion of thickened epithelium at the dorsal–ventral boundary of the limb that is essential for growth and patterning of the limb in the proximal–distal direction. In many experimental studies formation of the AER is presumed to be synonymous with the localized expression of the AER marker *Fgf8* along the distal limb ectoderm. This approach is based on that fact that discrete *Fgf8* expression correlates with the presence of the AER and that Fgf proteins can compensate for the surgical removal of the AER (Fallon et al., 1994; Niswander et al., 1993). However, it is important to recognize that a morphologically distinct AER is present even in the absence of *Fgf8* (Lewandoski et al., 2000; Moon and Capecchi, 2000; Sun et al., 2002). Moreover, in conditional *Fgf8*;*Fgf4* compound mutants, where ectodermal FGF signaling is significantly reduced, a morphologically distinct AER is present suggesting that ectodermal FGF signaling is not directly required for AER formation.

Formation of a single discrete AER is partially dependent on establishment of a dorsal–ventral axis in the limb. Prior to AER formation, many genetic markers of the future AER, including *Fgf8*, are initially expressed throughout the ventral ectoderm (Bell et al., 1998; Loomis et al., 1998). These markers eventually become restricted to the dorsal–ventral boundary of the limb where the AER will form. In mice lacking *En1*, expression of these AER markers persists in a broad domain of the ventral ectoderm (Loomis et al., 1996; Loomis et al., 1998). Occasionally more than one physically distinct AER will arise from the broad swath of cells expressing the AER markers. It has therefore been proposed *En1* regulates AER formation through the repression of an unidentified inducer of AER formation in the ventral ectoderm (Loomis et al., 1998).

The formation of the AER is also dependent on FGF10 signaling from the nascent limb mesenchyme. In *Fgf10* null embryos a thickening of the future limb ectoderm fails to occur, *Fgf8* is not expressed, and the limb development program is halted (Ohuchi et al., 1997; Sekine et al., 1999). The addition of cells expressing FGF10 to the flank of a chicken embryo results in the formation of an ectopic AER leading to the generation of an ectopic limb (Ohuchi et al., 1997). As shown in Fig. 3 and Fig. S2, *Fgf8* expression in the AER forms a positive feedback loop with *Fgf10* that is essential for the early initiation of limb growth (Ohuchi et al., 1997; Xu et al., 1998). The *Fgf10–Fgf8* loop is propagated through the Fgf receptor FGFR2 (Xu et al., 1998). Conditional ectodermal deletion of *FgfR2* following the initiation of limb bud development generates a forelimb with a normal stylopod and zeugopod but no autopod. This result suggests that the *Fgf8–Fgf10* feedback loop is active late into the limb development when the cells that will form the autopod are being generated (Lu et al., 2008). It has been proposed that *Twist1* might also regulate AER stability by mediating the *Fgf10–Fgf8* positive feedback loop (O'Rourke et al., 2002; Zuniga et al., 2002). *Twist1* null embryos have disrupted AER formation and maintenance that is most pronounced in the forelimb but the true cause of this defect is unclear (O'Rourke et al., 2002; Zuniga et al., 2002).

BMP and WNT signaling play a role in induction and maintenance of the AER that is independent of their role in regulating *En1* during establishment of dorsal–ventral polarity. While loss of *En1* can result in the formation of an additional AER, disruption of BMP or WNT signaling results in a loss of AER activity. Conditional ectodermal deletion of the BMP receptor *BmpR1a*, results in a complete loss of *Fgf8* expression and subsequent loss of distal limb structures (Ahn et al., 2001). Conditional deletion of *Wnt3* or *β-catenin* in the mouse limb ectoderm results in the failure to form a structurally distinct AER and disruption in *Fgf8* induction and maintenance (Barrow et al., 2003). Experimental evidence in chicken shows that *Wnt3a* performs the same functions as *Wnt3* in mice and also signals through *β-catenin* (Kawakami et al., 2001). *Wnt3*(*a*) is downstream of both ectodermal BMP signaling and mesenchymal FGF10 signaling (Kawakami et al., 2001; Soshnikova et al., 2003). The epistatic relationship between ectodermal BMP and mesenchymal FGF10 signaling in limb development is unknown.

The SP transcription factors regulate AER maintenance but not induction. Embryos in which *Sp8* has been deleted initially express *Fgf8* in the AER but this expression is not maintained resulting in severe distal limb truncations (Bell et al., 2003; Treichel et al., 2003). Likewise, both *Sp8* and *Sp9* have been shown to positively maintain the expression of *Fgf8* in chicken limbs (Kawakami et al., 2004). Both *Sp8* and *Sp9* are regulated by *Fgf10*; for *Sp9* this regulation is thought to be direct while for *Sp8* it is directed through WNT signaling (Kawakami et al., 2004).

Anterior–posterior axis formation

At the initiation of limb bud development an anterior–posterior axis is present within the nascent limb mesenchyme. The asymmetry is generated by the transcription factors *Gli3* and *Hand2* which mutually antagonize each other's expression as shown in Fig. 3 and Fig. S2. This co-repression results in *Gli3* expression being restricted to the anterior limb while *Hand2* expression is limited to the posterior limb (te Welscher et al., 2002a). Experimental evidence suggests that GLI3 might directly repress *Hand2* transcription but whether *Gli3* transcription is directly repressed by HAND2 in early limb development is unknown (Vokes et al., 2008). The loss of both *Gli3* and *Hand2* results in a limb without any distinct anterior–posterior identity (Galli et al., 2010). The initial events leading to the breaking of anterior–posterior symmetry of the limb remain unknown. Embryonic manipulations in chicken embryos indicate that the anterior–posterior axis is present in the lateral plate mesenchyme prior to the formation of the early limb (Michaud et al., 1997). For the forelimb the asymmetry is relayed by all four paralogous *Hox9* genes as when they are deleted there is a complete loss *Hand2* expression in the forelimb (Xu and Wellik, 2011). It is currently unknown whether the *Hox9* genes directly or indirectly regulate *Hand2* expression in the forelimb. It is tempting to speculate that other *Hox* genes regulate *Hand2* expression in the hindlimb, either in collaboration with or upstream of ISLET1, a hindlimb-specific regulator of *Hand2* (Itou et al., 2012).

Anterior *Gli3* expression not only restricts *Hand2* expression to the posterior limb but also represses the transcription of the genes at the centromeric end of the *HoxD* cluster (the 5′ *HoxD* genes—*Hoxd10, d11, d12 & d13*) (Litingtung et al., 2002; te Welscher et al., 2002b). This anterior repression is a critical event as *Hand2* and the 5′ *HoxD* genes have each been shown to be necessary and sufficient for the transcriptional activation of *Shh* which is expressed in a portion of the limb known as the zone of polarizing activity (ZPA) (Charite et al., 2000; Galli et al., 2010; Kmita et al., 2005; Knezevic et al., 1997; Tarchini et al., 2006). *Shh* expression from the ZPA regulates the number and identity of the digits formed in the limb, the ultimate readout of anterior–posterior patterning (Chiang et al., 1996; Riddle et al., 1993; Scherz et al., 2007; Towers et al., 2008; Yang et al., 1997; Zhu et al., 2008).

Both *Hand2* and the *Hox* genes are thought to directly activate *Shh* as they have been shown to bind the cis-regulatory element controlling *Shh* expression in the limb (Capellini et al., 2006; Galli et al., 2010). Transcription of *Hox* genes in the limb is positively regulated by the PBX transcription factors which also function as cofactors for Hox proteins in many developmental systems (Capellini et al., 2006; Capellini et al., 2011). Compound deletions of *Pbx* genes also result in loss of *Shh* expression. While PBX proteins also bind the regulatory element controlling *Shh* expression it is thought that their regulation of *Shh* primarily occurs indirectly via regulation of Hox transcription (Capellini et al., 2006). Interestingly *Shh* expression is also positively regulated by the dorsal–ventral patterning gene *Wnt7a* through an unknown mechanism (Parr and McMahon, 1995; Yang and Niswander, 1995). This data, represented diagrammatically in Fig. 3 and S2, demonstrates a clear genetic link between dorsal–ventral and anterior–posterior patterning.

Shh expression is restricted to the posterior limb in *wildtype* embryos but there is a discrete domain in the anterior limb that is also competent to expresses *Shh*. Deletion of *Gli3*, *Alx4*, *Etv4* & *Etv5*, *Twist1* and *EifC3* all result in ectopic expression of *Shh* in this anterior portion of the limb (Gildea et al., 2011; Krawchuk et al., 2010; Lettice et al., 2012; Mao et al., 2009; Masuya et al., 1995; Qu et al., 1997; Zhang et al., 2009). *Twist1* is expressed solely in the mesenchyme of developing limbs (Fuchtbauer, 1995). Conditional deletion of *Twist1* in the developing limb mesenchyme, driven by the limb specific *Prrx1-Cre*, results in a very different phenotype than that observed in the *Twist1* null embryos discussed above. While *Twist1* null embryos have AER defects, the later loss of *Twist1* results in ectopic *Shh* expression in the anterior limb and subsequent polydactyly (O'Rourke et al., 2002; Zhang et al., 2010; Zuniga et al., 2002). The difference in phenotype can be attributed to the fact that the role of *Twist1* in AER establishment occurs early, before *Prrx1-Cre* is active. In contrast, the role of *Twist1* in negatively regulating *Shh* expression occurs after *Prrx1-Cre* has removed the *Twist1* gene.

TWIST1 is capable of forming a homodimer with itself or a heterodimer with HAND2. Firulli and colleagues propose that TWIST1-HAND2 heterodimers positively regulate *Shh* expression while TWIST1–TWIST1 homodimers cannot (Firulli et al., 2007). This data fits with the fact that loss of *Twist1*, within the limb mesenchyme, results in ectopic anterior *Shh* expression and a polydactylous phenotype that is reduced in *Hand2* heterozygous mutants (Firulli et al., 2005; Krawchuk et al., 2010; Zhang et al., 2010). Likewise, over expression of *Hand2* causes pre-axial polydactyly and this effect is diminished by simultaneous over expression of *Twist1* (Firulli et al., 2005). *Etv4* and *Etv5*, as mentioned above, negatively regulate *Shh* expression (Mao et al., 2009; Zhang et al., 2009). This repression has been proposed to occur through direct and indirect mechanisms. Evidence for direct repression is the fact that ETV4 and ETV5 can bind the regulatory element controlling *Shh* expression in the limb (Lettice et al., 2012). Alternatively, in tissue culture assays ETV5 can also negatively regulate the formation of TWIST1–HAND2 heterodimers which are thought to activate *Shh* expression (Firulli et al., 2007; Zhang et al., 2010). Consistent with these roles, loss of *Etv4* and *Etv5* in the limb causes ectopic anterior *Shh* expression and polydactyly, that like the *Twist1* mutants, is reversed in *Hand2* heterozygous animals (Zhang et al., 2010; Zhang et al., 2009).

Ectodermal signaling and limb outgrowth

The following sections of the review discuss the transcriptional interactions driving the outgrowth of the developing limb. Fig. 4 and Fig. S3 display visual representations of these interactions for the forelimb and hindlimb, respectively. The outgrowth is partially regulated by signals emanating from two key signaling centers; the AER and ZPA (see Fig. 1). As we discuss here, non-AER ectoderm is an additional key signaling centre that is vital for limb bud development.

Surgical removal of the AER results in induction of apoptosis in the cells underlying the AER, leading to severe limb truncations (Dudley et al., 2002; Rowe et al., 1982). Ectopic application of FGF proteins to limbs in which the AER has been removed can largely restore outgrowth of the limb (Fallon et al., 1994; Niswander et al., 1993). There are four *Fgf* genes expressed in the AER of mice *Fgf4*, *8*, *9* and *17* (Sun et al., 2000). *Fgf8* is initially expressed throughout the ventral ectoderm but become restricted to the AER as it forms. *Fgf4, Fgf9* and *Fgf17* expression initiates during AER maturation and display a more restricted expression domain within a subset of AER cells. The expression domain of *Fgf4*, but not *Fgf9* or *Fgf17*, is expanded to cover the entire AER upon deletion of *Fgf8* (Lewandoski et al., 2000). Of the four *Fgf* genes *Fgf8* plays the most significant role in limb development (Lewandoski et al., 2000; Mariani et al., 2008; Moon and Capecchi, 2000). The key role of *Fgf8* is likely due to its extended temporal and spatial expression pattern as the product of the *Fgf* genes function in a largely identical manner in the developing limb (Lu et al., 2006; Mariani et al., 2008).

FGF proteins are secreted from the AER while WNT proteins are secreted from the entire limb ectoderm (Barrow et al., 2003; ten Berge et al., 2008; Witte et al., 2009). In the distal limb these two signaling cascades combine to maintain a population of pluripotent mesenchymal cells which will generate distal limb structures (see Fig. 4 and Fig. S3) (Cooper et al., 2011; Rosello-Diez et al., 2011; ten Berge et al., 2008). In the more proximal limb, ectodermal WNT signaling can promote connective tissue cell fates in the mesenchyme by signaling in the absence of FGF protein (ten Berge et al., 2008). Genetic disruption of FGF or WNT signaling in the limb ectoderm also causes significant apoptosis in the limb mesenchyme demonstrating their additional role as cell survival factors (Barrow et al., 2003; Boulet et al., 2004; Sun et al., 2002). Furthermore, WNT3 plays a role in driving mesenchymal cell proliferation through its transcriptional regulation of *Mycn* (ten Berge et al., 2008).

ZPA signaling and limb outgrowth

Loss of function mutations of *Shh* causes a loss of all posterior structures in the distal limb leaving only a single digit and the most anterior zeugopod element (Chiang et al., 2001; Chiang et al., 1996; Kraus et al., 2001). In contrast, ectopic SHH protein can induce ectopic growth in the anterior–posterior axis of the limb and subsequent polydactyly (Yang et al., 1997). SHH regulates transcription through the three vertebrate GLI transcription factors, GLI1-3. In the presence of Hedgehog signaling, nascent GLI proteins are processed to become transcriptional activators. In the absence of Hedgehog signaling GLI3, and to a limited extent GLI2, are cleaved to become transcriptional repressors (Dai et al., 1999; Pan et al., 2006; Ruiz i Altaba, 1999; Wang et al., 2000). GLI1, whose expression is positively regulated by Hedgehog signaling, functions solely as a transcriptional activator (Dai et al., 1999; Sasaki et al., 1999). *Gli1*;*Gli2* double null mouse embryos have limited limb malformations demonstrating only a limited role for these genes in limb morphogenesis (Park et al., 2000). In contrast, loss of *Gli3* results in significant polydactyly with up to 9 digits being present on a single limb (Hui and Joyner, 1993; Johnson, 1968).

Hedgehog target genes in the limb can be split into two categories. The first group requires SHH to relieve GLI mediated repression. These genes are down-regulated in *Shh* null embryos but rescued in *Shh*;*Gli3* double null embryos in which most, if not

all, GLI repression is removed. The second group of SHH target genes require the formation of GLI activator for expression. Only a few genes have been described that depend exclusively on GLI activator including *Gli1* itself and *Ptch1*, encoding the SHH receptor (Litingtung et al., 2002; te Welscher et al., 2002b). *Gli1* transcription is reduced in the developing limbs of *Gli3* null embryos demonstrating the ability of GLI3 to also function as a transcriptional activator (Hui and Joyner, 1993; Litingtung et al., 2002). A limited role for GLI3 activator in limb patterning has been postulated but this remains controversial (Hill et al., 2009; Wang et al., 2007).

A major functional role for SHH in the limb is to limit the repressive activity of GLI3. The additional loss of *Gli3* in *Shh*:*Gli3* double null embryos largely rescues the *Shh* null phenotype, resulting in a polydactyly that is similar to that observed in *Gli3* mutant embryos (Litingtung et al., 2002; te Welscher et al., 2002b). The identification of the direct transcriptional targets regulated by GLI3 has provided important insights into how SHH regulates anterior–posterior polarity and limb outgrowth. The truncated limb observed in *Shh* null mice can be partially explained by a significant increase in apoptosis and reduced cell proliferation in the limb mesenchyme (Chiang et al., 2001; te Welscher et al., 2002b; Zhu et al., 2008). Two positive regulators of the cell cycle, *Mycn* and *Cyclin-D1*, are downstream of SHH in the chicken limb (Towers et al., 2008). Limb mesenchyme specific deletion of *Mycn* in mice causes loss of autopod growth and subsequent soft tissue syndactyly (Ota et al., 2007). Mice carrying null mutations of *Cyclin-D1* do not have a limb phenotype but this could be due to redundancy with additional Cyclin proteins (Fantl et al., 1995). Genome-wide chromatin immunoprecipitation (ChIP) studies in the mouse limb with an epitope tagged GLI3-repressor protein, combined with gene expression profiling, identified 205 putative direct GLI target genes (Vokes et al., 2008). In addition to identifying GLI3 bound enhancer elements in proximity to known Hedgehog pathway targets such as *Ptch1* and *Gli1*, the study defined additional GLI3 bound cis-regulatory elements in proximity to *Gremlin*, *Hand2* and *Prdm1* (*Blimp1*). The study also identified putative GLI3 bound cis-regulatory elements in proximity to *Hox* genes and genes implicated in several major developmental signaling pathways (Vokes et al., 2008).

Given its pivotal role, it is not surprising that *Shh* functions in a number of auto-regulatory feedback loops that regulate *Shh* expression in a process that is essential for correct patterning of the limb (Sanz-Ezquerro and Tickle, 2000). Recent papers have described two negative feedback pathways through which this auto-regulation occurs. *Bmp* genes are positively regulated by *Shh* while BMP proteins function to repress *Shh* expression (Bastida et al., 2009; Chiang et al., 2001; Drossopoulou et al., 2000; Laufer et al., 1994; Yang et al., 1997). In support of this first feedback pathway, the *Shh* expression domain is expanded in *Bmp* compound mutant mice (Bandyopadhyay et al., 2006). The second negative feedback loop occurs through reception of mesenchymally expressed SHH which represses *Fgf* expression in the posterior AER (Bouldin et al., 2010). AER derived FGF proteins have been shown to positively regulate the expression of *Shh* (Boulet et al., 2004; Lu et al., 2006; Sun et al., 2002).

AER–ZPA feedback loop

A positive feedback loop between FGF signaling from the AER and SHH signaling in the ZPA plays an essential role in limb development by maintaining expression of *Shh* (see Fig. 4 and S3) (Bastida et al., 2009; Laufer et al., 1994; Niswander et al., 1994; Zuniga et al., 1999). This positive feedback loop contrast with the two negative feedback loops controlling *Shh* expression that were described above (Bastida et al., 2009; Bouldin et al., 2010). *Fgf4* is expressed in the AER close to the *Shh* expression domain. Deletion of *Fgf4* does not however disrupt *Shh* expression due to compensation by *Fgf8* (Moon et al., 2000; Sun et al., 2000). Ectodermal loss of both *Fgf4* and *Fgf8* results in a loss of *Shh* expression (Boulet et al., 2004; Sun et al., 2002). In many developmental systems FGF proteins exert their biological function through the ETS transcription factors (Brent and Tabin, 2004; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). The ETS transcription factors ETV4 and ETV5 are expressed in the limb mesenchyme in an FGF dependent manner. However, loss of ETV activity does not result in the reduced *Shh* expression nor the severe limb truncations observed in compound *Fgf* null mice (Boulet et al., 2004; Mao et al., 2009; Sun et al., 2002; Zhang et al., 2009). Recent data demonstrates that the two additional ETS proteins ETS1 and GABPA can directly bind and activate the regulatory elements controlling *Shh* expression in the limb (Lettice et al., 2012). Both proteins are expressed in the distal mesenchyme underlying the AER and therefore may mediate FGF induction of *Shh* transcription.

Shh maintains FGF signaling by inhibiting the activity of BMP proteins that would otherwise disrupt AER integrity (Laufer et al., 1994; Niswander et al., 1994; Pizette and Niswander, 1999; Zuniga et al., 1999). Specifically, SHH represses BMP activity by upregulating expression of *Gremlin*, a secreted glycoprotein that binds to and antagonizes BMP protein (see Fig. 4 and Fig. S3) (Khokha et al., 2003; Merino et al., 1999; Michos et al., 2004; Nissim et al., 2006; Sun et al., 2006; Vokes et al., 2008). Although *Gremlin* encodes a BMP antagonist, its transcription is actually induced by BMP proteins and then later maintained by SHH (Benazet et al., 2009; Capdevila et al., 1999; Nissim et al., 2006; Panman et al., 2006; Zuniga et al., 1999). SHH is thought to directly regulate *Gremlin* expression through a GLI dependent enhancer found in the *limb deformity* locus (Vokes et al., 2008; Zuniga et al., 2004).

There are two different models that explain the eventual termination of the AER–ZPA feedback loop in *wildtype* limbs. Work in the chicken demonstrates that the former *Shh* expressing cells, which originate from the ZPA, cannot express *Gremlin* (Scherz et al., 2004). As these ZPA descendents proliferate there is an increasing distance between the cells still expressing *Shh* and the cells able to express *Gremlin*. Eventually, the distance between the *Shh* and *Gremlin* expressing cells becomes greater than the diffusion distance of the SHH ligand and *Gremlin* expression is no longer activated (Scherz et al., 2004). This regulatory model suggests that *Gremlin* expression would decrease before loss of *Fgf4* expression, which is observed in chickens. However, in the mouse, *Fgf4* expression is found to decrease before *Gremlin* expression is reduced (Scherz et al., 2004; Verheyden and Sun, 2008). It has also been demonstrated in mice that high levels of FGF protein can inhibit *Gremlin* expression (Verheyden and Sun, 2008). This data has lead to an alternative model where increasing FGF signaling from the AER progressively increases the distance between the *Gremlin* expression domain and the distal limb. Eventually, *Gremlin* can no longer inhibit the negative effects of BMP proteins on AER maintenance in the distal limb leading to a loss of *Fgf4* expression (Verheyden and Sun, 2008). It is currently unclear whether these two different models truly represent interspecies difference or if they are the result of different experimental approaches.

A comparative timeline of *Shh*, *Fgf4* and *Fgf8* expression along with AER formation and regression, for mouse and chicken, is shown in Fig. S4. Note that *Fgf8* expression in the distal ectoderm is maintained for a number of days following the loss of both *Fgf4* and *Shh* expression (Jurand, 1965; Pajni-Underwood et al., 2007; Sanz-Ezquerro and Tickle, 2003; Wanek et al., 1989). This late distal *Fgf8* expression plays an important functional role in positively regulating the number and length of the forming phalanges

(Sanz-Ezquerro and Tickle, 2003). This data demonstrates that *Fgf8* expression becomes independent of the AER–ZPA feedback loop whose key function appears to be the temporal regulation of *Shh* expression.

Distinct patterning of the forelimb and hindlimb

A number of differences between the genetic networks regulating forelimb and hindlimb have been discussed in the previous sections. Genes that display differential expression or activity in regards to forelimb and hindlimb development in mice include *Raldh2*, *Rdh10*, *Islet1*, *Hox(a/b/c/d)9*, *Plzf*, *Tbx4* and *Tbx5* (Barna et al., 2000; Cunningham et al., 2011; Gibson-Brown et al., 1996; Kawakami et al., 2011; Niederreither et al., 2002; Xu and Wellik, 2011). While these genes play distinct roles in forelimb and hindlimb development they have yet to be proven to play a role in the distinct patterning of the two structures. One of the genes that is known to regulate hindlimb identity is *Pitx1*, a transcription factor that is expressed in the future hindlimb forming domain of the flank mesenchyme (Lanctot et al., 1997). Deletion of *Pitx1* in mice causes loss of the knee-cap and other skeletal structures that are unique to the hindlimb (Lanctot et al., 1999; Szeto et al., 1999). Likewise, ectopic expression of *Pitx1* in the developing forelimb of mice and chick generates novel bone and soft tissue articulations that are reminiscent of those observed in the hindlimb (DeLaurier et al., 2006; Szeto et al., 1999).

Tbx4 and *Tbx5* encode transcription factors that are specifically expressed in the developing hindlimb and forelimb, respectively (Gibson-Brown et al., 1996). *Pitx1* directly regulates the expression of *Tbx4* and it is currently a matter of contention whether *Pitx1* partially regulates hindlimb identity through this interaction (see Fig. S3) (Duboc and Logan, 2011; Menke et al., 2008; Ouimette et al., 2010). While *Tbx5* is important for the initiation of forelimb development it does not play a specific role in regulating the architecture of the forelimb. The forelimb truncations observed upon conditional deletion of *Tbx5* can be rescued by expression of a *Tbx4* transgene with no significant changes in forelimb patterning (Minguillon et al., 2005).

Proximal–distal skeletal patterning

The transcriptional mechanisms regulating proximal–distal development are still poorly understood. Experiments over the last decade have largely discredited the classical "progress zone" model in which the AER maintains a pool of proliferating cells in the distal mesenchyme that have an autonomous timing mechanism that promotes them to progressively more distal cell fates over time (Dudley et al., 2002; Fernandez-Teran et al., 2006; Galloway et al., 2009; Summerbell et al., 1973). In the absence of the "progress zone" model, new models have attempted to explain proximal–distal patterning on the basis of multiple inductive signals. The formation of distal structures can be partially explained by AER derived FGF and WNT signals which promote cells to progressively more distal cell fates dependent on the time under the influence of the signal (see Fig. 4 and Fig. S3) (Cooper et al., 2011; Mariani et al., 2008; Mercader et al., 2000; ten Berge et al., 2008).

It has been hypothesized that retinoic acid signaling from the flank mesoderm provides a key signal controlling the generation of proximal structures (Mercader et al., 2000; Tabin and Wolpert, 2007). In support of this idea, two publications have shown that retinoic acid signaling is required for the specification of proximal cell fates in cells from disaggregated chicken limb buds (Cooper et al., 2011; Rosello-Diez et al., 2011). In mouse embryos FGF signaling from the AER functions to limit retinoic acid signaling in the distal limb via the induction of *Cyp26b1*. The CYB26B1 protein functions to metabolize retinoic acid and thereby restrict the expression of retinoic acid target genes to the proximal limb (Probst et al., 2011). In agreement with this, mutation of *Cyp26b1* results in loss of distal markers and an expansion of proximal limb markers in both the forelimb and hindlimb (Yashiro et al., 2004). Additional experimental data, generated in mice, has however questioned the role of retinoic acid in proximal–distal patterning. Mutation of the enzymes responsible for retinoic acid synthesis in the developing limb of mouse embryos results in a limb with a normal proximal–distal pattern (Cunningham et al., 2011; Zhao et al., 2009). Various explanations have been proposed to resolve the seemingly conflicting results regarding the role of retinoic acid in proximal–distal patterning. It is possible that the experiments ablating retinoic acid synthesis in mice were not successful in eliminating all retinoic acid signaling, despite the widespread controls that were in place (Cunningham et al., 2011; Zhao et al., 2009). It is also possible that retinoic acid is not the sole signal from the flank to induce proximal cell fates despite the loss of proximal identities upon treatment of chick limbs with an antagonist of retinoic acid signaling (Rosello-Diez et al., 2011). Due to the lack of clearly defined role for retinoic acid in mouse proximal–distal patterning we have left it absent from the final temporal stage of our gene regulatory network (see Fig. 4 and Fig. S3).

Hox genes play a key role in the generation of the mature skeletal elements in the limb. There are four *Hox* clusters in mammals containing a variable contingent of the 13 paralogous *Hox* genes. Both *HoxA* and *HoxD* clusters play an overlapping role in the development and patterning of the limb (Davis et al., 1995; Dolle et al., 1993; Fromental-Ramain et al., 1996b; Kmita et al., 2005). Deletion of both *Hoxa13* and *Hoxd13* causes complete loss of mature digits in the autopod (Fromental-Ramain et al., 1996b). Deletion of the both *Hoxa11* and *Hoxd11* causes truncation of the zeugopod that is more severe in the forelimb than the hindlimb (Davis et al., 1995). Deletion of both *Hoxa9* and *Hoxd9* causes minor truncations of the stylopod in the forelimb only (Fromental-Ramain et al., 1996a). This pattern, where the position of the *Hox* gene in the *Hox* cluster correlates with location of the tissue in which the gene is active, is reminiscent of how *Hox* genes pattern the main body axis (Krumlauf, 1994). Further analysis of compound *Hoxa11*;*Hoxd11* mutants suggests that the *Hox* genes are involved in the maturation of skeletal elements within the limb rather than their initial specification (Boulet and Capecchi, 2004). How the HOX transcription factors regulate cell proliferation and/or differentiation is almost completely unknown due the lack of well defined target genes.

A number of genes have been shown to regulate *Hox* transcription in the limb. As discussed above PBX transcription factors positively regulate *Hox* transcription and compound *Pbx* mutants lose all distal limb structures (Capellini et al., 2006). PLZF is a DNA binding protein associated with transcriptional repression and *Plzf* null mice have limb deformities, primarily occurring in the hindlimb, that affect all three segments of the limb (Barna et al., 2000). The mutations are attributed to the fact that PLZF directly represses transcription of genes in the *HoxD* cluster (Barna et al., 2002). The predominance of the phenotypic defects in the hindlimb demonstrates that there is differential regulation of the *HoxD* genes between the forelimb and hindlimb (Barna et al., 2000; Barna et al., 2002). *Plzf*;*Gli3* double mutant embryos have an almost complete loss of the stylopod in the hindlimb (Barna et al., 2005). This genetic interaction might well be due to the fact that both *Plzf* and *Gli3* have overlapping roles in repressing *Hox* transcription in the developing limb (Barna et al., 2000; Barna et al., 2002; Litingtung et al., 2002; te Welscher et al., 2002b).

Digit specification

The ultimate readout of distal limb development is the formation of correctly specified digits. Despite intense interest,

genetic markers of each digit have not been found and seem increasingly unlikely to exist. Digit identity is a function of the number and length of the phalanges and these characteristics could be simply regulated by differential cell proliferation regulated by differential expression of non-unique genetic factors. The factors implicated in the growth and patterning of the digits are discussed below.

As discussed above, SHH signaling is a critical driver of anterior–posterior limb outgrowth and plays an essential role in regulating both digit number and identity (Chiang et al., 2001; Riddle et al., 1993; Yang et al., 1997). SHH controls digit patterning by acting as both a morphogen as well as a mitogen (Towers et al., 2008; Zhu et al., 2008). Data generated by the Mackem group has suggested that the morphogen and mitogen activities of SHH are separated in time. In this "biphasic model" SHH activity is required briefly to pattern the forming limb and then functions solely to generate a sufficient cell population with which to generate the digits (Zhu et al., 2008). The "biphasic model" is at odds with the "temporal model" of digit patterning which suggests that the SHH signal is integrated by cells over extended periods of time (Harfe et al., 2004; Scherz et al., 2007). Such an integrative morphogen model is consistent with recent studies examining how SHH patterns the neural tube (Balaskas et al., 2012; Dessaud et al., 2007). A recent review discusses the data utilized to generate both the biphasic and temporal models and attempts a synthesis of the two models (Towers and Tickle, 2009).

Despite the unequivocal role of SHH in regulating digit development, the identity of the developing digits in chicken embryos is still plastic at timepoints long after *Shh* has ceased to be expressed (Dahn and Fallon, 2000). At these later timepoints, digit identity can be changed by experimentally manipulating the interdigit mesenchyme (Dahn and Fallon, 2000). Presumably, the SHH signal is relayed by additional factors that ultimately control digit identity. While the identity of the interdigit-mesenchyme relay signal is currently unknown perhaps the best investigated candidates are the BMP proteins (Dahn and Fallon, 2000; Drossopoulou et al., 2000). Three *Bmp* genes, *Bmp2*, *Bmp4* and Bmp7, are expressed in the developing limb mesenchyme of which *Bmp2*, *Bmp7*, and to a limited extent, *Bmp4*, are dependent on *Shh* signaling (Chiang et al., 2001; Drossopoulou et al., 2000; Laufer et al., 1994; Yang et al., 1997). Ectopic application of the BMP antagonist Noggin to the interdigit mesenchyme of developing chicken limbs can alter digit identity (Dahn and Fallon, 2000). Moreover, each forming digit is associated with unique levels of phosphorylated SMAD1, 5 & 8, the transcription factors mediating canonical BMP signaling (Suzuki et al., 2008). It has been proposed that BMP signals may partially regulate phalange length and number by regulating *Fgf8* expression in the vestiges of the AER overlying the forming digits (Sanz-Ezquerro and Tickle, 2003). Arguing against a role for BMP signaling in digit specification is analysis of *Bmp* compound mutant mice. Mice limbs lacking *Bmp2* and *Bmp7*, the primary *Shh* responsive *Bmp* genes, are patterned normally with no overt changes in digit identity (Bandyopadhyay et al., 2006). A more definitive study would be provided by studies that completely ablate BMP signaling.

Another mechanism through which SHH might control digit specification is the regulation of the *Hox* transcription factors. Deletions of *Hox* genes *d11*, *d12*, *d13* and *a13* have all been linked to digit malformations in mice and the transcription of all these genes is regulated by SHH signaling (Chiang et al., 2001; Dolle et al., 1993; Fromental-Ramain et al., 1996b; Zakany and Duboule, 1996). The most pronounced effect on digit development is observed with a double deletion of *Hoxa13* and *Hoxd13* when an almost complete loss of the forming digits is observed (Fromental-Ramain et al., 1996b). Furthermore, mutation of *Hoxa13* or *Hoxd13* causes limb deformities in both humans and mice (Caronia et al., 2003; Johnson et al., 1998). Interestingly the HOXA and HOXD proteins have been suggested to regulate the transcription of *Bmp* genes and physically interact with SMAD transcription factors (Knosp et al., 2004; Salsi et al., 2008; Williams et al., 2005).

Character and function of cis-regulatory elements

In the previous sections we reviewed the scientific literature in order to generate networks describing three temporal phases of limb development. Of the large number of genetic interactions outlined less than 10 are known to be direct. In the vast majority of cases it is currently unknown whether there are intermediaries in the described genetic interaction and what the number and identity of these intermediaries might be. Much of the dynamic transcriptional activity occurring during development is regulated by distal cis-regulatory domains that function to activate or inhibit transcription (Chandler et al., 2007; Jeong et al., 2006; Montavon et al., 2011; Pennacchio et al., 2006; Ruf et al., 2011). The identification of cis-regulatory elements, the genes that they regulate and the interacting transcription factors is therefore vital for truly understanding the transcriptional regulation of limb development. The best demonstration of such detailed mapping of cis-regulatory inputs within vertebrate cells is the study of the transcriptional network regulating embryonic stem cell pluripotency (Boyer et al., 2005; Kim et al., 2008; Kim et al., 2010a; Wang et al., 2006). The following is a summary of what is currently known regarding the character and function of cis-regulatory elements within the developing limb and wider embryo.

Evolutionary conservation of enhancers

Since many of the genetic circuits regulating developmental processes, including the growth and patterning of the limb, are highly conserved it is not surprising that many of the cis-regulatory elements mediating this process are also highly conserved. Testing intergenic genomic sequences highly conserved between humans and *Fugu* and other elements perfectly conserved between human and rodents revealed that 42% and 61%, respectively, displayed tissue specific enhancer activity (Pennacchio et al., 2006). However, experimental work in *Drosophila* indicates that not all enhancer elements are evolutionarily conserved even when they are performing an evolutionary conserved function (Gompel et al., 2005; Swanson et al., 2011). Likewise, data from the mouse heart shows that many functional enhancer elements are not conserved through the mammalian lineage (Blow et al., 2010). So while cis-regulatory elements are far more likely to be evolutionary conserved than random intergenic sequences the absence of conservation in a section of DNA does not preclude enhancer activity.

Enhancer associated proteins and chromatin modifications

Strategies that identify active developmental enhancers, based on their association with distinct protein markers, represent a powerful and potentially unbiased way to understand gene regulation during development. A number of protein markers of active and inactive enhancers have been identified through extensive ChIP studies (summarized in Fig. 5). P300 is a member of enhancer associated protein complexes and as such has been used to identify active enhancer elements (Heintzman et al., 2007; Xi et al., 2007). Of the 2105 p300 binding sites identified in the limb using ChIP, 88% of the most highly conserved elements displayed reproducible enhancer activity (Visel et al., 2009). RNA polymerase II also binds to enhancer elements. This binding is thought to occur either through interactions with p300 or

because distal enhancers physically interact with the transcriptional complex assembled at a gene's transcriptional start site (Kim et al., 2010b). The presence of RNA polymerase II at enhancers results in transcription at the enhancer element that can also serve as a marker of active enhancer elements (Creyghton et al., 2010; Kim et al., 2010b).

Specific histone modifications have also been experimentally linked to the presence of enhancers in a wide variety of different cell types. One of the most widely used markers is monomethylation of the fourth lysine of histone H3 (H3K4me1) (Birney et al., 2007; Heintzman et al., 2007; Xi et al., 2007). The H3K4me1 modification is seemingly present at not only active enhancer elements but also inactive elements that are poised for future activity (Creyghton et al., 2010; Rada-Iglesias et al., 2011). Extragenic regions marked by acetylation of lysine 27 of histone H3 (H3K27ac) more closely associate with actively expressed genes than regions marked by H3K4me1 and/or p300 (Creyghton et al., 2010). A recent study using the H3K27ac histone modification identified 21,934 putative enhancer elements in the limb (Cotney et al., 2012). For a given locus, H3K27ac was comparatively enriched in specific portions of the limb in which proximal genes were transcriptionally active. For example, genes that were specifically transcribed in the posterior portion of the limb were found to associate with higher levels of H3K27ac in the posterior limb than the anterior limb (Cotney et al., 2012).

Functional redundancy of enhancer elements

The ultimate test of the contribution of a cis-regulatory element to the transcriptional regulation of a gene is to determine the consequence of its mutation or deletion. A number of distinct regulatory elements that are active in the developing limb have been analyzed through such mutational studies. These studies provide insights into the complexity of transcriptional regulation in the limb. Deletion of single regulatory elements associated with the transcriptional regulation of *Tbx4*, *HoxD* and *Prrx1* genes results in limited transcriptional and phenotypic effects (Cretekos et al., 2008; Menke et al., 2008; Montavon et al., 2011). This demonstrates that for these genes, and likely many others, there are multiple functionally-overlapping enhancer elements regulating gene expression. In mice, a number of genomic loci have been found to contain multiple enhancer elements that drive gene expression in overlapping domains (Jeong et al., 2006; Lee et al., 2011; Werner et al., 2007). Such functionally overlapping elements have also been identified and functionally assessed in invertebrates. In *Drosophila* multiple semi-redundant enhancer elements, termed shadow enhancers, provide robustness to the transcriptional process by stopping perturbations caused by environmental stresses (Frankel et al., 2010; Hobert, 2010; Hong et al., 2008; Perry et al., 2010). The presence of these redundant enhancers complicates mutational analysis as the consequence of deleting a putative cis-regulatory element may only be fully realized upon the deletion of additional elements (see Fig. 5).

Paradigms for the transcriptional regulation of limb development

The previous section describes the general character and function of developmentally active cis-regulatory elements. A great deal of work has already gone into identifying and characterizing the specific cis-regulatory elements active within the limb. We summarize these studies in the context of work done to understand the regulation of *Shh* and the *HoxD* transcription for which our knowledge is most advanced.

Transcriptional regulation of Shh

Shh expression in the limb is controlled by a regulatory element that lies 850 kb away from the *Shh* transcriptional start site in an intron of the *Lmbr1* gene (Lettice et al., 2002; Sagai et al.,

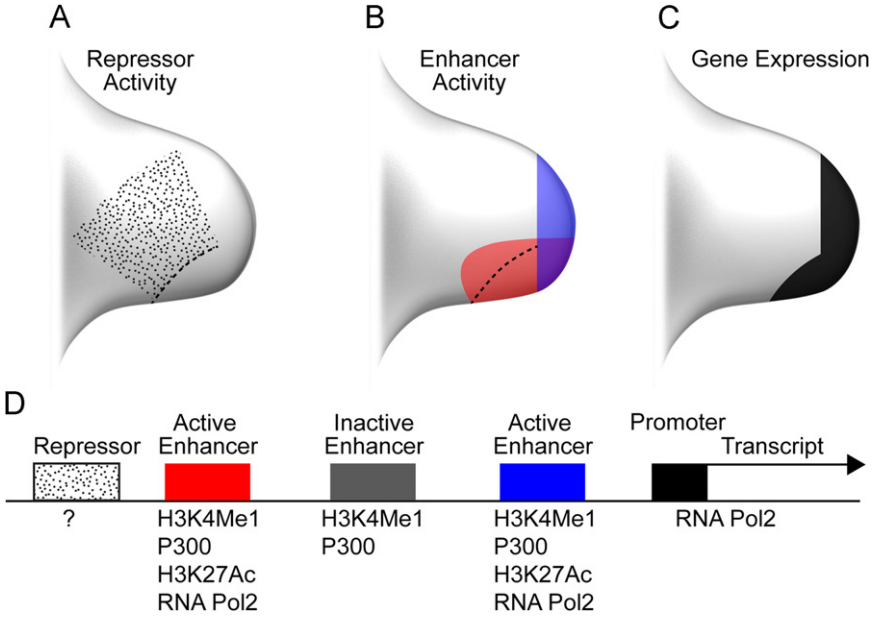

Fig. 5. Multiple cis-regulatory inputs coordinate transcription of developmental genes. (A)–(C) We present a generalized model for how multiple cis-regulatory elements are integrated to produce a transcriptional output. Gene transcription reflects the integration of positive and negative cis-regulatory inputs. Individual domains of enhancer activity are often larger than the corresponding gene expression domain (compare (B) to (C)), suggesting the presence of additional repressor domains (A). The two functioning enhancers (B) are active in a partially overlapping domain. Such semi-redundant enhancer elements have been frequently observed in the transcriptional regulation of genes in the developing limb. (D) Cis-regulatory elements can be identified by the localization of a number of protein markers. A number of protein markers initially linked to active enhancer elements have also been associated with inactive enhancer elements that are poised for activation at later developmental time points. Currently active enhancers associate with a number of protein markers not present at these poised enhancers. Specific protein markers capable of globally identifying repressor elements have yet to be described.

2005). This 2.2 kb regulatory element has been termed the ZRS (ZPA regulatory sequence). The presence of such distant cis-regulatory elements that lie within or beyond neighboring genes is not unique to *Shh* but it also found for cis-regulatory elements controlling *Gremlin* and *HoxD* expression (Gonzalez et al., 2007; Montavon et al., 2011; Vokes et al., 2008; Zuniga et al., 2004). The ZRS was identified by mapping mutations that cause ectopic expression of *Shh* in the anterior limb and pre-axial polydactyly in both mice and humans (Lettice et al., 2002). Similar point mutations in the ZRS have been found to cause polydactyly in a range of additional species including cats, dogs and chickens (Lettice et al., 2008; Maas et al., 2011; Park et al., 2008). In contrast, deletion of the entire ZRS element causes a complete loss of *Shh* expression in the limb (Maas et al., 2011; Sagai et al., 2005). The ZRS must therefore contain regulatory elements that both positively and negatively regulate *Shh* expression in the limb.

A number of transcription factors have been shown to activate *Shh* transcription and of these HAND2, HOXD10, HOXD13 and PBX proteins have been shown to bind directly to the ZRS (Capellini et al., 2006; Galli et al., 2010). Additional transcriptional activators ETS1A and GABPA bind the ZRS although their role in *Shh* regulation has not been fully confirmed (Lettice et al., 2012). Two known negative regulators of *Shh* expression, ETV4 and ETV5 also bind the ZRS (Lettice et al., 2012). Although the ZRS is by far the most densely characterized cis-regulatory domain in the limb (see Fig. 4 and Fig. S3) such clustering of transcription factors at a single cis-regulatory element is not unique. ChIP studies reveal that many key transcription factors involved in the development and maintenance of a given cell type can co-localize to common cis-regulatory regions (Boyer et al., 2005; Junion et al., 2012; Kim et al., 2008; Kim et al., 2010b). It is likely that such co-localization will be a common feature of many developmental systems.

Many of the transcription factors regulating *Shh* expression have also been shown to directly bind each other including TWIST1 and HAND2, as well as TWIST1 and ETV5 (Firulli et al., 2005; Zhang et al., 2010). These interactions are thought to play an essential role in modulating the transcriptional regulation of *Shh* (Firulli et al., 2007; Zhang et al., 2010). Many additional transcription factors that are active in the limb have been shown to be capable of binding to each other in at least some biological contexts, for example, HOX and SMAD proteins as well as HOX and GLI proteins (Chen et al., 2004; Williams et al., 2005). This suggests that some transcription factors may associate with specific cis-regulatory elements through protein-protein interactions. This is supported by observations that an intact DNA binding domain may not be required for all the biological activities of HAND2 and HOXD13 (Liu et al., 2009; McFadden et al., 2002; Williams et al., 2006).

Experiments examining *Shh* expression in the limb have shown that the *Shh* transcriptional start site physically associates with the ZRS through long-range chromosomal interactions. Interestingly, these chromosomal interactions occur both in the posterior and anterior limb but not in the more medial mesenchyme (Amano et al., 2009). The interactions in the anterior limb correlate with the ectopic expression of *Shh* in this domain in many mouse mutants (Gildea et al., 2011; Krawchuk et al., 2010; Lettice et al., 2012; Mao et al., 2009; Masuya et al., 1995; Qu et al., 1997; Zakany et al., 2004; Zhang et al., 2009). A further chromosomal looping event occurs only in the ZPA and is associated with *Shh* expression (Amano et al., 2009). This data demonstrates that physical association of cis-regulatory elements with transcriptional start site of the gene may be essential but not sufficient for transcriptional activation. Recent papers have suggested a role for Mediator and Cohesin complexes in transcriptionally associated changes in chromosomal conformation (Kagey et al., 2010).

Transcriptional regulation of the HoxD genes

The transcriptional regulation of *Hox* genes in the limb has been the most thoroughly investigated for the *HoxD* cluster. Genes in the *HoxD* cluster are expressed in two distinct, independently regulated, temporal phases that have been termed "early" and "late"(Kmita et al., 2002; Spitz et al., 2005; Tarchini et al., 2006). Expression of the *HoxD* genes during both these phases shows spatial collinearity; this means that the transcriptional domain of each *HoxD* gene correlate with the position of the gene within the *HoxD* cluster. Collinear expression occurs because *HoxD* genes are transcriptionally regulated by genetic elements that lie outside of the cluster itself.

The early phase of *HoxD* transcription is activated by an unidentified region, termed the early limb control region (ELCR), which lies telomeric of the *HoxD* cluster (Tarchini and Duboule, 2006; Zakany et al., 2004). Early phase *HoxD* transcription is simultaneously repressed by a repressor element, active in the anterior limb, that lies at the centromeric side of the *HoxD* cluster (Tarchini et al., 2006). The repression acts most strongly on genes at the centromeric end of the cluster so that most centromeric gene, *HoxD13*, shows the greatest spatial restriction and genes telomeric of *HoxD10* are expressed throughout the limb. GLI3 has been shown to repress *HoxD* transcription in the anterior limb suggesting that GLI3 may regulate the activity of the centromeric repressor (Litingtung et al., 2002; te Welscher et al., 2002b). Interestingly, GLI3 has been shown to bind a number of genomic loci centromeric of the *HoxD* cluster (Vokes et al., 2008).

The 5′ *HoxD* genes are positively regulated in the late phase by completely distinct enhancer elements located centromeric of the *HoxD* cluster (Gonzalez et al., 2007; Kmita et al., 2002; Spitz et al., 2003). Two genomic enhancer loci, termed Prox and GCR, identified through transgenic assays, were initially thought to be largely sufficient to drive late phase expression (Gonzalez et al., 2007; Spitz et al., 2003). More recent mutational analysis shows deletion of a 240 kb region containing GCR and Prox reduces *HoxD13* transcription by 40% with only minor resulting phenotypic defects. This is because GCR and Prox act in a partially redundant manner with additional enhancer elements spread over an additional 590 kb of genomic sequence (Montavon et al., 2011). Deletion of 830 kb of genomic sequence containing GCR, Prox and the additional enhancer elements causes a seemingly complete loss of late phase *HoxD* transcription and severe digit malformations (Montavon et al., 2011).

The mechanisms of *HoxD* transcriptional regulation provide many insights into possible transcriptional events in the limb. Like *Prrx1* and *Tbx4*, the *HoxD* genes are regulated by multiple redundant enhancer elements (Cretekos et al., 2008; Menke et al., 2008; Montavon et al., 2011). Additionally, *HoxD* genes are regulated by distinct cis-regulatory elements at distinct timepoints (Kmita et al., 2002; Spitz et al., 2005; Tarchini and Duboule, 2006). Given the dynamic transcriptional events occurring in the limb it is likely that many other genes may be regulated by distinct temporally limited cis-regulatory elements.

Conclusion

A great deal of work has already been performed to identify the key signals regulating limb formation and here we have attempted to integrate this data into three temporal gene networks. Application of modern genomic techniques will provide greater resolution of these networks and help fill-in the gaps in our knowledge. Data uncovered thus far has highlighted the likely complexity of the transcriptional events occurring in the limb. It is probable that many genes are regulated by multiple, often long-range, cis-regulatory elements, each of which may only be active

for discrete periods of time and are each regulated by the binding of multiple transcription factors. Integrating these processes represents a formidable challenge for developmental biologists in the post-genomic era. With the ease of embryonic and genetic manipulations, the vertebrate limb should be up to the task.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.05.035.

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