Seminars in Cell & Developmental Biology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Seminars in Cell & Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

Getting a handle on embryo limb development: Molecular interactions driving limb outgrowth and patterning

Caroline J. Sheeba[a,b,c,d], Raquel P. Andrade[a], Isabel Palmeirim[a,b,*]

[a] Regenerative Medicine Program, Department of Biomedical Sciences and Medicine, University of Algarve, 8005-139 Faro, Portugal
[b] Centre for Molecular and Structural Biomedicine, CBME/IBB, University of Algarve, 8005-139 Faro, Portugal
[c] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, 4710-057 Braga, Portugal
[d] ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal

ARTICLE INFO

Article history:
Available online xxx

Keywords:
Limb development
Patterning
Limb molecular clock
Molecular interactions
Signaling gradients

ABSTRACT

Development of the vertebrate embryo involves multiple segmentation processes to generate a functional, articulated organism. Cell proliferation, differentiation and patterning involve spatially and temporally regulated gene expression and signal transduction mechanisms. The developing vertebrate limb is an excellent model to study such fine-tuned regulations, whereby cells proliferate and are differentially sculptured along the proximal–distal, anterior–posterior and dorsal–ventral axes to form a functional limb. Complementary experimental approaches in different organisms have enhanced our knowledge on the molecular events underlying limb development. Herein, we summarize the current knowledge of the main signaling mechanisms governing vertebrate limb initiation, outgrowth, specification of limb segments and termination.

Contents

* Corresponding author at: Department of Biomedical Sciences and Medicine, University of Algarve, 8005-139 Faro, Portugal. Tel.: +351 289244481; fax: +351 289800076.
E-mail addresses: imesteves@ualg.pt, ipalmeirim@gmail.com (I. Palmeirim).

http://dx.doi.org/10.1016/j.semcdb.2015.01.007
1084-9521/

1. Introduction

The limb bud initiates from the lateral body wall as a small protrusion of mesenchymal cells within an ectodermal jacket, and is transformed into a three dimensional, functional adult limb. Spatiotemporally coordinated cellular and molecular interactions from the embryo flank, apical ectodermal ridge (AER), zone of polarizing activity (ZPA) and the non-ridge ectoderm sculpt the limb bud along the proximal–distal (PD), anterior–posterior (AP) and dorsal–ventral (DV) axes. The limb is a segmented structure [1], with the basic skeletal architecture of the proximal stylopod, middle zeugopod and distal autopod that are laid down in a PD sequence. While the number of bone elements in the stylopod (humerus or femur) and zeugopod (ulna and radius; tibia and fibula) is conserved across species, the autopod (carpals, metacarpals and phalanges) has been phylogenetically tweaked to adapt specific abilities [2–4]. The overall limb architecture across species, however, is set by conserved mechanisms and the key players are, the fibroblast growth factors (Fgfs), Wnts, sonic hedgehog (Shh), retinoic acid (RA) and bone morphogenetic proteins (Bmps). With the purpose of providing an overview on limb development and promoting its use as a model system for specialized studies, here we review the major molecular events during limb development, namely its initiation, PD, AP and DV outgrowth/patterning and termination.

2. The intermingled process of limb initiation and identity

The presumptive limb territory is molecularly specified at Hamburguer and Hamilton (HH) [5] stage HH13-HH14 in chick (48–50 h of egg incubation) although it only becomes visible to the eye at HH17 (after 53–60 h), or at embryonic day 9.5 in mouse (E9.5). After molecular specification of the forelimb (between somites 15–20 in chick and 7–12 in mouse) and hindlimb regions (somites 26–32 in chick and 23–28 in mouse) at precise AP positions, epithelial-to-mesenchymal transitions (EMT) and intense proliferation of the somatopleural lateral plate cells will cause the limb bud mesenchyme to protrude outward, enveloped into an ectodermal layer of cells [6,7]. The essential role of EMT in limb initiation was recently demonstrated in chick embryo [6]. These authors showed that at HH13, the somatopleure that eventually gives rise to the limb bud, is epithelial in nature, which in later stages become mesenchymal and generate the limb primordium. In addition to the two genes that control limb initiation, Tbx5 and Fgf10 [6], it is possible that more players are involved in the EMT of the somatopleure epithelium and this awaits further research.

2.1. Tbx genes in limb initiation

Although tetrapod fore- and hindlimb pairs look alike in early stages of development, they soon become morphologically and functionally distinct. This starts with the conserved expression of T-box transcription factors *Tbx5* and *Tbx4* in the lateral plate mesoderm (LPM) of prospective fore- and hindlimbs, respectively, and the expression of a paired-like homeodomain factor, *Pitx*, in the hindlimb mesenchyme. Misexpression studies of *Tbx5*, *Tbx4* and *Pitx1* in mouse, chick and zebrafish have corroborated their indispensable roles in limb initiation [8–14]. In *Tbx5* conditional knockout mice, forelimb buds were not formed [8,13]. Inhibition of *Tbx5* or *Tbx4* activity in the prospective fore- and hind-limb fields in chick also produced limbless embryos and their misexpression in the chick embryo flank produced ectopic limbs [14]. In both these scenarios, *Tbx* genes functioned through Fgf and Wnt signaling components, namely *fgf10*, *fgf8* and *wnt2b* or *wnt8c* [14], suggesting that *Tbx* genes function upstream of *fgf* and *wnt* expression (Fig. 1A, A′). However, in zebrafish, Wnt2b is reported to act upstream of *Tbx5* during limb induction [12], signifying that there might be variations in the molecular hierarchy between species.

Unlike *Tbx5* knockouts [8,13], *Tbx4*$^{-/-}$ mouse embryos displayed normal hindlimb induction and initial patterning, although they failed to develop further [15]. Subsequent studies revealed that while Tbx5 and Tbx4 are not necessary for limb outgrowth and skeletal element patterning [16,17], they are required for patterning of the limb muscles and tendons [18]. Employing limb-rescue assays, Minguillon et al. [11] showed that *Tbx4* is capable of replacing *Tbx5* in the forelimb without changing forelimb identity. This ability questions *Tbx5* and *Tbx4* as the molecules that provide limb-specific morphologies. However, the difference in the phenotypes observed in *Tbx5* [8,13] and *Tbx4* [15] null mutants still argues against the possibility of one *Tbx* gene being substituted by the other. Thus, the involvement of *T-box* genes in limb-specific morphologies is still elusive.

Pitx1 is reported to regulate *Tbx4* expression in the hindlimb [19] and contribute to hindlimb specific morphologies when misexpressed in place of Tbx5. Consistent with *Pitx1*'s role in hindlimb identity [19], its misexpression in mouse forelimb region transformed it into a hindlimb at the level of gene expression, bones, muscles and tendons [20].

2.2. Retinoic acid (RA) signaling in limb initiation

RA, the active derivative of vitamin A, has been shown to be critical in many aspects of limb development including its initiation. Although it is difficult to detect its precise location in the embryo, the distribution of RA synthesizing (Retinaldehyde dehydrogenases: *Raldh1-3*) and catabolizing (cytochrome P450 family members: *Cyp26a1*, *b1*, *c1*) enzymes is an approach to infer the location and relative amounts of RA. *Raldh2* is expressed in the somites and in the LPM during limb initiation stages [21,22]. Inserting an impermeable barrier between the somites and the presumptive forelimb LPM inhibited forelimb formation [23], suggesting the importance of somite-produced RA for limb initiation. In zebrafish, transplantation of wild-type paraxial mesoderm cells into *Raldh2* mutant embryos was able to rescue the absence of pectoral fins, further indicating the requirement of RA synthesized in the somitic mesoderm for pectoral fin induction [24].

While perturbation of RA signaling in chick, mouse and zebrafish prevented limb budding [25–27], maternal dietary RA supplementation rescued the absence of forelimbs in *Raldh2* null mice [27,28], clearly showing the involvement of RA in limb initiation. Both in mouse and zebrafish, RA is proposed to have an early role of inducing *Tbx5* expression [27,29]. Accordingly, *Tbx5* is absent in the forelimb field of mouse and zebrafish embryos lacking RA synthesis, and was rescued by RA supplementation [25,27–29]. Nevertheless, a RARE-lacZ reporter failed to detect RA activity in the presumptive limb mesenchyme of the rescued *Raldh2* mutant mouse, suggesting

Please cite this article in press as: Sheeba CJ, et al. Getting a handle on embryo limb development: Molecular interactions driving limb outgrowth and patterning. Semin Cell Dev Biol (2015), http://dx.doi.org/10.1016/j.semcdb.2015.01.007

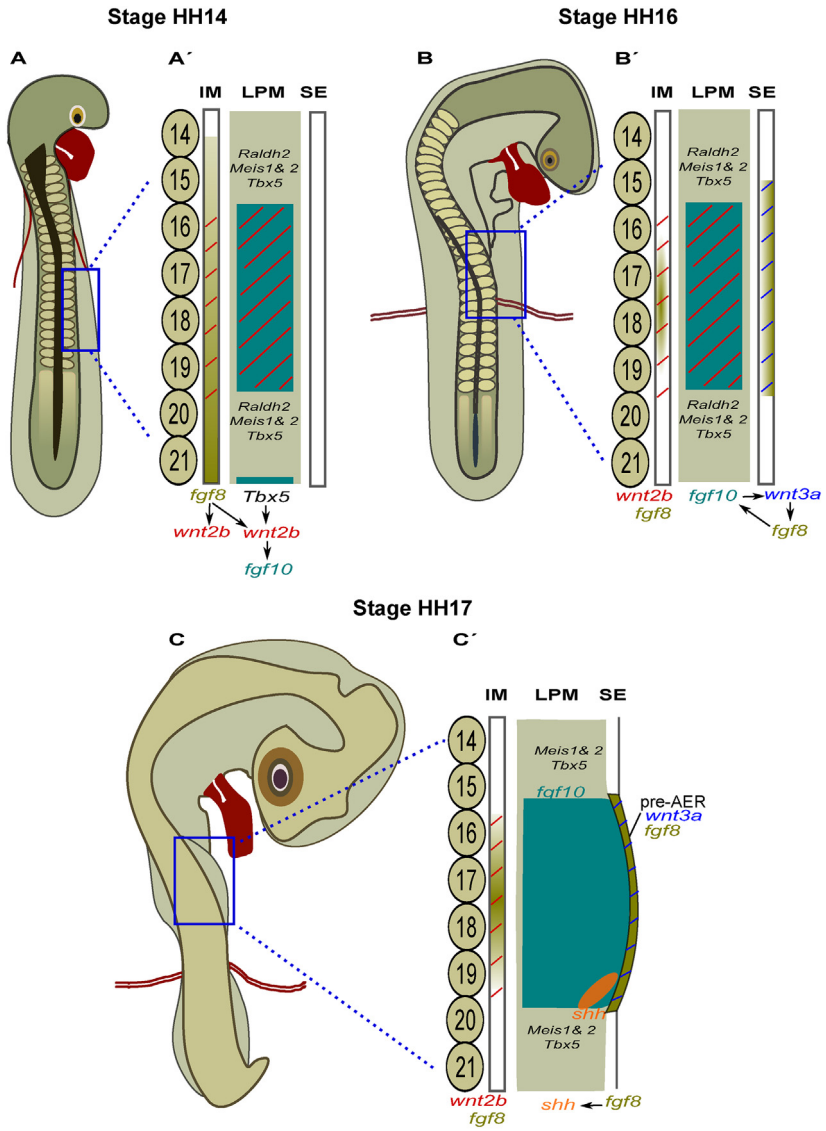

Fig. 1. Signaling interactions occurring during limb bud initiation. (A) Stage HH14 chicken embryo, where the blue box represents the presumptive forelimb field. (A′) Enlarged view of the presumptive forelimb region and molecular interactions operating therein. The intermediate mesoderm (IM) expresses *fgf8* (green) and *wnt2b* (red). Eventually, Fgf8 induces *fgf10* (teal) in the lateral plate mesoderm (LPM) through *wnt2b*. (B, B′) Representation of stage HH16 chick embryo and interactions in the presumptive limb field (enlarged). Fgf10 from the LPM relays *fgf8* expression from the IM to the surrounding ectoderm (SE) through the induction of *wnt3a* (blue). (C, C′) Stage HH17 chick embryo: initiation of *shh* (orange) expression at the posterior distal mesenchyme and the emergence of the pre-AER are the key events that take place at this stage of development.

that RA might be indirectly influencing *Tbx5* [28] and in zebrafish, this regulation is occurring through *Wnt2b* [12,26].

2.3. *Wnt and Fgf signaling in limb initiation*

Beads soaked in Fgfs and Wnts possess the ability to induce ectopic limbs in the embryo flank [30,31], positioning Fgf and Wnt as key molecules in limb induction. Fgf and Wnt signaling components interact with each other to implement the limb initiation program (Fig. 1). The expanded model proposed by Kawakami et al. [30] states that during chick forelimb initiation, *fgf8* expressed in the intermediate mesoderm (IM) [31,32] activates *wnt2b* expression in the IM and LPM, which then induces *fgf10* in the LPM (Fig. 1A′). From here, Fgf10 induces *wnt3a* in the surrounding ectoderm (SE), where it activates *fgf8* expression (Fig. 1B′) and this happens in parallel to the appearance of the AER at the distal tip of the limb bud, overlying the *fgf10* expressing limb mesenchyme (Fig. 1C′). Wnt3a helps in the maintenance of *fgf8* in the AER [30,33]. Expression of *fgf10* in chick presumptive hindlimb LPM is regulated by *wnt8c* [30]. Both Wnt2b and Wnt8c signal through canonical β-catenin pathway [30]. Although Wnt2b is a crucial component for chick and zebrafish forelimb initiation [12,26,30], its participation in mouse limb induction is questioned, since it is not expressed in the mouse limb [8]. Nevertheless, the crucial transcription factors of Wnt/β-catenin signaling, Lef1 and Tcf1 are known to be required for *fgf10* expression and limb development in mouse [8,34]. Induction and maintenance of Fgf10 is crucial for proper limb initiation as the knockout of *fgf10* generates limbless mice [35]. Also, absence of *fgf10* in the prospective forelimb bud mesenchyme of *Tbx5* knockout mice [13] and inability of *fgf10* knockout mice to maintain *Tbx5* expression [35], suggest the existence of a positive feedback loop between these molecules. Fgf10 is also known to maintain *Tbx5* expression in the forelimb and pectoral fin of chick and zebrafish, respectively [12]. Unlike *Tbx5* knockouts,

Please cite this article in press as: Sheeba CJ, et al. Getting a handle on embryo limb development: Molecular interactions driving limb outgrowth and patterning. Semin Cell Dev Biol (2015), http://dx.doi.org/10.1016/j.semcdb.2015.01.007

in *Tbx4*$^{-/-}$ mouse embryos *fgf10* expression in the hindlimb is initiated but not maintained [15], indicating that Tbx4 is not required to initiate *fgf10* expression in mouse hindlimb mesenchyme.

Among the FGF receptors (FgfR) that are tissue-specifically expressed in the developing limb, FgfR1 and FgfR2 are expressed in the limb bud from very early stages [36]. While the absence of FgfR1 didn't block limb initiation [37], *FgfR2* knockout mice lacked limbs [38], emphasizing the importance of FgfR2 for limb initiation. Activation of FgfR2 isoforms *FgfR2IIIc* and *FgfR2IIIb* in the mesenchyme and ectoderm by the ectoderm- and mesenchyme-expressed Fgf8 and Fgf10, respectively, is crucial for limb initiation [38,39].

3. Proximal-Distal (PD) limb outgrowth and patterning

3.1. The AER and Fgf signaling in outgrowth and patterning

PD outgrowth and patterning is mainly driven by Fgfs produced in the AER. Induction of *fgf8* in the SE by LPM-expressed Fgf10 is a crucial step in the establishment of the functional AER [38,40]. In addition to Fgf signaling mediated by FgfR1&2 [39,41,42], signaling from Wnt/β-catenin [43], Bmp/BmpR1a [44–46], RA [47] and ectoderm expressed-Shh [48] are implicated in the maintenance of the AER.

AER-Fgfs function as cell survival and proliferation factors for the subjacent mesodermal cells [49,50]. The mature chick and mouse AER expresses *fgf2*, *fgf4*, *fgf8*, *fgf9*, *fgf19* and *fgf4*, *fgf8*, *fgf9*, *fgf17*, respectively [51]. Both in chick and mouse, *fgf8* has the longest expression time-window, covering the entire AER tissue and other AER-*fgf*s appear relatively later in the posterior AER [51,52]. Accordingly, mice with conditional *fgf8* deletion displayed defective limbs [53,54] while KO mice for other AER-Fgfs, alone (*fgf4*, *fgf17* and *fgf9*) or in combination (triple KO for *fgf4*, *fgf9*, *fgf17*; Supplementary Table 1), did not show any limb abnormalities [52], revealing Fgf8 as the key AER-Fgf for normal limb development. But, double *fgf8*/*fgf4* knockouts had more severe forelimb defects and completely lacked hind limbs [50], indicating the requirement of cumulative AER-Fgf8 and Fgf4 action in this process. Furthermore, the triple *fgf8*/*fgf4*/*fgf9* knockout mice epitomize the contribution made by each AER-Fgf for the total AER-derived signal, by producing even more severe limb phenotypes [52].

In the distal limb mesenchyme of mouse and chick, Fgf signaling is mediated by Erk/MAPK and Akt/PI3K intracellular pathways, respectively [55,56]. However, p-Erk expression in chick AER is necessary to preserve AER integrity [56] where Flrt3 is involved [57], and to operate the epithelial-mesenchymal loop between the AER-Fgf and ZPA-Shh [58].

A series of *Cre*-mediated KO studies to delete *FgfR1* or *FgfR2* expression from the mesenchyme or ectoderm of mice [41,59,60] showed their importance for limb mesenchymal cells survival and proliferation, early and late PD-patterning events and for the establishment of proper chondrogenic primordia. Inactivation of *FgfR1IIIc* and *FgfR2IIIc* in the limb mesenchyme, either alone or in combination, demonstrated their partial redundancy in transducing Fgf signaling in the early limb mesenchyme [60]. Overall, FgfR1 and FgfR2 function as the predominant mesenchymal and ectodermal Fgf receptors, respectively [39,41,42]. The major functional studies carried out to decipher the function of Fgf signaling during limb development are summarized in Supplementary Table 1.

3.2. Wnt and Bmp signaling in outgrowth and patterning

Several Wnt family members are expressed in the limb mesenchyme and in the SE including the AER [61]. Like Fgf signaling, Wnt signaling is also required for cell proliferation and cell fate specification [62] and it negatively regulates chondrogenesis [62,63]. During limb development, the AER serves as the source of Fgf signaling and the ectoderm, including the AER, emanate Wnt signaling. While continuous exposure to Fgf8 or Wnt3A alone provided chondrogenic or connective tissue fate, respectively, their combined application to limb micromass cultures retained the cells in an undifferentiated proliferative state [62]. As per the model proposed by the authors, once the cells in the core of the limb bud escape the influence of both Fgf and Wnt signaling, they begin their chondrogenic differentiation program. But, in the periphery, the cells are still receiving Wnt signal from the SE, which maintains cell proliferation and respecifies them toward soft connective tissue fates. The limb outgrowth is more pronounced distally because of the combined strength of Fgf and Wnt signaling from the AER, compared to the strength of Wnt signaling alone from the non-ridge ectoderm [62].

Several Bmp ligands are expressed throughout limb development both in the AER and mesenchyme [64] particularly, *Bmp2*, *Bmp4* and *Bmp7*. Conditional inactivation of *Bmp2*, *Bmp4* and *Bmp7* either alone or in combination revealed that none of these Bmps are involved in limb patterning, but a threshold of Bmp signaling is necessary to form proper chondrogenic condensations [65]. The ubiquitously expressed Bmp receptor, BmpR1a, has high affinity for Bmp2 and Bmp4 [66,67] and *BmpR1a* mutant mice presented abnormalities in all the limb segments [68]. In these mutants, both AP and DV patterning genes displayed defective expression but not the PD-patterning genes [68]. BmpR1b does not play any role in limb patterning because mouse mutants for *BmpR1b* only display mild defects in cartilage differentiation [69,70].

3.3. RA signaling in PD patterning

AER-Fgf signaling from the distal limb is counteracted by the proximal, flank-RA signaling and this antagonism is proposed to instruct limb PD patterning [71–73]. However, whether this antagonism occurs at the limb PD level or in the LPM prior to limb budding is still not conclusive, because no RA activity was detected in the rescued forelimb buds of mouse mutants lacking RA synthesis [28,74]. According to these authors, RA-mediated inhibition of Fgf8 signaling in the presumptive forelimb flank creates a permissive condition for correct spatiotemporal induction of *Tbx5* expression and thus normal forelimb initiation [28,74].

RA activity is indicated by two closely related homeobox genes, *Meis1* and *Meis2*, which are expressed in the LPM before limb initiation, then in the entire nascent limb bud and later in the proximal limb region, up to the humerus-radius/ulna boundary [74,75]. Meis1 and Meis2 have been identified as determinants of proximal limb elements, because ectopic distal *Meis* expression inhibited progressive distalization and formed limbs with proximally shifted identities along the PD axis of chick and mouse [72,75,76]. When RA activity in the limb mesenchyme was distally expanded by inactivation of *Cyp26b1*, distal limb truncations were observed that were similar to the phenotype obtained by *Meis* overexpression [77]. Paradoxically, recent studies using *Rdh10* and *Raldh2*$^{-/-}$ mutant mice lacking RA activity has suggested that RA signaling is not required to establish *Meis1*/*2* expression during limb development [74]. Whether, RA acts as an instructive or permissive signal to proximalize the limb bone elements calls for further research in the field.

3.4. PD patterning models

Different models have been proposed to explain limb PD patterning (Fig. 2): the Progress Zone (PZ) model [78]; the Two Signal (TS) model [79] and, more recently, the Integrated Space-Time model for limb PD/AP patterning [80]. For the sake of

Please cite this article in press as: Sheeba CJ, et al. Getting a handle on embryo limb development: Molecular interactions driving limb outgrowth and patterning. Semin Cell Dev Biol (2015), http://dx.doi.org/10.1016/j.semcdb.2015.01.007

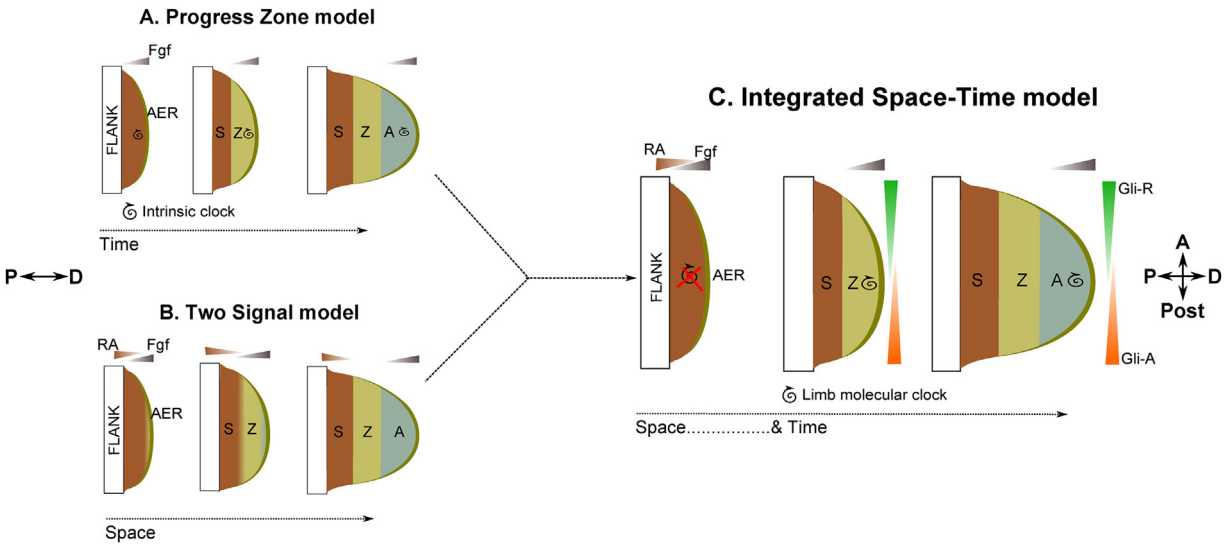

Fig. 2. Limb proximal–distal (PD) patterning models. (A) According to the Progress Zone (PZ) model [78], the distal most (~300 μm) limb mesenchymal cells under the influence of the AER-Fgfs, called the PZ, is maintained in a labile state to progressively acquire PD positional information provided by the intrinsic clock like mechanism operating in these cells (marked with spiral arrow). (B) The Two-Signal (TS) model is built on the basis of the influence of the opposing gradients of flank-RA (proximal–distal) and AER-Fgf (distal–proximal) signaling on the limb mesenchyme [79]. Over time, three distinct domains will be established: the proximal domain expressing *Meis* under the influence of flank-RA signaling, the distal domain experiencing AER-Fgf signal and expressing *Hoxa13* and the middle *Hoxa11* domain that is not under the influence of both signals. These domains represent the proximal-stylopd (S), middle-zeugpod (Z) and the distal-autopod (A) limb bone segments. (C) The Integrated Space-Time model: The limb molecular clock gene *hairy2* expression is regulated in the distal mesenchyme of chick forelimb bud by flank-RA (permissive and instructive signal), AER-Fgf (instructive signal) and ZPA-Shh (permissive signal) signaling. Since the entire early limb mesenchyme is under the combined influence of the permissive and instructive flank-RA signaling and the instructive AER-Fgf signaling, *hairy2* is persistently expressed in the entire early limb bud during which the proximal most limb segment, the stylopod is specified. Over time, limb outgrowth will displace the distal limb from the flank-RA signaling. Simultaneously, ZPA-Shh permissive signal get well established, creating different ratios of Gli-A/Gli-R along the AP axis. The combination of Gli-A/Gli-R with AER-Fgf signaling produces on/off *hairy2* expression in the chondrogenic precursor cells, endowing progressive positional information to form the zeugopod and autopod [80]. Thus, this mode of patterning proposes a transformation from the spatial signaling gradient based-to-temporal information based-PD patterning mechanism. Moreover, since the limb clock is integrating the signaling activities of the PD (RA and Fgf) and AP (Shh) axes patterning signaling molecules, we propose that it might be coordinating outgrowth and patterning along these axes. All limbs are represented anterior (A) on top and proximal (P) to the left.

understanding, the later model is provided in Section 5, after the section on limb AP patterning.

3.4.1. *The Progress Zone (PZ) model and the limb molecular clock*

Microsurgical experiments performed in the 1940s showed that the earlier the removal of the AER, the most proximal limb elements are truncated [81]. The PZ model was built on this foundation and proposed that the positional values in the distal mesenchyme freeze upon AER ablation and the resulting skeletal patterns reproduce the PD information acquired by the mesenchymal cells until AER ablation. The presumptive fate of the distal mesenchyme was further identified by swapping the tissue from younger to older and older to younger embryos [78,82], leading to the proposal of the PZ model in 1973 [78] (Fig. 2A). According to this model, the distal mesenchymal cells located in the PZ, corresponding to about 300 μm just beneath the AER, are maintained in an undifferentiated, proliferating state by the influence of the AER, which keeps them labile to acquire positional information about their future PD fate. The model proposes an intrinsic timer operating in the PZ that provides the cells the notion of time they spend in the PZ. Due to continuous cell proliferation and outgrowth of the limb, mesenchymal cells will be pushed out of the PZ and escape the influence of the AER. The amount of time each cell spends in the PZ, measured by the intrinsic timer, will determine its PD positional identity.

The first evidence for the existence of such a time counting mechanism was provided in 2007 based on the 6 h periodic *hairy2* gene (a Hairy-Enhancer-of-split (HES) family member) expression oscillations in stage HH20-28 chick distal limb chondrogenic precursor cells [83,84]. However, further work is required to substantiate the causality between the dynamics of limb bone element formation and the periodicity of the limb molecular clock [85].

3.4.2. *The Two Signal (TS) model*

The developing limb mesenchymal cells experience the opposing signaling activities of the flank-RA and the AER-Fgf [72,75] and this antagonism is the basis of the TS model [79] (Fig. 2B). As a consequence of limb outgrowth, these signaling gradients get distanced from each other, establishing three distinct domains, representing the three limb segments: the proximal stylopod domain under the influence of RA, the distal autopod domain influenced by Fgf signal and the middle zeugopod domain that is neither under the influence of RA or Fgf signaling [79] (Fig. 2B). These domains express specific markers, namely the proximal *Meis1* or *Meis2*, the middle *Hoxa11* and the distal *Hoxa13*. Except for *Meis* genes, none of these segment specific markers are directly involved in segment specification [79]. The TS model refers to the distal mesenchymal cells that are maintained in a proliferative undifferentiated state by the AER signal as the Undifferentiated Zone (UZ) [79,86]. Continuous proliferation in the UZ will push the cells out of this zone and from the influence of the AER-Fgf signaling, allowing them to enter the differentiation program. At the time of their exit from the UZ, the cells will only express one of the three limb segment markers which determine their fate. The proximal limit of the AER-Fgf signaling from where cells start their differentiation program is named as the 'Differentiation Front' (DF) [79]. The main difference of the TS model from the PZ model is that the TS model does not contemplate the operation of a clock mechanism and depends solely on the relative levels of proximalizing-RA *vs* distalizing-Fgf activity for

cell fate specification. By performing both *in vitro* and *in vivo* experiments in chick, a balance between the trunk-RA and the distal-Fgf signals was shown to be the key for limb PD patterning [71,73].

4. Limb Anterior-Posterior (AP) patterning

4.1. The ZPA and Shh signaling

The polarizing region or the ZPA, located at the posterior distal margin of the limb mesenchyme, was identified by grafting this tissue from a donor to the anterior mesenchyme of a host early wing bud which produced mirror-image symmetrical digit duplications [87]. The number and identity of the induced digits depends both on the strength/concentration and duration of the polarizing signal [87], suggesting that the ZPA signal should be mediated by a morphogen. Later, Shh was found to be this morphogen [88]. There are three digits in chick wing (digit 1, 2 and 3) [89] and five digits in mouse forelimb (digit 1, 2, 3, 4 and 5) [90]. In both species, the anterior most digit (digit 1) is patterned independently of Shh signaling [87].

A network of molecular signals functions to initiate *shh* expression in the ZPA (Fig. 3A). A *cis*-regulatory region known as the ZPA regulatory sequence (ZRS), located about 800 Kb up-stream of *shh* gene is also involved in this process [91]. Before *shh* induction, the limb is pre-patterned by mutually antagonizing anterior-*Gli3* and the posterior-*Hand2* expression (basic helix-loop-helix transcription factor) [92]. *Hand2* expression in the posterior limb is positively regulated by the concerted activity of all four Hox9 genes (*Hoxa9*, *Hoxb9*, *Hoxc9*, and *Hoxd9*) [93] and RA signaling [94]. The expression of *5′HoxA* and *HoxD* genes is also restricted to the posterior limb by Gli3 [95–97]. Together, the *5′*Hox and Hand2 initiate ZPA-*shh* expression, by directly interacting with the ZRS [98,99].

Establishment of ZPA-*shh* also requires AER-Fgf signaling through FgfR2 [32,38] and dorsal ectoderm-produced Wnt7a is necessary to maintain *shh* in the ZPA [100] (Fig. 3C). Shortly after AER starts to express *fgf8*, it induces *shh* expression in the ZPA [32] (Fig. 1C′), which is then maintained by the positive ZPA-Shh/Grem/AER-Fgf module throughout limb development [45,101] (Fig. 3A and B). This module comprises an initial fast loop (2 h), where Bmp induces its own antagonist, *Grem*, in the distal limb mesenchyme and a slower loop (12 h), where Grem antagonizes Bmp signaling allowing the rise of AER-Fgf, ZPA-Shh, and Grem activities [45] (Fig. 3A and B). This loop explains why AER-*fgf4* and -*fgf8* expression are abrogated in mouse and chick limbs developed in the absence of Shh signaling [102]. Accordingly, AER-*fgf8*/*fgf4*

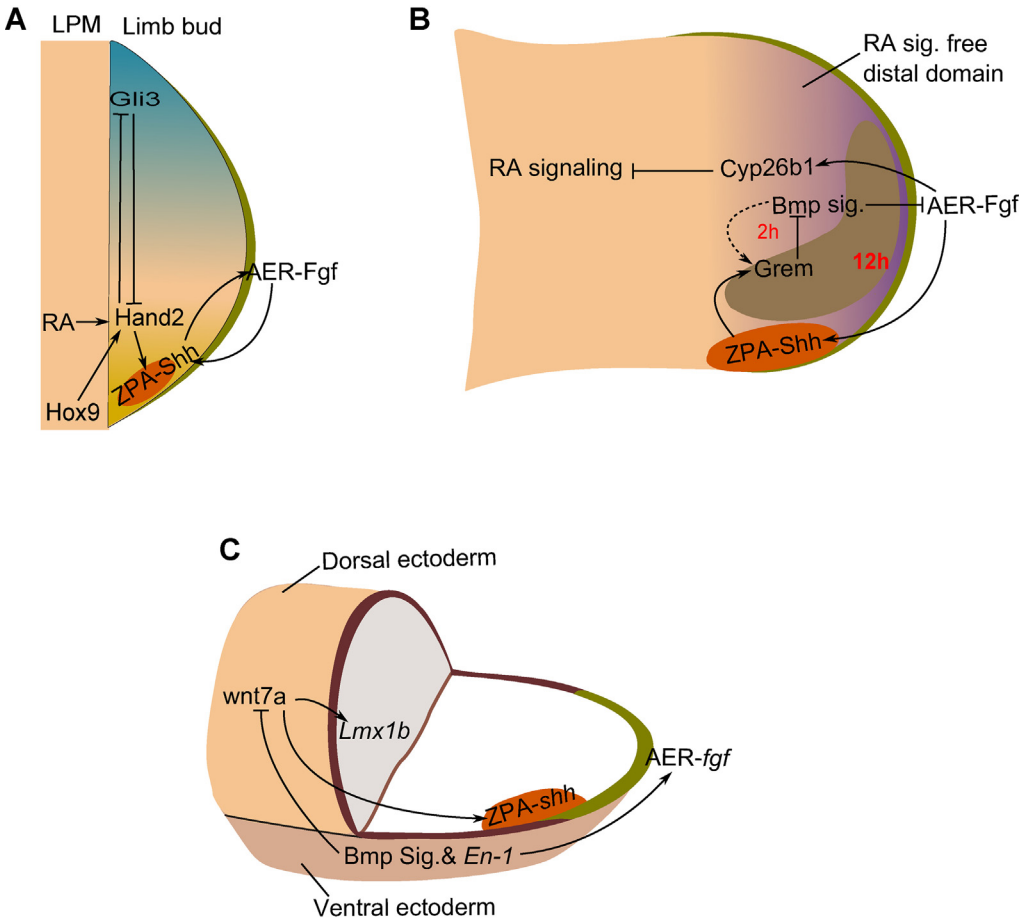

Fig. 3. (A) Establishment of ZPA-Shh: Molecular interactions involved in ZPA-Shh establishment are shown in an early stage limb bud (HH17 in chick or E9.5 in mouse). The limb is pre-patterned by mutually antagonizing anterior Gli3 (blue) and the posterior Hand2 (yellow). Positive cooperative regulations from RA, AER-Fgfs, Hand2 and 5′Hox genes facilitate *shh* induction in the ZPA and, in turn, Shh induces *fgf4* expression in the posterior AER. (B) The positive and negative modules ensuring limb outgrowth: In the early limb bud, Bmp signaling induces *Grem* expression in the mesenchyme through a fast module (2 h – [45]). In subsequent stages (as represented here), *Grem* expression will be ZPA-Shh dependent. By antagonizing Bmp signaling, Grem mediates the propagation of the positive ZPA-Shh/Grem/AER-Fgf module (12 h – [45]), enabling the rise of all its components. Simultaneously, a negative AER-Fgf/Cyp26B1/RA module will also be functional to ensure a RA free distal limb mesenchyme [47]. (C) Limb Dorsal–Ventral (DV) patterning: Wnt7a in the dorsal ectoderm induces *Lmx1b* exclusively in the dorsal mesenchyme while BMP signaling in the ventral ectoderm induces *En-1* expression and these players underlie limb DV patterning. Besides, Wnt7a is also necessary for proper ZPA-*Shh* expression and Bmp signaling for the establishment of the AER. All limbs are represented anterior on top and proximal to the left. The interactions that are not active are represented by dotted lines, arrows indicate positive transcriptional interaction, "T"-shaped lines represent inhibition.

Please cite this article in press as: Sheeba CJ, et al. Getting a handle on embryo limb development: Molecular interactions driving limb outgrowth and patterning. Semin Cell Dev Biol (2015), http://dx.doi.org/10.1016/j.semcdb.2015.01.007

double mutants have no ZPA-*shh* expression [50]. Along with the propagation of the positive ZPA-Shh/Grem/AER-Fgf module, ZPA-Shh signaling also indirectly enables the establishment of the antagonistic AER-Fgf/Cyp26B1/RA module in the distal limb mesenchyme (Fig. 3B), which eliminates the teratogenic activity of RA in the distal limb mesenchyme and promotes distal propagation [47].

In order to properly pattern the limb AP axis, Shh should be produced at the right level and its production must be strictly restricted to the posterior distal mesenchyme. The level of Shh within the limb mesenchyme is robustly maintained by various mechanisms including auto-regulation [58,103] and its expression in the AER [48]. Many factors, such as Bmp signaling [58], ETS transcription factors, Etv4 and Etv5 [104,105] and Tbx2 [106] contribute in restricting *shh* expression to the ZPA.

Canonical Shh signaling acts through Gli transcription factors (Gli1-3 in vertebrates). Gli1 is a target of Shh signaling and functions as an activator. Gli2 and Gli3 can either be activators or repressors, depending on the presence or absence of Shh [107]. Across the limb field Gli1 and Gli2 mediate the activator function while Gli3 mainly functions as a repressor [107,108]. Genetic analysis shows that Gli1 and Gli2 are dispensable for limb AP patterning [109,110], but inactivation of *Gli3* resulted in severe polydactyly [111], emphasizing the importance of Gli3 in specifying the number and identity of digits. While *shh* mutant limbs produce only one digit [102], the limbs of double *Gli3*/*shh* mutant mice are polydactylous, identical to single *Gli3* mutants [92,112], suggesting that Shh patterns the AP axis almost solely through Gli3 processing. Supportively, the non-processed full length Gli3 that functions as an activator was able to considerably rescue *shh* mutant limb phenotype [113]. Recently, Gli3 was reported to inhibit the expression of G1–S transition cell-cycle genes and *Grem1* in the anterior limb to ensure pentadactyly [114].

4.2. Limb AP patterning models

Several models have been proposed to explain Shh-mediated limb AP patterning (Supplementary Fig. 1). The very first is the French flag model proposed based on the spatial gradient of Shh across the chick wing bud [115] (Supplementary Fig. 1A), where each color represents a particular threshold of Shh that will give rise to a digit. Then, a Gli activity-based model was proposed showing that the anterior-most and posterior-most digits are specified by high Gli3-R and by the absence of Gli3-R activities, respectively [107] (Supplementary Fig. 1B). The Shh temporal gradient model, revealing the importance of both the spatial and temporal requirement of Shh signaling for limb AP patterning was proposed by Harfe et al. [90] (Supplementary Fig. 1C). According to this model, the whole digit 2 and half of digit 3 are formed by cells that experienced paracrine Shh signaling through diffusion while the other half of digit 3, digit 4 and digit 5 are created by cells that underwent high, autocrine Shh signaling, progressively for longer duration.

Patterning of the limb distal mesenchyme by Shh is also linked with cell proliferation [116,117]. Work performed in mouse allowed the proposal of the biphasic model, as per which Shh has an early transient role in the specification/patterning of digit progenitors and a later prolonged role in proliferative expansion of the specified progenitor pool [117] (Supplementary Fig. 1D). Both the proliferative role of Shh and its transient requirement in the early limb bud to pattern limb AP axis was validated in chick [116,118]. More recently, digit patterning was also explained by a Turing-type mechanism based on the dosage of distal Hox genes [119].

4.3. The role of Bmp and RA signaling in shaping the digits

Bmp signaling and its intracellular mediators – phosphorylated-SMADs – are implicated in digit specification [66,120,121]. Moreover, Bmp signaling has prominent role in shaping the digits through interdigital apoptosis [122,123]. RA signaling also accelerates cell death in the interdigital domain [124,125]. Although RA receptor *Rarβ* deficient limbs were normal, *Rarβ*/*Rarγ* double mutants showed interdigital webbing [126], supporting the role of RA signaling in interdigital cell death.

5. The Integrated Space-Time model for limb PD/AP patterning

The expression of the limb molecular clock gene *hairy2* is regulated by the key limb signaling molecules Fgf, RA and Shh [127,128]. Fgf and Shh are instructive and permissive signals for limb *hairy2* expression, respectively [127], whereas RA can have both instructive and permissive functions [128]. Since this regulatory network brings together the crucial components of both the PZ and TS models, a new model conciliating the previous ones was proposed, called the "Integrated Space-Time Model" [80] (Fig. 2C). According to this model, the early limb mesenchyme presents non-oscillatory *hairy2* expression due to simultaneous influence of flank-RA and AER-Fgf signaling and this would specify the proximal-most stylopod. Over time, the distal limb mesenchyme is distanced from flank-RA signaling and will be progressively influenced by combined AER-Fgf and ZPA-Shh signaling. Varying posterior–anterior gradients of Gli-activator to Gli-repressor ratio (Gli-A/Gli-R) established by ZPA-Shh signaling will allow on/off *hairy2* expression, constituting a time-counting mechanism underlying the progressive establishment of cell positional information for zeugopod and autopod specification [80] (Fig. 2C). The Integrated Space-Time model positions the limb molecular clock Hairy2 transcription factor as a crucial molecular component that integrates spatial morphogenic gradients with temporal precision along limb PD and AP axes, ensuring coordinated PD and AP limb outgrowth and patterning.

6. Limb Dorsal-Ventral (DV) patterning

DV axis specification in vertebrate limb occurs through a complex series of epithelial-mesenchymal interactions [129] (Fig. 3C). It has been suggested that the signals from the somitic mesoderm specify a dorsal fate to the neighboring LPM [130], which is transferred to the SE prior to limb budding and this results in the expression of *wnt7a* in the presumptive dorsal limb ectoderm. *En-1* is induced in the ventral ectoderm by Bmp signaling through BmpR1a. In *En-1* KO limbs, or when Bmp expression is impaired, *wnt7a* is misexpressed in the ventral ectoderm and the distal structures develop with bi-dorsal character [44,131]. In the absence of Wnt7a, the limb acquires bi-ventral identity at the expense of the dorsal pattern [132]. Wnt7a induces the expression of the LIM-homeodomain transcription factor *Lmx1b* specifically in the dorsal mesenchyme of the limb bud. Experiments in the chick and mouse indicated Lmx1b as necessary and sufficient to specify dorsal limb pattern [133,134].

7. Termination of limb outgrowth

Together with the inhibitory AER-Fgf/Grem loop [135], the ZPA-Shh/Grem/AER-Fgf positive module has been shown to terminate limb outgrowth in both chick and mouse, following different sequences [135,136] (Supplementary Fig. 2). In the early limb bud (chick: HH18-23 and mouse: E9.5-10.5), the level of AER-Fgf signaling is too low to inhibit *Grem* expression. Instead, the positive module of ZPA-Shh/Grem/AER-Fgf will facilitate limb outgrowth by increasing the strength of AER-Fgf signaling. By stage HH23-27 in chick and E10.5-12 in mouse, the strength of AER-Fgf signaling is

Please cite this article in press as: Sheeba CJ, et al. Getting a handle on embryo limb development: Molecular interactions driving limb outgrowth and patterning. Semin Cell Dev Biol (2015), http://dx.doi.org/10.1016/j.semcdb.2015.01.007

high enough to inhibit *Grem* expression in the mesenchyme [135]. As a consequence of this inhibition and continuous growth of the distal limb, *Grem* negative domain expands and triggers a sequence of termination mechanisms that differ in mouse [135] (Supplementary Fig. 2A) and chick [136] (Supplementary Fig. 2B). In mouse, the termination sequence starts with the inability of *Grem* negative domain to relay ZPA-Shh signal to AER-Fgf, which in turn will reduce ZPA-*shh* transcription and ultimately Shh-mediated induction of *Grem* [135]. Whereas, in chick, the *Grem* negative domain will first be out of range to receive ZPA-Shh signal because of the refractory nature of Shh producing cells to express *Grem* [136]. By stage HH27 in chick, cells competent to express *Grem* will be located too far from the ZPA to receive Shh signaling, terminating the loop from ZPA-Shh/Grem [136] (Supplementary Fig. 2B).

Recently, two other molecules were added to the limb termination loop, namely Twist and Tbx2. Overexpression of Twist in the chick hindlimb caused premature termination of limb outgrowth by repressing *Grem* [137]. Similarly, in mouse hindlimb, misexpression of Tbx2 also resulted in premature termination of the ZPA-Shh/Grem/AER-Fgf module [138].

8. Conclusions and perspectives

Although a long standing model for tissue patterning and outgrowth studies, the developing vertebrate limb continues to present exciting challenges to developmental biologists of all ages and specific fields of interest. Whether you are focused on structure formation, cell dynamics, stem cell properties, regeneration capacity, intricate gene expression regulation, ionic exchanges or mathematical modeling – you name it – the vertebrate limb continues to be an excellent model to pursue unexplored, daring paths. We hope the knowledge herein summarized will challenge the curious reader to embrace it!

Acknowledgements

C.J.S. was supported by Fundação para a Ciência e a Tecnologia, Portugal (grant SFRH/BPD/89493/2012); R.P.A. is funded by Programa Operacional Regional do Norte (ON.2) NORTE-07-0124-FEDER-000017. This work was supported by research grants from Institute for Biotechnology and Bioengineering/Centro de Biomedicina Molecular e Estrutural, LA (to I.P.), by Fundação para a Ciência e a Tecnologia (National and FEDER COMPETE Program funds: PTDC/SAU-OBD/099758/2008 and PTDC/SAU-BID/121459/2010 to I.P. and R.P.A., respectively) and by PEst-OE/EQB/LA0023/2011.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.semcdb.2015.01.007.

References

[1] Cohn MJ, Lovejoy CO, Wolpert L, Coates MI. Branching, segmentation and the metapterygial axis: pattern versus process in the vertebrate limb. BioEssays: News Rev Mol Cell Dev Biol 2002;24:460–5.
[2] Cooper KL, Sears KE, Uygur A, Maier J, Baczkowski KS, Brosnahan M, et al. Patterning and post-patterning modes of evolutionary digit loss in mammals. Nature 2014;511:41–5.
[3] de Bakker MA, Fowler DA, den Oude K, Dondorp EM, Navas MC, Horbanczuk JO, et al. Digit loss in archosaur evolution and the interplay between selection and constraints. Nature 2013;500:445–8.
[4] Lopez-Rios J, Duchesne A, Speziale D, Andrey G, Peterson KA, Germann P, et al. Attenuated sensing of SHH by Ptch1 underlies evolution of bovine limbs. Nature 2014;511:46–51.
[5] Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. Dev Dyn 1951;195:231–72.
[6] Gros J, Tabin CJ. Vertebrate limb bud formation is initiated by localized epithelial-to-mesenchymal transition. Science 2014;343:1253–6.
[7] Searls RL, Janners MY. The initiation of limb bud outgrowth in the embryonic chick. Dev Biol 1971;24:198–213.
[8] Agarwal P, Wylie JN, Galceran J, Arkhitko O, Li C, Deng C, et al. Tbx5 is essential for forelimb bud initiation following patterning of the limb field in the mouse embryo. Development 2003;130:623–33.
[9] Ahn DG, Kourakis MJ, Rohde LA, Silver LM, Ho RK. T-box gene tbx5 is essential for formation of the pectoral limb bud. Nature 2002;417:754–8.
[10] Marcil A, Dumontier E, Chamberland M, Camper SA, Drouin J. Pitx1 and Pitx2 are required for development of hindlimb buds. Development 2003;130:45–55.
[11] Minguillon C, Del Buono J, Logan MP. Tbx5 and Tbx4 are not sufficient to determine limb-specific morphologies but have common roles in initiating limb outgrowth. Dev Cell 2005;8:75–84.
[12] Ng JK, Kawakami Y, Buscher D, Raya A, Itoh T, Koth CM, et al. The limb identity gene Tbx5 promotes limb initiation by interacting with Wnt2b and Fgf10. Development 2002;129:5161–70.
[13] Rallis C, Bruneau BG, Del Buono J, Seidman CE, Seidman JG, Nissim S, et al. Tbx5 is required for forelimb bud formation and continued outgrowth. Development 2003;130:2741–51.
[14] Takeuchi JK, Koshiba-Takeuchi K, Suzuki T, Kamimura M, Ogura K, Ogura T. Tbx5 and Tbx4 trigger limb initiation through activation of the Wnt/Fgf signaling cascade. Development 2003;130:2729–39.
[15] Naiche LA, Papaioannou VE. Loss of Tbx4 blocks hindlimb development and affects vascularization and fusion of the allantois. Development 2003;130:2681–93.
[16] Hasson P, Del Buono J, Logan MP. Tbx5 is dispensable for forelimb outgrowth. Development 2007;134:85–92.
[17] Naiche LA, Papaioannou VE. Tbx4 is not required for hindlimb identity or post-bud hindlimb outgrowth. Development 2007;134:93–103.
[18] Hasson P, DeLaurier A, Bennett M, Grigorieva E, Naiche LA, Papaioannou VE, et al. Tbx4 and tbx5 acting in connective tissue are required for limb muscle and tendon patterning. Dev Cell 2010;18:148–56.
[19] Logan M, Tabin CJ. Role of Pitx1 upstream of Tbx4 in specification of hindlimb identity. Science 1999;283:1736–9.
[20] DeLaurier A, Schweitzer R, Logan M. Pitx1 determines the morphology of muscle, tendon, and bones of the hindlimb. Developmental biology 2006;299:22–34.
[21] Blentic A, Gale E, Maden M. Retinoic acid signalling centres in the avian embryo identified by sites of expression of synthesising and catabolising enzymes. Dev Dyn: Off Publ Am Assoc Anat 2003;227:114–27.
[22] Swindell EC, Thaller C, Sockanathan S, Petkovich M, Jessell TM, Eichele G. Complementary domains of retinoic acid production and degradation in the early chick embryo. Dev Biol 1999;216:282–96.
[23] Stephens TD, McNulty TR. Evidence for a metameric pattern in the development of the chick humerus. J Embryol Exp Morphol 1981;61:191–205.
[24] Linville A, Gumusaneli E, Chandraratna RA, Schilling TF. Independent roles for retinoic acid in segmentation and neuronal differentiation in the zebrafish hindbrain. Dev Biol 2004;270:186–99.
[25] Grandel H, Lun K, Rauch GJ, Rhinn M, Piotrowski T, Houart C, et al. Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior–posterior axis of the CNS and to induce a pectoral fin bud. Development 2002;129:2851–65.
[26] Mercader N, Fischer S, Neumann CJ. Prdm1 acts downstream of a sequential RA, Wnt and Fgf signaling cascade during zebrafish forelimb induction. Development 2006;133:2805–15.
[27] Mic FA, Sirbu IO, Duester G. Retinoic acid synthesis controlled by Raldh2 is required early for limb bud initiation and then later as a proximodistal signal during apical ectodermal ridge formation. J Biol Chem 2004;279:26698–706.
[28] Zhao X, Sirbu IO, Mic FA, Molotkova N, Molotkov A, Kumar S, et al. Retinoic acid promotes limb induction through effects on body axis extension but is unnecessary for limb patterning. Curr Biol: CB 2009;19:1050–7.
[29] Grandel H, Brand M. Zebrafish limb development is triggered by a retinoic acid signal during gastrulation. Developmental dynamics: an official publication of the American Association of Anatomists 2011;240:1116–26.
[30] Kawakami Y, Capdevila J, Buscher D, Itoh T, Rodriguez Esteban C, Izpisua Belmonte JC. WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. Cell 2001;104:891–900.
[31] Vogel A, Rodriguez C, Izpisua-Belmonte JC. Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. Development 1996;122:1737–50.
[32] Crossley PH, Minowada G, MacArthur CA, Martin GR. Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. Cell 1996;84:127–36.
[33] Kengaku M, Capdevila J, Rodriguez-Esteban C, De La Pena J, Johnson RL, Izpisua Belmonte JC, et al. Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. Science 1998;280:1274–7.
[34] Galceran J, Farinas I, Depew MJ, Clevers H, Grosschedl R. Wnt3a$^{-/-}$-like phenotype and limb deficiency in Lef1(−/−)Tcf1(−/−) mice. Genes Dev 1999;13:709–17.
[35] Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, et al. Fgf10 is essential for limb and lung formation. Nat Genet 1999;21:138–41.
[36] Sheeba CJ, Andrade RP, Duprez D, Palmeirim I. Comprehensive analysis of fibroblast growth factor receptor expression patterns during chick forelimb development. Int J Dev Biol 2010;54:1517–26.

Please cite this article in press as: Sheeba CJ, et al. Getting a handle on embryo limb development: Molecular interactions driving limb outgrowth and patterning. Semin Cell Dev Biol (2015), http://dx.doi.org/10.1016/j.semcdb.2015.01.007

[37] Deng C, Bedford M, Li C, Xu X, Yang X, Dunmore J, et al. Fibroblast growth factor receptor-1 (FGFR-1) is essential for normal neural tube and limb development. Dev Biol 1997;185:42–54.
[38] Xu X, Weinstein M, Li C, Naski M, Cohen RI, Ornitz DM, et al. Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. Development 1998;125:753–65.
[39] Revest JM, Spencer-Dene B, Kerr K, De Moerlooze L, Rosewell I, Dickson C. Fibroblast growth factor receptor 2-IIIb acts upstream of Shh and Fgf4 and is required for limb bud maintenance but not for the induction of Fgf8, Fgf10, Msx1, or Bmp4. Dev Biol 2001;231:47–62.
[40] Ohuchi H, Nakagawa T, Yamamoto A, Araga A, Ohata T, Ishimaru Y, et al. The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. Development 1997;124:2235–44.
[41] Li C, Xu X, Nelson DK, Williams T, Kuehn MR, Deng CX. FGFR1 function at the earliest stages of mouse limb development plays an indispensable role in subsequent autopod morphogenesis. Development 2005;132:4755–64.
[42] Lu P, Yu Y, Perdue Y, Werb Z. The apical ectodermal ridge is a timer for generating distal limb progenitors. Development 2008;135:1395–405.
[43] Barrow JR, Thomas KR, Boussadia-Zahui O, Moore R, Kemler R, Capecchi MR, et al. Ectodermal Wnt3/beta-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. Genes Dev 2003;17:394–409.
[44] Ahn K, Mishina Y, Hanks MC, Behringer RR, Crenshaw III EB. BMPR-IA signaling is required for the formation of the apical ectodermal ridge and dorsal–ventral patterning of the limb. Development 2001;128:4449–61.
[45] Benazet JD, Bischofberger M, Tiecke E, Goncalves A, Martin JF, Zuniga A, et al. A self-regulatory system of interlinked signaling feedback loops controls mouse limb patterning. Science 2009;323:1050–3.
[46] Pizette S, Abate-Shen C, Niswander L. BMP controls proximodistal outgrowth, via induction of the apical ectodermal ridge, and dorsoventral patterning in the vertebrate limb. Development 2001;128:4463–74.
[47] Probst S, Kraemer C, Demougin P, Sheth R, Martin GR, Shiratori H, et al. SHH propagates distal limb bud development by enhancing CYP26B1-mediated retinoic acid clearance via AER-FGF signalling. Development 2011;138:1913–23.
[48] Bouldin CM, Gritli-Linde A, Ahn S, Harfe BD. Shh pathway activation is present and required within the vertebrate limb bud apical ectodermal ridge for normal autopod patterning. Proc Natl Acad Sci U S A 2010;107:5489–94.
[49] Dudley AT, Ros MA, Tabin CJ. A re-examination of proximodistal patterning during vertebrate limb development. Nature 2002;418:539–44.
[50] Sun X, Mariani FV, Martin GR. Functions of FGF signalling from the apical ectodermal ridge in limb development. Nature 2002;418:501–8.
[51] Fernandez-Teran M, Ros MA. The Apical Ectodermal Ridge: morphological aspects and signaling pathways. Int J Dev Biol 2008;52:857–71.
[52] Mariani FV, Ahn CP, Martin GR. Genetic evidence that FGFs have an instructive role in limb proximal–distal patterning. Nature 2008;453:401–5.
[53] Lewandoski M, Sun X, Martin GR. Fgf8 signalling from the AER is essential for normal limb development. Nat Genet 2000;26:460–3.
[54] Moon AM, Capecchi MR. Fgf8 is required for outgrowth and patterning of the limbs. Nat Genet 2000;26:455–9.
[55] Corson LB, Yamanaka Y, Lai KM, Rossant J. Spatial and temporal patterns of ERK signaling during mouse embryogenesis. Development 2003;130:4527–37.
[56] Kawakami Y, Rodriguez-Leon J, Koth CM, Buscher D, Itoh T, Raya A, et al. MKP3 mediates the cellular response to FGF8 signalling in the vertebrate limb. Nat Cell Biol 2003;5:513–9.
[57] Tomas AR, Certal AC, Rodriguez-Leon J. FLRT3 as a key player on chick limb development. Dev Biol 2011;355:324–33.
[58] Bastida MF, Sheth R, Ros MA. A BMP-Shh negative-feedback loop restricts Shh expression during limb development. Development 2009;136:3779–89.
[59] Verheyden JM, Lewandoski M, Deng C, Harfe BD, Sun X. Conditional inactivation of Fgfr1 in mouse defines its role in limb bud establishment, outgrowth and digit patterning. Development 2005;132:4235–45.
[60] Yu K, Ornitz DM. FGF signaling regulates mesenchymal differentiation and skeletal patterning along the limb bud proximodistal axis. Development 2008;135:483–91.
[61] Loganathan PG, Nimmagadda S, Huang R, Scaal M, Christ B. Comparative analysis of the expression patterns of Wnts during chick limb development. Histochem Cell Biol 2005;123:195–201.
[62] ten Berge D, Brugmann SA, Helms JA, Nusse R. Wnt and FGF signals interact to coordinate growth with cell fate specification during limb development. Development 2008;135:3247–57.
[63] Geetha-Loganathan P, Nimmagadda S, Christ B, Huang R, Scaal M. Ectodermal Wnt6 is an early negative regulator of limb chondrogenesis in the chicken embryo. BMC Dev Biol 2010;10:32.
[64] Geetha-Loganathan P, Nimmagadda S, Huang R, Scaal M, Christ B. Expression pattern of BMPs during chick limb development. Anat Embryol (Berl) 2006;211(Suppl. 1):87–93.
[65] Bandyopadhyay A, Tsuji K, Cox K, Harfe BD, Rosen V, Tabin CJ. Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. PLoS Genet 2006;2:e216.
[66] Robert B. Bone morphogenetic protein signaling in limb outgrowth and patterning. Dev Growth Differ 2007;49:455–68.
[67] Yamaji N, Celeste AJ, Thies RS, Song JJ, Bernier SM, Goltzman D, et al. A mammalian serine/threonine kinase receptor specifically binds BMP-2 and BMP-4. Biochem Biophys Res Commun 1994;205:1944–51.
[68] Ovchinnikov DA, Selever J, Wang Y, Chen YT, Mishina Y, Martin JF, et al. BMP receptor type IA in limb bud mesenchyme regulates distal outgrowth and patterning. Dev Biol 2006;295:103–15.
[69] Baur ST, Mai JJ, Dymecki SM. Combinatorial signaling through BMP receptor IB and GDF5: shaping of the distal mouse limb and the genetics of distal limb diversity. Development 2000;127:605–19.
[70] Yi SE, Daluiski A, Pederson R, Rosen V, Lyons KM. The type I BMP receptor BMPRIB is required for chondrogenesis in the mouse limb. Development 2000;127:621–30.
[71] Cooper KL, Hu JK, ten Berge D, Fernandez-Teran M, Ros MA, Tabin CJ. Initiation of proximal–distal patterning in the vertebrate limb by signals and growth. Science 2011;332:1083–6.
[72] Mercader N, Leonardo E, Piedra ME, Martinez AC, Ros MA, Torres M. Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. Development 2000;127:3961–70.
[73] Rosello-Diez A, Ros MA, Torres M. Diffusible signals, not autonomous mechanisms, determine the main proximodistal limb subdivision. Science 2011;332:1086–8.
[74] Cunningham TJ, Zhao X, Sandell LL, Evans SM, Trainor PA, Duester G. Antagonism between retinoic acid and fibroblast growth factor signaling during limb development. Cell Rep 2013;3:1503–11.
[75] Capdevila J, Tsukui T, Rodriquez Esteban C, Zappavigna V, Izpisua Belmonte JC. Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. Mol Cell 1999;4:839–49.
[76] Mercader N, Selleri L, Criado LM, Pallares P, Parras C, Cleary ML, et al. Ectopic Meis1 expression in the mouse limb bud alters P-D patterning in a Pbx1-independent manner. Int J Dev Biol 2009;53:1483–94.
[77] Yashiro K, Zhao X, Uehara M, Yamashita K, Nishijima M, Nishino J, et al. Regulation of retinoic acid distribution is required for proximodistal patterning and outgrowth of the developing mouse limb. Dev Cell 2004;6:411–22.
[78] Summerbell D, Lewis JH, Wolpert L. Positional information in chick limb morphogenesis. Nature 1973;244:492–6.
[79] Tabin C, Wolpert L. Rethinking the proximodistal axis of the vertebrate limb in the molecular era. Genes Dev 2007;21:1433–42.
[80] Sheeba CJ, Andrade RP, Palmeirim I. Limb patterning: from signaling gradients to molecular oscillations. J Mol Biol 2014;426:780–4.
[81] Saunders J. The proximo–distal sequence of the origin of the parts of the chick wing and the role of the ectoderm. J Exp Zool 1948;108:363–403.
[82] Rubin L, Saunders Jr JW. Ectodermal–mesodermal interactions in the growth of limb buds in the chick embryo: constancy and temporal limits of the ectodermal induction. Dev Biol 1972;28:94–112.
[83] Andrade RP, Palmeirim I, Bajanca F. Molecular clocks underlying vertebrate embryo segmentation: a 10-year-old hairy-go-round. Birth Defects Res C: Embryo Today: Rev 2007;81:65–83.
[84] Pascoal S, Carvalho CR, Rodriguez-Leon J, Delfini MC, Duprez D, Thorsteinsdottir S, et al. A molecular clock operates during chick autopod proximal–distal outgrowth. J Mol Biol 2007;368:303–9.
[85] Shanmugasundaram M, Robert B. Timely digits. J Mol Biol 2014;426:777–9.
[86] Globus M, Vethamany-Globus S. An in vitro analogue of early chick limb bud outgrowth. Differ Res Biol Divers 1976;6:91–6.
[87] Tickle C. Making digit patterns in the vertebrate limb. Nat Rev Mol Cell Biol 2006;7:45–53.
[88] Riddle RD, Johnson RL, Laufer E, Tabin C. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 1993;75:1401–16.
[89] Tamura K, Nomura N, Seki R, Yonei-Tamura S, Yokoyama H. Embryological evidence identifies wing digits in birds as digits 1, 2, and 3. Science 2011;331:753–7.
[90] Harfe BD, Scherz PJ, Nissim S, Tian H, McMahon AP, Tabin CJ. Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. Cell 2004;118:517–28.
[91] Lettice LA, Heaney SJ, Purdie LA, Li L, de Beer P, Oostra BA, et al. A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. Hum Mol Genet 2003;12:1725–35.
[92] te Welscher P, Fernandez-Teran M, Ros MA, Zeller R. Mutual genetic antagonism involving GLI3 and dHAND prepatterns the vertebrate limb bud mesenchyme prior to SHH signaling. Genes Dev 2002;16:421–6.
[93] Xu B, Wellik DM. Axial Hox9 activity establishes the posterior field in the developing forelimb. Proc Natl Acad Sci U S A 2011;108:4888–91.
[94] Niederreither K, Vermot J, Schuhbaur B, Chambon P, Dolle P. Embryonic retinoic acid synthesis is required for forelimb growth and anteroposterior patterning in the mouse. Development 2002;129:3563–74.
[95] Kmita M, Tarchini B, Zakany J, Logan M, Tabin CJ, Duboule D. Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene function. Nature 2005;435:1113–6.
[96] Tarchini B, Duboule D, Kmita M. Regulatory constraints in the evolution of the tetrapod limb anterior–posterior polarity. Nature 2006;443:985–8.
[97] Zuniga A, Zeller R. Gli3 (Xt) and formin (ld) participate in the positioning of the polarising region and control of posterior limb-bud identity. Development 1999;126:13–21.
[98] Capellini TD, Di Giacomo G, Salsi V, Brendolan A, Ferretti E, Srivastava D, et al. Pbx1/Pbx2 requirement for distal limb patterning is mediated by the hierarchical control of Hox gene spatial distribution and Shh expression. Development 2006;133:2263–73.

Please cite this article in press as: Sheeba CJ, et al. Getting a handle on embryo limb development: Molecular interactions driving limb outgrowth and patterning. Semin Cell Dev Biol (2015), http://dx.doi.org/10.1016/j.semcdb.2015.01.007

[99] Galli A, Robay D, Osterwalder M, Bao X, Benazet JD, Tariq M, et al. Distinct roles of Hand2 in initiating polarity and posterior Shh expression during the onset of mouse limb bud development. PLoS Genet 2010;6:e1000901.
[100] Yang Y, Niswander L. Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. Cell 1995;80:939–47.
[101] Zeller R, Lopez-Rios J, Zuniga A. Vertebrate limb bud development: moving towards integrative analysis of organogenesis. Nat Rev Genet 2009;10:845–58.
[102] Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, et al. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 1996;383:407–13.
[103] Sanz-Ezquerro JJ, Tickle C. Autoregulation of Shh expression and Shh induction of cell death suggest a mechanism for modulating polarising activity during chick limb development. Development 2000;127:4811–23.
[104] Lettice LA, Williamson I, Wiltshire JH, Peluso S, Devenney PS, Hill AE, et al. Opposing functions of the ETS factor family define Shh spatial expression in limb buds and underlie polydactyly. Dev Cell 2012;22:459–67.
[105] Mao J, McGlinn E, Huang P, Tabin CJ, McMahon AP. Fgf-dependent Etv4/5 activity is required for posterior restriction of Sonic Hedgehog and promoting outgrowth of the vertebrate limb. Dev Cell 2009;16:600–6.
[106] Nissim S, Allard P, Bandyopadhyay A, Harfe BD, Tabin CJ. Characterization of a novel ectodermal signaling center regulating Tbx2 and Shh in the vertebrate limb. Dev Biol 2007;304:9–21.
[107] Ahn S, Joyner AL. Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. Cell 2004;118:505–16.
[108] Wang B, Fallon JF, Beachy PA. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell 2000;100:423–34.
[109] Bai CB, Auerbach W, Lee JS, Stephen D, Joyner AL. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. Development 2002;129:4753–61.
[110] Park HL, Bai C, Platt KA, Matise MP, Beeghly A, Hui CC, et al. Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. Development 2000;127:1593–605.
[111] Hui CC, Joyner AL. A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. Nat Genet 1993;3:241–6.
[112] Litingtung Y, Dahn RD, Li Y, Fallon JF, Chiang C. Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. Nature 2002;418:979–83.
[113] Wang C, Ruther U, Wang B. The Shh-independent activator function of the full-length Gli3 protein and its role in vertebrate limb digit patterning. Dev Biol 2007;305:460–9.
[114] Lopez-Rios J, Speziale D, Robay D, Scotti M, Osterwalder M, Nusspaumer G, et al. GLI3 constrains digit number by controlling both progenitor proliferation and BMP-dependent exit to chondrogenesis. Dev Cell 2012;22:837–48.
[115] Wolpert L. Positional information and the spatial pattern of cellular differentiation. J Theor Biol 1969;25:1–47.
[116] Towers M, Mahood R, Yin Y, Tickle C. Integration of growth and specification in chick wing digit-patterning. Nature 2008;452:882–6.
[117] Zhu J, Nakamura E, Nguyen MT, Bao X, Akiyama H, Mackem S. Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud. Dev Cell 2008;14:624–32.
[118] Francis-West P, Hill R. Uncoupling the role of sonic hedgehog in limb development: growth and specification. Sci Signal 2008;1:pe34.
[119] Sheth R, Marcon L, Bastida MF, Junco M, Quintana L, Dahn R, et al. Hox genes regulate digit patterning by controlling the wavelength of a Turing-type mechanism. Science 2012;338:1476–80.
[120] Dahn RD, Fallon JF. Interdigital regulation of digit identity and homeotic transformation by modulated BMP signaling. Science 2000;289:438–41.
[121] Suzuki T, Hasso SM, Fallon JF. Unique SMAD1/5/8 activity at the phalanx-forming region determines digit identity. Proc Natl Acad Sci U S A 2008;105:4185–90.
[122] Merino R, Rodriguez-Leon J, Macias D, Ganan Y, Economides AN, Hurle JM. The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. Development 1999;126:5515–22.
[123] Montero JA, Lorda-Diez CI, Ganan Y, Macias D, Hurle JM. Activin/TGFbeta and BMP crosstalk determines digit chondrogenesis. Dev Biol 2008;321:343–56.
[124] Lussier M, Canoun C, Ma C, Sank A, Shuler C. Interdigital soft tissue separation induced by retinoic acid in mouse limbs cultured in vitro. Int J Dev Biol 1993;37:555–64.
[125] Rodriguez-Leon J, Merino R, Macias D, Ganan Y, Santesteban E, Hurle JM. Retinoic acid regulates programmed cell death through BMP signalling. Nat Cell Biol 1999;1:125–6.
[126] Dupe V, Ghyselinck NB, Thomazy V, Nagy L, Davies PJ, Chambon P, et al. Essential roles of retinoic acid signaling in interdigital apoptosis and control of BMP-7 expression in mouse autopods. Dev Biol 1999;208:30–43.
[127] Sheeba CJ, Andrade RP, Palmeirim I. Joint interpretation of AER/FGF and ZPA/SHH over time and space underlies hairy2 expression in the chick limb. Biol Open 2012;1:1102–10.
[128] Sheeba CJ, Palmeirim I, Andrade RP. Retinoic acid signaling regulates embryonic clock hairy2 gene expression in the developing chick limb. Biochem Biophys Res Commun 2012;423:889–94.
[129] Chen H, Johnson RL. Dorsoventral patterning of the vertebrate limb: a process governed by multiple events. Cell Tissue Res 1999;296:67–73.
[130] Chen H, Johnson RL. Interactions between dorsal–ventral patterning genes lmx1b, engrailed-1 and wnt-7a in the vertebrate limb. Int J Dev Biol 2002;46:937–41.
[131] Loomis CA, Harris E, Michaud J, Wurst W, Hanks M, Joyner AL. The mouse Engrailed-1 gene and ventral limb patterning. Nature 1996;382:360–3.
[132] Parr BA, McMahon AP. Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. Nature 1995;374:350–3.
[133] Riddle RD, Ensini M, Nelson C, Tsuchida T, Jessell TM, Tabin C. Induction of the LIM homeobox gene Lmx1 by WNT7a establishes dorsoventral pattern in the vertebrate limb. Cell 1995;83:631–40.
[134] Vogel A, Rodriguez C, Warnken W, Izpisua Belmonte JC. Dorsal cell fate specified by chick Lmx1 during vertebrate limb development. Nature 1995;378:716–20.
[135] Verheyden JM, Sun X. An Fgf/Gremlin inhibitory feedback loop triggers termination of limb bud outgrowth. Nature 2008;454:638–41.
[136] Scherz PJ, Harfe BD, McMahon AP, Tabin CJ. The limb bud Shh-Fgf feedback loop is terminated by expansion of former ZPA cells. Science 2004;305:396–9.
[137] Wade C, Brinas I, Welfare M, Wicking C, Farlie PG. Twist2 contributes to termination of limb bud outgrowth and patterning through direct regulation of Grem1. Dev Biol 2012;370:145–53.
[138] Farin HF, Ludtke TH, Schmidt MK, Placzko S, Schuster-Gossler K, Petry M, et al. Tbx2 terminates shh/fgf signaling in the developing mouse limb bud by direct repression of gremlin1. PLoS Genet 2013;9:e1003467.

Please cite this article in press as: Sheeba CJ, et al. Getting a handle on embryo limb development: Molecular interactions driving limb outgrowth and patterning. Semin Cell Dev Biol (2015), http://dx.doi.org/10.1016/j.semcdb.2015.01.007