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Editor

Congenital Anomalies of the Upper Extremity

Etiology and Management

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To Don Sr., Judy, Susan, Dylan, Genevieve, Sharon, Jack, and Erik, with love and gratitude.

Preface

Hominem ad deos nulla re propius accedunt quam salutem hominibus dando.
In nothing do men more nearly approach the gods, than in giving health to men.

—Marcus Tullius Cicero

How much more do we approach divinity when we help a child to health?

I am very honored to be in the company of all of the authors of this text. Not only have they collaborated on this book you now hold in your hands but also we share one of the finest activities in the world: we have been trusted by parents to care for their children's limbs and health.

This text, like all texts, will become dated and be superseded by new knowledge. However, it stands as a milestone of where we are now in our knowledge and also as a direction sign of where our knowledge is heading.

It is my hope that readers of this text will join us in the care of children with congenital anomalies of the upper extremity and will be inspired to further advance our knowledge in this field.

Burlington, VT, USA — Donald R. Laub Jr.

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Part I

General Considerations

Embryology and Classification of Congenital Upper Limb Anomalies

1

Carlos Garrido-Allepuz Herrera, Michael A. Tonkin, and Kerby C. Oberg

Morphological Overview

In vertebrates, the limb bud starts as an accumulation of cells within the lateral plate mesoderm (LPM) forming an oblong ventrolateral bulge on the body wall. The limb is a composite structure of cells from the LPM (precursors of limb-associated skeletal tissues) and associated somites (muscle and vascular precursors). In humans, the upper limb bud appears during the fourth week of development around day 26 (Carnegie stage 12) and is located between somites 9 and 12 (Fig. 1.1a) [1, 2]. The limb emerges only in certain zones of the body known as limb fields. The position of limb fields are thought to be specified by a quantitative and/or qualitative combination of Hox transcription factors (see Fig. 1.1b) [3, 4].

By day 37 of development (Carnegie stage 16), the distal portion of the limb can be recognized as a handplate. At the same time there is progressive mesodermal condensation along the proximodistal axis forming the skeletal elements of the limb. By day 56 the major morphologic features of the limb are complete.

Limb Initiation

After the upper limb fields have been specified, induction of the limb bud occurs. The cells of the LPM located within the limb fields maintain active proliferation, while non-limb field LPM begins to divide more slowly [5]. Initially *Fgf10* is expressed broadly along the LPM, but just before the limb emerges, the domain of *Fgf10* expression becomes restricted to the limb fields. In chicken, the expression of *Tbx5* and *Wnt2b* in the LPM cells of the limb field are responsible for the induction of *Fgf10* in the presumptive limb (Fig. 1.2) [6–8]. Recent studies suggest that *Tbx5* expression can be induced and regulated by Hox transcription factors, suggesting a role for Hox genes in both positioning limb fields and initiating limb outgrowth [9]. Fgf10 through its receptor FgfrIIa has been shown to induce *Wnt3* and *Wnt3a* in prospective mouse and chick limb ectoderm, respectively. Concurrently, Bone Morphogenetic Protein (Bmp) signaling in the ventral ectoderm induces β-catenin competency in cells of the presumptive apical ectodermal ridge (AER) at the dorsal–ventral boundary [10, 11]. In turn, Wnt3 or Wnt3a induces *Fgf8* in a Wnt/β-catenin-dependent manner in the precursor cells of the AER [6, 12]. Fgf8 secreted from the recently formed AER maintains the expression of *Fgf10* in the mesoderm, establishing a positive regulatory loop that maintains proximal–distal growth [6, 12].

Another signaling molecule that is fundamental to the induction of the limb bud appears to be retinoic acid (RA), the active metabolite of vitamin A. This molecule is produced in the somites of the embryo by the enzyme Raldh2 [13–15]. RA restricts the early expression of Fgf8 within the heart field, which, in turn, permits the expression of *Tbx5* in the limb field to initiate forelimb development [16, 17]. Furthermore, RA has been shown to regulate the expression of *Hox* genes both in vitro and in vivo, which may contribute to limb field induction and/or positioning (see Fig. 1.2) [18, 19].

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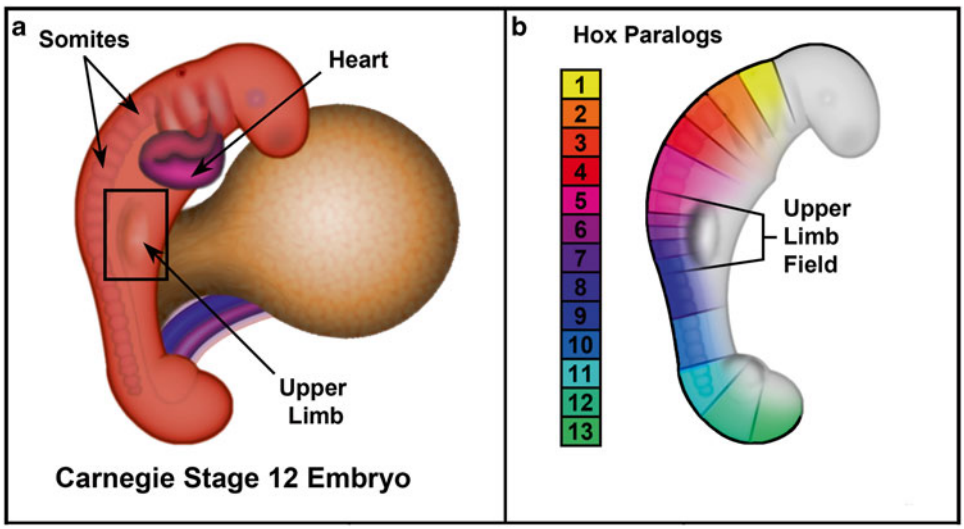

Fig. 1.1 Human embryo at stage of limb initiation and presumed Hox positioning. (**a**) Depiction of an emerging upper limb bud (boxed) in Carnegie stage 12 embryo. (**b**) Hox genes establish upper limb position and polarity. Courtesy of K.C. Oberg and Loma Linda University

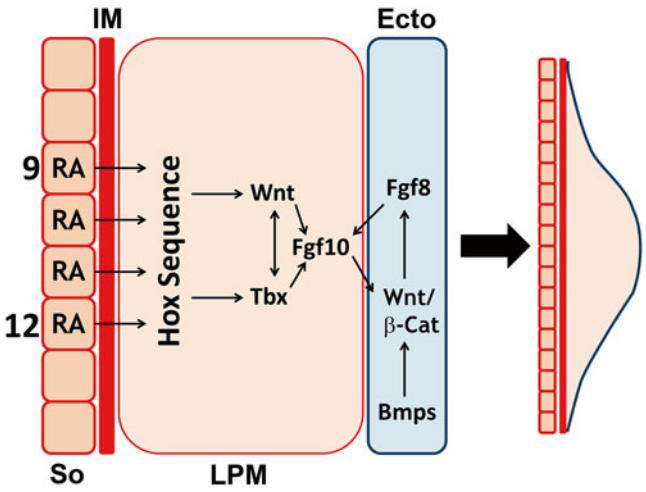

Fig. 1.2 Molecular pathways involved in limb induction. Depiction of the tissues involved in the initiation of the right upper limb bud emerging from lateral plate mesoderm (LPM) at somite (So) levels 9-12. Molecular interactions between LPM and ectoderm (Ecto) are also illustrated. IM-intermediate mesoderm. Courtesy of K.C. Oberg and Loma Linda University

Signaling Centers

Between the fourth and eighth weeks of development, the limb bud undergoes growth and differentiation to transform it into a fully patterned limb. This process can be described in terms of three coordinate axes: proximal–distal (P–D), anterior–posterior or radial–ulnar (A–P/R–U), and dorsal–ventral (D–V) modulated by three signaling centers [20].

Along the P–D axis, the AER appears as thickened ectoderm overlying the distal edge of the limb bud [21]. The AER is the signaling center that regulates the P–D growth and Fgfs are the signaling molecules that accomplish its function. Excision of the AER in chicken embryos at different stages of limb development results in limb truncations in a progressive fashion; the later the AER removal, the more distal the resulting truncation [22].

The signaling center for the A–P/R–U axis is the zone of polarizing activity (ZPA), a cluster of mesodermal cells located at the distal posterior (ulnar) margin. The ZPA directs A–P/R–U patterning and Shh is the signaling molecule that mediates its function. Both mice (*Shh* knock-out) that lack Shh function or mutant chickens (Oligozeugodactyly—*Ozd* mutants) that fail to have limb-specific Shh expression show marked loss of posterior (ulnar) elements [23, 24].

Dorsal non-AER ectoderm directs D–V patterning with Wnt7a as the signaling molecule that promotes dorsalization. Excision and rotation of the dorsal ectoderm results in the formation of dorsal structures within the ventral aspect of the limb [25].

Patterned Development Along Coordinate Axes

Pattern formation is a process by which the cells are sequentially specified, determined, and then differentiated to form the morphological structures of the limb. In this section we will focus on how the process of patterning is accomplished along each axis as directed by the signaling centers and the associated molecular pathways, recognizing that the molecular cascades of these three axes are operating concurrently and integrated together like a fine-tuned instrument.

Proximal–Distal Patterning (P–D)

The upper limb can be divided into three different segments along the P–D axis (Fig. 1.3): (1) the proximal segment or

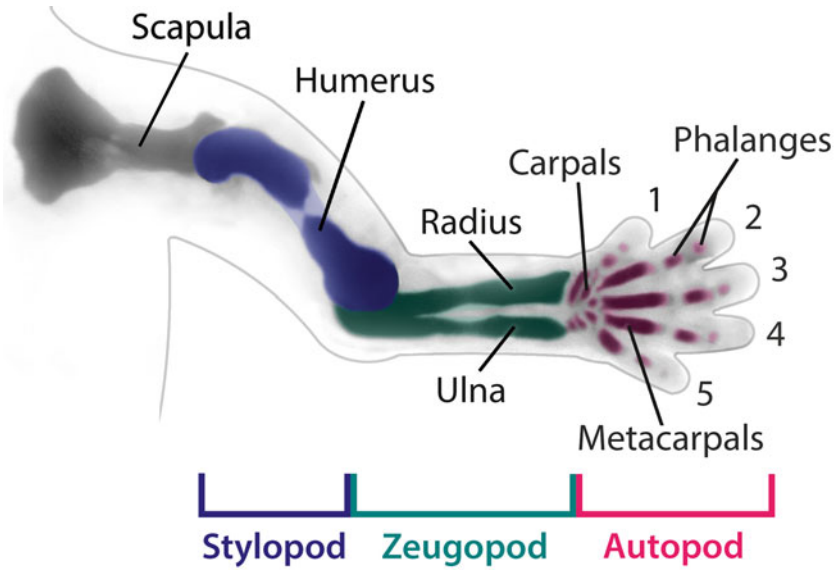

Fig. 1.3 Limb elements. The upper limb consists of a limb girdle or shoulder, and three limb segments known as the stylopod (humerus - colored blue), the zeugopod, which includes the radius and ulna (colored green) and the autopod or handplate (colored magenta). Courtesy of K.C. Oberg and Loma Linda University

stylopod where the skeletal elements of the humerus develop; (2) the intermediate segment or zeugopod where the radius and ulna form; and (3) the distal segment or autopod where the carpals, metacarpals, and digits form.

Patterning along the P–D axis begins during limb initiation with the formation of the AER, stratified ectoderm at the distal dorsal–ventral boundary of the developing limb bud. The AER secretes fibroblasts growth factors (Fgfs), the molecules primarily responsible for P–D patterning. Fgf8 is the first and functionally most important Fgf secreted from the AER during induction and maintained until the AER regresses, when the drafts of the last phalanges are formed. *Fgf4*, *Fgf9*, and *Fgf17* are activated sequentially in the posterior AER and expand to the anterior aspect as the limb develops [26, 27]. Classical experiments in chick embryos showed that AER removal abated distal limb outgrowth and resulted in truncations that corresponded to the timing of AER removal; in other words, the later the AER removal, the more distal the structures that were present [22]. Moreover, FGF-soaked beads were able to restore limb bud outgrowth and patterning after AER removal, indicating that Fgfs were the functional signaling factors of the AER [28, 29].

Among the different *Fgfs* expressed, *Fgf8* is thought to be the main AER signal, while *Fgf4*, *Fgf9*, or *Fgf17* are considered secondary or redundant [30, 31]. This concept is supported from experiments with *Fgf8* knock-out mice that showed smaller AERs, delayed limb bud outgrowth, and loss of some skeletal elements [26, 32]. In contrast, knock-out mice for *Fgf4*, *Fgf9*, and/or *Fgf17* did not develop limb anomalies. Interestingly, *Fgf4* expression in *Fgf8* knock-out mice was up-regulated, suggesting that redundant expression may have lessened the phenotype of these mutants. This was confirmed by the removal of both *Fgf4* and *Fgf8* that resulted in a worse phenotype with notably smaller limb buds [32, 33].

Several models have been proposed for P–D patterning. The progress zone model proposes that mesenchymal cell fate is determined by the length of time spent under the direct influence of the AER in a proliferative region called the progress zone (PZ) [34, 35]. The early specification model [36] postulates that the P–D identities are specified early and the different progenitor pools expand sequentially as the limb grows. The differentiation front model suggests that the AER maintains mesenchymal cells in an undifferentiated state; as the limb expands, the cells that are no longer under the influence of the AER differentiate [37].

However, the accumulating evidence supports an alternative model. The two signal model [30] proposes that two opposing signals pattern the limb along the P–D axis: RA emanating from the flank will specify a proximal fate, while Fgfs from the AER will specify a distal fate (Fig. 1.4a) [38, 39]. In somites, Raldh2 oxidizes Retinol to form RA which can act locally in the proximal limb buds to promote the expression of *Meis1* and *Meis2*. The expression of *Meis1*/2 defines the proximal limb segment and where the humerus (stylopod) will develop. Distally, Fgf signaling induces 5′*Hoxa* genes (*Hoxa11*, *Hoxa13*) and limits distal *Meis1*/*2* expression. Although the mechanism for this repression is not fully understood, it is known that Fgf8 signaling induces the expression of *Cyp26b1* in the distal mesenchyme of the limb bud; the product of this gene oxidizes RA into a non-active form, thus clearing the distal region of active RA (see Fig. 1.4c) [40]. Some have questioned RA role as a proximalizing agent [16], and further investigations are warranted to clarify whether RA or another factor influenced by RA is the proximalizing signal.

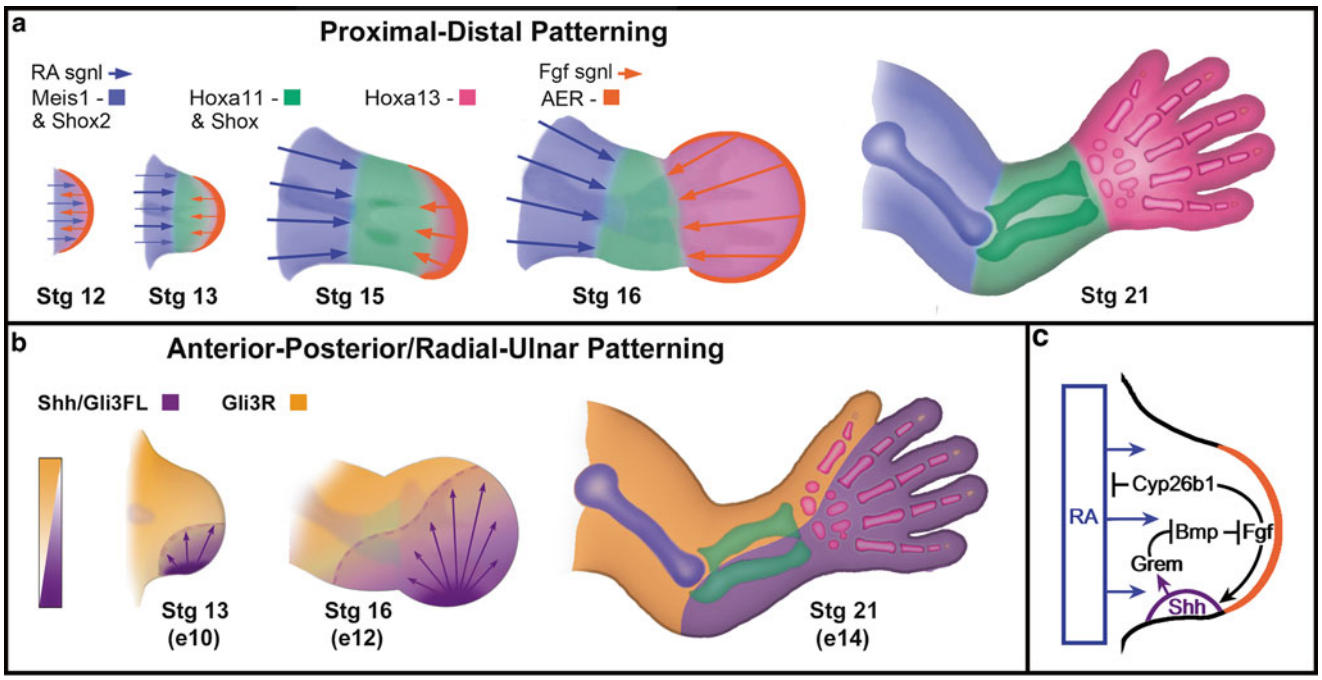

Fig. 1.4 Molecular pathways regulating proximal-distal & anterior-posterior/radial-ulnar axes. (**a**) Progressive segment specification along the proximal-distal axis based on the two signal model with RA-related proximalizing signals countered by distalizing Fgf signals. Outgrowth separates the signals and an intermediate zone is formed. Mies1 and Shox2 are restricted to the stylopod, Hoxa11 and Shox are markers for the zeugopod, and Hoxa13 delimits the handplate boundaries. (**b**) Opposing gradients of Gli3 repressor (Gli3R) and Shh-maintained full length Gli3 activator (Gli3FL) establish boundaries between the radius and ulna in the zeugopod and the thumb and ulnar digits in the autopod or hand. c) Some of the molecular interactions that maintain and terminate Shh expression in the ZPA (→ indicates positive regulation, while –| indicates inhibition). Courtesy of K.C. Oberg and Loma Linda University

Anterior–Posterior/Radial–Ulnar Patterning (A–P/R–U)

The limb along the A–P/R–U axis is divided into two segments: the anterior (radial), comprises the thumb and radius, and the posterior (ulnar) with the ulna and ulnar digits (digits two through five) [41].

Patterned development along the A–P/R–U axis is controlled by the zone of polarizing activity (ZPA), a cluster of mesenchymal cells maintained in the distal posterior/ulnar aspect of the developing limb (see Fig. 1.4b). The ZPA was discovered in 1968 through grafting experiments in chick limb buds [42]. In these experiments, grafts from the distal posterior/ulnar mesenchyme were excised from one group of chicks and then inserted into the distal anterior/radial aspect of another group of chicks. The limbs that developed from these grafts demonstrated mirror image duplication of structures [42–44]. RA was found to be the first molecule that mimicked ZPA grafts when applied to the distal anterior/radial aspect of the limb bud [45–48]. Later it was shown that Shh was the molecule responsible for the phenotype induced by RA [49].

Shh is critical to the correct developmental pattern of the limb, particularly for the forearm (zeugopod) and hand (autopod). This is demonstrated in *Shh* knock-out mice, which have a correctly developed stylopod, a single skeletal element (radius) for the zeugopod, and a minimal autopod. In the upper limb, the autopod forms as a small cartilage condensation [23, 50], while in the lower limb, the autopod consists of a single digit with two phalanges [23, 50].

A–P/R–U polarity and subsequent Shh expression is initiated by axial Hox gene segmentation [51] followed by several other factors that contribute to Shh induction and the establishment of the ZPA. Hand2, expressed in the posterior/ulnar half of the limb bud, is required for Shh induction [52, 53] and has been shown to directly interact with limb-specific Shh regulatory region [54]. Similarly, distal Hoxd (Hoxd10-13) transcription factors also interact with the limb-specific Shh regulatory region, and evidence suggests that their initial phase of limb bud expression helps to localize Shh expression (Fig. 1.5) [55].

The first or initial phase of distal Hoxd expression in the limb bud occurs in a nested collinear (corresponding to their gene order) fashion along the anterior–posterior axis, with Hoxd10 exhibiting the broadest initial expression domain. The expression of each successively more distal Hoxd gene is nested within the previous gene's expression domain (see Fig. 1.5). Hoxd13, the terminal transcription factor in the Hoxd cluster, has the most restricted expression domain within the distal posterior or ulnar aspect of the limb bud overlapping the ZPA. This first phase of distal Hoxd expression plays a role in localizing Shh expression and temporally corresponds to specification of the forearm or zeugopod.

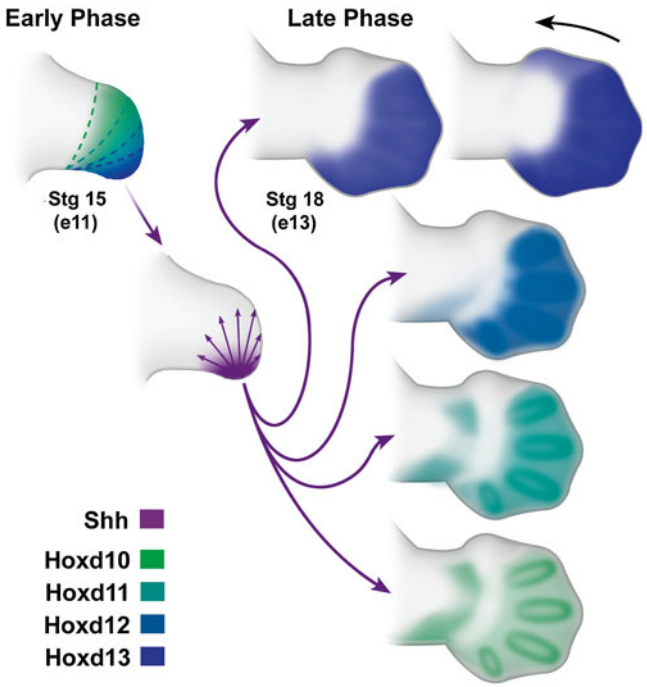

Fig. 1.5 Distal Hoxd genes are expressed in the limb bud in two phases. In the early phase, there is a nested collinear expression pattern. Dotted lines highlight the boundaries of expression, from broadest expression of Hoxd10 (green) to the most restricted of Hoxd13 (blue). In the late phase, Hoxd expressioin demonstrates quantitative colinearity with progressively more robust expression. Courtesy of K.C. Oberg and Loma Linda University

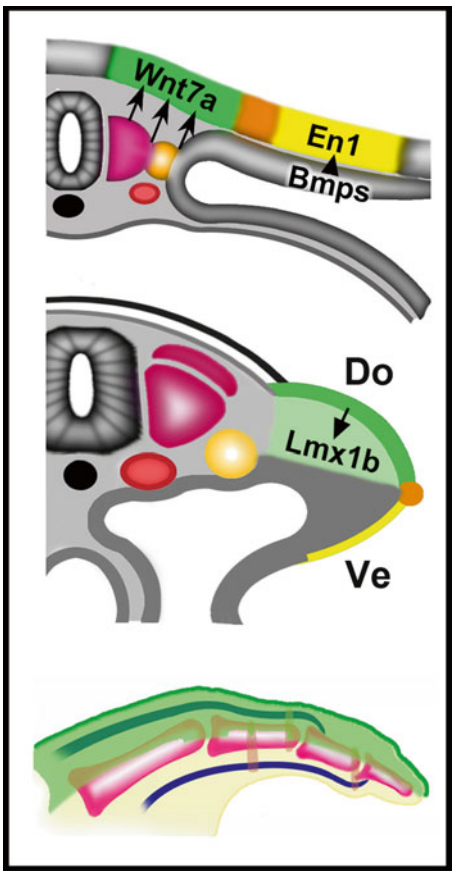

Fig. 1.6 Molecular pathways regulating the dorsal-ventral axis. From top to bottom, unknown factors in somites and/or intermediate mesoderm initiate Wnt7a expression in medial dorsal ectoderm. Bmps induce the expression of En1 in what will become the ventral ectoderm establishing the dorsal ventral boundary where the AER will form (orange). Wnt7a will induce Lmx1b in the underlying mesoderm to dorsalize developing tendons, joints and soft tissues. Courtesy of K.C. Oberg and Loma Linda University

The role of Shh in A–P/R–U axis patterning has been characterized largely through knock-out mice for members of the Gli protein family of transcription factors (Gli1, Gli2, and Gli3). *Gli3* mutant mice are polydactylous without digit identity while the zeugopod is perfectly formed [56, 57]. Remarkably, the limbs of the double knock-out mice for both *Gli3* and *Shh* were indistinguishable from the *Gli3* mutant alone [58, 59], suggesting that the principal function of Shh is mediated through Gli3. Molecular studies demonstrated that Shh signaling prevents the posttranslational processing of full-length Gli3 protein into a short form, which functions as a strong repressor of Shh target genes.

Secreted Shh diffusing from the ZPA establishes a posterior to anterior concentration gradient. A complementary gradient of Gli3R forms with high levels of Gli3R in the anterior zone where Shh signaling is minimal (see Fig. 1.4b) [59]. In the absence of *Shh*, the level of Gli3R is uniform along the A–P/R–U axis and the elevated levels of Gli3R, unopposed by Shh, are accompanied by an increase in the apoptotic rate of the limb mesenchyme [58, 60]. Thus, the A–P/R–U gradient of Gli3R and its reciprocal full length Gli3 activator are responsible for conveying pattern information along this axis. However, it remains unclear whether the critical patterning signal is the absolute level of GLi3R or the relative levels between the repressor and the activator forms [58, 59]. Collectively, these data help to characterize the role of Shh in A–P/R–U patterning which, at least, in part, is to regulate the form and function of its transcription factor, Gli3.

Dorsal–Ventral Patterning (D–V)

Patterning along this axis is regulated by signals from the non-AER ectoderm that surrounds the limb mesenchyme. The dorsal and ventral areas are defined by the expression of two different genes: *Wnt7a* in the dorsal ectoderm and *En-1* in the ventral ectoderm (Fig. 1.6). *Wnt7a* signaling defines the dorsal fate of the limb structures [61], while *En-1* restricts *Wnt7a* expression to the dorsal ectoderm, preventing the dorsalization of ventral limb tissues [62, 63]. It is not yet known how *Wnt7a* is induced in the presumptive limb ectoderm; however, there is evidence that BMP and WNT canonical signaling are responsible for the induction of *En-1* in the ventral ectoderm. Knock-out mice have further elaborated their functional roles. *Wnt7a* mutants have biventral limbs, while