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Chair: Kerby C. Oberg (USA)

Committee: Maria A. Ros (Spain)
Charles A. Goldfarb (USA)

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New Insights on Upper Limb Development: Digitizing the Hand

Kerby C. Oberg, Loma Linda University, Loma Linda, CA, USA;
Maria A. Ros, Instituto de Biomedicina y Biotecnología de Cantabria, Santander, Spain;
and Charles A. Goldfarb, Washington University School of Medicine, St. Louis, MO, USA

During the 5th week of human development, the distal end of the upper limb bud begins to flatten and expand forming the autopod or handplate. Over the next few days the handplate will transform into a predictable series of segmented digits. The molecular mechanisms underlying digit formation are not fully understood, however, recent investigations in animal models and clinical genetics provide some interesting insights into the process.

Limb Initialization, Outgrowth and Developmental Axes

The position of the upper limb along the cranial-caudal axis is established by Hox transcription factors (Fig. 1a and 1b)[3;42]. Within the presumptive upper limb field, Hox transcription factors up-regulate the T-box containing transcription factor 5 (TBX5) which, in turn, up-regulates fibroblast growth factor 10 (FGF10) secretion to promote upper limb bud initiation and outgrowth[1;15;20;24;31]. The subsequent morphogenesis of the emerging limb bud can be described in terms of three coordinate axes – the proximal-distal axis, the anterior-posterior (or radial-ulnar) axis and the dorsal-ventral (or dorsal-volar) axis as depicted in Figure 1. Each of these axes is controlled by a signaling center that initiates a cascade of axis-related pathways. Although all three axis-related pathways contribute to digit formation, the anterior-posterior axis establishes the number of digits and digit-specific morphology (phalange size and number) and will be the primary focus of this report.

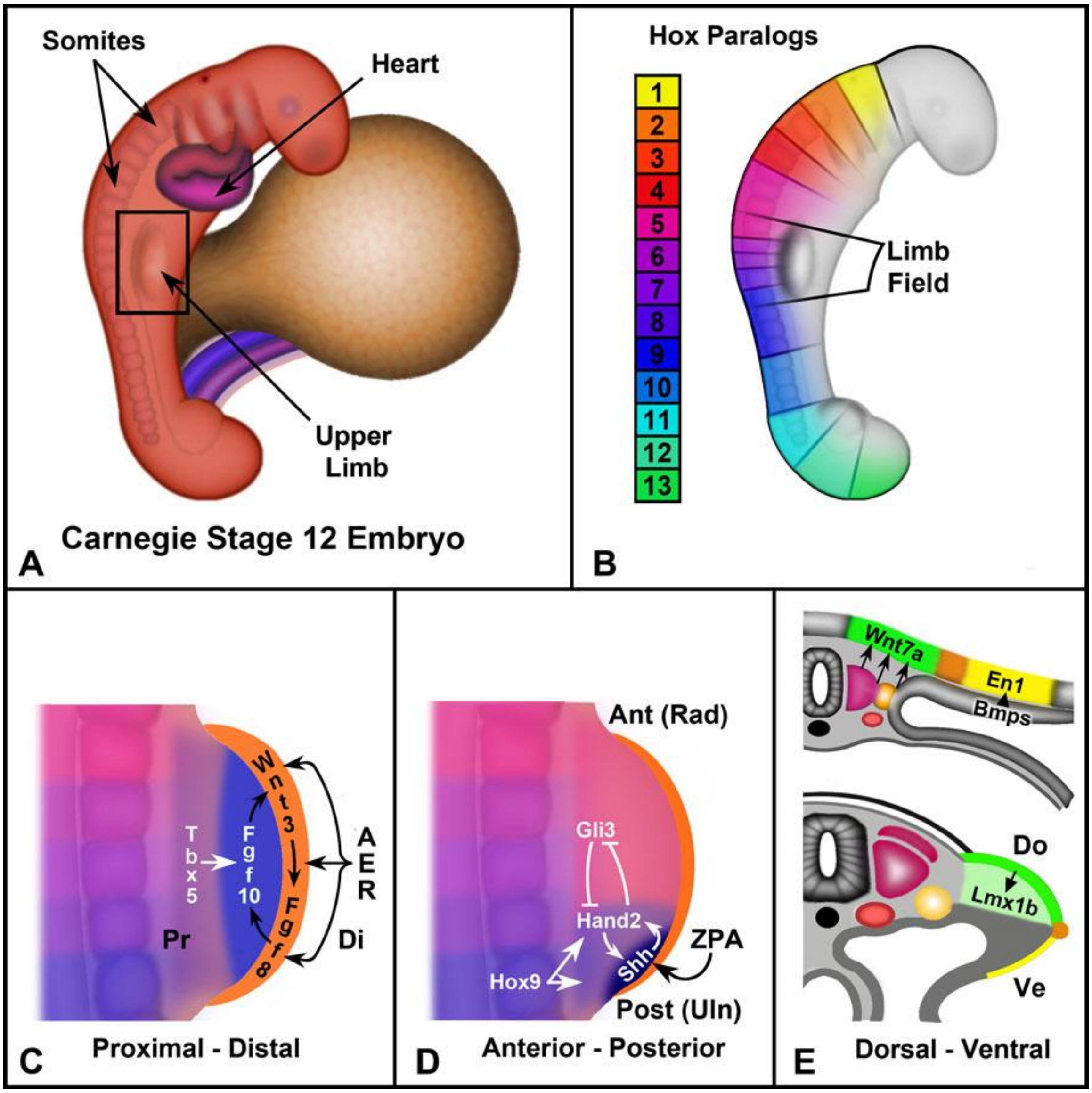

Figure 1. Limb Induction and Signaling Center Formation

A) Depiction of an emerging upper limb bud in Carnegie stage 12 embryo. B) Hox genes establish upper limb position and polarity. C) Cascade of events that initiate limb bud formation and proximal distal polarity with formation of the apical ectodermal ridge (AER) and induction of Fgf8. D) Cascade of events that induce the expression of Shh and formation of the zone of polarizing activity establishing radial-ulnar (anterior –posterior) polarity. E) Unknown factors in somites and/or intermediate mesoderm initiate Wnt7a expression in what will become the dorsal ectoderm. Bmps induce the expression of En1 in what will become the ventral ectoderm and thereby establishes the dorsal ventral boundary where the AER will form (orange).

Prior to handplate formation, expression of the first phase of distal HOXD transcription factors (HOXD10-13) occurs in a nested collinear fashion along the anterior-posterior axis, with HOXD10 exhibiting the largest initial expression domain. Each successively more distal HOXD transcription factor is nested within the previous gene's expression domain (See Figure 2). HOXD13, the terminal transcription factor in the HOXD cluster, has the smallest expression domain and overlaps the zone of polarizing activity (ZPA), within the distal posterior or ulnar aspect of the limb bud. This first phase of distal HOXD expression corresponds to the forearm or zeugopod specification.

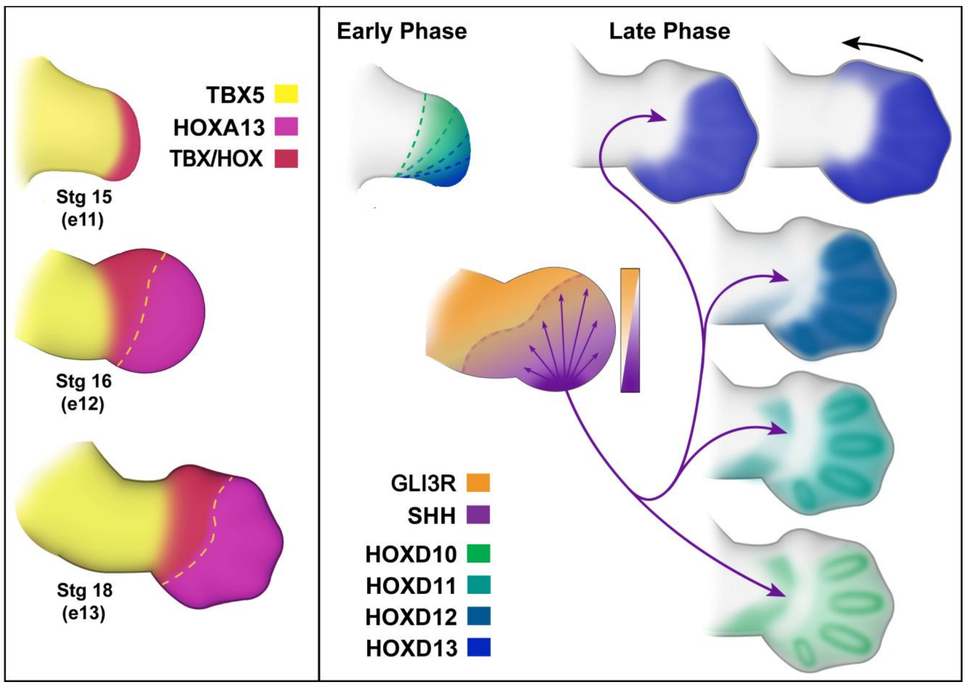

Figure 2. Molecular Pathways Regulating Digit Formation

TBX5 expression (yellow) persists as the autopod forms (Carnegie stage 16, post fertilization day 37; comparable to mouse embryonic day 12 or e12) and begins to differentiate (Carnegie stage 18, post fertilization day 44; comparable to mouse e13). However, expression of TBX5 in the autopod is limited to the anterior-proximal aspect (illustrated as a yellow-dashed line). HOXA13 is expressed within autopod cells (illustrated as magenta) and delineates the proximal autopod boundary. Interestingly, digit 1 or the thumb, is the only digit to have the combined expression both TBX5 and HOXA13. The distal HOXD complex (HOXD10-13) is expressed in a nested collinear pattern in early limb development (early phase, Stg 15 or mouse e11). This nested pattern is also thought to participate in the induction or maintenance of Shh expression. GLI3 processing by SHH (purple) sets up an anterior-posterior gradient of GLI3 repressor(GLI3R)(orange). SHH also regulates the expression of the distal HOXD transcription factors (HOXD10-13) (Carnegie Stg 18 or mouse e13) within increasing intensity (quatitative collinearity). These transcription factors appear to physically interact with GLI3 to refine digit identity (the SHH dependent boundary is highlighted by a purple dashed line). HOXD 10-12 have overlapping expression domains in presumptive digits 2-5, but are restricted from the thumb domain. HOXD13 in contrast is expressed in all of the digit domains including the presumptive thumb, though its late extension into the thumb domain is SHH independent.

Sonic hedgehog (SHH) is secreted from the ZPA and establishes a posterior to anterior gradient along the anterior-posterior (radial-ulnar) axis. SHH manifests its action via the family of GLI-Krupple zinc finger transcription factors (GLI1, GLI2 and GLI3). Of these GLI3 is the most important during limb development. In the absence of SHH, Gli3 is processed into a truncated form that is a strong transcriptional repressor (GLI3R) [27;38]. SHH inhibits this processing, thus the posterior-anterior SHH gradient is translated into a complementary anterior-posterior intracellular gradient of GLI3R (See Figure 2)[38]. SHH/GLI3 regulation is critical for posterior (ulnar) limb proliferation and distal patterning (forearm and hand) [33;44].

**A general summary of upper limb development can be reviewed at: http://www.llu.edu/central/faculty/koberg/limb.page? (Courtesy of the American Society for Surgery of the Hand)*

Digitizing the Handplate

The handplate is the last segment of the limb bud to form, appearing about 37 days post ovulation (Carnegie stage 16, Figure 2). As the handplate forms, several molecular pathways converge. HOXA13, the terminal Hox transcription factor of the HOXA cluster, is induced in the distal limb bud demarcating the handplate boundary[41;43]. Concurrently, a second "late", SHH-regulated phase of distal HOXD expression (that corresponds with digit formation) is generated that partially reverses their expression domains, i.e., reversed colinearity[19]. In addition, there is a graded expression intensity with HOXD13 exhibiting the most robust expression within the digits, and HOXD10 exhibiting the least intense expression, what has been termed quantitative colinearity[11].

Experimental evidence suggested that the ZPA produced a diffusible morphogen that established a spatial concentration gradient across the anterior-posterior limb bud axis[29]. This provided cells with a positional value according to their position within the gradient field. SHH was subsequently identified and validated as the morphogen secreted by the ZPA critical for limb patterning and digit identity[9;23]. More recently, Shh has been shown to have dual, separable functions in both patterning and growth[33;44].

The accumulated evidence suggests that these molecules work collectively to establish the five digit pattern common to most tetrapods (animals with four limbs). Although a molecular gradient has been a popular hypothesis, a gradient model does not fully explain the repeating digital-interdigital pattern. Recent analysis of compound deletions of distal *Hox* (*Hoxa13, Hoxd11-13*) and *Gli3* genes in mice, exposed an intrinsic self-organizing mechanism involved in patterning the digits[28]. With the progressive reduction of Hox gene dose in the absence of Gli3, there is a progressive increase in digit numbers (up to 14 digits) that is not accompanied by a corresponding increase in handplate size, thus the digits are increasingly thinner and shorter.

Alan Turing first proposed a mathematical diffusion-reaction model to account for repetitive self-organizing patterns, such as stripes or spots in animal skin and fur[34]. This model proposes two molecules, an activator and inhibitor, which diffuse into a field of cells. The activator auto-upregulates itself and upregulates its own inhibitor. In contrast, the model's inhibitor suppresses the activator and auto-inhibits its own expression (see Figure 3).

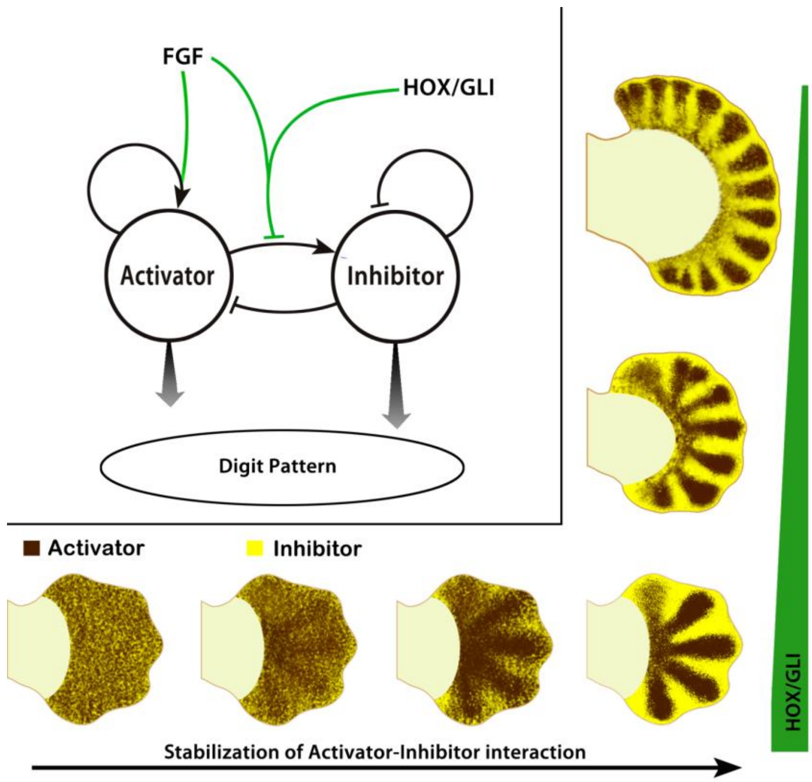

Figure 3. Turing-Like Patterning in Limbs
In the upper left-hand boxed region is a diagram of the diffusion-driven instability model with an activator and inhibitor. Modulation of this intrinsic self-organizing mechanism (ISOM) by FGF and HOX/GLI is also depicted. In the model described by Sheth et al. (2012), FGF from the apical ectodermal ridge (AER) promotes a radial stripe pattern from the ISOM and ultimately regulates digit length (blue), while FGF in concert with distal HOX and GLI transcription factors limit the number of digits (green). On the bottom of the figure, a series of handplates show the rapid progression from fluctuating activator-inhibitor interaction (noise) to a stabilized 5-digit pattern. On the right, progressive loss of digit suppressing HOX/GLI transcription factors (green bar) causes an increase in the number of digits patterned by the ISOM.

Small random molecular fluctuations of the activator and the inhibitor eventually lead to steady patterns, usually spots or stripes. The pattern is dependent upon the robustness of activator and inhibitor expression as well as their diffusion rates. This intrinsic self-organizing or Turing mechanism controls the initial alternating digit/non digit pattern in the handplate. Although the molecular identity of the activator and inhibitor are not yet known, these investigations indicate that the terminal HOXA/D transcription factors, in concert with Shh/Gli3 regulation, modulate the intrinsic self-organizing mechanism and are critical in resolving the common digit-interdigit pattern of pentydactyly.

Once the number of digits has been established, digit specific morphologies are determined. The mechanisms that regulate digit morphology are not full characterized, but at the distal end of each digit there is a thin cap of cells called the phalanx forming region (PFR) or digital cresent (Figure 4)[16;30]. Signals from the adjacent posterior interdigital tissue regulate digit morphology and function relatively late as the phalanges are progressively being formed [6]. Our current understanding suggests that SHH – GLI3R/GLI3FL counter gradients and SHH-regulated HOXD10-13 transcription factors (particularly for digits 2-5), likely through BMPs (predominantly BMP2, 4 &7) and from AER-related FGFs and WNTs instruct the PFR to form the appropriate number and size of phalanges[16;25;26;30;39;40].

The thumb domain is somewhat different, expressing factors that are thought to influence the specialized morphology of the thumb [21]. TBX5 expression extends into the proximal handplate, associated with the presumptive carpals and thumb, but does not extend into the ulnar digits (digits 2-5)(Figure 2)[12]. Moreover, the thumb domain is accentuated by the lack of HOXD10-12 expression and only the most terminal HOX transcription factors, i.e., HOXA13 and HOXD13, are expressed.

When the digit morphology has been established, the AER regresses and the terminal phalanges begin to form[5;26]. Formation of terminal phalanges is morphologically and mechanistically different from other phalanges. Although a cartilage model forms similar to other phalanges, ossification begins at the distal tapered tip rather than as a collar around the mid-shaft[4]. A keratinized nail also forms on the dorsal aspect of the terminal or ungual phalanx. In mice, the terminal phalanges are demarcated molecularly by the expression of Bambi, a Bmp inhibitor, and Sp8, a member of the specificity protein family of transcription factors that mediates Wnt signaling (Figure 4, last panel)[4;10]. In addition, the terminal phalanx retains the expression of MSX1, a transcription factor that is thought to convey the capacity for fingertip regeneration[2;7].

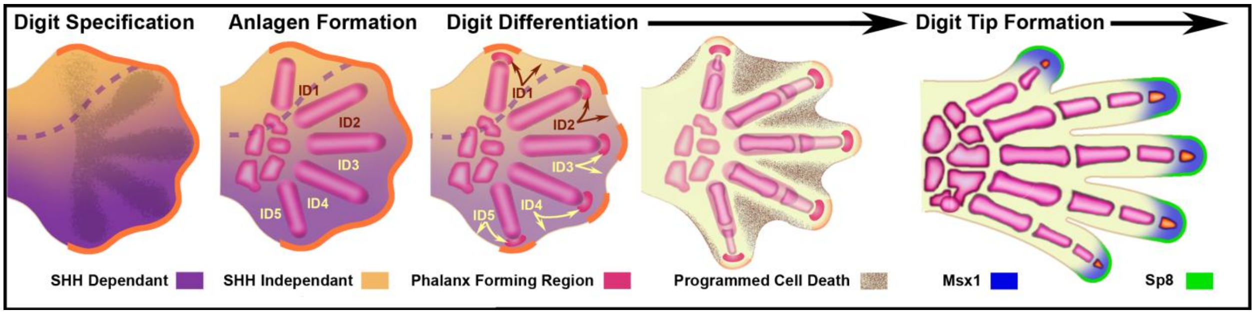

Figure 4. Digit Morphogenesis
After establishing digit number and the SHH dependant/independent domains, digit morphologies are specified. Digit morphologies are determined by the adjacent posterior interdigital mesoderm as illustrated (ID1–ID5). The inderdigital tissue conveys specified digit morphology to the phalanx forming region (PFR—magenta) capping the distal tip of each digital anlagen. The PFR, in concert with the AER (orange), determines phalanx size, length and positioning of joints. The interdigital tissue subsequently undergoes Bmp mediated programmed cell death (speckled regions). As the AER regresses the distal or ungual phalanx begins to form and is demarcated by expression of mesodermal Msx1 (blue) and ectodermal Sp8 (green). (Image adapted from Oberg et al., 2010)

Clinical Genetics

While animal models are important in characterizing many of the pathways that regulate digit formation, genetic analysis of patients with congenital upper limb anomalies are instrumental in providing relevant molecules to investigate and in confirming conservation of suspected pathways. For example, disruption of GLI3 was first recognized in patients with Greig cephalopolysyndactyly[37]. GLI3 was subsequently confirmed as the molecule disrupted in the mouse mutant extra-toes (Xt), that had some features akin to Greig cephalopolysyndactyly[36]. However, it was several years later before GLI3 was recognized as the transcription factor mediating SHH function[14]. Limb features in patients with this autosomal dominant mutation include broad thumbs with a central phalangeal defect/hole and distal phalangeal duplication, features similar to those seen in heterozygote mouse knockouts indicating functional conservation of GLI3 in vertebrate limb patterning[32]{personal observation, Ros MA).

Similarly, functional conservation of SHH in limb patterning and its limb specific regulation have also been demonstrated in humans. In fact, it was point mutations in patients with preaxial polydactyly type II or triphalangeal thumbs that unveiled the vertebrate ZPA regulatory sequence (ZRS){Lettice, 2002 1362 /id;Lettice, 2003 1360 /id}.

Based on animal models, loss of SHH function would limit ulnar expansion and affect the development of the ulna and ulnar digits, features known clinically as ulnar longitudinal deficiency. However, in humans, no syndromic or genetic basis of ulnar longitudinal deficiency has yet been identified.

Disruptions of distal HOXD and HOXA genes also have known mutations that demonstrate their involvement in hand development. Synpolydactyly has been linked to HOXD13 mutation and has central polydactyly supporting a role for HOXD13 in defining the number of digits formed[18]. While Hand-foot-uterus syndrome is caused by disruption in HOXA13 and is characterized by shortened thumbs and little fingers, occasionally with hypoplastic middle phalanges reducing the digital lengths of all of the fingers [17;35]. These findings support a role for HOXA13 in establishing digital lengths.

The thumb is an unusual digit and from a developmental biology standpoint, is differentiated from other digits by being the last to form, being independent of SHH, and lacking the expression of the distal HOXD genes. These features also appear to put the thumb at significant risk of disruption as over 1100 syndromes have hypoplastic thumbs as a feature[21]. Thus, it appears that thumb development (radial longitudinal deficiency) is most frequently impaired following any disruption that compromises the width of the handplate, particularly with persistent SHH function preserving the posterior or ulnar aspect of the limb bud[13;22].

Molecular contributions to the terminal phalanx proposed by animal models have also been demonstrated in humans[2] and implicated in the conserved regenerative capacity of digit tips[8].

These past recent successes in delineating the molecular basis of hand development encourage continued genetic evaluation of limb malformations to further characterize the involved pathways. Further discoveries are likely to occur from this synergistic relationship between clinical genetics and developmental biology.

References

(1) Agarwal P, Wylie JN, Galceran J et al. Tbx5 is essential for forelimb bud initiation following patterning of the limb field in the mouse embryo. *Development* 2003;130:623-633.

(2) Allan CH, Fleckman P, Fernandes RJ et al. Tissue response and Msx1 expression after human fetal digit tip amputation in vitro. *Wound Repair Regen* 2006;14:398-404.

(3) Burke AC, Nelson CE, Morgan BA, Tabin C. Hox genes and the evolution of vertebrate axial moprhology. *Development* 1995;121:333-346.

(4) Casanova JC, Badia-Careaga C, Uribe V, Sanz-Ezquerro JJ. Bambi and Sp8 expression mark digit tips and their absence shows that chick wing digits 2 and 3 are truncated. *PLoS One* 2012;7:e52781.

(5) Casanova JC, Sanz-Ezquerro JJ. Digit morphogenesis: is the tip different? *Dev Growth Differ* 2007;49:479-491.

(6) Dahn RD, Fallon JF. Interdigital regulation of digit identity and homeotic transformation by modulated BMP signaling. *Science* 2000;289:438-441.

(7) Han M, Yang X, Farrington JE, Muneoka K. Digit regeneration is regulated by Msx1 and BMP4 in fetal mice. *Development* 2003;130:5123-5132.

(8) Han M, Yang X, Lee J, Allan CH, Muneoka K. Development and regeneration of the neonatal digit tip in mice. *Dev Biol* 2008;315:125-135.

(9) 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;%20;118:517-528.

(10) Kawakami Y, Esteban CR, Matsui T, Rodriguez-Leon J, Kato S, Izpisua Belmonte JC. Sp8 and Sp9, two closely related buttonhead-like transcription factors, regulate Fgf8 expression and limb outgrowth in vertebrate embryos. *Development* 2004;131:4763-4774.

(11) Kmita M, Fraudeau N, Herault Y, Duboule D. Serial deletions and duplications suggest a mechanism for the collinearity of Hoxd genes in limbs. *Nature* 2002;420:145-150.

(12) Koshiba-Takeuchi K, Takeuchi JK, Arruda EP et al. Cooperative and antagonistic interactions between Sall4 and Tbx5 pattern the mouse limb and heart. *Nat Genet* 2006;38:175-183.

(13) Lee J, Tickle C. Retinoic acid and pattern formation in the developing chick wing: SEM and quantitative studies of early effects on the apical ectodermal ridge and bud outgrowth. *J Embryol Exp Morphol* 1985;90:139-69.:139-169.

(14) Marigo V, Johnson RL, Vortkamp A, Tabin CJ. Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. *Dev Biol* 1996;180:273-283.

(15) Minguillon C, Nishimoto S, Wood S, Vendrell E, Gibson-Brown JJ, Logan MP. Hox genes regulate the onset of Tbx5 expression in the forelimb. *Development* 2012;139:3180-3188.

(16) Montero JA, Lorda-Diez CI, Ganan Y, Macias D, Hurle JM. Activin/TGFbeta and BMP crosstalk determines digit chondrogenesis. *Dev Biol* 2008;321:343-356.

(17) Mortlock DP, Innis JW. Mutation of HOXA13 in hand-foot-genital syndrome. *Nat Genet* 1997;15:179-180.

(18) Muragaki Y, Mundlos S, Upton J, Olsen BR. Altered growth and branching patterns in synpolydactyly caused by mutations in HOXD13. *Science* 1996;272:548-551.

(19) Nelson CE, Morgan BA, Burke AC et al. Analysis of Hox gene expression in the chick limb bud. *Development* 1996;122:1449-1466.

(20) Ng JK, Kawakami Y, Buscher D et al. The limb identity gene Tbx5 promotes limb initiation by interacting with Wnt2b and Fgf10. *Development* 2002;129:5161-5170.

(21) Oberg KC. Review of the Molecular Development of the Thumb: Digit Primera. *Clin Orthop Relat Res* 2013.

(22) Raynaud A, Clergue-Gazeau M. [Chemically induced ectropodia in the Lacerta viridis embryo and formation of styliform limbs in reptiles]. *C R Acad Sci III* 1984;298:457-460.

(23) Riddle RD, Johnson RL, Laufer E, Tabin C. Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 1993;75:1401-1416.

(24) Rodriguez-Esteban C, Tsukui T, Yonei S, Magallon J, Tamura K, Izpisua Belmonte JC. The T-box genes Tbx4 and Tbx5 regulate limb outgrowth and identity. *Nature* 1999;398:814-818.

(25) Rowe DA, Cairns JM, Fallon JF. Spatial and temporal patterns of cell death in limb bud mesoderm after apical ectodermal ridge removal. *Dev Biol* 1982;93:83-91.

(26) Sanz-Ezquerro JJ, Tickle C. Fgf signaling controls the number of phalanges and tip formation in developing digits. *Curr Biol* 2003;13:1830-1836.

(27) Schweitzer R, Vogan KJ, Tabin CJ. Similar expression and regulation of Gli2 and Gli3 in the chick limb bud. *Mech Dev* 2000;98:171-174.

(28) Sheth R, Marcon L, Bastida MF et al. Hox genes regulate digit patterning by controlling the wavelength of a Turing-type mechanism. *Science* 2012;338:1476-1480.

(29) Summerbell D. The control of growth and the development of pattern across the anteroposterior axis of the chick limb bud. *J Embryol Exp Morphol* 1981;63:161-80.:161-180.

(30) 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;105:4185-4190.

(31) 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;130:2729-2739.

(32) te Welscher P, Zuniga A, Kuijper S et al. Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. *Science* 2002;298:827-830.

(33) Towers M, Mahood R, Yin Y, Tickle C. Integration of growth and specification in chick wing digit-patterning. *Nature* 2008;452:882-886.

(34) Turing AM. The chemical basis of morphogenesis. 1953. *Bull Math Biol* 1990;52:153-197.

(35) Verp MS, Simpson JL, Elias S, Carson SA, Sarto GE, Feingold M. Heritable aspects of uterine anomalies. I. Three familial aggregates with Mullerian fusion anomalies. *Fertil Steril* 1983;40:80-85.

(36) Vortkamp A, Franz T, Gessler M, Grzeschik KH. Deletion of GLI3 supports the homology of the human Greig cephalopolysyndactyly syndrome (GCPS) and the mouse mutant extra toes (Xt). *Mamm Genome* 1992;3:461-463.

(37) Vortkamp A, Gessler M, Grzeschik KH. GLI3 zinc-finger gene interrupted by translocations in Greig syndrome families. *Nature* 1991;352:539-540.

(38) Wang B, Fallon JF, Beachy PA. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* 2000;100:423-434.

(39) Winkel A, Stricker S, Tylzanowski P et al. Wnt-ligand-dependent interaction of TAK1 (TGF-beta-activated kinase-1) with the receptor tyrosine kinase Ror2 modulates canonical Wnt-signalling. *Cell Signal* 2008;20:2134-2144.

(40) Witte F, Chan D, Economides AN, Mundlos S, Stricker S. Receptor tyrosine kinase-like orphan receptor 2 (ROR2) and Indian hedgehog regulate digit outgrowth mediated by the phalanx-forming region. *Proc Natl Acad Sci U S A* 2010;107:14211-14216.

(41) Woltering JM, Duboule D. The origin of digits: expression patterns versus regulatory mechanisms. *Dev Cell* 2010;18:526-532.

(42) Xu B, Wellik DM. Axial Hox9 activity establishes the posterior field in the developing forelimb. *Proc Natl Acad Sci U S A* 2011;108:4888-4891.

(43) Yokouchi Y, Sasaki H, Kuroiwa A. Homeobox gene expression correlated with the bifurcation process of limb cartilage development. *Nature* 1991;353:443-445.

(44) 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;14:624-632.