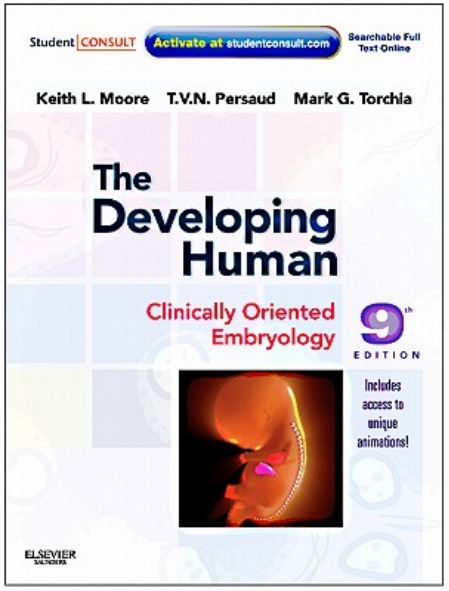
Limb Development

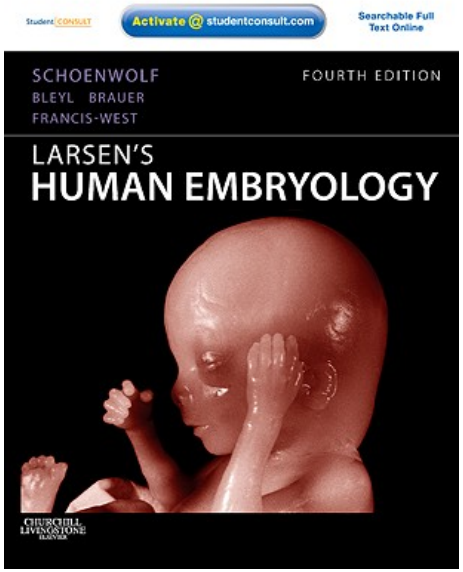
Involving the development of the appendicular skeleton and muscles

Objectives

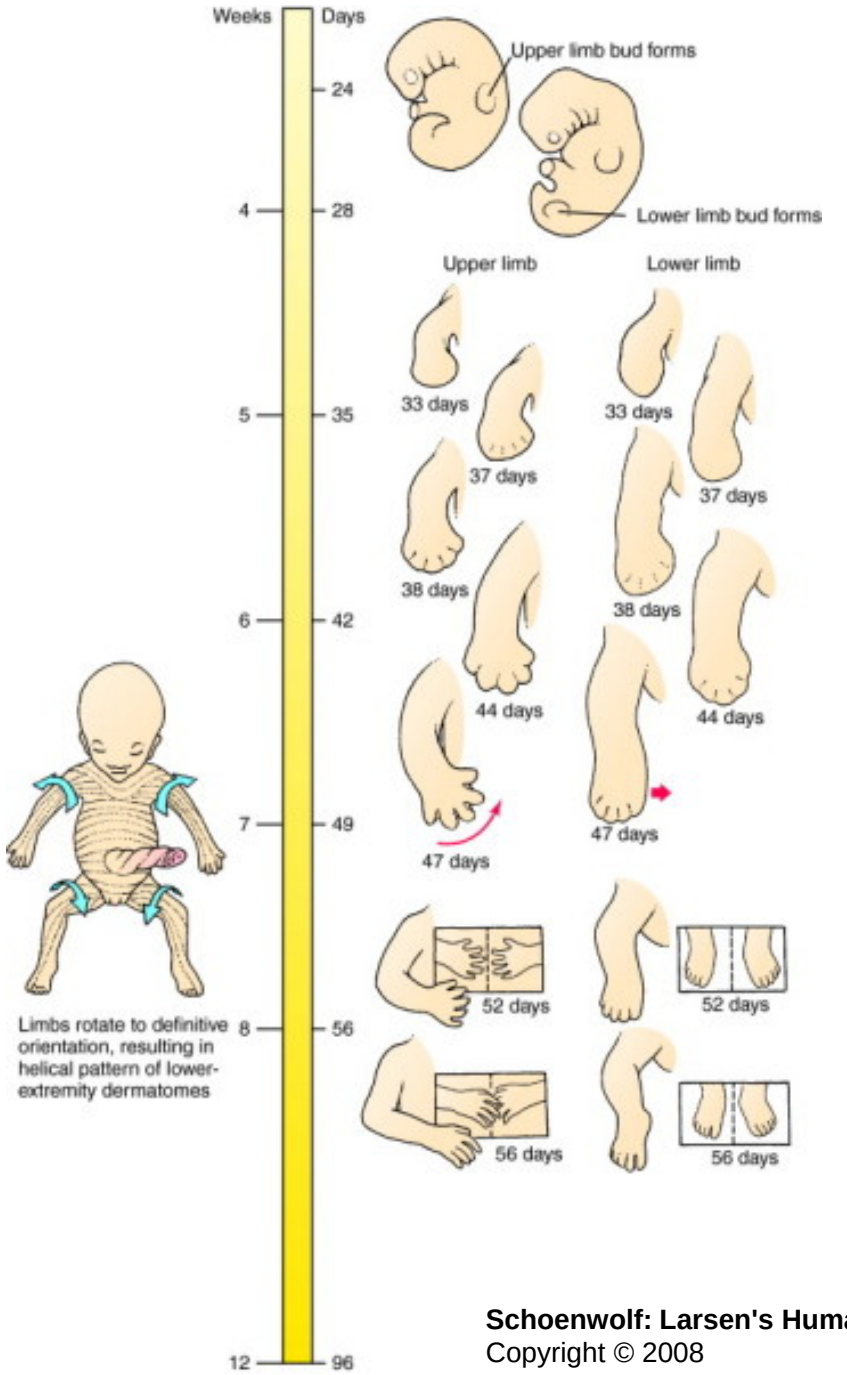
- Timing and location of limb bud development
- The tissues from which limb buds are made
- Determining the position of the limb buds on the body axis
- Signaling control mechanisms of early limb bud formation
- The control of forelimb and hindlimb specification
- Controlling the patterning of the limbs in 3 axes
- Rotation of the limbs and consequences for skin and innervation
- Shaping the hand and footplates through apoptosis



Citation: The Developing Human: clinically oriented embryology 9th ed. Keith L. Moore, T.V.N. Persaud, Mark G. Torchia. Philadelphia, PA: Saunders, 2011.
Chapter 16 – Development of Limbs



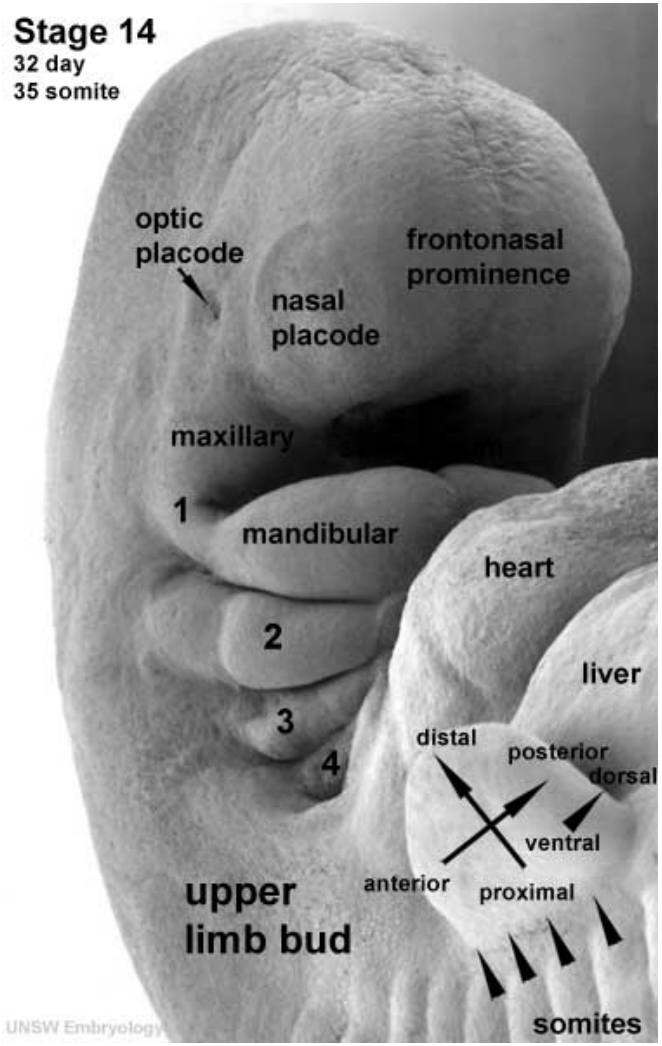
Citation: Larsen's human embryology 4th ed. Schoenwolf, Gary C; Larsen, William J, (William James). Philadelphia, PA : Elsevier/Churchill Livingstone, c2009.
Chapter 18 - Development of the Limbs



Timeline of limb development

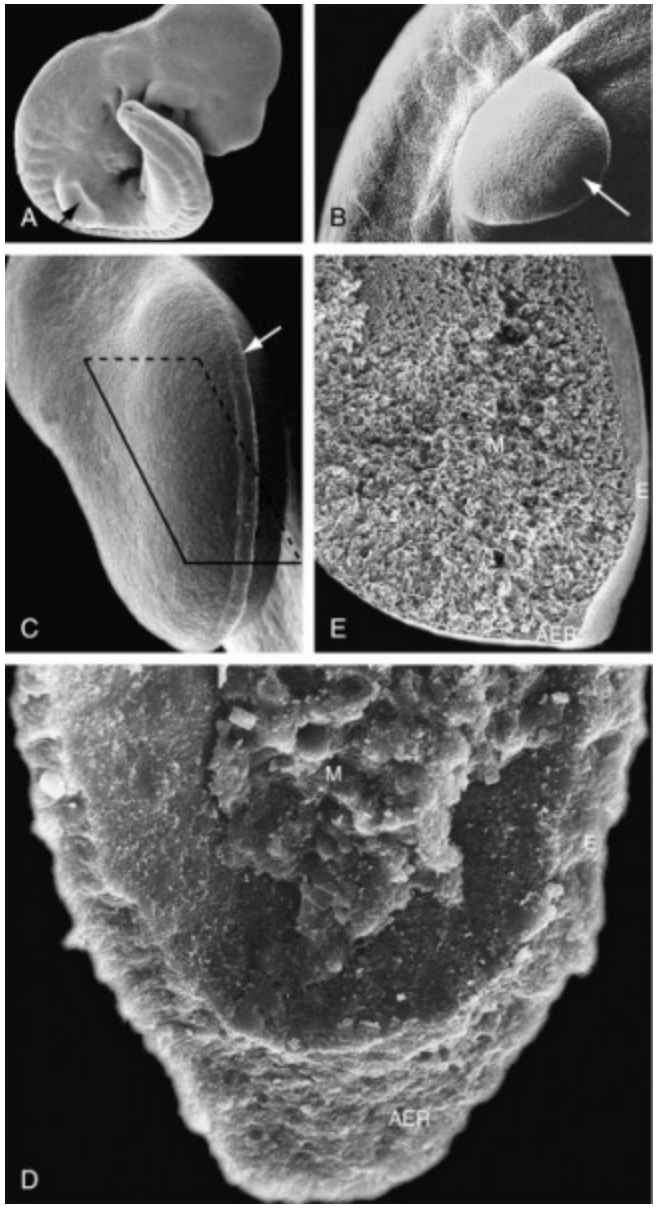
- Takes 5 weeks from week 4 to week 8
- Forelimb is slightly ahead of the hindlimb
- The upper limb bud appears at 24 days
- The lower limb bud appears at 28 days
- By the end they are nearly synchronized

Schoenwolf: Larsen's Human Embryology, 4th ed.



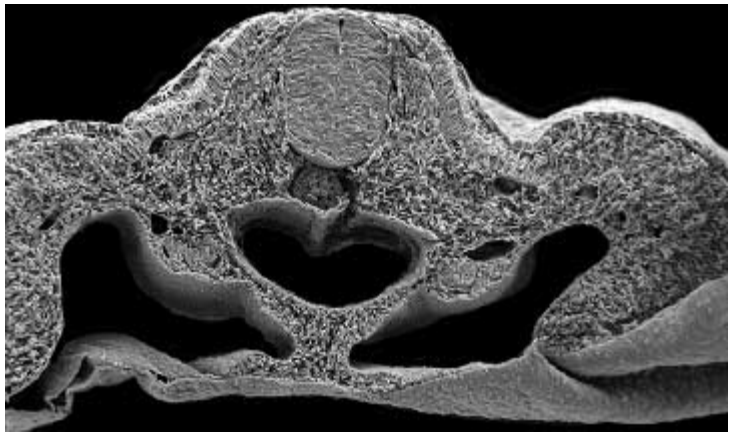
Limb buds

- Initial positioning looks caudal to final position due to the exaggeration of cranial structures during embryogenesis
- Forelimb - develops at the level of C5 to C8
- Hindlimb - develops at the level of L3 to L5

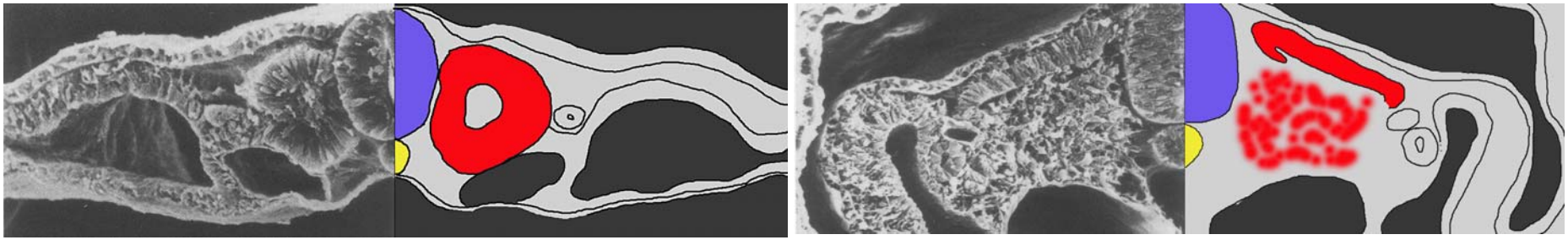


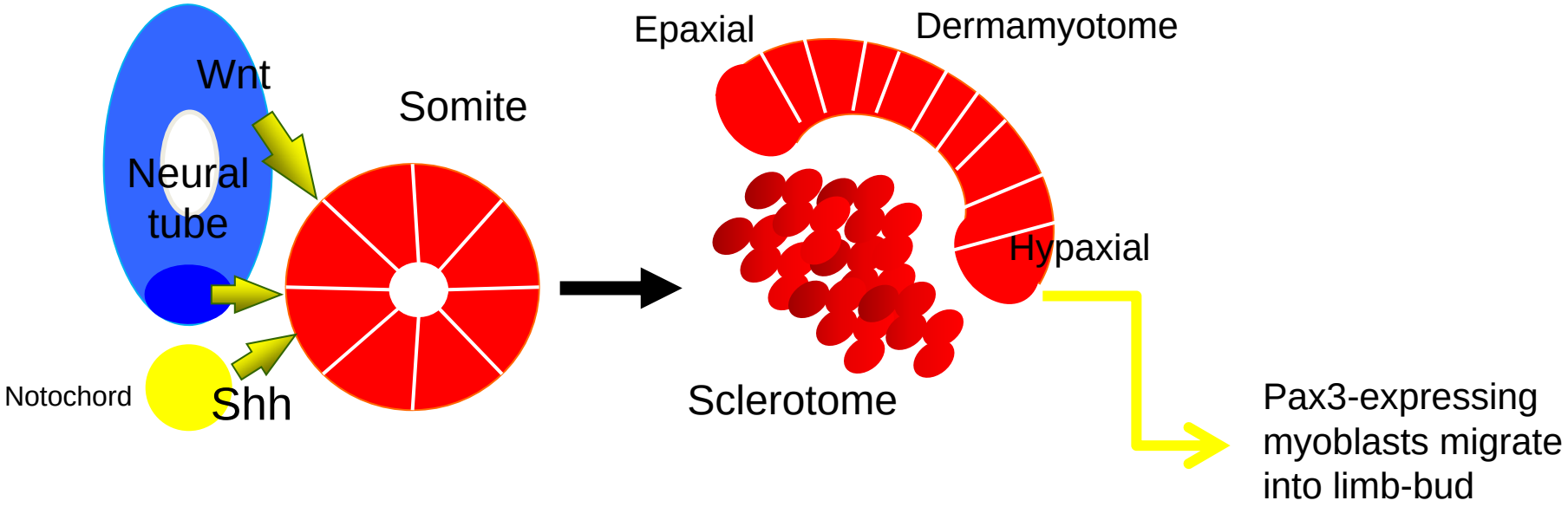
Scanning electron micrographs showing limb buds. The limb buds are formed from:

- Lateral plate mesoderm
- The overlying ectoderm which thickens at the edge to form the apical ectodermal ridge
- Invading myogenic mesoderm from the hypaxial region of the dermamyotome
- Dermal cells from the dermamyotome
- Innervation from spinal nerves
- Blood vessels

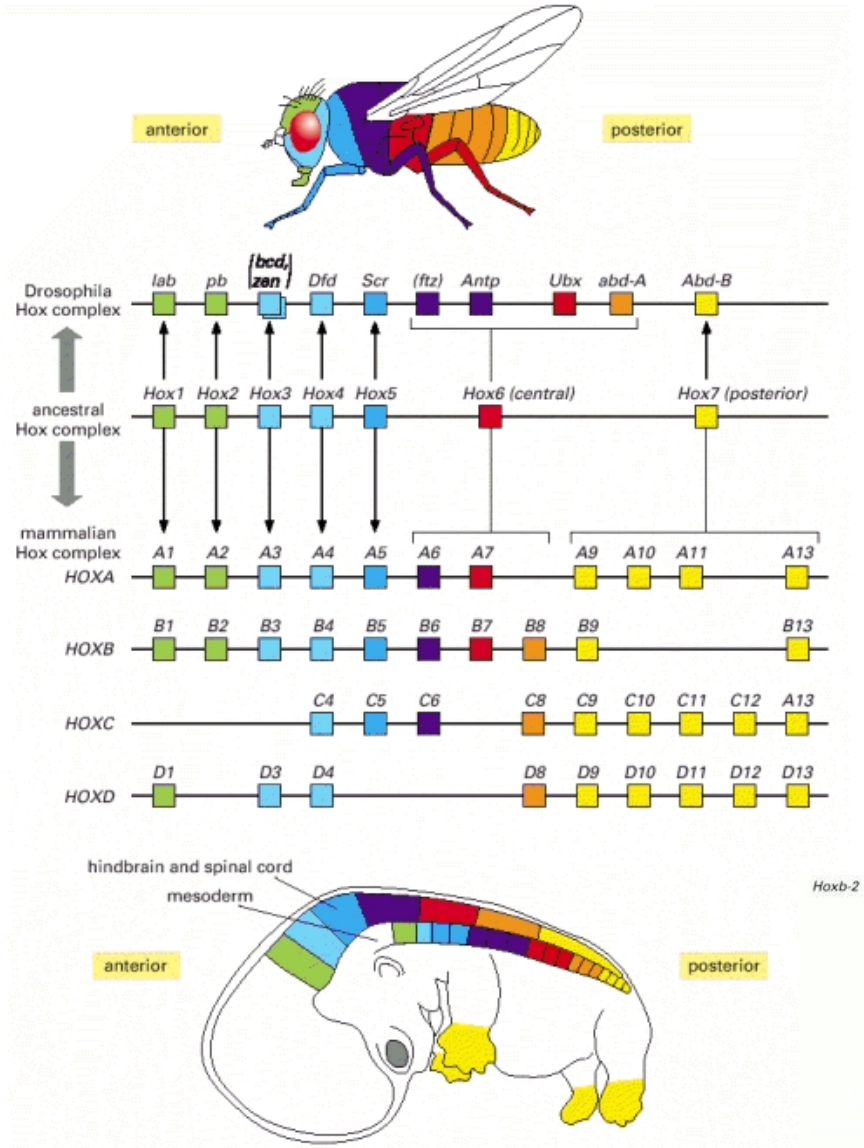


The origin of embryonic myoblasts in the chick





Limb development provides a good model of developmental patterning mechanisms



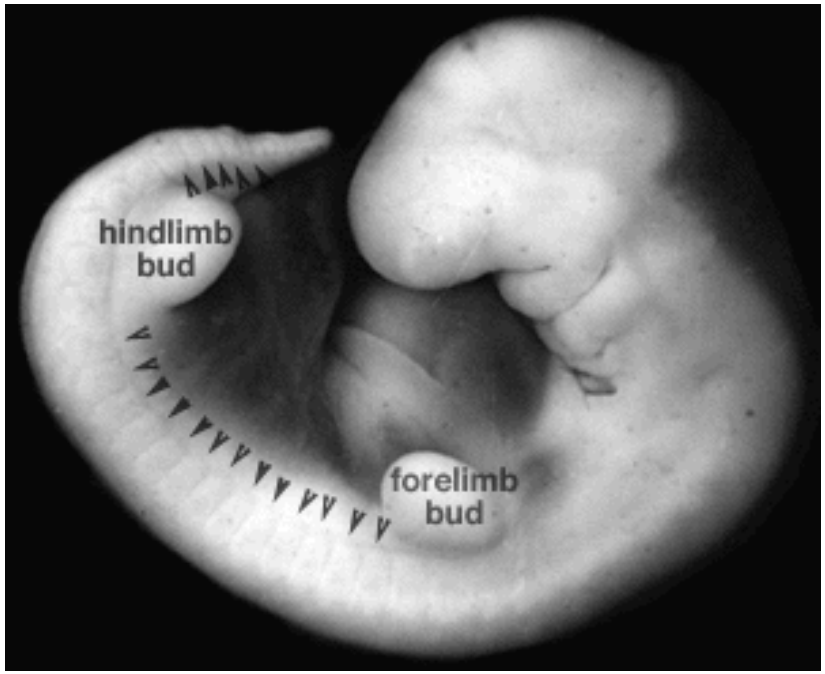
Gene expression becomes patterned in the embryo according to inductive signaling via diffusible morphogens or direct interaction at the cell surface.

For example the A/P axis shows patterning according to the activation of 4 HOX clusters of genes that have sharp anterior boundaries of expression.

Molecular Biology of the Cell. 4th edition.
Alberts B, Johnson A, Lewis J, et al.
New York: Garland Science; 2002.

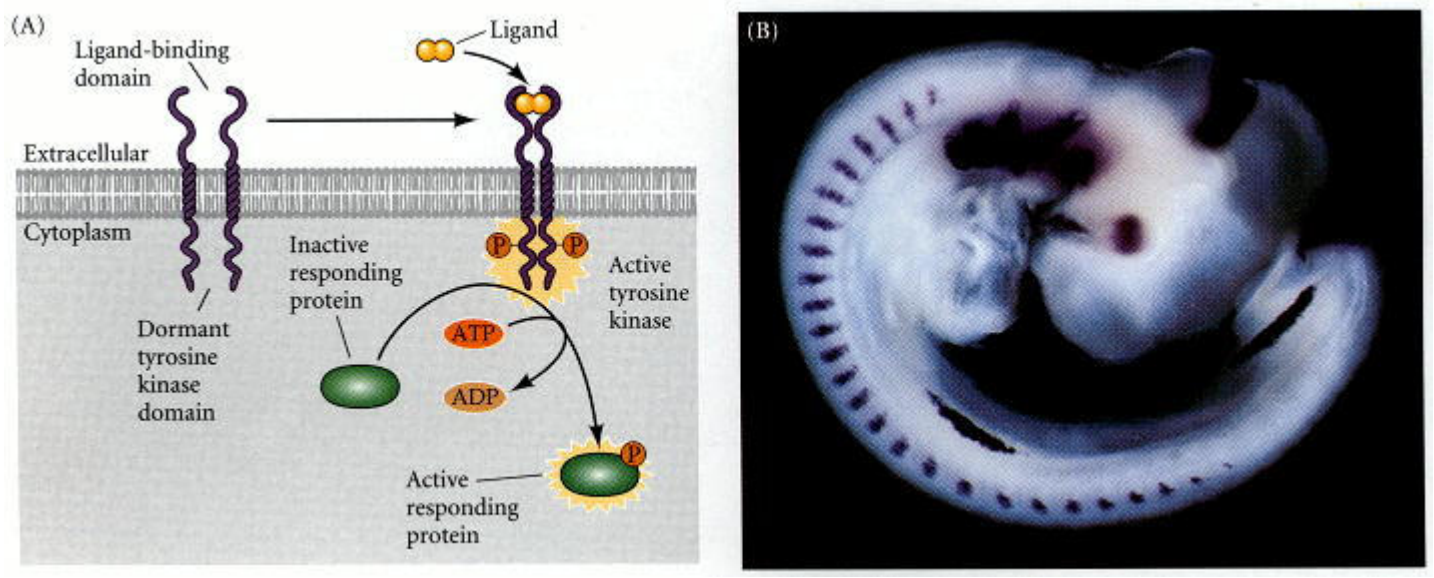
How does the embryo know where to position the limbs?

In vertebrates, from fish through to mammals, the position of the limbs varies with respect to the numbering of the somites but the limb positions correspond to boundaries of Hox gene expression. E.g. anterior boundary of *Hoxc6* expression always coincides with the position of forelimb development.



Limb Initiation

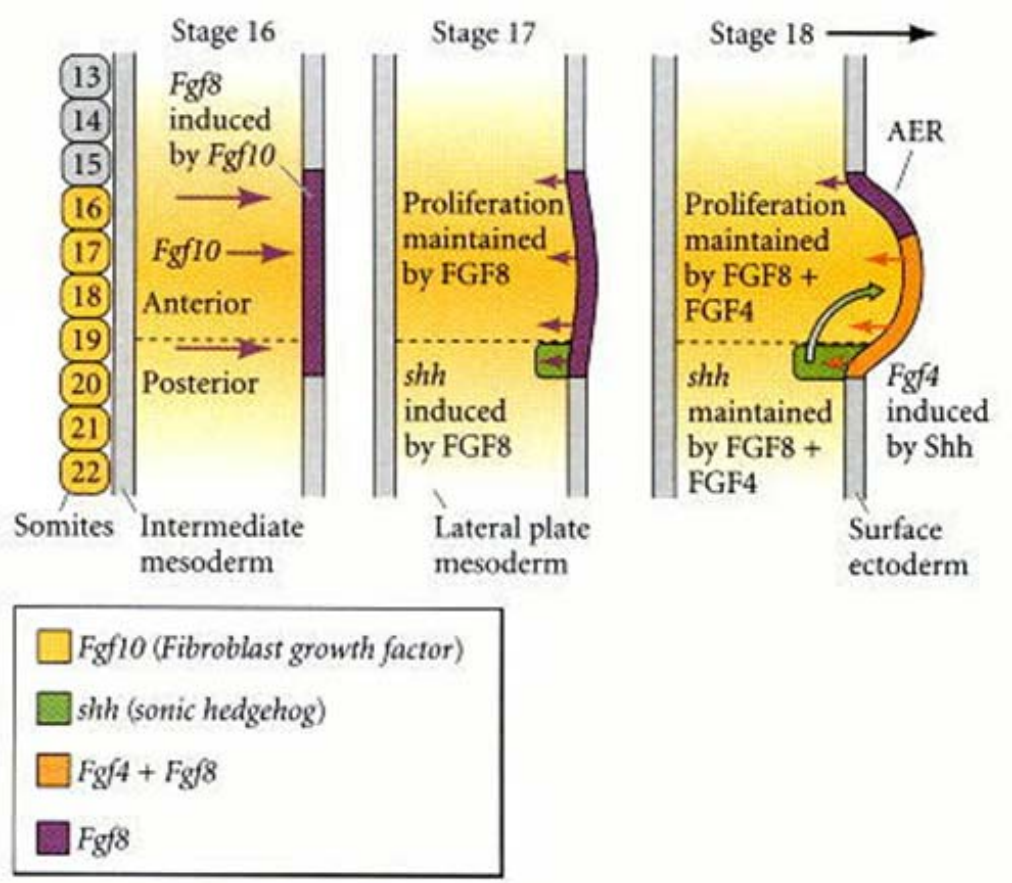
- FGF10 is expressed in lateral plate mesoderm prior to bud formation induces expression of FGF8 in the overlying ectoderm. FGF8 induces continued growth in the underlying mesoderm - thus a positive feedback loop



FGF expression. (A) Structure of a receptor tyrosine kinase. The dormant tyrosine kinase is activated by the binding of FGF by the extracellular portion of the receptor protein. This enzyme activity phosphorylates specific tyrosine residues of certain proteins. (B) FGF8 expression in the 3-day chick embryo, shown by in situ hybridization. FGF8 expression (dark areas) is seen in the most distal limb bud ectoderm, in the somitic mesoderm (the segmented blocks of cells along the anterior-posterior axis), in the branchial arches of the neck, at the boundary between the midbrain and hindbrian, and in the tail.

Developmental Biology. 6th edition.
Gilbert SF. Sunderland (MA): Sinauer Associates; 2000.

Initial limb development involves a feedback loop between the somatic component of the lateral plate mesoderm and the overlying ectoderm.



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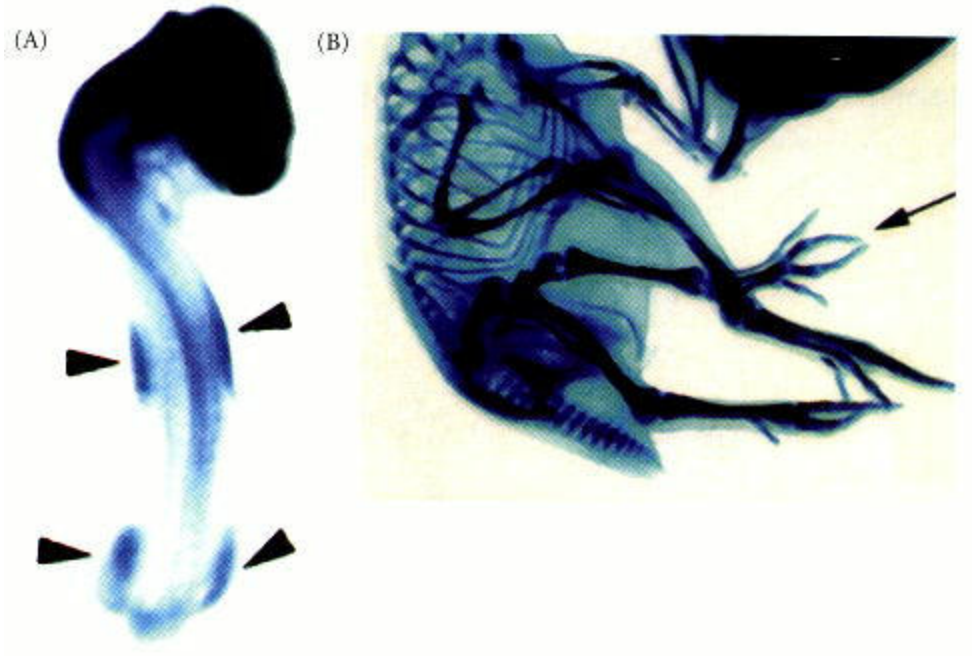
Fibroblast growth factor (FGF) coated beads or cells expressing FGF10 transplanted into chick embryos can induce an additional limb



FGF10 expression and action in the developing chick limb. (A) FGF10 becomes expressed in the lateral plate mesoderm in precisely those positions where limbs normally form. (B) When cells genetically constructed to secrete FGF10 are placed into the flanks of chick embryos, the FGF10 can cause the formation of an ectopic limb (arrow).

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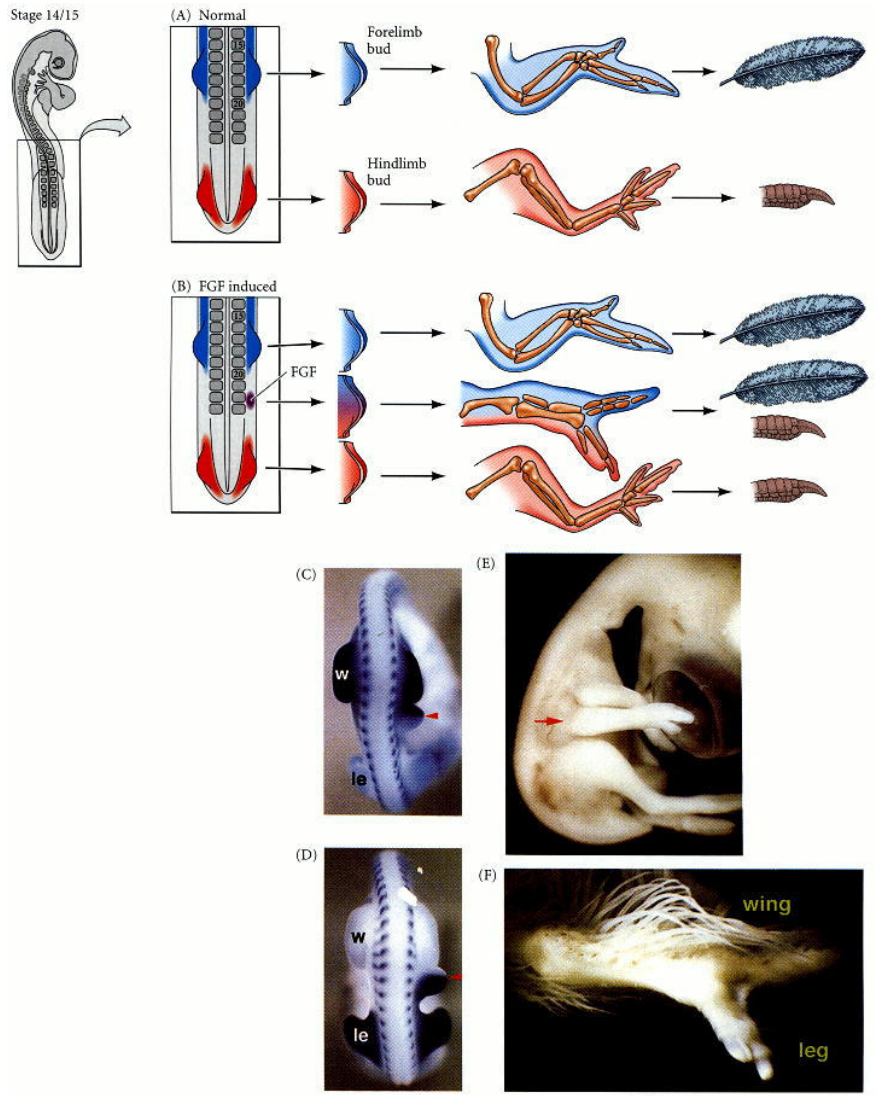
Gilbert SF. Sunderland (MA): Sinauer Associates; 2000.

How are forelimbs and hindlimbs determined?



Specification of limb type by Tbx4 and Tbx5. (A) During normal chick development, in situ hybridizations show that Tbx5 is found in the anterior lateral plate mesoderm, while Tbx4 is found in the posterior lateral plate mesoderm. Tbx5-containing limb buds produce wings, while Tbx4-containing limb buds generate legs. (B) If a new limb bud is induced with an FGF-secreting bead, the type of limb formed depends upon the *tbx* gene expressed in the limb bud. If placed between the regions of *Tbx4* and *Tbx5* expression, the bead will induce the expression of *Tbx4* posteriorly and *Tbx5* anteriorly. The resulting limb bud will also express *Tbx5* anteriorly and *Tbx4* posteriorly and will generate a chimeric limb. (C) Expression of *Tbx5* in the forelimb (w, wing) buds and in the anterior portion of a limb bud induced by an FGF-secreting bead. (The somite level can be determined by staining for *Mrf4* mRNA, which is localized to the myotomes.) (D) Expression of Tbx4 in the hindlimb (le, leg) buds and in the posterior portion of an FGF-induced limb bud. (E, F) A chimeric limb induced by an FGF bead contains anterior wing structures and posterior leg structures.

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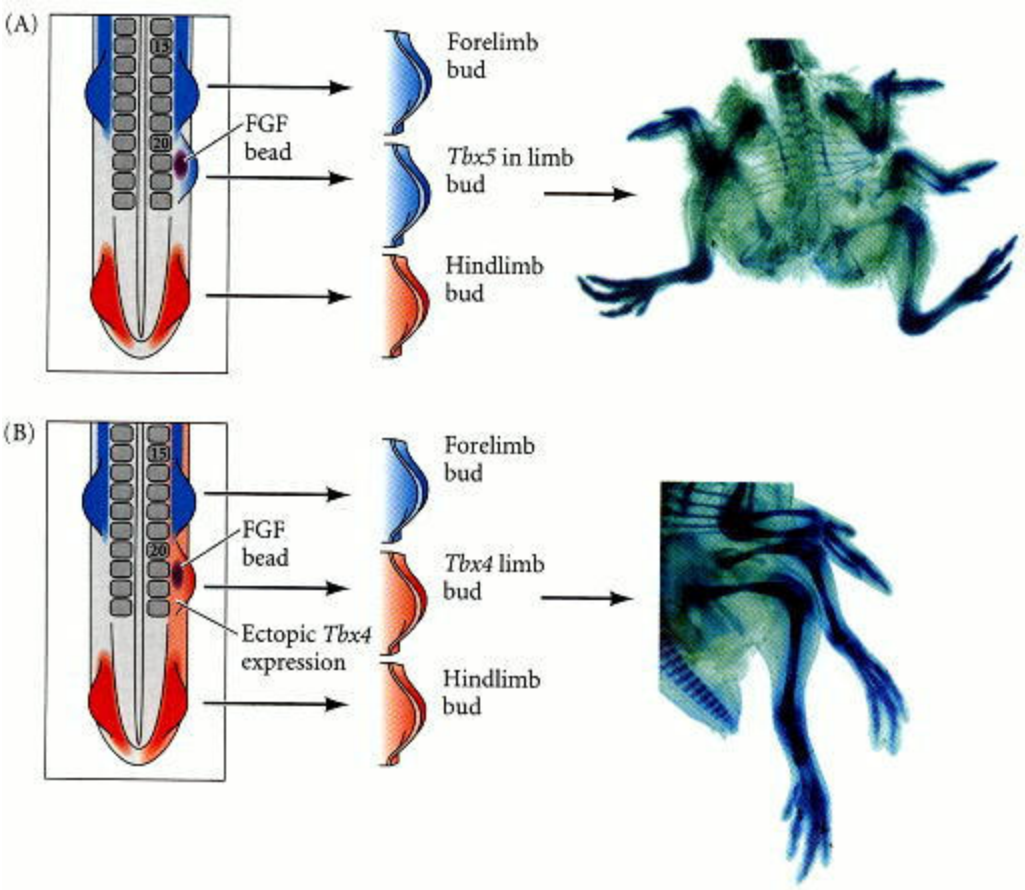
Gilbert SF. Sunderland (MA): Sinauer Associates; 2000.



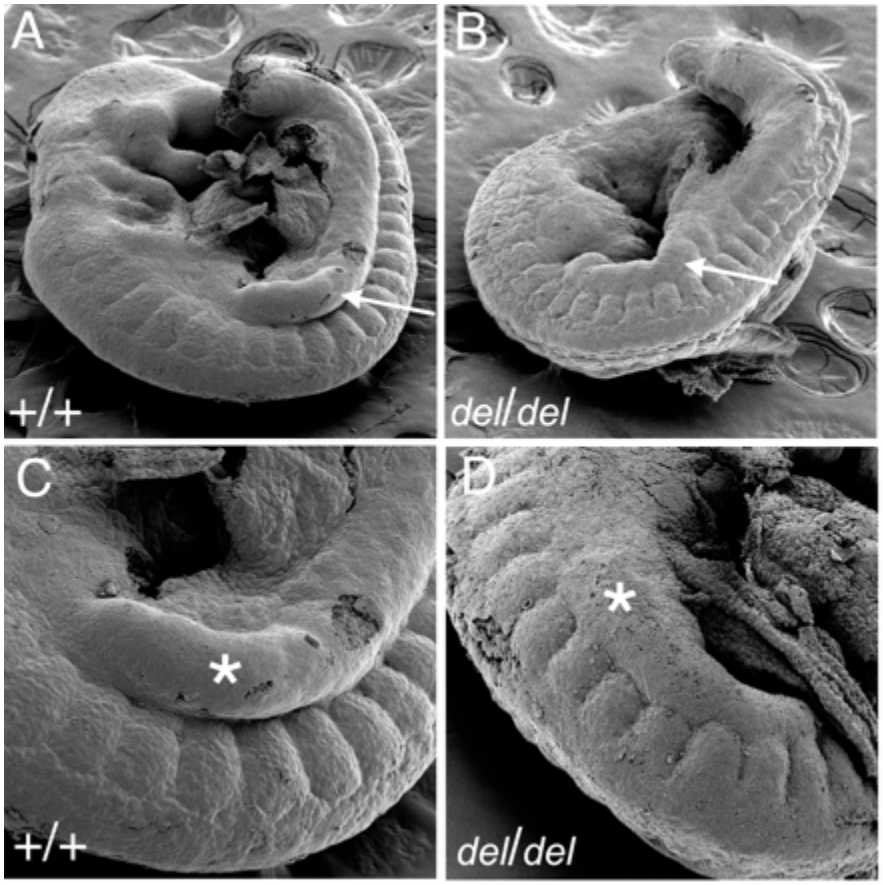
Respecification of forelimb into hindlimb by ectopic expression of Tbx4.

(A) An FGF-secreting bead opposite somite 21 usually induces a Tbx5-expressing limb bud that forms a new wing.

(B) If the entire flank is experimentally made to express Tbx4 (by infecting it with a Tbx4-expressing virus), the FGF-induced limb bud expresses Tbx4, and often becomes a leg.

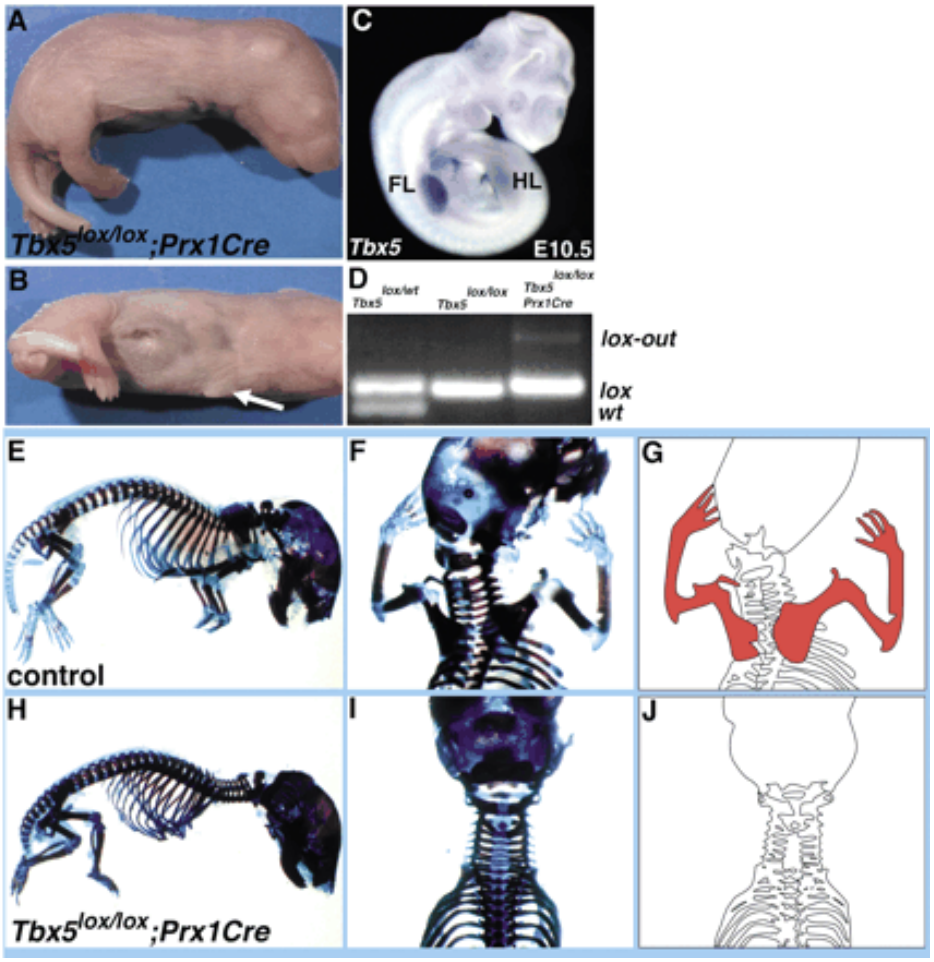
Developmental Biology. 6th edition.
Gilbert SF. Sunderland (MA): Sinauer Associates; 2000.

***Tbx5* is essential for forelimb bud initiation following patterning of the limb field in the mouse embryo.** Agarwal et al. 2003 Development 130:623



Scanning electron micrographs of wild-type (A,C) and *Tbx5*$^{del/del}$ (B,D) embryos at E9. Limb bud outgrowth is apparent in wild-type but not in *Tbx5*$^{del/del}$ embryos (arrows in A,B, asterisks in C,D). Lateral plate mesoderm is seen in embryo of either genotype as a ridge along the side of the embryo.

Tbx5 is required for forelimb bud formation and continued outgrowth.
Rallis et al. 2003 Development 130:2741



Absence of forelimb skeletal elements in newborn *Tbx5$^{lox/lox}$;Prx1Cre* pups. (A) *Tbx5$^{lox/lox}$;Prx1Cre* pup viewed from the side. (B) Ventral view of the pup shown in A. The arrow indicates a small flap of skin. (C) *Tbx5* expression in the developing forelimb but not the developing hindlimb of an E10.5 mouse embryo. (D) PCR analysis of the wild-type (wt) and conditional allele (*lox*) in heterozygous *Tbx5$^{lox/wt}$* embryos. (E) Skeletal preparation of a control littermate. (F) Dorsal view of the thoracic region of the embryo in E. (G) An outline of the skeletal preparation shown in F. (H) Skeletal preparation of a *Tbx5$^{lox/lox}$;Prx1Cre* pup. (I) Dorsal view of the thoracic region of the embryo shown in H. (J) An outline diagram of the skeleton shown in I. FL, forelimb; HL, hindlimb.

Controlling the organization of the limb - Axes and Morphogens

- **Anteroposterior** - (Rostrocaudal, Craniocaudal, Cephalocaudal) from the head end to opposite end of body or tail.

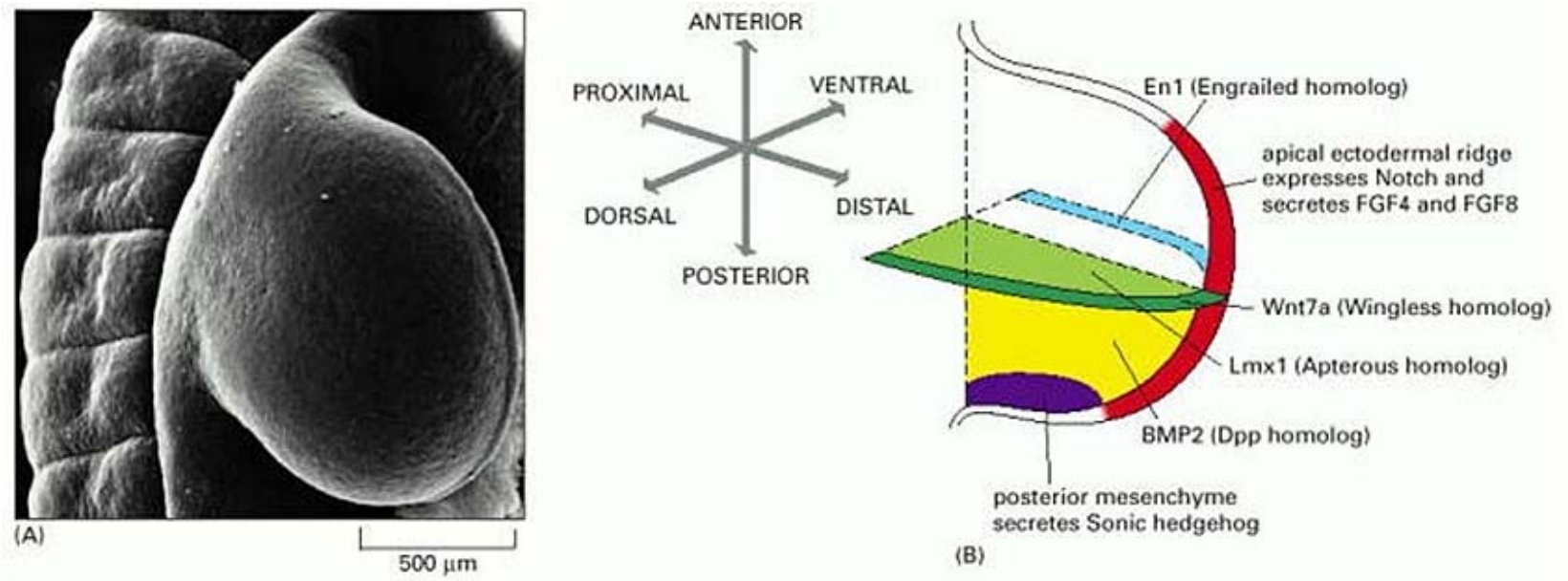
- **Dorsoventral** - from the spinal column (back) to belly (front).

- **Proximodistal** - from the tip of an appendage (distal) to where it joins the body (proximal).

Patterning in the limb bud

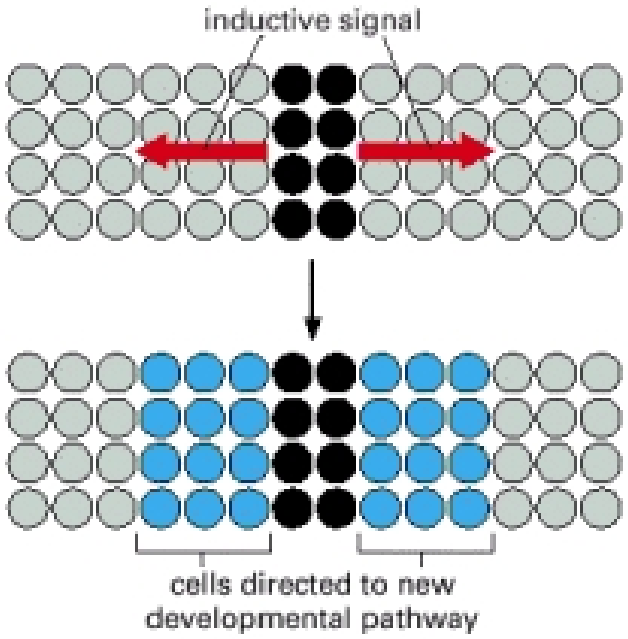
(A) A wing bud of a chick embryo at 4 days. At the distal margin of the limb bud a thickened ridge can just be seen—the apical ectodermal ridge (AER).

(B) Expression patterns of key **signalling proteins** and **gene regulatory factors** in the chick limb bud organized according the 3 axes. Sonic hedgehog, BMP2, and Lmx1 are expressed in the mesodermal core of the limb bud; the other molecules in the diagram are expressed in its epithelial covering. Some of these factors have drosophila homologs involved in wing patterning (in brackets).



Molecular Biology of the Cell. 4th edition.
Alberts B, Johnson A, Lewis J, et al.
New York: Garland Science; 2002.

Diffusible morphogens such as SHH, the BMPs, WNTs and the FGFs are synthesized from a discrete region of cells and the protein diffuses through the intercellular spaces. This creates a concentration gradient that is highest adjacent to source and declines with distance.



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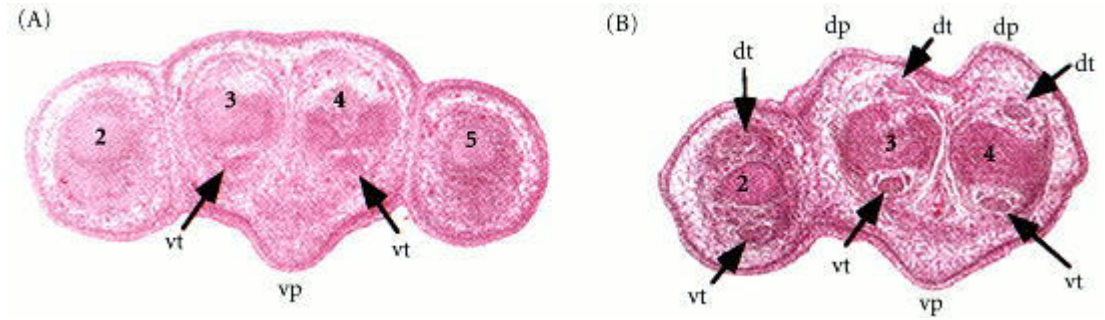
Dorsoventral (D/V) Axis of the limb bud

- Important for patterning muscles - ventral muscles - flexors; Dorsal muscles - extensors (see next slide)
- Early grafting experiments showed that the D/V signalling centre resided in the dorsal ectoderm
- Wnt7a is a diffusible morphogen that is secreted by dorsal ectoderm cells
- Wnt7a induces the expression of the homeobox gene Lmx1 in the underlying mesoderm adjacent to the dorsal surface
- The homeobox gene Engrailed (En1) is expressed in the opposite ventral ectoderm

Morphogen production from the dorsal ectoderm - Wnt7a

- name was derived from 'wingless' and 'int'
- Wnt gene first defined as a protooncogene, int1 (integration 1 of a mouse mammary tumour virus, MMTV)
- Humans have 19 Wnt genes
- Wnt7a gene is at 3p25 encoding a 349aa secreted glycoprotein
- patterning switch with different roles in different tissues
- One WNT receptor is called Frizzled (FZD) - named after a drosophila phenotype
- Frizzled gene family encodes a G protein-coupled receptor with 7-span transmembrane domains

Homozygous knockout of *Wnt7a* in mice leads to loss of limb D/V patterning
Therefore Wnt7a provides a **dorsalizing** signal to the limb bud



(A) Histological section (stained with hemotoxylin and eosin) of wild-type 15.5-day embryonic mouse forelimb paw. The ventral tendons and ventral footpads are readily seen.

(B) Same section through a mutant embryo deficient in *Wnt7a.* Tendons and footpads are duplicated on what would normally be the dorsal surface of the paw. dt, dorsal tendons; dp, dorsal footpad; vp, ventral footpad; vt, ventral tendon. Numbers indicate digit identity. (Parr and McMahon 1995)

Developmental Biology. 6th edition.
Gilbert SF. Sunderland (MA): Sinauer Associates; 2000.

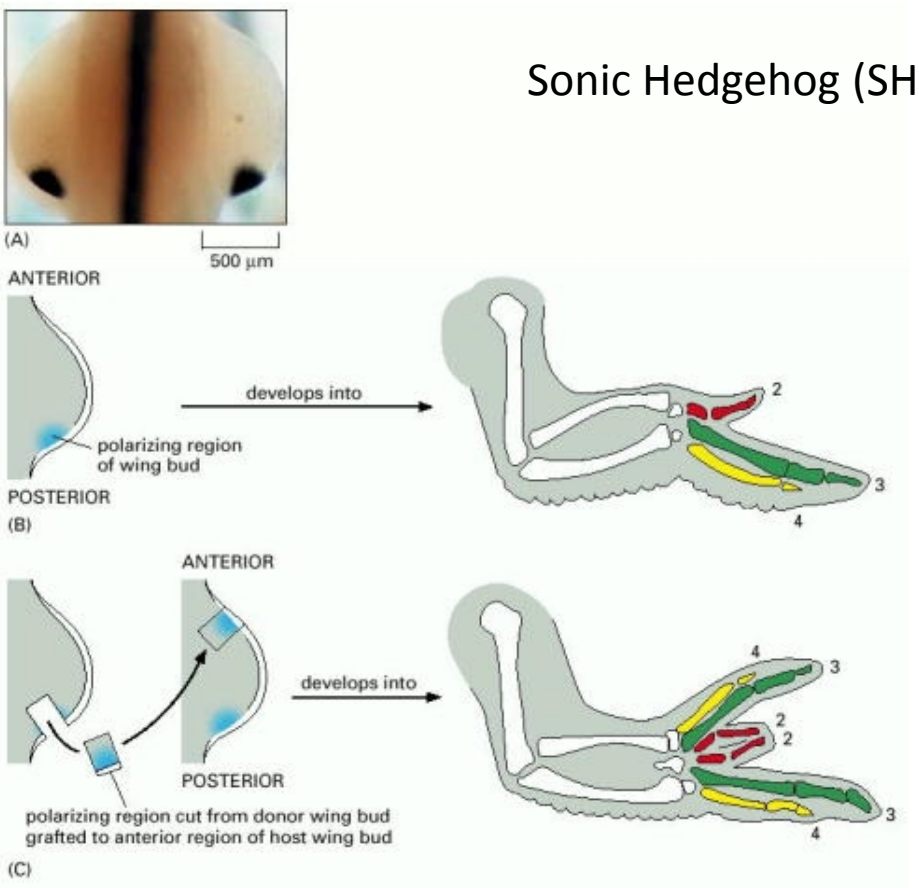
Anteroposterior (A/P) Axis of the limb bud

- Zone of polarizing activity (ZPA)
- a mesenchymal posterior region of limb
- secretes sonic hedgehog (SHH)

Morphogen production from the ZPA - Sonic Hedgehog (SHH)

- Sonic hedgehog (SHH) is a diffusible morphogen secreted from cells, the protein product of the SHH gene
- The protein is processed by cleavage of the preprotein and addition of a palmitate molecule to the amino terminus and cholesterol to the carboxy terminus
- The SHH receptor is a cell surface protein called Patched which interacts with another cell surface protein Smoothened.
- Binding of SHH to Patched blocks its inhibitory effect on Smoothened and allows it to initiate an intracellular signaling cascade

Sonic Hedgehog (SHH) is the key morphogen of the **ZPA**



(A) Dorsal view showing expression of the *Sonic hedgehog* gene in a chick embryo.
(B) Normal wing development.
(C) A graft of tissue from the polarizing region causes a mirror-image duplication of the pattern of the host wing. The type of digit that develops is thought to be dictated by the local concentration of Sonic hedgehog protein; different types of digit (labeled 2, 3, and 4) therefore form according to their distance from a source of Sonic hedgehog.

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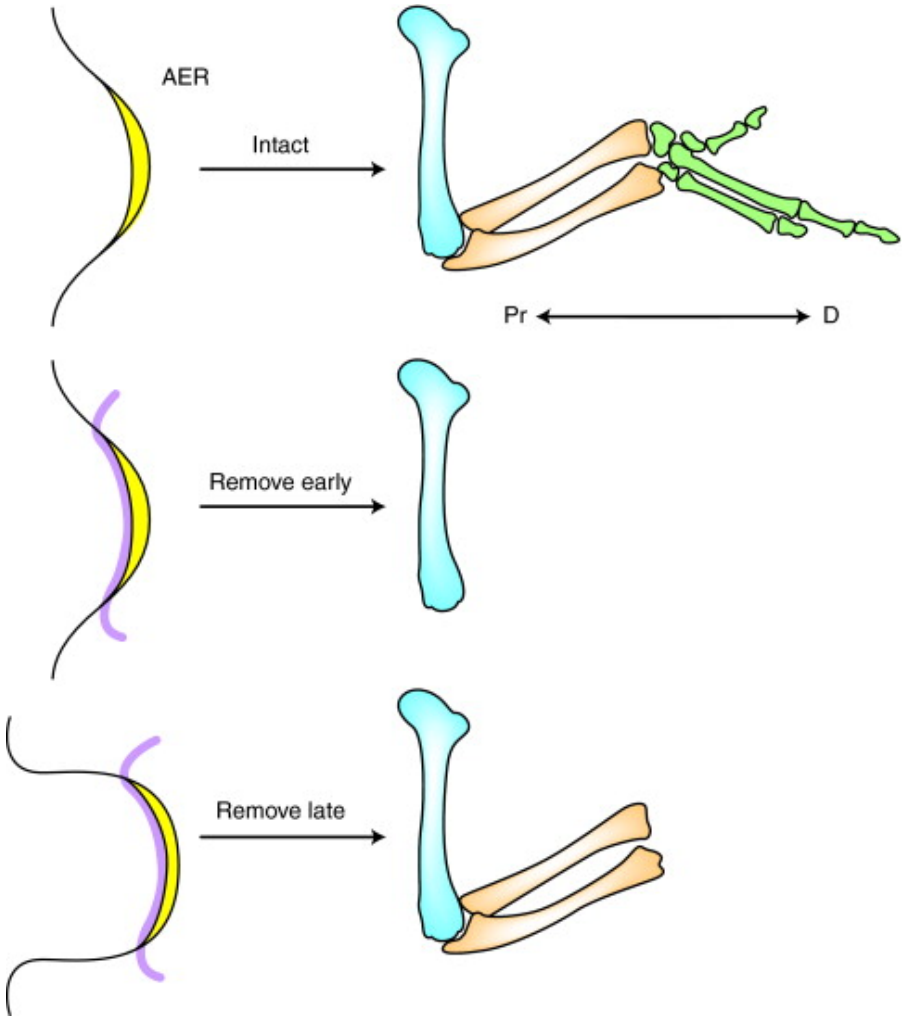
Proximodistal patterning is determined by time and space - Dynamic development and temporal gene expression



Removal of the AER at specific time points leads to the arrest of limb development at the stage at which it was removed.

Proximodistal Axis of the limb bud – The AER again

- Apical Ectodermal Ridge (AER) initially formed at the site of FGF10 induction
- AER forms and secretes FGF8 – then also FGF4 slightly later
- FGFs stimulate proliferation and outgrowth in the underlying mesenchyme

Morphogen production from the AER - The Fibroblast Growth Factors (FGFs)

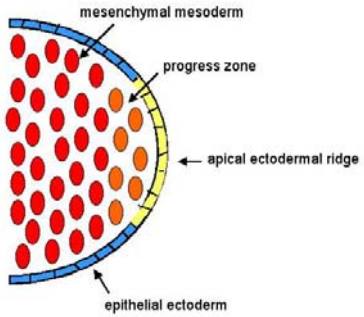
- 22 FGF genes identified in humans
- bind to membrane inserted tyrosine kinase receptors
- Patterning switch with many different roles in different tissues usually affecting cell division and differentiation

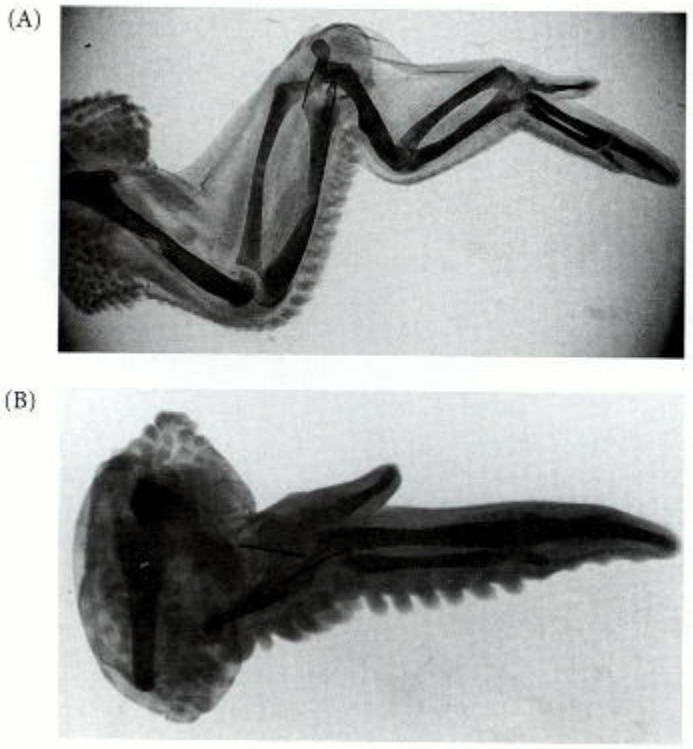
FGF receptors

- comprise a family of at least 4 related but individually distinct tyrosine kinase receptors (FGFR1- 4) similar protein structure
- 3 immunoglobulin-like domains in extracellular region
- single membrane spanning segment
- cytoplasmic tyrosine kinase domain

Proximodistal patterning is determined by time and space - Dynamic development and temporal gene expression

- Structures are determined in a proximal>distal direction with time, i.e. proximal structures such as the humerus bone are laid down first.

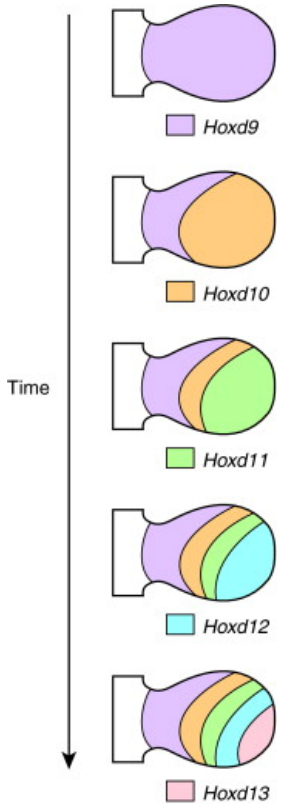




Control of proximal-distal specification by the cells of the **progress zone**. The progress zone is a narrow band of mesenchyme underlying the AER(A) Extra set of ulna and radius formed when an early-bud progress zone was transplanted to a late wing bud that had already formed ulna and radius. (B) Lack of intermediate structures seen when a late-bud progress zone was transplanted to an early limb bud.

Developmental Biology. 6th edition.
Gilbert SF. Sunderland (MA): Sinauer Associates; 2000.

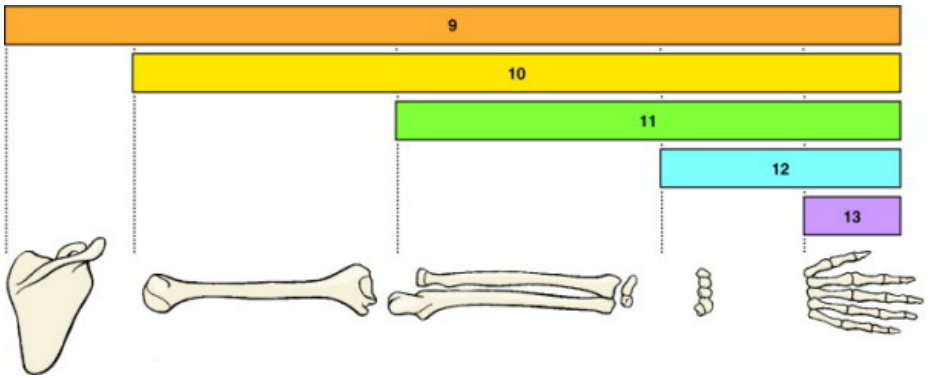
The Time Axis of limb development - Dynamic development, temporal gene expression and the proximodistal axis.



Understanding of the process is still unclear and a controversial issue. However, arguably the most popular model is called the **progress zone model**. Cells that spend the shortest time in the progress zone are destined to become proximal structures and those with the longest exposure, the most distal structures.

Regardless of whether this model is correct, it is recognized that a proximodistal gradient of Hox gene expression is set up that shows a similar colinearity to the A/P patterning events of the body axis.

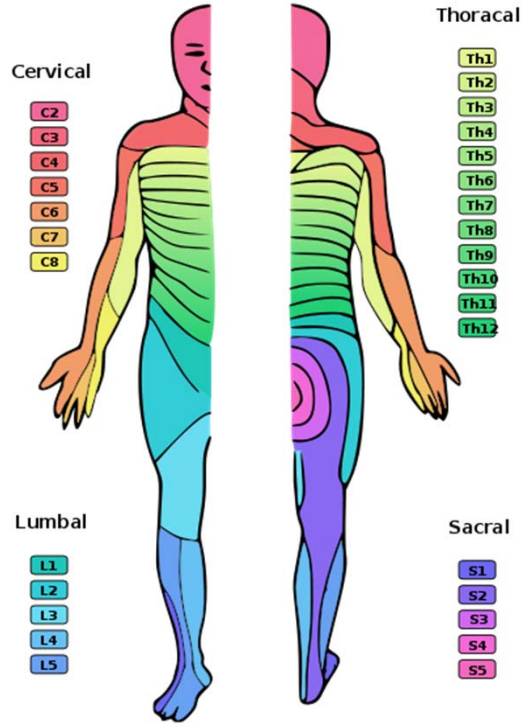
These expression boundaries correlate with the developing bony structures. For example – see the forelimb correlation below



Schoenwolf: Larsen's Human Embryology, 4th ed.

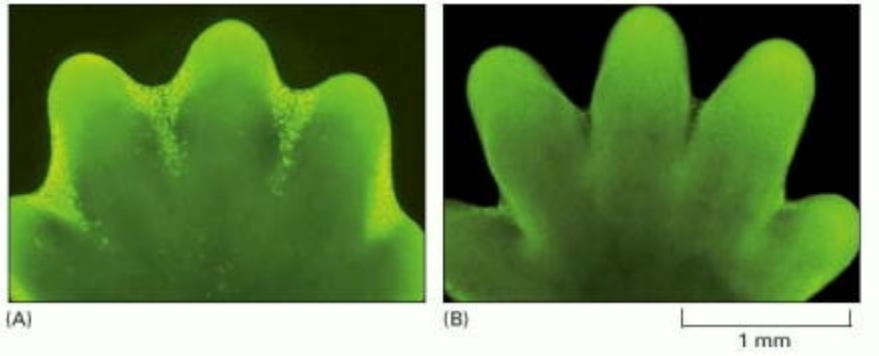
Limb muscle and dermis

- Skeletal muscle derived from somites, the hypaxial part of the myotome
- Pax3 positive migratory myoblasts invade the limb bud
- Similarly, dermal cells also invade derived from the dermomyotome
- Both maintain the identity of the somite from which they were derived so that innervation corresponds to the same spinal nerve root.
- Note that dermatomes are rotated due to embryonic limb rotations



Hand and footplate development

- 5th week- hand and footplates appear at the ends of limb buds and ridges form digital rays
- Cells between the digital rays are removed by programmed cell death (apoptosis)
- 3-5 day difference between hand and foot development



(A) The paw in this mouse embryo has been stained with a dye that specifically labels cells that have begun apoptosis. The apoptotic cells are *bright green*
(B) This interdigital cell death eliminates the tissue between the developing digits, as seen one day later, when few, if any, apoptotic cells can be seen.

Molecular Biology of the Cell. 4th edition.
Alberts B, Johnson A, Lewis J, et al.
New York: Garland Science; 2002.