# Limb Development

Ed Laufer elaufer@columbia.edu

## What we will cover...

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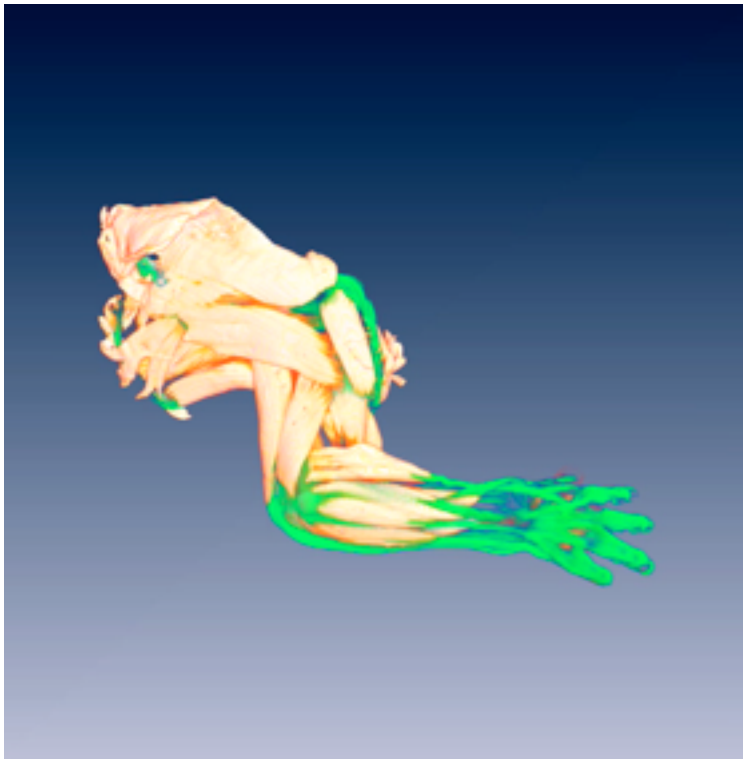
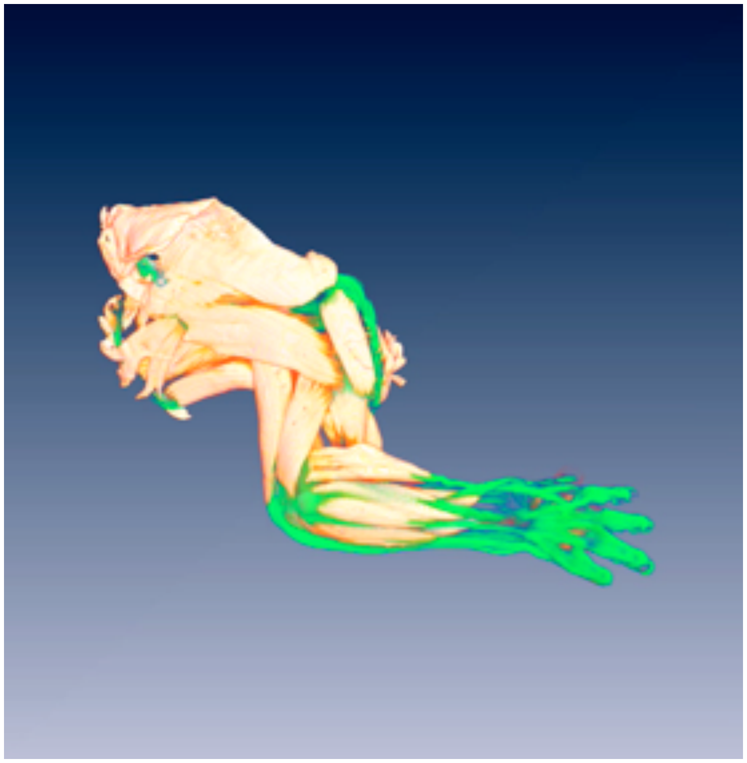
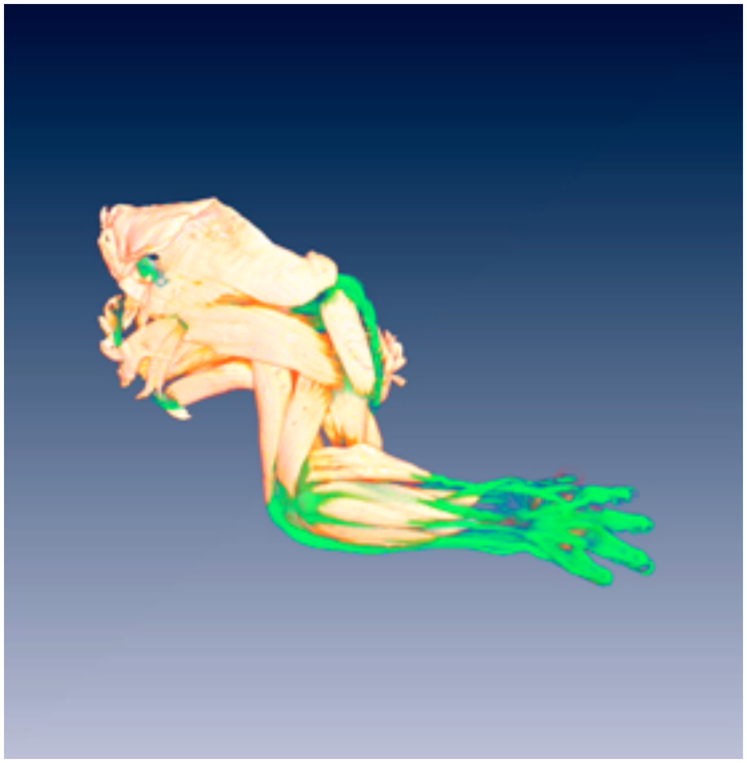


## What we will cover...



**What signals control initiation of limb bud formation ?**

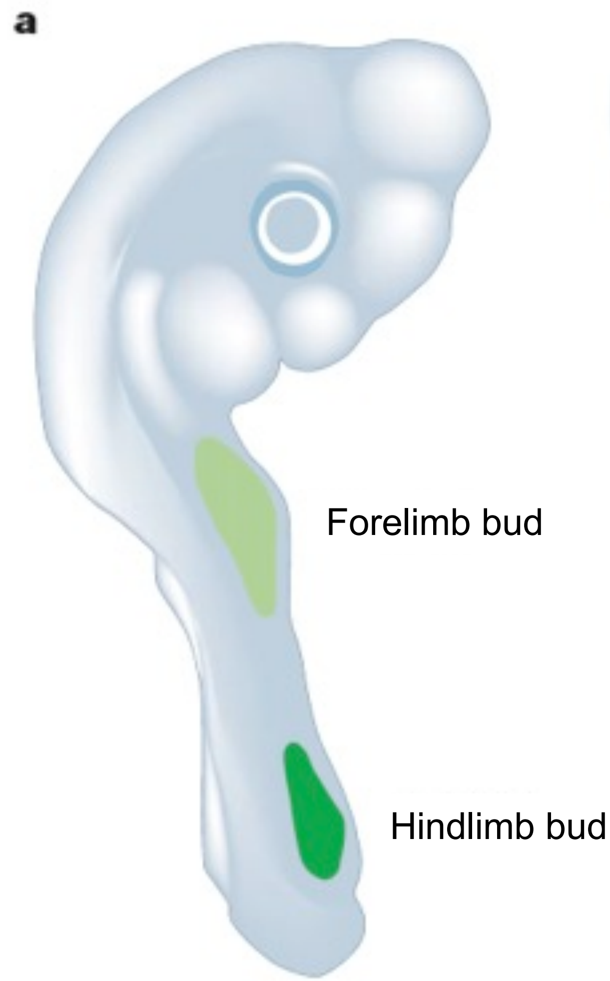
**What we will cover...**

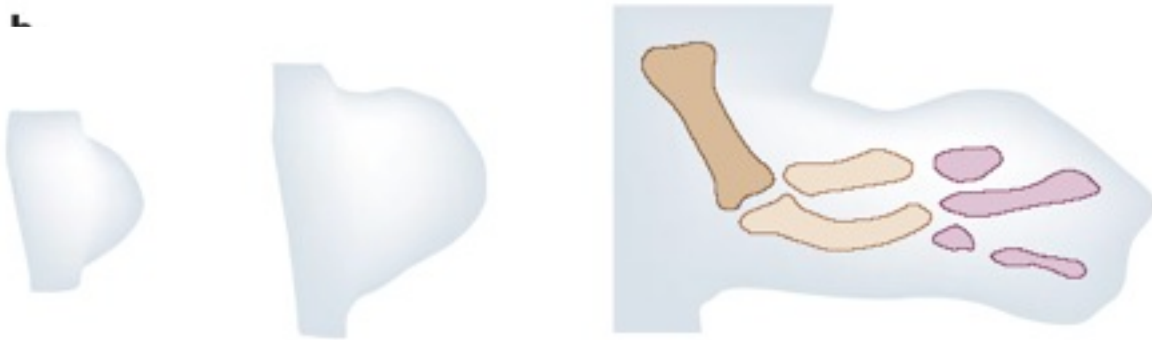


**What signals control initiation of limb bud formation ?**

**What signals transform the *embryonic limb bud* into a *mature limb* comprised of a precisely interconnected array of many different tissues?**

# EARLY LIMB PATTERNING:



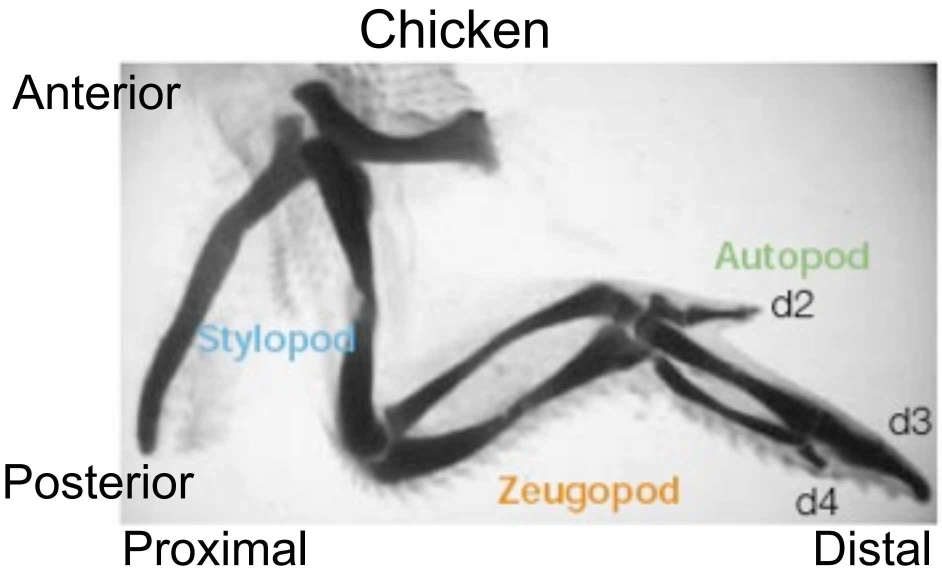



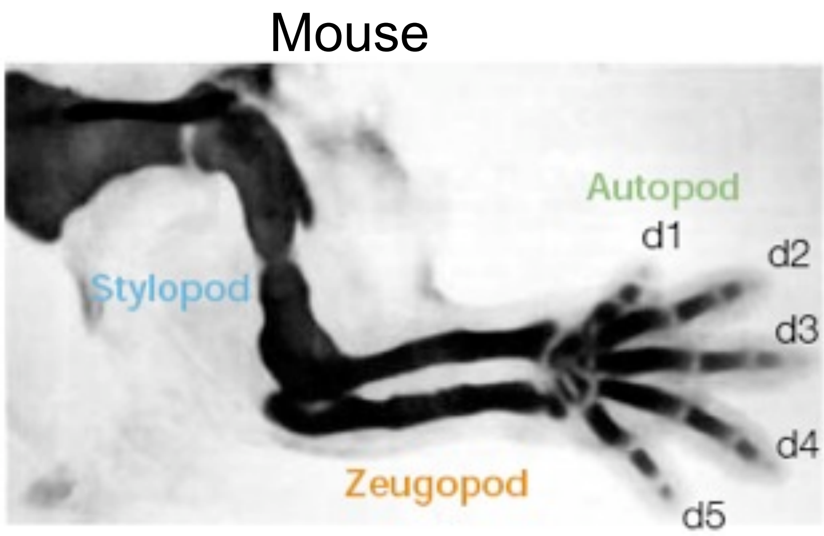
Limb formation initiates during the fourth week of development (E9.0 in mouse) as the primary axis (AP) is still elongating.  First the forelimb and then the hindlimb begin as protrusions from the lateral plate mesoderm at the sides of the embryo.  Limb buds consist of a core of mesenchyme and an outer covering of ectoderm.  Development proceeds in proximal-distal order.

1 in 200 live human births display limb defects.

# Limb skeletal elements:

<u>Dorsal</u>: top of hand/paw
<u>Ventral</u>: palm/sole



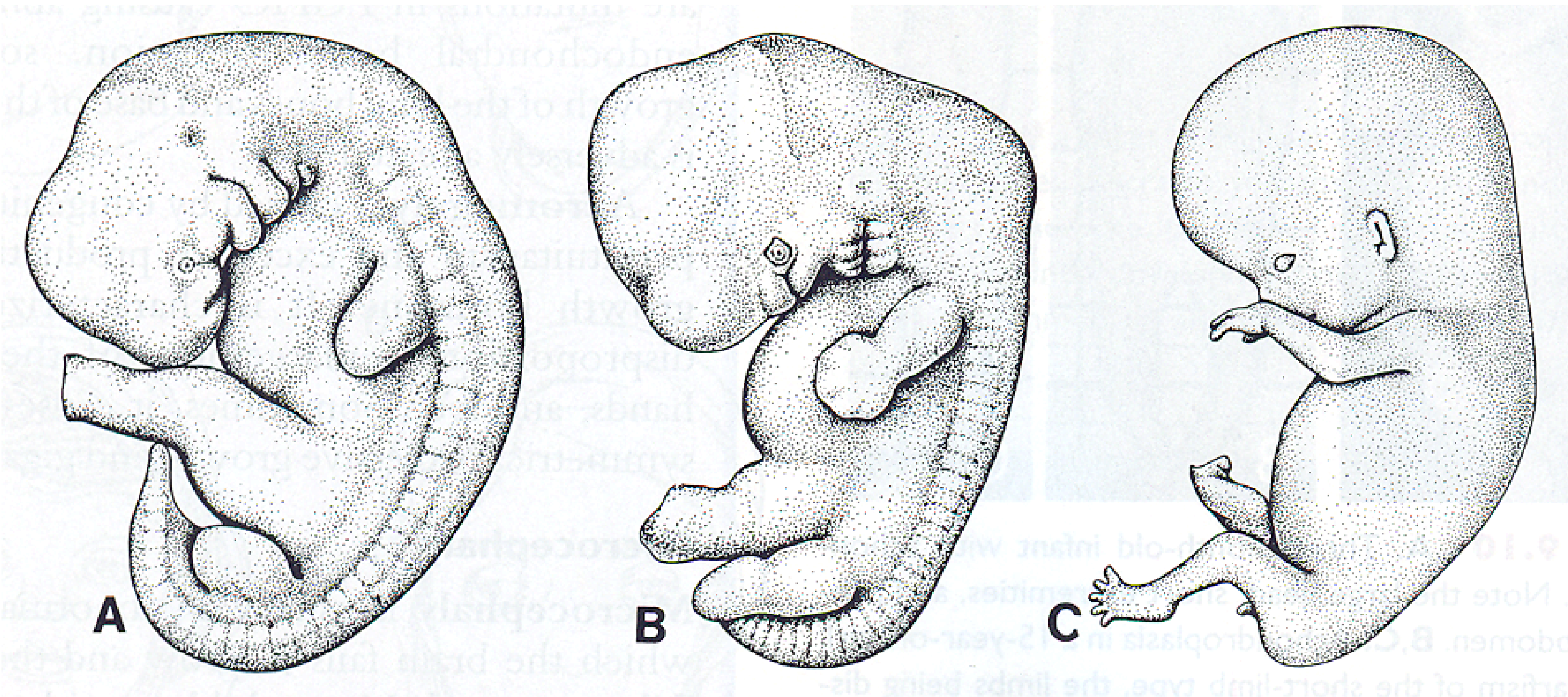




**Stylopod:** The proximal element of a limb.
The **humerus** in the forelimb; **femur** in the hindlimb

**Zeugopod:** The intermediate element of a limb.
The **radius** and **ulna** in the forelimb; **tibia** and **fibula** in the hindlimb

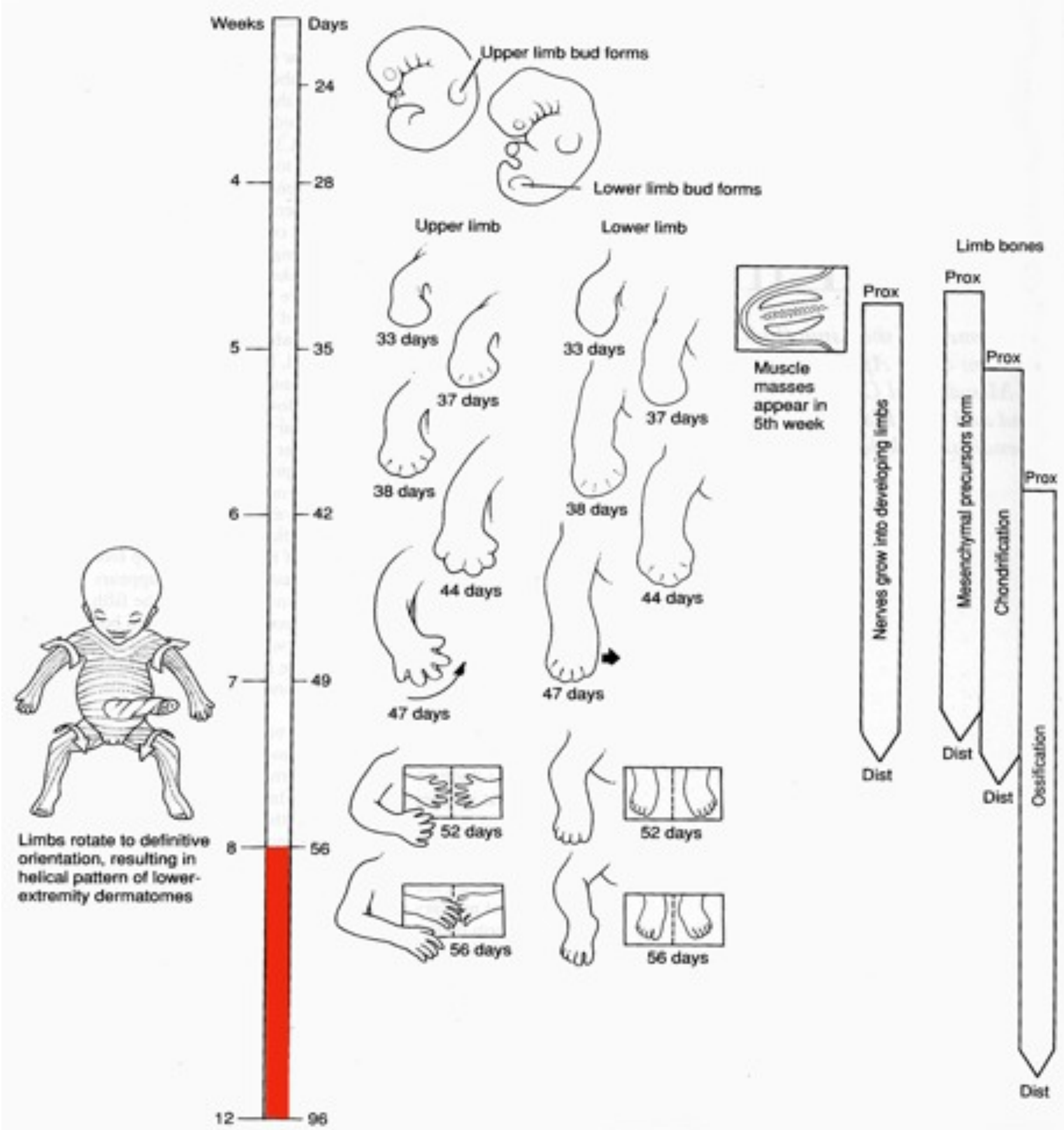
**Autopod:** The distal elements of a limb.
The **wrist** and the **fingers** in the forelimb; **ankle** and **toes** in the hindlimb

# Human Limb Development

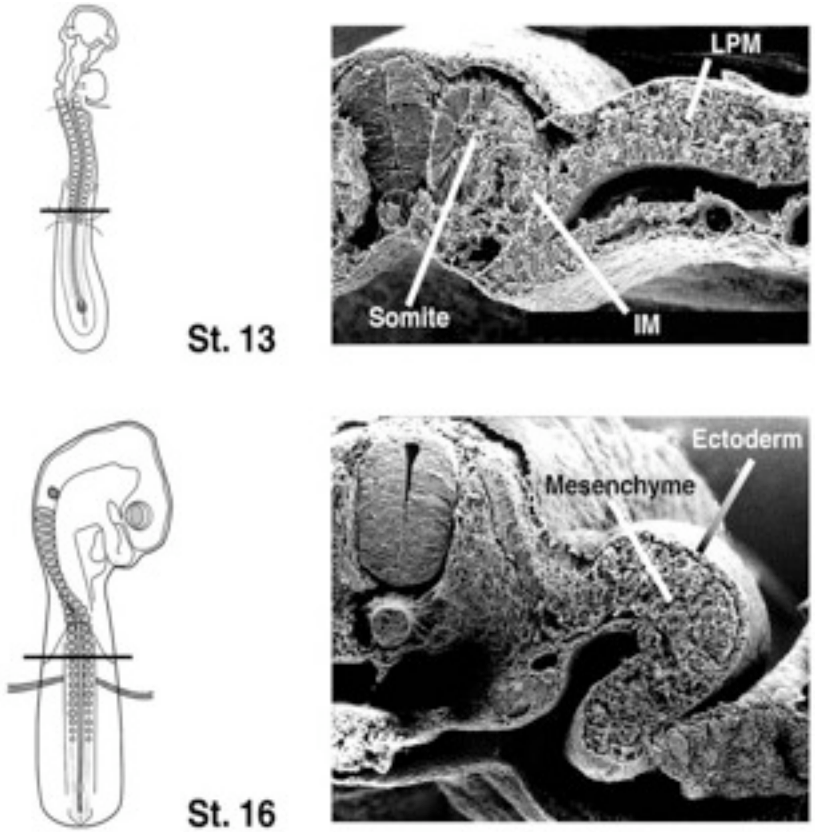



5 weeks 6 weeks 8 weeks

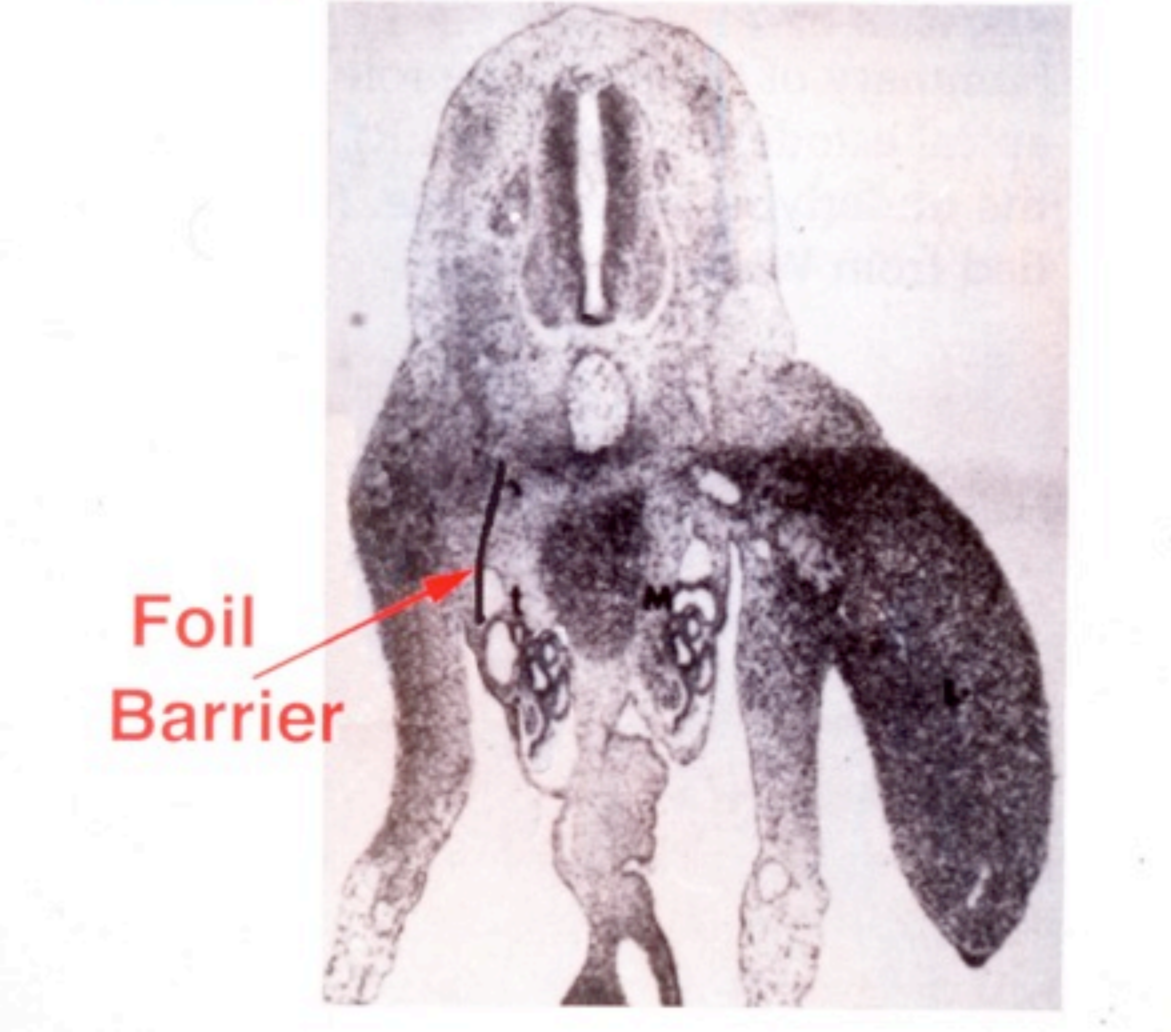
# Proximal to distal progression of limb development



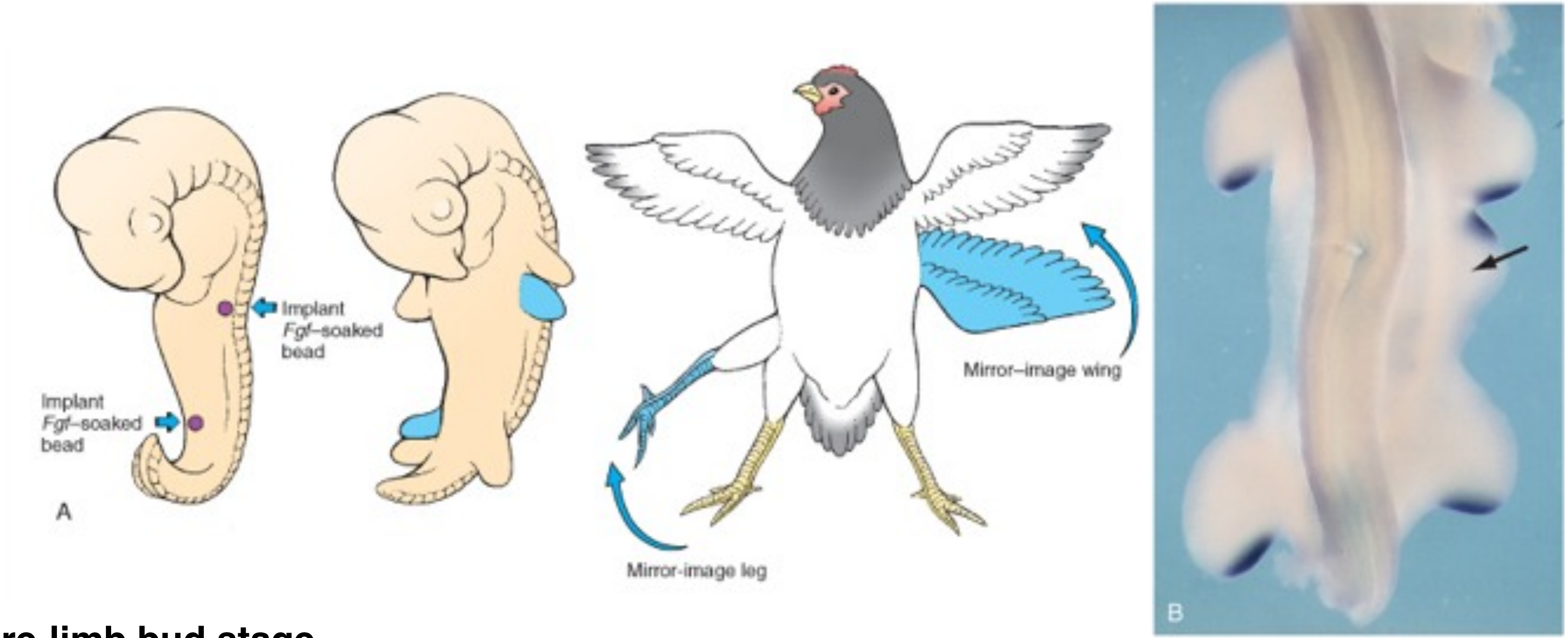
# How is limb initiation controlled?



# Barrier between the intermediate and lateral plate mesoderm prevents limb bud formation

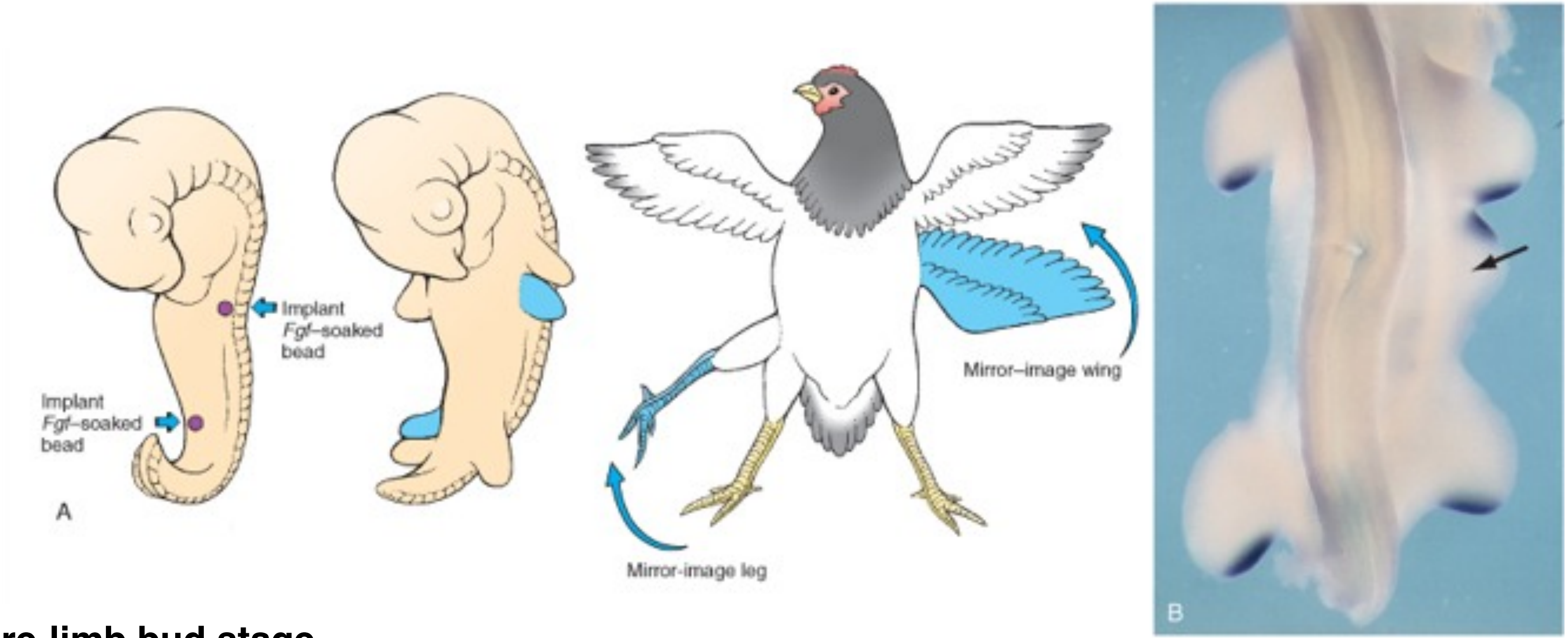


# Secreted signaling molecules, fibroblast growth factors (FGFs) can induce ectopic limb formation in the flank of the chick embryo




**Pre-limb bud stage chick embryo**

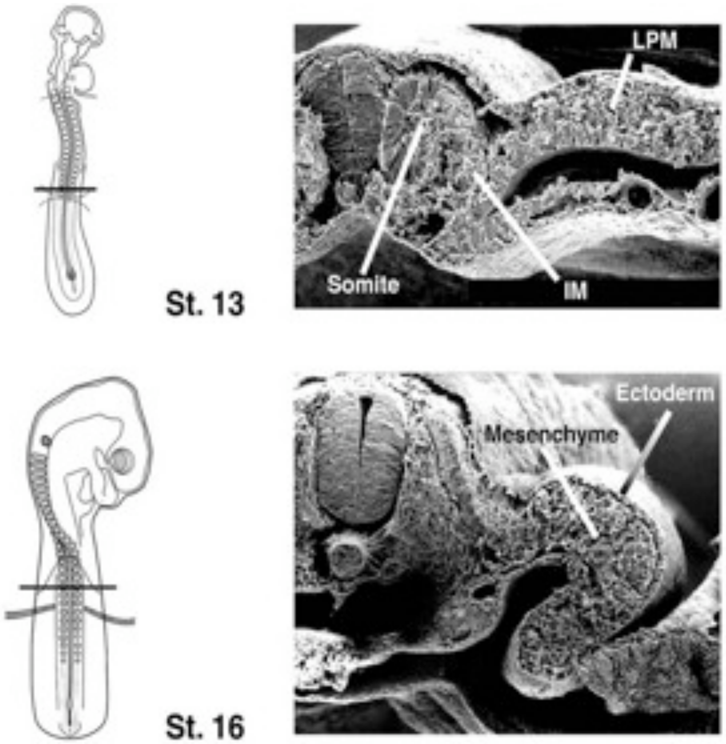
# Secreted signaling molecules, fibroblast growth factors (FGFs) can induce ectopic limb formation in the flank of the chick embryo




**Pre-limb bud stage chick embryo**

**-a single molecule (FGF) is capable of inducing cascade leading to limb formation**
**-cells in the flank are competent to respond to this secreted signal**

# Limb initiation



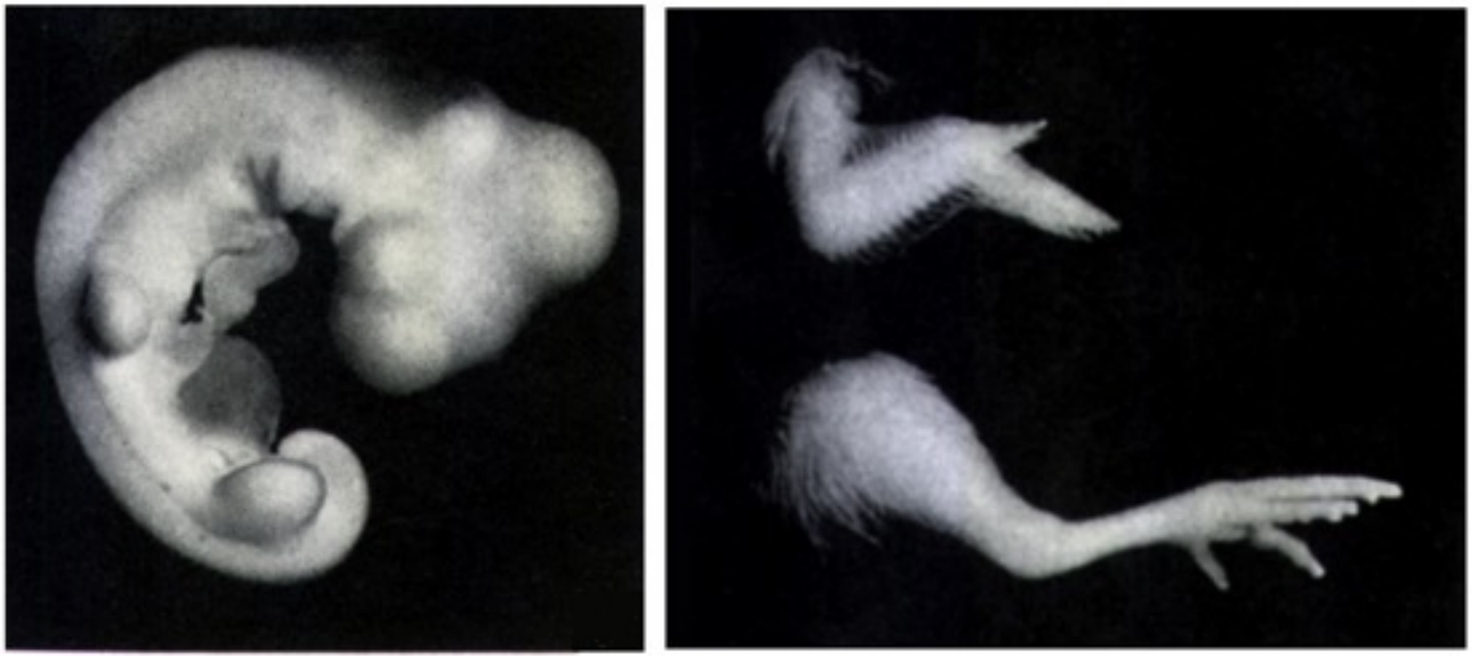

**lpm: lateral plate mesoderm**

**Axial cue** → **LPM** → **Ectoderm**
**Wnt?** **Fgf10** **Wnt3a, Fgf8**

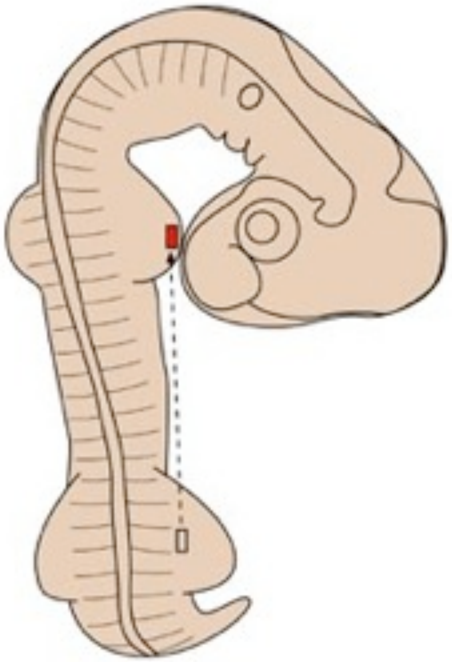
**Transfers information from the body to the limb axis**

## Limb-type specification

**Morphologically uniform limb buds develop to form morphologically distinct limb elements**



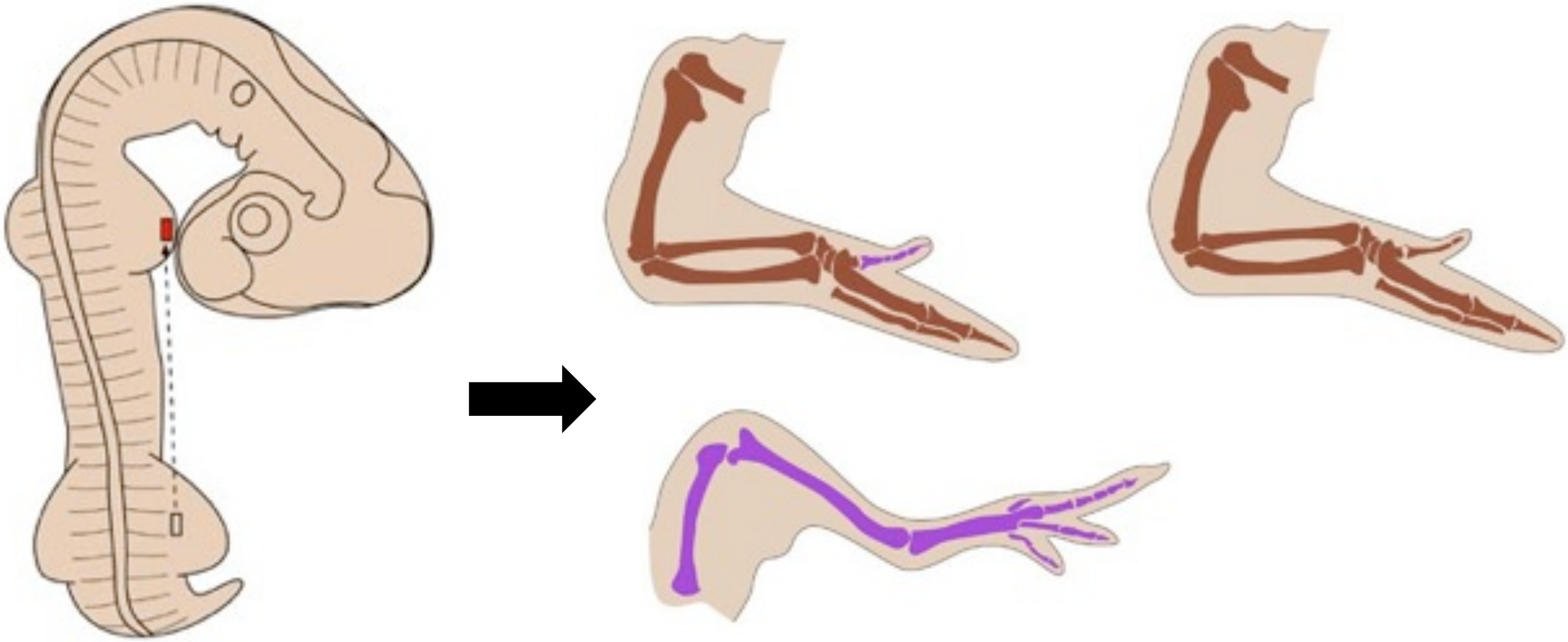
# Cell identity and plasticity in the developing limb bud



**Following heterotopic transplant, cells retain forelimb vs hindlimb identity**

Saunders et al., 1957, 1959

# Cell identity and plasticity in the developing limb bud



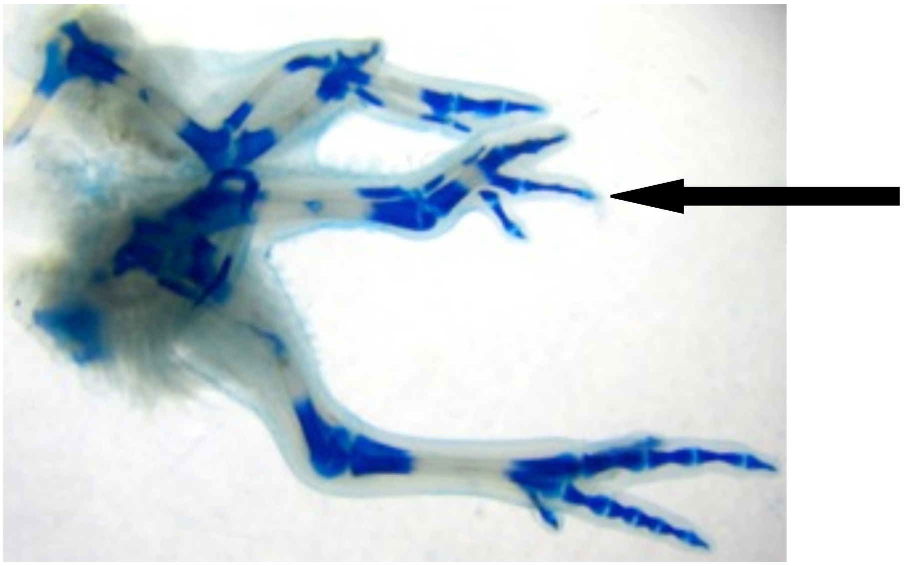
**Following heterotopic transplant, cells retain forelimb vs hindlimb identity**

Saunders et al., 1957, 1959

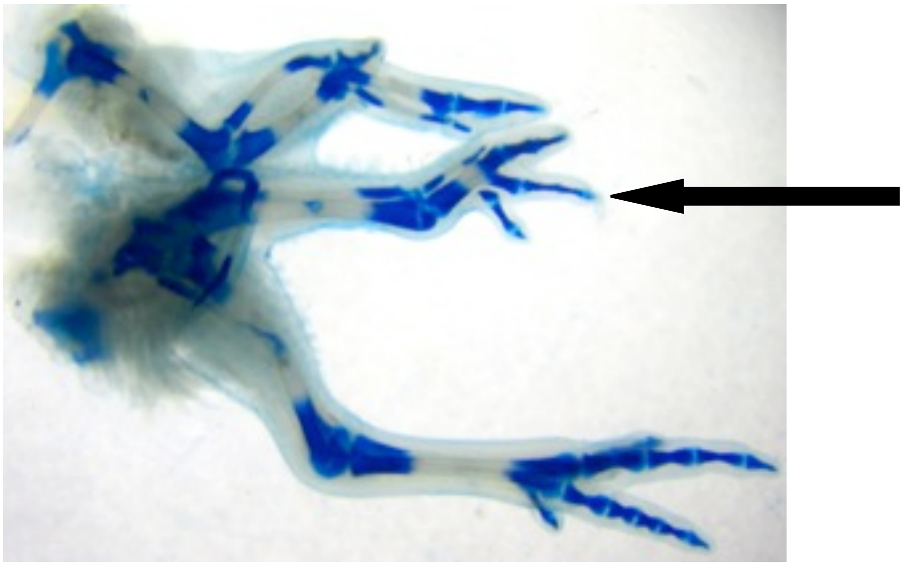
# Limb-type specification: candidate genes

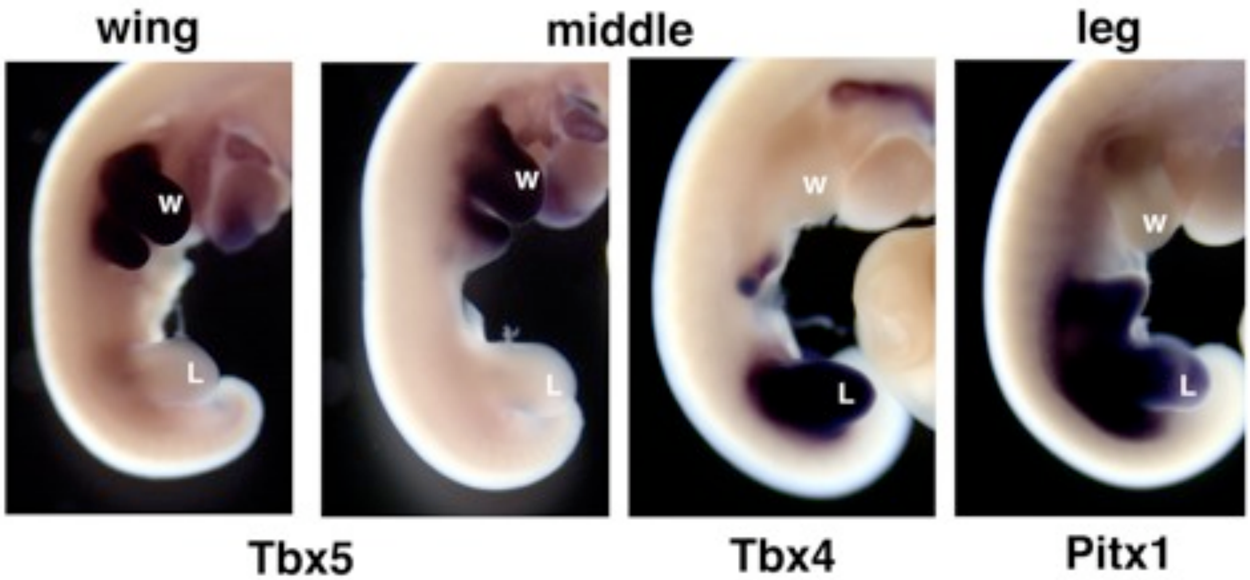

**chick st.29**

# A source of FGF applied to the interlimb flank induces ectopic limb formation in the chick embryo: Tbx expression correlates with morphology



# A source of FGF applied to the interlimb flank induces ectopic limb formation in the chick embryo: Tbx expression correlates with morphology



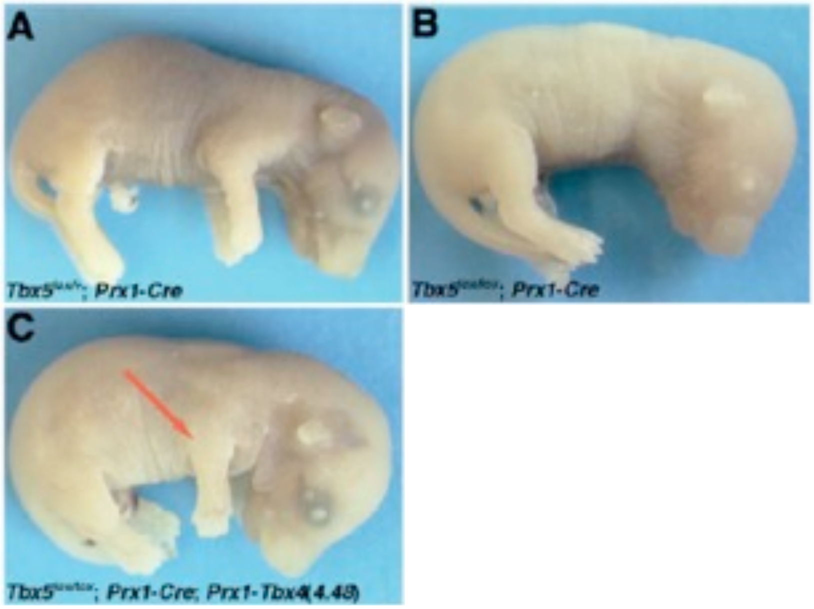


# Conditional knock-out of Tbx5 in the limbs leads to the absence of the forelimbs

*Tbx5$^{lox/lox}$;Prx1cre*

Replacement of Tbx5 with Tbx4 in the limb rescues limb outgrowth. The limb remains a forelimb.

Control no Tbx5 in limb




Tbx4 for Tbx5: Rescue

*Tbx5* and *Tbx4* Are Not Sufficient to Determine Limb-Specific Morphologies but Have Common Roles in Initiating Limb Outgrowth

Carolina Minguillon, Jo Del Buono, and Malcolm P. Logan*

Developmental Cell, Vol. 8, 75–84, January, 2005,

**Correlation is not causality: causality must be determined experimentally**

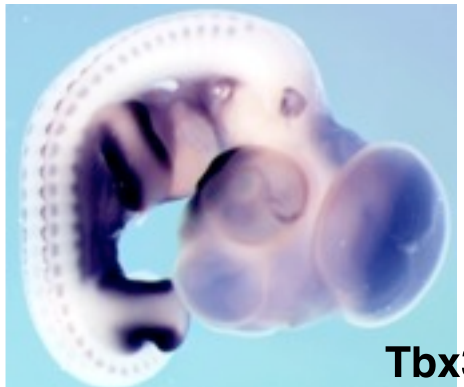
# Limb abnormalities are associated with many human congenital syndromes

**second most-common congenital abnormality in human live births**
**also common abnormality following environmental insult (eg Thalidomide)**

**Mutations in Human *TBX5* are associated with Holt-Oram Syndrome (HOS; OMIM 142900)**

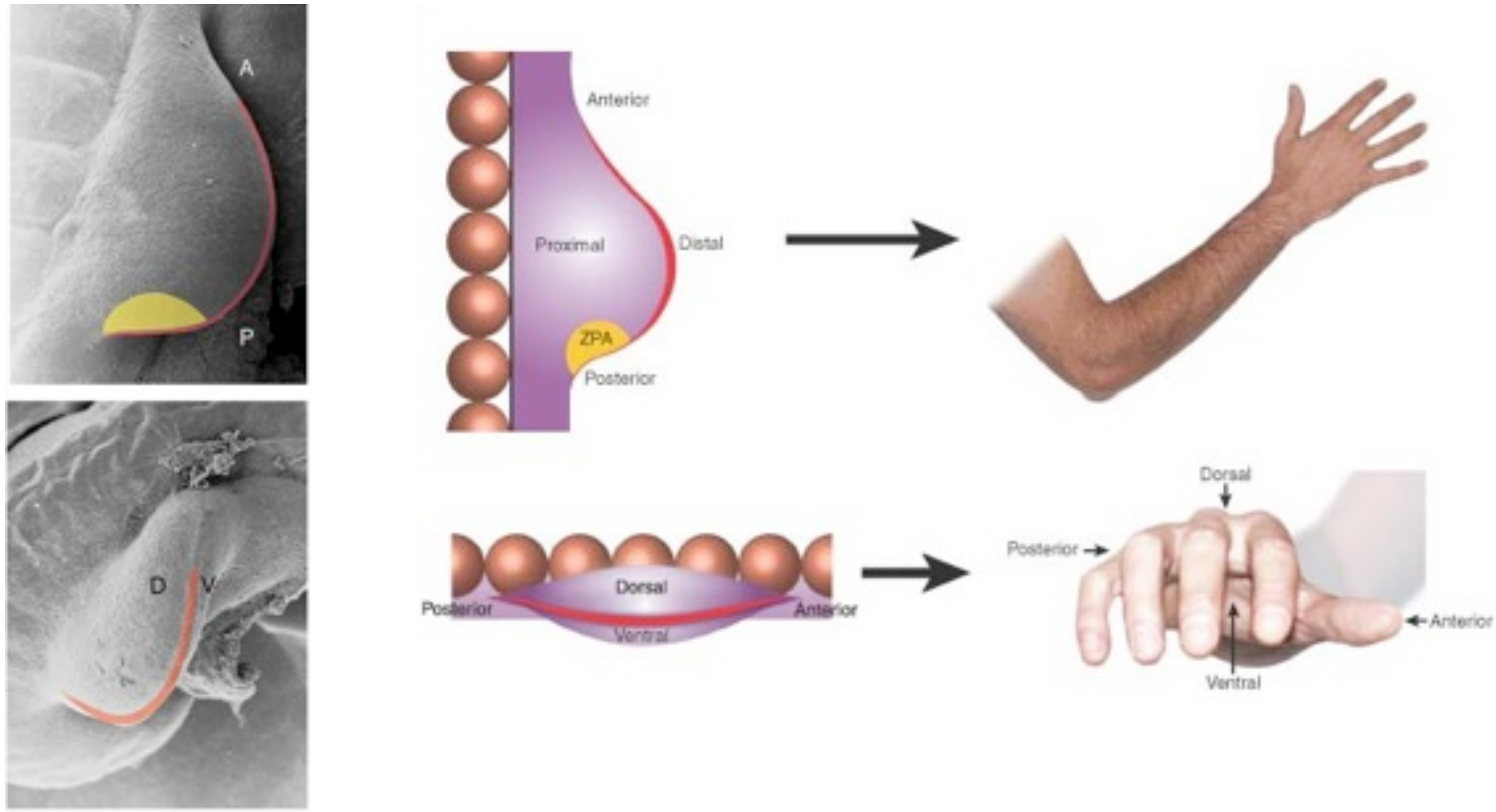



**Mutations in human *TBX3* are associated with Ulnar-Mammary Syndrome (UMS; OMIM 181450)**



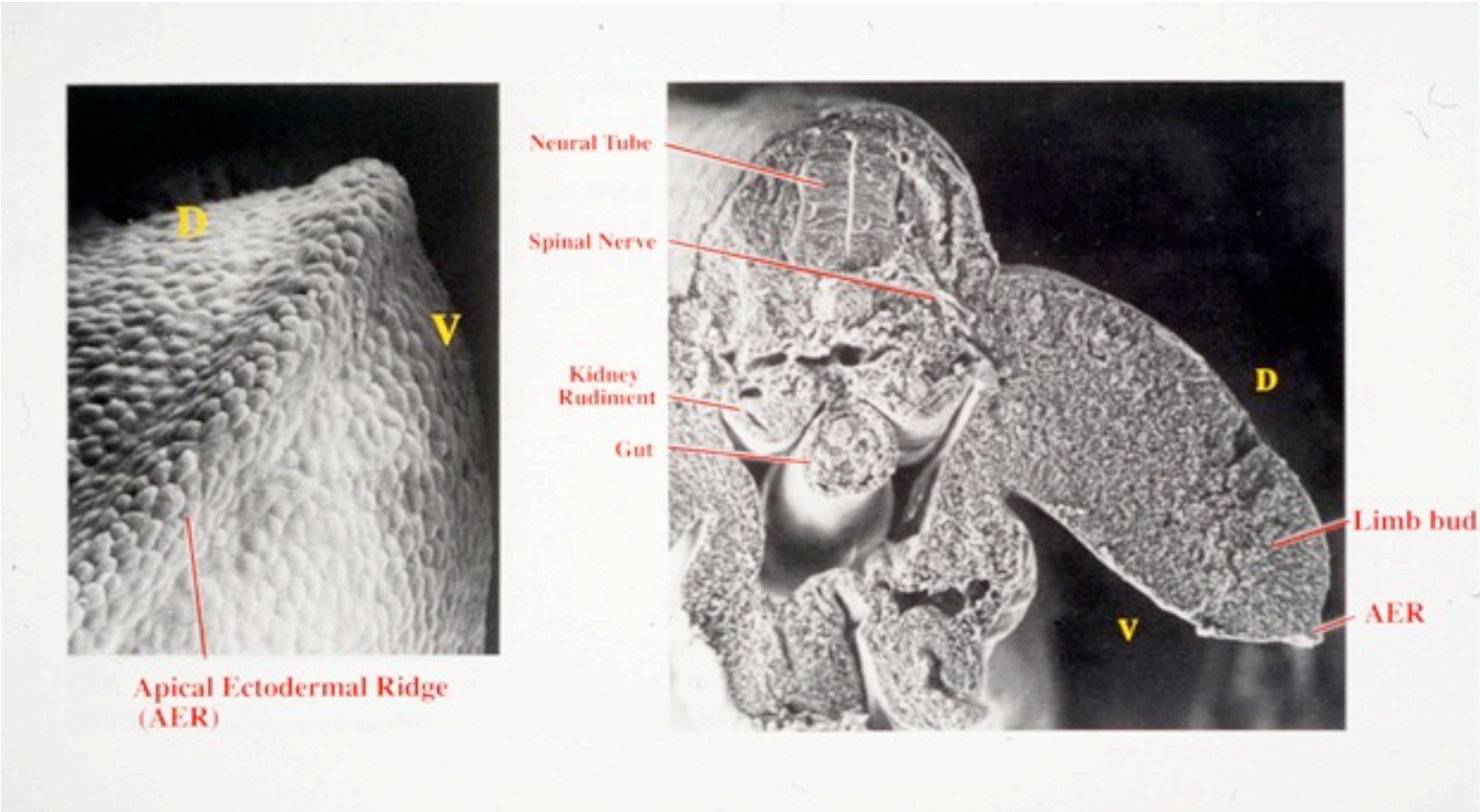
# Three signaling centers pattern the three primary limb axes

Apical ectodermal ridge (AER) - proximal-distal
Zone of polarizing activity (ZPA) - anterior-posterior
Dorsal ectoderm - dorsal-ventral

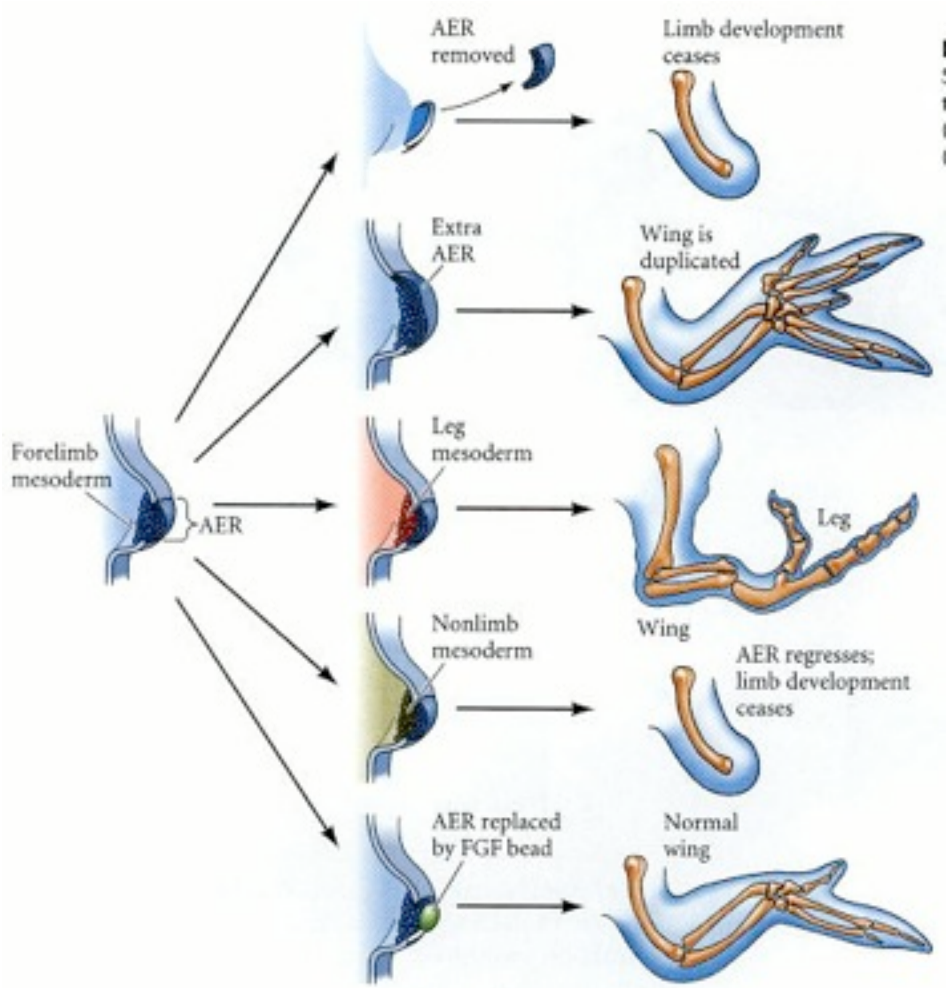


# Apical ectodermal ridge (AER) - proximal-distal axis

## Signals from the AER maintain limb outgrowth



# AER manipulations give insight into AER function




required

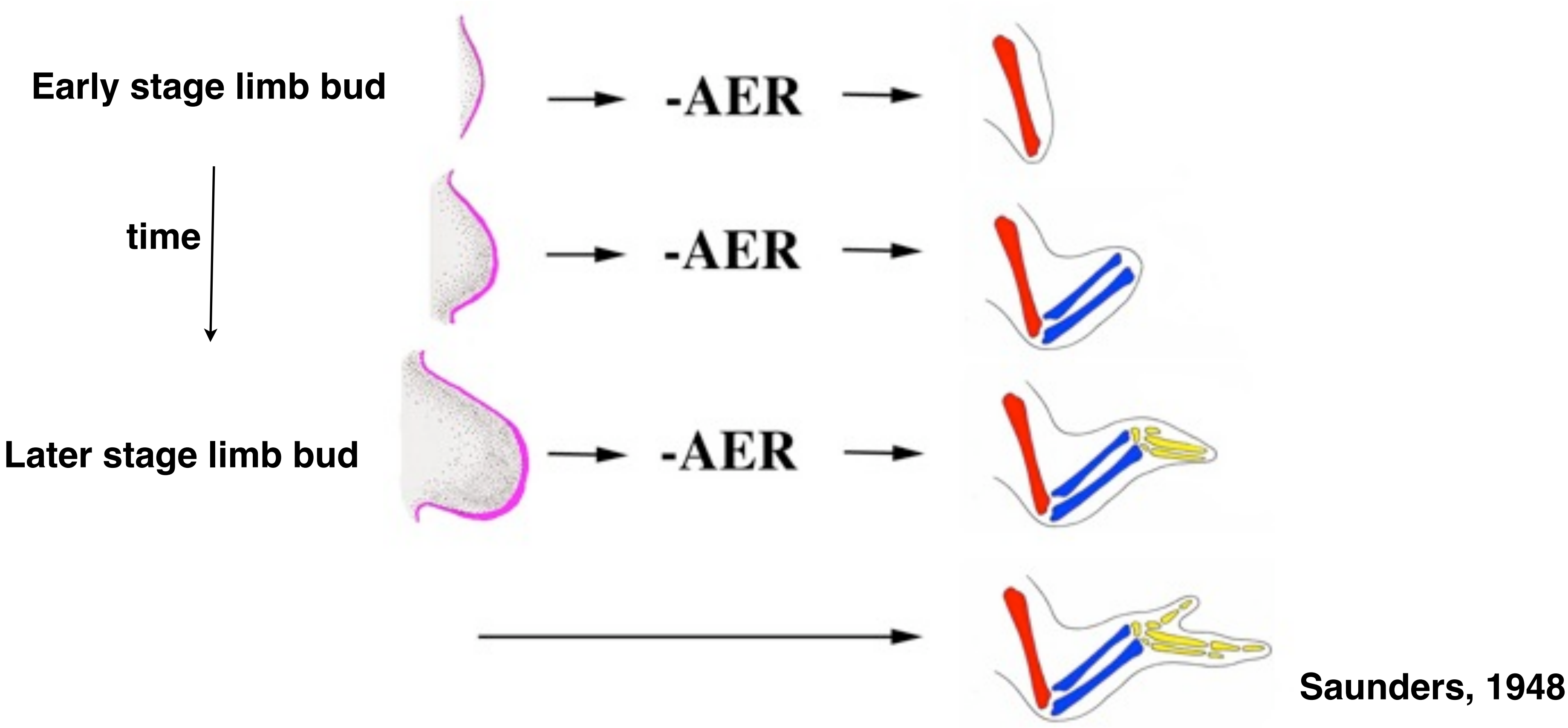
promotes outgrowth

not instructive

not instructive

FGF source

**Conclusions from 'classical' embryological studies**
**- the AER is required for normal proximal-distal pattern**

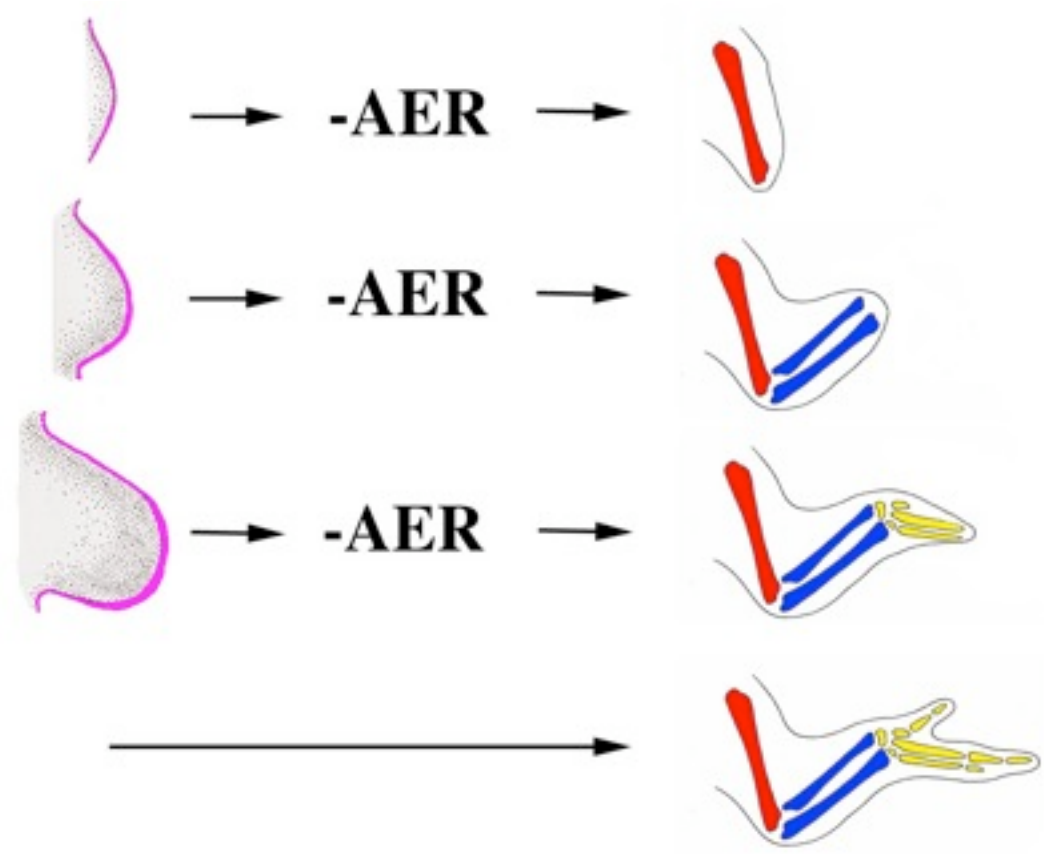


**Conclusions from ‘classical’ embryological studies**
**- the AER is required for normal proximal-distal pattern**

**Early stage limb bud**

**time**

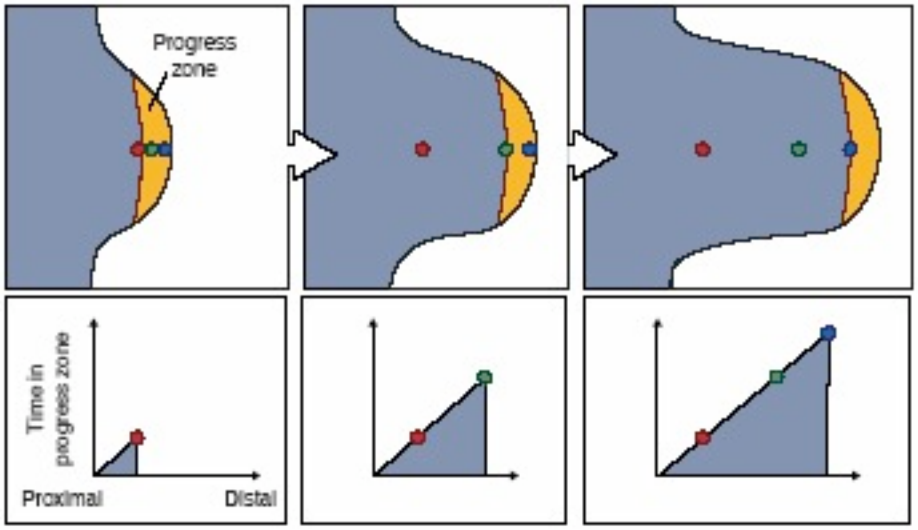
**Later stage limb bud**




**Saunders, 1948**

**- required for outgrowth, full P-D pattern**
**- elements laid down in proximal to distal progression**

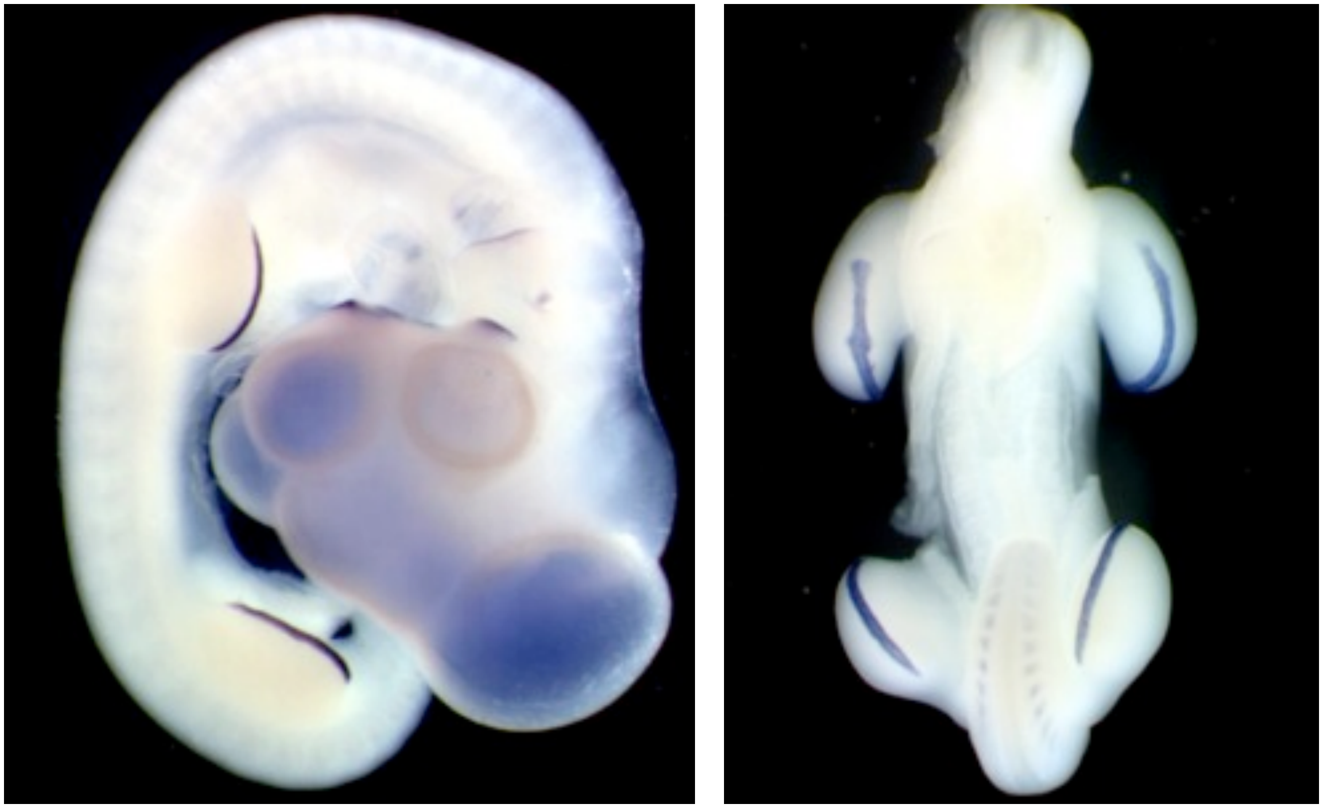
# The progress zone (PZ) model
**Summerbell et al. Nature 1973**




**The length of time a cell spends in the PZ may determine proximal-distal identity**

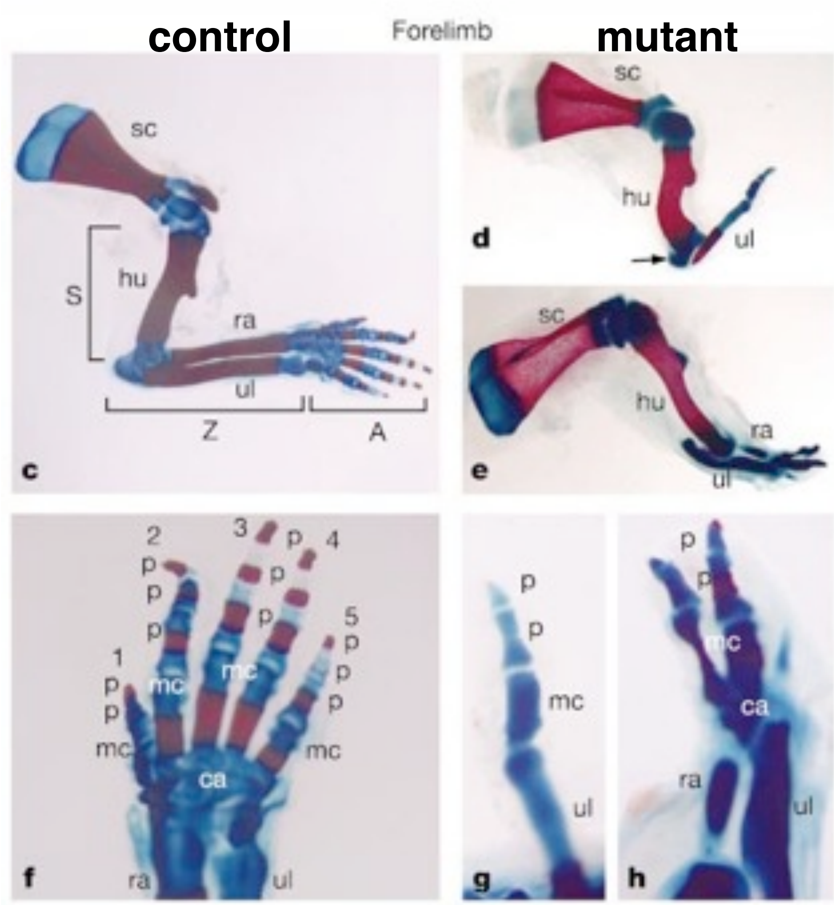
What factors are regulating outgrowth?

Evidence supporting a role for FGFs in proximal-distal patterning
- Fgf8 is expressed in the apical ectodermal ridge (AER)
  i.e. present at right time, in right place



Whole mount RNA *in situ* hybridization - chick (st23)

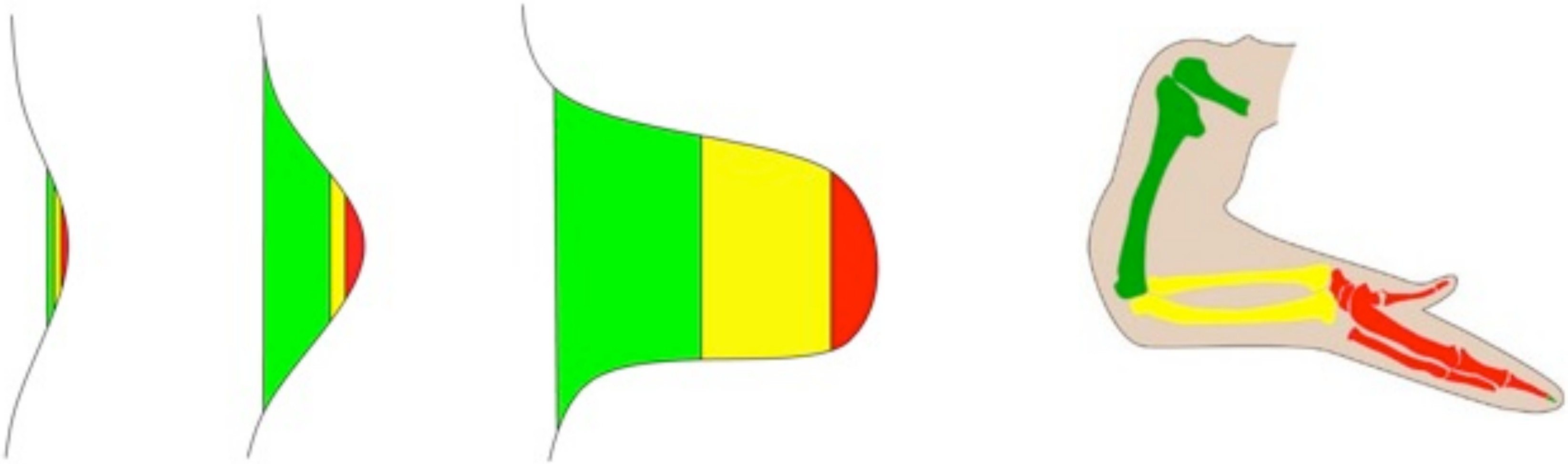
## Fgf8/Fgf4 double knock-out mouse: a genetic equivalent to AER removal in the chick




Sun et al., Nature 2002

**Limb outgrowth disrupted but distal structures do form. This would not be predicted by the progress zone model**

Alternative model: early allocation followed by expansion



progenitor pools are specified early during limb outgrowth

A signal (FGF) from the AER progressively expands these preexisting populations

# AER defects give rise to proximodistal outgrowth phenotypes

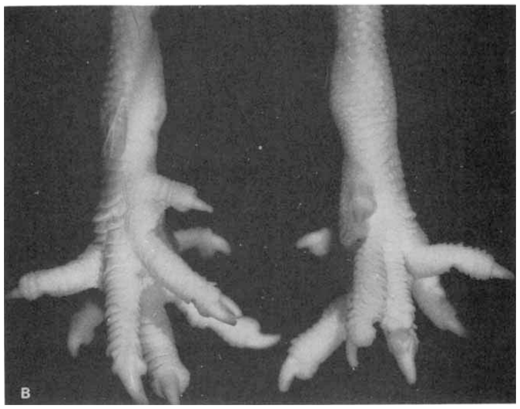
**Split-hand/split-foot malformation (SHFM) caused by p63 mutation: reduced AER maintenance**


Am J Hum Genet. 2000 July; 67(1): 59–66.
Published online 2000 June 5.

Pediatric Radiology 2003
10.1007/s00247-003-1017-3

**Diplopodia: ectopic AER?**

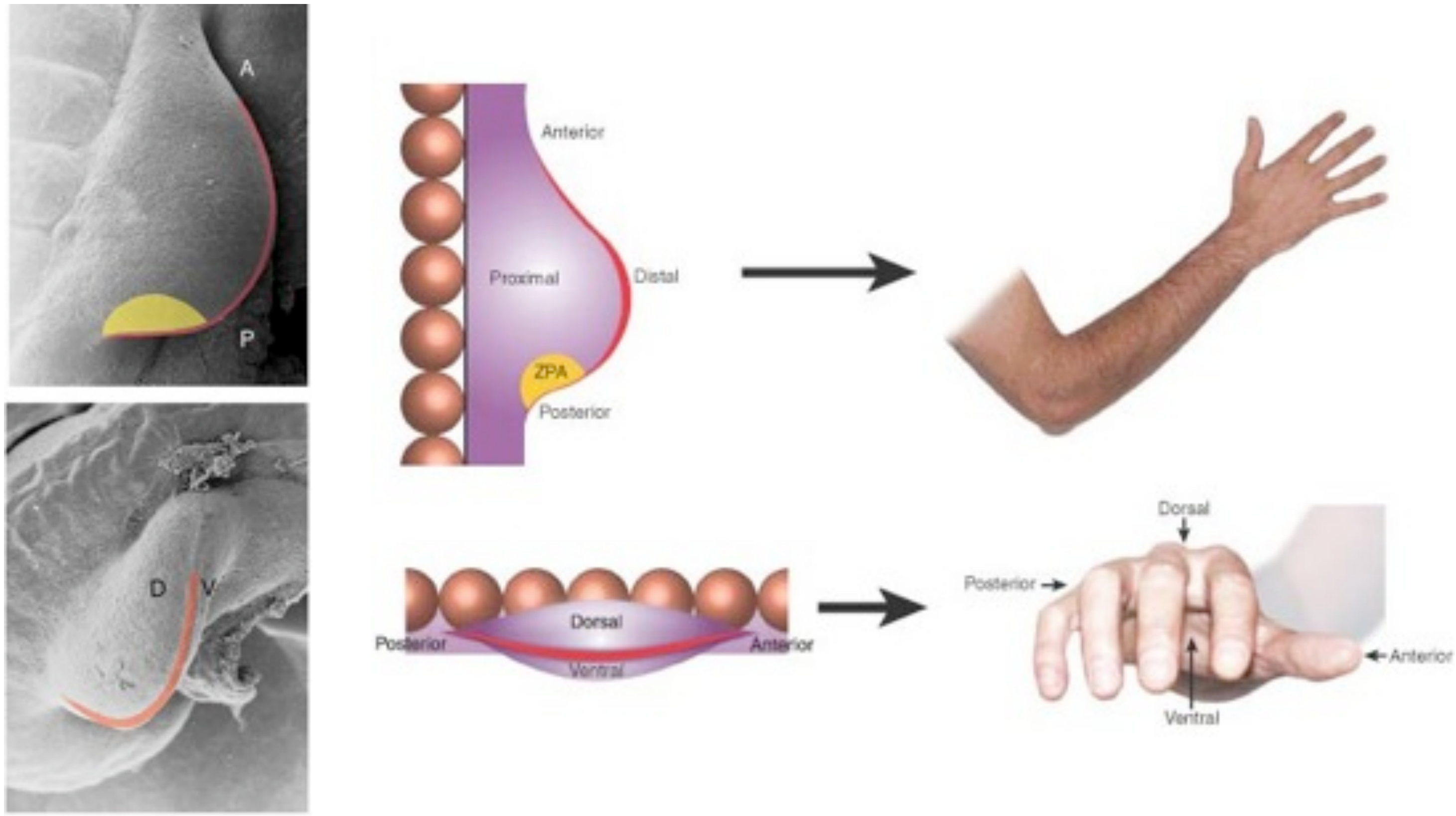
**Eudiplopodia: ectopic AER (chicken)**




J. Exp. Zool., *176*: 219-236.

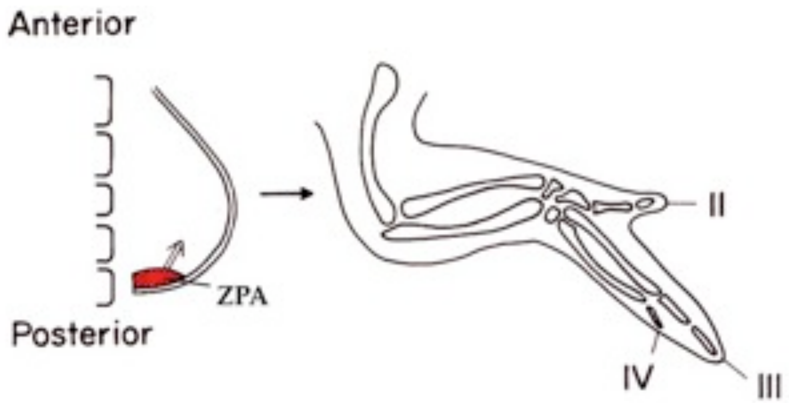
# The axes of the limb

## Zone of polarizing activity (ZPA) - anterior-posterior

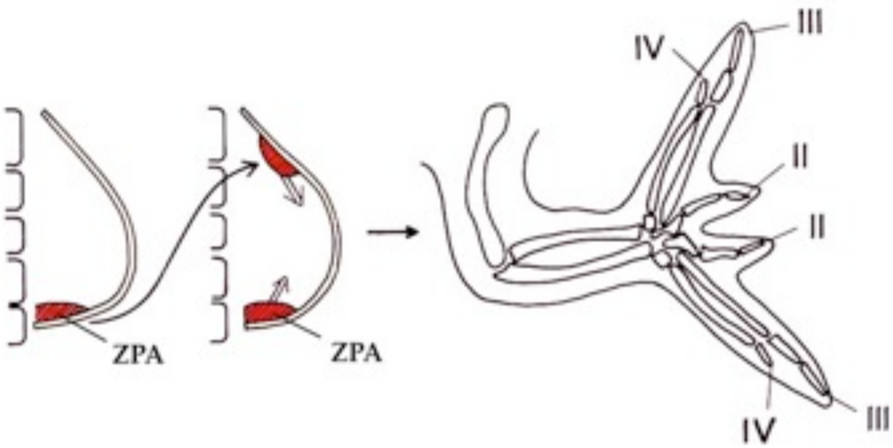


# A region of cells in the posterior limb bud, the zone of polarizing activity (ZPA) is important for patterning the anterior-posterior axis of the limb

**Again, an important signaling center in the limb was initially identified in 'classical' embryological experiments**

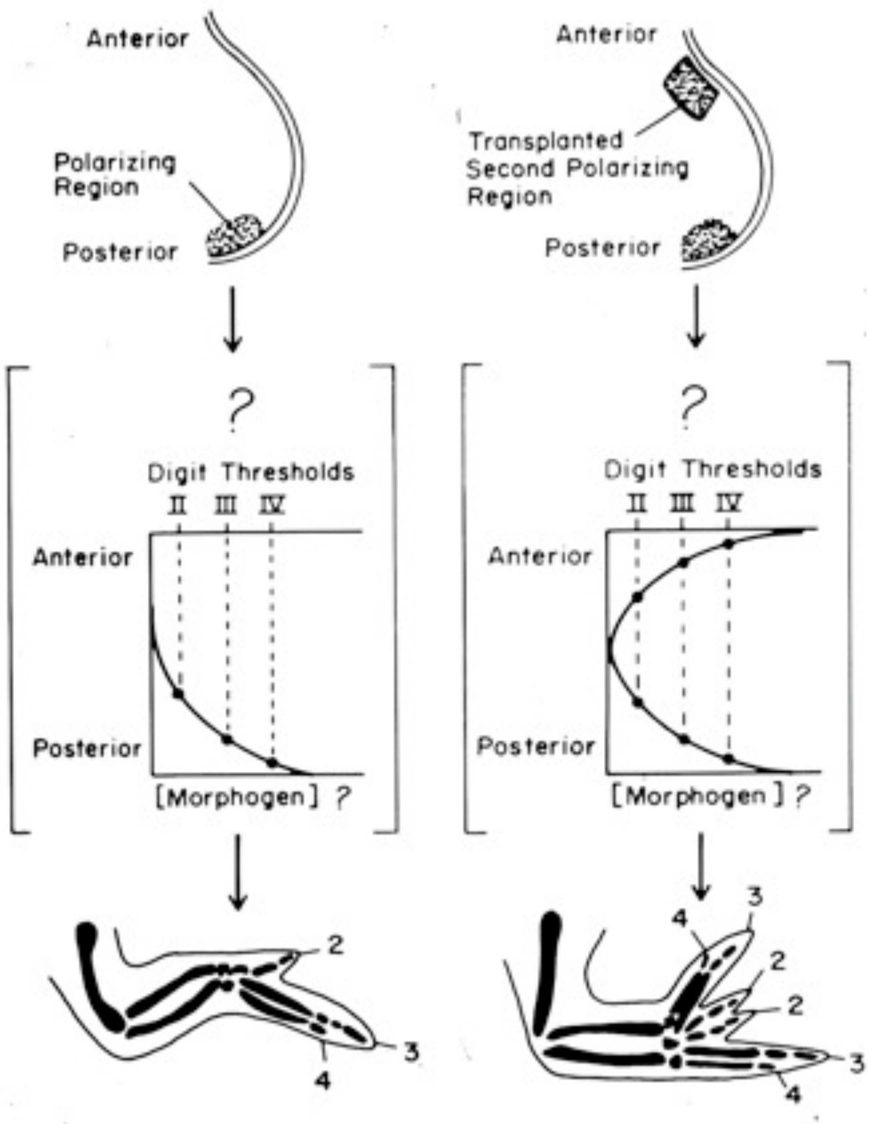



**Ectopic digits are not derived from the ZPA graft itself. They are induced in the host tissue. This is a non cell-autonomous phenotype.**



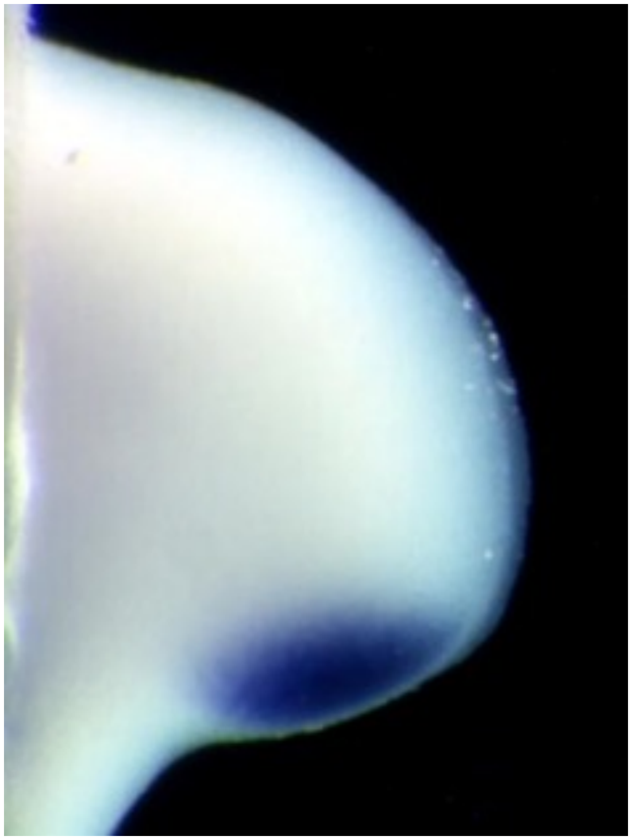

**Saunders and Gasseling 1968**

**Morphogen model: cell identity via threshold responses to a gradient of signaling molecule**

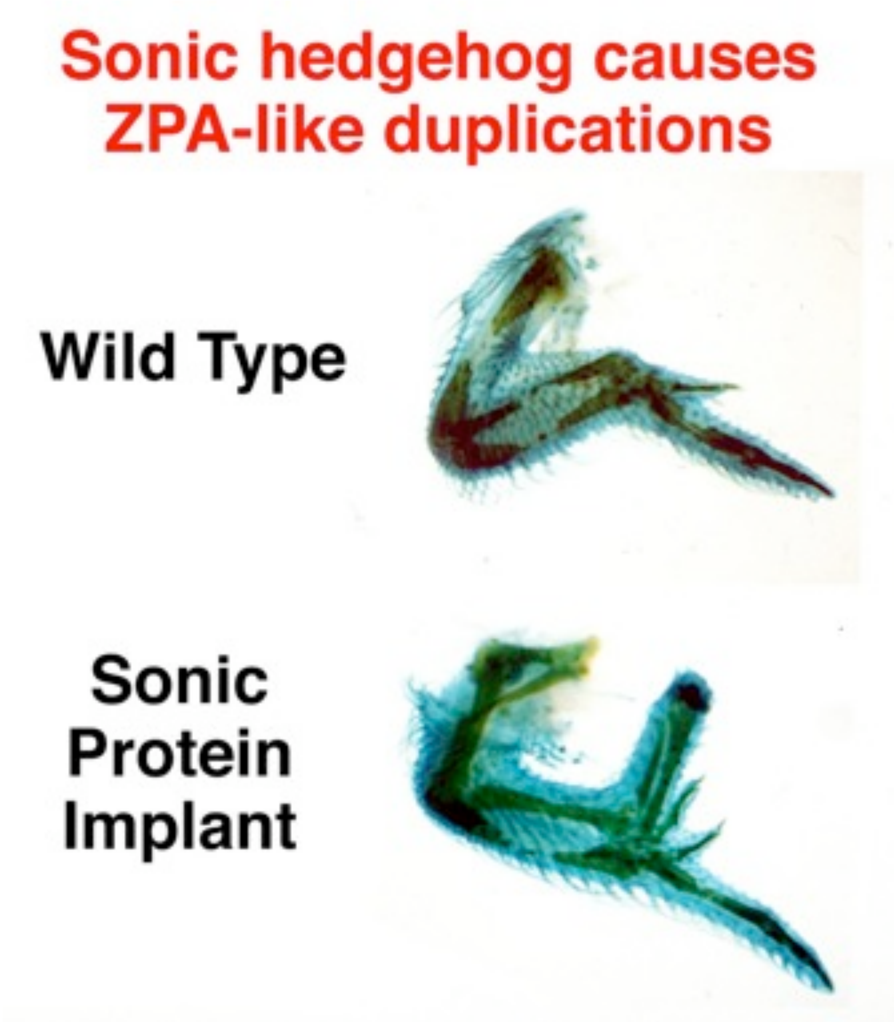



**circa 1969**

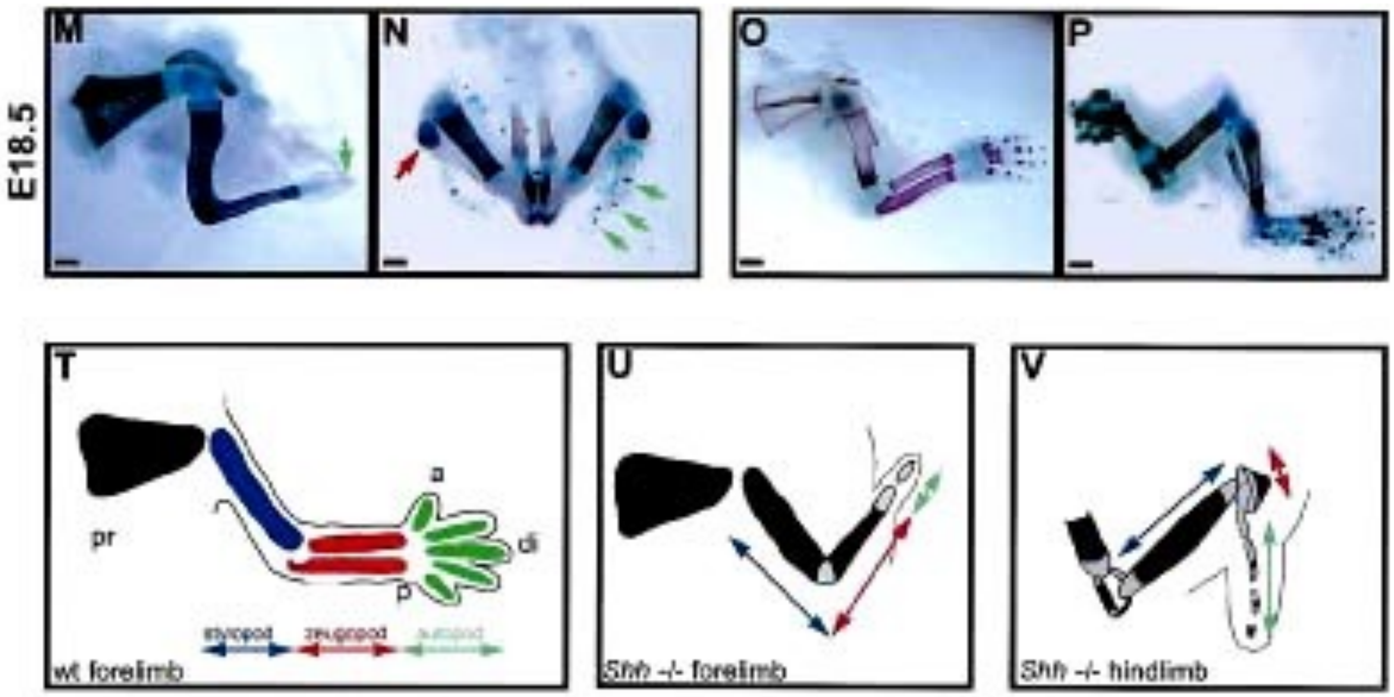
# Sonic Hedgehog (Shh) is expressed in the ZPA



# Direct evidence for a role of Shh in anterior-posterior patterning

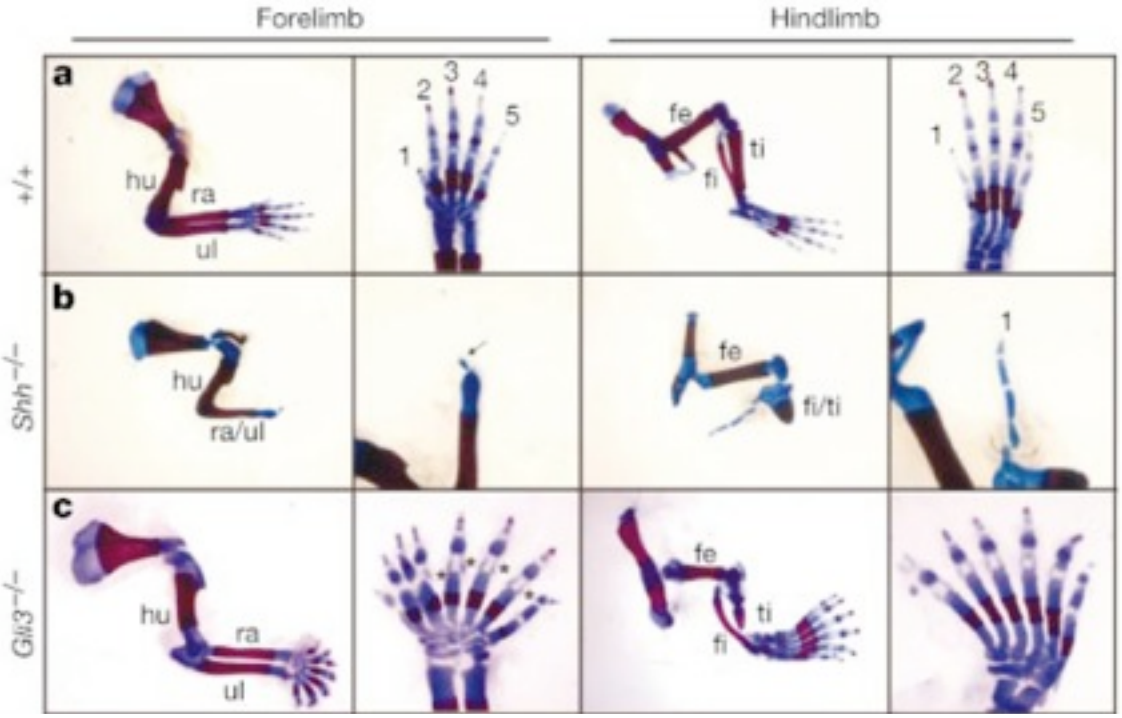


The complementary approach:
Deletion or ‘knock-out’ of the Shh gene disrupts anterior-posterior patterning of the limb



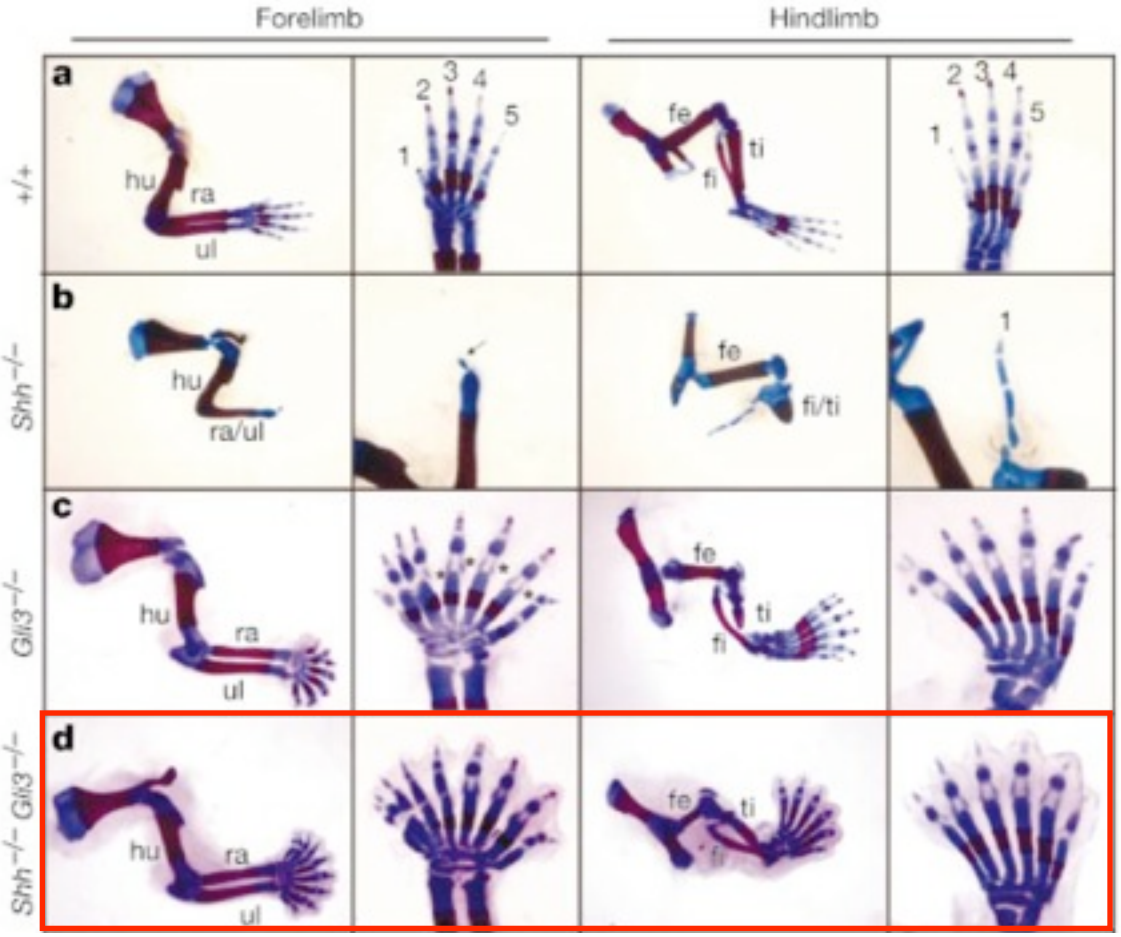

**Chiang et al., Nature 1996**
**Kraus et al., Mech Dev. 2001**

# Biochemistry: Gli3 transcription factor mediates Shh function




*Gli3* loss-of-function results in polydactyly.

Surprisingly, *Shh/Gli3* double mutants look identical to *Gli3* nulls




Shh modulates inherent polydactylous limb ‘ground state.’

Shh inhibits an inhibitor of digit formation and imposes polarity

# Regulation of AP identity by Shh involves complex integration of concentration and time dependent signaling

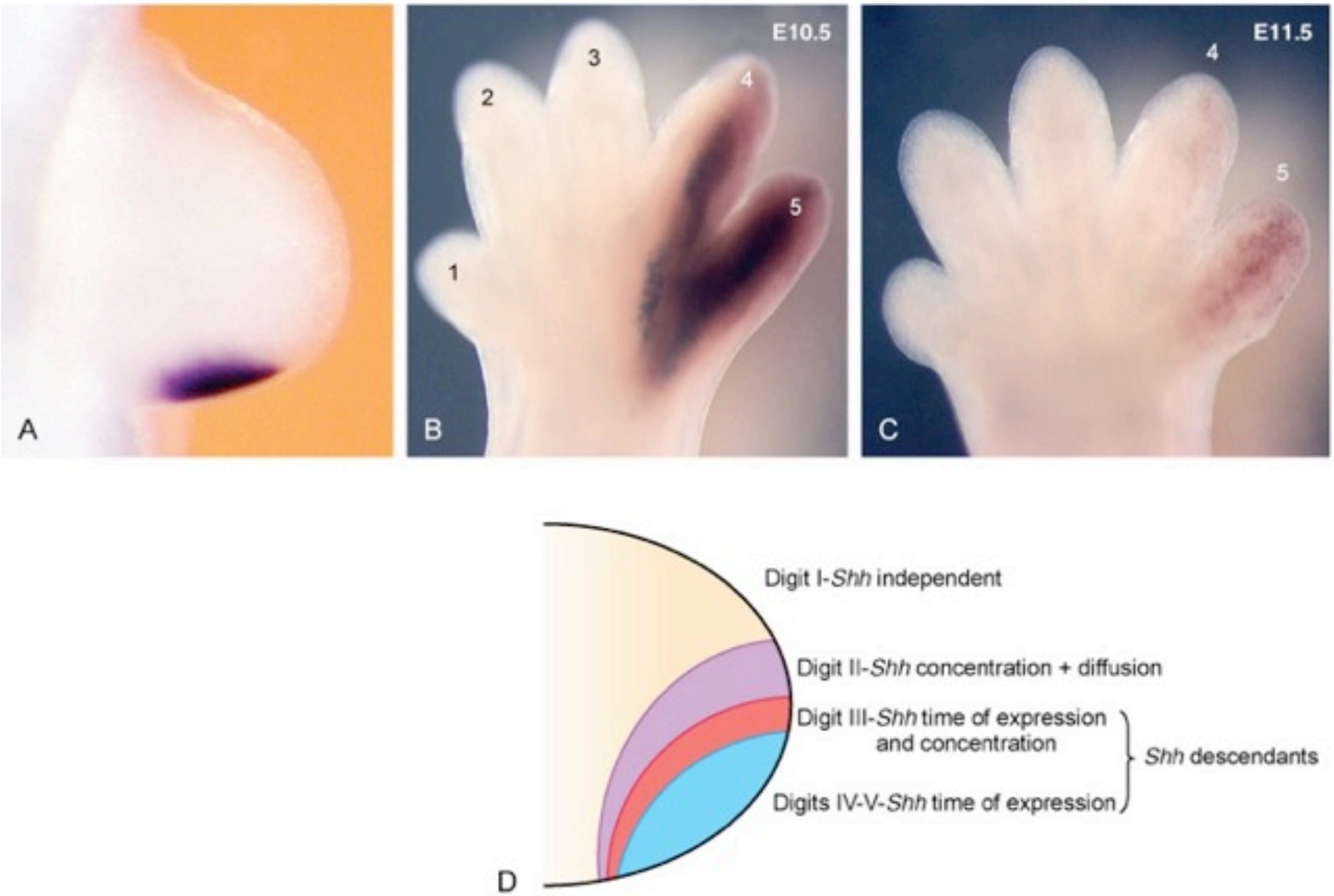
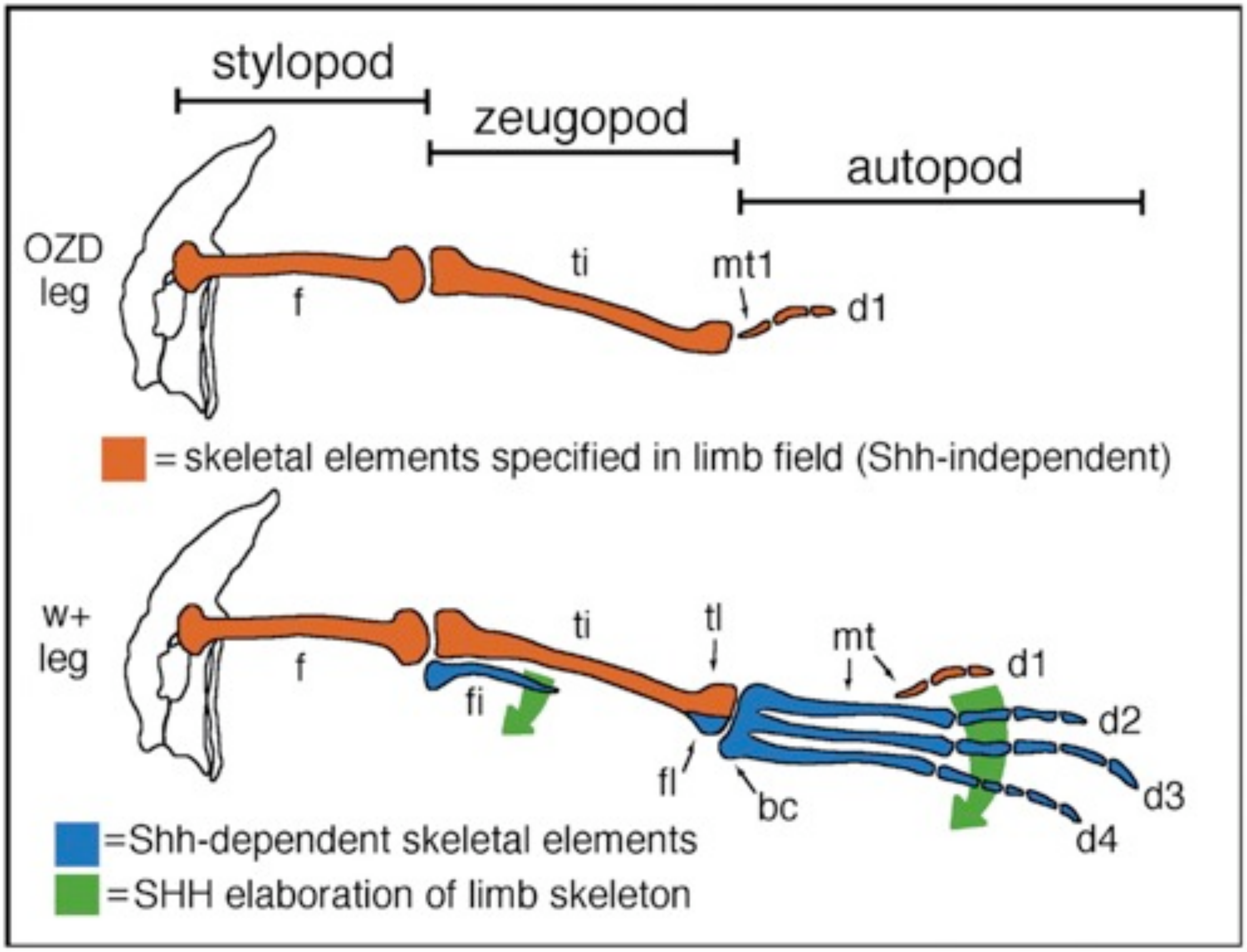







Fig. 18-14. Cranial-caudal patterning of the limb bud. *A*, Expression of *Shh* in the zone of polarizing activity (ZPA) of the limb bud of a mouse embryo. *B, C*, Fate maps of ZPA descendents when labeled at E10.5 and E11.5, respectively. Cells formerly in the ZPA express a reporter gene, allowing them to be traced over time. *D*, Model for explaining *Shh*-mediated cranial-caudal patterning of the digits.

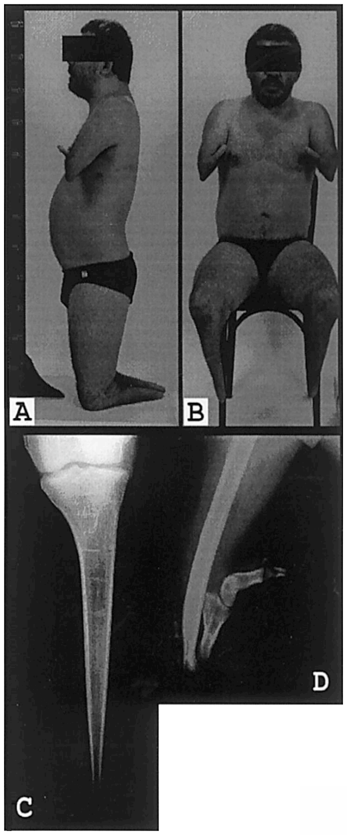
## SHH function in generating the amniote limb skeleton

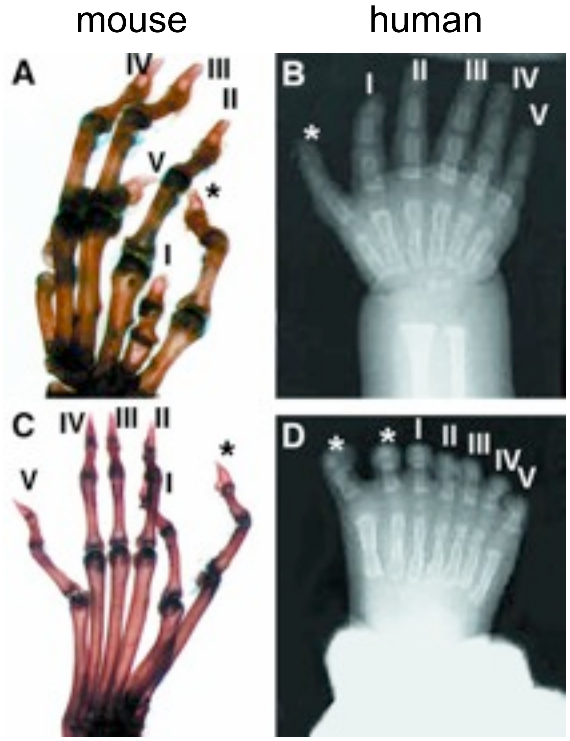


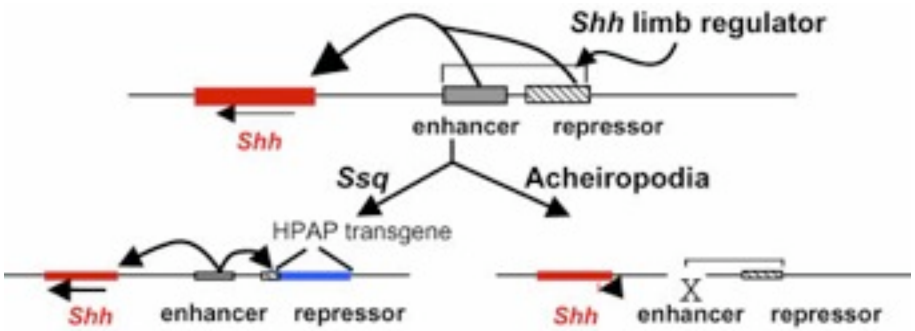

Ros, M. A. et al. Development 2003;130:527-537

Acheiropodia (OMIM 200500): deletion of the SHH limb enhancer

preaxial polydactyly (PPD2; OMIM 174500): ectopic anterior SHH activity




J Anat. 2003 January; 202(1): 13–20.
doi: 10.1046/j.1469-7580.2003.00148.x.



Human Figure 2 Affected individual (IV-18) from family 4. Note the small fingerlike appendages at the end of the arm, which are present bilaterally (A and B). C, Radiograph showing tapered amputation of the distal tibia. The proximal tibial epiphysis is well preserved. D, Radiograph showing dysplastic distal humerus articulating with a rudimentary forearm composed of three dysplastic long bones.

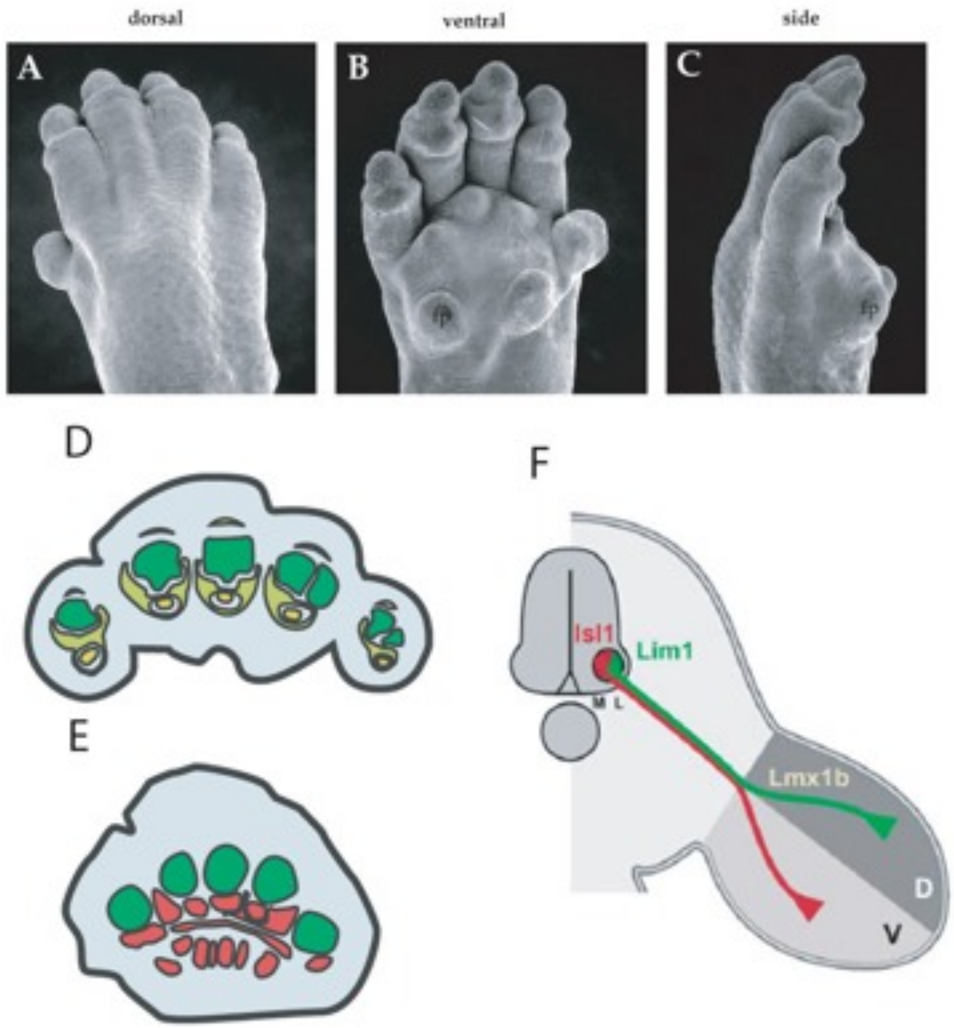
Region is in intron 5 of human LMBR1 locus, 1MB away from Shh coding sequence

Am J Hum Genet. 2001 January; 68(1): 38–45.

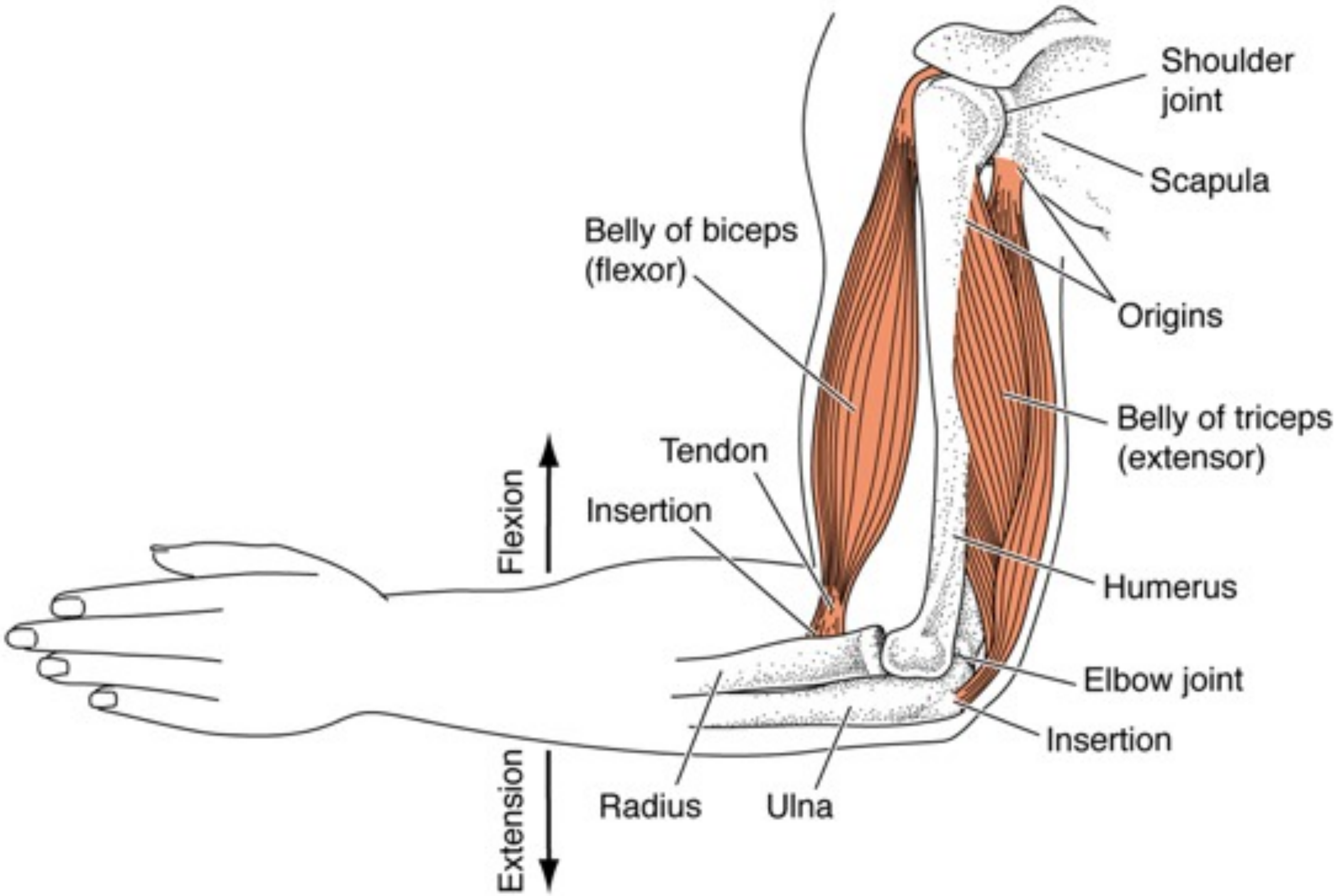
Published online 2000 November 22.

# The Dorsal-Ventral Axis

# Dorsal-ventral limb asymmetry

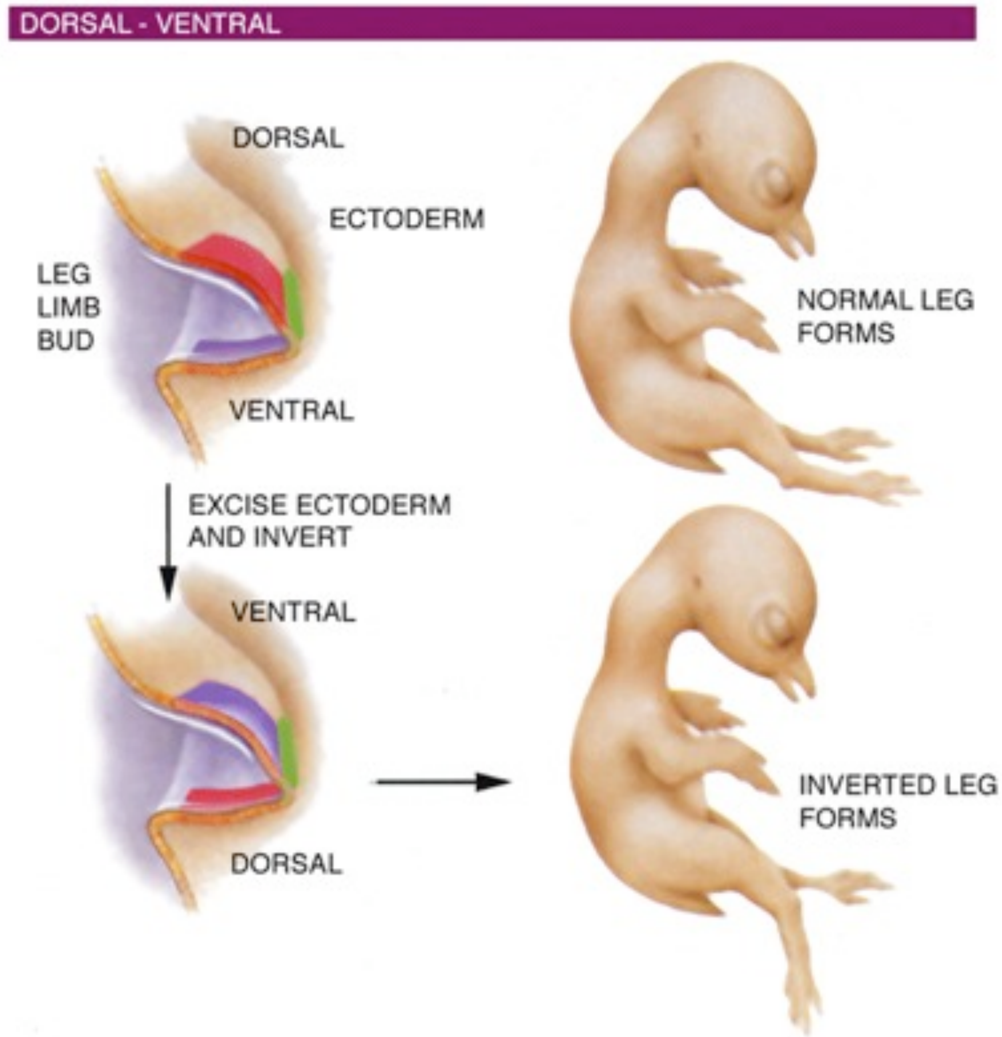


# Dorsal-ventral asymmetry is required for coordinated limb movement

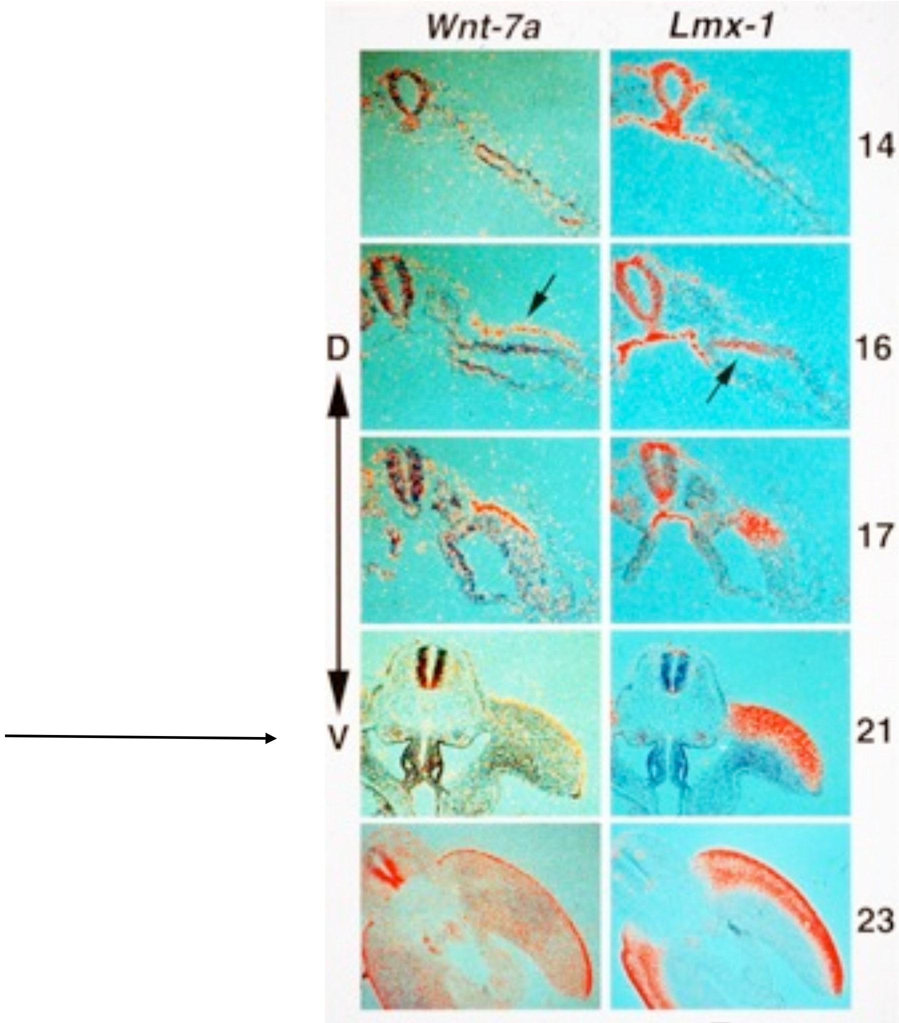


# Signals from the dorsal ectoderm play an important role in patterning the dorsal-ventral axis

**Initial observations in 'classical' embryological experiments**

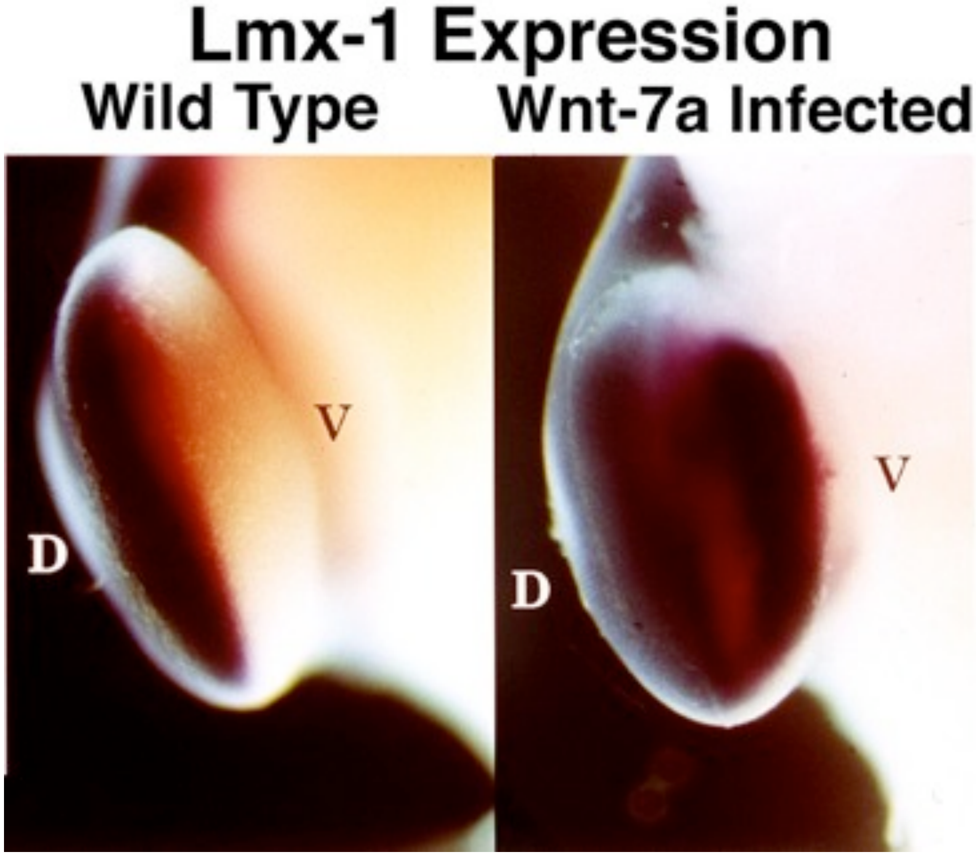


# The Wnt7a signal and Lmx1 transcription factor: candidates for DV pattern regulation

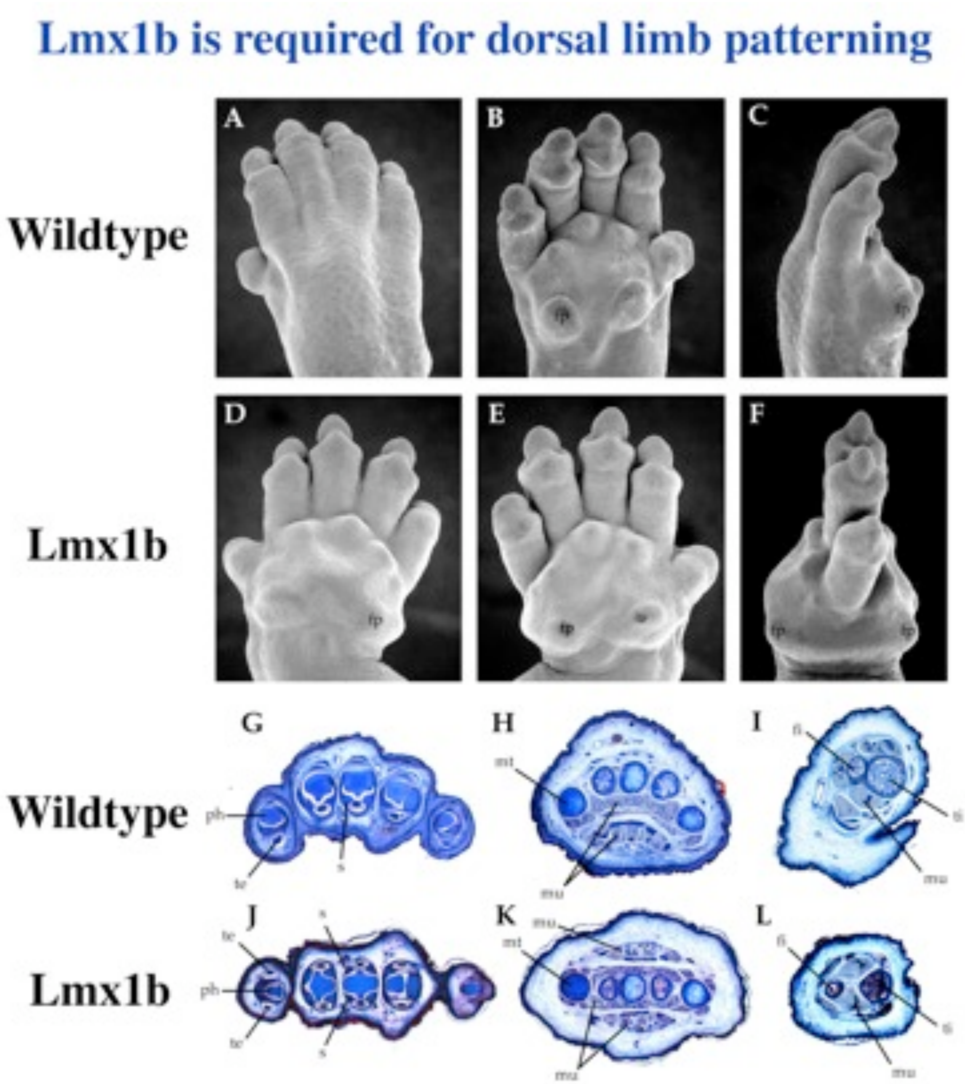



**Chick limb buds**

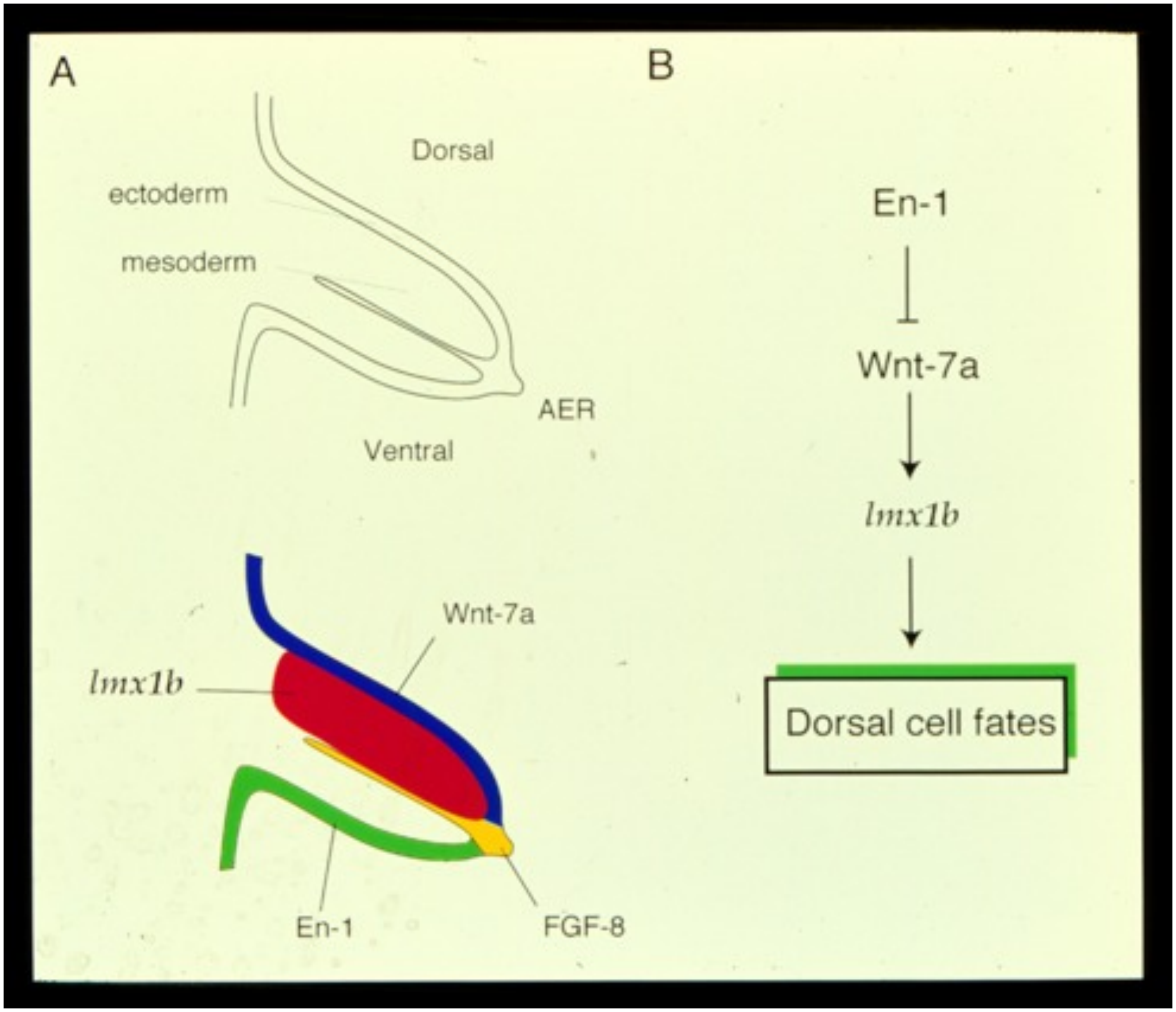
# Misexpression of Wnt7a in the ventral ectoderm induces Lmx-1 in the ventral mesenchyme



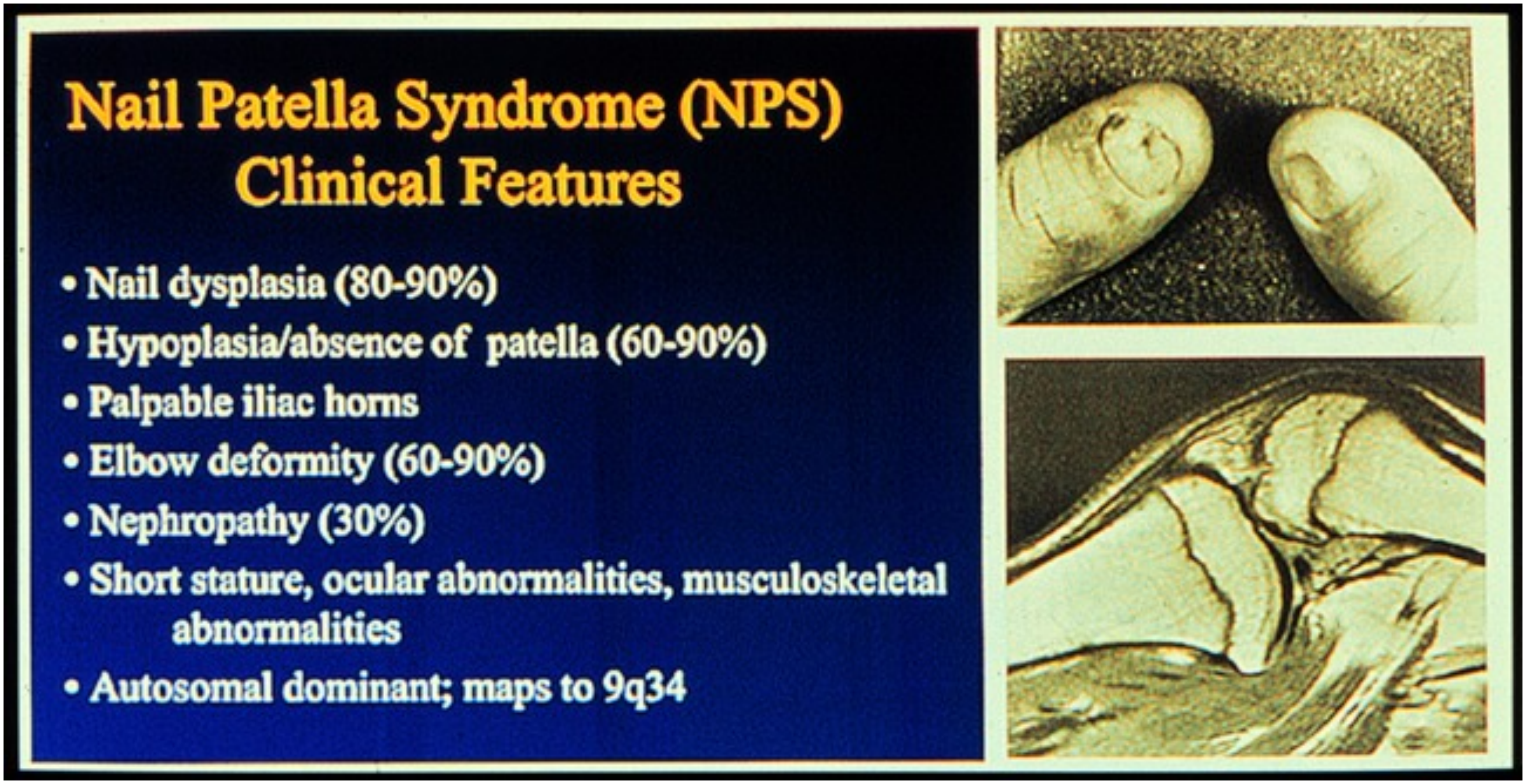
# Genetic deletion of Lmx1b in the mouse leads to a loss of dorsal limb pattern



# A cascade of factors in the ectoderm controls dorsal-ventral polarity in the mesoderm

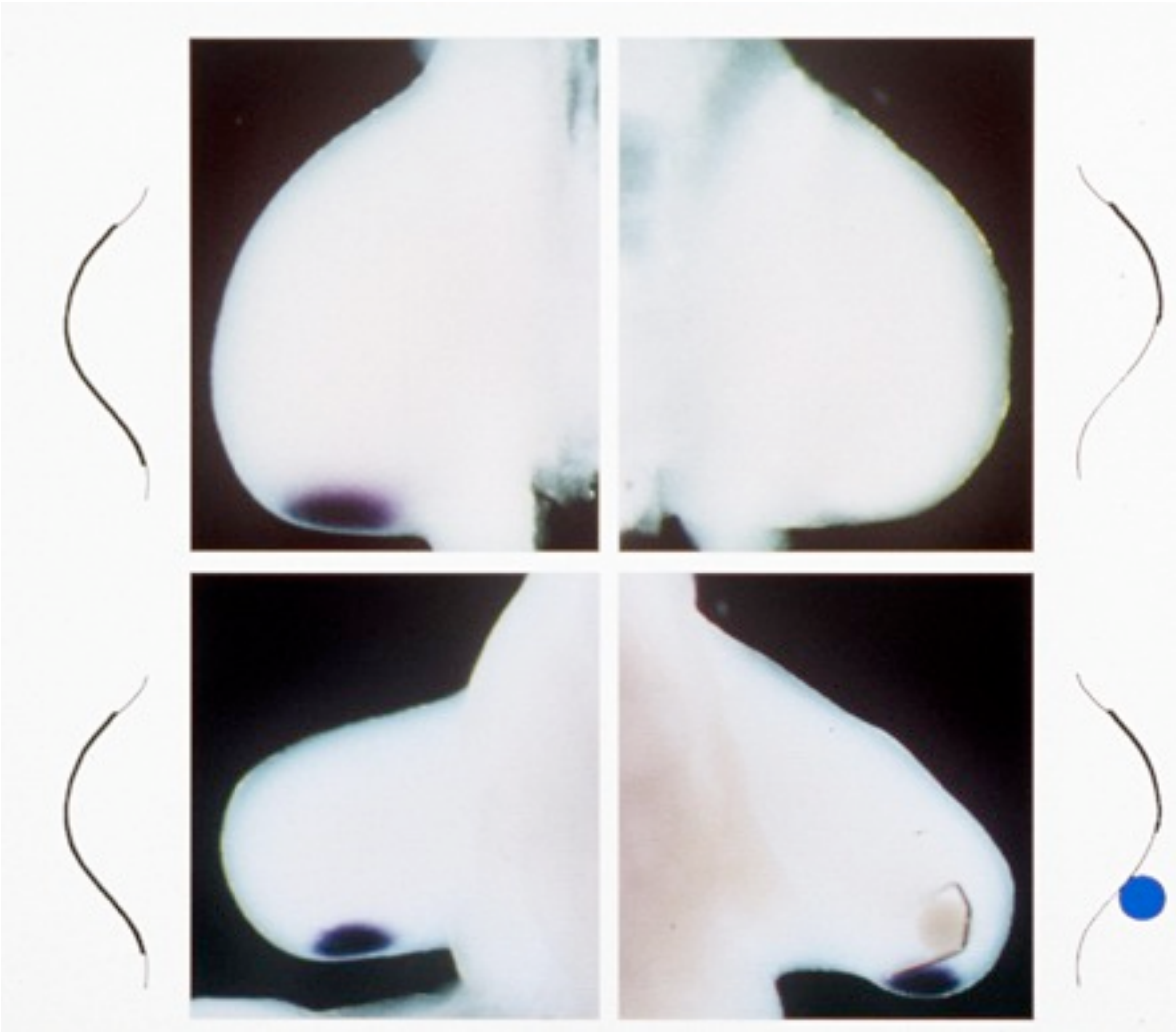


# Human LMX1B mutation:  Nail Patella Syndrome

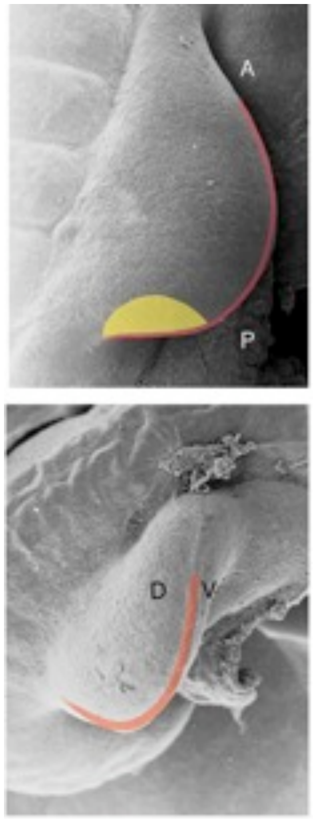


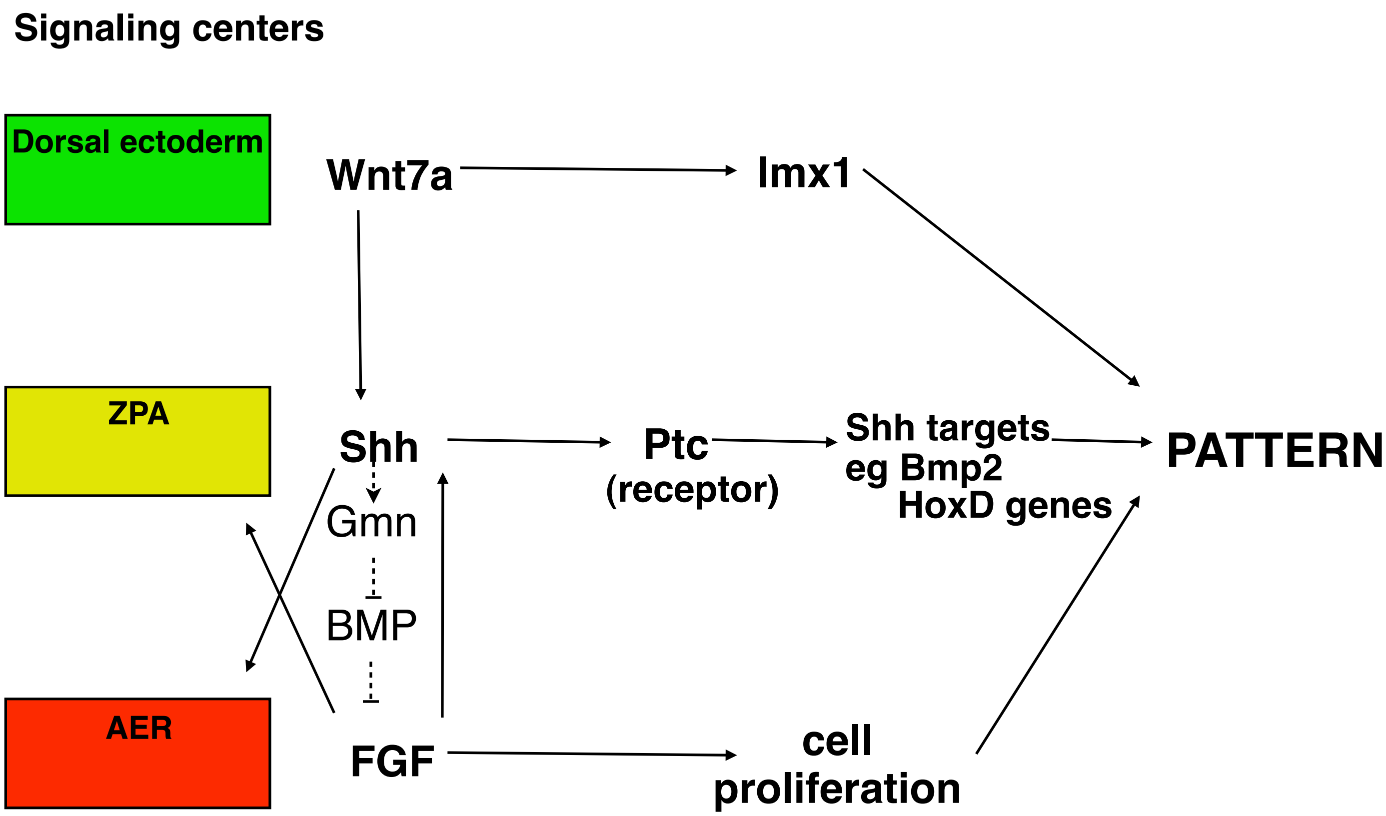

NPS; OMIM 161200

# Signaling center crosstalk:
# Signals from the AER (FGFs) maintain *Shh* expression

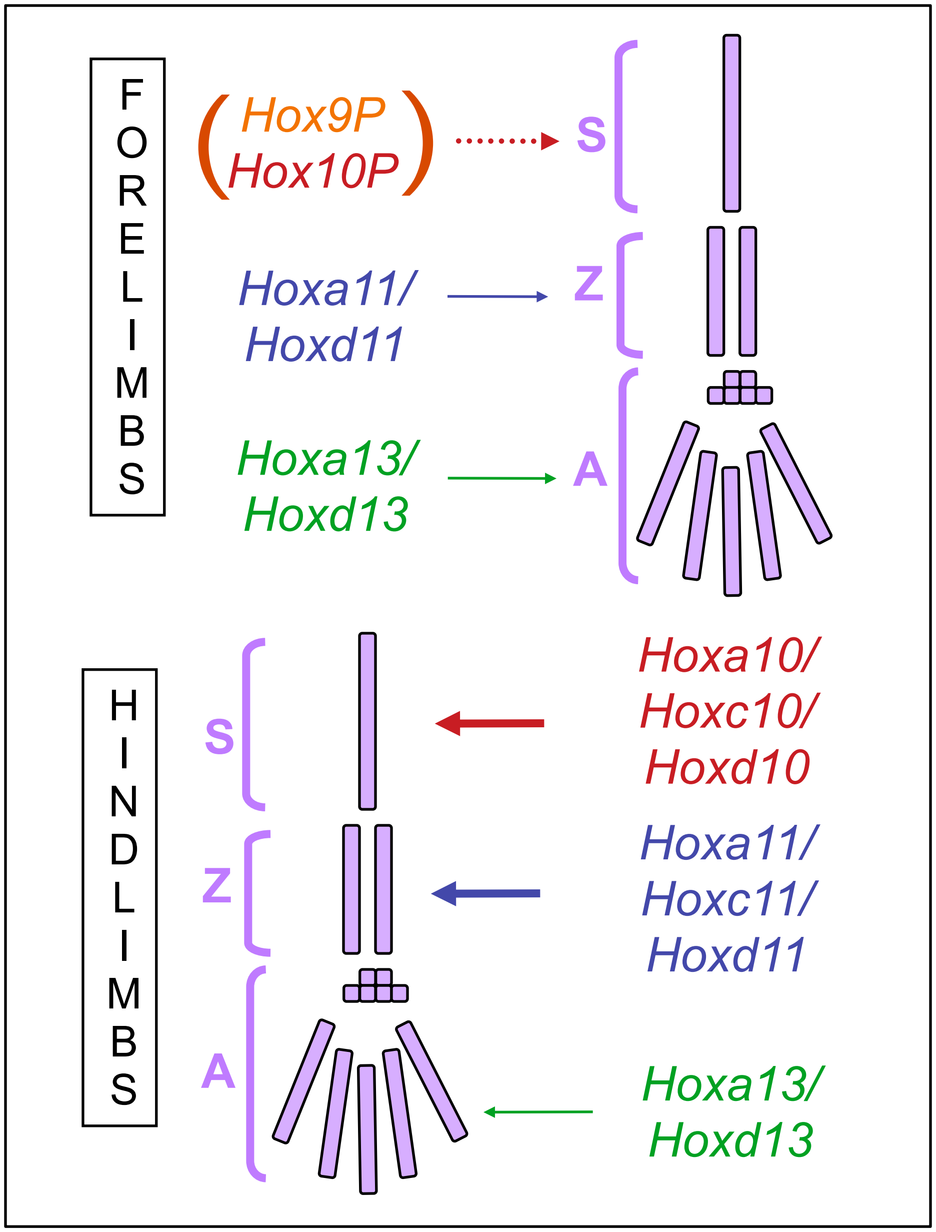


# Limb patterning occurs through the coordination of signals from three signaling centers



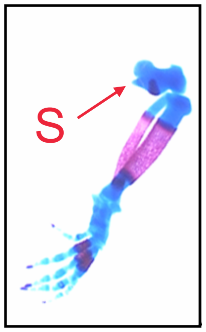



# Patterning of the limb elements: *Hox9* through *Hox13* paralogous groups are responsible for establishing morphological pattern




**Hox10**

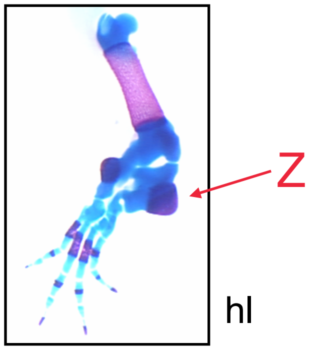
Wellik, *et al*, 2003




hl

**Hox11**

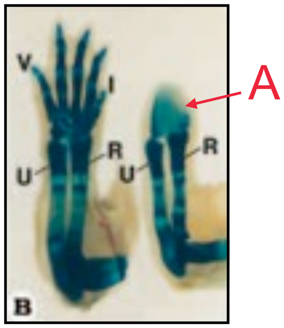
fl


hl

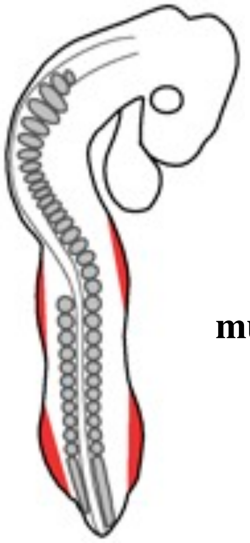
**Hox13**

fl & hl

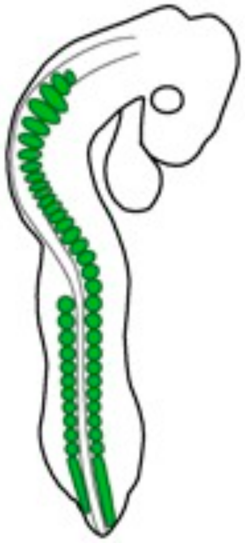



Fromental-Ramain, *et al*, 1996

# Development of the Limb Musculoskeleton



**lateral plate:**
**muscle connective tissue, tendons, ligaments, cartilage, bones**



**somites:**
**muscles**



The limb musculoskeleton derives from 2 developmental sources:

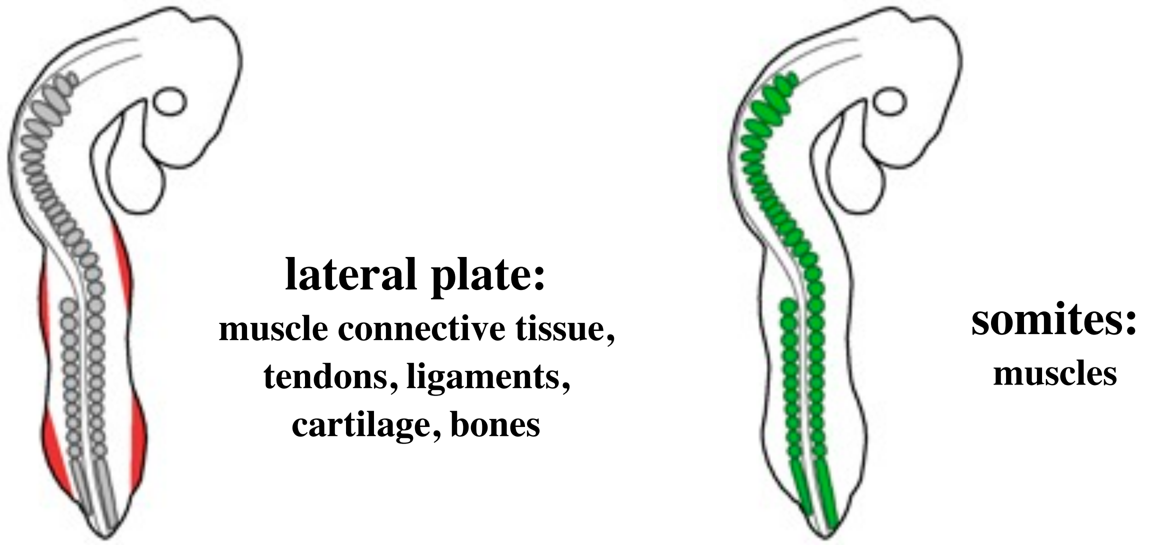
LATERAL PLATE (in red):

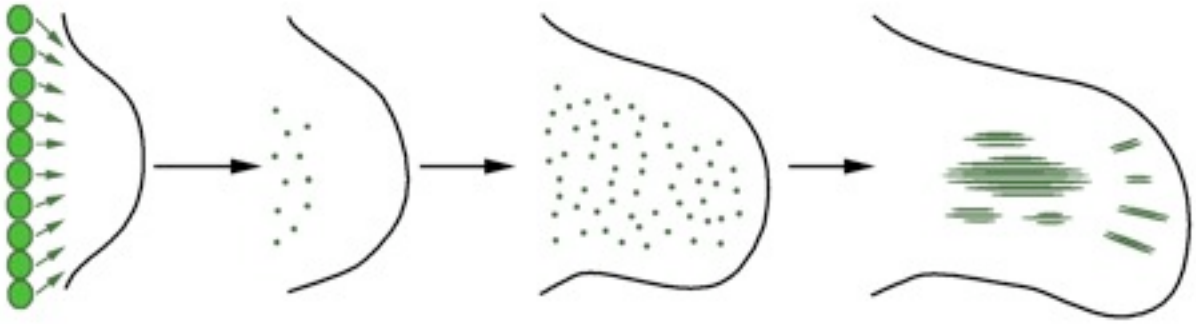
1. Proliferates and expands to produce the limb buds.
2. Cells differentiate to form the muscle connective tissue, tendons, ligaments, cartilage, and bones.

SOMITES (in green)

1. Segmental structures on either side of the neural tube.
2. Somitic cells migrate from the somites into the limb bud, and then in particular regions of the limb these cells become committed to a muscle cell fate and differentiate into myofibers. These regions of differentiated myofibers will form the nucleus for future anatomical muscles.

# Development of the Limb Musculoskeleton





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# Model of Muscle Patterning in the Limb



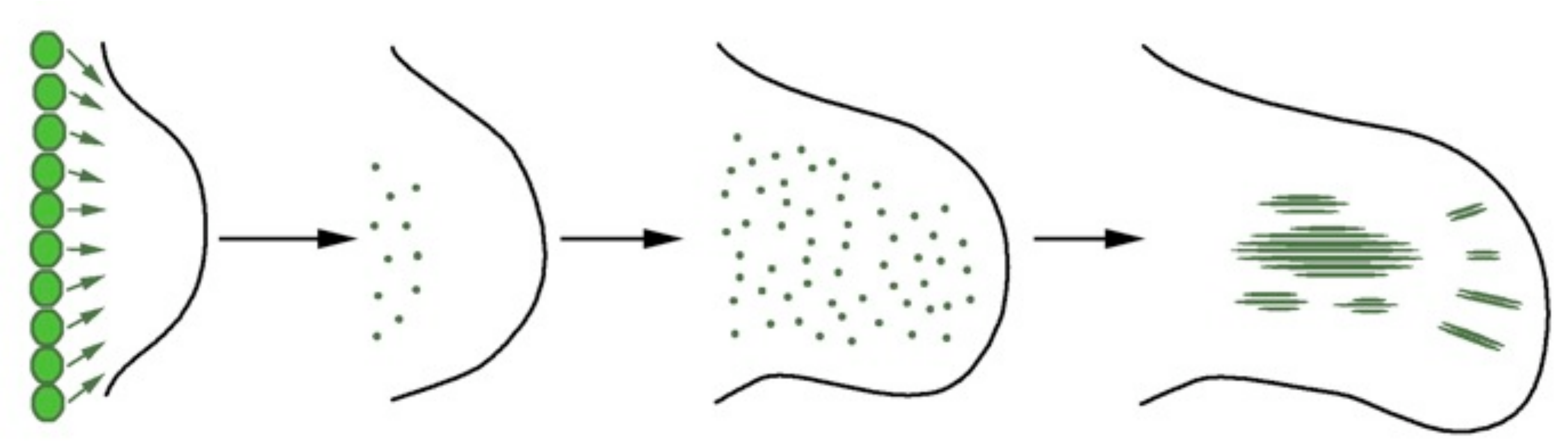
Model: Somitc cells migrate into lateral plate. Tcf4 is expressed in localized regions, forming a muscle prepattern. Migrating somitic cells differentiate in lateral plate regions where Tcf4 is expressed. Thus expression pattern of Tcf4 determines the pattern of muscles in the limb.

Several unanswered questions:

1. What determines the spatial expression of Tcf4 in the limb. If this model is correct, then understanding what controls Tcf4 expression is critical for understanding how muscle is patterned.
2. How are Tcf4+ cells communicating to muscle? Tcf4 is a transcription factor expressed within the fibroblasts. What are the downstream targets of Tcf4? Are any of these secreted molecules that may be signaling to to muscle? If so, what information are they communicating to the muscle? We know that the myofibers preferentially differentiate in close proximity to the Tcf4+ cells. Is this because the Tcf4+ provide signals necessary for commitment and/or differentiation of muscles cells? Cell survival signal?

# Model of Muscle Patterning in the Limb

**migration of myogenic cells into limb** → **muscle pattern** **formation of anatomical muscles**





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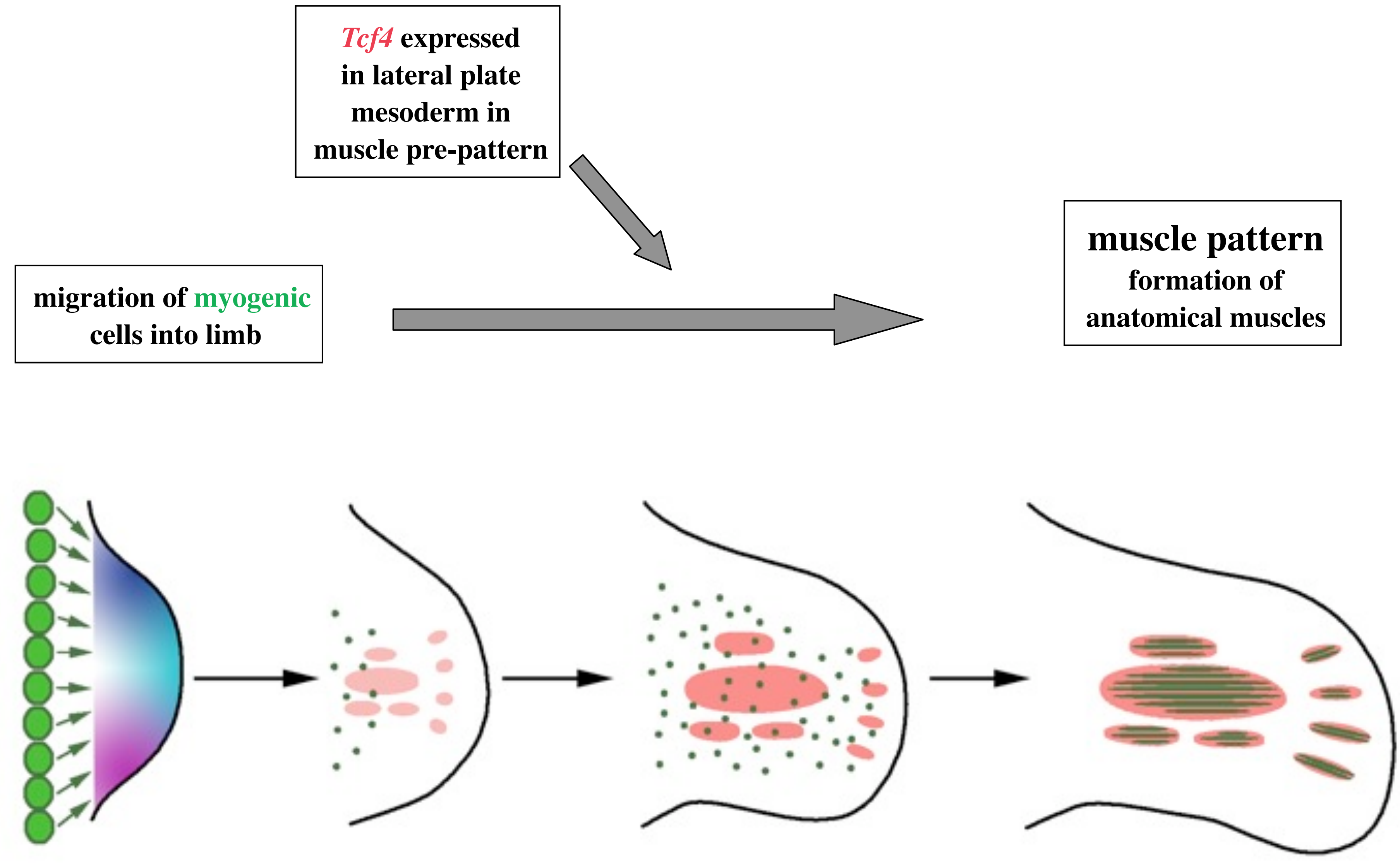




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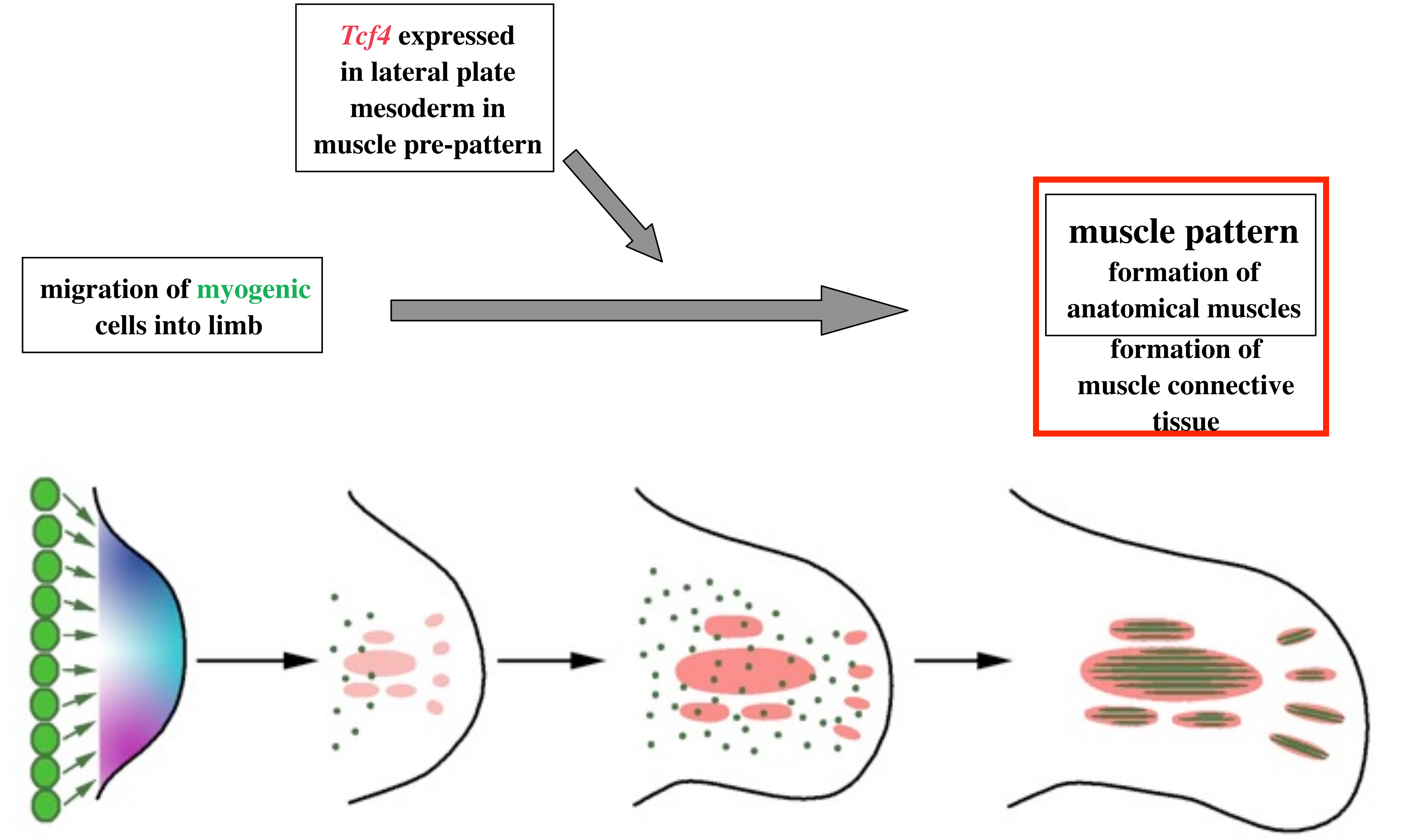




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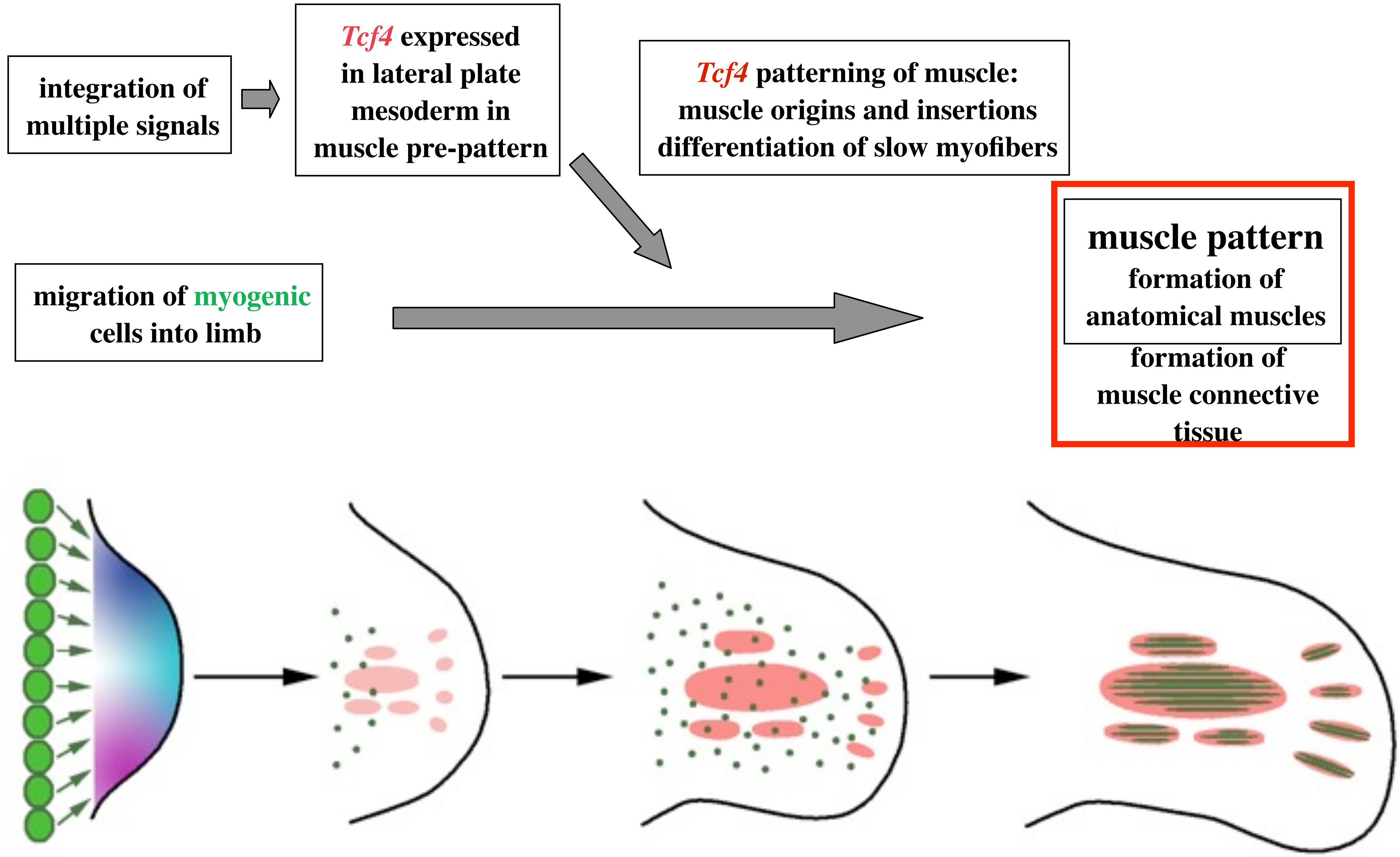


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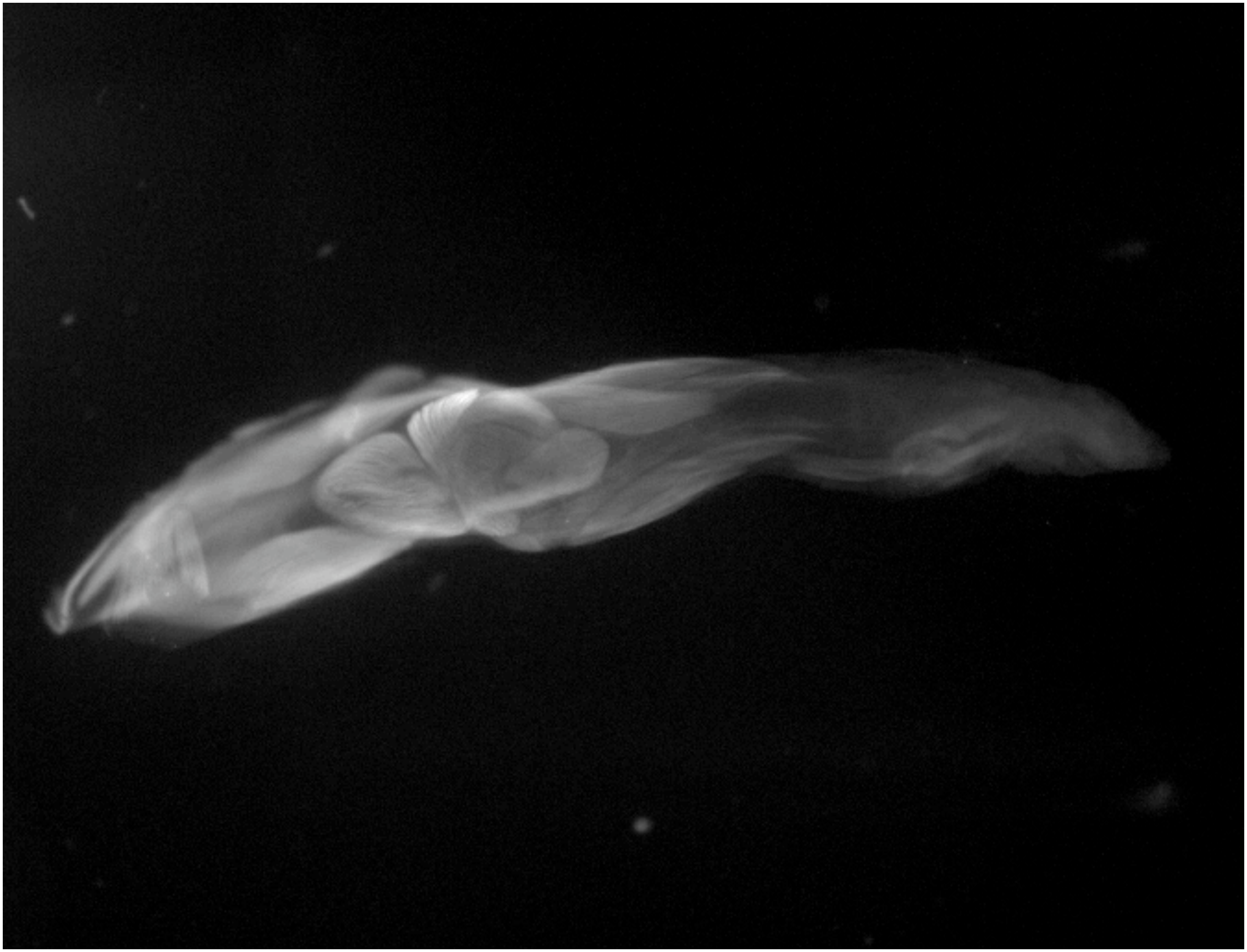
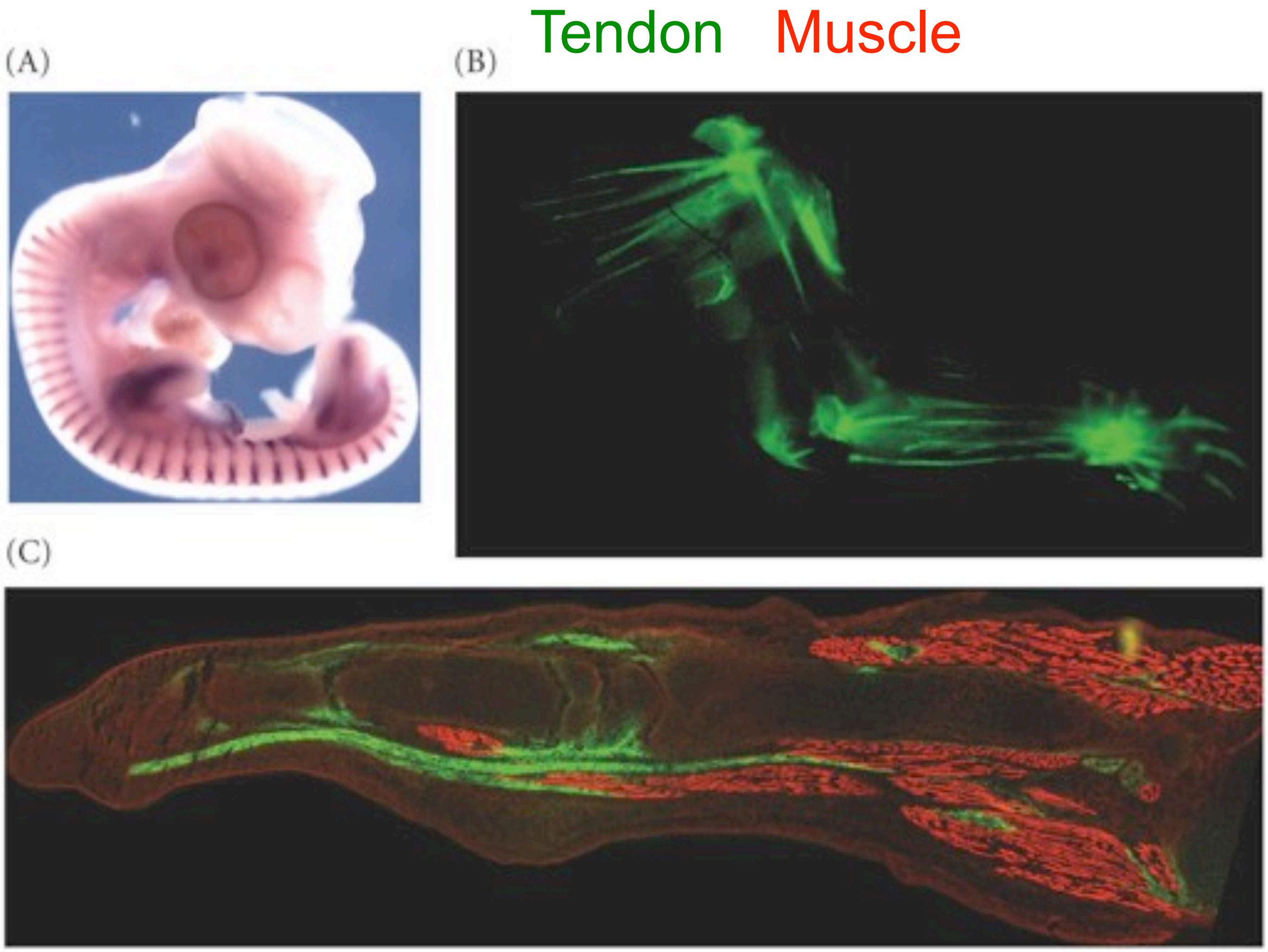
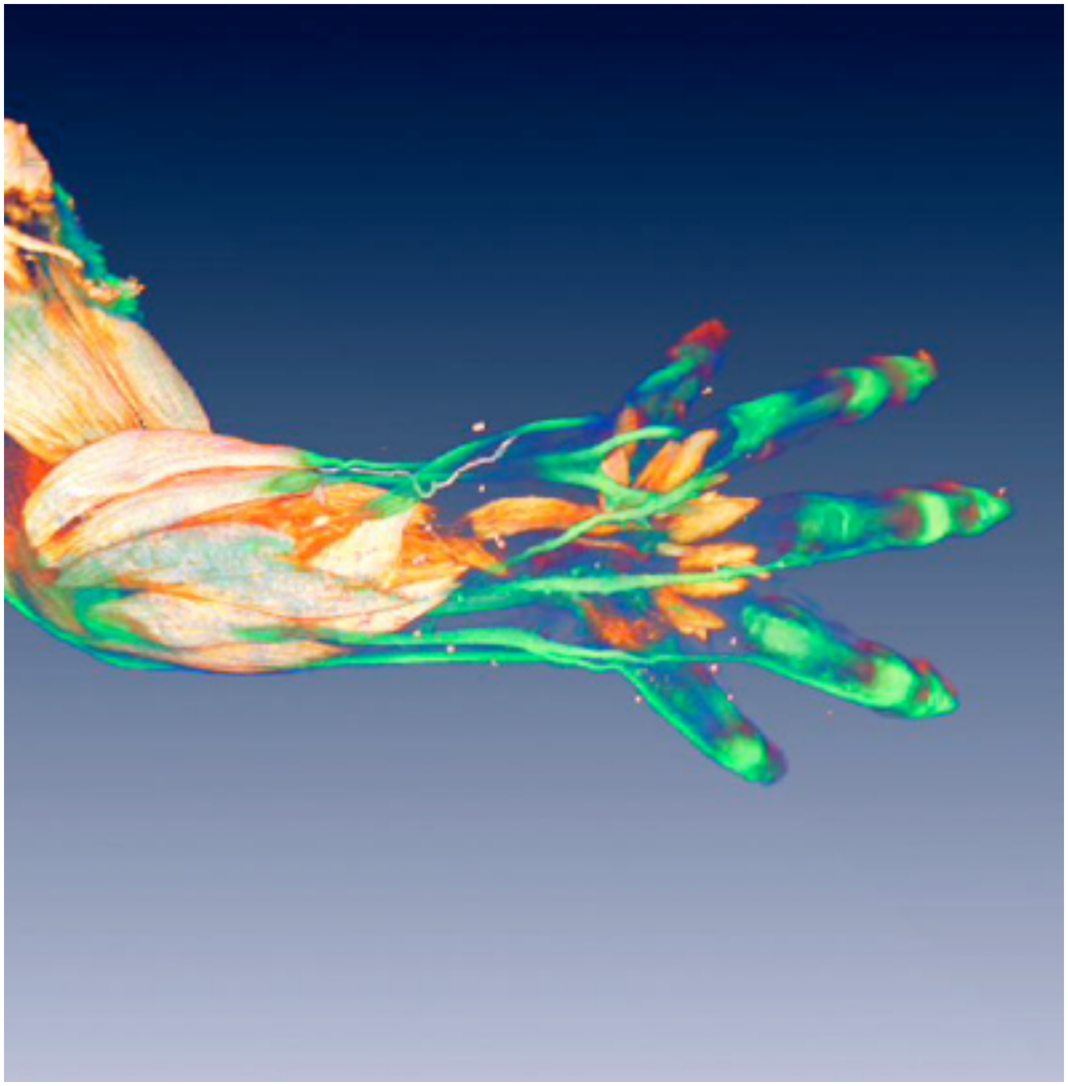


# Tendons develop in situ, attach to muscle as it develops



Integration of pattern and differentiation results in limb muscle-tendon connectivity



# Summary

- Initiation: localized FGF activity
- Outgrowth: FGFs produced by the AER
- Anterior-posterior patterning: ZPA/Sonic hedgehog
- Dorsal-ventral patterning: engrailed-wnt7a-lmx1b
- Integration of signaling centers
- Tbx genes are required for limb outgrowth
- Hox genes are essential regulators of limb development and control segmental development
- Myogenic precursors migrate into the limb, and follow prepattern laid down by connective tissue fibroblasts. Tendons develop in situ