The Timing of Bone Formation in Frog Skulls

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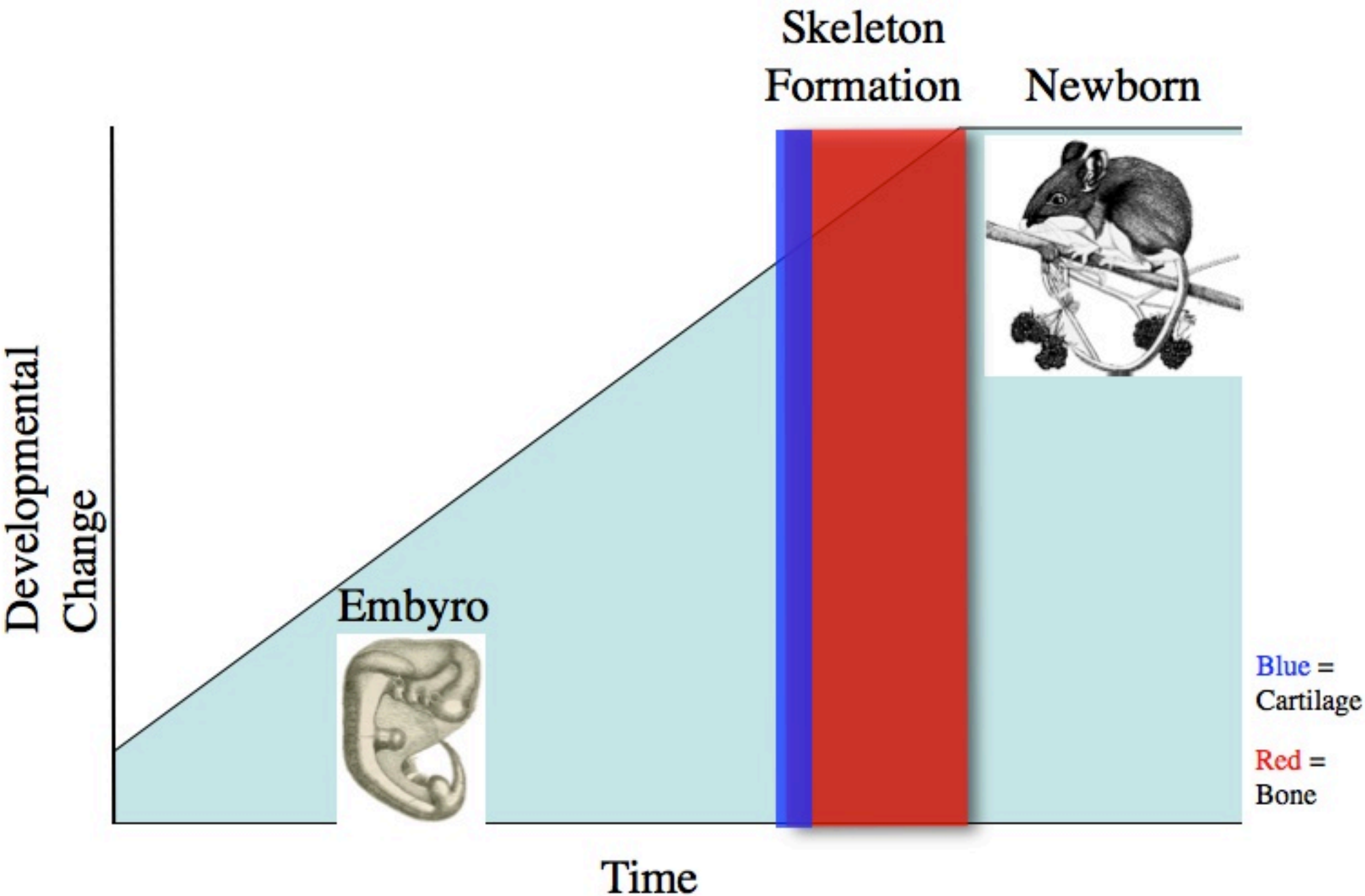
Outline

- Comparative studies on the order of bone formation in frog skulls.
- The role of thyroid hormones in frog skull metamorphosis.
- The development of transgene reporters to track skeletogenic cell populations.
- New research avenues to investigate the differentiation of the skull.

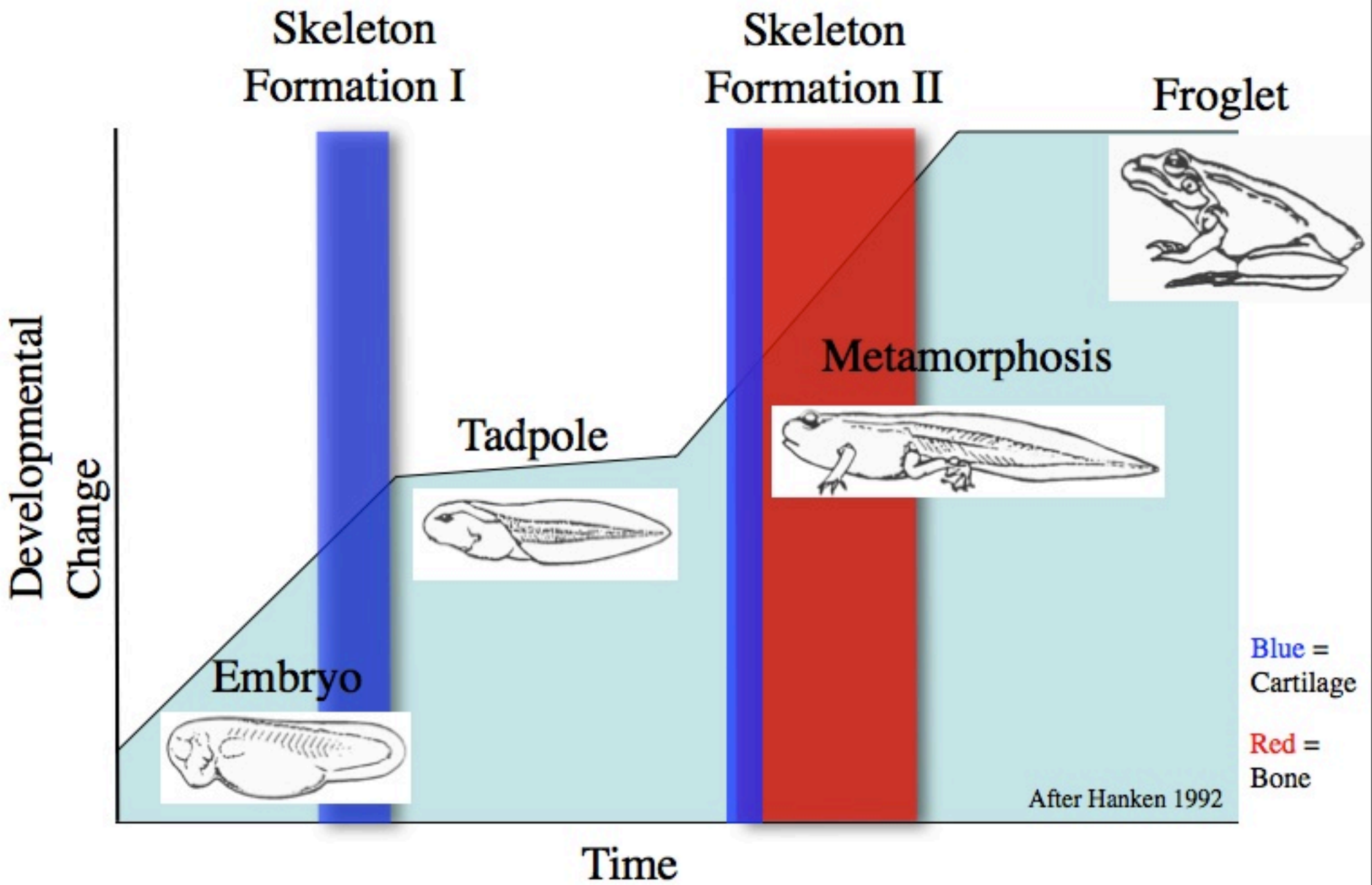
.....of Mice and Frogs

Photo: Pawan Kumar

Mouse Skeletal Development

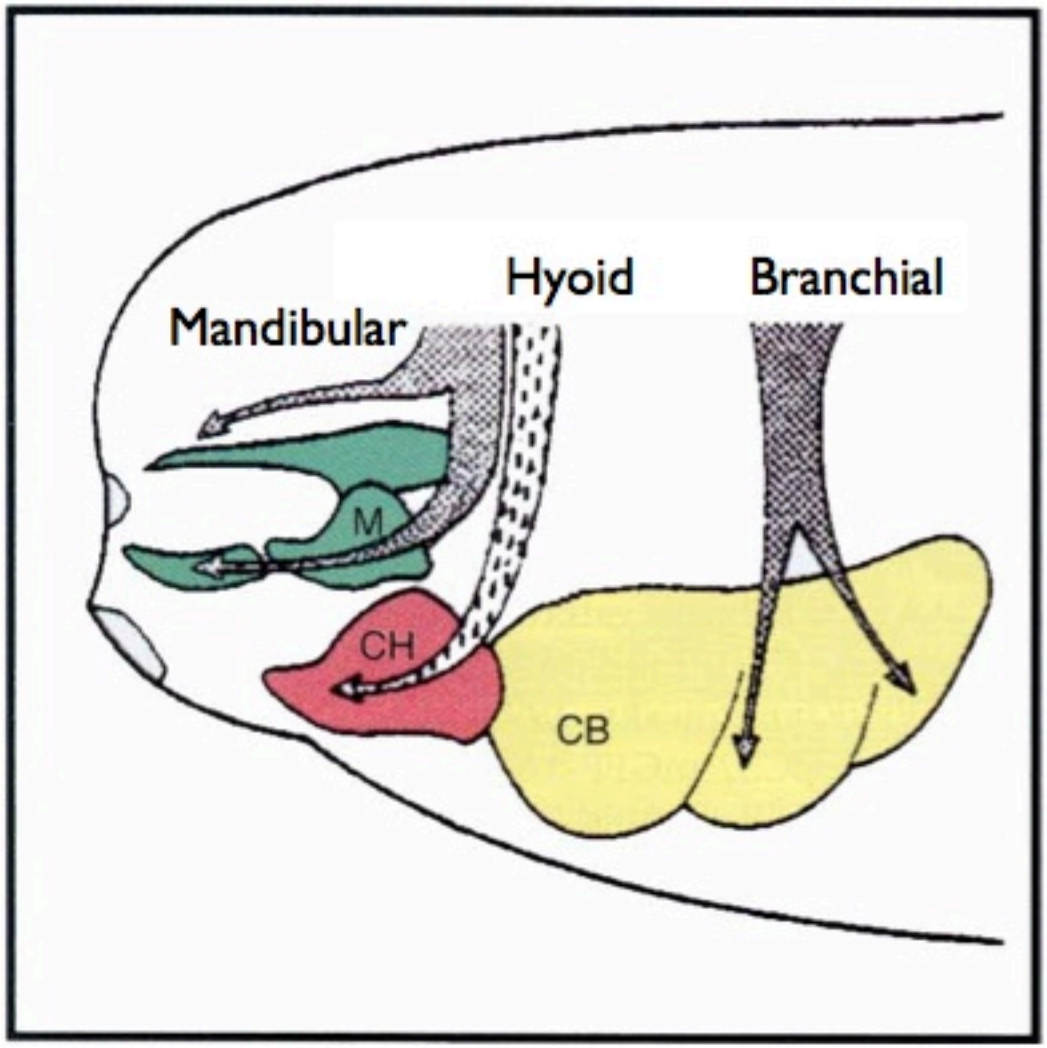


Metamorphosing Frog Skeletal Development



Introduction: Frog "Skeleton Formation I"

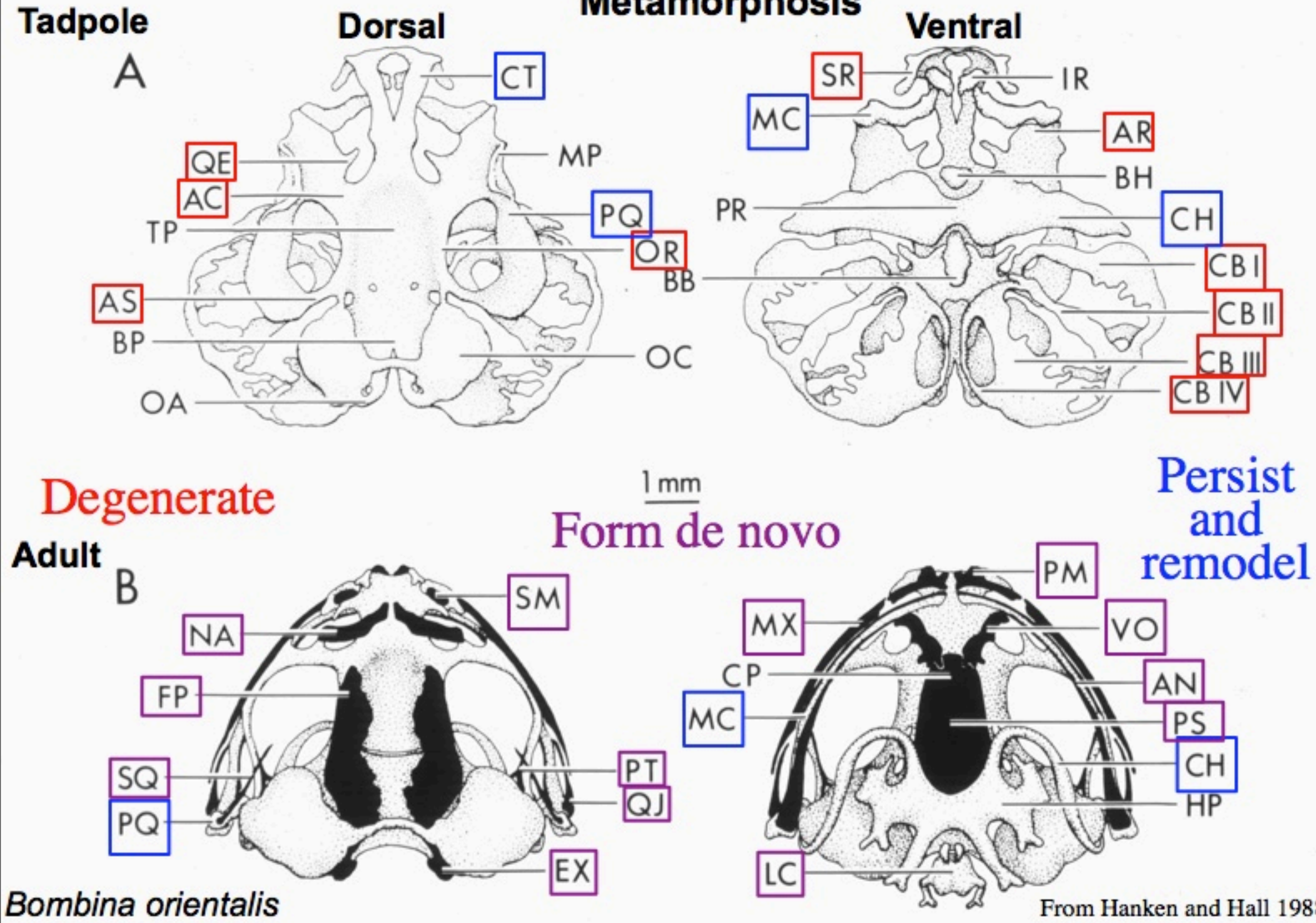
Cranial Neural Crest



Tadpole Skull

From Sadagihani
and
Thièbaud, 1987

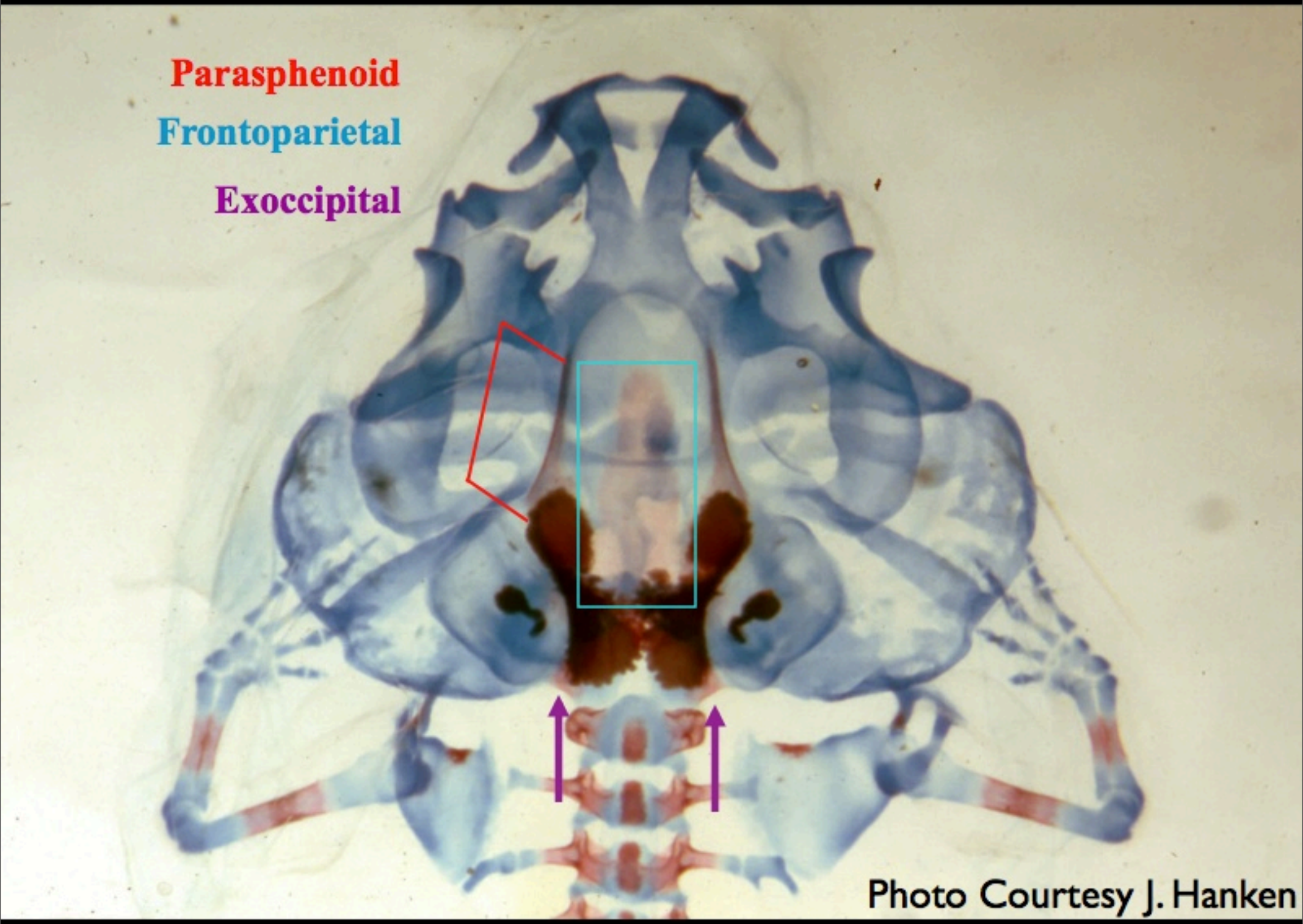
"Skeleton Formation II"
Metamorphosis



From Hanken and Hall 1988

Introduction: Frog "Skeleton Formation II" Metamorphosis

- http://www.youtube.com/watch?v=D5JZ0tYFOoc


Parasphenoid
Frontoparietal
Exoccipital
Photo Courtesy J. Hanken

Timing of differentiation associated with metamorphosis

TABLE 2. Comparison of cranial ossification sequences

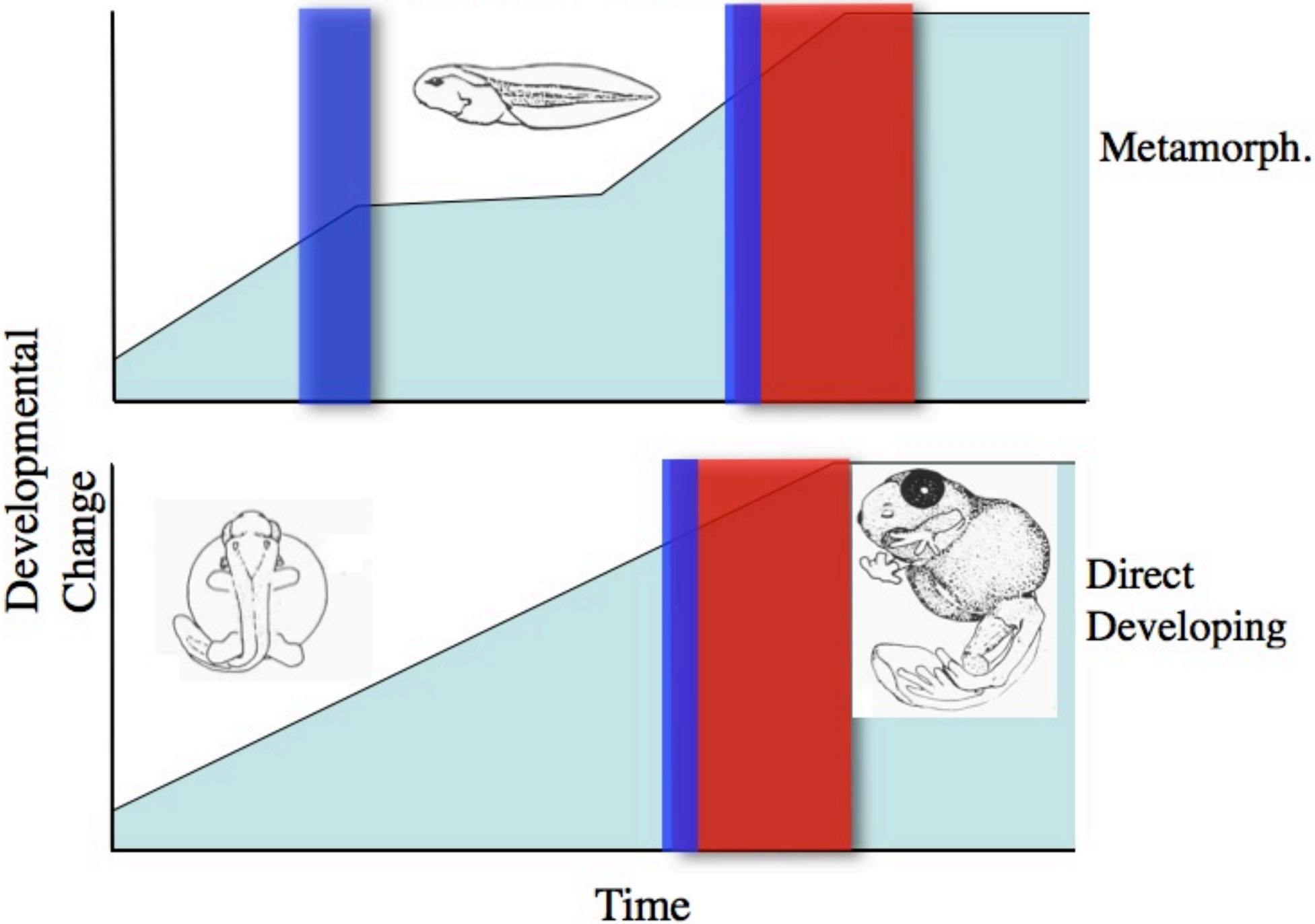
Bombina orientalis	*Discoglossus sardus*	*Spea bombifrons*	*Rhinophrynus dorsalis*	*Xenopus laevis*	*Pipa pipa*
[*Parasphenoid*	Parasphenoid	[Parasphenoid	Parasphenoid	Frontoparietal	[Parasphenoid
Frontoparietal	Frontoparietal	Exoccipital	Exoccipital	Parasphenoid	Frontoparietal]
Exoccipital]	Exoccipital	Frontoparietal]	Frontoparietal	Exoccipital	[Maxilla
Septomaxilla	[Premaxilla	Prootic	Maxilla	Prootic	Angulosplenial]
[Premaxilla	Septomaxilla	[Premaxilla	Prootic	Angulosplenial	[Exoccipital
Nasal	Nasal]	Nasal]	Nasal	Maxilla	Prootic
Vomer]	Maxilla	[Maxilla	[Premaxilla	[Premaxilla	Premaxilla
Maxilla	Vomer	Septomaxilla]	Sphenethmoid]	Nasal]	Dentary
METAMORPHOSIS	[Angulosplenial	[Angulosplenial	Angulosplenial	[Septomaxilla	Nasal]
[Angulosplenial	Dentary	Vomer	[Dentary	Dentary]	[Septomaxilla
Squamosal	Squamosal	Dentary	Quadratojugal]	[Pterygoid	Pterygoid]
Dentary]	Quadratojugal	Squamosal]	[Septomaxilla	Squamosal]	**METAMORPHOSIS**
[Quadratojugal	Teeth]	Pterygoid	Squamosal	Vomer	Sphenethmoid
Pterygoid]	**METAMORPHOSIS**	[Sphenethmoid	Vomer]	Sphenethmoid	[Squamosal
[Sphenethmoid	Sphenethmoid	Mentomeckelian	Pterygoid	**METAMORPHOSIS**	Stapes]
Prootic	Pterygoid	Quadrate]	**METAMORPHOSIS**		Tympanic annulus
Interfrontal	Prootic	**METAMORPHOSIS**			
Mentomeckelian]	Mentomeckelian				

Elements in brackets appear simultaneously. Data were collected from the literature as follows: *Bombina orientalis*, Maglia and Púgener (1998); *Discoglossus sardus*, Púgener and Maglia (1997); *Rhinophrynus dorsalis*, Trueb (1985); *Spea bombifrons*, Wiens (1989); *Xenopus laevis*, Trueb and Hanken (1992).

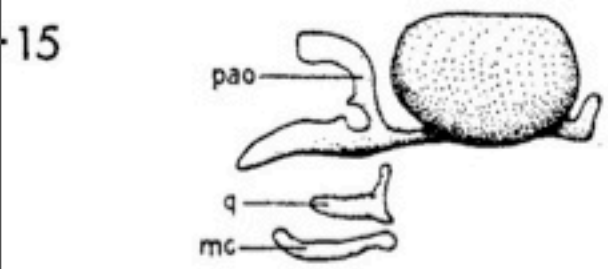
Trueb 1986

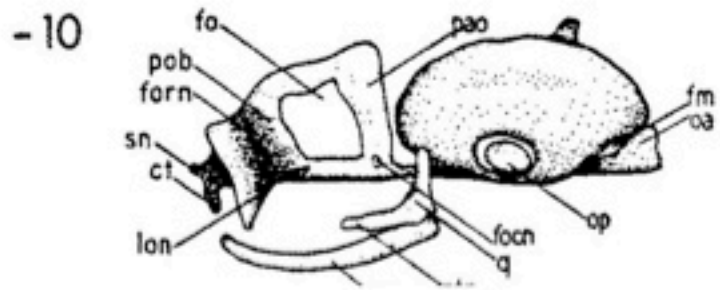
Eleutherodactylus coqui
(Puerto Rico)

Direct development has dramatic implications for the timing of skeletogenesis



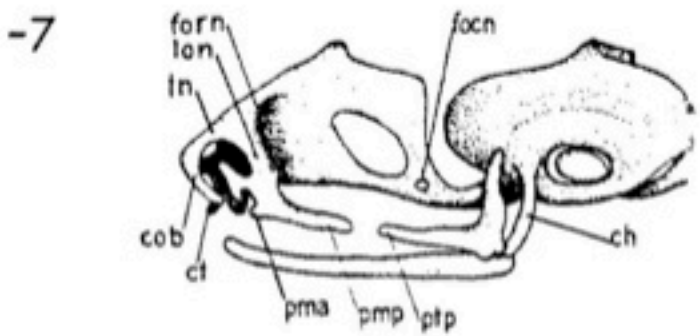
Skull Formation in *Eleutherodactylus*

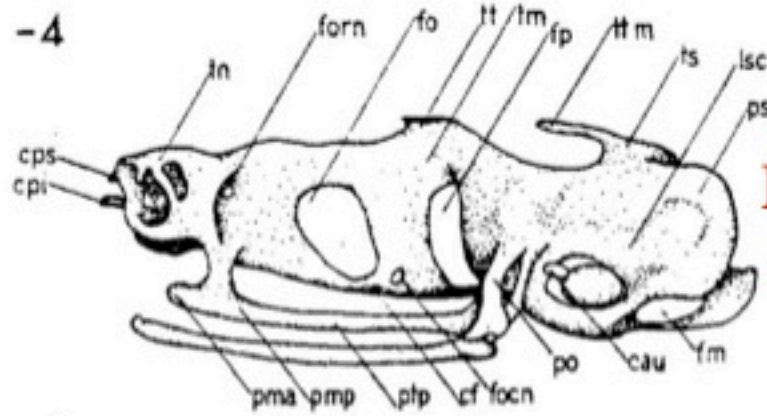




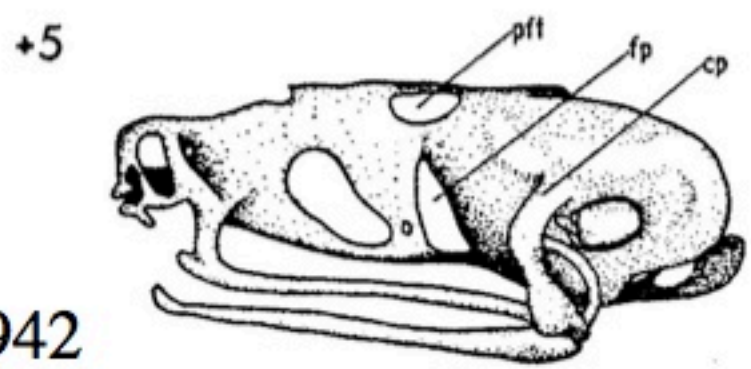
Upper Larval Jaw complete loss

Ceratobranchials complete loss





Meckel's Cartilage mid metamorphic emergence



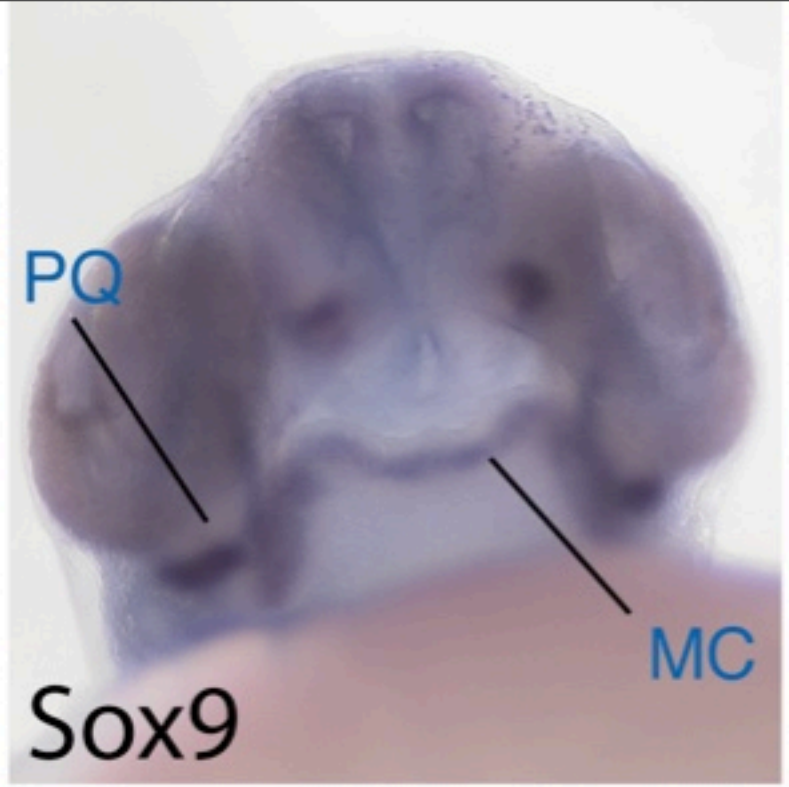
Lynn, 1942

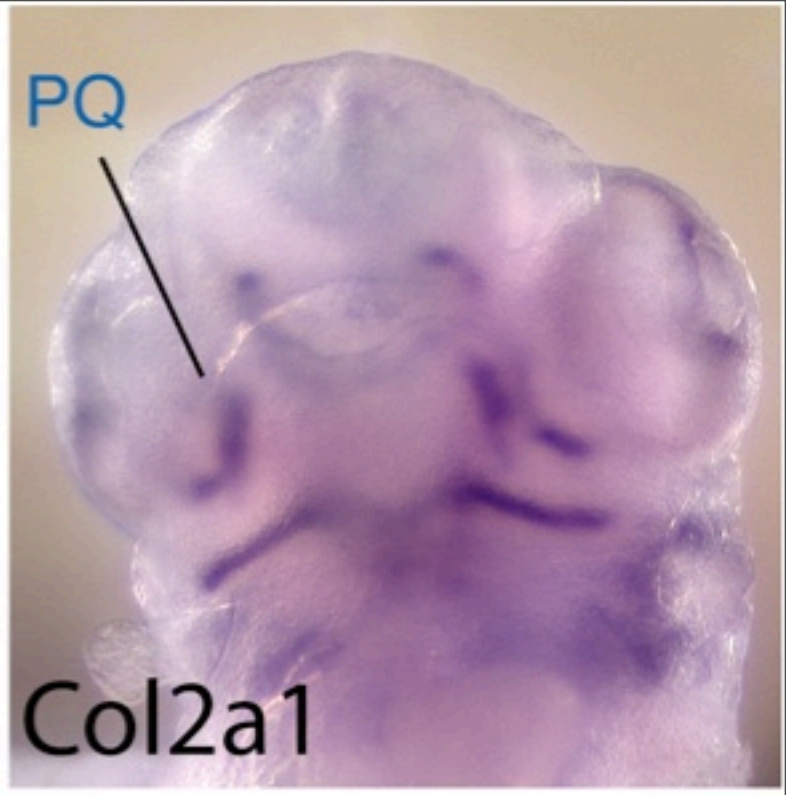
Re-created from Serial Sections *E. nubicola* - direct developing

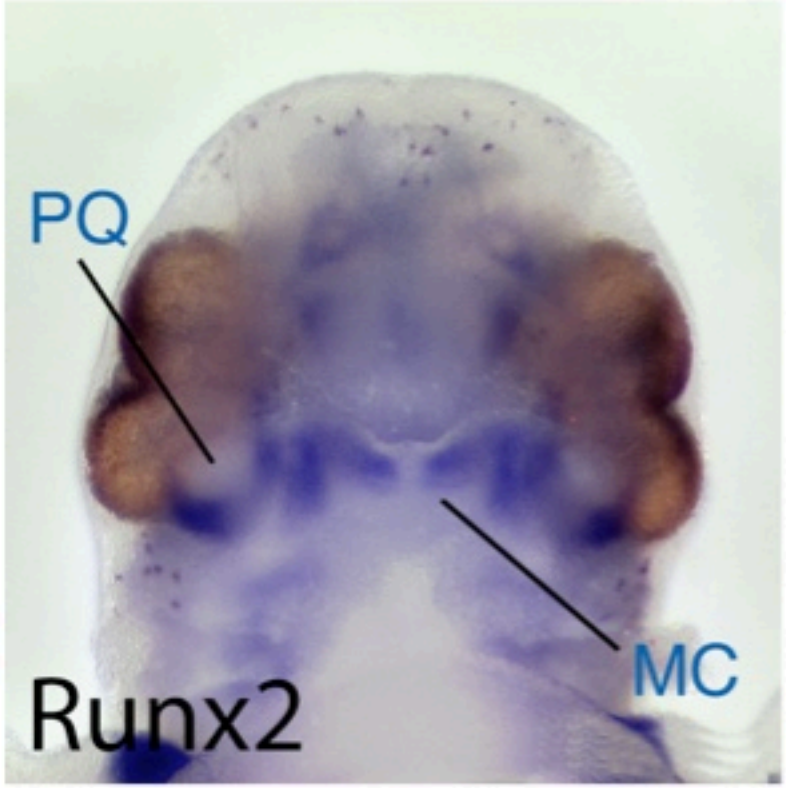
E. coqui
Gene Expression
Head - front view
Stage 7

PQ = Palatoquadrate

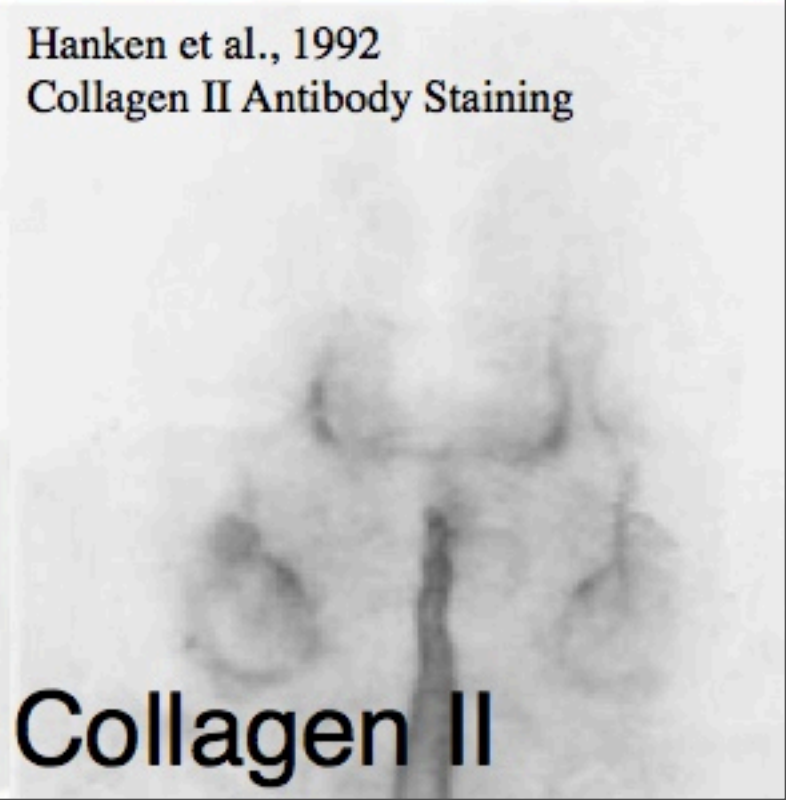
MC = Meckel's Cartilage







Hanken et al., 1992
Collagen II Antibody Staining



Kerney et al. 2010
Evol Dev

E. coqui
Gene Expression
Head - front view
Stage 9

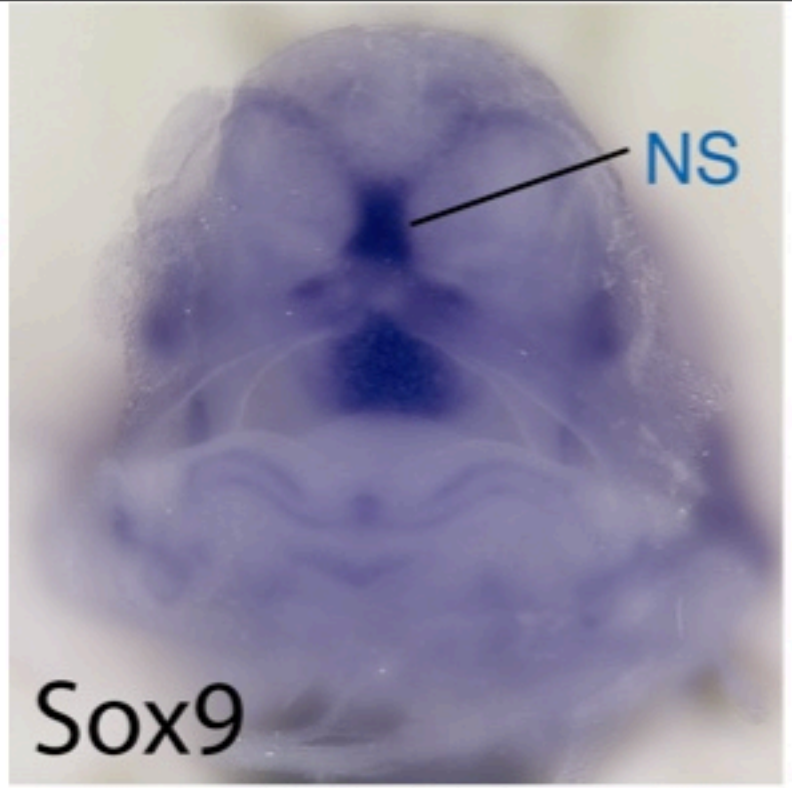
NS = Nasal Septum

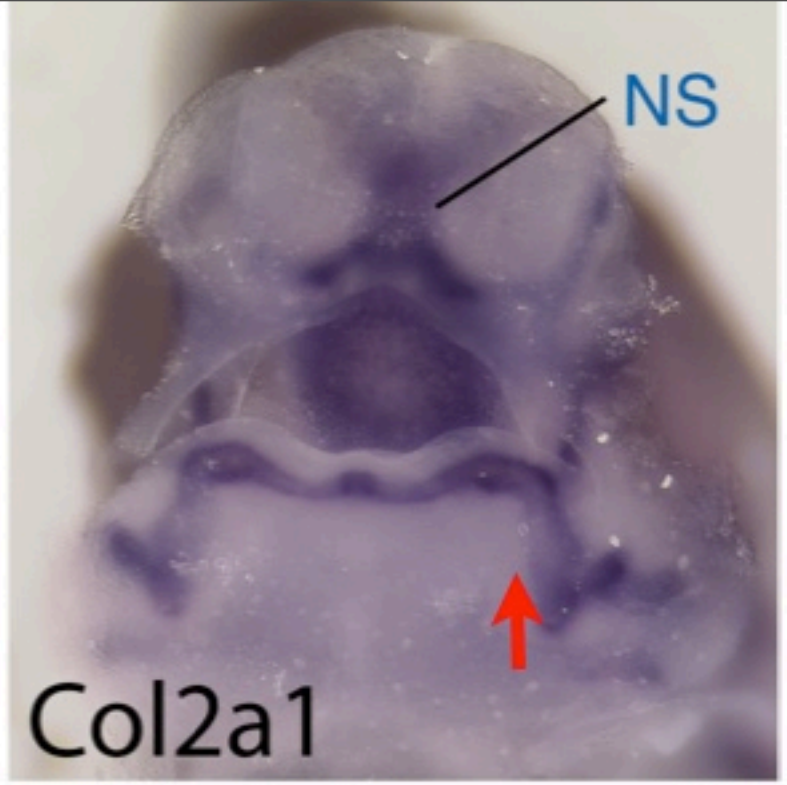
SQ = Squamosal

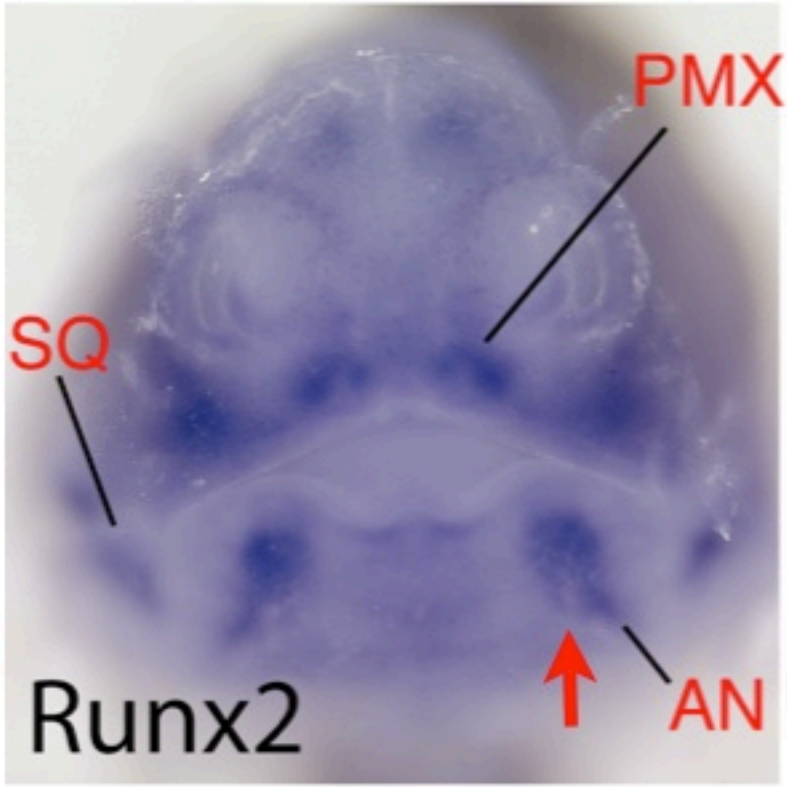
PMX = Premaxilla

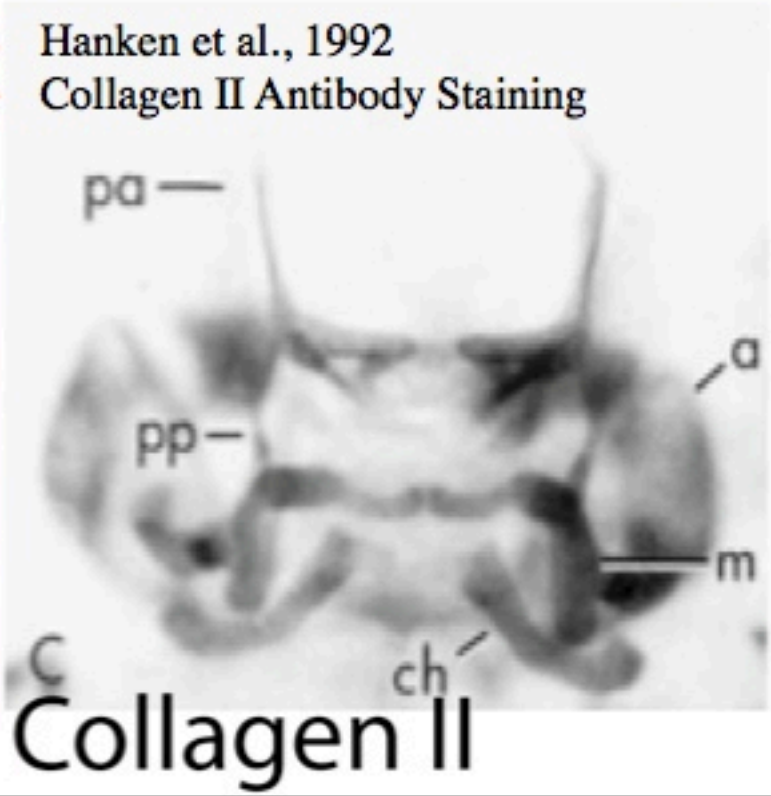
AN = Angulosplenial

Kerney et al. 2010
Evol Dev

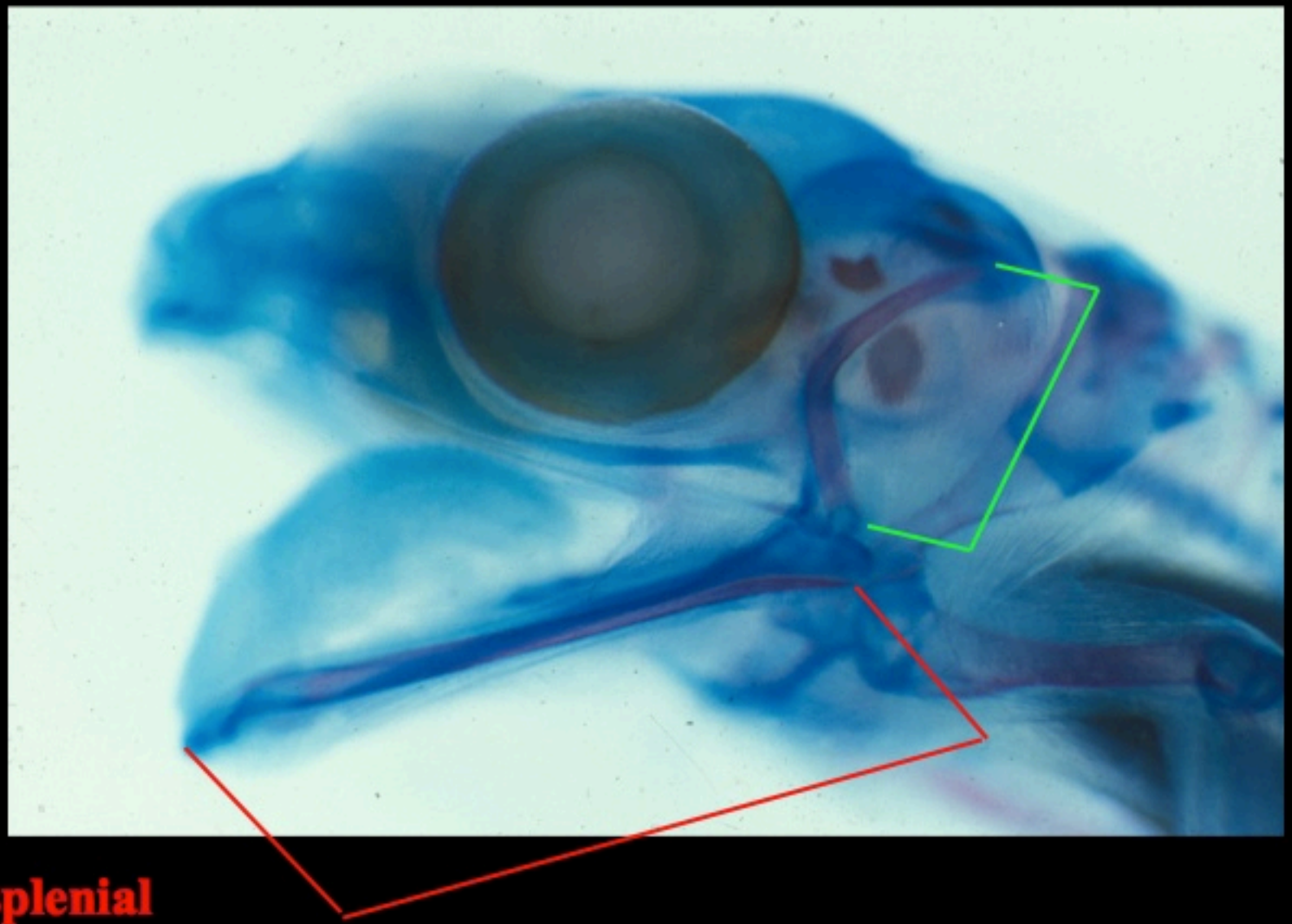






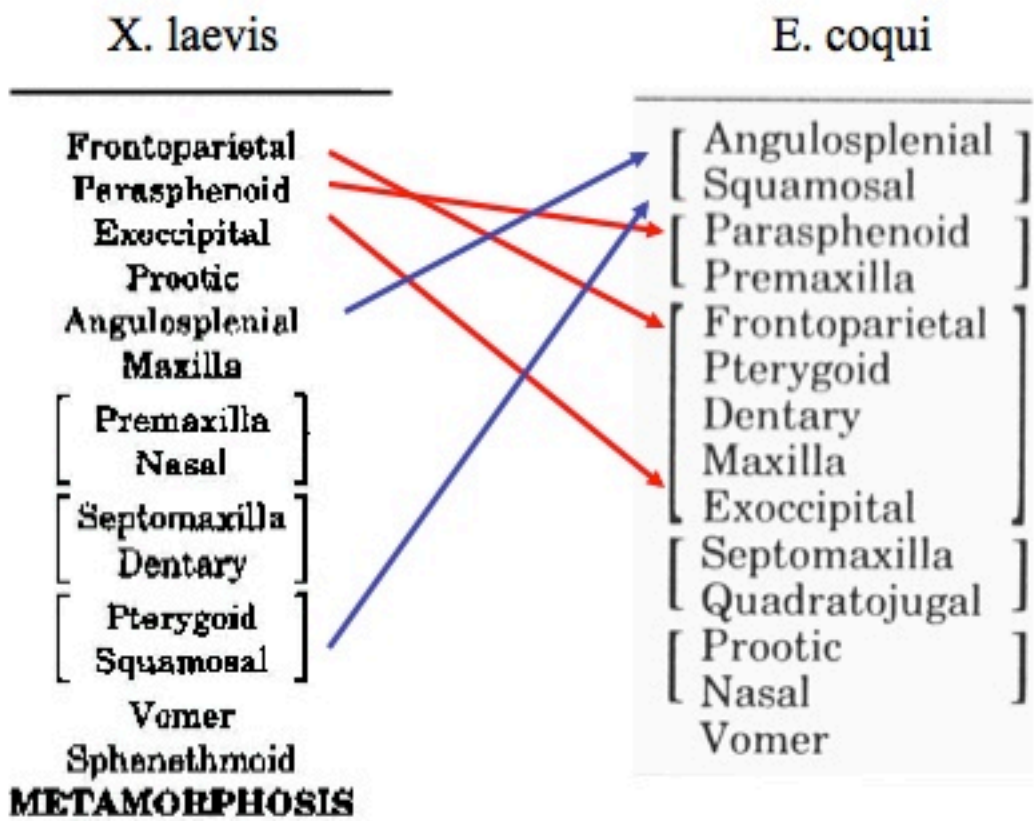


E. coqui
Embryo

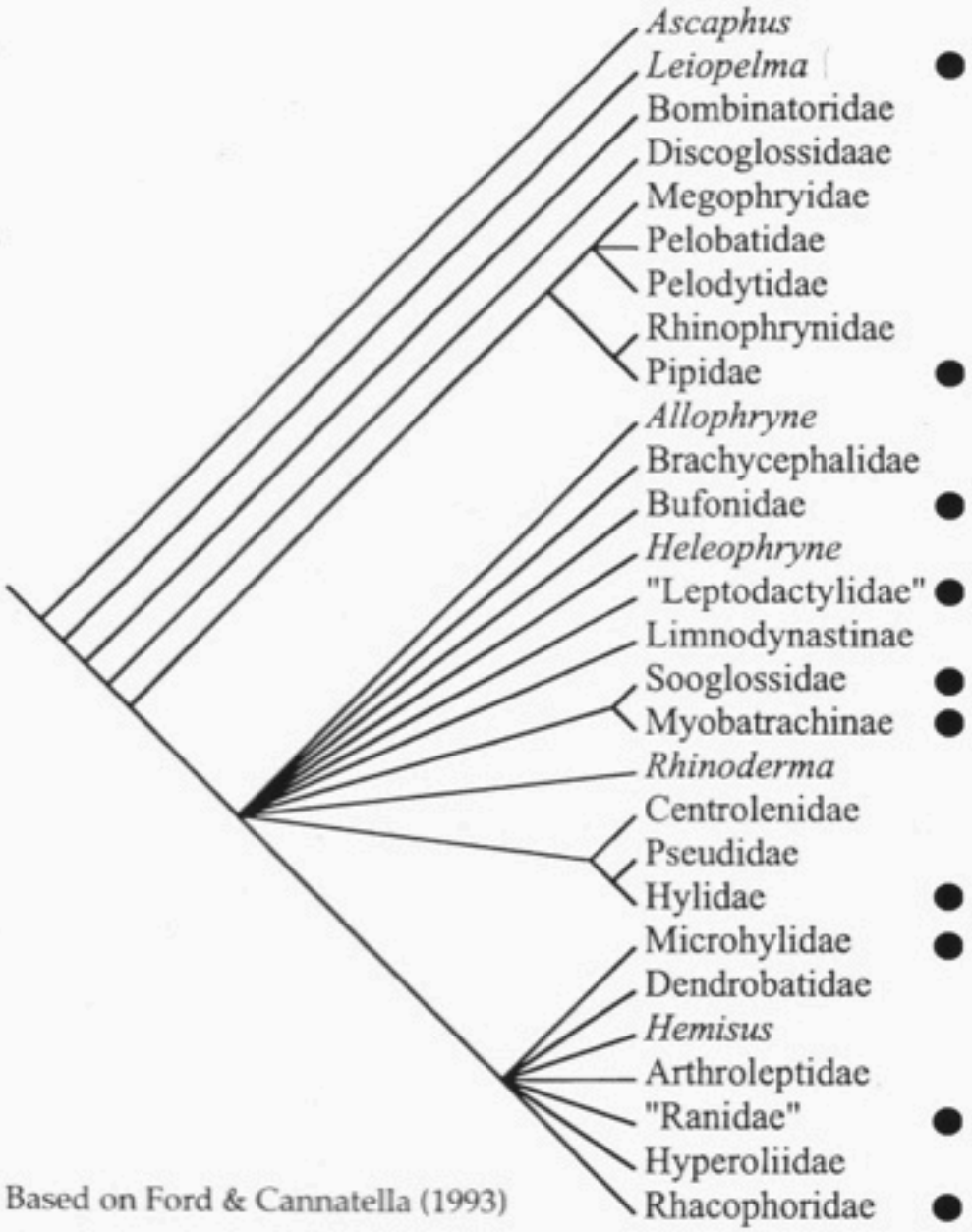


Angulosplenial
Squamosal

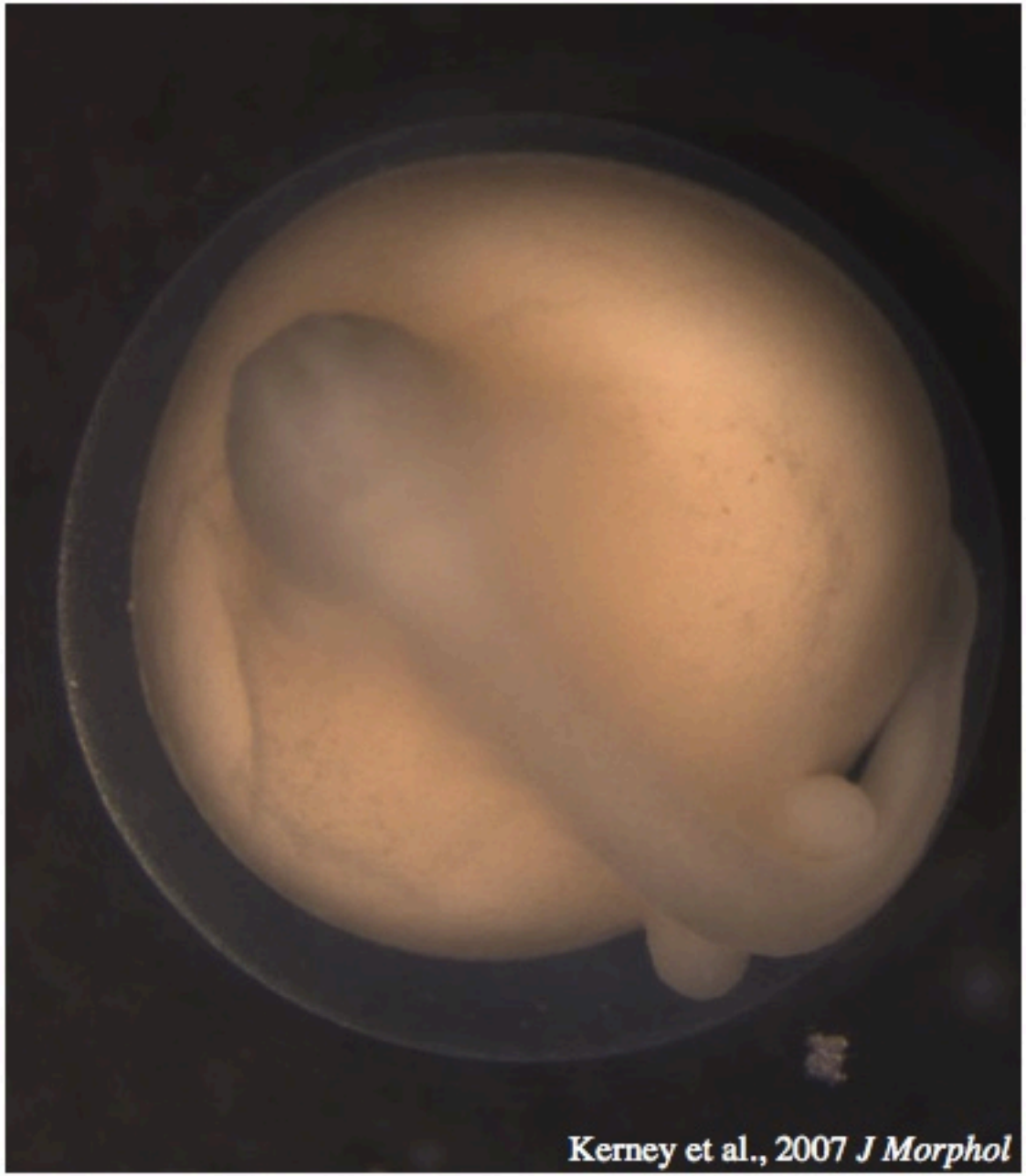
Photo Courtesy J. Hanken


X. laevis
Frontoparietal
Parasphenoid
Exoccipital
Prootic
Angulosplenial
Maxilla
Premaxilla
Nasal
Septomaxilla
Dentary
Pterygoid
Squamosal
Vomer
Sphenethmoid
METAMORPHOSIS
E. coqui
Angulosplenial
Squamosal
Parasphenoid
Premaxilla
Frontoparietal
Pterygoid
Dentary
Maxilla
Exoccipital
Septomaxilla
Quadratojugal
Prootic
Nasal
Vomer

Direct development has >10 independent origins in frogs



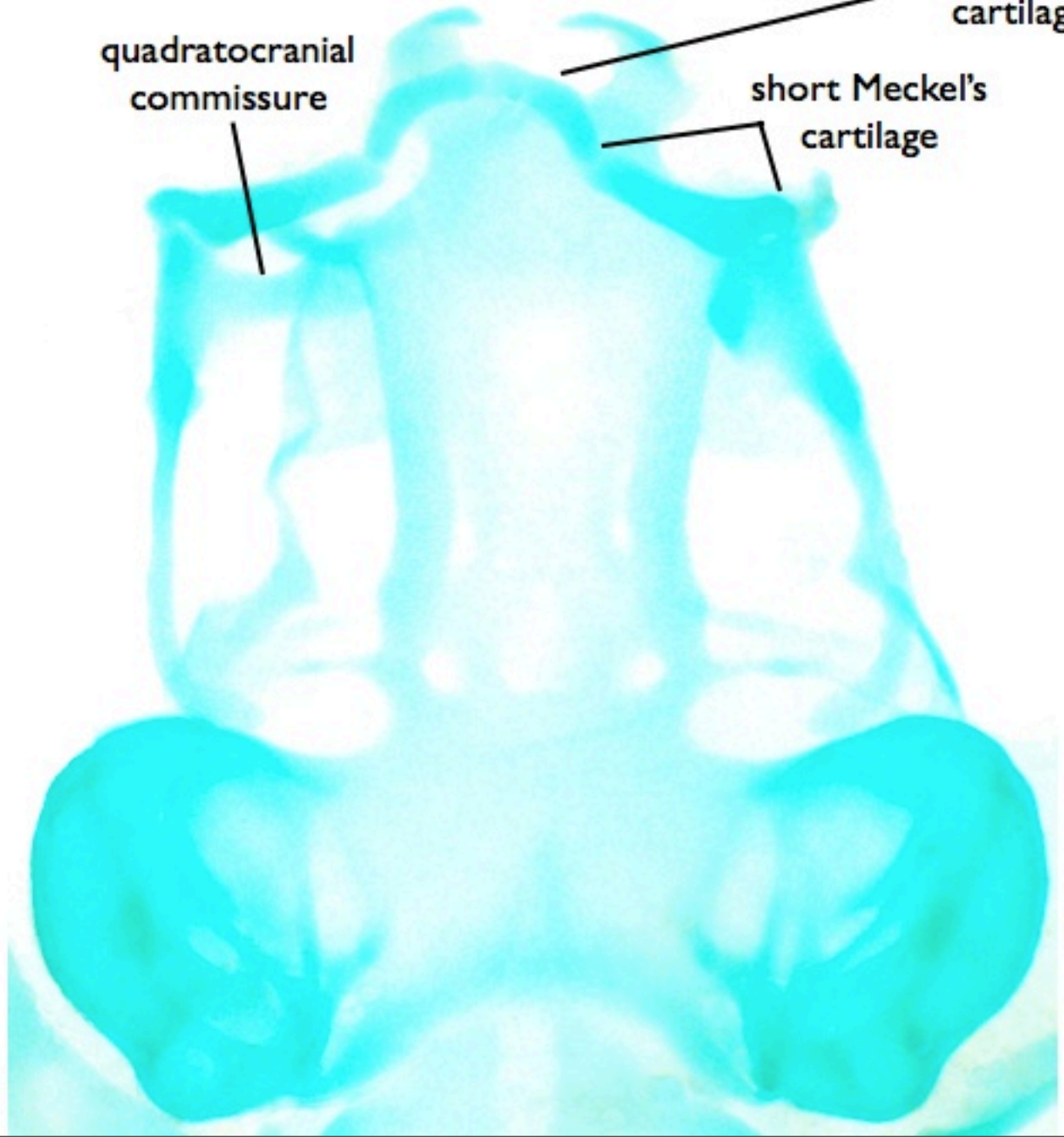
Based on Ford & Cannatella (1993)



Philautus temporalis
Sri Lanka

Eleutherodactylus coqui
Puerto Rico

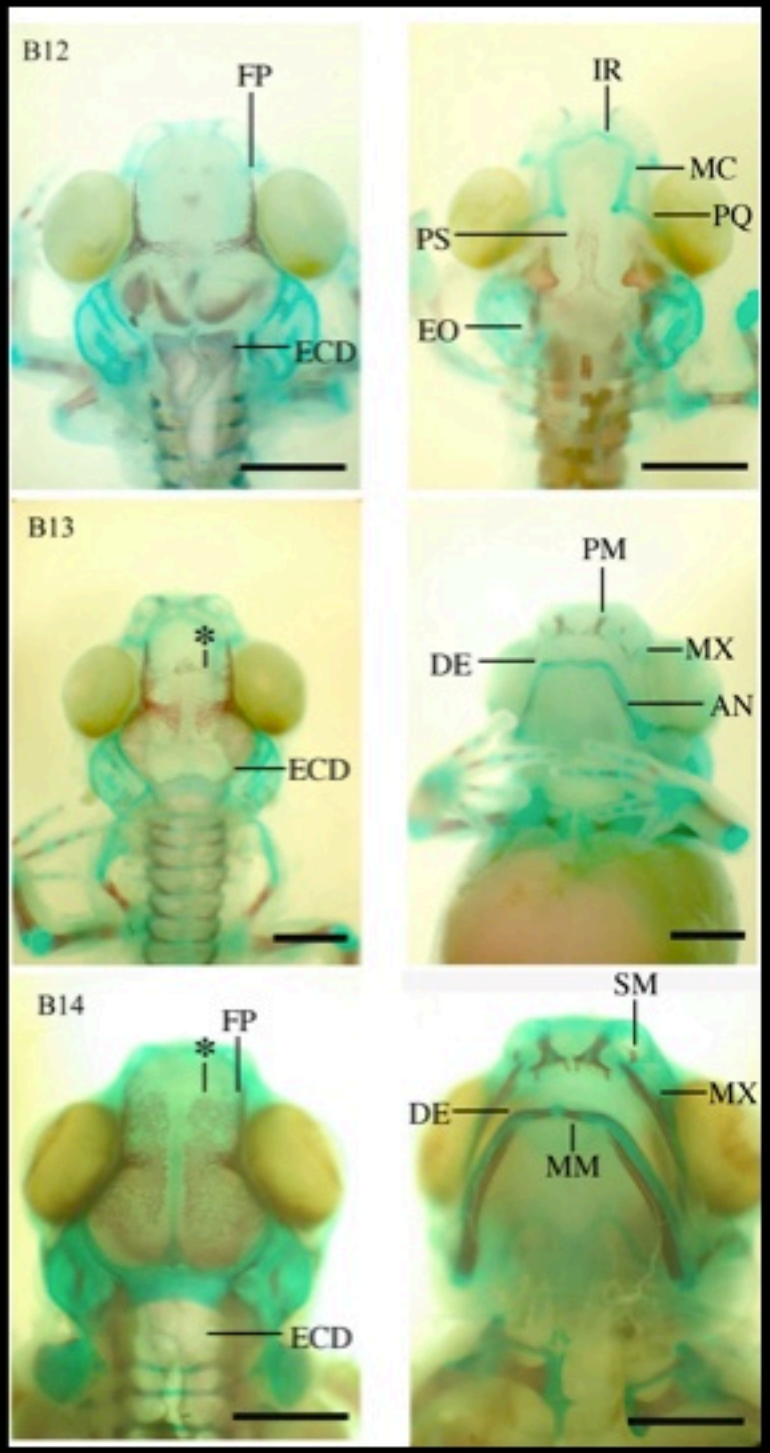
Philautus silus has Tadpole-Specific Features that are Lost in *E. coqui*



Kerney et al. 2007 *J Morphol*

Philautus silus

Clear and Stain to Determine Order of Bone Formation



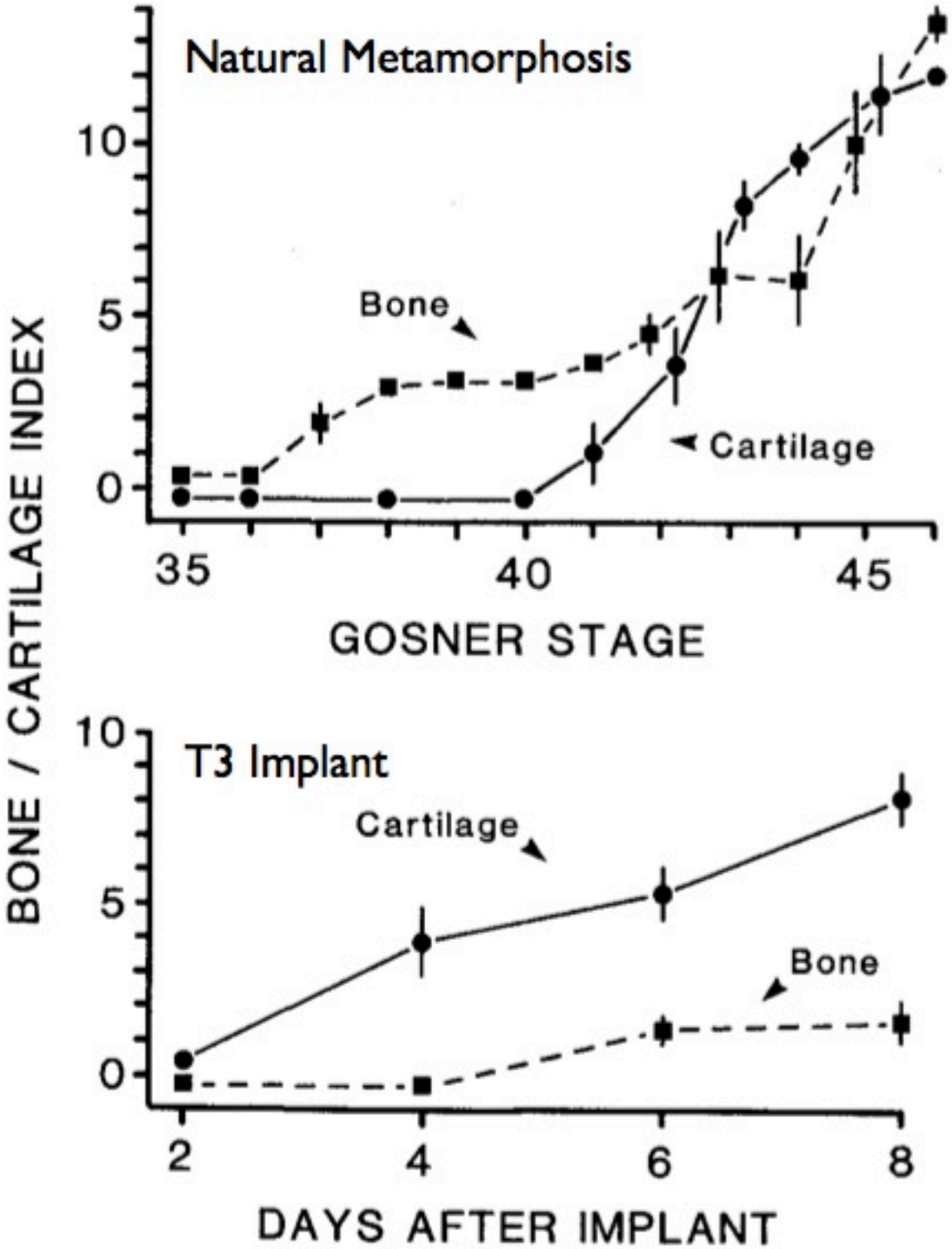
Kerney et al. 2007
J Morphol

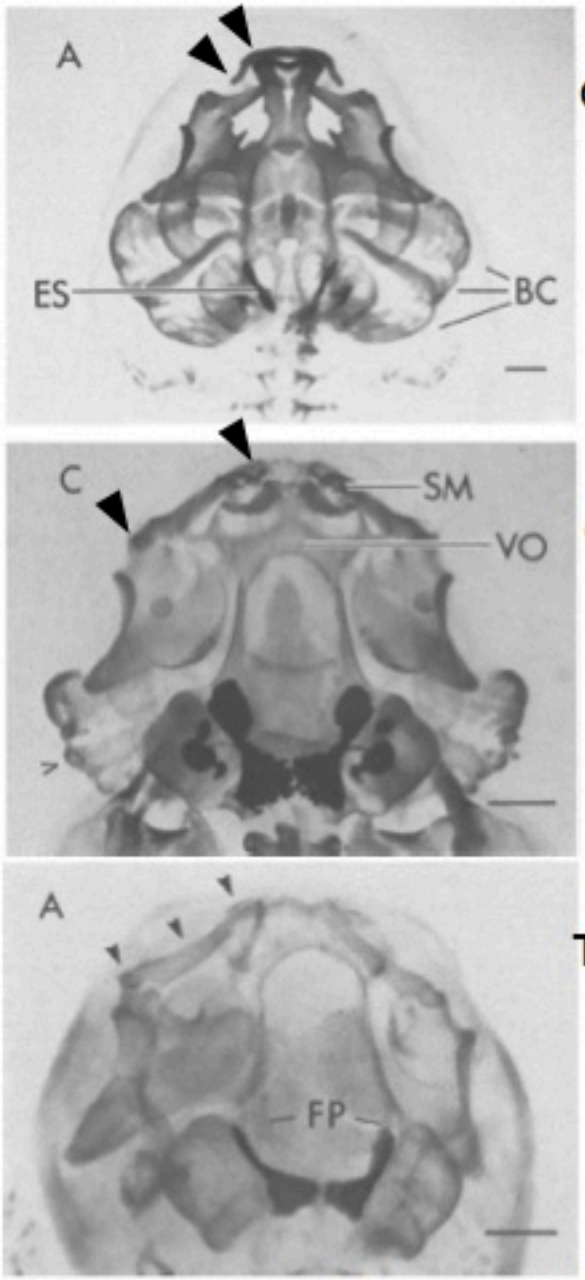
Bombina orientalis	*Philautus silus*	*Eleutherodactylus coqui*	*Pipa pipa*
Hatching	Frontoparietal	Angulosplenial	Frontoparietal
Frontoparietal	Parasphenoid	Squamosal	Parasphenoid
Parasphenoid	Exoccipital	Parasphenoid	Maxilla
Exoccipital	Premaxilla	Premaxilla	Angulosplenial
Septomaxilla	Maxilla	Frontoparietal	Exoccipital
Premaxilla	Angulosplenial	Exoccipital	Prootic
Nasal	Dentary	Maxilla	Premaxilla
Vomer	Squamosal	Pterygoid	Dentary
Maxilla	**Hatching**	Dentary	Nasal
Angulosplenial	Septomaxilla	Septomaxilla	Septomaxilla
Squamosal	Prootic	Quadratojugal	Pterygoid
Dentary	Mentomeckelian	**Hatching**	Sphenethmoid
Quadratojugal	Pterygoid	Prootic	Squamosal
Pterygoid	Nasal	Nasal	Stapes
Sphenethmoid	Columella	Vomer	Tympanic annulus
Prootic	Vomer		"**Hatching**"
Mentomeckelian	Quadratojugal		

Relative Timing of Bone Formation Conclusion

- The first three bones to form in metamorphosing frogs are the parasphenoid, frontoparietal, and exoccipital.
- These are also the first three to form in the direct-developing *Philautus silus* (Sri Lanka), which retains nearly all tadpole cartilages.
- However the angulosplenial and squamosal form first in the direct developing *E. coqui* (Puerto Rico), which never forms many tadpole-specific cartilages.
- These observations reveal a correlation between metamorphosis of the cartilaginous skull and the order of bone formation.

Metamorphic Changes in Cranial Cartilages are More Susceptible to Exogenous Thyroid Hormone than Bone





Stg 36
Control

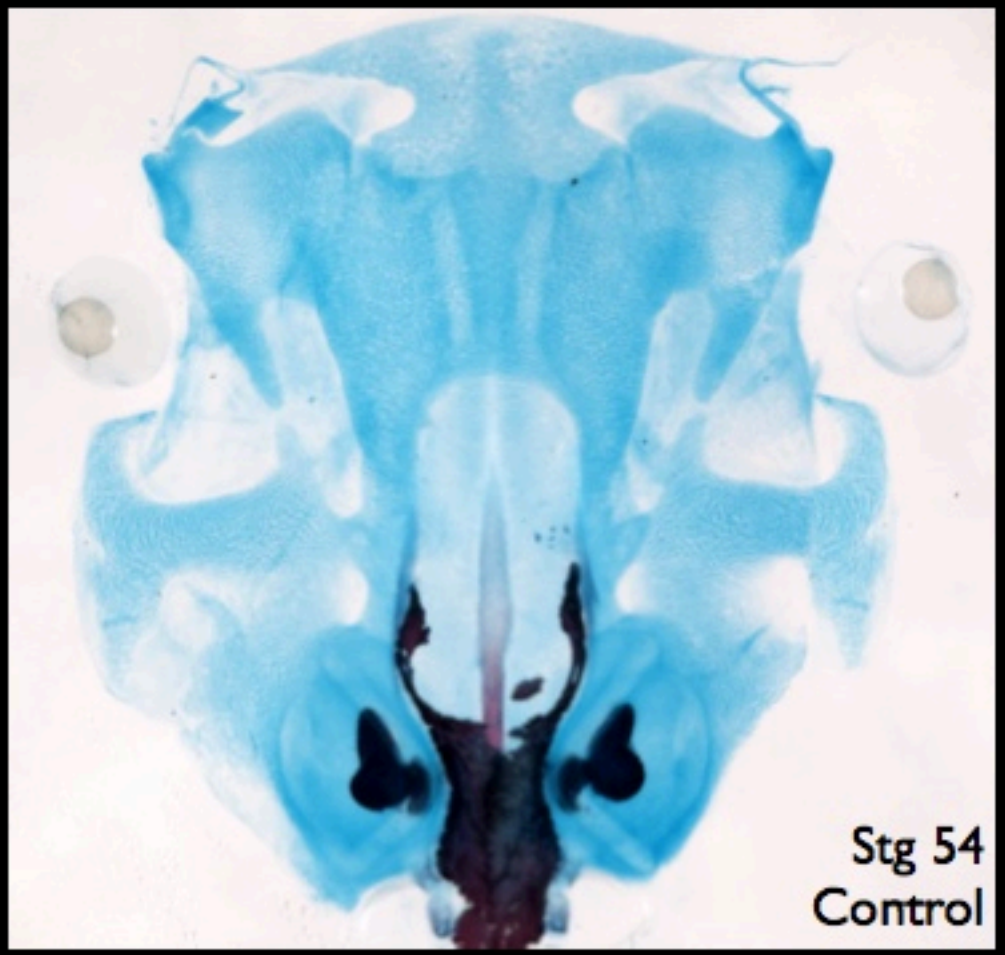
Stg 43
Control

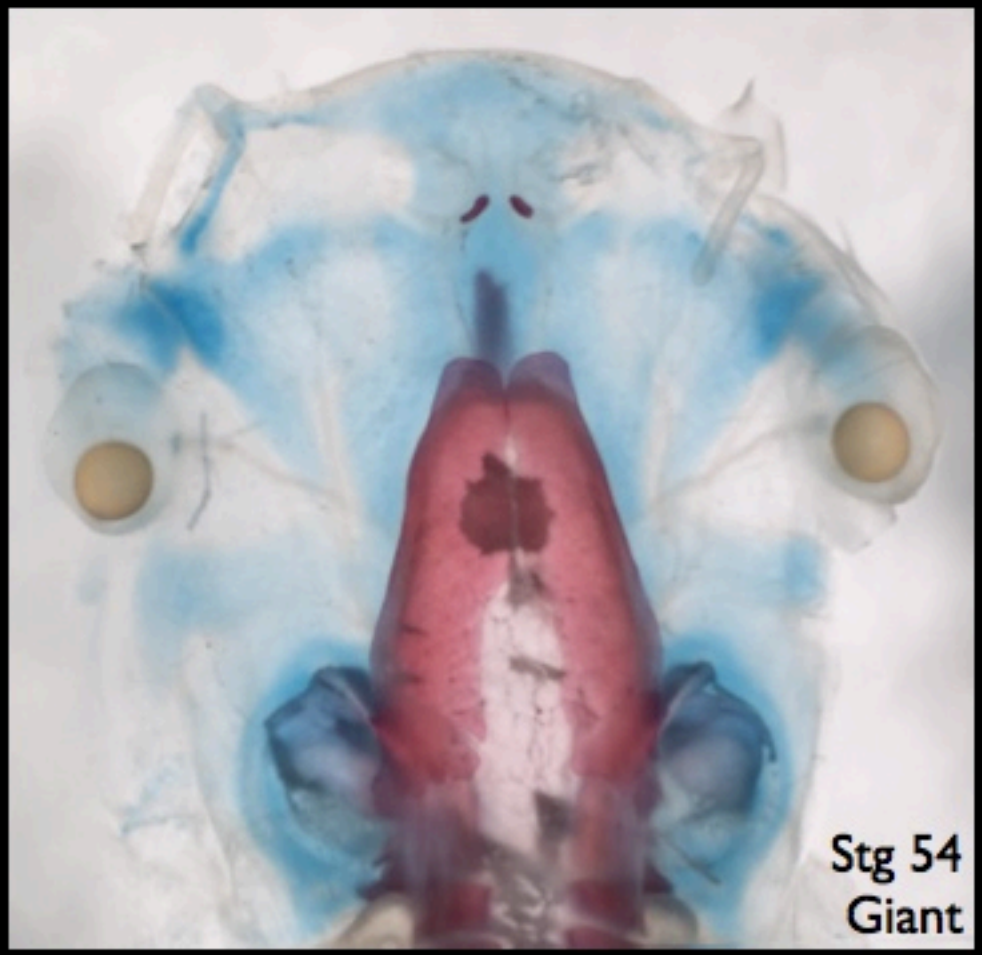
Stg 33/34
T3 Implant
8 days
Index =8

Hanken et al., 1989

Giant Non-Metamorphosing Tadpoles

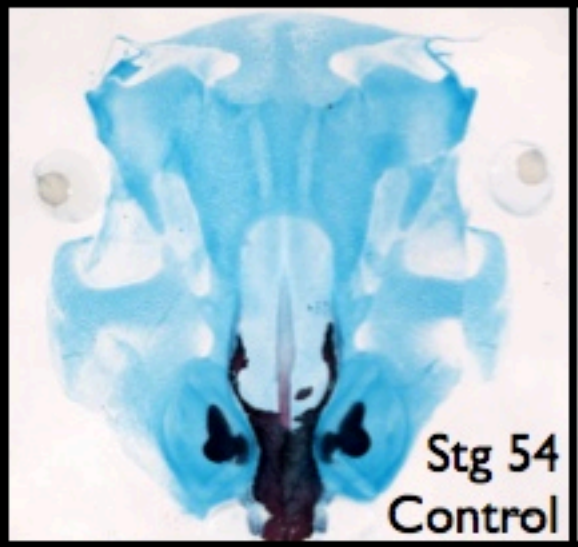
Rot-Nikcevic
& Wassersug 2003

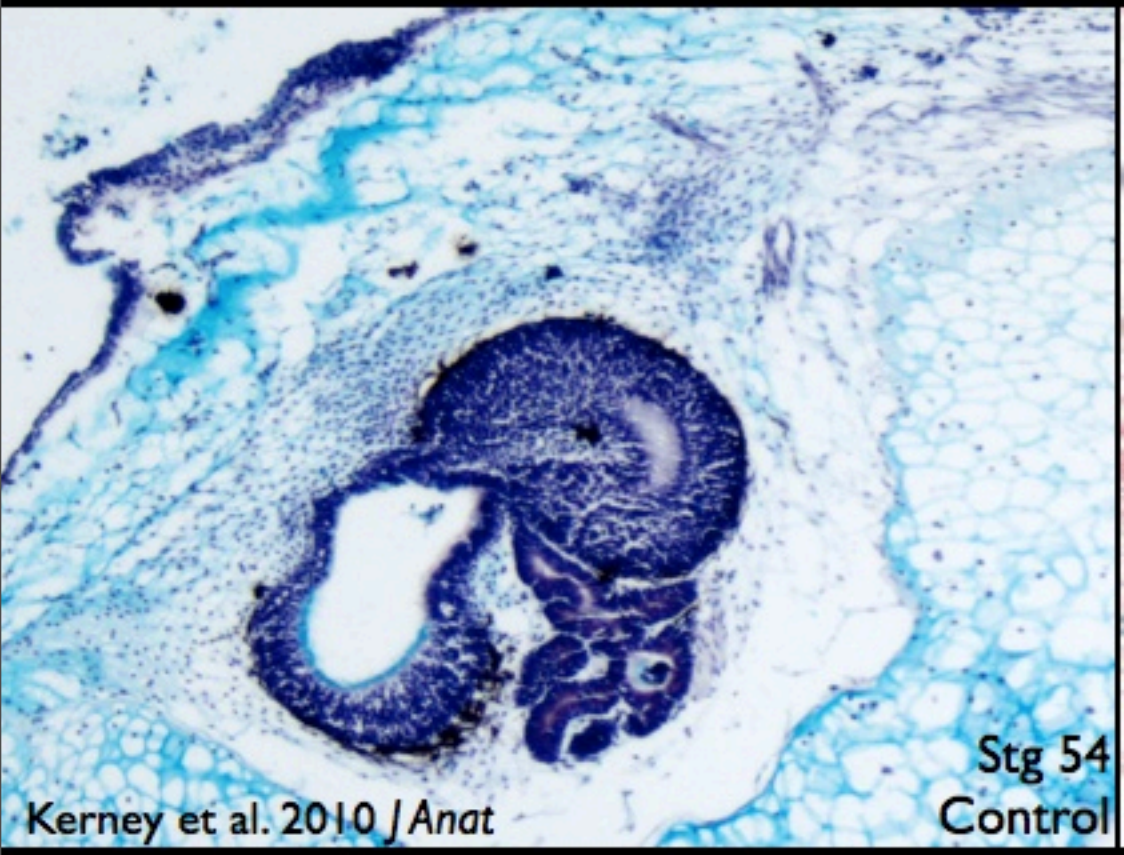




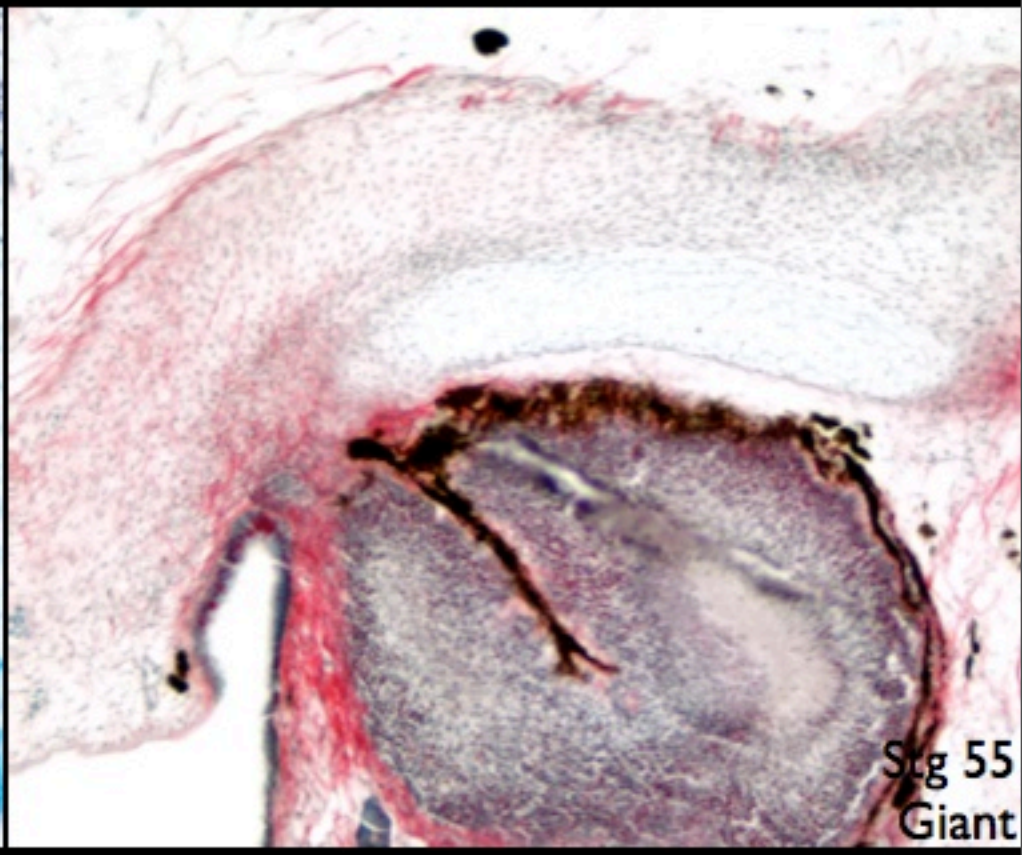
Kerney et al. 2010 *J Anat*

Arrest of Cartilage Maturation





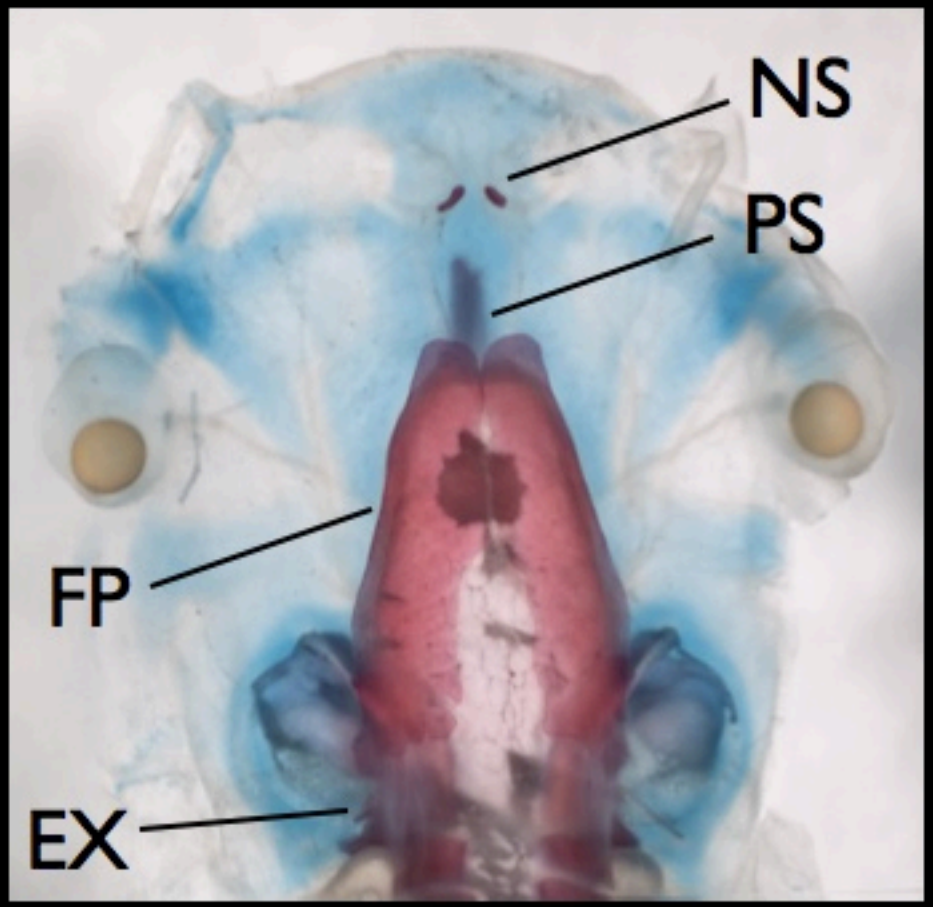
Kerney et al. 2010 *J Anat*

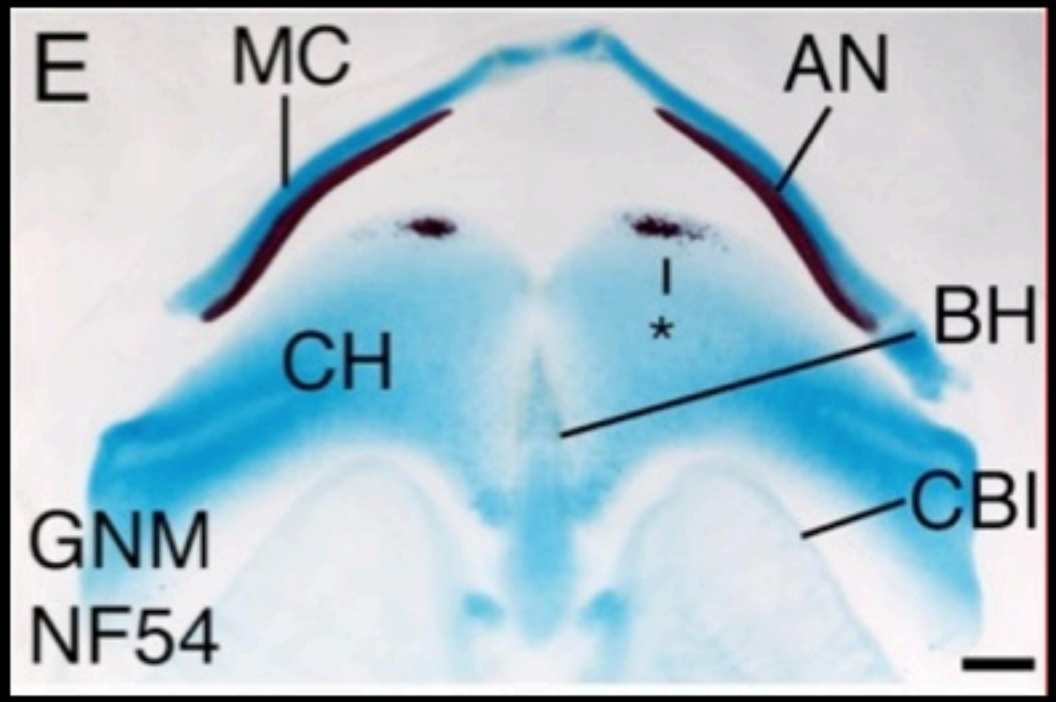


All of the giants (N=18) had ossified frontoparietals, exoccipitals (EX), parasphenoids (PS), and the medial angulosplenial (AN).

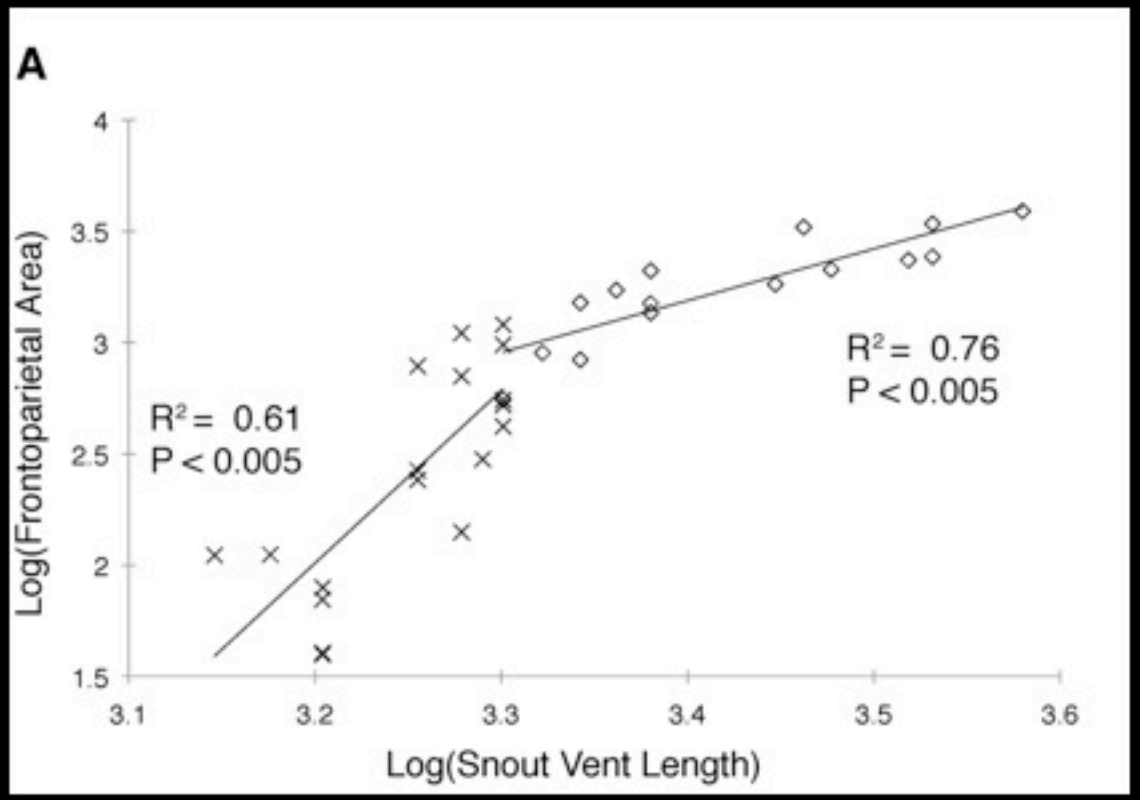
Three had the additional ossification of the nasal bone (NS), which typically does not appear until mid-metamorphosis in *Xenopus laevis*.

One had an additional unilateral ossification of the lateral angulosplenial, and five had ectopoic calcifications on the ceratohyals (CH).





Kerney et al. 2010 *J Anat*



X Controls (metamorphic series)

◇ Giant non-metamorphosing tadpoles

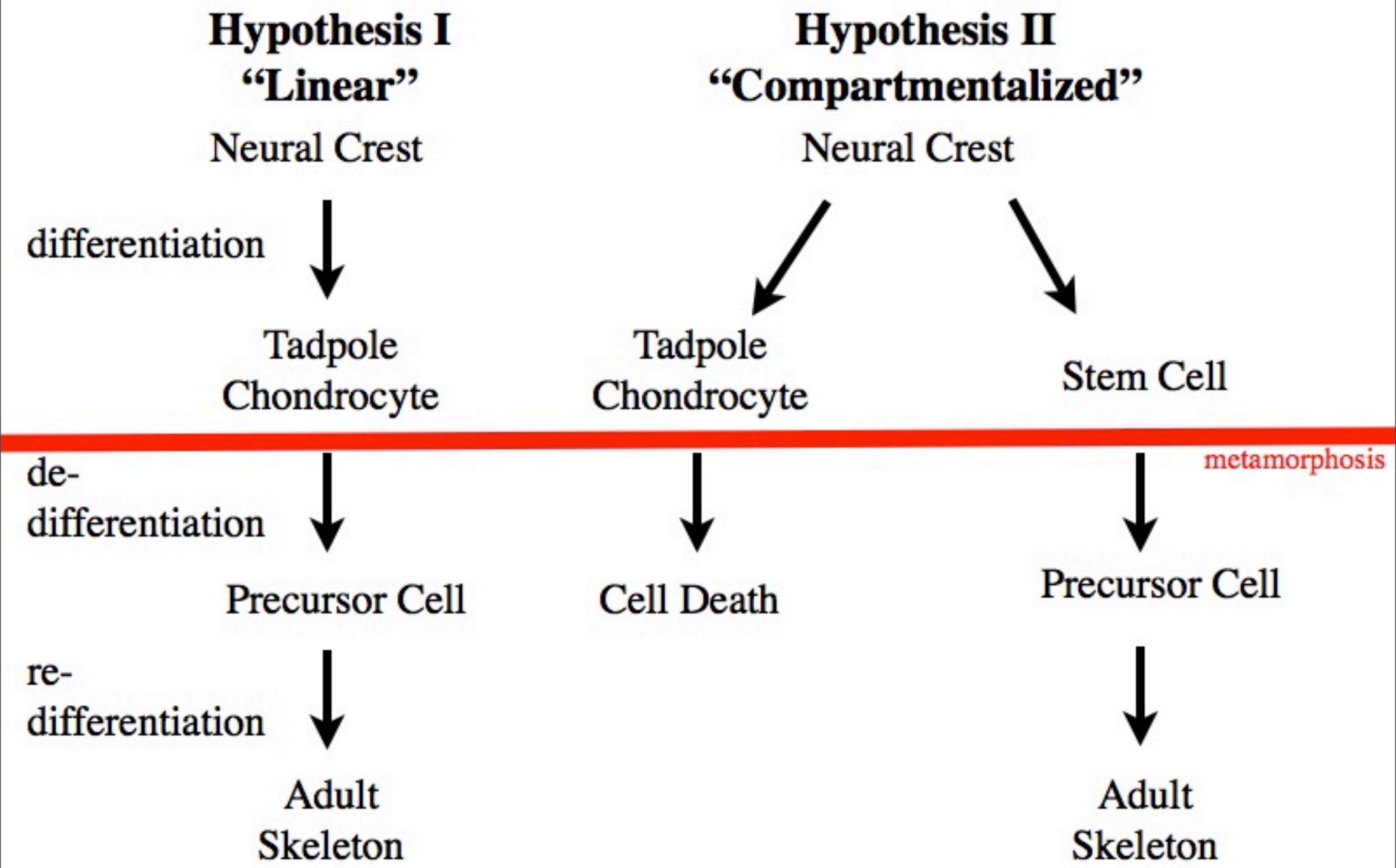
Giant's skull vault is larger than metamorphic control series - negative allometric growth

Kerney et al. 2010 *J Anat*

Thyroid Hormone Activity in Skull Bone Formation

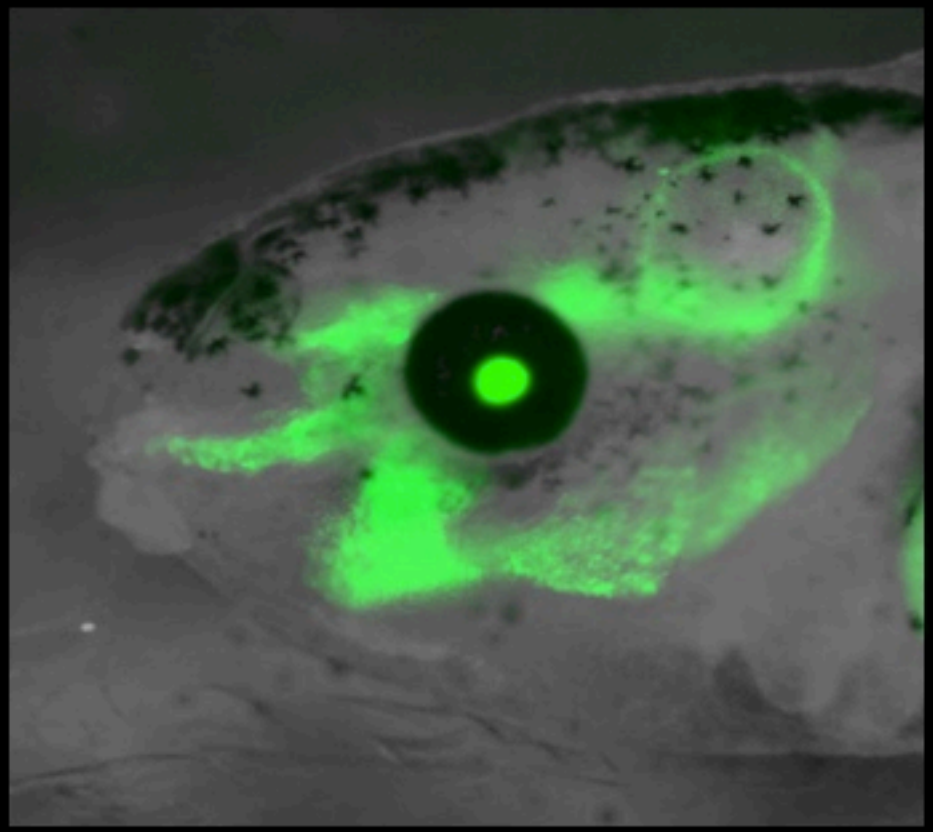
- Cartilage metamorphosis is more susceptible to exogenous thyroid hormone than bone.
- Athyroid giant tadpoles advance the formation of those first three bones that normally form during metamorphosis (frontoparietal, exoccipital, and parasphenoid).
- They arrest development before the formation of other cranial bones, with the occasional abberant exception of the nasals.

Compartmentalization



After Alberch and Gale, 1986

Col2a1 reporter driving EGFP in I-SceI Meganuclease-Mediated Transgenic Tadpole

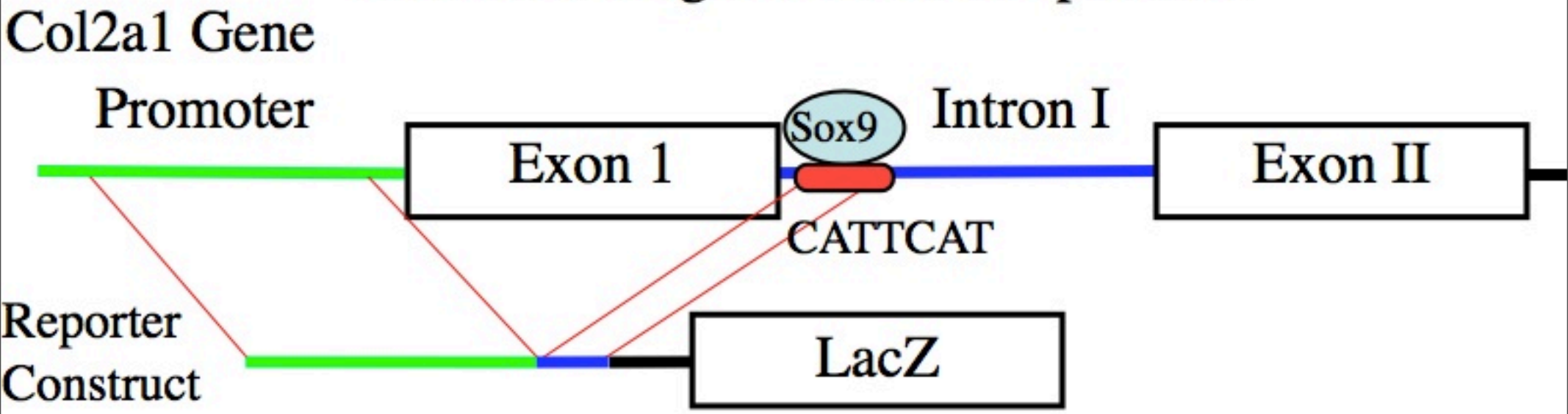


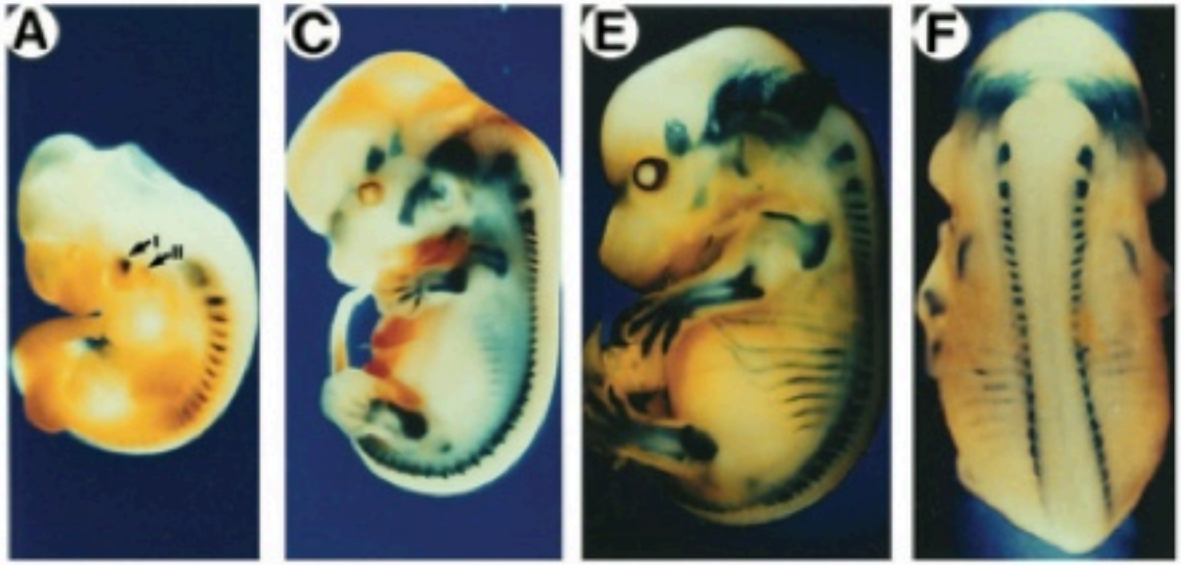
Xenopus laevis

A new tool in fate mapping larval chondrocytes

Kerney et al., 2010 *Int J Dev Biol*

Col2a1 Reporter Construct in Mouse Drives Cartilaginous Gene Expression





Blue X-gal = Cartilage

Alignment of the first intron of col2a1 from mouse and *Xenopus*

Lagan/Vista Alignment

Sox9 Binding Site

```
Mouse   ACTTTTTAATGTACGCATATTAA-----GTCTAGAGGTCCATCCACTGTATTTATAAAGG
Xenopus -TTGTTGGCCGCGAAGACATTGACCCACATCTGCATTTCTCAGCCCTAGCCTTCCAAAAG

Mouse   TGAT--TAAAATGGAAGGGCCTTGCTTAAAGGCTTCAATTCAGTATTGTGATGGTTTATT
Xenopus TGCTGCTGGTTCGGGAGGG---------GAGACCTCAGTCCTCCTTTGTGA-GACTTGTT

Mouse   TGCACTGGGAGGATTGGCAGCAATGGTTTCCAGATGGAGTTGAAATGCTGCCCATGTTTA
Xenopus TGCGTTGGG-GGATTGGCAGCGATGGCTTCCAGATGG-GCTGAAACCCTGCCCGTATTTA

Mouse   TTTAAACTGGCCCCTGACAGTGAAATGTGAATTGAGACCTCTGTGTATTTTACAAAAAGG
Xenopus TTTAAACTGGTTCCTCGTGGAGAGCTGTGAATCGGGCTCTGTATGCGCTTGA-GAAAAGC

Mouse   CCCATTCATTA-----AGGGTCCTCTGGGCTACCCAA----------------------
Xenopus CCCATTCATGAGAGGCAAGGCCCAGTGGGTCCCCCAACTCCCCGACCCCCCTCTCCCACA
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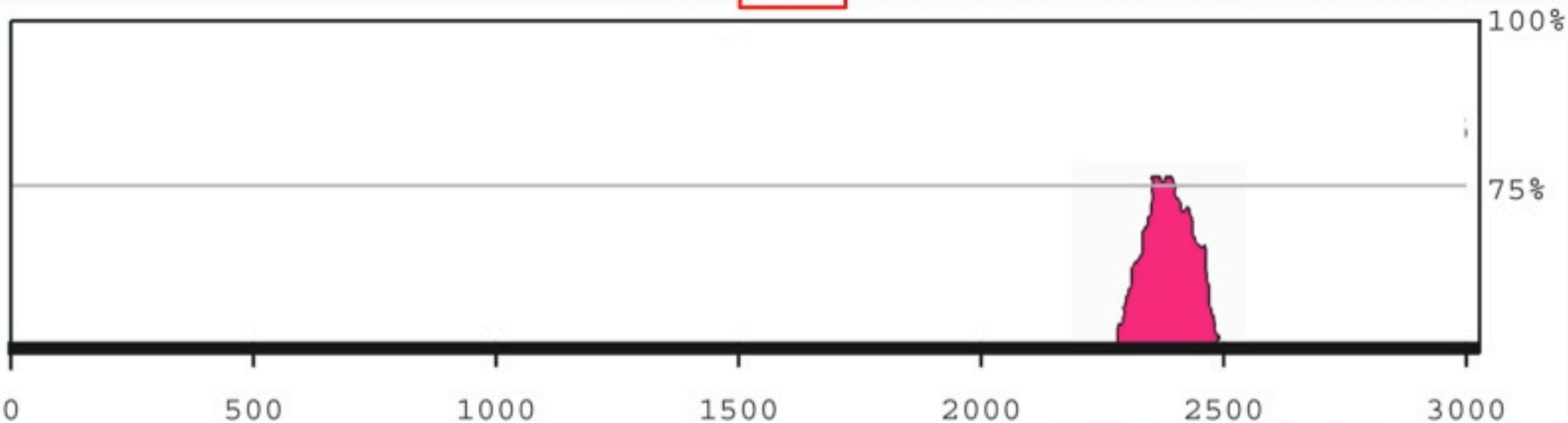
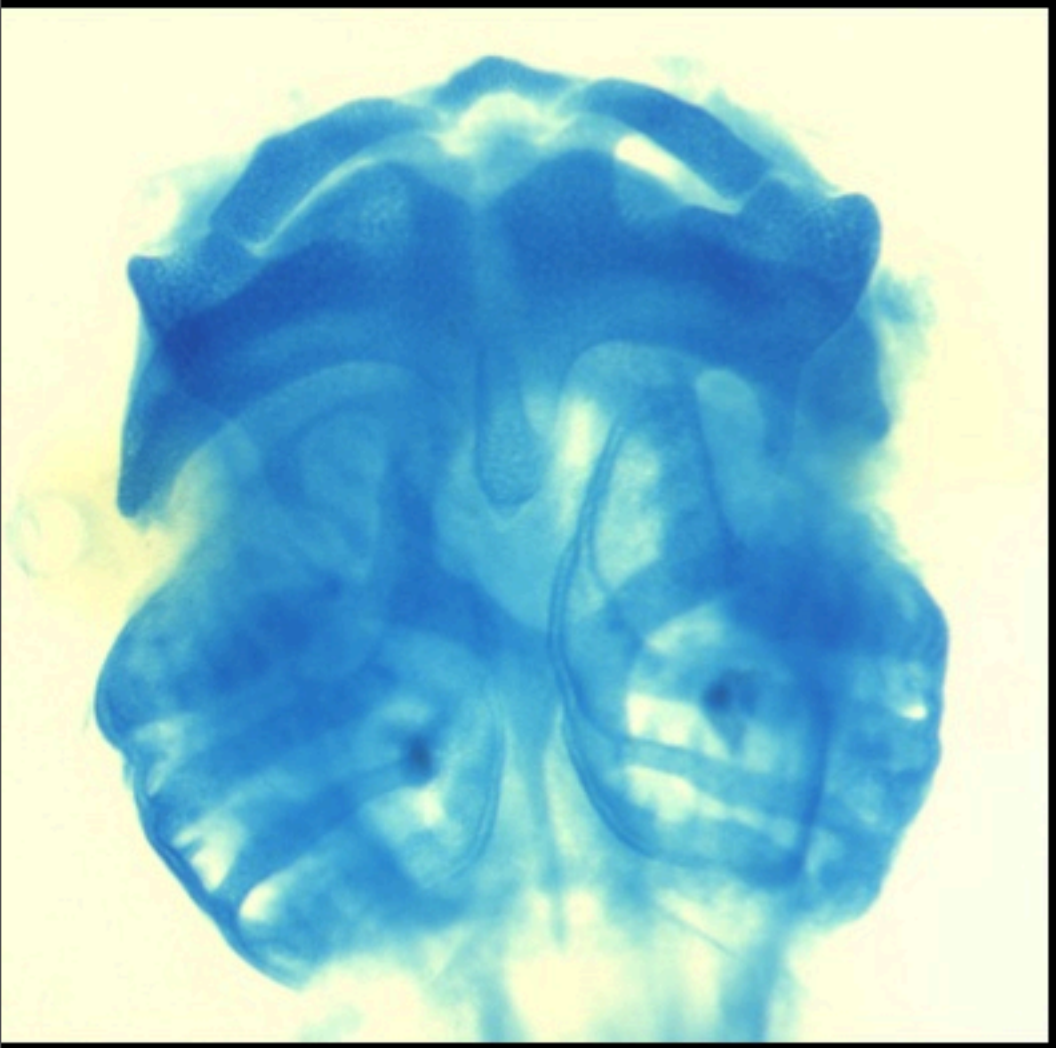
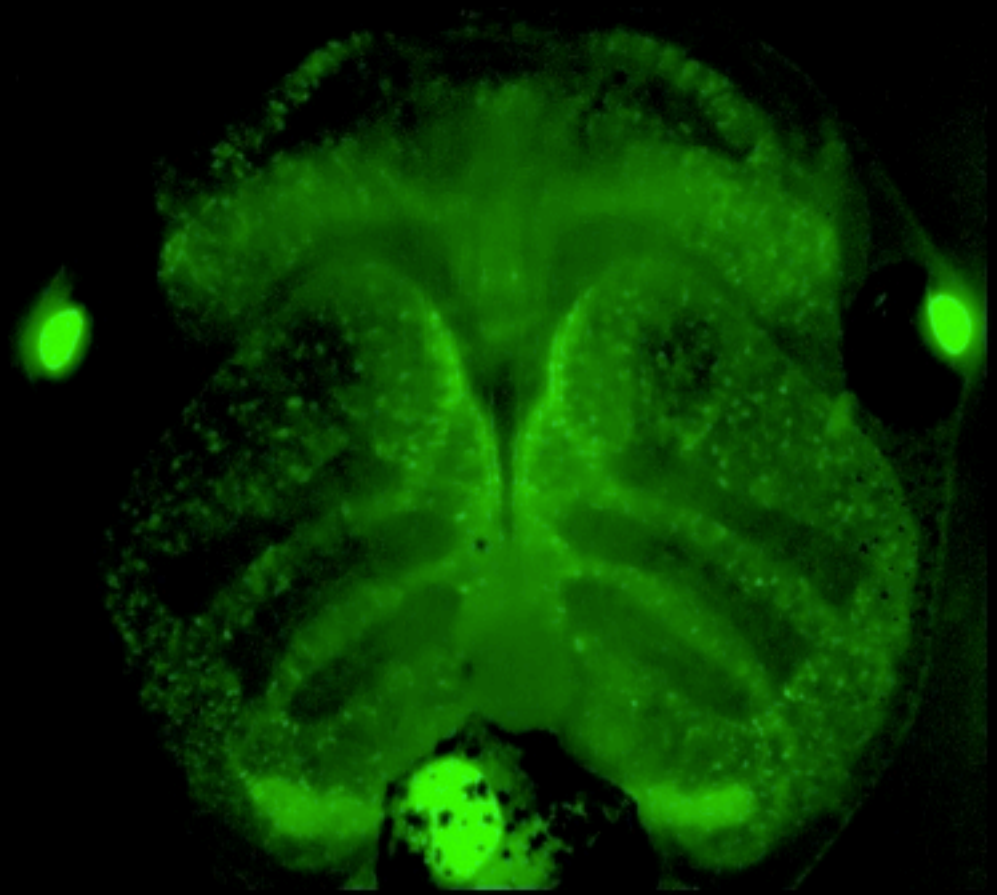


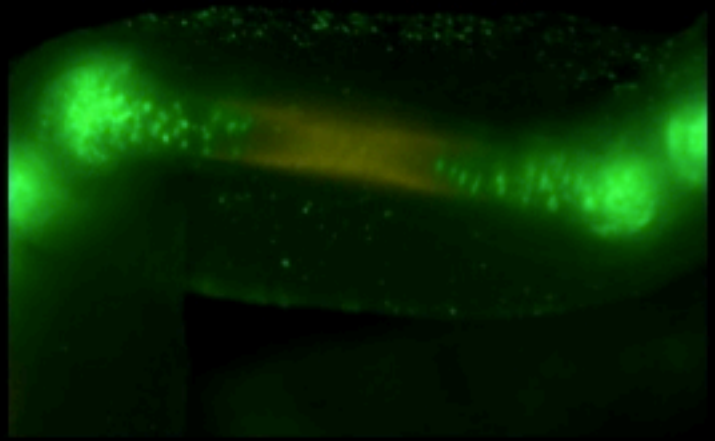
Kerney et al., 2010 *Int J Dev Biol*

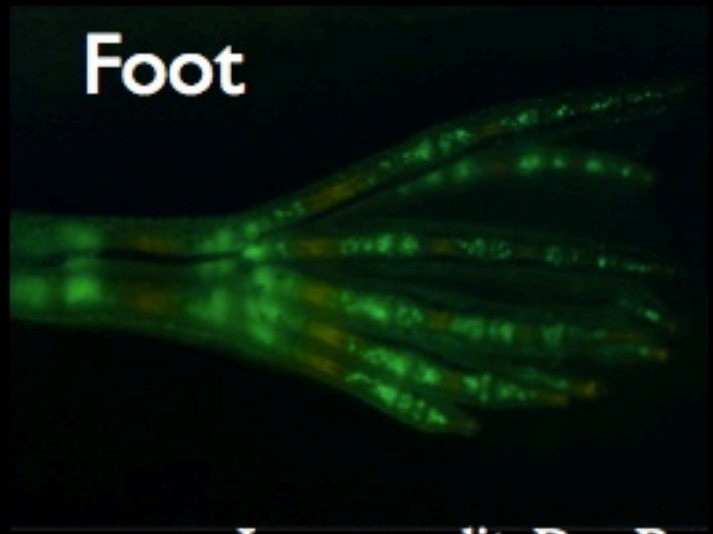




Stage 42

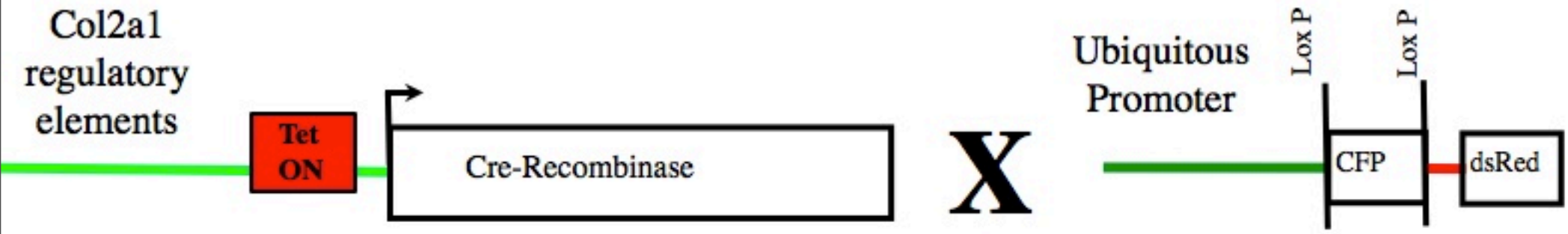
Kerney et al., 2010 *Int J Dev Biol*





Metamorphic tadpole (Stg 59)

Image credit: Dan Buchholz



Same tadpole before and after 3d of 10ug/mL Dox

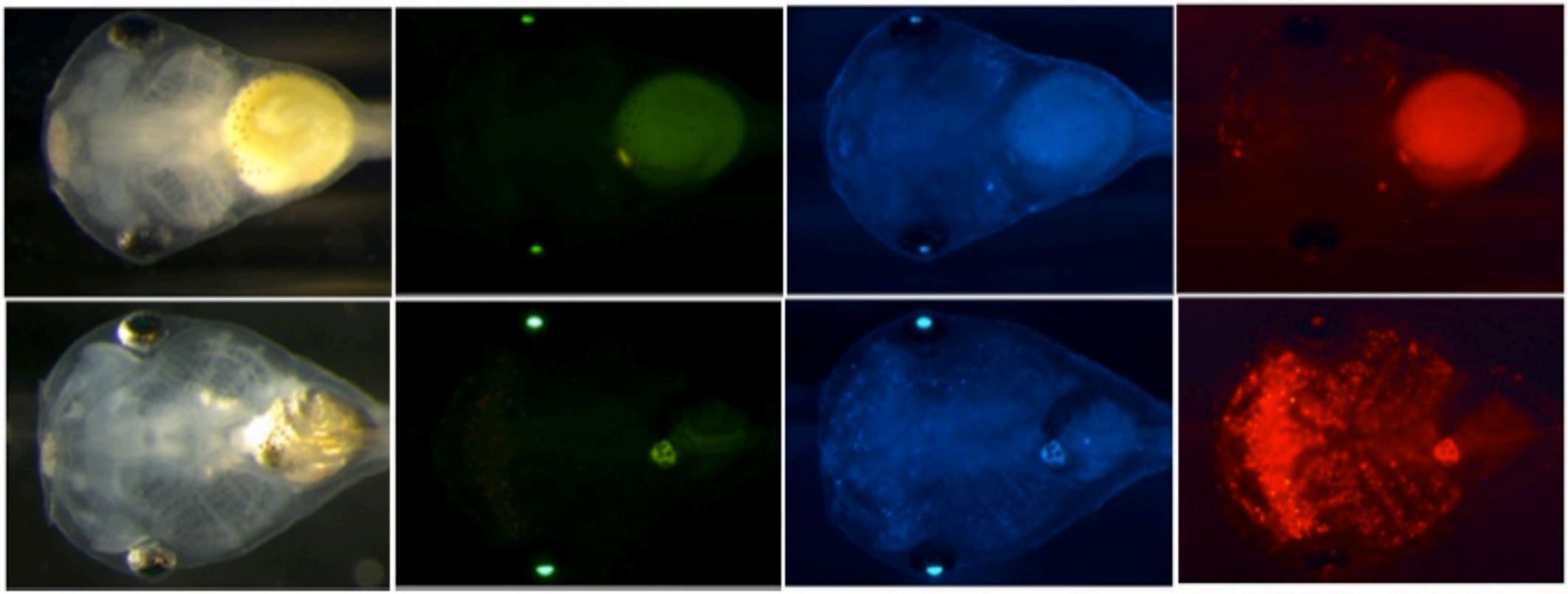
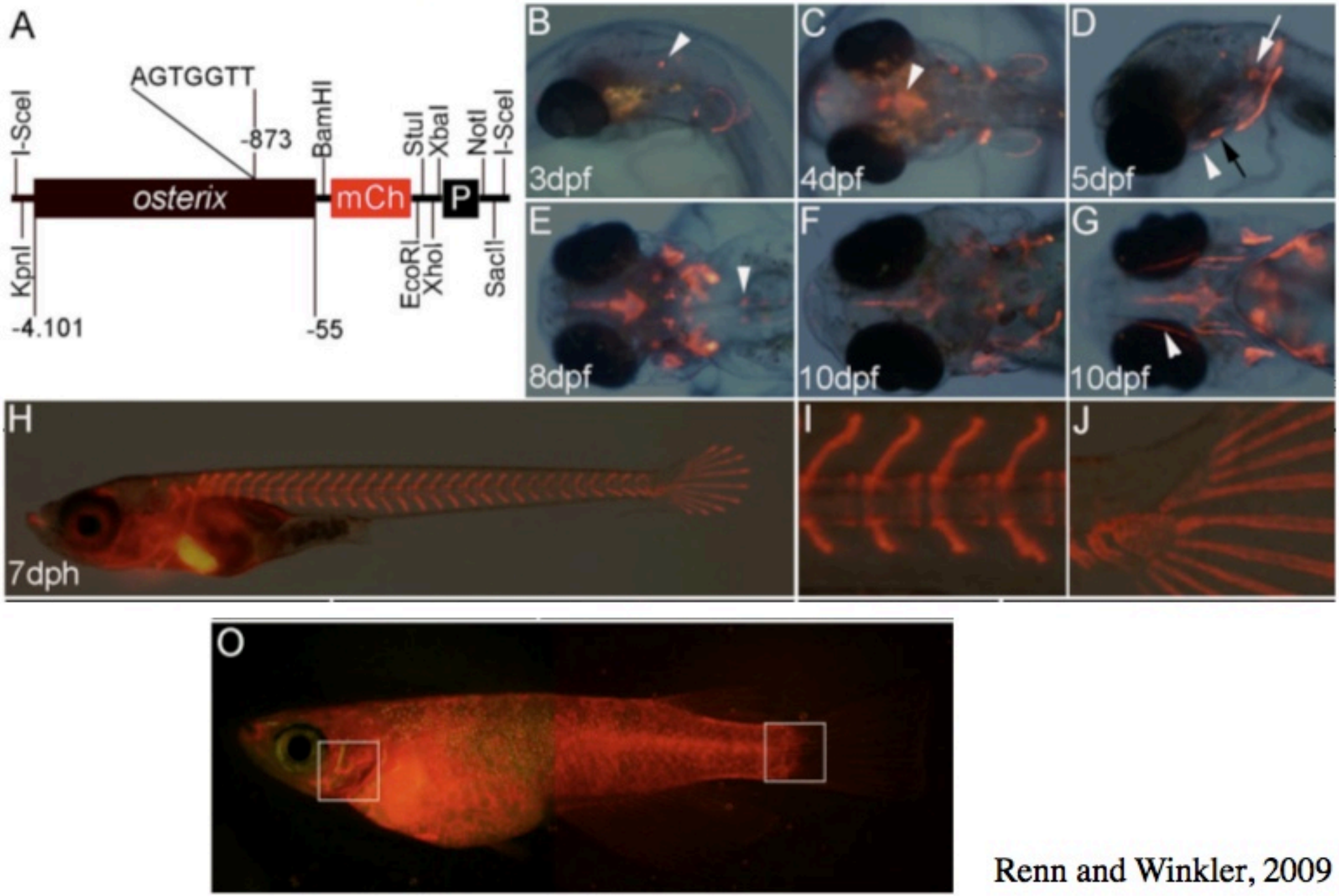
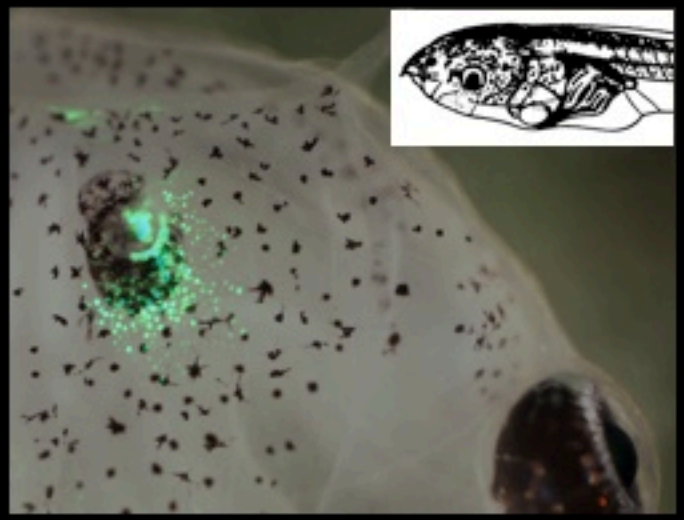


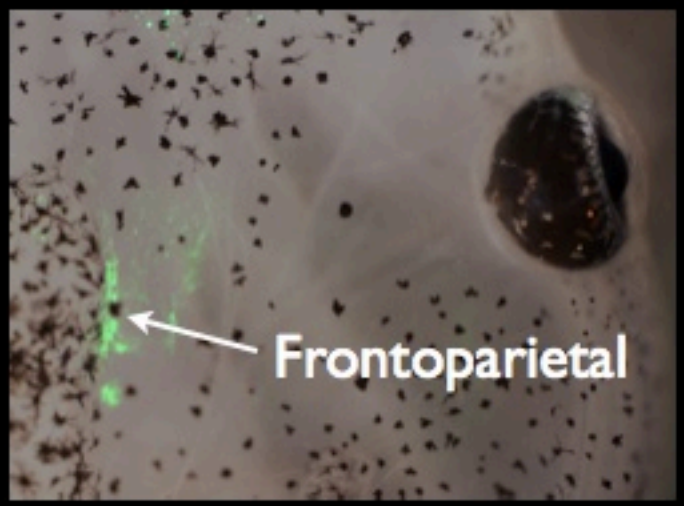
Image credit: Dan Buchholz

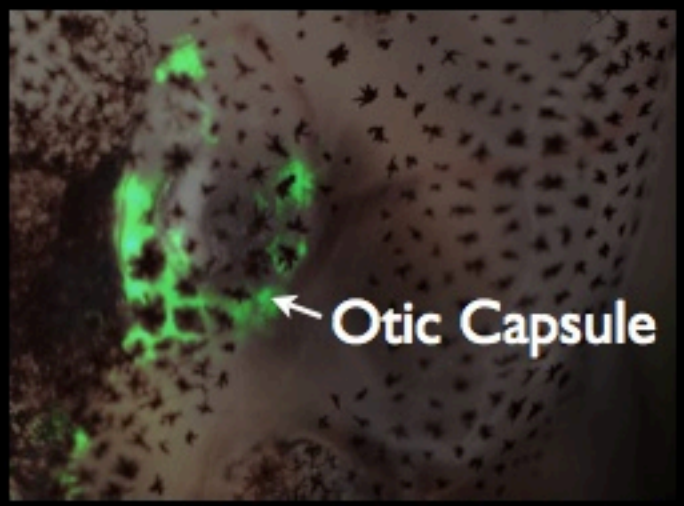
What about bone?



Renn and Winkler, 2009

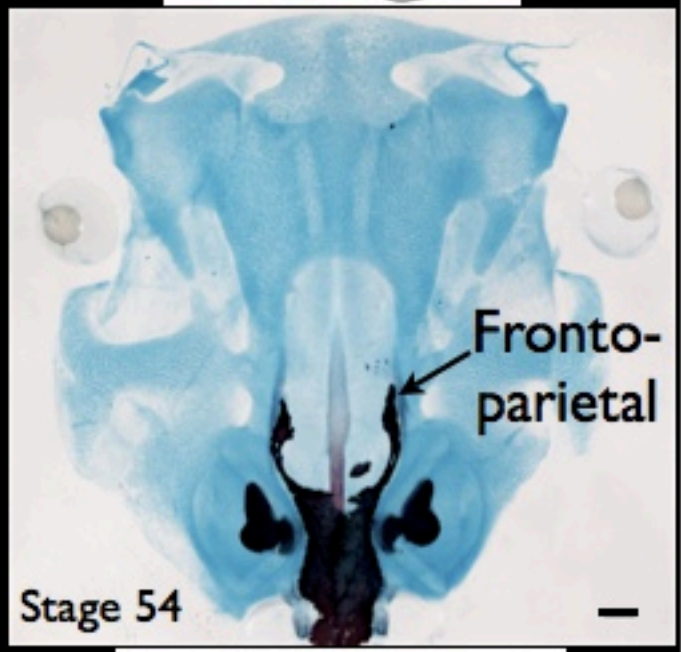


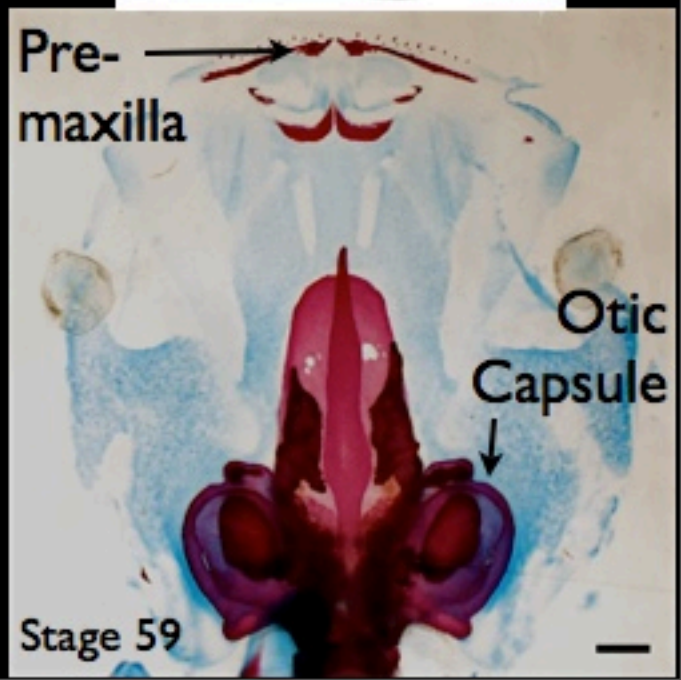




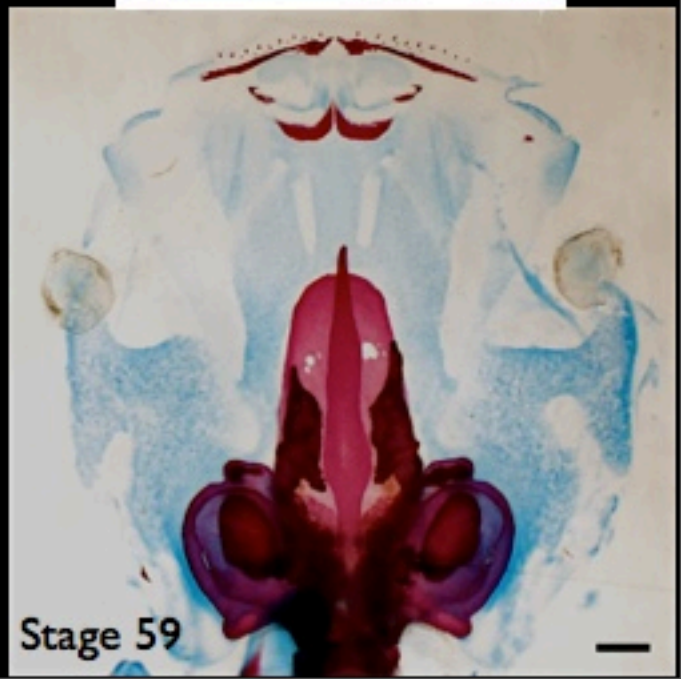
Osterix:EGFP Stage 50

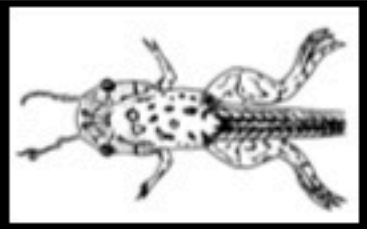
Osterix
5' Promoter EGFP

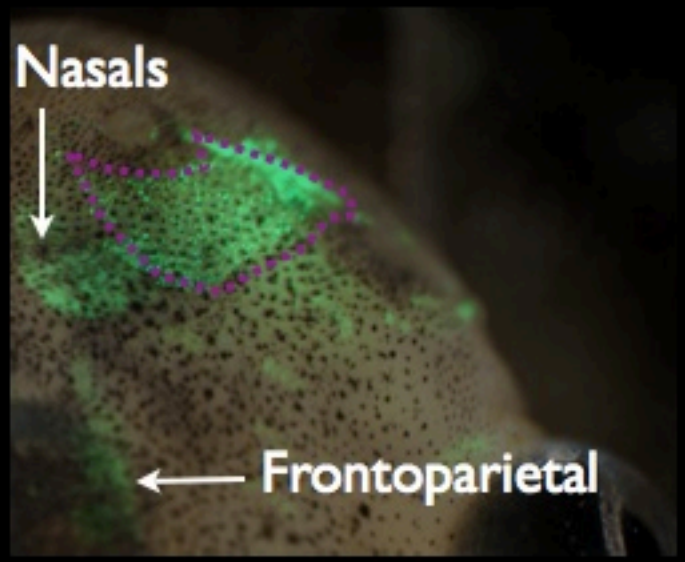


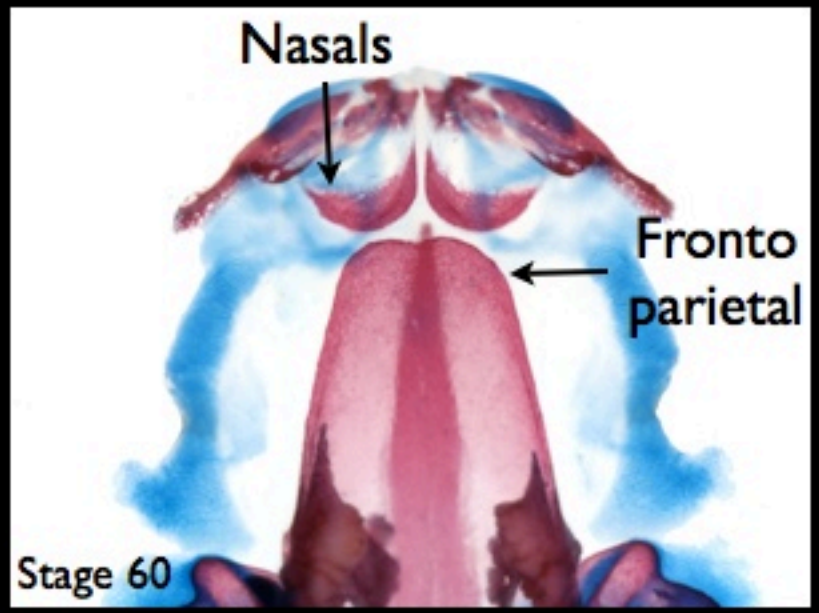


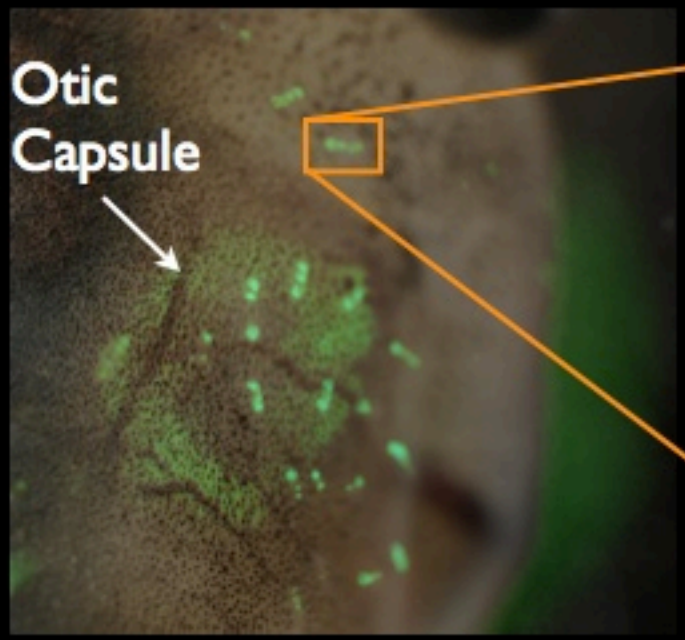

Premaxilla

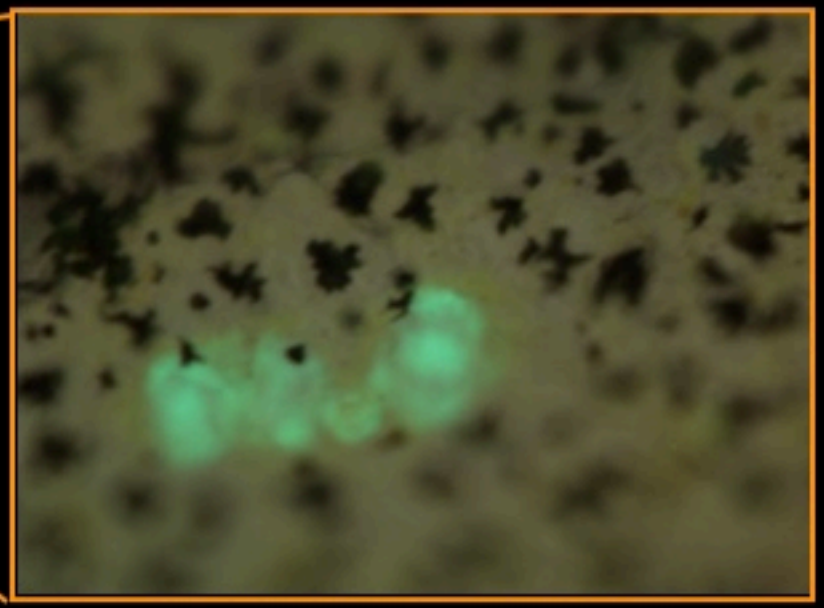

Stage 59



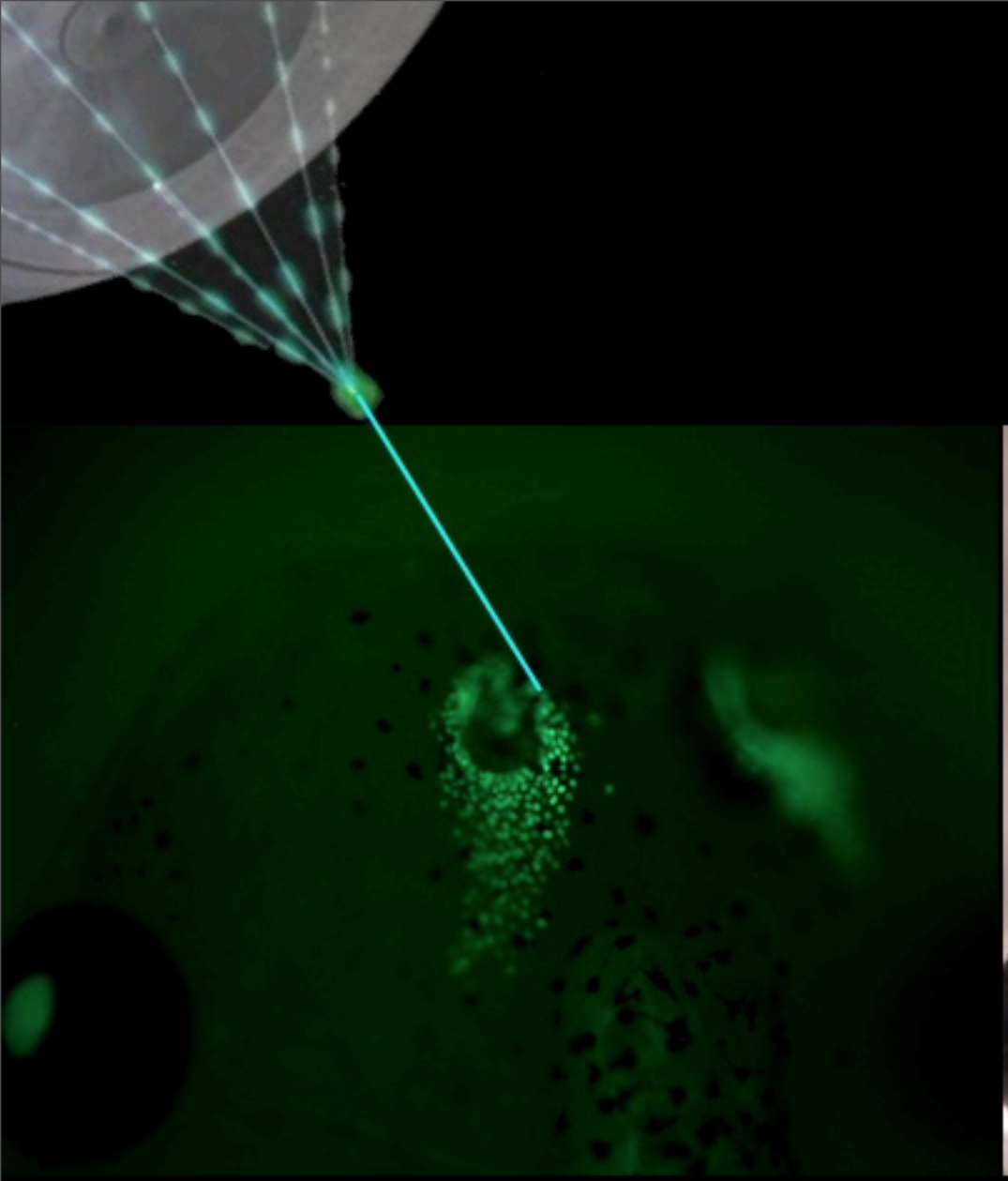








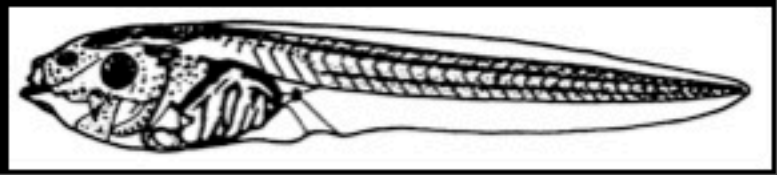
Osterix:EGFP Stage 60



Osterix:GFP

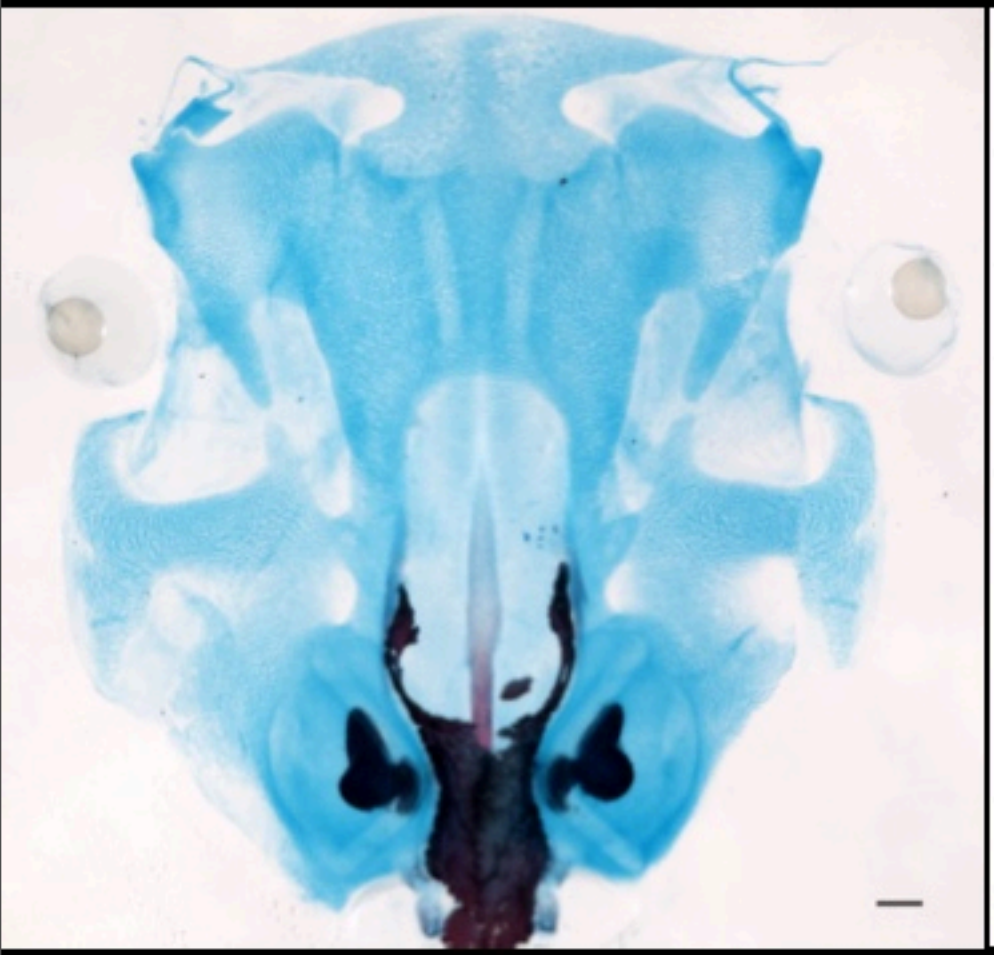
Xenopus laevis

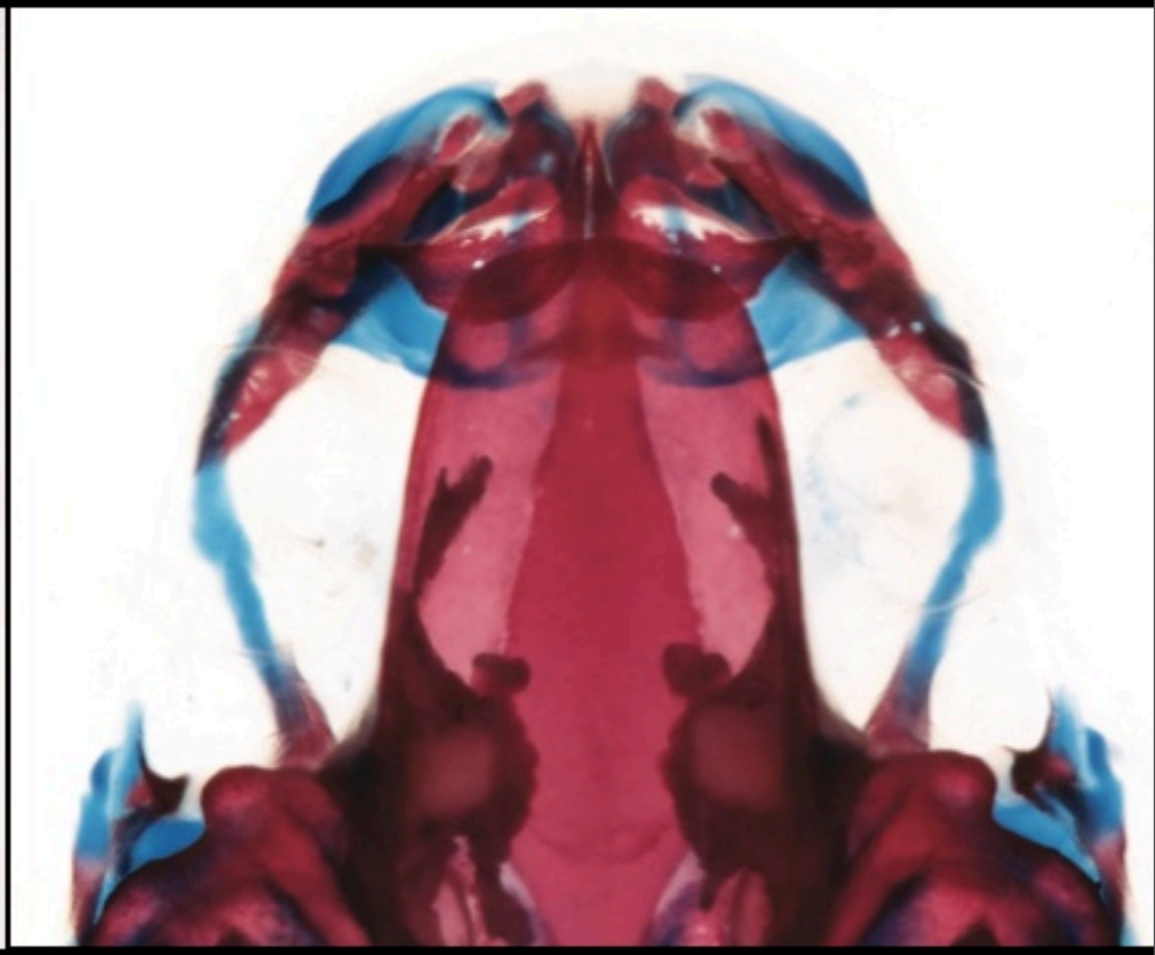
Stage 48



New Research Directions

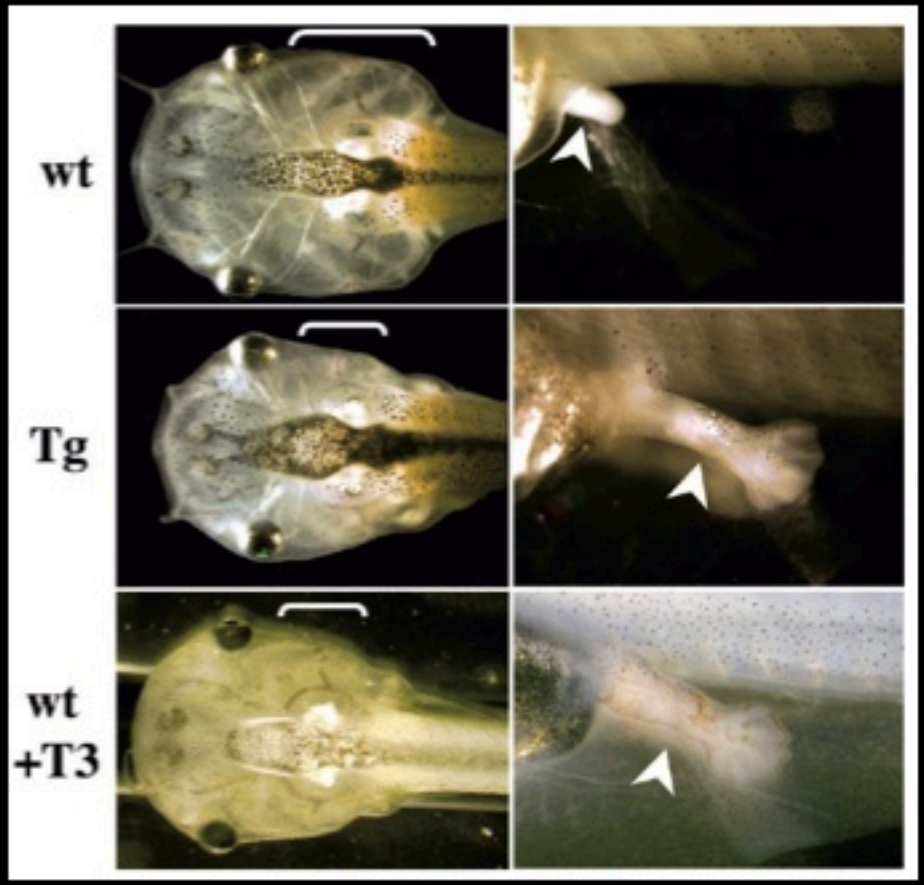
To what extent is the differentiation of bone contingent on shape changes in underlying cartilages?





Dominant-Negative & Dominant-Positive Thyroid Hormone Receptor Transgenes

- Currently working to replace reporter GFP with modified thyroid hormone receptor sequences.
- Should allow tissue-specific advance or arrest of cartilage or bone metamorphosis.
- May answer whether bone formation is contingent on changes to underlying cartilages.



HS-dpTR
Buchholz et al., 2004
Mol Cell Biol

Presentation Summary

- There is correlative evidence that suggests dermal bone formation is contingent on metamorphic re-modelling in frogs.
- This contingency may be due to changes in the physical environment experienced by pre-osteoblasts.
- However very little is known about the cell populations that give rise to the post-metamorphic skull.
- We are developing the tools to identify these cells, and experimentally challenge them in vivo.

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***Xenopus* Transgenics:**

DanielBuchholz (Univ. Cincinnati)

American Association of Anatomists Postdoctoral Fellowship