The vertebrate limb: An evolving complex of self-organizing systems

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Program on “Living Matter”

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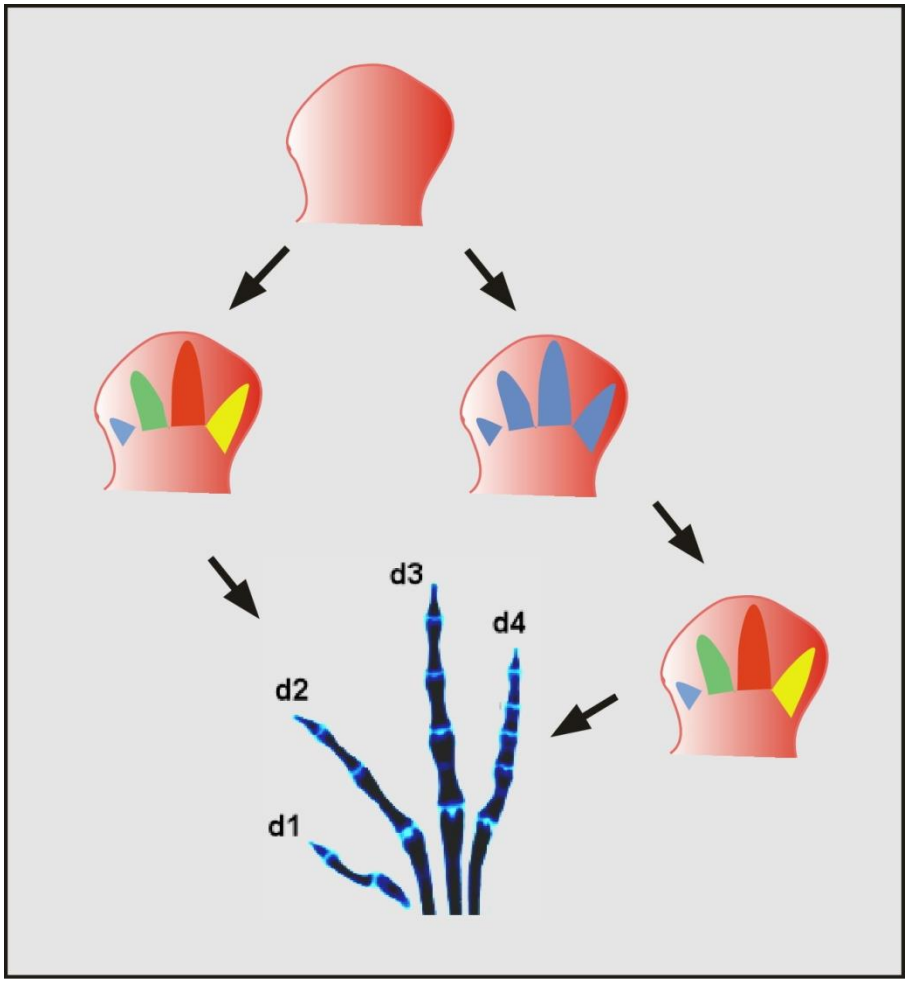
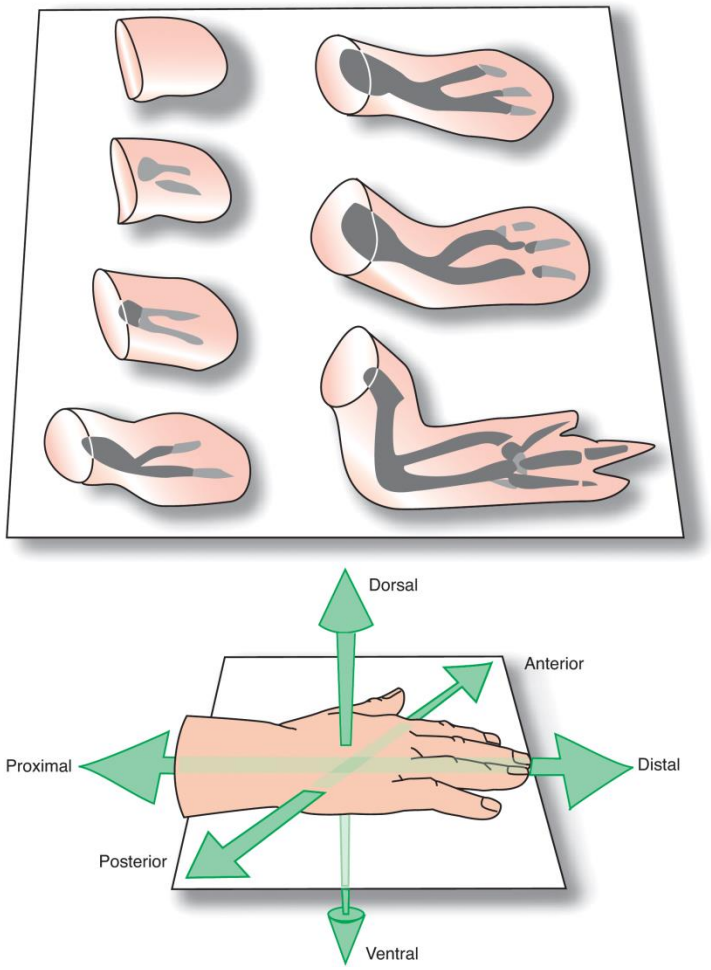
Positional information *vs.* isomorphic prepattern (e.g., Turing-type) mechanisms



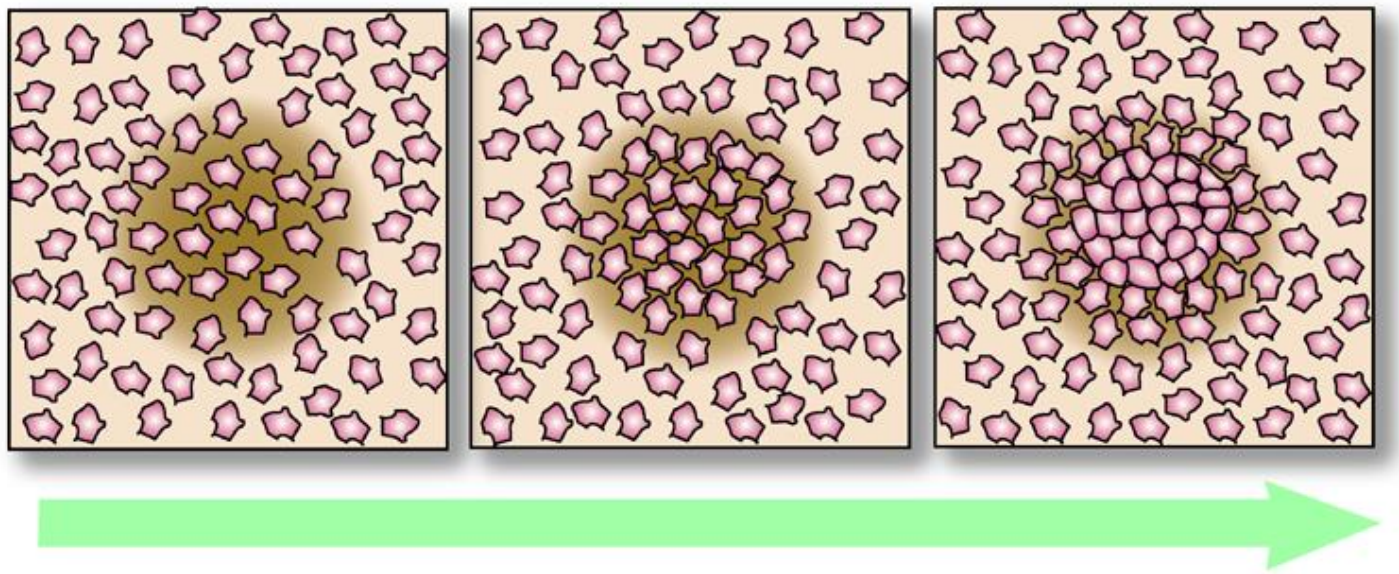
Based on Newman and Frisch *Science* 1979

Skeletal pattern formation in the avian limb

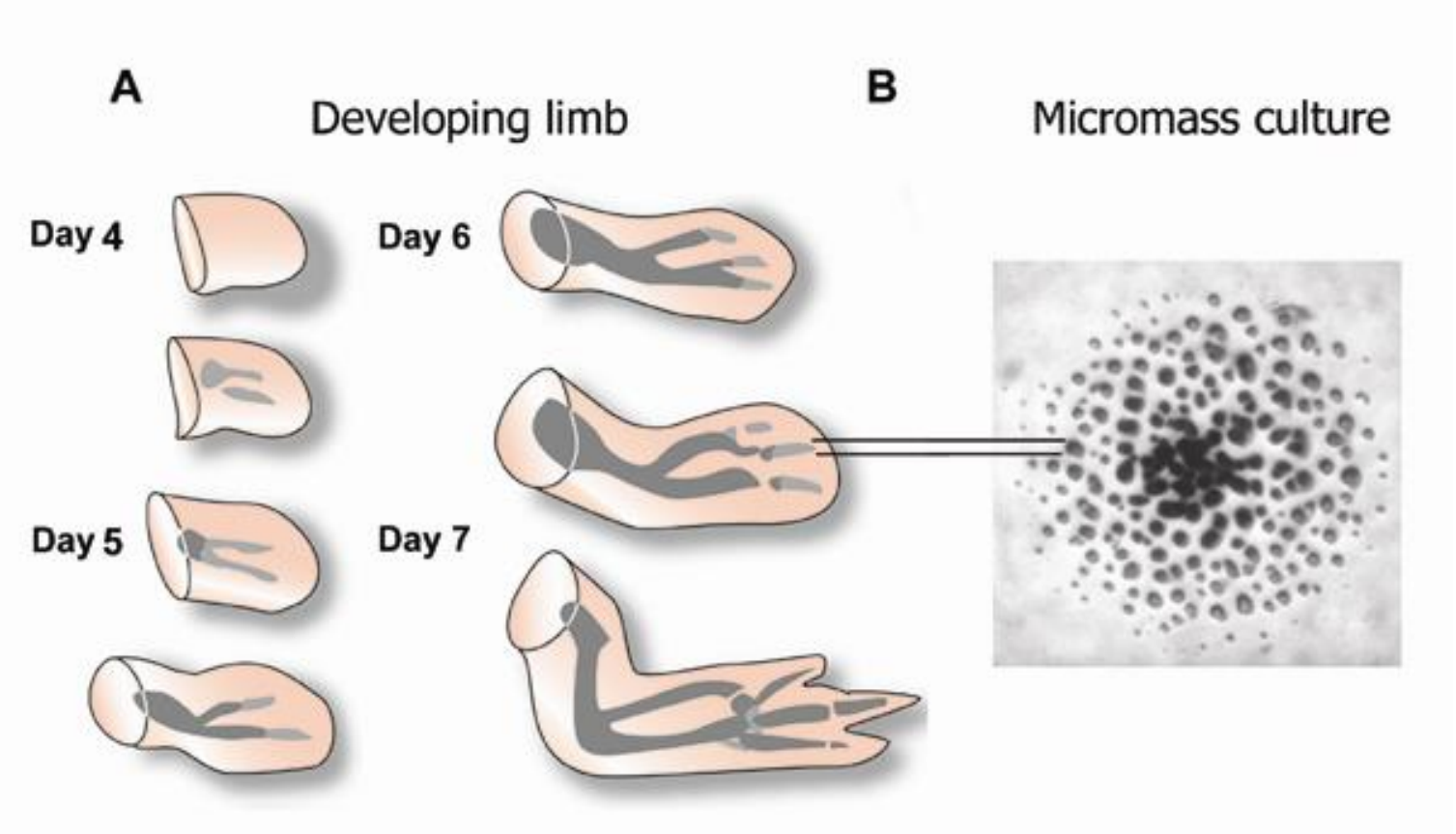


Forgacs & Newman, *Biological Physics of the Developing Embryo* 2005

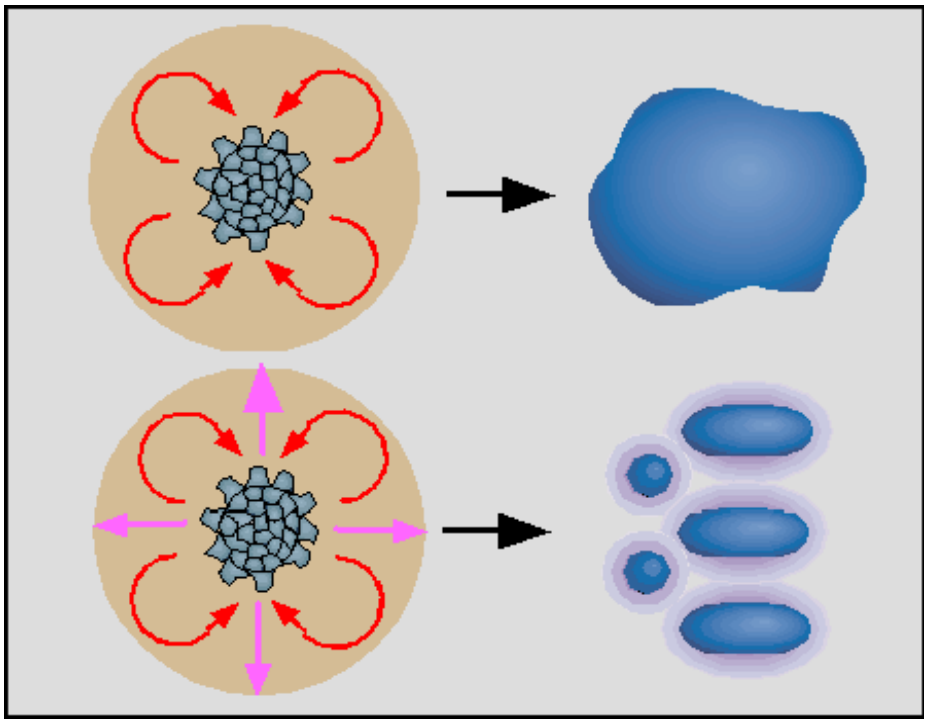
Mesenchymal condensation



Forgacs & Newman, *Biological Physics of the Developing Embryo* 2005


A
Developing limb
B
Micromass culture
Day 4
Day 5
Day 6
Day 7

Activator-inhibitor interactions in cartilage pattern formation



Moftah *et al*., *Dev Biol* 2002

RESEARCH ARTICLE Open Access

A regulatory network of two galectins mediates the earliest steps of avian limb skeletal morphogenesis

Ramray Bhat[1], Kenneth M Lerea[1], Hong Peng[1], Herbert Kaltner[2], Hans-Joachim Gabius[2], Stuart A Newman[1*]

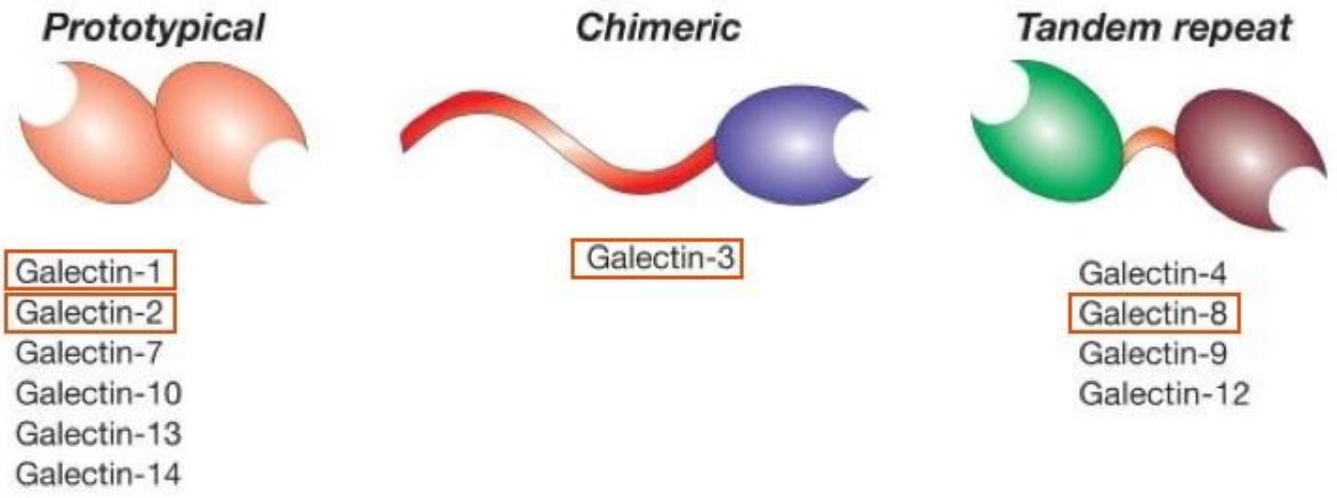
Abstract

Background: The skeletal elements of vertebrate embryonic limbs are prefigured by rod- and spot-like condensations of precartilage mesenchymal cells. The formation of these condensations depends on cell-matrix and cell-cell interactions, but how they are initiated and patterned is as yet unresolved.

Results: Here we provide evidence that galectins, β-galactoside-binding lectins with β-sandwich folding, play fundamental roles in these processes. We show that among the five chicken galectin (CG) genes, two, CG-1A, and CG-8, are markedly elevated in expression at prospective sites of condensation *in vitro* and *in vivo*, with their protein products appearing earlier in development than any previously described marker. The two molecules enhance one another's gene expression but have opposite effects on condensation formation and cartilage development *in vivo* and *in vitro*: CG-1A, a non-covalent homodimer, promotes this process, while the tandem-repeat-type CG-8 antagonizes it. Correspondingly, knockdown of CG-1A inhibits the formation of skeletal elements while knockdown of CG-8 enhances it. The apparent paradox of mutual activation at the gene expression level coupled with antagonistic roles in skeletogenesis is resolved by analysis of the direct effect of the proteins on precartilage cells. Specifically, CG-1A causes their aggregation, whereas CG-8, which has no adhesive function of its own, blocks this effect. The developmental appearance and regulation of the unknown cell surface moieties ("ligands") to which CG-1A and CG-8 bind were indicative of specific cognate- and cross-regulatory interactions.

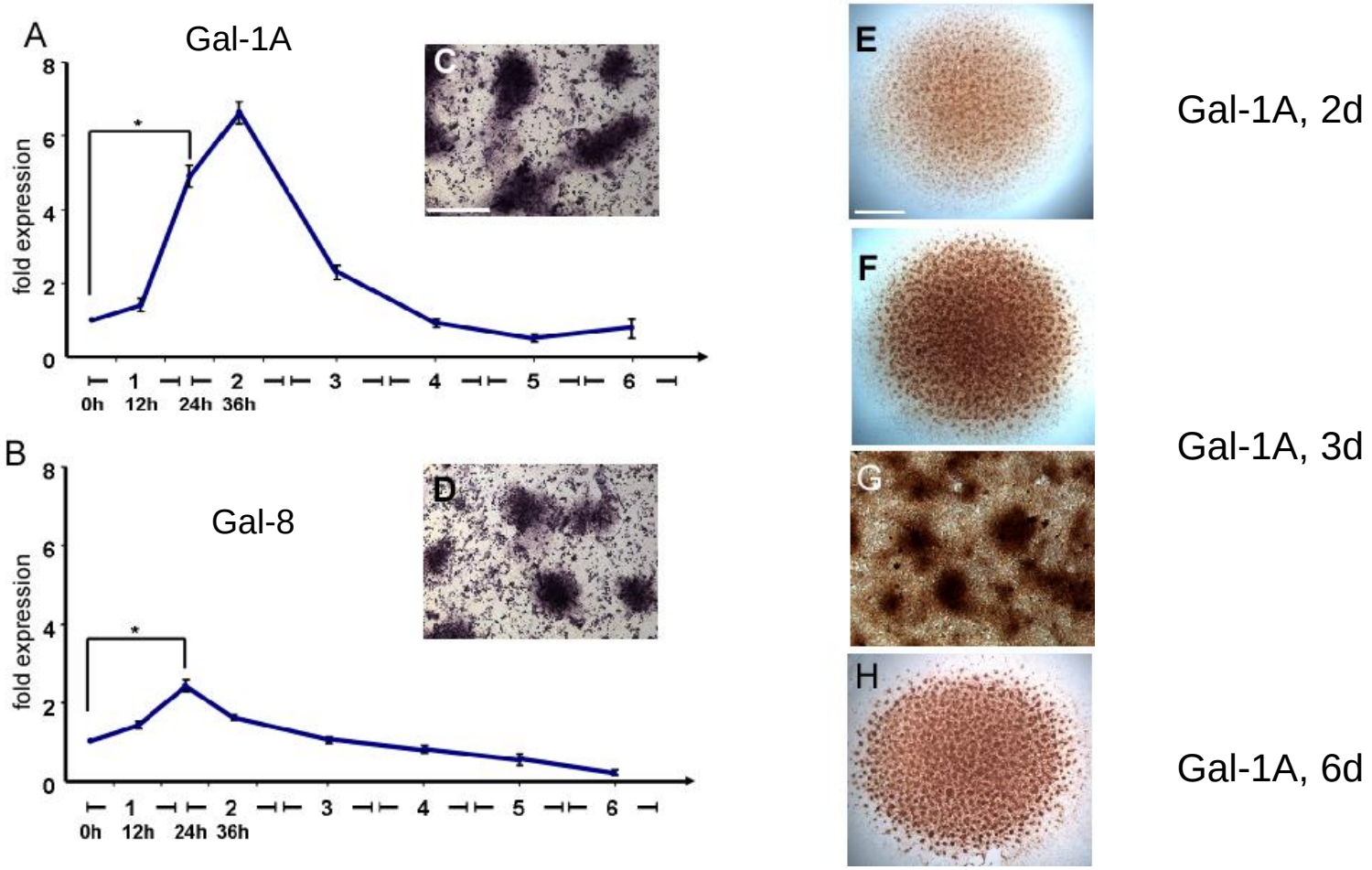
Conclusion: Our findings indicate that CG-1A and CG-8 constitute a multiscale network that is a major mediator, earlier-acting than any previously described, of the formation and patterning of precartilage mesenchymal condensations in the developing limb. This network functions autonomously of limb bud signaling centers or other limb bud positional cues.

Galectins bind to their glycan ligands with a conserved carbohydrate binding domain



Varki et al., *Essentials of Glycobiology* 2009

Gal-1A and Gal-8 are regulated spatiotemporally during chondrogenesis in limb bud micromass cultures



Gal-1A, 2d

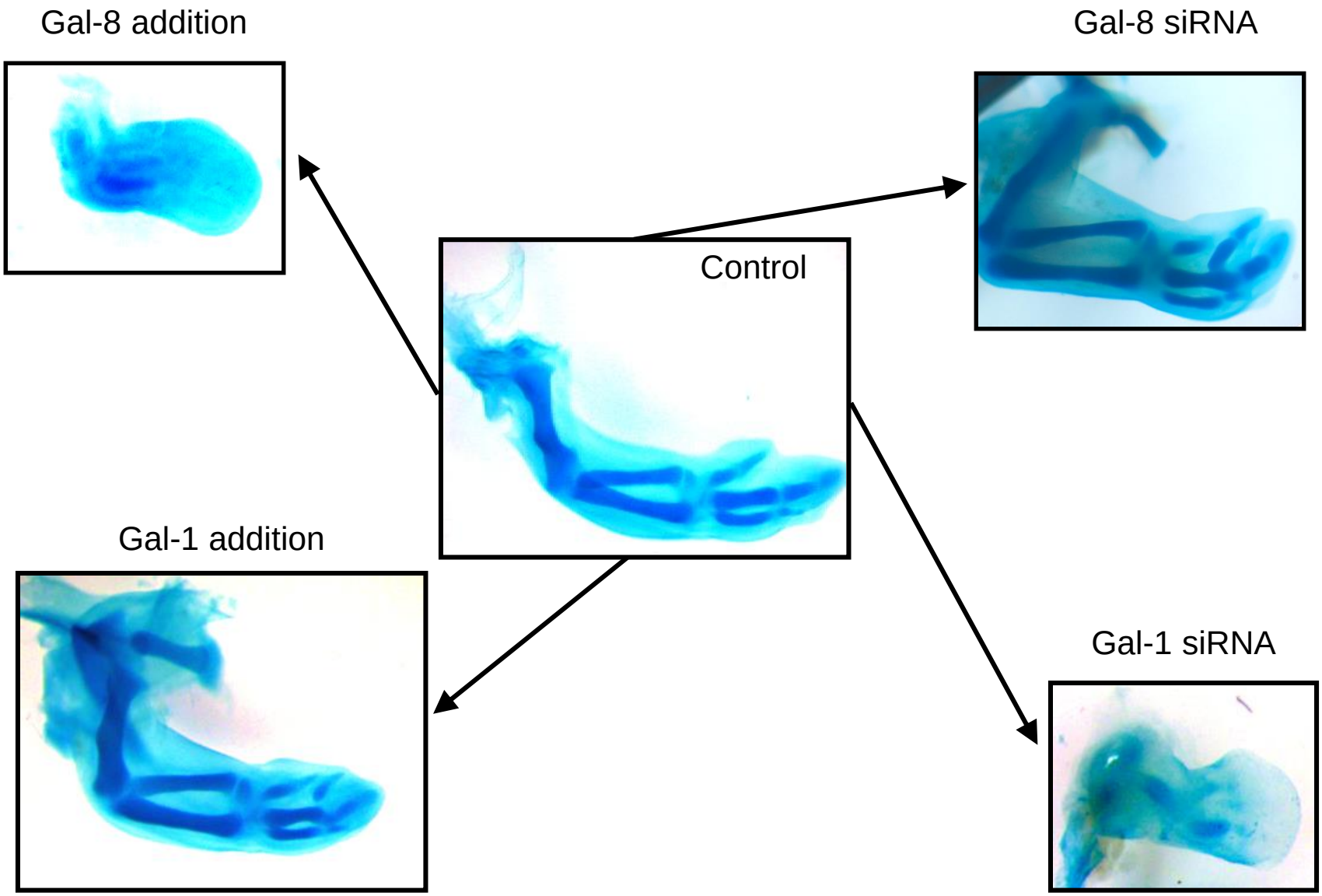
Gal-1A, 3d

Gal-1A, 6d

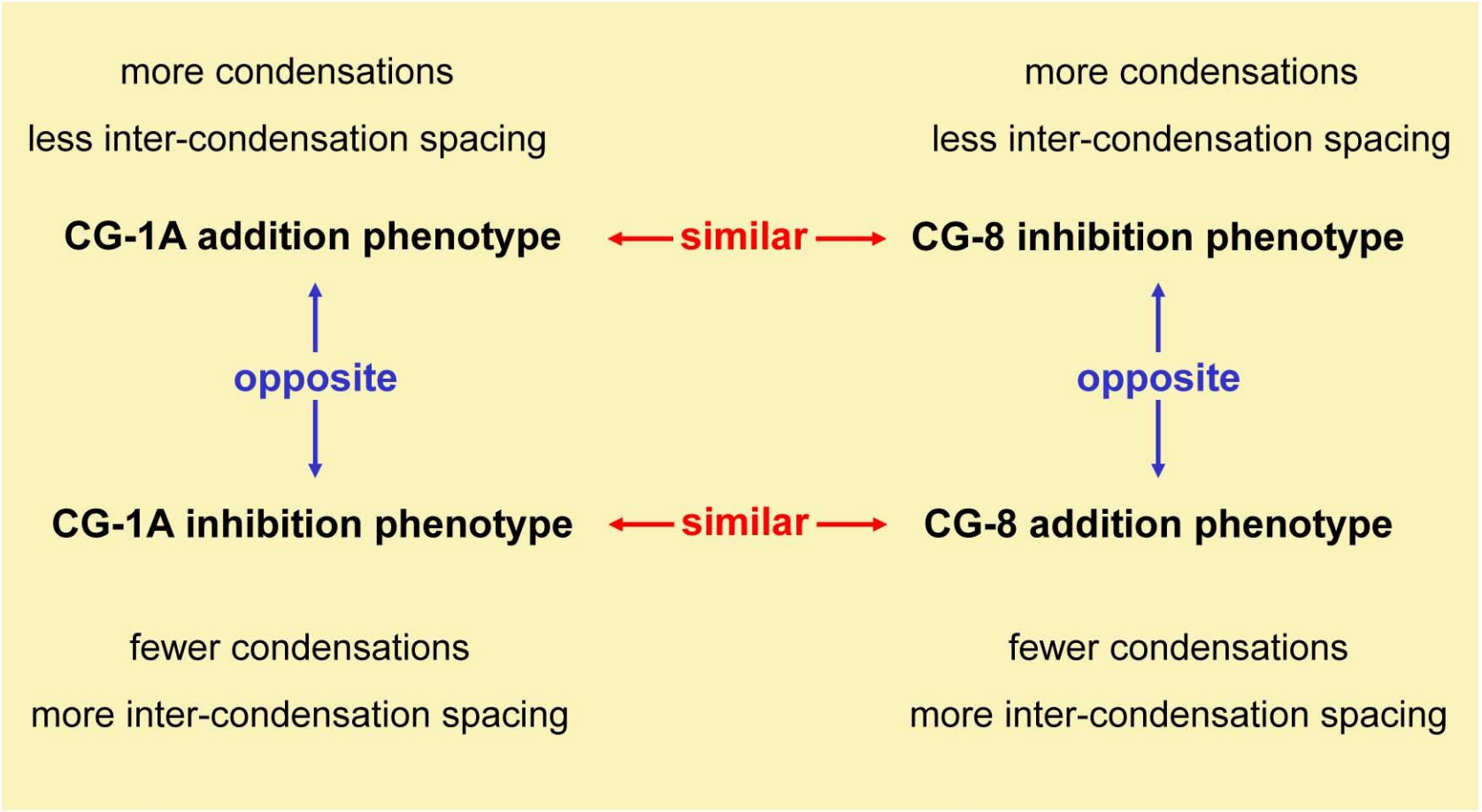
[Gal-1B, -2, and 3 are expressed to much lower or negligible extents during this period]

Bhat et al. *BMC Dev Biol* 2011

Gal-1A/Gal-8: opposing phenotypic effects



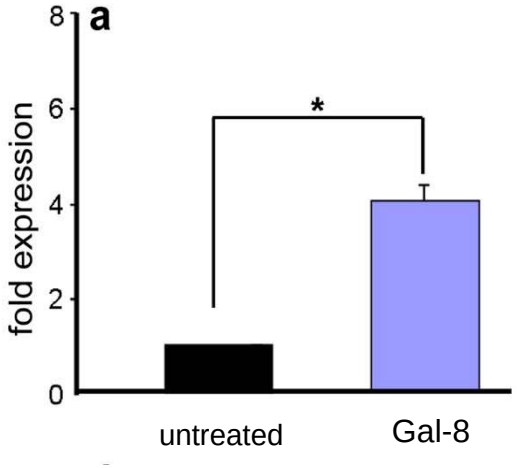
Reciprocal effects of Gal-1A (CG-1A) and Gal-8 (CG-8)



Bhat et al. *BMC Dev Biol* 2011

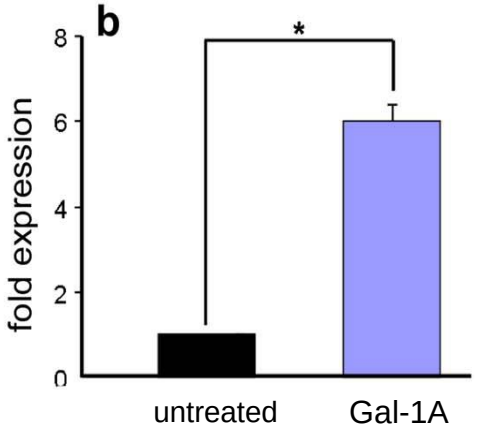
Gal-1A and Gal-8 activate each other's gene expression (despite having opposite effects on skeletogenesis)

Expression of Gal-1A (18 h)

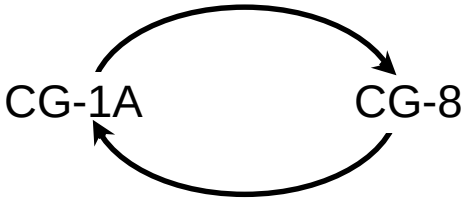


Cells treated with Gal-8

Expression of Gal-8 (18 h)

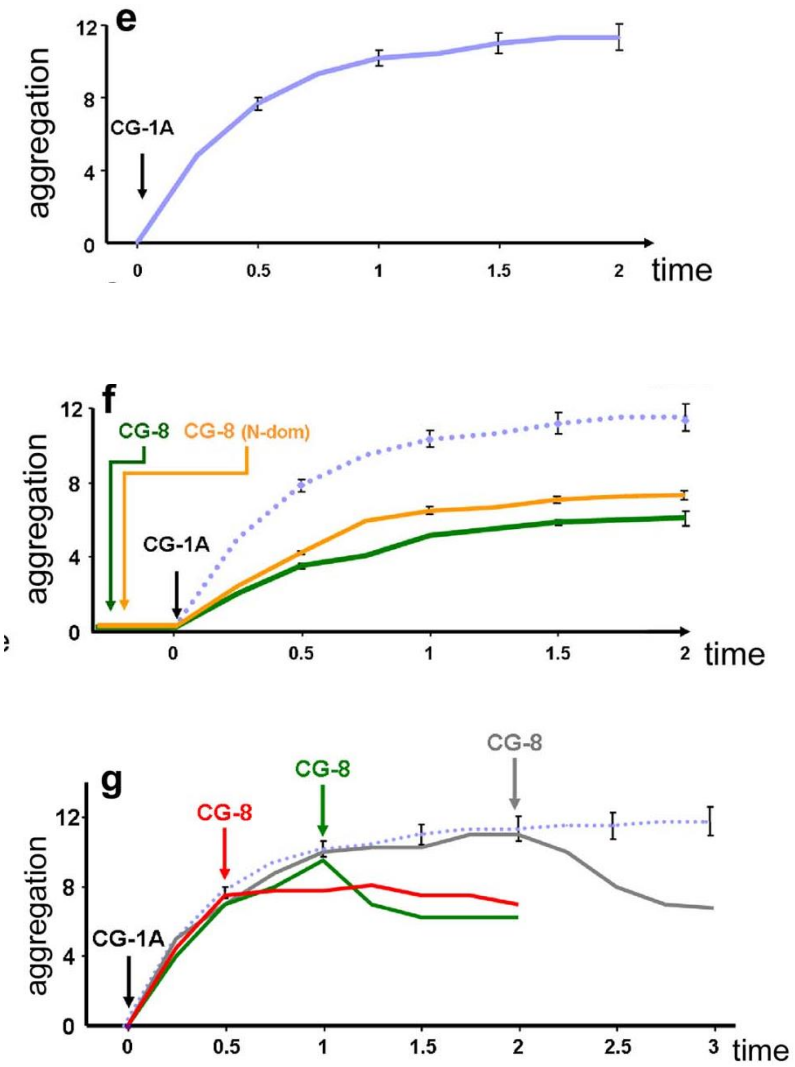


Cells treated with Gal-1A



Bhat et al. *BMC Dev Biol* 2011

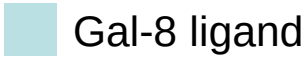
Gal-1A (but not Gal-8), aggregates limb cells; Gal-8 interferes



Bhat et al. *BMC Dev Biol* 2011

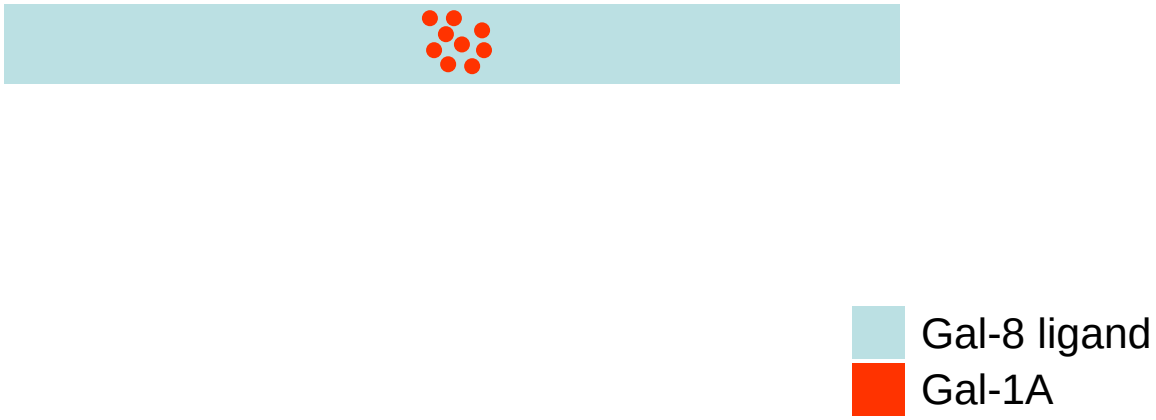
Mesenchymal cell interactions mediated by Gal-1A, Gal-8 and their glycan ligands constitute a pattern-forming network

1. Initial condition: Gal-8 ligand is uniformly distributed.



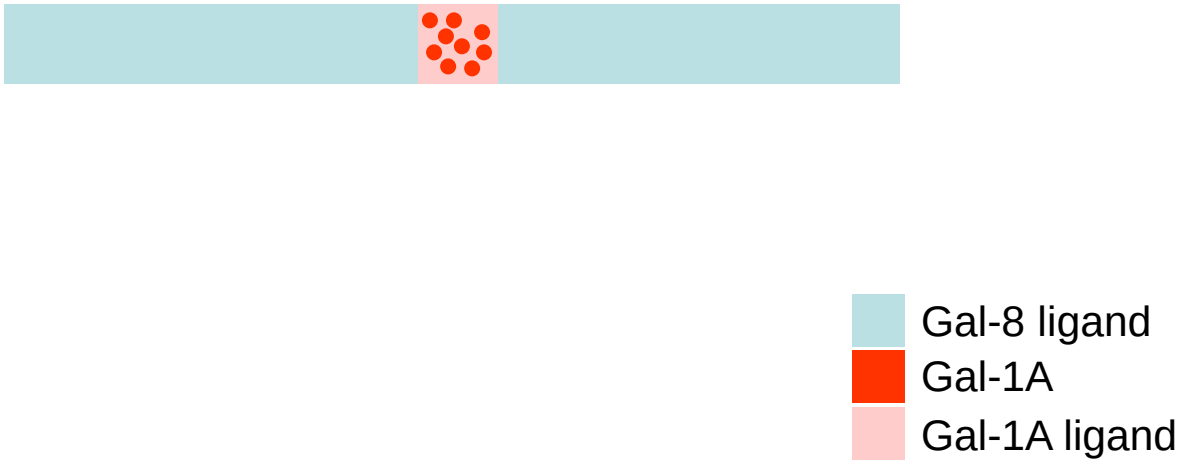
Mesenchymal cell interactions mediated by Gal-1A, Gal-8 and their glycan ligands constitute a pattern-forming network

2. Fluctuation leads to locally elevated Gal-1A expression.



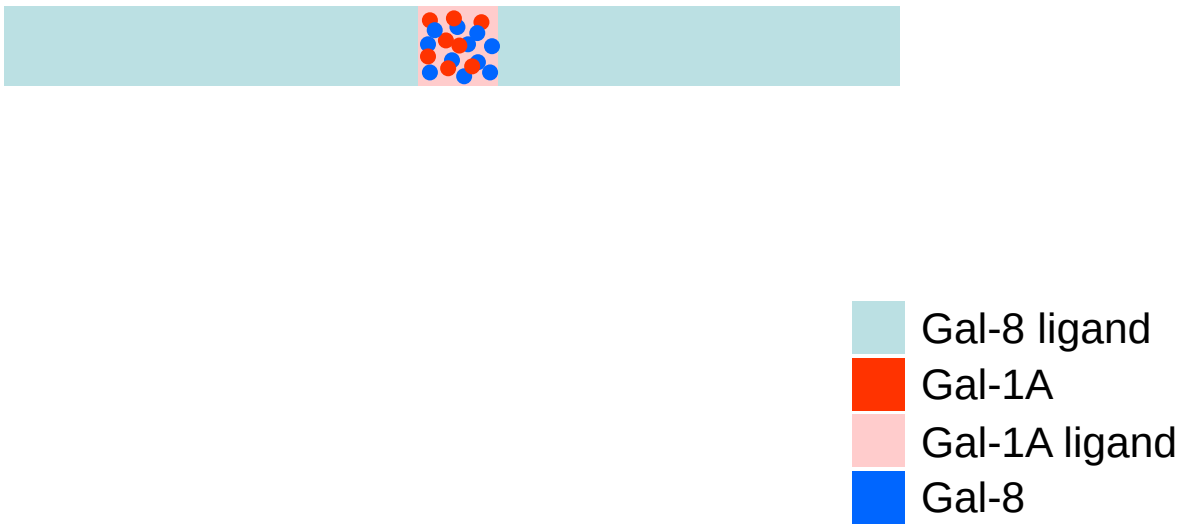
Mesenchymal cell interactions mediated by Gal-1A, Gal-8 and their glycan ligands constitute a pattern-forming network

3. Gal-1A induces its own ligand, which restricts its diffusion.



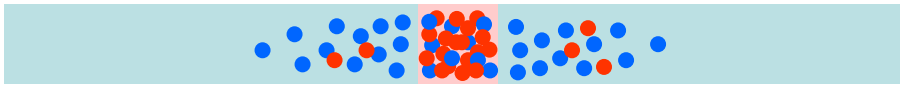
Mesenchymal cell interactions mediated by Gal-1A, Gal-8 and their glycan ligands constitute a pattern-forming network

4. Gal-1A induces the synthesis of Gal-8; Gal-8 induces the synthesis of Gal-1A.



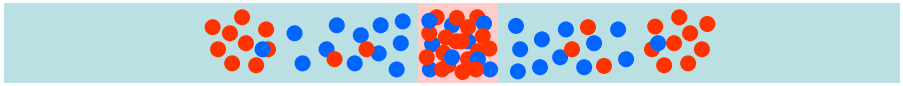
Mesenchymal cell interactions mediated by Gal-1A, Gal-8 and their glycan ligands constitute a pattern-forming network

5. Gal-8 diffuses from its site of production, laterally blocking activity of Gal-1A.



Mesenchymal cell interactions mediated by Gal-1A, Gal-8 and their glycan ligands constitute a pattern-forming network

6. Gal-1A becomes elevated and active at sites distant from original one.



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Modeling the morphodynamic galectin patterning network of the developing avian limb skeleton

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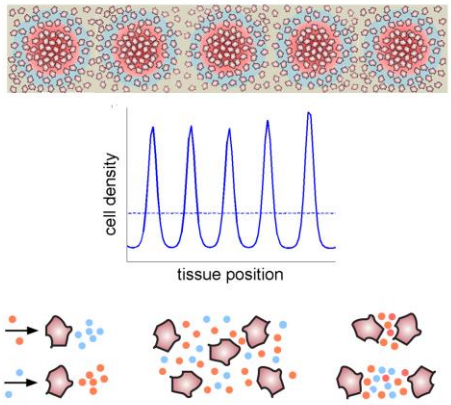


ABSTRACT

We present a mathematical model for the morphogenesis and patterning of the mesenchymal condensations that serve as primordia of the avian limb skeleton. The model is based on the experimentally established dynamics of a multiscale regulatory network consisting of two glycan-binding proteins expressed early in limb development: CG (chicken galectin)-1A, CG-8 and their counterreceptors that determine the formation, size, number and spacing of the "protocondensations" that give rise to the condensations and subsequently the cartilaginous elements that serve as the templates of the bones. The model, a system of partial differential and integro-differential equations containing a flux term to represent local adhesion gradients, is simulated in a "full" and a "reduced" form to confirm that the system has pattern-forming capabilities and to explore the nature of the patterning instability. The full model recapitulates qualitatively and quantitatively the experimental results of network perturbation and leads to new predictions, which are verified by further experimentation. The reduced model is used to demonstrate that the patterning process is inherently morphodynamic, with cell motility being intrinsic to it. Furthermore, subtle relationships between cell movement and the positive and negative interactions between the morphogens produce regular patterns without the requirement for activators and inhibitors with widely separated diffusion coefficients. The described mechanism thus represents an extension of the category of activator–inhibitor processes capable of generating biological patterns with repetitive elements beyond the morphostatic mechanisms of the Turing/Gierer–Meinhardt type.

"Morphodynamic" (i.e., including cell movement) reaction-diffusion-adhesion mechanism for precartilage condensation patterning

Dynamics of cell density

$$\frac{\partial R}{\partial t} = \underbrace{D_R \nabla^2 R}_{\text{cell diffusion}} - \underbrace{\nabla \cdot (R\mathbf{K}(R))}_{\text{cell–cell adhesion}}$$
$$\underbrace{-\frac{\partial}{\partial c_1}(\alpha R) - \frac{\partial}{\partial c_8^8}(\beta_8 R) - \frac{\partial}{\partial c_8^1}(\beta_1 R)}_{\text{binding/unbinding of galectins to counterreceptors}}$$
$$\underbrace{-\frac{\partial}{\partial \ell_1}[(\gamma - \alpha - \beta_1)R] - \frac{\partial}{\partial \ell_8}[(\delta - \beta_8)R]}_{\text{change in counterreceptors}}$$

Dynamics of Gal-1A

$$\frac{\partial c_1^u}{\partial t} = \underbrace{D_1 \nabla^2 c_1^u}_{\text{diffusion}} + \underbrace{\overline{\nu} \int c_8^8 R \, dP - \int \alpha R \, dP}_{\substack{\text{pos. feedback of CG–8 on prod. of CG–1A} \\ \text{binding of CG–1A to its counterreceptor}}} \underbrace{-\overline{\pi}_1 c_1^u}_{\text{degradation}}$$

$$\frac{\partial c_8^u}{\partial t} = \underbrace{D_8 \nabla^2 c_8^u}_{\text{diffusion}} + \underbrace{\overline{\mu} \int c_1 R \, dP - \int \beta_1 R \, dP - \int \beta_8 R \, dP}_{\substack{\text{pos. feedback of CG–1A on prod. of CG–8} \\ \text{binding of CG–8 to counterreceptors}}} \underbrace{-\overline{\pi}_8 c_8^u}_{\text{degradation}}$$

Dynamics of Gal-8

Glimm et al. *J Theor Biol* 2013

"Morphodynamic" (i.e., including cell movement) reaction-diffusion-adhesion mechanism for precartilage condensation patterning

Dynamics of cell density

$$\frac{\partial R}{\partial t} = \underbrace{D_R \nabla^2 R}_{\text{cell diffusion}} - \underbrace{\nabla \cdot (R\mathbf{K}(R))}_{\text{cell–cell adhesion}}$$

$$\underbrace{-\frac{\partial}{\partial c_1}(\alpha R) - \frac{\partial}{\partial c_8^8}(\beta_8 R) - \frac{\partial}{\partial c_8^1}(\beta_1 R)}_{\text{binding/unbinding of galectins to counterreceptors}}$$

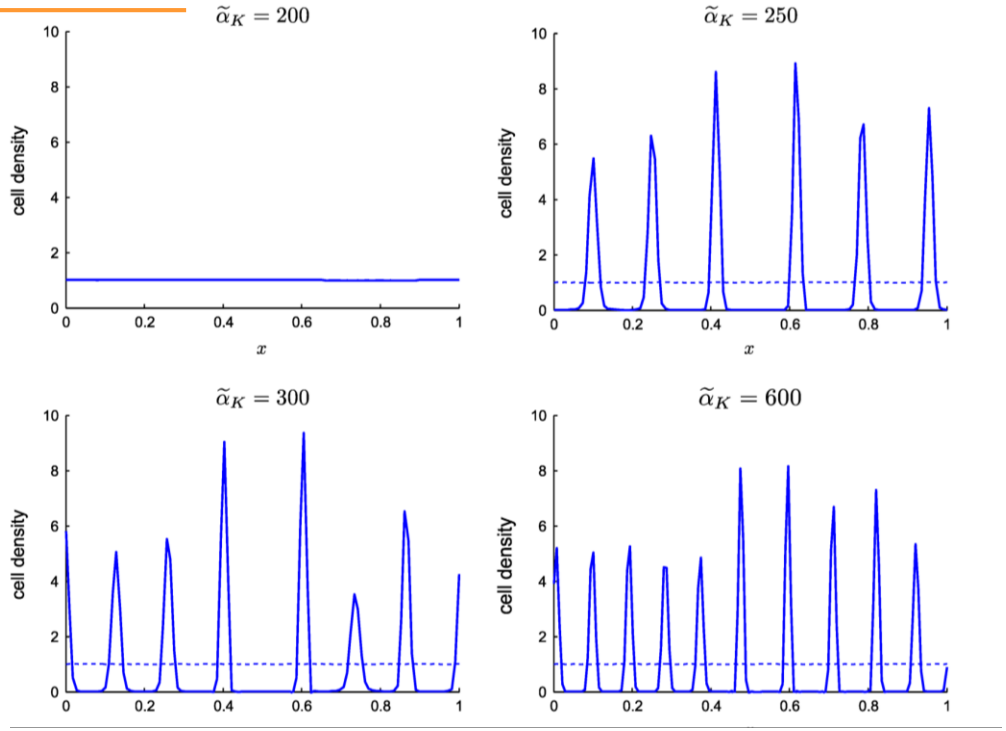
$$\underbrace{-\frac{\partial}{\partial \ell_1}[(\gamma - \alpha - \beta_1)R] - \frac{\partial}{\partial \ell_8}[(\delta - \beta_8)R]}_{\text{change in counterreceptors}}$$

Dynamics of Gal-1A

$$\frac{\partial c_1^u}{\partial t} = \underbrace{D_1 \nabla^2 c_1^u}_{\text{diffusion}} + \underbrace{\overline{\nu} \int c_8^8 R \, dP - \int \alpha R \, dP}_{\substack{\text{pos. feedback of CG–8 on prod. of CG–1A} \\ \text{binding of CG–1A to its counterreceptor}}} \underbrace{-\overline{\pi}_1 c_1^u}_{\text{degradation}}$$

Dynamics of Gal-8

$$\frac{\partial c_8^u}{\partial t} = \underbrace{D_8 \nabla^2 c_8^u}_{\text{diffusion}} + \underbrace{\overline{\mu} \int c_1 R \, dP - \int \beta_1 R \, dP - \int \beta_8 R \, dP}_{\substack{\text{pos. feedback of CG–1A on prod. of CG–8} \\ \text{binding of CG–8 to counterreceptors}}} \underbrace{-\overline{\pi}_8 c_8^u}_{\text{degradation}}$$



Dependence of patterning on adhesion term

Glimm et al. *J Theor Biol* 2013

Genome Biology and Evolution

SMBE

GBE

Structural Divergence in Vertebrate Phylogeny of a Duplicated Prototype Galectin

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Associate editor: Andreas Wagner

Accepted: September 20, 2014

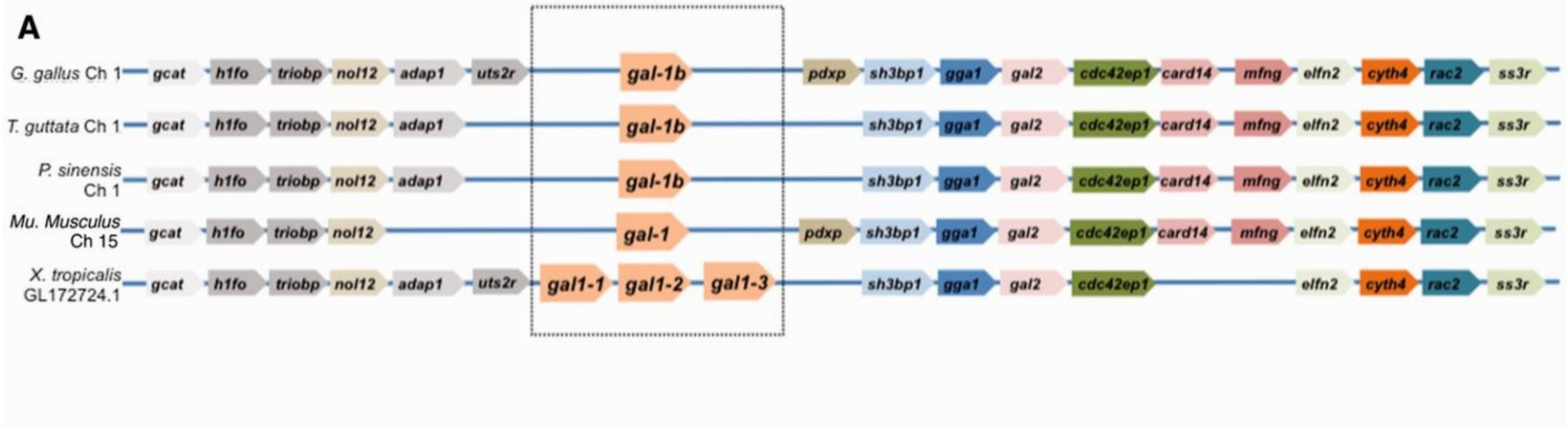
Data deposition: No new sequences have been obtained through empirical analysis in the course of study. Whatever sequences were used had been previously deposited and annotated in databases like ENSEMBL. In the supplementary file S1, Supplementary Material online, we have provided their ENSEMBL or GenBank IDs as well as the sequences.

Abstract

Prototype galectins, endogenously expressed animal lectins with a single carbohydrate recognition domain, are well-known regulators of tissue properties such as growth and adhesion. The earliest discovered and best studied of the prototype galectins is Galectin-1 (Gal-1). In the *Gallus gallus* (chicken) genome, Gal-1 is represented by two homologs: Gal-1A and Gal-1B, with distinct biochemical properties, tissue expression, and developmental functions. We investigated the origin of the Gal-1A/Gal-1B divergence to gain insight into when their developmental functions originated and how they could have contributed to vertebrate phenotypic evolution. Sequence alignment and phylogenetic tree construction showed that the Gal-1A/Gal-1B divergence can be traced back to the origin of the sauropsid lineage (consisting of extinct and extant reptiles and birds) although lineage-specific duplications also occurred in the amphibian and actinopterygian genomes. Gene synteny analysis showed that sauropsid *gal-1b* (the gene for Gal-1B) and its frog and actinopterygian *gal-1* homologs share a similar chromosomal location, whereas sauropsid *gal-1a* has translocated to a new position. Surprisingly, we found that chicken Gal-1A, encoded by the translocated *gal-1a*, was more similar in its tertiary folding pattern than Gal-1B, encoded by the untranslocated *gal-1b*, to experimentally determined and predicted folds of nonsauropsid Gal-1s. This inference is consistent with our finding of a lower proportion of conserved residues in sauropsid Gal-1Bs, and evidence for positive selection of sauropsid *gal-1b*, but not *gal-1a* genes. We propose that the duplication and structural divergence of Gal-1B away from Gal-1A led to specialization in both expression and function in the sauropsid lineage.

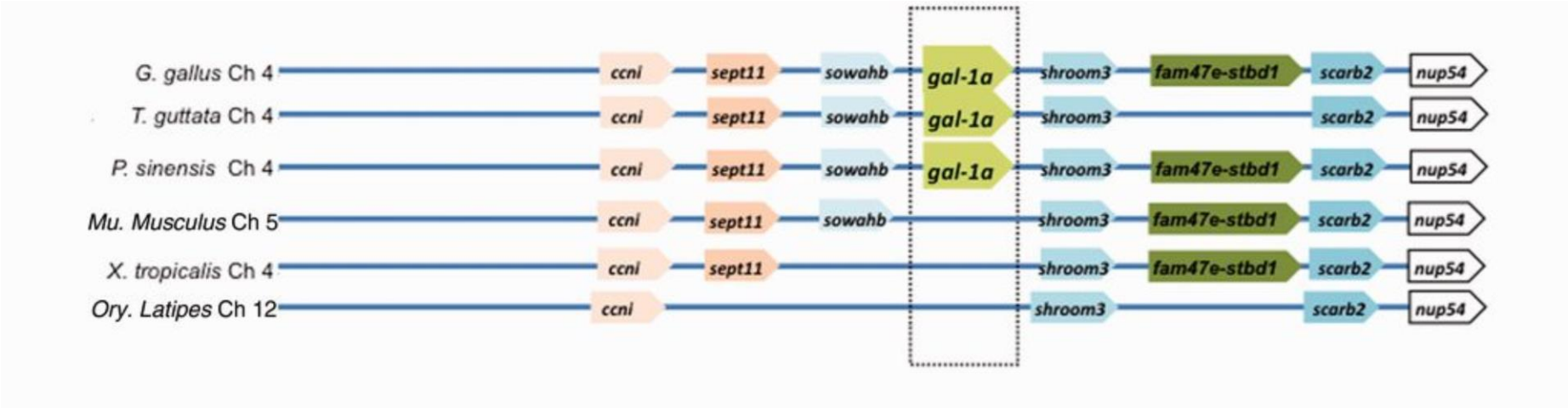
Key words: prototype galectin, galectin-1, sauropsids, protein fold, homology.

Synteny of the *gal-1b* gene and its non-sauropsid homologs



Bhat et al. *Genome Biol & Evol* 2014

Synteny of the sauropsid *gal-1a* genes



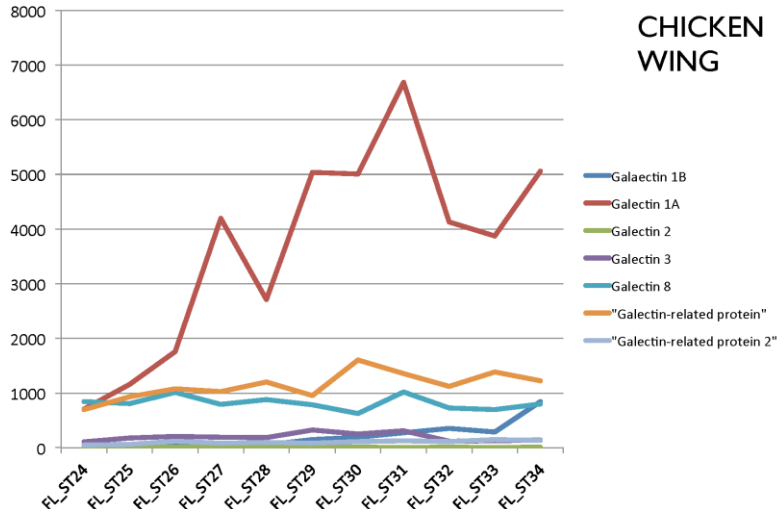
The *gal-1a* gene (sauropsids only) is on a different chromosome from other gal-1s

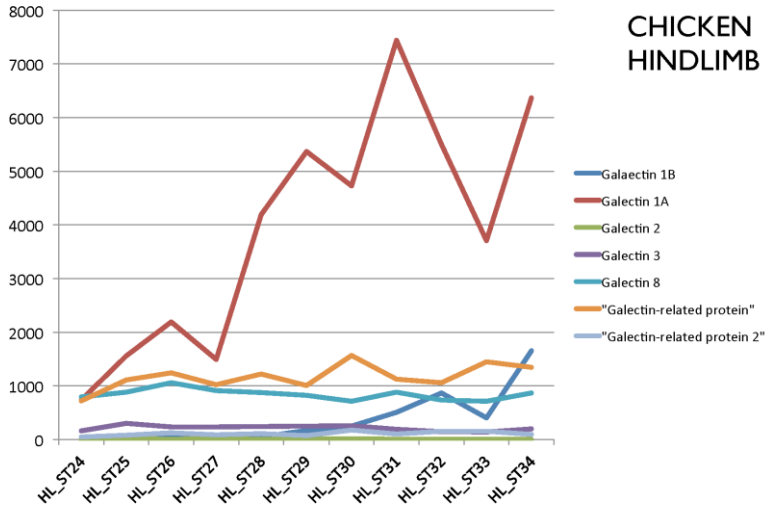
Bhat et al. *Genome Biol & Evol* 2014

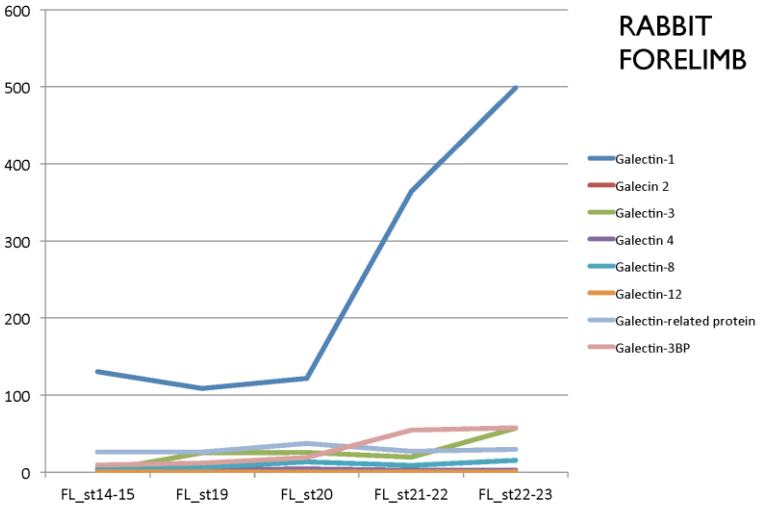
Conclusions - 1

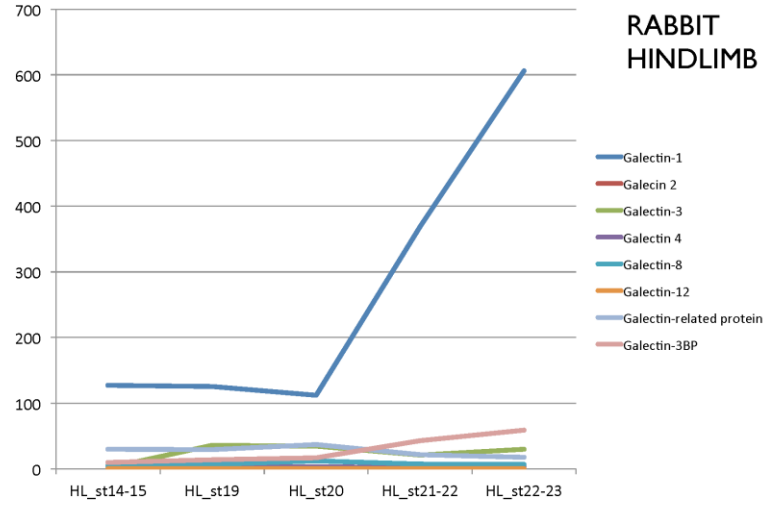
- The gal-1 gene underwent duplications and divergent evolution in the amphibians.
- One gal-1 gene (*gal-1a*) translocated to a new locus at the origin of the sauropsids. Its protein product, Gal-1A, retained the 3D structure found in at least one Gal-1 of all vertebrate species, and is **skeletogenic** in birds.
- The gene that remained at the ancestral locus in the sauropsids (*gal-1b*) evolved to specify a non-skeletogenic protein, Gal-1B.
- Mammals have a single gal-1 gene (no duplication or translocation) at the ancestral (*gal-1b*) locus. Its protein product retains the presumed skeletogenic 3D structure.

Rabbit Gal-1 is expressed similarly to chicken Gal-1A during limb development



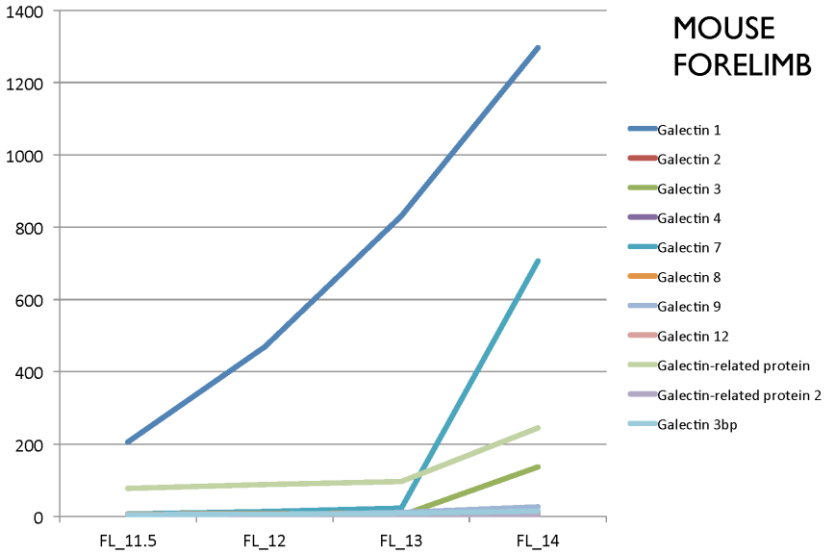


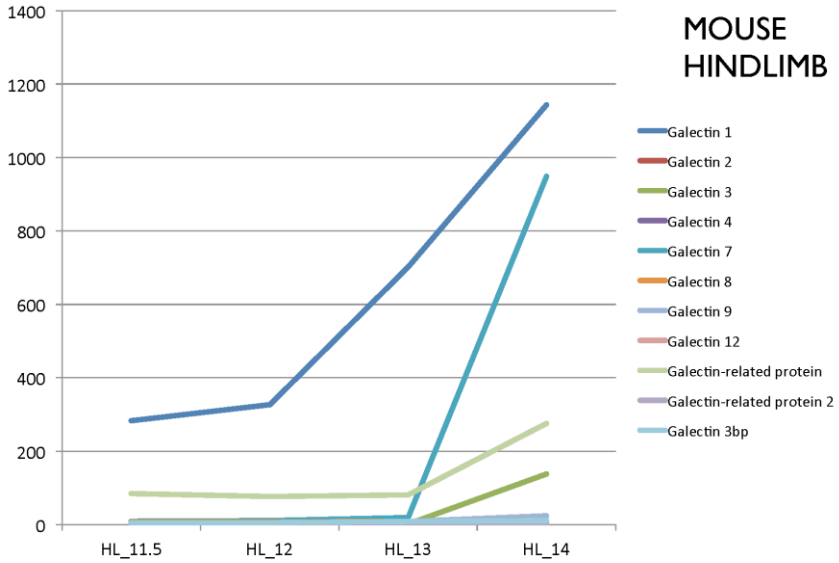




T. Stewart and G. Wagner

Mouse galectin-1 is expressed similarly to chicken galectin-1a during limb development, but so is galectin-7





Developmental system drift?

T. Stewart and G. Wagner

Bhat et al. BMC Evolutionary Biology (2016) 16:162
DOI 10.1186/s12862-016-0729-6

BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

Deep phylogenomics of a tandem-repeat galectin regulating appendicular skeletal pattern formation

Ramray Bhat[1,7*], Mahul Chakraborty[2], Tilmann Glimm[3], Thomas A. Stewart[4,5] and Stuart A. Newman[6*]

Abstract

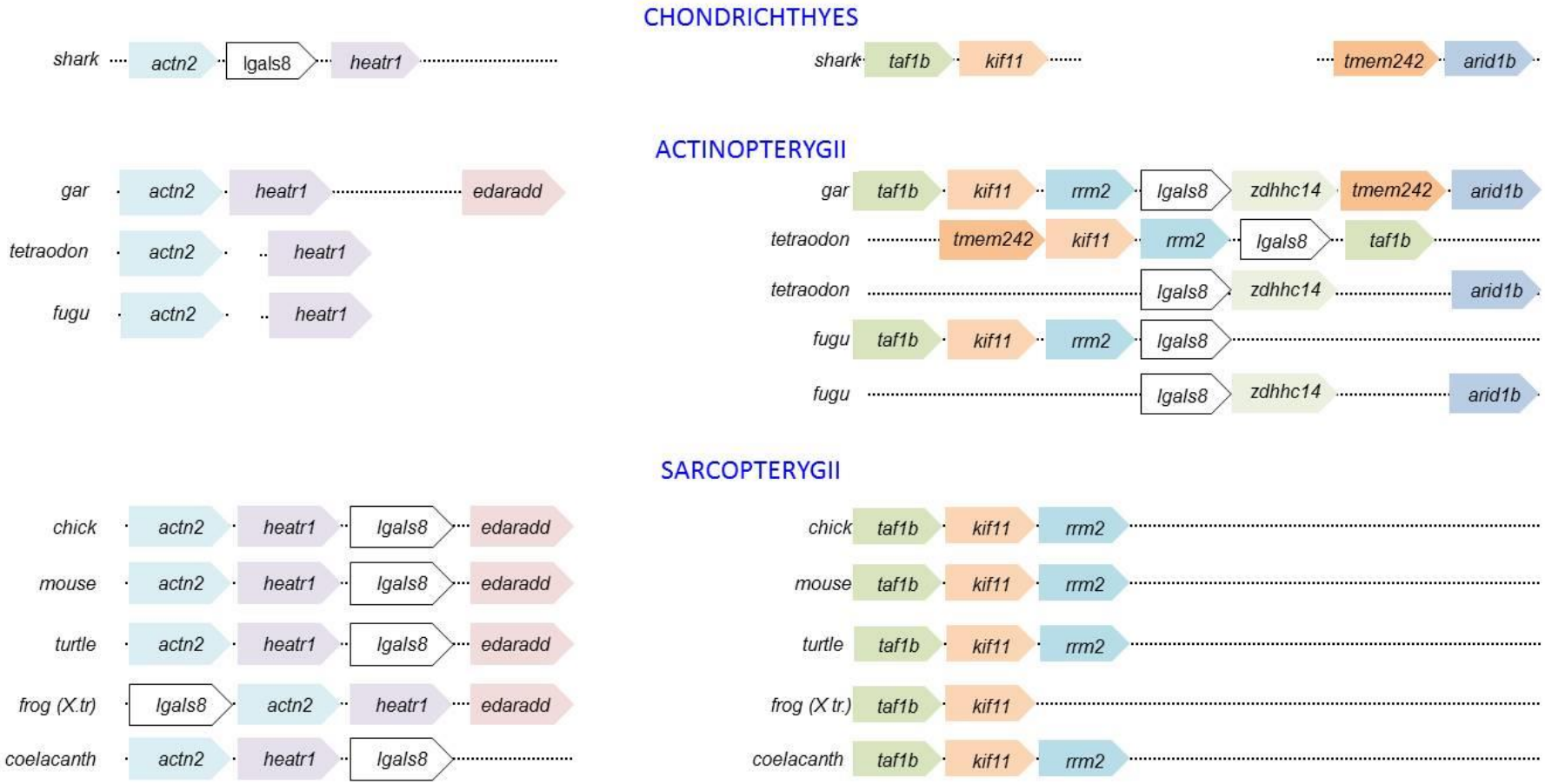
Background: A multiscale network of two galectins Galectin-1 (Gal-1) and Galectin-8 (Gal-8) patterns the avian limb skeleton. Among vertebrates with paired appendages, chondrichthyan fins typically have one or more cartilage plates and many repeating parallel endoskeletal elements, actinopterygian fins have more varied patterns of nodules, bars and plates, while tetrapod limbs exhibit tandem arrays of few, proximodistally increasing numbers of elements. We applied a comparative genomic and protein evolution approach to understand the origin of the galectin patterning network. Having previously observed a phylogenetic constraint on Gal-1 structure across vertebrates, we asked whether evolutionary changes of Gal-8 could have critically contributed to the origin of the tetrapod pattern.

Results: Translocations, duplications, and losses of Gal-8 genes in Actinopterygii established them in different genomic locations from those that the Sarcopterygii (including the tetrapods) share with chondrichthyans. The sarcopterygian Gal-8 genes acquired a potentially regulatory non-coding motif and underwent purifying selection. The actinopterygian Gal-8 genes, in contrast, did not acquire the non-coding motif and underwent positive selection.

Conclusion: These observations interpreted through the lens of a reaction-diffusion-adhesion model based on avian experimental findings can account for the distinct endoskeletal patterns of cartilaginous, ray-finned, and lobe-finned fishes, and the stereotypical limb skeletons of tetrapods.

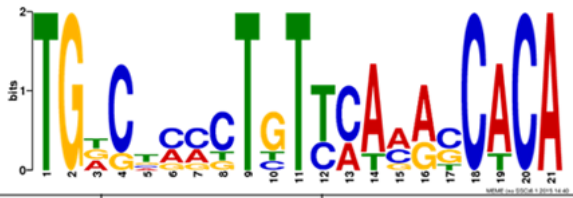
Keywords: Galectin-8, Limb skeleton, Pattern formation, Mathematical modeling, Homology, Phylogeny

The synteny of gal-8*s* in cartilaginous and lobe-finned fish (incl. tetrapods) are similar to each other, but *gal-8* jumped to a different chromosome in ray-finned fish



Bhat et al. *BMC Evol Biol* 2016

Gal-8s of lobe-finned fish (incl. tetrapods) acquired a limb-related conserved noncoding module (NCM) that is not present in cartilaginous or ray-finned fish



Clade	Species	P value of homolog(s)
Sarcopterygii	*G. gallus*	5.98e-10
	T. guttata	1.01e-08
	P. sinensis	5.27e-10
	M. musculus	9.46e-09
	X. tropicalis	1.29e-11
	L. chalumnae	4.50e-08
Actinopterygii	*G. aculeatus*	3.08e-01, 2.09e-01
	O. latipes	undetectable, undetectable
	T. rubripes	7.76e-01, 5.21e-01
	T. negroviridis	4.11e-01, 4.86e-01
	D. rerio	7.93e-01, undetectable
	L. oculatus	2.40e-01
Chondrichthyes	*C. milii*	4.04e-01

The NCM contains binding sites for Meis1, Tcfcp2l1, Runx1 and Runx2. All are mesenchymally expressed during limb development and Meis1 and Runx2 regulate PD patterning.

Bhat et al. *BMC Evol Biol* 2016

Conclusions - 2

- The gal-8 gene (arising in basal chordates), underwent a translocation around the origin of the ray-finned fishes.

- Gal-8 proteins in cartilaginous and lobe-finned fishes evolved 3D structures **similar that of skeletogenic Gal-1**, and thus capable of competing with it for its cell surface receptor. Ray-finned fish Gal-8s generally did not evolve to compete with their skeletogenic Gal-1 homologs.

- The gal-8 genes of lobe-finned fishes (including tetrapods), acquired a novel **conserved noncoding module** that enabled them to be quantitatively regulated in developing appendages.

Reaction-diffusion-adhesion mechanism for condensation pattern formation

Dynamics of cell density

$$\frac{\partial R}{\partial t} = \underbrace{D_R \nabla^2 R}_{\text{cell diffusion}} - \underbrace{\nabla \cdot (R\mathbf{K}(R))}_{\text{cell–cell adhesion}} \underbrace{-\frac{\partial}{\partial c_1}(\alpha R) - \frac{\partial}{\partial c_8^8}(\beta_8 R) - \frac{\partial}{\partial c_8^1}(\beta_1 R)}_{\text{binding/unbinding of galectins to counterreceptors}} \underbrace{-\frac{\partial}{\partial \ell_1}[(\gamma - \alpha - \beta_1)R] - \frac{\partial}{\partial \ell_8}[(\delta - \beta_8)R]}_{\text{change in counterreceptors}}$$

Dynamics of Gal-1A

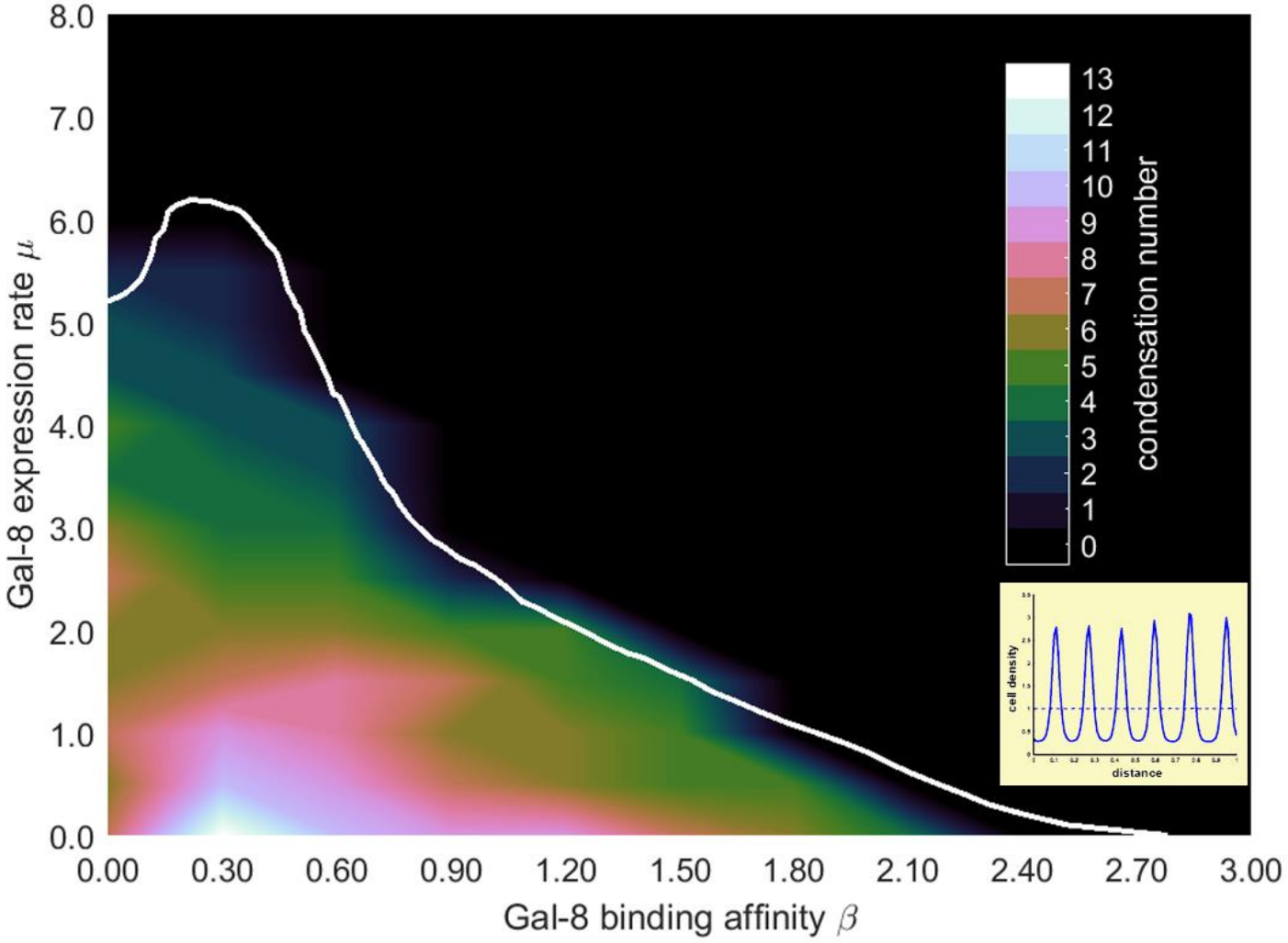
$$\frac{\partial c_1^u}{\partial t} = \underbrace{D_1 \nabla^2 c_1^u}_{\text{diffusion}} \underbrace{+\overline{\nu} \int c_8^8 R \, dP - \int \alpha R \, dP}_{\substack{\text{pos. feedback of CG–8} \\ \text{on prod. of CG–1A}} \quad \substack{\text{binding of CG–1A} \\ \text{to its counterreceptor}}} \underbrace{-\overline{\pi}_1 c_1^u}_{\text{degradation}}$$

$$\frac{\partial c_8^u}{\partial t} = \underbrace{D_8 \nabla^2 c_8^u}_{\text{diffusion}} \underbrace{+\overline{\mu} \int c_1 R \, dP}_{\substack{\text{pos. feedback of CG–1A} \\ \text{on prod. of CG–8}}} \underbrace{- \int \beta_1 R \, dP - \int \beta_8 R \, dP}_{\substack{\text{binding of CG–8} \\ \text{to counterreceptors}}} \underbrace{-\overline{\pi}_8 c_8^u}_{\text{degradation}}$$

Dynamics of Gal-8

Glimm et al. *J Theor Biol* 2013

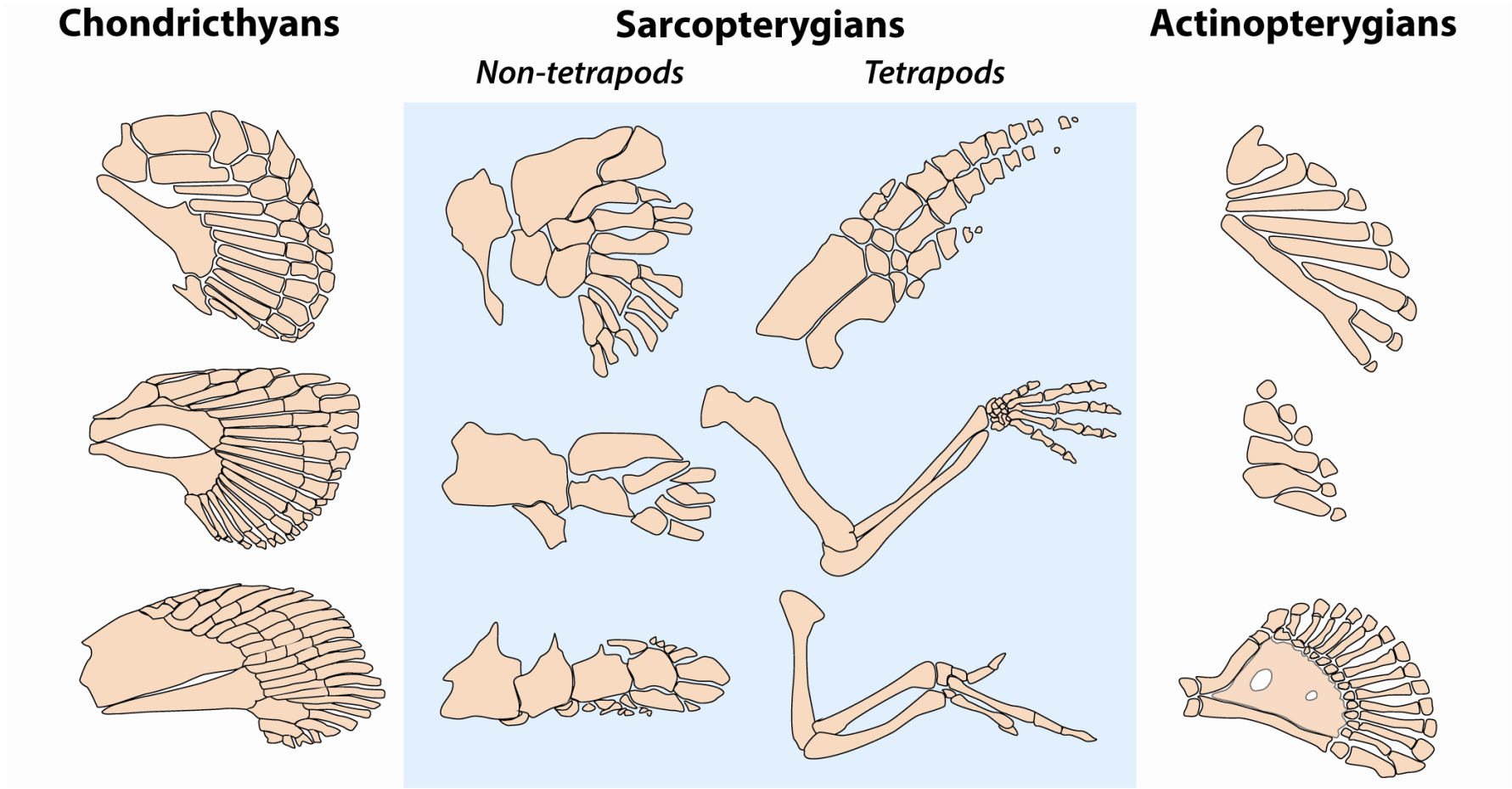
Condensation-permissive parameter space of two-galectin network: Dependence of patterns on μ (expression rate of Gal-8) and β (binding affinity of Gal-8 to its common receptor with Gal-1)



Bhat et al. *BMC Evol Biol* 2016

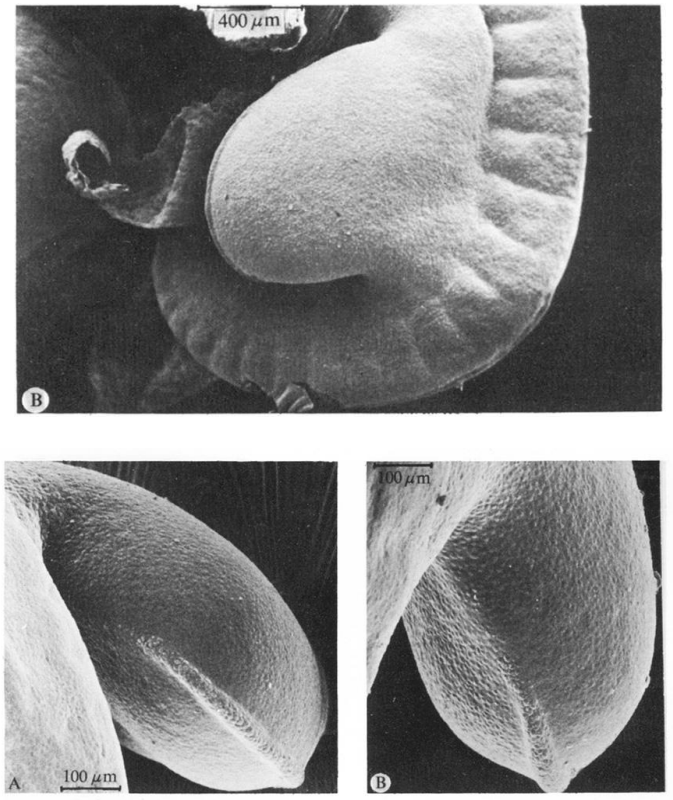
Conclusions - 3

- For Gal-8 proteins of intermediate binding affinity to their shared receptor to skeletogenic Gal-1 (specified by μ), the entire numerical range of parallel skeletal elements is possible.
- Different levels of *gal-8* expression (governed by β) lead to different numbers of skeletal elements.
- Only in sarcopterygians (lobe-finned fish, including tetrapods) has there evolved appropriate μ values and control of β to enable proximodistal increase in element number during development.



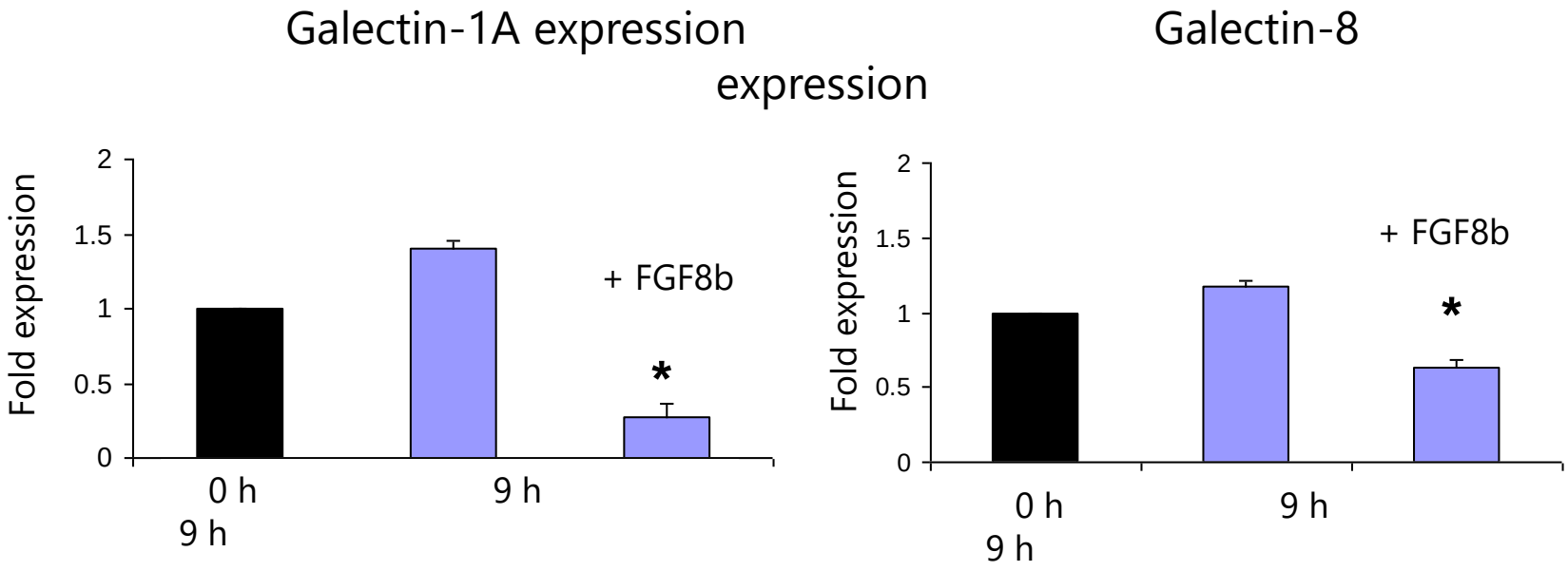
Bhat et al. *BMC Evol Biol,* in press

Dorsal and ventral limb ectoderm and apical ectodermal ridge (AER), a source of FGF



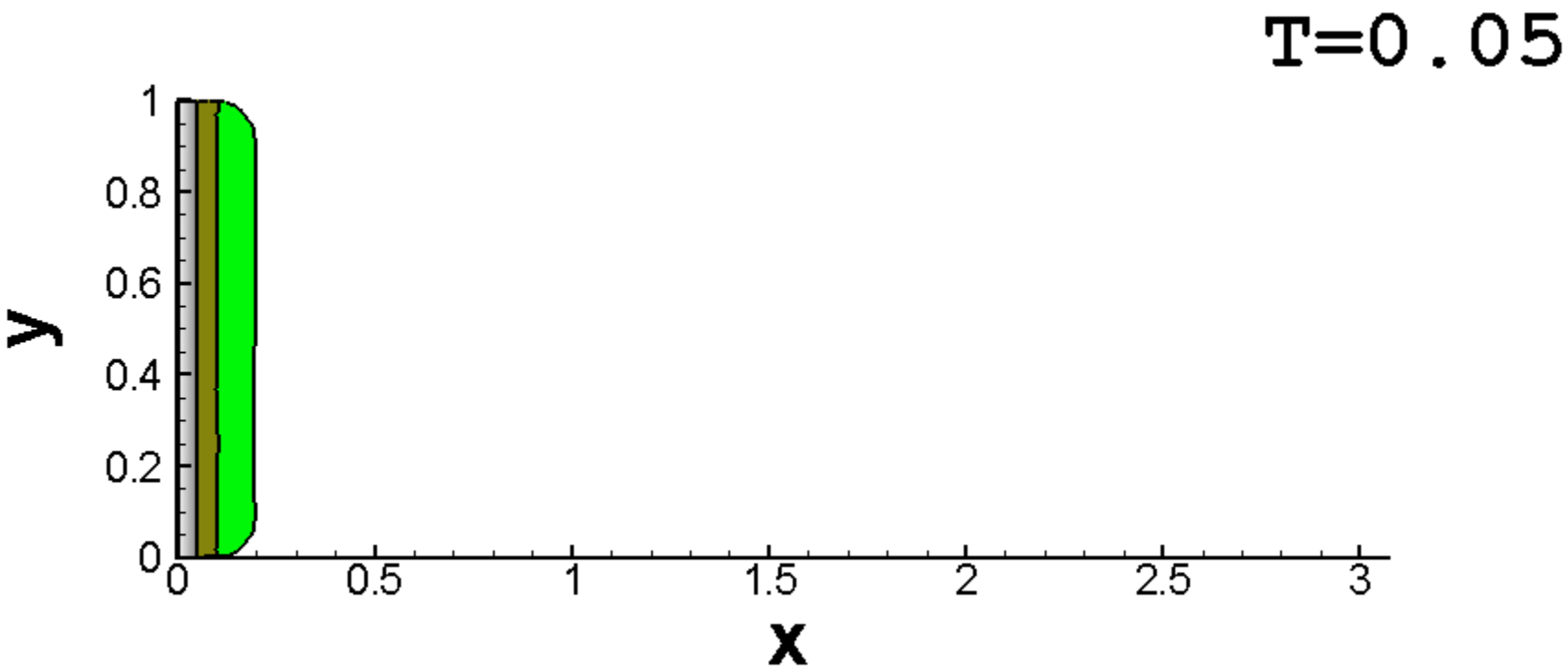
Hinchliffe & Johnson, 1980

Effect of FGF8 on expression of “pattern network” galectins



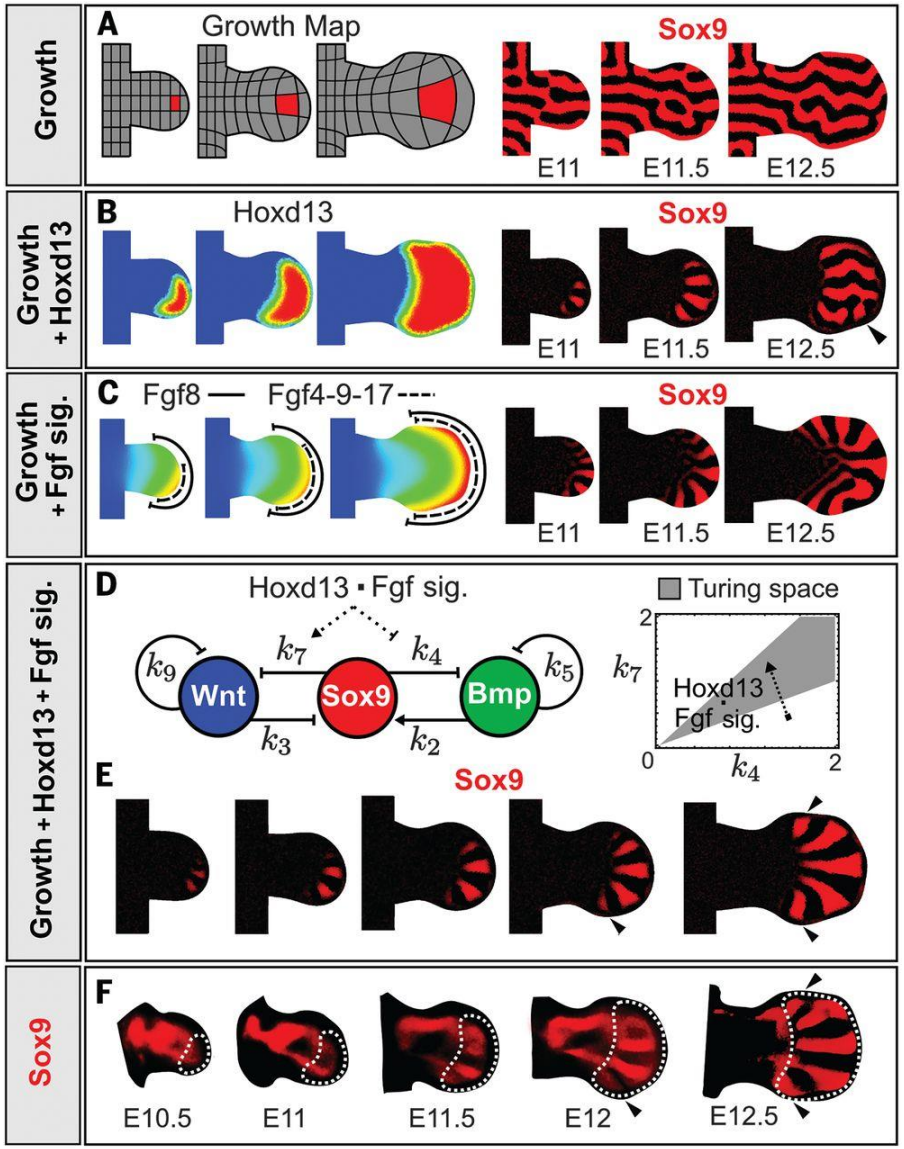
R. Bhat

Simulation of a skeletogenic R-D system with FGF-suppression of morphogenesis at the tip



Zhu et al., *PLoS One*; 2010

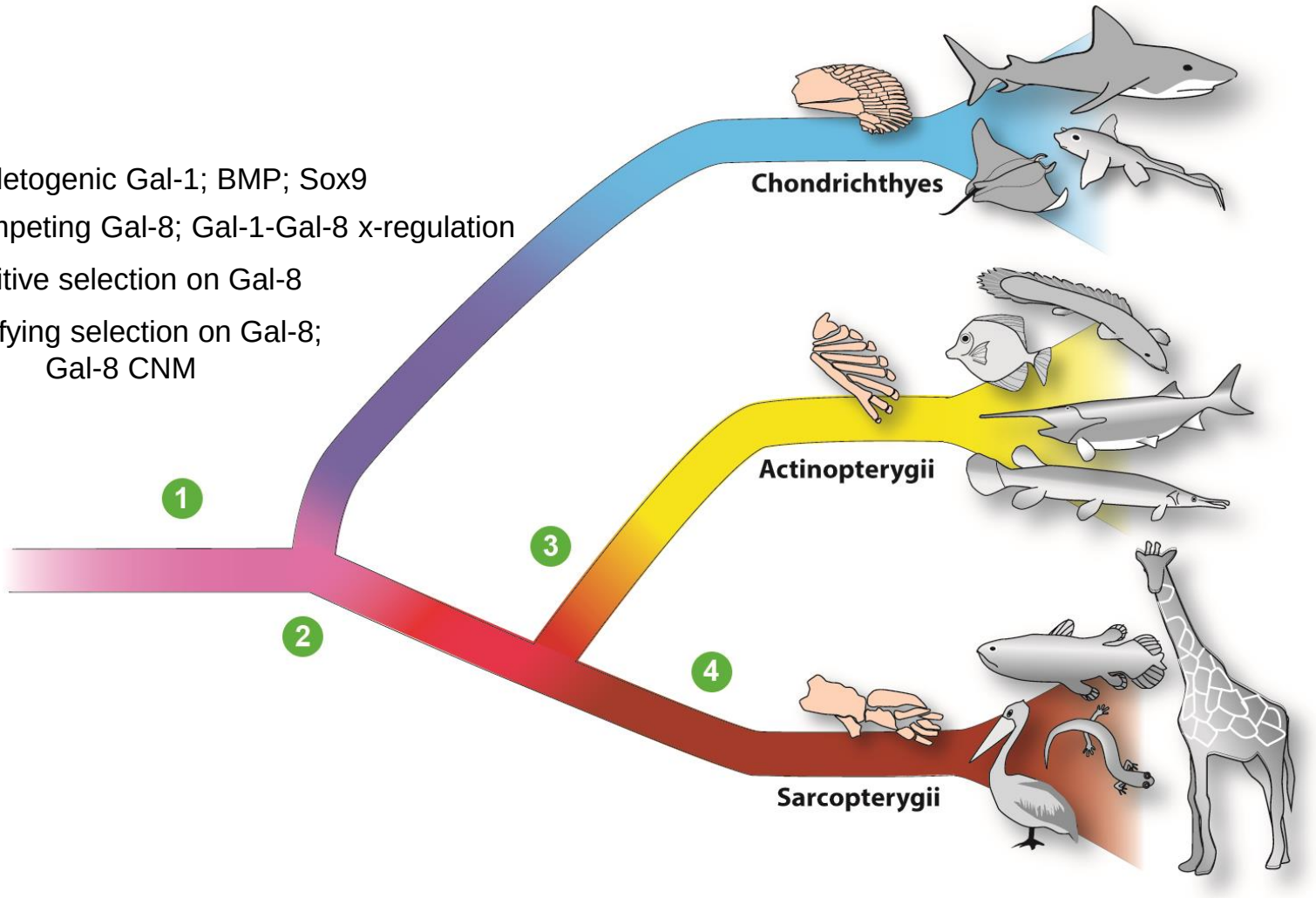
BMP-Sox9-Wnt (BSW) skeletal pattern-forming network



Raspopovic et al. *Science* 2014

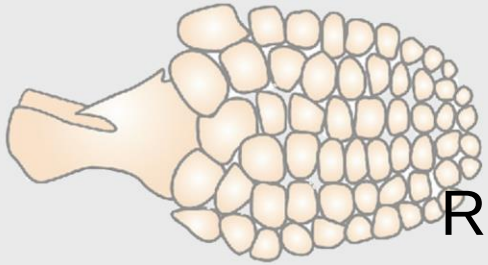
Hypothesized scenario for acquisition of regulatory networks leading to the fin-limb transition

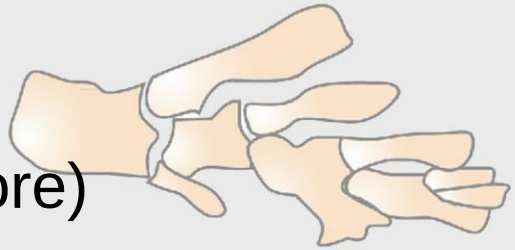
1. Skeletogenic Gal-1; BMP; Sox9
2. Competing Gal-8; Gal-1-Gal-8 x-regulation
3. Positive selection on Gal-8
4. Purifying selection on Gal-8; Gal-8 CNM



Newman et al., *PBMB* 2018

Collaborators





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