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

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ABSTRACT

The paired appendages (fins or limbs) of jawed vertebrates contain an endoskeleton consisting of nodules, bars and, in some groups, plates of cartilage, or bone arising from replacement of cartilaginous templates. The generation of the endoskeletal elements occurs by processes involving production and diffusion of morphogens, with, variously, positive and negative feedback circuits, adhesion, and receptor dynamics with similarities to the mechanism for chemical pattern formation proposed by Alan Turing. This review presents a unified interpretation of the evolution and functioning of these mechanisms. Studies are described indicating that protocondensations, compacted mesenchymal cell aggregates that prefigure the appendicular skeleton, arise through the adhesive activity of galectin-1, a matricellular protein with skeletogenic homologs in all jawed vertebrates. In the cartilaginous and lobe-finned fishes (and to a variable extent in ray-finned fishes) it additionally cooperates with an isoform of galectin-8 to constitute a self-organizing network capable of generating arrays of preskeletal nodules, bars and plates. Further, in the tetrapods, a putative galectin-8 control module was acquired that may have enabled proximodistal increase in the number of protocondensations. In parallel to this, other self-organizing networks emerged that acted, via Bmp, Wnt, Sox9 and Runx2, as well as transforming factor-β and fibronectin, to convert protocondensations into skeletal tissues. The progressive appearance and integration of these skeletogenic networks over evolution occurred in the context of an independently evolved system of Hox protein and Shh gradients that interfaced with them to tune the spatial wavelengths and refine the identities of the resulting arrays of elements.

Contents

1. Introduction

Paired appendages (fins or limbs) are found in the jawed vertebrates, or gnathostomes, other than those such as snakes that have secondarily lost them. Extant jawless vertebrates, cyclostomes such as the extant lamprey and hagfish lack these structures (Clack, 2012). The gnathostomes consist of two taxonomic subgroups, Osteichthyes or bony fish, which comprise ray-finned and lobe-finned fishes (respectively, Actinopterygii and Sarcopterygii, the tetrapods belonging to the latter clade), and Chondrichthyes, or

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cartilaginous fishes, such as sharks and rays. The fins or limbs of all these groups contain endoskeletal elements composed of cartilage or endochondral bone that arises by developmental replacement of cartilaginous primordia. The ray-finned fishes have, in addition, lepidotrichia (fin rays), parallel segmented bones that arise directly (i.e., non-endochondrally) from embryonic fin connective tissue and extend distally from the more proximal endoskeleton (Clack, 2012).

The patterning of the paired-appendage endoskeleton differs extensively among the gnathostome groups. Due to their shared ancestry (Sansom et al., 2013) however, and the conserved cell biology of chondrogenesis, there are likely to be common underlying mechanisms of fin and limb skeletogenesis. But whether this extends beyond cell differentiation to pattern formation – the developmental elaboration of taxon-associated arrangements of skeletal elements – has been more controversial.

For more than four decades the dominant model for vertebrate limb pattern formation was "positional information." This was the idea that a coordinate system composed of gradients of conserved gene products (e.g., Shh) or other widely shared molecules (e.g., retinoic acid) set up during the development of an organ primordium such as the limb bud, elicit all the structure's cell fates in a position dependent fashion. The detailed responses of pre-differentiated cells were presumed to be programmed as subroutines in an organism's computer-like genome (Wolpert, 1989). The process by which the positional coordinates were decoded ("interpreted") to produce developmental patterns was known as the "French Flag Problem," to emphasize the arbitrary relation of the distribution of positional signal to the final pattern. Thus, the pre-20th century doctrine of preformationism (Correia, 1997) was uncritically harnessed to mid-20th century cybernetics (Wiener, 1961) in a theoretical mélange that was to prove highly influential, particularly in studies of limb development.

Over the five decades since the positional information model was proposed as a "universal" mechanism of development (that is, employing a common set of informational signals in an instructional fashion independently of the output), it has increasingly been sidelined based on experimental evidence that embryonic patterning is inherently self-organizing and reciprocally interactive over multiple spatial and temporal scales. Somitogenesis in vertebrates (Dequéant and Pourquié, 2008) and segmentation of a wide range of arthropods, for example, have been shown to arise from the interplay of gradients and oscillations in gene expression (Brena and Akam, 2013; Salazar-Ciudad et al., 2001; Stollewerk et al., 2003). Reaction-diffusion networks of the type proposed by the mathematician Alan Turing as the "chemical basis of morphogenesis" (Turing, 1952) are mathematically related to the oscillatory mechanisms underlying segmentation, and appear to mediate other developmental processes with periodic or quasi-periodic outcomes, such as integumentary pigment (Inaba et al., 2012) and hair (Glover et al., 2017) or feather (Shyer et al., 2017) follicle patterns, and arrangements of tooth cusps (Salazar-Ciudad and Jernvall, 2010). Even the *Drosophila* pre-segment pair-rule stripe-forming system, originally thought to be an exemplar of the hierarchical positional information paradigm (Akam, 1989), was ultimately shown to employ reciprocally dynamical reaction-diffusion networks (Clyde et al., 2003; Manu et al., 2009).

The repetitive and near-harmonic pattern motifs exhibited by the tetrapod limb skeleton led to its being among the first among developmental systems to be modeled by a Turing-type process (Newman and Frisch, 1979). In addition, the fin skeletons of the wide range of extant fishes, which share ancestry with the tetrapods, and those of a rich fossil record of extinct gnathostomes, are consistent with the potential outcomes of this class of mechanism, suggesting evolutionary scenarios connecting these forms. All fin or limb endoskeletons are characterized by species-specific arrangements of cartilaginous nodules, rods and plates, with replacement by bone occurring to varying extents (or not at all), in different gnathostome species (Clack, 2012). At the higher taxonomic levels, some generalizations can be made. Lobe-finned fish and their fossil ancestors (although not the most basal of these; Zhu and Yu, 2009) characteristically have increasing numbers of parallel elements along the proximodistal axis. This arrangement is even more stereotypical in the tetrapods, where the proximodistal increase is usually in an arithmetic series. In this group, there is a single stylopod (humerus or femur) attached to the body, followed by a two-element zeugopod (the radius and ulna, or tibia and fibula), and a species- or limb type-characteristic number of wrist elements, culminating in a distal autopod (fingers or toes) (Fig. 1). This stereotypy was remarked on by Charles Darwin who noted the phenomenon of "similar bones in the same relative positions" despite adaptations for grasping, digging, running, swimming and flying (Darwin, 1859).

In groups that emerged earlier from the gnathostome stem– the ray-finned and cartilaginous fishes – there are different sets of characteristic endoskeletal motifs. In both subclasses of Chondrichthyes (which diverged from one another more than 400 million years ago), the elasmobranchs (sharks, skates and rays) and the holocephali (chimaeras, such as the elephant fish), the fin skeleton consists of one or more proximal cartilage rods or plates to which are appended numerous – as many as several dozen – parallel, jointed cartilage rods (Riley et al., 2017; Silva and Carvalho, 2015). In Actinopterygii, which diverged between 400 and 275 million years ago into several subclasses with extant species: polipterids (e.g., bichirs), acipenseriforms (e.g., sturgeons, paddlefish), holosteans (e.g., gars, bowfins), and teleosts (e.g., zebrafish, carp), there is less stereotypy of the fin skeleton across all groups than in the cartilaginous fishes and tetrapods (see, e.g., Cuervo et al. (2012); Mabee and Noordsy (2004)), and in some cases, there is pattern variability within a given species to an extent uncommon in tetrapods (Davis et al., 2004). Nonetheless, the endoskeletons of these ray-finned forms are all composed of cartilaginous or bony plates, nodules and varying numbers (typically many fewer than in chondrichthyans) of parallel or branched rods, all pattern motifs potentially generated by reaction-diffusion-type mechanisms (Fig. 1).

This review will describe recent ways in which Turing-type and related mechanisms[1] have been used to gain insight into the patterning of the tetrapod limb skeleton. The hypothesized role of such processes in the fin-to-limb transition and in the

[1] 'Turing-type' is increasingly used in the biological literature to designate a class of pattern formation mechanisms that involve production and diffusion of morphogens, with, variously, positive and negative feedback circuits, adhesion, and receptor dynamics (Love, A.C., Stewart, T.A., Wagner, G.P. and Newman, S.A., 2017. Perspectives on integrating genetic and physical explanations of evolution and development: an introduction to the symposium, Integr Comp Biol, Zhang, Y.T., Alber, M.S. and Newman, S.A., 2013. Mathematical modeling of vertebrate limb development, *Math. Biosci.*. **243**, 1–17.) Although they share similarities to the formalisms for chemical symmetry breaking suggested by Alan Turing, the core mechanism may differ from the reaction-diffusion instability Turing described, and thus lack some of the original features, e.g., the difference in diffusivities of the chemical species involved. (See, for example, Glimm, T., Bhat, R. and Newman, S.A., 2014. Modeling the morphodynamic galectin patterning network of the developing avian limb skeleton, *J. Theor. Biol.* **346**, 86–108, Madzvamuse, A., Ndakwo, H.S. and Barreira, R., 2015. Cross-diffusion-driven instability for reaction-diffusion systems: analysis and simulations, *J. Math. Biol.* **70**, 709-43, Nesterenko, A.M., Kuznetsov, M.B., Korotkova, D.D. and Zaraisky, A.G., 2017. Morphogene adsorption as a Turing instability regulator: Theoretical analysis and possible applications in multicellular embryonic systems, *PLoS One*. **12,** e0171212.).

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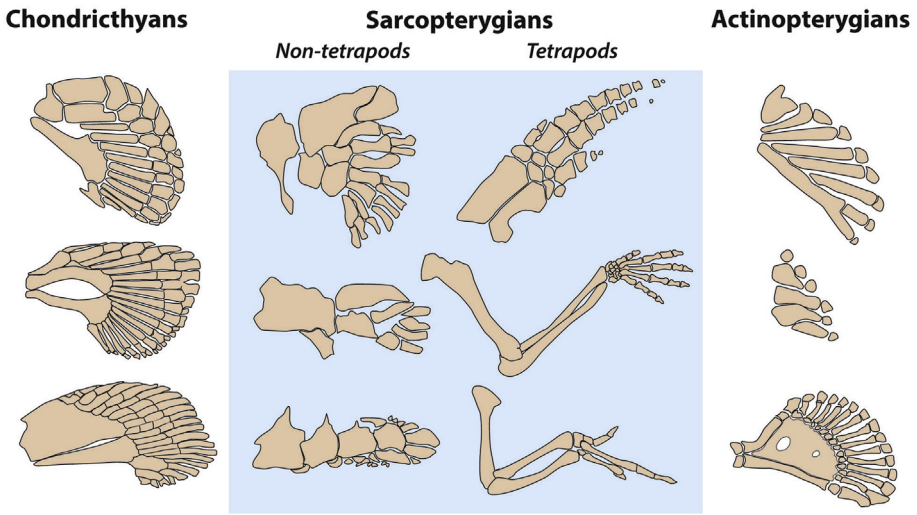

Fig. 1. Fin and limb endoskeletal morphologies of selected gnathostome species.
(First column) top, catshark - *Scyliorhinus canicula*; middle, shark *Hemiscyllium ocellatum*, bottom, shark *Centroscymnus owstoni*; (second column) top, lobe-finned fish fossil *Sauripteryus*, middle, lobe-finned fish fossil *Panderichthys*, bottom, coelacanth, *Latimeria*; (third column) top, pantropical spotted dolphin *Stenella attenuate*, middle, mouse *Mus musculus*, bottom, chicken. *Gallus gallus*; (fourth column) top, ray-finned paddlefish *Polyodon*, middle, ray-finned zebrafish *Danio rerio*; bottom, ray-finned fish *Polypterus*. Not to scale. Shaded region represents animals with limb skeletons putatively containing incipient or definitive forms of the 2GL network. From Bhat et al., 2016; see this paper for additional details, including the sources from which the drawings were adapted.

transformation of the ancestral sarcopterygian fin into the tetrapod limb, will also be discussed. While the earliest applications of such models to developmental patterning were necessarily abstract and nonspecific regarding the molecules involved e.g., (Gierer and Meinhardt, 1972; Kauffman et al., 1978), by the late 1970s limb developmental biology had advanced to the point that the first Turing-type model for limb development could incorporate roles for a few specific molecules (Newman and Frisch, 1979). With deepening knowledge of the cell biology and molecular genetics of limb development, and progress in mathematical and computational methods, biologically convincing models amenable to combined experimental and in silico testing have finally become available. Because the conceptual and technical challenges of the intervening decades can help guide future work, some of the steps toward our current understanding are presented chronologically in what follows.

It is the nature of Turing-type mechanisms (like any other physical wave-forming process), to generate repetitive elements whose number and general character (size, degree of elongation or curvature) are influenced by the dimensions and boundary properties of the domain in which they occur, but are otherwise indistinguishable from one another. Biology, however, tends toward individualizing initially generic entities over phylogeny, and this tendency is then reflected in ontogeny. Thus, the proposed Turing mechanisms that organize repeating endoskeletal elements, such as the radius and ulna or digits of the tetrapod limbs, do so against a background of concurrent developmental processes (e.g., establishment of gradients of Hox transcription factors, and of the diffusible morphogens Shh and Wnt) that result in the formed elements appearing in arrays with different wavelengths (Glimm et al., 2012; Sheth et al., 2012), or in their being "customized," i.e., different from one another in detail (Newman, 1988; Stewart et al, 2017). Less appreciated (though considered in the mathematical physics literature (Kyttä et al., 2007)) is the possibility that two or more Turing-type systems act coordinately, potentially in succession, mutually reinforcing the resulting pattern. The evidence on the mechanisms of limb skeletogenesis presented in the following sections points in this direction.

2. The Tgf-β-fibronectin-Fgf model of condensation patterning

The earliest application of the reaction-diffusion concept to tetrapod limb development was motivated by the recognition that distinct mesenchymal condensations — local increases in the packing of precartilage cells — precede the formation of the endoskeletal cartilages (Ede et al., 1977; Hall and Miyake, 2000; Thorogood and Hinchliffe, 1975). It was found that elevated deposition of the extracellular matrix glycoprotein fibronectin accompanied precartilage condensation in avian and mammalian embryos (Silver et al., 1981; Tomasek et al., 1982) and, due to its cell adhesive properties, this molecule was suggested to be a causal mediator of the process, a conjecture that was later confirmed experimentally (Frenz et al., 1989a, 1989b). The hypothesis was thus advanced that a Turing-type mechanism controlled expression of the fibronectin gene in the growing limb bud (Newman and Frisch, 1979). Since knowledge of the relevant determinants was incomplete, no explicit network for the underlying dynamics was presented. Rather, the geometric consequences of the hypothesis were explored under the assumption that the system achieves a sequence of quasi-stationary states as development proceeds (Newman and Frisch, 1979; Newman et al., 1988).

Physically plausible rate and diffusion constants were incorporated into a "dispersion relation" (in this case, the mathematical relationship between the distance between the fibronectin peaks and the system parameters, characterized by the dimensionless "Saunders number") that enabled determination of the dependence of the molecular prepatterns on the size and shape of the limb bud's distal zone at successive stages of development. This equation along with the experimentally measured progressive narrowing of this zone along the limb's proximodistal axis during embryonic development predicted the proximodistal (body wall to limb tip) increase in element number. Furthermore, any broadening of the limb bud distal tip (due, for example, to the *talpid*[2] mutation in the chicken, experimental grafting of an ectopic posterior margin of the limb bud — the zone of polarizing activity or ZPA, or species differences between chicken and mouse or human) increased the number of digits in the model, as it did in reality (Newman and

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Frisch, 1979).

Although this analysis established the feasibility of the reaction-diffusion approach to tetrapod limb development and evolution, it was not an actual dynamical model. What was missing was a molecular component that was diffusible and positively autoregulatory, and which induced the expression of fibronectin. This was satisfied by the identification several years later of the transforming growth factors (Tgf-β), secreted proteins that had all the required properties in a variety of mesenchymal tissues (Roberts et al., 1988; Van Obberghen-Schilling et al., 1988). Based on experimental evidence that members of this morphogen family indeed operated in this fashion in the developing limb bud (Leonard et al., 1991), this circuitry was incorporated into the model (Newman, 1988). A candidate inhibitory molecule was needed to complete the characterization of this model as an activator-inhibitor Turing-type mechanism, however, and this was still lacking.

Studies beginning in the 1990s on the role in limb development of fibroblast growth factors Fgfs and their receptors shed some light on this missing piece to the puzzle (Peters et al., 1993; Szebenyi et al., 1995). One member of this morphogen family (Fgf8) in the embryonic limb mediates the effect of the apical ectodermal ridge (AER) in promoting limb elongation (Moon and Capecchi, 2000). Since the work of John Saunders in the late 1940s it has been known that the successive appearance of skeletal elements along the developing proximodistal axis is entirely dependent on the presence of the AER and its presumed products (Saunders, 1948). This was the main experimental evidence for the order of determination of the endoskeletal primordia referred to above. Possibly related to its role in outgrowth and distal progression of the skeletal pattern is the effect of the AER in keeping the subjacent apical mesenchyme in a developmentally labile state (Kosher et al., 1979; Newman and Frisch, 1979).

The role of Fgf in condensation patterning is independent of its AER-related effects, however. The limb bud mesoderm expresses three different Fgf receptors during development (Szebenyi et al., 1995). FgfR1 appears in the distal tip mesenchyme (which remains unpatterned as it undergoes proximodistal narrowing, mentioned above, as the bud elongates), where it mediates the tip's growth and elongation response to Fgf8. FgfR3 is expressed by the developing cartilage in the more proximal regions of the bud, where it mediates growth control of the developing cartilage that arises subsequent to, and as a result of mesenchymal condensation (Peters et al., 1993) in response to the Fgfs (mainly Fgf2 in birds, and Fgf4 in mammals) produced by the limb bud's dorsal and ventral ectoderm (Mariani and Martin, 2003; Yu and Ornitz, 2007). FgfR2 is expressed in the region between the unpatterned and definitively patterned zones (the “active zone”), precisely at the sites of incipient condensations, where, in response to Fgf2 in the avian system it mediates a laterally acting inhibitory effect that confines the expansion of condensations (Moftah et al., 2002).

With the confirmed existence of a positively autoactivating morphogen that induces fibronectin production (i.e., Tgf-β), and a laterally acting inhibitor of condensation formation (i.e., the inferred effector released by activation of FgfR2), an explicit activator-inhibitor Turing-type network for chondrogenic pattern formation in a developing vertebrate limb could be delineated (Newman and Bhat, 2007). The extension of the model of Newman and Frisch to include the effects of Tgf-β and Fgf in addition to fibronectin, leads to its designation as the TFF model. In a study by Hentschel and coworkers (Hentschel et al., 2004) the TFF model took the form of eight coupled partial differential equations, representing spatiotemporal changes in concentrations of activator, inhibitor and Fgf, fibronectin, and density of mobile cells bearing R1- and R2-type Fgf receptors. The system, being too complicated to solve analytically, and too unwieldy for computational simulation in its full form, was studied in various heuristically simplified forms, where it generated realistic proximodistal patterning in an accurate spatiotemporal order (Chaturvedi et al., 2005; Cickovski et al., 2005; Izaguirre et al., 2004; Newman et al., 2008).

Despite the correspondence between mathematical expressions in the described dynamical system, the roles of known or inferred molecular species, and authentic-appearing development, questions can be raised about this approach from the mathematical side that pertain not only to this model, but any developmental model that claims to accurately represent the phenomena in question. For one thing, there is no guarantee that a system as elaborate as that of Hentschel and coworkers has mathematical solutions that are well-behaved, or indeed if solutions exist all. The fact that some ad hoc (though biologically plausible) approximations do not “blow up” with time begs the question of whether the full system can rigorously represent the developmental process.

To address this, conditions were sought under which the eight-equation system had smooth solutions that exist globally, that is, over the full spatiotemporal domain relevant to the developmental process (Alber et al., 2005). One assumption beyond those of the original model was necessary to ensure the parameter space of these solutions was not vanishingly small: that the fibronectin matrix is not entirely static after deposition, but that it diffuses slightly. This seems to be justified biologically (Li et al., 2013).

Establishing that the full system supports well-behaved mathematical solutions was just one of the conditions for using it in a reliable account of limb development. As mentioned, the system is not amenable to either analytically or computationally based predictions, so any application must rely on averaging and consolidating parameters, and other simplifications. Doing this in an informal fashion will produce a “toy model,” which may have utility in framing experimental strategies, but would not stand as a compelling causal explanation of the phenomena. It is possible to simplify such models in a more rigorous way, however, by making well-posed mathematical assumptions, so that the resulting set of equations, though reduced in number, inherit the well-behaved properties of the original system. Although many different modes of mathematical reduction are possible, the only ones suitable for developmental modeling will be those for which the simplifying assumptions are well-justified biologically.

This issue was first addressed by modeling the separation of certain developmental effects in time. In a general analysis of developmental patterning mechanisms, Salazar-Ciudad and coworkers distinguished between “morphostatic” and “morphodynamic” mechanisms (Salazar-Ciudad et al., 2003). In the first of these, a molecular prepattern (e.g., a gradient or a non-monotonic distribution of a morphogen) is established in a field of cells, after which the cells move and/or differentiate in response to different concentrations of the molecular cues. The long-held positional information model fits this description, as do the standard Turing-type models. However differently the morphogen patterns are generated in these two frameworks, their formation occurs independently of cell movement. In the second, morphodynamic, type of mechanism, cell movement occurs concomitantly and in reciprocal interaction with morphogen dynamics, leading to developmental patterns that are not straightforwardly templated by morphogen prepatterns.

From the description above, it is evident that the eight-equation limb patterning system of Hentschel et al. (2004) is morphodynamic: cell condensation, represented by focal increases in the density of FgfR2-expressing cells, occurs simultaneously with the changing profile of the morphogen Tgf-β. However, since morphostatic mechanisms lend themselves much more readily to simulation and in silico hypothesis testing, it was asked whether

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restrictions on relationships among the system's parameters could provide a version that functioned in this fashion. Mathematical analysis indeed identified such conditions, leading to a two-equation system for the patterning, independent of cell movement, of activator and inhibitor profiles (Alber et al., 2008). Condensation was treated as a downstream effect of the morphogen pattern. If the the required cell rearrangement was to be modeled computationally, it would require an add-on translocation module. Thus, a highly complex multiscale system was transformed into more standard Turing-type system by manipulations which, though sacrificing some of the cell and molecular authenticity of the full model, made these simplifications in a transparent fashion, subject to experimental evaluation.

A third issue (beyond the existence of well-behaved solutions and the desirability of analytically rigorous simplification) that is critical for implementing a complex limb model relates to the computational difficulty of performing reaction-diffusion simulation in growing domains and those with natural curvilinear boundaries (Zhang et al., 2013). In present-day tetrapods, variation in limb shape mainly occurs in the distal compartment at later stages of development (the prospective autopod), more proximal regions having roughly parallel anterior and posterior edges. This is reflected in variation in digit number in different species. In evolutionary antecedents, such as fossil sarcopterygians, in contrast, the disparity in numbers and shapes of proximal elements suggest that ancestral embryonic limb bud shapes might have differed from those of present-day embryonic limbs. To formulate and test hypotheses about limb origins and evolution thus requires simulating reaction-diffusion systems in domains with nonstandard, natural shapes.

A new finite element approach to simulating reaction-diffusion systems on moving and deforming domains, the discontinuous Galerkin (DG) method (Zhu et al., 2009), was therefore used to study the morphostatic limb model. Simulating the system in an elongating domain with an imposed proximodistal gradient representing Fgf showed that it could account for the normal proximodistal pattern of skeletogenesis as well as distal truncations resulting from AER removal (Zhu et al., 2010). Modifications of the model's parameters corresponding to the plausible effects of other developmental determinants, such as Hox protein and Shh gradients, alterations in core patterning effectors, and the reshaping of the model limb bud, yielded in silico phenotypes resembling experimentally manipulated and genetically aberrant forms, as well as skeletal morphologies with features of fossil limbs (Fig. 2).

The real-time simulations of vertebrate limb development of Zhu et al. (2010) was a proof-of-principle that these phenomena, including those obtained by experimental manipulation and from mutant forms, could be accounted for by a Turing-type system. But despite the empirical foundations of the TFF mechanism (from which the described morphostatic model was derived) these studies were insufficient to confirm it as the developmentally primary cell biological basis of limb skeletal patterning. Furthermore, the molecular nature of the inhibitor in the mechanism remained enigmatic.

3. The two-galectin-ligand mechanism of protocondensation patterning

In a study of chondrogenesis in the limb buds of chicken embryos, and cultured mesenchyme derived from them, Bhat et al. (2011) used probes directed at the five avian members of the galectin family, the most extensively studied class of endogenously expressed lectins with generic binding affinity to the β-galactosides prevalent among N-linked cell surface glycans (Kaltner and Gabius, 2012). They found that both Galectin-1a (Gal-1a) and Galectin-8 (Gal-8) transcripts and proteins, and respective cell surface ligands for the two proteins, localized to sites of prospective condensation well before fibronectin marked the definitive condensations (Bhat et al., 2011). This raised the possibility that a patterning mechanism distinct from the TFF system could precede it during development.

Further investigation led to the finding that Gal-1a promoted the aggregation of limb mesenchymal cells, an activity that was blocked by Gal-8. At the gene regulatory level, however, each of these two galectins induced expression of the other. This combination of antagonistic and reinforcing interactions, along with the effects each galectin has on the production (or mobilization at the cell surface) of its specific and shared ligands, constitutes a multiscale network with potential Turing-like pattern forming capability (Bhat et al., 2011). This inference was supported by a detailed mathematical model that explored the conditions under which this network can form regular patterns (Glimm et al., 2014). Surprisingly, the mechanism was inherently morphodynamic. That is, unlike the system of partial differential equations representing the TFF mechanism, which while morphodynamic in its most general form can function as a morphostatic pattern-forming mechanism in a highly restricted domain of parameter space (Alber et al., 2008), there is no pattern-forming version of the two-galectin-ligand (2GL) mechanism in which cells do not move simultaneously with changes in distribution of the involved molecules. This result was tied to the fact that Gal-1a functions in this mechanism both as a morphogen and a mediator of cell-cell adhesion. The modeled mechanism, therefore, in contrast to classic Turing-type reaction-diffusion systems, is a morphodynamic reaction-diffusion-*adhesion* process (Glimm et al., 2014).

The early-appearing foci of Gal-1a and Gal-8 enrichment were found to result from a subtle enhancement of cell association and were therefore termed “protocondensations” by Bhat et al. (2011). Protocondensations (which appear to be identical to the “compactions” identified using live imaging by (Barna and Niswander, 2007)), differ from the definitive condensations, tightly packed foci of rounded cells, which appear at least a day later. The mathematical representation of the 2GL mechanism enables protocondensation formation by modeling short-range cell movement up a cell adhesive Gal-1a concentration gradient (Glimm et al., 2014).

Two other galectins, Gal-1b and Gal-2 were both expressed, though to a substantially lower extent than Gal-1a and Gal-8, during the period of protocondensation formation, and both were capable of aggregating mesenchymal cells, though much less efficiently than Gal-1a. (The fifth avian galectin, Gal-3, was not detectably expressed during limb skeletogenesis; (Bhat et al., 2011)). Gal-1b, an isoform of Gal-1a which was produced by a gene duplication and translocation event and is only present in the sauropsids (birds and reptiles), has computationally predicted differences from Gal-1a in the folded structures of its galactoside-binding domains. It therefore serves as a natural control for the latter (Bhat et al., 2014). Specifically, it is possible to infer the likelihood that Gal-1s in other gnathostome groups have mesenchymal aggregating activity based on whether they share the fold structure of the chicken Gal-1a carbohydrate recognition domain (CRD) rather than that of Gal-1b. Such comparisons led to the proposal that all extant gnathostomes for which sequence information is available (except for the coelacanth, *Latimeria chalumnae*), have a presumptively skeletogenic Gal-1 (Bhat et al., 2014). (This includes the mammals, which have only one Gal-1 homolog.) Alhough L. *chalumnae* lacks any Gal-1 homologs the skeletogenic function of Gal-1 might be assumed by Gal-1b or Gal-2 in that species.

Because the mathematical complexity of the 2GL mechanism

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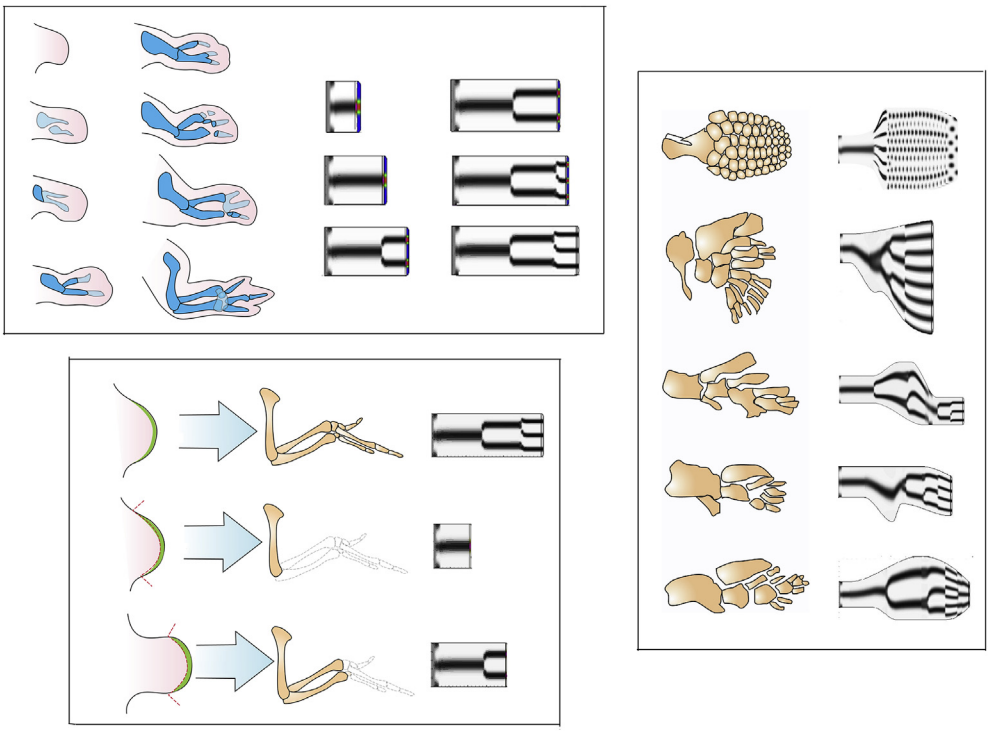

Fig. 2. Simulation of limb development with the TFF model. (Upper left panel) Developmental progression of chicken forelimb between days 3 and 7 of embryogenesis. Left, drawings of cleared stained specimens. Early cartilage, including precartilage condensations, shown in light blue; definitive cartilage shown in darker blue. Right, a sequence of snapshots from a computational simulation using a discontinuous Galerkin finite element method. (Stage progression for both the specimen drawings on the left of the panel and the simulation on the right are earlier-to-later, top-to-bottom, in each column). (Lower left panel) Simulations of removal of the AER. Left two columns, drawings of AER removal experiments, based on (Saunders, 1948). Top row images show an intact chicken wing bud at an early stage of development and the limb skeleton that it generates. Middle row images show a wing bud at the same early stage with the AER removed, and the resulting limb skeleton, which attains a normal size but is truncated at the elbow. Bottom row images show a later stage wing bud whose AER has been removed. The resulting skeleton is truncated at the wrist. (Right column) Simulations of limb development using standard parameters. Top, AER (i.e., the source of suppressive FGF morphogen) left intact; normal development results. Middle, AER function inactive from an early point in the simulation. Bottom, AER function inactive from a late point the simulation. (Right panel), simulation of fossil limb skeletons. A selection of limb skeletal patterns from fossil specimens (left drawings) were simulated by employing hypothetical but plausible scenarios for their embryonic development. The end-stages of the simulations of two lobe-finned fish, *Sauripterus* and *Eusthenopteron*, and two forms with skeletal patterns intermediate between those organisms and tetrapods, *Panderichthys* and *Tiktaalik*, are shown on the right. All panels adapted from Zhu et al. (2010), which can be consulted for additional details.

has thus far prevented its being analyzed computationally in a reshaping growth domain, or even in two dimensions, as has the TFF mechanism, all the predicted behaviors of 2GL mechanism up till now are based on one-dimensional simulations in which peak height and peak spacing are the only relevant outputs (Glimm et al., 2014). The finite element modeling approach used in the two-dimensional simulations of Zhu et al. (2010) showed that a Turing-type process is sufficient to generate natural and variant endoskeletal patterns (and is constrained to *not* produce non-limb-like patterns). Since the TFF and 2GL mechanisms are temporally overlapping during skeletogenesis, they might function and be amenable to computational analysis as a single, unified mechanism incorporating the two Turing-type processes in a complementary and mutually reinforcing fashion (Kyttä et al., 2007).

Even within the limits of one-dimensional modeling, the 2GL mechanism has guided inferences about the evolution of the fin and limb endoskeletons across all the gnathostome groups. It has also provided a hypothetical scenario for the successive emergence of the sarcopterygian paired appendages and the tetrapod limbs from the fins of ancestral bony fishes. This analysis begins with the presence, as mentioned, of skeletogenic galectins in all gnathostomes. The equations describing the dynamics of the 2GL system indicates that a galectin of this type can generate structures (peaks in one-dimensional space per the current analysis) with varying degrees of periodicity or aperiodicity. If the pattern is periodic, it can have different spatial wavelengths depending on certain features of Gal-8.

Gal-8 first appeared early during, or before, chordate evolution (Houzelstein et al., 2004) Phylogenomic and protein structure prediction analyses indicate that chondrichthyans and sarcopterygians (including tetrapods) have fold-structures of their CRDs that permit them to compete for shared cell surface receptors with skeletogenic Gal-1s. This is a necessary condition for regular (i.e., spatially periodic) patterns according to the 2GL model (Bhat et al., 2016). As described above, tetrapod limbs and chondrichthyan fins are both “regular” in this sense, with few or many parallel elements (in tetrapods an arithmetically increasing number of these proximodistally), with fossil and extant sarcopterygians tending, to varying extents, to the condition of the tetrapods in this respect. The broad endoskeletal plates in the proximal regions of cartilaginous fish fins would be interpreted as the limiting case of many parallel elements with vanishingly small spacing (Fig. 3).

In Actinopterygii, the gene specifying Gal-8 underwent a translocation to a different chromosomal environment (determined by contig analysis) from the one shared between Chondrichthyes and Sarcopterygii. There, instead of maintaining the Gal-1a-like CRD fold-structure required for regular patterning, the Gal-8s of ray-finned fishes underwent positive selection to conformations that were often noncompetitive with Gal-1a (Bhat et al., 2016). This variability in the structure of Gal-8, according to the 2GL model, can produce endoskeletal patterns that lack consistently organized arrays of elements (Bhat et al., 2016;

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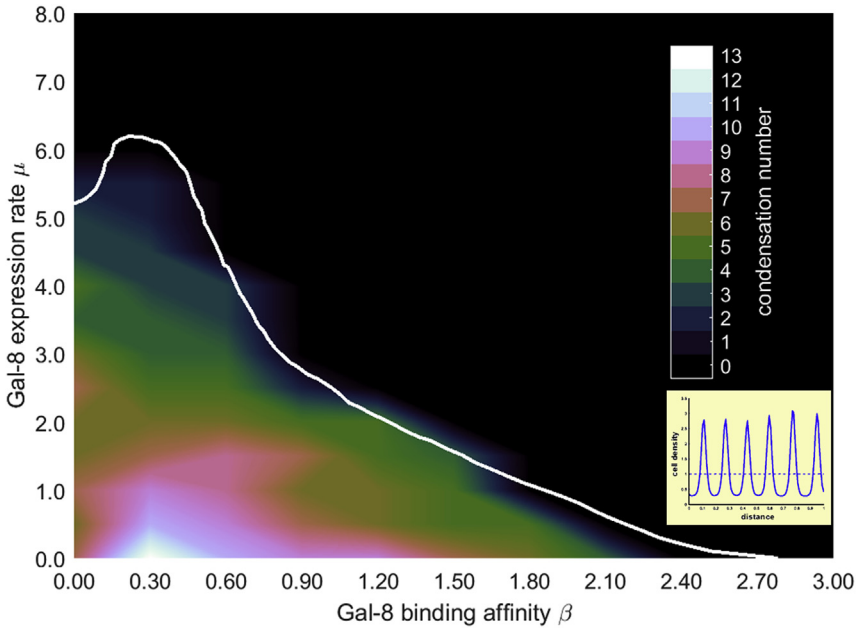

Fig. 3. Gal-8 dependence of pattern formation in the condensation-permissive parameter space of the 2GL model. Two-parameter bifurcation diagram showing the dependence of condensation patterns on μ, the expression rate of Gal-8, shown on the vertical axis, and binding affinity β shown on the horizontal axis. A single plane of the complete parameter space is shown in which all concentration values of Gal-1 are compatible with condensation formation. Gal-8 can participate in skeletogenic interactions with Gal-1 only if it is capable of reversibly competing with the condensation-promoting role of Gal-1. This competition thus corresponds to a range of β from about 0.0 to 2.10 in the relative units used to characterize the parameter space for the simulations mapped in the figure. Consistently generating small numbers of elements, as seen in the distal regions of sarcopterygian limbs, would involve a constraint on β to a range of values between 1.75 and 2.1 for $0.5 < \mu < 2$ and between 0.25 and 0.7 for $2 < \mu < 4.5$. Computations are based on the mathematical model of Glimm et al. (2014). Approximate contours demarcating the calculated number of distinct condensations are shown via a heat map within the condensation region. Inset, an example of a condensation pattern generated by the 2GL model. Main figure from Bhat et al. (2016). Inset from Glimm et al. (2014).

Glimm et al., 2014).

According to the 2GL model, the number of repeated elements in any limb bud domain (again, in one dimension, within the restrictions of the current analysis) depends on the relative expression levels of receptor-competing Gal-8 and skeletogenic Gal-1. It was therefore significant when comparative phylogenomics disclosed that the galectin-8 genes of the inferred common ancestor of modern sarcopterygians (including the tetrapods) acquired a novel 21 bp conserved non-coding motif (CNM) with canonical binding sites for transcription factors (e.g., Meis1, Tcfcp2l1, Runx1 and Runx2) known to be present during limb development. Ray-finned fishes lack this CNM, but so do cartilaginous fishes, despite their sharing with lobe-finned fishes (as mentioned above) the Gal-8 protein structural determinant that enables formation of regular repetitive patterns (Bhat et al., 2016).

This suggests an evolutionary scenario in which once the capacity to produce endoskeletal structures had emerged in the paired appendages of gnathostome ancestors it was followed by enhancement of the propensity to form stereotypical patterns in both cartilaginous and lobe-finned fishes, though less so in ray-finned fishes. The fin endoskeletons formed in these groups, moreover, were (according to this picture) originally under no constraints as to the order of their appearance during development.

With the presumed capacity for the galectin-8 gene to be quantitatively regulated in the sarcopterygian lineage (mediated by the described CNM), there would be a potential for the number of parallel elements to change as the limb develops. In fossil and extant sarcopterygians, this progression is one of general proximodistal increase, a pattern that is even more entrenched in the tetrapods. The establishment of this conserved spatiotemporal schedule in the limb could have been a consequence of evolutionary changes leading to the downregulation of Gal-8 levels during development (see Lorda-Diez et al. (2011)).

4. The BMP-Sox9-Wnt network and integration of the patterning processes

The two Turing-type mechanisms for limb skeletogenesis described above account for different stages of the process (protocondensation and definitive condensation), but overlap temporally. It is thus reasonable to treat them as phases of the same dynamical patterning network. Because both mechanisms are experimentally based and mathematically well-posed, testable hypotheses can be advanced for their functional interconnections.

For example, the earliest patterning step in the TFF mechanism according to the model of Hentschel et al. (2004) is the induction by Tgf-β of Fgf receptor 2 in a band of tissue (the subapical active zone) that has grown sufficiently distant from the AER to escape the suppressive effect of its secreted products, mainly Fgf8. While the inhibition of condensation formation by the AER is well-established experimentally, its molecular basis is unknown. Given the early role of the 2GL mechanism in organizing discrete condensations, it is therefore significant that both galectin-1a and galectin-8 gene expression are inhibited by Fgf8 (R. Bhat, unpublished). In addition, while the 2GL patterning process appears to be initiated before the TFF network, they are mobilized at the same locations with a significant temporal overlap.

The next step in the operation of the TFF mechanism is the production of a lateral inhibitor at sites of prospective condensation in the subapical mesenchyme. While the identity of the inhibitor has been elusive, Tgf-β has been found to induce Gal-1 (Lim et al., 2014) and Gal-8 to induce Tgf-β (Sampson et al., 2016), in some non-limb systems, suggesting that the TFF and 2GL systems may interact at these sites and that the self-organizing capacity of the 2GL network might be manifested as the lateral inhibition component of the TFF mechanism.

Once discrete protocondensations are established in the newly

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organizing (i.e., AER-remote) domain, with characteristic wavelengths determined by the domain's changing geometry and molecular gradients (Hox, Shh, etc.), they will begin producing fibronectin in response to Tgf-β (as per the TFF mechanism) and proceed to form definitive condensations. One important role of fibronectin is to suppress adipogenesis, the alternative cell fate of these mesenchymal cells (Mezentseva et al., 2008), in favor of chondrogenesis (Hudak and Sul, 2013; Wang et al., 2010). In this hypothetical scenario, the AER, as a source of Fgf8, provides the temporal and causal link between the 2GL and TFF mechanisms (which thereby become a single, complex one), by suppressing both until they are capacitated together in a way tied to limb bud outgrowth.

Another patterning network, described in the developing mouse limb (Raspopovic et al., 2014) and in a modified form in the fin of the catshark, a chondrichthyan (Onimaru et al., 2016), leads to the spatial expression of the transcription factor Sox9 in vitro and in vivo. In this network, Sox9 expression is under the control of two secreted morphogens, Bmp2 and Wnt, which also indirectly affect one another's expression via their effects on Sox9. Experiments that manipulated the levels of the two morphogens, in conjunction with computer modeling, led to the conclusion that this network (termed BSW) constituted a Turing-type process, though not of the familiar activator-inhibitor type (like the TFF model) where the peaks of the antagonistic components are in phase with each other. Rather, it behaves as a "substrate depletion" system, whereby an activator depletes the nearby capacity to form another center of activation by depleting its its generalized "substrate" (Meinhardt, 2006). Some of the main components in this scheme therefore occur spatially out-of-phase with one another (Fig. 4).

While the components of the TFF and 2GL networks (Tgf-β, fibronectin, FgfR2, Gal-1, Gal-8, and the galectins' receptors) operate in all regions of the developing avian limb (Newman and Bhat, 2007; Bhat et al., 2011), the tetrapod BSW mechanism has only been characterized in the mouse autopod, not in more proximal regions (Raspopovic et al., 2014). Correspondingly, in the catshark embryo the BSW network (with Bmp4 substituting for Bmp2) is involved in the formation of a rim of nodules along the distal contour of the developing pectoral fin, but apparently not the cartilage rods of the mid-fin which directly articulate with the distal nodules (Onimaru et al., 2016). Though present in both chondrichthyans and tetrapods, the BSW network is absent - potentially lost – in at least some actinopterygian groups (Onimaru et al., 2016).

Since Sox9 (the activator in the BSW mechanism) is an essential transcriptional regulator of the cartilage cell phenotype (de Crombrugghe et al., 2000), it is an authentic marker of early skeletal patterning, as are FgfR2, and Gal-1 and Gal-8, in the TFF and 2GL networks, respectively. However, unlike the latter proteins, Sox9 does not appear to be intrinsic to the condensation process. Specifically, while Bmp signaling is essential for the formation of condensations, this can occur in limb mesenchyme null for Sox9 (Barna and Niswander, 2007). Moreover, condensation is not restored by overexpression of Sox9 in limbs in which Bmp signaling is impaired (Lim et al., 2015), suggesting that sites of focal expression of Sox9 are not sufficient to induce cartilage elements. One or more factors deeper in the chondrogenic program, possibly Gal-1, FgfR2, or even Bmp itself independently of Sox9 (and therefore of the BSW mechanism) is evidently needed for endoskeletal pattern formation. Cartilage differentiation, which is indeed dependent on Sox9, is a later event.

5. Discussion and conclusions

Based on experimental studies in chicken and mouse embryos, the major conserved, and apparently functionally required, events of patterning of the tetrapod limb endoskeleton are:

1. Localized compaction of prechondrogenic mesenchyme, beginning within several hours of the limb bud's appearance.
2. Transformation of the protocondensations into definitive ECM-rich condensations, over 1–2 days.
3. Differentiation of the condensed mesenchyme into cartilage.
4. Progression of phenomena 1–3 as the limb bud elongates (proximodistally, in general, though with reversals of this mode in some amphibians Franssen et al. (2005); Kerney and Hanken (2008)), so that all are eventually in play concurrently, in successive regions, until the cartilaginous template of the skeleton is complete.

The paired appendage endoskeletons of Sarcopterygii (including the tetrapods) are, and were (in the case of organisms known only through their fossils), plausibly generated by the same processes. For the cartilaginous and bony fin endoskeletons of Chondrichthyes and Actinopterygii, which take their initial form as nodules, rods, and plates of cartilage, the cellular events in the formation of each of the elements are likely similar, although the spatiotemporal order of the elements' appearance will be different.

When the cell and molecular biology of endoskeletal development are considered, the relevant determinants are the gene products that mark or colocalize with the protocondensations, which are causally necessary for them to appear, and that directly mediate their formation. While these are not necessarily the same, we treat them here collectively as a "skeletogenic toolkit." The explanation of patterning of the array of endoskeletal elements in a fin or limb requires assigning these toolkit molecules to the various interacting Turing-type processes described above and specifying the timing of their deployment.

Based on experiments reviewed here, FgfR2 (Szebenyi et al., 1995) and Gal-8 (Bhat et al., 2011) are both early markers of prospective condensations. While each of these proteins is involved in keeping the preskeletal foci discrete, there is no suggestion that either mediates cell-cell associations. Furthermore, while Bmp2 is functionally required for early compaction and thus protocondensation, it is expressed between developing skeletal primordia, so it cannot be the primary mediator of changes in association among the cells within the latter. The Bmp receptor BmpR1b is indeed expressed in the digital primordia (Montero et al., 2008) and might thus be required for Bmp2's compaction-promoting activity, but like FgfR2 and Gal-8 it does not mediate cell-cell attachment. Of all the early-acting Turing process-related determinants of skeletogenesis, only skeletogenic Gal-1 has been shown to mark, and be required for, the formation of protocondensations. As a matricellular protein, moreover, it directly mediates their formation (Bhat et al., 2011).

The following represents a tentative synthesis of the current state of understanding of the origination of the gnathostome paired fin endoskeleton (Fig. 5). Skeletogenic Gal-1 arose early in vertebrate evolution, mediating the formation of endoskeletal elements in ancestral fins (Clack, 2012). Next, Gal-8 variants whose carbohydrate recognition domains (CRDs) had evolved to resemble those of skeletogenic Gal-1, and the evolution of mutual activation of gene expression of the two galectins, enabled the emergence of a two-galectin network that generated regular spots and stripes or bars (and plates, as fused bars) of cartilage (Bhat et al., 2016). This 2GL network, with possibly independent refinements of the Gal-1-Gal-8 interaction, characterized establishment of the fins of chondrichthyans and early sarcopterygians.

At some point, a translocation of the galectin-8 gene in a sublineage of this stem gnathostome population accompanied the

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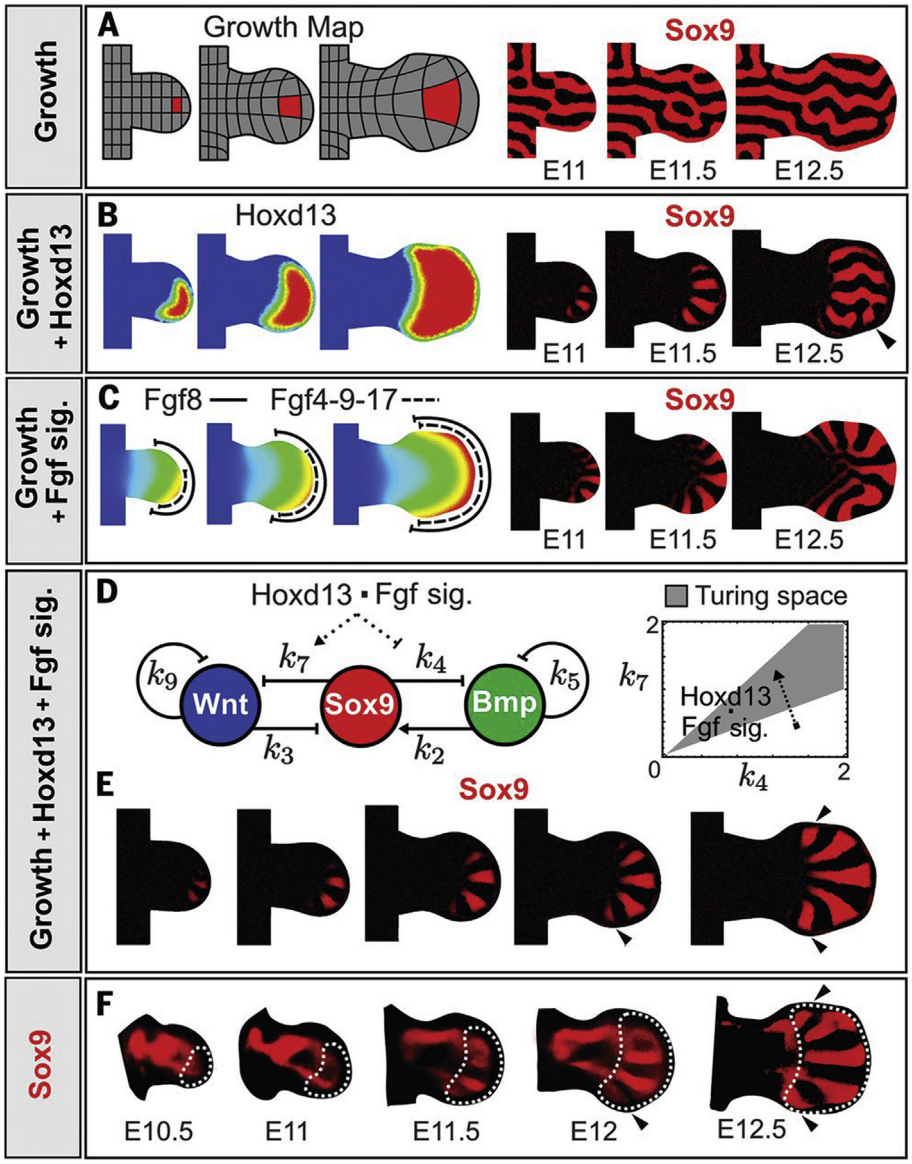

Fig. 4. Simulation of digit patterning with the BSW model. (**A**) Simulation of Sox9 patterns (right) inside an experimental limb growth map (left). (**B**) Mapping *Hoxd13* expression (left, heat-color map) into the growing limb bud, with corresponding modulation of the model's parameters led to a more digit-like pattern of Sox9 expression (right), although with abnormal digit bifurcation (arrowhead). (**C**) Expression of various Fgfs was mapped into the model (solid and dashed lines) and used to simulate an Fgf signaling gradient (left, heat-color map). With the Fgf modulation, the model predicted a radially oriented Sox9 pattern with larger wavelength toward the distal tip (right). (**D**) Joint modulation of the network's parameters by Hoxd13 and Fgf defines a "Turing space" (gray region) in which the system exhibits pattern-forming capability. (**E**) The simulated Sox9 pattern recapitulates the main features of (**F**) the experimental *Sox9* expression in the digits, outlined by the white dotted lines. From Raspopovic et al. (2014), used with permission from AAAS. See that article for additional details.

origination of the actinopterygians. In this lineage, Gal-8, and particularly its CRD, underwent positive selection away from competition with Gal-1. The resulting fin endoskeletons in many cases deviated from a plate and stripe format. In another gnathostome sublineage, one in which the galectin-8 gene did not undergo translocation, the gene acquired a conserved non-coding motif that enabled developmental regulation of the levels of Gal-8, leading (along with the evolution of Fgf-dependent limb bud elongation) to the proximodistal increase in the number of parallel elements of the sarcopterygians. Further purifying evolution of Gal-8 with respect to the 2GL network patterning constraints (Bhat et al., 2016) accompanied and possibly defined the highly conserved stereotypy of the tetrapod limb that caught Darwin's attention.

The morphogens of the Bmp family and their receptors were likely involved in this endoskeletal origination process, since this circuit is required for the compaction associated with protocondensations (Barna and Niswander, 2007). This involvement was not as part of the BSW network, however, since the Bmp circuit can act independently of, and even in the absence of, Sox9 (Lim et al., 2015). Bmp2 and its receptors have been proposed to act as a Turing-type system in their own right, via feedback of the morphogen on receptor synthesis (Badugu et al., 2012), but they would need to function in coordination with a developmentally early-acting morphogenetic (i.e., cell-adhesion-promoting) module. A plausible candidate for this module is the 2GL network. One possible connection is via the transcription factor Runx2, which is induced by Bmp2 and also induces it, forming an autoregulatory

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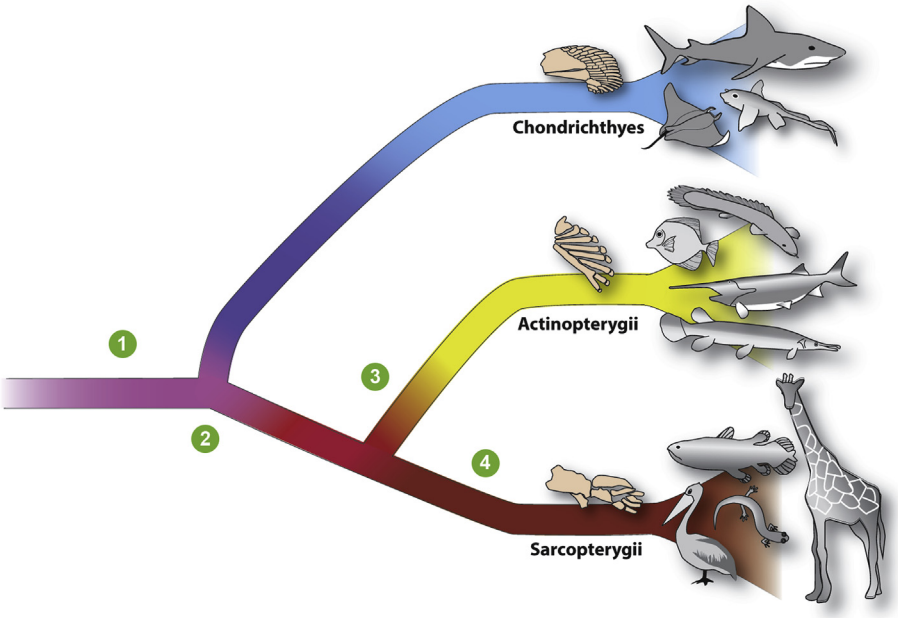

Fig. 5. Tree showing phylogenetic relationship of extant fish groups and hypothesized appearance of patterning mechanisms (1) Capacity for focal cartilage differentiation. A Gal-1 protein capable of mediating mesenchymal aggregation was present in ancestral gnathostomes. With the involvement of products of the conserved toolkit genes Bmp and Bmp receptor 1, and eventually Fgf and Fgf receptor 2, in "centripetal" (i.e., restricting expansion) roles these aggregates could progress to compactions or protocondensations. Coordinate induction of Sox9 and fibronectin (the latter under regulation of the positive autoregulatory TGF-β) at these sites converted them to cartilage elements. At some point on this branch of the evolutionary trajectory a Wnt gene was recruited to the Sox9-Bmp couple, forming the BSW Turing-type network, reinforcing the counter-chondrogenic distribution of Bmp. Although Gal-8 was present in these organisms, their carbohydrate-recognition domains (CRDs) were not competitive with those of Gal-1, so the full 2GL system was not in place. (2) Capacity for formation of nodules, stripes and plates of cartilage. Evolution of cross-activation of Gal-1 and Gal-8 and of the Gal-8 CRDs to competitive status with Gal-1 CRDs created the 2GL system. This generated numerous parallel cartilage rods and, with fusion, plates. The point represented as a trifurcation undoubtedly had a more complex branching pattern and the precise positioning of the described events along the respective trajectories is uncertain. (3) Variability of the endoskeleton under positive selection. In the ray-finned fishes all the focal chondrogenesis-enabling circuitry was carried forward and (depending on the species), possibly the BSW patterning system (although it appears to be absent in teleosts; Onimaru et al. (2016)). The galectin-8 gene underwent translocation around the inception of this group. In some ray-finned species, selection on the Gal-8 CRD reinforced the 2GL patterning circuitry, and in others not. (4) Capacity for regulated proximodistal patterning with small numbers of elements. In the sarcopterygians the ancestral focal chondrogenesis toolkit and skeletal patterning systems were carried forward, but sometime after their appearance Gal-8 also acquired a unique conserved noncoding motif (CNM) with cis-positioned transcription factor binding sites that permitted it, potentially, to be regulated in a quantitative fashion during elongation of the developing limb. Because of the inverse relationship between level of Gal-8 expression and number of elements (Bhat et al., 2016), a progressive decline in expression in the distal unpatterned mesenchyme during development will lead to the stereotypical proximodistal increase in parallel elements of the tetrapod limb. Purifying selection is proposed to have locked in the developmentally regulated 2GL mechanism, making its characteristic outcome a developmental constraint on further evolution. Examples of limb skeletons typical of the various groups are indicated on the respective branches. The pattern variability (by the criteria described here) among the actinopterygians is more extensive than among both the chondrichthyans and the sarcopterygians (see Fig. 1).

loop (Lian et al., 2006). As noted above, sarcopterygian galectin-8 genes (but not those of sequenced chondrichthyans or actinopterygians) contain Runx2 binding sites as part of their CNM, potentially integrating the Bmp loop with the inhibitory branch of the 2GL mechanism.

Another member of the Bmp superfamily, Tgf-β, acting later in the chondrogenic pathway, transforms the protocondensations into definitive condensations by inducing the production of fibronectin at the sites of protocondensations. Activation by Fgf of FgfR2, which is also expressed at these sites, mediates a lateral inhibitory effect on condensation formation (possibly with the participation of the Bmp-BmpR module and, as suggested above, the 2GL network), thus consolidating what may be transient protocondensation patterns by the operation of another superimposed Turing-type mechanism, the TFF network.

In this scenario, the BSW network was a later-evolving module, one which came to connect the protocondensation-condensation process to cartilage cytodifferentiation via recruitment of Sox9. By functional linkage to the ectodermal product Wnt, this differentiation-inducing module, rather than serving as a downstream readout of an existing patterning system, became a self-organizing system in its own right, dependent on the Bmp subsystem, but contributing robustness to the concerted outcome (Fig. 5).

The dissociation of the primary patterning module from the cytodifferentiation module can help explain the otherwise surprising finding of a relationship between the patterning of digits (an evolutionary innovation of tetrapods) and the patterning of the distal rays of actinopterygians (which are dermal bones, not endochondral bones like the digits). This can be appreciated by noting the part that Hox genes play in the interacting self-organizational mechanisms described here. Using gene modification techniques in mice, Sheth and coworkers concluded that the distally expressed Hox genes (Hoxa13 and Hoxd11-Hoxd13) regulate digit patterning by controlling the wavelength of a Turing-type mechanism (Sheth et al., 2012). This confirmed an earlier inference concerning the role of Hoxa13 based on the effect of misexpression of this gene in the chicken zeugopod (Newman, 1996; Yokouchi et al., 1995). From this perspective, a Turing-type mechanism (e.g., the early-evolved, early acting 2GL + Bmp/BmpR complex) must be in place for the Hox genes to exert their effect on patterning. Since Hoxa and Hoxd enhancers of the spotted gar, a ray-finned fish, drive reporter gene expression in the distal domain of its pectoral fins (Gehrke et al., 2015), and the distal dermal rays of the zebrafish are dependent on the expression of Hoxa13 (Nakamura et al., 2016), it was proposed that "digits originated via

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the transition of distal cellular fates," i.e., from osteocytes to chondrocytes.

We suggest that these results, seemingly in conflict with long-held ideas of both evolutionary morphology and developmental biology, can most straightforwardly be interpreted by considering the patterning role of the complex of Turing-type mechanisms described here. As noted, these appear to have evolved in relative independence of mechanisms of terminal cytodifferentiation. In the case of the paired appendages, the cartilage differentiation program was evidently recruited to an early-evolved Turing-type process (i.e., the 2GL + Bmp/BmpR network) in the sarcopterygian protoautopod and the chondrichthyan fin margin by the incorporation of Sox9 and required cofactors. (It is relevant that the spatiotemporal distribution of Sox9 alone does not determine where skeletal elements will form; Montero et al. (2017).) In the fin fold of actinopterygians, the same ancestral patterning systems could have recruited the bone, rather than cartilage differentiation program.

A few comments are also in order about the role of Shh, which is distributed as a gradient in avian and mammalian limb buds with its high point in the ZPA (see above). This molecule was assigned (and has long been referred to) as the primary anteroposterior determinant of digit formation within the positional information model (Riddle et al., 1993), but it was eventually recognized to have growth promoting activity independent of its role in specifying digit identity (Towers et al., 2008). Its effect on digit number is thus accounted for by the Turing framework, in which the number of peaks is proportional to the size of the tissue domain being organized. Importantly, however, neither Shh nor its antagonist Gli3 is required for the development of "generic" digits, which are more numerous in the absence of both factors (Litingtung et al., 2002; Sheth et al., 2012). The identity of digits is determined, variously by the concentration, duration of exposure to, or even absence of Shh (Tickle and Towers, 2017), but its spatial expression is controlled in an elaborate limb-specific fashion by enhancers responsive to distal Hoxd proteins (Lettice et al., 2017). The role of Shh, therefore is in the secondary fine-tuning of the Turing-organized pattern (Newman, 1988), rather than as an instance of a universally employed positional signal. (Additional considerations on the relation of Turing-type mechanisms to digits as evolutionary innovations can be found in (Stewart et al., 2017).)

The investigation of each of the component mechanisms of the multisystem complex discussed here has been limited to a very small selection of species, which will need to be expanded before any solid generalizations can be made. The studies up till now have also presented mathematical and computational challenges, including use of rigorous timescale and domain shape approximation methods, and variable reduction techniques to ensure analytical rigor of simulations. While the sophistication of experimental and visualization techniques, and the data already in hand, have made "toy" modeling approaches seem outmoded, simulating integrated systems and their changing relationships over evolution in authentic detail will be much more difficult, and only possible with new methodologies. Framing and testing increasingly specific hypotheses for the origination, development and evolution of gnathostome fins and limbs in the future will therefore require an unprecedented degree of interdisciplinary effort.

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