A sense of place, many times over – pattern formation and evolution of repetitive morphological structures

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Abstract

50 years ago, Lewis Wolpert introduced the concept of 'positional information' to explain how patterns form in a multicellular embryonic field. Using morphogen gradients, whose continuous distributions of positional values are discretized *via* thresholds into distinct cellular states, he provided, at the theoretical level, an elegant solution to the 'French Flag problem'. In the intervening years, many experimental studies have lent support to Wolpert's ideas. However, the embryonic patterning of highly repetitive morphological structures, as often occurring in nature, can reveal limitations in the strict implementation of his initial theory, given the number of distinct threshold values that would have to be specified. Here, we review how positional information is complemented to circumvent these inadequacies, to accommodate tissue growth and pattern periodicity. In particular, we focus on functional anatomical assemblies composed of such structures, like the vertebrate spine or tetrapod digits, where the resulting segmented architecture is intrinsically linked to periodic pattern formation and unidirectional growth. These systems integrate positional information and growth with additional patterning cues that, we suggest, increase robustness and evolvability. We discuss different experimental and theoretical models to study such patterning systems, and how the underlying processes are modulated over evolutionary timescales to enable morphological diversification.

KEYWORDS

Positional information, repetitive morphological structures, periodic pattern formation, patterning modules, self-organization, directional growth, morphological diversification

KEY FINDINGS

Through a combination of positional information, directed growth and additional periodic patterning modules, repetitive morphological structures can be specified during development, in a robust, yet flexible manner.

1 | INTRODUCTION

Repetitive structures are plentiful throughout nature – be it the juxtaposed leaves on the branch of a tree, the body segments of an insect, or the individual bones that make up the vertebrate spine. From an evolutionary perspective, such repetitive patterns can be explained by the adaptive value the repetition of a certain anatomical unit can provide in itself. Moreover, repeatedly re-deploying and modifying a pre-existing developmental patterning module can enable morphological diversification. For example, in case of the vertebrate spinal column, individual vertebrae are attached to one another in a stable yet movable fashion. This repetitive vertebral architecture ensures the overall suppleness of the structure that is required for body movement, while also providing a solid protective encasement of the delicate spinal cord it encloses. At the same time, by exploiting the inherent developmental modularity of these spinal building blocks, the overall number of vertebrae can differ substantially between species, and each individual vertebra along the anterior-posterior axis can be modified in its morphology.[1,2] The concept of modularity is thus central to our understanding of how repetitive patterns can arise on both developmental and evolutionary timescales.[3–5] How then, however, is a certain patterning module repeatedly specified during embryogenesis, in a reliable and robust manner, while at the same time allowing for slight deviations that eventually can be canalized into evolutionarily novel morphologies?

50 years ago, the theoretical biologist Lewis Wolpert introduced his concept of 'positional information' that, to this day, continues to influence the way we think about developmental pattern formation.[6] He hypothesized that cells in an embryonic field have their relative position specified through a coordinate system based on three essential features: boundaries, that define the field and to which the relative position of a cell needs to be specified; a scalar to measure the distance from said boundaries; and polarity, emergent from the juxtaposition of differing scalar values, to confer directionality to this measurement. Both scalar and polarity of the system have come to be associated most often with a diffusible substance or 'morphogen', a term originally introduced by Alan Turing,[7] even though Wolpert also alluded to other potential mechanisms.[8] To illustrate the concept of positional information, Wolpert first assumed a multicellular field with uniform progenitor identities. Through localized production and subsequent dispersal of a substance, i.e. a morphogen, cells in the embryonic field would be exposed to differing concentrations along a gradient, which in turn bestows upon them distinct 'positional values'. According to distinct 'thresholds' of morphogen concentration, this continuous distribution of positional values is then differentially interpreted by the cells in the field and translated into discretized cellular states (Figure 1A). Thus, over the course of development, an initial asymmetry in morphogen production would allow cells to acquire different, concentration-based positional values, categorize these values into a discontinuous distribution of changes in cell-

intrinsic parameters, and ultimately result in spatially distinct cell fate decisions. This is famously illustrated in the so-called 'French Flag problem', in which Wolpert's model posits the sub-division of a homogeneous population of cells into three discrete 'cell type domains' as a result of threshold-based interpretation of a continuous morphogen gradient (Figure 1A). In the decades since its initial proposal, the concept of positional information has accumulated support from a range of experimental observations, beginning with classical embryology approaches,[9,10] and followed by investigations into the underlying cellular, molecular and biochemical mechanisms.[11–13]

Despite its far-reaching implications and experimental validation, there remain certain common patterning motifs, as well as evolutionary variations therein, that Wolpert's initial theory alone cannot explain satisfactorily. These patterns include, as already Wolpert acknowledged himself, the ones underlying the formation of repetitive morphological structures (Figure 1B,C). He reasoned that for highly repetitive architectures the assumption of a pre-patterning mechanism would provide a more parsimonious explanation than a purely positional information-based system, given the increasingly high number of distinct thresholds that are to be defined in the latter.[8]

In this review, we focus on the repeated deployment of developmental patterning modules, and how positional information might work alongside other mechanisms to assure proper pattern formation and evolution. After a brief overview of repetitive pattern formation in both two- and one-dimensional domains, we will shift our focus to systems where the polarity of the resulting repetitive pattern is inherently linked to the directionality of tissue growth. We will highlight the role of positional information in defining the temporal and spatial dynamics of such directed growth and discuss the challenges of establishing morphogen gradients in non-static embryonic fields with high cellular turnover. At the same time, positional information can define windows of 'patterning competency', for proliferating progenitors to respond to additional, often self-organizing mechanisms, which eventually result in segmented architectures made of repetitive morphological structures. We emphasize the apparent ease with which evolutionary variations in segment repetitions can be achieved under such conditions – through modifications of positional information, growth parameters or the additional patterning modules – as evidenced by morphological extremes like the vertebral column of snakes or the number of digit bones in cetacean flippers. Finally, we will review experimental and theoretical approaches to study these processes *in vivo*, *ex vivo, in vitro* and *in silico*, and how results from such studies continue to contribute to our understanding of developmental pattern formation and evolutionary diversification.

2 | THE FORMATION OF REPETITIVE PATTERNS IN NATURE - POSITIONAL INFORMATION AND SELF-ORGANIZATION

Pattern formation is an essential feature of multicellular organism development, and variations in patterning mechanisms are thought to contribute substantially to morphological diversification. Consequently, pattern formation has fascinated scientists for centuries and, owing to its amenability to abstraction, has stimulated collaborations between experimental and theoretical biologists.[14–16] Formation of periodic patterns, in particular, has attracted mathematicians and computational modelers alike.[17] Two of the most prominent conceptual frameworks in the field of pattern formation are certainly Wolpert's theory on positional information, and Alan Turing's 'reaction-diffusion'-based mechanisms. Unlike positional information, Turing models do not explicitly require any polarized molecular asymmetries prior to pattern emergence. Rather, slight spatial imbalances in the initial distribution of a cross-regulatory pair of an 'activator' and an 'inhibitor' are accentuated over time, due to different diffusibilities of the two substances, and thereby give rise to essentially self-organizing patterns.[6,7,18] While positional information had found plenty of experimental support early on – owing in large part to the rise of molecular genetics that helped to elucidate the segmentation network in *Drosophila* or cell fate specification in the early frog embryo (see below) – Turing systems and other self-organizing models have recently gained renewed interest.[19] Examples include symmetry-breaking events that underlie the emergence of repetitive, two-dimensional patterns (Figure 1B), like the induction of ectodermal appendages in the amniote skin,[20] spacing of stripe-color patterns in fish,[21] bristles placement on the fruit fly thorax,[22] rugae formation in the mammalian palate,[23] or the formation of digits in the tetrapod autopod.[24] Additionally, rather than focusing exclusively on the self-organizing properties of reaction-diffusion-type molecular systems, the role of cellular and/or mechanical mechanisms is increasingly being acknowledged,[25–28] as well as the potential to rely on the inherent periodicity of molecular oscillators to generate repetitive patterns.[29] While the oscillatory nature of these latter systems can be an emergent property at the tissue level, and hence be referred to as self-organizing,[30,31] their impact on the formation of repetitive spatial patterns is less direct. Unlike Turing models, which can reach stable states inside static embryonic fields, the temporal dynamics of a molecular oscillator necessitate its coupling to other variables, e.g. polarized growth, to translate wave-like gene activities into a defined spatial pattern (Figure 1C).[32] Importantly, however, in most of the patterning scenarios investigated thus far, neither self-organizing principles nor positional information seem to function in an entirely isolated fashion. Rather, they frequently co-occur, in parallel or close temporal succession, and similar patterning principles might repeat themselves during the maturation of a particular morphological structure. For example, while the periodicity of a given two-dimensional pattern may rely on self-organizing properties, potential sub-types of the resulting units – e.g. different *Drosophila* sensory bristles or tetrapod digit homeotic identities – can be defined by pre-existing morphogen gradients (Figure 1B; blue to red).[22,33,34] Once initiated, these repetitive structures have the potential to act as morphogen sources themselves, to

refine the emerging pattern or instruct the fate of neighboring elements (Figure 1B,C; purple to orange).[22,35] Collectively, combining 'positional information' with growth and additional patterning modules alleviates many of the problems inherent to the establishment of highly repetitive structures, were they to be specified by 'positional information' only (e.g. setting up reliable long-range gradients or precisely defining multiple threshold values). Such combinatorial patterning modules can therefore contribute to increase pattering robustness as well as boost the potential for their evolutionary reshuffling.[33,36,37] Hence, it appears that the strict dichotomy often attributed to the deployment of these two distinct patterning concepts during embryogenesis – i.e. 'positional information' or 'self-organization' – is likely artificial and, as previously suggested, a more realistic approximation of development would entail various combinations of the two (Figure 1B,C).[8,18,19]

3 | ARTHROPOD SEGMENTATION – POSITIONAL INFORMATION AND THE SPECIFICATION OF REPETITIVE PATTERNS IN STATIC AND EXPANDING DOMAINS

It can been argued that part of the tremendous evolutionary success of arthropods, both in terms of taxonomic diversity and sheer abundance, is attributable to their segmented, metameric body plan organization. Indeed, functional specializations of different body segments have enabled the exploitation of a wide variety of different ecological niches.[38,39] The study of insect embryogenesis and segment formation, in particular, has substantially contributed to our understanding of how positional information can instruct the formation of repetitive patterns. For one, unlike for the aforementioned combinatorial patterning modes, during the early segmentation of the *Drosophila* embryo Wolpert's concept of positional information manifests itself most explicitly. Accordingly, anterior-posterior patterning in *Drosophila* was amongst the first experimental models to unequivocally prove some of Wolpert's key predictions. During *Drosophila* embryogenesis, all body segments are already contained within the length of the embryo's syncytial blastoderm. Fundamental to establishing positional information in this system, and by extension the specification of primary body axis segmentation and polarity, are two opposing gradients. Their presence had already been inferred from cytoplasmic constriction and transplantation experiments, and was predicted to rely on maternal gene products deposited on either end of the egg.[9] With the identification of *bicoid*, the causative anterior determinant, and subsequently *Nanos*, its posterior counterpart, the first molecules emerged to validate Wolpert's claims.[11,40] Downstream of *bicoid* and *Nanos*, a hierarchically organized gene regulatory network interprets the positional values of the two gradients, to sub-divide the anterior-posterior body axis, specify individual segments and establish segment polarity and identity.[41] Hence, within the static domain of the *Drosophila* embryo, two opposing gradients, with cross-regulatory interactions for increased precision, and their differential cell-intrinsic interpretation suffice to reliably

specify the positional values required for the formation of all body segments.

However, while the simultaneous specification of body segments is characteristic for long-germ band insects such as *Drosophila*, in short-germ band insects like the flour beetle *Tribolium casteneum* segments are formed sequentially, from anterior to posterior as the embryo elongates.[42] This mode of segmentation thus intimately links growth-based axis elongation to periodic pattern formation and is, in fact, considered to be the ancestral condition for arthropods in general.[43] The posterior region of short-germ band embryos contains a growth zone of proliferating progenitor cells that drives axis elongation. Positional information, based on a gradient of Wnt/β-catenin activity that delineates a posterior growth zone, and a molecular oscillator, involving the cyclic expression of 'Pair rule' genes, are required for axis extension and segmentation in short-germ band insects.[42,44–46] Unlike in *Drosophila*, where Pair-rule genes are concomitantly expressed in a striped pattern demarcating the future segments, dynamic waves of cyclic Pair-rule gene expression propagate along the *Tribolium* growth zone, to sequentially segment the emerging primary body axis. Additionally, *Caudal*, *Dichaete* and *Odd-paired* expression in *Tribolium* form spatiotemporally dynamic wavefronts that travel along the anterior-posterior axis of the elongating embryo, while in *Drosophila* their sequential activation acts as a timer of Pair-rule gene expression.[47] Hence, although displaying drastically different growth modes for axis elongation, in both long-germ and short-germ insect segmentation similar sets of orthologous genes are essential in anterior-posterior pattern formation. The underlying genetic circuitries thus seem to contain an ability to compute and execute analogous patterning functions, both within static embryonic fields as well as along progressively elongating domains.[47–49] Disparities in their regulatory architectures, though, between long-germ and short-germ insects, emphasize the importance of properly integrating temporally dynamic gene expression programs with positional information in directionally growing domains.[47,49,50] The fact that short-germ band insects do not seem to exploit their mode of axis elongation to increase overall segment number, like for example in the vertebral column of snakes (see below), may hint at an underlying developmental constraint, originating from molecular crosstalk between the two systems in insects.[42,51] Intriguingly, though, primary body axis patterning in other arthropod clades such as the *Myriapoda* clearly is more variable, with overall segment numbers in e.g. geophilomorph centipedes ranging from 27 to 191.[52] Hence, how seemingly similar genetic cassettes are cross-regulated in both space and time, and integrated with a particular growth dynamic, is what ultimately appears to determine the resulting segmented pattern and its evolvability.[43,53]

4 | POSITIONAL INFORMATION, DIRECTIONAL GROWTH, AND THE PERIODIC SPECIFICATION OF ONE-DIMENSIONAL PATTERNS

By explicitly decoupling the control of growth dynamics from a self-organizing mechanism, modular variations of periodic pattern formation – and hence segment numbers – can be achieved through evolutionary modifications altering either one or both of the two parameters. For the control of directional growth, morphogen-based positional information often delineates a pool of progenitor cells and, accordingly, gradient dynamics can define the spatial and temporal extent of proliferative axis elongation. Establishing accurate positional information within a directionally growing embryonic field, however, can present several challenges. Rather than cells being located statically within the field, and thus able to interpret a morphogen gradient both spatially and temporally, they dynamically traverse the domain to be patterned, as tissue elongation occurs. The history of positional cues that the cells experience thus directly relates to the directional growth dynamics they themselves help to establish.

There are numerous examples in nature where the creation of repetitive morphological structures depends on growth dynamics that can be approximated along a one-dimensional domain. While for much of the remainder of this review we focus on two iconic 1D-patterns in vertebrates – that of the somite-driven segmentation of the primary body axis and the individualization of phalangeal bones in tetrapod digits – it is worth mentioning that similar periodic patterns, some with striking similarities, have also arisen in the plant kingdom. Given the independent advent of multicellularity in the animal and plant kingdoms, the underlying mechanisms of these patterning systems must have evolved convergently. However, as previously argued by others, certain unifying design principles, as well as conserved molecular and/or cellular features implemented in these patterning systems, can emerge from such distant comparisons.[54–56]

4.1 | Plant shoot segmentation: repetitive patterns of phytomers

In most plants, above-ground growth relies on cell proliferation at the tip of the elongating shoot. This growth is sustained by a stem cell population that is located inside the so-called ‘shoot apical meristem’ (SAM).[57] The basic structure of the SAM can be roughly subdivided into a central zone - the reservoir containing the stem cells - a rib zone which forms the bulk of the plant stem, and a peripheral zone from which lateral organs such as leaves develop.[58] Importantly, shoot elongation occurs in a segmented fashion, through the successive addition of repetitive structures known as ‘phytomers’. Each phytomer is composed of a node carrying a leaf, an internode region and an axillary bud that allows for branching (Figure 2A).

Inside the SAM, stem cell proliferation *versus* differentiation needs to be tightly balanced. Genetic analyses in *Arabidopsis,* as well as comparative studies across species, have revealed the presence of multi-faceted regulatory cascades centered on the CLAVATA-WUSCHEL axis that maintain the

undifferentiated state of the SAM stem cells.[59,60] SAM stem cells provide the cellular building blocks to the different components of the phytomer, including the axillary buds. Axillary buds can act as meristems, just like the SAM, and give rise to secondary shoots that are either vegetative (e.g. lateral branches) or reproductive (i.e. flowers) in nature. They can thus be considered as several secondary 1D-growing fields connected to one major 1D-growing domain whose directionality is determined by the location of the SAM. By extension, spatiotemporal modulation of the patterning and positioning of these axillary buds along the apical-basal axis of the main shoot allows plants to diversify their overall architectures.[61]

Inside the main shoot, the repetitive deployment of the segmental phytomers depends on 'phyllotaxis', the process of periodic placement of plant lateral organs in regular intervals both around the central and apical-basal axes of the shoot.[62] Subsequent elongation of the phytomer then leads to the species-specific spacing patterns observed between the individual segments. For the radial patterns circumscribing the shoot, lateral organ placement can occur in whorled, distichous (alternate), decussate (opposite) as well as spiral arrangements – the latter invoking the famous Fibonacci sequence.[63–65] Auxin, a phytohormone produced in the SAM, has been shown to have a central role in lateral organ formation and thus phyllotaxis. Indeed, micro-manipulations of auxin concentration reveal that when auxin levels decrease, stem cells start to differentiate.[66,67] Thus, gradients of auxin concentration provide positional information along the apical-basal axis, and critically contribute to control the balance between cell proliferation and differentiation (Figure 2A).[66] During phyllotaxis, PIN proteins, a family of membrane bound efflux carriers, control the generation of new auxin maxima and polar transport of auxin by PIN proteins allows for periodic pattern formation of organ initiation on the plant shoot.[66,68,69] Several studies have suggested the presence of additional feedback mechanisms to ensure proper organ placement, both at the level of auxin transport or *via* inhibition from previously formed organ primordia.[70,71] In parallel to these mostly auxin-based inhibitory functions, cytokinin, another phytohormone, plays important roles in phyllotactic patterning.[72] Cytokinin is mainly produced in roots and is transported up the shoot, thus forming a basal-to-apical gradient of cytokinin and, in association with auxin, defining robust positional information along the shoot (Figure 2A). Cross-regulatory effects between the two hormones, at the level of their respective syntheses or transport modes, as well as intercellular movement of additional inhibitors seem to define this interaction at a molecular and cellular level.[71,73,74] Moreover, the fact that the eventual basal-to-apical 1D-pattern of the shoot involves - in its inception - a two-dimensional component, namely the circumferential positioning of lateral branches, has led to the consideration of different self-organizing properties involved in the process. For example, inhibitory fields of leaf primordia have been proposed to affect spacing during phyllotactic patterning,[64] and already Turing himself, and others, have argued that activator-inhibitor pairs might underlie the patterning

phenomenon of phyllotaxis.[7,62,75,76] How exactly such interplay of positional information and self-organizing principles is realized, however, and in which way the rate of apical-basal growth as determined by the SAM affects this balance, is an area of active investigation using both theoretical and experimental approaches.[62,67,77]

4.2 | Vertebrate primary body axis segmentation: repetitive patterns of somites

During vertebrate embryogenesis, the paraxial mesoderm, localized on both sides of the developing neural tube, is segmented into a series of repetitive structures that are known as 'somites'. Cells inside these somites give rise to a variety of tissues in the adult body, such as e.g. muscle, dermis, tendons or the progenitors of the axial skeleton.[78] Most somite-derived tissues lose their segmented appearance as they mature. Notably, although somite number determines vertebral count, even the separation into individual vertebrae is secondary to the original somite boundaries. Vertebrae form from the repeated fusion of the caudal and rostral halves of two consecutive somites, with additional patterning cues emanating from the notochord.[79–81] From an evolutionary perspective, overall somite number, and by extension vertebral count, can vary substantially between different vertebrate species.[1] Moreover, these skeletal somite derivatives appear highly regionalized along the anterior-posterior axis, with characteristic vertebral morphologies that reflect their distinct functions along the spine.[82,83] Somitogenesis initiates anteriorly, adjacent to the head mesoderm, and progresses along the primary body axis as the embryo elongates at its posterior end. Segmentation occurs periodically, with a species-specific temporal rhythm, with somites progressively forming from the paraxial mesoderm with a remarkably regular rate of segmentation.[29] The maintenance of this process critically depends on a posterior progenitor population, which in its unsegmented state is known as the 'presomitic mesoderm' (PSM) and acts as a unidirectional growth zone (Figure 2B).[84] As these mesenchymal cells approach the anterior margin of the PSM, an epithelium surrounding a mesenchymal core begins to form, thereby defining the individual somites. Hence, by controlling the elongation rate inside the PSM, as well as the temporal rhythmicity with which new boundaries are initiated, the basic pattern of somitogenesis is controlled.[85] Several models have been suggested to conceptualize the temporal and spatial aspects of this somitogenic process, most notably the 'clock and wavefront' model.[86] This model proposes two distinct mechanisms that, in combination, provide an explanation for the sequential formation of somites. First, a molecular oscillator, or 'segmentation clock', instructs the temporal periodicity with which new somites are formed. And second, a hypothetical gradient provides positional information in form of a 'wavefront', to define an anterior-posterior position inside the PSM where cells become responsive to the segmentation signals of the clock. This particular location is often referred to as the 'determination front'. Indeed, the clock and wavefront model has been

supported by numerous experimental observations. For example, cyclic expression of *Notch* target genes was reported in the PSM of chick embryos.[87,88] Moreover, mutations therein, as well as experimental perturbations in *Notch* modulators, were shown to affect the molecular clock and somitogenesis in various vertebrate species.[89–91] Following studies have revealed a substantially expanded oscillatory regime inside the PSM. Besides the *Notch* pathway, this includes members of the *Wnt* and *Fgf* signaling cascades,[92,93] both of which have also been implicated in the second major constituent of the model, the 'wavefront' (see below). Intriguingly, while the overall pathways of the oscillator seem conserved amongst vertebrates, the actual gene members that show cyclic behavior can vary considerably between species.[94] This argues for substantial stability when determining the net output of the respective signaling network, potentially conferred by multiple feedback loops, which in turn can explain the apparent drift in the developmental system at the molecular level.[95,96] The second major prediction in the model of Cooke and Zeeman is the presence of a wavefront at the anterior margin of the PSM, which acts as a traveling frontier of somite formation competency that moves posteriorly as the embryo elongates.[86] It was suggested that positional information by morphogen signaling gradients emanating from the PSM instructs the positioning of the wavefront. Indeed, posterior-to-anterior gradients of FGF and Wnt as well as an anterior-to-posterior gradient of Retinoic acid (RA) have been reported (Figure 2B). FGF signaling has been shown to determine wavefront position along the axis of the PSM and to be involved in the onset of the segmentation program.[97] High levels of FGF activity maintain an undifferentiated state and confer elevated levels of mobility in posterior cells.[98] As FGF production is restricted to the posterior end of the PSM, FGF levels decrease as the cells travel along to the PSM, allowing anteriorly located progenitors to start their segmentation program while at the same time contributing to axis elongation.[99,100] Additionally, graded *Wnt* activity contributes to the positioning of the wavefront, as well as providing a molecular link to the segmentation clock itself and the proliferative control of axial progenitors.[92,101–103] From the anterior end, a gradient of RA refines this boundary, while at the same time buffering for left-right asymmetries in the formation of somites on either side of the neural tube.[104–106] Hence, integrating the spatial and temporal dynamics of these gradients with the oscillations of a molecular clock, determines overall elongation and segmentation rate of the PSM, and provides a conceptual framework to contextualize somite size control.[85,107]

The development of models that are able to approximate important aspects of somite segmentation *ex vivo*, *in vitro* and/or *in silico* have empowered experimental and theoretical approaches to study the process at a more quantitative level. Many of them focus on some of the apparent self-organizing properties of the process, in particular for size scaling and the emergence of the molecular oscillator.[30,31,108–111] Some iterations abandon the notion of the importance of global positional information *via* gradients altogether, in favor of an oscillatory reaction-diffusion mechanism.[112]

Importantly, however, only by explicitly including termination of elongation and patterning in these models will the true evolutionary diversity in vertebral formulas be accounted for.[113] This would further entail the control to balance segmentation speed and progenitor pool size,[114] as well as incorporating the temporal and spatial effects of an axial *Hox* code on progenitor proliferation and somite identity.[115–118] Intriguingly, either modulations in the speed of the segmentation clock or, alternatively, changing the duration of progenitor pool persistence have been shown to alter the eventual number of segments in the vertebral column of different species.[114,115]

4.3 | Tetrapod digit segmentation: repetitive patterns of phalanges

Another striking example of repetitive pattern formation along a single axis of embryonic growth is the development of tetrapod digits. Tetrapod digits are segmented into individual digit bones called phalanges, which in adult hands and feet are connected to each other by synovial joints. Analogous to the somite-derived vertebral column, different numbers of phalanges per digit occur, both within and between species. According to the fossil record, early tetrapods already showed differences in phalanges count in their digits.[119] Once the pentadactyl 'ground state' of the autopod had been established, the ancestral phalanx numbers per digit are believed to be 2-3-4-5-3, for digits I to V.[4,120] However, these numbers have changed considerably in different tetrapod clades. For example, the majority of mammalian autopods display a 2-3-3-3-3 phalanx formula for their five digits,[4,120] while certain cetacean species have drastically increased the overall number of bones per digit. This resulted in an extreme variation of the ancestral phalanges pattern known as 'hyperphalangy'.[121] Moreover, phalanges in a given digit vary not only in number, but also differ markedly in individual size, both length- and girth-wise. As a consequence, within a given species, the number, size and shape of the phalanges are reflective of each digit's homeotic identity.[4,33]

At the onset of digit development the autopod plate is composed of alternating interdigit areas and digital rays, as previously specified by a Turing-like patterning mechanism.[24,34,122] While in the more proximal parts the metacarpals and metatarsals already start to condense, at the very distal tip of the autopod the actual outgrowth of the digits starts. Interestingly, the underlying molecular mechanisms for building these distal autopod elements are likely distinct from the more proximal ones, as demonstrated by the loss of phalange development in *Bmpr1b* knockout mice while metacarpals remain relatively unaffected.[123] Proliferation of a distal progenitor population, known as the 'phalanx-forming region' (PFR), or 'digital cresent' (DC),[124–126] allows for the growth of the digit to occur unidirectionally along its proximal-distal axis (Figure 2C). The PFR itself is thought to originate from the distal mesenchyme, localized just beneath a specialized epithelial structure called the apical ectodermal ridge (AER). Epithelial cells inside the AER are known to mediate overall limb growth, by

secreting FGF signals that promote proliferation in the underlying mesenchyme.[127] Consequently, a FGF gradient specifies a distal domain of growth competency, which is translated into digit elongation at the PFR (Figure 2C).[122,124,125,128] FGF signaling from the AER seems to have a role not only in the control of digit length, but also phalanx numbers. By examining *Fgf8* expression in the developing chicken foot - in which each digit is morphologically different, both length- and phalanx number-wise - a correlation of the duration of *Fgf8* expression at the digit tip and the resulting number of phalanges was observed.[129] Experimentally prolonging *Fgf8* expression at the digit tip induces the formation of an additional phalanx, while use of an FGF receptor inhibitor prevents formation of the most distal phalanx.[129] Temporal variations in AER persistence, and by extension duration of FGF signaling, have therefore the potential to explain even extreme deviations from an ancestral phalanx formula, such as for example seen in the hyperphalangy of cetacean flippers.[130] However, while the effect of FGF on cell proliferation suggests an obvious mechanism to control digit length, how can the segmentation into individual phalanges occur at the cellular and molecular level?

It is also within the PFR population that distinct cell fate decisions are thought to occur during digit elongation, instructing digit segmentation along its proximal-distal axis. In contrast to somite formation, where a change of tissue organization (i.e. mesenchymal-to-epithelial) drives segmentation, the partitioning of digits into individual phalanges involves the specification of two distinct cell types. Once proliferation of the PFR progenitor cells has displaced the source of the FGF gradient distally, the proximally located cells lose their progenitor state and undergo a divergent cell type specification. They differentiate accordingly into either chondrocytes - the cellular building blocks of the phalanges themselves - or prospective interzone cells that eventually form the synovial joints to connect the digit bones.[122,131] Hence, by controlling the temporal aspects of this divergent cell fate decision with respect to the overall growth rate, the digit segmentation pattern into individual phalanges can be determined. To faithfully execute this process, the PFR assimilates various signaling inputs that confer positional information and modulate additional, possibly self-organizing mechanisms, to result in correct digit segmentation patterns and thus homeotic identity. Most notably, it has been demonstrated in chicken embryos that the forming digits have their segmentation pattern specified by the interdigit mesenchyme that is located immediately posterior to them.[33] Interdigit mesenchyme “cut-and-swap” experiments result in homeotic transformations that corroborate the idea that the interdigit mesenchyme is involved in digit identity specification. Multiple lines of evidence implicate gradients of BMP signaling, originating from the interdigit tissue, to establish this positional information system at the molecular level. For example, implantation of a bead soaked with the BMP antagonist NOGGIN within the interdigit induces an anteriorization of digit identity.[33] Moreover, the PFRs of different digits were found to carry distinct levels of SMAD1/5/8 activity that correlate well with the eventual differences in their segmentation patterns.[125]

While the role of BMP signaling in determining phalanx numbers in each digit is well accepted, there is mounting evidence that it could also influence phalanx size. Within each digit the phalanges sizes do not vary randomly, but rather seem to change as an integral developmental module, separate from the rest of the autopod.[122,132] Capitalizing on a broad phylogenetic sampling covering multiple vertebrate clades, it was demonstrated that the ratios of measured areas of successive phalanges change in a predictable manner. Namely, the size of a proximally located phalanx is prognostic for the size of more distal phalanges, with the largest phalanges usually found at the proximal end of the digit.[132] Thus, despite the complexity and diversity of phalangeal morphology across digits and species, there appear certain remarkably conserved relationships amongst the distinct elements that point to the presence of conserved developmental modules. Moreover, the periodicity of the eventual pattern may hint at an underlying self-organizing property of the process, potentially Turing-like in nature, that acts concomitantly as digit elongation occurs. Indeed, individualized phalanges sizes are not merely the result of post-patterning events like, e.g., growth plate-mediated long bone elongation. Rather, they represent an integral part of the patterning process itself, as size differences are already apparent at early stages of phalanx specification. This corresponds to a timepoint when synovial joint interzones separating the successive phalanges are being initiated.[132,133] Barrier insertion and viral overexpression experiments in chicken, as well as genetic manipulations in mice, suggest that one or more diffusible cues from the previously formed phalanx and/or interzone may be instrumental in this process.[132,133] Several experimental observations also imply the presence of additional, partially self-organizing principles that may help to refine digit pattern periodicity.[122] For example, the ectopic induction of an interzone using retroviral overexpression of *Wnt9a* has been shown to inhibit formation of subsequent joint sites at a distance.[134] Likewise, insertion of a barrier into a proximal phalanx leads to an increased segment sizes in subsequently forming phalanges.[132] Based on its expression in maturing phalanges, as well as the lack of phalangeal joint formation in mutant embryos, *Noggin* has been proposed as the putative diffusible cue underlying these effects (Figure 2C).[135] A progressive build-up of NOGGIN protein, caused by the increasing phalanx expression domain, could instruct subsequent joint specification, once a critical threshold of BMP inhibition has been reached.[133] Using an allelic series in mice, NOGGIN-modulated BMP activity itself has been shown to lie downstream of a 5'Hoxd-Gli3 antagonism. Since both 5'Hoxd genes and Gli3 show quantitative differences in their expression levels along the pinky-to-thumb axis of the developing autopod, this model provides an elegant explanation of how anterior-posterior positional information could be translated into distinct digit identities.[129,133] However, based on the apparent dynamics of BMP signaling in the forming phalanges, across both space and time, additional modulators might be involved in the exact determination of digit-specific phalanx-joint patterns (Grall and Tschopp, unpublished observations).

5 | CONCLUSIONS AND FUTURE DIRECTIONS

As highlighted in the examples above, the three key components of a positional information-based coordinate system – boundaries, scalar, and polarity – face distinct challenges when we consider their implementation in a directionally growing domain. As for the non-expanding condition proposed in Wolpert's original model, morphogen gradients play an essential role in determining all three parameters. How they are established, however, can be quite different from their static counterparts. For phytomers, somites and phalanges, the position of a proliferating progenitor population defines one of the boundaries of the field to be patterned, as well as the directionality of tissue growth (Figure 2A-C). Localized production of a morphogen within (SAM, PSM) or nearby (AER) this progenitor population provides the source for establishing a molecular gradient. The time required for a cell to traverse the resulting gradient field, i.e. the interval a cell is displaced from the gradient's range of influence by the proliferation of more distally located cells, thus becomes central to the temporal integration of the signal.[8,136] Moreover, the distal production of the morphogen in phytomer and somite progenitors themselves, and the control of its polarized transport or stability, as the cells journey through the field, are essential to define the scalar of the gradient.[67,99] For the PFR, responsiveness to the FGF signals emanating from the overlaying AER alters proliferation rates and, by extension, the time the progenitors spend inside the gradient. The unidirectional nature of the growth, resulting from the distal location of the proliferating progenitor populations, inherently defines the polarity of these gradients. To ensure robustness in establishing and interpreting all of these primary gradients, secondary and opposing gradients act in conjunction (Figure 2A-C). In case of cytokinins (phytomers) and retinoic acid (somites), they function by directly counteracting the distal gradients,[72,105] whereas *Noggin* (phalanges) has been suggested to spatially modulate the induction of the following segments.[133,135] These proximally located gradients are also key to control secondary patterning events in the prospective segments, be it for a graded size control of the forming phalangeal segments,[132] to balance left-right asymmetries in somites,[106] or to control the spacing and orientation of subsequently forming secondary organs in plant shoots.[73,74] Importantly, in all three cases the production of these secondary gradients initiates in an already formed segment, i.e in cells that have been removed from the embryonic field to be patterned. As such, they help to determine and refine the proximal boundaries of the field, while at the same time contribute to segment size control.[5,74,107] Combining growth-driven displacement of molecular gradients, to establish positional information, with a secondary, self-organizing patterning module appears to be a common design principle in the establishment of periodic patterns.[32] In somitogenesis, the location of segment boundary formation famously depends on the combination of a gradient-dependent, moving 'determination front' and cell-intrinsic molecular oscillators.[29,30] Likewise, for root growth in plants – which relies on a SAM-like

arrangement of its proliferating progenitors, the root apical meristem (RAM) – oscillating gene expression networks have been reported to control the periodicity of lateral branching.[137] Above ground, however, phyllotaxis has been successfully approximated *in silico* by activator/inhibitor- and transport-based models.[67,75,138] While molecular similarities to the oscillator-based segmentation of the primary body axis have been proposed for overall tetrapod limb patterning,[139] self-organizing mechanisms in general await further experimental validation and quantitative data, in particular for the patterning of individual phalanges in the distal limb.[122,133] Clearly, however, it appears that the combination of positional information-based directional growth with additional patterning modules, often self-organizing in nature, might generally underlie the periodicity of repetitive morphological structures (see e.g. palate growth and Turing mechanisms during mammalian rugae formation[23]).

Indeed, by combining Wolpert's positional information with further patterning systems, either temporally or spatially, the overall robustness of the system might increase, and could thus be buffered against slight developmental deviations that eventually might transition into evolutionary novel patterns.[96,140] While variations in segment numbers are easily explained by alterations in the size or the temporal persistence of the progenitor pool, results from morphological extremes, like vertebral count in snakes or cetacean phalanges, suggest that different sub-modules of the system - for example the speed of an oscillator or proliferation-dependent feedback into the segmentation module - can be affected as well.[114,130] Moreover, size control between individual elements might be internally constrained by the molecular and/or cellular architecture of the ancestral segmentation process, thus restricting the exploration of the entire theoretically available morphospace.[5] And lastly, post-pattering processes, like discretized growth control of individual segments, may provide an additional layer of evolutionary diversification.[141,142] Importantly, all of these observations highlight the fact that rarely, if ever, a certain pattering module might function in a truly isolated fashion. It is therefore more likely that these tight interconnections, between positional information and additional systems hint at the existence of largely context-dependent patterning outputs.

While providing pattering robustness and evolvability, such combinatorial systems can render the acquisition of quantitative data cumbersome, as well as severely impede the design of clearly interpretable experimental perturbations in order to test certain hypotheses. Here, the development of dedicated *ex vivo* and/or *in vitro* models might prove invaluable to study a given patterning module in true isolation. This has already successfully been realized for important aspects of somitogenesis, or in different organoid systems.[31,109,143,144] In combination with microfluidic or optogenetic approaches controlling morphogen signaling, such *ex vivo*/*in vitro* methods are likely to contribute to a more quantitative understanding of the underlying molecular and cellular processes.[133,145,146] Furthermore, emerging techniques to measure and perturb various intrinsic parameters with cellular resolution will help to disentangle how virtually homogenous, extracellular positional information can be interpreted

differentially cell-intrinsically, to result in discretized cellular states.[128,147] Quantitative data from these newly available technologies should in turn result in the continuing refinement of mathematical and computational models, to approximate periodic pattering of repetitive morphological structures *in silico.*[17,19]

Finally, implementing these experimental and theoretical methods within the context of an evolutionary-comparative framework might turn out to be mutually beneficial. For example, *in silico* models may help to predict the causative parameter alterations that can transform one species-specific pattern into another, whereas contrasting repetitive pattern formation over different evolutionary timescales can instruct the design of improved models and experimental approaches alike. Here, studies at the micro-evolutionary level will reveal the degree of plasticity associated with a certain patterning process, while macro-evolutionary comparisons can inform us about potential development constraint. Indeed, embracing the power of comparative approaches may bring us full circle with Wolpert's initial proposition of 'positional information', where he discusses the problem in the context of species as diverse as hydra, *Drosophila* or chicken.[6] Such efforts have certainly contributed to our appreciation of two of the major underlying design principles in the patterning systems of highly repetitive structures: the fact that positional information seems to work preferentially in conjunction with additional, often self-organizing patterning modules, and that a decoupling of growth and segmentation control allows for modular alterations in segment numbers.

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FIGURE LEGENDS

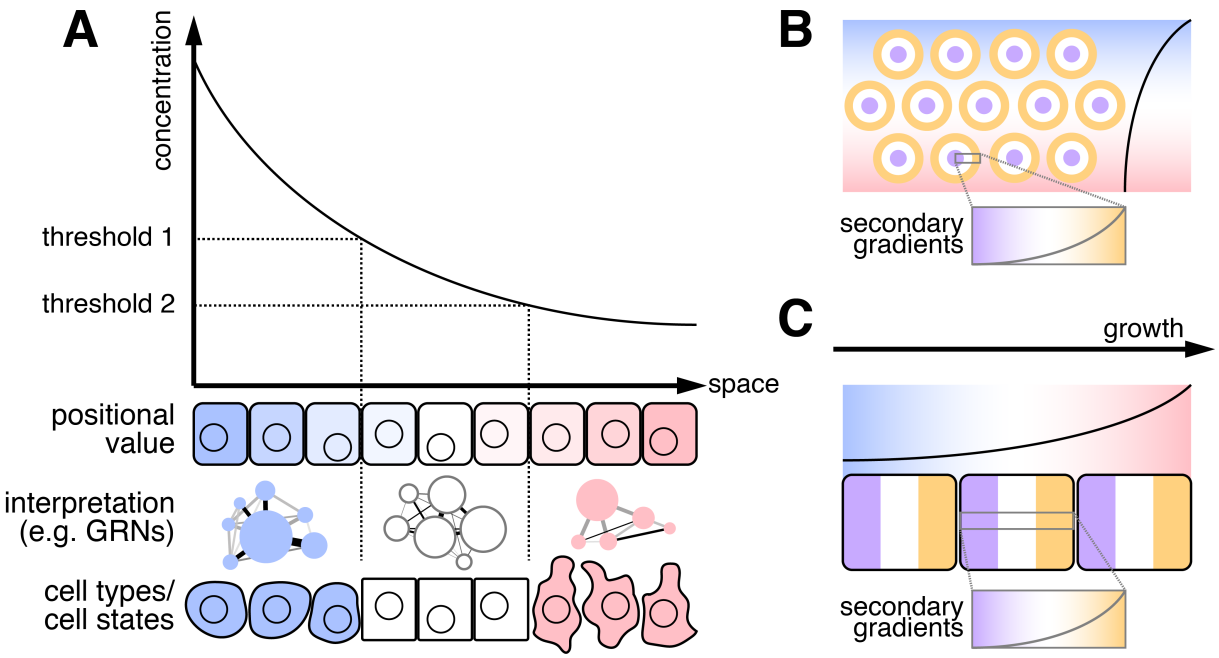

FIGURE 1. 'Positional information' and the emergence of repetitive patterns. A: Wolpert's classic illustration of positional information and its relation to the 'French Flag problem'. A morphogen is locally produced, secreted, and dispersed to establish a molecular gradient over an embryonic field. Cells are exposed to different molecular concentrations along the gradient, endowing them with distinct 'positional values'. According to distinct 'thresholds', these positional values are differentially interpreted by the cells (e.g. rewiring of gene regulatory networks (GRNs)) and result in distinct cell fate decisions. B: Positional information in two-dimensional, repetitive patterns. While the formation of many two-dimensional, repetitive patterns can be explained by self-organizing principles, their implementation is often constrained by additional, pre-existing positional information cues (blue to red gradient). Once initiated, repetitive elements may act as secondary morphogen sources and exert their effect on the surrounding tissue in a positional information-like manner (concentric patterns, purple to orange gradients). C: Positional information in one-dimensional, repetitive patterns driven by directional growth. Growth dynamics and their underlying progenitor populations rely on morphogen gradients that determine a field of competency (blue to red gradient). Moreover, previously formed segments may inherit positional information-containing polarity and establish secondary morphogen gradients themselves, to modulate the formation of successive elements (purple to orange gradients).

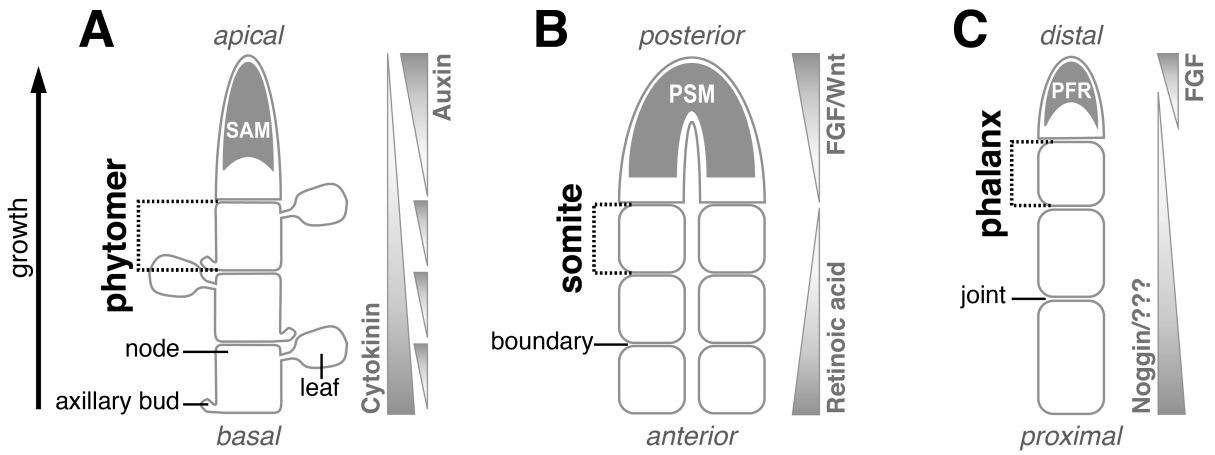

FIGURE 2. Formation of repetitive morphological structures across kingdoms. A: In plants, apical-basal growth depends on a proliferative zone at the apex of the shoot called the 'shoot apical meristem' (SAM). The elongating shoot is segmented into repetitive structures known as 'phytomers', which are composed of a node carrying the leaf, an internode region and an axillary bud. The integration of two opposing gradient systems of phytohormones - auxin, mainly synthetized in the meristems, and cytokinin, mainly synthetized in roots - provides positional information along the apical-basal axis, and helps to define a balance of proliferation and differentiation. B: Vertebrate axial elongation depends on the successive formation of 'somites', which originate from progenitors within the presomitic mesoderm (PSM). Posterior-to-anterior gradients of FGF and Wnt and an anterior-to-posterior gradient of Retinoic acid (RA) provide positional information along the primary body axis. These gradients delineate a 'determination front', at which progenitors respond to molecular oscillators to initiate segmentation. C: Tetrapod digits grow proximal-distally due to progenitor proliferation within the phalanx forming region (PFR), which relies on a distal FGFs. Once progenitors leave the PFR, cell fate decision into either joint- or cartilage-forming cells instruct the digit segmentation pattern into individual 'phalanges', potentially modulated by BMP inhibitors released from previously formed elements.

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