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Tissue tectonics and the multi-scale regulation of developmental timing

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Abstract

Development encompasses processes that occur at multiple length-scales, including gene regulatory interactions, cell movements and reorganisation, cell signalling and growth. It is essential that the timing of events in all of these different processes are coordinated to generate well patterned tissues and organs. However, how the timing of intrinsic cell state changes is coordinated with events at the multi-tissue and whole organism level is unknown. Here, we argue that an important mechanism which accounts for integration of timing across levels of organisation is provided by *tissue tectonics*: i.e. how morphogenetic events driving tissue shape change result in the relative displacement of signalling and responding tissues and coordinate developmental timing across scales. In doing so, tissue tectonics provides a mechanism by which the cell specification events intrinsic to cells can be modulated by the temporal exposure to extracellular signals. This exposure is in turn regulated by higher-order properties of the embryo such as their physical properties, rates of growth and the combination of dynamic cell behaviours impacting tissue morphogenesis. Tissue tectonics creates a downward flow of information from higher to lower levels of biological organisation, providing an instance of downward causation in development.

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1 Introduction

Time is central to biological phenomena: biological processes are inherently dynamic and this is true across all biological fields. Developmental biology provides a strong context to study biological time, as it allows for the study of developmental timing at many different levels of biological organisation - opening the possibility for the identification of mechanisms which coordinate these different length scales. Developmental timing can be thought of in terms of the absolute timing of a given event, the ordering of events relative to one another, the directionality of developmental processes, and the more general tempo (speed) at which development proceeds (Duboule, 2003; Ebisuya and Briscoe, 2018; Johnson and Day, 2000). We will focus specifically in this review on how the **absolute timing** of a given event in development is controlled, and propose a mechanism by which timing may be coordinated across different levels of organisation in the embryo.

As a cell moves through developmental time, it undergoes a series of cell state transitions that ultimately define its fate. In considering the mechanisms that regulate the timing of cell state transitions, a distinction has been made between intrinsic and extrinsic timing mechanisms: *intrinsic* timers function within the cell, whilst *extrinsic* mechanisms implicate the importance of the external cellular environment in providing inputs to the timer (*Figure 1)*. Whether a timer is controlled through intrinsic or extrinsic mechanisms has primarily been investigated using classical experimental embryological methods. For example, physically grafting cell populations between embryos of different ages (heterochronic grafting) allows any influence of external factors on a timer to be identified. If the timer of interest progresses as expected from the age of the donor tissue once placed in this novel environment, it suggests that the functioning of the timer is intrinsic to the cell population. Conversely, if the timer is accelerated, decelerated, reset or advanced in the host context, it suggests that external factors which the cell population is exposed to in this context are important for the normal functioning of the timer and that the mechanistic basis for the functioning of the timer is extrinsic.

To determine the contribution of extrinsic or intrinsic components to timing an event, an additional experimental embryological approach involves removing groups of cells from the embryonic environment and culturing them in a neutral environment (explant culture). If the timer is able to progress outside of the embryo, it may provide evidence that its underlying mechanisms act cell-intrinsically. Note that these experiments are very similar to those used to investigate cell specification and determination. A cell may be defined as specified to form a particular structure if, when isolated from the embryo and placed in a neutral environment, it will still form that structure in the absence of any external inputs

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(Slack, 1991). A cell is determined when it gives rise to this structure in any context, including any embryonic context (Slack, 1991). This differs somewhat to the employment of these assays in the field of timing, but the assays are nonetheless fundamentally the same and highlight the importance of understanding the mechanisms by which intrinsic timers are modulated by the local signalling environment that they encounter during development.

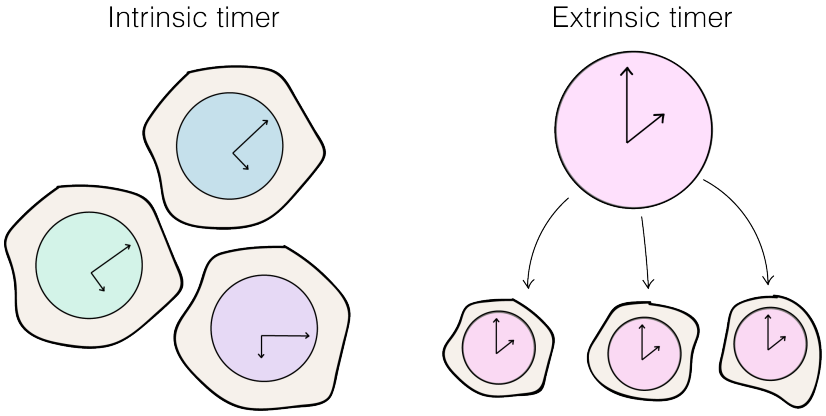

Figure 1: *Intrinsic and extrinsic timers.* Schematic summarising the distinction between intrinsic and extrinsic timers. Each of the 'blobs' represents either a cell population or cell, dependent upon context. In an intrinsic timer mechanism, each population (cell) has its own internal timer which is not affected by external information. In contrast, in an extrinsic timer mechanism, information from the surroundings is integrated by the population (cell) to infer the state of the timer.

The specification of distinct cell types during development is inherently linked to the timing at which cells receive either the inhibition or activation of extracellular signals. One example of this is in the patterning of the early ectoderm into epidermis, neural plate border or neural cell states, and the subsequent patterning of these embryonic territories. Neural specification requires a continued modulation of FGF, Wnt and BMP pathway activity from pre-gastrulation stages onwards (Linker and Stern, 2004; Streit et al., 2000; Tuazon and Mullins, 2015; Tucker et al., 2008), and neural plate border specification and regionalisation requires a distinct series of temporal exposure to these same pathways (Streit and Stern, 1999; Steventon et al., 2009; Steventon and Mayor, 2012; Patthey et al., 2009; Britton et al., 2019). Hence, an important unanswered question in developmental biology is how the temporal exposure to extracellular signals is regulated during early development, and how is this linked to alterations in the morphogenetic properties of tissues as they undergoes shape change and growth. This understanding is essential, as it likely holds the key to understanding the regulative and self-organising properties of the early embryo.

The timing at which cells receive external signals to modulate intrinsic timers of cell state transitions is in turn determined by when the two cell populations (i.e. signalling

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and responding populations) become apposed to one another in the embryo, or become shifted relative to the position of cells releasing secreted modulators of the signalling pathway activity. Therefore, a key regulator of developmental timing acts at the multi-tissue level, and is based on the progressive spatial re-positioning of tissues as they alter in size and shape through morphogenesis. To emphasize the importance of this higher-level regulation of developmental timing through the spatial displacement of signalling and responding tissues, we re-introduce the term *tissue tectonics*. This term has been introduced elsewhere in relation to the tension and stress forces acting within tissues to drive morphogenesis (Blanchard et al., 2009). Here, we extend the concept to consider how it can act as a important regulator of timing in development, and provides a mechanism by which intrinsic developmental timers can be regulated by morphogenetic events. While the direct regulation of cellular signalling and gene expression states by mechanochemical coupling has received increasing attention (Hannezo and Heisenberg, 2019; Naganathan and Oates, 2017), this review highlights tissue tectonics as an additional mechanism by which morphogenesis and patterning can be coordinated in development. We propose that changes to tissue tectonics in evolution, and more generally alterations to the timing of developmental events (heterochrony), are important for producing new forms.

As a complete coverage of the literature on developmental timing would be beyond the scope of a single review, we instead provide a series of case studies in which the concept of developmental timing has been approached at different levels of biological organisation. We will first present a series of studies in which the relative contributions of intrinsic and extrinsic timers to temporal control have been elucidated. Timers act at different rates (tempos) in different species, so we will follow this with a discussion of recent studies that have investigated the molecular basis for species-specific developmental tempos. We will then briefly discuss how alterations in the timing of one developmental process over another can act as a mechanism for evolutionary change in reference to a fundamental concept in evolutionary developmental biology: heterochrony. Finally, we will review some recent work that demonstrates the importance of tissue tectonics in coordinating multi-tissue morphogenetic events with the timing of cell fate decisions and the emergence of spatial patterning in development.

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2 Intrinsic and extrinsic timers in development

2.1 Single cell intrinsic timers

As as starting point to consider how developmental timing is regulated during embryonic development, we will first consider some examples where the intrinsic capability of individual cells has been demonstrated experimentally. The concept of cell intrinsic timers is inherently linked to the concept of competence in development, i.e. how cells change in their ability to respond to a given inductive cue over developmental time. Induction may be defined as the process in which an inducing tissue releases a signal that results in a change in the direction of differentiation of a responding tissue (Gurdon, 1987). In *Xenopus* animal cap explants, there is a clear delineation in time in the transition between a competent and a non-competent state for mesoderm induction: the competence of animal cap tissue to respond to contact with vegetal tissue is lost at the early gastrula stage (Gurdon et al., 1985). To investigate what determines the timing of competence in development, experiments have been performed which isolated animal cap ectoderm from the embryo and showed that even outside of the embryonic environment, competence is still lost at the same point in time (Grainger and Gurdon, 1989), indicating that competence loss is intrinsically timed. Further, even when cells are dissociated from the animal cap, single cells maintain the expected timing of competence: this suggests that timing acts in this case cell-autonomously (Grainger and Gurdon, 1989). By placing dissociated single cells in a solid gelatin matrix, the authors inhibited cell division and showed that loss of competence in this context is independent of cell division (Grainger and Gurdon, 1989). Together, these results implicate a cell-intrinsic timer in animal cap ectoderm cells that modulates the ability of the cell to respond to induction by vegetal signals.

An additional example of single-cell intrinsically timed developmental events comes from the *Drosophila* nervous system. Here, neuroblasts give rise to the precursors of the nervous system, ganglion mother cells (GMCs). GMCs are produced by asymmetric divisions of a neuroblast that produce a daughter neuroblast and a daughter GMC. Through the sequential expression by the neuroblast of 'temporal identity genes' (*hunchback, kruppel, pdm1, castor*), each of the sequentially generated GMCs has a specific identity (Isshiki et al., 2001). How are the transitions between each of these gene expression profiles controlled over time? Experiments that cultured isolated neuroblasts *in vitro* showed that gene expression transitions occur in isolated cells outside of the embryonic environment, suggesting that this gene expression timer is controlled by cell-intrinsic mechanisms (Grosskortenhaus et al., 2005). In G2-arrested embryos, the timing and order of expression of the temporal identity genes is maintained, suggesting that timing of these

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transitions is regulated by a mechanism independent of the cell cycle (Grosskortenhaus et al., 2005). This is also not a simple linear positive transcriptional cascade; mutations in the *hb* and *Kr* genes have little effect on later gene expression of the other temporal identity genes. More recently, mathematical modelling work has provided insights into the mechanistic basis for the *Drosophila* neuroblast temporal identity timer. Experimental data is consistent with a repressor-decay timer, where the decay of a previous timer component (e.g. *hb*) times the onset of expression of a later component (e.g. *pdm1*), through the relief of repressive interactions (Averbukh et al., 2018).

A final example of an intrinsic timer that functions in individual cells is given by the oscillatory component of the segmentation clock. During the production of the embryonic anteroposterior (head to tail) body axis, paraxial mesoderm on either side of the midline is sequentially segmented into blocks termed somites (somitogenesis). The primary model for somitogenesis is the Clock and Wavefront Model, in which an anteroposterior gradient of FGF and Wnt signalling (the wavefront) is combined with a cell-intrinsic oscillator based on Notch signalling to segment blocks of tissue (Cooke and Zeeman, 1976; Pourquié, 2011). A set of genes including those that encode transcription factors of the Hes/ Her family are expressed with oscillatory dynamics in the presomitic mesoderm (PSM) (Aulehla and Johnson, 1999; Lewis, 2003). Strikingly, when pre-somitic cells are removed from zebrafish embryos and cultured *in vitro*, oscillations in Her1 expression are still observed (Webb et al., 2016). Mathematical modelling was used to demonstrate that the observed patterns of gene expression are consistent with all of the cells essentially having the same oscillatory behaviour captured at different points in their dynamics (Webb et al., 2016). Thus, in development, individual cells have the capacity to generate oscillatory gene expression, which must be coordinated across the PSM population through extrinsic signals (recently reviewed by Oates, 2020). This cell-intrinsic oscillator is important, together with extrinsic signals (the wavefront) in segmenting blocks of paraxial mesoderm along the anteroposterior axis.

Together, these three examples provide good evidence for the existence of intrinsic timers that are able to operate in isolated single cells. Furthermore, they provide examples of the utility of experimental assays in investigating the intrinsic or extrinsic control of developmental timers. Together with methods for the improved imaging and analysis of real-time changes in single cell gene expression, experimental assays such as those described above are key in enabling questions to be asked relating to the degree to which single cell intrinsic timers can be coordinated across groups of cells in a given tissue. We will next consider three case studies in which the balance of intrinsic vs. extrinsic regulation of developmental timing has been investigated, before considering how these events can be regulated at the multi-tissue and whole organism level.

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2.2 Balancing intrinsic and extrinsic timing in avian limb development

Heterochronic grafting has provided good evidence for a number of population-level intrinsic timing mechanisms. A first example comes from avian limb development. Here, cells of the polarising region (or zone of polarizing activity, ZPA) express *Sonic Hedgehog* (*Shh*) for a defined duration between HH20–27 (approximately embryonic days 3.5–5.5) (Riddle et al., 1993). Sonic Hedgehog (Shh) protein functions in the specification of anteroposterior positional values and proliferation of limb bud cells (Towers et al., 2008; Yang et al., 1997). If the polarising region of a HH20 embryonic wing is grafted in place of the endogenous polarising region in a HH24 embryo, the donor polarising region continues to express *Shh* 32 hours post-graft. At this time point, the host polarising region in the contralateral wing has downregulated *Shh* expression and transcripts are not detectable by *in situ* hybridisation (Chinnaiya et al., 2014). This result suggests that the mechanisms controlling timing of *Shh* transcription in this tissue are not dependent upon extrinsic signalling. Population-level analyses of cell cycle parameters showed that there are distinct stereotyped changes to the progression of the cell cycle between HH20-30, including a marked increase in the proportion of cells in G1 (Chinnaiya et al., 2014). Inhibiting cell division in the limb bud using the drug colchicine leads to an extension in the period for which *Shh* is expressed (Chinnaiya et al., 2014). Together, these experiments point to an intrinsic timer for *Shh* expression in the polarising region, with cell cycle progression as an important input.

A second cell-population intrinsic timer acting in avian limb development controls the termination of limb bud outgrowth (Pickering et al., 2018). The limb field is initially specified as a portion of the lateral plate mesoderm, before outgrowth to form a bud beginning at HH18. The cells of the limb bud proliferate and drive outgrowth. The processes controlling the termination of outgrowth are important for regulating the size and shape of the limb. The classical model for limb outgrowth termination implicated the breakdown of a feedback loop between FGF signalling from the apical ectodermal ridge (AER) and BMP signalling in the underlying mesenchyme (Verheyden and Sun, 2008). A recent study has produced evidence that a cell intrinsic timer is key in controlling limb bud outgrowth termination (Pickering et al., 2018). When donor HH29 distal mesenchyme cells were grafted into a younger (HH20) host limb, grafted tissue had a cell cycle profile more similar to HH29 control (ungrafted) tissue than to HH24 contralateral tissue (Pickering et al., 2018). Furthermore, expression of genes involved in the *TGFß* pathway, as well as functional BMP signalling, was maintained in grafted tissue with a transcriptional profile similar to HH29 ungrafted tissue (Pickering et al., 2018). The authors argue that an intrinsic programme of increasing BMP signalling in distal mesenchyme is responsible

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for the termination of limb bud outgrowth.

An example of an extrinsically controlled developmental timer is also found in avian limb development. The proximodistal axis of the limb comprises three segments : the stylopod, zeugopod and autopod (from proximal to distal). The most proximal segments of the wing are specified early in its development (HH18-19) through an extrinsic mechanism (Saiz-Lopez et al., 2015). This has been demonstrated by grafting of HH20 distal mesenchyme into HH24 limb buds. The tissue-level contribution of the donor cells in these experiments was indistinguishable from HH24-HH24 homochronic grafts (Saiz-Lopez et al., 2015). That is, behaviour of the HH20 donor cells is reset by grafting into an older host environment, causing cell contribution only to structures distal to the autopod, rather than their normal contribution to the zeugopod. Interestingly, in this system, the extrinsic timer controlling proximal fate specification is combined with an intrinsic timer controlling the specification of more distal proximodistal values (Saiz-Lopez et al., 2015).

Taken together, these examples from avian limb development clearly demonstrate the utility of experimental embryological techniques in investigating the contribution of cell-intrinsic and extrinsic timers to the regulation of developmental events. Heterochronic grafting methods allow the external "time" experienced by a cell population to be manipulated, and these methods allow insights which would not be possible otherwise. The avian limb provides a rich example of how cell-population level intrinsic and extrinsic timers can work together to allow the patterning and morphogenesis of a higher-level structure in development.

2.3 Balancing intrinsic and extrinsic timing in mammalian neurogenesis

Population-level intrinsic timers have also been described in the development of the nervous system. The mammalian brain is complex and comprises a large repertoire of diverse cell types. In many cases, neural progenitor cells undergo temporal changes in the types of daughter cells that they produce, allowing for the production of a diverse set of daughter cells over time. For example, progenitor cells of the mouse cortex may be maintained in a culture system, where they express molecular markers with a similar timing to that observed in the embryo (Gaspard et al., 2008). As differential daughter cell identities are observed over time in the absence of any external signals, this suggests that the transcriptional progressions observed in normal development are mediated by an intrinsic timer. These cultured cells are functional: when grafted back into the embryo, their repertoire of projections closely resembles those found in normal development (Gaspard et al., 2008). These results are similar to those in the *Drosophila* neuroblasts described above, reveal-

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ing an important role in diverse species for cell-intrinsic timers in the generation of the nervous system.

The timing of differentiation of cells that make up the nervous system may also be determined through cell-intrinsic mechanisms. Experiments that removed rat optic nerve cells from the embryonic environment and cultured them in PDGF demonstrated that oligodendrocyte precursor cells (OPCs) divide and differentiate with a timing which closely replicates that observed within the embryo (Temple and Raff, 1986). If single OPCs are plated in individual microwells, they divide (with a maximum of eight divisions observed) before differentiating. If separated into individual wells, the daughters of the same OPC divide the same number of times before differentiating (Shen et al., 2006). What determines the timing of differentiation in these cells? It appears that the underlying mechanism is cell-intrinsic, given that cells, even in isolation, differentiate with replicable timing. In contrast to the aforementioned examples from limb development, however, this mechanism does not appear to be cell-cycle dependent. When OPC cultures are held at a reduced temperature (33°C as opposed to the standard 37°C), they divide more slowly. However, the timing of differentiation occurs earlier in these cultures (Gao et al., 1997). This suggests it is not the number of cell divisions which is important, so much as the absolute passage of time. Further investigation has led to a model for OPC differentiation that places gradual changes in the level of several proteins, including p27, p18 and Id4, at the centre of the intrinsic timer (reviewed by Raff, 2006).

Gradual changes in transcription over time have been implicated in another study focusing on mammalian cortex development. Here, apical progenitor cells (APCs), which give rise to cells of the dorsal cortex, were subjected to transcriptional analysis at several time points in development (Okamoto et al., 2016). Through computational manipulation of the resulting dataset, the authors were able to distinguish two orthogonal axes in the clustered dataset – a temporal axis and a differentiation axis. That is, a subset of genes was identified whose expression changed over time in a manner distinct from the phenomenon of differentiation. These genes show gradual changes in expression over developmental time (Okamoto et al., 2016). Manipulations which overexpressed a Cyclin dependent kinase (Cdk) inhibitor through electroporation did not disturb the temporal change in the temporal axis genes (Okamoto et al., 2016). This is evidence for another cell-intrinsic timer in mammalian cortex development, that is not dependent upon cell cycle progression for its function. These results, together with those of the studies described above, implicate an important role for cell intrinsic timers in generating the highly diverse cell type repertoire of mammalian brains.

In contrast to these examples of the ability of neurogenesis to be regulated by intrinsic

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mechanisms, heterochronic grafting has provided evidence for a number of population-level extrinsically controlled timer mechanisms in development. Progenitor cells of the mammalian neocortex produce different daughter cell fates sequentially during development. Radioactive labelling of progenitor cells in ferrets has shown that the deepest layers of the cerebral cortex are generated before the more superficial ones (Angevine and Sidman, 1961; Jackson et al., 1989; Luskin and Shatz, 1985; Rakic, 1974). In experiments which grafted dissociated cells from E29 embryos (which would normally contribute to layer 6 (L6) of the cortex) to embryos at a later stage (in the process of generating L2/3) it was found that dependent upon the cell cycle status of the donor cells, a different outcome was observed (McConnell and Kaznowski, 1991). If donor cells were transplanted into the older host embryo prior to cell cycle completion (during S-phase), daughter cells contributed to L2/3 of the cortex, like host progenitor cells at this stage. However, if cells were allowed to complete their cell cycle before transplantation (the graft was performed 24 hours later), daughter cells were found in L6 (McConnell and Kaznowski, 1991). These experiments suggest that there are extrinsic inputs to the timer mechanism that controls progressive generation of different neuronal fates by these progenitor cells. Further, it was found that the donor cells for the pre-cell cycle experiment had doubled DNA content (McConnell and Kaznowski, 1991). This was taken as evidence that the daughter cell contribution (status of the timer) is determined within the progenitor cell, not within the daughter cells themselves.

This case study of mammalian neurogenesis shows the importance of intrinsic timer mechanisms in generating a huge repertoire of neural cell types from a common progenitor population. The above studies have demonstrated that intrinsic mechanisms may regulate the transition between generation of different neural cell types, as well as the timing of differentiation of individual progenitor cells. In many cases, these transitions appear to be independent of cell division.

2.4 Balancing intrinsic and extrinsic timing during body axis elongation

An additional case study which demonstrates the importance of both intrinsic and extrinsic timer mechanisms in normal development is body axis elongation. During development of the vertebrate anteroposterior (AP, head to tail) body axis, progenitor cells found in the tailbud of the embryo contribute to various axial and paraxial tissues. These progenitor cells sequentially express *Hox* genes over time, with a timing corresponding to the organisation of these *Hox* genes on the chromosome – this is a phenomenon termed temporal collinearity (reviewed by Deschamps and Duboule, 2017). Vertebrate *Hox* genes exhibit spatial and temporal colinearity: the expression of the *Hox* genes within a cluster

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is organised both in space and in time in a manner that closely mirrors the organisation of genes on the chromosome (Izpisúa-Belmonte et al., 1991; Mallo et al., 2010). Genes at the 3' end of the cluster are expressed earlier in development and with more anterior domain boundaries than those found toward the 5' end of the cluster. Of particular interest to this review is the temporal aspect of colinearity (summarised in *Figure 2*). This schematic shows the generalised expression domains of various combinations of *Hox* genes in chicken embryos at three stages in development: HH4, HH8 and HH18. The more 3' *Hox* genes are expressed early in development (*Figure 2b*) whilst those which are found toward the 5' end of the cluster are not expressed until much later in development (*Figure 2d*). This expression pattern is particularly interesting given results which suggest a possible functional role for *Hox* gene expression in cell behaviours. Experiments which overexpressed various posteriorly-expressed *Hox* genes in chicken primitive streak cells suggest that this overexpression is sufficient to delay the ingression of cells that will contribute to mesoderm through the streak (Iimura and Pourquie, 2006). What are the mechanisms which account for the timing of *Hox* gene expression during body axis elongation?

To investigate the balance of intrinsic and extrinsic regulation in Hox gene expression during body axis elongation, heterochronic grafts were performed in a study conducted by McGrew *et al.* 2008. Chordoneural hinge (CNH) donor cells taken from a HH15 embryo were grafted to the location they would have been found in at HH8, in the posterior node region. Donor cells were assayed for expression of the *Hoxa10* gene, which is not expressed at HH8-10, but is expressed at HH15. It was found that within 8 hours post-graft, *Hoxa10* transcripts were no longer present in the donor tissue (nor the host) (McGrew et al., 2008). This result suggests that the mechanisms governing the progression of *Hox* gene expression by axial progenitor cells are under extrinsic control, because placing these cells in an 'earlier' host environment was able to 'reset' the expression of *Hox* genes to match host tissue time. However, the degree to which this is a common feature of Hox gene expression across the processes of embryo elongation and patterning has yet to be determined. In order to gain a more complete understanding of the mechanistic basis of temporal Hox colinearity, we will briefly review what is known about the regulation of *Hox* gene expression within the cell.

Analyses of *Hox* gene cluster chromatin structure have revealed changes that advance along the cluster over time, comprising a loss of H3K27 trimethylation coincident with a gain of H3K4 trimethylation (Soshnikova and Duboule, 2009). These histone modifications are widely associated with transcriptionally silent and active promoters respectively (Bannister and Kouzarides, 2011). In embryonic stem (ES) cells, silent *Hox* loci have both of these modifications, which are resolved during differentiation to one or the other (Azuara et al., 2006; Bernstein et al., 2006). Work that has analysed higher order

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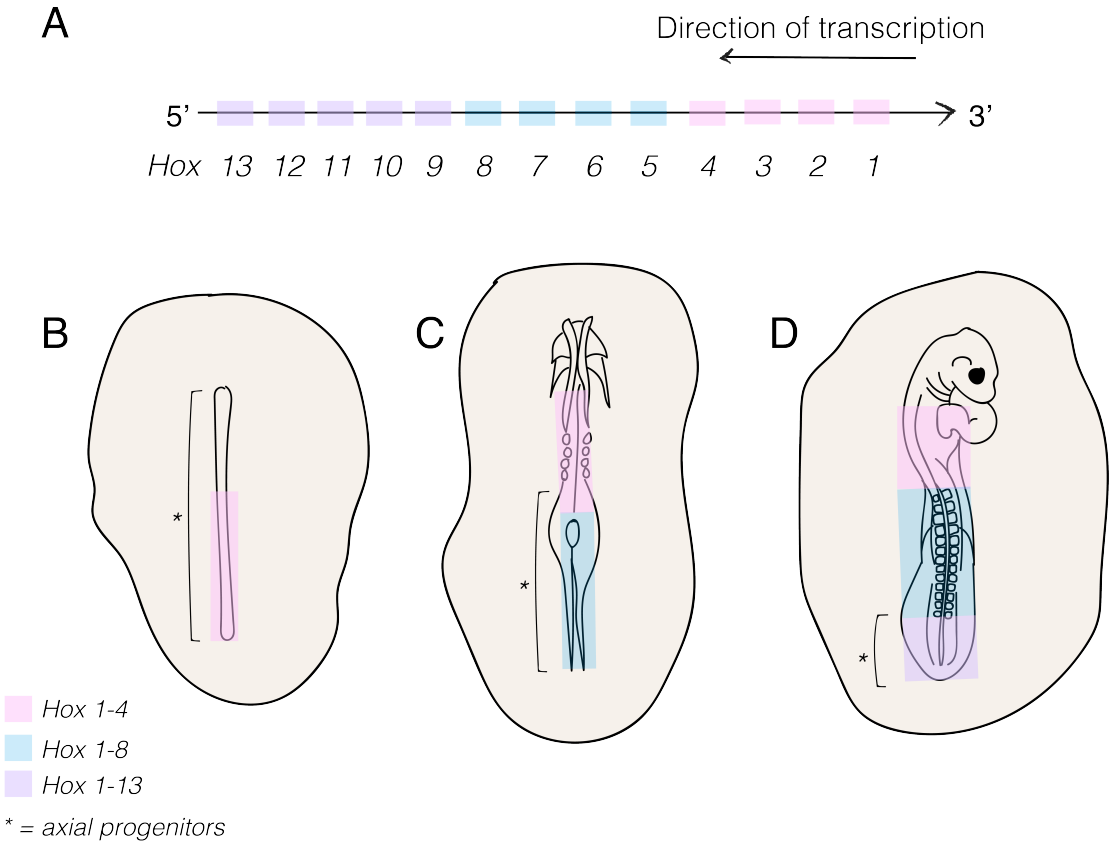

Figure 2: *Temporally colinear Hox gene expression in the chicken embryo.* (A) Schematic of a generalised *Hox* cluster. The direction of transcription is indicated. The schematics in (B)-(D) summarise the expression of *Hox* genes at three stages of chicken embryonic development: (B) HH4, (C) HH8 and (D) HH18. Each colour code represents a combination of different *Hox* genes. The axial progenitor domain is highlighted at each stage by the bracket labelled with an asterisk. As development proceeds, axial progenitor cells express more 5' *Hox* genes. This pattern of gene expression has been termed the 'Hox clock'. This schematic was inspired by a similar one in Deschamps and Duboule, 2017, which considered the Hox clock in mouse.

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structuring of chromatin in mice has found the segregation of active and silent genes to separate chromatin compartments during development (Nordmeer et al., 2011). In cells of the presomitic mesoderm (PSM), as transcriptional activity progresses along the *Hox* cluster toward the 5' end, genes are transferred into the active chromatin compartment (Nordmeer et al., 2011). This process has been suggested to be important for the timing of *Hox* gene regulation, through 'protection' of the most 5' (posteriorly-expressed) *Hox* genes from potential activating factors.

An important set of experiments in mouse used genetic modifications to insert a reporter gene at different locations within the *HoxD* cluster, in order to test the role of centromeric and telomeric influences on expression dynamics (Tschopp et al., 2009). They found evidence for a timer of *Hox* gene expression where the location of a gene within the cluster is sufficient to determine the timing with which it is expressed. If the gene was inserted closer to the 5' end of the *HoxD* cluster, it was expressed with a later timing (Tschopp et al., 2009). Note that the location within the cluster was not sufficient to predict the ultimate tissue-specific spatial gene expression pattern – it appears that spatial regulation is more complex and tissue-specific.

Studies have also shown that an important upstream factor for initial *Hox* gene expression is Wnt signalling. Addition of Chiron (a Wnt agonist) to cells in culture results in the sequential induction of expression of *Hox* genes (Neijts et al., 2016). Further, the exposure of pre-gastrulation mouse embryos to Chiron results in precocious expression of *Hox* genes (Neijts et al., 2016). A number of Beta catenin-binding regions have been identified in a region 3' to the *HoxA* cluster, which are in close contact with the most 3' genes of the cluster before their activation. These Wnt responsive enhancer regions are functionally required for the expression of *HoxA* genes in development (Neijts et al., 2016).

Together, these results support a chromatin organisation-based mechanism that governs the timing of *Hox* gene expression activation in development and is initially activated through Wnt signalling. As transcription of a given gene is activated, the locus is transferred from an inactive chromatin compartment to an active one. There are clearly extrinsic inputs into this timer, as demonstrated by heterochronic grafting experiments. It is not yet clear what the driving force for progressive chromatin opening is, or how the pace of gene activation is set. As developmental tempo varies widely between different vertebrates, it is likely that the rate of transfer of genes between the inactive and active compartments is also highly variable.

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3 Developmental tempo

The pace (tempo) and duration of embryonic development are highly variable amongst different animal species. For example, even amongst mammals, variation in gestation period is vast. Mouse embryonic development typically spans 20-30 days (dependent on the species), whilst human development takes nine months. Many of the developmental processes encompassed within embryogenesis differ in their pace between species, and so an active field of research focuses on asking how different tempos of development are achieved in different organisms. A study which differentiated mouse EpiS cells and human ES cells to neural fates in culture demonstrated that species-specific developmental timing is maintained in culture, with neural differentiation markers expressed with accelerated timing in the mouse cells (Barry et al., 2017).

A recent study used mouse and human cells in culture to investigate the basis for species-specific differences in the tempo of the segmentation clock (Matsuda et al., 2019). Matsuda and colleagues set up a cell culture system to differentiate pluripotent stem cells to a PSM-like state. They utilised a luciferase reporter gene under the control of the *HES7* promoter in order to image oscillations in gene expression over time, finding that the period of oscillations differs between human and mouse cells (in culture) - the period of oscillations is 2-3 times longer in human cells than mouse cells. This cell culture assay allowed the authors to ask how different species' cells, cultured in identical conditions, exhibit different oscillatory periods of gene expression. Experiments that swapped the *HES7* loci between cells of each species (inserting the mouse *HES7* locus into the human cell genome, and vice versa) demonstrated that the difference in period is not a result of sequence differences at this locus. Measurement of biochemical reaction parameters revealed differences in the rate of degradation of HES7 protein in human cells relative to mouse cells, regardless of whether this protein was encoded by the mouse or human gene sequence. Further, the delay in transcription and translation of *HES7* was greater in human cells than mouse cells. Using mathematical modelling, the authors showed that altering biochemical reaction parameters in line with these observations was sufficient to account for the different periods of oscillation in human and mouse cells (Matsuda et al., 2019). These results suggest that some fundamental difference in the status of biochemical reaction parameters may be responsible for differences in tempo of the segmentation clock.

Similar experiments which differentiated mouse and human spinal cord progenitors to form motor neurons in culture revealed global differences in protein stability between mouse and human cells (Rayon et al., 2019). Motor neuron differentiation in the embryo takes 3-4 days in the mouse and around two weeks in humans (Davis-Dusenbery et al.,

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2014). The pace of gene expression progression in progenitor cells in culture closely resembles the differences observed in embryos during this process. For example, expression of motor neuron marker genes including Isl1 occurs after 2-3 days of culture in mouse cells but not until approximately 6 days in human cells (Rayon et al., 2019). These differences do not result from genomic sequence differences, as introduction of the human *Olig2* gene into mouse cells reveals that timing of expression of this gene is determined by the cellular context: the human *Olig2* gene in mouse cells is expressed with the same timing as the mouse *Olig2* gene in mouse cells. Given that the observed differences in timing of motor neuron differentiation do not result from sequence differences in the known GRN components, the authors assayed kinetic parameters of gene expression in order to look for differences between mouse and human cells. They found that whilst mRNA half-life was similar in the two cell types, protein half-life was significantly shorter in mouse progenitors than in human progenitors. The authors argue that this is a general pattern, because introduction of an exogenous reporter protein showed that this protein also has a species-specific half-life dependent on its cellular context (Rayon et al., 2019).

These studies investigating the basis for species-specific developmental tempos are intriguing. A species-specific complement of transcription rate, translation rate, mRNA, and protein stability may determine the tempo of embryonic development. Interestingly, a study which compared eight different rodent species showed that protein degradation rates are negatively correlated with species lifespan (Swovick et al., 2018). An important open question remains: what is responsible for these differences in kinetics? Are genomic sequence differences in the machinery of transcription/ translation/ protein degradation responsible? Could there be inputs from metabolism? Metabolism and nutrition are key factors that vary dramatically between species: many species that have a prolonged period of embryogenesis also access a large nutrient supply. Studies in wild red deer have shown a modest extension of gestation time in mothers who had lower access to food, suggesting input from metabolism and nutritive factors to development (Asher et al., 2005; Verme, 1965). Further research effort is required in this area in order to understand the factors that cause differences in biochemical kinetics and give rise to species-specific tempos.

In summary, development at the organismic level occurs with a conserved timing and tempo in a given species. Our understanding of developmental tempo, which varies wildly between species, has been advanced by recent studies which have compared mouse and human cells in culture (Matsuda et al., 2019; Rayon et al., 2019). These studies have suggested an important role for global differences in biochemical reaction parameters in coordinating different tempos of developmental processes, though how these differences in reaction parameters are achieved in the evolution of species remains elusive. While changes in the global developmental tempo are no doubt important in driving evolution-

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ary change, there are also multiple examples of where the rate of development of one particular developmental process has been altered with respect to another to generate novel biological forms and functions.

4 Time in the evolution of development: heterochrony

Heterochrony, defined as a shift in the developmental timing of events, has long been recognised as an important concept in the field of evolutionary developmental biology (evo-devo). Both De Beer and Gould published influential texts arguing that changes in developmental timing of events relative to an ancestral form are an important force in the evolution of morphology (Gould, 1977; De Beer 1951). Dramatic changes to morphology may be achieved through changes in the timing of the development of specific organs relative to one another, as demonstrated by the following examples.

The classic example of developmental heterochrony is given by the Mexican axolotl, *Ambystoma mexicanum*. Most salamanders have a life history that involves progression via a metamorphosis event from an aquatic larval morph to a terrestrial adult morph. This metamorphosis involves considerable changes to body form and physiology, such that the adult form is specialised for life primarily on land. However, in the axolotl, reproducing adults closely resemble the larval form – this species does not undergo metamorphosis, but does have functional reproductive organs (Gould, 1977). This is an example of paedomorphosis, defined by Gould (1977) as the permanent retention of ancestral juvenile traits in the adult stage of a derived species. Many comparisons have focused on differences between *A. mexicanum* and its sister taxon, *A. tigrinum* (the tiger salamander). In axolotls, the production of functional gonads in an otherwise morphologically larval state is understood to occur through neoteny – a retardation in somatic development (Gould, 1977). Thus, a dramatically different morphological form is achieved through changes in the timing of some organs relative to others. In this case, the development of all organs but the gonads are temporally slowed.

Metamorphosis in salamander species closely related to the axolotl occurs through secretion of thyroid hormone (TH) from the thyroid gland (Gudernatsch, 1912). Experiments conducted by Laufberger that bathed axolotls in TH showed that exogenous TH is able to induce metamorphosis in the axolotl – an event that would never be observed during normal development (reviewed by Huxley and Hogben, 1922). Further, the axolotl has a functional thyroid gland, which has been observed to synthesise and release T4 if stimulated with thyroid stimulating hormone (TSH) (Taurog, 1974). However, during normal development, the axolotl does not release sufficient TSH into circulation to

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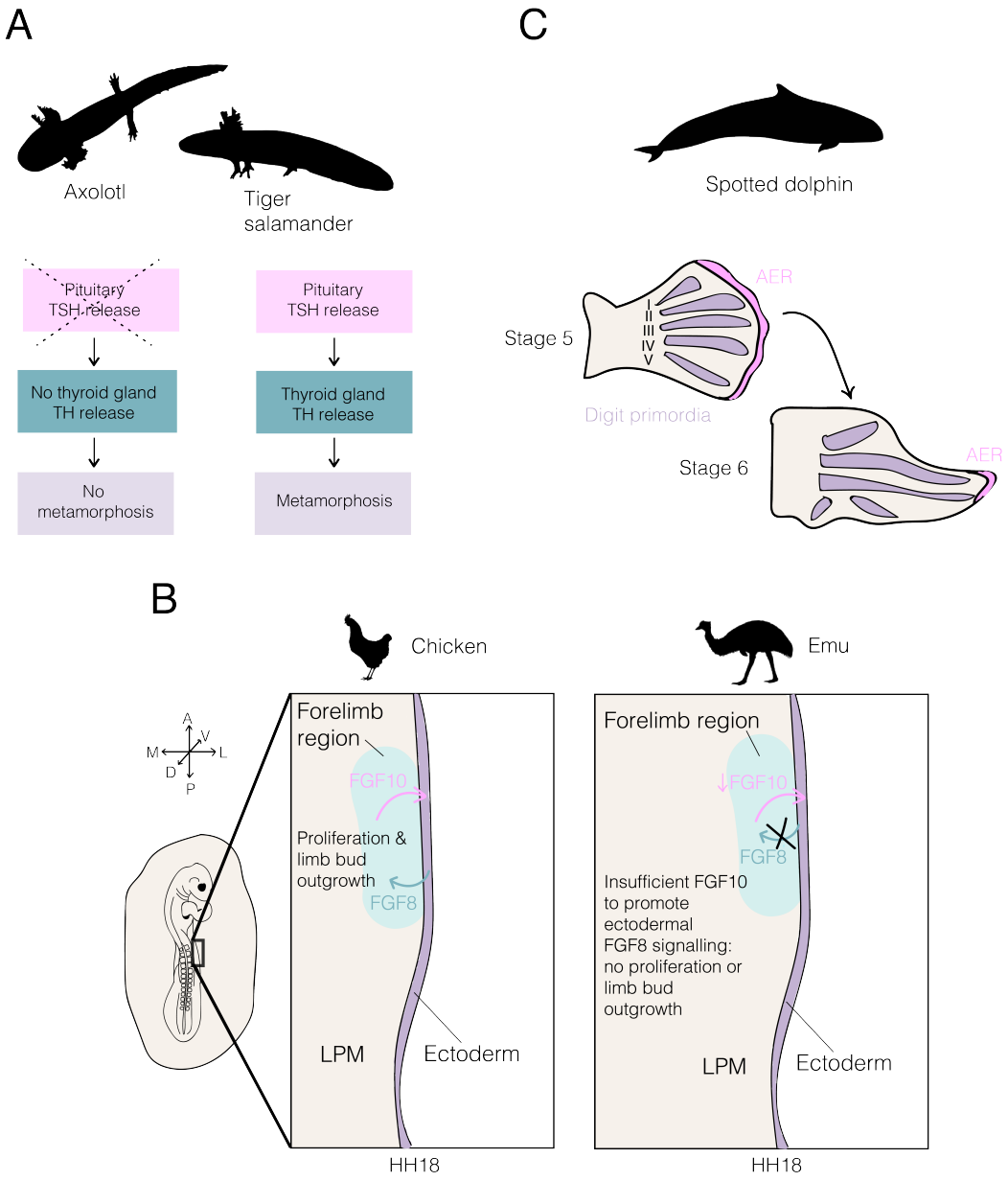

Figure 3: *Heterochrony between organs can produce new forms in evolution.* (A) Axolotl paedomorphosis results from a loss of the metamorphosis event. In a close relative of the axolotl, the tiger salamander, metamorphosis is triggered by the pituitary-thyroid axis. However, TSH release by the pituitary gland does not occur in the axolotl, causing a loss of the downstream events and associated metamorphosis. (B) Schematic of the chicken and emu lateral plate mesoderm (LPM) at HH18. In most avian species, including the chicken, reciprocal signalling between the forelimb region of the LPM and the overlying ectoderm (purple) promotes proliferation of LPM cells and limb bud outgrowth. However, the emu forelimb region of the LPM produces the signalling ligand FGF10 at an insufficient level to induce FGF8 production in the ectoderm (purple). Consequently, cells of the LPM forelimb region do not proliferate and the limb bud does not grow out from the body at HH17. (C) Analysis of spotted dolphin developing limb buds has shown that the apical ectodermal ridge (AER) persists over digits II and III for longer than over the other digits. These digits exhibit hyperphalangy (many finger bones) and it has been suggested that the local persistence of the AER may be related to this trait. *Silhouette images of animals were taken from PhyloPic: please see acknowledgements for attributions.*

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stimulate TH release (reviewed by De Groef et al., 2018) *(Figure 3a)*. Experimentally transplanting the pituitary gland of the axolotl into a host tiger salamander results in the inhibition of metamorphosis in the host (Blount, 1950). The reciprocal graft has also been performed, and induced metamorphosis in the axolotl (Blount, 1950). These results indicate that in the axolotl, the thyroid axis is disrupted and TSH is not released from the pituitary gland to stimulate TH release and metamorphosis. Genomic analyses have implicated the *met1*, *met2* and *met3* genes in these species differences. After axolotl treatment with exogenous T4, the effects of non-paedomorphic salamander alleles for these genes are additive in reducing time to metamorphosis (Voss and Smith, 2005). It is still not clear mechanistically how mutations in these genes in the axolotl result in a loss of TSH release. Nonetheless, this is a striking example of how a simple change to an endocrine pathway can shift the timing of development of one organ (the gonads) relative to the rest of the body, with striking morphological effects.

A second example in which developmental heterochrony has been implicated in morphological novelty is given by the emu, *Dromaius novaehollandiae*. The emu is one of many species of flightless bird within the ratites (a group within the Palaeognathae) and has very small wings that have a single digit. The timing of forelimb development in the emu is significantly delayed relative to that of other birds and other amniotes. The limb bud does not grow out from the flank of the emu embryo until Hamburger Hamilton stage 20 (HH20) – whilst in the vast majority of birds, outgrowth begins at HH17 (Hamburger and Hamilton, 1951). A recent study has revealed the underlying developmental basis for the delayed outgrowth of the emu wing bud (Young et al., 2019). Prior to limb bud outgrowth, limb precursor cells reside in the lateral plate mesoderm (LPM). In both the emu and the chicken, the epithelial to mesenchymal transition (EMT) of mesenchymal precursors from the somatopleure and their movement to the LPM is intact (Young et al., 2019). However, in the forelimb field of the emu LPM these precursors do not promote outgrowth of the limb bud at HH17. Analyses of proliferation in chicken and emu fore- and hindlimb regions of the LPM show that at stages at which chicken fore- and hindlimb regions and emu hindlimb regions are proliferating, the cells of the emu forelimb LPM do not proliferate (Young et al., 2019). This difference results from a disruption of reciprocal signalling between the LPM and overlying ectoderm. In chicken development, the production of FGF10 by the LPM induces reciprocal signalling via FGF8 from the ectoderm to the LPM (Ohuchi et al., 1997). FGF8 signalling of the ectoderm to the LPM is required to promote proliferation in the limb bud field (Ohuchi et al., 1997). However, in the emu, the ectoderm overlying the LPM does not express *Fgf8* at HH18 (Young et al., 2019). Grafting of donor chicken LPM into the emu limb field (under the host ectoderm) results in the development of a precocious limb bud, revealing that the

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difference in limb bud outgrowth timing results from changes to the LPM in the emu (Young et al., 2019). Though *Fgf10* is expressed by the emu LPM in the forelimb region, the authors suggest that the quantitative level of expression of this signalling ligand is insufficient to induce the expression of *Fgf8* in the ectoderm (*Figure 3b*). In support of this hypothesis, overexpression of *Fgf10* in the emu LPM results in *Fgf8* induction in the overlying ectoderm and precocious limb bud outgrowth (Young et al., 2019). An enhancer mutation responsible for the observed differences between *Fgf10* expression in the chicken and emu embryo was also identified. Together, these results reveal that subtle changes to the timing of expression of a signalling ligand in development are able to alter the timing of development of the emu forelimb, contributing to changes to the gross morphology of this structure.

A third example of heterochrony in morphological evolution is provided by the dolphin flipper. Dolphins are aquatic mammals possessing many adaptations for life in water, including the modification of the forelimb to form a flipper. The flippers act during swimming as rudders, and in many species the digits exhibit hyperphalangy: relative to the ancestral state, they possess numerous finger bones (phalanges) (Kukenthal, 1893). Careful study of spotted dolphin (*Stenella frontalis*) embryos over the period of limb development revealed that hyperphalangy is localised to digits II and III of the dolphin forelimb (Richardson and Oelschläger, 2002). This character correlates closely with the prolonged maintenance of an apical ectodermal ridge (AER) over digits II and III, suggesting that changes to the dynamics of AER development may be responsible for this morphological change (Richardson and Oelschläger, 2002) (*Figure 3c*). Thus, localised persistence of the AER over digits II and III may promote the formation of additional phalanges in these digits.

In summary, it is clear that alterations in phylogeny to the timing of development of specific organs relative to the rest of the body can allow for pronounced changes in form. This pattern was recognised by De Beer (1951) and Gould (1977), and the examples discussed demonstrate that organ-level heterochrony may occur in diverse contexts. The second and third examples in this section also provide good support for the importance of multi-tissue inductive interations in the timing of developmental events. In each of these cases (the emu forelimb and the spotted dolphin flipper), changes in signalling tissue dynamics cause a change to the timing of a developmental event. For example, in the emu forelimb, reduced FGF signalling from the LPM to the ectoderm results in substantial delay in time of a signalling event, and the outgrowth of the limb bud. It is clear that the apposition of signalling and responding tissues, as well as underlying signalling dynamics, are important points of control in timing of events. These signalling events can have effects which span levels of organisation: transcriptional changes in receiving tissues, population-

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level changes (e.g. the outgrowth of limb buds) and organ-level changes (e.g. the timing of development and final morphology of digits of the limb).

5 Tissue tectonics as a mechanism to coordinate developmental timing across scales

5.1 Pattern emergence in development: how intrinsic and extrinsic timing act together to generate spatial patterns of gene expression.

In the experiments enumerated above, we have seen multiple examples where cells display an intrinsic ability to move through successive gene expression states in the absence of extrinsic signals or cues. This demonstrates that cells are not passive entities that await exposure to extracellular signals, but set their own developmental pace of differentiation through a combination of mechanisms. Such mechanisms include the metabolic rate of the cell and the associated tempo of a cell's mRNA and protein turnover and also alterations in the accessibility of transcription factors to bind and regulate gene expression at the chromatin level. Ultimately, these biochemical alterations in a cell's physiology and nuclear architecture will impact the rates of transcription factor production and degradation, as well as the efficiency to regulate either activation or repression of target genes. The impact of these parameters can be modelled together with the higher level regulative structure of gene regulatory networks to generate predictions on the dynamics of cell state transitions through the use of sets of ordinary differential equations (Jaeger and Monk, 2014; Strogatz, 2014). When used to simulate the temporal changes in gene expression across a field of cells, this dynamical systems approach has been highly effective in determining how transcription factor networks operate as a function of these dynamic modulators to give rise to changes in gene expression states over time. Two well studied examples of how dynamical systems approaches have been used to investigate the function of gene regulatory networks are the gap gene system in dipteran insects, and the dorsal-ventral patterning of the vertebrate neural tube (Jaeger, 2018; Verd et al., 2019; Sagner and Briscoe, 2019; Kicheva et al., 2012). Locally, autocrine and paracrine signals pass between cells undergoing cell state transitions, allowing for non-cell autonomous regulation of gene expression dynamics across cell populations. When viewed at the tissue level through a series of snapshots of gene expression analyses, coupling cell intrinsic and extrinsic gene expresssion regulation in such a way results in the formation of gene expression patterns that are highly striking to the experimental observer. However, it is essential to remember that these gene expression patterns are not established at any one

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point in time, or through an instantaneous response of gene regulation to external signals. Rather they are an emergent property of cell intrinsic regulatory interactions coupled to cell extrinsic control of their their inherent dynamics.

Broadly speaking, extrinsic timers offer a mechanism to provide multi-tissue and multi-organ coordination of developmental processes. Extrinsic signals are inherently linked to the concept of induction in development, and ultimately to the role that morphogens play in patterning tissues as they develop. Historically, the study of morphogens has focused on their ability to generate gene expression patterns at a given fixed point in time, and ignores the dynamics of their exposure to cells and that of the emerging gene expression pattern in space. While much can be learned from asking how morphogens can provide sufficient precision in the spatial domain to generate a given pattern, the eventual fate of a cell is not determined by its expression state at any given point in time but is rather an output of the sum of all state transitions it undergoes during development (Verd and Jaeger, 2020). Caution against viewing pattern formation as a mere 'snapshot' of a continuous developmental process has been conceptualised as part of the "general relativistic positional information framework" (Jaeger et al. 2008). This framework places emphasis on our understanding of how biological systems may generate the full dynamic profile of a given set of gene expression states within a tissue of interest, and highlights cells as dynamic entities that integrate multiple sources of information through time (rather than receiving positional information at one critical timepoint). This consideration re-focuses the question of how gene expression patterns are established away from the generation and interpretation of concentration gradients and towards the regulation of the temporal exposure to morphogens during development.

5.2 Tissue tectonics as a higher-order regulator of morphogen exposure during multi-tissue morphogenesis.

The timing at which cells and cell populations receive signals is again a highly distributed phenomenon. Signal timing depends not only on when a signal reaches a cell (which itself is a composite of multiple mechanisms of both extracellular and intracellular transport, reviewed by Rogers and Schier, 2011; Sagner and Briscoe, 2017), but also on the rate of production and transport of signal inhibitors. Across longer time-scales, cells will also move relative to sources of these signals and inhibitors, creating a patterning mechanism that is acting at the multi-tissue level. The relative rate of tissue movement is an output of the state of the cells in question, but also of the mechanical properties of the environment in which it is moving. Here, we term the relative displacement of signalling and responding tissues '*tissue tectonics*' in reference to the relative sliding of the earth's plates

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in the lithosphere (*Figure 4*). The rate at which these multi-tissue interactions occur is inherently linked to alterations in the mechanical properties of tissues as they are formed during development. To fully illustrate how alterations in tissue tectonics can impact the spatial and temporal regulation of patterning in development, we will briefly review two examples.

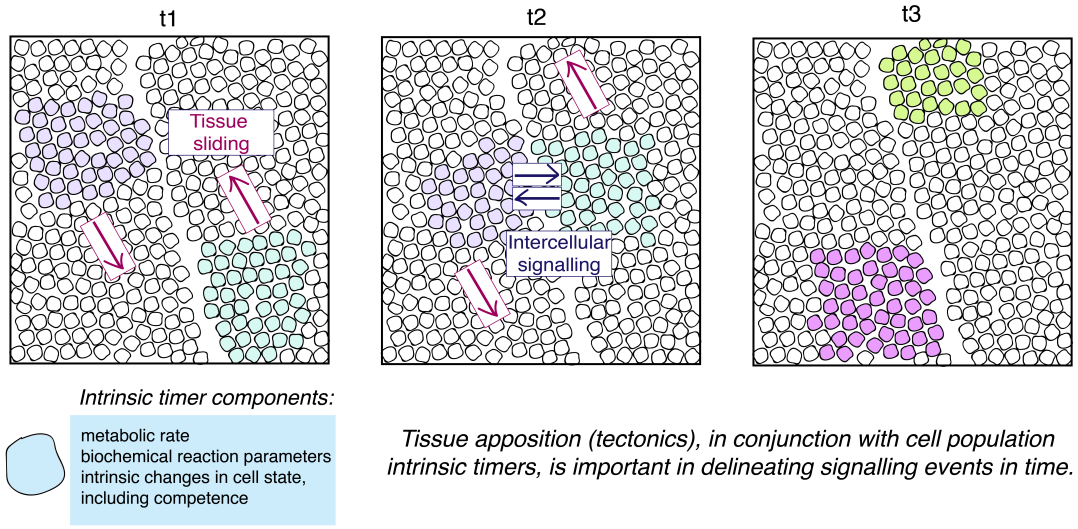

Figure 4: *Tissue Tectonics.* Three snapshots in time are shown in this schematic, at timepoints t1, t2 and t3. Two populations of cells are shown in purple and green, each of which is found in different tissue sheets. As the tissues slide relative to one another, the populations of interest come into close apposition, allowing intercellular signalling to occur. At t3, the inductive signalling event has occurred and both populations' cells have experienced a change in cell state. Clearly, tissue tectonics is an important determinant of the timing of signalling events in development.

5.2.1 Vertebrate gastrulation

During gastrulation, multiple tissue interactions act together to both specify and pattern the three principle germ layers along three principle axes of orientation: anterior-posterior, dorsal-ventral and left-right. In addition to establishing these essential coordinate systems of the body plan, a series of cell movements act in a well orchestrated manner to progressively separate layers of tissues, and to begin the process of embryo elongation along the anterior-posterior axis. A particularly well studied aspect of patterning during this process is the initial specification of neural tissue with the ectoderm, and its subsequent patterning along the anterior-posterior axis. While signals from the early gastrula-stage organiser are important for 'activating' the initial anterior character of neural tissue, subsequent 'transforming signals' then act to convert this character to more posterior neural tissue as gastrulation proceeds (for a recent review see Martinez Arias and Steventon, 2018). During both the initial specification of neural tissue and its subsequent patterning, multiple signals are required to integrate together that result in a precise temporal

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modulation of FGF, BMP, Wnt and retinoic acid signalling pathways (Streit et al., 2000; Linker and Stern, 2004; Stern et al., 2006). A conserved element of these interactions is a requirement for the down-regulation of BMP signalling during neural plate specification, and a subsequent posteriorisation by the Wnt signalling pathway (Niehrs, 2010). The temporal exposure of ectodermal cells to these pathways has been shown to be a key component of the patterning mechanism (Tuazon and Mullins, 2015; Tucker et al., 2008), highlighting the question of what regulates the temporal exposure of cells to patterning signals during gastrulation.

We propose that tissue tectonics is a key aspect of the temporal regulation of signal exposure, and it is essential for ensuring appropriate coordination between the morphogenetic and patterning aspects of gastrulation. One aspect of this coordination is well studied, and requires information to flow from gene-regulatory network activity through to the control of cell movements, tissue morphogenesis and embryo elongation. Critically however, it also requires information flow in the opposite direction, i.e. cells must be able to determine the state of embryo elongation and tissue morphogenesis to coordinate these processes with cell specification and patterning. A recent study has approached this question using explants of zebrafish embryonic cells that were cultured away from the yolk and yolk syncytial layer (Trivedi et al., 2019). Such aggregates go on to break morphological symmetry and generate multiple germ layers in an organised manner (Trivedi et al., 2019; Schauer et al., 2020). As the explants continue to elongate, progressive bands of the hindbrain marker Krox20 appear concomitantly with the movement of a pole of Wnt/beta-catenin away from a source of BMP4/7 expression at the opposite end, suggesting that the elongation itself may be an important upstream regulator to determine the timing of exposure to both BMP and Wnt signal activity. Indeed, blocking convergence and extension of the explants results in an alteration in the spatial-temporal exposure to these signalling pathways and the specification of hindbrain (Trivedi et al., 2019). Together, these results provide an initial insight into the role that tissue tectonics plays in providing a causal link between the mechanisms of global embryo elongation and the patterning of the nervous system during gastrulation.

5.2.2 Cavefish eyefield specification and the evolution of gastrulation.

Alterations in the morphogenesis of gastrulation are common and require a mechanism for such alterations to impact patterning in a manner that a conserved body plan can be generated at later developmental stages. A recent study examined differences between two different morphs of the characid fish during gastrulation: a wildtype river-dwelling morph and a cave morph. While the overall body plan is broadly similar, cavefish possess

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a number of morphological differences relative to surface fish, including a complete loss of eyes. This opens the question of how alterations in the morphogenesis aspect of gastrulation might impact the patterning aspect of gastrulation in the adaption of populations to new ecological environments. An examination of the expression of the homologs of various genes expressed by the organizer in the embryos of characid fish revealed substantial differences in the expression of these genes during gastrulation (Torres-Paz et al., 2019). For example, *dickkopf1b* (*dkk1b*) is expressed in two distinct populations at 50% epiboly in the river-dwelling morph, but in one continuous domain at the same stage in the cavefish morph. This difference in expression domain is associated with advanced internalisation of these cells (which contribute to the anterior prechordal plate) in the cavefish morph relative to the river-dwelling morph. Notably, the expression of *dkk1b* is also downregulated earlier in the cavefish morph than the river-dwelling morph. As a consequence of shifted timing of AP axis formation (heterochrony) in the cave morph, the eyefield which forms within the overlying neurectoderm is reduced in size. Through functional experiments which mimicked the impact of advanced Wnt signalling activation in the eyefield (through treatment with LiCl, because *dkk1b* is an antagonist of Wnt signalling), the authors showed that increasing Wnt signalling in early surface-dwelling embryos results in a reduced eyefield and later a misshaped retina. Together, these results give an example of a developmental signalling event which is altered in timing through changes to the timing of apposition of tissues (here, the anterior neurectoderm and the anterior prechordal plate). In this example, changes to the timing of these events have a marked morphological effect, accounting for the loss of eyes in the cavefish morph. This study opens a set of fascinating questions over the limits of developmental constraint and robustness in the evolution of gastrulation morphogenesis, and the causal role that tissue tectonics might play in linking these two aspects of body plan development and evolution.

5.3 Tissue tectonics as a mediator of downward causation in development.

Up until now, developmental biologists have focused on the emergent properties of development: how processes at a lower level of a complex system (i.e. at the level of a cell and the mechanisms driving its cell state transitions) can impact observable features at higher levels (i.e. the patterned expression of genes across a field of cells when observed at a fixed time-point). However, one of the salient properties of developmental systems is their ability to regulate pattern upon the loss or experimental removal of certain parts of the embryo. Some striking examples include, but are in no way limited to, the ability of halved sea urchin embryos to each give rise to a fully formed individual, the ability

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of the chicken embryo to develop normally after surgical removal of a large portion of the primitive streak, and the formation of monozygotic twins (Dreisch 1892; Psychoyos and Stern, 1996). This regulative (or self-organising) ability of developmental systems fascinates experimental embryologists to this day and has recently come back into focus through observations regarding the ability of embryonic cells to break symmetry and generate patterns when aggregated and cultured as multi-cellular aggregates (Beccari et al., 2018; van den Brink et al., 2020; Veenvliet et al., 2020; Warmflash et al., 2014).

Regulative development requires a mechanism that enables the sensing of changes to the properties of a system at higher levels (including alterations in the size and/ or shape of an embryo or primordium), and to convey changes in the state of the system at lower levels (i.e. alterations in the intrinsic state of cells and cell population) (*Figure 5*). To achieve this, there must be an element of downward causation in the system. Essentially, a mechanism by which information can be passed downwards to confer alterations in a cell's gene expression state in response to multi-tissue level perturbation. This downward causation runs in the opposite direction to the emergence of gene expression patterns (*Figure 5*), and similarly requires an understanding of how alterations in the timing of exposure to signals and their inhibitors is regulated through time. We propose that tissue tectonics is also an essential consideration in understanding the mechanisms of downward causation in development, because downward causation is intimately associated with signalling between cells. It is clear to see how manipulations to the embryo (for example, removal of the anterior primitive streak) would impact signalling events between cells, changing the apposition of different tissues. A full understanding of the mechanical properties of tissues will allow us to follow how tissues respond to injury, how this impacts the timing of exposure to extrinsic timers, and how this in turn regulates the operation of intrinsic timers and the emergence of patterns during regulative development and self-organisation.

5.4 Conclusions

In this review, we have given an overview of studies in developmental biology that have asked how developmental events are timed. These studies have focused at a variety of levels of organisation: from the level of chromatin modifications within single cells to the coordination of multi-tissue or multi-organ events. Cell-intrinsic and extrinsic timer mechanisms both contribute to the overall timing of events during development, and we have described the utility of experimental embryology (in particular, heterochronic grafting) in distinguishing between these modes of developmental timer control. Through changes in morphology and structure throughout ontogeny, tissues are brought into and

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out of close apposition, allowing for the controlled timing of developmental signalling events. Signalling events provide an important level of control for developmental timing, being converged on by both low-level events including gene expression as well as dramatic changes in the morphology of the embryo. We propose that tissue tectonics is a key mechanism that integrates timing information across scales of organisation within the embryo during development. As we have seen, changes to the timing of signalling events can have a dramatic effect on morphology; for example, in the development of the emu wing, in the development of the cavefish eyefield and in the development of the dolphin flipper. It is conceivable that the diversity of gastrulation-stage embryonic forms in vertebrates are associated with changes to the timing of developmental events, as tissue tectonics will be markedly different (Martinez Arias and Steventon, 2018). In summary, timing is a highly distributed phenomenon in developmental biology that is coordinated over diverse levels of organisation. A huge number of timer mechanisms, some intrinsic and others extrinsic, work together to reproducibly time developmental events in the embryos of a given species. We have shown the importance of inter-cell signalling events as a point of control and coordination across these levels of organisation, and have argued for the importance of tissue tectonics (the movement of signalling and responding tissues relative to one another) in timing events.

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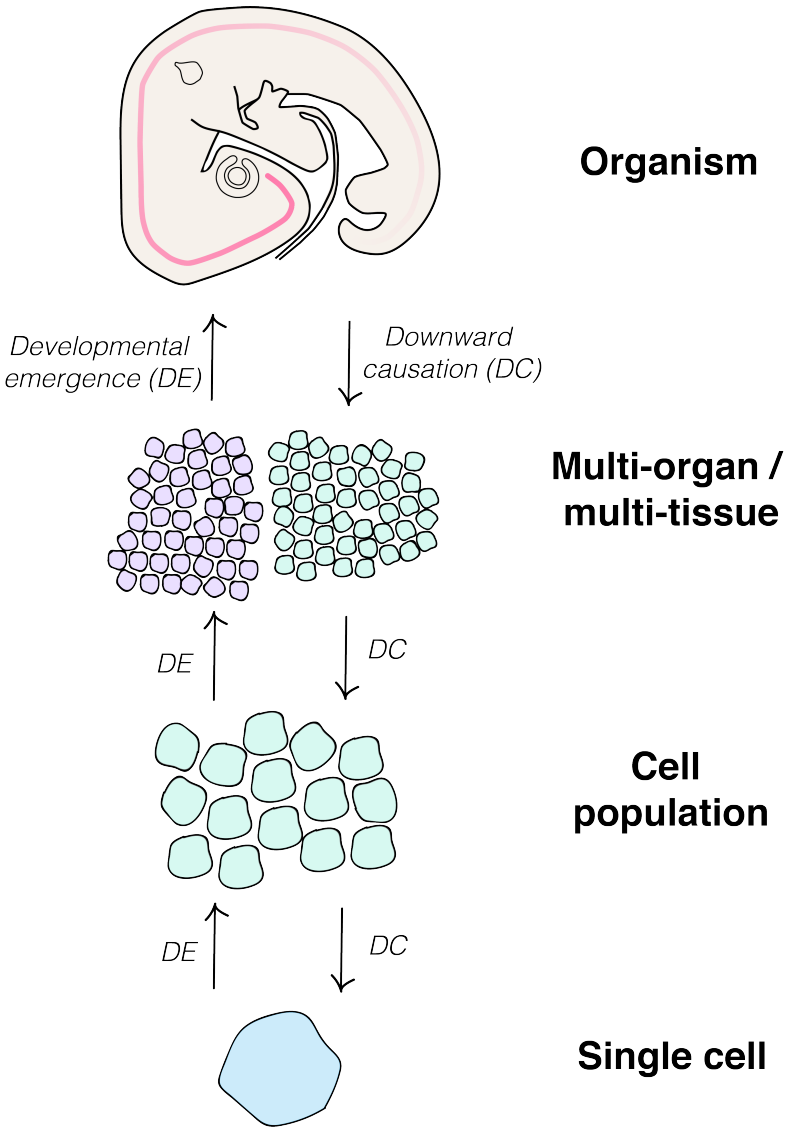

Figure 5: *Summary.* This schematic summarises the different levels of organisation where time has been studied in developmental biology. Interaction between the various levels of organisation occurs bidirectionally. Information passes from higher to lower levels through *downward causation*, exemplified by pattern regulation in the embryo (see text for examples). Information also passes from the lower levels to higher levels, through *developmental emergence*. For example, changes in cell state can lead to changes to inter-population signalling and ultimately direct higher-level changes to the embryo in morphogenesis. We argue that a link between these levels of organisation is provided by the concept of 'tissue tectonics'. The ways in which signalling and responding tissues are displaced relative to one another can influence the timing and location of signalling events between tissues.

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