CHAPTER EIGHT

Talking to your neighbors across scales: Long-distance Notch signaling during patterning

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Abstract

Tissue patterning is a critical part of animal development. Here we review the role that length- and timescales play in shaping patterns during development, focusing on the mechanisms by which Notch-mediated lateral inhibition signaling generates periodic tissue patterns. Because Notch ligands and receptors are membrane bound, the signaling that underlies lateral inhibition depends on direct cell-cell contacts. Nevertheless, there are many biological examples where effective Notch signaling occurs over distances larger than adjacent cells. Here, we summarize the theoretical and experimental evidence for mechanisms that modify the scale of Notch-mediated lateral inhibition.

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We focus on how cell protrusions, in addition to other cell behaviors like proliferation and neighbor exchange, allow for Notch signaling to both extend lateral inhibition beyond nearest neighbors and impact the timescale of patterning. Using recent examples, we examine how dynamic cell behaviors like the formation of protrusions affect the timing of Notch-mediated lateral inhibition as well as the density of the final tissue pattern. We suggest that mechanisms that affect the length and timescale of Notch signaling may have key implications for the evolution of patterns. This review highlights the role of cell behaviors in controlling the temporal and spatial dynamics of pattern formation across scales.

1. Introduction

The transformation of a single cell into an elaborate and reproducible body plan during development is achieved through a plethora of mechanisms that allow cells to dynamically organize themselves in time and space. This transformation requires cells in growing tissues to continuously transmit and gather information about timing and position, both locally and at the scale of the tissue or the entire organism. Although cells can directly sense only their local environment, mechanisms have evolved that allow cells to overcome this constraint and transcend the length and time scales imposed by their individual positions and life cycles.

Classically, one dominant theory of developmental patterning mechanisms is morphogen gradients, where molecules that are transcribed in localized regions can spread to form graded concentration profiles in a target tissue (Stapornwongkul & Vincent, 2021; Wolpert, 1969) (Fig. 1A). As a result, the morphogen concentration at any location becomes a readout of position that cells can use to turn on appropriate fates, generating spatial patterns at the scale of the tissue. However, more recently it is clear that tissue-scale gradients of gene activation in many cases are generated through signals mediated via local cell-cell contact alone (Bischoff et al., 2013; Fancher & Mugler, 2020; Hall et al., 2021; Kornberg, 2017; Zhang & Scholpp, 2019). Examples of long-range signaling via direct contact are the long range gradients of Vg1 and activin seen in early Xenopus embryos, which are formed through a signaling relay between adjacent cells that has the overall effect of a morphogen being transported over longer distances (Reilly & Melton, 1996). Alternatively, information transfer can occur rapidly between cells through force generation and mechanotransduction. When cells exert stress on their direct neighbors through cell junctions, these local stresses can lead to rapid mechanical waves that travel across cells and

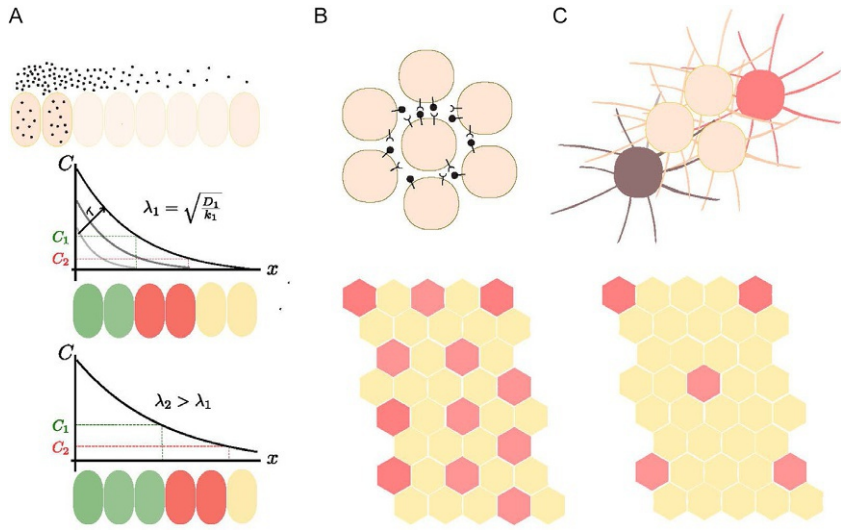


Fig. 1 Length and timescales of signaling and patterning. (A) Morphogen molecules diffuse away from a localized source to form a graded concentration profile in a target tissue. Morphogen profiles can often be described by exponential curves whose decay length, λ, defines the range of the gradient. The value of the decay length is given by $\lambda = \sqrt{\frac{D}{k}}$ where D is the morphogen effective diffusion coefficient and k is the effective degradation rate. The morphogen profile builds up and reaches steady state at a timescale $\tau = 1/k$. Cells take on different fates according to the morphogen concentration in space. Changes in the diffusion coefficient or degradation rate impact the morphogen decay length and the downstream pattern. (B) Local signaling interactions between cells can lead to symmetry breaking and periodic pattern formation at the level of the tissue. Without any mechanisms to expand signaling interactions beyond immediate neighbors, emerging spatial patterns are dense. (C) Cells can extend long cellular protrusions that mediate signaling through contact. A dense network of signaling protrusions emerges that leads to sparser tissue level patterns.

direct symmetry breaking and morphogenesis at the tissue level (Duque & Gorfinkiel, 2016; Serra-Picamal et al., 2012; Vasquez, Tworoger, & Martin, 2014).

Another example of tissue level patterning mediated by local cell-cell interactions is that of lateral inhibition. Lateral inhibition is a conserved juxtacrine signaling mechanism that, during the development of most organisms, drives the formation of diverse fine-grained patterns such as spots and striped boundaries in tissues that are, initially, nearly homogenous (Cohen, Georgiou, Stevenson, Miodownik, & Baum, 2010; Collier, Monk, Maini, & Lewis, 1996; Hamada et al., 2014). During this type of

patterning, each cell within the initial group has the potential to acquire either a signal receiving or signal sending fate. Small differences in the initial group of cells are amplified through feedback loops until one cell adopts a signal sending state. Since cells that have adopted the signal sending state inhibit the adoption of the same state in their contacting neighbors (Meinhardt & Gierer, 2000; Simpson, 1990), their neighbors adopt the signal receiving state, leading to periodic patterns at the tissue level.

In such cases, the effective length and time scales over which cells can interact are not immediately obvious. At first glance, only the signaling dynamics of cells that are in direct contact with one another should be expected to be coupled. However, cells can extend their zone of influence, for example through signaling filopodia or cellular rearrangements (Kornberg, 2017; Maroto, Dale, Dequéant, Petit, & Pourquié, 2005; Uriu, Morishita, & Iwasa, 2010). Furthermore, the spatiotemporal signaling dynamics of cells far from one another may become coupled even for cells that have never been in direct contact, for instance when relay mechanisms or trigger waves are in place so that the signaling states of cells at a distance come in or out of phase (Serra-Picamal et al., 2012).

Here, in exploring the power of this type of process to pattern tissues, we first introduce the role that length- and timescales play in patterning events in general. We will then focus on the Notch pathway, briefly reviewing examples of lateral inhibition and the processes that modify the range of Notch signaling. Next, we will summarize significant mathematical models of lateral inhibition and how they address the problem of scale. We next present specific examples where the spatiotemporal dynamics of patterning are affected by cellular protrusions. Finally, we discuss the potential of contact-mediated patterning for diversification in the course of evolution.

2. Patterning in space and time

2.1 Length scales

2.1.1 Length scale of positional information

The range of signaling at the molecular and cellular level ultimately specifies the length scale of spatial patterns generated at the tissue level (Fig. 1). This is most easily understood when we consider patterning along a single axis, e.g., along the anterior-posterior (AP) axis of a developing embryo. The positional information model of patterning posits that, in such cases, a cell's response to a morphogen depends on the concentration of morphogen and the cell state (which may define a threshold for response to the

morphogen) (Wolpert, 1969). In this model, morphogen is typically produced at a pattern boundary and is transported away from the source in order to generate a gradient of morphogen across a region of the tissue (Fig. 1A).

In the case of the establishment of the AP body plan during the initial stages of embryogenesis in *Drosophila melanogaster*, patterning occurs by the iterative use of morphogen gradients to drive the formation of successively smaller stripes of gene expression that confer segment identities to cells along the AP axis (Jaeger et al., 2004). Probably the best studied of these is the early Bicoid gradient (Ali-Murthy & Kornberg, 2016; Clark & Akam, 2016; Driever & Nüsslein-Volhard, 1988; Spirov et al., 2009). The transcriptional response of the nuclei in each segment depends on the morphogen's decay length—which in principle depends on protein diffusivity and degradation, and the spatial profile of mRNA which may itself form a gradient that is diffusion independent, e.g., through dispersion along cytoskeletal cables (Ali-Murthy & Kornberg, 2016; Spirov et al., 2009). Changes that impact events at the molecular level, such as mRNA production and transport, molecular diffusion and turnover, will be reflected at the tissue level through changes in the morphogen decay length and the spatial organization of boundaries (Drocco, Grimm, Tank, & Wieschaus, 2011) (Fig. 1A).

The Bicoid gradient that occurs in the embryonic syncytial blastoderm of Drosophila is essentially an intracellular event. In principle, gradients in multicellular tissues rely on similar processes, although, in the case of tissues, morphogens are secreted or presented externally. A well-studied example of this is the gradient of the TGFβ homolog Dpp in the Drosophila wing imaginal disc (Ben-Zvi, Pyrowolakis, Barkai, & Shilo, 2011; Kicheva et al., 2007; Stapornwongkul, de Gennes, Cocconi, Salbreux, & Vincent, 2020; Wartlick et al., 2011; Zhu, Qiu, Chen, Nie, & Lander, 2020). Here, the concentration of Dpp in the imaginal disc specifies the position of the veins in the adult wing (Bosch, Ziukaite, Alexandre, Basler, & Vincent, 2017; Campbell & Tomlinson, 1999). The concentration and decay length of Dpp increases over time to scale with the overall growth of the tissue, ensuring that the underlying pattern remains proportionate to the organ size (Wartlick et al., 2011). In principle, the ability of the Dpp gradient to scale with the tissue size may depend on a number of processes including extracellular ligand dispersion, the binding and unbinding of the ligand to receptors in the cell membrane, receptor-ligand internalization, recycling and active transport via cells (Hatori, Wood, Oliveira Barbosa, & Kornberg, 2021; Huang, Liu, & Kornberg, 2019; Zhu et al., 2020). Scaling,

in this case, is achieved by tuning the contributions that these processes play over time (Romanova-Michaelides et al., 2022).

2.1.2 Length scale of periodic patterns

The generation of periodic patterns, like those seen on the skin of animals from butterflies to birds, represent another type of challenge to developing organisms. Examples include the spacing between hair or feather follicles, pigmentation stripes, or sensory bristles (Cohen et al., 2010; Glover et al., 2017; Shyer et al., 2017; Yamaguchi, Yoshimoto, & Kondo, 2007). These patterns are characterized by the frequency at which motifs repeat themselves in space. In such cases, the spatial frequency itself depends on the effective length scale of the signaling molecules that pattern the tissue. In principle, periodic patterns can be achieved in an initially homogeneous tissue through reaction-diffusion processes, like those first described by Turing (Turing, 1952). This model proposes that an activator locally stimulates its own production together with that of an inhibitor. Differences in the relative rates of diffusion of the activator and inhibitor can lead to the emergence of stable, periodic patterns, where the wavelength of the spatial pattern depends on the diffusion coefficients of the activator and inhibitor, and the kinetic functions that specify interactions between the activator and inhibitor (Meinhardt & Gierer, 2000; Ouyang, Li, Li, & Swinney, 1995).

However, there are other ways to organize periodic tissue patterns. Periodic patterns can also arise from contact-mediated interactions without the need for prepatterns, diffusible molecules, or information at the global level (Kondo & Miura, 2010). One can show that local interactions between cells in a noisy environment are sufficient to lead to symmetry breaking and patterning in an initially homogeneous tissue (Collier et al., 1996) (Fig. 1B). In this case, the frequency of the developing pattern depends on the range over which cells can send and receive signals (Fig. 1B, C). When signaling only occurs between direct neighbors, patterning is expected to be denser whereas sparser patterns emerge when cells expand their sphere of influence through movement or signaling at protrusions (Fig. 1C). Contact dependent signaling at a distance can occur via the formation of cellular protrusions, including filopodia, cytonemes, and tunneling nanotubes. Cells in developing tissues and in culture have been observed to extend long, thin, processes that allow them to signal to distant cells (González-Méndez, Gradilla, & Guerrero, 2019). These protrusions are observed to participate in several signaling paradigms (e.g., TGFß, Wnt, Shh) (Bischoff et al., 2013; Hall

et al., 2021; Hsiung, Ramirez-Weber, David Iwaki, & Kornberg, 2005; Inaba, Buszczak, & Yamashita, 2015; Mattes et al., 2018). The primary evidence that they participate in the dispersion of local signals includes (1) the localization of signaling molecules along the length of the protrusion; (2) that interfering with protrusion length leads to the disruption of signaling gradients; and (3) that downstream effectors are activated in the vicinity of protrusion contacts. Indeed, the models of Turing-like patterns does not specify how the activator and inhibitor are transported in space, and cellular protrusions likely represents just one of the mechanisms by which morphogen movement occurs.

2.2 Time scales

Developmental patterning takes place during a finite window of time: patterning is initiated when certain signaling pathways become activated and ends when cells have received the appropriate signals and have become committed to their fate. The entire process depends on a range of events, each characterized by its own timescale. For example, sub-cellular timescales comprise events like metabolite turnover, transcription, or intracellular trafficking, while cellular timescales comprise events such as cell division and migration. Returning to the example of the Bicoid gradient in Drosophila embryos, multiple events associated to different timescales occur for the Bicoid transcription factor to form a gradient: this includes the translation of protein from the maternally deposited mRNA (minutes; Petkova, Little, Liu, & Gregor, 2014), diffusion or active transport of protein and mRNA away from the anterior pole (Ali-Murthy & Kornberg, 2016; Durrieu et al., 2018; Spirov et al., 2009), the binding of transcription factors to DNA targets (seconds), protein turnover lifetime ($t_{1/2}$–30 min; Durrieu et al., 2018), and nuclear division cycles (minutes; Foe & Alberts, 1983). Together, these events ensure a robust and reproducible morphogen gradient along the AP axis of the developing embryo that is initiated at fertilization and maintained until cellularization ~3 h later.

For multicellular patterns, similar considerations for transcription and translation can be made, but the timescale of trafficking, extracellular dispersion and degradation, as well as receptor dynamics must also be accounted for. Other cell behaviors in epithelia also need to be considered, for example the cell cycle for patterning tissues that are simultaneously growing; the timescale for the formation of cellular structures, like filopodia, which can deliver membrane-bound ligands and receptors; the timescale of cell movements—including

neighbor exchanges, and individual or collective migrations. Together these determine the spatiotemporal dynamics of pattern formation.

The development of cutting-edge live-imaging techniques have allowed researchers to quantify many of these processes. Some examples include, FRAP and FCS assays to quantify the diffusivity and degradation rate of morphogens (Kicheva et al., 2007; Zhou et al., 2012) or nanobody assays that can quantify rates of molecular internalization and recycling (Buser, Schleicher, Prescianotto-Baschong, & Spiess, 2018; Stapornwongkul et al., 2020). However, several questions remain. How do the multiple events occurring at the molecular, cellular and tissue level together dictate the time window in which patterning can happen? And can some processes be neglected when defining the timescale of patterning? Theoretical modeling can help shed light on these questions. For example, it can be shown that in a system where several processes control morphogen transport—such as extracellular diffusion, internalization, recycling and degradation of molecules—different effective timescales emerge that each depend on all these phenomena. Therefore, the timescale at which the morphogen profile is expected to reach steady state is not determined by a single process but is a nontrivial function of the rates at which molecules are trafficked and degraded (Aguilar-Hidalgo, Hadjivasiliou, Romanova-Michaelides, González-Gaitán, & Jülicher, 2019). At the same time, there are regimes in the parameter space that suggest certain events may dominate morphogen gradient formation and others can be neglected. A rigorous theoretical framework together with appropriate quantitative assays can help define which processes specify the timescale of gradient formation and patterning (Romanova-Michaelides et al., 2022). In the following section we approach this problem in the context of Notch signaling, then discuss how quantitative modeling has helped to integrate the cell and molecular complexities that drive to Notch-mediated tissue patterning.

3. Notch mediated patterning across scales

3.1 Notch signaling overview

Notch signaling is one of the best-studied examples of lateral inhibition, and is employed throughout development and across evolution to specify distinct signaling identities in neighboring cells. Examples of processes that depend on Notch signaling include, among many more, the selection of small sensory bristles in the Drosophila notum (Corson, Couturier, Rouault, Mazouni, & Schweisguth, 2017), the differentiation of cells into

neurons in the zebrafish spinal cord (Hadjivasiliou et al., 2019), blood vessel formation (Zakirov et al., 2021), hair cell patterning in the inner ear (Lanford et al., 1999) and the synchronization of oscillations between neighboring cells during vertebrate segmentation (Ozbudak & Lewis, 2008). Notch signaling requires that signal sending and receiving cells are in contact with one another (Fig. 2A). Notch mediated patterning can require the coordination

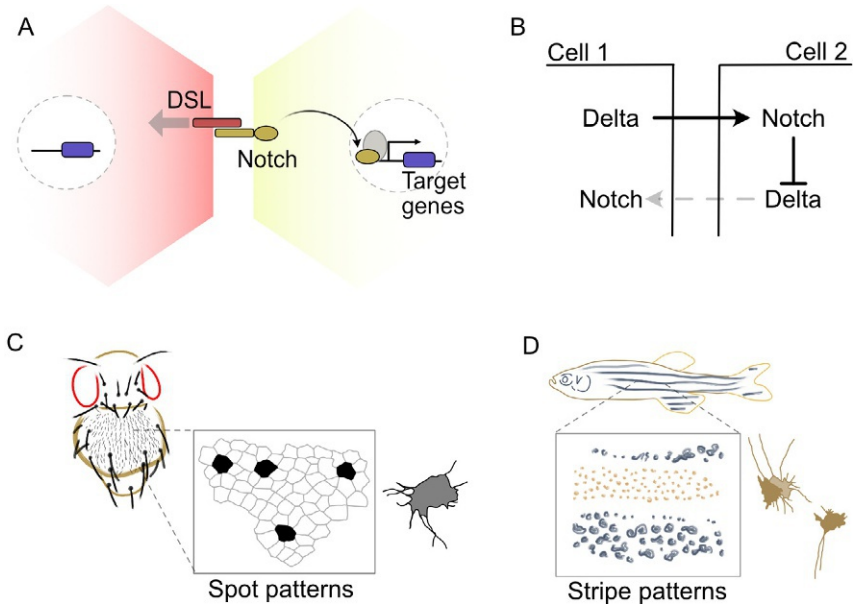


Fig. 2 Notch signaling during pattern formation. (A) A simplified schematic of Notch signaling. Pink cell to the left expresses high levels of DSL ligand, upon endocytosis (gray arrow) of DSL bound to Notch receptor in trans, Notch is cleaved and the intracellular domain translocates (black arrow) to the nucleus to regulate the expression of target genes with co-transcription factors (gray oval). Thus the yellow cell to the right becomes Notch activated. (B) The overall feedback loop of Notch signaling during lateral inhibition. Notch activation in Cell 2 leads to the repression of further Delta expression in Cell 2, which decreases the activation of Notch in Cell 1. Without Notch-mediated repression of Delta expression, Cell 1 maintains higher levels of Delta ligand. (C) The small sensory hairs on the dorsal thorax of *Drosophila melanogaster* is a model system for long-range lateral inhibition. Notch signaling mediates the spacing of Delta-expressing bristle precursor cells (black cells) among Notch activated epithelial cells (white cells). Cells which are more than one cell diameter away from each other may contact each other via actin-rich protrusions, shown to the right. (C) The pigment stripes of Zebrafish are generated by long-range lateral inhibition. Xanthophores (yellow) and melanophores (dark gray) extend protrusions towards each other (shown to the right) that support Notch signaling.

of many processes including feedback dynamics that underlie the signaling pathway, interactions between the Notch ligand and its receptor within and between cells, cell motility, and cellular protrusions. As such, Notch signaling offers an ideal framework to discuss how events at the molecular, cellular and tissue level together specify the length and timescale of patterning, and how signaling at the single cell level can effectively incorporate spatial and temporal information of a length and time scale that transcends those of individual cells.

Excellent reviews of the mechanisms of Notch activation have been written (e.g., Binshtok & Sprinzak, 2018; Bocci, Onuchic, & Jolly, 2020; Bray, 2016; Kopan & Ilagan, 2009), therefore we will only briefly introduce Notch signaling here. Notch is a type I transmembrane receptor whose canonical signaling pathway is activated upon binding with the type I transmembrane ligands Delta, Serrate, or Jagged (DSL) in a contacting cell. Cleavage of Notch, and thus its overall activation state, depends on the presence of (1) mechanical pulling forces orthogonal to the surface of the receptor expressing cell, provided by endocytosis of DSL by the DSL expressing cell (Gordon et al., 2015; Langridge & Struhl, 2017) and (2) proteases which cleave Notch first towards the C-terminal end of the extracellular domain and next in the transmembrane domain. The Notch intracellular domain (NICD) is released from the membrane via cleavage by the gamma-secretase complex in the transmembrane domain (Struhl & Greenwald, 1999). Once translocated to the nucleus, NICD is free to associate with other transcription factors (e.g., DNA-binding protein CBF-1/Suppressor of Hairless/LAG1; CSL) to collectively modulate the transcription of target genes. The downstream activity of NICD, and its co-factors, on gene expression is highly context dependent (Bray, 2016).

The presence of Notch protein on the cell surface is tightly controlled through the activity of the endosomal regulators (Johnson, Zitserman, & Roegiers, 2016). Movement of Notch from internal pools to the cell surface is dependent on signaling contexts, but the half-life for many signaling receptors at the cell surface occurs on the order of hours (Hervé, Derangeon, Bahbouhi, Mesnil, & Sarrouilhe, 2007). For Notch molecules actively engaging in signaling with a DSL ligand in trans, endocytosis (∼minutes), enzymatic cleavages (∼seconds) and translocation to the nucleus (∼minutes) occur rapidly relative to other steps in the signaling pathway (Ubezio et al., 2016). Once cleaved, the stability of the NICD fragment is in part regulated by post-translational modifications (e.g., phosphorylation of the PEST domain; NICD half-life ranges from minutes to hours) (Fryer, White, &

Jones, 2004). Interactions with NICD also leads to increased stability of CSL-DNA interactions, which may contribute to stabilizing the transcriptional response to Notch signaling (Falo-Sanjuan, Lammers, Garcia, & Bray, 2019). Negative feedback on the pathway occurs through the repression of pro-neural genes and DSL. This feedback loop occurs at longer timescales than other molecular events, in part because of the need to turnover existing Delta (Fig. 2B). All these steps in Notch signaling, as well as the cell behaviors that support the ability of cells to engage in cell-cell contact—motility (minutes–hours) protrusion formation (minutes), cell division (hours)—feed into the overall signaling dynamics and timescale of patterning at the tissue level.

In addition to activation in trans, the Notch signaling pathway is also subject to regulation in cis (i.e., ligand and receptor interactions in the same cell). *Cis*-inhibition is the sequestering of unactivated Notch receptor by higher concentrations of DSL ligand in the same cell. The details of the dynamics and regulation of this sequestration is unknown, but both experimental evidence and mathematical modeling results (del Álamo, Rouault, & Schweisguth, 2011; Palmer, Jia, & Deng, 2014; Sprinzak, Lakhanpal, LeBon, Garcia-Ojalvo, & Elowitz, 2011; Sprinzak et al., 2010) indicate that cis-inhibition contributes to efficient lateral inhibition, and the generation of sharp boundaries during certain pattern formation processes. It is important to note that molecular and cellular noise contributes to every step in the signaling pathway, and therefore stochastic noise in molecular processes need to be averaged over space and/or time in order to generate a precise patterning result. However, these heterogeneities may also help the progression of reproducible patterns in vivo. The ability of experimental approaches to measure the collective effect of different molecular and cellular timescales, as well as the noise averaging associated with them to achieve a coherent signal, is currently limited.

3.2 The interdependence of space and time in Notch signaling

A key aspect of Notch signaling is the negative feedback which exists downstream of receptor activation. One of the indirect targets of NICD transcriptional regulation is DSL itself, such that expression of ligand is repressed by activation of Notch (Fig. 2B). It follows that neighboring cells inhibit one another from producing the DSL ligands. As a result, patterns of cells of alternating fates emerge in an initially homogeneous tissue (Fig. 1B). The emerging pattern is expected to be dense with about 2–3 cell diameters between

subsequent DSL cells, although this also depends on the dimensionality of the tissue (Cohen et al., 2010). The spatial patterns observed in many experimental systems, however, are more sparse suggesting that the effective range over which Notch signaling takes place exceeds that of direct neighbors alone (Fig. 2C, D). What is more, the duration of the process of lateral inhibition relies on the transcriptional feedback that dictate the signaling pathway. For example, it is estimated that a single round of lateral inhibition in mouse cells would take up to 6 h, suggesting that the process of patterning an entire tissue may take several days (Zakirov et al., 2021). Nonetheless, patterning often takes effect in a substantially smaller time window. These observations suggest that additional mechanisms may be in place that expand the length and timescales over which Notch mediated patterning occurs. In the following sections we discuss the role of processes that impact the effective length scale and temporal dynamics of Notch signaling.

3.3 Mechanisms for the spatiotemporal control of Notch signaling

3.3.1 Protrusions

Evidence for Notch signaling through cellular protrusions include several examples from Drosophila and Zebrafish. Epithelial cells that are precursors to the small sensory bristles in the Drosophila pupal notum extend long, actin-rich, protrusions from their apical (De Joussineau et al., 2003; Renaud & Simpson, 2001) or basal surface (Cohen et al., 2010) (Fig. 2C). The former only have been suggested to appear under mechanical stress. Since Delta protein localizes to these protrusions, it was proposed that they might help increase the range of signaling for any given sensory bristle precursor cell. Indeed, the spacing between bristle precursor cells is coupled to protrusion length and mutations which disrupt protrusion length increase bristle density (Cohen et al., 2010; Georgiou & Baum, 2010; Hunter et al., 2019). The protrusions are present on all epithelial cells in the notum, in addition to the bristle precursor cells, which should further increase the range of lateral inhibition.

In the notum, protrusions also play a role in regulating the time window over which patterning occurs. This is achieved by coupling Notch signaling to the cell cycle that ultimately determines when cells become committed to their fates. Once cells reach a threshold of Notch activation, G2-exit is triggered; epithelial cells which have divided no longer participate in lateral inhibition (Hunter et al., 2016). Notch activation in cells that receive Delta signals through protrusions alone increases more slowly compared

to cells with larger contacts, leading to a longer time window over which patterning takes place. The formation of this pattern features a refinement process, whereby cells in the tissue adjust the pattern prior to the pattern being fixed. Adjustments include switching cell fates and apoptosis (Cohen et al., 2010; Koto, Kuranaga, & Miura, 2011), which help to ensure regular spacing but occur by yet unknown mechanisms. Therefore, allowing cells distant from the signal sending bristle precursor cell to have an increased window of time for plasticity may be a mechanism that promotes pattern refinement.

During development of the zebrafish pigment stripes, xanthophores (yellow pigment cells) express Delta ligands while melanophores (dark pigment cells) express Notch receptors, suggesting that Notch signaling plays a role in patterning the zebrafish skin (Hamada et al., 2014). Ectopic expression of Delta or Notch changes the boundaries and thickness of the stripes, supporting this hypothesis. Hamada et al. (2014) show that melanophores extend long protrusions towards xanthophores that mediate Notch signaling and contribute to the organization of the pigment stripes (Fig. 2D). Eom, Bain, Patterson, Grout, and Parichy (2015) demonstrated how Notch signaling via protrusions extending from xanthophore cells promotes the generation of stripes of alternating color that are several cell diameters wide. These long (~60 μm, or 5–6 cell diameters) rapid protrusions, or airinemes, are supported by both the actin and microtubule cytoskeleton, such that the genetic or pharmacological disruption of the cytoskeleton leads to the failure to segregate melanophores from interstripe regions. Delta ligand is observed to be carried in vesicles in airinemes and are released at the tip of the protrusion. In addition, disruption of xanthopore airinemes leads to a decreased Notch response in contacting melanophore cells. Finally, expression of constitutively active Notch in melanophores resulted in stripes that were broader than in wild type, supporting a role for Notch in promoting pigment sorting through melanophore migration. Although the role of airineme length and dynamics in the generation of the final pigment pattern is not yet known, these results demonstrate a role for long-range Notch signaling in the development of a Turing-like pattern.

Dynamic protrusions also specify the spatiotemporal dynamics of Notch signaling and subsequent neuronal differentiation in the zebrafish spinal cord (Hadjivasiliou et al., 2019; Moore & Alexandre, 2020), and the selection of tip cells in branching angiogenesis (Page et al., 2019). In these model systems, protrusion length and dynamics impact both the spatial and temporal dynamics of Notch signaling and pattern formation. We discuss these examples in more detail in the next section.

In other instances, it is less clear if the activity of protrusions is important for signaling length or time scales. Notch or Delta carrying protrusions have been shown to be essential for the development of the air sac primordium and stem cell niches in Drosophila (Huang & Kornberg, 2015; Yatsenko & Shcherbata, 2021). In the first example, the activation of Notch signaling in the air sac primordium is due to Delta on cytonemes, expressed in nearby myoblasts during wing disc development. In the second, the activation of Notch signaling in terminal filament cells during oogenesis is due to Delta presented on cellular projections from primordial germ cells. In both cases, the role of these protrusions is not a function of their length. In these examples, protrusion mediated signaling appears to ensure the targeted delivery of morphogens between cell types or tissues, comparable to the connectivity and function of neurons. When these extensions are shorter, for example, signaling simply fails.

The dynamic behavior of signaling protrusions can be measured using markers of cell shape visualized over time (González-Méndez, Seijo-Barandiarán, & Guerrero, 2017; Hunter et al., 2019). These studies show that protrusions have behaviors beyond simply extending and retracting, including pausing or trapezoid behavior, or even collective behaviors. Protrusions have lifetimes on the order of minutes to hours, and the signaling proteins on the protrusions often also appear motile. However, the tools needed to systematically and specifically manipulate protrusion mediated signaling have been lacking. Most evidence relies on cell-wide genetic manipulation of cytoskeleton regulators that also effect other essential processes in the cell that may contribute to signaling. For example, decreased Cdc42 activity leads to the decreased formation and maximum length of some protrusions, but Cdc42 is also can also play a role in endocytosis, which is essential for Notch signaling. Recently, promising optogenetic tools have been developed based on engineered myosin motors that allow the specific manipulation of morphogens along protrusions (Zhang et al., 2021). Tools such as these will help experimentalists address remaining questions about Notch signaling via protrusions. Direct evidence for Notch signaling via protrusions is still needed in most systems, comparable to the recent observations of Shh signaling via cytonemes in cell culture (Hall et al., 2021).

3.3.2 Cell division

For tissues undergoing proliferation during patterning, cell divisions can in principle affect both the length- and timescales of Notch signaling. First, proliferation can increase the distance between two signal sending cells.

Unless there is a mechanism to increase the range of Notch signaling and allow the two cells to continue contacting each other, this can lead to local Notch signal minima. In the case of repeating patterns, local Notch minima leads to "filling in" of the pattern. This mechanism may be a feature of pattern refinement. During bristle patterning, the onset of cell division signals the end of lateral inhibition between bristle precursors and epithelial cell neighbors; in order to create the appropriate pattern, Notch activated cells divide first, followed by the bristle precursor. Disruption of this relative timing leads to inappropriate filling-in of the pattern and errors in bristle placement (Cohen et al., 2010; Hunter et al., 2016; Nègre, Ghysen, & Martinez, 2003).

Second, changes in cell morphology and tissue organization associated with cell division may have an impact on the lengthscale of Notch signaling. Proliferation can modify connectivity through the formation of new contacts between daughter cells, and so increase Notch/Delta cell interactions within tissues. However, cell rounding associated with mitosis inhibits the ability of cells to extend protrusions. In the patterning notum epithelium, for example, basal protrusions that mediate Notch signaling are retracted as cells enter mitosis (Rosa, Vlassaks, Pichaud, & Baum, 2015). Changes in cell morphology can alter the contact area between neighboring cells, which can have a dramatic effect on the ability of cells to engage in Notch signaling (Shaya et al., 2017). For example, Shaya et al. have shown that smaller cell-cell contacts result in weaker Notch activation dynamics, dominated by the diffusion of ligand and receptor in and out of the contact area. In contrast, larger contacts maintain a stronger Notch activation. Therefore, impact of cell behaviors like rounding during mitosis on Notch signaling will be context dependent.

Finally, one feature of development is that the window of time during which cell fate decisions occur is often coordinated with extrinsic clocks like the cell cycle (Ayeni et al., 2016; Hunter et al., 2016; Slowik & Bermingham-McDonogh, 2016). Not only can the cell cycle help define the window of time during which Notch signaling may occur, it can also be used iteratively to pattern different cell fates in a lineage. Sensory bristle precursor cells in the notum undergo four rounds of cell division, and Notch signaling is critical in between each division in order to specify the supporting cell types of the adult sensory bristle (Guo, Jan, & Jan, 1996). Mistiming the divisions of the bristle precursor lineage leads to the transformation of daughter cells into the wrong cell fates (Ayeni et al., 2016).

3.3.3 Cell migration and rearrangements

If a signaling cell migrates in space the number of neighbor cells it contacts increases and the effective range over which lateral inhibition can act grows. For juxtacrine signaling-based patterning systems, this can play an essential role and impact both the spatial and temporal dynamics of resulting patterns. In the case of the mammalian intestinal crypts, Notch-mediated lateral inhibition is required for the generation of secretory and absorptive epithelial cells (Tóth, Ben-Moshe, Gavish, Barkai, & Itzkovitz, 2017). As cells acquire their fates, they collectively migrate towards the lumenal tip of the villi. Tóth et al. shows that a clear delineation between the region in which lateral inhibition can occur and the region in which cell migration can occur is essential for maintaining the correct numbers of each cell type. This separation of behaviors acts to restrict the range of Notch signaling.

In contrast to the villi model, Notch signaling during cell mixing is essential for development of the AP axis in vertebrates (Lawton et al., 2013). The increase in range of Notch signaling provided by movements of cells in the posterior pre-somitic mesoderm allows for the coordination of genetic oscillators (Uriu et al., 2010). Notch signaling plays a key role during vertebrate somatogenesis by synchronizing the oscillations of gene expression in neighboring cells (Liao & Oates, 2017). During somatogenesis, cell movement and division leads to dynamic rearrangements of relative cell position in the pre-somitic mesoderm, the part of the tissue where cells have not yet differentiated into somites. These cellular rearrangements imply that the range of Notch signaling increases as cells exchange neighbors and the number of neighbors they interact with expands. Theoretical work suggests that cell motility in this context promotes synchronization of the oscillations by making oscillations more robust to external perturbations and expanding the parameter space where cell synchronization can be achieved (Uriu et al., 2010).

In the context of epithelia where there is no individual cell migration, neighbor exchanges may instead play a role in increasing the signaling range of a given signal sending cell. For example, at the level of the apical junction, notum epithelial cells do not move large distances even though they may undergo T1 transitions and exchange neighbors (Curran et al., 2017). However, basal to these junctions, the bulk of the cell bodies do exhibit some shuffling (Renaud & Simpson, 2001) but it is unclear if this random motion is required for increasing the scale of sensory bristle patterning.

3.3.4 cis-Interactions

During lateral inhibition patterning events, cis-inhibition allows initial heterogeneities in an individual cell's surface levels of Notch or DSL ligand to

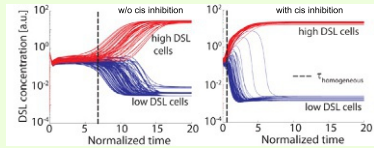
BOX 1 Mathematical descriptions of Notch signaling.

A general model of Notch mediated lateral inhibition can be defined by a set of coupled differential equations that describe the dynamical process of gene activation and inhibition as follows:

$$\frac{dR_i}{dt} = \beta_R \frac{\langle D\rangle_i^m}{q^m + \langle D\rangle_i^m} - \gamma_R R_i \quad (1) \qquad \frac{dD_i}{dt} = \beta_D \frac{p^l}{p^l + R_i^l} - \gamma_D D_i \quad (2)$$

Here R_i and D_i are the expression of Notch signaling and the Delta ligand in cell i, and $\langle D\rangle_i$ is the total amount of Delta received by cell i. The parameters β_R and β_D capture the baseline production rates for Notch signaling and Delta respectively and their ratio determines if heterogeneous spatial patterns are possible. The parameters γ_R and γ_D are the degradation rates of the Notch signal and Delta and specify the timescale at which the system reaches steady state. Finally, q, m and p, l capture the strength of the feedback between Notch signaling and Delta production.

A more detailed model describes the concentration of free ligand and receptors on individuals cells together with the levels of intracellular Notch singing (Eq. 3-4). Here, N_i, D_i, and R_i are the concentration of the free Notch receptors, free Delta and activated Notch in cell i. The parameters k_t and k_c capture the strength of receptor-ligand binding between and within cells respectively [ref].



Dynamics of cell signalling state in an initially homogeneous environment. The timescale of patterning ($\tau_{homogeneous}$) is substantially lower with cis inhibition. Figure adapted from Sprinzak et al (2012).

$$\frac{dN_i}{dt} = \beta_N - \gamma_N N_i - k_t N_i \langle D\rangle_i - k_c N_i D_i \quad (3)$$

$$\frac{dD_i}{dt} = \beta_D \frac{p^l}{p^l + R_i^l} - \gamma_D D_i - k_t D_i \langle N\rangle_i - k_c N_i D_i \quad (4)$$

$$\frac{dR_i}{dt} = \beta_R \frac{\left(\frac{k_t}{\gamma_s} N_i \langle D\rangle_i\right)^m}{q^m + \left(\frac{k_t}{\gamma_s} N_i \langle D\rangle_i\right)^m} - \gamma_R R_i \quad (5)$$

Eq. (3-5) explicitly consider events that happen at the molecular level, like binding at the cell membrane between and within cells, and associated timescales. Computational analysis of this model combined with experiments showed that cis binding without receptor activation speeds up patterning dynamics, promotes the formation of boundaries and expands the palette of possible patterns Notch signaling can sustain.

Signaling at protrusions can be implemented by expanding the range over which cells can come in contact with one another according to the range and directionality of protrusions [refs]. In addition, different assumptions about the efficiency of binding at protrusion versus cell body contacts can be made by assigning weights to the incoming signals (Eq. 6).

$$\langle D\rangle_i = w_b \left(\sum_{j \in \text{cell body contact}} D_j \right)_i + w_p \left(\sum_{j \in \text{protrusion contact}} D_j \right)_i \quad (6)$$

be quickly amplified without the need to complete several feedback loops (Box 1). In particular, cis-inhibition is thought to circumvent the slowest steps of the signaling pathway, which include the downregulation and degradation of DSL in the Notch activated cell. Experimental evidence in cell culture supports the findings of mathematical models demonstrating that cells with feedback from cis-inhibition spend *less* time in the pre-committed, bipotential state (Sprinzak et al., 2011, 2010), than cells without cis-inhibition. At larger length-scales, the less time is spent in pre-committed cell states, the faster overall patterning can occur. Furthermore, cis-inhibition promotes multistability of patterns and robustness via error minimization (Formosa-Jordan & Ibañes, 2014; Sprinzak et al., 2011). Error minimization in the overall pattern is a consequence of increasing the speed

of lateral inhibition: in the absence of cis-inhibition, the longer delays associated with the Notch signaling response in pre-committed cells with similar levels of Notch/DSL (in trans) can lead to scenarios where adjacent cells both initially adopt the same fate and fail to respond to each other before committing (Barad, Rosin, Hornstein, & Barkai, 2010). This may be especially problematic when cell fate commitment is linked to an extrinsic clock, like the cell cycle. Theoretical modeling also suggests an expansion in the phase space of possible patterns as well as parameters that lead to stable solutions is achieved when cis inhibition is in place (Formosa-Jordan & Ibañes, 2014).

3.3.5 Other regulators

Although the core Notch signaling pathway requires cell-cell contact, experimental evidence shows that Notch signaling can be modified, both directly and indirectly, by secreted regulators that directly bind to Notch. In principle, these regulators could help define the length or time-scales over which Notch signaling occurs. For example, there is evidence that *scabrous* acts as a direct regulator of Notch signaling, contributing to the length scale of lateral inhibition patterns. *Scabrous* encodes a secreted protein that has been shown to bind directly to Notch receptor and regulate its downstream signaling (Corson et al., 2017; Gavish et al., 2016; Mlodzik, Baker, & Rubin, 1990; Powell, Wesley, Spencer, & Cagan, 2001; Renaud & Simpson, 2001), however evidence suggests that *scabrous* may also be distributed via cellular protrusions (Lacoste et al., 2022). In the notum, *scabrous* mutants display decreased bristle spacing relative to wildtype flies (Renaud & Simpson, 2001) suggesting that the normal function of this protein is to promote long-range lateral inhibition, perhaps by increasing the sensitivity of distant cells to the weak DSL signals occurring in smaller, protrusion mediated, contacts. In the developing fly wing, ectopic expression of *scabrous* phenocopies wing defects in animals heterozygous for Notch, as well as decreasing the expression of Notch target genes (e.g., *Espl-m8*) (Lee, Yu, & Baker, 2000). These results are consistent with Scabrous as an antagonist of Notch activity. Together these studies indicate that the Scabrous can act as an antagonist or activator of Notch signaling, which may be context dependent.

Cells can release extracellular vesicles as a means of dispersing membrane-associated morphogens (McGough & Vincent, 2016). Evidence from cell culture experiments show that Notch ligand Delta-like 4 (Dll-4) can be packaged into exosomes and released into extracellular space (Sharghi-Namini, Tan,

Ong, Ge, & Asada, 2014; Sheldon et al., 2010). When a vesicle containing Dll-4 comes into contact with a Notch expressing cell, Notch target genes are expressed, indicating that the signaling pathway has been activated. Furthermore, in the developing vulva of *C. elegans*, several of Notch ligands and modifiers have been shown to be secreted, without the requirement of vesicles (Chen & Greenwald, 2004; Komatsu et al., 2008). Given evidence that a mechanical pulling force is required for the activation of Notch in trans (Langridge & Struhl, 2017), how effective signaling can occur via secreted Notch ligand is an open question. The distribution of these modifiers in extracellular space will determine the effect that they have on the range of lateral inhibition.

Other morphogens that prepattern tissues may also play an indirect role in the length and timescale of the Notch response. Continuing with the fly bristle pattern example, both Wg and Dpp signaling are required for the positioning of the invariant, large sensory bristle (Sato, Kojima, Michiue, & Saigo, 1999). In this example, Notch signaling occurs in the location specified by Wg and Dpp positional information. While prepatterning followed by lateral inhibition is a recurring theme in developmental patterning, in principle simultaneously combining a prepattern signal with a reaction-diffusion system can alter the length scale of the latter (Green & Sharpe, 2015). As will be discussed below, concurrent VEGF and Notch signaling during angiogenesis can speed up lateral inhibition, through the incorporation of additional feed forward loops.

4. Modeling long-range Notch signaling

The emergence of complex patterns during development has classically been attributed to molecules that disperse in tissues over large distances. However, recently theoretical modeling indicates that tissues can self-organize into diverse and complex patterns through contact mediated signaling alone, without the need to invoke diffusible factors (Binshtok & Sprinzak, 2018). Therefore, signaling pathways that require juxtacrine interactions such as Notch can in principle lead to a real diversity of spatial patterns of varying density, from spots to stripes and labyrinths (Formosa-Jordan & Ibañes, 2014; Hadjivasiliou, Hunter, & Baum, 2016). Theoretical work offers a framework that, combined with experiments, can improve our understanding of how the length and timescales involved in Notch signaling impact the spatiotemporal dynamics of patterning at the tissue level.

A mathematical description that captures the signaling dynamics of Notch was first introduced by Collier et al. (1996). This model describes Notch signaling by a pair of differential equations that capture the dynamics of activated Notch and Delta in individual cells (Box 1). In this context, the timescale of signaling interactions, and ultimately patterning at the tissue level, depend on key parameters like the degradation rate of the ligand and signal, and the strength of the negative feedback between Notch signaling and Delta production. A more detailed theoretical framework of the signaling dynamics was later put forward (Sprinzak et al., 2011, 2010). Here, a set of three differential equations describe the concentration of the Notch receptor, ligand and signaling levels over time (Box 1). This more explicit approach allows processes like binding and unbinding of ligands within and between cells to be directly considered. In this way, theoretical predictions about how the signaling and patterning dynamics depend on rates of molecular interactions can be obtained. Different approaches to model Notch signaling have been recently reviewed (Binshtok & Sprinzak, 2018). Below we focus on how cellular protrusions can be incorporated when modeling Notch, and the insights that such models can provide.

4.1 Modeling protrusion signaling

The role of protrusion mediated Notch signaling can be modeled by implementing an extended radius of influence for individual cells, so that the effective number of neighbors a cell is in contact with expands according to the protrusion length and polarization (Box 1). The strength of signaling at different contact types (cell body to cell body, protrusion to cell body, and protrusion to protrusion) can assume different weights to reflect variations in ligand or receptor concentrations at protrusions vs cell body, or variations in signaling efficiency. Furthermore, interactions between the Notch receptor and ligand can be modeled to incorporate inefficient receptor activation between cells (e.g., when cleavage of the receptor upon binding is not successful) leading to the effective sequestration of receptors (Hadjivasiliou et al., 2016). Exploring this framework suggests that tuning the interactions between Notch and its ligands at different contact types, as well as varying protrusion length and dynamics can substantially expand the phase space of patterns possible through Notch mediated lateral signaling alone to a range of patterns akin to those seen in diffusion-based systems. The spatial density of resulting patterns depends on protrusion length and the relative efficiency of activating Notch signaling at different cell contacts

(Hadjivasiliou et al., 2016; Vasilopoulos & Painter, 2016). Finally, the timescale of protrusion dynamics relative to transcriptional feedback, signal degradation and the duration of cell cycle together determine the scale of the spatial patterns and a time window over which cell fate determination becomes locked.

An alternative approach has been to capture protrusion mediated Notch signaling as structural noise (Cohen, Baum, & Miodownik, 2011). This has been done in the context of a cellular automaton model whereby the probability of becoming a signaling cell depends on the number of active neighbors, where direct and more distant neighbors are considered. In this context, the number of neighbors required to inactivate a signaling cell reflects a threshold in the incoming signal required for inactivation. Depending on the signaling range and inhibitory thresholds a range of spatial patterns become possible like those found in models that describe protrusion length and dynamics explicitly. In this study, the effects of spatial and temporal noise were explored and it was shown that intermediate levels of noise lead to optimized patterns. This result highlights that signaling noise, which is a feature of signaling systems and here introduced through signaling protrusions, can aid patterning by enabling cells to reverse their signaling state during pattern refinement. Importantly, the levels of noise must be finely tuned to the spatiotemporal dynamics of patterning and unsuitably high levels of noise lead to disordered spatial patterns (Cohen et al., 2011).

In other instances, protrusions are transient and their appearance correlates with the levels of Delta in a cell so that only cells that express high enough levels of the Delta ligand extend protrusions (Hadjivasiliou et al., 2019; Page et al., 2019). Here, protrusions can be modeled as dynamic processes that extend and retract as a response to Notch signaling. Cells that extend the long protrusions are able to upregulate Notch signaling in cells within their reach and so inhibit them from accumulating higher levels of Delta. In this context, theoretical modeling has shown that the speed of protrusion extension and restriction impacts the spatiotemporal dynamics of cell fate determination. Slow or shorter protrusions result in denser patterns that develop in a short period of time and vice versa (Hadjivasiliou et al., 2019).

Incorporating protrusions in models of Notch signaling can help make predictions about how changes in protrusion length, dynamics and signal efficiency impacts spatiotemporal patterns at the level of the tissue. Future theoretical models can integrate diffusible transported factors that operate together with cellular protrusions, for example to examine the putative role of factors such as *Scabrous* in promoting Notch signaling at a distance.

These approaches offer valuable tools to aid the design of appropriate experimental perturbations to test the role of protrusions in Notch signaling and tissue patterning in vivo.

5. Case studies of long-range Notch signaling

5.1 Spatial and temporal control of branching angiogenesis

Angiogenesis is the process by which new blood vessels are formed. During this process, endothelial cells (ECs) in preexisting blood vessels undergo sprouting as a response to vascular endothelial growth factor (VEGF). The conversion of ECs into tip cells that propagate new branches occurs in a spatially heterogeneous pattern regulated via Notch mediated lateral inhibition (Herbert & Stainier, 2011; Potente, Gerhardt, & Carmeliet, 2011).

The process is initiated when levels of VEGF increase as a response to hypoxia and induce Delta expression in ECs. Activated VEGF receptors (VEGFR) also promote the formation of dynamic filopodia through rapid, local polymerization of actin (Rousseau, Houle, Landry, & Huot, 1997). As cells express increasing levels of Delta, they begin to activate Notch in neighboring cells and the process of lateral inhibition is underway. At the same time, filopodia continue to reach further into the VEGF gradient and VEGFR activation on the filopodia increases (Fig. 3A). Activated Notch inhibits the expression of VEFG receptors which in turn inhibits VEGF signaling and filopodia production. This generates sharp positive feedback without the need for multiple rounds of transcription (Bentley & Chakravartula, 2017). Importantly, filopodia are formed within seconds following VEGFR activation and so provide a fast mechanism for feedback amplification that ultimately speeds up cell fate determination. Eventually, cells with higher VEGF and Delta signaling will be selected as tip cells and become migratory (Fig. 3A).

The role of filopodia in this process is primarily to speed up the process of lateral inhibition and tip cell selection. Multiple rounds of transcriptional feedback are required by Notch-Delta lateral inhibition to amplify the initially small differences in neighboring ECs and select for the heterogenous tip cell pattern necessary for the branching network that forms blood vessels (Collier et al., 1996). Each round of transcriptional feedback is estimated to take 3–4 h in vitro in mouse ECs and 2 h in zebrafish (Leslie et al., 2007; Ubezio et al., 2016). This would predict a timescale for the selection of a

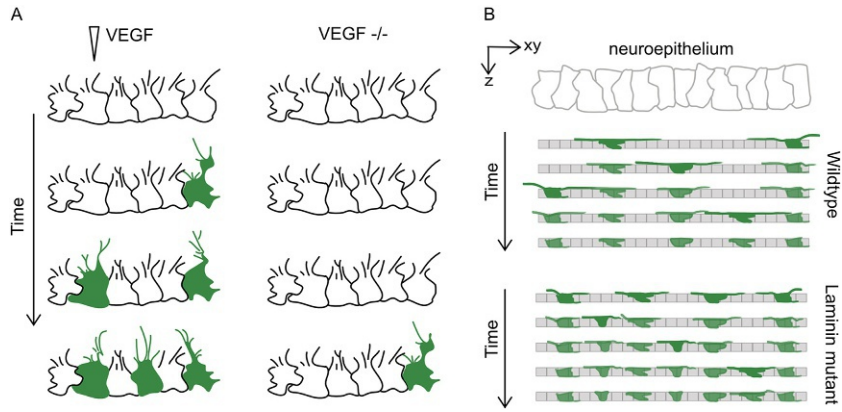


Fig. 3 Examples of Notch signaling across length and timescales. (A) Endothelial cells during angiogenesis. At early time points, all cells have filopodia activity. In the presence of a VEGF gradient (left) that promotes Notch signaling, tip cells (green, Notch inactive) are patterned in a timely manner, with correct spatial distribution. In the absence of a VEGF gradient (right) tip cell selection and lateral inhibition is delayed. (B) The spacing of neurons (green) selected from initially unpatterned neuroepithelial cells depends on Notch signaling occurring along transient protrusions extending in the xy-plane. Neurons express Delta, present in the extended protrusions, which activates Notch in neighboring cells (gray). In laminin mutants, protrusions are shorter, leading to an decrease in the spacing between neurons and overall increase in density of neurons.

single tip cell that is of the order of a several hours to a day, much slower than the selection window observed experimentally within which all new tip cells are selected (~8 h in zebrafish) (Zakirov et al., 2021). As such, Notch mediated lateral inhibition alone cannot account for the spatiotemporal patterns of tip cell selection during angiogenesis. The sharp feedback generated through filopodia extension in this system offers a cell-based mechanism that overcomes this constraint. By physically expanding the length scale over which they can sense their environment and employing local feedback mechanisms cells effectively change the time-scale of the patterning process. The temporal dynamics of tip cell selection in turn impacts the spatial patterning of the branching network. Fast feedback implies a shorter timescale for the signaling dynamics, more tip cells being selected per unit time, and ultimately a denser branching network (Bentley & Chakravartula, 2017). Slower signaling dynamics delays lateral inhibition and tip cell selection and results in more sparse branching (Fig. 3A). Theoretical modeling of the process has shown than the positive feedback generated by filopodia sensing the VEGF signaling generate an ultrastable bistable switch that underlies fast and robust tip cell selection. Experimental perturbation of

the VEGF levels in vivo and in vitro indicate that tip cell selection can be slowed down or sped up according to the levels of VEGF signaling. In agreement with theoretical predictions, higher levels of VEGF increased the number of ECs selected to sprout and, reversely, low level inhibition of VEGF signaling led to fewer tip cells being selected (Page et al., 2019).

This is an example whereby cell-based mechanisms, such as localized actin polymerization and filopodia, expand the range over which cells can access information, i.e., filopodia reaching further into the VEGF gradient. The fast, local feedback generated as a result impacts the dynamics of Notch signaling between neighboring cells allowing the timescale of fate determination to overcome the limitations set by the timescale of several rounds of gene transcription and activation required by the Notch pathway. Ultimately, the spatial and temporal scales achieved by these cellular processes impact the organization and length scale of patterning at the level of the tissue by specifying the duration of angiogenesis and density of the blood vessel branching network.

5.2 Spatiotemporal patterns of neurogenesis

During neurogenesis proliferative neuroepithelial cells gradually acquire proneural characteristics and generate specific neuron types. In the early stages of vertebrate neurogenesis, neurons of different subtype are born following a characteristic, non-random, spatiotemporal pattern in the spinal cord (Batista, Jacobstein, & Lewis, 2008; Dale, Roberts, Ottersen, & Storm-Mathisen, 1987; England, Batista, Mich, Chen, & Lewis, 2011; Higashijima, Mandel, & Fetcho, 2004; Roberts, Dale, Ottersen, & Storm-Mathisen, 1987). Quantitative analysis in vivo shows that neurons are rarely born close together in space and time. This pattern points to a local inhibitory mechanism that regulates the spatiotemporal appearance of new neurons. From a functional perspective, the sparse differentiation patterns observed experimentally may be important to allow for timely wiring of the central nervous system but this remains to be tested.

Live imaging in the zebrafish spinal cord has shown that neurons extend long protrusions along the apical-basal axis prior to differentiation (Fig. 3B) (Hadjivasiliou et al., 2019). The length of the protrusions resembles the spacing between neurons that are born at similar times. In addition, these cellular protrusions exhibit high levels of Delta, and Notch signaling is upregulated in their vicinity. Together these observations indicate that Notch mediated lateral inhibition may play a role in defining the spatiotemporal dynamics of

neurogenesis. In agreement with this hypothesis, experimental perturbation of the length and dynamics of cellular protrusions through manipulation of the extracellular matrix protein laminin result in shorter protrusions and neurons that are born closer together in space and time (Fig. 3B).

The dynamics and length of the protrusions feed back into the timing of neurogenesis and spatial density of neurons. For example, theoretical modeling shows that the maximal length of the protrusions specifies an upper limit for the average spacing between neurogenesis events that occur at a similar time (Hadjivasiliou et al., 2019). This is an idealized state that emerges when protrusions are effectively extended instantaneously and quickly inhibit Delta production in cells within their reach. This limit is attained if the speed at which protrusions are extended is fast relative to the timescale of neural fate acquisition at the absence of Notch signaling. Conversely, if protrusions extend slowly or are inefficient at signaling, the spacing between neurons that are born close in time to one another is smaller than the maximal protrusion length (Fig. 3B). It follows that the interplay between the timescale of Notch signaling and protrusion dynamics together specify the spatiotemporal density of neuron differentiation.

A question that emerges from this work is the role of Notch signaling via the transient protrusions in neuron generation in higher dimensions. For example, protrusions extend additional filopodia along the Dorsal/Ventral axis suggesting that differentiating neurons may be able to influence their environment beyond the A/P axis they lie in. In addition, it is not known whether signaling interactions occur between the protrusions in the rare cases when protrusions from different neurons come in contact with one another, and how these could affect protrusion dynamics or retraction. Finally, although the presence of protrusions correlates with high levels of Delta, how extension is initiated as a response to Delta levels in this system is not clear. Further experimental work investigating possible feedback between Delta expression and the extension and dynamics of protrusions, and interactions between protrusions will help pin down the feedback mechanism in place.

6. Evolvability of patterns

Mechanisms that affect the ability of patterns to form, scale to body size, or to complete during the appropriate time window are likely under selective pressure (Curantz & Manceau, 2021). Using the previous example of airinemes during stripe formation in Zebrafish, a closely related species of

fish (Pearl danio) does not feature stripes (Eom et al., 2015). Changes in the timing of xanthophore differentiation, due to the increased expression of a differentiation factor by neighboring interstripe cells, prohibits the formation of airinemes. Therefore, these cells cannot engage in long-range lateral inhibition, which is essential for stripe formation. These findings underline the importance of the interplay between gene regulation and the control of cell morphology for the evolution of patterning across species.

In another well-studied example, *Drosophila* species can exhibit a range of body sizes, and the organization of the thoracic sensory bristles varies with size (Simpson, Woehl, & Usui, 1999). There appears to be a mixture of scale invariant processes (e.g., the number and placement of large sensory bristles) in addition to the repeating processes that do not scale with tissue size (e.g., the number and organization of small sensory bristles). Scaling of the bristle patterns is important because the projection pattern of axons into the central nervous system is dependent on the location of the sensory bristle (Ghysen, 1980), and the behavioral response of the fly depends on the location of the stimulated bristles. Recent investigations of a mutant that alters both the spacing (Renaud & Simpson, 2001) and the timing of G2-exit in bristle precursors (Lacoste et al., 2022) indicates that mutations which disrupt the organization and timing of bristles and their neural projections lead to changes in cleaning reflexes. Despite the importance of the bristle organization for fly behavior, the regular array of sensory bristles on the dorsal thorax of Dipteran flies can be organized in a variety of ways, from randomly arranged small bristles to regularly spaced rows (Simpson et al., 1999). The genetic pathways and cell behaviors that control the placement of large and small sensory bristles was determined using Melanogaster, but in principle may extend to other Dipterans.

Both large and small sensory bristle patterns require Notch signaling, although in different contexts. The placement of the scale invariant large sensory bristles occurs during larval wing disc development and requires positional information generated by morphogen gradients (Yang, Hatton-Ellis, & Simpson, 2012). Notch signaling occurs after the placement of pro-neural clusters is determined, in order to generate the bristle cell lineages (Heitzler & Simpson, 1991). This is in contrast to the patterning of small sensory bristles, discussed previously, which is a more stochastic process that primarily relies on Notch signaling. Within the Drosophilidae family, larger flies tend to exhibit increased numbers of small bristles compared to the smaller Melanogaster, although the spacing between them does not always scale with size. There appears to be a conserved lateral inhibition process that

drives a spaced pattern such that bristles do not occur adjacent to one another. However, these observations suggest that the cell based mechanisms for determining the length scale of Notch signaling may vary or in some cases may be absent. It is unknown if mechanisms that modify the timing of Notch signaling, as outlined above, contribute to the variety of bristle patterns observed.

The capacity of lateral inhibition for diversification, and the role of cell-based processes such as protrusions in evolution can be explored theoretically. For example, this can be addressed by exploring how mutations that impact Notch signaling and protrusion function affect patterning at the tissue level. It would be interesting to explore this scenario in tissues of varying size under selection to maintain or alter patterning proportions relative to size. Theoretical work can also address what is the capacity for evolvability and robustness in patterning systems that utilize local cell-cell interactions versus dispersed morphogens. This can help explain what the evolutionary benefits and constraints of alternative patterning mechanisms are.

7. Conclusion

Notch signaling is a highly conserved signaling pathway that regulates binary cell fate decisions in a variety of contexts—from controlling neural stem cell populations in the developing brain, differentiation of enterocytes in the intestinal epithelium, to the formation of spot and stripe patterns in Drosophila and Zebrafish. In many cases Notch signaling achieves this through lateral inhibition, but the range of patterns generated by this process should be limited to alternating cell types. Indeed, this is what we observe for initial mathematical models of lateral inhibition (Collier et al., 1996). However, the repertoire of Notch-mediated patterns includes those that are more sparse than would otherwise be predicted. As we develop better tools to observe the dynamics of Notch signaling over time, we also see that lateral inhibition occurs more rapidly than would be predicted by the feedback loops that dominate the timescale of the Notch pathway. Therefore, there must be mechanisms for modulating the length- and timescale of Notch signaling. In this review we have discussed how cellular mechanisms, from protrusions to ligand-receptor interactions, cell division and cellular rearrangements, as well as the coupling between secreted regulators and Notch signaling allow cells and tissues to overcome these spatial and temporal constraints.

In light of mounting experimental evidence that cellular protrusions contribute to Notch signaling across model systems, we have focused on the role of these processes in determining the spatiotemporal dynamics of patterning at the tissue level. Because protrusions allow cells to activate Notch signaling at a distance, the spacing between Delta expressing cells in the emerging patterns increases. At the same time, protrusions can be polarized allowing not only spots but also stripes and labyrinth like patterns to emerge through Notch signaling. The dynamic nature of protrusions that can grow and shrink, possibly as a response to Notch signaling, also introduces a temporal aspect to their function. During many differentiation processes, changes in cell morphology follow as a consequence of adopting a new cell state. Further work is needed to establish how cellular protrusions and Notch signaling dynamically modulate each other. A combination of theoretical and experimental approaches can offer the quantitative tools needed to address this challenge.

Modulating the length of protrusions may also allow patterns to become sparser in larger tissues, but as organ size undergoes changes of several orders of magnitude, cell based adjustments are unable to adapt to the tissue size. This suggests that the ways in which patterns transform between species of different size may vary depending on the mechanism that underlies pattern formation. Theoretical work together with comparative approaches can explore the scaling potential of patterns that depend on extracellular transport as well as of cell-based mechanisms across evolution.

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