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REVIEW

Communication codes in developmental signaling pathways

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ABSTRACT

A handful of core intercellular signaling pathways play pivotal roles in a broad variety of developmental processes. It has remained puzzling how so few pathways can provide the precision and specificity of cell-cell communication required for multicellular development. Solving this requires us to quantitatively understand how developmentally relevant signaling information is actively sensed, transformed and spatially distributed by signaling pathways. Recently, single cell analysis and cell-based reconstitution, among other approaches, have begun to reveal the 'communication codes' through which information is represented in the identities, concentrations, combinations and dynamics of extracellular ligands. They have also revealed how signaling pathways decipher these features and control the spatial distribution of signaling in multicellular contexts. Here, we review recent work reporting the discovery and analysis of communication codes and discuss their implications for diverse developmental processes.

KEY WORDS: Communication codes, Signal processing, Pathway architecture

Introduction

Embryonic development depends on accurate, timely and specific communication between cells. Our understanding of cell-cell communication has evolved over many decades. In the early 20th century, classic tissue-grafting experiments revealed crucial roles for cell-cell communication in cell fate decision-making and other processes (Hörstadius, 1973; Spemann and Mangold, 1924). However, the identities of the communication signals remained elusive for more than half a century owing to the lack of molecular tools. Over several decades, however, researchers used molecular genetic approaches to uncover a set of highly conserved core pathways, including the Notch, Transforming Growth Factor beta (TGF-β), Wnt, Hedgehog (HH) and Receptor Tyrosine Kinase (RTK) signaling systems, which play pivotal roles across an astonishingly broad range of developmental processes (Fig. 1A). Genetic screens and biochemical studies helped to identify the molecular components and interactions that make up these pathways and elucidated their roles in numerous developmental contexts (Gerhart, 1999), providing a foundational framework for understanding developmental signaling.

A growing body of work, especially in single cells, increasingly suggests that these pathways signal through a set of 'communication codes'. More specifically, pathways sense different features of their ligand inputs, including molecular identity, concentration, combination with other ligands, and dynamics (Fig. 1B). Pathways actively sense and transform these features into the concentrations, states and dynamics of intracellular effectors, which, in turn, ultimately control target genes or proteins. Each processing step can depend on cell type or context. Furthermore, at the tissue level, pathways not only sense extracellular ligands but dynamically sculpt their spatial distribution to enable the generation of precise developmental patterns (Fig. 1C). From this point of view, each pathway can be thought of as a device that actively processes signals while changing their representation through a multi-tiered and spatially extended processing scheme.

Communication codes depend on pathway architecture. Pathways differ in the number and type of molecular interactions from receptor to target gene, the integration of parallel ligand and receptor variants, and feedback loops (Fig. 1A). Here, we review recent progress towards understanding how different pathway architectures implement a variety of communication codes, and discuss their functional implications from single cells to developing tissues. We organized the Review around four distinct input features: ligand identity, concentration, combinations and dynamics, exploring examples from selected pathways for each feature. We then discuss new approaches that are enabling quantitative and even single cell analysis of communication codes in spatio-temporally complex systems. Finally, we identify challenges and opportunities for future work.

Because of the stochastic and unsynchronized nature of signaling responses in different cells, single cell methods are essential for analyzing cell-cell communication. Therefore, throughout the Review, we highlight the roles of single cell tools and approaches, including fluorescent reporters, quantitative time-lapse imaging, microfluidics, synthetic biology, genome engineering and single cell gene expression profiling. We restrict our focus to the small set of core signaling pathways listed in Fig. 1A that play especially prevalent roles in embryonic development across the entire animal kingdom and exemplify signal encoding paradigms likely to generalize to other pathways. This Review therefore omits equally relevant work across many other pathways and contexts, such as NF-κB in immune signaling and cancer, which have been reviewed elsewhere (Colombo et al., 2018; Purvis and Lahav, 2013).

Discriminating ligand identity

During evolution, gene duplication and divergence produced multiple ligand variants that interact with multiple receptor variants in a many-to-many (promiscuous) fashion in many signaling pathways (Fig. 2A). For example, although each of the seven major mammalian fibroblast growth factor receptor (FGFR) isoforms preferentially binds to a subset of the 22 fibroblast growth factor (FGF) ligands, the binding affinities for different ligands tend to show a broad overlapping distribution (Ornitz et al., 1996; Zhang et al., 2006). In contrast to this extracellular complexity, diverse ligand-receptor complexes appear to converge intracellularly on a smaller set of overlapping effectors. Given this convergent architecture, it is puzzling to understand whether and

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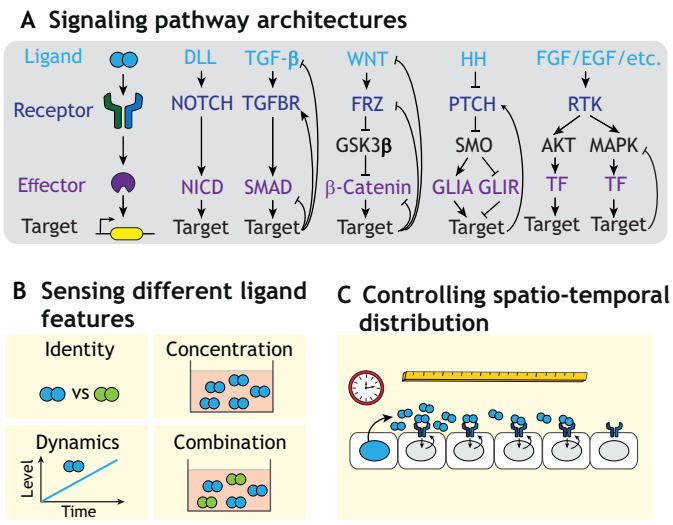

Fig. 1. Developmental signaling pathway architectures sense, process and control ligands in space and time. (A) Major developmental signaling pathways use diverse architectures to control cell-cell communication. In these pathways, ligand-receptor interactions activate intracellular effectors, which then regulate target gene expression. Intracellular signaling activity also induces a myriad of feedback loops to further modulate the signal processing capability of the pathway. TF, transcription factor. (B) At the single cell level, pathways can sense the molecular identity and concentration of individual ligands, the relative concentrations of multiple ligands (combinations) or the temporal dynamics of ligand concentrations. (C) At the tissue level, signaling pathways can actively modulate the distribution of extracellular ligands and intracellular signal perception in space and time. This spatio-temporal control occurs through direct ligand-receptor interactions, secreted inhibitors or modulators, and feedback loops (arrows inside each cell). The ruler and clock represent the spatial and temporal scales of signaling activity in morphogen gradients. Understanding the relationships between pathway architecture (A) and signal processing (B,C) is a fundamental challenge.

how cells activate different target programs in response to different ligands through a common set of intracellular effectors (Madhani and Fink, 1997).

Discriminating Notch ligands

The Notch pathway enables direct communication between neighboring cells and controls numerous cell fate decisions (Bray, 2016; Henrique and Schweisguth, 2019). Within the mammalian pathway, four Notch receptors interact in a promiscuous fashion with a set of Notch ligands, including delta like 1 (Dll1) and delta like 4 (Dll4). Signaling occurs when a ligand on one cell binds to a Notch receptor in a neighboring cell, inducing proteolytic release of the Notch intracellular domain (NICD) of the receptor. NICD translocates to the nucleus to activate target genes, such as Hes basic helix-loop-helix (bHLH) family repressors, which, in turn, control cell fate decisions (Kageyama et al., 2007) (Fig. 2B). This mechanism, along with the apparent equivalence among the different Notch receptor intracellular domains (Liu et al., 2015), provides no obvious way for a signal-receiving cell to qualitatively determine which ligand is responsible for its activation. Nevertheless, different ligands can trigger distinct Notch activity dynamics even through the same receptor. Using quantitative time-lapse microscopy to follow Notch signaling in cultured CHO cells, Nandagopal et al. discovered that Dll1 and Dll4 activate the Notch1 receptor with pulsatile or sustained dynamics, respectively (Nandagopal et al., 2018) (Fig. 2B). They further showed that direct modulation of NICD activation dynamics in cultured C2C12 cells differentially activates the Notch target genes *Hes1* and *Hey1/HeyL*; whereas pulsatile Notch activation is sufficient to induce *Hes1*, sustained Notch activity is required for *Hey1*/*HeyL* upregulation. Finally, Dll1 and Dll4 had opposite effects on cell fate, promoting or inhibiting myogenesis, respectively, when expressed in neural crest cells within developing chick embryos. These results showed that even the relatively 'direct' Notch pathway is capable of discriminating among similar ligands, processing ligand identity into effector dynamics, and then deciphering those dynamics into distinct target programs. In fact, using dynamics to discriminate among ligands or inputs appears to be a growing theme in many systems (Levine et al, 2013; Purvis and Lahav, 2013).

How is ligand identity processed into effector dynamics? Notch ligands and receptors are known to cluster at cell interfaces. Quantitative imaging of these clustered signaling complexes in cultured cells (Nandagopal et al., 2018) is consistent with a working hypothesis in which Dll1 preferentially and coordinately activates Notch receptors as clusters, thereby releasing a 'pulse' of many NICDs in a single event, whereas Dll4 activates Notch within smaller clusters or individual ligand-receptor complexes, resulting in a steady 'trickle' of NICDs to the nucleus (Nandagopal et al., 2018). Further studies should help to understand the ways in which clustering contributes to dynamic ligand discrimination.

The mechanism through which NICD dynamics are subsequently used to differentially activate *Hes1* and *Hey1*/*HeyL* remains unclear. However, several features of the Hes/Hey regulatory circuit could play a key role. *Hes1* and *Hey1*/*HeyL* are known to reciprocally inhibit each other's expression (Heisig et al., 2012; Noguchi et al., 2019), whereas Hes1 is unstable at the protein and mRNA level and negatively autoregulates its own expression. *Hes1* also has a unique Notch enhancer architecture, which enables it to respond faster than *Hey1*/*HeyL* to lower levels of NICD (Arnett et al., 2010; Nam et al., 2007; Ong et al., 2006). Mathematical modeling has suggested that these features together allow *Hes1* to respond rapidly to a sudden increase in NICD, whereas extended durations (~1-2 h) of sustained NICD activity are required to upregulate *Hey1*/*HeyL* (Nandagopal et al., 2018). Further studies will be necessary to disentangle the roles of these and other mechanisms in discriminating Notch ligand identities.

Discriminating RTK ligands

Dynamics also allow cells to discriminate among RTK ligands. Recent studies have revealed that different ligands can induce different epidermal growth factor receptor (EGFR) signaling dynamics by modulating the structural features of the receptor dimer (Fig. 2C). For example, epiregulin (EREG) and epigen (EPGN) promote differentiation of MCF-7 cells, whereas epidermal growth factor (EGF) promotes proliferation. These different outcomes correlate with sustained or transient phosphorylation of EGFR and its downstream effector ERK induced by EREG/EPGN or EGF, respectively (Freed et al., 2017). However, EREG/EPGN have a lower affinity for EGFR and induce shorter-lived receptor dimers than EGF. A possible explanation for this counter-intuitive result is that short-lived receptor dimers might fail to engage key negative feedback mechanisms and thus prevent the termination of the signal (Freed et al., 2017). Thus, structural alterations of receptors induced by different ligands could be crucial for determining signaling dynamics and discriminating ligands.

FGF and nerve growth factor (NGF) receptors also process ligand identity into effector dynamics, activating distinct target programs. For example, treating PC12 pheochromocytoma cells with EGF causes proliferation, whereas FGF or NGF treatment induces neural differentiation (Greene and Tischler, 1976;

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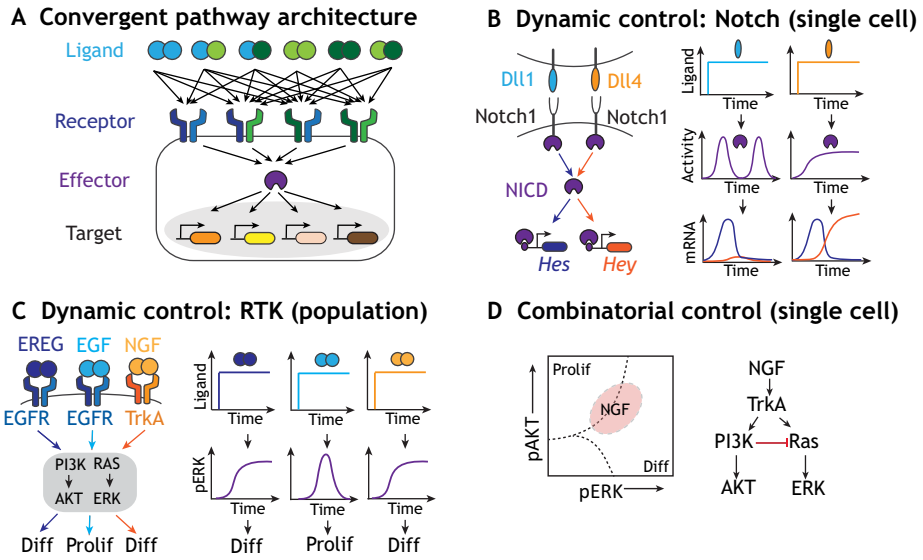

Fig. 2. Discriminating ligand identity. (A) Many signaling pathways share a convergent structure, with ligands interacting with receptors in a many-to-many fashion and information 'funneling' down to control a smaller number of intracellular effectors. However, the shared effector can discriminate ligand-receptor identity to induce differential gene expression programs. (B) The Notch pathway ligands Dll1 and Dll4 both bind to the Notch1 receptor but activate the downstream effector NICD with different dynamics. Dll1 induces pulsatile responses, which preferentially activate the transcriptional target *Hes1*, whereas Dll4 induces sustained responses, which are required for activating *Hey1*. (C) Different RTK pathways share a common set of intracellular signal transduction components, including PI3K/AKT and RAS/ERK, but induce different cellular responses. For example, both EREG and EGF share the same receptor EGFR, but EREG promotes differentiation (Diff) whereas EGF promotes proliferation (Prolif) in MCF-7 cells. Based on population-level analysis, EREG elicits sustained phosphorylation of ERK (pERK) whereas EGF induces transient pERK (Freed et al., 2017). Similarly, EGF/NGF treatment of PC12 cells induces transient/sustained pERK that correlates with proliferation/differentiation (Marshall, 1995). (D) Single cell analysis has revealed a heterogeneous response of PC12 cells to NGF treatment. At the single cell level, these cell fate decisions depend on both pERK and phosphorylated AKT (pAKT), with a curved boundary separating proliferation and differentiation (left). Feedback loops within the NGF pathway (right) maintain the distribution of pAKT and pERK activities within the cell population close to the boundary. Adapted from Chen et al. with permission (Chen et al., 2012).

Huff et al., 1981), although the two pathways signal through overlapping downstream effectors, such as AKT and ERK (Fig. 2C). Pioneering studies using synchronized populations of cells revealed that EGF induces transient ERK phosphorylation (pERK), whereas NGF induces sustained pERK (Fig. 2C; Traverse et al., 1992), leading to the hypothesis that ERK dynamics could represent the activating ligand and receptor identity. Numerous subsequent studies in the same experimental system have revealed how pERK duration encoding and decoding arise both from the activity of the ligand-receptor complexes and downstream signal processing circuits (Kao et al., 2001; Marshall, 1995; Murphy et al., 2002, 2004; Santos et al., 2007; Sasagawa et al., 2005; Traverse et al., 1994; Uhlitz et al., 2017; Whitmarsh, 2007). Nevertheless, it has remained unclear to what extent transient and sustained ERK activity can explain the distinct cellular phenotypes, especially at the single cell level.

More recent work has begun to analyze RTK signaling dynamics in individual cells. For example, using a fluorescence resonance energy transfer-based biosensor (FRET) to track ERK activity dynamics in individual cells in real time, Ryu et al. found that application of either EGF or NGF leads to a mixed population of PC12 cells with transient or sustained pERK activity, respectively (Ryu et al., 2016). Similar heterogeneity was also observed at the level of pERK and cell fate decision-making (proliferation versus differentiation) by assaying cell morphology and proliferation markers in fixed cells (Chen et al., 2012). Chen et al. further showed that pERK levels alone do not predict cell fate outcomes. Rather, cell fate depends on a combination of AKT and ERK activities. In fact, the decision between differentiation and proliferation can be discriminated by a curved boundary in this two-pathway signaling space (Fig. 2D). Interestingly, a feedback loop mediated by downregulation of Ras/ERK signaling in response to PI3K/AKT signaling appears to shift the signaling activities closer to this fate boundary, allowing a fraction of cells to remain in a proliferative state across a range of inputs. Functionally, this active signal processing system could thereby balance the number of cells in proliferating or differentiating states and thus the overall structure of the cell population. As EGF and NGF elicit different cellular phenotypes, it would be interesting to investigate whether different RTKs engage similar or distinct downstream feedback loops, or whether the different dynamics result from different structural features of various ligand-receptor complexes. Combinatorial control of a cell response by AKT and ERK has also been observed in individual human mammary gland epithelial cells stimulated with various growth factors (Sampattavanich et al., 2018). The RTK signaling system thus provides an important and intriguing example of how signaling pathways not only transmit inputs but actively process them to control population structure, and how quantitative, multi-dimensional, single cell measurements can provide insights into the relationship between signal processing and cell fate decision-making.

Modulating or programming ligand specificity

In the examples discussed above, multiple ligands activate different responses through the same pathway. But in other contexts, cells may selectively respond to some ligands but not others. When different ligands use distinct receptors, this could be achieved simply through differential receptor expression. However, cells appear to selectively sense specific ligands, even when multiple ligands can signal through the same receptors. One way this is achieved is through pathway modulators, such as co-receptors and enzymes that inhibit or facilitate binding between specific ligands

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and receptors, and thus alter communication specificity. For example, Fringe glycosyltransferases modify Notch receptors, altering their preference for Delta or Jagged ligands (LeBon et al., 2014; Moloney et al., 2000; Panin et al., 1997). Similarly, in the Wnt pathway, the cell-surface co-receptor Reck can specifically recognize Wnt7, but not other Wnts, and recruits Wnt7 into dynamic Wnt/Frizzled/Lrp5/6 signalosomes (Eubelen et al., 2018). This might explain how Wnt7 exclusively controls mammalian forebrain and ventral spinal cord angiogenesis within the neurovascular system.

Synthetic biology enables a complementary approach to understanding communication specificity by engineering synthetic 'orthogonal' (independent) communication channels (Elowitz and Lim, 2010). For example, the extracellular ligand-binding domains and the intracellular effector domains of Notch can both be replaced with engineered alternative domains to create synthetic receptors that sense arbitrary inputs and activate arbitrary target genes (Morsut et al., 2016). These and related synthetic communication systems have been used for controlling immune cell responses (Roybal et al., 2016), engineering synthetic multicellular patterns (Toda et al., 2018) and mapping direct cell-cell contact and neural connections (He et al., 2017; Huang et al., 2016, 2017). By demonstrating the possibility of orthogonal multiplexing, they provoke the question, which we address in the section 'Sensing ligand combinations': why do so many natural pathways appear to use many-to-many ligand-receptor specificities?

Sensing ligand concentration

For most developmental pathways, different ligand concentrations can trigger distinct cellular responses. For example, along a morphogen gradient, adjacent cells commit to distinct fates based on small differences in local ligand concentration. In the simplest scenario, higher concentrations of ligand generate correspondingly higher activities of intracellular effectors (Fig. 3A). However, recent work has begun to reveal more-complex signal processing schemes.

Fold change detection of ligand concentration

In some systems, ligand concentration controls the fold change (post-stimulus divided by pre-stimulus activity) of an intracellular effector, rather than its absolute level, and this fold change in turn determines cellular responses (Adler and Alon, 2018). Early evidence for fold change detection (FCD) in developmental signaling came from the observation in *Xenopus* that developmental outcomes depend on fold changes, rather than absolute levels, of β-catenin, the downstream effector of Wnt (Goentoro and Kirschner, 2009).

Analyzing FCD requires tracking signaling activity both before and after stimulation in the same cell. To achieve this, Frick et al. used quantitative single cell time-lapse imaging to demonstrate that FCD is used by the TGF-β pathway (Frick et al., 2017). The authors stimulated C2C12 cells with different concentrations of TGF-β ligand, quantified nuclear localization of the TGF-β effector Smad3 before and after the stimulation using time-lapse imaging, and analyzed the expression of downstream targets in the same cells by single molecule fluorescence *in situ* hybridization (FISH). Combining these single cell approaches, they discovered that different TGF-β concentrations induce different fold changes in nuclear Smad3 levels. They further observed that the expression of downstream transcriptional targets correlates more strongly with the fold change than the absolute level of nuclear Smad3 (Fig. 3B). Similar examples of FCD have also been suggested or reported in pathways employing ERK or NF-κB as intracellular effectors (Cohen-Saidon et al., 2009; Lee et al., 2014). Furthermore,

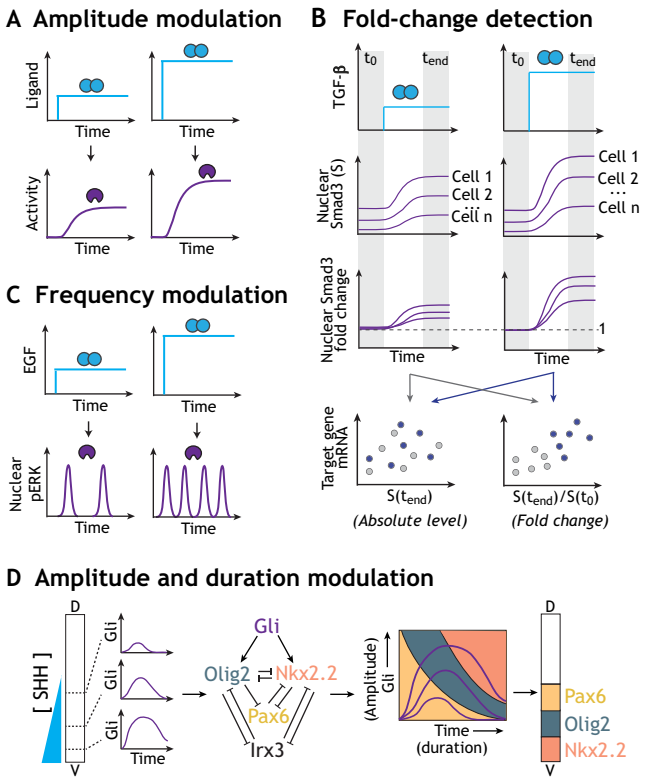

Fig. 3. Different strategies for sensing ligand concentration. (A) In the simplest 'amplitude modulation' systems, ligand concentrations are encoded in the concentrations of intracellular effectors. (B) The TGF-β pathway can encode ligand concentration in the fold change, rather than the absolute level, of its effector nuclear Smad3 (S). At the single cell level, cells display highly heterogeneous levels of nuclear Smad3 both before and after TGF-β treatment (top panels), and the distribution of absolute Smad3 level overlaps significantly between different concentration groups (second row). However, fold changes of nuclear Smad3 (post-stimulus divided by its pre-stimulus level in the same cell) show less heterogeneity and better separated distributions between different concentration groups (third row). Furthermore, target gene expression correlates better with the fold change, $[S(t_{end})/S(t_0)]$ than with the absolute level, $S(t_{end})$, of nuclear Smad3 (bottom row). Dots represent the number of the same transcripts in individual cells treated with either low (gray) or high (blue) concentrations of TGF-β. (C) EGF induces coherent oscillations of pERK shuttling between the nucleus and cytoplasm in certain cellular contexts, and the concentration of EGF regulates the frequency of these oscillations. (D) In developing mouse and chick embryos, SHH secreted from the ventral side of the neural tube forms a concentration gradient. Cells at different positions encode different SHH concentrations into Gli activity profiles with distinct amplitude and duration. Gli activity is then decoded by the downstream fate-decision circuits, composed of multiple transcription factors. A two-dimensional map based on both the amplitude and duration of Gli activity determines the cell fate output, and thus the spatial domains of distinct progenitor cell types. D, dorsal; V, ventral. Adapted from Briscoe & Small with permission (Briscoe and Small, 2015).

theoretical analysis and experimental perturbation have identified specific architectures of downstream circuits that could perform FCD, such as incoherent type-1 feedforward loops (Goentoro et al., 2009), non-linear integral feedback loops (Shoval et al., 2010) and logarithmic sensors (Olsman and Goentoro, 2016), as reviewed elsewhere (Adler et al., 2017). Together, these results support FCD as a prevalent mode of sensing and decoding ligand concentration.

What advantage does FCD provide compared with more directly sensing the absolute level of effector activity? One benefit is to reduce cell-to-cell variability in responses to homogenous ligand stimulation. Cells are inherently heterogeneous, or 'noisy', in the

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expression level of pathway components (e.g. receptors and effectors). Such heterogeneity could lead to high variability in the absolute level of effector activity both in the basal and ligand-stimulated states. However, by sensing the ratio of post- to pre-stimulation activity, many sources of variability effectively 'cancel out', providing a more accurate readout of input (Frick et al., 2017). FCD, therefore, can effectively reduce cell-to-cell variability and compensate for biological noise.

Frequency modulation by ligand concentration

Ligand concentration can be represented by the frequency, duration or amplitude of effector oscillations (Hao et al., 2013). For example, in human mammary epithelial cells (HMECs) and MCF-10A cells, EGF induces oscillatory shuttling of phosphorylated ERK between the nucleus and cytoplasm, with EGF concentration controlling the oscillation frequency (Albeck et al., 2013; Shankaran et al., 2009) (Fig. 3C). Similar ERK oscillations have also been reported in *Caenorhabditis elegans* (de la Cova et al., 2017). To test whether ERK dynamics generate differential cellular responses, Toettcher and colleagues used optogenetic approaches to directly control ERK activity in individual cells (Toettcher et al., 2013). They took advantage of the light-gated protein-protein interaction between Phytochrome B (Phy) and PIF (Shimizu-Sato et al., 2002). By anchoring Phy to the cell membrane, where Ras protein resides, and fusing the activator of Ras (SOScat) to the otherwise cytoplasmically localized PIF, they were able to use light to switch the SOScat-Ras interaction on and off within seconds, activating Ras, which in turn phosphorylates ERK. In this way, they showed that different ERK oscillation dynamics are sufficient to induce distinct gene expression programs (Wilson et al., 2017). Furthermore, they identified a network of immediate early genes that directly decipher ERK dynamics in NIH3T3 cells. As ERK oscillations are not synchronized between cells, these discoveries depended on quantitative single cell time-lapse imaging and the development of fluorescent reporters, such as an ERK-GFP fusion protein, a FRET-based ERK activity reporter and a phosphorylation-dependent kinase translocation reporter (Regot et al., 2014).

Frequency modulation has now been observed in diverse signaling systems, including p53 in cancer cells (Lahav et al., 2004; Purvis and Lahav, 2013), NF-κB in the immune system (Hoffmann, 2002; Nelson et al., 2004), calcium-NFAT signaling (Yissachar et al., 2013), Notch/Hes signaling in neurogenesis and stem cell maintenance (Hirata et al., 2002; Imayoshi et al., 2013; Manning et al., 2018 preprint; Shimojo et al., 2008) as well as in several yeast pathways (Cai et al., 2008; Hao and O'Shea, 2012; Lin et al., 2015). In yeast, a systematic movie-based screen found pulsing in ∼10% of transcription factors (Dalal et al., 2014), suggesting that pulsatile or oscillatory dynamics are likely to be prevalent.

Despite much work in individual systems, key questions about pulsing and oscillation remain unclear. For example, although recent theoretical work has identified candidate regulatory circuits that could decode dynamics (Gao et al., 2018) and examined mechanisms of dynamic decoding by target promoters in yeast (Hansen and O'Shea, 2013), decoding remains incompletely understood in most contexts. A second issue is context-dependence. ERK exhibits frequency-modulated oscillations in epithelial cells, but duration-modulated adaptive pulses in PC12 cells (Albeck et al., 2013; Marshall, 1995; Shankaran et al., 2009). Similarly, in the early *Drosophila* embryo, cellular responses depend on the integrated activity of ERK over time (Johnson and Toettcher, 2019), rather than on other features of the dynamics. Understanding how cell context affects decoding is a key challenge. Finally, why are oscillations often more sporadic than periodic (Levine et al., 2013)? One possibility is that the system functions mainly to control, e.g. by pulse frequency, the overall fraction of time that a given regulator is active. Further study is needed to reveal the principles of dynamic signal processing, tradeoffs between alternative processing schemes, and ways to rationally intervene and modulate signaling dynamics.

Amplitude and duration modulation in morphogen gradients

Ligand concentration can also be represented by the amplitude and duration of adaptive pulses of effector activity. A classic example occurs in neural tube development, during which multiple morphogens together specify complex tissue patterns. One of these, Sonic hedgehog (SHH), forms a concentration gradient on the ventral side of the neural tube, specifying several neural progenitor fate domains (Briscoe and Small, 2015). Here, SHH concentration controls the amplitude and duration of an adaptive pulse of intracellular SHH signaling activity (Cohen et al., 2015; Dessaud et al., 2007). Quantitative analysis of SHH signaling dynamics, as reported by the activity of the downstream effector Gli and the expression of cell fate markers (Pax6, Olig2 and Nkx2.2), has suggested that both the amplitude and duration of Gli activity collectively determine neural progenitor cell fates in chick and mice (Dessaud et al., 2007) (Fig. 3D). These features of effector activity are deciphered through a downstream gene regulatory network composed of the same transcription factors that mark progenitor cell fates (Balaskas et al., 2012). Recent work in zebrafish suggests that anterior and posterior regions of the developing zebrafish neural tube might be sensitive to distinct, but overlapping, features of the dynamic SHH activity signal (Xiong et al., 2018 preprint). These studies provoke the question of how many modes of signal processing exist, how they vary between tissue and species contexts, and how they are impacted by factors such as tissue geometry and developmental speed.

Sensing ligand combinations

Inside an embryo, cells are more often exposed to "cocktails" of multiple ligands rather than to a single ligand species. Recent studies suggest that cells can extract information encoded in multi-ligand combinations, both within a single pathway and among multiple pathways.

Ligand-receptor promiscuity enables combinatorial sensing

Within the Bone Morphogenetic Protein (BMP) pathway, multiple ligands and receptors interact in a promiscuous fashion (Fig. 2A). In most developmental processes, multiple BMP ligands are present in overlapping regions of the tissue and individual cells typically express multiple receptor variants (Danesh et al., 2009; Salazar et al., 2016). These observations provoke the general question of what functional capability these apparent redundancies might provide. Recent studies have therefore used mathematical modeling and experimental approaches to analyze the ways in which the BMP and larger TGF-β pathways process multi-ligand signals (Vilar et al., 2006; Antebi et al., 2017). By quantitatively studying the response of the BMP pathway to simultaneous presentation of multiple ligands, Antebi et al. showed that cells can respond to the relative (rather than absolute) concentrations of two ligands in complex ways (Antebi et al., 2017). For example, the pathway may respond to the ratio of two ligand concentrations, similar to other ratiometric sensing systems (Vilar et al., 2006; Escalante-Chong et al., 2015), or become activated when those

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ligands are either close to (‘balance detection’) or far from (‘imbalance detection’) a specific concentration ratio (Fig. 4A). Molecularly, these computations can emerge from competitive receptor-ligand interactions that form distinct protein signaling complexes, which then phosphorylate downstream effectors at different rates. In the simplest case, ratiometric sensing can emerge when two ligands compete to form active or inactive complexes with the same receptors. Furthermore, changing the profile of expressed receptors can alter the computation that the pathway performs on a given set of ligands (Fig. 4A). Consequently, altering the concentration of a single ligand could have opposite effects on cells exhibiting different receptor expression profiles. This result suggests a combinatorial solution to the problem of communication specificity in promiscuous systems: signaling occurs in a high-dimensional, combinatorial encoding space, with cells effectively ‘tuning in’ through their receptor expression profile to sense information encoded in ligand combinations. A recent theoretical study suggests additional functions for promiscuous ligand-receptor sensing, including improved sensing accuracy (Carballo-Pacheco et al., 2019). Given the prevalence of such promiscuous ligand-receptor interactions, it is tempting to speculate that these promiscuity-based combinatorial computations could be used more broadly to enable different cell types to respond in different ligand environments.

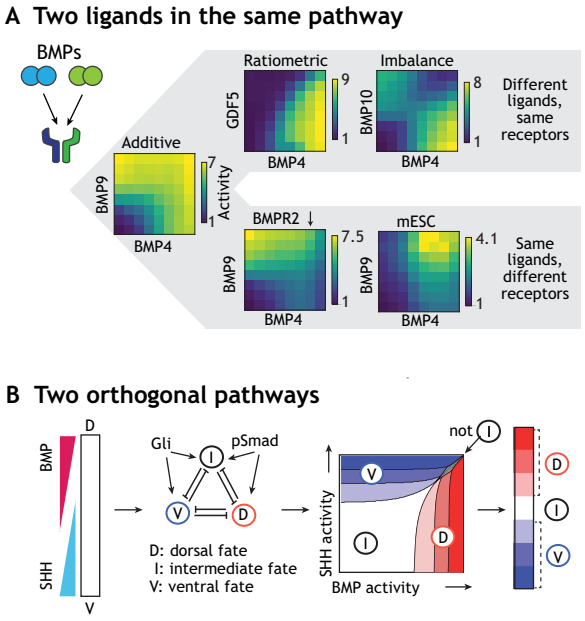

Fig. 4. Sensing combinations of multiple ligands in the same or orthogonal pathways. (A) Multiple BMP ligands can often bind to the same receptors. Pathway activity in this context can be a complex function of ligand combinations. Cells expressing the same receptors can compute different functions of distinct ligand combinations, including additive, ratiometric or imbalanced relationships between the two ligands (top row). Cells can also change their receptor expression profiles to compute different functions of the same ligand combinations (bottom row). For example, BMP4 and BMP9 exhibit an additive relationship in NMuMG cells, whereas knocking down BMPR2 in NMuMG cells or altering the BMPR expression profile entirely in a different cell type, such as mouse embryonic stem cells (mESC), completely changes the ligand relationship. (B) SHH and BMP set up anti-parallel gradients in the developing neural tube to specify several dorsal (D), intermediate (I) and ventral (V) neural progenitor fate domains. The ligand concentrations of the two orthogonal pathways control the activities of their canonical intracellular effectors, Gli and pSmad, which are subsequently decoded by a gene regulatory network, represented here in its abstract form. Different combinations of BMP and SHH concentrations lead to distinct cell fates: SHH-low/BMP-low produces intermediate fates, whereas SHH-high/BMP-high produces either dorsal or ventral fates in a stochastic manner but not intermediate fates, which suggests cells do not measure the relative level of the two ligands. Different dorsal progenitor fates are indicated by different shades of red, and different ventral progenitor fates are indicated by different shades of blue. Adapted from Zagorski et al. with permission (Zagorski et al., 2017).

Integrating information from orthogonal pathways

A recurring theme in development is the use of multiple signaling pathways simultaneously or sequentially in a given developmental process. However, the underlying logic of how information is integrated from orthogonal pathways often remains obscure. In the developing neural tube, BMP and SHH form antiparallel dorsal-ventral gradients that together pattern at least ten distinct cell fates along the dorsal-ventral axis. To understand how information from the two pathways is combined to control cell fates, Zagorski et al. used phosphorylated Smad (pSmad) staining and an SHH signaling reporter to quantitatively measure both pathway activities along the dorsal-ventral axis, and correlate these activities with the resulting positional identities (Zagorski et al., 2017). As expected, BMP-high/SHH-low elicited dorsal fates, BMP-low/SHH-high elicited ventral fates, and BMP-low/SHH-low specified fates in the intermediate zone (Fig. 4B). Interestingly, however, simultaneously treating chick neural tube explants with high BMP7 and high SHH concentrations produced a mixture of dorsal and ventral progenitors, but no intermediate fates. This behavior suggests that, at high ligand concentrations, cell fate decisions can be exclusive and stochastic. The authors further showed how a proposed gene regulatory circuit can recapitulate these experimentally measured signal-integration behaviors across different combinations of BMP/SHH concentrations (Fig. 4B). Importantly, acquiring positional information from both gradients appeared to help minimize the positional error, a principle that could generalize to other patterning systems.

Perceiving ligand dynamics

Experimentally, it is usually convenient to study responses to sudden increases in ligand concentration. However, within a developing embryo, ligand concentrations may be continually changing. How cells extract useful information from such constantly changing environments is an important but relatively understudied question. Recently, Sorre et al. applied microfluidics and quantitative time-lapse imaging to study the response of the TGF-β pathway to different ligand dynamics (Sorre et al., 2014). They analyzed the ability of TGF-β to block the differentiation of C2C12 cells into myotubes in low-serum conditions. Combining a GFP-Smad4 reporter to dynamically monitor Smad4 nuclear localization and a synthetic transcriptional reporter to quantify target promoter activity, they observed that a step-wise increase in TGF-β induces a strong adaptive signal response, as reported previously (Strasen et al., 2018; Vizán et al., 2013; Warmflash et al., 2012). Two interesting behaviors also emerged. First, they found that treating cells with a ‘pulse train’ of TGF-β (i.e. 1 h pulses separated by 6 h) blocks differentiation more efficiently compared with continuous (15 h) exposure to the same ligand at the same concentration (Fig. 5A) (Sorre et al., 2014). One crucial aspect is the duration of the interval between successive pulses, which must be longer than a minimal recovery time to produce the elevated effect. A similar effect of input intervals on cellular responses has been observed in PC12 cells treated with pulsatile EGF/NGF and in neuroblastoma cells treated with pulsatile TNF-α (Ashall et al., 2009; Ryu et al., 2016). Although the molecular mechanisms

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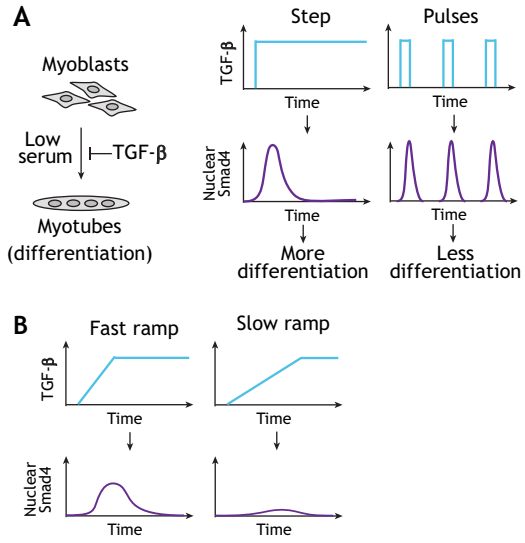

Fig. 5. Perceiving rates of change in ligand concentration. (A) Step-like but sustained TGF-β treatment leads to adaptive dynamics of intracellular signaling activity, which is measured by the level of nuclear Smad4. Sustained TGF-β exposure is less effective than pulsatile TGF-β exposure at inhibiting myoblast differentiation into myotubes, which is induced by a low level of serum in the culture. (B) In the same experimental system, gradually increasing TGF-β concentration activates the signal less strongly than a sudden step-like rise in the ligand concentration. The rate of change in ligand concentration correlates with response amplitude.

controlling the duration of the refractory periods remain unclear, negative feedback loops operating at specific timescales are likely involved. Second, the authors found that gradually ramping up the ligand concentration generates different outcomes compared with a sudden increase to the same final ligand concentration (Fig. 5B). In fact, the amplitude of the response correlates with the rate of increase in ligand concentration, with slower increase leading to dampened responses, similar to rate of change sensing in bacteria (Shimizu et al., 2010; Young et al., 2013). This could have interesting implications in morphogen gradients, in which cells at different positions could experience not only different ligand concentrations but also different rates of change in the ligand concentration. Lastly, it is interesting to contrast TGF-β with BMP4. Both ligands belong to the same superfamily of signaling ligands, but BMP4 induces sustained signaling activity whereas temporal adaptation appears to be built into the TGF-β pathway (Nemashkalo et al., 2017; Yoney et al., 2018). How and why the pathways exhibit such distinct dynamic behaviors remains unclear.

Spatio-temporal signaling codes for multicellular patterning

One of the key objectives of studies of intercellular communication codes is to understand, predict and engineer tissue-level behavior. To understand how multicellular behavior emerges from information processing in individual cells, it is necessary to first take the cells out of their spatial contexts, precisely control the ligand input and quantitatively measure the signal and cell fate outputs, as in the examples discussed above. However, studying cells in isolation neglects the roles of tissue geometry and polarity, as well as spatially organized ligand secretion and receptor expression, in the overall signaling and patterning behavior of a multicellular tissue (Butler and Wallingford, 2017; Chan et al., 2017). It is therefore essential to study the roles of signaling pathways in spatio-temporally organized multicellular contexts.

One of the most important types of information that ligands encode is spatial position within a developing tissue. In the simplest classic models of morphogenetic patterning, ligand concentrations diminish away from a source, such that signal-receiving cells can infer their position from local ligand concentration (Rogers and Schier, 2011). However, signaling pathways in receiving cells employ complex feedback loops (Fig. 1A), which can in fact actively modulate the spatial distribution of extracellular ligands and intracellular signaling activity (Freeman, 2000; Perrimon and McMahon, 1999). Although these systems are inherently challenging to analyze within complex developmental contexts, recent work has shown that many complex spatial patterning behaviors can be analyzed in reconstituted systems outside the embryo. Such studies are shedding light on the basic principles of how pathway activities within individual cells impact pattern formation at the multicellular scale. Here, we highlight examples of both spontaneous and synthetically engineered pattern formation in cultured cells.

Reconstituting spontaneous pattern formation

One of the most fascinating examples of tissue patterning is somitogenesis (Hubaud and Pourquié, 2014; Oates et al., 2012). Undifferentiated presomitic mesoderm (PSM) tissue progressively differentiates into a series of somites through a process involving tightly coordinated oscillations and spatial gradients of multiple signaling pathways that produce kinematic waves of activity (Soroldoni et al., 2014). Although somitogenesis has been imaged in spectacular detail within embryos, disentangling the dynamic roles of different signaling pathways during somitogenesis at the single cell level remains challenging in embryos. However, culturing individual PSM cells *in vitro* has allowed direct observation of signaling pathway activities, for example of Notch pathway activity.

Unlike their *in vivo* counterparts, isolated PSM cells do not sustain oscillations in Notch pathway targets (Masamizu et al., 2006; Palmeirim et al., 1997; Webb et al., 2016). However, reaggregation of unsynchronized PSM cells in 2D culture causes cells to self-organize in space and time to create synchronized Notch signaling waves that move across fields of cells, reminiscent of the segmentation clock *in vivo* (Hubaud et al., 2017; Lauschke et al., 2012; Tsiairis and Aulehla, 2016). More recently, Hubaud and colleagues used 2D *in vitro*-cultured PSM to study whether self-sustaining oscillations can occur in a cell-autonomous manner or whether they emerge from interactions among cells (Hubaud et al., 2017). By dissociating, re-aggregating and plating PSM cells at different densities, the authors showed that oscillations are a collective property of the cells that requires active intercellular signaling through the Yap and Notch signaling pathways (Fig. 6A). In a separate study, Sonnen and colleagues used cultured PSM together with microfluidics to investigate the role of the relative timing of Notch and Wnt oscillations (Sonnen et al., 2018). By directly forcing oscillations in one pathway, the other, or both, they were able to show that Notch oscillations could drive corresponding Wnt oscillations and vice versa. Using this system, they investigated the functional role of observed relative timing, or phasing, in patterning. It is known that the phase difference between the pathways naturally changes from anti-phase to in-phase along the posterior-anterior axis of the PSM. By driving oscillations of the two pathways either in or out of phase, they directly showed that the relative timing of these oscillations controls segmentation (Fig. 6B). Thus, cells can encode positional information in the relative timing of oscillatory pathway activities. It is interesting to compare these findings with recent work in yeast similarly showing that

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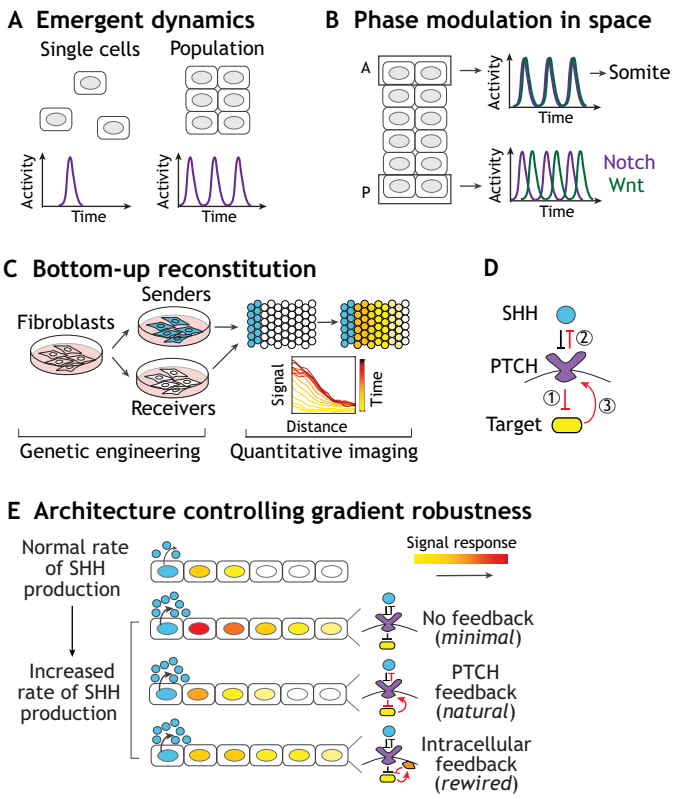

Fig. 6. Reconstituted systems enable quantitative analysis of communication codes in space and time. (A,B) A 2D culture system of PSM cells can be used as an *in vitro* model for somitogenesis. The activities of multiple pathways, including Notch, Wnt and FGF, oscillate in the PSM. Whereas isolated individual PSM cells exhibit pulses of signaling activation *in vitro*, populations of densely packed PSM cells display synchronized oscillations in a density-dependent manner (A). The relative phases between Notch and Wnt signal oscillation differ at different locations within the PSM, with an anti-phase relationship in the posterior and an in-phase relationship in the anterior, which triggers segmentation (B). The phasing between two oscillatory signals can therefore encode spatial information. (C) A bottom-up morphogen gradient reconstitution system enables quantitative analysis of the causal relationship between pathway architecture and tissue patterning. By engineering mouse fibroblasts into morphogen-sending and -receiving cells and plating the two populations under defined spatial arrangements, gradients can form within the cell layer in a petri dish. Spatio-temporal dynamics can be quantitatively measured using time-lapse imaging. (D) Unique architectural features of the SHH pathway. PTCH receptor (purple) inhibits downstream signal and transcriptional targets (yellow) in the absence of SHH (blue) (1). SHH-PTCH binding leads to inactivation of PTCH and SHH (2), and thus activation of the downstream targets. Signal activation induces an evolutionarily conserved negative feedback through upregulation of PTCH (3), which both sequesters ligand extracellularly and inhibits signal intracellularly, and therefore is bifunctional (red arrows). (E) Rewiring the SHH pathway to explore different architectures and measuring the resulting gradients revealed different degrees of robustness to variations in SHH production rate: without feedback (minimal), the amplitude (the response in the first cell next to the source) and length of the signaling gradient are sensitive to an increase in the ligand production rate (second row versus first row); with the evolutionarily conserved PTCH feedback (natural), both gradient amplitude and length become more robust (second row versus third row); with intracellular negative feedback (rewired) from a mutant PTCH (orange) that does not bind SHH but suppresses the intracellular signal (Briscoe et al., 2001), gradient amplitude but not lengthscale becomes more robust compared with no feedback (last row versus second row). These results directly link pathway architecture to patterning behavior.

the identities of different environmental stresses are encoded in the relative timing with which different transcription factors activate (Lin et al., 2015).

Similar reconstitution approaches have been applied to embryonic stem cells to enable spatially organized differentiation in 2D and 3D. Some of the 2D models are especially compatible with quantitative time-lapse imaging and spatio-temporally controlled perturbations, and hence are particularly suitable for studying signaling activities in space and time at the single cell level (Martyn et al., 2018; Morgani et al., 2018; Thorne et al., 2018; Warmflash et al., 2014; Yoney et al., 2018). Together, these systems are poised to address fundamental questions about how developmental signaling information is encoded in space and time during early embryonic development.

Synthetically engineering pattern formation from the bottom up

Despite our improved capability of analyzing natural genetic circuits, it is still difficult to identify which minimal circuits are sufficient to enable spatial patterning, what key parameters control system performance and what tradeoffs exist among alternative circuit designs. To address these issues, one would ideally like to systematically rewire circuit architecture, tune key parameters and quantitatively measure the resulting multicellular behaviors. In this respect, traditional genetic approaches of perturbing one or a few components at a time inside an embryo are limited. An alternative approach is to engineer genetic circuits into cells that normally do not express them, and test whether the reconstituted circuits are sufficient for enabling desired behaviors. Based on this rationale, we have recently reconstituted SHH signaling gradients in a petri dish using engineered mouse fibroblasts that can secrete and respond to SHH (Fig. 6C) (Li et al., 2018). In this system, gradients form primarily through ligand movement within the cell layer, similar to what happens inside an embryo. This reconstituted system avoids interference from upstream or parallel developmental processes, enables genetic rewiring of the pathway and is compatible with quantitative analysis of spatio-temporal gradient dynamics. It therefore provides a unique opportunity for studying how genetic circuits enable multicellular behaviors.

The SHH pathway has several unique architectural features (Fig. 6D). First, the pathway has a double-negative logic, in which free receptor PTCH inhibits the intracellular signal, and SHH-PTCH interaction leads to mutual inactivation and internalization of the complex, effectively removing the inhibition to enable pathway activation (Briscoe and Thérond, 2013). Second, pathway activation upregulates the expression of PTCH, forming an evolutionarily conserved feedback loop (Goodrich et al., 1996; Jeong and McMahon, 2005). Third, this feedback loop both sequesters ligands extracellularly and suppresses signal intracellularly (Chen and Struhl, 1996), and therefore acts as a bifunctional negative feedback. To understand how these architectural features impact gradient formation, we rewired the SHH pathway to implement alternative pathway variants that both do, and do not, exist in nature, and systematically analyzed the spatiotemporal dynamics of the resulting gradients (Li et al., 2018) (Fig. 6E). This analysis revealed that without the negative feedback (minimal design), signaling gradients are sensitive to variations in the ligand production rate. PTCH feedback (natural design) makes the signaling gradient more robust to variations in the ligand production rate, and it also accelerates the approach of gradients to steady states. Finally, PTCH feedback outperforms other alternative designs, such as an intracellular negative feedback that only inhibits signal inside the cells without affecting extracellular ligand distribution (rewired design). The performance of PTCH feedback requires its dual function, which enforces a tight coordination between intra- and extra-cellular modulation.

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These results directly demonstrate that the wiring of a pathway can determine the spatio-temporal dynamics and robustness of signaling gradients. They also pose new questions: how do other pathways achieve patterning robustness (Eldar and Barkai, 2005; Eldar et al., 2003, 2004)? Or, alternatively, have other pathways evolved to provide distinct capabilities? Examination of other spatial patterning systems reveals a rich set of patterning phenomena and pathway architectures that could be analyzed in a similar fashion. For example, work in embryos has shown that BMP ligands can be actively 'shuttled' from the location in which they are produced to generate specific developmental patterns (Shilo et al., 2013). Similarly, the ability to proportionally scale a pattern with tissue size can emerge from repression of a rapidly diffusible morphogen 'expander' or from a distinct mechanism (Ben-Zvi et al., 2011; Gregor et al., 2005; Inomata et al., 2013), which has been comprehensively reviewed (Umulis and Othmer, 2013). It will be interesting to see whether such complex, multi-protein behaviors can be understood through similar reconstitution approaches.

Synthetic approaches to development are now gaining traction. Engineering circuits using endogenous or orthogonal signaling components has shown that relatively simple circuits are sufficient for producing non-trivial population-level behaviors, such as forming concentric rings and periodic patterns (Matsuda et al., 2015; Sekine et al., 2018; Toda et al., 2018). These synthetic systems can generate patterns in roughly predictable ways, and it will be interesting to find out to what extent importing design principles from natural patterning systems is necessary or helpful in increasing their precision to the level of natural developmental systems. Together, these reconstituted systems provide an exciting opportunity for studying developmental processes at high spatio-temporal resolution.

Conclusions and future directions

As described above, developmental signaling systems are not passive transmitters of information from an extracellular ligand to an intracellular effector. The information they sense is represented in diverse ways, many of which could not be inferred from knowledge of molecular interactions alone. Experimentally, deciphering these codes will require systematic quantitative control of multiple ligand concentrations in space and time and simultaneous monitoring of pathway outputs in diverse cell contexts. It will also involve re-wiring pathways to understand which of their architectural features are necessary or sufficient for signal processing. A strong test of our understanding will be the ability to use synthetic biology approaches to program synthetic multicellular developmental behaviors that use these pathways.

Achieving these goals also requires that we grapple with thorny issues such as whether any single 'pathway' can be meaningfully isolated from any other, and how 'cell context' impacts the interpretation of signals. However, the payoff of learning these codes is likely to be immense. Developmental signaling pathways provide the most biologically relevant and powerful levers we have for controlling cells, explaining their frequent role as targets of drug development and their pivotal importance for regenerative medicine. Understanding how these pathways 'expect' their inputs to be encoded, whether in ligand concentrations, temporal dynamics, multi-ligand combinations or in other ways, could enable more specific control of cell fate and other responses, and provide crucial insight into the logic of the diverse developmental processes that they enable. An additional benefit of this approach could be conceptual. Currently, we represent pathways predominantly in molecular terms. But a complementary understanding will come from the ability to represent them as programs that address messages

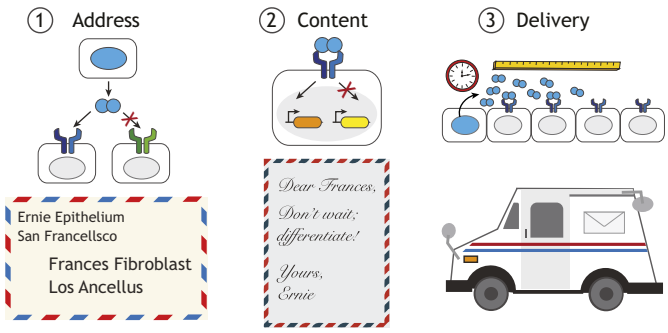

Fig. 7. Active signal processing allows specificity and precision in cell-cell communication. Developmental signaling pathways can be viewed as programs that control message addressing ('who can talk to whom'; 1), message content ('which target program to activate'; 2), and message delivery ('when and where the information should be received'; 3). The diverse signal processing schemes used by different pathways not only transduce signals but actively modulate them in ways that enable specificity and precision in multicellular development.

to specific cell types, control the 'content' (target program) of a message, and specify the precise spatial distribution of intercellular messages (Fig. 7). It appears that the approaches described above, together with ongoing revolutions in single cell analysis, are opening up powerful new opportunities for understanding and controlling cellular communication in the years ahead.

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Competing interests

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