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CHAPTER TWELVE

Toward a Systems Understanding of Signaling Pathway Function

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Contents

Abstract

A small number of developmental signaling pathways are used repeatedly throughout development in many different contexts. How these pathways interact with each other and the specific cell context to generate a wide range of appropriate responses remains an important question. The application of genomic and proteomic approaches and imaging at high spatiotemporal resolution are providing answers to this question and revealing new levels of complexity. Here, we discuss pathways as complex networks and examples of how signaling outcomes can be influenced by the temporal nature of the signal, its spatial regulation, and the cell context.

1. INTRODUCTION

When *Current Topics in Developmental Biology* was first published 50 years ago, the biochemical isolation of growth factors had begun to allow the effects of signals on cells to be studied. In the 1980s and 1990s, pioneering genetic screens in model organisms elucidated the major developmental signaling pathways' core components. Subsequent studies showed that these pathways are used repeatedly to perform different developmental, physiological, or

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pathological functions. It soon became clear that relatively few pathways, such as Notch, JAK–STAT, receptor tyrosine kinase, BMP, and Hedgehog were capable of giving rise to diverse cellular responses and that combinatorial signaling and cell context were critical to outcomes (Perrimon, Pitsouli, & Shilo, 2012). In many cases, we now have a good understanding of which pathways are involved in which processes. Today, two critical and interconnected questions in the field remain: (1) how do pathways signal together to generate diverse and robust outcomes, and (2) how does cellular context alter these responses? Over the last decade, a combination of genome-scale approaches and high-resolution imaging are offering answers to these questions and revealing new levels of complexity. Here, we discuss some of the ways in which our understanding of how signaling pathways function to regulate cell fate are changing and leading to a more complex view.

2. PATHWAYS AS COMPLEX NETWORKS

Genetic studies of signaling pathways led to relatively linear textbook views of canonical signaling (Fig. 1A). However, the fact that relatively few pathways can generate such diverse outcomes suggested a more complicated picture. The same pathway can give different outcomes in different cell types, so context plays an important role. Many of the classic examples of developmental signaling, such as the patterning of *Drosophila* ommatidia (Félix & Barkoulas, 2012) and the *Caenorhabditis elegans* vulva (Nagaraj & Banerjee, 2004), involve multiple signaling pathways working together with cross talk between them, so combinatorial signaling adds an additional layer of complexity (Housden & Perrimon, 2014). In addition, work on epidermal growth factor (EGF) and nerve growth factor (NGF) signaling through the mitogen-activated protein kinase (MAPK) pathway made it clear that a single pathway axis can generate different outcomes (Chao, 1992). Simple linear pathways, even acting in different combinations, would be insufficient to explain the diverse outputs generated. Enhancer and suppressor screens in model organisms have identified many additional regulators of signaling pathways, beyond the core components (Fig. 1B). Piecing together a more comprehensive picture of pathway complexity has required the combination of both proteomic approaches, to determine interaction partners of known components, and large-scale functional genomic approaches such as genome-wide RNAi. Functional screens have identified many novel pathway regulators, but in isolation do not distinguish between direct and indirect pathway regulation. Similarly, while proteomic approaches identify many proteins that interact with known pathway components these interactions may not be functional

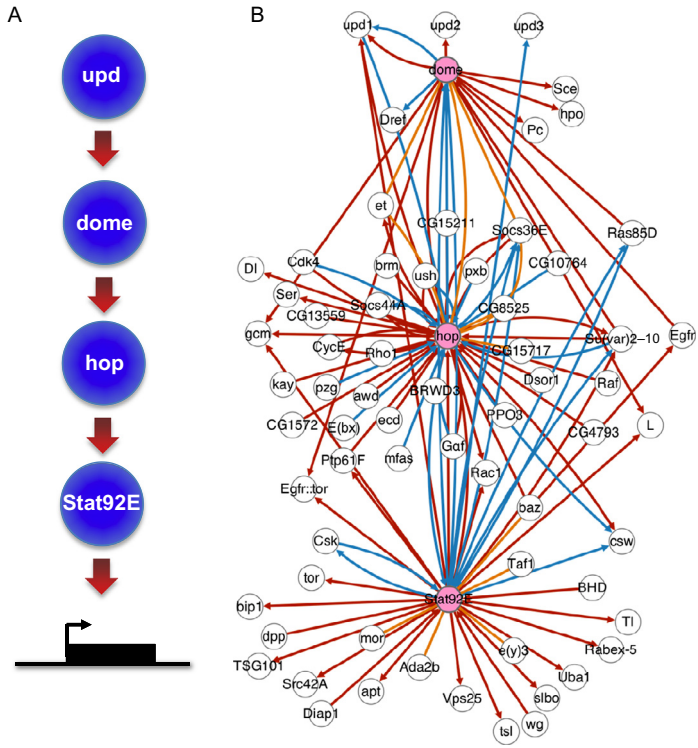

Figure 1 From linear pathways to complex networks. (A) Simple representation of the core Drosophila JAK/STAT pathway: cytokine ligands (upd1, 2, 3) bind to the receptor (dome), which activates JAK (hop) and in turn Stat (Stat92E) to regulate target gene expression. (B) An interaction network for dome, hop, and Stat92E (pink nodes) showing genetic interactions (red and blue lines with arrows) and physical interactions (orange lines). Network generated using EsyN (Bean et al., 2014).

regulatory interactions. The two approaches complement one another in characterizing the direct regulatory interactome of a pathway.

Proteomic approaches have allowed interactomes to be derived for several major signaling pathways. Large-scale yeast-two-hybrid approaches (Yu et al., 2008) in combination with siRNA functional analysis have been used to characterize the human MAPK interaction network, identifying a core of over 600 proteins including novel pathway chaperones and scaffolds (Bandyopadhyay et al., 2010). In a similar study across different *Drosophila* cell types, tandem affinity purification mass spectrometry (TAP-MS) and RNAi were used to identify functional RTK–Ras–ERK interactors (Friedman et al., 2011). Many of the novel regulators identified were found to be cell type specific,

suggesting that different network wiring offers a mechanism of generating context-specific outcomes. Recent work has also identified the hippo pathway interaction network in *Drosophila* and mammals (Couzens et al., 2013; Kwon et al., 2013; Wang, Li, et al., 2013). This allowed a link to be made between Hippo signaling and vesicle trafficking (Kwon et al., 2013), reflecting the importance of membrane biology and subcellular localization in signaling, as discussed below. In addition to targeted approaches, in which networks are derived by starting from known pathway components and working outward to their interactors, large-scale systematic interaction mapping by mass spectrometry has been performed in fly and mammalian cells (Guruharsha, Obar, & Mintseris, 2012; Guruharsha et al., 2011; Havugimana et al., 2012; Huttlin et al., 2015). While these studies were not focused on signaling, they identified many candidates for novel pathway regulators. Many genes remain without functional annotation and interaction maps allow functional annotations to be proposed based on their interaction partners, including attribution of possible roles in signal transduction. Conservation of interactions between species may indicate conserved functional significance and act as a filter to prioritize candidates for further study.

The combination of interaction and functional regulation from RNAi makes a strong case for a protein being a direct regulator but does not necessarily offer a mechanism. Phosphorylation events are often critical regulators of signaling cascades, but even when an interaction involves a kinase the systematic identification of kinase targets remains a work in progress. Large-scale attempts at mapping kinase targets by phospho-proteomics are beginning to address this and offer insights into the possible branching of signals through phosphorylation targets (Sopko et al., 2014). More generally, as interaction networks of hundreds of proteins are derived for different pathways, the presence of common components offers points for cross talk between pathways in signal transmission.

While the focus of signaling research has largely been on the role of proteins in pathways, nonprotein regulators have begun to take on greater importance, adding to the complexity and offering additional points for diversification of outcomes. As the function of noncoding RNAs has been elucidated in many processes, their roles in signaling networks have become apparent. miRNA regulation has been shown for pathways including Wingless (Silver, Hagen, Okamura, Perrimon, & Lai, 2007) and Hedgehog (Kim, Vinayagam, & Perrimon, 2014). The lin28-let-7 axis has been shown to be critical in a range of stem cell and oncogenic signaling pathways, and let-7 miRNA targets include components of the insulin signaling pathway (Frost & Olson, 2011; Zhu et al., 2011).

The metabolic status of the cell may also influence signaling and there are many examples of intersection between developmental signaling pathways and metabolism, with metabolites acting as regulators and readouts of signaling. For example, the eicosanoid PGE2 has been shown to regulate the Wnt pathway and EGF signaling in hematopoietic and embryonic stem cells, respectively (Goessling et al., 2009; Yun, Lee, Ryu, & Han, 2009), and Akt activity is regulated by TOR in response to amino acids (Saha et al., 2014). Metabolites either individually or collectively can also act as phenotypic sensors of signaling states, for example, the NAD/NADH sensor SoNar has been used as a readout for metabolic screening in cancer drug discovery (Zhao et al., 2015). AMP functions as an energy sensor, indicating low nutrient status by activating AMPK (Zhang et al., 2013), which is capable of cross talk with developmental signaling pathway components such as MEK–ERK via phosphorylation of BRAF (Shen et al., 2013). Physical factors may also have significant effects on signaling outcomes and there is an increasing appreciation of the role of mechanical forces in cellular communication (as reviewed in detail in Miller & Davidson, 2013).

Large interaction networks afford opportunities for context-specific outcomes and cross talk with other pathways. It has become clear that transmission of information through a network, even for a single pathway, is more complex than a linear flow. The differential expression or regulation of network components in distinct cell types offers a possible means to alter the flow of information through pathways in a cell type-specific manner (Kiel, Verschueren, Yang, & Serrano, 2013). Engineering approaches to information flow through a network such as modular response analysis, as opposed to qualitative assessment of inputs and outputs, may allow a better understanding of temporal pathway properties (Bruggeman, Westerhoff, Hoek, & Kholodenko, 2002). Understanding signal transmission in this way requires inputs and outputs of pathways to be measured at different points through the pathway in either real time or time series, and novel imaging approaches are allowing this.

3. TEMPORAL PROPERTIES OF SIGNAL TRANSDUCTION

Dissecting the temporal component of signaling requires tools to detect dynamic readouts and manipulate signaling at multiple levels. The toolset that would be required to do this in a systematic way across the major signaling pathways is very incomplete but, where approaches are available, they have offered new insights into the temporal properties of signaling pathways. Dynamic readouts coupled to inputs that can be adjusted for frequency,

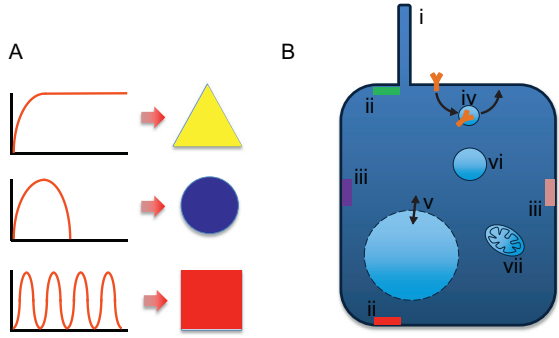

Figure 2 Spatiotemporal properties of signaling pathways. (A) The temporal properties of a single signal (graphs) may determine differential cellular responses (colored shapes). (B) Schematic of a cell showing some possible points of spatial regulation of signal transduction. Cells may localize surface signaling by: extension of short nanotube projections or longer cytonemes (i), apicobasal (ii), and planar (iii) cell polarization. Endocytosis and signaling endosomes (iv), lysosomes (vi), and mitochondria (vii) all have roles in subcellular localization of signaling, while nucleocytoplasmic transport (v) is an important step in many pathways.

amplitude, and duration have shown that the nature of the signal can be used to differentially transmit information (Fig. 2A; reviewed in Purvis & Lahav, 2013). In pheochromocytoma 12 (PC12) cells, EGF or NGF treatment results in the near-opposite responses of division and differentiation, respectively (Chao, 1992). Both of these pathways signal through the MAPK–ERK network but the dynamics of ERK activation differ according to the stimulus (Marshall, 1995). EGF triggers transient ERK signaling, whereas NGF causes sustained ERK activity. Differential dynamics of the same pathway effector's activity can therefore result in different cellular outcomes. Modular response analysis using RNAi to knockdown pathway components and phospho-specific antibody staining to detect the activation state of different points in the network has shown that different feedback responses underlie this difference in ERK activation dynamics (Santos, Verveer, & Bastiaens, 2007). EGF activation causes negative feedback within the network and hence transient ERK activation, whereas NGF signaling results in positive feedback and sustained ERK activation. Reversal of these feedback loops is sufficient to reverse the cellular responses to NGF and EGF.

In vivo, manipulating and tracking signaling at sufficient spatiotemporal resolution to study signaling dynamics remains challenging but a growing range of fluorescence-based tools are becoming available (Doupé & Perrimon, 2014). In some cases, subcellular localization of a fluorescently labeled component can be used to detect pathway activity. For example,

GFP tagged STAT translocation to the nucleus when the JAK/STAT pathway is activated (Chen & Reich, 2010). Where this is not possible changes in protein conformations or interactions can sometimes be detected by fluorescence resonance energy transfer (FRET) between donor and acceptor probes fused to signaling components such as MEK and ERK (Burack & Shaw, 2005). An intramolecular FRET probe has been used to successfully measure ERK activity *in vivo* in a *C. elegans* NaCl responsive sensory neuron with high temporal resolution (Tomida, Oda, Takekawa, Iino, & Saito, 2012). In this study, the nature of the upstream signal (NaCl) allowed a microfluidic device to be used to precisely control the signal and visualize the ERK activity response, revealing that cyclic stimulation with a periodicity in the tens of seconds resulted in sustained high levels of ERK activity whereas sustained signals or a shorter signal periodicity did not. The dynamics of an upstream signal rather than just the concentration of the particular signal can therefore be used as a determinant of the cellular response. Optogenetics (reviewed in Toettcher, Voigt, Weiner, & Lim, 2011) has also been used to manipulate Ras–ERK signaling with high temporal resolution demonstrating differential responses to different temporal activations in NIH 3T3 cells (Toettcher, Weiner, & Lim, 2013). Proteomics was used to assess downstream results, offering a nice demonstration of how the genome-scale approaches discussed above can be combined with high-resolution imaging to better understand signal transduction and responses. Dynamic signaling is not a unique feature of the Ras–ERK network. Oscillations in Notch signaling play a critical role in vertebrate somite formation and destabilized transcriptional reporters of Notch signaling have been used to image these oscillations directly (Aulehla et al., 2008; Masamizu et al., 2006). As tools become available to study the temporal properties of other pathways in other contexts, this may prove to be a general theme, providing additional ways to encode information with a finite number of pathways.

4. SPATIAL REGULATION OF SIGNAL TRANSDUCTION

Increasing resolution of signal imaging has also led to an increased understanding of the spatial properties and regulation of signal transduction at the subcellular level (Fig. 2B). In order to regulate gene expression nuclear translocation is an important process in many signaling pathways; for example, STAT6 has been shown to continuously shuttle between nucleus and cytoplasm but to only accumulate in the nucleus when activated (Chen & Reich, 2010). Many organelles have roles in signal transduction;

the mitochondrion is a signaling organelle in apoptosis and is an example of metabolites influencing signaling as mitochondrial reactive oxygen species can affect cellular responses (Chandel, 2014). The lysosome is the site of active TOR and AMPK signaling, representing a key point of intersection between metabolism and signaling (Bar-Peled, Schweitzer, Zoncu, & Sabatini, 2012; Zoncu et al., 2011). Endocytosis and membrane trafficking have particular importance to signal transduction by creating additional sites for signaling, and downregulating or recycling receptors (Di Fiore & von Zastrow, 2014; Gonnord, Blouin, & Lamaze, 2012). Combining RNAi with high-resolution imaging and multiparametric imaging analysis has allowed the complexity of endocytic traffic and its links to many signaling pathways to be revealed (Collinet et al., 2010). G-protein-coupled receptor signaling from endosomes may lead to extended pathway activation and the location of second messenger production is important to the cellular response (Tsvetanova & von Zastrow, 2014). Recycling endosome trafficking of the Notch ligand Delta is important for its function and plays a role in asymmetric fate of *Drosophila* sensory organ precursor (SOP) daughters (Emery et al., 2005). Segregation of endosomes at division can determine the signaling states and hence fates of the resulting cells. Specialized endosomes marked by the presence of Sara have been shown to be responsible for the equal distribution of TGFβ signal components at cell division in the *Drosophila* wing disc (Bökel et al., 2006). Sara endosomes are also critical for asymmetric notch signaling post cell division in both SOP cells and intestinal stem cells in *Drosophila* and in Zebrafish neural precursors (Coumailleau, Fürthauer, Knoblich, & González-Gaitán, 2009; Kressmann, Campos, Castanon, Fürthauer, & González-Gaitán, 2015; Montagne & Gonzalez-Gaitan, 2014).

Even at the plasma membrane, the localization of signaling receptors, ligands, and regulators to particular domains can be important for determining appropriate cellular responses. Apicobasal polarity of neuroblasts, for example, allows the asymmetric segregation of the Notch inhibitor Numb to the ganglion mother cell on division, resulting in biased Notch activity and asymmetric fate (Knoblich, 2010). Planar cell polarity components are responsible for noncanonical Wnt signaling in various development processes (Wansleeben & Meijlink, 2011). In addition to cell polarity, recent work in the *Drosophila* testes has shown that germline stem cells can extend nanotube projections, deforming the membrane of the hub cells that form their niche (Inaba, Buszczak, & Yamashita, 2015). These extensions were found to be strongly enriched for Dpp receptors, allowing highly localized

signaling that restricts the ability to self-renew to only the most proximal germline stem cells. On the larger scale of tissues and organs, imaging approaches are also giving new insights into classical questions about positional information. Cytonemes, cellular protrusions that may extend over several cell diameters, have been described in various systems and play important roles in morphogen gradients such as the distribution of Hedgehog in the *Drosophila* wing disc (Bischoff et al., 2013; Kornberg & Roy, 2014). Interpretation of positional information in response to signals is critical for determination of different cell fates in many developmental contexts (Kicheva, Cohen, & Briscoe, 2012; Wolpert, 2011). Many theoretical attempts have been made to model the establishment and maintenance of morphogen gradients and advances in imaging are allowing data to be generated to test these hypotheses, as for the bicoid gradient in the *Drosophila* embryo (Bergmann, Tamari, Schejter, Shilo, & Barkai, 2008; Gregor, Tank, Wieschaus, & Bialek, 2007; Gregor, Wieschaus, McGregor, Bialek, & Tank, 2007). Even a nonprotein gradient, that of retinoic acid in vertebrate hindbrain patterning, has been visualized using a modified retinoic acid receptor fused to a FRET donor and acceptor pair such that retinoic acid binding causes detectable changes in the FRET signal (Shimozono, Iimura, Kitaguchi, Higashijima, & Miyawaki, 2013).

While imaging has helped elucidate many of these processes on a subcellular to tissue scale, genomic and proteomic approaches can also inform studies of spatial aspects of signaling. Networks derived from large-scale proteomic approaches can reflect subcellular organization and where localization is known for a component of a complex, spatial annotation of less well-characterized proteins may be possible (Huttlin et al., 2015). The development of targeted proteomic approaches to profile the proteomes of specific organelles or cell compartments may also prove informative in signaling biology. Techniques such as BioID (Roux, Kim, Raida, & Burke, 2012) and Apex (Rhee et al., 2013), in which a protein-modifying enzyme is fused to a protein or tag that targets it to a specific subcellular localization, allow the proteome of that region of the cell to be identified by isolation of the modified proteins. BioID has already been used to help map the hippo pathway interactome by allowing profiling of proteins not readily extracted by FLAG AP–MS (Couzens et al., 2013). In principle, these approaches could be used both to obtain spatial information about signaling networks and to profile the changes in network component interactions following pathway stimulation. On the tissue, organ or developing embryo scale, recent advances in single-cell sequencing approaches also have

potential for mapping differences in the transcriptional outputs of signaling. Two recent studies developed droplet-based single-cell RNA sequencing techniques and applied them to characterize heterogeneous differentiated cell types in the retina, and heterogeneity among differentiating embryonic stem cells (Klein et al., 2015; Macosko et al., 2015). In principle, these approaches could be applied to questions of differential responses to signaling in development on a genome-wide scale at single-cell resolution. Positional information could be inferred retrospectively by imaging the expression patterns of differentially expressed genes *in situ*.

5. THE IMPORTANCE OF CONTEXT

Cell-type specificity is a key feature allowing relatively few pathways to generate the diverse outcomes observed in development. At the level of signal transduction, different cells will express different combinations of network components, allowing different flows of information through the same basic pathway. Altering the abundance of network components that exhibit mutually exclusive interactions with a pathway node, such as CRAF and RIN1 interactions with Ras, has been shown to alter signaling outcomes (Kiel et al., 2013). When modeling information flow within a network, it is therefore important to be aware of the specific cellular context in order to place appropriate constraints on the network. Spatial separation of components to distinct subcellular locations in specific contexts may also need to be considered. As techniques such as RNAseq and proteomics are used more widely, information on the expression of pathway components in specific cell types is increasingly becoming available. However, the need to collate this information and differences in experimental design, sample preparation, and data analysis present challenges to easy interpretation.

Ultimately, signaling effects on cell fate are most commonly mediated at the transcriptional level. The cell type and developmental context critically influence the outcome of signaling events at the level of transcriptional targets. The main effector transcription factors have been identified for the major pathways (Perrimon et al., 2012), but it is clear that their targets vary depending on the cellular context (for example, STAT (Wang, Chen, He, Zhou, & Luo, 2013) and Wnt (Vincent, 2014)). The cell's history and current state of other signaling pathways set a unique ground state upon which a signal can act. There are a number of ways in which context may influence outcomes at the level of transcription itself. Lineage-specific transcription factors (or effectors of other signaling pathways) may recruit signaling

pathway effectors, create a permissive chromatin state for their binding, or influence their function when bound (Fig. 3).

Approaches such as ChIPseq and DamID allow the profiling of transcription factor binding and chromatin states on a genome-wide scale and, in combination with expression profiling, can help dissect these mechanisms. Interaction with other transcription factors, which may be effectors of other pathways or lineage-specific factors, can influence both the recruitment to and function at enhancers and promoters across multiple species. Notch signaling, for example, generates different transcriptional responses in *Drosophila* muscle progenitors and hemocytes due to interaction with the lineage-specific factors Twist and Lozenge, respectively (Bernard, Krejci, Housden, Adryan, & Bray, 2010; Terriente-Felix et al., 2013). During vertebrate hematopoietic regeneration, both myeloid and erythroid lineages are generated in response to BMP and Wnt signaling. The effector transcription factors for BMP and Wnt in both lineages have been shown to be SMAD1

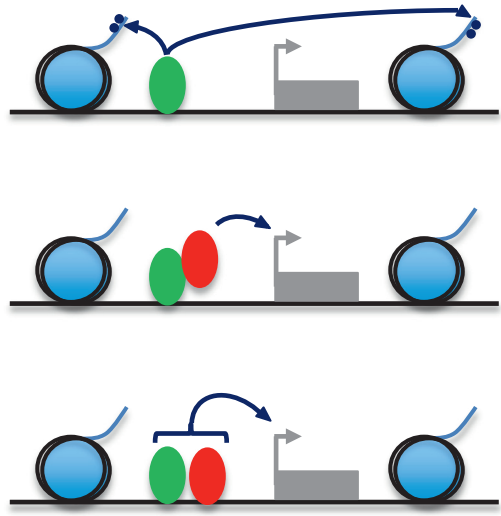

Figure 3 Transcriptional context in signaling. Lineage-specific regulators (or effectors of other pathways) (green) may regulate the targets of signaling pathways by: acting as pioneer factors to open chromatin into a permissive state (top panel, black line is DNA, light blue balls nucleosomes with histone tail extending, dark blue dots represent histone modifications); direct recruitment of effector transcription factors (red) to specific enhancer sites (middle panel); or by cooperative regulation, for example, by recruiting complementary chromatin modifiers or transcriptional apparatus (lower panel).

and TCFL2, respectively. However, despite the relatively close relationship between the lineages and the use of the same effector transcription factors, the responses show lineage specificity (Trompouki et al., 2011). In the myeloid lineage, the recruitment of SMAD1 and TCFL2 is mediated by the myeloid-specific transcription factor C/EBPα, whereas in the erythroid lineage GATA1 recruits the same pathway effectors to distinct targets. Interestingly, misexpression of the lineage-specific factor from one lineage in the other is sufficient to result in an exchange of targets.

While in some cases a single lineage-specific factor may act as a critical target determinant, it is likely that in many contexts the combination of factors is key. The binding of transcription factor PU.1 in macrophages and B cells correlates with C/EBPs and EBF1, respectively (Heinz et al., 2010). During macrophage activation, the combination of PU.1 and C/EBPs interact with NFKβ to determine its functional targets (Heinz, Romanoski, Benner, & Glass, 2015; Trompouki et al., 2011). PU.1 may act in this context as a pioneer factor, modifying histones and remodeling nucleosomes to create a permissive chromatin state for the recruitment of subsequent factors (Heinz et al., 2010; Iwafuchi-Doi & Zaret, 2014). In most developmental contexts, multiple signaling pathways may be active at once and a comprehensive understanding on how pathways regulate specific targets in specific cell types will therefore require both the lineage-specific factors and the effector transcription factors of multiple signaling pathways to be taken into account.

6. CONCLUSION

The field of intercellular signaling has developed rapidly and genomics and imaging approaches are offering a wide range of examples of how pathways generate diverse outcomes and how information is robustly transmitted. Complex networks of signaling regulators allow different ways of transmitting information through a single pathway, and provide points of cross talk with different pathways. Spatiotemporal aspects of signaling further increase the complexity of signal transduction and offer additional means to encode information. In addition to differential expression of pathway components, transcriptional context can act as a key determinant of cell type specificity in signaling. In many cases, new approaches are revealing examples of the varied ways in which diverse outcomes can be generated. However, in generating large datasets and describing aspects of signal transduction in ever-greater detail there is a risk of accumulating large quantities

of complex information without increasing understanding. It is important to dissect which regulatory processes occur in which context and which are the most functionally important. Moving forward, it will be important to use systematic approaches to generate coherent datasets that can be compared and used together, while anchoring findings to the biological outputs that they regulate. Just as the application of genetics to developmental biology led to the elucidation of many core signaling pathways, in this era of "omics" approaches, collaborative work between cell biology, biochemistry, computational biology, and physics is making substantial contributions to our understanding of signal transduction.

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