Published in final edited form as:

Curr Top Dev Biol. 2016 ; 117: 393–404. doi:10.1016/bs.ctdb.2015.10.016.

A thousand and one receptor tyrosine kinases: wherein the specificity?

Harish N. Vasudevan and Philippe Soriano*

Department of Developmental and Regenerative Biology, The Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029

Abstract

Over twenty years ago, a series of seminal studies in PC12 neurons provided a framework for how receptor tyrosine kinases generate many different outcomes despite activating a set of shared intracellular pathways. In this essay, we revisit the question of receptor tyrosine kinase specificity. We first examine the relationship between receptor phosphorylation and intracellular pathway activation. We then consider the mechanisms through which signaling dynamics encode distinct cellular outcomes and finally discuss how two different receptors drive divergent transcriptional responses within the same developmental context. Establishing the key parameters that dictate the response to growth factor stimulation is critical for determining how receptor tyrosine kinases orchestrate development, an essential prerequisite for understanding the pathological consequences when such signaling processes go awry.

Keywords

Cell signaling; ERK; PI3K; differentiation; proliferation; migration

Introduction

Receptor tyrosine kinases (RTKs) are a family of cell surface receptors important across a range of physiological and disease processes. Upon ligand treatment, RTKs undergo phosphorylation at intracellular tyrosine residues, leading to the binding of signaling proteins at these phosphotyrosine sites (Lemmon & Schlessinger, 2010). Many RTKs bind overlapping sets of signaling proteins, ultimately leading to the activation of many shared intracellular pathways, but the outcomes dictated by different RTKs can be quite distinct. The resulting puzzle of how distinct cellular outputs arise from a common set of signaling inputs has intrigued biologists for decades. Many models to explain signal specificity have been proposed (Marshall 1995; Hunter, 2000; Simon, 2000; Pawson, 2004; Volinsky & Kholodenko, 2013). In theory, each signaling event could determine a particular cellular output; under this linear model, perturbation of any single input would impair a specific outcome. For instance, epidermal growth factor (EGF) promotes osteoblast differentiation in human mesenchymal stem cells while platelet-derived growth factor (PDGF) represses this process; this effect is PI3K-dependent and explained at least in part by selective activation

*Corresponding author: philippe.soriano@mssm.edu.

of PI3K in response to PDGF, but not EGF, stimulation (Kratchmarova, Blagoev, Haack-Sorensen, Kassem, & Mann, 2005). However, the number of RTK-mediated outcomes is greater than the number intracellular pathways, making a strictly linear model insufficient to fully explain the range of behaviors controlled by RTKs. Alternatively, differences in the magnitude or duration of a signal could determine cellular output, and a common input would then specify multiple outputs based on distinct signaling dynamics. A classic example of such dynamic encoding is in PC12 neurons, where a transient phospho-ERK pulse in response to EGF treatment results in cell proliferation while a sustained phospho-ERK wave in response to NGF stimulation drives cell differentiation (Marshall, 1995). Further, the additive integration of multiple linear or dynamic inputs can generate an even greater range of outputs. Since RTK activation generally results in the concerted induction of many intracellular pathways, such combinatorial encoding likely contributes toward the outcomes mediated by RTK signaling. Given the limited number of intracellular pathways in comparison to the diversity of cell types and outcomes regulated by RTKs, both dynamic and combinatorial encoding are likely to play important roles in many contexts.

The framework and associated examples described above predominantly describe intracellular pathway activation in response to growth factor stimulation. However, it is important to distinguish between signaling events at the receptor (i.e. direct binding of proteins to phosphorylated tyrosine residues on the receptor) and intracellular pathway level (i.e. the consequent pattern of intracellular pathway activation in response to RTK signaling), as they constitute distinct steps at which information is encoded. Given that effector binding at the receptor drives the pattern of intracellular pathway activation, these two parameters are closely related; indeed, some effectors are themselves enzymes (e.g. RasGAP, PI3K, PLCY) that directly modulate downstream signaling, but others function as scaffolds (e.g. FRS2, IRS1), serving to recruit more signaling proteins. Nonetheless, events at the receptor and intracellular pathway level are mechanistically separate. For example, perturbation of PI3K binding at the receptor and chemical inhibition of the PI3K pathway directly can have different effects on signal transduction (Francavilla, Rigbolt, Emdal, Carraro, Vernet, et al., 2013), likely due to crosstalk between pathways and the ability of multiple effectors at the receptor to coordinately regulate a single downstream intracellular pathway.

In this essay, we explore the mechanisms underlying RTK specificity. We first discuss experiments examining the importance of individual phosphotyrosine residues on the receptor in dictating intracellular pathway activation and developmental outcomes. Focusing on phospho-ERK, we next consider a pair of recent studies elucidating the mechanisms through which differences in intracellular signaling dynamics are converted into distinct outcomes. Finally, we summarize our recent work examining how two different receptors elicit divergent transcriptional responses in a common context. We conclude by outlining two avenues for future investigation we find particularly intriguing: the systematic mapping of phosphotyrosine residues to intracellular pathway activation and the functional relevance of single cell heterogeneities within signaling responses.

Starting at the receptor: multifunctional phosphotyrosines

The first step upon ligand binding is phosphorylation of intracellular tyrosine residues on the receptor itself; therefore, a critical question is how effector binding at these phosphotyrosines shapes signal specificity. One can imagine two scenarios: individual binding events could modulate distinct signaling outcomes, or alternatively, the combined pattern of many binding events may function additively to drive the desired response. Using embryonic phenotypes as a readout for PDGF receptor α (PDGFR α) and PDGF receptor β (PDGFR β) signaling, analysis of mice harboring point mutations at tyrosine residues that abrogate binding of specific effector proteins suggest both models apply *in vivo*. An allelic series at the *Pdgfra* locus identified specific phenotypes associated with loss of particular binding events, suggesting each effector transmits a distinct biological signal with a central role for PI3K (Klinghoffer, Hamilton, Hoch, & Soriano, 2002). In contrast, an allelic series at the *Pdgfrb* locus did not identify distinct phenotypes when the binding of individual effectors was perturbed (Tallquist, French, & Soriano, 2003); instead, combinatorial loss of multiple effectors exacerbates a shared vascular smooth muscle phenotype, suggesting additive output across multiple binding events drives cellular outcomes downstream of PDGFR β . Interestingly, mutation of multiple PDGFR β intracellular tyrosines reduces the magnitude of phospho-ERK and phospho-Akt induction but does not completely abolish activation of these pathways, underscoring the phenotypic effect of quantitative differences in intracellular pathway activation.

While powerful in revealing the *in vivo* importance of specific effectors, these genetic experiments come with some important caveats. As with many loss-of-function approaches, these results do not distinguish between changes in signaling dynamics and total attenuation of a signaling event. Indeed, abrogation of PI3K binding to PDGFR α abolishes phospho-Akt induction entirely (Klinghoffer, Hamilton, Hoch, & Soriano, 2002), making it difficult to ascertain whether phospho-Akt induction or dynamics is critical. Alternatively, crosstalk with other signal transduction cascades may underlie the observed phenotypes, as disruption of one signaling event often leads to compensatory induction of others. Again taking the case of the *Pdgfra* allelic series, loss of PI3K binding leads to increased SHP-2 recruitment, which may be functionally relevant. Further, the temporal dynamics of tyrosine phosphorylation itself may be important for specifying different signals (Zheng, Zhang, Croucher, Soliman M, St-Denis, et al., 2013), and simple loss-of-function mutations do not provide insight regarding the function of phosphorylation kinetics. As discussed below, modern proteomic approaches show promise as a method to distinguish between these various possibilities, allowing systems-level measurements at high temporal resolution. Finally, the issue of cellular context is confounding: cell type specific requirements for an effector may occur independently of the receptor itself, perhaps based on the relative expression of a key signaling molecule (e.g. the receptor itself) or the local chromatin architecture permitting modulation of only a particular pathway's target genes in a given cell type. For example, the same receptor can induce different outcomes based on many context dependent parameters such as expression level, reflected by the observation that overexpressing the EGF receptor in PC12 neurons switches the effect of EGF stimulation from proliferation to differentiation (Traverse, Seedorf, Paterson, Marshall, Cohen, et al.,

1994). Nonetheless, these genetic experiments reveal that individual tyrosine residues (and the effectors that bind them) can function either independently or additively to specify developmental outcomes, hinting at the degree of mechanistic diversity at the very first step of signal transduction.

More recent phosphoproteomic approaches, in which the phosphorylation status of multiple residues can be simultaneously measured, have shed light on how different tyrosine phosphorylation patterns at the receptor encode distinct cellular outcomes. In one elegant study, the authors use proteomics to investigate the mechanisms underlying the specificity of fibroblast growth factor (FGF) signaling (Francavilla, Rigbolt, Emdal, Carraro, Vernet, et al., 2013). They demonstrate how two ligands, FGF7 and FGF10, drive different phosphorylation patterns at their shared receptor, FGFR2, thus modulating distinct intracellular pathway activation patterns and cellular outcomes within the same cell type. Harkening back to the importance of phospho-ERK duration established in the PC12 paradigm, FGF7 induces a sustained pERK response and drives cell proliferation while FGF10 results in a transient pERK response associated with cell migration in this system. This distinction originates from differential phosphorylation on a single FGFR2 intracellular tyrosine residue that binds PI3K subunits and acts as a switch between the two modes of signaling dynamics by regulating receptor degradation and recycling. Mutation of this tyrosine eliminates the differential response to FGF7 and FGF10, as FGFR2 is now 'locked' into the FGF7-proliferation axis and exhibits limited migration in response to FGF10. In addition, this single tyrosine mutation affects multiple signaling pathways in addition to PI3K, underscoring the high degree of crosstalk and complexity in linking individual phosphotyrosines to downstream pathway activation. This study provides an example in which a qualitative switch in tyrosine phosphorylation of a particular residue at the receptor results in altered signaling dynamics and cellular outcome, thus highlighting the distinction between information encoding at the receptor and intracellular pathway level. Such unbiased approaches to measure phosphorylation on a systems level will provide valuable insight into how patterns of intracellular tyrosine phosphorylation at the receptor encode downstream signaling.

With regard to the framework presented earlier, these results highlight the importance of dynamic and combinatorial encoding in the response to RTK activation, as evidenced by the requirement of multiple phosphotyrosines to coordinately regulate a shared set of pathways downstream of PDGFR β and the ability of FGF7 and FGF10 to induce distinct signaling kinetics through differential phosphorylation status of a single FGFR2 intracellular tyrosine residue. Further, when this FGFR2 phosphotyrosine residue is mutated, the signaling dynamics of multiple pathways are affected, arguing against a simple linear model in which each signaling event maps one-to-one to intracellular pathway activation and cellular outcome. In the case of PDGFR α , it is plausible that a similar mechanism may occur in mouse mutants where PI3K binding is disrupted at PDGFR α , and more generally, such mutations of a single tyrosine residue may have multiple effects on signal transduction that are difficult to predict *a priori*.

Intracellular signaling dynamics: all roads lead to ERK

The importance of quantitative differences in the kinetics and magnitude of intracellular pathway activation naturally leads to the question of how cells interpret such differences in signaling dynamics. Particular attention has been given to ERK, which can be activated in response to a diverse range of extracellular stimuli and result in many cellular outcomes, including proliferation, differentiation, and migration. The importance of differences in phospho-ERK duration for specifying distinct cellular outputs has been long appreciated in PC12 neurons; modern work investigating the basis for this phenomenon have illuminated the underlying mechanisms through which phospho-ERK dynamics are decoded to specify distinct outcomes. A recent study utilized optogenetics to selectively control ERK activation itself, allowing direct modulation of ERK signaling dynamics through control of upstream Ras activity (Toettcher, Weiner, & Lim, 2013); in contrast to loss of function studies (e.g. mutating individual tyrosine residues on the receptor or treating with pharmacological inhibitors), this type of approach defines signals for which ERK activation is sufficient and more generally, permits uncoupling of pathway activation from pathway dynamics. By combining their optogenetic tool with a reverse phase protein array, the authors delineate a proteomic ‘signature’ for distinct frequencies of ERK activity. Notably, not all proteins regulated by PDGF stimulation are ERK-responsive, indicating ERK alone is not sufficient to drive the full RTK response and highlighting the utility of this approach in delineating sufficiency from necessity. Thus, these other responses are either ERK-independent or more likely, require multiple intracellular pathway inputs, again underscoring the importance of combinatorial encoding.

The advent of computational models describing the RTK signaling network has advanced our understanding of how dynamic changes in an input encodes a desired output. The iterative refinement of such models based on experimental results designed to test model fidelity is a powerful method for understanding mechanisms of RTK specificity, as evidenced by a study focusing on how different phospho-ERK durations regulate downstream transcription (Nakakuki, Birtwistle, Saeki, Yumoto, Ide, et al., 2010). The authors initially build a model for how the transcription factor *Fos* distinguishes between transient and sustained phospho-ERK, with the primary parameters being (1) the combination of nuclear phospho-ERK and nuclear phospho-RSK (to drive *Fos* transcription) and (2) cytoplasmic phospho-ERK and Fos protein (to generate phosphorylated Fos protein). However, upon finding experimental results are not in full concordance with this model’s predictions, an updated model incorporating a transcriptional repressor is developed that does fit the observed data, making the system more robust to changes in input parameters and minimizing noise. Thus, the decoding of differences in ERK dynamics requires an intricate network integrating changes at the transcriptional and translational levels. Although the transcriptional repressor itself was not identified in this study, one can imagine the biological importance of such a gene that acts as a superintendent of the core transcriptional response to RTK signaling. Of note, both the optogenetics and computational modeling studies discussed above extrapolated their results to PC12 cells, highlighting the relevance of the PC12 paradigm over two decades later.

While elegant, a critical drawback to these studies is they have been primarily conducted *in vitro*, and it is not obvious how to extrapolate these parameters to an *in vivo* system. One potentially promising technology to interrogate these processes is live imaging using biosensors of ERK activity, which has revealed intriguing properties of phospho-ERK signaling patterns both *in vitro* (Aoki, Kumagai, Sakurai, Komatsu, Fujita, et al., 2013) and *in vivo* (Hiratsuka, Fujita, Naoki, Aoki, Kamioka, et al., 2015). In the first study (Aoki, Kumagai, Sakurai, Komatsu, Fujita, et al., 2013), the authors use a FRET biosensor to demonstrate that the frequency of ERK pulsing is correlated with cell proliferation in a density-dependent manner *in vitro*. By utilizing a light dependent ERK activation system, they then confirm that synthetically producing ERK oscillations drives a greater proliferative response than continuous ERK induction, demonstrating the functional outcome of alterations in ERK signaling dynamics. In a second study (Hiratsuka, Fujita, Naoki, Aoki, Kamioka, et al., 2015), the same group extended their FRET-based analysis of ERK dynamics to the skin, recording changes in ERK dynamics *in vivo*. At steady-state, the authors observed occasional ‘ripples’ of ERK activation, with spontaneous ERK pulses beginning at foci and then migrating radially outward. This radial propagation of ERK activity was associated with cell proliferation, and the authors use a combination of pharmacological agents to induce or inhibit mitotic activity to confirm this relationship. Such approaches to both visualize and control ERK dynamics *in vivo* will facilitate the assignment of functional roles for different modes of ERK signaling in developmental processes.

One context, two receptors, two responses: PDGF and FGF in the midface

The above examples center on the mechanisms through which a single receptor or intracellular pathway can mediate multiple cellular outcomes. However, an equally critical question is how two different RTKs elicit divergent responses. To address this issue, we recently compared the transcriptional responses to PDGF and FGF signaling in the palate (Vasudevan, Mazot, He, & Soriano, 2015), a context of particular interest given the palatal clefting phenotypes observed upon loss of either RTK. Interestingly, we found divergent gene expression programs and cellular outcomes in response to PDGF and FGF. These differences seem to stem from differences in intracellular pathway usage, with PDGF regulating a PI3K-dependent differentiation axis and FGF driving an ERK-dependent proliferation circuit. In addition to this qualitative delineation, the FGF-mediated phospho-ERK response displayed a greater magnitude and longer duration compared to the PDGF-mediated phospho-ERK response. Building upon the differential gene expression profiles observed upon PDGF and FGF stimulation in the palate, we further explored the function of a single shared target gene, Serum Response Factor (SRF) (Vasudevan & Soriano, 2014). Although both PDGF and FGF induce SRF mRNA and protein, the two RTKs promote different cofactor interactions and consequently, distinct gene expression programs, adding yet another layer of regulatory complexity.

These studies illustrate two important lessons. First, intracellular signaling dynamics are a critical parameter when comparing the effects of two different RTKs. For example, the PDGF and FGF-mediated phospho-ERK response in the palate is nearly identical after 5 minutes; only upon measuring phospho-ERK at multiple timepoints is a difference

appreciated, and this likely contributes to the ERK-dependence of FGF-regulated proliferation but not PDGF-mediated differentiation. Second, the integration of many parameters encodes the resultant gene expression program; in the case of SRF activity downstream of PDGF and FGF, intracellular pathway dynamics, mRNA induction, and protein-protein interactions are all key inputs delineating the ultimate transcriptional output. Further experimental and computational work is needed to fully define the full range and relative weights of the many input parameters that define the transcriptional response to RTK signaling.

Concluding remarks

We began this essay by putting forward two models for RTK signal specificity: a linear model where distinct intracellular pathways lead to particular cellular outcomes and a dynamic model in which the magnitude and duration of pathway activation encode distinct outcomes. As research into receptor tyrosine kinases and cell signaling has progressed over the last two decades, the linear model suggested by early genetic and pharmacologic experiments has given way to a more complex system in which receptor tyrosine kinases convert growth factor inputs into a myriad of intracellular signals with specific magnitudes and frequencies, which are further fine-tuned by a complex network of feedback regulation. In many respects, seminal studies in PC12 neurons were a harbinger of this shift, providing an early example of dynamic encoding that recent studies often return to for experimental validation. Despite the considerable progress toward unraveling the mechanisms underlying RTK signal specificity, our understanding remains far from complete. Two points of particular interest are how patterns of tyrosine phosphorylation at the receptor map to intracellular pathway activation and how signaling dynamics on a single cell level translate into behaviors within a population.

It is well appreciated that either loss of function or gain of function mutations in phosphotyrosine residues can have detrimental effects, but a systematic understanding of how these mutations alter intracellular pathway activation and dynamics is lacking. Can each loss of function mutation be linked to a particular set of intracellular pathways, and are any such dependencies 'hard-wired' into the signaling network or context dependent? Conversely, are all gain-of-function mutations created equal, or do different mutations at the receptor lead to distinct patterns of intracellular pathway activation? By applying phosphoproteomic approaches to obtain an unbiased picture of the signaling events mediated by each mutation, empirical answers to these questions are beginning to emerge. We believe combining this signaling data with genetic approaches to interrogate the developmental function of individual phosphotyrosine residues *in vivo* will provide a detailed understanding of how RTK mediated outcomes are generated.

Both the optogenetic and FRET-based studies discussed in this essay observed a degree of cellular heterogeneity, with the responses of individual cells within a population exhibiting high variability. Similarly, in the classic PC12 system, not all cells treated with NGF undergo cell differentiation. Thus, it is naturally of interest to examine how much variability in signaling dynamics is present during development and if there is an underlying organization to the heterogeneity of responses within a population. One could imagine the

multiple lineages that comprise a given tissue to be differentially primed for RTK responsiveness, providing an intuitive basis for such heterogeneity *in vivo*. Recently, a single cell RNA-seq study revealed marked variation within populations of dendritic cells identically treated with various stimuli *in vitro* (Shalek, Satija, Shuga, Trombetta, Gennert, et al., 2014), and we suspect the complexity of RTK signaling coupled with the high diversity of cell types within the embryo will only amplify this effect for growth factors utilized across multiple developmental processes. It will be enlightening to determine if particular transcriptional programs predispose a cell to respond in a specific manner and further, how the expression of various components within the transcriptional feedback/feedforward loops vary at the single cell level, both at baseline and in response to RTK signaling. To our knowledge, no such study comparing the response to different RTKs or simultaneously interrogating multiple intracellular pathways in addition to phospho-ERK has been carried out. Taken together, such information may contribute toward a predictive model utilizing gene expression, proteomic, and other data types as an input to identify those cells and tissues most likely to execute a particular RTK response.

Modern technologies allow elegant manipulation of specific nodes within an intracellular pathway, perturbation of pathway dynamics without affecting pathway activation, and the measurement of signaling parameters on a systems level, in many cases at single cell resolution. Applying these techniques to the developing embryo will elucidate the answer to a number of outstanding issues involving RTK specificity. The consequent knowledge will pay great dividends in understanding how growth factors dictate cellular behaviors, laying the requisite foundation for studying how such processes can be pathologically corrupted. More broadly, the conceptual frameworks and emerging technologies outlined above can likely be expanded beyond RTKs to any number of signaling events during development (Hoch & Soriano, 2015), and insights gleaned across the full range of signaling processes in the embryo will further our understanding of the critical parameters underlying cellular decision making.

Acknowledgments

Work in the author's laboratory is supported by grants to P.S. from NIH/NIDCR (RO1DE022363 and RO1 DE022778) and NYSYSTEM (IIRP N11G-131). H.N.V. was supported by NIH/NIDCR fellowship F31 DE023456.

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