MAPK signal specificity: the right place at the right time

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Although the mechanisms that lead to activation of the Ras, extracellular-signal-regulated kinase mitogen-activated protein kinase (Ras/ERK-MAPK) signaling pathway have been studied intensively, the fundamental principles that determine how activation of ERK signaling can result in distinct biological outcomes have only recently received attention. Factors such as cell-surface receptor density, expression of scaffolding proteins, the surrounding extracellular matrix, and the interplay between kinases and phosphatases modulate the strength and duration of ERK signaling. Furthermore, the spatial distribution and temporal qualities of ERK can markedly alter the qualitative and quantitative features of downstream signaling to immediate early genes (IEG) and the expression of IEG-encoded protein products. As a result, IEG products provide a molecular interpretation of ERK dynamics, enabling the cell to program an appropriate biological response.

Dynamics of ERK signaling and downstream interpretation

Hormonal activation of cell-surface receptors results in a cascade of events that promotes activation of Ras and, ultimately, dual phosphorylation and activation of the extracellular-signal-regulated kinases (ERKs) (Figure 1). Activated ERKs phosphorylate and activate downstream targets such as the family of ~90-kDa ribosomal S6 kinases (RSKs), which consists of RSK1 to RSK4 and mitogen- and stress-activated kinases 1 and 2 [1]. ERK1 and ERK2 (hereafter ERK1/2) and RSK1/2 can also translocate into the nucleus where, together with nuclear members of the RSK family, they phosphorylate several important transcriptional regulators including Elk-1, CREB and histone H3 [2]. The result is rapid transcriptional activation of IEGs, including transcription factors that control the cell cycle and/or cell survival.

Nearly all growth factors and cytokines activate ERK and RSK, induce their translocation to the nucleus, and promote activation of the same IEGs. Yet these growth factors and cytokines can evoke markedly different biological responses. How do specific cellular responses result from diverse extracellular stimuli that converge on the ERK/IEG signaling cassette? Here we propose that signal specificity is derived from the ability of IEG-encoded protein products to respond to changes in the spatial and temporal dynamics of ERK activity.

Why is signal duration important?

More than ten years ago, Marshall [3] proposed a model to explain how the inactivation kinetics or signal duration of ERK could regulate a cell fate decision. The basis for this model came from several studies showing that sustained, but not transient, activation of ERK signaling preceded the differentiation of rat PC12 pheochromocytoma cells into sympathetic-like neurons. This correlation between the duration of ERK signaling and distinct cell behavior has been also documented in fibroblasts [4,5], macrophages [6] and T lymphocytes [7,8]. Thus, normal cells use ERK signal duration as a way to drive many biological processes such as cell-cycle control.

Importantly, mutations in key pathway components that result in sustained ERK activation correlate with carcinogenesis. For example, the overall frequency of Ras mutations in cancer is ~30%, but >90% of pancreatic cancers have mutations in K-Ras and individuals affected with Costello syndrome have a high incidence of H-Ras mutations and are predisposed to tumor formation [9]. Mutations in B-raf are found in 67% of melanomas and at a lower frequency in colon and thyroid cancers. Not surprisingly, small-molecule inhibitors of the Ras/ERK pathway have been intensely pursued for therapeutic purposes. Such agents could inactivate constitutive oncogenic signaling, thereby leading to an increase in apoptosis and/or limiting the tumorigenic potential of the cancer cells. Thus, in cancers in which ERK activity is required for proliferation and survival, such as melanomas, inhibition of this pathway might be particularly beneficial [10].

Receptor density and trafficking

Studies using the PC12 model of neuronal differentiation provided the first evidence that receptor numbers can alter the outcome of ERK signaling. When treated with nerve growth factor (NGF), PC12 cells express neuronal markers and show neurite outgrowth. Such differentiation is preceded by sustained activation of ERK signaling for up to 24 h. When treated with epidermal growth factor (EGF) or insulin, by contrast, PC12 cells show a weak proliferative response and do not differentiate.
Importantly, EGF induces potent transient activation of the ERK pathway, whereas insulin causes weak activation (Box 1). Overexpression of either EGF receptors or insulin receptors, however, results in sustained ERK activation and differentiation [11,12].

In support of a hypothesis based on receptor signal strength, clonal isolates of PC12 cells expressing lower numbers of NGF receptors do not undergo differentiation in response to NGF [13]. Similarly, we have shown that changing the amount of receptor occupancy by lowering agonist concentrations (thereby lowering signal strength) also alters the duration of ERK signaling and the proliferative response in fibroblasts [14]. Furthermore, a frequent hallmark of tumor-derived cells is the overexpression and/or amplification of cell-surface growth factor receptors, resulting in the aberrant activation of downstream signaling in the absence of extracellular ligands.

In addition to the impact of receptor density on ERK signal strength and duration, the rate and extent of receptor internalization also contribute to ERK signaling. Thus, not only is receptor internalization an important control point for signal termination, but it can also result in further signaling by the internalized receptor–ligand complex from a different cellular location.

Receptor internalization and trafficking are best understood for members of the EGF receptor family [15].

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**Box 1. Unanswered questions**

- What causes the rapid inactivation of ERK signaling?
- What is the significance of Ras/ERK signaling at the ER and Golgi?
- What is the role of MKPs in controlling ERK activity during the cell cycle?
- As signal strength increases, what pathways become engaged that contribute to sustained ERK activity?
The EGF receptors ErbB1, ErbB2, ErbB3 and ErbB4 show basal levels of internalization into endosomes, but only the EGF receptor shows accelerated internalization after ligand binding [16]. Ligand-bound EGF receptors are internalized via the dynamin/clathrin-mediated endocytic pathway into early endosomes, where they are either recycled back to the plasma membrane or processed for lysosomal degradation (Figure 1). The process underlying the decision to degrade or to recycle is not well understood, but it might be determined by the nature of the ligand that is bound. For example, receptors associated with transforming growth factor-β are recycled back to the plasma membrane, whereas those associated with EGF are destined for lysosomal degradation [17].

Ligand-bound EGF receptors internalize into endosomes at a much faster rate than unbound receptors [16], but the key events that accelerate internalization of EGF receptors are poorly understood. Several studies have indicated that the E3 ubiquitin ligase c-Cbl binds to and monoubiquitinates the C terminus of the EGF, platelet-derived growth factor (PDGF), hepatocyte growth factor and colony-stimulating factor 1 receptors, thereby promoting the trafficking of these receptors to the lysosome [16,18]. The ErbB2 receptor does not bind c-Cbl, which could explain both its tendency to be recycled and its oncogenicity. The signals that control the choice between receptor recycling and degradation are unknown, but are likely to be chief determinants of signal duration, location and specificity.

Internalized activated receptors can also signal from the endosome. For example, expression of an endocytosis-defective dynamin mutant that blocks internalization of the EGF receptor has been found to prevent maximal EGF-induced ERK activation [19]. Recent observations show that internalized EGF, PDGF and insulin receptors colocalize with the endoplasmic reticulum (ER)--associated protein tyrosine phosphatase 1B (PTP1B), and this colocalization acts as the inactivation step before receptor degradation [20–22]. EGF receptors destined for lysosomal degradation are trafficked to late endosomes, but even in these structures the active receptors might still be able to regulate ERK signaling. The ERK scaffold protein MP1, which is a partner protein of MAPK kinase 1 (MEK1), is recruited to late endosomes by the adaptor p14 and can regulate ERK activation during sustained signaling [23,24].

**Ras**

The GTPase Ras is activated downstream of many receptor tyrosine kinases and is essential for ERK activation. All three Ras isoforms (N-Ras, K-Ras and H-Ras) are expressed ubiquitously and are modified by farnesyl groups, resulting in their association with membranes. Until recently, it was widely assumed that the active GTP-bound Ras was located mainly at the plasma membrane in close proximity to activated cell-surface receptors. Several studies indicate, however, that this model is too simplistic and that specific Ras isoforms have an important role in spatial and temporal ERK signaling.

A specific cysteine residue in the C terminus of each Ras isoform is farnesylated in the cytosol; the farnesylated Ras protein then associates with the ER, where the modified cysteine is methylated [25]. N- and H-Ras are additionally mono- and di-palmitoylated, respectively, at neighboring cysteines and transported to the plasma membrane via the secretory pathway. K-Ras bypasses the secretory pathway and integrates into the plasma membrane via a polybasic sequence adjacent to its farnesylated cysteine. Although all three Ras isoforms bind GTP and become activated at the plasma membrane within minutes of growth factor treatment, activation of H-Ras can also take place on the ER and Golgi in fibroblasts, PC12 and Jurkat T cells [26,27]. Interestingly, activation of H-Ras on the plasma membrane is more rapid than activation of Golgi-localized H-Ras, which is delayed but sustained by comparison [26].

In contrast to these studies, activation of glutamate receptors on hippocampal neurons results in a rapid redistribution of K- but not H-Ras from the plasma membrane to intracellular membranes including the Golgi [28]. Palmitoylation of N- and H-Ras can be reversed, resulting in their release from the plasma membrane and their relocation to Golgi membranes [29]. This retrograde transport of N- and H-Ras occurs irrespective of their activation state [29], but the exact components of the retrograde pathway are currently unknown [30].

Interestingly, Src signaling via phospholipase Cγ can activate H-Ras at the Golgi in a process that is independent of receptor tyrosine kinase activation at the plasma membrane [27]. Perhaps because of their proximity to the nucleus, the Golgi and ER signaling platforms are uniquely positioned to promote efficient ERK-dependent transcriptional responses long after the activation of cell-surface receptors (Box 1). Notably, in contrast to K-ras<sup>−/−</sup> mice, doubly deficient N-ras<sup>−/−</sup>/H-ras<sup>−/−</sup> mice develop normally and are viable [31–33]. Thus, assuming that there is no compensation from other Ras molecules in the N-ras<sup>−/−</sup>/H-ras<sup>−/−</sup> mice, these observations suggest that Ras signaling from the ER and Golgi is dispensable for normal development. Clearly, our understanding of the spatial and temporal aspects of Ras signaling remains limited.

In an alternative view of how Ras family proteins lead to variations in ERK activation kinetics, different Ras pathways could couple to distinct receptors. Data obtained from PC12 cells suggest that the Ras relative Rap1b shows sustained activation after treatment with NGF but not EGF [34,35], whereas activation of Ras is transient with EGF or NGF. Thus, a combination of transient Ras and sustained Rap1b activation could account for the generation of distinct ERK kinetics. NGF-regulated sustained activation of ERK signaling seems to be mediated by NGF-receptor-specific recruitment of the adaptor FRS2 and the Rap exchange factor C3G [34]. The general role of Rap1b in mediating sustained ERK signaling remains, however, controversial [36]. Indeed, if Rap1b couples to the NGF receptor, then it is unclear why reducing levels of the NGF receptor results in transient activation of ERK signaling [13]. We propose that receptor density, in combination
with recruitment of Rap1b or a similar regulator, could be responsible for generating distinct signal kinetics in PC12 cells.

**Modular interactions proximal to ERK**

Recent studies have established that activation of ERK1/2, phosphorylation of their downstream targets and their subsequent inactivation by phosphatases require distinct types of protein–protein or docking interaction. For MEK, RSK and some phosphatases, binding to ERK requires a ‘docking’ (D) domain class of docking site [37]; by contrast, for several physiological substrates, many of which are nuclear, binding to ERK requires a ‘docking site for ERK, [Phe/Tyr]-Xaa-[Phe/Tyr]-Pro’ (DEF) domain [38,39]. Whereas D domains bind to a region in ERK known as the ‘common docking’ (CD) domain [40], DEF domains bind to a set of residues that are proximal to the catalytic pocket in ERK [41].

An additional region in ERK, called the kinase interaction motif (KIM), is also important for binding to MEK [42]. Although not yet proved, it is likely that the binding of various D-domain and DEF-domain partner proteins to ERK is in constant equilibrium and thus can be altered by changes in localization and/or the subcellular concentration of ERK and its partner proteins (see later). Consistent with this notion, the binding of RSK1/2 to ERK1/2 before growth factor stimulation is disrupted after activation and relocation of ERK from the cytoplasm to the plasma membrane and then to the nucleus, which exposes the CD domain for a new round of interaction with other D-domain targets [43,44]. Importantly, binding of ERK to D and DEF domains is not mutually exclusive [45] and several ERK targets such as ELK-1 and JunD contain both classes of docking site [46,47], suggesting that an ERK dimer can engage these domains simultaneously.

The *in vivo* role of ERK signaling via D and DEF domains has been recently investigated [45]. Although early studies suggested that the CD domain and the KIM mediated efficient ERK activation [40,42], it is now known that the CD domain is not required *in vivo* [45]. It is, however, required for efficient activation of RSK *in vivo* [45], a finding consistent with previous results [43,48,49]. The induction of ERK target genes such as egr-1, and the phosphorylation of c-Fos, takes place independently of D-domain interactions but requires ERK signaling to DEF domains [45]. Importantly, it is possible to inhibit selectively either mode of docking without disrupting ERK-mediated phosphotransfer.

As mentioned, phosphatases containing D domains can bind directly to ERK and can dephosphorylate threonine and tyrosine residues in its activation loop. These enzymes, known as MAPK phosphatases (MKPs) are members of the dual specificity phosphatase family of PTPs [50]. Some PTPs, such as striatal enriched PTP (STEP) and hematopoietic PTP (He-PTP), can also bind to ERK but dephosphorylate only phosphotyrosine [50]. The significance of mono- versus di-dephosphorylation might be that negative regulators such as He-PTP generate a primed pool of ERK that is preferentially activated by MEK. If D-domain-containing phosphatases are required for inactivation of ERK signaling, then disrupting this interaction should result in prolonged ERK activation; however, only subtle changes in the inactivation kinetics of ERK have been observed *in vivo* [40,51]; J. MacKeigan and J. Blenis, unpublished). It is possible that, in addition to the CD domain that binds phosphatases, ERK has other interaction surfaces for these regulators – the KIM, for example – that can compensate for a loss of CD interactions.

Although it has been recognized for many years that MKPs such as MKP1/2 are encoded by IEGs that are transcriptionally upregulated in response to growth factors [52,53], it is unclear why ERK signaling is not completely inactivated in these situations [39] (Box 1). A likely explanation for this observation is that the strength of the MKP1/2 negative feedback is outbalanced by the still active upstream inputs to ERK. Because overactivation of Ras/ERK signaling in some contexts can result in senescence, cell-cycle arrest and/or apoptosis [54–56], it is also possible that induction of MKP1/2 might dampen ERK activity during the G1/S transition. In support of this idea, treatment with cycloheximide results in hyperactivation of ERK signaling 1–2 h after initial growth factor stimulation, indicating that *de novo* synthesis of gene products such as MKP1/2 is involved in reducing the strength and duration of ERK signaling [57].

It is important to remember, however, that in situations in which growth factors induce extremely transient activation of ERK signaling [39,58], the MKP1/2 gene products appear after signaling is terminated, indicating that these factors are not the key molecules that promote rapid inactivation. Furthermore, because MKP1/2 are localized to and dephosphorylate ERK in the nucleus [59,60], they are unlikely to be involved in the mechanisms that inactivate cytoplasmic ERK signaling. In these situations, constitutively expressed MKP3 might be responsible for inactivating ERK signaling. MKP3 shows selectivity for ERK1/2 over the stress-activated MAPKs [50], and its specific activity increases after docking to ERK1/2 [61]. In addition to the MKPs, the ratio of cytoplasmic to nuclear active ERK can be regulated by genes encoding Sef (similar expression to Fgf) [62], a transmembrane protein that is thought to localize to the Golgi. Sef prevents dissociation of the MEK–ERK complex, thereby inhibiting translocation of ERK to the nucleus; however, ERK can still signal to cytoplasmic targets [63]. Notably, additional negative feedback control of ERK activity can be mediated by the Sprouty family of proteins [64], as has been recently reviewed elsewhere [65].

In some cases, the docking of ERK to binding partners containing D domains can be regulated by phosphorylation in or around the D domain. For example, disruption of ERK binding to RSK1/2 occurs through ERK-mediated phosphorylation of the Ser749 residue that lies proximal to the C-terminal D domain in RSK, and mutation of this residue confers constitutive binding [44]. The MEK, ERK is maintained in an inactive state owing to its constitutive binding to the He-PTP phosphatase. On phosphorylation of the D domain in He-PTP by protein kinase A, however, ERK is released and is activated by MEK [66]. In primary striatal neurons, the STEP phosphatase is inactive owing
to phosphorylation of its D domain. After treatment with glutamate, which activates N-methyl-D-aspartate receptors and leads to an increase in intracellular Ca\(^{2+}\), STEP is rapidly dephosphorylated by the Ca\(^{2+}\)-dependent phosphatase calcineurin and then physically interacts with ERK1/2, resulting in dephosphorylation of its activation-loop tyrosine \([67]\). Interestingly, STEP has been reported to localize to the ER, lending further support to the idea that sustained ERK signaling emanates from intracellular membrane compartments and not the plasma membrane.

**Downstream sensing of transient and sustained signaling**

Although the importance of signal duration and strength is generally accepted, we have only recently gained a detailed understanding of how the dynamics of ERK signaling are interpreted at the molecular level. It was previously assumed that nuclear signaling by ERK would underlie signal specificity; however, growth factors that trigger transient and sustained signaling induce similar patterns of IEG expression \([39, 68–70]\). Thus, differences in the initial transcription program alone cannot account for cell fate decisions that occur downstream of ERK. Crucially, the expression kinetics of IEG-encoded protein products, such as c-Fos, is marked in response to agonists that induce sustained rather than transient ERK and RSK activation \([14, 39]\).

The c-Fos protein is very unstable (half-life, \(t_{1/2} \approx 30\) min) and will accumulate only if its C terminus is phosphorylated under conditions of sustained ERK activation (Figure 2). Because c-Fos is an important component of the dimeric AP-1 transcription factor, an increase in its stability results in greater promoter occupancy and expression of target genes \([39]\) (L.O. Murphy and J. Blenis, unpublished) and in cellular transformation \([71, 72]\). Accordingly, expression of the late-response gene \(Fra-1\), a target of c-Fos \([73]\), is sustained only after prolonged expression of c-Fos \([14]\). Thus, the behavior of c-Fos after transient or sustained ERK signaling enables the cell to distinguish among agonists that induce different activation kinetics. Therefore, by its ability to exist in unstable and stabilized states, the c-Fos transcription factor can function as a ‘sensor’ for ERK activation dynamics.

In addition to increasing stability, the C-terminal modification of c-Fos enhances the process of ERK-mediated phosphorylation of Thr325 and Thr331 within its C terminus \([39]\). These phosphorylation events are dependent on a DEF domain in c-Fos, and the integrity of this domain is required for cellular transformation \([39]\). Thus, ERK- and RSK-regulated phosphorylation at the extreme C terminus serves to ‘prime’ c-Fos for ERK docking and for further phosphorylation at Thr325 and Thr331 (Figure 2). Interestingly, the priming phosphorylation sites are located in a region of c-Fos that has been characterized as a transrepression domain \([74]\), and they are replaced by an irrelevant sequence in the oncogenic homolog p55 v-Fos. Although v-Fos has the same stability as phosphorylated c-Fos \([71, 72, 75]\), it does not contain a functional DEF domain because this is also replaced by an irrelevant sequence (L.O. Murphy and J. Blenis, unpublished).

On the basis of these observations, we propose that the DEF domain and phosphorylation of Thr325 and Thr331 are required to drive c-Fos into an activated state (during sustained signaling) and that the unique C terminus in v-Fos adopts a conformation that mimics the same activated state. Notably, unless it is constitutively expressed, the c-Fos sensor is never exposed to the maximal level of ERK activity detected in cells immediately after the addition of growth factors, because endogenous c-Fos is expressed minimally in most non-growing cells during the initial phase of ERK activation. As c-Fos accumulates, however, it becomes hyperphosphorylated during the submaximal phase of ERK signaling in G1. We suspect that the priming phosphorylation sites act as a gating mechanism to enable efficient hyperphosphorylation via the DEF domain only if and when signaling reaches a threshold strength and duration.

An apparent problem with this model is that fibroblasts that are deficient in \(c-fos\) proliferate normally \([76]\). This paradox is more apparent than real, however, because additional Fos-like IEG-encoded sensors of ERK can compensate for the lack of c-Fos expression \([14]\). Notably, fibroblasts deficient in both \(c-fos\) and \(fosB\) are compromised in their ability to undergo DNA synthesis \([77]\).
Although FosB does not have a DEF domain motif, it contains similar C-terminal priming residues and a phospho-mimetic aspartic acid in a position identical to Thr325 in c-Fos, which drives transformation [39]. In addition to FosB, the Fos-related transcription factors Fra-1 and Fra-2, and other IEG products, contain functional DEF domains and associated phosphorylation sites and, similar to c-Fos, propagate signals after growth factor treatment [14]. Notably, the induction kinetics of IEG products such as c-Fos, c-Myc, JunB and Fra-1 is non-identical, resulting in a cascade of potential IEG-encoded ERK sensors that coordinates progression through G1 (Figure 2).

Importantly, not all ‘sustained’ ERK signals are created equally. In response to different agonists, ERK signals sustained 4h after stimulation can be quantitatively different (e.g. 20% versus 40% maximal), leading to differential effects on the expression, phosphorylation and biological activity of IEG sensors during G1. This observation might explain, in part, why some mitogens are more potent than others in triggering DNA synthesis even though they induce similar IEGs. Thus, the sensitivity that IEG-encoded sensors show for ERK signal strength and duration, coupled with the fact that the transcriptional induction kinetics of IEGs is staggered, represents one of the principal specificity determinants downstream of ERK signaling (Figure 2). Although in mammalian cells ERK activation is graded in response to an increasing stimulus [78,79], induction and hyperphosphorylation of the ERK sensor c-Fos is ultrasensitive [78]. In contrast to mammalian cells, ERK activation in *Xenopus* oocytes can be bistable or irreversible owing to a strong positive feedback loop regulated by the Mos kinase [80]. Although the lack of a similar feedback loop in fibroblasts explains why ERK activity is graded in mammalian cells, we speculate that the switch-like behavior seen in *Xenopus* oocytes is conserved in this pathway but is located at the level of IEG induction and/or phosphorylation [78] (Figure 3). The identification of signaling mechanisms that control the ERK–IEG switch-like behavior deserves attention (Box 1).

**Concluding remarks**

Sustained activation of ERK signaling is involved in cell-cycle progression, cellular transformation and differentiation. The stabilization of IEG-encoded transcription factors takes place downstream of sustained signaling and results in an increase in target gene expression. Stabilization of IEG products containing DEF domains also might indirectly affect the dynamics of nuclear and/or cytoplasmic ERK signaling. The localization and subcellular concentration of ERK will dictate with which of the many binding partners the kinase will interact. Similarly, an increase in concentration of a binding partner, such as an IEG-encoded DEF-domain-containing sensor, will shift the equilibrium towards DEF binding and away from other classes of partner such as D-domain-containing phosphatases.

We propose that an increase in the abundance of nuclear IEG-encoded DEF domain targets after ERK activation will result in nuclear retention of active ERK, via DEF binding, and will sustain its activity in the nucleus by protecting it from nuclear MKPs (Figure 3). In support of this model, inhibition of protein synthesis with cycloheximide, which inhibits the expression of IEG products, decreases nuclear localization of ERK in serum-stimulated CCL39 fibroblasts [81]. Thus, we propose that IEG-encoded sensors have the ability not only to interpret ERK signal strength and duration but...
also to feedback positively to ERK by sustaining its presence and signaling in the nucleus.

Recent advances have revealed mechanisms that control temporal and spatial aspects of ERK signaling by modulating receptor activity, localization of Ras, Raf and phosphatases, and signaling in the nucleus where many ERK effectors are located. Differences in ERK kinetics and localization are then interpreted by several IEG products with DEF domains that physically interact with activated ERK. Many cancers are known to have manipulated these pathway control points to their advantage. In particular, activating mutations in Raf and overexpression of IEGs such as c-fos, c-jun and c-myc are a hallmark of several malignancies. In such cases, therapeutic targeting of the ERK/DEF domain signaling axis could be beneficial.

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