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Review

Regulation of MAPKs by growth factors and receptor tyrosine kinases☆

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Abstract

Multiple growth- and differentiation-inducing polypeptide factors bind to and activate transmembrane receptors tyrosine kinases (RTKs), to instigate a plethora of biochemical cascades culminating in regulation of cell fate. We concentrate on the four linear mitogen-activated protein kinase (MAPK) cascades, and highlight organizational and functional features relevant to their action downstream to RTKs. Two cellular outcomes of growth factor action, namely proliferation and migration, are critically regulated by MAPKs and we detail the underlying molecular mechanisms. Hyperactivation of MAPKs, primarily the Erk pathway, is a landmark of cancer. We describe the many links of MAPKs to tumor biology and review studies that identified machineries permitting prolongation of MAPK signaling. Models attributing signal integration to both phosphorylation of MAPK substrates and to MAPK-regulated gene expression may shed light on the remarkably diversified functions of MAPKs acting downstream to activated RTKs.

Keywords: Cancer; Growth factor; Signal transduction; Transcription; Tyrosine kinase

1. Introduction

Critical cellular decisions such as proliferation, migration and differentiation, are regulated by stimulatory cues from the extracellular environment, primarily growth factors, extracellular matrix (ECM) proteins and adhesion molecules presented on the surface of neighboring cells. These extracellular cues are converted to a cellular response through their binding to specific receptors present at the surface of the recipient cell. Many growth factors (GFs) bind and activate transmembrane glycoproteins of the receptor tyrosine kinase (RTK) family [1,2]. All RTKs contain an extracellular ligand binding domain, a single transmembrane domain, and an intracellular part that contains a tyrosine kinase domain and several regulatory tyrosines, which are modified through auto- or trans-phosphorylation. Upon binding to their respective receptors, GFs drive the formation of receptor dimers, leading to the activation of the intrinsic tyrosine kinase domain [3]. Subsequent phosphorylation of specific tyrosines enables the recruitment of various signaling adaptors containing Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains [4].

Much understanding has been gained in recent years, by solving the atomic structure of several RTKs, for how a growth factor may promote receptor activation. In this respect, one extensively studied RTK is the epidermal growth factor receptor (EGFR). According to the reported atomic structures [5,6], epidermal growth factor and transforming growth factor α (TGFα binding to two non-contiguous extracellular subdomains of the receptor, results in a conformational change that induces the release of a "dimerization loop", which is otherwise held in a closed conformation. As a result the "dimerization loop" protrudes from each ligand bound-monomer and facilitates the formation of a receptor dimer. In the intracellular side, the kinase of monomeric receptors is kept in an inactive state. This results from stabilization of the activation loop located at the carboxyl lobe of the kinase domain, through an intramolecular interaction with the amino-terminal lobe [7]. The induction of a receptor dimer induces kinase activation by driving the formation of an asymmetric kinase-dimer, in which the C-lobe of one kinase domain interacts with the N-lobe of the second kinase domain. This facilitates formation of a salt bridge

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in the N-lobe and the release of the intramolecular activation loop, resulting in its proper positioning for catalysis. Thus, one kinase in a dimer activates the other through an allosteric mechanism. Yet, for other RTKs, processes like trans-phosphorylation of the activation loop or of the juxtamembrane regions are required for the stabilization of the activation loop in an active conformation [8,9].

Kinase activation induces phosphorylation of tyrosine residues located at the cytoplasmic tail of the receptor, as well as phosphorylation of effector proteins, which are physically recruited to the active receptor. This step simultaneously initiates multiple signal transduction pathways, schematically depicted in Fig. 1. The four best characterized signaling pathways induced by RTKs are the mitogen-activated protein kinase cascades (MAPKs), the lipid kinase phosphatidylinositol 3 kinase (PI3K), a group of transcription factors called Signal Transducers and Activator of Transcription (STAT), and the phospholipase Cγ (PLCγ) pathway. Activation of these signaling pathways results in modulation of target proteins, as well as activation of transcription factors, leading to cellular alterations. After reviewing these pathways, we concentrate on GF-activated MAPKs, their linear organization and functional features, as well as effects on cell behavior and ability to integrate incoming signals.

2. Signaling pathways activated by growth factors

2.1. The PLCγ signaling pathway

PLCγ is recruited to an activated GF receptor through the binding of its SH2 domain with phosphorylated tyrosines on the receptor, and as a result it undergoes tyrosine phosphorylation [10]. This phosphorylation, along with a translocation to the plasma membrane, results in enzyme activation. Active PLCγ hydrolyzes phosphatidylinositol 4, 5 bisphosphate ($PtdIns(4,5)P_2$) to form two second messengers, diacylglycerol and $Ins(1,4,5)P_3$. Binding of $Ins(1,4,5)P_3$ to specialized receptors on the membrane of the endoplasmic reticulum (ER) leads to Ca^{2+} release. Free cytosolic Ca^{2+}, together with diacylglycerol, can then activate certain members of the protein kinase C (PKC) family, resulting in the phosphorylation of various effector proteins. In addition, high cytosolic Ca^{2+} initiates the activation of calcium/calmodulin-dependent protein kinases and phosphatases [11].

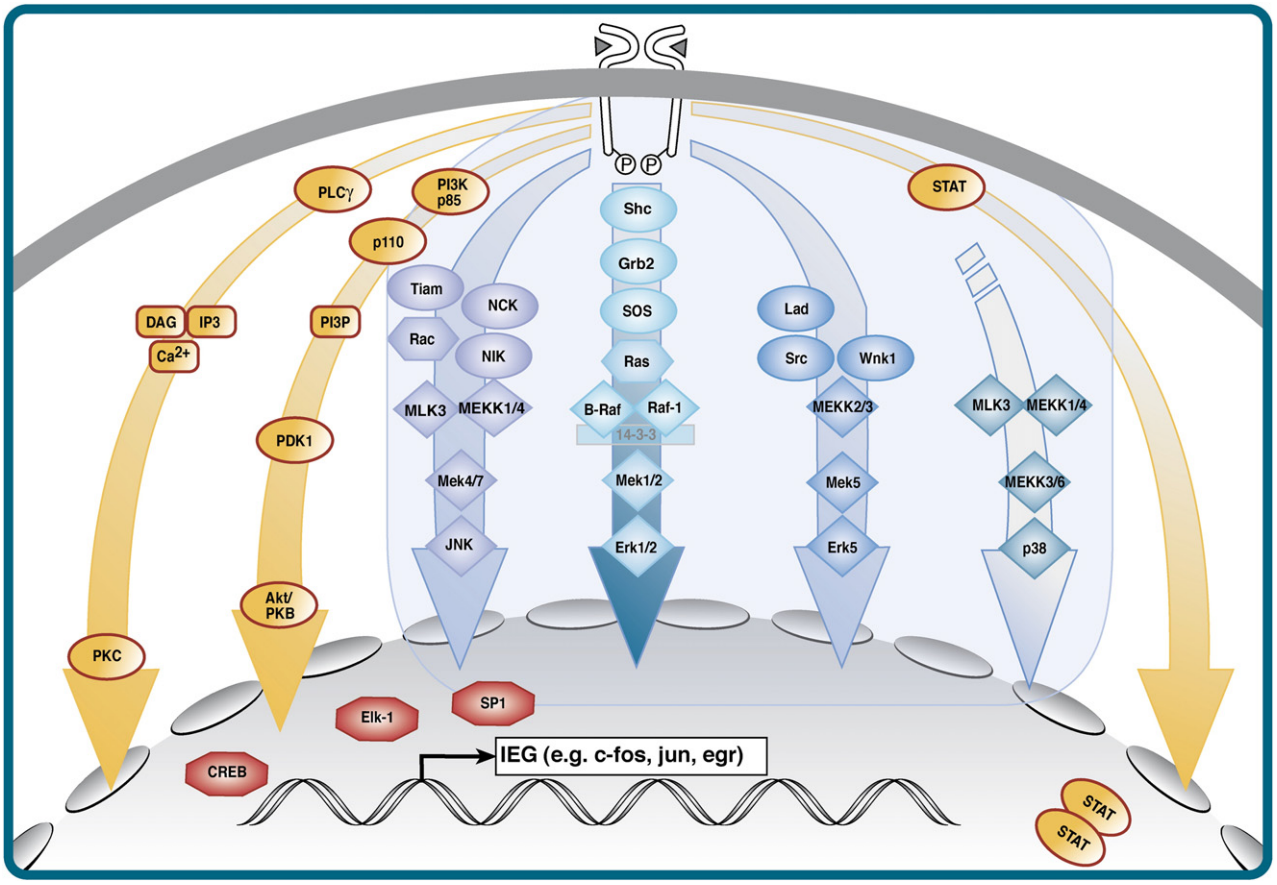

Fig. 1. Signaling pathways stimulated by growth factors and their receptor tyrosine kinases. Growth factor binding to and dimerization of transmembrane receptors is followed by trans-phosphorylation of the cytoplasmic portions of the receptors (*P* letters represent phosphate groups). The activated receptor physically recruits from the cytoplasm and from the plasma membrane a large variety of adaptors and enzymes, which subsequently put in motion several linear cascades, some of which are presented. The four canonical MAPK pathways are presented, although growth factors rarely activate p38-MAPK. Also shown are the PI3K-Akt, phospholipase C-PKC and the STAT pathways. All routes culminate in regulation of gene expression, such as rapid transcription of a group of immediate early genes (IEGs).

2.2. The PI3K/Akt signaling pathway

Another phospholipid modifying signaling pathway activated by RTKs is the PI3K pathway. This heterodimeric enzyme comprises two subunits, the p85 regulatory subunit harboring two SH2 domains, and the p110 catalytic subunit. PI3K activation may be achieved by binding of its p85 regulatory subunit to an activated receptor. Alternatively, RTK signaling may activate the small G protein Ras, which in turn recruits PI3K to the plasma membrane and induces a stimulatory conformational change in the lipid kinase [12]. Upon activation, PI3K induces PtdIns(3,4,5)P_3 formation at the inner leaflet of the plasma membrane, which serves as a docking site for various proteins containing phospholipid binding domains, such as the PH domain, including the kinases PDK1 and Akt/PKB. Recruitment of Akt to the plasma membrane, along with Akt phosphorylation by PDK1 and by a still unknown kinase, enables activation and subsequent phosphorylation of various substrate proteins, including major effectors of apoptosis, as well as several transcription factors [13].

2.3. The STAT signaling pathway

RTKs may directly phosphorylate and activate in the cytoplasm STAT-family transcription factors, thereby promoting their translocation to the nucleus. Originally identified as substrates activated by cytokines, several STATs have been shown to undergo phosphorylation by multiple RTKs [14]. Upon phosphorylation-induced dimerization and translocation to the nucleus, STAT proteins elevate transcription of genes involved in cell proliferation.

2.4. The MAPK signaling pathways

The MAPK pathway is a three layer signaling cascade, in which the MAPK elements (the most downstream tier) are activated upon tyrosine and threonine phosphorylation within a conserved Thr–Xxx–Tyr motif in the activation loop of the kinase domain. This phosphorylation is catalyzed by dual-specificity kinases, MAPK kinases (MAPKK, MEK). MAPKKs are regulated by serine/threonine phosphorylation within a conserved motif, located in the kinase activation loop, catalyzed by MAPKK kinases (MAPKKK or MEKK; the most upstream tier). The latter are activated by various upstream activators, including kinases and small GTP-binding proteins. The generic MAPK signaling pathway is shared by four distinct cascades, which are named according to their MAPK tier component; the extracellular signal-related kinases (Erk1/2), Jun amino-terminal kinases (Jnk1/2/3), p38-MAPK and Erk5. Although all these MAPK cascades are regulated by growth factor signaling, GFs are considered to be the major regulators of the Erk1/2 cascade, whereas Erk5 is regulated by both GFs and stress, and out of the two major stress-induced cascades, the JNK pathway is partially regulated by GFs, and only a few studies have documented activation of the p38-MAPK cascade by GFs.

3. Architectural features of MAPK cascades activated by growth factors

3.1. The Erk1/2 signaling cascade

The adaptor protein Grb2 can bind with activated RTKs through an SH2 domain-phosphotyrosine interaction, while through the SH3 domain (a binding domain specific to proline-rich sequences) Grb2 interacts with the guanine nucleotide exchange factor, Sos. Consequently, Sos is recruited to the vicinity of the plasma membrane, where it promotes the exchange of GDP for GTP on a small G protein, namely Ras. Alternatively, recruitment of the Grb2/Sos complex to an activated receptor may be mediated by the adaptor protein Shc, which interacts with activated RTKs by means of its PTB domain. GTP-bound active Ras can then bind with and activate the MAPKKK protein, Raf. Upon activation, Raf induces the phosphorylation of serine residue in the activation loop of Mek (MAPKK) [15]. Thereafter, activated Mek1/2 phosphorylates the MAPK protein, Erk, on adjacent threonine and tyrosine residues, spaced by a glutamic acid residue, at the activation loop. Active Erk phosphorylates multiple cytoplasmic and cytoskeletal proteins [16], including MAPK-activated protein kinases and the family of approximately 90-kDa ribosomal S6 kinases (Rsk). Additionally, active Erk and Rsk1/2 translocate to the nucleus, where Erk phosphorylates and activates various transcription factors including Sp1, E2F, Elk-1 and AP-1 (for a recent review see [17]). The latter comprises two short-lived proteins, namely Jun and Fos, which are the product of immediate early genes (IEGs), whose regulation and role in signal integration will be highlighted in another part of this review. Eventually, this pathway can control various cellular processes such as proliferation, migration and differentiation, as we will exemplify below.

3.2. The Erk5 signaling cascade

Erk5, which is also known as Big MAP kinase (Bmk1), contains in its activation loop a threonine–glutamate–tyrosine sequence, which is characteristic of the Erk1/2 MAPKs. However, in contrast to Erk1/2, Erk5 is specifically activated by the MAPKK tyrosine–threonine kinase, Mek5 [18], which is the substrate of the Mekk2/3 serine/threonine kinases [19]. The Erk5 cascade was initially found to be activated in vascular smooth muscle cells challenged by stress-inducing agents, such as oxidative stress and hyperosmolarity, but inflammatory cytokines (for example, the tumor necrosis factor; TNF) do not induce Erk5 activation [20], indicating that in contrast to the JNK and p38-MAPK pathways (see below), the Erk5 cascade is not a typical stress-induced MAPK. Indeed, significant Erk5 activation occurs in HeLa and in PC12 cells stimulated by various growth factors [18,21], and in contrast to the Erk1/2 pathway, Raf cannot activate Erk5 [22]. An exception is the PC12 pheochromocytoma cell line, in which Ras is involved in Erk5 activation following GF treatment [21]. Interestingly, Mekk2 (the MAPKKK of the Erk5 cascade) activation by EGF is a surprisingly complex process involving c-Src, the SH2-

containing adaptor Lad [23], the unique protein kinase Wnk1 [24], as well as inhibition by the cyclic AMP-activated protein kinase, PKA [25]. Upon activation, Erk5 phosphorylates several transcription factors, including Myc, MEF2 family members, Fos [26], as well as the serum- and glucocorticoid-inducible kinase (SGK) [27]. Collectively, these factors affect a variety of cellular outcomes, such as cell cycle progression [18] and nerve cell survival [28].

3.3. The JNK signaling cascade

The c-Jun amino-terminal kinase (JNK), also known as the stress activated protein kinase (SAPK), is stimulated primarily by a plethora of stress conditions, such as UV irradiation, DNA damage, heat shock and oxidants, as well as by inflammatory cytokines. Nevertheless, less efficient stimulants are GFs like insulin, EGF, PDGF and FGF [29]. The JNK family includes three isoforms, JNK-1 through -3, and like other MAPKs, JNKs are activated by phosphorylation on tyrosine and threonine residues, which in this case, are separated by a proline. JNK phosphorylation is catalyzed by the MAPKKs, MKK4 and MKK7, which are phosphorylated and activated by several MAPKKKs including MEKK1-4, MLK1-3 and Tak1 [30]. Several pathways have been identified through which GFs can activate the MAPKKK proteins of the JNK cascade. One pathway is mediated by the small G protein Rac, which can induce the activation of MLK3, MEKK1 and MEKK4 [31–33]. Growth factors can activate Rac either through a Ras-induced activation of the Rac-specific guanine exchange factor (GEF), Tiam1 [34], or by means of PI3K activation [35]. Indeed, PI3K activation downstream to EGFR signaling was found to mediate the activation of JNK [36], whereas knockdown of Shc in DT40 chicken B cells abolished the activation of JNK by EGF [37], implying that JNK activation by EGF may involve both Shc and PI3K. Alternatively, activation of Eph-family receptor tyrosine kinases may induce the JNK cascade through an Nck-mediated recruitment of the Ste20 kinase, namely NIK (NCK-interacting kinase) [38]. Thereafter, NIK induces the activation of MEKK1, a MAPKKK of the JNK pathway [39]. Activation of the JNK pathway results in the phosphorylation and activation of several transcription factors, including c-Jun, JunA, JunB, ATF2 and Elk. Finally, although JNK activation is predominantly associated with promotion of cell death, under certain conditions it enables cell survival and even tumor progression [30].

3.4. The p38-MAPK signaling cascade

The p38-MAPK is another stress-activated MAPK cascade. p38-MAPK activation is mediated by phosphorylation on a conserved motif that includes a glycine residue between the canonical threonine and tyrosine. This dual phosphorylation is catalyzed mainly by the MKK3 and MKK6 kinases, which are activated by several MAPKKKs, most of which are shared with those of the JNK cascade (e.g., MEKK1–4 and MLK1–4). In analogy to the JNK cascade, the p38-MAPK route is predominantly activated by stress conditions and inflammatory cytokines, but it seems almost insensitive to growth factor stimuli. Like in the JNK cascade, the reported GF- induced p38-MAPK activation is regulated by Rac [29]. On the other hand, GF withdrawal induces p38-MAPK and JNK activation, to stimulate death of PC12 cells [40]. Another interface between GFs and the p38-MAPK signaling cascade has recently been unraveled. Activation of p38-MAPK by stress stimuli, including DNA damage by platinum ions and UV irradiation, was found to induce serine/threonine phosphorylation of the EGFR. This phosphorylation serves as a signal that mediates a p38-MAPK-dependent endocytosis of EGFR, which unlike the ligand-induced endocytosis, does not result in receptor degradation [41,42]. These observations imply that p38 induction under stress conditions induces apoptosis on the one hand, and on the other hand it removes mitogenic receptors from the cell surface, thereby preventing GF-induced cell survival.

4. Functional features common to MAPK cascades activated by growth factors

A major open issue in the field of GF-to-MAPK signaling relates to the ability of the canonical linear pathway to generate different cellular outcomes in response to activation by various extracellular stimulants. For example, in PC12 cells FGF and NGF induce neurite outgrowth by stimulating the Erk1/2 pathway, along with other signaling routes, whereas EGF and insulin weakly induce cell proliferation, although these factors similarly recruit Erk1/2 [43]. Overexpression of the receptor for either EGF or insulin, resulted in prolonged Erk activation and a differentiated phenotype [44,45]. Accordingly, in NIH3T3 cells, low levels of Raf-1 stimulation resulted in cell proliferation, whereas sustained high activation of Raf-1 resulted in cell cycle arrest, through the p21Cip1 inhibitor [46]. Receptor expression levels, differential recruitment of the four MAPKs, co-lateral regulatory pathways, the presence of adaptor proteins, as well as sub-cellular compartmentalization of specific signaling molecules have been reported as potential mechanisms that confer output specificity to GF-induced MAPK activation (for a recent review see [17]; see also Fig. 2). What follows is a discussion of the major functional attributes of the MAPK pathway, which contribute to the ability of this kinase cascade not only to diversify signal output, but also store memory, integrate signals and transform a graded input into a binary output.

4.1. Linearity and cross-talks

Although linearity is a hallmark of all MAPK cascades, several cross-talks to other signaling pathways, including GF-stimulated small G proteins and kinases, richly regulate the MAPK signaling cascades. In the case of Erk1/2, most of this lateral input is apparent upstream to the Raf proteins (MAPKKK tier). Part of this complexity is contributed by the multiplicity of Raf proteins. B-Raf not only receives signals from several GF receptors [47], but according to recent reports, by means of hetero-oligomerization and trans-phosphorylation it activates Raf-1 in a mechanism that requires the scaffold

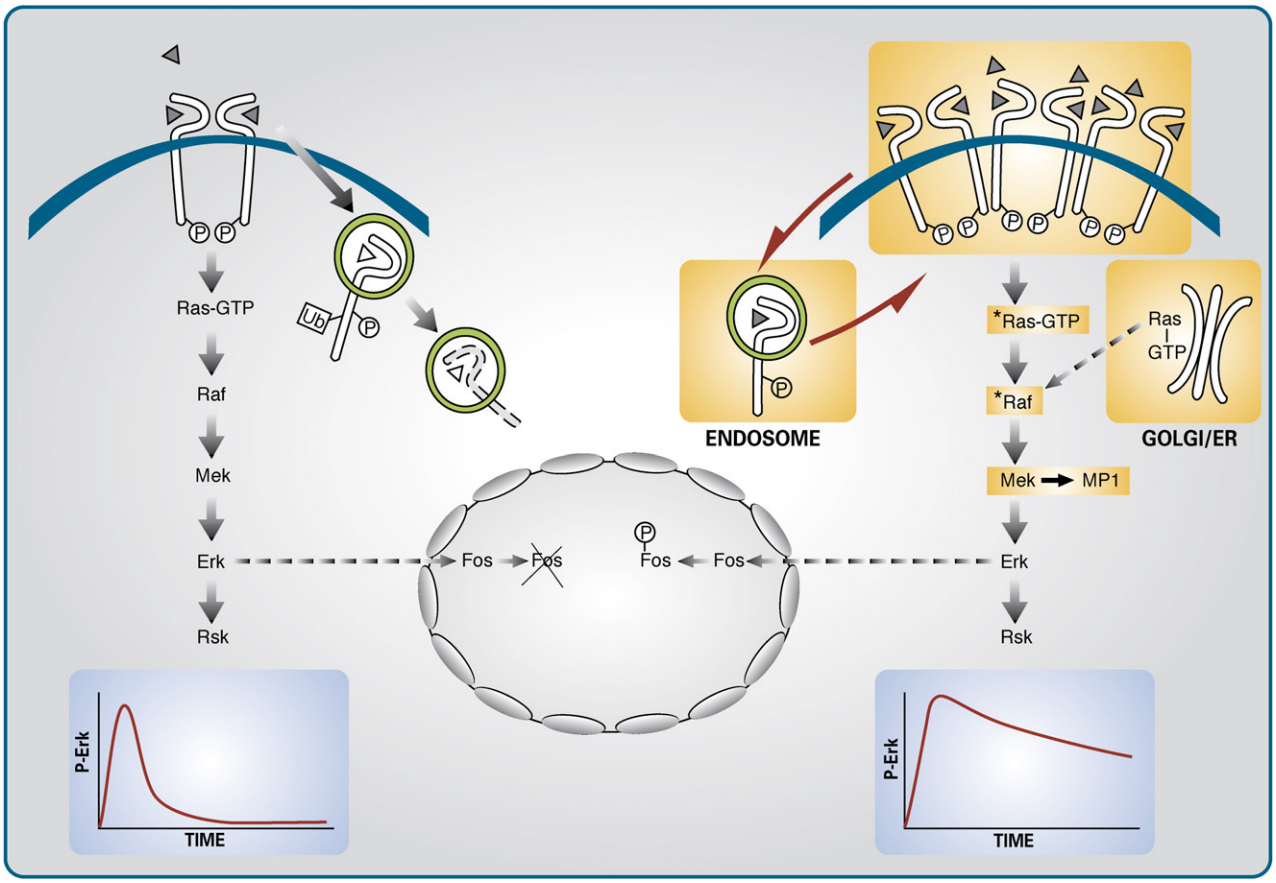

Fig. 2. Potential mechanisms regulating the duration of Erk (extracellular signal-regulated kinase) activation. The left part of the scheme presents transient activation of Erk. This pathway culminates in dual phosphorylation within the activation segment of the kinase, followed by translocation of Erk, as well as its substrates, the p90 ribosomal S6 kinases (Rsk1 and Rsk2), to the nucleus. The upstream canonical cascade links a growth factor-induced dimer of receptor tyrosine kinases to sequential activation of Ras, Raf, and Mek, which is a dual-specificity kinase that phosphorylates Erk. The phosphorylated form of Erk decays very rapidly, primarily through the action of double-specificity phosphatases. Concurrently, the phosphorylated (*P*), active receptors undergo endocytosis and they are sorted to degradation in lysosomes by means of a mechanism that conjugates ubiquitin molecules (*Ub*) to the cytoplasmic tail of the receptor. While in the nucleus, Erk mediates increased transcription of a large group of genes, including the immediate early gene c-*fos*, which encodes the short-lived Fos protein. Prolonged activation of Erk enables it to phosphorylate the newly synthesized Fos protein, thereby stabilizing Fos and other factors involved in gene expression. Several mechanisms may prolong Erk activation (right part of the scheme): gene amplification and autocrine loops can increase receptor numbers and receptor activation at the cell surface. Alternatively, mechanisms that derail active receptors, shunting them to a recycling endosome, extend growth factor signals. Recruitment of a MAPK scaffold protein, MP1, to late endosomes may also prolong Erk signaling. Naturally occurring mutations (represented by asterisks) of growth factor receptors, Ras proteins and the downstream tier, namely Raf kinases, similarly help sustain MAPK activation. Likewise, delayed but sustained activation of Ras may take place in the endoplasmic reticulum (ER) and Golgi.

protein 14-3-3 [48,49]. Interestingly, nerve growth factor (NGF)-induced activation of B-Raf in PC12 cells is mediated by both Ras and the small G protein Rap1. Activation of the Rap1-GEF protein, C3G, by RTKs is mediated by the Crk adaptor protein, and it leads to prolonged Erk activation, compared to the transient activation achieved through the Ras/Raf-1 pathway [50]. Alternatively, B-Raf stimulation downstream to EGFR may be mediated by the mixed lineage kinase-3 (MLK3) [51]. Likewise, in fibroblasts the Erk pathway may be regulated by a crosstalk with PKCα [52], in a mechanism mediated by PKC-induced Ras activation [53], or by a PI3K/Rac pathway that induces the activation of the p21 serine/threonine kinase, Pak, which regulates Raf-1 activity [54,55]. Moreover, in neuronal cells Erk-activation may be induced by various GFs that activate different PKC isoforms, while FGF stimulates Erk through PKCδ induction [56], and EGF induces Erk through the activation of PKCζ in a pathway involving PI3K and PDK1 [57]. On the other hand, activation of the PI3K/Akt pathway, in human colon cancer cell lines, may result in inhibition of the Erk pathway, through the activation of glycogen synthase kinase-3 (GSK3) and PKCδ [58].

4.2. Regulation of signal duration

Several studies that utilized fibroblasts [59], T lymphocytes [60] and megakaryocytes [61] concluded that sustained activation of the Erk1/2 pathway precedes cellular differentiation. As aforementioned, studies performed with PC12 pheochromocytoma cells found that transient activation of the Erk1/2 cascade results in cell proliferation, but prolonged Erk1/2 activation results in cell differentiation to sympathetic-like neurons, a response normally observed after stimulation with NGF or the fibroblast growth factor (FGF). These observations led Chris Marshall to propose that Erk1/2 inactivation, or the

duration of the signal, may dictate cellular outcomes [43]. According to one model, transient Erk1/2 activation results from recruitment of the Grb2–Sos complex to EGFR, but prolonged Erk activation is associated with the recruitment of the Shc–Grb2–Sos complex to the NGF-activated Trk receptor [62–64]. In addition, prolonged Erk1/2 activation results in Erk translocation to the nucleus, which broadens the repertoire of substrates compared to the transiently activated cytoplasmic Erk [45].

4.3. Control of MAPK compartmentalization

The sub-cellular localization of MAPKs is considered a major mechanism that controls signaling, because it can regulate the accessibility to specific substrates. All MAPKs can shuttle between the cytoplasm and the nucleus [65]. While in the nucleus, MAPKs may phosphorylate/activate various transcription factors, leading to induction of distinct sets of genes, whereas at the cytoplasm MAPKs can phosphorylate/activate proteins involved in processes such as metabolism, actin cytoskeleton organization and cell adhesion [16]. Blocking the transfer of Erk to the nucleus severely affects Erk-mediated cellular processes such as proliferation. Moreover, in primary foreskin fibroblasts, EGF induces neither the nuclear translocation of Erk, nor Fos induction [66]. Yet, if Erk is forced to shuttle to the nucleus following EGF-treatment, it will induce transcription of *FOS* [66]. The compartmentalization of signaling molecules is mediated by interactions with scaffold proteins (e.g., MP1, KSR and paxillin). For example the scaffold protein Sef, a transmembrane protein induced by FGF-signaling, interacts with the active Mek–Erk complex to retain it in the cytoplasm [67]. The complex may localize to sites of actin polymerization, thus affecting cell migration in cooperation with the scaffold protein IQGAP1. Accordingly, IQGAP1 expression levels strongly regulate Erk signaling [68]. Alternatively, paxillin-mediated targeting of the Mek–Erk complex to sites of focal adhesion may regulate cell adhesiveness [69].

4.4. Endocytosis of RTKs regulates MAPKs

Generally, endocytosis of GF-receptors is considered a major desensitization mechanism, as internalized receptors cannot bind to activating ligands, and the endocytic route leads receptors to degradation in lysosomes [70–72]. Nevertheless, several studies have defined the endosome as a compartment that enables assembly of signaling complexes. EGF treatment of rat livers leads to the association of activated Shc, Grb2 and Sos with the phosphorylated EGFR in endosomes [73]. The association of Grb2 and Shc with the activated receptor in endosomes has been confirmed by the use of fluorescence resonance energy transfer (FRET) [74]. According to an alternative model, endocytosis of activated MEK, rather than activated RTKs, is a critical event in the MAPK activation cascade [75]. Signals generated at endosomes seem sufficient for promotion of epithelial cell proliferation [76], probably through the activation of Erk [77]. Further, dissection of receptor-associated signaling complexes formed throughout the endocytic pathway defined different molecular assemblies at endosomes, compared to those formed on the cell surface [78]. For example, Rap1, which is activated by NGF in PC12 cells and associates with Erk-induced cellular differentiation, is localized primarily to endosomes [79], suggesting that spatial regulation of signaling complexes may affect cellular outcomes. Association of Erk proteins with activated signaling adaptors in endosomes can be mediated by the scaffolding protein MP1, which is recruited to endosomes by the adaptor protein p14 [80]. Interestingly, Erk is co-localized with activated Ras on rasosomes, which are Ras-enriched, fast moving small cytosolic nanoparticles [81]. Presumably, the relationships between early endosomes and Ras nanoparticles will shed light on the role played by endocystosis of RTKs and translocation of MAPK components in signal transmission across the cytoplasm.

4.5. How signals are interpreted by the MAPK circuitry?

The capacity of a cell to differentiate between true signals, which are meant to drive cellular outcome, and inconsequential noise, has been demonstrated to depend on mechanisms of feedforward and feedback regulation (reviewed in [82]), as will be discussed below. Essentially, the induction of positive cellular signals is balanced by pathways of negative regulation, resulting in stringent control of signal intensity and duration. Hence, hyperactivation of RTK signaling, due to loss of attenuation mechanisms, frequently associates with malignant transformation, as exemplified by EGFR/ErbB family proteins [83,84], and discussed in the end of this review. This highlights the importance of mechanisms of signal attenuation, which unlike the better-studied stimulatory pathways, remain poorly understood [85]. Negative regulators may pre-exist and undergo activation by means of either oligomerization or post-transcriptional modification (e.g., phosphorylation and ubiquitinylation). Alternatively, other negative regulators are newly synthesized as a part of negative feedback loops. Two examples of pre-existing negative regulators are RKIP, a Raf kinase inhibitor protein [86], and c-Cbl. This phosphotyrosine-activated mammalian E3 ubiquitin ligase plays a critical role in signal attenuation by tagging activated EGFRs with ubiquitin, thereby promoting receptor endocytosis and sorting for lysosomal degradation [71,72]. On the other hand, RALT is a newly synthesized feedback inhibitor of EGFR, which is up-regulated following stimulation of EGFR and the MAPK pathway [87]. More examples of inducible negative feedback regulators include Sprouty, Spred and Lrig-1 [82].

Feedback loop negative regulation is a central mechanism by which systems attain robustness [88,89]. One straightforward role for negative feedback is to limit the duration of a signal; once a predefined threshold is reached, the signal induces its own negative regulators. When incorporated in a pathway, the negative feedback circuit confers output stabilization, even when the system is challenged with a high degree of environmental noise. Several studies have shown that administration of protein synthesis inhibitors results in 'super-induction' of both MAPK and a set of IEGs, attesting to a

central role for transcription of negative regulators in limiting MAPK signaling and transcription of IEGs [90,91]. A direct negative feedback arm, which is sensitive to both transcription and translation inhibitors is the family of dual specificity phosphatases (Dusp or MKP) [92]. The Dusps are induced by multiple extracellular stimulants and inactivate specific MAPKs by dephosphorylating critical phosphate groups within the activation segment of MAPKs. One possible explanation for the unique targeting of the MAPK tier by Dusps is their pivotal position. At this distal point of the linear pathway, the otherwise narrow phosphorylation signal spreads to many substrates and it translates into transcription events, whereas upstream steps may be regulated by their downstream effectors, such as the regulation of Raf by MAPK [93].

4.6. Feedback loop negative regulation of immediate-early gene transcription

One important node, which is richly populated by feedback loops, is the early transcription network lying downstream to growth factor signaling and MAPK activation [94]. Within this transcription network, pre-existing negative regulators may be activated post-translationally. For example, Pointed is a pre-existing ETS transcription factor activated by the Drosophila EGFR. Pointed's activity is restricted by another Ets-family transcription factor, namely Yan [95], and their phosphorylation by Erk leads to antagonistic effects: whereas phosphorylation of Pointed is stimulatory, the repression activity of Yan is inhibited by phosphorylation, which results in a burst of transcription. Super-induction of IEGs upon treatment with an inhibitor of protein synthesis revealed another layer of control [91], that combines several types of negative feedback regulators. The MAPK-regulated transcription complex TCF–SRF–SRE is feedback regulated by the Id proteins, whose expression is driven in a delayed manner by the TCF factors themselves, thus forming a reliable time delayed negative feedback loop [96]. Another example relates to the cAMP signaling pathway: the transcription factor 'inducible cAMP early repressor' (ICER) is transcriptionally induced by cAMP in a delayed manner, to repress the activity of the cAMP pathway [97]. Similarly, the immediate early gene product Egr1, which is induced in response to NGF stimulation of PC12 cells [98], is inactivated by the delayed induction of the nuclear protein Nab2, and likewise both Fosl1/Fra1 and JunB are induced in a delayed manner to physically engage transcriptionally active complexes of Fos and Jun (AP-1 complex) and limit their activity [99,100].

4.7. The role for RNA-binding proteins in feedback loop negative regulation

An important component of the 'super induction' of IEGs comprises proteins able to bind AU-rich elements (AREs) in 3′ untranslated regions (UTR) of mRNAs. Characteristically, such transcripts are very unstable and relatively abundant in the population of mRNAs induced by GF signaling ([101] and our unpublished observations). The growing list of proteins that bind AREs and regulate mRNA turnover includes AUF1, HuR, BRF1 and KRSP ([102] and references therein). One of the major proteins responsible for binding, and later on, for targeting AU-rich transcripts for degradation is TTP/Zfp36 [103]. Originally, TTP was shown to be induced by various cytokines and to dramatically affect the expression levels of TNF-alpha, whose transcript is AU-rich. Further, a large number of the EGF-induced genes contain AU-rich sequences within their 3′-UTR, including c-*fos* [101]. Interestingly, in the case of c-*fos*, it has been shown that a segment of the 3′-UTR containing the AU-rich sequences is responsible for instability [104]; removal of this region converts c-*fos* to a transforming gene, presumably due to overproduction of the Fos protein [105]. Interestingly Zfp36 cooperates with a microRNA (miR16) in mRNA degradation [106]. Another microRNA (miR7) is induced by Pointed to degrade transcripts of Yan and activate EGFR signaling, as a part of a positive feedback loop [107]. These observations raise the possibility that microRNAs play essential roles in feedback regulation of GF signaling. In conclusion, IEGs contain regulatory elements in both 5′ and 3′ sequences proximal and distal to the coding region. The 5′-UTR elements are regulated by delayed induction of transcription factors or repressors, such as Id2, JunB and Nab2, whereas 3′-UTR regulatory elements are recognized by newly induced RNA-binding proteins, such as Zfp36, or microRNAs, that decrease RNA stability and/or translation. Thus, output's duration and amplitude are regulated in the nucleus by complex feedback inhibitory loops acting at the RNA level, which complements the rich cytoplasmic regulation at the level of substrate phosphorylation.

4.8. The circuitry of GF-to-MAPK regulation of cell division

While the signaling cascades and transcription events regulated by the GF-to-MAPK pathway have been studied thoroughly, the basic principles employed by mammalian cells to decode GF signals into distinct biological outcome have only recently received attention. According to one model, bistable circuits convert a graded input into a switch-like 'yes' or 'no' response (such as growth) [108]. Another model argues that cells must receive two sequential stimuli in order to mount a full response [109]. Adhering to the first model, the group of James Ferrell has shown that in the Erk-regulated maturation of Xenopus oocytes, a transient stimulus (in the form of progesterone) can be transformed into an irreversible biochemical response by means of multiple positive feedback machineries [110]. The group of John Blenis described another bistable circuit in proliferating mammalian fibroblasts, in which the switch-like all-or-none response resides at the level of IEGs [111]. Accordingly, sustained activation of Erk (1–2 h) permits phosphorylation-mediated protection of IEG products from degradation, thereby establishing a positive feedback loop. Unlike oocyte maturation, which is stimulated by a 2 h-long exposure to a steroid hormone, cell cycle progression in fibroblasts requires prolonged exposure to polypeptide growth factors until 2 h before they enter S phase. The group of Andrius Kazlauskas has shown that continuous exposure to GF may be

replaced by two intervals: the initial pulse of GF involves Mek activation and induction of c-Myc, whereas the essential second pulse may require PI3K activity [109].

5. Regulation of cellular outcomes by MAPKs

Growth factor signals regulate not only cell division, but also a variety of other outputs, ranging from cell migration to regulation of apoptosis, cellular adhesion and differentiation, as well as alterations in gene expression and intracellular metabolism. Below we review two of the most widely studied MAPK-regulated cellular outcomes, namely cell proliferation and cell migration, with the aim of exemplifying the functional diversity of GF-to-MAPK signaling. Notably, both outcomes are relevant to the process of malignant transformation. Hence, we separately discuss the ability of the MAPK pathway to contribute to the formation of malignant phenotypes.

5.1. Cell proliferation

The Erk1/2 cascade was initially identified as a growth-promoting pathway, whose major components associate with unleashed cell growth and cancer progression. For example, transforming forms of *RAS* and *RAF* are encoded by retroviral oncogenes, and the Erk cascade has been linked to cellular proliferation by using constitutively active, or dominant-negative mutants of Mek1, which promoted or suppressed cell transformation, respectively [112–114]. Several distinct mechanisms enable active Erk1/2 to induce cell cycle progression. Through the phosphorylation of carbamoyl phosphate synthetase II, which is involved in pyrimidine nucleotide biosynthesis, Erk1/2 can support DNA synthesis during the S phase [115]. Alternatively, Erk1/2 may remove obstructions of cell cycle progression either by activating p90Rsk, which mediates the inactivation of a cell cycle inhibitory kinase, MYT1 [116], or by promoting the degradation of the cyclin-dependent kinase (CDK) inhibitor, p27Kip1, thereby stimulating cyclin E/Cdk2 [117]. In addition, Erk1/2 can promote the transition from G1 into the S phase, by inducing transcription of cyclin D1, which in turn promotes DNA synthesis through the regulation of CDK4 and CDK6 [118]. Importantly, Erk1/2-signaling alone is insufficient for cyclin D1 induction, but requires also the activation of the PI3K pathway. The latter is probably induced by an Erk-dependent expression of autocrine growth factors [119]. In addition to the regulation of G1 to S phase transition, at least in some cell types Erk1/2 activation involves progression through the G2 phase [120]. Accordingly, activated Erk was found to be associated with kinetochores and spindle poles, as well as with the midbody in late stages of mitosis [121,122], implying multiple functions of Erk during mitosis. Surprisingly, however, sustained activation of the upstream regulators, Ras and Raf-1, may result in growth arrest rather than cell proliferation, perhaps because potent inhibitors of cell cycle progression (i.e., p53, p21Waf/Cip1 and p16) are up-regulated under these conditions [46,123]. Recently, it has been shown that hyperactivation of Erk may arrest the G1-S and the G2-M transitions during the cell cycle [124]. Moreover, hyperactivation may override spindle checkpoints, thereby causing chromosomal aberrations [125]. Likewise, sustained Raf activation in lung fibroblasts [126], or hyperactivation of Erk through depletion of the dual specificity phosphatase VHR [124], resulted in premature onset of senescence.

5.2. Cell migration

Cell migration is a cyclic process involving the formation of adhesion sites at the leading edge of the cell, and at the same time, disintegration of adhesion sites at the rear edge of migrating cells (reviewed in [127,128]). Directed polymerization of the actin cytoskeleton in lamellipodia or in fillopodia structures, along with actin stress fiber-dependent contractile forces leading to forward translocation of the cell body, are necessary for robust cell movement. In the case of epithelial cells, migration requires disintegration of intercellular adhesive contacts, as well as invasion through the extracellular matrix and endothelial cells lining blood vessels. Cell migration can be regulated by signals initiated by integrins (reviewed by [129]) and by RTKs, such as the hepatocyte growth factor (HGF) receptor (c-Met) (reviewed in [130]), EGF-receptor, PDGF-receptor and the IGF-l-receptor (reviewed in [131]). For example, HGF function as a scattering factor for various epithelial cells. During embryonic development, HGF regulates muscle cell migration [132], and serves as a chemoattractant for spinal motor axons [133]. Likewise, EGFR is involved in the regulation of trophoblast cell migration [134], motility of various nerve cells [135], and pancreatic islet cells [136], as well as in cell migration taking place in eyelid closure [137] and in wound re-epithelialization [138]. Moreover, hyper-activation of RTKs is highly associated with cancer progression, where the transition from localized primary tumor mass to invasive secondary metastasis is the result of unleashed cell migration (reviewed in [139]).

Several signaling pathways have been implicated in GF-induced cell migration, including the activation of PLCγ and the subsequent activation of gelsolin, profilin and cofilin. These proteins interact with the actin cytoskeleton, leading to formation of fillopodia and lamellipodia extensions [140,141]. Alternatively, GFs may stimulate alterations in membrane ruffling through the activation of Rac [142], which is a major target of the PI3K signaling pathway. Similarly, the Erk1/2 pathway has repeatedly been implicated in GF-induced cell migration and in tumor invasion. By using various Ras mutants, it was shown that Erk activation is sufficient for induction of lung metastasis [143]. On the other hand, inhibition of Erk activity impaired cell migration stimulated by different GFs, such as VEGF, EGF, FGF and insulin. Moreover, the migration ability of Mek-deficient ($Mek^{-/-}$) fibroblasts or vascular endothelial cells is impaired when plated on fibronectin [144]. Interestingly, activation of the PI3K pathway may prevent EGF-induced cell migration [145], probably through activation of Akt1 [146]. Yet, EGF activation can lead to reduction of Akt1 expression and to induction of Erk1/2 activity, resulting in enhanced cell migration [146].

5.2.1. Phosphorylation-mediated mechanisms of MAPK involvement in cell motility

A number of mechanisms have been identified through which the GF-to-Erk1/2 pathway may regulate cell migration (Fig. 3). EGF-activated Erk1/2 can mediate the phosphorylation of Calpain, which is an intracellular protease that modulates focal adhesion sites. Phosphorylation of Calpain stimulates its catalytic activity, resulting in turnover of adhesion sites and substrate detachment at the rear part of migrating cells [147]. In addition, Erk1/2 induces the phosphorylation and activation of the myosin light chain kinase (MLCK) following EGF treatment. Once phosphorylated, MLCK induces phosphorylation of the myosin light chain, thus promoting myosin's ATPase activity. Active myosin promotes the polymerization of actin fibers [148], and the protrusion of membranes at the front of polarized cells [149]. Moreover, Ras-induced activation of Erk and PI3K is required for the disassembly of adhesion sites following cell stimulation with HGF [150]. HGF-activated Erk can translocate from endosomes to adhesion complexes at the plasma membrane, where it becomes associated with adhesion proteins, such as vinculin, paxillin and actin, and promotes cell migration [69,151]. This translocation of Erk to adhesion sites is regulated by PKCε [151]. Paxillin can interact with Erk, Mek and Raf, thus serving as a scaffold protein. Formation of this complex, following HGF treatment, leads to the phosphorylation of paxillin. In turn, phosphorylated paxillin recruits the focal adhesion kinase (FAK) to adhesion sites, which activates Rac, resulting in rapid focal adhesion turnover and lamellipodia extension [69]. Interestingly, upon recruitment to adhesion sites, FAK promotes interactions between Erk1/2 and Calpain, and consequently serves as a proteolytic substrate for Calpain [152].

5.2.2. Transcription-mediated mechanisms of MAPK involvement in cell motility

In addition to the direct, phosphorylation-mediated effects of Erk activation on cell migration, MAPKs may regulate cellular motility by promoting specific gene transcription. For example, activation of the transcription factor complex AP-1, which is partly regulated by Erk, is required for EGF-induced migration of human epidermoid cancer cells [153]. Moreover, inhibition of either gene transcription or mRNA translation impairs endothelial cell migration [154,155]. Several transcriptional

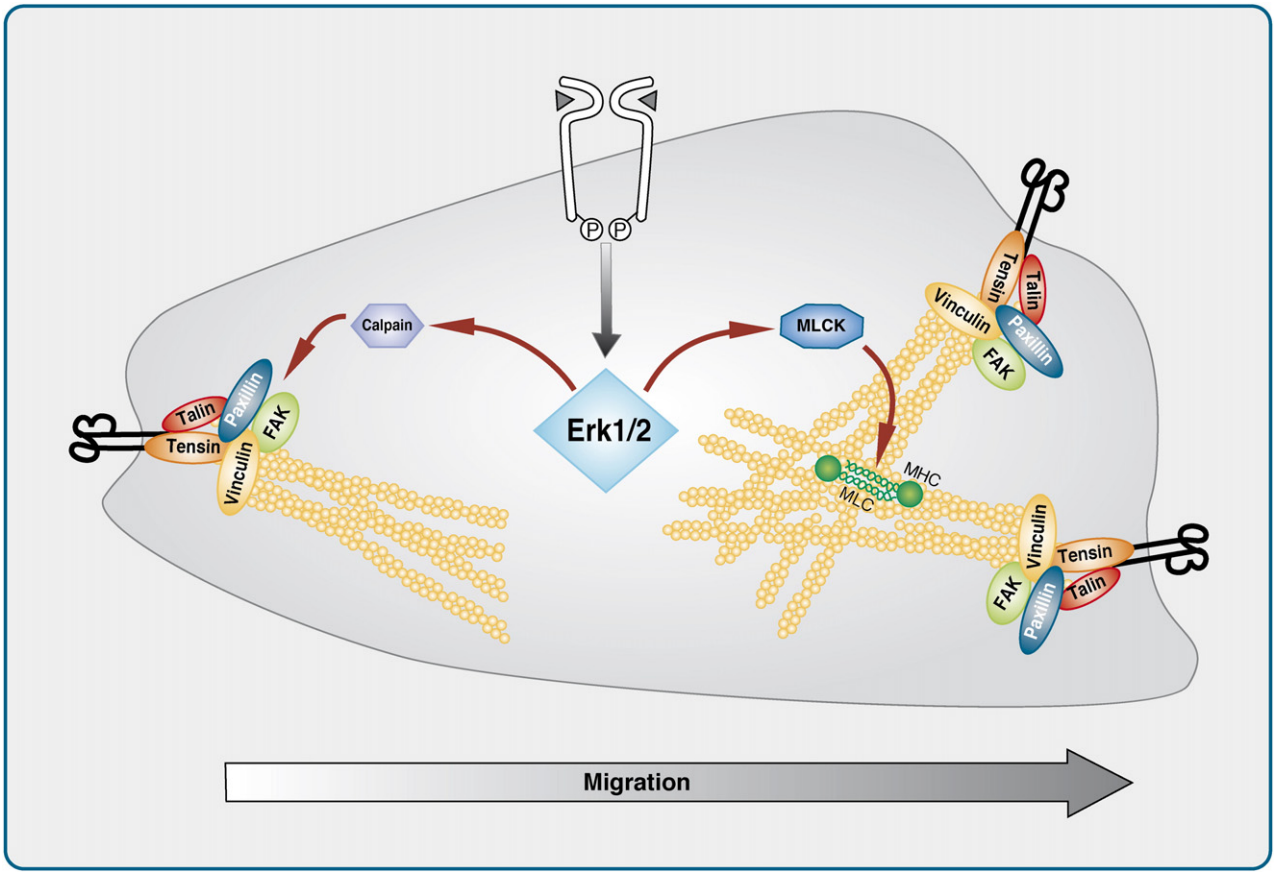

Fig. 3. Involvement of growth factor-regulated Erk in cell motility. Several signaling pathways underlie regulation of cellular movement by growth factors and cytokines. The Erk pathway controls transcription of multiple proteins, which engage in cell motility upon their synthesis and re-folding. In advance, Erk regulates pre-existing proteins that modulate the actin cytoskeleton. The scheme represents this aspect of Erk's action. A major target of Erk is the focal adhesion site, a junction of extracellular matrix (ECM) proteins and the actin cytoskeleton. Integrin, a major receptor of ECM proteins, engages several adaptors (e.g., Paxillin) and actin-binding proteins (e.g., Talin, and Tensin) just beneath the plasma membrane. Upon activation, Erk phosphorylates (thereby activates) an intracellular protease, Calpain, that disintegrates focal adhesion sites located at the rear part of migrating cells. Simultaneously, Erk phosphorylates the myosin light chain kinase (MLCK), which stimulates the ability of myosin light and heavy chains to polymerize actin at cellular extensions protruding from the front part of migrating cells. Erk itself translocates from endosomes to adhesion complexes, where it promotes recruitment of the focal adhesion kinase (FAK) and a GTP-binding protein, Rac.

targets, which lay downstream of the Erk pathway and promote cell migration, have been identified. Thus, a set of proteolytic enzymes that degrade the extracellular matrix (e.g., MMP-1, MMP-3, MMP-7 and MMP-9) have an AP-1 consensus sequence in the respective promoters, and they undergo up-regulation upon activation of Erk1/2 [156]. In addition, Erk1/2 may control cell migration through transcription-mediated regulation of the small G proteins Rac and Rho. Erk-dependent activation of the Fos family transcription factor, Fra-1, results in inhibition of integrin signaling. This leads to inhibition of RhoA activity, and consequently to deformation of actin stress fibers, which is a prerequisite for the formation of membrane protrusions [157]. Rho inhibition was also implicated in Erk-induced cell spreading on fibronectin; inhibition of Rho activity is mediated by a Pak1-induced Mek1 activation, which involves the scaffold MP1/p14 complex [158]. In addition, Erk1/2 activation promotes expression of uPAR, the receptor for urokinase-type plasminogen activator, thus leading to a signaling cascade that activates Rac and induces the formation of lamellipodia extensions [157].

5.2.3. Involvement of other MAPKs in cell motility

In addition to the Erk1/2 pathway, several other MAPK cascades were also implicated in GF-induced cell migration. The disruption of actin stress fibers, which is a prerequisite for motility, was apparent upon activation of Erk5 [159]. Moreover, recent studies indicate that the JNK cascade plays a crucial role in the regulation of cell migration. Activation of JNK by EGF or Ephrin B1 enhances cell migration [160,161]. The developmental process of eyelid closure, which is regulated by EGF signaling, involves the activation of MEKK1, an upstream kinase of JNK [162,163]. Several mechanisms have been identified through which JNK may regulate cell migration, including phosphorylation of paxillin at the focal adhesion site, phosphorylation of Dcx, a protein involved in neurite outgrowth, and phosphorylation of Spir, a protein involved in the regulation of actin reorganization. In addition, JNK regulates cell migration through activation of the transcription factor Jun (reviewed in [164]). EGF-induced JNK activation results also in the induction of insulin receptor substrate (IRS)-1 [165]. The increase in IRS-1 expression was found to be essential for the promotion of cell migration. Growth factors like PDGF, HGF, VEGF and EGF have been found to promote the migration of various cell lines through the induction of the p38 cascade. This pathway may involve the activation of specific p38 substrate proteins, like MAPKAPK 2/3, paxillin and caldesmon (reviewed in [164]). Alternatively, p38 may down-regulate the expression of E-Cadherin, thereby inducing cell migration in embryogenesis [166].

6. The oncogenic potential of the GF-to-MAPK signaling axis

The transforming ability of the MAPK pathway is best understood in the case of the Ras/Raf/Mek/Erk pathway. A constitutively active form of Mek can transform mammalian cells in culture [59]. Furthermore, naturally occurring mutations affecting certain components of the pathway result in sustained activation of Erk, and the mutations associate with progression of specific types of tumors. Examples include the majority (>90%) of pancreatic tumors, which carry mutations in K-Ras, and approximately 70% of melanomas (and 82% of nevi), which contain mutations in B-Raf. Many other components of the pathway are genetically or epigenetically modified in human tumors, as will be described below. It is interesting, however, that germline mutations affecting *KRAS*, *BRAF* and two downstream effectors, *MEK1* and *MEK2*, cause Cardio-facio-cutaneous Syndrome (CFC), a developmental disorder involving cardiac and craniofacial defects [167,168]. Unlike *HRAS* mutations of the autosomal dominant disease Costello Syndrome, CFC mutations are not cancer predisposing. Likewise, melanocytic nevi can be relatively indolent for many years, despite the presence of activating B-Raf mutations. Thus, although mutations leading to sustained activation of the pathway are important for the carcinogenic process, Erk activation may not be sufficient for tumorigenesis.

6.1. Oncogenic deregulation of RTKs

More than half of the known RTKs have been repeatedly found in either mutated or overexpressed forms in association with human cancer [2]. The discussion below concentrates on three out of more than 30 examples of oncogenic RTK mutants, which are coupled to MAPK activation.

6.1.1. c-Kit/SCF-receptor

Naturally occurring loss of function mutations affecting c-Kit (stem cell factor receptor) revealed the importance of this receptor for gametogenesis and melanogenesis, as well as for normal intestinal functions (reviewed in [169]). Multiple gain of function mutations or short deletions have been identified in *c-kit* in gastrointestinal tumors, as well as in myeloid and mast cell leukemias [170]. The mutations either activate the intrinsic tyrosine kinase catalytic function or remove an inhibitory component, like the SH 2 containing phosphatase, Shp-1, which dephosphorylates and inactivates RTKs [171].

6.1.2. Ret/GDNF-receptor

Similar to c-kit and gastrointestinal tumors, multiple endocrine neoplasm 2B (MEN2B) displays a somatic gain of function mutation in the gene encoding Ret, an upstream activator of Erk and a subunit of the glial-derived neurotrophic factor (GDNF). The mutation affects a conserved methionine within the kinase domain, causing kinase activation and altering substrate specificity [172]. An alternative mode of oncogenic activation of Ret is found in papillary thyroid carcinomas (PTCs). Somatic rearrangements result in fusions between the kinase domain of Ret and amino-terminal portions of many other proteins and cause constitutive, ligand-independent dimerization and activation of Ret.

6.1.3. EGFR and ErbB-2/HER2

Kinase domain mutations have been identified in EGFR of non-small cell lung cancer, and their presence predicts

significant clinical responses to a kinase inhibitor [173,174]. These aberrations include the deletion of a few amino acids, introduction of duplications, or insertion of single point mutations. In addition to MAPK activation, superior Akt and STAT signaling have been shown to occur with EGFR kinase mutants [174], such that kinase inhibition selectively induces apoptosis of mutant-expressing cells [175]. Interestingly, the presence of mutant receptors often associates with weak overexpression. In addition, the wild type form of EGFR is highly expressed in a variety of human tumors, which include non-small cell lung cancer, breast, colorectal and head and neck tumors [176]. In concurrence with a dramatic reduction in patient survival rates [177], high expression levels of EGFR have been associated with advanced tumor stage, as well as resistance to standard therapies [178]. Malignant gliomas display amplification of the *EGFR* gene in 40–60% of tumors [179]. Often, amplification of the EGFR gene is accompanied by gene rearrangements, which result in a number of variant EGFR transcripts [180]. The most common rearrangement is a genomic deletion of exons 2–7, which results in an in-frame deletion of 801 nucleotides of the coding sequence. This mutant receptor, EGFRvIII, is constitutively phosphorylated on the cell surface, although it lacks the ability to bind ligands [181]. Furthermore, the mutant receptor's ability to undergo endocytosis is defective, which ensures continuous and enhanced signaling [182]. EGFRvIII is not restricted to gliomas and has been reported in malignancies of the breast, lung, ovary and prostate [183,184]. Further, cancer-associated gene amplification is shared by the closest kin of EGFR, namely ErbB-2/HER2. Overexpression of ErbB-2 has been reported in breast, lung, pancreas, colon, endometrium and ovarian cancer (reviewed in [185,186]). Moreover, in breast and lung cancer over-expression of ErbB-2 predicts poor prognosis and resistance to chemotherapy. ErbB-2 is a ligand-less RTK that forms heterodimers not only with EGFR, but also with ErbB-3 and ErbB-4. ErbB-2-containing heterodimers are characterized by enhanced signaling and they evade negative regulation [83]. Hence, stimulation of tumors overexpressing ErbB-2 with EGF and related growth factors is thought to elicit formation of the more potent, ErbB-2-containing receptor complexes.

6.2. Autocrine activation of RTKs

Co-expression of RTKs and their respective ligands might result in the activation of an autocrine loop, leading to deregulated receptor activation and uncontrolled cell growth. Examples include synthesis and secretion of HGF, the vascular endothelial growth factor and isoforms of PDGF. Autocrine loops involving EGFR and possibly also ErbB-2, are shared by all carcinomas expressing an active mutant of Ras. In several other types of carcinomas, self-production of TGF-alpha or EGF leads to transformation by continuous receptor activation, in a manner associated with reduced patient survival [187,188]. In accordance, parallel analysis of both EGFR and its cognate ligands provides a strong predictive tool for survival in several types of human cancer (reviewed in [189]).

6.3. Mutations of RAS family members

Although Raf is the most studied target of the GTP-bound form of Ras, many additional direct effectors have been identified, including the enzymatic subunit of PI3K, a phospholipase and three exchange factors for the Ras-related Ral protein. Apparently, most effectors are aberrantly activated in tumors, primarily as a result of *RAS* mutations, although additional mechanisms, such as loss of GTPase activating proteins (GAPs) and RTK activation, also account for the unleashed Ras activity in cancer (reviewed in [190]). Approximately 25% of human tumors contain mutations in one of the three *RAS* genes. The most frequent mutations occur in *KRAS* (about 70–90% of all). Approximately 90% of pancreas adenocarcinomas carry such mutations, whereas tumors of the thyroid are the second most frequent targets of mutant *RAS* (all three genes display aberrations). The *KRAS* missense mutations affect codons 12, 13 and 61. These mutations reduce the GTPase activity of Ras, thereby enhance the accumulation of Ras-GTP and increase recruitment of Ras effector proteins.

6.4. RAF mutations

Early studies established the ability of viral *Raf* genes to transform murine and chicken cells, while another line of research has shown that B-Raf has a higher kinase activity, relative to the other two Raf proteins, towards Mek [191]. In addition, B-Raf has higher basal activity and is easier to activate, because it needs phosphorylation on a single site, unlike the double sites necessary for Raf1 activation [47]. Consistent with these lines of evidence, a survey of 923 cancer samples identified missense mutations of the *BRAF* gene in approximately 70% of human malignant melanoma and 15% of colorectal cancers [192]. Mutations were also detected at lower frequency in gliomas, lung cancers, sarcomas, ovarian carcinomas, breast and liver cancers. In addition, a survey of colorectal tumors identified *BRAF* mutations in 10% of tumors [193]. The major mutation identified in tumors is V599E, a replacement of a valine of the kinase activation segment for a glutamic acid. In addition to other mutations within the activation segment, the glycine-rich stretch within the nucleotide-binding cleft of B-Raf is also mutated at high frequency. Interestingly, although the profile of tumors harboring *RAS* and *BRAF* mutations are similar, fewer than 1% of cancer samples display both *BRAF* and *RAS* mutations. These observations suggest functional equivalence of the two types of mutations in respect to tumorigenicity, in line with the linear arrangement of the Ras-MAPK cascade. MAPK linearity explains another interesting observation, namely differential sensitivity of various tumor cell lines harboring *BRAF* mutations to Mek inhibitors [194]. Unexpectedly, this survey of tumor cell lines has shown that cells expressing a mutant *RAS* (G12V) are less sensitive to Mek inhibition than cells carrying an active *BRAF* mutant, which uncovers yet unknown aspects of the MAPK circuitry and possible pathway switching.

In summary, MAPKs emerge as a highway of signal transmission utilized by an enormously large variety of stimulants,

including growth factors. The cellular outcomes of MAPK activation are as divergent and complex. The challenges ahead include systems level understanding of how growth factor signaling makes use of MAPK circuitry to store memory crucial for cell fate determination, and for metabolic decisions. Along with better description of the MAPK road map, future experiments will resolve the molecular mechanisms, which translate graded MAPK inputs into many binary outcomes in mammalian model systems. Information gained in such endeavors will likely help deciphering ways to pharmacologically manipulate GF-to-MAPK signaling in a selective way, that evades the rich wiring of the pathway and effectively combat cancer and other diseases.

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