Review

The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells

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ABSTRACT

The mitogen-activated protein (MAP) kinase extracellular-signal-regulated kinases (ERKs) are activated by diverse mechanisms. These include ligation of receptor tyrosine kinases such as epidermal growth factor (EGF) and cell adhesion receptors such as the integrins. In general, ligand binding of these receptors leads to GTP loading and activation of the small GTPase Ras, which recruits Raf to the membrane where it is activated. Raf subsequently phosphorylates the dual specificity MAP/ERK kinase (MEK1/2) which in turn phosphorylates and thereby activates ERK. ERK is a promiscuous kinase and can phosphorylate more than 100 different substrates. Therefore activation of ERK can affect a broad array of cellular functions including proliferation, survival, apoptosis, motility, transcription, metabolism and differentiation. ERK activity is controlled by many distinct mechanisms. Scaffold proteins control when and where ERK is activated while anchoring proteins can restrain ERK localization to specific subcellular compartments. Meanwhile, phosphatases dephosphorylate and inactivate ERK thereby shutting off the pathway. Finally, several feedback mechanisms have been identified downstream of ERK activation. Here we will focus on the diverse mechanisms of ERK regulation in mammalian cells.

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Contents

1. Introduction

Extracellular-signal-regulated kinases (ERKs) are members of the larger family of mitogen-activated protein kinases that also includes ERK5, the c-JunNH2-terminal kinases (JNKs) and the p38 MAP kinases. The MAP kinases are conserved in all eukaryotes and have been studied intensely in a number of model organisms since their original discovery more than 20 years ago (for a brief history of the MAP kinase field see Avruch, 2007). Here we will focus specifically on the regulation of ERK1 and ERK2 in mammalian cells to present a detailed picture of the known mechanisms by which this MAP kinase is regulated. Many of these mechanisms are shared by other family members.

ERK1 and ERK2 are 84% identical and share many functions (Lloyd, 2006). For this reason they will be referred to here by the traditional designation ERK1/2. The functions attributed to ERK1/2 at both the cellular and physiological levels are diverse. ERK1/2 modulates cell cycle progression, proliferation, cytokinesis, transcription, differentiation, senescence, cell death, migration, GAP junction formation, actin and microtubule networks, neurite extension and cell adhesion. Physiologically, ERK1/2 is required for immune system development, homeostasis and antigen activation, memory formation, heart development, and the response to many hormones, growth factors and insulin. However, ERK1 and ERK2 are not entirely functionally redundant. For example, ERK2 null mice are embryonic lethal by E8.5 (Saba-El-Leil et al., 2003; Hatano et al., 2003) while ERK1 null mice have a generally normal phenotype (Nekrasova et al., 2005; Pages et al., 1999). Aberrations in ERK1/2 signaling are known for a wide range of pathologies including many cancers, diabetes, viral infection, and cardiovascular disease. Given such a diverse and potent collection of functions it is not surprising that an equally diverse collection of mechanisms for ERK regulation have evolved.

MAP kinases are activated in response to many different signals including those originating at growth factor receptors, integrins, src and fyn, and G-protein coupled receptors (Fig. 1). The activation of ERK has predominantly been found at the plasma membrane but ERK is also activated on endomembranes (Bivona and Philips, 2003; Teis et al., 2002). The canonical ERK MAP kinase cascade (see Fig. 1) is stimulated upon the binding of extracellular growth factors such as EGF and PDGF to their respective transmembrane receptor tyrosine kinases (RTKs). The subsequent autophosphorylation of the cytoplasmic tails of the receptor on tyrosine leads to the recruitment of Grb-2, which binds the guanine exchange factor SOS. Recruitment of SOS to the membrane promotes its interaction with the membrane localized small GTPase Ras and results in GTP loading and activation of Ras (Omerovic et al., 2007). This is followed by the sequential recruitment and activation of the kinases Raf, MEK, and ERK.

Rafs are a group of three serine/threonine kinases (A-Raf, B-Raf, C-Raf) whose regulation is complex and continues to be elucidated (Claperon and Therrien, 2007; Raabe and Rapp, 2003; Kolch, 2005). In brief, Raf is maintained in its inactive state in the cytosol by association with 14-3-3 and possibly connector enhancer of KSR (CNK). A 14-3-3 dimer binds the phosphorylated N-terminal (S259) and C-terminal sites (S621) on Raf, keeping Raf in a closed, catalytically inactive conformation. Upon growth factor stimulation, Raf translocates to the plasma membrane where it binds GTP-loaded Ras. Ras binding displaces the 14-3-3 interaction with the N-terminal site (S259) allowing this site to be dephosphorylated by the phosphatase PP2A. Dephosphorylation of this site causes the release of 14-3-3 thereby allowing conformational changes in Raf that open its kinase domain to further activating events. This includes phosphorylation of Raf at multiple sites by kinases such as PKC and src (for reviews see Leicht et al., 2007; Wellbrock et al., 2004). Heterodimerization of Raf isoforms via 14-3-3 also appears to play a critical role as B-Raf has been shown to augment C-Raf activation (Garnett et al., 2005; Rushworth et al., 2006). Once activated, Raf phosphorylates and activates MEK. Full activation of MEK is obtained only when it is phosphorylated by both Raf and PAK1 (Frost et al., 1997; Frost et al., 1996). MEK is a dual specificity kinase that binds to inactive ERK and retains it in the cytoplasm (Fukuda et al., 1997). Upon activation, MEK phosphorylates ERK at both the threonine and the tyrosine residues of the conserved Thr-Glu-Tyr (T^{183}-E-Y^{185}) motif in the phosphorylation loop. Active ERK is then released from MEK and can dimerize and translocate into the nucleus. In the nucleus ERK may phosphorylate many substrates including transcription factors. Alternatively, in the cytoplasm ERK can phosphorylate a separate set of substrates including kinases and cytoskeletal proteins (Fig. 2).

Upon activation ERK1/2 can phosphorylate over 100 possible substrates with diverse functions (Yoon and Seger, 2006). ERK1/2 are kinases that in general phosphorylate serine/threonine residues that are followed by a proline (S/T-P). There are two well-defined docking domains that mediate ERK binding to the various substrates. These are the D-domain (also known as the DEJL motif) and the DEF-domain (also known as the FxFP motif) (Biondi and Nebreda, 2003; Zhang and Dong, 2007). The DEF-domain is characterized by an S/T-P phosphorylation site adjacent to the sequence Phe-x-Phe-Pro (FxFP). Examples of substrates including this sequence are the transcription factor c-Fos and the phosphatase DUSP1 (MKP-1). The D-domain is a common binding site for many MAP kinases that was first identified for JNK substrates and includes the sequence Arg/Lys-x-x-Arg/Lys-x_{1-6}-Leu-x-Leu (Kallunki et al., 1994). This sequence can be found not only in ERK substrates such as the RSKs but also in other ERK interacting proteins such as the ERK kinase MEK1/2. Finally some ERK substrates contain both docking domains such as the transcription factor ELK-1 (Yang et al., 1998; Fantz et al., 2001). These docking sites are bound independently by different portions of ERK (Dimitri et al., 2005). The DEF domain binds to a region of ERK that includes residues 185–261 that are adjacent to the activation loop (containing the TEY sequence) while the D-domain interacts with residues 316 and 319 of the so-called CD domain of ERK (Tanoue et al., 2000; Lee et al., 2004). These docking domains thus help direct the activated ERK to specific available targets.

The output of ERK activation in a given cell is ultimately determined by the set of substrates that it phosphorylates in a specific context. Thus the results of ERK1/2 activa-
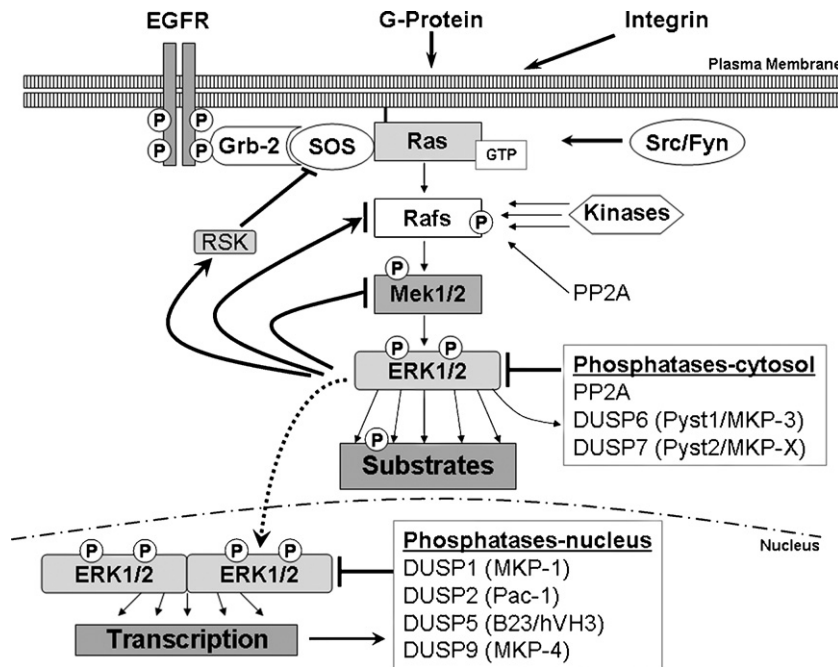
Fig. 1. ERK Pathway regulation by feedback loops and phosphatases. Activation of the ERK MAP kinase pathway by the EGF receptor is depicted in its simplest form. The ERK pathway can also be activated by G-protein coupled receptors, integrins, the tyrosine kinases Src/Fyn, and other receptor tyrosine kinases. Upon activation by EGF ligation, EGF receptor autophosphorylates at multiple tyrosine residues on its cytoplasmic face. This promotes the recruitment of the SH2 domain containing Grb-2, which in turn recruits the guanine nucleotide exchange factor SOS. SOS activates release of GDP from Ras which subsequently binds GTP. GTP bound Ras recruits RAF to the membrane where it is activated by multiple phosphorylations and de-phosphorylations. Raf phosphorylates two serine residues in the activation loop of MEK1/2. MEK is a dual specificity kinase that phosphorylates ERK on both threonine and tyrosine in the conserved TEY motif of the activation loop. PAK1 phosphorylation of MEK1/2 also contributes to its activation (not shown). Activated ERK can phosphorylate more than 100 substrates at various locations in the cell. The dimerized form of ERK actively translocates into the nucleus where it phosphorylates many transcription factors. ERK phosphorylation of MEK and possibly Raf can inactivate the pathway at those steps creating a negative feedback loop (indicated by the solid lines). ERK also activates the kinase RSK2 which can inhibit the ERK pathway by phosphorylating SOS. Interestingly ERK phosphorylation of Raf is also reported to enhance activation of the ERK pathway. Finally, there are several phosphatases including the dual specificity phosphatases (DUSPs) that can inactivate ERK in either the cytoplasm or the nucleus. ERK can upregulate transcription of some of these or alter their activity by direct phosphorylation. PP2A function is complex and it is reported to both inactivate MEK and ERK, while contributing to the activation of Raf.

tion are as diverse as the available substrates (Fig. 2). For example at the plasma membrane ERK phosphorylation of connexin43 downregulates GAP junction communication (Warn-Cramer et al., 1998; Warn-Cramer et al., 1996) whereas ERK activation of Myosin Light Chain Kinase (MLCK) regulates cell migration (Klemke et al., 1997). In the nucleus ERK can activate various transcription factors including ELK-1 (Yang et al., 1998; Rao and Reddy, 1994) and c-Fos (Chen et al., 1996; Chen et al., 1993; Murphy et al., 2002) in addition to kinases such as MSK-1 (Deak et al., 1998). In other cases the outcome of ERK activation involves as yet undefined substrates. For example ERK activity inactivates integrin-mediated cell adhesion by an undetermined mechanism (Hughes et al., 1997). Therefore the substrates available to ERK in a given cell can define the outcome of ERK activation for that cell.

One of the most potent mechanisms for shutting off MAP kinase signaling is by dephosphorylation of the threonine and tyrosine residues of the conserved TEY sequence in the activation loop. This is mitigated by a family of dual specificity threonine/tyrosine MAP kinase phosphatases (MKPs) also known as dual specificity phosphatases (DUSPs) (Owens and Keyse, 2007; Theodosiou and Ashworth, 2002). Of these MKPs, 7 can dephosphorylate

ERK1/2 (Fig. 1). MKPs can be localized to the cytoplasm, the nucleus, or travel between both. Some of these MKPs are encoded by genes that are transcriptionally activated by ERK (MKP1) and this can provide a feedback loop to downregulate ERK activity. ERK-directed phosphatases have been recently reviewed elsewhere (Owens and Keyse, 2007; Kondoh and Nishida, 2006; Junttila et al., 2008) and will not be discussed in detail here.

2. Regulation by feedback loops

Of particular interest to the regulation of ERK are ERK substrates that feedback on the ERK pathway to increase (positive feedback) or decrease (negative feedback) ERK activity. These substrates could exert control over the intensity and duration of ERK signaling. Signal duration is of primary importance in determining the outcome of ERK activation (Murphy and Blenis, 2006). Evidence for the importance of signal duration came from observations that only the sustained activation of ERK induced by NGF and not the transient activation induced by EGF, could cause differentiation of pheochromocytoma cells (PC12) and neurite extension (Nguyen et al., 1993; Traverse et al., 1992). Similar correlations were found for other ERK stimulated behaviors
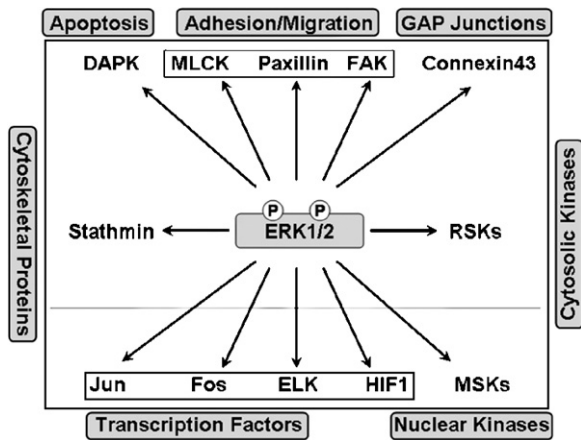
Fig. 2. The diversity of ERK1/2 substrates. ERK has more than 100 known substrates. The diversity of these substrates is indicated by showing examples of substrates with divergent functions. ERK targets both transcription factors and kinases in the nucleus. It can also phosphorylate various kinases and structural proteins in the cytosol, while at the plasma membrane it targets proteins that regulate cell adhesion, cell–cell communication, and cell survival. The outcome of activation of the ERK pathway in a given cell will therefore be determined in part by where the active ERK is targeted in the cell and which substrates it has access to at those locations.

in the transformation of fibroblasts (Mansour et al., 1994), and the development of T cells (Sharp et al., 1997) and macrophages (Whalen et al., 1997). These observations led to the first models to suggest that ERK specificity resulted in part from signal duration (Marshall, 1995).

There are several points of negative feedback in the ERK pathway (Figure 1). MEK is one target and can be inhibited by ERK phosphorylation of MEK1/2 at Thr292 (Eblen et al., 2004) and Thr212 (Sundberg-Smith et al., 2005). Phosphorylation prevents further enhancement of MEK activity by PAK1 (Slack-Davis et al., 2003) and thereby reduces activation of ERK. Phosphorylation of Raf at multiple sites by ERK provides a second possible feedback loop mechanism (Dougherty et al., 2005). Hyperphosphorylation of these sites prevents Raf interaction with the Ras GTPase and promotes its dephosphorylation by the phosphatase PP2A. Finally, one of the first negative feedback targets identified was the guanine nucleotide exchange factor SOS, which activates Ras (Corbalan-Garcia et al., 1996). ERK-dependent phosphorylation of SOS in proximity to its proline rich sequences inhibits SOS interaction with the SH3 domains of Grb-2. This prevents recruitment of SOS to the plasma membrane thereby reducing Ras activation. SOS phosphorylation is mediated in vivo predominantly by RSK2 (Douville and Downward, 1997) which is a serine/threonine kinase directly activated by ERK in the cytoplasm (Hauge and Frodin, 2006). The activation or transcriptional upregulation of phosphatases by ERK is an alternative negative feedback mechanism. These various ERK activated negative feedback loops provide one means by which to limit signal duration and to return the pathway to the basal state.

There are also examples of ERK substrates and downstream pathway targets that increase ERK activity by positive feedback. Interestingly, ERK phosphorylation of Raf at several sites is also reported to enhance RAF activity

(Balan et al., 2006). ERK phosphorylates already active Raf to increase its activity 4 fold. Interestingly, some of the sites identified in this work were the same as those involved in inhibition. What determines whether ERK phosphorylation results in Raf inhibition or activation remains unresolved. A second positive feedback loop involves the phosphorylation of the cytosolic phosphatase DUSP6 (MKP3). DUSP6 is a dual specificity phosphatase that inactivates ERK by dephosphorylating it (Muda et al., 1996). ERK phosphorylates DUSP6 at two serine residues (S159 and S197) and this targets DUSP6 for degradation in the proteasome (Marchetti et al., 2005). This is the opposite of the effect of ERK phosphorylation of the nuclear DUSP1 (MKP-1) which is stabilized by ERK phosphorylation contributing to a negative feedback loop (Brondello et al., 1999; Brondello et al., 1997). Thus regulation of phosphatases appears to be central in controlling ERK activity levels.

The importance of both positive and negative feedback loops in regulating ERK signal duration was re-iterated by a series of experiments that again utilized the differentiation of PC-12 cells in response to NGF. In these experiments the ERK signaling network was systematically perturbed by using RNAi to downregulate protein levels at each step of the signaling module. A method called modular response analysis (MRA) (Kholodenko et al., 2002) was then used to determine how each step in the pathway affected the other in response to either NGF or EGF. On EGF stimulation the ERK signaling network exhibited only negative feedback, while NGF stimulated only positive feedback. This corresponds to the observation that EGF activates only transient ERK activity, while NGF activates sustained ERK activity. When the pathways are rewired (by altering PKC activity) to allow EGF to activate sustained ERK activity and NGF to activate transient ERK activity, EGF is able to induce neurite outgrowth (Santos et al., 2007). This work illustrates the promise of combining mathematical modeling with experimentation to sort out the mechanisms that regulate signal transduction pathways.

Thus multiple signals can activate the core Raf/MEK/ERK signaling module. The specific signals that are active in a given context appear to be responsible for determining the intensity and duration of ERK signaling. Meanwhile active ERK has an extensive set of possible substrates and an equally extensive list of functional outcomes. We now turn to how this multi-functional pathway is regulated by accessory proteins of four general types: upstream scaffolds, downstream scaffolds, anchors, and inhibitors.

3. Upstream scaffolds: enhancing and targeting activation of ERK

The yeast MAP kinase scaffold Ste5p was the first MAP kinase scaffold identified and remains the prototypical scaffold (Schwartz and Madhani, 2004). Scaffold proteins bind two or more components of a signaling pathway to bring them into close proximity and thereby facilitate their functional interaction—for example enhancing kinase phosphorylation of a substrate (Dhanasekaran et al., 2007). In addition, scaffolds can target these multi-enzyme signaling modules to different cellular locations and thereby enhance phosphorylation of a specific subset of down-

stream substrates. Scaffolds also affect the duration of the signal, prevent crosstalk among similar pathways, and can link the activation of the signaling module to a specific upstream activating signal. Scaffolds themselves may be regulated and can in this way integrate the signaling of two or more signal transduction networks.

The relative stoichiometry of a scaffold to its binding partners can have dramatic and opposing effects on the signaling module (Levchenko et al., 2000; Yu et al., 1998). Scaffolds at relatively low levels do not sufficiently enhance kinase binding to substrate and so signaling is below optimum. In contrast when the scaffold is in significant excess to the kinase and substrate, signaling is impaired. This is the result of the scaffold binding the kinase and substrate independently and in this way preventing their functional interaction. The ERK scaffold KSR was one of the first proposed to behave in this way (Cacace et al., 1999; Yu et al., 1998). This effect is called "combinatorial inhibition" (Fig. 3) and has been observed for most ERK scaffolds and quantitatively modeled for a generic scaffold protein (Levchenko et al., 2000). The model predicts that scaffold-kinase complexes can effectively regulate signal specificity, efficiency, and amplitude. Moreover the location of the optimum signal enhancement is determined by the concentrations of the kinases rather than by their binding constants for the scaffold. Thus scaffolds can provide specificity to the multi-potent ERK MAP Kinase pathway. Some examples of the best studied of these are touched upon briefly below (Fig. 4).

Kinase suppressor of Ras (*KSR; binds Raf, MEK1/2, ERK1/2, 14-3-3*): Kinase suppressor of Ras (KSR) is the best studied scaffold in the ERK pathway (Claperon and Therrien, 2007). KSR proteins were initially identified in genetic screens in both *Drosophila* and *C. elegans* that were designed to identify proteins required for Ras-mediated phenotypes (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995). Two KSR proteins are present in mammals, KSR1 and KSR2. These are structurally similar to Raf and contain a similar kinase domain though it is unclear if this domain is functional (Ritt et al., 2005; Therrien et al., 1995). KSR binds to Raf (Roy et al., 2002), Mek (Denouel-Galy et al., 1998), and ERK (Yu et al., 1998). Moreover, like Raf, KSR also interacts with 14-3-3 and this may stabilize the interaction of KSR with Raf (Xing et al., 1997). Thus KSR can form a complex with all the components of the ERK module and this complex is present in vivo in normal mouse brain lysates but not in mKSR1-deficient mice (Nguyen et al., 2002). KSR expression is required for optimum ERK activation and works in a manner exactly analogous to that described for scaffolds above. That is, at optimum levels of expression KSR increases ERK activity whereas at expression levels in excess of its cognate ligands, it inhibits activation of ERK (Denouel-Galy et al., 1998; Yu et al., 1998). Moreover, mutations that prevent KSR interaction with Raf or MEK act as loss of function mutants (Roy et al., 2002; Stewart et al., 1999; Muller et al., 2000). Finally, KSR1 knockout mice have defects in T-cell activation (Nguyen et al., 2002) and are less prone to Ras-induced tumors than the wildtype mice (Kortum and Lewis, 2004; Lozano et al., 2003). These effects appear to result directly from KSR modulation of ERK signaling.

Regulation of KSR is mediated by the E3 ubiquitin ligase IMP1 (Matheny et al., 2004). IMP1 inactivates KSR by indirectly causing its hyperphosphorylation and sequestration in a triton-insoluble compartment. This limits the functional assembly of Raf/MEK complexes. Upon stimulation, RAS-GTP recruits IMP1 to the membrane where it auto-ubiquitinates and is degraded, allowing KSR to form
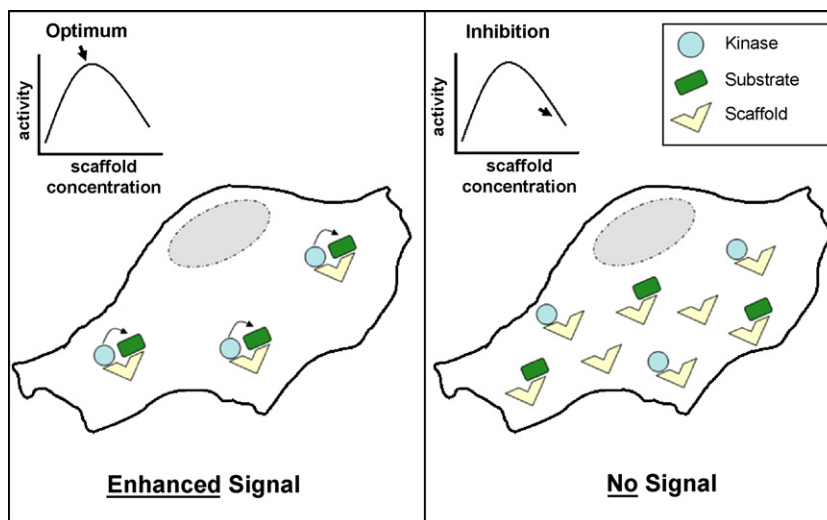
Fig. 3. Combinatorial inhibition of kinase activity by scaffolds. Scaffold proteins can promote the formation of a complex containing two or more components of a signaling pathway and thereby enhance signaling through that part of the pathway. In addition scaffolds can target the multi-enzyme signaling modules to particular sites in the cell (such as endosomes). The ability of scaffolds to regulate signal transduction is multi-faceted. Depicted is a binary scaffold model. At optimal concentrations of scaffold to kinase and substrate in this model, there is optimum kinase phosphorylation of substrate. However, when the scaffold is in excess of the kinase and substrate concentrations, it binds each individually and in this way prevents their interaction. This is called "combinatorial inhibition". This is important for understanding the potential conflicting functions of scaffolds in vivo as well as for interpreting data from experiments in which scaffolds are overexpressed.

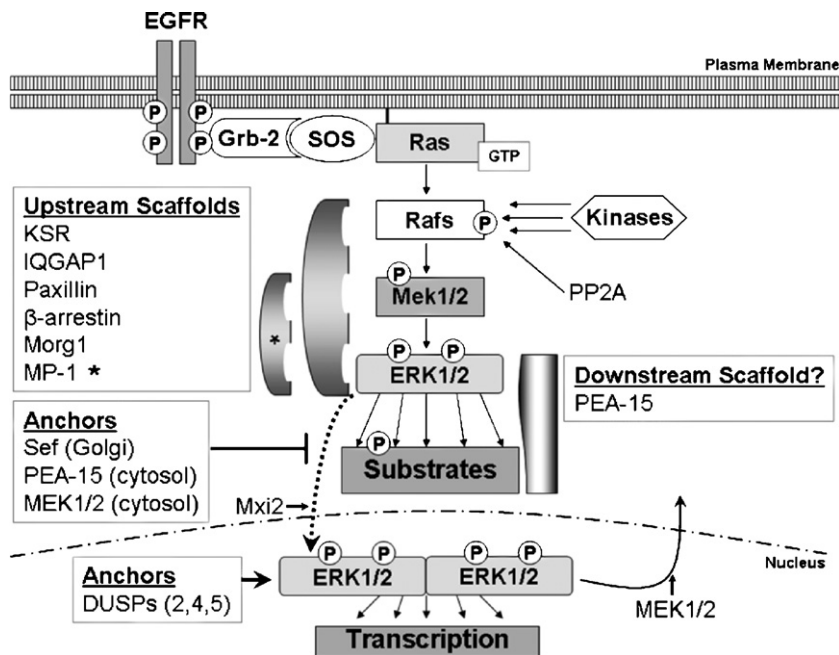**Fig. 4.** ERK regulation by scaffolds and anchors. The iconic ERK MAP kinase pathway is depicted. Scaffolds can form multi-enzyme complexes with upstream components of the pathway and thereby modulate ERK activation. Most of the upstream scaffolds identified thus far form complexes with Raf, Mek1/2, and ERK1/2. One exception is MP-1 which binds to Mek1 and ERK1 only (indicated by the "*"). PEA-15 is proposed to be a downstream scaffold that targets ERK1/2 to Rsk2. Many of these scaffolds also serve to target the ERK pathway to specific locations in the cell. For example MP-1 can target the MEK/ERK complex to endosomes. Other proteins serve as anchors to maintain ERK at specific locations in the cell such as the Golgi, cytosol or nucleus. Some of these proteins may also regulate ERK activity such as the nuclear phosphatases (DUSPs) and MEK1/2, while others appear to function solely as anchors (Sef). In contrast, Mxi2 promotes nuclear translocation of ERK by promoting ERK binding to the nuclear pore proteins, while MEK1/2 may aid in the removal of inactive ERK from the nucleus.

activating Raf/MEK complexes. KSR is therefore a regulated scaffold of the ERK MAP kinase pathway.

Connector enhancer of KSR (CNK1; binds Raf-1): Screens in *Drosophila* designed to identify functional partners of KSR led to the isolation of CNK (Therrien et al., 1998). CNK is essentially a large, non-catalytic adapter protein. There are three known CNK isoforms in mammals (Rabizadeh et al., 2004; Lanigan et al., 2003). In mammalian cells CNK binds only Raf and not other components of the ERK module suggesting that perhaps CNK can mediate non-ERK related activities of Rafs (Lanigan et al., 2003). CNK enhances Ras and Src activation of Raf-1 (Ziogas et al., 2005; Jaffe et al., 2004). In this way it enhances activation of ERK downstream of Raf. CNK binds to so many signaling proteins from different pathways that it remains difficult to determine precisely the mechanism by which it regulates the ERK module in mammals and the role of this activity in vivo. However experiments in *Drosophila* suggest that CNK is intimately involved in the activation of Raf by Ras (Douziech et al., 2006). In *Drosophila*, CNK recruits KSR and Raf before there is an activation signal. CNK recruits KSR through a novel protein named Hyphen (HYP) (Douziech et al., 2006) and binds directly to Raf. Upon activation of receptor tyrosine kinases, the kinase Src42 binds an RTK phosphorylated site on CNK (pY1163) thereby releasing an inhibitory effect of this site on Raf (Laberge et al., 2005) and this allows Raf activation by Ras. At this point Raf is in its fully open confirmation which may then interact with KSR (also tethered to CNK) to stabilize its activation and facilitate activation

of its target, MEK (Claperon and Therrien, 2007). It remains to be determined if CNK can perform a similar function in mammalian cells.

MEK Partner-1 (MP-1; binds MEK1 and ERK1) and Morg1 (binds Raf, MEK, ERK): MP1 was originally identified as a MEK1 binding protein in yeast two-hybrid screens (Schaeffer et al., 1998). MP1 selectively binds MEK1 and ERK1, but not MEK2. This enhances their interaction and leads to increased ERK activity (Schaeffer et al., 1998). This complex is targeted to late endosomes by the interaction of MP-1 with the endosome protein p14 (Wunderlich et al., 2001). Both MP-1 and p14 are required for MP-1 enhanced activation of ERK at the endosome (Teis et al., 2002). The MP-1/p14 complex also interacts with PAK1 and enhances PAK1 phosphorylation and activation of MEK during cell adhesion and spreading on fibronectin (Pullikuth et al., 2005). Whether MP-1 can form these molecular complexes and act as a scaffold in vivo is yet to be determined. MP-1 was used as bait in a yeast two-hybrid screen and was found to interact with a 35 KDa protein called MORG-1 (Vomastek et al., 2004). Morg-1 binds Rafs, MEK1/2, ERK1/2 and MP-1 in a high molecular weight complex. It enhances ERK activation in response to serum, lysophosphatidic acid (LPA), and phorbol ester (PMA), but has no effect on EGF-stimulation of ERK. Moreover, RNAi-mediated silencing of MORG-1 inhibited serum and LPA stimulation of ERK, but did not alter EGF stimulation of ERK (Vomastek et al., 2004). This suggests that MORG1 is specific for G-protein coupled receptor signaling. Though MP-1 and MORG1 co-localize at

vesicles, it remains unclear how they interact, if at all, to facilitate ERK activation.

IQGAP1 (binds B-Raf, MEK1 and ERK1): IQGAP interacts with a wide variety of proteins and can influence the actin-cytoskeleton, cell–cell adhesion and transcription as a result (Sacks, 2006). IQGAP binds B-Raf (Ren et al., 2007), MEK (Roy et al., 2005) and ERK (Roy et al., 2004) and facilitates activation of the Raf/ERK signaling module in response to EGF stimulation. B-Raf is not significantly activated by EGF in IQGAP1 null cells and a Raf-binding deficient IQGAP1 is likewise unable to promote ERK activation (Ren et al., 2007). IQGAP1 alone is able to activate B-Raf in vitro by an undetermined mechanism. IQGAP1 is overexpressed in breast cancer. Moreover, IQGAP1 contributes to proliferation, anchorage independent growth, and the invasive potential of MCF-7 breast cancer cells (Jadeski et al., 2008). Thus IQGAP1 scaffolding functions for the ERK pathway in addition to effects on cdc42/Rac signaling may contribute significantly to tumorigenesis. IQGAP1 scaffolding is also reported to mediate hippocampal N-cadherin memory consolidation (Schrick et al., 2007). Hence IQGAP1 is proving to be an intriguing new scaffold and it will be important to determine if it interacts with other scaffolds, for example MP1 which also modulates adhesion initiated ERK signals, in mediating its many functions.

β-Arrestin 1,2 (binds Raf-1, MEK1, ERK2): The Arrestins were originally isolated as proteins that inactivated GPCR signaling by mediating receptor internalization (Luttrell, 2006; DeWire et al., 2007). They have now been found to also act as scaffolds for ERK signaling (Luttrell, 2003). β-Arrestin 1 and 2 can bind to Raf, MEK and ERK (Defea et al., 2000a,b) and enhance cytosolic ERK activity at the expense of ERK-mediated transcription in the nucleus (Tohgo et al., 2002). The β-arrestin/ERK complex forms during endocytosis of the GPCR and the active ERK is present on the early endosomes (Defea et al., 2000b). The mechanism by which the ERK module is activated on the endosomes is not clearly defined, but is presumed to involve juxtaposition of the module with internalized growth factor receptors. In this way β-arrestins may provide a scaffold that acts specifically in GPCR signaling.

There are many other suggested upstream scaffolds for the ERK pathway including Sur-8 (binds Ras and Raf-1) (Li et al., 2000; Sieburth et al., 1998), MEKK1 (binds Raf-1, MEK1, ERK2) (Karandikar et al., 2000), Major Vault Protein (binds SHP-2 and ERK) (Kolli et al., 2004); and Paxillin (binds Raf, MEK, ERK, FAK) (Brown and Turner, 2004; Ishibe et al., 2003). With so many scaffolds available, very specific conduits for the relay of ERK signaling can be built that send signals from a specific receptor complex to specific cellular locations and perhaps also modulate signal duration. Thus scaffolds allow the cell to turn a multi-potent signal into a specific outcome and utilize a single kinase module to do more than one job.

4. Downstream scaffolds: targeting ERK to specific substrates

Given how effectively scaffolds provide control and specificity to the activation of ERK, it has been attractive to postulate that there may be scaffolds that target ERK to a specific subset of substrates. This would provide a mechanism by which the promiscuous ERK could be controlled to provide a specific outcome in a given cell. Until very recently there were no examples of these "downstream scaffolds" described in mammalian cells for any MAP kinase. The first candidate in this class was a protein called JNK-associated-leucine zipper protein (JLP) which tethers JNK or p38 to the transcription factors c-Myc and Max (Lee et al., 2002). JLP also binds the upstream kinases of the JNK module MEKK3 and MKK4. No similar protein has yet been identified for ERK activation of transcription.

Thus far the best candidate for a downstream scaffold for targeting ERK to a specific substrate is the phosphoprotein PEA-15. PEA-15 was originally characterized as a phosphoprotein enriched in astrocytes (Araujo et al., 1993) and was subsequently found to block H-Ras activated ERK signaling to integrins (Ramos et al., 1998). Moreover, PEA-15 generally suppresses ERK signaling in that it also blocks ERK activation of transcription when overexpressed (Formstecher et al., 2001). PEA-15 in these experiments binds to ERK1/2 and prevents nuclear accumulation of ERK in response to H-Ras or growth factor stimulation (see below). PEA-15 also binds RSK2 and can similarly affect RSK2 function when overexpressed (Vaidyanathan and Ramos, 2003). RSK2 is a substrate of ERK and so these observations immediately suggested that PEA-15 might act as a scaffold for ERK and RSK2. Indeed PEA-15 expression enhances ERK binding, phosphorylation and activation of RSK2 at low levels while at very high levels of expression PEA-15 blocks all of these events (Vaidyanathan et al., 2007) in a manner reminiscent of combinatorial inhibition (see above and Fig. 3). In this way PEA-15 is reminiscent of MP-1 and KSR and may be the first example of a downstream scaffold for ERK. As PEA-15 protein expression is primarily found in astrocytes and lymphocytes it might be that in these cells ERK activation in some contexts is predominantly directed to RSK2. The availability of PEA-15 for binding to ERK can be influenced by the phosphorylation of PEA-15 by PKC and CamK II (Renganathan et al., 2005). In this way, whether ERK is directed to RSK2 by PEA-15 could be controlled by signals from these pathways. This provides a model by which ERK targeting to a specific outcome may be due in part to the downstream scaffolds available. The targeting of ERK to particular substrates by scaffolds would provide a significant level of specificity to ERK signaling and perhaps provide focal points for the integration of other signal transduction pathways with the ERK pathway.

5. Regulation by localization

The concept of regulating kinases by anchoring them to specific cellular locations has been validated in many signaling systems. However, it has perhaps been shown most systematically for protein kinase A (PKA) where a diverse group of A-kinase anchoring proteins (AKAPs) has been defined (Smith et al., 2006). The localization of ERK is clearly an important component of controlling its activity. The translocation of ERK into the nucleus is important for many ERK functions. For example, active ERK2 retained in the cytoplasm is unable to promote neurite extension in PC12 cells, whereas its nuclear targeting results in neu-

rite outgrowth (Robinson et al., 1998). Similarly, ERK that is artificially localized to the cytoplasm is incapable of activating the c-fos promoter and progression into S phase in response to mitogenic stimulation (Brunet et al., 1999; Hochholdinger et al., 1999). It is therefore not surprising that several proteins have been found to restrict ERK localization or facilitate its targeting to specific cellular compartments (Fig. 4). In some cases a single protein can both target ERK localization to a specific cellular compartment and serve as a scaffold at that site (MP1 and PEA-15).

Rapid nuclear translocation of ERK requires phosphorylation of ERK by MEK. Part of the mechanism for the translocation is the phosphorylation-dependent dimerization of ERK2 (Khokhlatchev et al., 1998; Cobb and Goldsmith, 2000). Phosphorylation at the TEY motif by MEK1/2 results in a conformational change in ERK2 that allows the C-terminal portion (residues 309–358) to interact with the activation loop (Canagarajah et al., 1997). This exposes a dimer interface that includes leucines 333, 336, and 344 that has been identified in the crystal structure to mediate ERK2 homodimer formation (Canagarajah et al., 1997). This dimerization occurs at 7.5 nM which is below the intracellular concentrations of phosphorylated ERK2 (>100 nM) and so is likely to occur in cells (Ferrell and Machleder, 1998). The dimerization does not occur between different MAP kinases. The mechanism by which ERK dimerization promotes ERK nuclear translocation remains incompletely determined, though it may involve the exposure of a nuclear import sequence in ERK2 (Cobb and Goldsmith, 2000). Finally the import of active, phosphorylated ERK requires energy and cytosolic factors (Ranganathan et al., 2006). These observations regarding the active import of phosphorylated, dimerized ERK are in contrast to the energy- and carrier-independent, facilitated diffusion shown to mediate the nuclear accumulation of monomeric, non-phosphorylated ERK (Adachi et al., 1999; Ranganathan et al., 2006). In the case of monomeric ERK, nuclear localization may be the result of direct binding of ERK1/2 to the nuclear pore proteins (called Nups) (Matsubayashi et al., 2001; Yazicioglu et al., 2007). Aside form nuclear translocation, dimerization of ERK might also modulate ERK interaction with a substrate or other regulatory proteins such as scaffolds. This would be a mechanism to specify both ERK nuclear localization and enhance substrate specificity.

The ERK kinase MEK1/2 can prevent nuclear accumulation of inactive ERK (Rubinfeld et al., 1999). MEK was shown to mediate the export of inactive, monomeric ERK from the nucleus by a leptomycin B sensitive mechanism (Adachi et al., 2000). This was shown to be mediated by CRM1 interaction with the NES in MEK. Interestingly Cobb and Goldsmith have proposed that the dimerization of activated ERK may mask the MEK binding site and thereby contribute to the nuclear accumulation of the dimer (Cobb and Goldsmith, 2000). MEK-mediated export of ERK provides a mechanism by which the inactive ERK can re-associate with MEK and return to the cytoplasm for future activation.

The phosphoprotein PEA-15 described above as a possible downstream scaffold of ERK and RSK2 can also anchor ERK1/2 in the cytoplasm (Formstecher et al., 2001). PEA-15 is a 15 KDa phosphoprotein that consists of a death effector domain (DED) and an unstructured carboxy-terminal tail (Hill et al., 2002). PEA-15 binds tightly to ERK regardless of ERK phosphorylation state (Formstecher et al., 2001). The predominant binding site is the carboxy-terminal 15 residues of PEA-15 (Hill et al., 2002; Callaway et al., 2007) which interact with the MAP kinase insert of ERK which is structurally adjacent to the phosphorylation loop (Chou et al., 2003; Whitehurst et al., 2004). The mechanism by which PEA-15 prevents nuclear accumulation is not completely resolved. Leptomycin B promotes PEA-15 accumulation in the nucleus and PEA-15 has a NES that is required to maintain it in the cytoplasm suggesting CRM1-dependent export of PEA-15. When the NES is mutated both PEA-15 and ERK accumulate in the nucleus though PEA-15 binding to ERK is unchanged (Formstecher et al., 2001). These data indicate PEA-15 can mediate export of ERK from the nucleus via its NES in a manner analogous to that of MEK1/2. Alternatively, in experiments where the plasma membrane has been stripped away and exogenous PEA-15 in various combinations with ERK is added, PEA-15 prevents nuclear import into the nucleus (Whitehurst et al., 2004). PEA-15 also prevents ERK binding to nuclear pores in competition assays. Thus PEA-15 may prevent ERK translocation into the nucleus by blocking ERK binding to the pores (Whitehurst et al., 2004). The resolution of these differences has not been reported. It may be that the PEA-15/ERK complex in cells includes other components that alter the relationship of PEA-15 to the nucleoporin binding sites. Regardless, PEA-15 null cells exhibit dramatically increased levels of ERK in the nucleus in the absence of stimulus, and concomitant increases in c-Fos transcription and proliferation (Formstecher et al., 2001). This indicates an essential role for PEA-15 in regulating ERK distribution in the cells in which it is expressed. Moreover, it now appears that the cytoplasmic localization of ERK may also involve specific targeting of ERK to RSK2 as described above.

Sef is another spatial regulator of ERK signaling that has many functional similarities to PEA-15. Sef is thought to be a transmembrane protein and was originally identified as an inhibitor Ras-mediated FGF signaling in Zebrafish (Tsang et al., 2002; Furthauer et al., 2002). Sef blocks ERK nuclear translocation without altering ERK phosphorylation of its cytoplasmic targets such as RSK2. Sef does this by binding the activated forms of MEK and preventing the stimulus-dependent dissociation of ERK from MEK (Torii et al., 2004). Knockdown of Sef results in stimulus-induced accumulation of ERK in the nucleus and activation of ELK-1. The Sef/pMEK complex is localized both to membrane ruffles and to the Golgi complex. Sef may therefore contribute to control of ERK signaling by enhancing ERK activation at these specific subcellular compartments.

It is interesting to note here that in fibroblasts Ras is active in both the plasma membrane and at the Golgi (Quatela and Philips, 2006). Furthermore, Ras localization influences which pathways Ras activates in a cell. This point was convincingly supported by observations in fission yeast that plasma membrane-targeted Ras1 mediates signals regulating mating but not morphology. Conversely, endomembrane-targeted Ras1 is only able to mediate signals regulating morphology but not mating (Onken et al., 2006). It is therefore likely that proteins such as Sef enhance

Ras activation of the ERK pathway at the Golgi or other membranes by targeting ERK to these compartments.

In contrast to the effects of MEK, PEA-15 and Sef in promoting ERK retention in the cytoplasm a protein called Mxi2 has recently been identified that promotes stimulus-independent accumulation of ERK in the nucleus (Casar et al., 2007). Mxi2 is a p38α isoform that binds directly to ERK1/2 and prolongs its activation specifically in the nucleus (Sanz-Moreno et al., 2003). Mxi2 has no effect on cytoplasmic ERK activity. Knockdown of Mxi2 results in a reduction in both nuclear ERK1/2 and cellular proliferation. This is apparently due to Mxi2 binding to nucleoporins and thereby potentiating ERK proximity to the nuclear pores (Casar et al., 2007). Mxi2 also displaces PEA-15 from ERK thus perturbing PEA-15's localization of ERK to the cytoplasm. Mxi2 is the first example of a protein that promotes stimulus-independent import of ERK into the nucleus.

There is growing evidence that the dual specificity phosphatases (DUSPs) can anchor ERK in both the cytoplasm and the nucleus (Karlsson et al., 2006). For example, DUSP6 can anchor ERK2 in the cytoplasm by a mechanism dependent on the NES of DUSP6 (Karlsson et al., 2004). In contrast, several ERK phosphatases can localize ERK to the nucleus. DUSP5 contains a nuclear localization signal (NLS) adjacent to the KIM in its amino-terminus. Moreover the expression of DUSP5 causes the nuclear translocation and sequestration of inactive ERK2. This function is dependent on the NLS and the binding of DUSP to the CD domain or ERK2 (Mandl et al., 2005). Recently DUSP2 and 4 have also been shown to anchor ERK in the nucleus via their D-domains (Caunt et al., 2008). Aside from phosphatases, only kinetochores and topoisomerase II have been proffered as examples of nuclear anchors (Shapiro et al., 1998). Given the significant role of ERK in activating transcription in the nucleus, and the prolonged retention of ERK in the nucleus upon stimulation in some cells, it is expected that other nuclear anchors will be identified. Hence, MKPs directly modulate both ERK1/2 activity and localization.

We have already met MP-1 (a MEK/ERK scaffold) above. While MP-1 was originally shown to act as a MEK1/ERK1 scaffold it has subsequently been shown to be localized to late endosomes (Wunderlich et al., 2001). MP1 is localized to late endosomes through its interaction with p14. Interestingly loss of expression of either MP1 or p14 reduces MEK activation of ERK and the duration of the ERK1 signal (Teis et al., 2002). This indicates that MEK1 activation of ERK1 on endosomes is functionally significant. Hence, MP1 is a good example of a scaffold protein that also targets the scaffold to a specific cellular compartment.

Many viruses activate ERK during infection and require active ERK to replicate. Interestingly, the alphaherpesvirus Tegument protein Us2 binds ERK upon viral infection of a cell (Lyman et al., 2006). Us2-ERK binding prevents translocation of active ERK into the nucleus by sequestering it at the plasma membrane and in a perinuclear vesicular compartment. Us2 does not alter ERK activity and is required along with ERK for viral replication. In this way, inappropriate targeting of ERK in the cell may aid infection by localizing the ERK at sites where it promotes viral replication at the expense of host cell transcription and proliferation.

Several anchor proteins have been identified for ERK that can anchor ERK to various cellular compartments. These include the nucleus, cytoplasm, plasma membrane, Golgi and endosomes, thus far. Some of these proteins also serve as scaffolds. Furthermore, loss of expression of these proteins results in aberrant localization of ERK signaling complexes and changes in proliferation and transcription. Thus, anchors are an essential regulatory component of the ERK pathway.

6. Inhibitors of ERK/MAPK signaling

One final category of ERK regulators are the non-phosphatase inhibitors. The two best examples of these are Raf kinase inhibitor protein (RKIP) and Sprouty. RKIP binds to both Raf and MEK, but in doing so inhibits their interaction (Yeung et al., 1999). Downregulation of endogenous RKIP activates ERK signaling, supporting the hypothesis that it functions as a negative regulator of ERK signaling in vivo. In addition RKIP dissociates from Raf upon mitogen stimulation thereby permitting activation of MEK. Interestingly, RKIP is downregulated in a number of metastatic cancer cells (Fu et al., 2003; Chatterjee et al., 2004; Schuierer et al., 2006, 2004). RKIP has also been implicated in cell migration although the exact mechanism is uncertain (Zhu et al., 2005). Given these data, RKIP is currently thought to play a significant role as a metastasis suppressor (Keller, 2004). This is an excellent example of perturbations of an ERK regulator resulting in significant pathology and reiterates the importance of these controls.

Sprouty was originally identified as a feedback inhibitor of FGF signaling in *Drosophila* (Hacohen et al., 1998). Sprouty and the Sprouty-related proteins with EVH1 Domain (SPRED) appear to work in part by inhibiting protein interactions. For example Sprouty4 binds B-Raf and sequesters it to prevent Ras-independent activation of ERK in response to vascular endothelial growth factor (but not in response to EGF) (Sasaki et al., 2003). In another example, Sprouty blocks Ras activation by binding to Grb-2, in response to Src phosphorylation, and thereby impairs the recruitment of SOS to the growth factor receptor (Gross et al., 2001). Sprouty function is growth factor and cell type specific and provides a mechanism for blocking activation of Raf (and therefore ERK) in mammalian cells.

7. Concluding remarks

ERK1/2 are essential players in many cellular and physiological signaling responses. Aberrant ERK1/2 signaling is in part responsible for significant pathologies such as oncogenesis. Because of this many MEK and ERK inhibitors are being developed or are already in clinical trials for these diseases. The ubiquitous nature of ERK signaling however may result in significant side effects for these drugs. An alternative would be to develop drugs that specifically target accessory proteins that regulate ERK function. For example a drug that perturbs ERK interaction with a downstream scaffold (such as PEA-15) or substrate (such as RSK2) might block only that activity of ERK and permit other ERK functions. Indeed one such drug has been identified for JNK. A cell-penetrating, protease-resistant peptide was developed

that blocks JNK binding to many of its targets including c-Jun. This peptide effectively blocks JNK activation of c-fos transcription in neurons and thereby protects neurons during cerebral ischemia (Borsello et al., 2003). Drugs such as these for ERK might be expected to have fewer side effects and to better target specific ERK signaling anomalies. For these reasons identification and characterization of ERK regulatory proteins and their involvement in ERK related diseases remains a very active area of investigation.

Acknowledgements

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