COORDINATING ERK/MAPK SIGNALLING THROUGH SCAFFOLDS AND INHIBITORS

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Abstract | The pathway from Ras through Raf and MEK (MAPK and ERK kinase) to ERK/MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase) regulates many fundamental cellular processes. Recently, a number of scaffolding proteins and endogenous inhibitors have been identified, and their important roles in regulating signalling through this pathway are now emerging. Some scaffolds augment the signal flux, but also mediate crosstalk with other pathways; certain adaptors target MEK–ERK/MAPK complexes to subcellular localizations; others provide regulated inhibition. Computational modelling indicates that, together, these modulators can determine the dynamic biological behaviour of the pathway.

PC12 CELLS
A clonal line of rat adrenal pheochromocytoma cells which is used as model for neuronal differentiation as the cells respond to nerve growth factor and can synthesize, store and secrete catecholamines, much like sympathetic neurons. PC12 cells contain small, clear synaptic-like vesicles and larger dense-core granules.

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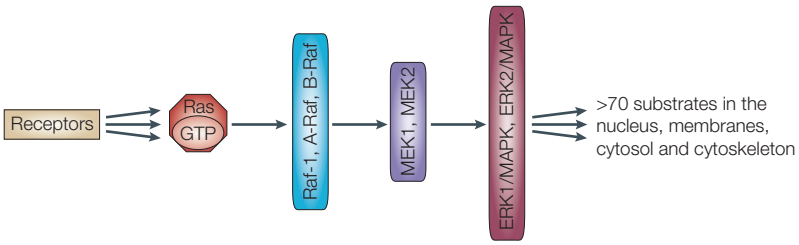
The Ras–Raf–MEK–ERK/MAPK pathway (MEK is MAPK and ERK kinase, MAPK is mitogen-activated protein kinase, and ERK is extracellular signal-regulated kinase) is an evolutionary conserved pathway that is involved in the control of many fundamental cellular processes that include cell proliferation, survival, differentiation, apoptosis, motility and metabolism[1–3]. It was one of the first roadmaps that described how extracellular molecules, such as growth factors and hormones, could generate signals that find their way to the nucleus to impinge on gene expression and alter the behaviour or even the biological programme of a cell. The essential biochemistry of the pathway is now established, but how the pathway can process a myriad of specific inputs into diverse biological outputs remains an enigma.

The ERK/MAPK pathway initially appeared as a linear pipeline that conveyed signals from cell surface receptors to ERK/MAPK, which distributed them to different effectors. Many receptors induce the activation of Ras, a small GTPase that binds to, and recruits, Raf kinases to the cell membrane for subsequent activation. Activated Raf kinases are the point of entry into a three-tiered kinase cascade in which Raf phosphorylates and activates MEK, and MEK phosphorylates and activates ERK/MAPK (BOX 1). Traditionally, ERK/MAPK was considered the sole effector of the pathway with substrates in the nucleus, cytosol, membranes and cytoskeletal compartments. This simplistic view of a linear pathway began to crumble as more and more functions of this pathway were discovered. For instance, the only commonly accepted substrate for all three isoforms of Raf (A-Raf, B-Raf and Raf-1) is MEK, but data from knockout mice clearly show that Raf has isoform-specific functions[1]. In particular, these studies indicate that B-Raf might be the predominant activator of MEK and that Raf-1 has another role in protection against apoptosis, which does not require its kinase activity or its ability to activate MEK[4,5].

There must, therefore, be branch points in the pathway that mediate different functions, but little is known how these branch points are coordinated. Some of these functions are mutually exclusive, such as proliferation versus cell-cycle arrest and differentiation. The classical example of PC12 CELLS shows that terminal differentiation into neuronal-like cells correlates with sustained activation of ERK/MAPK and its translocation into the nucleus, whereas proliferative signals cause a transient

Box 1 | **The ERK/MAPK pathway**



Most cell-surface receptors activate Ras GTPases. Ras GTPases comprise a large family of mostly membrane-resident proteins that shuttle between an inactive GDP-bound and active GTP-bound conformation[105]. The best-characterized family members are K-Ras, H-Ras and N-Ras, which are encoded by potent proto-oncogenes that are mutated in ~30% of human tumours[106]. The oncogenic mutations prevent them from hydrolysing GTP, which maintains them in the activated state. In its activated conformation, Ras•GTP can bind to a number of effector molecules, including the serine/threonine kinase Raf, phosphatidylinositol 3-kinase, RalGDS and others[106]. Ras•GTP recruits these proteins to the membrane compartment, which is crucial for their activation and signalling function. All three Raf family members, A-Raf, B-Raf and Raf-1, bind Ras•GTP as the first step in their activation process. Whereas Ras•GTP association might suffice to activate B-Raf, both Raf-1 and A-Raf undergo a complex series of activation steps that have not been entirely elucidated and involve changes in phosphorylation levels and protein interactions. For recent reviews on Raf isoforms and their activation mechanisms see REFS 1–3**. All Raf isoforms can activate MAPK and ERK kinase (MEK) by phosphorylating two serines in the MEK** ACTIVATION LOOP**, although B-Raf is much more effective at doing so than Raf-1, which is better than A-Raf. The gene that encodes B-Raf is also mutated in many cancers, mainly in melanoma and cancers of the thyroid, colon and ovaries[107]. MEK is a dual-specificity kinase, the only known substrate of which is extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), the prototypical MAPK that is activated by phosphorylation of both the threonine and tyrosine residues in a TEY motif that is in the activation loop. Both MEK and ERK/MAPK have two isoforms in mammals, which, in most instances, are co-regulated but might have different functions[108]. ERK/MAPK is considered the main effector of this pathway and has more than 70 known substrates that include nuclear transcription factors, cytoskeletal proteins, signalling proteins and receptors[109].**

activation, with ERK/MAPK mainly remaining in the cytosol[6]. By contrast, in fibroblasts nuclear translocation of ERK/MAPK is required for mitogenesis[7]. These examples show that biological information can be expressed by the kinetics of ERK/MAPK activation and its subcellular compartmentalization, but can also be interpreted differently in a cell-type specific context.

An emerging view is that signal transduction pathways are organized as communication networks that process and integrate information and their relay stations are formed by multiprotein complexes. Using the ERK/MAPK pathway as an example, this review explores our current understanding of the function of such signalling complexes. The focus will be on the role of protein interactions that regulate the signal flux and that integrate and distribute signals, with a view to how the diversity and specificity of ERK/MAPK signalling is generated.

Is Raf-1 a scaffolding protein?

Although the role of Raf-1 as a MEK kinase is well established, recent results imply that Raf-1 also functions as a scaffold. This view is supported by the finding that the lethal phenotype of Raf-1-knockout mice can be fully rescued by reconstitution with a mutant form of Raf-1 (Raf-1YY340/1FF) that has low kinase activity and that can not be activated by growth factors[4]. In fact, a mutant form of Raf-1 that completely lacks kinase activity can revert the susceptibility of *Raf-1*$^{-/-}$ cells to apoptosis[8].

Raf-1 and apoptosis. Raf-1 can inhibit apoptosis by binding to and suppressing the activity of the pro-apoptotic kinase MST2 (mammalian sterile-twenty-like-2) by preventing its dimerization and by recruiting a phosphatase that inactivates it[8]. B-Raf cannot compensate for Raf-1 in this function because it does not bind MST2. So B-Raf cannot prevent apoptosis of *Raf-1*$^{-/-}$ cells, although it is expressed in these cells and ensures the apparently normal activation of MEK and ERK/MAPK[4,5]. Raf-1 also binds ASK1 (apoptosis signal-regulating kinase), another pro-apoptotic kinase, and inhibits it independently of Raf-1 catalytic activity, although the molecular mechanism is unknown[9]. This interaction prevents cardiomyocyte apoptosis during heart development[10], which implies that Raf-1 controls several pro-apoptotic pathways in a tissue-specific manner by binding and inhibiting the activity of pro-apoptotic effectors. The signals that regulate these interactions are insufficiently characterized.

Intriguingly, the Raf-1–MST2 interaction is disrupted by both pro-apoptotic stress signals and growth factors[8]. However, the consequences for the cell are profoundly different. Whereas stress signals trigger MST2 activation and apoptosis, growth factors release MST2 from Raf-1 without activating MST2 (REF. 8), which indicates a potential model in which Raf-1 links mitogenic stimulation to the risk of apoptosis. Although counterintuitive at first glance, such connections constitute self-regulated mechanisms that can safeguard the cell against unlicensed proliferation. As multicellular organisms must constantly balance cell proliferation and cell death to stay healthy, such autoregulatory modules could convey an evolutionary advantage by providing robustness against faulty signals.

Raf-1 as a potential scaffold for the Rho pathway. It has been proposed that Raf-1 also has a scaffolding function in the regulation of the Rho pathway[11]. RhoA, RhoB and RhoC are Ras-related GTPases that, when activated by GTP binding, associate with several effectors that regulate transcription, cell cycle, cytokinesis and the cytoskeleton — in particular, the dynamics of microtubules and actin polymerization[12]. *Raf-1*$^{-/-}$ cells are compromised in their ability to form the actin STRESS FIBRES that are needed for cell migration. This might be an isozyme-specific effect, as *B-Raf*$^{-/-}$ cells show increased migration[13]. In *Raf-1*$^{-/-}$ cells the migration defect was traced to the hyperactivation and mislocalization of Rho-dependent kinase ROKα/ROCK2 (Rho-kinase/Rho-associated protein coiled-coil forming kinase), and could be corrected by reconstituting the cells with either Raf-1 or a catalytically inactive form of Raf-1. Raf-1 was found to associate with ROKα/ROCK2, presumably controlling its activity and its correct subcellular

ACTIVATION LOOP
A 20–25-residue segment in a protein kinase that functions to modulate kinase activity.

STRESS FIBRES
Also termed 'actin-microfilament bundles', these are bundles of parallel filaments that contain F-actin and other contractile molecules, and often stretch between cell attachments as if under stress.

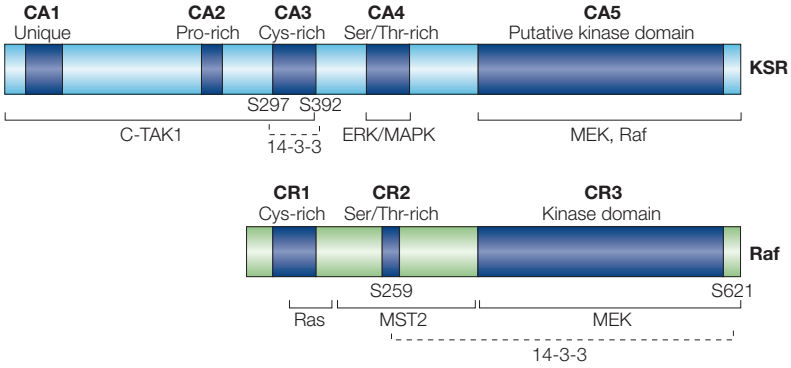


Figure 1 | **KSR and Raf domains.** KSR (kinase suppressor of Ras) proteins contain five conserved domains: CA1, which is unique to KSR1 and lacking in KSR2; CA2, a proline-rich sequence; CA3, a cysteine-rich domain that mediates interactions with membrane lipids; CA4, a serine/threonine-rich region that binds extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK); and CA5, the putative kinase domain. Binding sites for some interacting proteins are indicated. C-TAK1 binds to the N-terminal region and phosphorylates the 14-3-3 docking sites S297 and S392. MAPK and ERK kinase (MEK) and Raf bind to the C-terminal region that contains the CA5 domain. Raf kinases share three conserved regions: CR1, a cysteine-rich domain that contains part of the Ras-binding domain and also interacts with membrane lipids; CR2, a serine/threonine rich region that forms part of the MST2-binding region; and CR3, the kinase domain, which can bind MEK. Binding of 14-3-3 proteins to S392 in KSR or S259 in Raf-1 sequesters KSR1 and Raf-1 in the cytosol. MST2, mammalian sterile-twenty-like-2.

localization[11]. So, kinase-independent scaffolding functions of Raf-1 contribute to the physiological functions that connect Raf-1 with pathways that control both apoptosis and cytoskeletal dynamics.

Kinase suppressor of Ras-1

Kinase suppressor of Ras-1 (KSR1) was originally found in genetic screens in *Drosophila melanogaster* and *Caenorhabditis elegans* as a suppressor of an activated Ras phenotype[14]. KSR1 has high homology with Raf-1 (FIG. 1), but its kinase domain has mutations in key residues that are essential for catalytic activity in other protein kinases. Whether or not KSR1 possesses kinase activity has not been conclusively settled, although the bulk of evidence supports a kinase-independent function of KSR1 as a scaffold of the ERK/MAPK pathway.

KSR1 and the ERK/MAPK pathway. KSR1 can bind all kinase members of the ERK/MAPK pathway but, whereas MEK is associated constitutively, Raf-1 and ERK/MAPK might bind in a stimulus-dependent manner[14]. Strong evidence that KSR1 is a scaffold protein stems from experiments showing that cooperation of this protein with Ras to transform cells required the cysteine-rich CA3 domain in the non-catalytic region, but not the KSR1 kinase domain[15]. Titrating the levels of KSR1 expression affects ERK/MAPK pathway signalling in a bell-shaped-curve manner that is typical of scaffolding proteins[16]. That is, an increase in scaffold expression enhances the assembly of signalling-competent complexes until the concentration of the scaffold exceeds that of its client proteins, causing them to bind individually rather than to the same molecule at the same time, thereby effectively dissipating protein complexes and inhibiting signalling. In *Ksr1*$^{-/-}$ fibroblasts, re-expression of KSR1 at ~14-fold higher levels than normal resulted in maximal assembly of the Raf-1–MEK–ERK/MAPK complex and optimal ERK/MAPK signalling. However, even under these conditions, less than 5% of endogenous Raf-1, MEK or ERK/MAPK was associated with KSR1 (REF. 16), which indicates that, instead of globally enhancing ERK/MAPK signalling, KSR1 might affect only a subset of functions.

Insights from* Ksr-*knockout mice. In *C. elegans* both KSR-1 and KSR-2 isoforms are involved in distinct developmental pathways, and only their double knockout is lethal[17]. In mice, the knockout of *Ksr1* causes defects in antigen-triggered T-cell proliferation[18] and disorganized hair follicles[19]. The subtlety of the phenotype might be deceptive, as a recently identified relative, KSR2 (REF. 20), might compensate for the loss of KSR1. Importantly, *Ksr1*$^{-/-}$ mice are less susceptible to oncogene-induced tumours than their wild-type counterparts[16,18,19], which implies that KSR1 supports the proliferative and transforming functions of the ERK/MAPK pathway. This is consistent with the notion that ablation of KSR1 abolishes the sustained activation of ERK/MAPK that is required for mitogenesis[16,21]. *Ksr1*$^{-/-}$ mice also have an elevated basal level of apoptosis of colon epithelial cells, which manifests itself pathologically when these cells are challenged by tumour necrosis factor-α (TNFα)[22]. This correlates with a lack of ERK/MAPK activation by TNFα. Similarly, overexpression of B-KSR, a tissue-specific KSR1 splice form, converts epidermal growth factor (EGF) from a weakly proliferative signal into a differentiation signal for PC12 cells by extending the duration of ERK/MAPK signalling (which is required for neuronal differentiation)[23]. These data indicate that KSR1 can influence the biological fate of cells by regulating the activation kinetics of the part of the ERK/MAPK pathway that mediates proliferation, transformation, differentiation and apoptosis.

Regulating the scaffold. If KSR1 is the crucial denominator, the question then arises — what regulates the scaffold? In response to growth factors, KSR1 is translocated to the cell membrane (FIG. 2). This promotes MEK activation, presumably by presenting MEK to activated Raf[24]. In quiescent cells, KSR1 is retained in a Triton-insoluble compartment by the protein 'impedes mitogenic signal propagation' (IMP)[25] and in the cytoplasm by 14-3-3 PROTEINS that dock to the serine-phosphorylated residues 297 and 392 (REF. 24). S392 is phosphorylated by Cdc25C-associated kinase-1 (C-TAK1)[24] and nucleoside diphosphate kinase, mitochondrial-23 (Nm23)[26]. Mitogens induce the dephosphorylation of S392 by protein phosphatase-2A (PP2A)[24] and the destruction of IMP[25], which is sufficient to enable KSR1 to translocate to the cell membrane.

The KSR cycle. IMP is an E3 UBIQUITIN LIGASE that triggers its own degradation when it is bound by Ras•GTP[25]. However, in non-stimulated cells, instead of ubiquitylating KSR1, IMP causes KSR1 hyperphosphorylation,

14-3-3 PROTEINS
A large class of proteins that are involved in cell division, apoptosis, signal transduction, transmitter release, receptor function, gene expression and enzyme activation in eukaryotes. They function by binding to a wide range of specific target proteins, usually in response to phosphorylation of these targets.

E3 UBIQUITIN LIGASE
The final enzyme complex in the ubiquitin-conjugation pathway. E3 enzymes transfer ubiquitin from previous components of the pathway to the substrate protein to form a covalently linked ubiquitin–substrate conjugate.

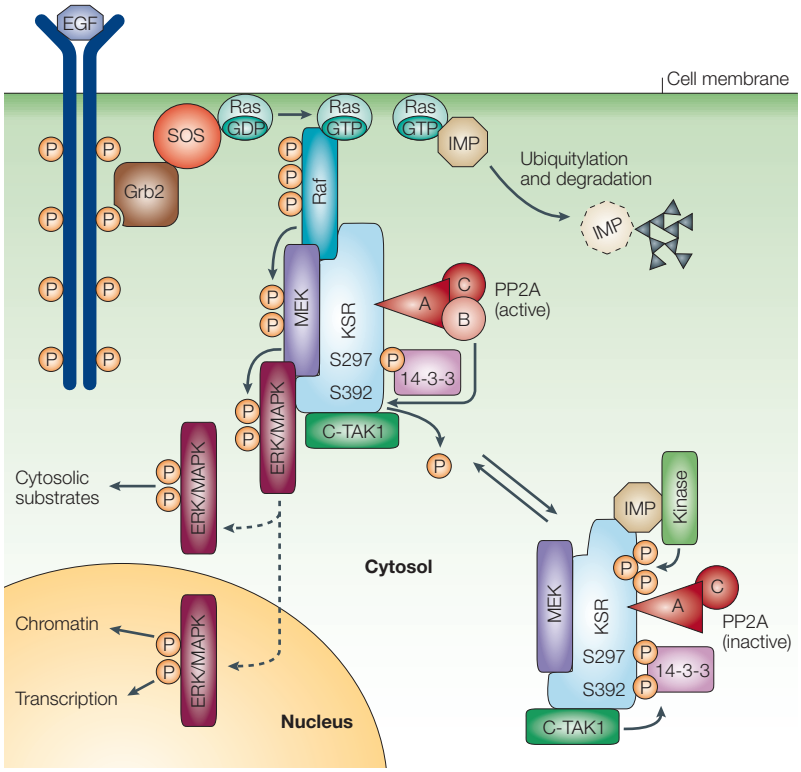


Figure 2 | **The KSR regulation cycle.** Inactive kinase suppressor of Ras (KSR) is sequestered in the cytosol and in a Triton-insoluble compartment by binding to 14-3-3 and by the protein 'impedes mitogenic signal propagation' (IMP)-induced phosphorylation. As the exact differences between KSR in these populations are unknown, KSR is depicted as one entity in the figure. On stimulation by mitogens such as epidermal growth factor (EGF), activated receptors recruit Ras guanine nucleotide-exchange factors, such as son of sevenless (SOS) through the adaptor protein growth-factor-receptor-bound-2 (Grb2), which generates Ras•GTP. Ras•GTP induces KSR dephosphorylation of S392 by stimulating the binding of the protein phosphatase-2A (PP2A) B subunit to its A and C subunits, which are constitutively associated with KSR. This results in the release of 14-3-3 from this KSR binding site and translocation of KSR to the cell membrane. The binding of active Ras to IMP triggers IMP autoubiquitylation and its subsequent degradation. KSR facilitates the phosphorylation of MAPK and ERK kinase (MEK) by Raf, and enhances the generation of activated extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), which can phosphorylate both nuclear and non-nuclear substrates. The inactivation of KSR involves Ras-induced phosphorylation (not shown), re-phosphorylation of the 14-3-3-binding site S392 by C-TAK1 or Nm23, and presumably the reassociation of KSR with IMP and phosphorylation by an unknown kinase that is recruited by IMP.

which sequesters KSR1 into a Triton-insoluble compartment[25]. Neither the phosphorylation site(s) nor the responsible kinase(s) are known. Whereas EGF induces the dephosphorylation of S392 (REF. 24), transfection of activated Ras induces KSR1 phosphorylation on T260, T274 and S443 (REF. 27). Mutation of S392 and T274 stabilizes KSR1, which implies that phosphorylation of these residues normally exerts negative feedback on protein stability[21]. So Ras coordinates several aspects of KSR1 function. Whereas activated Ras neutralizes the effect of the functional KSR1 inhibitors 14-3-3 and IMP, it also induces the phosphorylation of T274, a site that primes KSR1 for degradation. The twist is that phosphorylated T274 only promotes KSR1 degradation in conjunction with phosphorylated S392, but S392 is dephosphorylated in response to Ras activation. Re-phosphorylation of S392 enables 14-3-3 to bind again, thereby returning KSR1 to the cytosol. The concomitant presence of phosphorylated T274 would mark these KSR1 molecules for degradation, permitting the selective removal of KSR1 molecules that have participated in the mitogen-induced scaffolding and enhancement of the ERK/MAPK pathway. Mutating T274 and S392 to residues that cannot be phosphorylated extends ERK/MAPK activation and greatly enhances the mitogenic abilities of EGF[21]. The interplay and, in particular, the timing between these processes generates a combinatorial controller for modulating both the amplitude and duration of ERK/MAPK activity. This example demonstrates that even a simple system can generate complex behaviour when timing and spatial effects are taken into account.

Connector enhancer of KSR

Connector enhancer of KSR (CNK) was identified in a genetic screen in *D. melanogaster* as an enhancer of the DOMINANT NEGATIVE phenotype caused by the isolated KSR kinase domain[28]. CNK has no catalytic motifs, but it does have several protein interaction domains, including — starting from the N terminus — a STERILE α-MOTIF (SAM); a conserved region in CNK (CRIC); a PSD-95, Dlg and ZO1 (PDZ) DOMAIN; and, nearer the C-terminal, proline-rich Src-homology-3 (SH3)-binding sites and PLECKSTRIN HOMOLOGY (PH) DOMAINS (FIG. 3). This indicates a potential function as a multivalent adaptor protein.

CNK and ERK/MAPK signalling. *D. melanogaster* genetic analyses placed CNK function downstream of receptor tyrosine kinases (RTKs) and Ras, but upstream or parallel to Raf[28]. In *D. melanogaster*, CNK regulates Raf by a complex mechanism. In genetic EPISTASIS ANALYSES, the CNK C-terminal Raf-interaction domain, later narrowed down to the Raf inhibitory region, RIR (present only in *D. melanogaster* CNK (FIG. 3a))[29], prevented the disturbances in eye development that are caused by activated Ras and Raf. By contrast, the CNK N-terminal domain cooperated with activated Ras, but not with activated Raf, which implies that CNK exerts complex effects on Ras signalling that include Raf activation, and could also affect other Ras effector pathways[30]. Subsequent biochemical analysis in *D. melanogaster* S2 cells showed that *D. melanogaster* CNK mediates Raf activation by Ras through its SAM and CRIC domains in the N terminus, but inhibits Raf through the RIR in the C terminus[29]. This counterintuitive combination of a Raf-activating and Raf-inhibitory motif in the same scaffold presumably functions to filter signal from noise by suppressing background phosphorylation of MEK by Raf[29].

Activation of RTKs or Ras results in the release of RIR-mediated repression of Raf, which permits efficient Ras-mediated activation of Raf and MEK phosphorylation to proceed. The mechanism has been traced to the RTK-induced binding of SRC42 (a *D. melanogaster* homologue of mammalian Src) to a region of CNK that is just C-terminal to the RIR. Curiously, this derepression does not require SRC42 catalytic activity, but depends

DOMINANT NEGATIVE
A defective protein that retains interaction capabilities and so competes with normal proteins, thereby impairing protein function.

STERILE α-MOTIF
(SAM). Domain of ~70 amino acids roughly conserved in many proteins and thought to participate in protein–protein interactions.

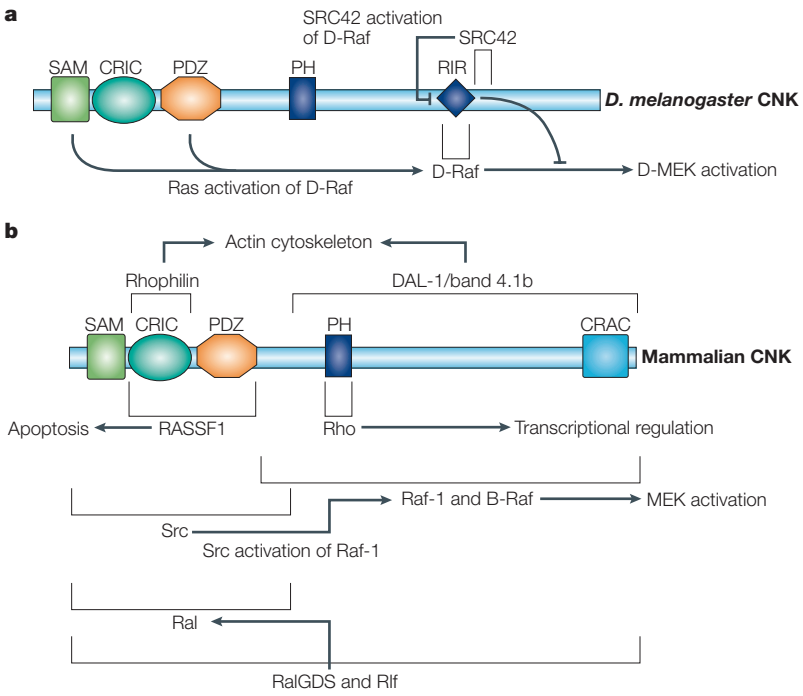


Figure 3 | **CNK interaction partners and signalling targets.** The domain structures of *Drosophila melanogaster* (**a**) and mammalian connector enhancer of KSR (CNK) (**b**) proteins are represented: sterile α-motif (SAM); conserved region in CNK (CRIC); PSD95, Dlg and ZO1 (PDZ) domain; pleckstrin homology (PH) domain; conserved region among chordate (CRAC), a domain of unknown function present only in vertebrate CNKs; Raf inhibitory region (RIR), which only exists in *D. melanogaster* CNK. CNK interaction partners, binding sites, activation, inhibition and affected signalling functions are indicated. In *D. melanogaster*, SAM and CRIC mediate Ras activation of D-Raf. Additionally, SRC42 — independently of its kinase activity — relieves D-Raf from repression by RIR enhancing D-Raf activity. In mammals, the CNK N terminus recruits Src to phosphorylate Raf-1, which associates with the CNK C terminus. CNK also binds to B-Raf, although the functional consequences of this interaction are unknown. In addition, mammalian CNK coordinates other signalling pathways by interacting with many proteins that include: Ral (Ras-like protein) and its exchange factors, Ral guanine nucleotide dissociation stimulator (Ral-GDS) and RalGDS-like factor (Rlf); Ras-association domain family-1 (RASSF1), which regulates apoptosis; Rhophilin and differentially expressed in adenocarcinoma of the lung-1 (DAL-1/band 4.1b), which regulate the actin cytoskeleton; and Rho, through which CNK1 selectively enhances Rho stimulated transcription.

on binding of the Src-homology-2 (SH2) and SH3 DOMAINS of SRC42 to CNK. The exact molecular mechanism is unknown, but does not involve the simple displacement of Raf from CNK[31] (FIG. 3a). In the absence of stimulation, Src is kept inactive by an intramolecular interaction — its SH2 and SH3 domains interact with its kinase domain. The engagement of SH2 and SH3 domains by other ligands can therefore lead to SRC42 activation[32]. So, CNK could coordinate Raf and Src activation. The RIR motif is not conserved in mammalian CNK, which seems to use a different mechanism to promote Raf activation by Src[33]. Mammalian CNK1 can bind Raf-1 and Src simultaneously, enhancing Raf-1 kinase activity and phosphorylation on Y341, a site that is implicated in Raf-1 activation. In contrast to SRC42, mammalian Src associates with the N-terminus of CNK, and efficient formation of the ternary complex requires activation of Raf-1 by Ras[33] (FIG. 3b).

PDZ DOMAIN
(Postsynaptic-density protein of 95 kDa, Discs large, Zona occludens-1). A protein-interaction domain that often occurs in scaffolding proteins and is named after the founding members of this protein family.

PLECKSTRIN-HOMOLOGY (PH) DOMAIN
A protein module of ~100 amino acids that is present in a range of proteins. Different PH domains interact with various phospholipids and are involved in the targeting of the proteins.

Mammalian CNK and its many binding partners. In mammals, three CNK isoforms are known[34]. CNK3 is more divergent and has not yet been functionally characterized. CNK1 is widely expressed and binds Raf-1, whereas CNK2 (also known as MAGUIN1, for membrane-associated guanylate kinase-interacting protein-1) is found mainly in neurons. CNK2 can bind to both Raf-1 and B-Raf, but fails to co-precipitate Ras, MEK, ERK/MAPK or KSR1 (REF. 35), which implies that, in mammalian cells, CNK2 might scaffold Raf signalling complexes that do not interact with components of the ERK/MAPK pathway.

However, the situation seems more complicated. In PC12 cells, CNK2 was required for ERK/MAPK activation by nerve growth factor (NGF), but not by EGF[36]. In addition, CNK2 made an ERK/MAPK-independent contribution to NGF-mediated neuronal differentiation by affecting actin remodelling through binding to 'differentially expressed in adenocarcinoma of the lung-1' (DAL-1)/band 4.1B, a protein that is involved in the regulation of the actin cytoskeleton and that has previously been identified as a tumour suppressor[37]. Intriguingly, CNK2 scaffolds a complex between densin-180 and PSD95, two proteins that are involved in the organization of cell polarity and nerve cell synapses[38]. This view of CNK2 as a multiadaptor protein was further substantiated by showing that CNK2 also interacted with the RAS-like protein (Ral) GTPase and RalGDS-like factor (RLF), which is a member of the Ral guanine nucleotide dissociation stimulator (RalGDS) family of exchange factors that activate Ral[36]. However, as overexpression of CNK2 did not activate Ral, the significance of these interactions remains to be elucidated. CNK1 also binds RalGDS, but it is unclear whether CNK1 affects any of the processes in which Ral is involved, such as vesicle trafficking, FILOPODIA formation, transcriptional regulation and cell transformation[39]. Interestingly, CNK1 has also been identified as an effector of the GTPase Rho[39,40]. In these studies, CNK1, binding activated Rho through its PH domain, mediated the transcriptional effects of Rho rather than its effects on the cytoskeleton.

The most recent addition to this kaleidoscope of CNK-binding partners is RASSF1A (Ras-association domain family-1)[34]. RASSF1A is the product of a prominent tumour suppressor gene, the pleiotropic activities of which are just beginning to be unravelled[41]. CNK1 augments the ability of RASSF1A to promote apoptosis through interaction with MST1/2 kinases[34]. Mammalian CNK therefore regulates at least four pathways (FIG. 3b): the ERK/MAPK pathway by enhancing Ras and Src-mediated activation of Raf-1; a cytoskeletal pathway by organizing cell polarity proteins; the transcriptional arm of the Rho pathway; and apoptosis through RASSF1A. The challenge is to find out whether these functions are independent or whether they intersect in a combinatorial fashion.

Compartmentalization of ERK/MAPK signalling

The staggering number of ERK/MAPK substrates and diverse biological processes that they regulate beg the question of how ERK/MAPK substrate specificity is

determined. An attractive possibility is compartmentalization and the role of the spatial arrangement of signalling pathways in the dynamic regulation of their function is rapidly emerging. ERK/MAPK rapidly accumulates in the nucleus after mitogenic stimulation. Preventing nuclear translocation in fibroblasts interferes with ERK/MAPK-induced gene expression and the mitogenic response[7]. Not surprisingly, then, the nucleocytoplasmic distribution of ERK/MAPK is used for regulating ERK/MAPK signalling. The importance of localizing ERK/MAPK signalling is reflected in the richness of mechanisms that accomplish it.

Cytosol versus nucleus: PEA15. PEA15 (phosphoprotein enriched in astrocytes) is a widely expressed death-effector-domain-containing protein with pleiotropic functions and means of regulation[42]. In the cytoplasm, PEA15 binds to ERK/MAPK, but not to other components of the ERK/MAPK pathway, and prevents the translocation of ERK/MAPK into the nucleus[43,44]. The mechanism is unclear. Whitehurst *et al.*[43] reported that PEA15 inhibits ERK/MAPK nuclear uptake by interfering with its binding to NUCLEOPORINS, whereas Formstecher *et al.*[44] showed that the nuclear export sequence of PEA15 keeps ERK/MAPK out of the nucleus, redirecting it to the cytosol. As a result, PEA15 interferes with proliferation, and *Pea15*-knockout mice exhibit hyperproliferation of hepatic and lymphoid tissues[44]. In addition, PEA15 also counteracts the suppression of INTEGRINS by activated Ras[45], which indicates that the nuclear localization of ERK/MAPK is required for this process. Interestingly, ERK/MAPK suppresses β-integrin activation through induction of *Fra1* gene expression[46], an event that requires ERK/MAPK nuclear signalling.

Cytosol versus nucleus: β-arrestin-2. A similar regulatory mechanism is mediated by β-arrestin-2. Although best known for their role in desensitizing and internalizing G-PROTEIN-COUPLED RECEPTORS (GPCRs), β-arrestins-1 and -2 have turned out to be versatile adaptor proteins for ERK/MAPK and other signalling pathways[47]. The phosphorylation of stimulated GPCRs by GPCR-kinases (GRKs) generates docking sites for β-arrestins, which terminate GPCR signalling by dissociating the heterotrimeric G-protein from the receptor and targeting the receptor to CLATHRIN-COATED PITS[48]. At the same time, β-arrestin-2 mediates the activation of ERK/MAPK by assembling new signalling complexes comprising Raf-1, MEK and ERK/MAPK[49,50]. The β-arrestin-2-scaffolded ERK/MAPK pathway accompanies the GPCR to EARLY ENDOSOMES, and ERK/MAPK activation is more efficient when stable β-arrestin–receptor complexes are formed[51]. In this situation, ERK/MAPK signalling is mostly excluded from the nucleus, probably due to the nuclear export sequence of β-arrestin-2. GPCRs that only bind transiently to β-arrestin-2 generate less activated ERK/MAPK, but permit stronger nuclear signalling[51,52], presumably because less ERK/MAPK is excluded from the nucleus. β-Arrestin-2 can therefore dispatch ERK/MAPK signalling to different subcellular compartments.

This versatility is further increased as GPCRs can also activate ERK/MAPK through their conventional effectors, the HETEROTRIMERIC G-PROTEINS[53]. This results in ERK/MAPK nuclear signalling with a more rapid onset and transient kinetics[52,54]. The intricate interplay between GPCR- and β-arrestin-mediated ERK/MAPK activation modulates the strength, kinetics and localization of ERK/MAPK signalling. Even more complexity is added by negative-feedback phosphorylation of β-arrestin-1 (REF. 55) and GRK2 (REF. 56) by ERK/MAPK, which inhibits GPCR internalization and decreases β-arrestin-mediated ERK/MAPK activation.

Cytosol versus nucleus: Sef. A further variation on this theme is afforded by the protein Sef (similar expression to FGF (fibroblast growth factor) genes). Sef is a transmembrane protein that was identified as the product of an FGF-induced gene and feedback inhibitor of the ERK/MAPK pathway in zebrafish[57,58]. The exact mechanism of inhibition remains elusive, but seems to involve pleiotropic interference at the level of phosphorylation of FGF receptor substrates and at the level of MEK-mediated ERK/MAPK phosphorylation[59]. A recent study added an intriguing twist by showing that Sef captures activated MEK–ERK/MAPK complexes at the Golgi apparatus[60]. Sef selectively bound to activated MEK and permitted ERK/MAPK activation. However, Sef retained activated ERK/MAPK in the complex, preventing its nuclear translocation and restricting ERK/MAPK signalling to cytosolic substrates. Ras proteins are also found at the Golgi and are activated by a pathway that is different from the canonical one (through growth-factor-receptor-bound-2 (Grb2) and the GUANINE NUCLEOTIDE-EXCHANGE FACTOR (GEF) son-of-sevenless (SOS)) that is used by RTKs to activate Ras at the cell membrane[61]. Localization at the Golgi could therefore dedicate the ERK/MAPK module to selectively connecting a distinct set of inputs with epigenetic outputs.

Cytoskeleton versus nucleus. Although primarily regulated by changes in the post-translational activity of cytoskeletal proteins, cytoskeletal remodelling often goes hand in hand with transcriptional responses that adapt the cell to the new environment caused by the cytoskeletal changes. An example of how this coordination is accomplished is provided by IQGAP1, a protein that contains several protein interaction domains and that regulates the cytoskeleton[62]. IQGAP1 is a potential effector of Cdc42, which — although it contains a region that resembles GTPase activating proteins (GAPs) — increases the levels of active Cdc42 by inhibiting its intrinsic GTPase activity[63,64] and thereby affects actin polymerization and promotes cell migration[65]. IQGAP1 also binds ERK/MAPK, and either the reduction or overexpression of IQGAP1 levels impaired ERK/MAPK stimulation by EGF or insulin-like growth factor-1 (REF. 66), which indicates that optimal ERK/MAPK activation requires a balanced stoichiometry of an IQGAP1–ERK2/MAPK complex. In ovarian cancer

EPISTASIS ANALYSIS
Epistasis is the masking of a phenotype caused by a mutation in one gene by a mutation in another gene. Epistasis analysis can therefore be used to dissect the order in which genes in a genetic pathway function.

SH2 DOMAIN
(Src-homology-2 domain). A protein motif that recognizes and binds tyrosine-phosphorylated sequences, and has a key role in relaying cascades of signal transduction.

SH3 DOMAIN
(Src-homology-3). A protein sequence of 50 amino acids that recognizes and binds sequences that are rich in proline.

FILOPODIA
Thin, transient actin protrusions that extend out from the cell surface and are formed by the elongation of bundled actin filaments in its core.

NUCLEOPORINS
Protein subunits of the nuclear pore complex.

INTEGRINS
A large family of heterodimeric transmembrane proteins that function as receptors for cell-adhesion molecules.

G-PROTEIN-COUPLED RECEPTOR
(GPCR). A seven-helix transmembrane-spanning cell-surface receptor that signals through heterotrimeric GTP-binding and GTP-hydrolysing G-proteins to stimulate or inhibit the activity of a downstream enzyme.

CLATHRIN-COATED PIT
(CCP). The initial site of invagination of a clathrin-coated vesicle.

EARLY ENDOSOME
An intracellular vesicular structure that is a precursor of the mature endosome and that has an important role in endocytosis.

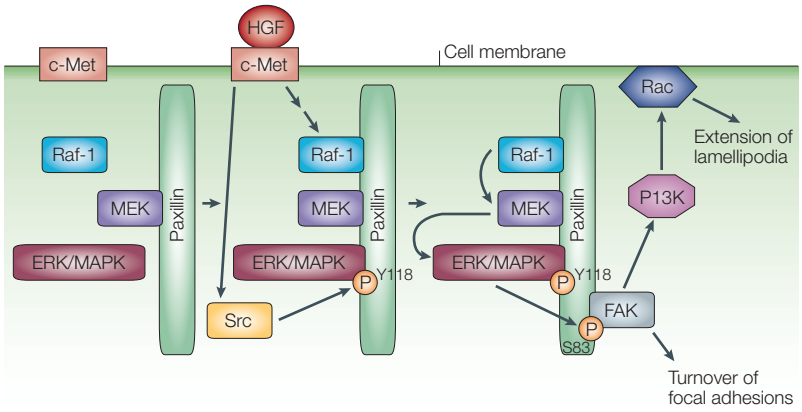


Figure 4 | **Paxillin connects the ERK/MAPK and FAK pathways.** Paxillin is an integral component of focal adhesions. Raf-1 and extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) are recruited to paxillin in response to the binding of hepatocyte growth factor (HGF) to its receptor c-Met, whereas MAPK and ERK kinase (MEK) is constitutively associated with paxillin. The binding of ERK/MAPK to paxillin requires Src-mediated phosphorylation of paxillin at Y118, which is induced by c-Met activation. Activated ERK/MAPK phosphorylates paxillin at S83, which induces the binding of focal adhesion kinase (FAK) to the scaffold and subsequent activation of phosphatidylinositol 3-kinase (PI3K) and Rac. This results in the extension of lamellipodia. FAK also causes the dissolution of focal adhesions, thereby coordinating detachment from the substrate with initiation of cell movement.

HETEROTRIMERIC G PROTEIN
A protein complex of three proteins (Gα, Gβ and Gγ). Gβ and Gγ form a tight complex, and Gα is part of this complex in its inactive, GDP-bound form but dissociates in its active, GTP-bound form. Both Gα and Gβγ can transmit downstream signals after activation.

GUANINE NUCLEOTIDE-EXCHANGE FACTOR
A protein that facilitates the exchange of GDP for GTP in the nucleotide-binding pocket of a GTP-binding protein.

FOCAL ADHESION
An integrin-mediated cell–substrate adhesion structure that anchors the ends of actin filaments (stress fibres) and mediates strong attachments to substrates. It also functions as an integrin signalling platform.

RHO-FAMILY GTPASE
A family of Ras-related GTPases that are involved in controlling the dynamics of the actin cytoskeleton.

LAMELLIPODIA
Flattened, sheet-like structures — which are composed of a crosslinked F-actin meshwork — that project from the surface of a cell. They are often associated with cell migration.

cells, the engagement of the hyaluronic acid receptor CD44 induced the formation of an IQGAP1 complex with ERK2/MAPK and Cdc42, which promoted cell migration through Cdc42-mediated actin binding, as well as a transcriptional response through ERK2/MAPK-mediated activation of the transcription factor Elk-1 and the α-oestrogen receptor[65]. The mechanism of ERK/MAPK activation by IQGAP remains to be elucidated, but because Cdc42 can also stimulate the activating phosphorylation event of Raf-1 on S338 (REF. 67), a role for IQGAP in the assembly of an activated ERK/MAPK module at the site of localized actin polymerization seems plausible.

In B cells, leukocyte-specific protein-1 (LSP1) targets a KSR–MEK–ERK/MAPK complex to the actin cytoskeleton[68]. This complex is required for the delayed phase of protein kinase CβI (PKCβI)-mediated ERK/MAPK activation in response to immunoglobulin M, a pro-apoptotic signal in these cells. ERK/MAPK is released after activation and might participate in cortical actin remodelling and apoptosis protection[68].

ERK/MAPK scaffolds at focal adhesions. The ERK/MAPK pathway can regulate cytoskeletal dynamics and cell migration through several routes[69]. The coordinated assembly and disassembly of protein complexes is at the heart of cytoskeletal remodelling, and mechanistic insights are emerging. Paxillin regulates cell spreading and migration through its central role as a multiadaptor protein in FOCAL ADHESION assembly[70]. Paxillin is constitutively associated with MEK and, in response to hepatocyte growth factor (HGF), also binds Raf-1 and ERK/MAPK[71]. Interestingly, HGF preferentially recruits activated Raf-1, but inactive ERK/MAPK, to paxillin, presumably to ensure that ERK/MAPK activation occurs specifically at newly forming focal adhesions. ERK/MAPK binding by paxillin requires phosphorylation of Y118 on paxillin by Src[71]. Once bound by paxillin and activated, ERK/MAPK phosphorylates paxillin on S83, which promotes the binding of focal adhesion kinase (FAK) to paxillin and, further downstream, the activation of the RHO FAMILY GTPASE Rac[72] (FIG. 4). FAK induces the local disassembly of focal adhesions, whereas Rac initiates migration through the extension of LAMELLIPODIA at the LEADING EDGE. So paxillin coordinates focal adhesion turnover and migration by functioning as a regulated scaffold for a localized connection between the ERK/MAPK and FAK signalling pathways.

Endosomal ERK/MAPK signalling. MEK partner-1 (MP1) is a widely expressed small scaffold protein that binds MEK1 to ERK/MAPK1, thereby enhancing ERK/MAPK1 activation[73]. Curiously, MP1 also promotes MEK activation by B-Raf *in vitro* although the mechanism has not been further investigated[73]. MP1 forms a tight heterodimer with p14 (REFS 74,75), a protein that targets MP1 and its binding partners to LATE ENDOSOMES. Downregulation of MP1 or p14 expression by SHORT INTERFERING RNA reduced ERK/MAPK activation, whereas overexpression increased ERK/MAPK activity in an additive manner. However, the enhancement of ERK/MAPK activation was strictly dependent on the endosomal localization of the MP1–MEK–ERK/MAPK complex, as its artificial mislocalization to the cell membrane abolished the positive effect of MP1. Notably, mislocalized MP1 dampened the duration of ERK/MAPK activation much more than the amplitude of the early activation peak, indicating that ERK/MAPK is initially activated at the cell membrane, but translocates to endosomes for sustained activation. In contrast to β-arrestin-1 and -2, which target ERK/MAPK signalling to early endosomes, MP1 enhanced ERK/MAPK nuclear signalling[73,75]. So, ERK/MAPK activation at endosomes could provide mechanisms for altering the quality, quantity or kinetics of ERK/MAPK signalling.

Interestingly, computational modelling of the EGF-activated ERK/MAPK pathway implies that the signalling from internalized — that is, endosomal — receptors is crucial for maintaining ERK/MAPK activity when the concentration of EGF is low[76]. MP1 constitutively binds MEK1, but releases ERK/MAPK after activation[77]. This turnover could provide an amplification mechanism that translates low MEK activity into sustained ERK/MAPK activation, in which MP1 continues to supply MEK1 with inactive ERK/MAPK for phosphorylation. The same study[77] also showed that MP1 is part of large signalling complexes, the components of which have begun to be unravelled. Intriguingly, MP1 recently was shown to coordinate the ERK/MAPK and Rho pathways by regulating p21-activated kinase-1 (PAK1)-mediated MEK1 phosphorylation at S298 and transiently suppressing Rho activation during cell spreading[78].

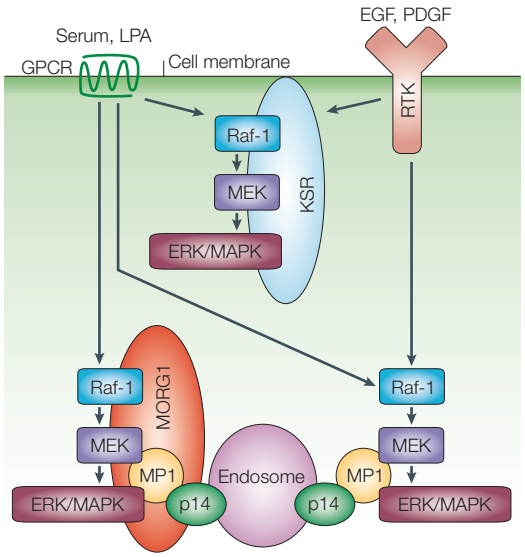


Figure 5 | **MORG1 and MP1.** MEK partner-1 (MP1) is a scaffold that coordinates the interaction between extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and MAPK and ERK kinase (MEK). The MP1–MEK–ERK/MAPK complex is targeted to late endosomes by p14. MP1 also binds to MAPK organizer-1 (MORG1). The exact composition and localization of MORG1 complexes are unknown. MORG1 also binds Raf-1 in addition to MP1, MEK and ERK/MAPK, and co-localizes with vesicles in cells. The MORG1-bound ERK/MAPK complex is selectively stimulated by serum and lysophosphatidic acid (LPA), which are agents that function mainly through G-protein-coupled receptors (GPCRs), but not through epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), which signal through receptor tyrosine kinases (RTKs). By contrast, ERK/MAPK complexes that are coordinated by the scaffold MP1–p14 on endosomes in the absence of MORG1 or by KSR1 at the cell membrane respond to both GPCR and RTK signals.

MAPK organizer-1 (MORG1), a recently isolated MP1-interaction partner that consists of WD40 DOMAINS, also associates with Raf-1, B-Raf, MEK and ERK/MAPK[79]. MEK binding to MORG1 is stabilized by Raf, ERK/MAPK and MP1, which implies that MP1 and MORG1 are modules of larger ERK/MAPK pathway complexes that are built from nested scaffolds[77]. MORG1 localizes to vesicles, although it is not known whether these are endosomes. MORG1 shows the typical properties of a scaffold, enhancing ERK/MAPK activation at low concentrations and inhibiting it at higher levels. Interestingly, MORG1 function seems to be stimulus-specific — it affects ERK/MAPK activation by serum, lysophosphatidic acid and PHORBOL ESTERS, but not by EGF or platelet-derived growth factor (PDGF)[79]. So, as CNK and KSR link the ERK/MAPK pathway to RTKs such as EGF and PDGF receptors, MORG1 could be an alternative scaffold that couples the ERK/MAPK pathway to other upstream activators (FIG. 5).

Endogenous inhibitors of ERK/MAPK signalling

The examples above illustrate the key role of protein interactions in the positive regulation of ERK/MAPK signalling. Equally important is inhibition and, instead of leaving this to chance, nature has developed elaborate mechanisms for its regulation. Although scaffolding proteins can function as inhibitors when overexpressed, dedicated inhibitors of protein interactions have been discovered.

RKIP. Raf kinase inhibitor protein (RKIP) inhibits MEK phosphorylation, and therefore activation, through Raf-1 (REF. 80) and B-Raf[81]. Rather than interfering with catalytic activity, RKIP binds to both Raf and MEK and prevents their physical interaction[80]. In addition, RKIP might also interfere with the PAK-mediated activating phosphorylation of Raf-1 on S338 by disrupting PAK binding to Raf-1 (REF. 82). RKIP dissociates from Raf during mitogen stimulation to allow MEK activation[80]. This dissociation is partly regulated by RKIP phosphorylation. PKC can phosphorylate RKIP on S153, which causes it to detach from Raf[83]. Interestingly, this phosphorylation enables RKIP to bind to GRK2 instead, which enhances GPCR signalling by inhibiting GPCR phosphorylation, desensitization and internalization[84]. This inhibition also seems to involve preventing the interaction of GRK2 with its receptor[84]. RKIP phosphorylation therefore enables cooperation between the GPCR- and RTK-activated ERK/MAPK pathways, by suppressing two brakes on ERK/MAPK activation (FIG. 6). RKIP is also involved in the regulation of other kinase-dependent signalling pathways such as nuclear factor-κB (NF-κB)[85], so it might function as a hub for coordinating different pathways.

The quest to unveil the intricacies of RKIP function is encouraged by recent discoveries that RKIP protein expression is downregulated in metastatic cancer cells[86–88]. Reconstitution with RKIP prevents metastasis in animal models[88] and sensitizes resistant cancer cells to chemotherapy[87]. Rituximab, an anti-CD20 antibody that is used in the treatment of non-Hodgkin's lymphoma, sensitizes cells to apoptosis by inducing RKIP expression, which leads to inhibition of the ERK/MAPK pathway and subsequent reduction of expression of the anti-apoptotic protein BCL-X[89]. It is unknown what causes the downregulation of RKIP expression in metastases, or how RKIP expression is normally regulated. Interestingly, RKIP protein levels increase during KERATINOCYTE differentiation[90]. In Down's syndrome model mice, RKIP expression seems to be repressed by a gene on human chromosome 21 (REF. 91) and, intriguingly, an extra copy of chromosome 21 (which causes Down's syndrome) increases childhood cancer risk[92]. However, it has not been investigated whether this is related to alterations of RKIP expression.

Sprouty and SPRED. Another intriguing class of inhibitor proteins contains Sprouty proteins and SPRED (Sprouty-related proteins with an EVH1 DOMAIN). These were recently reviewed[93], and are therefore only briefly discussed here. Identified as transcriptionally induced feedback inhibitors of FGF signalling, attempts to elucidate their mode of action revealed a multifaceted potential for interference at the level of the receptors Ras and Raf. A common theme is that they affect

LEADING EDGE
The thin margin of a lamellipodium that spans the area of the cell from the plasma membrane to a depth of about 1 μm into the lamellipodium.

LATE ENDOSOMES
These organelles mature from early endosomes and feature lower pH and different protein composition. They usually deliver cargo proteins to lysosomes for degradation.

SHORT INTERFERING RNA
A non-coding RNA (~21 nucleotides) that is processed from longer double stranded RNA during RNA interference. Such non-coding RNAs hybridize with mRNA targets, and confer target specificity to the silencing complexes that contain them.

WD40 DOMAIN
A poorly conserved repeat sequence of 40–60 amino acids, which usually ends with Trp-Asp (WD). Several consecutive repeats fold into a circular structure, a so-called β-propeller, in which each 'blade' is a four-stranded β-sheet. This domain is found in proteins that have different functions.

PHORBOL ESTER
A polycyclic ester that is isolated from croton oil. The most common are phorbol-12-myristate-13-acetate (PMA) and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). These are potent inflammatory agents and tumour promoters because they mimic diacylglycerol, and thereby cause sustained activation of protein kinase C.

KERATINOCYTE
An epithelial cell of the skin that has differentiated to produce keratin. Keratinocytes are the predominant cell type in the epidermis of the skin.

EVH1 DOMAIN
The Enabled/VASP homology domain-1 is a ~110 amino acid sequence that — similarly to SH3 domains — interacts with polyproline-rich regions. Structurally, EVH1 is similar to the PH domain despite low sequence homology and different binding partners.

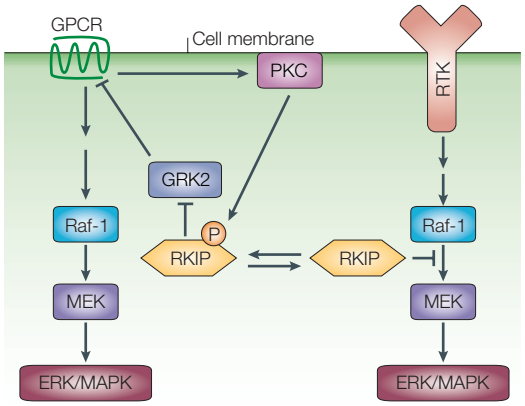


Figure 6 | **RKIP and ERK/MAPK activation.** In unstimulated cells, Raf kinase inhibitor protein (RKIP) is bound to Raf and prevents the phosphorylation of MEK (MAPK and ERK kinase) by Raf (MAPK is mitogen-activated protein kinase and ERK is extracellular signal-regulated kinase). G-protein-coupled receptors (GPCRs) are desensitized and internalized in response to phosphorylation by GPCR kinase-2 (GRK2). When they are active, however, GPCRs induce protein kinase C (PKC)-mediated phosphorylation of RKIP at S153. This phosphorylation inactivates RKIP as an inhibitor of Raf-1, and converts it to a GRK2 inhibitor. GPCR signalling through Ras and MEK to ERK/MAPK can therefore persist because a single phosphorylation event on RKIP blocks both GPCR inactivation by GRK2 and Raf inhibition. Non-phosphorylated RKIP would normally inhibit Raf and therefore block signalling from GPCRs (left) and from receptor tyrosine kinases (RTKs; right) to ERK/MAPK.

protein interactions. In response to RTK stimulation, Src phosphorylates a conserved tyrosine in the N-terminal region of Sprouty[94]. This phosphorylated tyrosine residue can bind and sequester Grb2, thereby impairing the recruitment of SOS to the RTK (and therefore impairing subsequent Ras activation)[95], or it can bind and sequester the ubiquitin ligase Cbl, thereby preventing EGF receptor ubiquitylation and degradation[96,97]. These opposite effects seem to be cell-type and stimulus specific. Sprouty4 can also directly bind to Raf-1 and B-Raf through a Raf-binding motif and inhibit ERK/MAPK stimulation by the PKC-dependent vascular endothelial growth factor (VEGF), but not by the Ras-dependent EGF RTK pathway[98]. Activation of PKC by phorbol esters dissociated the Raf–Sprouty complex in a dose-dependent manner, which was concomitant with ERK/MAPK activation, indicating that the inhibition might hinge on Raf sequestration. The Raf-binding motif is conserved in all four Sprouty and two SPRED isoforms[98], so Raf might be a common target for these proteins.

Computational analysis of scaffold function

Mathematical modelling has revealed that scaffolds have interesting contributions to the kinetic properties of signalling. The relative stoichiometric ratios of the scaffold and its targets determine whether the signal is enhanced or inhibited — this means that any scaffold has an optimal concentration for signalling. Cooperative binding of the targets shifts the optimum towards lower scaffold concentrations, but also broadens the optimal concentration range[99].

The sensitivity of pathway activity to scaffold concentration increases with the number of scaffold target components. For instance, in a three-component cascade — such as Raf–MEK–ERK/MAPK — a three-target scaffold yields faster and higher activation than a two-target scaffold. By decreasing the need for diffusion (and therefore eliminating diffusion rates), the scaffold accelerates the reaction rates between the kinases. So, scaffolding more reactions in a pathway results in faster and stronger responses. Conversely, an increased number of scaffold components increases the chance that incomplete complexes form when the scaffold exceeds its optimal concentration, which results in a higher sensitivity to inhibition by multi-component scaffolds[99]. This could be a reason that the same interactions — for example, between MEK and ERK/MAPK — are mediated by both a two-component (MP1) and a three-component (KSR) scaffold.

Scaffolds also can change the quality of the signal. Both MEK and ERK/MAPK require dual phosphorylation to become activated (BOX 1). Whereas MEK phosphorylation is processive — that is, Raf phosphorylates both activating sites on MEK during a single interaction[100] — ERK/MAPK phosphorylation is distributive[101] (MEK takes two interactions to phosphorylate the two activating sites in ERK/MAPK). When only a few MEK molecules are activated, this results in the accumulation of singly phosphorylated, but inactive, ERK/MAPK. If the concentration of singly phosphorylated ERK/MAPK is high, most of the new phosphorylation events are activating. This can give rise to switch-like activation kinetics of ERK/MAPK and bistability[102], which provide an ideal means to make biological decisions[103]. However, tethering to a scaffold should make all activation steps processive and eliminate this source of bistability in favour of a graded and fast dose-response curve. A three-chain kinase cascade is predicted to be optimal for fast and sharp signal output, because the distribution of signal amplification over a greater number of steps results in a faster gain of amplitude[104]. However, a fully scaffolded cascade will abrogate amplification, as the kinases are attached in 1:1 stoichiometry, but it will increase the speed of signalling by eliminating the limits that diffusion would usually impose. Some scaffolds, such as Sef, retain the activated kinases, whereas others, such as paxillin, release activated ERK/MAPK, ensuring turnover and thereby allowing amplification. So both non-amplifier and amplifier scaffolds can be distinguished.

Conclusion and outlook

The examples described here illustrate that the ERK/MAPK pathway is subject to many levels of regulation, and that scaffolds have an important function. By assorting individual components into distinct units, scaffolds provide a simple mechanism for the modular construction of signalling networks. The versatility is further increased by the dynamic regulation of scaffolds that affect assembly and turnover, as well as

super-scaffolds, such as MORG1, that connect different scaffolding complexes.

The advent of high-throughput genetics and proteomics screening methods has dramatically enhanced our ability to systematically map protein interactions and reconstruct the network topologies. Now the challenge is to integrate these findings into a higher-level framework of understanding of why and how the whole is more than the sum of its parts. This is emerging as a universal challenge in the signal transduction field as the initial perception of linear biochemical pathways gives way to the concept of intertwined communication networks.

We are struggling with the fact that a limited arsenal of signalling components that is capable of a comparatively small repertoire of biochemical reactions is used in a combinatorial fashion to specify different biological fates. Signalling resembles a game of chess where a few pieces that move by strictly defined rules can nevertheless generate an almost infinite variety of positions. Understanding the behaviour of these networks will require a systems-level approach that draws on the mathematical modelling of network function that is informed by both experimental measurements and theoretical elaboration of design principles.

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Competing interests statement

The author declares no competing financial interests.

Online links

DATABASES

The following terms in this article are linked online to:

Swiss-Prot: http://cn.expasy.org/sprot

A-Raf | B-Raf | β-arrestin-1 | β-arrestin-2 | CNK1 | CNK2 | EGF | FAK | Grb2 | GRK2 | HGF | IMP | KSR1 | MEK | MP1 | paxillin | PEA15 | Raf-1 | Ras | RASSF1 | RhoA | RhoB | RhoC | RKIP

FURTHER INFORMATION

Walter Kolch's laboratory: http://www.beatson.gla.ac.uk/research/index.html?topic_id=230

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Online Summary

- The Ras–Raf–MEK–ERK/MAPK (ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase) pathway mediates many different biological responses. It is still unclear how response fidelity and specificity are determined, although modulations of signal amplitude and duration, spatial constraints, as well as crosstalk with other pathways, have important roles.
- This pivotal regulation is mainly afforded through the manipulation of protein interactions by scaffolding, inhibitor and adaptor proteins that enhance, decrease or redirect the signal flux.
- Examples of signalling enhancers are scaffolding proteins such as kinase suppressor of Ras-1 (KSR1) and MEK partner-1 (MP1), which tether components together ensuring a fast and selective response.
- Inhibitors, such as Raf kinase inhibitor protein (RKIP), selectively disrupt the interaction between components of signalling pathways adding an additional dimension of regulation.
- Multidomain adaptor proteins, such as connector enhancer of KSR (CNK), integrate and distribute signals by engaging multiple signalling pathways.
- The combinatorial interplay between signalling proteins and pathways generates a rich diversity of regulation that can influence specific biochemical and biological responses. Unravelling these networks is an important challenge, which is facilitated by computational modelling.

Author Biography

Walter Kolch received an M.D. from the University of Vienna, Austria, but was led astray into basic research. Trying to understand Raf signalling as a postdoctoral fellow at the National Institutes of Health/National Cancer Institute, Bethesda, Maryland, USA, he still has not given up on it, now using wonderfully complicated technologies, such as proteomics, to dissect kinase signalling networks. He is a group leader at the Beatson Institute, Glasgow, UK, and Professor at the University of Glasgow.

Online Links

Swiss-Prot: http://cn.expasy.org/sprot

A-Raf
http://us.expasy.org/uniprot/P10398
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β-arrestin-1
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β-arrestin-2
http://us.expasy.org/uniprot/P32121
CNK1
http://us.expasy.org/uniprot/Q969H4
CNK2
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EGF:
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