# Organization and regulation of mitogen-activated protein kinase signaling pathways

Timothy P Garrington* and Gary L Johnson†

Mitogen-activated protein kinases (MAPKs) are components of a three kinase regulatory cascade. There are multiple members of each component family of kinases in the MAPK module. Specificity of regulation is achieved by organization of MAPK modules, in part, by use of scaffolding and anchoring proteins. Scaffold proteins bring together specific kinases for selective activation, sequestration and localization of signaling complexes. The recent elucidation of scaffolding mechanisms for MAPK pathways has begun to solve the puzzle of how specificity in signaling can be achieved for each MAPK pathway in different cell types and in response to different stimuli. As new MAPK members are defined, determining their organization in kinase modules will be critical in understanding their select role in cellular regulation.

**Addresses**
*†Program in Molecular Signal Transduction, Division of Basic Sciences, National Jewish Medical and Research Center, 1400 Jackson Street, Denver, CO 80206, USA
*Department of Pediatric Hematology/Oncology, The Children's Hospital, 1056 East 19th Avenue, Denver, CO 80218, USA
*e-mail: garringtont@njc.org
†e-mail: johnsong@njc.org

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**Abbreviations**

| | |
|---|---|
| **AKAP** | A-kinase anchoring protein |
| **CNK** | connector enhancer of Ksr |
| **ERK** | extracellular signal regulated kinase |
| **HPK-1** | hematopoietic progenitor kinase-1 |
| **JIP-1** | JNK interacting protein-1 |
| **JNK** | Jun amino-terminal kinase |
| **Ksr** | kinase suppressor of Ras |
| **MAPK** | mitogen-activated protein kinase |
| **MKK** | MAPK kinase |
| **MKKK** | MKK kinase |
| **MKKKK** | MKKK kinase |
| **MLK** | mixed lineage kinase |
| **MP1** | MEK partner 1 |
| **NIK** | Nck interacting kinase |
| **PH** | Pleckstrin homology |
| **RACK** | receptor for activated protein kinase C |
| **Ste** | sterile |
| **SUR-8** | suppressor of Ras |

## Introduction

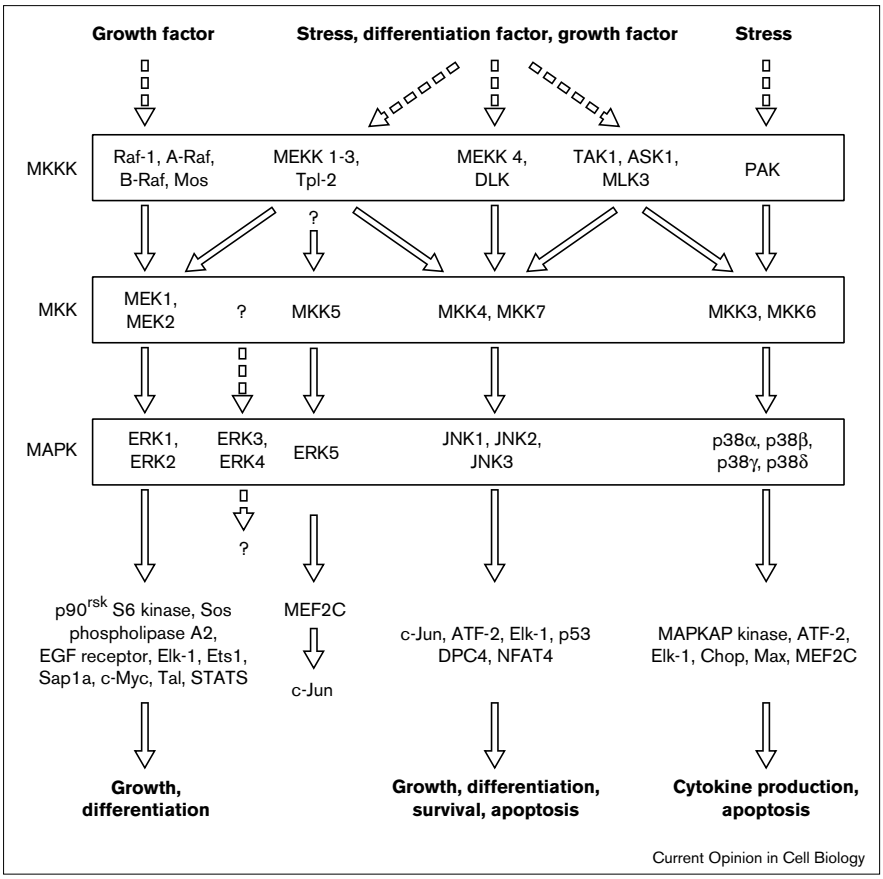
The core unit of mitogen-activated protein kinase (MAPK) pathways is a three-member protein kinase cascade. Within the three-kinase module, MAPKs are phosphorylated and activated by MAPK kinases (MKKs). MKKs are characteristically dual specificity kinases which catalyze the phosphorylation of MAPKs on both tyrosine and threonine residues. The MKKs are themselves phosphorylated and activated by serine/threonine kinases that function as MKK kinases (MKKKs). During evolution, many of the components of three kinase MAPK modules have been conserved in yeast and man. To date, 12 member proteins of the MAPK family have been identified in mammalian cells, and these can be grouped into five subfamilies, on the basis of sequence homology and function. Seven MKKs and fourteen MKKKs have been functionally identified in mammalian cells. Current knowledge suggests that low molecular weight GTP binding proteins (i.e., Ras, Rac, Cdc42) and specific kinases that could be considered to be MAPK kinase kinase kinases (MKKKKs) regulate the activity of MKKKs, thus controlling the activation of specific three kinase MAPK modules [1••,*2*–4].

Specificity of MAPK responses is achieved by activation of different MKKK–MKK–MAPK modules in response to different stimuli. MAPK modules are differentially activated by growth factors, hormones and cytokines. In addition, MAPK modules may be activated by cellular stresses including irradiation, heat shock, osmotic imbalance, DNA damage, and bacterial products such as lipopolysaccharide. Activation of MAPKs in response to these stimuli controls gene expression, metabolism, cytoskeletal functions and other cellular regulatory events. MAPKs contribute to complex regulatory events including mitogenesis, differentiation, survival and migration (Figure 1).

The combination of twelve MAPKs, seven MKKs and fourteen MKKKs, some with apparently redundant functions, seems extraordinarily complex. But, on closer inspection, certain themes start to emerge. First, the MKKs represent the fewest number of members in the MAPK module. MKKs also have high specificity for their MAPK substrates, allowing minimal variation of the MKK–MAPK part of the MAPK module. In contrast, the fourteen defined MKKKs are more diverse in structure. The MKKKs have different defined regulatory motifs that are not found in MKKs or MAPKs. These motifs include Pleckstrin homology (PH) domains, proline-rich sequences for binding SH (Src homology) 3 domains, binding sites for GTP-binding proteins, leucine-zipper dimerization sequences, and phosphorylation sites for tyrosine and serine/threonine kinases. Thus, MKKKs can be differentially regulated by a variety of upstream inputs for their selective regulation of MKKs.

The diversity of regulatory domains in different MKKKs gives the family of MAPK modules the flexibility to respond to a wide range of cellular stimuli. But what gives an MKKK the ability to act selectively in MAPK pathways, particularly MKKKs that can phosphorylate and activate multiple MKKs? Emerging evidence indicates that specificity is achieved, in part, by the use of scaffolding or anchoring proteins to coordinate MKKK binding to specific proteins for

**Figure 1**




Mitogen-activated protein kinase modules. The MAPK module consists of an MKKK, an MKK, and a MAPK. MKKKs respond to a variety of extracellular signals, including growth factors differentiation factors and stress. The activated MKKKs can then activate one or several MKKs. In contrast, the MKKs are relatively specific for their target MAPKs. Once activated, MAPKs can then phosphorylate transcription factors (for example ATF-2, Chop, c-Jun, c-Myc, DPC4, Elk-1, Ets1, Max, MEF2C, NFAT4, Sap1a, STATs, Tal, p53), other kinases (MAPKAP kinase, p90$^{rsk}$ S6 kinase), upstream regulators (EGF receptor, son of sevenless [Sos] Ras exchange factor), and other regulatory enzymes such as phospholipase A2. These downstream targets then control cellular responses including growth, differentiation, and apoptosis [1••,2,3].

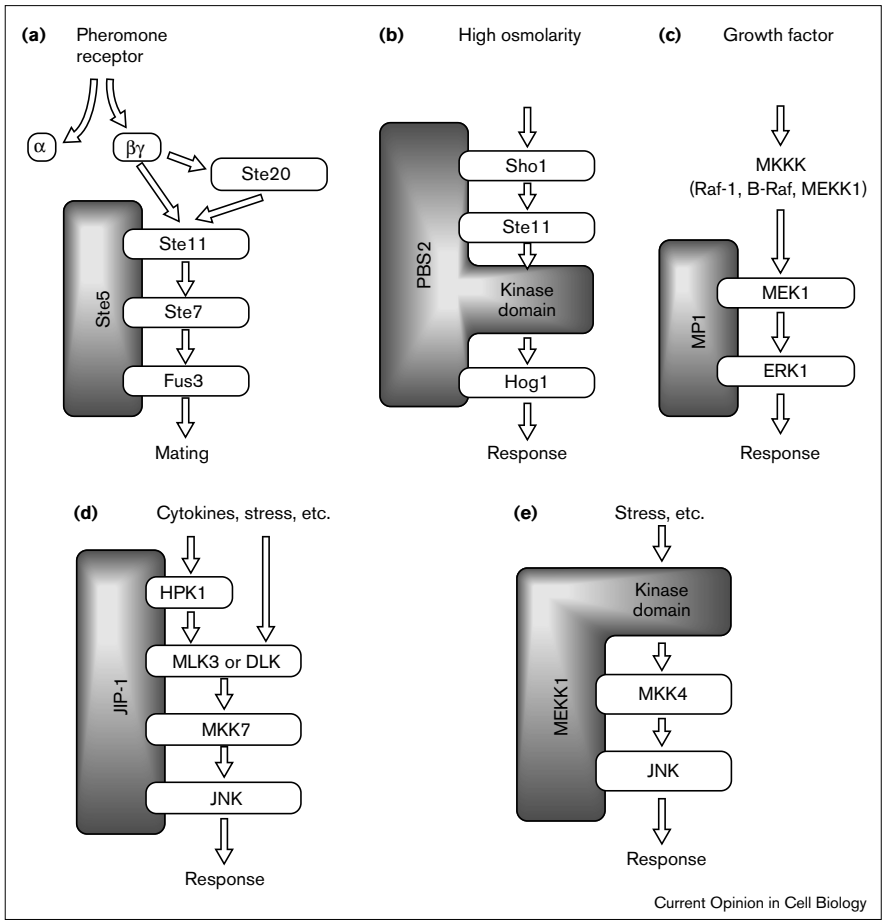
upstream inputs as well as specific downstream MKK–MAPK complexes. Scaffolding of multicomponent regulatory systems is now recognized as a major mechanism for controlling signal transduction pathways [5–8,9•]. The orchestration of MAPK modules by scaffolding or anchoring proteins can function by both positive and negative regulatory mechanisms. Scaffolding can bring together proteins for their interaction and regulation or conversely may sequester proteins so they do not interact with other proteins. Scaffolding can also control subcellular location, which can, in turn, be regulated by differential expression of specific scaffolding proteins. These protein interactions may also be post-translationally controlled by phosphorylation or proteolysis of scaffolding proteins or their binding partners. In this review we focus on the evidence for protein scaffolding in controlling MAPK modules.

## Scaffolding of mitogen-activated protein kinase modules in yeast

The role of scaffolding proteins in bringing together the components of a specific MAPK module has been most clearly demonstrated in the yeast *Saccharomyces cerevisiae* [10–13]. In *S. cerevisiae*, a specific MAPK cascade involved in the mating response of haploid cells is activated in response to binding of a mating pheromone to its receptor (Figure 2a). Because deletion or inactivation of component proteins in this pathway leads to sterility, the genes encoding the mating pathway proteins are referred to as sterile (Ste) genes. Binding of pheromone to its G-protein-coupled seven transmembrane receptor results in dissociation of the Gβγ subunit complex (Ste4/Ste18) from the GTP-bound Gα subunit (Gpa1). The Gβγ subunit complex activates the MKKK Ste20 and interacts with the scaffolding protein Ste5, resulting in the activation of the MAPK module Ste11 (MKKK), Ste7 (MKK), Fus3 (MAPK) [14]. It is believed that Ste20 regulates the activity of Ste11 by phosphorylating the Ste11 protein [14,15]. It should be noted, however, that although Ste11 is phosphorylated by Ste20, it has not yet been shown that this activates Ste11.

Ste5 is a scaffold for the mating response MAPK module. Using two-hybrid analysis, different regions of Ste5 have been shown to bind Ste11, Ste7, Fus3 and Ste4 [10,12]. These findings predict that Ste5 couples Gβγ to regulation of the mating MAPK pathway. The amino-terminal region of Ste5 encodes a cysteine-rich region that is a RING-H2 motif (these bind zinc and can mediate the interaction of proteins including the dimerization of two polypeptides). When this region of Ste5 is mutated the resulting Ste5 mutant protein cannot rescue deletions of the wild-type Ste5 gene [16•,17]. The Ring-H2 mutants still bind Ste11, Ste7 and Fus3 but do not bind Ste4 (Gβ subunit). The mutant Ste5 is also unable

**Figure 2**




Protein scaffolds. The scaffolding protein for each example is shaded. **(a)** The yeast signal transduction pathway involved in the mating response uses Ste5 as a scaffolding protein to bind the members of the MAPK module, Ste11 (MKKK), Ste7 (MKK), and Fus3 (MAPK). Ste20 is an MKKKK in this pathway. Activation of Ste11 and Ste20 occurs with pheromone binding to the seven-transmembrane protein pheromone receptor, which then leads to dissociation of the Gα subunit from the Gβγ subunit. The Gβγ subunit then activates Ste11 and Ste20 [10–13]. **(b)** The high osmolarity response pathway. In this pathway, the same MKKK (Ste11) is used. PBS2 acts as both the MKK and the scaffold protein. Hog1 acts as the MAPK [18•]. **(c)** MP1 is a recently described scaffolding protein which binds to MEK1 (an MKK) and ERK1 (a MAPK), enhancing the efficiency of MAPK cascades involving these proteins [19••]. **(d)** JIP-1 binds HPK1 (an MKKKK), MLK3 or DLK (MKKKs), MKK7, and JNK (a MAPK), leading to enhanced JNK activation [22••]. **(e)** MEKK1, in addition to acting as a kinase for MKK4, is able to bind JNK, allowing it to act as a scaffold to bring together all three components of this MAPK module [26••].

to dimerize. On the basis of these studies, a hypothesis was proposed that Ste5 dimerization is important in activation of the mating MAPK pathway [16•,17]. Gβγ interaction with Ste5 was proposed to induce this dimerization, which is inhibited when the Ring-H2 domain is mutated [16•]. Dimerization of Ste5 may induce Ste11 phosphorylation, leading to its activation. What is clear, however, is that by binding the component kinases of the mating MAPK molecule, Ste5 functions to organize a multicomponent signaling complex allowing regulation of signaling by the Gβγ subunit complex and hence regulation of the mating response.

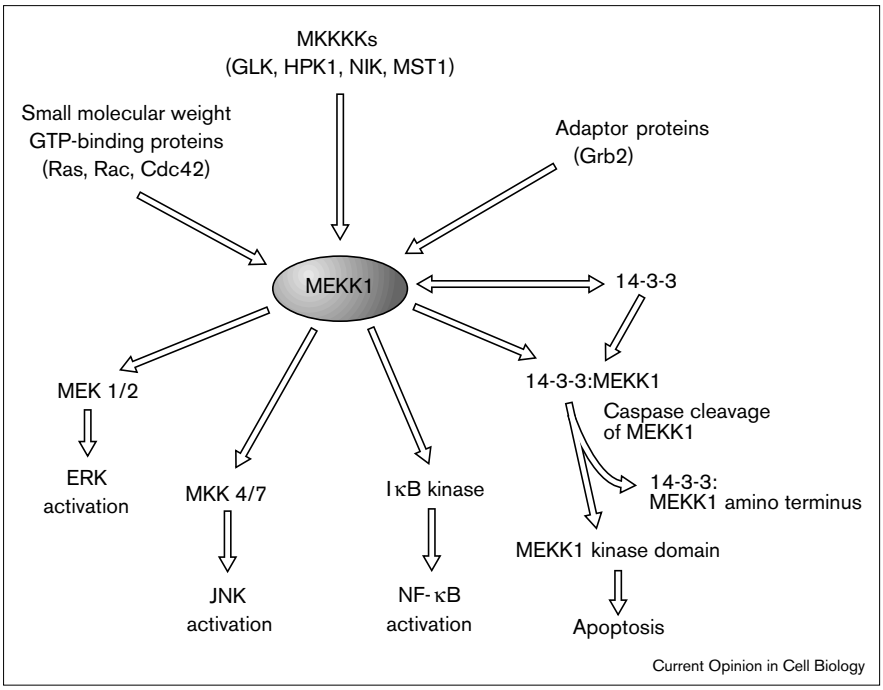
A second example of the organization of MAPK modules by scaffolding proteins is the high osmolarity response pathway in *S. cerevisiae* [18•]. This osmosensing response pathway includes Sho1 (an osmosensor), Ste11 (the same MKKK used in the mating pathway), Pbs2 (a MKK) and Hog1 (a MAPK). Pbs2 also acts as a scaffold, binding Sho1, Ste11 and Hog1 (Figure 2b). Despite the common use of Ste11 in the two MAPK modules, there is no crosstalk between the two pathways. The reason for this is that Pbs2 scaffolding of Ste11 selectively regulates its activation by Sho1 and Ste5 controls Ste11 activation by the Gβγ subunit complex. Thus, scaffolding proteins allow the use of a common MKKK for differential activation by upstream stimuli and regulation of different downstream MKK–MAPKs.

## Scaffolding of mitogen-activated protein kinase modules in mammalian cells

In 1998, a protein referred to as MP1 (MEK partner 1) was identified that appears to be a scaffold protein for the extracellular signal regulated kinase (ERK) pathway [19••,20]. MP1 was found to specifically bind MEK1 (a MKK) and ERK1 (a MAPK) (Figure 2c). When overexpressed in COS cells, MP1 enhances activation of ERK1. The prediction is that MP1 functions to increase the efficiency of the MKKK activation of the MEK1–ERK1 pathway. The predominant MKKK in this pathway is Raf-1 or B-Raf although MEKK1 also contributes to ERK activation.

A second mammalian cell MAPK scaffold protein is JIP-1 (Jun amino-terminal kinase [JNK] interacting protein-1) [19••,20,21,22•,23••] which is closely related to or encoded within the protein IB1. JIP-1 was initially described as a cytoplasmic protein which, when expressed by transfection, inhibited JNK activity; it was shown to bind JNK and

**Figure 3**




Multiple roles for MEKK1. MEKK1 is an MKKK that is able to interact with several upstream regulators, including small molecular weight GTP-binding proteins, MKKKKs, and adaptor proteins [27•,28,55–58]. In addition, MEKK1 is able to interact with 14-3-3 proteins, which may act as scaffolds or anchoring proteins to regulate the subcellular location and degree of activation of MEKK1 [44]. The 14-3-3 proteins bind to the amino-terminal regulatory region of MEKK1. With cleavage of MEKK1 by caspase 3, a 91 kDa carboxy-terminal fragment containing the kinase domain is released, leading to apoptosis [45,46]. 14-3-3 proteins have been postulated to play a role in sequestering the full-length MEKK1 protein to prevent its pro-apoptotic activity. Once activated, MEKK1 can activate at least three known downstream pathways [21]. It can phosphorylate the MKKs, MEK1 and MEK2, leading to ERK activation and affecting growth and differentiation. It can also phosphorylate MKK and possibly MKK7, leading to JNK activation, with downstream effects on growth, differentiation, survival and apoptosis. MEKK1 can also phosphorylate and activate IκB kinase. Prior to phosphorylation, IκB remains bound to the transcription factor NF-κB, acting as an inhibitor ofNF-κB by preventing its translocation from the cytoplasm to the nucleus. Upon phosphorylation by IκB kinase, IκB is proteolytically degraded, resulting in release of NF-κB, which is then able to translocate to the nucleus and regulate gene transcription. Thus, by phosphorylating and activating IκB kinase, MEKK1 activates NF-κB. Given the number of pathways MEKK1 can influence, some form of regulation is necessary to allow selectivity of response. Scaffolding proteins in this case may act to allow specificity of response for MEKK1 by controlling the upstream and downstream components with which it is able to interact in a given situation and cell type.

prevented its translocation to the nucleus when JIP-1 was overexpressed in COS cells [22•]. Subsequent studies suggested that JIP-1 probably functioned as a scaffolding protein for specific component kinases in the JNK pathway [23••]. JIP-1 was found to bind hematopoietic progenitor kinase-1 (HPK-1), a Ste20 homolog that functions as a MKKKK. JIP-1 also bound MLK3 and DLK, members of the mixed-lineage kinase (MLK) group of MKKKs, and MKK7, a known MKK in the JNK pathway. Coexpression of JIP-1 with these upstream component members of the JNK MAPK pathway enhanced JNK activation. Cumulatively, the results imply that JIP-1 is a scaffold that binds a MKKKK, MKKK and MKK for selective regulation of JNK activation. IB1, the rat homolog of JIP-1, was found to be a nuclear protein [21] suggesting that JIP-1/IB1 might localize this JNK regulatory module in the nucleus (Figure 2d).

A third potential MAPK scaffold in mammalian cells is a functional MKKK. MEKK1 is a large (196 kDa) protein that functions in the JNK and ERK pathways [24]. MEKK1 has been shown to interact with MKK4, its substrate in the JNK MAPK module [25]. MEKK1 also directly binds JNK, the MAPK downstream of MKK4 [26••]. Thus, MEKK1 could function as a MKKK that binds a specific MKK (MKK4) and MAPK (JNK) (Figure 2e). Two MKKKKs have been shown to phosphorylate MEKK1, HPK-1 and the Nck interacting kinase (NIK) [27•,28]. HPK-1 and NIK catalyzed phosphorylation of MEKK1 has not been shown to activate MEKK1, which is similar to the phosphorylation of Ste11 by Ste20 in the *S. cerevisiae* mating pathway. The findings are consistent with MEKK1 being a MKKK scaffold for regulation of the JNK pathway. Additional studies are required to quantify the binding of MKK4 and JNK to MEKK1 and to determine the affinities of these interactions.

## Other proteins that regulate mitogen-activated protein kinase signaling

Genetic screens in *Drosophila melanogaster* and *Caenorhabditis elegans* have identified several proteins that stimulate signaling of the Raf–MEK–ERK pathway. One of these proteins is the kinase suppressor of Ras (Ksr) [29–31]. In these two organisms, mutations in Ksr were found to attenuate Ras-mediated activation of the ERK pathway. Ksr is expressed in mammalian cells, and yeast two-hybrid analysis using mouse Ksr (mKsr-1) as bait showed interactions of mKsr-1 with both MEK1 and ERK [32•]. These interactions could also be shown in rat fibroblasts using transfection analysis. Activation of Ras has been shown to induce a translocation of mKsr-1 from the cytoplasm to the plasma membrane where it activates

**Table 1**

**MAPK pathway component proteins.**

MAPKs
- ERK – Extracellular-signal regulated kinase (ERK1 = p44MAPK, ERK2 = p42 MAPK).
- Fus3 – MAPK in *S. cerevisiae* mating pathway.
- Hog1 – High osmolarity glycerol response MAPK in *S. cerevisiae* osmosensing response pathway.
- JNK – c-Jun amino-terminal kinase.
- p38 – MAPK involved in stress response in higher eukaryotes (p38 MAPK, p38/HOG1).

MKKs
- MEK – MAPK/ERK kinase (MEK1 = MKK1, MEK2 = MKK2).
- PBS2 – MKK and scaffolding protein in *S. cerevisiae* osmosensing response pathway.
- SEK1 – SAPK/ERK kinase 1 (MKK4, JNKK).
- Ste7 – Sterile 7. MKK in *S. cerevisiae* mating pathway.

MKKKs
- ASK1 – Apoptosis signal-regulating kinase 1 (MKKK5).
- DLK – dual leucine-zipper bearing kinase (MUK).
- MEKK – MAPK/ERK kinase kinase (MEKK1-3, MEKK4 = MTK1).
- MLK3 – mixed-lineage kinase 3 (SPRK).
- PAK – p21-activated kinase.
- Ste11 – Sterile 11. MKKK in *S. cerevisiae* mating and osmosensor response pathways.
- TAK1 – TGFβ-activated protein kinase.
- Tpl2 – tumor progression locus 2 (also known as Cot).

MKKKKs
- GCK – germinal center kinase.
- GLK – GCK-like kinase.
- HPK1 – hematopoietic progenitor kinase 1.
- MST1 – mammalian Ste20-like protein kinase.
- NIK – Nck interacting kinase.
- Ste20 – Sterile 20. MKKKK in *S. cerevisiae* mating pathway.

Scaffolding/anchoring proteins
- AKAP – A-kinase anchoring protein.
- IB1 – Islet-Brain 1.
- JIP-1 – JNK interacting protein-1.
- MP1 – MEK partner 1.
- PBS2 – A scaffolding protein for the yeast osmosensing response pathway (see above).
- RACK – receptor for activated C-kinase.
- Ste5 – Sterile 5. Scaffolding protein for *S. cerevisiae* mating pathway.

Raf-1 activity [29•]. Interestingly, mKsr-1 enhancement of Raf-1 activity was independent of mKsr-1 kinase activity. The noncatalytic action of Ksr and its binding of MEK1 and ERK suggest that Ksr may have a scaffold function in regulating the Raf–MEK–ERK pathway. Ksr is evolutionarily conserved, suggesting it plays a critical role in controlling the activity of this MAPK module.

A second set of proteins that bind Raf-1 and are involved in regulating Raf-1 signaling are the 14-3-3 proteins [33–38,39•]. The 14-3-3 proteins are a family of conserved 30 kDa proteins encoded by eight different genes. 14-3-3 proteins recognize specific sequence motifs in proteins that sometimes involve phosphoserine and phosphothreonine residues. The dimeric nature of 14-3-3 proteins suggest that they bring together two proteins in a complex. Such complexes could function to regulate signaling cascades. 14-3-3 proteins bind Raf-1 in part via a sequence surrounding the phosphorylated Ser259 within the amino terminus of Raf-1 [40]. How 14-3-3 proteins regulate Raf-1 activity is poorly understood; there are differing reports that the 14-3-3 association with Raf-1 activates, inactivates or does not affect the kinase activity of Raf-1 [32•,33,34,41,42]. It has been proposed that 14-3-3 interaction with Raf-1 prevents Raf-1 dephosphorylation, thus prolonging its activation [37,43]. On the basis of the cumulative work of several laboratories, however, it is more likely that the 14-3-3 dimer is capable of binding a polypeptide that could organize signal complexes. How 14-3-3 proteins might do this is not understood. In particular, 14-3-3 proteins bind many different proteins, including many involved in signal transduction. A partial list of signaling proteins known to bind 14-3-3 proteins includes protein kinase C, Raf-1, Cbl, phosphatidylinositol 3-kinase, Bcr–Abl, polyoma middle tumor antigen, Ksr, Bad, MEKK1, MEKK2 and MEKK3. The binding of 14-3-3 protein to the amino terminus of MEKK1 has been proposed to control its location in the cell [44] (Figure 3). MEKK1 is a substrate for caspase 3 [45,46]. When MEKK1 is cleaved, the carboxy-terminal kinase domain is released from 14-3-3 tethering, changing its distribution in the cell. The free kinase domain is pro-apoptotic whereas the 14-3-3 tethered full length MEKK1 protein is not. This suggests

that 14-3-3 proteins function in part to tether or localize specific proteins in the cell and when this interaction is disrupted, as with caspase cleavage of MEKK1, redistribution can alter function.

The realization of the complexity of Ras-activated signaling pathways is focusing interest on the identification of additional modifiers of MAPK pathways, particularly the Ras–Raf–MEK–ERK pathway. Genetic screens in *D. melanogaster* have again identified potential candidates. New candidate scaffolding proteins in this pathway include SUR-8 (suppressor of Ras) and CNK (connector enhancer of Ksr) [47,48,49]. CNK interacts with Raf and SUR-8 interacts with Ras. SUR-8 and CNK both contain amino acid sequences consistent with protein interaction domains that could organize signaling complexes regulated by Ras. The biochemistry of these proteins is poorly understood. Undoubtedly, the characterization of these proteins will help us elucidate the role of scaffolding in the organization of macromolecular signaling complexes.

## Conclusions

The noncatalytic function of specific proteins to organize signaling complexes certainly extends beyond the MAPKs. In fact, cellomics — the definition of three dimensional organization of proteins in the cell — is one of the major problems to be solved in cell biology. One key to cellomics will be to identify protein scaffolds. Several examples of scaffolding proteins in signal transduction complexes other than MAPKs have been defined. Ina D is a protein in *D. melanogaster* photoreceptor neurons that has five PDZ (postsynaptic density protein, disc-large, zo-1) domains and functions as a scaffold for a phototransduction complex [50•]. Ina D associates, via its PDZ domains, with TRP (a calcium channel), phospholipase Cγ, eye protein kinase C, rhodopsin and calmodulin. Ina D organization of these proteins is involved in transmitting the response to photobleaching of rhodopsin. Other scaffolding proteins include the AKAPs (A-kinase anchoring proteins) that bind the regulatory subunit of cyclic AMP-dependent protein kinase and RACKs (receptors for activated protein kinase C) that bind protein kinase C [51,52].

A different sort of organizing protein is represented by caveolin, a membrane protein acting as major structural component of caveolae. Caveolae are islands in the plasma membrane that are believed to concentrate many signaling proteins, including the MAPKs ERK1 and 2. Transfection and overexpression of caveolin-1 inhibits signaling from epidermal growth factor (EGF) receptor [53]. Caveolin-1 overexpression also inhibits activation of the Raf-1–MEK1–ERK pathway [54•]. Thus, caveolin functions to form caveolae and to regulate the activity of specific signaling pathways.

The emerging vision of signal transduction is that cell pathways are regulated by their organization in macromolecular assemblies. This organization has long been appreciated in relation to subcellular organelles but it is now realized that macromolecular assembly is just as important for cytoplasmic proteins. The assembly of these signaling complexes involves proteins that have no apparent catalytic activity in addition to enzymes that are integral members of the pathway. Genetic screens in *D. melanogaster* and *C. elegans* are defining the component members and complexity of organization of MAPK pathways. Virtually every gene that is identified by mutagenesis screens in *D. melanogaster* or *C. elegans* has a mammalian counterpart that has been cloned. Because mammalian cells are available in large quantities relative to *D. melanogaster* and *C. elegans*, the biochemistry and cell biology of MAPK signaling complexes will be largely defined in mammalian cells. The role of so many interacting proteins in the Ras–Raf–MEK–ERK pathway will probably be mirrored in the JNK and p38 pathways. The interest in this subject will ensure rapid progress in defining the complex organization of MAPK modules in different cell types.

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