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EDITORIAL

Physiological roles of mitogen-activated-protein-kinase-activated p38-regulated/activated protein kinase

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Abstract

Mitogen-activated protein kinases (MAPKs) are a family of proteins that constitute signaling pathways involved in processes that control gene expression, cell division, cell survival, apoptosis, metabolism, differentiation and motility. The MAPK pathways can be divided into conventional and atypical MAPK pathways. The first group converts a signal into a cellular response through a relay of three consecutive phosphorylation events exerted by MAPK kinase kinases, MAPK kinase, and MAPK. Atypical MAPK pathways are not organized into this three-tiered cascade. MAPK that belongs to both conventional and atypical MAPK pathways can phosphorylate both non-protein kinase substrates and other protein kinases. The latter are referred to as MAPK-activated protein kinases. This review focuses on one such MAPK-activated protein kinase, MAPK-activated protein kinase 5 (MK5) or p38-regulated/activated protein kinase (PRAK). This protein is highly conserved throughout the animal kingdom and seems to be the target of both conventional and atypical MAPK pathways. Recent findings on the regulation of the activity and subcellular localization, *bona fide* interaction partners and physiological roles of MK5/PRAK are discussed.

Key words: Mitogen-activated protein kinase; p38-regulated/activated protein kinase; Extracellular signal-regulated kinase; Protein kinase A; Subcellular localization; Phosphorylation; Protein interaction

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INTRODUCTION

One of the major signaling pathways controlling a plethora of cellular processes is the mitogen-activated protein kinase (MAPK) pathway. The conventional mammalian MAPK pathway is composed of a cascade of three consecutive serine/threonine kinases referred to as MAPK kinase kinase, MAPK kinase and MAPK. Four conventional mammalian MAPK pathways exist: the extracellular signal-regulated kinases 1/2 (ERK1/2), the c-JUN N-terminal kinases 1-3 (JNK1-3) or stress-activated protein kinases (SAPKα, β and γ), the p38 MAPKs (p38α, β, γ and δ), and the big MAPK (BMK1/ERK5) modules.

The atypical MAPK pathways, which are not organized in the conventional tripartite module, include the ERK3/4, ERK7/8, and nemo-like kinase (NLK) pathways[1]. Both the conventional and the atypical MAPK pathways can phosphorylate non-protein kinase substrates or other protein kinases designated as MAPK-activated protein kinases (MAPKAPKs) (Figure 1). So far, 11 human MAPKAPKs have been identified. They are divided into four subfamilies and each subfamily contains several members. These MAPKAPK subfamilies comprises the ribosomal-S6-kinases (RSK1-4), the MAPK-interacting kinases (MNK1 and 2), the mitogen-and stress-activated kinases (MSK1 and 2), the MAPK-activated protein kinase (MK2/MK3) subgroup, and MAPK-activated protein kinase 5 (MK5)/p38-regulated/activated protein kinase (PRAK) (Figure 1). All MAPKAPKs can be activated by different conventional MAPK pathways, except MK5/PRAK which seems to be a unique substrate for the conventional $p38^{MAPK}$ and the atypical ERK3/4 pathways (Figure 1). No MAPKAPKs for ERK7/8 and NLK have been identified so far[1].

Although the amino acid sequence and the structural organization of MK5 show most similarity to MK2 and MK3 (approximately 33% homology[1,2]), several unique properties clearly distinguish MK5 from MK2/MK3. This review discusses recent insights into the pathways that convert to and regulate the subcellular distribution of MK5, and highlights novel biological functions of this protein kinase.

MK5 EXPRESSION AND ISOFORMS

In 1998, two groups reported the identification of a new member of the MAPKAPK subfamily[2,3]. Ni and colleagues used an expressed sequence tag clone of 414 bp with homology to MK2 to screen a murine spleen cDNA library to isolate the full-length cDNA sequence. Based on the amino acid homology with MK2, they named this novel protein MK5. Like MK2, MK5 could be phosphorylated and activated by $p38^{MAPK}$ and ERK2 *in vitro*, but not by SAPKβ/JNK3[3]. New and co-workers reasoned that novel homologs of MAPKAPKs could be found by screening a database using the peptide sequences LXTPCYTPYYVAP and LXTPCYTANFVAP containing the conserved phosphorylation motif LXTP. They isolated a new human protein that could be phosphorylated and activated by $p38^{MAPK}$, and consequently named it PRAK[2]. Northern blot analyses showed that the gene is ubiquitously transcribed, while the protein seems to be most abundant in brain and heart tissue[2-4]. The murine MK5 and its human homolog PRAK that were originally isolated in 1998, as well as the homologs found in other organisms are highly conserved (see next paragraph). In this review the name MK5 is used to refer to MK5 in general, while PRAK is exclusively used for studies that were specifically performed with the human MK5 protein.

The *mapkapk5* gene is conserved throughout the animal kingdom and has been identified in fish, amphibians, reptiles, birds and mammals (Table 1)[5,6]. Remarkably, the 14 exon structure of the gene has also been evolutionary conserved. Some species have a *mapkapk5* gene consisting of fewer exons, but this may be the result of incomplete identification of the genomic sequence. Table 1 provides an overview of the genomic organization and chromosomal localization of the *mapkapk5* gene and the number of amino acid residues in the encoded protein in all species in which the gene has been identified so far[5,6]. A homolog to the *mapkapk5* gene is absent from the *Caenorhabditis elegans* and the *Drosophila* genome, suggesting it is only present in vertebrates[7]. The human *mapkapk5* promoter lacks an obvious TATA box consensus sequence and is GC rich. Several putative binding sites for transcription factors are present, but *in vivo* binding to these sites remains to be confirmed[4]. The transcription of MK5 is responsive to increased cAMP levels, but no changes in protein levels were observed in cells exposed to different stress-inducing stimuli[4]. It was recently shown that c-MYC binds to the PRAK promoter *in vivo* and stimulates transcription[8].

Alignment of the primary structure of all reported MK5 sequences reveals that the protein is extremely well-conserved (Figure 2). Fish analogs of the human PRAK protein display 87%-98% amino acid identity, while the *Xenopus* variant 2 has 91% identity. The only known reptile MK5 protein (from the green anolis lizard *Aneolis carolinensi*) is 94% identical, while the known bird MK5 proteins possess 87%-95% amino acid identity with PRAK. Thus both ectothermic and endothermic animals express an MK5 protein with high sequence identity, suggesting that this protein possesses catalytic activity in a broad temperature spectrum. The functional motifs such as nuclear export signal (NES), nuclear localization signal (NLS), ERK3/4 binding motif, and the $p38^{MAPK}$ docking motif are extremely well conserved in all known MK5s. The threonine residue 182 in the activation loop, whose phosphorylation is regulated by $p38^{MAPK}$, ERK3 and ERK4, is also conserved. The protein kinase A (PKA) phosphoacceptor site Ser-115 is present in all MK5 sequences (Figure 2). Several species encode different isoforms. The human *mapkapk5* gene encodes two differently spliced transcript variants of 2060 and 2066 nucleotides, giving rise to a PRAK variant of 471 and a PRAK variant of 473 amino acids, respectively. The two isoforms differ in an additional two amino acids (G and K) in the C-terminal end of the larger variant, and these residues do not constitute part of any known functional domain (Figure 2). Whether these two isoforms exert different functions is not known, nor has it been examined whether they have different spatiotemporal or cell-specific expression patterns. Mouse (see below), rhesus macaque (*Macaca mulata*) and zebra finch (*Taeniopygia gutta*) also express isoform variants that vary by the deletion of the same

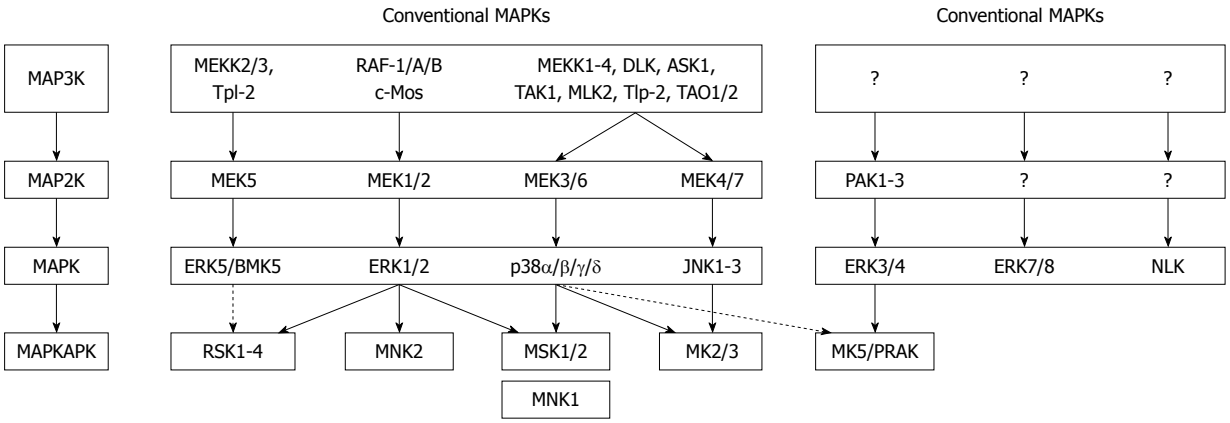


Figure 1 Mammalian mitogen-activated protein kinase pathways. The conventional mitogen-activated protein kinase (MAPK) pathway has a tripartite composition in which MAPK kinase kinase (MAP3K) phosphorylates and activates MAPK kinase (MAP2K), which in turn phosphorylates and activates MAPK. The atypical MAPK pathway lacks this typical three-tiered cascade. Both conventional and atypical MAPKs can phosphorylate different substrates, including other protein kinases referred to as MAPK-activated protein kinases (MAPKAPKs). The stippled lines indicate that a *bona fide in vivo* link between these MAPKs and their MAPKAPKs remains controversial. ERK: Extracellular signal-regulated kinase; RSK: Ribosomal-S6-kinase; MNK: MAPK-interacting kinase; JNK: c-JUN N-terminal kinase; PAK: p21-activated protein kinase; MK: MAPK-activated protein kinase; NLK: Nemo-like kinase; PRAK: p38-regulated/activated protein kinase.

residues (Figure 2). An additional PRAK isoforms of 467 amino acids, which lacks the N-terminal six amino acids, has also been described, but it is not know whether this isoform actually is expressed or if it represents a cloning artifact (Figure 2). Five MK5 mRNA splice variants (MK5.1-MK5.5) encoding proteins of 473, 471, 369, 324, and 322 amino acids, respectively, have been described in all mouse tissues examined[9]. MK5.1 was the most abundant transcript followed by MK5.2 in all tissues tested. Transcript variants 4 and 5 lack exons 2-6, while variant 3 skips exon 12. Exon 12 in transcript variant 2 and 5 has a six-base truncation at the 3' end. This removes the amino acids G and K. These amino acids correspond with those that are lacking in PRAK isoform 1. The isoforms 4 and 5 are truncated in their kinase domain, making them catalytically inactive, while MK5.3 lacks the C-terminal part of the protein including the ERK3/ERK4 binding motif. As a result, MK5.3 fails to bind ERK3. All isoforms have intact NLS and NES, and MK5.1, MK5.2 and MK5.3 were nuclear, but unexpectedly isoforms 4 and 5 were exclusively cytoplasmic when expressed in untreated HEK293 cells. While activation of the p38^{MAPK} pathway by anisomycin triggered nuclear export of MK5.1, MK5.2 and MK5.3, small amounts of MK5.4 and MK5.5 were transported into the nucleus after stimulation[9]. The mechanisms regulating MK5 mRNA splicing, subcellular localization of the protein and the functional role of these different isoforms are not known, but the relative abundance of the five mRNA variants was cell-specific and altered during postnatal cardiac development and under conditions of hypertrophy. These observations implicate different isoforms in distinct physiological processes. Furthermore, murine transcripts encoding two putative N-terminal truncated forms of 114 and 409 amino acid residues respectively have been described, but their existence has not been proven (Figure 2).

SUBCELLULAR LOCALIZATION OF MK5

Regulation of subcellular localization by p38^{MAPK}

Several studies have shown that endogenous as well as tagged MK5 resides predominantly in the nucleus of resting cells, but the protein seems to shuttle between the nucleus and the cytoplasm[10-20]. MK5 contains functional NLS and NES motifs. The latter partially overlaps with a p38^{MAPK} docking site (Figure 2). The accessibility of these opposing signals probably determines the location of the protein. The subcellular localization of MK5 is regulated by several proteins that interact with MK5 (Table 2). The first studies examined the effect of the p38^{MAPK} pathway on the subcellular distribution of MK5. Both our group and the group of Han have shown that activation of the p38 ^{MAPK} pathway causes nuclear exclusion of MK5[10,11]. This nucleocytoplasmic translocation was inhibited by leptomycin B, indicating a chromosome region maintenance 1-dependent mechanism[9-11]. We and others have shown that kinase dead MK5 mutants still translocate to the nucleus when p38^{MAPK} is overexpressed or when cells are exposed to arsenite[10,11,18]. Additional studies have pointed to a dual mechanism for p38 ^{MAPK}-mediated nuclear export of MK5: phosphorylation of Thr-182 by p38^{MAPK} exposes the NES probably because of conformational change of MK5, and binding of p38^{MAPK} to the p38^{MAPK} docking site masks the NLS[10,11]. Han's group has reported that different isoforms of p38^{MAPK} direct PRAK to different subcellular compartments. PRAK-p38α complexes are exclusively found in the nucleus, while PRAK-p38β complexes reside in the cytoplasm[21]. Mutation of the PRAK NES motif does not alter the subcellular localization of PRAK-p38β complexes, indicating that NES in PRAK is not required for cytoplasmic residence of this complex. Mutation of the NLS motif, however, redirects PRAK-p38α to the cytoplasm, emphasizing the importance of NLS in directing PRAK-

Table 1 Chromosomal localization and genetic organization of annotated and proven *mapkapk5* gene

Species	Class	Chromosome	Exons	Residues
Ailuropoda melanoleuca (giant panda)	Mammalians		14	471
Anolis carolinesis (green anole lizard)	Reptiles		14	473
Bos taurus (cow)	Mammalian	17	13	461
Callithrix jacchus (white-tufted-ear marmoset)	Mammalian	9	14	475
Canis familiaris (dog)	Mammalian	26	14	473
Cavia porcellus (guinea pig)	Mammalian		14	473
Choloepus hoffmanni (two-toed sloth)	Mammalian		9	311
Danio rerio (zebrafish)	Fish	5	14	471/475
Dasypus novemcinctus (nine-banded armadillo)	Mammalian		18	467
Dipodomys ordii (kangaroo rat)	Mammalian		15	467
Echinops telfairi (lesser hedgehog tenrec)	Mammalian		13	461
Equus caballus (horse)	Mammalian	8	15	467/473
Erinaceus europaeus (European hedgehog)	Mammalian		13	461
Felis catus (cat)	Mammalian		7	280
Gallus gallus (chicken)	Birds	15	14	474
Gasterosteus aculeatus (three-spined stickleback)	Fish		10	370
Gorilla gorilla (gorilla)	Mammalian	12	14	469
Homo sapiens (human)	Mammalian	12	14	471/473
Loxodonta africana (African bush elephant)	Mammalian		14	471
Macaca mulatta (rhesus macaque)	Mammalian	11	14	471
Macropus eugenii (Tammar wallaby)	Mammalian		13	461
Meleagris gallopavo (wild turkey)	Birds	17	14	476
Microcebus murinus (gray mouse lemur)	Mammalian		11	411
Monodelphis domestica (gray short-tailed opposum)	Mammalian	3	13	461
Mus musculus (house mouse)	Mammalian	5	14	322/324/369/471/473
Myotis lucifugus (little brown bat)	Mammalian		13	461
Ochotona princeps (American pika)	Mammalian		11	411
Oryctolagus cuniculus (rabbit)	Mammalian	21	14	473
Oryzias latipes (Japanese rice fish)	Fish	9	14	469
Otolemur garnetti (small-eared galago)	Mammalian		11	337
Pan troglodytes (chimpanzee)	Mammalian	12	14	467
Pongo pygmaeus (orang-utan)	Mammalian	12	14	467
Procavia capensis (rock hyrax)	Mammalian		15	448
Pteropus vampyrus (large flying dog)	Mammalian		12	467
Rattus norvegicus (rat)	Mammalian	12	9/11/13/14	324/369/409/473
Sorex araneus (shrew)	Mammalian		16	460
Spermophilus tridecemlineatus (thirteen-lined ground squirrel)	Mammalian		15	436
Sus scrofa (common wild pig)	Mammalian		14	473
Taeniopygia guttata (zebra finch)	Birds	15	13/14	486/473
Takifugu rubripes (Japanese pufferfish)	Fish	7	11/14	342/479
Tarsius syrichta (Philippine tarsier)	Mammalian		13	461
Tetraodon nigroviridis (spotted green pufferfish)	Fish	12	14	471
Tuparia belangeri (Northern treeshrew)	Mammalian		13	381
Tursiops truncatus (bottlenose dolphin)	Mammalian		15	467
Vicugna pacos (alpaca)	Mammalian		13	461
Xenopus tropicalis (clawed frog)	Amphibians		18	487

The number of residues in the mitogen-activated protein kinase-activated protein kinase 5 protein.

Table 2 Mechanisms of the subcellular distribution of mitogen-activated protein kinase-activated protein kinase 5 mediated by its interaction partners

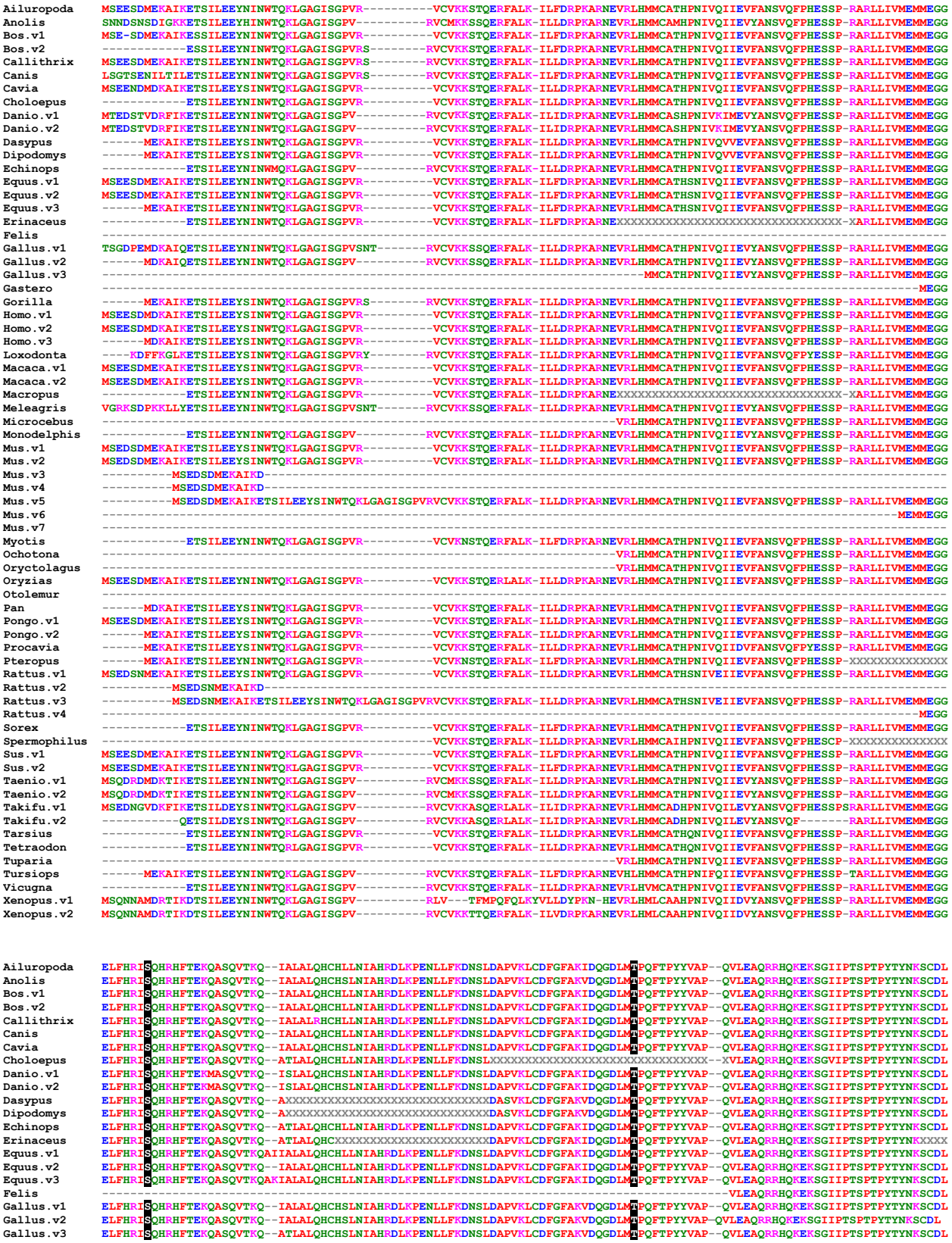
Nuclear export of MK5 mediated by	$p38^{MAPK}$	ERK3	ERK4	PKA	Cdc14A
Importin1/NES-dependent	Yes	Yes	No	Yes	Not tested
Kinase activity MK5	Not required	Not required	Not required	required	Not tested
Phosphorylation of MK5 at T182A	Not required	Not required	Not required	required	Not tested
Catalytic activity partner	Not required	Not required	Not required	required	Not required
Ser-115-dependent	No	No	No	Yes	Not tested

MK5: Mitogen-activated protein kinase-activated protein kinase 5; ERK: Extracellular signal-regulated kinase; PKA: Protein kinase A; NES: Nuclear export signal.

p38α complexes to the nucleus. Two residues (Asp-145 and Leu-156 in p38α, Gly-145 and Val-156 in p38β) are critical for regulating the localization of the p38-PRAK complex. A reciprocal swap of these residues switches


A
Ser-115
Thr-182
NES
NLS
1
Kinase domain
473
MK5
p38MAPK docking site
ERK3/4 docking site

B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Canis
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Echinops
Equus.v1
Equus.v2
Equus.v3
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Felis
Gallus.v1
Gallus.v2
Gallus.v3
Gastero
Gorilla
Homo.v1
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Homo.v3
Loxodonta
Macaca.v1
Macaca.v2
Macropus
Meleagris
Microcebus
Monodelphis
Mus.v1
Mus.v2
Mus.v3
Mus.v4
Mus.v5
Mus.v6
Mus.v7
Myotis
Ochotona
Oryctolagus
Oryzias
Otolemur
Pan
Pongo.v1
Pongo.v2
Procavia
Pteropus
Rattus.v1
Rattus.v2
Rattus.v3
Rattus.v4
Sorex
Spermophilus
Sus.v1
Sus.v2
Taenio.v1
Taenio.v2
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Tuparia
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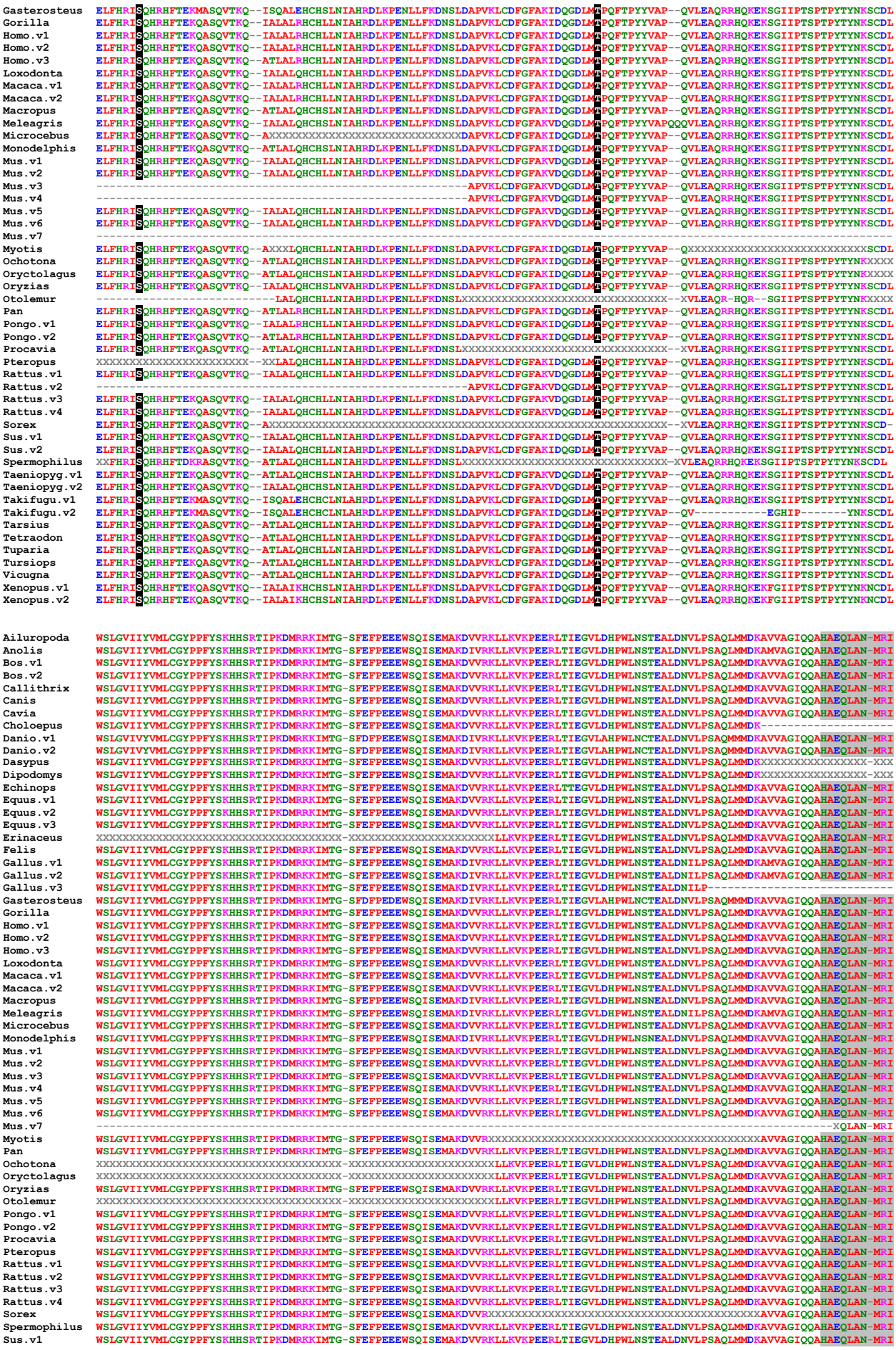
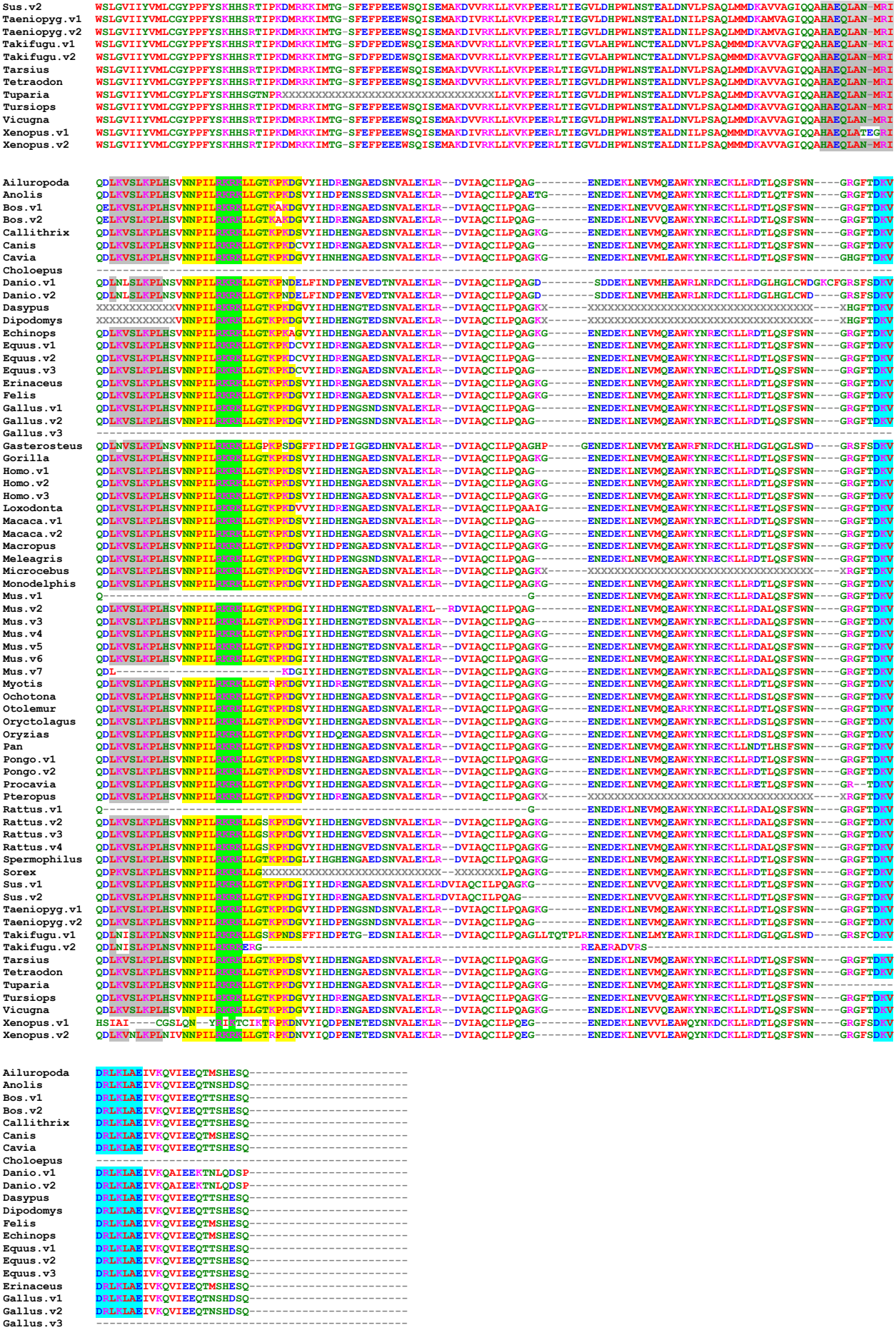
Xenopus.v2

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Taeniopyg.v1
Taeniopyg.v2
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Tetraodon
Tuparia
Tursiops
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Xenopus.v1
Xenopus.v2
Ailuropoda
Anolis
Bos.v1
Bos.v2
Callithrix
Canis
Cavia
Choloepus
Danio.v1
Danio.v2
Dasypus
Dipodomys
Echinops
Equus.v1
Equus.v2
Equus.v3
Erinaceus
Felis
Gallus.v1
Gallus.v2
Gallus.v3
Gasterosteus
Gorilla
Homo.v1
Homo.v2
Homo.v3
Loxodonta
Macaca.v1
Macaca.v2
Macropus
Meleagris
Microcebus
Monodelphis
Mus.v1
Mus.v2
Mus.v3
Mus.v4
Mus.v5
Mus.v6
Mus.v7
Myotis
Ochotona
Otolemur
Oryctolagus
Oryzias
Pan
Pongo.v1
Pongo.v2
Procavia
Pteropus
Rattus.v1
Rattus.v2
Rattus.v3
Rattus.v4
Spermophilus
Sorex
Sus.v1
Sus.v2
Taeniopyg.v1
Taeniopyg.v2
Takifugu.v1
Takifugu.v2
Tarsius
Tetraodon
Tuparia
Tursiops
Vicugna
Xenopus.v1
Xenopus.v2
Ailuropoda
Anolis
Bos.v1
Bos.v2
Callithrix
Canis
Cavia
Choloepus
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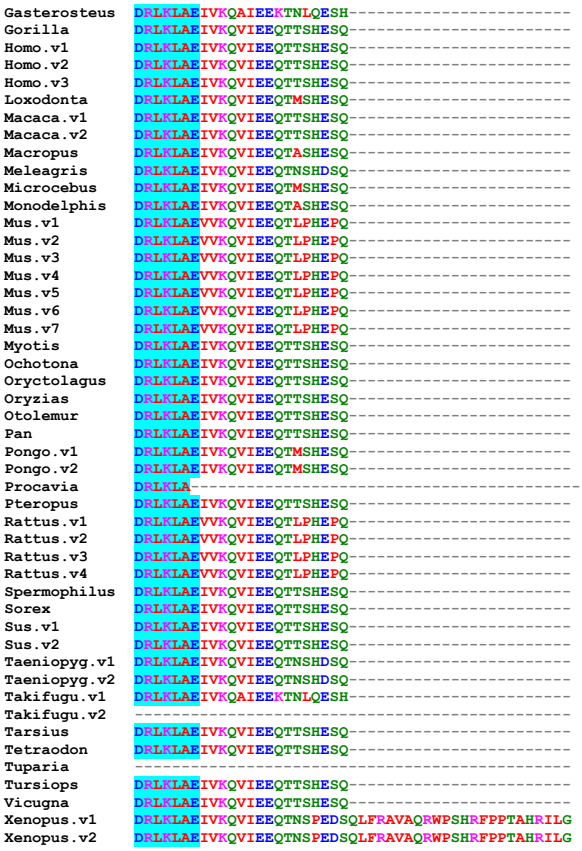

Figure 2 Structure of mitogen-activated protein kinase-activated protein kinase 5. A: Functional domains of mitogen-activated protein kinase-activated protein kinase 5 (MK5); B: Primary sequence of annotated and proven MK5. The $p38^{MAPK}$ docking motif is in yellow, the nuclear localization signal (NLS) is shaded in green, the protein kinase A inhibitor (PKI)-like and REV-like nuclear export signal (NES) motifs are depicted in green, and the extracellular signal-regulated kinases (ERK)3/4 interaction domain is shaded in light blue. Notice that the NLS and $p38^{MAPK}$ docking site are overlapping. Threonine residue 182 in the activation loop and the protein kinase A phosphoacceptor site Ser-115 are highlighted in black. The one-letter amino acid code is used. Red represents small and hydrophobic amino acids (A, V, F, P, M, I, L, W), blue symbolizes acidic amino acids (D, E), magenta corresponds to basic amino acids (R, K), while green stands for hydroxyl, amine and basic amino acids (S, T, Y, C, N, G, Q, H). Non-annotated residues are indicated by X, while gaps are shown by dashed lines. The abbreviation v after the name of the organism refers to different isoforms. Clustal series of programs was used for multiple sequence alignment[67].

PRAK-p38αG145/V156 complexes to the cytoplasm and PRAK-p38βD145/L156 to the nucleus. Structural modeling analysis of PRAK-p38α predicts Asp-145 to interact with importin, while Leu-156 seems to be involved in interaction with the NLS motif of PRAK. No such interactions seem to occur between PRAK and p38β[21]. Because of the different distribution of PRAK-p38α and PRAK-p38β complexes, relative amounts of p38α and p38β in cells determine the subcellular localization of PRAK.

The p38-regulated subcellular localization of MK5 resembles that of its most closely related MAPKAPKs MK2 and MK3. The subcellular distribution of MK2 is regulated by p38α and p38β in the same way as MK5[21], and phosphorylation of MK2 at Thr-317 is necessary for stress-induced nucleocytoplasmic relocation of MK2[22]. MK3 is also exported out of the nucleus by arsenite or sorbitol, and stress-induced nuclear exclusion is inhibited by SB203580[23]. Overexpression of $p38^{MAPK}$ also triggers nuclear export of MK3, and this redistribution requires MK3 and $p38^{MAPK}$ to interact with each other[24]. The differential role of the p38α and β isoforms in directing subcellular localization of MK3 has not been addressed so far. However, kinetic studies have revealed that cellular stress-induced export of MK2, MK3 and MK5 kinases differs. After 60 min of sorbitol or arsenite treatment, enhanced green fluorescent protein (EGFP)-MK2 is exclusively in the cytoplasm, while > 60% of MK5 is still in the nucleus. The kinetics of MK3 seem to be intermediate to those of MK2 and MK5 because predominant cytoplasmic EGFP-MK3 localization is not observed before 40 min after treatment[23].

Regulation of subcellular localization by ERK3 and ERK4

While ERK3 can be detected in both the nucleus and the cytoplasm, MK5 has a more profound nuclear localization[10-20,25,26]. Coexpression of ERK3, however relocalizes both proteins to the cytoplasm[12,13,16,19]. Ectopic expression of the ERK3 activation loop S189A mutant or catalytically inactive mutants can still trigger cytoplasmic translocation of MK5, while kinase-dead MK5 mutants and MK5 with mutation of Thr-182 in the activation loop are still excluded from the nucleus when ERK3 is overexpressed[12,13,16]. Similar to ERK3, ERK4

also modulates the subcellular distribution of MK5. When expressed separately, ERK4 is exclusively found in the cytoplasm, while MK5 is predominantly nuclear. Coexpression, however, results in exclusive cytoplasmic localization of both proteins[14,16,19,27]. ERK4-induced nucleocytoplasmic translocation of MK5 does not require kinase activity of ERK4 or MK5, indicating that interaction rather than kinase activity of either protein is responsible for the subcellular redistribution of MK5 by ERK4[14,16,27]. The group of Meloche has shown that overexpression of ERK4 with mutated Ser-186 in its activation loop markedly impairs cytoplasmic redistribution of MK5[16]. The reason for this remains elusive. The biological significance of the ERK3-MK5 and ERK4-MK5 interactions has not been resolved, but MK5 is implicated in regulating the role of ERK3 in cell cycle progression. Another unanswered question is whether $p38^{MAPK}$ and ERK3/4 bind the same or different pools of MK5, because MK5 possesses both $p38^{MAPK}$ and ERK3/4 binding sites (Figure 2A). Compartmentalization of the different proteins may allow spatial specificity and thereby channeling signals through the $p38^{MAPK}$-MK5 or the ERK3/4-MK5 pathways.

Regulation of subcellular localization by PKA

Our group has shown that activation of the cAMP/PKA pathway by the adenylate cyclase activator forskolin leads to transient nuclear export of endogenous and EGFP-tagged MK5. No such forskolin-induced nucleocytoplasmic redistribution is observed in PKA-deficient cells. Overexpression of the catalytic subunit of PKA (PKA-C) also triggers nucleocytoplasmic redistribution of MK5. PKA-induced nuclear export requires kinase activities of both PKA and MK5 and is sensitive to leptomycin[15,19]. Overexpression of PKA-C, to which an NES is tagged, or overexpression of the PKA inhibitor, which forces PKA out of the nucleus, does not induce nuclear exclusion of MK5, while expression of an NLS-tagged PKA-C induces nuclear export of MK5. The NLS-PKA-C fusion protein remains in the nucleus, suggesting that MK5 does not leave the nucleus in complex with PKA-C[15]. We identified Ser-115 in MK5 as a PKA phosphoacceptor site. Substitution of this residue into alanine generates an MK5 mutant that no longer translocates to the cytoplasm upon activation of the PKA pathway, whereas the phosphomimicking MK5 S115D mutant is distributed in both the nucleus and the cytoplasm in resting cells[19]. Activated PKA is unable to induce nuclear export of the MK5 T182A mutant and the kinase-dead MK5 K51E mutant[15], but the MK5 S115D/T182A and MK5 K51E/S115D mutants can enter the cytoplasm in untreated cells[19]. The reason why MK5 T182A and K51E mutants, which can still be phosphorylated at Ser-115, remain in the nucleus after PKA activation, while the S115D/T182A and K51E/S115D mutants are cytoplasmic, is unclear. One possible explanation is that PKA mediates posttranslational phosphorylation of MK5, while substitution of Ser-115 into Asp occurs during translation. The MK5 K51E and T182A mutants may possess a conformation that locks MK5 into a conformation that prevents exposure of the NES. MK5 K51E/S115D and MK5 S115D/T182A on the other hand contain amino acid substitutions introduced during protein synthesis. The nascent MK5 S115D polypeptide chain may adopt a structure that exposes the NES or/and masks the NLS independently of the incorporation of Lys at position 51 (respectively Ala at position 182), such that the synthesized MK5 K51E/S115D and MK5 S115D/T182A proteins enter the cytoplasm[19]. Furthermore, we have demonstrated that the S115A mutation does not abrogate the ability of $p38^{MAPK}$, ERK3 and ERK4 to induce nuclear export of MK5, suggesting a phosphoSer-115-independent mechanism of nuclear export by these proteins[19].

Regulation of subcellular localization by the dual-specificity phosphatase Cdc14A

Overexpression of the dual-specificity phosphatase Cdc14A also causes nucleocytoplasmic translocation of MK5[17]. The ability of Cdc14A to relocate MK5 does not require the catalytic activity of Cdc14A, because similar subcellular redistribution patterns of MK5 are observed in cells overexpressing wild-type or phosphatase-inactive Cdc14A. The authors did not investigate whether the catalytic activity of MK5 is required for Cdc14A-induced nuclear export. Cdc14A interacts with ERK3 *in vivo* and ERK3 regulates the subcellular distribution of MK5 (see above), therefore, Cdc14A-triggered MK5 relocation may be mediated by ERK3. Cdc14A overexpression does not lead to stabilization and increased ERK3 protein levels, so that enhanced ERK3 levels cannot account for Cdc14A-induced MK5 nuclear export. Cdc14A also forms a complex with ERK4 so that Cdc14A may be involved in ERK4-regulated nuclear export of MK5 as well[17]. The mechanism by which Cdc14A causes nuclear exclusion of MK5 has not been resolved, and the functional consequences of the Cdc14A-mediated nucleocytoplasmic translocation of MK5 remain to be elucidated.

ACTIVATION OF MK5

MK5 was originally shown to be phosphorylated by ERK2, JNK3 and p38δ *in vitro*. This *in vitro* phosphorylation by ERK2 and p38δ increases MK5 kinase activity towards a peptide substrate derived from the regulatory light chain of myosin Ⅱ by ninefold and 15-fold, respectively, compared to unphosphorylated MK5. JNK3 is unable to stimulate the catalytic activity of MK5[3]. Another study has revealed that PRAK can be phosphorylated *in vitro* by all four p38 isoforms, but not by ERK2 or JNK2[2]. Although PRAK is most efficiently phosphorylated by p38γ, it does not lead to activation of PRAK as measured in a coupled kinase assay with HSP27 as substrate. Similarly, p38δ efficiently phosphorylates, but

fails to activate PRAK. Only p38α and p38β are able to phosphorylate and activate PRAK. Phosphopeptide mapping has revealed that these two kinases preferentially phosphorylate Thr-182 *in vitro*. Remarkably, ERK2 and JNK2 also phosphorylate this site *in vitro*, although with different stoichiometry. This may explain why ERK2 and JNK2 are unable to activate PRAK *in vitro*. Studies with p38α- and p38β-phosphorylated PRAK mutants, in which the putative phosphoacceptor sites Ser-93, Thr-186, Ser-212 and Thr-214 have been replaced by Ala, have shown that these mutations have no effect on the catalytic activity. These results suggest that these sites are not required for PRAK activation. Mutating Thr-182 into Ala, however, completely abolishes the induction of PRAK by p38α/β. Phosphopeptide mapping of *in vitro* phosphorylated PRAK by p38β shows weak phosphorylation at Ser-212. However, mutation of this residue to Ala does not have any effect on PRAK activation by p38α/β. As previously mentioned, p38δ fails to activate PRAK, probably because p38δ does not phosphorylate the regulatory Thr-182 of PRAK[2]. Whether Thr-182 is a genuine *in vivo* phosphorylation site has been hampered by the lack of good phosphoThr-182 antibodies. Using such antibodies or quantitative mass spectrometry, p38β-, ERK3- and ERK4-mediated phosphorylation of this site in cells has been demonstrated[27-30]. Whether MK5 is regulated by the $p38^{MAPK}$ pathway *in vivo* remains a matter of dispute, and has been recently extensively reviewed by ourselves[31]. However, recent work by the group of Han has provided evidence that PRAK is a genuine substrate for p38β[30]. The regulation of MK2/MK3 activity by p38MAPK seems to be somewhat different form MK5 as multiple phosphorylation events are involved. Complete activation of human MK2 *in vivo* by $p38^{MAPK}$ requires phosphorylation of Thr-25, Thr-222, Thr-272 and Thr-334. Thr-222 and Thr-334 are the major activation sites, while the other sites probably represent minor autophosphorylation sites. All sites, except Thr-25, are conserved in MK3 and it is therefore postulated that this mechanism is also operational for MK3[32].

The atypical MAPK ERK3 and ERK4 (Figure 1) have been shown to induce phosphorylation of MK5 at Thr-182 and to activate MK5. The MK5 ATP-binding pocket mutant K51E is not phosphorylated at Thr-182 by ERK3, suggesting that ERK3 stimulates the autophosphorylation of MK5, rather than phosphorylation of MK5 at this residue. This is supported by the observation that catalytically inactive ERK3 still can activate MK5[12,13]. However, the group of Meloche has reported that the catalytically inactive ERK3 mutants K49A/K50A, D152A and S189A are unable to activate MK5[16]. The reason for this discrepancy remains unresolved, but Gaestel and his group have suggested that activation of MK5 by catalytically dead ERK3 may occur through recruitment of ERK4, which can form heterodimers with ERK3[14]. The finding that inactive ERK3 can activate MK5 suggests that conformational changes of MK5 are sufficient to trigger autophosphorylation and consequently activation of the protein. Structural changes in MK5 may be induced by interaction with other proteins (e.g. ERK3) or changes in residues. We have found that the L337A substitution in the NES motif generates an MK5 mutant that was approximately 6-fold more active than wild-type MK5 in an *in vitro* kinase assay[10]. The reason for this is not known, but it is tempting to speculate that the L337A substitution causes a conformational change that stimulates the autophosphorylation activity of MK5 and hence the enzymatic activity. MK5 can also form a complex with ERK4 and this is accompanied by activation of MK5, as well as increased ERK4 phosphorylation at Ser-186[14,27,33]. MK5 activation requires an intact Ser-186 residue because both the non-phosphorylatable S186A, as well as the phosphomimicking S186E ERK4 mutants, are unable to activate MK5, despite the fact that these mutants can still interact with MK5. Déléris and co-workers have reported that ERK4 S186A partially activates, while the kinase-dead variants ERK4 D149A and K49A/K50A fail to activate MK5[16]. The activation of MK5 by ERK3 and ERK4 differs in two ways. While wild-type ERK3 cannot induce phosphorylation of the kinase-dead MK5 K51E mutant at Thr-182, wild-type ERK4 is able to cause an increase of phosphorylation at this site. In contrast to kinase-inactivated ERK3, which can still activate MK5, the kinase-dead ERK4 D168A mutant fails to do this[13,27]. MK5 is, however, not a Ser-186 ERK4 kinase, because the inactive MK5 K51E and T182A mutants also increase pSer-186 ERK4 levels. The authors have proposed that MK5 may protect dephosphorylation of ERK4 by a yet-unidentified protein phosphatase or enhance the recruitment of an ERK4 kinase[33]. In light of the new findings, this ERK4 kinase could be group I p21-activated protein kinases (PAKs). Indeed, earlier this year, two groups reported that ERK3 and ERK4 are *bona fide* downstream targets of PAK1, PAK2 and PAK3. De La Mota-Peynado has used high-density protein microarrays containing almost 8300 different full-length proteins to identify ERK3 as a PAK2 substrate[34]. PAK2 phosphorylates Ser-189 within the ERK3 activation loop and this regulates the subcellular localization of ERK3 and the interaction with PRAK. The protein inhibitory domain, which corresponds to residues 83-149 of human PAK1, and acts as a highly specific inhibitor of class I PAKs, also modulates the interaction of ERK3 with PAK, suggesting that ERK3 is also a substrate for PAK1 and PAK3[34]. The group of Meloche has used extensive purification methods to identify ERK3 and ERK4 as class I PAKs substrates. Activation of ERK3/ERK4 by class I PAKs increases MK5 phosphorylation at Thr-182 and stimulates the catalytic activity of MK5[29].

Our group has shown that MK5 can be activated *in vivo* by PKA-C. MK5 that was *in vitro* phosphorylated by PKA-C displays higher kinase activity towards its substrate ERK3 compared to non-phosphorylated MK5. Moreover, MK5 immunoprecipitated from cells in which

PKA is activated by the adenylate cyclase activator forskolin has a higher catalytic activity toward the synthetic Praktide substrate than MK5 immunoprecipitated from untreated cells[15]. We have mapped Ser-115 as a PKA phosphoacceptor site, but have not directly investigated whether phosphorylation of this site potentiates the kinase activity of MK5. However, a phosphomimicking MK5 S115D mutant, but not wild-type MK5 is able to stimulate the transcriptional activity of p53 and trigger HSP27 phosphorylation in cells[19]. The observations suggest that phosphorylation of Ser-115 regulates the catalytic activity of MK5. Whether activation of MK5 by PKA requires an intact Thr-182 remains to be tested, nor is it known whether PKA stimulates the autophosphorylation activity of MK5.

SUBSTRATES OF MK5

MK5 has been shown to phosphorylate several substrates *in vitro*, but this has not been confirmed *in vivo*, nor have the functional implications of these phosphorylation events been tested. In the original papers describing the isolation of MK5 and PRAK, it was demonstrated that MK5 could phosphorylate the peptide KKRPQRATSNVFS derived from the regulatory light chain of myosin Ⅱ[3], while PRAK could phosphorylate mouse HSP25 and the human homologue HSP27, as well as glycogen synthetase[3]. Later, it was shown that MK5 is a genuine HSP27 kinase (see below). Tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of catecholamine, can be *in vitro* phosphorylated by MK5 on Ser-19[35]. Treatment of cells with arsenite induces phosphorylation of tyrosine hydroxylase at Ser-19, concomitant with an increase in $p38^{MAPK}$ phosphorylation. Both $p38^{MAPK}$ and tyrosine hydroxylase phosphorylation is inhibited by SB203580[36]. Although these results suggest the involvement of the $p38^{MAPK}$/MK5 pathway in tyrosine hydroxylase phosphorylation, the role of MK5 as genuine tyrosine hydroxylase kinases remains unexploited. *In vitro* assays have shown that MK5 binds to the large cytoplasmic loop of the major glucose transporter GLUT4, suggesting that MK5 phosphorylates GLUT4, thereby influencing the activity of this transporter. Experimental proof is required to buttress this paradigm[37]. PRAK has been shown to phosphorylate cytosolic phospholipase A2 (cPLA$_2$) at Ser-727 *in vitro*, and this site becomes phosphorylated in thrombin-treated platelets, a posttranslational modification that stimulates the release of arachidonate. Thrombin treatment of platelets has led to weak activation of MK5 (1.4-fold) and MNK1 (2.5-fold) activity, but not MSK1, MK2 and MK3[38]. These findings suggest that MK5 is partially involved in thrombin-induced phosphorylation of cPLA$_2$ at Ser-727. The known *in vivo* substrates of PRAK/MK5 so far include ERK3, ERK4, 14-3-3ε, HSP27, p53, FoxO3 and Rheb. Comparison of the phosphorylation motif of the *in vitro* and *in vivo* MK5 substrates does not reveal a clear consensus sequence, although there seems to be a preference for a nonpolar, hydrophobic residue (I, L, V, P) at position -1 and R at position -3 (Figure 3).

cPLA2 Ser-727:	NPSRC<u>S</u>VSLSN
FoxO3a Ser-215:	IRHNL<u>S</u>LHSRF
HSP27 Ser-15:	LLRGP<u>S</u>WDPFR
HSP27 Ser-78:	YSRAL<u>S</u>RQLSS
HSP27 Ser-82:	LSRQL<u>S</u>SGVSE
light chain myosin Ⅱ peptide:	KKRPQRAT<u>S</u>NVFS
p53 Ser-37:	LSPLP<u>S</u>QAMDD
PRAKtide:	KKLRRTL<u>S</u>VA
Rheb Ser-130:	MERVI<u>S</u>YEEGK

Figure 3 Amino acid alignment of the of the phosphoacceptor site motifs in mitogen-activated protein kinase-activated protein kinase 5 substrates. The phosphoacceptor site is underlined. The synthetic substrate peptide PRAKtide, which is derived from glycogen synthatese[40], and the peptide substrate derived from the regulatory light chain of myosin Ⅱ[3] are also shown.

Co-immunoprecipitation and glutathione-S-transferase pull-down studies have demonstrated a specific and direct interaction between ERK3 and ERK4[12,13]. Regions encompassing residues 383-393 and 460-465 of MK5 contribute to ERK3 and ERK4 binding, while the FRIEDE motif spanning residues 328-333 in ERK4 and the corresponding FHIEDE motif in ERK3 (residues 332-337) are required for MK5 interaction[28]. The exact physiological consequences of MK5/ERK3 and MK5/ERK4 complex formation are still unresolved (see next section). A yeast two-hybrid screen with 14-3-3ε as bait has identified MK5 as one of the binding proteins; an interaction that has been confirmed in mammalian cells by co-immunoprecipitation studies[39]. This interaction is independent of the enzymatic activity of MK5 as the MK5 T182A mutant can still bind 14-3-3ε. Unexpectedly, the unique C-terminal domain of MK5 is dispensable for interaction with 14-3-3ε, while the conserved catalytic domain is required. The authors have demonstrated that 14-3-3ε inhibits the MK5 kinase activity towards HSP27, and disrupts MK5-mediated actin rearrangement and cell migration. The authors have suggested that 14-3-3ε binding masks the phosphorylation site at Thr-182, thereby preventing activation of MK5[39]. The functional consequences of the link between MK5 and HSP27, 14-3-3ε, p53, FoxO3a and Rheb are discussed in the next section.

PHYSIOLOGICAL ROLES OF MK5

Lessons from animal studies

Knockout mouse models often provide valuable information on the biological function of a particular protein. The first MK5 knockout mice generated on a mixed 129 × C57/B6 genetic background were viable, fertile and displayed no obvious phenotype, therefore, the role of MK5 remained enigmatic[40,41]. However, both *mk5*$^{+/-}$ and *mk5*$^{-/-}$ mice are more susceptible to skin carcinogenesis induced by dimethylbenzanthracene, indicating that MK5 acts as a tumor suppressor[41]. Surprisingly, *mk5*$^{-/-}$ mice on

a C57BL/6 genetic background show embryonic lethality with incomplete penetrance around E11. The reasons for different phenotypes in different genetic backgrounds and for incomplete penetrance of the embryonic lethal phenotype are unknown[13]. Our group has generated a transgenic mouse that expresses a constitutive active variant of MK5 (MK5 L337A mutant). These mice exhibit changed anxiety behavior and locomotor differences compared to control littermates. The underlying causes for these differences are not known, but suggest that MK5 is involved in neurological processes[42].

MK5 as a scaffold protein or/and inducer of ERK3 and ERK4 activity

MK5 is the only known substrate for ERK3 and ERK4, therefore, *erk3* and *erk4* knockout models might provide some clues on the biological function of MK5. Similar to *mk5* null mice, ERK3$^{-/-}$ mice on a C57BL/6 background are non-viable[43]. Approximately 40% of the born offspring died within 15 min of delivery because of acute respiratory failure, while the remaining that survived displayed uncoordinated movements, lack of reflex on pinching, infirm vocalization, and diminished suckling reflex. These animals died within 24 h of delivery. The ERK3$^{-/-}$ mice had fetal growth restrictions and reduced body and organ weight, but no gross morphological changes. Moreover, they were characterized by pulmonary hypoplasia and incomplete differentiation of type Ⅱ pneumocytes, confirming a role of ERK3 in differentiation and proliferation. ERK3$^{-/-}$ mice had also defects in production and/or secretion of insulin-like growth factor Ⅱ[43]. The lethal effect of knocking out either the *mapk6* gene (encoding Erk3) or the *mapkapk5* gene on a C57BL/6 genetic background[13,43] and the observation that ERK3 and MK5 mRNA peak levels coincide at E11[13,44] underscore a functional link between ERK3 and MK5. Depletion or knockdown of MK5 also decreased the ERK3 levels, indicating that MK5 may stabilize ERK3 and act as a chaperone[12,13].

Whether MK5 is implicated in the ERK3$^{-/-}$ phenotype remains to be established. This could be examined by testing whether MK5 can rescue the Erk3-deficient mice, for example, by crossing them with mice that express constitutive active MK5.

Disruption of the *mapk4* gene encoding ERK4 had no obvious effects on viability, fertility, morphology and physiology of the mice on a mixed C57BL/6J × 129/Sv genetic background[45]. Moreover, knockout of ERK4 in ERK3$^{-/-}$ mice does not exacerbate their phenotype. However, ERK4-deficient animals on a C57BL/6J genetic background possess increased depression-like behavior, as monitored in the forced-swimming test, but no differences with wild-type littermates in locomotor activity and in anxiety-like behavior using the elevated plus maze test[45]. Our MK5 L337A transgenic mice on the same genetic background have displayed differences compared to wild-type mice in locomotor activity and behavior using the elevated plus maze and open field tests, but we have not examined their anxiety behavior by using the forced-swimming test[42]. Double knock out of the ERK3/ERK4 activators PAK1 and PAK3 on mixed C57BL/6J × CD1 background generates mice with increased locomotor activity, but these mice are more anxious[46]. Thus, inactivation of PAK1 plus PAK3 increases locomotor activity and anxiety, while these activities are unchanged in mice deficient in ERK4, compared with control littermates. Transgenic mice overexpressing activated MK5 have increased locomotor activity, but decreased anxiety behavior. The unchanged locomotor and anxiety behavior in ERK4-deficient mice is in contrast to increased locomotor and decreased anxiety behavior observed in MK5 L337A transgenic mice[42,45]. This may argue against a functional role of ERK4-MK5 in determining these phenotypical traits, but it may also be a result of a lack of ERK4-mediated activation of yet-unidentified targets, which are implicated in these properties. Another obvious explanation is that, in ERK4-deficient mice, no ERK4 protein is expressed, while in the transgenic MK5 337G mice, a constitutive active MK5, which mimics continuous activation by ERK4, is overexpressed.

MK5 may act as a scaffold protein for ERK3 and ERK4, which allows the recruitment of ERK3/ERK4 (e.g. group I PAK), or prevents dephosphorylation by ERK3/ERK4 phosphatase. This increases phosphorylation of ERK3 at Ser-189 and ERK4 at Ser-186, resulting in stabilization of the MK5-ERK3/4 complexes and increased phosphorylation of MK5 at Thr-182. The overall result is that the catalytic activity of all partners is boosted and the enzymes are then competent to phosphorylate other physiological substrates[16,33].

Role in F-actin remodeling and cell motility

Studies from our group and from Tak and co-workers have clearly established a role for MK5 in F-actin rearrangement[15,35,19,47]. The previous observations that the cAMP/PKA pathway can regulate F-actin polymerization[48] and interact with MAPK pathways[49], while HSP27 is an *in vitro* substrate of MK5, prompted us to investigate whether MK5 is involved in cAMP/PKA-induced F-actin remodeling, by catalyzing the phosphorylation of HSP27. We have found that activation of the cAMP/PKA pathway results in transient nuclear export of MK5. This process requires the activity of both kinases because kinase-dead PKA cannot induce nuclear export of MK5 and *vice versa,* the kinase-dead MK5 T182A mutant remains in the nucleus after activation of the cAMP/PKA pathway. PKA is able to phosphorylate MK5 at Ser-115 *in vitro* and to stimulate the kinase activity of MK5. The MK5 S115D mutant, which mimics the PKA phosphorylated form of MK5, can trigger HSP27 phosphorylation *in vivo* and cause F-actin rearrangements. Studies with siRNA and MK5 and HSP27 mutants have confirmed that MK5 and HSP27 are implicated in cAMP/PKA-induced F-actin rearrangements[15,19,47]. As described above, the findings by Tak and his colleagues have supported a role for MK5 in F-actin polymerization[39]. The authors also have shown

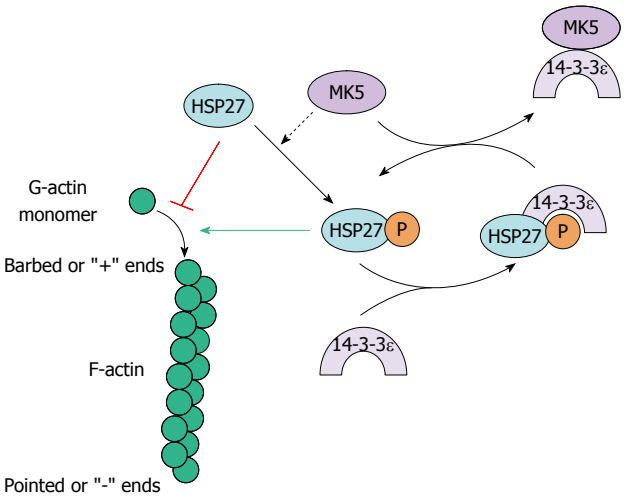

Figure 4 Mitogen-activated protein kinase-activated protein kinase 5 and F-actin remodeling through Hsp27. Mitogen-activated protein kinase-activated protein kinase 5 (MK5) can phosphorylate HSP27 and this stimulates F-actin polymerization. 14-3-3ε can bind and inhibit the enzymatic activity of MK5 and thus prevent F-actin remodeling. 14-3-3ε has also been shown to bind phosphorylated HSP27. MK5 may extricate 14-3-3ε from pHSP27 and releasing pHSP27 which in turn can mediate F-actin polymerization.

that MK5 can contribute to cell migration. The MK5-HSP27 link may be regulated by 14-3-3ε. Indeed, Tak and co-workers have found that MK5 and 14-3-3ε form complexes in cells, while the group of Gaestel has shown that phosphorylated HSP25, the mouse homolog of human HSP27, can interact with 14-3-3ε, at least *in vitro*[50]. MK5 can phosphorylate HSP27, which stimulates F-actin polymerization, but it may also bind 14-3-3ε, thereby extricating pHSP27 (Figure 4). On the other hand, 14-3-3ε can bind and inactivate MK5 and thus prevent MK5-catalyzed phosphorylation of HSP27. In resting cells, MK5 is predominantly in the nucleus. Activation of PKA causes nuclear export and activation of MK5, which can then phosphorylate HSP27 and trigger F-actin polymerization in the cytosol.

Group I PAKs have been implicated in several cellular processes including cytoskeletal remodeling[51]. The recent finding that group I PAKs can activate MK5 implicates MK5 in PAK-controlled cytoskeletal architecture[29,34].

MK5 as a tumor suppressor

Senescence, a permanent state of cell-cycle arrest, develops naturally in most cells after repeated cell division, but can be induced prematurely by DNA damage, telomere dysfunction, de-repression of the *INK4a/ARF* locus, and other stimuli. Senescence suppresses the development of cancer cells by arresting the proliferation of damaged cells that are at risk for malignant transformation[52-54]. Several observations have pointed to a role of MK5 in cell cycling and proliferation. Two independent research groups have reported that PRAK suppresses oncogenic RAS-induced proliferation. Chen and collaborators have found that ectopically expressed PRAK alone has little effect on cell proliferation, but almost completely abolishes oncogenic c-Ha-RAS-induced cell proliferation. A kinase-dead PRAK mutant has no inhibitory effect on RAS-induced proliferation. PRAK also blocks the activity of RAS-responsive promoters when oncogenic RAS is expressed[55]. Thus, PRAK can act as a negative regulator in oncogenic RAS proliferative signaling. The exact mechanism remains to be resolved, but the authors have shown that PRAK inhibits RAS-induced JNK activity by 85%. They have suggested that oncogenic RAS activates $p38^{MAPK}$, which subsequently activates PRAK. Activated PRAK may then interfere with RAS-induced JNK activation, resulting in inhibition of proliferation[55]. In another study, Li and colleagues have demonstrated that overexpression of wild-type PRAK strongly inhibits proliferation of NIH3T3 cells, and this requires nuclear localization of PRAK[21]. This is in contrast to the findings of Sun and collaborators who have found that overexpression of wild-type PRAK causes only a slight reduction in the proliferation rate of primary human fibroblast BJ cells. However, coexpression of oncogenic c-Ha-RAS with wild-type PRAK results in strong growth inhibition[41]. Sun and co-workers have demonstrated that PRAK can activate the transcription activity of p53 through phosphorylation of Ser-37. Increased activity of p53 may lead to upregulated expression of cyclin-dependent protein kinase inhibitor $p21^{WAF1/CIP1}$, a target of p53, and consequently arrest in cell proliferation. Oncogenic RAS-induced $p21^{WAF1/CIP1}$ expression is abolished in PRAK-deficient cells and reduced in cells overexpressing p53 S37A[41]. In summary, these findings indicate that PRAK mediates senescence by stimulating the transcriptional activity of p53 by phosphorylating residue Ser37, which enhances the expression of $p21^{WAF1/CIP1}$.

Marasa and colleagues have convincingly demonstrated that expression of MKK4 (MEK4), an upstream activator of JNK and $p38^{MAPK}$ (Figure 1), is increased in senescent human diploid cells because miRNAs miR-15b, miR-24, miR-25 and miR-141 that target *mkk4* mRNA are downregulated[56]. Enhancing MKK4 levels by overexpression of MKK4, or by simultaneous reduction in the concentration of the four miRNAs by antagomirs inhibits cell proliferation and triggers a senescent phenotype, as determined by augmented expression of senescence-associated proteins such as $p21^{WAF1/CIP1}$ and cyclin D1. Moreover, increased phosphorylation at Ser-93 and activity of PRAK towards HSP27 in senescent cells compared to young cells has been measured. Whether Ser-93 represents a genuine phosphoacceptor site remains to be determined, because both our own group and others have failed to map phosphorylation at this site[2,57].

Oxidative-stress-induced premature senescence of vascular smooth muscle cells (VSMCs) has been shown to play an important role in the development of atherosclerosis, while estrogens may partially counteract this. The protective properties of estrogens against atherosclerosis are, however, incompletely understood[58,59]. A

recent study has suggested a role for the $p38^{MAPK}$-MK5 pathway in H_2O_2-induced senescence of rat VSMCs[56]. The authors have shown that treatment of rat VSMCs with H_2O_2 induces premature senescence of the cells that is accompanied by an increase in RAS activity, $p38^{MAPK}$ phosphorylation levels, and MK5, p53 and pRb protein levels. However, a decrease in the expression of the cyclin-dependent protein kinase inhibitors $p16^{INK4A}$ and $p21^{WAF1/CIP1}$ and pRb phosphorylation levels has been observed in H_2O_2-treated cells compared to untreated cells[60]. Pretreatment of rat VSMCs with 17β-estradiol strongly represses H_2O_2-triggerd activation of RAS and inhibits H_2O_2-induced phosphorylation of $p38^{MAPK}$ and expression of MK5, p53, pRb, $p16^{INK4A}$ and $p21^{WAF1/CIP1}$. The exact role of MK5 in senescence of VSMCs remains elusive. The authors did not investigate whether MK5 actually becomes activated in H_2O_2-exposed cells, while we were unable to detect increased MK5 levels in rat pheochromocytoma cells (PC12 cells) with stress-inducing stimuli such as arsenite, vanadate, lipopolysaccharide, and NO donor spermine NONOate[4]. Future studies are necessary to investigate a possible role of MK5/PRAK in senescence of VSMCs and atherosclerosis, and senescence in general. MK5 null cell lines, siRNA-mediated depletion of MK5, overexpression of MK5 kinase-dead mutants, and specific MK5 inhibitors are indispensable tools for such studies.

Furthermore, MK5 may be involved in inhibition of cell proliferation through ERK3. ERK3 is involved in cell cycle regulation, because it has been reported that elevated ERK3 levels result in G_1 cell cycle arrest and inhibition of cell proliferation[25,26]. The fine molecular mechanisms by which ERK3 prevents cell cycle progression are incompletely understood, but ERK3 binds cyclin D3, as well as Cdc14A (an antagonist of cyclin-dependent kinase 1), and Cdc14A stabilizes complex formation between ERK3 and cyclin D3[17]. MK5 may, through targeting ERK3, affect the cell cycle. ERK3 levels are increased in MK5-deficient or -depleted cells, indicating that MK5 stabilizes ERK3[12,13]. Moreover, MK5-ERK3 complexes are exclusively anchored in the cytoplasm[12,13,19]. Thus, MK5 could, through retaining ERK3 in the cytoplasm, prevent ERK3 from inhibiting G_1/S phase transition.

An elegant recent study has revealed a novel mechanism for a tumor-suppressive role of MK5[8]. Using an siRNA screen of the human kinome, the authors have identified MK5 as a negative regulator of c-Myc expression, because Myc protein levels increased in cells transfected with siRNA that targeted MK5. Ectopic expression of active MK5 decreased Myc levels and suppressed cell proliferation. MK5 was shown to regulate Myc at the translational level, suggesting an miRNA-dependent mechanism. Indeed, the authors demonstrated that MK5 enhanced expression of miR-34b and miR-34c, which were processed from the same pre-miRNA, but not of the third member of the miR-34 family, miR-34a. Although the promoter of the pre-miRNA that encodes miR-34b and miR-34c is a p53-responsive promoter[61], and MK5/PRAK can phosphorylate and activate p53[19,41], MK5 does not seem to stimulate miR34-b/c expression in a p53-dependent manner, because depletion of MK5 in p53-deficient cells or cells overexpressing the dominant negative p53DD mutant still result in increased Myc protein levels. The pre-miRNA promoter also contains consensus binding sites for the FoxO family of transcription factors and chromatin immunoprecipitation studies confirmed the binding of FoxO3a to this promoter. Moreover, active FoxO3a upregulated expression of miR-34b/c. The authors went on to show that MK5 predominantly phosphorylates FoxO3a at Ser-215 *in vivo* (and at other sites *in vitro* as well) and that MK5-mediated phosphorylation of this site is required for the upregulation of miR-34b/c levels and downregulation of c-Myc levels. Myc protein seems to be engaged in a negative feedback loop by binding to the MK5 promoter and enhancing expression of MK5[8]. Myc is a central regulator of the cell cycle and aberrant Myc expression plays a central role in oncogenesis[62], therefore, Kress and colleagues have investigated the state of MK5 expression in tumor cells with increased Myc levels. MK5 expression was higher in normal colon mucosa than in colorectal carcinoma, whereas Myc expression was weak in normal colon epithelium, but strong in colorectal tumor tissues. Moreover, low levels of MK5 mRNA were associated with increased probability of the development of distant metastasis[8]. In conclusion, these exciting findings disclose a novel mechanism for the tumor-suppressive function of MK5. It would be interesting to validate the MK5-Myc link in *mk5*$^{-/-}$ mice or in transgenic mice overexpressing the active MK5 L337A mutant, by examining Myc levels and the development of colon cancer in these animals compared to wild-type mice.

MK5, cellular energy levels and cell growth

The serine/threonine protein kinase mammalian target of rapamycin (mTOR) plays a critical role in controlling cell growth and metabolism[63]. mTOR exists in two distinct complexes called mTORC1 and mTORC2. mTORC1 regulates cell growth in response to different signals, while mTORC2 modulates cytoskeletal organization[64,65]. One of the key regulators of mTORC1 is the small G-protein Rheb (Ras homolog enriched in the brain). In its GTP-bound state, Rheb activates mTORC1, while it cannot do so in its GDP-bound form. The group of Han has discovered a new role for PRAK in suppression of cell growth induced by energy starvation[30]. They have showed that certain forms of energy starvation cause activation of p38β and phosphorylation of Thr-182 and activation of PRAK. PRAK can bind and phosphorylate Rheb at Ser-130 *in vivo*; a modification that severely impairs the ability of Rheb to bind GTP or to retain already bound GTP. PRAK-mediated phosphorylation and inaction of Rheb leads to inhibition of mTORC1 and cell growth arrest. Thus, the p38β-PRAK pathway is involved

in energy-depletion-induced suppression of mTORC. However, either serum starvation or amino acid depletion do not result in p38β-PRAK activation. The same is true for carbon cyanide 4-(trifluoromethoxy) phenylhydrazone and for 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside treatment. It is unclear why p38β-PRAK has no role in mTORC inactivation under these conditions, but because several pathways can inactivate mTORC1, these pathways could operate in a stimulus-specific manner[30].

FUTURE PERSPECTIVES

The biological role of MK5 remains incompletely understood, partially due to the lack of a clear phenotype or embryonic lethality in knockout mice, and because no mutations in the gene have been described in a specific disease[13,40,66]. However, MK5 is highly conserved throughout the animal kingdom, suggesting that it plays a central role in cellular processes. During recent years, some of the functions of MK5 have started to emerge. One major role of MK5 seems to be that of a tumor suppressor. As a result of its cell growth-suppressive properties, MK5 appears an attractive therapeutic target in different forms of cancer. Proliferation of tumor cells with elevated Myc levels or with oncogenic RAS mutations may be treated with compounds that specifically activate MK5. Unfortunately, no such drugs have been developed yet. Solving the crystallographic structure may be a pivotal tool in drug design and may also provide valuable information on the regulation of MK5 activation and intracellular trafficking. On the other hand, MK5 seems to be involved in F-actin polymerization and cell motility, such that activation of MK5 may stimulate metastasis of tumor cells. The potential tumor-suppressing, but metastasis-stimulating properties of MK5 require caution when using MK5 targeting therapy. Other unresolved questions are the functional implication of the ERK3/ERK4-MK5 connections and the accurate nature of the $p38^{MAPK}$-MK5 connection. The recently described role of p38β-MK5 in inactivation of mTORC1 illustrates that MK5 can link up with specific $p38^{MAPK}$ isoforms under certain conditions.

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