

# Activation Loop Phosphorylation of ERK3/ERK4 by Group I p21-activated Kinases (PAKs) Defines a Novel PAK-ERK3/4-MAPK-activated Protein Kinase 5 Signaling Pathway<sup>\*[5]</sup>

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Classical mitogen-activated protein (MAP) kinases are activated by dual phosphorylation of the Thr-Xxx-Tyr motif in their activation loop, which is catalyzed by members of the MAP kinase kinase family. The atypical MAP kinases extracellular signal-regulated kinase 3 (ERK3) and ERK4 contain a single phospho-acceptor site in this segment and are not substrates of MAP kinase kinases. Previous studies have shown that ERK3 and ERK4 are phosphorylated on activation loop residue Ser-189/Ser-186, resulting in their catalytic activation. However, the identity of the protein kinase mediating this regulatory event has remained elusive. We have used an unbiased biochemical purification approach to isolate the kinase activity responsible for ERK3 Ser-189 phosphorylation. Here, we report the identification of group I p21-activated kinases (PAKs) as ERK3/ERK4 activation loop kinases. We show that group I PAKs phosphorylate ERK3 and ERK4 on Ser-189 and Ser-186, respectively, both *in vitro* and *in vivo*, and that expression of activated Rac1 augments this response. Reciprocally, silencing of PAK1/2/3 expression by RNA interference (RNAi) completely abolishes Rac1-induced Ser-189 phosphorylation of ERK3. Importantly, we demonstrate that PAK-mediated phosphorylation of ERK3/ERK4 results in their enzymatic activation and in downstream activation of MAP kinase-activated protein kinase 5 (MK5) *in vivo*. Our results reveal that group I PAKs act as upstream activators of ERK3 and ERK4 and unravel a novel PAK-ERK3/ERK4-MK5 signaling pathway.

ERK3 and ERK4 define a distinct subfamily of atypical MAP kinases that display structural and functional differ-

ences with conventional MAP kinases (1). First, they possess a single phospho-acceptor site (Ser-Glu-Gly) in their activation loop instead of the classical dual phosphorylation Thr-Xxx-Tyr motif (2, 3). Accordingly, they are not phosphorylated and activated by dual-specificity MAP kinase kinase family members (4, 5). Second, ERK3 and ERK4 bear the sequence Ser-Pro-Arg instead of Ala-Pro-Glu in subdomain VIII of the kinase domain, being the only kinases in the human kinome to have an arginine at this position. Third, contrary to conventional MAP kinases like ERK1/ERK2, which are multifunctional Ser/Thr kinases capable of phosphorylating a vast array of substrates, ERK3 and ERK4 appear to have a very narrow substrate specificity (6). Their only known physiological substrate is the protein kinase MK5<sup>5</sup> (7–10).

The regulation of ERK3 and ERK4 activity remains poorly understood. We and others have shown that the serine residue within the activation loop of ERK3/ERK4 (Ser-189/Ser-186) is phosphorylated *in vivo* (6, 11–13). Catalytically inactive forms of the kinases are similarly phosphorylated on this motif, indicating that activation loop phosphorylation is mediated by an upstream cellular kinase (12, 13). Phosphorylation of Ser-189/Ser-186 leads to enzymatic activation of ERK3/ERK4 and is required for binding to and for cytoplasmic relocalization of the substrate MK5 (12, 13). Recently, we have shown that phosphorylation of ERK3 in the C-terminal extension by CDK1 stabilizes the protein and leads to its accumulation in mitosis (14).

The lack of information on the stimuli and upstream regulatory events that control the activity of ERK3 and ERK4 has hampered the comprehensive study of these atypical MAP kinase signaling pathways. To get new insights into the regulation of ERK3/ERK4 activity, we have used a classical biochemical purification approach to isolate the kinase(s) responsible for ERK3 Ser-189 phosphorylation. Here, we identify group I PAKs as ERK3 and ERK4 activation loop kinases. We demonstrate that PAKs phosphorylate ERK3 and ERK4 *in vitro* and *in vivo*, leading to their catalytic activation and to downstream MK5 phosphorylation and activation.

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<sup>5</sup> The abbreviations used are: MK5, MAP kinase-activated protein kinase 5; CA, catalytically activated; KA, kinase-dead; MS/MS, tandem mass spectrometry; PAK, p21-activated kinase.

## EXPERIMENTAL PROCEDURES

**Reagents and Antibodies**—Antibodies to PAK1, PAK2, and PAK3 were from Cell Signaling Technology; anti-GAPDH was from Santa Cruz Biotechnology; HRP-conjugated goat anti-mouse and anti-rabbit IgG were from Bio-Rad; polyclonal anti-FLAG was from Sigma. Monoclonal anti-Myc antibody was prepared from 9E10 hybridoma-producing cells. Monoclonal rabbit anti-ERK3 antibody was from Epitomics. Polyclonal anti-phospho-ERK3(Ser-189) antibody has been described (11). This antibody also recognizes phosphorylated Ser-186 in ERK4. SMARTpool siRNAs targeted to PAK1 (L-003597-00), PAK2 (L-003521-00), and PAK3 (L-003614-00) were from Dharmacon.

**Plasmid Constructs**—pcDNA3-Myc<sub>6</sub>-ERK3 wild type (WT), kinase-dead (KA, K49A/K50A) and S189A (SA), pcDNA3-HA-ERK4, pcDNA3-FLAG-ERK4, and pcDNA3-FLAG-MK5 have been described (11, 12, 15). His<sub>6</sub>-ERK3 WT and SA, His<sub>6</sub>-ERK3(1–365)KA-GST, and His<sub>6</sub>-ERK3(1–365) (WT, KA, and SA) recombinant proteins were generated by PCR subcloning of the sequence corresponding to the first 365 residues of ERK3 (with or without a stop codon) in frame into the EcoRI site of pHGST.1 (16). pHGST-ERK3(365–721) plasmid encoding the C-terminal extension of ERK3 has been described (16). PAK1 cDNA (17) was obtained from J. Chernoff (Fox Chase Cancer Center) and subcloned into EcoRI/XbaI sites of pcDNA3-FLAG (12). Mouse PAK2 cDNA was purchased from Origene and was subcloned into EcoRI/XbaI sites of pcDNA3-FLAG. The PAK2KD (K279R/T402A) kinase-dead mutant was generated by mutagenesis using the QuikChange system (Stratagene). Mouse HA-PAK3 was obtained from E. Van Obberghen-Schilling (Université de Nice) and was subcloned into the XbaI site of pcDNA3-FLAG. For recombinant protein production, PAK2 WT and KD were subcloned into EcoRI/NotI sites of pHGST.2T (16). pRK5-Myc vectors encoding activated Rac1L61 (CA) and inactive Rac1N17 (DN) were provided by N. Lamarche (McGill University). The pGEX-hu27 prokaryotic vector coding for GST-Hsp27 protein was a gift from J. Landry (Laval University). All mutations and PCR products were verified by DNA sequencing. Sequence of primers used for PCR and details about cloning strategies are available upon request.

**Recombinant Protein Purification**—Recombinant Hsp27 and His<sub>6</sub>-MK5 were produced as described (12). Other recombinant proteins were expressed in BL21 pLys(DE3) strain and purified as described (16). Protein purity and yield were evaluated by SDS-PAGE and Coomassie staining using BSA as standard.

**Cell Culture and Transfections**—HEK 293 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum plus antibiotics. Cells were transiently transfected by the calcium phosphate precipitation method.

**Purification of ERK3 Ser-189 Kinase**—Exponentially proliferating HEK 293 cells were lysed with a Dounce homogenizer in hypotonic buffer B (50 mM  $\beta$ -glycerophosphate (pH 7.3), 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol (DTT), 100

$\mu$ M sodium orthovanadate, 1  $\mu$ M pepstatin A, 1  $\mu$ M leupeptin, 100  $\mu$ M PMSF). The lysate was clarified by centrifugation, and 50 mg of protein was fractionated on a Mono Q 10/100 GL column (GE Healthcare) using an AKTA Explorer FPLC system (GE Healthcare). Fractions were collected and assayed for ERK3 Ser-189 kinase activity as described below. Fractions 43–53 were pooled and further fractionated on a gel filtration Superdex 16/60 200 column (GE Healthcare). Superdex fraction 36 containing high Ser-189 kinase activity was then incubated with 1  $\mu$ g of His<sub>6</sub>-ERK3(1–365)KA-GST recombinant protein bound on glutathione-agarose beads for 2 h at 4 °C. The beads were washed four times with buffer B, and bound proteins were further separated by SDS-PAGE. The gel was stained with Coomassie Blue, and seven bands were excised, reduced with DTT, and alkylated with iodoacetamide prior to trypsin digestion. Peptides were extracted three times with 90% acetonitrile/0.5 M urea. Combined extracts were dried and resuspended in 5% acetonitrile, 0.1% trifluoroacetic acid. Tryptic peptides were analyzed by nanoLC-MS/MS using an Eksigent nano2D system coupled to a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Peptides were separated on a 150- $\mu$ m  $\times$  10-cm C<sub>18</sub> analytical column using a gradient of 5–40% acetonitrile (in 0.2% formic acid) over 65 min. The mass spectrometer was operated in a data-dependent acquisition mode with a 1-s survey scan at 60,000 resolution, followed by three ion trap product ion spectra (MS/MS) of the most abundant precursors. The centroided data were merged into single peak-list files and searched with the Mascot search engine version 2.10 (Matrix Science) against the combined forward and reverse human IPI protein data base version 3.24 containing 133,842 protein sequences.

**Immunoblotting, Immunoprecipitation, and Kinase Assays**—Cell lysis, immunoprecipitation, and immunoblot analysis were performed as described (11, 18, 19). For immunoprecipitation experiments, 750  $\mu$ g of lysate proteins were incubated with the indicated antibodies for 2–4 h at 4 °C. For the detection of endogenous ERK3 phosphorylation on Ser-189, HEK 293 cell lysate (5 mg of protein) was incubated for 4 h at 4 °C with monoclonal anti-ERK3 antibody precoupled to protein A-agarose beads. Immunoprecipitated proteins were analyzed by immunoblotting with the indicated antibodies.

For *in vitro* kinase assay of Ser-189 phosphorylation, cell lysate (50  $\mu$ g of protein) or recombinant PAK2 (1  $\mu$ g) was incubated with 1  $\mu$ g of the indicated purified recombinant ERK3(1–365) protein in 50  $\mu$ l of buffer A (50 mM Tris-HCl (pH 7.5), 1.5 mM KCl, 25 mM MgCl<sub>2</sub>, 1 mM DTT, 200  $\mu$ M ATP) at 37 °C for 45 min. The reaction was stopped with Laemmli buffer, and Ser-189 phosphorylation was analyzed by immunoblotting with a phospho-ERK3(Ser-189)-specific antibody. For analysis of FPLC fractions, 8% of each fraction was assayed in a final volume of 100  $\mu$ l of buffer A. For radioactive assays, 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP was added to buffer A, and the reaction was analyzed by autoradiography with a FLA5000 PhosphorImager (Fuji).

For *in vivo* assay of ERK3/ERK4 activity, immunoprecipitated proteins were incubated with 0.5  $\mu$ g of recombinant

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His<sub>6</sub>-MK5 and Hsp27 in kinase assay buffer (25 mM Hepes (pH 7.5), 25 mM MgCl<sub>2</sub>, 1 mM DTT) with 50 μM ATP and 20 μCi of [ $\gamma$ -<sup>32</sup>P]ATP. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and analyzed by autoradiography. Expression of transfected proteins was analyzed by immunoblotting. The same conditions were used to monitor MK5 activity, except that FLAG-MK5 protein was immunoprecipitated from cells, and no recombinant MK5 was added to the assay.

**Mass Spectrometry Analysis of MK5 Phosphorylation**—For the *in vivo* quantitative analysis of MK5 Thr-182 phosphorylation, FLAG-MK5 was immunoprecipitated from HEK 293 cell lysates using anti-FLAG M2 agarose beads (Sigma). After elution in 0.1 M glycine (pH 3.5), proteins were dialyzed in 8 M urea, 50 mM NH<sub>4</sub>HCO<sub>3</sub>, 0.5 mM TCEP using 10-kDa cut-off microcon devices (Millipore) as published (20). Prior to digestion, the urea concentration was diluted to 1.5 M in 50 mM NH<sub>4</sub>HCO<sub>3</sub>, 0.5 mM TCEP. Proteins were digested with sequence-grade trypsin (Promega) for 3 h and the eluted peptides dried in a SpeedVac. Peptides were analyzed on an Orbitrap mass spectrometer using similar settings as above. Peptide intensities were extracted from raw data, clustered with Mascot results, and validated using the in-house software ProteoProfile. Specifically, for the quantification of MK5 Thr-182 phosphopeptides, we detected four isoforms by MS/MS with peptide MOWSE scores of 57–114: double- and triple-charged IDQGDLMP TPQFTPYVAPQVLEAQR (*m/z* 1480.705 and 987.4684, respectively); triple-charged form of the methionine-oxidized IDQGDLMP TPQFTPYVAPQVLEAQR (*m/z* 992.8018), and the low abundant, triple-charged missed cleavage form IDQGDLMP TPQFTPYVAPQVLEAQR (*m/z* 1044.84). We extracted ion intensities of the four detected peptide isoforms containing the phosphorylated Thr-182 site from three independent replicates. The average intensities of each peptide isoform and the corresponding standard deviations were calculated. The summed peptide intensity of the MK5 control experiment (without ERK4) was set to 1, and the relative increase in MK5 phosphorylation was calculated from the ratio of peptide intensities over that of the control condition.

## RESULTS

**Purification of ERK3 Activation Loop Kinase**—To purify the ERK3 activation loop kinase(s), we developed a robust *in vitro* assay to monitor Ser-189 kinase activity using recombinant catalytically inactive His<sub>6</sub>-ERK3(1–365)KA protein (produced in *Escherichia coli*) as substrate and a phospho-ERK3(Ser-189)-specific antibody for detection. This assay readily detects the phosphorylation of wild-type and kinase-dead ERK3, but not that of the S189A mutant from a HEK 293 cell lysate (Fig. 1A). The strategy used for the purification of ERK3 Ser-189 kinase activity is summarized in Fig. 1B. Extracts of proliferating HEK 293 cells were sequentially fractionated on anion exchange and gel filtration columns (Fig. 1, C and D). Then, fraction GF1 of the Superdex column was incubated with recombinant His<sub>6</sub>-ERK3(1–365)KA-GST immobilized on glutathione-agarose beads. After washing, bound proteins were further separated by SDS-PAGE (Fig. 1E). The gel was stained

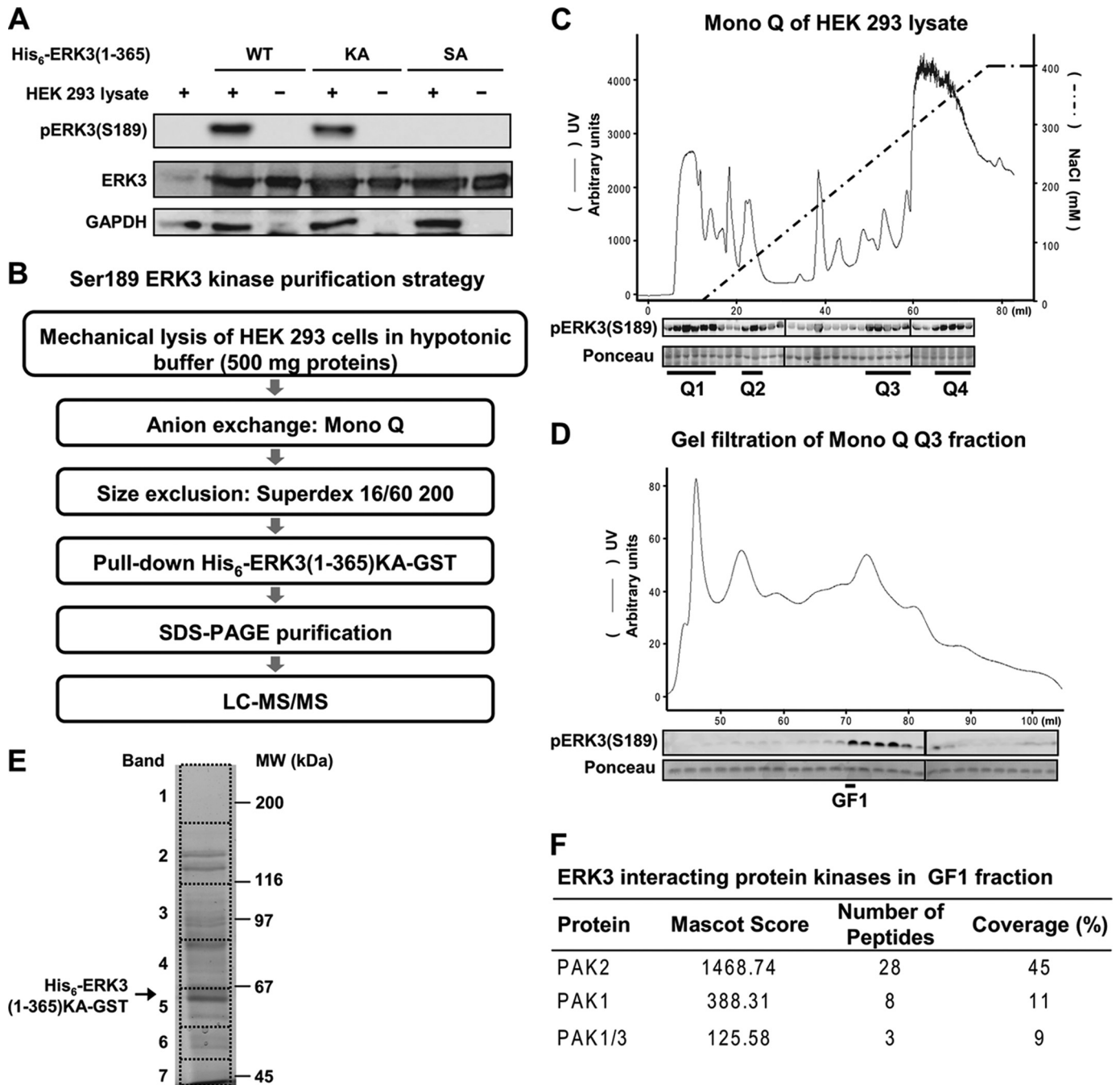
with Coomassie Blue and cut into seven bands that were subjected to in-gel digestion with trypsin and analyzed by LC-MS/MS. Among 142 proteins detected in the samples, two protein kinases were identified with a high Mascot score: PAK2 and PAK1 (Fig. 1F and supplemental Table S1). Three peptides shared by PAK1 and PAK3 were also identified from this analysis.

**Group I PAKs Phosphorylate ERK3 and ERK4 on Ser-189/Ser-186**—We first tested the ability of PAK2 to phosphorylate ERK3 on Ser-189 *in vitro* using purified recombinant PAK2 and full-length His<sub>6</sub>-ERK3KA protein as substrate. PAK2 wild type but not the catalytically inactive K279R/T402A (PAK2KD) mutant efficiently phosphorylated ERK3KA but not ERK3SA (Fig. 2A). Neither the S189A mutant of ERK3(1–365) nor the C-terminal extension of the kinase (residues 365–721) was phosphorylated by PAK2 *in vitro* (Fig. 2B), indicating that Ser-189 is the main PAK2 phosphorylation site. The *in vitro* phosphorylation of Ser-189 by PAK2 was further confirmed using the anti-phospho-ERK3(Ser-189) antibody for detection (Fig. 2C). Notably, PAK2 also phosphorylated the paralogous protein ERK4 on the activation loop residue Ser-186 (Fig. 2D).

To determine whether PAK2 phosphorylates ERK3 *in vivo*, we co-transfected HEK 293 cells with ERK3, PAK2, and an activated form of Rac1 (Rac1CA). Co-transfection of PAK2 increased the phosphorylation of ERK3 on Ser-189 (Fig. 2E, lane 3). The phosphorylation signal was further increased when activated Rac1 was transfected at the same time (Fig. 2E, lane 4). It should be noted that co-transfection of ERK3 with Rac1CA is sufficient to increase the Ser-189 phosphorylation signal compared with ERK3 alone (Fig. 2, E, lane 5, and G). This is likely due to the activation of endogenous PAK by Rac1. Similar results were obtained with the other group I PAK activator Cdc42 (Fig. 2G).

MS analysis identified both PAK2 and PAK1, and possibly PAK3, as candidate ERK3 Ser-189 kinases (Fig. 1F). We therefore wished to determine whether PAK1 and PAK3 are also able to phosphorylate ERK3 on Ser-189 in intact cells. Both PAK2 and PAK3 markedly increased the activation loop phosphorylation of ERK3, whereas PAK1 had a weaker albeit measurable effect (Fig. 2F). We conclude from these results that group I PAKs display ERK3/ERK4 activation loop kinase activity.

**Silencing of Group I PAKs Inhibits Rac1-Induced ERK3 Ser-189 Phosphorylation**—To address directly the contribution of endogenous group I PAKs to the activation loop phosphorylation of ERK3, we silenced the expression of PAK1, PAK2, and PAK3 by RNAi using SMARTpool siRNAs targeting each individual kinase. Silencing of a single PAK isoform partially reduced Rac1-stimulated ERK3 Ser-189 phosphorylation, with PAK2 and PAK3 siRNAs showing greater effects (Fig. 3, A and B). Importantly, a combination of siRNAs to all three group I PAKs completely abolished the effect of Rac1 on both ectopic and endogenous ERK3 phosphorylation (Figs. 3 and 4). These results confirm that group I PAKs are *bona fide* activation



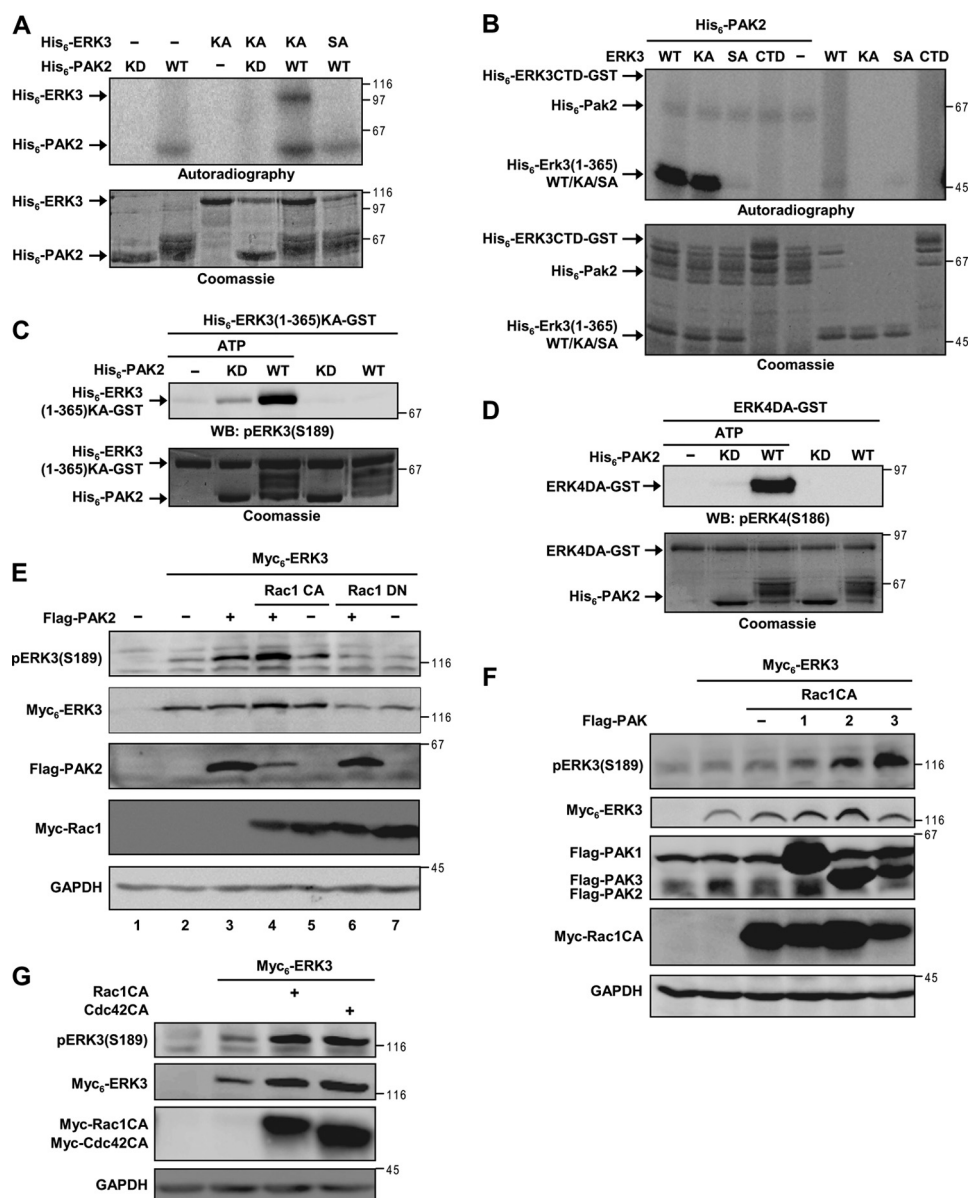
**FIGURE 1. Purification of ERK3 Ser-189 kinase.** *A*, ERK3 Ser-189 kinase assay. Purified recombinant His<sub>6</sub>-ERK3(1-365) wild type (WT), kinase-dead (KA), or nonphosphorylatable S189A (SA) mutant was incubated for 45 min at 30 °C with a lysate of HEK 293 cells. Ser-189 phosphorylation was analyzed by immunoblotting with anti-phospho-ERK3(Ser-189) and the indicated antibodies. *B*, strategy for the purification of ERK3 Ser-189 kinase. *C*, anion exchange chromatography. HEK 293 cell lysate was fractionated on a Mono Q column. Each individual fraction was assayed for Ser-189 kinase activity. *D*, fractions Q3 from the Mono Q column were fractionated by gel filtration chromatography and assayed as in *C*. *E* and *F*, fraction GF1 from the Sephadex column incubated with His-ERK3(1-365)KA-GST protein immobilized on glutathione-agarose beads. Bound proteins were further purified by SDS-PAGE and subjected to LC-MS/MS analysis. Protein kinases with the highest Mascot scores are presented in the table.

loop kinases for ERK3 and mediate the stimulatory effect of the small GTPases Rac1/Cdc42 on ERK3 phosphorylation.

*Group I PAKs Stimulate the Enzymatic Activity of ERK3 and ERK4*—We wanted to verify whether PAK-induced phosphorylation of ERK3 or ERK4 leads to their enzymatic activation. HEK 293 cells were co-transfected with ERK3/ERK4 and Rac1CA, in the absence or presence of PAK2 or a mixture of siRNAs targeting PAK1/PAK2/PAK3. Ectopi-

cally expressed ERK3 or ERK4 was immunoprecipitated, and its phosphotransferase activity was measured in a coupled kinase assay using recombinant His<sub>6</sub>-MK5 and its substrate Hsp27 in the presence of [ $\gamma$ -<sup>32</sup>P]ATP. Expression of activated Rac1 clearly induced the catalytic activation of ERK3 and ERK4 as demonstrated by the increased phosphorylation of Hsp27 (Fig. 5, *A* and *B*, lane 3). This activation was significantly attenuated in cells transfected with

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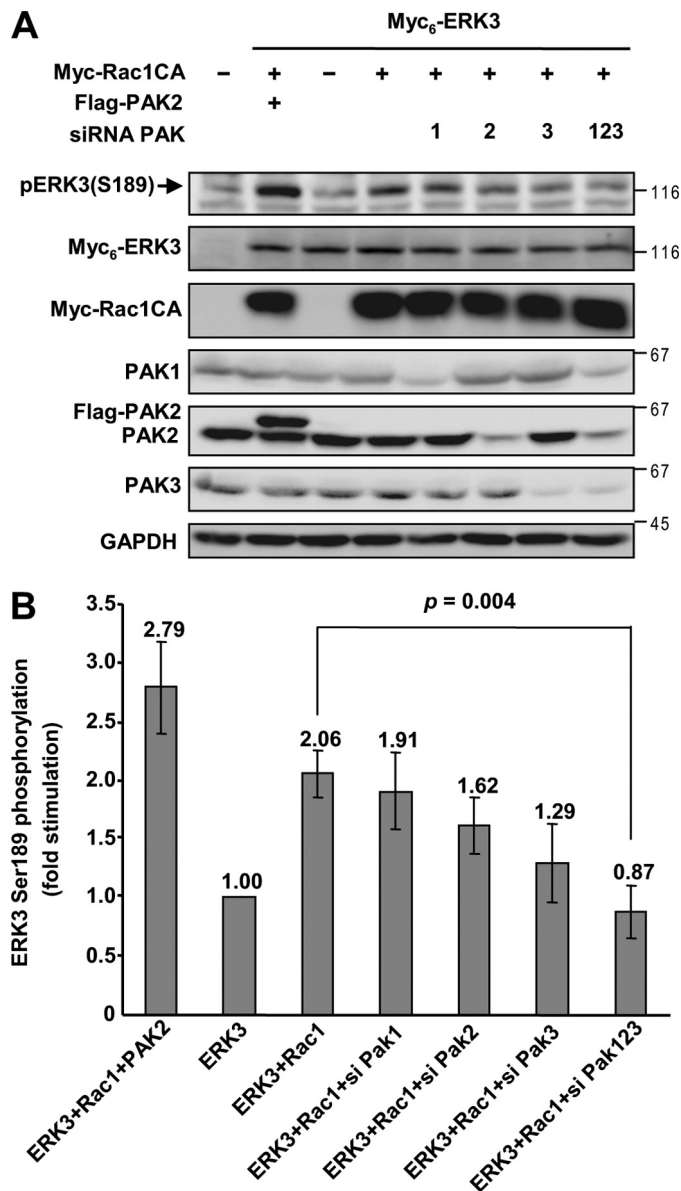


**FIGURE 2. Group I PAKs phosphorylate ERK3 and ERK4 in the activation loop *in vitro* and *in vivo*.** *A*, purified recombinant catalytically inactive His<sub>6</sub>-ERK3KA or His<sub>6</sub>-ERK3SA was incubated in kinase assay buffer with recombinant wild-type (WT) or kinase-dead (KD) His<sub>6</sub>-PAK2 and [ $\gamma$ -<sup>32</sup>P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. The Coomassie-stained gel is shown at the bottom. *B*, recombinant wild type His<sub>6</sub>-PAK2 was incubated with His<sub>6</sub>-ERK3(1–365) WT, KA, or SA mutant, or with His<sub>6</sub>-ERK3(365–721) C-terminal domain (CTD) GST fusion in the presence of [ $\gamma$ -<sup>32</sup>P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. *C* and *D*, panels are the same as in *A* except that [ $\gamma$ -<sup>32</sup>P]ATP was omitted from the reaction. The phosphorylation of recombinant ERK3 and ERK4 fusion proteins was analyzed by immunoblotting (WB) using a phospho-ERK3(Ser-189)/phospho-ERK4(Ser-186) antibody. A duplicate gel was stained with Coomassie Blue to control for protein loading. *E–G*, HEK 293 cells were transfected with the indicated constructs. After 36 h, the cells were lysed and analyzed by immunoblotting with anti-phospho-ERK3(Ser-189) and the indicated antibodies.

siRNAs to group I PAKs. These data indicate that PAK-mediated phosphorylation of ERK3 and ERK4 leads to their catalytic activation *in vivo*.

**Activation of ERK3/ERK4 by PAK3 Results in Downstream Activation of MK5 *In Vivo***—We next wished to determine whether the activation of ERK3/ERK4 by Rac1-PAK signaling translates into enzymatic activation of the downstream substrate MK5 *in vivo*. Because all of the anti-phospho-MK5(Thr-182) antibodies tested were found unsatisfactory, we analyzed the extent of MK5 Thr-182 (activation loop) phosphorylation by quantitative MS. HEK 293

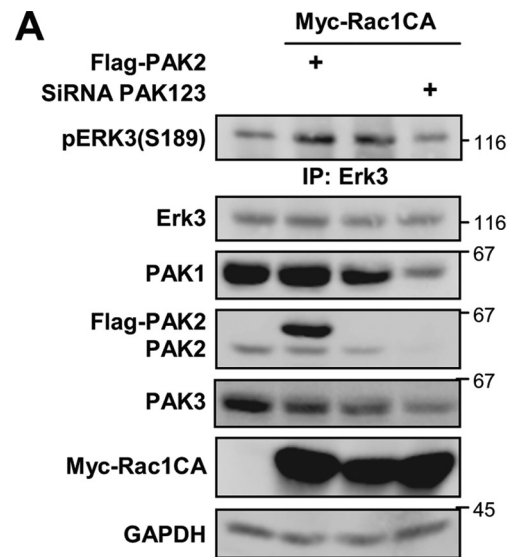
cells were transfected with FLAG-MK5 in the absence or presence of ERK4, activated Rac1, and PAK3. MK5 was immunoprecipitated with anti-FLAG M2 beads, eluted, digested with trypsin, and subjected to quantitative LC-MS/MS analysis. Activating Thr-182 phosphorylation of MK5 was increased by 26-fold following ERK4 overexpression and by 46-fold when ERK4 was co-expressed with activated Rac1 and PAK3 (Fig. 6A and supplemental Fig. S1). A comparison of the intensity of nonphosphorylated and phosphorylated peptide species around MK5 Thr-182 indicated that MK5 is phosphorylated at high stoi-



**FIGURE 3. Requirement of group I PAKs for Rac1-induced activation loop phosphorylation of ERK3.** *A*, HEK 293 cells were transfected with the indicated constructs in combination or not with SMARTpool siRNAs targeting group I PAKs. After 36 h, the cells were lysed and analyzed by immunoblotting with anti-phospho-ERK3(Ser-189) and the indicated antibodies. *B*, the ratio of Ser(P)-189 ERK3 to total ERK3 was determined by quantifying the anti-phospho-ERK3(Ser-189) and anti-Myc immunoblots by densitometry. The *bar graph* represents the mean  $\pm$  S.E. (error bars) of five independent experiments as shown in *A*.

chometry upon activation of the Rac1-PAK-ERK3/4 pathway.

To confirm that increased Thr-182 phosphorylation results in MK5 catalytic activation *in vivo*, the phosphotransferase activity of MK5 was measured by immune-complex kinase assay using recombinant Hsp27 as substrate. Expression of ERK3 or ERK4 augmented MK5 activity, and this activation was further potentiated by co-expression of Rac1CA and PAK3 (Fig. 6, *B* and *C*). These results confirm that activation of the Rac1-PAK-ERK3/4 signaling pathway leads to downstream activation of MK5 in cells.

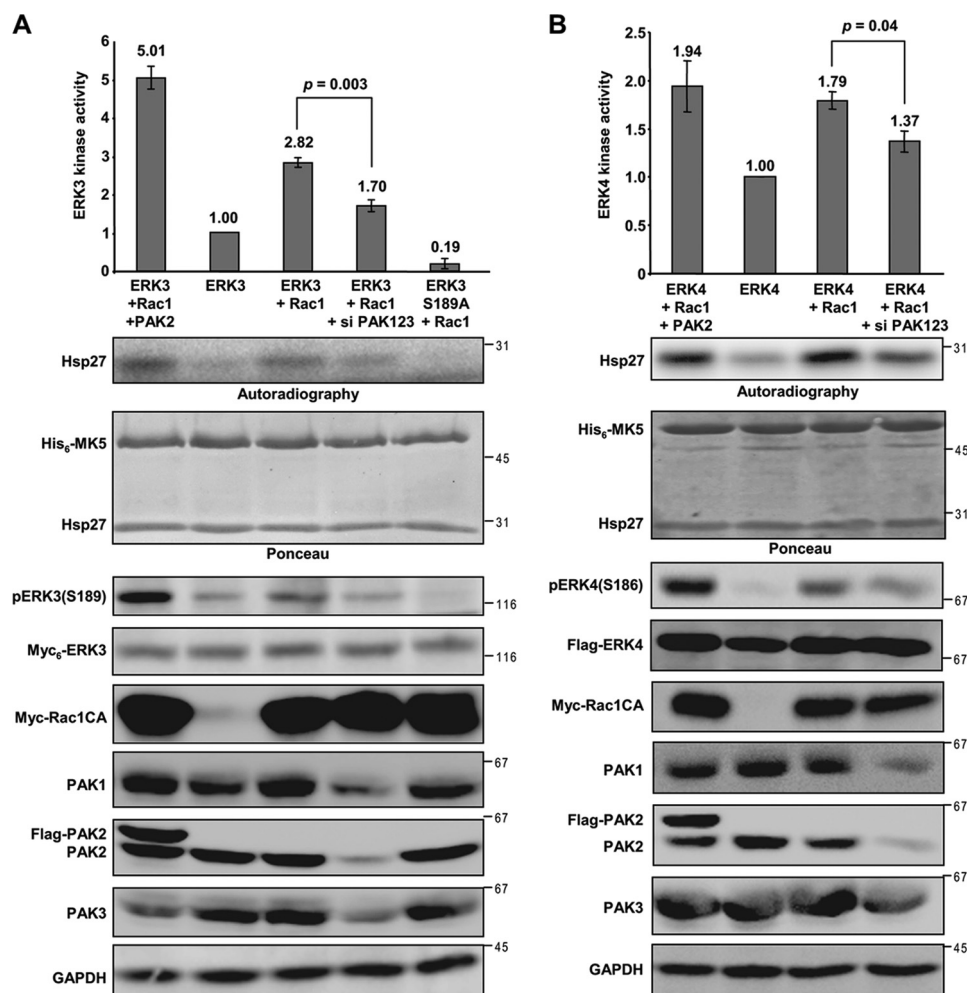


**FIGURE 4. Group I PAKs phosphorylate endogenous ERK3.** *A*, HEK 293 cells were transfected with the indicated constructs in combination or not with SMARTpool siRNAs targeting group I PAKs. After 36 h, ERK3 was immunoprecipitated from cell extracts and analyzed by immunoblotting with anti-phospho-ERK3(Ser-189) and the indicated antibodies. *B*, ratio of Ser(P)-189 ERK3 to total ERK3 was determined by quantifying the anti-phospho-ERK3(Ser-189) and anti-ERK3 immunoblots by densitometry. The *bar graph* represents the mean  $\pm$  S.E. (error bars) of three independent experiments as shown in *A*.

## DISCUSSION

A full understanding of the physiological functions of the atypical MAP kinases ERK3 and ERK4 will be greatly facilitated by the characterization of the upstream regulatory signals and pathways that control their activity. ERK3 and ERK4 are not components of classical MAP kinase modules, and their activator(s) has remained elusive so far. Of note, Cobb and colleagues have previously reported the partial characterization of a protein kinase immunologically distinct from MEK1 and MEK2 that phosphorylates ERK3 on Ser-189 (6). Here, we report the successful purifi-

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**FIGURE 5. PAKs stimulate the phosphotransferase activity of ERK3 and ERK4.** HEK 293 cells were transfected with the indicated constructs in combination or not with SMARTpool siRNAs to PAK1/2/3. After 36 h, the cells were lysed, and Myc<sub>6</sub>-ERK3 WT or SA (A) or FLAG-ERK4 (B) was immunoprecipitated with anti-Myc or anti-FLAG-antibody, respectively. Phosphotransferase activity was measured in a coupled kinase assay by incubating immunoprecipitated ERK3 or ERK4 with recombinant His<sub>6</sub>-MK5 and Hsp27 proteins in the presence of [ $\gamma$ -<sup>32</sup>P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography (upper panel). Ponceau staining was used to control for equal His<sub>6</sub>-MK5 and Hsp27 protein loading. The activation loop phosphorylation of ERK3/ERK4 and the expression of ERK3/ERK4, Rac1CA, and PAKs were analyzed by immunoblotting of cell lysates. The bar graph represents the mean  $\pm$  S.E. (error bars) of three independent experiments.

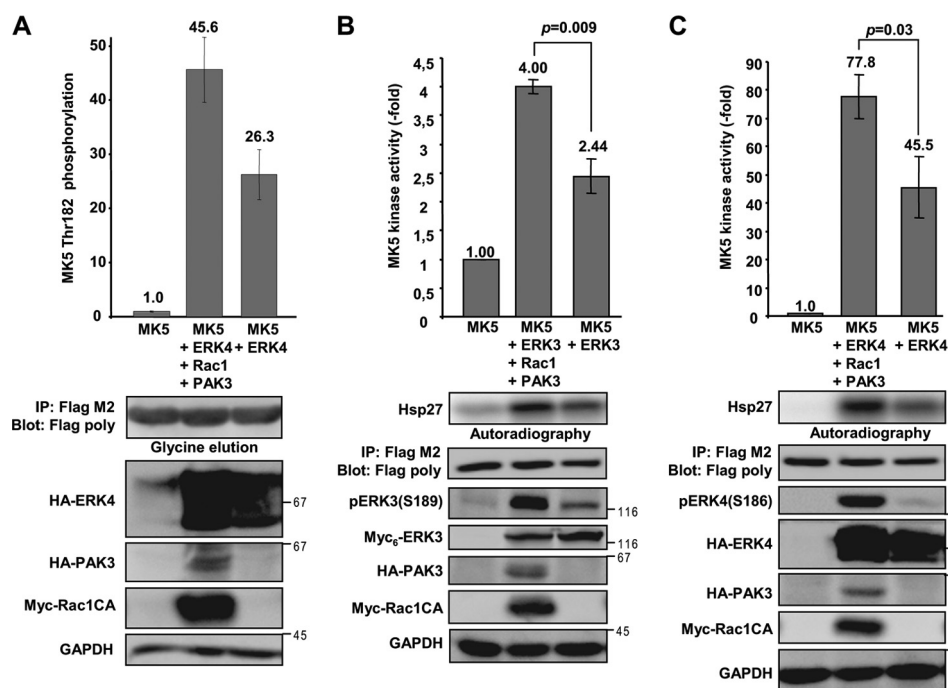
cation and identification of group I PAKs as ERK3 activation loop kinases. The PAK family is composed of six members divided in two subgroups of three members each based on their mechanism of regulation (21). All six members share a similar architecture with an N-terminal regulatory domain containing the Rac1/Cdc42 binding site and a C-terminal kinase domain. Group I PAKs include PAK1, PAK2, and PAK3, which are enzymatically activated by binding of Rac1 and Cdc42. PAKs belong to the STE (homologs of yeast Ste7, Ste11, Ste20 kinases) group of protein kinases, which also includes MAP kinase kinases.

We have used a three-step chromatographic procedure including an affinity step on ERK3(1–365)KA-agarose beads to isolate the ERK3 Ser-189 kinase. The affinity step was inspired by the observation that a candidate ERK3 Ser-189 kinase binds tightly to the catalytic domain of ERK3 (6). ERK3-binding proteins were further separated by SDS-PAGE and analyzed by LC-MS/MS, leading to the identification of PAK1 (and possibly PAK3) and PAK2. Overexpression and loss-of-function experiments confirmed that

PAK1, PAK2, and PAK3 efficiently phosphorylate ectopic and endogenous ERK3 on Ser-189 and that the three protein kinases are responsible for all the Ser-189 kinase activity induced by activated Rac1 in cells. We conclude from these results that group I PAKs are *bona fide* ERK3 activation loop kinases. However, we cannot exclude the possibility that other kinases can phosphorylate ERK3 on Ser-189 under different cellular conditions.

All ERK3 activation loop (from Leu-174 to Trp-216) amino acids but three are conserved in human ERK4 sequence. These residues are also highly conserved from zebrafish to human (12). In agreement with this observation, we found that group I PAKs similarly phosphorylate ERK4 on activation loop residue Ser-186, indicating a common mechanism of activation of ERK3 and ERK4 MAP kinases.

Rennefahrt *et al.* have used a degenerate peptide library to analyze the optimal phosphorylation sequence of group I and II PAKs (22). According to this work, the ERK3/ERK4 activation loop contains several favorable residues for PAK1 and PAK2 phosphorylation (Fig. 7). Notably, these



**FIGURE 6. Rac1-PAK signaling induces MK5 phosphorylation and activation.** HEK 293 cells were transfected with the indicated constructs. After 36 h, the cells were lysed, and FLAG-MK5 was immunoprecipitated (IP) with anti-FLAG M2 antibody coupled to agarose beads. **A**, following elution, proteins were trypsin-digested, and the relative abundance of MK5 Thr-182 phosphorylation was analyzed by LC-MS/MS. MK5 elution and expression of the transfected constructs were monitored by immunoblot analysis (Blot) with the indicated antibodies. The bar graph represents the mean  $\pm$  S.E. (error bars) of two independent experiments. **B** and **C**, MK5 kinase activity was assayed by incubating immunoprecipitated MK5 with recombinant MK5 with radiolabeled substrate in the presence of [ $\gamma$ - $^{32}$ P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography (upper panel). The Coomassie-stained gel is shown at the bottom. The phosphorylation of ERK3/ERK4 and the expression of transfected proteins were analyzed by immunoblotting. The bar graph represents the mean  $\pm$  S.E. (error bars) of three independent experiments.

**ERK3 activation loop sequence :** HKGHLSEGLV  
**PAK1 :** 5-531S2-4-  
**PAK2 :** 62625S3-5-

**FIGURE 7. Matching of ERK3 activation loop sequence to PAK1 and PAK2 consensus phosphorylation motifs.** The sequence of ERK3 activation loop was matched to the position-specific scoring matrices documented by Rennfahrt *et al.* (16) for optimal *in vitro* phosphorylation by PAK1 and PAK2. Green color corresponds to residues favoring phosphorylation, whereas red color indicates unfavorable residues. Numbers refer to the order of preference.

kinases clearly favor serine residues over threonine as substrates (22). It is noteworthy that the ERK3/ERK4 sequence appears to fit more closely the PAK2 consensus motif than PAK1, consistent with our observation that PAK2 is apparently a better activation loop kinase than PAK1.

Importantly, we have demonstrated that PAK-mediated phosphorylation of ERK3/ERK4 leads to their enzymatic activation and to downstream activation of MK5 *in vivo*. Group I PAKs have been implicated in many cellular processes, including transcription, apoptosis, cell proliferation, and cell motility (23–26). Specifically, multiple studies have documented the important role of PAKs in the regulation of actin dynamics and cytoskeletal remodeling (27, 28). In the past years, MK5 has also emerged as a regulator of F-actin polymerization and cell migration (29–31). In this study, we uncover a hitherto unrecognized link between Rac/Cdc42-PAK signaling and ERK3/4-MK5 activation, potentially explaining in part the common effect of group I PAKs and MK5 on the actin cytoskeleton. Future

studies will investigate how this new signaling branch of PAK signaling contributes to physiological responses such as cell motility, apoptosis, and senescence that can eventually impact cancer progression.

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