Supporting Information

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Methods

Antibodies. Antibodies to MEK1/2 and C-Raf were from BD Biosciences; antibodies to ERK1/2 and B-Raf were from Santa Cruz Biotechnology; antibodies to phosphotyrosine (pY), phospho-MEK, phospho-ERK, and phospho-MAPK substrates (pS/TP) were from Cell Signaling Technology; antibody recognizing the Flag M2 epitope was from Sigma-Aldrich; and antibody to the HA.11 epitope was from Covance. Antibodies to the Pyo epitope and the N terminus of KSR1 have been previously described (1).

DNA Constructs. Constructs encoding Flag-tagged MP1 and HA-WT-MEK1, ΔPRS-MEK1 (deletes residues 270–307), and WT-MEK2 (2, 3) were the gift of Dr. Michael Weber (University of Virginia). Flag-tagged and Pyo-tagged C-Raf, B-Raf, and KSR1 pcDNA3 constructs have been described previously (1, 4–6) or were generated by PCR amplification and subcloning. pBabe Pyo-tagged WT- and C′-KSR1 (residues 530–873) are described in ref.7. Sequences encoding Pyo-tagged N′-KSR1 (residues 1–529) or Flag-tagged B-Raf were subcloned into the pBabe retroviral vector. Point mutations were generated by site-directed mutagenesis and confirmed by DNA sequencing.

Metabolic Labeling and Phosphorylation Site Mapping. WT-KSR1 MEFs were incubated for 4–6 h at 37 °C in phosphate-free DMEM containing 2.5% dialyzed calf serum and [^{32}P]orthophosphate (1 mCi/mL media). Cells were stimulated with the appropriate agent and washed in Tris-buffered saline (TBS) [20 mM Tris (pH 7.4), 137 mM NaCl] before lysis in Nonidet P-40 lysis buffer. Labeled Pyo-tagged WT-KSR1 or endogenous B-Raf proteins were immunoprecipitated from cell lysates, separated by SDS/PAGE, eluted from the gel matrix, digested with trypsin, and analyzed by reverse-phase HPLC, phosphoamino acid analysis, Edman degradation, and mass spectrometry as previously described (8, 9).

In Vitro Kinase Assays. In experiments where KSR1 and B-Raf were used as in vitro substrates for ERK, FLAG-tagged kinase-dead (KD)-B-Raf or Pyo-tagged WT-KSR1 were affinity purified from serum-starved HeLa cells that had been lysed in RIPA buffer [20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate 0.15 U/mL aprotinin, 1 mM PMSF, 20 μM leupeptin, 5 mM sodium vanadate, 0.1 μM calyculin]. Purified KD-B-Raf or WT-KSR1 was then incubated in 40 μL kinase buffer [30 mM Hepes (pH 7.4), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM DTT, 1 mM $NaVO_4$, 15 mM ATP, 10 mM $MgCl_2$] containing 20 μCi [γ-^{32}P]ATP and recombinant activated ERK. After incubation for 30 min at 25 °C, the samples were resolved by SDS/PAGE and processed for phosphorylation site mapping. To determine MEK kinase activity, HA-tagged MEK proteins were immunoprecipitated from cells lysed in Nonidet P-40 lysis buffer. The MEK immunoprecipitates were washed extensively with Nonidet P-40 lysis buffer and incubated in 40 μL kinase buffer containing 20 μCi [γ-^{32}P]ATP and 0.1 mg of purified kinase-inactive ERK. After incubation for 30 min at 25 °C, the samples were resolved by SDS/PAGE and transferred to nitrocellulose and visualized by autoradiography.

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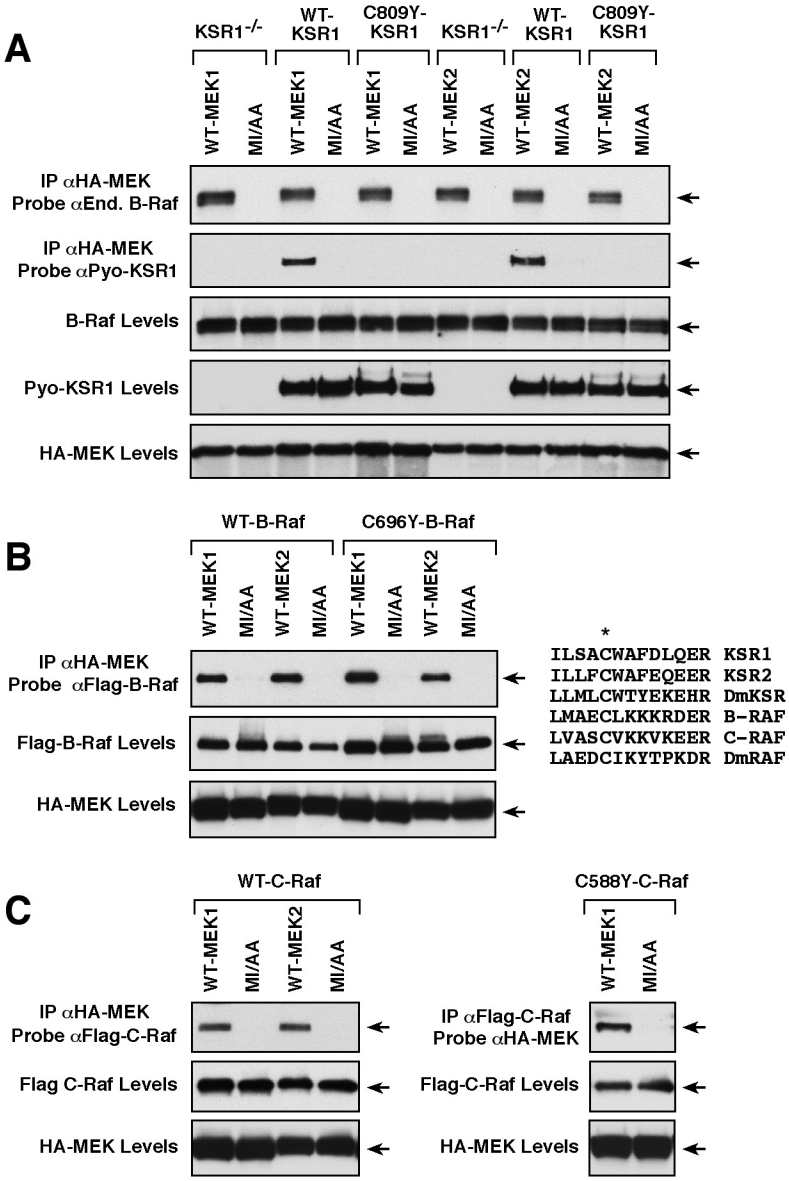

Fig. S1. MAI motif of MEK1/2 contributes to Raf binding. (*A*) HA-WT- or MI/AA-MEK proteins were expressed in KSR1$^{-/-}$, WT-, or C809Y-KSR1 MEFs. MEK1 proteins were immunoprecipitated and examined for KSR1 or endogenous B-Raf binding by immunoblot analysis. (*B*) HeLa cells were cotransfected with constructs encoding Flag-tagged WT- or C696Y-B-Raf and HA-tagged WT- or MI/AA-MEK proteins, and the MEK/B-Raf interaction was examined. Sequence alignment indicates that C809 of KSR1 is conserved in KSR and Raf proteins. (*C*) HeLa cells were cotransfected with constructs encoding Flag-tagged WT-or C588Y-C-Raf and HA-tagged WT- or MI/AA-MEK proteins, and the MEK/C-Raf interaction was examined. MI/AA: M308A, I310A in MEK1 and M316A, I318A in MEK2.

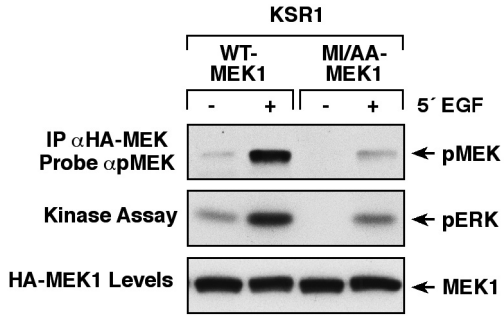

Fig. S2. The reduced in vitro kinase activity of MI/AA-MEK correlates with its activation state. Serum-starved WT-KSR1 MEFs expressing HA-tagged WT- or MI/AA-MEK1 were treated as indicated with EGF. HA-MEK1 proteins were immunoprecipitated and examined for their ability to phosphorylate inactive ERK in immune complex kinase assays and for their phosphorylation on activating sites using pMEK antibodies.

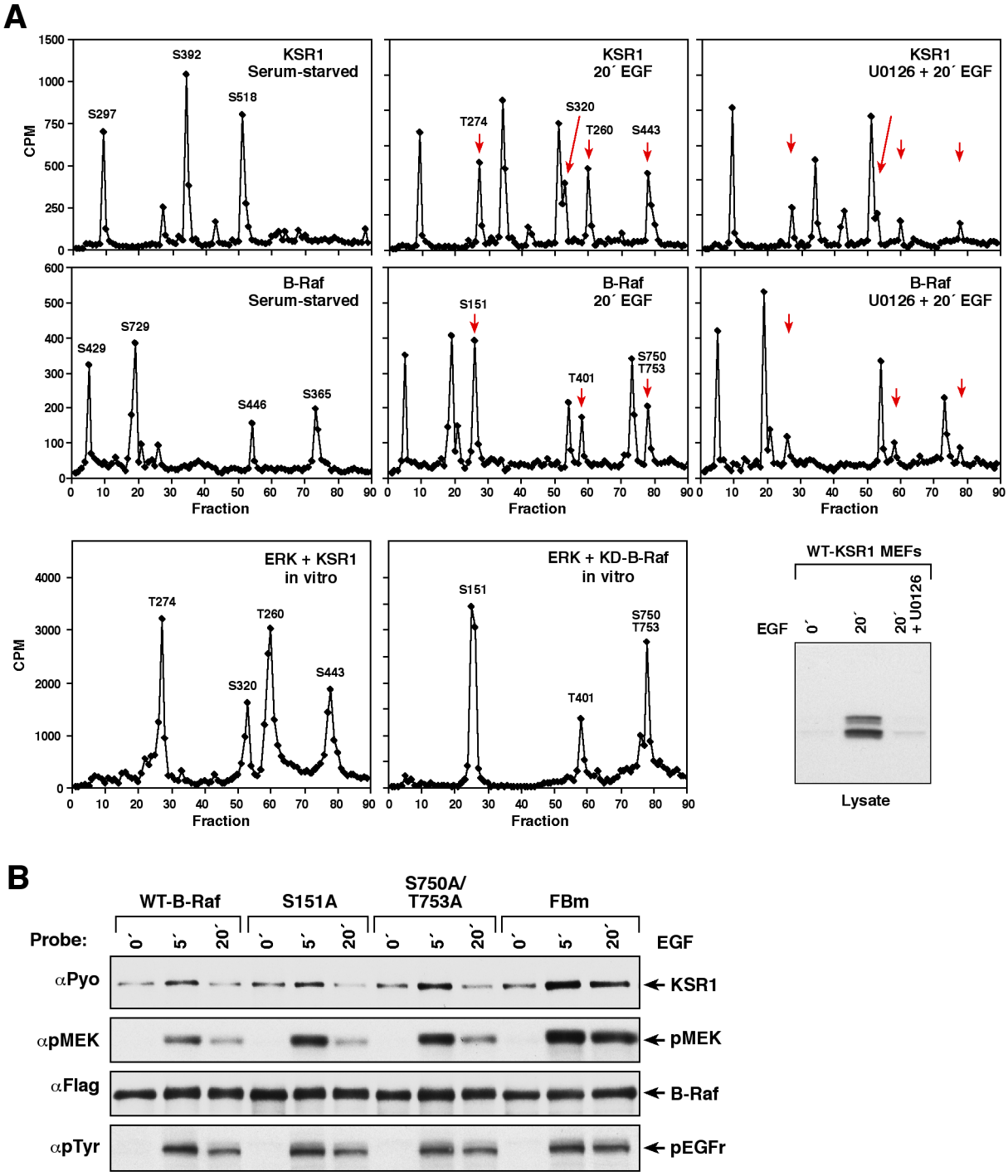

Fig. S3. Identification of ERK-dependent feedback phosphorylation sites on KSR1 and B-Raf. (*A*) For in vivo phosphorylation studies, serum-starved WT-KSR1 MEFs were metabolically labeled with [^{32}P]orthophosphate. Cells were left untreated or were stimulated with EGF for 20 min in the presence or absence of U0126 before lysis. Labeled Pyo-WT-KSR1 and endogenous B-Raf proteins were immunoprecipitated from cell lysates and isolated after SDS/PAGE. The labeled proteins were then digested with trypsin and the tryptic phosphopeptides were separated by HPLC. Shown are the profiles of radioactivity collected in the HPLC fractions. Phosphorylated residues are indicated. Total lysates were probed for pERK levels to confirm that U0126 treatment blocked ERK activation. For in vitro phosphorylation assays, purified WT-KSR1 and kinase-inactive B-Raf (KD-B-Raf) were incubated with activated ERK2 in the presence of γ[^{32}P]ATP. Labeled WT-KSR1 and B-Raf proteins were isolated and analyzed as described above. (*B*) WT-KSR1 MEFs infected with retroviruses encoding the indicated Flag-tagged B-Raf proteins were serum starved and then treated as indicated with EGF. Flag-B-Raf proteins were immunoprecipitated and binding of Pyo-KSR1 was determined by immunoblot analysis. Lysates were also examined for activated pMEK and tyrosine phosphorylated pEGFr.

Table S1. Analysis of KSR1 proteins

KSR1 protein	Growth factor (5′ EGF)	B-Raf binding	MEK binding	pERK binding	14–3–3 binding	Plasma membrane localization
WT	–	+(1)	+++ (1)	–	++ (1)	–
	+	++ (3.5)	+++ (0.95)	++ (1)	++ (0.98)	+
L56G/R57G	–	–	+++ (1.1)	–	++ (1.02)	–
	+	–	+++ (1.3)	+ (0.3)	++ (1.01)	+
C809Y	–	–	–	–	++ (0.95)	–
	+	–	–	–	++ (0.98)	+
AxAP	–	++ (4.2)	+++ (1.1)	–	++ (1.0)	–
	+	++++ (7.6)	+++ (1.15)	–	++ (1.03)	++
N′	–	–	–	–	++ (1.2)	–
	+	–	–	+ (0.4)	++ (1.17)	+
C′	–	–	+++ (0.7)	–	–	–
	+	–	+++ (0.8)	–	–	–

KSR1$^{-/-}$ MEFs stably expressing the indicated KSR1 proteins were evaluated for binding interactions. Binding data is compiled from 3 independent experiments. –, no binding; +, binding. The localization of the KSR1 proteins was determined by immunofluorescent staining. –, cytoplasmic; +, translocates to plasma membrane. Binding of the KSR1 proteins to B-Raf, MEK, and 14–3–3 were quantitated by densitometry and values indicated in parentheses are binding levels relative to WT-KSR1 isolated from serum-starved cells, which was arbitrarily given a value of 1. Binding of activated phospho-ERK to the KSR1 proteins was quantitated by densitometry and values indicated in parentheses are binding levels relative to WT-KSR1 isolated from EGF-treated cells, which was arbitrarily given a value of 1.