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# Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP

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**Raf-1 phosphorylates and activates MEK-1, a kinase that activates the extracellular signal regulated kinases (ERK). This kinase cascade controls the proliferation and differentiation of different cell types[1,2]. Here we describe a Raf-1-interacting protein, isolated using a yeast two-hybrid screen. This protein inhibits the phosphorylation and activation of MEK by Raf-1 and is designated RKIP (Raf kinase inhibitor protein). *In vitro*, RKIP binds to Raf-1, MEK and ERK, but not to Ras. RKIP co-immunoprecipitates with Raf-1 and MEK from cell lysates and colocalizes with Raf-1 when examined by confocal microscopy. RKIP is not a substrate for Raf-1 or MEK, but competitively disrupts the interaction between these kinases. RKIP overexpression interferes with the activation of MEK and ERK, induction of AP-1-dependent reporter genes and transformation elicited by an oncogenically activated Raf-1 kinase. Downregulation of endogenous RKIP by expression of antisense RNA or antibody microinjection induces the activation of MEK-, ERK- and AP-1-dependent transcription. RKIP represents a new class of protein-kinase-inhibitor protein that regulates the activity of the Raf/MEK/ERK module.**

In metazoans the Ras/Raf-1/MEK/ERK module is a ubiquitously expressed signalling pathway that conveys mitogenic and differentiation signals from the cell membrane to the nucleus[1]. This kinase cascade appears to be spatially organized in a signalling complex that is nucleated by Ras proteins[3]. The regulation of the Ras/Raf-1/MEK/ERK module is complex and may include associations with scaffolding and regulatory proteins[4]. To isolate such proteins we used the Raf-1 kinase domain, BXB[5], as bait in a yeast two-hybrid screen[6]. Screening 500,000 clones of a human T-cell library yielded nine clones that specifically interacted with BXB. Five clones corresponded to 14-3-3 proteins. One clone, RKIP, bound to both kinase-active and kinase-negative BXB, but not to control baits (Fig. 1a). Partial sequencing of the RKIP complementary DNA predicted a protein identical to the phosphatidylethanolamine

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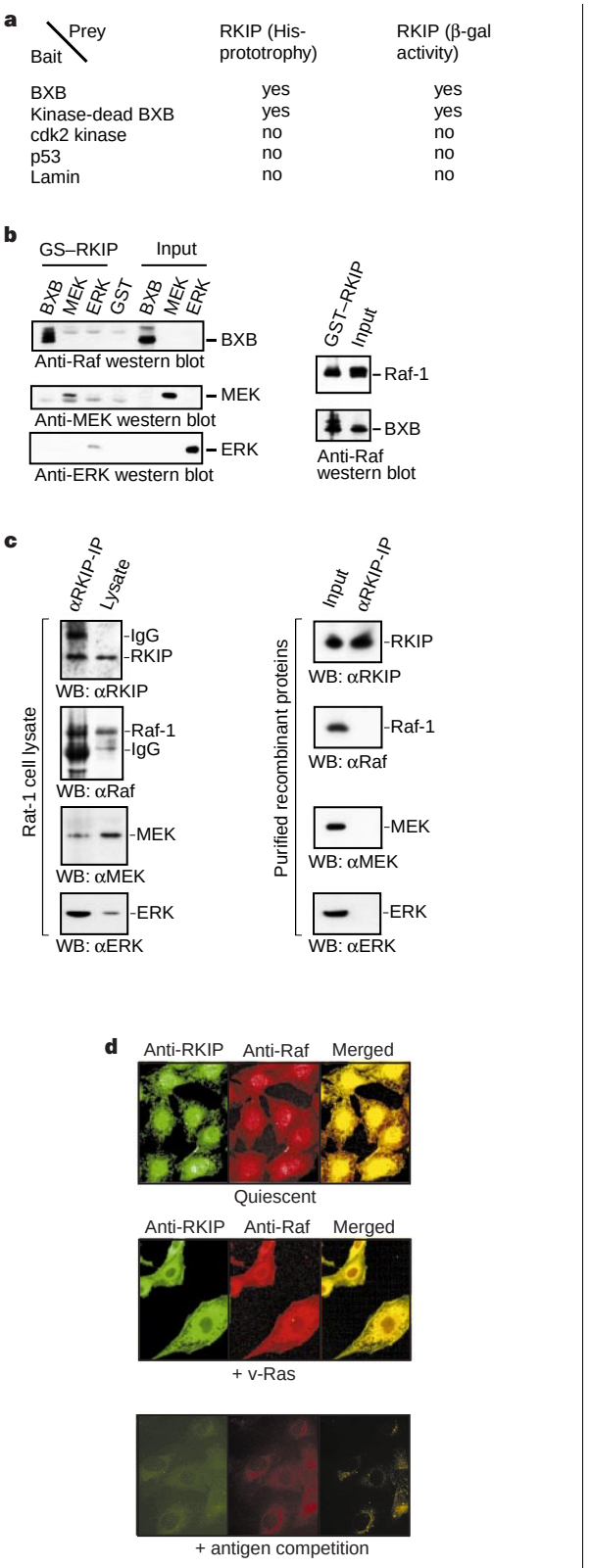


**Figure 1** *In vitro* interaction of RKIP with components of the ERK pathway. **a**, RKIP interacts with BXB but not with control baits in the yeast two-hybrid system. **b**, Binding of recombinant BXB, full-length Raf-1, MEK-1 and ERK-2 to GST–RKIP beads. Input: 1% of the respective proteins used in binding reactions; GST: GST-beads. **c**, Co-immunoprecipitation of Raf-1, MEK and ERK with RKIP in Rat-1 cells. The RKIP antiserum does not precipitate recombinant Raf-1, MEK-1 and ERK-2 proteins individually. **d**, Colocalization of Raf-1 and RKIP in 208F fibroblasts by confocal microscopy. Antigen competition: antisera were pre-absorbed with their cognate antigens.

binding proteins (PEBP) with relative molecular mass 23,000 ($M_r = 23K$) from humans and monkeys. These proteins are widely expressed and evolutionarily conserved, but their functions remain obscure[7].

Purified recombinant RKIP was tested for binding to the components of the Ras/Raf-1/MEK/ERK cascade. RKIP associated with BXB, full-length Raf-1, MEK-1 and (more weakly) with ERK-2, but not with Ras. RKIP binding was independent of Raf-1 kinase activity, not affected by phosphatidylethanolamine, and direct, as shown by the interaction of purified proteins produced in *Escherichia coli* (Fig. 1b and data not shown).

These interactions were also shown between endogenous mammalian proteins. An RKIP antiserum co-immunoprecipitated Raf-1, MEK and ERK from Rat-1 cells. This was not due to cross-reactivity, because the RKIP antiserum failed to immunoprecipitate purified Raf-1, MEK-1 or ERK-2 individually (Fig. 1c). These interactions were also observed in reciprocal immunoprecipitations with antisera to Raf-1, MEK or ERK (data not shown). Confocal microscopy revealed extensive colocalization between Raf-1 and RKIP, in both quiescent and Ras-transformed cells (Fig. 1d).

To investigate the relevance of the interaction between RKIP and the kinases of the Raf/MEK/ERK module in mammalian cells, we inhibited endogenous RKIP by antibody microinjection or expression of antisense RNA. As the AP-1 transcription factor is a major target of Raf signalling[8–10], we tested the influence of RKIP on AP-1 activity (Fig. 2a). Microinjection of affinity-purified anti-RKIP antibodies robustly activated a co-injected AP-1-dependent reporter gene in serum-deprived Rat-1 fibroblasts (Fig. 2a). This effect was highly specific, because the injection of control immunoglobulin (IgG) was ineffective, anti-RKIP IgG did not affect the expression of a cAMP-dependent reporter gene, and co-injection of an RKIP expression vector abolished AP-1 induction by anti-RKIP IgG. We also downregulated RKIP expression using an RKIP antisense vector, pAS-C143. This vector markedly reduced RKIP levels without affecting the expression of MEK-1 or actin (Fig. 2b). pAS-C143 substantially induced the AP-1 reporter gene in serum-starved NIH 3T3 cells (Fig. 2c). These data confirm the microinjection results and show that RKIP suppresses the Raf/MEK/ERK pathway.

Overexpression experiments further confirmed this conclusion. RKIP transfection diminished basal as well as BXB-induced AP-1 activity (Fig. 3a), and microinjection of an RKIP expression vector impaired AP-1 induction by BXB (Fig. 3b). Notably, RKIP did not interfere with AP-1 stimulation by ERK-1. Next, we tested the effects of RKIP overexpression in transformation assays. In contrast to transient reporter-gene assays, transformation assays accommodate the complexity of cellular responses to the chronic deregulation of a single signalling component. RKIP significantly reduced the transformation efficiency of BXB in three distinct assays: morphological transformation, focus formation and anchorage-independent growth (Fig. 3c). RKIP also decreased the total colony yield, albeit to a lesser extent than transformation, showing that RKIP interferes with Raf-mediated proliferation as well as transformation. In contrast, RKIP impaired the induction of foci by *v-fos* or mutationally activated MEK alleles only to a small extent, and failed to inhibit *v-src* transformation (Fig. 3d), indicating that RKIP may specifically block transformation by the Raf/MEK/ERK pathway, primarily by inhibiting Raf.

To investigate the effects of RKIP on individual activation steps, we reconstructed the Raf/MEK/ERK cascade *in vitro* using recombinant proteins (Fig. 4a). RKIP decreased the phosphorylation of MEK by Raf-1, but did not inhibit ERK phosphorylation by MEK or ELK phosphorylation by ERK. In addition, RKIP (1) failed to inhibit MEK-DD, a constitutively active mutant of MEK[11], or MEK activated by TPA treatment of cells (Fig. 4b); (2) did not

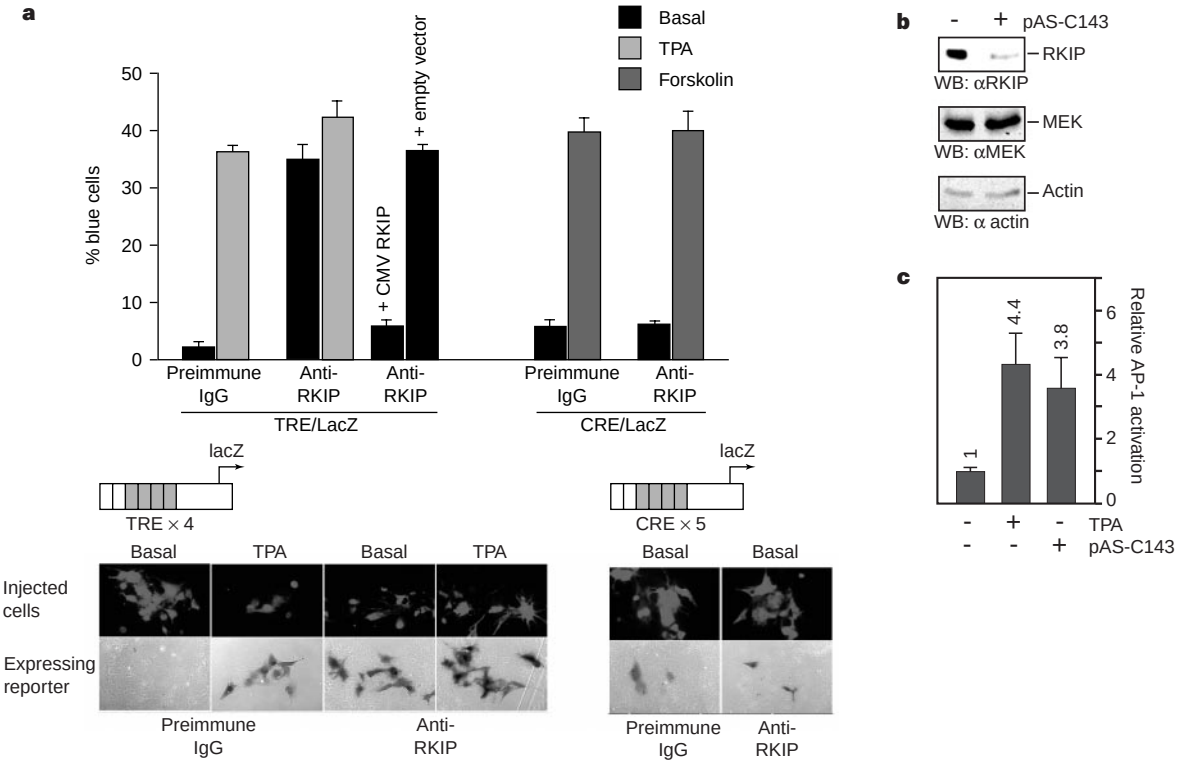


**Figure 2** Inhibition of endogenous RKIP activates AP-1-dependent transcription. **a**, Microinjection of anti-RKIP antibodies. Quiescent Rat-1 cells were microinjected with the indicated reporter plasmids and antibodies and either left unstimulated or treated with 200 ng ml$^{-1}$ TPA or 20 μg ml$^{-1}$ forskolin. **b**, The RKIP antisense vector, pAS-C143, downregulates expression of endogenous RKIP. NIH 3T3 cells were cotransfected with pAS-C143 and a GFP-expressing plasmid. GFP-positive cells were isolated by FACS and immunoblotted with the indicated antibodies. **c**, The activity of an AP-1 reporter gene was measured in serum-starved or TPA-stimulated NIH 3T3 cells following cotransfection with RKIP antisense (pAS-C143) or empty vectors.

prevent MEK phosphorylation by MEKK-1 (Fig. 4c); and (3) did not interfere with Raf-1 autophosphorylation or phosphorylation of myelin basic protein (MBP) by Raf-1 (Fig. 4d). These data indicate that RKIP is a very selective inhibitor that specifically blocks activation of MEK by Raf.

*In vitro*, RKIP disrupted the physical interaction between Raf-1 and MEK, which is required for MEK phosphorylation[12], and behaved like a competitive inhibitor for MEK (data not shown). Therefore, we investigated whether this mechanism also operated in cells. The downregulation of endogenous RKIP expression by the pAS-C143 antisense vector substantially enhanced phosphorylation of MEK on activation-specific sites (Fig. 5a). Similarly, microinjection of RKIP antibodies enhanced ERK activation in NIH 3T3 cells (Fig. 5b). In a complementary approach, RKIP was over-expressed. Cotransfection of RKIP had only a small influence on the activation of Raf-1 by TPA, but strongly inhibited the activation of MEK in a dose-dependent fashion (Fig. 5c). EGF produced the same results (data not shown). RKIP overexpression also downregulated the activation of ERKs by the *v-Ras* or *v-Src* oncogenes (Fig. 5d). Cotransfection of increasing amounts of an RKIP expression plasmid inhibited the BXB-induced activation of ERK in a dose-dependent manner. In contrast, RKIP did not affect activation by ERK by MEK-DD (Fig. 5e). These data confirm the *in vitro* results (Fig. 4), and show that RKIP regulates the ERK pathway primarily at the Raf/MEK interface *in vivo*.

To test whether the association between Raf and RKIP changes during mitogenic stimulation, we monitored the presence of RKIP in Raf-1 immunoprecipitates prepared from Rat-1 cells at different times after stimulation with serum (Fig. 6). The activation kinetics of the ERK pathway closely correlated with a decrease in RKIP co-precipitation. Furthermore, as the activity of the ERK pathway returned to basal levels at later times following mitogenic stimulation, the interaction between Raf-1 and RKIP returned to the level seen in quiescent cells.

What could be the physiological role of an inhibitor such as RKIP? Like the ERK pathway, RKIP is widely expressed. A quantification of Raf-1, MEK, ERK and RKIP protein levels in the cell lines used in this study showed a wide variation in RKIP expression relative to the kinases. The ratio Raf-1:MEK:ERK:RKIP was 1:1.6:2.4:14 in Rat-1; 1:1.4:3.5:27 in 208F; 1:0.7:9:4.2 in NIH 3T3; and 1:2.9:5.9:<1.9 in COS-1 cells. Thus, at least in the three fibroblast cells lines, RKIP is abundant enough to be stoichiometrically relevant as an inhibitor. RKIP also colocalized with Raf-1 in Ras-transformed cells (Fig. 1d), indicating that an appreciable fraction of Raf-1 and its inhibitor RKIP may remain associated even under conditions that promote Raf-1 activation. This may in part explain the observation that only a small fraction of Raf-1 can be activated[13]. We propose that RKIP could function like a rheostat that sets the sensitivity threshold for the activation of the Raf/MEK/ERK pathway. A quantitative analysis of the activation kinetics of the ERK pathway showed that this cascade operates like a switch

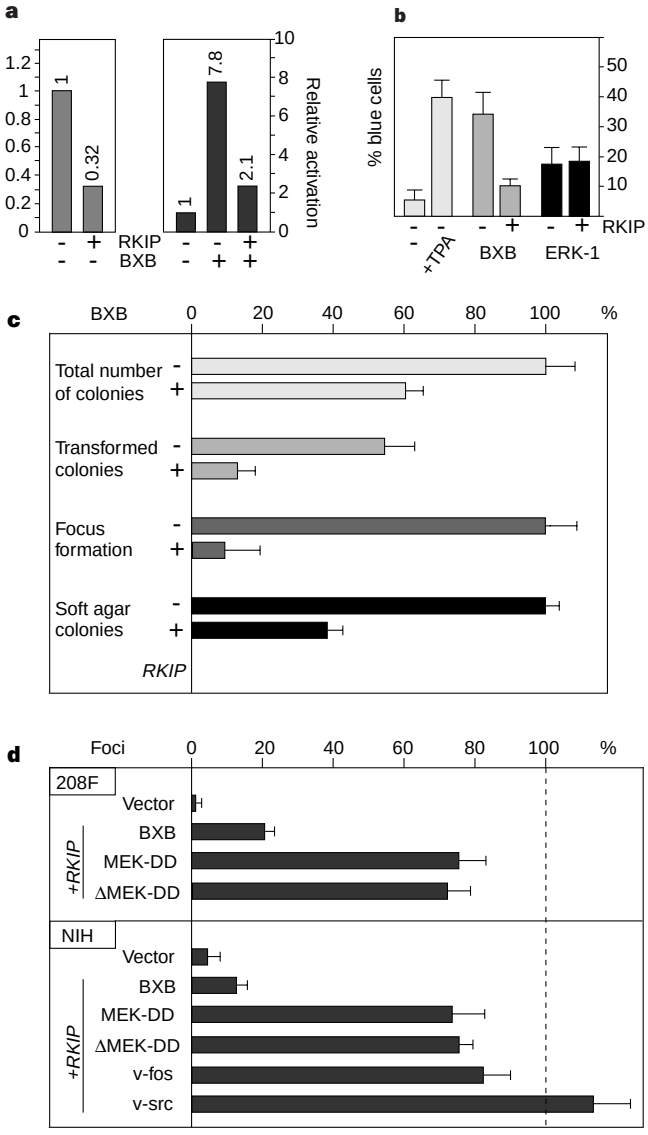


**Figure 3** RKIP inhibits Raf-induced AP-1 activation and transformation. **a**, RKIP reduces basal and BXB-induced AP-1 activity in NIH 3T3 cells cotransfected with a 3xTRE-CAT reporter and the indicated expression plasmids. **b**, RKIP blocks BXB- but not ERK-induced AP-1 activation. Rat-1 cells were co-microinjected with a 4xTRE-lacZ reporter and the indicated expression vectors. **c**, RKIP inhibits Raf-dependent proliferation and transformation. NIH 3T3 cells were transfected with BXB, alone or together with RKIP (linked to neo). G418-resistant colonies were counted and scored for morphological transformation. Aliquots of the same transfection were allowed to grow to confluency without drug and were scored for focus formation. A BXB-transformed cell line was infected with LXSH-RKIP retrovirus or LXSH (hygromycin resistant) and seeded in soft agar in the presence of hygromycin. **d**, RKIP does not inhibit transformation by *v-fos*, *v-src* or mutationally activated MEK[11] in 208F or NIH cells. Data are expressed as reduction in focus formation relative to cotransfection with empty vector (set to 100%).

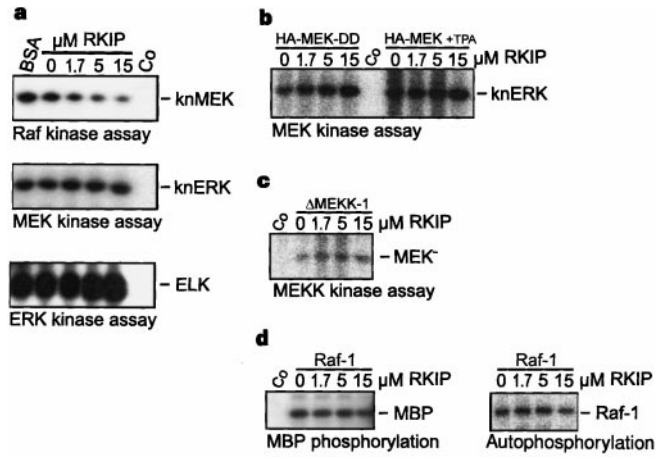


**Figure 4** RKIP specifically blocks MEK phosphorylation by Raf-1. **a**, Effect of RKIP on the activation steps of the Raf/MEK/ERK cascade reconstituted *in vitro* with purified recombinant proteins. BSA, 15 μM bovine serum albumin; Co, substrate alone; kn, kinase-negative mutant. **b**, RKIP does not inhibit activated MEK. HA–MEK-DD or HA–MEK-1 (ref. 11) expressed in COS-1 cells was immunoprecipitated with anti-HA antibodies from serum-starved cells or TPA-treated cells, respectively, and assayed for kinase activity. **c**, RKIP does not inhibit MEK phosphorylation by MEKK-1. ΔMEKK-1 (ref. 26) was immunoprecipitated from transiently transfected COS-1 cells and used to phosphorylate knMEK. **d**, RKIP does not inhibit Raf-1 autophosphorylation or phosphorylation of MBP.

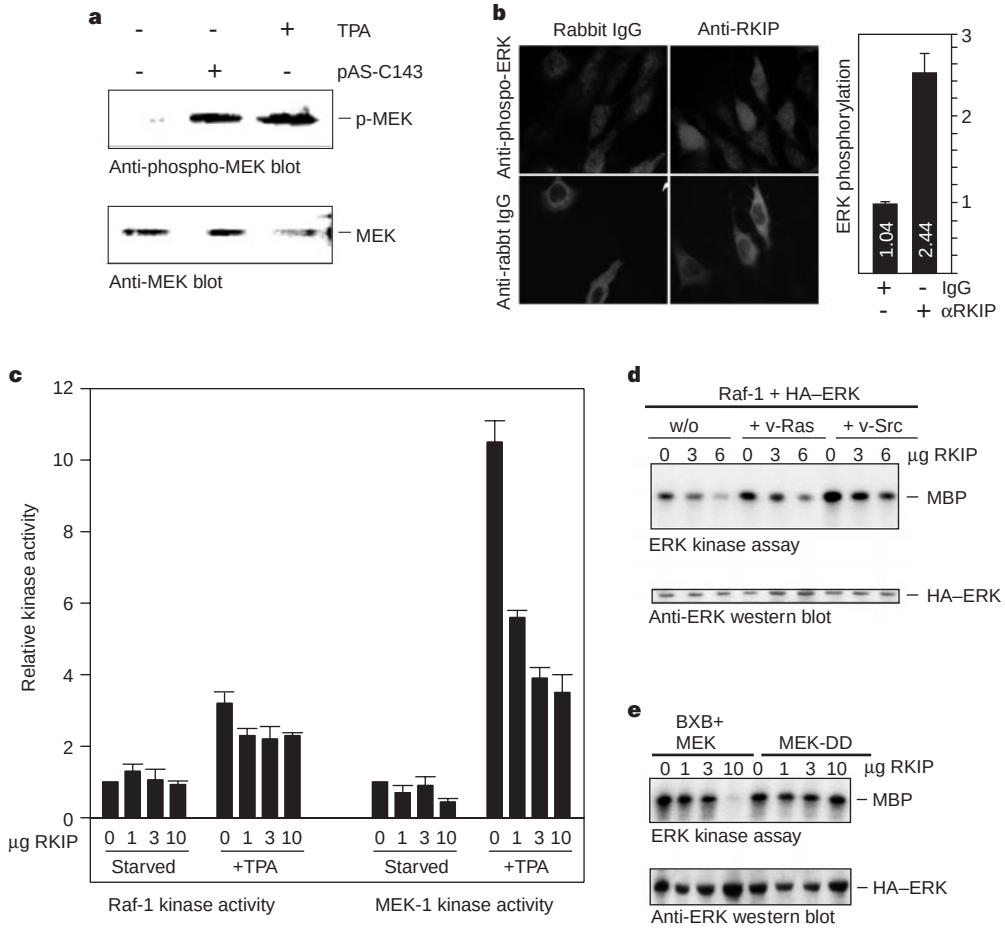


**Figure 5** RKIP regulates MEK and ERK activation *in vivo*. **a**, RKIP downregulation activates MEK. NIH 3T3 cells were cotransfected with GFP and the RKIP antisense plasmid, pAS-C143. GFP-positive cells were FACS sorted and immunoblotted with the indicated antisera. **b**, RKIP antibody microinjection enhances ERK activation. Quiescent NIH 3T3 cells were microinjected with anti-RKIP or control IgG and stimulated with 10 ng ml$^{-1}$ TPA for 30 min. ERK activation was visualized with a monoclonal anti-phospho-ERK antibody (Sigma) and quantified densitometrically. **c**, RKIP inhibits MEK-1 activation. COS-1 cells were transiently transfected with HA–MEK and increasing amounts of RKIP expression vectors. Serum-starved cells were stimulated with 100 ng ml$^{-1}$ TPA for 20 min, and the kinase activities of Raf-1 and HA–MEK immunoprecipitates were measured. **d**, RKIP inhibits stimulation of ERK by v-Ras and v-Src. COS-1 cells were transfected with the indicated expression plasmids plus increasing amounts of RKIP. HA–ERK-2 was immunoprecipitated and assayed with MBP. **e**, RKIP inhibits ERK activation by BXB, but not by MEK-DD. COS-1 cells were transfected with the indicated expression vectors and the kinase activity of HA–ERK immunoprecipitates was examined.

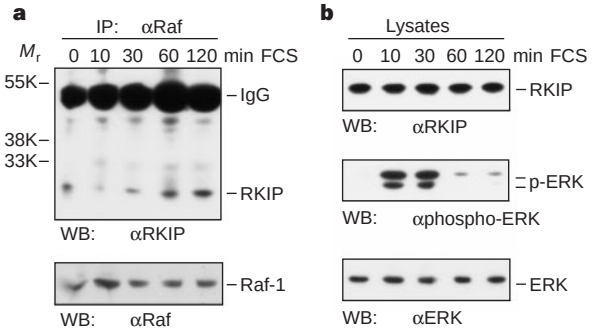


**Figure 6** During mitogenic stimulation RKIP binding to Raf-1 decreases. Serum-starved Rat-1 cells were treated with 20% fetal calf serum for the indicated times. **a**, Raf-1 immunoprecipitates were immunoblotted for associated RKIP. **b**, Cell lysates were examined for RKIP and ERK expression. ERK activation was monitored with a phospho-ERK specific antibody.

that suppresses background noise but strongly amplifies signals exceeding a certain threshold[14]. Overexpression of RKIP raises this threshold, whereas downregulation of RKIP lowers it. As the amplitude, kinetics and overall duration of ERK activity are known to vary between biological responses such as cell-cycle arrest, transformation, mitogenesis and differentiation[1,15–18], RKIP is expected to exert a profound influence on these parameters. □

## Methods

### Plasmids and protein expression

The rat RKIP cDNA[19] was cloned (1) into pcDNA3 to make p353/RKIP; (2) into pCMV5 with a triple HA-tag at the amino-terminus; and (3) into pGEX-KG to make GST–RKIP. pAS-C143 encompasses RKIP nucleotides 1–429 cloned into pCMVori in antisense orientation. pCMVori contains the CMV promoter, polylinker and polyadenylation sequences from pCMV5 inserted into pUCori upstream of the polyoma virus core origin[20]. 6xHis-tagged MEK- and GST-fusion proteins were expressed and purified as described[21]. RKIP of >95% purity was prepared from GST–RKIP by thrombin cleavage[22] and subsequent FPLC separation over Superose 12.

### *In vitro* binding assays

These contained 1–5 μg of GST-fusion protein immobilized on glutathione sepharose beads and 0.5–5 μg purified recombinant protein in PBS supplemented with 10% bovine serum as a nonspecific competitor. Sf-9 cell lysates were used as a source of Raf proteins[21]. After incubation for 1 h at 4 °C the samples were washed 4 times with PBS, resolved by SDS–PAGE and blotted. The blots were developed using ECL (Amersham).

### Immunoprecipitation

For co-precipitation experiments of endogenous proteins, $2 \times 10^7$ Rat-1 cells were lysed by sonication in PBS, and the immunoprecipitates were washed 4 times with PBS. Otherwise cells were lysed as described[21]. The antibodies used were crafVI, a peptide antibody against the 12 carboxy-terminal amino acids of Raf-1 (ref. 21); a Raf monoclonal antibody to the regulatory domain (Transduction Laboratories); anti-MEK H8 (Santa Cruz); anti-RKIP raised in rabbits immunized with purified GST–RKIP; anti-HA, 12CA5 monoclonal antibody; monoclonal anti-phospho-ERK (Sigma) and polyclonal anti-phospho-MEK antibodies (New England Biolabs); and anti-GST (Pharmacia).

### Kinase assays

These were done as described[21]. For *in vitro* reconstruction of the Raf/MEK/ERK cascade, activated Raf-1 was generated by co-expressing GST–Raf-1 with v-Ras and Lck in Sf-9 cells and collected on glutathione-sepharose beads[21]. Subsequent thrombin cleavage released Raf-1, which was fully active and >90% pure. To activate MEK and ERK *in vitro*, 20 ng of activated Raf-1 was incubated with 40 ng of purified His/MEK-1 and 250 ng of GST–ERK-2 in Raf kinase buffer containing 20 μM ATP for 20 min at 30 °C. To measure kinase activities at individual steps, the respective downstream components were omitted. The activation reactions were diluted into 50 μl Raf kinase buffer[21] containing 20 μM ATP to yield equimolar concentrations of the kinases to be assayed and incubated with increasing amounts of purified RKIP on ice for 10 min. Then, 2 μCi[$^{32}$P]-γ-ATP and recombinant substrates were added and incubated for 20 min at 30 °C. As substrates, we used 200 ng kinase negative His/MEK-1 for Raf, 1 μg kinase negative GST–ERK for MEK and 1 μg GST–ELK (New England Biolabs) for ERK. In some assays 1 μg GST–MEK was used as the Raf-1 substrate with identical results.

### Transfections

COS-1 cells were transfected as described[23] with 2 μg of HA–ERK-2, BXB, MEK and MEK-DD plasmids and the indicated amounts of p353/RKIP. The total amount of transfected DNA was kept constant using the appropriate vectors as carrier DNA. NIH 3T3 and 208F cells were transfected in 6-well plates with 1 μg of pCMV5-BXB and 3 μg of p353/RKIP using Superfect (Qiagen). For RKIP downregulation experiments, NIH 3T3 cells were transiently cotransfected using lipofectamine with 0.5 μg of pHACT[20] and 1.5 or 3 μg RKIP antisense expression vector (pAS-C143) or control vector (pCMVori) as indicated. pHACT expresses a truncated polyoma large-T construct with origin-binding activity, but does not bind Rb or p53, and boosts the expression of pAS-C143 to high levels. In addition, 0.1 μg of an AP1-Luc reporter was transfected for reporter-gene assays. 48 hours after transfection, cells were serum starved for 20 h and either left untreated or treated with TPA (200 ng ml$^{-1}$) or serum for 5 h before being collected. Cells were lysed and cell extracts were used for immunoblotting or assayed for luciferase activity. For the green fluorescent protein (GFP) sorting experiments, $5 \times 10^6$ NIH 3T3 cells were electroporated with either 100 μg pCMVori, 50 μg pCMV–GFP and 50 μg pHACT, or 100 μg pASC143, 50 μg CMV–GFP and 50 μg CMV–HAC. Two days later cells were trypsinized and sorted for green fluorescent cells by preparative FACS. 100,000 GFP-positive cells were lysed in SDS-gel sample buffer and immunoblotted.

### Microinjection

Microinjection of antibodies and reporter genes was done as described[24,25]. The RKIP antiserum was purified over a GST–RKIP affinity column. Cells were stained with an activation-specific anti-phospho-ERK monoclonal antibody (New England Biolabs). ERK phosphorylation was quantified by densitometry. For this purpose areas with micro-injected cells were randomly photographed, and the staining intensity of whole individual cells was measured using the PcBAS software.

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# *Saccharomyces cerevisiae* telomerase is an Sm small nuclear ribonucleoprotein particle

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**Activation of the chromosome end-replicating enzyme telomerase can greatly extend the lifespan of normal human cells[1] and is associated with most human cancers[2]. In all eukaryotes examined, telomerase has an RNA subunit[3], a conserved reverse transcriptase subunit[4] and additional proteins[5,6], but little is known about the assembly of these components. Here we show that the *Saccharomyces cerevisiae* telomerase RNA[7] has a 5′-2,2,7-trimethylguanosine (TMG) cap and a binding site for the Sm proteins, both hallmarks of small nuclear ribonucleoprotein particles (snRNPs) that are involved in nuclear messenger RNA splicing[8,9]. Immunoprecipitation of telomerase from yeast extracts shows that Sm proteins are assembled on the RNA and that most or all of the telomerase activity is associated with the Sm-containing complex. These data support a model in which telomerase RNA is transcribed by RNA polymerase II (ref. 10) and 7-methylguanosine-capped,**