es, 7 of which disrupted before completion of the experiment. Results from the nine stable control patches and six stable PI-PLC–treated patches were compared by Student's t tests. The average peak current after 4 min of recording was 67 ± 7 pA (SEM) in control patches and 51 ± 9 pA in PI-PLC treated patches. The difference was not significant ($P > 0.1$). During application of ATP, exchange current increased by 4.7 ± 0.9 times in control patches to 88 ± 14 pA and by 0.19 ± 0.05 times in treated patches to 14 ± 2 pA. The differences were highly significant ($P < 0.001$).
12. D. W. Hilgemann, unpublished data.
13. A. V. Smrcka and P. C. Sternwies, *J. Biol. Chem.* **268**, 9667 (1993).
14. D. W. Hilgemann, A. Collins, S. Matsuoka, *J. Gen. Physiol.* **100**, 933 (1992); S. Matsuoka *et al.*, *ibid.* **105**, 403 (1995); D. O. Levitsky, D. A. Nicoll, K. D. Philipson, *J. Biol. Chem.* **269**, 22847 (1994).
15. With continuous application of 10 μM free Ca^{2+}, ATP effects were small because the exchanger was more highly activated by Ca^{2+} (*14*). Reversal took place with a time constant of about 30 s, and guanosine 5′-*O*-(3′-triotriphosphate) (0.2 mM) did not affect this time course. With >20 μM free Ca^{2+}, the stimulatory effects of ATP were small and transitory, and subsequent effects of ATP with 0.5 μM free Ca^{2+} were small or absent. ATP effects were restored by PI.
16. Cardiac membranes were prepared by homogenization on ice of 2 g of isolated myocytes in 1 ml of solution containing 20 mM KCl, 10 mM Hepes (pH 7.0), 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μM leupeptin. Nuclei and cell debris were removed by centrifugation at 400*g* for 5 min at 4°C. The membrane fraction was recovered after centrifugation at 150,000*g* for 30 min at 4°C and resuspended at 8 milligrams of protein per milliliter in a solution containing 20 mM KCl, 20 mM Hepes (pH 7), and 1 mM EGTA. The PLC assay was done as described (*13*), except that sonicated vesicles containing 250 μM phosphatidylethanolamine and 50 μM (inositol-2-3H)-PIP_2were used, and the assay solution contained 20 mM Hepes (pH 7), 100 mM NaCl, 40 mM KCl, 10 mM EGTA, 0.5 mM $MgCl_2$, 20 mM CsCl, 20 mM TEA-Cl, and the indicated concentrations of free Ca^{2+}.
17. Fast reversal was blocked by EDTA (0.5 mM), fluoride (0.2 mM), 5 mM phosphate, and 5′-adenylimidodiphosphate (2 mM), which all bind polyvalent cations (*18, 19*).
18. A. E. Martell and R. M. Smith, *Critical Stability Constants*, vol. 3, (Plenum, New York, 1977).
19. R. B. Martin, *Biochem. Biophys. Res. Commun.* **155**, 1194 (1988). EDTA (2 mM) only partially reversed the aluminum effect. Usually, exchange current could be fully restimulated by second and third applications of ATP after brief applications of aluminum.
20. L. J. McDonald and M. D. Mamrack, *J. Lipid Mediat. Cell Signal.* **11**, 81 (1995); J. D. Birchall and J. S. Chappell, *Clin. Chem.* **34**, 265 (1988); C. Schofl *et al.*, *Biochem. J.* **269**, 547 (1990).
21. With 50 μM PIP_2 and 10 mM EGTA in the assay buffer, aluminum (50 μM) inhibited PLC-β1 and cardiac membrane PLC activities by 99% with 0.5 μM free Ca^{2+} and by 60% with 20 μM free Ca^{2+}. Aluminum (50 μM) had no effect on Ca^{2+}-activated PI hydrolysis in our assays, but inhibition has been reported for higher free aluminum concentrations, probably by aluminum binding to PI (*20*). As was consistent with a PI-aluminum interaction in cardiac patches, high concentrations of aluminum (100 to 500 μM with 10 mM EGTA) slowed development of the ATP effect and decreased its magnitude (*12*).
22. D. W. Hilgemann, *Nature* **344**, 242 (1990).
23. Phospholipid vesicles were prepared by sonication of pure PC, of 4:1 PC:PS, and of 4:1 PC:PIP_2 mixtures. The assay mixture contained 30 mM Hepes (pH 7 with NaOH); 20 mM NaCl; 10 mM EGTA; 450 μM total lipid; and one of three fluorescent probes: 20 μM ANS, 10 μM Di-8-ANEPPS (Molecular Probes) (a voltage-sensitive styryl dye), or 1 μM of a custom-prepared rhodamine-labeled hexalysine. $AlCl_3$ was added with twofold NaOH to compensate for protons released from EGTA. Fluorescence of ANEPPS increased in response to aluminum; fluorescence of rhodamine-labeled hexalysine decreased upon binding to vesicles, and the decrease was reversed by aluminum.
24. G. Weber and J. Slavik. *Biochim. Biophys. Acta* **694**, 1 (1982).
25. Voltage-activated sodium channel currents often ran down over 20 to 30 min in giant patches, whereas sodium pump currents are stable. ATP, PS, and PA were without effect on the sodium channel current and its run-down [A. Collins and D. W. Hilgemann, *Pflügers Arch.* **423**, 347 (1993)]. PS, PA, and PIP_2 were without effect on the sodium pump current. Pentalysine (50 μM) had no effect on either current.
26. C. G. Nichols and W. J. Lederer, *Am. J. Physiol.* **261**, H1675 (1991); N. Inagaki *et al.*, *Science* **270**, 1166 (1995).
27. T. Ohno-Shosaku, B. J. Zuenkler, G. Trube, *Pflügers Arch.* **40**, 133 (1987); R. Ribalet, S. Ciani, G. T. Eddlestone, *J. Gen. Physiol.* **94**, 693 (1989); M. Takano, D. Qin, A. Noma, *Am. J. Physiol.* **58**, H45 (1990).
28. In giant patches, ATP inhibited the potassium current with an inhibition constant (K_i) of 0.5 to 2 mM, which is a lower affinity than in most recordings in small patches. Usually, a component of current clearly could not be inhibited by ATP. Because current-voltage relations showed inward rectification, it is likely that inwardly rectifying potassium channels other than K_{ATP} channels are also activated by PIP_2.
29. P. A. Janmey, *Annu. Rev. Physiol.* **56**, 169 (1994); A. Hall, *Ann. Rev. Cell Biol.* **10**, 31 (1994); J. H. Hartwig *et al.*, *Cell* **82**, 643 (1995); A. P. Gilmore and K. Burridge, *Nature* **381**, 5311 (1996).
30. B. D. Johnson and L. Byerly, *Neuron* **10**, 797 (1993).
31. T. Furukawa, T. Yamane, T. Terai, Y. Katayama, M. Hiraoka, *Pflügers Arch.* **431**, 504 (1996).
32. The cardiac Na^+,Ca^{2+} exchanger may be linked to the cytoskeleton by ankyrin [Z. P. Li, E. P. Burke, F. W. Frank, V. Bennett, K. D. Philipson, *J. Biol. Chem.* **268**, 11489 (1993)], and disruption of the actin cytoskeleton inhibits exchange activity in a transfected cell line [M. Condrescu *et al.*, *ibid.* **270**, 9137 (1995)]. However, 10 μM cytochalasin D, 0.5 μM deoxyribonuclease, gelsolin, and G-actin were all without any evident influence on the effect of ATP on exchange current in giant patches, its reversal, or a series of ATP responses using aluminum (10 μM) to reverse the ATP effect (*12*). Gelsolin and actin were gifts of H. Yin.
33. D. Choquette *et al.*, *Biochem. Biophys. Res. Commun.* **125**, 908 (1984); A. G. Filoteo, A. Enyedi, J. T. Penniston, *J. Biol. Chem.* **267**, 11800 (1992)
34. M. Liscovitch, V. Chalifa, P. Pertile, C.-S. Chen, L. C. Cantley, *J. Biol. Chem.* **269**, 21403 (1994); A. P. Gilmore and K. Burridge, *Nature* **381**, 531 (1996).
35. S. Matsuoka, D. A. Nicoll, R. F. Reilly, D. W. Hilgemann, K. D. Philipson, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3870 (1993).
36. T. R. Shannon, C. C. Hale, M. A. Milanick, *Am. J. Physiol.* **266**, C1350 (1994).
37. H. Ito *et al.*, *J. Gen. Physiol.* **99**, 961 (1992); R. Ribalet and S. Ciani, *J. Memb. Biol.* **142**, 395 (1994).
38. L. Aguilar-Bryan *et al.*, *Science* **268**, 423 (1995).
39. R. W. Tsien, *Adv. Cyclic Nucleotide Res.* **8**, 363 (1977); M. Reiter, *Pharmacol. Rev.* **40**, 189 (1988).
40. We thank P. C. Sternweis, S. Muallem, and H. L. Yin for insightful discussions, encouragement, and reagents; and S. Feng and X. Xu for technical help. Supported by grants from NIH (5-R1-HL51323-03) and the American Heart Association (95014830) to D.W.H. and from NIH (GM49993) to P.C. Sternweis.

26 April 1996; accepted 24 June 1996

Coupling of the RAS-MAPK Pathway to Gene Activation by RSK2, a Growth Factor–Regulated CREB Kinase

Jun Xing,* David D. Ginty,* Michael E. Greenberg†

A signaling pathway has been elucidated whereby growth factors activate the transcription factor cyclic adenosine monophosphate response element–binding protein (CREB), a critical regulator of immediate early gene transcription. Growth factor–stimulated CREB phosphorylation at serine-133 is mediated by the RAS–mitogen-activated protein kinase (MAPK) pathway. MAPK activates CREB kinase, which in turn phosphorylates and activates CREB. Purification, sequencing, and biochemical characterization of CREB kinase revealed that it is identical to a member of the $pp90^{RSK}$ family, RSK2. RSK2 was shown to mediate growth factor induction of CREB serine-133 phosphorylation both in vitro and in vivo. These findings identify a cellular function for RSK2 and define a mechanism whereby growth factor signals mediated by RAS and MAPK are transmitted to the nucleus to activate gene expression.

Growth factors transmit signals from the plasma membrane to the nucleus to activate programs of immediate early gene (IEG) transcription that are critical for cell proliferation and differentiation (*1*). Growth factor binding to receptor tyrosine kinases produces stimulation of a RAS-dependent kinase cascade that includes the sequential phosphorylation and activation of RAF, MEK (MAPK or extracellular signal–regulated kinase kinase), mitogen-activated protein kinase (MAPK), and ribosomal protein S6 kinase ($pp90^{rsk}$ or RSK) (*2*). Once activated, MAPK translocates to the nucleus where it phosphorylates and activates the transcription factor ELK-1. ELK-1 binds,

J. Xing, Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, MA 02115, USA.
D. D. Ginty, Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205–2185, USA.
M. E. Greenberg, Division of Neuroscience, Department of Neurology, Children's Hospital, and Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed.

together with the serum response factor, to the serum response element (SRE) within IEG promoters and functions in the activation of IEGs in response to many growth factors (*3*). Like the MAPKs, members of the $pp90^{RSK}$ family translocate to the nucleus and phosphorylate several transcription factors in vitro (*4–7*). However, the in vivo function of RSKs in growth factor–stimulated cells remains unclear.

The interaction of the ELK-1–SRF complex with the SRE and the phosphorylation of ELK-1 in response to growth factors are crucial for the activation of the immediate early gene c-*fos*. However, these events are not sufficient to stimulate transcription of c-*fos* (*8–10*). Under some circumstances the SRE-binding proteins appear to mediate growth factor induction of c-*fos* transcription by cooperating with another transcription factor, the cyclic adenosine monophosphate response element–binding protein (CREB), which can bind to three distinct sequence elements within the c-*fos* promoter (*8*, *9*, *11*). Although CREB was first identified as a mediator of gene expression that occurs in response to increased concentrations of cAMP, CREB also regulates the cellular response to growth factor stimulation (*8*, *9*). Growth factors activate CREB's transcriptional activation potential by stimulating CREB phosphorylation at a specific amino acid, Ser^{133}. Phosphorylation of CREB at Ser^{133} is critical for growth factor induction of c-*fos* transcription (*8*). The kinase that catalyzes CREB Ser^{133} phosphorylation in growth factor–stimulated PC12 cells, termed CREB kinase, is a RAS-dependent enzyme distinct from RAF, MEK, MAPK, and the well-characterized member of the RSK family, RSK1 (*8*).

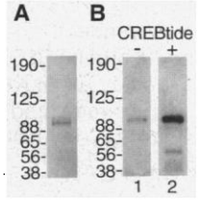

Fig. 1. Purification of a growth factor–inducible CREB kinase. (**A**) Coomassie blue staining of purified CREB kinase protein. CREB kinase purified from TPA-treated K562 cells (~1 μg) was subjected to SDS-PAGE. (**B**) Phosphorylation of CREBtide (*8*), a peptide corresponding to amino acids 123 to 136 of CREB, by purified CREB kinase. Purified CREB kinase was resolved by SDS-PAGE on gels prepared without (lane 1) or with (lane 2) CREBtide in the gel and subjected to an in-gel kinase assay (*8*). The low amount of phosphate incorporation into the 95-kD band in the absence of CREBtide is the result of autophosphorylation of CREB kinase on threonine residues (*12*). Because the purified CREB kinase used in this assay was electroeluted from gel slices containing only the 95-kD CREB kinase, the band detected at around 58 kD likely resulted from a small amount of degradation of the 95-kD CREB kinase that occurred during its isolation.

CREB kinase activity is induced in various cell types in response to a wide range of growth factors (*12*). In the pheochromocytoma cell line PC12, treatment with nerve growth factor (NGF), epidermal growth factor (EGF), or the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) activates CREB kinase, as does TPA treatment of HeLa cells and the human erythroleukemia cell line K562. CREB kinase was purified to homogeneity from TPA-treated K562 cells (Table 1) (*13*). The purified enzyme had an apparent molecular size of about 95 kD (Fig. 1A) and possessed both autophosphorylation and CREB kinase activity (Fig. 1B). In a BLAST search, peptide sequences from purified CREB kinase (*14*) showed 100% identity to a single protein, the protein kinase RSK2 (*15–17*), as well as similarities to the other RSK family members RSK1 (*15*, *16*, *18*, *19*) and RSK3 (*7*, *16*).

To confirm that the major CREB kinase present in extracts of growth factor–treated cells is RSK2, we fractionated extracts from control or NGF-stimulated PC12 cells on a Mono Q anion-exchange column and tested column fractions for CREB kinase activity and RSK2 immunoreactivity (Fig. 2, A and B). Treatment with NGF resulted in

Table 1. Purification of CREB kinase. Table of purification is from one typical preparation. Samples from several of such preps were pooled and subjected to preparative SDS-PAGE. A single band of CREB kinase identified by in-gel kinase assay was electroeluted. Estimated final purification was >20,000-fold.

Step	Procedure	Protein mass	Total activity (nmol min^{-1})	Specific activity (nmol min^{-1} g^{-1})	Purification (fold)	Yield (%)
1	Homogenate	7.0 g	290	42	1	100
2	100,000*g* supernatant	2.2 g	120	54	1.3	41
3	S-Sepharose	0.4 g	140	350	8.3	47
4	Phenyl-Sepharose	73 mg	56	770	18	19
5	Affi-Gel blue	30 mg	75	2,500	59	25
6	Mono Q	2.2 mg	24	11,000	260	8.2
7	Mono S	0.4 mg	18	45,000	1060	6.1

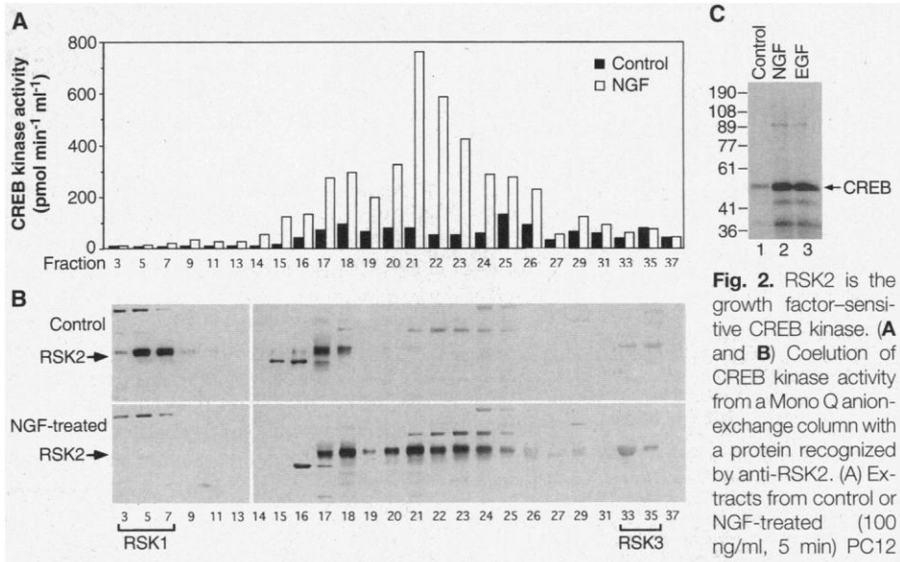

Fig. 2. RSK2 is the growth factor–sensitive CREB kinase. (**A** and **B**) Coelution of CREB kinase activity from a Mono Q anion-exchange column with a protein recognized by anti-RSK2. (A) Extracts from control or NGF-treated (100 ng/ml, 5 min) PC12 cells were applied to a Mono Q column, and proteins were eluted with a linear salt gradient (*8*). CREB kinase activity in column fractions was determined as described (*8*). (B) The same Mono Q column fractions were subjected to SDS-PAGE and protein immunoblot analysis with anti-RSK2 (UBI). In NGF-treated PC12 extracts, RSK2 eluted in fractions containing 0.09 to 0.21 M NaCl. In contrast, immunoblot analysis with anti-RSK1 (UBI) or RSK3 (*7*) showed that RSK1 immunoreactivity was present mainly in flow-through fractions, whereas RSK3 eluted in fractions containing 0.34 to 0.35 M NaCl (indicated by brackets). The failure to detect a complete correlation between CREB kinase activity and RSK2 immunoreactivity likely reflects differences in specific activity among differently modified (probably by phosphorylation) forms of RSK2. We confirmed the specificity of antibodies to RSKs by immunoblotting lysates of COS cells overexpressing individual isoforms of the RSKs (*12*). (**C**) Induction of CREB kinase(RSK2) activity by growth factors. RSK2 was immunoprecipitated from lysates of untreated, NGF-treated (100 ng/ml, 5 min), or EGF-treated (30 ng/ml, 5 min) PC12 cells. The immune complex was incubated with a kinase buffer containing [γ-^{32}P]adenosine triphosphate and baculovirus-expressed CREB protein. The phosphorylated CREB was resolved by SDS-PAGE and visualized by autoradiography.

the elution of RSK2 in higher concentration NaCl fractions, consistent with the possibility that RSK2 was modified by phosphorylation in response to growth factor stimulation. In NGF-treated extracts, CREB kinase and RSK2 copurified. Protein immunoblotting with antibodies specific for RSK family members indicated that the three RSK isoforms were well resolved by Mono Q chromatography, and that under the conditions of stimulation only column fractions containing RSK2 displayed CREB kinase activity. Similar results were obtained from TPA-treated K562 cells (*12*). The identity of CREB kinase and RSK2 was confirmed in an immune complex kinase assay. Antibodies to RSK2, but not antibodies to RSK1, immunoprecipitated the CREB kinase activity present in the Mono Q column fractions (*12*). In addition, the CREB kinase activity of RSK2 immunoprecipitated from extracts of PC12 cells was increased if cells were treated with NGF or EGF (Fig. 2C).

To determine whether RSK2 is a physiologically relevant growth factor–inducible CREB kinase in intact cells, we introduced hemagglutinin (HA) epitope–tagged wild-type (HA-RSK2) or catalytically inactive [HA-RSK2(KR100)] versions of RSK2 (*20*) into COS cells. The expression of recombinant forms of RSK2 in transfected COS cells was assessed by protein immunoblotting of COS cell extracts with an antibody to HA (Fig. 3A). The wild-type and mutant RSK2 proteins were expressed in comparable amounts in COS cells. The mobility on SDS–polyacrylamide gel electrophoresis (PAGE) of HA-RSK2, but not HA-RSK2(KR100), was decreased when extracts were prepared from EGF- or TPA-stimulated cells. This change in mobility is likely indicative of autophosphorylation of HA-RSK2 that accompanies its activation. In addition, the kinase activity of HA-RSK2 was increased when COS cells were treated with EGF (*12*) or TPA (Fig. 3B). By contrast, HA-RSK2(KR100) was inactive in kinase assays (*12*).

To determine whether RSK2 phosphorylates CREB Ser^{133} in vivo, we transfected COS cells with HA-RSK2 or HA-RSK2(KR100) and a recombinant form of CREB (GAL4-CREB) (*20*). The difference in mobility between GAL4-CREB and CREB on SDS-PAGE made it possible to distinguish between the Ser^{133}-phosphorylated forms of GAL4-CREB and the endogenous CREB. The transfected cells were stimulated with EGF or TPA, and the phosphorylation of CREB at Ser^{133} was monitored by protein immunoblotting with an antibody specific for CREB only when it is phosphorylated on Ser^{133} (anti-PCREB) (*21*). The amount of GAL4-CREB expressed was not affected by the presence of HA-RSK2 or the stimulation conditions (*12*). Upon treatment of the cells with either EGF or TPA, GAL4-CREB became phosphorylated on CREB Ser^{133} (Fig. 3C). When HA-RSK2 and GAL4-CREB were coexpressed in cells, the EGF- and TPA-induced increase in GAL4-CREB Ser^{133} phosphorylation was greater than that in cells expressing GAL4-CREB alone (Fig. 3C). Thus RSK2, when exogenously introduced into COS cells, appears to phosphorylate CREB at Ser^{133} upon growth factor stimulation. Because immunocytochemical analysis revealed that in transfected COS cells GAL4-CREB is localized exclusively in the cell nucleus (*12*), phosphorylation of CREB Ser^{133} appears to occur within the nucleus.

Expression of HA-RSK2(KR100) appeared to inhibit the activity of the endogenous CREB kinase in EGF- or TPA-stimulated cells (Fig. 3C), in that the amount of GAL4-CREB Ser^{133} phosphorylation in HA-RSK2(KR100)–transfected cells was less than that detected in cells transfected with GAL4-CREB alone. In contrast, growth factor–induced phosphorylation of ELK-1 Ser^{383}, an event catalyzed by MAPK (*3*), was not affected by the introduction into COS cells of either HA-RSK2 or the mutant HA-RSK2(KR100) (Fig. 3D). These findings suggest that HA-RSK2(KR100) functions in a specific manner as a dominant-interfering mutant and provide further evidence that RSK2 is a physiologically relevant CREB kinase in growth factor–stimulated cells.

Growth factor stimulation of CREB kinase activity and CREB Ser^{133} phosphorylation is inhibited by the expression of a dominant-interfering form of RAS (*8*), suggesting that the activation of CREB kinase may be mediated by a component of the RAS-MAPK signaling pathway. Purified MAPK phosphorylates and activates RSK

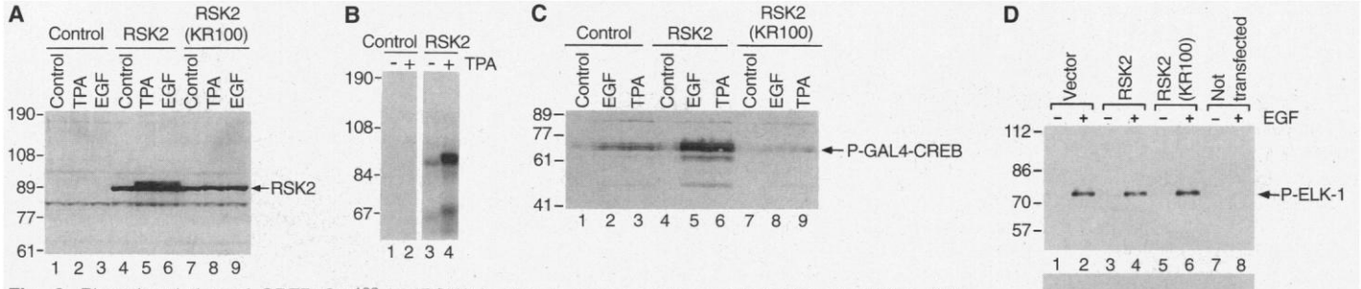

Fig. 3. Phosphorylation of CREB Ser^{133} by RSK2 in vivo in response to growth factor stimulation. (**A**) Expression of epitope-tagged RSK2 in COS cells. COS cells were transiently transfected (*27*) (3 μg of DNA per 60-mm dish) with an empty cloning vector pMT2 (lanes 1 to 3), an expression vector encoding HA-tagged wild-type RSK2 [pMT2-HA-RSK2] (lanes 4 to 6), or an expression vector encoding a catalytically inactive mutant of RSK2 [pMT2-HA-RSK2(KR100)] (lanes 7 to 9). Lysates were prepared from transfected COS cells that were untreated, treated with EGF (30 ng/ml, 10 min), or treated with TPA (100 ng/ml, 10 min), and subjected to SDS-PAGE. The immunoblot was probed with the monoclonal antibody to HA, 12CA5 (Boehringer Mannheim). The band migrating at ~84 kD was nonspecific and present in all COS cell lysates. (**B**) Activation of the CREB kinase activity of transfected RSK2 by treatment of cells with TPA. COS cells transfected (*27*) (5 μg per 10-cm dish) with pMT2 (lanes 1 and 2) or pMT2-HA-RSK2 (lanes 3 and 4) were either untreated (lanes 1 and 3) or treated with TPA (100 ng/ml, 10 min) (lanes 2 and 4). CREB kinase activity was measured with an in-gel kinase assay. Similar results were obtained when CREB kinase activity was measured with an immune complex kinase assay after immunoprecipitation with anti-HA (*12*). (**C**) Phosphorylation of GAL4-CREB by RSK2 in response to growth factor stimulation in vivo. COS cells were transfected (*27*) with 1 μg of CMV-GAL4- CREB together with 10 μg of pMT2 (lanes 1 to 3), pMT2-HA-RSK2 (lanes 4 to 6), or pMT2-HA-RSK2(KR100) (lanes 7 to 9). Transfected COS cells were left untreated (lanes 1, 4, and 7) or treated with EGF (30 ng/ml, 5 min) (lanes 2, 5, and 8) or TPA (100 ng/ml, 5 min) (lanes 3, 6, and 9). Lysates from transfected COS cells were separated on SDS-PAGE and immunoblotted with anti-PCREB. (**D**) Growth factor–induced phosphorylation of ELK-1 is not affected by wild-type or mutant RSK2. COS cells were transfected (*27*) with 1 μg of pCMV-ELK-1 together with 10 μg of pMT2 (lanes 1 and 2), pMT2-HA-RSK2 (lanes 3 and 4), pMT2-HA-RSK2(KR100) (lanes 5 and 6), or left not transfected (lanes 7 and 8). Cells were either untreated (lanes 1, 3, 5, and 7) or treated with EGF (30 ng/ml, 5 min) (lanes 2, 4, 6, and 8). Lysates were separated on SDS-PAGE and immunoblotted with anti–ELK-1 or with antibodies that recognize ELK-1 only when it is phosphorylated at Ser^{383} (New England Biolabs).

family members including RSK2 in vitro (*22*), and MAPK associates with RSK proteins in vivo (*23*). Therefore, we examined whether MAPK activates CREB kinase-(RSK2) in growth factor–stimulated cells.

We transfected a constitutively active form of MEK (the kinase that phosphorylates and activates MAPK) [MEK1(SE218/222)] (*20*), a dominant-interfering form of MEK [MEK1(KA97)] (*20*), or a MAPK phosphatase (MKP1) that selectively dephosphorylates and inactivates MAPK (*24*) into COS cells together with HA-RSK2 and GAL4-CREB. When constitutively active MEK1(SE218/222) was coexpressed in COS cells with CREB kinase(RSK2) and GAL4-CREB, a large amount of phosphorylation at GAL4-CREB Ser^{133} was detected even in the absence of growth factor treatment (Fig. 4A). By contrast, when a dominant-interfering MEK, MEK1(KA97), was coexpressed with CREB kinase(RSK2) and GAL4-CREB, EGF induction of phosphorylation of GAL4-CREB Ser^{133} was inhibited. Expression of MKP1 also inhibited the effect of EGF on phosphorylation of GAL4-CREB (Fig. 4B). These experiments indicate that the activities of MEK and MAPK are necessary and sufficient for growth factor–stimulated activation of CREB kinase(RSK2) and phosphorylation of CREB Ser^{133}.

To test whether activation of CREB Ser^{133} phosphorylation by the MEK-MAPK-RSK2 signaling pathway is important for CREB-dependent gene expression, we used a mutant c-*fos*–chloramphenicol acetyltrasferase (CAT) reporter (M2GfosCAT) gene in which the CREs are replaced by binding sites for the yeast transcription factor GAL4 (*8*). CREB binding to the promoter region of M2GfosCAT can be reconstituted in cells upon cotransfection of an expression vector encoding GAL4-CREB, a chimeric protein in which CREB is fused to the DNA binding domain of the yeast transcription factor GAL4. Thus, in the presence of GAL4-CREB, but not a GAL4-CREB mutant in which Ser^{133} is changed to an Ala (GAL4-CREBm1), NGF and other growth factors induce transcription of the M2GfosCAT reporter gene (*8*, *9*). Constitutively active [MEK1(SE218/222)] or dominant-interfering [MEK1(KA97)] MEK was introduced into COS cells together with RSK2, the M2GfosCAT reporter gene, and expression vectors encoding either GAL4-CREB or GAL4-CREBm1. In the presence of GAL4-CREB, MEK1(SE218/222) but not MEK1(KA97) stimulated M2GfosCAT expression. Stimulation of M2GfosCAT expression by the MEK-MAPK-RSK2 signaling pathway was apparently dependent on phosphorylation of GAL4-CREB Ser^{133} because expression of M2GfosCAT was not activated in cells expressing GAL4-CREBm1 (Fig. 5). These results indicate that when the MEK-MAPK pathway is activated, RSK2-mediated phosphorylation of CREB Ser^{133} contributes to the activation of gene expression. Such growth factor–induced phosphorylation of CREB Ser^{133} contributes to the activation of immediate early genes (*8*, *9*) and delayed response genes containing CREB binding sites (*9*).

Induction of c-*fos* transcription by growth factors results from the activation of multiple transcription factors associated with the SRE or the CRE elements. MAPK catalyzes the phosphorylation of the transcription factor ELK-1, which interacts with the SRE (*3*). Our findings demonstrate that MAPK also indirectly influences the transmission of growth factor signals to the nucleus by stimulating CREB kinase(RSK2) to phosphorylate CREB. The phosphorylated transcription factors CREB and ELK-1 then stimulate gene activation through a cooperative mechanism (*8*, *9*). Thus, a bifurcation exists in the RAS-MAPK signaling pathway, both branches of which contribute to growth factor induction of IEG transcription.

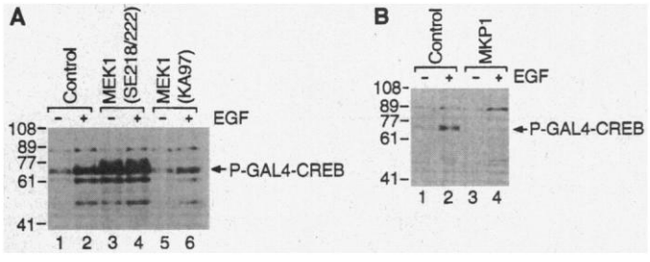

Fig. 4. Regulation of CREB kinase(RSK2) by the RAS-MAPK signaling pathway. (**A**) Regulation of RSK2 activity by MEK. COS cells were transfected (*27*) with 0.5 μg of CMV-GAL4-CREB, 2.5 μg of pMT2-HA-RSK2, and 5 μg of either an empty cloning vector (pCDNA3, lanes 1 and 2), an expression vector encoding a constitutively active form of MEK [pCDNA3-MEK1(SE218/222)] (lanes 3 and 4), or an expression vector encoding a dominant-interfering form of MEK [pCDNA3-MEK1(KA97)] (lanes 5 and 6). Transfected cells were either untreated (lanes 1, 3, and 5) or treated with EGF (30 ng/ml, 5 min) (lanes 2, 4, and 6). Cell lysates were separated on SDS-PAGE and immunoblotted with anti-PCREB. (**B**) Phosphorylation and activation of MAPK is required for RSK2 activation and phosphorylation of CREB Ser^{133}. COS cells transfected (*27*) with 0.5 μg of CMV-GAL4-CREB, 2.5 μg of pMT2-HA-RSK2, and 5 μg of either the empty cloning vector pSG5 or an expression vector encoding the MAPK phosphatase (pSG5-MKP1) were untreated (lanes 1 and 3) or treated with EGF (30 ng/ml, 5 min) (lanes 2 and 4). Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-PCREB.

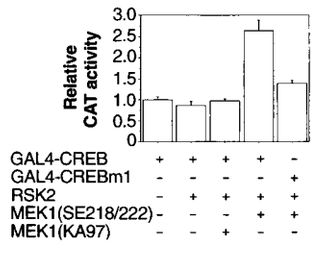

Fig. 5. CREB kinase(RSK2)–mediated CREB Ser^{133} phosphorylation contributes to gene activation. COS cells were transfected (*27*) with M2GfosCAT, GAL4-CREB, GAL4-CREBm1, RSK2, MEK1(SE218/222), or MEK1(KA97) as indicated. A pCMV-β-Gal construct encoding a β-galactosidase gene was cotransfected as an internal control for variations in transfection efficiency and sample handling. Extracts were prepared 2 days after transfection and measured for CAT and β-galactosidase activity (*28*). CAT activity was normalized against β-galactosidase activity from the same extracts. The data are averages from three independent experiments. Error bars, SEM.

REFERENCES AND NOTES

1. H. Herschman, *Annu. Rev. Biochem.* **60**, 281 (1991); R. Treisman, *Curr. Opin. Genet. Dev.* **4**, 96 (1994); R. A. Segal and M. E. Greenberg, *Annu. Rev. Neurosci.* **19**, 463 (1996).
2. J. Blenis, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5889 (1993); C. Marshall, *Curr. Opin. Genet. Dev.* **4**, 82 (1994); R. Seger and E. G. Krebs, *FASEB J.* **9**, 726 (1995).
3. R. Gille, A. D. Sharrocks, P. E. Shaw, *Nature* **358**, 414 (1992); R. Janknecht, W. H. Ernst, V. Pingoud, A. Nordheim, *EMBO J.* **12**, 5097 (1993); R. Marais, J. Wynne, R. Treisman, *Cell* **73**, 381 (1993); C. K. Miranti, D. D. Ginty, G. Huang, T. Chatila, M. E. Greenberg, *Mol. Cell. Biol.* **15**, 3672 (1995).
4. R. Chen, C. Sarnecki, J. Blenis, *Mol. Cell. Biol.* **12**, 915 (1992).
5. V. M. Rivera *et al.*, *ibid.* **13**, 6260 (1993).
6. R. Chen, C. Abate, J. Blenis, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10952 (1993).
7. Y. Zhao, C. Bjørbæk, S. Weremowicz, C. C. Morton, D. E. Moller, *Mol. Cell. Biol.* **15**, 4353 (1995).
8. D. D. Ginty, A. Bonni, M. E. Greenberg, *Cell* **77**, 713 (1994).
9. A. Bonni, D. D. Ginty, H. Dudek, M. E. Greenberg, *Mol. Cell. Neurosci.* **6**, 168 (1995).
10. L. M. Robertson *et al.*, *Neuron* **14**, 241 (1995).
11. L. A. Berkowitz, K. T. Riabowol, M. Z. Gilman, *Mol. Cell. Biol.* **9**, 4272 (1989).
12. J. Xing, D. D. Ginty, M. E. Greenberg, unpublished observations.
13. CREB kinase was purified from approximately 10^{11} K562 cells that were treated with TPA (100 ng/ml) for 5 min. Cells were collected by centrifugation at 4°C (all subsequent purification steps were done at 4°C or on ice) and resuspended in lysis buffer [50 mM β-glycerophosphate (pH 7.3), 1.5 mM EGTA, 1 mM Na_3VO_4, 1 mM dithiothreitol (DTT), 1 mM benzamidine, leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (20 μg/ml), 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Extracts were sonicated and centrifuged at 100,000*g* for 30 min. Supernatants were incubated with S-Sepharose resin [equilibrated in buffer A: 50 mM β-glycerophosphate (pH 7.3), 1.5 mM EGTA, 1 mM Na_3VO_4, 1 mM DTT, 1 mM benzamidine, leupeptin (1 μg/ml), pepstatin (1 μg/ml), aprotinin (2 μg/ml), 1 mM PMSF] for 45 min. The resin was then washed with buffer A, and proteins were eluted with 0.5 M NaCl in buffer A. Eluted fractions were then incubated with phenyl-Sepharose resin (equilibrated in buffer A with 0.4 M NaCl) for 45 min. The resin was washed

with buffer A containing 0.4 M NaCl, and proteins were eluted with buffer A containing 65% ethylene glycol. Fractions were diluted 1:10 in buffer B (buffer A with 0.1% Triton X-100) and incubated with Affi-Gel blue gel (equilibrated in buffer A) for 90 min. The resin was washed with buffer B, and proteins were eluted with 0.75 M NaCl in buffer B. Fractions containing protein were pooled and concentrated with Centriprep 30 (Amicon), and the concentrate was diluted with 10 volumes of buffer B and loaded on a Mono Q column. Proteins were eluted with a linear NaCl gradient (0 to 0.375 M) in buffer B, and column fractions were assayed for CREB kinase activity (*8*). Fractions with high CREB kinase activity were pooled, desalted by size exclusion chromatography, and applied to a Mono S column equilibrated in buffer B. Proteins were eluted with a linear gradient of 0 to 0.375 M NaCl in buffer B, and fractions were assayed for CREB kinase activity. Peak fractions were then pooled and concentrated in a Centricon-30, and proteins from the concentrated sample were separated on SDS-PAGE. The CREB kinase band (identified with an in-gel kinase assay) was excised and eluted from the gel by electroelution. The eluates from several preparations were pooled, concentrated, and subjected to SDS-PAGE. The CREB kinase polypeptide was then transferred to a polyvinylidene difluoride membrane for protein digestion and peptide sequencing.

14. Protease digestion of purified CREB kinase with the endoproteinase Lys-C, peptide isolation by high-performance liquid chromatography, and peptide sequencing were done by the Harvard Microchemistry Facility (Cambridge, Massachusetts). Sequences of four different peptides were obtained: EIAITHVK, ISG_DARQ_YAMK, L_YAFQTEGK, ATNMEF_V (*25*). These sequences matched 100% with those of amino acids 49 to 57, 88 to 100, 133 to 142, and 443 to 450 of human RSK2, respectively, and shared 75% overall identity with corresponding regions of human RSK1 or RSK3. Underlined blanks in peptide sequences represent residues that could not be determined unambiguously.
15. D. A. Alcorta *et al.*, *Mol. Cell. Biol.* **9**, 3850 (1989).
16. D. E. Moller, C. Xia, W. Tang, A. X. Zhu, M. Jakubowski, *Am. J. Physiol.* **266**, C351 (1994).
17. C. Bjørbæk *et al.*, *Diabetes* **44**, 90 (1995).
18. S. W. Jones, E. Erikson, J. Blenis, J. L. Maller, R. L. Erikson, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3377 (1988).
19. J. R. Grove *et al.*, *Biochemistry* **32**, 7727 (1993).
20. To generate the RSK2 expression vector pMT2-HA-RSK2, we cloned a sequence encoding the HA-tag (YPYDVPDYA) (*25*) into the NH_2-terminus of murine RSK2 using the polymerase chain reaction, and the HA-tagged RSK2 cDNA was cloned into the expression vector pMT2. We constructed the mutant RSK2 expression vector pMT2-HA-RSK2(KR100) by changing the coding determinants of Lys^{100} of RSK2 to Arg by site-directed mutagenesis [M. P. Weiner *et al.*, *Gene* **151**, 119 (1994)]. The following plasmids have been described: CMV-GAL4-CREB encoding a hybrid protein with the NH_2-terminal 147 amino acids of the yeast transcription factor GAL4 fused to the full-length CREB protein [L. A. Berkowitz and M. Z. Gilman, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5258 (1990)], pSG5-MKP1 (*24*), pCDNA3-MEK1(KA97) [R. Seger *et al.*, *J. Biol. Chem.* **269**, 25699 (1994)], and pCMV-Elk-1 [C. K. Miranti *et al.*, *Mol. Cell. Biol.* **15**, 3672 (1995)]. The constitutively active MEK expression vector pCDNA3-MEK1(SE218/222) was made by replacement of Ser^{218} and Ser^{222} with Glu residues.
21. D. D. Ginty *et al.*, *Science* **260**, 238 (1993).
22. T. W. Sturgill, L. B. Ray, E. Erikson, J. L. Maller, *Nature* **334**, 715 (1988); J. Chung, S. L. Pelech, J. Blenis, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4981 (1991); C. Sutherland, D. G. Campbell, P. Cohen, *Eur. J. Biochem.* **212**, 581 (1993).
23. J. C. Scimeca, T. T. Nguyen, C. Filloux, E. Van Obberghen, *J. Biol. Chem.* **267**, 17369 (1992); K. Hsiao, S. Chou, S. Shih, J. E. Ferrell Jr., *Proc. Natl. Acad. Sci. U.S.A.* **91**, 5480 (1994).
24. H. Sun, C. H. Charles, L. F. Lau, N. K. Tonks, *Cell* **75**, 487 (1993); C. H. Charles, H. Sun, L. F. Lau, N. K. Tonks, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5292 (1993).
25. Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.
26. For immune complex kinase assays, cells were washed once in cold phosphate-buffered saline (PBS) and then quickly collected in Triton X-100 lysis buffer [20 mM tris (pH 6.8), 137 mM NaCl, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 1% Triton X-100, 10% glycerol, 1 mM benzamidine, leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (20 μg/ml), and 1 mM PMSF]. The lysates were mixed and centrifuged at 13,000*g* for 10 min at 4°C. Antibodies to RSKs and protein A–Sepharose beads (washed in lysis buffer) were then added to the supernatants. After a 1-hour incubation at 4°C, the immune complex was collected by centrifugation and washed twice with lysis buffer and twice with kinase buffer [50 mM Pipes (pH 7.3), 10 mM $MgCl_2$, and 1 mM DTT]. Washed immune complexes were used to phosphorylate CREBtide or recombinant CREB in kinase reactions as described (*8*).
27. Transfection of COS cells was done by the DEAE-dextran method [F. M. Ausubel *et al.*, Eds., *Current Protocols in Molecular Biology* (Wiley, New York, 1994)]. Two days after transfection, cells were treated as described (Figs. 3 and 4). Cells were washed in ice-cold PBS and lysed in boiling SDS sample buffer for in-gel kinase assay or immunoblotting analysis. Alternatively, cells extracts were prepared and subjected to an immune complex kinase assay (*26*).
28. Assays for CAT and β-galactosidase were done as described [J. Sambrook *et al.*, Eds., *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)].
29. We thank D. E. Moller and Y. Zhao for providing antibodies to RSK3, R. L. Erikson for murine RSK2 cDNA, E. G. Krebs for plasmids pCDNA3-MEK1(SE218/222) and pCDNA3-MEK1(KA97), N. K. Tonks and H. Sun for pSG5-MKP1, M. Z. Gilman for CMV-GAL4-CREB, S. H. Orkin for cloning vector pMT2, members of the Greenberg laboratory for critical reading of the manuscript, A. Bonni and Z. Xia for helpful discussions, and S. E. Kim and Y. Zhang for technical assistance. D.D.G. is a recipient of a Klingenstein Award in Neuroscience, an American Cancer Society Junior Faculty Research Award, and an Alfred Sloan Research Fellowship. Supported by National Institutes of Health grants NS34814-01 (D.D.G.) and CA43855 (M.E.G.), Mental Retardation Research Center grant NIH P30-HD18655, and an American Cancer Society Faculty Research Award (FRA-379) (M.E.G.).

2 January 1996; accepted 24 June 1996

Control of MHC Restriction by TCR V_α CDR1 and CDR2

Bee-Cheng Sim, Loukia Zerva, Mark I. Greene, Nicholas. R. J. Gascoigne*

Individual T cell receptor (TCR) V_α elements are expressed preferentially in CD4 or CD8 peripheral T cell subsets. The closely related V_α3.1 and V_α3.2 elements show reciprocal selection into CD4 and CD8 subsets, respectively. Transgenic mice expressing site-directed mutants of a V_α3.1 gene were used to show that individual residues in either the complementarity-determining region 1 (CDR1) or CDR2 were sufficient to change selection from the CD4 subset to the CD8 subset. Thus, the germline-encoded V_α elements are a major influence on major histocompatibility class complex (MHC) restriction, most likely by a preferential interaction with one or the other class of MHC molecule.

Thymocytes that are positively selected on MHC class I proteins become peripheral $CD8^+$ cells and those positively selected on MHC class II proteins become $CD4^+$ peripheral T cells (*1*). An αβ TCR transgene from a $CD8^+$ T cell causes most T cells bearing that receptor to be positively selected into the $CD8^+$ compartment (*2*), whereas a transgenic TCR from a $CD4^+$ cell shows similarly skewed expression in the $CD4^+$ population (*3*). Less extreme skewing into the CD4 or CD8 peripheral T cell subset is also seen with individual V regions—most noticeably with V_α regions, which are preferentially expressed in one or the other subset (*4–9*). This phenomenon is largely independent of MHC haplotype and suggests either that individual V_α regions react preferentially with class I or class II molecules or that particular V_α regions associate with the CD4 or CD8 coreceptors.

The structure of the TCR V_α domain has recently been determined (*10*). The CDR3 segments of TCR V_α and V_β [produced by VJ or V(D)J recombination, respectively] are predicted to lie centrally in the combining site of the TCR (*10*, *11*). If CDR3 interacts with the peptide bound in the MHC groove, the less variable germline-encoded CDR1- and CDR2-equivalent regions would be available to interact with the MHC α helices. Thus, the skewed expression of V_α elements in CD4 and CD8 subsets suggests that the CDR1 and CDR2 of V_α could play a role in distinguishing between class I and II. Closely related members of the V_α3 family undergo selection by different MHC classes (*7*, *8*), allowing determination of the residues involved in MHC class discrimination. In B6 mice,

B.-C. Sim and N. R. J. Gascoigne, Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.
L. Zerva and M. I. Greene, Department of Pathology, University of Pennsylvania, Philadelphia, PA 19104, USA.

*To whom correspondence should be addressed. E-mail: gascoigne@scripps.edu