rest. Rsk-2 is required for Mos–MEK-1–p42 MAPK–induced suppression of Cdc2 inactivation and also for suppression of mitotic exit subsequent to Cdc2 inactivation. As shown in the accompanying report (*26*), an activated form of Rsk-1 is capable of causing a CSF arrest; thus, Rsk activation appears to be both necessary and sufficient for CSF arrest. Rsk proteins are therefore critical targets of p42 MAPK in the regulation of cell cycle progression and the development of fertilizable eggs.


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18. *Xenopus* Rsk-2 is 92% identical to human Rsk-2 (ISPK-1) and 79% identical to *Xenopus* Rsk-1. From quantitative immunoblotting experiments with purified recombinant Rsk-1 and Rsk-2 proteins as standards, we have estimated the oocyte concentration of Rsk-1 to be 2 to 6 nM and Rsk-2 to be ~100 nM. The *Xenopus* Rsk-2 sequence has been submitted to the National Center for Biotechnology Information (accession number AF165162).
19. Cycling extracts were prepared as described (*28*) with one modification; we waited 30 min after electrically activating the eggs before crushing them, rather than the usual 15 min. This resulted in extracts that were usually too far progressed to undergo $G_2$ arrest in response to added Mos (*7, 8*), making the extracts better suited for examining Mos-induced mitotic arrests. CSF extracts were prepared as described (*28*).
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21. Mos was expressed in bacteria as a maltose-binding protein (malE) fusion (*5*). The malE-Mos fusion protein was purified as described (*5*).
22. R. R. Bhatt and J. E. Ferrell Jr., unpublished data.
23. Rsk-2 immunodepletion was accomplished by incubating extracts for 75 min on ice with antibodies to Rsk-2 (Santa Cruz Biotechnology) that had been prebound to protein G agarose beads (Gibco-BRL). Mock depletions were carried out similarly, with the antibody omitted.
24. Immunodepletion of Rsk-1 yielded extracts that still underwent a CSF arrest in response to Mos. Thus, Rsk-1 appears not to be necessary for CSF arrest.
25. $(His)_6$-tagged Rsk-2 proteins (with His tag inserted directly after the putative translation initiation methionine codon) were produced in Sf9 cells and purified to homogeneity by nickel chelate chromatography. Catalytically inactive Rsk-2 (KR Rsk-2) was engineered by substituting arginine for lysine at residue 97.
26. We also titrated different concentrations of Rsk-1 or Rsk-2 into Rsk-2–depleted extracts. We found that 100 nM Rsk-1 or Rsk-2 was sufficient to restore the arrest, but 20 nM, 4 nM, and 1 nM were insufficient. These findings indicate that the Rsks are at least partially interchangeable, but by virtue of its higher normal concentration (*18*) Rsk-2 is the more important Rsk in this this context. Our results fit well with the recent finding that a gain-of-function form of Rsk-1 can cause CSF arrest [S. G. Gross, M. S. Schwab, A. L. Lewellyn, J. L. Maller, *Science* **286**, 1365 (1999)].
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29. We thank M. Murakami and G. Vande Woude for Mos plasmids, C.-Y. F. Huang for preparing Mos protein, and M. L. Sohaskey and S. A. Walter for comments on the manuscript. Supported by a grant from NIH (GM46383) and a National Research Service Award Predoctoral Fellowship (GM16415).

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# Induction of Metaphase Arrest in Cleaving *Xenopus* Embryos by the Protein Kinase $p90^{Rsk}$


**Stefan D. Gross, Markus S. Schwab, Andrea L. Lewellyn, James L. Maller***



**Before fertilization, vertebrate eggs are arrested in metaphase of meiosis II by cytostatic factor (CSF), an activity that requires activation of the mitogen-activated protein kinase (MAPK) pathway. To investigate whether CSF arrest is mediated by the protein kinase $p90^{Rsk}$, which is phosphorylated and activated by MAPK, a constitutively activated (CA) form of Rsk was expressed in *Xenopus* embryos. Expression of CA Rsk resulted in cleavage arrest, and cytological analysis showed that arrested blastomeres were in M phase with prominent spindles characteristic of meiotic metaphase. Thus, Rsk appears to be the mediator of MAPK-dependent CSF arrest in vertebrate unfertilized eggs.**


The unfertilized eggs of most vertebrates are naturally arrested at metaphase of meiosis II. Upon fertilization, an increase in free calcium activates the anaphase-promoting complex, which drives exit from mitosis and entry into the first embryonic cell cycle. The enzymatic activity that causes metaphase arrest is CSF; it appears in meiosis II during oocyte maturation and disappears shortly after fertilization (*1*). Microinjection of CSF into blastomeres of cleaving embryos causes arrest in metaphase of the next cell cycle (CSF arrest).

CSF has not been purified, and therefore its molecular composition is unknown. However, the MAPK pathway is required for the generation of CSF activity. The *c-mos* proto-oncogene product (Mos), which is a MAPK kinase kinase, is synthesized during meiosis in *Xenopus* and mice, and its expression is sufficient to produce CSF arrest in injected blastomeres (*2, 3*). CSF arrest can also be induced by a thiophosphorylated, phosphatase-resistant form of MAPK (*4*).

In *Xenopus* eggs and other systems, the 90-kD ribosomal protein S6 kinase ($p90^{Rsk}$) is directly phosphorylated and activated by MAPK (*5*); during oocyte maturation, activation of $p90^{Rsk}$ closely parallels that of MAPK, and both enzymes are dephosphorylated and deactivated after fertilization, when CSF activity also disappears (*3, 6*). Cloning of $p90^{Rsk}$ in *Xenopus* and mammalian systems revealed a structure with two distinct kinase domains (*7*). Three different isoforms of $p90^{Rsk}$, termed Rsk1, Rsk2, and Rsk3, are present in mammalian cells, and all have the same basic two-domain structure (*7, 8*). Activation of $p90^{rsk}$ requires phosphorylation at two specific sites ($Thr^{570}$ and $Ser^{362}$) by MAPK and autophosphorylation at a specific site ($Ser^{378}$) by the COOH-terminal kinase domain (Fig. 1A) (*9*). Activity also requires phosphorylation by an unidentified kinase of a site in the middle of the activation loop of the $NH_2$-terminal kinase domain ($Ser^{220}$) (*9*).

To evaluate a possible role for $p90^{Rsk}$ in mediating CSF arrest, we generated a constitutively active form of the enzyme. Inasmuch as the phosphorylation of exogenous substrates by $p90^{Rsk}$ is mediated by the $NH_2$-terminal kinase domain (*10*), we generated a construct in which the COOH-terminal domain was deleted completely with or without a truncation of the $NH_2$-terminal domain similar to that which causes constitutive activation of MAPK kinase (*11,*


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*12*). To evaluate their activation in vivo, we injected mRNAs encoding FLAG-tagged versions of these two constructs or the wild-type full-length enzyme (Fig. 1A) into resting stage VI oocytes. Some of the oocytes were then treated with progesterone to cause meiotic maturation, which activates endogenous Rsk to 75% of its maximum activity by the time of germinal vesicle breakdown (GVBD) (*6*). Rsk proteins were isolated from resting and GVBD oocytes with antibody to FLAG coupled to agarose beads and assayed for kinase activity in vitro (*13*). The $NH_2$- and COOH-terminally truncated enzyme (CA Rsk) was activated by progesterone treatment to a specific activity 4 times that of wild-type Rsk and 12 times that of Rsk with only the COOH-terminal truncation (ΔCT Rsk) (Fig. 1B).

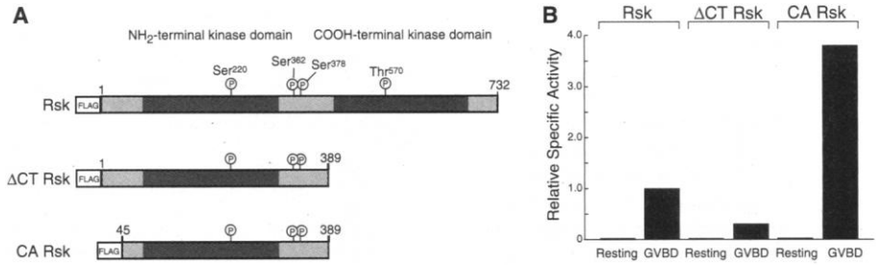


**Fig. 1.** A truncation mutant of $p90^{Rsk1}$ is hyperactivated in vivo. (**A**) Primary structure of $Xp90^{Rsk}$ and deletion constructs (*12*). For Rsk, the two kinase domains and known phosphorylation sites (P) are indicated. ΔCT Rsk denotes a construct in which the entire COOH-terminal kinase domain (ΔCT) has been removed. CA Rsk is a construct identical to ΔCT except that 44 amino acids at the $NH_2$-terminus were also deleted. All constructs have an identical $NH_2$-terminal FLAG tag. (**B**) Hyperactivation of CA Rsk in vivo. Messenger RNAs encoding the FLAG-tagged constructs were injected into resting oocytes. After incubation for 18 hours, one group of oocytes was treated with progesterone to induce maturation, and after GVBD, immunoprecipitates of the activated enzymes were assessed for kinase activity or blotted with antibodies to FLAG to determine the amount of expression of each construct (*13*). Specific activities were calculated by normalizing total kinase activity to densitometric measurements of FLAG immunoreactivity and are shown relative to the activity of Rsk at GVBD.

To assess whether MAPK could activate Rsk in vitro, we incubated with MAPK the FLAG-tagged wild-type protein recovered from a resting oocyte (*13*). MAPK activated the enzyme 70-fold and a $Ser^{220} \rightarrow Ala^{220}$ mutant was not activated at all (Fig. 2A). The sequence around $Ser^{220}$ is very closely related to a phosphorylation site in the $p70^{S6K}$ that is phosphorylated by 3-phosphatidylinositol–dependent kinase–1 (PDK-1) (*14*). When recombinant PDK-1 was incubated with FLAG-tagged Rsk from resting oocytes, activity increased minimally, by about twofold. However, when both PDK-1 and MAPK were incubated with Rsk, activity increased nearly 100-fold (Fig. 2). In contrast, the presence of both MAPK and PDK-1 caused only about a fivefold activation of CA Rsk and ΔCT Rsk, which lack the MAPK phosphorylation site at $Thr^{570}$. Recombinant Rsk lacking $Ser^{220}$ was not activated. These results indicate that the kinase mediating the required phosphorylation at $Ser^{220}$ in Rsk may be PDK-1 (*9*) and this site is highly phosphorylated even in resting oocytes. For a comparison of the activation in vitro with that in vivo, oocytes expressing these constructs that had undergone GVBD were assayed for Rsk activity, which was compared to that in resting oocytes (Fig. 2B). Rsk was activated 55-fold whereas ΔCT Rsk and CA Rsk were activated almost 25-fold despite their different specific activities (Fig. 1B). The activation of Rsk in vivo at GVBD may be less than that in vitro because only 50 to 75% of endogenous Rsk is activated at GVBD (*6*).

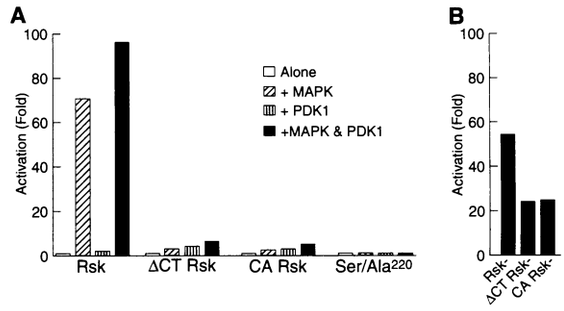


**Fig. 2.** Activation of $p90^{Rsk}$ by MAPK and PDK-1 in vitro or at GVBD. (**A**) In vitro activation of $p90^{Rsk1}$. Messenger RNAs encoding FLAG-tagged full-length wild-type Rsk, ΔCT Rsk, CA Rsk, or $Ser^{220} \rightarrow Ala^{220}$ Rsk (Ser/$Ala^{220}$) were injected into resting oocytes. After incubation for 18 hours, FLAG-tagged protein was immunoprecipitated and phosphorylated with either MAPK or PDK-1, or both, as indicated. Subsequently, the immunoprecipitates were washed and assayed for S6 peptide kinase activity (*13*). To control for differences in expression of each construct, we expressed activation as fold activation over activity present in the absence of phosphorylation by either MAPK or PDK-1. (**B**) In vivo activation at GVBD. Messenger RNAs encoding the indicated constructs were injected, and Rsk proteins were isolated as immune precipitates from resting and GVBD oocytes. Subsequently, kinase assays were performed, and fold activation is expressed in relation to that in resting oocytes.

Because CA Rsk had achieved a higher specific activity than the other Rsk variants in oocytes undergoing maturation, we evaluated its ability to cause CSF arrest after injection of mRNA encoding FLAG-tagged CA Rsk into one blastomere of a two-cell embryo (*15*). Injected blastomeres ceased dividing after two or three divisions, whereas the uninjected side continued to divide (Fig. 3). Expression of β-galactosidase (β-Gal), Rsk, or ΔCT-Rsk did not cause cleavage arrest. The arrest caused by CA Rsk was similar to that seen after injection of mRNA encoding Mos in both timing and appearance (Fig. 3) (*2*).

Confocal microscopy of arrested embryos stained for DNA and α-tubulin revealed metaphase spindles that exhibited a meiotic-like morphology in that they lacked prominent asters and exhibited the classical "barrel" morphology (Fig. 4) (*16*–*18*). In some cases, two spindles were seen in one large blastomere, perhaps reflecting failure of cytokinesis as previously reported for Mos overexpression in somatic cells (*18*). The spindles present in blastomeres arrested after the injection of Mos mRNA were very similar in morphology to those in blastomeres injected with CA Rsk (Fig. 4). These results indicate that arrested blastomeres are in metaphase and that Rsk may be a mediator of CSF activity.

During meiosis, MAPK is part of a feedback loop that promotes synthesis of Mos, thereby strengthening signal transduction through the pathway (*3, 19*). In arrested blastomeres expressing CA Rsk, Mos was undetectable, and MAPK and endogenous Rsk were not activated, whereas both MAPK and Rsk were activated in Mos-expressing blastomeres (Fig. 5A). This suggests that expression of CA Rsk did not induce expression of Mos to activate the MAPK pathway and produce CSF arrest but rather is the immediate downstream component of this pathway mediating the arrest. In CA Rsk- and Mos-arrested blastomeres, an increased fraction of cyclin B2 was present in a slower migrating form, characteristic of M phase (Fig. 5A) (*3*).

To compare the level of total Rsk activity in embryos expressing β-Gal, Rsk, ΔCT Rsk, CA Rsk, and Mos, we assayed extracts of these embryos for total S6 kinase activity in vitro. CA Rsk expression resulted in a level of Rsk activity equivalent to 50% of that caused by the expression of Mos (Fig. 5B). Kinase assays of FLAG immunoprecipitates from these extracts revealed that only CA Rsk produced substantial kinase activity (Fig. 5C), demonstrating the constitutive activation

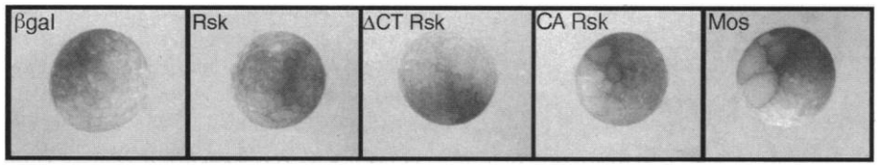


**Fig. 3.** Cleavage arrest in blastomeres caused by CA Rsk. One blastomere of a two-cell embryo was injected with mRNA encoding either β-Gal, Rsk, ΔCT Rsk, CA Rsk, or Mos, as indicated (*15*). Development was monitored with a dissecting microscope and photographed when control embryos had reached stage 7. Similar results were obtained in six independent experiments.

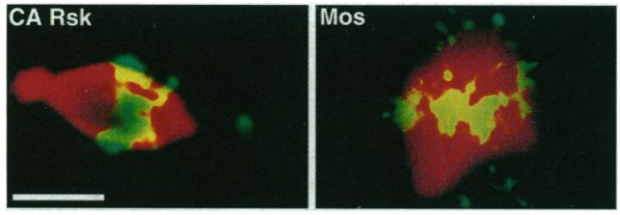


**Fig. 4.** Metaphase spindles in blastomeres arrested by CA Rsk or Mos. Blastomeres arrested after injection of mRNA for either CA Rsk or Mos (Fig. 3) were fixed 5 hours after injection and analyzed by confocal microscopy for spindles as described (*16*). Red indicates α-tubulin, green indicates DNA, and yellow indicates an overlapping signal. Scale bar, 10 μm.

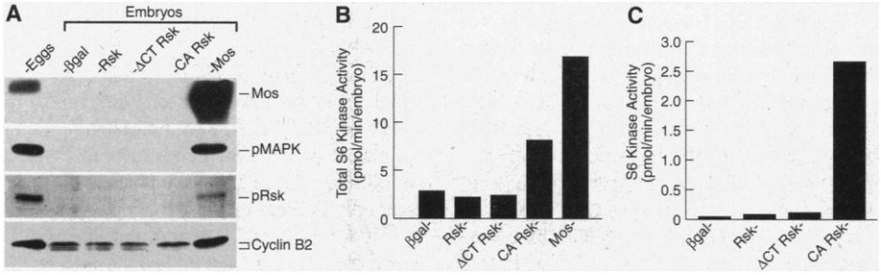


**Fig. 5.** CSF arrest induced by CA Rsk without activation of the endogenous MAPK pathway. (**A**) Biochemical analysis of CSF arrest. Both blastomeres of a two-cell embryo were injected with FLAG-tagged mRNA encoding β-Gal, Rsk, ΔCT Rsk, CA Rsk, or Mos, as indicated. Five hours after CSF arrest induced by either CA Rsk or Mos, arrested blastomeres were isolated and analyzed for the indicated individual components by protein immunoblotting. pMAPK, phosphorylated (active) MAPK; pRsk, Rsk phosphorylated on $Ser^{362}$, which denotes the activated state of endogenous Rsk (*9, 21*). (**B**) Total Rsk activity in eggs and embryos. Extracts from embryos expressing β-Gal, Rsk, ΔCT Rsk, CA Rsk, or Mos as in (A) were assayed for S6 peptide kinase activity. (**C**) S6 peptide kinase activity of FLAG immunoprecipitates from embryos expressing β-Gal, Rsk, ΔCT Rsk, and CA Rsk. The same extracts as in (B) were also immunoprecipitated with antibodies to FLAG coupled to agarose and assayed for S6 peptide kinase activity.

of CA Rsk in blastomeres. Whether altered signaling, changes in localization, or some other mechanism accounts for the high CA Rsk activity in blastomeres versus that in resting oocytes remains to be determined.

These results indicate that the meiotically activated Mos/MAPK pathway causes CSF arrest through the MAPK-dependent activation of $p90^{Rsk}$. Because Rsk expression did not activate the endogenous MAPK pathway, MAPK requires no other substrate for induction of CSF arrest. The importance of Rsk in CSF activity is supported by a study by Bhatt and Ferrell (*20*), who show that Rsk is necessary for expression of CSF activity in egg extracts. Our data indicate that CSF arrest is not a consequence of direct regulation of the spindle assembly checkpoint or the anaphase-promoting complex by MAPK.

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12. The parent *Xenopus* Rsk sequence (*7*) is closely related in overall sequence to mammalian Rsk1 and Rsk2 but is designated Rsk1 on the basis of a COOH-terminal region specific to mammalian Rsk1 isoforms. The following primers were used to generate deletion constructs of Rsk with the polymerase chain reaction: primer 1, GACGACGACAAGATGCCGCTGGCACAGCTGGTGAATCTTTGG; primer 2, GACGACGACAAGATGCCGATCACTCATCATGTTAAAGAAGGTTCG; primer 3, GAGGAGAAGCCCGGTGGCTCCAGCTAAAGAGTAGTAGAAGG; and primer 4, GAGGAGAAGCCCGGTCTAATCTTCCTCAACCAATGCTGGAGCCAC. Primers 1 and 3 were used to amplify the full-length wild-type $Xp90^{Rsk}$ (Rsk). Primers 1 and 4 and 2 and 4 were used to create constructs ΔCT Rsk and CA Rsk, respectively. Polymerase chain reaction products were subcloned into pOTV-FLAG-LIC with a ligation-independent cloning method (Novagen, Madison, WI). With the T7 promoter, this vector directs the synthesis of mRNA coding for a FLAG epitope fusion protein of the inserted gene. Messenger RNA was synthesized for each of the constructs with the T7 message machine kit (Ambion, Austin, TX).
13. Oocytes and embryos were isolated and prepared as described elsewhere (*3*). Oocytes were cultured in a 0.65× concentration of Dulbecco's minimum essential medium with gentamycin (50 μg/ml) (Gibco-BRL), microinjected with a 40-nl volume (50 ng each of Rsk, ΔCT Rsk, or CA Rsk mRNA in $H_2O$), incubated overnight at 18°C, induced with progesterone (100 ng/ml), and collected 3 hours after GVBD for immunoprecipitation/kinase assays and protein immunoblotting with the M2 FLAG monoclonal antibody to determine specific activity. Oocytes or embryos were resuspended in 20 μl of extraction buffer (EB) [20 mM Hepes (pH 7.5), 100 mM NaCl, 0.5% NP-40, 10 mM EDTA, 5 mM EGTA, 1 mM $Na_3VO_4$, 10 μM microcystin LR (Calbiochem, La Jolla, CA), and 1× Complete protease inhibitor set (Boehringer Mannheim)] per oocyte, homogenized, and spun for 5 min at 16,000*g*. For immunoprecipitation, supernatants were supplemented with 10 μg of FLAG antibody coupled to agarose beads incubated for 60 min at 4°C, and washed two times with EB, two times with wash buffer [50 mM tris (pH 7.5) and 750 mM NaCl], and one time with kinase assay buffer [50 mM tris (pH 7.5), 50 mM NaCl, 5 mM EGTA, and 1 mM β-mercaptoethanol]. For kinase assays, a 20-μl immunoprecipitate (one oocyte or embryo equivalent) was supplemented with 10 mM $MgCl_2$, 100 μM adenosine 5′-triphosphate (ATP), 5 μCi [$\gamma$-$^{32}$P]ATP, and 250 μM S6 peptide (Upstate Biotechnology, Waltham, MA). Where indicated in Fig. 2, assays also contained 10 units of MAPK (New England Biolabs, Beverly, MA) or 2.5 ng of activated PDK-1 (Upstate Biotechnology). Reactions were incubated at 30°C for 60 min and quenched by the addition of EDTA to 60 mM. The beads were removed by centrifugation at 16,000*g*, and 10 μl of the supernatant were spotted onto p81 paper in duplicate, washed, dried, and counted by the Cerenkov method. Protein immunoblots were performed essentially as described elsewhere (*3, 4*).
14. N. Pullen *et al.*, *Science* **279**, 707 (1998); D. R. Alessi *et al.*, *Curr. Biol.* **8**, 69 (1998).
15. Embryos were fertilized in vitro as previously described (*4*). Blastomeres of two-cell embryos were injected with 30 nl of mRNA (12.5 to 25 ng) encoding β-Gal, Mos, Rsk, ΔCT Rsk, or CA Rsk and then monitored with a dissecting microscope.
16. Confocal methods were performed essentially as described by Y.-W. Qian *et al.*, *Mol. Cell. Biol.* **18**, 4262 (1998).
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21. Phosphospecific MAPK antibodies were from New England Biolabs. Phosphospecific antibody to $Ser^{362}$ in Rsk was from P. Cohen (University of Dundee, Dundee, UK). Both antibodies were shown in other experiments to accurately reflect the activity state of MAPK and Rsk during maturation. $Mos^{xe}$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and antibody to cyclin B2 were used as previously described (*3*).


22. We thank P. Cohen for providing antibodies to $p90^{Rsk}$ (*9*) and E. Erikson for a critical reading of the manuscript. Supported by grant DK28353 from NIH. S.D.G. and M.S.S. are Associates and J.L.M. is an Investigator of the Howard Hughes Medical Institute.

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## References and Notes

[4] **Induction of Metaphase Arrest in Cleaving Xenopus Embryos by MAP Kinase**

Olivier Haccard; Boris Sarcevic; Andrea Lewellyn; Rebecca Hartley; Linda Roy; Tetsuro Izumi; Eleanor Erikson; James L. Maller

*Science*, New Series, Vol. 262, No. 5137. (Nov. 19, 1993), pp. 1262-1265.

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http://links.jstor.org/sici?sici=0036-8075%2819931119%293%3A262%3A5137%3C1262%3AIOMAIC%3E2.0.CO%3B2-L

[7] **A Xenopus Ribosomal Protein S6 Kinase Has Two Apparent Kinase Domains that are Each Similar to Distinct Protein Kinases**

Steven W. Jones; Eleanor Erikson; John Blenis; James L. Maller; R. L. Erikson

*Proceedings of the National Academy of Sciences of the United States of America*, Vol. 85, No. 10. (May 15, 1988), pp. 3377-3381.

Stable URL:

http://links.jstor.org/sici?sici=0027-8424%2819880515%2985%3A10%3C3377%3AAXRPSK%3E2.0.CO%3B2-O

[11] **Transformation of Mammalian Cells by Constitutively Active MAP Kinase Kinase**

Sam J. Mansour; Wayne T. Matten; April S. Hermann; Julian M. Candia; Sing Rong; Kenji Fukasawa; George F. Vande Woude; Natalie G. Ahn

*Science*, New Series, Vol. 265, No. 5174. (Aug. 12, 1994), pp. 966-970.

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http://links.jstor.org/sici?sici=0036-8075%2819940812%293%3A265%3A5174%3C966%3ATOMCBC%3E2.0.CO%3B2-5

# LINKED CITATIONS



[14] **Phosphorylation and Activation of p70 <sup> s6k</sup> by PDK1**

Nicholas Pullen; Patrick B. Dennis; Mirjana Andjelkovic; Almut Dufner; Sara C. Kozma; Brian A. Hemmings; George Thomas

*Science*, New Series, Vol. 279, No. 5351. (Jan. 30, 1998), pp. 707-710.

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[18] **Mos Overexpression in Swiss 3T3 Cells Induces Meiotic-Like Alterations of the Mitotic Spindle**

Kenji Fukasawa; George F. Vande Woude

*Proceedings of the National Academy of Sciences of the United States of America*, Vol. 92, No. 8. (Apr. 11, 1995), pp. 3430-3434.

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http://links.jstor.org/sici?sici=0027-8424%2819950411%2992%3A8%3C3430%3AMOIS3C%3E2.0.CO%3B2-2

[20] **The Protein Kinase p90 Rsk as an Essential Mediator of Cytostatic Factor Activity**

Ramesh R. Bhatt; James E. Ferrell Jr.

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