Bub1 is activated by the protein kinase p90^{Rsk} during *Xenopus* oocyte maturation

Markus S. Schwab*, B. Tibor Roberts†, Stefan D. Gross‡, Brian J. Tunquist, Frédéric E. Taieb, Andrea L. Lewellyn and James L. Maller

Background: The kinetochore attachment (spindle assembly) checkpoint arrests cells in metaphase to prevent exit from mitosis until all the chromosomes are aligned properly at the metaphase plate. The checkpoint operates by preventing activation of the anaphase-promoting complex (APC), which triggers anaphase by degrading mitotic cyclins and other proteins. This checkpoint is active during normal mitosis and upon experimental disruption of the mitotic spindle. In yeast, the serine/threonine protein kinase Bub1 and the WD-repeat protein Bub3 are elements of a signal transduction cascade that regulates the kinetochore attachment checkpoint. In mammalian cells, activated MAPK is present on kinetochores during mitosis and activity is upregulated by the spindle assembly checkpoint. In vertebrate unfertilized eggs, a special form of meiotic metaphase arrest by cytostatic factor (CSF) is mediated by MAPK activation of the protein kinase p90^{Rsk}, which leads to inhibition of the APC. However, it is not known whether CSF-dependent metaphase arrest caused by p90^{Rsk} involves components of the spindle assembly checkpoint.

Results: xBub1 is present in resting oocytes and its protein level increases slightly during oocyte maturation and early embryogenesis. In *Xenopus* oocytes, Bub1 is localized to kinetochores during both meiosis I and meiosis II, and the electrophoretic mobility of Bub1 upon SDS-PAGE decreases during meiosis I, reflecting phosphorylation and activation of the enzyme. The activation of Bub1 can be induced in interphase egg extracts by selective stimulation of the MAPK pathway by c-Mos, a MAPKKK. In oocytes treated with the MEK1 inhibitor U0126, the MAPK pathway does not become activated, and Bub1 remains in its low-activity, unshifted form. Injection of a constitutively active target of MAPK, the protein kinase p90^{Rsk}, restores the activation of Bub1 in the presence of U0126. Moreover, purified p90^{Rsk} phosphorylates Bub1 in vitro and increases its protein kinase activity.

Conclusions: Bub1, an upstream component of the kinetochore attachment checkpoint, is activated during meiosis in *Xenopus* in a MAPK-dependent manner. Moreover, a single substrate of MAPK, p90^{Rsk}, is sufficient to activate Bub1 in vitro and in vivo. These results indicate that in vertebrate eggs, kinetochore attachment/spindle assembly checkpoint proteins, including Bub1, are downstream of p90^{Rsk} and may be effectors of APC inhibition and CSF-dependent metaphase arrest by p90^{Rsk}.

Address: Howard Hughes Medical Institute and Department of Pharmacology, University of Colorado, School of Medicine, 4200 East Ninth Ave., Denver, Colorado 80262, USA

Correspondence: James L. Maller
E-mail: jim.maller@uchsc.edu

Present addresses: *CellZome GmbH, Meyerhofstr. 1, D-69117 Heidelberg, Germany. †Laboratory of Biochemistry and Genetics, NIDDK, NIH, Bethesda, Maryland 20892, USA. ‡Agouron Pharmaceuticals, La Jolla, California 92037, USA.

Received: **13 November 2000**
Revised: **22 December 2000**
Accepted: **5 January 2001**

Published: **6 February 2001**

Current Biology 2001, 11:141–150

0960-9822/01/$ – see front matter

Background

Recent evidence from many laboratories has identified the metaphase/anaphase transition in the cell cycle as a target of multiple checkpoint controls. Most extensively studied has been the spindle assembly checkpoint, also known as the kinetochore attachment checkpoint (for review see [1]). This checkpoint is thought to measure microtubule tension or occupancy on kinetochores [2, 3], and a single unattached kinetochore is sufficient to activate the checkpoint [4, 5]. Substantial genetic and biochemical evidence indicates that spindle assembly checkpoint activation blocks the metaphase/anaphase transition by preventing activation of the anaphase-promoting complex (APC) [6, 7]. The APC is an E3 ubiquitin ligase that targets mitotic cyclins and regulators of chromatid cohesion for degradation by the proteosome pathway at the metaphase/anaphase transition [8]. Regulation of the APC is complex. Early genetic screens in budding yeast identified seven genes, BUB1, 2, 3, (budding uninhibited in benzimidazole) [9], MAD1, 2, 3 (mitotic arrest deficient [10]), and MPS1 (monopolar spindle) [11, 12] that function in the checkpoint (for reviews see [13, 14]). Recently, most of the homologs in higher eukaryotes have been cloned. The Bub1 protein kinase is a crucial component

of the signaling cascade from unattached kinetochores that prevents activation of the APC (for review see [8, 13, 14]). In yeast, dominant Bub1 alleles block anaphase onset, and loss of function of Bub1 abrogates the spindle checkpoint [9, 15]. Bub1 and the kinetochore attachment checkpoint become activated not only upon disruption of the mitotic spindle but also early in mitosis during every cell cycle, which assures stable attachment of spindle microtubules to kinetochores and proper chromosome segregation [16, 17]. Bub1 and Bub3 are thought to interact directly and are upstream in the pathway, whereas Mad1, 2, and 3 are more downstream in the signaling cascade [15]. If kinetochores are not stably bound to microtubules, Mad2, Bub1, and Bub3 localize to kinetochores, and Mad2 interacts physically with Cdc20 (p55CDC, fizzy), an activator of the APC [16, 18, 19]. Mad2 binding to fizzy prevents APC activation and thereby inhibits degradation of chromatid cohesion regulators and cyclin B, halting exit from mitosis [6, 7, 20–22]. Although it has been shown recently that Bub1 and Bub3 form a complex with Mad1 [17], it is not clear how the signal from unattached kinetochores is transmitted to MAD2 and the APC.

In addition to Bub1, several other protein kinases are implicated in activation of the APC. One example is polo-like kinase, which localizes on kinetochores in metaphase [23–25]. In *Xenopus* egg extracts, kinase-dead Plx1 blocks activation of the APC [25, 26], and in mammalian cells, DNA damage in mitosis blocks APC activation in part by deactivation of Plk [27]. Other work has implicated the MAPK pathway in the spindle assembly checkpoint. In *Xenopus* egg extracts and in XTC cells, the ability of nocodazole to cause metaphase arrest depends on the MAPK pathway [28–30], and in mammalian cells, active MAPK enters the nucleus and localizes to spindle poles and kinetochores during mitosis despite low activity elsewhere in the cell [31, 32]. MAPK activity on kinetochores is high in prophase and is reduced once attachment of microtubules to kinetochores is complete [31, 32]; it remains high if spindles are disrupted by microtubule-depolymerizing agents. Although it has been proposed that MAPK promotes the spindle checkpoint by interfering with APC activation [31], it is not known if, or how, MAPK is functionally linked to proteins of the kinetochore attachment checkpoint such as Bub1 and 3; Mad1, 2, and 3; and fizzy.

During progesterone-induced *Xenopus* oocyte maturation, the MAPK pathway is activated (for review see [33]). Oocytes go through two meiotic divisions and arrest with an intact spindle in metaphase of meiosis II due to an activity defined as cytostatic factor (CSF) [34]. The MAPK pathway is required for establishing meiotic arrest in metaphase II by CSF [33, 35–37]. Recently, we and others have shown that a single direct downstream target of MAPK, $p90^{Rsk}$, mediates CSF arrest in oocytes [38, 39] Furthermore, in maturing oocytes, the MAPK pathway and $p90^{Rsk}$ inhibit activity of the APC and thereby prevent degradation of cyclin B [40].

In this report, we define a link between the MAPK pathway and a genetically defined component of the kinetochore attachment checkpoint, the protein kinase Bub1. Bub1 undergoes activation and a phosphorylation-dependent mobility shift during oocyte maturation that requires the activity of the MAPK pathway. Furthermore, $p90^{Rsk}$, a downstream target of MAPK, directly phosphorylates Bub1 and increases its protein kinase activity in vitro.

Results

Cloning of *Xenopus* Bub1

The *Xenopus* isoform of Bub1 (xBub1) was cloned by PCR from a λ ZAPII *Xenopus* oocyte library as described in Materials and methods. The sequence contains an open reading frame of 3408 nucleotides (1136 amino acids) and a 5′ untranslated region (UTR) of 89 nucleotides with stop codons upstream of the ATG in all reading frames. The 3′ UTR consists of 313 nucleotides including a poly(A) tail. Sequence comparison between the human and *Xenopus* isoforms reveals 54% similarity over the whole protein and 77% similarity in the kinase domain (Figure 1).

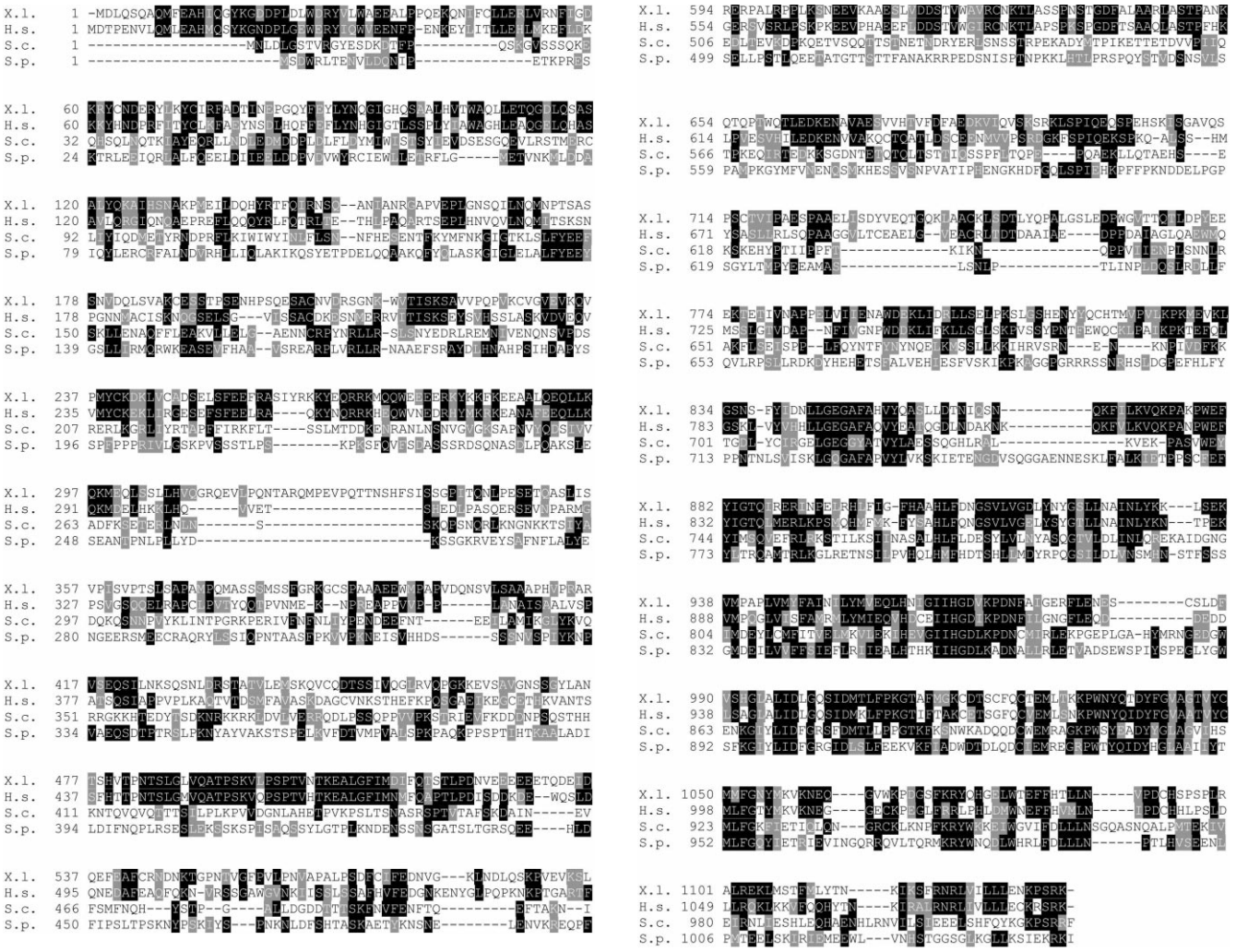
Bub1 localizes to kinetochores in mitosis and meiosis

To study xBub1 localization during mitosis, we immunostained *Xenopus* A6 cells (data not shown) and embryos (Figures 2a–c) with a polyclonal antibody against *Xenopus* Bub1. We observed Bub1 staining of kinetochores in mitotic prophase and metaphase in both A6 cells and embryos. No xBub1 staining was observed after metaphase in cultured cell lines, whereas greatly reduced Bub1 immunoreactivity was still evident on kinetochores of embryonic cells in anaphase (Figure 2c). Since embryos before the midblastula transition (MBT) are not able to execute a kinetochore attachment checkpoint arrest of the cell cycle in response to nocodazole [41, 42], we also compared kinetochore localization of xBub1 in embryos before and after the MBT. In both cases, a similar cell cycle–dependent localization of xBub1 to kinetochores during early mitosis was observed (data not shown), indicating that xBub1 localizes to kinetochores independently of the presence of a functioning checkpoint. To test whether xBub1 is present at potential sites of checkpoint control in meiosis, the localization of xBub1 during oocyte maturation was studied. In metaphase of both meiotic divisions, strong kinetochore staining was detected (Figure 2d,e).

xBub1 becomes phosphorylated and activated during oocyte maturation

We examined the abundance of xBub1 protein during oocyte maturation (Figure 3a) and early embryogenesis (data not shown). Bub1 was present in resting oocytes and the level of Bub1 protein increased slightly during

Figure 1

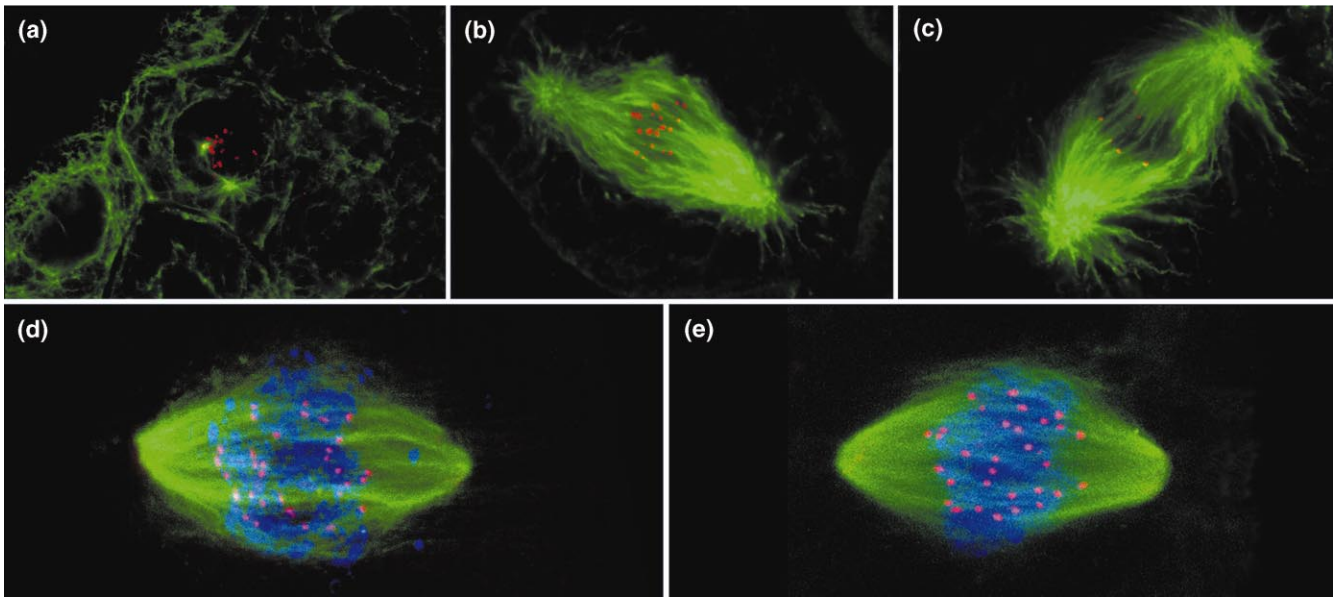


Sequence alignment of *Xenopus*, human, and yeast Bub1. The amino acid sequences of *Xenopus* (X.l.), human (H.s.), *S. cerevisiae* (S.c.), and *S. pombe* (S.p.) Bub1 are shown. Identical amino acids are underlayed with black; similar amino acids are in gray. The kinase domain of xBub1 in the C terminus of the protein (aa 838–1136) shows the highest degree of conservation. The sequence has been deposited in GenBank under accession number AF119789.

oocyte maturation, generally in the range of 2-fold (Figure 3a, upper panel). During oocyte maturation, we observed a decrease of Bub1 electrophoretic mobility upon SDS-PAGE, indicative of posttranslational modification (Figure 3a, compare 3 and 4 hr post-progesterone). The shift was more pronounced if the mobility of the C-terminal half of Bub1 encoding the kinase domain was compared in resting oocytes and those in M-phase that had undergone germinal vesicle breakdown (GVBD) (Figure 3b, lanes 1 and 2). In *S. cerevisiae*, Bub1 becomes transiently phosphorylated during mitosis or upon activation of the kinetochore assembly checkpoint with nocodazole, causing an electrophoretic shift that can be reversed by treatment with λ phosphatase [17]. Likewise, the shift of xBub1 was reversed by λ phosphatase (Figure 3b), indicating the shift during maturation is due to phosphorylation. The phosphorylation of xBub1 occurred slightly before GVBD and persisted throughout maturation to arrest at metaphase of meiosis II (Figure 3a). At the same time that Bub1 underwent phosphorylation, Mos protein started to accumulate (Figure 3a, second panel), which led to activation of the MAPK pathway, as detected with an antibody specific for the active phosphorylated form of MAPK (Figure 3a, third panel). Also at the same time, cyclinB/Cdc2 became activated, shown as a loss of inhibitory phosphorylation on Tyr15 (Figure 3a, lower panel), which correlates with an increase in histone H1 kinase activity.

To determine whether the phosphorylation and shift of

Figure 2



Immunolocalization of Bub1 in mitosis and meiosis. **(a–c)** Immunofluorescent staining of Bub1 and α-tubulin in stage 13 *Xenopus* embryos; Bub1 staining is shown in red, α-tubulin is in green. (a) Bub1 staining of kinetochores in early prophase. (b) Metaphase. (c) Anaphase. **(d,e)** Bub1 and α-tubulin immunoreactivity in oocytes. Bub1 and α-tubulin staining of a meiosis I spindle (45 min after GVBD) is shown in (d). A meiosis II spindle (120 min after GVBD) is shown in (e). DNA staining with Sytox green is in blue, α-tubulin is in green, and Bub1 is in red.

Bub1 reflects activation of the enzyme, we measured the kinase activity of immunoprecipitated endogenous Bub1 using histone H3 as a substrate (Figure 3c). Activity of Bub1 increased several-fold between stageVI and GVBD as shown both by increased autophosphorylation and by phosphorylation of histone H3. The increase in Bub1 activity at GVBD appeared to be greater than could be accounted for by the increase in level of Bub1. This result suggests that the electrophoretic shift of Bub1 reflects phosphorylation-dependent activation.

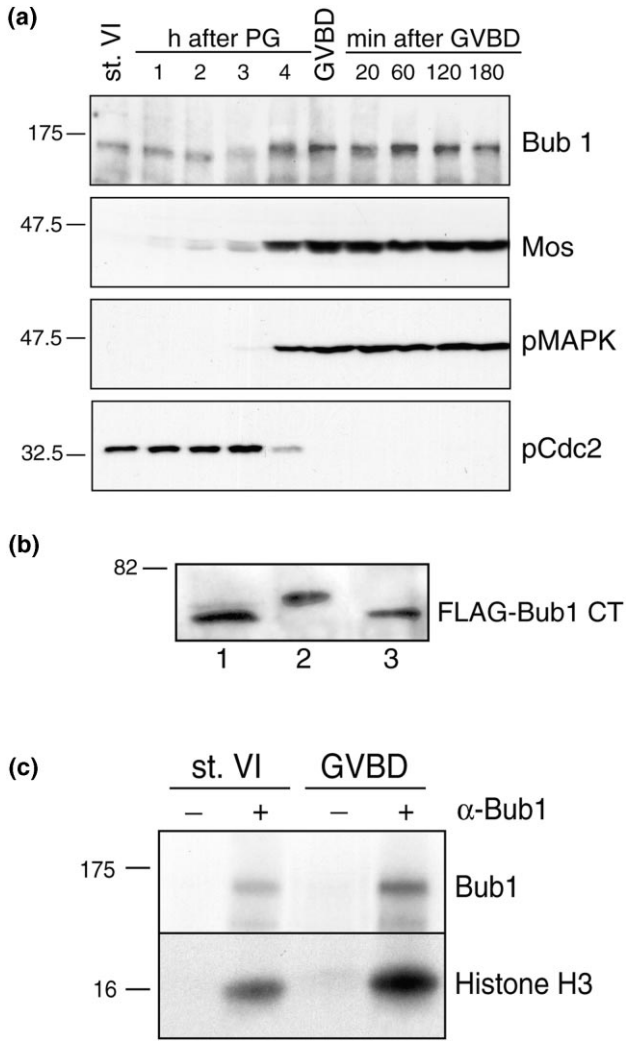
Since Bub1 has been reported to be in a complex with Bub3 in other systems [43], it was important to determine whether a physical interaction also exists in *Xenopus* oocytes. Therefore, we cloned xBub3 by PCR as described in Materials and methods, and we expressed in oocytes myc-tagged xBub3 either alone or with untagged xBub1. Expressed and endogenous xBub1 were precipitated with affinity-purified Bub1 antibodies, and the immunoprecipitates were Western blotted with anti-myc antibody to detect coprecipitated Bub3. Myc-tagged xBub3 was recovered in a complex with both ectopic and endogenous Bub1 (Figure 4). We conclude that, as previously reported for yeast and human cells, Bub1 exists as a complex with Bub3.

The MAPK pathway is responsible for activation of Bub1

Since Bub1 becomes phosphorylated during oocyte maturation at the time when the MAPK pathway becomes activated (Figure 3), and since activation of the spindle assembly checkpoint in egg extracts [28, 29] and XTC cells [30] is MAPK dependent, we reasoned that the MAPK pathway might be responsible for phosphorylation of xBub1, particularly since in mammalian cells MAPK activity on kinetochores declines once spindle assembly is complete and is increased during spindle checkpoint activation [31, 32]. We added ^{35}S-labeled Bub1 protein, produced in a reticulocyte lysate, to interphase egg extracts and monitored the time course of Bub1 phosphorylation by electrophoretic shift. In the control extract, exogenous Bub1 did not shift, the MAPK pathway was not activated, and cyclin B/Cdc2 histone H1 kinase activity remained low (Figure 5). When 50 ng bacterially produced Mos protein, an oocyte MAPKKK, was added, active MAPK reactivity was detected. p90^{Rsk}, a downstream target of MAPK, underwent a mobility shift, which is known to reflect activation [44], at the same time, and xBub1 shifted into its high activity, phosphorylated form. U0126, a potent and specific inhibitor of MEK1 [40, 45], prevented activation of the MAPK pathway by Mos protein and inhibited the shift of radiolabeled xBub1. These results indicate that Bub1 phosphorylation and activation are a downstream consequence of MAPK activation.

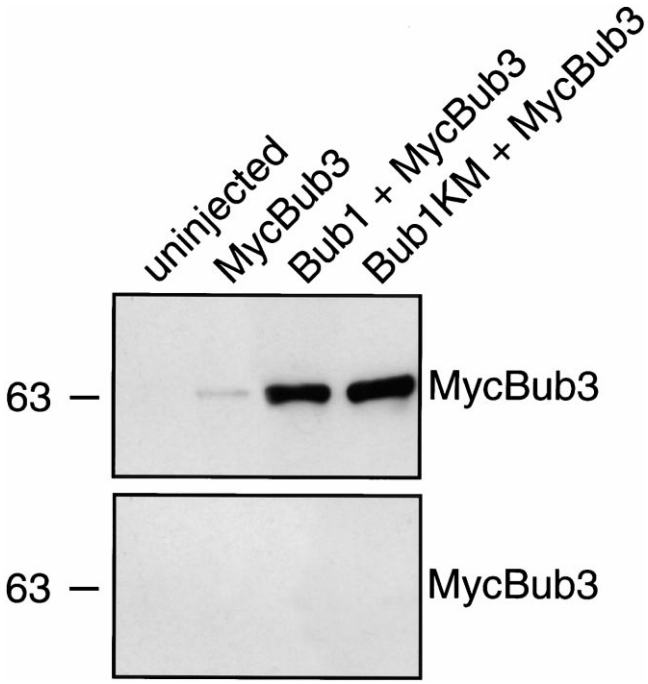
Previous results have shown that in oocytes, a downstream target of MAPK, p90^{Rsk}, is the sole mediator of several important functions of the MAPK pathway. These include

Figure 3



(a) Phosphorylation of Bub1 during oocyte maturation. Bub1 protein displays reduced electrophoretic mobility indicative of phosphorylation (upper panel) at the same time that the MAPK pathway becomes activated, as shown by the accumulation of Mos protein (second panel) and the appearance of phospho-MAPK reactivity (third panel), which reflects activation of the enzyme. Disappearance of inhibitory phosphorylation of Cdc2 on Tyr15 (lower panel) indicates that activation of MPF also correlates with phosphorylation of Bub1. The apparent slower mobility of Bub1 in St VI oocytes and 1–2 hr post-progesterone was an artefact of a "smile" in this gel and was not seen in other experiments [cf. (b)]. **(b)** The shift of Bub1 is due to phosphorylation. Oocytes were injected with mRNA encoding the FLAG-tagged C-terminal half of Bub1 (FLAG-Bub1 CT), which contains most of the phosphorylation sites. After overnight incubation, oocytes were treated or not with progesterone, lysed after GVBD, and supernatants incubated with 400 U/oocyte of λ phosphatase (lane 3) or buffer (lanes 1 and 2) for 30 min. Then SDS-PAGE sample buffer was added and Bub1 electrophoretic mobility was assessed by Western blotting with anti-FLAG antibody: lane 1, extract from control, St VI oocytes; lane 2, extract from oocytes at GVBD; lane 3, extract of oocytes at GVBD treated with phosphatase. **(c)** Endogenous Bub1 kinase activity in oocytes. The kinase activity of endogenous Bub1 immunoprecipitated from stage VI or GVBD oocytes (lanes 2 and 4) was measured by autophosphorylation (upper panel) or with histone H3 as an exogenous substrate as described in Materials and methods. Immunoprecipitation assays with beads only (no antibody) are shown in lanes 1 and 3 as controls.

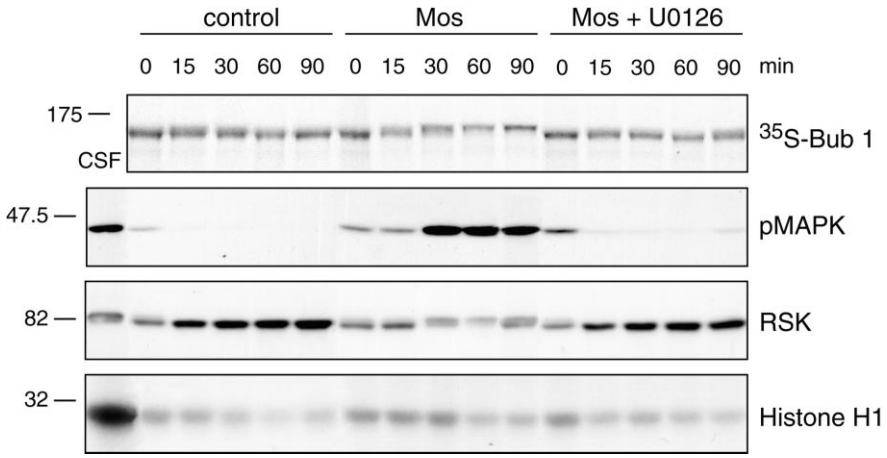
Figure 4



Bub1 and Bub3 exist in a complex. Bub1 was immunoprecipitated from uninjected oocytes, from oocytes injected with mRNA encoding myc-tagged Bub3 alone, or injected with Bub1 or Bub1 KM (catalytically inactive Bub1) mRNA together with myc-tagged Bub3 mRNA, as indicated. Bub3 was detected by immunoblotting with a monoclonal anti-myc antibody (clone 9E10). Bub3 co-precipitates with endogenous Bub1 and overexpressed wild type or catalytically inactive Bub1. No myc-tagged Bub3 was detectable in an immunoprecipitate using preimmune serum (lower panel).

generation of CSF activity for metaphase arrest at the end of meiosis II [38, 39] and inhibition of APC-mediated cyclin B degradation between meiosis I and II [40]. To investigate the role of p90^{Rsk} in mediating MAPK-dependent Bub1 activation, oocytes were injected with mRNA encoding a constitutively active form of p90^{Rsk} (CA-RSK), which does not require MAPK for activity [39, 40], and treated with progesterone. As reported before [40], CA-RSK was able to prevent cyclin B degradation by the APC in the presence of U0126. In addition, activation of Bub1 was evident in CA-RSK-expressing oocytes even when U0126 prevented activation of the MAPK pathway (Figure 6). Thus, activation of Bub1 might be due to either a direct interaction with p90^{Rsk} or through an as yet unidentified kinase downstream of p90^{Rsk}.

Figure 5



Phosphorylation of Bub1 is dependent on the MAPK pathway. CSF extracts were prepared as described [49, 50] and released from metaphase arrest by addition of 0.6 mM $CaCl_2$. To prevent reactivation of MPF, 50 μg/ml cycloheximide was added with the $CaCl_2$. At 45 min after the addition of Ca^{2+} and cycloheximide, ^{35}S-labeled Bub1 protein was added, indicated as the 0 min time point. The electrophoretic mobility shift of ^{35}S-labeled Bub1, which is indicative of phosphorylation, was followed over a period of 90 min in control extracts, in extracts in which the MAPK pathway was activated by the addition of 50 ng Mos protein, and in extracts supplemented with Mos protein and the MEK 1 inhibitor U0126 (50 μM) (upper panel). The activation of MAPK is shown in panel 2 by use of a phosphospecific antibody that detects only active MAPK, and in panel 3 the activity of $p90^{Rsk}$ is monitored by a shift known to reflect activation [44]. The antibody used (rabbit polyclonal antibody against $p90^{Rsk1}$ [Santa Cruz]) recognizes less well the phosphorylated and activated form of $p90^{Rsk}$. Total histone H1 kinase activity is shown in the lower panel.

$p90^{Rsk}$ phosphorylates and activates Bub1

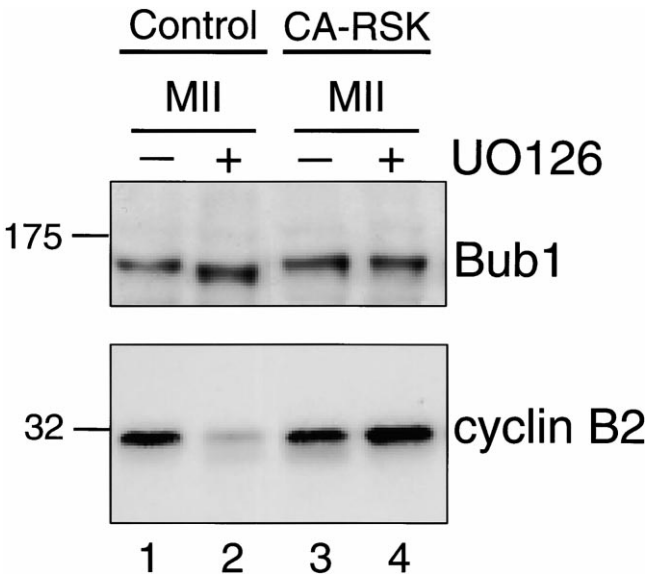
To determine if $p90^{Rsk}$ is a direct activator of Bub1, we used His_6-Bub1 protein purified from Sf9 cells as a substrate. Bub1 was incubated with $p90^{Rsk}$ or various other protein kinases active in M phase in oocytes (MPF, MAPK, Plx1, MEKDD) in the presence of [γ-^{32}P]ATP. Several of these kinases were able to phosphorylate Bub1 (Figure 7a). This is not surprising, inasmuch as a 160 kDa protein such as Bub1 contains consensus motifs for phosphorylation by multiple kinases. To determine if any of these phosphorylation events regulate Bub1 activity, Bub1 purified from Sf9 cells on Talon beads was first incubated with each of the above mentioned kinases in the presence of cold ATP to allow phosphorylation of Bub1. Subsequently, the activating kinases were removed, and Bub1 activity was assayed with histone H3 as substrate. Only $p90^{Rsk}$ activated Bub1WT significantly, whereas a kinase-inactive form of Bub1 (Bub1KM) did not become activated (Figure 7b). Also, incubation of xBub1WT with kinase-inactive $p90^{Rsk}$ did not result in activation of Bub1 activity toward histone H3 (Figure 7c). Treatment with λ phosphatase removed all radiolabel from Bub1 after phosphorylation by Rsk (data not shown) and deactivated the enzyme as judged by phosphorylation of histone H3 (Figure 7c). These results demonstrate that $p90^{RSK}$ directly activates Bub1 by phosphorylation.

Discussion

In this study, we focused on the relationship of the MAPK pathway, which regulates metaphase arrest by CSF in vertebrate eggs, to the kinetochore attachment/spindle assembly checkpoint. In budding yeast, this checkpoint has been defined as a genetic hierarchy of Bub and Mad proteins that regulate APC activation [1], an event required for exit from mitosis. These components and this pathway appear to be conserved in *Xenopus* and other eukaryotes [1]. However, biochemical studies in higher eukaryotes have implicated MAPK as another element that controls exit from mitosis in a variety of cell types. In particular, abundant evidence in *Xenopus* egg extracts indicates that MAPK activity is required for execution of the checkpoint. Although intact eggs and early embryos have insufficient DNA for checkpoint control by nocodazole addition, it can be obtained experimentally in egg extracts by addition of a large number of nuclei prior to treatment with nocodazole [28, 29], and under these conditions the MAPK pathway is activated. The addition of MAPK phosphatase to egg extracts in which the kinetochore attachment checkpoint is activated abrogates the checkpoint [28], as does immunodepletion of MAPK [29]. In addition, treatment of the extract with PD98059, an inhibitor of MAPK kinase [46], compromises establishment of the checkpoint. A similar role for MAPK is thought to operate in somatic cells, inasmuch as activated MAPK is present on kinetochores at metaphase [31, 32], and injection of MAPK phosphatase into tissue culture cells blocks checkpoint activation [30]. Moreover, it has been reported that MAPK activity on kinetochores is reduced once spindle assembly is complete but remains high if the spindle checkpoint is activated by microtubule-depolymerizing agents [31, 32]. At anaphase, no active MAPK can be detected on kinetochores.

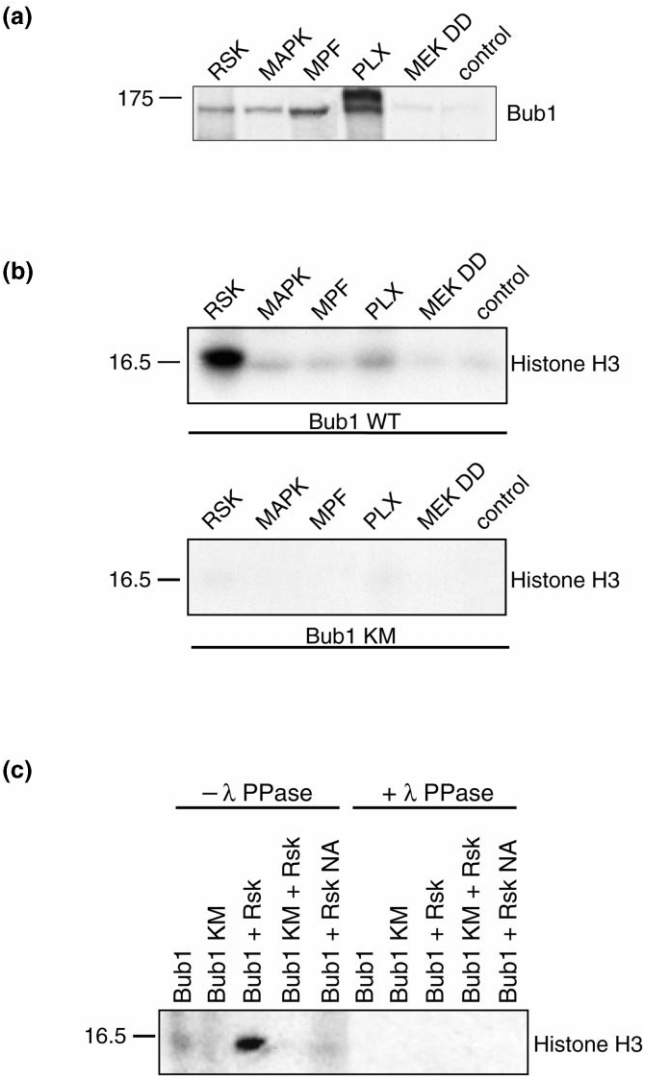
In meiosis II, vertebrate oocytes undergo a special form of metaphase arrest caused by CSF, an activity that is as yet uncharacterized on the molecular level. Although CSF activity normally disappears after fertilization [34], CSF-dependent metaphase arrest can be induced in cells after

Figure 6



CA-Rsk restores phosphorylation of Bub1 in U0126-treated oocytes. Uninjected oocytes and oocytes injected with CA-Rsk mRNA [39, 40] were incubated overnight at 18°C. The next day U0126 was added 1 hr before the addition of progesterone to induce oocyte maturation. Untreated and U0126-treated oocytes were collected at the time corresponding to meiosis II (MII), extracted, and immunoblotted for Bub1 (upper panel, lanes 1–4). U0126 prevented phosphorylation of Bub1 at meiosis II (MII, lanes 1 and 2, upper panel). Expression of constitutively active Rsk (CA-Rsk) reversed the effect of U0126, and full phosphorylation of Bub1 was evident (lanes 3 and 4, upper panel). A Western blot showing cyclin B2 protein reflects the cell cycle phase (lower panel), with CA-Rsk preventing APC-mediated cyclin B degradation and entry into S phase [40] after GVBD in the presence of U0126 (compare lanes 2 and 4).

Figure 7

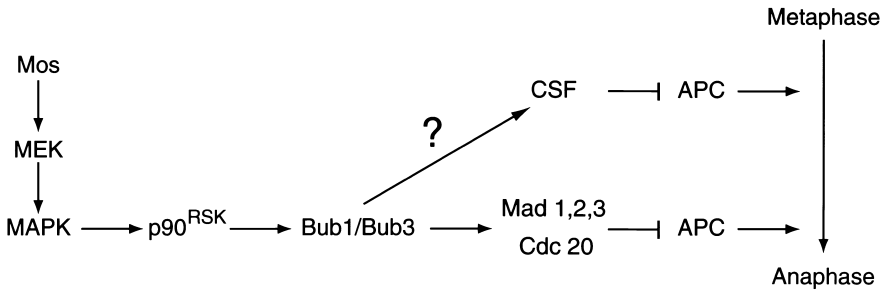


Phosphorylation and activation of Bub1 by p90^{Rsk}. **(a)** Bub1 purified from Sf9 cells was phosphorylated with various kinases in the presence of [γ-^{32}P]ATP for 30 min at 30°C. Proteins in the reaction mixture were separated by SDS-PAGE and analyzed by autoradiography. MEKDD, constitutively-active MAPK kinase; MPF, cyclin B/Cdc2. **(b)** Bub1wt (upper panel) and Bub1 KM (lower panel) bound to beads were phosphorylated for 30 min at 30°C with the same kinases as in (a), except that unlabeled ATP was used. The Bub1-containing beads were washed as described in Material and methods, and Bub1 kinase activity was measured by incorporation of radiolabel into histone H3. **(c)** Bub1 activation reactions were carried out as in (b) with the indicated combinations, except that after the activation reaction, beads either were or were not treated with λ phosphatase (as indicated) before assay of histone H3 kinase activity, as described in Materials and methods.

fertilization by Mos, MEKDD, thiophosphorylated MAPK, or a constitutively active form of p90^{Rsk}, even though a nocodozole-sensitive checkpoint is absent [35–37, 39]. Moreover, antibody depletion or inactivation of either MAPK or p90^{Rsk} from extracts removes CSF activity [38, 47]. The MAPK pathway and p90^{Rsk} have been shown to directly inhibit APC-mediated cyclin B degradation in meiosis II [40], suggesting CSF arrest involves APC inhibition. In oocytes treated with UO126 to remove MAPK activity, Cdc27, a component of the APC, fails to undergo a hypershift that correlates with low APC activity. This results in increased cyclin B degradation, and a failure to reaccumulate MPF in meiosis II (Figure 6; [40]).

Our results establish a link between the MAPK pathway and the protein kinase Bub1, a genetically identified element of the spindle assembly checkpoint. Bub1 undergoes a phosphorylation-dependent mobility shift corresponding to activation of the enzyme shortly before GVBD as a consequence of activation of the MAPK pathway, and it remains in this activated form throughout meiosis I and II. A hyperactive form of p90^{Rsk} caused activation even in the presence of UO126. Moreover, in vitro kinase assays using Bub1 as a substrate clearly showed that p90^{Rsk} directly phosphorylates Bub1 and increases its activity in vitro, indicating that Bub1 is a direct downstream target of p90^{Rsk}.

Figure 8



The pathway of CSF arrest at metaphase. The model proposes the activation of Bub1 as the target of the Mos/MAPK/Rsk pathway for inhibition of the APC and CSF arrest at metaphase. The action of Bub1 to promote metaphase arrest could involve established interactions with Mad proteins and Cdc20 (fizzy) to inhibit APC activity or, alternatively, could be mediated by interaction of Bub1 with other components in a novel pathway designated "CSF" that would also result in APC inhibition and metaphase arrest.

Although some of the protein components of the spindle assembly checkpoint, such as Mad1, 2, 3 and Fizzy, have previously been reported to be present in *Xenopus* eggs, it has been unclear what role, if any, they play in the cell cycle prior to appearance of the nocodazole-sensitive checkpoint following the midblastula transition [41, 42]. However, since the kinetochore attachment checkpoint prevents activation of the APC, it is possible that Bub1, as a target of $p90^{Rsk}$ required for generation of CSF activity (Figure 8), might act through known kinetochore attachment checkpoint components to prevent meiotic APC activation via targeting of Mad2 to Cdc20 (fizzy). In somatic cells, evidence indicates that MAPK activity in mitosis is restricted to spindle poles and kinetochores, and activity on kinetochores is reduced after completion of microtubule attachment [31, 32]. In the case of oocytes, however, Mos expression ensures that very high MAPK/Rsk activity is present throughout the cell in meiosis II despite the presence of a complete spindle; this pathway might activate the checkpoint to block cell cycle progression even though kinetochores are fully attached to an intact spindle and cell cycle progression is insensitive to nocodazole. Alternatively, Bub1 might exert its functions in a novel manner to generate a unique "CSF" pathway for APC inhibition (Figure 8).

Conclusions

Bub1 becomes activated during every cell cycle before all kinetochores are properly attached to spindle microtubules and after disruption of mitotic spindles with microtubule-destabilizing drugs. We have shown that xBub1 is also activated during the CSF-induced arrest of meiosis II and that the MAPK pathway mediates this activation through $p90^{Rsk}$, which directly phosphorylates and activates Bub1 in vitro. Thus, both inhibition of cyclin B degradation at the meiosis I to II transition and metaphase arrest by CSF may be mediated in part by $p90^{Rsk}$-dependent phosphorylation and activation of Bub1. These findings also provide a molecular mechanism for the proposal [31] that active MAPK on kinetochores in many somatic cell types promotes the spindle assembly checkpoint by interfering with APC activation.

Materials and methods

Cloning of Xenopus *Bub1 and Bub3*

The *Xenopus* homolog of Bub1 was cloned by PCR using the degenerate primers 5′-CAYGGIGAYITIAARCCIGA-3′ and 5′-SCIGCIAIICCRWAR TARTC-3′, which were designed against conserved regions in the kinase domain of the yeast [41] and mouse [14] Bub1 proteins. A 270 bp fragment obtained by PCR was used to screen a λ ZAPII *Xenopus* oocyte library. Among seven isolated positive cDNA clones, one contained the full open reading frame of Bub1. This sequence has been deposited in GenBank under accession number AF119789. A portion of the *Xenopus* Bub3 homolog was amplified by PCR from the same library using the degenerate primers 5′-TGGGAYCARACIGTIAARCTITGGG-3′ and 5′-TCIACIGCIACYCTICCYTC-3′, which were designed against conserved regions in the human and *Drosophila* homologs. The 271 bp fragment was then used to probe the library, and a full-length clone was obtained. Its sequence, which is almost identical to the recently published xBub3 homolog described by Goto and Kinoshita [48], has been deposited in GenBank under accession number AF119790

Plasmids and constructs

The open reading frame of xBub1 was amplified by PCR with primers 5′-GACGACGACAAGATGGATCTACAGAGTCAGGCA-3′ and 5′-GAG GAGAAGCCCGGTTTTGCGGGAAGGCTTGTTCTC-3′. The PCR product was cloned into pCS2+ or pCS2+Myc-tag (MT) vectors modified for ligation-independent cloning by insertion of the annealed primers 5′-AATTCTGACGACGACAAGAGCCCGGGCTTCTCCTCAC-3′ and 5′-TCGAGTGAGGAGAAGCCCGGGCTCTTGTCGTCGTCAG-3′ into the EcoRI/XhoI restriction site of pCS2+ or pCS2+MT, resulting in vectors pCSLIC and pCSMTLIC. An additional BseRI restriction site in pCS vectors was mutated by site-directed mutagenesis. PCSLICBub1 or pCSMTLICBub1 were used for in vitro transcription, pBac2cpLICBub1 for baculovirus expression of Bub1, and pT7Blue2LIC Bub1 for in vitro transcription/translation. The kinase inactive mutant of Bub1 was created by site-directed mutagenesis according to the Quick Change method (Promega). Lysines 871 and 874 were mutated to methionines in the ATP binding site of the Bub1 kinase domain using primers 5′-CAGAAGTTTAT ATTAATGGTTCAAATGCCGGCCAAGCCCTGGGAG-3′ and 5′-CTC CCAGGGCTTGGCCGGCATTTGAACCATTAATATAAACTTCTG-3′.

xBub3 was cloned into pCSMTLIC using the strategy described above for Bub1. The primers 5′-GACGACGACAAGATGACCGGGTCAAAT GAG-3′ and 5′-GAGGAGAAGCCCGGTCACTTGGGCTTTGTC-3′ were used to amplify xBub3. For some experiments (Figure 3c), the C-terminal half of Bub1 (amino acids 569–1136) was expressed as a FLAG-tagged fusion construct in pCSLIC for in vitro transcription.

RNA production and injection of oocytes

Bub1 and Bub3 mRNA were produced from NotI-linearized pCSLIC-Bub1, pCSMTLIC Bub1, or pCSMTLICBub3 plasmids using the SP6 mMessage mMachine kit (Ambion). mRNA (30–50 ng) in 30–50 nl water was injected into stage VI oocytes. Oocytes were allowed to express protein overnight at 18°C. To obtain oocytes that had undergone GVBD,

10 μg/ml progesterone was added the next morning to induce maturation. Oocytes were frozen at the desired time points and kept at −80°C until assay.

Antibodies and Western blotting

The antibody against xBub1 was produced by immunization of rabbits with bacterially produced His_6-tagged polypeptides containing amino acids 115–407 and 466–965 of Bub1. Serum was purified by affinity chromatography on a resin coupled to the polypeptides used for immunization. For SDS-PAGE and Western blotting, oocytes and embryos were lysed in EB (50 mM Tris-HCl [pH 7.4], 80 mM β-glycerophosphate, 20 mM EDTA, 1 mM DTT, 3μM microcystin LR, 0.2% Triton X-100) and centrifuged at 10,000 × g for 5 min. In general, extract supernatant corresponding to 1 oocyte was loaded per lane. For Bub1 Western blots, the proteins on the gels were transferred to nitrocellulose by wet transfer for 1 hr at 100V (transfer buffer: 25 mM Tris, 192 mM glycine, 0.005% SDS, 10% methanol). The membrane was blocked with 10% dry milk in TBS, 0.1% NP-40 for 30 min. Incubation with affinity-purified Bub1 antibody was overnight at 4°C, followed by three washes with TBS 0.1% NP-40, 500 mM NaCl. Secondary antibodies (goat anti-rabbit-HRP conjugate [Jackson]) were used at 1:20000 in TBS containing 0.1% NP-40. After five washes with TBS containing 0.1% NP-40, 500 mM NaCl, the signal was developed with the Renaissance chemiluminiscence reagent plus (Life Science Products).

Endogenous xBub1 protein was precipitated from oocyte lysates prepared with EB plus 1× Complete protease inhibitors (Roche Biochemicals). About 3 μg affinity-purified Bub1 antibodies were incubated with the lysate from 10 oocytes in a volume of 150 μl for 60 min on ice, 10 μl protein A-Sepharose beads (Pierce) were added, and the lysates further incubated for 60 min at 4°C on a rotator. Immunoprecipitates were washed twice with 50 mM Tris-HCl (pH 7.4), 80 mM β-glycerophosphate, 100 mM NaCl, 0.2% Triton X-100, twice with 50 mM Tris-HCl (pH 7.4), 80 mM β-glycerophosphate, 500 mM NaCl, 0.2% Triton X-100, and once with 1× kinase reaction buffer, if Bub1 was used for in vitro kinase assays.

Purification of Bub1 expressed in Sf9 cells

Sf9 cells were transfected with the Bub1 baculovirus an MOI of 1. The infected cells were harvested 48 hr later. Cell pellets were lysed with HBS (10 mM HEPES [pH 7.2], 150 mM NaCl, 0.01% Brij) by sonication. His-tagged Bub1 was purified by binding to Talon beads (Clontech) with two washes with HBS and 2 mM EDTA and two washes with HBS, 2 mM EDTA, and 500 mM NaCl. Purified proteins were eluted from the beads with 250 mM imidazole and dialyzed against HBS. The proteins were left on beads when used as substrate for in vitro kinase assays.

Kinase assays

For in vitro kinase assays, Bub1 on Talon beads was washed once with 1× kinase buffer (50 mM MOPS [pH 7.0], 15 mM $MgCl_2$, 0.75 mg/ml BSA, 1mM DTT). Bub1 protein (75 ng) was incubated with 0.5 mg/ml histone H3 (Roche Biochemicals) as a substrate in kinase buffer containing 100 μM cold ATP and 5 μCi [γ-^{32}P]ATP (ICN). Bub1 kinase reactions were incubated at 30°C for 30 min. For Bub1 phosphorylation experiments, xBub1 expressed in Sf9 cells was bound to Talon beads, washed as described above, and incubated with various kinases in kinase buffer in the presence of 100 μM ATP for 30 min at 30°C. The beads were washed twice with 50 mM Tris (pH 7.2), 80 mM β-glycerophosphate and twice with 50 mM Tris (pH 7.2), 80 mM β-glycerophosphate, 500 mM NaCl followed by one wash with kinase buffer. Then kinase buffer with histone H3 and 100 μM ATP containing 5μCi [γ-^{32}P]ATP was added, and the mixture was incubated for 30 min at 30°C. Samples were electrophoresed on a 4%–20% gradient SDS gel and the gel was dried and exposed to Kodak X-OMAT MR film (Kodak). In some experiments (Figure 7c), after phosphorylation by Rsk, beads were incubated for 30 min with buffer or 400 units of λ phosphatase and washed three times as above before addition of histone H3 and [γ-^{32}P]ATP.

Immunofluorescence

Embryos and oocytes were fixed in 100% methanol for 2 hr at room temperature and then transferred into 20% DMSO in methanol and incubated overnight at −20°C. Specimens were then rehydrated, bleached with 10% hydrogen peroxide in 50 mM Tris-HCl (pH 8) and preblocked with 5% BSA in TBS, 0.1% Triton X-100 for 3–4 hr at room temperature. Embryos and oocytes were incubated with primary antibodies in 5% BSA in TBS, 0.1% Triton X-100 overnight at room temperature, followed by five washes with TBS, 0.1% Triton X-100 within 8 hr. Secondary antibodies (goat anti-rabbit-Cy3, goat anti-mouse Cy5 [Jackson], and goat anti-mouse Alexa 488 [Molecular Probes]) were diluted in 5% BSA in TBS, 0.1% Triton and incubated with oocytes and embryos for at least overnight at room temperature. After five washes with TBS, 0.1% Triton the specimens were rinsed once with 0.5× TBS, followed by overnight incubation with Sytox green (Molecular Probes) 1:10 000 in 0.5× TBS. Embryos and oocytes were washed once with 0.5× TBS for 30 min before dehydration with increasing percentages of methanol. After three changes of 100% methanol, the oocytes and embryos were cleared with Murray's solution (benzylbenzoate:benzylalcohol 2:1) and mounted onto slides. The specimens were analyzed with a Nikon PCM 2000 confocal microscope.

Acknowledgements

This work was supported by a grant from the NIH (DK28353-19) to J.L.M. We thank the University of Colorado Comprehensive Cancer Center Sequencing Core Facility for sequencing the xBub1 and xBub3 genes and constructs, and the Tissue Culture Core Facility for generation of Bub1 and Bub3 baculoviruses and protein production (PO1-CA-46934). We also thank Brad Lattes, Guolin Chen, and Jan Kyes for expert technical assistance and Yue-Wei Qian and Eleanor Erikson for the λ ZAPII cDNA library. M.S.S., F.E.T., S.D.G., and B.T.R. are Associates and J.L.M. is an Investigator of the Howard Hughes Medical Institute.

References

1. Amon A: **The spindle checkpoint.** *Curr Opin Genet Dev* 1999, **9:**69-75.
2. Li X, Nichlas RB: **Tension-sensitive kinetochore phosphorylation and the chromosome obstruction checkpoint in praying mantid spermatocytes.** *J Cell Sci* 1997, **110:**537-545.
3. Waters JC, Chen R-H, Murray AW, Salmon ED: **Localization of Mad2 to kinetochores depends on microtubule attachment, not tension.** *J Cell Biol* 1998, **141:**1181-1191.
4. Rieder CL, Schultz A, Cole R, Sluder G: **Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle.** *J Cell Biol* 1994, **127:**1301-1310.
5. Rieder CL, Cole RW, Khodjakov A, Sluder G: **The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores.** *J Cell Biol* 1995, **130:**941-948.
6. Fang G, Yu H, Kirschner MW: **The checkpoint protein MAD2 and the mitotic regulators CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation.** *Genes Dev* 1998, **12:**1871-1883.
7. Kallio M, Weinstein J, Daum JR, Burke DJ, Gorbsky GJ: **Mammalian p55CDC mediates association of the spindle checkpoint protein MAD2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events.** *J Cell Biol* 1998, **141:**1393-1406.
8. Zachariae W, Nasmyth K: **Whose end is destruction: cell division and the anaphase-promoting complex.** *Genes Dev* 1999, **13:**2039-2058.
9. Hoyt MA, Totis L, Roberts BT: **S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule functions.** *Cell* 1991, **66:**507-517.
10. Li R, Murray AW: **Feedback control of mitosis in budding yeast.** *Cell* 1991, **66:**519-531.
11. Weiss E, Winey M: **The Saccharomyces cerevisiae spindle pole body duplication gene MPS1 is part of a mitotic checkpoint.** *J Cell Biol* 1996, **132:**111-123.
12. Lauze E, Stoelcker B, Luca FC, Weiss E, Schutz AR, Winey M: **Yeast spindle pole body duplication gene MPS1 encodes an**

essential dual specificity protein kinase. *EMBO J* 1995, **14:**1655-1663.
13. Burke DJ: **Complexity in the spindle checkpoint**. *Curr Opin Genet Dev* 2000, **16:**23-31.
14. Gardner RD, Burke DJ: **The spindle assembly checkpoint: two transitions, two pathways.** *Trends Cell Biol* 2000, **10:**154-158.
15. Farr KA, Hoyt MA: **Bub1p kinase activates the Saccharomyces cerevisiae spindle assembly checkpoint.** *Mol Cell Biol* 1998, **18:**2738-2747.
16. Taylor SS, McKeon F: **Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage.** *Cell* 1997, **89:**727-735.
17. Brady DM, Hardwick KG: **Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint functions.** *Curr Biol* 2000, **10:**625-678.
18. Taylor SS, Ha E, McKeon F: **The human homologue of Bub3 is required for kinetochore localization and a Mad3/Bub1-related protein kinase.** *J Cell Biol* 1998, **142:**1-11.
19. Hardwick KG, Johnston RC, Smith DL, Murray AW: **Mad3 encode a novel component of the spindle checkpoint which interacts with Bub3p, Cdc20p, and Mad2p.** *J Cell Biol* 2000, **148:**871-882.
20. Hwang CH, Law LF, Smith DC, Mistrot CA, Hardwick KG, Hwang ES, *et al.*: **Budding yeast Cdc20: a target of the spindle checkpoint.** *Science* 1998, **229:**1041-1044.
21. Fang G, Yu H, Kirschner M: **Direct binding of Cdc20 protein family members activates the anaphase-promoting complex in mitosis and G_1.** *Mol Cell* 1998, **2:**163-171.
22. Kim SH, Lin DP, Matsumoto S, Kitazono A, Matsumoto T: **Fission yeast Slp1: an effector of the Mad2-dependent spindle checkpoint**. *Science* 1998, **229:**1045-1047.
23. Charles J, Jaspersen R, Tenker-Kulberg L, Hwang L, Szidon A, Morgan D: **The polo-related kinase Cdc5 activates and is degraded by the mitotic cyclin machinery in S. cerevisiae.** *Curr Biol* 1998, **8:**497-507.
24. Shirayama M, Zachariae W, Clogh R, Nasmyth K: **The polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase-promoting complex in Saccharomyces cerevisiae.** *EMBO J* 1998, **17:**1336-1349.
25. Qian Y-W, Erikson E, Maller JL: **Mitotic effects of a constitutively active mutant of the *Xenopus* polo-like kinase Plx1.** *Mol Cell Biol* 1999, **19:**8625-8632.
26. Descombes P, Nigg E: **The polo-like kinase Plx1 is required for M phase exit and destruction of mitotic regulators in *Xenopus* egg extracts.** *EMBO J* 1991, **17:**1328-1335.
27. Smits VA, Klopmaker R, Arnaud L, Rijksen G, Nigg EA, Medema RH: **Polo-like kinase 1 is a target of the DNA damage checkpoint.** *Nat Cell Biol* 2000, **2:**672-676.
28. Minshull J, Sun H, Tonks NK, Murray AW: **A MAP kinase-dependent spindle assembly checkpoint in *Xenopus* egg extracts.** *Cell* 1994, **79:**475-486.
29. Takenaka K, Gotoh Y, Nishida E: **MAP kinase is required for the spindle assembly checkpoint but is dispensable for the normal M phase entry and exit in Xenopus egg cell cycle extracts.** *J Cell Biol* 1997, **136:**1091-1097.
30. Wang XM, Zhai Y, Ferrell JE Jr.: **A role for mitogen-activated protein kinase in the spindle assembly checkpoint in XTC cells.** *J Cell Biol* 1997, **137:**433-443.
31. Shapiro PS, Vaisberg E, Hunt AJ, Tolwinski NS, Whalen AM, McIntosh JR, *et al.*: **Activation of the MKK/ERK pathway during somatic cell mitosis: direct interactions of active ERK with kinetochores and regulation of the mitotic 3F3/2 phosphoantigen.** *J Cell Biol* 1998, **142:**1533-1545.
32. Zecevic M, Catling AD, Eblen ST, Renzi L, Hittle JC, Yen TJ, *et al.*: **Active MAP kinase in mitosis: localization at kinetochores and association with the motor protein CENP-E**. *J Cell Biol* 1998, **142:**1547-1558.
33. Nebreda AR, Ferby A: **Regulation of the meiotic cell cycle in oocytes.** *Curr Opin Cell Biol* 2000, **12:**666-675.
34. Masui Y, Markert CL: **Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes.** *J Exp Zool* 1971, **17:**129-145.
35. Sagata N, Watanabe N, Vande Woude GF, Ikawa Y: **The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs.** *Nature* 1989, **342:**512-518.
36. Haccard O, Sarcevic B, Lewellyn A, Roy L, Izumi T, Hartley RS, *et al.*: **Induction of metaphase arrest in cleaving *Xenopus* embryos by MAP kinase.** *Science* 1993, **262:**1262-1265.
37. Huang W, Kessler DS, Erikson RL: **Biochemical and biological analysis of Mek1 phosphorylation site mutants.** *Mol Biol Cell* 1995, **6:**237-245.
38. Bhatt RR, Ferrell JE Jr: **The protein kinase p90 Rsk as an essential mediator of cytostatic factor activity.** *Science* 1999, **286:**1362-1365.
39. Gross SD, Schwab MS, Lewellyn AL, Maller JL: **Induction of metaphase arrest in cleaving *Xenopus* embryos by the protein kinase p90 Rsk.** *Science* 1999, **286:**1365-1367.
40. Gross SD, Schwab MS, Taieb FE, Lewellyn AL, Qian Y-W, Maller JL: **The critical role of the MAP kinase pathway in meiosis II in *Xenopus* oocytes is mediated by p90 (Rsk).** *Curr Biol* 2000, **10:**430-438.
41. Clute P, Masui Y: **Regulation of the appearance of division asynchrony and microtubule-dependent chromosome cycles in Xenopus laevis embryos.** *Dev Biol* 1995, **171:**273-285.
42. Clute P, Masui Y: **Microtubule dependence of chromosome cycles in Xenopus laevis blastomeres under the influence of a DNA synthesis inhibitor, aphidicolin.** *Dev Biol* 1997, **185:**1-13.
43. Roberts BT, Farr KA, Hoyt MA: **The Saccharomyces cerevisiae checkpoint gene Bub1 encodes a novel protein kinase.** *Mol Cell Biol* 1994, **14:**8282-8291.
44. Erikson E, Maller JL: ***In vivo* phosphorylation and activation of Ribosomal protein S6 kinases during *Xenopus* oocyte maturation.** *J Biol Chem* 1989, **264:**13711-13717.
45. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, *et al.*: **Identification of a novel inhibitor of mitogen-activated protein kinase kinase.** *J Biol Chem* 1998, **273:**18623-18632.
46. Cross DA, Smythe C: **PD 98059 prevents establishment of the spindle assembly checkpoint and inhibits the G2-M transition in meiotic but mitotic cell cycles in *Xenopus*.** *Exp Cell Res* 1998, **241:**12-22.
47. Kosaka H, Gotoh Y, Nishida E: **Mitogen-activated protein kinase kinase is required for the mos-induced metaphase arrest.** *J Biol Chem* 1994, **269:**28354-28358.
48. Goto T, Kinoshita T: **Maternal transcripts of mitotic checkpoint gene, Xbub3, are accumulated in the animal blastomeres of Xenopus early embryo.** *DNA Cell Biol* 1999, **18:**227-234.
49. Lohka MJ, Maller JL: **Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts.** *J Cell Biol* 1985, **101:**518-523.
50. Murray AW: **Cell cycle extracts.** *Methods Cell Biol* 1991, **36:**581-605.