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Ca²⁺-promoted cyclin B1 degradation in mouse oocytes requires the establishment of a metaphase arrest

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Abstract

CDK1-cyclin B1 is a universal cell cycle kinase required for mitotic/meiotic cell cycle entry and its activity needs to decline for mitotic/meiotic exit. During their maturation, mouse oocytes proceed through meiosis I and arrest at second meiotic metaphase with high CDK1-cyclin B1 activity. Meiotic arrest is achieved by the action of a cytostatic factor (CSF), which reduces cyclin B1 degradation. Meiotic arrest is broken by a Ca²⁺ signal from the sperm that accelerates it. Here we visualised degradation of cyclin B1::GFP in oocytes and found that its degradation rate was the same for both meiotic divisions. Ca²⁺ was the necessary and sufficient trigger for cyclin B1 destruction during meiosis II; but it played no role during meiosis I and furthermore could not accelerate cyclin B1 destruction during this time. The ability of Ca²⁺ to trigger cyclin B1 destruction developed in oocytes following a restabilisation of cyclin B1 levels at about 12 h of culture. This was independent of actual first polar body extrusion. Thus, in metaphase I arrested oocytes, Ca²⁺ would induce cyclin B1 destruction and the first polar body would be extruded.

In contrast to some reports in lower species, we found no evidence that oocyte activation was associated with an increase in 26S proteasome activity. We therefore conclude that Ca²⁺ mediates cyclin B1 degradation by increasing the activity of an E3 ubiquitin ligase. However, this stimulation occurs only in the presence of the ubiquitin ligase inhibitor CSF. We propose a model in which Ca²⁺ directly stimulates destruction of CSF during mammalian fertilisation.

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Introduction

In mammalian oocytes, there are two rounds of chromosome segregation. During meiosis I, a reductional division segregates homologous chromosomes and then in meiosis II an equational division segregates sister chromatids. There is much interest in how these two events are regulated because meiotic nondisjunction is believed to cause the high rates of aneuploidy seen in human conceptuses (Angell, 1997; LeMaire-Adkins et al., 1997; Pellestor et al., 2002). Both

meiotic divisions are driven by changes in the activity of maturation (M-Phase) promoting factor (MPF); a heterodimer of a catalytic CDK1 and a regulatory cyclin B1 component (Dorée and Hunt, 2002; Jones, 2004; Masui, 2001; Pines, 1999).

In oocytes, MPF activity peaks twice, firstly before segregation of homologous chromosomes and secondly before segregation of sister chromatids (Hashimoto and Kishimoto, 1988). In oocytes, as in somatic cells, at anaphase onset, MPF activity declines by destruction of cyclin B1 (Glotzer et al., 1991; Holloway et al., 1993; Kubiak et al., 1993; Ledan et al., 2001; Nixon et al., 2002). Firstly cyclin B1 is polyubiquitinated by the anaphase-promoting complex (APC), an E3 ubiquitin ligase (Harper et al., 2002; King et al., 1995; Peters, 2002; Sudakin et al., 1995; Zachariae and Nasmyth, 1999), whose activity before full

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congression on the spindle is attenuated by an ‘anaphase wait’/spindle checkpoint’ signal (Musacchio and Hardwick, 2002; Yu, 2002). Secondly, polyubiquitinated cyclin B1 is then targeted for destruction by the 26S proteasome, a multisubunit protease specific for polyubiquitinated proteins (Baumeister et al., 1998; Coux et al., 1996; Hershko et al., 1991; Murray, 1995).

Mammalian oocytes arrest at metaphase II until they are fertilised. Thus, oocytes possess an activity, known as ‘cytostatic factor’ (CSF; Ciemerych and Kubiak, 1999; Kubiak et al., 1993; Masui and Markert, 1971; Tunquist and Maller, 2003; Watanabe et al., 1991) whose function is to inhibit anaphase onset by attenuating cyclin B1 and securin degradation. In mammalian oocytes, cyclin B1 degradation does occur during CSF-induced cell cycle arrest, with cyclin B1 having a half-life of 1–2 h (Nixon et al., 2002; Winston, 1997), and thus spontaneous activation is only prevented by continued cyclin B1 synthesis. Induction of an ‘anaphase wait’/spindle checkpoint’ by microtubule-disrupting agents greatly stabilises cyclin B1 (Nixon et al., 2002; Winston, 1997). In contrast, sperm have the capacity to accelerate the destruction of cyclin B1 about six-fold (Nixon et al., 2002) and at fertilisation a rise in intracellular Ca^{2+} levels, possibly caused by a sperm-specific PLC ζ (Saunders et al., 2002), is the physiological trigger to overcome CSF-induced cyclin B1 stabilisation. However, the exact mechanism by which Ca^{2+} stimulates cyclin B1 destruction is poorly understood. There is evidence that CaM-dependent kinase II (CaMKII) is the immediate Ca^{2+} transducer (Lorca et al., 1993; Markoulaki et al., 2003; Tatone et al., 2002; Winston and Maro, 1995) but the ultimate downstream target of Ca^{2+} action has not been identified. Ca^{2+} could act to stimulate the activity of an E3 ubiquitin ligase (the APC); the 26S proteasome; or indeed both. Inhibition of either one of these activities (APC and/or proteasome) before any Ca^{2+} rise would likely constitute CSF. Direct measurements of 26S proteasome activity have found it to increase in fertilizing eggs of three species: frog (Aizawa et al., 1996), ascidian (Kawahara and Yokosawa, 1994), and sand dollar (Chiba et al., 1999). However, ubiquitin ligation seems the more plausible control point since the two strongest CSF candidates, c-mos–MAPK (Colledge et al., 1994; Hashimoto et al., 1994; Sagata et al., 1989; Schwab et al., 2001; Tunquist et al., 2002) and Emi1 (Reimann and Jackson, 2002), have both been proposed to reduce APC activity, through Bub1 and inhibition of APC activator CDC20, respectively.

How universal is the ability of Ca^{2+} to trigger cyclin B1 degradation? Although Ca^{2+} signals have been observed during mitosis in somatic cells (Kao et al., 1990), they do not appear to be a universal phenomenon during mitosis of mouse embryos (Kono et al., 1996). Also, in sea urchin embryos, Ca^{2+} seems to play a specific role in sister chromatid disjunction rather than cyclin B1 destruction (Groigno and Whitaker, 1998). In mouse oocytes, little is understood about the controlling factors that regulate cyclin

B1 destruction during meiosis I. Despite the two meiotic divisions in mouse being separated by just a few hours, no study has set out to determine if Ca^{2+} plays a controlling factor in meiosis-I-associated cyclin B1 destruction, despite the observation that cyclin B1 destruction here also requires the APC (Herbert et al., 2003; Wassmann et al., 2003).

Here we have addressed the function of Ca^{2+} in stimulating cyclin B1 destruction in mouse oocytes by using a cyclin B1 construct fused to the enhanced GFP. We find that Ca^{2+} has a precise function in promoting cyclin B1 degradation only when oocytes are arrested and so have CSF activity. Furthermore, we have used the interaction of the E3 ligase SIAH1 (House et al., 2003), with one of its substrates T-STAR, to demonstrate that Ca^{2+} does not function through regulating 26S proteasome activity. A finding we confirmed by a fluorometric assay for proteasomal activity during oocyte activation. From the present findings, we conclude that Ca^{2+} has a specific role in cell cycle progression, stimulating the loss of an anaphase inhibitor (CSF) whose activity first appears at the completion of meiosis I.

Materials and methods

All chemicals were from Sigma (UK) unless otherwise stated and embryo tested or tissue culture grade where appropriate. MG132 was from Calbiochem.

Gamete collection and culture

In the present studies, random-bred four- to eight-week-old albino MFI mice (Harlan, UK) were used. GV stage oocytes were collected 44–52 h after mice were primed with an intraperitoneal injection of 7.5 IU pregnant mares’ serum gonadotrophin (Intervet, UK). Ovaries were punctured with a sterile needle and cumulus-enclosed oocytes were stripped of their cumulus cells mechanically using a mouth pipette. Oocytes were cultured at 37°C in medium M2, with the addition of 0.1 mM dibutyryl cAMP to arrest oocytes at prophase I where necessary. Mature oocytes were collected 12.5–15 h after intraperitoneal injection of 5 IU human chorionic gonadotrophin (Intervet) in mice that had been pregnant mares’ serum gonadotrophin-primed 44–52 h previously. Cumulus cells were removed with 0.3 mg/ml hyaluronidase.

In some studies, oocytes were treated with the parthenogenetic agent ethanol (Cuthbertson et al., 1981). Oocytes were placed in medium M2 containing 7% ethanol for 7 min at room temperature. Oocytes were then immediately washed in warm M2 before use.

Preparation of constructs

The cyclin B1::GFP plasmid used (gift of Dr J. Pines, Cambridge) consists of a full-length human cyclin B1 fused via a five-amino-acid linker (AGA QF) to the second amino

acid residue of mmGFP. This fusion had been cloned into a pRN3 vector specifically to produce cRNA transcripts with maximal stability. This is conferred by the presence of 5' globin UTR upstream and both 3'UTR and a poly(A)-encoding tract downstream of the gene to be transcribed.

Full-length human PLC ζ was cloned into pRN3 vector. RT-PCR was performed on human testis poly(A) RNA (Ambion, UK). Forward (GATCAGATCTACTATG GAAATGAGATGG) and reverse (GATCGCGGCCGCT-TATCTGACGTACCAAA CATA) primers to the predicted 5' and 3' end of the human PLC ζ were used. Following cutting with *Bgl*II and *Not*I, the vector with ligated insert was transformed into JM109 cells (Promega). Colonies forming on LB-agar plates supplemented with ampicillin were further cultured in ampicillin-containing LB medium and plasmid DNA extracted using WizardPlus SV Minipreps (Promega, UK). The complete insertion of full-length PLC ζ was confirmed by DNA sequencing.

Full-length human T-STAR was cloned into a pGFP3 vector by standard cloning procedures and the success of cloning was confirmed again by sequencing. For all constructs, cRNA was produced using the T7 mMES-SAGE mMACHINE (Ambion), and dissolved in nuclease-free water to a concentration of at most 1 $\mu\text{g}/\mu\text{l}$ before microinjection.

Microinjection and imaging of cells

Microinjection of cRNA constructs or fura2 dextran (Molecular Probes, OR) were made essentially as previously described (Jones et al., 1995, 1998; Nixon et al., 2002). Briefly fabricated micropipettes were inserted into cells by using a pulse of the negative capacitance overcompensation facility on an electrometer. A precise, bolus, injection corresponding to 1–3% of the total cell volume was achieved using a timed injection on a Pneumatic PicoPump (World Precision Instruments). Imaging of cells simultaneously for Ca $^{2+}$ and GFP is as described previously (Hyslop et al., 2001; Nixon et al., 2002). All recordings represent the mean GFP reading from a single oocyte tracked as a Region Of Interest by the MetaFluor software package (Universal Imaging Corp., PA).

For experiments involving ACMS and Suc-Phe-Leu-Arg-CAMS, oocytes were first microinjected with either a 120 mM KCl, pH 7.4, buffer or with 0.1 $\mu\text{g}/\mu\text{l}$ PLC ζ cRNA. PLC ζ -injected oocytes that had extruded their second polar body, and control oocytes that had not, were then further injected with a 1–3% volume injection of 33 mM bestatin (Chiba et al., 1997) to inhibit exopeptidase activity. Fifteen to sixty minutes later, pairs of oocytes were microinjected with a micropipette containing 5 mM Suc-Phe-Leu-Arg-CAMS, which is a proteasome substrate, in 100 mM HEPES, pH 7, containing 50 μM ACMS, used to monitor that exactly equal injection volumes were given to each pair of oocytes. The rate of formation of ACMS was then followed in the

two oocytes for a period of at least 10 min (Chiba et al., 1997, 1999). Pairs of oocytes at a time were imaged, such that one was a control metaphase II arrested oocyte, while the other had been injected with PLC ζ . The excitation filter was $360 \pm 12\text{nm}$ (Coherent, UK) and a 400-nm dichroic mirror was used fitted with a 435-nm-long pass emission filter (Nikon, UK).

Results

Ca $^{2+}$ -dependent cyclin B1 destruction during segregation of sister chromatids

Cyclin B1, the regulatory component of MPF, was tagged to GFP and cRNA injected into mature oocytes to follow its rate of destruction during fertilisation. Cyclin B1::GFP has previously been demonstrated to faithfully mimic endogenous cyclin and report cyclin B1 rates of proteolysis (Clute and Pines, 1999; Hagting et al., 1998). We have used it in mouse meiosis, and in agreement with our previous findings (Nixon et al., 2002), we consistently found an acceleration of cyclin B1 destruction associated with oocyte activation. In mammals, the sperm-induced signal for activation is a series of Ca $^{2+}$ spikes lasting several hours. Only about 2% of the total decrease in cyclin B1::GFP was observed during the first sperm-induced Ca $^{2+}$ spike. Instead, in the present study, we concentrated on the long-lasting decline in cyclin B1, which makes up the vast majority of the cyclin B1 degradation at fertilisation. The steady cyclin B1 decline began several minutes (13 ± 5 , $n = 20$) after the initiation of Ca $^{2+}$ spiking (Fig. 1A; starred) decreasing exponentially over the next 2 h (110 ± 35 min; $n = 20$), and terminating in extrusion of the second polar body following segregation of sister chromatids. It is highly probable, however, that newly synthesised cyclin B1 continues to be destroyed during the remaining period of meiotic exit (around 6 h) because cyclin B1::GFP levels only increased again once the oocyte had finished meiosis and pronuclei were visible in the one-cell embryo.

The sperm-induced decrease in cyclin B1 can be blocked by preincubation of mature oocytes with the Ca $^{2+}$ chelator BAPTA (Fig. 1B, $n = 7$), and can be mimicked by microinjection of cRNA to the sperm-specific oocyte-activating factor PLC ζ (Fig. 1C; Saunders et al., 2002, $n = 25$). This demonstrates that the promotion of cyclin B1 destruction during meiosis II, when sister chromatids are segregated, is dependent physiologically on the ability of sperm to generate a cytosolic rise in Ca $^{2+}$.

Ca $^{2+}$ -independent cyclin B1 destruction during segregation of homologous chromosomes

The above experiments show that the function of Ca $^{2+}$ is to increase the activity of the 26S proteasome and/or

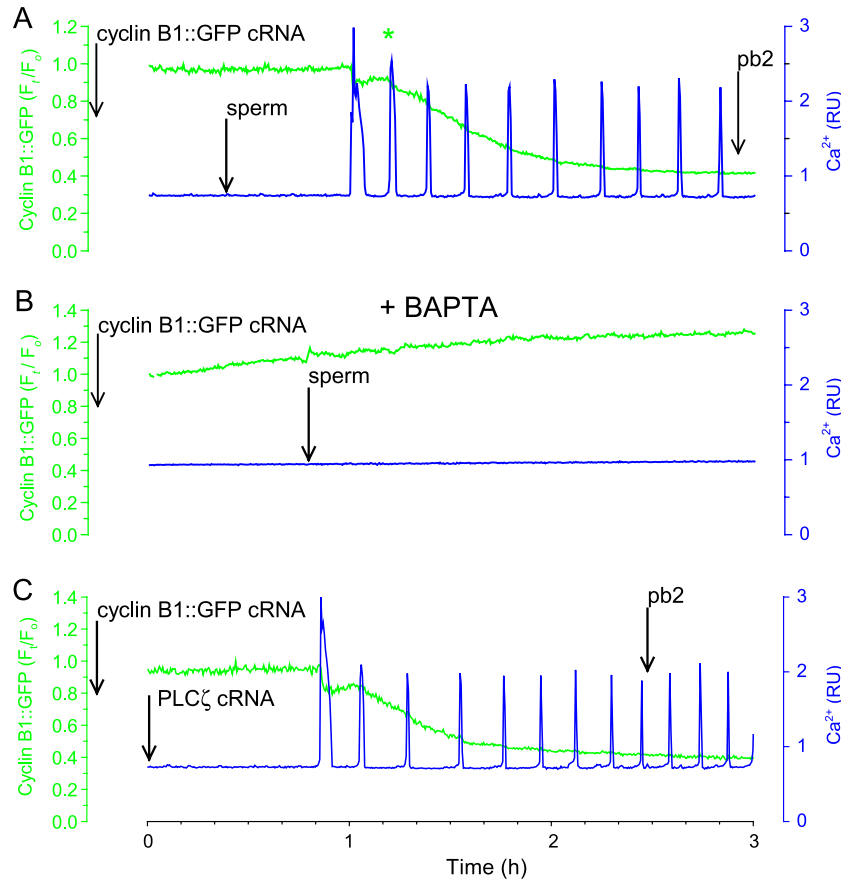


Fig. 1. Ca^{2+} promotes cyclin B1 destruction during meiosis II. Metaphase II mouse oocytes were coinjected with cyclin B1::GFP cRNA and fura2 dextran, and cultured for a further 2–3 h. At the times indicated by the arrow, oocytes were inseminated (sperm) in the absence (A) or presence (B) of BAPTA-AM (10 μM); or (C) microinjected with PLC ζ cRNA (0.25 $\mu\text{g}/\mu\text{l}$). In control oocytes (A), the initiation point in the steady decline of cyclin B1::GFP is marked by an asterisk and second polar body extrusion is indicated (pb2). In BAPTA-treated oocytes, there was no drop in cyclin B1 levels. Sperm fusion was confirmed by staining with Hoescht 33258 at the end of the recording period. Similar to the profile with inseminated oocytes, PLC ζ induced Ca^{2+} spiking and cyclin B1 degradation. GFP readings are represented by the quotient F_t/F_0 , where F_t is the fluorescence at time t hours, and F_0 is fluorescence at time 0 hours. F_0 was comparable for all three oocytes shown ($\pm 50\%$ of each other). Ca^{2+} is measured as fluorescence at excitation 340/380 nm (RU = ratio units). Intracellular Ca^{2+} and GFP fluorescence were monitored every 20 s. Time 0 h represents the time at which recording was started. In some oocytes, a steady state in cyclin B1 levels had been reached by the time sperm were added (A, C), while in others cyclin B1 levels were still rising (B). A steady state can be reached because metaphase II arrested oocytes have APC activity, and are therefore capable of degrading expressed cyclin B1 (Nixon et al., 2002).

APC during the period of metaphase II arrest. We then went on to examine if the segregation of homologous chromosomes is a process also associated with Ca^{2+} spiking. Tombes et al. (1992) previously commented on their inability to detect Ca^{2+} changes during oocyte maturation; however, in that study, they used fura2-AM, which because of its compartmentalisation into intracellular organelles makes it a problematic Ca^{2+} reporter over long time courses (Malgaroli et al., 1987). In the present study, we attempted to examine for Ca^{2+} changes in oocytes using dextran-coupled fura2, which is not compartmentalised, and also simultaneously monitor for cyclin B1::GFP degradation.

We microinjected cyclin B1::GFP cRNA into prophase I arrested oocytes that were then allowed to mature. Some oocytes, expressing very high amounts of cyclin B1 (Fig. 2A, black trace), failed to show any

obvious minimum in cyclin B1 levels during their period of maturation. However, the vast majority of oocytes ($n = 37/43$; red and green traces) showed a distinct period of cyclin B1 degradation, that in some ended with extrusion of the first polar body (Fig. 2A; green trace). At about 12 h of culture for all these maturing oocytes cyclin B1 levels stabilised, if maturation proceeded fully, then this restabilisation followed first polar body extrusion. Interestingly higher levels of expressed cyclin B1 appeared associated with a failure to extrude the first polar body (green vs. red; Fig. 2B and Table 1). This suggests a minimum CDK1 activity is required to complete meiosis I. The oocytes failing to extrude a polar body arrested at metaphase I with a fully aligned spindle (data not shown). Similar observations on cyclin B1-expressing oocytes have been made previously (Ledan et al., 2001) and this metaphase I arrest can also be observed in a

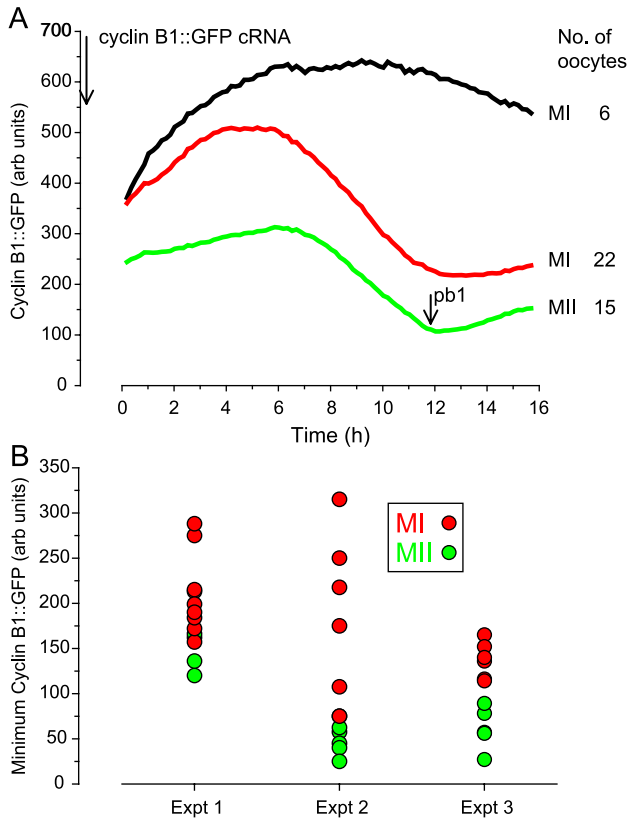


Fig. 2. Oocyte maturation in cyclin B1-expressing oocytes. Cyclin B1::GFP levels were monitored during oocyte maturation following injection of cRNA at the GV stage. (A) In the majority of oocytes, there was a period of cyclin B1 destruction (red and green traces), during 6–12 h of culture. In 15 of the 43 oocytes, the first polar body (pb1) was extruded during maturation, and in these oocytes pb1 was associated with the minimum in the cyclin B1 concentration (green trace). Only a few oocytes failed to show a minimum in their cyclin B1::GFP levels (black trace) and these oocytes expressed very high levels of cyclin. Stabilisation of cyclin B1 levels at about 12 h of culture was independent of segregation of homologous chromosomes since some oocytes (red trace) failed to extrude a pb1 but instead arrested at metaphase I. (B) In three separate experiments, the recorded minimum in the cyclin B1::GFP trace was a good indicator of whether oocytes would arrest at meiosis I (red) or meiosis II (green). Oocytes containing more cyclin B1 consistently arrested at meiosis I. GFP fluorescence was monitored every 10 min. Experiments were started 3 h after cyclin B1 microinjection by washing free of dbcAMP and at this time imaging was initiated.

smaller percentage of GV control oocytes (Table 1; Donahue, 1968; Eppig et al., 1994; Sorensen and Wassarman, 1976; Wickramasinghe et al., 1991). In the one third of oocytes expressing cRNA for cyclin B1::GFP that matured fully to a metaphase II oocyte, the chromatin when stained at the end of the recording was aligned on a metaphase II plate and chromosome spreads showed attached bivalents (not shown); therefore, they were indistinguishable from control *in vitro* fully matured oocytes.

In 11 oocytes, we were able to monitor cyclin B1 destruction during meiosis I, and then following oocyte

Table 1
Cyclin B1 overexpression reduces the rate of oocyte maturation

	Prophase I	Metaphase I [% (number)]	Metaphase II
Uninjected	4 (3)	18 (13)	78 (56)
KCl	1 (1)	21 (24)	78 (90)
eGFP	2 (1)	18 (11)	80 (48)
cyclin B1	7 (3)	61 (28) ^a	33 (15) ^a

Cyclin B1 expression in oocytes led to an increased arrest of oocytes at metaphase I (MI). Oocytes were classified as either at prophase I (no GV breakdown); at metaphase I (no first polar body extrusion); or at metaphase II (first polar body extruded). We confirmed metaphase I arrest by staining with Hoescht 33258 ($n = 12$) and also by Giemsa spreads ($n = 4$). At the GV stage, the following injections were made: KCl buffer (120 mM KCl, 10 mM HEPES pH 7.2); eGFP cRNA (1 $\mu\text{g}/\mu\text{l}$); or cyclin B1::GFP cRNA (1 $\mu\text{g}/\mu\text{l}$). Oocyte maturation was assessed at 16 h after washing free of dbcAMP, done to initiate maturation.

^a Significantly different from all other conditions (χ^2 test, $P < 0.01$).

activation at meiosis II (Fig. 3A). Oocyte activation was induced by addition of ethanol, a commonly used parthenogenetic agent (Cuthbertson et al., 1981) able to

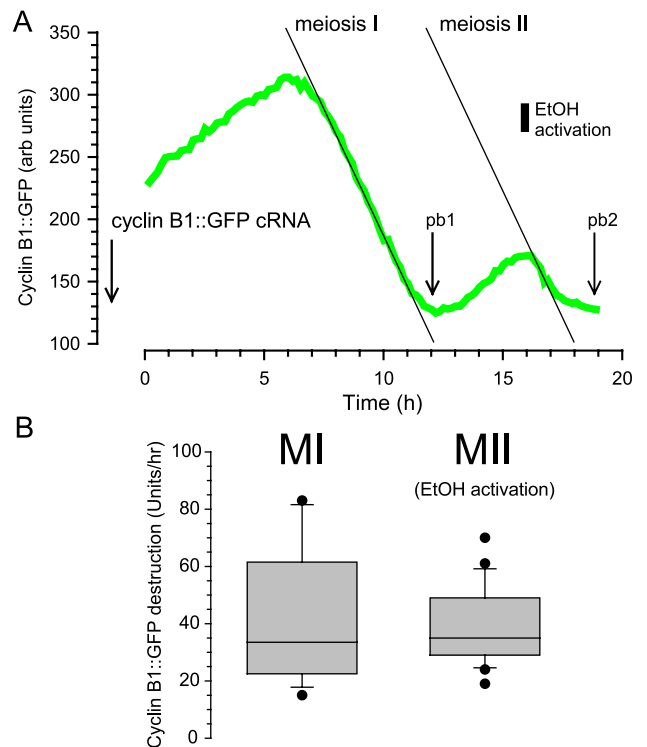


Fig. 3. Cyclin B1 destruction rates are similar during meiosis I and II. (A) GV stage oocytes were microinjected with cyclin B1::GFP cRNA and GFP fluorescence followed during oocyte maturation. At the time indicated, metaphase II arrested oocytes were activated by a brief incubation with 7% ethanol. Brightfield images were captured every 10 min so as to monitor extrusion of the first and second polar bodies (pb1 and pb2). The diagonal lines represent the maximum rates of cyclin B1 destruction during the two meiotic divisions. (B) By measuring the rate of cyclin B1 destruction in oocytes during meiosis I and then during meiosis II following ethanol-induced oocyte activation, it was possible to compare the two rates of destruction. The rates of cyclin B1 destruction during meiosis I and meiosis II were not statistically different ($n = 11$, $P = 0.68$, paired t test).

induce a large rise in intracellular Ca^{2+} . Interestingly, the rate of cyclin B1 degradation was similar during the two meiotic divisions despite the absolute levels of cyclin B1 being much less during meiosis II (Fig. 3B). In further control experiments, we examined the rate of cyclin B1 degradation at meiosis II for oocytes activated with ethanol and with strontium, to provide an oscillatory Ca^{2+} signal. We found no significant difference in the rates of cyclin B1 degradation (not shown).

Since the rate of cyclin B1 destruction was similar at meiosis II as at meiosis I, and cyclin B1 destruction is initiated by Ca^{2+} at meiosis II, we therefore wanted to establish if a Ca^{2+} signal also prompted cyclin B1 degradation during oocyte maturation. Oocytes were microinjected with cyclin B1::GFP cRNA (0.3 $\mu\text{g}/\mu\text{l}$) and fura2 dextran. Cyclin B1 levels and intracellular Ca^{2+} changes were then recorded during oocyte maturation in addition to brightfield images to monitor for polar body formation. Fifty-five percent of oocytes ($n = 18$) extruded a first polar body, a higher rate than in Table 1 because a lower cyclin B1 cRNA concentration was used, but in all oocytes no Ca^{2+} spiking was observed either at the initiation of cyclin B1 destruction (Fig. 4) or during the entire period of cyclin B1 destruction that terminated with polar body formation ($n = 10/10$). We confirmed the ability of the Ca^{2+} reporter dye fura2 to remain sensitive to Ca^{2+} changes throughout this very long period of recording by the addition of thimerosal at the end of the experiment (Fig. 4A). To examine if addition of exogenous cyclin B1 had a deleterious effect on the ability of oocytes to generate Ca^{2+} spikes, we performed the same experiments on oocytes microinjected with only fura2 dextran ($n = 22$) or Ca^{2+} green dextran ($n = 18$), another Ca^{2+} reporter. In these maturing oocytes, we monitored brightfield images for polar body formation simultaneously with intracellular Ca^{2+} . However, in all oocytes, we found that no Ca^{2+} changes were measurable for the entire period of recording, from the period of washing free of dbcAMP, done to initiate oocyte maturation, until the extrusion of the first polar body.

To further examine the role of Ca^{2+} during meiosis, we looked to see if Ca^{2+} would accelerate cyclin B1 destruction during meiosis I. Oocytes expressing cyclin B1::GFP were matured and cyclin B1 levels monitored. When a steady decline in cyclin B1 was observed, after about 6 h of culture, oocytes were treated with ethanol for a 7-min period to induce a large rise in cytosolic Ca^{2+} and then cyclin B1 further imaged to monitor for any acceleration of cyclin B1 destruction. However, in none of these maturing oocytes ($n = 5$) was any acceleration of cyclin B1 destruction observed (Fig. 4B). For ethanol activation, oocytes had to be removed from the stage of the microscope and placed in ethanol-containing medium. Therefore, there is a gap in the recording period during this time (Fig. 4B). We confirmed that ethanol is a good agent for inducing a large

Ca^{2+} rise by measuring the Ca^{2+} changes induced by it in maturing oocytes. All oocytes studied in this way responded to ethanol treatment ($n = 6$) with a Ca^{2+} rise that was greater than that induced by sperm (data not shown).

Cyclin B1 destruction during segregation of homologous chromosomes is Ca^{2+} -dependent if oocytes arrest at metaphase I

From the above data, we conclude that segregation of homologous chromosomes proceeds independently of observable Ca^{2+} spiking. Here we examined if maturing oocytes that had arrested at metaphase I (Fig. 2A) were Ca^{2+} -sensitive, as are oocytes arrested at metaphase II.

Oocytes were microinjected with cyclin B1::GFP cRNA and released from prophase I arrest for a period of 15 h. Oocytes that had not extruded a first polar body were then imaged for a further 10 h over which time cyclin B1::GFP and brightfield images were captured. During this time, no oocytes went on to extrude a first polar body, and cyclin B1 levels appeared stable (not shown). These observations were therefore consistent with a metaphase I arrest. In metaphase I arrested oocytes, intracellular Ca^{2+} was raised using the parthenogenetic agent ethanol and cyclin B1 destruction was observable in two-thirds of oocytes (Fig. 5; $n = 22/31$). Some of the oocytes ($n = 10$) went on to extrude a first polar body (Fig. 5) and then arrest at metaphase II. Therefore, in metaphase I arrested oocytes, there was a Ca^{2+} -stimulated decrease in cyclin B1, and in a proportion of these where the drop was sufficient, first polar body extrusion occurred. A simultaneous experiment performed with GV stage oocytes, at prophase I, expressing similar amounts of cyclin B1::GFP failed to show any decrease in cyclin B1 levels. Therefore, Ca^{2+} -stimulated cyclin B1 destruction appears specific to a metaphase arrest.

Ca^{2+} fails to stimulate the 26S proteasome during meiosis II

Cyclin B1 destruction is a two-step process, involving first its polyubiquitination through the APC, and second its proteolysis mediated by the 26S proteasome. Since we observe a Ca^{2+} -mediated acceleration of cyclin B1 destruction at metaphase I or metaphase II arrest, Ca^{2+} has to activate, directly or indirectly, either the APC and/or the 26S proteasome. Current models on the identity of CSF would predict that Ca^{2+} acts through the APC, yet there is some evidence in oocytes of other species that at fertilisation there is also an increase in 26S proteasome activity. We therefore thought it important to examine 26S proteasome activity before and during Ca^{2+} spiking. We used the E3 ligase SIAH1 as an APC-independent tool to examine the degradation of polyubiquitinated proteins (Fig. 6A). Our approach was to microinject cRNA to a GFP-tagged SIAH1 substrate, initiate its

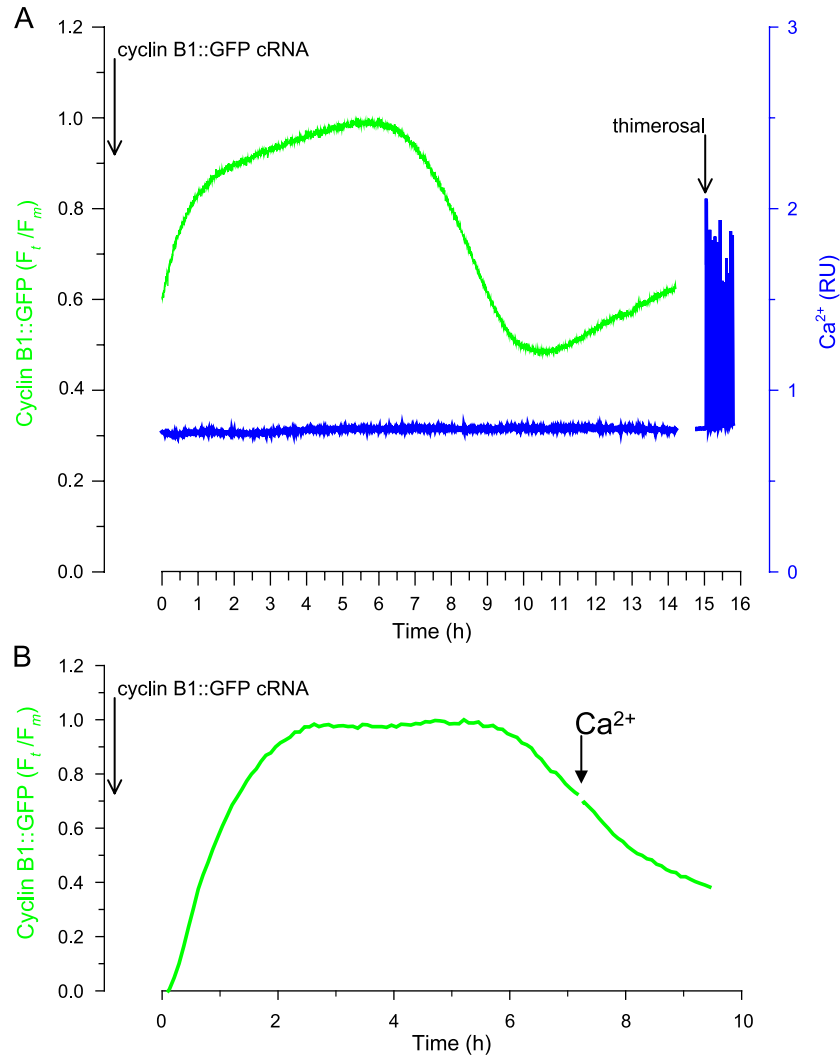


Fig. 4. Meiosis I proceeds independently of Ca^{2+} . (A) When intracellular Ca^{2+} levels were monitored in maturing oocytes, there were no observable changes in Ca^{2+} before or during the steady decline in cyclin B1 levels. GV-stage oocytes were microinjected with fura2 dextran and cyclin B1::GFP cRNA. Images were captured after 2–3 h of culture at which time oocytes were washed free of dbcAMP and allowed to mature. Cyclin B1 reached a minimum at the time of first polar body extrusion (pb1). In these oocytes, which had matured to metaphase II, the thiol-reagent thimerosal was added to induce Ca^{2+} spiking, and hence demonstrate that the Ca^{2+} reporter dye used was still responsive to cytosolic Ca^{2+} changes. (B) Raising cytosolic Ca^{2+} did not further promote cyclin B1 destruction once it had been initiated at meiosis I. Oocytes expressing cyclin B1::GFP were allowed to mature and when the initiation of cyclin B1 destruction was observed oocytes were treated with the parthenogenetic agent 7% ethanol to induce a large rise in cytosolic Ca^{2+} . No further increase in the rate of cyclin B1 destruction was observed following the rise in cytosolic Ca^{2+} . Images were captured every 20 s (A) or every 5 min (B). Ca^{2+} was measured as a ratio of 340/380 nm signal (RU). GFP readings are expressed by the quotient F_t/F_m , where F_t is the fluorescence at time t , and F_m is the maximum recorded fluorescence during the maturation period.

degradation by addition of cRNA to SIAH1, and then finally initiate Ca^{2+} spiking by a third microinjection of cRNA to PLC ζ . If Ca^{2+} was stimulating the 26S proteasome, then like cyclin B1::GFP, we would observe an acceleration in the destruction of the SIAH1 GFP-coupled substrate following initiation of Ca^{2+} spiking. We used the SIAH1 substrate, T-STAR/Salp/SLM-2/KHDRBS3 (Di Fruscio et al., 1999; Lee and Burr, 1999; Venables et al., 1999), a testis-specific protein related to Sam68, which is believed to regulate RNA splicing. When expressed in 293T cells, T-STAR is degraded following addition of the

E3 ligase SIAH1 (Venables and Elliott, unpublished). When cRNA to T-STAR::GFP was expressed in oocytes, it had no observable deleterious affect on oocyte morphology, and when injected into GV stage oocytes these went on to mature to metaphase II. T-STAR was a stable protein when expressed in oocytes, because the addition of cycloheximide to block protein synthesis did not lead to any rapid decline in T-STAR::GFP signal. In contrast, following the addition of the E3 ligase SIAH1, we observed a steady decline in T-STAR signal (Figs. 6B,C). In similar experiments, we observed no decline

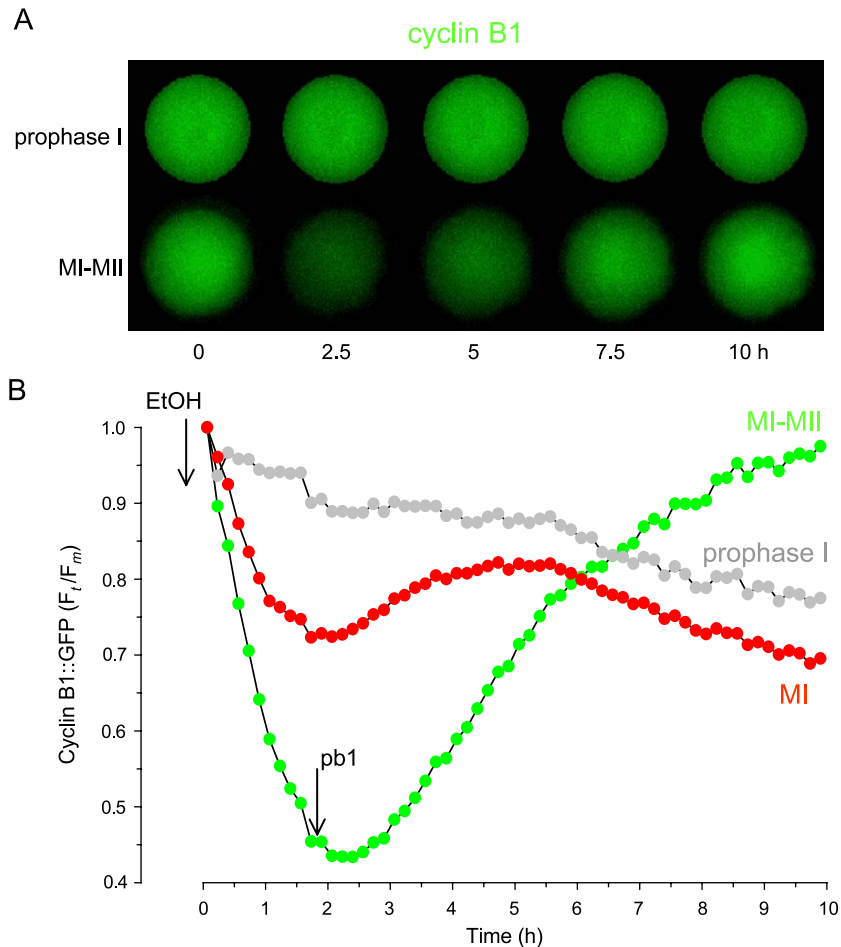


Fig. 5. Ca^{2+} promotes cyclin B1 destruction when oocytes arrest at metaphase I. Oocytes were matured following microinjection of cyclin B1::GFP cRNA and fura2 dextran. Oocytes that had failed to extrude a polar body by 25 h after release from dbcAMP were incubated with the parthenogenetic agent ethanol. Recording of cyclin B1::GFP began 10–11 min after oocytes were first cultured in ethanol-containing medium. (A) Represents pseudocolour images and (B) plots of the transient drop in cyclin B1::GFP levels recorded in metaphase I arrested oocytes ($n = 22$, red and green traces) but not in prophase I arrested oocytes ($n = 11$, grey) that were used as control. Some of the oocytes that were metaphase I arrested failed to extrude a polar body even though cyclin B1 levels decrease ($n = 12$, red trace). In these oocytes, the drop in cyclin B1 was small compared to oocytes that did extrude a polar body (decrease of 0.46 ± 0.11 versus 0.20 ± 0.08 , mean \pm standard deviation, $n = 22$, significantly different, t test, $P < 0.001$).

in GFP signal when T-STAR was replaced with cRNA to eGFP or a related splicing factor Sam68 (not shown). Furthermore, when oocytes were preincubated with the proteasome inhibitor MG132 (Lee and Goldberg, 1996), SIAH1 cRNA microinjection into oocytes failed to lead to degradation in T-STAR demonstrating that SIAH1 mediated T-STAR degradation requires the 26S proteasome. Importantly, in contrast to similar experiments performed with cyclin B1::GFP, no deflection in the rate of SIAH1-mediated T-STAR::GFP destruction was observed following the initiation of Ca^{2+} spiking (Fig. 7A; $n = 5$). These results therefore demonstrate that T-STAR is degraded through the action of the E3 ligase SIAH1 and the 26S proteasome, but the rate of destruction is not enhanced by Ca^{2+} . We also examined what happened to the degradation of T-STAR during extrusion of the first polar body. At this time, cyclin B1 levels become stable

for an extended period of time, due to the action of CSF (Fig. 2B). We observed an obvious inflexion in the rate of cyclin B1 degradation at polar body extrusion but not with SIAH1-mediated T-STAR destruction, which continued to decrease unabated (Fig. 7B). This confirms the above observations that CSF mediates its action solely through the APC in mammalian oocytes. If it had been affecting proteasome activity, then T-STAR, like cyclin B1, would have been stabilised at polar body extrusion.

How polyubiquitinated substrates are transferred to the 26S proteasome (Hartmann-Petersen et al., 2003) and what proteins constitute the complex (Leggett et al., 2002) are still being resolved. Because of this, it remains plausible that distinct proteasome complexes exist that are Ca^{2+} sensitive and preferentially degrade cyclins. Indeed, cell cycle components in budding yeast, notably cdc28, have been proven to interact directly with the 26S proteasome (Kaiser et al.,

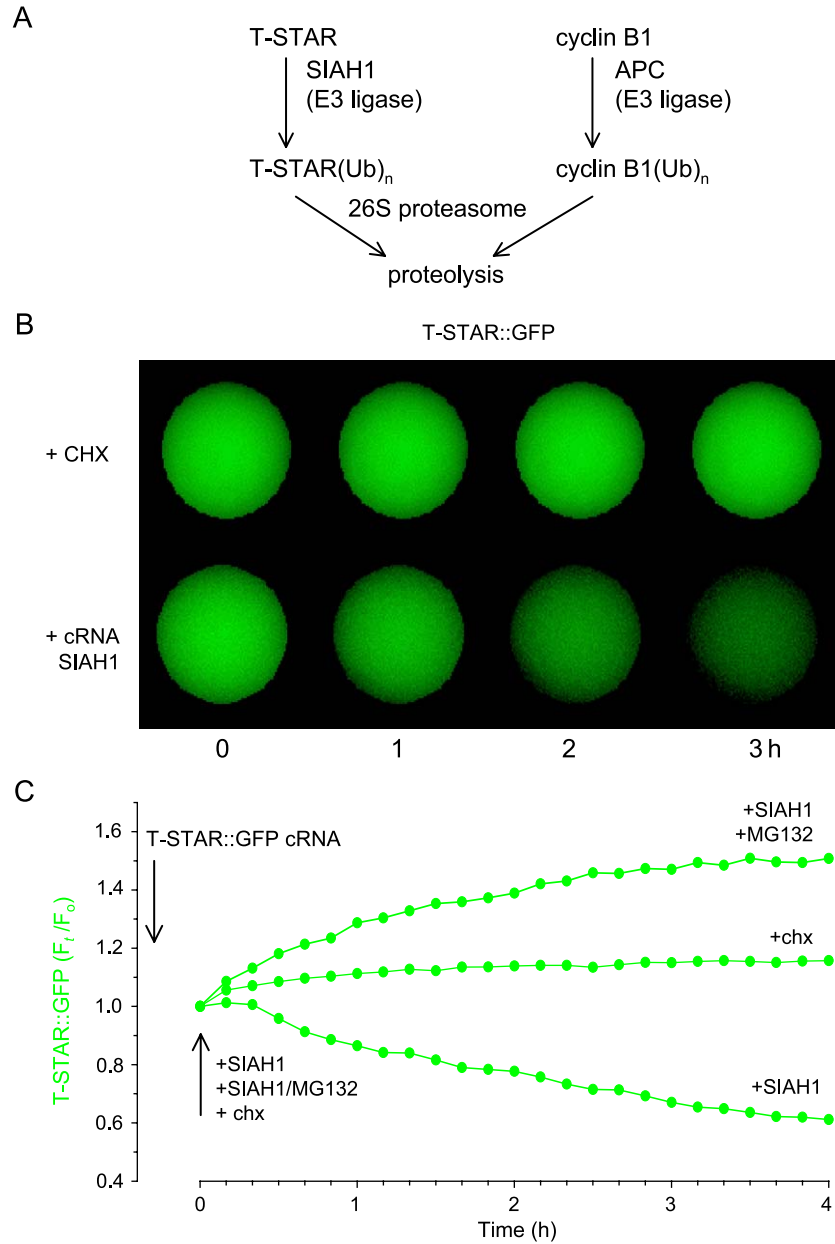


Fig. 6. SIAH1 mediates T-STAR destruction through the 26S proteasome. (A) A model to show the relationship between T-STAR, SIAH1, cyclin B1, the APC and the 26S proteasome. Here we show that T-STAR is a substrate of the RING finger E3 ligase SIAH1. In an analogous way, cyclin B1 is a substrate of the E3 ligase the APC. Both T-STAR and cyclin B1 become polyubiquitinated as a result of ubiquitin ligation, and as such are targets of the 26S proteasome. The ability of T-STAR to be degraded through SIAH1 in mouse oocytes is shown in B and C. (B) Pseudocolour images and (C) corresponding fluorescence readings taken from oocytes that had been microinjected with T-STAR::GFP cRNA 2–3 h previously. At time 0 h, oocytes were incubated with cycloheximide (chx, 1 μg/ml) or further microinjected with SIAH1 cRNA. The addition of the E3 ligase SIAH1 led to degradation of T-STAR, which otherwise was stably expressed. The 26S proteasome inhibitor MG132 completely blocked the degradation of T-STAR, demonstrating the need for the 26S proteasome in this pathway. The plots are representative of: 8 (+SIAH1 and MG132), 7 (+chx), or 12 (+SIAH1) oocytes. Images were captured every 10 min.

1999). Therefore, we measured total cellular proteasome activity by measuring the formation of fluorogenic ACMS. ACMS is generated by the proteolytic activity of the proteasome against peptide amides of AMC, in this case suc-Phe-Leu-Arg-CAMS (Chiba et al., 1997, 1999). This method has been used to measure increased proteasome

activity during starfish maturation and also during sand dollar egg fertilisation. However, in agreement with the observations with T-STAR here, we found no evidence for increased proteasome activity (Fig. 8; *n* = 8 pairs) during exit from meiosis II in mouse oocytes. We conclude that Ca²⁺ does not induce oocyte activation by increasing

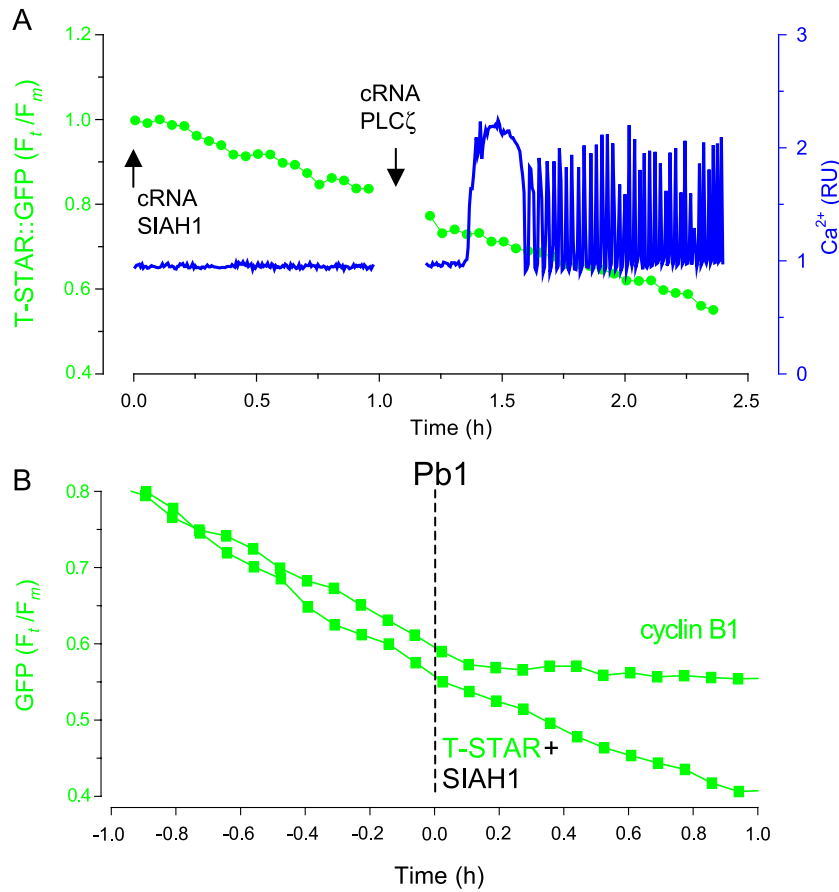


Fig. 7. SIAH1-mediated T-STAR destruction during meiosis I and meiosis II. (A) T-STAR degradation mediated by SIAH1 was not affected by PLC ζ -induced Ca^{2+} spiking. Metaphase II arrested oocytes expressing T-STAR::GFP were microinjected with SIAH1 cRNA to initiate T-STAR degradation. The rate of T-STAR degradation was unaffected by the initiation of Ca^{2+} spiking caused by the further microinjection of PLC ζ (microinjected during the recording gap). Fura2 images were captured every 10 s and T-STAR::GFP every 3 min. This recording is representative of four other oocytes. (B) T-STAR degradation mediated by SIAH1 was also not affected by extrusion of the first polar body (Pb1, $n = 12$); however, control oocytes in separate experiments expressing cyclin B1 showed an obvious inflexion in the rate of cyclin B1 destruction following Pb1 extrusion ($n = 15$). Times are given relative to Pb1 extrusion.

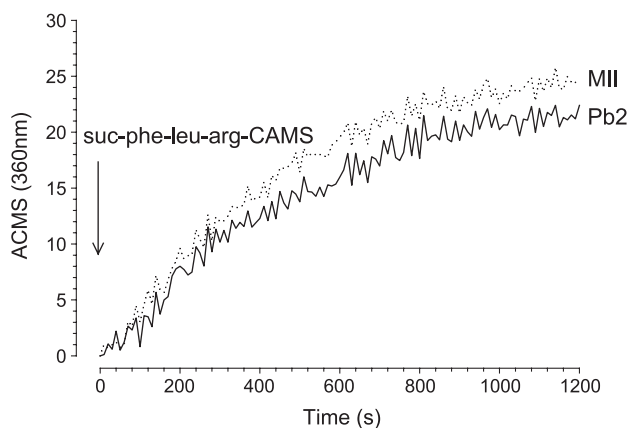


Fig. 8. Measurement of proteasome activity shows no increase following oocyte activation. Metaphase II oocytes and those activated by PLC ζ (cRNA, 0.1 $\mu\text{g}/\mu\text{l}$) were injected with the proteasomal substrate Suc-Phe-Leu-Arg-CAMS. Proteasomal activity was measured by the rate of formation of ACMS. Pairs of oocytes were imaged in any one experiment, such that the same micropipette was used for both a metaphase II oocyte and one that had extruded its second polar body (Pb2) following microinjection with PLC ζ . This experiment is typical of seven other pairs of microinjections.

proteasome activity, despite observations to the contrary in oocytes of lower species.

Discussion

Little is understood about the process by which Ca^{2+} controls cyclin B1 destruction during a CSF-induced cell cycle arrest; especially in mammalian oocytes. To further explore this process, cyclin B1 was coupled to GFP to visualise its destruction in mouse oocytes during the two meiotic divisions. Cyclin B1 was degraded at the same rate during the two meiotic divisions, suggesting that once initiated, destruction proceeds along a common pathway. Therefore, both periods of cyclin B1 destruction may be controlled by the same degradation pathway (APC activated by CDC20; and the 26S proteasome) present to the same amounts and working at the same rates.

During the second meiotic division, Ca^{2+} was the necessary and sufficient trigger for cyclin B1 destruction.

In contrast, during meiosis I, homologous chromosomes segregated in the absence of any measurable cytosolic Ca^{2+} changes and furthermore cyclin B1 degradation was not enhanced by Ca^{2+} . In maturing oocytes that failed to mature fully, and so arrested at metaphase I, Ca^{2+} stimulated cyclin B1 destruction and led to segregation of homologous chromosomes. Therefore, an activity appeared in oocytes at about 12 h of culture that stabilised cyclin B1 levels and conferred a sensitivity to Ca^{2+} -mediated cyclin B1 destruction. In contrast to the Ca^{2+} -induced acceleration in cyclin B1 destruction observed in mature oocytes, mediated by its E3 ligase the APC, there was no Ca^{2+} -induced acceleration in the rate of T-STAR degradation, mediated by its E3 ligase SIAH1. In addition, direct measurement of proteasome activity in oocytes showed no difference between mature oocytes and oocytes activated by PLC ζ . These observations show that Ca^{2+} is more likely to control APC activity than the 26S proteasome. Since physiological arrest at metaphase II and spontaneous arrest at metaphase I are both periods associated with a stabilisation of cyclin B1, it is argued that Ca^{2+} targets an activity (CSF) that is specifically generated at these times and arrests the cell cycle.

Ca^{2+} does not affect mouse oocyte 26S proteasome activity

The immediate Ca^{2+} transducer at fertilisation is CaM-dependent protein kinase II (Lorca et al., 1993; Markoulaki et al., 2003; Tatone et al., 2002; Winston and Maro, 1995). Since cyclin B1 degradation increases following the Ca^{2+} signal, then the Ca^{2+} signal provided by the sperm must ultimately increase the activity of the 26S proteasome and/or the APC. Proteasome activity has been seen to increase during the fertilisation of sand dollar (Chiba et al., 1999), ascidian (Kawahara and Yokosawa, 1994) and frog (Aizawa et al., 1996) eggs making the 26S proteasome a plausible target. However, there has been only one report of Ca^{2+} interacting with the proteasome and in this case Ca^{2+} decreases, rather than stimulates its activity, by binding a KEKE-containing REG subunit (Realini and Rechsteiner, 1995). In addition, in sand dollar eggs, it is the fertilisation-associated pH rise and not the Ca^{2+} rise that activates the 26S proteasome (Chiba et al., 1999), and in mammalian oocytes there is no pH rise at fertilisation (Kline and Zagray, 1995). Therefore, the only evidence that Ca^{2+} stimulates the 26S proteasome is from biochemical analyses on proteolysis rates in ascidian and frog eggs. The present data in mouse oocytes, however, present no evidence of a general increase in 26S proteasome activity, since there is a clear increase in the proteolysis of cyclin B1 but not of T-STAR, and direct measurement of proteasome activity fails to show any increase during fertilisation. In addition, the establishment of CSF activity during meiosis II was not associated with a decline in 26S proteasome activity (Fig. 7B).

An increase in APC activity at fertilisation is therefore the more likely pathway stimulated by Ca^{2+} . This is based on observation that proposed CSF candidates work through inhibiting APC action. Thus, the c-mos–MAPK cascade has the spindle checkpoint protein Bub1 as its downstream effector (Schwab et al., 2001). While Emi1, another proposed CSF candidate (Reimann and Jackson, 2002), binds CDC20/Fizzy to inhibit its interaction with the APC (Reimann et al., 2001) and thereby decreasing APC activity. In fact, in frog, immunodepletion of CDC20/Fizzy blocks CaMKII-mediated oocyte activation following CSF-induced oocyte arrest (Lorca et al., 1998). All these data taken together suggest that it is the APC rather than the 26S proteasome that is stimulated by Ca^{2+} .

Ca^{2+} -independent meiosis I

Here we observed that segregation of homologous chromosomes during meiosis I proceeded in the absence of any detectable change in intracellular Ca^{2+} . This is in agreement with earlier indirect observations that polar body extrusion would occur in BAPTA-treated oocytes (Tombes et al., 1992). If Ca^{2+} changes are present in maturing oocytes, they are certainly not to the same magnitude as those seen at fertilisation. Therefore, Ca^{2+} appears to play little function in stimulating cyclin B1 destruction during meiosis I and in support of this view we also found that Ca^{2+} would not accelerate loss of cyclin B1 during its normal window of destruction at meiosis I.

In mammalian oocytes, a CSF activity, assessed by fusion experiments between maturing oocytes and embryos, only appears shortly after first polar body extrusion (Ciemerych and Kubiak, 1998). In agreement with this observation, we (Figs. 2 and 3) and others (Ledan et al., 2001) find that expressed cyclin B1 becomes stable at this time. Interestingly, this CSF-mediated stabilisation appears independent of actual extrusion of the polar body, as it is observed in the majority of maturing oocytes that fail to extrude any polar body. The trigger for CSF is obviously not polar body extrusion. Instead, stimulation of its synthesis/activation may be initiated at the point at which cyclin B1 destruction begins during meiosis I. Efficient cyclin B1 destruction during meiosis I appears a necessary prerequisite for polar body extrusion (Fig. 2). Indeed, a nondestructible cyclin B1 arrests oocytes at metaphase I (Herbert et al., 2003). However, cyclin B1-expressing oocytes that failed to extrude a polar body and instead arrested at metaphase I showed Ca^{2+} -sensitive cyclin B1 destruction. These oocytes could be considered abnormal, since they should be at metaphase II. However, the important point here is that only when an arrest is imposed (naturally at metaphase II, or artificially at metaphase I) does cyclin B1 degradation become Ca^{2+} sensitive. It is clear that for the normal period of meiosis I (Fig. 4), Ca^{2+} has no role in cyclin B1 destruction, nor can it accelerate cyclin B1 destruction when artificially imposed on the

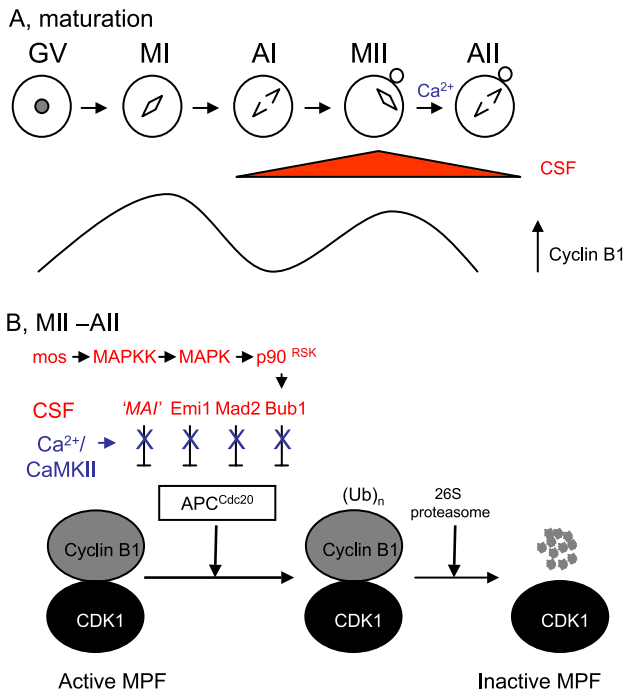


Fig. 9. Model of Ca^{2+} -stimulated CSF destruction during meiotic arrest. This model is based on the observed profile of cyclin B1 destruction in mouse oocytes. (A) Cyclin B1 destruction is initiated during meiosis I and reaches a minimum level at the time of first polar body extrusion. After this time, oocytes do not enter interphase but instead chromatin remains condensed and a second meiotic spindle is formed. Oocytes remain arrested at metaphase II until a Ca^{2+} signal provided by the sperm breaks this arrest. The arrest is initiated and maintained by the activity of CSF. (B) The molecular identity of CSF is unresolved but Emi1, Mad2 and Bub1 (the most downstream component of the c-mos–MAPK pathway) have all been suggested to constitute CSF. For completeness, we include MAI, a hypothetical meiosis-specific anaphase inhibitor. Ca^{2+} acts most likely through *CamKII* on these $\text{APC}^{\text{Cdc20}}$ inhibitors such that APC activity is accelerated and cyclin B1 destruction increases. Abbreviations, AI, AII, anaphase I and II; MI, MII, metaphase I and II; Ub, ubiquitin.

oocyte. Only when cyclin B1 is reestablished during oocyte maturation can Ca^{2+} stimulate cyclin B1 degradation, even when the oocyte has a metaphase I spindle. Thus, we argue that the main action of Ca^{2+} is to interfere with the function of a cyclin B1 stabilising activity (CSF) that develops in maturing oocytes at the end of meiosis I.

A model for Ca^{2+} stimulation of cyclin B1 degradation in mouse oocytes

We present the following model to explain the present data (Fig. 9). CSF activity is initiated by a period of cyclin B1 degradation during oocyte maturation. At the time when meiosis I normally completes during oocyte maturation, with extrusion of the first polar body, CSF activity becomes sufficient to stabilise cyclin B1 and so prevents entry into interphase at the end of meiosis I. The rise in CSF activity is triggered by initiation of cyclin B1 destruction; therefore, if there is a large excess of added exogenous cyclin B1 in an oocyte, CSF will become functional before cyclin B1 is at a

sufficiently low level to permit polar body extrusion. Ca^{2+} can stimulate the destruction of CSF and in so doing allows cyclin B1 to be degraded and the second polar body to be extruded. In mouse but not frog, this mechanism is possible since in mouse CSF, activity decreases quickly on oocyte activation (Ciemerych and Kubiak, 1999), whereas in frog CSF persists after MPF has declined (Watanabe et al., 1991).

CSF could constitute a component of the spindle checkpoint such as Mad2 (Kallio et al., 2000) or Bub1 (Schwab et al., 2001). However, arguing against this is the observation that induction of spindle checkpoint by microtubule-disrupting agents increases greatly cyclin B1 stability in oocytes (Nixon et al., 2002) and prevents the Ca^{2+} signal from triggering completion of meiosis II (Jones et al., 1995; Winston et al., 1995). Emi1 is an attractive alternative since it contains a potential consensus sequence for phosphorylation by *CaMKII* (Reimann and Jackson, 2002) making direct regulation by Ca^{2+} possible; however, it is not known if it is expressed in mammalian oocytes. Finally, CSF may be a novel Ca^{2+} -sensitive meiotic anaphase inhibitor (MAI, Fig. 6). This is not too speculative because not only are there meiosis-specific components of the c-mos–MAPK pathway (Lefebvre et al., 2002) but also in yeasts meiosis-specific regulators of the APC have already been identified (Bolte et al., 2002; Cooper et al., 2000).

In summary, we find that the Ca^{2+} signal provided by the sperm at fertilisation induces an increase in the rate of cyclin B1 degradation that is dependent on CSF-induced oocyte arrest. Given the immediate loss of CSF in mouse oocytes during activation and our finding that Ca^{2+} acts via the APC, one likely pathway is therefore the Ca^{2+} -stimulated loss of an APC inhibitor.

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