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Essential CDK1-inhibitory role for separase during meiosis I in vertebrate oocytes

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Separase not only triggers anaphase of meiosis I by proteolytic cleavage of cohesin on chromosome arms, but *in vitro* vertebrate separase also acts as a direct inhibitor of cyclin-dependent kinase 1 (Cdk1) on liberation from the inhibitory protein, securin. Blocking separase–Cdk1 complex formation by microinjection of anti-separase antibodies prevents polar-body extrusion in vertebrate oocytes. Importantly, proper meiotic maturation is rescued by chemical inhibition of Cdk1 or expression of Cdk1-binding separase fragments lacking cohesin-cleaving activity.

The cohesin complex holds sister chromatids together and, at anaphase, is cleaved by the protease separase[1,2]. Until this time, separase is kept inactive by mutually exclusive association with Cdk1 or securin[3–5]. At metaphase, the anaphase-promoting complex or cyclosome (APC/C) mediates degradation of securin and cyclin B1 (the regulatory subunit of Cdk1), thus freeing separase to cleave cohesin[6]. Biochemically, Cdk1 activity is itself switched off by separase–Cdk1 complex formation[4]. However, it is unclear whether separase acts as a Cdk1 inhibitor *in vivo*. During meiosis I of vertebrate eggs, decrease in cdk1 activity is transient and not associated with complete loss in cyclin B[7,8], raising the possibility that separase-dependent inactivation of Cdk1 may be necessary for meiotic maturation.

Cdk1 binding has only been demonstrated for human separase[4]. In addition to phosphorylation of Ser 1126, it requires a short sequence with weak homology to *Saccharomyces cerevisiae* Cdc6 and probably binding to cyclin B1. To investigate the role of separase-dependent Cdk1 inactivation in meiosis, antibodies were raised against the *Xenopus* sequences corresponding to the two known Cdk1-binding determinants (amino acids 1123–1154 and 1381–1422). Both antibodies recognized recombinant *Xenopus* separase (see Supplementary Information, Fig. S1a). The anti-amino acid 1381–1422 also detected and immuno-precipitated proteins with relative molecular masses of 240,000 and 180,000 (M_r 240 K and 180 K), from meiotic *Xenopus* egg extract. Consistent with these representing full-length and self-cleaved endogenous separase, respectively, the same bands were recognized by an anti-amino acid 1123–1154 antibody. Recombinant *Xenopus* separase–securin complexes were incubated in anaphase-arrested extracts to degrade securin. Separase was then re-isolated using amino-terminal HA-tags[3]. Cdk1 copurified with *Xenopus* separase, demonstrating that human and frog separase share Cdk1-binding despite low sequence conservation of Cdk1-binding determinants (CBDs; see Supplementary Information, Fig. S1b). A mixture of the two anti-CBD antibodies fully abolished separase–Cdk1 complex formation, but did not inhibit cleavage of separase, which is self-imposed and therefore serves as a read-out for proteolytic activity. Anti-CBD antibodies neither eluted securin from existing separase–securin complexes, nor affected binding of recombinant securin to securin-less separase (see Supplementary Information, Fig. S1c, d). We then investigated the effect of the anti-CBD antibodies on progesterone-induced meiotic maturation of surgically removed frog oocytes. Interestingly, microinjection of anti-CBD antibodies dramatically reduced the efficiency of polar-body formation compared with unspecific IgG (8.1-fold) or anti-CBD previously blocked with antigenic separase peptides (8.6-fold; see Supplementary Information, Fig. S1e). Taken together, these experiments indicate that transition from meiosis I to II requires the Cdk1-inhibitory activity of separase.

An antibody was also raised against the CBDs of mouse separase (amino acids 1120–1134 and 1340–1354), which detected an *in vitro*-translated mouse separase fragment (amino acids 1053–1382; Fig. 1a) and proteins with an M_r of 230 and 170 K in murine cell extracts that are likely to represent full-length and self-cleaved endogenous separase. Human separase cross-reacted with anti-mouse separase antibody because of its 83% sequence identity, and could therefore be used for further characterization. The anti-CBD antibody did not inhibit cohesin cleavage by separase, but counteracted separase–Cdk1 complex formation as it partially inhibited Cdk1-dependent inactivation of separase (Fig. 1b). Similarly to the observations mentioned earlier, anti-CBD antibody potently inhibited polar-body extrusion relative to unspecific IgG (5.7-fold) on microinjection into mouse oocytes (Fig. 1c). When anti-CBD-injected oocytes lacking polar bodies were stained with anti-tubulin

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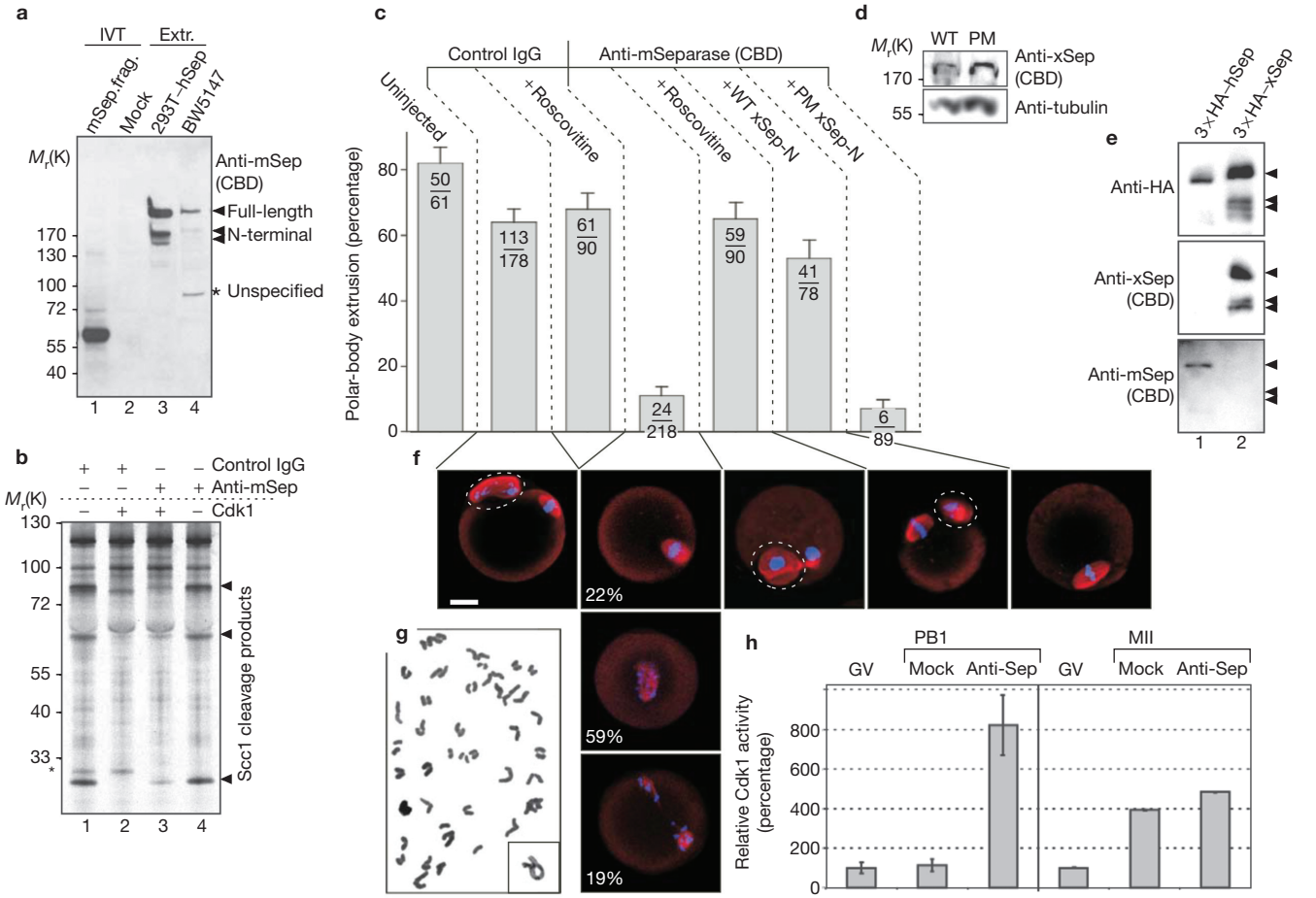

Figure 1 Block of polar-body extrusion by anti-separase antibody can be reversed by simultaneous inhibition of Cdk1. (**a**) *In vitro* translation (IVT) of mouse separase fragment (amino acids 1053–1382, mSep. frag.; lane 1) or negative control (mock; lane 2) and crude extracts of 293T cells expressing 3×HA hSeparase (lane 3) or a murine T lymphoma cell line (BW5147, lane 4) were used in western blot analysis to characterize an antibody raised against the CDB of mouse separase (amino acids 1120–1134 and 1340–1154). (**b**) The anti-mSeparase antibody does not impede proteolytic activity but counteracts separase-Cdk1 complex formation. Active human separase was pre-incubated with anti-mSeparase (lanes 3 and 4) or unspecific IgG (which always gave rise to an unexplained, unspecific band denoted by the asterisk, lanes 1 and 2), combined with Cdk1 (lanes 2 and 3) or reference buffer (lanes 1 and 4), and assayed for cohesin-cleaving activity. (**c**) Polar body extrusion in mouse oocytes injected with control IgG, anti-mSeparase, and mRNA coding for N-terminal fragments (amino acids 1–1552) of wild-type (WT) *Xenopus* separase or phosphorylation site mutant (PM; S1138A, S1139A). Where indicated, roscovitine (100 μM) was added 1 h before polar body formation. Number of polar bodies per total number of oocytes analysed is indicated (error bars represent mean ± s.d.). (**d**) Western blot analysis of mouse oocytes expressing wild-type or phosphorylation-site-mutant fragments of *Xenopus* separase. Tubulin served as loading control. (**e**) The anti-mSeparase antibody does not recognize *Xenopus* separase. Western blots of affinity purified HA-tagged human (lane 1) and *Xenopus* separase (lane 2) are shown. (**f**) Representative progesterone-treated oocytes imaged by confocal fluorescence microscopy are shown (spindles, red; chromosomes, blue). Dashed circles indicate the polar bodies. (**g**) Representative ($n = 11$ of 19 examined) Giemsa-stained chromosome spread from arrested, anti-CBD-injected oocyte lacking a polar body. Inset shows a typical bivalent for comparison. (**h**) Histone H1 assays on six pools of five control oocytes shortly after polar body extrusion (PB1) with the corresponding anti-CBD injected oocytes (left) and on two pools of 15 fully matured control oocytes (MII) with time-matched anti-CBD injected oocytes (right). The mean ± s.d. kinase activities normalized to corresponding oocytes in germinal-vesicle (GV) stage are shown. The scale bar in **f** represents 20 μm.

antibodies, the majority (59%) seemed to be arrested, with chromosomes scattered on elongated meiosis I spindles (Fig. 1f). Consistent with this observation, chromosome spreading revealed complete separation of all homologues in the majority of anti-CBD-injected oocytes without polar bodies ($n = 11$ from a total of 19; Fig. 1g). The homologue non-disjunction in these oocytes (42%) is similar to mock IgG-injected oocytes, where 37% of oocytes fail to extrude the first polar body (Fig. 1c) and arrest at metaphase I (with attached homologues, $n = 15$). This is a common maturation failure point[9,10] and here, it is probably due to microinjection damage. Thus, anti-CBD injection has no or little effect on cohesin cleavage, strongly suggesting that meiotic maturation failure is not due to inhibition of the proteolytic activity of separase.

To determine whether anti-CBD antibodies instead act by preventing separase-dependent inhibition of Cdk1, the Cdk1 inhibitor roscovitine was added to anti-CBD-injected oocytes 1 h before polar bodies normally extrude. This treatment fully abrogated the effect of anti-CBD on meiotic maturation, whereas it had no effect on polar-body formation in control-injected oocytes (Fig. 1c). Separase–Cdk1 complexes cannot be disassembled by treatment with roscovitine or phosphatase, making it unlikely that roscovitine indirectly leads to elevated proteolytic activity of Cdk1-inhibited separase that is kept inactive by association with Cdk1 (ref. 4 and data not shown). Next, we injected anti-CBD antibodies together with mRNAs encoding N-terminal separase fragments up to the self-cleavage site and thus lacking the carboxy-terminal protease

domain. A *Xenopus* separase mRNA that is not recognized by anti-mouse CBD-antibody was used (Fig. 1e), therefore, liberation of endogenous separase from antibody by the recombinant expression product could be excluded. Expression of wild-type *Xenopus* separase fragment efficiently reversed the effect of the anti-CBD antibody (Fig. 1c). This was in sharp contrast with separase proteins containing phosphorylation site mutations (S1138A, S1139A) and compromised Cdk1-inhibitory ability, which was unable to rescue polar-body formation despite being expressed at the same level as wild type (Fig. 1c, d). Finally, histone H1 kinase assays were performed on control oocytes that had just extruded polar body 1 and time-matched oocytes injected with anti-CBD. Although kinase activity in the controls was as low as that in the germinal vesicle stage, importantly, it was on average 7.3-fold higher in anti-CBD injected oocytes (Fig. 1h). This difference was transient, as fully matured control oocytes displayed only 18% lower kinase activity than corresponding anti-CBD injected oocytes. Taken together, these data demonstrate that the Cdk1-inhibitory activity of separase is required for polar body extrusion. Our results may explain why, after meiosis I, Cdk1 activity drops to its basal (germinal vesicle) level, despite persistence of some cyclin B1 (ref. 8).

Interestingly, *S. cerevisiae* separase also has a well-established meiotic role — it is essential for spindle disassembly after meiosis I (refs 11, 12). However, as part of the Cdc14 early anaphase release (FEAR) network it counteracts Cdk1 indirectly, by activating the antagonistic phosphatase Cdc14. Thus, meiotic Cdk1-inactivation seems to be an important conserved function of separase, despite the mechanistic differences between phyla. □

Note: Supplementary Information (including Methods) is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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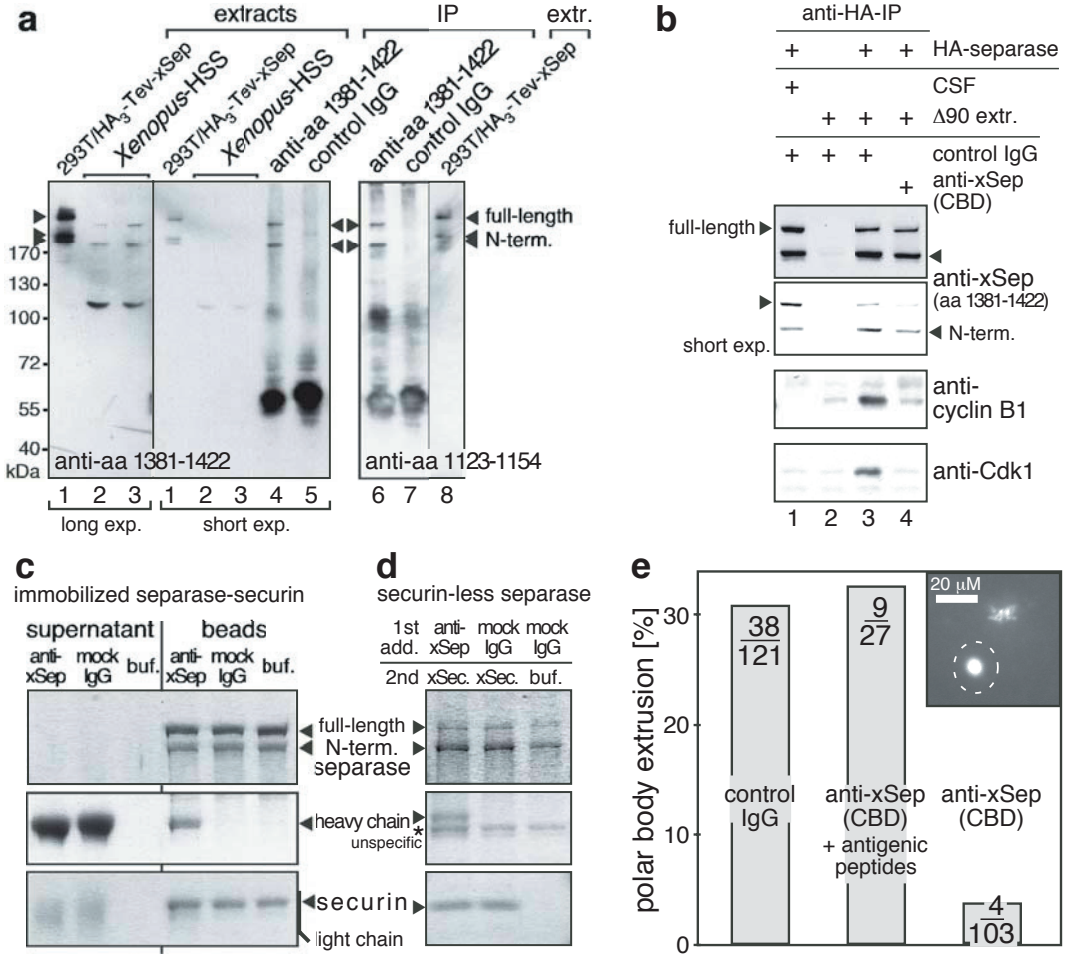

Figure S1 Antibodies, which block Cdk1-inhibitory but not proteolytic activity of separase, prevent polar body extrusion. **(a)** Crude extract of 293T cells expressing HA_3-Tev-xSeparase (lanes 1 and 8), high-speed supernatant (HSS) of crushed *Xenopus* eggs (lanes 2 and 3; 0.5 μl each), or material immunoprecipitated from 10 μl of *Xenopus* HSS (IP, lanes 4 to 7) were immunoblotted as indicated. **(b)** Recombinant HA_3-Tev-xSeparase-Securin was incubated with unspecific IgG (lanes 1 to 3) or antibodies against Cdk1-binding determinants (CBD; aa 1123-54, 1381-422; lane 4). Following re-isolation from metaphase- (CSF) or anaphase-like (Δ90) meiotic egg extracts Tev-protease eluates were analyzed by immunoblotting. **(c)** Isolated HA_3-Tev-xSeparase-Securin complex on anti-HA beads was challenged by incubation with anti-CBD. Eluted and bead-associated material was analyzed by Commassie-staining. **(d)** Isolated HA_3-Tev-xSeparase on anti-HA beads was incubated with anti-CBD or unspecific IgG before recombinant Δ90-xSecurin was added. After washing, bead-associated material was analyzed by Commassie-staining. **(e)** Stage VI oocytes were injected with 200 ng of unspecific IgG or anti-xSeparase antibodies (CBD) or anti-CBD blocked by incubation with 100 fold excess of antigenic peptides. Progesterone-matured oocytes were fixed, Hoechst 33258-stained, and inspected for polar bodies (marked on image by dashed circle). Number of polar bodies per total number of analyzed oocytes is indicated.

METHODS

Mutagenesis and *in vitro* transcription of *Xenopus* separase. Ser 1138 and Ser 1139 of *Xenopus* separase[1] were changed to alanines by gene-editor mutagenesis (Promega, Madison, WI). An T7-RNA promoter was attached by PCR to open reading frames (ORFs) encoding wild-type or phosphorylation-site mutant *Xenopus* separase up to its self-cleavage site. The PCR products were *in vitro* transcribed and given a polyA tail using the mMessage mMachine T7 Ultra kit from Ambion (#1345; Huntingdon, UK) and purified over MEGAclear columns (Ambion #1908). The products were confirmed by agarose gel electrophoresis and *in vitro* translation before being used for microinjections at a concentration of 150 ng μl^{-1}.

Recombinant proteins, *Xenopus* extracts and immunoprecipitation. Expression and purification of recombinant *Xenopus* separase–securin complexes, human cyclin $B1^{\Delta 90}$, and human Cks2–Cdk1–cyclin $B1^{\Delta 90}$ were as previously described[1,2,3]. *Xenopus* cytostatic factor (CSF) and Δ90 extracts were prepared as previously described[3,4]. High-speed supernatant (HSS) was prepared by spinning CSF extract at 100.000*g* for 1 h at 4 °C and harvesting the supernatant. For immunoprecipitation of *Xenopus* separase 10 µl protein-G–sepharose (Amersham Biosciences, Freiburg, Germany) were pre-absorbed with 2 µg of anti-xSeparase (amino acids 1381–1422), rotated with 100 µl HSS for 3 h at 4 °C, washed with extraction buffer (XB)[4] with 50 mM NaCl, 0.1% Tween 20, and eluted by boiling in SDS sample buffer.

Antibodies. A mixture of the peptides CETRDLLKAPESPTATS and SLEKNLPQFLSHTQDC was coupled to maleimide-activated mcKLH (Pierce, Rockford, IL) according to manufacturers' recommendations and was used to immunize a New Zealand rabbit. Antibody was affinity purified from serum using the peptides described above coupled to SulfoLink Coupling Gel (Pierce) according to manufacturers' recommendations, dialysed against 80 mM KCl, 10 mM HEPES–KOH at pH 7.5 and concentrated. The same procedure was performed using a mixture of the peptides CYNEESPVPEVLPRAPRRRKTR and CTVLKVDFNDSDLEVADNSEWE. Both affinity-purified antibodies were mixed 1:1 to give anti-xSeparase (CBD) used for microinjection. Anti-mSeparase (CBD) antibody against a mixture of the peptides CSSPVLKTKPPPNPGF and CTPKPPGRARQAGPR (Eurogentec, Seraing, Belgium) was affinity purified as described above, dialysed against 120 mM KCl, 10 mM HEPES–KOH at pH 7.4, and concentrated to 1 mg ml^{-1} by ultrafiltration before microinjection.

Competition experiments. 3×HA–Tev–xseparase–securin (2 µg) with 10 µl anti-HA beads (Roche) were incubated with 20 µg anti-CBD antibodies (or corresponding controls) for 30 min at room temperature before the supernatant was collected. Following extensive washing, beads were eluted with SDS. The same relative amounts of supernatant and SDS eluate were analysed by Coomassie staining. Alternatively, 2 µg of 3×HA–Tev–xseparase–securin on 10 µl anti-HA beads were first stripped of securin and Cdk1 by incubation in a *Xenopus* Δ90 extract followed by high-salt wash. As phosphorylation may affect binding of anti-CBD antibodies to separase, a λ-phosphatase treatment was also included before separase was incubated with 20 µg of anti-CBD antibodies (or corresponding controls) for 30 min at room temperature, then 2 µg of $xSecurin^{\Delta 90}$ (or reference buffer) were added for another 30 min at room temperature. Following extensive washing, bound material was eluted with SDS and analysed by SDS–PAGE and Coomassie staining.

***Xenopus* oocyte manipulations.** Surgically removed stage VI oocytes were collagenase-treated (Sigma, Taufkirchen, Germany; C-6885, 140 U ml^{-1}) in Oocyte Ringer 1 (OR1; 5.0 mM HEPES–KOH, 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM Na_2HPO_4 at pH 7.6) for 2 h. After washing with OR1, the oocytes were recreated in OR3 (5.0 mM HEPES–KOH, 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM Na_2HPO_4, 1 mM $CaCl_2$, 100 mg ml^{-1} pen–strep, 0.11 mg ml^{-1} sodium pyruvate at pH 7.6) overnight before injection with 25 nl of 8 mg ml^{-1} anti-xSeparase antibody or unspecific IgG in 80 mM KCl, 10 mM HEPES–KOH at pH 7,5. Oocytes were then treated with 10 µg ml^{-1} progesterone (Sigma P8783) for 10 h, fixed in 10 mM HEPES–KOH at pH 7.8, 100 mM KCl, 3 mM $MgCl_2$, 0.1% Triton X-100, 0.1% glutaraldehyde, 3.7% formaldehyde for 1 hour, stained in 10 mM HEPES–KOH at pH 7.8, 100 mM KCl,, 3 mM $MgCl_2$, 20 ng ml^{-1} Hoechst 33258 (Sigma), mounted in 48% glycerol, 1× Marc's modified ringer (MMR) and analysed by standard epifluorescence microscopy. All steps were performed at 18 °C.

Mouse oocyte culture. Four to six week old MFI mice (Harlan, Bicester, UK) were used. Germinal vesicle-stage oocytes were collected from primed females 44–52 hours after peritoneal injection of 7.5 IU pregnant mares' serum gonadotrophin (Calbiochem, Nottingham, UK). For bench handling, microinjections and imaging experiments, oocytes were cultured in M2 medium (Sigma). For long-term incubation, germinal-vesicle stage oocytes were cultured in MEM with 20% fetal calf serum (Invitrogen, Paisley, UK) in a 5% CO_2 humidified incubator at 37 °C. To arrest germinal-vesicle oocytes, the culture media was supplemented with milrinone (1 µM, Sigma).

Immunofluorescence microscopy. Oocytes were fixed and permeabilised by an incubation in 3.7% paraformaldehyde in PBS (30 min at room temperature), followed by 3.7% paraformaldehyde, 2% Triton X-100 in PBS (30 min at room temperature). Fixed oocytes were then washed extensively in 1% polyvinylpyrrolidone, 1% BSA in PBS. For spindle staining, oocytes were incubated with rat anti-anti-tubulin antibody (YL1/2, 1:40; Abcam, Cambridge, UK) for 1 h at 37 °C, washed and then incubated with anti-rat Texas Red IgG as a secondary antibody (5 µg ml^{-1}, Abcam) for 1 h at 37 °C. To stain chromatin, oocytes were incubated for a further 30 min in Hoechst 33258 (10 ng ml^{-1}).

Microinjection and imaging. All microinjections into germinal vesicle-arrested oocytes were made on the heated stage of a Nikon TE300 inverted microscope[5]. Briefly, micropipettes were inserted into cells using the negative capacitance overcompensation facility on an electrophysiological amplifier. This procedure ensures a very high rate of cell survival. A single 0.1–0.3% volume injection was achieved using a timed injection on a Pneumatic PicoPump (World Precision Instruments, Stevenage, UK). Following microinjection, oocytes were washed free of milrinone and *in vitro* matured. Chromosome spreads were prepared by the air-drying technique[6]. A Leica SP2 imaging system was used to acquire confocal sections in formalin-fixed oocytes. Metamorph software (Universal Imaging Corp., Downington, PA) was used for image preparation.

Histone H1 assay and western analysis on mouse oocytes. Histone H1 assays were performed on 5–15 oocytes as previously described[7]. The average values (plus s.d.) of 60 oocytes each are shown. For expression analysis, 55 oocytes were lysed in 10 µl of SDS-sample buffer and subjected to western analysis 5 h after microinjection of mRNAs coding for N-terminal fragments (amino acids 1–1552) of wild-type *Xenopus* separase or phosphorylation site mutant (S1138A, S1139A).

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