Methods

Mutagenesis and *in vitro*-transcription of *Xenopus* separase. Serine residues 1138 and 1139 of *Xenopus* separase (Fan et al., 2006) were changed to alanines by gene editor mutagenesis (Promega). An T7-RNA promoter was attached via PCR to ORFs encoding wild type or phosphorylation site mutant *Xenopus* separase up to its self-cleavage site. The PCR products were in vitro-transcribed and furnished with a polyA tail using the mMessage mMachine T7 Ultra kit from Ambion (#1345) and purified over MEGAclear columns (Ambion #1908). They were confirmed by agarose gel electrophoresis and *in vitro*-translation before using them for microinjections at a concentration of 150 ng/μl.

Recombinant proteins, *Xenopus* extracts, and immunoprecipitation. Expression and purification of recombinant *Xenopus* separase-securin complexes, human cyclin B1Δ90, and human Cks2-Cdk1-cyclin B1Δ90 were as described (Fan et al., 2006; Gorr et al., 2005; Stemmann et al., 2001). *Xenopus* CSF and Δ90 extracts were prepared as described (Murray, 1991; Stemmann et al., 2001). HSS was prepared by spinning CSF extract at 100.000 x g for 1 hour at 4°C and harvesting the supernatant. For immunoprecipitation of *Xenopus* separase 10 μl protein-G sepharose (Amersham Biosciences) were pre-absorbed with 2 μg of anti-xSeparase (aa 1381-1422), rotated with 100 μl HSS for 3 hours at 4°C, washed with XB plus 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) according to manufacturer's recommendations and used to immunize a New Zealand rabbit. Antibody was affinity purified from serum using the above peptides coupled to SulfoLink Coupling Gel (Pierce) according to manufacturer's recommendations, dialyzed against 80 mM KCl, 10 mM Hepes-KOH, pH 7.5, and

concentrated. The same was done 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) was affinity purified as above, dialysed against 120 mM KCl, 10 mM Hepes-KOH, pH 7.4, and concentrated to 1 mg/ml by ultrafiltration prior to microinjection.

Competition experiments. Two μg of HA_3-Tev-xSeparase-Securin on 10 μl anti-HA beads (Roche) were incubated with 20 μg of anti-CBD antibodies (or corresponding controls) for 30 minutes at room temperature before the supernatant was collected. Following extensive washing, beads were eluted with SDS. Same relative amounts of supernatant and SDS eluate were analyzed by Coomassie staining. Alternatively 2 μg of HA_3-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 might 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 minutes at room temperature. Then, 2 μg of xSecurin-Δ90 (or reference buffer) were added for another 30 minutes at room temperature. Following extensive washing, bound material was eluted with SDS and analyzed by SDS-PAGE plus Coomassie staining.

***Xenopus* oocyte manipulations.** Surgically removed stage VI oocytes were collagenase-treated (Sigma C-6885, 140 U/ml) in Oocyte Ringer 1 (5.0 mM Hepes-KOH, 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM Na_2HPO_4, pH 7.6) for 2 hours. After washing with OR1 they 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 Pen./Strep., 0.11 mg/ml sodium pyruvate, pH 7.6) over night before injection with 25 nl of 8 mg/ml anti-xSeparase antibody or unspecific

IgG in 80 mM KCl, 10 mM Hepes-KOH, pH 7,5. Oocytes were then treated with 10 μg/ml progesterone (Sigma P8783) for 10 hours, fixed in 10 mM Hepes-KOH (pH 7.8), 100 mM KCl, 3 mM $MgCl_2$, 0.1% Triton X100, 0.1% glutaraldehyde, 3.7% formaldehyde for 1 hour, stained in 10 mM Hepes-KOH (pH 7.8), 100 mM KCl, , 3 mM $MgCl_2$, 20 ng/ml Hoechst 33258 (Sigma), mounted in 48% glycerol, 1x MMR, and analyzed by standard epifluorescence microscopy. All steps are performed at 18°C.

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

Immunofluorescence microscopy. Oocytes were fixed and permeabilised by an incubation in 3.7% paraformaldehyde in phosphate-buffered saline (PBS, 30 minutes at room temperature), followed by 3.7% paraformaldehyde, 2% Triton X100 in PBS (30 minutes 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-α-tubulin antibody (YL1/2, 1:40, Abcam) for 1h at 37°C, washed, and then incubated with anti-rat Texas Red IgG as a secondary antibody (5 μg/ml, Abcam) for 1h at 37°C. To stain chromatin, oocytes received a further 30 min incubation in Hoechst 33258 (10 ng/ml).

Microinjection and imaging. All microinjections into GV arrested oocytes were made on the heated stage of a Nikon TE300 inverted microscope (Nixon et al., 2002). 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). Following microinjection oocytes were washed free of milrinone and *in vitro*-matured. Chromosome spreads were prepared by the air-drying technique of Tarkowski (1966). 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 done on 5 to 15 oocytes as described (Madgwick et al., 2004). Shown are average values (plus standard deviations) of 60 oocytes each. For expression analysis 55 oocytes were lysed in 10 μl of SDS-sample buffer and subjected to Western analysis 5 hours after microinjection of mRNAs coding for N-terminal fragments (aa 1 – 1552) of wild type *Xenopus* separase or phosphorylation site mutant (Ser1138,1139Ala).

References

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