SUPPLEMENTARY MATERIALS AND METHODS

Antibodies

The following antibodies were used for immunoblotting according to standard protocols: rabbit anti-separase (Stemmann et al, 2001), mouse anti-securin (1:1,000; MBL), rabbit anti-securin (1:1,000; Epitopes), mouse anti-Flag M2 (1:2,000; Sigma-Aldrich), mouse anti-Myc (1:50; 9E10, DSHB, hybridoma supernatant), rabbit anti-phosphoSer10-histone H3 (1:1,000; Millipore), rabbit anti-phosphoSer139-histone H2A.X (1:1,000; Millipore), mouse anti-cyclin B1 (1:1,000; Millipore), mouse anti-topoisomerase IIa (1:1,000; Enzo Life Sciences), mouse anti-Cdc20 (1:2,000; Santa Cruz Biotechnology), goat anti-Cdc27 (1:1000), mouse anti-cyclin A2 (1:200; Santa Cruz Biotechnology), mouse anti-phosphoSer (1:1,000; Invitrogen), mouse anti-PP2A-C (1:1,000; Millipore), rabbit anti-phosphoThr68-Chk2 (1:800; Cell Signaling) and mouse anti-α-tubulin (1:200; 12G10, DSHB, hybridoma supernatant). For immunoprecipitation (IP) experiments, the following affinity matrices and antibodies were used: mouse anti-Myc Agarose (Sigma-Aldrich), mouse anti-Flag M2-Agarose (Sigma-Aldrich), human IgG Sepharose (GE Healthcare), protein G Sepharose (GE Healthcare) loaded with mouse anti-separase (A302-214A, Bethyl; used for PP2A interaction experiments only), rabbit anti-separase (raised against a His<sub>6</sub>-tagged fragment (amino acids 1305-1573) of human separase), rabbit anti-securin (raised against His<sub>6</sub>-tagged human securin), goat anti-Cdc27 (raised against a peptide with the sequence CDADDTQLHAAESDEF) or unspecific rabbit IgG (Bethyl). For immunofluorescence (IF) staining of fixed cells, mouse anti-Hec1 (1:800; Genetex), rabbit anti-phosphoThr68-Chk2 (1:200; Cell Signaling) and mouse anti-phosphoSer139-histone H2A.X (1:2,500; Millipore) were used. Isolated centrosomes were stained with rabbit anti-centrin-2 and guinea-pig anti-C-Nap1 as previously described (Schockel et al, 2011). Secondary antibodies (all 1:500): Cy3 donkey anti-guinea pig IgG (Jackson Immunoresearch Laboratories), Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse IgGs (both Invitrogen).
**Cell treatments**

For synchronization at the G1/S boundary, cells were treated with 2 mM thymidine (Sigma-Aldrich) for 20 h, released into fresh medium for 10 h, and then exposed to 2 mM thymidine again for additional 18 h. Synchronization of cells in prometaphase was done by addition of nocodazole (Sigma-Aldrich) or taxol (Calbiochem) to 0.2 μg/ml each 6 h after release from a single thymidine block. G2 arrest was achieved by addition of RO3306 (Santa-Cruz Biotechnology) to 10 μM 4 h after release from a previous thymidine treatment. For the taxol-ZM override experiments, taxol-arrested mitotic cells were harvested by shake-off and released for the indicated times by replating into medium supplemented with ZM 447439 (5 μM, Tocris Biosciences), taxol (0.2 μg/ml) and, where indicated, cycloheximide (30 μg/ml, Sigma-Aldrich). For the long-term prometaphase arrest experiment shown in Figure 6C, caspase-1 and -3 inhibitors (5 μM each, Calbiochem) were additionally added after mitotic shake-off. To induce DNA-damage, doxorubicin (0.5 μM, Calbiochem) was added to cells 7 h after release from a double thymidine block. Transgenic securin and separase variants were induced using 0.2-1 μg/ml doxycycline (Sigma-Aldrich) from 10 h up to 3 d. For CaMKII inhibition, KN-93 (4 μM, Calbiochem) was added 4 h before ZM to taxol-arrested cells. For IP of Myc₆-separase (WT or ΔPP2A) as shown in Figure 2C, taxol-arrested cells were additionally treated with okadaic acid (100 nM; Sigma-Aldrich) 10 min prior to ZM addition. For time-lapse imaging transgenic securin HeLa cells were transiently transfected with plasmids coding for histone H2B-eGFP (BD Pharmingen) or H2B-mCherry-Scc1ₐa107-268-eGFP (separase-sensor). HEK293T and HeLa cells were transfected using calcium phosphate and Lipofectamine 2000 (Invitrogen), respectively.

**Immunofluorescence microscopy**

Stable transgenic HeLa cells grown on poly-lysine coated glass coverslips were washed once with 1x PBS, fixed with fixation-solution (1x PBS, 3.7% formaldehyde, 0.3% Triton X-
100) for 10 min at room temperature, and then washed twice with quenching-solution (1x PBS, 100 mM glycine). They were then further permeabilized by treatment with permeabilization-solution (1x PBS, 0.5% Triton X-100) for 5 min which was followed by a PBS wash and an incubation in blocking-solution (1x PBS, 1% (w/v) BSA) for 20 min at room temperature. Coverslips were transferred into a wet chamber and then incubated with primary antibodies for 1 h followed by 4 washes with PBS-Tx (1x PBS, 0.1% Triton X-100). After incubation with fluorescently labeled secondary antibodies for 40 min, samples were washed once, stained for 10 min with 1 µg/ml Hoechst 33342 in PBS-Tx and washed again 4 times. Finally, coverslips were mounted in 78% glycerol, 20 mM Tris-HCl pH 8.0, 2,33% (w/v) 1,4-diazabicyclo(2.2.2)octane on a glass slide. Immunofluorescence microscopy of fixed cells and isolated centrosomes was performed using a HCX PL APO 100x/1.40-0.70 oil objective. To detect DNA bridges or lagging chromosomes (Figure 4B) Z-stacks of 25-30 images at 0.35 µm increments were collected. For digital 3D deconvolution of Z-axis image series, the LAS-AF software was used (20 iterations; blind deconvolution algorithm). For quantification of DNA-damage foci (Figure 4C), Z-stacks series over 4 µM in 0.2 µM increments were collected and projected onto one focus plane using the LAS-AF software. Chromosome spreads were prepared using Carnoy's solution as described (McGuinness et al, 2005). To assess centriole engagement status, centrosomes were isolated from 4x10^6 cells and stained as previously described (Schockel et al, 2011).

**Live-cell imaging**

Cells were seeded into CO₂-dependent medium without phenol red onto µ-slide 8-well chambered coverslips (Ibidi). Cells were kept in an atmosphere of 37°C and 5% humidified CO₂ during microscopy on a DMI 6000 inverted microscope (Leica). For imaging of unperturbed mitosis, GFP and DIC images were captured in 180 s intervals over a period of 12 h, through a HCX PL APO 40x/0.85 CORR objective. For taxol-ZM override experiments separase sensor expressing cells were imaged at minimal light intensity and every 240-300 s
over a period of 90 min from ZM addition. Changes in focus plane due to mitotic rounding of the cells were compensated by collecting Z-stacks at each time point. Captured images from each experiment were analysed using the corresponding LAS-AF software (Leica).

CaMKII kinase assay

To activate CaMKII autophosphorylation reaction was performed in presence of 200 µM ATP, 1.2 µM calmodulin and 2 mM CaCl₂ in 1x protein kinase reaction buffer (all components from NEB) for 10 min at 30°C. For the radioactive phosphorylation assay 1.5 µg recombinant securin supplemented with 50 µM ATP and 40 µCi γ-32P-ATP (Hartmann Analytic) were combined with 500 U activated CaMKII and incubated for 1 h at 30°C. Samples were analysed by SDS-PAGE and autoradiography.

RNA interference

For knockdown of human Cdc20 or human securin, cells were transfected with 70-100 nM siRNA duplex (CDC20: 5’-CGGAAGACCUGCCGUUACAUU-3’, SECURIN: 5’-UCUUAGUGCUUCAGAUUGUGUAU-3’) and RNAiMax (Invitrogen) for 48 h according to the manufacturers instructions. Luciferase siRNA (GL2) was used as negative control.

SILAC and mass spectrometry analysis

HEK293T cells were calcium phosphate transfected to co-express securin with either separase-WT or separase-ΔPP2A and arrested in prometaphase by nocodazole (0.2 µg/ml). Prior to transient transfection the cells were SILAC labeled as previously described (Cox & Mann, 2008). Briefly, DMEM medium without arginine and lysine (Invitrogen) was supplemented with either 13C/15N labeled arginine and 13C/15N labelled lysine (R10/K8) or
with the unlabeled amino acids (R0/K0) (Sigma-Aldrich). Cells that were later transfected to express separase-WT and securin were cultivated in R0/K0 medium, while cells that were to express separase-ΔPP2A and securin were grown in R10/K8 medium over 6 generations prior to transfection.

Following two days of expression, the SILAC-labeled, mitotic HEK293T cells were lysed in lysis buffer (20 mM Tris-HCl at pH 7.7, 100 mM NaCl, 10 mM NaF, 20 mM β-glycerophosphate, 5 mM MgCl₂, 0.1 % Triton X-100, 5 % glycerol, 1 mM EDTA) supplemented with 1 μM okadaic acid and 1 μM microcystin LR (both from Alexis Biochemicals) to preserve phosphorylations. Lysates were cleared by centrifugation (45 min, 160,000 g, 4°C) and then rotated with IgG sepharose for 4 h at 4°C to capture separase-securin complexes via the N-terminal ZZ-Tev₄-tag on separase (Z = IgG binding domain of protein A). Beads were washed three times with lysis buffer before bound separase-securin complexes were eluted by Tev-protease treatment for 2 h at 18°C. Eluates were mixed at a 1:1 ratio and separated by SDS-PAGE. Coomassie-stained separase and securin bands were subjected to in-gel digestion with trypsin or Asp-N according to standard protocol (Shevchenko et al, 2006). Phosphorylated peptides were then enriched by titansphere chromatography. Sample analysis was done by reversed phase LC-MS/MS using the Agilent Technologies 1200 nanoflow system connected to an LTQ Orbitrap XL system (Thermo Electron) with a nanoelectrospray ion source (Proxeon Biosystems) as described (Olsen et al, 2005). Peptides were identified using the Mascot-MatrixScience LLC software and quantified using MSQuant.

Extract experiments

³⁵S-labeled securin variants (WT, 4A, 4D or mKEN/mDB) were in vitro expressed by combining corresponding pCS2 plasmids with SP6 RNA polymerase supplemented rabbit reticulocyte lysate (TNT SP6 Coupled Transcription/Translation System from Promega) in presence of ³⁵S-methionine (Hartmann Analytic) according to the manufacturers' instructions.
CSF-arrested *Xenopus* egg extracts were prepared as previously described (Murray, 1991) and combined with cycloheximide (100 μg/ml), recombinant human cyclin B1Δ90 (10 ng/μl), sperm nuclei (2.000 μl⁻¹) and ³⁵S-labeled securin (16 μl/ml). After 20 min at room temperature, extracts were additionally supplemented either with 0.6 mM CaCl₂ and 1 μM okadaic acid (Figure S4B) or with CaCl₂ only (Figure S6). At indicated times thereafter, 3 μl each were analysed by SDS-PAGE and autoradiography. For λ-phosphatase treatment of selected samples (Figure S4B) 3 μl aliquots were treated for 30 min at 30°C with 400 U of λ-phosphatase (NEB) in presence of 2 mM MnCl₂.

For λ-phosphatase treatment of immunoprecipitated proteins (Figure S4A), the corresponding beads were washed twice with EDTA-, NaF- and β-glycerophosphate-free but MnCl₂ (2 mM) containing lysis buffer and incubated with 400 U λ-phosphatase (NEB) for 30 min at 30°C prior to SDS-PAGE and immunoblotting.

**Flow cytometry**

For analysis of DNA content, cells were fixed with 70% ethanol (-20°C), washed twice with 1x PBS, 0.1% (w/v) BSA and resuspended in 69 μM propidium iodide, 38 mM tri-sodium citrate, 100 μg/ml RNase A (Qiagen). After incubation for 30 min at 37°C, the cell cycle profiles were analysed on a Cytomics FC 500 flow cytometer using CXP Analysis software (Beckman Coulter).
SUPPLEMENTARY REFERENCES


