Online Methods

Cell culture

Stably expressing cell lines were derived from the HeLa 'Kyoto' cell line obtained from S. Narumiya (Kyoto University, Japan) or from hTERT-RPE1 obtained from P. Meraldi (University of Geneva, Switzerland). Monoclonal reporter cell lines were generated as previously described[36] and cultured in Dulbecco's modified eagle medium (DMEM; GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS) (PAA Laboratories), 1% (v/v) penicillin–streptomycin (Invitrogen), 500 µg/ml G418 and 0,5 µg/ml puromycin. For live-cell microscopy, cells were grown either in 96-well plastic-bottom plates (µclear; Greiner Bio-One Ltd.), or on LabTek II chambered coverslips (ThermoScientific). Live cell imaging was performed in DMEM containing 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin, but without phenolred and riboflavin to reduce autofluorescence[36]. Laser microsurgery experiments were performed in Leibovitz L-15 medium (GIBCO) containing 10% FBS.

RNAi and drug treatment

Cells were transfected ~ 24 h before imaging in 96-well plastic-bottom plates (µclear; Greiner Bio-One Ltd.) with HiPerfect (Qiagen) or Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. Mad2 siRNA targeting sequences: 5'-AAGAGTCGGGACCACAGTTTA-3' (Qiagen) (Supplementary Fig. 1) and 5'-TTACTCGAGTGCAGAAATA-3' (Dharmacon) (Fig. 5). The non-targeting control siRNA was AllStars (Qiagen). Final siRNA concentration was 10 nM. Nocodazole (Sigma) was dissolved to final concentrations of 100 ng/ml to disrupt the entire spindle, or 6-25 ng/ml to induce metaphase plates with persistent unaligned chromosomes (Fig. 5d-f).

Indirect immunofluorescence staining

For correlative microtubule immunofluorescence staining after laser microsurgery (Fig. 1b-e) 16% (w/v) formaldehyde solution (methanol-free, ThermoScientific) was added directly to the medium to a final concentration of 4%. Cells were fixed for 7:30 min:s and then washed followed by 5 min permeabilization with 0.5% Triton X-100 in PBS. Cells were blocked for 10 min with 10% FCS in PBS with 0.05% Tween20 and stained for 1 h with mouse monoclonal anti-α-tubulin antibody (1:1,000; clone DM1A, mouse ascites fluid; Sigma T9026, lot #: 052M4837). Cells were then stained for 30 min with goat anti-mouse IgG Alexa Fluor 633 (1:600; Molecular Probes).

To stain Mad2 after low-dose nocodazole treatment (Fig. 5d, e), cells were fixed and simultaneously extracted with 4% formaldehyde diluted in PTEM buffer (20 mM PIPES, pH 6.8, 0.2%Triton X-100, 10 mM EGTA, 1mM $MgCl_2$) for 7 min. Cells were blocked for 30 min with 3% BSA in PBS before staining for 1 h with rabbit polyclonal anti-Mad2 (1:1,000; Bethyl, lot #: 300-301 A-1) and human anti-centromere antibodies (CREST) (1:400; Antibodies Incorperated). Subsequently cells were stained for 30 min with donkey anti-rabbit IgG Alexa Fluor 488 and goat anti-human IgG Alexa Fluor 594 (1:600; Molecular Probes, respectively). DNA was stained with 2 µg/ml Hoechst 33342 (Sigma).

Microscopy and laser microsurgery

Automated wide-field fluorescence microscopy was performed on a Molecular Devices ImageXpressMicro XL screening microscope equipped with reflection-based laser autofocus and a 10x 0.5 N.A. S Fluor dry objective (Nikon), controlled by in-house developed Metamorph macros[25]. Cells were maintained in a microscope stage incubator at 37ºC in humidified atmosphere of 5% CO_2.Confocal microscopy was performed on a customized Zeiss LSM780 microscope using a 40x 1.4 N.A. Oil DIC Plan-Apochromat objective (Zeiss), controlled by ZEN 2011 software and an autofocus macro (AutofocusScreen) kindly provided by J. Ellenberg, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany. The microscope was equipped with an EMBL incubation chamber, providing a humidified atmosphere at 37°C with 5% CO_2.

Laser microsurgery was performed on a Zeiss LSM710 confocal microscope equipped with a tuneable Chameleon Ti:Sapphire laser for multi-photon excitation using a 63x 1.4 N.A Oil DIC Plan-Apochromat objective (Zeiss), controlled by a ZEN 2010 software. A 37°C atmosphere was provided by a stage incubator and an objective heater. Single chromosome were detached from the spindle by tuning the Ti:Sapphire laser to 915 nm and 100% power, and scanning with slow speed in a highly elongated polygon resembling a single line.

Image analysis

Microscopy images were processed and analyzed using Fiji[37]. 3D-confocal time-lapse images contained 5-10 z-sections. 3D-confocal microscopy of immunofluorescence samples contained 7-30 z-sections (Fig.1c-f; Fig. 5d, e), and sampled the entire metaphase plate and all unaligned chromosomes in the experiment shown in Fig. 5d, e. To display fine image structures, maximum intensity projection was applied to selected z-sections that contained the

relevant structures (e.g., laser-displaced chromosomes), or the entire cell. Figures or Videos display single z-sections (Fig. 1a), or projections of 2 z-sections (Fig. 3a, 4a, b; Supplementary Video 4-6), 3 z-sections (Fig. 1b-e, 2a-c, Supplementary Fig. S2a-c Supplementary Fig. S1d, e, Supplementary Video 1-3), 9 z-sections (Fig. 5f, Supplementary Video 7)), or 30 z-sections (Fig. 5d, e). To preserve quantitative information of securin-mEGFP also in the displayed image frames, full stacks were projected by mean intensity (Fig. 4a, b and 5f, Supplementary Video 5-7).

Cell fate analysis after laser-induced chromosome detachment (Fig. 2d) was performed with cells expressing H2B-mCherry and mEGFP-α-tubulin, or H2B-mCherry and securin-mEGFP.

To measure Mad2-EGFP levels on sister kinetochores after laser induced chromosome displacement and subsequent fixation (Fig. 1d-f, Supplementary Fig. S1e, f), Mad2 intensity was measured in individual z-sections on chromosomes containing two Mad2 spots, within a circular region with 0.91 µm diameter for each sister kinetochore. To calculate Mad2-EGFP increase over cytoplasm the cytoplasmic EGFP background and image background were measured in the corresponding z-section. Mad2 peak levels (Fig. 3c "Laser-detached") and recruitment kinetics (Fig. 3b) were measured on displaced chromosomes that remained unaligned for at least 5 min in cells, and only in cells that did not enter anaphase within 3 min after cutting. Mad2 accumulation was measured on individual displaced chromosomes that could be followed throughout the time series. Mad2-EGFP fluorescence was measured in single z-sections in a circular region of interest with 1.2 µm diameter. Prior to laser microsurgery, EGFP fluorescence was measured in the central z-slice at the chromatin to cytoplasm interface near the cut. Following laser microsurgery, the Mad2-EGFP spot appearing on the first sister kinetochore was followed over time in a single z-section. Cytoplasmic EGFP and image background were measured as described before. The onset of Mad2 recruitment was determined based on Mad2-EGFP increase more than 3 s.d. above the mean Mad2-EGFP fluorescence before the cut. Exact timing for onset of Mad2 recruitment was derived by intersection of the detection threshold and a linear interpolation between two Mad2-EGFP measurements. Mad2-EGFP intensities were also measured in cells entering mitosis in presence of 100 or 500 ng/ml nocodazole (Fig. 3c) about 3-10 min after NEBD. All spots visible within the middle z-section were quantified in 3D movies, using identical imaging settings and analysis procedures as before.

Securin-mEGFP fluorescence after laser microsurgery (Fig. 4c, d) was measured in mean intensity projections of confocal 3D time-lapse images. First, the rate of photobleaching was measured in a fast time-lapse recording of a cell treated with 100 or 200 ng/ml nocodazole. A single exponential function was fitted to the mean fluorescence, serving as reference to compensate for acquisition photo-bleaching (Supplementary Fig. S3c). Securin-mEGFP

levels were normalized to prometaphase. To determine the dynamic range of APC/C activity (Fig. 5a-c), total securin-mEGFP intensity was measured in wide field time-lapse images and bleach corrected as described above. Control and Mad2 RNAi data were normalized to 8-0 min before prometaphase. Cells treated with 100 ng/ml nocodazole were normalized to 0-300 min after prometaphase onset to compensate for cell rounding artifacts. Rates of securin-mEGFP degradation were determined by linear regression of data points 12-0 min before anaphase onset (control and Mad2 RNAi), or 20 min after prometaphase onset until mitotic exit (100 ng/ml nocodazole). A small fraction of cells that died in mitosis was omitted from the measurement of mitotic slippage timing.

In the experiments shown in Fig. 5f-h, the number of unaligned chromosomes was determined by visual inspection of confocal 3D image stacks. Securin-mEGFP fluorescence in these movies was measured in mean intensity projections, normalized to 8-12 min after prometaphase onset. Securin-mEGFP degradation rates were calculated by linear regression at time frames in which either 1, 2-5, or >5 unaligned chromosomes persisted longer than 22 min.

Statistical analysis

All data in this manuscript were tested for normality with D'Agostino-Pearson omnibus test ($\alpha = 0.05$) using the software GraphPad Prism and for equal variances with Levene's test ($\alpha = 0.05$) using the software R. Data with normal distribution were presented as mean ± s.d. or mean ± s.e.m, whereas data that had no normal distribution were presented as median values. The appropriate statistical test was chosen accordingly: Normal distributed data with different variances were tested with a two-tailed unpaired t-test with Welch's correction (Fig. 4e; comparison of "5-10 min" in Fig. 4e with "Noc" in Fig 5c; Fig. 5h). Data with non-normal distribution, but equal variances were tested with a two-tailed Mann-Whitney U test (Fig.2d). Statistical significance was determined at $\alpha = 0.01$ using the software R.

Experimental replicates and sample numbers were as follows: Fig. 1b-e; Supplementary Figure S1d-f: 18 cells, each an independent laser microsurgery experiment. Fig. 2: 56 cells, each an independent laser microsurgery experiment. Mitotic slippage timing in presence of 100 ng/ml was determined in 3 independent experiments. Fig. 3b: 16 cells, each an independent laser microsurgery experiment. Fig. 3c: 20 cells, each an independent laser microsurgery experiment, and 11 cells treated with 100 or 500 ng/ml nocodazole from 3 independent experiments, respectively. Fig. 4; Supplementary Fig. S4: 11 cells, each an independent laser microsurgery experiment). Fig. 5a-c; Supplementary Fig. S3: 3 independent experiments and quantified in 10 cells per condition. Fig. 5d,e: 34 cells in 3 independent

experiments. Fig. 5f-h; Supplementary Fig. S5: 13 cells (100 ng/ml nocodazole) and 26 cells (6-25ng/ml nocodazole) from 3 independent experiments. Supplementary Fig. S1: 30 cells per condition from 3 independent experiments. Supplementary Fig. S2: 35 cells,each an independent laser microsurgery experiment.

References

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37. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682 (2012).