**Figure S1**

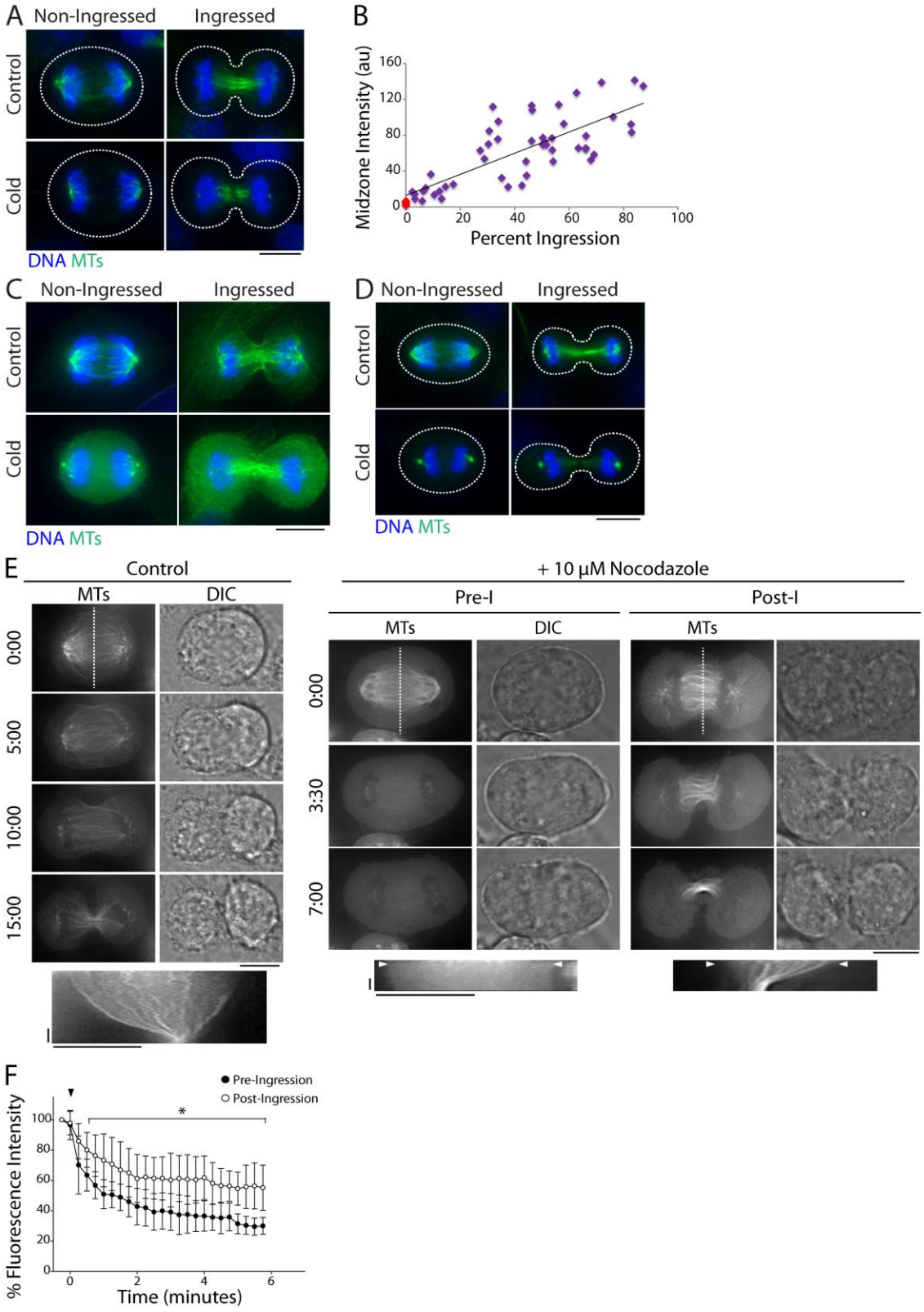
A
Non-Ingressed
Ingressed
Control
Cold
DNA MTs
B
Midzone Intensity (au)
160
120
80
40
0
0
20
40
60
80
100
Percent Ingression
C
Non-Ingressed
Ingressed
Control
Cold
DNA MTs
D
Non-Ingressed
Ingressed
Control
Cold
DNA MTs
E
Control
+ 10 µM Nocodazole
MTs
DIC
Pre-I
Post-I
MTs
DIC
MTs
0:00
5:00
10:00
15:00
0:00
3:30
7:00
I
I
F
Pre-Ingression
Post-Ingression
*
% Fluorescence Intensity
100
80
60
40
20
0
0
2
4
6
Time (minutes)

**Figure S1, Related to Figure 1.** A) Maximum z-projections of untreated (top) and cold-treated (bottom) HeLa cells in C phase with non-ingressed or ingressed cleavage furrows. Cells were permeabilized for 30 seconds prior to fixation to extract free tubulin and stained with antibodies against tubulin (green). Dashed lines indicate cell boundary. DNA (blue) was counterstained with Hoechst 33342. B) Quantification of midzone MT fluorescence intensity after cold treatment in cells with non-ingressed or ingressed cleavage furrows plotted as a function of the percent furrow ingression in cells that were fixed without extraction of free tubulin (n=52). C) Maximum z-projections of untreated (top) and cold-treated (bottom) RPE-1 cells in C phase with non-ingressed or ingressed cleavage furrows. Cells were stained with antibodies against tubulin (green). DNA (blue) was counterstained with Hoechst 33342. Scale bar, 10 µm. D) Maximum z-projections of RPE-1 cells permeabilized for 30 seconds prior to fixation to extract free tubulin. Cells were otherwise treated, fixed, and stained as described in C). Dashed lines indicate cell boundary. Scale bar, 10 µm. E) Top: Single z-plane micrographs taken from time lapse movies of C phase HeLa cells expressing mCherry-tubulin in the presence of DMSO (control) or 10 µM nocodazole. Time is indicated in min:seconds relative to the initial frame. Dashed lines were used to generate kymographs. Scale bars, 10 µm. Bottom: Kymographs of GFP-tubulin fluorescence across the division plane during C phase. Arrowheads (white) indicate time point of nocodazole addition. Scale bars, 2.5 min (x axis) and 10 µm (y axis). F) Quantification of midzone MT fluorescence intensity in pre- and post-ingression cells treated with 10 µM nocodazole as described in E). Arrowhead (black) indicates the time point of nocodazole addition. Data represent mean ± SD. n=8 (pre-ingression) and 9 (post-ingression) from three independent experiments, * = $p < 0.05$.

**Figure S2**

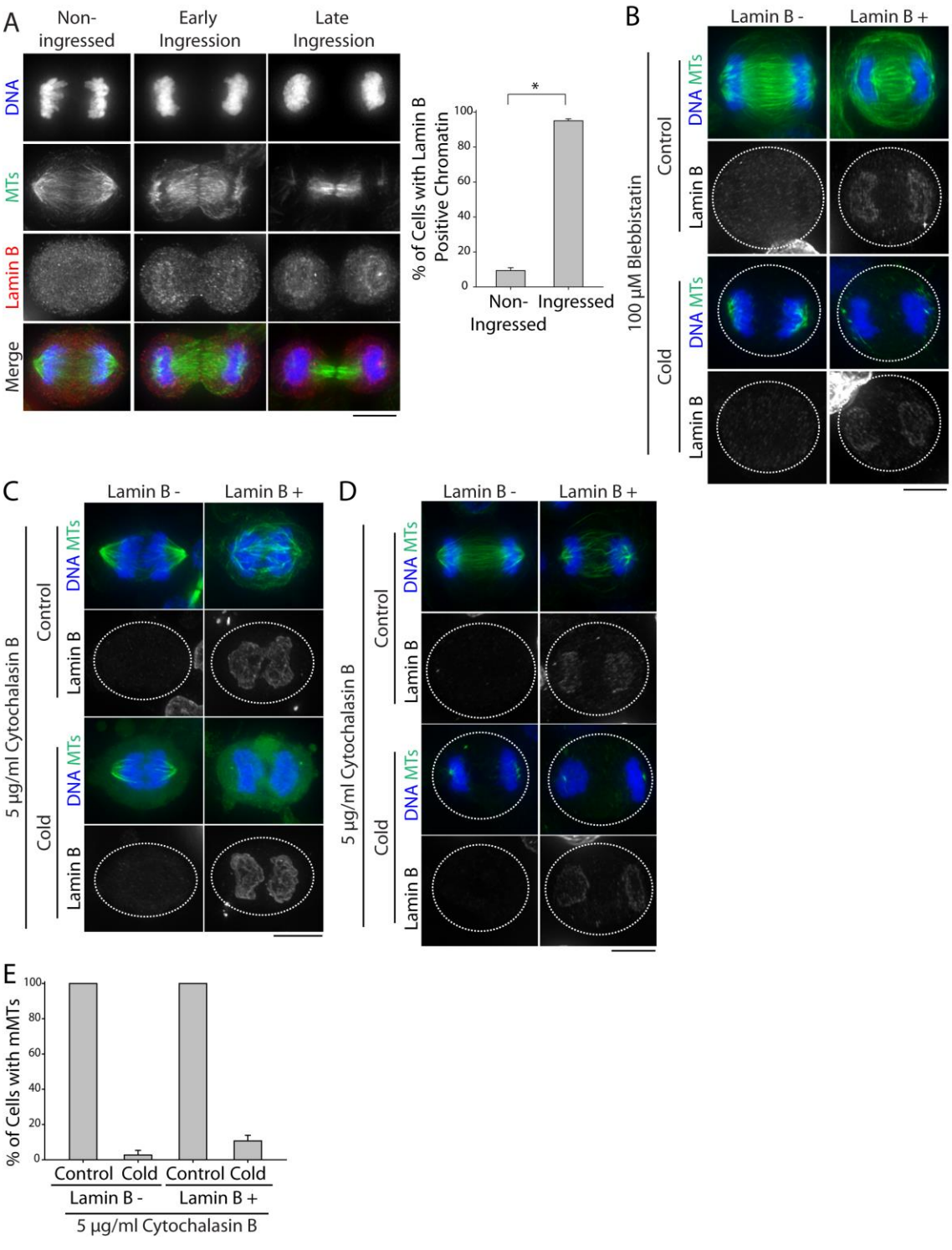
A
Non-ingressed
Early Ingression
Late Ingression
DNA
MTs
Lamin B
Merge
% of Cells with Lamin B Positive Chromatin
Non-Ingressed
Ingressed
B
Lamin B -
Lamin B +
100 µM Blebbistatin
Control
Cold
DNA MTs
Lamin B
C
5 µg/ml Cytochalasin B
D
E
% of Cells with mMTs
Control
Cold
Lamin B -
Lamin B +
5 µg/ml Cytochalasin B

**Figure S2, Related to Figure 2.** A) Left: Maximum z-projections of HeLa cells in C phase with non-ingressed or ingressed cleavage furrows showing nuclear envelope staining. Cells were stained with antibodies against tubulin (green) and lamin B (red). DNA (blue) was counterstained with Hoechst 33342. Scale bar, 10 μm. Right: Quantification of the percent of cells with lamin B staining in C phase with non-ingressed or ingressed cleavage furrows. Data represent mean ± SE. $n > 200$ cells from three independent experiments, * = $P < 0.005$. B) Maximum z-projections of cells treated with 100 μM blebbistatin prior to fixation (top) or cold treatment (bottom). Cells were permeabilized to remove free tubulin and stained with antibodies to lamin B (single channels) to mark the nuclear envelope and tubulin (green). DNA was counterstained with Hoechst 33342 (blue). Dashed lines indicate cell boundary. Scale bar, 10 μm. C) Maximum z-projections of HeLa cells treated with 5 μg/ml cytochalasin B prior to fixation (top) or cold-treatment (bottom). Cells were stained with antibodies to tubulin (green) and lamin B (single channels). DNA (blue) was counterstained with Hoechst 33342. Scale bar, 10 μm. D) Maximum z-projections of HeLa cells permeabilized for 30 seconds prior to fixation to extract free tubulin. Cells were otherwise treated, fixed, and stained as described in C). Dashed lines indicate cell boundary. Scale bar, 10 μm. E) Quantification of the percent of cytochalasin B treated cells with midzone MTs before and after cold treatment as described in C). Data represent mean ± SE, $n > 150$ cells from three independent experiments.

**Figure S3**

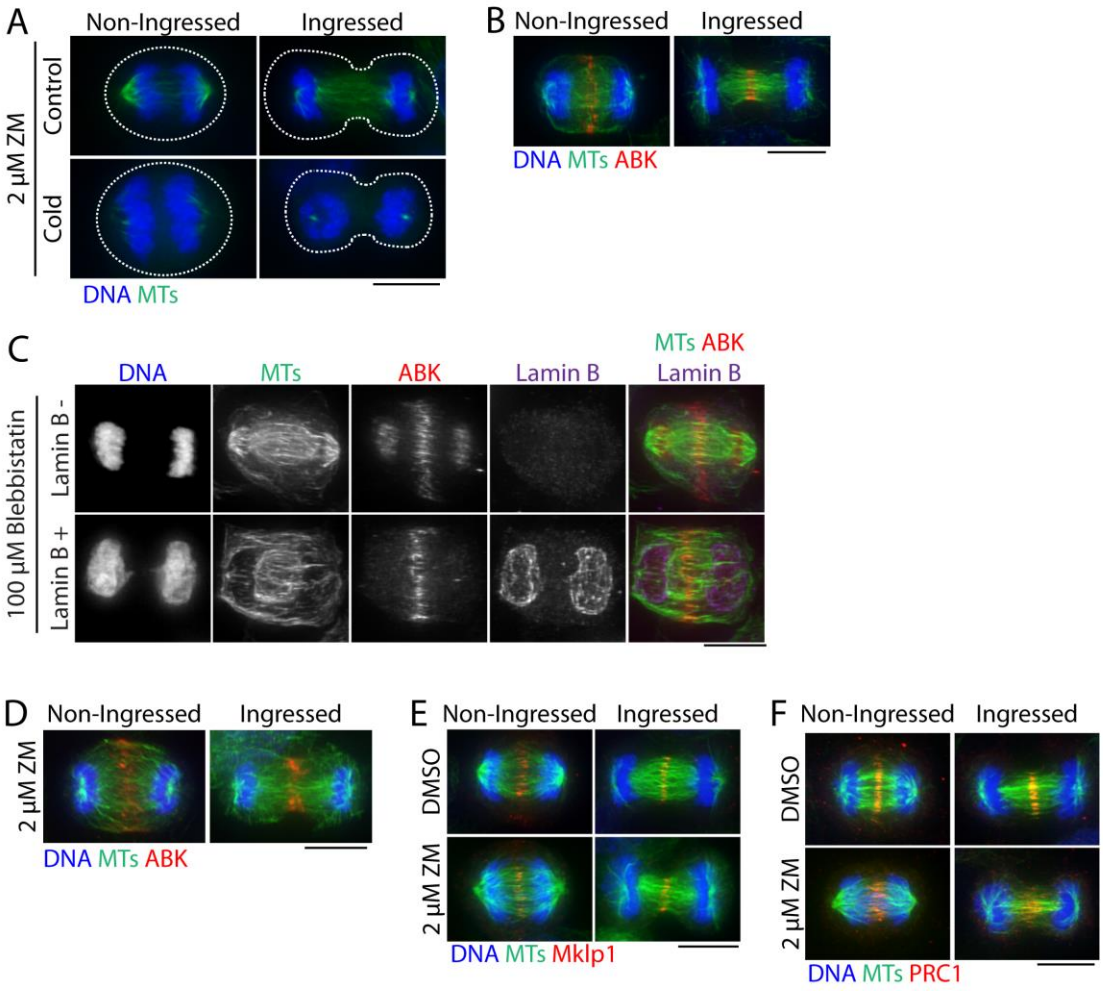


**Figure S3, Related to Figure 3.** A) Maximum z-projections of HeLa cells treated with 2 µM ZM447439 (ZM) prior to fixation (top) or cold treatment (bottom). Cells were permeabilized to remove free tubulin and stained with antibodies to tubulin (green) and DNA was counterstained with Hoechst 33342 (blue). Dashed lines indicate cell boundary. Scale bar, 10 µm. B) Maximum z-projections of HeLa cells stained with antibodies to Aurora B kinase (ABK, red) and tubulin (green). DNA was counterstained with Hoechst 33342 (blue). Scale bar, 10 µm. C) Maximum z-

projections of cells treated with 100 μM blebbistatin. Cells were stained with antibodies to ABK (red), lamin B (purple) to mark the nuclear envelope, and tubulin (green). DNA (blue) was counterstained with Hoechst 33342. Scale bar, 10 μm. D) Maximum z-projections of cells treated with 2 μM ZM447439. Cells were stained with antibodies to ABK (red) and tubulin (green). DNA (blue) was counterstained with Hoechst 33342. Scale bar, 10 μm. E) Maximum z-projections of cells treated with DMSO (top) or 2 μM ZM447439 (bottom). Cells were stained with antibodies to Mklp1 (red) and tubulin (green). DNA (blue) was counterstained with Hoechst 33342. Scale bar, 10 μm. F) Maximum z-projections of cells treated with DMSO (top) or 2 μM ZM447439 (bottom). Cells were stained with antibodies to PRC1 (red) and tubulin (green). DNA (blue) was counterstained with Hoechst 33342. Scale bar, 10 μm.

**Figure S4**

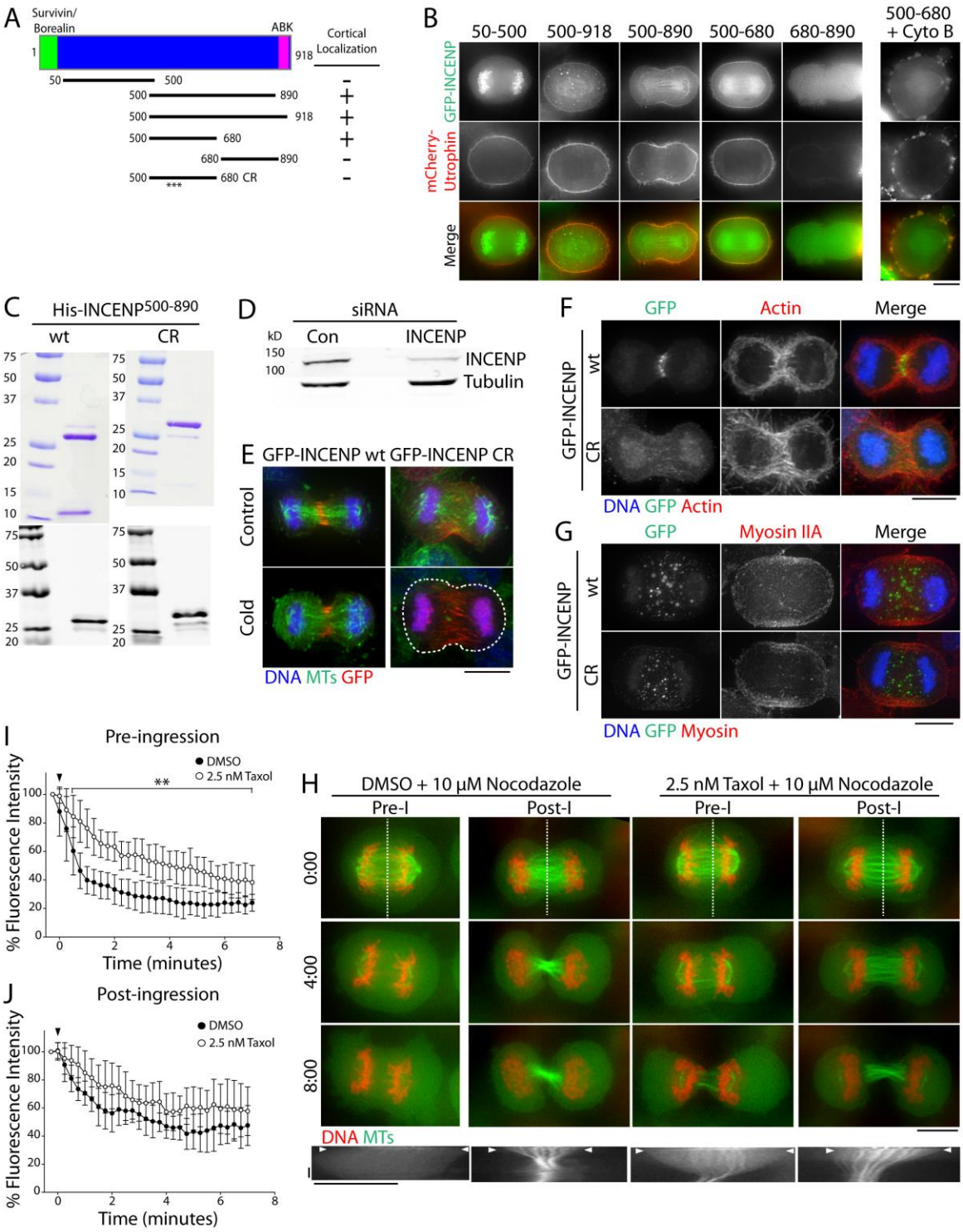


**Figure S4, Related to Figure 4.** A) Schematic of GFP-INCENP truncations used to map the actin binding domain in cells. B) Single plane micrographs of HeLa cells expressing GFP-INCENP (green)

truncations and mCherry-Utrophin (red). Cells expressing GFP-INCENP 500-680 and mCherry-Utrophin cells were treated with 10 μg/ml cytochalasin B to confirm that colocalization was specific. Scale bar, 10 μm. C) Top: Coomassie-stained gels showing recombinant $His_6$-$INCENP^{500-680}$ and $His_6$-$INCENP^{500-680\ CR}$. Bottom: Anti-His immunoblots of recombinant $His_6$-$INCENP^{500-680}$ and $His_6$-$INCENP^{500-680\ CR}$. Molecular weight standards are indicated in kDa. D) Immunoblots of cells extracts prepared from HeLa cells transfected with a control (left) or INCENP-targeting (right) siRNA. Tubulin is shown as a loading control. Molecular weight standards are indicated in kDa. E) Maximum z-projections of HeLa cells depleted of endogenous INCENP and rescued with GFP-INCENP (wt or CR) prior to fixation or cold-treatment. Cells were permeabilized for 30 seconds prior to fixation to extract free tubulin and stained with antibodies against tubulin (green) and GFP (red). DNA (blue) was counterstained with Hoechst 33342. Dashed lines indicate cell boundary. Scale bar, 10 μm. F) Maximum z-projections of HeLa cells depleted of endogenous INCENP and transfected with GFP-INCENP (wt or CR). Cells were stained with antibodies against GFP (green) and actin was visualized using phalloidin-TRITC (red). DNA (blue) was counterstained with Hoechst 33342. Scale bar, 10 μm. G) Maximum z-projections of HeLa cells depleted of endogenous INCENP and transfected with wt GFP-INCENP or the charge reversal mutant (CR). Cells were stained with antibodies against GFP (green) and myosin IIA (red). DNA (blue) was counterstained with Hoechst 33342. Scale bar, 10 μm. H) Top: Single z-plane micrographs taken from time lapse movies of HeLa cells expressing GFP-tubulin (green) and mCherry-H2B (red) incubated for > 30 mins in DMSO or 2.5 nM Taxol prior to exposure to 10 μM nocodazole. Time is indicated in min relative to the initial frame. Dashed lines were used to generate kymographs. Scale bars, 10 μm. Bottom: Kymographs of GFP-tubulin fluorescence across the division plane

during C phase. Arrowheads (white) indicate time point of nocodazole addition. Scale bars, 2.5 min (x axis) and 10 µm (y axis). I) Quantification of midzone MT fluorescence intensity in pre-ingression cells incubated with DMSO or 2.5 nM Taxol before exposure to 10 µM nocodazole. Arrowhead (black) indicates the time of nocodazole addition. Data represent mean ± SD. n=11 (DMSO) and 10 (2.5 nM Taxol) from three independent experiments, ** = $p < 0.01$. J) Quantification of midzone MT fluorescence intensity in post-ingression cells incubated with DMSO or 2.5 nM Taxol before exposure to 10 µM nocodazole. Arrowhead (black) indicates the time of nocodazole addition. Data represent mean ± SD. n=9 (DMSO) and 10 (2.5 nM Taxol) from three independent experiments.

**Supplemental Experimental Procedures**

**Cell culture and transfections**

Cell lines were cultured in media supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin 37°C, 5% $CO_2$. HeLa "Kyoto" cells were cultured in DMEM. HeLa cells stably expressing GFP-Tubulin and mCherry-H2B (a gift from Dr. Dan Gerlich) additionally contained 500mg/ml G418. RPE-1 cells were cultured in 50% DMEM/50% F-12 media. Transfections were performed using Lipofectamine 2000 (Invitrogen) for plasmid DNA or siRNAs according to manufacturer instructions. Cells were cultured in Opti-MEM or DMEM supplemented with 10% FBS following transfection. siRNA sequences used in this study were: Stealth Lo GC negative control (LifeTechnologies); and INCENP 3'UTR, 5'-AAGGCTTGGCCAGGTGTATAT (Qiagen). Cells were fixed for immunofluorescence or used for live cell imaging 24 hours after transfection.

**Cold and Pharmacological Perturbations**

For fixed cell experiments, cells were treated with the following small molecules at 37$^{o}$C for the lengths of time indicated: Blebbistatin (Tocris Bioscience), 100 μM for 30 minutes; Cytochalasin B (Sigma), 5 μg/ml for 30 minutes or 10 μg/ml for 10 minutes; ZM447439 (Tocris Bioscience), 2 μM for 10 minutes. Drug stocks in DMSO were diluted in DMEM immediately before use. To destabilize MTs, cells were incubated in DMEM (without small molecules) chilled to < 4$^{o}$C and placed on ice for 10 minutes immediately after exposure to small molecules as indicated. For live cell "nocodazole shock" experiments cells were treated with the same small molecules at 37$^{o}$C for the lengths of time indicated prior to imaging or addition of 10 μM Nocodazole (Sigma): Blebbistatin, 100 μM for 20 minutes, and ZM447439, 2 μM for 5 minutes, 2.5 nM Taxol for 30 minutes. To destabilize MTs an equivalent volume of movie medium was added during imaging containing 2X nocodazole ([Final] = 10 μM) and 1X Blebbisatin, ZM447439, or Taxol.

**Immunofluorescence and fixed-cell imaging**

For most experiments, HeLa and RPE-1 cells were fixed with methanol at -20$^{o}$C for 5 or 10 min. The following primary antibodies were used in this study: mouse anti-tubulin (DM1α; Vanderbilt Antibody and Protein Resource), 1:500; rat anti-tubulin (YL1/2; Accurate Chemical and Scientific Corporation), 1:500; anti-AIM (BD Transduction Labs), 1:500; anti-INCENP (Abcam), 1:500; anti-lamin B (a gift from Dr. Susan Wente), 1:200; anti-Mklp1 (Santa Cruz Biotechnology), 1:500; anti-PRC1 (Santa Cruz Biotechnology) 1:50; anti-GFP conjugated to DyLight 488 (Rockland), 1:1000. Secondary antibodies conjugated to Alexa 488, Alexa 594, or Alexa 647 (Invitrogen) were used at

1:1000. Primary and secondary antibody incubations were for 1 hour each at room temperature. DNA was counterstained with 5 µg/ml Hoechst 33342. Stained cells were mounted in Prolong Gold (Invitrogen)

To quantify midzone MT fluorescence after cold treatment, cells were fixed in 2% glutaraldehyde in cytoskeleton buffer (CB, 10 mM MES pH, 6.1, 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA) plus 11% sucrose at room temperature for 10 minutes. To visualize actin, cells were fixed with 1% glutaraldehyde in CB plus 11% sucrose at room temperature for 10 minutes. Actin was stained with phalloidin-TRITC (Sigma) at 500 ng/µl. To visualize PRC1, cells were fixed in 4% formaldehyde and Perm-Fix (100 mM K-PIPES, 0.2% TX-100, 10 mM K-EGTA, 1 mM $MgCl_2$). To visualize myosin IIA, cells were fixed with 4% paraformaldehyde (PFA) in PBS at room temperature for 10 min and stained with anti-myosin IIA antibody (a gift from Dr. Dylan Burnette) at 1:1000. Where indicated, cells were permeabilized with 100 mM K-PIPES (pH 6.8), 1 mM MgCl2, and 1% TX-100 for 30 seconds prior to fixation to extract free tubulin.

Cells were visualized using either a 60X 1.4 NA or 100X 1.4 NA objective (Olympus) on a DeltaVision Elite imaging system (GE Healthcare) equipped with a Cool SnapHQ2 CCD camera (Roper). Optical sections were collected at 200 nm intervals and processed using ratio deconvolution in SoftWorx (GE Healthcare). Images were prepared for publication using Image J and NIS-Elements AR.

To quantify midzone MT fluorescence following cold treatment, cells were fixed in glutaraldehyde with or without pre-extraction as described above. Sum intensity projections were generated from 200 nm z-slices of tubulin fluorescence and a boxed region within the midzone was used to measure the integrated fluorescence intensity. To measure fluorescence from polymer only, background fluorescence was measured from an equivalently sized region outside the midzone where no detectable polymer was observed, and subtracted from midzone MT fluorescence. To determine the extent to which cleavage furrows had ingressed, the following formula was used: distance furrow ingressed $\div$ total cell width $\times$ 100 = % ingression.

**Live cell imaging**

All live cell imaging was performed at 37$^{o}$C with 5% $CO_2$ and a 60X 1.4 NA objective on a DeltaVision Elite imaging system equipped with a WeatherStation Environmental Chamber. For live imaging of HeLa cells stably expressing GFP-Tubulin and mCherry-H2B, cells were plated on glass bottom dishes (MatTek Corporation) 24 hours prior to imaging. For live imaging of HeLa cells transiently expressing mCherry-Tubulin, cells were plated on glass bottom dishes 48 hours prior to imaging and transfected 24 hours prior to imaging. For live imaging of HeLa cells depleted and rescued with GFP-INCENP wt or CR cells were plates on glass bottom dishes 48 hours prior to imaging and transfected with the appropriate siRNA and vector 24 hours prior to imaging. Immediately before imaging, cells were incubated in movie medium (L-15 medium without Phenol Red supplemented with 10% FBS, penicillin/streptomycin, and 7 mM K-HEPES, pH 7.7) with the appropriate concentration of drug as indicated. One optical section was selected and cells were imaged at 15 second intervals.

To quantify midzone MT fluorescence in live cells after exposure to nocodazole the midzone was defined as the region between the chromosome plates in the X dimension, and the chromosome edges in Y dimension. The fluorescence intensity of GFP-tubulin within a boxed region encompassing the midzone from a single optical section was measured over time. Similar to fixed cells, a region outside the midzone lacking detectable polymer was used to measure background fluorescence for each time point, which was subtracted from the midzone MT fluorescence values at corresponding time points. To normalize for changes in the size of the midzone over time (due to ingression or movement of the chromosome plates), we calculated the integrated fluorescence intensity per pixel at each time point and then scored these values relative to the initial fluorescence intensity (before nocodazole addition) to determine the percent fluorescence decay over time.

**Immunoblotting**

For preparation of whole cell lysates, cells were washed three times with PBS and resuspended in 2X Laemmli buffer. For pure protein, samples were mixed with 2X Laemmli buffer. After heating to 95$^{o}$C for 5 min, proteins were resolved by SDS-PAGE and transferred to nitrocellulose (Whatman). Immunoblots were blocked with 5% w/v milk in PBST and then probed with primary antibodies: anti-His (GE Healthcare), anti-INCENP, or DM1α to detect tubulin at 1:500 for 1 hour. Blots were then probed with species-appropriate fluorescently-tagged secondary antibodies at 1:1000 for 45 minutes. Fluorescence was measured using an Odyssey fluorescence detection system (LI-COR Biosciences).

**Molecular Biology**

A full-length human INCENP ORF (Source Bioscience, IOH62774:pDEST15) was used to prepare all INCENP DNA constructs. Amplification of the INCENP ORF was performed using PrimeStar GXL polymerase (Takara). All constructs were prepared using isothermal assembly [S1]. PCR fragments were cloned into pEGFP-C1 (Clontech) restricted with *Kpn*I and *Nde*I or into pET15 (Novagen) restricted with *Xho*I and *Nde*I.

Charge reversal mutagenesis was performed using Gblocks (IDT) containing the desired mutations and a 4-part Isothermal Assembly of INCENP Gblock gene fragments. The following mutations were made to codons corresponding to amino acids 563-580 to change lysine and arginine residues to glutamic acid (mutations underlined): G<u>GAAGAAGAA</u>CTGGAGGAGGTG<u>G</u>AGCTG<u>G</u>AG<u>GAA</u>GAGGAA<u>GAA</u>CTC<u>GAAGA</u>G.

**Protein Expression and Purification**

$His_6$-INCENP$^{500-680}$ and $His_6$-INCENP$^{500-680\ CR}$ were expressed in BL21 DE3 RIPL cells with 0.4 mM IPTG for 16 hours at 16$^{o}$C. For purification, cells were pelleted and resuspended in lysis buffer (PNI [50 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole], 5 mM β-mercaptoethanol (β-ME), 1% NP40, and protease inhibitors [1 mM phenylmethylsufonyl fluoride, 1 mM benzamidine, and 10 μg/ml each of leupeptin, pepstatin, and chymostatin]). Cells were enzymatically lysed by incubation in 1 mg/ml lysozyme (Sigma) for 30 minutes. The lysate was sonicated, and clarified by centrifugation at 35K rpm for 1 hour in a Ti-45 rotor (Beckman). 4 ml of $Ni^{++}$-NTA agarose

(Qiagen) was incubated with the supernatant for 1 hour at 4°C, and then washed extensively with wash buffer (PNI, 5 mM β-ME). Protein was eluted with PNI, 5 mM β-ME, and 180 mM imidazole. Peak fractions were desalted using a PD-10 column (GE Healthcare) equilibrated in 10 mM K-HEPES pH 7.7, 50 mM KCl, 1 mM DTT. Powdered sucrose was added to 20% w/v. Protein was aliquoted, frozen in liquid nitrogen and stored at -80°C.

**Actin polymerization and co-sedimentation**

Rabbit skeletal muscle actin (Cytoskeleton) was resuspended in 5 mM Tris-HCl pH 8.0 and 200 µM $CaCl_2$, and stored at -20°C until use. Actin was polymerized in 500 mM KCl and 20 mM MgCl2 for 10 minutes at room temperature.

Prior to use, $His_6$-INCENP$^{500-680}$ and $His_6$-INCENP$^{500-680\ CR}$ were pre-clarified by centrifugation at 90K rpm for 30 minutes at 4°C in a TLA-100 Ultracentrifuge (Beckman). For co-sedimentation with actin, $His_6$-INCENP$^{500-680}$ or $His_6$-INCENP$^{500-680\ CR}$ (5 µM) were mixed with F-actin (10 µM) in 100 µl reaction buffer (10 mM K-HEPES pH7.7, 50 mM KCl, 1 mM DTT, 1 mM $MgCl_2$-ATP). Reactions were incubated at room temperature for 15 min and centrifuged at 90K rpm for 20 minutes at 22°C. 100 µl of supernatant was collected and mixed with and equivalent volume of 2X Laemmli buffer. Pellets were resuspended in 200 µl of 2X Laemmli buffer. Fractions were heated to 95°C for 5 min and 40 µl of each fraction was run on a 12% SDS-PAGE gel and stained using Coomassie Brilliant Blue.

**Statistical Analysis**

Statistically relevant differences in experimental data were determined using the T.TEST function in Excel (Microsoft). In all cases, P-values report the two-tailed distribution of a two-sample Student's t-Test assuming unequal variance. For all quantitative analysis, n = total number of cells analyzed over at least three independent experiments. For nocodazole shock experiments, data from three independent experiments was pooled so that the values reported represent the mean of the pooled data along with the standard deviation. For cold treatment assays and the analysis of cleavage furrow ingression, the percent value was first determined for each independent experiment and the mean of those independent values is reported along with the standard error of the mean

**Supplemental References**

S1. Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., 3rd, and Smith, H.O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods *6*, 343-345.