Supporting Material

Sensing chromosome bi-orientation by spatial separation of Aurora B kinase from kinetochore substrates

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Supplemental Material and Methods

Cell culture, transfection and synchronization

Hela and U2OS cells were cultured in growth medium: DMEM with 10% fetal bovine serum and penicillin-streptomcyin, at 37 ºC in a humidified atmosphere with 5% CO_2. Hela cells were transfected with plasmid DNA using Fugene (Roche Diagnostics), following the manufacturers instructions, and U2OS cells according to a standard calcium phosphate protocol. Where indicated, cells were synchronized at the G1/S boundary by addition of 2.5 mM thymidine and released from the block 24 h later.

Construction of INCENP fusion proteins and FRET sensors

The plasmid termed CENP-B encodes the centromeric DNA-binding domain of CENP-B (amino acids 1-158 (S*1*)). It was PCR amplified from pGAdGH-CENPB (a kind gift of H. Masumoto, Nagoya University, Japan) and cloned into pCR3 containing an N-terminal vsv-tag to generate vsv-CENP-B or into pEGFP-C1 (Clontech) to generate GFP-CENP-B. Full-length hMis12 was PCR amplified from pOTB7-hMis12 and ligated into pCR3 containing an N-terminal vsv-tag to obtain vsv-hMis12. The plasmids encoding the CENP-B- and Mis12-INCENP fusion cDNAs were generated as follows: Full-length human INCENP (S*2*) was digested with EcoRI/XhoI to generate $INCENP_{47\text{-}920}$. This fragment was cloned into pCR3 containing a N-terminal vsv-tag. The centromeric DNA-binding domain of CENP-B was subsequently ligated in frame with $INCENP_{47\text{-}920}$ to generate vsv-CB-INCENP. Full-length Mis12 was ligated in frame with $INCENP_{47\text{-}920}$ to generate vsv-Mis12-INCENP. The vsv-CB-INCENP and vsv-Mis12-

INCENP encoding cDNAs were subsequently cloned into pEGFP-N1 (Clontech) to create a GFP-tag at the C-terminus of the fusion proteins. For some experiments (Fig. 3-4) GFP was replaced with mCherry to tag the INCENP fusion proteins. mCherry is spectrally distinct from the fluorophores used in the FRET sensors (S*3, S4*), so that all three can be imaged quantitatively in the same cell.

The centromere-targeted Aurora-B phosphorylation sensor has been previously described (S*5*). To construct the kinetochore-targeted sensor, full-length hMis12 was used in place of CENP-B for targeting. Because there are a limited number of Mis12 binding sites at the kinetochore (S*6*), we modified the sensor design to get sufficient fluorescence intensity for ratiometric FRET measurements: CFP was replaced with mTFP1, which is significantly brighter and also functions as a FRET donor to YFP (S*3*). Localization of CENP-B and Mis12 does not depend on either kinetochore-microtubule attachment or tension (S*7, S8*), so our measurements are not limited by sensor loss at metaphase. To test the sensor responses to changes in Aurora B activity, cells were treated with or without the Aurora B inhibitor ZM447439 in the presence of nocodazole to eliminate any effects of microtubule attachment. We also mutated the phosphorylated threonine in the substrate peptide to alanine to prevent substrate phosphorylation and mimic the dephosphorylated state.

Drug treatments

The Aurora B re-activation assay allowed us to examine both correct and incorrect attachments simultaneously in a single cell (Fig. 1 and 2), as previously described (S*9*). Hela cells were incubated with the reversible kinesin-5 inhibitor monastrol (100 μM) for 3 hours to arrest them in mitosis with monopolar spindles. Monastrol was washed out, and cells were incubated with MG132 (10 μM) and the Aurora B inhibitor ZM447439 (1 μM) for 1.5 hrs, leading to formation of bipolar spindles with a high frequency of syntelic attachment errors, in which both sister chromatids are connected to a single pole. ZM447439 was washed out to re-activate Aurora

B, and cells were incubated with MG132. For phospho-CENP-A staining, cells were fixed 10 min after ZM447439 washout and processed for immunofluorescence. Since attachment errors are not yet corrected in this time, correct (bi-oriented) and incorrect (syntelic) attachments can be directly compared in a single cell. As a measure of relative CENP-A Ser-7 phosphorylation, we calculated the ratio of phospho-CENP-A to CREST fluorescence intensity (Fig. 1).

The same assay was performed with cells expressing the centromere-targeted or kinetochore-targeted phosphorylation sensors, except that cells were imaged live at the indicated timepoints relative to ZM447439 washout. For steady-state phosphorylation measurements, cells transfected with the centromere-targeted or kinetochore-targeted phosphorylation sensors were incubated with nocodazole (1 μg/ml), monastrol (100 μM) or MG132 (10 μM) for 2 hours, then imaged live. For measurements of sensor dephosphorylation (Fig. S1B-C), 2 μM ZM447439 was used to ensure rapid kinase inhibition.

Flow cytometry

Cells were plated in 10 cm^2 dishes (U2OS) or 6-wells culture plates (Hela) and transfected with spectrin-GFP, the indicated plasmids encoding vsv-tagged proteins and with or without BubR1 or Mad2 shRNA plasmids (S*2, S10*).Twelve hours after transfection thymidine was added for 24 h. Fourteen hours after thymidine release (where indicated 3 μM ZM447439 was added at the onset of the release) cells were harvested and fixed in ice-cold 70% ethanol. To detect mitotic cells, fixed cells were washed in PBS containing 0.05% Tween 20 and incubated with anti-MPM2 mAb (Upstate Biotechnology). Cells were subsequently incubated with a Cy5-conjugated goat-anti-mouse antibody (Jackson ImmunoResearch Labs) and counterstained with propidium iodide (Sigma). Fluorescent cells were detected by a FACScalibur (BD Biosciences) and data analyzed with CellQuest (BD Biosciences). Appropriate gates were set to select the GFP^+ (i.e. transfected) cells and the percentage of MPM2 positive cells within the 4N DNA population was determined.

Immunofluorescence

For phospho-CENP-A staining cells were fixed in cold methanol for 10 minutes. For Mad1 staining cells were pre-extracted in buffer containing 100 mM K-PIPES, 10mM EGTA, 1mM $MgCl_2$ (PEM, pH to 6.9 by KOH) and 0.5% Triton-X, then washed once with PEM buffer and fixed in 4% formaldehyde for 10 min. For all other staining cells were fixed in PEM buffer containing 0.2% Triton-X and 4% paraformaldehyde (pH to 6.9 by KOH) or 10 minutes. For analysis of cold-stable microtubules, cells were incubated in L15 media containing 20 mM Hepes (pH 7.3) on ice for 10 minutes and then fixed as described above. The following antibodies were used: mouse anti-tubulin monoclonal (DM1α, Sigma) 1:2000, rat anti-tubulin monoclonal (Serotec) 1:1000, rabbit anti-phospho-CENP-A ser-7 polyclonal (Upstate Biotech) 1:1000, mouse anti-Aurora-B monoclonal (BD Transduction labs) 1:1000, mouse anti-Hec1 monoclonal (9G3, Abcam) 1:1000, mouse anti-VSV monoclonal 1:5000 (Sigma), CREST anti-sera 1:10,000, mouse anti-Mad1 mAb 1:20 (a kind gift of A. Musacchio, Milan, Italy), Alexa-594, Alexa488 and Alexa647 secondary antibodies (Invitrogen) 1:500.

Imaging, data acquisition and processing

For live imaging of the FRET sensors Hela cells were plated on 22x22 mm no. 1.5 glass coverslips (Fisher Scientific) coated with Poly-D-lysine (Sigma). Coverslips were mounted in custom designed Rose chambers, using L-15 medium without phenol-red (Invitrogen). Temperature was maintained at 35-37 °C, using either an air stream incubator (ASI 400, Nevtek) or an environmental chamber (Pecon). All images (live and fixed) of Hela cells were acquired on a Leica DM4000 microscope with a 100x 1.4 NA objective, an XY-piezo Z stage (Applied Scientific Instrumentation), a spinning disk confocal (Yokogawa), an electron multiplier CCD camera (Hamamatsu ImageEM), and an LMM5 laser merge module (Spectral Applied Research), controlled by IP Lab software (BD Biosciences). Images of fixed Hela cells are presented as

maximal intensity projections of confocal stacks, and insets are optical sections (Fig. 1 and 4). For analysis of cold-stable microtubules, kinetochores were examined by visual inspection of confocal image stacks to determine whether a microtubule fiber was attached.

For live imaging of phosphorylation sensors, TFP was excited at 440 nm, and TFP and YFP emissions were acquired simultaneously with a beamsplitter (Dual-View, Optical Insights). Custom software written in Matlab (Mathworks) was used for image analysis. Individual centromeres or kinetochores were defined automatically from confocal image stacks, and the YFP/TFP emission ratio was calculated at each centromere/kinetochore identified by our algorithm (S*5*). Because there are few Mis12 binding sites at each kinetochore (S*6*), the signal-to-noise ratio in our FRET measurements did not allow us to track phosphorylation changes at individual kinetochores over time. Note that the fluorescence signal is considerably dimmer for the kinetochore-targeted sensor than for the centromere-targeted sensor (Fig. 2C and Fig. S1F). To address this limitation, we averaged all of our results over multiple kinetochores or centromeres from multiple cells. For steady-state measurements, the emission ratios from all kinetochores/centromeres were averaged over the entire cell. To measure phosphorylation kinetics after washing out ZM447439, each centromere/kinetochore was characterized as aligned or unaligned based on visual inspection, and the emission ratios were averaged within the two groups. For measurements of phosphorylation (Fig. 3B) and inter-kinetochore distance (Fig. S1D-E) in cells expressing CB-INCENP together with the kinetochore-targeted sensor, we selected kinetochores that were aligned at the metaphase plate.

For live imaging of H2B-dsRed in cells expressing the INCENP fusion proteins, U2OS cells were plated on 0.17 mm glass-bottom multi-well culture dishes (Willco Wells BV) and transfected the next day with plasmids encoding the GFP-tagged INCENP fusion proteins and Histone H2B dsRed (S*11*). Eight hours after release from a thymidine block wells were transferred to a heated stage (37 ºC) in an environmental chamber of a Zeiss Axiovert 200M microscope equipped with a 0.55 N.A. condenser and 40x1.3 N.A. F-Fluar objective and a

Lambda DG-4 Ultra high speed wave-length switcher (Sutter Instruments). Eight bits fluorescence images were captured every 5 min. by a Photometrics Coolsnap HQ CCD camera (Roper Scientific). Images were processed with Metamorph software (Universal Imaging). Images of fixed U2OS cells were acquired on a Zeiss 510 Meta confocal microscope with a 63x 1.3 N.A. objective and analyzed with the Zeiss LSM Meta 510 software and presented as single z sections, with insets that show magnifications of individual centromere pairs used for linescans (Fig. 3).

Supplemental Figure Legends

Figure S1. (**A**) Spindle schematic showing correct and incorrect (syntelic) attachments. Blow-up: relative positions of Aurora B, CENP-B, and Mis12 at the centromere/kinetochore and FRET sensor targeting. The kinetochore is the proteinaceous structure that assembles on centromeric DNA and binds spindle microtubules. Morphologically, the centromere is positioned between the two sister kinetochores. (**B**) Hela cells expressing either the kinetochore-targeted or centromere-targeted sensors were treated as indicated and imaged live. The YFP/TFP emission ratio increases in cells treated with the Aurora kinase inhibitor ZM447439 for 1 hr, indicating sensor dephosphorylation for this sensor design. Each bar represents an average over multiple cells, ≥30 kinetochores/centromeres analyzed per cell. A Thr-Ala mutation in the phosphorylated residue in the substrate peptide mimics the dephosphorylated state. (**C**) Cells expressing either the centromere-targeted or kinetochore-targeted sensor were incubated with nocodazole and imaged live before and after addition of ZM447439 at t=0. The increase in the YFP/TFP emission ratio over time indicates sensor dephosphorylation kinetics. (**D**-**E**) For cells expressing the kinetochore-targeted sensor, the inter-kinetochore distance was calculated from the YFP emission images (D). Cells were treated with nocodazole, monastrol, or MG132 as in (B). Each bar (E) represents N>50 kinetochore pairs from multiple cells. (**F**) Sensor localization (YFP) and a

color-coded representation of the emission ratio before ZM447439 washout, for cells treated and analyzed as in Fig. 2C. Scale bar 5 μm. Note that both sensors are dephosphorylated, though there is variation between kinetochores due to low signal intensity for the kinetochore-targeted sensor, as discussed in Supplemental Methods

Figure S2. (**A**) Schematics of INCENP fusion proteins: wt, CENP-B fusion, or Mis12 fusion. (**B**) Images of U2OS cells expressing vsv-tagged CENP-B and vsv-tagged Mis12. Note that in contrast to the CB-INCENP and Mis12-INCENP fusion proteins (Figure 3), mere overexpression of CENP-B and Mis12 does not affect the inner centromere localization of endogenous Aurora B. Images are single z sections, insets are blow-ups of individual centromere/kinetochore pairs (**C**) Hela cells expressing CB-INCENP at different levels were fixed and stained for Aurora B. Images are maximal intensity projections, and insets are blow-ups of individual centromere pairs used for linescans. Scale bars 5 μm. (**D**-**E**) The inter-kinetochore distance was measured for cells expressing the kinetochore-targeted sensor (green) together with CB-INCENP (red). Images (D) show cells with low and high CB-INCENP expression levels, and inter-kinetochore distances (E) were calculated for cells expressing different levels of CB-INCENP.

Figure S3. (**A**) Hela cells expressing the indicated proteins were released from a G1/S block with or without ZM447439 for 14 hrs. The mitotic index was determined by propidium iodide/MPM2 mAb labeling and FACS analysis. (**B**) Mitotic index of U2OS cells expressing the indicated INCENP fusion proteins with or without shRNAs specific for Mad2 or BubR1. (**C**-**D**) Video microscopy of U2OS cells expressing GFP-tagged wt-INCENP, Mis12-INCENP (a) or CB-INCENP (b) and H2B-dsRed (shown). Cells expressing Mis12-INCENP or CB-INCENP are severely delayed in metaphase and undergo aberrant anaphases with many lagging chromosomes. (**E**) Video microscopy of U2OS cells expressing GFP-tagged CB-INCENP. Cells are severely delayed in metaphase and paired sister-chromatids sometimes leave and re-enter the metaphase plate (arrow). (**F**) Hela cells expressing vsv-tagged wt-INCENP were analyzed for cold-stable microtubules (green) and vsv immunofluorescence (red) as in Fig. 4C. Scale bar 5 μM.

Figure S4. U2OS cells were transfected with the indicated GFP-tagged INCENP fusion proteins and fixed 12.5 h after release from a thymidine block, with MG132 present during the final hour of the release. (**A**) Metaphase cells were scored for the presence of misaligned chromosomes based on DAPI (DNA) and crest (centromere) staining. (**B**) The number of metaphase cells with at least one Mad1 positive kinetochore was scored (asterisks indicate spindle poles). Most metaphase cells expressing either CB-INCENP or Mis12-INCENP have at least one Mad1 positive kinetochore, which indicates loss of microtubule attachment. Misaligned chromosomes were frequently observed in cells expressing either CB-INCENP or Mis12-INCENP, consistent with the live imaging data showing that chromosomes frequently exit and re-enter the metaphase plate (Fig. S3). Even when all chromosomes appear aligned in a fixed image, our live imaging data together with the Mad1 staining indicate that the alignment is not stable, and chromosomes will exit the metaphase plate. The unattached (Mad1 positive) kinetochores are likely frequent enough to arrest cells in mitosis (Fig.4A-B and Fig. S3) but are in a small enough minority to have little effect on the average inter-kinetochore distance (Fig. S2E-F).

Supplemental movies

Movie S1: U2OS cells co-expressing GFP-tagged INCENP-wt and H2B-dsRed. H2B-dsRed images are shown (stills: Fig. S3C).

Movie S2: U2OS cells co-expressing GFP-tagged Mis12-INCENP and H2B-dsRed. H2B-dsRed images are shown (stills: Fig. S3Da).

Movie S3: U2OS cells co-expressing GFP-tagged CB-INCENP and H2B-dsRed. H2B-dsRed images are shown (stills: Fig. S3Db).

Movie S4: Similar to Movie S3 but now the GFP images are shown (stills: Fig. S3E).

Fig. S1

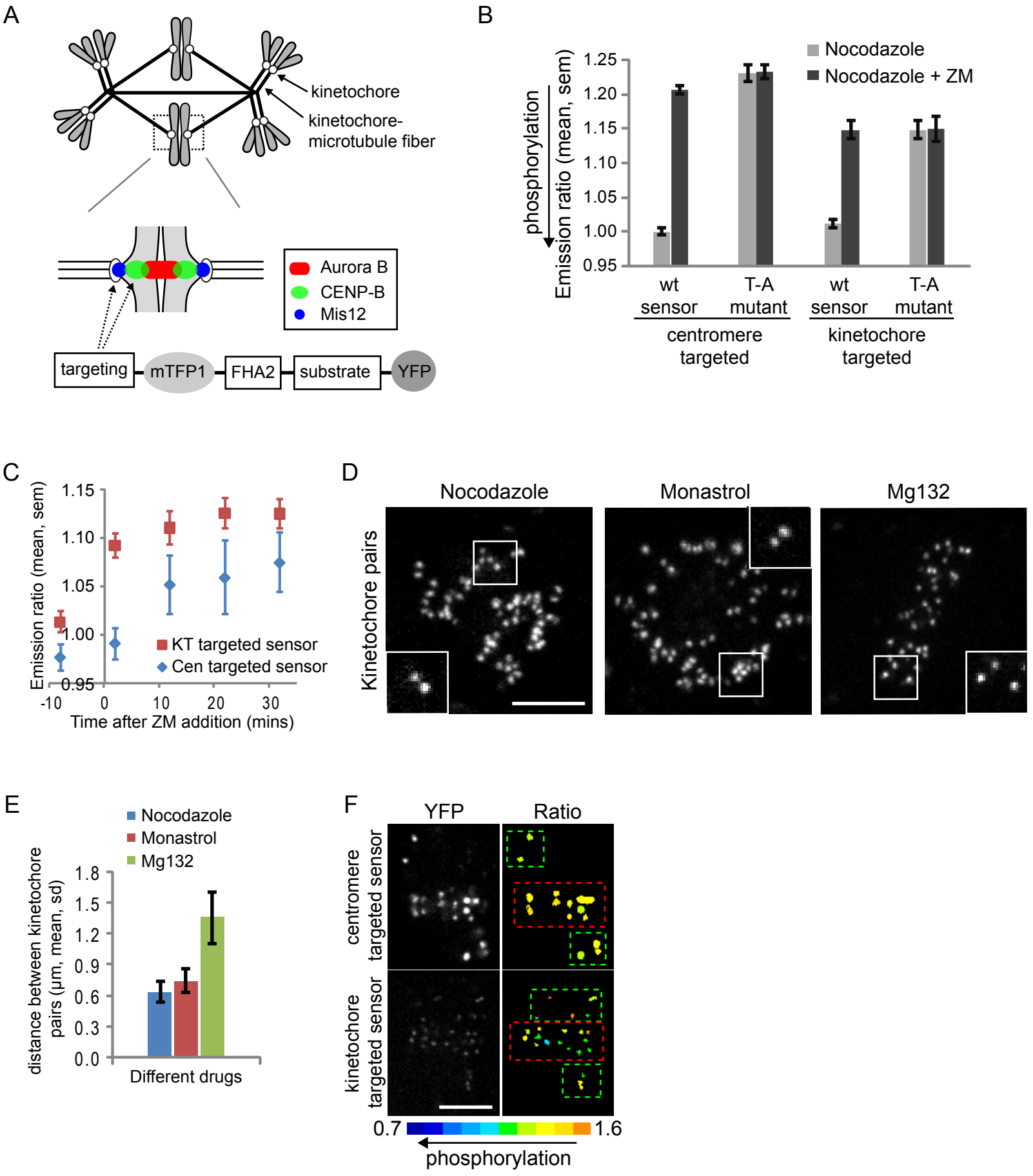

A
kinetochore
kinetochore-
microtubule fiber
Aurora B
CENP-B
Mis12
targeting
mTFP1
FHA2
substrate
YFP
B
Nocodazole
Nocodazole + ZM
phosphorylation
Emission ratio (mean, sem)
1.25
1.20
1.15
1.10
1.05
1.00
0.95
wt sensor
T-A mutant
wt sensor
T-A mutant
centromere targeted
kinetochore targeted
C
Emission ratio (mean, sem)
1.15
1.10
1.05
1.00
0.95
KT targeted sensor
Cen targeted sensor
-10
0
10
20
30
Time after ZM addition (mins)
D
Nocodazole
Monastrol
Mg132
Kinetochore pairs
E
Nocodazole
Monastrol
Mg132
distance between kinetochore pairs (µm, mean, sd)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
Different drugs
F
YFP
Ratio
centromere targeted sensor
kinetochore targeted sensor
0.7
1.6
phosphorylation

Fig. S2

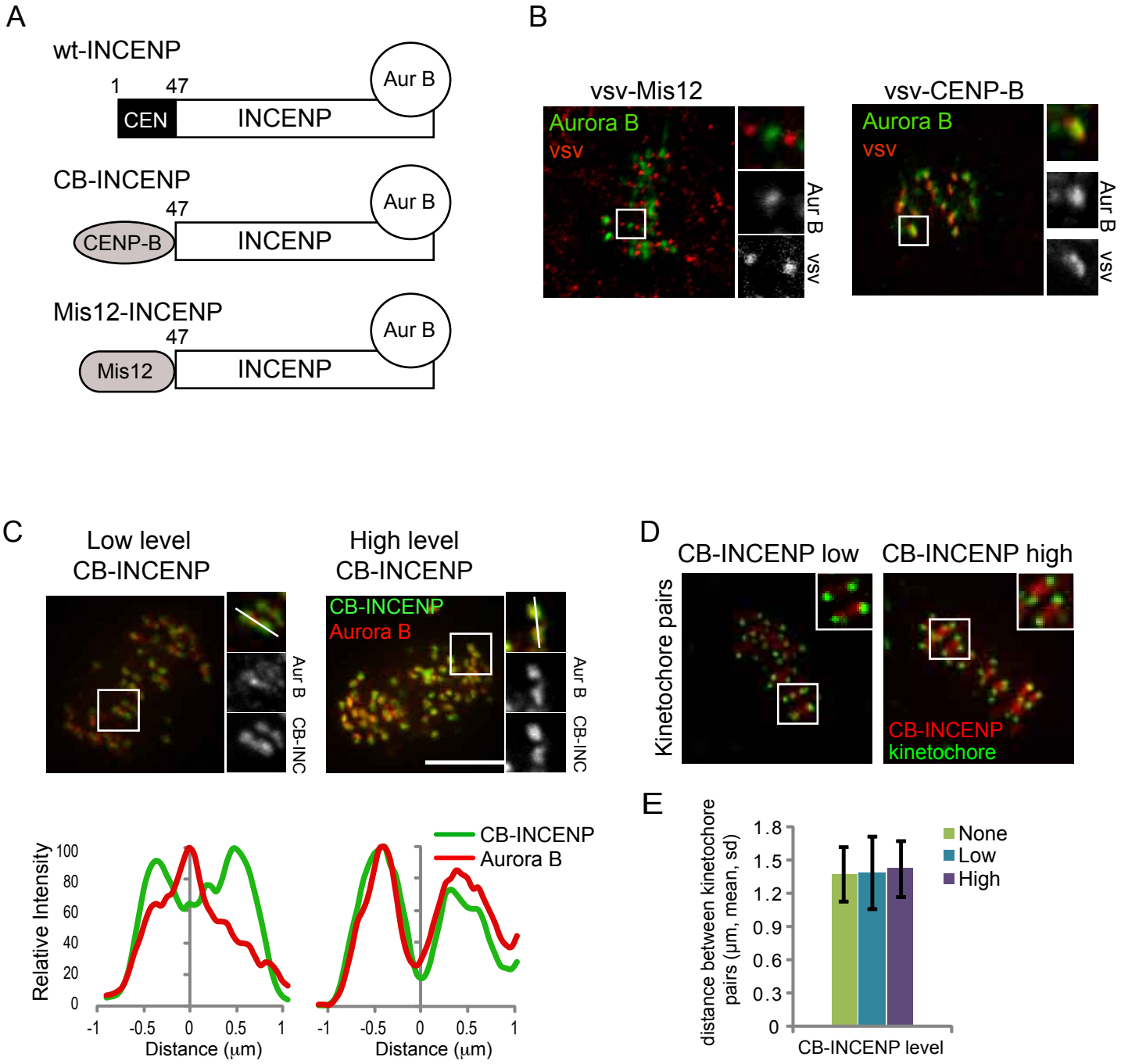
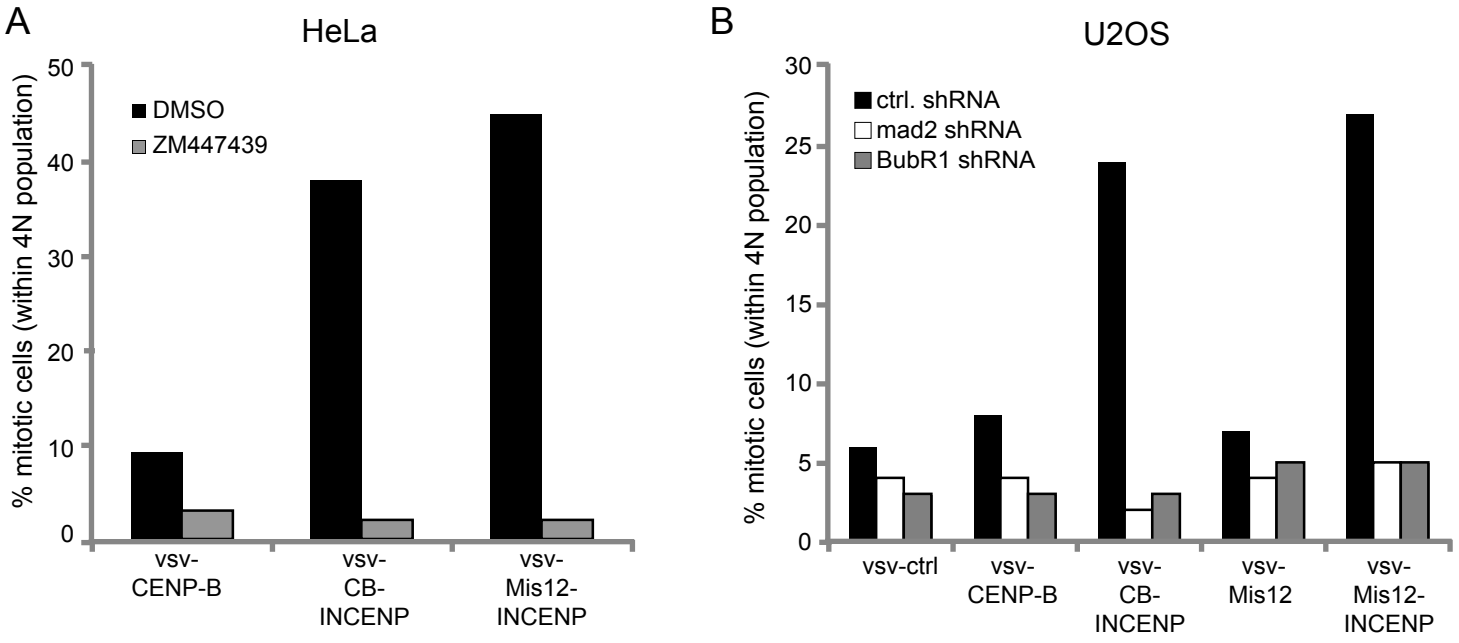
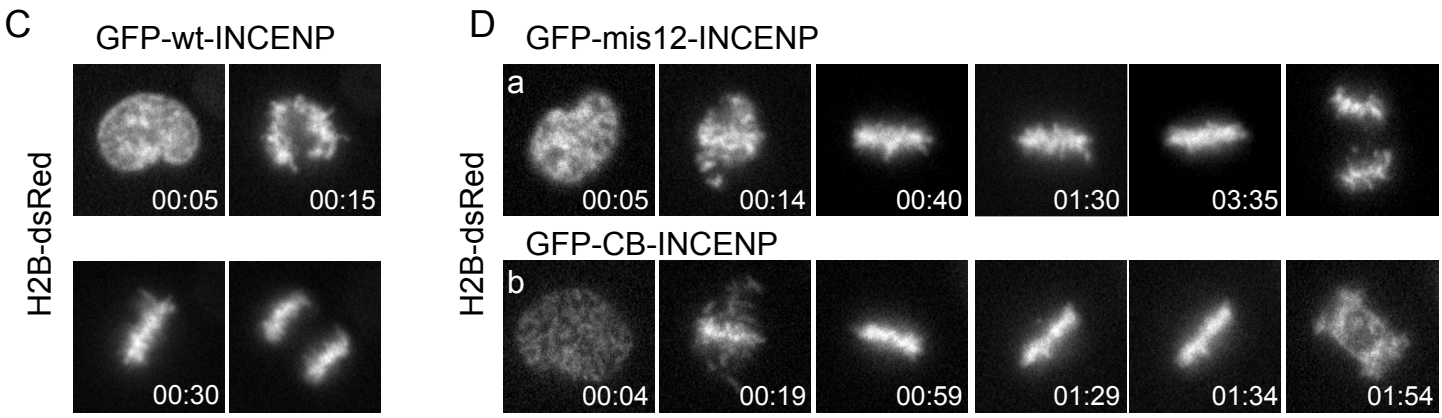
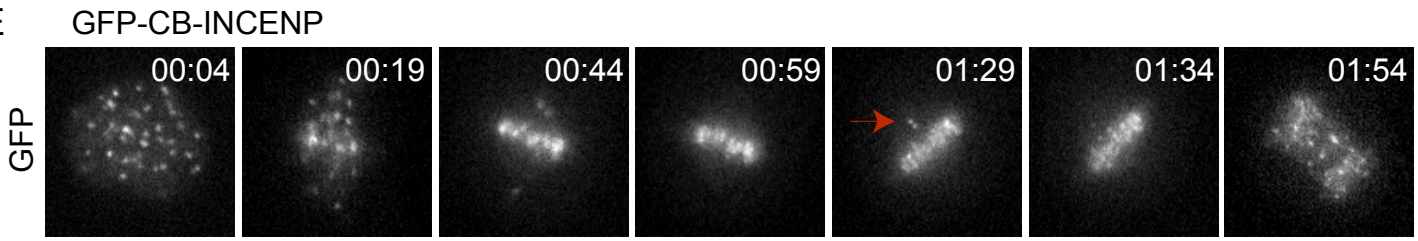

A
wt-INCENP
1
47
CEN
INCENP
Aur B
CB-INCENP
47
CENP-B
INCENP
Aur B
Mis12-INCENP
47
Mis12
INCENP
Aur B
B
vsv-Mis12
Aurora B
vsv
Aur B
vsv
vsv-CENP-B
Aurora B
vsv
Aur B
vsv
C
Low level
CB-INCENP
High level
CB-INCENP
CB-INCENP
Aurora B
Aur B
CB-INC
Relative Intensity
100
80
60
40
20
0
-1
-0.5
0
0.5
1
Distance (μm)
CB-INCENP
Aurora B
D
CB-INCENP low
CB-INCENP high
Kinetochore pairs
CB-INCENP
kinetochore
E
distance between kinetochore pairs (μm, mean, sd)
1.8
1.5
1.2
0.9
0.6
0.3
0
CB-INCENP level
None
Low
High

Fig. S3


A
HeLa
% mitotic cells (within 4N population)
DMSO
ZM447439
vsv-CENP-B
vsv-CB-INCENP
vsv-Mis12-INCENP
B
U2OS
ctrl. shRNA
mad2 shRNA
BubR1 shRNA
vsv-ctrl
vsv-CENP-B
vsv-CB-INCENP
vsv-Mis12
vsv-Mis12-INCENP


C
GFP-wt-INCENP
H2B-dsRed
00:05
00:15
00:30
D
GFP-mis12-INCENP
a
00:05
00:14
00:40
01:30
03:35
GFP-CB-INCENP
b
00:04
00:19
00:59
01:29
01:34
01:54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GFP-CB-INCENP
GFP
00:04
00:19
00:44
00:59
01:29
01:34
01:54

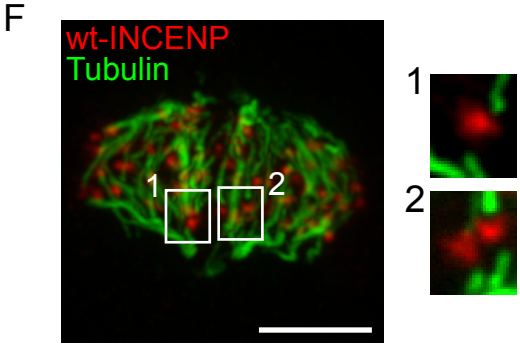
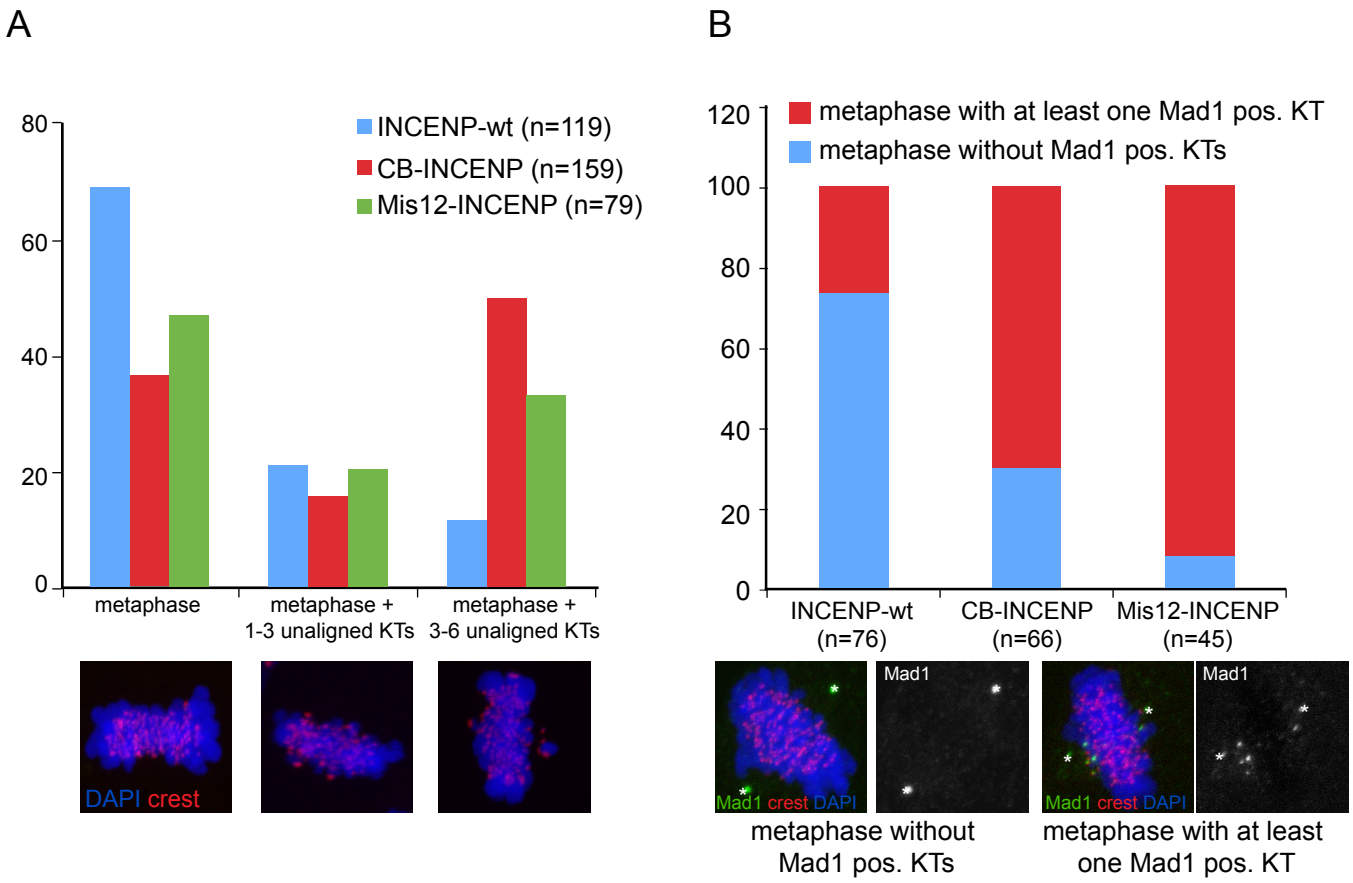

F
wt-INCENP
Tubulin
1
2

Fig. S4


A
80
60
40
20
0
INCENP-wt (n=119)
CB-INCENP (n=159)
Mis12-INCENP (n=79)
metaphase
metaphase +
1-3 unaligned KTs
metaphase +
3-6 unaligned KTs
DAPI crest
B
120
100
80
60
40
20
0
metaphase with at least one Mad1 pos. KT
metaphase without Mad1 pos. KTs
INCENP-wt
(n=76)
CB-INCENP
(n=66)
Mis12-INCENP
(n=45)
Mad1
Mad1 crest DAPI
Mad1
Mad1 crest DAPI
metaphase without
Mad1 pos. KTs
metaphase with at least
one Mad1 pos. KT

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