Supplemental Information

Figure S1, CENP-E T424 (*Xenopus*) is phosphorylated by Aurora A and B *in vitro*, Related to Figure 1

Figure S2, pT422 kinetochore staining is specific for CENP-E, Related to Figure 1

Figure S3, Imaging CENP-E single molecules, Related to Figure 2

Figure S4, CENP-E phosphorylation at T422 is required for chromosome alignment, Related to Figure 3.

Figure S5, CENP-E phosphorylation at T422 is required for chromosome alignment, Related to Figure 3.

Figure S6, Phosphorylation of T422 is not required for CENP-E's kinetochore localization, and an alternative mutation that prevents phosphorylation at CENP-E T422, Related to Figure 3.

Figure S7, Phosphorylation of CENP-E T422 is required for the congression of polar chromosomes, Related to Figure 4

Movie S1. Related to Figure 2

Movie S2. Related to Figure 3

Movie S3, Related to Figure 3

Movie S4, Related to Figure 4

Movie S5, Related to Figure 6

Movie S6, Related to Figure 6

Supplemental Information

Supplemental Figure Legends

Supplemental Figure 1. CENP-E T424 (*Xenopus*) is phosphorylated by Aurora A and B *in vitro*, Related to Figure 1 (A) Diagram of the *Xenopus* CENP-E motor fragments used as substrates in the *in vitro* kinase assay shown in (B). (B) Aurora A or Aurora B/INCENP kinase assays using various CENP-E motor fragments as substrates. Coomassie staining shows the purified protein and autoradiogram shows the incorporation of ^{32}P-γATP. (C) Phosphopeptide used to generate a rabbit polyclonal pT422 antibody. (D) Aurora A kinase assays performed using recombinant WT or T424A *Xenopus* CENP-E$^{1\text{-}473}$ as substrates. Samples taken at 0, 10 and 20 min time points were immunoblotted with the pT422 antibody.

Supplemental Figure 2. pT422 kinetochore staining is specific for CENP-E, Related to Figure 1 (A) HeLa cells were arrested with nocodazole for 3 hours and fixed and stained for CENP-E (Green), pT422 (Red), Bub1, and DNA (Blue). (B) Immunofluorescnce of nocodazole-arrested DLD-1 cells transfected with CENP-E siRNA. Cells were fixed and stained for CENP-E (Green), Bub1, pT422 (Red) and DNA (Blue). The cell on the left was not transected with the CENP-E siRNA and serves as an internal control.

Supplemental Figure 3. Imaging CENP-E single molecules, Related to Figure 2 (A) The experimental setup for imaging CENP-E single motors using TIRF microscopy. *Xenopus* CENP-E$^{1\text{-}473}$ was tagged with TagRFP-T, and Oregon Green 488-labeled GMPCPP microtubules were immobilized on a coverslip using a tubulin antibody. A low concentration of CENP-E$^{1\text{-}473}$-RFP was flowed into the chamber. (B) Kymograph showing microtubule-bound CENP-E$^{1\text{-}473}$-RFP in

the presence of apyrase. CENP-E$^{1-473}$-RFPs were photobleached in one or two steps 89% of the time (75 double steps and 68 single, out of 160 total).

Supplemental Figure 4. CENP-E phosphorylation at T422 is required for chromosome alignment, Related to Figure 3. (A) Immunoblot showing tetracycline inducible expression of various MycGFP-CENP-E transgenes. (B) Cells were transfected with control (GAPDH) or CENP-E siRNA and WT or T422A MycGFP-CENP-E was expressed for 24 hours. Cells were arrested with MG132 for 1.5 hours, fixed and processed for immunofluorescence. Representative immunofluorescence staining of cells. Green, GFP; Red, Tubulin. (C) Graph quantifying the proportion of cells with polar chromosomes. ***, $P < 0.0001$ by *t* test. (D) Graph showing the number of polar chromosomes in cells that displayed chromosome misalignment. Bars represent the mean of at least four independent experiments. Error bars represent the SEM.

Supplemental Figure 5. CENP-E phosphorylation at T422 is required for chromosome alignment, Related to Figure 3. (A, B) Endogenous CENP-E was replaced with either WT or T422A MycGFP-CENP-E and cells imaged by time-lapse microscopy every 3 minutes from nuclear envelope breakdown. Green, MycGFP-CENP-E; Red, histone H2B-mRFP. Stills are taken from Movie S2 and S3. Time is marked in the upper right of each panel and numbered arrowheads track the movement of individual kinetochore pairs. Arrow head 1 shows a mono-oriented kinetochore pair that moves to the pole of the spindle, while 2 and 3 show kinetochore pairs that successfully congress from the pole. Note, that a number of chromosomes remain stuck at the pole throughout the duration of imagining in cells expressing T422A MycGFP-CENP-E.

Supplemental Figure 6. Phosphorylation of T422 is not required for CENP-E's kinetochore localization, and an alternative mutation that prevents phosphorylation at CENP-E T422, Related to Figure 3. (A) Cells were transfected with control (GAPDH) or CENP-E siRNA and WT or T422A MycGFP-CENP-E expressed. Representative immunofluorescence images showing similar levels of WT or T422A MycGFP-CENP-E at the kinetochore. Green, GFP; Red, ACA; Blue, DAPI. (B) Quantitation of the fluorescence intensity of MycGFP-CENP-E at kinetochores. Bars represent the mean of at least 22 cells imaged in two independent experiments. Error bars represent the SEM. (C) Sequence alignment of human and *Xenopus* CENP-E showing the position of the T422A or T424A and RR:KK mutations. (D) *In vitro* kinase assays using Aurora B/INCENP to phosphorylate recombinant WT, T424A or RR:KK *Xenopus* CENP-E^{1-428} motor domain fragments for 0, 5, 10, 15, 20, 25, 30 and 60 minutes. Coomassie stained gel shows the purified protein and autoradiogram shows the incorporation of γ-^{32}P ATP. (E) WT, T422A or RR:KK MycGFP-CENP-E was immunoprecipitated from nocodazole-arrested DLD-1 cells with Myc antibody and immunoblotted with the pT422 antibody. (F) Box and whisker plots showing the time spent in mitosis for cells expressing MycLAP-CENP-E following transfection of control (GAPDH, green) or CENP-E (red) siRNA. 150 cells per condition are plotted from at least two independent experiments.

Supplemental Figure 7. Phosphorylation of CENP-E T422 is required for the congression of polar chromosomes, Related to Figure 4 (A) Endogenous CENP-E was replaced with either WT or T422A MycGFP-CENP-E and cells exposed to a drug treatment scheme to enrich mono- or syntelically oriented chromosomes near spindle poles. (B) and (C) Cells were released from the Aurora kinase inhibitor ZM and imaged every 3 minutes for 1.5 hours. Green, MycGFP-CENP-

E; Red, histone H2B-mRFP. Stills are taken from Movie S4. Time is marked in the upper right of each panel and numbered arrowheads track the movement of individual kinetochore pairs. Arrow head 1 shows a mal-oriented kinetochore pair that moves to the spindle pole and then congresses. Arrow heads 2, 3 and 4 mark kinetochore pairs that move from the pole towards the equator, but fail to congress. These movements may represent CENP-E-mediated lateral sliding along astral microtubules that subsequently undergo catastrophe.

Supplemental Movie Legends

Movie S1. (Left) Processive movements of CENP-E$^{1-473}$-TagRFP-T (green) along single, Oregon488-labeled GMPCPP-stabilized microtubules (red). (Right) Processive movements of CENP-E$^{1-473}$-TagRFP-T (green) phosphorylated by Aurora A, along GMPCPP-stabilized microtubules (red). 0.5 sec per frame displayed at 30 frames per second. Movie corresponds to kymographs shown in Figure 2E.

Movie S2. Histone H2B-mRFP (red) expressing cell in which endogenous CENP-E has been replaced with WT MycGFP-CENP-E (green). 3 min per frame displayed at two frames per second. Movie corresponds to Figure S5A.

Movie S3. Histone H2B-mRFP (red) expressing cell in which endogenous CENP-E has been replaced with T422A MycGFP-CENP-E (green). 3 min per frame displayed at two frames per second. Movie corresponds to Figure S5B.

Movie S4. (Left) Histone H2B-mRFP (red) expressing cell in which endogenous CENP-E has been replaced with WT MycGFP-CENP-E (green) Movie corresponds to Figure S7B. (Right) Histone H2B-mRFP (red) expressing cell in which endogenous CENP-E has been replaced with T422A MycGFP-CENP-E (green). Cell was treated with monastrol for 2 hours, released into ZM and MG132 for 1 hour and finally released into MG132 before imaging began. 3 min per frame displayed at two frames per second. Movie corresponds to Figure S7C.

Movie S5. Histone H2B-EYFP (pink) expressing HeLa cell microinjected with rhodamine-labeled rabbit IgG antibody (green). 5 min per frame displayed at two frames per second. Movie corresponds to Figure 6C.

Movie S6. Histone H2B-EYFP (pink) expressing HeLa cell microinjected with rhodamine-labeled pT422 antibody (green). 5 min per frame displayed at two frames per second. Movie corresponds to Figure 6C.

Extended Experimental procedures

Phosphopeptide antibody production and affinity purification

A synthetic phospho-peptide (KRKRRVpTWCLGK) based on the human CENP-E sequence flanking threonine 422 was generated using standard methods (Biomatik). The phospho-peptide was coupled to KLH (Keyhole limpet hemacyanin, Sigma) using EDC (1-ethyl-3-[3 dimethylaminopropyl] carbodiimide hydrochloride, Pierce) and injected into rabbits (Covance). Polyclonal pT422 antibodies were affinity-purified using the phosphopeptide coupled to a

HiTrap NHS-activated HP column (GE Healthcare). For immunofluorecnce and microninjection studies, the pT422 was conjugated to Rhodamine Red-X using standard methods.

Antibodies for Immunofluorescence

Staining was performed with the following antibodies: HpX (rabbit anti CENP-E, 1:200) (Yao et al., 1997), 1H12 (mouse monoclonal anti-CENP-E, Abcam, 1:500), ACA (human anti-centromeres, Antibodies Inc, 1:500;), DM1A (mouse anti-α-tubulin, 1:1000), SB1.1 (sheep anti-Bub1, 1:1000) (Taylor et al., 2001), SBR1.1 (Sheep anti-BubR1, 1:1000) (Taylor et al., 2001), Cy5-conjugated hKNL (1 μg/ml) (Cheeseman et al., 2008) and Rhodamine Red-X-conjugated anti-pT422 (2.5 μg/ml).

Immunoblots and immunoprecipitations

For immunoblot analysis, protein samples were separated by SDS-PAGE, transferred onto nitrocellulose membranes (BioRad) and then probed with the following antibodies: 4A6 (mouse anti-Myc, Upstate, 1:1000), HpX (rabbit anti CENP-E, 1:200) (Yao et al., 1997), DM1A (mouse anti-α-tubulin, 1:1000), P-Histone H3 (mouse anti pS10-Histone H3, Cell Signaling, 1:1000), P-TACC3 (rabbit anti pS558-TACC3, a kind gift of Patrick Eyers, the University of Sheffield, UK, 1:1000), anti-pT422 (1μg/ml), and PP1 (mouse monoclonal E-9, Santa Cruz, 1:200). For immunoprecipitations, cells were lysed in lysis buffer (10 mM Tris pH 7.5, 0.1% triton X-100, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 20 mM β-glycerophosphate, 0.1 mM DTT plus protease inhibitors), sonicated and soluble extracts prepared. Anti-Myc (4A6) antibodies were added to the supernatant and retrieved using protein A/G beads (Santa Cruz biotechnology). Beads were washed 5 times in lysis buffer prior to analysis. λ-phosphatase

treatment was performed as described previously (Holland and Taylor, 2006).

Live cell microscopy

For high-resolution spinning-disk fluorescence microscopy, cells were plated on poly-L-lysine coated 35 mm glass-bottomed tissue culture dishes (MatTek Inc). 24-48 hours later cells were transferred to CO_2 independent media (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 units U/ml streptomycin and 2 mM L-glutamine and placed in a heat-controlled stage set at 37°C. Cells were imaged using a 60× 1.4 NA PlanApochromat oil lens on a spinning disk confocal mounted on a Nikon TE2000-E inverted microscope equipped with a krypton-argon 2.5 W water-cooled laser (Spectra-Physics). Images were acquired on a Hamamatsu Orca ER CCD camera controlled by MetaMorph software (Molecular Devices). 5 X 2 μM z sections were acquired at 3 min time intervals for GFP and RFP and maximum intensity projection created using MetaMorph. Movies were assembled and analyzed using QuickTime™ (Apple).

To determine mitotic timing, cells were seeded into 8 well poly-L-lysine coated coverglass chamber slides (Nunc) and 24 hours later transferred to supplemented CO_2 independent media. Cells were maintained at 37°C in an environmental control station and images collected using a Deltavision RT system with a 40× 1.35 NA oil lens at 5 min time intervals. For each time point, 4 X 2.5 μM z sections were acquired for RFP and maximum intensity projection created using softWoRx. Movies were assembled and analyzed using QuickTime™ (Apple).

Microinjection

HeLa cells stably expressing H2B-YFP were plated on poly-L-lysine coated 35 mm glass-bottomed tissue culture dishes (MatTek Inc). 48 hours later cells were transferred to CO_2 independent media (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 units U/ml streptomycin and 2 mM L-glutamine. Cells were placed on a Nikon Eclipse 300 inverted microscope and viewed with phase contrast using a 20× 0.4 NA long working distance objective. Microinjections were performed using an Eppendorf FemtoJet/InjectMan NI 2 system. The cytoplasm of interphase cells were microinjected to a maximum of ~10% of their volume with either Rhodamine Red-X-conjugated pT422 antibody or Rhodamine Red-X-conjugated Rabbit IgG (Jackson ImmunoReserach). Antibodies were injected at 1 mg/ml in microinjection buffer (10 mM Na_2PO_4, pH 7.4, 100 mM KCl, 1 mM $MgCl_2$). Injected cells were transferred to a Deltavision RT system and progression through mitosis was monitored by live cell microscopy as described above. For Figure 7B, imaging of the localization of Rhodamine Red-X-conjugated pT422 antibody was obtained using a 100× 1.4 NA PlanApochromat oil lens on a spinning disk confocal mounted on a Nikon TE2000-E as described above. 15 X 0.5 μM z sections were acquired for GFP and RFP and maximum intensity projection created using MetaMorph.

Protein purification

Recombinant CENP-E^{1-473}-TagRFP-T and CENP-E^{1-473}-Myc-6His proteins were expressed in *E.coli* [strain Rosetta (DE3)] and purified as previously described (Kim et al., 2008; Wood et al., 1997). 6His-tagged *Xenopus* Aurora A was expressed from pET28 vector and purified using Ni-NTA agarose (Qiagen). *Xenopus* Aurora B and full-length INCENP were co-expressed from a bicistronic vector in *E.coli* [strain BL21(DE3) pLys] and purified as described (Rosasco-Nitcher et al., 2008). 6His-tagged *Xenopus* PP1γ was purified using Ni-NTA agarose (Qiagen) from

uninduced *E.coli* [strain Rosetta(DE3)] grown for 48 hours at room temperature in SOB medium supplemented with 1 mM $MnCl_2$.

In vitro kinase and phosphatase assays

In vitro kinase assays were performed at room temperature in 20 mM Tris pH 7.5, 25 mM KCl, 1 mM $MgCl_2$, 1 mM DTT in the presence of 0.2 mM MgATP and 50 μCi/ml γ^{-32}P ATP. 2 μM of CENP-E^{1-473}-myc-6His was incubated with 0.2 μM of bacterially purified Aurora A or B at room temperature for 30 min, with the exception of kinetics studies. Kinase reactions were stopped with sample buffer and analyzed by SDS-PAGE. For quantification, individual bands were excised and the incorporation of ^{32}P was quantified using a scintillation counter (Beckman).

In vitro phosphatase assay was performed at room temperature by adding either 2 μM of purified PP1γ or 2 μM of PP1γ preincubated with 5 μM of Microcystin-LR (EMD) for 10 min, into the kinase reaction containing CENP-E^{1-473}-myc-6His (2 μM) and Aurora A (0.5 μM). The phosphatase reaction buffer was supplemented with $MnCl_2$ and KCl to have 25 mM Tris, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 0.1 mM MgATP final. The phosphatase reaction was stopped with sample buffer and analyzed by immunoblot using pT422 antibody.

In vitro pulldown experiments

5 μM of bacterially purified PP1γ was mixed with 5 μM, 10 μM, or 25 μM of wild-type or W425A CENP-E^{1-473} in 50 mM Tris pH 7.5, 150 mM KCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.1 mM MgATP, 1 mM DTT, 0.05% NP-40, 10% Glycerol and incubated for 30 min at room temperature and additional 30 min following addition of Microcystin-agarose (Millipore). PP1-

bound complexes were washed five times in binding buffer and eluted from the beads in SDS sample buffer.

ATPase assays

Steady-state ATPase rate was measured at room temperature in a coupled enzyme system based on 2-amino-6-mercapto-7-methyl-purine riboside (MESG) and purine nucleoside phosphorylase (PNP) (EnzChek Phosphate Assay Kit, Invitrogen). 2 μM of freshly prepared protein was pre-incubated with or without 0.2 μM of active Aurora A for 30 min at room temp and the reaction was kept on ice until use. To measure ATPase rate, 20 nM of CENP-E was incubated with varying concentrations of microtubules (0 – 4 μM) in assay buffer (10 mM Imidazole pH 6.8, 10 mM $MgCl_2$, 5 mM K-acetate, 4 mM Mg-acetate, 2 mM EGTA, 0.1 mM bovine serum albumin, 2 mM ATP) and 0.2 mM MESG and 1 U/ml of PNP. Prism (Graphpad) was used for hyperbola curve fitting to derive k_{cat} and K_mMT.

Microtubule pelleting assays

Assembly of taxol-stabilized microtubules and measurement of polymerized tubulin concentration were performed as described (http://mitchison.med.harvard.edu/protocols.html). 2 μM of wild-type or T424A *Xenopus* CENP-E^{1-473} was incubated at room temperature with or without 0.2 μM of Aurora A for 15 min in BRB80 buffer (80 mM K-PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) with 0.1 mM MgATP. CENP-E^{1-473} was subsequently mixed with varying concentrations of microtubules in the presence of 2 mM MgADP and incubated for 15 min at room temperature. 40 μl of the reaction was layered onto 100 μl of BRB80 + 40% glycerol and sedimented for 5 min at 90,000 rpm in a TLA100 rotor. 20 μl was removed from the top for the

supernatant sample and the remaining supernatant was aspirated. 40 µl of BRB80 containing 5 mM $CaCl_2$ was added to the pellet for 10 min on ice. Equivalent volumes of supernatant and pellet fractions were analyzed by SDS-PAGE.

Supplemental References

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Supplemental Figure

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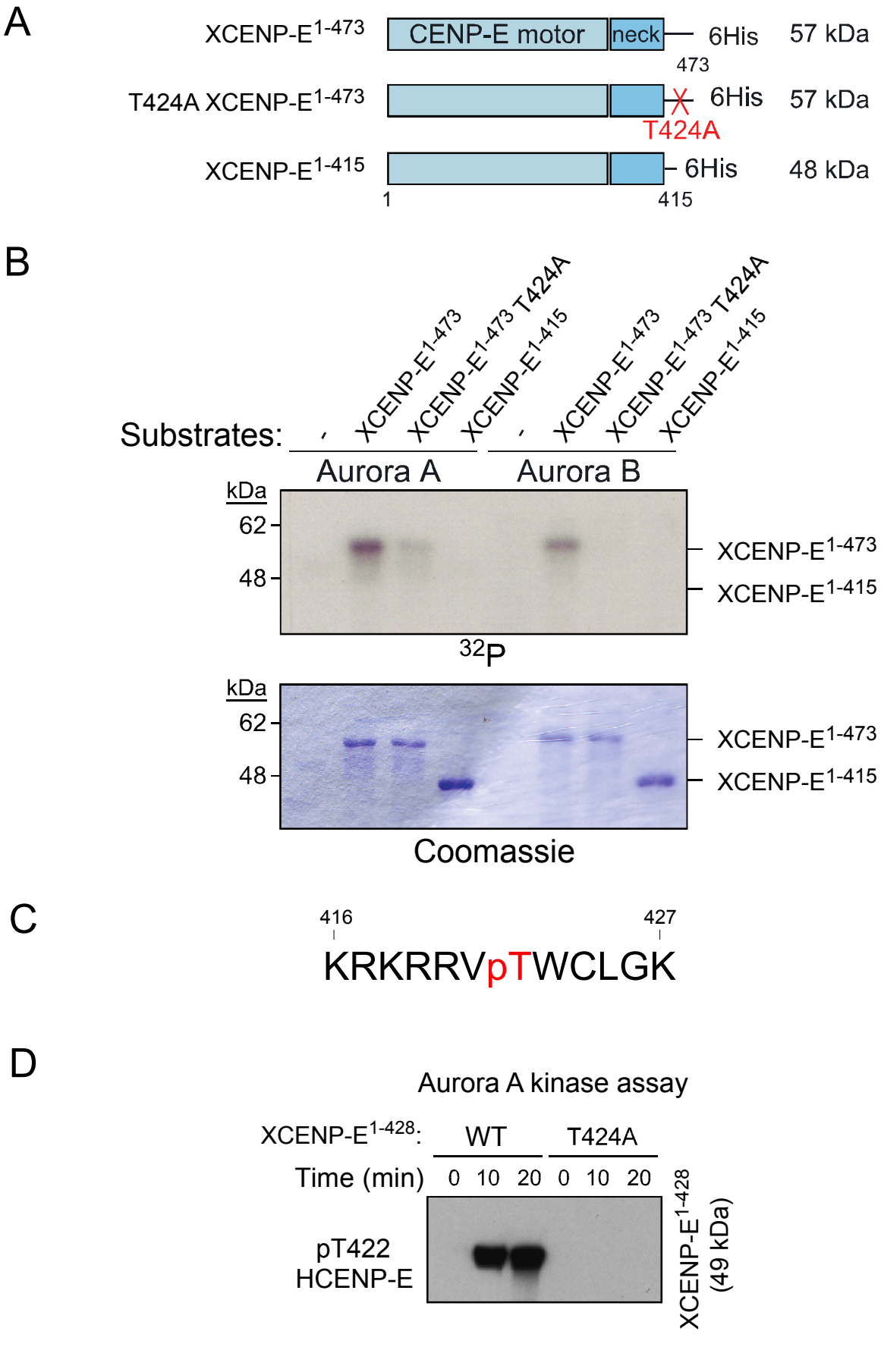

Figure S1

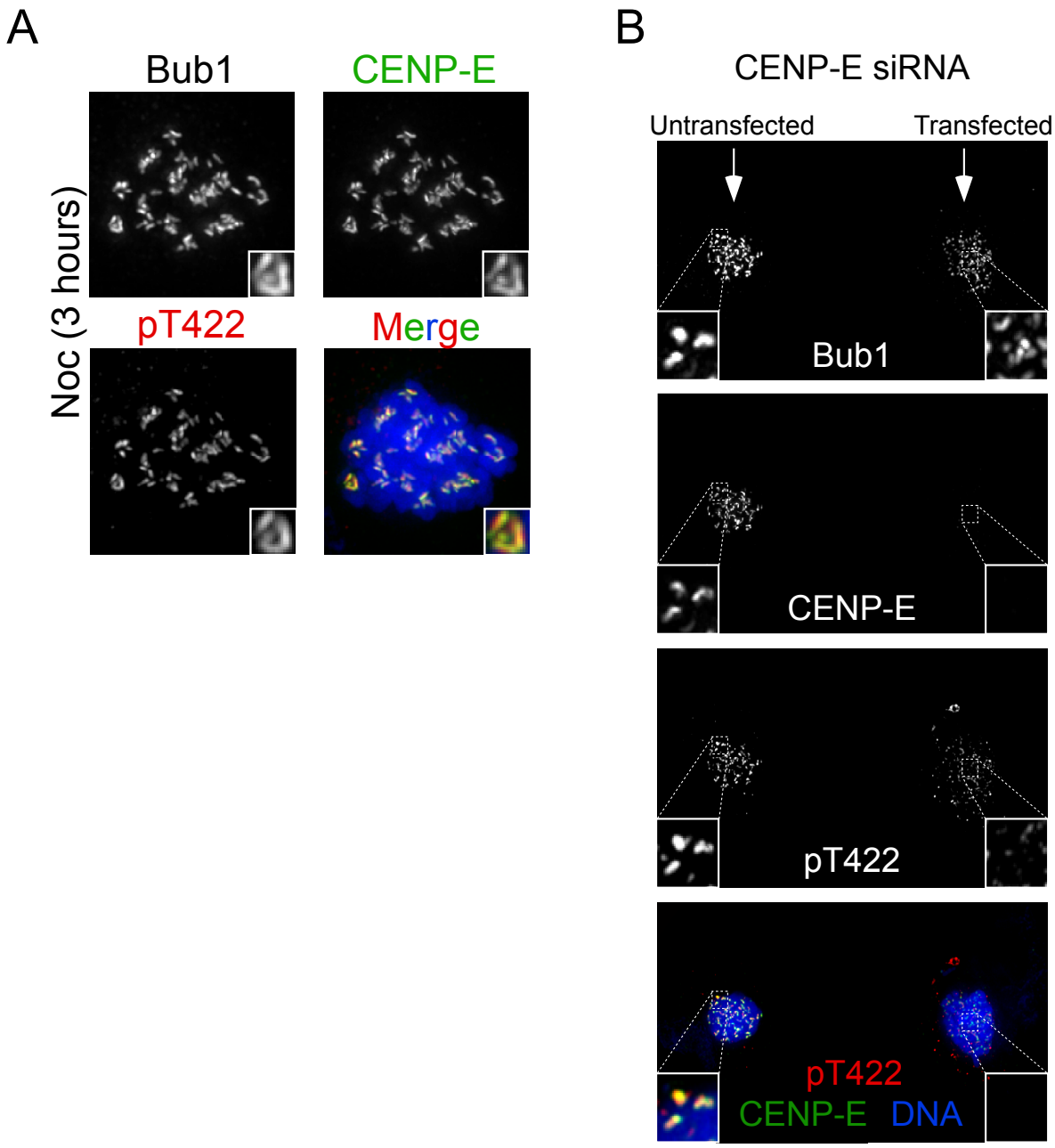
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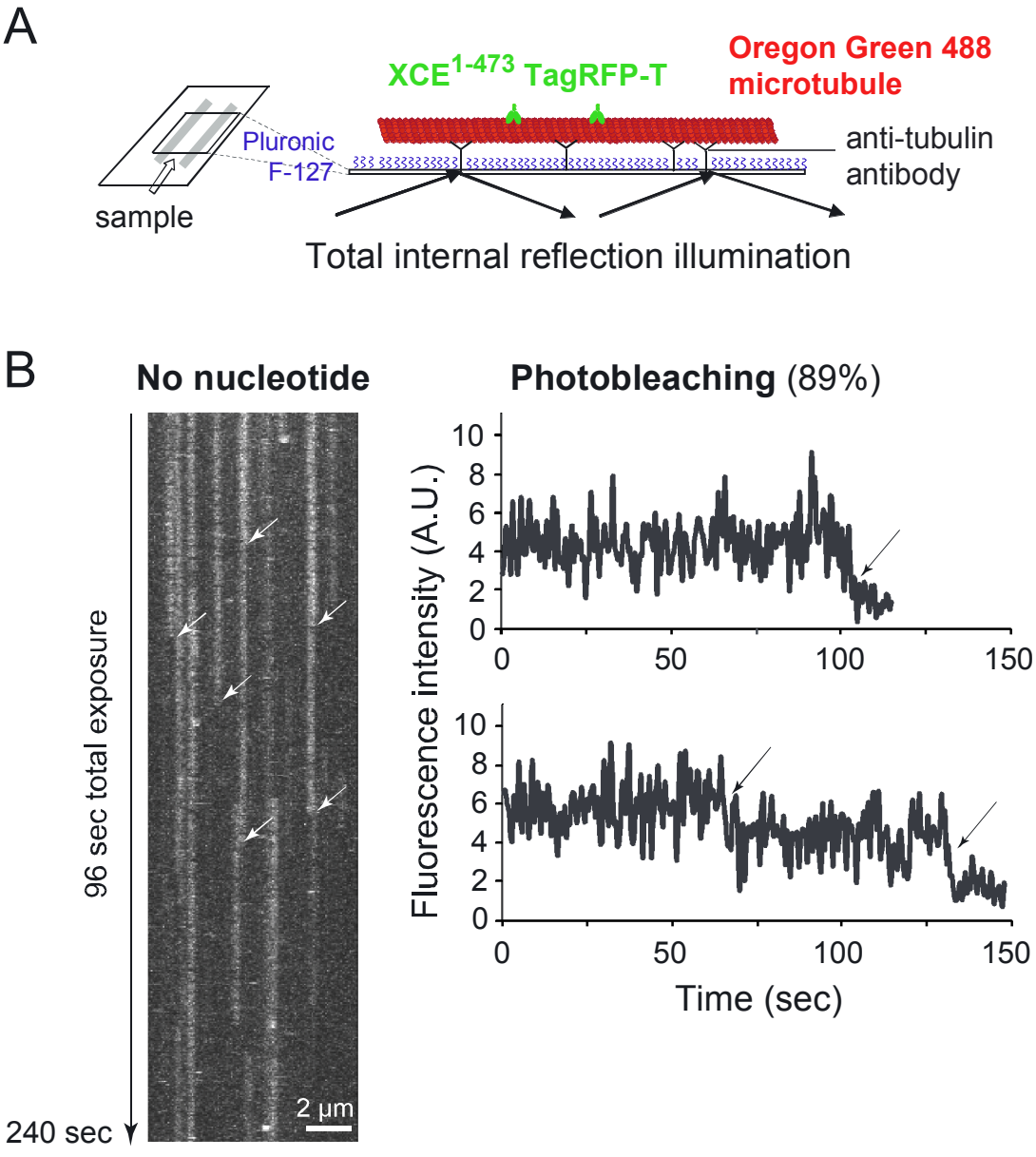
Figure S2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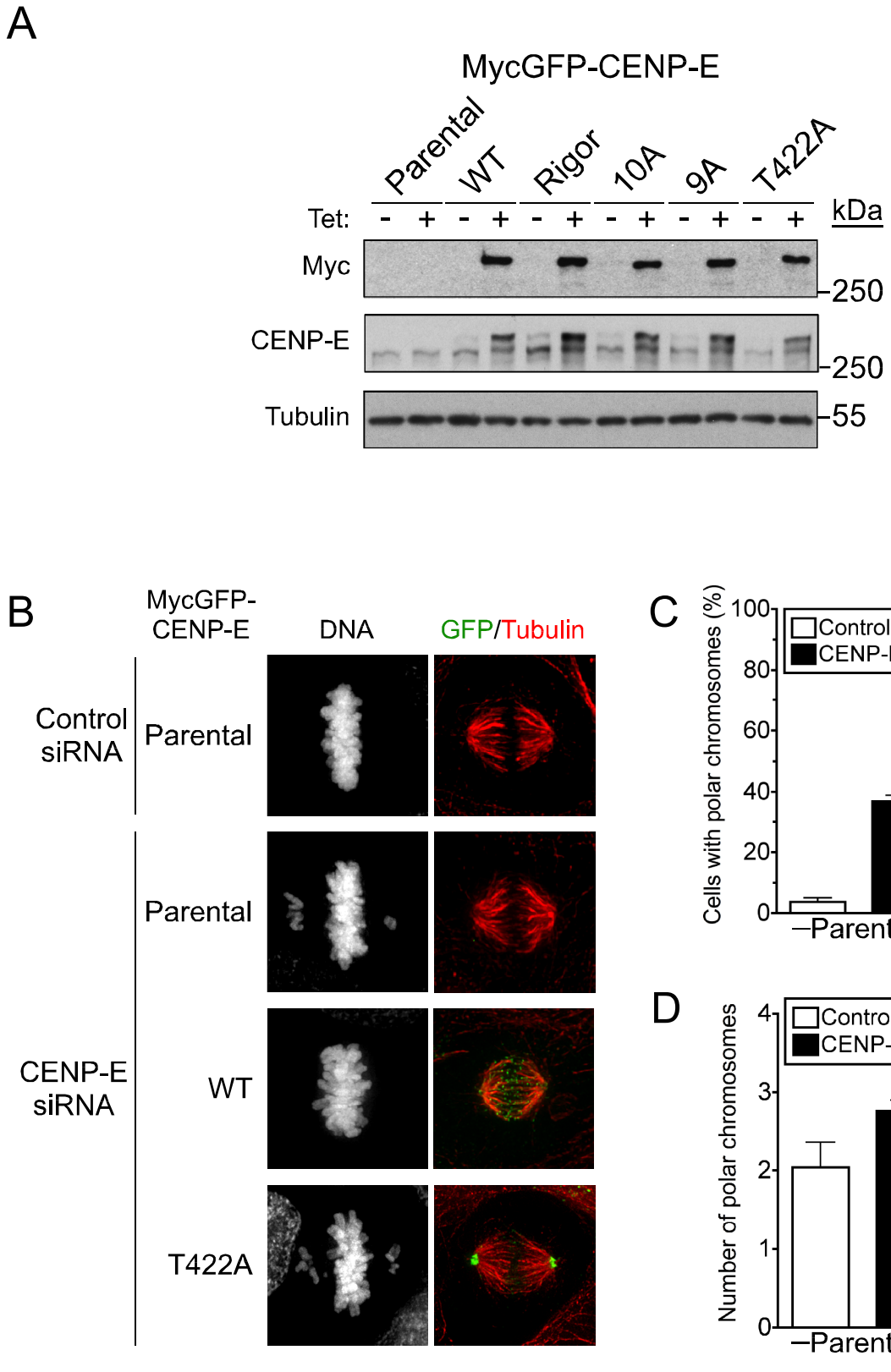
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250
Tubulin
55
B
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DNA
GFP/Tubulin
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CENP-E siRNA
Parental
WT
T422A
C
Control siRNA
CENP-E siRNA
***
Cells with polar chromosomes (%)
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60
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20
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Figure S4

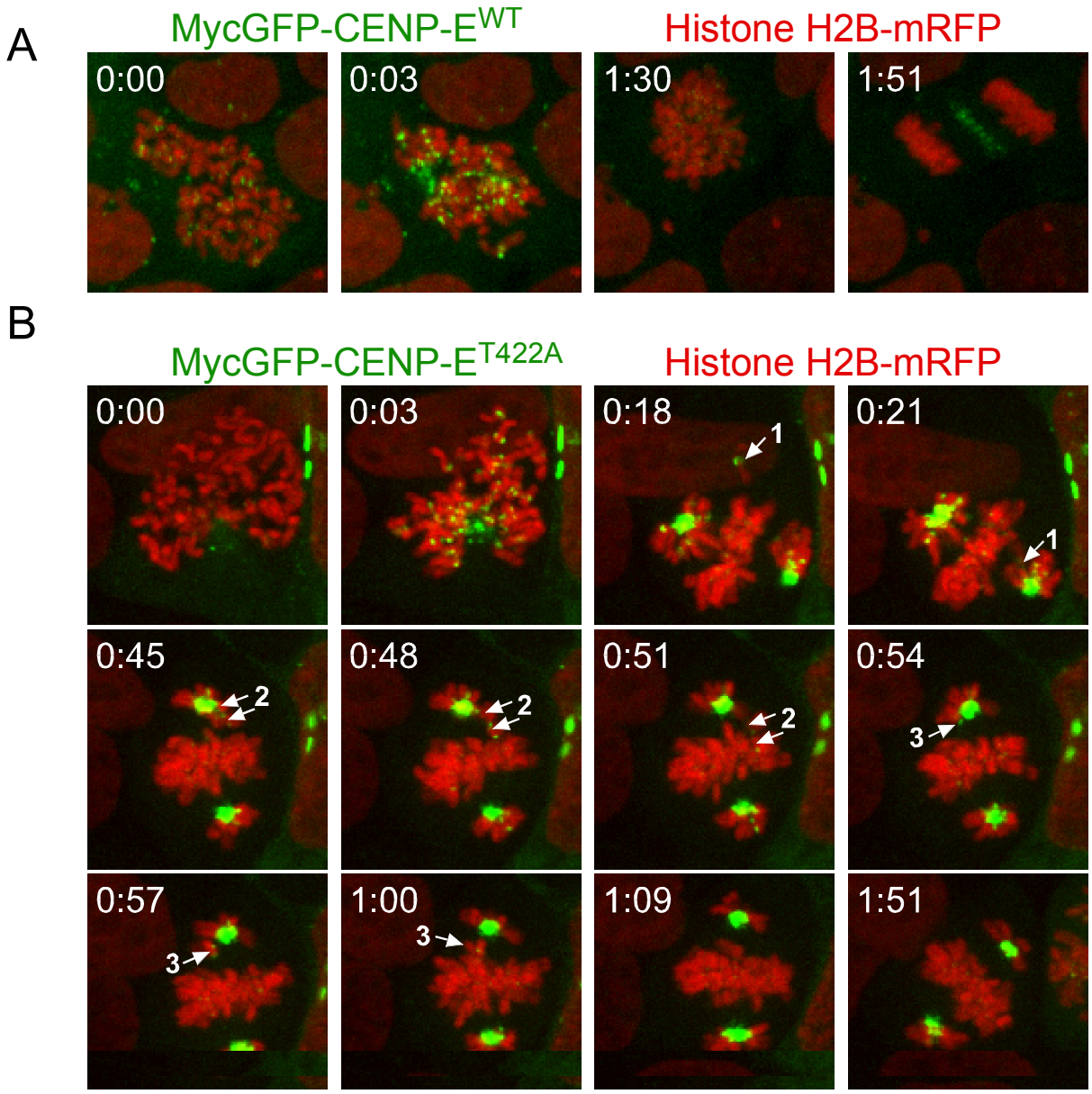
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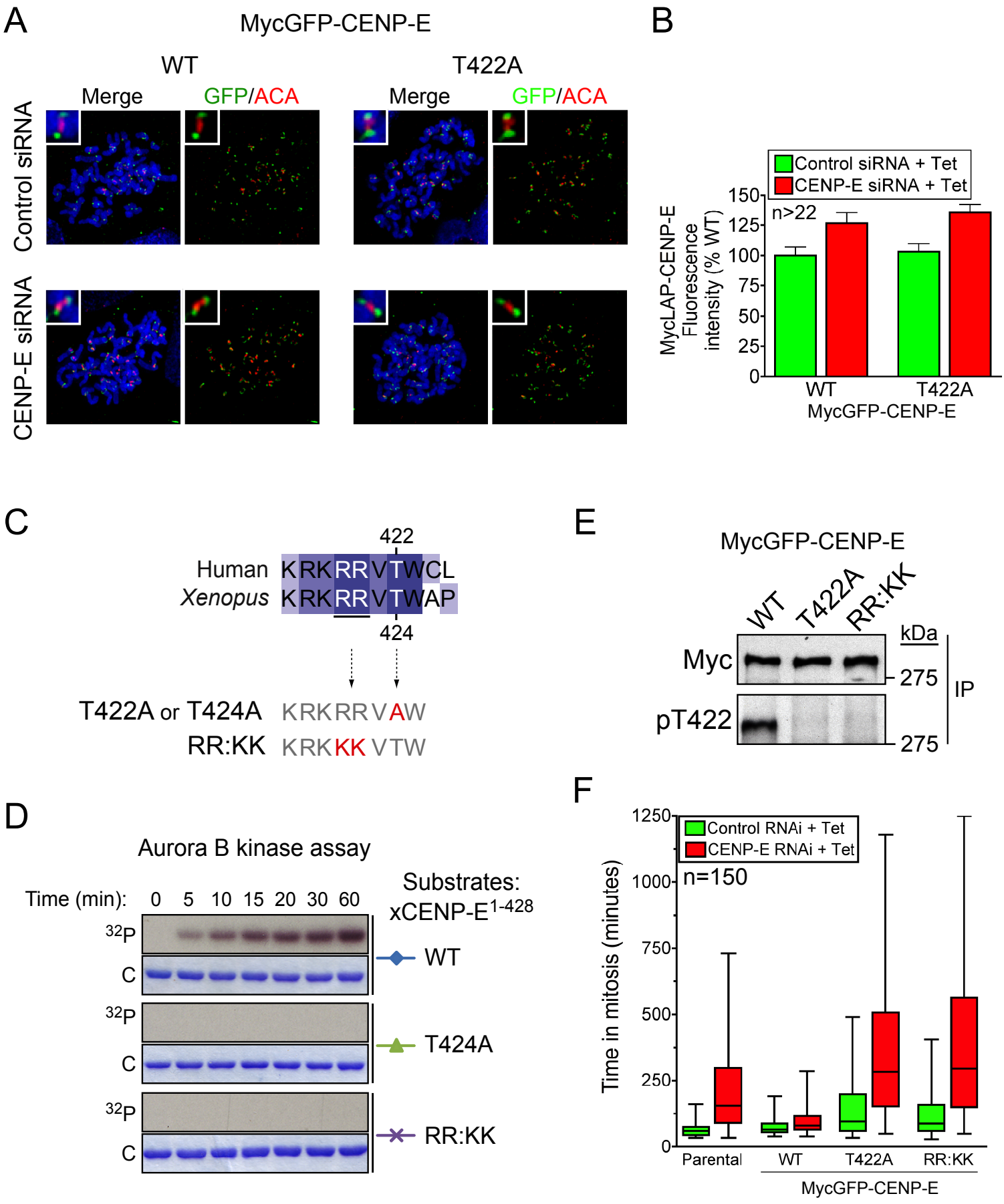
Figure S5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125
100
75
50
25
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C
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424
T422A or T424A KRKRRVAW
RR:KK KRKKKVTW
D
Aurora B kinase assay
Time (min): 0 5 10 15 20 30 60
Substrates:
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32P
C
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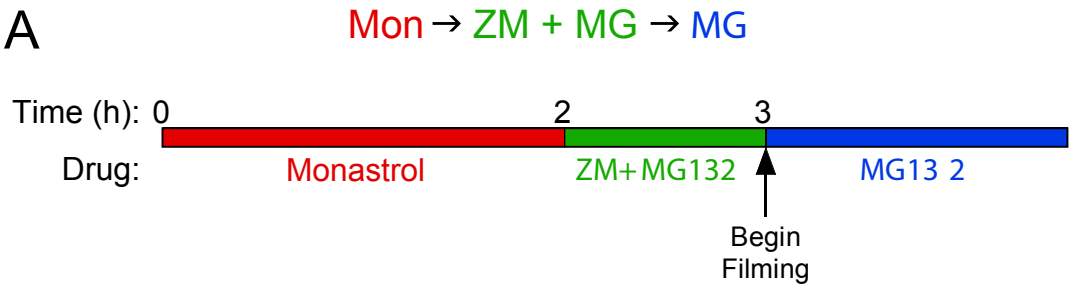
Figure S6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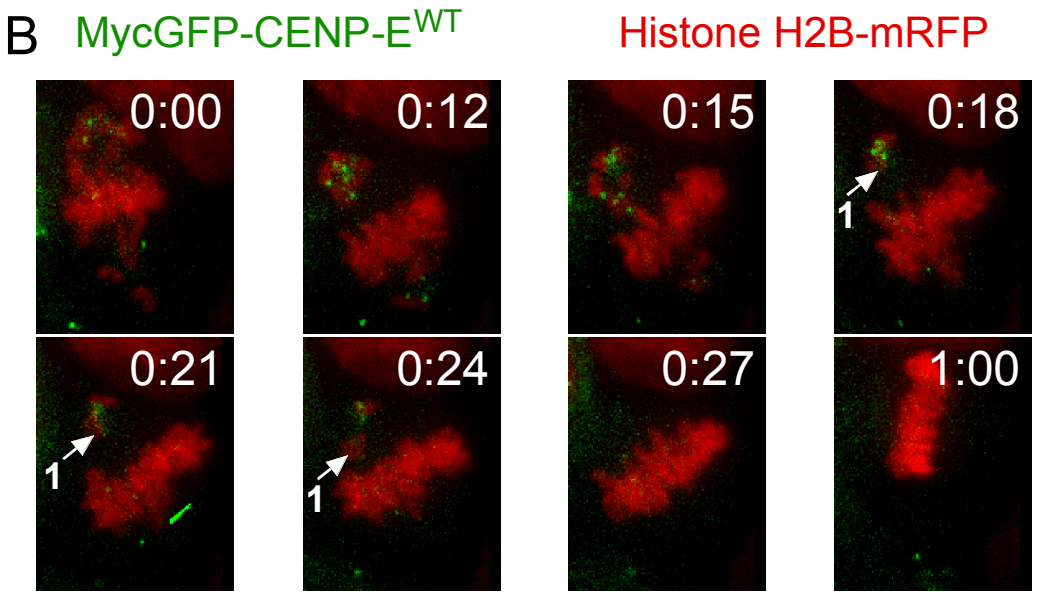
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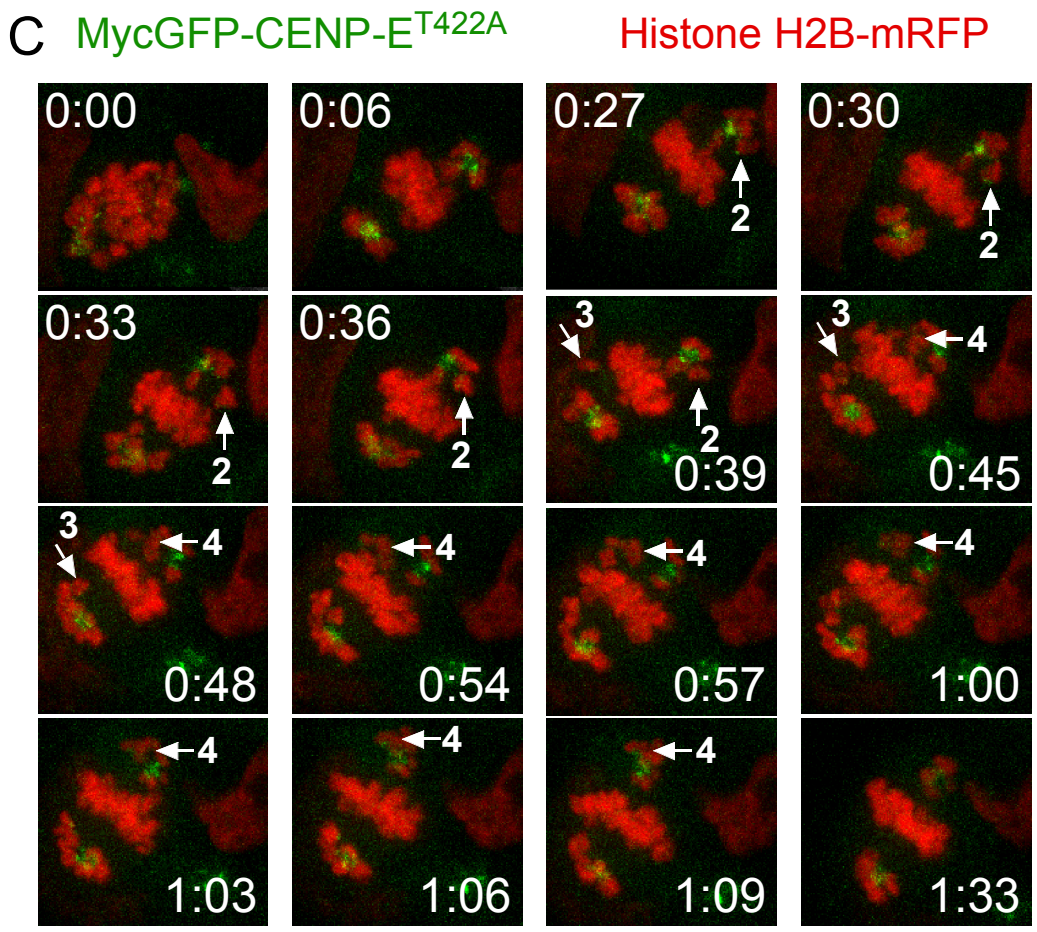
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0:57
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1:06
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1:09
4
1:33

Figure S7