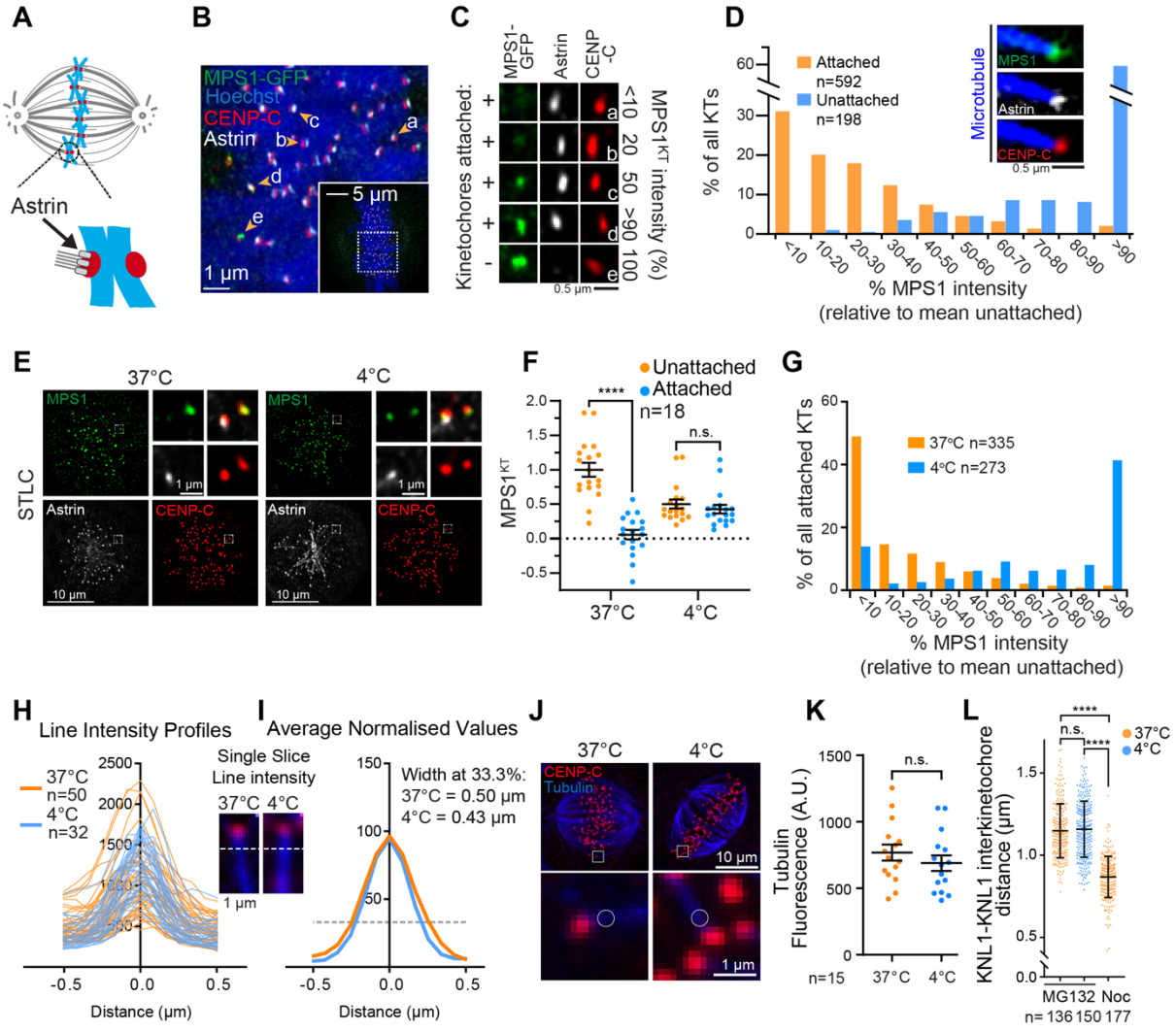

Figure S1. MPS1 can be observed at end-on attached kinetochores. Related to Figure 1. (**A**) Diagram of a late prometaphase cell with a monotelically attached chromosome. (**B**) A late prometaphase HeLa MPS1-GFP cell, stained as indicated. Arrows with letters indicate kinetochores displayed in (C). (**C**) Attached and unattached kinetochores from (B) with varying levels of MPS1. (**D**) Histogram of attached kinetochores (Astrin +ve) and unattached kinetochores in prometaphase cells with the indicated MPS1-GFP intensities relative to MPS1-GFP levels at unattached kinetochores. The inset shows an example of an attached kinetochore with high levels of GFP-MPS1. K-fibers were visualized by anti-HURP staining. (**E**) STLC-arrested HeLa MPS1-GFP cells were either fixed directly or cold-treated for 9 mins before fixation and staining with the indicated antibodies. (**F**) Cell average kinetochore-MPS1 intensities from (E), normalized to the mean unattached signal for the given temperature. Bars represent mean ± S.E.M. (**G**) Histogram of MPS1 intensities at attached kinetochores in control or cold-treated cells. (**H**) Raw tubulin intensities (A.U.) across a 1 µm line drawn 0.5 µm from the kinetochore center and perpendicular to the microtubule K-fiber in metaphase cells at 37°C or cold-treated for 9 min. Example immunofluorescence images are shown with Tubulin (blue) and CENP-C (red). (**I**) Average normalized line profiles from (H) with values at 0.0 µm normalized to 100%. (**J**) Metaphase HeLa cells immunostained for Tubulin (blue) and CENP-C (red) at 37°C or cold-treated for 9 min. (**K**) Cell average Tubulin intensity at K-fibers taken 0.5 µm away from kinetochores as shown in (J). Bars represent mean ± S.E.M. (**L**) The interkinetochore distances (KNL1 to KNL1) of control or cold-treated MG132-arrested cells or nocodazole-treated cells were plotted. The bars represent mean ± S.D.

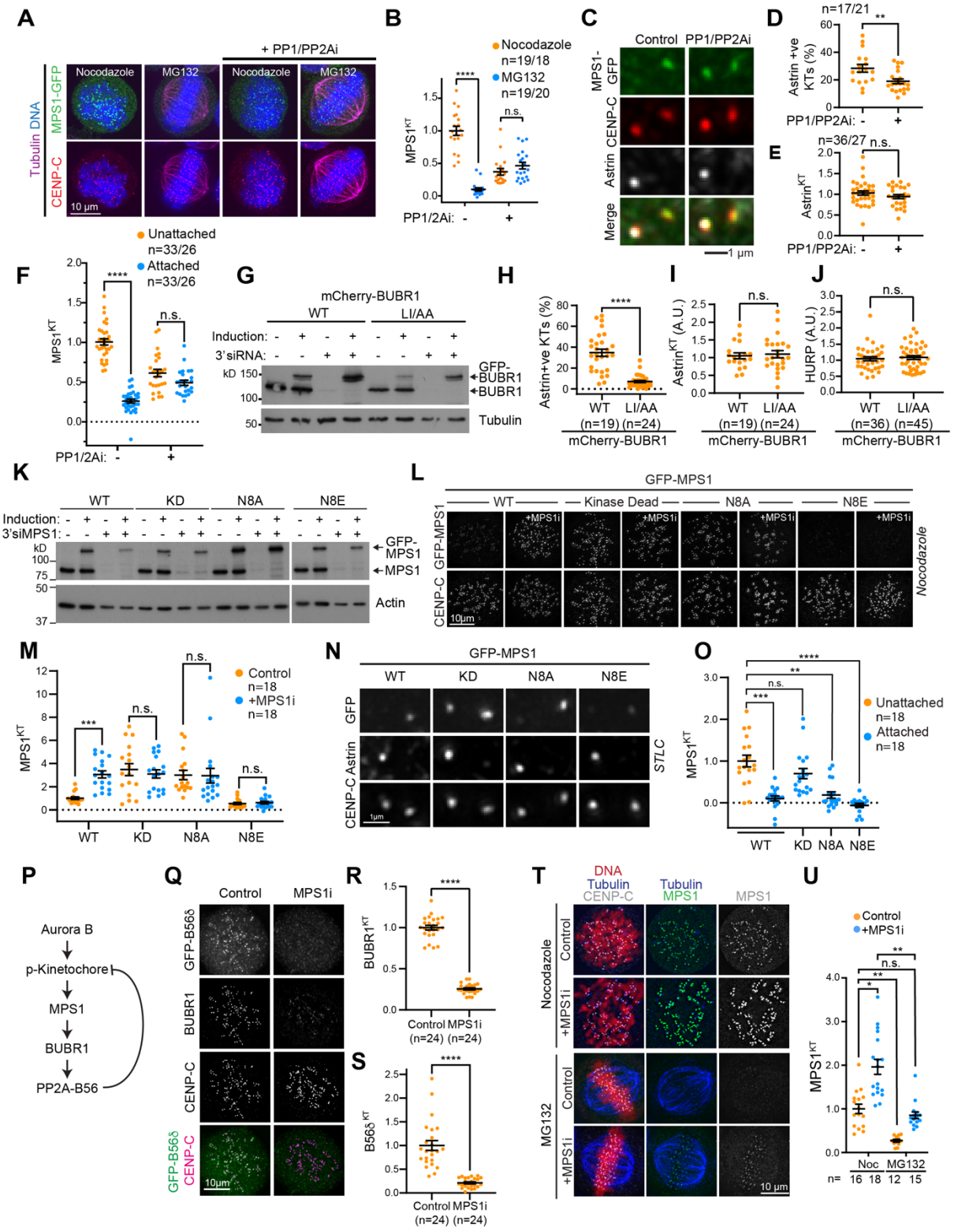

Figure S2. **Influence of kinetochore PP2A-B56 or MPS1 N-terminal autophosphorylations on MPS1 recruitment**. **Related to Figure 2.** (**A**) Extended version of Figure 2A. HeLa MPS1-GFP cells were arrested with either Nocodazole or MG132 and then briefly treated with calyculin A (PP1/2Ai) and immuno-stained as indicated. (**B**) Cell averages of kinetochore-MPS1 in cells in (A). Bars represent mean± S.E.M. Values were normalized to levels of MPS1 at unattached kinetochores without calyculin A treatment. (**C**) STLC arrested HeLa MPS1-GFP cells treated with calyculin A were immunostained as indicated. Representative monotelic kinetochore pairs are shown. (**D**) The per cell proportion of attached (Astrin +ve) kinetochores in cells in (C). Bars indicate mean± S.E.M. (**E**) Average cell Astrin intensities at attached kinetochores in cells in (C). Bars show mean± S.E.M. (**F**) Cell average intensities

of kinetochore-MPS1 at unattached and attached kinetochores in cells in (C). Values were normalized to the mean of the unattached control. Bars show mean± S.E.M. (**G**) Representative BUBR1 western blot of HeLa Flp-In TREx cells expressing mCherry-BUBR1^{WT} or mCherry-BUBR1$^{LI/AA}$ demonstrating the depletion of endogenous BUBR1 and induction of the transgenes for the cells used in Figure 2C. Tubulin is a loading control. (**H**) The proportion of attached (Astrin +ve) kinetochores, in cells in Figure 2C. (**I**) Cell average Astrin intensities at attached kinetochores in cells in Figure 2C. Bars show mean± S.E.M. (**J**) Cell averages of K-fiber HURP intensities in STLC arrested cells in Figure 2C. Values were normalized to the mean of the WT control. (**K**) Anti-MPS1 Western blot of HeLa Flp-In TREx cells treated with siMPS1 or siControl ± GFP-MPS1 transgene expression. Actin is a loading control. (**L**) HeLa Flp-In TREx cells depleted of endogenous MPS1 and expressing GFP-MPS1 mutants arrested with nocodazole and MG132 and treated with or without MPS1i were immuno-stained as indicated. (**M**) Cell averages of MPS1 kinetochore intensity from (L). Bars show mean ± S.E.M. (**N**) Monotelic kinetochore pairs from HeLa Flp-In TREx cells depleted of endogenous MPS1 and expressing GFP-MPS1 mutants, arrested with STLC and MG132. (**O**) Cell averages of MPS1 kinetochore intensities from cells in (N). Bars show mean± S.E.M. (**P**) Schematic diagram depicting negative feedback of PP2A-B56 on MPS1 recruitment (**Q**) Control or MPS1i treated HeLa-Flp-In TREx cells expressing PP2A-B56δ, stained as indicated. (**R**) Plot of kinetochore BUBR1 intensities or (**S**) kinetochore PP2A-B56δ intensities of cells in (Q). Bars show mean ± S.E.M. (**T**) MPS1-GFP cells arrested with nocodazole and MG132 or MG132 alone, treated with or without MPS1i. (**U**) Plot of MPS1 kinetochore intensities in (T). Bars indicate mean± S.E.M.

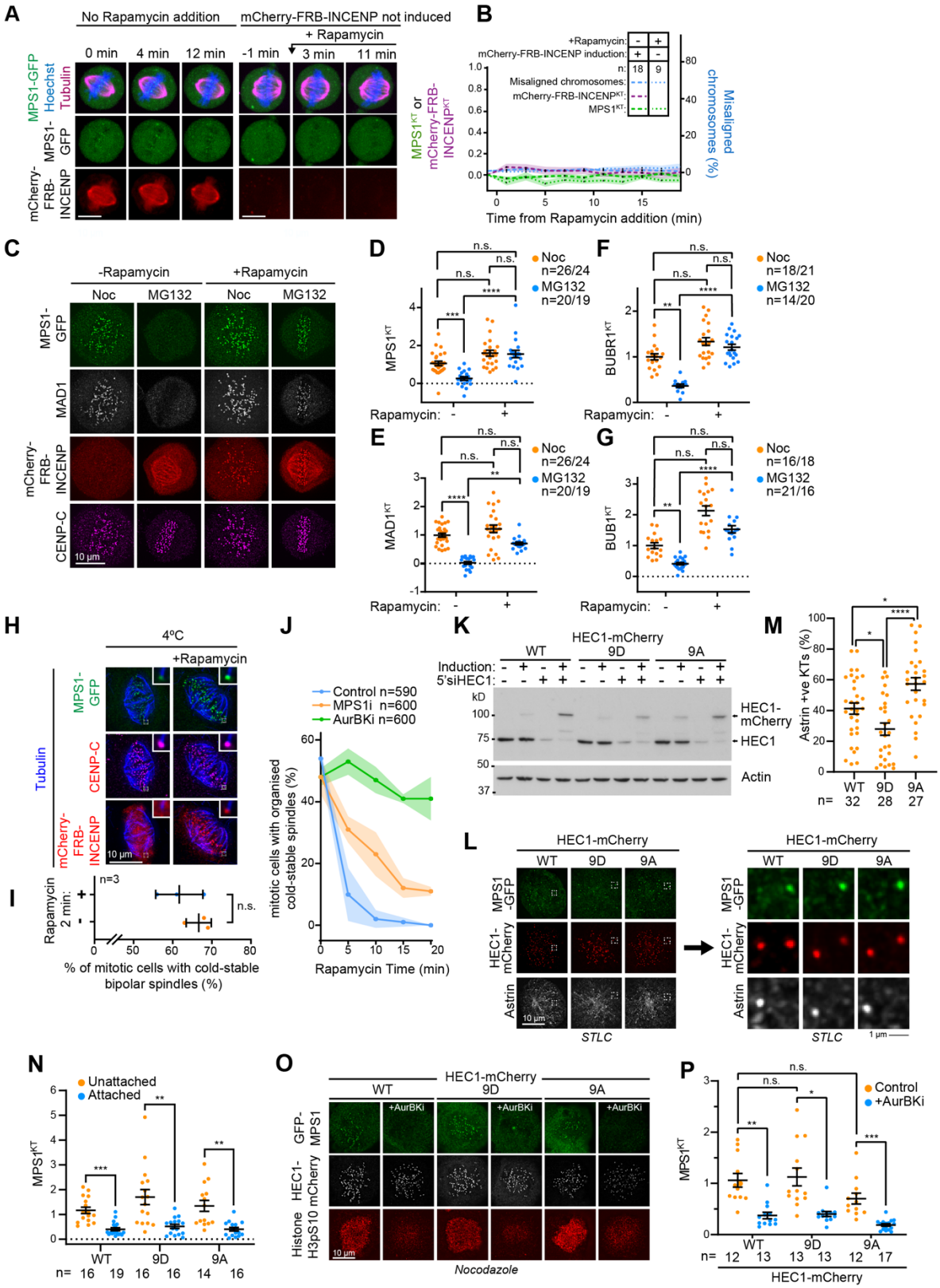

Figure S3. Analysis of Aurora B-induced MPS1 kinetochore recruitment. Related to Figure 3. (**A**) Live cell imaging stills of MG132-arrested HeLa MPS1-GFP cells either with the Aurora-B kinetochore targeting system but without Rapamycin (left), or without the Aurora-B kinetochore targeting system but with Rapamycin (right). DNA and Tubulin were visualised with Hoechst and SiR-tubulin. Cell at -1 min is at metaphase before addition of Rapamycin. (**B**) Kinetochore MPS1-GFP or mCherry-FRB-INCENP intensities and level of chromosome misalignment in cells from (A). Lines represent mean, coloured bands represent S.E.M. (**C**) Cells as in (A), arrested with Nocodazole or MG132, and treated with

Rapamycin for 2 min prior to fixation, immuno-stained as indicated. (**D**) Plot of MPS1 kinetochore mean cell intensities from cells in (C). Bars show mean± S.E.M. (**E**) Plot of MAD1 kinetochore mean cell intensities from cells in (C). Bars show mean± S.E.M. (**F**) Plot of BUBR1 or (**G**) BUB1 intensities in cells treated as in (C) and stained for BUBR1 or BUB1. (**H**) Cells as in (A), treated with Rapamycin for 2 mins and then cold-treated for 9 min before fixation and staining. (**I**) Plot of the proportion of mitotic cells with cold-stable bipolar spindles in cells in (H). Error bars show S.D. (**J**) Plot of the proportion of mitotic cells with cold-stable bipolar spindles in the indicated conditions at different times after rapamycin addition. (**K**) Anti-HEC1 Western blot of HeLa GFP-MPS1 Flp-In TREx cells treated with siHEC1 or siControl ± HEC1-mCherry transgene expression. Actin is a loading control. (**L)** STLC arrested HeLa Flp-In TREx GFP-MPS1 cells, depleted of endogenous HEC1 and expressing HEC1 mutants were immuno-stained for Astrin. Representative monotelic kinetochores pairs, indicated by dashed boxes, have been enlarged on the right-hand side. (**M**) Proportion of attached (Astrin +ve) kinetochores, in cells from (L). Bars show mean ±S.E.M. (**N**) Cell average kinetochore-MPS1 intensities from cells in (L). Bars show mean± S.E.M. (**O**) Nocodazole arrested HeLa Flp-In TREx GFP-MPS1 cells, depleted of endogenous HEC1 and expressing HEC1 mutants were treated with AurBKi and fixed and stained as indicated. (**P**) Cell average MPS1 kinetochore intensities for cells in (O) were plotted. Bars indicate mean± S.E.M.

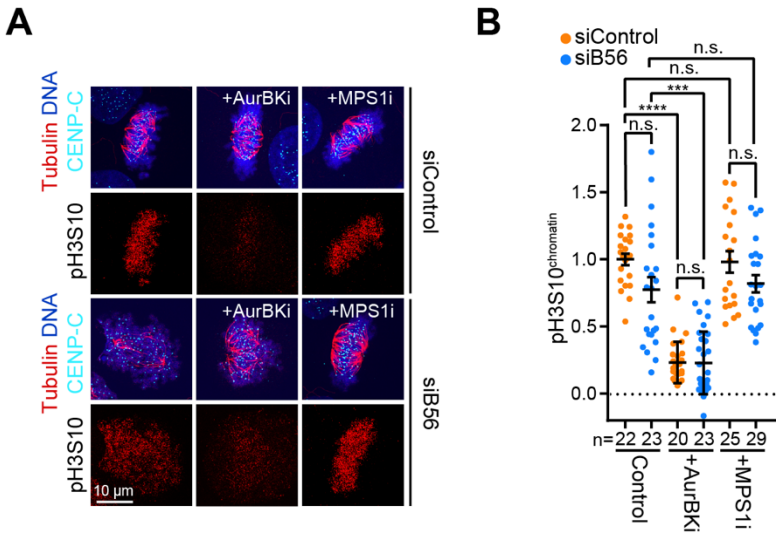

Figure S4. Effective Aurora B inhibition in PP2A-B56 depleted cells. Related to Figure 4G. (**A**) Immunofluorescence analysis of control or PP2A-B56 depleted HeLa cells treated with the indicated kinase inhibitors before cold-treatment, as in Figure 4G. Cells were immunostained as indicated. Histone H3 pSer10 (pH3S10) staining serves as an indicator of Aurora B activity. (**B**) Quantitation of (A). Bars show mean± S.E.M.