Cell Reports

Supplemental Information

Shugoshin-1 Balances Aurora B Kinase Activity via PP2A to Promote Chromosome Bi-orientation

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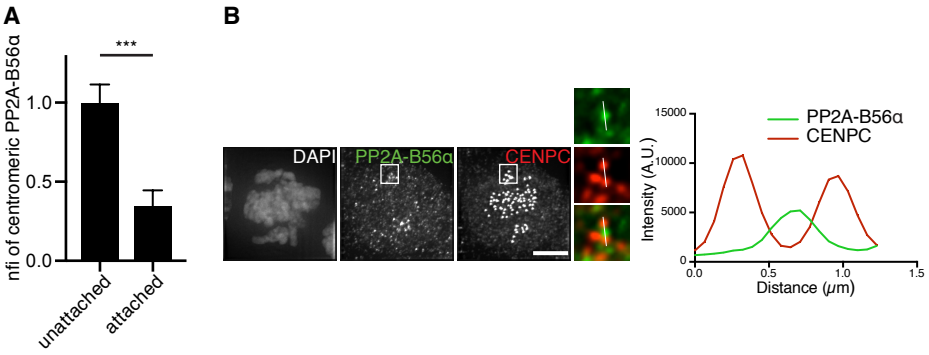

Figure S1, related to Figure 1: Centromere levels of PP2A-B56α on unattached and attached chromosomes of RPE-1 cells

(A) Quantification of PP2A-B56α in RPE-1 cells, treated with a low dose (0.069 μM) of nocodazole to increase the frequency of cells with both bi-oriented (attached) and unattached chromosomes. Centromeric Fluorescence Intensities (CFIs) were measured on centromeres of attached and unattached chromosomes and normalized for the unattached centromeres (nfi), using an in-house-written macro for ImageJ. Error bars are SEM between cells from 2 independent experiments (10-15 cells/experiment).

(B) IF of PP2A-B56α and CENPC in cells treated as in (A). Insets show kinetochore pairs in the boxed regions used for line plot analysis.

nfi, normalized fluorescence intensity. A.U. Arbitrary units. Scale bars, 5μm. *** = $p < 0.001$; (unpaired t-test).

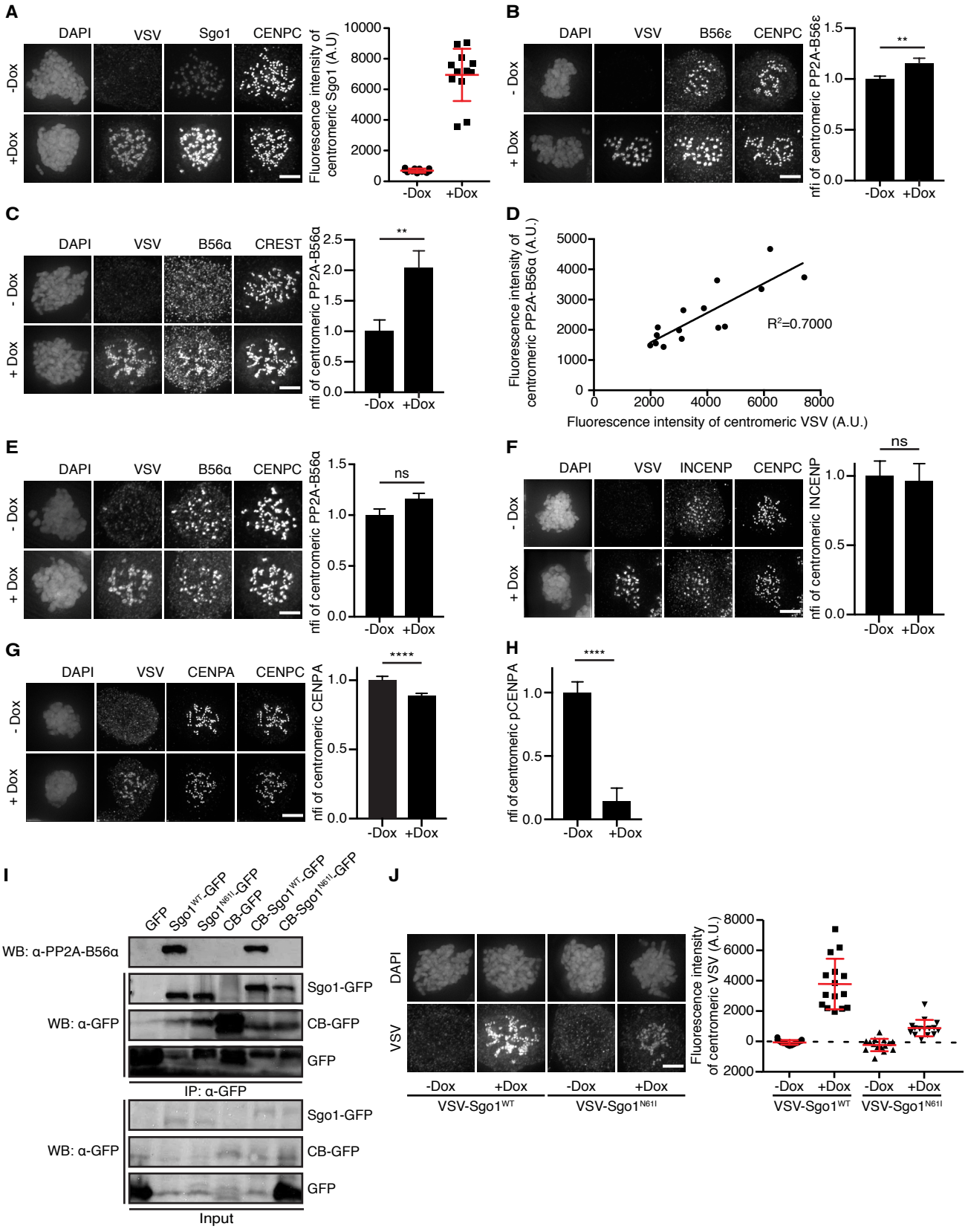

Figure S2, related to Figure 2 and Figure 3: Sgo1 overexpression recruits extra PP2A-B56 to centromeres in both transformed and non-transformed cells

(A) IF and quantification of VSV, Sgo1 and CENPC in U2OS cells, stably expressing VSV-Sgo1 under control of a Tet-inducible promotor, treated with 0.83 μM nocodazole and doxycycline where indicated. CFIs of Sgo1 were measured using ImageJ, each dot represents the CFI in a single cell. Error bars represent SD.

(B) IF and quantification of PP2A-B56ε in RPE-1 cells stably expressing VSV-Sgo1 under control of a Tet-inducible promotor, treated with 0.83 µM nocodazole and doxycycline where indicated. Error bars are SEM between cells from 2 independent experiments (15-30 cells/ experiment).

(C) IF and quantification of PP2A-B56α in U2OS cells treated as in (A). Error bars represent SD. 10-15 cells were analyzed.

(D) Pearson Correlation between absolute CFIs of VSV and PP2A-B56α from (C).

(E) IF and quantification of PP2A-B56α in RPE-1 cells, treated as in (B). Error bars represent SD. 15 cells were analyzed.

(F-G) IF and quantification of INCENP and CENPA in RPE-1 cells treated as in (B). Error bars are SEM between cells from 2 independent experiments (15 cells/experiment).

(H) Quantification of phosphorylated CENPA (pCENPA) in U2OS cells treated as in (A). Error bars represent SD. 10-15 cells were analyzed.

(I) An anti-GFP-immunoprecipitation was performed in mitotic cell lysates from U2OS cells transiently transfected with the indicated GFP constructs. After SDS-PAGE, the Western blot was probed for PP2A-B56α and subsequently for GFP. 10% of input was loaded.

(J) IF and quantification of VSV in U2OS cells stably expressing VSV-Sgo1WT and VSV-Sgo1N61I under control of a Tet-inducible promotor, treated with 0.83 µM nocodazole and doxycycline where indicated. Each dot represents the intensity measured for all centromeres in one cell. Error bars represent SD.

nfi, normalized fluorescence intensity. A.U. Arbitrary units. Scale bars, 5µm. ** = $p < 0.01$; **** = $p < 0.0001$; ns = not significant (unpaired t-test).

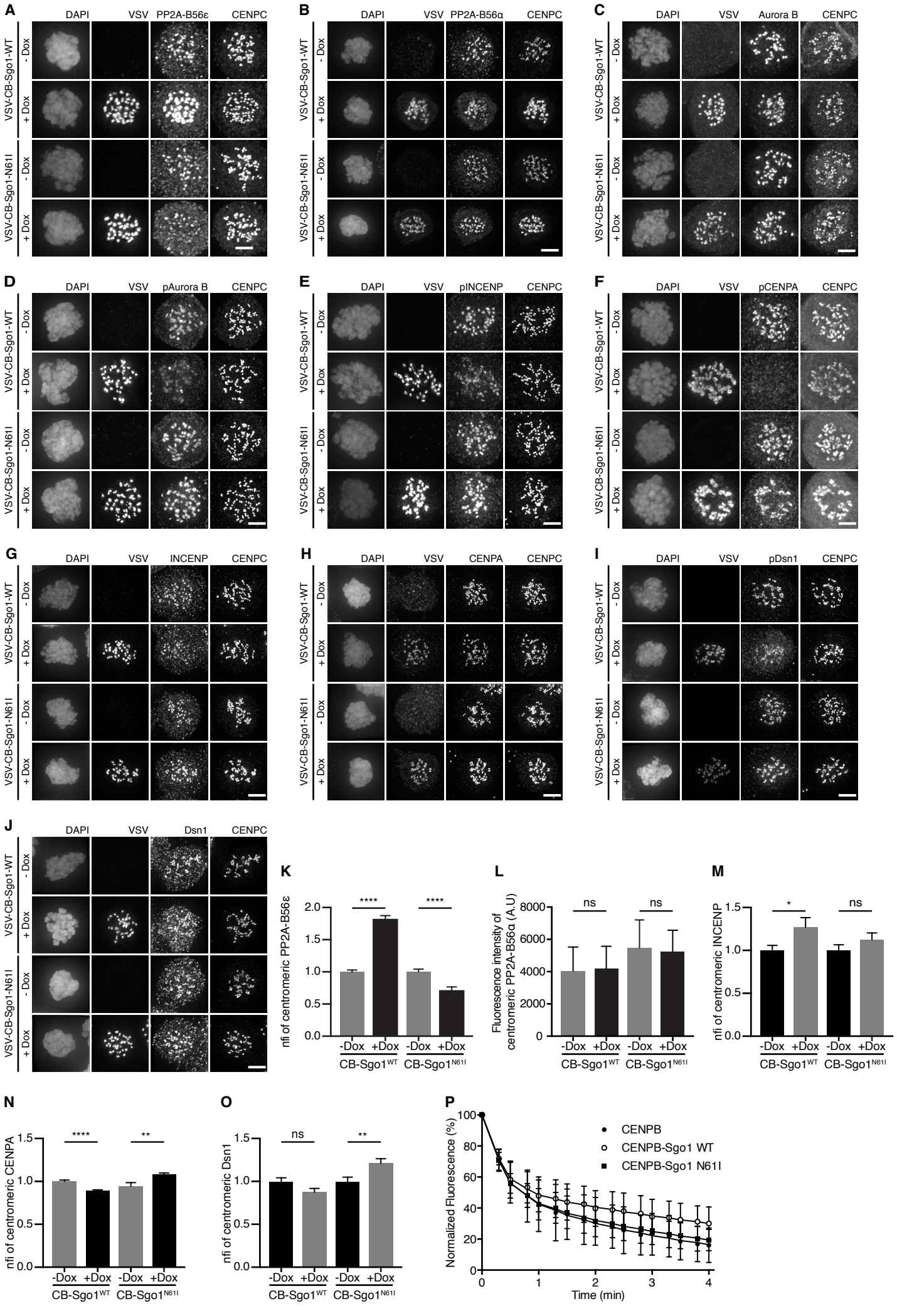
A
DAPI
VSV
PP2A-B56ε
CENPC
B
PP2A-B56α
C
Aurora B
D
pAurora B
E
pINCENP
F
pCENPA
G
INCENP
H
CENPA
I
pDsn1
J
Dsn1
VSV-CB-Sgo1-WT
VSV-CB-Sgo1-N61I
- Dox
+ Dox
K
nfi of centromeric PP2A-B56ε
L
Fluorescence intensity of centromeric PP2A-B56α (A.U)
M
nfi of centromeric INCENP
N
nfi of centromeric CENPA
O
nfi of centromeric Dsn1
P
Normalized Fluorescence (%)
Time (min)
CENPB
CENPB-Sgo1 WT
CENPB-Sgo1 N61I
-Dox
+Dox
CB-Sgo1WT
CB-Sgo1N61I
****
**
*
ns

Figure S3, related to Figure 3: Sgo1 overexpression recruits excessive PP2A-B56 to centromeres that counteracts Aurora B substrate phosphorylation and hyperstabilizes kinetochore microtubules

(A-J) Representative IF images of RPE-1 cells stably expressing VSV-CB-Sgo1WT or VSV-CB-Sgo1N61I under control of a Tet-inducible promotor, treated with 0.83 μM nocodazole and doxycycline where indicated, belonging to Figures S3K, S3L, 3E, 3B, 3C, 3D, S3M, S3N, 3H, and S3O, respectively.

(K) Quantifications of PP2A-B56ε in cells treated as in (A) and normalized for the non-induced condition. Error bars are SEM between cells from 3 independent experiments (10-15 cells/experiment).

(L) Quantification of absolute CFIs of PP2A-B56α in RPE-1 cells treated as in (A). Error bars represent SD (n= 15 cells).

(M-O) Quantifications of INCENP, CENPA, and Dsn1 in RPE-1 cells treated as in (A). Error bars are SEM between cells from 2 independent experiments (10-15 cells/experiment.

(P) Normalized fluorescence intensity over time after photoactivation of spindles in VSV-CB (filled circles), VSV-CB-Sgo1WT (open circles), and VSV-CB-Sgo1N61I (filled squares) overexpressing prometaphase cells. Data represents mean ± SD, n >10 cells.

nfi, normalized fluorescence intensity. A.U. Arbitrary units. Scale bars, 5μm. * = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$; ns = not significant (unpaired t-test).

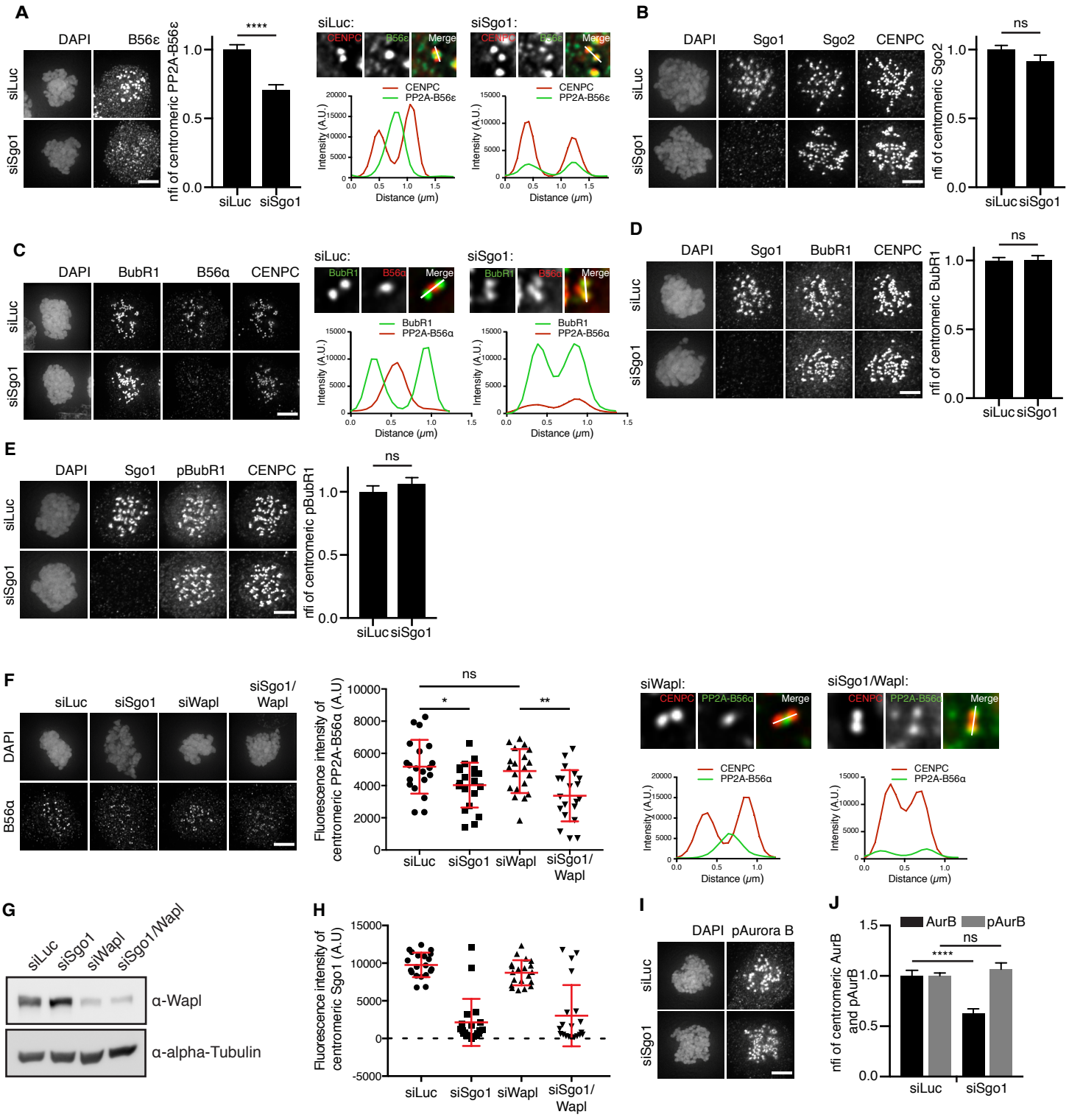

Figure S4, related to Figure 4: Depletion of Sgo1 reduces PP2A-B56ε and PP2A-B56α at centromeres, but does not affect the centromere/kinetochore levels of Sgo2, BubR1 and Plk1-phosphorylated BubR1

(A) IF and quantifications of PP2A-B56ε and CENPC in RPE-1 cells transfected with siRNAs for Luciferase or Sgo1 and treated with 0.83 μM nocodazole, CFIs were normalized for siLuciferase. Enlargements show single kinetochore pairs in one z-plane used for line plot analysis using ImageJ. Error bars are SEM between cells from 3 independent experiments (10-15 cells/experiment).

(B) IF and quantifications of Sgo1, Sgo2 and CENPC in RPE-1 cells treated as in (A). Error bars are SEM between cells from 2 independent experiments (10-15 cells/experiment).
(C) IF and quantifications of BubR1, PP2A-B56α and CENPC in RPE-1 cells treated as in (A). Enlargements show single kinetochore pairs in one z-plane used for line plot analysis.
(D-E) IF and quantifications of Sgo1, BubR1 and pBubR1 (pT680) in RPE-1 cells treated as in (A). Error bars are SEM between cells from independent experiments (n=3 for BubR1 and n=2 for pBubR1, 10-15 cells/experiment).
(F) IF and quantification of PP2A-B56α in RPE-1 cells transfected with siRNAs for Luciferase, Sgo1, Wapl or Sgo1 and Wapl combined. Cells were treated with 0.83 μM nocodazole. Absolute CFIs were quantified and each dot represents the intensity measured for all centromeres in one cell. Error bars represent SD. Enlargements show kinetochore pairs in one z-plane used for line plot analysis.
(G) Western blot of cells treated as in (F) probed for Wapl to confirm Wapl depletion. Alpha-tubulin was used as a loading control.
(H) Quantifications of Sgo1 in RPE-1 cells treated as in (F) to confirm Sgo1 depletion. Each dot represents the intensity measured for all centromeres in one cell. Error bars represent SD.
(I) RPE-1 cells treated as in (A) and immunostained for pAurora B.
(J) Quantification of CFIs of pAurora B and normalized for the siLuc condition. Error bars represent the SEM between cells from independent experiments (n=3, 10-15 cells per experiment). For comparison the nfi's for total Aurora B of figure 4A were included in the graph.
nfi, normalized fluorescence intensity. A.U. Arbitrary units. Scale bars, 5μm. * = $p < 0.05$; ** = $p < 0.01$;**** = $p < 0.0001$ ns = not significant (unpaired t-test).

Supplemental Experimental Procedures

Antibodies

Primary antibodies used were Aurora B mouse mAb (AIM-1, BD Tranductions, 611083, 1:500), phospho-Aurora B-T232 rabbit pAb (Rockland, 600-401-677, 1:500), SgoL1 mouse mAb (Abnova, h00151648, 1:1000), PP2A-B56α mouse mAb (Santa Cruz, sc-136045, 1:500), PP2A-B56δ mouse mAb (Santa Cruz, sc-271363 (H-11), 1:250), PP2A-B56ε rabbit pAb (Gift from B. Hahn, (Chen et al., 2005)), phospho CENPA-S7 rabbit pAb (Upstate, 07-232, 1:500), CENPC guinea pig pAb (MBL, PD-030, 1:1000), CREST (Cortex Biochem, CS-1058, 1:500), phospho INCENP-S893/894 rabbit pAb (Gift from M. Lampson (Salimian et al., 2011), 1:1000) Mad1 mouse mAb (Gift from A. Musacchio (De Antoni et al., 2005), 1:20), VSV mouse mAb (Sigma, V5507, 1:500), VSV rabbit pAb (Abcam, Ab19257, 1:1000), Sgo2 rabbit pAb (Gift from A. Losada, 1:1000), BubR1 rabbit pAb (Bethyl, a300-386a, 1:1000), phospho-BubR1-T680 rabbit pAb (Gift from G. Kops (Suijkerbuijk et al., 2012), 1:1000), CENPA mouse mAb (Abcam, Ab13939, 1:500), INCENP rabbit pAb (Sigma, I5238, 1:10.000), Dsn1 rabbit pAb (Genetex, GTX120402, 1:1000), phospho Dsn1-S109 rabbit pAb (gift from I. Cheeseman (Welburn et al., 2010), 1:500) GFP rabbit pAb (1:000), alpha-tubulin rat mAb (For IF, Thermo Scientific, MA1-80017, 1:500), alpha-tubulin mouse mAb (for WB, Sigma, T5168, 10:10.000), WAPL mouse pAb (Abnova, h00023063, 1:1000), Secondary Antibodies used were goat anti-human or anti-guinea pig IgG-alexa 647; goat anti-mouse or anti rabbit IgG-Alexa 488; goat anti-mouse or rabbit IgG-Alexa 568 (Invitrogen 1:500), donkey anti-mouse IgG-HRP, donkey anti-rabbit IgG-HRP and rabbit anti-goat IgG-HRP (Dako 1:2500).

siRNA and plasmid transfection

siRNA target sequences: siLUC (Luciferase GL2 duplex;Dharmacon/D-001100-01-20), siSgoL1 (Dharmacon/J-015475-12: GAUGACAGCUCCAGAAAUU), siWAPL (Dharmacon/J-026287-10: GAGAGAUGUUUACGAGUUU). They were transfected at 20 nM each using Hiperfect (Qiagen). The siPP2A-B56 pool was composed of 5 siRNAs targeting the 5 individual B56 subunits (Foley et al., 2011); B56α: 5′-UGAAUGAACUGGUUGAGUA-3′; B56β: 5′-GAACAAUGAGUAUAUCCUA-3′; B56γ:

5′-GGAAGAUGAACCAACGUUA-3′; B56δ: 5′-UGACUGAGCCGGUAAUUGU-3′; B56ε: 5′-GCACAGCUGGCAUAUUGUA-3′). The PP2A-B56 siRNA pool was reverse transfected at a total concentration of 50nM using RNAiMax. For the immunoprecipitation experiment, U2OS cells were transiently transfected with the indicated GFP tagged proteins using the calcium phosphate method. U2OS cells stably expressing photoactivatable tubulin were transiently transfected with the indicated mCherry tagged versions of Sgo1 using fuGENE 6 (Roche diagnostics) and analyzed 12 hours later.

Cell synchronization

For IF analysis of mitotic U2OS cells, 0.83 μM nocodazole (Sigma-Aldrich) was added 45 min after RO3306 release and cells were fixed 75 minutes later. For IF analysis of mitotic RPE-1 cells, cells were blocked in mitosis using either a high (0.83 μM) or low (0.069 μM) concentration of nocodazole for 14-16 hours. Where indicated, doxycycline was added 14-16 hours prior to treatment with 7.5μM RO3306 (U2OS) or nocodazole (RPE-1). The capacity to correct k-MT attachment errors was assayed by blocking RPE-1 cells in prometaphase with the Eg5 inhibitor monastrol (100μM, Sigma-Aldrich). After 14-16 hours cells were released from the monastrol block into medium containing 10μM MG132 (Calbiochem). Cells were fixed 60 min later.

Immunofluorescence Microscopy

Cells grown on 12 mm High Precision coverslips (Superior-Marienfeld GmbH & Co) were fixed with 4% PFA in PBS followed by ice-cold methanol. Alternatively, cells were pre-extracted with 0.2% Triton X-100 in PEM buffer (PEM-T) for 30-60 seconds, then PFA was added to a final concentration of 2% for 4 min. After this, cells were treated with 4% PFA in PBS for 4 min. For the cold-induced microtubule depolymerization assay cells were subjected to ice-cold media and kept on ice for 5 minutes. Then cells were washed with PEM-T, followed by a quick wash with PEM, followed by fixation in 4% PFA in PBS. Primary and secondary antibodies were incubated in PBS 3% BSA 0.1% Tween. DNA was stained using DAPI. Coverslips were mounted using ProLong Antifade (Molecular Probes) and imaged on a DeltaVision RT or Elite system (Applied Precision) with

a 100X/1.40NA UPlanSApo objective (Olympus). Images were deconvolved in Softworx. For each experiment all images were acquired with identical illumination settings. Images are projections of deconvolved Z-stacks, unless stated otherwise, and ImageJ was used for quantification as described in (Saurin et al., 2011). Graphs and statistical analysis were done using Graphpad Prism (GraphPad Software, Inc.).

Kinetochore-microtubule stability assay

U2OS cells stably expressing photoactivatable tubulin were used to assay kinetochore-microtubule half-life as described in (Kabeche and Compton, 2012).

Western blot sample preparation

siRNA transfected RPE-1 cells were blocked in mitosis using 0.83 μM nocodazole for 16 hours. Cells were collected by mitotic shake-off, lysed in Laemmli buffer and subjected to standard immunoblotting protocol.

Immunoprecipitation

U2OS cells were transiently transfected with the indicated GFP constructs using calcium phosphate and blocked in mitosis for 16 h using nocodazole after release from a 24 h thymidine (2.5 mM, Sigma-Aldrich) block. Mitotic cells were collected by mitotic shake-off and lysed in buffer A (20 mM TRIS (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 0.2% NP-40, 10% glycerol, 1 mM NaF, 1 mM vanadate, 20 mM B-Glycerol phosphate, 10 mM beta-mercaptoethanol, 0.2 mM PMSF, protease inhibitors (Complete)) followed by two freeze-thaw cycles in liquid nitrogen. Insoluble material was pelleted by high-speed centrifugation. Supernatant was collected and the pellet was resuspended in buffer B (10 mM TRIS (pH7.5), 150 mM NaCl, 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 0.25 M Sucrose, 0.2 mM PMSF, protease inhibitors (Complete) + 8U MNase/ml). After incubation at 30˚C insoluble material was pelleted by high-speed centrifugation at 4˚C. Supernatants were combined and added to agarose beads coupled to anti-GFP-antibody for 2 hours at 4°C while rotating. Beads were washed three times with buffer B, boiled for 5 minutes in standard SDS sample buffer and subjected to immunoblotting according to standard protocol.

Supplemental References

Chen, W., Arroyo, J.D., Timmons, J.C., Possemato, R., and Hahn, W.C. (2005). Cancer-associated PP2A Aalpha subunits induce functional haploinsufficiency and tumorigenicity. Cancer Res *65*, 8183-8192.

De Antoni, A., Pearson, C.G., Cimini, D., Canman, J.C., Sala, V., Nezi, L., Mapelli, M., Sironi, L., Faretta, M., Salmon, E.D.*, et al.* (2005). The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint. Curr Biol *15*, 214-225.

Foley, E.A., Maldonado, M., and Kapoor, T.M. (2011). Formation of stable attachments between kinetochores and microtubules depends on the B56-PP2A phosphatase. Nat Cell Biol *13*, 1265-1271.

Kabeche, L., and Compton, D.A. (2012). Checkpoint-independent stabilization of kinetochore-microtubule attachments by Mad2 in human cells. Curr Biol *22*, 638-644.

Salimian, K.J., Ballister, E.R., Smoak, E.M., Wood, S., Panchenko, T., Lampson, M.A., and Black, B.E. (2011). Feedback control in sensing chromosome biorientation by the Aurora B kinase. Curr Biol *21*, 1158-1165.

Saurin, A.T., van der Waal, M.S., Medema, R.H., Lens, S.M., and Kops, G.J. (2011). Aurora B potentiates Mps1 activation to ensure rapid checkpoint establishment at the onset of mitosis. Nat Commun *2*, 316.

Suijkerbuijk, S.J., Vleugel, M., Teixeira, A., and Kops, G.J. (2012). Integration of kinase and phosphatase activities by BUBR1 ensures formation of stable kinetochore-microtubule attachments. Dev Cell *23*, 745-755.

Welburn, J.P., Vleugel, M., Liu, D., Yates, J.R., 3rd, Lampson, M.A., Fukagawa, T., and Cheeseman, I.M. (2010). Aurora B phosphorylates spatially distinct targets to differentially regulate the kinetochore-microtubule interface. Mol Cell *38*, 383-392.