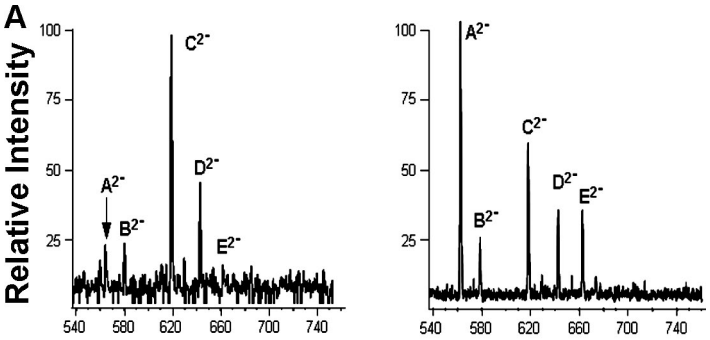
Huang et al., http://www.jcb.org/cgi/content/full/jcb.200805163/DC1



B

Ion Signal	BubR1 Sequence	Phospho site
A	1041-1050 + P	Ser 1043
B	540-550 + P	Ser 543
C	668-677 + P	Ser 670, Ser676
D	541-550 + P	Ser 543
E	429-439 + P	Ser 453

C

pS435

Human BubR1:	E L L T S A E K R
Drosophila BubR1:	S V Q E E D E K K
Zebrafish BubR1:	E I N Q S G S D A
XBubR1:	D L Q A S A L R R
mBubR1:	E L L T S A K K R
Chick BubR1:	E I Q T I A Q K K

pS543

Human BubR1:	K K N K S P P A D
Drosophila BubR1:	W E P N A P S V P
Zebrafish BubR1:	E D N A Q T S K T
XBubR1:	S S E A L P S M S
mBubR1:	K K D K S P A T G
Chick BubR1:	S S T S A N Q N T

pS670

Human BubR1:	I K K L S P I I E
Drosophila BubR1:	K I K F S N D S M
Zebrafish BubR1:	E K K L S P I L E
XBubR1:	I K K L S P I L E
mBubR1:	I K K L S P I I E
Chick BubR1:	V N K L S P I M E

pS1043

Human BubR1:	G K L T S P G A L
Drosophila BubR1:	N T I L Q K - - -
Zebrafish BubR1:	- - - - - - - - -
XBubR1:	E M R L N P L - -
mBubR1:	G K T I S P E A L
Chick BubR1:	G D V F F L E K F

Figure S1. **Analysis of BubR1 phosphorylation by mass spectrometry.** (A) Partial precursor ion mass spectrum of trypsin-digested BubR1 immunoprecipitated from asynchronous (left) and mitotic HeLa cells (right). Phosphopeptides are identified in the unenriched samples by detection of the marker ion PO_3^-, which is released from the side chain of phosphorylated amino acids upon collision-induced dissociation. Superscripted numbers in each mass spectrum refer to the detected charge state for each peptide in the negative ion mode. The mass spectrometry data shown here are not quantitative between the two samples. (B) BubR1 peptide sequences assigned to the phosphopeptides identified in the precursor ion scans. Sequences were confirmed, and the exact site of phosphorylation was determined by tandem mass spectrometry. The peptide 668–677 +P was found to be monophosphorylated on the two different sites shown. (C) Phosphorylation sites in human BubR1 occur on residues conserved among vertebrates. Red rectangles indicate the position of the BubR1 phosphorylation site in humans and the relative position of the same site in other species.

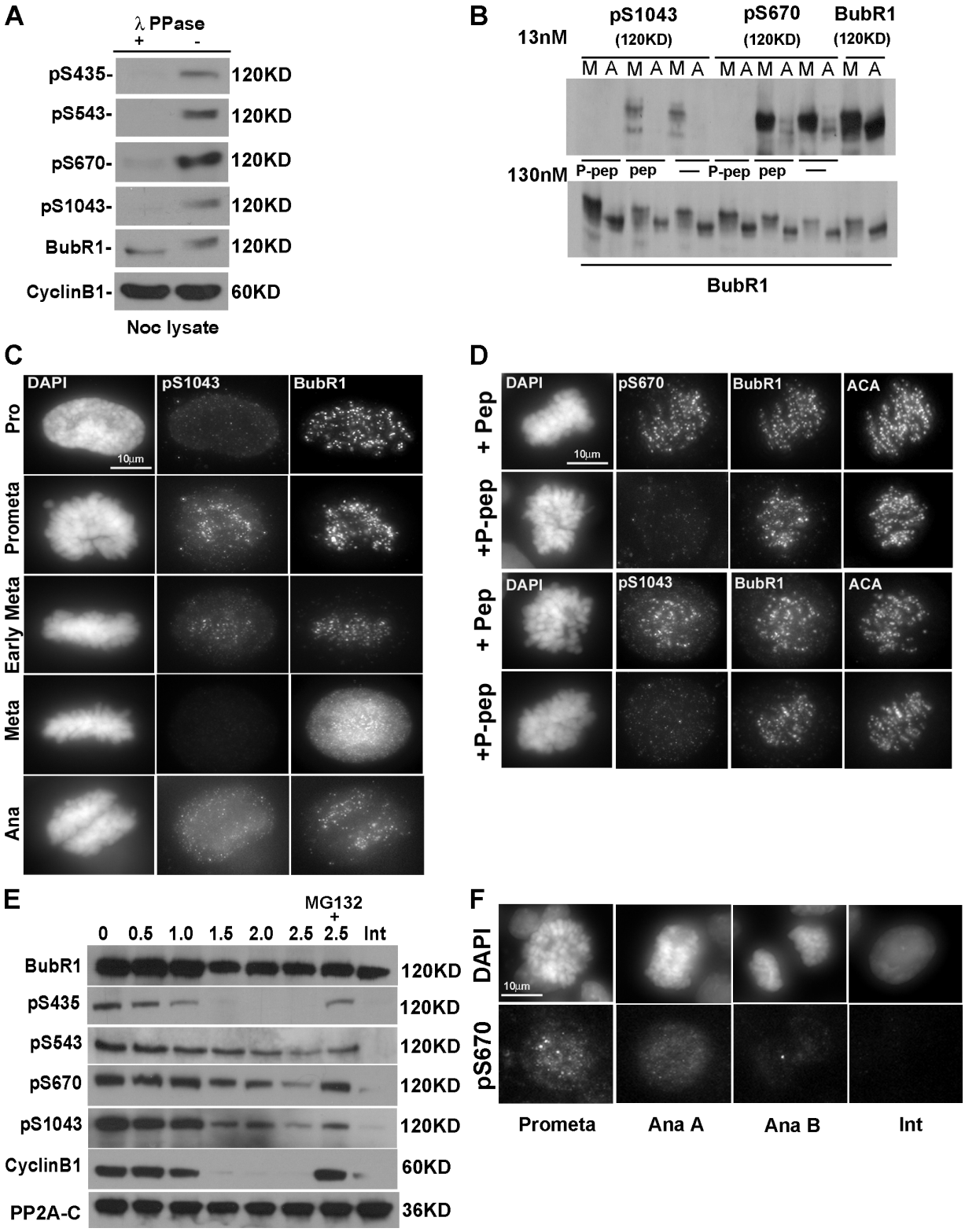


Figure S2. **Verification of the specificity of BubR1 phosphoantibodies.** (A) Mitotic lysates were treated and untreated with λ protein phosphatase (PPase) and probed with the phosphoantibodies. (B) Mitotic (M) and asynchronous (A) HeLa lysates were probed with the indicated concentrations of pS670, pS1043 and BubR1 antibodies, and peptides. P-pep and pep denote phosphopeptides and nonphosphopeptides, respectively. (C) HeLa cells were stained for pS1043, BubR1, and DAPI. (D) HeLa cells were extracted, fixed, and costained with DAPI, BubR1, ACA, and pS670/pS1043 antibody + 300 nM nonphosphopeptide or 300 nM phosphopeptide. (E) Mitotic cells were released from a nocodazole arrest, and samples were collected every 30 min for 2.5 h. MG132 was added to one sample at the time of drug washout to prevent mitotic exit. Lysates were probed with affinity-purified BubR1 antibodies. Cyclin B1 was used to monitor activation of APC/C. PP2Ac serves as a loading control. (F) Cells were fixed first and stained with pS670 antibody to visualize the cytosolic pool of the antibody. Ana, anaphase; Ana A, early anaphase; Ana B, late anaphase; Int, interphase; Meta, metaphase; Pro, prophase; Prometa, prometaphase.

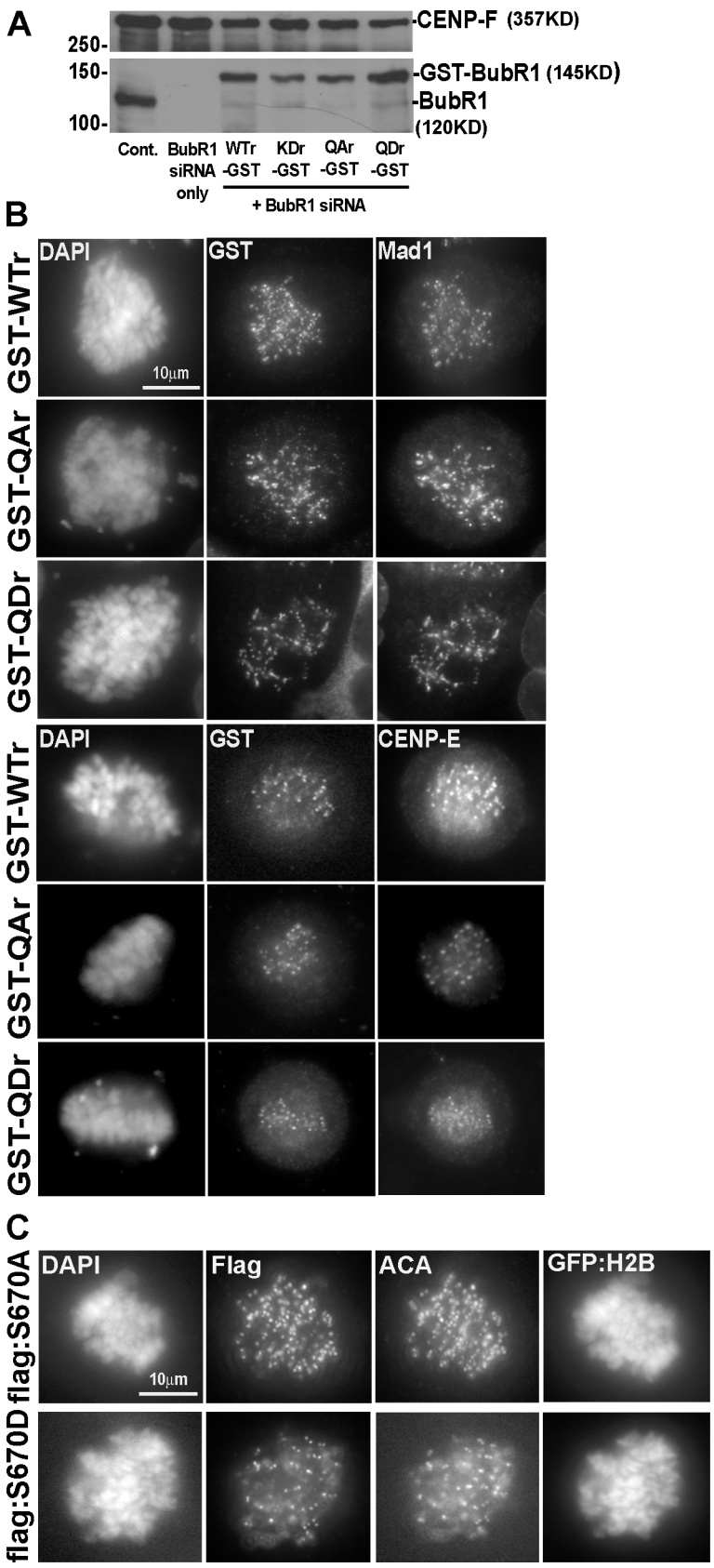


Figure S3. **Phospho-BubR1 mutants do not disrupt Mad1 and CENP-E localization at kinetochores.** (A) Cells depleted of BubR1 by siRNA were transfected with GST–wild type (WT) as well as GST-KD, GST-QA, and GST-QD mutants. Lysates were probed with BubR1 antibodies that detect both endogenous and GST/BubR1. CENP-F was used as a loading control. (B) Cells depleted of BubR1 by siRNA were transfected with GST–wild type, and GST-QA and GST-QD were fixed and stained for DAPI, GST, Mad1, and CENP-E. (C) HeLa cells transfected with BubR1 siRNA were injected with 3×Flag BubR1^{RNAi} S670A and S670D mutant constructs and stained for Flag, ACA, and DAPI. H2B/GFP was used to identify transfected cells.

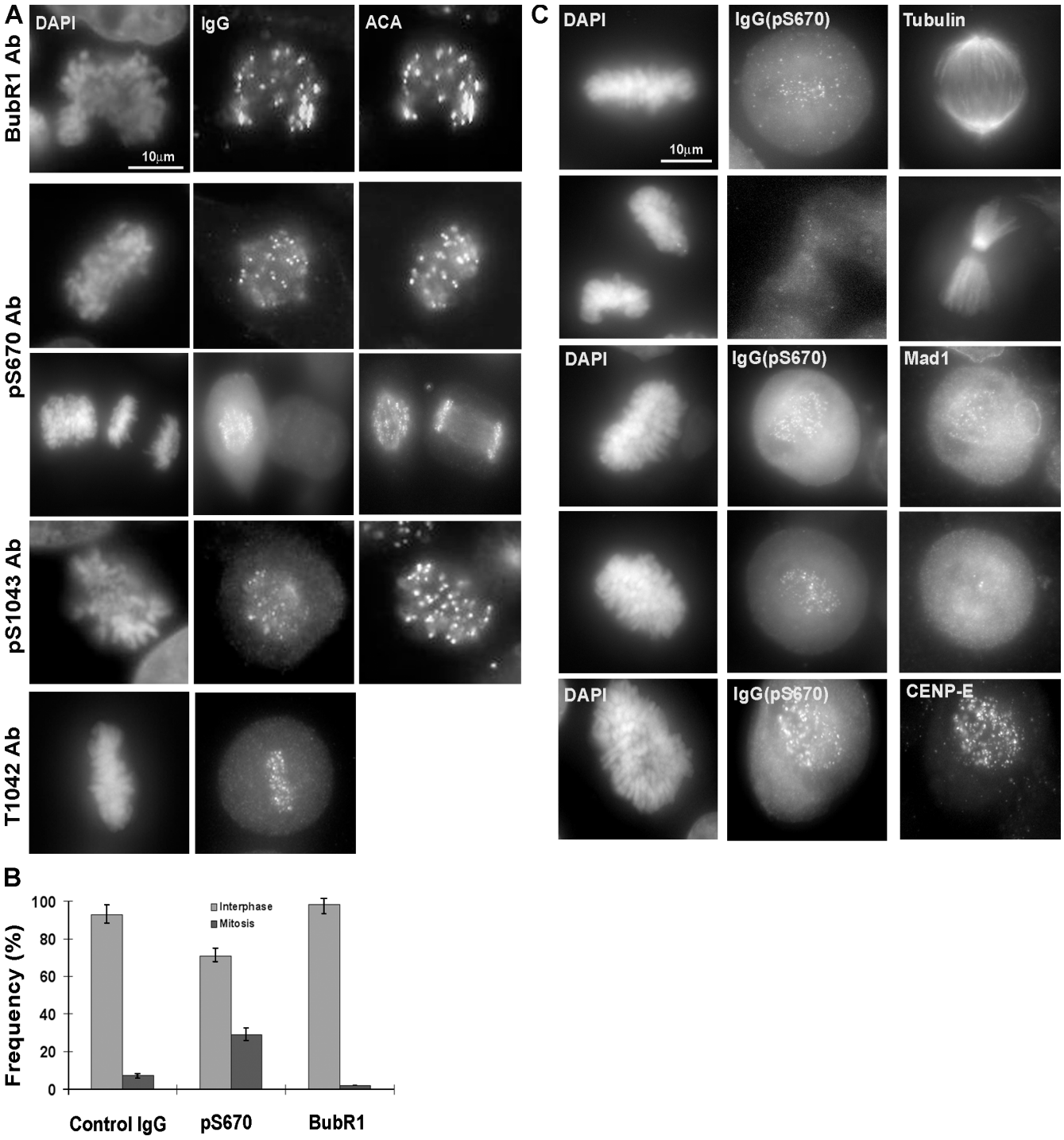


Figure S4. **Characterization of microinjected BubR1 antibodies.** (A) HeLa cells were injected with the indicated antibodies 4–5 h after release from a thymidine block. 10 h after thymidine release, cells were fixed and stained for DAPI, ACA, and anti–rabbit IgG to visualize the injected antibodies. Note the lack of pS670 signal in the anaphase cell. (B) Comparison of the mitotic indices of cells that were injected with control, pS670, and BubR1 antibodies. Error bars indicate the highest and lowest values in the data set. (C) Injected cells were fixed as described and stained for DAPI, anti–rabbit IgG, tubulin, Mad1, and CENP-E.

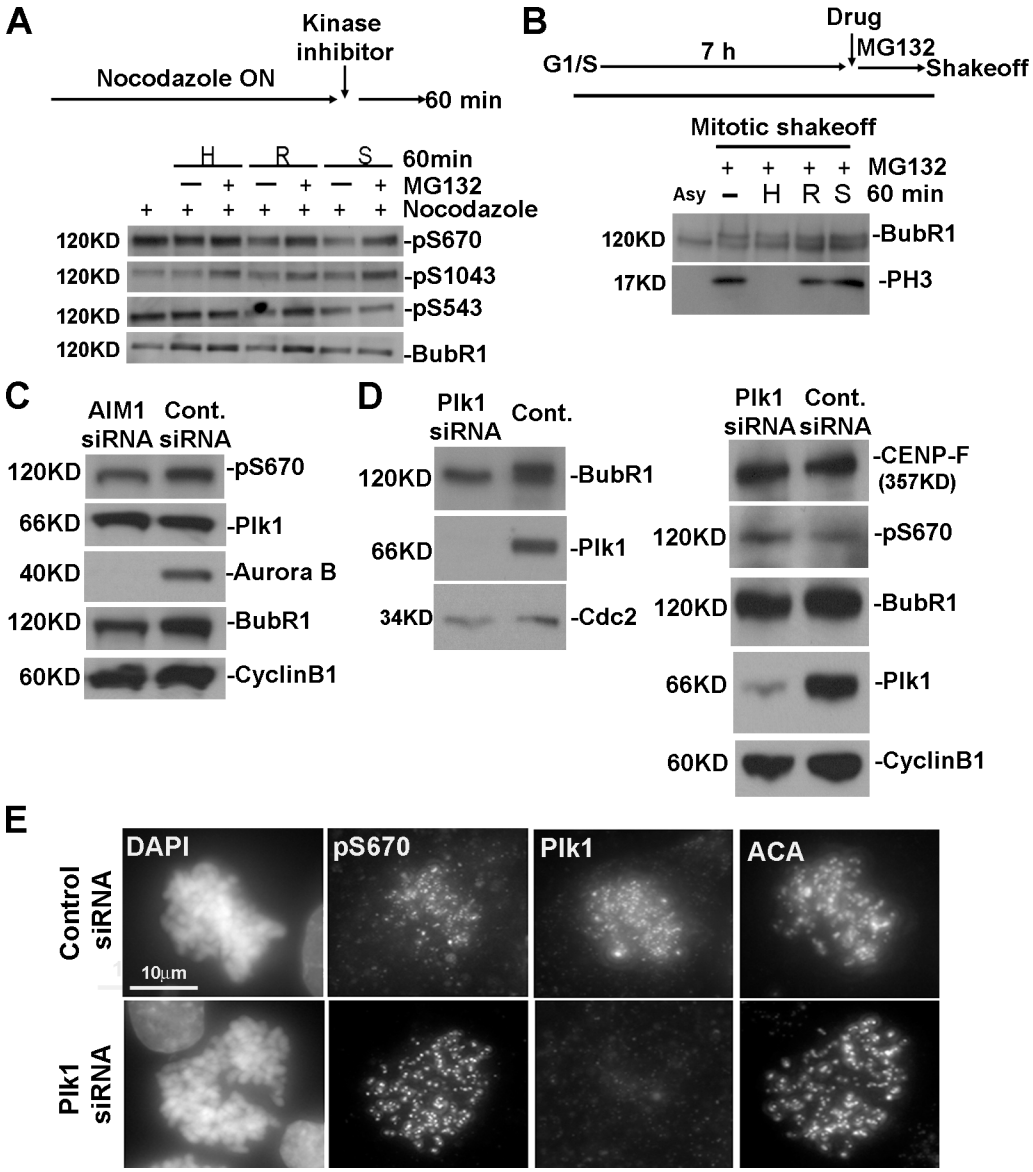


Figure S5. **Aurora B and Plk1 are not required for S670 phosphorylation.** (A) Cells were arrested in mitosis by nocodazole, and kinase inhibitors, hesperadin (H), roscovitine (R), and staurosporine (S) were added with and without MG132 for 60 min before harvesting. Lysates were probed for various phospho-BubR1 along with BubR1. (B) Cells were released from thymidine block, and inhibitors were added in the presence of MG132 at 7 h after release. Mitotic cells were shaken off after a further 60-min incubation, and the lysates were probed for BubR1 and phosphohistone3. Asy, asynchronous lysates. (C) Cells were transfected with control (Cont.) and aurora B siRNA and treated with nocodazole and MG132. Lysates from mitotic cells were probed for pS670, Plk1, aurora B, BubR1, and cyclin B1. (D) Untransfected cells (Cont.) and cells that were transfected with control and Plk1 siRNAs and blocked in mitosis as in C were probed with the indicated antibodies. Depletion of Plk1 increases the mobility of BubR1. (E) Mitotic cells transfected with control siRNA and Plk1 siRNA were fixed and stained for DAPI, pS670, Plk1, and ACA.