Figure S1

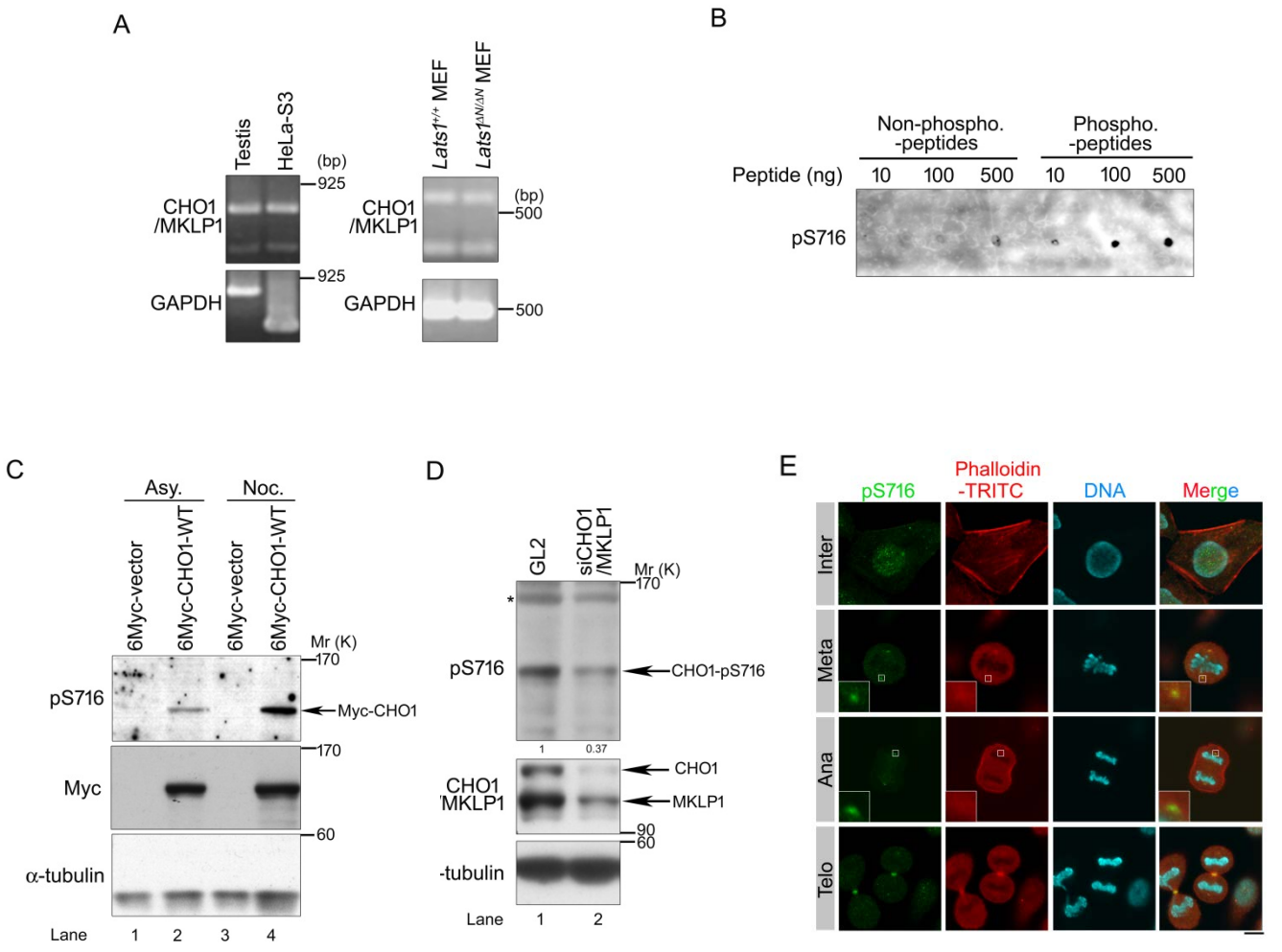

Figure S1. Expression of the *Mklp1* gene in mouse embryo fibroblasts (MEFs) and analysis of the anti-CHO1-pS716^{S717} antibody.

(A) RT-PCR analysis showing the expression of the *Mklp1* (332 bp) and *Cho1* (620 bp) mRNAs in mouse testis, HeLa-S3 cells, and MEFs (*Lats1*$^{+/+}$ [wild type; WT] and *Lats1*$^{\Delta N/\Delta N}$ [ΔN]; Yabuta et al., 2013). The expression level of *Gapdh* was used as a loading control. (B) A dot blot analysis confirming the quality of the anti-CHO1-pS716^{S717} polyclonal antibody (1:250 dilution). A PVDF membrane was spotted with the indicated amounts of non-phosphorylated and phosphorylated peptides. (C) Immunoblot analyses of exogenous CHO1-pS716^{S717} in HeLa-S3 cells transiently expressing 6Myc-tagged vector alone or full-length WT CHO1. The cells were asynchronous (Asy) or synchronized by treatment with nocodazole (Noc). The expression level of α-tubulin was used as a loading control. (D) Recognition of endogenous CHO1 in HeLa-S3 cells by the anti-pS716^{S717} antibody. The cells were transfected with a control (GL2) or CHO1/MKLP1-specific siRNA and synchronized by thymidine single block-and-release (10 h). Immunoblotting was performed using anti-CHO1-pS716^{S717} and anti-CHO1/MKLP1. The expression level of α-tubulin was used as a loading control.

(E) Colocalization of CHO1-pS716^{S717} with F-actin at the centrosomes during various stages of the cell cycle. HeLa-S3 cells were synchronized at each stage, stained with anti-CHO1-pS716^{S717} (green) and phalloidin-TRITC (red), and counterstained with Hoechst 33258 to detect DNA (blue). Scale bar, 10 μm. The insets show enlarged images of the signals at the centrosome.

Figure S2

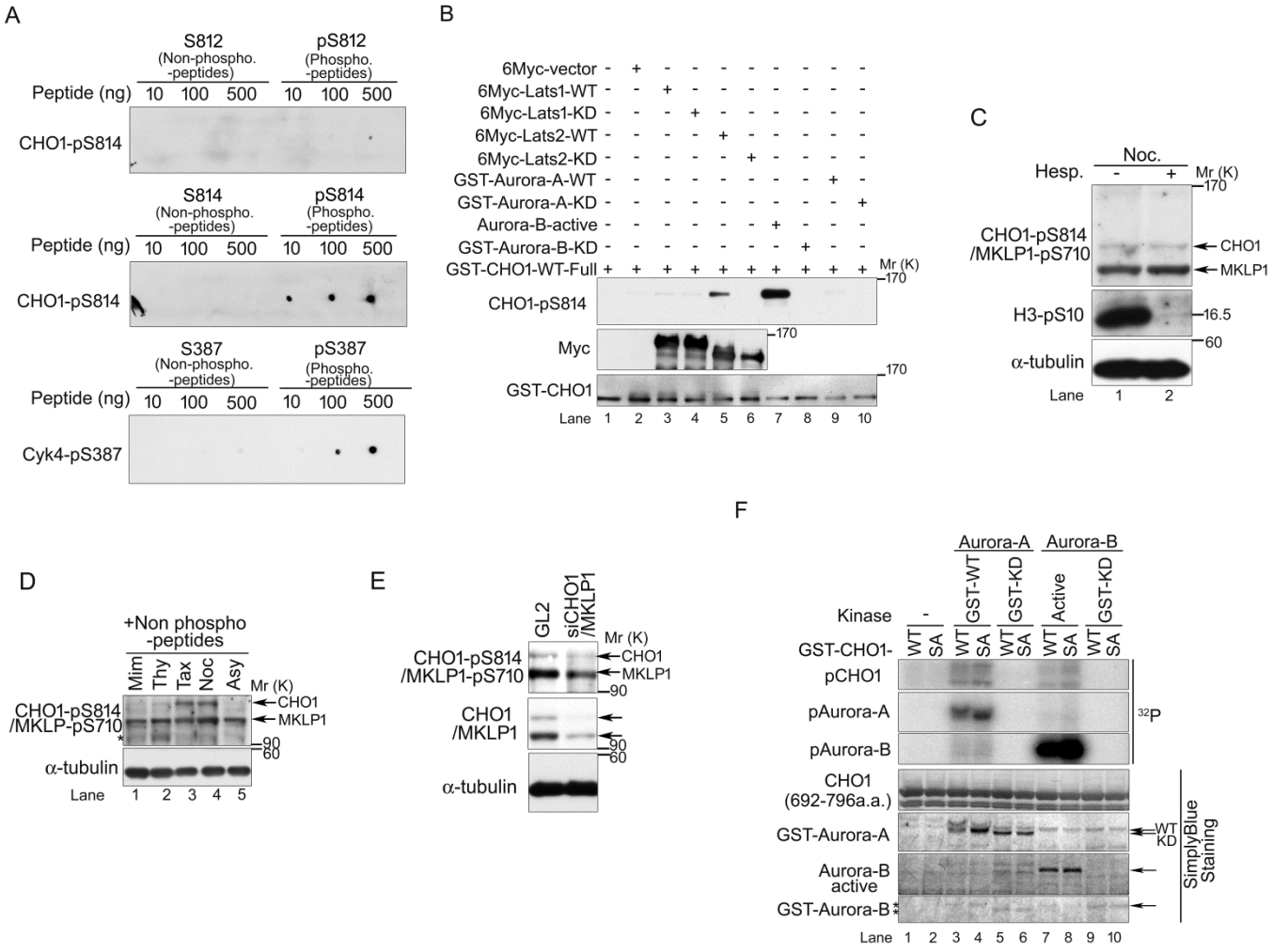

Figure S2. CHO1-S814^{S807} and MKLP1-pS710 are phosphorylated by Lats2.
(A) Dot blot analyses confirming the quality of the anti-CHO1-pS814^{S807} and anti-HsCyk4-pS387 polyclonal antibodies (1:250 dilution). A PVDF membrane was spotted with the indicated amounts of non-phosphorylated and phosphorylated peptides. (B) *In vitro* kinase assays using immunoprecipitates of 6Myc-tagged WT and KD Lats1 and Lats2, active (WT) and kinase dead (KD) forms of Aurora-A and Aurora-B as kinases, and recombinant GST-tagged full-length WT CHO1 as a substrate in the absence of [γ-^{32}P] ATP. Immunoblotting was performed using anti-CHO1-pS814^{S807}, anti-Myc and anti-GST. (C) Immunoblot analysis showing that the mitotic phosphorylation of CHO1-S814^{S807}/MKLP1-pS710 are independent of Aurora-B. HeLa-S3 cells were treated for 10 h with nocodazole (80 ng/ml) with or without the Aurora-B inhibitor hesperadin (Hesp. 100 nM). Lysates were detected using anti-CHO1-S814^{S807} and anti-histone H3-pS10. The intensity of the histone H3-pS10 bands represents the level of activation of Aurora-B. (D) Immunoblot analysis showing the mitotic phosphorylation of CHO1-S814^{S807} and MKLP1-S710. HeLa-S3 cells were treated with mimosine (Mim), thymidine (Thy), taxol (Tax), or nocodazole (Noc). Asynchronous cells (Asy) were used as a control. To prevent cross-reactions with non-phosphorylated CHO1-S814^{S807} and MKLP1-S710, immunoblotting was performed using an anti-CHO1-pS814^{S807} antibody together with non-phosphorylated peptides. The expression level of α-tubulin was used as a loading control. Asterisks show non-specific bands. (E) The cells were transfected with a control (GL2) or CHO1/MKLP1-specific siRNA and synchronized by Taxol (18 h). Immunoblotting was performed using anti-CHO1-pS814^{S807} and anti-CHO1/MKLP1. α-tubulin was used as a loading control. (F) *In vitro* Aurora-A and Aurora-B kinase assays performed using GST-tagged WT or S717A (SA) mutant MmCHO1 fragments (amino acids 692–796) in the presence of [γ-^{32}P] ATP. SimplyBlue staining was used as a loading control. Arrows show the indicated proteins. Asterisks show non-specific bands.

Figure S3

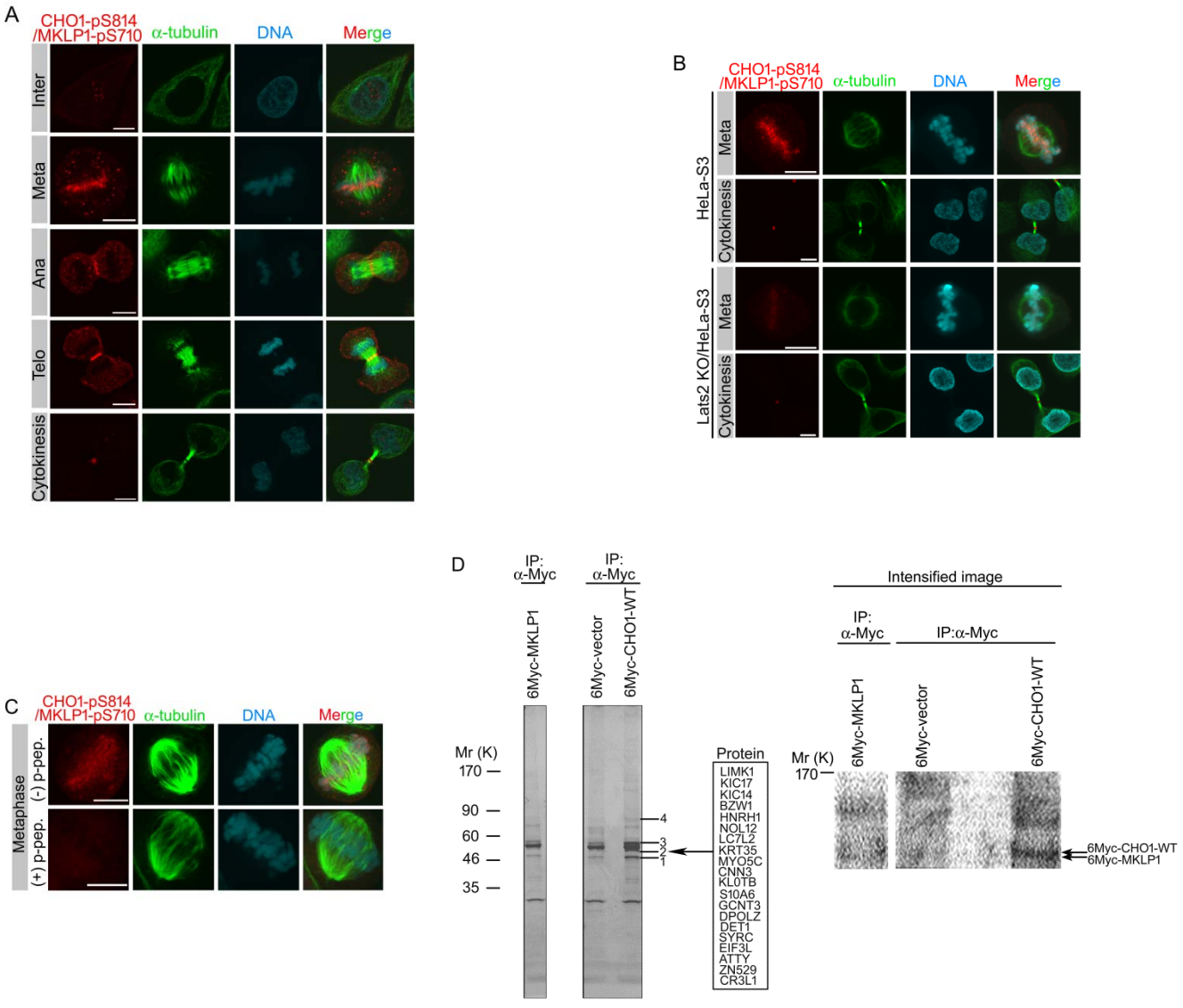

Figure S3. The subcellular localizations of CHO1-pS814^{S807}/MKLP1-pS710 throughout the cell cycle and Identification of LIM-kinase 1 (LIMK1) is an association partner of CHO1.
(A) The localizations of CHO1-pS814/MKLP1-pS710 at different phases of the cell cycle. Synchronized HeLa-S3 cells were immunostained with anti-pS814^{S807} (red), or anti-α-tubulin (green) and counterstained with Hoechst 33258 (blue). Scale bar, 10 μm. (B) Immunostaining of CHO1-pS814^{S807}/MKLP1-pS710 in parental and Lats2 knockout HeLa-S3 cells performed as described for (A).(C) Peptide competition assays in which the anti-pS814^{S807} antibody was pre-incubated with phosphorylated (+) or non-phosphorylated (-) peptides prior to immunostaining.(D) The identification of CHO1 interacting proteins. HeLa-S3 cells were transfected with 6Myc-tagged full-length CHO1-WT, MKLP1, or vector alone. The cell lysates were immunoprecipitated with anti-Myc, separated by SDS-PAGE, and stained with SimplyBlue. The four specific bands were digested, excised from the gel, and then analyzed by MALDI-TOF and LC-ESI-MS/MS. LIMK1 was identified as a candidate CHO1-interacting partner based on the presence of specific peptide fragments in band 2. The other peptides identified in band 2 are also listed. Right panel shows the image intensified by Photoshop.

Figure S4

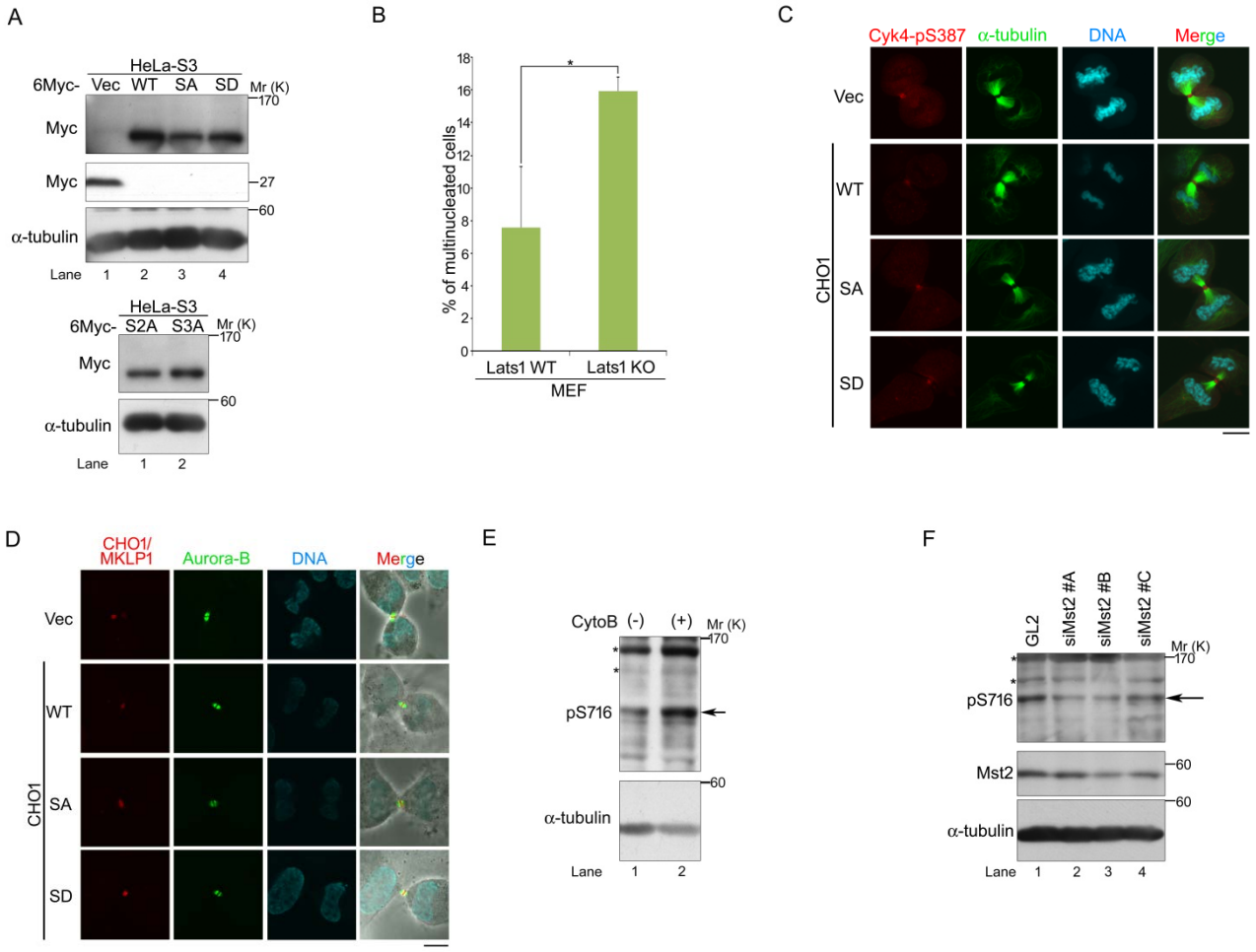

Figure S4. The midbody localization of HsCyk4 and Aurora-B in HeLa-S3 cells expressing CHO1 mutants and F-actin disassembly promotes phosphorylation of CHO1 at S716^{S717}.

(A) Immunoblot analyses of isolated HeLa-S3 clones stably expressing vector alone or 6Myc-tagged CHO1-WT (wild type), -SA (S717A), -SD (S717D), -S2A (S805A/S807A), or -S3A (S717A/S805A/S807A). The expression level of each CHO1 mutant was detected using anti-Myc. The expression level of α-tubulin was used as a loading control. (B) The percentages of multinucleated cells in Lats1 wild-type (WT) and knockout (KO) mouse embryo fibroblasts (MEFs). The MEFs were stained with anti-α-tubulin and Hoechst 33258. Data are represented as the mean ± SD of n=3 experiments (100 cells per experiment). (C) The midbody localization of HsCyk4-pS387 in HeLa-S3 cells stably expressing 6Myc-tagged vector alone or CHO1-WT, -SA, or -SD. The cells were synchronized by thymidine single block-and-release (11 h), immunostained with anti-HsCyk4-pS387 (red) and anti-α-tubulin (green), and counterstained with Hoechst 33258 (blue). Scale bar, 10 μm.

(D) The midbody localization of CHO1/MKLP1 and Aurora-B in HeLa-S3 cells stably expressing 6Myc-tagged vector alone or CHO1-WT, -SA, or -SD. The cells were synchronized by thymidine single block-and-release (11 h), immunostained with anti-CHO1/MKLP1 (red) and anti-Aurora-B (green), and counterstained with Hoechst 33258 (blue). Scale bar, 10 μm. (E) Immunoblot analysis showing the level of endogenous HsCHO1-pS716^{S717} in cells treated with cytochalasin B (CytoB) (5 μg/ml) for 24 h. Cell lysates were separated by SDS-PAGE and detected using anti-pS716^{S717}. The expression level of α-tubulin was used as a loading control. Asterisks indicate non-specific bands. (F) The effect of knockdown of Mst2 kinase on the phosphorylation of S716^{S717} of CHO1. HeLa-S3 cells were transfected with three different Mst2-siRNAs and synchronized by thymidine single block-and-release (10 h). Lysates were detected using anti-S716^{S717} and anti-Mst2. The expression level of α-tubulin was used as a loading control. Asterisks indicate non-specific bands.

Figure S5

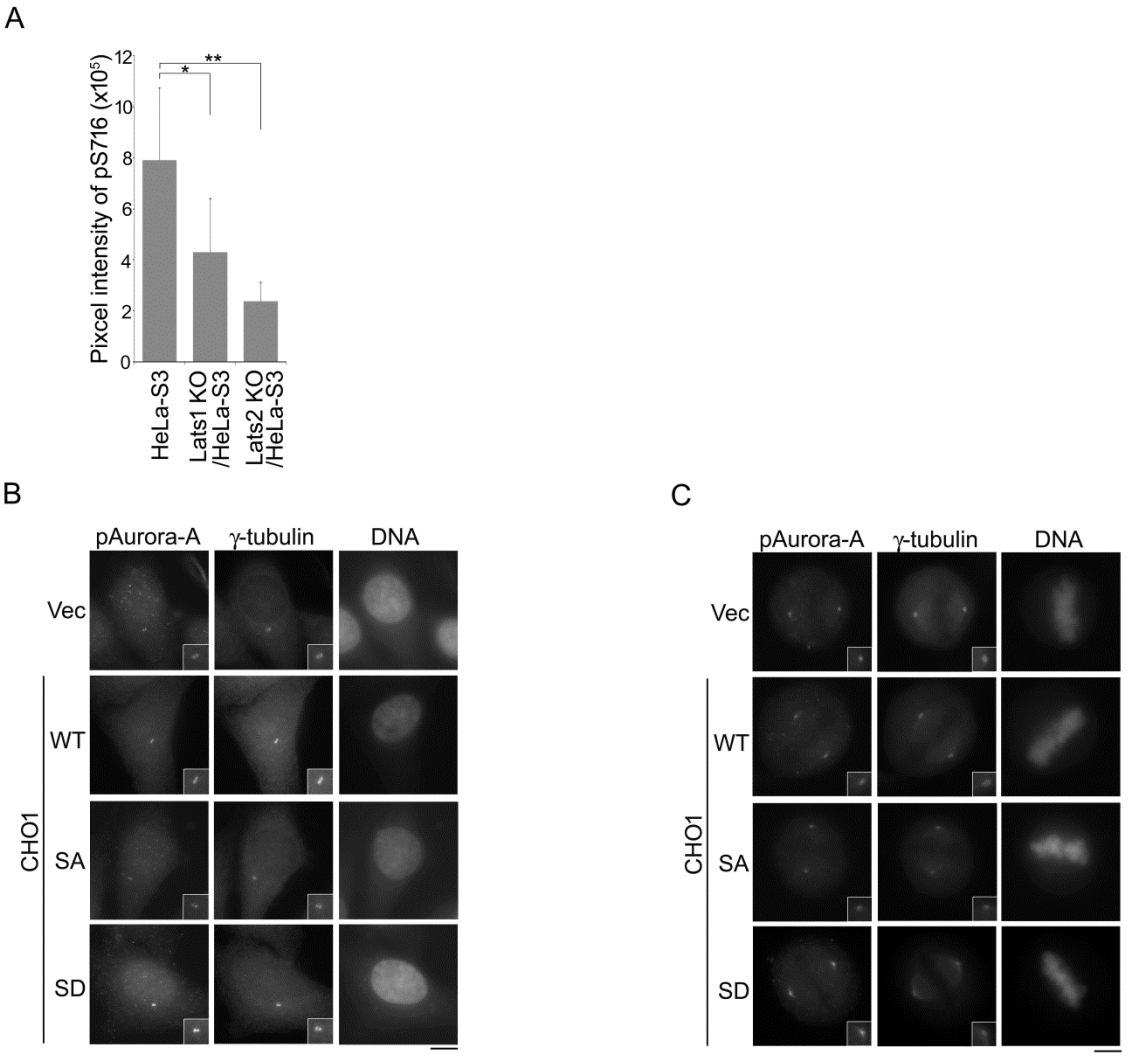

Figure S5. Signal intensities of CHO1-pS716 on centrosomes in Lats1 KO and Lats2 KO cells and the centrosomal localization of Aurora-A-pT288 in HeLa-S3 cells expressing CHO1 during G2 and metaphase.

(A) The signal intensities of CHO1-pS716 on centrosomes in Lats1-KO/HeLa-S3 and Lats2-KO/HeLaS3 cells during metaphase. Data represent the mean ± SD of n=3 independent experiments (30 cells per experiment).

(B, C) Centrosomal localization of Aurora-A-pT288 in HeLa-S3 cells stably expressing vector alone or 6Myc-tagged CHO1-WT, -SA (S717A), or -SD (S717D) during G2 phase (B) and metaphase (C). The cells were synchronized with a single thymidine block-and-release (10 h). The insets show enlarged images of signals at the centrosome. γ-tubulin is a centrosomal marker. Scale bar, 10 μm.

Figure S6

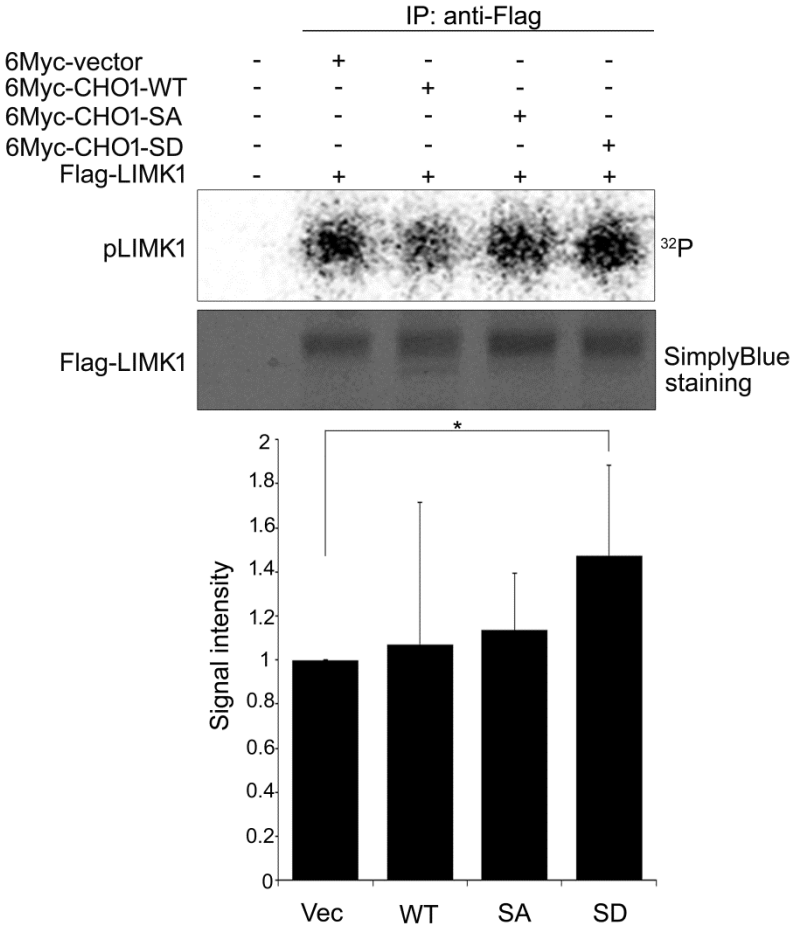

Figure S6. CHO1-S716D increases the kinase activity of LIMK1 *in vitro*.
HeLa-S3 cells stably expressing 6Myc-tagged CHO1 (WT, SA, or SD) were transfected with 3FLAG-tagged LIMK1, followed by immunoprecipitaion with an anti-Flag antibody. *In vitro* kinase assays were performed using the immunoprecipitates in the presence of [γ-^{32}P] ATP. SimplyBlue staining was used as a loading control. The bar graph shows the signal intensity of the pLIMK1/Flag-LIMK1 ratio. Data are represented as the mean ± SD of n=3 experiments.