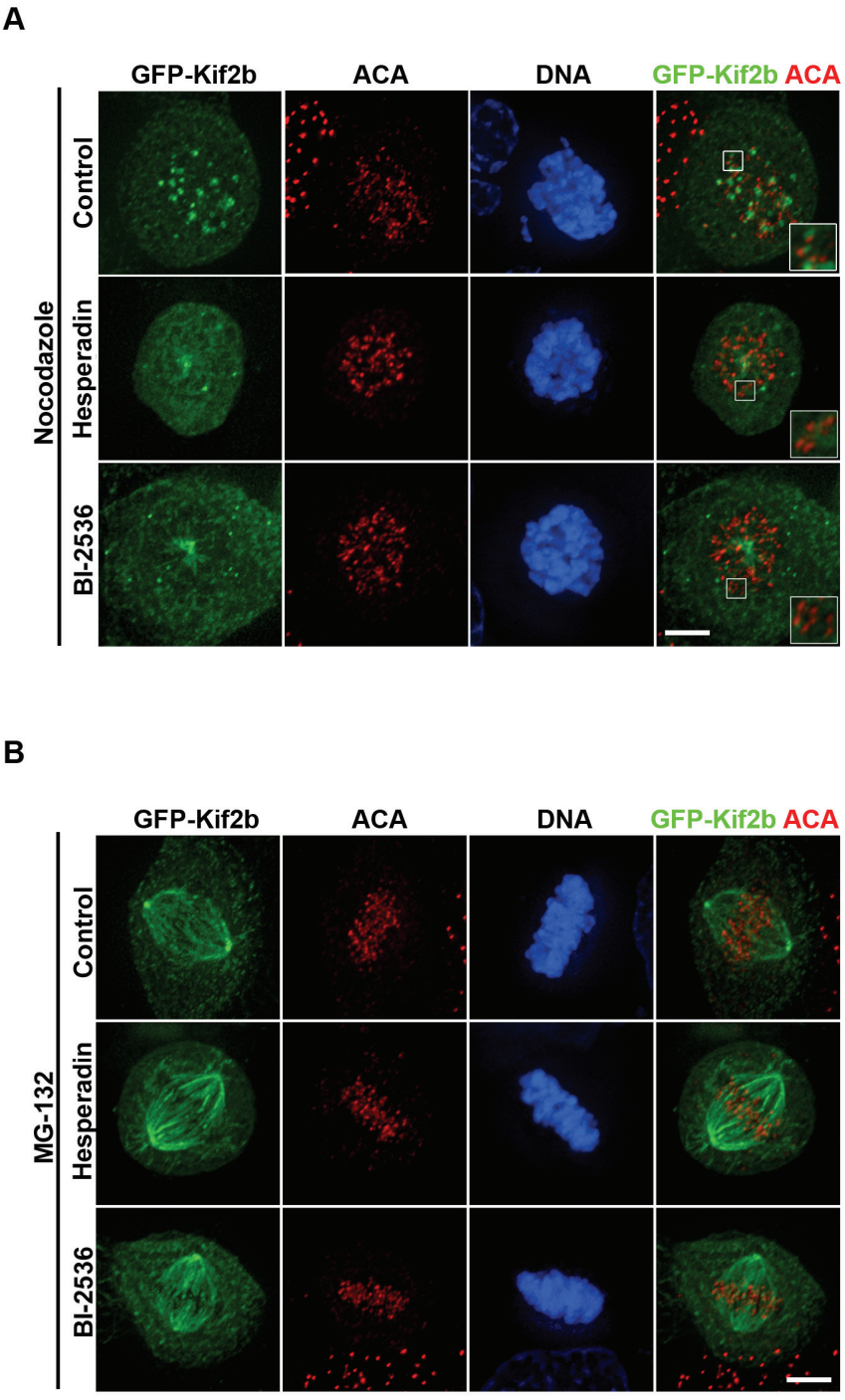
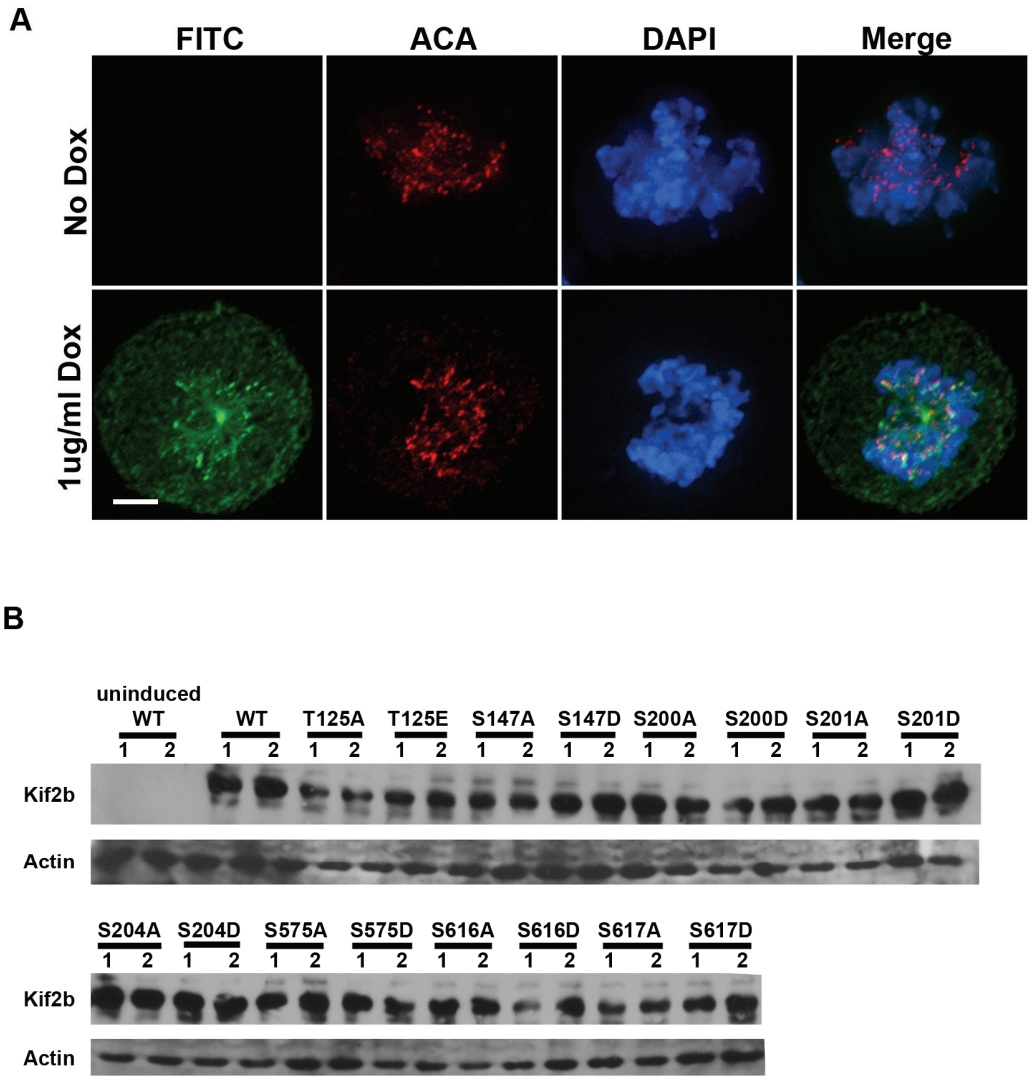
Supplementary Figure Legends



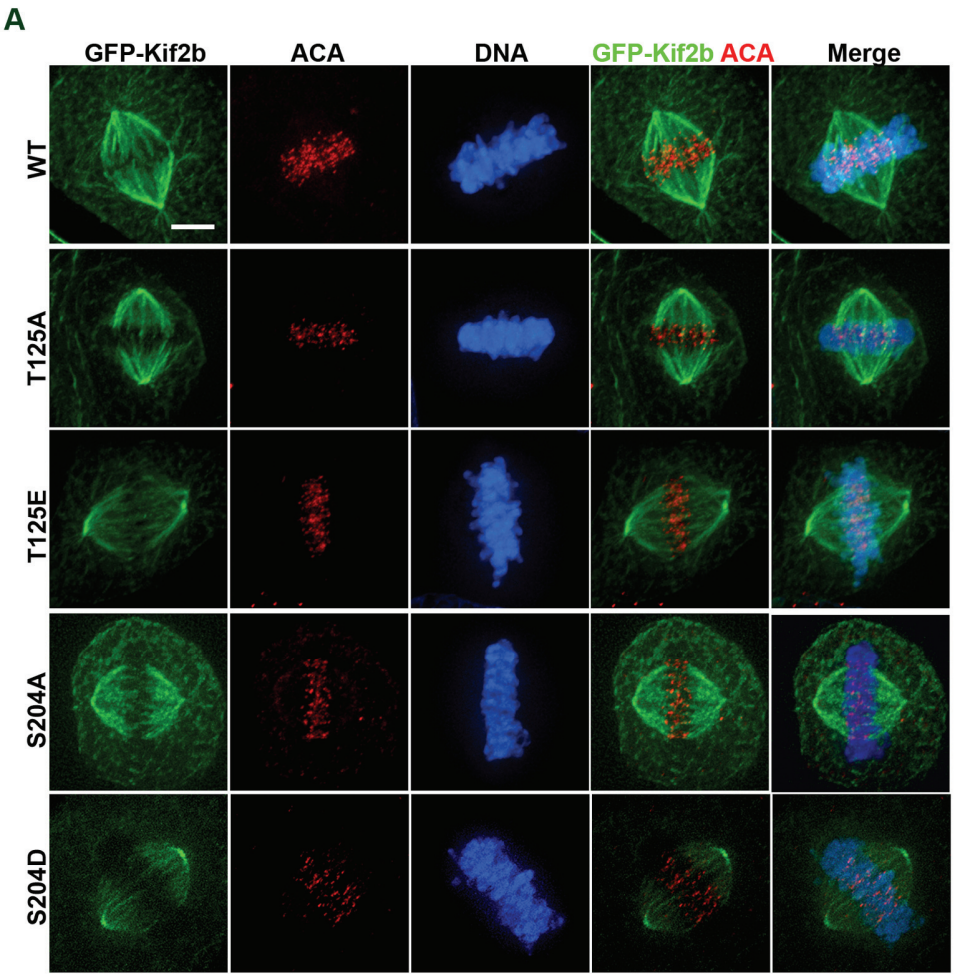
Supplementary Figure 1. GFP-Kif2b kinetochore localization is sensitive to chemical inhibition of Aurora kinase and Plk1 kinase. Representative images of nocodazole-

treated (A) and MG-132-treated (B) control, Aurora inhibited (Hesperadin), and Plk1 inhibited (BI-2536) Human U2OS cells stably expressing GFP-Kif2b (green) and stained for DNA (blue) and centromeres (red). Scale bar: 5 μm.

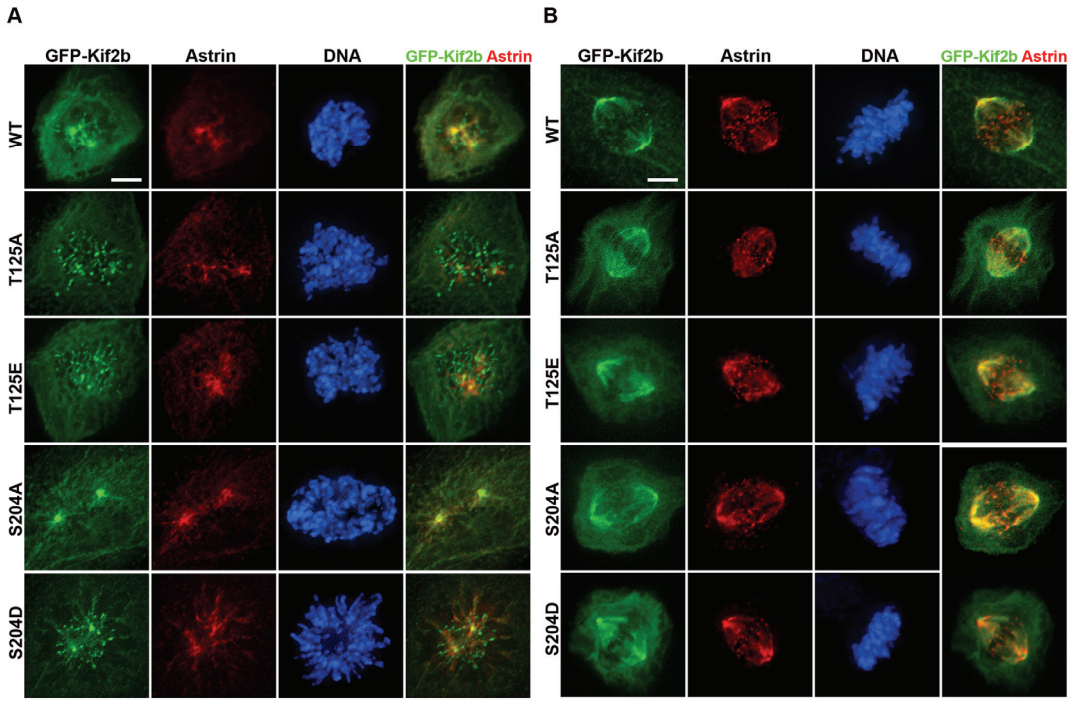
Supplementary Figure 2. Scheme of SILAC strategy to identify Kif2b phosphorylation sites. U2OS cells stably expressing GFP-Kif2b were labeled with either isotopically normal or “heavy” arginine and lysine were arrested in prometaphase (nocodazole) or metaphase (MG-132) and treated with BI-2536 or hesperadin. Light cells were arrested with nocodazole or MG-132 and without kinase inhibitors. Afterwards, cells were counted, mixed, lysed, and Kif2b was immunoprecipitated with anti-Kif2b antibody. Immunoprecipitates were separated by SDS-PAGE, visualized with Coommassie stain, and the band corresponding to Kif2b was excised, tryspin digested, and analyzed by LC-MS/MS.



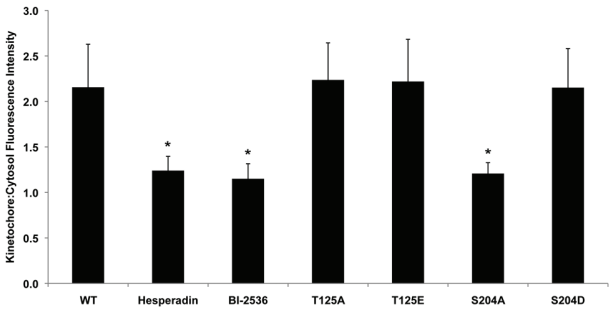
Supplementary Figure 3. Inducible expression of wild-type and mutant GFP-Kif2b protein. (A) Representative images of U2OS cells stably carrying wild-type GFP-Kif2b (green) with and without doxycycline. Cells were fixed and stained for DNA (blue) and centromeres (red). Scale bar: 5 μm. (B) Kif2b protein from U2OS cells expressing wild-type or mutant GFP-Kif2b. Total cell lysates were resolved by SDS-PAGE, and GFP-Kif2b and actin were detected by immunoblot with anti-Kif2b and anti-actin. Two clones of each cell type are represented.



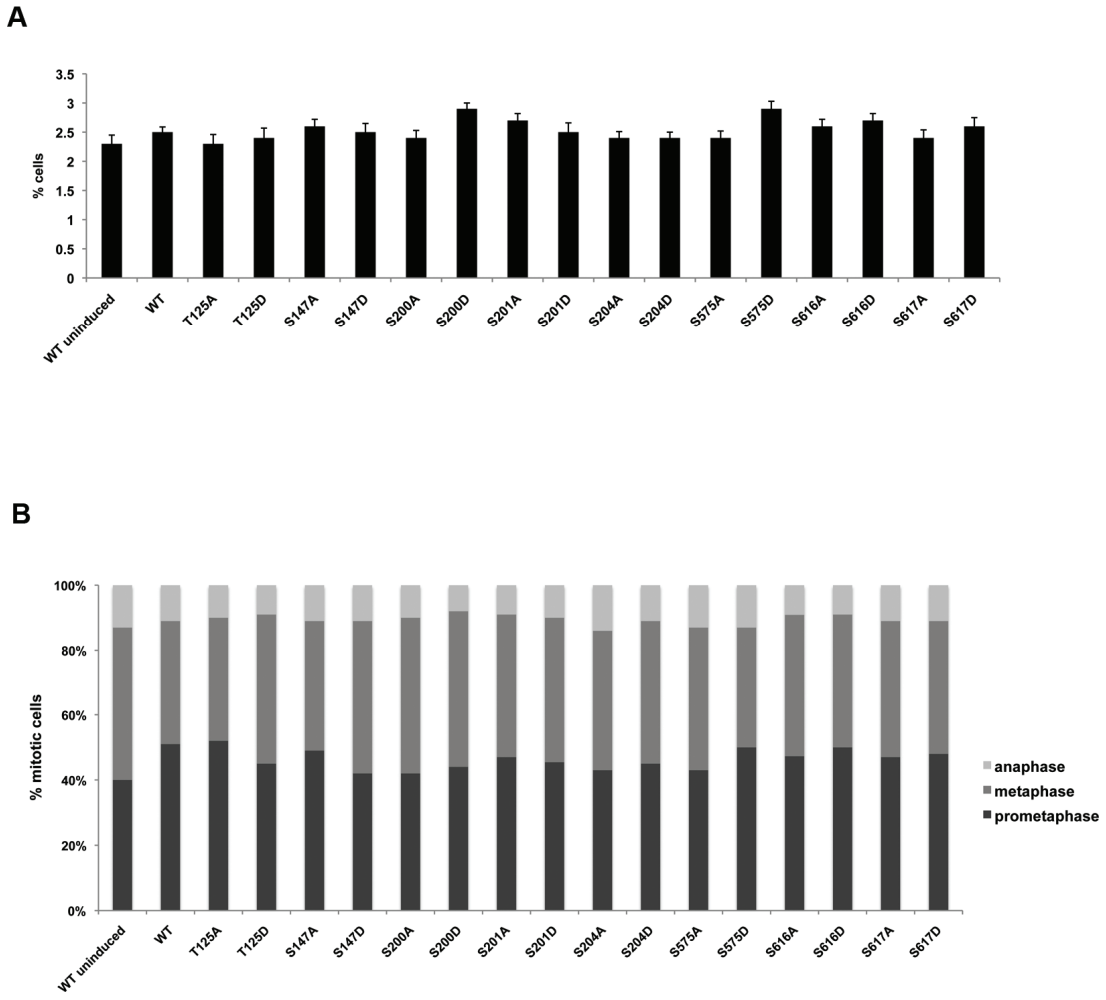
Supplementary Figure 4: GFP-Kif2b phospho-mutants are efficiently removed from kinetochores in metaphase. (A) Representative images of metaphase U2OS cells expressing wild-type or mutant GFP-Kif2b (green). Cells were fixed and stained for DNA (blue) and centromeres (red). Scale bar: 5 μm.



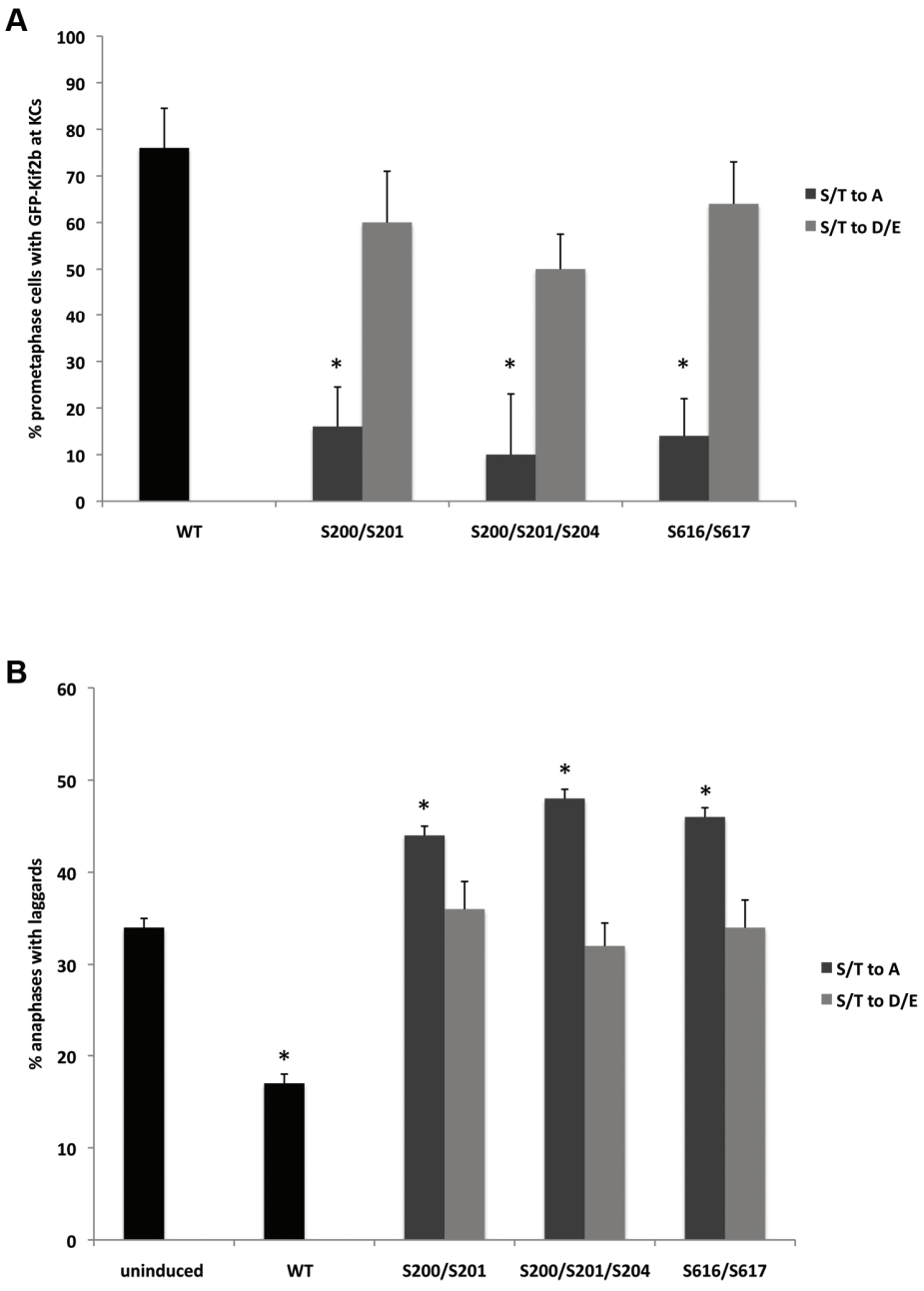
Supplementary Figure 5: Astrin localizes normally in cells expressing GFP-Kif2b mutants. Representative images of (A) prometaphase and (B) metaphase U2OS cells expressing wild-type or mutant GFP-Kif2b (green). Cells were fixed and stained for Astrin (red) and DNA (blue). Scale bar: 5 μm.



Supplementary Figure 6: Quantification of fluorescence intensity of GFP-Kif2b mtuants at kinetochores. Fluorescence intensity for GFP-Kif2b and CREST are reported as ratios of kinetochore fluorescence to cytosolic fluorescence. Error bars represent SD for each data set. *p < 0.0001, t-test, n = at least 75 kinetochores, 10 cells per condition.



Supplementary Figure 7: Mitosis progresses normally in cells stably expressing GFP-Kif2b mutants. (A) Percent of expressing cells in mitosis. Error bars represent mean ± SEM. *$p < 0.05$, t-test, n = 4000 cells from two clonal populations, two experiments each. (B) Percent mitotic cells in prometaphase, metaphase, and anaphase. n = 400 cells from two clonal populations, two experiments each.



Supplementary Figure 8. Localization and functional activity of GFP-Kif2b with multiple mutations. (A) Percent of prometaphase cells with GFP-Kif2b localized to kinetochores. Error bars represent mean ± SEM. *p < 0.05, chi-squared test, n = 400 cells from two clonal populations, two experiments each. (B) Percent of anaphase cells with lagging

chromosomes in cells expressing wild-type or mutant GFP-Kif2b compared to U2OS cells in which expression has not been induced. Error bars represent mean ± SEM. *$p < 0.05$, chi-squared test, n = 400 cells from two clones, two experiments each.

Supplementary Tables (see attached spreadsheets)

Supplementary Table 1: Phosphorylation sites and peptides identified by LC-MS/MS of GFP-Kif2b protein isolated from mitotically synchronized cells. PPM, mass measurement accuracy in parts per million; XCorr, SEQUEST cross-correlation score; dCn, SEQUEST delta-correlation score.

Supplementary Table 2: Phosphorylation sites, peptides, and phosphorylation ratios identified by LC-MS/MS of GFP-Kif2b protein from SILAC experiments. PPM, mass measurement accuracy in parts per million; XCorr, SEQUEST cross-correlation score; dCn, SEQUEST delta-correlation score.

Supplementary Table 3: Summary of endogenous Kif2b phosphorylation sites identified by LC-MS/MS of protein isolated from mitotically synchronized cells.

Supplementary Table 4: Summary of recombinant Kif2b phosphorylation sites identified by LC-MS/MS from *in vitro* Plk1 kinase assay.