Supplementary Materials

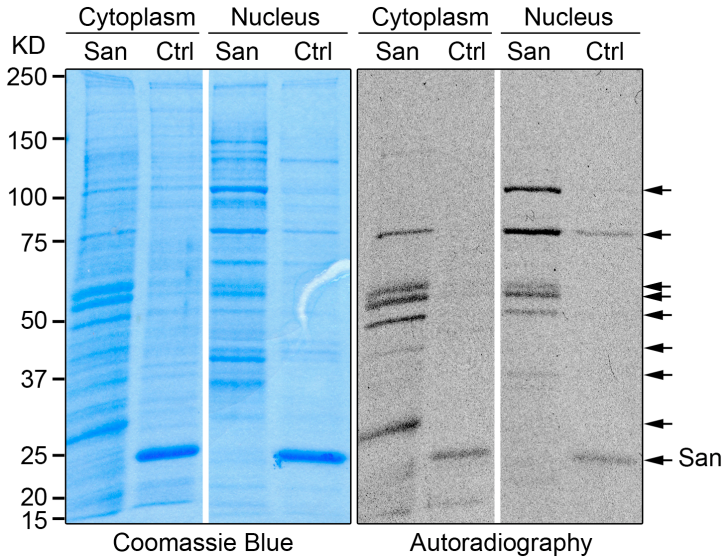

Figure S1. Affinity purification of San substrates. 293T cell fractions were incubated with either San-conjugated beads (San) or empty beads (Ctrl). The beads were washed three times with buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT), followed by one wash with the acetylation buffer omitting NaCl. Proteins bound to the beads were subjected to in vitro acetylation by San, with additional recombinant San added to the empty beads sample. After resolving the proteins by SDS-PAGE, the gel was stained by Coomassie Blue (left panel) and exposed to X-ray film (right panel). Arrows mark the proteins radiolabeled by San. In the San beads sample, San proteins were covalently linked to the beads and therefore not visible on the gel.

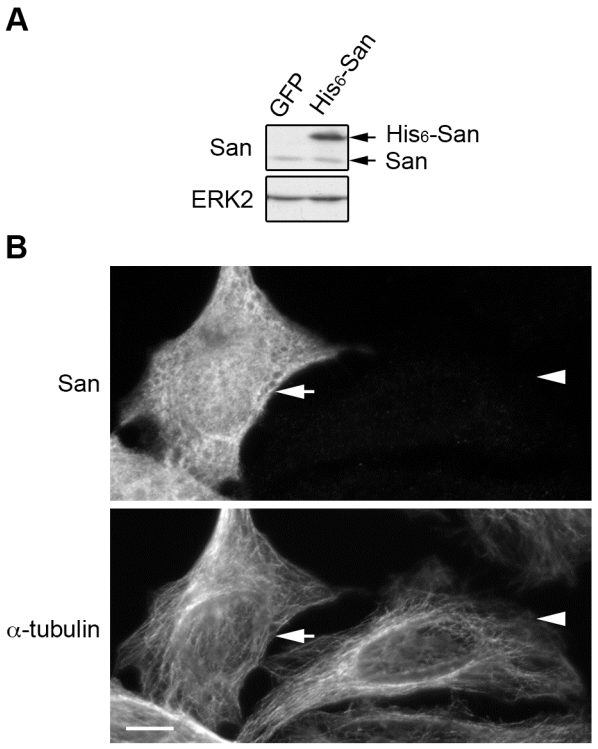

Figure S2. Overexpression of San has no effect on the MT organization in HeLa cells. (A) HeLa cells transiently expressing GFP or His-tagged San were analyzed by immunoblot. ERK2 serves as the loading control. (B) HeLa cells expressing His-tagged San were stained for San and α-tubulin. Arrows indicate the cell strongly expressing His-tagged San. Arrowheads indicate a cell not overexpressing San, and the staining of endogenous San is barely visible with the exposure time used here. Scale bar, 10 μm.

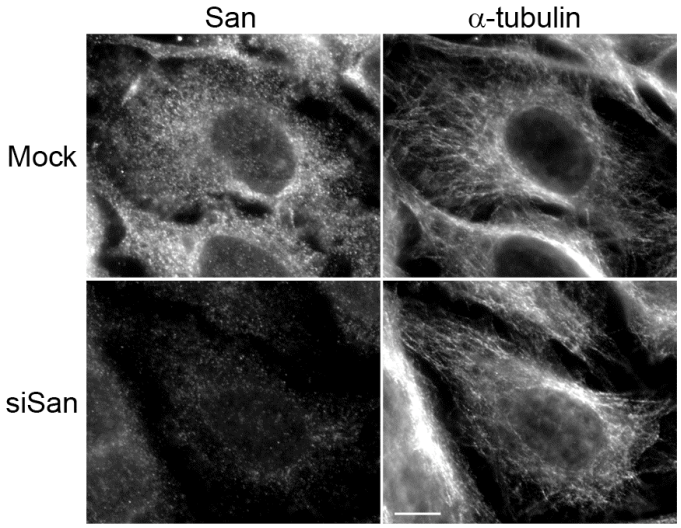

Figure S3. Depletion of San has no effect on the MT organization in HeLa cells. HeLa cells transfected with mock or siRNA oligo targeting San (oligo B) were stained for San and α-tubulin. Scale bar, 10 μm.

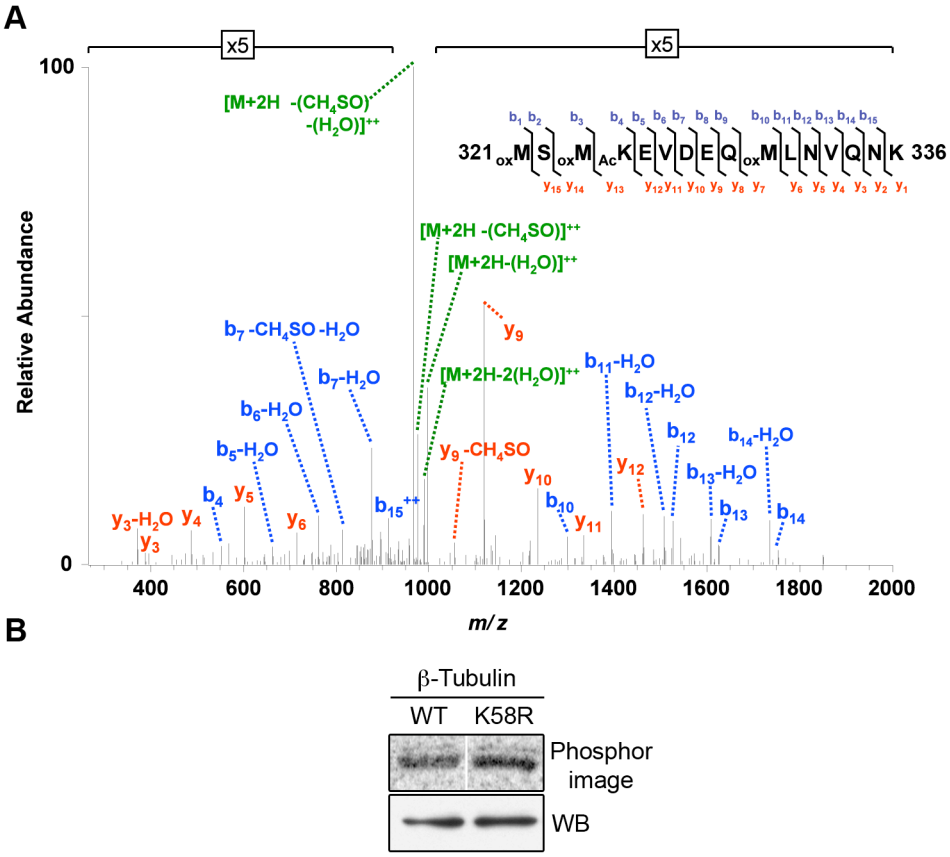

Figure S4. (A) Tandem MS spectra demonstrating in vivo acetylation of β_I-tubulin at K324 as described in Fig. 5. (B) San does not acetylate β-tubulin K58 in vitro**.** The K58R tubulin mutant expressed in 293T cells were purified on anti-flag affinity gel and eluted with TEV protease. The acetylation of the mutant was analyzed by the in vitro San acetylation assay.

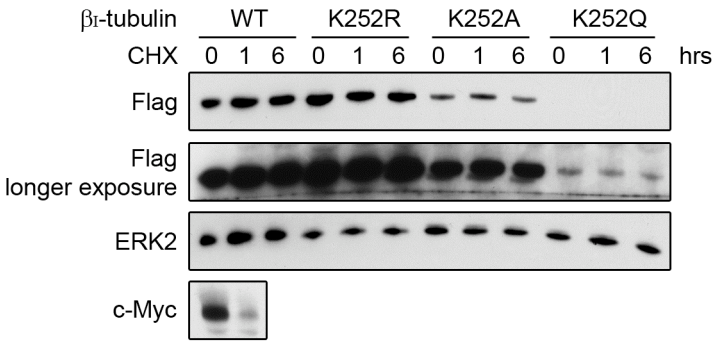

Figure S5. The β-tubulin K252 mutants are stable for 6 hours. HeLa cells expressing flag-tagged tubulin mutants were treated with cycloheximide (CHX) for indicated time and analyzed by immunoblot. ERK2 served as the loading control. The reduced level of c-Myc indicates that protein synthesis was effectively halted by cycloheximide.

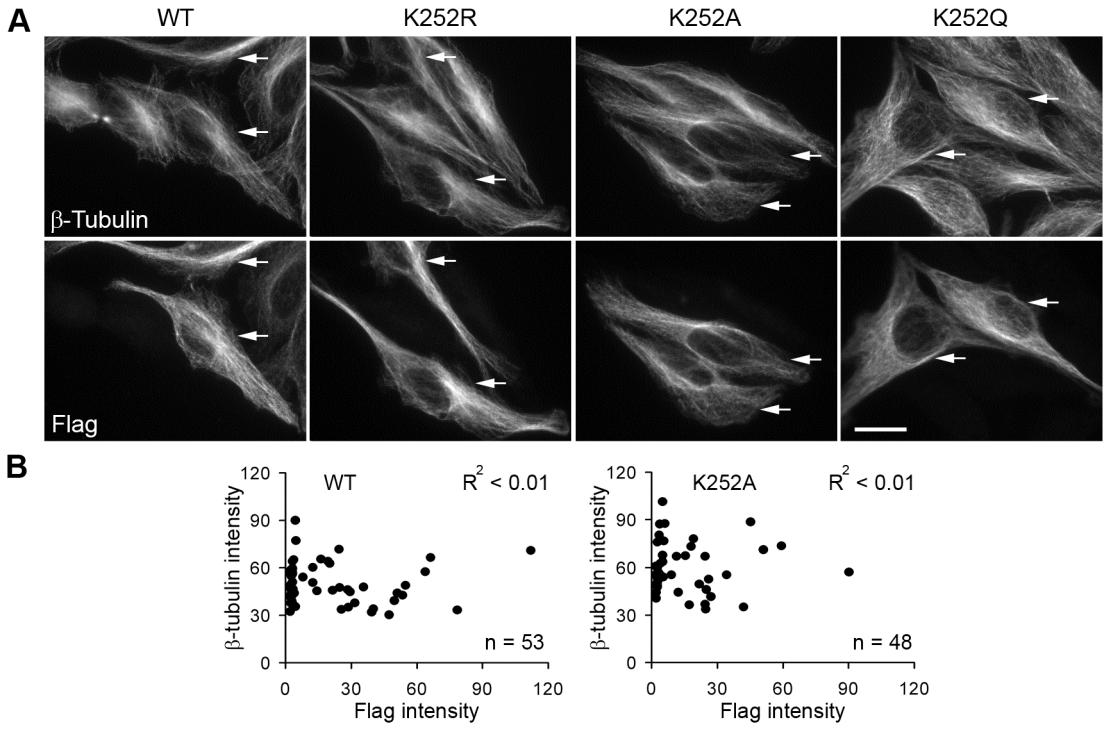

Figure S6. Flag-tagged β-tubulins were not overexpressed in HeLa cells. Cells transiently transfected with indicated tubulin constructs were stained for β-tubulin and flag. Representative images are shown in (A). Arrows indicate cells strongly expressing flag-tagged tubulins. Note that expression of the flag-tagged tubulin did not significantly increase the staining intensity of total β-tubulin. Scale bar, 20 μm. (B) The protein levels of total β-tubulin and flag-tagged β-tubulin were measured by quantitative image analysis. After applying a fixed threshold to the image to subtract the background, the mean fluorescence intensity (counts/pixel) within a cell was determined by outlining the cell edge in ImageJ 1.42q (National Institutes of Health, Bethesda, MD). Each data point represents an individual cell, with its β-tubulin staining intensity plotted as y and flag staining intensity as x. Linear regression analysis of the scatter plots was performed using Microsoft Excel with the correlation coefficient (R^2) indicated. No significant correlation is detected between the intensities of total β-tubulin and flag-tagged β-tubulin.

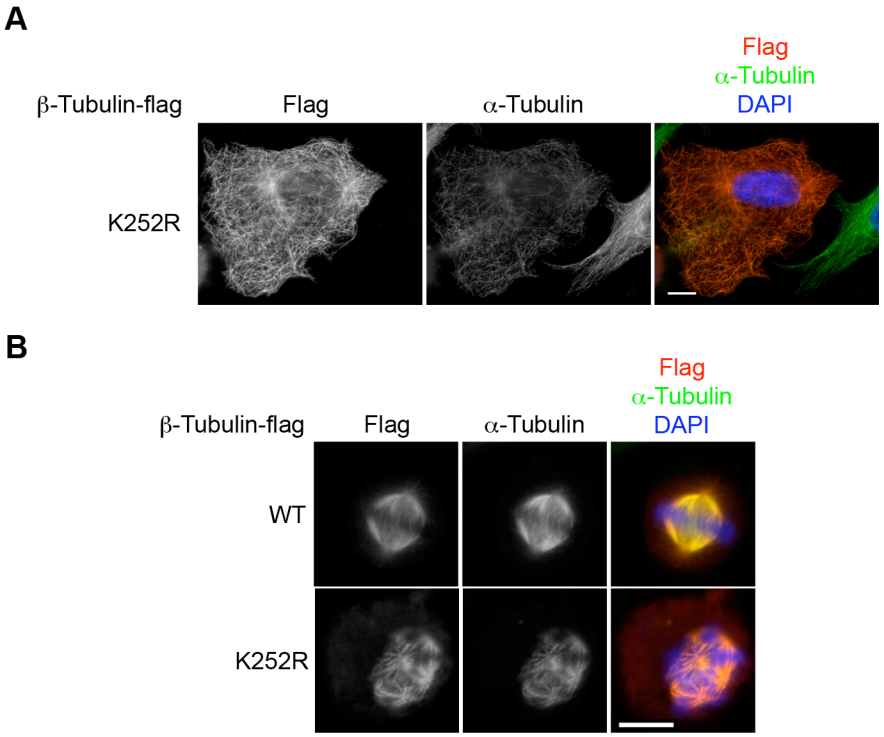

Figure S7. The K252R mutant disrupts microtubule cytoskeleton in interphase and mitotic cells. HeLa cells transiently expressing K252R mutant were stained for tubulin mutants (flag, red), microtubules (α-tubulin, green) and DNA (DAPI, blue). Representative images are shown. Expression of the K252R mutant had two distinct effects on interphase microtubule organization (A) and affected mitotic spindle organization (B). Scale bar, 10 μm.

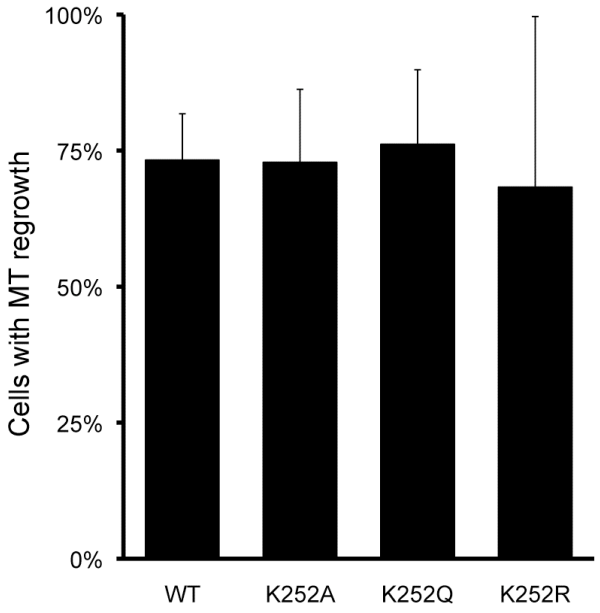

Figure S8. Microtubule (MT) regrowth rate is not affected by expression of the β-tubulin K252 mutants. HeLa cells expressing indicated flag-tagged tubulin mutants were subjected to microtubule regrowth assay for 2.5 minutes of regrowth, and the average percentage of flag-stained cells with microtubule regrowth was determined from three independent experiments. Error bars indicate SD.

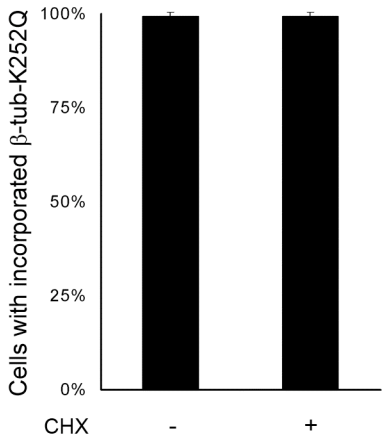

Figure S9. Incorporation of the β-tubulin K252Q mutants is not affected by cycloheximide treatment. HeLa cells expressing flag-tagged K252Q mutants were subjected to microtubule regrowth assay for 60 minutes of regrowth in the presence or absence of cycloheximide (CHX). The average percentage of flag-stained cells with flag-positive microtubules was determined from three independent experiments. 50 cells were counted in each experiment. Error bars indicate SD.

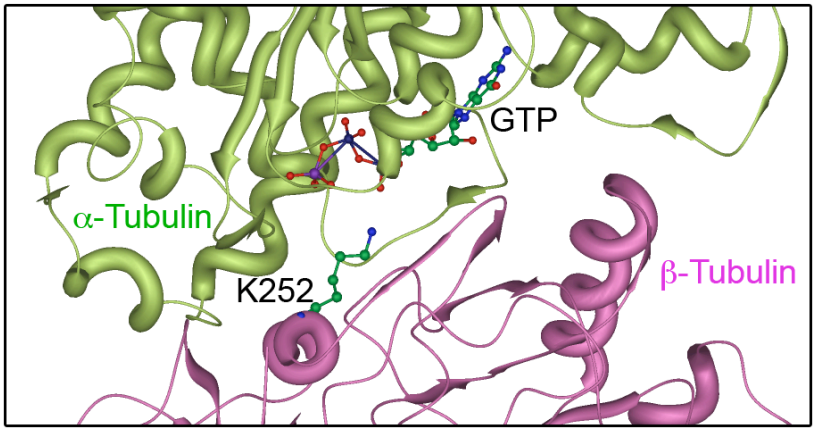

Figure S10. The location of β-tubulin K252 on the structure of tubulin heterodimer. Tubulin structure is obtained from zinc-induced tubulin sheets (PDB: 1JFF). The α-tubulin-bound GTP and β-tubulin K252 are shown as ball-and-stick models.