Chan et al., http://www.jcb.org/cgi/content/full/jcb.200812167/DC1

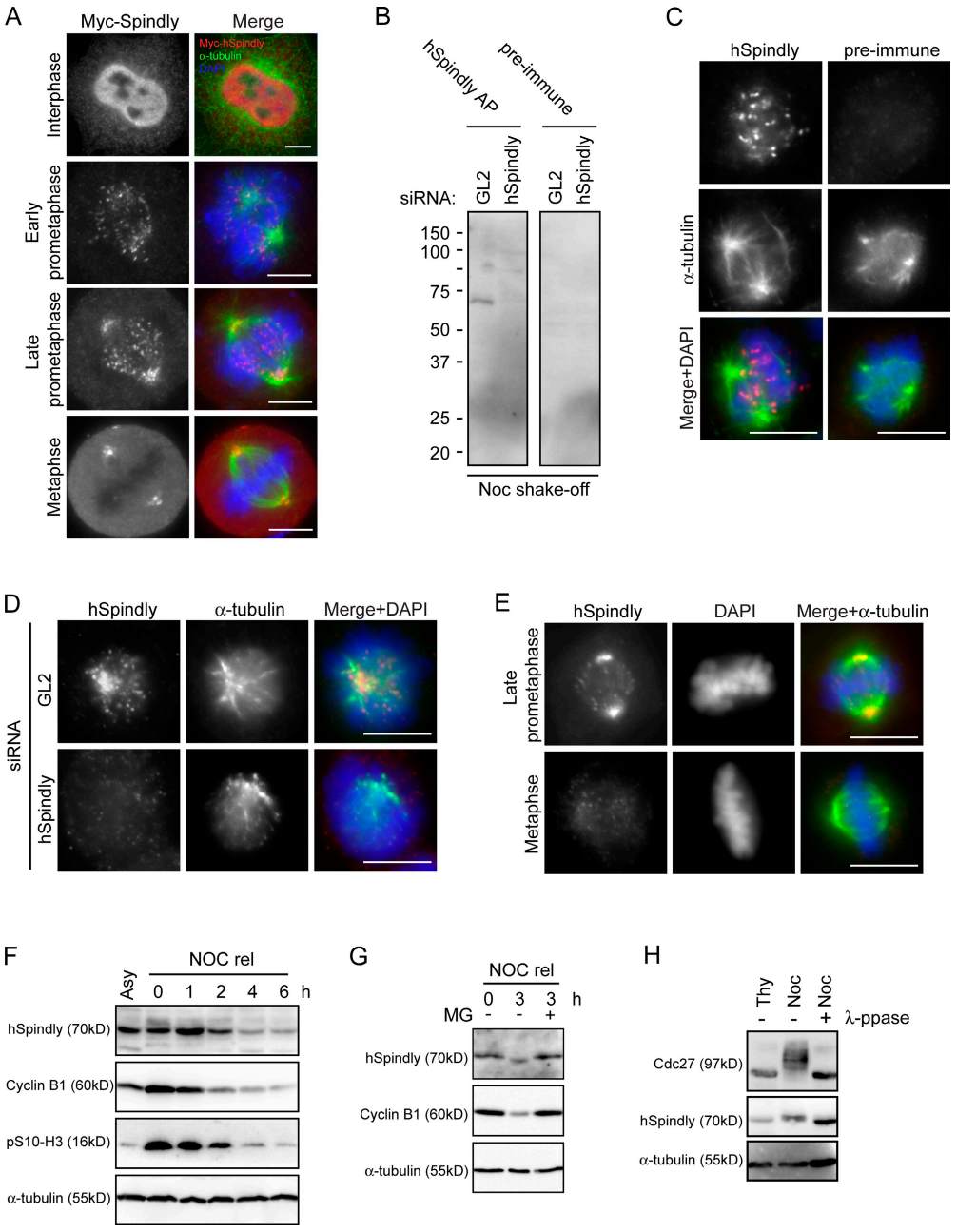


Figure S1. **Characterization of hSpindly.** (A) HeLa S3 cells were transfected with myc-tagged hSpindly construct for 48 h and stained with anti-myc 9E10 serum (red), anti–α-tubulin antibody (green), and DAPI (blue). (B) Mitotic cells (nocodazole shake-off) treated with GL2 or hSpindly siRNAs for 48 h were collected, and equal amounts of cell extracts were separated by SDS-PAGE. Then, proteins were probed by Western blotting with either affinity-purified anti-hSpindly antibody (AP) (left) or pre-immune serum (right). (C) Cells were stained with either anti-hSpindly antibody or pre-immune serum (red), together with anti–α-tubulin antibody (green) and DAPI (blue). (D) Cells were treated with GL2 or hSpindly siRNAs for 48 h and stained with anti-hSpindly (red) and anti–α-tubulin antibodies (green) and DAPI (blue). (E) Cells were stained with anti-hSpindly (red) and anti–α-tubulin antibodies (green) and DAPI (blue). (F) Mitotic cells (nocodazole shake-off) were washed with PBS, released into fresh medium, and collected at indicated time points. Lysates were prepared and separated by SDS-PAGE and probed by Western blotting with indicated antibodies. Asynchronous cells were used as control. (G) Mitotic cells (nocodazole shake-off) were washed with PBS, released into fresh medium in the presence or absence of MG132, and collected after 3 h. Lysates were prepared and separated by SDS-PAGE and probed by Western blotting with indicated antibodies. (H) Cells were treated with thymidine or nocodazole overnight. Lysates were prepared and treated with λ-phosphatase as indicated, before proteins were separated by SDS-PAGE and probed by Western blotting with indicated antibodies. Bars = 10 μm.

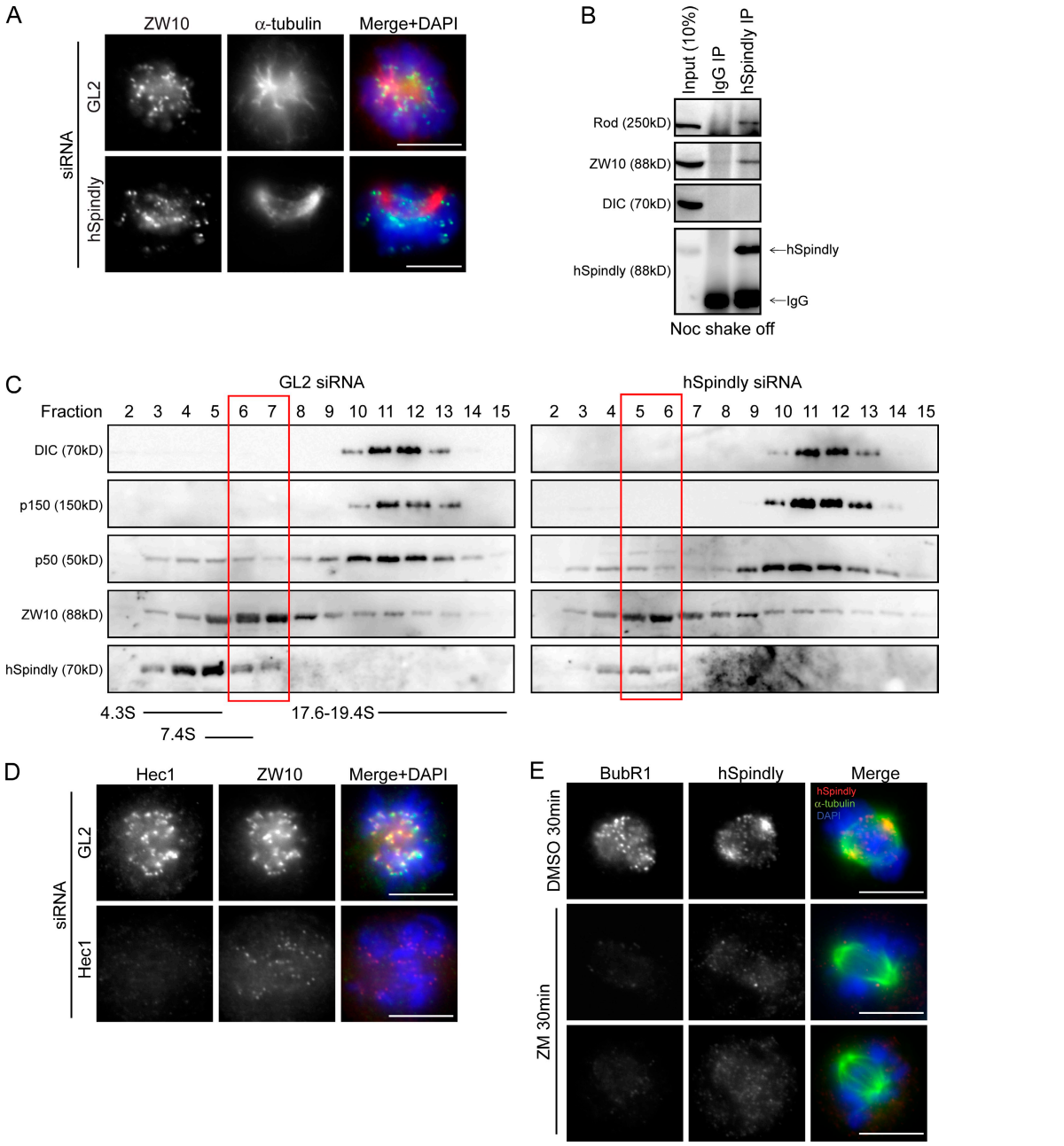


Figure S2. **hSpindly interacts with the RZZ complex.** (A) HeLa S3 cells were treated with GL2 or hSpindly siRNAs for 48 h and stained with anti-ZW10 (red) and anti–α-tubulin antibodies (green) and DAPI (blue). (B) Mitotic cells were collected by nocodazole shake-off. Cells were resuspended in Hepes buffer and opened by nitrogen cavitation. Immunoprecipitation was performed with either IgGs as control or the anti-hSpindly antibody. (C) GL2- or hSpindly siRNA–treated mitotic cells (nocodazole shake-off) were analyzed by glycerol gradient centrifugation (10–25%). Each sample was separated into 27 equal fractions. Fractions 2–15 were resolved by SDS-PAGE and probed by Western blotting with indicated antibodies. Sedimentation coefficients were determined using standard proteins. Red rectangles indicate fractions containing maximum amounts of ZW10. (D) Cells were treated with GL2 or Hec1 siRNAs for 48 h and stained with anti-Hec1 (green), anti-ZW10 antibodies (red), and DAPI (blue). (E) Cells were treated with either DMSO or ZM447439 (ZM) for 30 min and stained with anti-BubR1, anti-hSpindly (red), and anti–α-tubulin antibodies (green), and DAPI (blue). Bars = 10 µm.

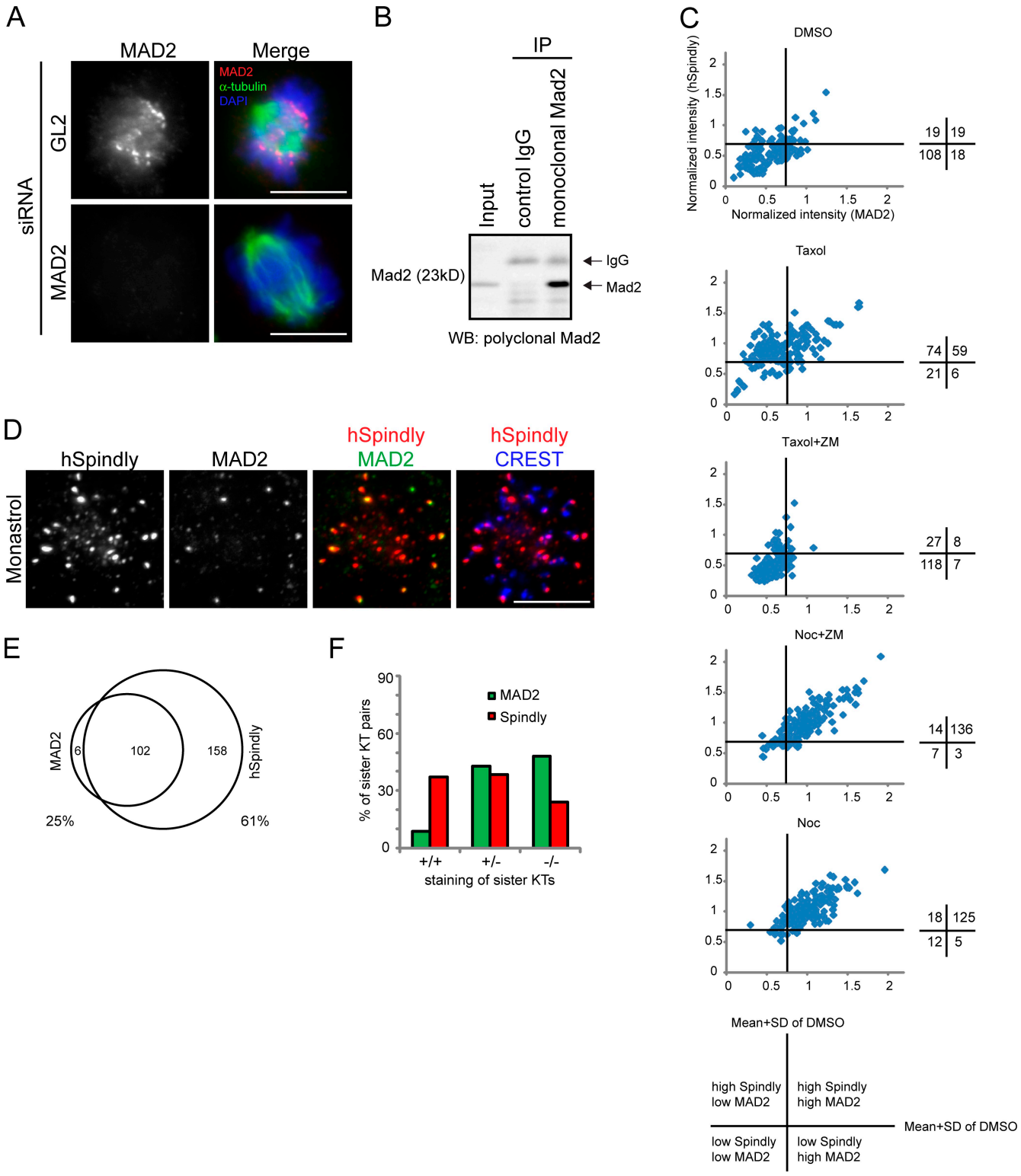


Figure S3. **KT localization of hSpindly in taxol- or monastrol-treated cells.** (A) HeLa S3 cells were treated with GL2 or MAD2 siRNAs for 48 h and stained with anti-MAD2 serum (red), anti–α-tubulin antibody (green), and DAPI (blue). (B) Mitotic cells (nocodazole shake-off) were lysed and immunoprecipitation was performed with either IgGs as control or the newly generated monoclonal MAD2 antibody. Membranes were probed with a polyclonal MAD2 antibody. Note that the newly generated monoclonal antibody can be used for immunofluorescence and immunoprecipitation but not for Western blotting. (C) The normalized intensities of hSpindly and MAD2 staining of cells in Fig. 2 C were plotted in X-Y graphs. The horizontal and vertical lines in the graphs represent the mean + SD of control cells treated only with MG132 and DMSO. The number of KTs in corresponding categories is shown to the right. (D–F) Cells were treated with monastrol for 16 h and analyzed as in Fig. 2, C–E, except that they were stained with anti-MAD2 antibody (green) instead of anti-BubR1 antibody. In E, 428 KTs from 10 cells were counted in total. Percentages of KTs positive for MAD2 or hSpindly are shown. Bars = 10 µm.

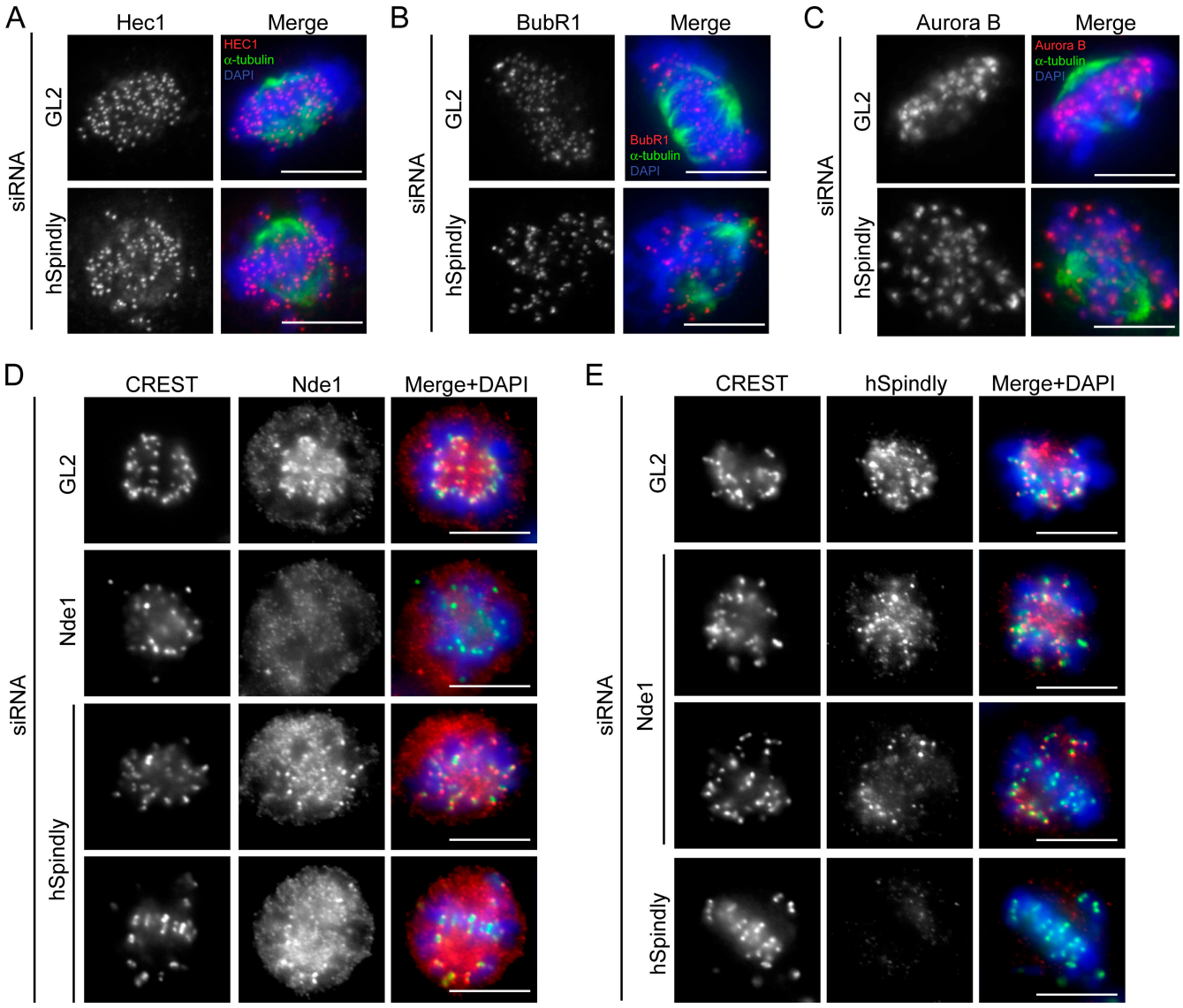


Figure S4. **Localization of KT/centromere proteins upon hSpindly depletion.** HeLa S3 cells were treated with GL2 or hSpindly siRNAs for 48 h and then stained with anti-Hec1 antibody (A), anti-BubR1 serum (B), or anti-Aurora B antibody (C, red), together with anti–α-tubulin (green) antibody and DAPI (blue). (D) Cells were treated with GL2, hSpindly, or Nde1 siRNAs for 48 h and stained with CREST serum (green), anti-Nde1 antibody (red), and DAPI (blue). (E) Cells treated as in D, but stained with anti-hSpindly antibody (red) instead of anti-Nde1 antibody. Bars = 10 μm.

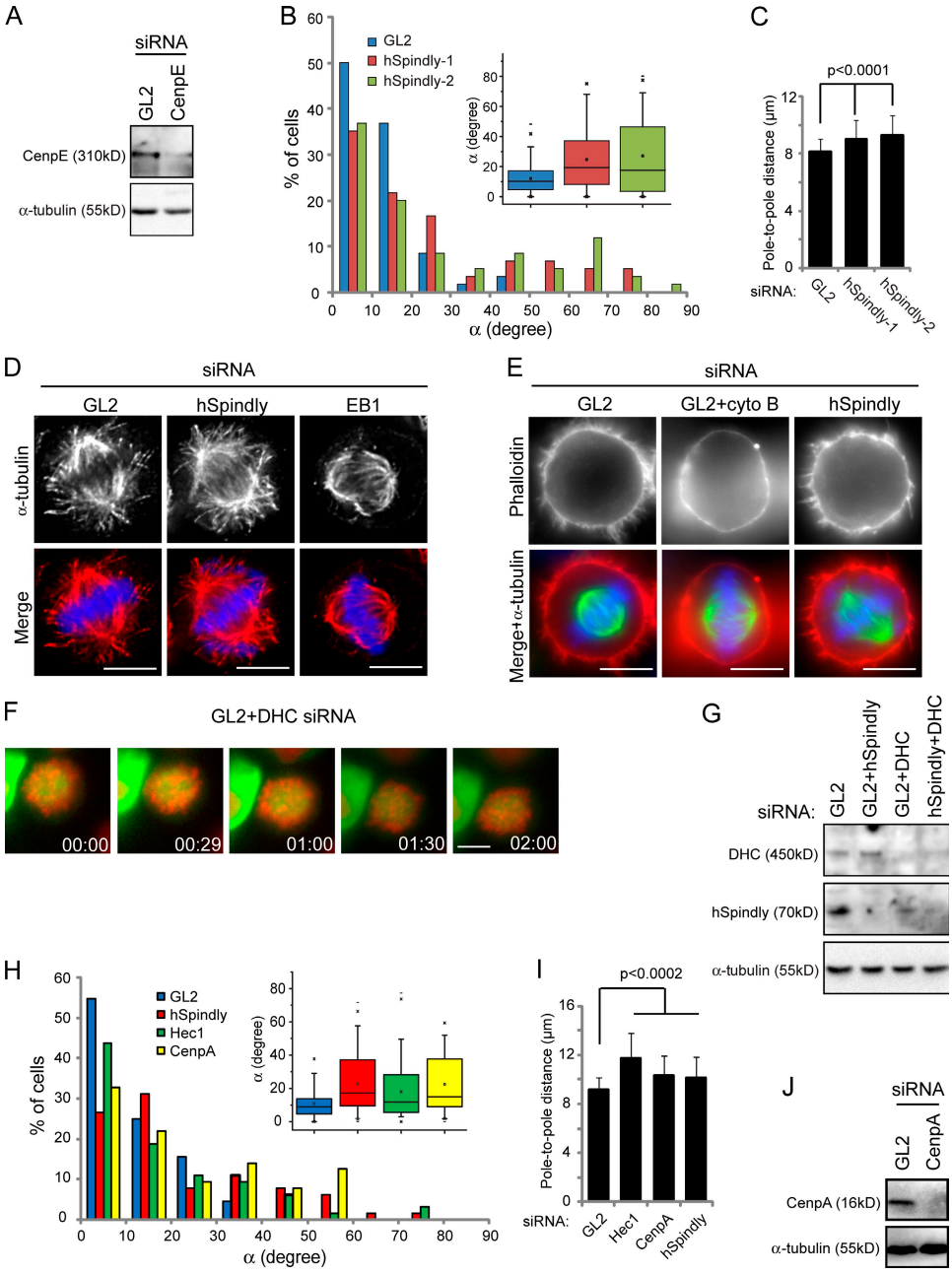
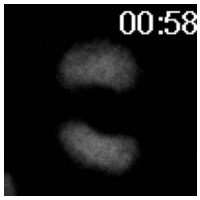
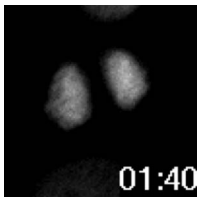


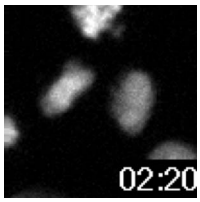
Figure S5. **hSpindly depletion induces spindle misorientation and increases spindle length.** (A) HeLa S3 cells were treated with GL2 or CenpE siRNAs for 48 h. Lysates were separated by SDS-PAGE and probed by Western blotting with anti-CenpE antibody; α-tubulin is shown as loading control. (B) Cells were treated with GL2, hSpindly-1, or hSpindly-2 siRNAs for 48 h. MG132 was added for 2 h before fixation. Spindle angles were calculated by the same method as used in Fig. 8 C, except that centrin-3 was used as a centriolar marker and Z-stacks were taken every 0.2 μm. Only bipolar mitotic cells with well-separated spindle poles (x > 6 μm) were counted (60 cells, P < 0.0002). (C) Bar graph showing the pole-to-pole distances (x) of the cells in B. Error bars show the SD after performing measurements on 60 cells. (D) Cells were treated with GL2, hSpindly, or EB1 siRNAs for 48 h and stained with anti–α-tubulin (red) and DAPI (blue). (E) Cells were treated with GL2 or hSpindly siRNAs for 48 h. Cytochalasin B (cyto B) or DMSO was added to the cells 1 h before they were stained with anti–α-tubulin (red) and DAPI (blue). (F) Stills of a DHC-depleted cell displaying defects in bipolar spindle formation. Time is shown in h:min. (G) HeLa Kyoto cells stably expressing GFP-α-tubulin/cherry-H2B were treated with different combinations of siRNAs as indicated for 48 h. Lysates were prepared and equal amounts of cell extracts were separated by SDS-PAGE and probed by Western blotting with indicated antibodies. (H) Cells were treated with GL2, hSpindly, Hec1, or CenpA siRNAs for 48 h and MG132 was added for 1 h before fixation. Spindle angles were calculated by the same method as used in Fig. 8 C. ≥60 cells were measured (P < 0.0002). (I) Bar graph shows the pole-to-pole distances (x) of the cells in H. Error bars show the SD after performing measurements on ≥60 cells. (J) Cells were treated with GL2 or CenpA siRNAs for 48 h. Western blotting was performed with anti-CenpA antibody; α-tubulin is shown as loading control. Bars = 10 μm.



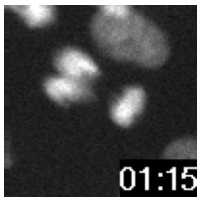
Video 1. **Mitotic timing of HeLa S3 cells depleted of GL2.** Time-lapse imaging of histone H2B-GFP expressing HeLa S3 cells treated with GL2 siRNA. Imaging was performed using a 20x objective and images were captured using 10-ms exposures for GFP every 3 min for 16 h.



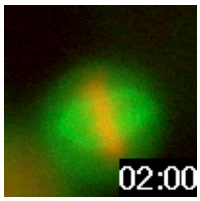
Video 2. **Mitotic timing of HeLa S3 cells depleted of hSpindly (hSpindly-1 siRNA).** Time-lapse imaging of histone H2B-GFP expressing HeLa S3 cells treated with hSpindly-1 siRNA. Imaging was performed using a 20x objective and images were captured using 10-ms exposures for GFP every 3 min for 16 h.



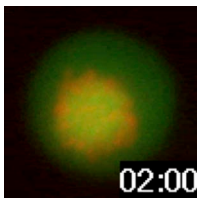
Video 3. **Mitotic timing of HeLa S3 cells depleted of hSpindly (hSpindly-2 siRNA).** Time-lapse imaging of histone H2B-GFP expressing HeLa S3 cells treated with hSpindly-2 siRNA. Imaging was performed using a 20x objective and images were captured using 10-ms exposures for GFP every 3 min for 16 h.



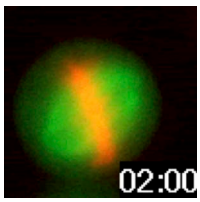
Video 4. **Mitotic timing of HeLa S3 cells depleted of ZW10 (ZW10-1 siRNA).** Time-lapse imaging of histone H2B-GFP expressing HeLa S3 cells treated with ZW10-1 siRNA. Imaging was performed using a 20x objective and images were captured using 10-ms exposures for GFP every 3 min for 16 h.



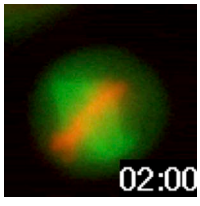
Video 5. **Spindle rotation in HeLa Kyoto cells depleted of GL2.** Time-lapse imaging of HeLa Kyoto cells stably expressing GFP-α-tubulin/cherry-H2B treated with GL2 siRNA and exposed to MG132. Imaging was performed using a 40x objective and images were captured using 10- and 80-ms exposures for cherry and EGFP, respectively, every 3 min for 16 h.



Video 6. **Spindle rotation in HeLa Kyoto cells depleted of GL2 and hSpindly (hSpindly-2 siRNA).** Time-lapse imaging of HeLa Kyoto cells stably expressing GFP-α-tubulin/cherry-H2B treated with GL2+hSpindly-2 siRNA and exposed to MG132. Imaging was performed using a 40x objective and images were captured using 10- and 80-ms exposures for cherry and EGFP, respectively, every 3 min for 16 h.



Video 7. **Spindle rotation in HeLa Kyoto cells depleted of hSpindly (hSpindly-2 siRNA) and dynein heavy chain (DHC).** Time-lapse imaging of HeLa Kyoto cells stably expressing GFP-α-tubulin/cherry-H2B treated with hSpindly-2+DHC siRNA and exposed to MG132. Imaging was performed using a 40x objective and images were captured using 10- and 80-ms exposures for cherry and EGFP, respectively, every 3 min for 16 h.



Video 8. **Spindle rotation in HeLa Kyoto cells depleted of GL2 and dynein heavy chain (DHC).** Time-lapse imaging of HeLa Kyoto cells stably expressing GFP-α-tubulin/cherry-H2B treated with GL2+DHC siRNAs and exposed to MG132. Imaging was performed using a 40x objective and images were captured using 10- and 80-ms exposures for cherry and EGFP, respectively, every 3 min for 16 h.