Supplemental material

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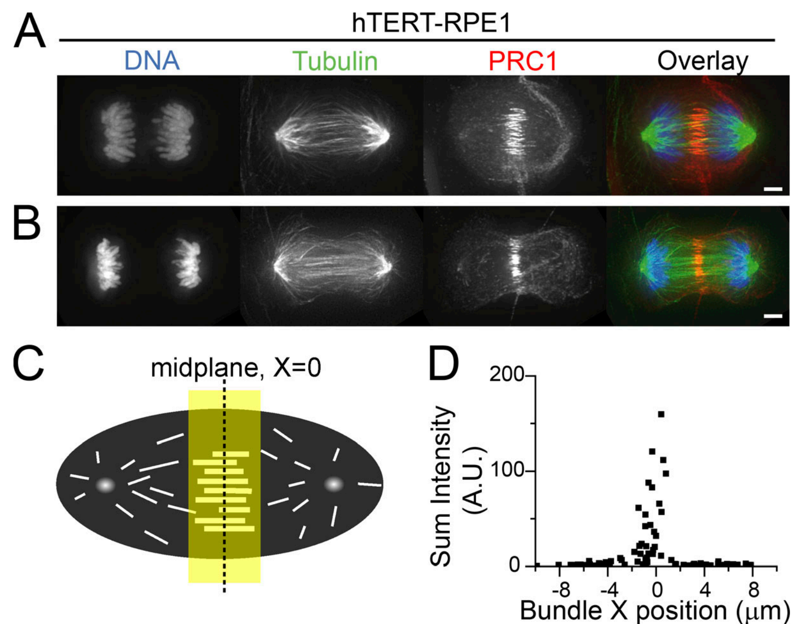


Figure S1. **Analysis of PRC1 localization in hTERT-RPE1 cells. (A and B)** Immunofluorescence analysis of PRC1 localization in hTERT-RPE1 cells. Single-channel images (maximum-intensity projections) and overlays show chromosomes (blue), tubulin (green), and PRC1 (red) in a mid anaphase (A) and late anaphase (B) cell. Scale bar, 3 µm. **(C and D)** Analysis of microtubule bundle position within the spindle. X = 0 was defined as the position of the spindle midplane, the plane orthogonal to the pole-to-pole axis and equidistant from the two poles. **(C)** Schematic highlighting position of analyzed midzone microtubule bundles (yellow box). The position of the spindle midplane (dotted line) is indicated. **(D)** Plot of sum intensity per microtubule bundle as a function of position along the pole-to-pole axis. An example hTERT-RPE1 cell, treated with a DNA dye and expressing GFP-PRC1, was imaged using LLSM. Analysis was performed at T = 0, the frame immediately prior to that with detectable chromosome separation. The centroid of each bundle was determined and its X coordinate calculated relative to X = 0.

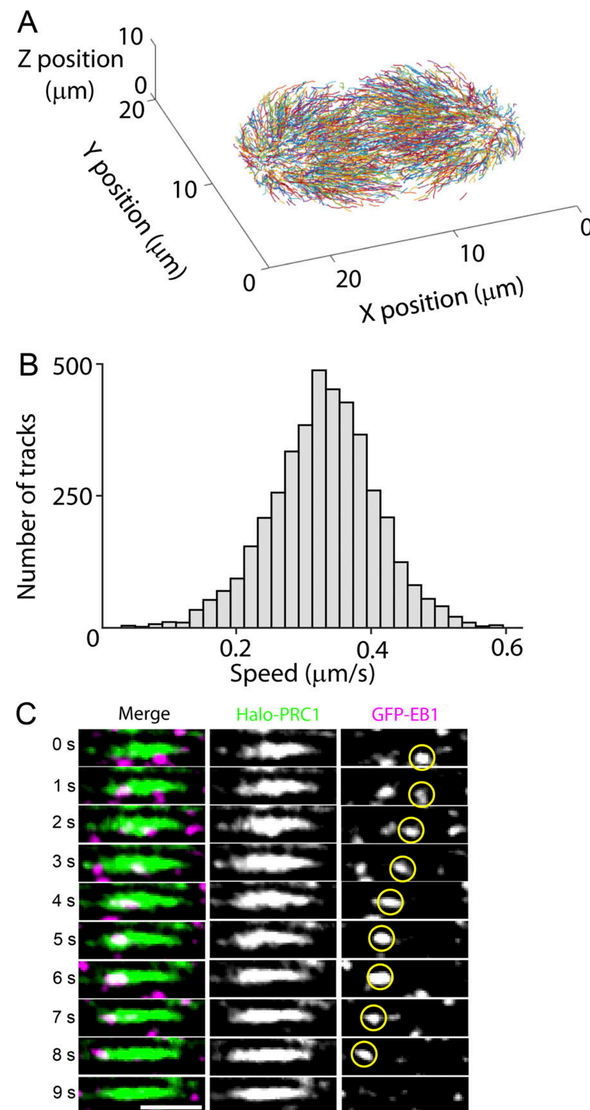


Figure S2. **Analysis of GFP-EB1 and Halo-PRC1 in dividing cells. (A)** GFP-EB1 trajectories generated by automated 3D comet tracking (see Materials and methods). **(B)** Histogram of GFP-EB1 track velocities during anaphase. Data were pooled from from *n* = 3 cells. Mean velocity: 0.33 ± 0.08 µm/s (±SD). **(C)** Single-channel images (single plane) and overlays show Halo-PRC1 (green) and GFP-EB1 (magenta) in consecutive frames. Example shown is the only instance of long-lived (more than three consecutive frames) GFP-EB1 colocalization with Halo-PRC1–tagged microtubule bundles. Scale bar, 3 µm.

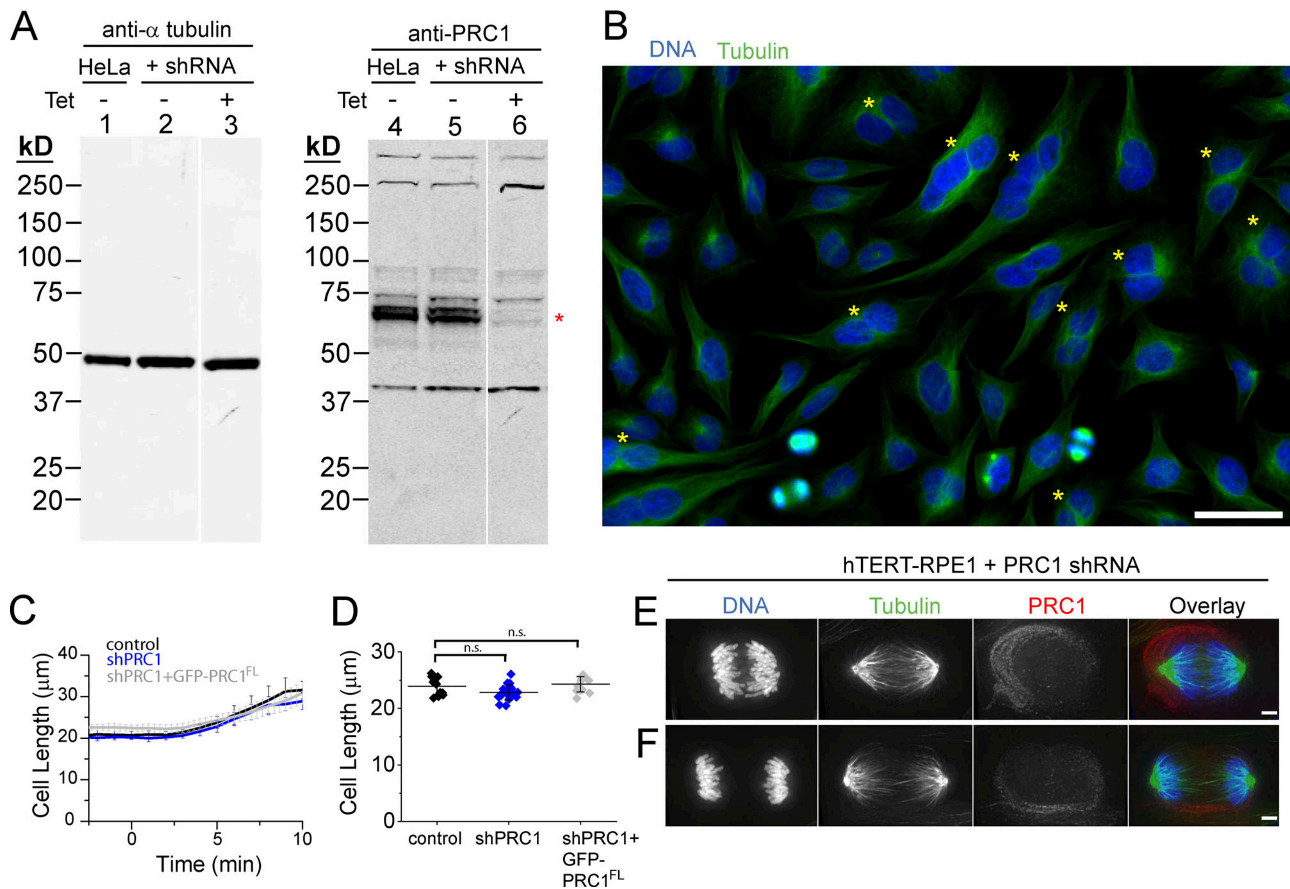


Figure S3. **Western blot and immunofluorescence analysis of cells expressing shRNA to PRC1. (A)** Western blot analysis of cell lysates of HeLa control cells (lanes 1 and 4), and HeLa cells containing shRNA to PRC1 before (lanes 2 and 5) and after (lanes 3 and 6) tetracycline induction of shRNA. Antibodies against α-tubulin and PRC1 are indicated. Expected position of PRC1 protein is indicated (red asterisk). **(B)** Immunofluorescence analysis in HeLa cells 72 h after induction of shRNA to PRC1. Overlay image shows tubulin (green) and DNA (blue). Interphase cells with more than one nucleus are indicated (yellow asterisks). Scale bar, 50 µm. **(C and D)** Analysis of cell length in HeLa control cells (black), HeLa cells expressing shRNA to PRC1 (shPRC1, blue), and HeLa cells expressing shRNA to PRC1 and shRNA-resistant GFP-PRC1 (shRNA+GFP-PRC1^{FL}, gray). Cell length of HeLa cells during anaphase over time (C) and at T = 5 min (D; control: 23.9 ± 1.4 µm; shPRC1: 22.8 ± 1.4 µm; shPRC1+GFP-PRC1^{FL}: 24.3 ± 1.3 µm; $P > 0.07$); mean ± SD. **(E and F)** Immunofluorescence analysis of PRC1 localization in hTERT-RPE1 cells expressing shRNA to PRC1. Single-channel images and overlays show DNA (blue), tubulin (green), and PRC1 (red) in an early anaphase (E) and late anaphase (F) cell. Scale bar, 3 µm. n.s., not significant.

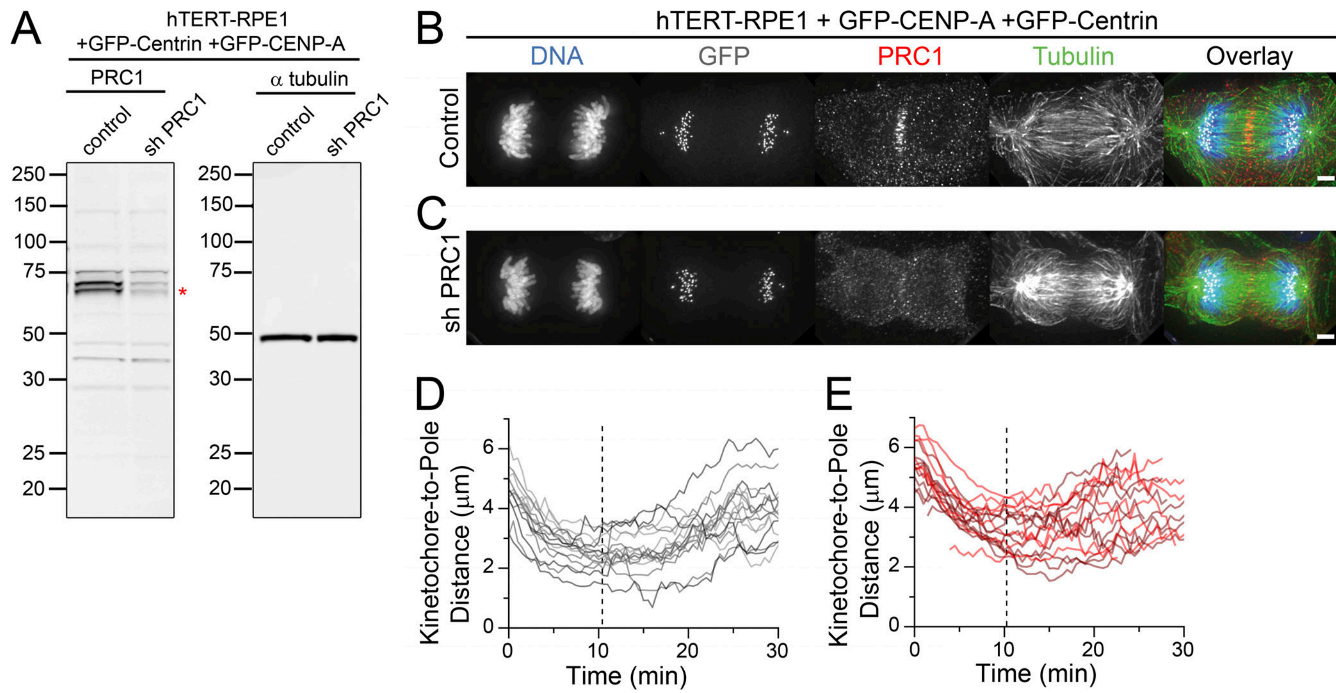


Figure S4. **Western blot and immunofluorescence analysis of hTERT-RPE1 cells expressing GFP-CENP-A, GFP-Centrin, and shRNA to PRC1. (A)** Western blot analysis of metaphase-arrested cell lysates from hTERT-RPE1 cell lines expressing GFP-centrin and GFP-CENP-A. Samples from control cells (ctr) and cells expressing shRNA to PRC1 (sh PRC1). Antibodies against α-tubulin and PRC1 are indicated. Expected position of endogenous PRC1 is indicated (red asterisk). **(B and C)** Immunofluorescence analysis of fixed cells. Single-channel images and overlays show chromosomes (blue), GFP (gray), tubulin (green), and PRC1 (red) in a control cell (B) and a cell expressing shRNA to PRC1 (C). Scale bar, 3 µm. **(D and E)** Analysis of kinetochore-to-pole distance. Traces from individual kinetochores from a control cell (D) and a cell expressing shRNA to PRC1 (E) are shown. Time of centrosome release is indicated (dotted line).

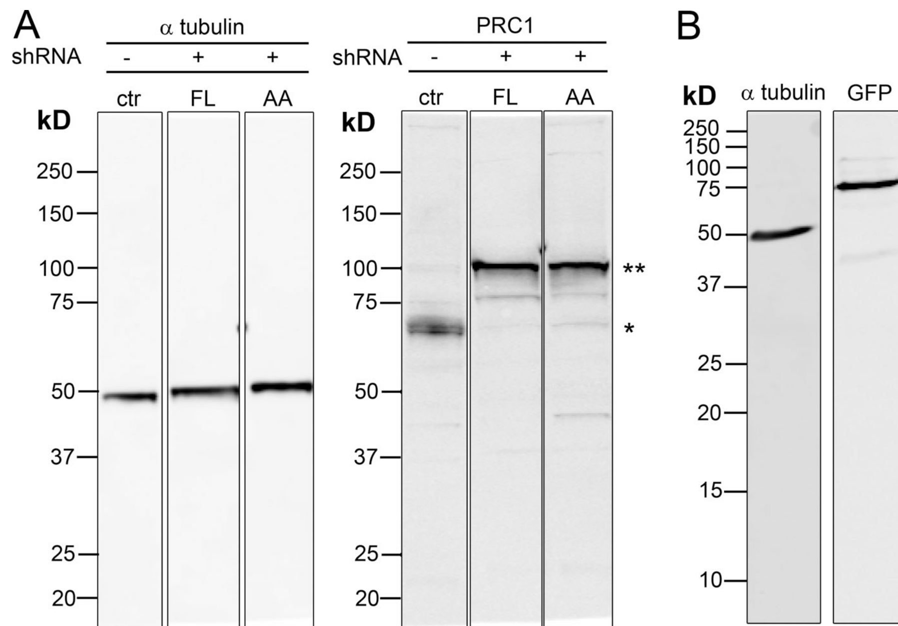
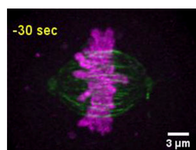
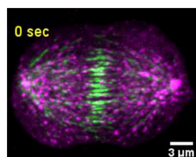


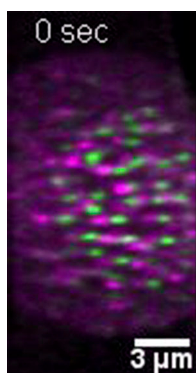
Figure S5. **Western blot analysis of cells expressing shRNA to PRC1 and shRNA-resistant GFP-tagged PRC1 constructs. (A and B)** Western blot analysis of metaphase-arrested cell lysates from HeLa cell lines. **(A)** Samples from HeLa control cells (ctr) and HeLa cells expressing shRNA to PRC1 and shRNA-resistant GFP-PRC1^{FL} (FL) or GFP-PRC1^{AA} (AA) 72 h after adding tetracycline. Antibodies against α-tubulin and PRC1 are indicated. Expected position of endogenous PRC1 (single asterisk) and GFP-tagged PRC1 (double asterisk) is indicated. **(B)** Samples from HeLa cells expressing shRNA to PRC1 and GFP-PRC1$^{\Delta C}$ 72 h after adding tetracycline. Antibodies against α-tubulin and GFP are indicated.



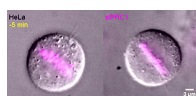
Video 1. **Near-simultaneous two-color LLSM was used to image GFP-PRC1 and chromosomes during anaphase in hTERT-RPE1 cells.** Overlays (maximum-intensity projections) show GFP-PRC1 (green) and chromosomes (magenta). T = 0 was assigned to the frame immediately before that with detectable chromatid separation. Scale bar, 3 µm. Video frame rate is 10 frames per second.



Video 2. **Near-simultaneous two-color LLSM was used to image Halo-PRC1 and GFP-EB1 during anaphase in hTERT-RPE1 cells.** Overlays (maximum-intensity projections) show Halo-PRC1 (green) and GFP-EB1 (magenta). T = 0 was assigned to the first frame of the movie. Scale bar, 3 µm. Video frame rate is 10 frames per second.



Video 3. **Near-simultaneous two-color LLSM was used to image Halo-PRC1 and GFP-EB1 during anaphase in hTERT-RPE1 cells.** Overlays show Halo-PRC1 (green) and GFP-EB1 (magenta) in the spindle midplane (see Fig. 3 C). T = 0 was assigned to the first frame of the movie. Scale bar, 3 µm. Video frame rate is 10 frames per second.



Video 4. **Spinning disk confocal microscopy was used to image HeLa cells.** Single-channel (single z slice) and overlay images show differential interference contrast images (gray) and chromosomes (magenta) in HeLa control cells (left) and cells expressing shRNA to PRC1 (right). T = 0 was assigned to the frame immediately before that with detectable chromatid separation. Scale bar, 3 µm. Video frame rate is 5 frames per second.

Video 5. **Spinning disk confocal microscopy was used to image HeLa cells expressing shRNA to PRC1 and shRNA-resistant GFP-PRC1.** Single-channel (single z slice) and overlay images show differential interference contrast images (gray), chromosomes (magenta), and GFP-PRC1 (green). T = 0 was assigned to the frame immediately before that with detectable chromatid separation. Scale bar, 3 µm. Video frame rate is 7 frames per second.

Video 6. **Spinning disk confocal microscopy was used to image control hTERT-RPE1 cells expressing GFP-CENP-A to label the kinetochores and GFP-Centrin to label the centrosomes.** Maximum-intensity projections are show a control cell in anaphase. T = 0 was assigned to the frame immediately before that with detectable chromatid separation. Scale bar, 3 µm. Video frame rate is 10 frames per second.

Video 7. **Spinning disk confocal microscopy was used to image hTERT-RPE1 cells expressing shRNA to PRC1, GFP-CENP-A to label the kinetochores, and GFP-Centrin to label the centrosomes.** Maximum-intensity projections show a cell in anaphase. T = 0 was assigned to the frame immediately before that with detectable chromatid separation. Scale bar, 3 µm. Video frame rate is 10 frames per second.