Supporting Information

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SI Materials and Methods

Cell Culture. The SMC2 conditional knockout cell line, SMC2:CENP-H:GFP, and SMC2:GFP:CENP-A were cultured as previously described (1, 2). The construct Dronpa-CENP-A was obtained by substituting the GFP in the GFP:CENP-A construct. Dronpa was kindly provided by J. Lippincott-Schwartz (Bethesda, MD).

The CENP-C (3), CENP-H (4), CENP-N (5), and CENP-W (6) knockout cell lines stably expressing GFP:CENP-A were prepared by cotransfection with puromycin, hygromycin, neomycin, and neomycin-resistant markers. Clones were selected based on GFP:CENP-A localization. GFP-CENP-A was expressed from the CMV promoter. Stable cell lines were chosen in which levels of fluorescence resembled those seen with CENP-H and diffuse background was minimal.

Electron Microscopy. Metaphase $SMC2^{ON}$ DT40 cells were identified by fluorescence light microscopy and processed for EM as described previously (1). Length measurements were performed on kinetochores with distinct outer plates using the ruler tool in Photoshop (Adobe Systems). Lengths of curved or bent outer plates were measured by subdividing the plate into segments that were approximately linear and summing the lengths of each segment in the plate. The measurements were analyzed in GraphPad Prism. The histograms of frequency distribution were done with a bin of 8.

Indirect Immunofluorescence and Conventional Light Microscopy. For the analysis of $SMC2^{ON/OFF}$, cells were seeded in Polysine slides for 20 min, followed by 4% paraformaldehyde (in PBS solution) fixation during 5 min. Cell permeabilization was achieved by incubating slides for 2 min in 0.1% Triton X-100 PBS solution. Cells were blocked during 30 min at 37 °C in 1% BSA PBS solution. Antibody incubation was done in 1% BSA-PBS solution for 1 h at 37 °C. The following antibodies were used: anti–CENP-A at 1:1,000 (5), anti–CENP-H at 1:200 (4), anti–CENP-T at 1:1,000 (6), anti–CENP-C at 1:200 (7) anti-H3K9me3 (Upstate), anti-H3K4me2 (Upstate). Fluorescence-labeled secondary antibodies 1:200 were used (Jackson ImmunoResearch Laboratories). Slides were mounted in Vectashield with DAPI.

TEEN experiments were performed as previously described (1, 8). Stains were performed as described for nontreated cells.

$SMC2^{ON/OFF}$ immunostaining image stacks were taken using a microscope (IX-70; Olympus) with a charge-coupled device camera (CH350 or HQ; Photometrics) controlled by DeltaVision SoftWorx (Applied Precision) and a ×100 S Plan apochromat with an N.A. 1.4 objective using a Sedat filter set (Chroma Technology) and running at romm temperature. Image stacks were deconvolved, and maximum projections were generated using SoftWorx. All files were saved as TIFF files and exported to Photoshop (Adobe) for final presentation. Levels were adjusted similarly for each experimental dataset to lower nonspecific background haze using the standard Photoshop adjust levels tool. The line profile was drawn in ImageJ, the values of intensity of each wavelength exported to Excel (Microsoft), and graphs of each channel superimposed.

The fiber length was measured using the SoftWorx system. The length was determined in the 528 nm channel. The data were analyzed using GraphPad Prism. The histograms of frequency distribution were done with a bin of 1.0 and the relative frequencies tabulated as percentages. The values represented in the graph come from the analysis of two or three independent experiments. In each experiment, between 10 and 30 images were analyzed.

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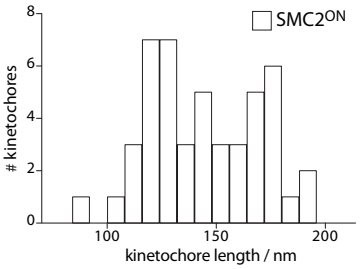

Fig. S1. Frequency distribution of kinetochore diameter measured by EM, with an average value of 145 ± 27 nm (n = 48; average ± SD).

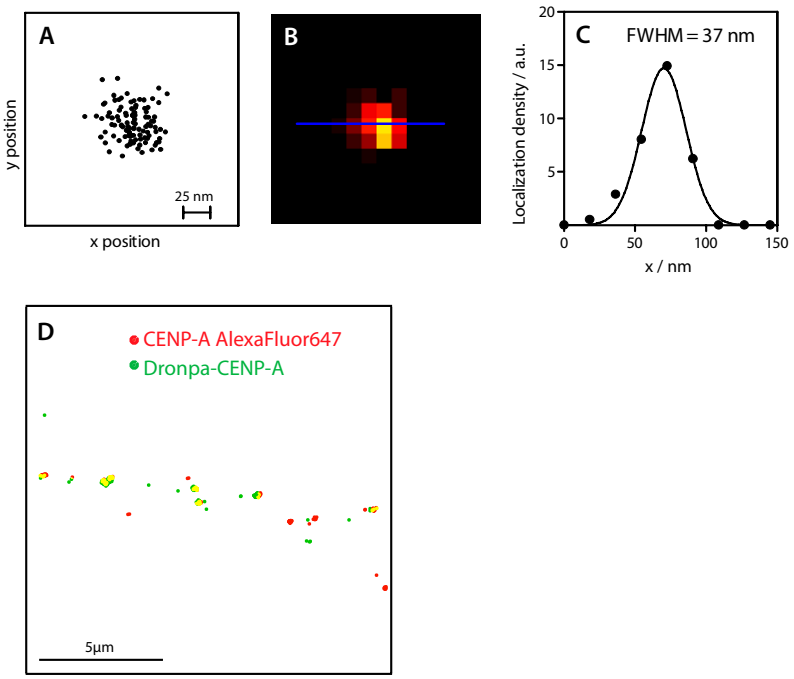

Fig. S2. (*A*–*C*) Localization error determination for super-resolution microscopy experiments. (*A*) Multiple localizations of the same molecule of Alexa Fluor 647; (*B*) Density image constructed from data in *A* with line cross section (*Materials and Methods*); (*C*) Gaussian fit to the cross section in *B* showing an estimated spatial resolution of 37 nm. (*D*) Super-resolution image of Dronpa-CENP-A unfolded fiber immunostained with CENP-A antibody. There is colocalization between both, demonstrating that the endogenous pool of unlabeled CENP-A is not significant.