Expanded View Figures

Figure EV1. **(related to Fig 1).** *De novo* **CENP-A reloading follows the canonical CENP-A deposition pathway.**　　　►

A　Image of IAA-treated cells. IAA escaper is highlighted with a dashed yellow circle, and CENP-A depleted cells are contoured with red dashed lines. Scale bar, 10 μm.

B　Schematic for the experiments shown in C.

C　Quantification of centromeric CENP-A levels normalized to non-treated level. Each dot represents one experiment, and error bars represent SD. Unpaired *t*-test: *P* = 0.0493.

D　Quantification of the relative number of DLD-1 (square) and U-2OS (circle) cells with centromeric CENP-A at the indicated timing of IAA treatment and recovery. Each dot represents one experiment with at least 20 cells per condition. Error bars represent standard deviation (SD) from 3 independent experiments.

E　Schematic for the experiments shown in F-H.

F　Left panel: representative images to confirm M18BP1 knock-down in late M phase cells. Scale bar, 10 μm. Yellow dashed lines highlight nuclei of daughter cells. Right panel: relative M18BP1 levels in late M/early G1 phase after siRNA knock-down using M18BP1 antibody. Each dot represents one centromere, and error bars represent standard deviation.

G　Representative images of *de novo* CENP-A reloading upon M18BP1 knock-down. Nuclei are highlighted with white dashed lines. Scale bar, 5 μm.

H　Quantification of centromeric CENP-A intensities in the indicated conditions (relative intensities normalized to CENP-A level in untreated cells). Each dot represents one experiment (> 30 cells per condition per experiment), and error bars represent SD of 2 independent experiments.

I　Schematic representation for the experiments shown in J-K.

J　Bar graphs showing quantification of centromeric CENP-A intensities following the indicated treatment. Each dot represents one experiment with at least 30 cells. Error bars represent SD of 2 independent experiments.

K　Immunoblot of total protein levels in the indicated cell lines and conditions.
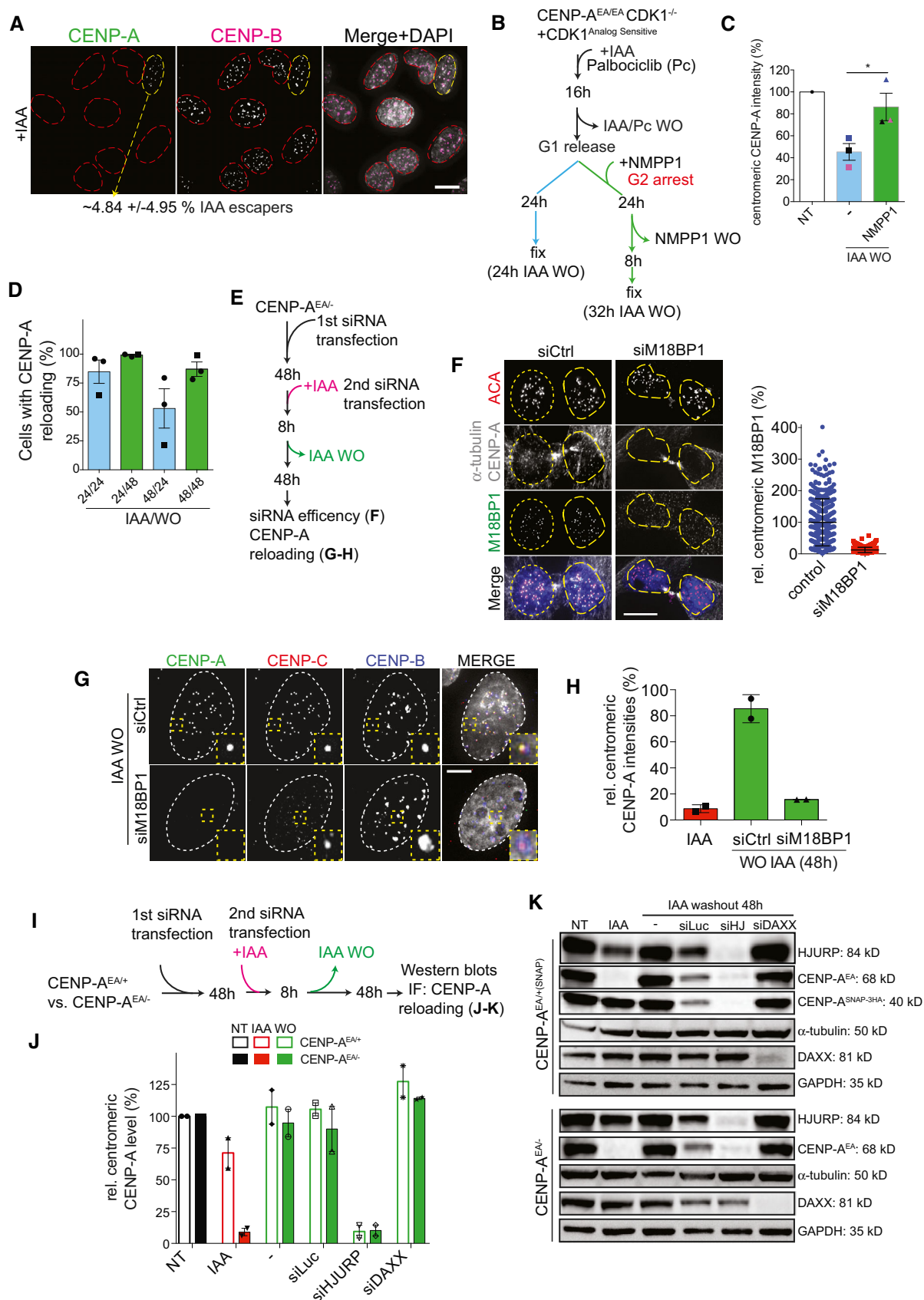
Source data are available online for this figure.

Figure EV1.

Figure EV2. (related to Fig 2). Complete centromeric CENP-A^{EA} depletion with the AID system.

A   Schematic illustration of experiment shown in B, C.

B   Representative images showing complete depletion of CENP-A^{EA} despite doxycycline (DOX)-induced overexpression in DLD-1 cells. Nuclei are contoured with white dashed lines. Scale bar, 5 μm.

C   Quantification of CENP-A intensities in the nucleus and at the centromere in the presence or absence of IAA/DOX. Endogenous (End.) CENP-A$^{EYFP-AID}$ (CA) or overexpressed (OE) CA is depleted to non-detectable background-level in the presence of IAA. Each dot represents a cell. Mean CA intensities are indicated by a black line.

D   Schematic illustration of the single molecule microscopy (SMM) experiments shown in E, G.

E   Representative microscopy images from live cell imaging and corresponding 3D surface plots showing single molecule GR^{EYFP} detection in I and following IAA treatment CENP-A^{EA} signal absence at CENP-B^{mcherry} marked centromeres in II, using SMM acquisition settings.

F   Examples of background-corrected EYFP signal intensities quantified over time (as shown in D) for single GR^{EYFP} molecules (in magenta), centromeric EYFP signals in IAA-treated CENP-A$^{EA/EA}$ cells (in green), and in the absence of EYFP molecules (in black).

G   Signal quantification as shown in D in the indicated conditions. Unpaired t-test, ns ($P = 0.88$), ****$P < 0.0001$, error bars represent standard deviation. Each dot represents the quantification of one GR^{EYFP} signal (GR^{EYFP}, $n = 13$) or one centromere, respectively (No EYFP CENP-C^{mcherry}, $n = 85$ and CENP-B^{mcherry} CENP-A^{EA}, $n = 52$).

H   CENP-A levels at the indicated HOR arrays quantified by CUT&RUN sequencing. CENP-A levels 48 h after IAA wash-out recover at the original HOR.

I   Quantifications of CENP-A occupancy at the DXZ1 HOR array after one CENP-A$^{OFF/ON}$ cycle in DLD-1 cells using IF-FISH on chromatin fibers. Each dot represents a single chromatin fiber. Error bars show standard deviation. P-values from unpaired t-test.

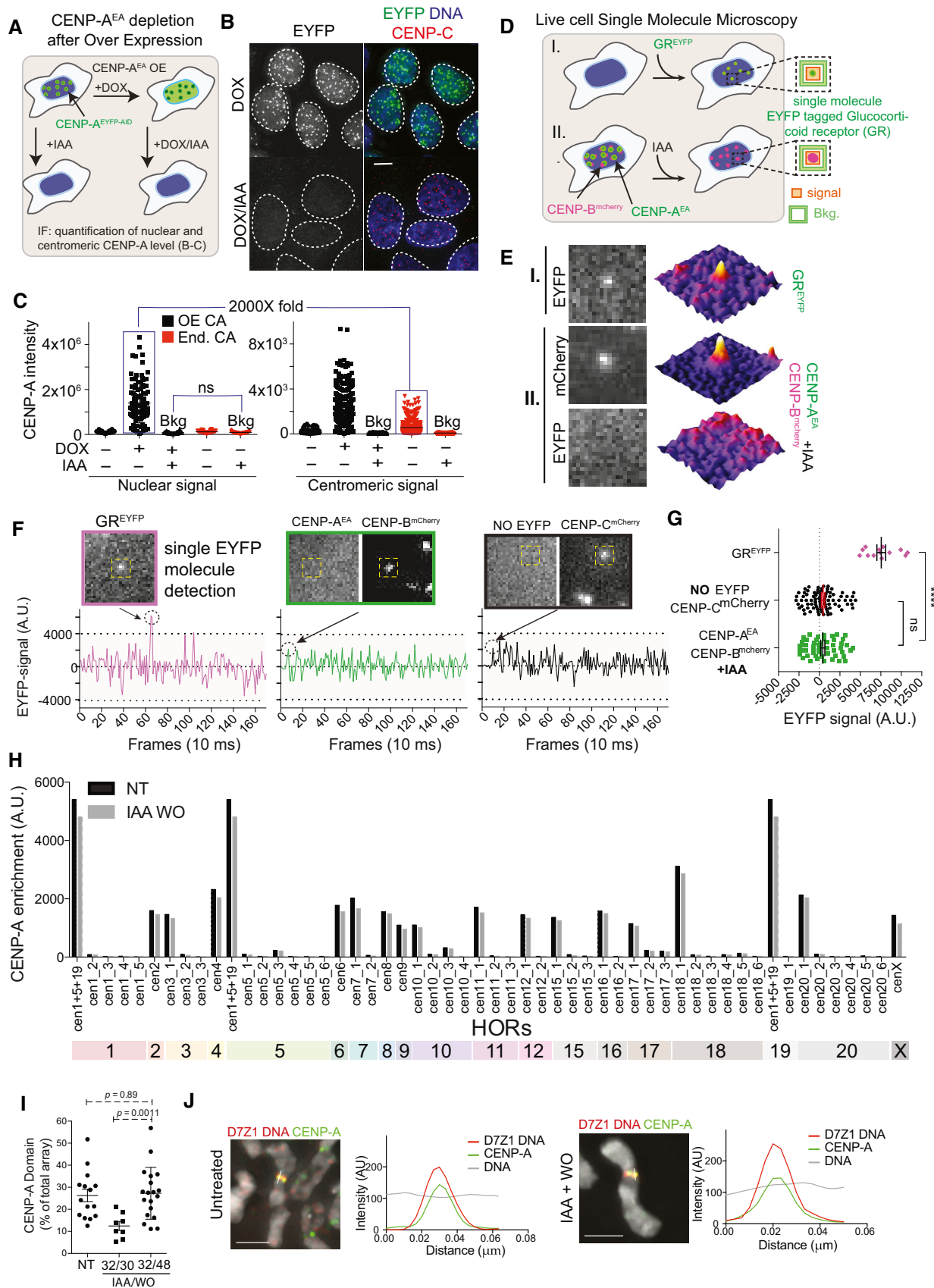J   Line scan analysis at the D7Z1 array on chromosome spreads in the indicated treatment. Scale bar, 2 μm.
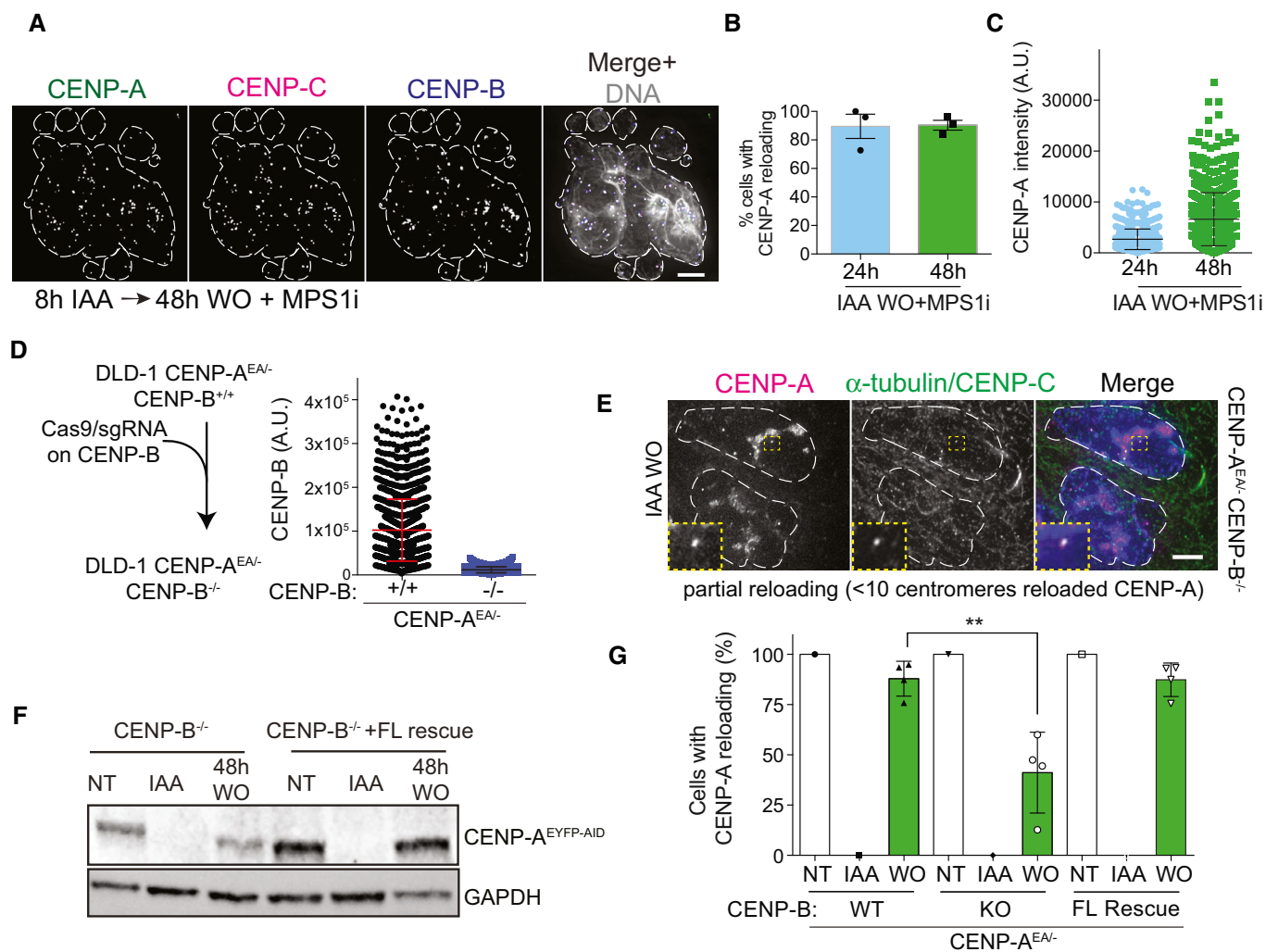
Figure EV2.

Figure EV3. (related to Fig 3). CENP-B is a key factor for efficient *de novo* CENP-A reloading.

A   Representative immunofluorescence showing *de novo* CENP-A deposition in CENP-B (+/+) DLD-1 cells after reversine-induced chromosome mis-segregation. DAPI staining is contoured by a white dashed line. Scale bar, 5 μm.

B, C Bar graphs showing the relative number of CENP-A-positive cells (B) and the level of centromeric CENP-A level (C) in the presence of reversine. Each dot represents one experiment with more than 20 cells per condition. Error bars represent SD of 3 independent experiments.

D   Left: schematic of the CRISPR/Cas9 strategy to deplete CENP-B in DLD-1 cells as measured in the dot plot on the right. Each dot represents one centromere, and error bars show standard deviation.

E   Immunofluorescence images showing partial *de novo* CENP-A[EA] reloading (< 10 centromeres) in CENP-B^{−/−} DLD-1 cells in late M phase. Nuclei of daughter cells are highlighted with white dashed lines. Scale bar, 5 μm.

F   Immunoblot of total protein levels in the indicated conditions in the indicated cell line. FL = full length.

G   Bar graphs showing the relative number of DLD-1 cells with centromeric CENP-A in indicated conditions in the indicated cell line. Each dot represents one single experiment, > 30 cells per condition. Unpaired *t*-test, **P = 0.0053, error bars represent SD of 4 independent experiments.

Source data are available online for this figure.

Figure EV4. (related to Fig 7). Exogenously expressed CENP-C, but not CENP-A, reloaded at the original centromere position in absence of endogenous CENP-A/C.

A Models of CENP-B-induced CENP-A reloading via (I.) initial Mis18 recruitment or (II.) initial CENP-C recruitment.

B Schematic of the experiment analyzed in C, D.

C Quantification of M18BP1 foci in late M phase/early G1 (eG1) in both daughter cells in the indicated conditions. Each dot represents two daughter cells. Error bars show SEM. Unpaired *t*-test: ****$P < 0.0001$.

D Representative immunofluorescence images showing M18BP1 foci in different cell lines after 24-h IAA treatment. Cells with centromeric CENP-A are marked with a red dashed contour line, while a yellow contour line marks cell without centromeric CENP-A. Scale bar, 5 μm.

E Illustration of the genomic make-up of DLD-1 cells used to test CENP-A or CENP-C reloading in the absence of endogenous CENP-A/C.

F Immunoblot showing exogenous CENP-A (left) and CENP-C (right) expression upon addition of doxycycline (DOX) and TMP to induce CENP-A$^{DHFR-mRFP}$ or CENP-C$^{DHFR-mRFP}$ overexpression in DLD-1 cells.

G Experimental design of experiments shown in Fig 7C–E and Appendix Fig S7H and I.

H Bar graphs showing the control of CUT&RUN–qPCR on CENP-C antibody and primers binding to the centromere of chromosome 4 in the indicated conditions. Enrichment is measured relative to the IgG control and normalized to Alu repeats. Error bars represent SD of 3 independent experiments.

I Representative immunofluorescence image showing 24 h CENP-A$^{mRFP-DHFR}$ stability in the absence of endogenous CENP-A and CENP-C in G1 arrested cells.

J Experimental design of experiments shown in Fig 7F–H and Appendix Fig S7K–M.

K Representative immunofluorescence image showing CENP-C reloading in interphase cells (nuclei are highlighted by a dashed red contour line) in the presence (control) or absence (sample) of endogenous CENP-A/C. Scale bar, 5 μm.

L Quantification of relative number of exogenous CENP-C-positive centromeres per cell in the indicated conditions quantified on chromosome spreads.

M Bar graph showing the control of CUT&RUN–qPCR on CENP-A antibody and primers binding to the centromere of chromosome 4 in the indicated conditions. Enrichment is measured relative to the IgG control and normalized to Alu repeats.

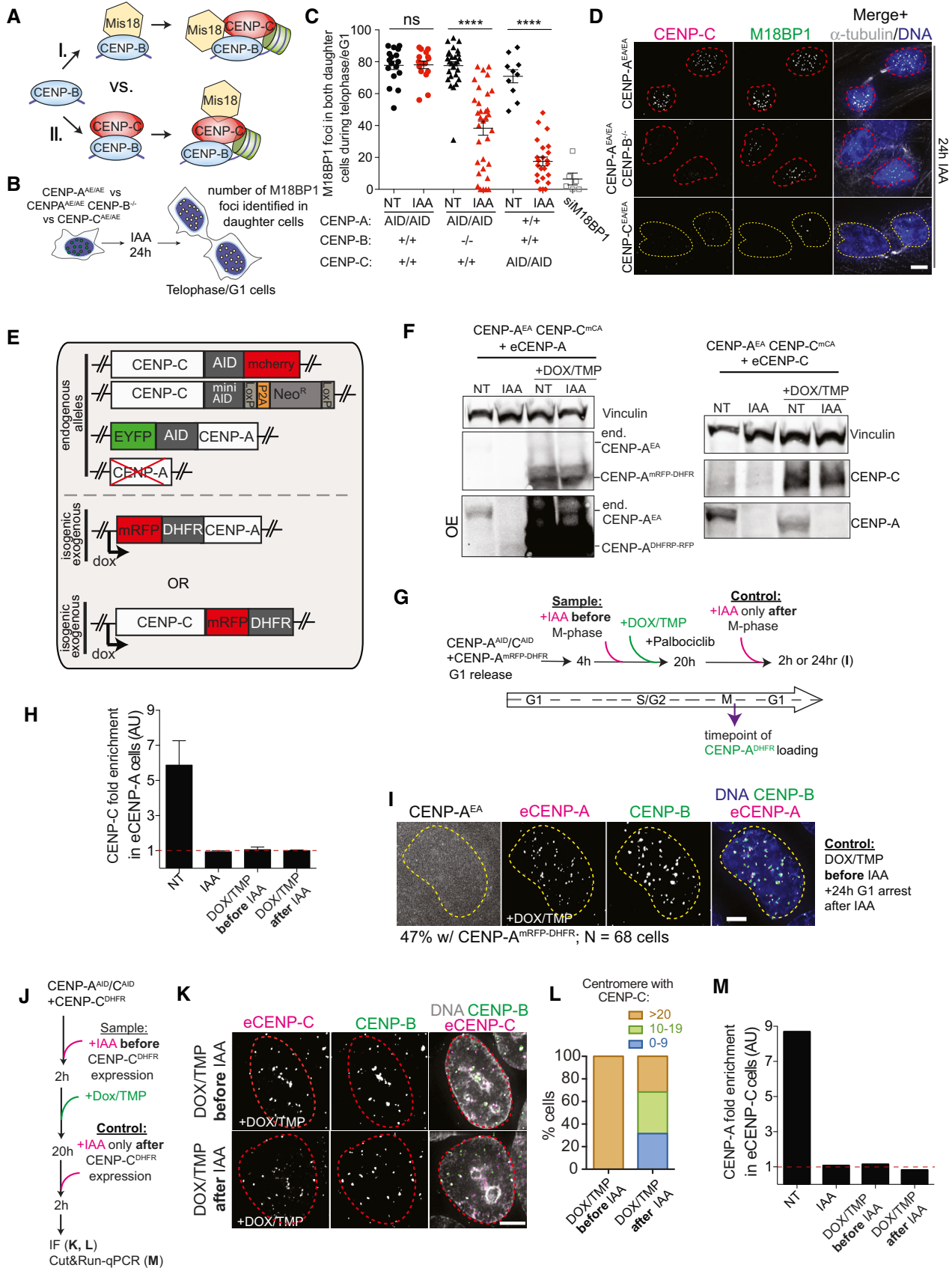Source data are available online for this figure.

Figure EV4.

Figure EV5. (related to Fig 8). CENP-A-deprived CD4+ T cells are found in human blood samples but disappear upon T-cell activation.

A   Representative FACS plots showing the efficiency of total CD4+ T-cell purification from human blood PBMCs.
B   Representative FACS plots showing gating of CENP-B-positive/CENP-A-high and CENP-B-positive/CENP-A-low populations of freshly purified human CD4+z T cells based on isotype controls. Ms = mouse; Rb = rabbit.
C   Graph showing CENP-A foci identified in high or low CENP-A expressing cell colocalizing with CENP-B foci in two donors. Each dot represents the percentage of CENP-A/B colocalizing in one cell. Error bar shows SEM.
D   Quantification of centromeric CENP-A level in high or low CENP-A expressing CD4+ T cells. Each dot represents one centromere. Error bars show standard deviation.
E   Representative immunofluorescence images showing CENP-B- and CENP-C-positive centromeres, but lacking CENP-A in a CD4+ T cell. Nucleus is contoured by a dashed yellow line. Scale bar, 5 μm
F   Representative plots showing CENP-A expression vs. forward scatter area (FSC-A), which determines the relative size of CD4+ T cells after activation. Gates represent the frequency of CENP-A high and low populations in total CD4+ T (shaded gates represent CENP-A^low cells).
G   Graph representing the absolute number of CFSE-high/CENP-A-low CD4+ T cells during the experimental kinetics. One-way ANOVA, multiple comparisons, n = 6 (each symbol represents a different donor). **P < 0.01, ***P < 0.001.
H   Graph showing the frequency of dead cells in CD4+ T cell cultures over time. One-way ANOVA, multiple comparisons, n = 6 (each symbol represents a different donor). Error bars show SEM. *P < 0.05
I   CFSE dilution and CENP-A expression at day 3 post-activation. Representative FACS plots showing CENP-A expression in CD4+ T cells that have not divided (div 0) and those that have divided once or more times (div ≥ 1), gated based on CFSE dilution. Gates in CENP-A plots were set based on isotype control for each specific population (cells in shaded gates are CENP-A-low CD4+ T cells).
J   Graph representing the frequency of CENP-A low cells (shaded gate in I). One-way ANOVA, multiple comparisons, n = 6 (each symbol represents a different donor). Error bars show SEM. ****P < 0.0001.
K   Representative immunofluorescence images showing CENP-A, CENP-C, and CD4 staining after FACS. Cells with centromeric CENP-A are marked with a red dashed contour line, while a yellow contour line marks cell without centromeric CENP-A. Scale bar, 5 μm.
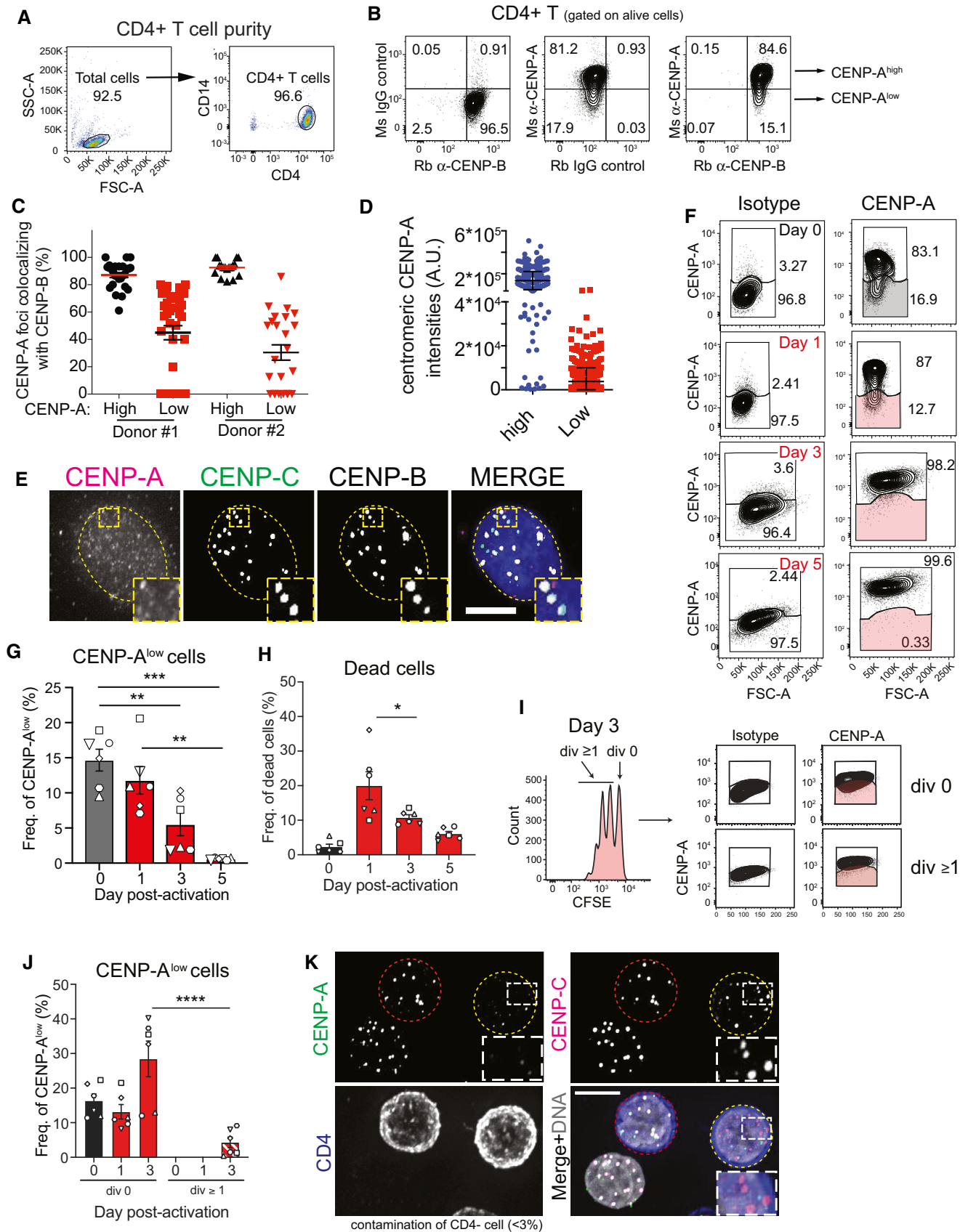
Figure EV5.