JCB

Supplemental material

Kuhn and Dumont, https://doi.org/10.1083/jcb.201902105

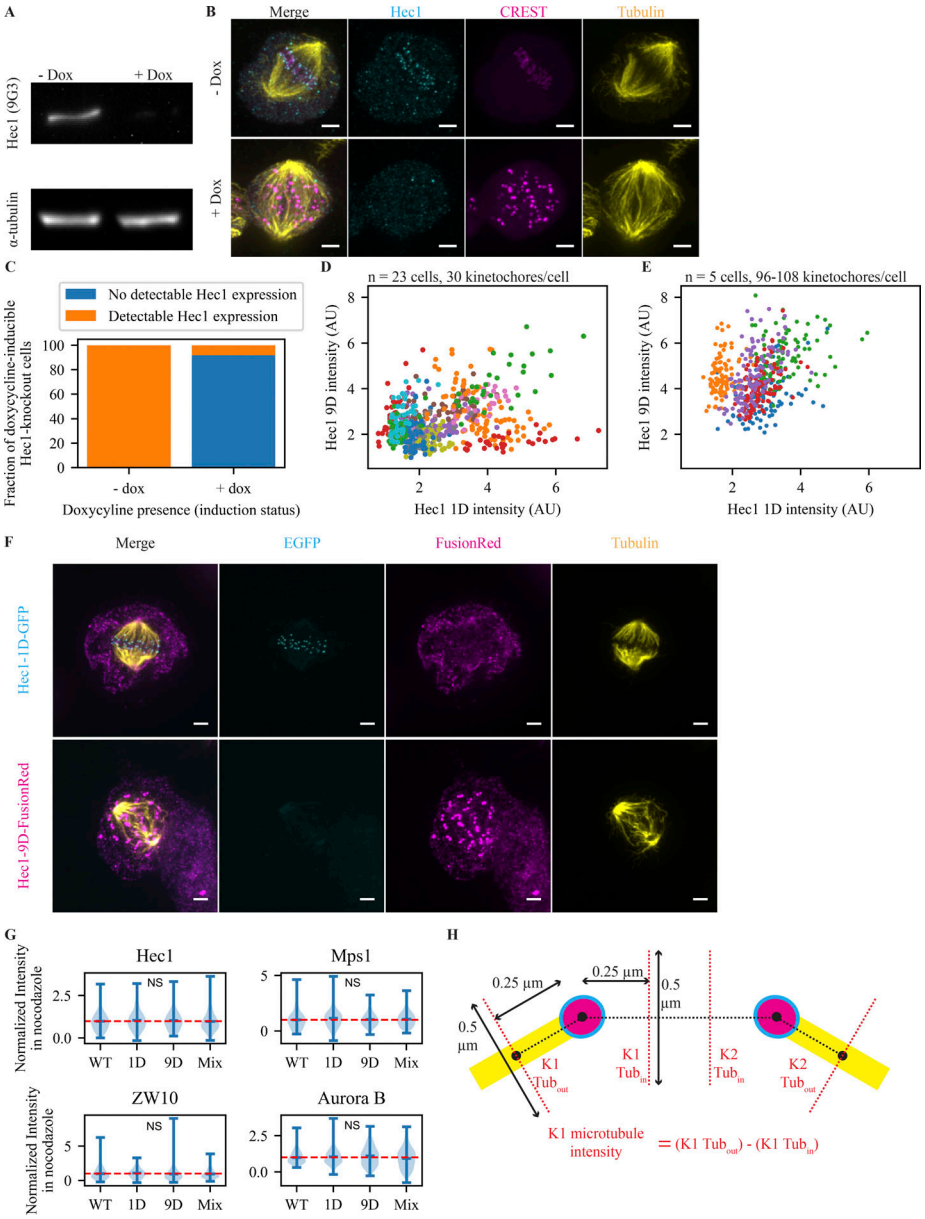
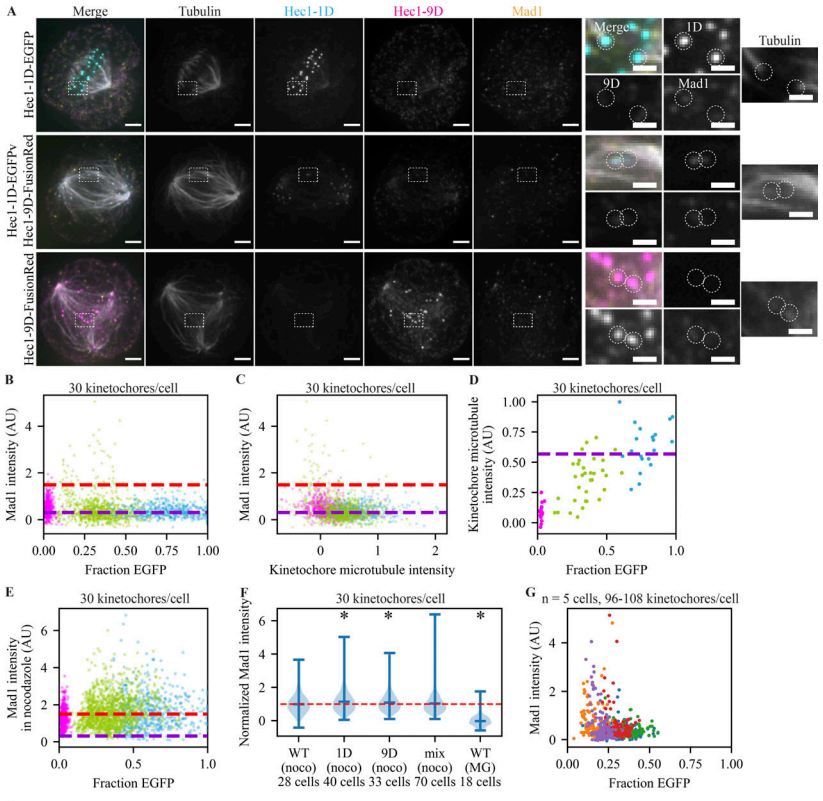


Figure S1. **Hec1-1D, but not -9D, rescues spindle defects after Hec1 depletion.** Related to Fig. 1. **(A)** Western blot of Hec1 and tubulin abundance in cells with a stably expressed doxycycline-inducible Cas9 and Hec1 sgRNA (McKinley and Cheeseman, 2017) 48 h after induction (equivalent to experiments in Figs. 1, 2, S1, and S2). **(B)** Immunofluorescence imaging (maximum-intensity projection) of microtubules (tubulin), kinetochores (CREST), and Hec1 intensity in Hec1 knockout cells with and without doxycycline (+dox and −dox) 48 h after induction (equivalent to experiments in Figs. 1, 2, S1, and S2). Scale bars = 3 µm. **(C)** Hec1 depletion quantified by immunofluorescence of mitotic cells +dox or −dox fixed after 1 h of 5 µM MG132 treatment to prevent premature anaphase onset in Hec1-depleted cells. 92 of 100 induced cells exhibited no detectable Hec1 kinetochore localization 48 h after induction (equivalent to experiments in Figs. 1, 2, S1, and S2). **(D)** Hec1-1D-EGFP and Hec1-9D-FusionRed intensity at individual kinetochores in Hec1-depleted cells expressing both mutants. Colors indicate different cells (n = 23 cells, with 30 kinetochores/cell, 1 coverslip). The variation between cells is far higher than variation within cells (F = 175, P = 10^{-220} for EGFP; F = 59, P = 10^{-139} for Fusion Red, one-way ANOVA). **(E)** Hec1-1D-EGFP and Hec1-9D-FusionRed intensity at all visible individual kinetochores in five randomly chosen Hec1-depleted cells expressing both Hec1-1D and Hec1-9D (n = 508 kinetochores, five cells). Colors indicate different cells. The variation between cells is far higher than variation within cells (F = 170, P = 10^{-92} for EGFP; F = 50, P = 10^{-35} for Fusion Red, one-way ANOVA). **(F)** Immunofluorescence imaging (maximum-intensity projection) of microtubule attachments (tubulin), Hec1-1D intensity (anti-EGFP), and Hec1-9D intensity (anti-mKate, binds to FusionRed) in Hec1 knockout +dox cells expressing Hec1-1D-EGFP or Hec1-9D-FusionRed. Cells were treated with 5 µM MG132 to accumulate them at metaphase. Hec1-1D, but not -9D, expression rescues the spindle structure defects in B. **(G)** Distribution and average (lines) kinetochore intensities after 1 h in 5 µM nocodazole for listed kinetochore proteins in −dox cells (WT, n = 300 kinetochores, five cells) and +dox cells transfected with Hec1-1D-EGFP (1D, n = 300 kinetochores, five cells), Hec1-9D-FusionRed or Hec1-9D-mRuby2 in the Aurora B dataset (9D, n = kinetochores, five cells), or both (Mix, n = 600 kinetochores, 10 cells). WT cells were stained with CREST, Hoechst, and the indicated protein, while all other cells were stained for EGFP, FusionRed, and the indicated protein. In the Aurora B dataset, an anti-RFP antibody was substituted for an anti-FusionRed antibody. Intensities are normalized to the average intensity in WT cells. Red dashed line indicates average intensity in WT cells. No transfected conditions have significantly different intensities from WT cells (P > 0.05; NS). **(H)** Schematic of how kinetochore attachment intensity is calculated for data in Figs. 1 and S2. Red dashed lines indicate intensity line scans, which are summed to attain Tub_{in} and Tub_{out} (see Materials and methods).



H

Method of tubulin calculation	Wild type attachment source ("high") (X in Fig. 2D)	Low attachment source ("low") (X in Fig. 2D)	% of wild type number of attached microtubules (low/high)	Number of microtubules at the weakest binding kinetochores without Mad1 (% * 17.1)
Plugging in fraction EGFP to linear fit	Fraction EGFP = 1	Fraction EGFP = lowest cellular average in Hec1-1D + Hec1-9D population, Fig. 2C	22.4%	3.84
Plugging in fraction EGFP to linear fit	Fraction EGFP = average of whole Hec1-1D population, Fig. 2C	Fraction EGFP = lowest cellular average in Hec1-1D + Hec1-9D population, Fig. 2C	24.9%	4.26
Plugging in fraction EGFP to linear fit	Fraction EGFP = average of whole Hec1-1D population, Fig. 2C	Fraction EGFP = average of whole Hec1-9D population, Fig. 2C	12.5%	2.14

Figure S2. **Hec1-1D, but not -9D, allows for robust Mad1 loss after Hec1 depletion.** Related to Fig. 2. **(A)** Immunofluorescence imaging (maximum-intensity projection) of SAC activation (Mad1), Hec1-1D intensity (anti-EGFP), and Hec1-9D-FusionRed intensity (anti-mKate, binds to FusionRed), and microtubule attachments in Hec1 knockout cells expressing Hec1-1D-EGFP, Hec1-9D-FusionRed, or both in a four-color imaging experiment combining labels from Figs. 1 and 2. Cells were treated with 5 µM MG132 to accumulate them at metaphase. Hec1-1D, but not -9D, expression allows Mad1 loss to undetectable levels on most kinetochores. Some kinetochores in mixed kinetochore cells with low Hec1-1D expression and cells expressing only Hec1-9D are capable of losing Mad1. Scale bars = 3 µm (large) and 1 µm (zoom). **(B and C)** Fraction EGFP versus Mad1 intensity (B) or kinetochore–microtubule intensity versus Mad1 intensity (C) from mixed kinetochores (n = 990 kinetochores, 33 cells; green), 1D alone (n = 600 kinetochores, 20 cells; blue), and 9D alone (n = 600 kinetochores, 20 cells; pink) from A. Dashed lines correspond to the average Mad1 intensity at kinetochores without doxycycline (–dox) in a parallel experiment where cells were treated with 5 µM nocodazole (n = 300 kinetochores, 10 cells; red) or 5 µM MG132 (n = 240 kinetochores, 8 cells; purple). **(D)** Mean of cellular EGFP fraction for each cell versus mean cellular kinetochore–microtubule intensity from Hec1 knockout cells in A with mixed kinetochores (n = 990 kinetochores, 33 cells; green) and cells with control 1D alone (n = 600 kinetochores, 20 cells; blue) and 9D alone coverslips (n = 600 kinetochores, 20 cells; pink). Purple dashed line corresponds to the average kinetochore–microtubule intensity at –dox kinetochores in a parallel experiment where cells were treated with 5 µM MG132 (n = 240 kinetochores, 8 cells). Error bars = SEM. **(E)** Fraction EGFP versus Mad1 intensity in mixed kinetochores (n = 1,200 kinetochores, 40 cells; green), 1D alone (n = 600 kinetochores, 20 cells; blue), and 9D alone (n = 540 kinetochores, 18 cells; pink) treated with 5 µM nocodazole in parallel to the cells in A–D. Dashed lines correspond to the average Mad1 intensity at –dox kinetochores in a parallel experiment where cells were treated with 5 µM nocodazole (n = 300 kinetochores, 10 cells; red) or 5 µM MG132 (n = 240 kinetochores, 8 cells; purple). **(F)** Distribution and average (lines) kinetochore Mad1 intensity in –dox cells treated with 5 µM nocodazole (WT [noco]; n = 840 kinetochores, 28 cells) or 5 µM MG132 for 1 h (WT [MG]; n = 540, 18) or cells with doxycylcine (+dox) treated with 5 µM nocodazole for 1 h expressing Hec1-1D-EGFP (1D; n = 1,200 kinetochores, 40 cells), Hec1-9D-FusionRed (9D; n = 990 kinetochores, 33 cells), or both (Mix; n = 2,100 kinetochores, 70 cells). Intensities are normalized to the average intensity in WT cells. Red dashed line indicates average intensity in WT cells. Individual kinetochores in our assay were able to recruit Mad1 at WT levels (mixed kinetochores, 104% of WT, P = 0.155) or slightly above WT (Hec1-1D alone, 115%, P = 10^{-6}; Hec1-9D alone, 109%, P = 10^{-5}). **(G)** Fraction EGFP versus Mad1 intensity in mixed kinetochores (n = 508 kinetochores, five cells) cells treated with 5 µM MG132 where all visible kinetochores were measured. Different colors indicate different cells; *, P < 0.05 versus WT. **(H)** Table displaying different possible assumptions for choosing the parameters to estimate the number of microtubules at the weakest binding kinetochores without Mad1. Varied parameters include where the WT (high) and minimum (low) attachment quantities are set. The first parameter set is identical to Fig. 2 D. While these assumptions produce slightly different values, they are all significantly lower than previous estimates and likely represent a maximum, not a minimum, threshold value.

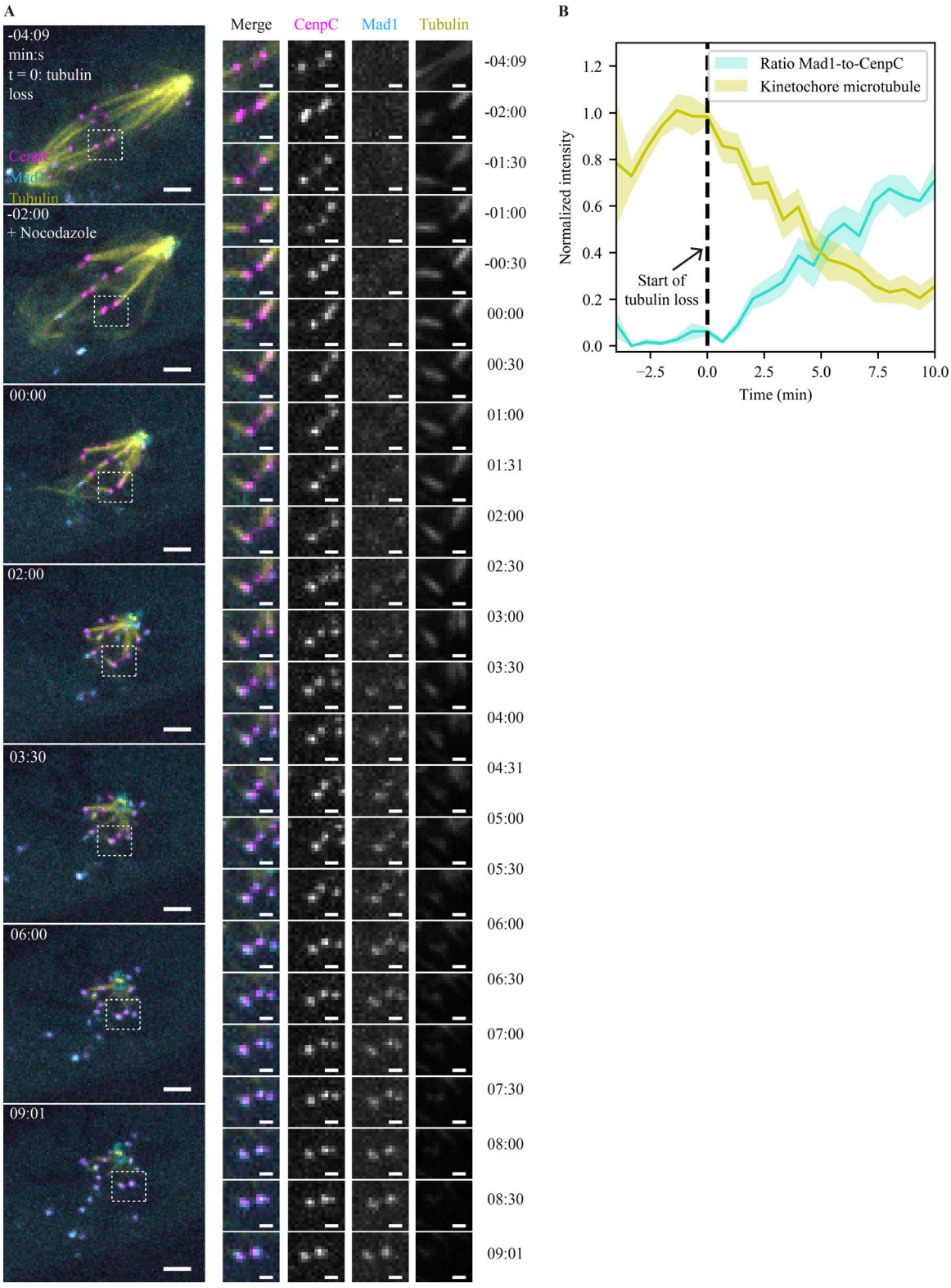
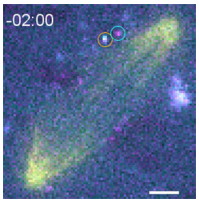
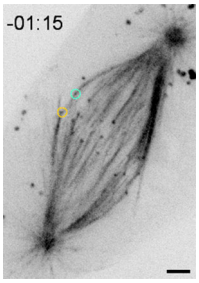


Figure S3. **Mad1 rerecruitment is associated with microtubule loss.** Related to Fig. 4. **(A)** Time-lapse imaging (maximum-intensity projection) of representative SAC activation kinetics (EYFP-Mad1) and microtubule attachment (SiR-Tubulin) on kinetochores (CenpC-mCherry) in PtK2 cells after treatment with 10 µM nocodazole. Nocodazole addition does not immediately depolymerize microtubule attachments. Mad1 is rerecruited on individual kinetochores rapidly only once attachments start to disappear at t = 0. Scale bars = 3 µm (large) and 1 µm (zoom). **(B)** Mean and SEM of kinetochore–microtubule attachment intensity and Mad1-to-CenpC ratio at kinetochores in nocodazole-treated cells. Mad1 starts to relocalize to kinetochores only once tubulin depolymerizes, and it does so rapidly (<1 min; *n* = 3 cells, 8 kinetochores).



Video 1. **Lowering microtubule occupancy at a kinetochore slows down the process of Mad1 loss.** Time-lapse spinning-disk confocal imaging (maximum-intensity projection) of a representative kinetochore pair's Mad1 loss kinetics (EYFP-Mad1, green) and attached microtubules' geometry (SiR-Tubulin, yellow) at kinetochores with reduced microtubule affinity (Hec1-9D-FusionRed, magenta) during spindle assembly in a PtK2 cell with endogenous Hec1 depleted by RNAi. Prior to t = 0, one kinetochore is Mad1 negative and has a weak microtubule end-on attachment (cyan circle), while its sister is Mad1 positive and unattached (orange circle). Mad1 loss (orange arrow) on the orange-circled kinetochore begins at t = 0 as the pair moves toward the metaphase plate, consistent with acquiring an end-on attachment. Mad1 remains detectable until t = 7:26, significantly longer than in WT cells. Four planes spaced 350 nm apart were imaged every 20 s. Playback is 10 frames/s. Scale bar = 3 µm. Time is in min:s. Video corresponds to the still images from Fig. 3 B.



Video 2. **Mechanical isolation of a kinetochore fiber from a metaphase spindle.** Time-lapse spinning-disk confocal imaging (maximum-intensity projection) of representative mechanical isolation of a kinetochore by severing its k-fiber (Hec1-EGFP and EGFP-Tubulin, black) at an aligned kinetochore pair in a PtK2 cell with NuMa partially depleted and endogenous Hec1 depleted by RNAi. Upon initial laser ablation (red asterisks, t = 0), the kinetochore whose k-fiber was severed (cyan circle) recoils toward its sister (orange circle). After ablation, the K-K distance remained low and the ablated k-fiber remained isolated from other spindle microtubules, never moving back toward its former pole. Periodically, the free minus-end was reablated (red asterisks) to prevent reincorporation of the k-fiber into the spindle. The cell was fixed 16 s after the last frame. Three planes spaced 700 nm apart were acquired every 15 s (before ablation and periodically after) or 7.5 s (after ablation). On time points with 15-s intervals, duplicate frames were added. Playback is 10 frames/s. Scale bar = 3 µm. Time is in min:s. Video corresponds to images from Fig. 4, B and E.