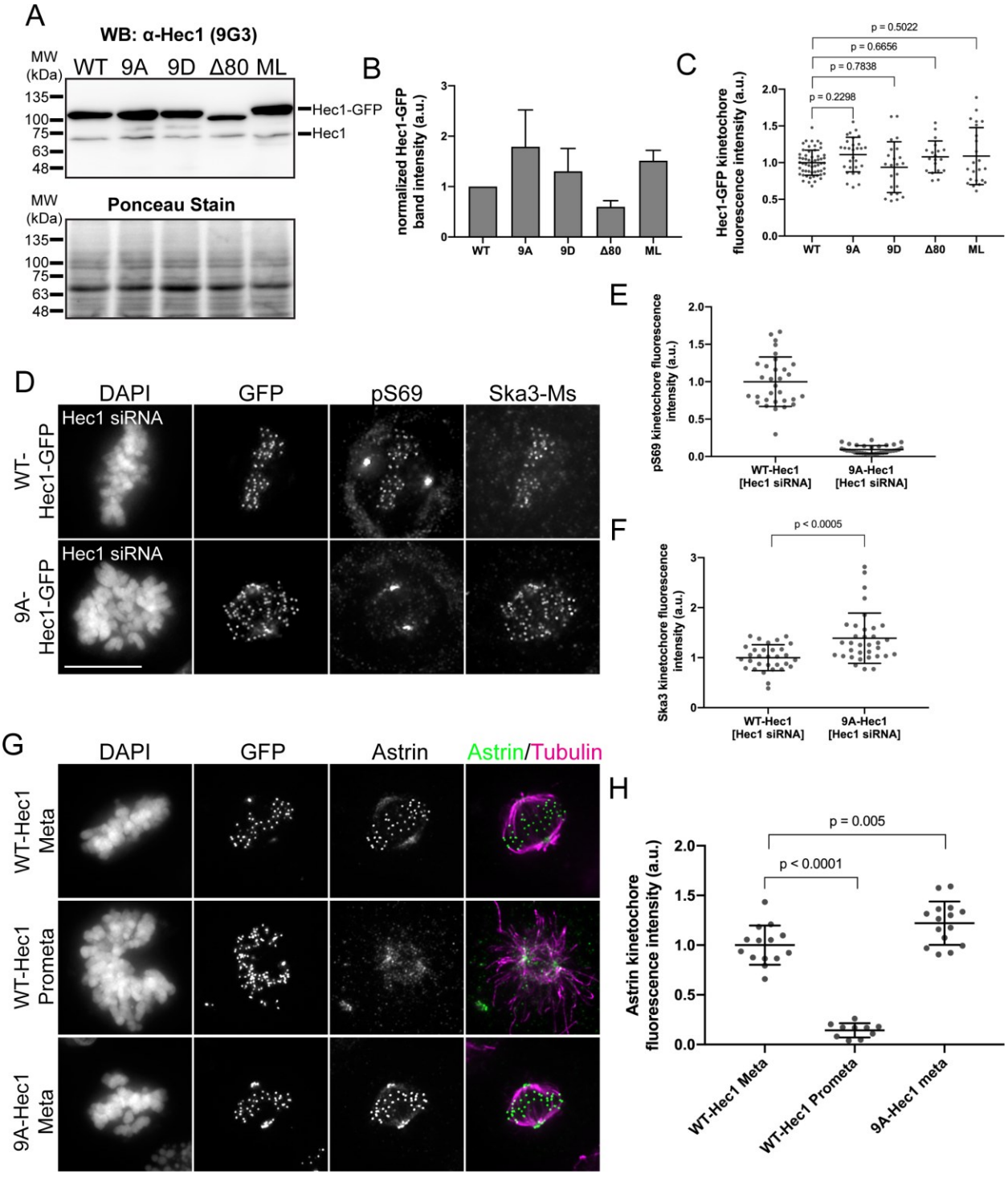
Supplemental Materials

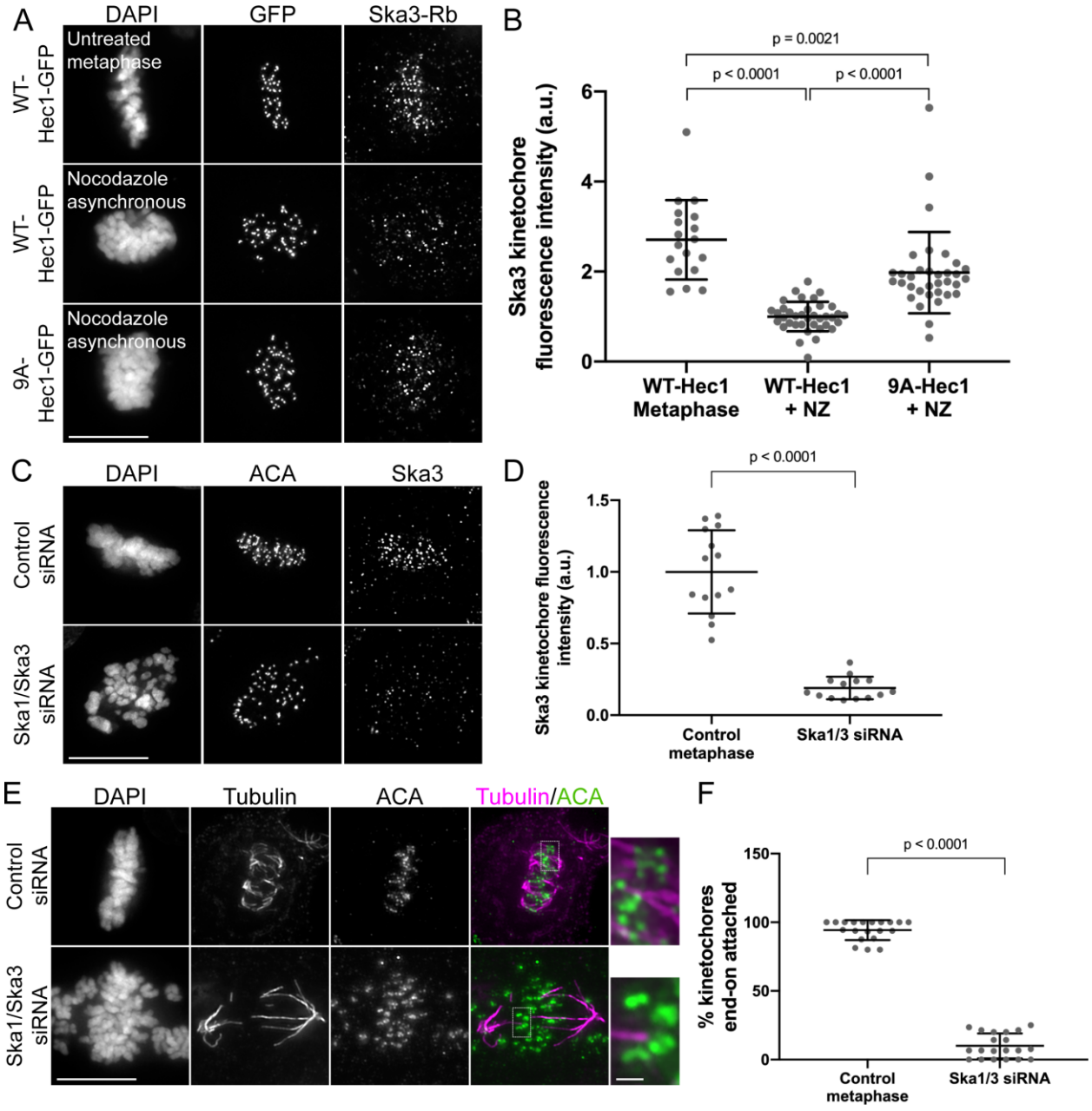
Molecular Biology of the Cell

Wimbish et al.

Supplemental Figure Legends

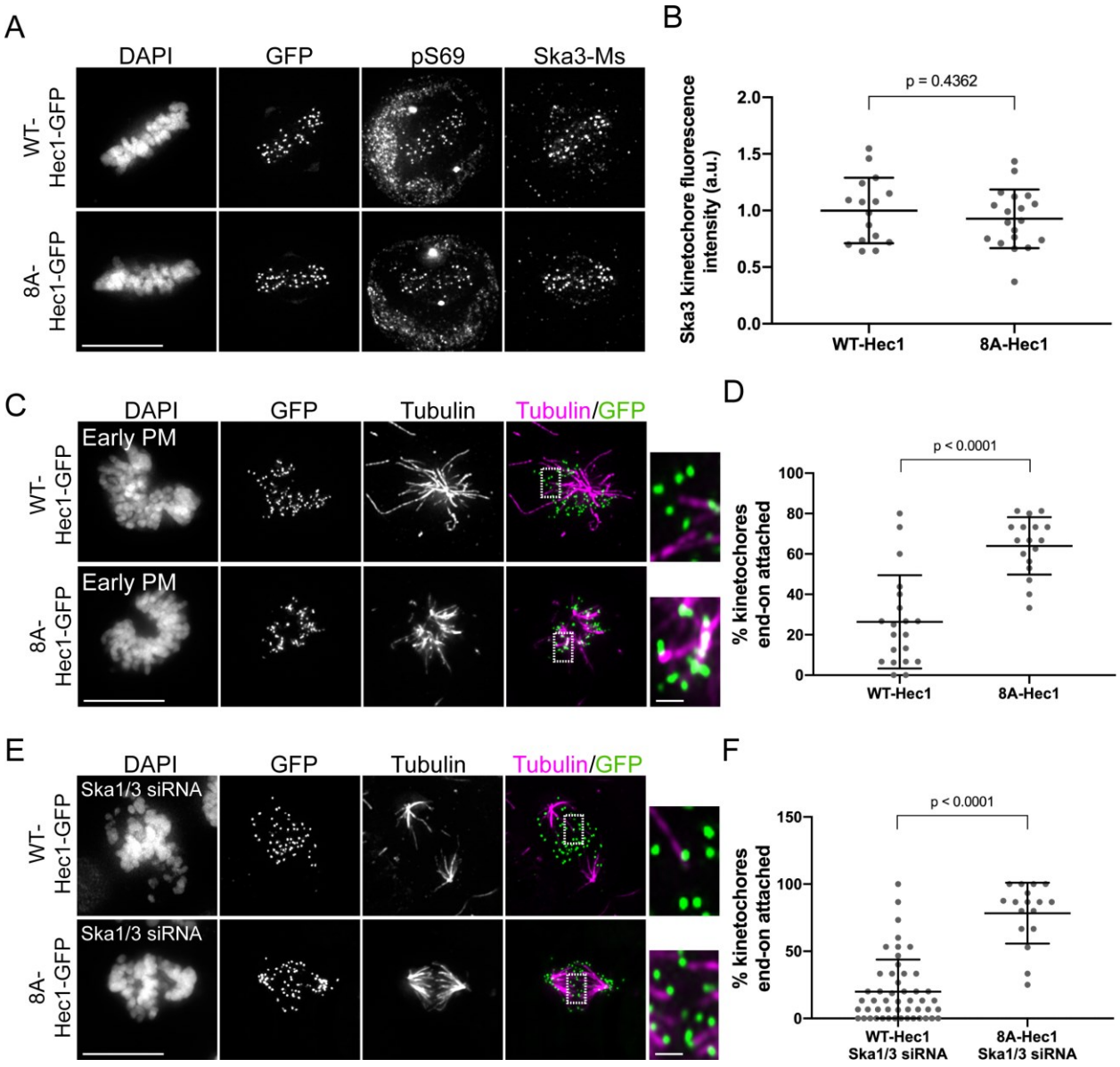


Supplemental Figure 1. Hec1-GFP transgene expression is sufficient to elicit a dominant phenotype and 9A-Hec1 hyper-recruits Astrin to kinetochores. (A) Representative Western blot showing Hec1-GFP expression levels in HeLa Kyoto cells. Prominent bands correspond to endogenous Hec1 (72 kDa) and Hec1-GFP (97 kDa, or ~90 kDa for ∆80-Hec1). Ponceau stained blot is shown as a loading control. (B) Quantification of average Hec1-GFP expression levels from 3 independent Western blot experiments. Hec1-GFP band intensity was normalized to WT-Hec1 expression and corrected for protein loading by Ponceau stain (see Materials and Methods). (C) Hec1-GFP kinetochore fluorescence intensity levels measured from RO/NZ experiment (Figure 2D). For experiments in which kinetochore fluorescence intensity was not quantified, cells with similar GFP expression were analyzed qualitatively (see Materials and Methods). (D) Immunofluorescence images of WT- and 9A-Hec1 expressing cells depleted of endogenous Hec1 with siRNA and stained with antibodies to Hec1-pS69 and Ska3 (generated in mouse). (E) Quantification of pS69 kinetochore fluorescence intensity from Hec1 siRNA-treated cells expressing WT- and 9A-Hec1-GFP. For each condition, at least 20 kinetochores per cell were measured from at least 9 cells per experiment from 3 separate experiments. (F) Quantification of Ska3 kinetochore fluorescence intensity from Hec1 siRNA-treated cells expressing WT- and 9A-Hec1-GFP. For each condition, at least 20 kinetochores per cell were measured from at least 9 cells per experiment from 3 separate experiments. A Student's t-test was carried out to determine statistical significance. (G) Immunofluorescence images of WT- and 9A-Hec1 expressing cells stained with antibodies to Astrin and tubulin. (H) Quantification of Astrin kinetochore fluorescence intensity from cells expressing WT- and 9A-Hec1. For each condition, at least 20 kinetochores per cell were analyzed from at least 5 cells per condition from 2 separate experiments. Statistical significance was determined by a one-way Anova analysis. On all dot plots (C, E, F, and H), each dot represents the average value for all kinetochores from a single cell. Scale bar: 10 μm.



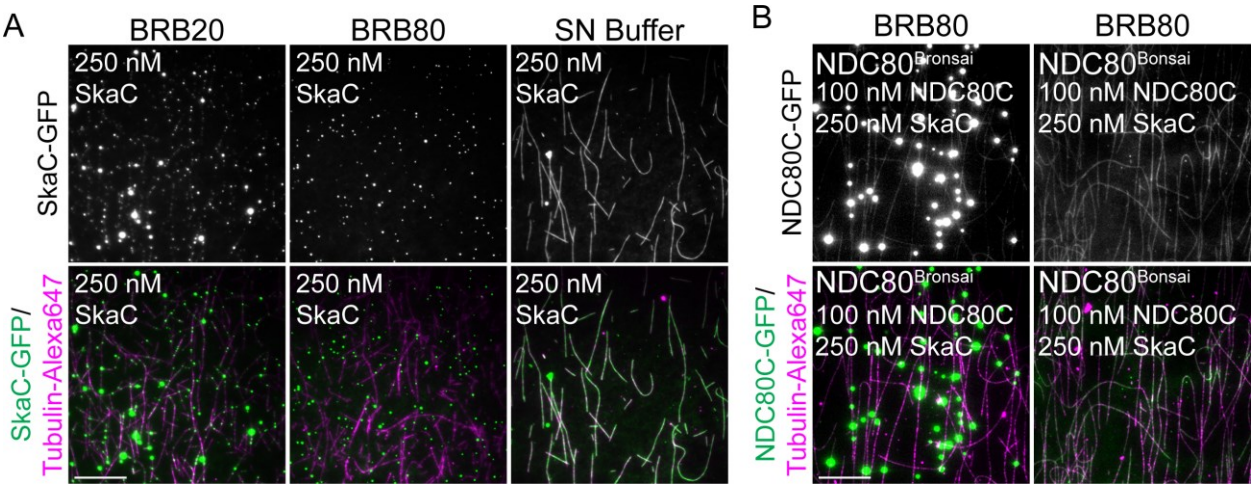
Supplemental Figure 2. 9A-Hec1 expression causes hyper-recruitment of Ska3 in nocodazole, and Ska depletion disrupts end-on attachment formation in cells. (A) Immunofluorescence images of asynchronous cells expressing WT- and 9A-Hec1-GFP treated with or without 10 μm nocodazole for 1h prior to fixation. Cells were fixed and stained with antibodies to Ska3 (rabbit). (B) Quantification of Ska3 kinetochore fluorescence intensity from cells expressing WT- and 9A-Hec1-GFP treated with or without nocodazole. For each condition, at least 20 kinetochores per cell were measured from at least 5 cells per experiment from 4 separate experiments. Statistical significance was determined by a one-

way Anova analysis. (C) Immunofluorescence images of control cells or cells treated with Ska1 and Ska3 siRNA. Cells were fixed and stained with anti-centromere antibodies (ACA) or antibodies to Ska3 (mouse). (D) Quantification of Ska3 kinetochore fluorescence intensity from control cells or cells treated with Ska1 and Ska3 siRNA. For each condition, at least 20 kinetochores per cell were analyzed from at least 7 cells per experiment from 2 independent experiments. Statistical significance was determined by a Student's t-test. (E) Immunofluorescence panels of control cells or cells treated with Ska1 and Ska3 siRNA. Cells were incubated in ice-cold DMEM for 12 minutes prior to fixation, permeabilized, fixed, and stained with antibodies to tubulin and an anti-centromere antibody (ACA). Insets are enlargements of the region indicated by the dashed box. (F) Quantification of end-on attachment in control cells and cells treated with Ska1 and Ska3 siRNA. For each condition, at least 15 kinetochores per cell were measured from 10 cells per experiment from 2 separate experiments. Statistical significance was determined using a Student's t-test. On all graphs, each dot represents the average value for all kinetochores from a single cell. Scale bars: 10 μm and 1 μm for panels and insets, respectively.

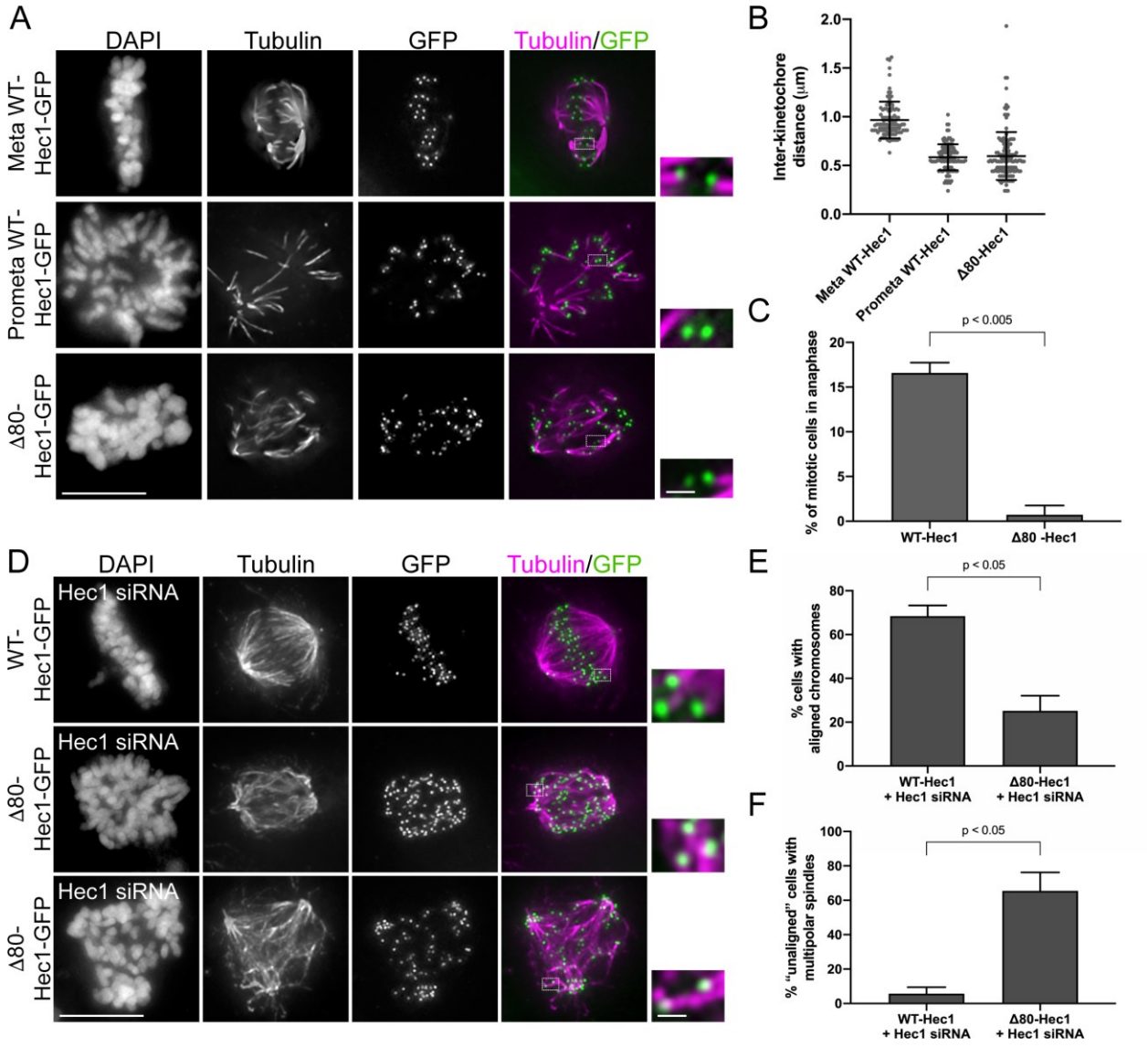


Supplemental Figure 3. 8A-Hec1 expression phenocopies 9A-Hec1 in kinetochore-microtubule attachment formation and WT-Hec1 in Ska recruitment. (A) Immunofluorescence images of cells expressing WT- and 8A-Hec1-GFP. Cells were fixed and stained with antibodies to Hec1-pS69 and Ska3 (mouse). (B) Quantification of Ska3 kinetochore fluorescence intensity from metaphase cells expressing WT- and 8A-Hec1-GFP. For each condition, at least 20 kinetochores per cell were quantified for at least 7 cells per experiment from 2 independent experiments. (C) Immunofluorescence images of early prometaphase cells expressing WT- and 8A-Hec1-GFP. Cells were incubated in ice-cold DMEM for 12 minutes prior to fixation, permeabilized, fixed and stained using antibodies to

tubulin. Insets are enlargements of the region indicated by the dashed box. (D) Quantification of end-on attachments in early prometaphase cells expressing WT- and 8A-Hec1-GFP. For each condition, at least 15 kinetochores per cell were measured from at least 8 cells per experiment from 2 independent experiments. Data for WT-Hec1 are from Figure 1D. (E) Immunofluorescence images of cold-treated cells expressing WT- and 8A-Hec1-GFP and treated with Ska1 and Ska3 siRNA. Cells were incubated in ice-cold DMEM for 12 minutes prior to fixation, permeabilized, fixed and stained using antibodies to tubulin. Insets are enlargements of the region indicated by the dashed box. (F) Quantification of end-on attachments in cells expressing WT- and 8A-Hec1-GFP and treated with Ska1 and Ska3 siRNA. For each condition, at least 15 kinetochores per cell were measured from at least 8 cells per experiment from at least two independent experiments. Data for WT-Hec1 are from Figure 2F. For all quantifications, statistical significance was determined using a Student's t-test. On all graphs, each dot represents the average value for all kinetochores from a single cell. Scale bars: 10 μm and 1 μm for panels and insets, respectively.



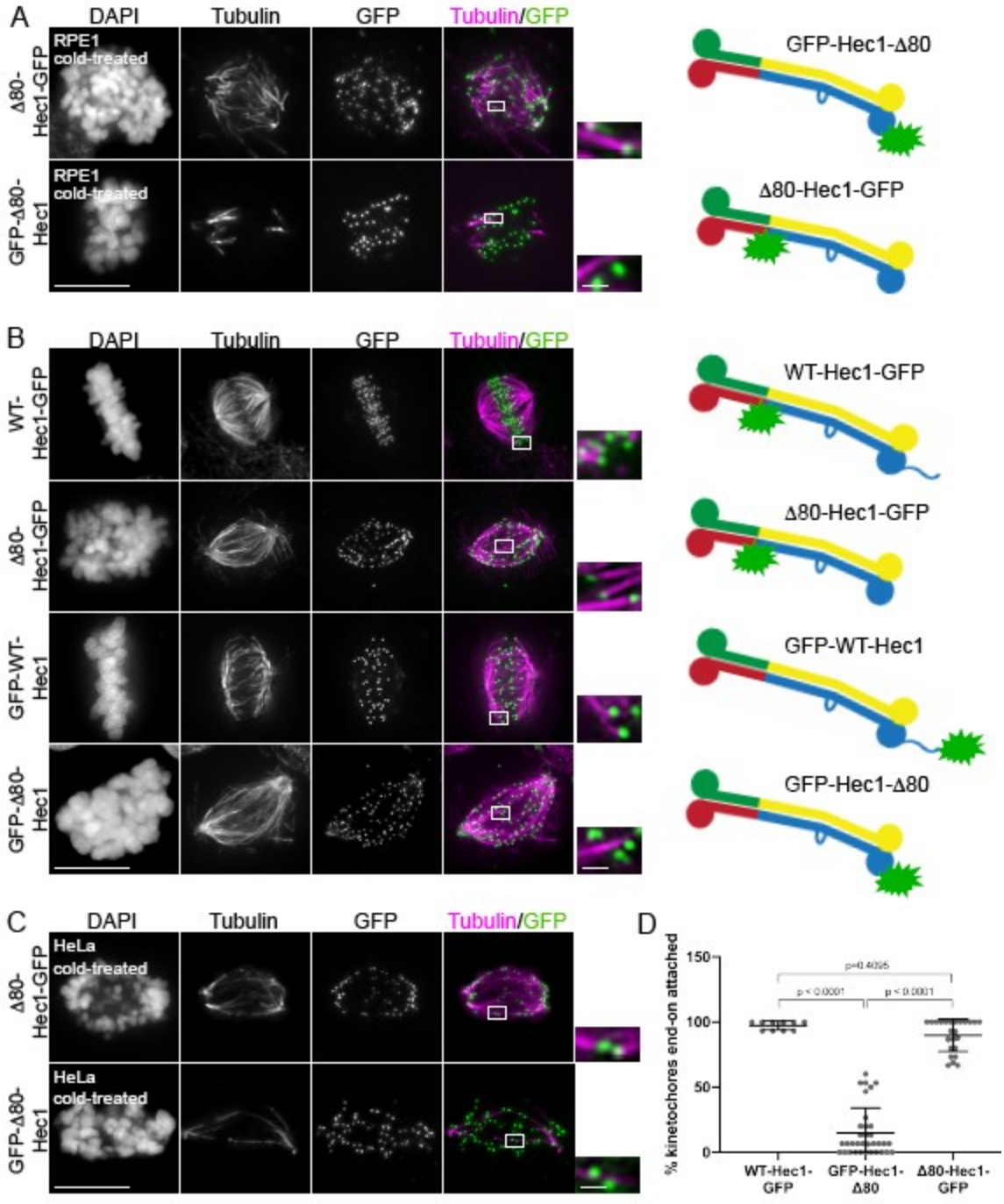
Supplemental Figure 4. Oligomerization of Ska and NDC80 complexes *in vitro* is buffer dependent. (A) GFP fluorescence (top row) and overlay with Alexa647-tubulin (bottom row) images of GFP-tagged Ska complex (SkaC-GFP) diluted to the noted concentrations in buffers indicated above each column. SkaC-GFP microtubule binding reactions were carried out in the same manner as experiments from Figure 3 (see Materials and Methods). (B) GFP fluorescence (top row) and overlay with Alexa647-tubulin (bottom row) images of indicated NDC80C-GFP constructs incubated with unlabeled Ska complex (SkaC) in BRB80 buffer. Scale bars: 20 μm.



Supplemental Figure 5. Hec1 tail deletion impacts tension generation at kinetochores. (A) Immunofluorescence images of HeLa cells expressing WT- and Δ80-Hec1-GFP. Cells were incubated in ice-cold DMEM for 12 minutes prior to fixation, permeabilized, fixed, and stained with antibodies to tubulin. Insets are enlargements of the region indicated by the dashed box. (B) Quantification of inter-kinetochore distances in metaphase and prometaphase cells expressing WT-Hec1-GFP, and cells expressing Δ80-Hec1-GFP. For each condition, inter-kinetochore distances were measured from at least 10 kinetochores per cell in at least 10 cells per experiment from at least 3 independent experiments. Each dot represents the distance measured for a single kinetochore pair. (C) Quantification of anaphase index in cells expressing WT- or Δ80-Hec1-GFP. For each

condition, anaphase index was assessed for at least 100 mitotic cells per experiment in 2 separate experiments. (D) Immunofluorescence images of HeLa cells expressing WT- and Δ80-Hec1-GFP and depleted of endogenous Hec1 stained with antibodies to tubulin. Insets are enlargements of the region indicated by the dashed box. (E) Quantification of chromosome alignment in cells expressing WT- and Δ80-Hec1-GFP. For each condition, chromosome alignment was assessed in at least 100 cells per experiment in 2 separate experiments. Cells were scored as “aligned” if they had a metaphase plate with < 5 chromosomes off the plate. (F) Quantification of multipolarity observed in cells expressing WT- and Δ80-Hec1-GFP. Cells with unaligned chromosomes were scored for containing bi- vs multi-polar spindles, and the percent of cells with multipolar spindles is shown. For each condition, at least 100 cells per experiment from two separate experiments were assessed. On all bar graphs (C, E, F), statistical significance was determined using a Student’s t-test. Scale bars: 10 μm and 1 μm for panels and insets, respectively.

Supplemental Figure S6



Supplemental Figure 6. Location of the GFP tag differentially affects end-on attachment formation. (A) Immunofluorescence images of cold-treated RPE1 cells expressing N- and C-terminally GFP-tagged Δ80-Hec1 constructs. Cells were incubated in ice-cold DMEM for 15 minutes prior to fixation, permeabilized, fixed, and stained with

antibodies to tubulin. Insets are enlargements of the region indicated by the dashed box. Schematics of the constructs used are indicated on the right. (B) Immunofluorescence images of HeLa cells expressing N- and C-terminally GFP-tagged WT- and Δ80-Hec1 constructs. Insets are enlargements of the region indicated by the dashed box. Schematics of the constructs used are indicated on the right. (C) Immunofluorescence images of cold-treated HeLa cells expressing N- and C-terminally tagged Δ80-Hec1 constructs. Cells were incubated in ice-cold DMEM for 12 minutes prior to fixation, permeabilized, fixed, and stained with antibodies to tubulin. Insets are enlargements of the region indicated by the dashed box. (D) Quantification of end-on kinetochore-microtubule attachment in cold-treated HeLa cells expressing WT-Hec1-GFP, and N- and C-terminally tagged Δ80-Hec1 constructs. For N- and C-terminally tagged Δ80-Hec1 constructs, at least 15 kinetochores per cell were quantified from at least 8 cells from 3 independent experiments. For the WT-Hec1-GFP metaphase data, 15 kinetochores per cell were quantified from 5 cells per experiment from 2 independent experiments. The data for C-terminally tagged Δ80-Hec1-GFP and WT-Hec1-GFP are from Figure 4C. Statistical significance was determined using a Student’s t-test. Scale bars: 10 μm and 1 μm for panels and insets, respectively.

A

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WT      : 420 RKLKLIPKGAENSKGYDFEIKFNPEAGANCLVKYRAQVYVP 460
WT420-424A: 420 AAAAAIPKGAENSKGYDFEIKFNPEAGANCLVKYRAQVYVP 460
WT425-429A: 420 RKLKLAAAAAENSKGYDFEIKFNPEAGANCLVKYRAQVYVP 460
WT430-434A: 420 RKLKLIPKGAAAAAAYDFEIKFNPEAGANCLVKYRAQVYVP 460
WT435-439A: 420 RKLKLIPKGAENSKGAAAAAKFNPEAGANCLVKYRAQVYVP 460
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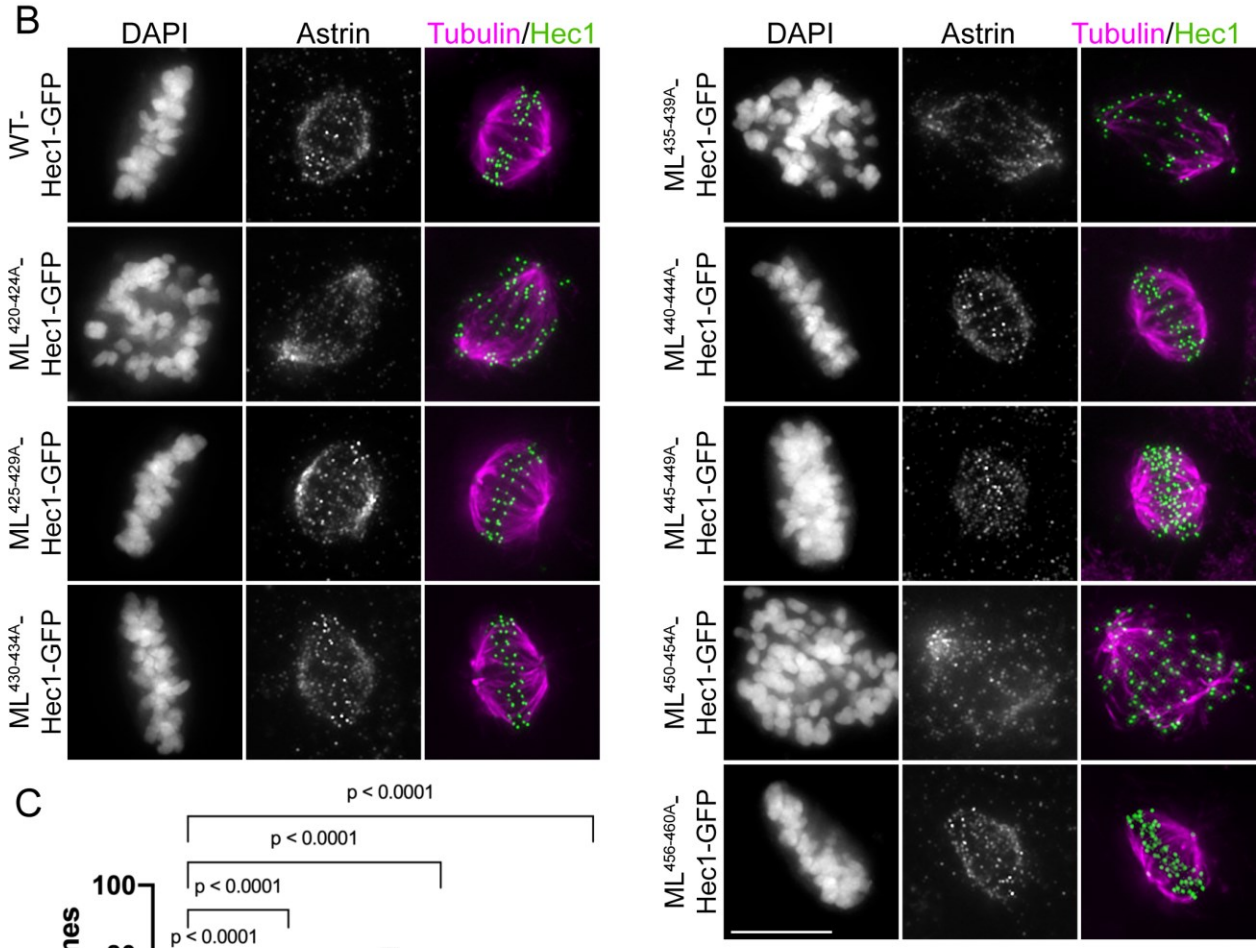
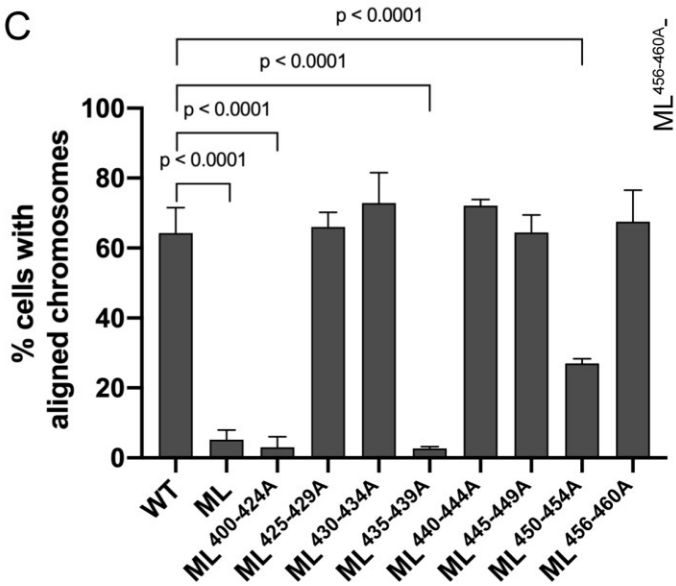
B



C



Supplemental Figure 7. Specific regions of the Hec1 loop are required for chromosome alignment in cells. (A) List of loop mutants used. (B) Immunofluorescence images of cells expressing WT- or ML-Hec1-GFP constructs and stained with Astrin and tubulin antibodies. (C) Quantification of chromosome alignment in cells expressing WT- or ML-Hec1-GFP constructs. For each condition, at least 120 cells were analyzed from at least 2 separate experiments. Cells were scored as “aligned” if they had a metaphase plate with < 5 chromosomes off the plate. Statistical significance was determined using a one-way Anova analysis. Scale bar: 10 μm.