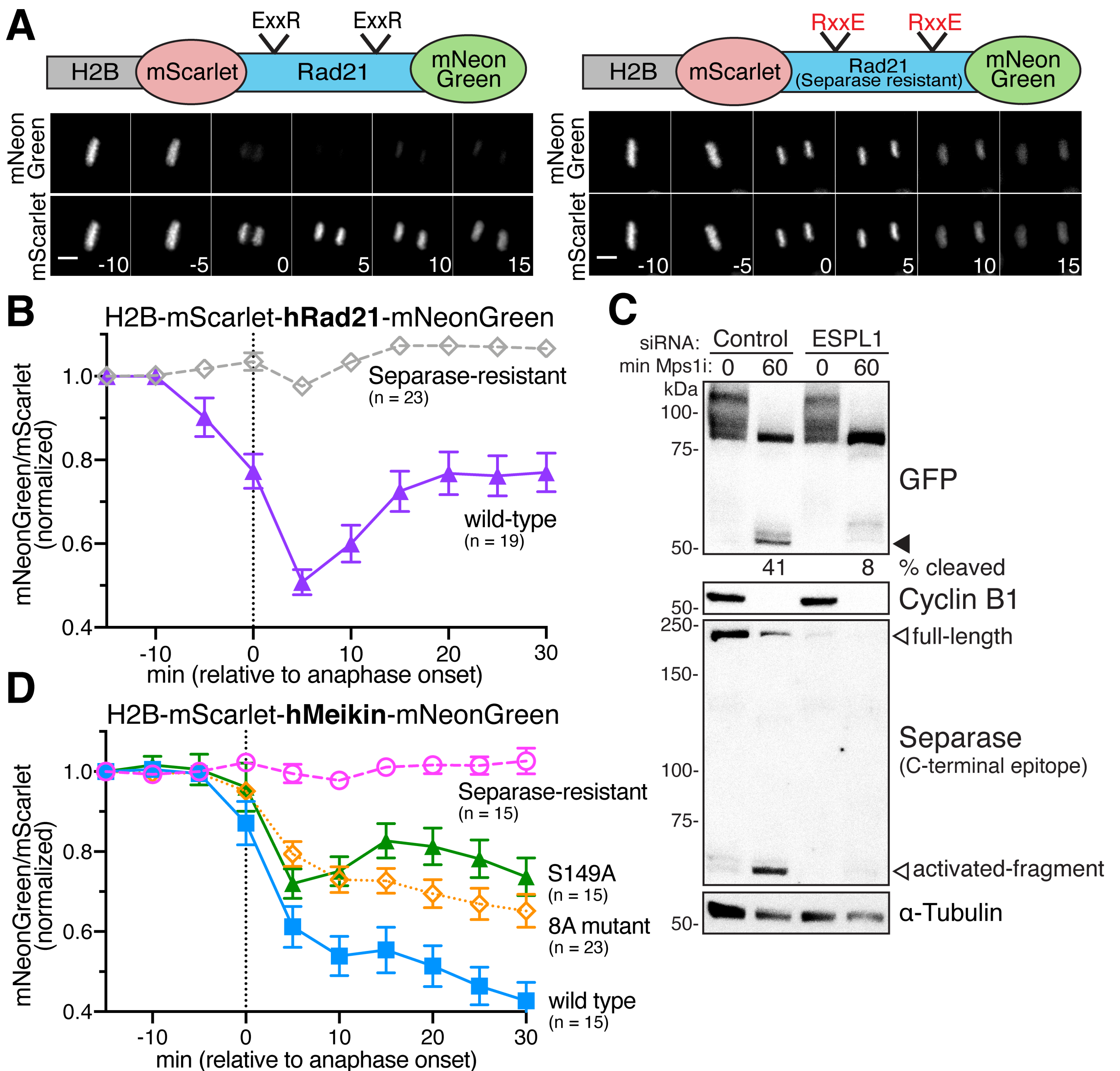

Figure S1. Dual color H2B-cleavage sensors monitor Separase activity at anaphase. Related to Figure 1.

(A) Schematic of dual-color hRad21 H2B-cleavage sensor. A fragment of hRad21 including the predicted Separase cleavage sites was used. Montage of time lapse images of cells expressing the indicated sensor. Numbers indicate minutes relative to anaphase onset. Scale bars, 10 µm.

(**B)** Quantification of the mNeonGreen:mScarlet ratio of Rad21 H2B cleavage sensors. The increase in mNeonGreen signal after +10 min in the wild-type sensor coincides with DNA decondensation and likely reformation of the nuclear envelope. Thus, we hypothesize that this increase is due to nuclear import of the cleaved C-terminal fragment.

(C) HeLa cells were treated with the indicated siRNA for 24 hr then induced to express EGFP-hMeikin and arrested in mitosis by treatment with nocodazole. Cells were forced to exit from

mitosis by treatment with Mps1i. At the indicated timepoint, cells were analyzed by Western blotting. Percent cleaved was calculated by densitometry analysis of the GFP blot.

(**D)** Quantification of the mNeonGreen:mScarlet ratio of Meikin mutant H2B cleavage sensors as in Figure 1. n = number of mitoses analyzed. Error bars indicate 95% confidence intervals.

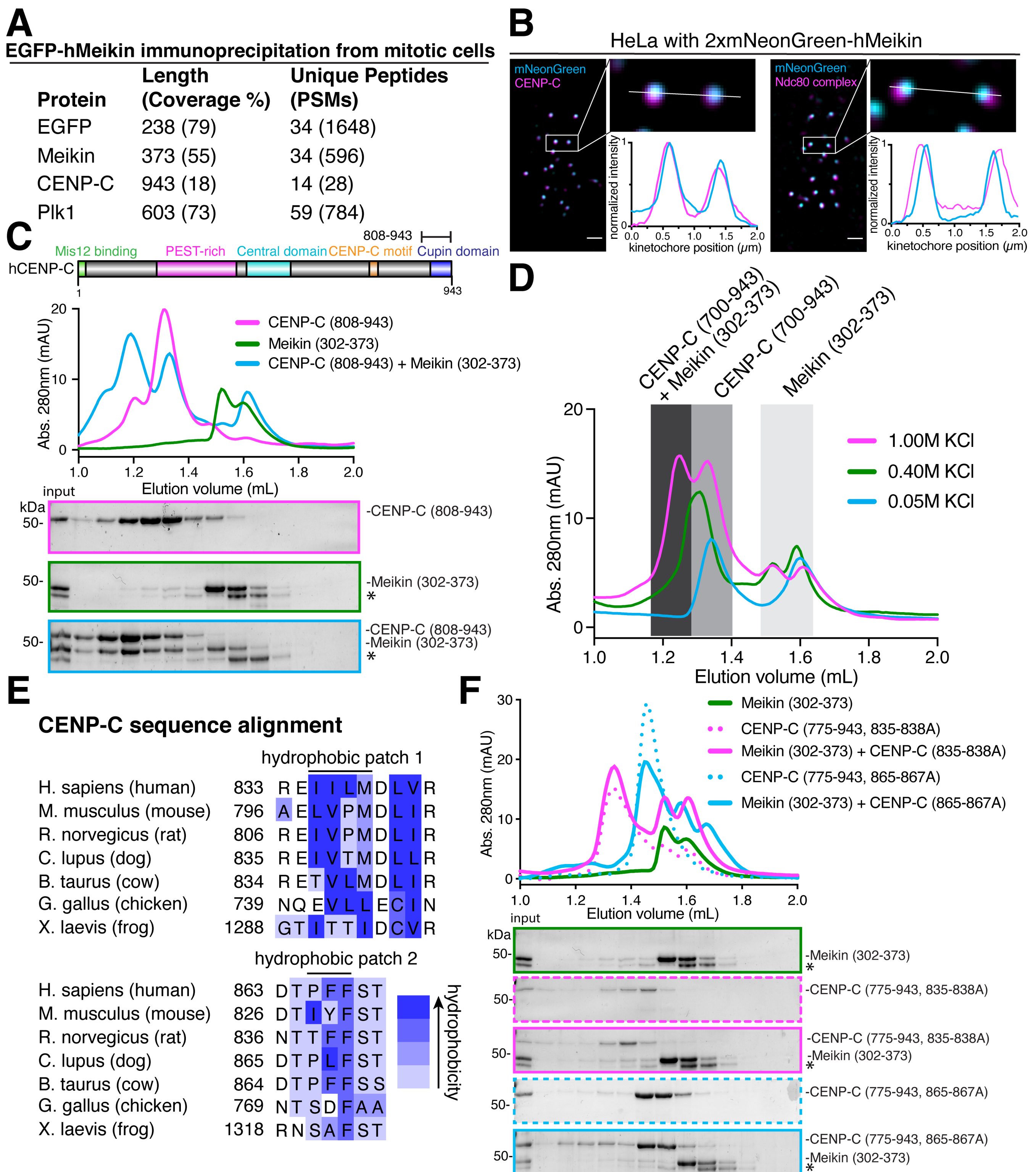

Figure S2. Meikin binds to CENP-C through hydrophobic patches on both proteins. Related to Figure 2.

(A) GFP-immunoprecipitates from HeLa cells expressing EGFP-hMeikin were analyzed by mass-spectrometry. The percent sequence coverage and number of total peptides of the indicated proteins are shown. Data are the sum of multiple mass-spectrometry experiments. A full list of proteins identified is included in Table S1.

(**B)** Metaphase HeLa cells stably expressing 2xmNeonGreen-hMeikin were fixed in ice cold methanol for 10 min then stained for CENP-C or the Ndc80 complex. Linescan analysis of the boxed sister-kinetochore pairs is shown. Meikin localizes to the inner kinetochore overlapping with CENP-C. Scale bar is 1 μm. Deconvolved immunofluorescence images are shown. Linescan analysis was performed before deconvolution.

(C) Diagram of known kinetochore protein interaction sites within CENP-C. Recombinant sfGFP-tagged Meikin and GST-tagged CENP-C protein fragments were bound and complexes analyzed by gel filtration. Fractions corresponding to elution volumes of 1.0 to 2.0 mL were analyzed by SDS-Page and Coomassie staining.

(**D)** Recombinant sfGFP-tagged Meikin and GST-tagged CENP-C protein fragments were bound and complexes analyzed by gel filtration. Binding reactions and gel filtration were performed in buffer with the indicated KCl concentration.

(E) Sequence alignment of CENP-C from selected vertebrates with conserved hydrophobic patches indicated. Amino acid hydrophobicity is indicated in blue.

(**F)** Recombinant Meikin and CENP-C fragments containing mutations in conserved hydrophobic patches analyzed by gel filtration as above.

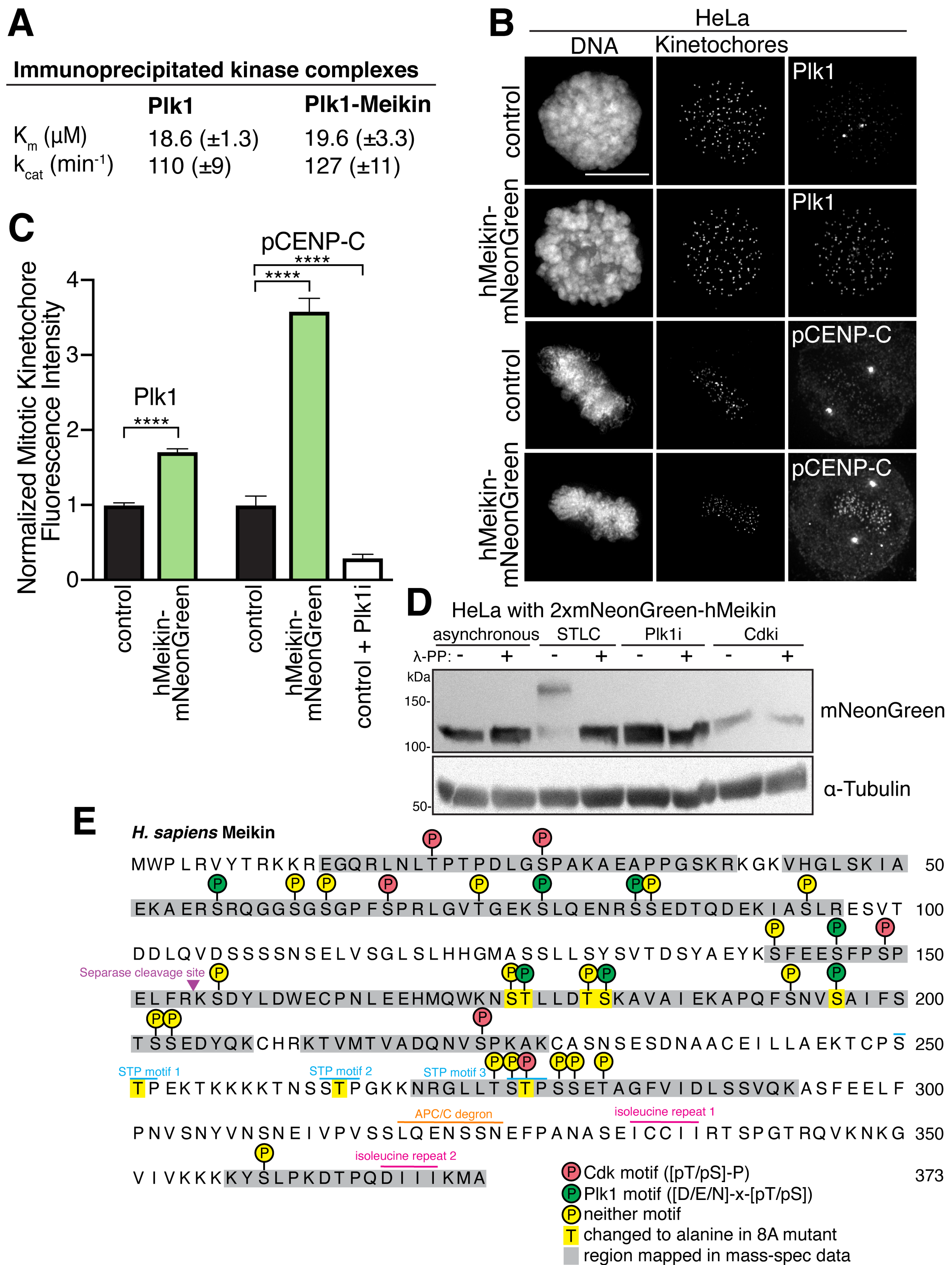

Figure S3. Meikin binds active Plk1. Related to Figure 3.

(A) Michaelis-Menten parameters derived from kinase assays of Plk1 or Plk1-Meikin complexes immunoprecipitated from HeLa cells. Standard error is represented in brackets.

(**B**) Deconvolved immunofluorescence images of HeLa cells (control) or HeLa cells stably expressing hMeikin-mNeonGreen and stained for Plk1 (pre-extracted in PBS + 0.5% Triton-X100 for 5 min; pre-extraction disrupts the bipolar spindle and centrosome staining of Plk1; kinetochores are co-stained with CENP-C antibody) or phosphorylated S311 on CENP-C (fixed in ice-cold methanol at -20°C for 20 min; centrosome pCENP-C staining is non-specific; kinetochores are co-stained with CENP-A antibody). Images of similarly stained cells are scaled identically. Scale bars, 10 μm.

(C) Quantification of kinetochore intensity of mitotic HeLa cells stably expressing hMeikin-mNeonGreen. Values were normalized to the mean of the control. Means and 95% confidence intervals are presented. Specificity of the pS311 CENP-C antibody was demonstrated by treatment of cells with Plk1i for 2 hr prior to staining. ****$P < 0.0001$, two-tailed *t*-test. n = Plk1: control (27 cells, 2,911 kinetochores), hMeikin (22 cells, 2,626 kinetochores). n = pCENP-C: control (21 cells, 1,093 kinetochores), hMeikin (27 cells, 1,185 kinetochores), control + Plk1i (25 cells, 2,302 kinetochores). Quantification was performed before deconvolution.

(**D**) HeLa cells stably expressing 2xmNeonGreen-hMeikin were treated with the indicated inhibitors. Whole cell lysates were incubated with or without lambda-phosphatase and analyzed by Western blot.

(E) GFP-immunoprecipitates from HeLa cells expressing EGFP-hMeikin were analyzed by mass-spectrometry. Mapped peptides and phosphorylation sites identified across multiple experiments are indicated on the Meikin protein sequence. Sequence motifs discussed in this work are marked.

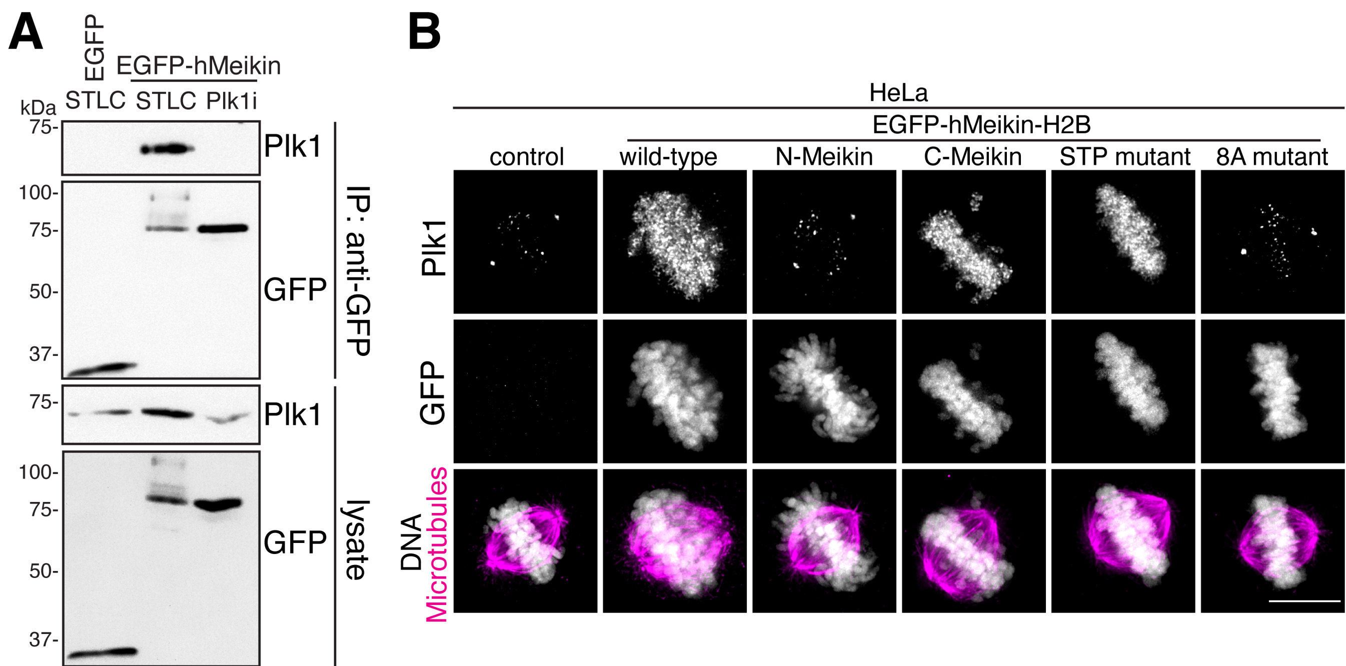

Figure S4. Full length Meikin and the C-Meikin cleavage fragment bind Plk1. Related to Figure 3.

(A) HeLa cells expressing EGFP-hMeikin were induced with doxycycline and arrested in mitosis by STLC or Plk1i treatment. Cells were lysed and GFP-immunoprecipitation (IP) was performed and analyzed by Western blot.

(**B)** Deconvolved immunofluorescence images of HeLa cells induced to express EGFP-hMeikin-H2B by treatment with doxycycline. A Meikin fragment excluding the C-terminal kinetochore binding domain was used. Cells were stained for Plk1. Localization was consistent across the cell population in multiple independent experiments. Images are not scaled equivalently. Scale bars, 10 μm.

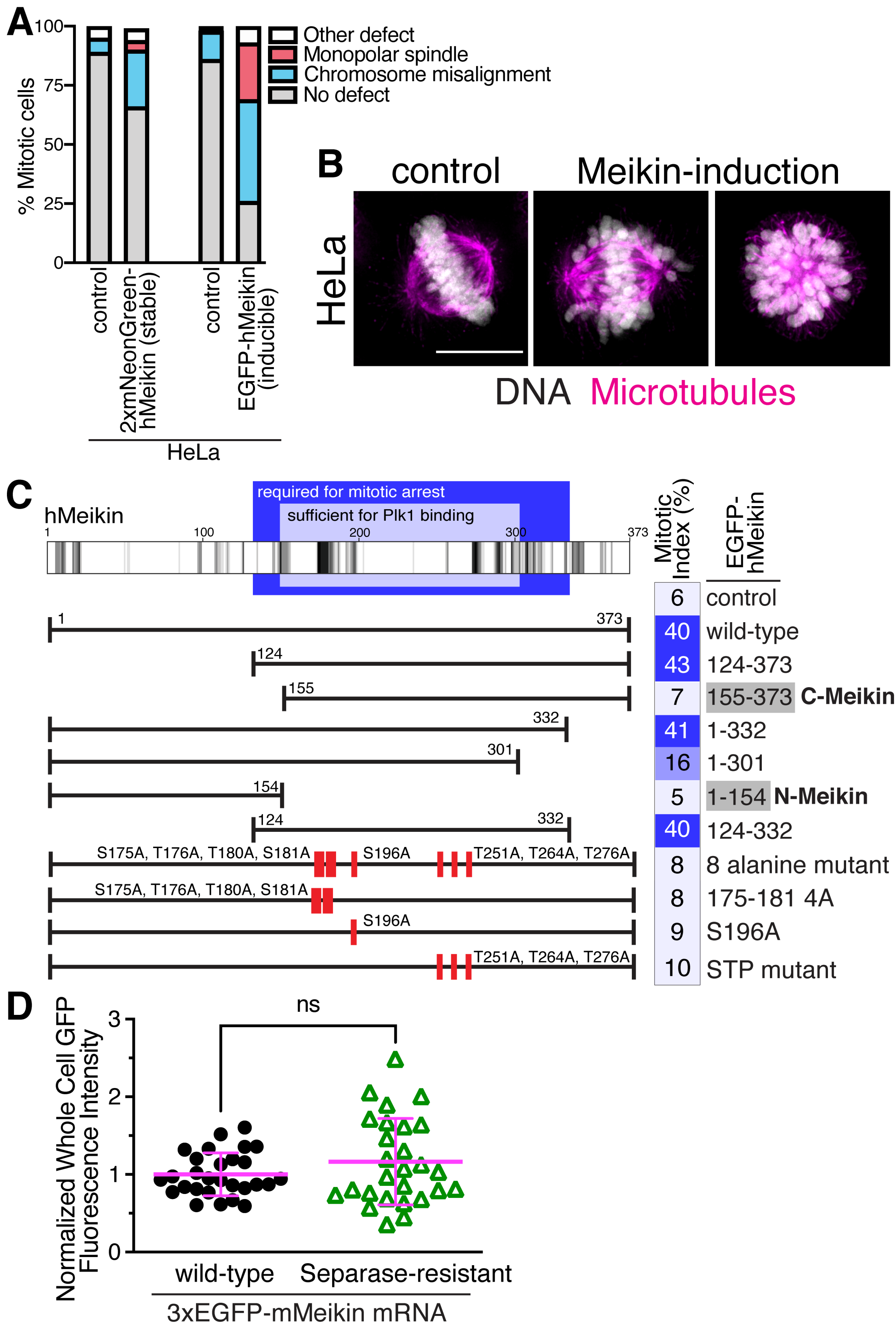

Figure S5. Meikin expression causes chromosome misalignment and monopolar spindles. Related to Figure 4.

(A) Quantification of mitotic defects observed in HeLa cells stably expressing Meikin or acutely induced to express Meikin from a doxycycline-inducible promoter. 100 mitotic cells were analyzed for each condition.

(**B)** Representative immunofluorescence images of mitotic HeLa cells stained for DNA and microtubules. High Meikin levels were induced in HeLa cells using a doxycycline inducible promoter. Scale bars, 10 μm.

(C) Schematic of the human Meikin protein with conserved residues (as measured by Consurf (Ashkenazy et al., 2016)) indicated in black. The indicated EGFP-Meikin constructs were expressed in HeLa cells by doxycycline induction. Cells were fixed and stained for the mitotic marker phosphorylated histone 3. The mitotic index of GFP-positive cells was measured by flow cytometry. The minimal region sufficient for Plk1 binding and for the mitotic arrest phenotype are indicated.

(D) Quantification of whole cell GFP intensity in oocytes injected with the indicated EGFP-mMeikin mRNA. Germinal vesicle intact (18 hr post-injection) and meiosis I (24 hr post-injection) stage oocytes were analyzed. Values were normalized to the mean of wild-type oocytes at the same stage. Mean and standard deviation is indicated. P = 0.1709 (non-significant, n.s.), two-tailed t-test. n = wild-type (28 oocytes), Separase-resistant (27 oocytes).

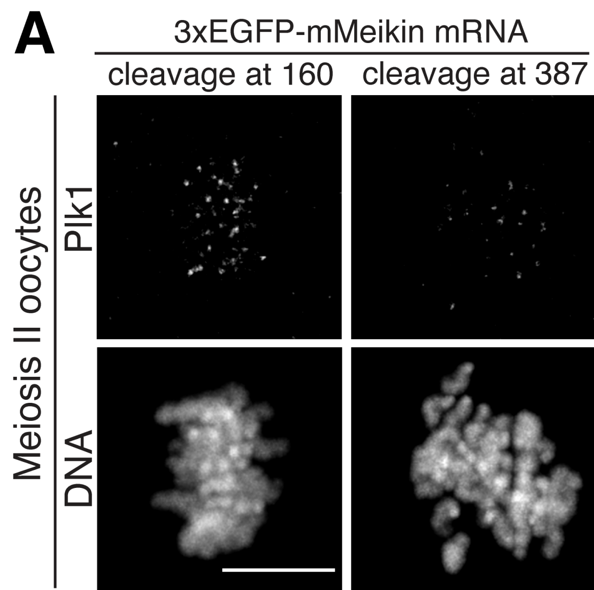

Figure S6. C-Meikin localizes Plk1 to meiosis II kinetochores. Related to Figure 5.

(A) Mouse oocytes injected with the indicated mMeikin mRNA construct were matured to meiosis II and stained for Plk1. Images are scaled identically. Scale bars, 10 μm.