JCB

Supplemental material

Bonner et al., https://doi.org/10.1083/jcb.201901004

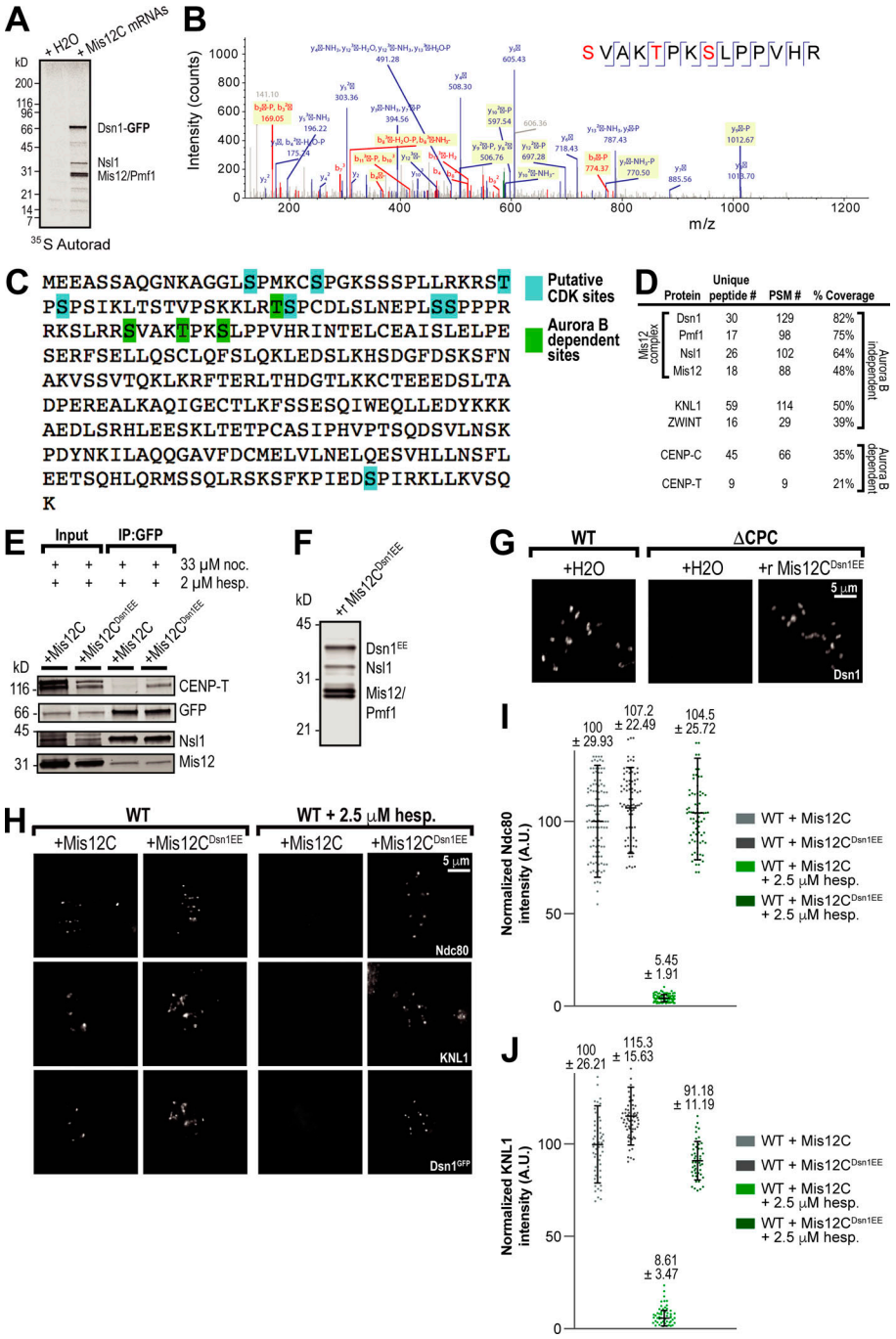

Figure S1. **Aurora B–dependent phosphorylation of Dsn1 controls outer kinetochore assembly in *Xenopus* egg extracts. (A)** ^{35}S Autorad shows stoichiometric expression of Mis12C mRNAs in cycled egg extract. Dsn1 is tagged at the C-terminus with a LAP tag. **(B)** Tandem mass spectrometry spectrum of the *Xenopus* Dsn1 peptide pSVAKpTPKpSLPPVHR, corresponding to residues 77–90. The peptide was detected as a +3 charge state, with a mass error of −1.17 ppm. The ptmRS confidences for phosphorylation of S77, T81, and S84 were all 100. The identified b (red) and y (blue) ions are denoted. **(C)** Amino acid sequence of Dsn1 showing all phosphorylation sites identified by mass spectrometry. Constitutive sites, which display characteristic CDK sequences, are highlighted in blue, and the Aurora B–dependent phosphorylation sites are highlighted in green. **(D)** Proteins that interact with the Mis12$^{Dsn1\text{-}LAP}$ complex expressed from mRNAs in extracts in the presence or absence of active Aurora B, as identified by mass spectrometry. All purifications were performed in the absence of chromosomes. **(E)** Western blot for proteins that copurify with Mis12C or Mis12C^{Dsn1EE} expressed from mRNA in extracts in the presence of 2 µM hesperadin. IP, immunoprecipitate. **(F)** Coomassie staining of purified recombinant Mis12C^{Dsn1EE} consisting of Mis12, Pmf1, Nsl1, and Dsn1^{EE}. **(G)** Representative immunofluorescence images of replicated chromosomes in WT and ΔCPC metaphase extracts with recombinant Mis12C^{Dsn1EE}. Chromosomes were stained for Dsn1. **(H)** Representative immunofluorescence images of replicated chromosomes in WT metaphase extracts with either Mis12C or Mis12C^{Dsn1EE} expressed from mRNAs and treated as indicated with hesperadin. Chromosomes were stained for Ndc80, KNL1, and GFP for Dsn1–LAP. **(I)** Quantification of fluorescence intensity of Ndc80 on replicated chromosomes in metaphase extracts in indicated conditions shown in H, normalized to WT. *n* = 96 kinetochores. A.U., arbitrary units. **(J)** Quantification of fluorescence intensity of KNL1 on replicated chromosomes in metaphase extracts in indicated conditions shown in (H), normalized to WT. *n* = 96 kinetochores. Error bars represent SD.

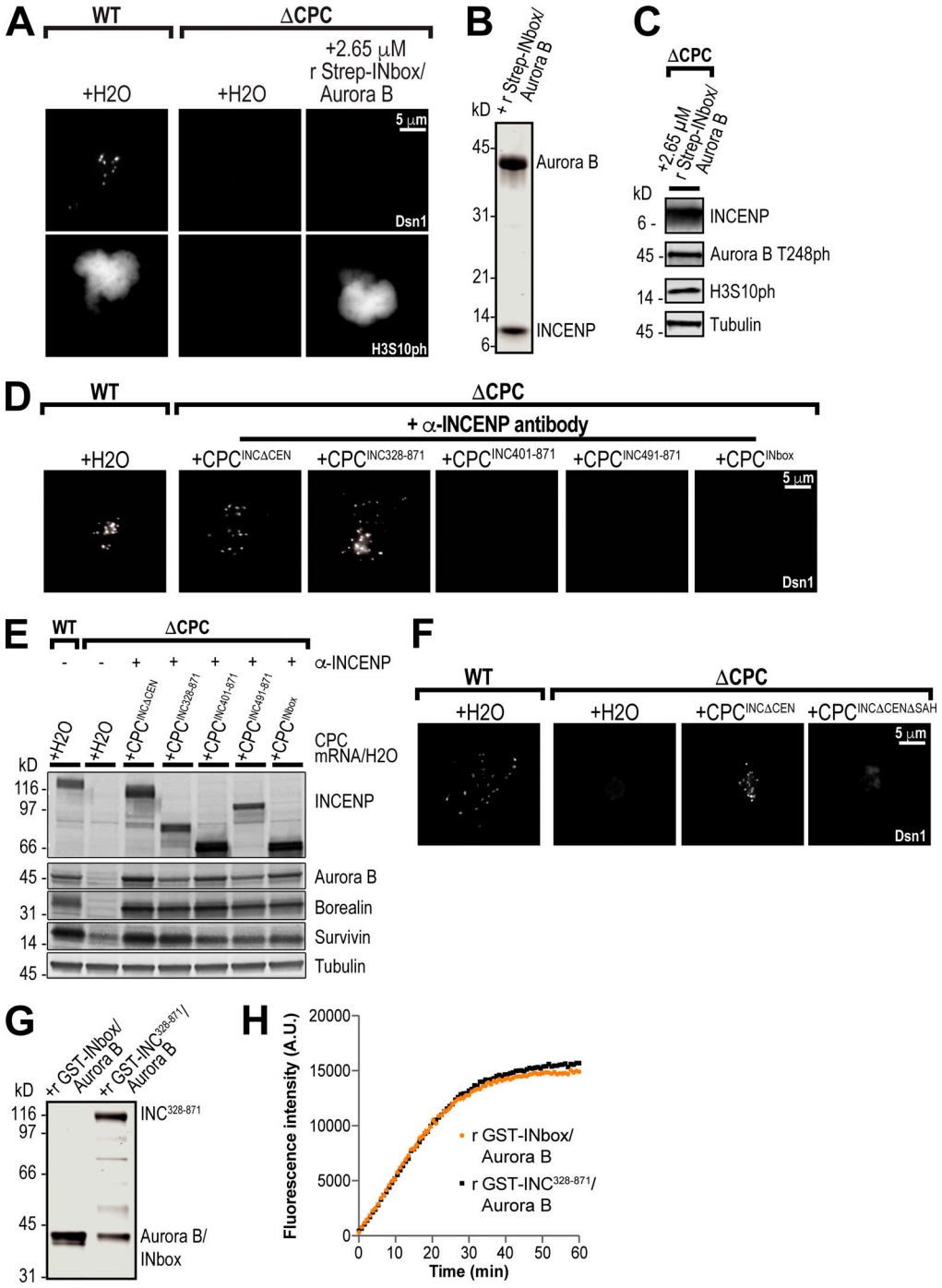

Figure S2. **Kinetochore assembly requires Aurora B kinase activity and the central region of INCENP. (A)** Representative immunofluorescence images of replicated chromosomes in WT and ΔCPC metaphase extracts reconstituted with or without preactivated recombinant (r) Strep-INbox–Aurora B. Chromosomes were stained for Dsn1 or histone H3S10ph. **(B)** Coomassie staining of recombinant protein complex consisting of Aurora B kinase and a fragment of INCENP containing the INbox domain (amino acids 788–871) fused to a Strep tag. **(C)** Western blot for INCENP, Aurora B T248ph, histone H3 phosphorylation (H3S10ph), and tubulin for samples shown in Fig. 2 A and panel A here. **(D)** Representative immunofluorescence images of replicated chromosomes in WT and ΔCPC metaphase extracts reconstituted with CPC containing the indicated INCENP construct with anti-INCENP antibody added where specified. Chromosomes were stained for Dsn1; a scatter plot of fluorescence intensities is shown in Fig. 2 E. **(E)** Western blot for CPC components and tubulin for samples shown in Fig. 2 E and panel D here. **(F)** Representative immunofluorescence images of replicated chromosomes in WT and ΔCPC metaphase extracts reconstituted with CPC containing the indicated INCENP construct with anti-INCENP antibody added to $CPC^{INCΔCEN}$ and $CPC^{INCΔCENΔSAH}$. Chromosomes were stained for Dsn1. **(G)** Coomassie staining of recombinant protein complex consisting of Aurora B kinase and a fragment of INCENP containing the INbox domain (amino acids 788–871) or the central region and the INbox domain (amino acids 328–871) fused to GST. **(H)** Chemosensor phosphorylation over time (minutes) for each indicated CPC complex. To compare the phosphorylation kinetics of the purified, preactivated protein complexes, 5 nM recombinant GST-INbox–Aurora B or recombinant $GST\text{-}INC^{328–871}$–Aurora B was incubated with 24 μM chemosensor and phosphorylation was measured by fluorescence intensity every 45 s. Note the absence of a lag time at early time points. Graph represents data from three independent experiments. A.U., arbitrary units.

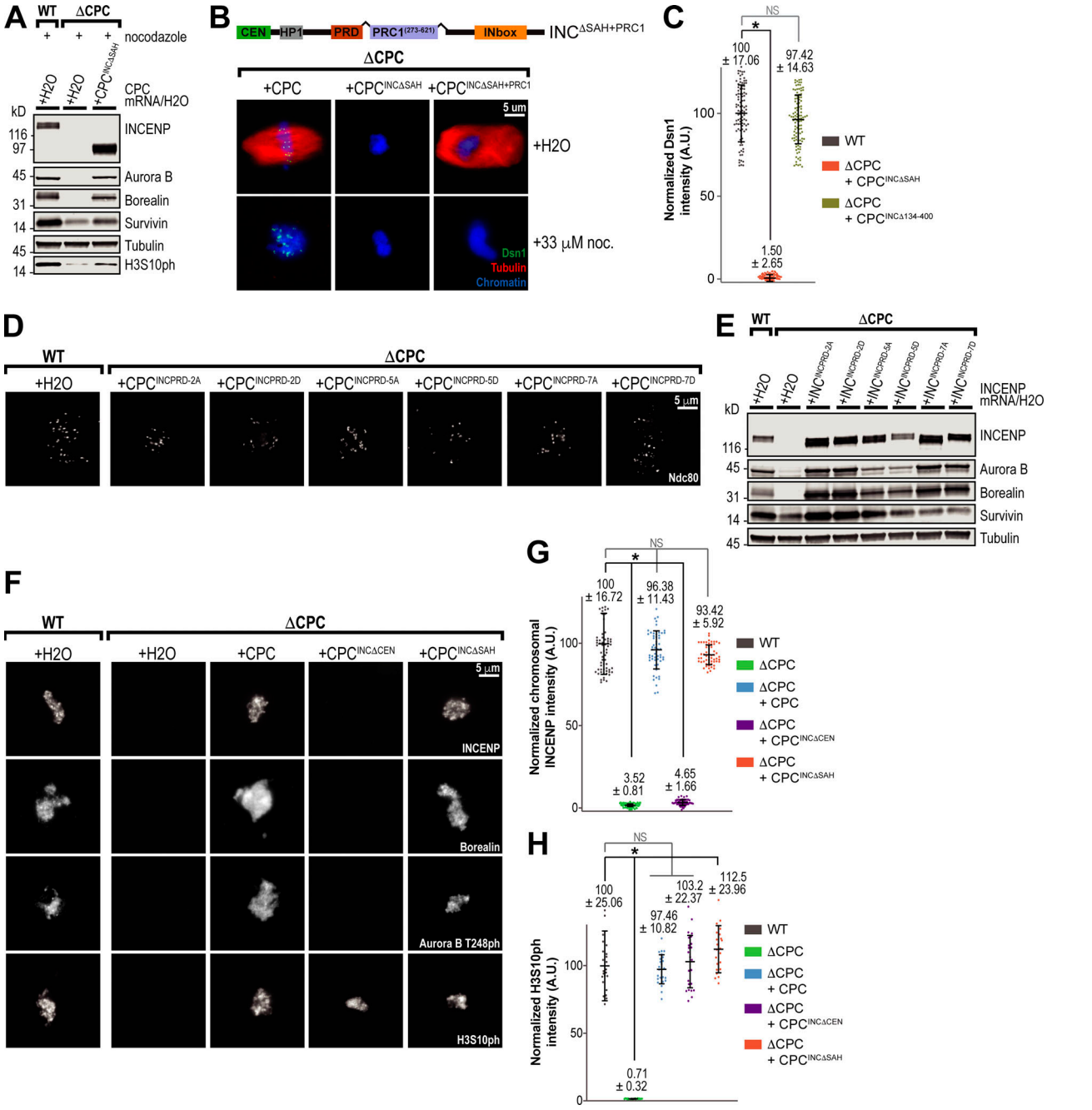

Figure S3. **The central region of INCENP promotes outer kinetochore assembly in a microtubule-independent manner. (A)** Western blot for CPC components, histone H3 phosphorylation (H3S10ph) and tubulin for samples shown in Fig. 3, A and B. **(B)** Schematic of an INCENP construct with the SAH domain replaced with the microtubule binding domain of human PRC1 (residues 273–621). This construct was expressed from mRNA in extract, along with full-length Borealin, Survivin, and Aurora B, to reconstitute CPC extracts. Top: Representative immunofluorescence images of spindles formed in WT and CPC extracts with indicated CPC conditions. Chromosomes were stained for Dsn1 (green), chromatin was stained with Hoechst (blue), and rhodamine-labeled tubulin was added to visualize microtubules (red). Note the dearth of microtubules near chromosomes in the PRC1-substituted condition. Bottom: Same as upper panel, but with nocodazole treatment. **(C)** Quantification of fluorescence intensity of Dsn1 on replicated chromosomes in metaphase extracts in indicated conditions, normalized to WT. *n* = 96 kinetochores. A.U., arbitrary units. **(D)** Representative immunofluorescence images of replicated chromosomes in WT and ΔCPC metaphase extracts reconstituted with CPC containing the indicated INCENP construct containing mutations of conserved CDK sites to either alanine or aspartic acid. PRD-5A/D denotes mutation of residues S430, T436, T438, T469, and T487 within the PRD; PRD-2A/D denotes mutation of residues S751 and T760 C-terminal to the SAH domain; and PRD-7A/D denotes a combination of 2A/D and 5A/D mutants. Chromosomes were stained for Ndc80. **(E)** Western blot for CPC components and tubulin for samples shown in D. **(F)** Representative immunofluorescence images of replicated chromosomes in WT and ΔCPC metaphase extracts reconstituted with CPC containing the indicated INCENP construct with anti-INCENP antibody added for the $CPC^{INCΔCEN}$ sample. Chromosomes were stained for INCENP, Borealin, Aurora B T248ph, and H3S10ph. **(G)** Quantification of total fluorescence intensity of INCENP on chromosomes in WT and ΔCPC metaphase extracts with the indicated conditions (shown in F), normalized to WT. *n* = 50 chromosome masses per condition. **(H)** Quantification of fluorescence intensity of H3S10ph in WT and ΔCPC metaphase extracts with the indicated conditions (shown in F), normalized to WT. *n* = 50 chromosome masses per condition. Error bars represent SD. *, P < 0.001.

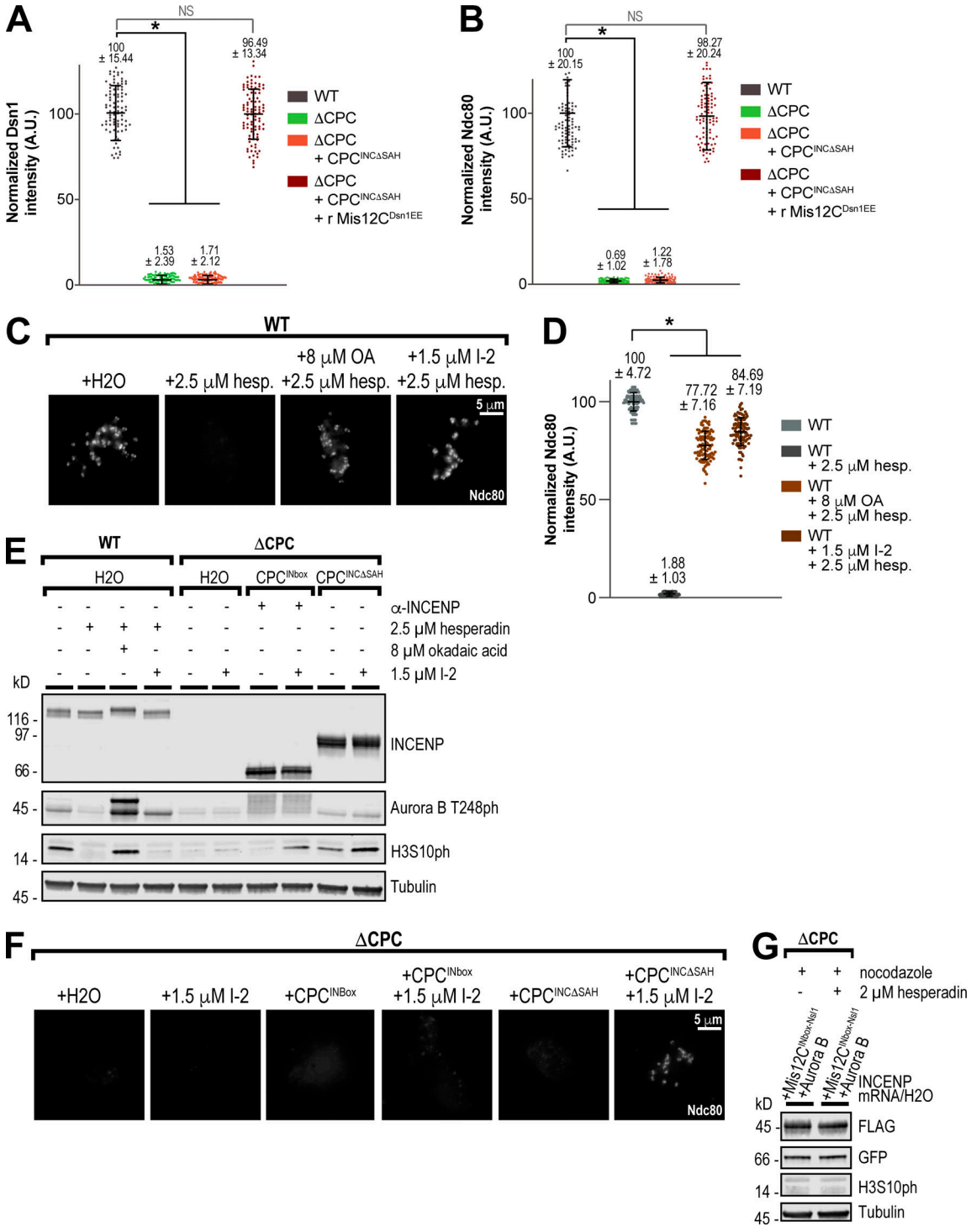

Figure S4. **Phosphomimetic Dsn1 or phosphatase inhibition bypasses the requirement for the SAH domain in kinetochore assembly. (A and B)** Quantification of fluorescence intensity of Dsn1 and Ndc80, respectively, in WT and ΔCPC metaphase extracts with indicated conditions and recombinant (r) Mis12C^{Dsn1EE} as specified, shown in Fig. 3 C, normalized to WT. *n* = 96 kinetochores per condition. A.U., arbitrary units. **(C)** Representative immunofluorescence images of replicated chromosomes in WT metaphase extracts treated with 2.5 μM hesperadin, 8 μM okadaic acid (OA), and 1.5 μM I-2 as indicated, 30 min into metaphase, after kinetochore assembly is established, to confirm that Aurora B activity is not required for kinetochore maintenance in the absence of phosphatase activity. Chromosomes were stained for Ndc80. **(D)** Quantification of fluorescence intensity of Ndc80 in WT metaphase extracts treated with 2.5 μM hesperadin, 8 μM okadaic acid, and 1.5 μM I-2 as indicated (shown in C), normalized to WT. *n* = 96 kinetochores per condition. **(E)** Western blot for INCENP, Aurora B phosphorylation (T248ph), histone H3 phosphorylation (H3S10ph), and tubulin for samples shown in Fig. 3 E (and C and D here). Clustering anti-INCENP antibody was added to the CPC^{INbox} sample to ensure full activation of Aurora B kinase. **(F)** Representative IF images of replicated chromosomes in ΔCPC metaphase extracts with indicated CPC conditions and anti-INCENP antibody added to the INbox sample to ensure kinase activation, treated as indicated with 1.5 μM I-2. Chromosomes were stained for Ndc80. Quantification of fluorescence intensity of Ndc80 shown in Fig. 3 E. **(G)** Western blot for FLAG, histone H3 phosphorylation (H3S10ph), and tubulin for samples shown in Fig. 3, F and G. Error bars represent SD. *, P < 0.001.

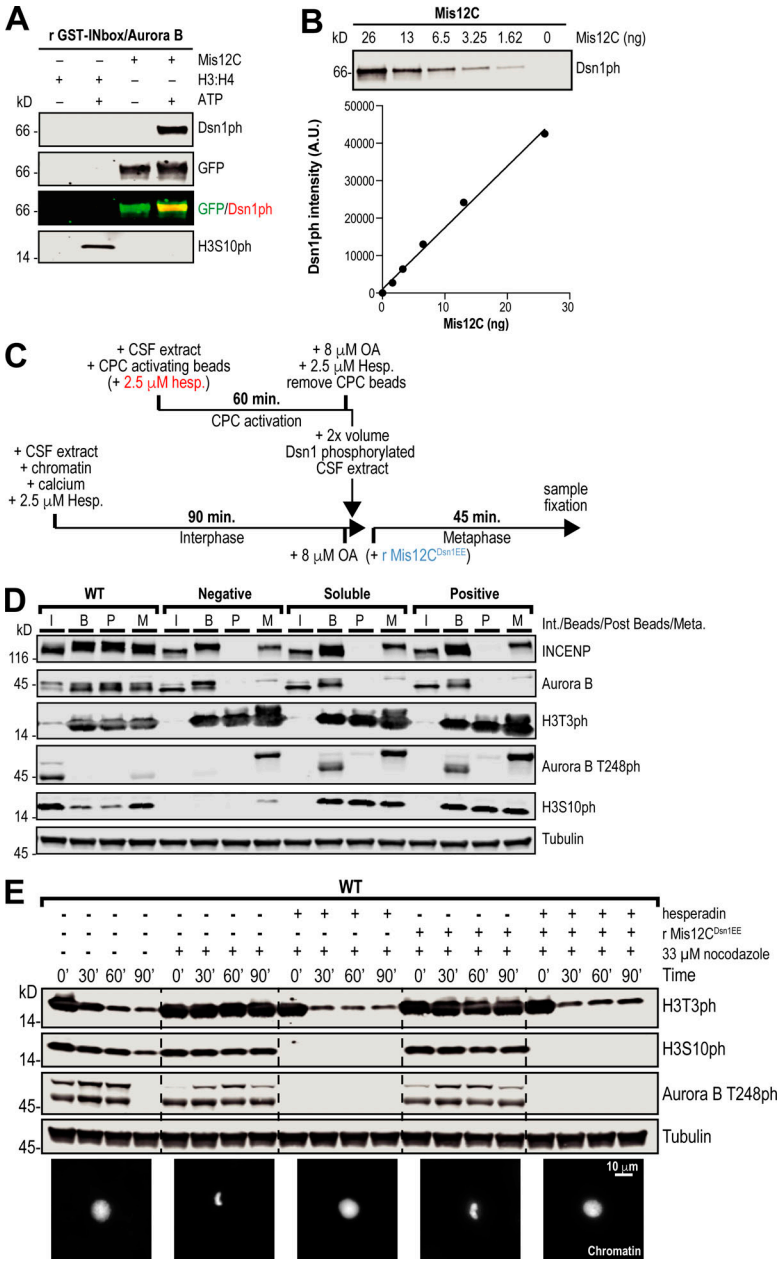

Figure S5. **Mis12C assembly and the SAC require localized Aurora B kinase activity. (A)** Western blot to demonstrate specificity of custom phospho-specific Dsn1 antibody. 1 μM preactivated recombinant INbox–Aurora B was incubated with 150 nM recombinant (r) Mis12C or 150 nM recombinant H3–H4 complex in the presence or absence of 0.7 mM ATP for 8 h. Phosphorylation of Dsn1 was only detected in the presence of ATP, similar to phosphorylation of histone H3S10. This confirms that phosphorylation of Dsn1 requires Aurora B kinase activity and that the antibody is specific to Ser77 of Dsn1. **(B)** Western blot demonstrating linearity of custom phospho-specific Dsn1 antibody. A dilution series was prepared from an in vitro kinase assay reaction of purified 190 nM recombinant Mis12C and 2 μM preactivated recombinant GST-INbox–His-Aurora B incubated with 4 mM ATP for 8 h to give fully phosphorylated product. Intensity of Dsn1ph bands were quantified and plotted against concentration of Mis12C. A.U., arbitrary units. **(C)** Detailed schematic of the soluble Dsn1 assay shown in Fig. 5 A to test if Mis12C phosphorylated away from chromatin could be incorporated into kinetochores (see Materials and methods for the detailed protocol). Briefly, CSF extract lacking chromatin was incubated with anti-INCENP beads for 60 min to activate the CPC to phosphorylate substrates, followed by kinase and phosphatase inhibition and bead removal. This extract, containing soluble phosphorylated Mis12C, was then added to interphase extract with chromatin in the presence of hesperadin to drive the reaction into metaphase. Kinetochore assembly was evaluated after 45 min. Positive and negative control reactions are indicated with blue and red, respectively. **(D)** Western blot for CPC components, histone H3 phosphorylation (H3T3ph and H3S10ph), Aurora B phosphorylation (T248ph), and tubulin for samples from soluble Dsn1 assay shown in Fig. 5, A–C, and in panel C here (see Materials and methods for detailed protocol). Western blot samples were taken at the end of interphase (I), from the CSF extract at the end of incubation with anti-INCENP beads (B), from the CSF extract after removal of anti-INCENP beads (P), and at 45 min into metaphase after the CSF extract (after bead removal) was added to the interphase extract to drive the reactions into metaphase (M). **(E)** Western blot for global checkpoint assay for histone H3 phosphorylation (H3T3ph and H3S10ph), Aurora B phosphorylation (T248ph), and tubulin. WT metaphase extracts treated with 2 μM hesperadin, 33 μM nocodazole, or recombinant Mis12C^{Dsn1EE} as specified were incubated with sperm nuclei and then challenged with the addition of calcium chloride. Samples were taken at indicated time points after calcium addition, shown with representative images of chromatin morphology at the end of the assay.