

### **Supplemental Experimental procedures**

Cloning Mis18BP1 was cloned into a modified pMal-c2 vector containing an attR Gateway® cloning cassette inserted into the BamHI cut site after the N-terminal MBP gene. Mis18BP1 harboring a C-terminal 6X-His tag was cloned into the modified pMal-c2 vector using LR Clonase II (Cat# 11791020, Life Technologies). A StrepHA Gateway® vector was made from an existing 6X-his Gateway® vector (Addgene, pDest-527) by removing the 6X-His tag with NdeI and BglII and replacing it with a StrepHA tag digested with the same enzymes. Entry clones of Mis18 $\alpha$  and Mis18 $\beta$  were generated by recombination from PCR products according to the Gateway® cloning protocol (Life Technologies) and were recombined into the StrepHA acceptor vector with LR Clonase II. The CENP-C fragment (a.a. 694-943) was recombined into a HisNusA Gateway® vector (Addgene, pDest-544) using LR clonase II.

Tissue Culture HeLa derived cells lines were cultured in DMEM High Glucose (Life Technologies) supplemented with 10% heat inactivated FBS (Optima, Atlanta Biologicals) and 1X Penicillin/Streptomycin (Life Technologies). U2OS derived cell lines were cultured in DMEM High Glucose GlutaMAX™ (Life Technologies) supplemented with 10% FBS and 1X Penicillin/Streptomycin. Cells were incubated at 37°C in 5% CO<sub>2</sub> and 85% humidity. A stable GFP-Mis18BP1 expressing cell line was generated using the Flp-In system (Life Technologies) in a previously established HeLa T-REX Flp-In cell lines (S. Taylor, University of Manchester). Recombined cell lines were maintained in 200 µg/mL Hygromycin B-supplemented media.

Cells were transfected using Lipofectamine 2000 (Life Technologies). For centromere localization experiments cells were transfected with a 10:1 ratio of GFP construct to RFP-H2B plasmid, as a transfection marker. Cells were analyzed 48 hours post-transfection and analysis was restricted to cells expressing the RFP-H2B.

siRNA depletion Simple siRNA depletions of Mis18 proteins in cells that stably expressed GFP-Mis18BP1 were accomplished by plating cells at a density of 10,400 cells/cm<sup>2</sup> in a 6-well format and letting the cells attach to the plate and coverslips. The following day, cells were treated with 20 nM Silencer® Select siRNA (Mis18BP1, 5'-GGAUAUCCAAAUAUCUCAAtt-3'; Mis18 $\alpha$ , 5'-GAAGAUGUCUUGAAAGCAUtt-3'; Mis18 $\beta$  5'-GCACAAUCGCUUAAAUAUCAAtt-3', Negative Control, Cat# 4390846, Ambion) using RNAiMax in Opti-MEM. After 12 – 24 h, 1 mL of full growth media was added to supplement the reduced serum media. Cells were then harvested 48 h post-siRNA transfection using 3mM EDTA-PBS or fixed and stained using the previously stated protocol. For siRNA depletion and rescue experiments, cells were transfected with Lipofectamine 2000 with 30 pmol Silencer® Select siRNA (Mis18BP1 5'-GGAUCUGAUAAGACAAAUAAtt-3' or GAPDH siRNA, Cat# 4390850, Ambion) and 1  $\mu$ g of the GFP-Mis18BP1<sup>2-721</sup> plasmid and 0.1ug RFP-H2B plasmid.

Immunofluorescence. Cells on coverslips were fixed in 4% formaldehyde in PBS. and blocked 1 h in blocking buffer (2% FBS, 2% BSA, 0.1% Triton-X100, PBS). Primary antibodies were incubated for 1 h in blocking buffer: anti-CENP-A mouse monoclonal antibody (ab13939, Abcam) at 1  $\mu$ g/mL and anti-CENP-T rabbit polyclonal antibody

serum (D. Cleveland, Ludwig Institute for Cancer Research) at 1:2000. Secondary antibodies were diluted in blocking buffer and were incubated with the coverslips for 1 h (Jackson ImmunoResearch Laboratories, Inc.). Cells were stained with 0.2  $\mu\text{g}/\text{mL}$  DAPI for 5 min. and mounted using Prolong Gold Antifade (Life Technologies).

Images were acquired using a DeltaVision microscope (GE Healthcare Lifesciences). Images within an experiment were acquired using identical exposure times. Images shown in figure panels are deconvolved maximum projections and scaled equally. The integrated intensity at each array was divided by the average background intensity from three non-centromeric sites within the nucleus cell to generate a ratio of the GFP signal at the array over the average nuclear GFP background.. > 20 arrays were analyzed per condition.

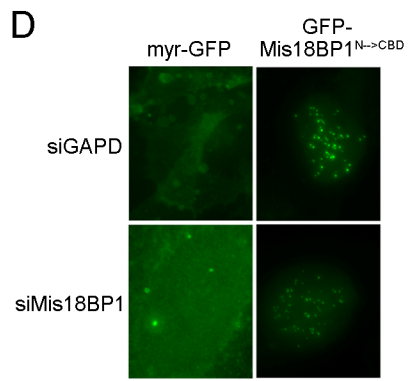
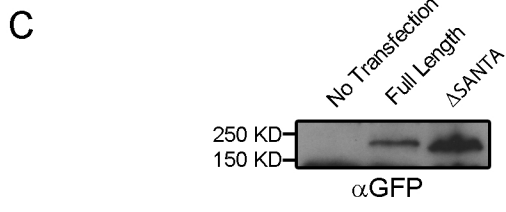
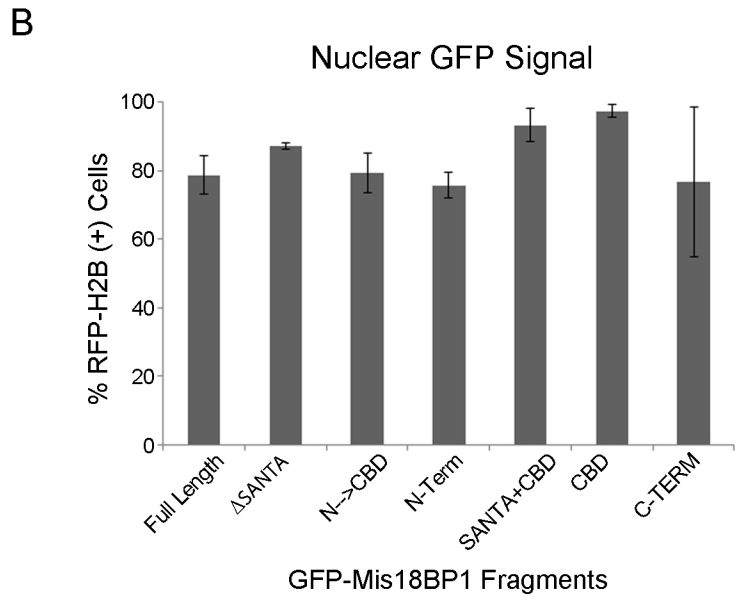
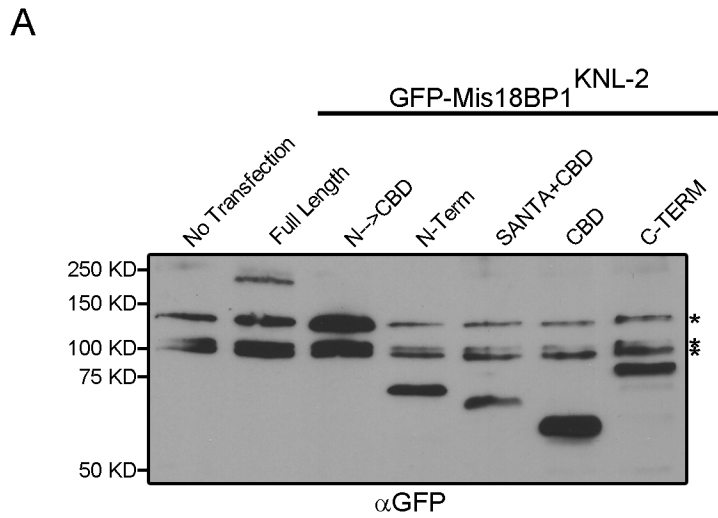
Image Quantitation The CENP-A intensity at centromeres in Figure S2 was measured from non-deconvolved maximum projections, using ImageJ. The background intensity for an individual nucleus was determined by averaging the integrated intensity of at least three non-centromeric locations within the nucleus. The integrated intensity of at least five random centromeres in each nucleus was measured, and the average background for that nucleus was subtracted from each measurement.

For siRNA experiments, at least 95 nuclei were analyzed in each siRNA condition. Background-corrected centromere intensities were then averaged across each condition. Standard deviations were calculated between the averaged centromere intensities for each condition, across three replicates.

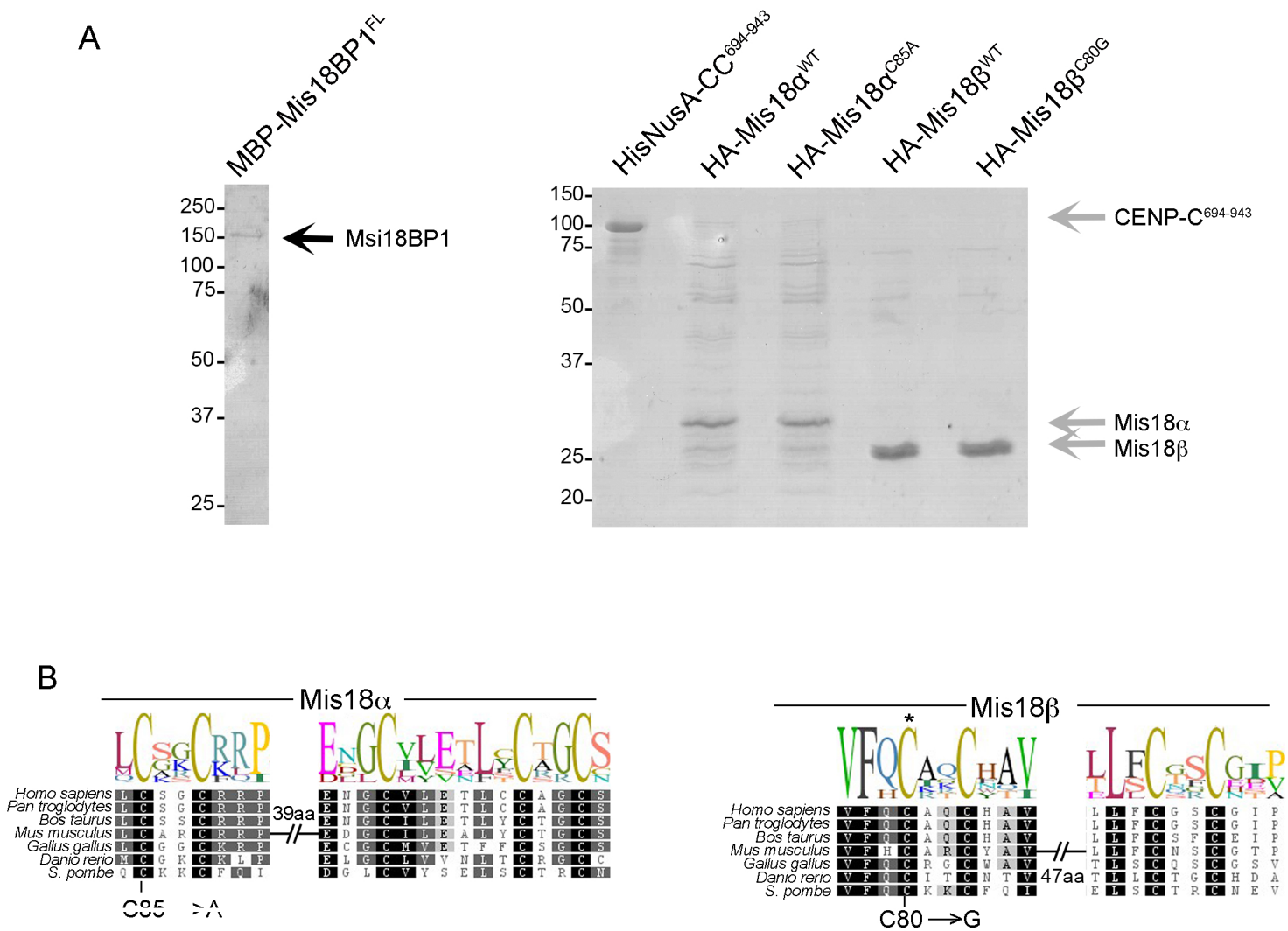
Western blotting. Immunoblots were blocked for 1 h in blocking buffer (5% milk in TBS with 0.1% tween20). Primary antibodies were incubated overnight in blocking buffer at 4°C [anti-GFP (Custom made by Covance), c-Myc 9E10 (Santa Cruz Biotechnology, sc-40), anti- $\beta$  Tubulin clone AA2, Mis18BP1 (Bethyl Laboratories, Inc., A302-825A and A302-824A), and Mis18 $\beta$  (BL10295, Bethyl Laboratories, Inc.). 6X-His tags (sc-803, Santa Cruz Biotechnologies), MBP tag (New England Biolabs), HA.11 (Covance), and NusA (Novus Biologicals)] HRP conjugated secondary antibodies were incubated in blocking buffer at 40 ng/mL for 1 h at room temperature (Jackson ImmunoResearch Laboratories, Inc.). HRP was detected using the West Pico ECL Regent (Pierce).

Recombinant Protein expression Recombinant MBP-tagged Mis18BP1, StrepHA-Mis18 $\alpha$ , StrepHA-Mis18 $\beta$ , and His-NusA-CENP-C (a.a. 694-943) proteins were expressed in the Rosetta™ (DE3) pLysS bacterial strain. Transformed bacteria were grown in LB media to an OD of 0.6 at 37°C, cooled to 18°C, and protein expression was induced with 0.1 mM IPTG for 16 h at 18°C. Pellets were lysed in 50 mM Tris-HCl, 350 mM NaCl, 0.5 mM CaCl<sub>2</sub>, 10% glycerol, 0.1% NP-40, 5 mM  $\beta$ -mercaptoethanol, LPC, and 1mM PMSF using a steel Wheaton dounce homogenizer. For His-tagged proteins, 20 mM imidazole was added to the lysis buffer and 40 mM imidazole was added to the wash buffer. Strep-tagged proteins were purified with Strep-Tactin Superflow Plus (Qiagen) and eluted in a buffer containing 2.5 mM d-desthiobiotin. His-tagged proteins were purified using Ni-NTA agarose (Qiagen) eluted with 250 mM imidazole. Mis18BP1 was initially purified using its C-terminal His-tag using the protocol above. It was further

purified using a Superdex 200 10/300 column (GE-Healthcare). Briefly, elutions were pooled and run over the column in the above buffer (without LPC and PMSF). Peak fractions were pooled and concentrated on Ni-NTA agarose and eluted in the same buffer containing 250 mM imidazole.

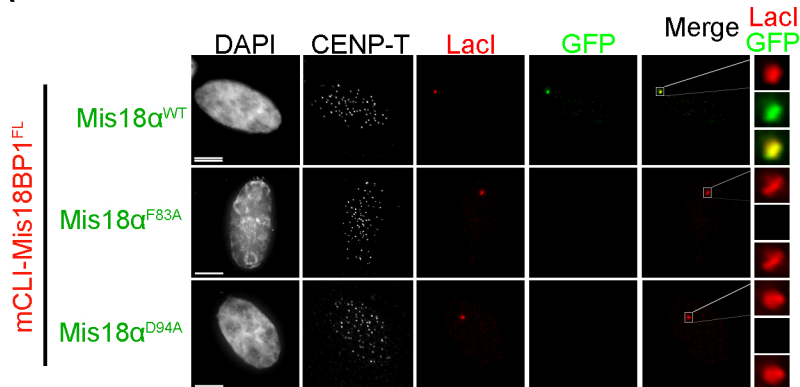


**Supplemental Figure 1.** Related to Figure 1. Endogenous Mis18BP1 is not required for Mis18BP1<sup>N->CBD</sup> to localize to centromeres. (A) Anti-GFP immunoblot of whole cell lysates from U2OS cells transiently expressing the GFP-Mis18BP1 constructs used in the immunofluorescence subcellular localization analysis in Figure 1. (B) Graph of the percentage of cells expressing the transfection marker (RFP-histone H2B) that have the indicated GFP-Mis18BP1 fragment localized in the nucleus. Error bars indicate S.D. between replicates. (C) Immunoblot of GFP-Mis18BP1<sup>ΔSANTA</sup> expressed in U2OS cells. (D) GFP signal for control myrGFP (myristolated GFP, plasma membrane associated) or GFP-Mis18BP1<sup>N->CBD</sup> observed in siRNA rescue experiments.

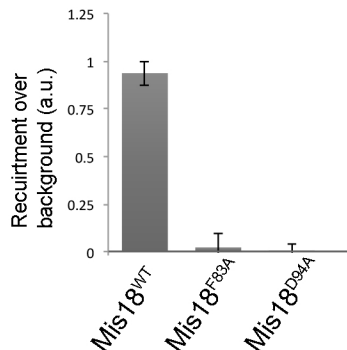


**Supplemental Figure 2.** Related to Figure 3 and Figure 4. (A) Coomassie stained SDS-PAGE gel of the recombinant protein used in this study. (B) ClustalW2 alignment of CXXC motifs of Mis18 $\alpha$  and Mis18 $\beta$  homologs. Darker highlighting and larger letters indicate highly conserved residues.

A

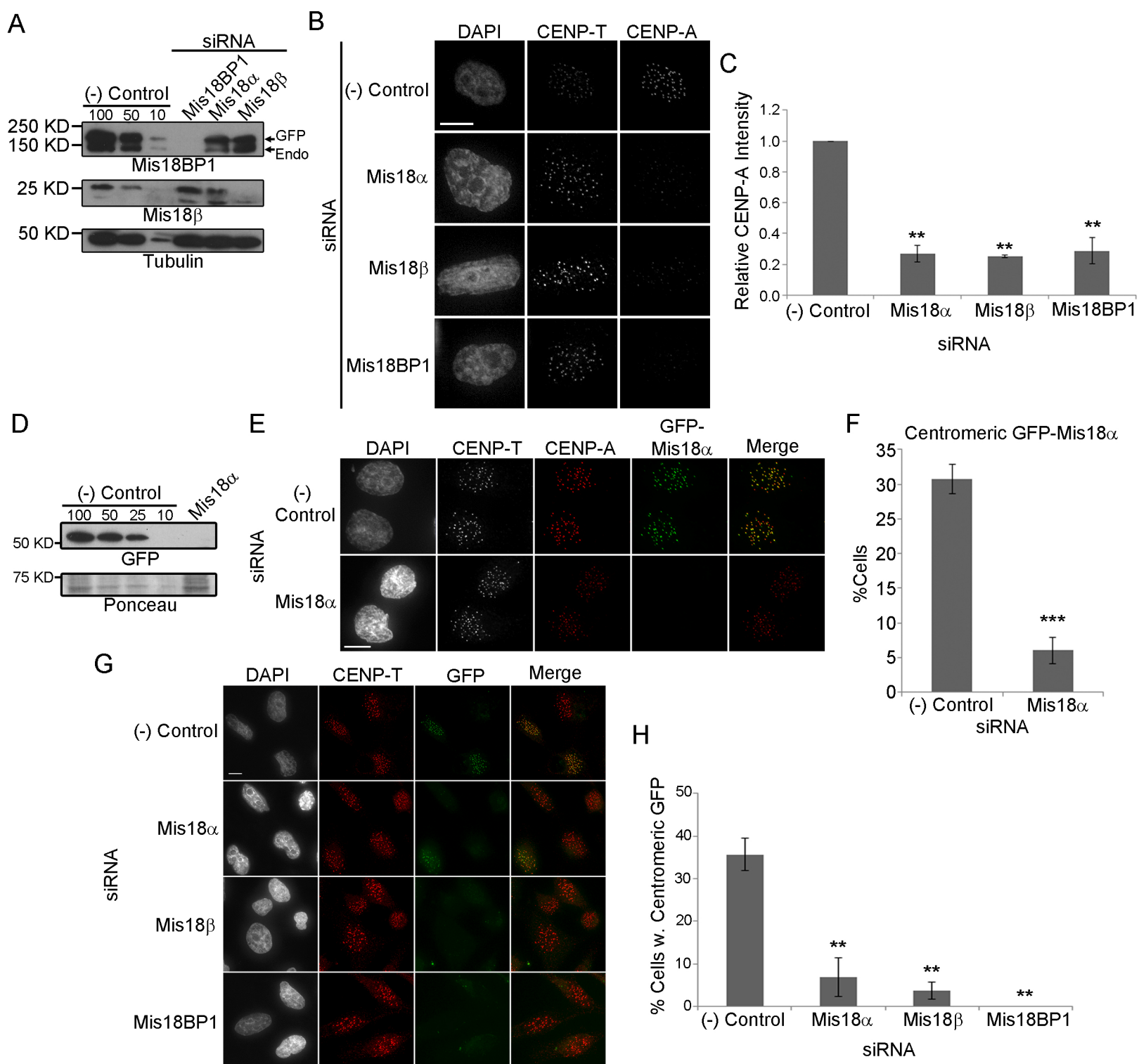


B



**Supplemental Figure 3.** Related to Figure 3. Mutations in the YIPPEE domain of Mis18α eliminate interaction between Mis18α and Mis18BP1 at the LacO array. (A) mCherry-LacI tagged Mis18BP1 and Mis18α wild type or mutant Mis18α were transiently expressed in LacO-containing U2OS cells. Alanine mutations in Mis18α were made in the conserved amino acids F83 and D94 within the YIPPEE domain. (B) Quantification of Mis18α recruitment was conducted by measuring the fold recruitment over background (n=10).





Supplemental Figure 4. Related to Figure 3 and Figure 4. Mis18BP1 requires Mis18 $\alpha$  and Mis18 $\beta$  to localize to the centromere. (A) Whole cell lysates of HeLa T-Rex cells that stably express GFP-Mis18BP1, which were treated with negative control siRNA or siRNA against Mis18 $\alpha$ , Mis18 $\beta$  or Mis18BP1 for 48 h. Lysates were blotted with antibodies against Mis18 $\beta$  and Mis18BP1. Two bands are present in the Mis18BP1 immunoblot, the upper band correlates to GFP-Mis18BP1. An anti-tubulin antibody was used as a loading control. (B) Representative images of the GFP-Mis18BP1 expressing cells treated as in A. Cells were stained with antibodies against CENP-T to mark centromere location. CENP-A centromeric protein levels were assayed by staining with a monoclonal antibody. (C) Bar graph depicts the background corrected, integrated intensity of CENP-A at centromeres, relative to the negative control condition. Error bars indicate the standard deviation across three replicates. Double asterisks represent a p-value of < 0.01 with respect to the negative siRNA control, as calculated by the Student's t-test. (D) Anti-GFP immunoblot of whole cell lysates from HeLa cells stably expressing GFP-Mis18 $\alpha$ . Cells were treated with negative control siRNA or siRNA against Mis18 $\alpha$  for 48 h. Ponceau staining serves as a loading control. (E) Representative images of HeLa GFP-Mis18 $\alpha$  cells, which were treated as in D. Centromeres were visualized with antibodies against CENP-T (white) and CENP-A (red). (F) Graph of the average percentage of randomly cycling cells that recruited GFP-Mis18 $\alpha$  to endogenous centromeres. Error bars indicate the standard deviation across three replicates. Triple asterisks represent a p-value of < 0.001, as calculated by the Student's t-test. (G) Representative images of HeLa T-Rex cells stably expressing GFP-Mis18BP1 that were treated as in A. Centromeres were visualized using anti-CENP-T antibodies. (H) Graph shows the percentage of cells in which GFP-Mis18BP1 is localized to endogenous centromeres with respect to siRNA treatment. Double asterisks indicate a p-value of < 0.01 with respect to the negative control siRNA condition, as calculated by the Student's t-test. Scale bars represent 5  $\mu$ m.