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Supplemental Information

Licensing of Centromeric Chromatin Assembly through the Mis18α-Mis18β Heterotetramer

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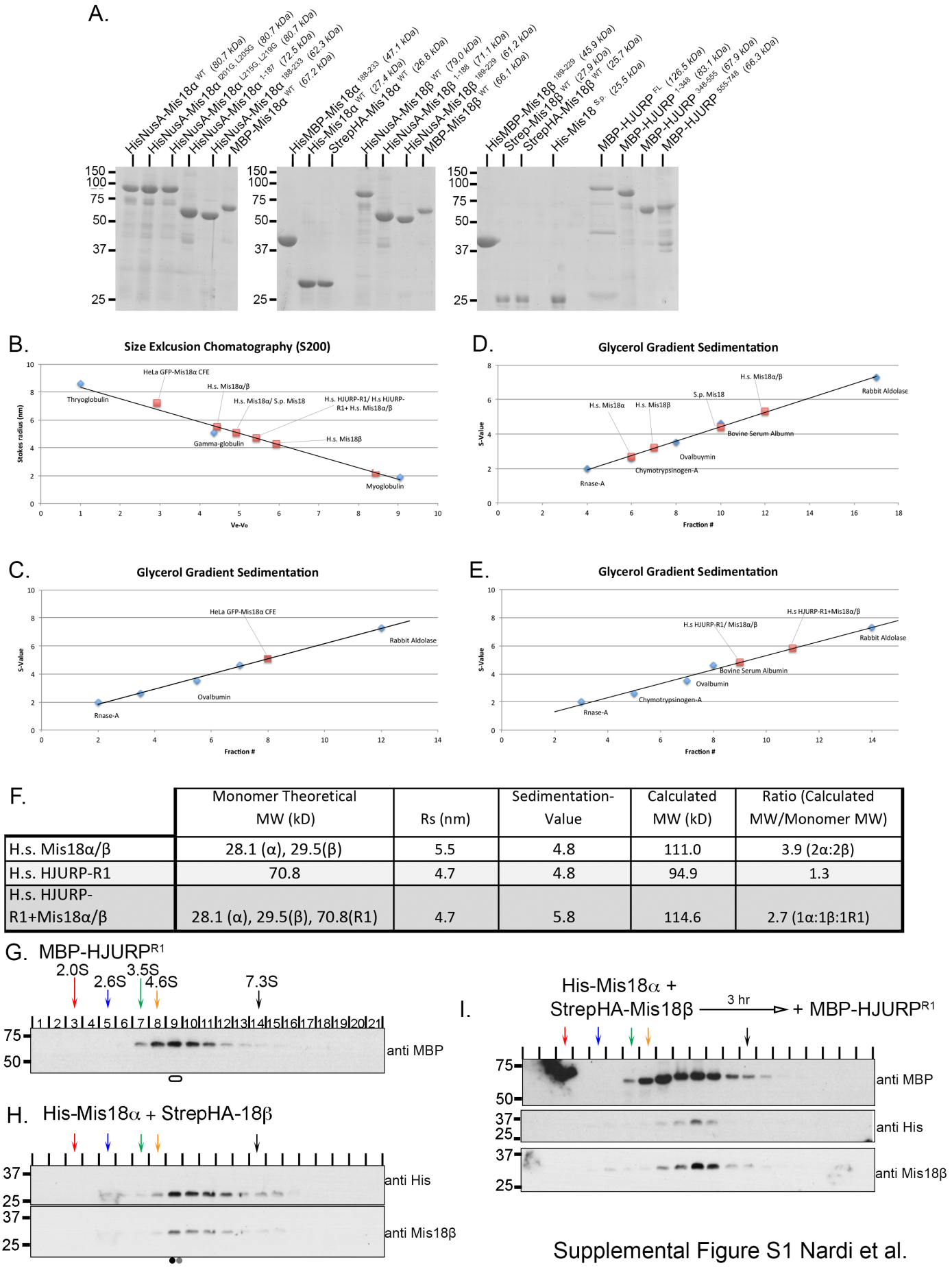
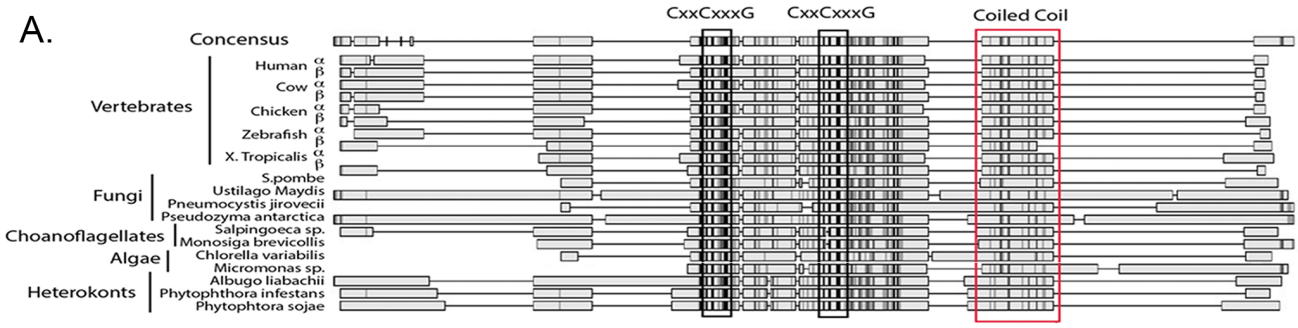


F.

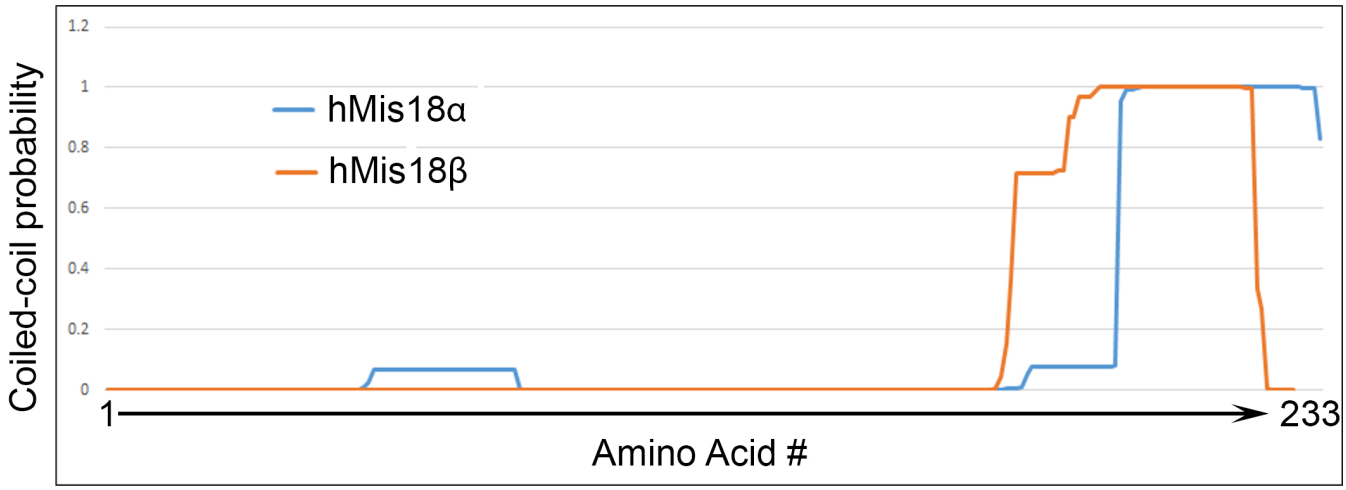
	Monomer Theoretical MW (kD)	Rs (nm)	Sedimentation-Value	Calculated MW (kD)	Ratio (Calculated MW/Monomer MW)
H.s. Mis18α/β	28.1 (α), 29.5(β)	5.5	4.8	111.0	3.9 (2α:2β)
H.s. HJURP-R1	70.8	4.7	4.8	94.9	1.3
H.s. HJURP-R1+Mis18α/β	28.1 (α), 29.5(β), 70.8(R1)	4.7	5.8	114.6	2.7 (1α:1β:1R1)

Supplemental Figure S1 Nardi et al.

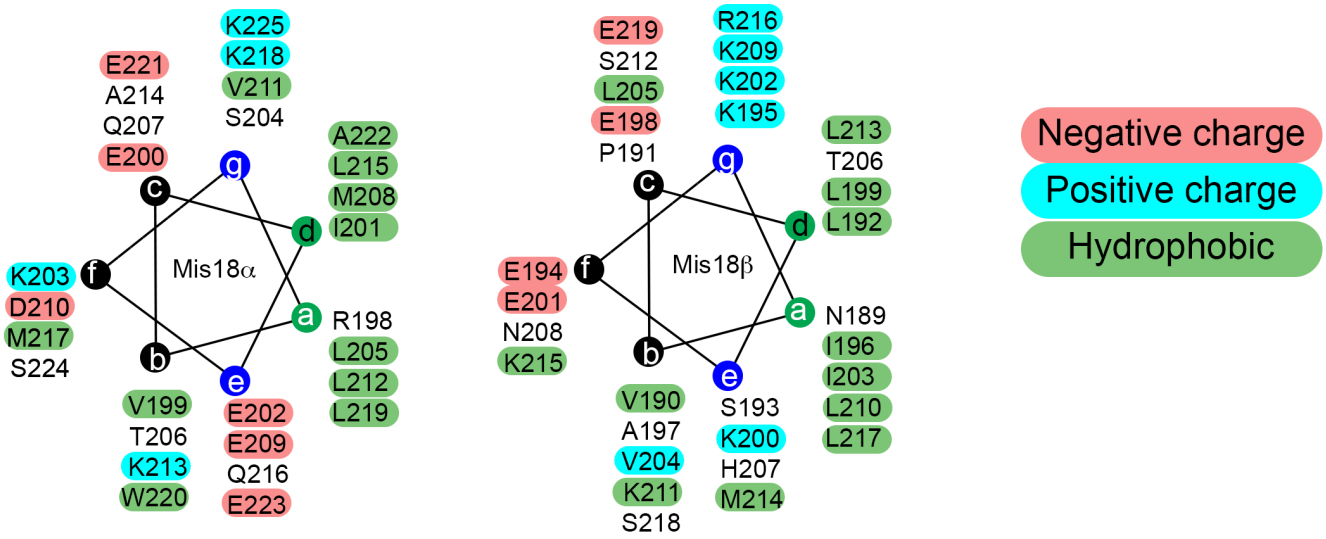
A.



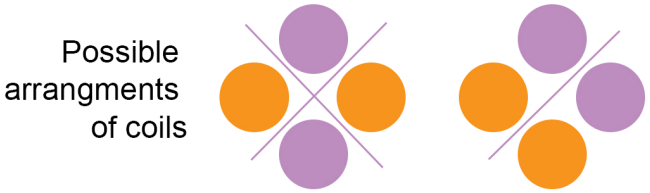
B.



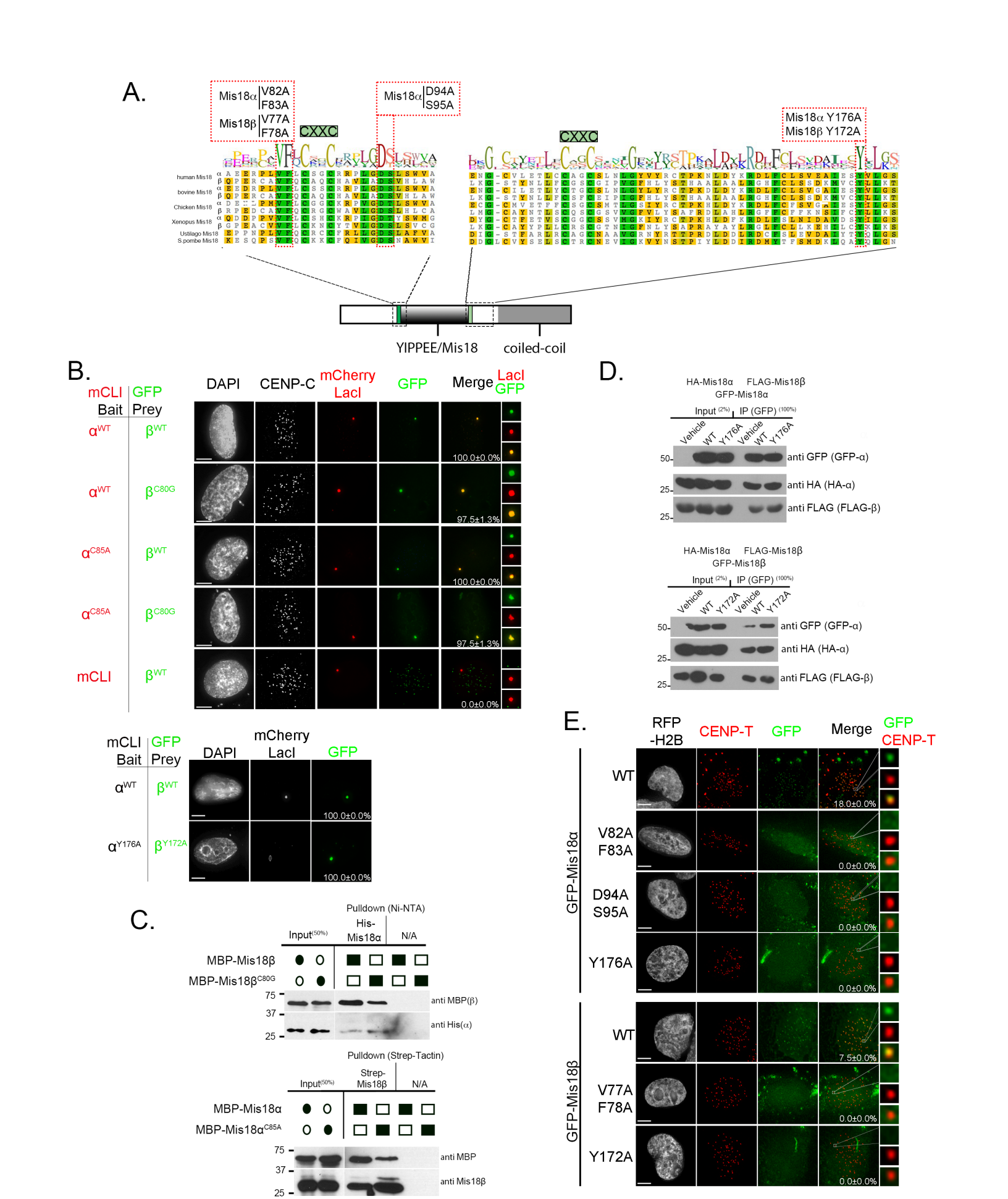
C.



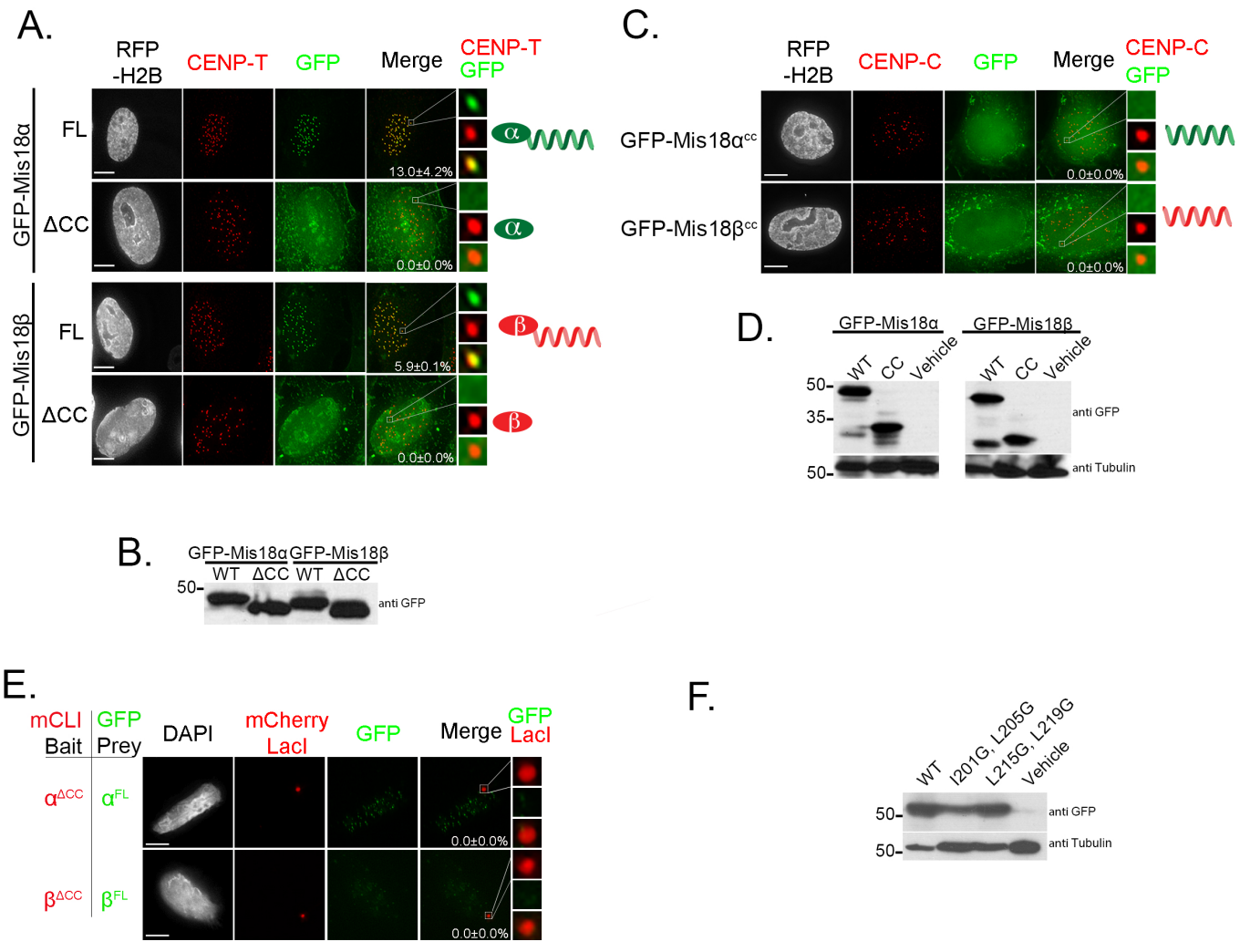
D.



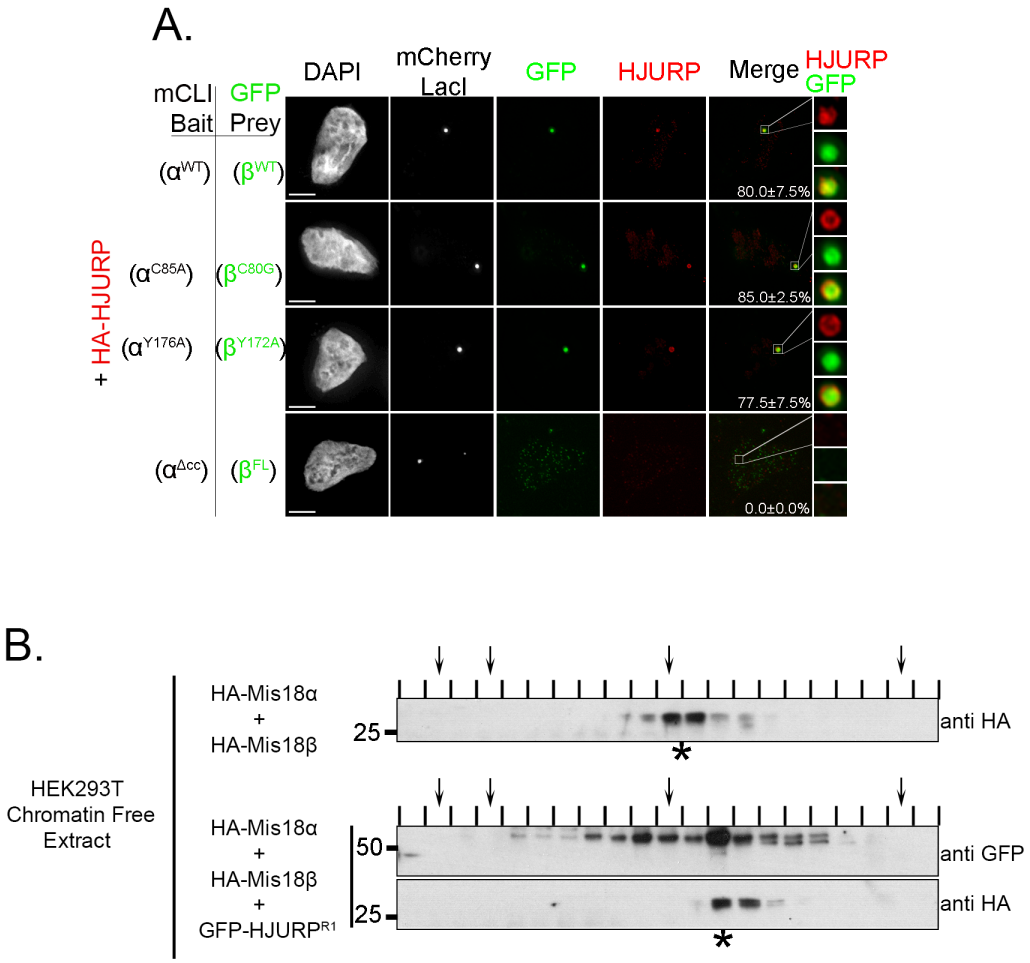
Supplemental Figure S2 Nardi et al.



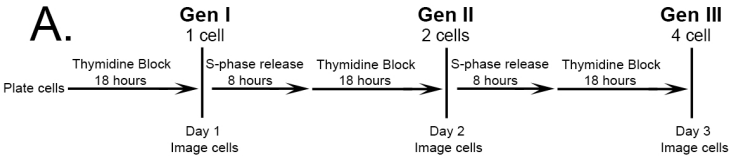
Supplemental Figure S3 Nardi et al.

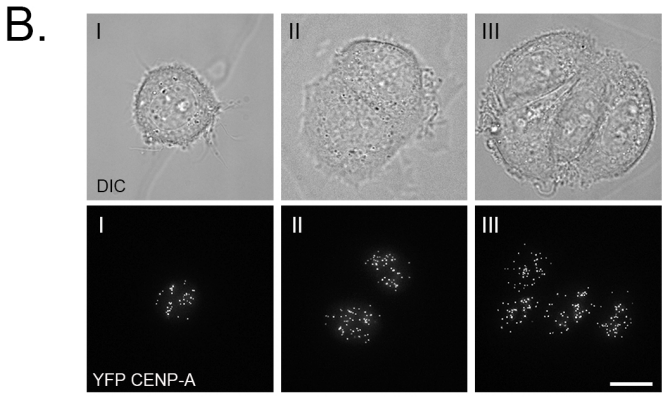


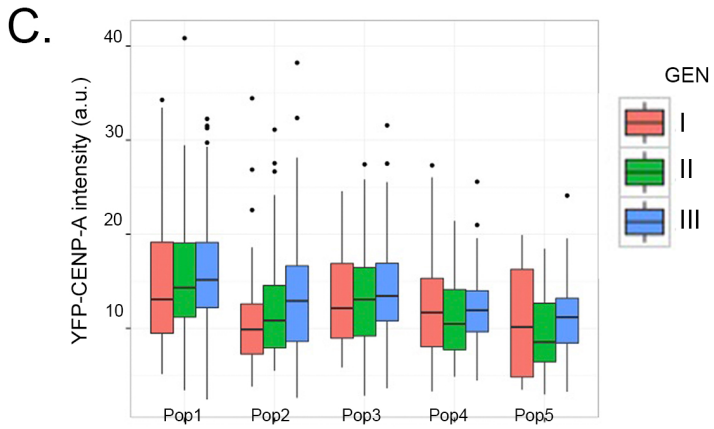
Supplemental Figure S4 Nardi et al.

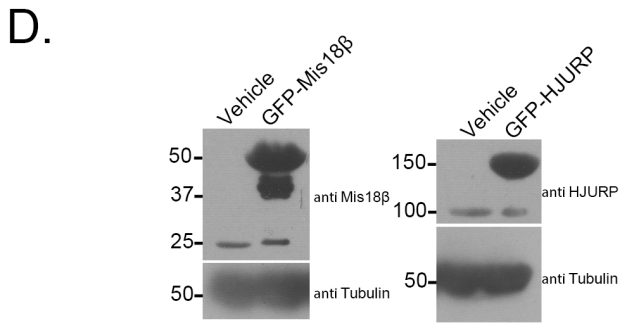


Supplemental Figure S5 Nardi et al.









Supplemental Figure S6 Nardi et al.

Supplemental Figure Legends

Figure S1 Recombinant proteins and hydrodynamic analysis, Related to Figures 1,2,4,5. A. Coomassie gel of recombinant proteins used for *in-vitro* analysis. Predicted Molecular weights are indicated for each protein in parentheses next to each name. Molecular weight markers used on the gel are indicated on the left sides (kDa). B. Linear curve with line of best fit for size exclusion standards thyroglobulin (Rs=8.6), Gamma globulin (Rs=5.1), and myoglobulin (Rs=1.9) (Biorad, blue dots, unboxed labels). The X-axis is calculated by subtracting the elution volume (Ve) by the void volume of the column (Vo). The y-axis is the Stokes radii of each standard and calculated protein. The proteins of interest were (boxed labels) overlaid onto the line of best fit independently and ther Rs values were calculated from the three original standards. C-E Linear curves with a line of best fit for glycerol gradient sedimentation. Standards used were Rnase-A (2.0S), Chymotrypsinogen-A (2.6S), Olvalbumin from hen egg (3.5S), Bovine Serum Albumin (4.6S), and Rabbit Aldolase (7.3S) (Blue dots, unboxed labels). From these standards a line of best fit was made and used to calculate the S-values of the proteins of interest (Red dots, boxed labels). The X-axis is the fraction # of the peak elution for each standard and protein of interest while the Y-axis corresponds to the S-value. The proteins of interest were overlaid on the line of best fit independently. Graphs S1C was used to calculate the S-values of the recombinant Mis18 proteins (Figure 1A-D,F). Graph S1D was used to calculated the S-value of the HeLa GFP-Mis18α CFE (Figure 1E,F). Graph S1E was used to calculate the S-values of the $HJURP^{R1}$ and the Mis18 complex alone and in complex together (Figure 5A-C,E). F. Table summarizing hydrodynamic analysis of $HJURP^{R1}$ and the Mis18α/β separately and mixed together from Figure 5 A-C. Stokes

radii and sedimentation values were calculated from linear curves (Figure S1B,E) and were used to calculate the native molecular weights (Siegel and Monty 1966). Values are the average of two replicates. Stoichiometry of the individual complexes was calculated by dividing the calculated predicted molecular weights of the complex by the calculated molecular weights of the monomeric proteins. G-I. Glycerol gradient sedimentation performed on recombinant HJURP fragment (aa 348-555) MBP-HJURP^{R1}, Mis18α-β and MBP-HJURP^{R1}. Sedimentation-values of standards are indicated by arrows. Proteins were detected by Western blot. Shapes under the blots identify the peak fractions represent and the proteins present. This analysis was done in conjunction with S.E.C analysis to determine stokes radii of the complexes as in Figure 5A-C.

Figure S2. Mis18 coiled-coil conserved across species, Related to Figure 2. A. ClustalW alignment was conducted of Mis18 sequences from a broad range of phylogeny. The coiled-coil domains are boxed in red. Shading indicates the degree of amino acid similarity, with black indicating 100% similarity. B. Mis18 proteins were run through the MARCOIL prediction program showing they both contain C-terminal predicted coiled-coil domains. C. Alpha-helical representation of the amino acids present within the Mis18α and Mis18β coiled coils. a-g indicate the position with the heptad repeat. D. Possible configuration of a two-component four helical coiled-coil. Lines indicate the axes of separation that would yield a heterodimer.

Figure S3. Conserved residues in the YIPPEE domain of Mis18 necessary for centromere recruitment, **Related to Figures 2,5** A. Sequence alignment of Mis18 across species. Green indicates residues that are highly conserved and yellow indicates residues that are less conserved across species. B. U2OS LacO cells were transfected

with Mis18 wild-type or YIPPEE mutants as bait (mCLI) and prey (GFP). Boxed regions are magnified to the right. Percentages of cells with GFP-Mis18 co-localized with the array are indicated; ± S.D. Scale bars, 5μm. C. *In vitro* pull-downs using recombinant Mis18WT and cysteine mutants. D. Immunoprecipitation conducted from U2OS cells transfected with wild-type or distal YIPPEE domain mutants of Mis18α or Mis18β. E. Localization of transiently transfected GFP-tagged wild-type Mis18 or the indicated mutants in parental U2OS cells. Cells were co-transfected with RFP-H2B to identify transfected cells and stained with anti-CENP-T to mark centromeres. Boxed regions are magnified to the right. Percentages of cells with Mis18 localized to centromeres are indicated ± S.D. Scale bars, 5μm.

Figure S4. Mis18 heterotetramer must form for proper centromere recruitment, Related to Figures 2,5 A. Parental U2OS cells were transfected with GFP-tagged wild-type Mis18 or ΔCC mutants. Cells were co-transfected with RFP-H2B to identify transfected cells and stained with anti-CENP-T to mark centromeres. Boxed regions are magnified to the right. Percentages of cells with Mis18 localized to centromeres are indicated ± S.D. Scale bars, 5μm. B. Western blot of U2OS cells transiently transfected with the Mis18 full-length or YIPPEE domain to show expression. Anti-tubulin is a loading control. C. Parental U2OS cells transfected with the indicated Mis18 coiled-coil domain. Cells were co-transfected with RFP-H2B to identify transfected cells and stained with anti-CENP-T to mark centromeres. Boxed regions are magnified to the right. Percentages of cells with Mis18 localized to centromeres are indicated ± S.D. D. Western blot of U2OS cells transiently transfected with the Mis18 full-length or coiled-coil

domain to show expression. Anti-tubulin is a loading control. E. Western blot of Mis18 WT and mutants transfected U2OS cells. Anti-tubulin is a loading control.

Figure S5. Mis18 heterotetramer disrupted into heterodimers upon HJURP binding, Related to Figures 4,6 A. U2OS LacO cells were transfected with Mis18 wild-type or YIPPEE mutants as bait (mCLI) and prey (GFP). Boxed regions are magnified to the right. Percentages of cells with GFP-Mis18 co-localized with the array are indicated; ± S.D. Scale bars, 5μm. B. S.E.C. of HEK293T cells transfected with HA-Mis18α/β +/- GFP-HJURP^{R1}. Chromatin free extracts were made and run over an S200 column. Proteins were detected by western blot. Asterisk's under blots represent peak fractions and the Stokes radii of the standards and void are noted above the fractions. C. Averaged centromeric intensities for the stably expressing GFP-Mis18α HeLa cells with either HJURP or control siRNA from Figure 6C.

Figure S6. **CENP-A stable at centromeres across generations, Related to Figure 6** A. Flow chart of the protocol used to plate, block, release, and image cells at successive G1/S boundaries B. Representative images of cells at each generation (generation I, II, and III). Cells were imaged to assess the changes in YFP-CENP-A intensity across cell generations C. Vertical box plots of integrated YFP-CENP-A fluorescence across generations for individual cells and their progeny. Bars indicate standard deviation while horizontal lines in the boxes indicate the mean. D. Immunoblot of overexpressed GFP-tagged Mis18β and HJURP from figure 6F.

Table S1. Related to Materials & Methods DNA cloning, Figures 1-6, S1, S3, S4, S5, S6. Cloning description of destination vectors and subsequent grouping of constructs by which destination vector they were put into for analysis.

Table S1. DNA Construct used in this study.

Name/tag	Tag	Destination/Parent Vector	Cloning Description (If Applicable)
Destination Vectors			
pIN12	MBP	pMal c2	pMal c2 vector cut with BamH1. Gateway cassette containing ccdB and chloremphenical resistance markers flanked by aatR sites created by PCR with BglII overhangs ligated into the cut pMal c2 vector
pDF270	mCLI	pCS2 mCherry Dest	Destination vector was cut with BglII and a LacI PCR product was dropped in with compatible overhangs
pcDNA3-Dest	none	pcDNA3	pcDNA3 was cut with Kpn1. Gateway cassette containing ccdB and chloremphenical resistance markers flanked by aatR sites created by PCR and ligated into Kpn1 site.
pDF275	3XHA	pcDNA3-Dest	pcDNA3 Gateway Destination vector was cut with BstB1. An oligo coding for a tandem 3X HA tag with Bstb1 overhangs was ligated in. This creates fusion proteins harboring a C-terminal HA-tag
pIN257	3X-Flag	pcDNA3-Dest	pcDNA3 Gateway Destination vector was cut with BstB1. An oligo coding for a tandem 3X FLAG tag with BstB1 overhangs was ligated in. This creates fusion proteins harboring a C-terminal FLAG-tag
pIN109	Strep-HA	Custom	pDEST527 (Addgene #11518) was cut with Nde1 and BglII and an Oligoendcoding for a Streptactin-HA tandem affinity tag with compatible overhangs was ligated into the same site.
Name		**Destination/Parent Vector**	**Cloning Description**
MBP Plasmids			
HJURP FL		pIN12	LR reaction
HJURP (aa1-348)		pIN12	LR reaction
HJURP (aa348-555)		pIN12	LR reaction
HJURP (aa555-748)		pIN12	LR reaction
Mis18α FL		pIN12	LR reaction
Mis18α (aa1-187)		pDEST566 (Addgene #11517)	LR reaction
Mis18α (aa188-233)		pDEST566 (Addgene #11517)	LR reaction
Mis18α FL		pDEST566 (Addgene #11517)	LR reaction
Mis18β FL		pIN12	LR reaction
Mis18β (aa1-188)		pDEST566 (Addgene #11517)	LR reaction
Mis18β FL(aa189-229)		pDEST566 (Addgene #11517)	LR reaction
Mis18β FL		pDEST566 (Addgene #11517)	LR reaction
HisNusA Plasmids			
Mis18α FL		pDest544 (Addgene #11519)	LR reaction
Mis18α (aa1-187)		pDest544 (Addgene #11519)	LR reaction
Mis18α (aa188-233)		pDest544 (Addgene #11519)	LR reaction
Mis18α (aa188-233)		pDest544 (Addgene #11519)	LR reaction
Mis18α I201G,L205G		pDest544 (Addgene #11519)	LR reaction
Mis18α L215G,L219G		pDest544 (Addgene #11519)	LR reaction
Mis18β FL		pDest544 (Addgene #11519)	LR reaction
Mis18β (aa1-188)		pDest544 (Addgene #11519)	LR reaction
Mis18β FL(aa189-229)		pDest544 (Addgene #11519)	LR reaction
Strep-HA Plasmids			
Mis18α FL		pIN109	LR reaction
Mis18β FL		pIN109	LR reaction
HA Plasmids			

Mis18α FL	pDF375	LR reaction
Mis18β FL	pDF375	LR reaction
HJURP FL	pDF375	LR reaction
HJURP (aa1-348)	pDF375	LR reaction
CENP-A	pDF375	LR reaction
FLAG Plasmids		
Mis18β FL	pIN257	LR reaction
GFP Plasmids		
Mis18α FL	pcDNA-DEST53	LR reaction
Mis18α (aa1-187)	pcDNA-DEST53	LR reaction
Mis18α (aa188-233)	pcDNA-DEST53	LR reaction
Mis18α I201G	pcDNA-DEST53	LR reaction
Mis18α I201G, L205G	pcDNA-DEST53	LR reaction
Mis18α (L215G, L219G	pcDNA-DEST53	LR reaction
Mis18β FL	pcDNA-DEST53	LR reaction
Mis18β (aa1-188)	pcDNA-DEST53	LR reaction
Mis18β (aa189-229)	pcDNA-DEST53	LR reaction
Mis18β C80G	pcDNA-DEST53	LR reaction
Mis18β Y172A	pcDNA-DEST53	LR reaction
HJURP FL	pcDNA-DEST53	LR reaction
Tetracycline	pcDNA-DEST53	LR reaction
mCherry-LacI Plasmids		
Mis18α FL	pDF370	LR reaction
Mis18α (aa188-233)	pDF370	LR reaction
Mis18α (aa1-187)	pDF370	LR reaction
Mis18α C85A	pDF370	LR reaction
Mis18α Y176A	pDF370	LR reaction
Mis18β FL	pDF370	LR reaction
Mis18β (aa189-229)	pDF370	LR reaction
Mis18β (aa1-188)	pDF370	LR reaction
Mis18BP1 FL	pDF370	LR reaction
Special		
His-*sp*MIs18 FL	IN67	Custom made from Genewiz (Sequence below, codon optimized for E.coli)
ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGAGCCAGACAGAAACCAGCCACAGCGGCTACATCGAC TTCAAGAAGGAGAGCCAGCCGAGTGTGTTCCAGTGCAAGAAGTGCTTTCAGATCGTGGGTGACAGCAACGCCTGGGTGATCAGCCACCGCGAGTACCTG AGCTTCACACTGAGCGATGCCGTTGAGAACAGTGTTCGCGTGGAGGATACCTTCAAGCGCAGTGACGACGGCCTGTGCGTGTACAGCGAGCTGAGTTGC ACCCGCTGCAACGAGGTGATCGGCAAAGTGTACAACAGCACCCCGATCTACCTGGATGACATCCGCGACATGTACACCTTCAGCATGGACAAGCTGCAGG CCTACCAACTGGGCAACAAGACAGTGAACCCGGAGGGTCTGACCCGCTACCAGGTGGATCTGGAGATGCGCGAAGACATCATCAAGCTGAAGAGTTTCT GCCTGAGCCTGTACGAGAAGTTCGAGCTGCACGACGAGACCCTGCGCAGCGTGAAGGAGACCATCAGCAGCCTGAAGAAGCCGAAGATTGAGGGTAAA GAAGGTAAGAAGGAGAAGGCCCGCACCTACAGTAAACGTACCCGTAAA		

Supplemental Experimental Procedures

DNA constructs. DNA constructs created using gateway cloning and the vectors used are listed in supplemental table S1. Mis18α was created by PCR amplification with a forward primer that added a 6X-His tag flanked with an Nde1 cut site and a reverse primer that was flanked by a Kpn1 cut site. Mis18β was amplified with a forward primer harboring an Nde1 cut site with no affinity tag built on and a reverse primer harboring a BamH1 cut site. Both PCR products were cut with the respective restriction enzymes and ligated into pST50trc1 (Mis18α) and pst39 (Mis18β) expression plasmids. Two complimentary oligos harboring flanking Nde1 compatible overhangs that encoded for a Strep-TEV tag were ligated together and ligated into the pST39 harboring Mis18β that had been cut with Nde1.

Cell culture. Parental U2OS and LacO-TRE were transfected with 1 μg of GFP containing Mis18 plasmid and 0.02 μg RFP-H2B (transfection marker) using Lipofectamine-2000 (Invitrogen) in serum-free Optimem Media (Gibco). U2OS-LacO-TRE cells were transfected with 0.2μg mCherry-LacI construct, 0.4 μg GFP construct, and 0.4 μg HA-HJURP construct (1:2:2). For immunocytochemistry U2OS-LacO-TRE cells were pre-extracted with 0.1% Triton X-100 in PBS for 3 min, fixed with 4% formaldehyde in PBS for 10 min, and quenched with 100 mM Tris, pH 7.5, for 10 min at room temperature. Coverslips were blocked in 2% FBS, 2% BSA and 0.1% Triton X-100 in PBS. Parental U2OS cells were not pre-extracted. Coverslips were mounted with ProLong (Invitrogen).

Antibodies used. Cells were stained with rabbit anti–CENP-T (1:3000, D. Cleveland, UCSD) mouse anti-CENP-C (1:3000) and mouse anti-HA monoclonal antibody (1:1000,

Covance), and a mouse monoclonal CENP-A antibody (1:1000, Sigma). Secondary antibodies used were donkey anti–rabbit and goat anti-mouse Cy5-conjugated (1:6000, Jackson ImmunoResearch). DNA was stained with 0.2 ug/ml DAPI.

Protein expression and purification. Recombinant proteins were expressed in the Rosetta (DE3) pLysS bacterial strain. Bacteria were grown in LB media to an OD of 0.6 at 37^{o}C and induced at 18^{o}C with 0.1mM IPTG for 16 hr. His-tagged HJURP fragments were purified on Ni-NTA (Qiagen). Bacteria were lysed using a steel Wheaton-dounce in buffer contained 50mM Tris-HCl pH 7.5, 250mM NaCl, 20mM MgCl2, 0.5mM CaCl2, 10% glycerol, 0.1% NP-40, 5mM BME, LPC, 1mM PMSF, 20mM imidazole, and 0.15mg/ml RNase-A. DnaseI was added and lysates were centrifuged at 22,000x*g* for 20 min. Supernatants were collected and pellets were re-extracted with a second round of lysis. Cleared lysates were incubated with Ni-NTA agarose (Qiagen) for 1 hour and washed twice in lysis buffer with 40mM imidazole. Proteins were eluted in lysis buffer containing 250mM imidazole and no protease inhibitors. Full-length HJURP was further purified on a Superdex 200 10/300 column (G.E. Healthcare) and re-concentrated on Ni-NTA agarose (Qiagen). Mis18 proteins were purified as described in lysis buffer containing: 50mM Tris-HCl, 350mM NaCl, 0.5mM CaCl2, 10% glycerol, 0.1% NP-40, 5mM BME, LPC, and 1mM PMSF, and imidazole. MBP tagged proteins were purified using maltose agarose (Qiagen) and eluted in buffer containing 10mM maltose. Strep–tagged proteins were purified using Strep-Tactin Super-flow plus (Qiagen) and eluted in buffer containing 2.5mM d-desthiobiotin.

Live cell imaging. YFP-CENP-A expressing HeLa cells were plated onto gridded coverslips (MatTek) 1.0x10^{4} cells per well and treated 24 hrs later with 01.ug/ml

thymidine for 18 hrs. Thymidine was washed out and new media containing deoxycytidine at 24uM was added. After this, thymidine was again added so that the single cells would progress through mitosis and then be blocked at the G1/S boundary again for imaging. This process was repeated for a total of three cell generations, and the same cells were imaged at each generation during each G1/S block. Fluorescence intensities were measured from raw-imaged files and images were then deconvolved and presented as stacked images.

Immunoprecipitations. Cells were lysed 24 h post transfection in RIPA buffer (150 mM NaCl, 1% NP-40, 0.3% deoxycholate, 0.15% SDS, 50 mM Tris HCl pH 7.5, 1 mM EDTA, 10% glycerol, Protease Inhibitors (Roche), 200 μM NaV, 0.5 mM PMSF, 5 mM NaF, 50 mM β-mercaptoethanol, 5 μM microcystin) on ice for 15 min with occasional vortexing. Extracts were DNAseI (1:200, NEB Biolabs) treated and sonicated where indicated. Lysates were centrifuged at 18 000 *g* for 10 min at 4°C and pre-cleared with Protein A agarose (Biorad) for 2 h at 4°C. Pre-cleared lysates were incubated with anti-GFP antibody (1:1000, Cell Signaling) at 4°C overnight. Antibody-bound complexes were recovered on Protein A Dynabeads (Invitrogen) at room temperature for 45 min, washed with RIPA buffer followed three times in PBS including 0.1–0.5% Tween-20. Complexes were eluted by boiling in SDS sample buffer.