Supplemental Information

Supplemental Figures

Figure S1

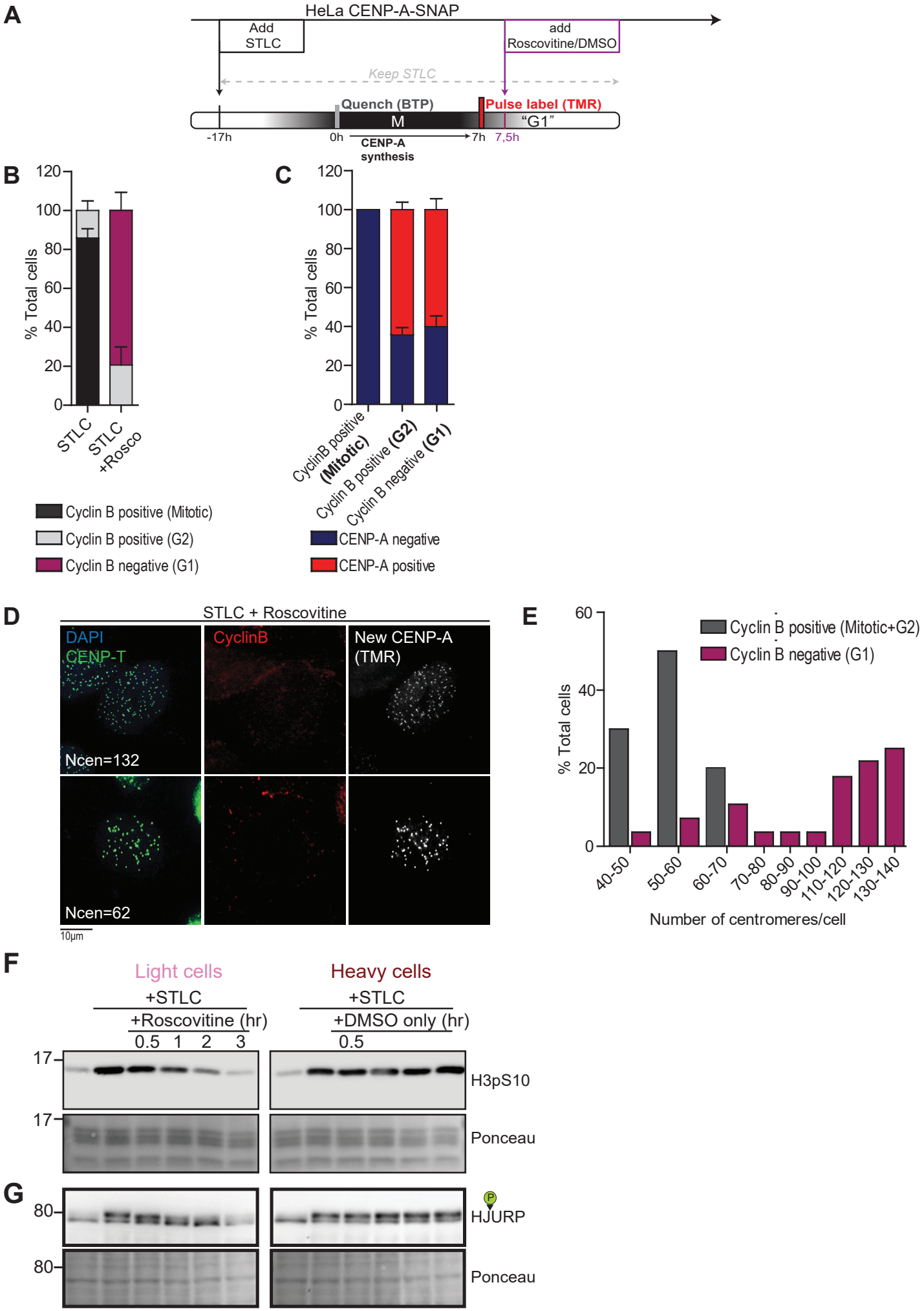

A
HeLa CENP-A-SNAP
Add STLC
add Roscovitine/DMSO
Keep STLC
Quench (BTP)
Pulse label (TMR)
M
"G1"
-17h
0h
7h
7,5h
CENP-A synthesis
B
% Total cells
STLC
STLC +Rosco
Cyclin B positive (Mitotic)
Cyclin B positive (G2)
Cyclin B negative (G1)
C
% Total cells
CyclinB positive (Mitotic)
Cyclin B positive (G2)
Cyclin B negative (G1)
CENP-A negative
CENP-A positive
D
STLC + Roscovitine
DAPI
CENP-T
CyclinB
New CENP-A (TMR)
Ncen=132
Ncen=62
10µm
E
% Total cells
Cyclin B positive (Mitotic+G2)
Cyclin B negative (G1)
40-50
50-60
60-70
70-80
80-90
90-100
110-120
120-130
130-140
Number of centromeres/cell
F
Light cells
Heavy cells
+STLC
+Roscovitine (hr)
0.5 1 2 3
+STLC
+DMSO only (hr)
0.5
H3pS10
Ponceau
G
HJURP
Ponceau

Figure S1. (supplement to Figure 1) Conditions used for SILAC analysis are permissive for CENP-A deposition.

(A) To enrich for mitotic phase, HeLa CENP-A-SNAP cells were treated with Eg5 inhibitor (STLC) for 24h. 17h into mitotic arrest, pre-existing CENP-A-SNAP pool was quenched with BTP, followed by 7h of chase. 30min before pulse labeling of newly synthesized pool of CENP-A-SNAP and fixation, cells were treated either with Roscovitine or DMSO.

(B) Quantification of frequencies of Cyclin B positive (G2 and mitotic) cells and Cyclin B negative (tetraploid cells which exited mitosis without cytokinesis due to Cdk1 inhibition, see panel D, E) in the experiment described in (A).

(C) Quantification of frequencies of CENP-A-SNAP (TMR) positive cells in each cell cycle stage present. Cells arrested in mitosis do not assemble CENP-A (left bar). Degree of CENP-A assembly in either G2 or G1 cells is assayed following Roscovitine treatment.

(D) Representative images of Roscovitine-treated cells in experiment described in (A). Cells were counterstained with Cyclin B, CENP-T and DAPI to indicate cell cycle status, centromeres and DNA respectively.

(E) Distribution of number of centromeres under experimental conditions described in (A). Doubling of centromere number indicates formation of tetraploid cells due to forced mitotic exit in the presence of STLC and Roscovitine.

(F) Western blots for the mitotic marker H3pS10 indicating cell cycle position of HeLa S3 cells used in SILAC experiment in Figure 1. Cells were arrested in mitosis with the Eg5 inhibitor STLC followed by treatment with DMSO control or Roscovitine (light cells) to force mitotic exit caused by Cdk inhibition.

(G) Western blots for HJURP (isolated from soluble fraction) from HeLaS3 cells showing dephosphorylation (as seen by shift in SDS-PAGE mobility of phosphorylated HJURP) upon Roscovitine treatment of "light" cells. Based on this, we harvested cells after 30 min of Roscovitine (or DMSO) treatment, balancing between HJURP dephosphorylation and completion of HJURP-mediated centromeric chromatin assembly.

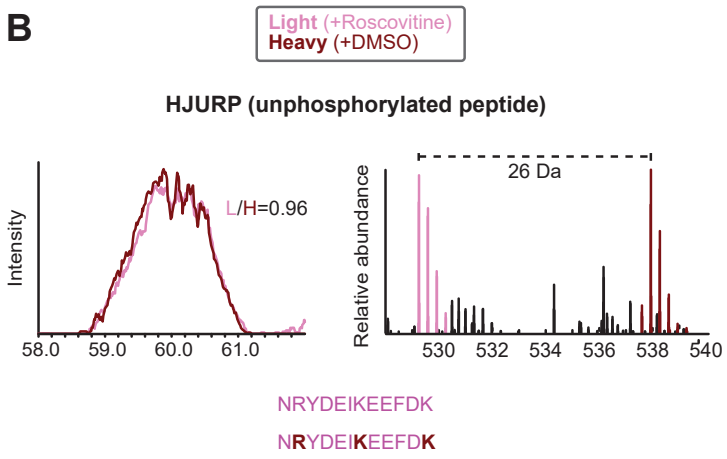
Figure S2

A

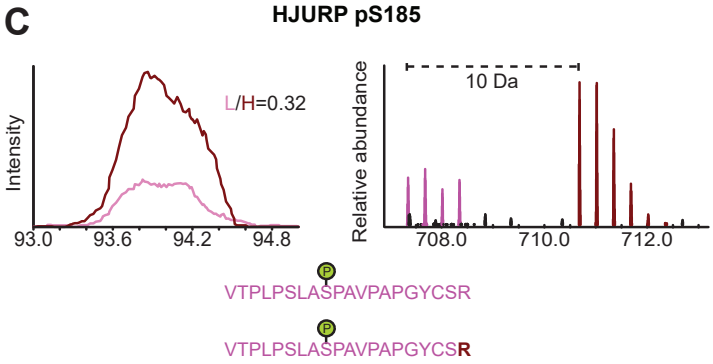
Phospho-site(s) covered	Phosphopeptide	(Roscovitine-treated)/(Mock-treated) Ratio: Experiment #1 (Forward labeling)	Experiment #2 (Reverse labeling)	Experiment #3 (Reverse labeling)
HJURP pS185	VTPLPSLA**pS**PAVPAPGYCSR	**0.32**	nd	**0.30**
HJURP pS412	WLI**pS**PVK	**0.72**	nd	**0.62**
HJURP pS448	EYCL**pS**PR	**0.53**	nd	nd
HJURP pS473	GGPA**pS**PGGLQGLETR	**0.48**	**0.37**	**0.42**
HJURP pS595	**pS**PGQMTVPLCIGVSTDK	**0.23**	nd	**0.25**
	YCLK**pS**PGQMTVPLCIGVSTDK	**0.28**	nd	**0.30**
HJURP pS642	LPS**pS**PLGCR	**0.46**	**0.48**	**0.39**
CENP-A pS16+pS18	**pS**P**pS**PTPTPGPSR	nd	**0.93**	nd
	R**pS**P**pS**PTPTPGPSR	nd	**1.01**	nd
	RR**pS**P**pS**PTPTPGPSR	**0.87**	**0.98**	nd
CENP-A pS16	RR**pS**PSPTPTPGPSR	**0.94**	**1.13**	nd

nd = no data

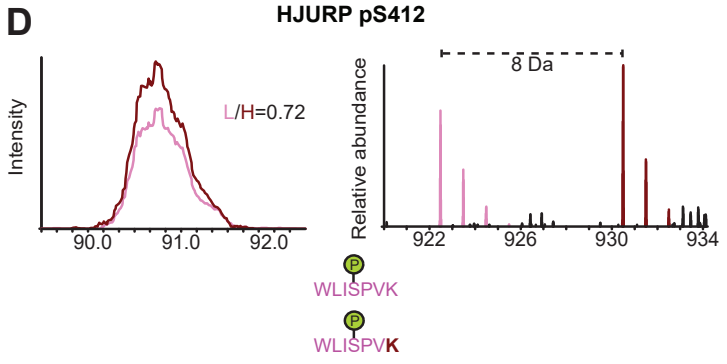
B



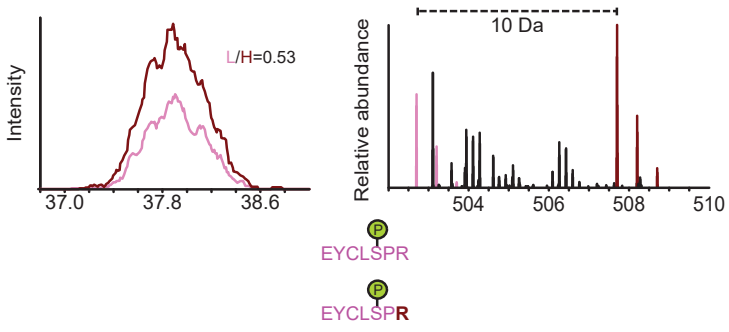
C



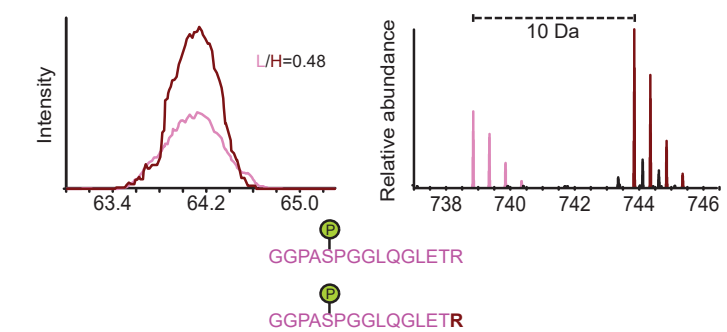
D



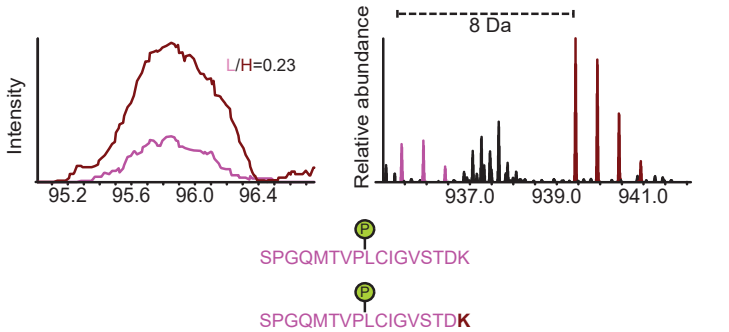
E



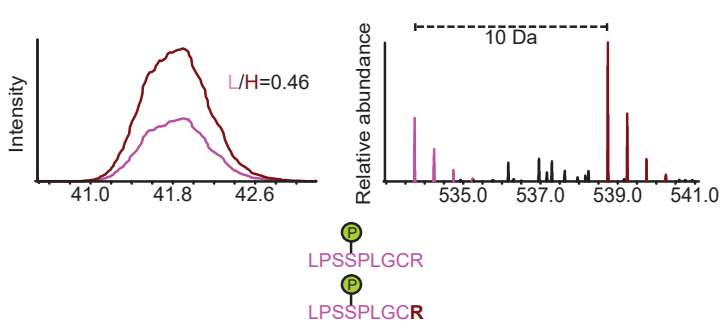
F



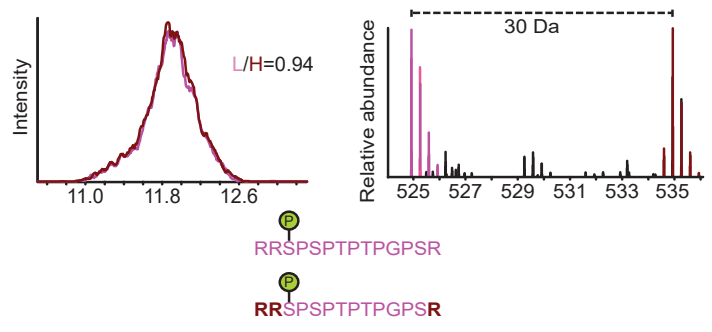
G



H



I



J

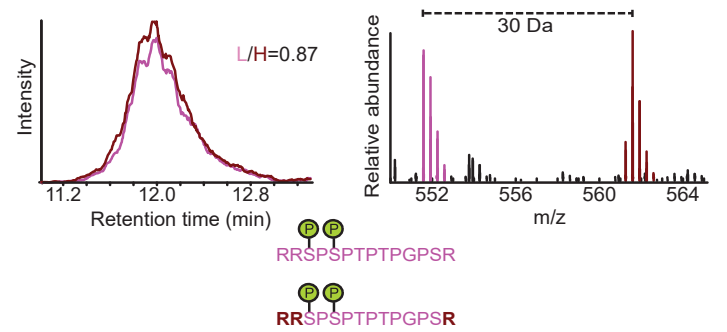


Figure S2 (supplement to Figure 1) G1/Mitotic Ratios of HJURP and CENP-A phosphopeptides are reproducible.

(A) Table summarizing data from three independent SILAC experiments (the data described in Figures 1B, are displayed in the column labeled "Experiment #1"). For the forward labeling experiment, the "light" cells are treated with Roscovitine while the "heavy" cells are mock-treated. For reverse labeling experiments, the "light" cells are mock-treated while the "heavy" cells are treated with Roscovitine. Red residues are the sites where phosphate groups were unambiguously mapped. Bolded residues are the Cdk consensus motifs in each peptide. (nd=no data).

(B-J) Representative chromatograms and spectra of HJURP and CENP-A phosphopeptides in SILAC. Extracted ion chromatograms of all phosphopeptides from Figure 1C and 1D, showing co-elution of phosphopeptide pairs (left panels), and representative mass spectra showing isotopic envelopes of light vs. heavy peptides (right panels). Phosphopeptides from "light" (Roscovitine-treated) cells are coloured in pink, while the phosphopeptides from "heavy" (mock-treated) cells are coloured in dark red. Each peptide pair is separated by a predictable mass difference (calculated from the number of lysines and arginines in the peptide), which is labeled with dotted lines between the monoisotopic peaks of light and heavy peptides.

(B) A representative unphosphorylated HJURP peptide as internal control.

(C-H) HJURP phosphopeptides.

(I-J) CENP-A phosphopeptides.

Figure S3

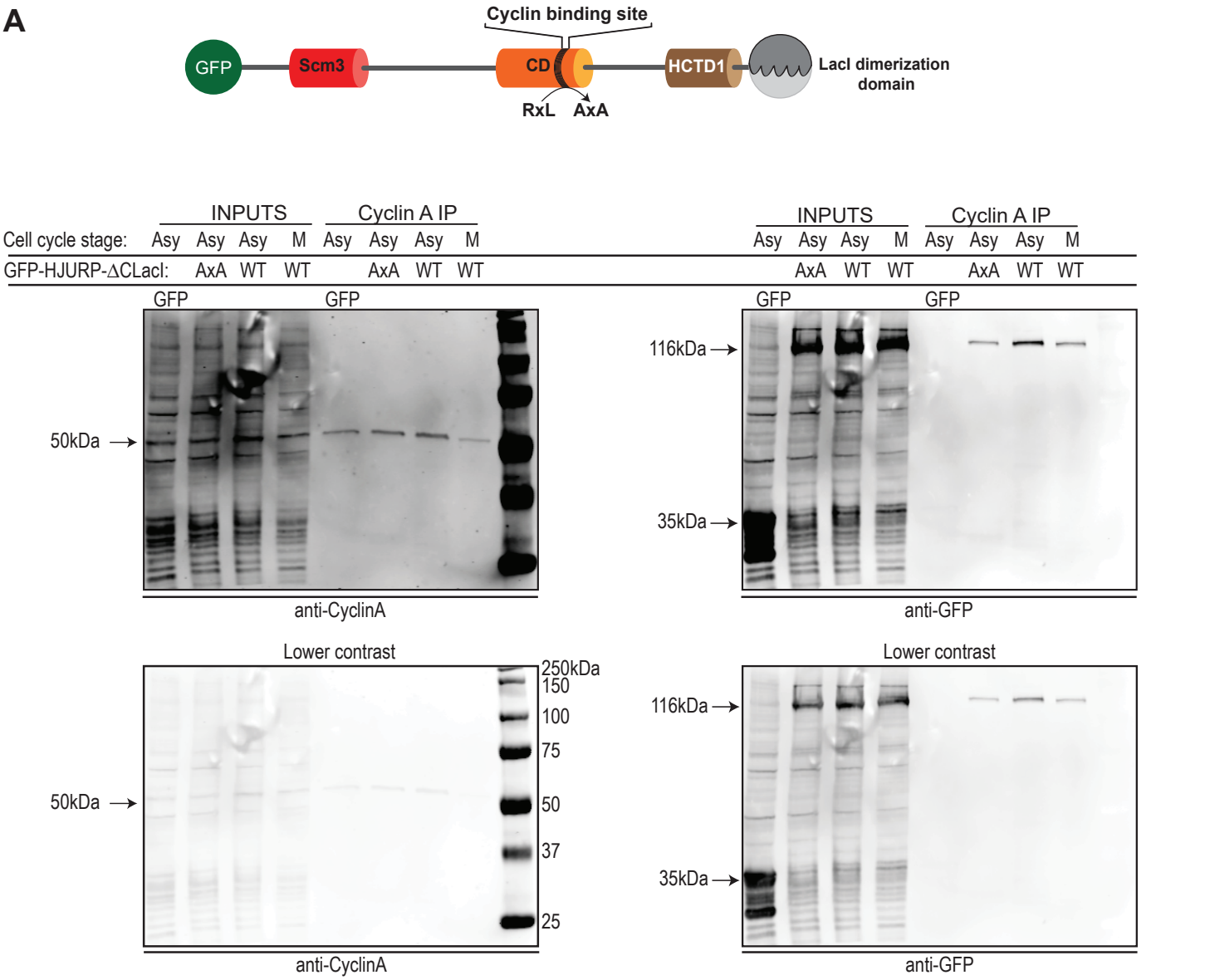

A
Cyclin binding site
GFP
Scm3
CD
HCTD1
LacI dimerization domain
RxL
AxA
INPUTS
Cyclin A IP
Cell cycle stage:
Asy Asy Asy M Asy Asy Asy M
GFP-HJURP-ΔCLacI:
AxA WT WT AxA WT WT
GFP
GFP
50kDa
anti-CyclinA
Lower contrast
250kDa
150
100
75
50
37
25
116kDa
35kDa
anti-GFP
B
INPUTS
Cyclin B IP
Cell cycle stage:
M M M M M M
GFP-HJURP-ΔCLacI:
AxA WT AxA WT
GFP AxA WT GFP AxA WT
55kDa
anti-CyclinB

Figure S3. (supplement to Figure 1) Cyclin A and Cyclin B bind to HJURP.

(A) Top: Schematic of GFP-HJURP-ΔCLacI protein with relevant domains depicted. Experiments were performed with a HJURP construct in which the C-terminal homodimerization domain is replaced with that of LacI to prevent dimerization with wild type HJURP. Bottom: Raw images of a Western blot shown in Figure 1E. Upper and lower panels are identical except for different contrasting.

(B) Cyclin B interacts with HJURP in mitosis independently of Conserved Domain. Randomly cycling HEK293T cells were transiently transfected either with GFP alone, GFP-HJURP-ΔCLacI or GFP-HJURP^{AxA}-ΔCLacI. 24h post-transfection cells were treated overnight using DME III inhibitor to induce mitotic arrest. 48h post-transfection, cells were lysed and Cyclin B was immunoprecipitated using Anti-Cyclin B coated beads. Bound complexes were separated using SDS-PAGE followed by immunoblotting with indicated antibodies (raw images of Westerns are shown).

Figure S4

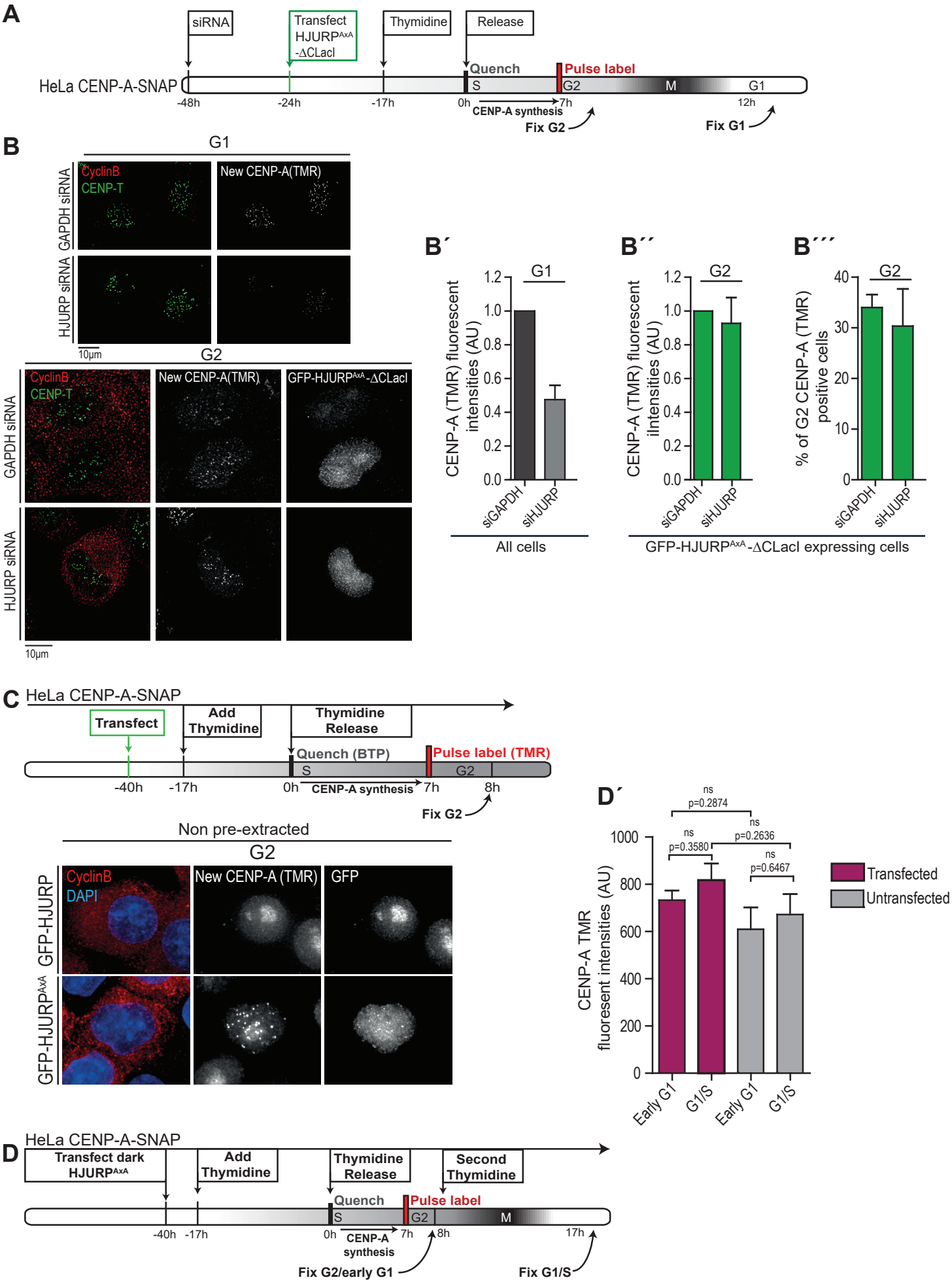

A
siRNA
Transfect HJURP^AxA -ΔCLacI
Thymidine
Release
HeLa CENP-A-SNAP
Quench
Pulse label
S
G2
M
G1
-48h
-24h
-17h
0h
7h
12h
CENP-A synthesis
Fix G2
Fix G1
B
G1
GAPDH siRNA
HJURP siRNA
CyclinB
CENP-T
New CENP-A(TMR)
10µm
G2
GFP-HJURP^AxA-ΔCLacI
B´
CENP-A (TMR) fluorescent intensities (AU)
siGAPDH
siHJURP
All cells
B´´
B´´´
% of G2 CENP-A (TMR) positive cells
GFP-HJURP^AxA-ΔCLacI expressing cells
C
HeLa CENP-A-SNAP
Transfect
Add Thymidine
Thymidine Release
Quench (BTP)
Pulse label (TMR)
-40h
-17h
0h
7h
8h
Fix G2
Non pre-extracted
GFP-HJURP
GFP-HJURP^AxA
DAPI
New CENP-A (TMR)
GFP
D´
CENP-A TMR fluoresent intensities (AU)
ns
p=0.2874
p=0.3580
p=0.2636
p=0.6467
Transfected
Untransfected
Early G1
G1/S
D
Transfect dark HJURP^AxA
Second Thymidine
Fix G2/early G1
Fix G1/S

Figure S4. (supplement to Figure 2) Centromeric targeting of HJURP and timing of CENP-A assembly is controlled by the HJURP Conserved Domain.

(A) HeLa CENP-A-SNAP cells were treated with siRNAs against HJURP or GAPDH and synchronized by double thymidine arrest and release combined with SNAP quench-chase-pulse labeling as indicated. Cells were transfected with GFP-HJURP^{AxA}-ΔCLacI 24 hours prior to synchronous release into S phase. Cells were either fixed at G2 or cycled into the next cell cycle and collected at early G1 phase, following canonical CENP-A assembly.

(B) Representative images of experiment described in (A). Cells were counterstained with Cyclin B, CENP-T and DAPI to indicate cell cycle status, centromeres and DNA respectively.

(B´) CENP-A SNAP (TMR) fluorescent signal intensities of G1 cells are plotted in grey scale. Signals are normalized to siGAPDH.

(B´´) CENP-A SNAP (TMR) fluorescent signal intensities of GFP-HJURP^{AxA}-ΔCLacI expressing G2 cells are plotted in green. Signals are normalized to siGAPDH.

(B´´´) Percentage of total G2 cell population positive for CENP-A-SNAP for indicated siRNA conditions were determined from 3 replicate experiments, plotted in green. All error bars indicate SEM.

(C) Timing of CENP-A deposition is controlled by Conserved Domain of HJURP. Experiment identical to the one described in Figure 2A, B with exception of transfection with GFP-HJURP or GFP-HJURP^{AxA}. (i.e. wild type HJURP C-terminus instead of LacI dimerization domain) Following fixation, cells are counterstained for cyclin B and DAPI to indicate G2 status, centromere localization and DNA, respectively.

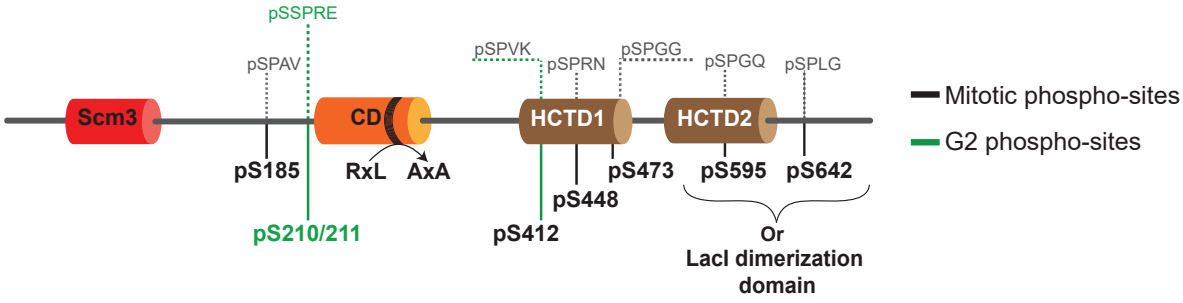
(D) The apparent partiality of HJURP^{AxA}-induced G2 phase loading is not a consequence of subsequent HJURP^{AxA}-induced overloading in G1 phase to which G2 phase signals are normalized. As in Figure 2A, HeLa CENP-A-SNAP cells were either transiently transfected for 48h with untagged HJURP^{AxA} or left untransfected. 17h post-transfection cells were arrested in Thymidine for 17h followed by 7h of release, in which S phase synthesized CENP-A-SNAP was labeled by TMR. Subsequently, a set of cells were fixed (in this way, CENP-A-SNAP positive G2 and early G1 cells were obtained, as in Figure 2A, B), or Thymidine was re-added for additional 17h followed by fixation (this allowed for collection of cells arrested at G1/S phase transition). In this way, the size of pulse labeled pool of CENP-A-SNAP is identical between early G1 and G1/S arrested cells, but the time window given for CENP-A-SNAP loading is extended to the full extent of G1 phase.

(D´) Cells from the experiment described in (D) were counterstained for Cyclin B, CENP-T and Tubulin, to indicate G2 status, centromeres and the presence of a mid-body (early G1), respectively (not shown). Fluorescent (TMR) signals from all CENP-A-SNAP positive

centromeres of early G1 or G1/S cells from either condition (transfected or untransfected) were quantified using the Centromere Recognition and Quantification (CRaQ) method (Bodor et al., 2012). Error bars indicate SEM. P values are indicated on the graph (ns, Student´s t test). These results demonstrate that canonical CENP-A-SNAP loading efficiency is near its maximum in early G1 phase in HeLa CENP-A-SNAP cell line and is minimally influenced by $HJURP^{AxA}$ expression.

Figure S5

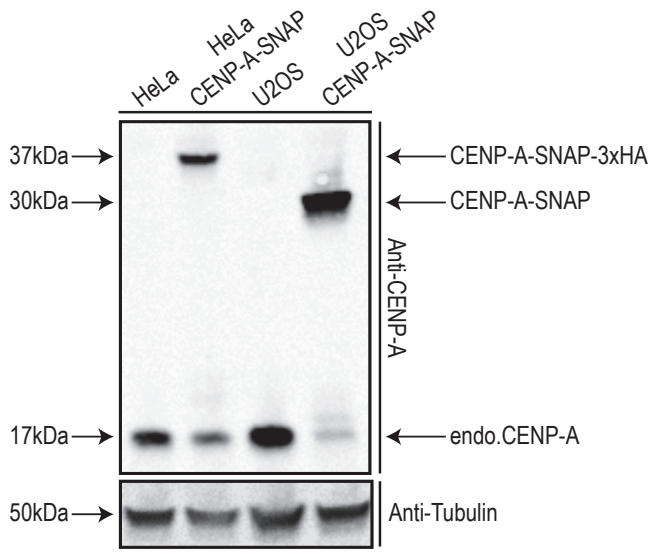
A



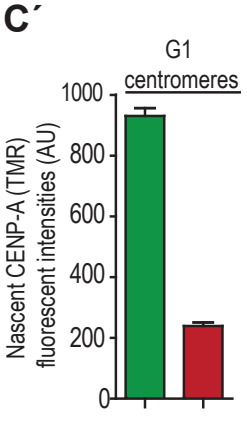
B

HJURP	G2 phase CENP-A assembly % intensity of G1 (% of G2 cells assembling)	
Mitotic phospho-site mutations	**C-terminal domain**	
	endogenous	**ΔC-LacI**
S185A	undetectable	undetectable
S185A, S412A, S448A,S473A	undetectable	undetectable
S595,S642A	undetectable	n.a.
S185A,S595A,S642A	undetectable	n.a.
S412,S448A,S473A	undetectable	undetectable
S185A,S412A,S448A,S473A,S595A,S642A	undetectable	n.a.
G2 phospho-site mutations		
S210A/S211A/S412A	~10% (~7%)	~10% (~15%)
S210A/S211A	undetectable	undetectable
Conserved domain mutation		
RxL>AxA	~30% (~25%)	~40% (~30%)
Other		
RxL>AxA, S412A	~30 (~50%)	n.d.

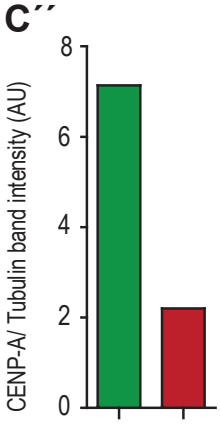
C



C´



C´´



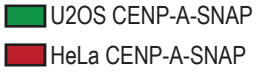


Figure S5. (**supplement to Figure 2 and 3**). **Summary of premature CENP-A assembly phenotypes of HJURP phospho-site mutants.**

(A) Schematic representation of HJURP protein, along with previously recognized domains (CENP-A binding domain (Scm3), Conserved Domain (CD), HJURP C-Terminal Domain 1 and 2 (HCTD1 and 2)) and the position of phospho-sites identified by mass spectrometry in mitosis (black lines; Figure 1, S2) or in G2 enriched cells (green lines; Figure 3). Amino acid sequences flanking phospho-sites are annotated. Position of LacI dimerization domain replacing the endogenous C-terminal dimerization domain is indicated.

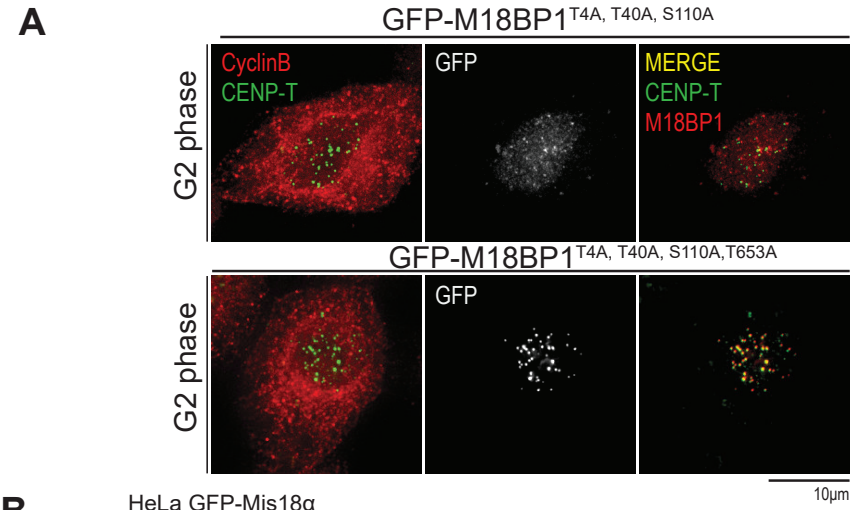
(B) Table summarizing premature CENP-A assembly phenotypes upon expression of indicated mutant HJURP proteins. Experiments were performed as in Figure 2A, B. HeLa CENP-A-SNAP cells were transiently transfected with either GFP-HJURP or GFP-HJURP-ΔCLacI and congenic point mutations thereof. 23h-post transfection, cell were enriched in G2 phase by a single Thymidine block, followed by 7h of release and subsequent fixation. CENP-A assembly was assayed using SNAP TMR-labeling of its S phase synthesized pool. Following fixation, cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA, respectively. Efficiency of CENP-A assembly is indicated as % of G1 phase CENP-A intensities and % of transfected cells loading. undetectable: no centromere signals were discernable. n.d. not determined. n.a. not applicable.

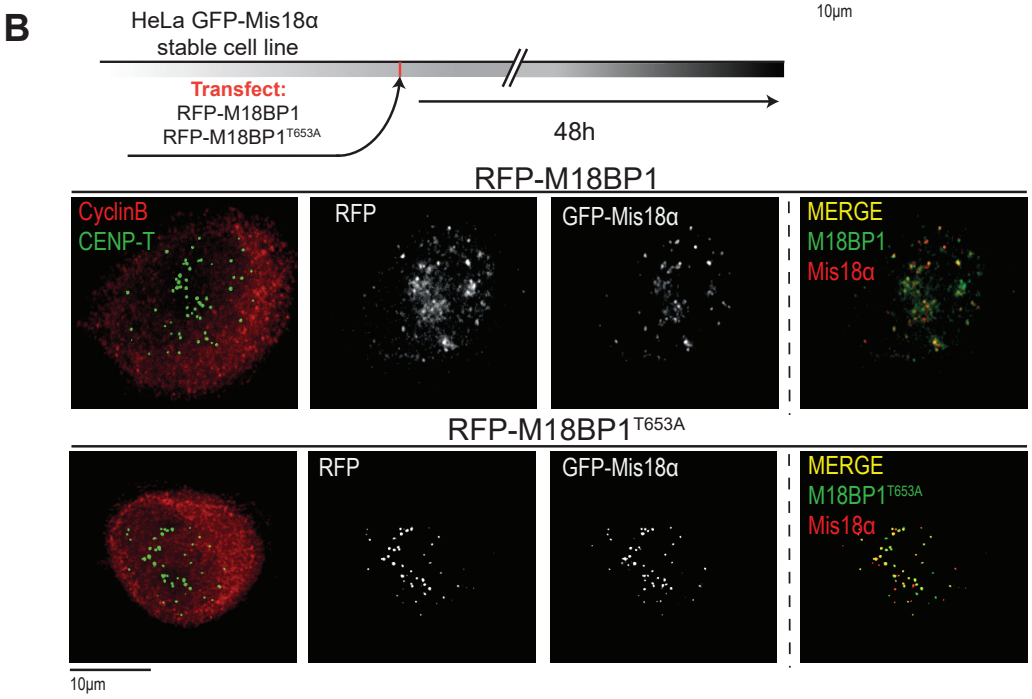
(C) Western blot showing higher levels of CENP-A-SNAP transgene in U2OS cell line (Müller et al., 2014) compared to HeLa cell line (used in this study). Extracts of randomly cycling U2OS CENP-A-SNAP and HeLa CENP-A-SNAP cell lines together with respective parental cell lines (carrying no transgene) were separated by SDS-PAGE followed by immunoblotting with anti-CENP-A and anti-Tubulin antibodies.

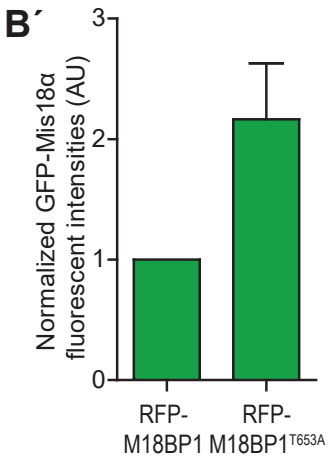
(C´) Quantification of CENP-A-SNAP band intensities of Western blot showed in (C) using Odyssey infrared scanner. Band intensities of CENP-A-SNAP were normalized to tubulin (loading control).

(C´´) Quantification of G1 CENP-A-SNAP fluorescent intensities from HeLa CENP-A-SNAP and U2OS CENP-A-SNAP. Randomly cycling HeLa CENP-A-SNAP and U2OS CENP-A-SNAP were subjected to Quench-Chase-Pulse experiment (as in Figure 2A, B). CENP-A-SNAP (TMR) fluorescent signal intensities were determined using CRaQ method.

Figure S6


A
GFP-M18BP1T4A, T40A, S110A
G2 phase
CyclinB
CENP-T
GFP
MERGE
CENP-T
M18BP1
GFP-M18BP1T4A, T40A, S110A,T653A
G2 phase
GFP
10μm


B
HeLa GFP-Mis18α
stable cell line
Transfect:
RFP-M18BP1
RFP-M18BP1T653A
48h
RFP-M18BP1
CyclinB
CENP-T
RFP
GFP-Mis18α
MERGE
M18BP1
Mis18α
RFP-M18BP1T653A
RFP
GFP-Mis18α
MERGE
M18BP1T653A
Mis18α
10μm


B´
Normalized GFP-Mis18α fluorescent intensities (AU)
0
1
2
3
RFP-M18BP1
RFP-M18BP1T653A

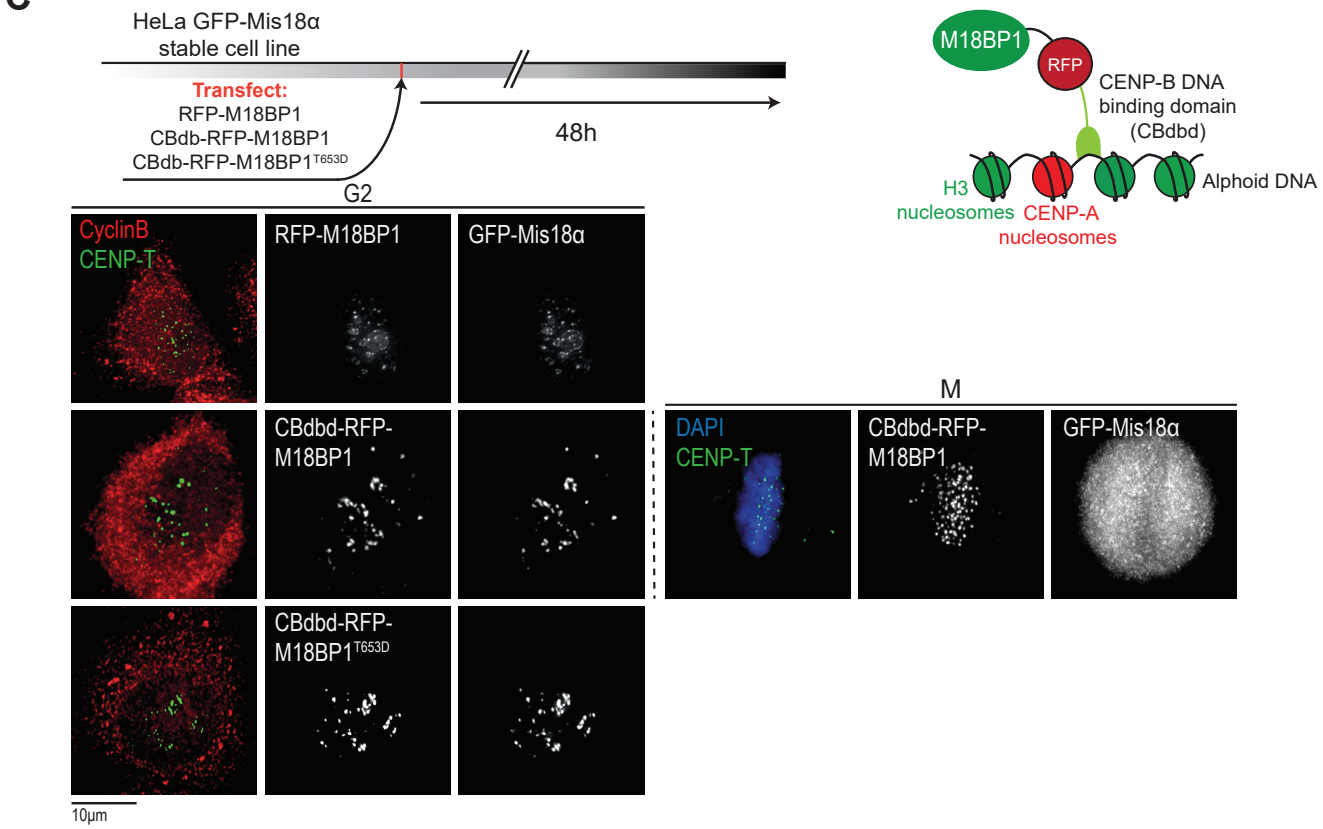

C
HeLa GFP-Mis18α
stable cell line
Transfect:
RFP-M18BP1
CBdb-RFP-M18BP1
CBdb-RFP-M18BP1T653D
48h
G2
M18BP1
RFP
CENP-B DNA
binding domain
(CBdbd)
H3
nucleosomes
CENP-A
nucleosomes
Alphoid DNA
CyclinB
CENP-T
RFP-M18BP1
GFP-Mis18α
CBdbd-RFP-
M18BP1
CBdbd-RFP-
M18BP1T653D
M
DAPI
CENP-T
CBdbd-RFP-
M18BP1
GFP-Mis18α
10μm

Figure S6. (supplement to Figure 5) Cell cycle control of M18BP1/Mis18α complex formation

(A) M18BP1^{T4,T40,S110} triple mutant is enriched at G2 centromeres, whereas M18BP1^{T4,T40,S110,T653A} quadruple mutant is strongly enriched at G2 centromeres. Constructs expressing M18BP1^{T4,T40,S110} or M18BP1^{T4,T40,S110,T653A} were transfected into asynchronous HeLa cells 48hr prior to fixation, followed by counterstaining for cyclin B, CENP-T and DAPI to indicate G2 status, centromeres and DNA, respectively.

(B) T653 residue in M18BP1 does not determine Mis18 complex formation. Left: Asynchronously cycling HeLa cells stably expressing GFP-Mis18α were transfected with constructs expressing RFP-M18BP1 or RFP-M18BP1^{T653A} 48hr prior to fixation, followed by counterstaining for cyclin B and CENP-T to indicate G2 status and centromeres respectively. Right: Average centromeric GFP fluorescent signals from Cyclin B positive cells were determined from 3 replicate experiments. Intensities were normalized to GFP-M18BP1. Error bars indicate standard error of the mean (SEM).

(C) M18BP1/Mis18α complex formation is not inhibited by Cdk activity in G2 phase. Asynchronously cycling HeLa cells stably expressing GFP-Mis18α were transfected with constructs expressing RFP-M18BP1, CBdbd-RFP-M18BP1 or CBdbd-RFP-M18BP1^{T653D} 48hr prior to fixation, followed by counterstaining for cyclin B and CENP-T to indicate G2 status and centromeres respectively. To enrich for mitotic stages, cells were treated with Nocodozole for 5h.

Figure S7

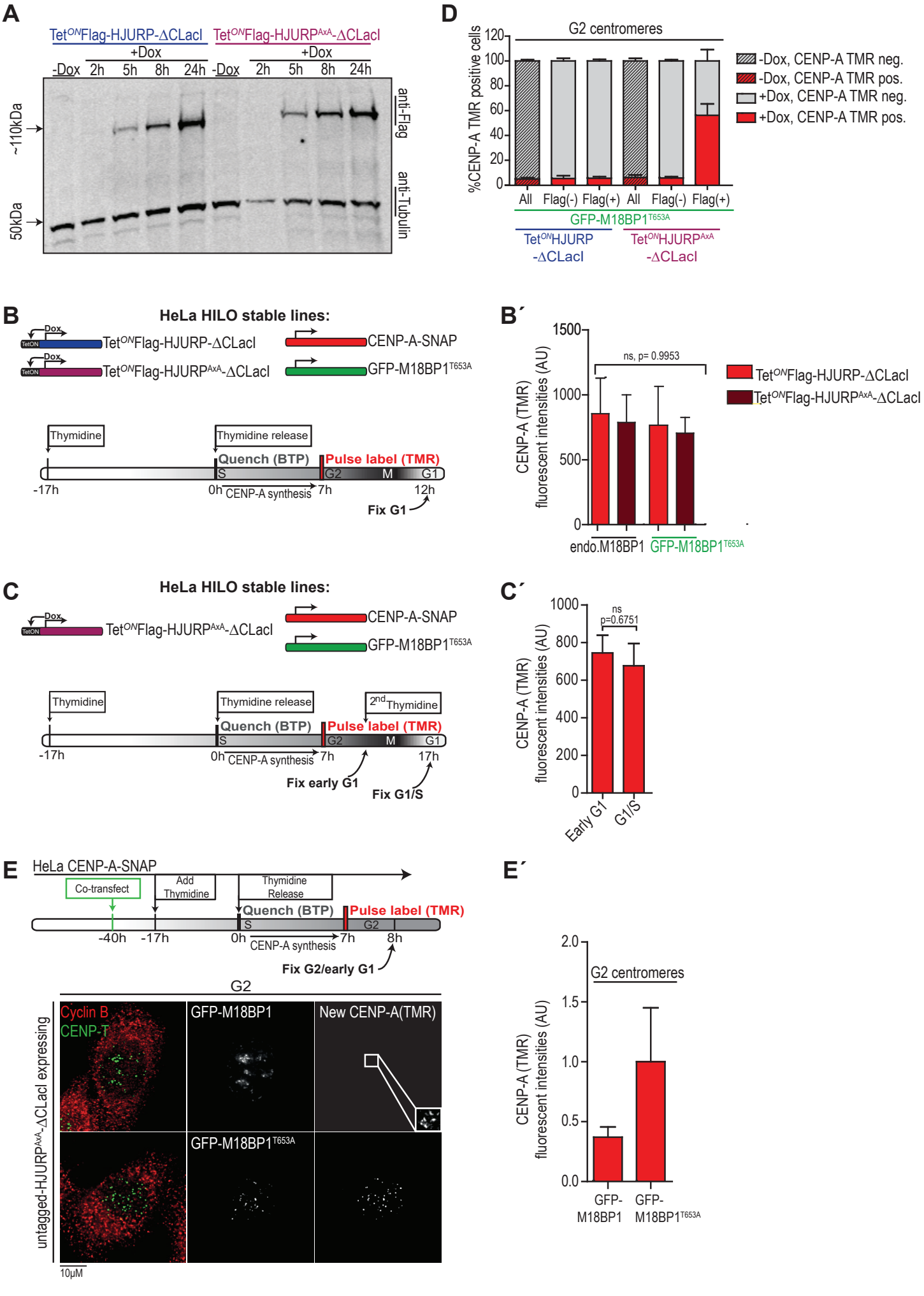

A
Tet^ON Flag-HJURP-ΔCLacI
Tet^ON Flag-HJURP^AxA-ΔCLacI
+Dox
-Dox 2h 5h 8h 24h -Dox 2h 5h 8h 24h
~110kDa
50kDa
anti-Flag
anti-Tubulin
D
G2 centromeres
%CENP-A TMR positive cells
-Dox, CENP-A TMR neg.
-Dox, CENP-A TMR pos.
+Dox, CENP-A TMR neg.
+Dox, CENP-A TMR pos.
All Flag(-) Flag(+) All Flag(-) Flag(+)
GFP-M18BP1^T653A
Tet^ON HJURP -ΔCLacI
Tet^ON HJURP^AxA -ΔCLacI
B
HeLa HILO stable lines:
Dox
Tet^ON Flag-HJURP-ΔCLacI
Tet^ON Flag-HJURP^AxA-ΔCLacI
CENP-A-SNAP
GFP-M18BP1^T653A
Thymidine
Thymidine release
Quench (BTP)
Pulse label (TMR)
S G2 M G1
-17h 0h CENP-A synthesis 7h 12h
Fix G1
B´
CENP-A (TMR) fluorescent intensities (AU)
ns, p= 0.9953
Tet^ON Flag-HJURP-ΔCLacI
Tet^ON Flag-HJURP^AxA-ΔCLacI
endo.M18BP1
GFP-M18BP1^T653A
C
HeLa HILO stable lines:
Dox
Tet^ON Flag-HJURP^AxA-ΔCLacI
CENP-A-SNAP
GFP-M18BP1^T653A
Thymidine
Thymidine release
2nd Thymidine
Quench (BTP)
Pulse label (TMR)
S G2 M G1
-17h 0h CENP-A synthesis 7h 17h
Fix early G1
Fix G1/S
C´
CENP-A (TMR) fluorescent intensities (AU)
ns
p=0.6751
Early G1
G1/S
E
HeLa CENP-A-SNAP
Co-transfect
Add Thymidine
Thymidine Release
Quench (BTP)
Pulse label (TMR)
S G2
-40h -17h 0h CENP-A synthesis 7h 8h
Fix G2/early G1
G2
untagged-HJURP^AxA-ΔCLacI expressing
Cyclin B
CENP-T
GFP-M18BP1
New CENP-A(TMR)
GFP-M18BP1^T653A
10μM
E´
G2 centromeres
CENP-A (TMR) fluorescent intensities (AU)
GFP-M18BP1
GFP-M18BP1^T653A

Figure S7. (supplement to Figure 6) Characterization of HeLa HILO inducible HJURP cell lines and CENP-A assembly dynamics.

(A) 3xFlag-HJURP-ΔCLacI and 3xFlag-HJURP^{AxA}-ΔCLacI proteins are induced to a similar extent. HeLa HILO CENP-A-SNAP, GFP-M18BP1^{T653A} carrying tetracycline-inducible 3xFlag-HJURP-ΔCLacI or3xFlag-HJURP^{AxA}-ΔCLacI were synchronized by a single Thymidine block, and released into G2 phase. Doxycycline (Dox) was added for 2h, 5h, 8h or 24h. Following fixation, whole cell lysates were separated using SDS-PAGE followed by immunoblotting with indicated antibodies.

(B) CENP-A-SNAP loading efficiency in G1 is equal in all HeLa HILO cell lines used in this study. Overview of HILO transgenes are shown. Uninduced HeLa HILO CENP-A-SNAP cells carrying indicated M18BP1 and or HJURP transgenes were synchronized by addition of Thymidine for 17h, followed by a release for 7h during which a nascent CENP-A-SNAP pool was fluorescently labeled. Cells were allowed to cycle into early G1 phase, 12h post release and fixed (early G1 as in Figure 6). This experimental set-up allows for labeling of an equal pool of nascent CENP-A-SNAP and compares the degree of assembly across cell lines used (conditions analogous to the ones in Figure 6).

(B´) CENP-A-SNAP fluorescent intensities were quantified using CRaQ method and tested for statistically significant differences (ns, one-way ANOVA). Results indicate that G1 phase levels are similar across all cell lines used. Error bars indicate SEM.

(C) CENP-A loading in G1 in 3xFlag-HJURP^{AxA}-ΔCLacI / GFP-M18BP1^{T653A} expressing cells as near its maximum in early G1 phase. Relevant transgenes are shown. Experimental condition is identical to (B) except that in addition to fixation in early G1 phase, a subset of cells were allowed to cycle through G1 and were collected at the G1/S boundary following a second Thymidine addition.

(C´) CENP-A-SNAP fluorescent intensities from early G1 cells and cells collected at the end of G1 were directly compared and tested for statistically significant differences (ns, one-way ANOVA). Results indicate that early and late G1 phase levels are similar. CENP-A-SNAP fluorescent intensities were quantified using CRaQ method. Error bars indicate SEM. P values are indicated on the graph (ns, Student´s t test).

(D) Fraction of cells assembling CENP-A upon mutant M18BP1 and/or mutant HJURP expression. Data from experiment shown in Figure 6. Here the frequency of CENP-A-SNAP G2 centromeres is plotted as a fraction of total Flag positive cells, except for un-induced samples, in which the fraction of total cells was determined. Averages of 3 independent experiments are shown. Error bars indicate SEM.

(E) Over-expression of GFP-M18BP1 does not enhance HJURP^{AxA}-driven precocious CENP-A deposition. HeLa CENP-A-SNAP cells were enriched in G2 phase by a single Thymidine arrest, followed by 7 h release. 40 h prior to synchronized S phase release, cells were co-transfected with untagged-HJURP^{AxA}-ΔCLacI combined with either GFP-M18BP1 or GFP-M18BP1^{T653A}. S phase synthesized CENP-A-SNAP was pulse labeled in G2 phase to assay CENP-A assembly. Cells were counterstained for cyclin B and CENP-T to indicate G2 status and centromeres, respectively.

(E´) Quantification of CENP-A-SNAP (TMR) fluorescent signal intensities of experiment in A. Average centromeric CENP-A-SNAP fluorescent signals from G2 cells (Cyclin B positive) and G1 cells (Cyclin B negative) were determined from 4 replicate experiments using CRaQ method. Signal intensities were normalized to CENP-A-SNAP in G1 cells expressing GFP-M18BP1. Error bars indicate SEM. GFP-M18BP1 force expression in combination with HJURP^{AxA}-ΔCLacI results in similar of CENP-A assembly as in the presence of endogenous M18BP1 levels (see also Figure 2B, B´)

Supplemental Experimental Procedures

DNA constructs

HJURP-GFP (pLJ381) and GFP-HJURP-ΔCLacI (pLJ632) (Zasadzińska et al., 2013), in which amino acids 483-743 (C-terminus) were replaced by dimerization domain of LacI (Zasadzińska et al., 2013) were a gift from Dan Foltz (Northwestern University of Chicago). GFP-HJURP (pLJ380) and GFP-HJURP-ΔCLacI (pLJ632) were converted to GFP-HJURP^{AxA} (pLJ600) and GFP-HJURP^{AxA}-ΔCLacI (pLJ654) by quick exchange PCR replacing R276 and L278 by Alanine. GFP-HJURP$^{S210A, S211A, S412A}$ (pLJ828) or GFP-HJURP$^{S210A, S211A, S412A}$-ΔCLacI (pLJ830) was made via quick exchange PCR. pLJ591, HJURP-CBdbd-GFP was created by PCR amplification of the first 158 N-terminal amino acids of CENP-B protein [CENP-B DNA binding domain (CBdbd)] and ligation to the N terminus of pLJ383 (a GFP-Mis18α construct). Subsequently, Mis18α was excised using EcoRI/BamHI restriction, followed by T4 end blunting and ligation, resulting in CBdbd-GFP vector. The CBdbd fragment was subsequently inserted in frame in between HJURP and GFP of pLJ381. For the creation of the HeLa HILO 3xFlag-HJURP-ΔCLacI /HJURP^{AxA}-ΔCLacI inducible cell lines, the donor plasmid pRD-RIPE (Khandelia et al., 2011) was digested with AgeI and BsrGI to remove EGFP followed by insertion of the LacI dimerization domain. Next, PCR amplified 3xFlag-HJURP-ΔC (aa 1-482) or 3xFlag HJURP^{AxA}-ΔC (aa 1-482) was inserted into an AvaI/BsrGI digested vector, resulting in an in-frame fusion of 3xFlag HJURP/HJURP^{AxA}-ΔCLacI (designated pLJ745 and pLJ746, respectively). GFP-M18BP1^{T653A} (pLJ649) was created by quick exchange PCR using GFP-M18BP1 as a template (pLJ415 (Silva et al., 2012)). An analogous procedure was employed to generate GFP-M18BP1^{T653D} (pLJ699). CBdbd-GFP-M18BP1 (pLJ592) was generated by ligation of CBdbd fragment between GFP and M18BP1 of the GFP-M18BP1 plasmid (pLJ415) mRFP-M18BP1^{T653D} (pLJ705) was created by NotI and AfeI replacement of GFP with mRFP (from pLJ287). CBdbd-mRFP-M18BP1/M18BP1^{T653D}/M18BP1^{T653E} (pLJ697, pLJ700 and pLJ642, respectively) were created by PCR insertion of CBdbd fragment from pLJ591 (CBdbd-GFP-HJURP) into pLJ534 (mRFP-M18BP1) or pLJ705 (mRFP-M18BP1^{T653D}) or pLJ641 (mRFP-M18BP1^{T653E}). PCR amplified CBdbd was subsequently fused to N-terminal portion of mRFP. All plasmid inserts were verified by DNA sequencing.

Cell lines and culturing conditions

All human cell lines used were grown at 37°C, 5% CO_2. Cells were grown in DMEM (Bio West) supplemented with 10% fetal bovine serum (FBS) (BioWest)), 2 mM glutamine, 1 mM sodium pyruvate (SP) (Thermo Fischer Scientific), 100 U/ml penicillin, and 100 µg/ml streptomycin, with the exception of HeLa HILO derived cell lines in which 10% tet-free (BioWest) FBS was used. HeLa HILO RMCE cell lines were a gift from E.V. Makeyev, Nanyang Technological University, Singapore, and contain a single genomic recombination

which allows for the insertion of a tetracycline responsive expression cassette (Khandelia et al., 2011). The four lines outlined in Figure 6 were assembled as follows: HeLa HILO RMCE clone #10 (Khandelia et al., 2011), was transfected with (pLJ649) that constitutively drives GFP-M18BP1^{T653A} expression. Positive clones were selected with 500 µg/ml of Neomycin (Gibco). A polyclonal population was sorted based on GFP fluorescence. A single clone of HeLa HILO GFP-M18BP1^{T653A} as well as the parental HILO RMCE clone #10 were transduced with pBABE-CENP-A-SNAP-3xHA retrovirus (pLJ718) (Bodor et al., 2012). Infected cells were selected by 300 µg/ml of Hygromycine (Invitrogen). Individual resistant cells were sorted by FACS. CENP-A-SNAP-3xHA clones #9 and #10, respectively were selected for further analysis. This selection was based on equal expression of CENP-A-SNAP-3xHA between different cell lines, as determined by immunoblot using rabbit anti-CENP-A (Cell Signaling technology) and by TMR fluorescent intensities. Both clones were then transfected with 2,5 ng/µl of pLJ745 and pLJ746, vectors carrying two loxP sites flanking the Doxycycline (Dox) inducible 3xFlag-HJURP or 3xFlag-HJURP^{AxA} expression construct. Cre recombinase (Khandelia et al., 2011) was added at 1% of total DNA content. Positive clones were selected using 1 µg/ml of Puromycin (MERCK). Expression of 3xFlag-HJURP/HJURP^{WT}/^{AxA} was induced by 10 µg/ml of Doxycycline (Sigma-Aldrich) and assayed for equal expression by western blot using FlagM2 antibody (Sigma-Aldrich). U2OS CENP-A-SNAP cell lines were gift from Genevieve Almouzni (Institut Curie, France).

DNA transfection and siRNA treatment

Transient transfection of HeLa CENP-A-SNAP and HEK293T was performed using Lipofectamine LTX (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. All siRNAs were obtained from Dharmacon. Mis18α and HJURP were depleted as previously reported (Silva et al., 2012; Zasadzińska et al., 2013, respectively).

Co-Immunoprecipitation

HEK293T cells were transiently transfected either with GFP alone, GFP-HJURP-ΔCLacI or GFP-HJURP^{AxA}-ΔCLacI. 24h post-transfection cells were either allowed to continue to cycle or were treated overnight in DME III to induce mitotic arrest. 48h post-transfection, 10^7 cells were harvested in ice-cold PBS for 5 min at 3,200 x g and lysed in buffer containing 3,75mM Tris pH 7,5, 20mM KCl,0,5mM EDTA, 0,1% digitonin and 0,4 µM DTT. Lysates were homogenized using a 27G needle, and spun at 300 x g for 5 min. This was repeated two times, followed by combining two supernatants and spin clarification at 10,000 x g for 15 min. Soluble fraction was collected and KCl concentration was adjusted to 150 mM. 5µg/ml of anti-Cyclin A coated agarose beads (Santa Cruz Biotechnology) or anti-Cyclin B (Santa Cruz Biotechnology) was equilibrated in lysis buffer prior to pulldown. After immunoprecipitation, beads were washed once in wash buffer A (20mM HEPES, 20Mm M KCl, 0,4m M DTT and 0,4mM EDTA) and two times in wash buffer B (wash buffer A with

225mM NaCl for Cyclin A pulldown, and 150mM NaCl for Cyclin B). Complexes bound to the beads were eluted using 2% SDS for 20 min, followed by immunoblotting with anti-Cyclin A (Santa Cruz) (Figure 1E) or anti-Cyclin B (Santa Cruz) (Figure S3B) and anti-GFP (Chromotek) antibodies. IRDye800CW-coupled anti-rat (Licor Biosciences) and DyLight680-coupled anti-rabbit (Rockland Immunochemicals, Gilbertsville, PA) secondary antibodies were used prior to detection on an Odyssey near-infrared scanner (Licor Biosciences, Lincoln, NE). Immunoblot signals were quantified using the Odyssey software (see also (Bodor et al., 2014)). GFP signal values were normalized to their respective Cyclin A signals and to corresponding GFP input values.

SILAC and affinity purification of prenucleosomal HJURP/CENP-A/H4 complex

SILAC labeling medium (MEM Eagle Joklik Modification) deficient in lysine and arginine was reconstituted according to manufacturer's instructions (Sigma-Aldrich), and supplemented with normal lysine and arginine (Sigma-Aldrich) for “light” medium, and 50 mg/ L $^{13}C_6$,$^{15}N_2$-lysine and 50 mg/L $^{13}C_6$, $^{15}N_4$-arginine (Silantes) for “heavy” medium. Both media were supplemented with 10% dialyzed FBS (Gemini), GlutaMax (Gibco), 1 mM HEPES, 1% Pen/Strep, MEM non-essential amino acids (Gibco), and 120 mg/L proline to prevent arginine-to-proline conversion. Two parallel cultures of previously characterized HeLaS3 cells stably expressing localization and purification (LAP)-tagged CENP-A (Bailey et al., 2013) were cultured in spinner flasks for at least 6 cell doublings to allow full incorporation of the stable isotope-containing amino acids. Heavy isotope labeling efficiency of ~98% was confirmed by mass spectrometry after trypsin digestion of proteins extracted from heavy-labeled cells. To enrich for mitotic cells, both cultures were treated with 50 μM S-trityl-L-cysteine for 17 h. Subsequently, the "light" cells were treated with 100 μM R-Roscovitine (AdipoGen) for 30 min while the "heavy" cells were mock-treated with DMSO. Cell cycle status and HJURP phospho-status was monitored by immunoblotting for H3pS10 (Upstate) and an anti-HJURP antibody generated against a C-terminal fragment (1 μg/ml) (Bassett et al., 2012, Dev Cell), respectively. Cell pellets from 1.4 x 10^9 of "light" and "heavy" cells were combined in 1:1 ratio. Affinity purification of the prenucleosomal HJURP/CENP-A/H4 complex was performed as previously described (Bailey et al., 2013) except that protein elution was performed with 2% SDS and heating at 95°C.

Mass spectrometry and data analysis

Purified CENP-A and associated proteins were precipitated using pre-chilled acetone (4 X volume) followed by successive washing. Dried protein pellets were reconstituted with 0.1% RapiGest SF Surfactant (Waters) in 100 mM NH_4HCO_3, pH 8.0. Resuspended proteins were reduced using DTT, alkylated with iodoacetamide, and digested using trypsin. Since trypsin cleaves only after lysines and arginines, this ensures that every resulting peptide will contain at least one lysine or arginine, so that all heavy peptides are distinguishable

from their corresponding light peptides by predictable mass differences. Rapidgest was removed by adding 0.5% TFA and incubation for 30min at 37°C. The peptides were desalted with StageTips (Rappsilber et al., 2007), followed by phosphopeptide enrichment by TiO2 prior to analysis by Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). The pFind search engine was used to search the UniProt human protein database to identify peptides (Wang et al., 2007). Quantification was done using extracted-ion chromatograms (XICs) of each light and heavy peptide pair, and L/H ratio represents the ratio of total area under each elution peak. Mass spectra of a representative non-phosphorylated HJURP peptide from the flow through in Figure 3 of samples from cells containing HJURP and HJURP^{AxA} had a retention time range of 28.75-29.89min, which includes all scans in both runs in which the peptide was detectable. Mass spectra of the phosphopeptide containing pS210/pS11, from the elution of the phospho-enrichment from cells containing HJURP and HJURP^{AxA} had a retention time range of 24.00-25.48min, which includes all scans from both runs in which the peptide was detectable.

Phospho-specific anybody generation, application and phosphatase treatment

Phospho-specific rabbit antibody for M18BP1 was produced by immunization of 2 rabbits with phosphorylated peptide ((NH2-)CKAYILV (pT)PLKSRK (-CONH2)), and subsequent affinity purification of both sera (Innovagen AB, SE-22370 Lund, Sweden). 10^6 of transiently transfected HEK293T carrying either GFP M18BP1 or GFP M18BP1^{T653A} were lysed in buffer containing 75mM HEPES pH 7,5, 150mM NaCl, 2mM MgCl2, 0,1% NP-40, 5% Glycerol, 2mM EDTA supplemented with Roche complete protease and phosphatase inhibitors. Lysates were spun at 4°C for 5 min at 15,000 x g. Supernatants were either left untreated or 300 units of Lambda phosphatase was added. All samples were incubated for 30 min at 30°C. Reaction was stopped by addition of 4 x Orange sample buffer. For assaying Cdk dependent phosphorylation of M18BP1, transiently transfected Hek293T were treated with 100μM of Roscovitine for 30min, or treated for DMSO. Protein extracts were separated by SDS-PAGE and probed with pT653 and GFP (Chromotek) antibodies. Fluorescence activated cell sorting (FACS) for cell cycle profile was performed based on propidium iodide staining as described (Silva et al., 2012).

Immunofluorescence and pre-extraction procedure

Procedures are essentially as described (Bodor et al., 2012). Briefly, all cell lines were grown on glass coverslips coated with poly-L lysine (Sigma-Aldrich) and fixed with 4% formaldehyde (Thermo Scientific) for 10 min followed by permeabilization in 0.1% Triton X-100. HeLa cells were stained with anti-cyclin B1 (1:50; sc-245, Santa Cruz) and anti-CENP-T (Barnhart et al., 2011). Secondary antibodies used were either FITC-conjugated anti-mouse (Jackson Immunoresearch Laboratories) or Dy680 conjugated anti-rabbit antibody (Rockland Immunochemicals). Cells were stained with DAPI (40, 6-diamidino-2-

phenylindole; Sigma-Aldrich) before mounting in Mowiol. For detecting GFP HJURP^{AxA} on G2 centromeres, HeLa CENP-A SNAP cells transiently expressing the construct were pre-extracted for 5min using CSK buffer (10mM Pipes-KOH pH7, 100mM NaCL, 300mM Sucrose and 3mM $MgCl_2$) supplemented with 0.3% of Titon X-100. Subsequently, cells were washed one time with CSK buffer, followed by PBS wash and fixation for 22 min with 4% formaldehyde. Cells were counterstained using anti-CENP-T (Barnhart et al., 2011) and anti-Aurora B (1:100; BD transduction laboratories). GFP HJURP^{AxA} signal was amplified using GFP-Booster Atto488 (Chromotek).

DAPI area as a measure of cell cycle position

To identify G2 cells in experiment presented in Figure 6 we synchronised cells in early S phase (by double Thymidine block), G2 phase (by double Thymidine block and 7h of release), late G2 (by an overnight treatment with RO3306 (Roche)) or left them asynchronous. Using these synchronized populations we established a cut-off for DAPI area size of G2 cells for each experiment. Following image acquisition, thresholding parameters selecting isolated DAPI areas were manually adjusted using ImageJ software. Subsequently, these parameters were propagated to all data sets, and an average DAPI area of each cell cycle stage was determined. Cells were identified as G2 if DAPI area was at least two standard deviations above the average DAPI area size of the S phase population. We confirmed that these values completely overlapped to the averages of DAPI area size coming from G2 synchronized populations (double thymidine released or RO3306 treated). All cells that had an equal or smaller DAPI area size from average values of S phase population were excluded from the analysis.

Supplemental References

Bailey, A.O., Panchenko, T., Sathyan, K.M., Petkowski, J.J., Pai, P.-J., Bai, D.L., Russell, D.H., Macara, I.G., Shabanowitz, J., Hunt, D.F., et al. (2013). Posttranslational modification of CENP-A influences the conformation of centromeric chromatin. Proc. Natl. Acad. Sci. *110*, 11827–11832.

Barnhart, M.C., Kuich, P.H.J.L., Stellfox, M.E., Ward, J.A., Bassett, E.A., Black, B.E., and Foltz, D.R. (2011). HJURP is a CENP-A chromatin assembly factor sufficient to form a functional de novo kinetochore. J. Cell Biol. *194*, 229–243.

Bodor, D.L., Rodríguez, M.G., Moreno, N., and Jansen, L.E.T. (2012). Analysis of Protein Turnover by Quantitative SNAP-Based Pulse-Chase Imaging. Curr. Protoc. Cell Biol. *Chapter 8*, Unit8.8.

Foltz, D.R., Jansen, L.E.T., Bailey, A.O., Yates, J.R., Bassett, E. a., Wood, S., Black, B.E., and Cleveland, D.W. (2009). Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP. Cell *137*, 472–484.

Khandelia, P., Yap, K., and Makeyev, E. V (2011). Streamlined platform for short hairpin RNA interference and transgenesis in cultured mammalian cells. Proc. Natl. Acad. Sci. U. S. A. *108*, 12799–12804.

Müller, S., Montes de Oca, R., Lacoste, N., Dingli, F., Loew, D., Almouzni, G., Montes de Oca, R., Lacoste, N., Dingli, F., Loew, D., et al. (2014). Phosphorylation and DNA Binding of HJURP Determine Its Centromeric Recruitment and Function in CenH3(CENP-A) Loading. Cell Rep. *8*, 190–203.

Rappsilber, J., Mann, M., and Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat. Protoc. *2*, 1896–1906.

Silva, M.C.C., Bodor, D.L., Stellfox, M.E., Martins, N.M.C., Hochegger, H., Foltz, D.R., and Jansen, L.E.T. (2012). Cdk Activity Couples Epigenetic Centromere Inheritance to Cell Cycle Progression. Dev. Cell *22*, 52–63.

Wang, L.-H., Li, D.-Q., Fu, Y., Wang, H.-P., Zhang, J.-F., Yuan, Z.-F., Sun, R.-X., Zeng, R., He, S.-M., and Gao, W. (2007). pFind 2.0: a software package for peptide and protein identification via tandem mass spectrometry. Rapid Commun. Mass Spectrom. *21*, 2985–2991.

Zasadzińska, E., Barnhart-Dailey, M.E.C., Kuich, P.H.J.L., and Foltz, D.R. (2013). Dimerization of the CENP-A assembly factor HJURP is required for centromeric nucleosome deposition. EMBO J. *32*, 2113–2124.