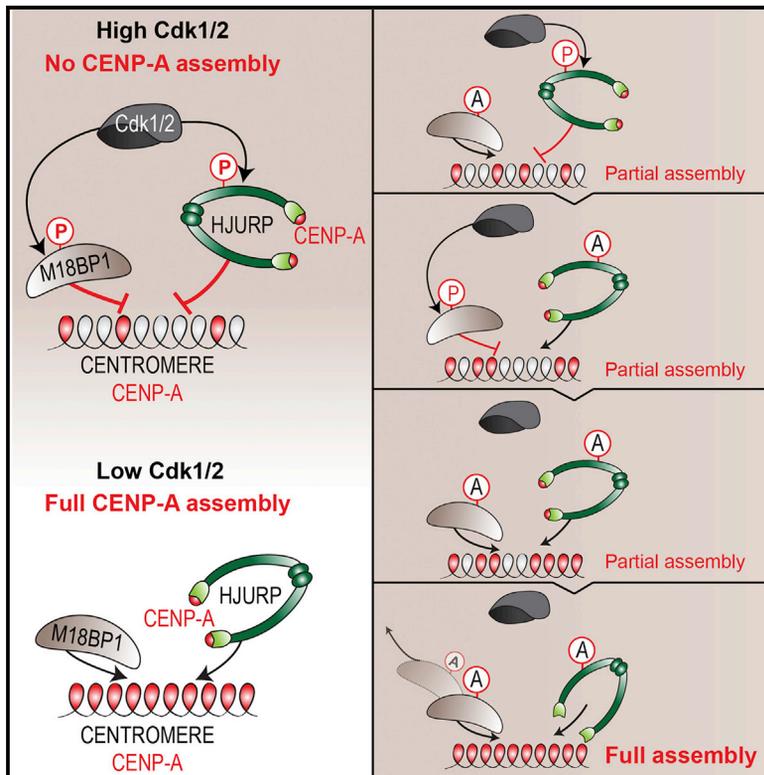


# A Dual Inhibitory Mechanism Sufficient to Maintain Cell-Cycle-Restricted CENP-A Assembly

## Graphical Abstract



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## In Brief

Centromeres are epigenetically defined by the heritable histone H3 variant CENP-A, whose assembly is restricted to G1 phase. Stankovic et al. show that cell-cycle coupling is achieved through inhibitory Cdk-mediated phosphorylation of two key CENP-A assembly factors, M18BP1 and HJURP, preventing unscheduled centromeric chromatin assembly outside of the G1 phase.

## Highlights

- The HJURP conserved domain interacts with cyclin A
- Timing of M18BP1 localization is controlled by T653 phosphorylation
- Control of HJURP and M18BP1 suffices to cell-cycle restrict CENP-A assembly
- CENP-A assembly results in functional removal of M18BP1 from centromeres



# A Dual Inhibitory Mechanism Sufficient to Maintain Cell-Cycle-Restricted CENP-A Assembly

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## SUMMARY

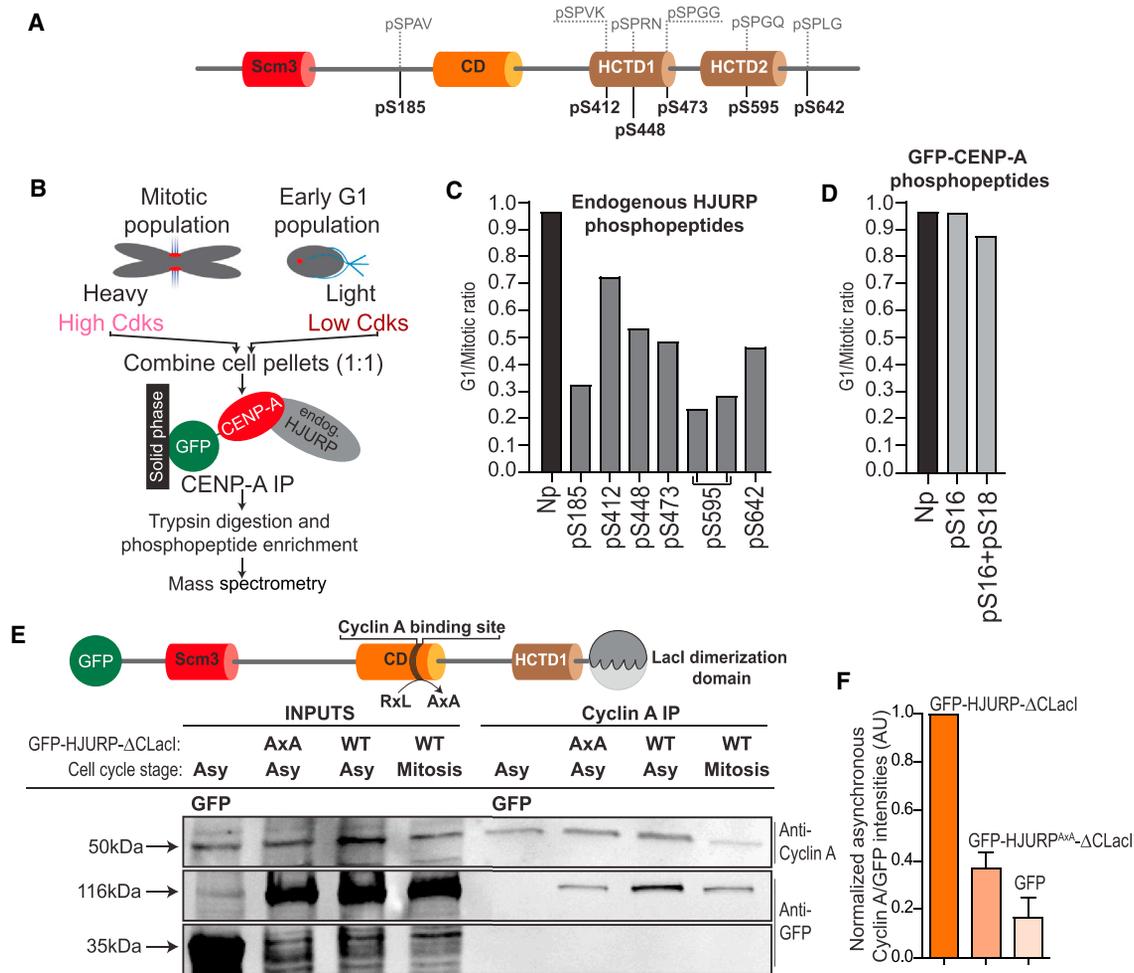
Chromatin featuring the H3 variant CENP-A at the centromere is critical for its mitotic function and epigenetic maintenance. Assembly of centromeric chromatin is restricted to G1 phase through inhibitory action of Cdk1/2 kinases in other phases of the cell cycle. Here, we identify the two key targets sufficient to maintain cell-cycle control of CENP-A assembly. We uncovered a single phosphorylation site in the licensing factor M18BP1 and a cyclin A binding site in the CENP-A chaperone, HJURP, that mediated specific inhibitory phosphorylation. Simultaneous expression of mutant proteins lacking these residues results in complete uncoupling from the cell cycle. Consequently, CENP-A assembly is fully recapitulated under high Cdk activities, indistinguishable from G1 assembly. We find that Cdk-mediated inhibition is exerted by sequestering active factors away from the centromere. Finally, we show that displacement of M18BP1 from the centromere is critical for the assembly mechanism of CENP-A.

## INTRODUCTION

Centromeres are chromosomal loci that drive faithful genome segregation during mitotic division (Allshire and Karpen, 2008). The functional foundation of the centromere is established by a specialized chromatin structure that features the histone H3 variant CENP-A (Black and Cleveland, 2011). This CENP-A-based chromatin domain provides a structural platform for formation of the kinetochore, which links chromosomes to spindle microtubules during mitosis (Cheeseman and Desai, 2008; Foltz et al., 2006; Okada et al., 2006). In addition, CENP-A ensures stable maintenance of the centromere position through an epigenetic

self-propagating feedback loop (Black and Cleveland, 2011; Gómez-Rodríguez and Jansen, 2013). Support for the epigenetic nature of the centromere comes from naturally occurring neocentromeres (Amor et al., 2004; Marshall et al., 2008), where centromere proteins vacate the original centromeric DNA sequence and assemble heritably on previously naive chromatin. In addition, ectopic targeting of CENP-A or proteins of the centromere complex to a non-centromeric locus was shown to be sufficient to initiate a functional and heritable centromere (Barnhart et al., 2011; Hori et al., 2013; Mendiburo et al., 2011). Consistent with a key role at the core of a positive epigenetic feedback loop, CENP-A nucleosomes are long lived and are maintained through multiple cell divisions (Bodor et al., 2013; Jansen et al., 2007). The unusually slow turnover of CENP-A at each centromere (Falk et al., 2015) indicates that replenishment is either equally slow or limited in time and tied to CENP-A redistribution following DNA replication. Indeed, in metazoans, assembly of newly synthesized CENP-A is directly linked to cell-cycle progression and is initiated during mitotic exit and restricted to the early G1 phase of the cell cycle (Jansen et al., 2007; Schuh et al., 2007).

Previously, we showed that brief inhibition of cyclin-dependent kinase 1 and 2 (Cdk1/2) activities is sufficient to drive CENP-A deposition prior to mitotic exit (Silva et al., 2012). This has led to a model where the CENP-A assembly machinery is present and poised for activity but is kept inactive throughout S, G2, and M phase until mitotic exit, when activities of Cdk1/2 drop, concomitant with the onset of CENP-A deposition. Key proteins necessary for the process of CENP-A deposition include the Mis18 complex and the CENP-A chaperone HJURP, which bears CENP-A-specific nucleosome assembly activity (Dunleavy et al., 2009; Foltz et al., 2009; Fujita et al., 2007). HJURP and M18BP1 (also known as HsKNL2), a member of the Mis18 complex, are phosphoproteins (Bailey et al., 2016; Dephoure et al., 2008; Kato et al., 2007; McKinley and Cheeseman, 2014; Müller et al., 2014; Silva et al., 2012; Wang et al., 2014) and localize to centromeres in a cell-cycle-controlled manner in the early G1 phase (Dunleavy et al., 2009; Foltz et al., 2009; Fujita et al., 2007; Maddox et al., 2007), indicating they are putative



**Figure 1. HJURP Is Phosphorylated in a Cdk-Dependent Manner and Interacts with Cyclin A**

(A) Schematic representation of HJURP protein (Scm3, CENP-A binding domain; CD, conserved domain; HCTD, HJURP C-terminal domain). Position of phospho-sites identified by SILAC in (C) are indicated. Amino acid sequences flanking phospho-sites are annotated in gray.

(B) Schematic of SILAC experiment (see Supplemental Experimental Procedures for details). Light cells were released into G1 by Roscovitine treatment for 30 min. At this stage, HJURP is partially dephosphorylated (see Figures S1F and S1G).

(C) The L/H ratios of phosphorylated Cdk sites detected on endogenous HJURP are listed. A representative non-phosphorylated peptide (Np) is shown as internal control. Note: pS595 was detected on two independent peptides.

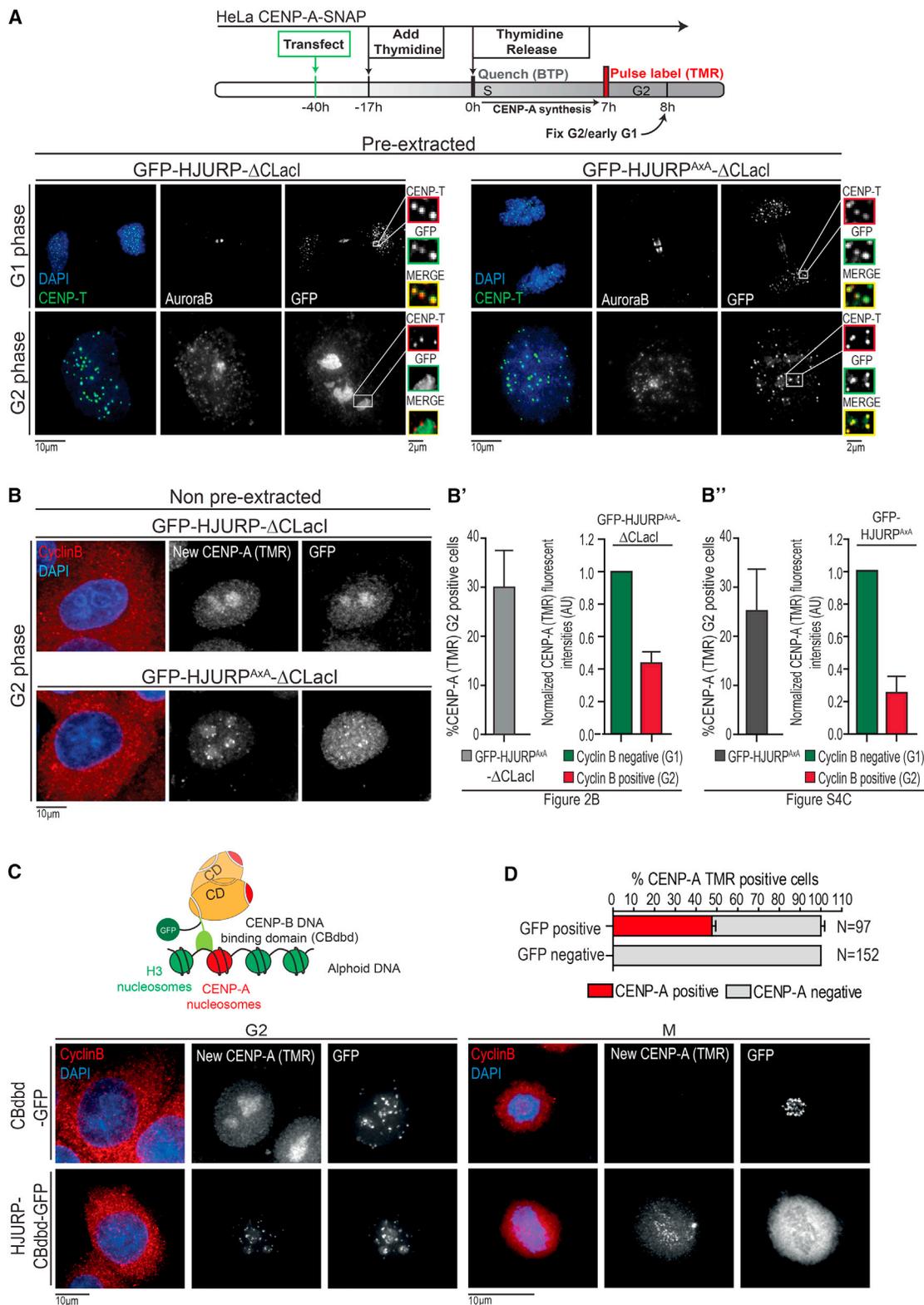
(D) L/H ratios of Cdk consensus sites within the N-terminal tail of CENP-A (see Figure S2 for data from two additional replicate experiments).

(E) HJURP CD mediates interaction with cyclin A. Top: schematic representation of HJURP protein. The mutation of conserved RxL motif to AxA is annotated with a black arrow. Experiments are performed with an HJURP construct in which the C-terminal homodimerization domain is replaced with that of Lacl to prevent dimerization with wild-type HJURP. Bottom: co-immunoprecipitation (IP) of extracts expressing indicated constructs either from asynchronous or mitotically enriched cells. Bound complexes were separated using SDS-PAGE, followed by immunoblotting with indicated antibodies.

(F) Quantification of IP experiments. The GFP signal from each IP was normalized to corresponding cyclin A signal and input GFP signal in order to control for IP efficiency and GFP fusion protein expression level, respectively. GFP-HJURP signals were set to 1. Error bars indicate SEM from three independent experiments. See also Figures S1 and S2.

targets for Cdk regulation. In addition, recent work has identified the mitotic kinase Plk1 as a critical component to drive CENP-A assembly (McKinley and Cheeseman, 2014). However, while Plk1 is itself a cell-cycle-controlled kinase, it does not restrict CENP-A assembly to the G1 phase, because it is required for both canonical assembly in G1 phase as well as premature assembly upon Cdk inhibition. In addition, several residues on CENP-A itself are phosphorylated (Bailey et al., 2013; Yu et al., 2015; Zeitlin et al., 2001). One of these, serine 68, is proposed

to be phosphorylated by mitotic Cdk activity (Yu et al., 2015), but the relevance of this is being disputed (Fachinetti et al., 2017), and mutation of this residue does not lead to a change in the timing of CENP-A deposition. In contrast, mutations of phospho-residues in HJURP or artificial recruitment of M18 $\alpha$  to centromeres has been reported to result in premature centromere recruitment of CENP-A (McKinley and Cheeseman, 2014; Müller et al., 2014). While these studies point to a contributing role for these factors, they leave open the critical questions of



**Figure 2. Timing of HJURP Targeting and CENP-A Deposition Is Controlled by HJURP CD**

(A) HeLa CENP-A-SNAP cells were transiently transfected with indicated constructs, and thymidine synchronized to enrich cells in the G2 phase. Cells were permeabilized prior to fixation and counterstained for Aurora B, CENP-T, and DAPI to distinguish between G2 and early G1 cell-cycle phases, centromere localization, and DNA, respectively. GFP booster was used to amplify the GFP-HJURP fluorescent signal.

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which factors are necessary, which are sufficient, how Cdk-mediated control is exerted, and how key proteins are functionally inhibited.

To resolve the specific molecular steps that ensure cell-cycle-restricted CENP-A assembly, we report full uncoupling of CENP-A assembly from the cell cycle and Cdk regulation. To achieve this, we identified a functional cyclin-interacting domain in HJURP and a critical phospho-site in M18BP1. Simultaneous uncoupling of these factors from cell-cycle progression results in a complete premature reconstitution of CENP-A assembly process in the G2 phase prior to mitotic exit. Our results identify a dual inhibitory mechanism that is sufficient to maintain cell-cycle-restricted centromere propagation and define the molecular underpinnings of how assembly is turned on and subsequently turned off.

## RESULTS

### HJURP Is Phosphorylated in a Cell-Cycle-Dependent Manner

HJURP, the CENP-A-specific chaperone, is a phospho-protein and features several putative Cdk sites (Figure 1A; Bailey et al., 2016; Dephoure et al., 2008; Kato et al., 2007; Müller et al., 2014; Wang et al., 2014), making it a prime candidate for cell-cycle control of CENP-A assembly. To quantitatively measure HJURP phosphorylation, we used stable isotope labeling by amino acids in cell culture (SILAC) coupled to mass spectrometry. This allowed us, in an unbiased manner, to precisely determine which residues are phosphorylated under high Cdk conditions and how they respond to changes in Cdk activity. Cdk1 levels differ most dramatically between mitosis and the G1 phase. We therefore compared levels of phospho-peptides on the prenucleosomal GFP-CENP-A/HJURP complex between populations of mitotically arrested cells and cells that were released from mitotic arrest by Roscovitine-mediated Cdk inhibition (Figure 1B). Normal timing and efficiency of CENP-A assembly was preserved under these conditions (Figure S1). We detected six phosphorylated residues corresponding to putative Cdk consensus sites within HJURP, all of which were dephosphorylated upon mitotic exit, ranging from a 25%–70% decrease relative to mitotic values (Figures 1C and S2). Although three of these sites (S412, S448, S473) correspond to reported phospho-sites (Müller et al., 2014; Wang et al., 2014), our analysis shows that these are neither the sole nor the most responsive

sites to inactivation of Cdks, at least in mitosis. In contrast, no change was observed at unphosphorylated peptides of HJURP (Figure 1C) or at Cdk-consensus phospho-sites on the CENP-A N-terminal tail (Bailey et al., 2013) after forced mitotic exit (Figure 1D), indicating that protein levels of CENP-A and HJURP remain unaffected (see also Figures S1F and S1G) and that HJURP is selectively dephosphorylated.

### The HJURP-Conserved Domain Interacts with Cyclin A and Controls the Timing of CENP-A Assembly

Our findings from SILAC experiments led us to focus on HJURP in particular and determine how its phospho-regulation is coupled to the control of cell-cycle timing of CENP-A chromatin assembly. Although the canonical consensus site for Cdks is (S/T)PX(K/R) (Hagopian et al., 2001; Holmes and Solomon, 1996), five of the six phospho-sites in HJURP that are affected by Cdk inactivation display a shorter (S/T)P motif (Figure 1A) (Errico et al., 2010). Phosphorylation of such truncated motifs often requires additional cyclin-binding sites for enhanced substrate recognition (Adams et al., 1996; Russo et al., 1996). Indeed, we found a typical cyclin A-binding RxL motif (Brown et al., 2007) within a vertebrate conserved domain (CD) of HJURP, which had no previously described function (Sanchez-Pulido et al., 2009). We tested whether HJURP interacts with cyclin A and B, the major drivers of Cdk activity in S/G2 phase and mitosis, respectively, all stages at which CENP-A assembly is inhibited (Silva et al., 2012). We performed either cyclin B or cyclin A co-immunoprecipitation from HEK293T cells in which we ectopically expressed either GFP-tagged HJURP with a mutated RxL motif (RLL > ALA, henceforth referred to as HJURP<sup>AXA</sup>), or with a wild-type CD. HJURP forms a homodimer (Zasadzińska et al., 2013). To avoid cross-dimerization with endogenous HJURP, we replaced its C-terminal domain with that of LacI (henceforth named HJURP-ΔCLacI), which does not interfere with the CENP-A chaperoning and assembly activity of HJURP, as described by Zasadzińska et al., 2013. Cyclin A robustly co-immunoprecipitated GFP-tagged HJURP-ΔCLacI (Figures 1E and S3A). In contrast, GFP-HJURP<sup>AXA</sup>-ΔCLacI pull-down was reduced by 70% compared to HJURP-ΔCLacI, carrying a wild-type CD (Figure 1F). Mitotically enriched cells (low cyclin A) were used as a control to demonstrate that HJURP pull-down is cyclin A dependent. Consistent with the fact that inhibition of CENP-A assembly is maintained in mitosis (Jansen et al., 2007), even though cyclin A is degraded in early mitosis (den Elzen and Pines, 2001; Geley

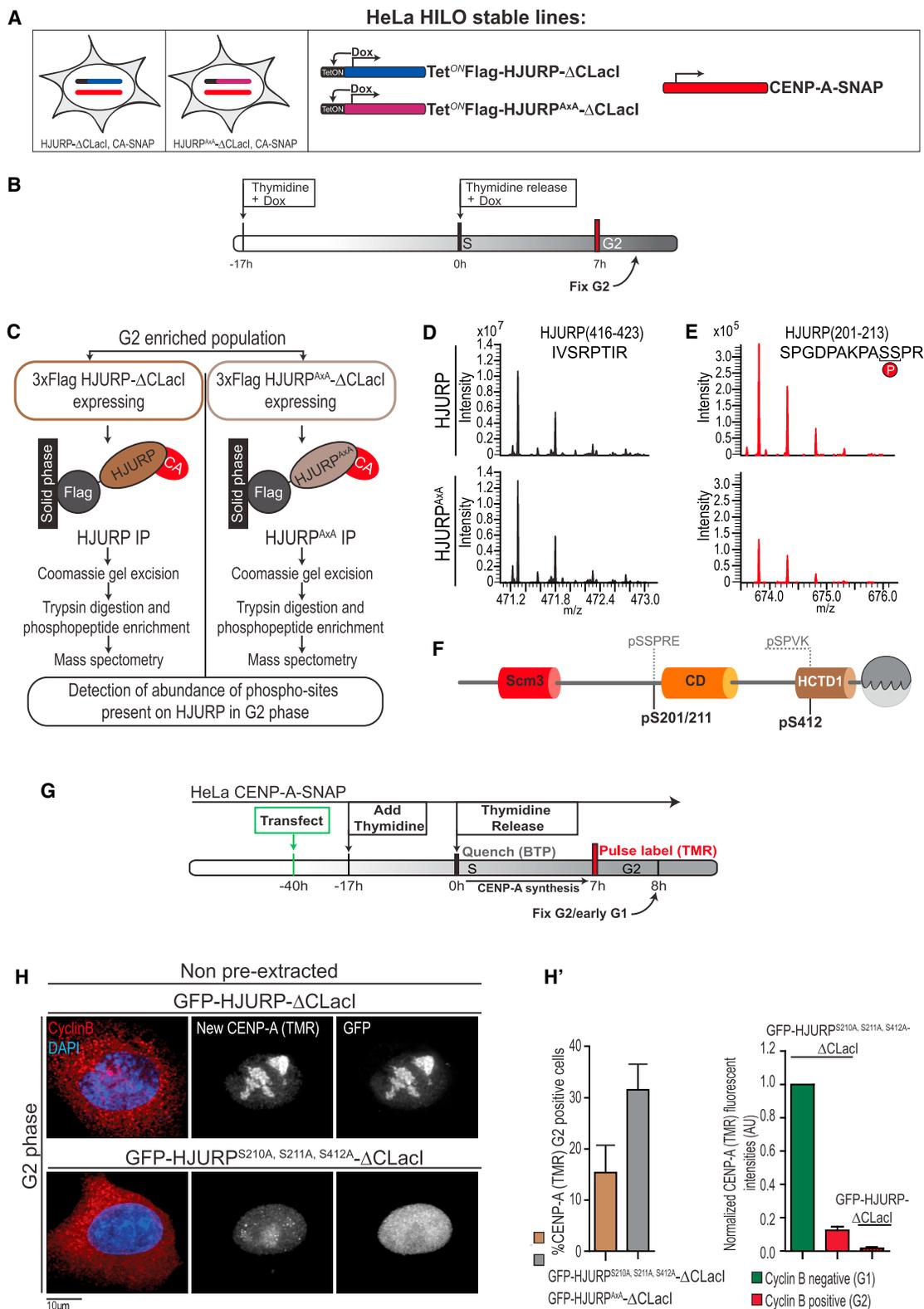
(B) Experiments were performed as in Figure 2A, except here, CENP-A assembly was assayed using SNAP TMR-labeling of its S-phase-synthesized pool. Following fixation, cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA, respectively. (See Figure S4C for extended analysis of GFP-HJURP- and GFP-HJURP<sup>AXA</sup>-induced assembly.)

(B') Left: quantification of frequency of premature CENP-A loading in cyclin B-positive cells expressing GFP-HJURP<sup>AXA</sup>-ΔCLacI. Right: quantification of CENP-A-SNAP (TMR) fluorescent signal intensities of cells from the experiment on the left in the G2 phase (cyclin B positive) and the G1 phase (cyclin B negative) using CENP-T signal as a centromere reference (not depicted). Centromeric CENP-A-SNAP fluorescent signals were normalized to the average of G1 cell signals in each experiment (not considering the difference in replicated sister G2 centromeres versus segregated G1 centromeres). Three replicates, error bars indicate SEM.

(B'') Left: quantification of frequency of premature CENP-A loading in cyclin B-positive cells expressing GFP-HJURP<sup>AXA</sup> from three replicate experiments (see Figure S4C for images). Right: quantification of CENP-A-SNAP (TMR) fluorescent signal intensities from the same experiment.

(C) Top: schematic of relevant domains in centromere-targeted HJURP. Bottom: HeLa CENP-A-SNAP cells were transfected with indicated constructs. 7 hr post thymidine release, cells were either fixed in the G2 phase or collected in nocodazole to enrich for mitotic cells. Cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA, respectively.

(D) Quantification of frequency of premature CENP-A-SNAP deposition in cyclin B-positive cells driven by expression of Cbdb-HJURP-GFP. Error bars indicate SEM. See also Figure S3 and S4.



**Figure 3. HJURP Serine 210/211 Is Functionally Phosphorylated in G2 Phase Cells**

(A) Schematic of cell lines used for a label-free mass spectrometry analysis.

(B) HeLa HILO cells carrying indicated doxycycline-inducible HJURP constructs were enriched in G2 cells by thymidine arrest and release during Dox induction.

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et al., 2001), we found that, like cyclin A, cyclin B can interact with HJURP (Figure S3B). However, this interaction is not dependent on an intact CD within HJURP, indicating inhibitory control in mitosis is exerted through a different mechanism.

Our mapping of the principal cyclin A interaction site on HJURP allowed us to determine the consequences of the loss of this interaction for the timing of its localization along the cell cycle. Upon removal of soluble HJURP by pre-extraction, we revealed that the stably chromatin-bound pre-mitotic HJURP- $\Delta$ CLaCl was enriched in nucleoli (as observed previously by Dunleavy et al., 2009). In contrast, HJURP<sup>AxA</sup>- $\Delta$ CLaCl targeted to centromeres prematurely in the G2 phase, the time of the cell cycle in which cyclin A is the principal cyclin (Figure 2A). In addition, we analyzed CENP-A deposition using a SNAP tag-based fluorescent quench-chase-pulse labeling protocol that we described previously (Figure 2B) (Bodor et al., 2012; Silva et al., 2012). Remarkably, expression of the cyclin A binding mutant of HJURP, but not its wild-type counterpart, resulted in a precocious deposition of nascent CENP-A in the G2 phase. We performed these experiments using HJURP- $\Delta$ CLaCl to force homodimerization of HJURP<sup>AxA</sup>. In this way, we showed that HJURP<sup>AxA</sup> itself is a functional assembly factor, independent of wild-type HJURP copies. Consistent with this, downregulation of endogenous HJURP showed no effect on either the efficiency or the frequency of premature CENP-A loading following GFP-HJURP<sup>AxA</sup>- $\Delta$ CLaCl expression (Figures S4A and S4B). Either GFP-HJURP<sup>AxA</sup>- $\Delta$ CLaCl (Figure 2B) or GFP-HJURP<sup>AxA</sup> (carrying the endogenous C-terminal HJURP dimerization domain) (Figures 2B' and S4C) expression result in a similar level of precocious deposition of CENP-A, demonstrating that uncoupling is not an artifact of Lacl-mediated dimerization. Quantitative analysis showed that precocious CENP-A assembly at the centromere reached ~40% of the G1 levels (Figure 2B; see also Figure S4D) (i.e., 20% assembly per centromere, considering the replicated state of sister centromeres in the G2 phase, unresolvable by microscopy). We conclude that the CD of HJURP is a cell-cycle control element that interacts with cyclin A. Disruption of this site is sufficient to alleviate at least part of the Cdk-mediated inhibition of HJURP.

### Cdk Activity Controls HJURP Localization, Not Its Chaperoning Activity

Phosphorylation of HJURP could directly interfere with its chaperoning activity, thereby inactivating the key function of

the protein. Alternatively, it may sequester an otherwise active HJURP away from the centromere, preventing its untimely recruitment. To distinguish between these possibilities, we fused HJURP to the DNA-binding domain of CENP-B (CBdbd) (Figure 2C). This domain binds specifically to centromeric  $\alpha$ -satellite DNA and allowed us to drive HJURP to centromeres in G2-synchronized cells, while likely bearing inhibitory phosphorylation due to high Cdk activity. We detected nascent CENP-A-SNAP at G2 centromeres after expression of HJURP-CBdbd-GFP (Figures 2C and 2D), but not CBdbd-GFP alone, indicating centromeric localization of HJURP is sufficient to enable unscheduled CENP-A loading. Although HJURP is removed from mitotic chromatin (a process that apparently overrides the DNA binding activity of the CENP-B DNA binding domain), newly loaded CENP-A-SNAP remained associated with centromeres upon entry into mitosis, suggesting it is assembled into centromeric nucleosomes rather than part of an HJURP-associated prenucleosomal complex (Figure 2C, right). Based on these results, we conclude that Cdk-driven phosphorylation does not interfere with HJURP chaperoning activity; rather, it results in sequestering HJURP away from the centromere, preventing its untimely recruitment.

### HJURP Serine 210/211 Is Functionally Phosphorylated in G2 Phase Cells

Next, we determined whether the uncoupling of HJURP from its cell-cycle control involves specific phosphorylation sites. Expression of HJURP in which the six identified putative mitotic Cdk phospho-residues (Figure 1C) were mutated to alanine (either all six or combinations thereof) did not result in changes in the timing of CENP-A assembly (Figure S5B), despite previous reports implicating three of these residues (S412, S448, and S472; Müller et al., 2014). Because we observed premature CENP-A assembly in the G2 phase, during which cyclin A is the major cyclin, we aimed to identify additional potentially relevant phospho-residues in this cell-cycle window. We expressed doxycycline (Dox)-inducible 3 $\times$ Flag-HJURP- $\Delta$ CLaCl or 3 $\times$ Flag-HJURP<sup>AxA</sup>- $\Delta$ CLaCl in the G2 phase-enriched HeLa high-efficiency and low-background (HILO) cells (Khandelia et al., 2011) (Figures 3A and 3B; see also the section below). Following 3 $\times$ Flag-HJURP- $\Delta$ CLaCl immunoprecipitation, titanium dioxide (TiO<sub>2</sub>) phospho-enrichment, and mass spectrometry (Figure 3C), we identified S210/S211 phosphopeptides (the proximity of these residues prevented us from differentiating S210 versus S211 as the site of phosphorylation). These phosphopeptides

(C) Cell pellets obtained from the experiment in (B) were subjected to immunoprecipitation using Flag-coupled agarose beads to isolate 3 $\times$ Flag-HJURP- $\Delta$ CLaCl, separated on SDS-PAGE, and followed by Coomassie-based excision of HJURP proteins. Purified proteins were subjected to trypsin digestion and phosphopeptide enrichment, followed by liquid chromatography-mass spectrometry (LC-MS/MS) analysis.

(D) Mass spectra of a representative non-phosphorylated HJURP peptide from the flow through of the phospho-enrichment and of samples from cells containing WT HJURP- $\Delta$ CLaCl (top) and HJURP<sup>AxA</sup>- $\Delta$ CLaCl (bottom).

(E) Mass spectra of the phosphopeptide containing pS210/pS211 from the elution of the phospho-enrichment from cells expressing indicated constructs. Because the two serines are adjacent, it was not possible to differentiate between S210 and S211 as the site of phosphorylation.

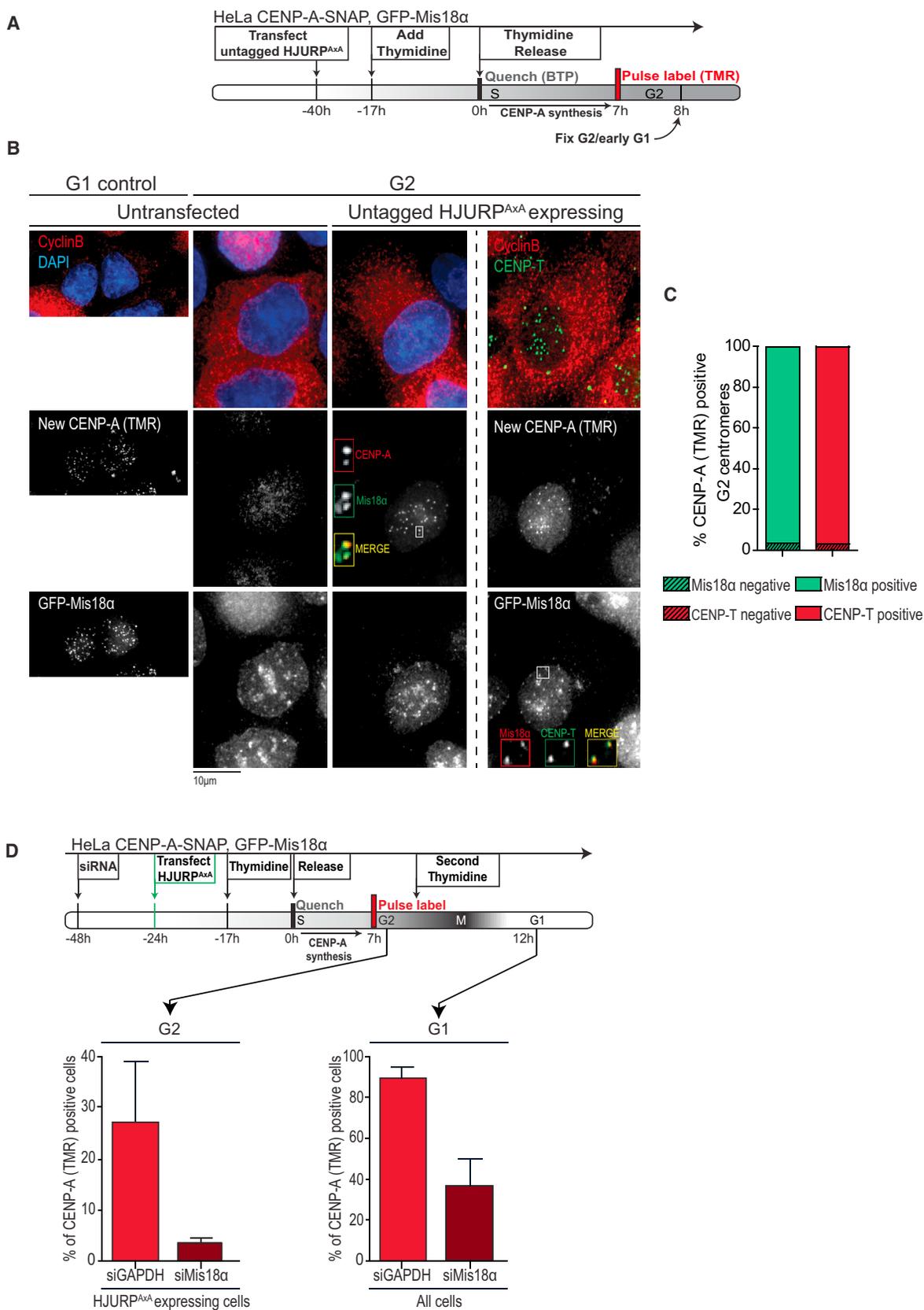
(F) Schematic representation of Cdk-consensus phospho-sites detected on HJURP in the G2 phase.

(G) Experiment analogous to Figure 2A assaying indicated HJURP constructs for localization and CENP-A assembly in the G2 phase.

(H) Representative images of cells from the experiment in (G). 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.

(H') Left: quantification of frequency of premature CENP-A loading in cyclin B-positive cells expressing indicated constructs from three replicate experiments. Right: quantification of CENP-A-SNAP (TMR) fluorescent signal intensities.

See also Figure S5.



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were not detected in mitotically synchronized cells (Figure 1C), suggesting differential phosphorylation of HJURP, consistent with our finding that cyclin B also interacts with HJURP, but in a CD-independent manner (Figure S3B). Further, we found S412 to be the only common phospho-residue between G2 and mitotically synchronized cells (Figure 3F). Importantly, the relative abundance of S210/S211 phospho-peptides was substantially reduced on the HJURP<sup>AxA</sup> mutant in which cyclin A binding was reduced compared to wild-type (Figure 3E). This suggests that the cyclin A/Cdk complex interaction with HJURP results in phosphorylation of this site.

To test the functional significance of these residues, we mutated serines 210 and 211 in combination with serine 412 and expressed HJURP<sup>S210A,S211A,S412A</sup><sub>ΔCLaCl</sub> mutants in the G2 phase cells. Quench-chase-pulse labeling of CENP-A-SNAP showed that mutation of these residues to alanine results in low, but detectable, levels of nascent CENP-A at centromeres (Figures 3G and 3H). This indicates that cyclin A binding to HJURP in the G2 phase results in phosphorylation, at least on serines S210/211 and S412, and that these modifications contribute to preventing premature CENP-A assembly.

#### HJURP<sup>AxA</sup>-Induced CENP-A Assembly in G2 Phase Is Mis18 Dependent

Although HJURP<sup>AxA</sup> is capable of inducing unscheduled CENP-A assembly, it does so with a relatively low efficiency and centromere specificity as compared to canonical G1 loading (Figures 2B' and 2B''). This indicates that an additional level of cell-cycle control exists. A candidate for this is the Mis18 complex, which includes Mis18 $\alpha$ , Mis18 $\beta$ , and the associated protein M18BP1 (Fujita et al., 2007). All subunits share a common localization pattern, with highly enriched and centromere-specific localization in anaphase, followed by disappearance in mid-G1 (Fujita et al., 2007; Silva and Jansen, 2009). Interestingly, we found that premature HJURP<sup>AxA</sup> driven CENP-A assembly in the G2 phase correlates with low levels of stably expressed GFP-Mis18 $\alpha$  at centromeres (Figures 4A–4C). Moreover, small interfering RNA (siRNA)-mediated depletion of Mis18 $\alpha$  leads to a loss of both canonical assembly in the G1 phase as well as premature assembly of CENP-A in the G2 phase (Figure 4D). This demonstrates that HJURP<sup>AxA</sup>-induced assembly occurs through the canonical assembly pathway and suggests that the partial nature of this assembly is possibly due to low levels of Mis18 complex members at G2 centromeres.

#### Recruitment of the Mis18 Complex to the Centromere Is Controlled by Phosphorylation of M18BP1<sup>T653</sup>

Previously, we reported that a phospho-dead M18BP1 mutant, in which 24 known phospho-sites are mutated to alanine, re-

sulted in its premature centromere targeting (Silva et al., 2012), suggesting that at least 1 of these sites is regulated by Cdk. We have now identified four putative Cdk motifs that are highly conserved among vertebrates, three of which are clustered close to the N terminus of M18BP1 (T4, T40, and S110), while a fourth (T653) is located between the highly conserved SANTA and SANT domains (Maddox et al., 2007) (Figure 5A). Mutation of all four sites to alanine leads to a loss of cell-cycle controlled localization of M18BP1 (Figure S6A). Interestingly, mutation of T653 alone was sufficient to result in premature centromere targeting of M18BP1, with an  $\sim$ 3-fold increase in centromeric levels relative to wild-type protein (Figure 5B). We generated a phospho- and site-specific antibody against the T653 site and show that pT653 levels rise as cells accumulate in S/G2 and mitosis, correlating with increasing levels of Cdk1 and -2 activities (Figure 5C). A brief treatment with the Cdk1/2 inhibitor of cells expressing GFP-M18BP1 caused a strong reduction in phosphorylation of T653, suggesting that M18BP1 is a direct target of these kinases (Figure 5D).

Further, the M18BP1<sup>T653A</sup> mutant co-recruited Mis18 $\alpha$  to G2 centromeres, indicative of ongoing Mis18 complex formation independent of T653 phosphorylation (Figure S6B). An N-terminal 490-amino acid fragment of M18BP1 was reported to be functional in supporting CENP-A assembly in the G1 phase (McKinley and Cheeseman, 2014), consistent with our finding that mutation of the T653 residue does not abrogate M18BP1 localization, but we now add that this residue controls cell-cycle dependent localization. To test whether M18BP1 phosphorylation of T653 results in disruption of the Mis18 $\alpha$  interaction, we expressed a translational fusion of wild-type or mutant M18BP1 to the CBdbd in cells synchronized in the G2 phase (analogous to artificial HJURP tethering; Figure 2C). Forced recruitment of M18BP1 to centromeres leads to strong co-recruitment of Mis18 $\alpha$  to G2 centromeres, suggesting that the Mis18 complex can form under inhibitory Cdk activity, at least at this stage in the cell cycle (Figure S6C), but not in mitosis, as observed previously (McKinley and Cheeseman, 2014). Similarly, forced recruitment of a phosphomimetic M18BP1<sup>T653D</sup> (Figure S6C) or M18BP1<sup>T653E</sup> mutant (data not shown) is capable of co-recruitment of Mis18 $\alpha$ . Thus, we find that mutation of the T653 residue does not disrupt the M18BP1/Mis18 $\alpha$  interaction. Rather, its phosphorylation prevents centromere targeting of the Mis18 complex in the G2 phase until mitotic exit, when Cdk1/2 activities are low.

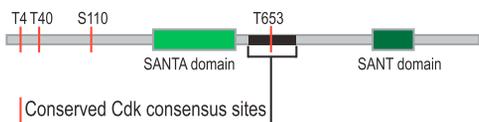
#### Cdk-Mediated Control of M18BP1 and HJURP Is Sufficient to Ensure Tight Cell-Cycle Timing of Centromere Propagation

Our results indicate that centromere localization of both HJURP and M18BP1 is blocked by Cdk-mediated phosphorylation,

#### Figure 4. HJURP<sup>AxA</sup>-Induced CENP-A Assembly Is Mis18 $\alpha$ Dependent

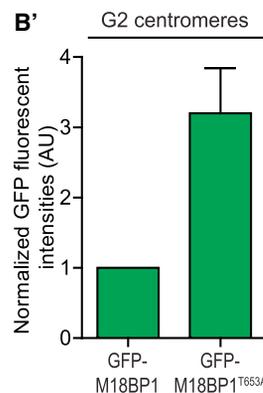
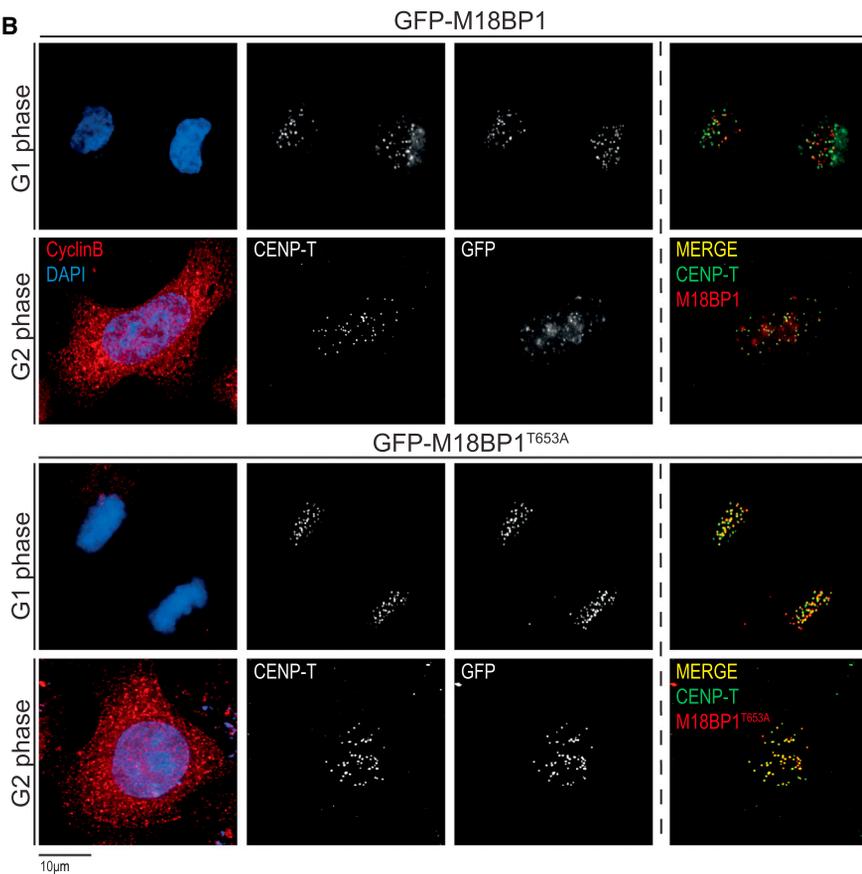
(A) Stable GFP-Mis18 $\alpha$ , CENP-A-SNAP double transgenic HeLa cells were transfected with untagged HJURP<sup>AxA</sup>, synchronized, and assayed for nascent CENP-A assembly by SNAP quench-chase-pulse labeling, followed by immunostaining for cyclin B and DAPI to indicate G2 status and DNA, respectively. (B) Representative images of experiment described in (A). (C) Quantification of frequency of CENP-A (TMR)-positive G2 centromeres of the experiment described in (A). Cells were scored in relation to whether GFP-M18 $\alpha$  (green) or CENP-T (red) signals were simultaneously detected together with CENP-A (TMR) or not. (D) Top: scheme outlining RNAi against Mis18 $\alpha$  or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), synchronization, and quench-chase-pulse labeling of CENP-A-SNAP, GFP-Mis18 $\alpha$  cells. Bottom: quantification of CENP-A-SNAP (TMR)-positive cells from three independent experiments. Error bars indicate SEM.

**A** M18BP1

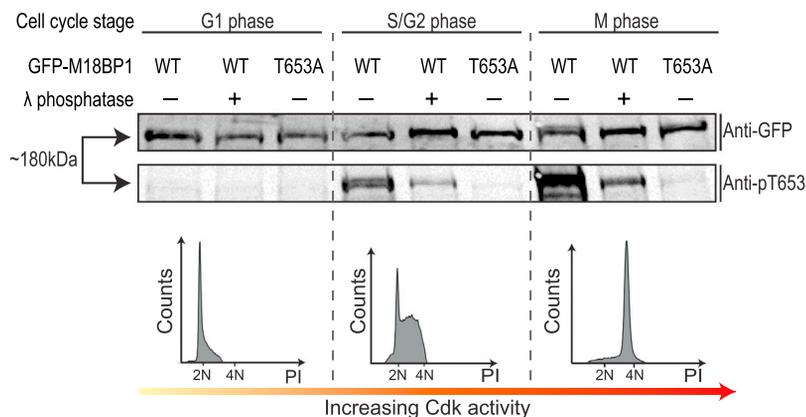


Human	633	FSDEERK	YMAINQ	KKAYILV	TP	PLKSRK	VIEQRC	MRYNLS	SAG	673
Mouse	496	TSGKERR	HPLLSQ	KRAYVLI	TP	LRNKKL	IEQRCI	DYSLS--	534	
Rat	499	TSNKER-	HPPLGQ	KEAYVLM	TP	LRTRTK	LIEQRC	MEHSLS--	536	
Chimpanzee	633	FSDEERK	YMAINQ	KKAYILV	TP	PLKSRK	VIEQRC	MRYNLS	SAG	673
Wolf	639	FSDEERK	CMTLNQ	KEVCVLV	TP	LKSKKI	IEQKCM	QYDLSD	679	
Bovine	543	FSDEERK	YMTVSQ	KKPCILV	TP	LKSKKI	IEQKCM	DYNLSSD	583	
Tarsius	647	ILSPKKE	QMVASD	CKKNTRL	TP	PKLKKI	ENQVAM	SFHKHQS	687	
Opossum	650	SDTEKME	CINNIEK	KLAVLV	TP	MNSRKT	LEQKCK	EHNLIS	719	
Zebrafish	501	STPKKP	ATSQIAE	EKSFRPK	TP	IERGRC	ITSSSE	DELSVPR	541	
Sole	650	RKASQT	KLSDVQ	MSTTQPV	TP	PAETNG	STGNSF	VTVSTRSK	690	
Latimeria	535	STEYWE	EKNQG	CKKTLVLI	TP	PMSTYE	KMKDR	CKYNTLFS	572	

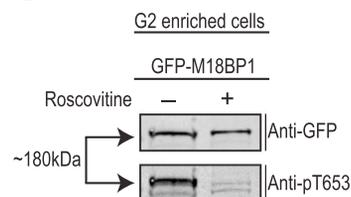
**B**



**C**



**D**



(legend on next page)

suggesting that combined phospho-control of these protein complexes contributes to cell-cycle-specific loading of CENP-A. To directly test this, we constructed HeLa HILO cells expressing equal levels of either HJURP- $\Delta$ CLaCl or HJURP<sup>AxA</sup>- $\Delta$ CLaCl (Figure S7A) under the control of a doxycycline-inducible promoter at a defined locus using recombination-mediated cassette exchange (RMCE) (Khandelvia et al., 2011). HJURP induction was performed in cells either stably expressing GFP-tagged M18BP1<sup>T653A</sup> or expressing endogenous M18BP1 along with CENP-A-SNAP to assay for CENP-A assembly (Figure 6A). We compared the efficiency of G2 phase loading to the normal level of assembly in the G1 phase. CENP-A assembly in uninduced control G1 cells was equal across all cell lines and essentially completed at the time of fixation (Figures S7B and S7C, respectively). As observed after transient expression, induction of HJURP<sup>AxA</sup> alone resulted in low levels (~20% of G1, when corrected for centromere replication in G2 phase) of CENP-A assembly (Figures 6B, 6C, and S7D). Forced expression of otherwise wild-type but GFP-tagged M18BP1 does not enhance the degree of premature CENP-A assembly (Figures S7E and 7E'). Conversely, constitutive M18BP1<sup>T653A</sup> expression led to infrequent and inefficient recruitment of nascent CENP-A to G2 centromeres (Figure 6C). Remarkably, induction of HJURP<sup>AxA</sup> combined with stably expressed M18BP1<sup>T653A</sup> resulted in highly efficient and centromere-restricted CENP-A assembly in the G2 phase, reaching 93% of G1 control levels (Figure 6C). In sum, disrupting the timing of centromere targeting of either HJURP or M18BP1 results in a limited deregulation of CENP-A assembly, as has been shown previously (McKinley and Cheeseman, 2014; Müller et al., 2014). We now show that simultaneous uncoupling of both of these proteins leads to full-fledged CENP-A assembly, indistinguishable from canonical G1 phase assembly. These findings strongly suggest that M18BP1 and HJURP are the two principal targets of Cdk-mediated inhibition.

### Efficient CENP-A Assembly Requires Displacement of M18BP1 from the Centromere

During the course of these experiments, we observed that induction of CENP-A assembly in the G2 phase resulted in concomitant loss of centromeric GFP-M18BP1<sup>T653A</sup> levels to <30%, on average, relative to the uninduced control (Figures 6C and 6D). Expression of HJURP<sup>AxA</sup>- $\Delta$ CLaCl, but not wild-type HJURP, re-

sults in GFP-M18BP1<sup>T653A</sup> loss, showing that displacement is directly dependent on CENP-A assembly. This suggests that M18BP1 removal is an active CENP-A loading-dependent process and not a passive consequence of cell-cycle progression. To test this directly in G1 cells, we either overexpressed wild-type M18BP1 or artificially tethered it to G1 centromeres (using the CBdbd tether) while measuring nascent CENP-A chromatin assembly (Figures 7A and 7B). We observed an ~40% reduction in nascent CENP-A fluorescent intensities in both of these conditions (Figure 7C). We conclude that, while M18BP1 is an essential positive regulator of CENP-A assembly, preventing its turnover by overexpression or by rendering it unable to be removed from G1 centromeres results in defects in CENP-A assembly.

### DISCUSSION

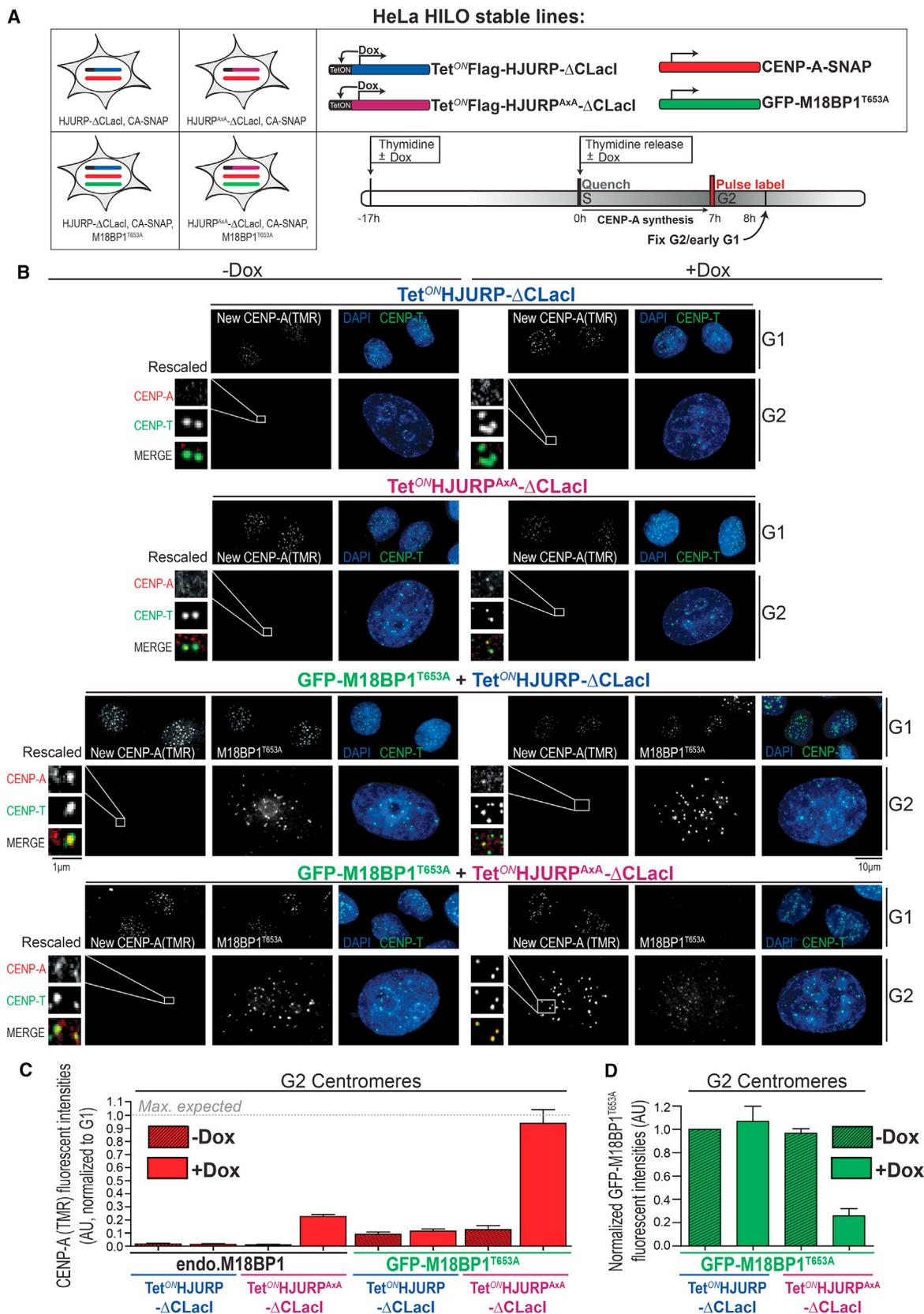
We have identified the licensing factor M18BP1 and the CENP-A chaperone HJURP as the two key targets of Cdk-based inhibition sufficient for maintenance of strict cell-cycle control of CENP-A assembly (Figure 7D). However, we do not exclude that additional levels of regulation exists (e.g., in chromatin maturation steps or in mitotic inhibition, which we find to be controlled in a distinct manner from the G2 phase).

Inhibition of CENP-A assembly prior to mitosis at the level of HJURP or M18BP1 alone is incomplete. This is in agreement with previous studies that showed that mutation of HJURP phospho-sites within the HJURP C-terminal domain 1 (HCTD1) (Müller et al., 2014) or forced recruitment of Mis18 $\alpha$  resulted in precocious CENP-A assembly (McKinley and Cheeseman, 2014). We note that, in our system, mutation of the HCTD1 phosphosites did not result in precocious CENP-A assembly (Figure S5B). This discrepancy is likely the result of expression level differences between the cell types used in each study (Figure S5C and Bodor et al., 2014).

We provide evidence that the primary mechanism of Cdk-mediated inhibition is to prevent otherwise active factors from reaching the centromere (Figures 2C, S6B, S6C and S7D). We propose that phosphorylation blocks the ability of M18BP1 and HJURP to bind to a partner(s) already docked at the centromere. M18BP1 interacts with CENP-C, which is a constitutive core component of the centromere (Dambacher et al., 2012; Hori

### Figure 5. Cdk-Mediated T653 Phosphorylation of M18BP1 Controls Its Centromere Recruitment

(A) M18BP1 T653 is conserved among vertebrates. Left: schematic of M18BP1 protein. Relevant domains and conserved Cdk sites are indicated. Right: conservation of the human T653 residue across species. Conserved threonine or serine is highlighted in gray.  
 (B) T653 residue controls cell-cycle-dependent M18BP1 centromere recruitment. Indicated constructs were transfected into asynchronous HeLa cells 48 hr prior to fixation, followed by counterstaining for cyclin B, CENP-T, and DAPI to indicate G2 status, centromeres, and DNA, respectively.  
 (B') Average centromeric GFP fluorescent signals from cyclin B-positive cells were determined using the centromere recognition and quantification (CRaQ) method (Bodor et al., 2012) and normalized to GFP-M18BP1. Error bars indicate SEM from three replicates.  
 (C) T653 is phosphorylated in a cell-cycle-dependent manner. HEK293T cells were transiently transfected with GFP-Mis18BP1 (wild-type [WT]) or GFP-Mis18BP1<sup>T653A</sup> as a non-phosphorylatable control. 24 hr later, cells were synchronized in indicated cell-cycle stages and lysed. Extracts were either left untreated or treated with lambda phosphatase, separated by SDS-PAGE, and followed by immunoblotting with indicated antibodies (see also Supplemental Experimental Procedures). Apparent molecular weight is indicated. Cells were assayed for cell-cycle position by fluorescence-activated cell sorting (FACS) using propidium iodide (PI) to indicate DNA content.  
 (D) T653 is phosphorylated by Cdk1/2. HEK293T cells were transiently transfected with GFP-Mis18BP1 and enriched in the G2 phase by a single thymidine block, followed by 7 hr of release. 30 min before fixation, cells were treated with 100  $\mu$ M Roscovitine. Extracts were separated by SDS-PAGE followed by immunoblotting with anti-GFP and anti-pT653 antibodies.  
 See also Figure S6.



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et al., 2013; Moree et al., 2011; Nardi et al., 2016; Stellfox et al., 2016). In turn, the prenucleosomal HJURP/CENP-A complex binds to the Mis18 complex (Nardi et al., 2016; Wang et al., 2014). Our proposal is consistent with a recent report describing an interaction between HJURP and the Mis18 complex subunit Mis18 $\beta$ , which is reduced upon Cdk phosphorylation in vitro (Wang et al., 2014).

Recent studies have reported cell-cycle-regulated phosphorylation of CENP-A itself (Yu et al., 2015) or Plk1-mediated modification of M18BP1 (McKinley and Cheeseman, 2014). Although the latter is required for Mis18 complex localization upon mitotic exit, none of these modifications directly dictates the G1-restricted CENP-A assembly. Therefore, while key positive regulatory events also involve phospho-regulation (which may include some of the novel phosphorylation sites that we identified on HJURP), we defined the specific targets and mechanisms of the inhibitory control that is responsible for limiting CENP-A assembly to the G1 phase. Rather than relying on a single tightly regulated factor, the combinatorial action of two layers of control synergizes to efficiently restrict CENP-A assembly to the early G1 phase.

The designation of the Mis18 complex as a priming (licensing) factor was originally inspired by its temporal centromere localization that initiates in anaphase, before the onset of CENP-A assembly (Fujita et al., 2007). This is analogous to the licensing of DNA replication by the assembly of the pre-replication complex (pre-RC) in the early G1 phase (Nishitani and Lygerou, 2002), the S-phase removal of which ensures a single round of genome duplication per cell cycle (Blow and Dutta, 2005; Blow and Hodgson, 2002). Analogously, we find that removal of M18BP1 from the centromere is directly coupled to the onset of CENP-A deposition, at least under induced conditions in the G2 phase, providing a causal link between efficient CENP-A assembly and M18BP1 displacement from the centromere.

These results reveal novel parallels between DNA replication and CENP-A-chromatin, manifested in consumption of the licensing factor, which is directly instigated by the start of duplication of the heritable mark. These findings are consistent with a recent study showing that nascent CENP-A/HJURP binding to the Mis18 complex in vitro leads to the disassembly of this complex (Nardi et al., 2016), suggesting that Mis18 complex disassembly could be a mechanism to turn off CENP-A chromatin assembly. We show that CENP-A assembly not only results in Mis18 complex removal (as shown by Nardi et al., 2016), but that this is a requirement for efficient loading of CENP-A. Two

possible implications follow from these observations. First, while M18BP1 is required for recruitment of nascent CENP-A to centromeres, its presence may physically block completion of the assembly process. By direct binding to CENP-C (Dambacher et al., 2012; Moree et al., 2011; Shono et al., 2015; Westhorpe et al., 2015), which in turn interacts with CENP-A (Falk et al., 2015; Guse et al., 2011; Kato et al., 2013; Logsdon et al., 2015), it is possible that M18BP1 physically marks the site of incorporation for nascent CENP-A. The inability to remove M18BP1 would therefore provoke steric inhibition, resulting in low rates of CENP-A incorporation. Second, given the key role in initiation of CENP-A loading, removal of M18BP1 from centromeres provides an “OFF” switch for the process of assembly, thereby contributing to a tight cell-cycle window, ensuring a single round of CENP-A incorporation per cell cycle.

## EXPERIMENTAL PROCEDURES

### Cell Synchronization

Double thymidine-based synchronization was performed as described (Bodor et al., 2012). For mitotic synchronization, 2.4  $\mu$ M of EG5 inhibitor III Dimethylnastron-DMEIII (Calbiochem) was used for 24 hr. For synchronous mitotic exit, following DMEIII washout, HeLa and HEK293T cells were released for 5 hr and 7 hr, respectively. For Figure 2C, nocodazole was used at 100 ng/mL.

### Co-immunoprecipitation

HEK293T cells were transiently transfected with GFP-tagged constructs. 24 hr post-transfection, cells were either allowed to continue to cycle or were treated overnight in DME III to induce mitotic arrest. Cells were harvested 48 hr post-transfection and subjected to cyclin A or B immunoprecipitation (see also Supplemental Experimental Procedures). Isolated complexes were separated by SDS-PAGE, followed by immunoblotting with anti-cyclin A (Figure 1E) or anti-cyclin B (both Santa Cruz) (Figure S3B) and anti-GFP (Chromotek) antibodies, detected on an Odyssey near-infrared scanner, and quantified using the Odyssey software (see also Bodor et al., 2014).

### SILAC and Affinity Purification of Prenucleosomal HJURP/CENP-A/H4 Complex

SILAC-labeling medium was supplemented with normal lysine and arginine (Sigma-Aldrich) for “light” medium, and 50 mg/L  $^{13}\text{C}_6$ ,  $^{15}\text{N}_2$ -lysine and 50 mg/L  $^{13}\text{C}_6$ ,  $^{15}\text{N}_2$ -arginine (Silantes) for “heavy” medium (see also Supplemental Experimental Procedures). Two parallel cultures of previously characterized HeLaS3 cells stably expressing (LAP)-tagged CENP-A (Bailey et al., 2013) were grown in either heavy or light medium until reaching ~98% labeling efficiency. To enrich for mitotic cells, both cultures were treated with 50  $\mu$ M S-trityl-L-cysteine for 17 hr. Subsequently, the “light” cells were treated with 100  $\mu$ M R-Roscovitine (AdipoGen) for 30 min while the “heavy” cells

## Figure 6. A Dual Inhibitory Mechanism Restricts CENP-A Deposition to G1 Phase

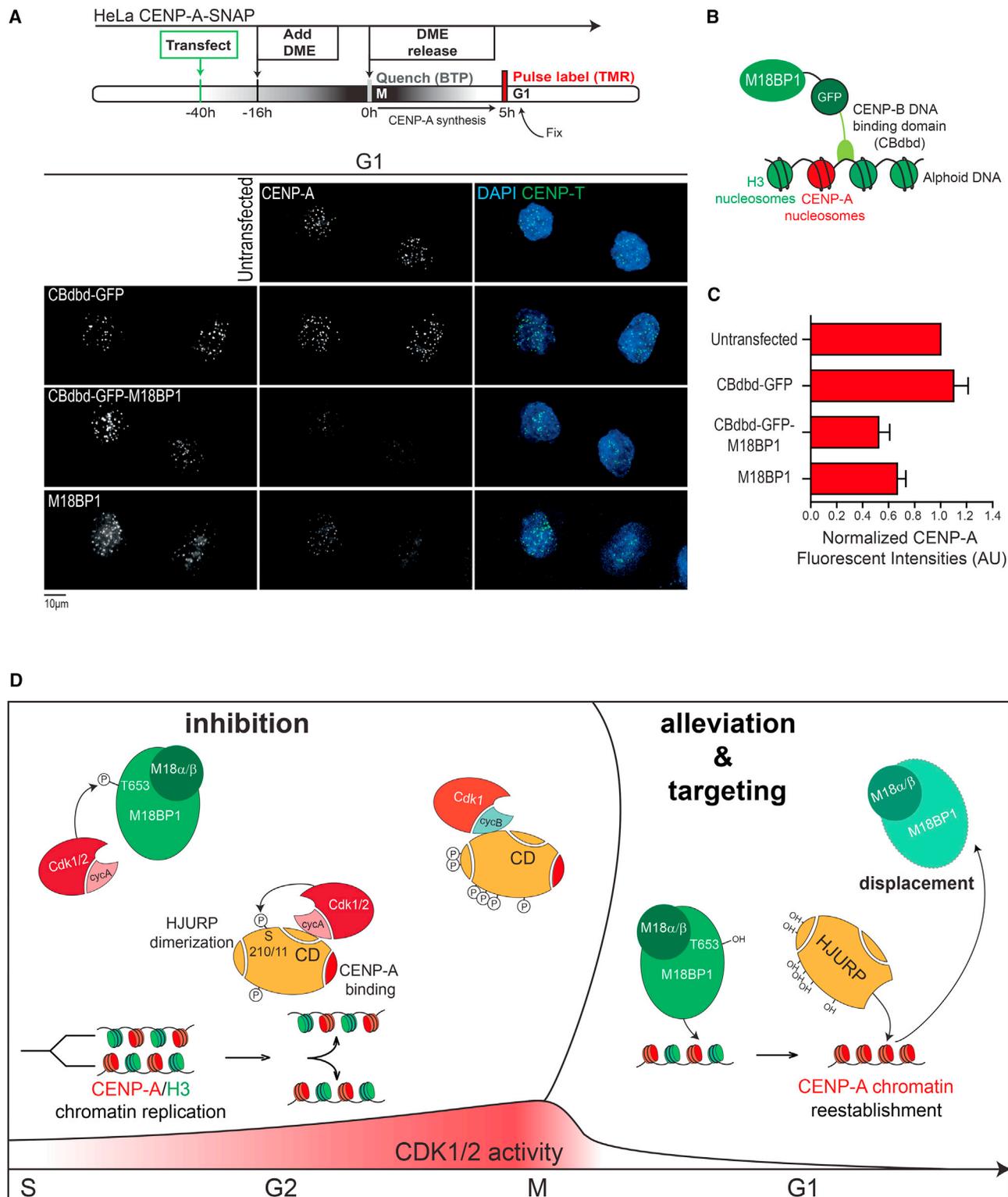
(A) Schematic representation of HeLa HILo cells carrying low levels of constitutively expressed CENP-A-SNAP (red) with or without stable expression of GFP M18BP1<sup>T653A</sup> (green), along with doxycycline-inducible 3 $\times$ Flag-HJURP- $\Delta$ CLaCl (blue) or 3 $\times$ Flag-HJURP<sup>AxA</sup>- $\Delta$ CLaCl (purple). Cells were processed as indicated in the scheme.

(B) Representative images of the experiment described above. Following fixation, cells were counterstained for CENP-T and DAPI to indicate centromeres and DNA, respectively. Cell-cycle status was determined by measuring total DAPI area (see Supplemental Experimental Procedures).

(C) Quantification of CENP-A-SNAP fluorescent signals from (B). Average CENP-A-SNAP signals from G2 centromeres were normalized to respective G1 centromeres and corrected for centromere number (assuming signal intensity per focus represents one and two centromeres in G1 and G2, respectively). Error bars indicate SEM of four independent experiments.

(D) CENP-A assembly drives M18BP1 displacement from centromeres. Quantification of centromeric GFP-M18BP1<sup>T653A</sup> fluorescent signals from (B) using CRaQ method. Average GFP-M18BP1<sup>T653A</sup> signals were normalized to uninduced 3 $\times$ Flag-HJURP- $\Delta$ CLaCl-expressing cells. Error bars indicate SEM of four independent experiments.

See also Figure S7.



**Figure 7. M18BP1 Removal from G1 Centromeres Is Necessary for Efficient Canonical CENP-A Assembly**

(A) HeLa CENP-A-SNAP cells were transfected with indicated constructs and synchronized in mitosis by an overnight treatment with Eg5 inhibitor (DMEIII). Newly synthesized CENP-A pool was quenched in mitosis, followed by 5 hr of release in early G1 when nascent CENP-A-SNAP was labeled with TMR (G1-specific pool).

(legend continued on next page)

were mock-treated with DMSO. Affinity purification of the prenucleosomal HJURP/CENP-A/H4 complexes from 1:1 mixed “light” and “heavy” cells 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, washed, and dried. Following reconstitution, proteins were cleaved with trypsin and phosphopeptides and enriched by TiO<sub>2</sub> prior to mass spectrometry analysis (see Supplemental Experimental Procedures for details). Extracted-ion chromatograms (XICs) of each light and heavy peptide pair were used for quantification. The light/heavy (L/H) ratio represents the ratio of total area under each elution peak.

### Affinity Purification of 3XFlag-HJURP<sup>wt/AxA</sup>-ΔCLaCl and Mass Spectrometry

HeLa HILO RMCE cell lines carrying either 3×Flag-HJURP-ΔCLaCl or 3×Flag-HJURP<sup>AxA</sup>-ΔCLaCl were enriched in the G2 phase as described (see Cell Synchronization) and induced with 10 μg/mL of doxycycline (Sigma) for 24 hr. HJURP was affinity purified using anti-Flag M2 mouse agarose beads (Sigma-Aldrich) as described (see Co-immunoprecipitation), followed by SDS-PAGE separation of bound complexes, staining (by Instant Blue, Expedon), and subsequent HJURP band excision in-gel trypsin digestion and phosphopeptide enrichment by TiO<sub>2</sub>. Samples were run on a Q Exactive mass spectrometer coupled with Easy nLC 1000 HPLC. MaxQuant was used to search the human protein database, identify peptide sequences, and extract their ion chromatograms.

### SNAP Quench-Chase-Pulse Labeling

Cell lines expressing CENP-A-SNAP were pulse labeled as previously described (Bodor et al., 2012), with the exception of HeLa HILO-derived cell lines, where bromothynylpteridine (BTP; New England Biolabs) concentration was adjusted to 0.5 μM.

### Immunofluorescence and Pre-extraction Procedure

Procedures are essentially as described (Bodor et al., 2012) (see also Supplemental Experimental Procedures). To detect GFP-HJURP<sup>AxA</sup>-ΔCLaCl on G2 centromeres, HeLa CENP-A-SNAP cells transiently expressing the construct were pre-extracted for 5 min prior to fixation. Cells were counterstained using anti-CENP-T (Barnhart et al., 2011) and anti-Aurora B (1:100; BD Transduction Laboratories). GFP-HJURP<sup>AxA</sup>-ΔCLaCl signal was amplified using GFP-Booster Atto488 (Chromotek).

### Microscopy

Imaging was performed using a DeltaVision Core system (Applied Precision) inverted microscope (Olympus, IX-71) coupled to a Cascade2 EMCCD camera (Photometrics). Images (1024 × 1024) were acquired at 1× binning using a 100× oil objective (NA 1.40, UPlanSApo) with 0.2 μm z sections.

### ACCESSION NUMBERS

Unprocessed image data used to prepare the figures in this manuscript have been deposited in Mendeley Data and are available at <http://dx.doi.org/10.17632/8833hjzmnf.1>.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2016.11.021>.

### AUTHOR CONTRIBUTIONS

A.S. performed the experiments. L.Y.G. performed CENP-A/HJURP co-purifications for SILAC experiments and helped write the manuscript. J.F.M. generated constructs and performed experiments in Figure 4. D.L.B. generated constructs for experiments in Figures 6 and S7. A.O.B., J.S., and D.F.H. performed the initial mass spectrometry analysis of HJURP phosphorylation sites. L.Y.G., X.-J.C., B.E.B., and B.A.G. performed mass spectrometry experiments and analysis. L.E.T.J. and B.E.B. directed the research. B.E.B. helped write the manuscript. A.S. and L.E.T.J. conceived of the study and wrote the manuscript.

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(B) Schematic of relevant domains in centromere-targeted M18BP1.

(C) GFP-positive cells were selected and CENP-A TMR fluorescent intensities were determined using CRaQ, with the exception of the untransfected control, where all cells were analyzed.

(D) Model summarizing the key molecular steps that are sufficient to restrict CENP-A assembly to the G1 phase. CD: HJURP vertebrate-conserved domain.

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**Supplemental Information**

**A Dual Inhibitory Mechanism Sufficient to Maintain  
Cell-Cycle-Restricted CENP-A Assembly**

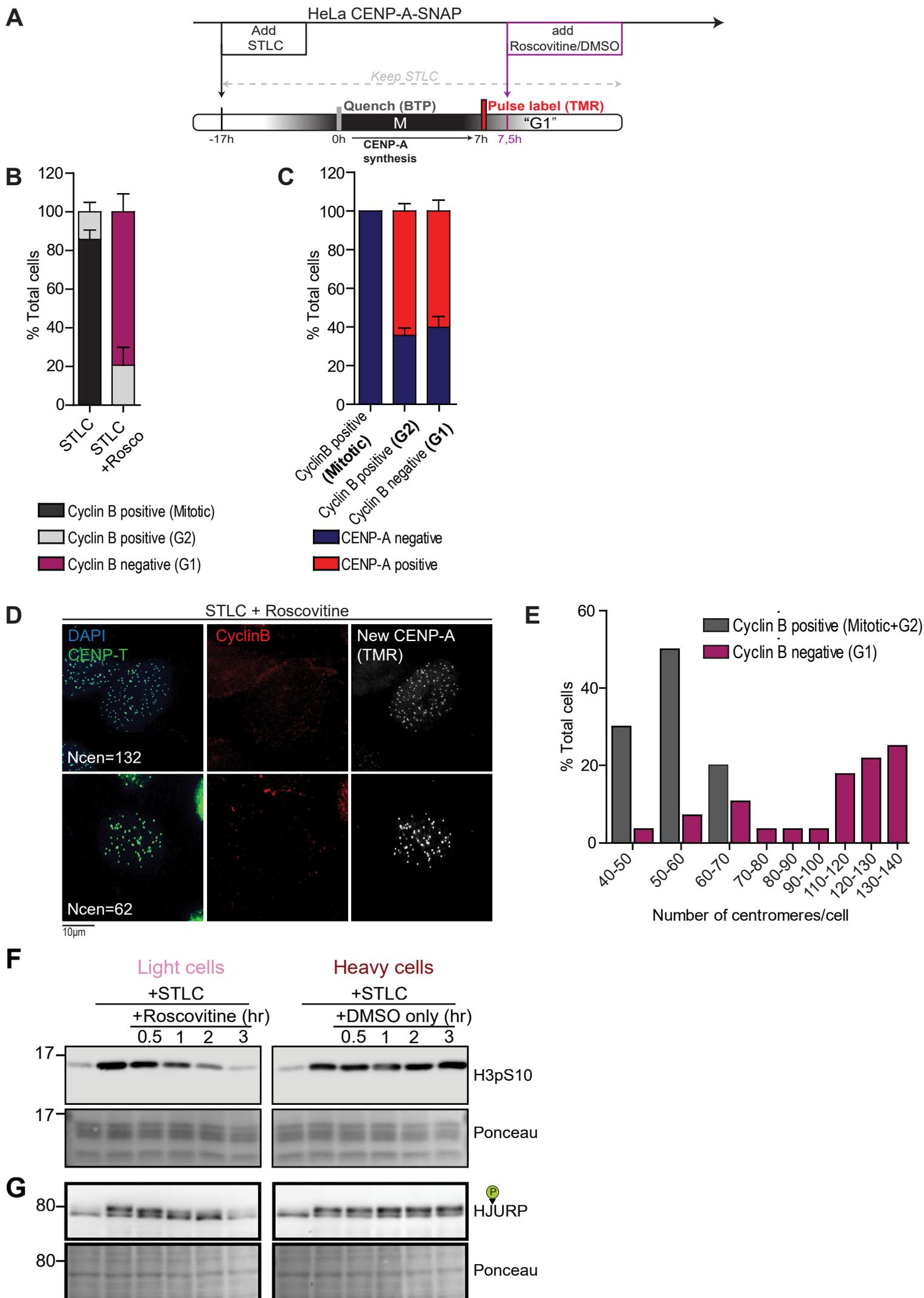
**Ana Stankovic, Lucie Y. Guo, João F. Mata, Dani L. Bodor, Xing-Jun Cao, Aaron O. Bailey, Jeffrey Shabanowitz, Donald F. Hunt, Benjamin A. Garcia, Ben E. Black, and Lars E.T. Jansen**

## **Supplemental Information**

7 supplemental figures with corresponding legends

Supplemental Experimental Procedures

Supplemental References



**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.

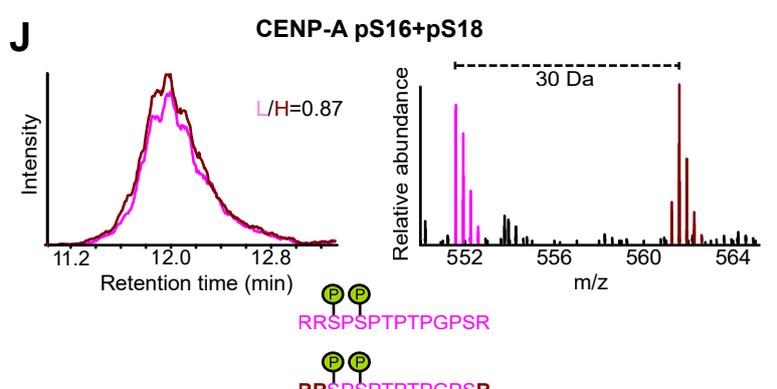
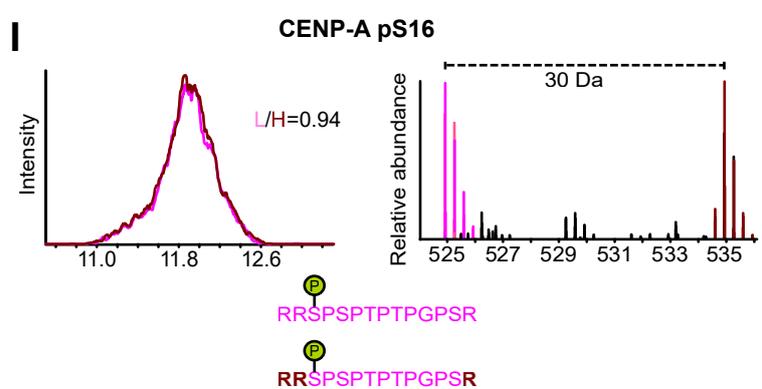
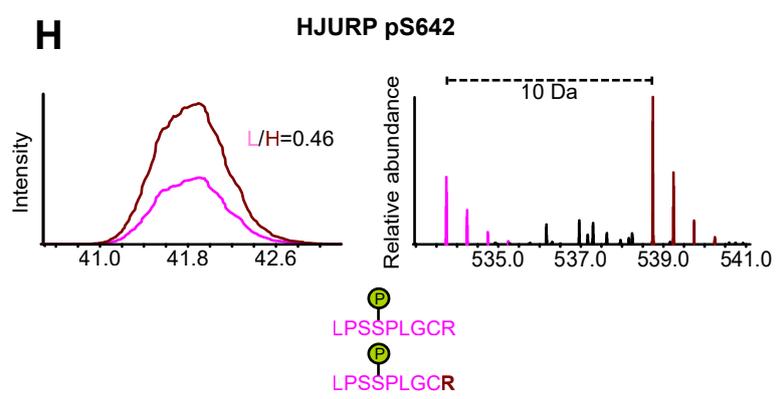
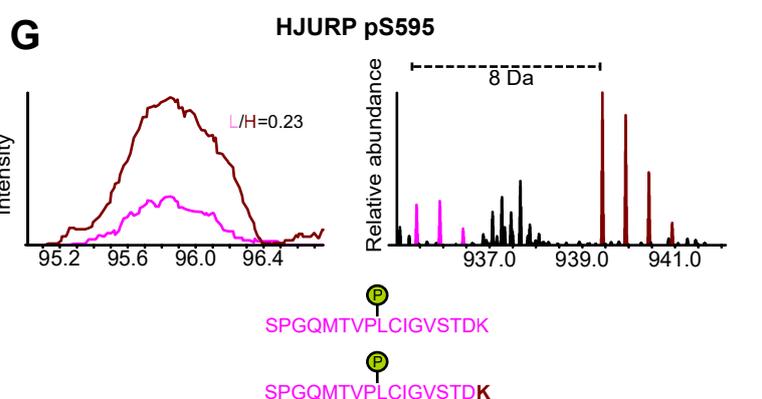
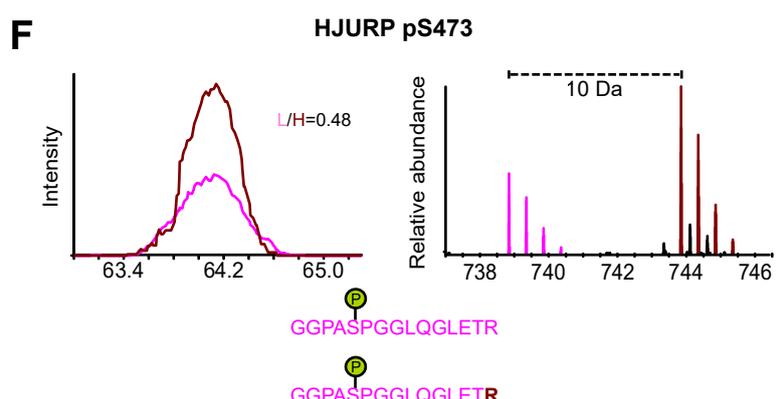
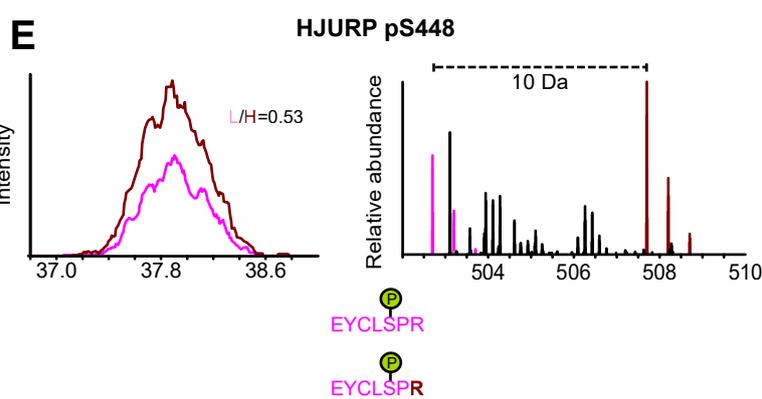
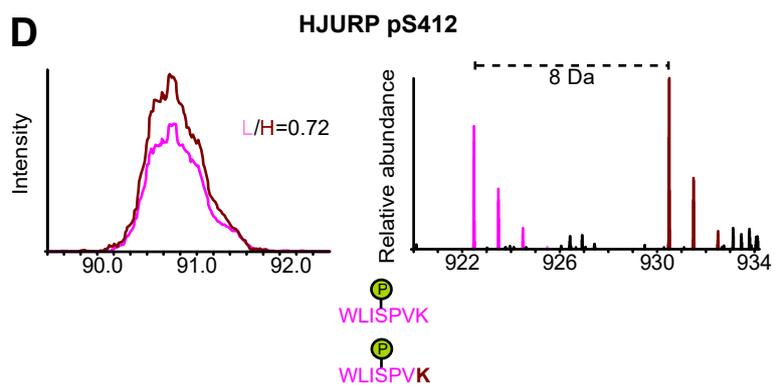
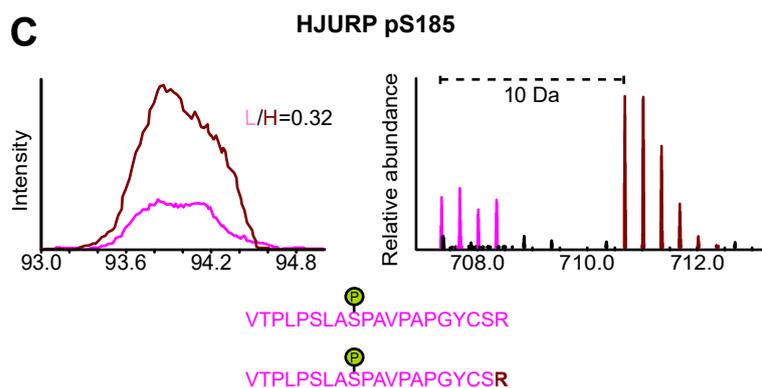
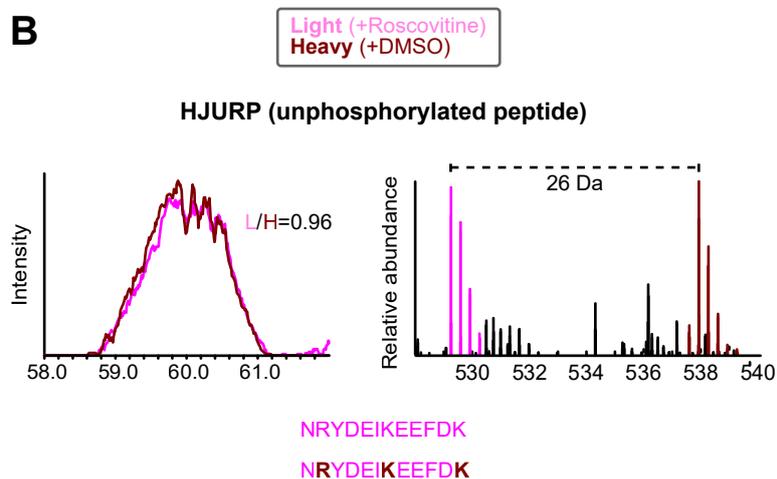
(F) Western blots for the mitotic marker H3pS10 indicating cell cycle position of HeLa S3 cells used in SILAC experiment in Figure 1. Cells were arrested in mitosis with the Eg5 inhibitor STLC followed by treatment with DMSO control or Roscovitine (light cells) to force mitotic exit caused by Cdk inhibition.

(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.

**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 <b>p</b> SPAVPAPGYCSR	0.32	nd	0.30
HJURP pS412	WLI <b>p</b> SPVK	0.72	nd	0.62
HJURP pS448	EYCL <b>p</b> SPR	0.53	nd	nd
HJURP pS473	GGPA <b>p</b> SPGGLQGLETR	0.48	0.37	0.42
HJURP pS595	<b>p</b> SPGQMTVPLCIGVSTDK	0.23	nd	0.25
	YCLK <b>p</b> SPGQMTVPLCIGVSTDK	0.28	nd	0.30
HJURP pS642	LPS <b>p</b> SPLGCR	0.46	0.48	0.39
	<b>p</b> SPpSPPTTPGPSR	nd	0.93	nd
	R <b>p</b> SPpSPPTTPGPSR	nd	1.01	nd
CENP-A pS16+pS18	RR <b>p</b> SPpSPPTTPGPSR	0.87	0.98	nd
	RR <b>p</b> SPPTTPGPSR	0.94	1.13	nd

nd = no data



**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).

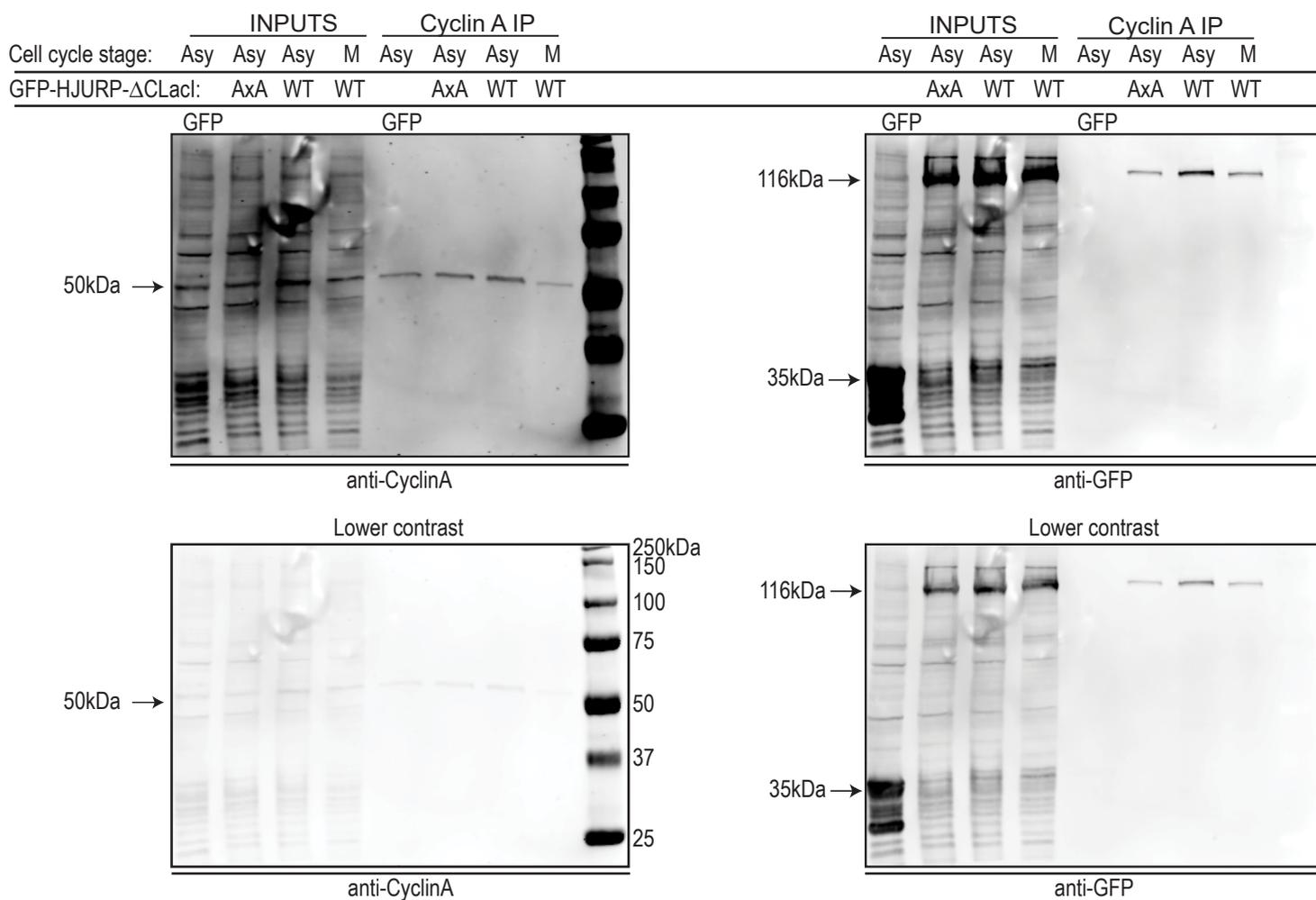
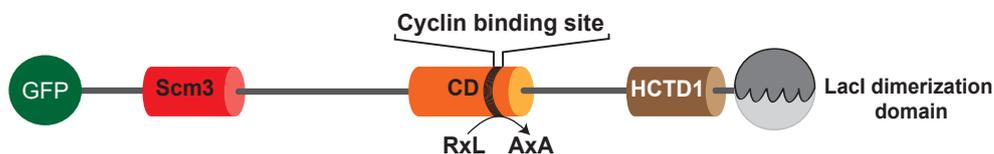
(B-J) Representative chromatograms and spectra of HJURP and CENP-A phosphopeptides in SILAC. Extracted ion chromatograms of all phosphopeptides from Figure 1C and 1D, showing co-elution of phosphopeptide pairs (left panels), and representative mass spectra showing isotopic envelopes of light vs. heavy 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.

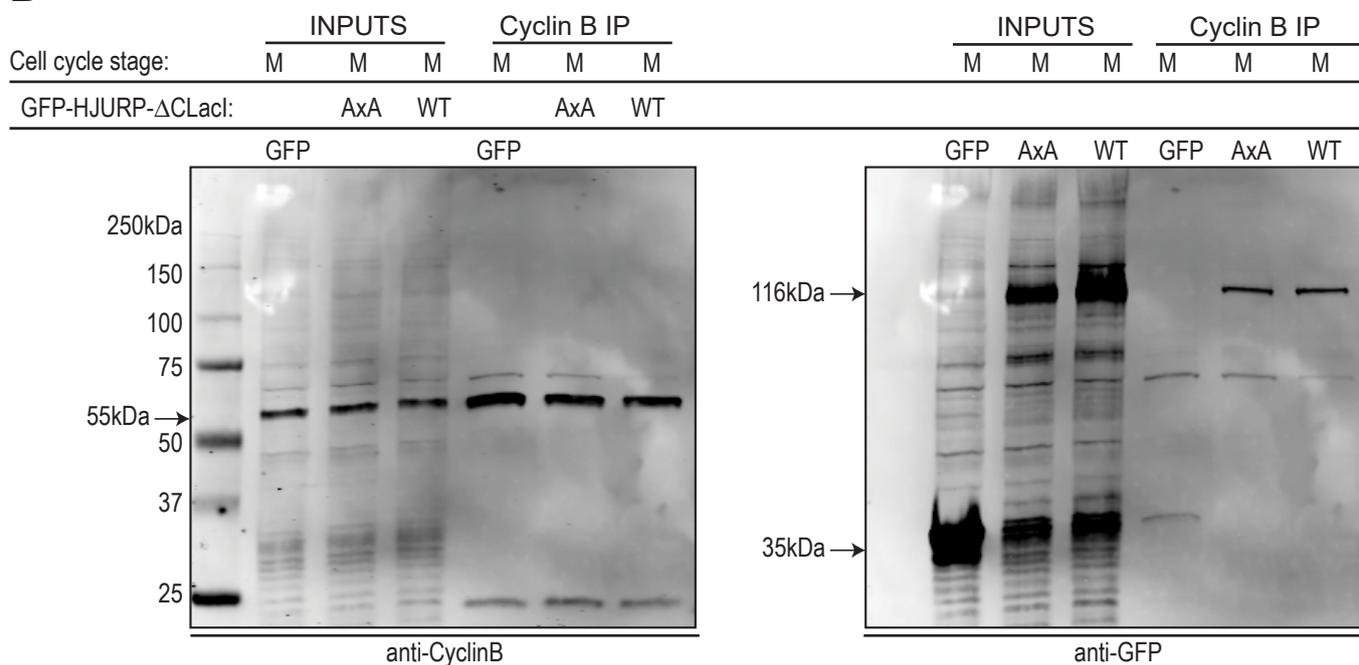
(C-H) HJURP phosphopeptides.

(I-J) CENP-A phosphopeptides.

**A**



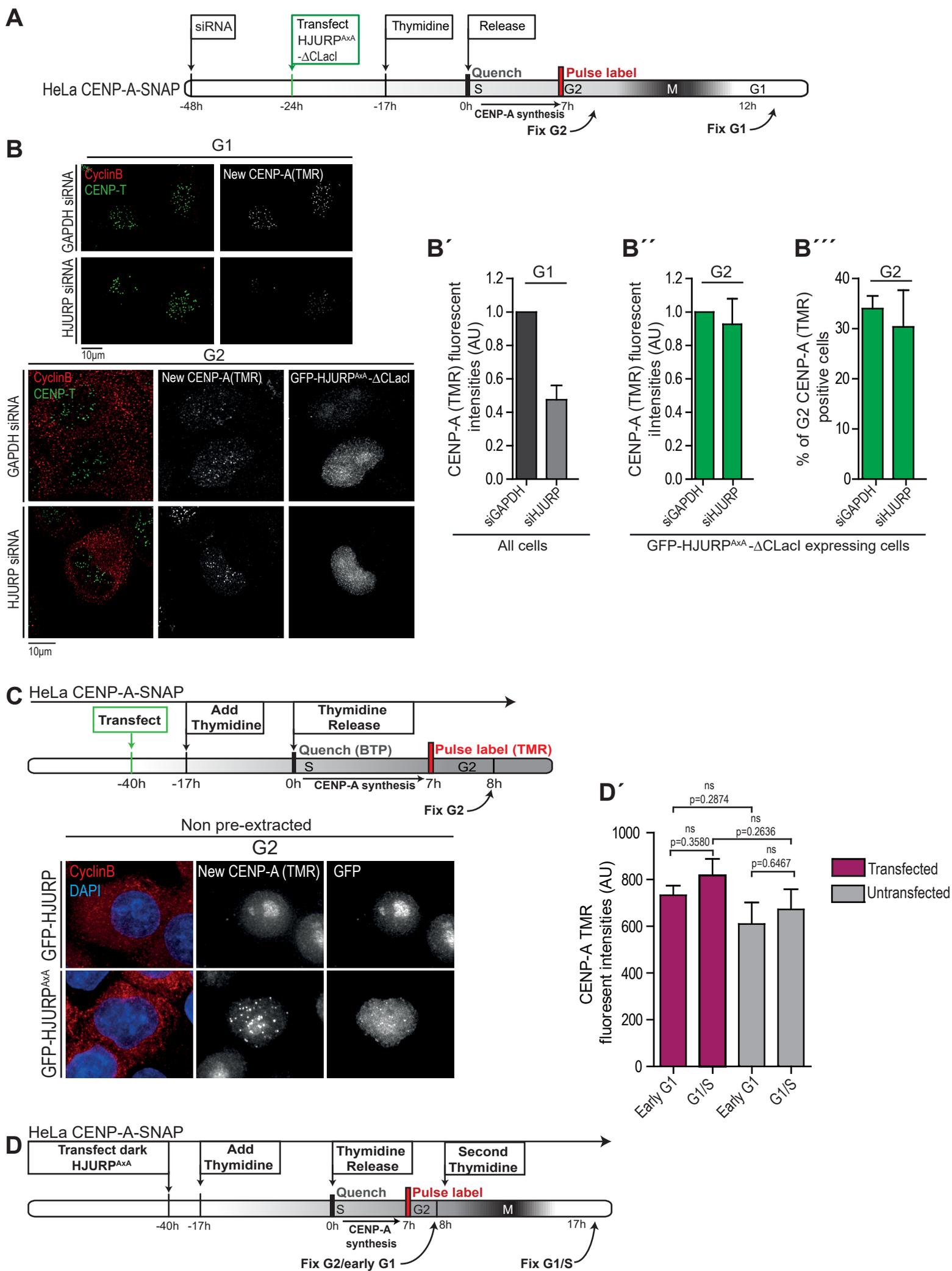
**B**



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

(A) Top: Schematic of GFP-HJURP- $\Delta$ CLaI protein with relevant domains depicted. Experiments were performed with a HJURP construct in which the C-terminal homodimerization domain is replaced with that of LaI 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- $\Delta$ CLaI or GFP-HJURP<sup>AxA</sup>- $\Delta$ CLaI. 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. (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<sup>AxA</sup>- $\Delta$ CLacI 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<sup>AxA</sup>- $\Delta$ CLacI 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.

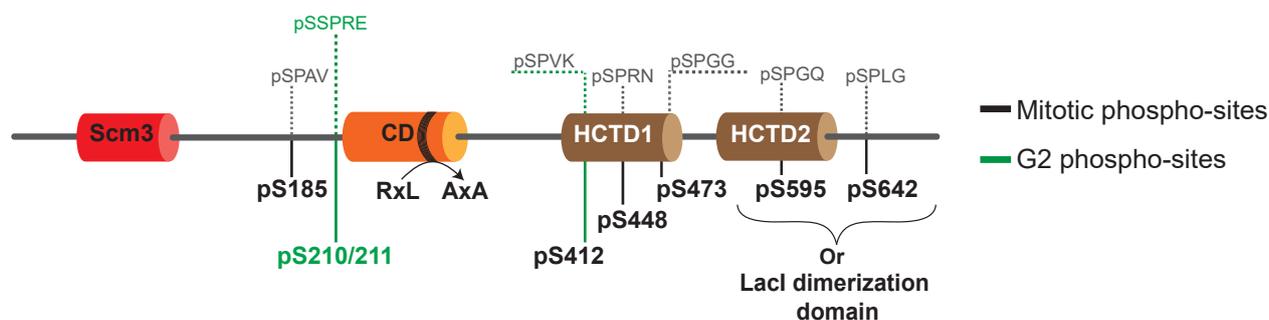
(C) Timing of CENP-A deposition is controlled by Conserved Domain of HJURP. Experiment identical to the one described in Figure 2A, B with exception of transfection with GFP-HJURP or GFP-HJURP<sup>AxA</sup> (i.e. wild type HJURP C-terminus instead of LacI dimerization domain). Following fixation, cells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

(D) The apparent partiality of HJURP<sup>AxA</sup>-induced G2 phase loading is not a consequence of subsequent HJURP<sup>AxA</sup>-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<sup>AxA</sup> 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<sup>AxA</sup> expression.

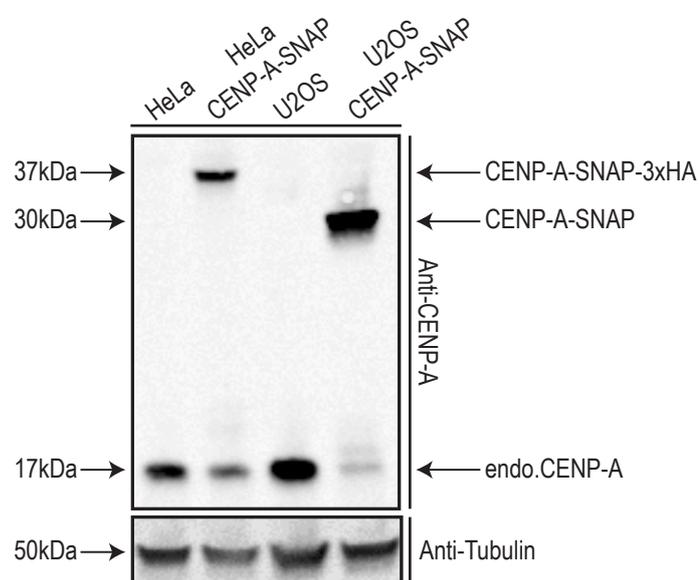
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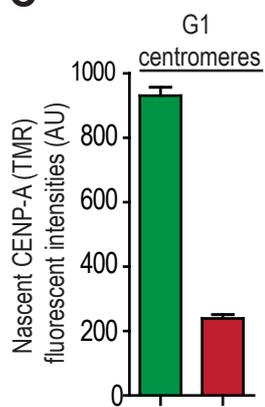
B

HJURP	G2 phase CENP-A assembly % intensity of G1 (% of G2 cells assembling)	
Mitotic phospho-site mutations	C-terminal domain	
	endogenous	$\Delta$ C-Lacl
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.

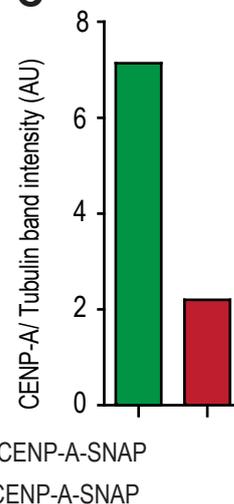
C



C'



C''



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

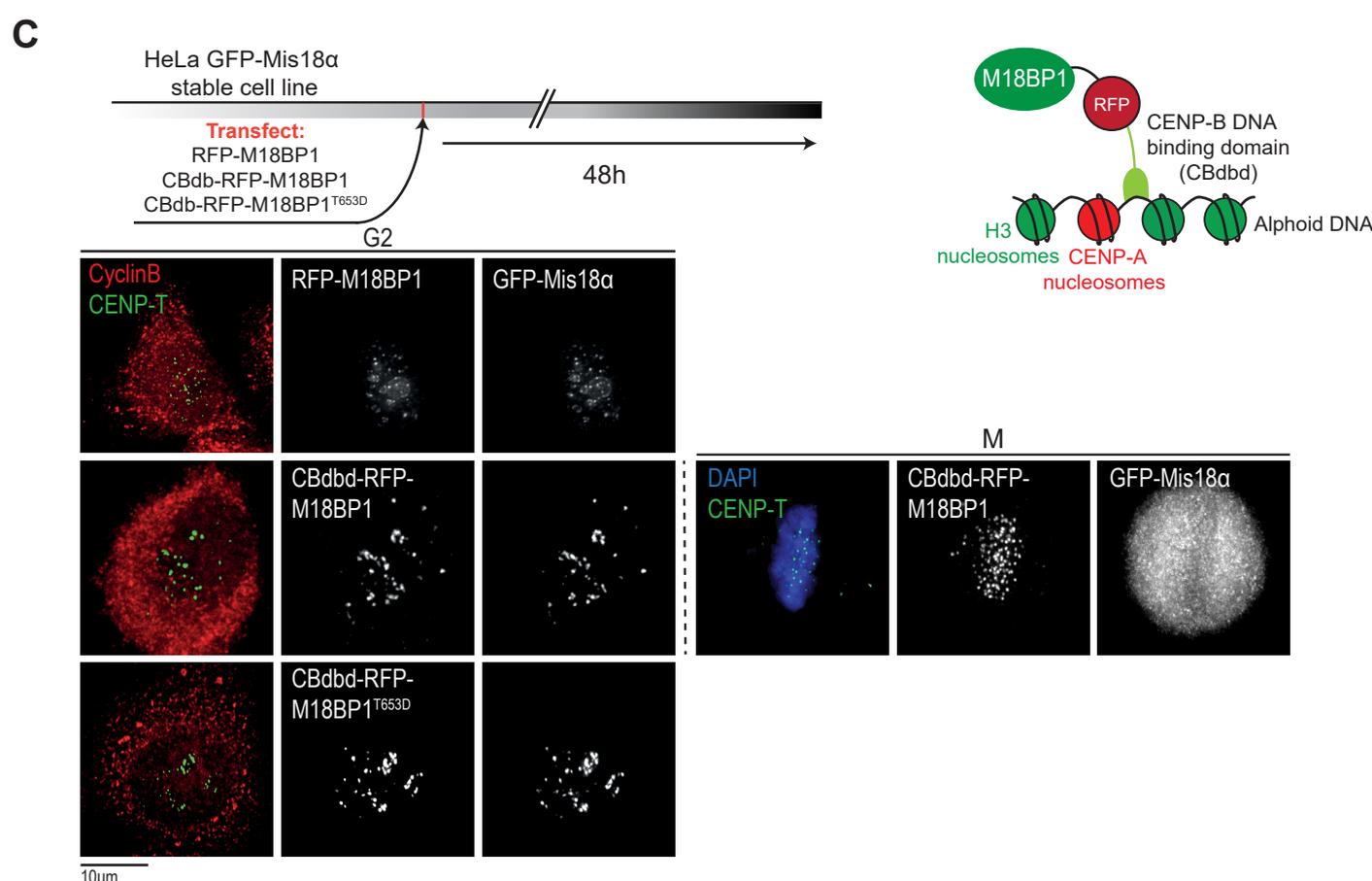
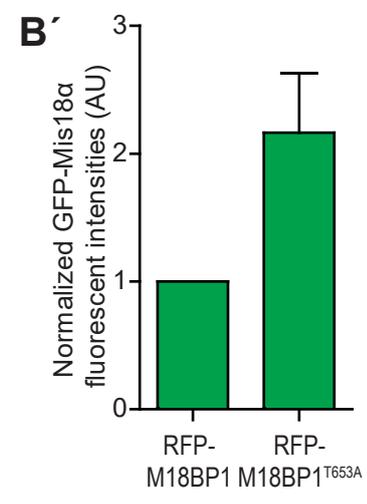
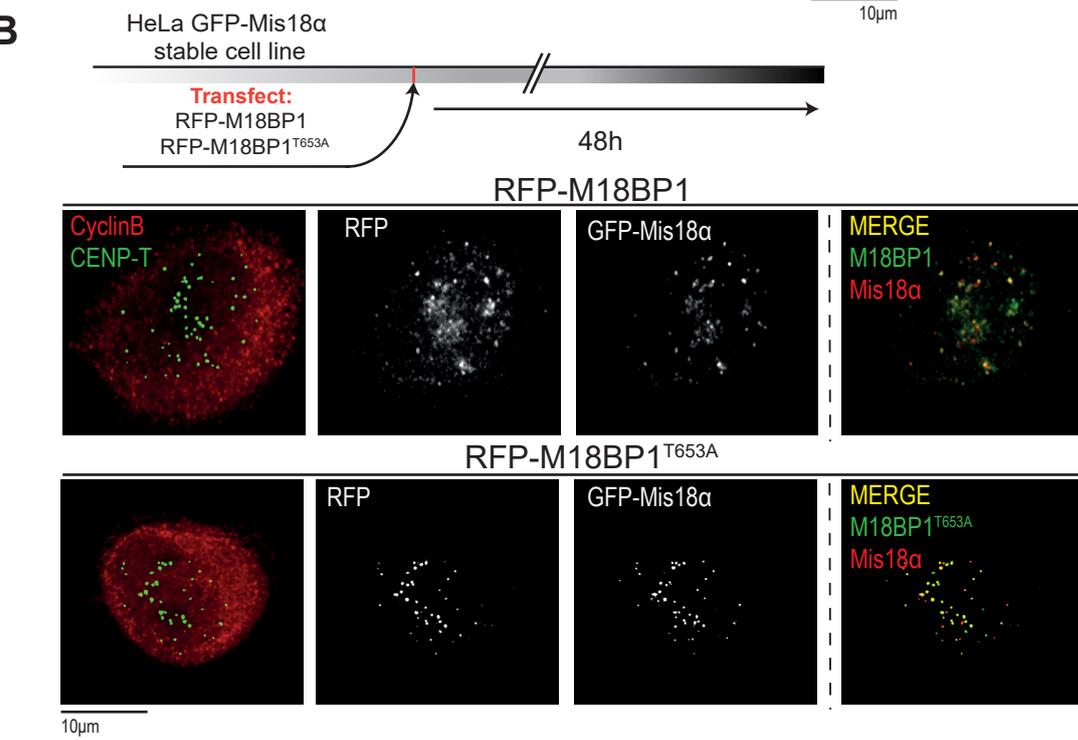
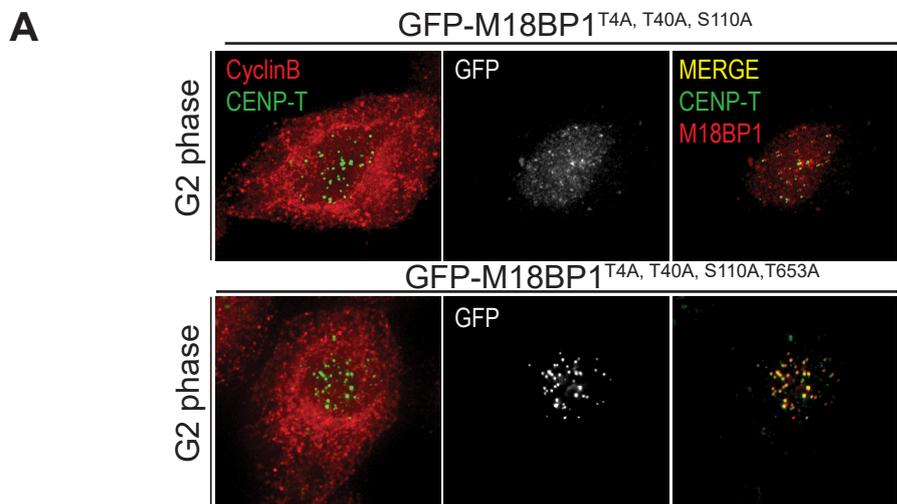
(A) Schematic representation of HJURP protein, along with previously recognized domains (CENP-A binding domain (Scm3), Conserved Domain (CD), HJURP C-Terminal Domain 1 and 2 (HCTD1 and 2)) and the position of phospho-sites identified by mass spectrometry in mitosis (black lines; Figure 1, S2) or in G2 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- $\Delta$ LacI 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.



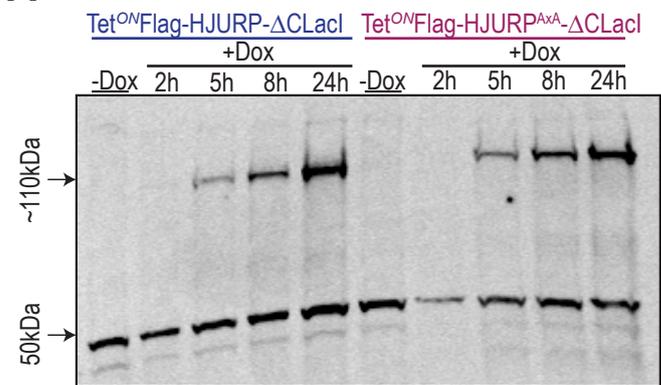
**Figure S6. (supplement to Figure 5) Cell cycle control of M18BP1/Mis18 $\alpha$  complex formation**

(A) M18BP1<sup>T4,T40,S110</sup> triple mutant is enriched at G2 centromeres, whereas M18BP1<sup>T4,T40,S110,T653A</sup> quadruple mutant is strongly enriched at G2 centromeres. Constructs expressing M18BP1<sup>T4,T40,S110</sup> or M18BP1<sup>T4,T40,S110,T653A</sup> 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.

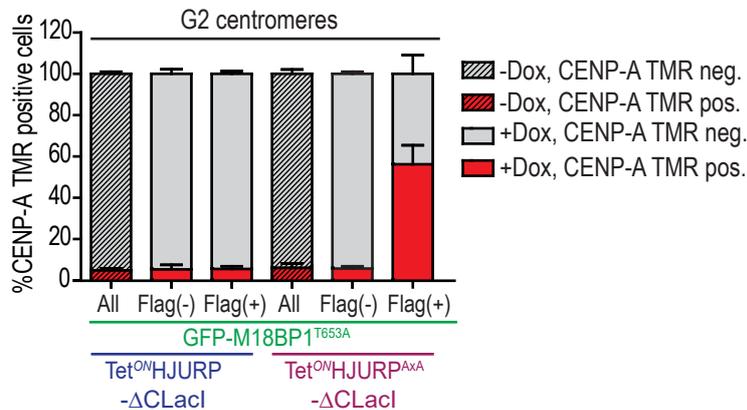
(B) T653 residue in M18BP1 does not determine Mis18 complex formation. Left: Asynchronously cycling HeLa cells stably expressing GFP-Mis18 $\alpha$  were transfected with constructs expressing RFP-M18BP1 or RFP-M18BP1<sup>T653A</sup> 48hr prior to fixation, followed by counterstaining for cyclin B and 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/Mis18 $\alpha$  complex formation is not inhibited by Cdk activity in G2 phase. Asynchronously cycling HeLa cells stably expressing GFP-Mis18 $\alpha$  were transfected with constructs expressing RFP-M18BP1, CBdbd-RFP-M18BP1 or CBdbd-RFP-M18BP1<sup>T653D</sup> 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 Nocodazole for 5h.

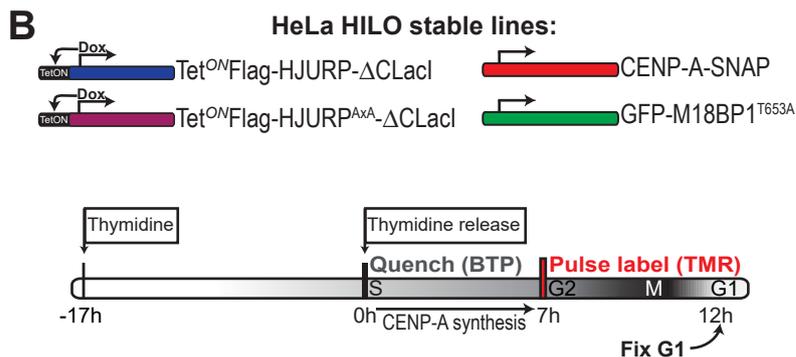
**A**



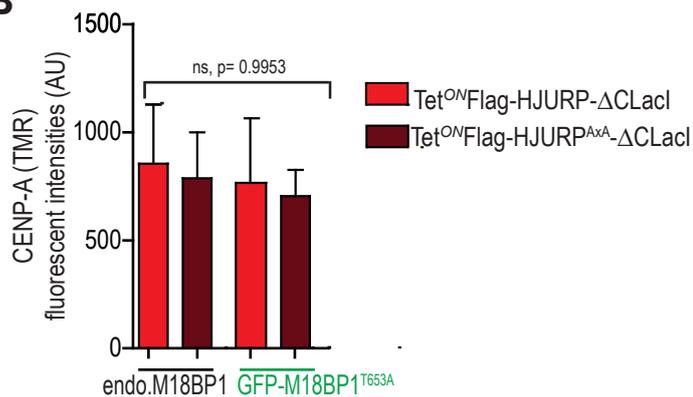
**D**



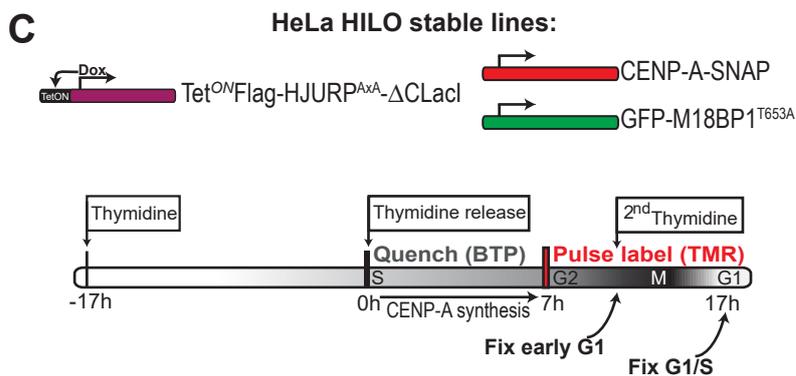
**B**



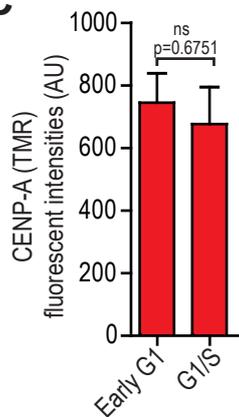
**B'**



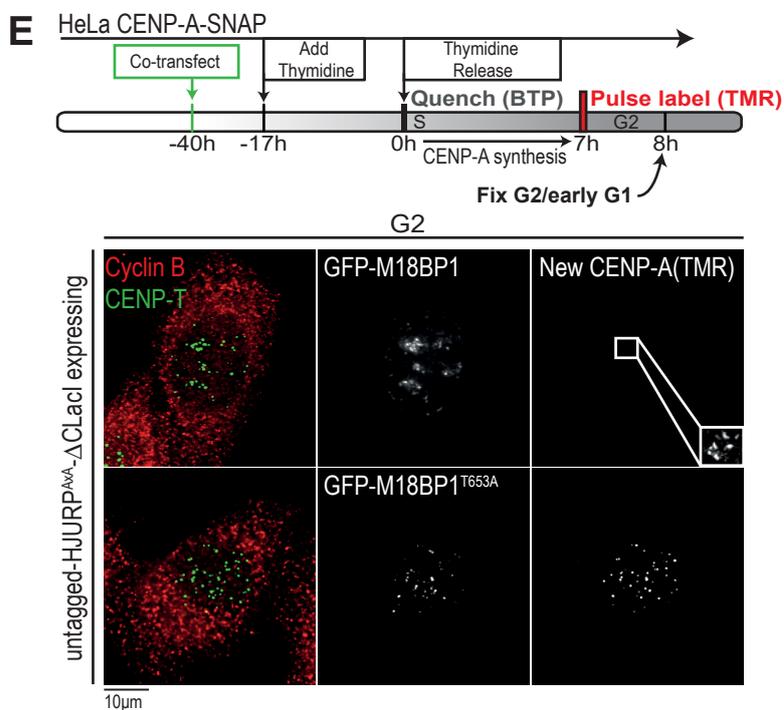
**C**



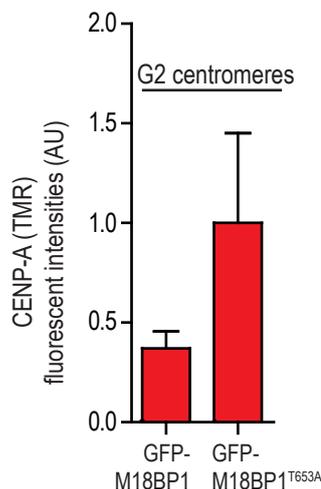
**C'**



**E**



**E'**



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

(A) 3xFlag-HJURP- $\Delta$ CLacI and 3xFlag-HJURP<sup>AxA</sup>- $\Delta$ CLacI proteins are induced to a similar extent. HeLa HILO CENP-A-SNAP, GFP-M18BP1<sup>T653A</sup> carrying tetracycline-inducible 3xFlag-HJURP- $\Delta$ CLacI or 3xFlag-HJURP<sup>AxA</sup>- $\Delta$ CLacI 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<sup>AxA</sup>- $\Delta$ CLacI / GFP-M18BP1<sup>T653A</sup> 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<sup>AxA</sup>-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<sup>AxA</sup>- $\Delta$ CLacI combined with either GFP-M18BP1 or GFP-M18BP1<sup>T653A</sup>. 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 E. 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<sup>AxA</sup>- $\Delta$ CLacI 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- $\Delta$ CLacI (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- $\Delta$ CLacI (pLJ632) were converted to GFP-HJURP<sup>AxA</sup> (pLJ600) and GFP-HJURP<sup>AxA</sup>- $\Delta$ CLacI (pLJ654) by quick exchange PCR replacing R276 and L278 by Alanine. GFP-HJURP<sup>S210A, S211A, S412A</sup> (pLJ828) or GFP-HJURP<sup>S210A, S211A, S412A</sup>- $\Delta$ CLacI (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-Mis18 $\alpha$  construct). Subsequently, Mis18 $\alpha$  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- $\Delta$ CLacI /HJURP<sup>AxA</sup>- $\Delta$ CLacI 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- $\Delta$ C (aa 1-482) or 3xFlag HJURP<sup>AxA</sup>- $\Delta$ C (aa 1-482) was inserted into an Aval/BsrGI digested vector, resulting in an in-frame fusion of 3xFlag HJURP/HJURP<sup>AxA</sup>- $\Delta$ CLacI (designated pLJ745 and pLJ746, respectively). GFP-M18BP1<sup>T653A</sup> (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<sup>T653D</sup> (pLJ699). CBdbd-GFP-M18BP1 (pLJ592) was generated by ligation of CBdbd fragment between GFP and M18BP1 of the GFP-M18BP1 plasmid (pLJ415) mRFP-M18BP1<sup>T653D</sup> (pLJ705) was created by NotI and AfeI replacement of GFP with mRFP (from pLJ287). CBdbd-mRFP-M18BP1/M18BP1<sup>T653D</sup>/M18BP1<sup>T653E</sup> (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<sup>T653D</sup>) or pLJ641 (mRFP-M18BP1<sup>T653E</sup>). 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<sub>2</sub>. 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  $\mu$ g/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<sup>T653A</sup> 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<sup>T653A</sup> 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<sup>AxA</sup> 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<sup>WT/AxA</sup> 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. Mis18 $\alpha$  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- $\Delta$ CLaI or GFP-HJURP<sup>AxA</sup>- $\Delta$ CLaI. 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<sup>7</sup> 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  $^{13}\text{C}_6$ ,  $^{15}\text{N}_2$ -lysine and 50 mg/L  $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$ -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  $\mu\text{M}$  S-trityl-L-cysteine for 17 h. Subsequently, the "light" cells were treated with 100  $\mu\text{M}$  R-Roscovitin (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  $\mu\text{g}/\text{ml}$ ) (Bassett et al., 2012, Dev Cell), respectively. Cell pellets from  $1.4 \times 10^9$  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  $\text{NH}_4\text{HCO}_3$ , 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. RapiGest 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 TiO<sub>2</sub> 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<sup>AxA</sup> 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<sup>AxA</sup> 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 antibody generation, application and phosphatase treatment**

Phospho-specific rabbit antibody for M18BP1 was produced by immunization of 2 rabbits with phosphorylated peptide ( (NH<sub>2</sub>-)CKAYILV (pT)PLKSRK (-CONH<sub>2</sub>)), and subsequent affinity purification of both sera (Innovagen AB, SE-22370 Lund, Sweden). 10<sup>6</sup> of transiently transfected HEK293T carrying either GFP-M18BP1 or GFP-M18BP1<sup>T653A</sup> were lysed in buffer containing 75mM HEPES pH 7,5, 150mM NaCl, 2mM MgCl<sub>2</sub>, 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<sup>AxA</sup> 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<sub>2</sub>) 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<sup>AxA</sup> 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.

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