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Cdk Activity Couples Epigenetic Centromere Inheritance to Cell Cycle Progression

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SUMMARY

Centromeres form the site of chromosome attachment to microtubules during mitosis. Identity of these loci is maintained epigenetically by nucleosomes containing the histone H3 variant CENP-A. Propagation of CENP-A chromatin is uncoupled from DNA replication initiating only during mitotic exit. We now demonstrate that inhibition of Cdk1 and Cdk2 activities is sufficient to trigger CENP-A assembly throughout the cell cycle in a manner dependent on the canonical CENP-A assembly machinery. We further show that the key CENP-A assembly factor Mis18BP1^{HsKNL2} is phosphorylated in a cell cycle-dependent manner that controls its centromere localization during mitotic exit. These results strongly support a model in which the CENP-A assembly machinery is poised for activation throughout the cell cycle but kept in an inactive noncentromeric state by Cdk activity during S, G2, and M phases. Alleviation of this inhibition in G1 phase ensures tight coupling between DNA replication, cell division, and subsequent centromere maturation.

INTRODUCTION

Accurate segregation of newly replicated chromosomes during mitosis is essential for the maintenance of genome integrity. Central to preserving fidelity of this process is the kinetochore, which forms the chromosomal attachment site for spindle microtubules and is required for chromosome movement and mitotic checkpoint signaling (Cheeseman and Desai, 2008). The centromere is a unique constitutive chromatin domain that assembles the kinetochore during mitosis and is essential for mitotic progression (Allshire and Karpen, 2008). Centromeres are propagated epigenetically, largely independent of any particular DNA sequence (Warburton et al., 1997; Vafa and Sullivan, 1997; Warburton, 2004). Key to the structure and maintenance of the centromere is the specific assembly of the histone H3 variant Centromere Protein A (CENP-A) into centromeric nucleosomes (Silva and Jansen, 2009; Olszak et al., 2011; Barnhart et al., 2011; Guse et al., 2011). Inheritance and replication of this mark is essential to ensure epigenetic propagation of centromere identity (Olszak et al., 2011; Barnhart et al., 2011). Indeed, CENP-A containing nucleosomes are extremely stable and maintained throughout the cell cycle, being redistributed only during S phase (Jansen et al., 2007; Hemmerich et al., 2008; Shelby et al., 2000; Régnier et al., 2005). Importantly, centromeric chromatin replication is uncoupled from centromeric DNA replication and, at least in metazoans, restricted to late mitosis/early G1 phase of the cell cycle (Jansen et al., 2007; Hemmerich et al., 2008; Schuh et al., 2007; Bernad et al., 2011; Moree et al., 2011). Assembly strictly depends on passage through mitosis (Jansen et al., 2007; Schuh et al., 2007; Bernad et al., 2011; Moree et al., 2011), which ensures tight coupling of centromere duplication to cell cycle progression. However, the mitotic trigger that initiates centromere propagation has not been identified. Possible candidates for this have been previously proposed (Figure 1A), including changes in nuclear architecture (Jansen et al., 2007), anaphase promoting complex/cyclosome (APC/C)-mediated destruction of a specific inhibitor of CENP-A assembly (Erhardt et al., 2008), or assembly of a proper kinetochore-microtubule interface (Mellone and Allshire, 2003; Jansen et al., 2007; Allshire and Karpen, 2008).

Here, we sought to identify the molecular signal that temporally controls CENP-A assembly. We find that inhibiting Cdk1 and Cdk2 in any phase of the cell cycle is sufficient to trigger rapid CENP-A assembly in a canonical fashion. Thus, our results point to a simple mechanism that excludes the need for any active involvement of mitosis in subsequent CENP-A assembly, other than the concomitant downregulation of Cdk activity upon mitotic exit.

RESULTS

Cdk Inhibition Triggers CENP-A Assembly prior to Mitosis

In order to identify the molecular mechanism controlling the unusual timing of CENP-A assembly, we employed SNAP-based fluorescent quench-chase-pulse labeling (see Experimental Procedures and Jansen et al., 2007) to uniquely and directly track the fate of nascent proteins. One defining feature of mitotic exit is APC/C-mediated destruction of cyclin B and concomitant loss of associated cyclin-dependent kinase (Cdk) activity. We therefore hypothesized that the CENP-A assembly process

might be controlled directly by Cdk activity, without a strict need for APC/C activation, destruction of APC/C targets apart from the Cdk activator cyclin B, or any other aspect of mitosis. To test this hypothesis, we synchronized HeLa cells in G2 phase, upon which a nascent pool of CENP-A-SNAP, synthesized during the preceding S phase, was fluorescently pulse labeled. A brief (1 hr) treatment with the pan-Cdk inhibitors Roscovitine or Purvalanol A induced CENP-A assembly into the centromere in nearly half the cyclin B positive (G2 phase) population, whereas assembly is never observed in control cells at this stage in which the nascent pool remained diffusely nuclear (Figures 1B–1E; see Figures S1A and S1B available online). We confirmed these results in nontransformed, hTERT immortalized RPE cells, without thymidine-mediated cell synchronization (Figure S2). Assembly under transient Roscovitine-treated conditions in G2 phase resulted in stable incorporation of CENP-A into chromatin, as it is retained at centromeres on condensed mitotic chromosomes following Roscovitine washout (Figure S1F). However, a brief, 1 hr induction of CENP-A assembly in this phase is likely incomplete as normal CENP-A accumulation at centromeres continues for the duration of G1 phase (~10 hr) (Lagana et al., 2010).

Roscovitine treatment of G2 cells did not change cell cycle position as treatment prevents mitotic entry and cyclin B levels remained high (Figures 1B and 1E; Figure S1C). Preservation of high cyclin B levels suggests that Roscovitine-treated cells do not enter a precocious G1-like state by premature activation of APC/C-mediated protein destruction. Indeed, treatment of cells with the proteasome inhibitor MG132 or protein synthesis inhibitor Cycloheximide did not interfere with G2-induced CENP-A assembly. Therefore, the destruction of a specific CENP-A assembly inhibitor or de novo synthesis of an assembly factor is unlikely to be required for CENP-A loading (Figures 1E and 1F; Figures S1D and S1E). These results suggest that the CENP-A assembly machinery is present and poised for activation prior to mitosis. In addition, they argue against a role for APC/C-mediated destruction of a putative inhibitor of CENP-A assembly other than the Cdk activator cyclin B.

Cdk1 and Cdk2 Are Sufficient to Maintain Cell Cycle Control of CENP-A Assembly

Our small molecule inhibitor experiments indicate that, prior to mitosis, the CENP-A assembly machinery is present but kept in an inactive state by Cdk activity. The predominant Cdks that are active during G2 phase and mitosis are Cdk2 and Cdk1, respectively. Both kinases are naturally deactivated upon mitotic entry and mitotic exit, respectively (Pines, 2006), and both are strongly inhibited by Roscovitine and Purvalanol A (Wesierska-Gadek and Krystof, 2009). However, due to the broad substrate specificity of these inhibitors (Wesierska-Gadek and Krystof, 2009), we cannot determine which Cdk (if any) is responsible for controlling CENP-A assembly. To address this directly, we turned to chicken DT40 cells that harbor defined mutations in Cdk1 and/or Cdk2. We utilized cells that carry either a homozygous *CDK2* deletion (*cdk2*$^{-/-}$) and/or express analog-sensitive Cdk1 in a homozygous *cdk1* null background (*cdk1as*) (Hochegger et al., 2007). Cdk1as can be selectively inhibited by addition of the ATP analog 1NM-PP1. 1NM-PP1 does not affect cell cycle progression of wild-type DT40 cells, underscoring the specificity to the *cdk1as* mutation (data not shown and Hochegger et al., 2007). These cells were further retrofitted to stably express subendogenous levels of SNAP-tagged chicken CENP-A, which results in a centromeric fluorescent signal following pulse labeling with TMR-*Star* (Figure 2A and 2A′).

Assembly of nascent SNAP-CENP-A in DT40 cells occurred only in cells with low cyclin B2 levels and can be fully blocked when cells are prevented from entry into G1 by nocodazole-induced mitotic checkpoint arrest (Figures 2A–2B). This demonstrates that, like in human cells (Figure 1B) (Jansen et al., 2007; Hemmerich et al., 2008), *Drosophila* embryos (Schuh et al., 2007) and *Xenopus* extracts (Bernad et al., 2011; Moree et al., 2011), chicken DT40 cells assemble CENP-A at centromeres only upon mitotic exit. Cdk2 protein is nonessential in DT40 cells (Hochegger et al., 2007) and mice (Berthet et al., 2003; Ortega et al., 2003), possibly due to compensation by cyclin A- and E-mediated Cdk1 activity (Hochegger et al., 2007; Santamaría et al., 2007). Consistently, timing of assembly did not change in the *cdk2*$^{-/-}$ mutant background (Figures 2A and 2B), indicating that inhibition of Cdk2 alone is not sufficient to induce unscheduled CENP-A assembly. To test for the involvement of Cdk1 we synchronized *cdk1as* single or *cdk1as/cdk2*$^{-/-}$ double mutants in low (1 μM) levels of 1NM-PP1. At this concentration Cdk1 activity is sufficient to drive S phase progression but not high enough to allow entry into mitosis, resulting in a G2 arrest (Figure S3A) (Hochegger et al., 2007). These cells were either maintained in G2 in low inhibitor concentrations, or released into G1 phase by inhibitor removal, or shifted to high (10 μM) doses of ATP analog to completely abolish Cdk1 activity (Figure 2C). While centromeric CENP-A assembly was detected in G1 phase in cells of either genotype, *cdk1as* single mutants showed little centromere assembly in G2 (Figures 2C′ and 2D). Strikingly, G2 arrested *cdk1as/cdk2*$^{-/-}$ double mutants readily incorporated CENP-A at centromeres in virtually all cells in both low and high 1NM-PP1 concentrations (Figures 2C and 2D). These results were confirmed by pan-Cdk inhibition using Roscovitine, consistent with our observations in HeLa cells (Figure S3D). Clearly, loss of both Cdk1 and Cdk2 activities is necessary and sufficient to trigger premature CENP-A loading, which indicates that these are responsible for suppressing the CENP-A assembly machinery prior to mitotic exit.

CENP-A Assembly Can Be Induced in S Phase

We next determined whether CENP-A assembly, induced by the loss of Cdk activity, was restricted to G2 phase. Randomly cycling DT40 cells spend up to 60% of their time in S phase (Zhao et al., 2007). However, Cdk1/2 double inactivation in these cells resulted in CENP-A assembly in ~78% of the population (Figures 3A and 3A′; Figure S3B) suggesting that CENP-A loading can be induced also in S phase cells. To directly test this possibility, we synchronized both *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cells in S phase with Hydroxyurea (HU) and assayed centromere assembly of a nascent CENP-A-SNAP pool during HU arrest. In the presence of Cdk2 activity little or no CENP-A assembly was observed, even when Cdk1 was fully inhibited (Figures 3B and 3B′; Figure S3C) indicating that Cdk2 is sufficient to block CENP-A assembly. However, in the absence of Cdk2 ~37% of the cells with active Cdk1 (no 1NM-PP1) assembled

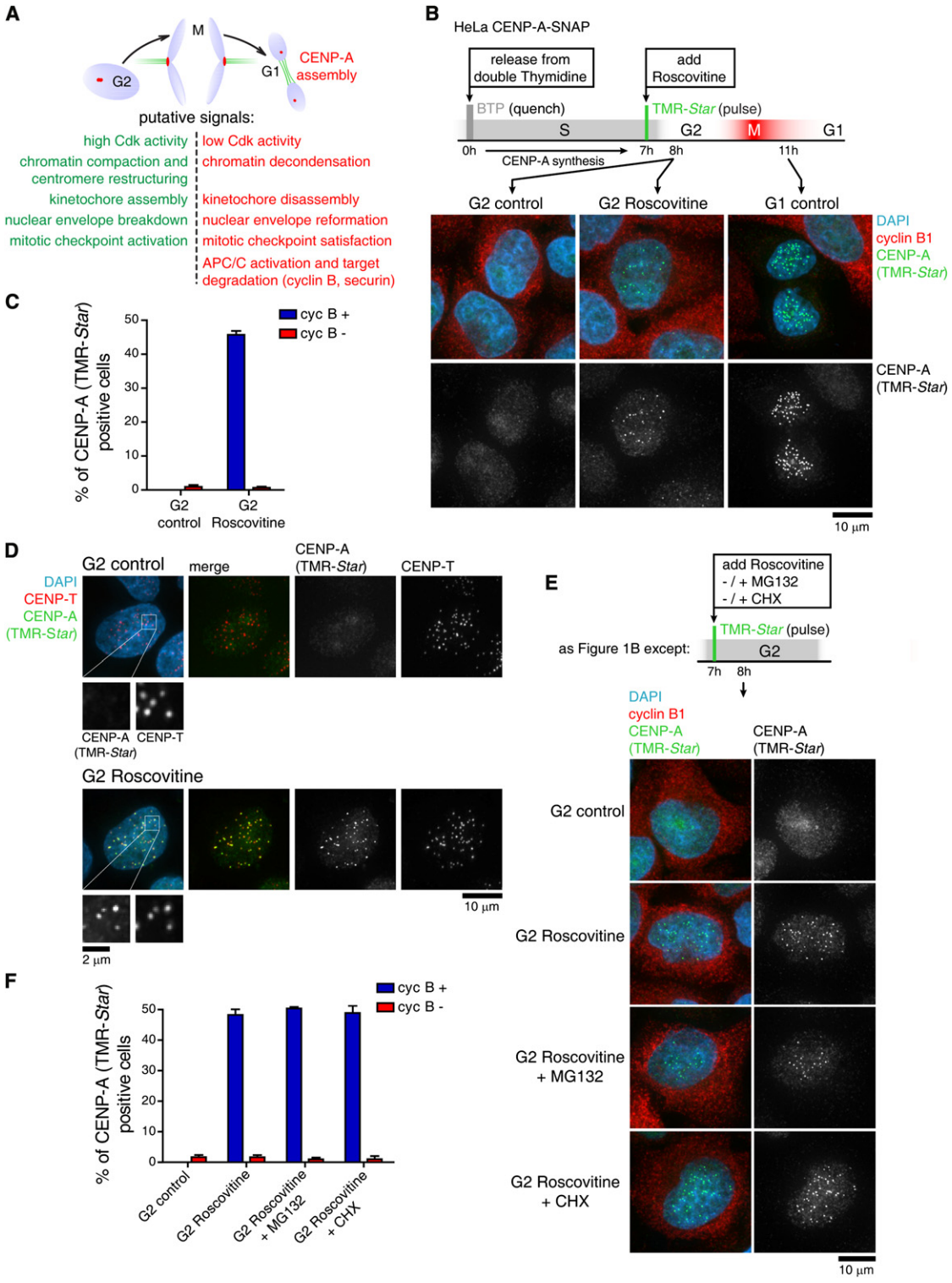

Figure 1. Cdk Inhibition Induces Premature CENP-A Assembly at Centromeres

(A) Schematic representing mitotic passage with key steps during either mitotic entry (green) or mitotic exit (red) that are potential signals for subsequent CENP-A assembly in early G1.

(B) HeLa CENP-A-SNAP cells were synchronized at the G1/S boundary by double thymidine arrest. S phase synthesized CENP-A-SNAP was subsequently pulse labeled in G2 phase, 7 hr after release from thymidine. G2 cells were mock treated (G2 control), treated with Roscovitine for 1 hr, or allowed to cycle through mitosis (G1 control) prior to fixation. Cells were counterstained for cyclin B and with DAPI to indicate G2 status and DNA, respectively.

(C) Quantification of (B).

CENP-A, which increased to ~80 when Cdk1 was further inhibited (10 μM 1NM-PP1), indicating that Cdk1 can contribute but is not sufficient to prevent CENP-A assembly in S phase. These results demonstrate that cells arrested in S phase are competent for CENP-A assembly and that assembly at this stage is prevented primarily by Cdk2 activity. This is consistent with recent findings highlighting nonredundant roles for Cdk2 in cell cycle progression (Merrick et al., 2008, 2011).

Cells held in S phase by HU treatment are not actively replicating DNA due to HU-induced stalling of DNA replication. To test whether CENP-A assembly can occur in actively replicating cells we synchronized *cdk1as/cdk2*$^{-/-}$ cells in early S phase by HU treatment followed by release in the presence of BrdU to mark actively replicating cells. A nascent CENP-A-SNAP pool was synthesized and labeled during progression through S phase in the presence or absence of 10 μM 1NM-PP1. Three hours following the release from HU, cells were scored for CENP-A assembly and cell cycle position. Upon inhibition of Cdk2 and Cdk1, 85% of cells assembled nascent CENP-A at centromeres under conditions where 70% are in S phase and BrdU positive (Figures 3C and 3C′). These results demonstrate that cells undergoing active DNA replication are competent for CENP-A assembly.

Unscheduled CENP-A Assembly Requires the Canonical Assembly Factors

We next determined whether unscheduled CENP-A assembly induced prior to mitosis resulted from the activation of the canonical CENP-A assembly pathway. To address this, we returned to the HeLa cell system. Mis18α, Mis18β, and the Myb-domain containing protein Mis18BP1^{HsKNL2} (collectively named the Mis18 complex) are essential for CENP-A assembly in G1 phase and are recruited to centromeres during anaphase, just prior to the onset of CENP-A assembly (Silva and Jansen, 2009; Fujita et al., 2007; Maddox et al., 2007). Strikingly, inhibition of Cdk activity in G2 cells resulted in rapid recruitment of GFP-Mis18α and Mis18BP1^{HsKNL2} to centromeres (Figures 4A–4D). Cells displayed either centromere localized GFP-Mis18α alone (Figure 4A, red arrow) or both GFP-Mis18α and nascent CENP-A-SNAP (Figure 4A, green arrow) but never CENP-A-SNAP alone (Figure 4B). This suggests that, as for canonical G1 loading of CENP-A, Mis18α arrives prior to, and is required for subsequent CENP-A assembly under induced conditions in G2 phase. Consistently, siRNA-mediated depletion of Mis18α, Mis18BP1^{HsKNL2}, or the CENP-A-specific chaperone HJURP (Dunleavy et al., 2009; Foltz et al., 2009) resulted in a reduction of both G1 phase assembly of nascent CENP-A as well as unscheduled assembly in G2 phase (Figure 4E). Note that partial depletion of assembly factors under these conditions (to or below 50% of unperturbed levels; Figure S4) is sufficient to impair CENP-A assembly, indicating that these are rate limiting for assembly in both G1 phase and G2 phase.

Molecular Mechanism Maintaining Cell Cycle Control of CENP-A Assembly

Our results thus far identify two upstream cell cycle control kinases that maintain the CENP-A assembly machinery in an inactive state. Aurora B is a mitotic kinase downstream of Cdk activity that is responsible for pericentric H3 phosphorylation from late S through G2 phase into mitosis (Monier et al., 2007) and phosphorylates CENP-A during mitosis (Zeitlin et al., 2001), potentially implicating Aurora B in maintaining cell cycle control of CENP-A assembly. However, treatment of HeLa cells expressing CENP-A-SNAP with inhibitors of either Aurora A or Aurora B (Hoar et al., 2007; Ditchfield et al., 2003) did not alter the G1 phase timing of CENP-A assembly (Figure S5A) nor did they significantly block Roscovitine-induced CENP-A assembly in G2 phase (Figure S5B). These observations suggest that the Aurora kinases are unlikely to be involved in cell cycle control of CENP-A assembly.

Rapid and early recruitment of members of the Mis18 complex suggests that Cdk activity acts either directly or indirectly on these components. Indeed, previous phosphoproteome screens have found that Mis18α, Mis18β, and Mis18BP1^{HsKNL2} are phosphorylated on at least 1, 4, and 20 positions, respectively, including Cdk consensus sites in the latter (Olsen et al., 2006; Dephoure et al., 2008; Wang et al., 2008; Mayya et al., 2009). Mutation of all known S/T phosphorylation sites in Mis18α or Mis18β had no discernible effect on centromere localization (not shown). Strikingly, however, conversion of 24 S/T sites in Mis18BP1^{HsKNL2} to alanine (20 known sites plus an additional 4 S/T residues immediately adjacent to known phospho sites; Mis18BP1^{HsKNL2}-Ala^{24}; Figure 5A) resulted in a precocious recruitment of this protein to centromeres in G2 phase and mitotic cells (Figures 5B and 5C). We find Mis18BP1^{HsKNL2} to be phosphorylated in a cell cycle-dependent manner peaking in cells enriched in mitosis (Figure 5D). Treatment of phosphorylated protein by phosphatase in vitro or by mutation of known sites (Mis18BP1^{HsKNL2}-Ala^{24}) resulted in the loss of high molecular weight species, indicating that most, if not all, phosphorylation events are removed in this mutant (Figure 5D). These results strongly suggest that Mis18BP1^{HsKNL2} is kept in a noncentromeric state by phosphorylation, a state alleviated by loss of phosphorylation during mitotic exit upon diminishment of Cdk activity. While Mis18BP1^{HsKNL2}-Ala^{24} targeting did not result in premature CENP-A assembly (not shown), our results indicate that Cdk-mediated inhibition of CENP-A assembly is likely exerted through controlling centromere localization of key CENP-A assembly factors, including Mis18BP1^{HsKNL2}.

DISCUSSION

Our combined results demonstrate that the CENP-A assembly machinery is present and poised for activity throughout most of the cell cycle, but is kept in an inactive state by Cdk1 and Cdk2 activities until after completion of DNA replication and

(D) Nascent CENP-A-SNAP colocalizes with centromeres (CENP-T) after Roscovitine-induced assembly in G2 phase HeLa CENP-A-SNAP cells.
(E) Experiment as in (B) but with the inclusion of MG132 or cycloheximide (CHX) to block proteolysis or protein synthesis, respectively (see also Figures S1C–S1E).
(F) Quantification of (E). Mean and standard error of the mean (SEM) of three replicates are shown in (C) and (F). Percentage of total cells positive for centromeric CENP-A-SNAP (TMR-*Star*) signal was scored and represented according to cyclin B status. See also Figures S1 and S2.

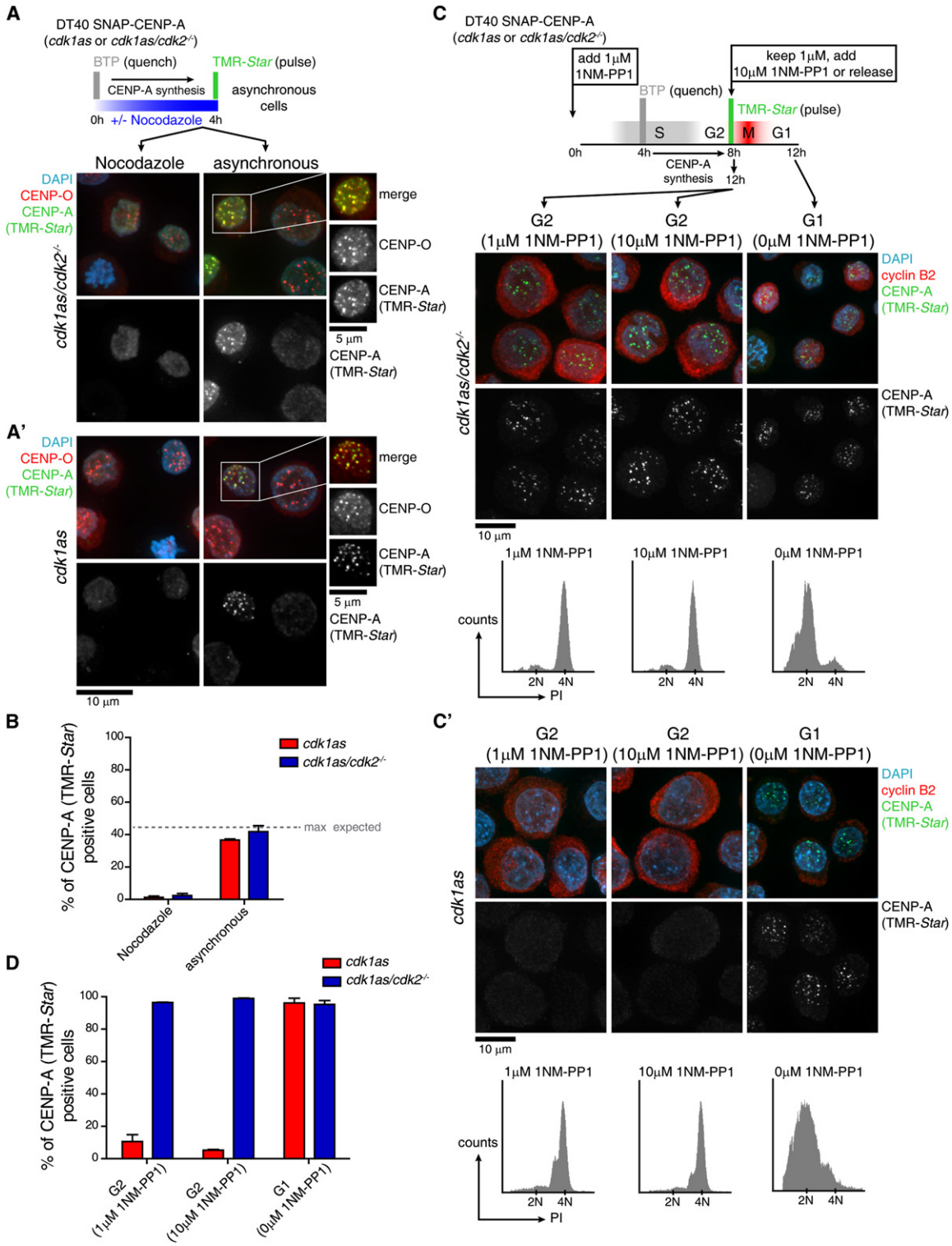

Figure 2. CENP-A Assembly Is Suppressed by Cdk1 and Cdk2 Activity

(A) DT40 *cdk1as/cdk2*$^{-/-}$ double mutant cells (i.e., Cdk2 null but active Cdk1) stably expressing SNAP-CENP-A were assayed for assembly of a nascent pulse labeled pool in either asynchronous cultures or cells prevented from entering G1 phase by nocodazole treatment. Insets show nascent SNAP-CENP-A (TMR-*Star*) colocalization with centromeres (CENP-O).

(A′) as A but for *cdk1as* single mutant (i.e., active Cdk1 and Cdk2).

(B) Quantification of (A) and (A′). Dotted line represents the maximally expected percentage of cells assembling CENP-A (3.5 hr synthesis/8 hr cell cycle [Zhao et al., 2007] × 100 = 44%).

chromosome segregation (Figure 5E). We further demonstrate that the only aspect of mitotic passage that is essential for subsequent CENP-A assembly is the resulting loss of mitotic Cdk activity. This does not exclude the possibility that other aspects of mitosis fine-tune CENP-A homeostasis as previously suggested (Brown and Xu, 2009). Because APC/C-mediated destruction of proteins, other than the Cdk1 activator cyclin B, is not required for CENP-A assembly we argue that the trigger initiating assembly likely depends directly on a phosphoswitch mediated by overlapping Cdk1 and Cdk2 kinase activity. The requirement for exit from mitosis to trigger CENP-A assembly is broadly conserved across transformed and untransformed human cells (Figure 1B; Figure S2) (Jansen et al., 2007; Hemmerich et al., 2008), *Drosophila* embryos (Schuh et al., 2007), and *Xenopus* extracts (Bernad et al., 2011; Moree et al., 2011), implying cell cycle control is maintained in an analogous manner. Intriguingly, however, a recent study demonstrated that unlike *Drosophila* embryos, $CID^{CENP\text{-}A}$ assembly in fly cell lines occurs in metaphase (Mellone et al., 2011). Possibly, in these cells, CID assembly is held in check exclusively by cyclin A-mediated Cdk activity during S and G2 phases. Cyclin A is degraded in early mitosis (den Elzen and Pines, 2001; Geley et al., 2001), potentially triggering the centromere targeting of CID in this system.

Phosphorylation of $Mis18BP1^{HsKNL2}$ is inversely correlated with its centromere localization as previously observed for the fission yeast CENP-A assembly factor Smc3 (Pidoux et al., 2009). We show here that preventing phosphorylation is sufficient to drive $Mis18BP1^{HsKNL2}$ centromere location. This suggests that the phosphoswitch controlling CENP-A assembly mediates CENP-A assembly factor activity or localization that includes, but is likely not limited to, $Mis18BP1^{HsKNL2}$.

Two general implications follow from these findings. First, as outlined in Figure 5E, our results provide a logical explanation for the cell cycle coupling between DNA replication, mitosis and CENP-A assembly. CENP-A is redistributed onto sister centromeres in S phase (Jansen et al., 2007; Dunleavy et al., 2011) during which histone H3 is assembled at neighboring positions (Dunleavy et al., 2011). The recently identified CENP-T/CENP-W complex specifically interacts with chromatin containing H3 nucleosomes directly adjacent to CENP-A nucleosomes (Hori et al., 2008; Ribeiro et al., 2010), suggesting that neighboring H3 and CENP-A nucleosomes make up an integral part of the centromeric complex. Importantly, such a mixed H3/CENP-A mitotic chromatin state can be achieved by delaying assembly of CENP-A until after mitosis, providing a possible explanation for the temporal disconnect between DNA replication and CENP-A loading. Second, inhibition of CENP-A loading is mediated exclusively by Cdk1 and Cdk2, which are in turn essential for the initiation of DNA replication (Bell and Dutta, 2002) and entry into mitosis. Conversely, loss of Cdk1 activity triggers licensing of the next round of DNA replication (Mailand and Diffley, 2005) coinciding with the temporal window during which CENP-A assembly is permitted (Figure 5E). We hypothesize that the Cdk-mediated molecular switch that turns the CENP-A assembly machinery "ON" in early G1 and then "OFF" after S phase entry is one and same. Consistent with this notion, quantitative live cell measurements have recently shown that CENP-A assembly in human cells continues until ~10 hr after anaphase, which coincides with late G1/early S phase (Lagana et al., 2010). This temporal restriction of CENP-A assembly activity during G1 phase may represent an important mechanism to maintain a proper centromere size and architecture.

EXPERIMENTAL PROCEDURES

Cell Lines and Constructs

HeLa cells and their derivatives were cultured in DMEM medium (GIBCO) supplemented with 10% newborn calf serum (GIBCO) at 37°C 5% CO_2. A HeLa cell line stably expressing both CENP-A–SNAP and LAP-(GFP)-Mis18α was generated using the previous established stable cell line expressing CENP-A-SNAP (Jansen et al., 2007). A construct containing *LAP-(GFP)-Mis18α* (a gift from I. Cheeseman, MIT, Cambridge, MA) was stably integrated into this cell line via Moloney murine leukemia retroviral delivery. Cells stably expressing CENP-A-SNAP and LAP-(GFP)-Mis18α (referred to as GFP-Mis18α throughout this paper) were selected by Blasticidin S (5 μg/ml; Invitrogen) and Puromycin (1.5 μg/ml; Calbiochem) and single-cell sorted by flow cytometry. The resulting monoclonal lines were expanded and selected by fluorescence microscopy. DMEM-F12 (GIBCO) medium supplemented with 10% fetal bovine serum (FBS; GIBCO) and 0.348% Sodium Bicarbonate was used to culture hTERT-RPE cells stably expressing CENP-A-SNAP at 37°C 5%CO_2. This cell line was generated by retroviral delivery of a construct carrying *CENP-A-SNAP-3XHA* (Jansen et al., 2007) and selected by Blasticidin S (10 μg/ml; Invitrogen) analogous to HeLa cell lines described above.

A *3xHA-SNAP-CENP-A* construct named pLJ404 was generated by inserting a PCR-generated fragment carrying the chicken *CENP-A* open reading frame (gift from T. Fukagawa, NIG, Mishima, Japan) flanked by BamHI and XbaI sites into corresponding sites of pSS26m (Covalys) containing an additional triple HA tag at its N terminus. The resulting 3xHA-SNAP-CENP-A fusion protein (referred to as SNAP-CENP-A throughout this paper) was subcloned into p3XFLAG-CMV-14 (Sigma) resulting in pLJ410 (including a STOP codon, excluding FLAG from the ORF). DT40 cell lines were cultured in RPMI1640 medium (GIBCO) supplemented with 50 μM β-mercaptoethanol, 10% FBS and 1% chicken serum (GIBCO) at 39°C 5%CO_2. Stable lines expressing SNAP-CENP-A were created in DT40 *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cells (Hochegger et al., 2007) by electroporation with a Gene Pulser apparatus (Bio-Rad) at 550 V and 25 μF as previously described (Sonoda et al., 1998). Puromycin (0.5 μg/ml, Calbiochem) and Zeocin (500 μg/ml, Invivogen) were used to select *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cell lines, respectively. Clonal lines expressing SNAP-CENP-A at subendogenous levels were selected by fluorescence microscopy after TMR-*Star* labeling and by western blot using an anti-chicken CENP-A antibody (gift from T. Fukagawa, NIG, Mishima, Japan).

The *Mis18BP1*^{HsKNL2} ORF was amplified from cDNA (kind gift from Paul Maddox) by PCR and cloned into the XhoI/EcoRI sites of pIC113 (kind gift from Iain Cheeseman) creating pLJ415 expressing a GFP-TEV-S-tag-$Mis18BP1^{HsKNL2}$ fusion protein. The construct expressing the $Mis18BP1^{HsKNL2}$-Ala^{24} mutant (pLJ451) was identical to pLJ415 except that residues S110, S134, S135, S191, S192, T260, T261, S263, S299, S365, S541, T653, T821, S824, S914, S991, T992, T993, S1004, S1008, S1086, S1087, S1089, and S1104 are mutated to alanine.

(C) DT40 *cdk1as/cdk2*$^{-/-}$ cells were synchronized in G2 phase with a low concentration (1 μM) of 1NM-PP1, followed by synthesis of a nascent SNAP-CENP-A pool. Cells were then either kept arrested in G2 with low (1 μM) or high (10 μM) 1NM-PP1 or released into G1 followed by fixation and processing for imaging or FACS (PI: propidium iodide). Cyclin B2 staining indicates G2 phase.
(C′) As in (C) but for *cdk1as* single mutant cells.
(D) Quantification of (C) and (C′). Mean and SEM of three replicates of each condition are shown in (B) and (D).

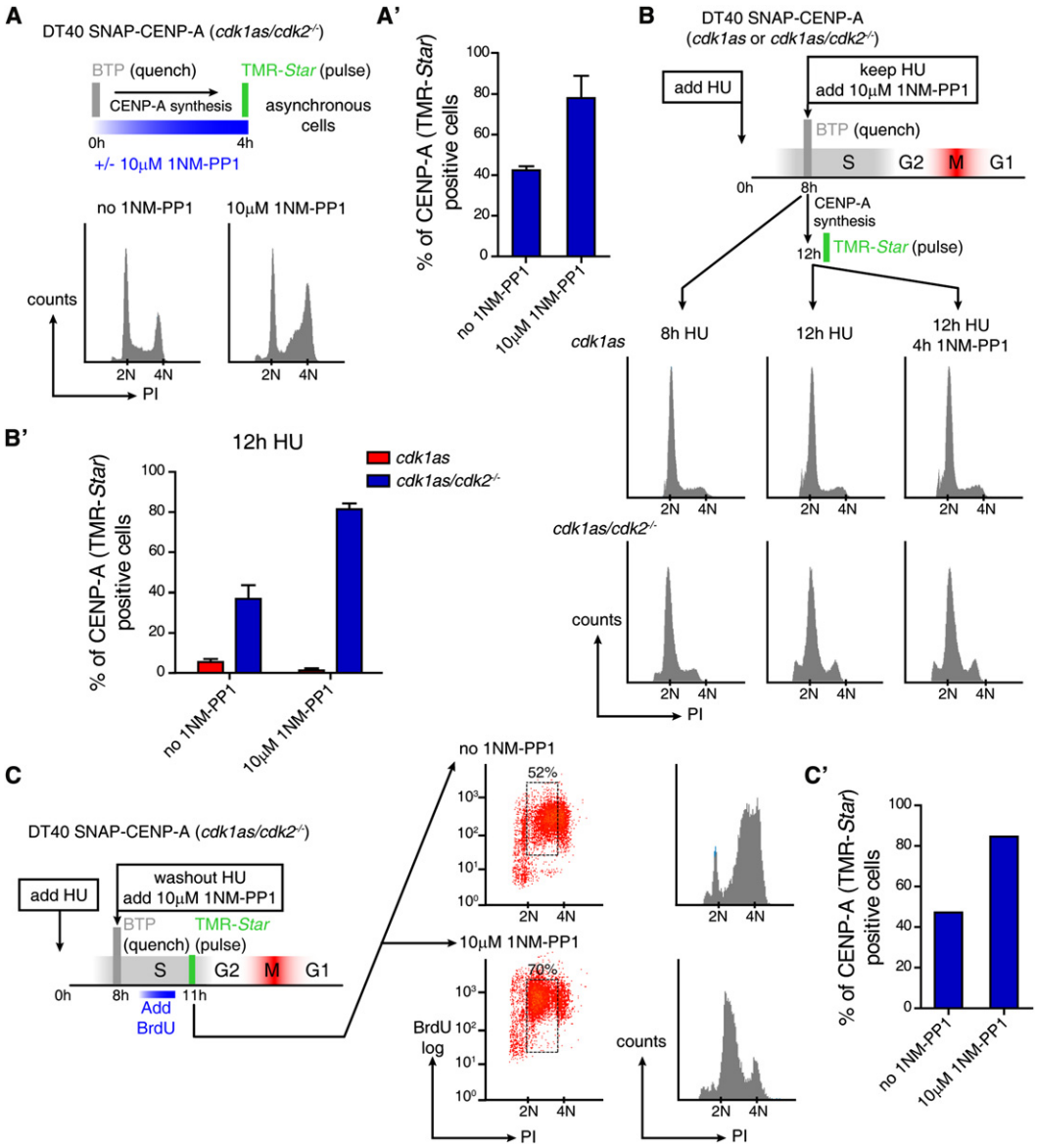

Figure 3. S Phase Cells Are Competent for CENP-A Assembly

(A) Asynchronous cultures of DT40 *cdk1as/cdk2*$^{-/-}$ SNAP-CENP-A cells were treated with DMSO or 10 μM 1NM-PP1 to induce G2 arrest. During the arrest, a nascent pool of SNAP-CENP-A was synthesized and pulse labeled. Cells were then assayed for assembly by microscopy (Figure S3B) and for cell cycle position by FACS. (A′) Percent cells assembling CENP-A at centromeres.

(B) DT40 *cdk1as* and *cdk1as/cdk2*$^{-/-}$ SNAP-CENP-A cells were arrested in S phase by HU treatment. A nascent SNAP-CENP-A pool was labeled in the presence or absence of 1NM-PP1 under continued HU arrest and scored for centromere assembly (Figure S3C). Samples were collected for FACS analysis before (8 hr) and after (12 hr) nascent SNAP-CENP-A synthesis to monitor continued S phase arrest.

(B′) Quantification of B. Percent cells assembling CENP-A at centromeres is scored.

(C) Experiment as in B except that *cdk1as/cdk2*$^{-/-}$ cells were released from HU-induced S phase arrest in the presence of BrdU to monitor active DNA replication during which a new SNAP-CENP-A pool was synthesized. Cells were scored for CENP-A centromere assembly in actively replicating cells in the absence or presence of 1NM-PP1. Fraction of cells undergoing DNA replication is indicated (boxed region). PI: propidium iodide.

(C′) Quantification of percentage of cells assembling CENP-A. Mean and SEM of 3 replicates of each condition are shown in (A′) and (B′). See also Figure S3.

Cell Synchronization, Transfection, and Drug Treatments

HeLa cells were synchronized by a double thymidine block. Cells were treated with thymidine (2 mM, Sigma) for 17 hr, washed twice in medium, and released in medium containing deoxycytidine (24 μM, Sigma) for 9 hr. Subsequently, cells were treated again with thymidine for 16 hr, and finally released into medium containing deoxycytidine and assayed.

HeLa cells were transfected with 250 ng of DNA, 1 μl Plus Reagent and 1.25 μl of lipofectamine (Invitrogen) in Optimem (GIBCO) according to manufactures instructions for Figure 5B. For Figure 5D, HeLa cells were transfected with 400 ng of plasmid DNA using Effectene transfection reagent (QIAGEN) in Opti-MEM reduced serum media (Invitrogen) according to the manufacturer's instructions.

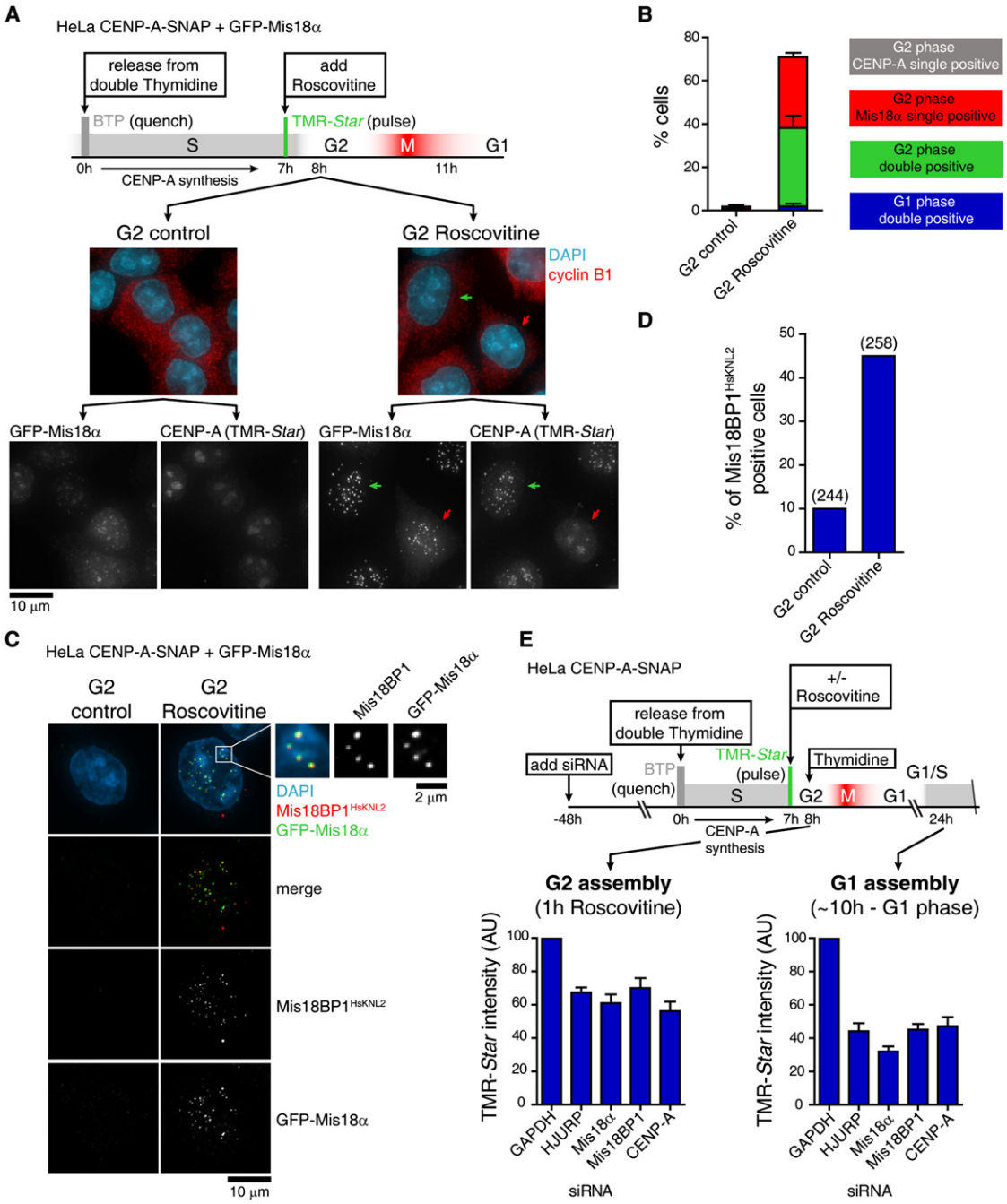

Figure 4. Unscheduled CENP-A Assembly in G2 Phase Occurs through the Canonical Assembly Pathway

(A) CENP-A-SNAP, GFP-Mis18α double-tagged HeLa cells were treated as in Figure 1B. G2 control or Roscovitine-treated cells were counterstained for cyclin B to confirm G2 status and imaged to determine GFP-Mis18α and nascent CENP-A-SNAP centromere localization.

(B) Quantification of (A).

(C) Experiment as in A but stained using antibodies against endogenous Mis18BP1^{HsKNL2}.

(D) Quantification of (C). Number of cells analyzed is indicated between brackets.

(E) HeLa CENP-A-SNAP cells were treated with siRNAs against indicated targets (GAPDH and CENP-A serve as negative and positive controls, respectively), synchronized by double thymidine block combined with SNAP quench-chase-pulse labeling. Cells were treated with Roscovitine for 1 hr in G2 to induce CENP-A assembly or were cycled into the next cell cycle and collected at the next G1/S boundary following canonical CENP-A assembly. Cells were imaged and TMR-*Star* centromere intensity was determined. More than 1,200 centromeres were quantified per condition. Mean and SEM of three replicates of each condition are shown in (B) and (E). See Figure S4 for siRNA efficiency controls.

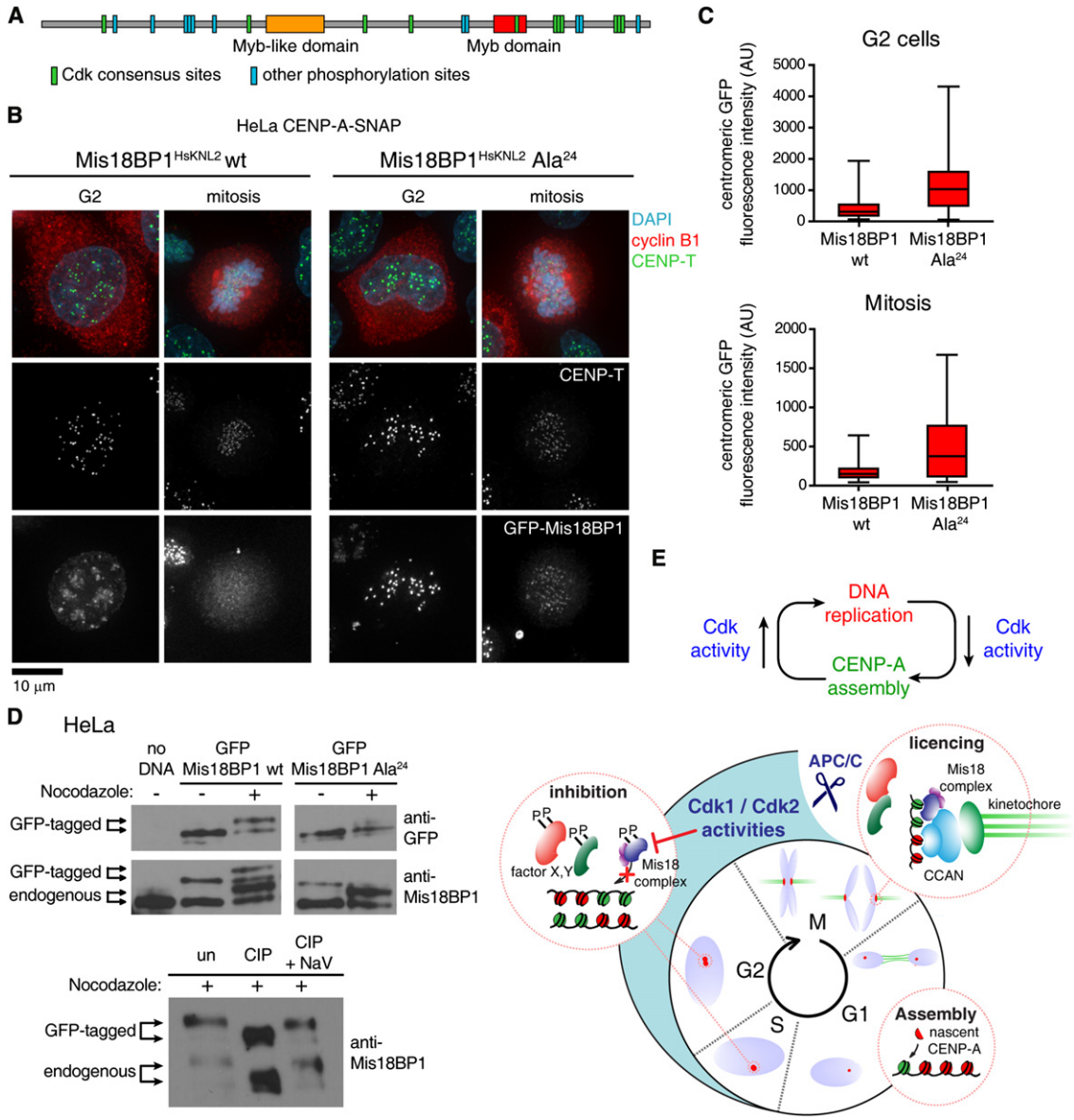

Figure 5. Phosphorylation of Mis18BP1^{HsKNL2} Controls Its Centromere Localization

(A) Schematic of Mis18BP1^{HsKNL2} protein. Relevant domains and known phosphorylation sites are indicated.

(B) Constructs expressing wild-type GFP-tagged Mis18BP1^{HsKNL2} or Mis18BP1^{HsKNL2}-Ala^{24} were transfected into asynchronous HeLa cells 32 hr prior to fixation followed by counterstaining for cyclin B, CENP-T, and with DAPI to indicate G2 status, centromeres and DNA, respectively.

(C) Box and whisker plots of relative GFP-Mis18BP1^{HsKNL2} fluorescent signal per centromere in G2 phase (high cyclin B) and mitotic cells. CENP-T was used as a reference for centromere position. More than 300 and more than 90 centromeres were quantified in G2 and mitotic cells, respectively.

(D) Top: HeLa cells transiently expressing GFP-tagged Mis18BP1^{HsKNL2} or Mis18BP1^{HsKNL2}-Ala^{24} for 29 hr were left untreated or were treated with nocodazole for another 12 hr to enrich for mitotic cells, followed by processing for SDS-PAGE and immunoblotting. Endogenous and GFP-tagged Mis18BP1^{HsKNL2} is detected by indicated antibodies. High molecular weight species are detected in nocodazole treated cells. Bottom: GFP-tagged Mis18BP1^{HsKNL2} was pulled down from mitotic cell extracts that were either untreated (un), treated with calf intestinal alkaline phosphatase (CIP) alone or in combination with sodium orthovanadate (NaV), followed by SDS-PAGE and immunoblotting for Mis18BP1^{HsKNL2}.

(E) Top: Schematic outlining inverse relationship between DNA replication and CENP-A chromatin assembly driven by the Cdk activity cycle. Bottom: Cartoon illustrates Cdk1/Cdk2-mediated inhibition of CENP-A assembly, exerted in part through phosphorylation (P) of Mis18BP1^{HsKNL2} (member of the Mis18 complex) during S, G2, and M phases. Factors X and Y symbolize the involvement of other, yet to be identified, components. Inhibition is alleviated through APC/C-mediated loss of Cdk1 activity in anaphase, targeting the Mis18 complex to the centromere (licensing) followed by CENP-A assembly in G1 phase. Canonical (H3 containing) nucleosomes are shown in green, CENP-A nucleosomes in red. See also Figure S5.

Roscovitine, Purvalanol A, MG132 and cycloheximide (Sigma) were used at 100 μM, 25 μM, 24 μM, 10 μg/ml, respectively. DMSO (Sigma) was used in control conditions. TNFα (R&D Systems) was used at 50 ng/ml (Seldon et al., 2007). MLN8054 (Selleck Chemicals) and ZM447439 (Enzo Life Sciences) were used at 1 and 2 μM, respectively. DT40 cells were treated with 1 or 10 μM of 1NM-PP1 (synthesized by Chris Larch and Hansjoerg Streicher, Sussex University, UK) for partial or strong inhibition of Cdk1as activity, respectively. DT40 cells were synchronized with 100 ng/ml nocodazole or 2 mM hydroxyurea (HU) (both from Sigma). Following release from an HU-induced S phase arrest (Figure 3C), DT40 cells were allowed to enter S phase for 30 min prior to 1NM-PP1 addition to avoid rearrest due to Cdk1 requirement to enter S phase.

SNAP Quench-Chase-Pulse Labeling

HeLa or hTERT-RPE cells expressing CENP-A-SNAP were pulse labeled by addition of 2 μM BTP (Covalys) in growth medium for 30 min at 37°C, 5% CO_2, for irreversible, nonfluorescent labeling of preexisting SNAP pool. We refer to this step as "quench." Following quenching, cells were chased for 6 hr and 30 min to allow synthesis of new, unlabeled CENP-A-SNAP and were then pulse labeled with 2 μM TMR-*Star* (New England Biolabs) in growth medium for 15 min at 37°C, 5% CO_2, thereby fluorescently labeling the nascent SNAP pool, specifically. DT40 cells were quenched as described for HeLa cells, except that the chase time was 3 hr and 30 min and kept at 39°C 5% CO_2 and the cells were pulse labeled with 5 μM of TMR-*Star*. After each labeling step (both fluorescent and nonfluorescent), cells were washed twice with medium and reincubated at the appropriate temperature to allow excess SNAP substrate to be released from cells. After 30 min, cells were washed again once in medium.

Immunofluorescence

HeLa or hTERT-RPE cells were grown on glass coverslips coated with poly-L-lysine (Sigma) and fixed with 4% formaldehyde (Thermo Scientific) for 10 min. DT40 cells were resuspended in PBS at a concentration of 2×10^5 cell/ml and cytospun at 800 rpm during 5 min. Cells were fixed with 4% formaldehyde. HeLa cells were stained with anti-cyclin B1 (1:50; sc-245, Santa Cruz), anti-CENP-T (Barnhart et al., 2011) and anti-Mis18BP1^{HsKNL2} (1 μg/ml; gift from P. Maddox, Université de Montréal, Montreal, Canada). hTERT-RPE cells were stained with anti-α-tubulin (1:2500; clone YL1/2, Serotec), anti-HA (1 μg/ml; clone HA11, Covance). DT40 cells were stained with anti-chicken CENP-O (1:3000; gift from T. Fukagawa, National Institute of Genetics, Mishima, Japan), anti-chicken cyclin B2 (1:50; gift from E. Nigg, University of Basel, Basel, Switzerland). Secondary antibodies (Cy5- or FITC-conjugated anti-mouse, FITC- or Cy3-conjugated anti-rabbit and FITC-conjugated anti-rat) were obtained from Jackson Immunoresearch Laboratories. Dy680 conjugated anti-mouse antibodies were from Rockland Immunochemicals. Cells were stained with DAPI (4′,6-diamidino-2-phenylindole; Sigma) before mounting in Mowiol.

siRNA Transfection

All siRNAs were obtained from Dharmacon. Smart pools were used to deplete Mis18BP1^{HsKNL2}, HJURP, and GAPDH. CENP-A and Mis18α were depleted with siRNAs: 5′-ACAGUCGGCGGAGACAAGGdTdT-3′ and 5′-CAGAAGCU AUCCAAACGUGdTdT-3′, respectively. Sixty picomoles of siRNAs was used for each depletion in a 24-well format according to the manufacturer's instructions.

Flow Cytometry

DT40 cells (10^6) were harvested and fixed during 1 hr at 4°C with 70% ethanol. Cells were washed twice in PBS containing 3% BSA (Sigma) and incubated for 3 hr at room temperature with 5 μg/ml propidium iodide (PI; Sigma) and 200 μg/ml of RNaseA in PBS containing 3% BSA. Subsequent flow-cytometric analysis was performed on a FACScan (Becton Dickinson) or FACS Canto (Becton Dickinson) using CellQuest and FACSDiva software, respectively. For BrdU staining we used an anti-BrdU antibody (347580, Becton and Dickinson). Cells were fixed as described above and processed for staining according to manufactures' instructions. Cells were subsequently stained with a Cy5 secondary antibody from Jackson Immunoresearch and with PI as described above. Cells were analyzed on a CyAn ADP (Beckman Coulter).

Immunoblotting

Extracts of 10^5 (HeLa) or 2×10^6 (DT40) cells were separated in a 6% (Figure 5D) or 12% (Figures S1 and S4) SDS-PAGE gel and transferred to a PVDF membrane. Blots were probed with anti-human-cyclin B1 (sc-245, Santa Cruz) at 1:500 dilution, anti-Actin (A2066, Sigma) at 1:1,000 dilution, anti-IκB-α (sc-371, Santa Cruz) at 1:1,000 dilution, anti-HJURP and anti-GFP (Foltz et al., 2009) at 1:2,000 and 1:10,000 dilution, respectively. For blot shown in Figure 5D, anti-GFP was used at 1:1,000 dilution overnight at 4°C. Anti-Mis18BP1$^{hsKNL-2}$ antibody (A302-824A, Bethyl Labs) was a 1:5,000 dilution overnight at 4°C. To screen DT40 monoclonal lines stably expressing subendogenous levels of 3xHA-SNAP-CENP-A we used anti-chicken CENP-A (gift from T. Fukagawa, NIG, Mishima, Japan) and anti-HA (HA11, Covance Research Products, Inc.) antibodies at dilution of 1:3,000 and 1:1,000, respectively. Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories.

Phosphatase Treatment

Lysates were prepared from a HeLa cell line stably expressing LAP-Mis18BP1^{HsKNL2} that had been blocked for 12 hr with 100 ng/ml nocodazole. Cells enriched in mitosis were harvested with 3 mM EDTA-PBS for 10 min at room temperature. Cells were resuspended in buffer containing 75 mM HEPES (pH 7.5), 1.5 mM EGTA, 1.5 mM $MgCl_2$, 150 mM KCl, 15% glycerol, 0.075% IGEPAL, 10 mM imidazole, 200 μM sodium orthovanadate (NaV), 5 mM sodium fluoride, 50 mM β-glycerophosphate, and Complete EDTA-free Protease Cocktail (Roche) and sonicated on ice in 30 s cycles for a total of 2 min. Lysates were centrifuged for 10 min at 1,000 × g at 4°C and the supernatant was passed five times over a column containing His-tagged GFP binding protein (GBP) bound to Ni-NTA agarose. Proteins bound to the GBP beads were washed, resuspended in 30 μl wash buffer and incubated with 10 mM sodium orthovanadate (MP Biomedicals) and/or 60 units of calf intestinal alkaline phosphatase (CIP; New England Biolabs) for 1 hr at 37°C. Reactions were stopped by the addition of SDS sample buffer and separated by SDS-PAGE.

Microscopy

Digital images were captured using a DeltaVision Core system (Applied Precision) that controls an inverted microscope (Olympus, IX-71), coupled to a Cascade2 EMCCD camera (Photometrics). Images (512 × 512) were collected at 1× binning using a 100× oil objective (NA 1.40, UPlanSApo) with 0.2 μm z sections scanning the entire nucleus. Images were subsequently deconvolved and maximum signals were projected as 2D images using softWoRx (Applied Precision). Centromeric TMR intensity was quantified on non-deconvolved, maximum projection images by placing a 7 × 7 pixel square on each centromere using an unrelated centromere marker (CENP-T). Local background corrected intensity values were obtained by subtracting minimum intensity values from maximum values for each centromere measurement.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.devcel.2011.10.014.

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