Cdk activity couples epigenetic centromere inheritance to cell cycle progression

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Running title: Cell cycle control of CENP-A assembly

Abstract

Centromeres are chromosomal loci that drive chromosome segregation during mitosis. Structure and identity of centromeres are maintained epigenetically by nucleosomes containing the histone H3 variant CENP-A. Propagation of CENP-A chromatin is uncoupled from DNA replication and initiates following mitotic exit. However, the nature of the signal that triggers assembly of CENP-A is unknown. We now show that neither entry into mitosis, nor protein destruction upon mitotic exit is required for CENP-A assembly. Instead, direct inhibition of Cdk1 and Cdk2 activities is sufficient for rapid CENP-A assembly at centromeres without mitotic passage, in a manner dependent on the canonical CENP-A assembly factors. Our 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 state by Cdk activity during S, G2 and M phases, ensuring tight coupling between DNA replication, cell division and subsequent centromere maturation.

Highlights for Silva et al:

- Onset of CENP-A assembly in G1 phase is triggered by loss of Cdk1 activity

- Cdk1 and Cdk2 are sufficient to inhibit CENP-A assembly outside of G1 phase

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 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. Centromeres are propagated epigenetically, largely independent of any particular DNA sequence (Allshire and Karpen, 2008; Amor et al., 2004). Key to the maintenance of centromere identity is the specific assembly of the histone H3 variant Centromere Protein A (CENP-A) into centromeric nucleosomes (Silva and Jansen, 2009; Black and Bassett, 2008). Inheritance and replication of this mark is essential to ensure epigenetic propagation of centromere identity. 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 is restricted to late mitosis / early G1 phase of the cell cycle (Jansen et al., 2007; Schuh et al., 2007; Hemmerich et al., 2008). Assembly strictly depends on passage through mitosis (Jansen et al., 2007; Schuh et al., 2007) 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 chromatin architecture (Jansen et al., 2007; Mellone and Allshire, 2003), APC-mediated destruction of a specific inhibitor of CENP-A assembly (Erhardt et al., 2008), or

assembly of a proper kinetochore-microtubule interface (Allshire and Karpen, 2008; Jansen et al., 2007). Here, we sought to identify the molecular signal that temporally controls CENP-A assembly. Our results point to a simple mechanism that excludes the need for any active involvement of mitosis in subsequence CENP-A assembly, other than the concomitant down regulation of Cdk activity upon mitotic exit.

Results

CDK inhibition is sufficient to induce CENP-A assembly

In order to identify the molecular mechanism controlling the unusual timing of CENP-A assembly, we employed SNAP-based quench-chase-pulse labeling [see Methods and (Jansen et al., 2007)] to uniquely and directly track the fate of nascent proteins. One defining feature of mitotic exit is APC mediated destruction of cyclin B and concomitant loss of associated cyclin dependent kinase (Cdk1) activity. We therefore hypothesized that the CENP-A assembly process might be controlled directly by Cdk activity, without a strict need for APC activation, destruction of APC 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 hour) treatment with the pan-Cdk inhibitors Roscovitine or Purvalanol A induced assembly in nearly half the G2 population whereas assembly in G2 phase is never observed in control cells in which the nascent pool remained diffusely nuclear (Figures 1B, C, and S1A-C). Assembly under these transiently induced 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 S2). However, a brief, 1 hour induction of CENP-A assembly in this phase is likely incomplete as normal CENP-A

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accumulation at centromeres continues for the duration of G1 phase [~10 hours (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 S1D). Preservation of high cyclin B levels suggests that Roscovitine treated cells do not enter a precocious G1-like state by premature activation of APC mediated protein destruction. Indeed, blocking global proteolysis by the proteasome inhibitor MG132 did not interfere with G2 induced CENP-A assembly. Similarly, treatment with Cycloheximide shows that *de novo* synthesis of proteins is not required for unscheduled CENP-A loading (Figures 1D and E). These results argue against a role for APC-mediated destruction of a putative inhibitor of CENP-A assembly other than the Cdk activator cyclin B.

Unscheduled CENP-A assembly requires the canonical assembly factors

We next determined whether unscheduled CENP-A assembly induced in G2 phase resulted from the activation of the canonical CENP-A assembly pathway. Mis18α, Mis18β and the Myb-domain containing protein HsKNL2/M18BP1 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 HsKNL2/M18BP1 to centromeres (Figures 2A, B, and S3). Cells displayed either centromere localized GFP-Mis18α alone (Figure 2A, red arrow) or both GFP-Mis18α and nascent CENP-A-SNAP (Figure 2A, green arrow) but never CENP-A-SNAP alone (Figure 2B). 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, depletion of Mis18α,

HsKNL2/M18BP1 or the CENP-A specific chaperone HJURP (Dunleavy et al., 2009; Foltz et al., 2009) by siRNA treatment resulted in a reduction of both G1 phase assembly of nascent CENP-A as well as unscheduled assembly in G2 phase (Figure 2C).

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 are Cdk2 and Cdk1, both of which are naturally deactivated upon mitotic entry and mitotic exit, respectively (Pines, 2006), and both of which are strongly inhibited by Roscovitine and Purvalanol A (Wesierska-Gadek and Krystof, 2009). However, due to the broad substrate specificity of pan-Cdk 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 Cdk2. We utilized cells that carry either a homozygous Cdk2 deletion (*cdk2-/-*) and/or express analogue sensitive Cdk1 in a homozygous *cdk1* null background, which can be selectively inhibited by addition of the ATP analog 1NM-PP1 (*cdk1as*) (Hochegger et al., 2007). 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 3A).

Assembly of nascent SNAP-CENP-A in DT40 cells occurred only in cells with low cyclin B levels and can be fully blocked when cells are prevented from entry into G1 by Nocodazole induced mitotic checkpoint arrest (Figures 3A, A´, and B). This

demonstrates that, like in human cells [Figure 1A and (Jansen et al., 2007; Hemmerich et al., 2008)] and fly embryos (Schuh et al., 2007), chicken DT40 cells assemble CENP-A at centromeres only upon mitotic exit. Cdk2 is non essential 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 3A and B), 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 these concentrations Cdk1 activity is sufficient to drive S phase progression but not high enough to allow entry into mitosis resulting in a G2 arrest. 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 analogue to completely abolish Cdk1activity. While CENP-A assembly in G1 was normal in cells of either genotype that were released from the G2 arrest, *cdk1as* single mutants showed little centromere assembly in G2 (Figures 3C′ and D). 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 3C and D). Clearly, loss of both Cdk1 and Cdk2 is sufficient to induce premature CENP-A loading, which indicates these activities are responsible for suppressing the CENP-A assembly machinery prior to mitotic exit.

CENP-A assembly can be induced in S phase

Assembly of CENP-A occurs even at low doses of 1NM-PP1 in *cdk1as/cdk2-/-* cells which maintain sufficient Cdk1 activity to progress through S phase [Figure S4A and

(Hochegger et al., 2007)]. This suggests different thresholds of Cdk activity for the inhibition of CENP-A loading and initiation of DNA replication and that unperturbed assembly can occur as late as early S phase (Figure 4D). Consistent with this notion, quantitative live cell measurements have recently shown that CENP-A assembly in human cells continues until ~10 hours after anaphase which is consistent with early S phase (Lagana et al., 2010). Cdk1/2 double inactivation in randomly cycling cells resulted in CENP-A assembly in ~80% of the cells (Figures 4A and S4B). The FACS profile in these experiments indicates that approximately 30% of cells are in S phase. This suggests that loss of Cdk activity leads to CENP-A assembly not only during G1 and G2 phases, but possibly also in S phase. To directly test this possibility, we synchronized *cdk1as/cdk2$^{-/-}$* cells in S phase with hydroxyurea (HU) and assayed a nascent CENP-A-SNAP pool in these cells. ~25% of the cells with active Cdk1 (no 1NM-PP1) assembled CENP-A. This likely represents cells in early S phase, which have low Cdk1 activity and may therefore be intrinsically competent for assembly. Inhibition of Cdk1 activity in *cdk1as/cdk2$^{-/-}$* cells arrested throughout S phase induced CENP-A assembly in the majority of cells (~70%). This demonstrates that Cdk mediated inhibition of CENP-A assembly is maintained not only in G2 but also in S phase cells, which are otherwise competent for assembly (Figures 4B and S4C). We further validated this result by allowing cells to proceed into S phase in the presence of BrdU (to label DNA synthesis prior to 1NM-PP1 mediated induction of CENP-A assembly) confirming cell cycle position of these cells (Figure 4C).

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 completion of DNA replication and chromosome segregation. 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. Because APC-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 phospho-switch mediated by overlapping Cdk1 and Cdk2 kinase activity.

Several implications follow from these findings. First, as outlined in Figure 4D, our results provide a logical explanation for the cell cycle coupling between DNA replication, mitosis and CENP-A assembly. The recently identified CENP-T/CENP-W complex specifically and functionally interacts with histone H3 containing 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 only 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, since inhibition of CENP-A loading requires a higher Cdk activity threshold compared to S phase entry it is possible that cells are permissive for CENP-A assembly throughout G1 up to early S phase where cyclin A associated Cdk activity is still low. We propose 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. This temporal restriction of CENP-A assembly activity during G1 may represent an important mechanism to maintain a proper centromere size.

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.

A 3xHA-SNAP-CENP-A construct 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) (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% fetal bovine serum (Gibco) 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$^{-/-}$* backgrounds (Hochegger et al., 2007) by electroporation with a Gene Pulser apparatus (BioRad) at 550 V and 25 μF as 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 sub-endogenous 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).

Cell synchronization and drug treatments

HeLa cells were synchronized by a double thymidine block. Cells were treated with thymidine (2 mM, Sigma) for 17 h, washed twice in medium and released in medium containing deoxycytidine (24 μM, Sigma) for 9 h. Subsequently, cells were treated again with thymidine for 16h, and finally released into medium containing deoxycytidine and assayed. Roscovitine, Purvalanol A, MG132 and Cyclohexamide (Sigma) were used at 100 μM, 25 μM, 24 μM, 10 μg/mL respectively. DMSO (Sigma) was used in control conditions.

DT40 cells were treated with 1 μM 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).

SNAP quench-chase-pulse labeling

HeLa 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, non-fluorescent labeling of pre-existing SNAP pool . We refer to this step as "quench". Following quenching, cells were chased for 6h and 30min 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 3h and 30 min and kept at 39 ºC and the cells were pulse labeled with 5 μM of TMR-*Star*.

After each labeling step (both fluorescent and non-fluorescent), 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 cells were grown on glass coverslips coated with poly-L-Lysine and fixed with 4% formaldehyde for 10 min. DT40 cells were resuspended in PBS at a concentration of 2 x 10^5 cell/mL and cytospun at 800 rpm during 5 min. Cells were fixed with 4% formaldehyde for all experiments, except when staining for BrdU, in which case cells were fixed in MetOH/Acetone (1:1, Sigma), acid treated with 2 mM HCl during 10 min at RT and then washed 3 x 10 min with 100 mM Borax (Sigma). HeLa cells were stained with anti-CyclinB1 (1:50; sc-245, Santa Cruz), anti-CENP-T (crude serum 1:1000, gift from D. Foltz, University of Virginia, VA, USA) and anti-HsKNL2/M18BP1 (1 μg/mL; gift from P. Maddox, Université de Montréal, Montreal, Canada). DT40 cells were stained with anti-chicken CENP-O (1:3000; gift from T. Fukagawa, National Institute of Genetics, Mishima, Japan), anti-chicken CyclinB2 (1:50; gift from E. Nigg, University of Basel, Basel, Switzerland) and anti-BrdU (1:100; MoBU-1, Santa Cruz). Secondary antibodies (Cy5- or FITC-conjugated anti-mouse and FITC- or Cy3-conjugated anti-rabbit) were obtained from Jackson Immunoresearch Laboratories. Cells were stained with DAPI (Sigma) before mounting in Mowiol.

siRNA transfection

All siRNAs were obtained from Dharmacon. Smart pools were used to deplete HsKNL2/M18BP1, HJURP and GAPDH. CENP-A and Mis18α were depleted with siRNAs: 5'-ACAGUCGGCGGAGACAAGGdTdT-3' and 5'-CAGAAGCUAUCCAAACGUGdTdT-3' respectively. 60 pmoles of siRNAs were used for each depletion in 24 well format according to the manufacturer's instructions.

Flow cytometry

DT40 cells (10^6) were harvested and fixed during 1 h at 4°C with 70% ethanol. Cells were washed twice in PBS containing 3% BSA (Sigma) and incubated for 3 h at RT with 5 μg/ml propidium iodide (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.

Immunoblotting

Extracts of 10^5 (HeLa) or 2 x 10^6 (DT40) cells were separated in a 12% SDS-PAGE gel and transferred to a PVDF membrane. Blots were probed with anti-human-CyclinB1 (sc-245, Santa Cruz) at a dilution of 1:500. An anti-Actin (A2066, Sigma) antibody was used at a dilution of 1:1000 as a loading control. 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 1:3000 and 1:1000, respectively. Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were purchased from Jackon Immunoresearch Laboratories.

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). 512 x 512 images were collected at 1x binning using a 100x 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 x 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.

Figure legends

Figure 1. Cdk inhibition induces premature CENP-A assembly in a proteolysis independent manner. (A) Schematic representing mitotic transition 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 hours after release from thymidine. G2 cells were either mock treated (G2 control), treated with Roscovitine for 1 hour or allowed to cycle through mitosis (G1 control) prior to fixation. Cells were counterstained with cyclin B and DAPI to indicate G2 status and DNA respectively. (C) Quantification of B. (D) Experiment as in B but with the inclusion of MG132 or cycloheximide to block proteolysis or protein synthesis, respectively. (E) Quantification of D. Mean and SEM of 3 replicates are shown in C and E. Percentage of total cells positive for centromeric CENP-A-SNAP (TMR-*Star*) signal was scored and represented according to cyclin B status. See also Figure S1 and S2.

Figure 2. 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 1. 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) HeLa CENP-A-SNAP cells were treated with siRNAs against indicated targets (GAPDH and CENP-A serve as negative and positive controls, respectively) followed by synchronization by double thymidine block. Cells were treated with Roscovitine for 1 hour in G2 to induce CENP-A assembly or were cycled into the next cell cycle and collected at the next G1/S boundary

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following canonical CENP-A assembly. Cells were imaged and TMR centromere intensity was determined. >1200 centromeres were quantified per condition. Mean and SEM of 3 replicates of each condition are shown in B and C. See also Figure S3.

Figure 3. CENP-A assembly is suppressed by both Cdk1 and Cdk2 activity. (A) DT40 *cdk1as/cdk2-/-* double mutant cells (i.e. Cdk2 null but Cdk1 WT) stably expressing SNAP-CENP-A were assayed for assembly of a nascent pool in either asynchronous cultures or cells prevented from entering G1 phase by Nocodazole treatment. Blowups show nascent SNAP-CENP-A (TMR-*Star*) colocalization with centromeres (CENP-O). (A´) as A but for *cdk1as* single mutant (i.e. Cdk1 and Cdk2 WT). (B) Quantification of A and A´. Dotted line represents the maximally expected percentage of cells assembling CENP-A (3,5 hour synthesis / 8 hour cell cycle (Zhao et al., 2007) x 100 = 44%). (C) DT40 *cdk1as/cdk2-/-* cells were synchronized in S/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 (1µM) or high (10µM) 1NM-PP1 or released into G1 followed by fixation and processing for imaging or FACS. CyclinB2 staining indicates G2 phase. (C´) as C but for *cdk1as* single mutant cells. (D) Quantification of C and C´. Mean and SEM of 3 replicates of each condition are shown in B and D.

Figure 4. S phase cells are competent for CENP-A assembly. (A) Asynchronous cultures of DT40 *cdk1as/cdk2-/-* SNAP-CENP-A cells were treated with 10 µM 1NM-PP1 to induce G2 arrest. During the arrest, a nascent pool of SNAP-CENP-A was synthesized. Cells were then assayed for assembly and cell cycle position (FACS): No 1NM-PP1: 44% G1, 27% S, 26% G2; 10 µM 1NM-PP1: 29% G1, 30% S, 38% G2. Graph represents Mean and SEM of 3 replicates of each condition. (B) DT40 *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. Samples were collected for FACS analysis before (8h) and after (12h) nascent SNAP-CENP-A synthesis to monitor continued S phase arrest. (C) Experiment as in B except that cells were released from HU induced S phase arrest for 3 hours in the presence of BrdU to monitor DNA replication. This was followed by synthesis of a new SNAP-CENP-A pool and re-arrest in HU to prevent exit from S phase. Cells were scored for CENP-A centromere assembly. Partial BrdU staining confirms S phase positioning of cells assayed for assembly. (D) Schematic outlining how Cdk1/Cdk2 uncouples CENP-A assembly from DNA replication and mitosis. See also Figure S4.

Acknowledgements

References

Allshire, R. C., and Karpen, G. H. (2008). Epigenetic regulation of centromeric chromatin: old dogs, new tricks? Nat. Rev. Genet *9*, 923-937.

Amor, D. J., Bentley, K., Ryan, J., Perry, J., Wong, L., Slater, H., and Choo, K. H. A. (2004). Human centromere repositioning "in progress". Proc. Natl. Acad. Sci. U.S.A *101*, 6542-6547.

Berthet, C., Aleem, E., Coppola, V., Tessarollo, L., and Kaldis, P. (2003). Cdk2 knockout mice are viable. Curr. Biol *13*, 1775-1785.

Black, B. E., and Bassett, E. A. (2008). The histone variant CENP-A and centromere specification. Curr. Opin. Cell Biol *20*, 91-100.

Cheeseman, I. M., and Desai, A. (2008). Molecular architecture of the kinetochore-microtubule interface. Nat. Rev. Mol. Cell Biol *9*, 33-46.

Dunleavy, E. M., Roche, D., Tagami, H., Lacoste, N., Ray-Gallet, D., Nakamura, Y., Daigo, Y., Nakatani, Y., and Almouzni-Pettinotti, G. (2009). HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres. Cell *137*, 485-497.

Erhardt, S., Mellone, B. G., Betts, C. M., Zhang, W., Karpen, G. H., and Straight, A. F. (2008). Genome-wide analysis reveals a cell cycle-dependent mechanism controlling centromere propagation. J. Cell Biol *183*, 805-818.

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

Fujita, Y., Hayashi, T., Kiyomitsu, T., Toyoda, Y., Kokubu, A., Obuse, C., and Yanagida, M. (2007). Priming of centromere for CENP-A recruitment by human hMis18alpha, hMis18beta, and M18BP1. Dev. Cell *12*, 17-30.

Hemmerich, P., Weidtkamp-Peters, S., Hoischen, C., Schmiedeberg, L., Erliandri, I., and Diekmann, S. (2008). Dynamics of inner kinetochore assembly and maintenance in living cells. J. Cell Biol *180*, 1101-1114.

Hochegger, H., Dejsuphong, D., Sonoda, E., Saberi, A., Rajendra, E., Kirk, J., Hunt, T., and Takeda, S. (2007). An essential role for Cdk1 in S phase control is revealed via chemical genetics in vertebrate cells. J. Cell Biol *178*, 257-268.

Hori, T., Amano, M., Suzuki, A., Backer, C. B., Welburn, J. P., Dong, Y., McEwen, B. F., Shang, W., Suzuki, E., Okawa, K., et al. (2008). CCAN makes multiple contacts with centromeric DNA to provide distinct pathways to the outer kinetochore. Cell *135*, 1039-1052.

Jansen, L. E. T., Black, B. E., Foltz, D. R., and Cleveland, D. W. (2007). Propagation of centromeric chromatin requires exit from mitosis. J. Cell Biol *176*, 795-805.

Lagana, A., Dorn, J. F., De Rop, V., Ladouceur, A., Maddox, A. S., and Maddox, P. S. (2010). A small GTPase molecular switch regulates epigenetic centromere maintenance by stabilizing newly incorporated CENP-A. Nat. Cell Biol *12*, 1186-1193.

Maddox, P. S., Hyndman, F., Monen, J., Oegema, K., and Desai, A. (2007). Functional genomics identifies a Myb domain-containing protein family required for assembly of CENP-A chromatin. J. Cell Biol *176*, 757-763.

Mellone, B. G., and Allshire, R. C. (2003). Stretching it: putting the CEN(P-A) in centromere. Curr. Opin. Genet. Dev *13*, 191-198.

Ortega, S., Prieto, I., Odajima, J., Martín, A., Dubus, P., Sotillo, R., Barbero, J. L., Malumbres, M., and Barbacid, M. (2003). Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. Nat. Genet *35*, 25-31.

Pines, J. (2006). Mitosis: a matter of getting rid of the right protein at the right time. Trends Cell Biol *16*, 55-63.

Régnier, V., Vagnarelli, P., Fukagawa, T., Zerjal, T., Burns, E., Trouche, D., Earnshaw, W., and Brown, W. (2005). CENP-A is required for accurate chromosome segregation and sustained kinetochore association of BubR1. Mol. Cell. Biol *25*, 3967-3981.

Ribeiro, S. A., Vagnarelli, P., Dong, Y., Hori, T., McEwen, B. F., Fukagawa, T., Flors, C., and Earnshaw, W. C. (2010). A super-resolution map of the vertebrate kinetochore. Proc. Natl. Acad. Sci. U.S.A *107*, 10484-10489.

Santamaría, D., Barrière, C., Cerqueira, A., Hunt, S., Tardy, C., Newton, K., Cáceres, J. F., Dubus, P., Malumbres, M., and Barbacid, M. (2007). Cdk1 is sufficient to drive the mammalian cell cycle. Nature *448*, 811-815.

Schuh, M., Lehner, C. F., and Heidmann, S. (2007). Incorporation of Drosophila CID/CENP-A and CENP-C into centromeres during early embryonic anaphase. Curr. Biol *17*, 237-243.

Shelby, R. D., Monier, K., and Sullivan, K. F. (2000). Chromatin assembly at kinetochores is uncoupled from DNA replication. J. Cell Biol *151*, 1113-1118.

Silva, M. C. C., and Jansen, L. E. T. (2009). At the right place at the right time: novel CENP-A binding proteins shed light on centromere assembly. Chromosoma *118*, 567-574.

Sonoda, E., Sasaki, M. S., Buerstedde, J. M., Bezzubova, O., Shinohara, A., Ogawa, H., Takata, M., Yamaguchi-Iwai, Y., and Takeda, S. (1998). Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J *17*, 598-608.

Wesierska-Gadek, J., and Krystof, V. (2009). Selective cyclin-dependent kinase inhibitors discriminating between cell cycle and transcriptional kinases: future reality or utopia? Ann. N. Y. Acad. Sci *1171*, 228-241.

Zhao, G. Y., Sonoda, E., Barber, L. J., Oka, H., Murakawa, Y., Yamada, K., Ikura, T., Wang, X., Kobayashi, M., Yamamoto, K., et al. (2007). A critical role for the ubiquitin-conjugating enzyme Ubc13 in initiating homologous recombination. Mol. Cell *25*, 663-675.
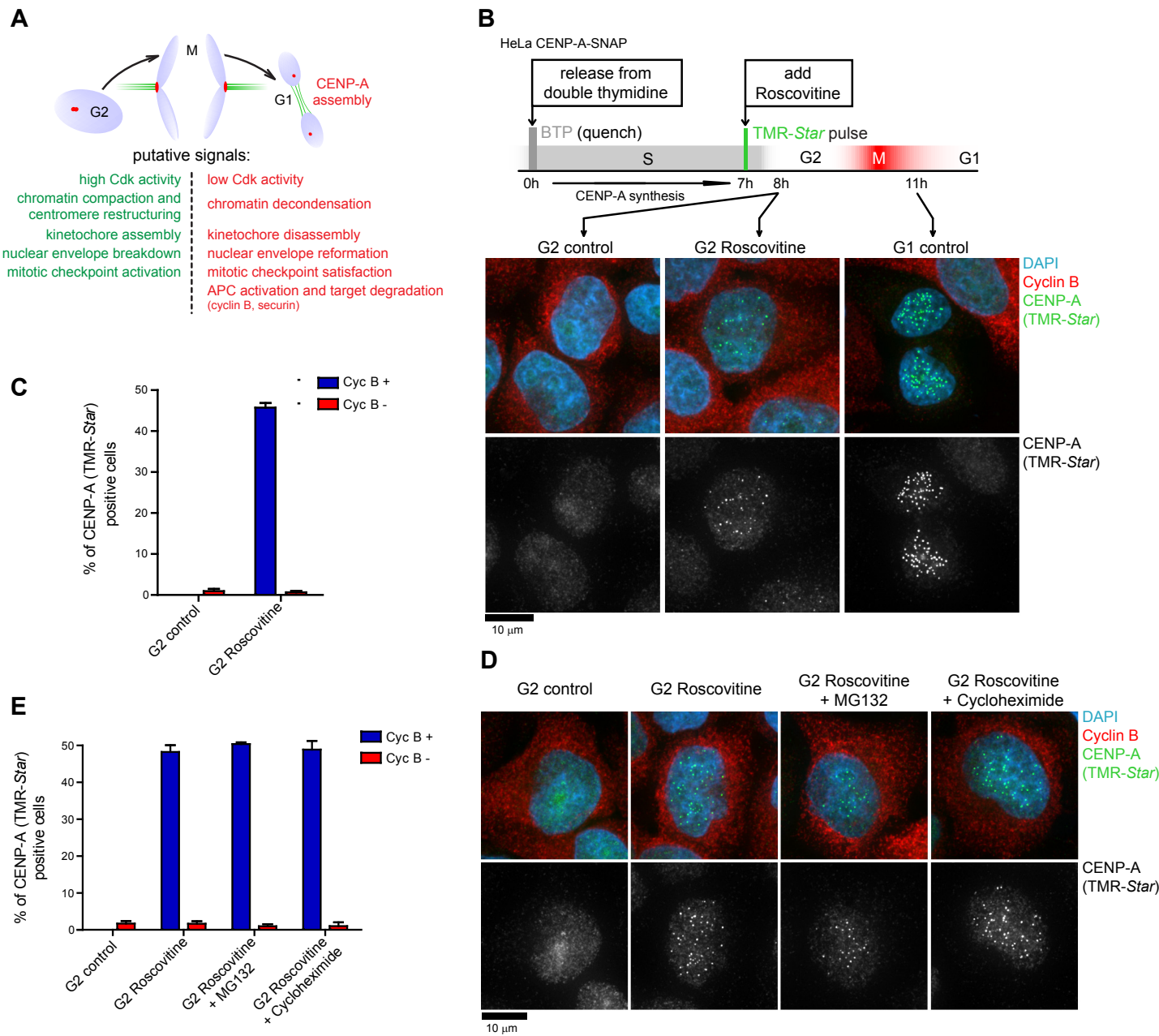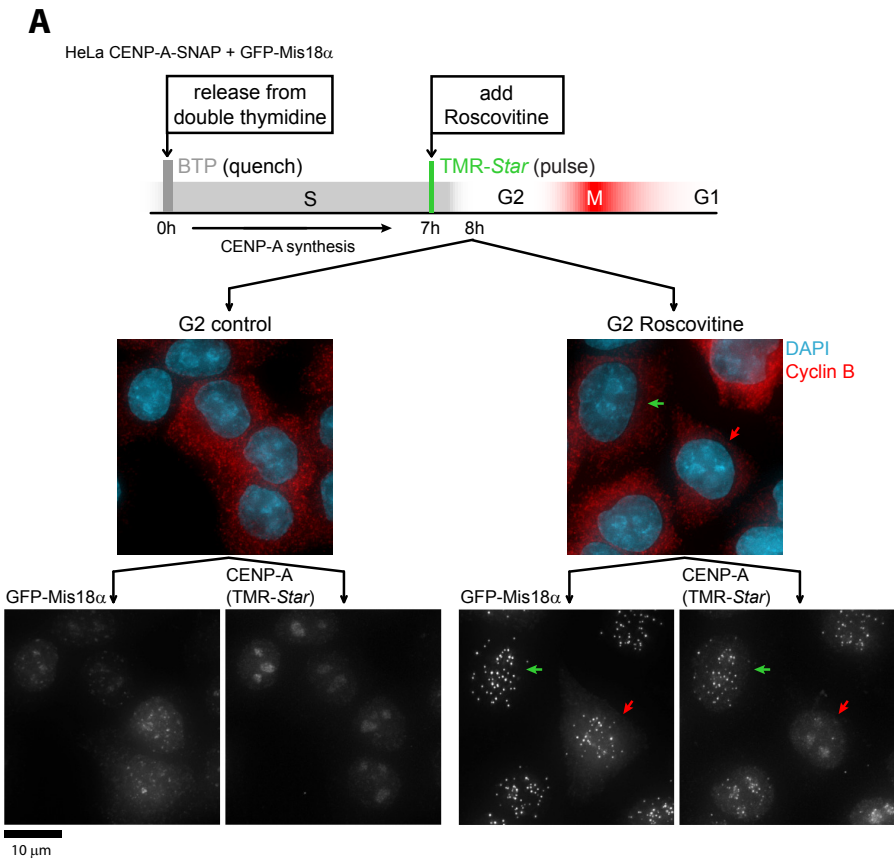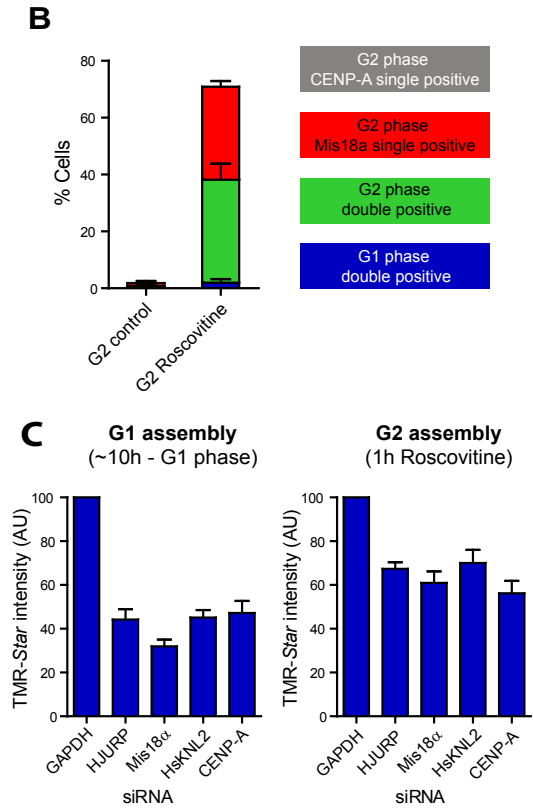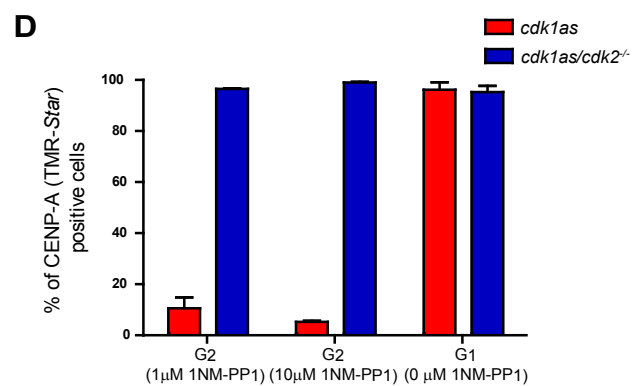
Figure 1

Figure 1

Figure 2

Figure 3
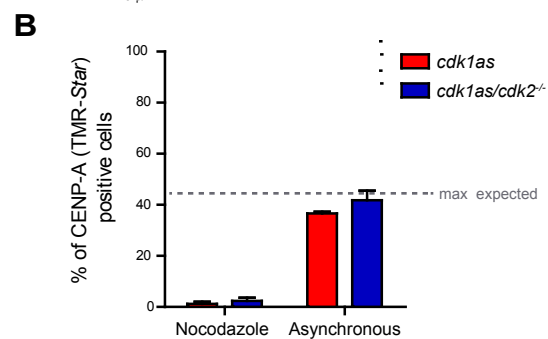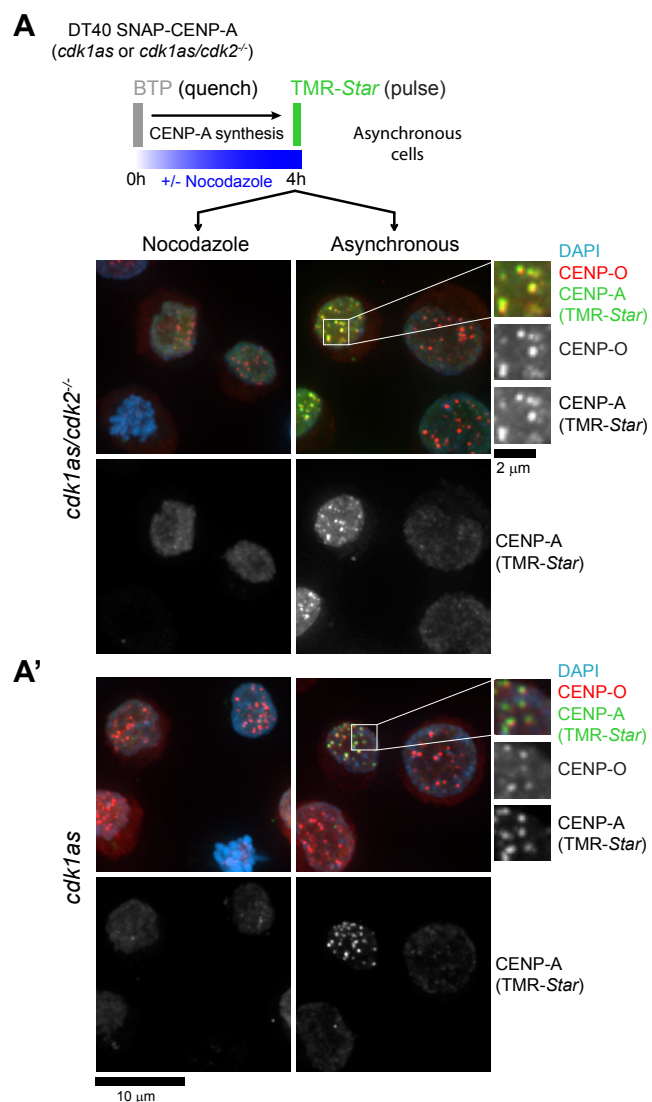
Figure 3

Figure 4

Figure 4

A

DT40 SNAP-CENP-A (*cdk1as/cdk2⁻ᐟ⁻*)

B

DT40 SNAP-CENP-A (*cdk1as/cdk2⁻ᐟ⁻*)

C

DT40 SNAP-CENP-A (*cdk1as/cdk2⁻ᐟ⁻*)

D
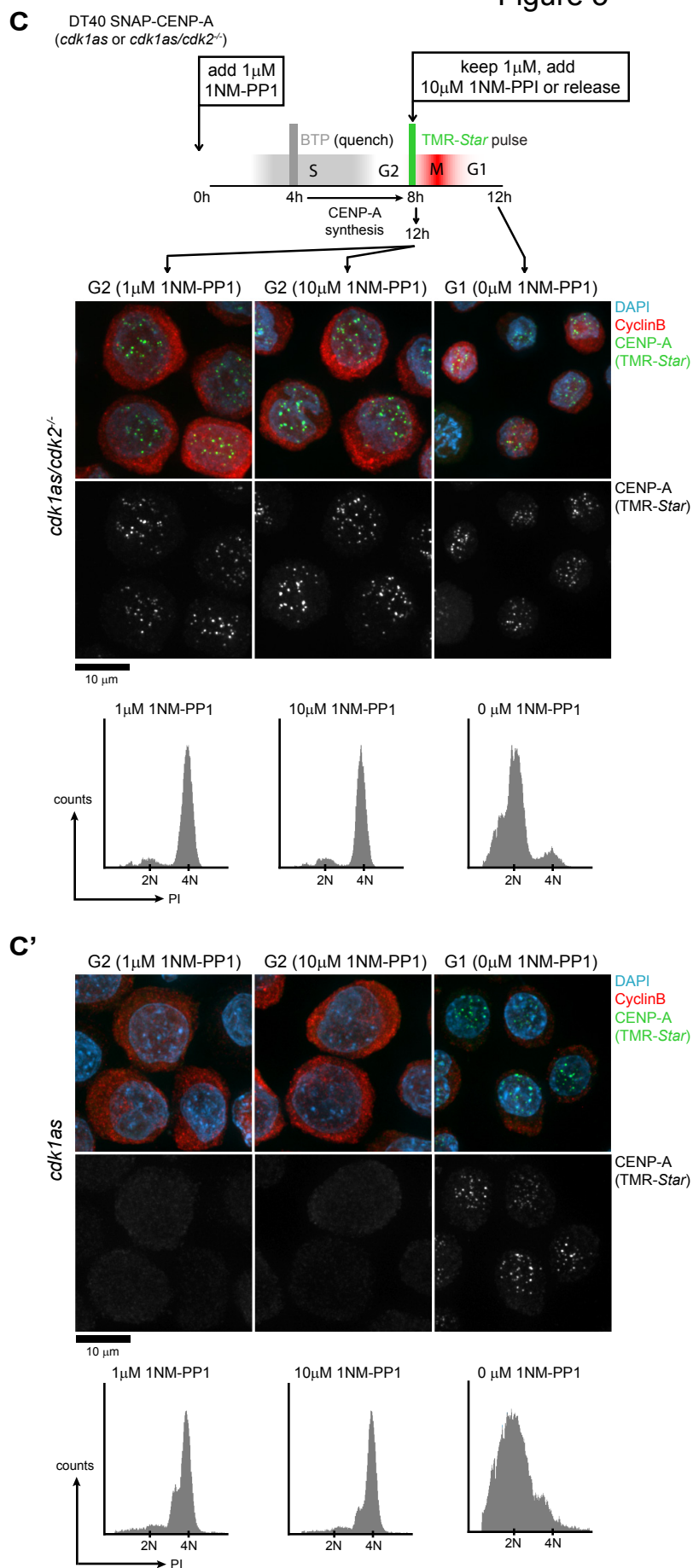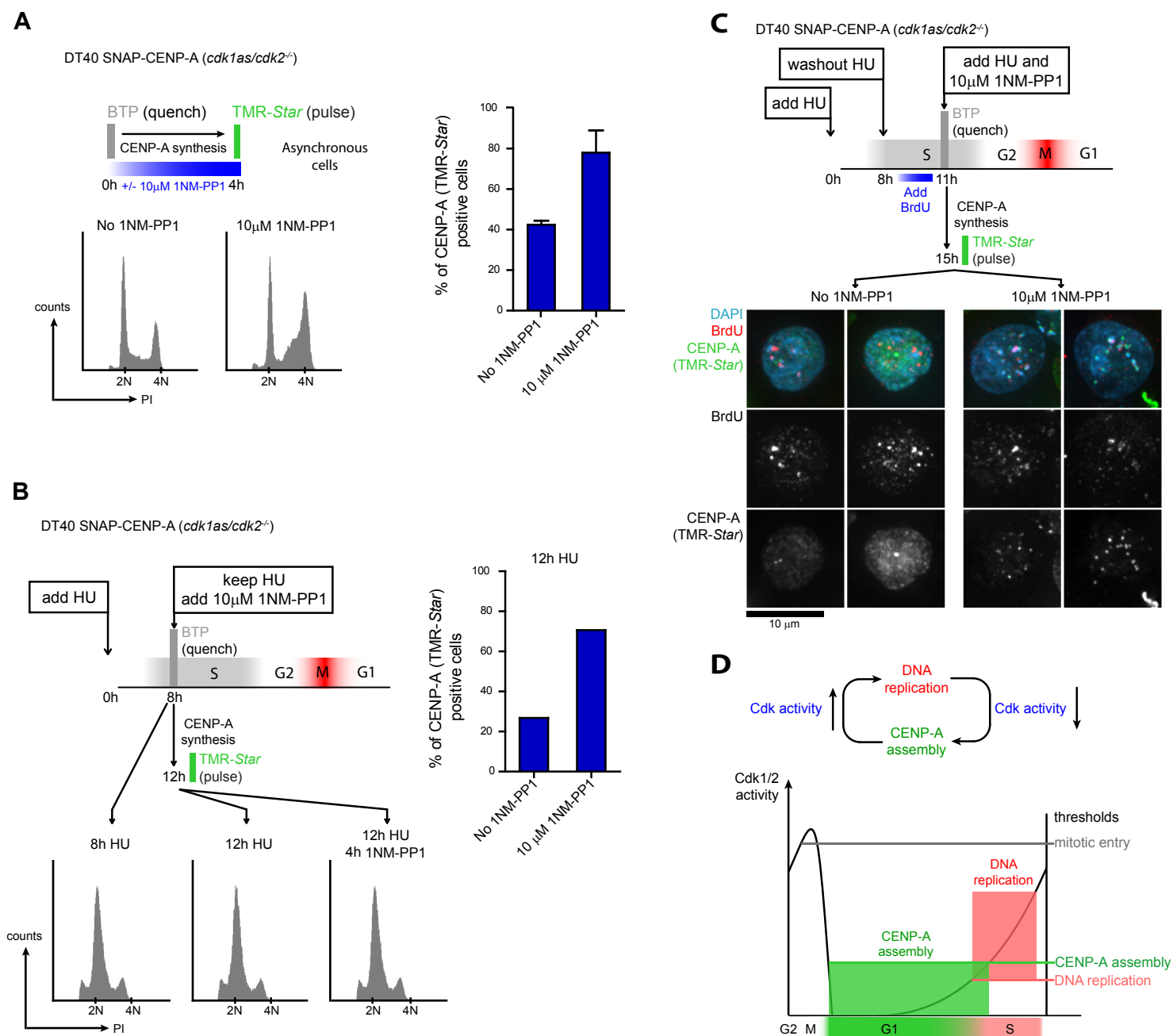
Inventory of supplemental Information

Supplemental figures:

Figure S1 provides data supporting Figure 1

Figure S2 provides data supporting Figure 1

Figure S3 provides data supporting Figure 2

Figure S4 provides data supporting Figure 4

Supplemental figures for Silva et al.

Figure S1. Roscovitine or Purvalanol A treatment induces CENP-A assembly in G2 phase. (A) Nascent CENP-A-SNAP colocalizes with centromeres (CENP-T) after Roscovitine induced assembly in G2 phase cells. (B) Experiment as in Figure 1, except treatment of cells in G2 with either Roscovitine or Purvalanol A. (C) Quantification of B. (D) G2 phase Cyclin B levels remain unchanged in MG132 and/or Roscovitine at concentrations used in Figure 1D. HeLa CENP-A-SNAP cells were synchronized in G2 by release from a double thymidine block followed by a 1 hour treatment with indicated drugs. Cells were processed for immunoblot and probed for Cyclin B1 levels or Actin (as a loading control) (D′), MG132 is sufficient to prevent cyclin B degradation upon mitotic exit. Experiment as in D except cells were arrested in mitosis by 6 hour treatment with nocodazole. Mitotic cells were collected by mitotic shake-off and either kept arrested, released into medium or medium containing MG132.

Figure S2. CENP-A-SNAP assembled in G2 phase is stable during mitosis. Experiment as in Figure 1 except cells were released from Roscovitine after a 1 hour induction of CENP-A assembly in G2 phase. Cells were analyzed in mitosis (1 hour after washout) and scored for retention of nascent CENP-A-SNAP on mitotic chromosomes.

Figure S3. Roscovitine induces premature centromere targeting of Mis18α and HsKNL2/M18BP1. (A) Experiment as in Figure 2 but stained using antibodies against endogenous HsKNL2/M18BP1. (B) Quantification of A. Number of cells analyzed is indicated between brackets.

Figure S4. Loss of Cdk1 and 2 activities in S phase results in unscheduled CENP-A assembly. (A) DT40 *cdk1as* or *cdk1as/cdk2$^{-/-}$* mutant cells were treated with either low (1 μM) or high (10 μM) 1NM-PP1 for 12 hours followed by processing for FACS. Note that whereas *cdk1as* single mutants arrest in G2 at both 1NM-PP1 concentrations used, *cdk1as/cdk2$^{-/-}$* double mutants can enter S phase and progress to G2 in low but not in high 1NM-PP1. (B) Images of cells quantified in Figure 4A. (C) Images of cells quantified in Figure 4B, demonstrating centromere assembly of nascent SNAP-CENP-A during S phase arrest.

A

G2 control

DAPI
CENP-T
CENP-A
(TMR-*Star*)

CENP-A   CENP-T
(TMR-Star)

G2 Roscovitine

2 µm

10 µm

B

HeLa CENP-A-SNAP

release from
double thymidine

add Roscovitine
or Purvalanol A

BTP (quench)   TMR-*Star* pulse

S   G2   M   G1

0h   7h   8h

CENP-A synthesis

G2 control   G2 Roscovitine   G2 Puvalanol A

DAPI
Cyclin B
CENP-A
(TMR-*Star*)

CENP-A
(TMR-*Star*)

10 µm

C

% of CENP-A (TMR-*Star*) positive cells

■ Cyc B +
■ Cyc B -

D   **D'**

	G2 Cells		Cells reseed after mitotic shake off		
Roscovitine	-	+	+	-	-	-
Nocodazole	-	-	-	+	-	-
MG132	-	-	+	-	-	+

Cyclin B1

Actin

HeLa CENP-A-SNAP

A

HeLa CENP-A-SNAP + GFP-Mis18α

B

A

DT40 *cdk1as*

1µM 1NM-PP1   10µM 1NM-PP1

counts

2N 4N   2N 4N

PI

DT40 *cdk1as/cdk2⁻ᐟ⁻*

1µM 1NM-PP1   10µM 1NM-PP1

2N 4N   2N 4N

B

Representative images of experiment in fig. 4a

No 1NM-PP1   10µM 1NM-PP1

DAPI
CENP-O
CENP-A
(TMR-*Star*)

CENP-O

CENP-A
(TMR-*Star*)

10 µm

C

Representative images of experiment in fig. 4b

No 1NM-PP1   10µM 1NM-PP1

DAPI
CENP-O
CENP-A
(TMR-*Star*)

CENP-O

CENP-A
(TMR-*Star*)

10 µm