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Centromere-Specific Assembly of CENP-A Nucleosomes Is Mediated by HJURP

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SUMMARY

The centromere is responsible for accurate chromosome segregation. Mammalian centromeres are specified epigenetically, with all active centromeres containing centromere-specific chromatin in which CENP-A replaces histone H3 within the nucleosome. The proteins responsible for assembly of human CENP-A into centromeric nucleosomes during the G1 phase of the cell cycle are shown here to be distinct from the chromatin assembly factors previously shown to load other histone H3 variants. Here we demonstrate that prenucleosomal CENP-A is complexed with histone H4, nucleophosmin 1, and HJURP. Recruitment of new CENP-A into nucleosomes at replicated centromeres is dependent on HJURP. Recognition by HJURP is mediated through the centromere targeting domain (CATD) of CENP-A, a region that we demonstrated previously to induce a unique conformational rigidity to both the subnucleosomal CENP-A heterotetramer and the corresponding assembled nucleosome. We propose HJURP to be a cell-cycle-regulated CENP-A-specific histone chaperone required for centromeric chromatin assembly.

INTRODUCTION

The ability of cells to properly apportion a complete set of chromosomes to each daughter cell during mitosis is dependent on a unique chromatin domain known as the centromere. It is this locus on the chromosome, through its recruitment of a large macromolecular protein complex, that mediates the attachment of chromosomes to spindle microtubules as well as the transient recruitment of proteins involved in the mitotic or spindle assembly checkpoint (Cleveland et al., 2003; Musacchio and Salmon, 2007), the major cell-cycle control pathway in mitosis. Centromeric chromatin incorporates a unique centromeric nucleosome containing *Cen*tromere *P*rotein-*A* (CENP-A). In humans, CENP-A assembles into centromeric nucleosomes that recruit a CENP-A nucleosome-associated complex (CENP-A^{NAC}) present throughout the cell cycle (Foltz et al., 2006) as part of a larger group of proteins that make up a constitutive centromere complex (Foltz et al., 2006; Izuta et al., 2006; Okada et al., 2006). Distinct from the CENP-A^{NAC}, the centromeric CENP-A nucleosome also interacts with three additional components, HJURP (*H*olliday *Ju*nction *R*ecognition *P*rotein, previously known as hFLEG1) and Nucleophosmin1 (NPM1) as well as the FACT complex (Foltz et al., 2006; Obuse et al., 2004). The consequence of the interaction of the CENP-A nucleosome with HJURP is explored below.

Human centromeric DNA is primarily comprised of 171 base pair alpha-satellite elements arranged in tandem repeats (Manuelidis and Wu, 1978; Willard, 1985). However centromere identity in mammals is primarily defined epigenetically, with the underlying DNA sequence neither necessary nor sufficient (Marshall et al., 2008; Vafa and Sullivan, 1997; Warburton et al., 1997). The 0.5–5 megabases of alpha-satellite DNA that are present within human centromeres (Cleveland et al., 2003) are packaged into chromatin by the assembly of centromere-specific nucleosomes in which CENP-A replaces histone H3 (Palmer et al., 1987; Sullivan et al., 1994; Yoda et al., 2000). The centromere-specific nucleosomes are interspersed with canonical histone H3 containing nucleosomes (Blower et al., 2002). It is this unique CENP-A-containing chromatin that is the most likely candidate to constitute the epigenetic mark of the centromeres. Obviously, each round of DNA synthesis presents a challenge for the stable propagation of a centromeric epigenetic mark, including deposition at replicated centromeres of new CENP-A nucleosomes.

Various compositions of CENP-A nucleosomes (or nucleosome-like complexes) have been suggested including tetrameric and hexameric complexes that could distinguish the CENP-A nucleosome from the canonical H3.1-containing octameric nucleosome (Dalal et al., 2007; Mizuguchi et al., 2007). The predominant form of CENP-A in chromatin in vertebrate cells (Blower et al., 2002; Foltz et al., 2006), as well as in *Drosophila* (Blower et al., 2002), is a nucleosome containing both H2A and H2B in addition to H4 and CENP-A. Recombinant CENP-A combines with histone H4 to spontaneously form a heterotetramer containing two copies each of CENP-A and histone H4 (Black et al., 2004), similar to the

subnucleosomal $(H3:H4)_2$ heterotetramer. Further, in the presence of a DNA template, CENP-A nucleosomes are formed in vitro into octameric nucleosomes with equal stoichiometries of CENP-A, H4, H2A, and H2B (Black et al., 2007b; Yoda et al., 2000), containing a conformationally more rigid core (Black et al., 2007b), and accompanied by a steady-state unwrapping of 7 base pairs at the DNA entry/exit site, relative to H3-containing nucleosomes (Conde e Silva et al., 2007). On the other hand, in budding yeast, a hexameric nucleosome-like structure containing Cse4, the CENP-A homolog, and H4 (Camahort et al., 2007; Mizuguchi et al., 2007; Stoler et al., 2007) in which Scm3 replaces histones H2A and H2B has been proposed.

Assembly of histone H3.1-containing nucleosomes is coincident with DNA replication and is accomplished through a stepwise mechanism (Jackson, 1990; Smith and Stillman, 1989, 1991). Soluble, prenucleosomal histones H3.1 and H4 associate with the *c*hromatin *a*ssembly *f*actor-*1* (CAF-1) complex consisting of CAF-1 p150, CAF-1 p60, and CAF-1 p46/48 and as a dimer with the *a*nti-silencing *f*actor *1* chaperone (ASF1) (English et al., 2005, 2006; Groth et al., 2007; Natsume et al., 2007). Assembly of the H3 and H4 heterotetramer along with two H2A:H2B dimers into the nucleosome is facilitated through its interaction with the CAF-1 complex (Kaufman et al., 1995; Smith and Stillman, 1989; Verreault et al., 1996). In contrast, while the histone H3.3 variant also interacts with ASF1 outside of S phase, it is incorporated into chromatin independent of DNA synthesis through the action of a distinct prenucleosomal complex that includes HIRA and CAF-1 p48 but is devoid of CAF-1 p150 and CAF-1 p60 (Ahmad and Henikoff, 2002; Tagami et al., 2004).

Very surprisingly, recruitment of new CENP-A to centromeric chromatin is not contemporaneous with replication of centromere DNA. Rather, it is restricted to a brief interval in G1 immediately following mitosis in human cells (Hemmerich et al., 2008; Jansen et al., 2007) and slightly earlier in anaphase in the rapidly dividing *Drosophila* syncytial embryo (Schuh et al., 2007). The assembly of new CENP-A nucleosomes in early G1 is coincident with the accumulation of the Mis18 complex (Mis18α, Mis18β, and Mis18BP1/hsKNL2) at the centromere (Fujita et al., 2007; Hayashi et al., 2004; Maddox et al., 2007). CENP-A loading is dependent on this complex for assembly, although no direct interaction has been observed between CENP-A and Mis18.

Although members of the CAF-1 complex have been implicated in CENP-A nucleosome assembly in yeast, flies, and humans (Furuyama et al., 2006; Hayashi et al., 2004; Sharp et al., 2002), no direct interaction has been demonstrated between human CENP-A and members of the CAF-1 complex. We now use affinity tagging to identify prenucleosomal complexes containing human CENP-A. One component, HJURP, is shown to be a CENP-A-selective histone chaperone required for assembly of CENP-A nucleosomes.

RESULTS

Identification of a CENP-A-Associated Prenucleosomal Complex

Prenucleosomal CENP-A or histone H3.1 and their associated proteins were purified from chromatin-depleted extracts of cells stably expressing tandem affinity purification (TAP) tagged versions of CENP-A or histone H3.1 (Figures 1A and 1B). Since the chromatin-bound CENP-A-TAP in these cells had been demonstrated previously to directly bind a collection of centromere proteins that comprise the $CENP\text{-}A^{NAC}$ (Foltz et al., 2006) and localize properly to centromeres (Figure 1A), we reasoned that it must participate in the appropriate protein-protein interactions required for targeting and assembling new CENP-A nucleosomes at centromeres. Comparable proportions of endogenous CENP-A, TAP-tagged CENP-A, or histone H3 were in the initial chromatin-free extracts (Figure 1C). Affinity purification of CENP-TAP or H3.1-TAP (using IgG-coupled beads) yielded complexes that were devoid of histone H2B (Figure 1D), consistent with complexes representing prenucleosomal forms. This is in contrast to the purification of histone H3.1 and CENP-A from nucleosome-containing chromatin extracts where stoichiometric amounts of histone H2A and H2B were present (Foltz et al., 2006).

A combination of silver staining (Figure 1E) and mass spectrometry (Table S1 available online) was used to demonstrate that the CENP-A and histone H3.1 prenucleosomal complexes obtained were almost completely distinct. Only histone H4, part of the final nucleosome, and HSP70 were common to both CENP-A and histone H3.1 prenucleosomal complexes. Consistent with previous reports (Kaufman et al., 1995; Smith and Stillman, 1989; Tagami et al., 2004), prenucleosomal TAP-tagged histone H3.1 was associated with members of the CAF-1 complex including CAF-1 p150, CAF-1 p60, CAF-1 p48, and CAF-1 p46, as well as importin 4, *h*istone *a*cetyl *t*ransferase-*1* (HAT1), and ASF1 (Figure 1E; Table S1).

The most prominent proteins uniquely associated with prenucleosomal CENP-A were the 32 kDa phosphoprotein NPM1 and the 83 kDa protein HJURP (Kato et al., 2007; Figure 1E). Homologs of HJURP were identified in several mammals (human and mouse share only 40% sequence identity; Figure S1B). Both NPM1 and HJURP were completely absent from prenucleosomal histone H3.1-TAP complexes. Both were also previously found to be associated with CENP-A nucleosomes present within centromeric chromatin, albeit at substantially lower levels as judged by silver staining and mass spectrometry (Foltz et al., 2006).

The CENP-A prenucleosomal complex did not contain any of the known constitutive centromere components. Inspection of the HJURP sequences revealed five highly conserved tryptophan residues (Figures S1A and S1C) resembling the tryptophan-aspartate (WD40) repeats found in chromatin assembly factors such as CAF-1 p60, RbAp46, RbAp48, and HIRA (Figure S1D). Two additional proteins, *RuvB l*ike-*1* (RuvBL1) and *r*eplication *p*rotein *A1* (RPA1), were identified by solution mass spectrometry in association with prenucleosomal CENP-A, although with low sequence counts (Table S1). Neither were observable by silver staining (Figure 1E), and mass spectrometry of isolated silver-stained bands failed to reidentify these proteins, indicating that these proteins are, at best, substoichiometric components of the CENP-A prenucleosomal complex.

Direct Association of HJURP and CENP-A

Sucrose gradient sedimentation of chromatin-free extracts from untransfected HeLa cells was used to characterize the prenucleosomal complexes of endogenous CENP-A. The majority of CENP-A and HJURP migrated together with a 10 S sedimentation

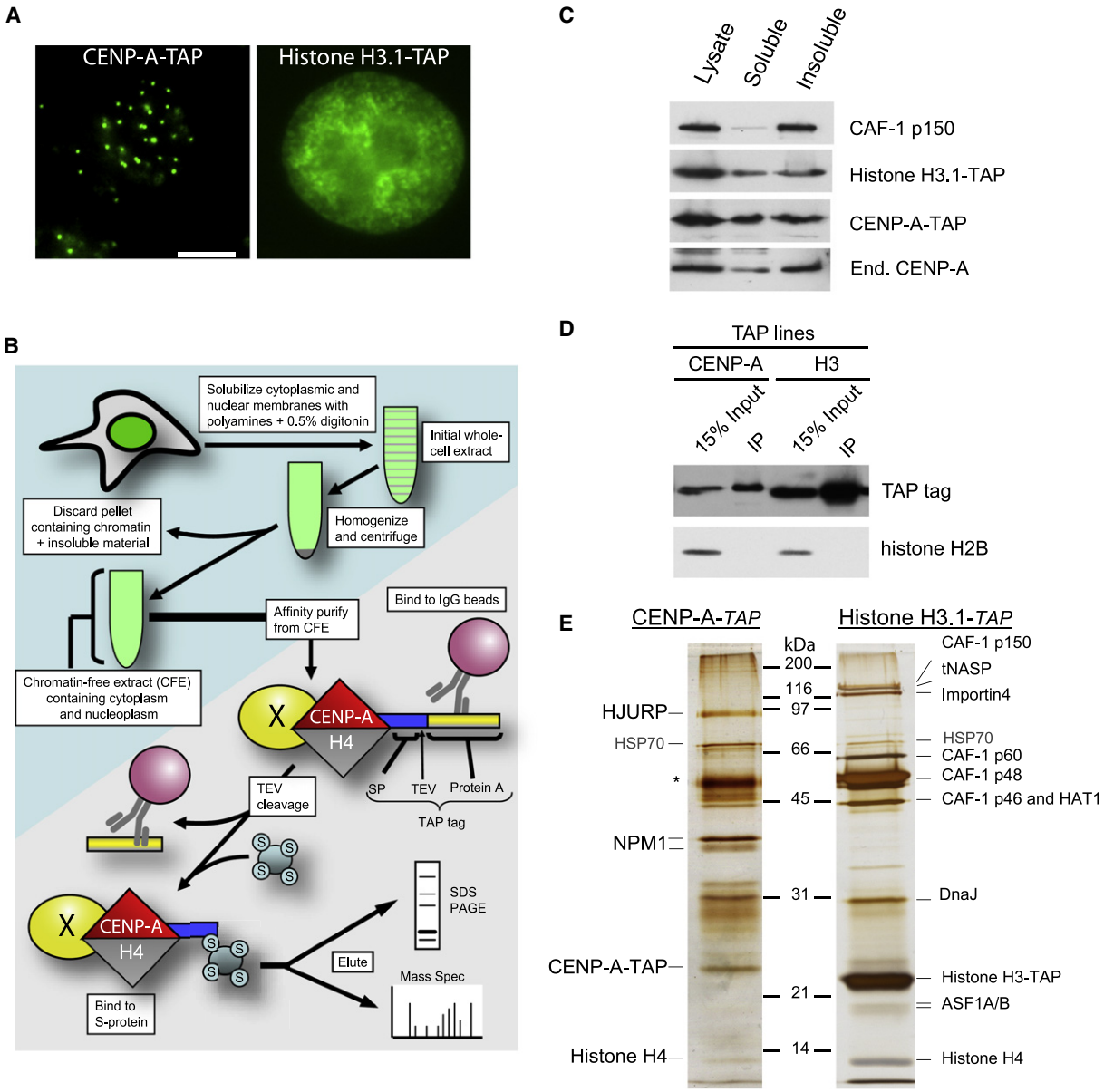

Figure 1. Affinity Purification of the CENP-A Prenucleosomal Complex

(A) Localization of CENP-A-TAP and histone H3.1-TAP in stable cell lines to centromeres and chromatin, respectively. The scale bar = 5 μm.

(B) Purification scheme for identification of a soluble CENP-A prenucleosomal complex by production of a chromatin-free extract followed by tandem affinity purification.

(C) Immunoblots of chromatin-free extracts derived from parental HeLa cells and stable cell lines expressing TAP-tagged CENP-A or histone H3.1 demonstrate the presence of tagged and endogenous histones as well as chromatin assembly factors.

(D) Single-step affinity purifications of TAP-tagged CENP-A and histone H3.1 immunoblotted for the affinity tag as well as histone H2A.

(E) Tandem affinity purified CENP-A and histone H3.1 and the associated complexes from chromatin-free extracts were visualized by silver stain. Asterisk indicates contaminant present in both preparations. Proteins associated with the soluble complexes were identified in solution by MudPIT mass spectrometry.

coefficient (Figure 2A). No significant pool of free CENP-A:H4 heterotetramer (3.2 S; Black et al., 2004) or dimer was present and no histone H2B sedimented with CENP-A in this prenucleosomal fraction. A minority of NPM1 cofractionated with prenucleosomal CENP-A, consistent with only a small proportion of total NPM1 stably associated with CENP-A. The partial overlap of NPM1 and HJURP with each other supports their formation of distinct prenucleosomal complexes with CENP-A. The peak of soluble CENP-A fractions did not contain an enrichment of RPA1 or RuvBL1 (data not shown), further suggesting that these proteins may be associated with only a small subset of the CENP-A prenucleosomal complex.

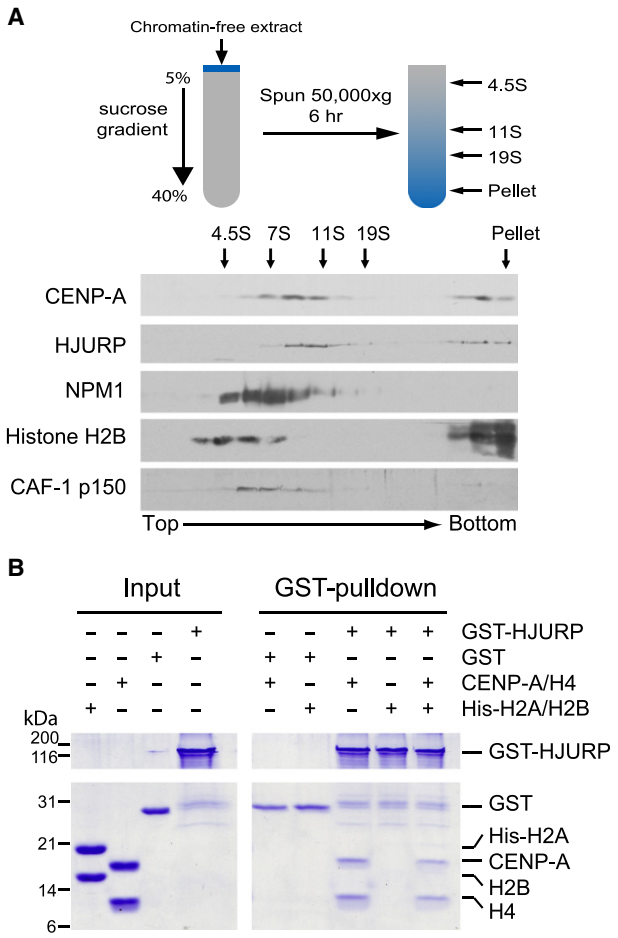

Figure 2. Identification of an Endogenous CENP-A Prenucleosomal Complex

(A) Chromatin-free extract derived from HeLa cells was subjected to sucrose gradient sedimentation. The bottom of the gradient appears on the right. The migration of sedimentation coefficient standards bovine serum albumin, aldolase, catalase, and thyroglobulin are indicated by arrows at the top.

(B) A direct interaction was observed between recombinant GST-HJURP and recombinant untagged CENP-A:H4 but not recombinant His-H2A:H2B dimer. Following purification on glutathione agarose, proteins associated with GST-HJURP were separated by SDS-PAGE and stained with Coomassie blue.

To determine whether HJURP directly binds CENP-A, GST-HJURP, CENP-A, and histone H4 were expressed and purified from *E. coli*. In assays where the CENP-A and histone H4 hetero-tetramer was combined with GST-HJURP or with GST, a complex with equimolar levels of CENP-A and H4 was selectively recovered with GST-HJURP (Figure 2B). When added to these binding assays, recombinant his-tagged H2A:H2B dimers did not interact with HJURP either alone or as part of the CENP-A:H4-HJURP complex. Thus, association of CENP-A:H4 and HJURP reflects a direct interaction that is independent of H2A:H2B and can form spontaneously in the absence of other cellular factors.

HJURP Is Required for CENP-A Centromeric Localization

To test if HJURP is required for CENP-A localization to centromeres, HJURP levels were reduced by transfection of siRNA targeting HJURP mRNA. Within 72 hr, HJURP protein levels were reduced to below 5% of their initial levels (Figure 3A). Cell-cycle distribution of the resulting cell population was not affected (Figure S2). By three cell cycles after initiating HJURP depletion, the majority of cells had substantially reduced levels of endogenous CENP-A (Figures 3B and 3D) or YFP-CENP-A (Figures 3C and 3E) at individual centromeres. Since expression of YFP-CENP-A is controlled by the 5′ LTR of the virus used to produce the stable lines, this latter finding demonstrates that CENP-A loss is not due to cell-cycle-dependent transcriptional regulation of CENP-A. Cells reduced in HJURP developed a higher proportion (accumulating to more than a third of the total by 72 hr) of misshapen, multilobed nuclei or contained micronuclei (Figure 3F). Both morphological abnormalities were phenocopies of siRNA-mediated reduction in CENP-A itself (Black et al., 2007a; Goshima et al., 2003) that drives chromosome missegregation events underlying the interphase nuclear defects. Our attempts to alter CENP-A nucleosome assembly by reducing NPM1 protein levels by siRNA showed no effect on overall levels of CENP-A at the centromere following a 72 hr treatment (data not shown). As we were only able to obtain modest suppression of NPM1 (70% reduction), we cannot rule out that the degree of NPM1 knockdown is insufficient to alter CENP-A assembly, especially given that NPM1 is a highly expressed protein. However, it is also possible that NPM1 plays a nonessential role in the assembly of CENP-A nucleosomes or that the nucleophosmin paralogs NPM2 and NPM3 may compensate for the absence of NPM1.

Long-term reduction of HJURP resulted in a reduction of the level of CENP-A protein overall (Figures 3A and S3B), consistent with failure to load new CENP-A and/or loss from centromeres and suggesting instability of the pool of CENP-A that is not associated with the prenucleosomal complex or incorporated into centromeric chromatin. To test if putative histone chaperone activity of HJURP was limited to stabilizing prenucleosomal CENP-A, but not directly involved in its centromeric loading, CENP-A was expressed at high levels. If HJURP was required for stability but not loading, CENP-A should be incorporated into centromeres in the absence of HJURP. However, this was not the case. When cells were treated with siRNA against HJURP for 24 hr and subsequently transfected with YFP-CENP-A for the following 48 hr (Figure S3B), few cells were still able to load YFP-CENP-A at centromeres (Figure S3C). Indeed, most cells with reduced HJURP along with a sustained high accumulation of CENP-A showed a pattern of YFP-CENP-A staining consistent with CENP-A's inclusion into general chromatin.

Cell-Cycle-Regulated Accumulation of HJURP at Centromeres

Chromatin-free extracts derived from synchronized HeLa cells were immunoblotted to determine when in the cell cycle the CENP-A prenucleosomal complex was present (Figure 4A). Levels of nonchromosomal CENP-A and HJURP were at their lowest in S phase and early G2 phase but rose together to peak levels in mitosis and early G1 phase. This is consistent with previous reports on CENP-A mRNA levels, which rise during G2 (Shelby et al., 1997). Comparison of asynchronous cells with those blocked in mitosis (by treatment with nocodazole)

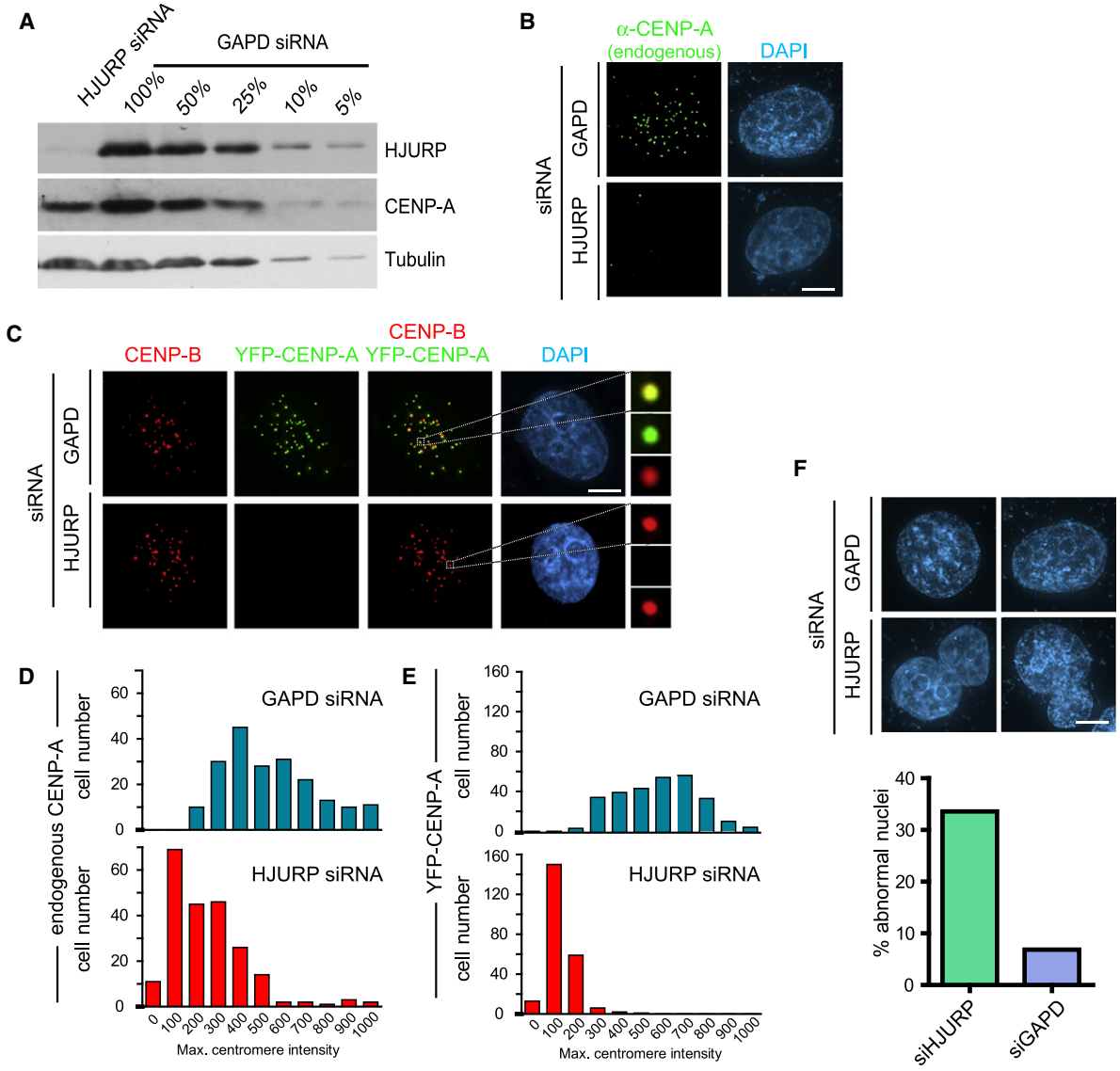

Figure 3. Loss of CENP-A Recruitment in HJURP-Depleted Cells

(A) Extracts from HeLa cells depleted of endogenous HJURP by treatment with siRNA pools for 72 hr were subjected to immunoblot. Serial dilution of GAPD control treated cell extracts was used to determine the degree of HJURP knockdown.

Parental HeLa cells (B and D) or HeLa cells stably expressing YFP-CENP-A (C and E) were treated with HJURP siRNA pools. Cells were pre-extracted and fixed 72 hr after the initiation of siRNA treatment and CENP-A and YFP-CENP-A were detected by immunofluorescence. Centromeres were identified by using anti-CENP-B monoclonal antibody in (C). Reduction of endogenous (B, n = 200) and YFP-CENP-A (C, n = 250) in response to HJURP siRNA was assessed by measuring the maximum pixel intensity per nucleus. All values are background corrected. Scale bar = 5 μm. (F) Abnormally shaped nuclei were observed by DAPI stain in HJURP siRNA-treated cells at a greater frequency than in controls. Abnormal nuclei included those that were multilobed or contained micronuclei.

confirmed that mitotic cells had a 2.5-fold higher level of non-chromatin-bound CENP-A and HJURP than asynchronous cells and a 4.5-fold high level than cells at the G1/S boundary. Single-step affinity purifications of CENP-A-TAP from these different points in the cell cycle demonstrated that HJURP and CENP-A remained associated at all times (Figure 4A), even though loading of CENP-A is restricted to early G1 (Jansen et al., 2007).

HJURP localization was undertaken in cells stably expressing GFP-Mis18α. The Mis18 complex is recruited to centromeres beginning in late anaphase and persists for approximately 3 hr after metaphase, which overlaps with the time during which new CENP-A is recruited to centromeres (Fujita et al., 2007; Jansen et al., 2007). HJURP was found to be strongly localized to centromeres during the period in early G1 when CENP-A nucleosomes are being assembled (Figure 4B). HJURP colocalized to centromeres with CENP-A in 15% of cells (Figures 4B and 4C). In all cells where HJURP was present at centromeres, GFP-Mis18α was also centromere bound (Figure 4D). In contrast, only a subset, 57%, of cells with GFP-Mis18α present at centromeres also recruited HJURP. During anaphase and telophase HJURP

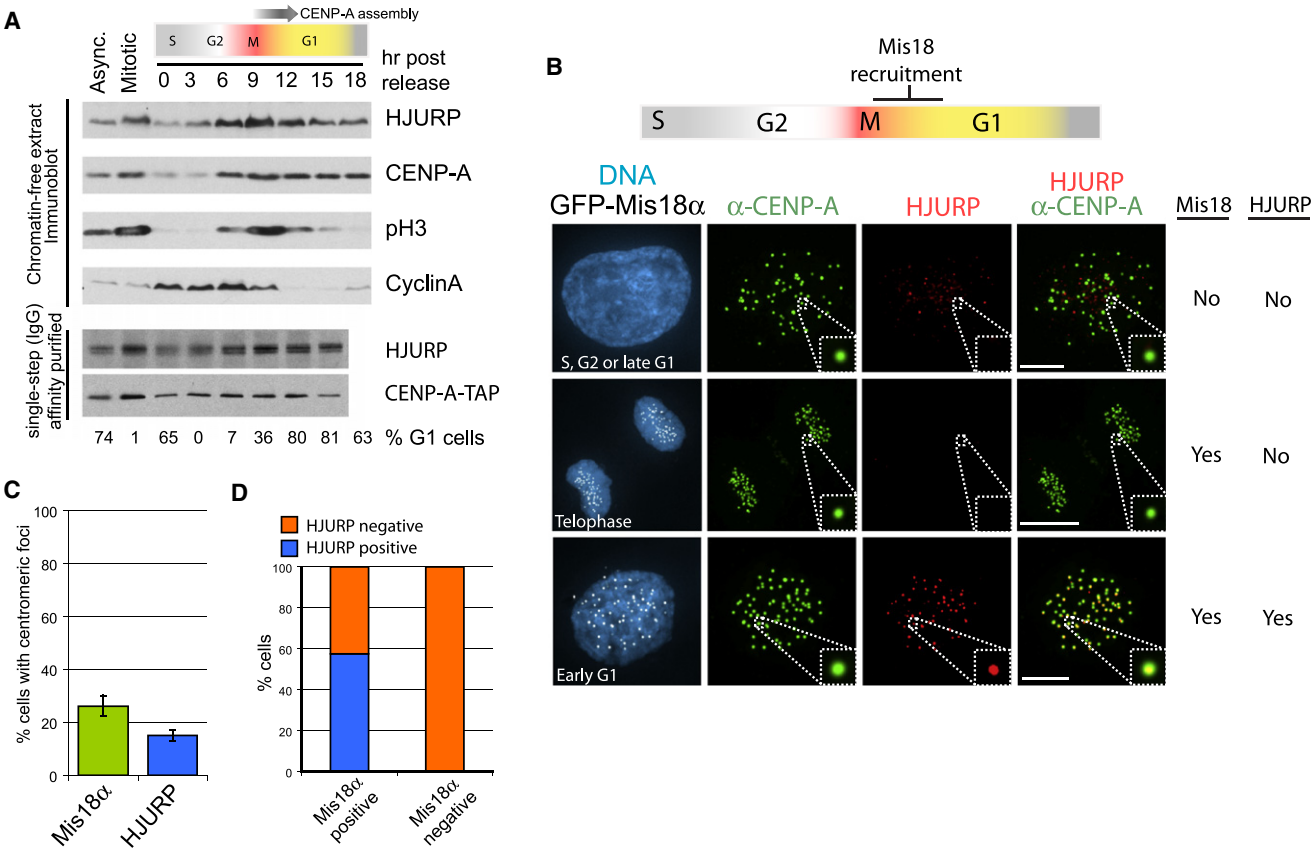

Figure 4. Cell-Cycle Regulation of HJURP Accumulation and Recruitment

(A) The levels of soluble CENP-A and HJURP across the cell cycle were assessed in chromatin-free extracts from synchronized cells by immunoblot. Each lane contains extract from 1 × 10^5 cells. The percent of cells in G1 was determined by FACS analysis.

(B) Cells stably expressing GFP-Mis18α were pre-extracted and fixed prior to immunostaining for HJURP and CENP-A.

(C and D) Cells were scored for the accumulation of HJURP and GFP-Mis18α at CENP-A foci (n = 280 cells). Data are represented as the mean ± standard deviation (SD); Scale bar = 5 μm.

was never visible at centromeres, demonstrating that HJURP is recruited to centromeres during early G1, slightly later than Mis18. In the 85% of interphase cells where HJURP was not present at centromeres (Figures 4B and 4C), it was distributed throughout the nucleus and accumulated to varying levels consistent with the cell-cycle regulation shown in Figure 4A. The noncentromeric localization of HJURP was often punctate and may represent a pool of protein involved in a DNA-damage response (as characterized by Kato et al., 2007).

Centromeres cluster around nucleoli throughout the cell cycle (Figures S4A and S4B); however, during G1, NPM1 was also found in extranucleolar foci following the disassembly of the nucleoli during mitosis (Boisvert et al., 2007). During G1, a subset of NPM1 foci was found in close association with centromeres (Figures S4A and S4B), although these foci were usually larger and more diffuse than the centromere itself.

HJURP Is Required for Loading of New CENP-A Nucleosomes

To determine if HJURP is required for cell-cycle-dependent incorporation of CENP-A into centromeric nucleosomes after mitotic exit into G1, as opposed to HJURP acting only to maintain already assembled CENP-A nucleosomes, loading of newly synthesized CENP-A was tested during the first cell cycle after depletion of HJURP by siRNA. We have previously developed a pulse-chase labeling technique by fusing a SNAP tag to CENP-A (Jansen et al., 2007). Cell lines in which a CENP-A-SNAP fusion protein is localized to centromeres (Jansen et al., 2007) were partially synchronized with a first thymidine arrest and then released, transfected with siRNA to HJURP or to a control (GAPD), and arrested at the next G1/S boundary (Figure 5A). Under these conditions, HJURP is reduced to below 10% of initial levels (data not shown). Visualization of all existing CENP-A-SNAP was blocked with nonfluorescent benzylguanine. Newly synthesized CENP-A-SNAP was labeled with TMR-*Star* (the fluorescent benzylguanine) during the subsequent G2 phase, and assembly of new labeled CENP-A nucleosomes was then assessed at the subsequent G1/S boundary. Postmitotic loading of new CENP-A into centromeres was severely diminished in cells with reduced HJURP levels compared with the GAPD siRNA-treated control cells where TMR-*Star*-labeled CENP-A-SNAP was easily apparent (Figures 5B and 5C).

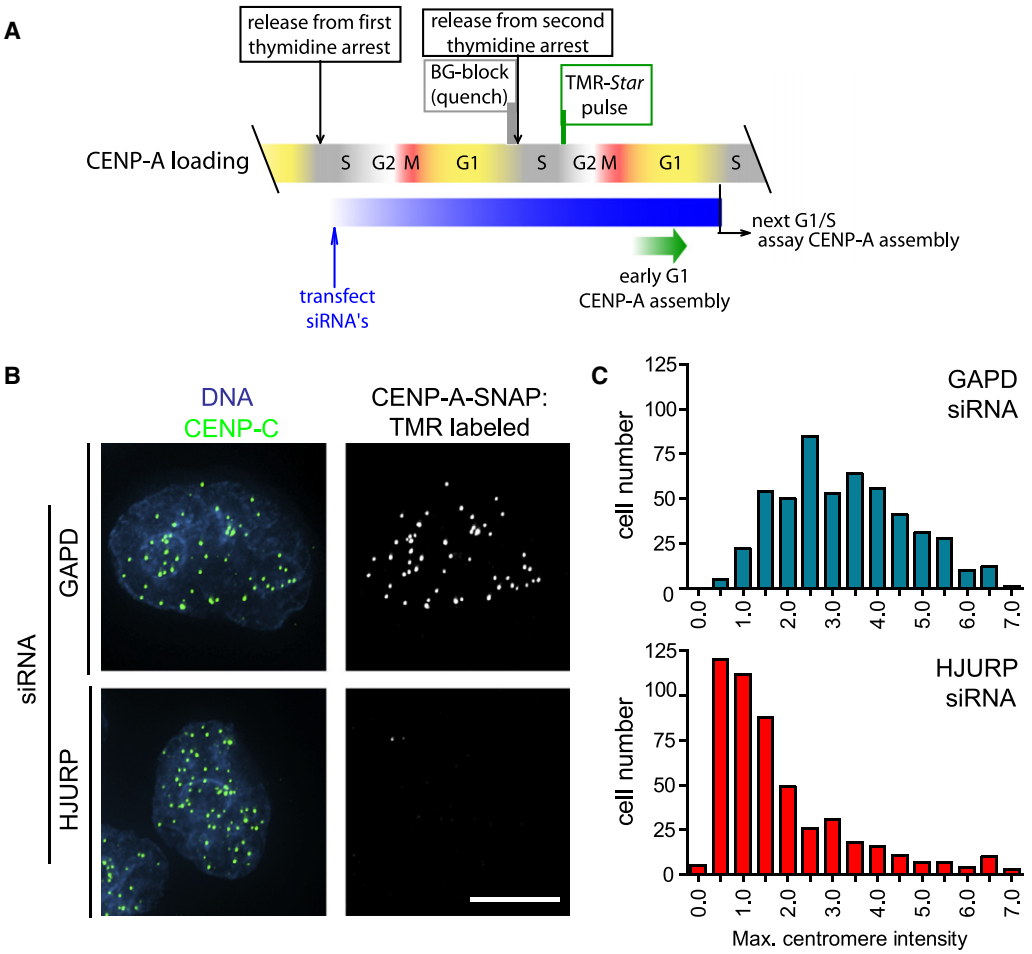

Figure 5. HJURP Is Required for Recruitment of New CENP-A Nucleosomes

(A) Scheme for siRNA treatment and SNAP labeling to visualize loading of newly synthesized CENP-A-SNAP in the absence of HJURP.

(B) Loading of newly synthesized CENP-A was assessed by the ability of cells to recruit TMR-*Star*-labeled (i.e., newly synthesized) CENP-A to centromeres in cells treated with HJURP or GAPD siRNA and SNAP labeled as described in (A). Centromeres are shown by immunostaining for CENP-C. Scale bar = 5 μm.

(C) The degree of TMR-labeled CENP-A-SNAP loading into centromeres in siRNA-treated cells was quantified by measuring the maximum pixel intensity per nucleus in >500 cells per condition.

The CENP-A Targeting Domain Mediates the Interaction with HJURP

We have previously established that the *cis*-acting element within CENP-A required for its assembly at centromeres is the centromere targeting domain (CATD) (Black et al., 2004, 2007a), which is comprised of the loop 1 and α2 helix of the histone-fold domain (Figure 6A). Swapping the 22 amino acids of CENP-A that differ between the two variants within the CATD into histone H3.1 is sufficient to convert the corresponding heterotetramers (Black et al., 2004) or corresponding nucleosomes (Black et al., 2007b) into structures that have an increased conformational rigidity relative to the corresponding complexes assembled with histone H3. Moreover, $H3^{CATD}$ not only assembles at centromeres but also provides an essential role of CENP-A in centromere maintenance (Black et al., 2007a).

Because these observations suggested that proteins important for targeting CENP-A must also associate with $H3^{CATD}$, we isolated proteins associated with $H3^{CATD}$ in cell lines stably expressing a TAP-tagged version of this chimeric histone. $H3^{CATD}$-TAP localized to centromeres as expected (Figure 6A). After double thymidine arrest and release to produce cells synchronized to be in the G1 cell-cycle phase (Figure 6B), proteins associated with prenucleosomal CENP-A, $H3^{CATD}$, and histone H3.1 were purified by tandem affinity (Figure 6C). $H3^{CATD}$ bound a dual set of proteins that reflected both its histone H3 and CENP-A characteristics. CAF-1 p60, CAF-1 p46, and CAF-1 p48 were bound to prenucleosomal $H3^{CATD}$, although the levels were clearly reduced relative to authentic H3.1 and the CAF-1 p150 subunit was absent. More importantly, despite its complete absence from histone H3.1 purifications done in parallel, swapping of the CATD region into histone H3 was sufficient for recognition and binding by HJURP, producing sufficient levels of bound HJURP to be clearly visible by silver stain (Figure 6C) or immunoblotting (Figure 6D). Inclusion of the

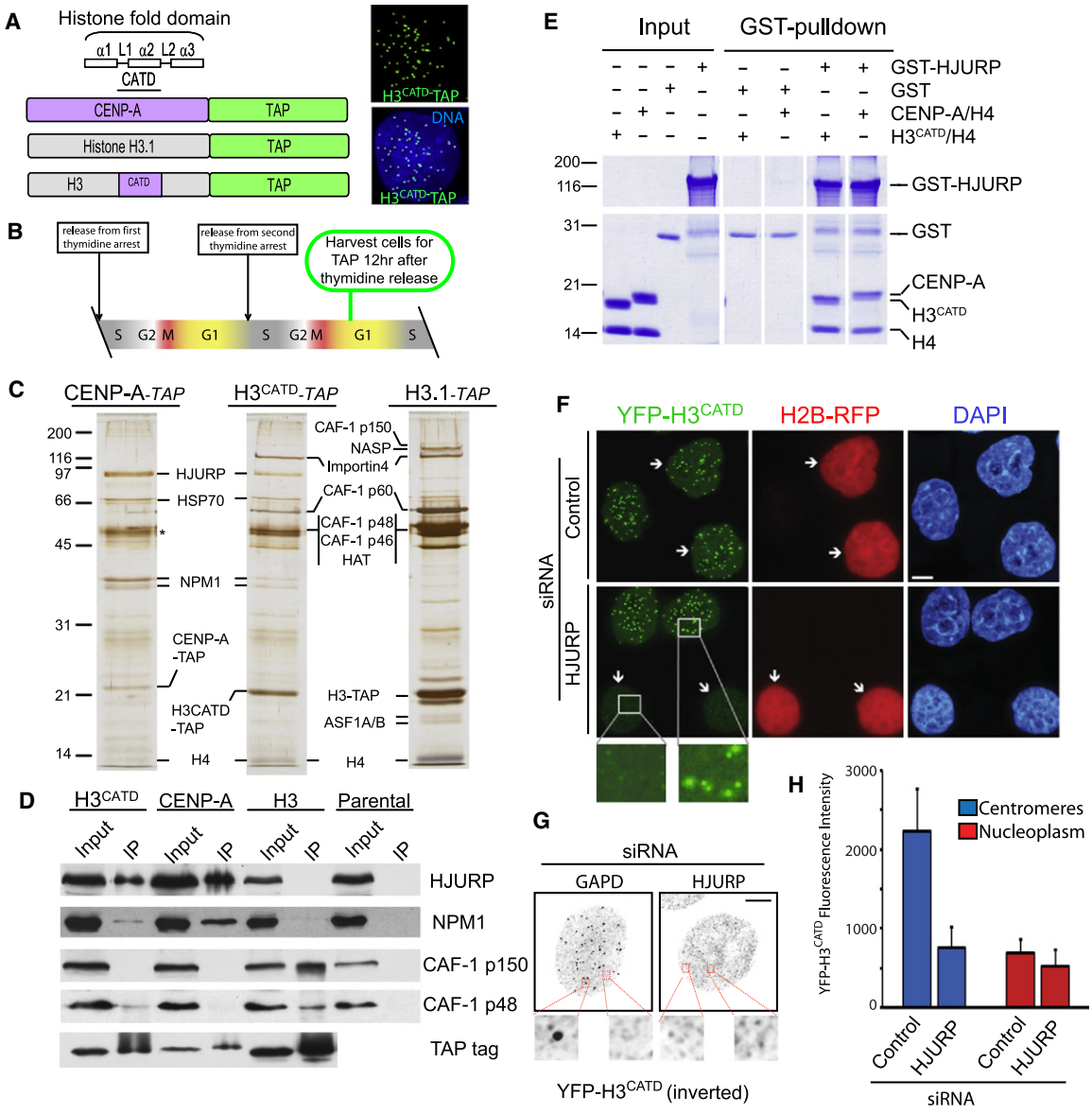

Figure 6. Histone H3^{CATD} Chimeric Protein Recruits HJURP

(A) Stable cell lines expressing a chimeric H3^{CATD} in which the loop 1 and α2 helix of CENP-A were swapped into histone H3 and fused to the TAP tag. H3^{CATD}-TAP localizes to discreet centromeric foci.

(B) Cells were harvested for tandem affinity purification (TAP) 12 hr after release from double thymidine arrest when the majority of cells are in the G1 phase.

(C) Tandem affinity purifications conducted from stable cell lines during the G1 phase of the cell cycle. Proteins were identified by in-gel trypsin digestion and mass spectrometry.

(D) Immunoblots of single-step affinity purifications.

(E) Recombinant coexpressed chimeric H3^{CATD} and histone H4 directly interacts with GST-HJURP in vitro.

(F) A cell line that stably expresses YFP-H3^{CATD} was treated with control or HJURP-directed siRNA and processed for immunofluorescence 72 hr after transfection. Arrowheads indicate cells cotransfected with the indicated siRNA plasmids and a plasmid encoding H2B-RFP.

(G) Inverted grayscale images of YFP-H3^{CATD} in which soluble protein was pre-extracted prior to fixation. Scale bars = 5 μm.

(H) Quantitation of the effect of HJURP siRNA or control treatment on YFP-H3^{CATD} fluorescent intensity. Centromeres and nucleoplasm were distinguished based on ACA immunostaining (n = 12). Data are represented as the mean maximum intensity (± SD).

CATD also recruited NPM1 to the prenucleosomal H3^{CATD}, although not as efficiently as authentic CENP-A (Figure 6D). The inclusion of the CATD within histone H3 is sufficient to mediate a direct interaction between H3^{CATD} and HJURP, as the recombinant GST-HJURP was able to pull down H3^{CATD}:H4 in an equimolar ratio to a degree comparable to that of CENP-A:H4 (Figure 6E).

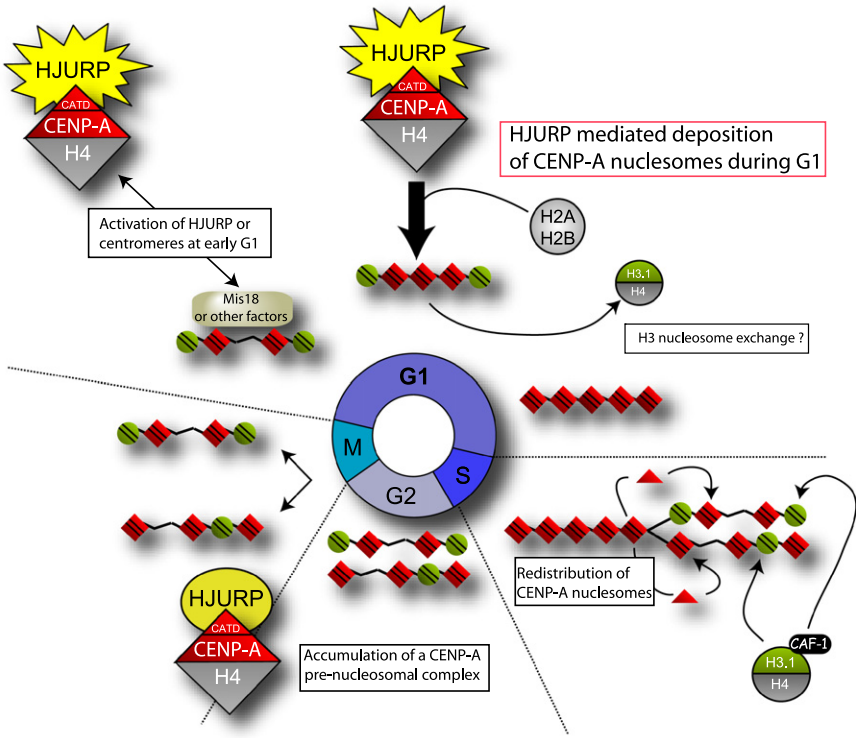

Figure 7. Model of CENP-A Nucleosome Deposition at Centromeres

Replication of sister chromatids requires that new CENP-A be deposited specifically at centromeric loci in order to maintain the epigenetic mark of the centromere. Existing CENP-A nucleosomes are distributed to sister chromosomes during DNA replication. Cells progress through G2 and mitosis with half the maximal centromeric complement of CENP-A nucleosomes. CENP-A nucleosomes are assembled in a HJURP-dependent manner during the G1 phase of the cell cycle. HJURP and CENP-A levels accumulate during G2 and peak during M even though active assembly of CENP-A is restricted to G1. Restriction of CENP-A assembly may be achieved by modification of the CENP-A prenucleosomal complex or through the recruitment of other proteins recruited to the centromere during G1, such as the Mis18 complex, that may serve to prime the centromere and restrict CENP-A nucleosome assembly.

Consistent with dual CENP-A and histone H3 characteristics, when YFP-H3^{CATD} was stably expressed in a monoclonal cell line (Black et al., 2007a) at a level comparable to endogenous CENP-A (2.4×10^6 copies per cell versus 2.0×10^6 copies per cell, respectively), it localized primarily to centromeres (as seen previously with ~85% of the selectivity of bona fide CENP-A; Black et al., 2004) (Figure S5). In addition, YFP-H3^{CATD} also incorporated at low levels into general chromatin (Figures 6F–6H). After siRNA-mediated depletion of HJURP in these YFP-H3^{CATD}-expressing cells, a large majority (84%) lost YFP-H3^{CATD} from centromeres (Figures 6F and 6H). In contrast, treatment with HJURP siRNA did not reduce the incorporation of the H3^{CATD} into general chromatin, and this pool was stable in cells that were pre-extracted to remove soluble nuclear proteins (Figures 6G and 6H). Therefore, although its incorporation into general chromatin persisted, most probably through its association with CAF-1 components, H3^{CATD} was unable to be loaded onto, or maintained at, centromeres in the absence of HJURP.

DISCUSSION

Epigenetic inheritance of the centromere requires that CENP-A nucleosomes are incorporated into centromeric chromatin preferentially over the other histone H3 variants, H3.1 and H3.3. We have identified HJURP as a unique CENP-A histone chaperone required for new CENP-A nucleosome assembly at centromeres. With other chaperones known for the other H3 variants, this demonstrates that all histone H3 variants depend on different assembly factors to achieve distinct temporal and spatial patterns of deposition within chromatin. The complexes responsible for assembling histone H3.1 and H3.3 include CAF-1 p150 and CAF-1 p60 for H3.1 and HIRA for H3.3. The CENP-A prenucleosomal complex does not include the canonical CAFs. The interaction of prenucleosomal CENP-A with the HJURP histone chaperone that is unique for it and the absence of canonical histone chaperones support a model in which CENP-A is specifically targeted to centromeres by HJURP-dependent deposition (Figure 7). In an accompanying paper, Dunleavy et al. (2009) also demonstrate HJURP to be an essential CENP-A-specific chaperone. Other proteins may facilitate the process of CENP-A nucleosome assembly, including cofactors in the nucleosome assembly process and proteins that dictate the specific targeting of HJURP-mediated CENP-A nucleosome deposition.

While CENP-A nucleosomes are quantitatively redistributed to daughter centromeres contemporaneous with DNA replication, as shown by pulse-chase labeling of CENP-A with SNAP tagging (Jansen et al., 2007), new CENP-A incorporation is delayed until the subsequent telophase or early G1, and we have shown this to require HJURP. Consequently, cells must progress through G2 and mitosis with only 50% of the maximal CENP-A nucleosome complement. It is not known whether histone H3 nucleosomes are assembled in place of CENP-A nucleosomes following their redistribution during DNA synthesis or whether these sites remain unoccupied through G2. Certainly H3.1 nucleosomes are able to occupy alpha-satellite DNA and can be found interspersed with CENP-A nucleosomes (Blower et al., 2002). If H3.1 nucleosomes are assembled with the centromeres during S phase, HJURP-dependent assembly of new CENP-A nucleosomes in late M/early G1 occurs via a reaction in which histone H3 nucleosomes are exchanged for CENP-A nucleosomes.

A previous report suggested a role for HJURP in a double-strand DNA damage break response and that in vitro it can interact with a synthetic Holliday junction-like structure (Kato et al., 2007), an observation based upon which the HJURP

name was proposed. HJURP was also reported to interact with the mismatch repair protein hMSH5 and the MRN complex component NBS1 involved in double-stranded break processing, consistent with a role for HJURP-mediated CENP-A nucleosome assembly in chromatin remodeling accompanying DNA repair. In addition, HJURP was independently identified as a 14-3-3 interacting protein by a yeast two-hybrid screen (Luhn et al., 2007). That the serine threonine kinase Akt/PKB is able to phosphorylate HJURP in vitro (leading to the additional proposed name FAKTS [*f*ourteen-three-three *Akt* substrate]) and may regulate its binding to 14-3-3 proteins has suggested a possible mechanism of HJURP regulation.

NPM1 is a highly abundant phosphoprotein that acts as a histone chaperone for both H3:H4 and H2A:H2B, in addition to playing many other cellular roles (Frehlick et al., 2007; Grisendi et al., 2006). NPM1 can bind ATP and the *Drosophila* homolog functions as an ATP-dependent chromatin remodeler, suggesting that NPM1 may provide ATPase activity in the CENP-A histone deposition/exchange reaction (Chang et al., 1998; Ito et al., 1996). The requirement for ATP hydrolysis may be an important aspect of histone variant exchange. Deposition of *Drosophila* histone H3.3 is also dependent on both HIRA and the ATPase CHD1 (Konev et al., 2007). Two other potential ATPases with roles in CENP-A nucleosome assembly include RuvBL1, identified in this study as a substoichiometric prenucleosomal CENP-A-associated component, and the hSNF2H component of the remodeling and spacing factor (RSF) found associated with CENP-A containing chromatin but not the prenucleosome (Obuse et al., 2004).

CENP-A assembly is a tightly regulated process. Levels of HJURP and CENP-A protein are cell cycle regulated, accumulating in G2 and showing maximal levels during mitosis (Figure 2C). The coregulation of CENP-A and HJURP levels and the loss of CENP-A protein in the absence of HJURP suggest that an important role for HJURP in CENP-A chromatin assembly is to stabilize prenucleosomal CENP-A. Recruitment of HJURP to centromeres and the subsequent assembly of new CENP-A nucleosomes only occur during G1 following mitosis, suggesting an additional telophase/early G1-dependent activation event. G1 phase initiation of CENP-A deposition may be regulated by modification of the CENP-A prenucleosomal complex or centromeric chromatin, or by the activation and recruitment of chromatin-bound, G1-specific assembly-promoting factors. The Mis18 complex is a good candidate to fulfill this role in centromere assembly as it is localized to centromeres only during early G1 (Fujita et al., 2007; Maddox et al., 2007), coincident with the packaging of new CENP-A into nucleosomes. Mutations and reductions of components of the Mis18 complex (Mis18α or Mis18BP1/hsKNL2) profoundly reduce the accumulation of CENP-A nucleosomes at the centromere (Fujita et al., 2007; Hayashi et al., 2004; Maddox et al., 2007). The Mis18 complex is not, however, found as part of the CENP-A prenucleosomal complex (Figure 1D) or in association with CENP-A nucleosomes (Foltz et al., 2006; Obuse et al., 2004), nor are CENP-A or HJURP associated with the Mis18 complex purified from nuclease-digested chromatin (Fujita et al., 2007). A simple view would be that Mis18 may alter centromeric chromatin structure possibly by promoting priming modifications in late mitosis or early G1 that are required for CENP-A nucleosome incorporation (a possibility supported by complementation of a Mis18 requirement by an inhibitor of histone deacetylation [Fujita et al., 2007]) or by affecting the removal of histone H3 from the centromere as an initiating step for HJURP-mediated deposition of new CENP-A nucleosomes. This scenario would be consistent with positioning of the Mis18 complex and CENP-A nucleosomes near to one another along centromeric chromatin (Maddox et al., 2007). Alternatively, the Mis18 complex when bound to chromatin may facilitate the deposition of CENP-A nucleosomes by directly recruiting CENP-A assembly factors such as HJURP.

Results differ between organisms as to the role of the canonical chromatin assembly and remodeling factors in CENP-A deposition. In budding and fission yeast, components of the canonical CAF-1 complex have been shown to be involved in CENP-A loading (Hayashi et al., 2004; Sharp et al., 2002). In fission yeast the chromatin remodeler Hrp1 (Walfridsson et al., 2005) and the histone-binding protein NASP1-related protein Sim3 (Dunleavy et al., 2007) have been shown to alter spCENP-A assembly at the centromere. Sim3 interacts with nonchromosomal CENP-A, possibly fulfilling a partially overlapping chaperone role with HJURP. In contrast, Sim3 is not found concentrated at centromeres and interacts with histone H3, and human NASP is found only in the H3 prenucleosomal complex, suggesting that HJURP may play a more inclusive role in CENP-A nucleosome deposition. CAF-1 p48/RpAb48 (a.k.a., Mis16 in *S. pombe*) can interact directly with the *Drosophila* CENP-A homolog CID/CenH3 (Furuyama et al., 2006). While CAF-1 p46 and p48 have an effect on mammalian CENP-A accumulation (Hayashi et al., 2004), a direct interaction has not been documented, and as we have shown here, it is not found in prenucleosomal CENP-A complexes. While this does not rule out a substoichiometric or transient interaction between CENP-A and CAF-1 p46 or p48, it seems unlikely especially given that interactions between CAF-1 p46 and p48 and other histone variants are easily seen by similar approaches (Tagami et al., 2004). It is possible that the tagging approach we have employed disrupts interaction of CAF-1 p46/48 with CENP-A; however, if this is the case, it is clear that CAF-1 p46/48 is not required for the specific recruitment of CENP-A to centromeres.

With our discovery of HJURP as a unique CENP-A histone chaperone, it is now clear that distinct chromatin assembly factor complexes are used for the unique spatial and temporal accumulation of H3 variant nucleosomes in mammals. Assembly of histone H3.1 nucleosomes is coupled to replication through interaction between histone H3 and the MCM replicative helicase (Groth et al., 2007) and between CAF-1 p150 and PCNA (Moggs et al., 2000; Shibahara and Stillman, 1999). Both MCM and PCNA are major components of the replication machinery, such that new histone H3.1 nucleosome assembly occurs in close proximity to DNA synthesis. Existing CENP-A nucleosomes may direct the incorporation of new CENP-A nucleosomes either directly or through the recruitment of intermediate factors that could include the covalent modification of surrounding centromeric chromatin. In turn HJURP must recognize either existing CENP-A nucleosomes or the intermediate factors or modifications that they induce in order to direct the deposition of new CENP-A nucleosomes only into active centromeres.

EXPERIMENTAL PROCEDURES

Cell Culture, Synchronization, and Transfection

CENP-A-TAP, H3.1-TAP, YFP-CENP-A, and YFP-H3^{CATD} stable expressing cells were described previously (Black et al., 2007a; Foltz et al., 2006; Kops et al., 2004). Stable H3^{CATD}-TAP cell lines were produced by retroviral infection as described (Foltz et al., 2006). Synchronization was achieved as described by Jansen et al. (2007). For siRNA treatment, 1.5 × 10^5 cells were plated on glass coverslips in a 6 well plate and duplexed siRNAs were introduced into cells using Oligofectamine (Invitrogen, Carlsbad, CA, USA). siRNA-encoding plasmids were cotransfected with RFP-tagged histone H2B (H2B-RFP; Black et al., 2007a) at a ratio of 20:1 (siRNA:H2B-RFP) using the Effectene transfection reagent (QIAGEN, Valencia, CA, USA). Cells were fixed and processed for immunofluorescence 72 hr after transfection. SNAP labeling was conducted as described previously (Jansen et al., 2007).

Affinity Purification

Chromatin-free extracts were produced from 5 × 10^8 cells expressing histone H3.1-TAP or 1 × 10^9 cells expressing either CENP-A-TAP or H3^{CATD}-TAP. Cells where dounce homogenized in buffer A (3.75 mM Tris, pH 7.5, 20 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.05 mM spermidine, 0.125 mM spermine, 0.1% digitonin, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml chymostatin). Homogenized extracts were centrifuged at 300 × g for 5 min and the pellet was resuspended in buffer A, homogenized, and centrifuged for 5 min at 300 × g. Supernatants from these two centrifugations were combined and centrifuged at 12000 × g for 10 min to produce a chromatin-free extract. Tandem affinity purifications were conducted as described previously (Foltz et al., 2006). In the case of single-step purification, proteins were eluted from the first affinity step (IgG-bound beads) by boiling in SDS sample buffer.

Mass Spectrometry

Total eluates from tandem affinity purifications were analyzed by mass spectrometry using MudPIT analysis as described previously (Foltz et al., 2006) or by excision and in-gel digestion of proteins from silver-stained polyacrylamide gels. Gel bands were dehydrated in 50% acetonitrile, rehydrated in 50 mM ammonium bicarbonate pH 8 including 1 μg/ul trypsin (Promega, Madison, WI, USA), and incubated overnight at 37°C. Peptides were eluted from the gel in 50% acetonitrile, 5% formic acid, dehydrated, resuspended in 0.1% acetic acid, and analyzed by separation on a 10 cm C18 column (Michrom BioResources, Auburn, CA, USA) coupled to a Thermo Finnigan LCQ ion trap mass spectrometer (Waltham, MA, USA). Data-dependent collection of MS spectra was conducted by taking a single MS scan followed by MS/MS scans of the top three most intense ions. Peptide identification was conducted using COMET.

Sucrose Gradient Sedimentation

Chromatin-free extracts were prepared as described above from 6 × 10^7 randomly cycling HeLa cells and applied to the top of a 2 ml 5%–40% sucrose gradient in buffer A. Sucrose gradients were centrifuged at 4°C for 6 hr at 50,000 × g in a Beckman TLS55 swinging bucket rotor and the gradient was separated into 150 μl fractions. Proteins were separated by SDS-PAGE and blotted to nitrocellulose and detected by immunoblot in 25 mM Tris, pH 7.4, 150 mM NaCl, 5% dry milk.

Protein Purification and In Vitro Binding

All proteins were expressed in the Rosetta BL21 (DE3) pLysS bacteria strain and purified using either Ni-NTA affinity resin (QIAGEN) for 6×His proteins or Glutathione sepharose (GE Healthcare, Piscataway, NJ, USA) for GST fusions. CENP-A:H4 heterotetramers were produced as described (Black et al., 2004). Histone H2A-containing amino-terminal tandem 6×His and S tags (His-H2A) and untagged histone H2B were expressed in bacteria and initially purified as monomers. His-H2A:H2B dimers were reconstituted and purified as described (Luger et al., 1999). In vitro binding assays were conducted in 100 mM NaH_2PO_4, pH 7.4, 300 mM NaCl, 0.05% NP-40, 10% glycerol, and 1 mM DTT. Recombinant proteins were combined and incubated at room temperature for 30 min and an additional 30 min following addition of glutathione sepharose. Bound protein complexes were washed twice in binding buffer, once in low-salt buffer (100 mM NaH_2PO_4, pH 7.4, 100 mM NaCl, 0.05% NP-40, 10% glycerol, and 1 mM DTT), eluted from the beads in SDS sample buffer, separated on a 15% SDS-PAGE gel, and stained with Coomassie.

Immunocytochemistry

Cells were pre-extracted using 0.3% Triton X-100 in PBS, fixed in 4% formaldehyde for 10 min, and quenched in 100 mM Tris, pH7.7. Cells were preblocked in PBS containing 2% FBS, 2% BSA, and 0.2% tween 20. Incubations with primary antibodies (see Supplemental Experimental Procedures) were conducted in blocking buffer for 1 hr at room temperature. DNA was detected using DAPI and cells were mounted in Prolong Antifade (Invitrogen). Images were collected using a Deltavision microscope (Applied Precision, Issahquah, WA, USA) and deconvolved z-projections were presented. Images of plasmid-based siRNA-treated cells were acquired using a Leica DMI 6000 B microscope using Leica LAS software. Quantification of siRNA effect was conducted using Metamorph (Molecular Devices, Sunnyvale, CA, USA) on nondeconvolved images collected on the same day with identical exposure times by measuring maximum pixel intensity per nucleus with background subtracted.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, one table, and five figures and can be found with this article online at http://www.cell.com/supplemental/S009-8674(09)00253-0.

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Supplemental Data

Centromere-Specific Assembly of CENP-A Nucleosomes Is Mediated by HJURP

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Supplemental Experimental Procedures

DNA Constructs amd siRNAs. Full length HJURP (accession number NM_018410) flanked by SpeI and EcoRI was generated by PCR from the IMAGE clone 2820741 (BC001940) using the forward primer '5-GGACTAGTATGCTGGGTACGCTGC-3' and reverse primer 5'-GGGAATTCCTACACACTTTTAGTTTCC-3'. PCR fragments were cloned into the pGEX6T vector to create a GST-HJURP fusion protein. For antibody production, a 786 bp fragment of HJURP encoding amino acids 481-743 was cloned into pET28 fused in frame to a His6 tag at its amino terminus. The $H3^{CATD}$ chimera (Black et al., 2004) was fused to the TAP tag, consisting of the S-protein-TEV-protein A tandem affinity tag at its carboxy terminus, as described previously for CENP-A-TAP and H3.1-TAP (Foltz et al., 2006).

siRNAs directed against HJURP were purchased from Dharmacon (Layafette, CO) as presynthesized pools of four separate duplexed siRNAs targeting nucleotides 1135-1153, 1225-1243, 1815-1833 and 2017-2033 of the HJURP open reading frame. Duplexed GAPD siRNA targeted the sequence 5'-UGGUUUACAUGAUCCAAUA-3'. Plasmid-based shRNA targeting nucleotides 1288-1306 of HJURP were cloned into pSuperior-Retro-Puro (OligoEngine, Seattle, WA).

FACS analysis. Adherent synchronized cells were detached from the cell cuclture plate using 3mM EDTA in PBS and fixed in 70% ethanol. DNA was stained with 10 μg/ml propidium iodide and 250 μg/ml RNaseA for 30 minutes at 37^{o}C. DNA content was analyzed using a Becton Dickinson LSRII (Franklin Lakes, NJ) FACS diva software.

Antibodies. Rabbit polyclonal antibodies against HJURP were raised against amino acids 481-743 with a 6XHis tag at the amino terminus (Covance, Denver PA). The following antibodies were used for immunocytochemistry: anti-CENP-A (1:100, Gift from K. Yoda), anti-CENP-B (1:1000, 2D7 mAb), anti-HJURP (1:1000, Covance,Denver PA), and anti-CENP-C (1:100, mAb). For immunoblot, the following antibodies were used: HJURP (1:1000, Covance, Denver PA), Tubulin (1:5000, mAb DM1A), histone H2B (1:500, Millipore,Billerica MA), NPM1 (1:5000, Sigma, St. Louis, MO), CAF1p150 (1:500, Santa Cruz Biotech., Santa Cruz, CA), CAF1p48 (a.k.a. RbAp48) (1:1000, Oncogene Cambridge, MA), ACA (1:500, Antibodies Inc., Davis, CA), and cyclin A (1:100, Becton Dickinson, San Jose CA).

CENP-A-TAP

Name/Gene symbol		Protein ID	MW	Sequence Count	Spectrum Count	Sequence Coverage
HJURP	-	NP_060880	83539	36	134	56.6%
RPA1	-	NP_002936	68138	8	11	18.3%
RuvBL1	-	NP_003698	50228	10	11	31.1%
NPM1	-	NP_002511	32575	31	609	41.80%
CENP-A	-	NP_001800	15991	4	5	22.9%
Histone H4	**	-	11367	13	91	61.2%

Histone H3.1-TAP

Name/Gene symbol		Protein ID	MW	Sequence Count	Spectrum Count	Sequence Coverage
Importin-4	IPO4	NP_078934	118717	3	3	3%
CAF1p150	CHAF1A	NP_005472	106926	8	12	8%
NASP		NP_751896	85238	45	177	41%
CAF1p60	CHAF1B	NP_005432	61493	4	5	11%
CAF1p46	RBBP7	NP_002884	52314	18	40	22%
HAT1	-	NP_003633	49513	15	106	27%
C14orf130	UBR7	NP_786924	47999	33	173	49%
CAFp48/RbAp48	RBBP4	NP_005601	47525	14	28	19%
DnaJ	DNAJC9	NP_056005	29910	6	11	20%
ASF1A	-	NP_054753	22951	3	28	11%
ASF1B	-	NP_060624	22434	3	3	19%
Histone H3.1	**	-	15404	6	10	35%
Histone H4	**	-	11236	9	20	48%

Table S1. Mass spectrometry of tandem affinity purified CENP-A and histone H3.1. Official gene symbol is noted where it differs from the common gene name. *Histone H3.1 and histone H4 exist as multiple gene clusters the members of which could not be distinguished based on their protein sequence.

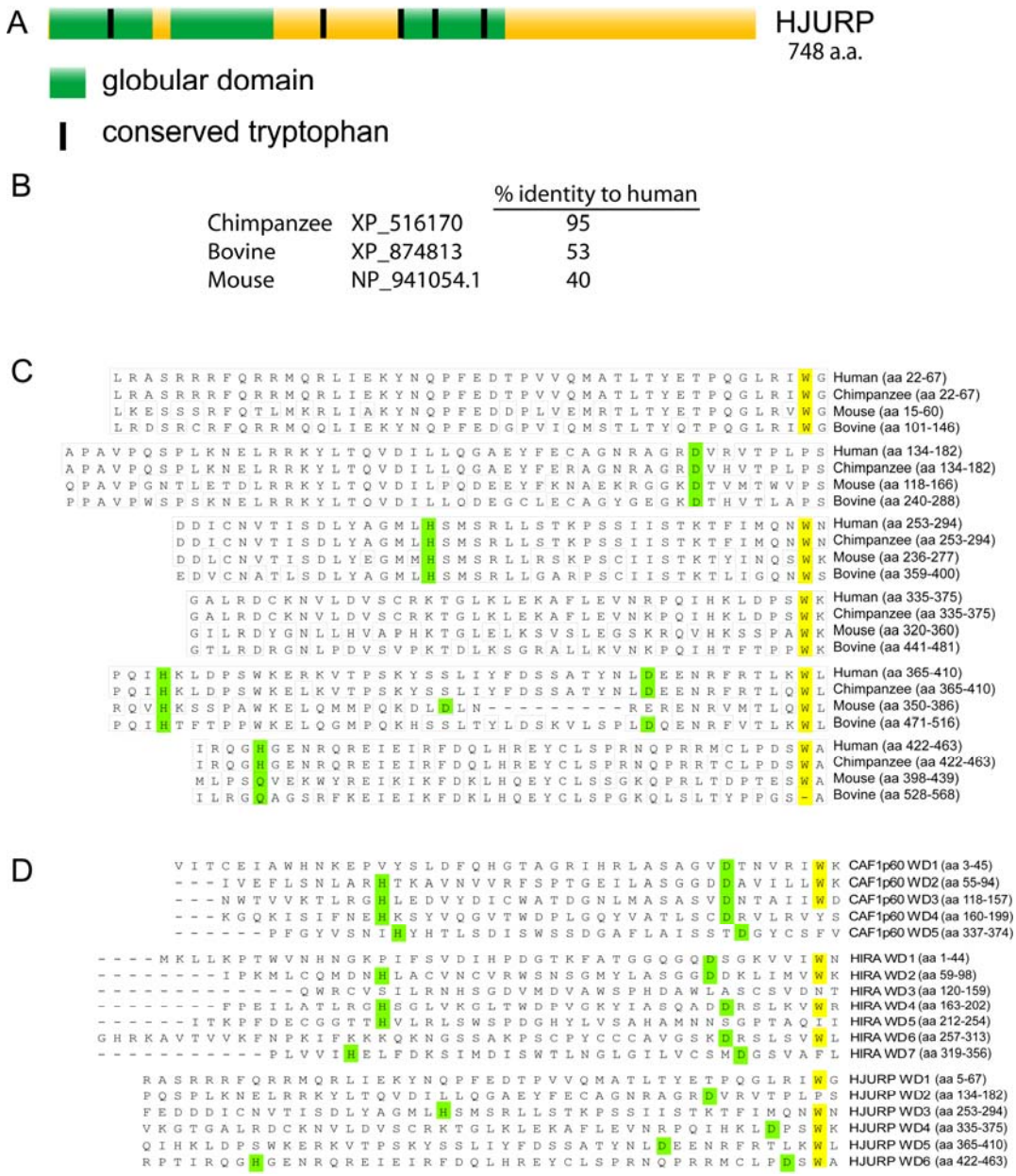

Figure S1. (A) Schematic of HJURP showing the location of conserved tryptophan residues and predicted globular domains. (B) Newly identified mammalian HJURP homologues. (C) Alignment of putative WD40-like repeats present in the known mammalian homologues. Yellow indicates the conserved tryptophan residues. Green indicates other conserved amino acids known to be part of the WD40 repeat. (D) Alignment of known WD40 repeats from other chromatin assembly factors.

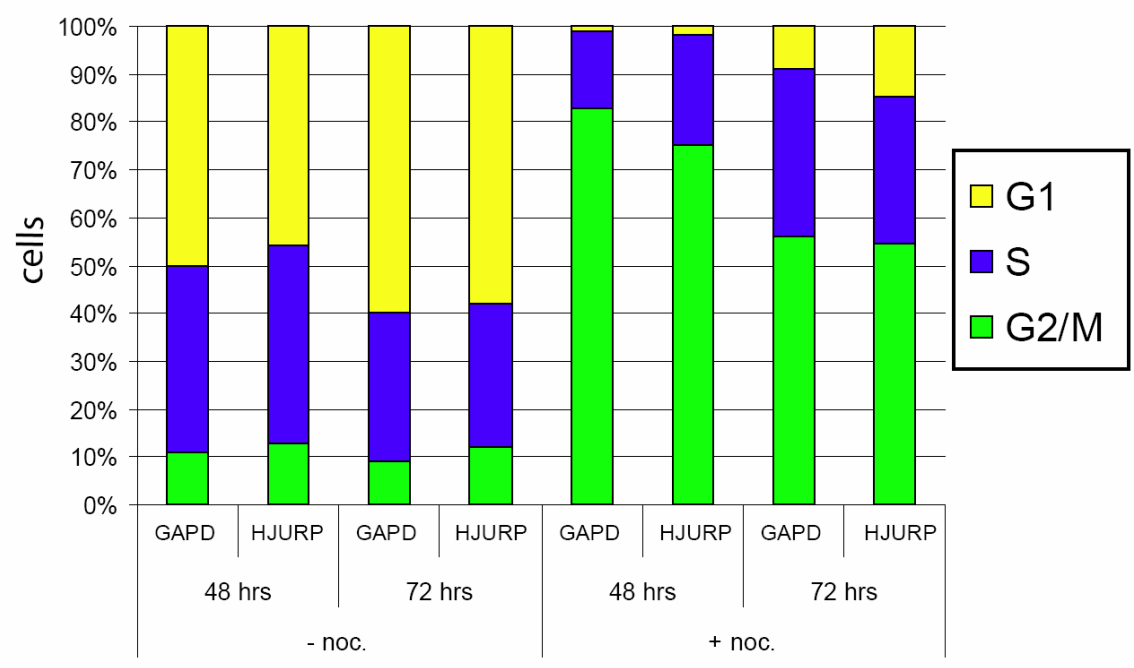

Figure S2. Cell cycle analysis in cells reduced for HJURP. Cells were treated with siRNA directed against GAPD or HJURP and cell cycle position was determined by FACS analysis for DNA content. Nocodazole treatments were conducted for 16 hours prior to fixation.

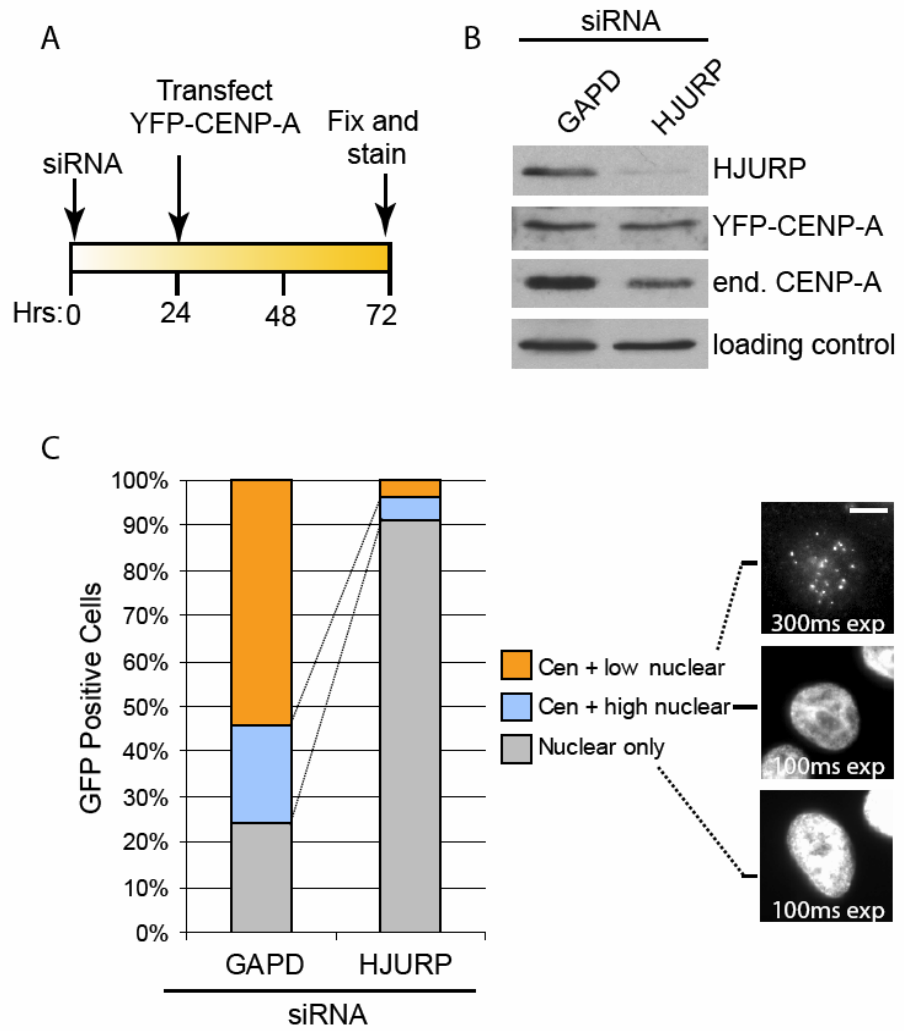

Figure S3. Overexpression of CENP-A does not rescue CENP-A accumulation or assembly after reduction of HJURP. (A) After 24 hours of HJURP or GAPD siRNA treatment cells were transfected with a construct encoding YFP-CENP-A and harvested for immunofluorescence after 72 hours. (B) Immunoblots of whole-cell extracts from either HJURP siRNA treated cells or GAP treated cells expressing exogenous YFP-tagged CENP-A. (C) YFP-CENP-A cells were pre-extracted and YFP-CENP-A was visualized with GFP antibody. Cells were divided into three classes based on the level of expression and subcellular localization of YFP-CENP-A in HJURP and GAPD siRNA treated cells. For each condition, 200 cells were scored. Scale bar equals 5μm.

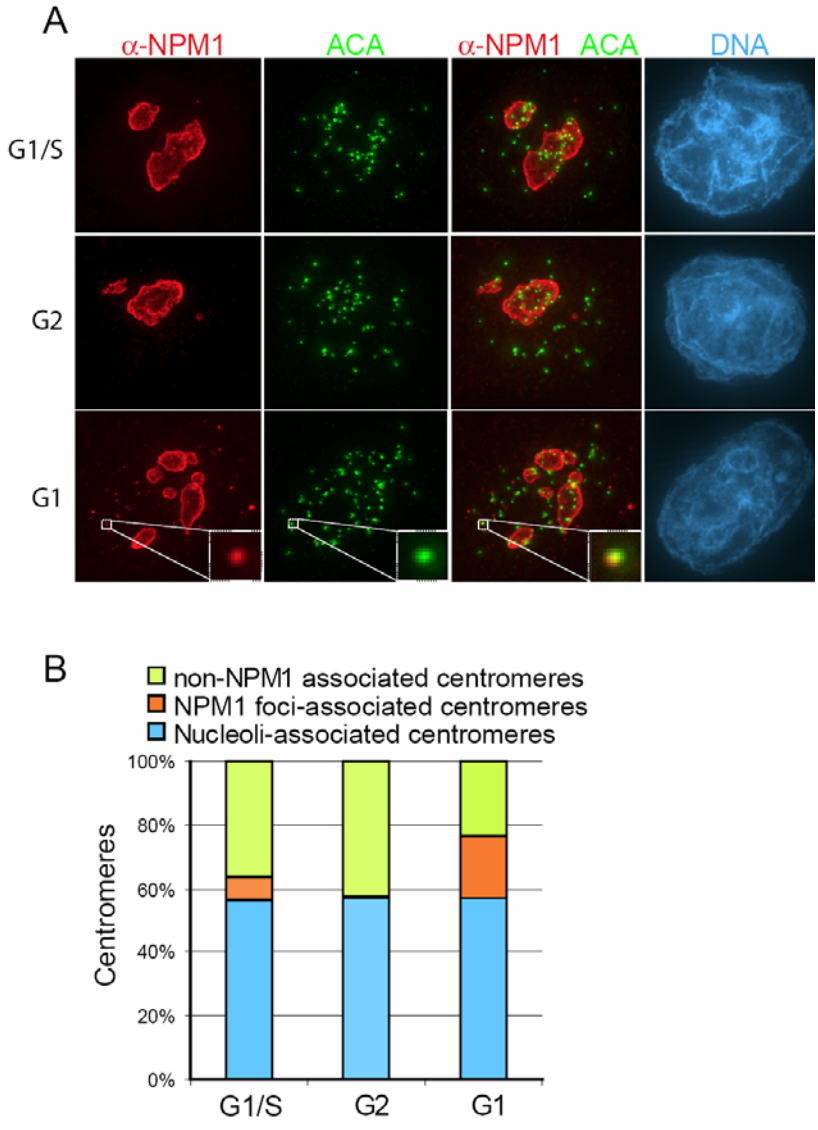

Figure S4. Localization of NPM1 and centromeres to foci during G1. (A) HeLa cells were synchronized by double thymidine block and released for 0 hours (G1/S boundary) or 5 hours (G2 phase), or cells were released into nocodazole, mitotic cells were isolated by shake-off and replated for 2 hours in nocodazole free media to obtain cells in G2. Cells were pre-extracted, fixed and immunostained for centromeres and NPM1. (B) Centromeres were scored as to whether they were found associated with nucleoli (either peripherally or embedded within the nucleolus), associated with smaller non-nucleolar NPM1 foci or not associated with NPM1 (n=9).

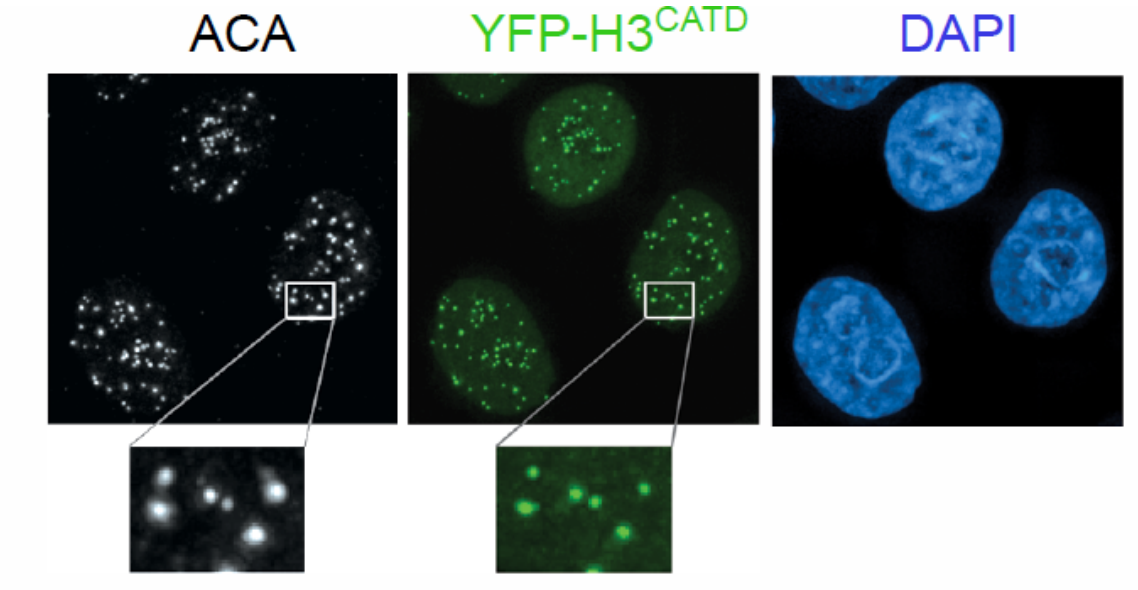

Figure S5. YFP-H3^{CATD} localization to centromeres. Foci in the stable YFP-H3^{CATD} expressing cell line correspond to centromeres as shown by colocalization of YFP-H3^{CATD} with anti-centromere antibodies (ACA).