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HJURP Is a Cell-Cycle-Dependent Maintenance and Deposition Factor of CENP-A at Centromeres

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SUMMARY

The histone H3 variant CenH3, called CENP-A in humans, is central in centromeric chromatin to ensure proper chromosome segregation. In the absence of an underlying DNA sequence, it is still unclear how CENP-A deposition at centromeres is determined. Here, we purified non-nucleosomal CENP-A complexes to identify direct CENP-A partners involved in such a mechanism and identified HJURP. HJURP was not detected in H3.1- or H3.3-containing complexes, indicating its specificity for CENP-A. HJURP centromeric localization is cell cycle regulated, and its transient appearance at the centromere coincides precisely with the proposed time window for new CENP-A deposition. Furthermore, HJURP downregulation leads to a major reduction in CENP-A at centromeres and impairs deposition of newly synthesized CENP-A, causing mitotic defects. We conclude that HJURP is a key factor for CENP-A deposition and maintenance at centromeres.

INTRODUCTION

The centromere is the highly specialized chromosomal locus that ensures the delivery of one copy of each chromosome to each daughter cell at cell division. Centromeres form a platform upon which the kinetochore, the multiprotein complex that mediates spindle microtubule attachment during mitosis, is assembled (Cleveland et al., 2003). Intriguingly, the site of centromere formation is not governed by DNA sequence except in budding yeast where a specific centromeric sequence has been defined (Cheeseman et al., 2002). In humans and flies, centromeres can arise at ectopic sites on chromosomes called neocentromeres that are devoid of repetitive DNA sequences usually found at centromeres (Amor and Choo, 2002). Moreover, dicentric chromosomes can arise with two regions capable of acting as centromeres with only one active (Earnshaw et al., 1989; Sullivan and Willard, 1998). Hence centromeric DNA sequences are neither necessary nor sufficient to mark a functional centromere whose identity is epigenetically regulated (Karpen and Allshire, 1997). One hallmark of functional centromeres is the rapidly evolving histone H3 variant CenH3 (Allshire and Karpen, 2008; Malik and Henikoff, 2003). Present in centromeric nucleosomes, these variants define the site of kinetochore assembly dedicated to efficient chromosome segregation in mitosis (Black and Bassett, 2008; Palmer et al., 1991). In budding yeast, a simple "point" centromere consisting of only one nucleosome has been extensively characterized (Cheeseman et al., 2002; McAinsh et al., 2003). In humans, chromatin fiber analysis showed a more complex organization with CENP-A nucleosomes interspersed with H3 nucleosomes (Blower et al., 2002). Our knowledge of human centromere function has been greatly enriched by examining proteins interacting with CENP-A on chromatin (Foltz et al., 2006; Izuta et al., 2006; Obuse et al., 2004), including a six-component CENP-A nucleosome-associated complex (CENP-A^{NAC}) that acts as a foundation for assembly of the CENP-A-nucleosome distal complex (CENP-A^{CAD}) (Foltz et al., 2006). Human artificial chromosome technology further helped in dissecting functional centromere composition (Nakano et al., 2008; Okada et al., 2007). However, how CENP-A is targeted to and assembled at centromeres and excluded from flanking pericentromeric heterochromatin is poorly understood. Intrinsically, CenH3 can go to any site on the chromosome as shown by transient overexpression experiments in different organisms (Collins et al., 2004; Henikoff et al., 2000; Heun et al., 2006; Van Hooser et al., 2001). Thus, targeting mechanisms must exist that restrict CENP-A deposition to centromeres.

One important mechanism thought to contribute to CENP-A incorporation at centromeres is its specific cell-cycle timing. Unlike canonical histones that are deposited during DNA replication, the deposition of histone variants can occur outside of S phase (Henikoff et al., 2004; Loyola and Almouzni, 2007). During S phase, dilution of parental CENP-A occurs, and either gaps, replicative H3 as placeholders, or hemisomes could be generated in the wake of replication fork passage (Dalal et al., 2007; Henikoff and Dalal, 2005; Sullivan, 2001). Thus CENP-A incorporation at centromeres is delayed and proceeds in

a replication-independent fashion. A first hypothesis proposed this would occur in G2 when CENP-A levels peak in expression (Ahmad and Henikoff, 2001; Shelby et al., 2000). More recent evidence in human cells, by elegant use of SNAP-tagging methodology, showed that new CENP-A deposition is restricted to a discrete cell-cycle window in late telophase/early G1 (Jansen et al., 2007). While the precise mechanism of CENP-A incorporation during that time remains to be elucidated, how the inheritance of an essential epigenetic mark occurs outside of S phase has generated much interest.

Genetic screens in fission yeast have identified candidate factors that assist in CENP-A deposition and based on homology have increased our understanding of how human CENP-A incorporation may be controlled. In fission yeast, Mis16 and Mis18 are required for the proper loading of Cnp1 (*S. pombe* CenH3), possibly by maintaining adequate acetylation of histones at the inner centromere region (Fujita et al., 2007; Hayashi et al., 2004). Their human counterparts, RbAp46/48 (or RBBP4/7) and the hMis18 complex (hMis18α, hMis18β, and M18BP1/KNL-2), also impact on the localization of CENP-A at centromeres (Fujita et al., 2007; Hayashi et al., 2004), and RbAp48 in *Drosophila* promotes the reconstitution of CID (fly CenH3)-containing nucleosomes in vitro (Furuyama et al., 2006). While the human hMis18 complex is required for CENP-A association with centromeres, it does not interact with CENP-A, and like in *S. pombe* it is thought to prepare centromeres to accept CENP-A by altering histone acetylation (Fujita et al., 2007; Maddox et al., 2007). In addition, although it does not localize to centromeres, a NASP-(N1/N2)-related protein Sim3 interacts with Cnp1 (*S. pombe* CenH3) and is required for its deposition at centromeres in fission yeast (Dunleavy et al., 2007). While studies in model organisms have been crucial to developing a candidate approach for CENP-A deposition factors in human cells, they have the limitations of missing less conserved components. This is particularly critical in the case of CEN-histone variants that have evolved a lot faster than other H3 variants (Dalal et al., 2007; Malik and Henikoff, 2003). Thus, other strategies to gain insight into new factors important for the dynamics and timing of CENP-A incorporation should be considered.

Inspired by powerful biochemical strategies combined with proteomics used to identify chaperones important for deposition of H3 variants in human cells (Tagami et al., 2004), we searched for new human CENP-A chaperones. The definition of histone chaperones states that they associate with histones and stimulate a reaction involving histone transfer, without being a necessary part of the final product (De Koning et al., 2007). A key property is that they should be found in a complex with histones before they are assembled into nucleosomes. Previously, specific chaperones for predeposited histone H3 variants H3.1 and H3.3 were identified (Tagami et al., 2004): H3.1 associates with CAF-1 (chromatin assembly factor-1), which mediates DNA synthesis-dependent chromatin assembly during replication and repair, whereas H3.3 interacts with HIRA, which mediates replication-independent chromatin assembly (De Koning et al., 2007). However, a CENP-A-dedicated chaperone awaited discovery.

To investigate the molecular players participating in CENP-A delivery to centromeres, we identified partners interacting with predeposited CENP-A in both cytosolic and nuclear soluble fractions. Among the different histone chaperones studied here, we propose that HJURP has a key role for both maintenance and incorporation of CENP-A at centromeres.

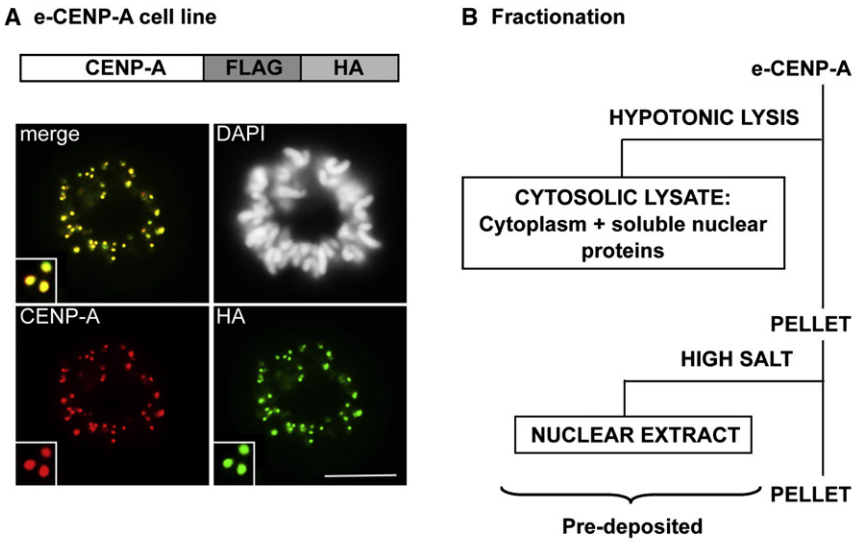
RESULTS

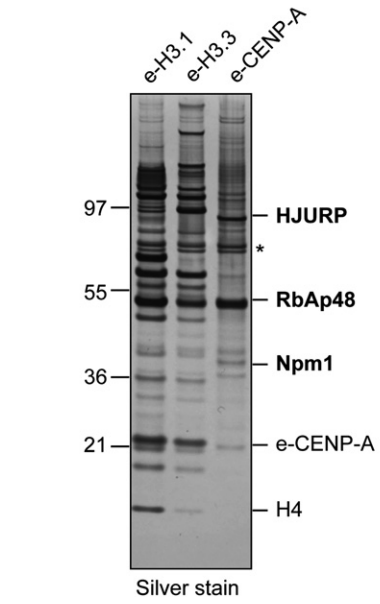
Soluble CENP-A Complexes and Identification of Partners

To search for a CENP-A chaperone, we established a HeLa cell line (as in Nakatani et al., 2003; Tagami et al., 2004) in which CENP-A fused with a C-terminal FLAG- and HA-epitope tag is stably expressed (Figure 1A). Immunofluorescent staining with anti-HA antibody showed that e-CENP-A localizes to centromeres indicating that the presence of the tag does not prevent the deposition of CENP-A in vivo (Figure 1A). Tagged CENP-A was expressed at an ~4-fold higher level in this cell line compared to CENP-A in the parental cell line leading to a major reduction of endogenous CENP-A (Figure S1 available online). Thus the majority of CENP-A in this cell line was the tagged version. A similar downregulation consistent with competition between exogenous and endogenous CENP-A for assembly at centromeres was reported for cell lines expressing tagged versions of CENP-A (Foltz et al., 2006; Jansen et al., 2007). Remarkably, to adapt to the unusual level of tagged CENP-A, upregulation of key CENP-A partners occurred in parallel, thereby facilitating their biochemical identification (see text later in Results).

Using this cell line, we followed a fractionation scheme previously used to assess posttranslational modifications of predeposited and deposited H3 (Loyola et al., 2006). In this way (Figure 1B), we purified predeposited e-CENP-A from both soluble cytosolic extracts (hypotonic cell lysis) and soluble nuclear extracts (high-salt extraction) in parallel with e-H3.1 and e-H3.3 to reveal factors differentially represented. To control for nonspecific interactors, we performed a mock purification from untransduced HeLa S3 cells. From silver staining, three major bands in the e-CENP-A soluble nuclear complex were analyzed by mass spectrometry (Figure 1C). In the highest migrating band, we identified a mammalian-specific 83 kDa protein as a new candidate for a CENP-A chaperone. This protein, first annotated as hFLEG1 (human fetal liver expressing gene 1, GenBank accession number AB101211), is also known as HJURP (Holliday Junction-Recognizing Protein) (Kato et al., 2007). In addition we found two known histone chaperones. The first chaperone, RbAp48, a retinoblastoma-binding protein (Qian and Lee, 1995), is part of the human CAF-1 complex and interacts with H4 (Murzina et al., 2008; Song et al., 2008; Verreault et al., 1998). The second, Npm1 (Nucleophosmin 1 or B23), has affinity for all core histones (Namboodiri et al., 2004; Okuwaki et al., 2001).

While HJURP was listed among many proteins copurifying with CENP-A-containing nucleosomes (Foltz et al., 2006), here it was a prominent component of e-CENP-A soluble complexes from cytosolic and nuclear fractions (Figures S2B, 1C, and 1D). Higher levels were reproducibly present in nuclear fractions. Intriguingly, in the tagged cell line where e-CENP-A levels are upregulated, HJURP levels also increased pointing to possible coregulation of CENP-A and this interacting partner (Figure S1).





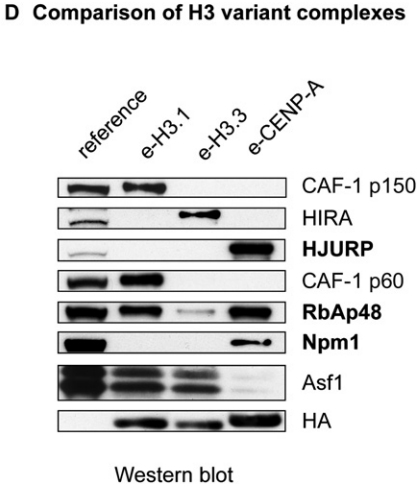


Figure 1. Purification of e-CENP-A Complexes

(A) e-CENP-A localizes at centromeres. Scheme showing CENP-A tagging on C terminus with FLAG and HA. HeLa S3 cell line expressing e-CENP-A stained with anti-HA antibody (green) and anti-CENP-A (red) antibody reveals tagged CENP-A localization at centromeres in a mitotic cell. Scale bar, 5 μm.

(B) Purification scheme of e-CENP-A complexes shows soluble cytosolic and nuclear fractions corresponding to predeposited e-CENP-A complexes. Pellet fraction after nuclear extraction contains chromatin-bound proteins including deposited histones.

(C) e-CENP-A complex by mass spectrometry. Silver-stained gel corresponds to e-H3.1, e-H3.3, and e-CENP-A complexes isolated from nuclear fraction (see also Figure S1D). *Bands present in all preparations.

(D) Comparison of H3 variant e-H3.1, e-H3.3, and e-CENP-A complexes by western blotting. We revealed hCAF-1 p150, HIRA, HJURP, hCAF-1 p60, RbAp48, Nmp1, Asf1a/b, and HA as indicated.

Weak histone H4 staining in the e-CENP-A complex suggests cell-cycle variation, as we could detect it in complexes from late G1 synchronized cells (Figure S1D), or the presence of an H4 variant (Ekwall, 2007). We further compared H3.1, H3.3, and CENP-A complexes by western analysis (Figure 1D). Consistent with previous findings (Tagami et al., 2004), we found p150 and p60 subunits of CAF-1 in the e-H3.1 complex and not e-H3.3; HIRA present only in the e-H3.3 complex; and Asf1a/b in both, although at somewhat different levels. Neither of these H3 chaperones was detected in the e-CENPA complex showing that it is a distinct entity. Importantly, we confirmed that HJURP and Npm1 were specifically enriched in the e-CENP-A complex and not detected in e-H3.1 and e-H3.3 complexes (Figure 1D) while RbAp48 copurified with all three complexes (Figure 1D). HJURP and RpAp48 remained stably associated with e-CENP-A, while Npm1 was lost from the complex under conditions of high salt (400 mM KCl, Figure S2A). Thus, we conclude that among the three partners, only Npm1 and HJURP were specifically found in the e-CENP-A complex and HJURP showed a more stable association.

HJURP and Interactions with CENP-A

The fact that HJURP was not detected in H3.1-CAF-1 or H3.3-HIRA complexes, whereas RbAp48 was present in all three complexes (Figure 1C), was an initial indication of a specific association with predeposited CENP-A. We used reciprocal immunoprecipitations with nuclear extracts from a cell line stably expressing GFP-HJURP to confirm that HJURP and CENP-A were together in a complex. Using both anti-GFP and anti-HJURP antibodies, endogenous CENP-A readily coimmunoprecipitated with GFP-HJURP (Figure 2A). We also prepared nuclear extracts from the e-CENP-A cell line and showed that anti-HJURP antibodies specifically immunoprecipitated e-CENP-A and not histone H3 (Figure 2B), a further argument for specificity. Importantly, neither RbAp48 nor Npm1 were detectable in the HJURP immunoprecipitated fraction, indicating that they are not in the same complex as CENP-A and HJURP. Finally, we used e-CENP-A and e-H3.1 octamers derived from cell lines (see Experimental Procedures) for a pull down with full-length recombinant GST-HJURP. Western blot with anti-HA antibody shows that GST-HJURP only interacted with the fraction involving e-CENP-A and not e-H3.1 (Figure 2C). Thus we conclude that HJURP is a specific component of the CENP-A

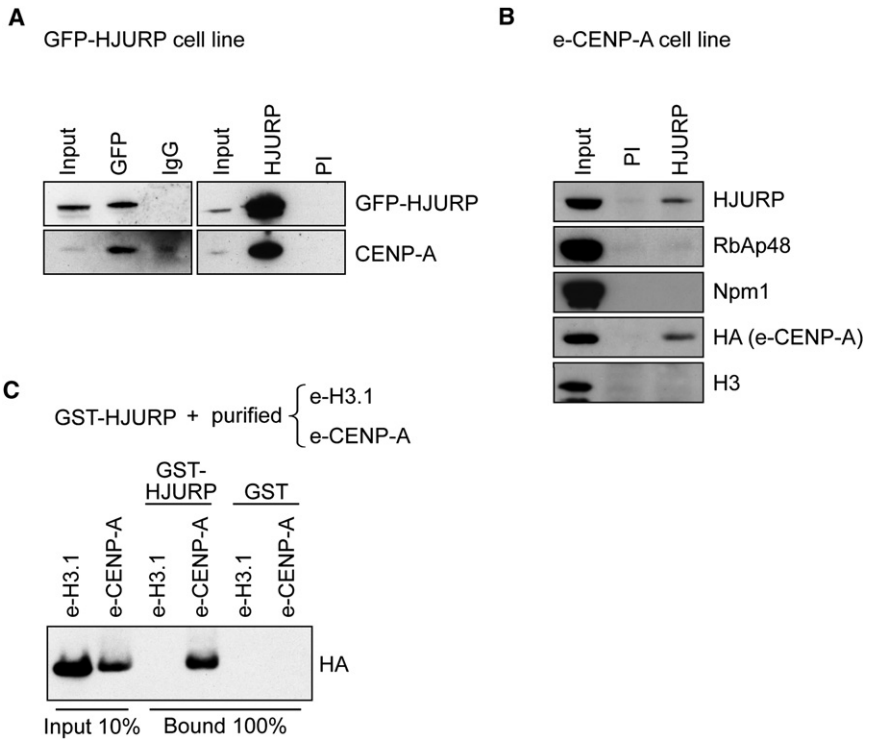


Figure 2. HJURP and CENP-A Interactions

(A) GFP-HJURP immunoprecipitates endogenous CENP-A. We used anti-GFP (or IgG control) (left) or anti-HJURP antibodies (or preimmune (PI) control) (right) to immunoprecipitate GFP-HJURP from nuclear extracts derived from stable cell line expressing GFP-HJURP (input). Immunoblots on precipitates (IP) show GFP and CENP-A.

(B) HJURP immunoprecipitates e-CENP-A. Nuclear extracts from e-CENP-A cell line (input) were immunoprecipitated with rabbit anti-HJURP or preimmune (PI) serum. Western blotting shows HJURP, RbAp48, Npm1, HA (e-CENP-A), and H3.

(C) Specific interaction between HJURP and CENP-A in vitro. GST-HJURP was used as bait for a pull-down experiment with purified-e-CENP-A and e-H3.1 octamers (Experimental Procedures). Western analysis reveals HA.

complex in vivo. Furthermore, our in vitro interaction studies using recombinant HJURP and CENP-A purified octamers exclude other bridging partners to mediate the interaction. Taken together, these properties make HJURP an ideal dedicated chaperone and prompted further investigation into the ability of HJURP to promote CENP-A deposition in vivo.

HJURP Localization during the Cell Cycle

Considering that the loading of new CENP-A at centromeres occurs during late telophase/early G1 phase (Jansen et al., 2007), enrichment of key CENP-A deposition factors at centromeric chromatin is expected at this time. A first localization of HJURP, consistent with earlier observations (Kato et al., 2007), showed a diffuse localization pattern in the nucleus (Figure 3A, −TX). Cells displayed varying levels of HJURP expression, suggesting cell-cycle regulation. Removal of the soluble HJURP pool by triton extraction (Martini et al., 1998) showed that different levels of nucleolar staining remained, colocalizing with the nucleolar marker fibrillarin (Figure 3A, +TX). Thus, like Npm1, HJURP can localize to the nucleolus, a site often related to storage for a variety of proteins. To explore a more direct connection to centromeres, we focused on HJURP localization through the cell cycle. First, we used triton-extracted cells costained with antibodies against HJURP and the CAF-1 subunit p150 (1) for a comparison with a distinct H3.1 loading factor and (2) for marking cells in S phase with the typical early, mid, and late S phase profile in a manner that parallels BrdU incorporation or PCNA staining (Figure 3B) (Krude, 1995; Martini et al., 1998). HJURP localization differed from CAF-1 p150, emphasizing their different roles. HJURP nucleolar staining was detected in early, mid, and late S phase with increasing intensity as cells progressed through S phase. Notably, in a small fraction of cells (4% ± 0.4%), HJURP localized on chromatin in a particular spotted pattern always observed in two cells side by side, likely to have just completed cell division. Cells displaying this distinctive HJURP localization were always negative for CAF-1 p150 and thus out of S phase. To further delineate the exact stage outside of S phase, we used costaining with antibodies against histone H3 phosphorylated on serine 10 (pH3ser10) marking cells in late G2 phase, prophase, metaphase, and late anaphase of mitosis. The negative pH3ser10 staining in cells displaying the HJURP spotted pattern showed that they were in late telophase/early G1 phase (Figure 3C). Using anti-tubulin antibody to mark cells undergoing division, we further refined this time window to the point in mitosis when the mid-body is visible (Figure 3D). We conclude that HJURP is enriched in nuclear spots on chromatin during late telophase of mitosis just prior to or at the moment of cytokinesis, when cells enter into early G1 phase.

HJURP Associates with Centromeres during Late Telophase/Early G1 Phase

As HJURP localization onto chromatin appeared to coincide precisely with the short time window when new CENP-A is loaded at centromeres (Jansen et al., 2007), we assessed whether HJURP associates with centromeres at this time. Remarkably, costaining of late telophase/early G1 cells with anti-CENP-A antibodies revealed that HJURP spots were at or slightly adjacent to centromeres (Figure 4A, left). A confocal section of G1 cells also showed this overlap of HJURP with CENP-A on chromatin (Figure 4A, right), consistent with the fact that HJURP copurifies with CENP-A-containing nucleosomes (Foltz et al., 2006). This partial overlap with HJURP staining always extended on one side of CENP-A staining (Figures 4A and 4B). We further examined the dynamics of HJURP localization at centromeres selectively in a G1 population, after synchronization in mitosis using nocodazole and release into G1 (Figure 4B). Cells in G1, fixed at various time points, were costained with anti-HJURP antibody

and either anti-CENP-A antibody to mark centromeres (Figure 4B) or anti-tubulin antibody to mark the mid-body (Figure 3D). An enrichment for cells with centromeric HJURP (73% of total cells counted, n = 1000) was found in early G1 (2 hr). At this time, cells with mid-body staining were also enriched (38% of total cells counted, n = 1000) with one mid-body visible for every two cells with centromeric HJURP. At later time points in G1 (3 and 4 hr), the number of cells showing the centromeric HJURP pattern decreased, along with cells positive for mid-body staining. We conclude that in U2OS cells HJURP localizes transiently at centromeric chromatin for 2 to 3 hr in late telophase/early G1, which coincides with the precise timing of new CENP-A deposition at centromeres. Interestingly, FRAP experiments showed that CENP-A, normally stably associated with centromeres through the cell cycle, is dynamic during that same period of 3 to 4 hr in early G1 (Hemmerich et al., 2008). These similar dynamics between HJURP and CENP-A prompted further investigation into the ability of HJURP to act as a CENP-A localization/deposition factor.

HJURP Is Required for CENP-A Localization to Centromeres and for Accurate Chromosome Segregation

To assess the role of HJURP in CENP-A localization/deposition at centromeres, we transfected human U2OS cells with two independent siRNAs against HJURP (#1 and #2) to downregulate its expression. Both immunostaining (Figure 5A) and western blot showed reduced HJURP signals although slightly less pronounced with si#2 (Figure 5B). Remarkably, immunostaining with anti-CENP-A antibody showed a dramatic reduction in CENP-A localization at centromeres 72 hr after treatment with HJURP siRNAs in most cells (Figure 5A). This reduction already detected between 36 and 48 hr after transfection with si-HJURP was progressive with time (data not shown). Reduced CENP-A on depletion of HJURP and as cells undergo division suggests that either the stability of CENP-A already incorporated or the lack of provision of new CENP-A at centromeres is compromised, or both, which ultimately leads CENP-A to become diluted out of chromatin. Indeed, western analysis showed that the total level of CENP-A was also reduced on depletion of HJURP (Figure 5C), while the level of histone H3 was unaffected (Figure S3A). Western blot shows that downregulation of HJURP 144 hr after transfection did not result in an alteration of RbAp48 or Npm1 levels, suggesting that the dramatic effects on CENP-A localization were not indirectly due to downregulation of these factors (Figure 5C). Cell-cycle analysis of HJURP-depleted cells by FACS and detection of comparable numbers of early G1 cells in si-HJURP- and si-control-treated cells by immunostaining with anti-tubulin antibody indicate that the loss of CENP-A from centromeres is not a result of failure to proceed into G1, the time at which CENP-A should be loaded (Figures S3B and S3C). Reduced CENP-A localization at centromeres was also observed on treatment of HeLa cells with HJURP siRNAs (Figure S4B), indicating that this reduction in CENP-A localization is not a peculiarity of the U2OS cell line. A more detailed analysis by DNA FISH where the number of alpha-satellite signals per nucleus was quantified showed that reduced levels of CENP-A staining upon reduction of HJURP did not reflect a general disruption or dispersal of centromeric domains that could have occurred as a consequence of major genomic rearrangements or breakages (Figure S5). Taken together, these data argue for a defect in CENP-A localization at centromeres.

After a number of rounds of cell division, HJURP-depleted cells accumulated in mitosis (1.65% ± 0.35% in control siRNA transfected cells and 6% ± 0.25% in si-HJURP transfected cells, n = 400) (Figure 5D). Similar to phenotypes reported for other factors that disrupt CENP-A association with centromeres (Fujita et al., 2007; Maddox et al., 2007), cells exhibited defects in chromosome segregation, most likely reflecting a need for a critical amount of CENP-A to complete a successful mitosis (Black et al., 2007). Indeed, immunofluorescent staining with CENP-A antibody confirmed that cells with segregation defects had reduced CENP-A levels (Figure 5D). si-HJURP-treated cells primarily displayed defects in anaphase, where 79% (n = 70) of anaphase cells analyzed were abnormal, with lagging chromosomes or anaphase bridges. Mitotic defects in metaphase were less pronounced, where 43% (n = 100) of metaphases analyzed were abnormal, with misaligned chromosomes on the metaphase plate. While the kinetochore protein CENP-B that binds to centromeres in a sequence-specific manner remained localized upon HJURP downregulation, some decrease in CENP-C association with centromeres on metaphase chromosomes was observed (Figure S6). We conclude that HJURP downregulation results in a dramatic loss of CENP-A from centromeres, likely impacting on kinetochore assembly and microtubule attachment, which can explain defects in chromosome segregation that ensue during mitosis.

Npm1, RpAp48, and CENP-A Localization

To determine whether CENP-A defects observed after HJURP depletion were unique to HJURP or could arise after any other chaperone in our soluble complex was depleted, we also downregulated Npm1 and RbAp48 chaperones. Npm1's diverse functions include ribosome biogenesis, centrosome duplication, and maintenance of genome stability (Grisendi et al., 2006). Interestingly, Npm1 was previously found to associate with CENP-A-containing nucleosomes (Foltz et al., 2006); however its role in relation to centromeres had not been explored. We could downregulate the Npm1 level using a pool of four siRNAs, partially by western blot but comparable to HJURP (si-HJURP#2, Figure 5B), but Npm1 depletion was clearly visible by immunofluorescence. However this siRNA treatment did not result in any obvious defect in CENP-A localization at centromeres that was comparable to HJURP depletion (Figure 6A). Using immunofluorescent staining with anti-tubulin antibody that marks the mid-body, we verified that Npm1-depleted cells progress into G1 when new CENP-A is loaded (Figure 6B), and thus we could exclude a major role for Npm1 in directing CENP-A deposition in vivo.

In yeast and HeLa cells, interference with RbAp48 along with RbAp46 led to defects in CENP-A localization at centromeres (Hayashi et al., 2004). Importantly, we reproduced these results in U2OS cells (Figure S7). While downregulation of RbAp48 alone resulted in reduced CENP-A at centromeres, codepletion of RbAp46 worsened the effect as reported (Hayashi et al., 2004) (Figure S7B). As RpAb48 is part of several chromatin-modifying

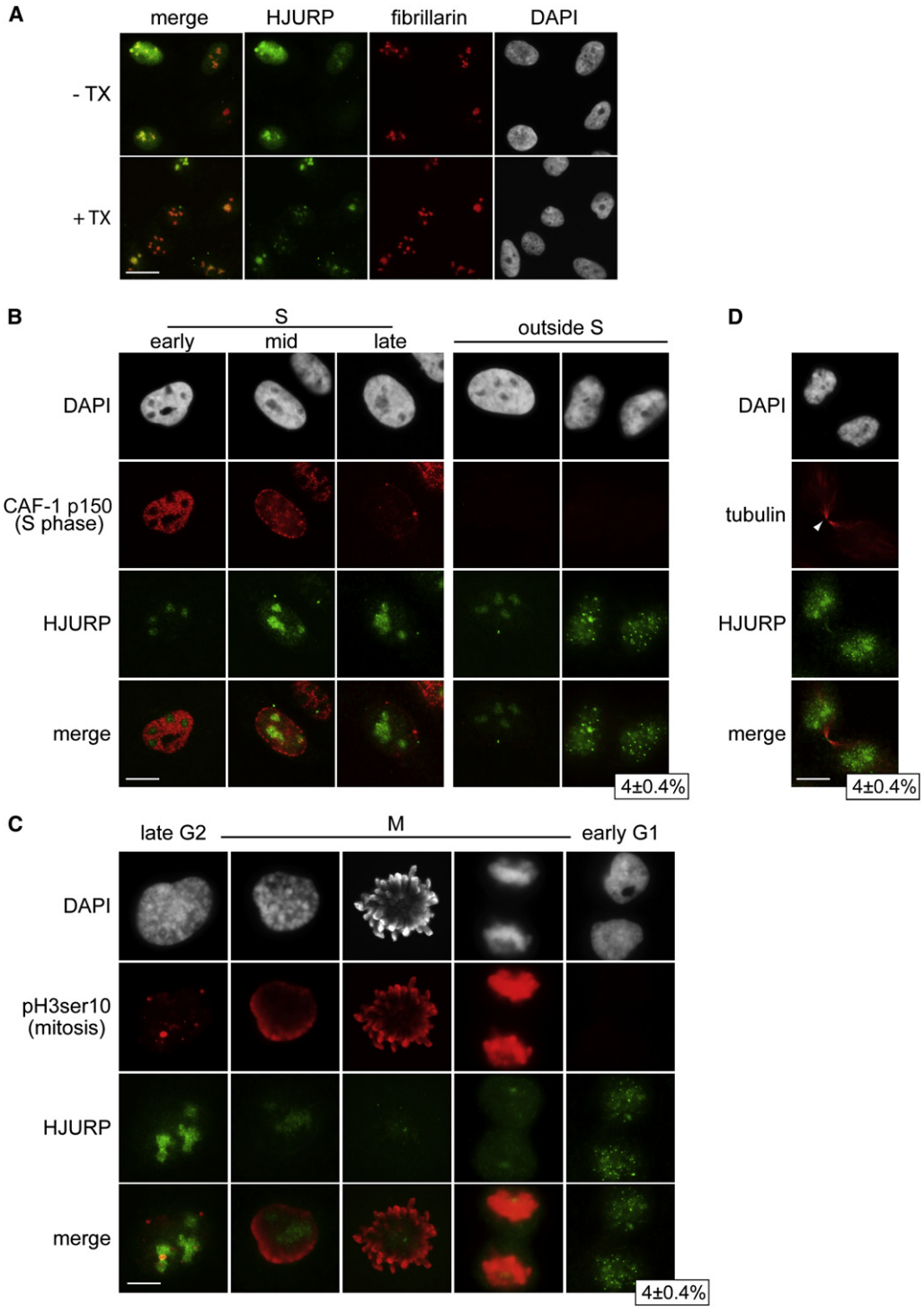


Figure 3. Cell-Cycle Localization of HJURP

(A) U2OS cells fixed without triton (−TX) to visualize the whole pool of proteins and with triton (+TX) to remove the soluble pool were stained to reveal HJURP (green), fibrillarin (nucleolar marker in red), and DAPI. Scale bar, 20 μm.

(B) Cell-cycle localization of HJURP during and out of S phase by immunofluorescence microscopy. After removal of soluble HJURP pool, we revealed HJURP (green) and CAF-1 p150 (red). HJURP shows a nucleolar staining pattern in S phase (CAF-1 p150-positive in early, mid, and late) and out of S phase (CAF-1 p150-negative). HJURP shows a spotted nuclear staining in 4% ± 0.4% of cells that were CAF-1 p150 negative, i.e., out of S phase. Scale bar, 10 μm.

(C) Cell-cycle localization of HJURP during mitosis. As in (B), we revealed HJURP (green) and phosphoH3ser10 (red) as a marker of late G2/mitotic cells. Late telophase/early G1 cells showing spotted HJURP pattern in 4% ± 0.4% of total population were pH3ser10 negative. Scale bar, 10 μm.

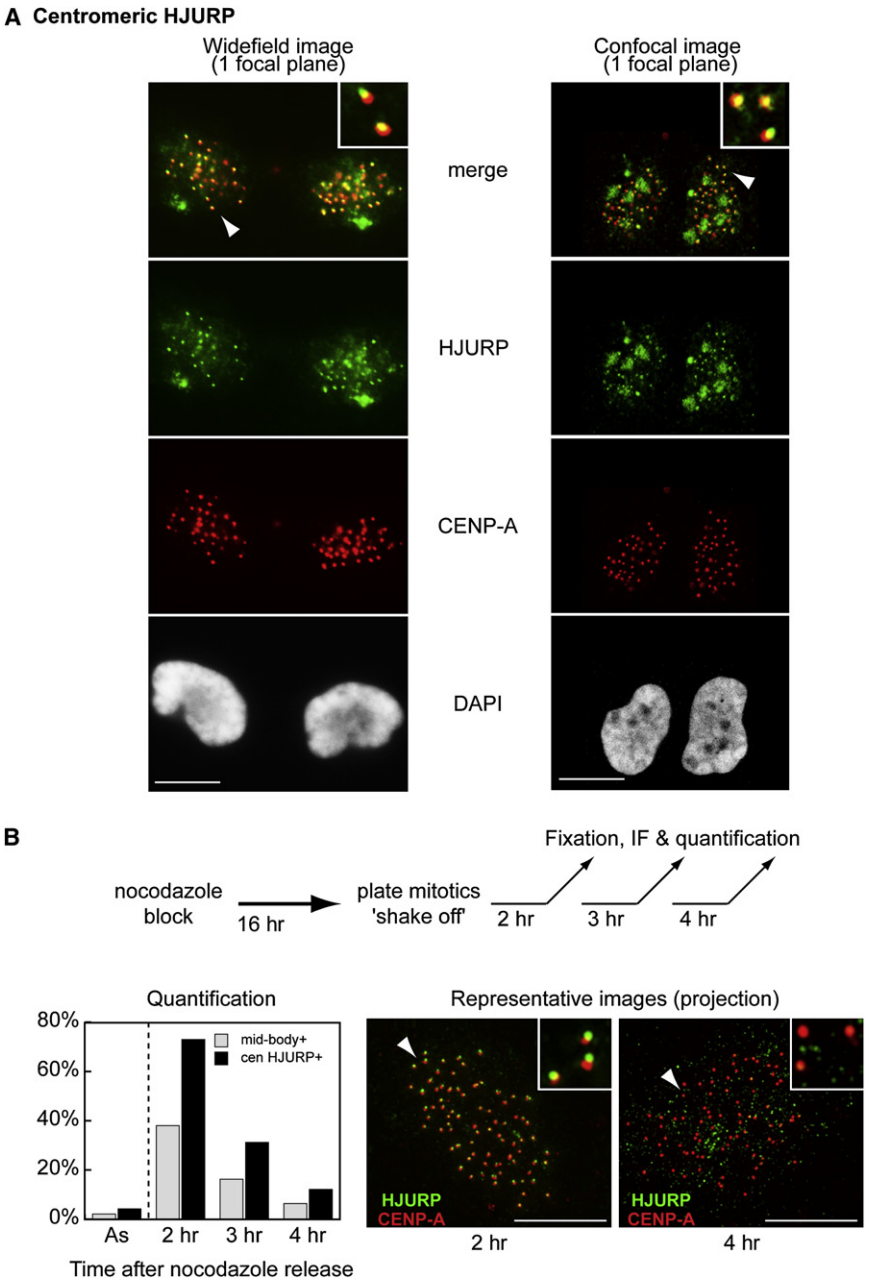


Figure 4. Centromeric Localization of HJURP

(A) Centromeric HJURP in late telophase/early G1 cells. Left panel: widefield Leica image (one focal plane) of triton-extracted G1 cells. Staining reveals HJURP (green) and CENP-A (red). Scale bar, 10 μm. Inset represents enlarged image indicated by the arrow. Right panel: confocal image (one focal plane) of two G1 cells showing HJURP (green) and CENP-A (red) costaining. With high resolution, CENP-A and HJURP signals colocalize (estimation of resolution in Supplemental Data). Scale bar, 10 μm. We note that HJURP marks the centrosome.

(B) Transient association of HJURP with centromeres during late telophase/early G1. Scheme shows synchronization of cells in mitosis with nocodazole and release into G1. Left panel: Graph shows quantification of cells with mid-body staining (mid-body+) and HJURP at centromeres (cen HJURP+) in asynchronous (As) cells or cells 2, 3, and 4 hr after release into G1 (n = 500 for each time point). One hundred percent represents total number of cells and percentages given are averages of two independent experiments. Right: projection of deconvolved Delta Vision images representative of HJURP (green) and CENP-A (red) localization in cells 2 and 4 hr after release into G1. Insets represent enlarged images indicated by arrows. Quantification of colocalization after 2 hr shows that in 73% of cells, HJURP and CENP-A colocalize and in only 12.1% after 4 hr. Scale bar, 5 μm.

complexes, including CAF-1 (De Koning et al., 2007), it is not surprising that depletion of RbApA8 gives a complex phenotype, including cell-cycle defects where few cells progress into late telophase/G1 when new CENP-A should be loaded (Figure S7B). Remarkably, downregulation of both RpAp48 and RbAp46 reduced levels of HJURP in these cells (Figure 6D). Thus, this observation supports some link between HJURP and RbAp48, and the effect on CENP-A localization observed could possibly relate to reduced HJURP. We conclude that neither Npm1 nor RbAp48/46 depletion leads to defects strictly comparable to those observed with HJURP depletion.

HJURP Is Required for Incorporation of Newly Synthesized GFP-CENP-A to Centromeres

Our results show that downregulation of HJURP results in reduced CENP-A association at centromeres and implicates HJURP in CENP-A stability. However, this does not exclude an additional role in the specific targeting or deposition of new CENP-A at centromeres. To investigate this possibility, we designed a transient transfection assay, based on a previous approach, to follow the incorporation of new histones in vivo (Polo et al., 2006) (Figure 7A). Importantly, removal of soluble GFP-CENP-A by pre-extraction before fixation enabled us to reveal only chromatin-bound GFP-CENP-A. We verified that GFP-CENP-A localizes during mitosis to centromeres in vivo although overexpressed

(D) Cell-cycle localization of HJURP during late telophase/early G1. Triton-extracted U2OS cells show HJURP (green). Tubulin (red) detects the mid-body (white arrow) that appears in late telophase just prior to or at the point of cytokinesis. Mid-body stained cells showed spotted HJURP pattern (4% ± 0.4% of total population). Scale bar, 10 μm.

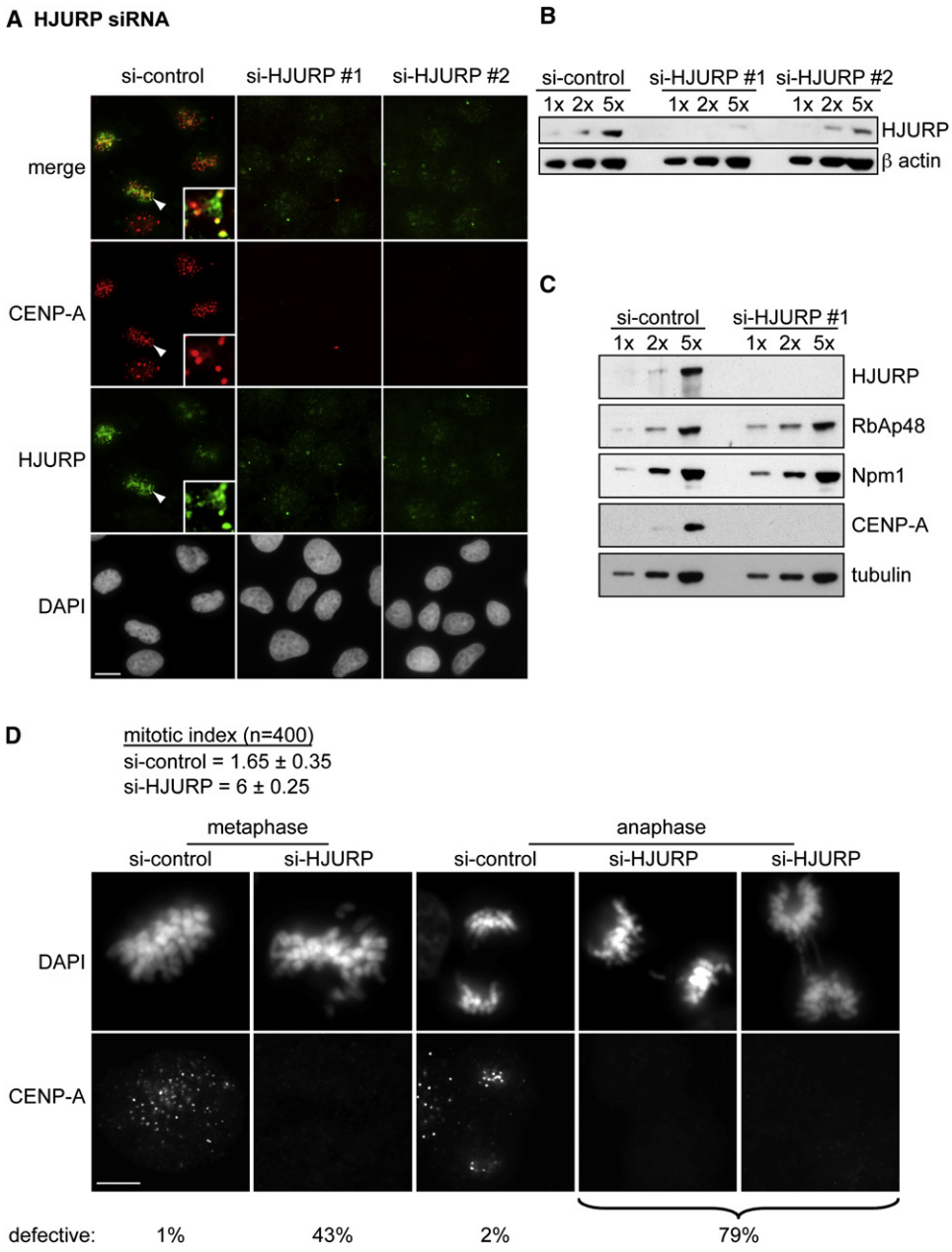


Figure 5. siRNA Strategy to Downregulate HJURP Expression

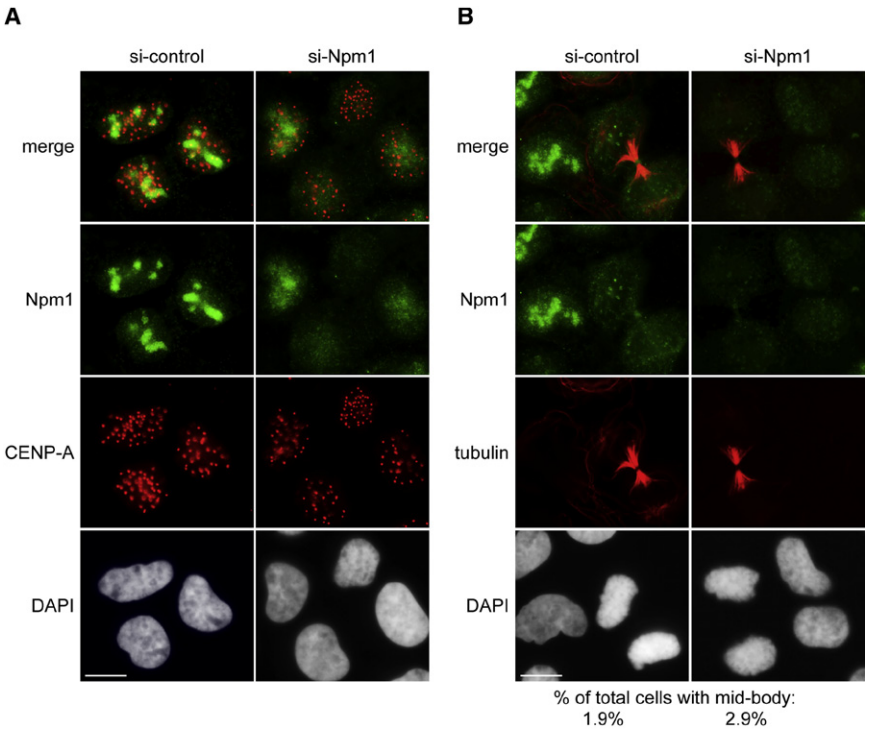
(A) Downregulation of HJURP by two independent siRNAs (si-HJURP#1 & #2) in U2OS cells results in reduced association of CENP-A with centromeres. Seventy-two hours after transfection with siRNAs (HJURP or control), we detected and visualized CENP-A (red) and HJURP (green) by immunofluorescence. Inserts show images for centromeric HJURP/CENP-A staining in G1 cell. Scale bar, 10 μm.

(B) HJURP downregulation on transfection of siRNAs as in (A) assessed by western analysis on total extracts to reveal HJURP and actin as loading control.

(C) Western blotting of total extracts from si-HJURP#1-treated cells 144 hr after transfection. 1x, 2x, 5x are relative amounts of protein loaded. Levels of CENP-A, RbAp48, and Nmp1 were assessed with tubulin as loading control.

(D) Defects in chromosome segregation at mitosis after siRNAs against HJURP#1 (144 hr after transfection). Mitotic index was calculated by counting prophase, metaphase, and anaphase cells (n = 400). DAPI staining of si-HJURP-treated cells show metaphase (43%, n = 100) and anaphase defects (79%, n = 70) defects. CENP-A staining confirmed CENP-A reduction in si-HJURP-depleted cells. Control siRNA treated cells show a baseline missegregation rate in 3.1% of mitoses (n = 200). Scale bar, 10 μm.

(Figure S8A). Interestingly, we noticed that overexpression of GFP-CENP-A showed, in addition to the typical dotty pattern in interphase cells, a global triton-resistant staining reminiscent of overexpression patterns reported in other organisms (Collins et al., 2004; Henikoff et al., 2000; Heun et al., 2006; Van Hooser et al., 2001). Transfection efficiency was similar between cells



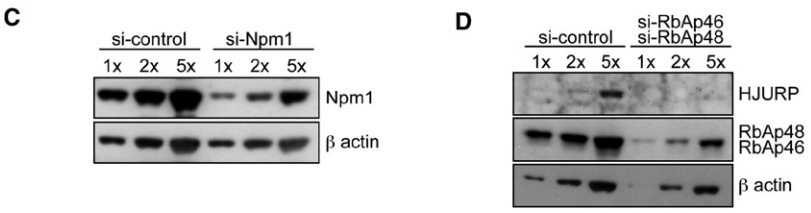


Figure 6. siRNA to Downregulate Npm1 and RbAp48, 46 Expression

(A) Npm1 siRNA and CENP-A localization at centromeres. Immunofluorescent staining of si-control- and si-Npm1-treated cells, CENP-A (red), and Npm1 (green) analyzed 72 hr after transfection. Scale bar, 10 μm.

(B) Numbers of cells in late telophase/early G1 in si-control (1.9%) and si-Npm1 (2.9%) treated cells. Immunofluorescent staining of si-control- and si-Npm1-treated cells revealing tubulin marking mid-body that appears at late telophase/early G1. Percentages indicate numbers of cells with mid-body out of total cells counted (n = 500), and cells were analyzed 72 hr after transfection. Scale bar, 10 μm.

(C) Western blotting of total extracts from si-control- and si-Npm1-treated cells. Anti-Npm1 antibody reveals Npm1 reduction to 20% of control levels 72 hr after transfection. Beta-actin served as loading control.

(D) HJURP downregulation in si-RpAp48,46-treated cells. Western blotting of total extracts from si-control- and both si-RbAp46- and si-RbAp48-treated cells revealing RbAp46,48 and HJURP 72 hr after transfection and beta-actin as loading control.

transfected with control or HJURP siRNAs (average of 47% and 51%, respectively) and new GFP-CENP-A expressed at similar levels in si-control- and si-HJURP-treated cells 48 hr after transfection (Figure 7B). Forty-eight hours after transfection, when cells should have completed at least one cell cycle, control siRNA-treated cells repeatedly showed that newly synthesized GFP-CENP-A, expressed at low levels, was properly targeted to centromeres (Figure 7C). In contrast, in si-HJURP-treated cells, newly synthesized GFP-CENP-A was inefficiently targeted to centromeres and was incorporated all over chromatin (Figures 7C and S8B). Using tubulin to stain the mid-body we detected similar numbers of cells in late telophase/early G1 in control (1.8% ± 0.2% of transfected cells, n = 500) and HJURP (1.7% ± 0.3% of transfected, n = 500) treated cells. Coupled with the earlier observation that HJURP-depleted cells can proceed into G1 (see Figure S3), these results indicate that defects in new CENP-A incorporation are not merely due to defects in cell-cycle progression. Notably, GFP-CENP-A was efficiently transported into the nucleus in HJURP-depleted cells ruling out a role for HJURP in nuclear import. Importantly, although stability of endogenous CENP-A was compromised in si-HJURP-treated cells, newly synthesized GFP-CENP-A protein was here stable (Figure 7B). Thus the loss of CENP-A incorporation at centromeres can be dissociated from degradation because GFP-tagged CENP-A remained stable upon HJURP depletion, yet it could not be incorporated specifically at centromeres. We propose that HJURP is a critical component of the assembly line that chaperones newly synthesized CENP-A to supervise its safe deposition into chromatin at centromeres in a cell-cycle-dependent fashion.

DISCUSSION

Soluble Human CENP-A Complex Reveals HJURP as a Key Partner of Major Importance in Mitosis

Our biochemical fractionation to search for factors interacting with soluble CENP-A identified HJURP, with a key role in the specific localization and inheritance of CENP-A at centromeres that impacts chromosome segregation (Foltz et al., 2009). Specific to the CENP-A complex, HJURP was not detected in H3.1- and H3.3-containing complexes (Figures 1C and 1D). So far we did not find obvious HJURP homologs outside of mammals based on sequence conservation. Thus, our biochemical strategy has proved powerful to identify novel nonconserved partners of CenH3 in mammals, yet functional homologs may exist. In this respect, HJURP may overlap functionally with Scm3, a yeast factor shown to bind CenH3 and to be required for its association with centromeres in both budding and fission yeasts (Camahort et al., 2007; Mizuguchi et al., 2007; Stoler et al., 2007; Pidoux

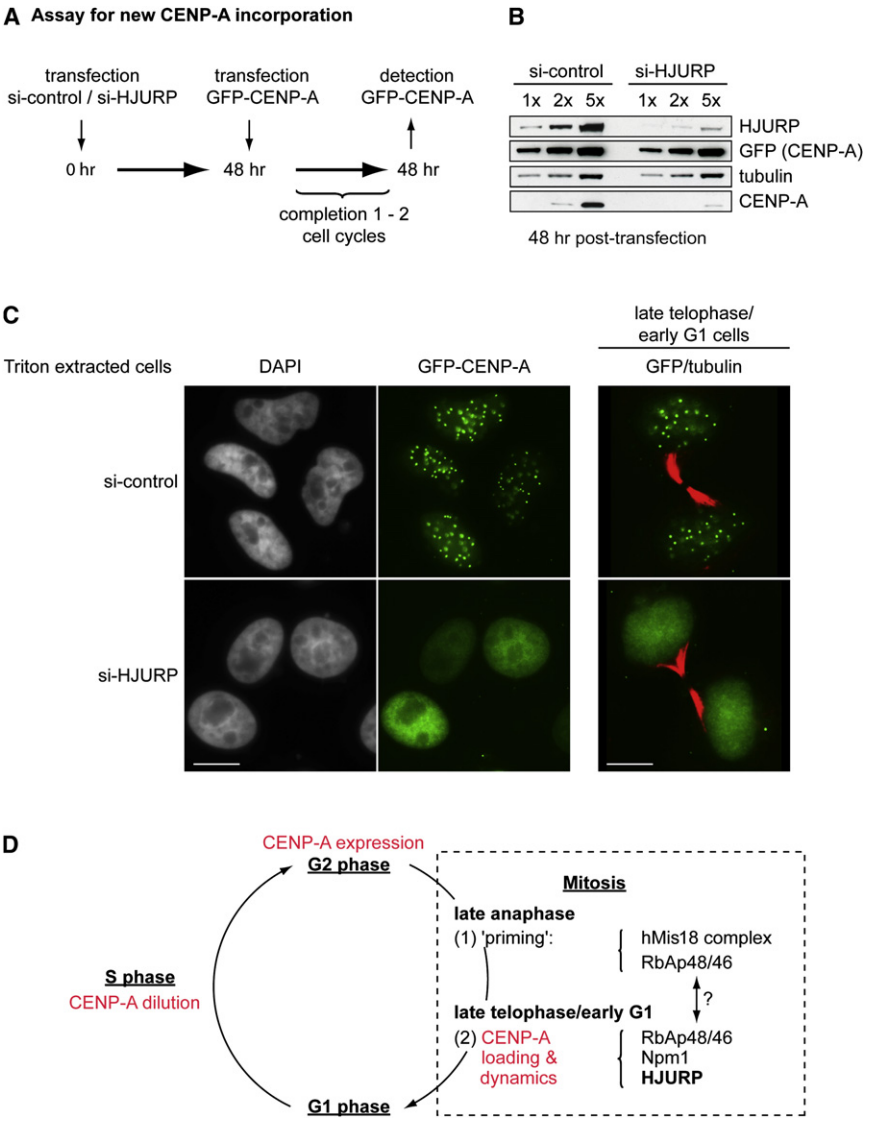


Figure 7. Assay for Incorporation of Newly Synthesized CENP-A

(A) Scheme shows experimental procedure. Cells were pre-extracted to remove soluble GFP-CENP-A pool before fixation and visualization.

(B) Western analysis comparing si-control- and si-HJURP-treated cells. Immunoblotting of total extracts 48 hr after transfection with GFP-CENP-A revealed HJURP, GFP (GFP-CENP-A), endogenous CENP-A, and tubulin as a loading control.

(C) HJURP is required for the deposition of newly synthesized GFP-CENP-A at centromeres. Left: fluorescent images of new GFP-CENP-A expression in si-control- or si-HJURP-treated cells. HJURP depletion results in loss of centromere staining of new GFP-CENP-A. We show cells expressing low levels of GFP-CENP-A along with DAPI images (Figure S8B). Right: merged images of new GFP-CENP-A signal and immunostaining of late telophase/early G1 cells using tubulin antibody in si-control- or si-HJURP-treated cells. Similar numbers of late telophase/early G1 cells were observed in si-control (1.8% ± 0.2% of transfected cells, n = 500) and si-HJURP (1.7% ± 0.3% of transfected cells, n = 500) treated cells. Scale bar, 10 μm.

(D) HJURP and CENP-A deposition during late mitosis: a stepwise model. Cell-cycle dynamics of CENP-A highlight late mitosis/early G1 as the key window for CENP-A incorporation whereas parental CENP-A is diluted at centromeres in S phase (Jansen et al., 2007) and CENP-A expression peaks in G2 (Shelby et al., 2000). We place HJURP and partners in CENP-A deposition according to a stepwise mechanism where (1) centromeres are "primed" to accept CENP-A during late anaphase possibly involving the hMis18 complex (hMis18α, hMis18β, and M18BP1/KNL2) through alteration of histone acetylation status (Fujita et al., 2007; Maddox et al., 2007). This then allows (2) HJURP-specific localization at late telophase to centromeres to promote CENP-A loading. In this process, HJURP plays a dominant role in CENP-A incorporation at centromeres and its stabilization while Npm1 may play an accessory role. RbAp proteins may act as a dynamic bridge between the two steps: in (1) in connection with histone acetylation and chromatin remodeling events, possibly through interactions with H4-H3 to mediate histone exchange, and in (2) by association in the soluble e-CENP-A complex that contains HJURP.

et al., 2009; Williams et al., 2009). Sequence analysis of HJURP did not reveal obvious motifs reminiscent of known histone chaperones. However one coiled-coil structure at the N terminal end could be predicted. We also isolated the known histone chaperones Npm1 and RbAp48 in the CENP-A complex (Figures 1C and 1D). However neither Npm1 nor RbAp48 presence in the complex was necessary for HJURP interaction with CENP-A, nor were they sufficient to compensate for the dominant effect of HJURP depletion (Figures 6 and S7). Downregulation of Npm1 on its own had no obvious effect on CENP-A association with centromeres; however we cannot exclude that it may play a less important role than HJURP or simply that there are redundant factors. We confirmed that downregulation of RbAp48 in conjunction with RbAp46 does compromise CENP-A association with centromeres (Hayashi et al., 2004). But, we also noted that downregulation of RbAp48 and RbAp46 impacts HJURP stability, raising the possibility that the effect of RbAp48 on CENP-A association with centromeres could reflect reduced HJURP function. RbAp48 is a member of a variety of complexes including those with chromatin-related functions and transcriptional repression (De Koning et al., 2007). Accordingly, depletion of RbApA8 gives a complex phenotype, including an abnormal nuclear morphology reminiscent of defects in lamins (Burke and Stewart, 2002) and cell-cycle defects. RpAb48 also interacts with H4 (Verreault et al., 1998) and, in light of more recent structural analysis (Murzina et al., 2008; Song et al., 2008), it is also possible that depletion of both RbAp48 and RbAp46 affects CENP-A incorporation indirectly via interaction with H4. Thus, we conclude that HJURP is a key CENP-A partner that plays a dominant role in the efficient targeting of CENP-A to centromeres, while Npm1

and RbAp48/46 in cooperation with HJURP play more auxiliary roles.

HJURP Function at Centromeres and CENP-A Dynamics during the Cell Cycle

Our data show that HJURP localizes transiently to centromeric chromatin during a 2 to 3 hr window at late telophase/early G1 (Figures 3 and 4), corresponding precisely to the time when CENP-A is most dynamic (Hemmerich et al., 2008) and when new CENP-A is loaded (Jansen et al., 2007). Such dynamics would fit with a role for HJURP in the deposition of new CENP-A. This unique localization of HJURP may provide a new cell-cycle marker to pinpoint the particular time when CENP-A is highly dynamic. Interestingly, on chromatin, CENP-A and HJURP partially colocalize and are juxtaposed where the HJURP signal extends always on one side of the centromere, revealing a polarity to the interaction, and the proportion of overlap diminishes with time. The functional significance of this polarity will be interesting to examine further in the context of events associated with telophase/cytokinesis, a particular point in the cell cycle (Glotzer, 2005; Straight and Field, 2000) during which control mechanisms are just being explored.

Depletion of HJURP showed a major impact on CENP-A localization at centromeres that is paralleled with a decrease in CENP-A levels (Figure 5). These data support a role for HJURP as a chaperone that promotes CENP-A stability, whereby it impacts both the maintenance of parental CENP-A and incorporation of new CENP-A. In light of proteolytic pathways reported in other systems that degrade excess CENP-A (Collins et al., 2004; Moreno-Moreno et al., 2006), HJURP may block CENP-A degradation specifically at the site of incorporation. Aside from its important role in stability, through its particular cell-cycle localization, HJURP likely contributes more directly to CENP-A targeting/assembly. Our assay for de novo incorporation using GFP-tagged CENP-A that remained stable upon HJURP depletion shows that a loss of CENP-A incorporation at centromeres can be dissociated from degradation (Figure 7). Whichever mechanisms permit stabilization of GFP-CENP-A, it proved convenient to separate a strict role in CENP-A stabilization from one in specific targeting/incorporation at centromeres.

Model for Specific Targeting of HJURP-CENP-A to Centromeres

How HJURP is recruited to centromeres and then dissociates remains unclear. Two general means can be considered by modulation of either (1) recipient chromatin to make it competent for HJURP association and/or (2) HJURP and CENP-A interactions and properties. For the first hypothesis, "priming" prepares centromeres to accept CENP-A during late anaphase possibly involving the hMis18 complex that alters histone acetylation (Fujita et al., 2007; Maddox et al., 2007). Remarkably, CENP-A mislocalization upon hMis18α depletion is rescued by addition of the histone deacetylase inhibitor TSA (Trichostatin A) (Fujita et al., 2007). HJURP localization should be examined under these conditions. Furthermore, flanking regions with heterochromatic properties may create the correct environment for deposition (Folco et al., 2008; Greaves et al., 2007). Chromatin remodeling events, possibly mediated by RbAp48 present in complexes like Mi-2/NURD with both remodeling and histone-modifying activities (Denslow and Wade, 2007), may also be important. For the second part, identification of HJURP modifications and/or interacting partners at this particular time could be key. Of note, phosphorylation of HJURP by ATM kinase may be activated at this time, possibly in relation to more DNA damage/repair in these regions (Kato et al., 2007). Also, in early mitosis, phosphorylation of CENP-A by the chromosomal passenger protein Aurora B kinase (Kunitoku et al., 2003; Zeitlin et al., 2001) may be critical. Our e-CENP-A cell line will prove useful in identifying such modifications using mass spectrometry of complexes at different times.

In summary, the stepwise mechanism (Figure 7D), first with a priming event and subsequently with the late telophase/early G1 transient localization of HJURP to centromeres, could account for the timing of new CENP-A deposition, when CENP-A is most dynamic and when parental CENP-A is vulnerable. In such a scheme RbAp proteins could bridge the two steps. We hope that this working model can stimulate further work to better understand how a key event for genome stability can be connected to the intricate network of factors acting to promote cell-cycle progression.

Taken together we propose that HJURP represents a key chaperone for newly synthesized CENP-A that through its particular cell-cycle dynamics facilitates the safe delivery, incorporation, and maintenance of CENP-A at centromeres. This new piece in the puzzle of "CENP-A incorporation at centromeres at telophase" stands out as the first CENP-A-specific chaperone, emphasizing the importance of histone chaperones that we are only beginning to unravel. Interestingly, HJURP was originally identified due to its overexpression in lung cancer cells (Kato et al., 2007), and this link to genome instability in the context of mitosis and cell proliferation should be a focus of future studies. This discovery should open many avenues for cell-cycle studies and broaden our views concerning pathways involved in histone variant deposition independently of DNA synthesis.

EXPERIMENTAL PROCEDURES

Cell Lines and Cloning

CENP-A stably expressed as a fusion protein with C-terminal FLAG- and HA-epitope tags (e-CENP-A) in HeLa S3 cells (Nakatani et al., 2003) was characterized (Figure S1). Full-length *HJURP* was amplified from cDNA (ImaGenes, Germany), cloned with GFP tag into pcDNA5/FRT vector (Invitrogen, V6010-20), and transfected in Flp-In-293 human cell line (Invitrogen, R750-07) for selection of clones stably expressing GFP-HJURP and cloned into p-GEX vector (Amersham) for recombinant GST-HJURP fusion protein expression. For transient GFP-CENP-A expression, we cloned full-length human *CENP-A* amplified from cDNA (ImaGenes, Germany) into pEGFP-C1 vector (BD Clontech).

Purification of e-CENP-A Complex

We purified e-H3.1, e-H3.3, and e-CENP-A complexes (Tagami et al., 2004) and prepared cytosolic and nuclear extracts as in Loyola et al. (2006). After immunoprecipitation on anti-FLAG antibody-conjugated agarose (Sigma) in 250 mM KCl (Loyola et al., 2006), bound polypeptides were eluted with the FLAG peptide and when indicated further purified using anti-HA conjugated agarose (Nakatani et al., 2003).

GST Pull Down and Immunoprecipitations

We prepared e-CENP-A and e-H3.3 octamers (Loyola et al., 2006). Nuclear pellets digested with micrococcal nuclease were fractionated by glycerol

gradient centrifugation to isolate mononucleosomes for immunoprecipitation with anti-FLAG antibody (Supplemental Data).

Immunofluorescence Microscopy

Cells were processed for paraformaldehyde fixation as in Martini et al. (1998), involving for detection of chromatin-bound protein a pre-permeabilization with 0.5% Triton prior to fixation. For immunofluorescence detection, see Supplemental Data.

RNAi, Cell Culture, and Synchronization

U2OS cells were transiently transfected using Lipofectamine 2000 (Invitrogen) and using Oligofectamine (Invitrogen) for siRNAs (OnTarget, Dharmacon) and analyzed 72 or 144 hr later (double round of transfection). For siRNA sequences, see Supplemental Data. Synchronization in mitosis was with nocodazole (50 ng/ml, Sigma) for 16 hr.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and eight figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00254-2.

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