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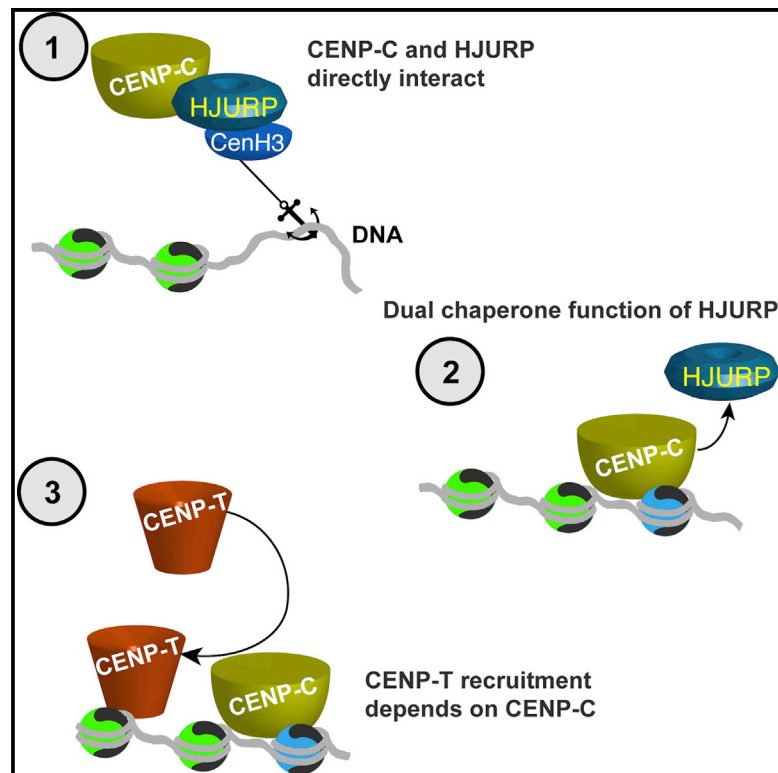
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HJURP Involvement in De Novo CenH3^{CENP-A} and CENP-C Recruitment

Graphical Abstract



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In Brief

Using a synthetic centromere system, Tachiwana et al. show that CENP-C and CENP-T form a single axis of kinetochore establishment, with CENP-C upstream of CENP-T. CENP-C recruitment requires the CAC and CATD of CenH3^{CENP-A}. HJURP directly interacts with CENP-C and has a dual chaperone function, enabling coordination of CenH3^{CENP-A} and CENP-C recruitment.

Highlights

- De novo CENP-T accumulation at an ectopic locus depends on CENP-C
- Two distinct domains in CenH3^{CENP-A} are necessary for de novo CENP-C accumulation
- CENP-C recruitment entails a direct interaction between CENP-C and HJURP
- HJURP is involved in recruiting CENP-C at synthetic centromeres



HJURP Involvement in De Novo CenH3^{CENP-A} and CENP-C Recruitment

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SUMMARY

Although our understanding of centromere maintenance, marked by the histone H3 variant CenH3^{CENP-A} in most eukaryotes, has progressed, the mechanism underlying the de novo formation of centromeres remains unclear. We used a synthetic system to dissect how CenH3^{CENP-A} contributes to the accumulation of CENP-C and CENP-T, two key components that are necessary for the formation of functional kinetochores. We find that de novo CENP-T accumulation depends on CENP-C and that recruitment of these factors requires two domains in CenH3^{CENP-A}: the HJURP-binding region (CATD) and the CENP-C-binding region (CAC). Notably, HJURP interacts directly with CENP-C and is critical for de novo accumulation of CENP-C at synthetic centromeres. On the basis of our findings, we propose that HJURP serves a dual chaperone function in coordinating CenH3^{CENP-A} and CENP-C recruitment.

INTRODUCTION

Centromeres are defined by specific chromatin features and maintained during cell division, which is achieved by a centromere-specific network of proteins in a cell-cycle-controlled manner (Cheeseman et al., 2004; Hori et al., 2008; reviewed in Müller and Almouzni, 2014). In most eukaryotes, centromere identity is maintained by a specific histone H3 variant (Palmer et al., 1987), as its depletion or downregulation leads to impaired accumulation of other components (Howman et al., 2000; Fachinetti et al., 2013). It was originally co-purified as CENP-A together with CENP-B and CENP-C (Earnshaw and Rothfield, 1985), and was recently called CenH3^{CENP-A} according to a nomenclature that is currently being debated (Earnshaw et al., 2013; Müller and Almouzni, 2014; Talbert et al., 2012; Filipescu

et al., 2014). To maintain CenH3^{CENP-A} at centromeres, it is escorted to the locus by its dedicated chaperone, HJURP (Dunleavy et al., 2009; Foltz et al., 2009), which is involved in CenH3^{CENP-A} loading to the chromatin mediated by its DNA-binding domain (Müller et al., 2014). Previous studies proposed that human CenH3^{CENP-A} and fission yeast CenH3^{Cnp1} contribute to centromeric maintenance (Black et al., 2004; Fachinetti et al., 2013) via a mechanism whereby CENP-C levels are maintained at endogenous centromeres through the six C-terminal amino acids of CenH3 (CAC), and CENP-B levels are maintained through its N terminus. Although we have learned a lot about centromere maintenance, the mechanism of de novo centromere formation remains elusive.

Neocentromeres contain all centromere-specific components except for CENP-B (Voullaire et al., 1993; Saffery et al., 2000). It is possible to experimentally engineer neocentromeres upon removal of endogenous centromeres (Hori et al., 2013; Shang et al., 2013). However, using this method, the neocentromeres have already been established and are being maintained, and thus it is not suitable for studying the initial steps of their formation. In contrast, recruiting CenH3 to an ectopic locus, which leads to the formation of a functional kinetochore (Barnhart et al., 2011; Mendiburo et al., 2011; Hori et al., 2013), is useful for studying the mechanism of de novo centromere formation. Tethering fragments of CENP-C or another crucial kinetochore component, CENP-T, to an ectopic locus can bypass the requirement for CenH3^{CENP-A} to form a functional kinetochore (Gascoigne et al., 2011), indicating that CenH3^{CENP-A} may ensure the recruitment of these components. Indeed, human CENP-C binds to CenH3^{CENP-A}-containing nucleosomes via the CAC on the surface of the particle (Carroll et al., 2010; Tachiwana et al., 2011; Kato et al., 2013; Fachinetti et al., 2013). The CAC can promote de novo accumulation of CENP-C to reconstituted nucleosomes using a H3 mutant carrying the CAC (H3^{CAC}) in *X. laevis* egg extracts (Guse et al., 2011). CENP-T forms a complex with CENP-W, which interacts with centromeric H3 nucleosomes in chicken (Hori et al., 2008; Ribeiro et al., 2010). Recent work proposed that CENP-T/W recruitment to centromeres

could depend on kinetochore components downstream of CENP-C (Basilico et al., 2014). Taken together, these findings suggest that CenH3^{CENP-A} could play a key role in the de novo recruitment of CENP-C and CENP-T.

We explored de novo CENP-C and CENP-T recruitment in vivo using the lacO system in human cells. We found that CENP-T accumulation depends on CENP-C at the ectopic locus. To dissect this mechanism further, we tethered CenH3^{CENP-A} or chimera proteins of CenH3^{CENP-A} and H3.1 at lacO, and found that both the HJURP-binding region (CATD) and the CAC of CenH3^{CENP-A} are required for de novo CENP-C recruitment. Furthermore, we found that HJURP directly interacts with CENP-C and is required for efficient recruitment of CENP-C at lacO independently of its function in escorting CenH3^{CENP-A}. We propose that HJURP has a dual functionality: (1) it is involved in CenH3^{CENP-A} loading and (2) it plays a role in the stable incorporation of CENP-C by coordinating the spatial reordering of key centromeric components.

RESULTS

Tethered CenH3^{CENP-A} Recruits CENP-C and CENP-T De Novo at an Ectopic Locus

To investigate de novo CENP-C and CENP-T recruitment, we used the lacO-lacI system in a U2OS cell line with a lacO followed by a tetO array (lacO-tetO U2OS). CenH3^{CENP-A} and H3.1 were transiently expressed as LacI and EGFP fusion proteins (Figure 1A). We investigated whether the lacO-tethered histones are incorporated into chromatin by nucleosome immunoprecipitation (IP) in the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by Southern blot (Figure 1A). We detected lacO DNA in H3.1 and CenH3^{CENP-A} precipitates, but not in LacI-EGFP. As a control, we performed the experiment in the absence of IPTG (Figure S1A), where we detected lacO DNA for LacI-EGFP. We observed a stronger signal for LacI-EGFP than for LacI-EGFP-CenH3^{CENP-A} as a result of LacI-EGFP-CenH3^{CENP-A} also localizing to centromeres (Figure 1B). Taken together, these findings indicate that the tethered histones are incorporated into the lacO chromatin.

Next, we investigated de novo CENP-C and CENP-T accumulation at lacO, tethered with CenH3^{CENP-A} or H3.1 (Figure 1B). A TetR-mCherry fusion protein was co-expressed as a control to indicate the position at lacO. Tethering with CenH3^{CENP-A}, but not H3.1, resulted in CENP-C and CENP-T recruitment to lacO. The recruitment of LacI-EGFP-CenH3^{CENP-A} and CENP-C did not change upon addition of IPTG (Figure S1B), suggesting that LacI-EGFP-CenH3^{CENP-A} is incorporated at lacO in vivo. We observed the recruitment of NDC80 during mitosis, indicating that our system can form kinetochores (Figure S1C). This demonstrates that LacO-EGFP-histones H3 are incorporated at lacO and that our system is suitable for investigating de novo recruitment of factors downstream of CenH3^{CENP-A}.

De Novo CENP-T Accumulation at an Ectopic Locus Depends on CENP-C

Recent work suggested that CENP-T recruitment is linked to kinetochore components downstream of CENP-C (Basilico

et al., 2014). We examined the interdependence of de novo CENP-T and CENP-C recruitment following CenH3^{CENP-A} accumulation. For this purpose, we performed co-immunostaining against CENP-C and CENP-T in cells with CenH3^{CENP-A} tethered to lacO (Figure 2A). We found three patterns of LacI-EGFP-CenH3^{CENP-A} at lacO: (1) neither CENP-C nor CENP-T enriched, (2) only CENP-C enriched, and (3) both CENP-C and CENP-T enriched. We could not detect CENP-T at lacO in the absence of CENP-C. This suggests that de novo CENP-T recruitment depends on CENP-C.

Next, we tested whether CENP-C can recruit CENP-T in the absence of CenH3^{CENP-A} by tethering CENP-C at lacO. Since tethering CENP-C can lead to the accumulation of CenH3^{CENP-A} in chicken (Hori et al., 2013), we tethered full-length CENP-C and four fragments (aa 1–235, 1–425, 236–703, and 704–943), and assessed de novo CENP-T and CenH3^{CENP-A} accumulation (Figure 2B). Tethering full-length CENP-C and the aa 236–703 fragment led to the accumulation of both CENP-T and CenH3^{CENP-A} (Figure 2B). We detected CENP-T accumulation at lacO when we used the aa 1–425 CENP-C fragment, which lacks CenH3^{CENP-A} binding, and found that CENP-T was recruited without CenH3^{CENP-A} (Figure S2A), in contrast to the aa 236–703 fragment. This suggests that CENP-T recruitment is directly dependent on CENP-C, but not on CenH3^{CENP-A}, and that residues 236–425 of CENP-C are important for recruiting CENP-T. This reinforces previous observations in human cells (Basilico et al., 2014), which may be distinct from the chicken system (Hori et al., 2013). When we performed a homology comparison between human and chicken CENP-C, we found no conservation in the region we identified to be important for CENP-T recruitment (Figure S2B). Therefore, the independence of the CENP-T and CENP-C pathways may be a singularity of the chicken system, and in human cells CENP-T recruitment is downstream of CENP-C.

The CAC and CATD of CenH3^{CENP-A} Are Required for De Novo CENP-C Accumulation at lacO

Next, we investigated how CENP-C is recruited de novo to an ectopic locus containing CenH3^{CENP-A}. We took advantage of our lacO-lacI system to determine which regions of CenH3^{CENP-A} are responsible for de novo CENP-C accumulation. The chimera proteins we constructed were H3^{CAC}, H3^{CATD}, and H3^{CATD_CAC} carrying the CAC, which mediates CENP-C recruitment to CenH3^{CENP-A}-containing nucleosomes in vitro (Carroll et al., 2010; Guse et al., 2011), and/or the CATD, which interacts with HJURP (Shuaib et al., 2010; Hu et al., 2011) and is sufficient to recruit CenH3^{CENP-A} to centromeres (Black et al., 2004; Figure 3). H3.1-CenH3^{CENP-A} chimera proteins were transiently expressed as LacI and EGFP fusion proteins in lacO-tetO U2OS, as evidenced by western blot (Figure S3A). We confirmed that H3^{CAC}, H3^{CATD}, and H3^{CATD_CAC} were also incorporated into chromatin (Figure S3A). We then investigated de novo CENP-C and CENP-T accumulation at lacO tethered with these H3 chimeras. Strikingly, tethering H3^{CAC} or H3^{CATD} did not lead to CENP-C or CENP-T accumulation at the locus, suggesting that neither of these domains is sufficient to recruit CENP-C (Figure 3). Tethering H3^{CATD_CAC}

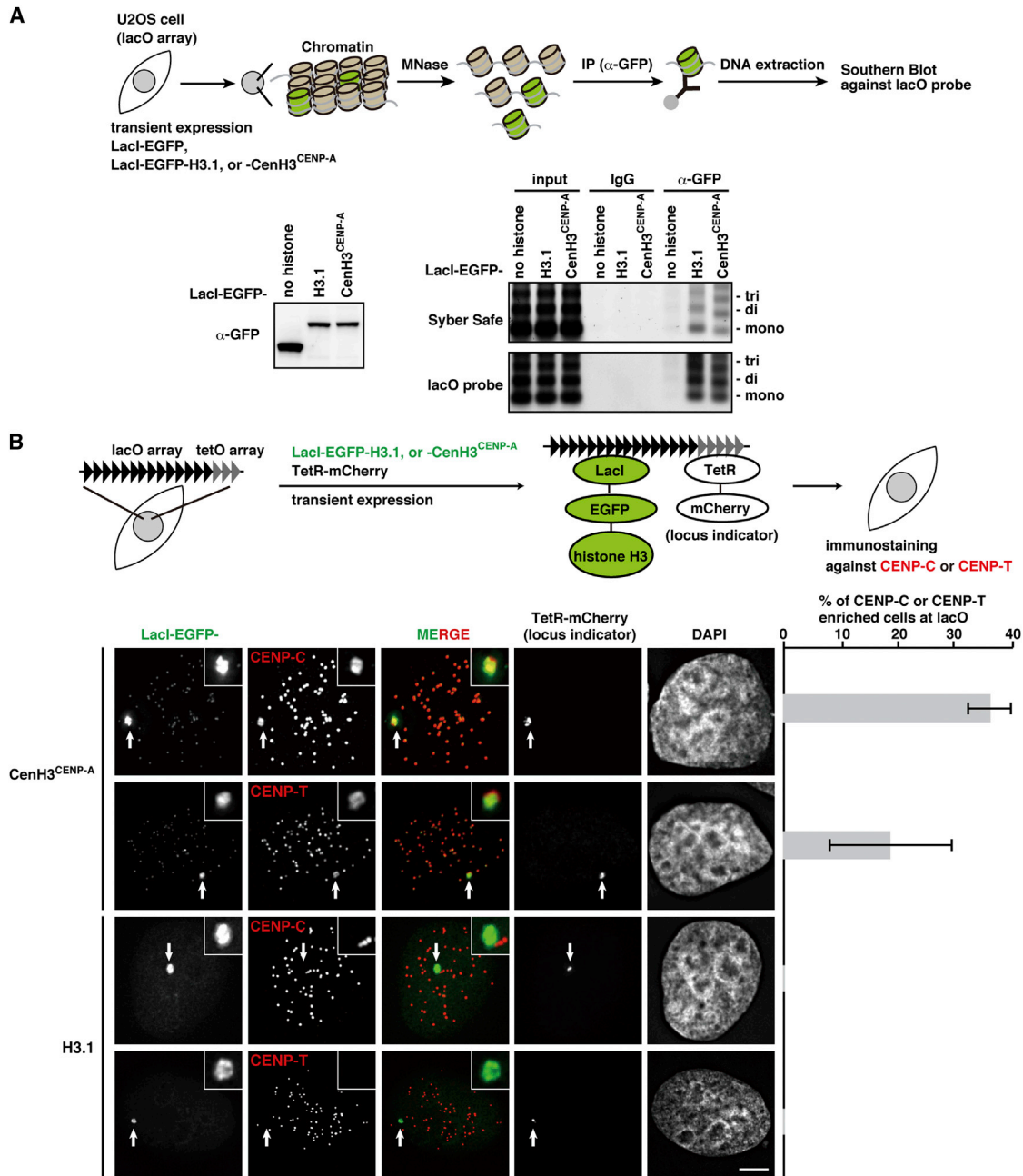


Figure 1. Tethered CenH3^{CENP-A} Recruits CENP-C and CENP-T De Novo at an Ectopic Locus

(A) Top: experimental scheme. Nucleosomes were prepared from lacO-tetO U2OS cells transiently expressing LacI-EGFP-H3 at 48 hr post-transfection, and immunoprecipitated with an anti-GFP antibody. We added IPTG during nucleosome preparation and IP, and then extracted precipitated DNA for Southern blotting. We used the lacO sequence as probe DNA. Bottom left: transiently expressed proteins detected by western blot. Bottom right: Southern blot of precipitated DNAs. We detected total DNA (Syber Safe, top) and lacO DNA (labeled lacO probe, bottom).

(B) Top: scheme of the assay. LacI-EGFP fused CenH3^{CENP-A} or H3.1 was transiently expressed in lacO-tetO U2OS. TetR-mCherry fusion protein was co-expressed (position of lacO). Bottom: immunostaining against CENP-C or CENP-T. CENP-C and CENP-T were detected 48 hr after transfection of LacI-EGFP-histones. Arrows indicate lacO. Scale bar, 5 μ m. Percentages of CENP-C and CENP-T enriched cells at lacO are shown on the right with SDs (three independent experiments with more than 60 cells each).

See also Figure S1.

led to CENP-C accumulation to the same levels as observed for CenH3^{CENP-A}, indicating that both the CAC and CATD are required for de novo CENP-C and subsequent CENP-T recruit-

ment. We found that fewer cells tethered with H3^{CATD_CAC} recruited CENP-T than CENP-C, suggesting that CENP-T follows CENP-C accumulation.

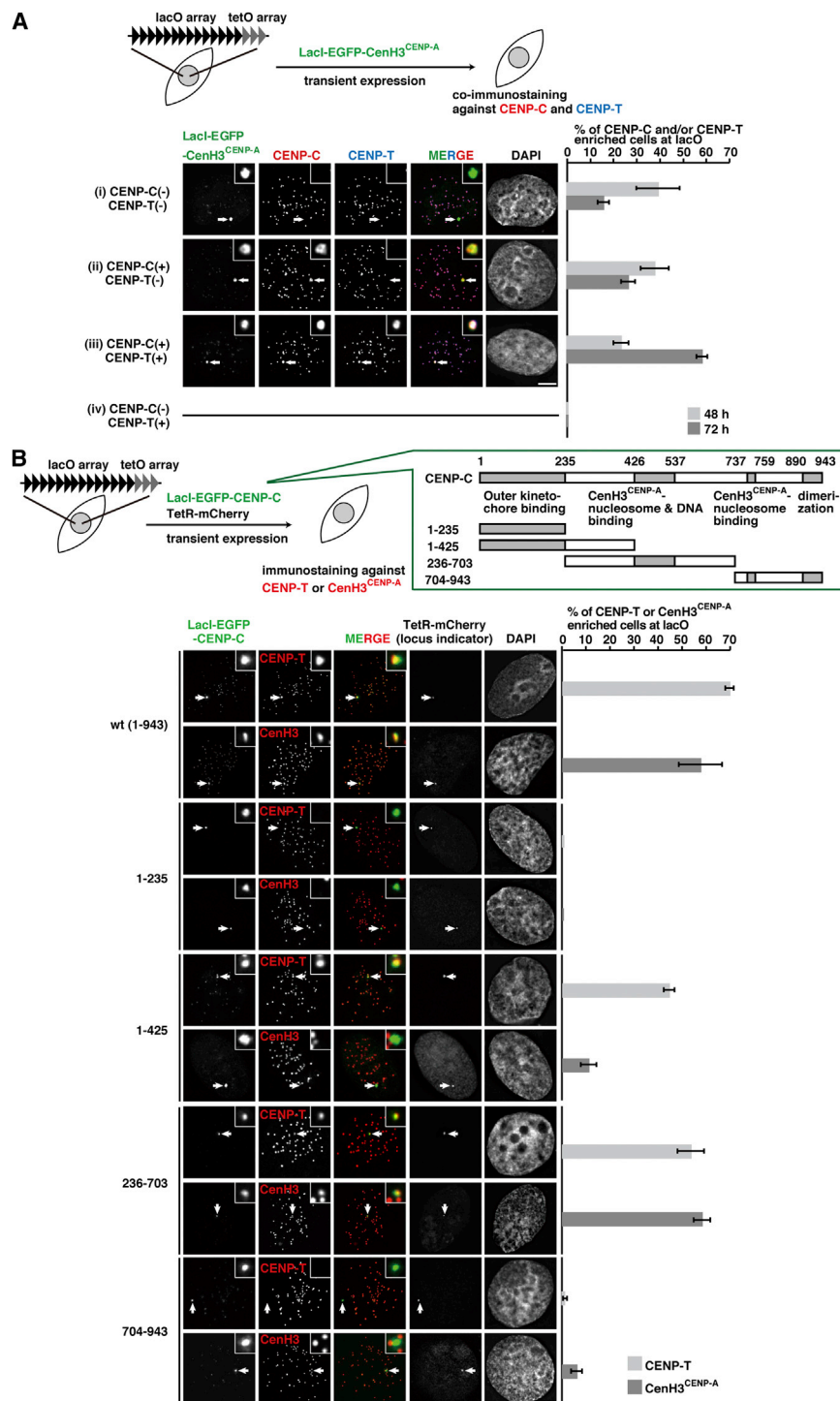


Figure 2. De Novo CENP-T Accumulation at an Ectopic Locus Depends on CENP-C

(A) LacI-EGFP-CenH3^{CENP-A} was expressed in lacO-tetO U2OS 48 or 72 hr after transfection, and CENP-C and CENP-T were detected at lacO. Arrows indicate lacO. Scale bar, 5 μ m. Percentages of CENP-C- or CENP-T-enriched cells at lacO are shown on the right with SDs. More than 60 cells were counted per experiment and the SD was calculated from three independent experiments. (B) Top: scheme of the de novo CENP-T or CenH3^{CENP-A} accumulation assay by CENP-C. CENP-C domains and constructs are indicated. LacI-EGFP-fused CENP-C or its fragments were transiently expressed in lacO-tetO U2OS. A TetR-mCherry fusion protein was co-expressed to indicate lacO (top right). Bottom: immunostaining against CENP-T or CenH3^{CENP-A}. CENP-T and CenH3^{CENP-A} were detected 48 hr after transfection. Arrows indicate the position of lacO. Scale bar, 5 μ m. Percentages of CENP-T or CenH3^{CENP-A} enriched cells at lacO are shown on the right with SDs (three independent experiments with more than 60 cells each). See also Figure S2.

sent in H3^{CATD_CAC} (Figure S3B). We could not detect endogenous CenH3^{CENP-A} at lacO tethered with H3^{CATD_CAC} (Figure S3C), suggesting that de novo CENP-C accumulation does not rely on the presence of endogenous CenH3^{CENP-A}. We further investigated CenH3^{CENP-A} and CENP-C accumulation at lacO tethered with H3^{CATD_CAC} at the same time by co-immunostaining (Figure S3D). We detected CenH3^{CENP-A} at endogenous centromeres, but not at lacO, even when CENP-C was detected at both endogenous centromeres and lacO, indicating that tethering H3^{CATD_CAC} leads to the accumulation of CENP-C in the absence of endogenous CenH3^{CENP-A}. Our data show that both the CAC and CATD of CenH3^{CENP-A} are required for de novo CENP-C and CENP-T accumulation at lacO.

HJURP Recruits the C-Terminal Part of CENP-C to lacO

Since HJURP binds to the CATD (Shuaib et al., 2010; Hu et al., 2011), we wondered whether it could be involved in the mechanism of de novo CENP-C recruitment. We verified HJURP accumulation at lacO tethering our histone H3 constructs (Figure S4A), showing that CenH3^{CENP-A}, but not H3.1, can recruit HJURP to the locus. Tethering H3^{CATD} or H3^{CATD_CAC}, but not H3^{CAC}, leads to HJURP recruitment, confirming that the CATD is essential to recruit HJURP. As a control, we tested the

CenH3^{CENP-A} particles can form octamers containing two molecules of CenH3^{CENP-A} (Tachiwana et al., 2011; Hasson et al., 2013; Lacoste et al., 2014). Thus, we investigated the presence of endogenous CenH3^{CENP-A} together with tethered H3^{CATD_CAC} at lacO. To distinguish CenH3^{CENP-A} from H3^{CATD_CAC}, we used a monoclonal antibody against CenH3^{CENP-A}, whose epitope is ab-

sent in H3^{CATD_CAC} (Figure S3B). We could not detect endogenous CenH3^{CENP-A} at lacO tethered with H3^{CATD_CAC} (Figure S3C), suggesting that de novo CENP-C accumulation does not rely on the presence of endogenous CenH3^{CENP-A}. We further investigated CenH3^{CENP-A} and CENP-C accumulation at lacO tethered with H3^{CATD_CAC} at the same time by co-immunostaining (Figure S3D). We detected CenH3^{CENP-A} at endogenous centromeres, but not at lacO, even when CENP-C was detected at both endogenous centromeres and lacO, indicating that tethering H3^{CATD_CAC} leads to the accumulation of CENP-C in the absence of endogenous CenH3^{CENP-A}. Our data show that both the CAC and CATD of CenH3^{CENP-A} are required for de novo CENP-C and CENP-T accumulation at lacO.

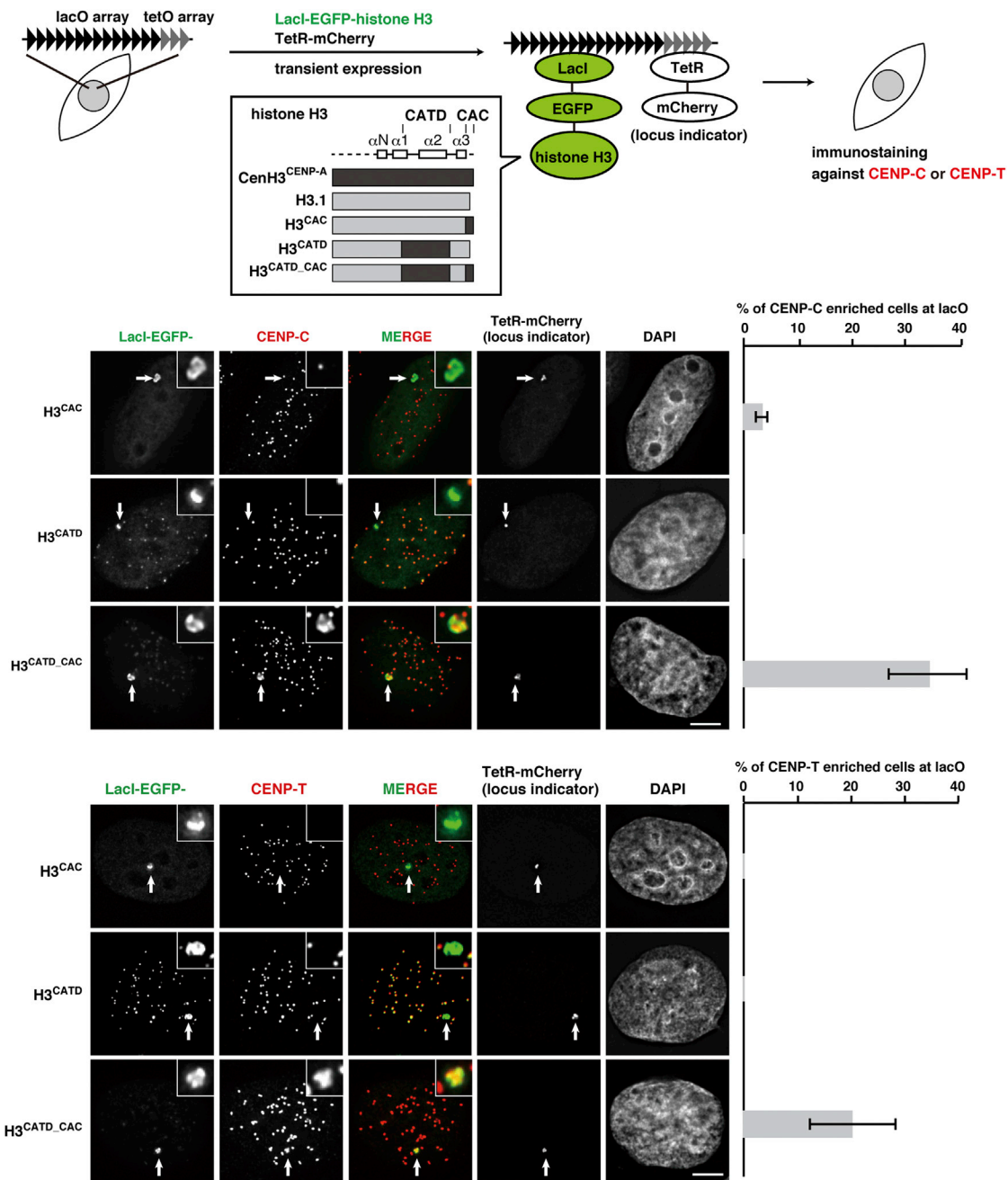


Figure 3. The CAC and CATD of CenH3^{CENP-A} Are Required for De Novo CENP-C Accumulation at lacO

Top: experimental scheme. LacI-EGFP-fused histones H3 were transiently expressed in lacO-tetO U2OS. CenH3^{CENP-A} domains and chimera proteins are indicated. In this assay, a TetR-mCherry fusion protein was co-expressed to indicate lacO. Bottom: CENP-C and CENP-T were detected 48 hr after transfection of LacI-EGFP histones. Arrows indicate lacO. Scale bar, 5 μ m. Percentages of CENP-C- or CENP-T-enriched cells at lacO are shown on the right with SDs (three independent experiments with more than 60 cells each).

See also Figure S3.

recruitment of an HJURP mutant (aa 79–748), lacking the CenH3^{CENP-A}-binding domain (Shuaib et al., 2010; Hu et al., 2011) to lacO tethered with our histone H3 constructs (Figure S4A). The percentage of HJURP (aa 79–748)-enriched cells at lacO tethered with CenH3^{CENP-A}, H3^{CATD}, or H3^{CATD_CAC}

was significantly decreased compared with the full length, confirming that the CATD is responsible for the recruitment of HJURP.

Next, we examined whether HJURP could directly recruit CENP-C. Since tethering HJURP leads to the accumulation of

CenH3^{CENP-A} at lacO (Barnhart et al., 2011), we tethered HJURP (aa 75–748) to eliminate HJURP-dependent CenH3^{CENP-A} accumulation. LacI-HJURP did not recruit endogenous CENP-C. We tested full-length CENP-C and various constructs in the same assay (Figure 4A). Human CENP-C binds to the outer kinetochore via its N-terminal region (aa 1–235) (Przewloka et al., 2011; Gascoigne et al., 2011), to CenH3^{CENP-A} nucleosomes via the central region (aa 426–537), and via aa 737–759 (Kato et al., 2013), and dimerizes through the C-terminal region (aa 890–943) (Sugimoto et al., 1997). CENP-C has a DNA-binding domain in the central region (Yang et al., 1996) and the C-terminal region is highly conserved (Talbert et al., 2004). We found that tethering HJURP (aa 75–748) led to the accumulation of the fragments CENP-C (aa 704–943) and CENP-C (aa 538–943). However, we did not detect the recruitment of full-length CENP-C, CENP-C (aa 1–703), or CENP-C (aa 236–943) (Figure 4A). We investigated HJURP accumulation at lacO tethering CENP-C or the mutants (Figure S4B) and found the same recruitment pattern. This suggests that HJURP can recruit the C-terminal part of CENP-C. Because full-length CENP-C has a DNA- and nucleosome-binding domain in its central region (aa 426–537), we hypothesized that our observations could be linked to how it interacts with chromatin. To test this hypothesis, we prepared fractionated extracts by modifying a previously described method (Martini et al., 1998) that allows cellular extracts to be separated into low-salt (soluble), salt-extractable (nuclear and chromatin bound), and pellet (tightly chromatin bound) fractions. We performed salt extraction at 150, 300, 450, and 600 mM NaCl, and stained for endogenous CENP-C in western blot (Figure S4C). We could not detect CENP-C in the soluble fraction, and could only detect it in the salt-extractable fractions at concentrations of ≥ 450 mM NaCl. This suggests that CENP-C is predominantly associated with chromatin and exhibits tight binding. We tested transiently transfected full-length or mutants of HA-CENP-C and performed salt extraction at 300 mM NaCl (Figure 4B). We found that, like endogenous CENP-C, full-length HA-CENP-C, CENP-C (aa 1–703), and CENP-C (aa 236–943) were present in the pellet extract. However, we detected CENP-C (aa 704–943) and CENP-C (aa 538–943) predominantly in the salt-extractable fraction, indicating that these mutants are more loosely bound to chromatin. Thus, our data suggest that HJURP is involved in CENP-C accumulation at lacO, but this interaction is impaired when CENP-C is tightly bound to chromatin.

A Direct Interaction between CENP-C and HJURP Is Required for Efficient CENP-C Recruitment at lacO

We investigated the interaction between full-length CENP-C and HJURP independently of its chromatin context by IP. First, we prepared cell extracts fractionated into low-salt, high-salt, and pellet extracts using the protocol of Martini et al. (1998). Whereas we found HJURP in all three fractions, we found CenH3^{CENP-A} mainly in the pellet fraction, and CENP-C in the pellet and salt-extractable fractions (Figure S5A). Then, we performed IP analyses using the pellet and high-salt extracts (Figure 5A). We found that CENP-C and HJURP co-IPed in both extracts. We also found that CenH3^{CENP-A} co-immunoprecipitated with HJURP and CENP-C, apart from the CENP-C IP

with the high-salt extracts. We also performed an IP using a CenH3^{CENP-A}-specific antibody and found that HJURP and CENP-C precipitated in both extracts. We found very little CENP-C in the low-salt fraction, but it could be immunoprecipitated with the HJURP antibody (Figures S5A and S5B), demonstrating that HJURP and CENP-C can interact in a soluble complex. We then performed sucrose gradients using our different extracts and found a co-migration of HJURP and CENP-C in all three fractions (Figure S5C). Taken together, these results suggest that HJURP and CENP-C can form a complex in vivo.

Next, we examined whether HJURP could directly interact with CENP-C. Based on our previous results, we used the aa 631–943 fragment of recombinant CENP-C and glutathione S-transferase (GST)-fused HJURP in an in vitro pull-down (Figure 5B). We detected the CENP-C fragment (aa 631–943) in the GST-HJURP pull-down, indicating that HJURP directly binds to C-terminal CENP-C. We also performed this experiment in the presence of CenH3^{CENP-A}-H4 and found that HJURP, CENP-C, and CenH3^{CENP-A}-H4 can form a complex in vitro.

Next, we tested whether HJURP is required for the recruitment of CENP-C to centromeres. For this purpose, we treated cells with an siRNA against HJURP or a control (luciferase) (Figure S5D). We found that HJURP is required for the accumulation of CENP-C and CenH3^{CENP-A} at centromeres. Next, we tested whether HJURP is involved in CENP-C recruitment to lacO tethered with CenH3^{CENP-A}. We assessed recruitment of LacI-EGFP-CenH3^{CENP-A} to lacO upon treatment with siRNA. We found that HJURP is not required to recruit LacI-CenH3^{CENP-A} to lacO; therefore, its function of targeting CenH3^{CENP-A} is bypassed in our system. We next explored CENP-C recruitment to lacO tethered with CenH3^{CENP-A} or H3^{CATD_CAC}. We found a reduction in the number of cells that recruited CENP-C in the presence of siHJURP compared with the control, both by tethered CenH3^{CENP-A} and H3^{CATD_CAC} (Figure 5C). As a control, we performed this experiment in the presence and absence of IPTG, and found no difference in the number of cells that recruited CENP-C (Figure S5E). Thus, our data suggest that HJURP is required for loading of CENP-C to lacO tethered with CenH3^{CENP-A} or H3^{CATD_CAC}.

DISCUSSION

In this work, we studied the de novo recruitment of two key kinetochore components: CENP-C and CENP-T (Barnhart et al., 2011; Gascoigne et al., 2011; Hori et al., 2013). Previous work demonstrated that placing fragments of CENP-C or CENP-T at an ectopic locus could bypass the requirement for CenH3^{CENP-A} for kinetochore formation (Gascoigne et al., 2011). We addressed how CenH3^{CENP-A} recruits CENP-C and CENP-T de novo by tethering CenH3^{CENP-A}, H3.1, or chimera proteins. Our work demonstrates that CENP-T is recruited to lacO following CENP-C via a mechanism that requires residues 236–425 of CENP-C. This is in agreement with a recent report showing that recruitment of the CENP-T/W complex depends on factors downstream of CENP-C (Basilico et al., 2014). Previous studies indicated that CENP-T interacts with canonical centromeric H3 nucleosomes (Hori et al., 2008; Ribeiro et al., 2010). Thus,

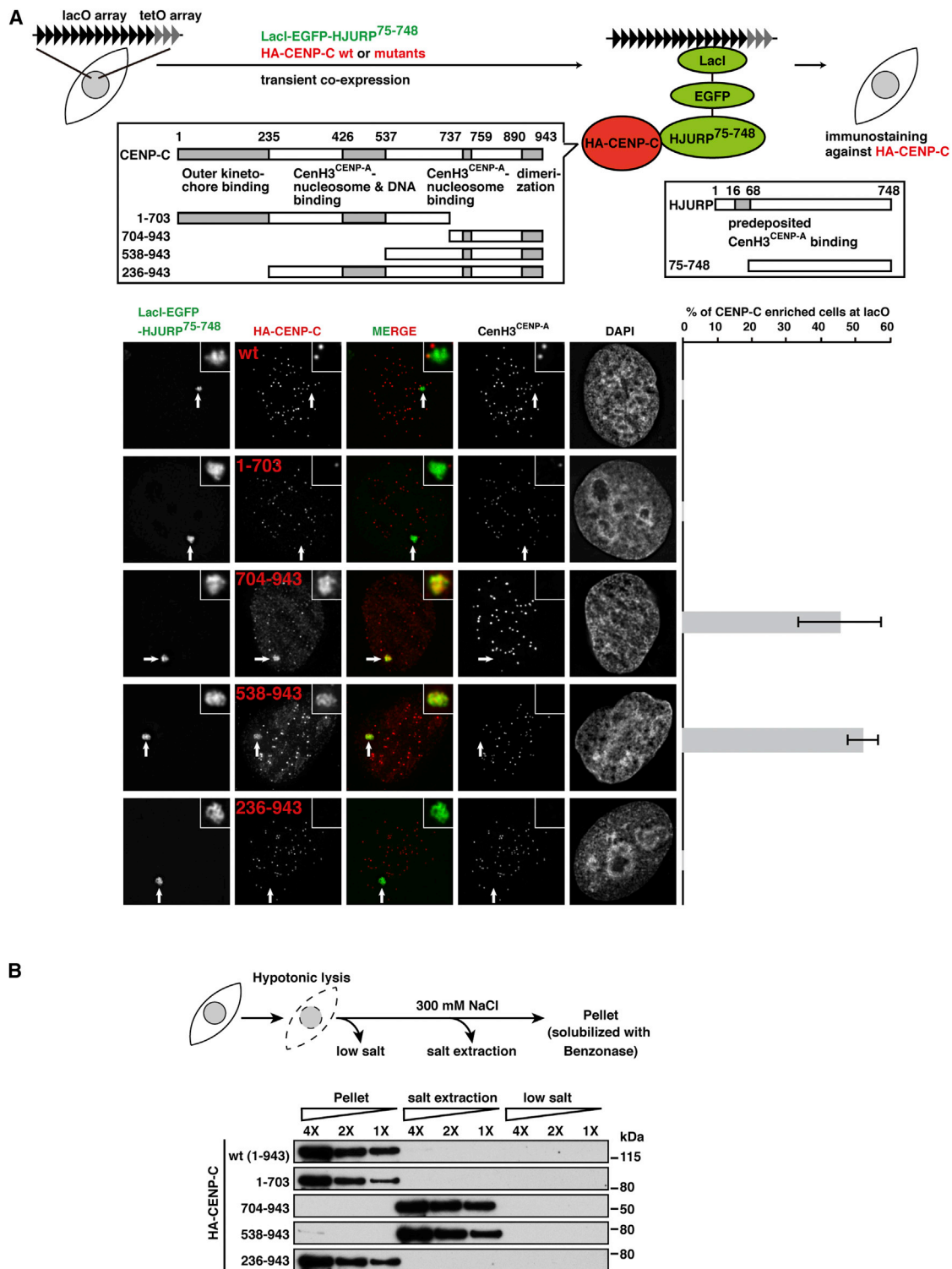


Figure 4. HJURP Recruits the C-Terminal Part of CENP-C to lacO

(A) Top: experimental scheme. LacI-EGFP-HJURP (aa 75–748) and HA-CENP-C full length (wt) or mutants were transiently co-expressed in lacO-tetO U2OS. Known functional domains of human CENP-C and HJURP are illustrated. Bottom: HA-CENP-C full length (wt) or mutants were detected 24 hr after transfection. Arrows indicate lacO. Scale bar, 5 μ m. Percentages of CENP-C-enriched cells at lacO are shown on the bottom right with SDs (three independent experiments with more than 50 cells counted in each).

(legend continued on next page)

CENP-C may mark centromeres for recruitment of CENP-T specifically to centromeric H3 nucleosomes.

We found that the CAC and CATD of CenH3^{CENP-A} are required for CENP-C recruitment to lacO. The CATD cannot interact with CENP-C in a nucleosomal context (Tachiwana et al., 2011). However, in a CenH3^{CENP-A} pre-deposition complex, HJURP binds to CenH3^{CENP-A} through the CATD (Shuaib et al., 2010; Hu et al., 2011). This mechanism is distinct from the one that governs CENP-C maintenance at endogenous centromeres (Fachinetti et al., 2013), where the CAC maintains CENP-C levels at centromeres, and the N-terminal tail of CenH3^{CENP-A} recruits CENP-B. At endogenous centromeres, CENP-B can recruit CENP-C, constituting a CAC-independent pathway of CENP-C recruitment. However, neocentromeres are devoid of CENP-B (Voullaire et al., 1993; Saffery et al., 2000), which suggests that de novo recruitment of CENP-C involves a CAC-dependent pathway at neocentromeres.

HJURP interacts directly with the C terminus of CENP-C, and these proteins form a complex in vivo. The C terminus of CENP-C is conserved among most eukaryotes (Talbert et al., 2004), and a previous study employing a yeast two-hybrid screen identified an interaction between the C-terminal part of *D. melanogaster* CENP-C and Cal1, the functional HJURP homolog (Schittenhelm et al., 2010). This suggests there could be a conserved interaction between CENP-C and the dedicated CenH3 chaperone. We find that HJURP is required for accumulation of CENP-C at lacO tethered with CenH3^{CENP-A} or H3^{CATD_CAC}, and that HJURP can recruit the C-terminal fragment of CENP-C. Interestingly, we observe that HJURP cannot recruit full-length CENP-C or fragments carrying the CAC and DNA-binding domains, which are tightly bound to chromatin. Our data suggest a transient interaction between CENP-C and HJURP. Thus, HJURP may be involved in loading CENP-C, but not bring it to the centromere per se.

We recently demonstrated that HJURP plays a role in CenH3^{CENP-A} deposition through its DNA-binding activity (Müller et al., 2014). This may involve non-canonical DNA structures (Kato et al., 2007). This function of HJURP may be akin to the DNA chaperone function of HMGB proteins (reviewed in Stros, 2010), which are involved in chromatin remodeling through nucleosome binding and interactions with non-canonical DNA structures (Bianchi et al., 1989). In this context, future studies should further unravel the contribution of HJURP to CENP-C loading and the related cell-cycle timing.

A previous report on *D. melanogaster* embryos documented concomitant centromeric CENP-C and CenH3^{CID} loading at centromeres in anaphase (Schuh et al., 2007). In human cells, HJURP recruitment to centromeres may involve the Mis18 complex (Wang et al., 2014), which in turn is recruited by CENP-C (Dambacher et al., 2012). HJURP may exploit its CENP-C binding activity to load an old or a new CENP-C molecule onto a CenH3^{CENP-A} nucleosome. Since CENP-C is actively turned over in G1 and G2 (Hemmerich et al., 2008),

and HJURP forms foci at centromeres between telophase and early G1 (Dunleavy et al., 2009; Foltz et al., 2009), loading of CENP-C by HJURP may represent a fraction of the CENP-C pool. LacI-CENP-A-containing nucleosomes can assemble in the absence of HJURP at lacO. It is possible that anchoring by the LacI of the fusion protein is sufficient to direct its incorporation into chromatin. Alternatively, other chaperones, such as DAXX, could play a role in this process (Lacoste et al., 2014). Taken together, our results demonstrate a mechanism whereby HJURP coordinates CenH3^{CENP-A} and CENP-C recruitment.

EXPERIMENTAL PROCEDURES

Plasmid Constructions, Transfection, Immunofluorescence, and Microscopy

U2OS or lacO-tetO U2OS was cultured in DMEM (Life Technologies) supplemented with 10% (v/v) fetal calf serum (Eurobio), 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies) in a humidified atmosphere with 5% CO₂ at 37°C. We made LacI-EGFP-histone fusion genes using the vector pEGFP-N1. We inserted the TetR-mCherry sequence into this plasmid after the gene with the internal ribosome entry site (IRES). Human histone H3.1, CenH3^{CENP-A}, and chimera protein H3^{CATD} genes were a generous gift from Dr. H. Kurumizaka (Waseda University). For transfection, we used Effectene (QIAGEN) according to the manufacturer's protocol. We obtained siRNAs from MWG Eurofins and the sequences used were siHJURP 5'-GAG-AUA-ACC-UCG-AGU-UCU-UTT-3' and siControl (control) 5'-CGU-ACG-CGG-AAU-ACU-UCG-A-3'. Cells were co-transfected with plasmids and siRNA using jetPRIME (Polyplus). Immunofluorescence and microscopy are detailed in Supplemental Experimental Procedures.

Cell Extracts, Western Blotting, and IP

We prepared fractionated extracts as described previously (Martini et al., 1998) and used 150, 300, 450, or 600 mM NaCl. Western blotting and IP are detailed in Supplemental Experimental Procedures.

Recombinant Protein Purification and Pull-Down Assay

Pull-down was carried out with 15 pmol of GST or GST-HJURP with 150 pmol of CENP-C (aa 631–943) in 50 µl of reaction buffer (20 mM Tris-HCl pH8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2% NP-40, and 10 µg/ml bovine serum albumin [BSA]). Then, we added 3 µl of Glutathione-Sepharose 4B beads (50% slurry; GE Healthcare) and incubated the samples at 4°C for 60 min on a wheel. After incubation, we washed the beads three times with 500 µl of reaction buffer without BSA. Then, we analyzed the samples on NuPAGE Bis-Tris 4%–12% gels (Invitrogen) in MOPS buffer (Invitrogen) followed by Coomassie brilliant blue (CBB) staining or western blotting. Western blotting was performed with a rabbit anti CENP-C antibody against GST-CENP-C732-849 at the IFOM-IEO Campus Antibody Facility (Milan). Original bleeds from rabbits were affinity purified against the same CENP-C construct used for immunization. Purification of the recombinant protein is detailed in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.03.013>.

(B) Top: scheme of cell extract preparation. Cells expressing HA-CENP-C or mutants were subjected to hypotonic lysis and the supernatant (low salt) was separated. The remaining cells were further treated with 300 mM NaCl and separated into a salt-extracted fraction (high salt) and pellets (pellet fraction). The remaining pellets were treated with benzonase. Bottom: the protein amounts in each fraction were investigated by western blot. See also Figure S4.

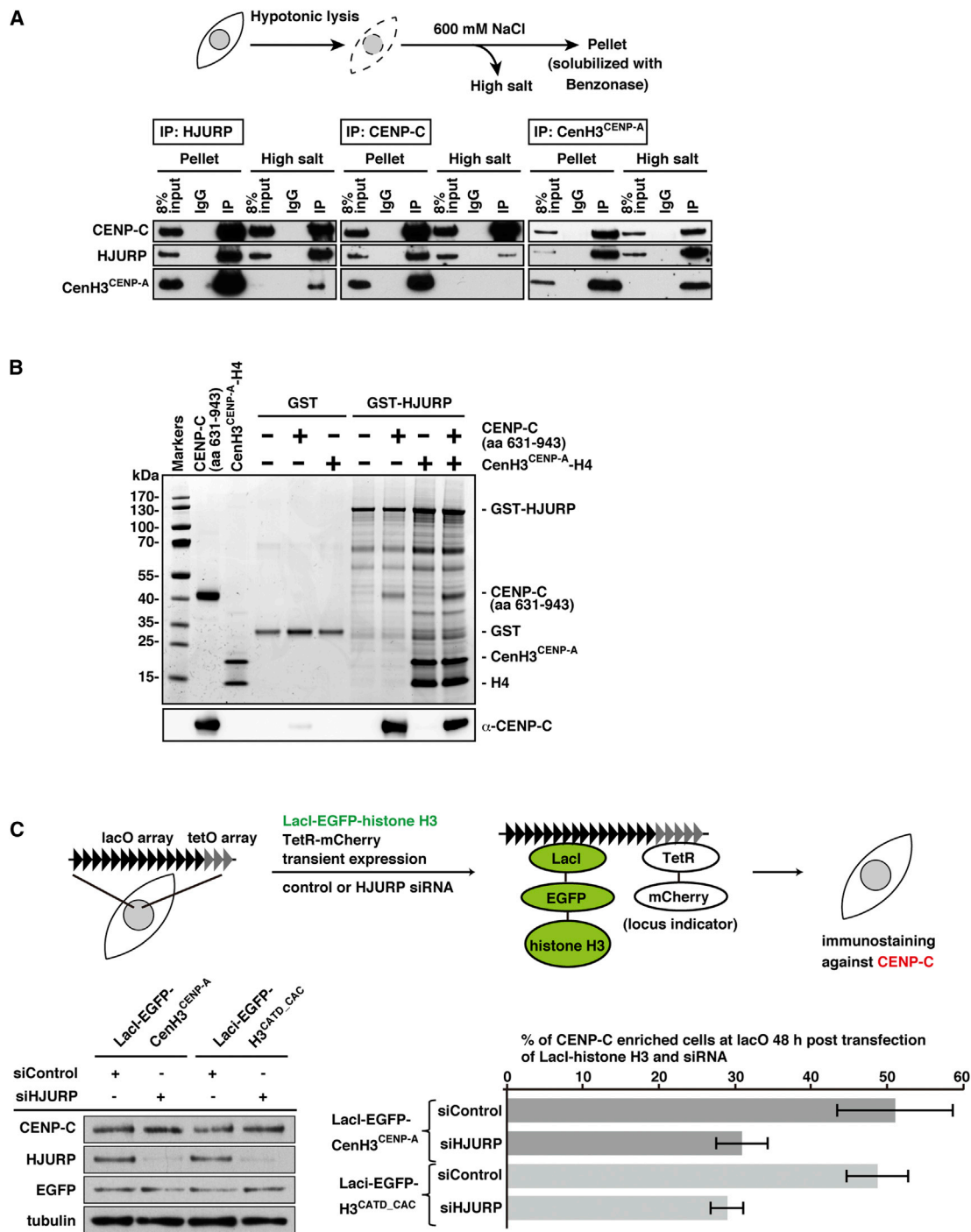


Figure 5. A Direct Interaction between CENP-C and HJURP Is Required for Efficient CENP-C Recruitment at lacO

(A) Top: scheme of cell extract preparation. Cells were treated with hypotonic lysis and the supernatant (low salt) was separated. The remaining cells were treated with 600 mM NaCl and separated into a salt-extracted fraction (high salt) and pellets. The remaining pellets were treated with benzonase (pellet fraction). Bottom: IP with an anti-HJURP (left), anti-CENP-C (middle), or CenH3^{CENP-A} (right) antibody using pellet and high-salt extracts.

(B) GST pull-down assay. GST or GST-HJURP was mixed with CENP-C (aa 631-943) or CenH3^{CENP-A}-H4, or both, with Glutathione-Sepharose beads. After incubation, GST and GST-HJURP were pelleted and proteins were analyzed by SDS-PAGE. CENP-C was visualized with CBB staining (top) or CENP-C antibody (bottom).

(C) Top: experimental scheme. Left: western blot showing the expression of LacI-EGFP-CenH3^{CENP-A} and LacI-EGFP-H3^{CATD_CAC} in the presence of siHJURP or siControl. Right: graphs representing the number of TetR-positive cells at lacO recruiting LacI-EGFP-histone H3 or CENP-C by tethered LacI-EGFP-histone H3 (CenH3^{CENP-A} or LacI-EGFP-H3^{CATD_CAC}) in the presence of siHJURP or siControl. Error bars denote SD (three independent experiments with at least 100 cells each). See also Figure S5.

AUTHOR CONTRIBUTIONS

H.T., S.M., J.B., and K.K. performed the experiments. H.T., S.M., A.M., and G.A. designed the experiments. H.T., S.M., and G.A. wrote the paper. G.A. supervised all of the work. All authors discussed all of the data and commented on the manuscript.

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