The phosphorylation of the CENP-A NH$_2$-terminus in mitotic centromeric chromatin is required for kinetochore function

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Abstract

The role of the mitotic phosphorylation of the NH$_2$-terminus of CENP-A, the histone variant epigenetic marker, remains elusive. Here, we show that the NH$_2$-terminus of human CENP-A is essential for mitotic progression and that localization of CENP-C, another key centromeric protein, requires only phosphorylation of the CENP-A NH$_2$-terminus, and is independent of the CENP-A NH$_2$-terminus length and amino acid sequence. Mitotic CENP-A nucleosomal complexes contain CENP-C and phospho-binding 14-3-3 proteins. In contrast, mitotic nucleosomal complexes carrying non-phosphorylatable CENP-A-S7A contained only low levels of CENP-C and no detectable 14-3-3 proteins. Direct interactions between the phosphorylated form of CENP-A and 14-3-3 proteins as well as between 14-3-3 proteins and CENP-C were demonstrated. Taken together, our results reveal that 14-3-3 proteins could act as specific mitotic "bridges", linking phosphorylated CENP-A and CENP-C, that are necessary for the platform function of CENP-A centromeric chromatin in the assembly and maintenance of active kinetochores.

1 Introduction

Histone variants are non-allelic isoforms of conventional histones. It is widely accepted that the incorporation of histone variants generally confers novel structural and functional properties to the nucleosome (Doyen et al., 2006). CENP-A is a histone variant, which replaces the canonical histone H3 at the centromeres (Palmer et al., 1991) and marks epigenetically the centromeres and the kinetochores (for reviews, see Henikoff and Dalal, 2005; Buscaino et al., 2010). The presence of CENP-A is required for the assembly of active kinetochores and its

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depletion results in numerous mitotic problems, such as chromosome misalignments and segregation defects, generation of chromosome bridges, aneuploidy, etc. (Régnier et al., 2005). The resulting mitotic defects, following CENP-A depletion, were associated also by notable alterations in the composition and organization of the kinetochore including the delocalization of the inner kinetochore proteins CENP-C, CENP-I and CENP-H as well as the outer kinetochore components HEC1, Mad2 and CENP-E (Régnier et al., 2005).

During last years the studies on CENP-A were focused mainly on its histone-fold domain. The NH$_2$-terminus of CENP-A, which is not required for centromeric targeting (Shelby et al., 1997; Vermaak et al., 2002) appeared, however, to play an important role in both mitosis and meiosis. In yeast, the NH$_2$-tail of Cse4p (the homologue of mammalian CENP-A) has an essential function distinct from that of the histone-fold domain in chromosome assembly and segregation (Chen et al., 2000). The reported data in _A. thaliana_ suggested the existence of meiosis-specific loading pathway for CENP-A requiring its NH$_2$-terminus (Ravi et al., 2011). In addition, human CENP-A is phosphorylated in its NH$_2$-terminus at serine 7 in mitosis but the role of this phosphorylation is far from being clear (Kunitoku et al., 2003; Zeitlin et al., 2001).

Sequence alignments of the NH$_2$-termini of CENP-A from different species show very low sequence conservation in terms of amino acid composition, sequence and length (Figure 1a). However, expression of CENP-A with its NH$_2$-terminus deleted in lethal in yeast (Morey et al., 2004) and plants (Ravi and Chan, 2010). These data create a paradox since on one hand, the NH$_2$-terminus of CENP-A, in certain organisms, is required for their survival and on the other hand, it appears to be non essential since there is no evolutionary pressure to conserve at least some specific elements.

Here, we have analyzed the mitotic function of the NH$_2$-terminus of human CENP-A and its phosphorylation. We show the mere phosphorylation of CENP-A, but not its length and aminoacid sequence, is required for the localization of CENP-C, a key mediator between centromeric chromatin and the outer kinetochore components. Our data reveal that in mitosis the phosphorylated CENP-A nucleosome are “bridged” to CENP-C via the phosho-binding 14-3-3 proteins. These 14-3-3 mitotic “bridges” are essential for assembly of active kinetochores.

## 2 Results

### 2.1 The CENP-A NH$_2$-terminus per se, but not its amino acid sequence composition and length, is essential for mitotic progression

In order to address the paradox regarding the essential role of the NH$_2$-tail of CENP-A in human cells, we asked first whether its deletion results in the generation of specific cell phenotypes. We have established stable HeLa cell lines which express siRNA-resistant forms of either GFP-CENP-A or CENP-A NH$_2$-tail deletion mutant (GFP-ΔN-CENP-A) or GFP-H3-CENP-A (a swapped tail mutant with the tail of conventional H3 fused to the histone fold domain of CENP-A). The expression of endogenous CENP-A was then suppressed by using siRNA treatment and the resulting cell phenotypes were observed and quantified by fluorescence microscopy. Importantly, all of the GFP-CENP-A fusions localize to the centromeres and the expression of any one of them in control cells, but not siRNA treated cells, had no phenotypic effect, i.e. the cells behave identically to the naïve parental HeLa cells (Figures 1b, 2a, 3, and S1).

SiRNA treatment led to very efficient suppression of CENP-A expression: more than 85–90% of the endogenous CENP-A was depleted in each of the cell lines used and, as expected,
Figure 1. The NH$_2$-tail of CENP-A is required for mitosis and the H3 swapped NH$_2$-tail CENP-A chimera rescues the CENP-A null cell phenotype. (a) Sequence alignment of CENP-A NH$_2$-termini from different species. The names of species are indicated in the left part of the panel. The serine residues are indicated in blue. The conservation between the different species is shown on the lower part of the panel. (b) Cell cycle visualization, after CENP-A suppression by siRNA treatment of naïve HeLa cells (second row) or HeLa cells stably expressing siRNA-resistant full length GFP-CENP-A (third row) or tailless GFP-ΔN-CENP-A (fourth row) or GFP-H3-CENP-A swapped tail mutant (fifth row). The first row shows naïve cells not treated with siRNA. A CREST antibody was used to visualize the centromeres in naïve cells; GFP fluorescence was used to visualize CENP-A in GFP-fusion expressing cells. An antibody against INCENP and anti-lamina antibody were used to detect the midbody during cytokinesis and the nuclear envelope in interphase cells (showed in red). Blue, DNA; white arrowheads point to misaligned or lagging chromosomes and to chromatin bridges; scale bar, 5 μm. (c) Detection of the GFP-CENP-A fusions and endogenous CENP-A in control (−) and siRNA treated (+) cells at 72 hours posttransfection by Western blot. The cells used are indicated above the panel. Arrows indicate the position of the GFP-CENP-A fusions or endogenous CENP-A. (d) Histograms showing the percentage of mitotic defects at 72 hours posttransfection with siRNA against CENP-A in the indicated cell lines; HeLa, naïve cell line. (e) Same as (d), but showing the percentage of multinucleate cells. For each experiment at least 400 cells were counted. Data are means and s.e.m. of 5 independent experiments.
the expression of the siRNA-resistant GFP-CENP-A fusions was unaffected (Figure 1c). In agreement with the reported data (Régnier et al., 2005), the depletion of CENP-A in naive HeLa cells has deleterious effects on mitosis and cytokinesis (Figure 1b). Multiple mitotic and cytokinesis defects, such as chromosome misalignment, presence of lagging chromosomes, chromatin bridges, multiple nuclei in interphase, etc. were observed in cells depleted of CENP-A (Figure 1b). The presence of stably expressed GFP-CENP-A in the siRNA treated HeLa cells was sufficient to completely rescue the severe mitotic (Figures 1b, d) and cytokinesis defects (Figures 1b, e). Importantly, no rescue was detected in CENP-A siRNA treated HeLa cells, stably expressing the GFP-ΔN-CENP-A deletion mutant (Figures 1b, d, e). These data illustrate the crucial role of the CENP-A NH$_2$-tail in mitosis in human cells.

A complete rescue was, however, found in HeLa cells depleted of endogenous CENP-A which stably express the swapped tail mutant GFP-H3-CENP-A (Figures 1b, d, e). This result demonstrates that the NH$_2$-tail of histone H3 can function identically to the NH$_2$-tail of CENP-A in mitosis in human cells if it is targeted to the centromeric chromatin.

2.2 The phosphorylation of the NH$_2$-terminus of CENP-A is required for its mitotic and cytokinetic functions

Taken together the data described above show that neither the amino acid sequence composition nor the length of the NH$_2$-terminus of human CENP-A are essential for its mitotic and cytokinesis function. Examination of the alignments of the CENP-A NH$_2$-termini from different species shows that they all contain potentially phosphorylatable serines (Figure 1a). We hypothesized that serine phosphorylation of CENP-A is a general feature required for mitosis. To analyze this, we established HeLa cell lines stably expressing either a siRNA-resistant non-phosphorylatable GFP-CENP-A-S7A mutant (serine 7 was substituted with alanine, Zeitlin et al., 2001), or a siRNA-resistant non-phosphorylatable GFP-H3SA-CENP-A swapped-tail mutant (in which the two phosphorylatable serines in the H3 tail of the fusion mutant, S10 and S28, were substituted with alanines). These cell lines were treated with CENP-A siRNA to determine the capacity of the mutants to rescue the CENP-A depletion phenotypes (Figure 2). When the stable cell lines were synchronized in mitosis with taxol the GFP-H3-CENP-A fusion (which contains phosphorylatable serines in its NH$_2$-terminus) was phosphorylated (Figure 2b). On contrast, the GFP-H3SA-CENP-A mutant was, as expected, not phosphorylated (Figure 2b). Importantly, neither GFP-CENP-A-S7A nor GFP-H3SA-CENP-A were able to rescue the severe cell phenotype due to the depletion of endogenous CENP-A (Figures 2a, c, d). Thus, the absence of phosphorylation of the GFP-fusions was responsible for their inability to fulfill their mitotic roles. We conclude that upon phosphorylation the tail of CENP-A acquires novel properties essential for mitotic progression.

One interesting property became apparent when one compares the phosphorylation levels of the GFP-H3-CENP-A chimera in relation to those of the endogenous H3 (Figure 2b). The number of molecules of GFP-H3-CENP-A chimera in the transfected cells is extremely low compared to total wild type H3 and yet the levels of phosphorylation at S10 and at S28 in the chimera appear to be comparable to those of H3. This suggests that the NH$_2$-terminus of H3 is much more heavily phosphorylated when it is located in the centromeric chromatin as opposed to the bulk of chromatin.
Figure 2. Both non-phosphorylatable NH$_2$-tail mutants GFP-CENP-A-S7A and GFP-H3SA-CENP-A are unable to rescue the CENP-A knocked down cell phenotype. (a) Cell cycle microscopy visualization (after CENP-A suppression by siRNA treatment) of HeLa cells stably expressing siRNA-resistant either non-phosphorylatable NH$_2$-tail GFP-CENP-A-S7A (first row) or GFP-H3SA-CENP-A (second row) mutants. GFP fluorescence was used for the visualization of CENP-A. An antibody against INCENP and anti-lamina antibody were used to detect the midbody during cytokinesis and the nuclear envelope in interphase cells, respectively; blue, DNA; white arrowheads point to misaligned or lagging chromosomes and to chromatin bridges; scale bar, 5 μm. (b) Phosphorylation status of both GFP-H3-CENP-A and GFP-H3SA-CENP-A fusions visualized by antibodies against histone H3 phosphorylated either at serine 10 or at serine 28; anti-histone H2B antibody was used to show that equal amounts of proteins were loaded. The types of cells used are indicated on the top part of the panel. Arrows indicate the positions of the GFP fusions or histone H3. (c) Histograms showing the percentage of mitotic defects at 72 hours post-transfection with siRNA against CENP-A in the indicated cell lines. (d) Same as (c), but for the percentage of multinuclear cells. For each experiment at least 400 cells were counted. Data are means and s.e.m. of 5 independent experiments.
Figure 3. The absence of either the NH$_2$-terminus of CENP-A or its phosphorylation affects the localization of CENP-C, but not this of CENP-T. (a) Localization of CENP-C in CENP-A depleted cells stably expressing the indicated siRNA-resistant GFP-CENP-A fusions (rows 3–7). The first two rows show the localization of CENP-C in naive HeLa cells treated with control (first row) or CENP-A siRNA (second row). Centromeres in naïve cells were stained with a CREST antibody; blue, DNA; scale bar, 5μm. Note the complete delocalization of CENP-C from the centromeric chromatin. (b) Same as (a), but for CENP-T.

2.3 The phosphorylation of the CENP-A NH$_2$-terminus determines the CENP-C centromeric localization

CENP-C and CENP-T, two members of the Constitutive Centromere Associated Network (CCAN), form the chromatin-based platform required for vertebrate kinetochore assembly (Buscaino et al., 2010). In addition, depletion of CENP-A from TD40 chicken cells leads to mislocalization of inner kinetochore components including CENP-C (Régnier et al., 2005).

Thus, based on the already published data we made the hypothesis that phosphorylation of the CENP-A NH$_2$-tail might be required for CENP-C localization at centromeres. To test this, we first determined how expression of the GFP-fusions affects the localization of CENP-C and CENP-T in our HeLa stable cell lines depleted of endogenous CENP-A. We found that depletion of CENP-A by siRNA resulted in a complete mislocalization of CENP-C in interphase cells; the CENP-C labelling did not colocalize with the CREST centromeric staining (Figure 3a). Note that the delocalized non-centromeric CENP-C shows a punctuated pattern, a result in agreement with the reported data (Régnier et al., 2005). Expression of the siRNA-resistant GFP-
CENP-A rescued the colocalization of CENP-C with GFP-CENP-A (Figure 3a). In contrast to the wild-type CENP-A, the expression of neither the NH₂-tail CENP-A deleted mutant (GFP-ΔN-CENP-A) nor of the other two non-phosphorylatable CENP-A mutants (GFP-CENP-A-S7A and GFP-H3SA-CENP-A) were able to rescue the mislocalization pattern of CENP-C. Indeed, in all three cases CENP-C does not colocalize with the centromeres (Figure 3a). These data demonstrate that the CENP-A NH₂-terminus and its phosphorylation are required for the centromere localization and function of CENP-C. The importance of the phosphorylation per se of the NH₂-terminus was underlined even more by the observation that the phosphorylation of the H3 tail of the swapped tail mutant GFP-H3-CENP-A was necessary and sufficient for the rescue of the CENP-C localization and function.

It should be noted that we have been able to study the localization of CENP-C in interphase cells only, since the antibody used to detect CENP-C failed to mark centromeres even in control untreated mitotic cells. However, CENP-A is phosphorylated in mitosis, and thus one should expect CENP-A phosphorylation to be essential for the CENP-C centromeric localization in mitosis. To test this we have used biochemistry approaches and have quantitatively analyzed the composition of both CENP-A and the non-phosphorylatable CENP-A-S7A mitotic nucleosomal complexes. Briefly, we used mitotic stable HeLa cell lines expressing double HA-FLAG tagged CENP-A (e-CENP-A) or identically tagged CENP-A with serine 7 mutated to alanine (e-CENP-A-S7A) to isolate the respective CENP-A nucleosomal complexes (e-CENP-A.com). Mass spectrometry analysis showed, in agreement with the reported data (Foltz et al., 2006), that the mitotic wild-type complex contained proteins from the constitutive centromere associated network (CENP-B, CENP-I, CENP-C), the CENP-A histone chaperones HJURP and NPM1, the chromatin remodeler RSF-1, as well as several other proteins (Figure 4a and results not shown). Importantly, the number of CENP-C peptides identified by mass spectrometry decreased dramatically (from 25 to 5) in the mutated CENP-A-S7A mitotic nucleosomal complex compared to the wild-type one (Figure 4a). In agreement with this, the Western blotting analysis reveals that the amount of CENP-C associated with the mutated CENP-A-S7A nucleosomal complex was very strongly decreased compared to wild-type CENP-A nucleosomal complex (Figure 4b). We conclude that phosphorylation of CENP-A is also required for the centromeric localization of CENP-C in mitosis.

As for CENP-T, the depletion of CENP-A does not affect its centromeric distribution pattern (Figure 3b). This is in agreement with the reported data showing that CENP-T and CENP-C use distinct pathways for assisting the assembly of active kinetochores (Gascoigne et al., 2011).

2.4 14-3-3 proteins act as mitotic “bridges” required for the assembly of the phospho-CENP-A nucleosome/CENP-C complex

How does the phosphorylated tail of CENP-A function in mitosis? One plausible hypothesis is that CENP-A phosphorylation may directly or indirectly, through some intermolecular bridges, stabilize the association of CENP-C with the centromere in mitosis. If such protein bridges exist, they should mediate a mitosis-specific interaction between CENP-A and CENP-C and thus, would be associated with the CENP-A mitotic nucleosomal complex. And indeed, mass spectrometry identified the phospho-binding proteins 14-3-3-θ, -ε and -ζ associated with the e-CENP-A complex (Figure 4a). Importantly, all three isotypes of 14-3-3 proteins were absent from the mutated mitotic e-CENP-A-S7A complex (Figure 4a). These results were further confirmed by Western blot analysis (Figure 4c). In addition, the Western blot analysis showed that the amount of 14-3-3 proteins in the complex isolated from asynchronous cells
was much smaller than that present in the complex isolated from mitotic cells (Figure 4c), further confirming the specific association of these proteins with mitotic centromeric CENP-A chromatin. Since 14-3-3 are phospho-binding proteins (Aitken, 2011), this suggests that these proteins may act as intermolecular protein bridges that couple phosphorylated CENP-A with CENP-C in mitosis. If this was the case, CENP-C should also associate with 14-3-3 proteins in vivo. To address this, we have purified by immuno-chromatography, the CENP-C complex (e-CENP-C) from HeLa cell lines stably expressing HA and FLAG double-tagged CENP-C (Shuaib et al., 2010) and, as expected, we found 14-3-3 proteins to be members of the e-CENP-C complex (Figure 4d). This results is in agreement with a previous report showing that 14-3-3 proteins bind to the N-terminal part of Drosophila CENP-C in vivo (Przewloka et al., 2011).

The above described in vivo for the “bridging” role of 14-3-3 proteins were further supported by a series of in vitro pull-down experiments using highly purified components (Figure 4e–g). The data reveal that recombinant purified from insect cells CENP-C binds directly to 14-3-3 proteins (Figure 4e). A direct interaction was also observed between phosphorylated CENP-A and 14-3-3 proteins. The detected interaction was highly specific and phosphorylation dependent, since neither unphosphorylated CENP-A nor histone H4 were found to bind to 14-3-3 (Figure 4f, g). Taken as a whole, the data reveal that 14-3-3 proteins act as key mediator factors required for the assembly of the tripartite phospho-CENP-A/14-3-3/CENP-C complex, which is essential for mitotic progression.

3 Discussion

In this work we have studied the role of the CENP-A NH$_2$-terminus and its phosphorylation in mitosis. We addressed this problem by using stable cell lines expressing siRNA-resistant either mutated CENP-A or CENP-A/H3 swapped tail chimeras. Importantly, the controlled expression of the different mutants has any effect on the stable HeLa cell lines, i.e. they behave identically to the parental naïve HeLa cells. This has allowed to carry out the experiments in endogenous CENP-A depleted cells, a sine qua non condition for understanding the implication of the CENP-A NH$_2$-terminus and its phosphorylation in mitosis.

We have first shown that the tail of CENP-A was required for mitosis in human cells, a result in agreement with the data in yeast (Morey et al., 2004). The same finding, i.e. the requirement of “some kind” of CENP-A NH$_2$-terminus for proper mitosis proceeding, was also recently reported in plants (Ravi and Chan, 2010). The data for both non-phosphorylatable GFP-CENP-A-S7A and GFP-H3SA-CENP-A fusions illustrate that the phosphorylation per se and not the amino acid composition and sequence of the CENP-A tail is required for mitosis. These results could explain why the NH$_2$-termini of different species are completely divergent in length, amino acid composition and sequence, but contain always serine residues (see Figure 1a). We speculate that during mitosis the NH$_2$-tail of CENP-A is phosphorylated in the different species (probably at different site(s) along their tail) and that this phosphorylation is required for proper mitosis proceeding.

Earlier studies on the role of S7 phosphorylation of CENP-A by Aurora B resulted in contradictory results. In one study, the non-phosphorylatable mutant of CENP-A did not interfere with kinetochore formation, spindle assembly, or cell cycle progression, but a notable delay at the terminal stage of cytokinesis, probably at the abscission, was detected (Zeitlin et al., 2001). In contrast, in a later study, expression of the same CENP-A S7A mutant resulted in defective kinetochore function (Kunitoku et al., 2003). However, an important caveat applying to both of these studies is the fact that the endogenous CENP-A was still present.

We further analyzed in detail the role of the CENP-A NH$_2$-terminus phosphorylation in
Figure 4. 14-3-3 proteins act as bridges between phosphorylated CENP-A and CENP-C in mitosis. (a) The mitotic WT e-CENP-A nucleosomal complex, but not the mutated e-CENP-A-S7A nucleosomal complex, contains 14-3-3 proteins. Nucleosomal complexes containing HA-FLAG epitope-tagged wild type CENP-A or CENP-A mutated at serine 7 were isolated by double immunoaffinity from either cycling or mitotic stable HeLa cells expressing either CENP-A or the CENP-A-S7A mutant. Proteins present in both the mitotic WT CENP-A and in the CENP-A-S7A mutant complexes together with the number of identified peptides are indicated. CENP-C and the 14-3-3 proteins are shown in blue. (b) Western blot analysis of CENP-C associated with CENP-C or CENP-A-S7A mitotic nucleosomal complexes. Identical amounts of the two complexes were analyzed by SDS PAGE, blotted and detected with anti-CENP-C antibody (lower panel). The same blot was also probed with anti-FLAG antibody to detect CENP-A to show that equal amounts of complex had been loaded (upper panel). (c) Western blot analysis of the 14-3-3 proteins associated with wild type CENP-A nucleosomal complexes (isolated from either mitotic or non-synchronized cells) or with mutated CENP-A-S7A nucleosomal complex (lower panel). Pan-anti-14-3-3 antibodies were used. An anti-FLAG antibody was used to detect CENP-A to show that equal amounts of complex had been loaded (upper panel). (d) 14-3-3 proteins are members of the soluble CENP-C complex. HA-FLAG epitope-tagged CENP-C (e-CENP-C.com) soluble complex was isolated from a stable HeLa cell line expressing e-CENP-C; upper panel, SDS PAGE of the complex; middle panel, Western blot analysis of the complex using anti-FLAG antibody (for detection of e-CENP-C); lower panel, Western blot analysis of the complex with pan anti-14-3-3 proteins. (e) Pull-down experiments show that CENP-C binds to 14-3-3 proteins. Recombinant HA-tagged CENP-C isolated from insect cells was mixed with GST-14-3-3-ζ proteins. Glutathione agarose beads were used to pull-down the GST-14-3-3-ζ proteins. CENP-C associated with the 14-3-3 in the pull-down was revealed by Western blotting using anti-HA antibody, while 14-3-3 were detected by pan anti-14-3-3 antibodies. Input, 1/10 of the 14-3-3-ζ / HA-tagged CENP-C mixture used for the pull-down. (f, g) 14-3-3 proteins bind to phosphorylated, but not to un-phosphorylated CENP-A. Purified to homogeneity (f, upper panel) un-phosphorylated or phosphorylated CENP-A-H4 tetramers were mixed with purified GST-14-3-3-ζ (f, upper panel, and g) and incubated with Glutathione agarose beads. The CENP-A attached to the beads (IP) was analyzed by autoradiography (f, lower panel) or by Western blotting using anti-CENP-A antibodies (g). (h) Schematics describing how phosphorylated CENP-A and CENP-C act in concert with 14-3-3 proteins in the assembly and maintenance of kinetochores.
mitosis. Our in vivo data revealed that the centromeric localization of CENP-C was determined by the NH$_2$-terminus phosphorylation of CENP-A. Immuno-affinity purification of the wild type CENP-A and non-phosphorylatable CENP-A S7A nucleosomal complexes together with a series of biochemistry experiments demonstrated the key role of the 14-3-3 proteins proteins in maintaining the association of phosphorylated CENP-A nucleosomes with CENP-C and the assembly of a tripartite phosphorylated CENP-A nucleosomes/14-3-3 proteins/CENP-C complex in mitosis. The schematic scenario presented in Figure 4h suggests a tentative mechanism for the assembly and function of this complex. The heavily phosphorylated population of CENP-A molecules acts as a sink to recruit 14-3-3 molecules to centromeres in the beginning of mitosis. Once recruited to centromeres, 14-3-3 proteins interact simultaneously with both CENP-C and the phosphorylated NH$_2$-terminus of CENP-A and stabilize the already existing CENP-A nucleosome-CENP-C interaction (Carroll et al., 2010). This allows CENP-C to be stably attached to the inner centromeres, and to serve as a scaffold for the building of a functional kinetochore. This scenario implies a much higher level of CENP-A phosphorylation compared to this of histone H3 in order to recruit the 14-3-3 proteins specifically to the centromeres and not to bulk chromatin. This appears to be the case, since even though cells contain amount of GFP-H3-CENP-A comparable to this of endogenous CENP-A (Figure 1c) which is estimated to be several orders of magnitude smaller than the amount of conventional H3 (Yoda et al., 2000), they nevertheless exhibited an NH$_2$-terminus phosphorylation level comparable to that of endogenous H3 (Figure 2b). Higher levels of phosphorylation may be achieved by the local activity of kinases, such as Aurora B, or by local inhibition of phosphatases (PP1s or PP2A), or both.

4 Experimental procedures

4.1 Plasmids

The full-length human cDNA clones of CENP-A and CENP-C were purchased from Invitrogen. The complete coding sequence from each clone was subcloned into the XhoI-NotI sites of the pREV-HTF retroviral vector. CENP-A serine 7 mutant (S7A) was constructed by PCR based mutagenesis and subcloned into pREV-HTF retroviral vector containing the Flag-HA. For protein expression in insect cells, the cDNA of CENP-C was cloned into pFastBac, which encodes HA tag at the N-terminus. The human histones CENP-A/H4 were cloned in a homemade bicistronic pET28b vector as described previously (Shuaib et al., 2010). Silent-resistant GFP-CENP-A fusions were constructed from a CENP-A coding sequence from Tanaka et al., 2004 and cloned into a pBABE-puro vector (Addgene #1764).

4.2 Cell culture and synchronization

The cells were maintained in standard DMEM media containing 10% fetal bovine serum, 1% penicilin and streptomycin, and 1% glutamine at 37 °C in a 5% CO$_2$ atmosphere. For generation of stable HeLa cell lines expressing Flag-HA epitope-tagged CENP-A.wt, CENP-A-S7A or CENP-C, the cells were transfected with calcium phosphate. The HeLa cell lines stably expressing e-CENP-A (WT and S7A) were synchronized in mitosis by treatment with thymidine-nocodazole. Cell lines stably expressing the various GFP-CENP-A fusions were established by retroviral infection with Moloney murine leukemia viruses (MMLV) produced by amphotropic Phoenix packaging cells (Swift et al., 2001).
Supplementary figure S1. Cells expressing the GFP-CENP-A fusions behave identically to the naïve parental HeLa cells. (a) The siRNA-resistant GFP-CENP-A fusions, stably expressed in HeLa cells, are incorporated into centromeric chromatin. Stable HeLa cell lines, expressing the indicated GFP-CENP-A fusions were established. The natural fluorescence of the GFP and CREST antiserum were used for detection of CENP-A fusions and the centromeres, respectively; blue, DNA; scale bar, 5 μm. Note the complete co-localization of the GFP and CREST signals. (b) The distribution patterns of the centromeric protein Zwint in stable HeLa cells expressing the studied GFP fusions are identical to these in the naïve HeLa cells. (c) Same as (b), but for Bub1 protein. (d) The stable expression of the siRNA-resistant GFP-CENP-A fusions does not affect the duration of mitosis. Stable cell lines expressing the indicated GFP fusions were synchronized by double thymidine block and collected at the specified time points after release. The Western blot analysis of the phosphorylation status of histone H3 serine 10 is shown. Antibody against phosphorylated at serine 10 histone H3 was used. Note that for all studied stable cell lines the peak of H3 phosphorylation is at 11 hours after release.
4.3 Tandem affinity purification

The chromatin extracts were prepared from stable HeLa cell lines expressing either CENP-A, CENP-A-S7A or CENP-C proteins fused to FLAG and HA epitope tags. The protein complexes were purified from asynchronous or mitotic cells by double immunoaffinity purification procedure with anti-Flag M2 antibody-conjugated agarose (Sigma), followed by anti-HA purification. Interacting partners of purified complexes (CENP-A.com, CENP-A-S7A.com, and CENP-C.com) were identified by an iron-trap mass spectrometer.

4.4 Recombinant protein purification

Recombinant histones CENP-A/H4 were expressed in Escherichia coli strain BL21-Codon-Plus-RIL (Stratagene) for 3 hours at 25 °C in the presence of 0.5 mM isopropyl-d-thiogalactopyranoside (IPTG). Bacterial cells from one liter cultures were then harvested and resuspended in 30 ml lysis buffer containing 1 M NaCl, 0.4 M ammonium acetate, 50 mM Tris-HCl pH 7.65, 2 mM DTT, 0.2 mM PMSF, 10 % glycerol, 0.01 % NP40, and 20 mM imidazole. The supernatants containing the soluble proteins were subjected to chromatography with Ni-NTA resin (0.5 ml, Qiagen) pre-equilibrated with lysis buffer; elution was performed with 150 mM imidazole.

Bacterially expressed GST-tagged 14-3-3-ζ protein was purified as described elsewhere (Winter et al., 2008). Recombinant baculoviruses encoding the full-length human HA-tagged-CENP-C were generated. The N-terminal HA fusion CENP-C was expressed in Sf9 insect cells for 48 hours. The soluble proteins were purified on HA-agarose beads by standard procedure.

For in vitro interaction the recombinant GST-tagged-14-3-3-ζ proteins were pre-bound to glutathione Sepharose 4B beads (Amersham) and then incubated with either HA-CENP-C, phosphorylated His-CENP-A, or non-phosphorylated His-CENP-A for 1 hour at room temperature. After the beads were washed 5 times with washing buffer (20 mM Tris-HCl pH 7.65, 250 mM NaCl, 0.01 % Triton X100, and 5 mM EDTA) and 1 time with low salt buffer (20 mM Tris-HCl pH 7.65, 150 mM NaCl, 0.01 % Triton X100). The eluted proteins were separated by 12 % SDS-PAGE and either visualized by Coomassie brilliant blue (CBB) staining or blotted and probed with appropriate antibodies.

4.5 RNA interference

Endogenous CENP-A expression was silenced by transient transfection with CENP-A siRNAs (Dharmacon). Transfections were carried out in 6 wells plates with 100 nM of siRNA mixed with 12 μl of HiPerfect (Qiagen), following the provider’s instructions. The following day, the transfection medium was replaced by fresh medium and cells were allowed to grow for two days before being fixed or collected.

4.6 Image acquisition

All microscopy was performed on fixed cells with a Zeiss Axio Imager Z1 microscope with a Plan-Apochromate 63× objective. GFP, cyanine-3 and Hoechst 33342 were used as fluorochromes. Images were acquired with a Zeiss AxioCam camera piloted with the Zeiss Axivision 4.8.10 software. Acquired images were exported as greyscale 8-bits TIFF files which were loaded into the GIMP 2.8.2 image manipulation software, where false colorization and merging of channels were performed with the “Colorify” tool and the “Lighten only” layer mode, respectively.
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References


