RESEARCH COMMUNICATION

Histone deacetylase 3 is required for centromeric H3K4 deacetylation and sister chromatid cohesion

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We describe here the role of histone deacetylase 3 (HDAC3) in sister chromatid cohesion and the deacetylation of histone H3 Lys 4 (H3K4) at the centromere. HDAC3 knockdown induced spindle assembly checkpoint activation and sister chromatid dissociation. The depletion of Polo-like kinase 1 (Plk1) or Aurora B restored cohesion in HDAC3-depleted cells. HDAC3 was also required for Shugoshin localization at centromeres. Finally, we show that HDAC3 depletion results in the acetylation of centromeric H3K4, correlated with a loss of dimethylation at the same position. These findings provide a functional link between sister chromatid cohesion and the mitotic "histone code".

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Histone deacetylase (HDAC) activity is involved in controlling mitosis progression. Histones are mostly deacetylated in mitosis and the treatment of cells with HDAC inhibitors leads to impaired mitotic progression and defects in kinetochore assembly (Sandor et al. 2000; Cimini et al. 2003; Mikhailov et al. 2004; Robbins et al. 2005). HDAC inhibition also results in spindle assembly checkpoint (SAC) defects, leading to premature mitosis exit (Warrener et al. 2003; Dowling et al. 2005; Magnaghi-Jaulin et al. 2007). HDAC inhibitors are a new class of powerful anti-tumor drugs currently yielding promising results in clinical trials (Bolden et al. 2006). Identification of the individual HDACs involved in mitosis may therefore facilitate the identification of new therapeutic targets and the design of more specific anti-cancer drugs,

overcoming the toxicity of nonspecific HDAC inhibitors.

HDAC3 has histone and nonhistone substrates (for review, see Karagianni and Wong 2007). It deacetylates all histone lysines tested in *in vitro* assays, with a preference for acetylated H4K5, H4K12, and H2AK5 (Johnson et al. 2002). It has been suggested that HDAC3 deacetylates histone H3 tails to provide an optimal recognition site for the phosphorylation of histone H3 serine 10 (H3S10) by the mitotic kinase Aurora B (Li et al. 2006). Despite reports that *HDAC3* gene deletion in mouse cells does not lead to mitosis defects (Bhaskara et al. 2008) (see Supplemental Note S1), a recent study reported the localization of HDAC3 to the mitotic spindle and showed that HDAC3 knockdown leads to chromosome misalignment, impaired kinetochore-microtubule attachment, and mitotic spindle collapse (Ishii et al. 2008).

During mitosis, the sister chromatids remain attached by cohesin until the metaphase-anaphase transition (Uhlmann 2004; Nasmyth 2005). In vertebrate cells, sister chromatid dissociation involves two mechanistically distinct steps. First, during prophase, the chromosome arms dissociate in a process dependent on Polo-like kinase 1 (Plk1) and Aurora B (Losada et al. 2002; Gimenez-Abian et al. 2004), whereas Shugoshin (Sgo1) protects the cohesin at centromeres (Watanabe 2005). Thus, on SAC release, centromeric sister chromatid cohesion is abolished by separase-mediated cleavage of the Scc1 cohesin subunit. Separase is maintained in an inactive state by binding to securin until SAC inactivation, which leads to the anaphase promoting complex (APC)/proteasome-dependent degradation of securin and the release of an active form of separase (Musacchio and Salmon 2007).

Centromeric chromatin contains the specific histone H3 variant CENP-A and displays specific histone post-translational modification patterns (Sullivan and Karpen 2004), but little is known about the histone modifications required to control centromeric chromatid cohesion. We describe here the role of HDAC3 in centromeric cohesion control and the deacetylation of histone H3 Lys 4 (H3K4) at the centromere.

Results and Discussion

HDAC3 knockdown leads to sister chromatid separation and SAC activation

We reported recently that the treatment of mitotic cells by the broad-range HDAC inhibitor trichostatin A (TSA) leads to overriding of the SAC (Magnaghi-Jaulin et al. 2007). In an attempt to identify the individual HDAC involved in mitosis progression, we designed specific siRNAs for classes I, II, and IV HDAC and investigated the mitotic phenotype associated with the down-regulation of each HDAC. We could not identify a single HDAC as involved in SAC function, and it therefore seems likely that the overriding of the SAC observed when mitotic cells are treated with broad-range HDAC inhibitors depends on the simultaneous inhibition of several individual HDACs. However, transfection with two independent HDAC3 siRNAs (HDAC3-1 and HDAC3-2) significantly increased the proportion of mitotic cells (Fig. 1A). This phenomenon was not observed

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Figure 1. HDAC3 down-regulation induces SAC-dependent mitotic arrest. (A) HeLa cells were transfected with HDAC3 siRNA or with control siRNA (GFP) on days 1 and 2, and mitotic cells were scored by microscopy on day 4. At least 1000 cells were counted for each experimental point. Data are means and SD from three independent experiments. (B) Western blotting analysis of mitosis markers in cells transfected with HDAC3 siRNA and control siRNA. HDAC1 was used as a loading control. Transfection conditions were as in A. (C) Cotransfection with HDAC3-1 siRNA and MAD2 siRNA. Cells were transfected with HDAC3-1 or control (GFP) siRNA on days 1 and 2 and transfected with MAD2 siRNA on day 3. Mitotic index was determined on day 4. At least 1000 cells were scored for each experimental point. Data are means and SD from three independent experiments. (D) Quantification of chromosome alignment defects. At least 200 mitoses were counted for each experimental point. Data are means and SD from four independent experiments.

after the depletion of HDAC1 or HDAC2 (Supplemental Fig. S1A). Transfection with the HDAC3-1 and HDAC3-2 siRNAs also induced the accumulation of mitotic chromatin markers (H3S10Ph, H3S28Ph, and H3T3Ph) and of the essential APC targets cyclin B1 and securin (Fig. 1B). H3S10Ph fluorescence signals were quantified with two independent anti-H3S10Ph antibodies in individual mitotic cells and revealed no HDAC3-depletion specific decrease in labeling (Supplemental Fig. S2A), in contrast to previous reports (Li et al. 2006) (see Supplemental Note S2).

We investigated whether the mitotic block induced by HDAC3 knockdown was a consequence of SAC activation by determining the mitotic index in cells cotransfected with the HDAC3-1 siRNA and an siRNA targeting Mad2, an essential SAC component (Fig. 1C; Supplemental Fig. S3). SAC inhibition due to Mad2 depletion abolished the HDAC3-1-dependent increase in mitotic index, indicating that HDAC3 knockdown activates the SAC, consistent with previous observations that cells in which HDAC3 is depleted are blocked in the G2/M phase (Li et al. 2006; Wilson et al. 2006) and in mitosis in a SAC-dependent manner (Ishii et al. 2008).

Cells transfected with HDAC3-1 or HDAC3-2 siRNAs frequently displayed inaccurate mitosis, with 10%–30% of mitoses showing congression defects with groups of isolated chromosomes failing to align on the metaphase plate (Fig. 1D; Supplemental Fig. S4). These alignment defects may result from defective sister chromatid cohesion. We therefore examined chromosome spreads from HDAC3-1 siRNA-transfected cells. We found that a high

proportion of these cells displayed premature sister chromatid separation (PSCS) (Fig. 2A). This phenomenon was not observed following the depletion of HDAC1 or HDAC2 (Supplemental Fig. S1B). As observed for mitotic block, a loss of chromatid cohesion was also observed for cells transfected with HDAC3-2 siRNA (Fig. 2B). The SAC is activated in these cells. Chromatid separation must therefore result from a cohesion defect rather than early entry into anaphase. SAC activation in HDAC3-depleted cells may result from the detection of a lack of tension induced by sister chromatid dissociation. However, HDAC3 has been reported to be involved in microtubule-kinetochore attachment (Ishii et al. 2008) and impaired kinetochore occupancy may also be responsible for checkpoint activation in HDAC3-depleted cells. HDAC3 depletion, inducing PSCS, may account for the impaired chromosome alignment previously reported in HDAC3-depleted cells (Ishii et al. 2008). It remains unclear whether the loss of cohesion and mitotic spindle collapse following HDAC3 knockdown are independent events.

We investigated the involvement of separase in the PSCS induced by HDAC3 depletion, by treating cells

Figure 2. HDAC3 depletion leads to separase-independent sister chromatid dissociation. (A) Typical chromosome spreads from HeLa cells transfected with control siRNA (left panel) or with HDAC3-1 siRNA (middle and right panels). (B) Quantification of cells displaying separated chromatids following transfection with HDAC3 siRNA. (C) HeLa cells were transfected with the indicated siRNA on days 1 and 2. On day 4, cells were treated with MG132 (3 μ M) for 1 h, and mitotic cells were removed by mitotic shake-off to eliminate cells that had entered mitosis before the addition of MG132. Cells were incubated for another 3 h in the presence of MG132, and chromatid separation was then quantified on chromosome spreads. Data are expressed as percentages of total mitoses and are means and SD from at least three independent experiments. (D) At least 400 mitoses were scored for each experimental point. (D) Plk1 and Aurora B depletion restored sister chromatid cohesion in HDAC3-depleted cells. Cotransfection with HDAC3-1 siRNA and Plk1 or Aurora B siRNA. Cells were transfected with HDAC3-1 or control (GFP) siRNA on days 1 and 2, and with Plk1 or Aurora B siRNA on day 3. Chromatid separation was scored on chromosome spreads prepared on day 5. Data are expressed as percentages of total mitoses and are means and SD from two independent experiments. At least 300 mitoses were counted for each experimental point.

transfected with HDAC3-1 siRNA with MG132, a specific proteasome inhibitor. Under these conditions, securin, which inhibits separase action, cannot be degraded, and separase remains inactive. MG132 treatment induced a modest but reproducible increase in loss of cohesion in control cells, consistent with previous reports (Lee et al. 2008), but had no effect on HDAC3-induced PSCS, indicating that this process does not involve separase (Fig. 2C).

Aurora B and Plk1 knockdown partially restore cohesion in HDAC3-depleted cells

The other known mechanism of chromatid dissociation in vertebrate cells takes place on chromosome arms during prophase and is dependent on Aurora B and Plk1 activities (Losada et al. 2002; Gimenez-Abian et al. 2004). We investigated whether knockdown of these mitotic kinases could rescue the loss of cohesion caused by HDAC3 depletion, by depleting cells of both HDAC3 and Aurora B or Plk1 and assessing sister chromatid cohesion. Cotransfections with HDAC3-1 siRNA and Aurora B siRNA or Plk1 siRNA partially restored sister chromatid cohesion (as shown by comparison with transfections with HDAC3-1 alone) (Fig. 2D; Supplemental Fig. S5). However, the depletion of Plk1 or Aurora B prevents cohesin removal from chromosome arms, thereby preventing chromatid separation, regardless of centromeric cohesion status. However, the continued absence of centromeric cohesion in AurB/HDAC3- or Plk1/HDAC3-depleted cells would lead to chromosomes with attached arms displaying a loss of cohesion in the centromere region only. We never observed such a phenotype in AurB/HDAC3- or Plk1/HDAC3-depleted cells, suggesting that loss of centromeric cohesion provoked by HDAC3 depletion took place through the Aurora B- and Plk1-mediated prophase pathway.

HDAC3 is required for correct Sgo1 localization and centromeric H3T3 phosphorylation in mitosis

In normal mitosis, Sgo1 protects centromeric cohesins from phosphorylation by Plk1 and Aurora B (Watanabe 2005). We tested whether knocking down HDAC3 affected the centromeric localization of Sgo1 in mitosis. We labeled cells with both the antibody of interest and a CREST human autoimmune serum that specifically recognizes kinetochore proteins (Moroi et al. 1980). Mitotic cells with attached chromatids display paired CREST-labeled dots, whereas the disruption of cohesion results in the scattering of CREST signals (see Fig. 3A, CREST column, top and bottom panels, respectively).

HDAC3 depletion resulted in the almost complete loss of Sgo1 from the centromere in mitotic cells displaying PSCS (Fig. 3A). Fluorescence analyses showed the Sgo1 signal in HDAC3-depleted cells to be only about one-tenth as strong as that in control cells (Fig. 3B), whereas the intensity of the CREST signal was unaffected.

The HDAC3 knockdown phenotype resembled that observed in Haspin-depleted cells. Haspin is a kinase with only one known substrate (apart from Haspin itself): the threonine residue in position 3 of histone H3 (H3T3). The centromeric region is enriched in H3T3Ph (the phosphorylated form) during mitosis (Dai et al. 2005,

2006). Haspin knockdown leads to a loss of H3T3Ph staining, PSCS, and mitotic block, but has no effect on the centromeric localization of Sgo1. Furthermore, Sgo1 depletion leads to diffuse H3T3Ph staining on chromatid arms, but has little effect on the overall intensity of H3T3Ph staining during mitosis (Dai et al. 2006). We investigated the phosphorylation status of H3T3 in HDAC3-depleted cells and found that HDAC3 knockdown impaired centromeric H3T3 phosphorylation during mitosis (Supplemental Fig. S6). We observed two distinct phenotypes in cells displaying PSCS: (1) cells in which H3T3Ph staining spread along chromosome arms, with an overall H3T3Ph fluorescence intensity not significantly different from that measured in control cells ("spread" phenotype), similar to that in cells in which Sgo1 levels had been knocked down (Supplemental Fig. S6A [middle panel], B [gray bars]); and (2) cells apparently lacking H3T3 phosphorylation ("null" phenotype), resembling Haspin-depleted cells (Supplemental Fig. S6A [bottom panel], B [black bars]). H3T3Ph-null cells had not exited mitosis, as shown by the intense H3S10Ph labeling of these cells (Supplemental Fig. S6C,D). The intensity of the CREST fluorescence signal was not affected by HDAC3 depletion (Supplemental Fig. S6B). We measured the ratio of null to spread H3T3Ph phenotypes at various times after transfection with HDAC3 siRNA and found that the proportion of H3T3Ph spread cells decreased with time, whereas the proportion of H3T3Ph-null cells increased (Supplemental Fig. S7A). This strongly suggests that the two phenotypes are actually one, and that in HDAC3-depleted cells, H3T3 phosphorylation on chromosome arms is progressively lost when cells remain blocked in mitosis for extended periods of time.

The total loss of H3T3Ph in a fraction of cells following Sgo1 depletion has not been reported. We investigated whether Sgo1-depleted and HDAC3-depleted cells had the same H3T3Ph phenotype by depleting Sgo1 and assessing H3T3Ph status at various times after depletion. We found that the spreading of H3T3Ph labeling predominated shortly after transfection (48 h), but that this phenotype was gradually lost over time. We found that 96 h after transfection with Sgo1 siRNA, H3T3Ph labeling had been lost from >60% of the cells displaying PSCS (Supplemental Fig. S7B). H3T3Ph delocalization and eventual loss therefore appear to be a consequence of Sgo1 depletion and the observed phenotypes in HDAC3-depleted cells are consistent with the loss of Sgo1 localization alone.

The deacetylation and dimethylation of centromeric H3K4 require HDAC3

According to centromere models, CENP-A-containing nucleosomes are located at the outer region of the centromere and build the kinetochore, whereas H3-containing nucleosomes are located in the inner centromere region, which contains Sgo1, and in which cohesion occurs (Blower et al. 2002). H3K4 has been found to be dimethylated in the centromeric region of human mitotic chromosomes, but no such methylation is observed in pericentromeric regions (Sullivan and Karpen 2004). It is not known whether H3K4 dimethylation is required for sister chromatid cohesion. H3K4 methylation has been extensively studied, particularly as concerns its role in gene transcription (Martin and Zhang 2005). In contrast, H3K4 acetylation has been studied in much less detail,

Figure 3. HDAC3 depletion prevents the centromeric localization of Sgo1 and induces H3K4 acetylation and a loss of H3K4 dimethylation at the centromere. (A) Immunofluorescence microscopy on chromosome spreads obtained by cyto centrifugation, showing staining for Sgo1, CREST, and DNA in HeLa cells transfected with control or HDAC3-1 siRNAs. Single chromosome enhancements are shown. Cells were transfected with HDAC3-1 siRNA or with a control siRNA on days 1 and 2. Immunofluorescence was analyzed on day 5. (B) CREST and Sgo1 signal intensities were measured in 15 HDAC3-depleted cells showing chromatid separation (black bars) and compared with those in 15 control cells (white bars). Data are means and SD for comparisons with controls. (C) Immunofluorescence microscopy on chromosome spreads obtained by cyto centrifugation of HeLa cells transfected with HDAC3-1 or control siRNAs. Staining for H3K4Ac, CREST, and DNA is shown. Transfection variables were as in A. (D) Quantification of centromeric fluorescence signals for CREST and H3K4Ac in 10 control cells (white bars) and in 10 HDAC3-depleted cells displaying PSCS (black bars). Data are means and SD. (E) Immunofluorescence microscopy on individual chromosomes obtained by cyto centrifugation of HeLa cells transfected with HDAC3-1 or control siRNAs. Staining for H3K4diMe, CREST and DNA is shown. Arrows indicate centromeric H3K4diMe labeling lost upon HDAC3 depletion. Transfection variables were as in A. (F) Quantification of fluorescence signals for CREST and H3K4diMe in 15 centromeres from control cells (white bars) and in 15 centromeres from HDAC3-depleted cells displaying PSCS (black bars). Data are means and SD.

although it has been described in human cells (Garcia et al. 2007). Our preliminary experiments showed that H3K4 undergoes active deacetylation, as the treatment of cells with an HDAC inhibitor leads to a strong H3K4Ac signal on Western blots (G. Eot-Houllier, unpubl.).

No H3K4Ac labeling was detected in normal mitotic cells on immunofluorescence microscopy (Fig. 3C, top panel). In the absence of HDAC3, a centromere-specific H3K4Ac signal became visible on mitotic chromosomes, strongly suggesting that HDAC3 deacetylates H3K4 at the centromere (Fig. 3C, bottom panel). Quantification of the H3K4Ac fluorescence signal at the centromere showed a dramatic increase associated with HDAC3 down-regulation (Fig. 3D). Consistent with these findings, HDAC3 deacetylated H3K4Ac in an *in vitro* deacetylase assay, whereas a mutated version of HDAC3 lacking HDAC activity did not (Supplemental Fig. S8). Background levels of H3K4 acetylation were high in interphase nuclei, but we detected specific centromeric H3K4Ac labeling in HDAC3-depleted interphase cells,

suggesting that HDAC3-dependent H3K4 deacetylation takes place before the cell enters mitosis (Supplemental Fig. S9). This is consistent with the observation that HDAC3 is associated with chromatin in interphase and prophase, but dissociates from chromatin before metaphase (Bhaskara et al. 2008). The specificity of the anti-H3K4 antibody was checked (Supplemental Note S3; Supplemental Fig. S10). No centromeric H3K4Ac labeling was observed following the depletion of HDAC1 or HDAC2, and the acetylation of other histone lysines (H3K9 and H4K5) was not detected at the centromere following the depletion of HDAC1, HDAC2, or HDAC3 (Supplemental Fig. S11).

We investigated whether the acetylation of H3K4 at the centromere could prevent its dimethylation by looking for centromeric H3K4 dimethylation in HDAC3-depleted and control cells. We detected a centromeric H3K4diMe signal in control cells (as described in Sullivan and Karpen 2004) with three independent H3K4diMe antibodies (Fig. 3E, top panel). No such H3K4diMe cen-

centromeric signal was detected on separated chromatids in HDAC3-depleted cells. Thus, despite the change in shape observed with HDAC3 knockdown-induced chromatid dissociation (chromatids are shorter and more compact than those in the control), CREST labeling never colocalized with a discrete H3K4diMe signal, in contrast to what was observed in control cells (Fig. 3E, bottom panel). Quantification of the H3K4diMe fluorescence signal at the centromere showed an almost complete loss of signal associated with HDAC3 knockdown (Fig. 3F; Supplemental Fig. S12). Therefore, centromeric H3K4 acetylation is associated with a loss of centromeric H3K4 dimethylation in HDAC3-depleted cells.

H3K4 acetylation may directly prevent Shugoshin from binding to the centromere. Alternatively, although we were unable to detect a H3K4diMe/Sgo1-specific interaction *in vitro* to date (L. Magnaghi-Jaulin, unpubl.), the deacetylation of centromeric H3K4 by HDAC3 may be required for H3K4 dimethylation, providing a docking site for Shugoshin (or a Shugoshin-recruiting factor). The docking of Shugoshin facilitated by HDAC3-mediated deacetylation and the subsequent dimethylation of centromeric H3K4 would therefore be a key element of a new mitosis-specific “histone code.”

Materials and methods

Detailed experimental procedures are provided in the Supplemental Material.

Antibodies

Commercial primary antibodies are listed in the Supplemental Material. Human CREST serum was a gift from Dr. C. Johanet (Hôpital Saint-Antoine, Paris). Secondary antibodies were obtained from Jackson Immunoresearch Laboratories.

Cell culture and siRNA

HeLa S3 cells were cultured in DMEM (Gibco) supplemented with 10% FCS (Invitrogen) and standard additives. Cells were transfected with siRNA duplexes using Hiperfect (Qiagen) according to the manufacturer's instructions.

Immunoblotting

Immunoblots were performed using standard techniques. Proteins were detected with the Western Lightning Chemiluminescence Plus kit (Perkin-Elmer) used according to the manufacturer's instructions.

Immunofluorescence microscopy

Cells were grown on poly-L-lysine-coated coverslips (Sigma-Aldrich), fixed by incubation in 3% paraformaldehyde solution, and sequentially incubated with primary and secondary antibodies. Signals were quantified with ImageJ (NIH, <http://rsb.info.nih.gov/ij>).

Mitotic chromosome spreads

Chromosome spreads were obtained by hypotonic lysis of mitotic cells followed by ethanol/acetate fixation. For chromosomes immunostaining, cells were cytocentrifuged in a CYTOSPIN (Shandon) before fixation in 3% paraformaldehyde solution. Immunostaining was carried out as described above.

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