Histone Deacetylase 3 Regulates Cyclin A Stability*

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Background: Cyclin A is a regulatory subunit of cyclin-dependent kinases that are key enzymes in the regulation of cell cycle progression.

Results: Histone deacetylase 3 (HDAC3) regulates cyclin A deacetylation.

Conclusion: HDAC3 regulates cyclin A stability by modulating cyclin A acetylation.

Significance: HDAC3 regulates cell cycle progression by controlling cyclin A levels.

Histone deacetylase 3 (HDAC3) directly interacts with and deacetylates cyclin A. HDAC3 interacts with a domain included in the first 171 aa of cyclin A, a region involved in the regulation of its stability. In cells, overexpression of HDAC3 reduced cyclin A acetylation whereas the knocking down of HDAC3 increased its acetylation. Moreover, reduction of HDAC3 levels induced a decrease of cyclin A that can be reversed by proteasome inhibitors. These results indicate that HDAC3 is able to regulate cyclin A degradation during mitosis via proteasome. Interestingly, HDAC3 is abruptly degraded at mitosis also via proteasome thus facilitating cyclin A acetylation by PCAF/GCN5, which will target cyclin A for degradation. Because cyclin A is crucial for S phase progression and mitosis entry, the knock down of HDAC3 affects cell cycle progression specifically at both, S phase and G2/M transition. In summary we propose here that HDAC3 regulates cyclin A stability by counteracting the action of the acetyl-}

netic gene silencing as EZH2 (7). Thus cyclin A-cdk complexes play a crucial role in the regulation of gene expression during cell cycle progression.

Cyclin A levels are low during G1 but they increase at the onset of S phase, when it contributes to the stimulation of DNA synthesis (8, 9). Its levels remain elevated until early mitosis when, by associating with and activating cdk1, it drives the initiation of chromosome condensation and nuclear envelope breakdown (10–12). Another cyclin, cyclin B, also activates cdk1 at mitosis. Cyclin B levels rise during G2, and then it binds to cdk1. This complex promotes the completion of chromosome condensation and spindle assembly, thus driving cell cycle progression until metaphase (13).

To proceed with metaphase to anaphase transition, the inactivation of both cyclin A-cdk1 and cyclin B-cdk1 complexes is necessary. Their inactivation is accomplished by degradation of both cyclins. Cyclin A is destroyed during prometaphase by the Anaphase Promoting Complex/Cyclosome (APC/C) via proteasome (14) whereas cyclin B is degraded during metaphase, significantly later than cyclin A (15). The ordered destruction of these different cyclins is important for maintaining the correct sequence of events in late mitosis (16). Thus, non-degradable mutants of cyclin A cause cell cycle arrest at metaphase, whereas those of cyclin B block cells at anaphase (17, 18).

In general, cyclins have a “destruction box,” which is a sequence that is recognized by the ubiquitylation machinery in order to degrade these proteins (19). Additionally, cyclin A also has an extended “destruction box” that includes aa 47–72 (20). However, to totally avoid cyclin A ubiquitylation and degradation the first 171 aa of cyclin A must be eliminated, revealing that in addition to the extended “destruction box” more sequences from the N terminus are needed for cyclin A degradation (21).

Cyclin A degradation is induced by APC/C bound to the targeting subunit Cdc20 (APC/C\(^{Cdc20}\)) that is activated by phosphorylation by cyclin B-cdk1. It is spindle-checkpoint independent, and thus, it starts as soon as APC/C\(^{Cdc20}\) is activated (14, 22). In contrast, cyclin B degradation by APC/C\(^{Cdc20}\) is sensitive to the spindle assembly checkpoint. This different behavior of cyclin A and cyclin B degradation by the same APC/C complex indicates that distinct signals participate in...
targeting these cyclins for ubiquitylation and the subsequent degradation during mitosis (22).

It has been reported that the cyclin A-cdc complex must bind a Cks protein to be degraded at prometaphase. The cyclin A-cdc-Cks complex is recruited to the phosphorylated APC by its Cks protein (23). Moreover, cyclin A directly associates with cdc20 by its amino-terminal domain. Cyclin A associated with cdc20 is also able to bind to APC (24). Thus, Cyclin A associates with APC/C through at least two different ways: by its associated Cks and through cdc20. This association with APC/C causes cyclin A to be degraded regardless of whether the spindle checkpoint is active or not (23). Its insensitivity to the spindle checkpoint is due to the fact that cyclin A interacts with cdc20 with much higher affinity that the spindle checkpoint proteins as BubR1 and Bub3 (24). Thus, cyclin A-cdc-Cks complexes competes and displaces these proteins for binding to cdc20, and under these conditions, cyclin A is degraded (25).

The signals that trigger cyclin A degradation at prometaphase have been recently elucidated. We previously reported that, at mitosis, cyclin A is acetylated by the acetyltransferase PCAF at specific lysine residues: K54, K68, K95, and K112 (26). These lysines are located on the N-terminal domain of cyclin A and specifically at domains implicated in the regulation of the stability of the protein (23, 27). This acetylation subsequently leads to cyclin A ubiquitylation through APC/C and finally to the proteasome-dependent degradation. A more recent report validated this mechanism by showing that the ATAC acetyltransferase complex regulates mitotic progression by acetylating cyclin A and targeting it for degradation (28). Interestingly, this complex contains GCN5, an acetylase highly homologous to PCAF (29).

Protein acetylation is reversible because of the action of deacetylases, commonly named histone deacetylases (HDACs) that eliminate the acetyl group thus counteracting the action of acetyltransferases. Until now, eighteen HDACs have been identified. They are classified in two families: classical HDACs and sirtuins. Classical HDACs include those grouped in class I, II, and IV whereas Sirtuins corresponded to class III. HDACs 1–3 and 8 belong to class I whereas HDACs 4–7 and 9–10 are included in class II. Class IV only contains one member namely HDAC11 (30). Sirtuins are included in a different family of deacetylases because of their dependence on NAD+. Most of these enzymes act deacetylating a high diversity of substrates that include histones and non-histone proteins localized in different cellular compartments.

Here we report that the histone deacetylase 3 (HDAC3) participates in the regulation of cyclin A stability by modulating the acetylation status of cyclin A. HDAC3 directly associates with cyclin A through its N-terminal region during cell cycle until mitosis. At this moment of the cell cycle, HDAC3 is degraded, thus facilitating the PCAF-dependent acetylation of cyclin A that targets it for degradation.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—HA-cyclin A, Flag-cyclin A-WT, Flag-cyclin A-4R, and GST-cyclin A-WT were described elsewhere (26). GST–cyclin A 1–171, and GST–cyclin A 171–432 were described elsewhere (31). HDAC1–Flag, HDAC2–Flag, and HDAC3–Flag were in pcDNA3 (32). GST–HDAC1 51–482 was in pGEX (32). ShRNAs against HDAC1 (NM-004964.2), HDAC2 (NM-001527.1) and control shRNA were purchased from Sigma. Sure Silencing™ shRNA plasmids against human HDAC3 (clone ID2 and 3) was purchased from Superarray Biosciences (KH05911P). pcDNA3 Flag-cyclin A 171–432 was subcloned from pGEX cyclin A 171–432. pGEX HDAC3 and pGEX-HDAC2 were subcloned from pcDNA3 Flag-HDAC3 and pcDNA3 Flag-HDAC2, respectively.

**Antibodies and Reagents**—Antibodies against cyclin A (H-432), cyclin A (BF-683), cdk2 (M2), HDAC1 (H-51), HDAC2 (H-54), and HDAC3 (H-99) were purchased from Santa Cruz Biotechnology. Anti-acetyl lysine (9441), mouse anti-HDAC3 (7G6C5), and anti-phospho-histone 3 (9713) were from Cell Signaling. Anti-acetyl lysine antibody (401–939) was purchased from Rockland. Antibodies against Flag (F7425) and HA (H6908) were purchased from Sigma. Monoclonal antibody against cyclin A (611268) was from Becton Dickinson. Monoclonal antibody against histones (MAB052) was from Millipore. For IP we used monoclonal anti-FA-agarose and monoclonal anti-Flag M2 affinity gel from Sigma. Anti-GFP (ab290) was from Abcam. Thymidine, nocodazole, cycloheximide, roscovitine, sodium fluoride, okadaic acid, propidium iodide, and TSA were from Sigma. ALLN was from Calbiochem. For pull down experiments, purified proteins were coupled to CNBr-Sepharose 4B beads (GE Healthcare).

**Cell Culture, Transfection, and Synchronization**—Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Transfection experiments were performed using Lipofectamine 2000 from Invitrogen and Polyfect from Qiagen. Transfected synchronized cells were obtained as described (33). Briefly, to obtain cells at metaphase, cells were cultured in the presence of 80 ng/ml of Nocodazol (Sigma) for 16 h. Then, cells were washed with fresh medium and collected. To obtain cells at G1/S, they were blocked with nocodazol as mentioned above, and then after washing, they were cultured with fresh medium for 9 h and subsequently collected. Finally, to obtain cells at G2/M, they were cultured in the presence of 2 mm thymidine (Sigma) for 16 h. Then, the culture medium was changed by normal fresh medium, and cells were subsequently cultured in the absence of thymidine for 8 h. After this incubation, the first step (incubation with thymide for 16 h) was repeated. Finally, cells were washed with fresh medium and left in culture with normal medium 4 more hours and subsequently collected.

**Protein Purification, Pull Down, and Immunoprecipitation**—Protein expression and purification were performed as described (31). For pull down experiments, GST, GST–cyclin A 1–171, or GST-cyclin A 171–432 were bound to glutathione-Sepharose beads (glutathione-Sepharose 4B; GE Healthcare) and washed with NETN (20 mm Tris-HCl, pH 8, 1 mm EDTA, 0.5% Nonidet P-40, and 100 mm NaCl). Beads were then incubated for 1 h at room temperature with HDAC1 (51–482 aa), HDAC2, or HDAC3. Beads were washed with NETN containing 150 mm NaCl, and the bound material was analyzed by SDS-polyacylamide gel electrophoresis followed by Western blot (WB). For affinity chromatography experiments, GST-HDAC1, GST-HDAC2, or GST-HDAC3 were loaded onto a
cyclin A-Sepharose 4B column or a control column. Then, after extensive washing, proteins were eluted with 3 M KCl buffer or 200 mM glycine, pH 2.5. For IP, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 mM Na3VO4, 0.5 μg/μl aprotinin, and 10 μg/μl leupeptin) for 30 min on ice. Lysates (0.2–2 mg of protein) were incubated with anti-Flag or anti-HA-agarose beads for 2 h at 4 °C. After three washes with RIPA buffer, Laemmli buffer was added to the samples that were subsequently electrophoresed.

Immunofluorescence—To detect cyclin A, HDAC1, HDAC2, and HDAC3, cells were grown in coverslips, fixed in 4% paraformaldehyde/PBS for 15 min at room temperature, washed with PBS, and blocked with 1% BSA, 0.1% Triton X-100 in PBS for 15 min at room temperature. Then, cover slips were incubated with anti-cyclin A (mouse monoclonal) and anti-HDAC3 (rabbit polyclonal) antibodies for 1 h at 37 °C. They were then washed with PBS and incubated for 45 min at 37 °C with Alexa-Fluor 594 (goat anti-mouse, dilution 1:500) and Alexa-Fluor 488 (goat anti-rabbit, dilution 1:500). After that, coverslips were washed, mounted on glass slides with Mowiol (Calbiochem), and analyzed by confocal microscopy.

Flow Cytometry Analysis—Cells were fixed with 70% cold ethanol for 2 h at 4 °C, washed with PBS, and finally incubated with 20 μg/ml of propidium iodide and 200 μg/ml RNase for 30 min at room temperature. Analysis of DNA content was carried out in a Becton Dickinson FACS Calibur. Data were analyzed with the WinMDI 2.9 software.

Determination of HDAC3 Activity—To determine HDAC3 activity at different stages of the cell cycle HeLa cells were firstly treated overnight with 3 μM TSA to increase the acetylation levels of endogenous histones. These acetylated histones were used as a substrate in the experiments. On the other hand, HeLa cells were transfected with Flag-HDAC3 and subsequently synchronized as described (33). To analyze HDAC3 activity at the

Figure 1. Cyclin A directly interacts with HDAC3. A, HeLa cells were transfected with HA-cyclin A and Flag-HDAC1, Flag-HDAC2 or Flag-HDAC3. Cell extracts were subjected to IP using anti-HA (left panel) or anti-Flag (right panel). IP with IgG was used as a control. The immunoprecipitates were subjected to WB with anti-HA or anti-Flag. A sample of cell lysate (input) was used as a control. B, cells were transfected with Flag-cyclin A. Cell extracts were subjected to IP using anti-Flag with IgG that was used as a control. The immunoprecipitates were subjected to WB with anti-cyclin A or anti-HDAC4, HDAC9, or HDAC11. A sample of cell lysate (input) was used as a control. C, HeLa cell extracts were subjected to IP using anti-cyclin A or anti-HDAC3 to analyze the interaction between endogenous cyclin A and HDAC1, HDAC2, or HDAC3. IgG was used as a control. A sample of cell lysate (input) is shown on the left. D, endogenous cyclin A, HDAC1, HDAC2, and HDAC3 were visualized by immunofluorescence as described under “Experimental Procedures.” E, Sepharose 4B beads coupled to cyclin A WT (CYCA) or control beads were incubated with HDAC1 51–482, HDAC2, or HDAC3. Then, the proteins associated with the beads were eluted and the bound (B) or not-bound (NB) proteins were detected by WB using specific antibodies. F, Sepharose 4B beads coupled to GST, GST-cycA 1–171, or GST-cycA 171–482 were incubated with HDAC1 51–482 or HDAC3. Then, the proteins associated with the beads were eluted and the bound (B) or not-bound (NB) proteins were detected by WB using specific antibodies.
different stages of the cell cycle, synchronized cell extracts were subjected to IP using anti-Flag. The immunoprecipitated HDAC3 was then mixed with 20 μg of cell lysates containing acetylated histones and then incubated at 30 °C for 30 min in 15 μl of HDAC buffer. Finally, the acetylation status of histones was analyzed by WB with anti-acetyl lysine antibodies.

**RESULTS**

**HDAC3 Directly Interacts with Cyclin A**—To analyze the putative interaction of cyclin A with different members of the class I family of classical HDACs, cells were transfected with HA-cyclin A together with Flag-HDAC1, Flag-HDAC2, or Flag-HDAC3. Lysates from these cells were subjected to immunoprecipitation (IP) with anti-HA or anti-Flag, and the immunoprecipitates analyzed by Western blotting (WB). Results showed that all these three HDACs, HDAC1, -2, and -3 interacted with cyclin A (Fig. 1A). We also studied the putative interaction of cyclin A with several members of class II (HDAC4 and HDAC9) and the unique member of class IV (HDAC11). In these experiments, cells were transfected with Flag-cyclin A and then, cell extracts were subjected to IP with anti-Flag. Results indicated that HDAC4 but not HDAC9 and HDAC11 interacted with cyclin A (Fig. 1B).

We next studied the interaction among the endogenous proteins HDAC1, -2, and -3 and cyclin A. We excluded from these studies HDAC4 because despite its interaction with cyclin A, it has been reported that HDAC4 activity depends on its association with HDAC3. Thus, HDAC4 alone cannot play a direct role on the regulation of cyclin A acetylation (34). Fig. 1C shows that endogenous cyclin A interacts with all these three HDACs. The putative cellular co-localization of cyclin A with HDAC1, -2, or -3 was then analyzed by immunofluorescence. As shown in Fig. 1D all these three HDACs co-localized with cyclin A in the nucleus. To analyze whether cyclin A directly interacts with these three HDACs, affinity chromatography experiments using cyclin A-Sepharose columns and purified recombinant HDACs were performed. Results revealed that HDAC1 and HDAC3 directly interacted with cyclin A whereas HDAC2 did not (Fig. 1E). Because the cyclin A domain involved in its degradation is included in the first 171 aa of its sequence, we aimed to study the direct interaction of this domain with HDAC1 and HDAC3 by pull down. As it can be observed in Fig. 1F, HDAC3 but not HDAC1 interacted with the fragment 1–171aa of cyclin A. Because of this interaction, we subsequently focused our attention on the relationship between cyclin A and HDAC3.

**HDAC3 Regulates the Levels and the Acetylation of Cyclin A**—We subsequently studied the effect of knocking down HDAC3 on cyclin A levels. As observed in Fig. 2A, the decrease of HDAC3 induced a clear reduction of cyclin A. Interestingly, this effect was highly specific since knocking down (KD) HDAC1 or HDAC2 with specific shRNAs did not modify cyclin A levels (Fig. 2, B and C). Because HDAC3 is involved in the regulation of transcription, we also analyzed the effects of knocking down HDAC3 on the level of cyclin A mRNA. As shown in Fig. 2D, the decrease of HDAC3 did not reduce cyclin A mRNA but, in contrast, it induced a significant increase of cyclin A mRNA. Thus, the decrease of cyclin A protein levels in HDAC3 knock-down cells cannot be attributed to a defect in cyclin A transcription.

We subsequently aimed to analyze whether HDAC3 was able to modify the acetylation status of cyclin A. Thus, HeLa cells overexpressing HA-cyclin A were transfected with Flag-HDAC3 or with an empty vector. Then, the levels of acetylated HA-cyclin A were analyzed by IP followed by WB with anti-acetyl lysine antibody. As shown in Fig. 2E, overexpression of...
HDAC3 reduced cyclin A acetylation. Moreover, knocking down HDAC3 in cells overexpressing HA-cyclin A resulted in a significant increase of acetylated cyclin A (Fig. 2F).

**HDAC3 Regulates Cyclin A Stability**—We studied whether the increased acetylation observed in HDAC3 knocked down (HDAC3-KD) cells induces cyclin A degradation via proteasome. To this purpose, cyclin A levels were determined by WB in HDAC3-KD cells in the presence or absence of the proteasome inhibitor ALLN. As shown in Fig. 3A, ALLN treatment inhibits cyclin A degradation in HDAC3-KD cells. We also determined the half-life of cyclin A in these cells. For these experiments HDAC3-KD cells were synchronized at G1/S, by a double thymidine blockade (because at this stage cyclin A is highly stable). Then, cells were released from the block, and cycloheximide was added to the culture. Finally, cells at different times after cycloheximide addition were collected and subjected to WB with anti-HDAC3, anti-cyclin A, and anti-actin, the latter used as a loading control. Results clearly revealed that HDAC3-KD cells presented a much more reduced cyclin A half-life (t1/2 = 4 h) than control cells (t1/2 > 6 h) (Fig. 3B).

We subsequently studied the effect of HDAC3 knockdown on the stability of a cyclin A mutant in which 4 lysines (K54, K68, K95, and K112) were substituted for arginines. It has been previously shown that this cyclin A mutant (cyclin A-4R) cannot be acetylated (26). Thus, HDAC3-KD cells were transfected with Flag-cyclin A WT, Flag-cyclin A 4R, or Flag-cyclin A 171–432 and subsequently synchronized at metaphase with nocodazole. Then, synchronized and asynchronously growing cells were analyzed by WB with anti-Flag. WB with anti-actin was used as a loading control.
determined. Results indicated that the half-life of cyclin A-4R is higher than cyclin A-WT (Fig. 3D).

Such type of experiments were also performed using a cyclin A lacking the first 171 aa (cyclin A 171–432). Similarly to that observed with cyclin A-4R, in HDAC3-KD cells the levels of cyclin A 171–432 were not reduced (Fig. 3C). It is known that cyclin A is degraded during mitosis, and that this degradation is necessary for triggering anaphase. Thus, we analyzed here the behavior of these two non-acetylatable mutants, cyclin A-4R and cyclin A 171–432 at mitosis. As shown in Fig. 3E both mutants were more stable than cyclin A-WT at this stage of the cell cycle.

**HDAC3 Is Degraded during Mitosis via Proteasome and Regulates Cell Cycle Progression**—To investigate the behavior of HDAC3 at different times of cell cycle progression cells were transfected with Flag-HDAC3 and HA-cyclin A and synchronized at different phases of the cell cycle. Then, the levels of both proteins were determined by WB. As shown in Fig. 4A, the amount of HDAC3 behaved quite similar to that of cyclin A at the different phases of the cell cycle: high at G1/S and G2/M and very low at metaphase. Fig. 4A also revealed that cyclin A and HDAC3 interacted at these two stages of the cell cycle but not at metaphase (probably due to the low levels of both proteins). Then, the activity of HDAC3 at G1/S and G2/M was determined in cells transfected with Flag-HDAC3 by IP with anti-Flag using acetylated histones as a substrate. Results revealed that HDAC3 activity is high at these two stages of the cell cycle (Fig. 4B).

To analyze whether HDAC3 degradation at metaphase was produced via proteasome, cells were transfected with Flag-HDAC3, and its levels analyzed in cells cultured in the presence or absence of the proteasome inhibitor ALLN for 16 h. Then, the levels of HDAC3, phosphorylated histone H3 and actin were determined by WB. D, HeLa cells were transfected with Flag-HDAC3 and treated with 20 μM roscovitine overnight. Then, the levels of Flag-HDAC3 were analyzed by WB in treated (ROS) versus untreated (C) cells. Actin was used as a loading control. E, HeLa cells were synchronized with nocodazol to obtain cells at metaphase. At the same time cells were treated with 5 mM NaF overnight or 20 μM OA for 3 h. Levels of endogenous HDAC3 and cyclin A were then determined by WB in treated versus untreated cells. Actin was used as a loading control. On the left, cyclin A levels in asynchronously growing cells can be observed.
FACS analysis. Quantification data indicated that at 14 h after release, a 20% of HDAC3-KD cells were at G2/M and an 18% at S phase. In contrast, in control cells these percentages were of only a 4.5 and 9%, respectively (Fig. 4F). These results indicate that HDAC3 regulates the progression of cells through G1/S.

**DISCUSSION**

Cyclin A degradation occurs at metaphase independently of the spindle checkpoint and this fact is essential for cdk1 inactivation and subsequently for mitosis exit. A recent report described that the signal triggering cyclin A destruction at that time of the cell cycle is its acetylation in at least 4 specific lysine residues (K54, K68, K95, and K112) (26). All these residues are located at the N-terminal region of cyclin A that includes the destruction box and the extended destruction box, both involved in its degradation. Cyclin A acetylation is carried out by PCAF but also by ATAC complexes that contain the PCAF homologue GCN5 (26, 28). Here we report that cyclin A stability during cell cycle progression is not only regulated by the acetylases PCAF/GCN5 but also by HDAC3 that temporally counteracts the effect of these acetylases.

We found that HDAC3 directly associates with the N-terminal region (aa 1–171) of cyclin A and that cyclin A is deacetylated by HDAC3. Our results also revealed that HDAC3 levels varied along the cell cycle in a similar manner than those of cyclin A: they were low at G1, then, increased at G1/S and remained high until mitosis when both proteins were degraded. Interestingly, HDAC3 associated with cyclin A during cell cycle follows a similar kinetics: their interaction was low at G1 and higher during G1/S, S and G2/M.

It is worth noting that cyclin A associates with PCAF and cdk2 during the same period of time (26, 35), suggesting the existence of putative protein complexes including these four proteins (cyclin A, cdk2, PCAF, and HDAC3) during G1/S, S and G2/M. Interestingly, it was reported that cyclin A acetylation was very low at G1 phase, slightly increased at S phase and subsequently was high at G2/M (26). Additionally, our results indicate that at G1/S and G2/M HDAC3 displays a significant deacetylase activity. Thus, altogether these results suggest that in this putative quaternary complex (cyclin A, cdk2, PCAF, and HDAC3) the activity of HDAC3 could counteract the PCAF induced acetylation of cyclin A during G1/S, S and G2/M. Moreover, the observation that cyclin A acetylation progressively increases at G2/M, despite that at this time the HDAC3 activity remained high, suggests that PCAF/GCN5 activity has to be progressively increased during this period of the cell cycle.

**FIGURE 5.** HDAC3 regulates cell cycle progression. A, HeLa cells were transfected with a shRNA control (shΦ) or with a specific shRNA against HDAC3 (shHDAC3). At 60 h post-transfection, levels of endogenous HDAC3 and cyclin A were determined by WB. WB anti-actin was used as a loading control. B, HeLa cells transfected with shΦ or shHDAC3 were subjected to fluorescence-activated cell sorting (FACS) analysis. Results were represented in a graph showing the number of cells in each cell cycle phase. C, HeLa cells were transfected with shΦ or shHDAC3. At 24 h post-transfection, cells were synchronized with a double thymidine blockade to obtain cells at G1/S transition. Then, cells were released from the blockade and at different times after the release cells were fixed, stained with propidium iodide, and analyzed by FACS. The percentage of cells in each cell cycle phase was plotted in a graph.

**FIGURE 6.** Cyclin A stability is regulated by acetylation. During G1 and S phases of the cell cycle there is a balance between acetylated and non-acetylated forms of cyclin A due to the opposing actions of PCAF and HDAC3. During this period of time, the non-acetylated form of cyclin A would be predominant, thus allowing its association with cdk2 that would be activated. Cells can then progress through S phase. At G2, the acetylated form of cyclin A would be predominant and this would lead to its ubiquitylation and degradation during mitosis.
increased acetylation of cyclin A would subsequently induce its ubiquitylation and the subsequent degradation via the ubiquitin/proteasome pathway (26).

The role of HDAC3 in this process is supported by a number of evidences reported here. We showed that knocking down HDAC3 clearly reduced the half-life of cyclin A and consequently cellular cyclin A levels were decreased, probably due to its increased acetylation. In contrast, the non-acetylatable mutant cyclin A-4R is much more stable in HDAC3-KD cells.

The observation that HDAC3 is degraded via proteasome during mitosis, just at the time of cyclin A destruction, is especially relevant because it suggests that HDAC3 dissociation from cyclin A could be necessary to proceed with cyclin A degradation. Despite a number of reports indicating that HDAC3 activity is regulated by different mechanisms as by interacting with SMRT/N-CoR (36), by phosphorylation and dephosphorylation by CK2 and PP4c (37) or by phosphorylation by DNA-PK (38), not much is known about the regulation of its stability. Our preliminary results showed that treatment of cells with the cdk inhibitor roscovitine decreased the amount of HDAC3, suggesting that cdk-dependent phosphorylation could stabilize HDAC3. However, the mechanisms participating in HDAC3 degradation at mitosis still remain to be elucidated.

Interestingly, it has been reported that the interaction of cyclin A with cdc20, essential for cyclin A destruction, is performed through the N-terminal domain of the protein (24). Moreover, it has been shown that cyclin A degradation is insensitive to the spindle checkpoint because cyclin A directly interacts with the N-terminal region of cyclin A with much higher affinity than the spindle checkpoint proteins BubR1 and Bub3 (24). Thus, all these observations suggest the possibility that HDAC3 binding to the N-terminal region of cyclin A could interfere with the association of cyclin A with cdc20. Thus, dissociation of HDAC3 from cyclin A or its degradation at mitosis would facilitate the interaction of cyclin A with cdc20 and subsequently its destruction.

Results reported here are compatible with those observed in HDAC3−/− MEFs showing a delay in cell cycle progression due to alterations in S phase progression and DNA damage (39). Under the light of our observations we can interpret that the absence of HDAC3 in MEFs must produce a decrease of cyclin A levels. Because of the fact that cyclin A is necessary for DNA replication, its reduction could be the responsible for the S phase delay observed in these cells.

In summary, our results reported here reveal that HDAC3 regulates the stability of cyclin A by modulating its acetylation status (Fig. 6). These results are in complete agreement with those previously reported demonstrating that cyclin A acetylation by PCAF/GCN5 at specific lysine residues targets it for degradation at mitosis (26, 28).

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