Histone Deacetylase 3 Regulates Cyclin A Stability

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ABSTRACT

PCAF and GCN5 acetylates cyclin A at specific lysine residues targeting it for degradation at mitosis. We report here that 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 and in thus targeting 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 G_2/M transition. In summary we propose here that HDAC3 regulates cyclin A stability by counteracting the action of the acetylases PCAF/GCN5.

INTRODUCTION

Cyclin A is the regulatory subunit of several members of the cyclin dependent kinase family (cdks) that play an important role during cell cycle progression. Specifically, cyclin A associates with and activates cdk2 thus driving S phase progression. Moreover, it also binds to and activates cdk1, a kinase necessary for G_2/M transition (1). The role of cyclin A-cdk complexes during cell cycle is to phosphorylate a plethora of substrates that include a significant number of transcription factors as for instance Sp1, NF-Y, FOXK2 and PR (2-5), transcriptional repressors as pRb and RBP1 (6) or proteins involved in epigenetic gene silencing as EZH2 (7). Thus cyclin A-cdk complexes plays a crucial role in the regulation of gene expression during cell cycle progression.

Cyclin A levels are low during G_1 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 G_2 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).

In order to proceed with metaphase anaphase transition, the inactivation of both, cyclin A-cdk1 and cyclin B-cdk1 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, in order 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 amino 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 behaviour 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-cdk complex must bind a Cks protein to be degraded at prometaphase. The cyclin A-cdk-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. The cyclin A associated with cdc20 it 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-cdk-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 paper validated this mechanism by showing that the ATAC acetyl transferase 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 deacetylates, 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 4-5 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 amino 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.

MATERIAL AND METHODS

Plasmids

HA-cyclin A, Flag-cyclin A-WT and -4R, GST-cyclin A-WT and YFP-PCAF were described elsewhere (26). GST-cyclin A 1-171, and GST-cyclin A 171-432 were described elsewhere (31). HDAC1-Flag, HDAC2-Flag, HDAC3-Flag and HDAC4-Flag were in pcDNA3. GST-HDAC1 51-482 and GST-HDAC2 were in pGEX. ShRNAs against HDAC1, HDAC2, HDAC3 and control shRNA were purchased from Sigma. pcDNA3 Flag-cyclin A 171-432 was sub-cloned from pGEX cyclin A 171-432 and pGEX HDAC3 was sub-cloned from pcDNA3 Flag-HDAC3.

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 Signalling. Anti-acetyl lysine antibody (401-939) was purchased from Rockland. Antibodies against Flag (F7425) and HA (H6908) were purchased from Sigma. Antibody against HDAC8 (3608) was purchased from BioVision. Monoclonal antibody against cyclin A (611268) was from Becton Dickinson. Monoclonal antibody against histones (MAB052) was from Millipore. For immunoprecipitation (IP) we used monoclonal anti-HA 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 growth in Dulbeccos'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 (32).

Protein purification, pull down and immunoprecipitation

Protein expression and purification was performed as described (31). For pull down experiments, GST, GST-cyclin A-WT, 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, pH8, 1 mM EDTA, 0.5% NP-40 and 100 mM NaCl). Beads were then incubated for 1 h at room temperature (RT) with GST-HDAC1 (51-482 aa), GST-HDAC2 or GST-HDAC3. Beads were washed with NETN containing 150 mM NaCl and the bound material was analysed by SDS-polyacrilamide gel electrophoresis followed by western blot (WB). For affinity chromatography experiments, proteins were eluted with 3 M KCl buffer or 200 mM glycine pH2.5. For IP, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1

mM Na_3V0_4, 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 4ºC. 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 cover slips, fixed in 4% paraformaldehyde/PBS for 15 min at RT, washed in PBS an blocked with 1% BSA, 0.1% Triton X-100 in PBS for 15 min at RT. Then, cover slips were incubated with anti-cyclin A (mouse monoclonal) and anti-HDAC1 (rabbit polyclonal) or anti-HDAC2 (rabbit polyclonal) or anti-HDAC3 (rabbit polyclonal) for 1 h at 37ºC. They were then washed in PBS and incubated for 45 min at 37ºC with Alexa-Fluor 594 (goat anti-mouse, dilution 1:500) and Alexa-Fluor 488 (goat anti-rabbit, dilution 1:500). After that, cover slips were washed, mounted on glass slides with Mowiol (Calbiochem) and analysed by confocal microscopy.

Flow cytometry analysis

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

Determination of HDAC3 activity

To analyze whether cyclin A is a direct substrate of HDAC3, we first obtained HDAC3 from HeLa cells previously transfected with Flag-HDAC3. It was purified from these cells with agarose beads coupled to anti-Flag antibodies and used as an enzyme source. As a substrate we used HA-cyclin A that was acetylated *in vivo*. To obtain acetylated HA-cyclin A, HeLa cells were transfected with HA-cyclin A and subsequently treated with TSA (3 µM) to inhibit cellular HDACs. Then, acetylated HA-cyclin A was purified with agarose beads coupled to anti-HA antibodies. Deacetylation reaction was started by mixing purified Flag-HDAC3 on 30µl of RIPA buffer with the acetylated cyclin A containing agarose-beads. Reaction tubes were incubated at 30ºC for 45 min. Beads were washed once with RIPA buffer, and then the bound proteins were eluted. Acetylation levels of eluted proteins were detected by WB with anti-acetyl lysine antibodies.

To determine HDAC3 activity at different stages of the cell cycle HeLa cells were firstly treated overnight with 3µM TSA in order 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 (32). To analyze HDAC3 activity at the different stages of the cell cycle, synchronized cell extracts were subjected to IP using anti-Flag. The immunoprecipitated was then mixed with 20 µg of cell lysates containing acetylated histones and then incubated at 30ºC for 30 min in 15

μl of HDAC buffer (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl and 1 mM $MgCl_2$). 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 HDACs, cells were transfected with HA-cyclin A or Flag-cyclin A, together with Flag-HDAC1, Flag-HDAC2 or Flag-HDAC3. Cell lysates were subsequently subjected to IP with anti-HA or anti-Flag and the immunoprecipitates analyzed by Western Blotting (WB) with anti-HA, anti-Flag or anti-HDAC8. Results showed that HDAC1, 2 and 3 interacted with cyclin A whereas in contrast HDAC8 did not (Figures 1A and 1B). Further, we studied the interaction of Flag-cyclin A with endogenous HDACs 4, 9 and 11, members of other HDAC families. In these experiments we observed that only HDAC4 interacted with cyclin A (Supplementary Figure S1). We subsequently studied by immunofluorescence the putative cellular co-localization of cyclin A with HDAC1, 2 or 3. As shown in Figure 1C all these three HDACs co-localized with cyclin A in the nucleus. In order 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 (Figure 1D). Because the cyclin A domain involved in its degradation is included in the first 171 aa of its sequence, we subsequently analyzed the direct interaction of this domain with HDAC1 and HDAC3 by pull down. As it can be observed in Figure 1E, 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. Thus, we studied by IP the association of endogenous cyclin A with HDAC3 in cells. Figure 1F shows that endogenous cyclin A and HDAC3 interacted in cells.

HDAC3 regulates the levels of cyclin A

The evidence showing that cyclin A directly interacts with HDAC3 through its 1-171aa domain, a region involved in cyclin A stability, aimed us to study the effect of knocking down HDAC3 on cyclin A levels. As observed in Figure 2A, cells transfected with specific shRNA against HDAC3 showed a reduced expression of this HDAC but also of cyclin A. Interestingly, this effect was highly specific since knocking down (KD) HDAC1 or HDAC2 did not modify cyclin A levels (Figures 2B and 2C).

HDAC3 regulates cyclin A acetylation

We subsequently aimed to analyze whether HDAC3 was able to modify the acetylation status of cyclin A. Thus, HeLa cells over-expressing HA-cyclin A and YFP-PCAF were subsequently 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 Figure 3A, over-expression of HDAC3 reduces cyclin A acetylation. Moreover, knocking down HDAC3 in cells over-expressing HA-cyclin A resulted in a significant increase of acetylated cyclin A (Figure 3B). Finally, we studied whether HDAC3 could directly deacetylate cyclin A. For this purpose,

cells transfected with Flag-HDAC3 were subjected to IP with anti-Flag in order to obtain active HDAC3. Then, this HDAC3 was incubated with acetylated cyclin A and the status of cyclin A acetylation was subsequently determined by WB with anti-acetyl lysine. As shown in Figure 3C, HDAC3 directly deacetylates cyclin A.

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, the levels of cyclin A were determined by WB in HDAC3 KD cells in the presence or absence of the proteasome inhibitor ALLN. As shown in Figure 4A, 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 G_1/S, by a double thymidine blockade, because at this stage cyclin A is highly stable. Then, cells were released 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 later being used as a loading control. Results clearly revealed that HDAC3 KD cells presented a much more reduced cyclin A half-life ($t1/2 \approx 4h$) than control cells ($t1/2 > 6h$) (Figure 4B). We subsequently studied the effect of HDAC3 knock down 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 or Flag-cyclin A-4R. Then, cyclin A levels were determined by WB. As shown in Figure 4C in HDAC3 KD cells the levels of cyclin A-WT were clearly reduced whereas those of the mutant cyclin A-4R were not. 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 (Figure 4C). 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 Figure 4D both mutants were more stable than cyclin A-WT at this stage of the cell cycle.

HDAC3 is degraded during mitosis via proteasome

To investigate the behavior of HDAC3 at different times of the 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 Figure 5A, the amount of HDAC3 behaved quite similarly to that of cyclin A at the different phases of the cell cycle: high at G_1/S and G_2/M and very low at metaphase. Figure 5A 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).

We subsequently analyzed the activity of HDAC3 at G_1/S and G_2/M. HDAC3 activity was determined in cells transfected with Flag-HDAC3 by IP with anti-Flag. The activity was

measured in the immunoprecipitates by using acetylated histones as a substrate. Interestingly, we observed that HDAC3 activity is high at these two stages of the cell cycle (Figure 5B).

To analyze whether HDAC3 degradation at metaphase was produced via proteasome, cells were transfected with Flag-HDAC3 and its levels analyzed in cell cultures in the presence or absence of the proteasome inhibitor ALLN. Figure 5C shows that at mitosis, cells treated with ALLN have higher levels of HDAC3 than untreated cells, suggesting that HDAC3 is degraded at mitosis via proteasome.

Preliminary results we have obtained, suggest that HDAC3 degradation at mitosis could be regulated by phosphorylation. Specifically, we have observed that the addition of a cyclin-cdk inhibitor (roscovitine) to the cell cultures decreased HDAC3 levels (Supplementary Figure S1). On the contrary, treatment of cells with two different phosphatase inhibitors namely okadaic acid (OA) or NaF increased the levels of HDAC3 (Supplementary Figure 2A). Nevertheless, to clarify the exact mechanism operating in the process of HDAC3 degradation at mitosis much work has to be performed.

HDAC3 regulates cell cycle progression at S and G_2/M transition

Taking into account that HDAC3 regulates cyclin A stability and that cyclin A degradation is essential for mitosis progression, we aimed to study the effect of HDAC3 knock down on cell cycle progression. Thus, HDAC3-KD cells that display reduced levels of cyclin A (Figure 6A) were transfected with shØ or shHDAC3 and subsequently subjected to FACS analysis Results revealed a clear accumulation of these cells at S and G_2/M (Figure 6B). We also studied the effect of HDAC3 decrease on cell cycle progression in synchronized cells. Thus, cells transfected with shØ or shHDAC3 were synchronized by a double thymidine block and subsequently released. Samples were collected at different times after release and subjected to FACS analysis. Quantification data indicated that at 14h after release, a 20% of HDAC3-KD cells were at G_2/M and an 18% at S phase. In contrast, in control cells these percentages were of only a 4.5% and 9%, respectively (Figure 6C).

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 directly 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 G_1, then, increased at G_1/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 G_1 and higher during G_1/S, S and G_2/M.

It is worth noting that cyclin A associates with PCAF and cdk2 during the same period of time (26,33), suggesting the existence of putative protein complexes including these four proteins (cyclin A, cdk2, PCAF and HDAC3) during G_1/S, S and G_2/M. Interestingly, it was reported that cyclin A acetylation was very low at G_1 phase, slightly increased at S phase and subsequently was high at G_2/M (26). Additionally, our results indicate that at G_1/S and G_2/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 G_1/S, S and G_2/M. Moreover, the observation that cyclin A acetylation progressively increases at G_2/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. The 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 levels of the non-acetylatable mutant cyclin A-4R did not decrease in HDAC3-KD cells.

The observation that HDAC3 is degraded via proteasome during mitosis, just at the time of cyclin A destruction, is specially 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 (34), by phosphorylation and dephosphorylation by CK2 and PP4c (35) or by

phosphorylation by DNA-PK (36), not much is known about the regulation of its stability. Although our preliminary results, showing that treatment of cells with the cdk inhibitor roscovitine decreased the amount of HDAC3, suggest that cdk phosphorylation could stabilize HDAC3, in fact 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 as 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 (37). Under the light of our observations we can interpret that the absence of HDAC3 in MEFs must produce a decrease of cyclin A levels. Due to 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. 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).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online:
Supplementary Figures S1 and S2

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LEGENDS TO THE FIGURES

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**) NIH 3T3 cells were transfected with Flag-cyclin A. Then, cell extracts were subjected to IP using anti-Flag or IgG that was used as a control. The immunoprecipitates were subjected to WB with anti-Flag or anti-HDAC8. A sample of cell lysate (input) was used as a control. (**C**) Endogenous cyclin A, HDAC1, HDAC2 and HDAC3 were visualized by immunofluorecence as described under material and methods. (**D**) 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. (**E**) 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. (**F**) Extracts from HeLa cells were subjected to IP with anti-HDAC3 or with IgG as a control. Then, the presence of endogenous cyclin A and HDAC3 in the immunoprecipitates were detected by WB. A sample of cell lysate (input) is shown on the left.

Figure 2. HDAC3 regulates the levels of cyclin A. (**A**) HeLa cells were transfected with a control shRNA (shØ) or with a specific shRNA for HDAC3 (shHDAC3). Then, the levels of HDAC3 and cyclin A were determined by WB (left panel). The amount of HDAC3 and cyclin A was quantified and represented in a graph. Results are the mean ± SD of three independent experiments. ** p>0.01; *** p>0.001 (**B**) Experiments similar to (A) were performed using shRNA against HDAC1 (**C**) Experiments similar to (A) were performed using shRNA against HDAC2.

Figure 3. HDAC3 deacetylates cyclin A. (**A**) HA-cyclin A and YFP-PCAF expression vectors were transfected with or without a vector expressing Flag-HDAC3 on HeLa cells. Then, cell extracts were subjected to IP with anti-HA. Total cyclin A and acetylated cyclin A in the immunoprecipitates were detected by WB with anti-HA or anti-acetyl lysine, respectively. WB performed on samples from cell lysates (input) were shown on the left. (**B**) HeLa cells were non-transfected (Ø), transfected with a control shRNA (shØ) or with a specific shRNA for HDAC3 (shHDAC3). 24h later, cells were additionally transfected with HA-cyclin A. Then, cell extracts were subjected to IP with anti-HA. Total cyclin A and acetylated cyclin A in the immunoprecipitates were detected by WB with anti-HA or anti-acetyl lysine, respectively. WB performed on samples from cell lysates (input) were shown on the left. (**C**) Flag-HDAC3 was obtained by IP from Flag-HDAC3 transfected HeLa cells as described under material and methods and acetylated HA-cyclin A was obtained from HA-cyclin A transfected HeLa cells also

as described under material and methods. Then, acetylated HA-cyclin A and Flag-HDAC3 were mixed an incubated for 45 min. After that, acetylated HA-cyclin A was determined by WB with anti-acetyl lysine antibodies.

Figure 4. HDAC3 regulates cyclin A stability. (**A**) HeLa cells were transfected with a shRNA control (shØ) or with a specific shRNA against HDAC3 (shHDAC3). At 48h post- transfection cells were treated with ALLN (100 μM) for 16h. Untreated cells were used as a control. Then, cyclin A levels were determined by WB. Actin was used as a loading control. (**B**) HeLa cells were transfected with shHDAC3 or shØ. At 24h post-transfection cells were synchronized with a double thymidine blockade to obtain cells at G_1/S transition of cell cycle. At this moment cells were released from thymidine blockade and cycloheximide (CHX) (10 μg/ml) was added to the cell culture. Samples were collected at different times after CHX treatment and cyclin A and HDAC3 levels were then determined by WB. WB with anti-actin was used as a loading control (upper panels). Cyclin A levels were quantified and represented in a graph (bottom panel). Results are the mean ± SD of three independent experiments. (**C**) HeLa cells were transfected with shHDAC3 or shØ. 24h later, cells were additionally transfected with Flag-cyclin A WT, Flag-cyclin A 4R or Flag-cyclin A 171-432. Then, the amount of the different forms of cyclin A and that of HDAC3 were determined by WB. WB anti-actin was used as a loading control. (**D**) HeLa cells were transfected with the mentioned vectors and subsequently synchronized at metaphase with nocodazole. Then, synchronized and asynchronously growing cells were analysed by WB with anti-Flag. WB with anti-actin was used as a loading control.

Figure 5. HDAC3 interacts with cyclin A at G_1/S and G_2/M phases of the cell cycle and is degraded at metaphase. (**A**) HeLa cells were transfected with HA-cyclin A and Flag-HDAC3.Then, cells were synchronized at different stages of the cell cycle as described under material and methods section and levels of HDAC3 and cyclin A were determined by WB (left panel). Cell extracts were subjected to IP with anti-Flag and the amount of HDAC3 and cyclin A in the immunoprecipitates was determined by WB. (**B**) HeLa cells were transfected with Flag-HDAC3 and subsequently synchronized at G_1/S and G_2/M as described under material and methods. Then, the levels of Flag-HDAC3 in asynchronously growing and synchronized cells were determined by WB with anti-Flag (left panel). Cell extracts were subjected to IP with anti-Flag or IgG used as a control. The immunoprecipitates were used as a source of HDAC3 and were subsequently incubated for 30 min with acetylated histones that were obtained as described under material and methods. After that, the total levels of histone H4 and the levels of acetylated histone H4 were determined with anti-histones and anti-acetyl lysine respectively. (**C**) HeLa cells were transfected with Flag-HDAC3 and subsequently synchronized at metaphase as described under material and methods. Asynchronously growing and synchronized cells were incubated in the presence or absence of the proteasome inhibitor ALLN for 16 h. After that, the levels of HDAC3, phosphorylated histone H3 and actin were determined by WB.

Figure 6. HDAC3 regulates cell cycle progression. (**A**) HeLa cells were transfected with a shRNA control (shØ) or with a specific shRNA against HDAC3 (shHDAC3). At 60h 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 shHDAC3 or shØ 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 shHDAC3 or shØ. At 24h-post transfection cells were synchronized with a double thymidine blockade to obtain cells at G_1/S transition. Then, cells were released from the blockade and at different times after the release cells were fixed, stained with propidium iodide and analysed by FACS. The number of cells in each cell cycle phase was plotted in a graph.

Figure 1

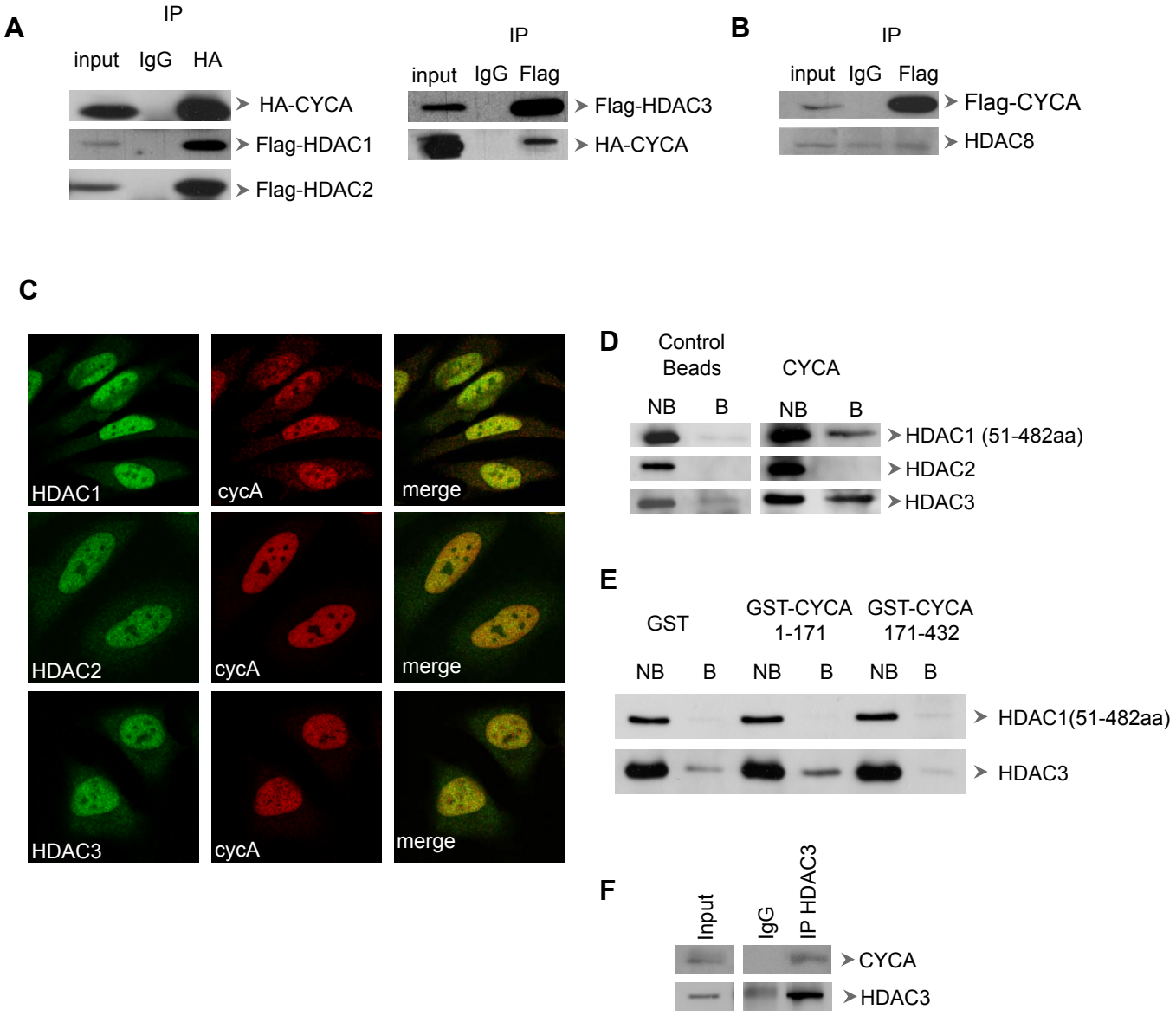
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Figure 2

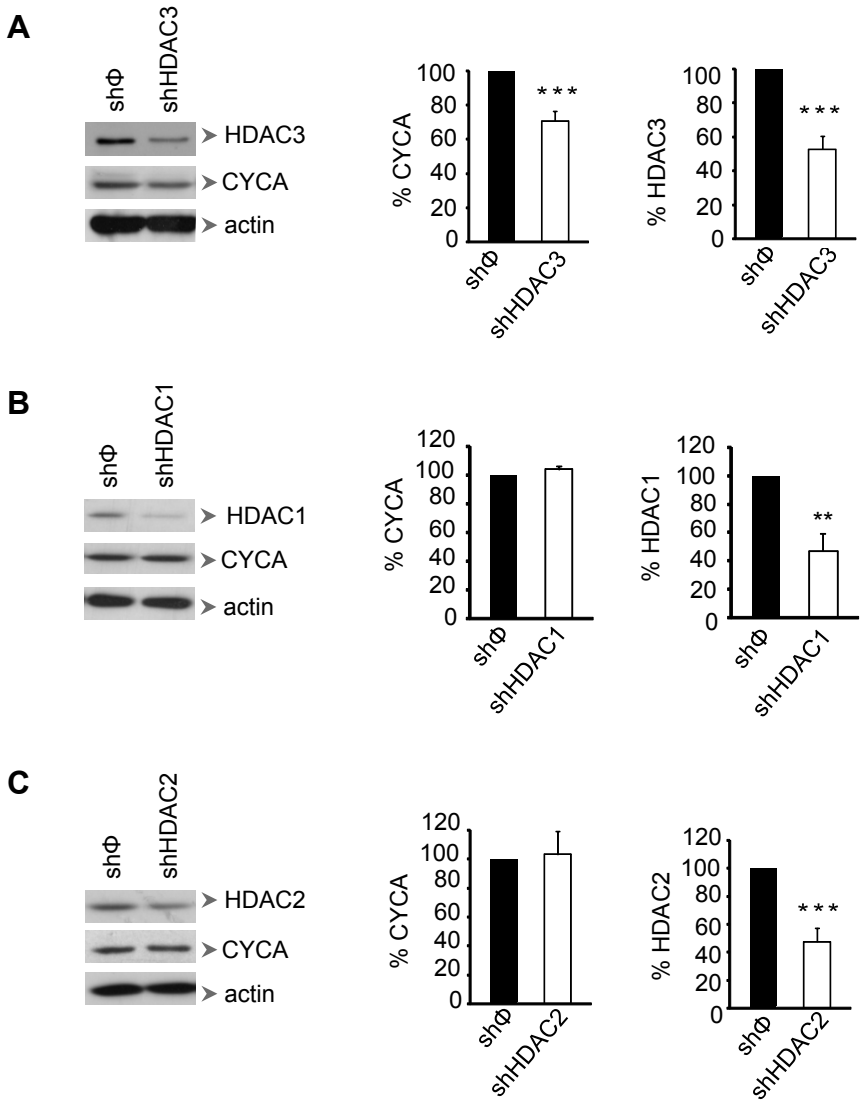
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Figure 3

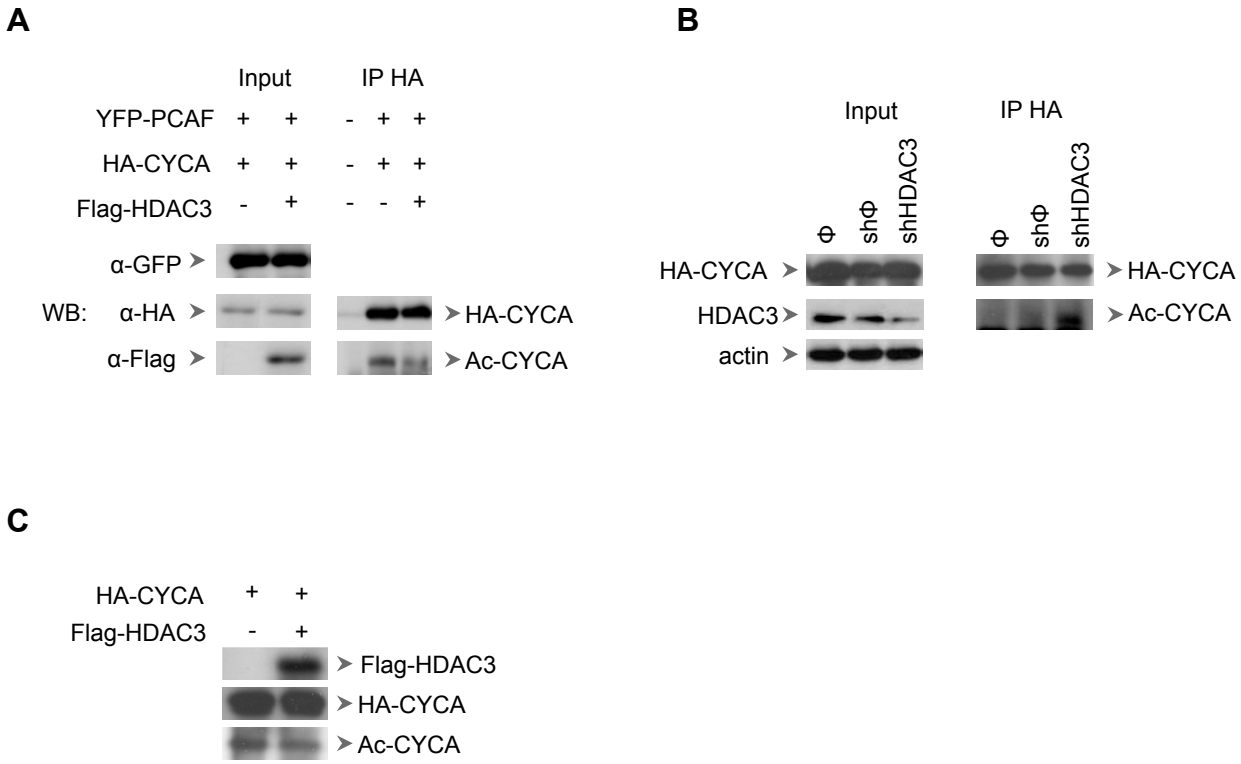
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Figure 4

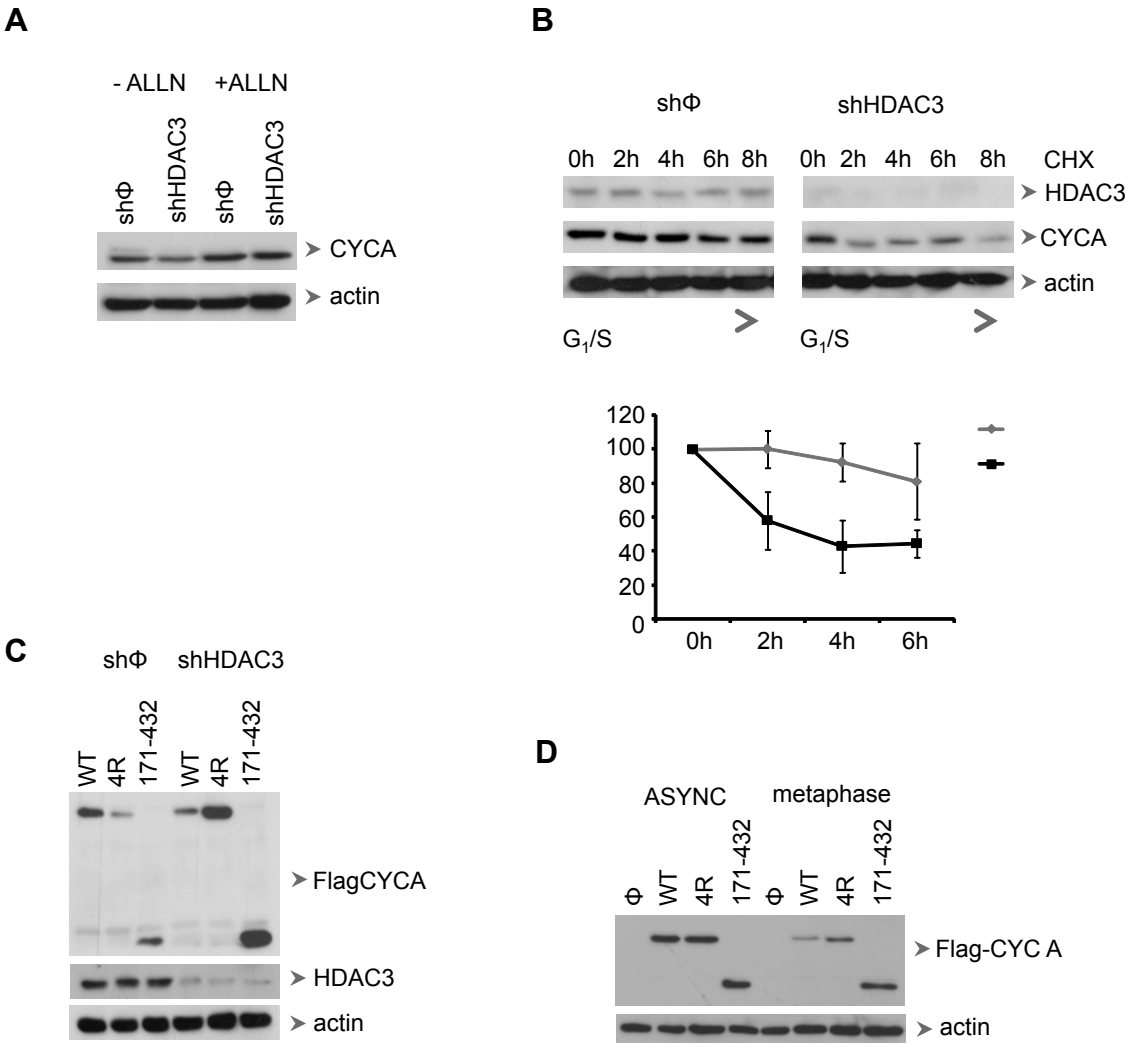
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Figure 5

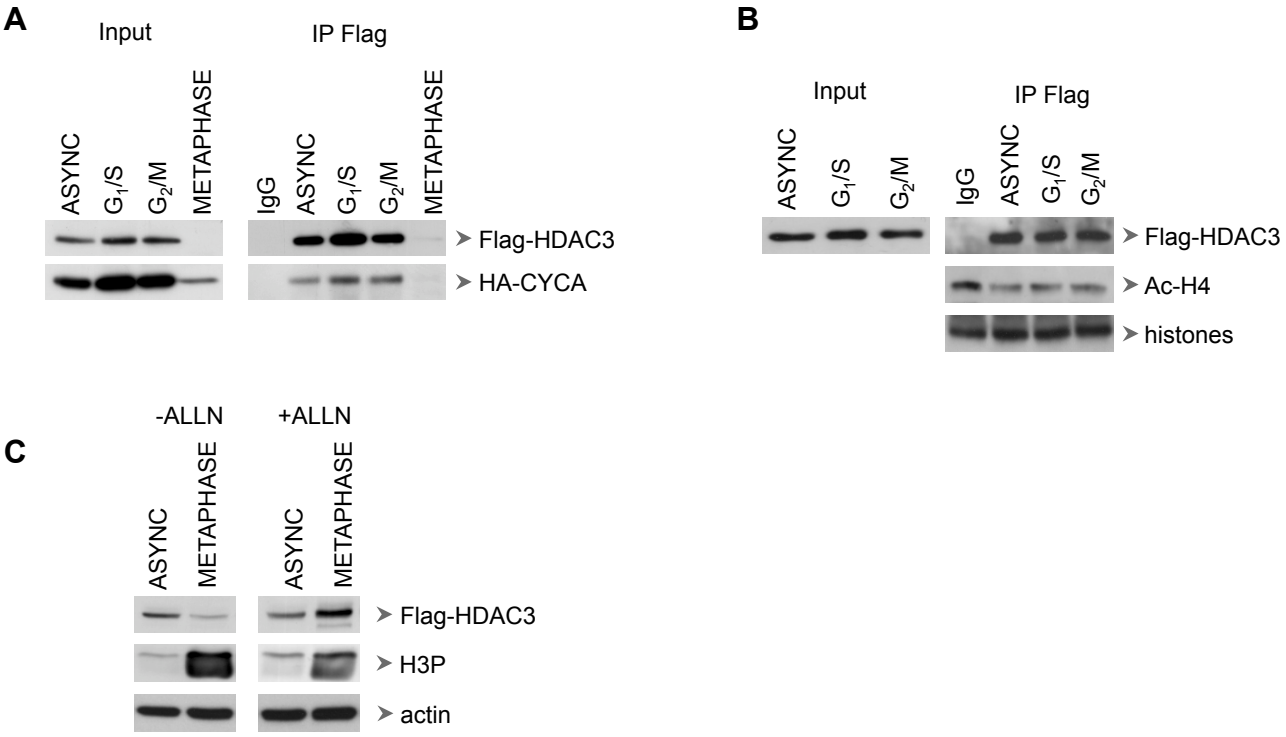
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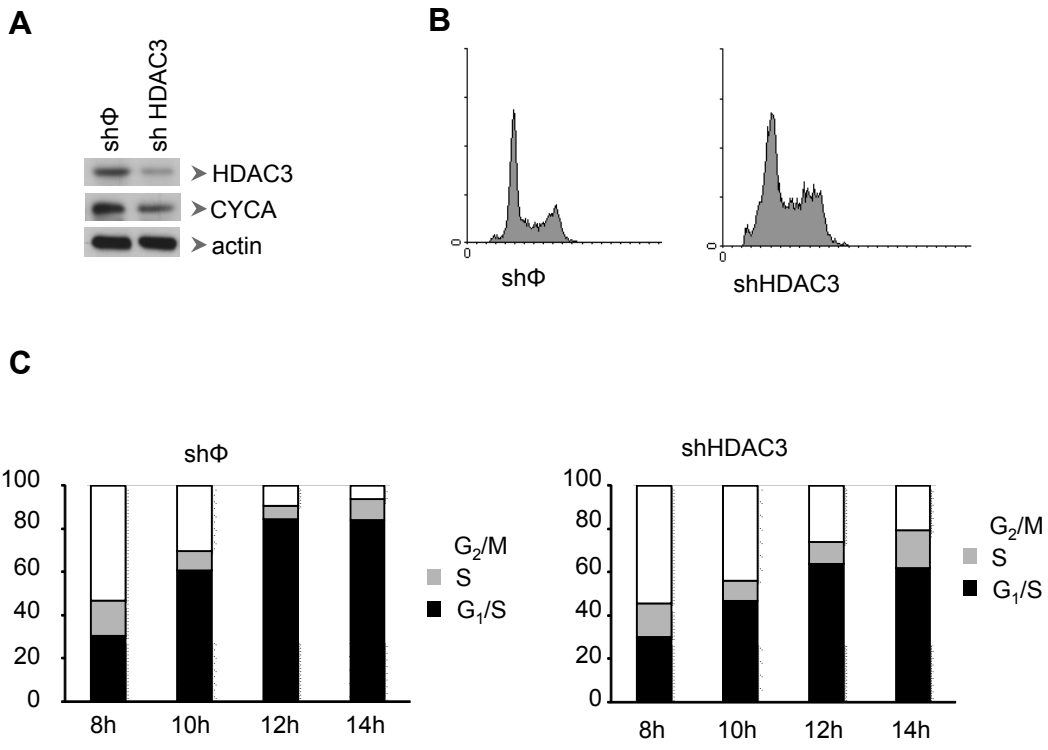
Figure 6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