AUTOACETYLATION REGULATES P/CAF NUCLEAR LOCALIZATION

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Abbreviations: HAT, histone acetyltransferase; HDAC, histone deacetylase; CBP, CREB binding protein; LMB, leptomycin B; TSA, trichostatin A; NES, nuclear export signal; NLS, nuclear localization signal; GST, glutathione S-transferase; GFP, green fluorescent protein; DAPI, 4',6-diamidino-2-phenylindole; FL, full length.

Running title: Regulation of P/CAF nuclear localization
SUMMARY

Acetylation is a posttranslational modification that alters the biological activities of proteins by affecting their association with other proteins or DNA, their catalytic activities, or their subcellular distribution. The acetyltransferase P/CAF is autoacetylated and acetylated by p300 in vivo. P/CAF autoacetylation is an intramolecular or intermolecular event. Intramolecular acetylation targets five lysines within the nuclear localization signal at the P/CAF C terminus. We analyzed how the subcellular distribution of P/CAF is regulated by intramolecular autoacetylation, and found that a P/CAF mutant lacking histone acetyltransferase activity accumulated primarily in the cytoplasm. This cytoplasmic fraction of P/CAF is enriched for nonautoacetylated P/CAF. In addition, P/CAF deacetylation by HDAC3 and in minor degree by HDAC1, HDAC2 or HDAC4, leads to cytoplasmic accumulation of P/CAF. Importantly, our data show that P/CAF accumulates in the cytoplasm during apoptosis. These results reveal the molecular mechanism of autoacetylation control of P/CAF nuclear translocation, and suggest a novel pathway by which P/CAF activity is controlled in vivo.

INTRODUCTION

p300/CBP-associated factor (P/CAF, also known as PCAF) was initially identified as a CBP/p300 binding protein, due to its sequence similarity to the yeast histone acetyltransferase (HAT) GCN5 (yGCN5) (1). Both P/CAF and GCN5 belong to the GCN5 family of HATs, and show sequence conservation in the regions responsible for HAT activity, the bromodomain and the ADA2 region (responsible for binding the yADA2 cofactor) (2,3). In addition, P/CAF activates transcription; to do that it requires its intrinsic HAT activity (4,5).

P/CAF is found in a complex comprising more than 20 polypeptides. Some of these polypeptides are similar to TATA box binding protein (TBP)-associated factors (TAFS), while others contain a histone fold domain (6). P/CAF has an intrinsic ability to acetylate nucleosomal histones in vitro and in vivo, and also acetylates nonhistone proteins such as p53 (7), E2F (8,9), YY1 (10), NFκB (11), SREBP (12), Ku70 (13), and Smad7, 2 and 3 (14,15). Acetylation affects the in vivo functions of these proteins. Moreover, P/CAF exists as an acetylated protein in vivo. P/CAF is autoacetylated and acetylated by p300, but not by CBP. P/CAF autoacetylation takes place via intramolecular and intermolecular mechanisms (16). Intramolecular acetylation targets five lysines in the nuclear localization signal (NLS) at amino acids 416–442 of the C terminus. Autoacetylation leads to an increase in P/CAF HAT activity in vitro (16). Furthermore, P/CAF physically associates with HDAC1, SIRT1, and HDAC3 histone deacetylases (HDACs) (17-19). HDAC3 deacetylates P/CAF (17). This suggests that multiple HDACs target P/CAF and other factors that are not histones (17,19-22). In addition to its acetyltransferase activity, P/CAF has intrinsic ubiquitination activity, which is important in controlling Hdm2 protein levels (23).

P/CAF may have a role in DNA damage response and apoptosis due to its ability to acetylate p53, E2F1, and Ku70 (8,9,13,24-27). Moreover, specific modifications of chromatin are likely to play an essential role in apoptosis induction. In support of this role, treatment with HDAC inhibitors induces apoptosis (28). Recent data have shown that during apoptosis, global changes in histone modifications occur (29-34), including histone acetylation (34,35). At the same time, the activity of many HAT and HDAC enzymes is affected during apoptosis. For example, HAT CBP/p300 is cleaved during apoptosis in the central nervous system (36). More recently, it was shown that HDAC-4 and HDAC-3 nuclear–cytoplasmic shuttling promotes apoptosis (35,37).

Although P/CAF is a nuclear protein, its nuclear localization was reported to change in response to cellular or extracellular signals (17,38). However, the molecular mechanisms governing this transition were not known. Here, we report that P/CAF localization is regulated by intramolecular autoacetylation.

EXPERIMENTAL PROCEDURES
**Cell culture and transfections**

NIH3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), supplemented with 10% calf serum (CS, Invitrogen). HeLa, CV1COS, HTC119, and 293T cells were grown in DMEM supplemented with 10% fetal calf serum (FCS, Invitrogen). Transient transfections were performed either by standard calcium phosphate coprecipitation with 3 µg of each expression vector, or with lipofectamine reagent (Invitrogen). Transfected cells were washed after 14 h and harvested 24 h after transfection. Cells were then treated with either a HDAC inhibitor, trichostatin A (TSA) (0, 100, or 150 mM for 12 h), an inhibitor of nuclear export, leptomycin B (LMB) (20 nM added to the culture medium for 2–8 h), a proteasome inhibitor, MG132 (10 µM for 12 h), or dexamethasone 2 µM, followed by collection and analysis.

**Plasmids and recombinant proteins**

P/CAF(352-832) and P/CAF(352-832)ΔHAT were cloned from PCX-GFP-P/CAF(FL)-Flag and PCX-GFP-P/CAFΔHAT-Flag (16) by PCR into the pcDNA3 vector (Invitrogen). A mutation was introduced at leucine 606 in P/CAF cDNA (Stratagene) and following the manufacturer’s instructions. The mutation was verified by DNA sequencing. pcDNA3-HDAC1-Flag, pcDNA3-HDAC4 and pcDNA3-HDAC2 were previously described (39). pcDNA3-Flag-HDAC3 and pSuper-shRNAHDAC3 were a gift from Dr. X.-J. Yang, and pcDNA3-Sirt1 and pcDNA3-GFP-Sirt2 were gifts from Dr. M. Gorospe and Drs. Y. Yoneda and T. Sekimoto, respectively.

**Indirect immunofluorescence**

Cells on coverslips were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized with methanol for 10 min. After blocking with 3% BSA in PBS and 0.1% Tween 20 for 1 h at room temperature, coverslips were incubated with a 1:200-500 anti-P/CAP (Upstate Biotechnology), 1:500 anti-acetylated histone H3 (Upstate Biotechnology), and/or 1:100 anti-Flag (Upstate Biotechnology) antibodies in PBS and 3% BSA for 2 h. This was followed by incubation for 1 hr with goat anti-rabbit IgG, used at a 1:250 dilution in PBS and 3% BSA. After each antibody incubation, the coverslips were washed four times for 10 min each time with PBS and 0.05% Tween 20 at room temperature. Cells fixed in 4% paraformaldehyde in PBS for 30 min at room temperature were incubated with fluorescent Annexin V (Roche) for 2 h. The nuclei were stained with DAPI. To distinguish endogenous and ectopic, transfected P/CAP immunofluorescence signals different laser intensities were used at the confocal microscope. In addition, transfected cells were followed by GFP coexpression (see Supplementary Figure 1).

**Cell extract preparation and immunoblotting and immunoprecipitation assays**

Total cell extracts were prepared in IPH buffer (40) by keeping the cells on ice for 20 min or for the indicated time, followed by centrifugation at 12 000xg for 10 min at 4 °C. All buffers contained protease inhibitors (Boehringer). Immunoblotting was performed using standard procedures and visualized using an ECL kit (Amersham). The antibodies were acetyl-lys antibody (Chemicon) used at a 1:1,000 dilution, anti-P/CAP antibody (Santa Cruz Biotechnology) used at a 1:1,000 dilution, and anti-Flag M2 antibody (Upstate Biotechnology) used at a 1:100. Immunoprecipitation analyses were performed as described elsewhere (41), except for the in vivo acetylation detection and IP-HAT assays, in which the IPH buffer was changed for RIPA buffer.

**HAT and AT assays**

In vitro HAT and AT assays were performed as described elsewhere (4,8). P/CAP autoacetylation was performed under the same conditions than HAT assays, but in the absence of histones (16).

**In vitro translation, recombinant proteins, and pull-down assay**

In vitro translations and GST pull-downs were performed essentially as described previously (41). GST and GST fusion proteins were expressed in Escherichia coli XA90 using the pGEX (Pharmacia) vector system for 4 h at 30 °C after 0.1mM IPTG was added. Purification from crude bacterial lysates was performed as in (41). The buffer used for the pull-downs was a variation of Z’ (25 mM HEPES pH 7.5, 12.5 mM MgCl2, 20% glycerol, 0.1% NP40, and 250 mM KCl).
RESULTS

P/CAF lacking HAT activity localizes to the cytoplasm

Previous work has shown that amino acids 428–442 of P/CAF contain the NLS. Mutations of lysines 428, 430, 441, and 442 to arginines leads to P/CAF cytoplasmic accumulation (16). These lysines are the targets of intramolecular autoacetylation (16). However, mutations of the NLS lysines are not direct proof of the autoacetylation requirement, as the lysines themselves are essential for NLS function. Here, we investigated the role of P/CAF intramolecular autoacetylation on P/CAF subcellular distribution using a HAT-domain mutant. P/CAF (352-832)ΔHAT cannot be autoacetylated (Figure 1A and 1C), because it lacks the N-terminal part of the protein, the region required for intermolecular autoacetylation (16) and HAT catalytic activity (Figure 1B). HAT catalytic activity is required for intramolecular autoacetylation (Figure 1C). However, P/CAF (352-832)ΔHAT retains the NLS (Figure 1A).

First, we analyzed the cellular localization of P/CAF(352-832) and P/CAF(352-832)ΔHAT. To do this, we transfected CV1COS cells with either P/CAF(352-832) or P/CAF(352-832)ΔHAT. The cellular distribution of P/CAF was analyzed by immunostaining using an antibody that specifically recognizes P/CAF. P/CAF(352-832) signal was found exclusively in the nucleus, whereas P/CAF(352-832)ΔHAT was located predominantly in the cytoplasm (Figure 1D).

To confirm these results, we constructed a P/CAF HAT mutant (L606A) (Figure 1A) that had only a limited ability to acetylate histones and it is not able to autoacetylate (Figure 1B and 1C). We then tested the cellular localization of this mutant. CV1COS cells were transfected either with P/CAF(352-832) or P/CAF(352-832)ΔHAT. The cellular distribution of P/CAF was analyzed by immunostaining using an antibody that specifically recognizes P/CAF. P/CAF(352-832) signal was found exclusively in the nucleus, whereas P/CAF(352-832)ΔHAT was located predominantly in the cytoplasm (Figure 1D).

To determine whether this result was specific to the CV1COS cell line used in these experiments, we examined the subcellular localization of P/CAF(352-832) and P/CAF(352-832)ΔHAT in different cell lines. P/CAF(352-832) was found mainly in the nuclei in all analyzed cell types (Table 1). In contrast, varying levels of the P/CAFΔHAT mutant were distributed between the nucleus and the cytoplasm (Table 1). Taken together, these results suggest that P/CAF requires HAT activity and the NLS to localize in the nucleus.

We next tested whether intramolecular autoacetylation might regulate the localization of full-length P/CAF. To do this, we transfected CV1COS cells either with P/CAF full length (FL) or with P/CAFΔHAT-FL and revealed their localization by immunostaining using an antibody that specifically recognizes P/CAF. As expected, P/CAF-FL was mainly nuclear (68% of the analyzed cells) and P/CAFΔHAT-FL partially cytoplasmic (57% of the analyzed cells were cytoplasmic, 28% were distributed between nucleus and cytoplasm and 15% were nuclear) (Figure 1E). We next examined the subcellular localization of P/CAF-FL and P/CAFΔHAT-FL in different cell lines (Hela, NIH3T3 and 293T). P/CAF-FL was found mainly in the nuclei in all analyzed cell types. In contrast P/CAFΔHAT-FL was distributed between the nucleus and the cytoplasm at similar levels than P/CAF(352-832)ΔHAT (data not shown). These results indicate that P/CAF autoacetylation might be involved in its cellular distribution. We performed some experiments using P/CAF(352-832)ΔHAT or P/CAFΔHAT-FL fused to GFP and Flag epitopes at the N-terminus of the protein. Surprisingly, in these cases, P/CAF(352-832)ΔHAT was enriched in the nucleus (data not shown), probably due to (i) a conformational change in P/CAF protein induced by the epitopes or (ii) a change in the accessibility of the P/CAF NLS in the fusion proteins.

P/CAF nuclear localization correlates with autoacetylation

As HAT activity is required for P/CAF nuclear accumulation, we next sought to confirm that P/CAF nuclear localization correlates with autoacetylation. To that end, we transfected P/CAF(352-832), P/CAF(352-832)ΔHAT, and P/CAF(352-832)L606A into CV1COS cells. The transfected proteins were recovered by immunoprecipitation and their acetylation status
was analyzed by immunoblot using an antibody that specifically recognizes acetylated lysines. Only nuclear P/CAF was acetylated in vivo (Figure 2A, lane 1). In contrast, cytoplasmic P/CAFs lacking HAT activity were not acetylated in vivo (Figure 2A, lanes 2 and 3).

We then treated P/CAF(352-832) and P/CAF(352-832)ΔHAT-transfected cells with varying amounts of the HDAC inhibitor TSA. Subsequently, the P/CAF location was analyzed by immunostaining. As expected, TSA did not modify the P/CAF cellular distribution (Figure 2B), probably because cytoplasmic P/CAF(352-832)ΔHAT is a nonacetylated protein, and therefore is not affected by changes in HDAC activity.

Proteasome inhibition partially reverses P/CAF cytoplasmic accumulation

Once we had confirmed that P/CAF acetylation correlates with localization to the nucleus, we sought to understand the molecular mechanisms responsible for this cellular distribution.

Autoacetylation could affect the localization of P/CAF(352-832) by affecting nuclear import or nuclear export. The recognition of NLS-containing proteins in the cytoplasm is mediated by the heterodimeric importin-α/β receptor, in which the importin α subunit is the saturable component of the receptor, and the importin β targets the NLS-containing protein in the nuclear pore complex (42,43). Thus, we asked whether P/CAF(352-832) directly bound importin α or β, and whether autoacetylation affected this interaction in vitro. As shown in Figure 3A, GST-P/CAF(352-832) interacted with importin-α1 in an in vitro pull down assay. Moreover, P/CAF(352-832) bound to GST-importin-β in vitro (Figure 3B). In addition, P/CAF(352-832) autoacetylation decreased these interactions (Figure 3A, lanes 3 and 4; Figure 3B, lane 3). To confirm these results, we transfected CV1COS cells with P/CAF or P/CAFΔHAT, prepared whole cell extracts from the transfected cells, and used them for pull-down experiments with GST, GST-importin α1, and GST-importin β columns. Both P/CAF and P/CAFΔHAT interacted efficiently with importin α1 and GST-importin β (Figure 3C). These results suggest that nuclear import was not the cause of the observed P/CAF cellular distribution, probably because cytoplasmic P/CAF, which recognized the heterodimeric importin-α/β receptor, was always nonacetylated, as we showed in Figure 2.

To gain further insight into how P/CAF autoacetylation regulates subcellular distribution, we analyzed whether nuclear export is affected by autoacetylation. To do this, we transfected CV1COS cells with P/CAF(352-832), P/CAF(352-832)ΔHAT, or GFP-Sirt2 (as a control) and treated them with leptomycin B (LMB), which impairs CRM1-dependent nuclear export. P/CAF localization was determined by immunostaining with an antibody that specifically recognizes P/CAF. Blocking nuclear export did not affect either P/CAF(352-832) (nuclear localization) or P/CAF(352-832)ΔHAT (cytoplasmic localization), but nuclear export of GFP-Sirt2 was affected [as described in (44)] (Figure 4A).

The above results suggest that cytoplasmic P/CAF is efficiently imported into the nucleus. What happens to nonacetylated nuclear P/CAF? The results shown in Figure 4A suggest that is not actively exported to the cytoplasm. Thus, nuclear nonacetylated protein may be degraded by proteolytic processing. We therefore tested whether the proteasome inhibitor MG132 could reverse the cytoplasmic localization of P/CAFΔHAT. To this end, we transfected CV1COS cells with P/CAF(352-832) or P/CAF(352-832)ΔHAT, and then treated them with MG132, which blocks proteasome-mediated degradation. The P/CAF localization was determined by immunostaining with an antibody that specifically recognizes P/CAF. Blocking protein degradation induced the accumulation of P/CAF in PML bodies (data not shown). However, it did not significantly alter the nuclear/cytoplasmic distribution of P/CAF (Figure 4B). In contrast, MG132 treatment allowed a partial accumulation of P/CAF(352-832)ΔHAT in the nucleus; the protein was distributed between the nucleus and cytoplasm in 70% of the cells (Figure 4B). Under these conditions, nonautoacetylated P/CAF also accumulated in the PML and in other cell bodies (data not shown).

P/CAF deacetylation by HDAC3 promotes P/CAF cytoplasmic accumulation
Our results thus far suggested that autoacetylation was important in promoting the nuclear localization of P/CAF. On the other hand, it had previously been shown that some HDACs bind P/CAF (17-19), and that HDAC3 inhibits P/CAF autoacetylation (17). Therefore, we tested whether deacetylation of P/CAF by HDAC1, HDAC2, HDAC3, HDAC4 or SIRT1 affected P/CAF nuclear localization. To do this, we transfected CV1COS cells with P/CAF(352-832) in the presence or absence of coexpressed Flag-HDAC1, HDAC2, Flag-HDAC3, HDAC4 or SIRT1. The cellular localization of P/CAF was then analyzed by immunostaining with antibodies specific to P/CAF. Although HDACs 1, 2, and 4 caused P/CAF localization to switch from primarily nuclear to both nuclear and cytoplasmic, only coexpression of HDAC3 efficiently promoted the cytoplasmic localization of P/CAF (Figure 5A). The specific effect of HDAC3 was confirmed by using shRNA which partially blocks the expression of HDAC3 (shRNA HDAC3) (Fig 5B) and control shRNA (shRNA C). The results in Fig. 5B show that in the presence of low levels of HDAC3, P/CAF cytoplasmic accumulation decreases. These results were consistent with our idea that intramolecular acetylation is required for P/CAF nuclear localization. This transition correlated with P/CAF deacetylation (Figure 5C, lane 2). To further confirm that the observed effect on P/CAF localization was due to deacetylation, we added the HDAC inhibitor TSA to the transfected cells 10 h before collection. We then analyzed the P/CAF localization. HDAC3 in the presence of TSA could not promote the P/CAF cytoplasmic transition (Figure 5D). This correlated with high levels of P/CAF autoacetylation in vivo (Figure 5D, lane 5). Finally, we sought to establish whether cytoplasmic P/CAF was active. To that end, we transfected CV1COS cells with P/CAF(352-832) in the presence or absence of coexpressed Flag-HDAC3. Then, we immunoprecipitated P/CAF and determined the HAT activity associated with the immunopellets in an in vitro HAT assay. The results show that both nuclear and cytoplasmic P/CAF were capable of acetylating histones, although a slight decrease in HAT activity was observed in cytoplasmic nonautoacetylated P/CAF, (Figure 5E) in agreement with previous results (16). We next tested whether HDAC3 expression might promote cytoplasmic accumulation of full-length P/CAF. To do this, we transfected CV1COS cells with P/CAF-FL and HDAC3 and revealed their localization by immunostaining using an antibody that specifically recognizes P/CAF and Flag epitope. As expected, coexpression of HDAC3 promoted the cytoplasmic or nuclear and cytoplasmic localization of P/CAF (64% of the analyzed cells) (Figure 5F). On the other hand, HDAC3 in the presence of TSA could not induce P/CAF cytoplasmic accumulation (Figure 5F).

Of the assayed HDACs (HDAC1, HDAC2, HDAC3, HDAC4 and SIRT1), HDAC3 was the enzyme that altered more efficiently P/CAF cellular localization (Figure 5A). It had been shown previously that HDAC3 binds P/CAF in C2C12 cells (17). We sought to establish whether the P/CAF–HDAC3 interaction also took place under our experimental conditions. Thus, we transfected CV1COS cells with P/CAF(352-832) and/or Flag-HDAC3 DNA plasmids and analyzed the localization of P/CAF and HDAC3 by immunostaining with antibodies against P/CAF and the Flag epitope. The proteins colocalized when they were expressed together (Figure 6A). To confirm this result, we immunoprecipitated Flag-HDAC3 and analyzed for the presence of P/CAF in the immunopellet by immunoblot. The results indicate that under our experimental conditions, P/CAF and HDAC3 interacted in vivo (Figure 6B, lane 3). The same results were obtained using P/CAF-FL (data not shown).

### Apoptosis induces P/CAF cytoplasmic accumulation

Although P/CAF is known to be a nuclear protein (there are, however, a few examples of P/CAF cytoplasmic localization) [see (17,38)], we investigated whether endogenous P/CAF would localize to the cytoplasm under different conditions. It has been proposed that P/CAF may have a role in apoptosis and DNA damage response due to its ability to acetylate p53, E2F1, and Ku70 (9,13,24-27). Thus, we investigated whether P/CAF could localize in the cytoplasm during apoptosis. To do this, we UV irradiated CV1COS cells. We then determined endogenous P/CAF localization by immunostaining with an antibody that specifically recognizes P/CAF. Apoptosis induction was followed by staining with fluorescent annexin V. In apoptosis,
endogenous P/CAF partially accumulated in the cytoplasm (Figure 7A). Then, we analyzed P/CAF localization throughout the apoptotic progression. To do that endogenous P/CAF localization was analyzed at different times after UV irradiation. The results shown that 10 minutes after UV irradiation P/CAF is clearly distributed between nucleus and cytoplasm. After 30 min, P/CAF levels increase and it is still localized between nucleus and cytoplasm. Finally, after 1 h, P/CAF is distributed between nucleus and cytoplasm and in most condensed cells is completely cytoplasmic (Figure 7A and B, see arrowheads). Similar results were obtained when apoptosis was induced by treatment with dexamethasone (Figure 7C and D). These results suggest that apoptosis induces P/CAF cytoplasmic accumulation.

**DISCUSSION**

The results show that P/CAF intramolecular acetylation is required for P/CAF nuclear localization. Lysines are essential components of NLS (45), and are often acetylated. It has been proposed that lysine acetylation regulates the nuclear/cytoplasmic distribution of several proteins [see reviews by (46,47)]. This modification could regulate NLS function by affecting NLS interactions with nuclear import machinery. Alternatively, acetylation within the NLS could induce conformational changes that affect NLS or nuclear export signal (NES) functions and alter protein subcellular distribution, as has been described for several proteins (48-54). Our results indicate that P/CAF NLS acetylation does not increase P/CAF’s ability to recognize importin machinery. In addition, blocking nuclear export did not affect either P/CAF(352-832) (nuclear) or P/CAF(352-832)/HAT (cytoplasmic) localization. This suggests that some additional mechanisms (probably related to protein degradation) are responsible for the observed P/CAF intracellular distribution.

Although P/CAF is a histone acetyltransferase, like CBP and p300, it has also been recognized as a critical regulator of nonhistone proteins involved in many cellular processes, such as differentiation and apoptosis (55). In yeast, loss of the P/CAF homologous Gen5l2 leads to high levels of apoptosis (56). The apoptosis regulators p53, p73, and E2F1 have been shown to be regulated by P/CAF acetylation (9,24-27,57) in response to DNA damage. In addition, P/CAF associates with Ku70 *in vivo*, acetylates its C-terminal linker, and blocks the ability of Ku70 to suppress Bax-mediated apoptosis, indicating that acetylation negatively regulates the antiapoptotic function of Ku70 (13). Interestingly, the Ku70 pool responsible for Bax sequestration is cytoplasmic (58), suggesting that P/CAF acetylation takes place in the cytoplasm. Relocalization of P/CAF from the nucleus to the cytosol following cellular damage might be a key regulatory step in Bax-mediated apoptosis. Our results provide evidence for a molecular mechanism that regulates P/CAF subcellular redistribution by autoacetylation following DNA damage. Additional experiments will be needed to determine what signals regulate P/CAF autoacetylation under proapoptotic conditions. It is possible that P/CAF interacts with the apoptotic regulator PKCd, which has been shown to inhibit the HAT activity of p300 (59). It should also be noted that cytoplasmic relocalization of HDAC3 is important for apoptosis progression (35).

In addition to a possible P/CAF cytoplasmic function, the ability of cytoplasmic P/CAF to interact with the transcriptional machinery in the nucleus is reduced. It would be interesting to address whether the cytoplasmic localization of nonautoacetylated P/CAF has a function, such as repressing transcription by potentially sequestering positive transcriptional components. Alternatively, it may be part of a mechanism that prevents P/CAF outside of the nucleus from taking part in any further action, such as global histone H3 acetylation/deacetylation, after apoptosis is induced (34, 35).
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FIGURE LEGENDS

Figure 1. P/CAF lacking HAT activity localizes in the cytoplasm

(A–C) Schematic representation of P/CAF proteins (A), showing the NLS and acetylated lysines. Relative HAT activity (B) and P/CAF autoacetylation (C) of GST (control), GST-P/CAF(352-832), GST-P/CAF(352-832)ΔHAT, and GST-P/CAF(352-832)L606A. Acetylated proteins were visualized by fluorography. Ac-P/CAF, acetylated P/CAF.

(D) Localization in transfected CV1COS cells of P/CAF(352-832), that we named as P/CAF, P/CAF(352-832)ΔHAT, that we named as P/CAFΔHAT, or P/CAF(352-832)L606A, that we named as P/CAF-L606A.

(E) Localization in transfected CV1COS cells of P/CAF-FL and P/CAFΔHAT-FL.

For (D) and (E), the bar graphs show the percentage of cells with predominantly nuclear (N) or cytoplasmic (C) localization or both (N+C) for over 200 cells from four independent experiments.

Figure 2. P/CAF nuclear localization correlates with autoacetylation

(A) Immunoprecipitation of P/CAF, P/CAFΔHAT, P/CAF-L606A, or vector alone from transfected CV1COS cells. P/CAF proteins were immunoprecipitated using an anti-P/CAF antibody, and immunocomplexes were tested for P/CAF acetylation status by immunoblot using antibodies that recognize acetylated lysines (Anti-AcLys, top blot) and P/CAF (Anti-P/CAF, bottom blot).

(B) CV1COS cells were transfected as in (A) and treated with 0, 100, or 150 nM TSA for 18 h. Twenty-four hours after transfection, the localization of the expressed proteins was visualized by immunofluorescence staining using anti-P/CAF antibody (red). Cells were costained with DAPI to reveal DNA (blue). The results shown are representative of at least three independent experiments.

Figure 3. P/CAF nuclear import is not regulated by autoacetylation

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(A) *In vitro* pull-down assay of GST, GST-P/CAF(352-832) (P/CAF), and *in vitro* autoacetylated GST-P/CAF(352-832) (Ac-P/CAF) fusion proteins bound to *in vitro*–translated and radiolabelled importin α1. Input represents 50% of total importin α1 input. The diagrams show the percentage of importin α1 bound to P/CAF.

(B) *In vitro* pull-down assay of GST and GST-importin β fusion proteins bound to *in vitro*–translated and radiolabelled P/CAF and Ac-P/CAF. The input represents 5% of the total P/CAF input.

(C) Pull-down assay with whole-cell extracts of CV1COS cells transfected with P/CAF or P/CAFΔHAT and loaded on the following columns: GST (lane 2), GST-importin α1 (lane 4), and GST-importin β (lane 3). The bar graphs show the percentage of importin α1 or β bound to transfected P/CAF and P/CAFΔHAT.

Figure 4. Proteasome inhibition partially reverses P/CAF cytoplasmic accumulation

(A) Localization of P/CAF, P/CAFΔHAT, and GFP-Sirt2 (control) in transfected CV1COS cells treated with 0 or 20 nM LMB. Localization of the expressed proteins was analyzed by immunofluorescence staining using anti-P/CAF antibody (red). Cells were costained with DAPI to reveal DNA (blue).

(B) Localization of P/CAF and P/CAFΔHAT in transfected CV1COS cells treated with 0 or 10 μM MG132. Localization of the expressed proteins was analyzed by immunofluorescence staining using anti P/CAF antibody (red). Cells were costained with DAPI to reveal DNA (blue). The bar graphs show the percentage of cells with predominantly nuclear (N) or cytoplasmic (C) localization or both (N+C) for over 200 cells from three independent experiments.

Figure 5. P/CAF deacetylation by HDAC3 promotes P/CAF cytoplasmic accumulation

(A) CV1COS cells were transfected with P/CAF and one of the following: Flag-HDAC1, HDAC2, Flag-HDAC3, HDAC4, or SIRT1. Localization of the expressed proteins was visualized by immunofluorescence staining using anti-P/CAF antibody (red). The bar graphs show the percentage of cells with predominantly nuclear (N) or cytoplasmic (C) localization or both (N+C) for over 300 cells from four independent experiments. The expression levels of Flag-HDAC1, HDAC2, Flag-HDAC3, HDAC4, and SIRT1 were similar (data not shown).

(B) CV1COS cells were transfected with P/CAF, Flag-HDAC3, shRNAHDAC3 or shRNA Control. Localization of the expressed P/CAF and Flag-HDA3 proteins was visualized by immunofluorescence staining using anti-P/CAF antibody and anti-Flag antibodies respectively. Cells were costained with DAPI to reveal DNA (blue).

(C) CV1COS cells were transfected with P/CAF (lane 1), P/CAF and Flag-HDAC3 (lane 2), or Flag-HDAC3 alone (lane 3). P/CAF was immunoprecipitated using an anti P/CAF antibody. The immunocomplexes were tested for P/CAF acetylation status by immunoblot using antibodies that recognize acetylated lysines (anti-AcLys, top blot) and P/CAF (anti-P/CAF, bottom blot).

(D) CV1COS cells were transfected as in (B) and treated with 0 or 150 nM TSA. P/CAF localization was visualized by immunofluorescence staining using anti-P/CAF antibody (red). TSA activity was followed by immunofluorescence staining using anti-Ac-H3 antibody (green) (increased Ac-H3 levels are indicated by white arrowheads). Cells were costained with DAPI to reveal DNA (blue). P/CAF proteins were immunoprecipitated using an anti P/CAF antibody. The immunocomplexes of P/CAF were tested for P/CAF acetylation status by immunoblot, using antibodies that recognize acetylated lysines (anti-Ac-Lys, top blot) and P/CAF (anti-P/CAF, bottom blot). The results shown are representative of at least three independent experiments.

(E) CV1COS cells were transfected with P/CAF and/or Flag-HDAC3. P/CAF proteins were immunoprecipitated using an anti-P/CAF antibody in RIPA buffer. The immunocomplexes were tested for P/CAF HAT activity in an *in vitro* HAT assay and for the presence of P/CAF protein by immunoblot analysis.

(F) CV1COS cells were transfected with P/CAF-FL and Flag-HDAC3 in the presence or absence of 150 nM TSA for 12 h. Localization of the expressed P/CAF and Flag-HDA3 proteins was visualized.
by immunofluorescence staining using anti-P/CAF and anti-Flag antibodies respectively. Cells were costained with DAPI to reveal DNA (blue).

Figure 6. P/CAF binds HDAC3 to promote P/CAF deacetylation
(A) CV1COS cells were transfected with P/CAF and/or Flag-HDAC3 and the localization of the expressed proteins was analyzed by immunofluorescence staining using anti-P/CAF (red) and anti-Flag (green) antibodies. Cells were costained with DAPI to visualize DNA (blue).
(B) CV1COS cells were transfected as in (A). P/CAF was immunoprecipitated using an anti-P/CAF antibody. The immunocomplexes were tested for the presence of Flag-HDAC3 protein by immunoblot using antibodies against P/CAF (top blot) and the Flag epitope (bottom blot).

Figure 7. Apoptosis induces P/CAF cytoplasmic accumulation
(A) CV1COS cells were irradiated with 200 J/m² UV for 0 or 60 min. Localization of endogenous P/CAF was analyzed by immunofluorescence staining using anti-P/CAF antibody (red). Cells were costained with DAPI to visualize DNA (blue) and with fluorescent annexin V to follow apoptotic cells.
(B) Cells were treated as in (A) and P/CAF localization was analyzed at different times.
(C) CV1COS cells were treated with 2 μM dexamethasone for 0 or 60 min. Localization of endogenous P/CAF was analyzed by immunofluorescence staining using anti-P/CAF antibody (red). Cells were costained with DAPI to visualize DNA (blue) and with fluorescent annexin V to follow apoptotic cells.
(D) Cells were treated as in (C) and P/CAF localization was analyzed at different times. The results shown are representative of at least three independent experiments. Arrowheads mark cells with complete P/CAF cytoplasmic localization.
### Table 1

**Subcellular distribution of P/CAF on different cell lines**

| Cell line Construct | COS | 293T | HCT116 | Hela | NIH3T3 |
|--------------------|-----|------|--------|------|--------|
| **P/CAF**          | 79% N | 74% N | 66% N | 75% N | 71% N |
|                    | 21% N+C | 26% N+C | 34% N+C | 25% N+C | 29% N+C |
|                    | 0% C     | 0% C     | 0% C     | 0% C     | 0% C     |
| **P/CAFΔHAT**      | 0% N     | 0% N     | 0% N     | 0% N     | 0% N     |
|                    | 17% N+C  | 4% N+C   | 33% N+C  | 37% N+C  | 66% N+C  |
|                    | 83% C    | 96% C    | 67% C    | 63% C    | 34% C    |

N = Nuclear  
C = Cytoplasmic  
N+C = Nuclear and cytoplasmic
Figure 1

A

P/CAF (352-832)  

\[ \begin{array}{cccc}
352 & 489 & 658 & 832 \\
\end{array} \]

\[ \begin{array}{c}
HAT \\
\end{array} \]

\[ \begin{array}{cccc}
416 & 428 & 430 & 441 \\
\end{array} \]

\[ \begin{array}{cccc}
442 \\
\end{array} \]

\[ \begin{array}{c}
\Delta HAT \\
\end{array} \]

\[ \begin{array}{cccc}
352 & 489 & 527 & 547 \\
\end{array} \]

\[ \begin{array}{c}
HAT \\
\end{array} \]

\[ \begin{array}{cccc}
352 & 489 & L606A & 658 \\
\end{array} \]

\[ \begin{array}{c}
HAT * \\
\end{array} \]

B

HAT Activity

C

P/CAF Autoacetylation

Fluorograph

D

Transfected:

- Anti-P/CAF
- DAPI
- MERGE

- GST-P/CAF
- GST-P/CAF ΔHAT
- GST-P/CAF L606A

- Ac-P/CAF

Graphs showing percentage of cells with different transfected proteins compared to control (N) and combined (N+C) conditions.
Figure 1

Transfected:

- **P/CAF-FL**
- **P/CAF-ΔHAT-FL**

**Bar Graphs**

- **P/CAF-FL**
  - % Cells
  - N: Nuclear
  - C: Cytoplasmic
  - N+C: Nuclear and Cytoplasmic

- **P/CAF-ΔHAT-FL**
  - % Cells
  - N: Nuclear
  - C: Cytoplasmic
  - N+C: Nuclear and Cytoplasmic

**Legend**

- N = Nuclear
- C = Cytoplasmic
- N+C = Nuclear and Cytoplasmic
Figure 2

A

| Transfected       | IP: α-P/CAF |
|-------------------|------------|
| P/CAF             | +          |
| P/CAF-ΔHAT        | -          |
| P/CAF(L606A)      | -          |

Immunoblot: Anti-AcLys

Immunoblot: Anti-P/CAF

B

| Transfected       | IP: α-P/CAF |
|-------------------|------------|
| P/CAF             | +          |
| P/CAF-ΔHAT        | -          |
| P/CAF(L606A)      | -          |

Immunoblot: Anti-AcLys

Immunoblot: Anti-P/CAF

1 2 3 4

55 KD
Figure 3

A

35S-Importin α

Immunoblot α−AcLys

1     2     3    4

50% Input  GST  Ac-P/CAF  P/CAF

% Importin α bound

P/CAF  Ac-P/CAF

B

35S-P/CAF

Ac−35S-P/CAF

1     2     3

5% Input  GST  Import β

% Importin β bound

P/CAF  Ac-P/CAF

C

Transfected:
P/CAF  P/CAF−ΔHAT

20% Input  GST  GST-import β  GST-import α

% Importin β bound

P/CAF  P/CAF−ΔHAT

Immunoblot α−P/CAF

% Importin α bound

P/CAF  P/CAF−ΔHAT
Figure 4

A

|          | Anti-P/CAF | DAPI | MERGE |
|----------|------------|------|-------|
| 0nM LMB  |            |      |       |
| 20nM LMB |            |      |       |

P/CAF

B

|          | Anti-P/CAF | DAPI | MERGE |
|----------|------------|------|-------|
| MG132 0 uM |            |      |       |
| MG132 10 uM |            |      |       |

P/CAF

GFP-SIRT2

|          | GFP         | DAPI | MERGE |
|----------|-------------|------|-------|
| 0nM LMB  |             |      |       |
| 20nM LMB |             |      |       |

MG132

N=C Nuclear and Cytoplasmic

N=N Nuclear

C=C Cytoplasmic
Figure 5

A

Transfected:

|                | Anti-P/CAF | DAPI | MERGE |
|----------------|------------|------|-------|
| P/CAF          | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |
| P/CAF+ HDAC1   | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |
| P/CAF+ HDAC2   | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |
| P/CAF+ HDAC3   | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |
| P/CAF+ HDAC4   | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |
| P/CAF+ Sirt1   | ![Image](https://example.com) | ![Image](https://example.com) | ![Image](https://example.com) |

N=Nuclear      C=Cytoplasmic
N+C= Nuclear and Cytoplasmic
Figure 5

**B**

| Transfected: | Anti-P/CAF | Anti-Flag | DAPI | MERGE |
|--------------|------------|-----------|------|-------|
| P/CAF        |            |           |      |       |
| P/CAF+HDAC3  |            |           |      |       |
| P/CAF+HDAC3 shRNA HDAC3 |            |           |      |       |
| P/CAF+HDAC3 shRNA C |            |           |      |       |

**C**

| Transfected | IP: α-P/CAF |
|-------------|-------------|
| P/CAF       | + + -       |
| Flag-HDAC3  | - + +       |

**Inmunoblot:** Anti-AcLys

55 KD

**Inmunoblot:** Anti-P/CAF

55 KD

1 2 3
Figure 5

D

Transfected:

| Condition                | Anti-P/CAF | Anti-Ac-H3 | DAPI | MERGE |
|--------------------------|------------|------------|------|-------|
| P/CAF                    | -          | -          | -    | -     |
| P/CAF + HDAC3            | -          | -          | -    | -     |
| P/CAF + HDAC3 + 150 nM TSA | -          | -          | -    | -     |

IP: α-P/CAF

| Transfected | 1 | 2 | 3 | 4 | 5 |
|-------------|---|---|---|---|---|
| P/CAF       | - | + | + | + | + |
| Flag-HDAC3  | - | - | - | + | + |
| TSA         | - | - | + | - | + |

Immunoblot: Anti-AcLys

55 KD

Immunoblot: Anti-P/CAF

55 KD

E

Transfected:

| Condition          | Relative HAT Activity |
|--------------------|-----------------------|
| P/CAF              | -                     |
| Flag-HDAC3         | +                     |

F

Transfected:

| Condition          | Anti-P/CAF | Anti-Flag | DAPI | MERGE |
|--------------------|------------|-----------|------|-------|
| P/CAF-FL           | -          | +         | -    | -     |
| P/CAF-FL + HDAC3   | -          | -         | -    | -     |
| P/CAF-FL + HDAC3 + TSA | -          | -         | -    | -     |
Figure 6

**A**

| Transfected         | Anti-P/CAF | Anti-Flag | DAPI | MERGE |
|---------------------|------------|-----------|------|-------|
| P/CAF               | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| P/CAF+ Flag-HDAC3   | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| Flag-HDAC3          | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |

**B**

| Transfected | IP: α-P/CAF |
|-------------|-------------|
| P/CAF       | -  +  +  - |
| Flag-HDAC3  | -  -  +  + |

**Immunoblot**

- Anti-P/CAF: ![Image](image13) 55 KD
- Anti-Flag: ![Image](image14) 55 KD
Figure 7

A

UV 200 J/m²
0 min

UV 200 J/m²
60 min

Time post irradiation (200 J/m²)

Non-irradiated 5 min 10 min 30 min 40 min 1 h 4 h

Anti-P/CAF

DAPI

MERGE
Supplementary Figure 1

CV1COS cells were transfected with P/CAF and GFP expression vectors. Localization of the expressed P/CAF protein was visualized by immunofluorescence staining using anti-P/CAF antibody. Cells were costained with DAPI to reveal DNA. To distinguish endogenous and ectopic, transfected P/CAF immunofluorescence signals different laser intensities were used at the confocal microscope. In addition, transfected cells were followed by GFP coexpression.
Autoacetylation regulates P/CAF nuclear localization
Noemí Blanco-García, Elena Asensio-Juan, Xavier de la Cruz and Marian A. Martínez-Balbás

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