hCAF1, a new regulator of PRMT1-dependent arginine methylation

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Summary

Protein arginine methylation is an emergent post-translational modification involved in a growing number of cellular processes, including transcriptional regulation, cell signaling, RNA processing and DNA repair. Although protein arginine methyltransferase 1 (PRMT1) is the major arginine methyltransferase in mammals, little is known about the regulation of its activity, except for the regulation induced by interaction with the antiproliferative protein BTG1 (B-cell translocation gene 1). Since the protein hCAF1 (CCR4-associated factor 1) was described to interact with BTG1, we investigated a functional link between hCAF1 and PRMT1. By co-immunoprecipitation and immunofluorescence experiments we demonstrated that endogenous hCAF1 and PRMT1 interact in vivo and colocalize in nuclear speckles, a sub-nuclear compartment enriched in small nuclear ribonucleoproteins and splicing factors. In vitro methylation assays indicated that hCAF1 is not a substrate for PRMT1-mediated methylation, but it regulates PRMT1 activity in a substrate-dependent manner. Moreover, small interfering RNA (siRNA)-mediated silencing of hCAF1 in MCF-7 cells significantly modulates the methylation of endogenous PRMT1 substrates. Finally, we demonstrated that in vitro and in the cellular context, hCAF1 regulates the methylation of Sam68 and histone H4, two PRMT1 substrates. Since hCAF1 and PRMT1 have been involved in the regulation of transcription and RNA metabolism, we speculate that hCAF1 and PRMT1 could contribute to the crosstalk between transcription and RNA processing.

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Introduction

Methylation of arginine residues in proteins is a post-translational modification that increases the structural diversity of proteins and modulates their function in the cell. The enzymes responsible for this modification are the protein arginine methyltransferases (PRMTs) classified in two groups, according to the generation of either asymmetric or symmetric dimethylarginine. In humans, the PRMT family comprises so far nine members (PRMTs 1-9) displaying different substrate specificities (Bedford and Richard, 2005; Cook et al., 2006). PRMT1 is a type-I methyltransferase (MT) that transfers a methyl group from S-adenosylmethionine to the guanido nitrogens of arginine residues to form monomethylarginine and asymmetric dimethylarginine (McBride and Silver, 2001). More than 85% of the arginine methylation activity is catalyzed by PRMT1, the predominant PRMT in human cells, which is located both in the nucleus and the cytoplasm (Tang et al., 2000; Pawlak et al., 2000; Cote et al., 2003; Herrmann et al., 2005). Disruption of the *PRMT1* gene has shown that PRMT1 plays an essential and non-redundant role, as *PRMT1*$^{-/-}$ embryos die shortly after implantation and embryonic stem (ES) cells derived from these embryos are defective in their ability to differentiate in vitro (Pawlak et al., 2000). However, these ES cells are viable and able to assemble normal heterogenous ribonucleoprotein (hnRNP) complexes, suggesting that arginine methylation is not required for vital processes under cell culture conditions. This is consistent with findings in yeast, where the disruption of the only known MT RMT1 does not affect cell viability (Gary et al., 1996). PRMT1 targets a wide array of different proteins for post-translational modifications in glycine-arginine-rich (GAR) domains, preferentially in an arginine-glycine (RG) or arginine-glycine-glycine (RGG) context, a common feature of RNA-binding proteins, suggesting that arginine methylation might modulate protein-RNA or protein-protein interactions (Liu and Dreyfuss, 1995). For example, methylation of the RNA-binding protein Sam68 at arginine residues decreases its binding to SH3 domains (Bedford et al., 2000). Moreover, arginine methylation has been implicated in a variety of processes such as signal transduction, protein trafficking or transcriptional regulation (reviewed by Bedford and Richard, 2005) and, more recently, in DNA damage checkpoint control and DNA base excision repair (BER) (Boisvert et al., 2005; El Andaloussi et al., 2006). In addition, PRMT1, as well as PRMT4/CARM1, acts as a transcriptional co-activator of nuclear receptors (Koh et al., 2001), probably in part because both proteins methylate histones H4 and H3, respectively, and facilitate histone acetylation and chromatin remodeling (Strahl et al., 2001; Wang et al., 2001).

Little data exist about the regulation of PRMT activities:

PRMTs have been found to interact with proteins that are often not methyl-accepting substrates but can modulate the MT activity of PRMTs. Binding of the tumor suppressor DAL-1/4 to PRMT3 acts as an inhibitor of enzyme activity (Singh et al., 2004); by contrast, DAL-1/4 has been found to regulate PRMT5 activity by either inhibiting or enhancing protein methylation (Jiang et al., 2005). Methyltransferase activity of PRMT5 is also positively regulated by its association with the hSWI/SNF chromatin remodelers BRG and BRM (Pal et al., 2004). PRMT1 has been identified as an interactor of antiproliferative proteins BTG1 (B-cell translocation gene 1) and TIS21/BTG2, which also stimulate its activity towards selected substrates (Berthet et al., 2002; Lin et al., 1996). BTG1 and BTG2 are both involved in cell growth, differentiation and survival (Matsuda et al., 2001). We previously demonstrated that these proteins are involved in transcriptional regulation (Prevot et al., 2000; Prevot et al., 2001), and that they interact, through hCAF1, with the mammalian CCR4-NOT complex whose subunits CCR4 and CAF1, in addition to being involved in transcription, represent the major cytoplasmic mRNA deadenylases in various species (Bianchin et al., 2005; Daugeron et al., 2001; Tucker et al., 2001; Viswanathan et al., 2004).

We report here for the first time that hCAF1 and PRMT1 interact in vivo and colocalize in nuclear speckles. In addition, hCAF1 acts as a cofactor of PRMT1, and regulates its enzymatic activity in vitro and in cellular systems, with different effects according to the substrate. We show that small interfering RNA (siRNA) knockdown of hCAF1 affects asymmetric PRMT1-dependent dimethylation in MCF-7 cells. Furthermore, we report that hCAF1 regulates the methylation of two PRMT1 substrates, Sam68 and histone H4, in vitro and in the living cells. Altogether, our data suggest that hCAF1, by regulating arginine methylation, might contribute to the crosstalk between processes such as transcription and mRNA metabolism.

Results

PRMT1 colocalizes and associates with hCAF1 in vivo

PRMT1 is a type I arginine MT responsible for methylating the terminal guanidine nitrogens of arginine residues to produce mono- and asymmetric dimethylarginine in proteins. However, whereas arginine methylation represents an emerging regulator of protein function, the mechanisms by which PRMT1 is regulated are still for the most part unknown. Analyzing its partners and substrates will be a major challenge for elucidating PRMT1 physiological roles. PRMT1 has been identified as an interacting protein of the immediate early gene product BTG1 (Lin et al., 1996), which has also been described by our group and others as a functional partner of hCAF1 (Rouault et al., 1998; Bogdan et al., 1998). Interestingly, PRMT1 and hCAF1, in addition to being BTG1 partners, are both involved in estrogen receptor α (ERα) transcriptional regulation (Prevot et al., 2001; Koh et al., 2001). These findings raised the possibility of a functional relationship between these proteins. As a first step to test this hypothesis, we analyzed the subcellular localizations of endogenous PRMT1 and hCAF1 using immunofluorescence experiments. The localization of endogenous PRMT1 was determined in MCF-7 cells using a monoclonal antibody. We observed that PRMT1, as previously described, resides in the

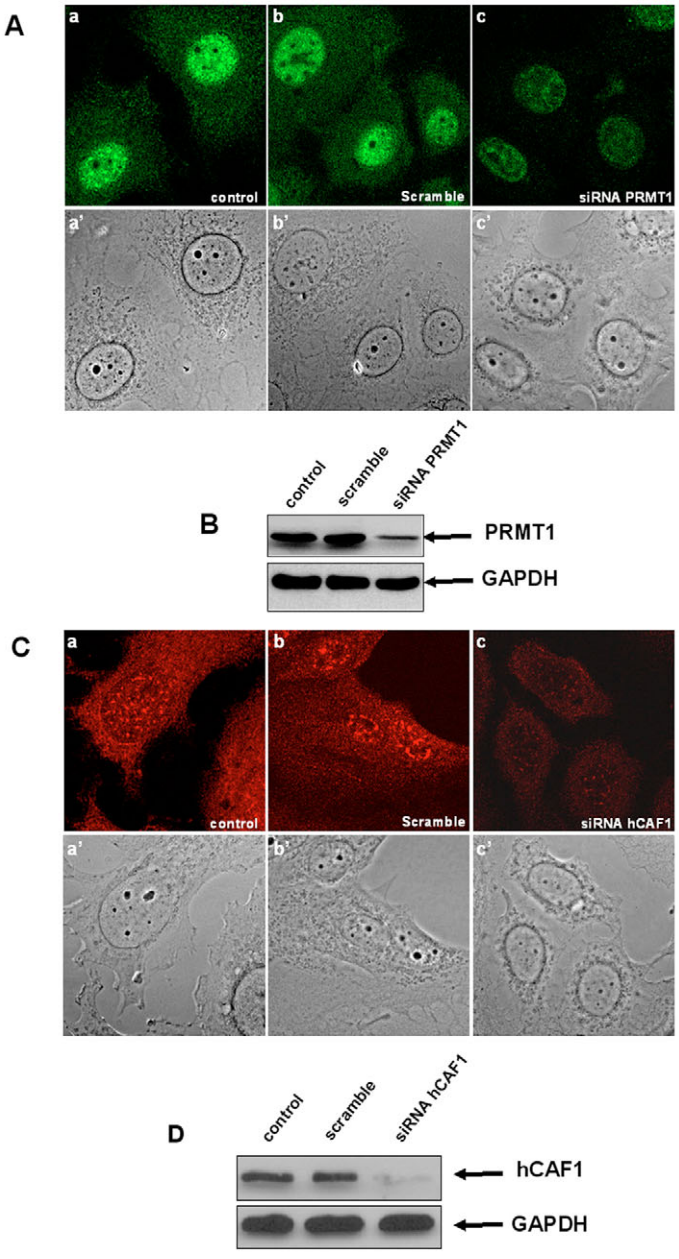

Fig. 1. Distribution of PRMT1 and hCAF1 in MCF-7 cells. Confocal fluorescence microscopy experiments showing the subcellular distribution of endogenous PRMT1 and hCAF1. (A) PRMT1 was visualized using a monoclonal anti-PRMT1 antibody in MCF-7 cells (a) control or transfected with (b) scramble siRNA duplexes or (c) specific siRNA PRMT1 duplexes. The corresponding phase-contrast micrographs are shown in panels a′-c′. (B) Suppression of endogenous PRMT1 was checked by western blotting with polyclonal anti-PRMT1. A loading control was performed with an anti-GAPDH antibody. (C) Immunofluorescence analysis using a rabbit polyclonal anti-hCAF1 antibody in MCF-7 cells (a) control or transfected with (b) scramble siRNA duplexes or (c) specific siRNA hCAF1 duplexes. The corresponding phase-contrast micrographs are shown in panels a′-c′. (D) Suppression of endogenous hCAF1 was checked by western blotting. A loading control was performed with an anti-GAPDH antibody.

cytoplasm and the nucleus (Fig. 1Aa). Nevertheless, the enzyme mainly showed a nuclear localization and was distributed in a punctuate pattern over a diffuse background throughout the nucleus. This unexpected staining was confirmed by using a specific commercial polyclonal antibody that gave similar results (see supplementary material Fig. S1). In addition, treatment of MCF-7 cells with PRMT1-specific siRNAs strongly abolished this staining pattern, thus confirming the identity of the signal and the specificity of the antibody (Fig. 1Ac). The discrepancy of the labeling we obtained with other studies could be explained by the fixation method used. Indeed, when we fixed the cells with paraformaldehyde, we could not see the speckle staining for PRMT1 (data not shown). This staining was further demonstrated using several different cell lines (data not shown). Western blot analysis with the polyclonal anti-PRMT1 antibody confirmed that the PRMT1 knockdown efficiency was approximately 75% and that no change in protein expression level was observed in knockdown cells as assessed by glyceraldehyde phosphate dehydrogenase (GADPH) western blotting (Fig. 1B).

We also analyzed the localization of endogenous hCAF1 using an affinity-purified polyclonal antibody previously described (Morel et al., 2003). As shown in Fig. 1C, in MCF-7 cells, hCAF1 appeared expressed in the cytoplasm and in different structures of the nucleus. In the nucleus, the fluorescence signal accumulated in dot-like structures in the majority of the cells, a pattern that is similar to PRMT1 nuclear labeling (Fig. 1Aa). Knockdown by siRNA directed against hCAF1 decreased hCAF1 protein levels compared with mock MCF-7 cells transfected with scramble siRNA (Fig. 1C, compare panels a and b with panel c). Protein immunoblotting analysis showed that the hCAF1 knockdown efficiency was approximately 90% (Fig. 1D). Double-labeling experiments showed a perfect overlap of hCAF1 and PRMT1 signals in nuclear foci (Fig. 2Ac). We also verified that PRMT1 localization was not altered in hCAF1 siRNA-treated cells and vice versa (data not shown). Quantitation of fluorescence images indicated that in the nucleus the colocalization was more than 50% (supplementary material Fig. S2). Although PRMT1 and hCAF1 showed almost 100% overlap in the bright foci, revealing the concentration of both proteins in the same structures. The total nuclear colocalization was less conspicuous (~50%) because of a higher background of inhomogeneous signal throughout the nucleus. Similar distribution patterns were also shown in HeLa and MRC5 cells (data not shown).

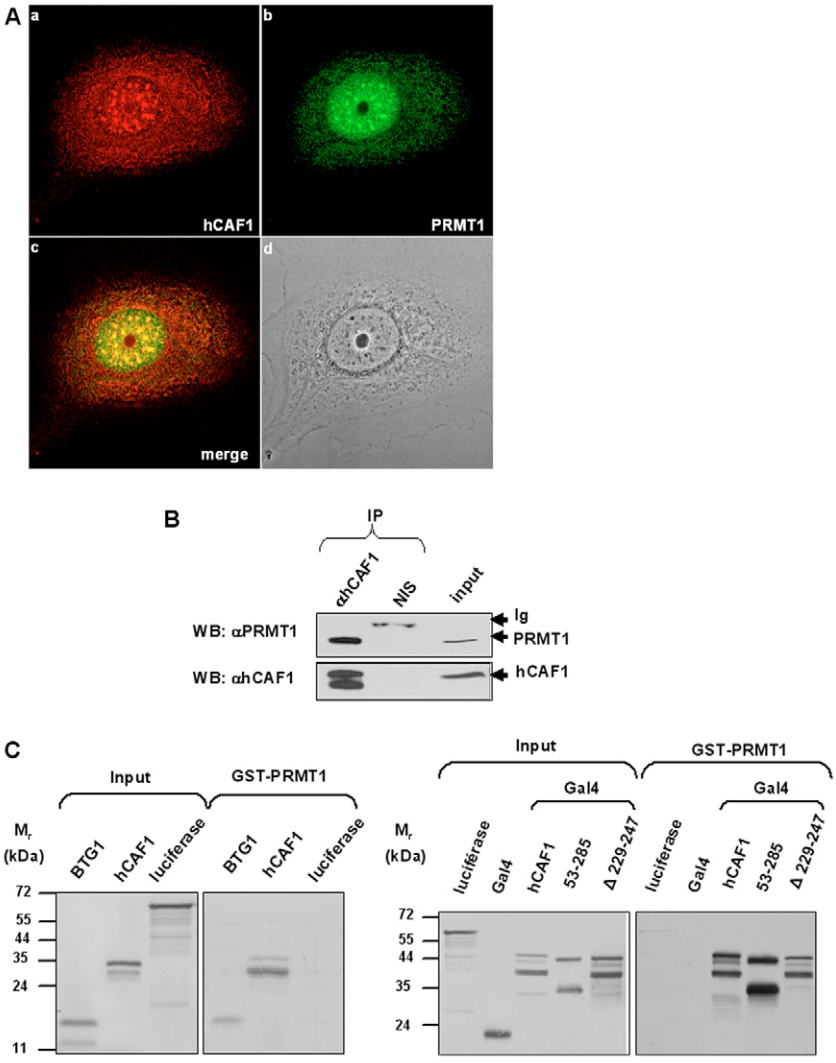

Fig. 2. PRMT1 colocalizes and interacts with hCAF1. (A) Laser-scanning confocal microscopy was used to compare the distributions of (a) hCAF1 and (b) PRMT1 in MCF-7 cells using the polyclonal anti-hCAF1 and the monoclonal anti-PRMT1 antibodies. The corresponding overlay is shown in panel c and the corresponding phase-contrast micrograph is shown in panel d. (B) MCF-7 cell extract was immunoprecipitated using anti-hCAF1 polyclonal antibody or non-immune serum (NIS). Total cell lysate (input) as well as immunoprecipitants (IP) were analyzed by western blotting using the polyclonal anti-PRMT1 or anti-hCAF1 antibodies. Input represents 4% of the total cell extract used for immunoprecipitation experiments. (C) Direct interaction between hCAF1 and PRMT1 was analyzed by GST-pull-down experiments. ^{35}S-labeled in vitro translated hCAF1, BTG1 and luciferase (left) or deletion mutants (right) were incubated with GST-PRMT1–glutathione-Sepharose beads. The eluted proteins and 1/50 of input radiolabeled proteins were analyzed by SDS-PAGE and visualized by autoradiography.

To further analyze the relationship of PRMT1 and hCAF1 in the context of intact cells, we performed immunoprecipitation experiments on MCF-7 cell lysates using a polyclonal anti-hCAF1 antibody, followed by immunoblotting with anti-PRMT1 monoclonal antibody. Results showed that endogenous PRMT1 is immunoprecipitated with the anti-hCAF1 antibody but not with the non-immune serum (Fig. 2B). Of note, hCAF1 was detected as two distinct bands in the immunoprecipitate but not in the cellular extract input, as visualized by immunoblotting

with anti-hCAF1 antibody (Fig. 2B). The faster band, detected only after immunoprecipitation, could represent a second isoform or a post-translational modification of hCAF1, present at a low and undetectable concentration in the cellular extract, and enriched by immunoprecipitation. Bogdan and colleagues had previously described two bands with similar sizes for hCAF1 (Bogdan et al., 1998). When PRMT1 was immunoprecipitated, we did not detect co-immunoprecipitated hCAF1 (data not shown). One possible explanation could be that the antibody recognizes the region that is involved in the interaction with hCAF1 or that modifies the folding of PRMT1.

Glutathione S-transferase (GST) pull-down assays were then performed to investigate whether PRMT1 directly interacted with hCAF1. GST-PRMT1 protein was coupled to glutathione-Sepharose beads and incubated with [^{35}S] methionine-labeled hCAF1, BTG1 (used as positive control) or luciferase (used as negative control). As shown in Fig. 2C (left), a specific retention of hCAF1 and BTG1 was observed. These results point to a direct physical interaction of hCAF1 and PRMT1. As hCAF1 interacts with both PRMT1 and BTG1, we examined whether these interactions were exclusive. We had previously shown that two regions of hCAF1, corresponding to the residues 11-31 and 229-247, are crucial for interaction of the protein with BTG1 (Prevot et al., 2001). As the GAL4-hCAF1 chimeric mutants lacking these regions are still able to interact with PRMT1 (Fig. 2C, right), we conclude that different regions of hCAF1 are involved in hCAF1 interaction with BTG1 and PRMT1.

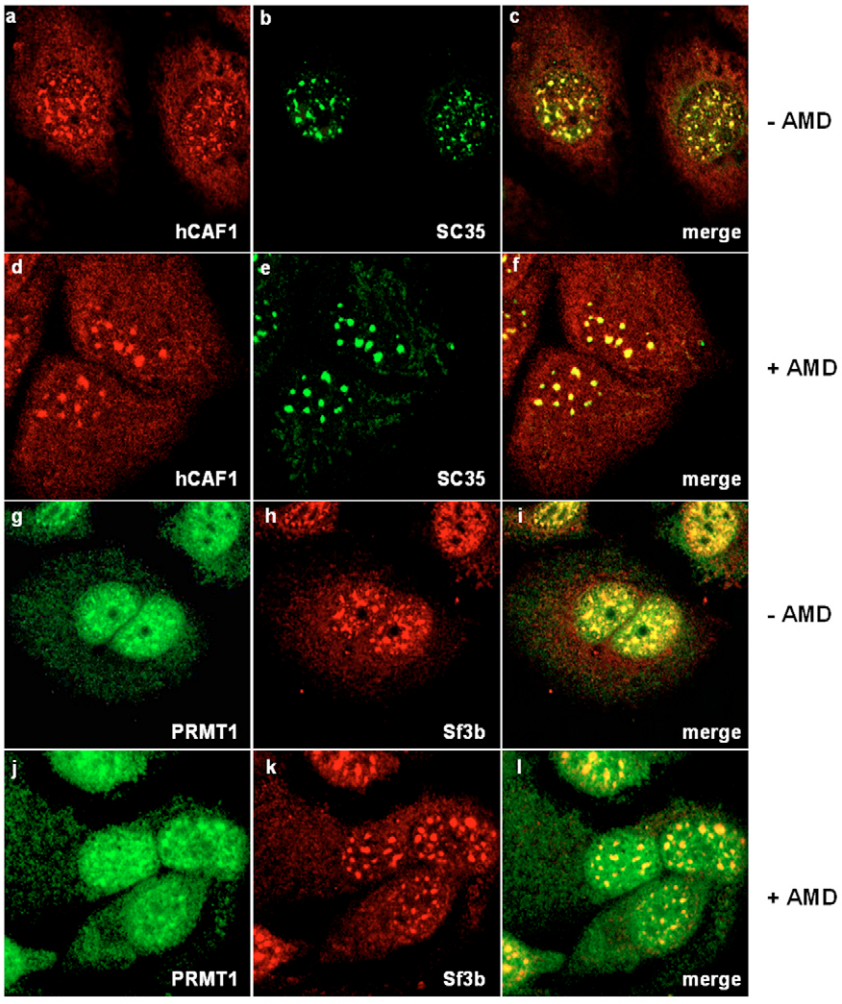

Fig. 3. Colocalization of hCAF1 and PRMT1 with nuclear speckle marker proteins is sensitive to AMD treatment. Laser-scanning confocal microscopy of double-labeling experiments before and after treatment with AMD (5 μg/ml). Distribution in MCF-7 cells of endogenous hCAF1 (a) and PRMT1 (g) along with specific compartment proteins such as SC35 (b) and Sf3b (h) was analyzed by double-labeling experiments using laser-scanning confocal microscopy. Panels (c) and (i) show overlays of the two signals. Distribution of hCAF1 (d) and PRMT1 (j) along with SC35 (e) and Sf3b (k) on MCF-7 cells treated with AMD (5 μg/ml). The corresponding overlays are shown in panels (f) and (l).

PRMT1 and hCAF1 colocalize in nuclear speckles

Given the localization of PRMT1 and hCAF1 in the same nuclear structures, we questioned whether these could correspond to previously described nuclear bodies.

The nucleus contains morphologically distinct and dynamic structures that include the nucleoli, Cajal bodies, splicing speckles, nuclear gems and PML bodies (Matera, 1999). To identify the dots containing PRMT1 and hCAF1, we compared the localization of endogenous PRMT1 and hCAF1 with several proteins reported as markers of nuclear structures in immunolabeling experiments (data not shown). We found a perfect colocalization only with the widely recognized marker proteins for nuclear speckles, the splicing factors SC35 and Sf3b (Caceres et al., 1997; Schmidt-Zachmann et al., 1998) (Fig. 3c,i). Quantitation of the fluorescence signals confirmed these qualitative observations. Interestingly, analysis of fluorescence images indicated that in the nucleus SC35 and Sf3b largely (more than 95%) colocalized with the foci containing hCAF1 and PRMT1, but only approximately 25% of nuclear hCAF1 and PRMT1 were localized with SC35 or Sf3b.

Effect of cell transcriptional activity on PRMT1 and hCAF1 localization

It is known that the nuclear speckles, like other nuclear bodies, are dynamic structures that are influenced by transcriptional or translational inhibitors (O'Keefe et al., 1994). For example, when cells are treated with transcriptional inhibitors, splicing activity is reduced and speckles, as indicated by SC35 labeling, become fewer in number, enlarged and rounded. We investigated whether the distribution of hCAF1 and PRMT1 in the nuclear speckles was dependent on the transcriptional activity of the cell. MCF-7 cells were treated with actinomycin D (AMD), which blocks Pol II-dependent transcription, and were simultaneously stained for hCAF1 and SC35 or for PRMT1 and Sf3b. Redistribution from

connected speckles in transcriptionally active cells to unconnected and large speckles (Fig. 3, compare panels b with e and h with k) was seen, consistent with published studies reporting that a variety of transcriptional inhibition treatments can cause such an alteration of the speckle morphology. The redistribution of hCAF1 and PRMT1 after AMD treatment appeared to be the same as that of splicing factors SC35 and Sf3b, thus confirming the identification of hCAF1 and PRMT1 as speckle components in transcriptionally active and inactive cells (Fig. 3, compare panels a with d and g with j). In the cells treated with AMD, hCAF1 completely coalesced into larger speckles coinciding with SC35 (Fig. 3f). The redistribution of PRMT1 after treatment appeared less evident probably because the fluorescent signal was more dispersed in the nucleus (Fig. 3j). Quantitation of the fluorescence signals indicated that, as for hCAF1, SC35 and Sf3b, the PRMT1 bright foci were larger (1.8×) than in untreated cells. Although PRMT1 was present at speckles and colocalized with Sf3b (Fig. 3i), speckles did not appear enriched in PRMT1 after AMD treatment (Fig. 3l).

hCAF1 regulates in vitro PRMT1 activity in a substrate-dependent manner

Although the precise functions of nuclear speckles are still unclear, there is accumulating evidence that they may constitute dynamic specialized structures either involved in essential processes such as transcription and splicing, or functioning as scaffolds into which transcription complexes assemble, or as sites of post-translational modifications or, finally, as 'storage sites' that can titrate the concentration of soluble factors in the nucleus (Lamond and Spector, 2003). As a step towards understanding the relationships between PRMT1 and hCAF1, we tested whether hCAF1 could be a substrate for arginine methylation by PRMT1. An in vitro methylation assay was performed using [^{3}H]AdoMet and recombinant GST-PRMT1, which is enzymatically active when incubated with GST-Sam68P3, a known PRMT1 substrate (Cote et al., 2003) used as positive control (Fig. 4A). In the same in vitro assay, PRMT1 did not methylate recombinant hCAF1 (data not shown), indicating that hCAF1 is not a substrate for PRMT1, at least in vitro. Since BTG1, which interacts with but is not methylated by PRMT1, plays a role as a PRMT1 co-regulator (Lin et al., 1996), we tested whether hCAF1 has the same effect. To test this hypothesis, GST-PRMT1 was incubated in the presence of GST-Sam68P3 substrate, along with increasing amounts of either GST-BTG1 or GST-hCAF1. Addition of GST-BTG1 or GST-hCAF1 showed a dose-dependent inhibition of PRMT1-mediated Sam68 methylation (Fig. 4A). Increasing amounts of GST, used as control, had no effect on PRMT1-mediated Sam68 methylation. We tested hCAF1 regulation of PRMT1 activity for other known PRMT1 substrates, namely hnRNPA1 and histone H4 (H4). Both hCAF1 and BTG1 had no effect on hnRNPA1 methylation (Fig. 4B). Lin et al. have demonstrated that BTG1 activates PRMT1-dependent methylation, particularly on hnRNPA1 (Lin et al., 1996). This discrepancy may be attributed to differences in assay conditions, as we used only pure recombinant proteins, whereas Lin and colleagues performed methylation experiments on cytosolic extracts. As shown in Fig. 4C, addition of GST-hCAF1 induced a dose-dependent inhibition of PRMT1-mediated methylation of H4. By contrast, BTG1 induced only an enhancement of the

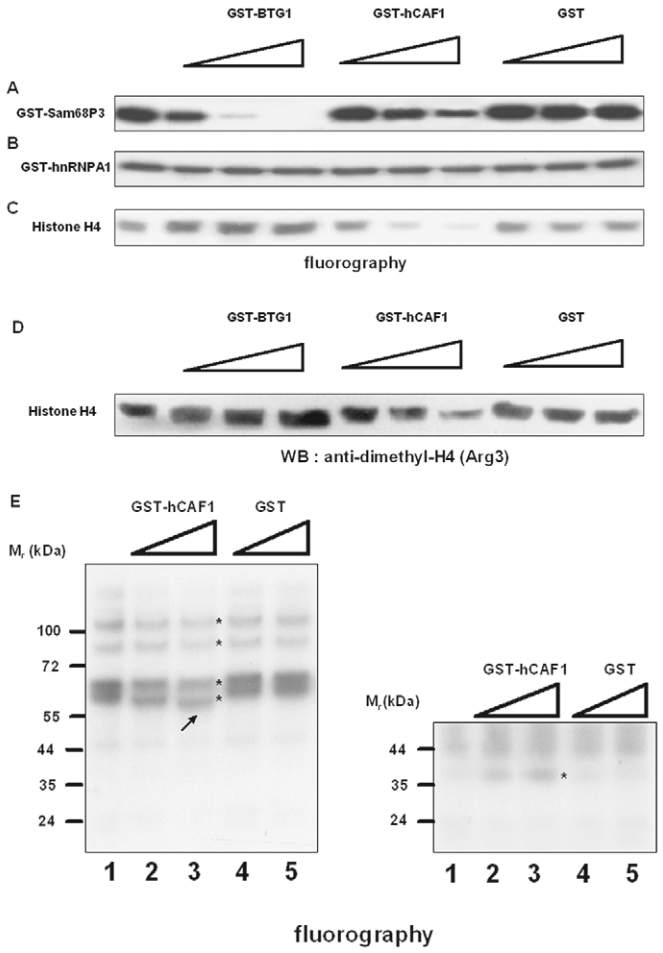

Fig. 4. Effect of hCAF1 and BTG1 on PRMT1 enzymatic activity in vitro. GST-PRMT1 (1 μg) was incubated with 1 μg of (A) GST-Sam68P3, (B) GST-hnRNPA1 or (C) recombinant histone H4 in the presence of [^{3}H]AdoMet without or with increasing amounts of GST-hCAF1, GST-BTG1 or GST for 90 minutes at 37°C. The reaction mixtures were resolved on SDS-PAGE and visualized by autoradiography. (D) GST-PRMT1 (1 μg) was incubated with 1 μg of recombinant histone H4 in the presence of cold AdoMet without or with increasing amounts of GST-hCAF1, GST-BTG1 or GST for 90 minutes at 37°C. The reaction mixtures were resolved on SDS-PAGE and revealed by western blot using the anti-dimethyl-Histone H4 (Arg3) antibody. Results are representative of several individual experiments. (E) GST-PRMT1 (1 μg) was incubated with [^{3}H]AdoMet, 20 μg of hypomethylated extracts prepared from MCF-7 cells without or with increasing amounts of GST-hCAF1 or GST as described above. The reaction mixtures were resolved on SDS-PAGE and visualized by autoradiography. A longer exposition of the gel is shown in the right-hand panel.

methylation signal. Given that PRMT1 has been shown to selectively methylate H4 at Arg3, we investigated whether hCAF1 specifically regulates this post-modification. We performed experiments identical to those described above in the presence of cold AdoMet. The reaction mixtures were then analyzed by western blotting using the anti-dimethyl-Histone H4 (Arg3) antibody. As expected, immunoblots showed that GST-PRMT1 efficiently methylated H4 at Arg3 in vitro (Fig. 4D). Addition of GST-hCAF1 induced a dose-dependent

inhibition of this methylation activity. These results indicate that hCAF1 can regulate the PRMT1-dependent methylation of Arg3 on H4.

To confirm the effect of GST-hCAF1 on endogenous substrates, we prepared hypomethylated cellular extracts from MCF-7 cells grown in the presence of the methylation inhibitor adenosine dialdehyde (Adox). Hypomethylated cell extracts were then incubated with recombinant PRMT1 along with [^{3}H]AdoMet alone or with recombinant hCAF1 protein. As can be seen in Fig. 4E, addition of exogenous GST-hCAF1 was found to inhibit the methylation of several substrates (indicated with asterisks in lane 3) compared with that found in cells having no GST-hCAF1 (lane 1). GST alone had no effect on the methylation profile (compare lanes 4 and 5 with lane 1). When we analyzed the methylation profile in the presence of GST-hCAF1 (lane 3) we found that one band, indicated with an arrow, migrated faster than the corresponding band in lane 1. This suggests that either the lesser amount of methylated arginines in the protein is responsible for the faster migration or this band represents a different protein, not detectable in lane 1, whose methylation was enhanced by hCAF1. A longer exposition of the gel (Fig. 4E, right) revealed that hCAF1 was able to enhance the methylation of an additional substrate of PRMT1.

Based on the above results, we conclude that hCAF1 regulates PRMT1 enzymatic activity by both enhancing and inhibiting arginine methylation in a substrate-specific manner.

hCAF1 regulates asymmetric dimethylation of proteins in MCF-7 cells

Given the ability of hCAF1 to regulate PRMT1 activity in vitro, we next sought to determine whether hCAF1 regulates asymmetric arginine dimethylation under physiological conditions. We analyzed the effect of silencing hCAF1 on asymmetric arginine methylation of endogenous cellular proteins in MCF-7 cells. Following siRNA transfections with scrambled control siRNA and two distinct hCAF1-specific siRNAs (hCAF1-1 and hCAF1-2), cell lysates were immunoblotted with ASYM24 antibody, which specifically recognizes proteins that contain asymmetric dimethylated arginines (aDMA) in (RGRG)-containing sequences (Boisvert et al., 2005). As shown in Fig. 5A lane 1, ASYM24 recognized four major bands in the cell lysate from cells transfected with the scrambled siRNA. Interestingly, knockdown of hCAF1 had a strong effect on the amount of dimethylated proteins. Fig. 5B shows the intensity of band signals shown in Fig. 5A quantified by densitometric analysis. Among the proteins recognized by the antibody, the level of the band indicated as (a) remained identical to the control, the signal of band (b) sensibly increased, whereas the levels of bands (c) and (d) were clearly reduced (Fig. 5A, compare lanes 2 and 3 with lane 1 and Fig. 5B). This result strongly indicated an effect of hCAF1 on the regulation of protein arginine methylation. To assess whether the proteins detected by ASYM24 antibody were putative PRMT1 substrates and because we cannot exclude the implication of other PRMTs

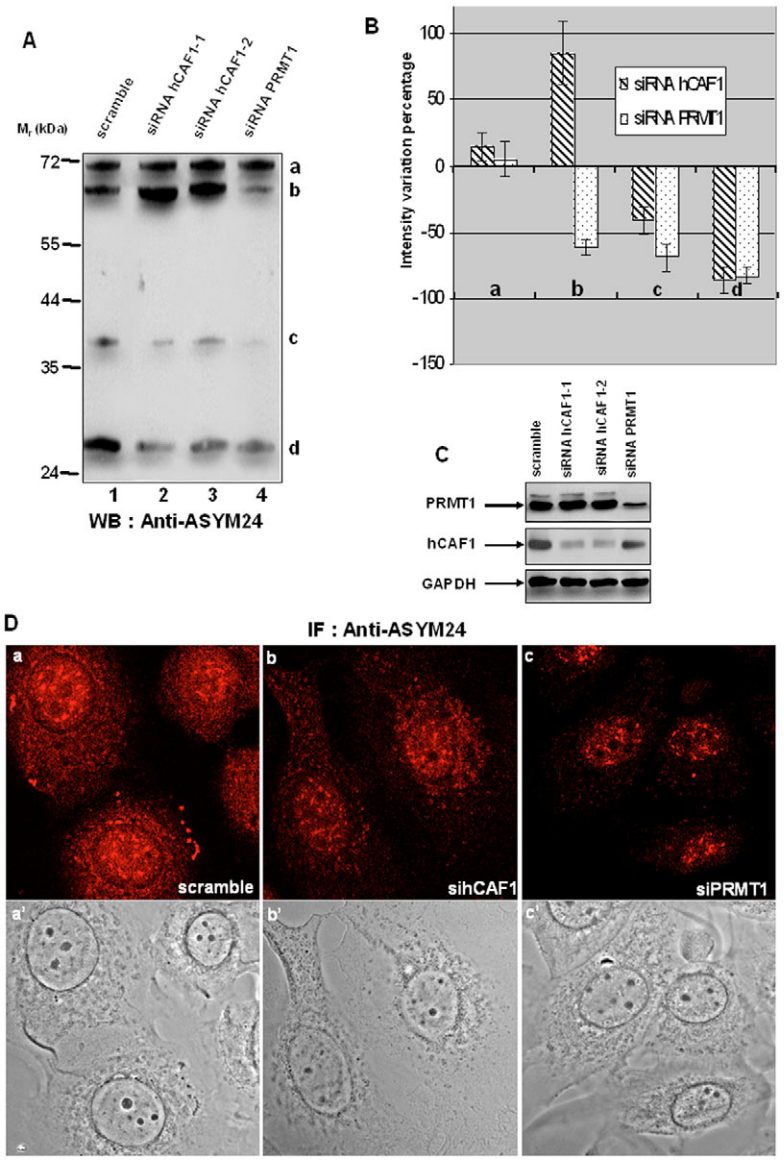

Fig. 5. hCAF1 regulates asymmetric dimethylation of proteins in MCF-7 cells. (A) Lysates of MCF-7 cells transfected either with control (scramble) or specific hCAF1 or PRMT1 siRNA duplexes were analyzed by western blotting using the pan antibody ASYM24. Results are representative of several individual experiments. (B) Quantification of arginine dimethylated bands revealed with ASYM24 antibody. Data represent the changes in arginine dimethylated bands after hCAF1 and PRMT1 knockdown, expressed as the ratio of the quantity present in knockdown cells to that found in cells treated with scrambled control siRNA. Data representing the mean of three independent experiments are shown with error bars. (C) Western blot analysis showing the expression of the indicated proteins in MCF-7 cell extracts after transfection with control (scramble) or hCAF1 or PRMT1 siRNA duplexes. GAPDH served as a loading control. (D) Confocal fluorescence microscopy experiments showing the subcellular distribution of arginine dimethylated proteins in MCF-7 cells transfected either with control (scramble) (a) or specific hCAF1 (b) or PRMT1 (c) siRNA duplexes revealed with ASYM24 antibody.

in the methylation of (RGG)-containing proteins, we inhibited PRMT1 expression in MCF-7 cells by siRNAs. Knockdown of PRMT1 resulted in a decreased detection of three of the four proteins recognized by ASYM24, indicating that PRMT1 activity was significantly reduced (Fig. 5A, compare lanes 1 and 4) and that the methylation of bands (b), (c) and (d) is PRMT1-dependent. Like hCAF1 knockdown, no effect on the signal of band (a*)* was observed (Fig. 5A, compare lanes 1 and 4). Western blot analysis demonstrated that the depletion of hCAF1 (87%) and PRMT1 (75%) did not affect the level of total proteins, as indicated by the control performed with an anti-GAPDH antibody (see Fig. 5C).

Together, these results provide strong evidence for a regulatory role of hCAF1 in PRMT1-dependent methylation in vivo, with different effects according to the substrate. We show that hCAF1 positively regulated the methylation of the proteins indicated as (c) and (d), which decreased after hCAF1 extinction by siRNA, and inhibited the methylation of protein (b) in the same experimental conditions.

We next examined the effect of hCAF1 and PRMT1 silencing on the cellular distribution of aDMA-containing proteins. MCF-7 cells were assayed by indirect immunofluorescence using the ASYM24 antibody. As shown in Fig. 5D, aDMA-containing proteins localize to the cytoplasm and throughout the nucleus where they are distributed in bright foci over a diffuse background. Knockdown of PRMT1 resulted in an important decrease of signals with ASYM24 antibody (Fig. 5Dc), confirming that this antibody recognizes most PRMT1 substrates. The ASYM24 signals were also affected in cells knocked down for hCAF1 (Fig. 5Db), substantiating its role in the regulation of PRMT1-dependent methylation.

As ASYM24 bright focal nuclear staining (Fig. 5Da) resembled nuclear speckles, MCF-7 cells were simultaneously stained using SC35 and ASYM24 antibodies (Fig. S3). The merged image demonstrated that the nuclear bodies stained with ASYM24 coincide with the nuclear speckles detected by SC35 antibody. These observations indicate that several aDMA-containing proteins concentrate with PRMT1 and hCAF1 in these structures.

hCAF1 regulates methylation of Sam68 and Histone H4 in MCF-7 cells

We next examined the effect of hCAF1 on Sam68 and H4 methylation in living cells. Cell lysates from MCF-7 cells transfected with scrambled control siRNA or with the two distinct hCAF1-specific siRNAs or grown in the presence of the methylation inhibitor Adox were immunoprecipitated using anti-Sam68 antibody and revealed with ASYM24 antibody. Fig. 6A shows that Sam68 methylation increased in cells transfected with hCAF1-specific siRNAs compared with scrambled control siRNA. Western blot with anti-Sam68 antibody confirmed that a similar amount of protein was immunoprecipitated at each point (Fig. 6A). These results strengthen the results obtained in vitro showing that hCAF1 regulates Sam68 methylation (Fig. 4A).

The effect of hCAF1 depletion on Sam68 methylation was highly reminiscent of that observed for band b in Fig. 5A. Indeed, band b comigrates with immunoprecipitated Sam68 revealed with anti-Sam68 antibody (supplementary material Fig. S4). These results suggest that band b represents or at least contains Sam68.

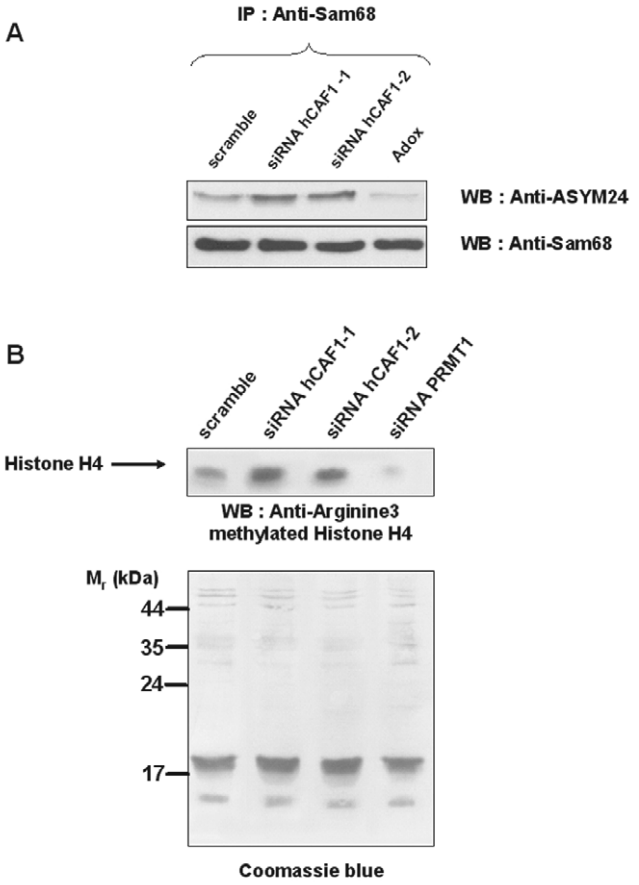

Fig. 6. hCAF1 regulates Sam68 and histone H4 methylation in MCF-7 cells. (A) Extracts from MCF-7 cells transfected with scramble or specific hCAF1 siRNA duplexes or treated with Adox were immunoprecipitated using anti-Sam68 antibody, then analyzed by western blotting with ASYM24 after stripping with anti-Sam68 antibody. (B) Acid-extracted histones from MCF-7 cells transfected either with scramble or specific hCAF1 or PRMT1 siRNA duplexes were resolved on SDS-PAGE and revealed by western blotting using the anti-dimethyl-Histone H4 (Arg3) antibody.

We also tested the effect of hCAF1 depletion on H4 methylation in the living cells. Acid-extracted histones from MCF-7 cells transfected either with scramble or specific hCAF1 or PRMT1 siRNAs were resolved on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and revealed by western blot using the anti-dimethyl-Histone H4 (Arg3) antibody. Again, H4 methylation was improved in cells transfected with hCAF1 siRNAs compared with control siRNA (Fig. 6B), confirming the results obtained in vitro.

Together with in vitro experiments, these results demonstrated that hCAF1 inhibited Sam68 and H4 methylation, which in fact increased after hCAF1 extinction by siRNA.

Discussion

Protein arginine methylation is a general post-translational modification that regulates diverse processes such as transcription and RNA metabolism. PRMT1 represents the predominant arginine MT of human cells. The major targets of

arginine methylation include nuclear RNA-binding proteins, such as components of the hnRNP particle involved in pre-mRNA processing and transport, or nucleolar components such as fibrillarin and nucleolin, which are involved in pre-rRNA processing and ribosome biogenesis. Whereas much data has accumulated about PRMT1 substrates, little is known about the regulation of MT activity. BTG1 and BTG2 are the only coregulators of PRMT1 described so far (Berthet et al., 2002; Lin et al., 1996). As hCAF1 is a partner of BTG1 and BTG2 and is involved in transcription and RNA metabolism regulation, we wondered whether we could establish a putative link between the two proteins hCAF1 and PRMT1. Our results demonstrate that, in MCF-7 cells, endogenous hCAF1 and PRMT1 co-immunoprecipitate and colocalize in nuclear structures, which we identified as the splicing speckles, suggesting that the two proteins might have a common role in these structures. Although the speckles proteins SC35 and Sf3b showed almost 100% overlap with both hCAF1 and PRMT1, a significant portion of the latter proteins remained associated with the 'nonspeckle nucleoplasm', which is heterogeneous, as it contains chromatin, nuclear bodies and parts of the speckle-like compartments. Two alternative explanations, which are not mutually exclusive, can be proposed on the basis of our findings. The mammalian cell nucleus contains numerous subcompartments and the mechanisms by which nuclear compartments are formed and maintained are unclear. In the first model, hCAF1 and PRMT1 could exhibit multiple nuclear localizations, not yet identified. Nevertheless, results using fluorescence recovery after photobleaching (FRAP) techniques suggest that proteins localized in nuclear structures are highly mobile and are rapidly exchanged between the different cellular subcompartments and the nucleoplasm. This high mobility of proteins suggests that nuclear proteins continuously dissociate from their compartments and that movement of these proteins is not restricted to particular nuclear domains (Phair and Misteli, 2000). Determination of the relative mobilities of hCAF1 and PRMT1 using FRAP techniques should be informative.

A speckled localization is diagnostic for proteins involved in pre-mRNA splicing. The presence of PRMT1 in structures related to mRNA splicing is not surprising as several results indicate a role of arginine methylation in splicing regulation. In fact, the spliceosomal complex does not assemble properly in hypomethylated nuclear extracts (Boisvert et al., 2002). In addition, proteins involved in pre-mRNA processing and/or in mRNAs transport, such as hnRNPs (Liu and Dreyfuss, 1995) or the Sm proteins D1 and D3 (Brahms et al., 2000), are methylated on arginine residues in vivo. In agreement with these observations, we have shown (supplementary material Fig. S3) that a significant portion of aDMA proteins, detected with the ASYM24 antibody, were localized in the nuclear speckles, indicating that arginine methylation regulates components of the nuclear speckles. Speckles are located in the interchromatin regions of the nucleoplasm of mammalian cells. A sequence motif, characterized by a region rich in arginine/serine dipeptide, sufficient for targeting a protein to nuclear speckles has been described (Schmidt-Zachmann et al., 1998), but neither PRMT1 nor hCAF1 contain this motif although they clearly localize to speckles. It is possible that other motifs for this specific intranuclear localization exist, or the proteins may be directed to speckles through their interaction with other proteins carrying a serine-arginine-rich (SR)-domain, as previously described for the splicing factor SC35 (Spector et al., 1991). The localization of speckle proteins in the nucleus is highly dynamic and influenced by several parameters such as the rate of transcription and heat shock treatment. The use of the RNA transcription inhibitor AMD, known to affect nuclear/nucleolar structure, showed that the distribution of hCAF1 and PRMT1 varied from connected speckles in transcriptionally active cells to unconnected and enlarged speckles, which is similar to the distribution described earlier for various splicing factors such as SC35 and Sf3b (Carmo-Fonseca et al., 1991; Schmidt-Zachmann et al., 1998). Arginine methylation could be involved in regulating the pool of factors that are accessible to the transcription/pre-mRNA processing machinery. However, a proteomic analysis has revealed that speckles also contain transcription factors and 3′-end RNA-processing factors (Saitoh et al., 2004). It is also conceivable that PRMT1 and hCAF1 can be stored in these areas for assembly into larger macromolecular structures. Interestingly, hCAF1 is part of the mammalian CCR4-NOT complex (Morel et al., 2003), which regulates transcription (Denis and Chen, 2003) and mRNA turnover (Bianchin et al., 2005; Daugeron et al., 2001; Tucker et al., 2001) in yeast and in mammalian cells. We also investigated a putative functional role of PRMT1-hCAF1 interaction. Despite direct interaction between the two proteins, hCAF1 was determined not to be a methyl-accepting substrate for PRMT1, as assayed by in vitro methylation (data not shown), but rather appeared to regulate PRMT1-mediated methylation in a substrate-dependent manner (Figs 4-6). Indeed, we have demonstrated that hCAF1 inhibits Sam68 and histone H4 methylation, but has no effect on hnRNPA1 methylation (Fig. 4). The effects of hCAF1 depletion on asymmetric dimethylated proteins (Fig. 5A) and specifically on H4 Arg3 and Sam68 methylation (Fig. 6) strongly indicate that, in the cellular context, hCAF1 regulates PRMT1 activity. Moreover, in vitro experiments, using purified recombinant proteins, ruled out the possibility that hCAF1 might regulate the expression or the activity of regulatory proteins other than PRMT1, with downstream effects on PRMT1 activity.

Of particular interest is our finding that hCAF1 regulates the methylation of histone H4 at Arg3. It has been shown that PRMT1 is the major H4 Arg3 methyltransfase in human cells (Strahl et al., 2001) and that histone H4, when methylated by PRMT1, becomes a better substrate for p300, whereas acetylation of H4 by p300 inhibits its methylation by PRMT1 (Wang et al., 2001; Huang et al., 2005). This crosstalk has been described to contribute to the complex 'histone code' in hormone signaling, providing control marks for the specific targeting of trans-acting factors to chromatin (Bauer et al., 2002; Daujat et al., 2002; Huang et al., 2005; Wang et al., 2001). As both hCAF1 (Prevot et al., 2001) and PRMT1 (Koh et al., 2001) have been described as coactivators of the nuclear receptor response, these results suggest a putative role for hCAF1 in the transcription process through participation to the 'histone code' regulation that modulates the genetic information transmitted by the DNA. It is currently believed that the fidelity and efficiency of gene expression relies on the interconnection between transcription, mRNP assembly, mRNA processing and cytoplasmic mRNA metabolism. In addition, proteins regulating these different pathways are often part of multimeric

complexes, directly or indirectly involved in the control of the different steps of gene expression (for a review, see Guthrie and Steitz, 2005). We can speculate that PRMT1 and hCAF1, through their selective recruitment to multimeric complexes, may be recruited to the promoter, modulate transcription by regulating histone modifications and then associate with nascent transcripts and accompany the mRNA to the cytoplasm to control translation and/or RNA decay. Further work will be required to test this attractive hypothesis.

Materials and Methods

Cell culture

HeLa and MCF-7 cell lines obtained from the American Type Culture Collection were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% SVF and non-essential amino acids for MCF-7 in a humidified atmosphere of 5% CO_2 at 37°C.

Antibodies

Rabbit polyclonal antibody to hCAF1 was generated at Agrobio Laboratory (France) using the purified recombinant protein as an antigen. hCAF1 antibody was purified by affinity chromatography using NHS columns (Amersham Pharmacia Biotech) coupled with GST-hCAF1. The monoclonal antibody directed against PRMT1 was produced using the purified recombinant GST-PRMT1 as an antigen by Covalab (Lyon, France). The rabbit polyclonal anti-PRMT1 (07-404), the polyclonal anti-Sam68, the ASYM24 (07-414) and the anti-dimethyl-Histone H4 (Arg3) (07-213) antibodies were purchased from Upstate Biotechnology. The anti-PRMT1 monoclonal antibody (clone 171) and anti-SC35 (S4049) were purchased from Sigma, and GADPH (clone 6C5) from Biodesign International. The rabbit polyclonal anti-Sf3b antibody was kindly provided by M. S. Schmidt-Zachmann (German Cancer Research Center, Heidelberg, Germany).

Cell transfections

The siRNA sequences targeting hCAF1 corresponded to the coding regions 470-481 (sihCAF1-1) and 697-715 (sihCAF1-2). For PRMT1, the targeted sequence was located within region 753-771 (siPRMT1). Transfections were performed on 1×10^5 MCF-7 cells in six-well plates with a final concentration of 100 nM siRNA duplex using lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's guidelines. After 72 hours, cells were lysed in modified RIPA buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate) supplemented with protease inhibitor tablets (Roche Molecular Biochemicals) and phosphatase inhibitors (1 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate) and cell lysates were tested for the suppression of endogenous PRMT1 or hCAF1.

Confocal laser-scanning microscopy and image processing

MCF-7 cells (9×10^4) were grown on coverslips into 12-well plates. For drug treatment, cells were incubated for 4 hours in fresh medium containing 5 μg/ml of AMD. Cells were then fixed in methanol for 2 minutes at –20°C, washed twice in PBS and incubated with primary antibodies for 1 hour at 37°C. After PBS washes, the cells were incubated for 30 minutes at 37°C with the appropriate secondary antibodies Alexa Fluor 488 and Alexa Fluor 568 from Molecular Probes (1:3000) in Dako diluent, then washed in PBS and mounted on glass slides in mounting solution (Dako).

Confocal laser-scanning immunofluorescence microscopy was done on a Zeiss LSM 410 UV instrument (Zeiss). Images were acquired under identical conditions, ensuring that the maximal signal was not saturating, and were subjected to contrast stretching, excluding the top and bottom 1% of pixels. Acquisition of images and measurements were performed using either LSM software (Zeiss) or the ImageJ software Release 1.36 (Framasoft). Frequency distributions of intensities were collected either over a region of interest or through binary masks. Two features of interest were compared, which were the hCAF1 and PRMT1 signals or each hCAF1 and PRMT1 signal in speckles (corresponding to the SC35 and Sf3b domains).

Immunoprecipitation and western blotting

Protein extracts from MCF-7 cells were prepared in modified RIPA buffer described above. For histone extraction, cell extracts were centrifuged and insoluble fractions were isolated by acid extraction with 0.2 N HCl overnight at 4°C and dialyzed against 0.1 N HCl and then H_2O. For immunoprecipitation experiments, protein extracts (1 mg) were incubated with rabbit polyclonal hCAF1 antibody overnight at 4°C with shaking. Protein A-Sepharose beads (Upstate Biotechnology) were then added and the mixture was incubated for 2 hours at 4°C. The beads were subsequently washed three times with lysis buffer. The immunoprecipitates were denatured by boiling in 40 μl of Laemmli sample buffer and separated on SDS-PAGE. The gels were electroblotted onto a PVDF membrane and incubated with primary antibodies, then with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse immunoglobulins (Dako). The proteins were visualized by an enhanced chemiluminescence kit (Roche Molecular Biochemicals) following the manufacturer's instructions.

GST-fusion protein expression

GST-BTG1 and GST-hCAF1 have already been described (Rouault et al., 1998; Prevot et al., 2001). For GST-PRMT1, GST-Sam68 (P3 fragment) and GST-hnRNPA1, cultures of transformed BL21 (Stratagene) were grown to an A_{600} of 0.6, induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), incubated at 28°C for 3 hours and harvested by centrifugation. Cells were lysed for 30 minutes in 50 mM Tris, pH 8, 1 mM DTT containing protease inhibitors and 0.5 mg/ml lysozyme. The crude lysate was sonicated four times for 30 seconds, and Triton X-100 was added to a final concentration of 1% (v/v). Cell debris was removed by centrifugation (13,000 ***g*** for 10 minutes at 4°C). GST-fusion proteins were isolated by incubation of the supernatant for 2 hours at 4°C with glutathione-Sepharose beads (Amersham Pharmacia Biotech), and eluted in 20 mM glutathione in 50 mM Tris-Cl, pH 8.

In vitro protein-protein interaction assays

^{35}S-labeled in vitro translated proteins were synthesized with the combined in vitro transcription/translation (TnT) kit from Promega used following the manufacturer's instructions. The vectors pSG5-Flag-BTG1, pSG5-Flag-hCAF1, pGal, pGal-hCAF1 and the pGAL-hCAF1-deleted mutants used in the assays, as well as the GST pull-down experiments, have been described previously (Prevot et al., 2001).

In vitro methylation assays

The in vitro methylation assay was performed as described by Bedford (Bedford et al., 2000). Briefly, GST-PRMT1 and the different substrates GST-Sam68 proline-rich motifs P3 (GST-Sam68P3) (Bedford et al., 2000), GST-hnRNPA1 and recombinant histone H4 (Upstate Biotechnology) were incubated with either GST-hCAF1, GST-BTG1 or GST in the presence of S-adenosyl-L[methyl-^{3}H]methionine ([^{3}H]AdoMet; 85 Ci/mmol from a 10.4 μM stock solution in dilute HCl/ethanol 9/1, pH 2.0-2.5; Amersham Biosciences) for 90 minutes at 37°C. Methylation reactions were quenched by the addition of an equal volume of 2× Laemmli sample buffer, heated at 100°C for 5 minutes and separated on SDS-PAGE. Following electrophoresis, gels were soaked in Amplify fluorographic reagent (Amersham Biosciences) according to the manufacturer's instructions, and visualized by fluorography. The cold methylation assay of Histone H4 was performed in the same experimental conditions in the presence of 0.5 mM AdoMet. The reaction mixtures were subjected to SDS-PAGE and then analyzed by western blot using the anti-dimethyl-Histone H4 (Arg3) antibody (Upstate Biotechnology).

For methylation experiments of hypomethylated cell extracts, MCF-7 cells were cultured for 24 hours with 40 μM of the general methylation inhibitor, Adox. Protein extracts were prepared in RIPA buffer and 20 μg of proteins were incubated with radiolabeled [^{3}H]AdoMet and GST-PRMT1, either alone or in the presence of GST-hCAF1 or GST, and analyzed as described above.

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