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H3K9 histone methyltransferase G9a-mediated transcriptional activation of *p21*

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ABSTRACT

We report that H3K9 HMTase G9a activates transcription of the cell cycle regulatory gene, *p21*, in *p53*-null H1299 cells. Positive regulation of *p21* by G9a is independent of its HMTase activity. We demonstrate that G9a upregulates *p21* via interaction with PCAF, and provide evidence that the activating complex is recruited to the *p21* promoter upon DNA damage-inducing agent etoposide treatment. Our study suggests that G9a decreases proliferation and cell viability by increasing the level of p21-mediated apoptosis. Our results suggest that G9a functions as a coactivator for *p21* transcription, and directs cells to undergo apoptosis.

Structured summary of protein interactions:
G9a physically interacts with **PCAF** by anti tag coimmunoprecipitation (1, 2) **G9a** physically interacts with **PCAF** by anti bait coimmunoprecipitation (View interaction)

1. Introduction

Methylation at Lys 9 of histone H3 (H3K9me1, 2, or 3) is involved in gene repression and heterochromatin formation [1]. G9a (EHMT1, KMT1C) is the major histone lysine methyltransferase acting on both H3K9me1 and H3K9me2 in euchromatin [2]. Knockout of *G9a* leads to embryonic lethality in mice [3]. G9a is a component of many repressive transcription complexes, including Gfi1, UHRF1, CDP/Cut, and WIZ, and therefore has been intensively studied in the context of its transcriptional corepressor activity [4–7]. Furthermore, G9a has been shown to be overexpressed in many types of cancers, with knockdown or inhibition of G9a decreasing cellular proliferation [8–10]. In contrast to its repressive role in gene expression, G9a can also act as a transcriptional coactivator for certain nuclear receptor target genes, thereby inducing cellular differentiation [11–14].

The regulatory protein, *p21*, belongs to the Cip/Kip family of cyclin dependent kinase (cdk) inhibitors and thus plays a major role in cell cycle control [15]. Moreover, *p21* also contributes to the DNA damage response in processes such as cell cycle arrest and apoptosis [16]. The main regulator of *p21* is the tumor suppresser gene, *p53*; *p21* expression is upregulated by p53 in response to DNA damage [17]. However, *p21* can also be regulated by a *p53*-independent mechanism. For example, *p21* is upregulated during differentiation of murine erythroleukemia cells, which express a mutated version of *p53* [18]. Furthermore, *p21* can also be induced at the transcriptional level upon DNA damage by etoposide, in a manner similarly independent from *p53* [19].

In this study, we hypothesized that G9a functions as a positive regulator of *p21* expression via interaction with PCAF, and that G9a is recruited to the *p21* promoter upon cellular stress. Our results indicate that etoposide-mediated induction of *p21* expression in *p53*-null cells is mediated by G9a, and that induction of *p21* in this context eventually inhibits cellular proliferation and leads to apoptosis. These results suggest that G9a can positively regulate expression of *p21* upon cellular stress, and thereby inhibit cellular proliferation, through a p53-independent mechanism.

2. Materials and methods

For details of 'Materials and methods' section; see Supplementary Data.

2.1. Plasmid constructs

pEGFP-C1, pEGFP-hG9a, pEGFP-hG9a-ΔSET, pcDNA3.0, pcDNA 3.0-Flag-G9a, Flag-G9a-685C, Flag-G9a-936C, pCMX-p300, pCMX-CBP, and Flag-PCAF were used. The construct expressing short hairpin RNA (shRNA) directed against G9a (RHS4533-NM-006709) was purchased from Open Biosystems.

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2.2. Cell culture and transient transfection

H1299, *G9a* knockdown stable H1299, K562, HCT116, and HCT116 (p53−/−) cells were grown in RPMI 1640, and HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat inactivated fetal bovine serum and 0.05% penicillin–streptomycin at 37 °C in a 5% CO_2 atmosphere. Cells were treated either doxorubicin (3 μM) or etoposide (Sigma) (5 μM) for 24 h. At 24 h post-transfection, cells were treated with the indicated amount of BIX-01294 (Ann Arbor) for an additional 24 h, or at 48 h post-transfection, cells were treated with either 5 μM etoposide or DMSO for an additional 24 h.

2.3. Luciferase assay

Luciferase assays were conducted using a *p21* promoter reporter system. H1229 cells were cotransfected with the *p21* promoter reporter construct and the indicated DNA constructs using Lipofectamine 2000, or treated with 5 μM etoposide, for 24 h. After transfection, cells were harvested and assayed for luciferase activity using a luciferase assay system (Promega). Each value was expressed as the mean of three replicates from a single assay, and experiments were performed at least three times.

2.4. Immunoprecipitation (IP)

For the interaction assays, cells were lysed in lysis buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1X protease inhibitor cocktail, and 1 mM PMSF) at 4 °C. Proteins were immunoprecipitated with anti-GFP, anti-G9a, or anti-PCAF antibodies overnight at 4 °C, and then protein A/G agarose beads (GenDEPOT) were added for 2 h with rotation at 4 °C. Bound proteins were analyzed via Western blotting with anti-GFP, anti-G9a, anti-PCAF, anti-Flag, anti-p300, and anti-CBP antibodies.

2.5. Chromatin immunoprecipitation assay

H1299, K562, and HEK293T cells were treated with either 5 μM etoposide or DMSO and harvested 24 h later. H1299 cells were treated with 5 μM BIX-01294 and harvested 24 h later. Cells were cross linked with 1% formaldehyde, which was added to the medium for 10 min at RT, followed by the addition of 125 mM glycine for 5 min at RT. Cells were then lysed in SDS lysis buffer, after which samples were sonicated and immunoprecipitated using the indicated antibodies. Immunoprecipitates were eluted and cross links were reversed; subsequently, DNA fragments were purified using a PCR purification kit (Axygen). The following PCR primers were used: *p21* (forward, 5′-GTG GCT CTG ATTGGC TTT CTG-3′; reverse, 5′-CTG AAA ACA GGCAGC CCA AG-3′). Disassociation curves were generated after each PCR run to ensure that a single product of the appropriate length was amplified. The mean threshold cycle (C_T) and standard error values were calculated from individual C_T values, obtained from duplicate reactions per stage. The normalized mean C_T value was estimated as ΔC_T by subtracting the mean C_T of the input from that of *p21*.

2.6. TUNEL assay

HEK293T cells were seeded in 4-well chamber slides and then transiently cotransfected with pcDNA3.0-Flag-G9a and sh-G9a. After 2 days, cells were treated with 10 μM etoposide for 24 h and subsequently fixed with 4% paraformaldehyde for 1 h at RT. TUNEL (TdT-mediated dUTP nick end labeling) reactions were then carried out using a DeadEndTM Fluorometric TUNEL System, according to the manufacturer's instructions (Promega).

3. Results

3.1. G9a upregulates p21 expression

To gain insight into the role of the H3K9 HMTase, G9a, in cellular proliferation, we began by investigating whether G9a regulates genes involved in cell cycle regulation. Initially, we examined the effect of G9a overexpression on the levels of *ING1*, *p27*, *GADD45β*, and *p21* using real time PCR. As expected, G9a overexpression downregulated *ING1*, *p27*, and *GADD45β*; in contrast, G9a overexpression upregulated *p21* (Fig. 1A). Since G9a is well known for its role in transcriptional repression [1], we further tested the regulation of *p21* expression by G9a through shRNA-mediated gene silencing. Using two different shRNA sequences, G9a knockdown decreased both the mRNA and protein levels of *p21* in H1299 cells (Fig. 1B and C). Consistent result was obtained when *p21* expression was tested in H1299 cells stably expressing *G9a* shRNA (Fig. 1D). Intriguingly, a mutant version of G9a lacking the SET domain (G9a ΔSET) still upregulated *p21* expression (Fig. 1B). Different cell lines such as K562 and HEK293T were tested and showed the consistent results (Supplementary Fig. S1A and S1B). To confirm the role of G9a HMTase activity for positive regulation of *p21*, we examined the effect of BIX-01294, a G9a-specific inhibitor. No apparent changes in p21 expression were measured by increasing the concentration of BIX-01294 (Fig. 1E and Supplementary Fig. S1C). These observations indicate that G9a-mediated upregulation of *p21* expression is independent of its HMTase activity.

3.2. Transcriptional activation of p21 by G9a

To investigate the mechanism by which G9a upregulates *p21* expression, we determined whether G9a activates the *p21* promoter using transient transfection of G9a *in vitro*. To this end, we established a reporter assay using a *p21*-driven luciferase (luc) reporter system which enables quantification of G9a-mediated transcriptional modulation of *p21*. Consistent with our real time PCR and Western blot results, *p21* transcription was repressed by G9a knockdown and activated by G9a overexpression in H1299 cells (Fig. 2A). Using the G9a SET domain deletion mutant (G9a ΔSET), we next verified that transcriptional activation of *p21* by G9a was independent of its HMTase activity (Fig. 2A). Since H1299 cells are *p53*-null cells, harboring a homozygous partial deletion of *p53*, these findings indicate that G9a positively regulates *p21* transcription by directly acting on the *p21* promoter, independent of *p53*. Using the same reporter assay in *p53*-positive HCT116 cells, we observed that neither overexpression nor knockdown of G9a had an effect on *p21* expression (Fig. 2B and Supplementary S1D). Next, we tested the effect of G9a on *p21* activation induced by etoposide-mediated DNA damage. When H1299 cells were treated with etoposide for 24 h, transcription of *p21* was increased (Fig. 2C and Supplementary S2A). Surprisingly, *p21* activation by etoposide was significantly reduced by shRNA-mediated G9a knockdown (Fig. 2C). This experiment was performed with two independent shRNA sequences, to ensure that the observed effects were not sequence-specific (Fig. 2C). Conversely, overexpression of G9a further increased etoposide-mediated upregulation of *p21* expression (Fig. 2C). These results were also confirmed on the protein level by Western blot analysis, which showed that etoposide treatment increased p21 protein levels and that G9a knockdown and overexpression in the presence of etoposide treatment either decreased or further increased p21 levels, respectively (Fig. 2D). Western blot analysis of HCT116 cell lysates revealed that p21 protein levels were not altered in any of these conditions (data not shown). When another DNA damaging agent doxorubicin was treated in H1299 and HEK293T cells, same induction of p21 was observed

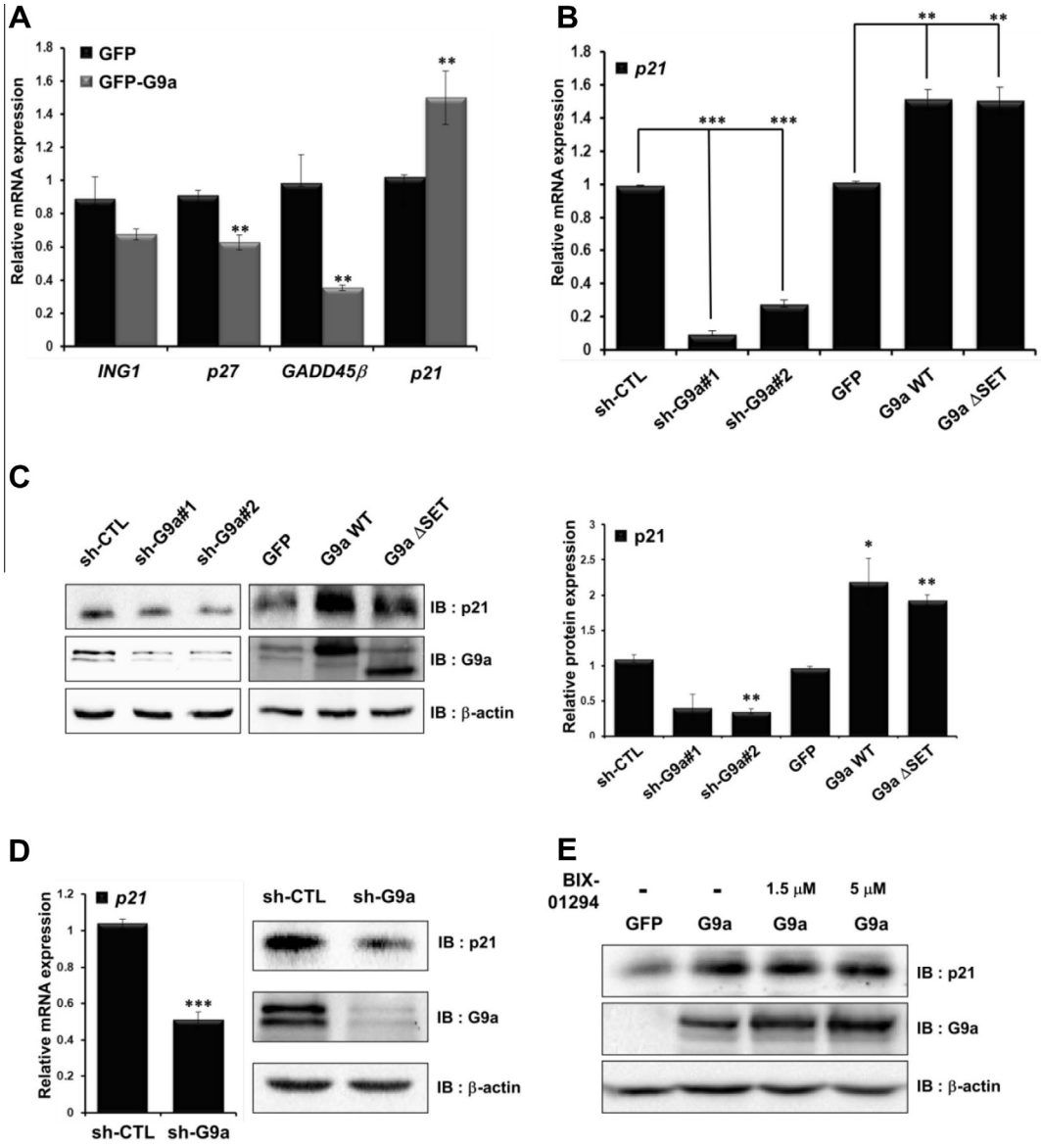

Fig. 1. Upregulation of *p21* expression by G9a. (A) H1299 cells were transfected with GFP or GFP-G9a; subsequently, mRNA expression levels of *ING1*, *p27*, *GADD45β*, and *p21* were examined by real time PCR. Results are shown as means ± S.D.s; $n = 3$. $^{**}P < 0.01$. (B and C) H1299 cells were transfected with sh-CTL, sh-G9a#1, sh-G9a#2, GFP, GFP-G9a (G9a WT), or GFP-G9a ΔSET, followed by analysis of mRNA and protein levels of *p21* by real time PCR (B) and Western blotting (C). Protein expression levels of p21 were quantified and normalized to the levels of β-actin. Results are shown as means ± S.D.s; $n = 3$. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. (D) The mRNA and protein levels of *p21* in H1299 cells stably expressing *G9a* shRNA were analyzed by real time PCR and Western blotting. Results are shown as means ± S.D.s; $n = 3$. $^{***}P < 0.001$. (E) H1299 cells were transfected with the indicated constructs. At 24 h post-transfection, cells were treated with BIX-01294 (1.5 or 5 μM) for 24 h. Cell extracts were then analyzed by Western blotting with the indicated antibodies.

suggesting upregulation of p21 might be DNA damage specific (Supplementary Fig. S2B). Taken together, these results demonstrate that G9a has a *p53*-independent role in the DNA damage-mediated transcriptional activation of *p21*.

3.3. G9a interacts with PCAF and is recruited to the p21 promoter

Previous studies have reported that G9a positively regulates gene expression by acting as a transcriptional coactivator for nuclear hormone receptors such as the androgen receptor, the glucocorticoid receptor, and the estrogen receptor [11,14]. Since our results indicated that G9a functions as a transcriptional activator for *p21*, we decided to investigate whether G9a works in concert with coactivators such as p300, CBP, and PCAF to activate *p21*. To test this hypothesis, reporter assays were carried out to investigate whether G9a works in concert with HATs. As shown in Fig. 3A, *p21* transcription was activated by G9a overexpression. When cells were cotransfected with G9a and PCAF, *p21* transcription was further activated, but no apparent changes in cotransfected cells with p300 or CBP (Fig. 3A). To further test whether G9a interacts with HATs, IP assays were conducted. These assays clearly showed that PCAF, but not p300/CBP, strongly interacted with G9a, suggesting that G9a and PCAF form an activator complex during *p21* transcriptional activation (Fig. 3B). *In vivo* interaction between endogenous G9a and PCAF was confirmed by IP using antibodies (Fig. 3C). We next performed domain mapping of G9a and PCAF interaction. *In vivo* interactions between G9a and PCAF were confirmed by IP with transiently transfected cell extracts using PCAF antibodies (Fig. 3D). Interestingly, the G9a 685C (685–1210) deletion mutant with an intact ankyrin domain

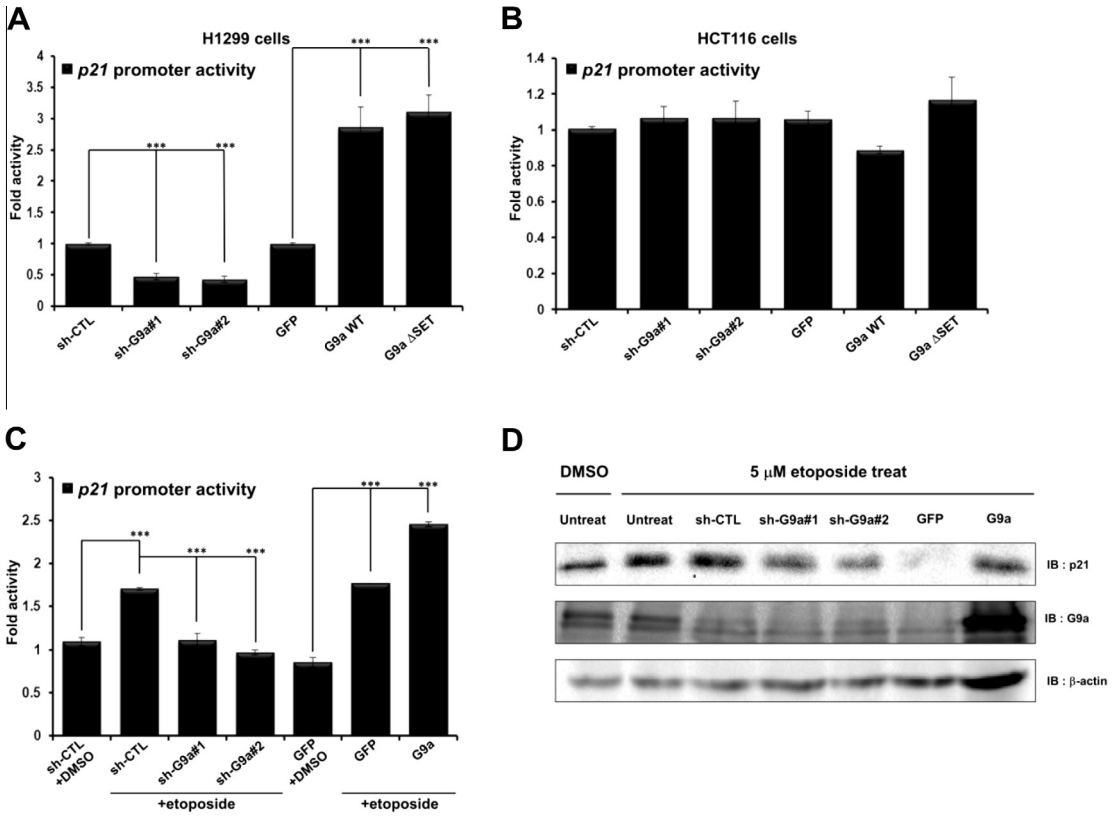

Fig. 2. Transcriptional activation of *p21* mediated by G9a in the presence of etoposide. (A and B) H1299 cells and HCT116 cells were cotransfected with the *p21* promoter-luc construct and either the sh-CTL, sh-G9a#1, sh-G9a#2, GFP, GFP-G9a WT, or G9a ΔSET construct. (C) H1299 cells were cotransfected with the *p21* promoter-luc construct and either the sh-CTL, sh-G9a#1, sh-G9a#2, GFP, or GFP-G9a. At 48 h post-transfection, cells were treated with either 5 μM etoposide or DMSO for 24 h. Cell extracts were then assayed for luciferase activity. Luciferase activities were normalized to those of β-galactosidase. Results are shown as means ± S.D.s; $n = 3$. $^{***}P < 0.001$. (D) H1299 cells were transfected with the indicated constructs. At 48 h post-transfection, cells were treated with either 5 μM etoposide or DMSO for 24 h. Cell extracts were then analyzed by Western blotting with the indicated antibodies.

showed strong interactions with PCAF. G9a deletion mutant without ankyrin domain lost interaction with PCAF indicating that the binding is dependent on the ankyrin domain of G9a (Fig. 3D). To investigate further whether or not interaction between G9a and PCAF is etoposide dependent, we monitored the interaction of the two proteins with and without etoposide treatment. We have been detected the basal level of interaction was strongly induced by etoposide treatment (Fig. 3E and Supplementary Fig. S3A). Intriguingly, very weak interaction was detected before etoposide treatment and there was no induction of interaction in p53 intact HCT116 cells which indicates the interaction is p53 independent (Supplementary Fig. S3B).

To further elucidate the mechanism underlying *p21* transcriptional regulation by G9a and PCAF, we performed ChIP assay followed by real time PCR. G9a and PCAF recruitment to the *p21* promoter increased upon treatment with etoposide, as did the level of H3 acetylation (Fig. 3F and Supplementary Fig. S3C and S3D). Interestingly, the level of H3K9me2 on the *p21* promoter significantly decreased with etoposide treatment, suggesting that H3K9 methyltransferase activity is not involved in this process (Fig. 3F). These results indicate that G9a activates *p21* expression in a manner independent of its HMTase activity. Rather, G9a-mediated activation of *p21* expression involves interaction with PCAF; this activating complex is recruited to the *p21* promoter, at least in the context of etoposide-induced cell stress. When we tested H3K9me2 recruitment on *p21* promoter upon BIX-01294 treatment, H3K9me2 level was unchanged because of the basal level of G9a recruitment in the absence of etoposide (Supplementary Fig. S3E).

3.4. G9a inhibits cellular proliferation in the presence of etoposide

G9a is known to be overexpressed in many types of cancers, and knockdown or inhibition of G9a has been shown to decrease cellular proliferation [8–10]. However, in our experiments, G9a positively activated *p21* expression in *p53*-null cells. This finding prompted us to verify that G9a regulates *p21* and cellular proliferation upon etoposide-induced cellular stress in HEK293T cells, in which p53 is inactivated by viral proteins [20]. First, we conducted BrdU incorporation assays to measure cellular proliferation in the presence of etoposide. As shown in Fig. 4A, overexpression of G9a significantly inhibited cellular proliferation, whereas knockdown of G9a increased cellular proliferation. The observed inhibitory effect on cell proliferation mediated by G9a was hardly detected in the absence of etoposide treatment, indicating that G9a is involved in the cellular stress response. Consistent results were obtained in cell viability analyses, in which overexpression of G9a decreased cell viability and shRNA-mediated knockdown of G9a increased cell viability (Fig. 4B). The *p53*-null HCT116 cells showed the same results in BrdU and cell viability assays (Supplementary Fig. S4A and S4B). In contrast, no apparent changes were observed when p53 intact HCT116 cells were used suggest that p21-mediated apoptosis induction upon etoposide is p53 independent (Supplementary Fig. S4C and S4D). Positive regulation of p21-mediated apoptosis by G9a was also investigated by TUNEL assays. The amount of apoptotic nuclei in DNase I-treated control and etoposide-treated cells decreased upon knockdown of G9a by different two shRNA sequences, a finding which supports the hypothesis that G9a positively regulates p21-mediated apoptosis

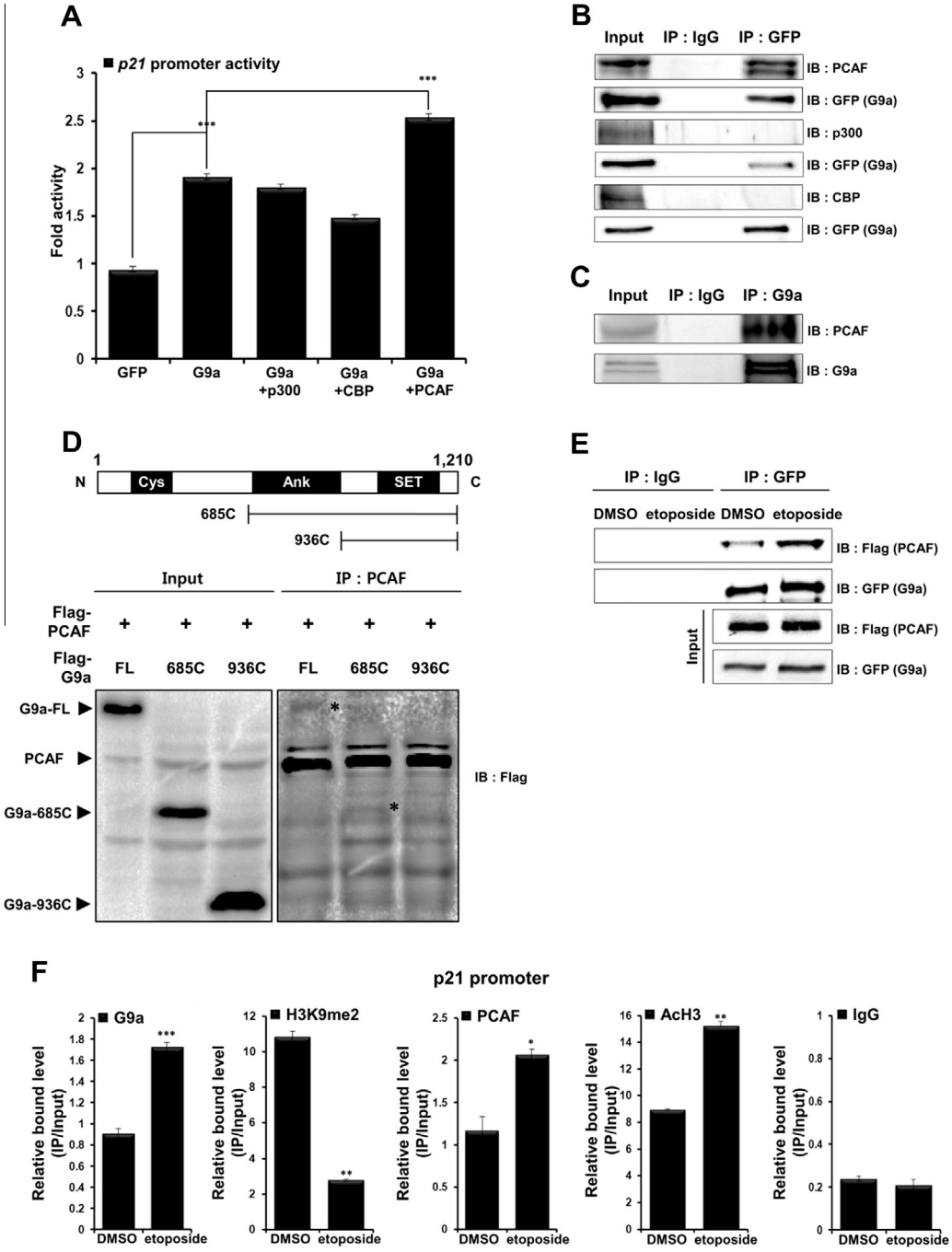

Fig. 3. G9a interacts with PCAF and is recruited to the *p21* promoter. (A) H1299 cells were cotransfected with the *p21* promoter-luc construct and either GFP, GFP-G9a, GFP-G9a and pCMX-p300, GFP-G9a and pCMX-CBP, or GFP-G9a and Flag-PCAF. Cell extracts were then assayed for luciferase activity. Luciferase activities were normalized to those of β-galactosidase. Results are shown as means ± S.D.s; $n = 3$. $^{***}P < 0.001$. (B) HEK293T cells were cotransfected with GFP-G9a and Flag-PCAF, GFP-G9a and pCMX-p300, or GFP-G9a and pCMX-CBP, and G9a was immunoprecipitated with anti-GFP antibodies. Associated proteins were analyzed by Western blotting with the indicated antibodies. (C) Endogenous interaction between G9a and PCAF was examined in HEK293T cells. Cell lysates were immunoprecipitated with anti-G9a antibodies and associated protein was analyzed by Western blotting with anti-PCAF antibodies. (D) HEK293T cells were cotransfected with Flag-PCAF and either full length (FL) or truncated mutants of Flag-G9a (685C or 936C) constructs. Cell lysates were immunoprecipitated with anti-PCAF antibodies and associated protein was analyzed by Western blotting with anti-Flag antibodies. (E) HCT116 (p53−/−) cells were transfected with GFP-G9a and Flag-PCAF. At 24 h post-transfection, cells were treated with either 5 μM etoposide or DMSO for 24 h. Anti-GFP immunoprecipitates were analyzed via Western blotting with anti-Flag antibodies. (F) H1299 cells were treated with either 5 μM etoposide or DMSO for 24 h. ChIP analysis was then conducted using anti-G9a, anti-H3K9me2, anti-PCAF, and anti-AcH3 antibodies. Recruitment of G9a, H3K9me2, PCAF, and AcH3 to the *p21* promoter region was normalized to the assay inputs. Anti-IgG antibodies were used as a negative control. Results are shown as means ± S.D.s; $n = 3$. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

(Fig. 4C). Altogether, our data suggest that G9a inhibits cellular proliferation and cell survival via upregulation of *p21* transcription, and induces p21-mediated apoptosis upon etoposide-induced stress.

4. Discussion

G9a is a member of the Suv39h subgroup of the SET domain-containing proteins, and exhibits methylation specificity for

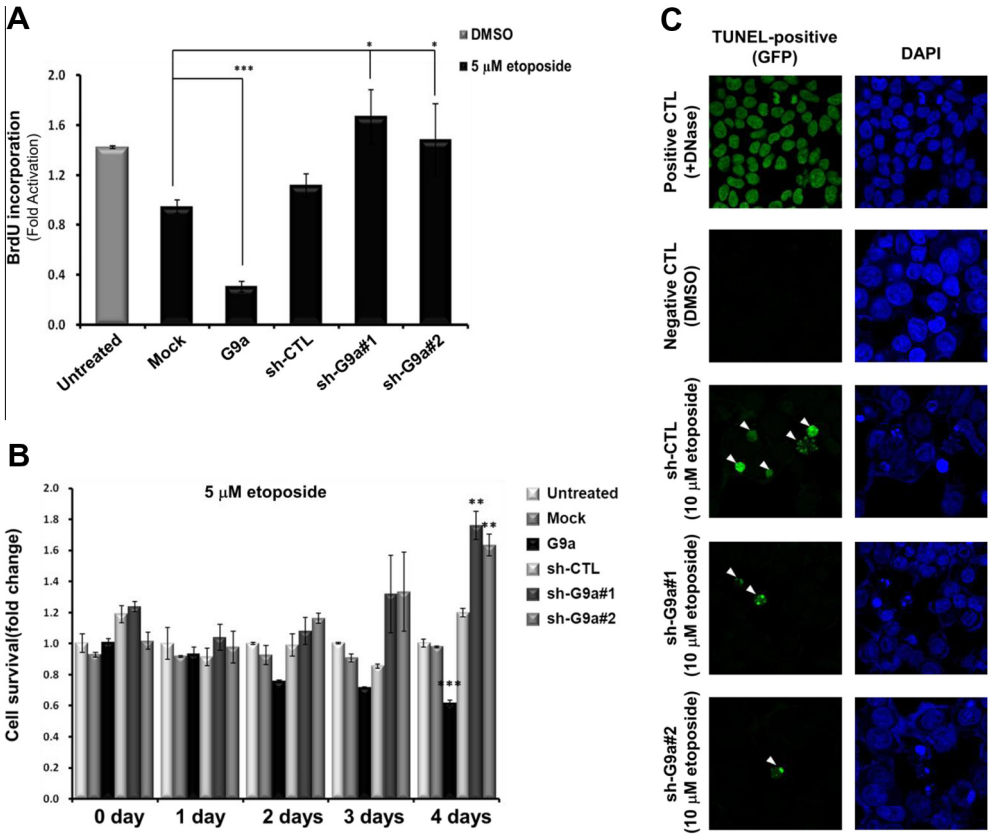

Fig. 4. Effect of G9a on etoposide-mediated apoptosis. (A) HEK293T cells were transfected with the indicated constructs upon etoposide treatment (5 μM). At 72 h post-transfection, cells were fixed and BrdU assays performed. Results are shown as means ± S.D.s; $n = 3$. $^{*}P < 0.05$; $^{***}P < 0.001$. (B) Cell viabilities were determined with the MTT assay. HEK293T cells were treated with etoposide (5 μM) and subsequently transfected with the indicated constructs. Results are shown as means ± S.D.s; $n = 3$. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with untreated cells. (C) HEK293T cells, transiently transfected with the indicated constructs upon etoposide treatment (5 μM), were subjected to TUNEL assays. DNase I-treated cells are shown as a positive control.

H3K9me1 and H3K9me2. G9a and GLP are homologous HMTases which mediate H3K9me1, H3K9me2, and H3K27 methylation and are also linked with heterochromatin formation and transcriptional repression [1,2]. Although various roles of G9a have been proposed in diverse biological processes and human diseases, the function of G9a in cellular proliferation remains poorly understood. In this study, we discovered that G9a positively regulates *p21* expression, at the transcriptional level, in *p53*-null cells, indicating that G9a-mediated activation of *p21* is *p53*-independent. Furthermore, knockdown of G9a decreased *p21* promoter activity in a SET-domain-, HMTase activity- independent manner.

It is intriguing that G9a has different domains which mediate activation and inhibition of transcription. G9a positively regulates transcription mediated by various nuclear receptors, including the steroid, estrogen, and glucocorticoid receptors, through a HMTase-independent mechanism [11,13]. It is possible that the previously characterized N-terminal domain of G9a, containing its transcriptional activation domain, mediates its transcriptional activation of *p21*. We further investigated the role of G9a in *p21* activation upon etoposide-induced DNA damage. Using *p21*-luc reporter and ChIP assays, we showed that G9a increased the promoter activity and expression of *p21* in *p53*-null H1299 cells; furthermore, we demonstrated that G9a was recruited to the *p21* promoter upon etoposide treatment. We also identified an interaction between G9a and PCAF which contributes to the transcriptional activation of *p21*, and showed that this complex is recruited to the *p21* promoter upon etoposide treatment. These results suggest that G9a and the PCAF HAT complex are both physically and functionally associated in the transcriptional activation of *p21*. Both ChIP assays and real time PCR analysis showed that the level of H3K9me2 on the *p21* promoter significantly decreased, even though G9a recruitment was increased, in the presence of etoposide. This result indicates that activation of *p21* expression by G9a does not depend on its H3K9 HMTase activity, rather, suggests possible involvement of H3K9me2 demethylase.

Coordinated epigenetic repression of *p21* has previously been reported, involving a transcriptional regulator, Gfi1. Gfi1 has been shown to recruit G9a and HDAC1 to the *p21* promoter, thereby repressing its expression in HL-60 cells [4]. Another study suggested that the RING-finger E3 ubiquitin ligase, UHRF1, is a transcriptional corepressor that functions to recruit G9a and other chromatin-modifying enzymes to the *p21* promoter [5]. In these studies, H3K9 HMTase activity was shown to play a critical role in the recruitment of corepressors such as HDAC and DNMT and thereby facilitate transcriptional repression of target genes.

The effect of *p21* upregulation by G9a was also monitored via cellular viability, proliferation, and apoptosis analyses. These experiments indicated that G9a negatively regulates cellular viability and proliferation by inducing p21-mediated apoptosis in the presence of etoposide. These observations suggest that the role of G9a in upregulation of *p21* is potentiated under stress conditions. In summary, our data demonstrate an additional role of G9a as a transcriptional coactivator that is recruited with PCAF to the *p21* promoter, and suggest that G9a plays dual functions in transcriptional regulation, which are in turn regulated by various chromatin modifiers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.01.039.

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