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PARP-2 regulates cell cycle-related genes through histone deacetylation and methylation independently of poly(ADP-ribosyl)ation

Ya-Chen Liang [a], Chiao-Yu Hsu [a], Ya-Li Yao [b], Wen-Ming Yang [a,*]

[a] Institute of Molecular Biology, National Chung Hsing University, Taichung 40227, Taiwan
[b] Department of Biotechnology, Asia University, Taichung 41354, Taiwan

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ABSTRACT

Poly(ADP-ribose) polymerase-2 (PARP-2) catalyzes poly(ADP-ribosyl)ation (PARylation) and regulates numerous nuclear processes, including transcription. Depletion of PARP-2 alters the activity of transcription factors and global gene expression. However, the molecular action of how PARP-2 controls the transcription of target promoters remains unclear. Here we report that PARP-2 possesses transcriptional repression activity independently of its enzymatic activity. PARP-2 interacts and recruits histone deacetylases HDAC5 and HDAC7, and histone methyltransferase G9a to the promoters of cell cycle-related genes, generating repressive chromatin signatures. Our findings propose a novel mechanism of PARP-2 in transcriptional regulation involving specific protein–protein interactions and highlight the importance of PARP-2 in the regulation of cell cycle progression.

1. Introduction

Poly(ADP-ribose) polymerases (PARPs) are enzymes involved in various cytoplasmic and nuclear processes, including inflammation, mitochondrial metabolism, DNA damage repair [1], and transcriptional regulation [2]. PARPs exert their functions by poly(ADP-ribosyl)ation (PARylation). In PARylation, the ADP-ribose moiety of donor NAD^+ molecule is transferred to acceptor proteins, resulting in functional changes [2]. Recent studies indicate that PARPs have emerging roles in transcriptional regulation, but the studies are limited to a few PARPs such as PARP-1 [3].

PARP-1 serves as a transcriptional co-regulator, which regulates transcription in a PARylation-independent or PARylation-dependent manner. In the PARylation-independent mode, PARP-1 represses transcription by binding and compacting chromatin [4,5]. In this reaction, the binding of PARP-1 on target chromatin restricts access of the transcription machinery [6] or blocks the binding of activators to promoters [7]. However, in the PARylation-dependent mode, PARylation of PARP-1, histone proteins [8] and transcription factors [9] causes PARP-1 to depart from chromatin, causing a loosening of chromatin [4,5,8,10], deposition of transcriptional complexes [11], and activation of transcription factors [9,12]. These two modes coordinate together to complete the regulation [13]. However, although PARP-2 shares a high sequence conservation with PARP-1 and contains PARylation activity [14], there is no direct evidence to prove that PARP-2 controls transcription in the same manners as PARP-1.

Recent studies prove that PARP-2 represses transcription by decreasing the activities or the amounts of transcription factors [15]. Through genetic studies, depletion of PARP-2 decreases expressions of transcription factor KAP1-dependent genes [16]. Depletion of PARP-2 also alters the transcriptional activities of TTF1 [17], ERα, PPARα, PPARβ, and PPARγ [18] in luciferase reporter systems. In addition, PARP-2 occupies the SIRT1 promoter and decreases the expression of SIRT1 [19]. These results suggest that PARP-2 might function as a transcriptional co-repressor. However, the molecular basis of how PARP-2 directly represses promoters and the cellular outcomes remain largely unknown.

Through molecular biochemistry approaches, here we demonstrate that PARP-2 is targeted to promoters by DNA-binding factor YY1 and recruits histone modifiers to alter chromatin signatures independently of its PARylation activity, resulting in transcriptional repression. Our study suggests that protein–protein interactions constitute a novel mechanism of PARP-2 in transcription regulation, and this finding also expands the roles of PARP-2 to cell cycle regulation.

2. Materials and methods

2.1. Plasmid constructs

pBC-mPARP-2 [20] was provided by Dr. Gilbert de Murcia, and subcloned to obtain HA/Flag/Gal4-tagged proteins. Serial Gal4-

* Corresponding author. Address: Institute of Molecular Biology, National Chung Hsing University, 250 Kuo Kuang Rd., Taichung 40227, Taiwan. Fax: +886 4 2287 4879.
E-mail address: yangwm@nchu.edu.tw (W.-M. Yang).

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PARP2 deletion constructs were made by insertion of PCR-amplified truncated region of PARP-2 into pM1 vector. Enzyme-defective mutant PARP2(E534A) and acetylation site mutants were generated by the site-directed mutagenesis procedure. Human cDNA clones of SUV39h1 and G9a were obtained from Open Biosystems and subcloned into pcDNA3.1(+)-HA(3). Native promoter plasmids of c-MET-Luc and MyoD-Luc were constructed by inserting the PCR-amplified sequences into pGL3-Basic vector. Constructs of Flag-YY1, Flag-HDAC1, 2, 3, 8, 10, TRP-1-Luc, and MITF-Luc have been described [21]. HA-HDAC7 was provided by Dr. Michael Downes [22], Flag-HDAC5 was provided by Dr. Stuart L. Schreiber [23], c-MYC-Luc was from Dr. Mark Groudine [24], p21-Luc was provided by Dr. Xiao-Fan Wang [25], Rb-Luc and CAD-Luc were provided by Dr. Peggy J. Farnham [26], and BAX-Luc was provided by Dr. M. Lienhard Schmitz [27].

2.2. Cell culture, transfection, and reporter assays

Human embryonic kidney 293 (HEK293) cells were grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and 1% penicillin–streptomycin. Transfection was done by the standard calcium phosphate procedure. Treatments were performed by refreshing the culture media with reagent-containing media. Working concentrations of reagents: 10 μM trichostatin A (TSA); 5 mM 3-aminobenzamide (3AB); 200 μM β-nicotinamide adenine dinucleotide (NAD^+). In reporter assays, 1.1×10^5 HEK293 cells were seeded into 60-mm cell culture dishes, and followed by co-transfection of 0.5 μg of pRL-TK vector, 5 μg of G3G5TK [21] luciferase reporter, and indicated expression vectors. After 48 h, luciferase assays were performed by the dual-luciferase reporter assay protocol (Promega).

2.3. shRNA methods and antibodies

Small hairpin RNA (shRNA) constructs of pSM2-PARP-2-1 (V2HS_68371), pSM2-PARP-2-2 (V2HS_68372), pGIPZ-YY1 (V2HS_219592), and pGIPZ-Pc2 (V2LHS_240091) shRNA were obtained from GenDiscovery Biotechnology. The mouse monoclonal anti-HA (H9658) and anti-FLAG M2 (F1804) were from Sigma–Aldrich and phosphatase-conjugated goat anti-mouse IgG (SAB-101) antibody was from Stressgen Biotechnology. For ChIP assays, anti-histone H3 (tri-methyl K9) antibody (ab8898) was from Abcam plc., and anti-acetyl-lysine antibody (06-933) was from Upstate Biotechnology.

2.4. Co-immunoprecipitation (co-IP)

Transfected HEK293 cells were collected and lysed in PBS (0.1% NP-40). For IP anti-Flag, the supernatants of lysates were incubated with 20 μl of anti-FLAG M2-agarose (Sigma, A2220), washed, and eluted by adding 80 μl of 2× SDS-sample buffer into the agarose.

2.5. Chromatin immunoprecipitation (ChIP) assay

HEK293 cells were transfected with indicated plasmids. After 48 h, cross-linking was performed by adding formaldehyde and stopped by glycine. After sonication, 25 μg of DNA was used in IP with indicated antibodies. After phenol–chloroform DNA extraction, precipitated and input DNA fragments were amplified by PCR using indicated primer pairs. Details of primer sequences are available upon request.

2.6. MTT assay

HEK293 cells were grown in 24-well plates and transfected with indicated shRNA plasmids. After 48 h, cells were refreshed with 0.5 ml of media containing 0.5 mg/ml of 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and cultured for 1 h. The culture media was discarded and 1 ml of dimethyl sulfoxide (DMSO) was added for 10 min. One hundred microliters of supernatant was transferred into a 96-well plate for measuring absorbance on an ELISA reader at 570 nm.

2.7. Cell cycle analysis by flow cytometry

HEK293 cells were transfected with indicated shRNA or expression constructs. After 24 h, cells were passed into 100-mm cell culture dishes and incubated for another 24 h. Cells were then treated with indicated reagents. After fixing in 70% ethanol, 1×10^6 cells were stained in 400 μl of PI staining solution and measured by flow cytometer (Becton Dickinson FACScalibur). The results were analyzed with a modeling program, ModFit LT V3.0 (Verity Software House, Inc.), for estimation of percentages of cells in G1, S, and G2/M phases.

3. Results and discussion

3.1. PARP-2 represses transcription independently of PARylation

To explore whether PARP-2 processes transcriptional activity, Gal4-based luciferase assays were used. PARP-2 was fused to a Gal4 DNA-binding domain and co-transfected into HEK293 cells with a luciferase reporter construct (G3G5TK) containing five Gal4 binding sites. As shown in Fig. 1A, Gal4-PARP2 had a significant transcriptional repression activity compared to Gal4 alone, and co-transfection with PARP-2 shRNA (shPARP2) eliminated the repressional activity. To further identify the domain responsible for transcriptional repression, truncated Gal4-PARP2 proteins were generated and used in the Gal4 system (Fig. 1B). Our result indicated that the catalytic domain (401–451 a.a.) of PARP-2 [28] had a significant transcriptional repression activity compared to Gal4 alone, Gal4-PARP2(280–400), and Gal4-PARP2(452–559). Furthermore, to elucidate whether PARylation activity was involved in the repressional activity, PARP-2 enzyme-defective mutant, PARP-2(E534A), was used in the Gal4 system. As shown in Fig. 1D, the transcriptional repression activity of Gal4-PARP2(E534A) was not significantly different from that of Gal4-PARP2. To further confirm that PARylation cannot affect the transcriptional repression activity of PARP-2, PARP inhibitor 3-aminobenzamide (3AB) and donor nicotinamide adenine dinucleotide (NAD^+) were added in the luciferase assay. Treatments of 3AB and NAD^+ made no significant changes to the repressional activity of Gal4-PARP2 (Fig. 1D). These results demonstrated that, unlike PARP-1, which regulates transcription through PARylation [29], PARP-2 regulated transcription independently of its PARylation activity, although its catalytic domain is still required.

To determine which sites regulate the transcriptional activity of PARP-2, PARP-2 lysine 36 and 37 mutants were used in the Gal4 system. PARP-2 K36 and K37 are the well-documented sites for modifications such as acetylation and auto-mono(ADP-ribosyl)ation [30]. As shown in Fig. 1E (left panel), Gal4-PARP2(K36R) and Gal4-PARP2(K36/37R), but not Gal4-PARP2(K37R), had significantly reduced repressional activity compared to Gal4-PARP2. This result indicated that PARP-2 K36 played a critical role in transcription regulation. To elucidate whether the acetylation of K36 affected the transcriptional repression, mutants that mimic PARP-2 acetylation, K36Q and K37Q were used. Gal4-PARP2(K36Q), but not Gal4-PARP2(K37Q), showed significantly reduced repressional activity compared to Gal4-PARP2 (Fig. 1E, right panel). This result indicated that acetylation cannot alter the transcriptional repression activity of PARP-2 and confirmed that K36 is the key site

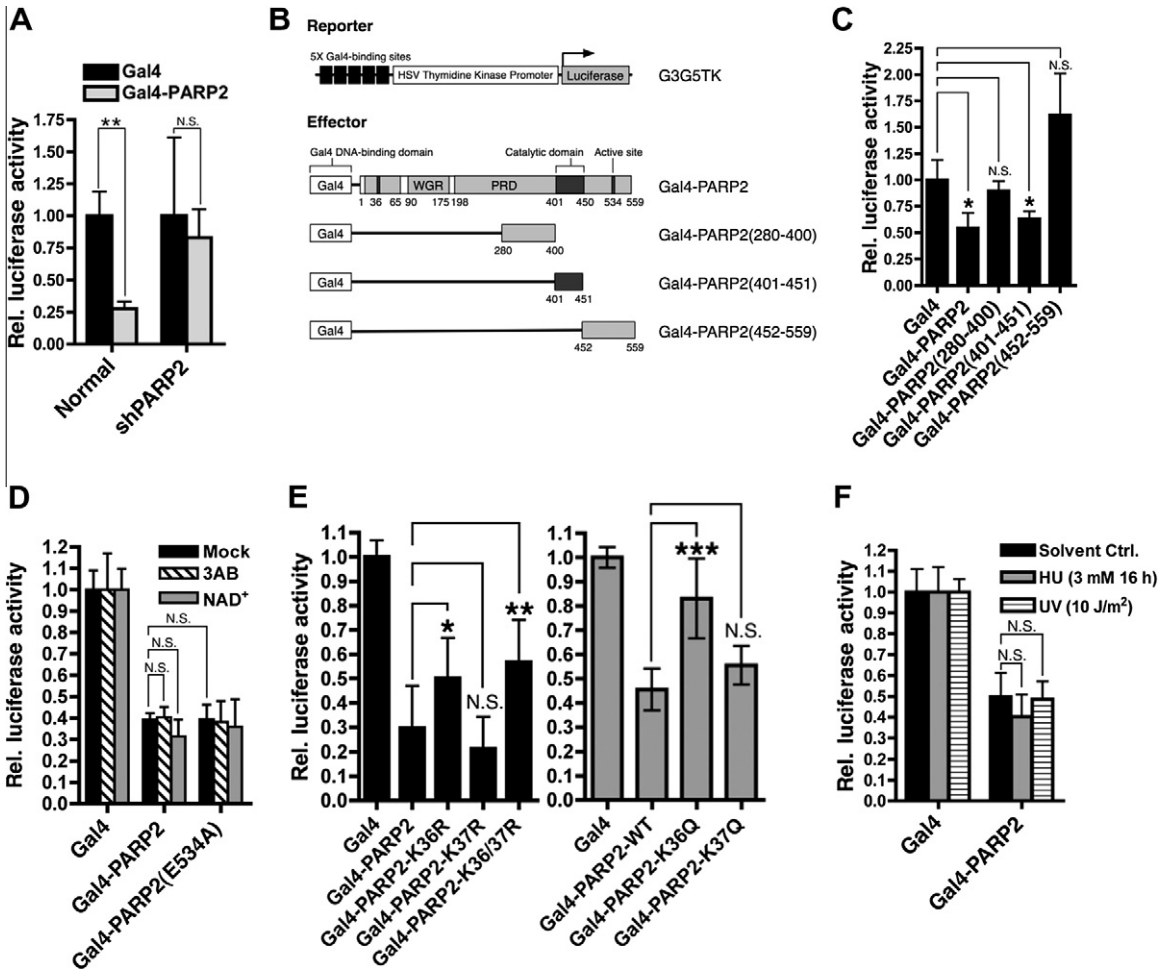

Fig. 1. The transcriptional repression activity of PARP-2 is independent of PARylation. (A) PARP-2 processes transcriptional repression activity. Gal4-PARP2 or Gal4 expression vectors were co-transfected with the reporter construct (*G3G5TK*) and/or shPARP2. After 48 h, luciferase activities were measured as described [34]. Values were normalized to Gal4 alone. The results are represented as mean + SD from at least two separate transfections. Statistical significance was evaluated by Student's *t*-test. $^{**}p < 0.01$; N.S., non-significant, $p > 0.05$. (B) Illustration of the reporter construct (*G3G5TK*) and Gal4-PARP2 truncated fusion proteins. WGR, tryptophane-, glycine- and arginine-rich domain. PRD, PARP regulatory domain. (C) PARP-2 represses transcription through amino acid positions 401–451. Gal4-PARP2 truncated proteins or Gal4 expression vectors were co-transfected with the *G3G5TK*. The luciferase activity was determined and analyzed as described in the legend of panel A. $^{*}p < 0.05$. (D) PARP-2 represses transcription independently of PARylation activity, 3AB, and NAD^+. Gal4-PARP2, Gal4-PARP2(E534A), or Gal4 expression vectors were co-transfected with *G3G5TK*. After 24 h, the cells were treated with 5 mM 3AB or 200 μM NAD^+ for 24 h. (E) PARP-2 lysine 36 affects the transcriptional activity. PARP-2 acetylation site mutants (K36R, K37R, K36/37R, K36Q and K37Q) or Gal4 vector was co-transfected with *G3G5TK*. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; N.S., non-significant. (F) HU treatment and UV irradiation have no effect on the transcriptional activity of PARP-2. Gal4-PARP2 or Gal4 vector was co-transfected with *G3G5TK*. After 24 h, cells were treated with 3 mM HU for 16 h or 10 J/m^2 UV irradiation.

conferring the repressional efficiency of PARP-2. These results implicated that PARP-2 K36 might be regulated by other post-translational modifications.

PARP-2 has long been characterized as a DNA damage-dependent PARP [28]. To clarify whether DNA damage-induced PARylation activity alters the transcriptional repression activity of PARP-2, hydroxyurea (HU) treatment and UV irradiation were applied during the Gal4-based transcriptional analysis. Our result indicated that HU treatment and UV irradiation made no significant differences in the transcriptional repression activity of PARP-2 compared to Gal4-PARP2 alone (Fig. 1F).

3.2. HDAC5, HDAC7, and G9a are involved in the transcriptional repression of PARP-2

Transcriptional repression by histone deacetylases (HDACs) and methyltransferases (HMTs) has been thoroughly investigated, and previous studies indicate connections between PARPs and HDACs [31]. We hypothesized that PARP-2 functions as a transcriptional co-repressor, which recruits HDACs and HMTs to target promoters to generate repressive chromatin. To examine the hypothesis, several HDACs and HMTs were screened for their interactions with PARP2. Co-IP results indicated that HDAC1, 2, 3, 5, 7, 8, 10, SirT1 and HMT SETDB1, SUV39H1, and G9a interacted with PARP-2 (Fig. 2A and B, and data not shown). To identify HDACs and HMTs involved in the transcription regulation of PARP-2, we further screened these HDACs and HMTs for their ability to enhance the transcriptional repression activity of PARP-2. Our results showed that only HDAC5, HDAC7, and G9a significantly enhanced the transcriptional repression activity of PARP-2 (Fig. 2C and D, and data not shown). To confirm PARP-2 recruited HDACs and HMTs to a target promoter, ChIP assays were performed. As shown in Fig. 2E, *G3G5TK* was pulled-down by anti-Flag antibody when Flag-HDAC5 and Gal4-PARP2 were co-expressed, showing that HDAC5 was recruited to the PARP-2 target promoter.

These results demonstrated that PARP-2 serves as a transcriptional co-repressor, which recruited HDAC5, HDAC7, and histone methyltransferase G9a to target promoters through protein–protein interactions, resulting in transcriptional repression. Although PARP-2 interacted with numerous histone modifiers,

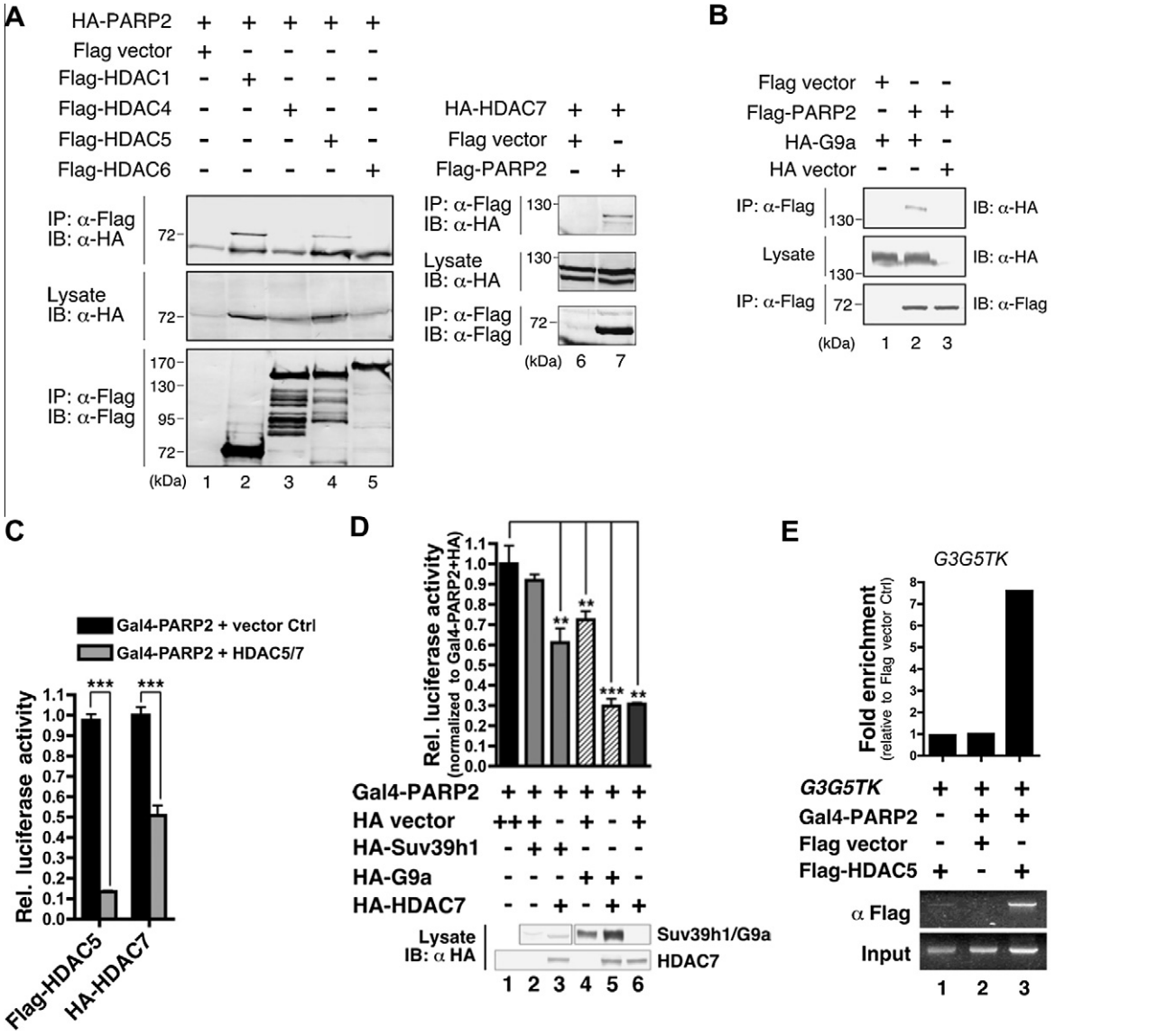

Fig. 2. HDAC5, HDAC7, and G9a are involved in the transcriptional repression activity of PARP-2. (A and B) PARP-2 interacts with HDAC5, HDAC7, and G9a. Expression plasmids, as indicated, were transfected into HEK293 cells. Anti-Flag immunoprecipitates of cell lysates were immunoblotted with anti-Flag or anti-HA antibodies as indicated. (C) HDAC5 and HDAC7 enhance the transcriptional repression activity of PARP-2. Gal4-PARP2 expression vector was co-transfected with *G3G5TK* and/or expression constructs of Flag-HDAC5, HA-HDAC7, or Flag/HA vector controls (vector Ctrl). $^{***}p < 0.001$. (D) G9a, but not SUV39H1, enhances the repressional activity of PARP-2. Gal4-PARP2 was co-transfected with *G3G5TK*, and HA-SUV39H1, HA-G9a, HA vector, and/or HA-HDAC7 expression constructs. The protein expressions are presented in the bottom panel. (E) The recruitment of Flag-HDAC5 on the target promoter of PARP-2 was assessed by ChIP assay, using anti-Flag antibody. The Flag vector expression set serves as negative control.

only HDAC5, HDAC7, and G9a were involved in the transcriptional regulation of PARP-2 in the Gal4-based luciferase system. We postulate that PARP-2 might interact with different histone modifiers or associate with different DNA-binding factors in different transcriptional systems.

3.3. PARP-2 is recruited to c-MYC promoter by YY1 and establishes repressive chromatin

To screen responsive promoters of PARP-2, a panel of native promoter constructs was used in the luciferase reporter assay. As shown in Fig. 3A, cell cycle-related promoters of *p21*, *RB*, *E2F1*, and *c-MYC* were significantly repressed and the *c-MET* promoter was significantly activated when Flag-tagged PARP-2 was co-transfected. Other cell cycle-unrelated promoters such as *MITF*, *CAD*, and *BAX* were not significantly regulated by PARP-2. To address the role of PARP-2 in the transcriptional repression of cell cycle-related genes, we selected the *c-MYC* promoter as a model for our study. A previous study indicates that DNA-binding factor YY1 coordinates HDAC5 to regulate transcription [32]. Therefore, we proposed that PARP-2 coordinated YY1 in repressing *c-MYC* promoter. To determine if YY1 contributed to the transcriptional repression activity of PARP-2, Flag-YY1 was co-transfected with HA-PARP2 in a *c-MYC* promoter-based reporter assay (*c-MYC-Luc*). As shown in Fig. 3B, the *c-MYC* promoter was significantly repressed with co-expression of Flag-YY1 and HA-PARP2, indicating that YY1 enhanced the transcriptional repression activity of PARP-2. To demonstrate that PARP-2 coordinated with YY1 through protein–protein interactions, co-IP was performed. Our result showed that Flag-YY1 pulled down HA-tagged PARP-2 in HEK293 cells through both the *N*- and the *C*-termini (Fig. 3C and data not shown). To confirm that YY1 recruited PARP-2 to the

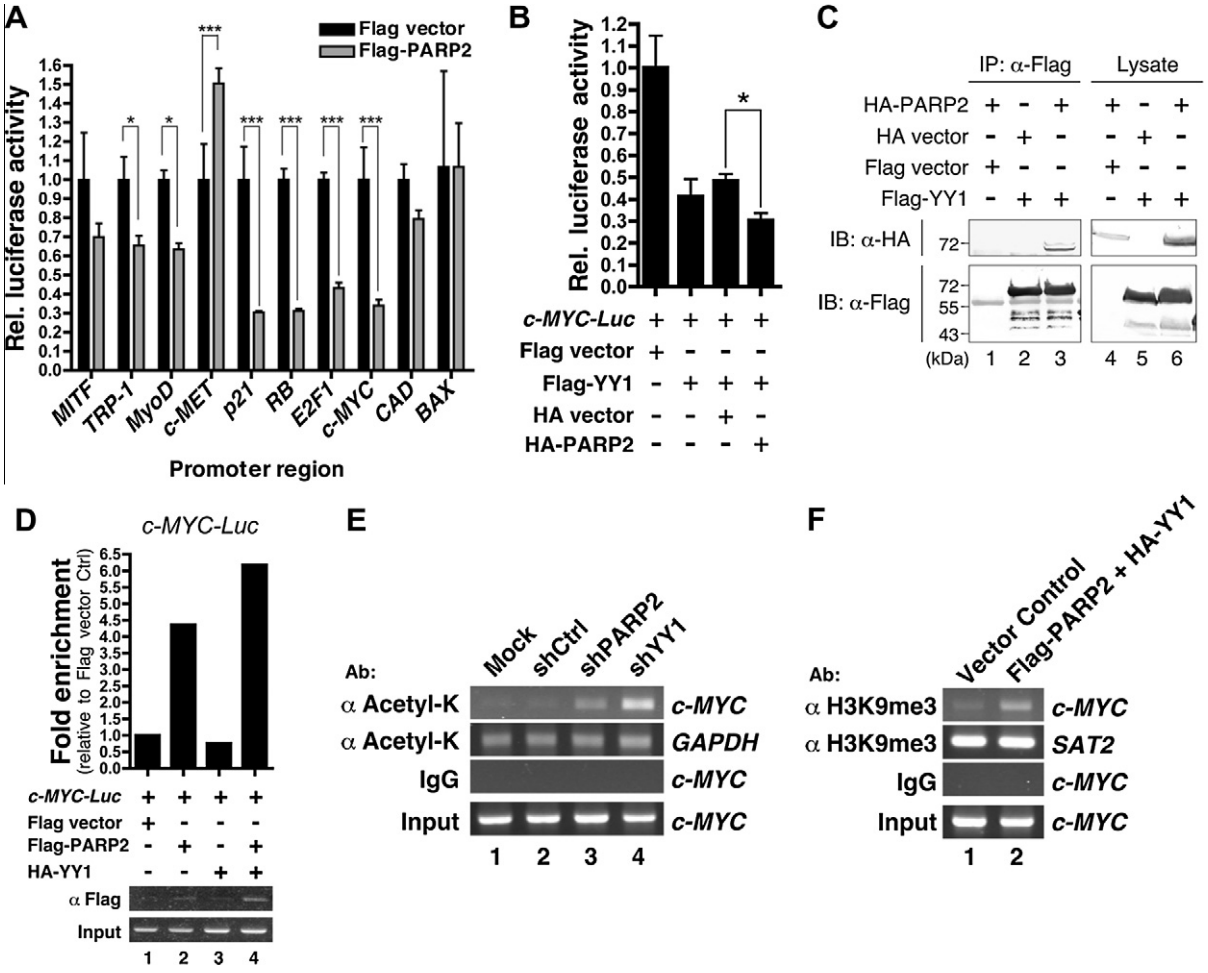

Fig. 3. PARP-2 is recruited to *c-MYC* promoter by YY1 and establishes repressive chromatin. (A) PARP-2 represses cell cycle-related promoters. Flag vector alone or Flag-PARP2 expression vector was co-transfected with a panel of native promoter reporter constructs. $^{*}p < 0.05$; $^{***}p < 0.001$. (B) The repressional activity of PARP-2 on *c-MYC* promoter is enhanced by YY1. Flag vector alone or Flag-YY1 expression vector was co-transfected with HA-PARP2 and *c-MYC-Luc*. $^{*}p < 0.05$. (C) Flag-YY1 pulls down HA-PARP2. Expression plasmids, as indicated, were transfected into HEK293 cells. Anti-Flag immunoprecipitates of cell lysates were immunoblotted with anti-Flag or anti-HA antibodies as indicated. (D) ChIP assay shows that PARP-2 occupies *c-MYC* promoter, and YY1 enhances the occupancy. The intensities of bands in DNA agarose gels were quantified, normalized to vector control, and presented as fold enrichment. (E) ChIP assay with indicated antibodies presents that knockdown of PARP-2 or YY1 increases the acetyl-K levels of *c-MYC* promoter. GAPDH primer set serves as positive control, and ChIP with anti-GFP antibody (IgG) serves as negative control. (F) ChIP assay with indicated antibodies shows that co-expression of PARP-2 and YY1 increases the H3K9me3 levels of *c-MYC* promoter. SAT2 primer set serves as positive control, and ChIP with anti-GFP antibody (IgG) serves as negative control.

c-MYC promoter, ChIP assay with anti-Flag antibody was used. As shown in Fig. 3D, Flag-PARP2 pulled down *c-MYC-Luc* (lane 2) and HA-YY1 increased the pull-down intensity (lane 4). These results indicate that YY1 increased the occupancy of PARP-2 on *c-MYC* promoter through protein–protein interactions, enhancing the transcriptional repression activity of PARP-2.

To further examine if the chromatin structure is affected by PARP-2 and YY1, ChIP assays detecting the status of endogenous *c-MYC* promoter were performed using anti-acetyl-lysine (acetyl-K) and anti-histone H3 lysine 9 tri-methylation (H3K9me3) antibodies. These results demonstrated that the depletion of PARP-2 or YY1 by shRNA increased the acetyl-K levels of *c-MYC* promoter compared to transfection of control shRNA (Fig. 3E, lanes 3 and 4 compared to lane 2). Moreover, the H3K9me3 levels were increased with co-overexpression of PARP-2 and YY1 compared to parental expression vectors (Fig. 3F).

In summary, our results support a transcriptional co-repressor model of PARP-2 in which YY1 targets PARP-2 to the cell cycle-related *c-MYC* promoter, and PARP-2 further recruits HDAC5, HDAC7, and G9a to decrease the histone lysine acetylation levels and to increase H3K9me3 levels, causing transcriptional repression. These results indicate that although PARP-2 contains a DNA-binding domain, selections of target promoters and efficient bindings still require specific DNA-binding factors such as YY1. Our results also suggest that other target promoters of PARP-2 might be regulated by different DNA-binding factors, but the mechanisms need to be further clarified.

3.4. PARP-2 mediates the cell cycle

Our results showed that PARP-2 regulated cell cycle-related genes (Fig. 3). To further elucidate the outcomes of transcriptional regulations by PARP-2 in cell cycle regulation, we assayed cell viability by MTT assay with the depletion of PARP-2 or YY1. Our result demonstrated that cell viability decreased significantly ($p < 0.001$) with transfection of shPARP2 or shYY1 compared to transfection of water or control shRNA. The depletion of PARP2 was efficient as shown in Fig. 4B. To address the function of PARP-2 in regulating the cell cycle, cell cycle profile was analyzed by flow cytometry after propidium iodide (PI) staining. As shown in Fig. 4C (left panel), depletion of PARP-2 results in a minor G2/M arrest (12–15%) compared to non-transfected cells, and this result was

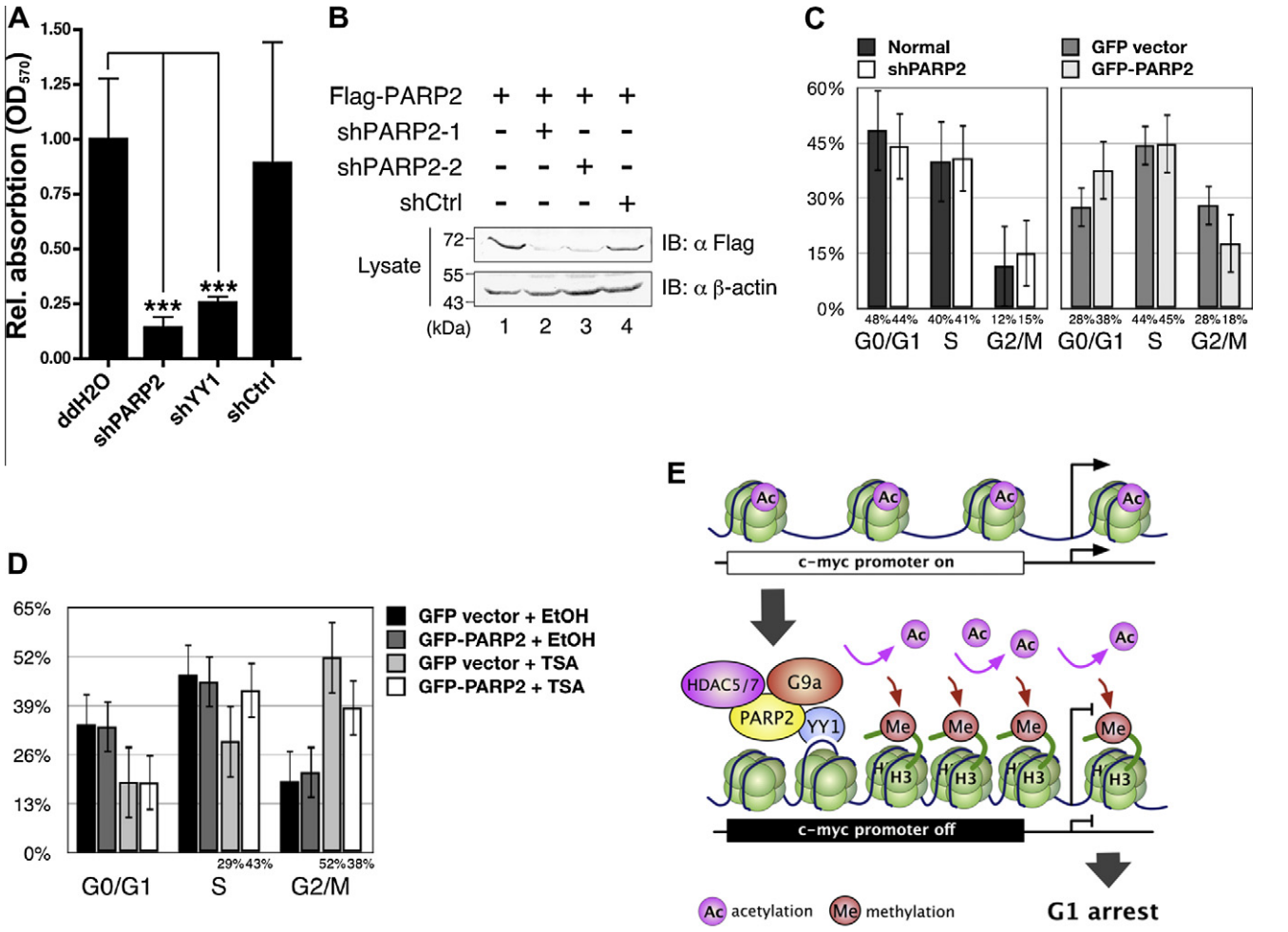

Fig. 4. PARP-2 mediates the cell viability and the cell cycle. (A) MTT assay shows that cell viability is decreased under the depletion of PARP-2 or YY1. All values were normalized to transfection with buffer. The results are mean + SD from at least two separate transfections. $^{***}p < 0.001$. (B) Flag-PARP2 was co-transfected into HEK293 cells with or without shPARP2-1, shPARP2-2, or shPC2 (shCtrl) constructs. After 48 h, lysates were immunoblotted with anti-Flag or anti-β-actin antibodies as indicated. (C) Depletion of PARP-2 results in a minor G2/M arrest, while overexpression of PARP-2 causes prolonged G0/G1 accumulation. Cell cycle profiles were analyzed after propidium iodide staining of cells that had been transfected with shPARP2 (left panel) or expressed GFP-PARP2 (right panel). The results are represented as mean ± SE from at least two independent transfections. (D) Cell cycle analysis by flow cytometry shows that PARP-2 eliminates the G2/M arrest caused by HDAC inhibitor TSA. (E) Summary of the role of PARP-2 in transcription regulation of cell cycle-related genes.

consistent with a previous study in *Parp2*$^{-/-}$ cells [33]. Moreover, cells expressing GFP-tagged PARP-2 had a prolonged G0/G1 accumulation (28–38%) compared to cells expressing the GFP tag (Fig. 4C, right panel). To demonstrate that PARP-2 regulated the cell cycle in an HDAC-mediated manner, HDAC inhibitor Trichostatin A (TSA) was added during the cell cycle analysis. As shown in Fig. 4D, cells expressing GFP-tagged PARP-2 eliminated the G2/M arrest caused by TSA (52–38%).

These results suggested that PARP-2 controls G1 progression by negatively regulating *c-MYC*, preventing premature G1 exit. These findings implicate that the co-repressor function of PARP-2 prevents the inappropriate induction of *c-MYC* and apoptosis. We propose a transcriptional co-repressor model of PARP-2 (Fig. 4E) in which PARP-2 is recruited by DNA binding factor YY1 to the *c-MYC* promoter, binds HDAC5, HDAC7, and G9a to generate repressive chromatin, and prevents premature cell cycle progression.

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