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PARP1 controls KLF4-mediated telomerase expression in stem cells and cancer cells

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ABSTRACT

Telomerase is highly expressed in cancer and embryonic stem cells (ESCs) and implicated in controlling genome integrity, cancer formation and stemness. Previous studies identified that Krüppel-like transcription factor 4 (KLF4) activates telomerase reverse transcriptase (TERT) expression and contributes to the maintenance of self-renewal in ESCs. However, little is known about how KLF4 regulates TERT expression. Here, we discover poly(ADP-ribose) polymerase 1 (PARP1) as a novel KLF4-interacting partner. Knockdown of PARP1 reduces *TERT* expression and telomerase activity not only in cancer cells, but also in human and mouse ESCs. Recruitment of KLF4 to *TERT* promoter is reduced in PARP1-suppressed cells. The poly(ADP-ribose) polymerase activity is dispensable, while the oligo(ADP-ribose) polymerase activity is required for the PARP1- and KLF4-mediated *TERT* activation. Repression of Parp1 in mouse ESCs decreases expression of pluripotent markers and induces differentiation. These results suggest that PARP1 recruits KLF4 to activate telomerase expression and stem cell pluripotency, indicating a positive regulatory role of the PARP1–KLF4 complex in telomerase expression in cancer and stem cells.

INTRODUCTION

Telomeres are mainly elongated by the telomerase complex, a telomerase reverse transcriptase (TERT) and an integral RNA subunit (TERC) (1). Transcriptional regulation of TERT is a major limiting factor of telomerase activity in human cells (2). Embryonic and other stem cells maintain high levels of telomerase activity, which are essential for long-term stem cell self-renewal (3). A proper telomere maintenance system is necessary for its replicative potential (4–6), as shortened telomeres are associated with differentiation and aging (7). During the reprogramming of differentiated cells into pluripotent stem cells, telomeres are elongated by telomerase and telomeres of induced pluripotent stem cells (iPSCs) acquire similar epigenetic marks of mouse embryonic stem cells (ESCs) (8).

Krüppel-like factors (KLFs) are a family of DNA-binding transcriptional factors linked by a triple zinc finger DNA-binding domain (DBD) that modulates diverse and essential functions in multiple cellular processes, including proliferation, differentiation, migration, inflammation and pluripotency (9,10). Among them, Krüppel-like transcription factor 4 (KLF4) received significant attention due to the discovery that expression of KLF4 and other three transcription factors can reprogram somatic cells into iPSCs (11–17). KLF4 is expressed in a variety of tissues, including intestinal epithelium and skin, and is important for development, differentiation and maintenance of normal tissue homeostasis (18). KLF4 can both activate and repress transcription, depending on the contents of target promoters and its interacting partners (19–21). Also, KLF4 func-

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tions as an oncogene or a tumor suppressor depending on the types of cancers (18).

Previous studies demonstrated that KLF4 is required for maintaining *hTERT* expression in human ESCs and cancer cells (22). β-Catenin was further identified to be recruited by Klf4 to the promoter of *Tert* to activate telomerase expression in cancer and mouse ESCs (23). Klf4 also activates pluripotent gene *Nanog* (24) and represses endoderm differentiation genes *Gata6* and *Sox17* (25). These findings may explain why KLF4 maintains ESC renewal. However, whether other key components modulate KLF4-mediated *TERT* expression and pluripotency preservation is still not clear.

Here, we identified PARP1 as a novel KLF4-interacting protein. As the founding member of the PARP enzyme family, PARP1 is a nuclear enzyme responsible for post-translational poly(ADP-ribosyl)ation (or PARylation) modification that covalently transfers mono- or oligomeric ADP-ribose moieties from NAD^+ to itself and other acceptor proteins (26). Its structure contains an N-terminal segment of DBD, nuclear localization signal, a breast cancer type 1 susceptibility protein (BRCA1) C-terminus (BRCT)/Automodification domain (AMD) for protein–protein interaction and self-inhibitory modification and a C-terminal catalytic domain (CAT) for PARylation. PARP1 participates in a broad range of critical cellular processes including chromatin remodeling, DNA repair, genome integrity and cell death (27). It also collaborates with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or p53 for transcriptional regulation (28).

In this study, we demonstrate that PARP1 modulates telomerase expression and stemness maintenance. PARP1 controls the recruitment of KLF4 to the *TERT* promoter, and is important for Klf4-mediated *Tert* expression. These results delineate PARP1 as a key regulator for KLF4 recruitment to thereby enhance telomerase expression and stemness.

MATERIALS AND METHODS

Cell culture and transfection

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone). FaDu (squamous cell carcinoma) and oral epidermoid carcinoma (OECM1) cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 Medium containing 10% FBS. Transfection of the plasmid DNAs was performed using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. NTU1 (hESCs) (29) were maintained as undifferentiated cells on inactivated mouse embryonic fibroblast (MEF) feeder in DMEM/F12 supplemented with 20% Knockout Serum Replacement (Invitrogen), 1 mM glutamine, 0.1 mM nonessential amino acid, 4 ng/ml basic fibroblast growth factor and 0.1 mM β-mercaptoethanol. D3 mouse ESCs were cultured on inactivated SNLP 76/7-4 feeders (a puromycin resistant derivative of SNL76/7) in DMEM supplemented with 15% FBS, 1 mM L-glutamine, 100 μM non-essential amino acid, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 1-fold penicillin/streptomycin and 1000 U/ml leukemia inhibitory factor (LIF) (Millipore). In feeder-free cultured cells, mouse ESCs were grown on culture dishes coated with 0.2% bovine gelatin (Sigma-Aldrich). Mouse ESCs cells were maintained in DMEM supplemented with 15% Knockout Serum Replacement with 1000 U/ml LIF. The cells were passaged every 2–3 days, and the medium was changed daily. Transfected cells were selected with 1 μg/ml puromycin and 5 μg/ml blasticidin. For PiggyBac transposition, 10^7 mouse ES cells were electroporated with 20 μg of donor plasmids (pXL-T3-Neo-UGm-cHS4X) and 4 μg of helper plasmids (pTriEx-NP-mPB) (30). Electric pulses were provided by a BTX Electro Square Porator EM830 (Harvard Apparatus, Inc.; 230 V, 0.77 ms). After electroporation, mouse ES cells from each cuvette were seeded onto a 10-cm plate with a feeder layer. One day after electroporation, drug selection was conducted with 400 μg/ml G418 for 2 weeks.

Plasmid constructions and viral transduction

pcDNA3-KLF4 was obtained as previously described (22). KLF4 cDNAs were inserted into the BamH1 sites of the pcDNA3-HA vector. The pGEX4T-1-KLF4 and pET28a-KLF4 mutants were constructed by cloning the PCR-amplified fragment of pcDNA3-KLF4 into the vectors (Supplementary Table S1). pcDNA3.1/myc-His-PARP1 was kindly provided by Dr Masashi Idogawa (31). pGEX4T-1-PARP1 mutants were constructed by cloning the polymerase chain reaction (PCR)-amplified fragments of pcDNA3.1/myc-His-PARP1 into pGEX4T-1. PARP1-specific shRNA and control shRNA (shLuc) were obtained from Academia Sinica, RNAi core. We followed their protocol for lentiviral transduction. About 7.5 μg of shRNA plasmid, 6.75 μg of lentiviral vector pCMVD8.91 and 0.75 μg of envelope plasmid pMD.G (VSV-G expressing plasmid) were co-transfected into HEK293T cells in the 10 cm tissue culture plate. Eighteen hours later, the medium was changed to bovine serum albumin (BSA)-containing media, and harvested in 24 and 48 h for clearance by centrifugation (3000 rpm for 5 min) and filtration (0.22 μM).

Immunoprecipitation and mass spectrometry analysis

293T cells were transfected with pcDNA3-KLF4 for 48 h. Cells were washed twice with phosphate-buffered saline (PBS) and lysed in immunoprecipitation (IP) buffer (150 mM NaCl, 1% NP-40, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris–HCl [pH 7.5]), supplemented with Complete EDTA-free Protease Inhibitor Cocktail (Roche). Anti-KLF4 antibody (Santa Cruz) was added to a final concentration of 2 μg/ml lysate and incubated for overnight at 4°C. Lysates were then incubated with protein G Sepharose (GE Healthcare) for 2 h with end-to-end mixing at 4°C. After extensive washing with IP buffer for three times, the bound proteins were eluted with 50 μl of 2× sodium dodecyl sulphate (SDS) sample buffer. To identify the potential KLF4-interacting proteins, a gel-based proteomic approach was used. Briefly, comparing to the control lane, the additional bands from the KLF4-pulldown were cut and subjected to in-gel tryptic digestion followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. All the MS and MS/MS

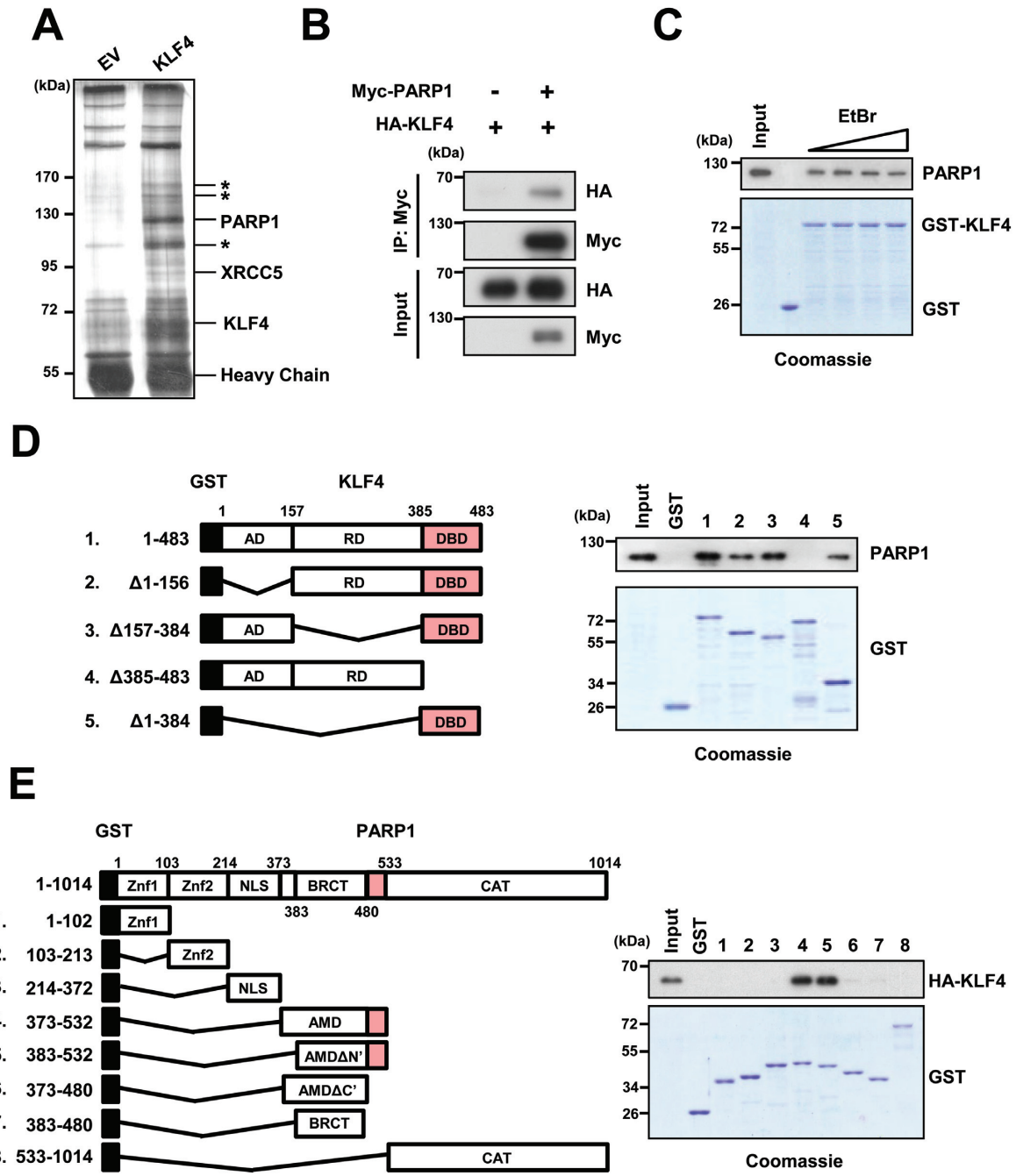

Figure 1. PARP1 associates with KLF4 through protein–protein interaction. (**A**) Identification of PARP1 as a KLF4-interacting protein by IP combined with mass spectrometry analysis. A representative silver-stained gel obtained from IP and two interacting proteins, PARP1 and XRCC5, identified by MS are shown. Asterisks indicate additional sliced gel bands (No. 1–5 in Supplementary Table S4) prepared for MS analysis. EV stands for the empty vector. (**B**) PARP1 co-immunoprecipitates KLF4. HEK293T cells were transfected with HA-KLF4 and Myc-PARP1. Anti-Myc precipitates were analyzed by western blotting with HA and Myc antibodies. (**C**) GST pull-down assay demonstrates the protein–protein interaction between KLF4 and PARP1 in the absence of contaminating DNA. GST or GST–KLF4 fusion proteins were incubated with HEK293T lysates containing endogenous PARP1 in the presence or absence of EtBr (0, 50, 100 and 200 μg/ml). KLF4 was pulled down with glutathione–agarose beads, and co-purified PARP1 was analyzed by western blotting (top panel). Coomassie blue staining indicates the expression level of recombinant proteins (lower panel). (**D**) A schematic diagram of GST-fused KLF4 constructs illustrates its domains. Full-length KLF4 contains an N-terminal activation domain (AD), a central repression domain (5) and a C-terminal DBD (left panel). GST-tag is marked in black and the interacting region is colored differently. Pull-down assay was conducted with the indicated GST fusion proteins and lysates of HEK293T cells. Co-purified PARP1 was analyzed by western blotting, and the expression level of each purified GST–KLF4 fusion protein was detected by Coomassie blue staining (right panel). (**E**) A schematic diagram of GST-fused PARP1 illustrates its domains: zinc finger 1 (Znf1), zinc finger 2 (Znf2), nuclear localization signal (NLS), AMD, BRCA1 C-terminus (BRCT) and CAT (left panel). GST-tag is marked in black and the interacting region is colored differently. Pull-down assay was conducted with the indicated GST fusion proteins and HA-KLF4 expression HEK293T lysates. Co-purified KLF4 was analyzed by western blotting with an anti-HA antibody, and purified GST–PARP1 fragments were detected by Coomassie blue staining (right panel).

data were analyzed and processed using Proteome Discoverer (version 1.2; Thermo Scientific). The top six fragment ions per 100 Da of each MS/MS spectrum were extracted for a protein database search using the Mascot search engine (version 2.2.03, Matrix Science) against the UniProtKB/Swiss-Prot sequence database. For co-IP assay, 293T cells were transfected with pcDNA3.1-His/Myc-PARP1 and pcDNA3-HA-KLF4 and IP was conducted as the procedure described above using an anti-Myc antibody (Roche). For mouse ESC co-IP assay, IP was performed with D3 mouse ESC lysates as described above using an anti-KLF4 antibody (Santa Cruz). Precipitates were analyzed by western blotting using antibodies against Klf4 (Abcam), Parp1 (Cell Signaling) and β-catenin (Cell Signaling).

In vitro binding assay and detection of poly(ADP-ribose) chains

For *in vitro* binding assay, purified Glutathione S-Transferase (GST) tagged fusion KLF4-truncated domains or GST fusion PARP1-truncated domains were coupled to glutathione–agarose beads in IP buffer (150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 10 mM Tris–HCl [pH 7.5]), supplemented with Complete EDTA-free Protease Inhibitor Cocktail (Roche). Glutathione–agarose-coupled GST proteins were subsequently incubated for overnight at 4°C with an equal amount of the 293T or 293T expressing HA-KLF4 lysates. The beads were washed several times, and bound proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and followed by western blotting. For *in vitro* binding assay between recombinant *Escherichia coli* expressed proteins, purified GST, the GST–PARP1 AMD or its C-terminal domain truncated fusion was co-incubated with an equal amount of His_6 fusion KLF4 DBD proteins, and GST pull-down was conducted as described above. Poly(ADP-ribose) (PAR) was detected by using an antibody against PAR (Genetex).

RNA purification and quantitative reverse transcription PCR (qRT-PCR)

RNA was extracted using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized using SuperScript III Cells Direct cDNA Synthesis Kit (Invitrogen). Quantitative reverse transcription PCR (qRT-PCR) was performed on an ABI PRISM7500 Sequence Detection System or Biorad CFX Connect Real-Time PCR Detection System. All primer sequences for PCR are listed in Supplementary Table S2.

siRNA and telomeric repeat amplification protocol (TRAP)

KLF4 and PARP1 siRNAs were synthesized by Silencer Select Predesigned siRNA system (Ambion), targeting the coding regions of the human KLF4 and PARP1 genes, respectively. Silencer Select Negative Control #1 siRNA (NCsi) was used as a negative control. All oligo sequences for siRNA are available upon request. For transient transfections, NTU1 (hESCs) were seeded on the inactivated MEF feeder in 6-well plates. On the following day, specific siRNA (10 nM) duplexes or control duplexes transfection was performed by using Lipofectamine RNAiMAX. Cells were harvested 48 h after transfection. Telomeric repeat amplification protocol (TRAP) assay was conducted and quantified as previously described (22). Whole-cell extracts were prepared by detergent lysis and assayed by the PCR-based telomere amplification protocol using 50 ng of FaDu and OECM1 lysates and 12.5 ng of mouse ESC lysates. The PCR reactions were conducted for 35 cycles. Telomerase activity was calculated by the ratio of the sum intensity of the entire ladder to the signal of the amplified internal control. Telomerase activity was normalized between experiments using the sum intensity values of each particular cell line.

Luciferase reporter assay

About +18 to +77 of the *hTERT* promoter region was PCR amplified and inserted into the luciferase reporter vector, pXP2 (22). This reporter construct (0.5 μg) and pcDNA3 or pcDNA3-KLF4 (1 μg) were transfected into control or PARP1 knockdown 293T cells using the calcium phosphate transfection method. Renilla luciferase plasmid (0.1 μg) was transfected to serve as an internal control for transfection efficiency. Dual luciferase reporter assay was performed as manufacturer's instruction (Promega).

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) assay was performed as previously described (22). The following antibodies were used for the IP reactions: anti-KLF4 antibody (SC-20691, Santa Cruz) and anti-PARP1 antibody (46–0705, Invitrogen). DNA was analyzed by qPCR using the primers at the *hTERT* promoter, and the *CCNA1* promoter was used as a negative control. All primer sequences for PCR are listed in Supplementary Table S3.

Alkaline phosphatase staining

Mouse ESCs were seeded on a gelatin-coated plate for 5 days selection with puromycin. Cells were washed with PBS, fixed with paraformaldehyde for 3 min and stained with the Alkaline Phosphatase Substrate Kit III (SK-5300, Vector, Burlingame).

Statistical analysis

All of the continuous variables are expressed as the mean ± standard deviation (s.d.). Differences between groups were tested using Student's *t*-test. A P-value < 0.05 was considered statistically significant.

RESULTS

Identification of PARP1 as a KLF4-interacting protein

KLF4 functions as a transcriptional activator or repressor depending on the target genes and its interacting partners (18,32). Furthermore, KLF4 is associated with several interacting proteins, such as p53, CBP/P300 and β-catenin (19–21,33). To identify additional factors that may regulate KLF4-mediated telomerase expression, KLF4 IP

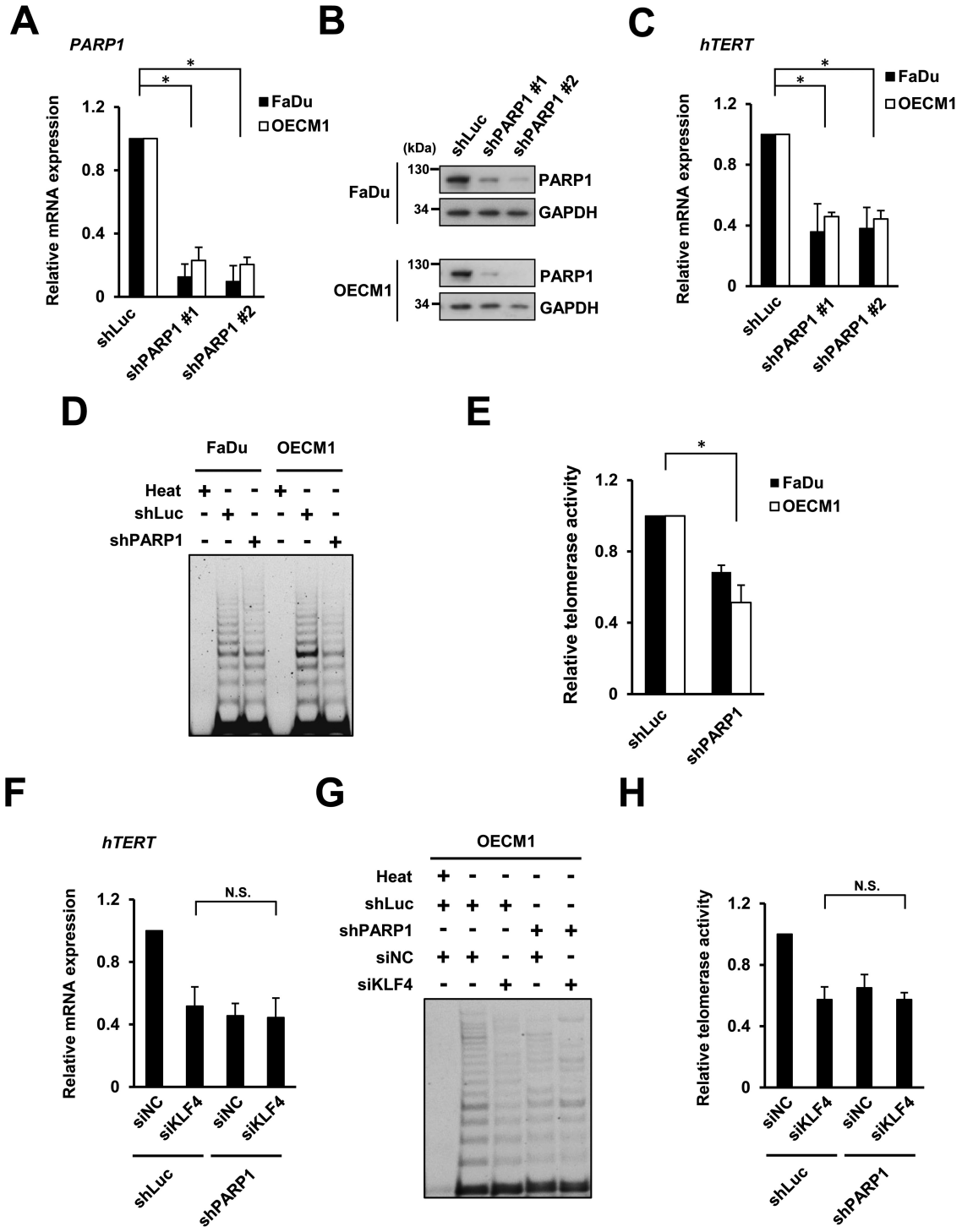

Figure 2. (**A**) Knockdown of endogenous PARP1 reduces KLF4-mediated *hTERT* mRNA expression and activity. qRT-PCR analysis of endogenous *PARP1* mRNA levels in control (shLuc) and shPARP1 knockdown cell lines. Black bars represent FaDu *PARP1* expression, and white bars represent OECM1 *PARP1* expression. Values are means ± s.d. of triplicates.(**B**) Western blots show PARP1 protein levels in control and shPARP1 knockdown FaDu and OECM1 cells. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as a loading control. (**C**) Endogenous *PARP1* knockdown represses the expression of *hTERT*. *hTERT* mRNA levels in control and shPARP1 knockdown cell lines were analyzed by qRT-PCR. Black bars represent FaDu *hTERT* expression, and white bars represent OECM1 *hTERT* expression. The standard deviations were calculated from triplicates. (**D**) Telomerase activity was measured by the TRAP assay performed using protein extracts from control (shLuc) and shPARP1 knockdown FaDu or OECM1 cells. (**E**) TRAP assay results were quantified. Black bars represent FaDu telomerase activity, and white bars represent OECM1 telomerase activity. (**F**) qRT-PCR analysis of *hTERT* mRNA levels in control (shLuc) and shPARP1 knockdown OECM1 cell lines with additionally transfected negative control (siNC) or *KLF4* siRNA oligo (siKLF4). (**G**) Telomerase activity was measured by the TRAP assay using protein extracts from shPARP1 and siKLF4 knockdown OECM1 cells. (**H**) Quantitative analysis of the TRAP results is displayed. Each bar represents the means ± s.d. $*P < 0.05$ indicates statistical significance; N.S., non-significant.

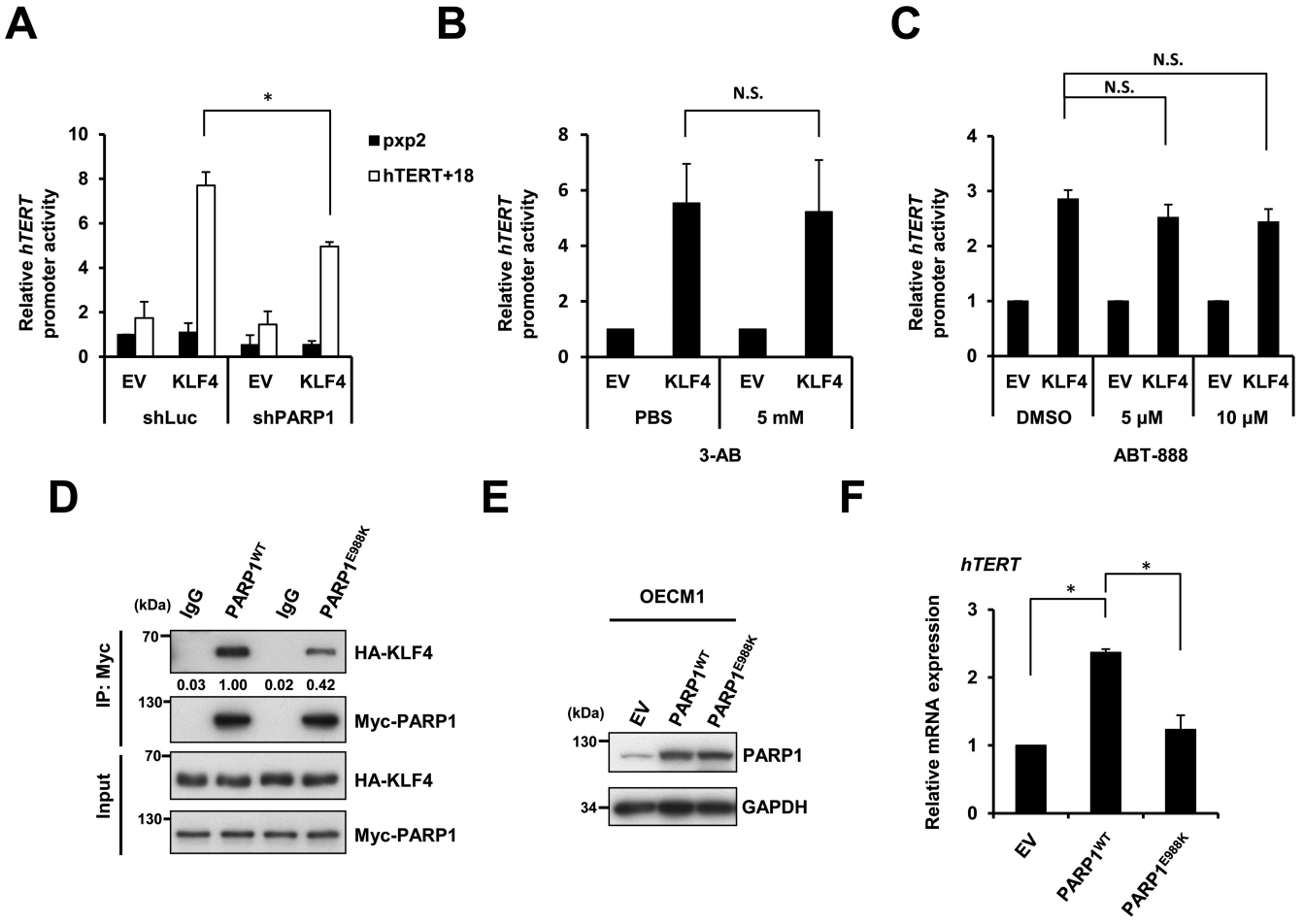

Figure 3. The PARP1 oligo(ADP-ribose) polymerase activity, but not poly(ADP-ribose) polymerase activity, is required for KLF4-mediated *hTERT* expression. (**A**) Knockdown of PARP1 decreases *hTERT* promoter activity. Control (shLuc) and PARP1 knockdown (shPARP1) HEK293T cells were co-transfected with luciferase reporter constructs and the pcDNA3 (EV, empty vector) or pcDNA3-KLF4 expression plasmid. Black bars represent luciferase reporter vector, pXP2, and white bars represent luciferase reporter fused to the +18 *hTERT* promoter. (**B** and **C**) PARP1 promoting KLF4-activated *hTERT* expression is independent of the PAR activity of PARP1. HEK293T cells were co-transfected with *hTERT* promoter luciferase reporter plasmid and the pcDNA3 (EV) or pcDNA3-KLF4 expression plasmid and grew in media with or without 3-AB (5 mM) and ABT-888 (5 and 10 μM). $*P < 0.05$; N.S., non-significant. (**D**) The oligo(ADP-ribosyl)ation dead mutation of PARP1 reduces the KLF4–PARP1 interaction. HEK293T cells were co-transfected with HA-KLF4 and Myc-PARP1 wild-type or the E988K mutant. Anti-Myc precipitates were analyzed by western blotting with HA and Myc antibodies. (**E**) Western blotting shows the PARP1 protein levels in control and PARP1 overexpressing OECM1 cells. GAPDH was used as a loading control. (**F**) qRT-PCR analysis of *hTERT* mRNA levels in control (EV), PARP1 wild-type or the E988K mutant overexpressing OECM1 cells. Each bar represents the means ± s.d. $*P < 0.05$ indicates statistical significance; N.S., non-significant.

and MS analysis were conducted and multiple interacting proteins were revealed (Figure 1A and Supplementary Table S4). PARP1 drew our attention due to its role in transcriptional regulation and other capabilities (34). Moreover, among these potential KLF4-associated proteins, XRCC5, also known as KU80, was reported to interact with PARP1 (31). To validate the interaction between KLF4 and PARP1, co-IP and GST pull-down assays were performed. KLF4 was co-immunoprecipitated with PARP1 (Figure 1B). Pull-down assay demonstrates that *E. coli*-expressed recombinant KLF4 directly interacted with *E. coli*-expressed recombinant PARP1 (Supplementary Figure S1). GST–KLF4 was able to pull down endogenous PARP1 protein both in the presence and absence of DNA-intercalating agent ethidium bromide (EtBr) (35) (Figure 1C). These results suggest that the interaction between KLF4 and PARP1 is direct and independent from their DNA-binding abilities. To determine the interacting domain of KLF4 binding to PARP1, various KLF4 deletion mutants were employed for GST pull-down assay (Figure 1D). Endogenous PARP1 associated specifically with the C-terminal DBD of KLF4. Likewise, by using several GST–PARP1 mutants for pull-down assay, we uncover that the C-terminal region of the PARP1 AMD, residues 480–532, specifically associated with HA-KLF4 (Figure 1E).

PARP1 regulates KLF4-mediated *hTERT* transcription

Because KLF4 contributes to telomerase *TERT* expression in both cancer and stem cells (22,23), we examined whether *hTERT* is governed by the KLF4-interacting protein PARP1. PARP1 was repressed in telomerase-positive cancer cells, FaDu and OECM1, by shRNA viruses (Figure

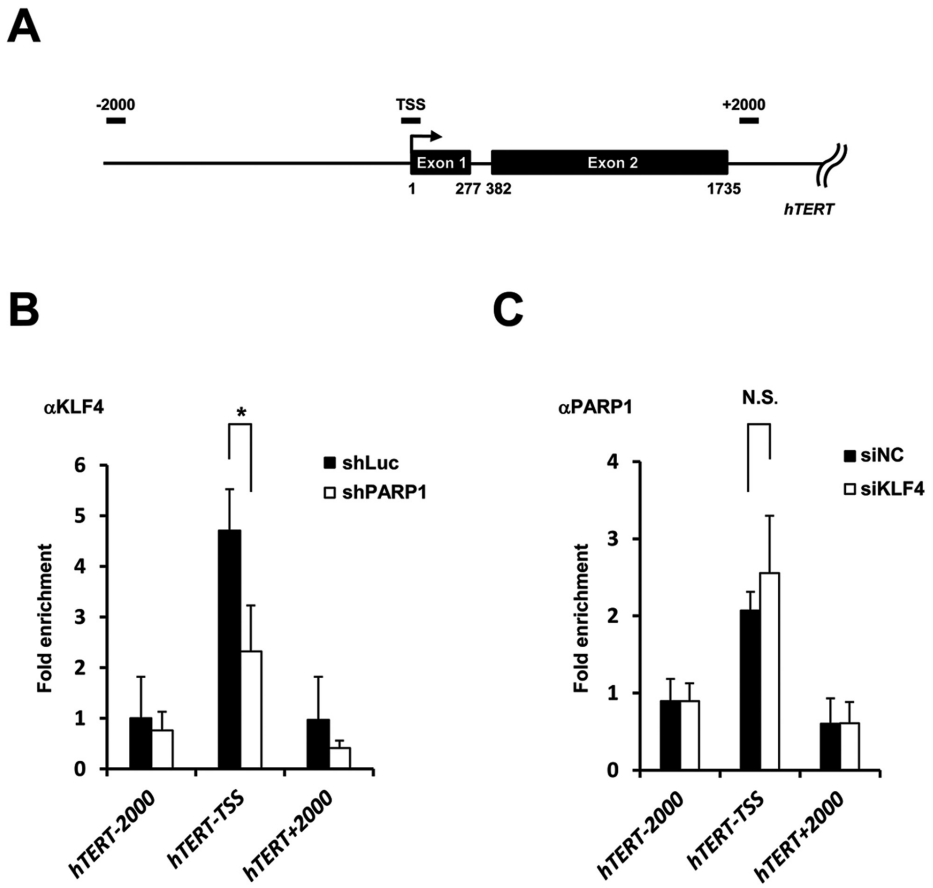

Figure 4. PARP1 promotes KLF4-mediated *hTERT* expression by modulating KLF4 binding to the *hTERT* promoter. (**A**) A schematic diagram shows the *hTERT* locus and locations of PCR primers for ChIP assay. (**B**) ChIP assays were conducted using an anti-KLF4 antibody in control (shLuc) and PARP1 knockdown (shPARP1) FaDu cells. (**C**) ChIP assays were performed using an anti-PARP1 antibody in control (siNC) and KLF4 knockdown (siKLF4) FaDu cells. DNA was analyzed by qPCR using the primers at the *hTERT* promoter. *CCNA1* promoter was used as a negative control. Values are means ± s.d. of triplicates. *$P < 0.05$ versus shLuc control; N.S., non-significant.

2A and B), and *hTERT* expression was decreased in these PARP1 knockdown cells (Figure 2C). Consistently, endogenous telomerase activity was reduced in the PARP1 knockdown cells (Figure 2C and D). These results suggest that PARP1 regulates *hTERT* expression in human cancer cells. To verify whether both KLF4 and PARP1 are involved in the same pathway, OECM1 cells were infected with shRNA viruses targeting PARP1 and co-transfected with KLF4 siRNA oligo (Supplementary Figure S2A). Knockdown of KLF4 or PARP1 significantly decreased *hTERT* expression and double depletion of KLF4 and PARP1 did not further reduce *hTERT* expression and telomerase activity (Figure 2F–H). These data indicate that PARP1 regulates KLF4-activated *hTERT* transcription.

PARP1 controls KLF4 binding to the *hTERT* promoter to facilitate KLF4-dependent transcriptional activation

To gain insight into the PARP1–KLF4 interaction on *hTERT* gene expression, we performed the luciferase reporter assay. Previous evidence showed that KLF4 binds to 18–77 of the *hTERT* promoter region (22). PARP1-suppressed 293T cells were transfected with luciferase reporter constructs and a KLF4 expression plasmid (Supplemental Figure S2B). Overexpression of KLF4 led to an increase of the *hTERT* promoter activity in control (shLuc) cells. Silencing of PARP1 reduced the enhancement of *hTERT* promoter activity (Figure 3A). At least two different mechanisms were proposed for PARP1-mediated transcriptional regulation: PARP1 may either modulate chromatin structure or act as part of gene-specific enhancer/promoter-binding complexes (34). For each case, the PARP1 enzymatic activity may or may not be essential for the regulation. We then examined whether the PARylation activity is required for activating *hTERT* expression. KLF4-induced *hTERT* promoter activity was not changed after treatment with a poly(ADP-ribosyl)ation inhibitor, 3-aminobenzamide (3-AB, $K_i \sim 500$ nM), while 3-AB effectively blocked PARylation induced by hydrogen peroxide (H_2O_2), a well-established activator of PARP1 catalytic activity (36) (Figure 3B; Supplementary Figure S3A and B). The similar results were observed when another poly(ADP-ribosyl)ation inhibitor (ABT-888, $K_i \sim 5$ nM) was utilized in the reporter assay (Figure 3C and Supplementary Figure S3C). These findings suggest that the poly(ADP-ribosyl)ation activity of PARP1 is not required for KLF4-dependent transcriptional activation at the *hTERT* pro-

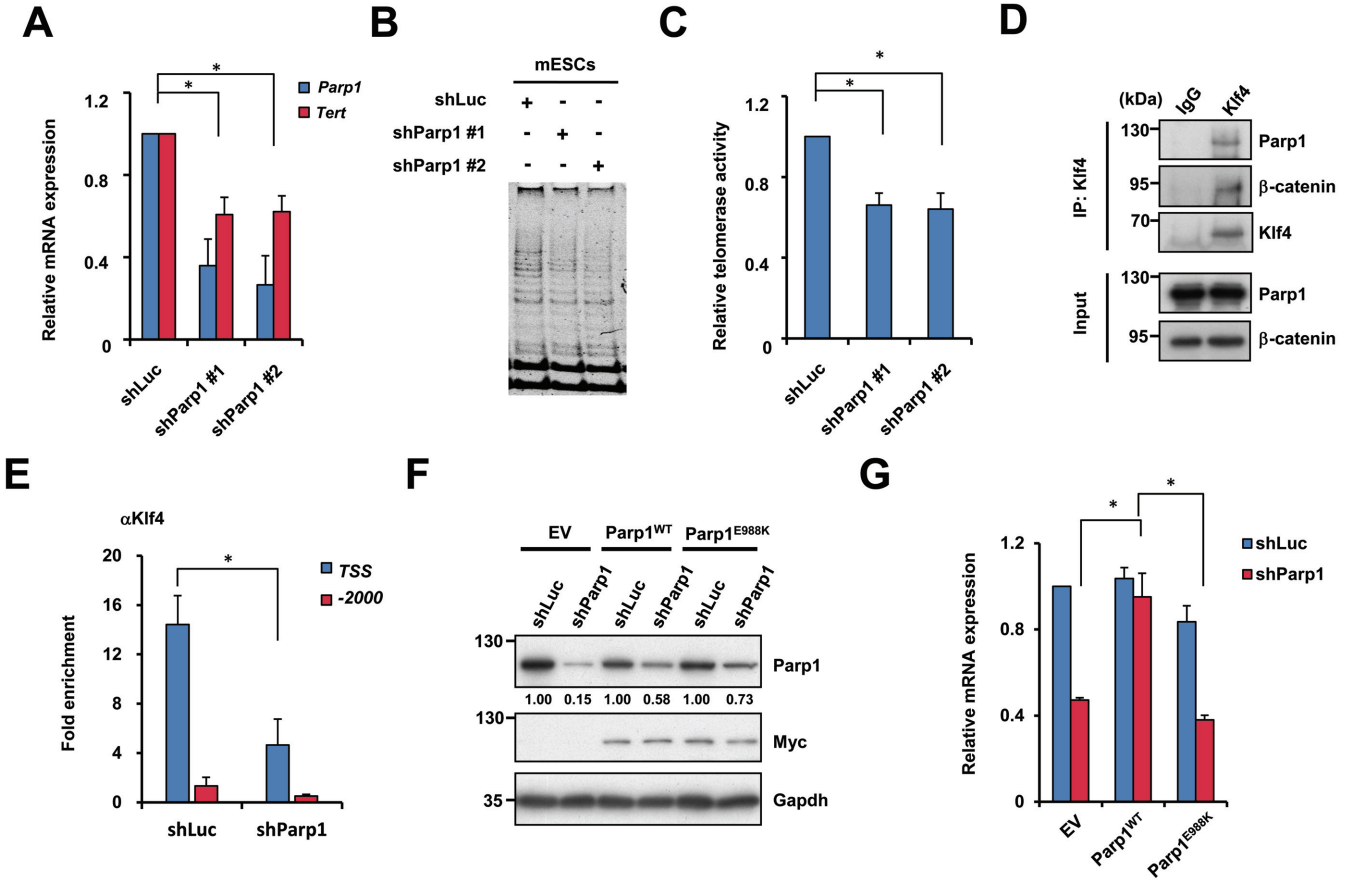

Figure 5. Parp1's oligo(ADP-ribose) polymerase activity is required for *Tert* expression in mouse ESCs. (**A**) qRT-PCR analysis of endogenous *Parp1* mRNA levels in shLuc and shParp1 knockdown D3 mouse ESCs. (**B**) Telomerase activity was measured by the TRAP assay using protein extracts from shLuc and shParp1 knockdown cells. (**C**) Quantitative analysis of the TRAP results is displayed. (**D**) Klf4 immunoprecipitates of mouse ESC extracts contain Parp1 and β-catenin. Endogenous Klf4 was immunoprecipitated using an anti-Klf4 antibody with D3 mouse ESC extract, and precipitates were analyzed by western blotting using indicated antibodies. (**E**) ChIP assays were conducted using an anti-Klf4 antibody in shLuc and shParp1 D3 mouse ESCs. *Hprt* was used as a negative control. Values are means ± s.d. of triplicates. *$P < 0.05$ versus shLuc control. (**F**) Western blotting shows the Myc and Parp1 protein levels in control (EV), Parp1 wild-type or the E988K mutant expressing shParp1-knockdown D3 mESCs. GAPDH was used as a loading control. (**G**) qRT-PCR analysis of *Tert* mRNA levels in control (EV), Parp1 wild-type or the E988K mutant expressing shParp1-knockdown D3 mESCs. Each bar represents the means ± s.d. *$P < 0.05$ indicates statistical significance.

moter. To discriminate whether mono- and oligo(ADP-ribosyl)ation activity of PARP1 participates in this regulation, we generated a catalytically dead mutant of PARP1 (E988K), which lacks the PAR elongation activity and converts PARP1 into a mono(ADP-ribosyl)transferase (37). KLF4 exhibited higher binding affinity to wild-type PARP1 than the E988K mutant (Figure 3D). Overexpression of wild-type, but not the catalytically dead PARP1, increased the *hTERT* expression in cancer cells (Figure 3E and F). Collectively, these results indicate that PARP1 oligo(ADP-ribose) polymerase activity, but not poly(ADP-ribose) polymerase activity, is required for KLF4-stimulated *TERT* expression. Given that PARP1 interacts with KLF4 through its AMD, we examined whether this interaction is preserved upon PARP1 inhibition or stimulation. PARP1 inhibitor ABT-888 treatment did not alter the KLF4–PARP1 interaction. However, H_2O_2 treatment reduced the KLF4–PARP1 interaction (Supplementary Figure S3D).

Previous studies found that KLF4 directly binds to the *hTERT* promoter to drive its expression (22). We, therefore, performed ChIP assay to analyze whether PARP1 regulates KLF4 binding to the *hTERT* promoter *in vivo*. KLF4 binding at the *hTERT* transcription start site (TSS) was significantly reduced in PARP1 knockdown cells (Figure 4A and B). Conversely, KLF4 knockdown did not lessen PARP1 binding to the *hTERT* promoter (Figure 4C). The PARP1 ChIP assay also successfully pulled down several well-known PARP1-regulated elements (38) (Supplementary Figure S4). These data demonstrate that PARP1 binding is required for KLF4 to localize to the *hTERT* promoter.

PARP1 is required for maintaining *TERT* expression and pluripotency in ESCs

Because KLF4 activates *TERT* expression in both cancer and ESCs (22,23), we determined whether PARP1 is involved in telomerase activation in ESCs. By transient transfection of KLF4 and PARP1 siRNA oligos in NTU1 human ESCs, both KLF4 and PARP1 were knocked-down and *hTERT* expressions were declined (Supplementary Figure S5A and B). Next, we examined whether the

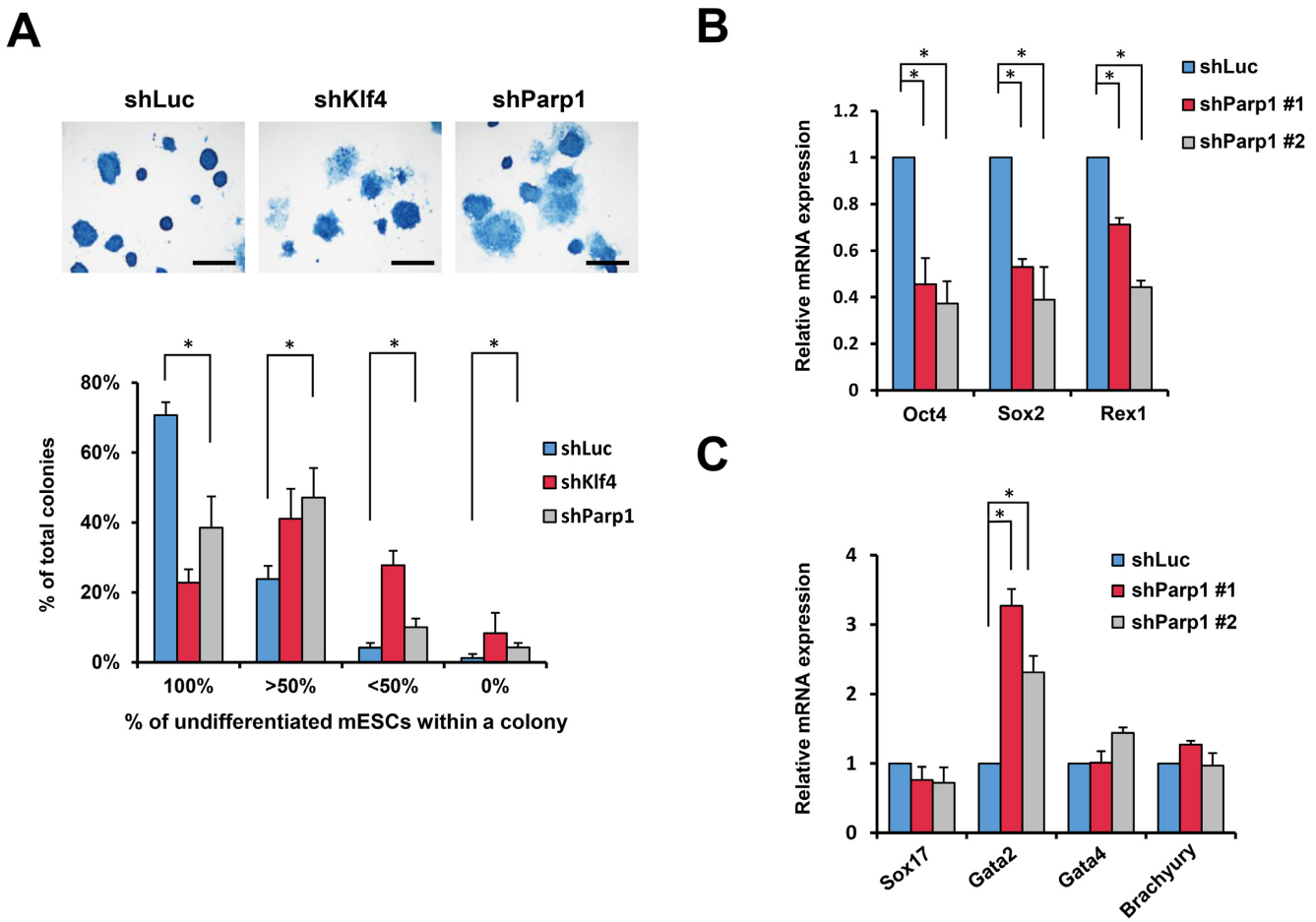

Figure 6. Knockdown of Parp1 reduces pluripotency and increases differentiation in mouse ESCs. (**A**) Representative images of the AP activity in D3 mouse ESCs**.** Experiments were evaluated by microscope imaging to assess the percentage of four categories (100, >50, <50 and 0% undifferentiation) of mouse ESCs. (**B** and **C**) qRT-PCR analysis of pluripotent markers and differentiation markers in control and shParp1 cells. Parp1 was depleted via lentiviral vector-mediated shRNA transduction for 12 days. Values are means ± s.d. of triplicates. *$P < 0.05$ versus shLuc control.

mouse Parp1 may regulate telomerase expression in mouse ESCs. Toward this aim, mouse ESCs were transduced with shRNA lentiviral particles targeting Parp1, which caused a decrease in Parp1 expression (Figure 5A). Concordantly, telomerase expression and activity were reduced in Parp1-depleted mouse ESCs (Figure 5A–C). The *Tert* mRNA expression levels did not display any difference between wild-type and Parp1-null mESCs (Supplementary Figure S6A and B). The interaction between endogenous Klf4 and Parp1 in mouse ESCs was confirmed by co-IP (Figure 5D). β-Catenin also associated with endogenous Klf4 (Figure 5D) as previously reported (23,33). Furthermore, to test whether Klf4 binding to the *Tert* promoter in mouse ESCs relies on Parp1 as observed in cancer cells, ChIP was performed against the endogenous Klf4 with shRNA targeting Parp1. The binding of Klf4 was reduced at the *Tert* promoter in Parp1 knockdown cells (Figure 5E). To discriminate whether the catalytic activity of Parp1 participates in this regulation in mouse ESCs, we utilized a PiggyBac system with insulators flanked Parp1 to express wild-type and catalytically dead Parp1. Wild-type Parp1, but not the catalytically dead Parp1, maintained the *TERT* expression in Parp1-depleted mESCs (Figure 5F and G). Thus, Parp1 modulates Klf4 recruitment at the *Tert* promoter in mouse ESCs.

Alkaline phosphatase (AP) is highly expressed in undifferentiated ESCs but is rapidly downregulated as they differentiate (39). Additionally, when ESCs differentiate, they undergo extensive morphological changes (40). Following Parp1 knockdown, mouse ESCs exhibited morphologies of differentiated cells and 100% undifferentiated AP-positive colonies were significantly decreased (Figure 6A), indicating that Parp1 is required for maintaining mouse ESC pluripotency. Knockdown of Parp1 reduced the pluripotent markers *Oct4*, *Sox2* and *Rex1* expression (Figure 6B), and enhanced the hematopoietic stem cells marker and trophectoderm-associated gene *Gata2* expression (Figure 6C). These findings establish that Parp1 expression contributes to the maintenance of pluripotency of mouse ESCs.

DISCUSSION

Telomerase expression is crucial for maintaining replicative potential and providing self-renewal of ESCs. Oct4, Sox2, c-Myc and Klf4 can convert adult fibroblasts to pluripo-

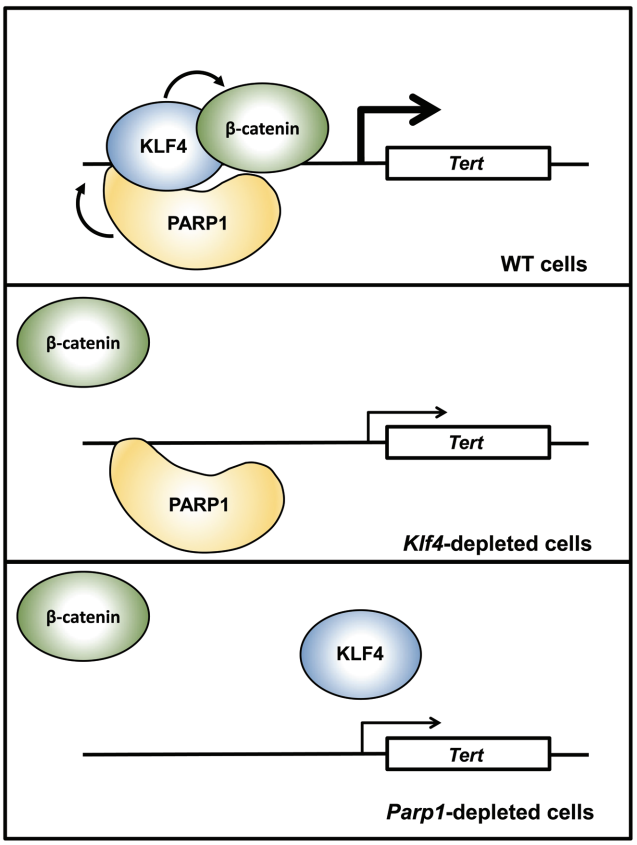

Figure 7. A model of PARP1–KLF4-mediated *TERT* expression. PARP1 is the first factor to be recruited to the *TERT* promoter region. In concert with PARP1, KLF4 and β-catenin are subsequently recruited to the promoter. In the absence of KLF4, PARP1 retains on the *TERT* promoter, but β-catenin cannot bind to the *TERT* promoter. Once PARP1 is depleted, both KLF4 and β-catenin dissociate from the *TERT* promoter. PARylation activity is dispensable for this regulation.

tent stem cells (17). Among them, c-Myc is dispensable for telomerase expression (8), while KLF4 directly binds to the *TERT* promoter and activates telomerase expression in ESCs (22). KLF4 also plays a significant role in stem cell self-renewal (10). However, how cells modulate KLF4 loading to the *TERT* promoter remains as an interesting question. Here, we reveal that PARP1 recruits KLF4 to the *TERT* promoter. This interaction may facilitate and strengthen the loading of KLF4 to the telomerase promoter. Although *in vitro* binding assay unveiled a direct KLF4–PARP1 interaction, it remains to be determined whether this interaction occurs before or after their DNA bindings.

PARP1 regulates KLF4-mediated *TERT* transcription by promoting KLF4 directly localizing to the promoter of *TERT*. On the contrary, KLF4 does not control the PARP1 recruitment to the *TERT* promoter, implying that PARP1 loading may occur before the KLF4 binding to its target element. Our results revealed that the PARylation activity is not required for KLF4-dependent *hTERT* activation, suggesting that PARP1 does not PARylate vicinal substrates to induce telomerase expression. Interestingly, the oligo(ADP-ribose) polymerase activity is involved in the interaction between PARP1 and KLF4, which further regulates *TERT* expression. Two plausible hypotheses may explain this observation: (i) oligo(ADP-ribose) polymerase activity may remodel chromatin structure for KLF4 binding (41,42) or (ii) oligo(ADP-ribosyl)ation on PARP1 may directly enhance the KLF4–PARP1 interaction (43,44). According to the previous studies, loading of KLF4 further helps the recruitment of β-catenin to the promoter region of *Tert* to activate telomerase expression in mouse ESCs (23). All these findings uncover a sequential loading mechanism to stimulate the expression of telomerase (Figure 7).

Telomerase activity and telomere length maintenance are critical for the pluripotency and self-renewal. High-level telomerase activity in ESCs decreases rapidly during differentiation (45). Overexpression of Tert can augment self-renewal ability and proliferation efficiency of mouse ESCs (3). In our results, knockdown of PARP1 in ESCs decreases *TERT* expression. But knockout of PARP1 in ESCs did not change the TERT levels. This is not entirely unexpected because previous studies demonstrated that different patterns of phenotype frequently occur between knockdown and knockout cells (46). A genetic compensatory pathway can be induced in the knockout cells for them to adapt and survive. PARP1 is critical for cells to maintain stemness. That might explain why Tert expression is induced in long-term cultured Parp1$^{-/-}$ mES cells. Together, these results suggest that Parp1- and KLF4-mediated *Tert* expression may play a critical role in regulating pluripotency of mESCs.

A growing number of studies indicate that Parp1 participates in both pluripotency and differentiation in pluripotent stem cells. Recent reports have highlighted that Parp1 regulates somatic cell reprogramming and promotes iPSC formation (47–49). Parp1 and Tet2 are epigenetic modification factors that are recruited to the *Nanog* and *Esrrb* loci during somatic cell reprogramming. Parp1 induction further promotes accessibility for Oct4 to localize to the *Nanog* and *Esrrb* promoters (47). Parp1 and Parp7 also safeguard pluripotent state and protect mouse ESCs from progressive epigenetic repression (50). Besides, Parp1 has been known to regulate the differentiation potential of mouse ESCs (51). Parp1 is recruited to the *FGF4* promoter and poly(ADP-ribosyl)ates Sox2, which stabilizes Sox2 to stimulate *FGF4* expression during the differentiation of mouse ESCs (52). Here, we uncover a novel coactivator role of Parp1 in mESCs. The role is quite similar to the recently identified cooperative function of Parp1 in Sox2-mediated gene expression (53). These discoveries suggest that PARP1 plays multiple roles in ESC self-renewal and differentiation. In summary, all these findings indicate that Parp1 and *Tert* expression may be required for the early stage embryo to sustain pluripotency, and Parp1 serves as a modulator for sufficient *Tert* expression in ESCs. This novel KLF4–PARP1 interaction provides new insights into the mechanisms underlying the regulation of KLF4 in cancer cells and ESCs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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