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Heng Lu
University of Central Florida

Xianhui Wang
University of Central Florida

Tianshu Li
University of Central Florida

Alison M. Urvalek
University of Central Florida

Lin Yu
University of Central Florida

See next page for additional authors

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Identification of Poly (ADP-ribose) Polymerase-1 (PARP-1) as a Novel Krüppel-like Factor 8-interacting and -regulating Protein*[S]

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Heng Lu[‡], Xianhui Wang[‡1], Tianshu Li[‡], Alison M. Urvalek[‡2], Lin Yu[‡], Jieli Li[§], Jinghua Zhu[¶], Qishan Lin[¶], Xu Peng[§], and Jihe Zhao[‡3]

From the [‡]Burnett School of Biomedical Sciences, University of Central Florida College of Medicine, Orlando, Florida 32827, the [¶]Center for Functional Genomics, University at Albany, Rensselaer, New York 12144, and the [§]Department of Systems Biology and Translational Medicine, College of Medicine, Texas A&M Health Science Center, Temple, Texas 76504-7105

Krüppel-like factor 8 (KLF8) regulates critical gene transcription and cellular events associated with cancer. However, KLF8-interacting proteins remain largely unidentified. Using co-immunoprecipitation (co-IP), mass spectrometry, and GST pulldown assays, we identified poly(ADP-ribose) polymerase-1 (PARP-1) as a novel KLF8-interacting protein. Co-IP and Western blotting indicated that KLF8 is also a PARP-1 substrate. Mutation of the cysteines in the zinc finger domain of KLF8 abolished PARP-1 interaction. Surprisingly, immunofluorescent staining revealed a cytoplasmic mislocalization of KLF8 in PARP-1$^{-/-}$ cells or when the interaction was disrupted. This mislocalization was prevented by either PARP-1 re-expression or inhibition of CRM1-dependent nuclear export. Interestingly, co-IP indicated competition between PARP-1 and CRM1 for KLF8 binding. Cycloheximide chase assay showed a decrease in the half-life of KLF8 protein when PARP-1 expression was suppressed or KLF8-PARP-1 interaction was disrupted. Ubiquitination assays implicated KLF8 as a target of ubiquitination that was significantly higher in PARP-1$^{-/-}$ cells. Promoter reporter assays and chromatin immunoprecipitation assays showed that KLF8 activation on the cyclin D1 promoter was markedly reduced when PARP-1 was deleted or inhibited or when KLF8-PARP-1 interaction was disrupted. Overall, this work has identified PARP-1 as a novel KLF8-binding and -regulating protein and provided new insights into the mechanisms underlying the regulation of KLF8 nuclear localization, stability, and functions.

KLF8 is a Krüppel-like transcription factor (KLF)[4] family member that plays a critical role in the regulation of important cellular processes including cell cycle progression (1–4), oncogenic transformation (5), epithelial-to-mesenchymal transition, migration, and invasion (6). The transcription of KLF8 is regulated by cell signaling molecules such as Src and PI3K downstream of focal adhesion kinase (1, 7) and transcriptional activators such as Sp1 (2), KLF1, and KLF3 (8). The cellular function of KLF8 is regulated by post-translational sumoylation (3) and acetylation (9). As a typical KLF family member, KLF8 contains three highly conserved C2H2 zinc fingers on the C terminus, which can bind to the GT-box (CACCC) promoter sequence (10). KLF8 binds directly to the cyclin D1 promoter and activates the transcription (1) by recruiting the p300 and p300/CBP-associated factor transcription co-activators (11). KLF8 also functions as a transcriptional repressor on the promoters of γ-globin (10), KLF4 (3), and E-cadherin (6). Two nuclear localization signals on KLF8 were identified within the activation domain and the zinc finger domain, respectively (4, 11).

Poly(ADP-ribose) polymerase-1 (PARP-1), the founding member of the PARP enzyme family, is a nuclear enzyme responsible for post-translational modification poly(ADP-ribosyl)ation (or PARylation). It has been implicated in the regulation of a wide range of important cellular processes including chromatin organization, transcription, DNA repair, genomic integrity, differentiation, proliferation, cell death, and cancer progression (12–14). Pharmacologic inhibition of PARP-1 has shown a strong antitumor effect in preclinical and clinical trials (12, 15–18).

Significant progress has been made in the understanding of KLF8-regulated cellular events and target genes. However, its interacting proteins have been understudied. In this study, we performed mass spectrometric screening of KLF8-binding proteins and identified PARP-1 as a novel interacting and regulating protein of KLF8. We present evidence showing that interaction and PARylation of KLF8 by PARP-1 are critical for maintaining the proper subcellular localization, transcription-regulating function, and protein stability of KLF8 in the nucleus.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The anti-HA antibody (F-7), anti-Myc antibody (9E10), and anti-PARP-1 antibody (sc-25780) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-HA and anti-Myc antibody-conjugated agarose beads, leptomycin B (LMB), and PJ-34 were purchased

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[1] Present address: Ge*NY*sis Center for Excellence in Cancer Genomics, University at Albany, Rensselaer, NY 12144.

[2] Present address: Dept. of Pharmacology, Weill Medical College of Cornell University, New York, NY 10065.

[3] To whom correspondence should be addressed: Burnett School of Biomedical Sciences, Room BBS-313, University of Central Florida College of Medicine, Orlando, FL 32827. Tel.: 407-266-7099; Fax: 407-266-7002; E-mail: jizhao@mail.ucf.edu.

[4] The abbreviations used are: KLF8, Krüppel-like factor 8; PARP-1, poly(ADP-ribose) polymerase-1; PARylation, poly(ADP-ribosyl)ation; IP, immunoprecipitation; co-IP, co-immunoprecipitation; IB, Western blot; MEF, mouse embryonic fibroblast; CHX, cycloheximide; LMB, leptomycin B; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; ZF, zinc finger.

from Sigma. Anti-PAR antibody was purchased from Travigen, Inc. (Gaithersburg, MD). IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Nicotinamide was purchased from Acros Organics (NJ). Cycloheximide and doxorubicin were purchased from Calbiochem.

Plasmid Construction—The mammalian expression plasmids pKH3, pKH3-KLF8, pHAN, and pHAN-KLF8 were previously described (1, 3). All the KLF8 mutants were generated by site-directed mutagenesis PCR or overlapping PCR using pKH3-KLF8 or pHAN-KLF8 as the template and mutation-specific primers paired with one of the master primers (forward, 5′-CCCAAGCTTCTGCAGGTCG-3′, and reverse, 5′-GGACAAACCACAACTAGAATGCAG-3′). dN100, dN200, dC26, dC38, dC56, dC82, H294/8L, H324/8L, mZF1dC, mZF2dC, dZF1, and dZF2 mutants were previously described (4). The primer pairs forward and reverse used to generate the rest of the KLF8 point mutants are as follows: C276A, 5′-tgc aaa gtc agc ttg gtg aat c-3′ and 5′-ttc acc aag ctg act ttg c-3′; C281A, 5′-act ttg ctg gct cct gca aag tc-3′ and 5′-ttg cag gag cca gca aag-3′; C306A, 5′-tcc cag gtg gct tta taa ggc-3′ and 5′-agc ctt ata aag cca cct ggg-3′; C311A, 5′-tcc agg agg cgc cat ccc-3′ and 5′-tgg gat ggc gcc tcc tgg-3′. ZF1mCs is the double mutant of C276A and C281A. ZF2mCs is the double mutant of C306A and C311A. ZF1,2mCs is the double mutant of ZF1mCs and ZF2mCs. Human PARP-1 expression vector was kindly provided by Dr. Zhao-qi Wang (Leibniz Institute For Age Research–Fritz Lipmann Institute, Jena, Germany) (19). Myc-PARP-1 was generated by cloning PARP-1 into the pHAN vector using the primers forward and reverse: 5′-agt aga tct gcg gag tct tc-3′ and 5′-act ccc ggg tta cca cag g-3′. Myc-E988K (PARP-1 mutant lacking poly(ADP-ribosylation)) was generated by PCR-directed mutagenesis using the primers (forward/reverse): 5′-atc gat atc ttt aag ata g/atg tac ttg tta tat ag-3′.

Cell Culture and Transfection—HEK293T and MCF10A cells were purchased from the American Type Culture Collection. T80, the immortalized human ovarian epithelial cell line, was kindly provided by Dr. Jinsong Liu (20). PARP-$1^{-/-}$ and PARP-$1^{+/+}$ mouse embryonic fibroblast (MEF) cells were kind gifts from Dr. Zhao-qi Wang (19). Primary MEFs were isolated as described previously (21, 22) and kindly provided by Dr. Hamid Boulares of Louisiana State University. HEK293T, T80, and MEF cells were maintained in DMEM supplemented with 10% fetal bovine serum (Mediatech) and 100 units/ml penicillin-streptomycin (Invitrogen). MCF10A cells were cultured as described previously (6). Transfections of the plasmid DNAs were performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions.

Parallel Affinity Precipitation and Mass Spectrometry—HEK293T cells were transfected with pKH3-KLF8, pHAN-KLF8, or one of the control vectors for 24 h. The transfected cells from 100-mm dishes were lysed with Nonidet P-40 buffer supplemented with protease inhibitors (1 mM Na_3VO_4, 1 mM PMSF, 20 μg/ml leupeptin, and 0.06 trypsin inhibitor unit/ml aprotinin). Precleared cell lysates were incubated with anti-HA- or anti-Myc-conjugated beads for 24 h in a cold room. The immunoprecipitates were washed three times, eluted with SDS sample buffer, resolved on an SDS-PAGE gel, and stained with the Bio-Safe™ Coomassie Blue stain solution (Bio-Rad). In addition to the HA-KLF8 and Myc-KLF8 bands, protein bands common to HA-KLF8- and Myc-KLF8-expressing cells but missing from the control cells were excised from the gel. The gel pieces were then washed, reduced, alkylated, and in-gel tryptic or chymotryptic digested. Proteolytic peptides were extracted from the gel, and peptides were concentrated and reconstituted in 10 μl of 5% formic acid followed by 5% liquid chromatography tandem mass spectrometry analysis using a Waters ESI Q-TOF2 system. In-house MASCOT 2.2 from Matrix Science (London, UK) was used to assist in the interpretation of tandem mass spectra.

Promoter Reporter Assays—Luciferase reporter assays were performed essentially as described previously (1, 2). Briefly, cells were plated to ~60% confluency in a 12-well plate. The −962-bp cyclin D1 promoter-luciferase reporter construct (0.2 μg) and 4 ng of pRISV40 *Renilla* luciferase reporter vector were co-transfected into PARP-$1^{-/-}$ or PARP-$1^{+/+}$ cells with 0.2 μg of the expression vectors encoding KLF8 or KLF8 mutants with or without 1.0 μg of PARP-1, E988K, or control vector. In some experiments, 24-h transfected cells were treated with 3 μM PJ-34 or 10 mM nicotinamide for 12 h.

Fluorescent Microscopy—PARP-$1^{-/-}$ and PARP-$1^{+/+}$ cells were transfected or co-transfected with KLF8, KLF8-mut, PARP-1, or E988K for 24 h and processed for indirect immunofluorescence staining as described (23). Briefly, the primary antibodies used were anti-HA (Y-11) and/or anti-Myc (9E10) antibodies (1:200). The secondary antibodies used were FITC-conjugated goat-anti rabbit antibody and/or Texas Red-conjugated goat-anti mouse (1:200, Jackson ImmunoResearch Laboratory, West Grove, PA). The nuclei were stained with Hoechst 33258 dye. Images were acquired with an Olympus BMX-60 microscope equipped with a cooled charge-coupled device sensi-camera (Cooke, Auburn Hills, MI) and Slidebook software (Intelligent Imaging Innovations, Denver, CO). At least 200 positively transfected cells were examined for each of multiple experiments.

RNA Interference—PARP-1 and GFP ON-TARGET*plus* siRNAs from Dharmacon (J006656-05, 5′-GAU UUC AUC UGG UGU GAU A-3′; J006656-06, 5′-GAA AAC AGG UAU UGG AUA U-3′; J006656-07, 5′-GUU CUU AGC GCA CAU CUU G-3′; J006656-08, 5′-CCA AUA GGC UUA AUC CUG U-3′) were transfected into PARP-$1^{+/+}$ MEF cells by using Oligofectamine according to the manufacturer's instructions (Invitrogen). After 48–72 h, the cells were further transfected with either wild-type or mutant KLF8 constructs.

Ubiquitination Assay—PARP-$1^{-/-}$ and PARP-$1^{+/+}$ cells were grown to ~60% density in 6-well plates. The cells were transfected with a combination of 1.0 μg of pKH3-KLF8 or pKH3-KLF8 mutant and 1.0 μg of Myc-ubiquitin. At 48 h after transfection, cells were treated with 20 μM of the proteasomal inhibitor MG132. Then anti-HA precipitates from the cell lysates were analyzed by Western blotting using anti-HA and anti-Myc antibodies.

Cycloheximide (CHX) Chase Assays—PARP-$1^{-/-}$ and PARP-$1^{+/+}$ cells were transfected with either plasmids or siRNAs. At 48 h after transfection, the cells were treated with 3 μM PJ-34 for 12 h, 10 mM nicotinamide for 12 h, or 0.6 μg/ml doxorubicin for 8 h. Then the cells were treated with 50 μg/ml

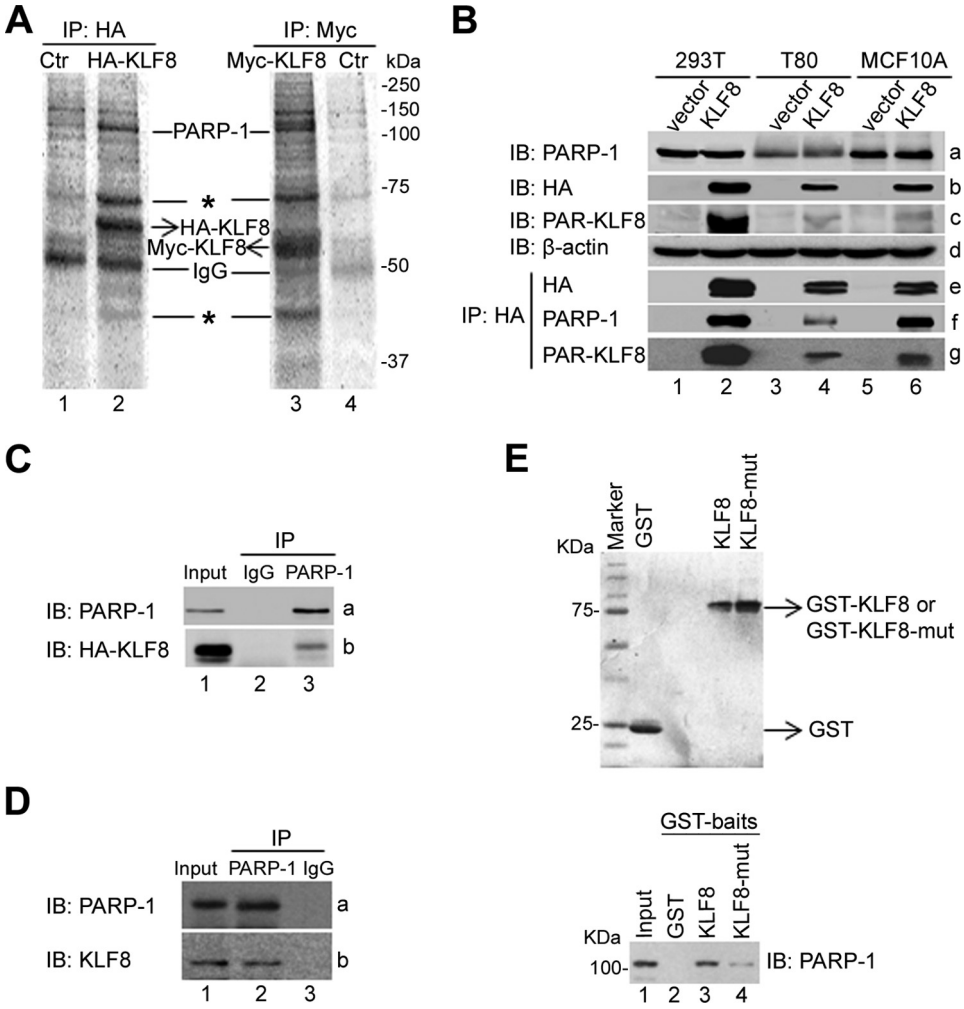

FIGURE 1. **KLF8 interacts with PARP-1 and is PARylated.** *A*, identification of PARP-1 as a KLF8-interacting protein by co-IP and mass spectrometry. 293T cells were transfected with HA-KLF8, Myc-KLF8, or control (*Ctr*) vectors for 24 h. Whole cell lysates were prepared for IP with anti-HA- or anti-Myc-conjugated agarose beads. The precipitated proteins were resolved by SDS-PAGE and revealed by Coomassie Blue staining. *B*, PARP-1 was co-immunoprecipitated with PARylated KLF8 in multiple cell types. 293T, T80, or MCF-10A cells were transfected with HA-KLF8 or control vector. Whole cell lysates or anti-HA precipitates (IP) were analyzed by Western blot (IB) with anti-PARP-1, anti-HA, or anti-PAR antibody. Anti-β-actin blotting was used as a loading control. *C*, KLF8 was co-immunoprecipitated with PARP-1. 293T cells were transfected with HA-KLF8, and whole cell lysate was subjected to IP with anti-PARP-1 or control IgG followed by the indicated IB. *D*, endogenous KLF8 was co-immunoprecipitated with PARP-1. Hs579T breast cancer cell lysate was subjected to IP with anti-PARP-1 or control IgG followed by anti-KLF8 IB. *E*, KLF8 and PARP-1 interact directly. Recombinant PARP-1 protein was pulled down with GST, GST-KLF8, or GST-KLF8-mut fusion protein conjugated to glutathione agarose beads (*top panel*, Coomassie Blue staining) followed by anti-PARP-1 blotting (*bottom panel*).

CHX before lysates were collected at different time points and analyzed by anti-HA Western blotting.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed essentially as we previously described (2, 3, 6, 11, 24), using the Millipore EZ-ChIP^{TM} kit according to the manufacturer's protocol with minor modifications. pKH3 vector, pKH3-KLF8, or its ZF1,2mCs mutant (KLF8-mut) was transiently transfected into primary PARP-$1^{+/+}$ or PARP-$1^{-/-}$ MEF cells. Sonicated lysates prepared from 2×10^6 cells were subjected to immunoprecipitation overnight at 4 °C using the anti-HA, anti-PARP-1, or anti-p300 antibody or control IgGs followed by a rotation for 1 h at 4 °C with 60 μl of protein A/G agarose beads. Primers are specific for the mouse cyclin D1 promoter to amplify the KLF8 binding region (11).

GST Pulldown Assays—Purified GST, the GST fusion with the dN50-KLF8 (4) (GST-KLF8), or its ZF1,2mCs mutant (GST-KLF8-mut) was coupled to glutathione-agarose beads from Thermo Scientific for 1 h. After three washes with PBS buffer containing protease inhibitors, glutathione-agarose-coupled GST proteins were subsequently incubated for 2 h at 4 °C with equal amount of the recombinant PARP-1 protein (Enzo Life Sciences, ALX-201-250-C010), washed three times, resolved on SDS-PAGE, and followed by anti-PARP-1 blotting.

RESULTS

Identification of PARP-1 as a Novel KLF8-interacting Protein—KLF8-interacting proteins in the cells remain largely unknown. To identify and characterize these proteins, we coupled parallel co-IP and mass spectrometry (Fig. 1*A*). We overexpressed HA-KLF8 or Myc-KLF8 in HEK293T cells. The empty vector (pKH3 for HA or pHAN for Myc) transfected cells were used as negative controls. The whole cell lysates prepared from these transfected cells were subjected to IP with either anti-HA or anti-Myc antibody-conjugated agarose beads. After being resolved by SDS-PAGE, the precipitated proteins were visualized by Coomassie Blue staining. The protein bands that

were pulled down from both HA-KLF8-expressing and Myc-KLF8-expressing cells (Fig. 1*A*, *lanes 2* and *3*) but not from the vector transfected cells (Fig. 1*A*, *lanes 1* and *4*) were excised for further analysis by mass spectrometry. PARP-1 was strongly and consistently present in the ~120-kDa band and thus chosen for further characterization (Fig. 1*A*). To investigate whether PARP-1 modifies KLF8 protein by PARylation, we blotted the anti-HA precipitated KLF8 protein with a specific anti-PAR antibody. We found that the KLF8 protein was PARylated in all three cell types (Fig. 1*B*, *row g*). We confirmed that the interaction between KLF8 and PARP-1 occurs not only in HEK293T cells, but also in T80 and MCF10A cells that expressed ectopic HA-KLF8 by anti-HA co-IP (Fig. 1*B*, *rows e* and *f*) and anti-PARP-1 co-IP (Fig. 1*C*). These results indicated that the interaction might be general in epithelial cell types. The interaction was further verified by anti-PARP-1 co-IP of endogenous KLF8 (Fig. 1*D*) and GST-KLF8 pulldown of recombinant PARP-1 protein (Fig. 1*E*). Inclusion of ethidium bromide in the co-IP reaction did not affect the interaction (supplemental Fig. 2). These results clearly indicate that KLF8 directly interacts with PARP-1 probably independent of DNA binding in a variety of cell types and that the PARylation of KLF8 by PARP-1 may be important in regulating KLF8 function in the cells.

KLF8 Interacts with PARP-1 through the First and Second Zinc Finger Domains, and the Interaction Is PARylation-independent—To map the PARP-1 binding site(s) on KLF8, we took advantage of the KLF8 truncation mutants we made previously (4) and performed similar transfection and co-IP (Fig. 2*A*). We found that the N-terminal truncation up to 200 amino acid residues did not affect the interaction with PARP-1 (Fig. 2*A*, compare *lanes 7* and *8* with *lane 6*). Further N-terminal truncations were not tested as those mutants are known to mislocalize to the cytoplasm instead of nucleus (4). In contrast, the C-terminal deletion of zinc finger (ZF) domain, particularly the complete deletion of all three ZFs (dC82), disrupted the interaction (Fig. 2*A*, compare *lanes 2–5* with *lane 6*).

Using the internal ZF deletion mutants and point mutants of KLF8 (19) (Fig. 2, *B* and *C*) for the co-IP experiments, we determined that deletion of ZF1 resulted in partial loss of the interaction and that deletion of ZF2 almost abolished the interaction (Fig. 2*B*, *lanes 1* and *2*). Interestingly, mutation of the DNA contact motifs of ZF2 or both the cysteines of ZF2 reduced the interaction to the same content as the deletion of ZF2 (Fig. 2*B*, *lane 5*, and Fig. 2*C*, *lane 7*), whereas mutation of two histidines (Fig. 2*B*, *lane 6*) or the individual cysteines (Fig. 2*C*, *lanes 4* and *5*) of ZF2 or any of the cysteines, histidines, or DNA contact motifs of ZF1 (Fig. 2*B*, *lanes 3* and *4*, and Fig. 2*C*, *lanes 2*, *3*, and *6*) did not affect the interaction.

Anti-PAR blotting indicated that the ZF2mCs mutant had significantly reduced levels of PARylation (Fig. 2*C*, *row c*, compare *lane 7* with *lane 9*). Interestingly, the ZF1mCs mutant displayed a similar degree of reduction in PARylation (Fig. 2*C*, *row c*, compare *lane 6* with *lane 7*), and mutation of all the cysteines in both ZF1 and ZF2 did not cause a further reduction of the PARylation (Fig. 2*C*, *row c*, compare *lane 8* with *lanes 6* and *7*). The PARylation of KLF8 was totally abolished by the PARP-1 specific inhibitors (supplemental Fig. 3*A*).

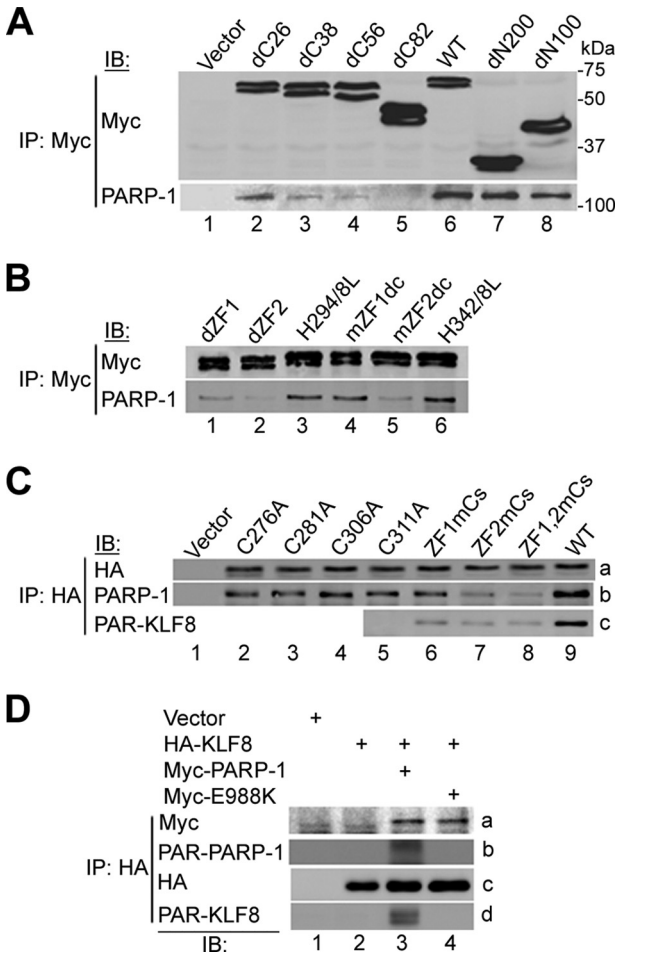

FIGURE 2. **KLF8 interacts with PARP-1 through its ZFs independent of the catalytic activity of PARP-1.** *A*, the ZF1 and ZF2 region is required for KLF8 interaction with PARP-1. HEK293T cells were transfected with the indicated Myc-tagged KLF8 or mutants or with empty vector. The protein expression was verified by anti-Myc IB of the whole cell lysates (supplemental Fig. 1*A*). The anti-Myc IP followed by IB with either anti-Myc or anti-PARP-1 was performed as in Fig. 1*B*. *B*, the histidine residues and DNA contact motifs in ZF1 or ZF2 are not required for KLF8 binding to PARP-1. The indicated Myc-tagged KLF8 mutants were used for co-IP similarly as in *A*. The protein expression was verified by anti-Myc IB of the whole cell lysates (supplemental Fig. 1*B*). *C*, the cysteine residues in ZF2 are most critical for KLF8 binding to PARP-1. The indicated HA-tagged KLF8 point mutants were used for similar co-IPs. The protein expression was verified by anti-HA IB of the whole cell lysates (supplemental Fig. 1, *C* and *D*). PARylation of KLF8 (PAR-KLF8) was determined by anti-PAR blotting (in *row c*, *lane 5* represents vector-transfected cells). *D*, KLF8 interaction with PARP-1 does not require PARP-1 catalytic activity. PARP-1$^{-/-}$ MEFs were transfected with the indicated expressing plasmids for co-IP assays. The protein expression was verified by anti-Myc and anti-HA IB of the whole cell lysates (supplemental Fig. 1*E*).

To determine whether KLF8 interaction with PARP-1 depends upon the catalytic activity of PARP-1 on modification of KLF8, we co-expressed KLF8 and either wild-type PARP-1 or its catalytic dead mutant E988K in the PARP-1-null MEF cells and performed co-IP analysis (Fig. 2*D*). We found that like the wild-type PARP-1, the E988K mutant remained capable of interacting with KLF8 (Fig. 2*D*, *row a*, compare *lane 4* with *lane 3*) despite the incapability of autoPARylation (Fig. 2*D*, *row b*, compare *lane 4* with *lane 3*) and PARylation of KLF8 (Fig. 2*D*, *row d*, compare *lane 4* with *lane 3*). Taken together, these results suggest that KLF8 interaction with PARP-1 is mainly through the cysteine residues in ZF2 of KLF8 independent of

the catalytic activity of PARP-1 and that the PARylation of KLF8 is specifically mediated by PARP-1, which depends on both the ZF2-mediated interaction between the two proteins and the presence of the ZF1 cysteines in KLF8.

Interaction with PARP-1 Is Essential for the Nuclear Localization of KLF8—Next, we sought to verify the nuclear localization of the newly generated KLF8 mutants described in Fig. 2*C*. We transiently expressed these mutants in HEK293T and NIH3T3 cells and carried out immunofluorescent staining. All these mutants were localized in the nucleus with the exception of the PARP-1 binding-deficient mutants ZF2mCs and ZF1,2mCs, which were localized in both the nucleus and the cytoplasm (data not shown). This surprising finding was confirmed when the ZF1,2mCs mutant was transiently expressed in the PARP-$1^{+/+}$ MEF cells and compared with the wild-type KLF8 (Fig. 3, *A* and *C*, compare KLF8-mut and KLF8 in the PARP-$1^{+/+}$ MEF cells). Even the wild-type KLF8 was dramatically mislocalized in the cytoplasm when PARP-1 expression in the PARP-$1^{+/+}$ MEF cells was silenced by RNAi (Fig. 3*A*, c_1–c_3, and Fig. 3*D*, compare siPARP-1 with siCtrl). Interestingly, treatment of the cells with the PARP-1 inhibitor PJ-34 only resulted in a slight KLF8 mislocalization in the cytoplasm (Fig. 3*A*, *panels* d_1–d_3, and Fig. 3*D*, compare PJ-34 with siCtrl). This result was further verified using the PARP-$1^{-/-}$ MEF cells. In these cells, KLF8, like the KLF8-mut, was clearly and completely mislocalized in the cytoplasm (Fig. 3*B*, compare KLF8 with KLF8-mut, and Fig. 3*C*, compare PARP-$1^{-/-}$ with PARP-$1^{+/+}$ MEF cells for KLF8). Also, this mislocalization was prevented by re-expression of either the wild-type PARP-1 (Fig. 3*B*, panels c_1–c_3) or its catalytically dead mutant (Fig. 3*B*, *panels* d_1–d_3). These results were reproduced using the freshly isolated PARP-$1^{+/+}$ and PARP-$1^{-/-}$ primary MEF cells, additional PARP-1 inhibitors, and various PARP-1 siRNAs (supplemental Fig. 4, *A* and *B*). These results suggest that KLF8 interaction with PARP-1 is required for the nuclear localization of KLF8, whereas PARylation is dispensable.

PARP-1 Binding and PARylation Inhibit the Interaction between KLF8 and CRM1—To test whether the KLF8 mislocalization to the cytoplasm is associated with the nuclear exporting protein CRM1-dependent nucleus-to-cytoplasm trafficking mechanism, we treated the PARP-$1^{-/-}$ MEF cells with the CRM1 inhibitor LMB. We found that this treatment significantly reduced the cytoplasmic presence of KLF8 (Fig. 4, *A* and *B*, compare *LMB*+ with *LMB*−). This result suggests that KLF8 interaction with CRM1 may be critical for KLF8 cytoplasmic mislocalization in the absence of PARP-1. To test this possibility, we re-expressed KLF8 and its mutant KLF8-mut in the PARP-$1^{-/-}$ MEF cells and performed co-IP analysis. As expected, we found that KLF8 and its mutant interacted with CRM1 equally (Fig. 4*C*, *row a*, *lanes 2* and *3*). The CRM1 interaction with KLF8 was completely blocked by co-expression of either wild-type PARP-1 or its E988K mutant (Fig. 4*C*, *row a*, *lanes 4* and *6*), whereas the CRM1 interaction with the KLF8-mut was only slightly prevented by PARP-1 re-expression (Fig. 4*C*, *row a*, *lane 5*). On the other hand, similar co-IP experiments showed that KLF8 interaction with CRM1 was most remarkably increased in the PARP-$1^{+/+}$ MEF cells when PARP-1 expression was silenced (Fig. 4*D*, *row a*, compare *lane 5* with *lane 2*). It should be noted that the affinity of KLF8 binding to PARP-1 and CRM1 was reversely correlated (Fig. 4, *C* and *D*, compare *row a* and *row b*). These results support the notion that PARP-1 maintains

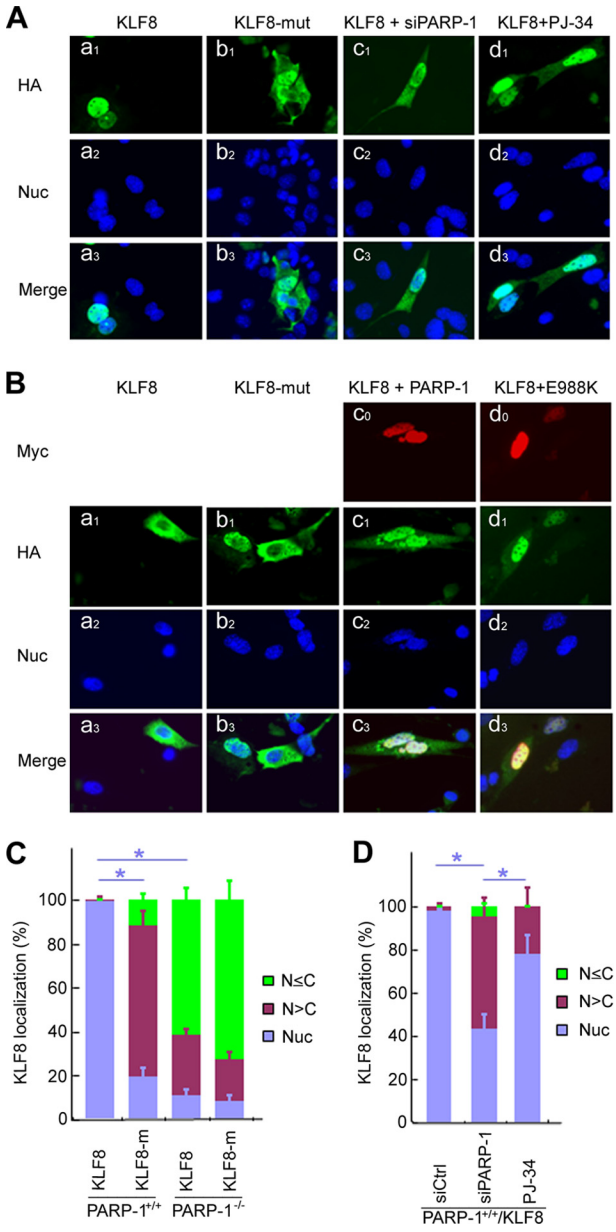

FIGURE 3. **Interaction with PARP-1 is essential for the nuclear localization of KLF8.** *A*, KLF8 mislocalizes in the cytoplasm when its PARP-1 binding site is disrupted or PARP-1 is knocked down but not when PARP-1 activity is inhibited. PARP-$1^{+/+}$ MEFs were transfected with KLF8 or its PARP-1 binding-deficient mutant (KLF8-mut) individually or in combination with treatment with PARP-1 siRNA (*siPARP-1*) or inhibitor (PJ-34, 3 μM). GFP siRNA was used as negative control. After 34–48 h, cells were analyzed by anti-HA staining of the KLF8 proteins (*green*) and Hoechst staining of the nuclei (*blue*) followed by fluorescent microscopy. *Scale bar*, 20 μm. *B*, cytoplasmic mislocalization of KLF8 in PARP-$1^{-/-}$ MEFs can be prevented by ectopic expression of both wild-type PARP-1 and its PARylase dead mutant (E988K). PARP-$1^{-/-}$ cells were transfected with KLF8 or KLF8-mut alone or along with PARP-1 or its dead mutant. After 24 h, cells were analyzed by anti-HA staining of the KLF8 (*green*), anti-Myc staining of PARP-1, and Hoechst staining of the nuclei (*blue*) followed by fluorescent microscopy. *C* and *D*, statistical analysis of data represented in *A* and *B*. The data represent the mean ± S.E. of at least three independent experiments. For each experiment, 200 cells were examined. *, $p < 0.01$ for difference in nuclear localization (*Nuc*) only.

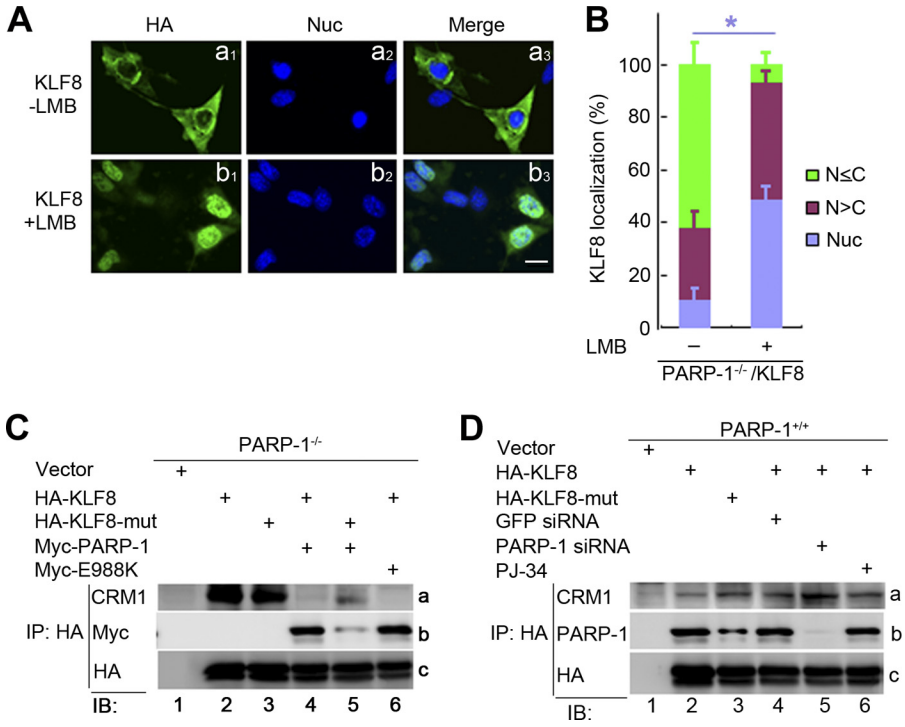

FIGURE 4. **PARP-1 competes with nuclear exportin protein CRM1 to regulate KLF8 subcellular localization.** *A*, cytoplasmic mislocalization of KLF8 in PARP-1$^{-/-}$ MEFs can be prevented by inhibiting CRM1. PARP-1$^{-/-}$ MEFs were transfected with KLF8 with or without LMB (3 nM) treatment. After 24 h, the cells were analyzed by anti-HA staining of the KLF8 (*green*) and Hoechst staining of the nuclei (*blue*) followed by fluorescent microscopy. *B*, statistical analysis of data represented in *A*. Data represent the mean ± S.E. of at least three independent experiments. For each experiment, 200 cells were examined. *, $p < 0.01$ for difference in nuclear localization (*Nuc*) only. *C*, KLF8 interacts with CRM1 in PARP-1$^{-/-}$ MEFs, which can be prevented by ectopic expression of PARP-1 or its PARylase dead mutant. PARP-1$^{-/-}$ MEFs were transfected with empty vector, with KLF8 or KLF8 mutant alone, or along with PARP-1 or its E988K mutant. After 24 h, whole cell lysates were prepared for anti-HA IP followed by IB with anti-PARP-1, anti-CRM1, and anti-HA. *D*, KLF8 interaction with CRM1 is enhanced by PARP-1 knockdown. PARP-1$^{+/+}$ MEFs were transfected for 24 h with empty vector, KLF8, or KLF8-mut with or without prior treatment with PARP-1 siRNA or GFP siRNA as control for 48 h or PJ34 for 4 h (6 μM). Whole cell lysates were prepared for IP as in *C*. Effective overexpression and knockdown were confirmed (supplemental Fig. 1, *F–H*).

the nuclear localization of KLF8 by competing with CRM1 for KLF8 binding.

PARP-1 Is Required for the Stability of KLF8 Protein in the Cell—Export of a nuclear protein to the cytoplasm usually results in either the cytoplasmic destruction or the cytoplasmic function of the protein. Assuming that the function of KLF8 is restricted in the nucleus given that it is a transcription factor, we first tested whether the nuclear export of KLF8 leads to its degradation in the cytoplasm. We overexpressed KLF8 or its mutant KLF8-mut in either the PARP-1$^{+/+}$ or the PARP-1$^{-/-}$ MEFs and performed CHX chase assays (Fig. 5). We found that the half-life of KLF8 was reduced from ~16 h in the PARP-1$^{+/+}$ MEFs to only ~4 h in the PARP-1$^{-/-}$ MEFs (Fig. 5, *A* and *C*). The KLF8-mut had an even shorter half-life of ~4 h in the PARP-1$^{+/+}$ cells and was further shortened to only ~1 h in the PARP-1$^{-/-}$ cells (Fig. 5, *B* and *C*). In the PARP-1$^{+/+}$ cells, the half-life of KLF8 was reduced to 4–8 h when PARP-1 expression was silenced or its activity was inhibited (Fig. 5, *D* and *E*). Based on these results, we concluded that both PARP-1 interaction and PARylation are important for maintaining the stability of KLF8 protein in the cell.

The Interaction with and PARylation by PARP-1 Affect the Ubiquitination of KLF8—Ubiquitination-mediated proteasomal degradation of proteins is a most common mechanism that regulates protein stability. To determine whether this mechanism regulates KLF8 protein turnover in the cell, we co-expressed KLF8 or KLF8-mut and ubiquitin in the MEFs, treated the cells with the proteasome inhibitor MG132, and examined the ubiquitination status of KLF8 protein by co-IP analysis (Fig. 6*A*). We found that the levels of ubiquitinated KLF8 were significantly higher in the PARP-1$^{-/-}$ MEFs than the PARP-1$^{+/+}$ MEFs (Fig. 6*A*, compare *lane 2* with *lane 7*) and that the KLF8-mut was more ubiquitinated than KLF8 in the presence of PARP-1 (Fig. 6*A*, compare *lane 10* with *lane 7*) but not in the absence of PARP-1 (Fig. 6*A*, compare *lane 5* with *lane 2*). These results suggest that PARP-1 protects KLF8 from ubiquitination.

To test whether nuclear export is required for the ubiquitination of KLF8, we treated the PARP-1$^{-/-}$ MEF cells with LMB and performed a similar ubiquitination analysis (Fig. 6*B*). We found that the ubiquitination of KLF8 was strongly inhibited by LMB (Fig. 6*B*, compare *lane 3* with *lane 2*). This result suggests that loss of PARP-1 interaction results in KLF8 ubiquitination primarily subsequent to its export to the cytoplasm.

To determine whether the PARylation of KLF8 affects its ubiquitination, we examined the ubiquitination status of KLF8 in the PARP-1$^{+/+}$ MEFs in the presence of the PARP-1 inhibitor PJ-34 (Fig. 6*C*). We found that the levels of ubiquitinated KLF8 were significantly increased by the PJ-34 treatment (Fig. 6*C*, compare *lane 2* with *lane 3*). In a separate project, we unexpectedly found that doxorubicin treatment increased the catalytic activity of PARP-1 (data not shown). Taking this advantage, we enhanced the levels of PARylated KLF8 in the

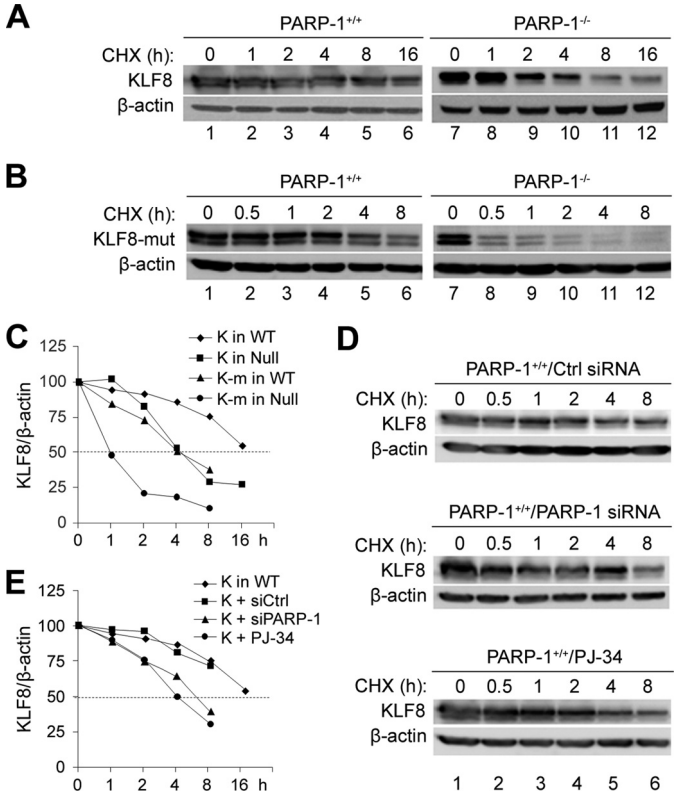

FIGURE 5. **PARP-1 is required for stabilizing KLF8 protein in the cell.** *A–C*, KLF8 protein has a shorter half-life in PARP-1$^{-/-}$ MEFs than in PARP-1$^{+/+}$ MEFs. PARP-1$^{+/+}$ or PARP-1$^{-/-}$ MEFs were transfected with KLF8 (*A*) or KLF8-mut (*B*) for 36 h and then treated with CHX (50 μg/ml) for the indicated periods of time. Whole cell lysates were prepared for anti-HA and anti-β-actin IB, and relative intensity of KLF8 protein (*K*) in the blots was graphed (*C*). *D* and *E*, the half-life of KLF8 protein is shortened when PARP-1 expression is knocked down or activity is inhibited. PARP-1$^{+/+}$ MEFs were transfected for 36 h with KLF8 after the prior transfection with the indicated siRNAs for 48 h or treatment with PJ-34 (3 μM, 12 h). After being chased with CHX treatment for the indicated periods of time, whole cell lysates were prepared for anti-HA and anti-β-actin IB (*D*), and relative intensity of KLF8 protein in the blots was graphed (*E*).

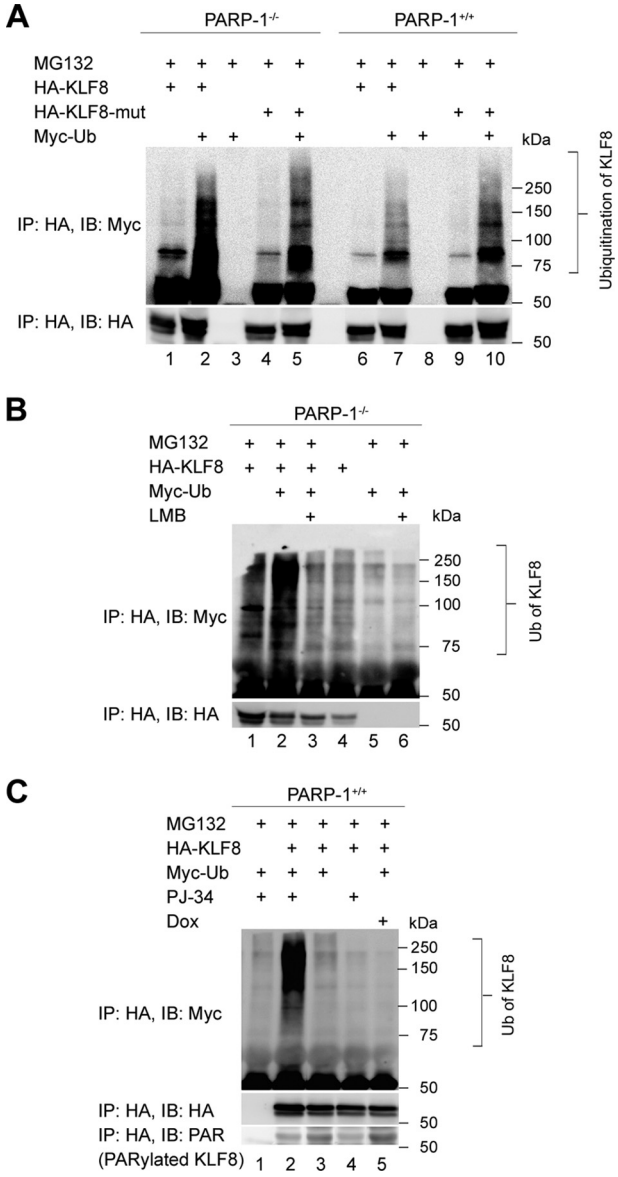

FIGURE 6. **KLF8 can be ubiquitinated, which can be inhibited by PARP-1.** *A*, KLF8 ubiquitination level is higher in PARP-1$^{-/-}$ MEFs than in PARP-1$^{+/+}$ MEFs. PARP-1$^{+/+}$ or PARP-1$^{-/-}$ MEFs were co-transfected with HA-KLF8 or its KLF8-mut mutant and Myc-ubiquitin (*Myc-Ub*) for 48 h. The cells were then treated with MG132 (20 μM, 8 h). Whole cell lysates were prepared for IP with anti-HA followed by IB with anti-Myc or anti-HA. *B*, KLF8 ubiquitination (*Ub*) is prevented by LMB treatment. PARP-1$^{-/-}$ MEFs were transfected, treated, and analyzed as in *A* except for the treatment with LMB (3 nM) during the last 10 h. *C*, KLF8 ubiquitination level is inversely correlated with its PARylation level and PARP-1 catalytic activity in the cell. PARP-1$^{+/+}$ MEFs were transfected, treated, and analyzed as in *A* except for the treatment with PJ-34 (3 μM) or doxorubicin (*Dox*) during the last 10 h.

PARP-1$^{+/+}$ MEFs using doxorubicin treatment and found that this treatment protected KLF8 from ubiquitination (Fig. 6*C*, compare *lane 5* with *lane 3*). These results indicated an inverse correlation between the PARylation and ubiquitination of KLF8 and suggested an opposing cross-talk between these potentially important post-translational modification of KLF8.

Interaction with and PARylation by PARP-1 Are Required for the Transcriptional Activity of KLF8—KLF8 was previously shown as a transcription activator of cyclin D1 gene promoter (1, 11). To determine the effect of PARP-1 on the transcriptional activity of KLF8, we performed cyclin D1 promoter luciferase reporter assays (Fig. 7). We found that the activation of cyclin D1 promoter by KLF8 was dramatically reduced when PARP-1 was deleted (Fig. 7*A*, *left panel*, compare *column 2* with *column 8*) or catalytically inhibited (Fig. 7*A*, *left panel*, compare *columns 10* and *12* with *column 8*). This reduction was well correlated with the levels of PARylated KLF8 in the cells (Fig. 7*A*, *right panel*, *row g*, compare *lanes 3* and *4* with *lane 2*). Similarly, the promoter activation was attenuated by disruption of KLF8 interaction with PARP-1 (Fig. 7*B*, *left panel*, compare *column 3* with *column 2*) or by silencing PARP-1 expression (Fig. 7*B*, *left panel*, compare *column 5* with *column 2*) in the PARP-1$^{+/+}$ MEFs. Importantly, the activation of cyclin D1 promoter by KLF8 in the cells was consistent with the change in cyclin D1 protein levels (Fig. 7*B*, *right panel*, *row b*, compare *lanes 3*, *5*, and *6* with *lane 2*). Conversely, re-expression of PARP-1 (but not its E988K mutant) in the PARP-1$^{-/-}$ MEFs restored the ability of KLF8 (but not its KLF8-mut mutant) to activate the cyclin D1 promoter (Fig. 7*C*, *left panel*, compare *column 6* with *columns 4*, *7*, and *8*) and promote cyclin D1 protein expression in the cells (Fig. 7*C*, *right panel*, *row b*, compare *lane 4* with *lanes* 2, *5*, and *6*). Inter-

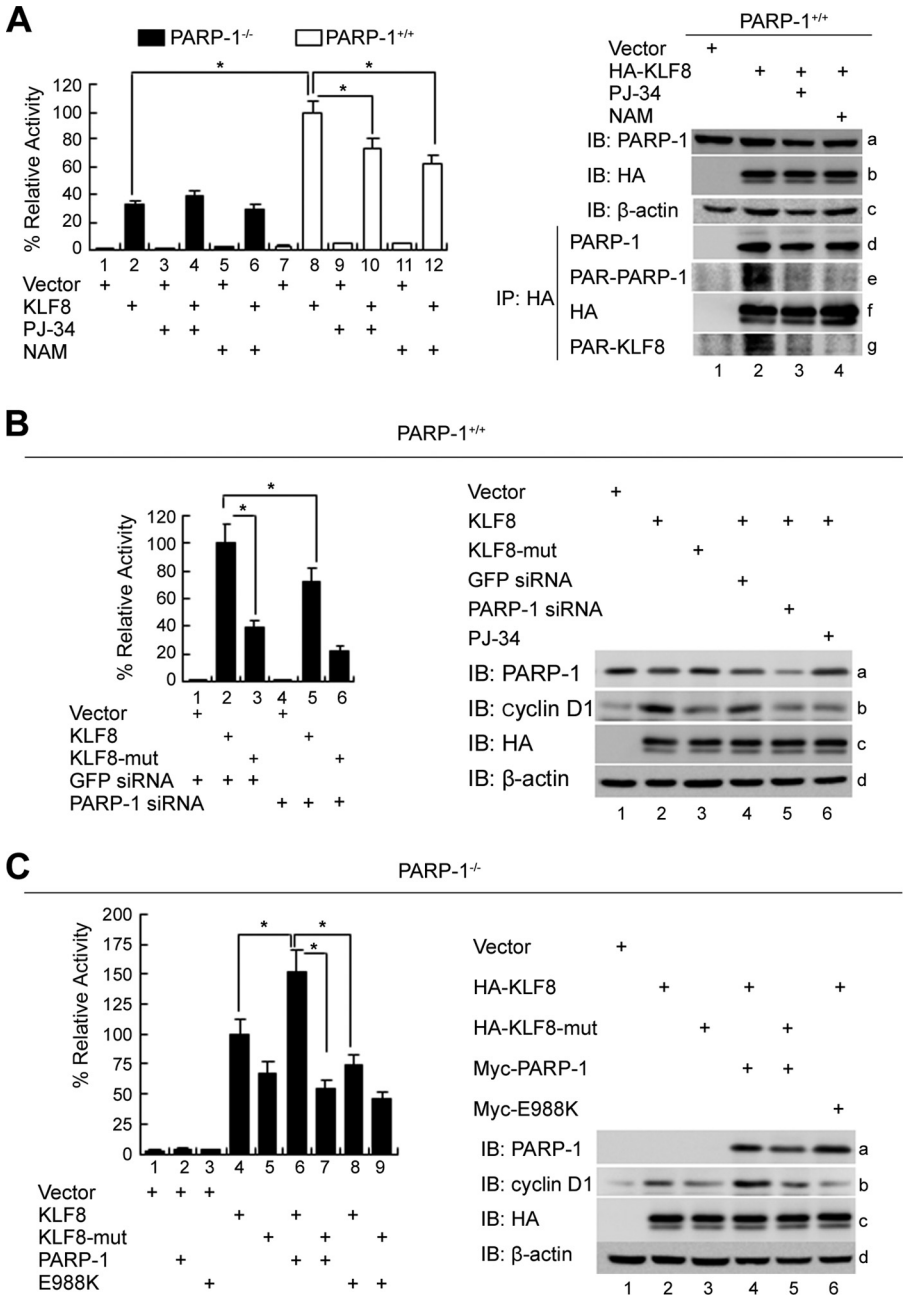

FIGURE 7. **KLF8 transcriptional activity depends partially on interaction with and modification by PARP-1.** *A*, KLF8 activates cyclin D1 promoter more potently in the presence than in the absence of PARP-1 activity. PARP-1$^{-/-}$ or PARP-1$^{+/+}$ MEFs were transfected with KLF8 or control vector along with cyclin D1 promoter reporter for 24 h with or without further treatment with PJ34 (3 μM) or nicotinamide (*NAM*, 10 mM) for an additional 12 h. Luciferase assays were performed as described under "Experimental Procedures" (*left panel*). KLF8 interaction with and PARylation by PARP-1 in the PARP-1$^{+/+}$ MEFs were confirmed by anti-HA IP followed by IB with anti-PARP-1 and anti-PAR (*right panel*). *B*, KLF8 activation of cyclin D1 transcription requires PARP-1 interaction and activity. PARP-1$^{+/+}$ MEFs were transfected with PARP-1 siRNA or GFP siRNA for 48 h followed by transfection with KLF8, KLF8-mut, or control vector along with the cyclin D1 promoter reporter. Luciferase assays (*left panel*) and IB for indicated proteins (*right panel*) were performed as in *A*. *C*, up-regulation of cyclin D1 expression by KLF8 in PARP-1$^{-/-}$ MEFs is enhanced by ectopic expression of PARP-1 in an interaction- and PARylation-dependent manner. PARP-1$^{-/-}$ MEFs were co-transfected with KLF8, KLF8-mut, or control vector and either PARP-1 or its E988K mutant for 24 h. Luciferase assays (*left panel*) and IB for indicated proteins (*right panel*) were performed as in *B*. Data in all the graphs represent the mean ± S.E. of at least three independent duplicate experiments. *, $p < 0.01$.

estingly, ChIP assays did not detect PARP-1 protein at the cyclin D1 promoter region where both KLF8 and p300 bind, although this promoter binding by KLF8 and p300 largely depends upon PARP-1 activity (supplemental Fig. 5). These results clearly indicated that PARP-1 plays a critical role in the regulation of KLF8 transcriptional activity through interaction with and PARylation of KLF8 in the nucleus.

DISCUSSION

Both KLF8 and PARP-1 play an important role associated with human diseases including cancer. Our novel findings that PARP-1 binds KLF8 in various cell types and regulates KLF8 in various aspects shed new light on both KLF8 and PARP-1 research with potentially significant clinical impact. We propose that KLF8 needs to stay in the nucleus to regulate target

gene transcription and possibly PARP-1-associated nuclear events. The nuclear localization of KLF8 requires its interaction with PARP-1 in the nucleus, which prevents KLF8 from being exported to the cytoplasm and subsequent degradation.

The fact that PARP-1 interaction with KLF8 prevents the nuclear exportin CRM1 from binding to KLF8 suggests that PARP-1 serves as a nuclear retention factor of KLF8. In agreement with this work, other groups have recently demonstrated a role for PARP-1 to regulate the nuclear localization of p65 NF-κB and p53 (22, 25). We have previously identified two nuclear localization signals for KLF8, one of which is located within the ZF DNA binding domain (4). That work also demonstrated that both nuclear importin-β and CRM1 are involved in the regulation of KLF8 nuclear localization (4). It is plausible to consider that the nuclear localization signal in the ZF domain serves as a platform for PARP-1 to keep KLF8 in the nucleus after KLF8 is carried into the nucleus by the nuclear importin. Because the ZF DNA binding domain is required for KLF8 interaction with both importin-β and PARP-1, it is also possible that PARP-1 relays KLF8 from the importin and helps send KLF8 to promoter DNA of target genes in the nucleus. PARP-1 has been suggested to function as a *bona fide* transcriptional co-activator, as in the case of PARP-1 regulation of NFκB-mediated transcription (26). In contrast, our results show that PARP-1 does not stay together with KLF8 at the promoter of cyclin D1, suggesting that PARP-1 likely works as a messenger to pass KLF8 from the nuclear importin to another co-activator, such as the histone acetyltransferases p300 and p300/CBP-associated factor transcription (11), at the promoter and lets the latter do the job of transcriptional co-activation. Indeed, we have shown that the histone acetyltransferase-mediated acetylation on both histones (11) and KLF8 protein (9) is required for the activation of the cyclin D1 promoter, and the binding to the cyclin D1 promoter by both KLF8 and p300 heavily depends upon PARP-1 activity. These results suggest an interesting notion that PARP-1 promotes an exchange of a co-repressor complex with a co-activator complex at the target promoter in a dynamic manner (14). Consistently with this notion, our result shows that only a fraction of KLF8 protein molecules interacts with PARP-1 (see supplemental Fig. 3*B*). Therefore, PARP-1 interaction with KLF8 may have multifold significance. First, it prevents CRM1-dependent nuclear export of KLF8. Second, it helps the PARylation of KLF8, which in turn helps formation of a co-activator complex at a KLF8 target promoter. Third, it helps to temporarily put KLF8 into sleep when the cell needs KLF8 to be away from its target gene promoters, or fourth, it may help switch KLF8 from one group to another group of target gene promoters to meet the cellular requirement. Nevertheless, constitutive PARP-1 interaction could restrict the ability of KLF8 to regulate its target gene expression, as in the case of PARP-1 regulation of Sp1 (28).

Nevertheless, all the possibilities described above have pointed to a conclusion that in the nucleus, KLF8 is localized to at least two compartments, at the target gene promoters together with its transcriptional co-regulators or away from the promoter and likely together with PARP-1. This subnuclear compartmentation dictates where and how KLF8 protein is modified and possibly where and how KLF8 functions in the nucleus. It will be interesting to test whether KLF8 is differentially modified post-translationally by the histone acetyltransferase-mediated acetylation and PARP-1-mediated PARylation. We have recently demonstrated that KLF8 is also modified by sumoylation in the nucleus and that this modification delimits the ability of KLF8 to activate transcription and to promote cell proliferation (3) and is counteracted by the acetylation of KLF8 (9). Whether or not KLF8 sumoylation and PARylation are also mutually associated or regulated and take place at the same subnuclear compartment is not known but a very interesting question to address. Does KLF8 play any active role in this compartment containing PARP-1, and how is this role affected by these post-translational modification statuses? Experiments are in progress to answer these interesting questions.

In addition to KLF8, ubiquitination-mediated proteasomal degradation has been reported to regulate other KLF family members including KLF5 (29, 30), KLF2 (31), and KLF10 (32). One KLF protein can be targeted by more than one ubiquitin E3 ligase, such as the homologous to the E6-AP carboxyl terminus domain E3-ubiquitin ligase WWP1 (29) and the F-box ubiquitin E3 ligase Fbw7 (30, 33). On the other hand, one ubiquitin E3 ligase such as WWP1 can target more than one KLF protein for proteasomal degradation (29, 31). The E3 ligase that targets KLF8 for ubiquitination and subsequent proteasomal degradation is currently unknown. WWP1 or Fbw7 does not appear to be the one as there is not an evident potential targeting site for either of the E3 ligases in KLF8, suggesting that the ubiquitination of KLF8 is likely mediated by a different E3 ligase still to be identified. It appears likely that the ubiquitination of KLF8 takes place primarily in the cytoplasm given that LMB treatment prevents it in the PARP-$1^{-/-}$ MEF cells. Because E3 ligases usually target the activation domains of transcriptional factors and frequently involve phosphorylation of the target protein, it will be interesting to test whether KLF8 is targeted by any E3 ligase at its activation domain (11) and whether any phosphorylation events within this domain are involved.

The fact that interaction between KLF8 and PARP-1 takes place in several different types of cells suggests a general importance of this regulation. In addition, consistent with our results, another KLF family member KLF5 has recently been reported to interact with PARP-1 in a ZF-dependent manner (34). Because the ZF domains are well conserved among the KLF family proteins, the PARP-1-mediated regulatory mechanism identified in this work could apply to other KLF family proteins as well. Like KLF8, several other KLF family members including KLF4, KLF5, KLF6, KLF8, KLF9, KLF10, KLF11, and KLF12 are involved in cancer pathobiology (27, 35). PARP-1 has long been considered as a molecular target to enhance anti-cancer therapeutic efficiency (12, 15–18). Therefore, our novel results presented in this work highlight the clinical significance of co-targeting PARP-1 and KLF8 (or other potential PARP-1-interacting KLFs) as a novel anti-cancer therapeutic strategy.

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