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

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Kruppel-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 cysteine 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 (CACC) 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...
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, pHK3-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 pHK3-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'-GGACAACACCACTAGAATGCG-3'). 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'-tgg gat gcc gcc tcc tgg-3' and 5'-ttg cag gag cca gca aag-3'; C306A, 5'-tcc cag gct gct tta taa ggc-3' and 5'-agc ctt ata-3'; C311A, 5'-tcc agg agg cgc cat ccc-3' and 5'-tggt gat ggc gcc tcc tgg-3'. ZF1mCs is the double mutant of C276A and C278A. 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'-aga tgt aca ttg gac gct tct tc-3' and 5'-tgt cgg gag cca gca aag-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 Na3VO4, 1 mM PMSF, 20 μg/ml leupeptin, and 0.06 trypsin inhibitor unit/ml aprotnin). 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 pRSV40 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-TARGETplus 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'-GUG CCU AGC GCA CAU CUU G-3'; J006656-08, 5'-CCA AUU GCC 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...
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® 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/H11001/H11001 or PARP-1/H11002/H11002 MEF cells. Sonicated lysates prepared from 2 x 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. 1A). We overexpressed HA-KLF8 or Myc-KLF8 in HEK293T cells. The whole cell lysates prepared from these transfected cells were subjected to IP with anti-HA- or anti-Myc-conjugated agarose beads. The precipitated proteins were resolved by SDS-PAGE and revealed by Coomassie Blue staining. PARP-1 was co-immunoprecipitated with PAR-ylated KLF8 with similar efficiency (Fig. 1B). To confirm the direct interaction, recombinant PARP-1 protein was pulldown 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).

FIGURE 1. KLF8 interacts with PARP-1 and is PAR-ylated. 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 PAR-ylated 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 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. Hs578T 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 pulldown 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).
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were pulled down from both HA-KLF8-expressing and Myc-KLF8-expressing cells (Fig. 1A, lanes 2 and 3) but not from the vector transfected cells (Fig. 1A, 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. 1A). 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. 1B, 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. 1B, rows e and f) and anti-PARP-1 co-IP (Fig. 1C). 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. 1D) and GST–KLF8 pulldown of recombinant PARP-1 protein (Fig. 1E). 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. 2A). We found that the N-terminal truncation up to 200 amino acid residues did not affect the interaction with PARP-1 (Fig. 2A, compare lanes 7 and 8 with lane 6). Further N-terminal truncations were not tested as those mutants are known to mis-localize 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. 2A, 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. 2B, 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. 2B, lane 5, and Fig. 2C, lane 7), whereas mutation of two histidines (Fig. 2B, lane 6) or the individual cysteines (Fig. 2C, lanes 4 and 5) of ZF2 or any of the cysteines, histidines, or DNA contact motifs of ZF1 (Fig. 2B, lanes 3 and 4, and Fig. 2C, 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. 2C, row c, compare lane 7 with lane 9). Interestingly, the ZF1mCs mutant displayed a similar degree of reduction in PARylation (Fig. 2C, 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. 2C, 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. 3A).

![FIGURE 2. KLF8 interacts with PARP-1 through its ZFs independent of the catalytic activity of PARP-1.](image_url)

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. 2D). We found that like the wild-type PARP-1, the E988K mutant remained capable of interacting with KLF8 (Fig. 2D, row a, compare lane 4 with lane 3) despite the incapability of autoPARylation (Fig. 2D, row b, compare lane 4 with lane 3) and PARylation of KLF8 (Fig. 2D, 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. 2C. 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. 3A, c1–c3, and Fig. 3D, 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. 3A, panels d1–d3, and Fig. 3D, 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. 3B, compare KLF8 with KLF8-mut, and Fig. 3C, 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. 3B, panels e1–e3) or its catalytically dead mutant (Fig. 3B, panels d1–d3). 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. 4C, 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. 4C, row a, lanes 4 and 6), whereas the CRM1 interaction with the KLF8-mut was only slightly prevented by PARP-1 re-expression (Fig. 4C, 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. 4D, 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.
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FIGURE 4. PARP-1 competes with nuclear export 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 μM) 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. 6A). We found that the levels of ubiquitinated KLF8 were significantly higher in the PARP-1−/− MEFs than the PARP-1+/+ MEFs (Fig. 6A, compare lane 2 with lane 7) and that the KLF8-mut was more ubiquitinated than KLF8 in the presence of PARP-1 (Fig. 6A, compare lane 10 with lane 7) but not in the absence of PARP-1 (Fig. 6A, 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. 6B). We found that the ubiquitination of KLF8 was strongly inhibited by LMB (Fig. 6B, compare lane 3 with lane 2). This result suggests that loss of PARP-1 interaction results in KLF8 ubiquitination.

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. 6C). We found that the levels of ubiquitinated KLF8 were significantly increased by the PJ-34 treatment (Fig. 6C, 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
Regulation of KLF8 by PARP-1

PARP-1+/+ MEFs using doxorubicin treatment and found that this treatment protected KLF8 from ubiquitination (Fig. 6C, 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. 7A, left panel, compare column 2 with column 8) or catalytically inhibited (Fig. 7A, 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. 7A, 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. 7B, left panel, compare column 3 with column 2) or by silencing PARP-1 expression (Fig. 7B, 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. 7B, 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. 7C, left panel, compare columns 4, 7, and 8) and promote cyclin D1 protein expression in the cells (Fig. 7C, right panel, row b, compare lane 4 with lanes 2, 5, and 6). Inter-
Interestingly, 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 expression.
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. 3B). 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 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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