Polycomb Group Protein PHF1 Regulates p53-dependent Cell Growth Arrest and Apoptosis*

Yang Yang†, Chenni Wang†, Pingzhao Zhang‡§, Kun Gao‡, Dejie Wang‡, Hongxiu Yu‡, Ting Zhang‡, Sirui Jiang‡, Saiyin Hexige‡, Zehui Hong‡, Akira Yasui**, Jun O. Liu†, Haojie Huang‡‡, and Long Yu†§,‡

From the †State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, 220 Handan Road, Shanghai 200433, China, the ‡Institutes of Biomedical Sciences, Fudan University, Shanghai 200032, China, the ‡§Key Laboratory of Developmental Genes and Human Disease, Ministry of Education, Southeast University, Nanjing 210009, China, the ‡¶Department of Molecular Genetics, Institute of Development, Aging and Cancer, Tohoku University, Aobaku, Sendai 980-8575, Japan, the ‡‡Departments of Pharmacology and Molecular Sciences and Oncology, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205, and the **Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905

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†‡ Both authors contributed equally to this work.
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‡‡ To whom correspondence should be addressed: State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, 220 Handan Rd., Shanghai 200433, China. Tel.: 86-21-65643954; Fax: 86-21-65643250; E-mail: longyu@fudan.edu.cn.

Background: PHF1 is a component of a novel PRC2 complex and plays important roles in H3K27 methylation and Hox gene silencing.

Results: PHF1 stabilizes p53 to promote cell growth arrest and apoptosis by protecting p53 from MDM2-mediated ubiquitination and degradation.

Conclusion: PHF1 is a novel regulator of p53 pathway.

Significance: We unraveled a new function of PHF1 in p53 pathway regulation.

Polycomb group protein PHF1 is well known as a component of a novel EED-EZH2-Polycomb repressive complex 2 complex and plays important roles in H3K27 methylation and Hox gene silencing. PHF1 is also involved in the response to DNA double-strand breaks in human cells, promotes nonhomologous end-joining processes through interaction with Ku70/Ku80. Here, we identified another function of PHF1 as a potential p53 pathway activator in a pathway screen using luminescence reporter assay. Subsequent studies showed PHF1 directly interacts with p53 proteins both in vivo and in vitro and co-localized in nucleus. PHF1 binds to the C-terminal regulatory domain of p53. Overexpression of PHF1 elevated p53 protein level and prolonged its turnover. Knockdown of PHF1 reduced p53 protein level and its target gene expression both in normal state and DNA damage response. Mechanically, PHF1 protects p53 proteins from MDM2-mediated ubiquitination and degradation. Furthermore, we showed that PHF1 regulates cell growth arrest and etoposide-induced apoptosis in a p53-dependent manner. Finally, PHF1 expression was significantly down-regulated in human breast cancer samples. Taken together, we establish PHF1 as a novel positive regulator of the p53 pathway. These data shed light on the potential roles of PHF1 in tumorigenesis and/or tumor progression.

The p53 tumor suppressor is critical for maintenance of genome stability and protection against malignant transformation (1). p53 mutations have been documented in more than half of all human tumors (2). Defects in other components of the p53 pathway such as the ARF tumor suppressor are observed in tumor cells that retain wild-type p53 (3). Thus, inactivation of the p53 pathway appears to be a common event in human cancer. The cellular functions of p53 are rapidly activated in response to various stresses to cause cell cycle arrest and apoptosis (1). p53 is tightly regulated by multiple signaling pathways through distinct mechanisms. Its turnover is mainly controlled by MDM2 that binds to p53 and functions as an ubiquitin E3 ligase to promote p53 ubiquitination and degradation by the proteasome (1). Thus, factors that affect the physical interaction of MDM2 with p53 or MDM2 E3 ligase activity will directly impact p53 steady-state level. Posttranslational modifications such as phosphorylation and p300-dependent acetylation of C-terminal lysine of p53 have been shown to regulate p53 stability in part by controlling the accessibility of p53 to MDM2 and blocking MDM2-mediated ubiquitination and degradation (1). A number of oncoproteins or tumor suppressor proteins affect protein stability and activity by modulating the MDM2-p53 axis. For instance, the tumor suppressor p14ARF directly interacts with and functionally inactivates MDM2, by either relocating MDM2 to the nucleolus and/or forming a tripartite complex with MDM2 and p53, which abrogates MDM2-mediated ubiquitination and degradation of p53 (4). Other proteins such as MdmX, YY1, gankyrin, VHL, and several ribosomal proteins also participated in MDM2-p53 axis modulation by multiple mechanisms (5–8).

Polycomb group proteins play an essential role in the epigenetic maintenance of repressive chromatin states (9). The gene-silencing activity of the Polycomb repressive complex 2
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(PRc2)³ depends on its ability to trimethylate lysine 27 of histone H3 (H3K27) (10). The core components of PRc2 complex contains four components, EZH2, EED, SUZ12, and RbAp46/48, and has been demonstrated to have intrinsic histone methyltransferase activity toward H3K27 (10). The Polycomb group protein PHF1 has been shown to interact with EZH2 (11), PHF1 positively regulates the H3K27 methyltransferase activity of the PRc2 complex both in vitro and in vivo (12, 13). In addition, PHF1 is also important for H3K27 methylation and Hox gene expression in vivo (12). PHF1 directly contributes to HOXA10 silencing by facilitating the recruitment of the PRc2 complex and subsequent H3K27 methylation at its promoter (12). In addition to the roles in gene repression, PHF1 is also involved in the response to DNA double-strand breaks in human cells. PHF1 is rapidly recruited to double-strand break sites, promoting non-homologous end-joining processes by directly interacting with Ku70/Ku80 (14). Among other proteins implicated in DNA damage response, p53 was previously found to coimmunoprecipitate with PHF1 in a proteomics analysis, although it was not determined whether the interaction is direct and what functional consequence this interaction has on p53 (14).

Here, we demonstrated that PHF1 is a novel activator of the p53 signaling pathway. We verified the interaction between PHF1 and p53 both in vitro and in vivo. PHF1 was found to protect p53 protein from MDM2-mediated ubiquitination and degradation. Furthermore, we showed that PHF1 promoted p53-dependent cell growth arrest and etoposide-induced apoptosis. Taken together, these findings unravel a novel mechanism of p53 regulation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—U2OS, H1299, and HepG2 cells were obtained from the American Type Culture Collection. HCT116 p53¹/² and HCT116 p53⁻/⁻ cells were generous gifts from Dr. Bert Vogelstein (The Johns Hopkins University). U2OS, HepG2, HCT116 p53¹/², and HCT116 p53⁻/⁻ cells were maintained in DMEM with 10% FBS, whereas H1299 cells were cultured in RPMI 1640 with 10% FBS. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Expression Constructs**—Human PHF1 cDNA were described previously (14). All other PHF1 and p53 deletion mutants were generated using the KOD-Plus mutagenesis kit (Toyobo, Japan). USP7 and USP10 were from Dr. Wei Gu (Columbia University).

**Transcriptional Reporter Assay**—Luciferase assay (Promega) was conducted in cells that were transiently transfected with the reporter constructs (AP1-Luc, CRE-Luc, DRS-Luc, E2F-Luc, GRE-Luc, HSE-Luc, NFAT-Luc, NfκB-Luc, p53-Luc, Rb-Luc, and SRE-Luc; purchased from Clontech), pTK-galactosidase (Promega) construct, and the PHF1 expression constructs and/or with the empty vector alone. Luciferase activity in cell lysates was measured using the luciferase assay system in a Berthold Lumat LB 9507 luminometer (Promega). Luciferase activity was normalized to galactosidase activity as an internal control. Each assay was performed in triplicate, and results were confirmed by at least three individually repeating experiments.

**RNA Interference**—The HuSH 29-mer shRNA constructs against PHF1 in pGFP-V-RS vector were purchased from OriGene, Inc. The non-effective scrambled pGFP-V-RS construct (TR30013, OriGene) was used as negative control (NC). The sequences for PHF1-specific shRNA expression and scrambled cassettes are as follows: KD-1, 5’-ACATCTGAGCAACCGACAGCAGAGTTACT-3’; KD-2, 5’-TGTCACGTGTAGAGAAGTGTCGCCCATGCT-3’; and NC, 5’-GCACTACAGAGCTAATCAGATGTACT-3’.

**Antibodies**—The following antibodies were used in this work: PHF1 (sc-130646; Santa Cruz Biotechnology), PHF1 (AT3294a; Abgent), p53 (Do-1; sc-126; Santa Cruz Biotechnology), p53 (FL-393; sc-6243; Santa Cruz Biotechnology), acetyl-p53 (Lys-382; 2525S; Cell Signaling), phospho-Ser-15-p53 (9286S; Cell Signaling), p53 (FL-393), phospho-Ser-15-p53 (9286S; Cell Signaling), p53 (Do-1; sc-126), Myc (9E10; Sigma), FLAG (M2; Sigma), HA (MM5–101R; Convance), GFP (sc-8334; Santa Cruz Biotechnology), and actin (AC-74; Sigma).

**Immunoprecipitation**—Cells were lysed with 1× cell lysis buffer (Cell Signaling), and the lysate was centrifuged. The supernatant was precleared with protein A/G beads (Sigma) and incubated with indicated antibody overnight. Thereafter, protein A/G beads were applied, all at 4 °C. After 2 h of incubation, pellets were washed five times with lysis buffer and resuspended in sample buffer and analyzed by SDS-PAGE.

**Western Blot**—Cell lysates or immunoprecipitates were subjected to SDS-PAGE and proteins were transferred to nitrocellulose membranes (GE Healthcare). The membrane was blocked in Tris-buffered saline (TBS, pH 7.4) containing 5% nonfat milk and 0.1% Tween 20, washed twice in TBS containing 0.1% Tween 20, and incubated with primary antibody for 2 h and followed by secondary antibody for 1 h at room temperature. Afterward, the proteins of interest were visualized using ECL chemiluminescence system (Santa Cruz Biotechnology).

**GST Pulldown Assay**—293T cells were lysed 40 h after transfection with 1× cell lysis buffer at 4 °C. GST fusion proteins were immobilized on glutathione-Sepharose beads (Amersham Biosciences). After washing with pull-down buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM DTT, 10% glycerol, 1 mM EDTA, 2.5 mM MgCl₂, and 1 µg/ml leupeptin), the beads were incubated with lysates of 293T cells or Escherichia coli expressed and purified recombinant His-p53 protein for 2 h. The beads were then washed five times with binding buffer and resuspended in sample buffer. The bound proteins were subjected to SDS-PAGE analysis.

**Immunofluorescent Cytochemistry**—Cells cultured and transiently transfected on coverslips were fixed in 4% paraformaldehyde for 10 min and permeabilized in 0.2% Triton X-100 for 15 min at room temperature and blocked with 10% normal horse serum plus 1% BSA (Amersham Biosciences) for 1 h. The treated cells on the coverslips were incubated overnight at 4 °C with mouse anti-HA or Myc antibody (1:500 dilution). After being washed three times in PBS containing 0.1% Tween 20, the cells were incubated with rhodamine red-conjugated goat anti-mouse secondary antibody (1:300 dilution) for 1 h and stained.
RESULTS

Identification of PHF1 as a Potential p53 Pathway Regulator—The earlier observation that p53, along with a number of other proteins involved in DNA damage response, coimmunoprecipitated with PHF1 suggested a tantalizing functional link between the two proteins (14). To further explore the potential functional interaction between PHF1 and p53, we performed reporter gene assays to determine whether PHF1 affected the transcriptional activity of p53. In addition, we also included reporter genes for a number of other transcription factors, including AP1, CRE, DR5, E2F, GRE, HSE, NFAT, NFκB, p53, Rb, and SRE. These reporter constructs contain the luciferase gene under the control of tandem response elements of the corresponding transcription factors. The PHF1 expression construct was co-transfected with each of the reporter constructs into HepG2 cells. The luminescence outputs were measured and used to survey the effects of PHF1 on the different transcription factors. Expression of PHF1 significantly increased the p53-luciferase reporter activity (18.6-fold, p < 0.001). In contrast, PHF1 had much smaller or no effects on all other reporters tested (Fig. 1A). To further confirm that PHF1 increased p53 reporter activity is dependent on endogenous p53, we repeated the reporter activation experiment in cell lines with or without wild-type p53. As shown in Fig. 1B, PHF1 significantly increased p53 reporter activity in a dose-dependent manner in U2OS (p53 wild type) and HCT116 p53+/+ cells but had no effect on the p53 reporter in H1299 (p53 null) and HCT116 p53−/− cells, suggesting that activation of the p53 reporter by PHF1 is dependent on p53. We also determined the effects of PHF1 on the promoter activity on two well-established p53 target genes: the cell cycle regulator p21 and the proapoptotic gene Bax. PHF1 increased activities of both p21 and Bax promoters in a dose-dependent manner in U20S and HepG2 cells (Fig. 1C). These results suggest that PHF1 potentiates p53 transactivation activity and PHF1 may be a novel regulator of p53.

PHF1 Interacts with p53 in Vitro and in Vivo—We determined whether PHF1 directly interacts with p53. We co-expressed HA-p53 and Myc-PHF1 in H1299 cells and immunoprecipitated p53 by anti-HA antibody. As shown in Fig. 2A (upper panel), HA-p53 coimmunoprecipitated with Myc-PHF1. Similar results were obtained in a reciprocal coimmunoprecipitation experiment using anti-Myc antibody (Fig. 2A, lower panel). Importantly, when endogenous p53 was immunoprecipitated from U2OS cells by anti-p53 antibody (FL-393), endogenous PHF1 was detected in the immunoprecipitate by Western blot (Fig. 2B). To examine the interaction between PHF1 and p53 in vivo, we determined whether these two proteins are localized to the same subcellular compartments. As shown in Fig. 2C, PHF1 was exclusively localized in nucleus. p53 was predominantly localized in nucleus, but a small fraction was also localized in cytoplasm. When p53 and PHF1 were coexpressed, the two proteins were exclusively co-localized in nucleus. To determine whether the interaction between p53 and PHF1 is direct, we assessed their interaction in vitro using purified recombinant proteins. Recombinant His-p53 and GST-PHF1 were expressed and purified from bacteria. The
GST-PHF1, but not GST alone, bound to His-p53 in a pulldown assay (Fig. 2D). Taken together, these results indicate that PHF1 interacts with p53 both in vitro and in vivo.

**Delineation of the Domains Mediating the Mutual Interactions between PHF1 and p53**—p53 is modular with multiple domains, including a transactivation domain, a proline-rich domain, a DNA-binding domain, a tetramerization domain, and a C-terminal regulatory domain. To determine which domain mediates its interaction with PHF1, we generated a series of truncation mutants of p53 (C1-C3, N1-N4). A total of seven mutants were generated as GST fusion proteins and expressed and purified from bacteria. A pulldown assay with GST-p53 and the various truncation mutants was used to assess their interactions with Myc-PHF1 overexpressed in H1299 cells. As shown in Fig. 3A, the C-terminal regulatory domain of p53 is sufficient for PHF1 binding. A similar strategy was used to map the p53-interacting sequences of PHF1. We generated a series of N- or C-terminal deletion mutants of PHF1 and determined the minimal region that mediates its interaction with p53. As shown in Fig. 3B and C, there exist two discrete domains (named BD1 and BD2) that were capable of p53 binding, which are localized to the N and C termini of PHF1, respectively.

**PHF1 Promotes p53 Stability in Vivo**—p53 is a short-lived protein, and its stability is tightly controlled under normal and stressed conditions. First, we tested whether overexpression of PHF1 affected the level of p53 protein. As shown in Fig. 4A, the protein level of p53 was dramatically elevated when PHF1 was co-expressed in H1299 cells. Two deletion mutants, PHF1/H9004 BD1 or BD2, only moderately increased p53 protein levels. However, the deletion mutant lacking both binding domains (PHF1/H9004 BD1/2) had no effect on the steady-state level of p53, suggesting PHF1 stabilizes p53 through direct interaction. The increase in p53 protein level in the presence of PHF1 can be attributed to either enhanced expression at the mRNA or protein levels or an increase in protein stability. To distinguish
between those possibilities, we determined the half-life of p53 in the absence or the presence of coexpressed PHF1. We found that PHF1 significantly increased the half-life of p53 (Fig. 4B). In a complementary experiment, we knocked down the endogenous PHF1 and determined the changes in p53 protein level in p53 wild-type cells. As shown in Fig. 4C, knockdown of PHF1 in U2OS and HepG2 cells by two independent shRNAs resulted in a marked decrease in the protein levels of endogenous p53. To exclude the possibility that p53 protein decrease resulted from transcriptional down-regulation, we performed qRT-PCR to measure the mRNA levels of PHF1 and p53 in PHF1 knockdown U2OS cells. In contrast to the significant decrease in mRNA of PHF1, the mRNA level of p53 in PHF1 depleted U2OS cells stayed at a level similar to that of control cells (Fig. 4D). Furthermore, knockdown of PHF1 in U2OS cells also decreased the half-life of endogenous p53 (Fig. 4E). Taken together, these results further support the notion that PHF1 regulates p53 protein stability in vivo.

We also tested whether knockdown of PHF1 affected the expression of p53 target genes. As shown in Fig. 4F, knockdown of PHF1 in U2OS cells resulted in a marked decrease in the mRNA levels of a subset of p53 target genes examined, including p21, 14-3-3δ, MDM2, PUMA, PIG3, and cyclin G1. But knockdown of PHF1 had no obvious effect on the mRNA level of GADD45, suggesting that PHF1 may selectively regulates certain p53 target genes. Given that DNA damage is one of the major stresses that provoke p53 action, we examined the p53 protein stabilization in PHF1 knockdown U2OS cells treated with genotoxic drug etoposide. As shown in Fig. 4G, in control knockdown cells, p53 protein levels gradually increased in a time-dependent manner, followed by an increase in endogenous p21 protein levels. In contrast, knockdown of PHF1 resulted in a marked delay in the accumulation of p53 and p21. The etoposide-induced Lys-382 acetylation and Ser-15 phosphorylation of p53 were also reduced in PHF1 knockdown U2OS cells (Fig. 4G), and two post-translational mod-

FIGURE 3. Delineation of the domains mediating the mutual interactions between PHF1 and p53. A, schematic presentation of p53 domains (left panel, top) and deletion mutants (left panel, bottom) and the results of GST pulldown experiments (right panel). Bacterially expressed GST fusion proteins of wild-type p53 and the deletion mutants were bound to glutathione-Sepharose beads, respectively, and incubated with lysates of H1299 cells transfected with a Myc-PHF1 expression construct. Bound Myc-PHF1 was detected by Western blot with anti-HA antibody, and various GST-p53 proteins were detected by ponceau S staining. B and C, schematic model of PHF1 domains (left panel, top) and deletion mutants (left panel, bottom) and the results of GST pulldown experiments (right panel). Bacterially expressed GST fusion proteins of wild-type PHF1 and the deletion mutants were bound to glutathione-Sepharose beads, respectively, and incubated with lysates of H1299 cells transfected with a Myc-p53 expression construct. Bound Myc-p53 was detected by Western blot with Do-1 antibody, and GST-PHF1 deletion mutant proteins were detected by ponceau S staining. M.W., molecular weight.
PHF1 Controls p53 Protein Stability

Modifications are critical for p53-mediated transactivation activities. These results suggest that PHF1 controls p53 stability and activity both in normal states and DNA damage response.

PHF1 Protects p53 from MDM2-mediated Ubiquitination and Degradation—p53 stability is mainly regulated by ubiquitin-dependent degradation. MDM2 is an E3 ubiquitin ligase that plays a major role in regulating p53. Other E3 ubiquitin
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PHF1 binds to the C-terminal domain of p53 that contains several lysine residues that can be post-translationally modified by the small ubiquitin-like protein SUMO. Unlike ubiquitination, p53 sumoylation does not target proteins for rapid degradation but rather regulates its transcriptional activities and localization. We examined whether PHF1 affects the sumoylation status of p53. As shown in Fig. 5, PHF1 overexpression in vitro and in vivo reversed the deubiquitination of FLAG-p53, but none of the other ubiquitin ligases or deubiquitinases (USP7 and USP10) were each co-expressed with Myc-PHF1 in 293 cells and extracts of the transfected cells were immunoprecipitated with the anti-FLAG antibody. Western blots revealed that PHF1 was clearly detected in the immunoprecipitates of FLAG-p53 (Fig. 5A), but none of the other ubiquitin ligases or deubiquitinases were able to co-immunoprecipitate PHF1 (Fig. 5A). These results demonstrate the specificity of the PHF1-p53 interaction. We also demonstrated that PHF1 proteins significantly abrogated MDM2-mediated p53 ubiquitination in vivo.

FIGURE 4. PHF1 regulates p53 protein stability. A, the increasing amount of WT or various mutant expression constructs of Myc-PHF1 were co-transfected with same amounts of HA-p53 into H1299 cells. The protein levels of PHF1 and p53 were determined by Western blot. The pGFP-N3 expression construct was included as a transfection efficiency control. The mean value (± S.D.) of three independent experiments is shown. B, H1299 cells were co-transfected with HA-p53, along with empty vector, or Myc-PHF1 constructs. At 48 h after transfection, cells were treated with 30 μM cycloheximide (CHX) for the indicated lengths of time. Equal amounts of cell lysates were detected by Western blot, and for each experimental condition, the blot corresponds to one representative experiment, and the graph in the lower panel shows the quantification of p53 protein levels using GFP for standardization. The mean values (± S.D.) of three independent experiments are shown. C, U2OS cells and HepG2 cells were transiently transfected with two Hush 29-mer shRNA constructs (KD-1 and KD-2) against PHF1 or control constructs (NC), and at 72 h after transfection, the protein levels of endogenous PHF1 and p53 were detected by Western blot. The quantification of immunoblot is shown in the right panel. The mean values (± S.D.) of three independent experiments are shown. D, qRT-PCR measurements of the mRNA levels of PHF1 and p53 in PHF1 knockdown U2OS cells. The left three columns are relative mRNA levels of PHF1, and the right three columns correspond to those of p53. The mRNA level of GAPDH was used for normalization. E, U2OS cells were transiently transfected with shRNA constructs against PHF1 (KD-1) or control constructs (NC), at 48 h after transfection, cells were treated with 30 μM cycloheximide (CHX) for the indicated lengths of time. Equal amounts of cell lysates were detected by Western blot. Right panel shows the quantification of p53 protein levels. F, qRT-PCR measurements of the expression of p53 target genes in control and PHF1 knockdown U2OS cells. The mean values (± S.D.) of three independent experiments are shown. G, U2OS cells transiently transfected with PHF1-specific shRNA (KD-1) or control shRNA (NC) were treated with 20 μM etoposide for the indicated time. Cell lysates were detected by Western blot with indicated antibodies.
observed in PHF1 expressing cells in comparison with control cells in p53 wild-type cell lines U2OS and HCT116 p53+/+ (Fig. 6A). However, PHF1 overexpression had no obvious effect on the colony formation of two p53 null cell lines, H1299 and HCT116 p53−/− (Fig. 6A). Furthermore, PHF1-ΔBD1/2 overexpression had no effect on the colony formation of U2OS and HCT116 p53+/+ cells, suggesting that the p53-binding capacity of PHF1 were essential for its cell growth arrest activities (Fig. 6A). BrdU incorporation assay results also showed that PHF1 overexpression reduced the DNA synthesis activities in U2OS and HCT116 p53+/+ cells but not in H1299 and HCT116 p53−/− cells (supplemental Fig. 3).
In a complementary experiment, we knocked down PHF1 by two independent shRNAs in these cell lines. As shown in Fig. 6B, knockdown of PHF1 significantly enhanced the colony formation of U2OS and HCT116 p53+/− cells but not in H1299 and HCT116 p53−/− cells (Fig. 6B). Furthermore, BrdU incorporation assay results also showed that knockdown of PHF1 expression enhanced the DNA synthesis activities in a p53-dependent manner (Fig. 6C).

As PHF1 was found to promote p53 protein stability and activation in cells treated with genotoxic drug etoposide, we speculated that the attenuation of p53 activation by PHF1 knockdown might be translated into a phenotypic change in apoptosis. To this end, we transfected HCT116 p53+/− and HCT116 p53−/− cells with either control shRNA or PHF1 shRNA followed by etoposide treatment. Cells were collected at 24 h, stained with PI, and analyzed by flow cytometry for apoptotic cells according to DNA content. As shown in Fig. 6D, basal level sub-G1 content is minimally affected by PHF1 knockdown. However, 24 h after etoposide treatment, an average of 60.6% of HCT116 p53+/− cells transfected with control shRNA (NC) were apoptotic, whereas only 23.0% (KD-1) or 33.4% (KD-2) of cells transfected with PHF1 shRNA were apoptotic (Fig. 6D). Importantly, the differential effect of PHF1 knockdown on cell apoptosis was not observed in HCT116 p53−/− cells (Fig. 6D). Furthermore, introduction of HA-p53 vector into HCT116 p53−/− cells resulted in resensitization to PHF1 knockdown as measured by BrdU incorporation assay and PI staining assay (Fig. 6, E and F). Taken together, these results suggest that PHF1 is important for p53-dependent cell growth and apoptosis.

**PHF1 Expression Is Significantly Down-regulated in Breast Cancer**—Because p53 plays an important role in tumor suppression and PHF1 potentiates p53 function by protecting p53 from E3 ligase-mediated ubiquitination and degradation, it is possible that PHF1 also acts as a tumor suppressor and dysregulated in human cancers. Our results shown in Fig. 6 demonstrated the ability of PHF1 to promote p53-dependent cancer cell growth arrest and apoptosis, which lend support to the hypothesis that PHF1 functions as a tumor suppressor in *vivo*. However, the relative expression of PHF1 in various human cancers samples has not been reported yet. To further test this hypothesis, we examined the expression of PHF1 in a panel of breast cancer tissue samples. Tissue microarrays containing 57 primary ductal breast carcinoma and adjacent non-tumor tissues were immunostained using a specific anti-PHF1 antibody. Substantial PHF1 immunostaining was detected in the non-tumor samples (Fig. 7), whereas very little to moderate PHF1 staining was observed in breast cancer samples (Fig. 7). PHF1 expression was detected predominantly in the nucleus of normal mammary gland cells, which is consistent with the immunofluorescence analysis data shown in Fig. 2C. This result suggests that PHF1 may act as a tumor suppressor in breast cancer.

**DISCUSSION**

In this study, we unraveled a novel function of PHF1 in p53 regulation. PHF1 is a Polycomb group protein and a component of PRC2 complex. PHF1 positively regulates the H3K27 methylation activity of the PRC2 complex *in vitro*, and PHF1 is important for H3K27 methylation and Hox gene expression *in vivo* (13, 14). PHF1 was also found to be involved in the response to DNA double-strand breaks in human cells. It has been shown to interact with Ku70/Ku80 and promote homologous end-joining process. Furthermore, a proteomics approach to identify interacting proteins of PHF1 in 293 cells showed SMC1, DHX9, p53 and Rad50 as potential interacting partners of PHF1 (14). But the physiological roles of these potential interactions have remained unexplored. In this study, we uncovered a novel function of PHF1 as a regulator of p53. We found PHF1 can transactivate p53 through pathway screen assays. Subsequent studies showed that PHF1 binds to p53 *in vivo* and *in vitro*. Mechanically, PHF1 can promote p53 protein stability and transactivation by protecting p53 from MDM2-mediated ubiquitination and degradation. PHF1 reduced cell proliferation and promoted DNA damage reagent-induced apoptosis in a p53-dependent manner. These results demonstrated that PHF1 is a novel p53 regulator.

The potential roles of PHF1 in tumorigenesis and tumor progression have not been clearly characterized. Our results that PHF1 expression is significantly down-regulated in breast cancer shed light on PHF1 function as a tumor suppressor in human. Moreover, PHF1 abnormality was reported in endometrial stromal sarcomas, which were rare malignancies accounting for less than 10% of uterine sarcomas (17). Recently, specific translocation events leading to the fusion of PHF1 with other two genes clearly defines a new pathogenetic subgroup of endometrial stromal sarcomas (16). PHF1 was found to be recombined with two different partners, with the JAZF1 gene in the two tumors showing a (t6p;7p) rearrangement and with the

**FIGURE 5.** PHF1 stabilizes p53 by protecting from MDM2-mediated ubiquitination and degradation. A, H1299 cells were transfected with Myc-PHF1 and/or FLAG-tagged ubiquitin ligases (MDM2, Pirh2, and COP1) and deubiquitinases (USP7 and USP10) as indicated. Cells lysates were prepared and subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitated proteins were detected with the indicated antibodies. B, Myc-PHF1, FLAG-ubiquitin, and Myc-PHF1 were co-transfected into H1299 cells. 48 h after transfection, the cells were treated with 20 μM etoposide for 8 h, the polyubiquitinated forms of endogenous p53 were detected by Western blot with anti-p53 antibody. C, HA-MDM2, pcDNA3.0-p53, Myc-PHF1, and FLAG-ubiquitin were co-transfected into H1299 cells. Cells were treated with of the proteasome inhibitor MG132 (20 μM) for 4 h before harvest. The polyubiquitinated forms of p53 were detected by Western blot with anti-p53 antibody. D, shRNA construct (KD-1) against PHF1 or control construct (NC) were co-transfected into U2OS cells. 48 h after transfection, the cells were treated with 20 μM etoposide for 8 h, the polyubiquitinated forms of endogenous p53 were detected by Western blot with anti-p53 antibody. E, HA-p53, FLAG-SUMO1, and Myc-PHF1 were co-transfected into H1299 cells. 53 was immunoprecipitated by anti-HA antibody. The sumoylated forms of p53 were detected by Western blot with anti-p53 antibody. F, the increasing amount of WT or ΔBD1/2 Myc-PHF1 expression constructs were co-transfected with HA-MDM2 and untagged p53 constructs into H1299 cells. The protein levels of PHF1, MDM2, and p53 were determined by Western blot. G, HA-MDM2, pcDNA3.0-p53, Myc-PHF1, or PHF1-ΔBD1/2 mutants were co-transfected into H1299 cells. 36 h after transfection, cells were lysed and subjected to immunoprecipitation using anti-p53 antibody. Immunoprecipitated proteins were detected with the indicated antibodies. H, HA-MDM2, FLAG-ubiquitin, and Myc-PHF1 constructs were co-transfected into H1299 cells. Cells were treated with MG132 for 4 h before harvest. MDM2 was immunoprecipitated by anti-HA antibody. The polyubiquitinated forms of MDM2 were detected by Western blot with anti-FLAG antibody. M.W., molecular weight.
EPC1 (enhancer of Polycomb) gene from 10p11 in the third tumor, which had a t(6p;10q;10p) translocation as the sole karyotypic abnormality (18). JAZF1/PHF1 fusion gene was also presented in a low-grade endometrial stromal sarcoma cell line JHU-ESS1 (19). The underlined pathological functions of PHF1 fusion proteins were not fully understood. Our data showed that wild-type PHF1 promoted p53 protein stability and transactivation under normal and stressed conditions. It will be interesting to test whether p53 pathway is compromised in endometrial stromal sarcomas harboring JAZF1/PHF1 and

**FIGURE 6. PHF1 regulates cell proliferation and apoptosis in a p53-dependent manner.**

A. H1299, U2OS, HCT116 p53−/−, and HCT116 p53+/+ were transfected with pcDNA3.1-PHF1 or control vector and selected with G418 for 2 weeks, and outgrowth colonies were stained by crystal violet. Representative photographs of cell colonies were shown. Total numbers of the colonies from three independent experiments were counted. An asterisk indicates statistical significance (*, p < 0.05; **, p < 0.001). B. Two HuSH 29-mer shRNA constructs (KD-1 and KD-2) against PHF1 or control construct (NC) were transfected into cells, and colony formation assay was performed same as described in A. C. Two HuSH 29-mer shRNA constructs (KD-1 and KD-2) against PHF1 or control construct (NC) were transfected into cells, at 72 h after transfection, the proliferative capacity of four cell lines was measured by BrdU ELISA assay. D. HCT116 p53−/− and HCT116 p53+/+ cells were transfected with PHF1 knockdown (KD1) and control shRNA (NC) constructs. At 72 h after transfection, the cells were treated with etoposide (40 μM). At 24 h after treatment, apoptosis was measured using PI staining assay. E. pcDNA3.0-p53 was co-transfected with shRNA constructs into HCT116 p53−/− cells, and etoposide-induced apoptosis was measured the same as D.

EPC1 (enhancer of Polycomb) gene from 10p11 in the third tumor, which had a t(6p;10q;10p) translocation as the sole karyotypic abnormality (18). JAZF1/PHF1 fusion gene was also presented in a low-grade endometrial stromal sarcoma cell line JHU-ESS1 (19). The underlined pathological functions of PHF1
EPC1/PHF1 chimeras. In future, pathological analysis of various human cancer specimens and genetically engineered mouse models are warranted to determine whether PHF1 is a bona fide p53-dependent tumor suppressor in animals.

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