Identification of Akt Interaction Protein PHF20/TZP That Transcriptionally Regulates p53*

Sungman Park†, Donghwa Kim†, Han C. Dan†, Huihua Chen†, Joseph R. Testa§, and Jin Q. Cheng∥

From the †Department of Molecular Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida 33612 and the §Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Background: Akt exerts its cellular function through interaction/phosphorylation of downstream proteins.
Results: Akt interacts with and phosphorylates PHF20, which regulates p53 and inhibits cell proliferation and survival, resulting in loss of its function.
Conclusion: PHF20 up-regulates p53. Akt phosphorylates and abrogates PHF20 function.
Significance: We identified a novel interactor/substrate of Akt and an additional link between Akt and p53 cascades.

WITHDRAWN

This article has been withdrawn by the authors. The same data were used to represent different experimental conditions. Specifically, the p53 panel from HCT116 cells in Fig. 4F was reused in the p53 panel from Fig. 5C. The authors state that the overall conclusions of the study are not affected.

Akt regulates cell survival. Although a number of molecules have been identified as upstream regulators and downstream targets of Akt, the mechanisms by which Akt regulates these cellular processes remain elusive. Here, we demonstrate that a novel transcription factor, PHF20/TZP (referring to Tudor and zinc finger domain) is a downstream target of Akt. PHF20 containing protein), binds to Akt and inhibits p53 expression at the transcription level. Knockdown of PHF20 significantly reduces transactivation of p53 reporter and attenuation of PHF20 function. These data indicate that PHF20 is a substrate of Akt and plays a role in Akt cell survival/growth signaling.

Akt is also known as protein kinase B (1, 2). Viral Akt is highly activated and oncogenic because of the fact that viral Akt is constitutively associated with the cell membrane through a myristoylated Gag protein sequence fused to the N terminus of Akt (3). The important role of Akt in cellular transformation and tumorigenesis was discovered by the cloning of the human AKT2 gene (4) and by the discovery that AKT2 is frequently amplified and overexpressed in human cancers (4–6). Akt is activated by various stimuli in a phosphatidylinositol 3-kinase (PI3K)-dependent manner (7–10). Activation of Akt results in its translocation from the nucleus to the cytoplasm and at Thr308 accounts for this translocation. Akt phosphorylates Thr308 and Ser473 of Akt (23–25) and that IKBKE phosphorylates Akt (19), PKCII (20), DNA-dependent kinase (21), and other kinases (22). Akt is also known as protein kinase B

† These authors contributed equally to this work.
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mechanisms keep a strong check on p53 in normal circumstances but allow rapid activation in response to cellular stress that might be caused by or contribute to oncogenic progression (28, 30). However, little is known about the transcriptional regulation of the p53 gene and the contribution of this transcriptional control to DNA damage-induced cell cycle checkpoints. Previous studies have shown that p53 is transcriptionally up-regulated by the homeobox protein HOXA5 (36, 37), p53 itself (38), and death-promoting factor Btf (39). Recently, the Bcl6 oncprotein was found to suppress p53 expression through binding to p53 and inhibiting p53 promoter activity (40). Several studies have raised the possibility that p53 may also be regulated at the transcriptional level in response to genotoxic stress (41, 42). However, the underlying mechanism and functional consequences remain unclear.

A link between Akt and p53 pathways was established by the identification of Akt phosphorylation of MDM2 (43). MDM2 is an E3 ubiquitin ligase that negatively regulates p53 transcriptional activity (44). Phosphorylation of MDM2 by Akt stimulates translocation of MDM2 to the nucleus, where it binds to p53 and targets it for degradation by the proteasome (45–47). Here, we have identified a novel transcription factor, PHF20, that interacts with Akt. PHF20 directly binds to the p53 promoter and up-regulates p53 at the mRNA level. As a result, PHF20 inhibits cell proliferation, DNA synthesis, and cell survival. Akt phosphorylates PHF20 on serine 291 within a nuclear localization signal, which leads to PHF20 translocation from the nucleus to the cytoplasm and loss of its biochemical and cellular functions.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—Yeast two-hybrid system was used to identify interaction protein(s) using the C-terminal regulatory region of Akt as bait following the manufacturer’s instructions (Clontech). A human fetal brain library (Clontech) was screened. Full-length cDNA of PHF20 was amplified from a human Marathon-ready skeletal muscle cDNA library (Clontech) by PCR, subcloned into 3×FLAG-pcDNA3, EGFP-C1, and pTRE-tight vectors. PHF20 mutants were created with the QuikChange multiple site-directed mutagenesis kit (Stratagene). The cytomegalovirus-based expression constructs encoding Akt and p53 as well as pGL3-p53-Luc have previously been described (48, 49).

Cell Culture and Transfection—HEK293, MCF7, and HCT116 cells were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. Lipofectamine Plus (Invitrogen) was used for transfection.

Glutathione S-transferase (GST) Fusion Protein and Generation of PHF20 Antibody—Different portions of PHF20, including AT-hook region, C3H2 zinc finger domain, C-terminal motif, and the regions containing each Akt phosphorylation site, were subcloned into pGEX-4T1. Expression and purification of the GST fusion protein were carried out as described previously (50). Polyclonal PHF20 antibody was raised in New Zealand White rabbits. Approximately 300 μg of GST fusion protein (GST-PHF20/AT-hook and GST-PHF20/C-terminal) was used to immunize rabbits every 2 weeks; rabbits were bled 10 days after each booster injection. The anti-PHF20 antibodies were affinity-purified with Affi-Gel protein A (Bio-Rad). The phosphi-PHF20-Ser291 antibody was produced by New England Peptide.

Northern Blot, Immunoprecipitation, and Immunoblotting Analysis—Northern blot analysis of total cellular RNA was performed according to standard procedures (51). Cell lysate was prepared in lysis buffer and subjected to immunoprecipitation and immunoblot analysis as described previously (52). Briefly, lysates were precleared with protein A/protein G (2:1)-agarose beads. Following the removal of the beads by centrifugation, lysates were incubated with appropriate antibodies in the presence of protein A/protein G (2:1)-agarose beads for 2 h. After washing, the immunoprecipitates were subjected to immunoblotting. Protein expression was determined by probing Western blots of total cell lysates with the appropriate antibodies as noted in the figure legends.

In Vitro Kinase Assay—Akt kinase assay was performed as described previously (46). Briefly, reactions were carried out in the presence of 32P-ATP and 2 μM cold ATP in a final volume of 50 μl containing 100 μM MgCl2, 2 mM MnCl2, and 1 mM dithiothreitol. GST-phosphi-PHF20 was added to a final concentration of 1 μg/ml. After incubation at 30 °C for 30 min, reactions were stopped by boiling and then separated in SDS-polyacrylamide gels. Autoradiography was performed to detect phosphorylated PHF20 bands of incorporated radioactivity.

In Vivo [32P]Orthophosphate Cell Labeling—HEK293 cells were transfected with FLAG-PHF20 and constitutively active Akt or pcDNA3 and labeled with [32P]orthophosphate (54). After incubation for the desired number of generations were stopped and then separated in SDS-PAGE. Autoradiography was performed to detect phosphorylated PHF20 bands of incorporated radioactivity.

Cell Proliferation, Viability, and DNA Synthesis Assay—Cells were plated in 35-mm dishes at a density of 1.0 × 103 cells/dish. Cell number was measured with a Coulter Counter (Coulter Electronics, FL) daily for up to 3 days. MTS 4 assays were performed according to the manufacturer’s recommendations (Promega). The cells were plated in a 96-well plate at a density of 1.0 × 103 cells/well. The number of cells at 1–3 days was determined using cell counter and the colorimetric CellTiter96 Aqueous (MTS) assay. Results were depicted as absorbance at 490 nm as a function of time. Cell viability was examined by staining with trypan blue.

Thymidine incorporation was used to investigate the effect of PHF20 on DNA synthesis. The cells were grown to 80% confluence in 6-well plates, and during the last 16 h of growth, they were subjected to 5 μCi/ml of [3H] thymidine. After rinsing with ice-cold serum-free medium, the cells were incubated with the presence of 100 μM of dexamethasone, doxycycline (1 μg/ml), or the vehicle control (54). Integration of the GST fusion protein were carried out as described previously (50). Polyclonal PHF20 antibody was raised in New Zealand White rabbits. Approximately 300 μg of GST fusion protein (GST-PHF20/AT-hook and GST-PHF20/C-terminal) was used to immunize rabbits every 2 weeks; rabbits were bled 10 days after each booster injection. The anti-PHF20 antibodies were affinity-purified with Affi-Gel protein A (Bio-Rad). The phosphi-PHF20-Ser291 antibody was produced by New England Peptide.

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5 ml of 10% TCA for 10 min on ice and then lysed in 500 μl of 1% SDS in 0.3 M NaOH for 30 min at 37 °C. Incorporated radioactivity was quantified with a spectrometer.

Luciferase Reporter Assay—Cells were cultured in 12-well plates and were transiently transfected with pGL3/p53-Luc, PHF20, and/or Akt. The amount of DNA in each transfection was kept constant by the addition of empty vector. After transfection for 36 h, luciferase activity was measured using a luciferase assay reagent (Promega). Transfection efficiency was normalized by co-transfection with β-galactosidase expressing vector. The β-galactosidase activity was measured using Galato-Light (Tropix). Luciferase activity was expressed as relative luciferase activity.

Chromatin Association—Chromatin was isolated as described previously with small modifications (supplemental Fig. S1A) (55). Briefly, MCF7 cells stably transfected with FLAG-PHF20 were resuspended in buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 mM sucrose, 10% glycerol, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride). Nonidet P-40 (0.1%) was added, and the cells were incubated for 5 min on ice. Nuclei (P1) were collected by low speed centrifugation (5 min, 1,300 × g, 4 °C). The supernatant (S1) was further clarified by high speed centrifugation (15 min, 20,000 × g, 4 °C). Nuclei were washed once in buffer A and then lysed in buffer B (3 mM EDTA, 0.2 M EGTA, 1 mM DTT, protease inhibitors as described above). Insoluble chromatin was collected by centrifugation, 1,700 × g, 4 °C and washed once in buffer B. The pellet (P3) was resuspended in solvent (10 mM Hepes, pH 8.1, 10 mM EDTA, 1% SDS) and sonicated in a Branson sonicator using a microtip at 10% amplitude. Chromatin was collected and analyzed for shared sequence patterns by visual inspection and by weblogo software.

ChIP Assay—ChIP was performed essentially as described previously. Solubilized chromatin was prepared from a total of 2 × 107 asynchronously dividing HCT116 cells. The chromatin solution was divided and utilized for immunoprecipitation with PHF20 antibody. Following washing, the chromatin-DNA complex was eluted from the beads by treatment of the pellets in 1% SDS, 0.1 M NaHCO3 at room temperature for 20 min. After cross-linking, protein and RNA were removed by incubation with 10 μg of proteinase K and 10 μg of RNase A at 42 °C for 3 h. Purified DNA was subjected to PCR with primers spanning putative PHF20-binding sites of the p53 promoter. Amplified PCR products were resolved by 1.2% agarose gel electrophoresis and visualized by BioImage. The sequences of oligonucleotides used for ChIP assays are 5'-CAATTTCGCCCCTCAGCTCCTGGTGTC-3' and 5'-CTCAAAACCTTTTATGCAGCAGTCTTGGAC-3'.

RESULTS

Identification of a Novel Akt-binding Protein, PHF20/TZP—In an attempt to identify proteins that interact with Akt, the C-terminal regulatory domain of Akt (amino acids 410–480) was used as bait in a yeast two-hybrid screening. A human fetal brain cDNA library was used for this screen because Akt is highly expressed in brain (57, 58). Altogether, 32 clones that specifically interacted with the bait were identified. Sequence analysis revealed that three of the clones contained overlapping sequences of a cDNA. The largest clone contained a 262-amino acid open reading frame with a C-terminal region of PHF20/TZP. Additional cDNA clones were isolated from a human skeletal cDNA library by plaque hybridization using the largest clone as radiolabeled probe. Sequence analysis revealed that the full-length open reading frame of cDNA encodes a 1,012-amino acid protein composed of two Tudor domains, an A-T hook motif, a C2H2 zinc finger domain, and a PHD finger motif (Fig.

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Cyclic Amplification and Selection of Targets (CASTing)—A 76-bp oligonucleotide containing 26 random nucleotides in the center flanked by sequences complementary to primers A and B was synthesized (Invitrogen). The sequences of the oligonucleotides are as follows: 76-base oligonucleotide, 5'-CAGGTCGTTGCCATCTGC(N)26GAGGCGAATTCTAGTGCAACTGGC-3'; primer A, 5'-GCTGCGCTTTGACTTGATTTGGCACTC-3'; primer B, 5'-CAGGTGCTATTGCGCCCGATCTGTCG-3'. A random sequence library of double-stranded radiolabeled oligonucleotides was prepared by annealing the oligonucleotide to 5-fold excess of primer B followed by extension with Klenow enzyme. Electrophoretic mobility shift assay (EMSA) was performed by adding 5 μg of FLAG-PHF20 containing nuclear extract to radiolabeled DNA in DNA binding buffer (5% glycerol, 10 mM Hepes, pH 7.9, 75 mM KCl, 1 mM DTT, 2.5 mM MgCl2, 1 mM EDTA) in the presence of 0.5 μg of poly(dA-dT) and 1 μg of bovine serum albumin. The reaction was incubated at room temperature for 30 min, and subsequently the DNA-protein complexes were resolved by electrophoresis. The complexes formed specifically in the presence of FLAG-PHF20 proteins were detected by autoradiography, excised from gels, and eluted overnight at 37 °C in DNA elution buffer containing 0.3 M NaCl, 1 mM EDTA, and 0.1% SDS. The eluted DNA was extracted once in phenol/chloroform and then precipitated with ethanol. Purified DNA was subjected to re-amplification by PCR in the presence of [α-32P]dCTP. The amplified radiolabeled DNA was purified using G-50 Nick columns (Amersham Biosciences) and was used in subsequent EMSA experiments. After four cycles of CASTing, the amplified DNA was cloned directly using pGEM-T cloning kit (Promega). Nucleotide sequences of 60 independent clones were determined (supplemental Fig. S1, B and C). The degenerated portion of the sequences was compiled and analyzed for shared sequence patterns by visual inspection and by weblogo software.
1A). Therefore, we initially named it as TZP (referring to Tudor and zinc finger domain containing protein; GenBank™ accession number AY027523). In addition, PHF20 contains three nuclear localization signals and two putative Akt phosphorylation consensus motifs (229\textit{KRGRPPSSA}237 and 285\textit{LRRKISKK}293, Fig. 1A). The expression pattern of PHF20 is similar to that of Akt, being abundant in skeletal muscle, brain, pancreas, and heart (Fig. 1B). To confirm the association of Akt with PHF20 identified by yeast two-hybrid screening, HEK293 cells were co-transfected with FLAG-PHF20 and HA-Akt. Immunoprecipitation was performed with anti-FLAG and detected with anti-HA antibody or vice versa. As shown in Fig. 1C, HA-Akt was detected in the FLAG-PHF20 immunoprecipitates, and PHF20 was co-immunoprecipitated with HA-Akt. Their interaction at endogenous protein levels was confirmed in HCT116 cells (Fig. 1D). To define the binding region of PHF20 to Akt, we created deletion mutant constructs of FLAG-PHF20 and of GST-Akt. Immunoprecipitation and GST pull-down assays revealed that interaction of PHF20 and Akt occurs through their C-terminal regions (Fig. 1, E and F).

Akt Phosphorylates PHF20-Serine 291 in Vitro and in Vivo—Because PHF20 contains two putative Akt phosphorylation consensus sites, serine 265 and serine 291 (Fig. 1A), we next determined whether PHF20 is phosphorylated by Akt. HEK293 cells were co-transfected with FLAG-PHF20 and constitutively active Akt or pcDNA3 vector. After labeling with [\textsuperscript{32}P]orthophosphate, PHF20 was immunoprecipitated with FLAG antibody, separated, and blotted on a membrane. PhosphoImager quantification analysis revealed that the incorporation of \textsuperscript{32}P into PHF20 was 8-fold higher in cells co-transfected with Akt/PHF20 as compared with the cells transfected with pcDNA3/PHF20 (Fig. 2A). Furthermore, in vitro kinase assay revealed Akt phosphorylation of PHF20 on serine 291 but not serine 265 (Fig. 2B). We also generated phospho-PHF20-Ser291 antibody. Western blot analysis of in vitro kinase products using phospho-PHF20-Ser291 antibody showed that phospho-
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PHF20-Ser291 is elevated by incubation with Akt (Fig. 2C). Moreover, expression of constitutively active Akt significantly induced phospho-PHF20-Ser291 (Fig. 2D), whereas knockdown of Akt considerably decreased phospho-PHF20-Ser291 in MDA-MB-468 cells in which PTEN is mutated (Fig. 2E). We also observed that phospho-PHF20-Ser291 was induced by IGF1 and serum and that the Igf1-induced phospho-PHF20-Ser291 was inhibited by knockdown of Akt (Fig. 2F and supplemental Fig. S1). Taken collectively, we conclude that Akt phosphorylates PHF20-Ser291 in vitro and in vivo.

Akt Phosphorylation of PHF20 Results in PHF20 Translocation from the Nucleus into the Cytoplasm—We further determined whether Akt phosphorylation of PHF20 affects its interaction. Phosphomimic and nonphosphorylatable PHF20 were created by mutation of serine 291 into aspartic acid (PHF20-S291D) and alanine (PHF20-S291A), respectively. Co-immunoprecipitation analysis revealed that wild-type PHF20 and phosphomimic PHF20-S291D bound to Akt, whereas non-phosphorylatable PHF20-S291A failed to interact with Akt (Fig. 3A), implying that phosphorylation of PHF20 by Akt is important for their interaction.

Because the serine 291 of PHF20 locates at a nuclear localization signal (Fig. 1A), we next tested whether Akt phosphorylation of PHF20 affects its subcellular localization. MCF7 cells were transfected with FLAG-PHF20 and/or Red-Myr-Akt. PHF20 was localized exclusively in the nucleus in cells transfected with FLAG-PHF20 alone. However, co-transfection of constitutively active Red-Myr-Akt resulted in PHF20 redistribution from the nucleus to the cytoplasm (Fig. 3B). Furthermore, IGF1 stimulation also induced PHF20 translocation from the nucleus to the cytoplasm, which was blocked by pretreatment with PI3K inhibitor LY294002 and Akt inhibitor AP2-TCN (59) but not by MEF inhibitor PD98059 (Fig. 3C). Interestingly, Akt was primarily co-localized with PHF20 in the cytoplasm (Fig. 3, B and C). To further examine if PHF20 subcellular localization is dependent on Akt phosphorylation of PHF20-Ser291, HCT116 cells were transfected with FLAG-PHF20 and/or Myc-Akt and/or a nonphosphorylatable PHF20-S291A or a phosphomimic PHF20-S291D. Immunofluorescence analysis showed that PHF20-S291D was predominantly localized in the cytoplasm, whereas wild-type PHF20 localized in the nucleus. Knockdown of Akt with siRNA increased the expression of PHF20 localized in the cytoplasm under "Experimental Procedures." Briefly, HEK293 cells were transfected with FLAG-PHF20 and constitutively active HA-Akt or HA-Myr-Akt labeled with [32P]orthophosphate (0.5 mCi/ml) for 4 h. Cell lysates were immunoprecipitated with FLAG antibodies transferred to membrane. The phosphorylated bands were detected by autoradiography (top). Middle and bottom: Western blotting of transfected plasmids. B and C, Conventional kinase assay was performed with GST-fused PHF20 amino acids 200–280 (Ser265) and 270–330 (Ser291) and recombinant active Akt. The phosphorylated band was examined by autoradiography (B, top) and phospho-PHF20-Ser291 antibody (B, bottom). D, Akt phosphorylates PHF20 in vitro. GST-fused PHF20 amino acids 200–280 (Ser265) and 270–330 (Ser291) and recombinant active Akt were incubated for 30 min, and then kinase assay was performed by incubation of recombinant active Akt with [32P]ATP-labeled PHF20 consensus sequence containing oligonucleotides. As shown in Fig. 4B, PHF20 bound to the oligo-
FIGURE 3. Akt induces PHF20 translocation from the nucleus to the cytoplasm. A, phosphomimic PHF20-S291D but not nonphosphorylatable PHF20-S291A preferentially binds to Akt. MCF7 cells were transfected with indicated plasmids, immunoprecipitated (IP) with FLAG antibody, and detected with Akt antibody (top). Middle and bottom panels show the expression of the transfected plasmids. IB, immunoblot. B, PHF20 localizes in the nucleus, and Akt induces PHF20 nuclear-cytoplasm translocation. MCF7 cells were transfected with FLAG-PHF20 together with/without Red-myr-Akt. After 48 h, cells were stained with FITC-conjugated FLAG antibody and DAPI. C, PI3K or Akt inhibitors but not MEK inhibitor blocked IGF1-induced PHF20 nuclear-cytoplasm translocation. MCF7 cells were transfected with GFP-PHF20. After 36 h, cells were deprived from serum for 12 h and treated indicated drugs for 30 min prior to IGF1 stimulation for 1 h. Cells were stained with Akt antibody and Texas Red-conjugated secondary antibody.
nucleotides could be competed by excess of cold probe but not by its mutant oligonucleotides. Furthermore, we cloned three repeats of PHF20 binding consensus sequence (PBCS) into pGL3 luciferase vector (pGL3–3PBCS-Luc). A reporter assay was performed in HEK293 cells that were transfected with pGL3–3PBCS-Luc and increasing amounts of PHF20. Fig. 4C shows that promoter activity was induced by PHF20 in a dose-dependent manner. These data suggest that PHF20 associates...
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with DNA in the form of chromatin and induces transcription by binding to a specific DNA element.

Because PHF20 is a putative transcriptional factor and inhibits cell proliferation and survival (see Fig. 6), we established a MCF7/Tet-off PHF20 cell line and examined the effects of PHF20 on expression of a dozen molecules that involve cell survival and cell proliferation. Immunoblot analysis revealed that PHF20 was induced upon withdrawal of doxycycline and that p53 as well as p53 downstream targets p21, Bax, and GADD45 were also elevated (Fig. 4D). The p53 mRNA level was also induced by PHF20 (Fig. 4E). Furthermore, knockout of PHF20 resulted in significantly reduced p53 expression (Fig. 4F and supplemental Fig. S4A). In addition, sequence analysis revealed two putative PHF20-binding consensus sites within the −110–bp region of the p53 promoter, e.g. −29/–34 and −79/–84 bp (Fig. 4H and supplemental Fig. S5A). Luciferase reporter assay was performed in MCF7 cells that were transfected with pGL3-p53 (−110/+13) and increasing amounts of PHF20. As shown in Fig. 4G, PHF20 considerably induced the promoter activity of p53. Moreover, mutation of the two putative PHF20-binding sites by converting the core sequence GTG to AAA largely abrogated the pGL3-p53 promoter activity induced by PHF20 (supplemental Fig. S5B).

To determine whether PHF20 directly binds to the PHF20-binding site of the p53 promoter in vivo, we carried out ChIP assay, which detects specific genomic DNA sequence fragments associated with a particular transcription factor in intact cells. HEK293 cells were transfected with FLAG-FLAG-FLAG-FLAG-tagged PHF20 and chromatin was subjected to PCR using oligonucleotide primers that amplify the region spanning two PHF20-binding sites within the p53 promoter. The anti-FLAG antibody pulldown PHF20 from the chromatin of HEK293 cells but not FLAG-tagged anti-IgG resulted in the absence of a band. Furthermore, we demonstrated that endogenous PHF20 bound to the p53 promoter in MCF7 and MEF cells. The affinity is much higher in Akt1-null MEF than wild-type MEF (supplemental Fig. S6). Because the two PHF20-binding sites (−29/–34 and −79/–84) are very close to each other, ChIP assay is unable to distinguish which site directly binds to PHF20. Taken collectively, our data indicate that PHF20 transcriptionally regulates p53 by directly binding to and activating the p53 promoter.

Akt Inhibits PHF20 Transcriptional Activity and p53 Expression—Having demonstrated that Akt phosphorylates and induces PHF20 nuclear-cytoplasm translocation and that PHF20 transactivates p53, we next examined the effect of Akt on PHF20 transcriptional activity and on p53 expression at the mRNA level. Luciferase reporter assay revealed that PHF20-induced pGL3–3×PBCS-Luc activity was largely abrogated by expression of constitutively active Akt (Fig. 5A). Furthermore, the p53 promoter activity induced by PHF20 was also inhibited by Akt in a dose-dependent manner (Fig. 5B). Northern blot and semi-quantitative RT-PCR analysis showed that the p53 mRNA level was higher in Akt1−/− MEFs than wild-type MEFs. Reintroduction of Akt into Akt1−/− MEFs repressed p53 expression (Fig. 5C). In contrast, the p53 mRNA level was increased by expression of kinase-dead Akt or knockdown of Akt in MCF7 and HCT116 cells (Fig. 5, D and E). Moreover, expression of constitutively active Akt repressed PHF20-induced p53 expression (Fig. 5F).

To investigate if Akt inhibits PHF20 binding to the p53 promoter and if Akt regulation of p53 depends on phosphorylation of PHF20-Ser291, ChIP assay was performed and showed that Akt-nonphosphorylatable PHF20-S291A increased binding activity to the p53 promoter compared with wild-type PHF20, whereas phosphomimic PHF20-S219D did not bind to p53. Moreover, expression of constitutively active Akt abrogated the DNA binding activity of wild-type PHF20 toward p53 (Fig. 5G). Accordingly, expression of PHF20-S291A, but not PHF20-S291D, resulted in increased expression of p53, and Akt had no significant effect on p53 expression in both PHF20-S291A- and PHF20-S291D-transfected cells (Fig. 5H). These results indicate that Akt inhibits PHF20 transcriptional activity and regulates p53 transcription through phosphorylation of PHF20.

PHF20 Inhibits Cell Proliferation and Survival, and Akt Abrogates PHF20 Activity—To examine the cellular function of PHF20, colony formation, DNA synthesis, and cell survival were examined. Off cells. As shown in Fig. 6A, Akt inhibited by induction of the Mdm2. The phosphorylation of Mdm2 by Akt leads to its retention to the nucleus where Mdm2 inhibits p53 transactivation and DNA synthesis, and cell survival. Moreover, PHF20-S291A- and PHF20-S291D-transfected cells (Fig. 6A) with DNA, but not PHF20-S291D, failed to inhibit DNA synthesis and cell survival (Fig. 6G). In addition, anchorage-independent growth in soft agar was used to assess the effects of PHF20 and Akt on transforming activity. As shown in Fig. 6H, expression of PHF20 significantly reduced colony formation of MCF7 cells, and this action was overridden by co-transfection of Myr-Akt. Phosphomimic PHF20-S291D lost and nonphosphorylatable PHF20-S291A retained inhibitory activity. These data suggest that Akt inhibits PHF20 cellular function and that phosphorylation of PHF20 by Akt represents a key regulation of PHF20 function.

DISCUSSION

Akt was shown to regulate p53 through phosphorylation of Mdm2. The phosphorylation of Mdm2 by Akt leads to its retention to the nucleus where Mdm2 inhibits p53 transactivation function and targets p53 protein for degradation (43, 45). In this study, we have identified PHF20 as a transcription factor that interacts with Akt. PHF20 transactivates the p53 promoter,
resulting in induction of p53 mRNA. Akt phosphorylates PHF20 leading to its translocation from the nucleus to the cytoplasm and consequent abrogation of PHF20 transactivation of p53. Moreover, we have shown that knockdown of PHF20 significantly reduces the basal level of p53 mRNA and protein. These findings suggest a pivotal role of PHF20 in both Akt and...
FIGURE 6. PHF20 exhibited cell growth inhibitory function that was overridden by Akt. A–C, PHF20 inhibited cell proliferation, DNA synthesis, and cell survival. MCF7/Tet-Off PHF20 and MCF7/Tet-Off control cells were seeded in 24-well plate. After withdrawal of doxycycline (Dox) for indicated times, cell growth (A), DNA synthesis (B), and cell survival (C) were assayed with accounting cell number, thymidine incorporation, and MTS as described under "Experimental Procedures." Right panel of A shows expression of PHF20. D, PHF20 reduced DNA synthesis in HCT116 cells. E–G, Akt inhibits PHF20 cellular function. MCF7/Tet-Off PHF20 and MCF7/Tet-Off control cells were transfected with myr-Akt and cultured in the absence and presence of doxycycline. Cell number was accounted at indicated times (E). F and G, MCF7 cells were transfected with indicated plasmids and then assayed for DNA synthesis (F) and cell viability (G). H, effects of PHF20 and Akt phosphorylation of PHF20 on cell anchorage-independent growth. MCF7 cells were transfected with indicated plasmids and then grown in soft agar for 3 weeks (left panel). The colony was accounted and quantified in three plates/transfectant (right panel).
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In summary, PHF20 is a novel transcriptional factor and both a binding partner and substrate of Akt. It exhibits putative tumor suppressor activity and plays a role in maintaining basal levels of p53. Based on our findings, we propose the following model of Akt regulation of PHF20. In the absence of cell growth and survival signals, PHF20 transactivates its target genes, including p53, which lead to cell growth arrest and cell death. Upon Akt activation, a fraction of the activated Akt translocates to the nucleus and phosphorylates PHF20, resulting in attenuation of PHF20 transcriptional activity and translocation into the cytoplasm where it co-localizes with Akt (Fig. 7). Further investigations are required to characterize PHF20 tumor suppressor function and its possible involvement in human malignancy. In addition, the genome-wide PHF20 target genes need to be identified to fully understand the normal cellular function of PHF20.

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Akt Interacting Protein PHF20 That Regulates p53

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WITHDRAWN

September 20, 2016

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Supplemental Figures

Figure S1. Serum induces phosphorylation of PHF20-S291. Following serum starvation for 12 h, MCF7 cells were stimulated with fresh serum for 30 min and 1 h and subsequently were immunoblotted with indicated antibodies.

Figure S2. Subcellular localization of PHF20 depends on phospho-S291. MCF7 cells were transfected with indicated plasmids. Following treatment with and without IGF1, cells were stained with FITC conjugated Flag antibody and DAPI.

Figure S3. Schematic procedures of chromatin isolation (A) and CASTing (B) assays. Sequence alignment of PHF20 binding consensus sequence (C).

Figure S4. Knockdown of PHF20 induces cell growth and cell survival. MCF7 cells were transfected with increasing dose of siRNA-PHF20 and then immunoblotted with indicated antibodies (A). Two shRNAs of PHF20 and control shRNA were introduced to MCF7 cells (insert figure). Cell growth was examined for indicated time (B), and cell death was evaluated after treatment with and without VP16 for 12 h. Single and double asterisks indicate p<0.05 between PHF20 knockdown and control cells in basal and VP16-induced cell death, respectively (C). Panel D shows cell proliferation in MCF7 cells transfected with constitutively active Akt together with/without siRNA-PHF20.

Figure S5. PHF20 activation of p53 promoter depends on the PHF20 binding sites. The p53 promoter sequence and putative PHF20-binding motifs highlighted in red (A). Luciferase assay was performed in MCF7 cells transfected with mutant pGL3-p53-luc and PHF20 (B).

Figure S6. Endogenous PHF20 binds to p53 promoter. ChIP assay was performed with antibody against endogenous PHF20 in MCF7 cells, which had been treated with 2 different doses of siRNA-PHF20 (A and Figure S4A), as well as in Akt-null and wild type MEF (B).
Figure S1

| Serum:          | 0 | 30’ | 1h |
|-----------------|---|-----|----|
| p-PHF20-S291    |   |     |    |
| PHF20           |   |     |    |
| p-Akt-T308      |   |     |    |
| Akt             |   |     |    |

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Figure S2

| Vehicle | Flag-PHF20-D | Flag-PHF20-A | Flag-PHF20 |
|---------|-------------|-------------|------------|
| Flag    | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| DAPI    | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
|         | 95% (cyto)  | 95% (cyto)  | 93% (nuc)  |

**IGF-1**

| Vehicle | Flag-PHF20-D | Flag-PHF20-A | Flag-PHF20 |
|---------|-------------|-------------|------------|
| Flag    | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| DAPI    | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
|         | 96% (cyto)  | 98% (nuc)   | 86% (cyto) |

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**A**

- **Cell lysis with Non-ionic detergent**
  - **Spin**
  - **S1 (spin)**
  - **S2 (soluble proteins)**
  - **P2**
  - **P1 (nuclei)**
    - [Optional: treatment with MNase to release chromatin-bound proteins]
    - Resuspension in no-salt buffer
  - **S3**
  - **P3 (chromatin enriched fraction)**

**B**

**76-mer degenerated oligo library**

- **In vitro translated Flag-PHF20**
  - Hind III
  - ---AAGCTT---
  - ---TTCCGA---

- **Binding reaction** (oligo library + Flag PHF20)
  - α-FLAG antibody conjugated to magnetic beads
  - Wash beads and retrieve oligo DNA/PHF20 complex

- **PCR**
  - Subclone PCR product into plasmid and examine inserted oligo sequence

**C**

- **Figure S3**
  - AGTGAGCGATGGATGCGGGCGATG
  - TGTTGTGCTATTTTTCTCT
  - GCTTGTGGGGATGCTGGGCTGATT
  - AGGAGACGTGGGGCGAGCTCA
  - TAGCTGGCGAGCAGACTTAAAC
  - CTACAGTAGCTGGCACAGCT
  - TACGAGCGGCTGGATATG
  - GTAGCTGGATATGGTGGG
  - ACTATTGTCGTGGAAGGAGTTTTG
  - CTACGATAGGTGAAGGAGCTTC
  - CTTCCTAGGGGTGCCTGGGTGTAGGG
  - GTGTGTGTGATGTCTTCTAGTGGGAA
  - CTACGGGCGCGGGTACATGGTG
  - GGCGTCCTTACGGCGTGGCGATATG
  - GTAGCTGGATATGGTGGG
  - ACTATTGTCGTGGAAGGAGTTTTG
  - CTACGATAGGTGAAGGAGCTTC
  - CTTCCTAGGGGTGCCTGGGTGTAGGG
  - GTGTGTGTGATGTCTTCTAGTGGGAA

- **Consensus:** RGTGNNR

*Withdrawn: September 20, 2016*
Figure S4

(A) Western blot analysis showing the expression levels of PHF20, p53, p21, and β-actin in cells treated with siRNA-PHF20.

(B) Graph illustrating the cell number over time for different treatments: cont, shPHF20 (#2), shPHF20 (#3), C, #2, #3.

(C) Bar graph comparing total cell death between shCont, shPHF20 (#2), and shPHF20 (#3).
**Figure S5**

### A

```
-268 CAATTCTGCCCTCACAGCTCTGGCTTGCGAGAATTTTCCACCCCAA -224
AATGTATTAGTACTTGAGGGCACCAGTGCGGAGAATCTGACTCTG -179
CACCCCTCTCCCCAATATTTCCCTTTGCTTTCTCCGGGGCAGGCG -134
GATTACTTGGCCCTTACTTGCCCTTCATTGCTGACTGTCCA -89
GCTTTGTGCCAGGAGCCTCGCGAGAATGATGGGATTGGGTTTT -44
CCCCTCCCATGTCCTCAGACGTGGCGCTAAAAGTTTTGAGCTTCT +2
CAAAAGGTCTAG                                    +13
```

### B

![Bar chart showing luciferase activity (fold)](chart.png)

Luciferase activity (fold) compared across different PHF20 conditions: PHF20 - (control), PHF20 +, PHF20 ++, and PHF20 +++.
Figure S6

A

| Input | α-IgG | α-PHF20 |
|-------|-------|---------|
|       | -     | ++      |

:siRNA-PHF20

p53 promoter

B

| Input | α-PHF20 |
|-------|---------|
|       | MEF/Akt1-null |
| α-IgG | α-PHF20 |

p53 promoter

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September 20, 2016
Identification of Akt Interaction Protein PHF20/TZP That Transcriptionally Regulates p53

Sungman Park, Donghwa Kim, Han C. Dan, Huihua Chen, Joseph R. Testa and Jin Q. Cheng

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