Signaling and Regulation

Pirh2, a Ubiquitin E3 Ligase, Inhibits p73 Transcriptional Activity by Promoting Its Ubiquitination

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Abstract

p73, a homolog of the tumor suppressor p53, transactivates many p53 target genes, leading to apoptosis or cell-cycle arrest. p73 has recently been reported to play an important role in tumor suppression in a mouse model. Here, we show that Pirh2 physically interacted with p73 and downregulated p73 function through its E3 ligase activity. Pirh2 promoted p73 ubiquitination in vivo and in vitro. Intriguingly, Pirh2 primarily used K63-linked chains to ubiquitinate p73 in vitro, but in vivo, Pirh2 utilized K11-, K29-, K48-, and K63-linked chains to promote p73 ubiquitination. Depletion of Pirh2 by siRNA significantly reduced the ubiquitination of p73 in p53 null cells. Ectopic expression of Pirh2 repressed p73-dependent transcriptional activity, but the levels of p73 were not decreased. We consistently showed that ablation of endogenous Pirh2 restored p73-mediated transactivational activity. We found that Pirh2 repressed p73 transcriptional activity by directly inhibiting the p73 transcript, and p73 repression by Pirh2 was required for p73-dependent transcriptional activity and G1 arrest but not for apoptosis. This study provides evidence that the ubiquitination of p73 mediated by Pirh2 represents an important pathway for controlling the suppressive function of p73. Furthermore, the data suggest a link between the transcriptional activity of p73 and its ubiquitination. Mol Cancer Res; 9(12); 1780–90. ©2011 AACR.

Introduction

p73 is a homolog of p53 and is capable of inducing apoptosis in cells or cell-cycle arrest (1–4). Although p73 has apoptotic activity, it is rarely mutated in human tumors, and p73-deficient mice are not tumor prone and do not display an increase in tumor incidence (5, 6). However, a number of studies have recently shown that p73 expression is lost or reduced in certain human tumors (through LOH or allelic silencing), implying that p73 has tumor suppressor capabilities (7–9). Flores and colleagues (2005) have shown that p73+-/– mice develop spontaneous tumors, and loss of p73 can cooperate with loss of p53 in tumor suppression. In addition, mutation in p73 combined with p53 mutations lead to a more aggressive tumor phenotype, indicating that p73 plays an important role in tumor suppression in a mouse model (10). The protein Itch has been reported to function as an E3 ligase for p73 (11), but multiple pathways are involved in controlling p73.

Pirh2 has also been reported to promote ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor p27Kip1 (24).

In this study, we found that Pirh2 physically interacted with p73 in vitro and in vivo, and we showed that Pirh2 repressed p73-dependent transcriptional activity. Our findings revealed that Pirh2-mediated ubiquitination of p73 was linked to the inhibition of the transactivational activity of p73. We showed that depletion of Pirh2 by siRNA markedly reduced the ubiquitination of p73 in p53 null cells. We...
further showed that Pirh2 repressed p73 transcriptional activity by directly inhibiting the p73 transcript, and p73 repression by Pirh2 was required for p73-dependent transcriptional activity and G1 cell-cycle arrest but not for apoptosis.

Materials and Methods

Plasmids and antibodies

pcDNA3-Pirh2, GST-Pirh2, and p21-Luc have previously been described (12). Myc-Pirh2 and Myc-Pirh2 mutants were generated by PCR amplification and subcloned into the pcDNA3.1-Myc vector. Myc-AIP4 and the Myc-AIP4 mutant (C830A) were provided by Dr. T. Pawson. Histagged and His-p73β were PCR amplified and subcloned into pET15b. All ubiquitin and ubiquitin mutants were PCR amplified and subcloned into pET28a. Flag-p73β, Flag-p73β, and Flag-p73 mutants were generated by PCR and subcloned into pCMV-Tag1 (Agilent Technologies). All PCR products were confirmed by sequencing. The p73-specific antibodies (ER-15, Abcam; H-79, Santa Cruz Biotechnology), anti-p73 delta N antibody (38C6, Abcam), anti-Myc antibody (9E10, Roche), anti-Flag antibody (M5, M2, Sigma), anti-GST antibody (B-14; Santa Cruz Biotechnology), anti-HA antibody (12CA5; Roche), anti-ubiquitin antibody (BD Biosciences), and anti-actin antibody (Sigma) were used according to manufacturer’s instructions. The Pirh2 polyclonal antibody has previously been described (12).

Cell culture and DNA transfection

All cells were maintained in α-minimal essential medium supplemented with 10% FBS. H1299 cells and Saos-2 cells were transfected using the previously described calcium phosphate method (12).

siRNA experiments

For siRNA experiments, HCT116 p53+/−/− cells were transfected with the indicated siRNA constructs using Lipofectamine 2000 (Invitrogen). The following sequences were used: Pirh2 siRNA1, CCTTGCTGTGACAAGCTTT; Pirh2 siRNA2, GCTTTAAGTGAAGGAGAT; and control siRNA, CTATGTGTGACACGCTGTT. The following AIP4 target sequences (11) were used: AAGTGCTTCTCGTGCAGA; GACGATAGGTGGGCGCGATG; and scrambled sequence was AATTCTCCGAAACGTTACTT.

Expression and recombinant protein preparation

All GST- or His-tagged recombinant proteins were expressed in E. coli strain BL21 (DE3; Novagene), treated with isopropyl-β-D-thiogalactoside to induce fusion protein expression, and purified using Glutathione Sepharose 4B (GE Healthcare) for glutathione S-transferase (GST) fusion proteins or Ni2+-NTA agarose (Qiagen) for His fusion proteins.

Immunoprecipitation and measurement of p73 half-life

Cells were lysed in 50 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP-40, and a protease inhibitor tablet (Roche) and immunoprecipitated with the indicated antibodies. The immune complexes were collected with protein A agarose beads and washed 4 times with the same lysis buffer. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography. To measure the p73 half-life, Saos-2 cells were cotransfected with plasmids expressing Pirh2, AIP4, and p73. Twenty-four hours after transfection, the cells were treated with 25 μg/mL cycloheximide (CHX) to inhibit de novo protein synthesis, and the protein levels were monitored by immunoblotting with the ER-15 antibody (to measure p73) at the indicated time points. The relative amount of p73 protein was determined by densitometry and normalized to β-actin.

In vitro ubiquitination assay

The in vitro ubiquitination assay was done as previously described with some modifications (12, 25). For Pirh2-mediated ubiquitination, the reactions were done by adding E1 (20–40 ng; Calbiochem), E2 (UbcH5b, 100 ng; Calbiochem), ubiquitin or His-ubiquitin (3–5 μg; Sigma), His-p73 and GST-Pirh2 (0.2–0.5 μg) in ubiquitination buffer [50 mmol/L Tris-HCl (pH 7.4), 2 mmol/L ATP, 5 mmol/L MgCl2, and 2 mmol/L DTT] to a final volume of 30 μL. The reactions were incubated at 30°C for 1.0 to 1.5 hours. The reactions were stopped with 2 x SDS loading buffer, resolved by SDS-PAGE gels and analyzed by Western blot.

To eliminate possible autoubiquitination of Pirh2 in the coupled in vitro ubiquitination/immunoprecipitation (IP), the mixtures were incubated for 2 hours, immunoprecipitated with the ER-15 (anti-p73) monoclonal antibody (mAb) and protein A agarose beads, rotated at 4°C for 2 to 3 hours, washed with radioimmunoprecipitation assay (RIPA) buffer, separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by Western blot.

In vivo ubiquitination assay

Cells were transfected with expression plasmids encoding p73, Pirh2, AIP4, and HA-tagged ubiquitin or various HAUbiquitin mutants either alone or in combination. After 30 hours, the cells were harvested, lysed, and immunoprecipitated with the indicated antibodies. The immune complexes recovered by protein A Sepharose were washed 4 times with RIPA buffer, separated by 10% SDS-PAGE, and analyzed by immunoblotting as previously described (12, 25).

Chromatin immunoprecipitation

Chromatin IP (ChIP) was done as previously described (12).

Luciferase assay

As previously described (12), pGL3-E1bTATA contains a minimal promoter consisting of a TATA box downstream of one copy of the p73 binding site from the 5′ end of the p21WAF1 promoter; this construct is referred to as p21-Luc.
A β-galactosidase reporter construct, pCMV-β-gal (Promega), was included in all of the transfection reactions. Luciferase activity was measured 2 days posttransfection in samples containing equivalent amounts of protein using an LB9507 luminometer and a luciferase assay reagent (Promega). The values were normalized to β-galactosidase activity.

**Statistical analysis**

Statistical significance was determined using the 2-tailed Student t test and expressed as a P value.

**Results**

**Pirh2 physically interacts with p73**

In our previous study, Pirh2 was shown to bind to the p53 DNA binding domain (12), which is highly conserved in p73 (60%–80%, 1–4). On the basis of these data, we first investigated whether Pirh2 interacted with p73 in vivo. p53 null Saos-2 cells were transfected with plasmids expressing Flag-p73α and Flag-p73β or with these plasmids combined with Myc-Pirh2, immunoprecipitated with the indicated antibodies, and analyzed by Western blot. The data showed that Pirh2 coimmunoprecipitated with p73α and p73β (Fig. 1A and B). To determine whether endogenous p73 and Pirh2 interacted under physiologic conditions in the absence of overexpression, an IP/Western blot experiment was done using extracts prepared from human HEK293 cells. We observed that (i) p73 was present in the anti-Pirh2 immunoprecipitates, but not in the control mouse IgG sample, and that (ii) Pirh2 was present in the anti-p73 immunoprecipitates, but not in the control rabbit IgG sample (Fig. 1C). Next, we carried out an in vitro Ni²⁺ pull-down assay, which revealed that GST-Pirh2 bound to His-p73α and His-p73β but not to His alone (Fig. 1D). Thus, we concluded that p73 and Pirh2 physically interact in vivo and in vitro.

**Mapping the Pirh2 and p73 interaction sites**

To identify the region(s) of Pirh2 that interacted with p73, a number of Pirh2 deletion mutants were generated with Myc-tagged Pirh2 or various Myc-tagged Pirh2 mutants (Fig. 2A). Saos-2 cells were cotransfected with plasmids expressing Myc-tagged Pirh2 or Myc-Pirh2 mutants and p73α. Twenty-four hours after transfection, the cells were lysed and the cell extracts were mixed with an affinity-purified His-p73α protein. Ni²⁺-NTA agarose was added to the mixture, and it was rotated for 2 to 3 hours at 4°C. Following extensive washing, complexes of His-p73α and Pirh2 were isolated on Ni²⁺-NTA agarose and detected by Western blot (Fig. 2B). All of the Pirh2 fragments, with the exception of an N-terminal fragment containing the first 60 amino acids, were capable of binding to p73. These results were validated upon mixing of affinity purified GST-Pirh2 and various truncated Pirh2 fusion proteins with the purified His-p73α and analysis using a GST pull-down assay with a His-specific antibody against p73 (His-p73; Fig. 2C). We further confirmed that a region of Pirh2 encompassing residues 100 to 137 was required for binding to p73 (Fig. 2C, right). To map the p73 binding site on the Pirh2 protein, Flag-tagged full-length p73 or a series of Flag-tagged p73 deletion mutants were generated and expressed in Saos-2 cells (Fig. 2E). An in vitro GST pull-down assay was done, and cell extracts prepared from Saos-2 cells expressing p73 or various p73 deletion mutants were mixed with purified GST-Pirh2. As shown in Fig. 2F, full-length p73 and p73 123–313 (DNA binding domain) bound to immobilized GST-Pirh2 but not to other p73 deletion mutants. Thus, these data indicated that the DNA binding domain of p73 is important for its interaction with Pirh2.

**Pirh2 promotes the ubiquitination of p73 in vivo**

We next investigated whether Pirh2 promoted p73 ubiquitination in vivo. Saos-2 cells were cotransfected with plasmids encoding Flag-p73α and Flag-p73β, either alone or in combination with pcDNA3-Pirh2 and together with HA-tagged ubiquitin. p73 was immunoprecipitated with a specific mAb against the Flag epitope (M5) and analyzed by immunoblotting with an HA-specific antibody (12CA5) to detect ubiquitinated p73 (Fig. 3A, top) or with a p73-specific mAb (ER-15) to detect total p73 (Fig. 3A, bottom). Similar results were obtained using the ER-15 antibody (Fig. 3B). We further found that Pirh2ARING (deleted RING domain in Pirh2) was unable to promote p73 ubiquitination, indicating that the RING domain of Pirh2 is required to mediate p73 ubiquitination (Fig. 3C). Similar results were obtained with p73β ubiquitination (data not shown).

To determine which lysine residue(s) of ubiquitin (Ub) was required for p73 ubiquitination by Pirh2 in vivo, we examined a number of Ub mutants. p53 null H1299 cells were cotransfected with plasmids encoding Pirh2, Flag-p73α, or Flag-p73β and HA-ubiquitin or ubiquitin mutants. p73 was immunoprecipitated with a Flag-specific antibody (M5) and immunoblotted with an HA-specific antibody to detect ubiquitinated p73 (Fig. 3D, top) or with ER-15 to detect total p73 (Fig. 3D, bottom). p73α was more heavily ubiquitinated in the presence of Pirh2 with K11, K29, K48, and K63 Ub when compared with wild-type Ub. Immunoprecipitated p73β was ubiquitinated to a lesser extent in the presence of Pirh2 with K11, K29, K48, and K63 Ub when compared with p73α ubiquitination. These data indicate that Pirh2 preferentially uses Lys-11, -29, -48, and -63 of Ub to mediate p73 ubiquitination in vivo.

**Pirh2 is required for the ubiquitination of p73 in vivo**

Because overexpression of Pirh2 mediated p73 ubiquitination in vivo, we next tested whether endogenous Pirh2 played a role in regulating p73 ubiquitination. To determine whether Pirh2 was required for p73 ubiquitination in vivo, p53⁻/⁻ HCT116 cells were treated with the indicated siRNAs. Forty hours later, the cells were transfected with an HA-Ub expression plasmid and immunoprecipitated with an ER-15 antibody. The levels of ubiquitinated p73
were significantly lower upon treatment with Pirh2-siRNA2 when compared with treatment with the control-siRNA (Fig. 4A). Stronger polyubiquitination of p73 was detected when the cells were treated with MG132, indicating that Pirh2 promoted p73 ubiquitination (Fig. 4B). Similar results were observed in Pirh2-shA or control-shA cell lines expressing HA-Ub (Fig. 4C). Notably, the levels of p73 did not significantly change. These data showed that Pirh2 is required for p73 ubiquitination in vivo.

**Pirh2 promotes the ubiquitination of p73 in vitro**

Given that Pirh2 promoted p73 ubiquitination in vivo, we sought to determine whether p73 was a direct substrate for Pirh2 in vitro. An in vitro ubiquitination assay was done (12, 25, 26) using GST-Pirh2 and His-p73α or His-p73β purified from *Escherichia coli*. The reactions were done in the presence of E1 and E2 (UbcH5b) and ubiquitin or the following ubiquitin mutants (KO: mutants in which the 7 lysine residues, K6, K11, K27, K29, K33, K48, and K63, were replaced with arginine; mutant in which only lysine K48 was replaced with arginine (K48R); and mutant in which only lysine K63 was replaced with arginine (K63R). The ubiquitination of purified p73 was analyzed by Western blot. Surprisingly, the UbK63R mutant markedly reduced the Pirh2-mediated ubiquitination of p73α and p73β when compared with UbK48R or wild-type Ub, suggesting that Pirh2-mediated p73 ubiquitination occurred primarily through the K63 chain of ubiquitin in vitro (Fig. 5A and B). To confirm these results, a series of ubiquitin mutants containing 1 lysine with the remaining 6 lysine residues...
mutated to arginine (K6, K11, K29, K48, and K63) were generated, purified from *Escherichia coli*, and used in the *in vitro* ubiquitination assay. In the presence of Pirh2 and p73, we detected high levels of Ub-Lys-63 and wild-type Ub conjugation, moderate levels of Ub-Lys-11 and Ub-Lys-29 conjugation, and low levels of Ub-Lys-6 and Ub-Lys-48 conjugation (Fig. 5C, top). The p73 immunoblots revealed differences in the pattern of ubiquitination between p73α and p73β; whereas Pirh2 primarily used Lys-63 to promote the ubiquitination of p73α, it used multiple lysine residues to mediate the ubiquitination of p73β (Fig. 5C, bottom). To eliminate the possible autoubiquitination of Pirh2, a coupled *in vitro* ubiquitination/IP was done (25). After the reactions proceeded for 2 hours, the mixtures were immunoprecipitated with the ER-15 antibody and analyzed by immunoblotting with the anti-Ub mAb to detect ubiquitinated p73 (Fig. 5D, top) or the ER-15 antibody to detect total p73 (Fig. 5D, bottom). Taken together, Pirh2 catalyzed K11-, K29-, K48-, and K63-linked Ub chains to promote p73 ubiquitination in vivo; in vitro, Pirh2 only used Lys-11, -29, and -63 Ub to mediate p73 ubiquitination, suggesting that additional factors may be involved.

To determine the physiologic consequence of the p73-Pirh2 interaction, we tested whether the ectopic expression of Pirh2 regulated the levels of endogenous p73 in HEK293 cells. We observed that overexpression of Pirh2 did not result...
in a reduction in the steady-state levels of endogenous p73 protein in HEK293 cells (data not shown). To further test whether Pirh2 negatively regulated p73 levels, Saos-2 cells were cotransfected with plasmids expressing an anti-Flag (M5) mAb and analyzed by immunoblotting with an HA antibody (top) or with the ER-15 antibody for p73 (bottom). Direct Western blots for p73a, p73b, and Pirh2 are shown in the bottom panels. B, similar to (A) except that p73 was immunoprecipitated with CHX to inhibit de novo protein synthesis. Notably, in the presence of Pirh2, the half-life of p73 was not significantly altered when compared with its half-life in the presence of the empty vector, pcDNA3 (data not shown). These data were further confirmed by pulse chase with [35S]-methionine/cysteine analysis of ectopically expressed p73 in the presence or absence of Pirh2 in HEK293 (data not shown). Together, the data indicated that Pirh2 is unable to directly regulate the stability of p73.

Pirh2 represses p73-dependent transcriptional activity
To gain insight into the functional consequences of the interaction of Pirh2 with p73, we examined the effect of Pirh2 or AIP4 expression on p73-mediated transcriptional activity. H1299 cells were cotransfected with a luciferase reporter construct (p21-luc) containing the p73 binding site from the p21WAF promoter and either p73 alone or p73 in combination with Pirh2 or AIP4. Both Pirh2 and AIP4 repressed p73-dependent transcriptional activity. Unlike p53 (12), the Pirh2DING mutant was unable to repress p73-dependent transcriptional activity, suggesting that the RING domain of Pirh2 was required inhibiting the p73-mediated transcriptional activity (Fig. 6A). Similarly, the AIP4 mutant (AIP4C830A) lost the ability to repress p73b transcriptional activity. These results indicate that the
E3 ligase activity of Pirh2 is linked to the repression of p73 transactivation. To further assess the involvement of endogenous Pirh2 in the regulation of p73 ubiquitination, HCT p53−/− cells were pretreated with control siRNA, Pirh2 siRNA2, AIP4 siRNA, or a combination of Pirh2 siRNA2 and AIP4 siRNA. The cells were then transfected with the p21WAF1 luciferase reporter. As shown in Fig. 6B, p21WAF1-luc activity was greatly increased in cells depleted of Pirh2 or AIP4 by siRNA. The transcriptional activity of p73 was further increased when both Pirh2 and AIP4 were depleted (Fig. 6B). These data are consistent with the results showing that p73 transcriptional activity was inhibited by overexpression of Pirh2. We also tested the binding of p73 to intron 3 of Pirh2 using a ChIP assay. As shown in Fig. 6C, similar to p53 (12), p73 bound to intron 3 of Pirh2 in p73+/− MEFs in vivo but not in p73−/− MEFs.

To determine the mechanisms of Pirh2 suppression of p73 transcriptional activity, reverse transcriptase PCR (RT-PCR) was done to examine p73 transcript levels in H1299 clones that stably expressed Pirh2, Pirh2ΔRING, or an empty vector. As shown in Fig. 6D (top), p73 transcript levels were decreased in the two H1299 clones stably expressing Pirh2 but not in the empty vector or Pirh2ΔRING expressing cells. This reduction was highly relative to the overexpression of Pirh2 and was dependent on the RING domain of Pirh2. Similar data were also obtained in Saos-2 cells that stably expressed Pirh2 (data not shown), indicating that the Pirh2 repression of p73 transcript levels was not restricted to one cell line and was dependent on the Pirh2 E3 ligase activity. The p73 protein levels were consistently unaffected by ectopic expression of Pirh2 (Fig. 6D, bottom).

To determine whether endogenous Pirh2 was critical in regulating p73 transcript levels, siRNA experiments were conducted. We observed that Pirh2-siRNA1 and Pirh2-siRNA2 effectively knocked down the levels of Pirh2 RNA and protein in H1296 p53−/− cells (Fig. 6E). The p73 transcript levels increased, but the p73 protein levels were not affected. Together, our findings indicated that Pirh2 represses p73 transcriptional activity possibly by directly inhibiting p73 transcripts and is dependent on Pirh2 E3 ligase activity.

Because wild-type p73 can be negatively regulated by delta Np73 (27–29), we tested whether delta Np73 levels were affected by the presence or absence of Pirh2. H1299 cells were transfected with delta Np73 alone or delta Np73 in combination with Pirh2. As shown in Fig. 6F, ectopic expression of Pirh2 did not affect delta Np73 protein levels. We consistently observed that delta Np73 protein levels did not change when cells were depleted of Pirh2 using siRNA in MCF-7 cells (Fig. 6G). In contrast, p53 protein levels increased when cells were depleted of Pirh2 (Fig. 6G). Together, the data indicate that p73 transcriptional repression is differently regulated by Pirh2 and delta Np73.

To add to suppression of p73 transcriptional activity, we examined whether Pirh2 inhibited p73-dependent apoptosis. Transient expression experiments and fluorescence-activated cell sorting analysis (using Annexin V staining) were done to determine whether Pirh2 expression could...
rescue cells from p73-dependent cell death. The expression of p73 alone resulted in apoptosis; however, apoptosis could not be prevented by coexpression of Pirh2 (data not shown). In addition, in long-term colony assays, Pirh2 did not inhibit p73-dependent cell death (data not shown). Cell-cycle arrest mediated by p73 is important for its tumor suppressor function. To determine whether Pirh2 inhibited p73-induced G1 arrest, human bladder carcinoma EJ cells, which lack functional p53 (30, 31), were transfected with p73 alone or p73 in combination with Pirh2 or Pirh2

**Discussion**

The p73 protein has been identified as a homolog of the tumor suppressor protein p53 and is capable of inducing apoptosis or cell-cycle arrest. Here, we report that Pirh2 physically interacts with p73 and inhibits p73-dependent transcriptional activity by promoting its ubiquitination. Our findings suggest that the regulation of p73 transcriptional activity and degradation can be controlled by different mechanisms.

Intriguingly, Pirh2 catalyzed K11-, K29-, K48- and K63-linked Ub chains to ubiquitinate p73 in vivo and markedly used K63-linked chains to promote p73 ubiquitination in vitro. Substrates with 4 or more Ub moieties, linked via Lys-48, Lys-29, or Lys-11 for p73 ubiquitination in vitro. Affinity purified GST-Pirh2, His-p73α, or His-p73β were added to bacterial extracts containing recombinant E1 and E2 (UbcH5b) and wild-type Ub or ubiquitin mutants as indicated and analyzed by immunoblotting with anti-Ub to reveal ubiquitinated products (top) or anti-p73 (ER-15) to reveal ubiquitinated p73 species (bottom). D, similar to (C); samples after the ubiquitination reaction were immunoprecipitated with anti-p73 (ER-15) and analyzed by immunoblotting with anti-Ub (top) or anti-p73 (ER-15, top) as indicated. IB, immunoblotting.
K63R mutation of Ub in yeast degrades short-lived proteins and abnormal proteins, which are the canonical substrates of the Ub-proteasome system at a normal rate (37). Recent studies have suggested that K63-linked chains also support proteasomal degradation (38, 39). Rad6 has been reported to be an E2 that normally supports Lys-48 chain-dependent degradation in yeast, but Rad6 also supports Lys-63 chain-mediated degradation, suggesting that linkage specificity may depend on either the target proteins or the E3 used for conjugation (40). As shown by Parkin (41), Pirh2 may act as a dual E3 Ub ligase that uses K48-linked chains to promote p53 degradation and K63-linked chains to repress p73 transactivation. The possibility that Pirh2 uses different E2 proteins through distinct mechanisms exists.

Wu and colleagues (2004) proposed that p73 transcriptional activity is linked to its degradation (42). We observed that Pirh2 repressed p73-dependent transcriptional activity by promoting p73 ubiquitination without targeting its degradation. Depletion of Pirh2 by siRNA restored p73 transcriptional function in p53 null cells. Moreover, we found that Pirh2 repressed p73 transcriptional activity by directly inhibiting p73 transcripts, and p73 repression by Pirh2 was

**Figure 6.** Pirh2 represses p73-dependent transactivation. A, H1299 cells were cotransfected with a p21-Luc reporter plasmid and the p73 expression construct in combination with Pirh2, AIP4, or their mutants or an empty vector (pcDNA3.1). The luciferase activity was measured. Error bars indicate the SEM (n = 3). P < 0.01 (2-tailed Student t test). B, HCT116 p53−/− cells were treated with Pirh2-siRNA2, AIP4-siRNA, or control-siRNA for 30 hours. The cells were then transfected with the p21-Luc reporter expressing plasmid. The luciferase activity was measured. Error bars indicate the SEM (n = 3). P < 0.01 (2-tailed Student t test). C, ChiP assay to assess p73 DNA binding activity in p73−/− MEFs and parental p73+/+. PCR analysis (using primers to intron 3 of Pirh2, the Pirh2 promoter, or intron 1 of Mdm2) is shown using input DNA (1/30 of ChiP) or DNA after ChiP. The amplification products ranged from 200 to 250 bp. D, H1299 clones stably expressing the empty vector (pcDNA3), Pirh2-1, Pirh2-2, and Pirh2ΔRING were subjected to semiquantitative RT-PCR to measure p73, Pirh2 and GAPDH mRNA levels (top two panels) and immunoblotting (IB, bottom) with the indicated antibodies to detect p73, Pirh2, or Pirh2ΔRING and actin.
required for p73-dependent transcriptional activity and G1 arrest but not for apoptosis. Our results provide the molecular mechanism for understanding how Pirh2 negatively regulates p73 function. Recently, Jung and colleagues reported that Pirh2 promoted p73 ubiquitination and degradation in RKO and MCF7 cells (43), which suggests that the regulation of p73 protein stability by Pirh2 seems to be dependent on cell type. Notably, MDM2 did not promote p73 degradation in most cell lines (44, 45). Kubo and colleagues reported that MDM2 mediated p73 degradation through the interaction with Itch in HeLa cells (46). Therefore, it will be of interest to further investigate whether additional factors may be involved in p73 degradation mediated by Pirh2.

In summary, our findings indicate that p73-dependent transcriptional activity is regulated by ubiquitination. In addition, our data reveal that p73-mediated transactivation and apoptosis are controlled by different mechanisms.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Figure 6.** (Continued) E, HCT116 p53/−/− cells were transfected with the Pirh2-siRNA1, Pirh2-siRNA2, or control-siRNA as indicated. Semiquantitative RT-PCR was used to measure p73, Pirh2, and GAPDH mRNA levels. The amounts of endogenous p73, Pirh2, and actin proteins were determined by Western blot using p73-specific, Pirh2-specific, and actin-specific antibodies. F, H1299 cells were transfected with plasmids for the empty vector (pcDNA3), Flag-DNp73, or a combination of these with Myc-Pirh2 as indicated. The levels of ectopically expressed Pirh2 and DNp73 proteins were determined by immunoblotting with an anti-Flag antibody for DNp73 and an anti-Myc antibody for Pirh2. An antibody against β-actin was used as the loading control. G, MCF-7 cells were treated with control-siRNA, Pirh2-siRNA1, or Pirh2-siRNA2 for 40 hours. The levels of endogenous Pirh2, DNp73, and p53 proteins were determined by immunoblotting with Pirh2-specific, DNp73-specific (38C674, Abcam), and p53-specific (DO-1) antibodies. An antibody against β-actin was used as the loading control. H, human bladder carcinoma EJ cells were transfected with p73α alone or p73α in combination with Pirh2, Pirh2ΔRING, or an empty vector (pcDNA3) as indicated, and the cell-cycle profile was determined by propidium iodide staining and flow cytometry. The results represent the average of triplicate experiments.
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Pirh2, a Ubiquitin E3 Ligase, Inhibits p73 Transcriptional Activity by Promoting Its Ubiquitination

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