Tumor and Stem Cell Biology

ING5 Is a Tip60 Cofactor That Acetylates p53 in Response to DNA Damage

Nansong Liu, Jiadong Wang, Jifeng Wang, Rukai Wang, Zhongle Liu, Yao Yu, and Hong Lu

Abstract

Posttranslational modification of p53 is a critical event in regulating the expression of its target genes. p53 is acetylated at lysine 120 (K120) by acetyltransferases Tip60 (KAT5) and hMOF (KAT8) in response to DNA damage. Identification of cofactors for these two enzymes will shed light on the mechanism by which cells make a choice between cell-cycle arrest and apoptosis. It has been reported that ING5, a member of the inhibitor of growth (ING) family, is involved in p53-dependent pathways, but its exact role is unknown. In this study, we found that ING5 expression was significantly increased and that ING5 assisted Tip60, but not hMOF, in acetylating p53 at K120 in response to DNA damage. ING5 had no effect on acetylation of p53 at K373/382, but it formed a complex with p53 and Tip60. ING5 was required for acetylation of p53 at K120, and p53 acetylated at K120 subsequently bound to the promoters of its target apoptotic genes, BAX and GADD45, to promote their expression and lead to apoptosis. Mutation of K120 to K120R abolished the effects of ING5 on p53-induced gene expression. Thus, we conclude that ING5 functions as a cofactor of Tip60 in the acetylation of p53 at K120 in response to DNA damage. Cancer Res; 73(12): 3749–60. ©2013 AACR.

Introduction

The inhibitor of growth (ING) family of proteins was identified more than a decade ago as potential tumor suppressors, and these proteins play an important role in numerous cellular processes and signaling pathways, such as cell-cycle progression, cell senescence, apoptosis, DNA repair, and NF-kappa B signaling (1–3). ING proteins play a role in transcriptional regulation with various binding partners, including histone modifiers such as histone deacetylase (HDAC) and histone acetyltransferase (HAT) (4–7).

All ING proteins share a similar phylogenetic conserved structure. However, ING3 is relatively distant from other INGs, whereas ING1 is closely related to ING2 and ING4 is closely related to ING5 (8). Biochemical studies support these phylogenetic findings. ING1 and ING2 are part of the histone deacetylase complex Sin3A (7). ING4 and ING5 are part of the HBO1-containing HAT complex and are involved in acetylation of histone H4 and activation of p53 (4, 9). In contrast, ING3 is one part of the hNuA4 complex, which regulates the acetylation of histones H2A and H4 (4, 5).

Although ING5 appears to overlap with ING4 in most biochemical aspects and biologic functions, ING5 has also been found to have functions that differ from those of ING4. For example, ING5 is a subunit of a histone H3-specific HAT complex that includes MOZ/MORF leukemic proteins (4). ING5 expression has also been reported to be decreased in certain tumors, such as gastric carcinoma (10), human head and neck squamous cell carcinoma (11), and oral squamous cell carcinoma (12). However, the biologic functions of ING5 are not as well understood as those of other members of the ING family.

p53 is a tumor suppressor and transcriptional factor that is regulated by numerous posttranslational modifications. In response to DNA damage, p53 is modified and binds to promoters of its specific target genes, such as p21, PUMA, BAX, GADD45, and NOXA, to activate expression of these genes, which are involved in either cell-cycle arrest or apoptosis (13). Acetylation of p53 at various sites plays an important role in regulating the transcriptional activity of p53, and acetylation at different sites results in distinct biologic outcomes. For example, acetylation of p53 at 3 lysines in the C terminus (i.e., K373, K382, and K320) by p300/CPB or PCAF increases the stability of the protein and enhances p53-dependent transcription, thus promoting growth arrest or apoptosis (14, 15).

Recently, 2 independent groups reported simultaneously that MYST family proteins, Tip60 (KAT5) and hMOF (KAT8), directly acetylated p53 at lysine 120 (K120) within the DNA-binding domain (16, 17). Consequently, acetylated p53 activated expression of apoptotic genes and led to cell apoptosis. However, DNA damage induced p53-dependent cell-cycle arrest, rather than p53-dependent apoptosis, when acetylation at K120 was eliminated by mutation of K120 to arginine, a nonacetylatable residue. Furthermore, acetylation of p53 at K120 has been found to be caused specifically by Tip60 and...
hMOF, not by either CBP/p300 or PCAF. However, the regulator of p53 acetylation at K120 after DNA damage is still unclear (16, 17). All ING proteins except ING3 have been reported to coimmunoprecipitate with p53 and induce G1-phase cell-cycle arrest or apoptosis in a p53-dependent manner (18–20). ING5 may induce apoptosis and/or cell-cycle arrest through the p53 pathway (19), but the molecular mechanism for this process is not clear. In this study, we have provided experimental evidence of how ING5 functions in the p53 pathway in response to DNA damage. Our results indicate that ING5 serves as a cofactor of Tip60, but not hMOF, to acetylate p53 at K120 and subsequently activates the expression of p53-dependent apoptotic genes in response to DNA damage.

Materials and Methods
Plasmids, cell lines, and transfection
For cell transfection, ING5, Tip60, and hMOF were cloned into the pCMV-Myc vector (Clontech), and p53 and its point mutations (K120R, K373/382R) were cloned into the pCMV-HA vector (Clontech). We also used the SFB vector with Tip60 or empty SFB vector, as described previously (21). For subcellular localization, ING5 was cloned into the pDsRed vector, and Tip60 was cloned into the pEGFP vector (Clontech). For shRNA-mediated silencing, ING5 was cloned into the pSilencer vector (Ambion), and its truncated deletions were cloned into the pGEX-5X-1 vector (GE Healthcare) to express GST-fusion proteins.

Hepatocellular carcinoma cell line HepG2 and osteosarcoma cell lines U-2 OS (p53+) and Saos-2 (p53-null) were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum (Hyclone). Cells were transfected with Lipofectamine 2000 reagent (Invitrogen) or FUGENE-HD (Promega) with 10% fetal bovine serum. Cells were transfected with plasmids containing Myc-Tip60 and Myc-ING5, and pCMV-HA-p53. Forty-eight hours after transfection, cells were collected and lysed in TPER Lysis Buffer (Pierce) for analysis, as described previously (23).

Detection of posttranslational modifications of p53
In U-2 OS cells transfected with pCMV-ING5, pCMV-Tip60, or pCMV-hMOF, acetylation of p53 at K120 or at K373/382 was analyzed using anti-p53 acetyl-K120 antibody (Abcam ab78316; Abcam) or anti-p53 acetyl-K373/382 antibody (Millipore, 06-758). Densities of the bands of interest were quantified using BandScan 5.0 software (www.seekbio.com/soft/254.html) and normalized to the densities of β-actin.

Chromatin immunoprecipitation
U-2 OS cells were transfected with pSilencer-ING5 or pSilencer void vector and treated with doxorubicin (DOX). Cells were cross-linked using 1% formaldehyde for 10 minutes at room temperature and then subjected to the chromatin immunoprecipitation (ChIP) assay following the protocol of the kit (Millipore 17-295). Purified immunoenriched samples were used for sequence-specific polymerase chain reaction (PCR) reaction mixtures, and genomic DNA in the lysate served as the input.

Analysis of apoptosis by flow cytometry
U-2 OS cells transfected with pSilencer-ING5 or pSilencer void vector were harvested using trypsinization and fixed in 70% cold ethanol. Cells were resuspended in 1 mL of PBS containing 200 μg/mL RNase A (Sigma) and 20 μg/mL propidium iodide (Sigma), and the cells were then incubated at room temperature for 15 minutes. Ten thousand cells were measured using flow cytometry and apoptotic cells were measured by counting sub-2N population (24). Data were analyzed using CellFIT software (Becton-Dickinson).

Real-time PCR
Total RNA was extracted using Trizol (Invitrogen) and reverse-transcribed into cDNA. Real-time PCR was conducted using SYBR Premix Ex Taq II (Takara), using cDNA as the template in an iCycler IQ real-time PCR system (Bio-Rad). Reactions started at 95°C for 1 minute, followed by 40 cycles of 5 seconds at 95°C alternating with 30 seconds at 60°C. Primers used in real-time PCR were as follows:

- Bcl-2 Forward: 5’ AGATGGCAAAATGACCACAGAT 3’
- Bcl-2 Reverse: 5’ TGGGACGAGTACGCAAGG 3’
- MDM2 Forward: 5’ TACAGGCGGCAATCGAATC 3’
- MDM2 Reverse: 5’ TGAAGTGCATTTCCAATAGTCAGC 3’
- BAK Forward: 5’ GCTGACAGGCCAAGTTAAGG 3’
- BAK Reverse: 5’ GTGTAACAGCCGTGAAAGG 3’
- p21 Forward: 5’ GATTAGCACCAGCAGAGAGT 3’
- p21 Reverse: 5’ TACAGTCTAGGTTGAAAGG 3’

Immunofluorescence and protein subcellular localization
In brief, U-2 OS cells were fixed with 100% methanol, permeabilized with 0.01% Triton X-100 in phosphate-buffered saline (PBS), and then incubated with anti-ING5 antibody for 1 hour at room temperature. The cells were washed with PBS and incubated with fluorescein isothiocyanate conjugated with a secondary antibody for 1 hour in a humidified chamber at room temperature. The cells were then washed with PBS and stained with 1 μg/mL 4’,6’-diamidino-2-phenylindole (DAPI) to visualize nuclei. Images were captured under an Olympus IX71 laser microscope (Olympus).

Knockdown of ING5, Tip60, and hMOF
Tip60 was knocked down by transfection of U-2 OS cells with a siRNA duplex (GGACAGCUCUGAUGGAAUtt) corresponding to the human Tip60 mRNA. A different siRNA duplex (CCAUCCUCUCUUUGAGAAUtt) was used to ablate endogenous hMOF. A nontarget siRNA duplex (UUCUCCGAACGU-GUCAGUtt) was used as a negative control. Cells were transfected with pSilencer-ING5 to knock down ING5 or with a void vector as a negative control (22).
**Results**

**ING5 expression increased and ING5 accumulated in the nucleus in response to DOX-induced DNA damage**

To investigate how ING5 is involved in the response to DNA damage, U-2 OS cells were first treated with DOX, a chemical that can induce DNA double-strand breaks (25). As shown in Fig. 1, both mRNA and protein expression levels of endogenous ING5 increased after treatment with DOX. The mRNA level of ING5 was about 3 times as high in cells treated with 1 μmol/L DOX for 24 hours as it was in cells that were not treated with DOX (Fig. 1A). The increased protein levels of ING5 were dose-dependent within tested concentrations of DOX ranging from 0.5 to 10 μmol/L and time-dependent for treatment with 1 μmol/L DOX over durations ranging from 12 to 60 hours (Fig. 1B and C). In addition, ING5 accumulation in the nucleus increased in response to DOX-induced DNA damage (Fig. 1D).

Interestingly, the level of endogenous ING4 appeared to decrease after treatment of cells with 5 or 10 μmol/L DOX, suggesting that ING4 may be involved in a different DNA damage response pathway than ING5 (Fig. 1B).

Considering that DNA damage can activate p53 and increase acetylation of p53 (16, 17, 26), we analyzed the changes in
expression levels of endogenous p53 and p53 acetylated at K120 or K373/382 after DOX treatment. As expected, expression levels of endogenous p53 increased, and levels of p53 acetylated at K120 also increased after treatment with DOX. Acetylation of p53 at K373/382 also increased after treatment with 0.5 μmol/L DOX (Fig. 1B), but expression of p53 acetylated at K373/382 decreased with prolonged treatment with DOX (Fig. 1C).

**ING5 is required for acetylation of p53 at K120**

As shown in Fig. 1, expression levels of ING5 and p53 acetylated at K120 both increased in response to DOX-induced DNA damage, suggesting a potential relationship. We next used a short-hairpin RNA method to knock down ING5 in U-2 OS cells to examine the role of ING5 in the p53 pathway during DNA damage. As shown in Fig. 2A, knockdown of endogenous ING5 substantially repressed acetylation of p53 at K120 but not acetylation of p53 at K373/382 in response to DNA damage; expression of p53 was affected by treatment with DOX, but almost no effects were observed from knockdown of endogenous ING5. On the other hand, expression of Tip60 was not affected by either DNA damage or knockdown of endogenous ING5 (Fig. 2A).

Consistent with previous reports (16, 17), overexpression of either Tip60 or hMOF increased expression levels of p53 acetylated at K120 in U-2 OS cells (Fig. 2B). Similarly, overexpression of ING5 enhanced acetylation of p53 at K120 (Fig. 2B). These results combined with those in Fig. 1B and

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C strongly suggest the involvement of ING5 in acetylation of p53 at K120.

To address whether ING5 is involved in acetylation of p53 at K120 through Tip60 and/or hMOF, endogenous TIP60, hMOF, or ING5 was knocked down individually or in combination. Knockdown of TIP60 or hMOF reduced the levels of p53 acetylated at K120 to about 60% of the levels in the control cells, whereas coknockdown of TIP60 and hMOF reduced the levels of p53 acetylated at K120 to 6% of the levels in the control cells, suggesting that TIP60 and hMOF mediate acetylation of p53 at K120 through different pathways (Fig. 2C). Similarly, coknockdown of ING5 and hMOF reduced the levels of p53 acetylated at K120 to 9% of the levels in the control cells, suggesting that ING5 and hMOF function in different pathways (Fig. 2C). In contrast, coknockdown of ING5 and TIP60 reduced the levels of p53 acetylated at K120 to 46% (Fig. 2C). This level is modestly lower than that in the Tip60 knockdown cells (63%), which might be due to the relatively lower level of Tip60 in the coknockdown cells. These data suggest that ING5 mediates acetylation of p53 at K120 in the same pathway with Tip60, but not with hMOF.

In addition to ING5, the protein PDCD5 is also involved in Tip60-mediated acetylation of p53 at K120. PDCD5 has been found to promote the stability of Tip60 and increase acetylation of p53 at K120 after DNA damage (27). As shown in Fig. 2D, overexpressing either ING5 or PDCD5 increased acetylation of p53 at K120, and overexpressing both proteins led to an additive increase. Therefore, ING5 stimulation and PDCD5 stimulation of Tip60-mediated acetylation of p53 at K120 may occur through different pathways.

**ING5 associates with Tip60, but not hMOF, to form a complex with p53**

We further have shown the involvement of ING5 in the acetylation of p53 at K120 through Tip60, but not hMOF, by investigating the interaction among ING5, Tip60, and hMOF. As shown in Fig. 3A, a clear association between Tip60 and ING5 was visible after treatment with DOX, whereas hMOF had no interaction with ING5 regardless of DOX treatment. The association between Tip60 and ING5 was also suggested by the colocalization of fusion proteins GFP-Tip60 and RFP-ING5 in HepG2 cells (Fig. 3B). Furthermore, endogenous association between ING5 and Tip60 was identified, and that association was significantly strengthened after treatment with DOX (Fig. 3C). These results strongly suggest that ING5 is closely associated with Tip60, but not hMOF, after DNA damage. We also found that Myc-ING5 can immunoprecipitate endogenous p53 in our Co-IP analysis (Fig. 3D), which is consistent with a previous report (4), and this result indicates an interaction between ING5 and p53.

To determine whether ING5 forms a protein complex with Tip60 and p53, an in vitro pull-down assay was conducted. GST-p53 or GST expressed in *Escherichia coli* was immobilized on sepharose beads and mixed with the lysate of U-2 OS cells cotransfected with pCMV-Myc-ING5 and pCMV-Myc-Tip60. As shown in Fig. 3E, both Tip60 and ING5 interacted with GST-p53, but not with GST. This was confirmed by the Co-IP result in Fig. 3G. U-2 OS cells were cotransfected with Myc-ING5 and Myc-Tip60 as well as HA-p53, and Myc-ING5 and Myc-Tip60 could be detected in the immunoprecipitates of HA-p53.

The semiendogenous Co-IP assay was also conducted to analyze the interaction between p53, Tip60, and ING5 before and after treatment with DOX. U-2 OS cells were transfected with SFB-Tip60 and immunoprecipitated with S protein sepharose. As shown in Fig. 3F, endogenous p53 interacted with SFB-Tip60 regardless of DOX treatment, whereas endogenous ING5 associated with SFB-Tip60 apparently after treatment with DOX, suggesting that ING5 forms a complex with Tip60 and p53 in vivo in response to DNA damage.

To locate the region of p53 that binds with Tip60 or ING5, serial truncated fragments of p53 fused with GST were expressed in *E. coli* and mixed with the lysate of U-2 OS cells containing Myc-ING5 and Myc-Tip60. As shown in Fig. 4, Tip60 bound to the region of p53 from amino acid 50 to 250, whereas ING5 bound to the fragment from amino acid 250 to 393. These results showed that p53 could use different regions to interact with ING5 and Tip60, which suggests a possible ternary p53-ING5-Tip60 complex in vivo.

**ING5 is required for p53-mediated apoptosis in response to DNA damage**

The involvement of ING5 in acetylation of p53 at K120 prompted us to identify the effects of ING5 on the expression of p53 target genes, including the apoptosis-related genes Bcl-2, BAK, BAX, and GADD45; the cell-cycle–related gene p21; and the negative-feedback gene MDM2. ING5 was knocked down by shRNA in U-2 OS cells and its level was determined by Western blotting (Fig. 5A). In the cells transfected with the shRNA void vector, mRNA levels of BAX, GADD45, p21, and MDM2 increased after 24 hours of treatment with DOX, mRNA levels of Bcl-2 decreased, and mRNA levels of BAK did not change (Fig. 5B, columns 1 and 3). The expression of BAX and GADD45 after treatment with DOX was substantially lower in cells with ING5 knockdown than in cells transfected with control vector, whereas the expression of other tested genes after treatment with DOX was the same in ING5-knockdown and control vector-transfected cells (Fig. 5B, columns 3 and 4).

The effect of ING5 on cell apoptosis was further analyzed. As shown in Fig. 5C, more than 15% of cells were apoptotic when normal U-2 OS cells were treated with DOX for 24 hours. A similar apoptotic rate was observed after treatment with DOX in the control cells transfected with the shRNA void vector. However, ING5-knockdown cells were more resistant to apoptosis; the apoptotic rate was only about 5% after treatment with DOX. These results indicate that ING5 is required for the expression of p53-targeted apoptotic genes BAX and GADD45 and subsequent apoptosis after DNA damage.

**ING5 is recruited to promoters of the p53 target genes after DNA damage**

It has been reported that selective acetylation of p53 by specific acetyltransferases and cofactors can regulate the binding of p53 to the target promoters (16, 28). Thus, we investigated the occupancies of ING5, p53, and Tip60 at promoters of BAX, GADD45, and p21 using the ChIP assay (Fig. 6). As expected, p53 targeted all of the promoters in
Figure 3. Interactions among ING5, p53, and Tip60. A, results of a coimmunoprecipitation (Co-IP) assay to detect interaction between ING5 and Tip60 or between ING5 and hMOF in U-2 OS cells. The level of endogenous ING5 in the anti-Myc immunoprecipitate (IP) from cells transfected with pCMV-Myc-Tip60 or pCMV-Myc-hMOF was detected by Western blot (WB). B, colocalization of GFP-ING5 and Tip60 in the nuclei of HepG2 cells. C, results of Co-IP and reverse Co-IP assays to detect association between endogenous ING5 and Tip60. U-2 OS cells were lysed before or after 24-hour treatment with 1 μmol/L DOX and precipitated with anti-Tip60. ING5 in the anti-Tip60 IP was detected by WB. For reverse Co-IP, cell lysate was precipitated with anti-ING5, and Tip60 in the IP was detected by WB. IgG was used to precipitate ING5 or Tip60 as a negative control. D, results of a Co-IP assay to detect the interaction between ING5 and p53 in U-2 OS cells. The level of endogenous p53 in the anti-Myc IP from cells transfected with pCMV-Myc-ING5 was detected by WB. E, results of a pull-down assay to detect the complex containing ING5, p53, and Tip60. GST-p53 or GST was immobilized on glutathione sepharose beads and mixed with the lysate of U-2 OS cells containing pCMV-Myc-ING5 and pCMV-Myc-Tip60. After the beads were thoroughly washed, proteins conjugated to the beads were detected by WB using the anti-Myc antibody. F, results of a semiendogenous Co-IP assay to detect the complex containing ING5, p53, and Tip60. U-2 OS cells transfected with SFB-Tip60 or SFB void vector were lysed and precipitated with S protein sepharose. Endogenous p53 and ING5 conjugated to the beads were detected by WB. G, results of a Co-IP assay to detect the complex. Myc-ING5 and Myc-Tip60 in the anti-HA IP from the cells cotransfected with pCMV-Myc-Tip60, pCMV-Myc-ING5, and pCMV-HA-p53 were detected by WB.
response to DNA damage, and the same occupancy pattern was observed for Tip60, suggesting a constitutive interaction between p53 and Tip60. p53 acetylated at K120 accumulated at the promoters of BAX and GADD45, but not at the promoter of p21, and the same binding pattern was observed for ING5 (Fig. 6). However, in ING5-knockdown cells treated with DOX, recruitment of p53 and Tip60 to the promoters of the apoptotic genes BAX and GADD45 decreased significantly, and p53 acetylated at K120 at these 2 promoters was almost undetectable. In contrast, p53 acetylated at K373/382 specifically targeted the promoter of p21 (29), but not the promoters of BAX and GADD45, and this binding pattern was not affected by ING5 (Fig. 6). These results indicate that ING5 is required for the acetylation of p53 at K120 after DNA damage, and this acetylation is necessary for the recruitment of p53, Tip60, and ING5 to the promoters of apoptotic genes but not to the promoters of cell-cycle genes.

ING5 contributes to the p53-mediated activation of BAX and GADD45 through acetylation of p53 at K120

The results presented above reveal that ING5 is required for the acetylation of p53 at K120 and for the subsequent
activation of apoptotic genes after DNA damage. To address the question of whether ING5 is involved in other modifications of p53 that contribute to p53-mediated transcriptional activation, we constructed a mutant p53K120R, which cannot be acetylated at site 120 owing to the replacement of K with R. A mutant p53K373/382R was also constructed and used as a control. p53-null Saos-2 cells were cotransfected with ING5 shRNA and pCMV-HA-p53wt, p53K120R, or p53K373/382R and then treated with DOX for 24 hours. As expected, no detectable p53 expression was found in the vector control group, whereas expression of p53 was observed in cells transfected with p53wt, p53K120R, or p53K373/382R, and expression of endogenous ING5 was substantially inhibited by ING5 shRNA (Fig. 7A).

Figure 5. ING5 is required for p53-mediated transcriptional activation and apoptosis in response to DNA damage. A, level of ING5 in U-2 OS cells transfected with ING5 shRNA or shRNA vector. Cells were treated with DOX for 24 hours as indicated. The level of ING5 was determined by Western blot. B, mRNA levels of p53-target genes in ING5-knockdown U-2 OS cells after DNA damage. mRNA levels were quantified by real-time PCR, and the average of 3 independent experiments is shown. Error bars, SD. C, flow cytometry analysis of apoptosis in ING5-knockdown U-2 OS cells. Average percentage of apoptotic cells from 3 independent experiments is shown by the chart. Error bars, SD.
suggest that acetylation of p53 at K120 did not contribute to the activation of p21, nor did ING5. However, the cells transfected with p53K373/382R exhibited the same transcriptional pattern as cells transfected with p53wt or p53K120R. This may indicate that other modifications of p53, such as acetylation at K164 and K292, play a role in the activation of p21 (30, 31).

Expression of p53-target apoptotic genes GADD45 and BAX did not detectably change when cells were transfected with p53K120R, regardless of ING5 (Fig. 7C and D). p53K373/382R, depletion of ING5 notably reduced expression of these 2 apoptotic genes. These results indicate that ING5 plays a role in transcriptional activation of p53-target genes through acetylation of p53 at K120, but not through acetylation at other sites, such as K373/382.

Furthermore, apoptosis of the transfected Saos-2 cells was analyzed by flow cytometry as shown in Fig. 7E. The more than 15% of cells were apoptotic in Saos-2 cells cotransfected with pCMV-p53wt and the empty shRNA vector after treatment with DOX for 24 hours, whereas the percentage of apoptotic cells was only about 6% after knockdown of ING5. In Saos-2 cells cotransfected with p53K120R and the empty shRNA vector, about 3% of cells were apoptotic after treatment with DOX, and the same percentage of apoptotic cells was observed in the cells cotransfected with p53K120R and ING5 shRNA. These results further support the hypothesis that ING5 is involved in the acetylation of p53 at K120 and consequently affects the expression of p53-target genes BAX and GADD45, as well as p53-dependent apoptosis.

Discussion

ING proteins have recently received more and more attention because of their involvement in p53-dependent pathways and tumorigenesis of multiple tumors (32, 33). To the best of our knowledge, this is the first report to show that ING5 assisted acetyltransferase Tip60 in modifying p53 at K120 in response to DNA damage. Knockdown of endogenous ING5 by shRNA led to decreased acetylation of p53 at K120 (Fig. 2A), which led to inhibited expression of the apoptotic genes BAX and GADD45, which are regulated by p53 acetylated at K120, and consequently reduced apoptosis of DNA-damaged cells (Figs. 5, 6). When K120 was mutated to K120R, no induction was observed for apoptotic genes BAX and GADD45 after DNA damage, whether endogenous ING5 was inhibited or not (Fig. 7). In addition, ING5 was not found to affect acetylation of p53 at K373/382 (Fig. 2A). These results suggest that the involvement of ING5 in the modification of p53 is site specific.

Both of the acetyltransferases Tip60 and hMOF can acetylate p53 at K120 (16). However, we found that ING5 can function as a cofactor only for Tip60, not for hMOF (Figs. 2C and 3A). Although both Tip60 and hMOF responded to DNA damage by modifying p53 at K120, they may work through different pathways (Fig. 2C). Thus, if one of the pathways is defective, cells can still respond to DNA damage by using the alternative pathway.

Previously, ING5 has been defined as a cofactor that assists HAT in modifying chromatin. Previous studies have shown that ING5 may stimulate acetylation of histone H4 via HBO1 and acetylation of histone H3K9 via MOZ/MORF (4). Our data suggest that ING5 stimulates Tip60-mediated acetylation of nonhistone substrates such as p53. Previous studies have shown that PDCD5 may also promote acetylation of p53 at K120 (27). Our results suggest that ING5 and PDCD5 function through different pathways (Fig. 2D). Interestingly, another factor, TRIM29, has been shown to accelerate the degradation of Tip60 and negatively regulate the acetylation of p53 at K120 (34). Therefore, several carefully coordinating factors appear to be involved in the regulation of acetylation of p53 at K120, reflecting the essential role of this modification in the apoptosis induced by DNA damage.

Posttranslational modification of p53 at its DNA-binding region directly affects its transcriptional activity with cell-cycle or apoptotic genes (31). p300/CREB-mediated acetylation of p53 at K164 activates the expression of the cell-cycle gene p21 and promotes cell-cycle arrest at the G1/S phase for DNA repair (31). p53 acetylated at K373/382 binds to the promoter of cell-cycle gene p21, but not to promoters of BAX and GADD45 (Fig. 6). p53 acetylated at K120 tends to bind to promoters of
apoptotic genes BAX (Fig. 6), GADD45 (Fig. 6), and PUMA (16, 17) under stress, which activates expression of these genes and promotes cell apoptosis (Fig. 5). In contrast, p53 acetylated at K120 does not bind to the promoter of p21 (Fig. 6). It is possible that, after DNA damage in vivo, ING5, p53, and Tip60 form a complex (Fig. 3E–G) by ING5 binding to the C-terminus of p53 and Tip60 binding to the N-terminus of p53 (Fig. 4). Subsequently, ING5 assists Tip60 in acetylating p53 at K120 and promotes p53-dependent apoptosis (Figs. 5 and 7). However, the process by which acetylation of p53 at various sites is regulated and balanced after DNA damage remains to be determined.

Figure 7. ING5 mediated the p53-dependent activation of apoptotic genes through acetylation of p53 at K120. A, expression of wild-type p53, p53 point mutants, and endogenous ING5 in the transfected cells. p53-null Saos-2 cells were transfected with various plasmids, and cell lysates were subjected to Western blot. B to D, mRNA levels of p21 (B), GADD45 (C) and BAX (D) in the transfected cells. Levels were quantified by real-time PCR, and the average of 3 independent experiments is shown. Error bars, SD. E, flow cytometry analysis of transfected Saos-2 cells. Bar chart showing the average percentage of apoptotic cells from 3 independent experiments. Error bars, SD.
Expression of ING5 increased and ING5 was found to be localized in the nucleus in response to DNA damage in the present study (Fig. 1D). ING proteins are not always in the nucleus; they enter the nucleus after external stimuli such as DNA damage signals (35, 36). Translocation of ING5 has also been observed in tumorigenesis and progression (10–12). Various ING proteins enter the nucleus by different mechanisms. ING1 depends on NLS for entering the nucleus (36), and ING2 enters the nucleus via the combination of PHD and phosphatidylinositol 5-phosphate, a phospholipid molecule induced by DNA damage (35). Unlike ING2 PHD, ING5 PHD cannot bind to the phospholipid for activation (35), and thus ING5 cannot enter the nucleus in the same way as ING2. Analysis at http://expasy.org/ has revealed the existence of potential sites for acetylation and phosphorylation in ING5. However, we did not find any detectable acetylation and/or phosphorylation of ING5 itself after DNA damage by using general or site-specific acetyl- or phospho-antibodies (data not shown). Thus, it is plausible that ING5 enters the nucleus through NLS. However, this hypothesis awaits confirmation.

In summary, in response to DNA damage, ING5 is required for Tip60-modified, but not hMOF-modified, acetylation of p53 at a specific site K120.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: N. Liu, H. Lu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Liu, J. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Liu, J. Wang, J. Wang, Y. Yu, H. Lu
Writing, review, and/or revision of the manuscript: N. Liu, J. Wang, Y. Yu, H. Lu
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