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The Notch Regulator MAML1 Interacts with p53 and Functions as a Coactivator*

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Members of the evolutionarily conserved Mastermind (MAM) protein family, including the three related mammalian Mastermind-like (MAML) proteins MAML1–3, function as crucial coactivators of Notch-mediated transcriptional activation. Given the recent evidence of cross-talk between the p53 and Notch signal transduction pathways, we have investigated whether MAML1 may also be a transcriptional coactivator of p53. Indeed, we show here that MAML1 is able to interact with p53. We show that MAML1-p53 interaction involves the N-terminal region of MAML1 and the DNA-binding domain of p53, and we use a chromatin immunoprecipitation assay to show that MAML1 is part of the activator complex that binds to native p53-response elements within the promoter of the p53 target genes. Overexpression of wild-type MAML1 as well as a mutant, defective in Notch signaling, enhanced the p53-dependent gene induction in mammalian cells, whereas MAML1 knockdown reduced the p53-dependent gene expression. MAML1 increases the half-life of p53 protein and enhances its phosphorylation/acetylation upon DNA damage of cells. Finally, RNA interference-mediated knockdown of the single *Caenorhabditis elegans* MAML homolog, Lag-3, led to substantial abrogation of p53-mediated germ-cell apoptotic response to DNA damage and markedly reduced the expression of *Ced-13* and *Egl-1*, downstream pro-apoptotic targets of the *C. elegans* p53 homolog Cep-1. Thus, we present evidence for a novel coactivator function of MAML1 for p53, independent of its function as a coactivator of Notch signaling pathway.

The tumor suppressor protein p53 plays a crucial role in coordinating cellular responses to genotoxic and other stresses, hence its designation as the "guardian of the genome" (1). At a biochemical level, p53 protein functions as a sequence-specific transcriptional activator to coordinate the expression of a variety of cell cycle, apoptosis, and senescence-associated genes during cellular stress (2, 3). The p53-regulated genes determine the alternative cellular fates upon DNA damage and other stress stimuli. A key mechanism to tightly control p53-dependent transcriptional responses is the strict control of p53 protein levels and its activity by post-translational modifications, including ubiquitination, acetylation, phosphorylation, sumoylation, and methylation (4, 5).

In addition to modifications that result in an increase in p53 levels and activation of its specific DNA binding, recruitment of transcriptional coactivators to target gene promoter-associated p53 plays an essential role in p53-mediated transcriptional responses (6, 7). Coactivators typically facilitate transcription by bridging transcription factors to basal transcription machinery as well as to chromatin-modifying complexes, in particular the histone acetyltransferase machinery (8). Indeed, coactivators with histone acetyltransferase activity, such as p300, CBP,[6] and PCAF, have been demonstrated to interact with and regulate p53-dependent transcription (7–9). Recent studies have identified additional p53 coactivators, such as ADA3, that stabilize p53 and apparently recruit the ADA complex-associated histone acetyltransferase activities of GCN5 and PCAF (6, 10). In turn, well documented p53 coactivators, such as p300/CBP, also interact and functionally regulate other transcription factors. For example, p300 plays an important role in regulating Notch-dependent transcriptional activation (11, 12). Given the fundamental importance of elucidating biochemical mechanisms of p53-mediated transcriptional responses, the potential role of novel coactivators of p53 is of substantial interest.

In this study, we have investigated the potential role of MAML1 as a p53 coactivator. MAML1 is a member of the *Dro-*

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[6] The abbreviations used are: CBP, CREB-binding protein; GST, glutathione *S*-transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; RNAi, RNA interference; cRNA, control RNAi; Gy, gray; FACS, fluorescence-activated cell sorter; siRNA, short interfering RNA; GFP, green fluorescent protein; RT, reverse transcription.

sophila Mastermind-like (MAML) gene family (MAML1–3) that functions as coactivators for Notch-dependent transcription (13). Although mammals express three distinct MAML genes (*MAML1–3*) (14, 15), a single gene is found in *Drosophila* (mastermind) and *Caenorhabditis elegans* (*Lag-3/Sel-8*), the latter coding for two alternatively spliced transcripts (16, 17). The role of mastermind and MAML proteins in canonical Notch signaling has been documented in substantial detail. Interaction of a Notch ligand (of which there are five in mammals, including Jagged-1, Jagged-2, and Delta-1/2/3) with a Notch receptor (four in mammals, Notch 1–4) leads to a 2-step proteolytic cleavage of Notch receptors, first by an ADAM protease and subsequently by γ-secretase (12). The latter cleaves within the transmembrane region, thereby releasing the active intracellular domain of Notch (ICN). The ICN then translocates to the nucleus where it binds to the CSL (CBF1/RBP-Jκ in mammals, Su(H) in *Drosophila*, and Lag-1 in *C. elegans*) family of DNA-binding transcription factors (18). MAML proteins form a ternary complex with CBF1-ICN (19) via a direct interaction and play a critical role in transcriptional activation of Notch targets by recruiting p300/CBP, which in turn mediates chromatin remodeling via histone acetylation (11).

A number of observations suggested the possibility that MAML1 may function as a p53 coactivator. Although MAML proteins have been considered Notch-specific coactivators, recent mouse knock-out data demonstrated that MAML1 also functions as a coactivator of muscle differentiation-related transcription factor MEF2 independent of Notch; in fact, Notch and MEF2 function antagonistically in myoblast differentiation, yet MAML1 can play a coactivator role for both pathways (20, 21). Furthermore, the observations that MAML1 recruits p300 to Notch and that p300 is a known p53 coactivator (8, 18) raised the possibility that MAML1 may also function as a p53 coactivator. Importantly, recent findings have suggested a cross-talk between Notch and p53, as activation of Notch signaling was shown to inhibit p53-mediated apoptosis (22, 23). Furthermore, Notch activation in neural progenitor cells led to elevated levels of p53 and its targets, and p53-deficient cells showed a suppression of Notch-mediated apoptosis (23). Finally, similar to ADA3 (6), MAML1 was identified as a human papilloma virus E6-binding protein[7,8]; given the ability of ADA3 to function as a p53 coactivator, we considered the possibility that MAML1 may also play such a role aside from its known function as a Notch coactivator.

Here, we demonstrate that MAML1 interacts with p53 *in vitro* and *in vivo*, associates with p53 transcriptional complexes bound to native p53-reponsive promoters, and upon overexpression markedly enhances p53-mediated gene transcription as well as biological function. Overexpression of p53 inhibits MAM-induced CBF-luciferase activity, suggesting that p53 and CBF may compete for MAML1 under certain conditions. Complementary *in vivo* studies utilizing RNAi-mediated knockdown demonstrate that *Lag-3*, the sole MamL ortholog in *C. elegans*, demonstrates an important role in a p53 (Cep-1 in *C. elegans*)-mediated pathway that leads to apoptosis in the germ line (24). Taken together, our data demonstrate a novel and evolutionarily conserved role for the Notch coactivator MAML1 (and Lag-3) in p53-mediated transactivation and biological function.

EXPERIMENTAL PROCEDURES

Cells and Media—293T cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). MCF-7, U2OS, and Saos2 cell lines were grown in α-minimum Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum. These cell lines were obtained from the ATCC. 76N-derived immortal mammary epithelial cell lines 76N-TERT (p53-positive) and 76R-30 (p53-negative) were grown in DFCI medium (25).

Plasmid Constructs—Plasmids encoding FLAG-tagged full-length MAML1 (FLAG-MAML1) and truncated mutants 1–302 (FLAG-MAML1-(1–302)) been described earlier (13). FLAG-MAML-(742–1016) was derived by subcloning MAML1 sequences encoding amino acids 742–1016 from a pGAD10 construct isolated in a yeast two-hybrid interaction with HPV16 E6.[8] FLAG-MAML1 was further subcloned into pEF-HisB vector (Invitrogen) for mammalian expression using BamHI and DraI and into pet14b vector for bacterial expression using a BamHI site. Retroviral constructs of MAML1 and its mutant 742–1016 were derived by subcloning the corresponding fragments from PEF-His constructs into the pMSCVpuro vector (Invitrogen). GST-MAM-CT was constructed by cloning the 742–1016 fragment from the original pGAD10 clone into pGEX-4T1 vector (Roche Applied Science). Wild-type p53 and its truncation mutants were derived by subcloning cDNA inserts from pCMV-p53 (obtained from Bert Vogelstein, The Johns Hopkins Medical Center) into PCR3.1 plasmid. Additional truncation mutants of p53 containing residues 1–101, 102–292, and 292–393 were generated by the PCR cloning using the primer sets caagaattcatggaggagccgcagtcagatcctagcgtc and cggctcgagtcatttctgggaagggacagaagatgac, cgagaattcatgacctaccagggcagctacggtttc and cggctgcaggcggccgctcatttcttgcggagattctcttcctc, and cgagaattcatgggggagcctcaccacgagctgcc and cggctcgagtcagtctgagtcaggcccttctgtcttgaac, respectively. Luciferase reporter plasmids containing the p53-responsive elements of Bax, Gadd45, and p21 were obtained from Dr. Carol Prives (Columbia University, New York) and the pG13 reporter originally obtained from Dr. Bert Vogelstein (The Johns Hopkins School of Medicine, Baltimore), was provided by Dr. Wafik El-Deiry (University of Pennsylvania).

In Vitro Binding Assays—^{35}S-Labeled p53 or its truncation mutants were generated using [^{35}S]methionine and the TnT T7-coupled rabbit reticulocyte system (Promega). *In vitro* translated proteins were treated with 1 unit of DNase I for 30 min prior to the binding reaction. One μg each of GST or GST-MAML1, noncovalently bound to glutathione beads in 500 μl of lysis buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40), were incubated with the *in vitro* translated proteins for 1 h at 4 °C and washed five times with lysis buffer. The ^{35}S-labeled p53 or its truncation mutants bound to GST fusion products were analyzed by SDS-PAGE and subsequent autoradiography. Binding reactions using cell lysates were carried out

[7] L. Wu and J. D. Griffin, manuscript in preparation.
[8] I. Bhat, L. Delmolino, Y. Zhang, Y. Zhao, Q. Gao, D. E. Wazer, H. Band, and V. Band, manuscript in preparation.

with 1 μg of His-MAML1 immobilized on His-bind resin (Novagen), or His-Bind resin alone, in 200 μl of binding buffer (100 mM Tris, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 30 mM imidazole) for 5 min at 4 °C and washed six times with binding buffer. Bound proteins were resolved by SDS-PAGE and visualized by fluorography or analyzed by immunoblotting followed by ECL (reagents from Amersham Biosciences).

In Vivo Association between MAML1 and p53—For coimmunoprecipitation analyses, 5×10^5 U2OS cells expressing endogenous wild-type p53 were transfected with 5 μg of FLAG-MAML1 or vector, using the FuGENE 6 reagent, according to the supplier's instructions (Roche Applied Science). 48 h later, the cells were harvested in lysis buffer, and 1-mg aliquots of cell lysate protein were pre-cleared with protein G-agarose, subjected to immunoprecipitation with an anti-p53 antibody (ab-1, Oncogene Research), and immunoblotted for MAML1 using an anti-FLAG antibody (M2, Sigma) followed by ECL detection. For His tag pulldown analyses, 5×10^5 293T cells were transfected with 5 μg of His-MAML1 and 1 μg of FLAG-p53 using the calcium phosphate method. 48 h post-transfection, the cells were lysed in the binding buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 0.5% Nonidet P-40), and 400 μg of protein was incubated with His-Bind resin (Novagen) for 5 min at 4 °C. Beads were washed six times with binding buffer, and bound proteins were subjected to immunoblotting with anti-FLAG and anti-His (Santa Cruz Biotechnology) antibodies to detect p53 and MAML1, respectively.

To demonstrate the interaction of endogenous p53 with MAML1, U2OS cells were treated with 10 ng/ml leptomycin B (Sigma) or an equal volume of 70% methanol (as control) for 4 h, and the cells were lysed in NETN buffer (100 mM Tris, pH 8.0; 100 mM NaCl; 0.5% Nonidet P-40; 1 mM phenylmethylsulfonyl fluoride). Cell lysates were pre-cleared with protein-G beads, and immunoprecipitation from aliquots was carried out with a mixture of six anti-p53 monoclonal antibodies (p53 (DO-1), p53 (pAb1801), p53 (pAb 240) from Santa Cruz Biotechnology; p53 (polyclonal antibody 421) from Calbiochem; p53 (pAb122) from NeoMarkers, or normal mouse IgG (negative control)). p53-associated MamL1 was analyzed by immunoblotting with an anti-MamL1 monoclonal antibody and visualized using ECL.

Transactivation Assays—For the p53-mediated transactivation, 5×10^5 Saos2 (a p53-negative osteosarcoma cell line) or MCF-7 cells per 100-mm dish or 2×10^5 per 60-mm dish were transfected with the indicated luciferase reporters, MAML1, p53, as indicated in figure legends. Each dish also received 20 ng of SV40-*Renilla* luciferase reporter (pRL-SV40) to normalize for differences in transfection. Total DNA was kept constant by addition of vector DNA. Luciferase activity was measured 24 h later, using a dual-luciferase kit (Promega, WI). For the ICN-mediated transactivation, 1×10^5 cells/well of a 6-well plate were transfected with various constructs (100 ng of CBF1 reporter, 10 ng each of ICN1 and SV40 *Renilla* reporter, and different doses of p53 and MamL1, as indicated in the figures legends) using the FuGENE 6 reagent.

Chromatin Immunoprecipitation (ChIP) Assay to Detect the Native p21, Bax, and GADD45 Promoters-bound MamL1 and p53—Subconfluent MCF-7, a wild-type p53-containing cell line, was used for ChIP analysis. The native protein-DNA complexes were cross-linked by treatment with 1% formaldehyde for 15 min. The ChIP assay was carried out as reported earlier (26). Briefly, equal aliquots of isolated chromatin were subjected to immunoprecipitation with an anti-p53 (DO1; Santa Cruz Biotechnology) anti-MamL1 (13), anti-polymerase II, or IgG monoclonal antibodies. DNA associated with immunoprecipitates was used as a template for PCR to amplify different p53 target promoters containing the p53-binding site. The primers used were as follows: p21, 5′ primer 5′-TCCATCCCTATGCTGCCTGCT and 3′ primer 5′-CAAGGATCCTGCTGGCAGATC; GADD45, 5′ primer 5′-GGATCTGTGGTAGGTGAGGGTCAGG and 3′ primer 5′-GGAATTAGTCACGGGAGGCAGTGCAG; and Bax, 5′ primer 5′-GATTGGGCCACTGCACTCCAG and 3′ primer 5′-TGACTAAAAACTGAGTGG. As a specificity control, the GAPDH promoter was amplified from the same templates using the following primers: 5′ primer 5′-AAAAGCGGGGAGAAAGTAGG and 3′ primer 5′-CTAGCCTCCCGGGTTTCTCT.

Antibodies—Monoclonal antibodies against p53 (DO1), p21, and GFP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-acetylated p53 antibody was obtained from Upstate (Temecula, CA); anti-FLAG antibodies were from Sigma; β-actin antibodies were from Abcam Inc.(Cambridge, MA), and anti-phospho-p53 (Ser-15) was from Cell Signaling Technology (Boston).

Transient Transfection and Western Blotting—For transfection experiments, Saos-2 cells were plated in100-mm dishes and transfected with the indicated expression constructs (see figure legends) using FuGENE 6 (Roche Applied Science). The cells were lysed 40 h post-transfection in lysis buffer (50 mM Tris-Cl, 400 mM NaCl, 0.2% Nonidet P-40, 10% glycerol) and protease inhibitors from Roche Applied Science, and equivalent amounts of whole cell extracts were processed for SDS-PAGE and subjected to immunoblotting with the appropriate antibodies.

RNA Extraction and RT-PCR—The total RNA was extracted using TRIzol reagent (Invitrogen). The semiquantitative RT-PCR was performed with 200 ng of total RNA using the following primers: (*a*) p53, forward primer 5′-CAGCCAAGTCTGTGACTTGCACGTAC-3′ and reverse primer 5′-CTATGTCGAAAAGTGTTTCTGTCATC-3′; (*b*) p21, forward primer 5′-GTGAGCGATGGAACTTCGACTT-3′ and reverse primer 5′-GGCGTTTGGAGTGGTAGAAATC-3′; (*c*) GAPDH forward primer 5′-ACCTGACCTGCCGTCTAGAA-3′ and reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′; (*d*) MamL1 forward primer 5′-CAGCATCAGTTGCTTTTGGA-3′ and reverse primer 5′-CTGCTCTGAGGCATGTTTTG-3′. The RT-PCR products were visualized under UV light after running on ethidium bromide-stained 2% agarose gel.

Analysis of the p53 Protein Turnover—100-mm dishes of Saos-2 cells were transfected with plasmids expressing p53 alone or in combination with MamL1. At 16 h post-transfection cells were trypsinized, and equal numbers of cells were replated on five 6-well plates. After 16 h, 100 μg/ml cycloheximide (Sigma) was added to the medium, and cells were harvested at the indicated time points. Total cell extracts were prepared, and equivalent amounts were run on SDS-PAGE and analyzed by Western blotting. Densitometry analysis was carried out on

scanned images using Scion Image for Windows software (Scion Corp., Frederick, MD).

Apoptosis Assay (FACS Analysis of Pre-G_1 Cells)—76NTERT cells (containing wild-type p53) and 76R30 cells (containing mutant p53) were infected with retroviruses expressing full-length MAM and various fragments of MAML1 for 48 h and selected in 100 μg/ml G418 for 48 h. The cells were then plated into 100-mm dishes for Western blotting and in 6-well plates for fluorescence-activated cell sorting (FACS) analysis. 48 h later cells were treated with adriamycin (0.25 μg/ml) for 18 h. Cells were then harvested and resuspended in 1 ml of phosphate-buffered saline containing 5 mM EDTA, and ice-cold 100% ethanol was gently added to make the final ethanol concentration to 50%. The cell solution was incubated at room temperature for 30 min in the dark. The cells were spun down and resuspended in 0.5 ml of RNase solution (40 μg/ml), and the tubes were vortexed and incubated at room temperature for 30 min. 0.5 ml of diluted propidium iodide solution (final concentration 0.1 mg/ml) was added to each tube, and the samples were analyzed for the pre-G_1 phase of the cell cycle.

Construction of RNAi against p53 and MAML1—To construct a retrovirus-based vector expressing MamL1 RNAi, we used Oligoengine software and used the sequence gaggaatcttgacagcgcc (336–354) for MAML1 RNAi 1 and sequence gcagcctttgagcaagaac (913–931) for MamL1 RNAi 2 to synthesize complementary strands of a 64-bp oligonucleotide with BglII cohesive site at the 5′ end and HindIII cohesive site at the 3′ end. Cloning was done into the retroviral vector pSuper Retro-Neo (pSRNeo) from Oligoengine (Seattle, WA). RNAi against p53 was designed according to the literature (27) and cloned into pSRNeo BglII and HindIII sites.

Duplex RNAi oligonucleotides were also utilized in our experiments. The RNA oligonucleotides used to generate 21-nucleotide siRNA duplexes were synthesized through Dharmacon, corresponding to the human MAML1 sequences in open reading frame siRNA 1 (nucleotides 336–354) (5′-GAGGAATCTTGACAGCGCC-3′), siRNA 2 (nucleotides 913–931) (5′-GCAGCCTTTGAGCAAGAAC), and control siRNA (5′-CCAAGATCATCACCGCCTG-3′).

C. elegans Strains and Culture Conditions—*C. elegans* worms were cultured at 22 °C, unless otherwise indicated, under standard growth conditions (28). Strains used in this study were Bristol strain N2 (wild-type) and dpy-20 (el282); grls50 expresses a GFP-tagged Lag-3 and was kindly provided by Iva Greenwald (see Ref. 17).

C. elegans Feeding RNAi—The RNAi construct used to target Lag-3 was constructed by cloning into the BamHI and HindIII sites of plasmid L4440(2xT7) PCR amplified Lag-3 cDNA. cDNA was synthesized from total RNA from N2 worms using One-step PCR (Invitrogen) and the following primers. The forward primer sequence was 5′-CGCGGATCCATGAAACCGTCGACA and the reverse primer was 5′-CGGGGTACCTCAATTGATATTAGC. Feeding RNAi experiments were performed under the same conditions as those described by Timmons *et al.* (29). Following overnight egg laying at 22 °C by gravid hermaphrodites, the plates were kept at 15 °C.

C. elegans Germ Line Apoptosis Assay—Germ line apoptosis assays were performed similarly to previously described experiments (24). Briefly, synchronized L4 stage worms fed with the indicated RNAi plasmid transformed bacteria were either left untreated or were subjected to 120 Gy of γ-irradiation using a ^{137}Cs source. Following irradiation, the animals were maintained at 22 °C for 21 h at which time the worms were collected for staining with 33 μM SYTO 12 (Invitrogen) for 2 h. The worms were then washed with M9 (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85.5 mM NaCl, 1 mM $MgSO_4$) buffer and then transferred to seeded plates to allow for clearing of SYTO 12-stained bacteria from the guts of the worms for 45 min. The worms were then mounted onto coverslips with agar pads to be observed with both Normarski optics and fluorescence microscopy. SYTO 12-stained cells were counted and averaged from about 15–25 animals per condition.

Real Time RT PCR—Worms were treated with γ-irradiation as indicated above. Twenty four hours after irradiation, adult worms were picked from the plates, and total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized using random primers and Moloney murine leukemia virus reverse transcriptase (Stratagene). Real time PCR was performed using custom TaqMan gene expression assays (Applied Biosystems) directed against Ced-13, *Egl-1*, and *C. elegans* actin as an internal control. The assays were performed using Mx3000P QPCR machine (Stratagene, TX). Expression of Ced-13 and egl-1 was normalized to actin expression, and fold induction was calculated.

RESULTS

p53 and MAML1 Interact in Vitro—Given the apparent cross-talk between Notch and p53 and the regulation of multiple transcription factors by coactivators, we assessed if Notch coactivator MAML1 interacts with p53. To assess the interaction of p53 with MAML1 *in vitro*, ^{35}S-labeled p53 protein was translated *in vitro* using TnT system (Promega) and incubated with bacterially expressed GST alone (a negative control) or GST fusion proteins of full-length MAML1, MAML1 fragments, and the tetramerization domain of p53 (a positive control). Although GST alone did not show any interaction with p53 (Fig. 1, *lane 2*), a clear interaction was observed with full-length MAML1 (*lane 3*). Furthermore, although no binding was observed with the MAML1 fragments 302–806 or 742–1016, the N-terminal fragment of MAML1 (MAML1-(1–302)) showed a clear binding to the *in vitro* translated p53 protein (Fig. 1*A*). The *bottom panels* of Fig. 1*A* show that comparable amounts of GST or the fusion proteins were used in the binding reactions. These results demonstrated that MAML1 specifically interacts with p53 and that the interaction is mediated by the N-terminal region of MAML1.

To further characterize the interaction of MAML1 with p53 and to identify the p53 domain that interacts with MAML1, *in vitro* binding experiments were performed with *in vitro*-translated ^{35}S-labeled p53 protein or its fragments and the bacterially expressed GST-MAML1 or GST protein alone. As expected, GST alone showed no binding (Fig. 1*B*), whereas clear binding was seen between the full-length p53 and GST-MAML1 (Fig. 1*B*). Notably, the N terminus (amino acids 1–101) or the C terminus (tetramerization and regulatory domains) of p53 showed no binding to MamL1. In contrast, the DNA binding domain of p53

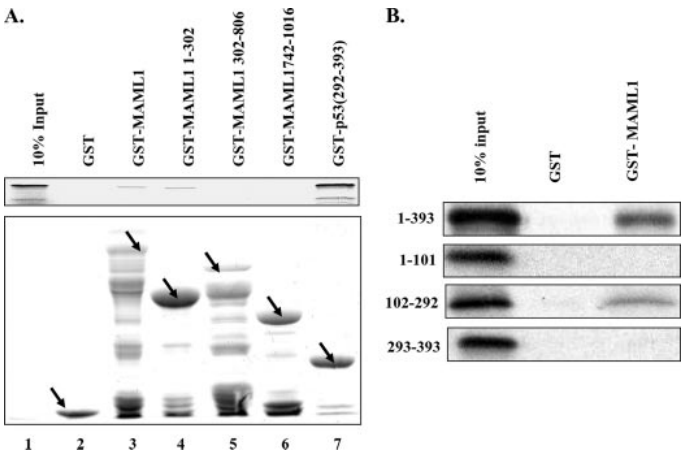


FIGURE 1. **MAML1 and p53 interact *in vitro* and *in vivo*.** *A,* N-terminal residues 1–302 of MAML1 interact with p53. The indicated ^{35}S-labeled proteins were generated from their pCR3.1 expression constructs by using lysate-based coupled *in vitro* transcription-translation system in the presence of [^{35}S]methionine. Aliquots of ^{35}S-labeled proteins were incubated with 1 μg of GST or indicated GST fusion proteins (GST-MAML1, and mutants and GST-p53-(293–393)) noncovalently bound to glutathione beads and bound proteins were resolved by SDS-PAGE and visualized by fluorography. Coomassie Blue staining of GST fusion proteins is shown. *B,* MAML1 binds to DNA binding domain (102–292) of p53. Binding reaction included [^{35}S]methionine-labeled *in vitro*-translated p53 and its truncated mutants and GST-MAML1 or equivalent GST protein. Bound p53 mutants were detected as above.

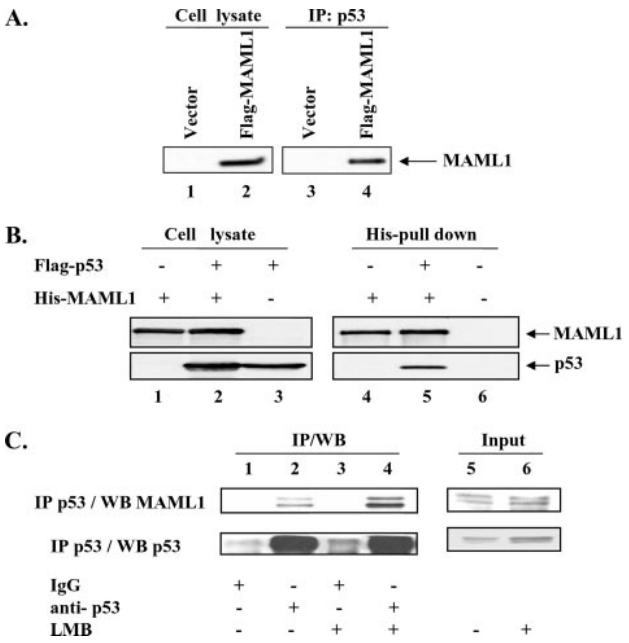


FIGURE 2. *A,* overexpressed MAML1 associates with endogenous p53. 5×10^5 U2OS cells (contain wild-type p53) were transfected with 4 μg of pCR3.1 vector or FLAG-MAML1, using FuGENE reagent. 48 h later, 1-mg aliquots of cell lysate proteins were subjected to anti-p53 immunoprecipitation (*IP*) and immunoblotted with an anti-FLAG antibody. Whole cell lysates (50 μg) were directly blotted with anti-FLAG antibody to assess the expression of FLAG-MAML1. *B,* overexpressed MAML1 and p53 associate in mammalian cells. 293T cells were transfected with His-MAML1 or FLAG-p53 alone or together using FuGENE reagent. After 48 h, 400 μg of protein from each lysate was incubated with His Bind Beads for 5 min followed by five washes with lysis buffer. Proteins were resolved by SDS-PAGE and immunoblotted with anti-FLAG and anti-His antibodies. *C,* endogenous MAML1 binds to endogenous p53. Logarithmically growing U2OS cells were treated with either leptomycin B (*LMB*) or solvent for 4 h, harvested and lysed in NETN buffer, followed by immunoblotting with either normal mouse IgG or anti-p53 antibody mixture. Antigen-antibody complexes were resolved on 8% SDS-PAGE, transferred to polyvinylidene difluoride membrane, blotted with either MamL1 monoclonal antibody or p53 polyclonal antibody, and visualized with chemiluminescence. *WB,* Western blot.

(amino acids 102–292; Fig. 1*B*) showed binding to MAML1. Together, the *in vitro* binding results are consistent with specific binding between MAML1 to p53 via an interaction between the N-terminal region of MAML1 and the DNA binding domain of p53. The stickiness of the DNA binding region of p53 was ruled out by performing the reactions after DNase I digestion of the *in vitro* translated proteins.

p53 Associates with MAML1 in Vivo—Given the *in vitro* interaction between MAML1 and p53, we examined whether these proteins associate *in vivo* using two different cell types. First, p53-positive U2OS osteosarcoma cells were transfected with empty vector or with FLAG-tagged MAML1. Cell lysates were immunoprecipitated with p53 antibody followed by immunoblotting with an anti-FLAG antibody to detect p53-associated MAML1. As expected, no protein was detected in vector-transfected cells (Fig. 2*A*, *lane 3*), whereas MAML1 was clearly coimmunoprecipitated with endogenous p53 in MAML1-transfected U2OS cells (Fig. 2*A, lane 4*).

In a second experiment, we cotransfected His-tagged MAML1 and FLAG-tagged p53 into 293T cells, affinity-isolated MAML1 on His-Bind resin, and assessed the presence of associated FLAG-p53 using anti-FLAG immunoblotting. As with the endogenous p53 (above), a clear association was observed between the exogenously expressed p53 and MAML1 (Fig. 2*B*, *lane 5, lower right panel*). An immunoblot of the whole cell lysates showed the expected expression of the transfected proteins (Fig. 2*B*, *left upper* and *lower panels*). These results clearly demonstrate that MAML1 and p53 can associate *in vivo*.

Endogenous MAML1 and p53 Associate with Each Other—Because the overexpressed MAML1 interacted with both the overexpressed (Fig. 2*B*) and the endogenous p53 (Fig. 2*A*), we set out to examine if the endogenous MAML1 and p53 proteins associate with each other. U2OS cells were treated with the nuclear export inhibitor leptomycin B, and cell lysates were subjected to immunoprecipitation with a control IgG or anti-p53 antibodies (using a mixture of anti-p53 monoclonal antibodies) followed by an immunoblot with an anti-MAML1 monoclonal antibody. No MAML1 signal was seen in control IgG lane (Fig. 2*C*, *lane 1*), whereas a clear MAML1 band was observed in anti-p53 immunoprecipitation lane (*lane 2*). As anticipated, treatment with leptomycin B enhanced the association between p53 and MAML1 (Fig. 2*C*, *lane 4*). The immunoblotting of input lanes and anti-p53 immunoblotting of immunoprecipitation lanes demonstrated the expected proteins bands in appropriate lanes. These results demonstrate that endogenously expressed p53 and MAML1 proteins form a complex.

MAML1 Is Present in Activator Complexes Bound to the Native Promoter of the p53 Target Genes p21, Bax, and GADD45—Given the *in vitro* and *in vivo* MAML1-p53 interaction and the known role of MAML1 as a Notch coactivator, we asked if MAML1 functions as a p53 coactivator. An essential property of coactivators is their ability to assemble into transcriptional activator complexes on specific promoters where transcription factors bind in a DNA sequence-specific manner (30–32). To directly assess if MAML1 is assembled into transcriptional activator complexes at native p53 target promoters, we performed ChIP analysis of the *p21*, *Bax*, or *GADD45* pro-

moter regions that harbor only the p53-binding site. The ChIP analysis was carried out using chromatin isolated from MCF-7 cells that endogenously express a wild-type p53 protein. The chromatin immunoprecipitations were carried out using IgG (negative control), anti-MAML1, and anti-p53 (positive control) and anti-polymerase II (positive control) antibodies. The ChIP-associated DNA was used as a template for the PCR to specifically amplify the p53-binding regions of the p21, Bax, or GADD45 promoters. As shown in Fig. 3, a clear PCR product was observed in the anti-MAML1 but not in anti-hemagglutinin ChIP (compare *lane 2* with *lane 4*), with an expected PCR product seen in anti-p53 antibody ChIP (*lane 2*). Amplification of non-p53 target promoter GAPDH demonstrated the specificity of the results. The results of the ChIP analyses clearly demonstrate that MAML1 is a part of protein complex bound to the p53-binding site in the *p21*, *Bax*, or *GADD45* promoters in its native chromatin configuration.

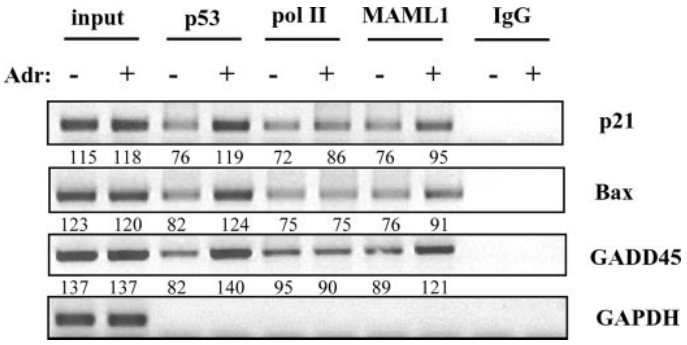


FIGURE 3. **MamL1 binds to p21, Bax, and Gadd45 promoters, targets of p53 protein.** Soluble chromatin was prepared from MCF-7 cells treated with adriamycin (*Adr*) (0.5 μg/ml) and immunoprecipitated with anti-p53, anti-MamL1, anti-RNA polymerase II, or IgG monoclonal antibodies. The final DNA extractions were amplified using pairs of primers that cover the region of p21, Bax, and Gadd45 promoters containing p53-binding sites. PCR for the region of GAPDH promoter was used as a negative control.

MAML1 Enhances the p53-dependent Transactivation of p53-responsive Luciferase Reporter—In view of the p53-MamL1 association together with the ChIP results demonstrating the presence of MAML1 in p21 promoter-bound coactivator complexes, we examined if MAML1 influences p53-mediated transactivation. For this purpose, p53-negative Saos-2 cells were cotransfected with a fixed amount of p53 and increasing amounts of MAML1, together with the Bax gene promoter-luciferase reporter, and the luciferase activity was measured. As expected, low luciferase activity was observed in vector-transfected cells, with a 2-fold increase in Bax-luciferase activity upon cotransfection with p53 under the experimental conditions (Fig. 4*A*, *lane 5*). Significantly, cotransfection of MAML1 resulted in a dose-dependent increase in the luciferase activity (Fig. 4*A*, *lanes 6–8*). No activity was observed when MAML1 was transfected without p53 (Fig. 4*A*, *lanes 2–4*) demonstrating that the MAML1-induced increase in transactivation is p53-dependent. No transactivation of MG_{15}-luciferase (containing a mutated p53-binding consensus and used as a negative control) (33) was seen upon transfection with p53 or p53 together with MAML1 (data not shown). Notably, Western blot analysis of cell lysates used in the luciferase assay showed a MAML1 dose-dependent increase in p53 protein levels (Fig. 4*A*, *lower panel*). These data suggest that similar to other coactivators, such as p300 (7–9) and hADA3 (6, 10), MAML1 coactivator function may also involve stabilization of the p53 protein.

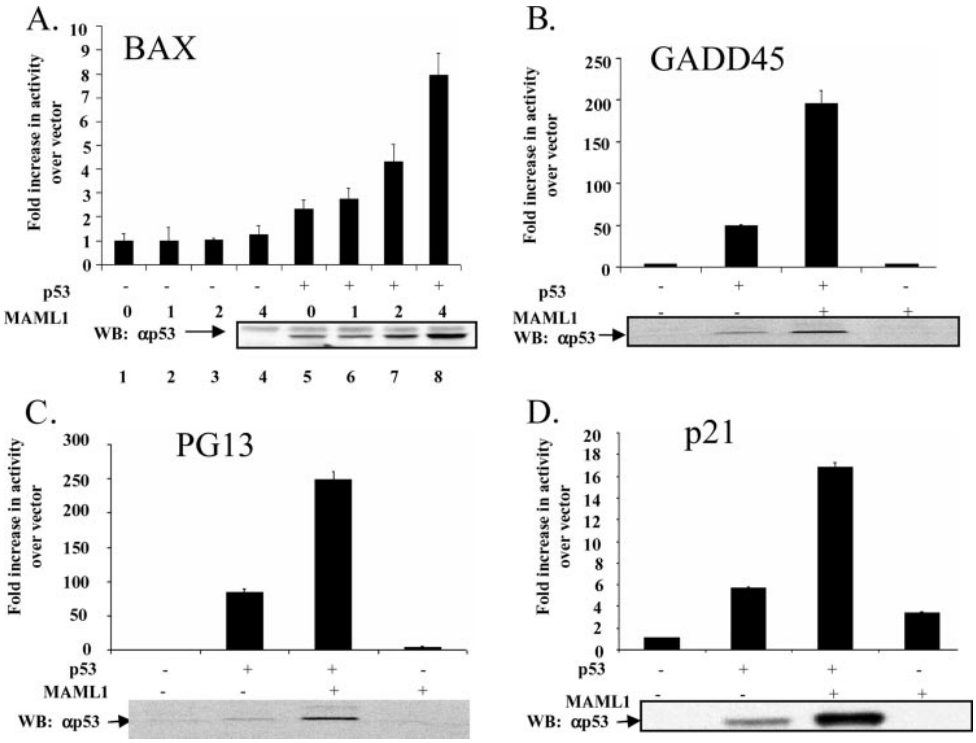


FIGURE 4. **Enhancement of p53-mediated transactivation by MAML1.** *A,* MAML1 increases p53-mediated Bax transactivation in a dose-dependent manner. Saos-2 cells were cotransfected with 50 ng of p53 and 1, 2, or 4 μg of MAML1, together with 1 μg of Bax-luciferase (the promoter of p53-responsive gene cloned upstream of luciferase) and 20 ng of SV40-driven *Renilla* luciferase (as a transfection control). The cells were lysed, and the luciferase activity was measured. Results are shown as fold activity over vector alone after normalizing for transfection efficiency against *Renilla* luciferase activity. The lysates from the cells used in the luciferase assays were analyzed for p53 levels by immunoblot (*WB*) (*lower panels*). *B–D, MAML1* increases p53-mediated transactivation of its target genes, *Gadd45* (*B*), *Pg13* (*C*), or p21 (*D*) in a dose-dependent manner. Similar experiments, as above, were performed using luciferase reporters linked to promoters of p53 target genes *Gadd45* (1 μg), *p21* (1 μg), and pG_{13}-luciferase (0.1 μg) (a p53-responsive reporter containing 13 repeats of a consensus p53-binding site upstream of firefly luciferase), 10 ng of p53, and/or *MAML1*. Results are shown as above.

We further confirmed the ability of MAML1 to enhance the p53-mediated transactivation using luciferase reporters linked to promoters of several additional p53 target genes, including *GADD45* and *p21*, as well as the pG_{13}-luciferase (a p53-responsive reporter containing 13 repeats of a consensus p53-binding site upstream of the firefly luciferase) (33). In each case, MAML1 enhanced the p53-mediated transactivation of the reporters, whereas little or no transactivation was observed in the absence of p53

cotransfection (Fig. 4*B*, *C* and *D*). Taken together, these results demonstrate that MAML1 functions as a coactivator for p53-dependent transactivation in mammalian cells.

Ectopic MamL1 Expression Increases the Stability of p53 Protein—Given the importance of the regulated p53 protein degradation in the cells, it appeared likely that MAML1-dependent increase in p53 levels may reflect a post-translational mechanism. However, we wanted to first rule out any influence of MAML1 expression on p53 mRNA levels. For this purpose, Saos-2 cells were transfected with p53 and Vector or p53 with MAML1 plasmid. Parallel samples were processed for Western blotting of protein lysates and RT-PCR analysis of p53 mRNA levels. Although MAML1 increases p53 protein levels, RT-PCR analysis showed that p53 mRNA levels remained unchanged similar to mRNA levels of the housekeeping gene GAPDH, used as a loading control (Fig. 5, *A* and *B*). In contrast, the increase in the level of p21 protein, a transcriptional target of p53, was accompanied by an expected MAML1-dependent increase in p21 mRNA levels (Fig. 5*B*). These observations indicated that the accumulation of p53 protein upon MAML1 overexpression is likely to be due to a post-transcriptional mechanism.

To investigate if the MAML1-dependent increase in p53 levels reflected an increase in the stability of p53 protein, we assessed the half-life of p53 protein following cycloheximide block of new protein synthesis. For this purpose, p53-null Saos-2 cells were transfected with p53 expression vector pCMV-p53 alone or together with MAML1 expression vector. The transfected cells were incubated in the presence of cycloheximide, and p53 levels were assessed by immunoblotting of cellular extracts prepared at various times after cycloheximide addition followed by densitometry to assess the half-life. As shown in Fig. 5*C*, the overexpression of MAML1 resulted in a slower loss of p53 signals compared with cells without MAML1 overexpression. The half-life of the p53 protein increased from 20 min in cells without MAML1 overexpression to 70 min in cells with MAML1 overexpression (Fig. 5*D*). This finding, together with lack of any effect on p53 mRNA levels, supports our conclusion that ectopic MAML1 overexpression increases p53 protein levels by promoting its stability.

MAML1 Overexpression Enhances the Level of p53 Phosphorylation and Acetylation—Stability of p53 is controlled by post-translational modifications with ubiquitination signaling protein degradation and a number of other modifications such as acetylation and phosphorylation. We hypothesized that MAML1 may stabilize p53 by promoting its phosphorylation or acetylation. To test this possibility, Saos-2 cells were transfected with p53 alone or cotransfected with MAML1. 16 h after transfection, each transfected plate was split into two plates. One plate in each set was used as a control, whereas the other plates were exposed to adriamycin for 2, 4, and 8 h to induce DNA damage, and cell lysates were analyzed for total, phosphorylated, and acetylated p53 levels using immunoblotting.

As expected, total as well as phosphorylated and acetylated p53 levels increased (about 2-fold) upon adriamycin treatment (Fig. 6, *A–C*). Notably, acetylated p53 levels were higher in cells overexpressing MAML1, and these levels increased further upon adriamycin treatment; the MAML1-dependent increase

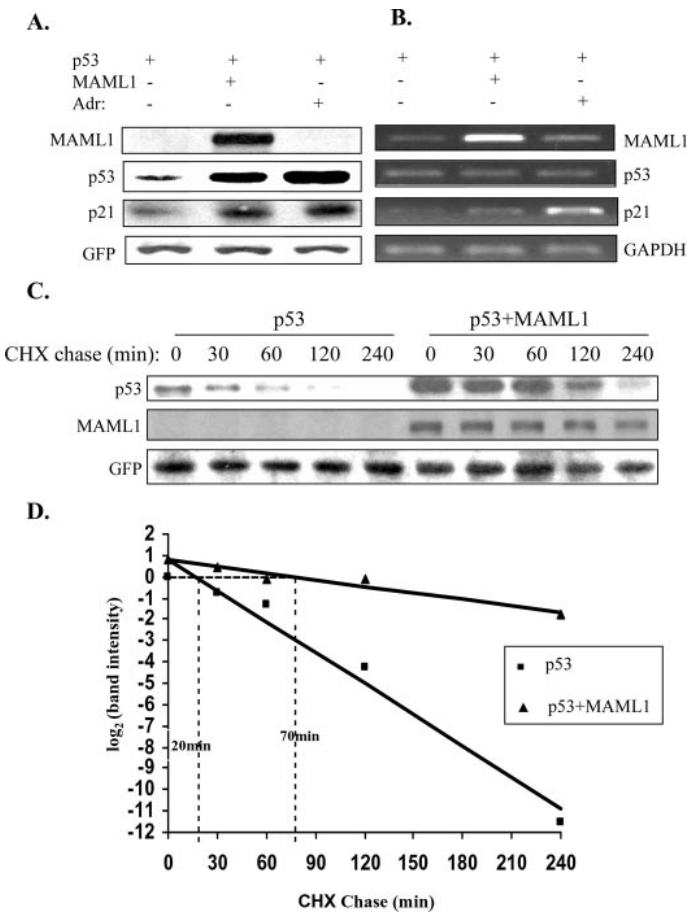


FIGURE 5. **MamL1 stabilizes p53 protein at post-transcriptional level.** Saos-2 cells were transiently transfected with either pCMV-vector or pCMV-p53 (four 100-mm dishes) or pCMV-p53 along with MamL1 plasmid (two 100-mm dishes). After 24 h, cells were either mock-treated or treated with adriamycin (*Adr*) (0.5 μg/ml) for 16 h as indicated in the figure. Cells were then harvested for protein or mRNA analysis. Equal amounts of whole cell extracts were subjected to SDS-PAGE followed by Western blotting using anti-MamL1, anti-p53, or anti-β-actin antibodies (*A*). Total RNAs were analyzed by RT-PCR to detect the levels of p53, p21, MamL1, or GAPDH (*B*). *C*, MamL1 overexpression extends p53 half-life. Saos-2 cells were transfected with either pCMV-p53 and vector or pCMV-p53 along with MamL1. GFP was transfected in each set as a transfection control. Twenty four hours after transfection, each set of cells was replated into five equal parts. After 16 h of seeding, cells were treated with cycloheximide (*CHX*) (100 μg/ml) and harvested at the indicated time intervals. Cell lysates were analyzed by Western blotting using anti-p53, anti-MamL1, or anti-GFP antibodies. The intensity of p53 bands was quantified by densitometry against GFP using Scion Image software and plotted against time of cycloheximide treatment (*D*). Each decrease of 1 unit of $\log_2$ is equivalent to 1 half-life.

in phosphorylated and acetylated p53 paralleled the increase in total p53 levels (Fig. 6, *A–C*).

Overexpression of MAML1 Enhances the DNA Damage-induced p53-mediated Apoptosis—Given our results that MAML1 functions as a coactivator for p53-mediated transcription, we next asked whether overexpression of MAML1 is able to enhance the magnitude of p53-mediated biological responses. For this purpose, we transfected the vector, wild-type MAML1, or MAML1 fragment 742–1016 into 76N-TERT (contains wild-type p53) and 76R-30 cells (derived from the same parental cell but contains no p53 protein), and quantified the proportion of cells in sub-G_1 (apoptotic) fraction following adriamycin treatment using propidium iodide staining and FACS analysis. As depicted in Fig. 7, *A* and *B*, overexpression of

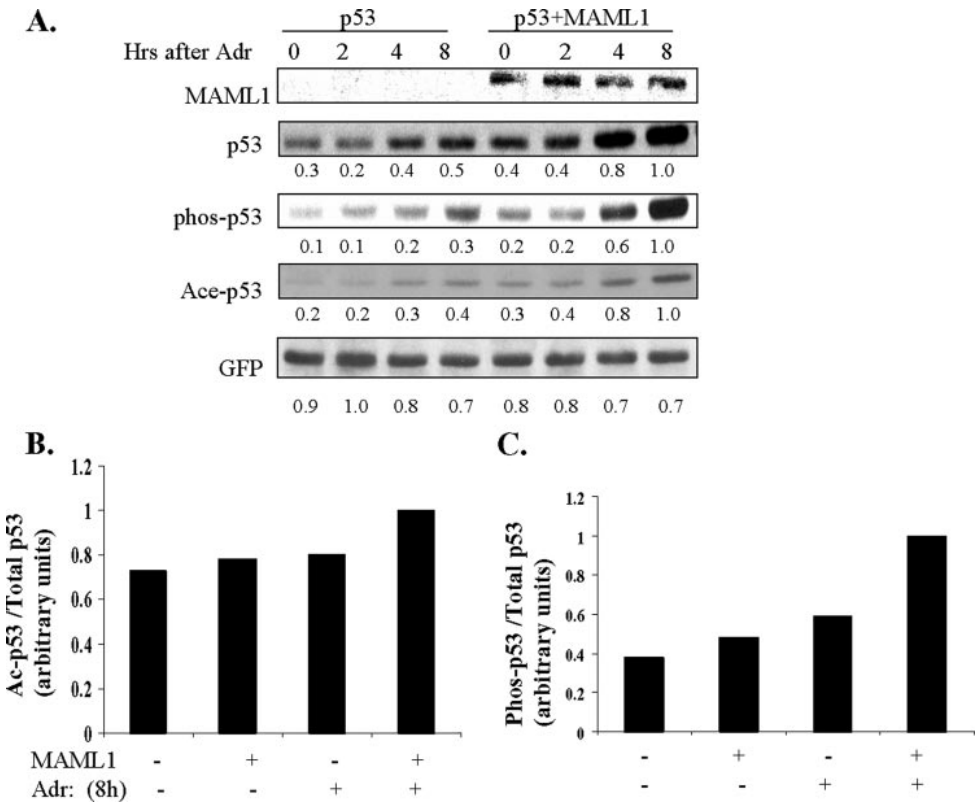


FIGURE 6. **MamL1 promotes p53 phosphorylation and acetylation.** *A,* Saos-2 cells were transfected with either p53 and vector or p53 together with MamL1. GFP was transfected in each set as a transfection control. Next day, each set of cells were split into four equal parts. After 24 h, cells were either treated with Me_2SO or with adriamycin (*Adr*) (0.5 μg/ml) for different time points as indicated in the figure. Cells were harvested, and equivalent amounts of cell lysates were subjected to Western blotting using indicated antibodies. The *numbers* below the acetylated p53 (*Ace-p53*), phosphorylated p53 (*phos-p53*), total p53, and GFP bands are the arbitrary values for relative intensity of the respective signals. *B,* intensity of the phosphorylated p53, acetylated p53, and the total p53 signals of 0 and 8 h adriamycin were quantitated and equalized against GFP using Scion image software. The figure shows the histogram plotted with the ratio of acetylated *versus* total p53 with or without MamL1 overexpression of either no treatment or 8-h treatment with adriamycin from *A*. *C,* phosphorylated p53 was plotted similar to *B*.

the full-length MAML1 but not the 742–1016 fragment in 76N-TERT cell line increased the proportion of apoptotic cells. Furthermore, MAML1 enhanced the degree of apoptosis to an even greater degree when the cells were treated with adriamycin (Fig. 7, *A* and *B*). The MAML1-dependent enhancement of apoptosis was p53-dependent as no apoptosis was observed under any conditions in 76R-30 cells lacking p53 protein expression (Fig. 7). In addition, MAML1 overexpression was associated with an increase in the level of the p53 target protein p21 (Fig. 7*B*). Similar results were obtained with two other mammary epithelial cell lines (76N and MCF-7 cells; data not shown). Similar to our results with overexpressed p53 (Fig. 4), we observed an increase in the levels of endogenous p53 protein upon MAML1 overexpression (*lanes 2* and *5*), consistent with p53 stabilization. Overall, our results demonstrate that MAML1 enhances the p53-mediated biological responses (apoptosis and p53 target p21 expression) in mammalian cells.

RNAi-mediated MAML1 Knockdown Decreases p53-mediated Transactivation—Next, we addressed if the endogenous MAML1 was required for the p53-mediated transcriptional response. For this purpose, we constructed two RNAi against MAML1 and tested their ability to decrease MAML1 protein by expressing RNAi in MCF-7 cells. A clear decrease in the levels of MAML1 protein was seen upon RNAi 1 expression and slight decrease in MAML1 was seen with RNAi 2 (Fig. 8*A*). As control p53 RNAi was expressed and showed a decrease in p53 protein levels in these cells (Fig. 8*A*). Next, cells expressing control RNAi or MAML1 RNAi 1 or p53 RNAi were transfected with p53 target genes (*pG13* and *GADD45*) reporter-luciferase or CBF1-luciferase reporter (as control) and analyzed for luciferase activity. Indeed, reduced pG_{13}- and GADD45-luciferase activity was seen in cells expressing both RNAi (Fig. 8, *B* and *C*). As control p53 RNAi also showed significant decrease in luciferase activity (Fig. 8, *B* and *C*). Furthermore, RNAi against MAML1 but not p53 showed dramatic decrease in ICN/MAML1-induced CBF-luciferase activity. These results support the idea that MAML1 plays a critical role in promoting the function of p53 as a coactivator.

Requirement of the C. elegans MAML1 Ortholog in p53-mediated Germ Cell Apoptosis as Demonstrated by RNAi Knockdown—Given the expression of three MamL family members in mammalian cells and the associated complexities, we used a simpler *C. elegans* system in which a single MamL1 ortholog, Lag-3/Sel-8, is expressed (16, 17) so that its biological role in p53-mediated functional responses could be assessed in an *in vivo* stetting. Lag-3, like other MAM proteins, is known to function as a coactivator of Notch signaling (Lin-12 and GLP-1 in *C. elegans*) (16, 17).

To assess the role of Lag-3 as a p53 coactivator in *C. elegans*, we examined the effect of knocking down Lag-3 expression by RNAi feeding on germ cell apoptosis. It is well documented that the *C. elegans* p53 ortholog, Cep-1, is required for apoptosis of germ line cells in the region of the hermaphrodite gonad where these cells complete the pachytene stage of meiosis, and DNA damage is known to induce p53-dependent apoptosis of these cells (34, 35). Given the lethality of *lag-3* knockdown because of its effects on Notch signaling (16, 17), we utilized a feeding RNAi protocol that allowed us to obtain viable F1 generation adult worms with a reduction in Lag-3 expression in tissues where Lag-3 is normally expressed, such as in the vulva and in developing embryos (Fig. 9*A*); as expected in these worms, Lag-3 expression in neuronal cells was not reduced. Accordingly, the majority of F2 generation eggs failed to hatch, and those that did had similar developmental defects as reported previously and were unable to develop to adulthood. Furthermore, as compared with the N2 wild-type worms, the age-1

worms exhibit an extended life span, and daf-16 worms exhibit a shortened life span (36, 37), and the Lag-3 RNAi F1 adults exhibited a normal life span (data not shown). Thus, we used the F1 generation Lag-3 RNAi to assess the role of Lag-3 in p53-mediated germ cell apoptosis.

The F1 generation N2 (wild type) worms grown on control RNAi (cRNAi) bacteria, Lag-3 RNAi, or Cep-1 RNAi throughout their development were treated with 120 Gy of γ-irradiation to induce the DNA damage (34, 35, 38). Twenty four hours after irradiation, the number of SYTO 12-stained (apoptotic) cells or corpses were counted using fluorescent microscopy. As shown in Fig. 9*B*, N2 control RNAi worms treated with 120 Gy of γ-irradiation exhibited an ~4-fold increase in germ line corpses per gonad arm compared with untreated worms. As expected, no change in the number of apoptotic cells was observed in Cep-1 RNAi worms indicating that apoptosis is p53-dependent. Notably, Lag-3 RNAi-treated F1 generation worms showed a partial decrease in induction of apoptotic germ line cells (Fig. 9*B*). These data are consistent with the role of Lag-3 in Cep-1 (p53)-mediated apoptosis in germ line cells.

RNAi-mediated Lag-3 Knockdown Reduces the Expression of p53 Target Genes—Schumacher *et al.* (38) recently showed that Cep-1 mediates the DNA-damage induced expression of *Ced-13* and *egl-1*, the *C. elegans* orthologs of the Bcl-2 homology 3 domain containing human proteins Puma and Noxa, respectively, that are involved in the apoptotic response (38). Thus, we asked if Lag-3 is required for Cep-1-dependent expression of *ced-13* and *egl-1*. Quantitative real time PCR analysis of RNA isolated from untreated *versus* 120 Gy γ-irradiation-treated worms showed that RNAi-mediated reduction of Lag-3 expression resulted in a significant decrease in *Ced-13* and *egl-1* induction upon γ-irradiation (Fig. 10, *A* and *B*, respectively). These results demonstrate that MamL1/Lag-3 is required for p53/Cep-1-mediated transcriptional activation in the *in vivo C. elegans* system.

Taken together, the results presented above indicate that Lag-3/MamL1 functions as a transcriptional coactivator for p53 and thus enhances the p53-mediated transactivation and apoptosis in both *in vitro* and *in vivo* systems.

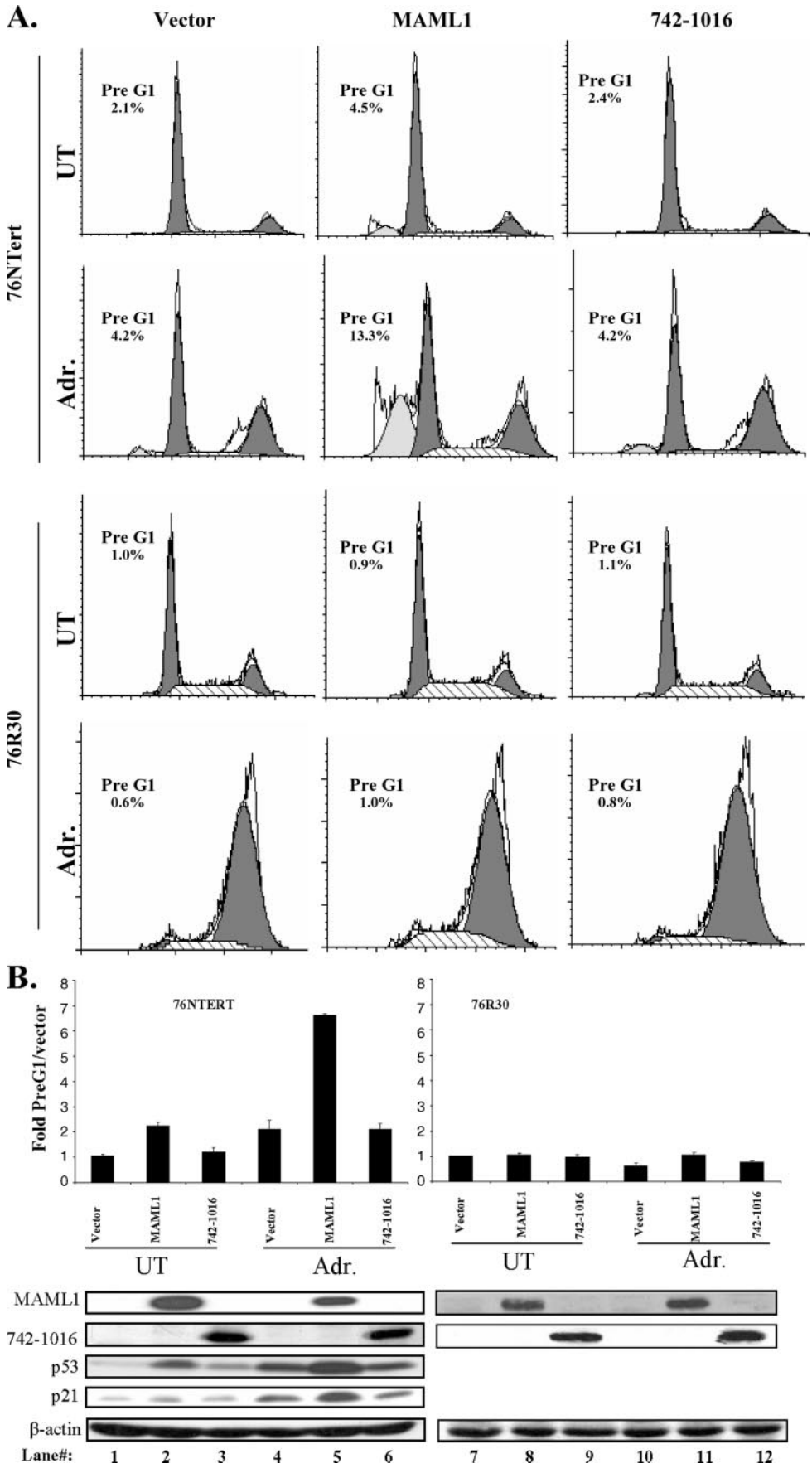
DISCUSSION

Incorporation of coactivators into transcriptional activator complexes at specific target promoters has emerged as a crucial determinant of the function of various transcription factors, including p53 (6). The p53 gene is the single most frequently inactivated gene in human cancers and is mutated in ~50% of all cancers (39). p53 is a sequence-specific homotetrameric transcription factor that becomes activated in response to many forms of cellular stress such as irradiation, hypoxia, drug-induced DNA damage, and oncogene activation; in response p53 orchestrates the transcription of many genes to either arrest cell proliferation or, more dramatically, to induce apoptosis. Substantial evidence points to a critical role for the p300/CBP coactivators in p53 responses to DNA damage (5, 8). p300/CBP and the associated protein P/CAF bind to and acetylate p53 during the DNA damage response in addition to histone acetylation, and they are needed for full p53 transactivation as well as for downstream p53 effects of growth arrest and/or apoptosis (4). Through physical interaction, the p300/CBP histone acetyltransferase/ubiquitin ligase can positively or negatively regulate p53 transactivation as well as p53 protein turnover, depending on cellular context and the environmental stimulus (4, 5). Recent work has also identified another coactivator ADA3 that binds to p53 and positively regulates its protein level and transcriptional activity (6, 10). Given the versatile functional roles of p53 as a tumor suppressor and its ability to control the expression of a diverse set of genes, identification of additional p53 coactivators continues to be an important area of research in p53 biology.

Here we have used both *in vitro* and *in vivo* assays to demonstrate, for the first time, that the Notch coactivator MAML1 interacts with p53. We defined the N terminus of MAML1 as the binding domain for p53 and reciprocally the DNA-binding domain on p53 as the interacting domain for MAML1. Our ChIP assay findings that MAML1 is a part of the activator complex that binds to the native p53 target promoters strongly suggested that the MAML1-p53 interaction is functionally significant.

Similar to other p53 coactivators, such as p300 and ADA3, we also demonstrate that interaction with MAML1 stabilizes the p53 protein. p300 has been reported to stabilize p53 by interfering with Mdm2 interaction; whether a similar mechanism might mediate MAML1-dependent increase in p53 levels will need to be addressed through future studies. Interestingly, it has also been reported that MAML1 has a p300-binding domain (11). Thus, it is possible that p53, MAML1, and p300 function together in one protein complex. Previous studies have shown that the human papilloma virus E6 protein interacts with the p53 coactivator p300 as well as human ADA3 and inhibits their coactivator function for p53. It is notable that we and others (unpublished) isolated MAML1 in a screen for HPV16 E6-interacting proteins in a yeast two-hybrid screen, suggesting the role of MAML1 in HPV16E6-induced transformation and in cervical cancer (15). The recently elucidated structure of a ternary Notch-CSL-MAML1 complex (19, 40) indicates that the N terminus of MAML1 binds as a kinked α-helix to a composite surface formed by the interaction of the ankyrin domain of Notch-1 and CSL. Our data suggest that the N terminus of MAML1 is also involved in the interaction with p53.

One of the important functions of p53 is to induce apoptosis under DNA damage conditions. We demonstrate here that wild-type MAML1 promotes p53-dependent apoptosis during DNA damage, whereas cells lacking wild-type p53 or expressing a mutant of MAML1 that does not interact with p53 showed no response. We also demonstrate that MAML1 enhances p53 acetylation/phosphorylation upon DNA damage. Furthermore, our analyses using the *C. elegans* model system with a single MAML1 homolog, Lag-3, support the conclusion that MAML1 functions as a transcriptional coactivator of p53. We demonstrate that knocking down Lag-3 expression in the worm mimics a p53-deficient response to radiation-induced DNA damage. The result that Lag-3 RNAi incompletely abrogated the germ cell apoptotic response, in contrast to a nearly complete abrogation with Cep-1 (p53) knockdown, is likely to be due to residual Lag-3 expression in the F1 generation worms or



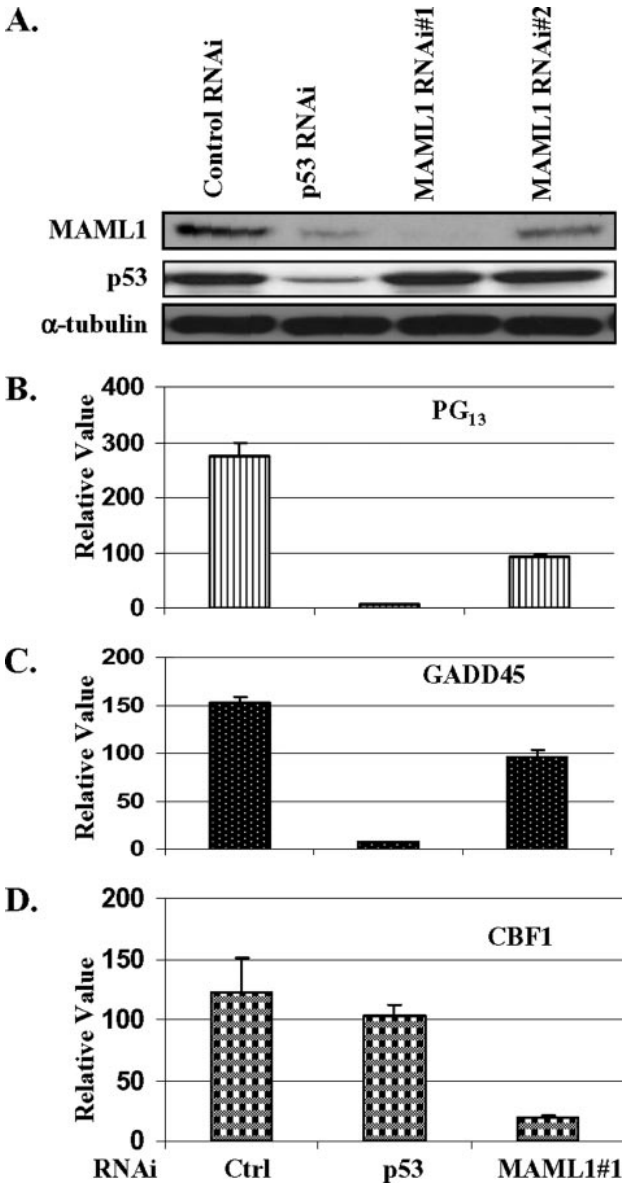


FIGURE 8. **RNAi-mediated knockdown of MAML1 decreases the expression of MAML1 and consequently inhibits p53-mediated transactivation.** Two RNAis (designated as MAML1 RNAi 1 and MAML1 RNAi 2) were designed to target two MAML1 open reading frame sequences (336–354 (gaggaatcttgacagcgcc) and (913–931) (gcagcctttgagcaagaac). One scrambled sequence was used as a cRNAi. The targeted sequence of p53 RNAi is gacuccagugguaaucuac. A pair of 64-nucleotide oligonucleotides, each containing a unique 19-nucleotide sequence derived from the target transcript, were annealed and ligated into pSUPER.retro.neo BglII and HindIII sites. MCF-7 cells were infected with viral supernatant containing control RNAi, MamL1 RNAi 1 and 2, p53 RNAi. 48 h after infection, stable cells were established by G418 selection (250 μg/ml). Stable cell lines expressing various constructs were analyzed for MAML1 and p53 protein levels by Western blotting after 24 h (Fig. 7*A*) or plated in 12-well plates as triplicate experiments at the density of 3×10^4. Twenty four hours later cells were transfected with indicated luciferase reporters and plasmid DNAs and analyzed for luciferase activity as shown in Fig. 6.

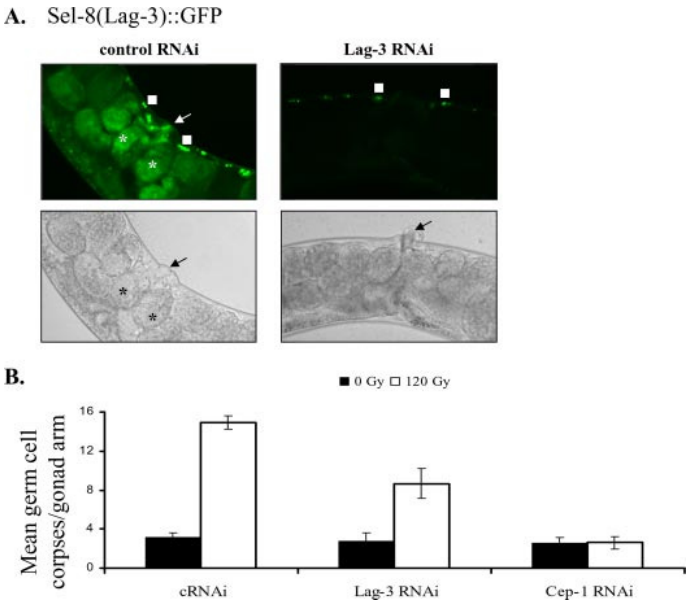


FIGURE 9. **Lag-3 RNAi abrogates DNA damage-induced germ cell apoptosis in *C. elegans*.** Gravid transgenic worms expressing GFP-tagged Lag-3 were plated on control or Lag-3 RNAi bacteria-seeded plates overnight to lay eggs. F1 generation adults were examined for expression of the Lag-3::GFP transgene by fluorescence microscopy (*A*). F1 generation worms were grown on control RNAi, Lag-3 RNAi, or Cep-1 RNAi-seeded plates. L4 synchronized worms were either left untreated or were subjected to 120 Gy of γ-irradiation. 21 h after treatment, adult worms were stained with the dye SYTO 12 and then examined at 24 h after treatment by fluorescence microscopy. SYTO 12-positive cells in the distal tip of the gonad arms representing apoptotic cells were counted from an average of 15–25 worms (*B*). The data represent the average of three independent experiments; mean ± S.E. is shown.

because of the continued function of other transcriptional coactivators of Cep-1. Consistent with our conclusions, Lag-3 down-regulation led to reduced induction of the Cep-1 target genes *Egl-1* and *Ced-13* that are involved in the apoptotic response. Thus, our *C. elegans* studies strongly suggest that Lag-3 is an important transcriptional coactivator of Cep-1, further supporting our conclusion from mammalian cell studies that MAML1 is a coactivator of p53. In addition to providing an *in vivo* biological system, the *C. elegans* studies also circumvented the complexities of investigating the functional roles of a three-member gene family in mammalian cells. Importantly, these results strongly suggest that the p53 coactivator role of MAML1 identified here is evolutionarily conserved.

The ability of MAML1 to function as a coactivator for a tumor suppressor protein, p53, suggests that its well known role as a coactivator of the Notch pathway may also serve a tumor suppressor role in defined contexts. Notch signaling can be both oncogenic and tumor-suppressive (41). In humans, aberrant *NOTCH1* expression has been identified as a causative factor in the development of T-cell acute lymphoblastic leukemia and lymphoma (42). Deregulated Notch receptor expression has been reported in a growing number of solid human

FIGURE 7. **Overexpression of MAML1 induces p53-dependent apoptosis.** 1×10^6 76NTERT (p53 wild type) or 76R-30 cells (p53-null) in 100-mm dishes were infected with retroviral supernatants containing pMSCV, MAML1/pMSCV, MAML-1-(742–1016)/pMSCV for 48 h, followed by selection for 48 h. The cells were then plated into 100-mm dishes for Western blotting and in 6-well plates for FACS analysis. Two days later cells were treated with adriamycin (*Adr*) (0.25 μg/ml) for 18 h, harvested, fixed in ethanol, RNase A-digested, stained with propidium iodide, and analyzed for FACS analysis using the FACSCalibur and CELLQuest 3.3 (BD Biosciences). The data were represented as the mean ± S.D. of triplicate experiments. Experiments were repeated at least three times. A representative experiment is shown. FACS analysis of cells in pre-G_1, G_1, S, and G_2/M are shown in *A*. Quantitative data of pre-G_1 (apoptotic cells) is shown in *upper panels* of *B*. The *lower panels* show the Western blotting of various proteins. *UT*, untreated.

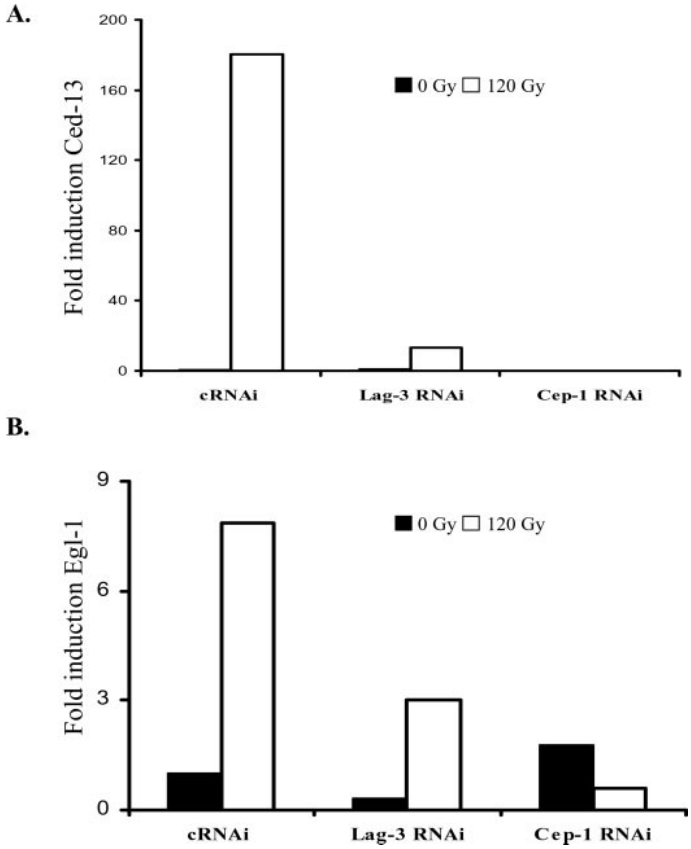


FIGURE 10. **Lag-3 RNAi inhibits expression of downstream targets of Cep-1 (p53) following γ-irradiation in *C. elegans*.** L4-synchronized, F1 generation worms that were grown on control RNAi, Lag-3 RNAi, or Cep-1 RNAi-seeded plates were either left untreated or treated with 120 Gy of γ-irradiation. Twenty four hours later RNA was extracted from adult worms picked from the plates. Real time RT-PCR was performed to detect and quantify the fold induction of Ced-13 (*A*) and Egl-1 (*B*) expression following γ-irradiation. Samples were normalized relative to actin expression; representative data of three experiments are shown.

tumors (43). Increased Notch1 protein expression is observed in human cancers of the cervix, colon, lung, pancreas, skin, and brain (12). Recent studies have highlighted a potential role for Notch signaling in human and mouse breast cancer development (44). Despite increasing experiments indicating that Notch is oncogenic, there is also emerging evidence that Notch signaling could be tumor-suppressive as has been observed for Notch1 in the mouse skin (44, 45). When Notch1 function is inactivated specifically in the mouse skin, the epidermis undergoes hyperproliferation with subsequent development of skin tumors. These mice also have increased sensitivity to chemical-induced skin carcinogenesis. In normal differentiated human epidermis, Notch signaling is activated and functions to promote keratinocyte differentiation. Human basal cell carcinomas, in contrast, lack activated Notch1 signaling. A tumor-suppressive role for activated Notch signaling has also been suggested in the prostate, lung, brain, and liver (43). Future studies will need to address if MAML proteins serve a coactivator role in these Notch functions.

There are conflicting reports regarding the interaction of p53 and Notch function. Some experiments have shown that Notch activation can promote p53-mediated transactivation and lead to apoptosis (23). Other reports, in contrast, have shown that Notch is able to inhibit p53-mediated function (22). The function of Notch receptors is complicated and can depend on the cellular context (46). MAML1 functions as a coactivator of the Notch pathway. It is possible that the interaction of MAML1 with p53 could influence its availability to form a ternary complex with CBF1-Notch intracellular component and thus determine the balance between gene regulation by p53 and Notch. One important feature of Notch signaling is its striking dose dependence in *Drosophila* (47) and mammalian cells (48). It is tempting to speculate that p53 and Notch may regulate each other's transcriptional activity by competing for MAML1. Thus, one of the factors that control the "strength" of a Notch signal may be the available level of free MAML1, which in turn is determined by the level of nuclear p53.

This idea is consistent with recent data indicating that alternative association of MamL1 with Notch or MEF2 transcription factor controls distinct and apparently opposite fates of myoblast cells (21). Our findings that a deletion mutant of MAML1 that is known to be deficient in Notch-mediated transactivation but still functions as a p53 coactivator are reminiscent of MAML1 association with MEF2 *versus* Notch. These similarities further suggest that the interaction of MAML1 with p53 may regulate distinct transcriptional programs and the associated biological responses from those mediated through Notch, and these may be cooperative or antagonistic with Notch-mediated responses. In this regard, it is quite possible that coactivator roles of MAML1 for Notch *versus* p53, MEF2, and other uncharacterized transcription factors are exerted in distinct cellular or tissue contexts or at different stages of development, as is well known for Notch (49).

In conclusion, our identification of MAML1 as a coactivator of p53 provides a new insight into p53 biology as well as a novel function of MAML1, which has previously been thought to only function as a Notch coactivator but is now emerging as a multifaceted regulator of transcriptional programs distinct from Notch (13, 21). Further studies should help determine the relative importance of the roles of MAML1 and its family members that are mediated via Notch *versus* other transcriptional pathways.

REFERENCES

1. Lakin, N. D., and Jackson, S. P. (1999) *Oncogene* **18,** 7644–7655
2. Vousden, K. H., and Lu, X. (2002) *Nat. Rev. Cancer* **2,** 594–604
3. Dimri, G. P. (2005) *Cancer Cell* **7,** 505–512
4. Bode, A. M., and Dong, Z. (2004) *Nat. Rev. Cancer* **4,** 793–805
5. Brooks, C. L., and Gu, W. (2003) *Curr. Opin. Cell Biol.* **15,** 164–171
6. Kumar, A., Zhao, Y., Meng, G., Zeng, M., Srinivasan, S., Delmolino, L. M., Gao, Q., Dimri, G., Weber, G. F., Wazer, D. E., Band, H., and Band, V. (2002) *Mol. Cell. Biol.* **22,** 5801–5812
7. Lill, N. L., Grossman, S. R., Ginsberg, D., DeCaprio, J., and Livingston, D. M. (1997) *Nature* **387,** 823–827
8. Avantaggiati, M. L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A. S., and Kelly, K. (1997) *Cell* **89,** 1175–1184
9. Gu, W., Shi, X. L., and Roeder, R. G. (1997) *Nature* **387,** 819–823
10. Wang, T., Kobayashi, T., Takimoto, R., Denes, A. E., Snyder, E. L., el-Deiry, W. S., and Brachmann, R. K. (2001) *EMBO J.* **20,** 6404–6413
11. Wallberg, A. E., Pedersen, K., Lendahl, U., and Roeder, R. G. (2002) *Mol. Cell. Biol.* **22,** 7812–7819
12. Miele, L. (2006) *Clin. Cancer Res.* **12,** 1074–1079
13. Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., and Griffin, J. D. (2000) *Nat. Genet.* **26,** 484–489

14. Lin, S. E., Oyama, T., Nagase, T., Harigaya, K., and Kitagawa, M. (2002) *J. Biol. Chem.* **277,** 50612–50620
15. Wu, L., Sun, T., Kobayashi, K., Gao, P., and Griffin, J. D. (2002) *Mol. Cell. Biol.* **22,** 7688–7700
16. Petcherski, A. G., and Kimble, J. (2000) *Nature* **405,** 364–368
17. Doyle, T. G., Wen, C., and Greenwald, I. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7877–7881
18. Fryer, C. J., Lamar, E., Turbachova, I., Kintner, C., and Jones, K. A. (2002) *Genes Dev.* **16,** 1397–1411
19. Wilson, J. J., and Kovall, R. A. (2006) *Cell* **124,** 985–996
20. Wu, L., and Griffin, J. D. (2004) *Semin. Cancer Biol.* **14,** 348–356
21. Shen, H., McElhinny, A. S., Cao, Y., Gao, P., Liu, J., Bronson, R., Griffin, J. D., and Wu, L. (2006) *Genes Dev.* **20,** 675–688
22. Nair, P., Somasundaram, K., and Krishna, S. (2003) *J. Virol.* **77,** 7106–7112
23. Yang, X., Klein, R., Tian, X., Cheng, H. T., Kopan, R., and Shen, J. (2004) *Dev. Biol.* **269,** 81–94
24. Gumienny, T. L., Lambie, E., Hartwieg, E., Horvitz, H. R., and Hengartner, M. O. (1999) *Development* (*Camb.*) **126,** 1011–1022
25. Band, V., Zajchowski, D., Kulesa, V., and Sager, R. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 463–467
26. Christova, R., and Oelgeschlager, T. (2002) *Nat. Cell Biol.* **4,** 79–82
27. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) *Science* **296,** 550–553
28. Brenner, S. (1974) *Genetics* **77,** 71–94
29. Timmons, L., Court, D. L., and Fire, A. (2001) *Gene* (*Amst.*) **263,** 103–112
30. Coutts, A. S., and La Thangue, N. B. (2005) *Biochem. Biophys. Res. Commun.* **331,** 778–785
31. Meng, G., Zhao, Y., Nag, A., Zeng, M., Dimri, G., Gao, Q., Wazer, D. E., Kumar, R., Band, H., and Band, V. (2004) *J. Biol. Chem.* **279,** 54230–54240
32. Zeng, M., Kumar, A., Meng, G., Gao, Q., Dimri, G., Wazer, D., Band, H., and Band, V. (2002) *J. Biol. Chem.* **277,** 45611–45618
33. Kern, S. E., Kinzler, K. W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., and Vogelstein, B. (1991) *Science* **252,** 1708–1711
34. Schumacher, B., Hofmann, K., Boulton, S., and Gartner, A. (2001) *Curr. Biol.* **11,** 1722–1727
35. Derry, W. B., Putzke, A. P., and Rothman, J. H. (2001) *Science* **294,** 591–595
36. Dorman, J. B., Albinder, B., Shroyer, T., and Kenyon, C. (1995) *Genetics* **141,** 1399–1406
37. Henderson, S. T., and Johnson, T. E. (2001) *Curr. Biol.* **11,** 1975–1980
38. Schumacher, B., Schertel, C., Wittenburg, N., Tuck, S., Mitani, S., Gartner, A., Conradt, B., and Shaham, S. (2005) *Cell Death Differ.* **12,** 153–161
39. Olivier, M., Eeles, R., Hollstein, M., Khan, M. A., Harris, C. C., and Hainaut, P. (2002) *Hum. Mutat.* **19,** 607–614
40. Nam, Y., Sliz, P., Song, L., Aster, J. C., and Blacklow, S. C. (2006) *Cell* **124,** 973–983
41. Radtke, F., and Raj, K. (2003) *Nat. Rev. Cancer* **3,** 756–767
42. Grabher, C., von Boehmer, H., and Look, A. T. (2006) *Nat. Rev. Cancer* **6,** 347–359
43. Leong, K. G., and Karsan, A. (2006) *Blood* **107,** 2223–2233
44. Politi, K., Feirt, N., and Kitajewski, J. (2004) *Semin. Cancer Biol.* **14,** 341–347
45. Nicolas, M., Wolfer, A., Raj, K., Kummer, J. A., Mill, P., van Noort, M., Hui, C. C., Clevers, H., Dotto, G. P., and Radtke, F. (2003) *Nat. Genet.* **33,** 416–421
46. Louvi, A., and Artavanis-Tsakonas, S. (2006) *Nat. Rev. Neurosci.* **7,** 93–102
47. Heitzler, P., and Simpson, P. (1991) *Cell* **64,** 1083–1092
48. Lathion, S., Schaper, J., Beard, P., and Raj, K. (2003) *Cancer Res.* **63,** 8687–8694
49. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) *Science* **284,** 770–776

The Notch Regulator MAML1 Interacts with p53 and Functions as a Coactivator

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