

Activated Notch1 interacts with p53 to inhibit its phosphorylation and transactivation

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We propose a biochemical mechanism for the negative role of Notch signaling on p53 transactivation function. Expression of the intracellular domain of human Notch1 (Notch1-IC) inhibits the expression of p53-responsive genes *p21*, *mdm2*, and *bax* in HCT116 *p53*^{-/-} cells. Furthermore, Notch1-IC expression inhibits the phosphorylation of ectopically expressed p53 in HCT116 *p53*^{-/-} cells as well as the phosphorylation of endogenous p53 in UV-treated HCT116 *p53*^{+/+} cells. Transcriptional downregulation of p53-responsive genes by Notch1-IC was confirmed both by chromatin immunoprecipitation assay and Northern blot analysis. We found the intracellular interaction between Notch1-IC and p53 in HCT116 *p53*^{+/+} cells and suggest that activated Notch1 interaction with p53 is an important cellular event for the inhibition of p53-dependent transactivation. The N-terminal fragment of Notch1-IC, which can interact with p53, inhibits p53 phosphorylation and represses p53 transactivation. In addition, Notch signaling downregulated p53-dependent apoptosis induced by UV irradiation.

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The *Notch* gene family, first identified in *Drosophila melanogaster*, has been identified and characterized in a variety of animal models.¹ Notch proteins are large but highly conserved transmembrane receptor proteins that mediate cellular signals involved in cell fate decisions.² In vertebrates, Notch proteins comprise a family of four transmembrane receptors (Notch1–4) that are highly related to each other and to homologues in *Drosophila* and *Caenorhabditis elegans*. The extracellular domain of each Notch receptor includes EGF-like repeats followed by the Cys-rich Notch/Lin12 domain, whereas the intracellular domain contains a RAM domain, six ankyrin repeats, a transactivation domain, and a PEST sequence. Thus far, six mammalian Notch ligands have been identified including Delta-like homologues DLL1, DLL2, DLL3, and DLL4 and Serrate-like homologues JAG1 and JAG2.²

Notch signaling is initiated by interaction between the receptor and its ligand. Ligand binding leads to proteolytic cleavage near the transmembrane region of Notch intracellular domain, resulting in release of the intracellular domain (Notch-IC). Notch-IC enters the nucleus and binds to a CSL transcription factor. CBF1 (also known as RBP-J κ), the mammalian homologue of *D. melanogaster* Suppressor of Hairless, is an important CSL transcription factor in mammalian cells. After association with CBF1, Notch-IC stimulates the expression of target genes through recruitment of transcriptional coactivators such as histone acetyl transferases.³ Among the best-characterized downstream targets of Notch signaling in mammals are *HES* gene family members, which are known to inhibit the expression of genes involved in neuronal differentiation.⁴ Activated Notch1 also

induces p21 expression either by CBF1-dependent or CBF1-independent transcriptional regulation in primary mouse keratinocytes.⁵ Increased expression of the cell cycle regulator p21 causes cell cycle arrest and helps to trigger terminal differentiation. Thus, Notch signaling can either activate or inhibit a variety of cellular processes including cell cycling, differentiation, proliferation, and death. These Notch-influenced processes are all important in the context of the roles of Notch signaling in tumorigenesis.

The oncogenic role of Notch was first illustrated in T-cell acute lymphoblast leukemia.⁶ Whether Notch signaling plays a critical role in neoplastic transformation is unknown, but there is evidence to suggest oncogenic properties of Notch signaling. Overexpression of activated human Notch family members was observed in translocation-associated T-cell acute lymphoblast leukemia.^{7,8} Activated Notch1 can induce neoplastic transformation in mouse mammary and salivary glands and in rat kidney cells *in vitro*.^{9,10} Notch signaling was reported to be required for neoplastic transformation in Ras-transformed human breast cancer cells.¹¹ Increased expression of activated Notch proteins and enhanced Notch signaling were shown in human colon adenocarcinoma.¹² The establishment of an immortalized hematopoietic cell line by constitutive Notch1 signaling strongly illustrates the oncogenic properties of Notch. However, controversial roles for the involvement of Notch signaling in tumorigenesis have been presented. Studies have shown that Notch signaling is not exclusively oncogenic but can also be tumor-suppressive. For example, Notch1-deficient mice develop basal-cell-carcinoma-like tumors and are highly susceptible to

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Abbreviations: ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; PARP, poly(ADP-ribose) polymerase; GST, glutathione S-transferase

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chemical-induced carcinogenesis, suggesting a tumor suppressor function for Notch.¹³ In addition, activated Notch1 inhibits the growth of mouse prostate cancer cells and functions as a tumor suppressor.¹⁴

p53 is a transcription factor and a tumor suppressor protein that controls cell proliferation and counteracts the effects of oncogene activation.¹⁵ Activation of wild-type p53 in response to DNA damage results in either growth arrest or apoptosis by inducing the expression of downstream target genes including *p21*, which encodes an inhibitor of cyclin-dependent kinase to block cell cycle progression¹⁶ and proapoptotic gene such as *bax*.¹⁷ In this study, we propose a mechanism by which Notch signaling represses the p53-dependent transactivation based on the observations that activated Notch1 interacts with p53, inhibits p53 phosphorylation, and interferes with sequence-specific p53 DNA binding resulting in the inhibition of p53-dependent transactivation and subsequent inactivation of p53-dependent apoptotic pathway in human cancer cells.

Results

Activated Notch1 Represses p53 Transactivation. To determine whether Notch signaling is capable of counteracting the tumor-suppressive properties of p53, we evaluated the effects of transiently expressed intracellular domain of human Notch1 (Notch1-IC) on p53 transactivation. HCT116 *p53*^{-/-} cells were cotransfected with p53 expression vector, varying amounts of plasmid encoding Notch1-IC, and luciferase reporter plasmid containing one of the promoters of *p21*, *mdm2*, and *bax*. Subsequent luciferase assay revealed a dose-dependent inhibition of p53 transactivation by transient expression of Notch1-IC (Figure 1a). Notch1-IC expression also reduced endogenous expression of p53 target genes such as *p21*, *mdm2*, and *PUMA* in HCT116 *p53*^{-/-} cells, suggesting the downregulation of p53 transactivation by Notch signaling (Figure 1b). The transcriptional downregulation of p53-responsive *p21* gene was confirmed by Northern blot analysis. The transcription of *p21* gene in UV-treated HCT116 *p53*^{+/+} cells was evidently reduced by Notch1-IC expression (Figure 1c). The negative role of Notch signaling on the regulation of tumor-suppressive p53 transcriptional activity does not seem to be restricted to HCT116 human colon cancer cells, as Notch1-IC expression also inhibited p53 transactivation in COS monkey kidney cancer cells and HeLa human cervical cancer cells (Figure 1d).

Notch1-IC Inhibits p53 phosphorylation and p53 DNA binding. We examined the effects of Notch signaling on p53 phosphorylation and DNA binding. The phosphorylation of p53 was analyzed using phospho-specific antibodies against p53 phosphorylated at specific Ser residues. Expression of Notch1-IC in HCT116 *p53*^{-/-} cells inhibited the phosphorylation of ectopically expressed p53 at Ser 6, 9, 15, 37, and 46 in the N-terminal region of p53 (Figure 2A). Notch1-IC expression also inhibited the phosphorylation of endogenous p53 induced by DNA damage in HCT116 *p53*^{+/+} cells irradiated with 30 J/m² UV (254 nm). The phosphorylation of p53 at Ser residues was analyzed

immediately or 8 h after UV irradiation. Cellular levels of endogenous p53 increased after UV irradiation, and became phosphorylated at Ser 15 and 37 in response to UV DNA damage. However, the phosphorylation of endogenous p53 was decreased by Notch1-IC expression in UV-treated HCT116 *p53*^{+/+} cells (Figure 2B). We sought to confirm the inhibition of p53 phosphorylation by Notch signaling in Notch1 knockdown cells transfected with siRNA. The silencing of Notch1 was evident in UV-treated HCT116 *p53*^{+/+} cells after transfection with siRNA, specific for human Notch1. The phosphorylation of p53 at Ser 15 and 37 was restored by Notch1 siRNA transfection, but transfection of control siRNA did not affect p53 phosphorylation (Figure 2C). To determine whether the activated Notch1 interferes with the ability of p53 to bind its recognition sites on DNA, p53 binding to the double-stranded oligonucleotide probes containing the recognition site either in *GADD45* or *p21* promoter was analyzed by electrophoretic mobility shift assay (EMSA). The ability of p53 to bind both recognition sites was markedly affected by Notch1-IC expression in HCT116 *p53*^{-/-} cells (Figure 2D). To test whether Notch signaling interferes with the binding of p53 to cellular promoter regions of p53 downstream target genes in cells, chromatin immunoprecipitation (ChIP) analysis was performed. Notch1-IC expression impaired the binding of p53 to the promoters of *p21* and *PUMA* in cotransfected cells (Figure 2E).

Notch1-IC Interacts with p53. Coimmunoprecipitation experiments were performed to examine whether activated Notch1 physically interacts with p53. Notch1 was present in both HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cells, whereas p53 was detected only in HCT116 *p53*^{+/+} cells (Figure 3A, a, left). The interaction between endogenous Notch1 and p53 was detected by coimmunoprecipitation. Notch1 was coimmunoprecipitated with p53 from HCT116 *p53*^{+/+} cells (Figure 3A, a). Likewise, endogenous p53 protein from HCT116 *p53*^{+/+} cells was also coimmunoprecipitated with endogenous Notch1 protein (Figure 3A, b). Intermolecular association between FLAG-tagged Notch1-IC deletion derivatives and glutathione *S*-transferase (GST)-fused p53 was also examined. Results from the GST pull-down assay suggest that the N-terminal region of FLAG-tagged Notch1-IC binds effectively with GST-fused p53 in cotransfected HCT116 *p53*^{-/-} cells (Figure 3B). Examination of intermolecular association between FLAG-tagged p53 deletion derivatives and GST-fused Notch1-IC in cotransfected HCT116 *p53*^{-/-} cells showed that the N-terminal region of p53 protein is important for the interaction between Notch1-IC and p53 (Figure 3C). Collectively, these data suggest that the N-terminal region of Notch1-IC interacts with the N-terminal region of p53.

The N-terminal fragment of Notch1-IC represses p53 transactivation. In an attempt to understand the relationship between Notch1-IC interaction with p53 and the repression of p53 transactivation, the effects of the N-terminal and C-terminal fragment of Notch1-IC on the repression of p53 transactivation were studied. Our data demonstrate that the ability of Notch1-IC to interact with p53

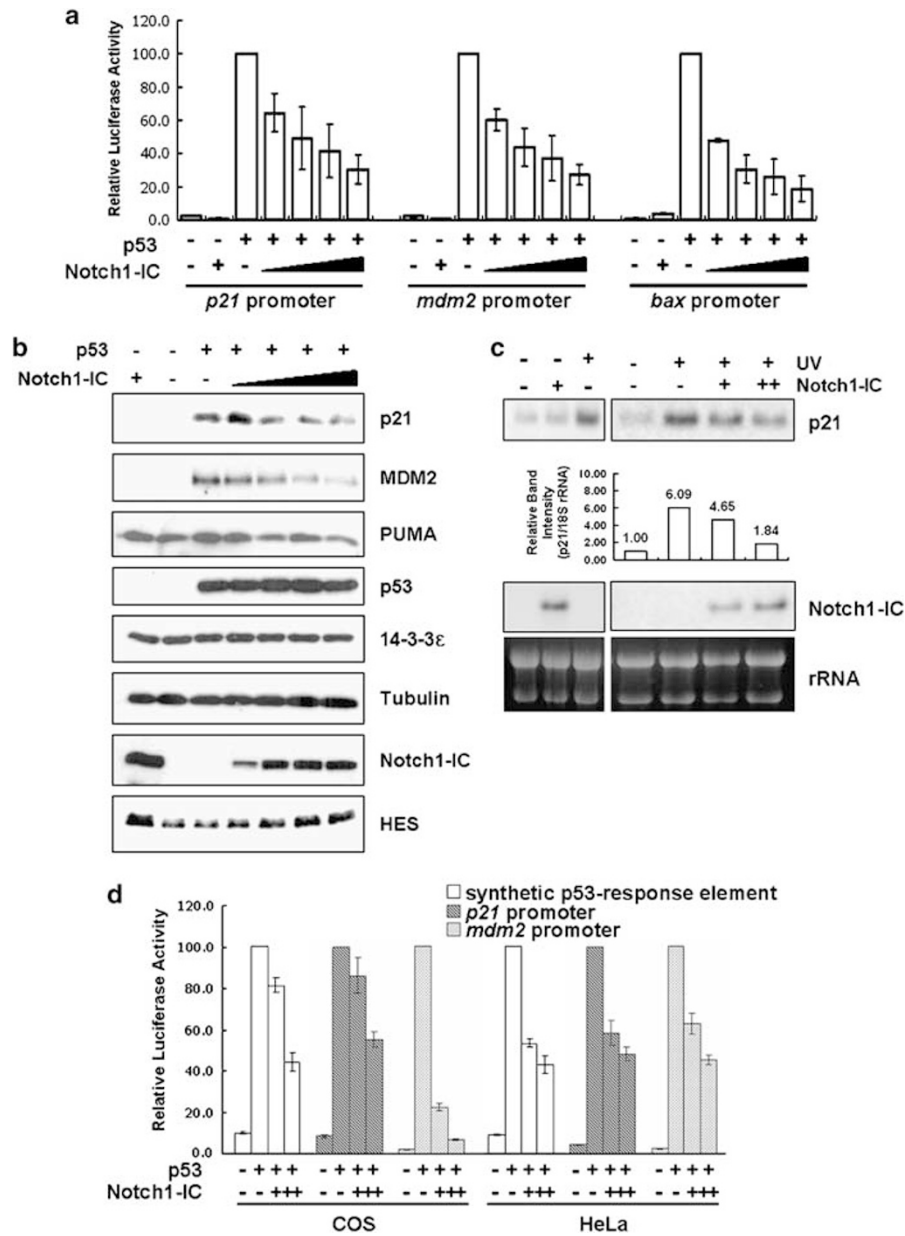


Figure 1 Notch signaling represses p53 transactivation. **(a)** Effects of transiently expressed Notch1-IC on p53 transactivation. HCT116 $p53^{-/-}$ cells (2×10^5) were cotransfected with p53 expression vector (100 ng) and luciferase reporter (100 ng each) containing one of the promoters of *p21*, *mdm2*, and *bax* together with increasing amounts (100, 200, 300, and 400 ng) of Notch1-IC expression vector. Cells were harvested 24 h after cotransfection. Luciferase activity driven by each p53-responsive promoter in the presence of p53 expression but in the absence of Notch1-IC expression was set to 100 units. In this and subsequent luciferase assays, results from at least three independent experiments are presented. **(b)** Effects of transiently expressed Notch1-IC on intracellular expression of p21, MDM2, and PUMA. HCT116 $p53^{-/-}$ cells (2×10^6) were cotransfected with p53 expression vector (400 ng) and increasing amounts (400, 800, 1,200, and 1,600 ng) of Notch1-IC expression vector. After incubation for 24 h, whole-cell lysate was analyzed by immunoblotting for the expression of endogenous p21, MDM2, and PUMA. The cellular levels of α -Tubulin or 14-3-3 ϵ in cotransfected cells were also examined as internal controls. The endogenous levels of Notch downstream target gene HES were also monitored. **(c)** Northern blot of endogenous *p21* mRNAs from UV-treated HCT116 $p53^{+/+}$ cells. Cells (2×10^6) were transfected with Notch1-IC expression vector (500 and 1000 ng) and were treated with 30 J/m² UV. After incubation for 8 h, total RNAs were extracted and probed with DIG-labeled oligonucleotides specific for *Notch1* or *p21* gene. The band intensity of *p21* in control cells, which were neither treated with UV nor transfected with Notch1-IC, was set to 1.00 unit. The relative band intensities of *p21* mRNA in Northern blot were determined by densitometric scanning of RNA bands using Quantity One software (Bio-Rad) and normalized by the amounts of 18S rRNA in each sample. The expression of transfected Notch1-IC was also examined by Northern blotting. Ribosomal RNAs were used as a loading control. **(d)** Effects of transiently expressed Notch1-IC on p53 transactivation in COS and HeLa cells. Cells (2×10^5) were cotransfected with p53 expression vector (100 ng) and Notch1-IC expression vector (100 and 400 ng) together with luciferase reporter plasmids containing p53-responsive promoters

is important for the modulation of p53 transcriptional activity. The expression of the N-terminal fragment of Notch1-IC, which interacts with p53, results in the repression of p53

transactivation as well as the inhibition of p53 phosphorylation on Ser 15, 37, and 46 in cotransfected HCT116 $p53^{-/-}$ cells (Figure 4a and b). However, the

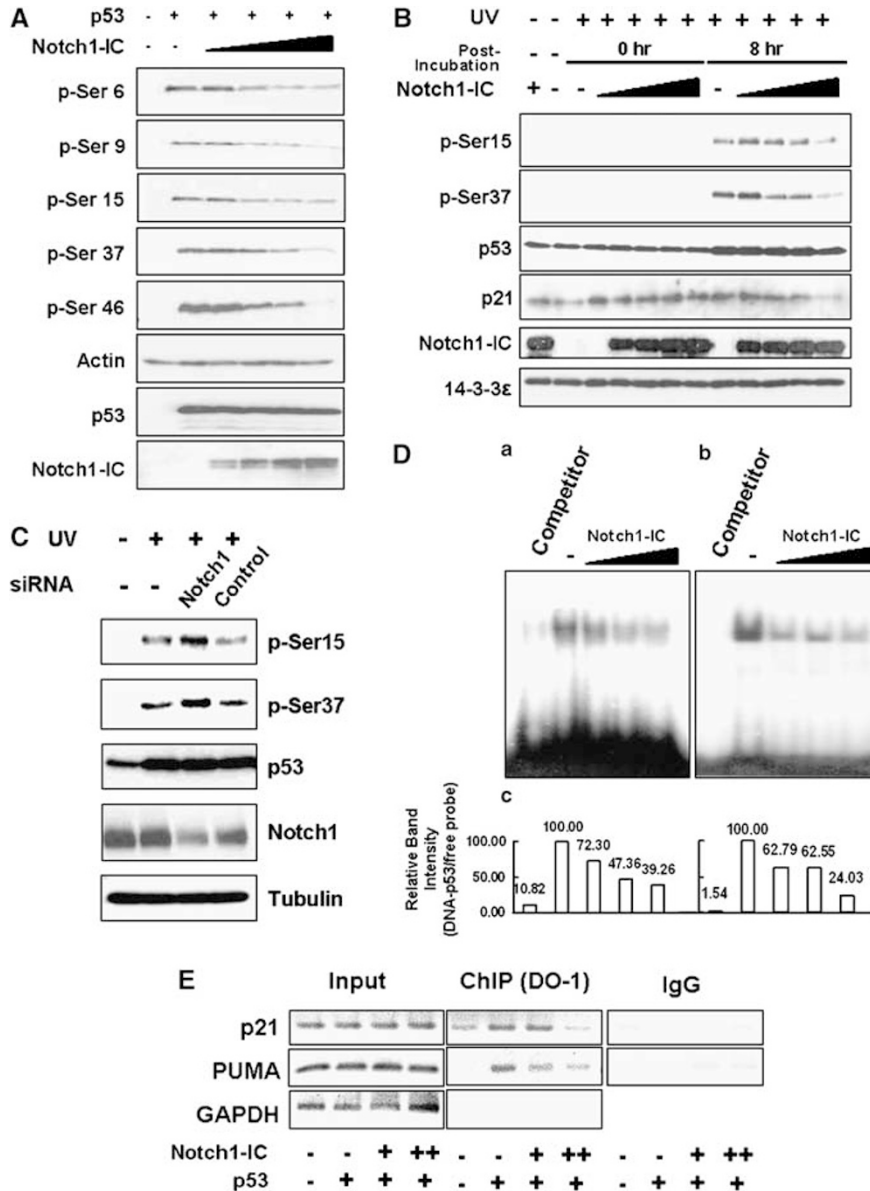


Figure 2 Notch signaling inhibits p53 phosphorylation and DNA binding. **(A)** HCT116 $p53^{-/-}$ cells (2×10^6) were cotransfected with FLAG-tagged p53 expression plasmid (400 ng) and increasing amounts (400, 800, 1200, and 1600 ng) of Notch1-IC expression vector. At 24 h after cotransfection, the phosphorylation of transiently expressed p53 on Ser residues was determined by immunoreactivity using antibodies that specifically recognize p53, phosphorylated at specific Ser residues. **(B)** HCT116 $p53^{+/+}$ cells (2×10^6) were transfected with increasing amounts (0.5, 1, 1.5, and 2 μ g) of Notch1-IC expression vector, incubated for 24 h, and exposed to 30 J/m² UV. Ser phosphorylation of endogenous p53 was determined 8 h after UV irradiation. Control cells were transfected with 2 μ g of Notch1-IC expression vector alone. The levels of endogenous p21 and ectopically expressed Notch1-IC were also monitored. **(C)** HCT116 $p53^{+/+}$ cells treated with 0.13 μ M Notch1 siRNA or control siRNA for 24 h were exposed to 30 J/m² UV and the phosphorylation of endogenous p53 on Ser residues was examined 8 h after UV irradiation. **(D)** Notch1-IC expression inhibits p53 DNA binding. HCT116 $p53^{-/-}$ cells (2×10^6) were cotransfected with p53 expression vector (1 μ g) and increasing amounts (1, 2, and 4 μ g) of Notch1-IC expression vector. Nuclear extracts were incubated with ³²P-end-labeled 26-mer double-stranded oligonucleotides containing either the p53-binding site from *p21* promoter (a) or the p53-binding site from *GADD45* promoter (b). Summary of EMSA data is shown. Relative band intensities of DNA–p53 complex in EMSA were determined by densitometric scanning using Quantity One software (Bio-Rad) and normalized by the amount of free probe. The relative band intensity of DNA–p53 protein complex from HCT116 $p53^{-/-}$ cells transfected with p53 alone was set to 100 units (c). **(E)** p53 binding to endogenous promoters of *p21* and *PUMA* was assessed by ChIP. HCT116 $p53^{+/+}$ cells (2×10^6) were transfected with p53 expression vector (400 ng) together with Notch1-IC expression vector (400 or 1600 ng) and processed for ChIP with anti-p53 antibody (DO-1) or control IgG (Upstate Biotechnology). The immunoprecipitates were analyzed by PCR using the specific primer for *p21* or *PUMA* promoter. The control PCR reactions were performed using the specific primer for *GAPDH*. The image of PCR products was presented in reversed black and white in which the DNA band is in black

expression of the C-terminal fragment of Notch1-IC, which lacks the ability to interact with p53, neither repressed p53 transactivation (Figure 4a) nor inhibited p53 phosphorylation (Figure 4b).

Notch transcriptional activity is not required to repress p53 transactivation. Experiments were conducted to determine whether transcriptional activity of Notch1 interferes with p53 transactivation function. The experiments

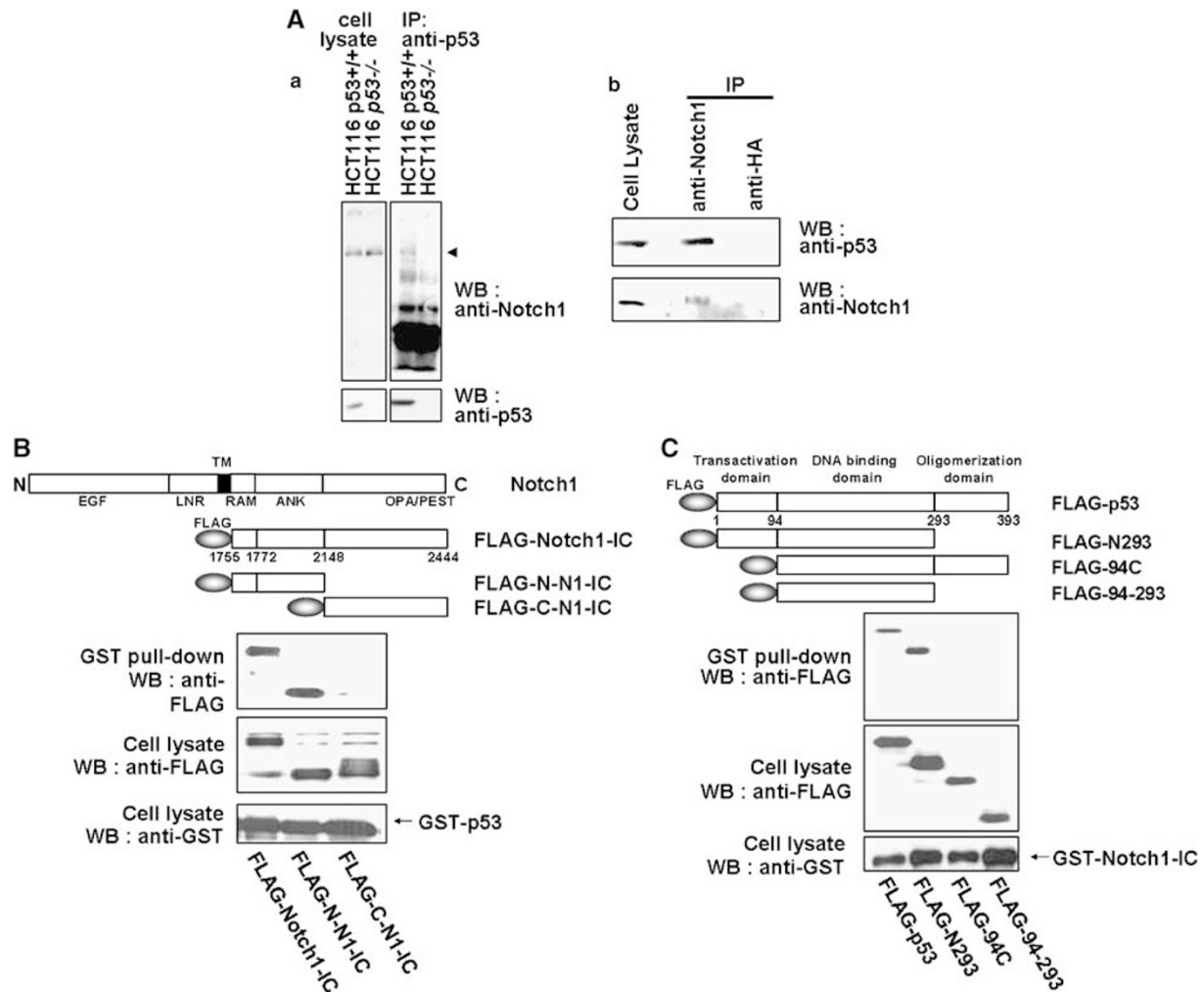


Figure 3 Notch1-IC interacts with p53. **(A)** (a) Notch1-IC interacts with p53 in HCT116 $p53^{+/+}$ cells. Endogenous expression of Notch1-IC (upper left) and p53 (lower left) in HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cells was examined by immunoblot analysis. Equal amounts of cell lysate protein from HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were immunoprecipitated with anti-p53 (C-19) antibody. The immune complexes were resolved by immunoblot analysis using an antibody directed against the N-terminal region of p53 (lower right) or Notch1 (upper right). The arrowhead indicates Notch1-IC associated with p53. (b) Equal amount of cell lysates from HCT116 $p53^{+/+}$ cells were immunoprecipitated with anti-Notch1 antibody. The precipitates were resolved in SDS-PAGE and the associated proteins were analyzed by immunoblotting using anti-p53 or anti-Notch1 antibody. Anti-HA antibody was employed for the negative control experiment. **(B)** The N-terminal region of Notch1-IC interacts with p53. HCT116 $p53^{-/-}$ cells (2×10^6) were cotransfected with expression vector for FLAG-tagged Notch1-IC deletion derivatives (1 μ g) and GST-fused p53 (1 μ g). After incubation for 24 h, GST-fused p53 in cell lysates was purified by GST pull-down. The binding of FLAG-tagged Notch1-IC derivatives to GST-fused p53 was examined by SDS-PAGE and immunoblotting using anti-FLAG monoclonal antibody (top). The expression of FLAG-tagged Notch1-IC derivatives (middle) and GST-fused p53 (bottom) in cell lysates was verified. **(C)** The N-terminal region of p53 interacts with Notch1-IC. HCT116 $p53^{-/-}$ cells (2×10^6) were cotransfected with expression vector for GST-fused Notch1-IC (1 μ g) and FLAG-tagged p53 derivatives (1 μ g). The binding of GST-fused Notch1-IC to FLAG-tagged p53 derivatives was determined.

utilized Δ RAM-N1-IC mutant, a Notch1-IC deletion mutant lacking a RAM domain which interacts with CBF1 to activate Notch target genes such as *HES1* and *HES2*.^{18,19} In accordance with previous reports, Δ RAM-N1-IC expression failed to transactivate luciferase reporter containing either 6 \times NRE (Notch Responsive Element) or *HES* promoter (Figure 5a). In contrast, both Δ RAM-N1-IC mutant and wild-type Notch1-IC reduced luciferase reporter activity driven by the promoters of the p53-responsive genes *p21*, *mdm2*, and *bax* (Figure 5b). Like wild-type Notch1-IC, the expression of Δ RAM-N1-IC inhibited the phosphorylation of endogenous p53 at Ser 15 and 37 in transfected cells treated with 30 J/m² UV (Figure 5c). Moreover, ectopically expressed Δ RAM-N1-IC was also capable of robust binding with p53 (Figure 5d).

Notch signaling downregulates p53-dependent apoptosis. In an effort to gain more insight into the biological function of Notch signaling in the regulation of p53-dependent pathways, apoptotic responses resulting from UV irradiation were evaluated. Expression of Notch1-IC resulted in the relative resistance to UV-induced apoptosis, which is reportedly known as a p53-dependent cellular event.²⁰ The negative role of Notch signaling on UV-induced apoptosis was further supported by the result showing the elevated level of apoptosis by Notch1 siRNA in UV-treated HCT116 $p53^{+/+}$ cells (Figure 6a). Notch1-IC expression also plays negative roles on caspase-3 activation and PARP cleavage in UV-treated HCT116 $p53^{+/+}$ cells (Figure 6b). Like wild-type Notch1-IC, the expression of

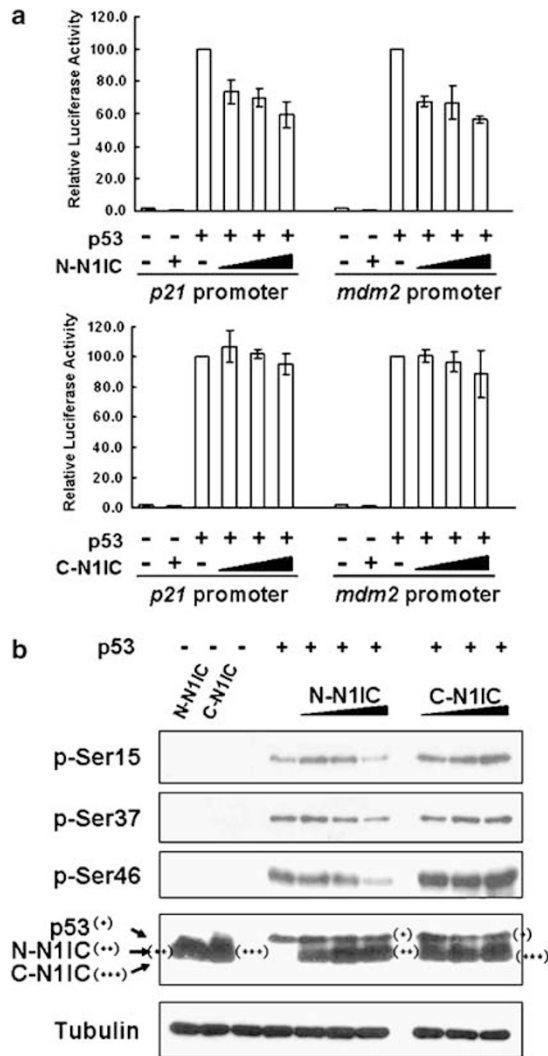


Figure 4 The N-terminal region of Notch1-IC is responsible for both the inhibition of p53 phosphorylation and transactivation. (a) HCT116 $p53^{-/-}$ cells (2×10^5) were cotransfected with plasmids for p53 (100 ng) and luciferase reporter (100 ng) together with increasing amounts (100, 200, 300, and 400 ng) of the expression vector for the N-terminal or C-terminal fragment of Notch1-IC. After incubation for 24 h, luciferase activity driven by p53-responsive promoter of *p21* or *mdm2* was determined. (b) HCT116 $p53^{-/-}$ cells (2×10^6) were cotransfected with p53 expression vector (400 ng) and increasing amounts (400, 800, and 1600 ng) of expression vector for the N-terminal or C-terminal fragment of Notch1-IC. After incubation for 24 h, p53 phosphorylation was determined by immunoreactivity using antibodies against phosphorylated p53 on specific Ser residues. The level of intracellular α -Tubulin was expressed as internal controls

Δ RAM-N1-IC, which is capable of interacting with p53, also inhibited caspase-3 activation (Figure 6c). In addition, Notch1 silencing by Notch1 siRNA transfection restored UV-induced apoptosis as evidenced by the increased PARP cleavage in UV-irradiated HCT116 $p53^{+/+}$ cells (Figure 6d).

Discussion

Previously, conflicting conclusions on the reciprocal relationship between Notch signaling and p53 function have been reported. First, p53 may negatively regulate Notch signaling.

The intracellular levels of Notch1-IC and its downstream target HES1 were elevated in $p53^{-/-}$ mouse thymocytes as compared with $p53^{+/+}$ thymocytes.²¹ Evidence for the competition between p53 and Notch1-IC for binding to the coactivator p300 also supports the negative regulation of Notch signaling by p53.²² The common transcriptional coactivator p300 interacts with the EP domain of Notch1 through the CH3 region to enhance Notch1-mediated transactivation. p53 interferes with Notch1-mediated transactivation by competing for binding to p300. p53 was also shown to downregulate Notch1 activation by enhancing the transcription of *p21* whose expression represses the transcription of the gene for Presenilin1, which is necessary for Notch activation.²³ Second, members of the tumor-suppressive p53 family may activate the Notch signaling pathway. p53 family members p63 and p73 upregulate transcription of *Jag-1* and *Jag-2* genes, which encode Notch ligands.²⁴ Third, Notch signaling may upregulate p53 transactivation. The expression of Notch1-IC elevated the levels of nuclear p53 and its target gene transcription in several mammalian cell lines including human hepatocellular carcinoma cells, mouse neural progenitor cells, and human cervical cancer cells.^{25–27} Increased p53 function by Notch signaling has also been demonstrated by an increase in p53-dependent apoptosis in cells expressing activated Notch1.²⁷ Fourth, Notch signaling may negatively regulate p53 function and inhibit apoptosis. Overexpression of activated Notch1 decreased p53 transcriptional activity and p53-dependent apoptosis in cultured mammalian cells^{28,29} and in mice.³⁰ Suggested roles for Notch1 in the downregulation of p53 function involve the amplification of cell survival signals through PI3K/Akt/PKB pathway^{28,29} or ARF/MDM2 pathway.³⁰

The present study supports the negative roles for Notch signaling in p53 function. We have shown that activated Notch1 interferes with p53 transactivation of downstream target genes (Figure 1). The protein stability and biochemical activity of p53 are tightly regulated by a variety of mechanisms. One mechanism likely to mediate both the stability and activity of p53 is phosphorylation. Kinases responsible for p53 phosphorylation have been identified, including casein kinase I and II, DNA-PK, ATM, ATR, CDK-activating kinase, cyclin-dependent kinases, and protein kinase C.³¹ Our data indicate that Notch signaling inhibited phosphorylation of various Ser residues in both ectopically expressed p53 (Figure 2A) and DNA damage-induced endogenous p53 (Figure 2B). Correspondingly, we found that inactivation of Notch signaling by Notch1 siRNA restores p53 phosphorylation at Ser 15 and 37 in UV-treated HCT116 $p53^{+/+}$ cells (Figure 2C). The function of p53 protein is largely regulated by its stability. p53 is normally maintained at low levels in unstressed mammalian cells by MDM2-induced p53 ubiquitination and subsequent degradation by 26S proteasomes. When cells encounter stress such as DNA damage or oncogenic gene expression, p53 ubiquitination is suppressed, and p53 is stabilized in the nuclei in order to induce the expression of p53 target genes.³¹ Several studies highlight the importance of p53 phosphorylation to its stability. Phosphorylation of p53 on Ser 20 has been proposed to stabilize both ectopically expressed and endogenous p53 in cultured mammalian cells by weakening the interaction with MDM2.^{32,33} JNK-mediated phosphorylation of

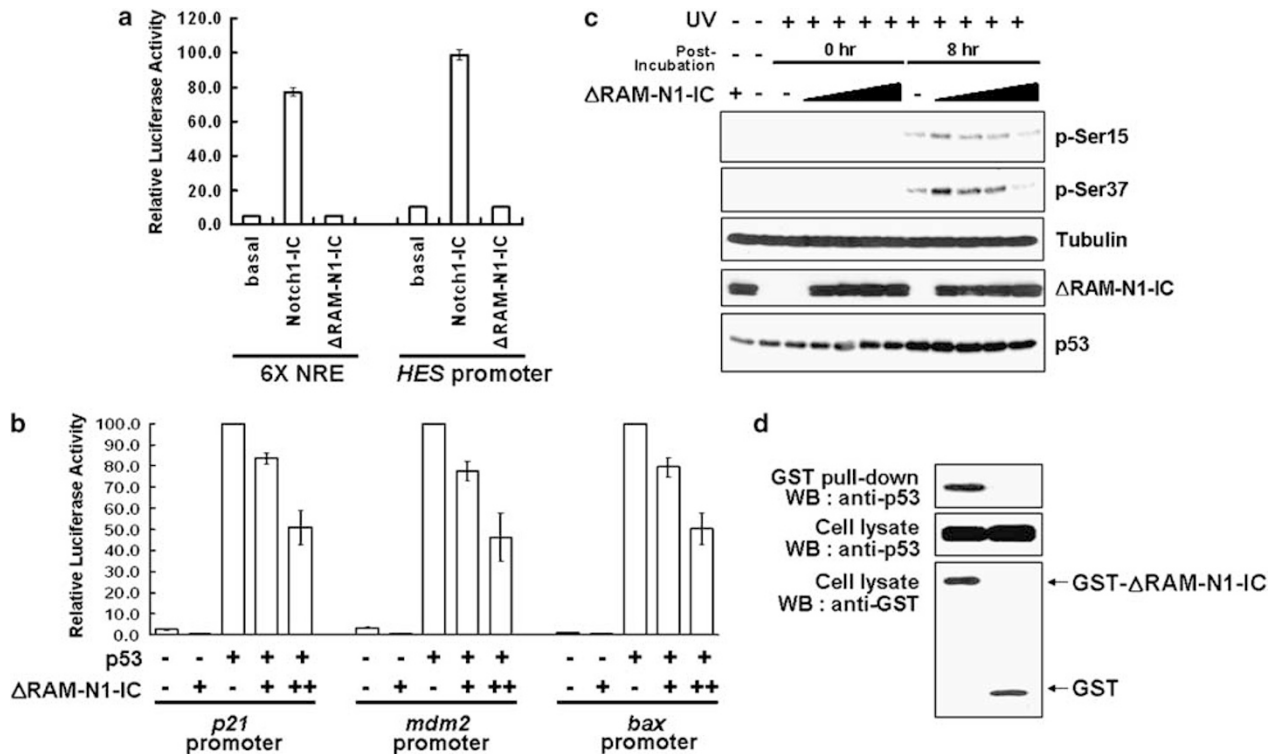


Figure 5 Notch1 transcriptional activity is dispensable for the repression of p53 transactivation. (a) HCT116 $p53^{-/-}$ cells (2×10^5) were cotransfected with luciferase reporter plasmid (100 ng) containing $6 \times$ NRE (Notch Response Element) and the expression vector for Notch1-IC or Δ RAM-N1-IC (100 ng each) (left). Luciferase activity driven by *HES* promoter in cotransfected cells was determined (right). (b) HCT116 $p53^{-/-}$ cells (2×10^5) were cotransfected with plasmids encoding Δ RAM-N1-IC (100 and 400 ng), p53 (100 ng), and luciferase reporter plasmid containing each p53-responsive promoter (100 ng). (c) HCT116 $p53^{+/+}$ cells (2×10^6) were transfected with increasing amounts (0.5, 1, 1.5, and 2 μ g) of plasmid encoding Δ RAM-N1-IC. At 24 h after transfection, cells were irradiated with 30 J/m² UV. After additional incubation for 8 h, the phosphorylation of endogenous p53 in transfected cells was examined as in Figure 2B. (d) The interaction between GST-fused Δ RAM-N1-IC and ectopically expressed p53 was examined in cotransfected HCT116 $p53^{-/-}$ cells

p53 on Thr 81 was also shown to increase p53 stability and transcriptional activity in cultured human cells.³⁴ However, the present study does not support the biochemical mechanism for Notch-mediated repression of p53 transactivation by reduced p53 stability. We investigated the effects of Notch signaling on p53 stability but did not observe any differences in p53 levels in the presence or absence of Notch1-IC expression (Figures 1b, 2A–C, and 6b). A recent study has proposed that Notch suppresses tumor-suppressive p53 function through ARF/MDM2-dependent proteolytic degradation of p53 based on the almost undetectable levels of p53 and a lack of the MDM2 inhibitor ARF protein in transgenic mice that express human Notch1-IC under the transcriptional control of the tetracycline operator sequences.³⁰ However, HCT116 human colon cancer cells do not express functional ARF protein.³⁵ Therefore, it is unlikely that Notch1 signaling interferes with the expression of ARF which promotes MDM2 degradation and elevates cellular levels of p53 by increasing its stability. A more recent report in the literature has demonstrated the inhibition of p53 phosphorylation and its transactivation by Notch1-IC in human cancer cells and proposed a biochemical mechanism in which activated Notch1 downregulates p53-dependent transactivation and p53-induced apoptosis by the aspects of PI3K/Akt/PKB pathway,²⁹ but we failed to prove this conclusion in our studies. Our results showed that the inhibition of PI3K, which

signals cell survival through PKB/Akt pathway, did not affect Notch1-mediated downregulation of p53 transactivation in HCT116 $p53^{+/+}$ cells (data not shown). While the precise biochemical mechanism remains exclusive, our results propose that the expression of activated Notch1 interferes with p53 binding to the promoters of its target genes (Figure 2D and E), leading to the downregulation of p53-dependent transactivation.

Previous reports have identified several ankyrin repeat proteins such as 53BP1 and 53BP2 that contain ankyrin repeats and bind to p53. These proteins are also known to regulate the tumor suppressor function of p53.³⁶ We were interested to know whether Notch protein containing six ankyrin repeats interacts with p53 and confirmed the intracellular association of Notch with p53 (Figure 3). Immunofluorescence analysis using confocal microscope reveals that p53 colocalizes with Notch1-IC in the nucleus of cotransfected HCT116 $p53^{-/-}$ cells (data not shown). This rules out the possibility that Notch1 may repress the p53 function by altering the subcellular distribution of p53 in HCT116 cells. This physical interaction between Notch1-IC and p53 seems to provide a functional framework for the inhibition of p53 phosphorylation and transactivation by Notch signaling. Overexpression of the N-terminal fragment of Notch1-IC, which is capable of interacting with p53, inhibited both p53 phosphorylation and its transcriptional activity. In

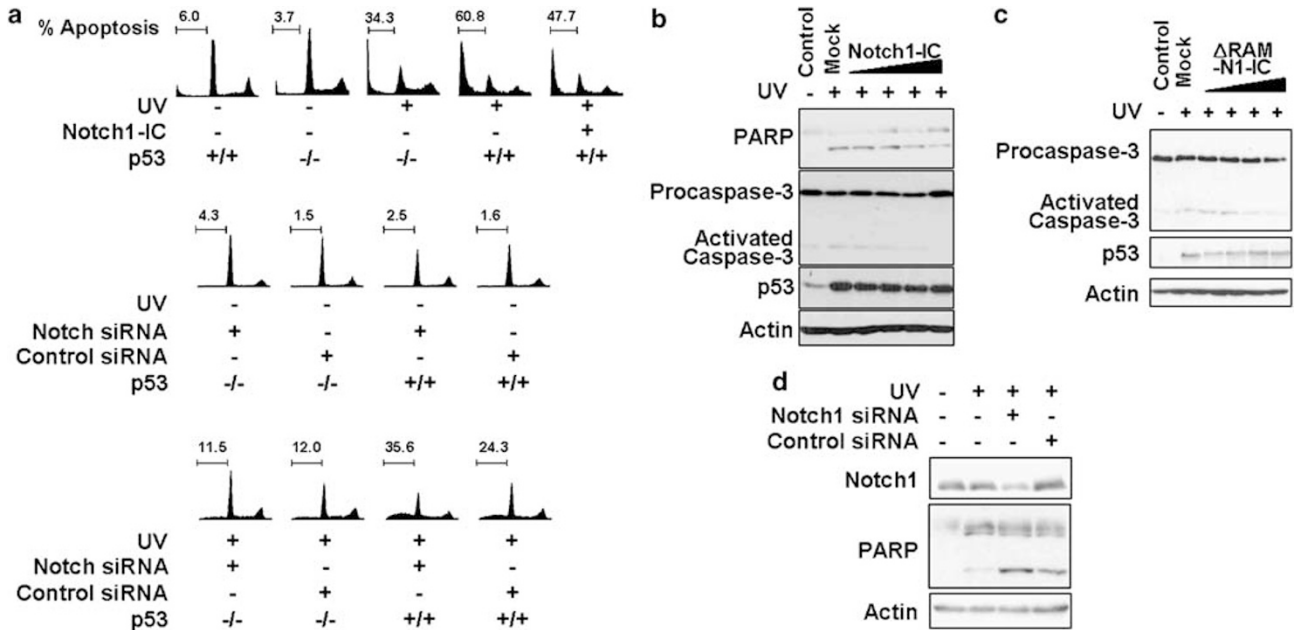


Figure 6 Notch1-IC inhibits UV-induced apoptosis. (a) HCT116 *p53*^{+/+} cells (2×10^6) were transfected with 2 μ g of Notch1-IC expression vector. Transfected cells were irradiated with 80 J/m² UV. Irradiated cells were postincubated for 24 h, harvested, and stained with PI followed by ethanol fixation. DNA contents were measured by FACS. The DNA contents of cells at sub-G1 were used as the index for the degree of apoptosis (upper). HCT116 *p53*^{+/+} or *p53*^{-/-} cells were transfected with 0.13 μ M of Notch1 siRNA or control siRNA. At 24 h after transfection, cells were irradiated with 30 J/m² UV treatment, postincubated for 24 h, harvested, stained with PI and analyzed for apoptosis by FACS (lower). (b) The effect of Notch1-IC on the cleavage of procaspase-3 and PARP in 30 J/m² UV-treated HCT116 *p53*^{+/+} cells was examined. HCT116 *p53*^{+/+} cells (2×10^6) were transfected with increasing amounts (500, 1000, 1500, and 2000 ng) of Notch1-IC expression vector. Transfected cells were irradiated with 30 J/m² UV. The proteolytic cleavage of procaspase-3 or PARP was examined by SDS-PAGE and immunoblot analysis using anti-PARP or anti-caspase-3 antibody. (c) The effect of Δ RAM-N1-IC on UV-induced apoptosis was assessed by the cleavage of procaspase-3. HCT116 *p53*^{+/+} cells (2×10^6) were transfected with varying amounts (500, 1000, 1500, and 2000 ng) of expression vector for Δ RAM-N1-IC. Proteolytic cleavage of procaspase-3 was examined by immunoblot analysis using anti-caspase-3 antibody. (d) siRNA-induced knockdown of Notch1 abrogates the inhibitory effects of Notch1-IC on PARP cleavage in UV-treated HCT116 *p53*^{+/+} cells. Cells were transfected with Notch1 siRNA or control siRNA. After incubation for 24 h, cells were irradiated with 30 J/m² UV and incubated for additional 24 h. The cleavage of PARP was analyzed by immunoblot analysis using anti-PARP antibody

contrast, the C-terminal fragment of Notch1-IC, which lacks the ability to interact with p53, failed to inhibit p53 phosphorylation and its transactivation (Figure 4). Whether or not the interaction between Notch1-IC and p53 plays an essential function in Notch-mediated repression of p53 transactivation still remains to be established. However, it is unlikely that the binding of Notch1-IC to p53 directly hinders the binding of p53 to its recognition sites of DNA and represses p53 transactivation. As the central DNA-binding domain of p53 (amino acids 94–293) lacks the ability to interact with Notch1-IC. Notch1-IC bound to the N-terminal domain of p53 might not be directly responsible for the inhibition of the binding of central p53 DNA-binding domain to DNA. It is noteworthy that post-translational modifications such as phosphorylation and acetylation have been implicated in the regulation of p53 sequence-specific DNA binding.³¹ In addition, there are studies suggesting that the C-terminal domains of p53 could indirectly influence the sequence-specific p53 DNA binding.^{37,38}

The results in Figure 5 rule out the possibility that Notch signaling modulates p53 transactivation function through Notch target genes. HES family proteins are transcriptional repressors that act as Notch effectors by negatively regulating the expression of downstream genes such as tissue-specific transcription factors that induce neuronal differentiation.⁴

Expression of Δ RAM-N1-IC mutant lacking the RAM domain, which is the main CSL interaction site, failed to activate *HES-1* promoter linked to luciferase reporter in transfected HCT116 *p53*^{-/-} cells (Figure 5a). Nonetheless, Δ RAM-N1-IC, which lacks Notch1 transcriptional activity, repressed p53-dependent transactivation as much as wild-type Notch1-IC (Figure 5b). Moreover, overexpression of Δ RAM-N1-IC also inhibited endogenous p53 phosphorylation at Ser 15 and 37 in UV-treated HCT116 *p53*^{+/+} cells (Figure 5c). Both wild-type Notch1-IC and Δ RAM-N1-IC deletion mutant were capable of binding to p53 (Figure 5d). These results support our interpretation that physical interaction between activated Notch1 and p53 is important for both the inhibition of p53 phosphorylation and repression of its transactivation by Notch signaling.

Previous studies have defined the antiapoptotic role of Notch signaling in both animal^{30,39} and cultured *in vitro* cell models.^{28,29} Our results in Figure 6 suggest that the negative impact of Notch signaling on p53-dependent apoptosis correlates with decreased activation of caspase-3 and reduced PARP cleavage. Although the precise mechanism by which the physical interaction between p53 and activated Notch modulates p53 function remains to be elucidated, our findings offer an alternative role for Notch signaling in counteracting p53 tumor suppressor activity during tumorigenesis.

Materials and Methods

Cells and antibodies. Human colon cancer HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cells grown in DMEM supplemented with 10% FBS (Gibco BRL) were transfected using Lipofectamine Plus reagent (Invitrogen) as specified by the manufacturer. Antibodies against PUMA, caspase-3, and PARP, and phospho-specific antibodies that recognize phosphorylated p53 on each Ser residue were purchased from Cell Signaling. Antibodies against FLAG, GST, MDM2, α -Tubulin, and β -Actin were supplied by Sigma. Antibodies directed against C-terminal Notch1, p21, HES1, HA, N-terminal p53 (Bp53-12 and DO-1), C-terminal p53 (C-19), and 14-3-3 ϵ were from Santa Cruz. FITC-conjugated anti-CD20 antibody was from Becton Dickinson.

Plasmids. cDNAs encoding full-length human p53 (amino acids 1 – STOP) and p53 fragments N293 (amino acids 1–293), 94C (amino acids 94 – STOP), and 94-293 (amino acids 94–293) were cloned in-frame to *KpnI* and *BamHI* sites of pFLAG-CMV-2 vector (Sigma) to generate FLAG-tagged p53 constructs. cDNAs encoding full-length human Notch1-IC (amino acids 1762 – STOP) and Notch1-IC deletion mutants N-N1-IC (amino acids 1762 – 2148), C-N1-IC (amino acids 2147 – STOP), and Δ RAM-N1-IC (amino acids 1877 – STOP) were cloned in-frame to *HindIII* and *BamHI* sites of pFLAG-CMV-2 vector (Sigma) to generate FLAG-tagged Notch1-IC constructs. cDNAs encoding *Notch1-IC* and *p53* were cloned into *BamHI* and *NotI* sites of mammalian GST fusion vector, pEBG, to generate expression vectors for GST-Notch1-IC and GST-p53. Δ RAM-N1-IC was cloned into *BamHI* and *Clal* sites of pEBG to produce GST- Δ RAM-N1-IC.

Luciferase assay. For the p53 reporter assay, cells seeded on a 12-well plate (2×10^5 cells/well) were transfected with p53-responsive reporter gene construct (100 ng) driven by a synthetic p53 responsive element or one of the promoters of *p21*, *mdm2*, or *bax* with or without p53 expression vector (100 ng), together with increasing amounts of plasmid encoding Notch1-IC, Δ RAM-N1-IC, N-N1-IC, or C-N1-IC. For the Notch1 reporter assay, HCT116 *p53*^{-/-} cells were cotransfected with a reporter plasmid containing either $6 \times$ NRE (Notch Response Element) or *HES* promoter together with plasmid for each Notch1 construct. Luciferase activity was assayed 24 h after transfection using Luciferase Assay System (Promega). The values of luciferase activity were normalized for transfection efficiencies by measuring β -galactosidase activities using Luminescent β -galactosidase detection kit II (Clontech).

Coimmunoprecipitation. HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cells were grown in 100 mm dishes and lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 200 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Equal amounts of protein lysate were mixed with the antibody recognizing C-terminal p53 (C-19) or C-terminal Notch1. After incubation for 16 h at 4°C, 50 μ l 50% Protein G Sepharose (Amersham Biosciences) was added and mixed at 4°C for an additional 2 h. The bound proteins were washed six times with phosphate-buffered saline (PBS) and analyzed by 12% SDS-PAGE followed by immunoblotting using antibodies directed against C-terminal Notch1 or N-terminal p53 (Bp53-12).

GST pull-down assay. HCT116 *p53*^{-/-} cells (2×10^6) were transfected with 2 μ g total plasmid DNA. After incubation for 24 h, transfected cells were lysed in the lysis buffer described above. Cell lysates were mixed with Glutathione-Sepharose 4B beads (Amersham-Pharmacia) by rocking at 4°C for 16 h. The bound proteins were washed three times with PBS containing 500 mM NaCl and three times with PBS. The proteins bound to GST fusion proteins were analyzed by 10% SDS-PAGE followed by immunoblotting using antibodies directed against the respective epitopes.

EMSA. An aliquot (30 μ l) of reaction mixture containing 10 μ g nuclear proteins, 10 mM HEPES (pH 7.5), 50 mM KCl, 0.5 mM EDTA, 0.2 mM BSA, 1 mM DTT, 10% glycerol, 3 μ g poly(dI-dC), and approximately 0.5 ng of ³²P-labeled DNA probe was incubated for 30 min at room temperature. The oligonucleotides used to probe p53 DNA-binding sites had the following sequences: 5'-AGC TTA GGC ATG TCT AGG CAT GTC TA-3' from *p21* promoter and 5'-TAG AGC GAA CAT GTC TAA GCA TGC TGG CGT CG-3' from *GADD45* promoter. The oligonucleotides were annealed with their complementary strands and end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase. The reaction mixture was then subjected to electrophoresis in $0.5 \times$ TBE buffer ($1 \times$ TBE: 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) on a 5% native polyacrylamide gel at 120 V. Gels were dried and DNA-protein complexes were visualized by autoradiography.

ChIP. ChIP experiments were carried out as described.⁴⁰ Briefly, chromatin from HCT116 *p53*^{+/+} cells overexpressing p53 and Notch1-IC was crosslinked using 1% formaldehyde for 10 min at 37°C. After phenol-chloroform extraction and ethanol precipitation, the DNAs recovered from the immunocomplex and input material were PCR-amplified using primers designed to amplify the p53-binding site in *p21* gene (forward primer: 5'-GTG GCT CTG ATT GGC TTT CTG-3', reverse primer: 5'-CTG AAA ACA GGC AGC CCA AG-3') or *PUMA* gene (forward primer: 5'-CTG TGG CCT TGT GTC TGT GAG TAC-3', reverse primer: 5'-CCT AGC CCA AGG CAA GGA GGA C-3'). PCR was also performed using primers for *GAPDH* gene (forward primer: 5'-TAC TAG CGG TTT TAC GGG CG-3', reverse primer: 5'-TCG AAC AGG AGG AGC AGA GAG CGA-3') as an internal control experiment.

Northern blot analysis. HCT116 *p53*^{+/+} cells (2×10^6) were transfected with Notch1-IC expression vector (500 and 1000 ng). After incubation for 24 h, transfected cells were treated with 30 J/m² UV and harvested after postincubation for 8 h. Total RNAs were extracted using Trizol Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. RNA samples (10 μ g each) were fractionated on a 1.2% agarose-formaldehyde gel, and transferred to a positively charged nylon membrane. Specific primer for *Notch1* gene (forward oligonucleotide: 5'-AGC TCT GGT TCC CTG AGG GCT T-3', reverse oligonucleotide: 5'-ATG CAG TCG GCG TCA ACC TCA C-3') or *p21* gene (forward oligonucleotide: 5'-GTG TGA GCA GCR GCC GAA GT-3', reverse oligonucleotide: 5'-TTT TCG ACC CTG AGA GTC TCC A-3') was used for PCR. Whole-cell RNA was hybridized with DIG-labeled probes and assayed according to the manufacturer's protocol at 50°C for 12 h (Roche).

RNA interference. HCT116 *p53*^{+/+} cells were transfected with 0.13 μ M *Notch1* siRNA or control siRNA (Santa Cruz) using Oligofectamine (Invitrogen) as described by the manufacturer's instruction. Experiments were carried out 24 h after siRNA transfection.

Apoptosis assay. HCT116 *p53*^{+/+} cells (2×10^6) were transfected with Notch1-IC expression vector (2 μ g) together with expression vector for CD20 (200 ng), a marker of transfected cells. After incubation for 24 h, cells were treated with specified doses of UV, postincubated for 24 h, harvested and mixed with FITC-conjugated anti-CD20 antibody (Becton Dickinson) and incubated for 20 min on ice. Cells were then fixed with ice-cold 70% ethanol and incubated on ice for 30 min. The effect of Notch silencing on UV-induced apoptosis was carried out using Notch1 siRNA. Cells were transfected with Notch siRNA or control siRNA and incubated for 24 h. Cells were irradiated with 30 J/m² UV. Cells were incubated for additional 24 h and harvested. Fixed cells were treated with RNaseI and stained with 10 μ g/ml propidium iodide (PI). DNA contents were determined by FACS. Cells with DNA content at sub-G0/G1 phase were considered as apoptotic cells. Apoptotic cell death was also examined by monitoring the proteolytic cleavage of PARP and procaspase-3. The immunoblot analysis was carried out using antibodies against each protein.

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