Suppression of p53 by Notch in Lymphomagenesis: Implications for Initiation and Regression

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Abstract

Aberrant Notch signaling contributes to more than half of all human T-cell leukemias, and accumulating evidence indicates Notch involvement in other human neoplasms. We developed a tetracycline-inducible mouse model (Top-Notch^{ic}) to examine the genetic interactions underlying the development of Notch-induced neoplastic disease. Using this model, we show that Notch suppresses p53 in lymphomagenesis through repression of the ARF-mdm2-p53 tumor surveillance network. Attenuation of Notch expression resulted in a dramatic increase in p53 levels that led to tumor regression by an apoptotic program. This shows that continued Notch activity is required to maintain the disease state. However, all tumors relapsed with rapid kinetics, most of which, by reactivation of Notch expression. Furthermore, by directly inhibiting the mdm2-p53 interaction by using either ionizing radiation or the novel small molecule therapeutic Nutlin, p53 can be activated and cause tumor cell death, even in the presence of sustained Notch activity. Therefore, it is the suppression of p53 that provides the Achilles heel for Notch-induced tumors, as activation of p53 in the presence of Notch signaling drives tumor regression. Our study provides proof-of-principle for the rational targeting of therapeutics against the mdm2-p53 pathway in Notch-induced neoplasms. Furthermore, we propose that suppression of p53 by Notch is a key mechanism underlying the initiation of T-cell lymphoma. (Cancer Res 2005; 65(16): 7159-68)

Introduction

The mammalian Notch gene family consists of four closely related members (*Notch1-4*). Notch genes are critically important for determining cell fate decisions of many cell types during development. Substantial evidence exists that all Notch family members play a role in neoplastic transformation, in both human and animal models (1–7). Notch was first implicated in human T-cell acute lymphoblastic leukemia (T-ALL) by the finding that the *Notch1* gene was involved in a chromosomal translocation t(7;9)(q34;q34.3) in cells derived from a T-ALL patient (3). This translocation fuses the *T-cell receptor* β locus to a portion of the *Notch1* gene leading to expression of a Notch protein that lacks most of the extracellular domain, termed N^{ic}. A recent report identified point mutations and deletions that has extended the involvement of Notch in human T-ALL to >50% of all cases (8). It is now clear that N^{ic} is a bona fide oncogene that is associated with initiation and progression of neoplastic disease. However, the precise biochemical mechanism for how N^{ic} is able to elicit cellular transformation is not well understood. The current model suggests that N^{ic} drives transformation by direct transcriptional activation of genes through a multiprotein complex containing CSL and Mastermind (Maml-1; refs. 9–12).

The tumor suppressor protein p53 plays a critical role in maintaining cellular homeostasis both in normal development and following various cellular stresses, including DNA damage and nutrient deprivation (13–15). Activation of p53 directs cells to undergo cell cycle arrest and/or apoptosis depending on the type and extent of the stimulus. Regulation of p53 turnover is largely mediated by the ubiquitin ligase mdm2, which is the primary negative regulator of p53 (16–18). Furthermore, expression of ARF, which binds to and inhibits the activity of mdm2, leads to activation of p53 (19, 20). It is thought that the ARF-mdm2-p53 pathway acts as a tumor surveillance mechanism that is activated in response to inappropriately sustained proliferative signals (21, 22). Therefore, many oncogenes cause an inherent apoptotic response that places a selective pressure on mutating this surveillance mechanism. This is apparent from the observation that all three genes in the pathway are commonly mutated in cancers, which ultimately leads to inactivation of p53 function (23–26).

Herein, we report the finding that Notch signaling suppresses p53 activity during leukemogenesis. Following inactivation of Notch signaling, p53 protein levels accumulate and cells rapidly initiate an apoptotic program. Inhibition of the mdm2-p53 interaction by ionizing radiation or Nutlin is sufficient to activate p53 in N^{ic} lymphoma. This indicates that p53 protein levels are suppressed by a mechanism involving mdm2-dependent degradation of p53. In support of this model, we show that Notch-induced lymphomas do not express ARF. Furthermore, we show that efficient regression of N^{ic}-induced lymphoma requires p53 activity. Although inactivation of the N^{ic} transgene leads to tumor regression, 100% of mice relapse within 6 weeks. Interestingly, in a majority of the relapsed tumors the Notch transgene is reactivated, indicating that there is a strong selective pressure to reacquire Notch activity. Therefore, suppression of p53 by Notch is an important event in the development of lymphoma and the activation of p53 mediates regression of disease.

Materials and Methods

Mice. Top-Notch^{ic} transgenic mice were engineered by subcloning human N^{ic} cDNA, encoding amino acids 1758 to 2556 into the UHD 10-3 vector (kindly provided by H. Bujard, Heidelberg, Germany). EμSR-tTA mice (tTA) were described previously (27).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Tumorigenesis assays. Mice that were either compound hemizygous for tTA-N^{ic} or hemizygous Top-Notch^{ic} mice were monitored for up to 1 year for signs of hematopoietic disease. Mice that were given doxycycline were maintained on 100 μg/mL in the drinking water. Single-cell suspensions of leukemia cells were prepared by standard procedures. RBC were lysed in RBC lysis buffer (150 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 10 μmol/L EDTA) for 10 minutes on ice.

Flow cytometry. Single-cell suspensions were used for all fluorescence-activated cell sorting (FACS) analysis. Antibodies for flow cytometry were obtained from PharMingen (San Jose, CA) and used as indicated by supplier. Cells were analyzed in the FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) using Cellquest software. Data shown is representative of multiple mice.

Protein analysis. Tissues from indicated mice were pulverized in liquid nitrogen using mortar and pestle. Protein extracts were prepared by lysis of cells in standard lysis buffer and proteins were detected using standard techniques as previously described (28). Size exclusion chromatography was done as described using an AKTA fast protein liquid chromatography system, as previously described (10).

Southern blot analysis. Southern blot analysis was done using standard techniques.

Reverse transcription-PCR analysis. RNA was extracted from tumor samples using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Total RNA (2 μg) was used to produce cDNA in a 20 μL reaction using MLV reverse transcriptase (Promega, Madison, WI) according to manufacturer's specifications.

Cell culture and *in vitro* studies. Molt-3, SUP-T1, and Jurkat were obtained from American Type Culture Collection (Manassas, VA). NA1 were provided by K. Georgopoulos (Massachusetts General Hospital, Charlestown, MA). Cell lines were maintained in RPMI supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine. Cells were treated with 5 μmol/L Nutlin (Cayman Chemical, Ann Arbor, MI), 200 nmol/L wortmanin (Sigma, St. Louis, MO), 10 μmol/L UO126 (EMD Biosciences, San Diego, CA), or 25 μmol/L MG132 (EMD Biosciences) all prepared in DMSO for the indicated times.

Results

Inducible tumorigenesis by N^{ic}. We engineered transgenic mice that express human N^{ic} (amino acids 1758-2556) under the transcriptional control of the tetracycline operator sequences (Top-Notch^{ic}). Hemizygous Top-Notch^{ic} mice were born with the expected Mendelian frequency, develop normally, and do not express the Top-Notch^{ic} transgene (Fig. 1*A-D*; Supplementary Fig. S1*A*). Mice that are double hemizygous for the Top-Notch^{ic} transgene were generated by backcrossing hemizygous siblings. Double hemizygous Top-Notch^{ic} mice were then mated to hemizygous transgenic mice that express the tetracycline transactivator (tTA) in T-cell progenitor cells under the control of the immunoglobulin heavy-chain enhancer and Srα promoter (EμSR-tTA). The EμSR-tTA has been previously described and expresses the tTA in T cells, leading to tissue type–specific activation of genes placed under the control of the tetracycline operator sequences (27). Resulting offspring included hemizygous Top-Notch^{ic} and compound hemizygous Top-Notch^{ic}/EμSR-tTA (tTA-N^{ic}) mice on an inbred FVB/n background. Offspring were monitored for up to 1 year for signs of hematopoietic disease. A cohort of compound hemizygous mice was given doxycycline in their drinking water to suppress expression of the Top-Notch^{ic} transgene. tTA-N^{ic} mice not treated with doxycycline developed aggressive leukemia/lymphoma with a median onset of disease of ~30 weeks with the earliest onset of 7 weeks after birth (Fig. 1*A*). By 1 year of age, 60% of tTA-N^{ic} mice succumb to disease in the absence of doxycycline. Visual autopsy of leukemic tTA-N^{ic} mice revealed gross splenomeglia, enlarged lymph nodes, and thymic involvement compared with disease-free Top-Notch^{ic} mice (Fig. 1*B*). Western blot analysis of thymocytes (*t*) and splenocytes (*s*) from leukemic tTA-N^{ic} mice using an affinity purified Notch^{ic} antibody showed high levels of N^{ic} expression (Fig. 1*C*). FACS analysis of splenocytes from Top-Notch^{ic} mice revealed normal ratios of mature $CD4^+$ and $CD8^+$ single-positive T cells in the periphery (Fig. 1*C*). In contrast, nearly all splenic-derived lymphocytes in the tTA-N^{ic} mice were immature $CD4^+/CD8^+$ double-positive T cells, which is a diagnostic characteristic of leukemia (Fig. 1*C*). Histologic analysis of tissues obtained from tTA-N^{ic} leukemic mice showed effacement of the spleen, bone marrow, and thymus with leukemic T cells. The liver also contained numerous focal sites of lymphoma (see Supplementary Fig. S1*A*).

To show that *in vivo* tumor formation in the mouse is biochemically analogous to transformation by N^{ic} in cell culture and in human samples, we analyzed N^{ic} nuclear complex formation (10, 28). N^{ic} from tTA-N^{ic} leukemia cells was predominantly in a large molecular weight complex with Mastermind-1 and CSL, as determined by size exclusion chromatography (Fig. 1*D*; data not shown). Taken together, these data show that this is an authentic model for Notch-induced T-ALL.

tTA-N^{ic} leukemia cells require sustained Notch expression. To address if leukemic cells derived from tTA-N^{ic} mice are dependent on the continuous expression of the Top-Notch^{ic} transgene, we did leukemia transplant experiments. tTA-N^{ic} mice that were moribund due to lymphoma were euthanized and single-cell suspensions were prepared from lymphoid organs. For these experiments, 5×10^5 cells were transplanted i.v. into the tail vein of multiple nontransgenic FVB/n mice. Cohorts of recipient mice were either treated with doxycycline or received no treatment. Recipient mice that were not treated with doxycycline developed T-cell leukemia by 12 weeks of age, whereas mice maintained on doxycycline in the drinking water remained disease free. Moreover, a single dose of doxycycline (250 μg) injected i.p. on the day following transplant, was sufficient to inhibit tumor formation in all experiments ($n = 4$, data not shown). Gross autopsy of mice that succumb to disease in the absence of doxycycline displayed a similar phenotype compared with the primary leukemic mice (Fig. 2*A*). This result also shows that the disease is an authentic lymphoma and not simply a lymphoproliferative disease. Western blot analysis showed the absence of N^{ic} expression from the transgene in the doxycycline-treated mice, whereas nontreated mice expressed the transgene (Fig. 2*B*). As before, examination of CD4 and CD8 T-cell surface markers on splenocytes displayed an accumulation of immature $CD4^+/CD8^+$ double-positive T cells (Fig. 2*C*). Leukemic cells acquired from this analysis were then transplanted into 3- to 6-week-old nontransgenic recipients in the presence or absence of doxycycline for up to five serial passages. For each passage, leukemogenesis in the transplanted animals maintained the requirement for N^{ic} expression (data not shown). This finding is consistent with our previous observation, *in vitro*, that tamoxifen-regulated ER-N^{ic}-transformed RKE cells maintain the requirement for activated Notch (29).

To determine if a primary, late-stage N^{ic}-induced lymphoma can be reverted by inhibiting N^{ic} expression, we treated tTA-N^{ic} mice that displayed visible signs of lymphoma with doxycycline. On the initial day of treatment, mice were moribund and presented with multiple enlarged lymph nodes in the neck and legs, enlarged thymus, and palpable lymphoma in the abdomen, which represents involvement of the spleen and liver (Fig. 2*D*, day 0). Three days following doxycycline treatment, all mice were rejuvenated with a striking reduction in the size of the lymph nodes, thoracic and abdominal

cavities (Fig. 2*D*, day 3). In the period between 5 and 17 days, most mice typically displayed no signs of disease and were virtually indistinguishable from disease-free mice. (Fig. 2*D*, day 17). These data show that tTA-N^{ic} leukemia have a strict requirement for Notch activity to maintain the leukemic phenotype.

Rapid regression is accompanied by apoptosis. To address the mechanism of regression, multiple mice were injected i.v. with 1×10^6 cells from the same primary lymphoma and allowed to develop signs of advanced disease. Mice were then given doxycycline and regression of disease was analyzed over a time course beginning at 6 hours after administration of doxycycline. The most striking sign of disease regression is the marked decrease in the overall size of the spleen within 4 days following doxycycline treatment, as shown by gross autopsy at each time point (Fig. 3*A*). N^{ic} expression is extinguished within 6 hours of administration of doxycycline, as determined by the analysis of both protein and mRNA levels (Fig. 3*B*). Furthermore, regression was analyzed at a cellular level using flow cytometry to follow both apoptosis and T-cell differentiation over a time course following administration of doxycycline (Fig. 3*B*). We observed a wave of apoptotic cells as measured by an increase in Annexin V–positive staining cells that peaked at 36 hours after initiation of continuous doxycycline treatment. The increase in the percentage of cells undergoing apoptosis was observed in all hematopoietic organs including thymus, spleen, lymph nodes, and bone marrow (Fig. 3*B*; data not shown). Notch-induced leukemias have a characteristic CD4$^+$/CD8$^+$ dual-positive immature T-cell

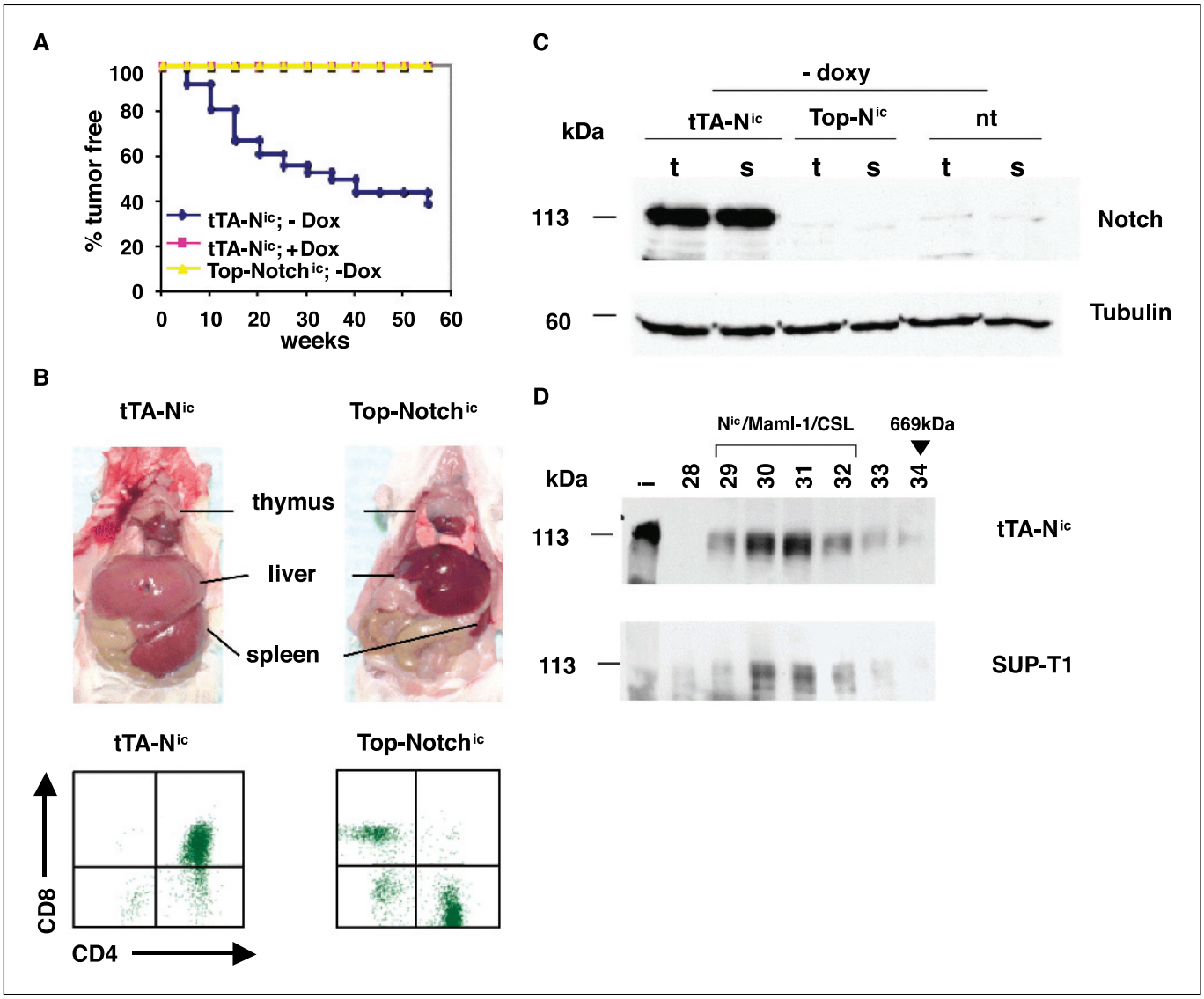

Figure 1. Characterization of tTA-N^{ic} leukemia. *A,* survival curves for mice monitored in this study. *Triangles,* Top-Notch^{ic} hemizygous mice (n = 15); *squares,* compound hemizygous tTA-N^{ic} mice administered doxycycline (*doxy, n* = 15); *diamonds,* compound hemizygous tTA-N^{ic} mice on normal drinking water (n = 36). *B,* gross autopsy of Top-Notch^{ic} and tTA-N^{ic} lymphoma. Lines indicate location of thymus, liver, and spleen (*top*). Splenocytes from Top-Notch^{ic} and tTA-N^{ic} leukemia were stained with FITC-TCRβ, Cy-Chrome-CD8α, and PE-CD4α followed by three-color FACS analysis. Data shown is gated on TCRβ-positive cells. *C,* Western blot analysis of Notch expression (*top*) from thymocytes (*t*) or splenocytes (*s*) from nontransgenic (*nt*), Top-Notch^{ic} (*Top-N*), and tTA-N^{ic} leukemic mouse. Tubulin expression for each lysate is shown as a loading control (*bottom*). *D,* nuclear lysates were prepared from tTA-N^{ic} leukemic cells (*top*) and from the SUP-T1 cell line (*bottom*). (*bottom*). Nuclear lysates were separated on a Superose6 gel filtration column followed by Western blot analysis using an affinity-purified Notch antibody (*927*). Column fractions are indicated above (*28-34*), approximate molecular weights are indicated to the left (kDa) and arrowhead at fraction 34 indicates the migration of the size standard, thyroglobulin (669 kDa). *i*, 5% of total input loaded onto the column.

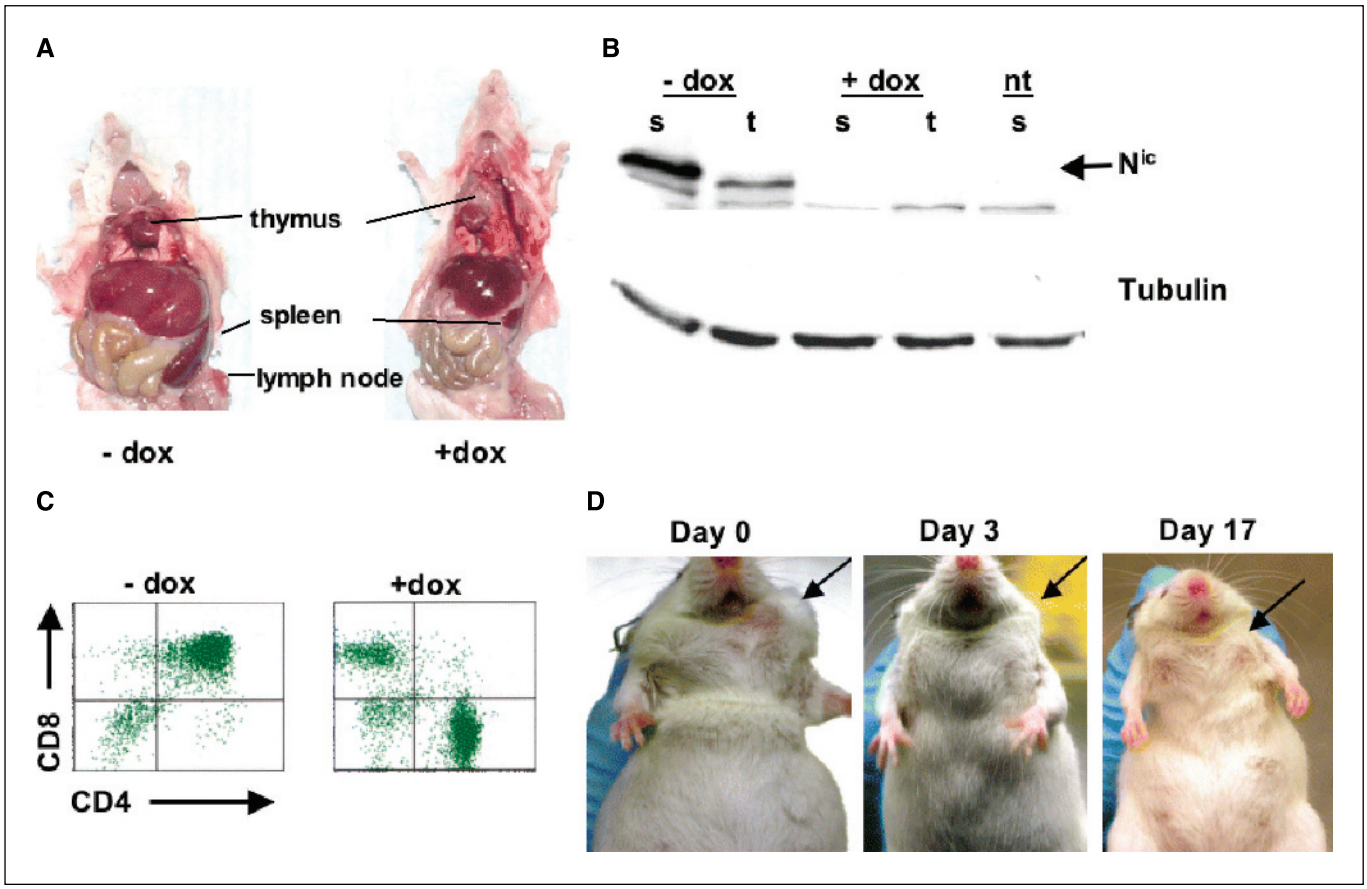

Figure 2. Notch induced leukemia is dependent on activated Notch expression. *A,* gross autopsy of transplant recipient mice that received 5×10^5 leukemia cells. Mice were either placed on doxycycline water (+ *doxy*) or left on normal water (− *doxy*). *B,* Western blot analysis of tissues from transplant recipient mice treated with doxycycline (+ *doxy*) or untreated with doxycycline (− *doxy*) following transplantation and a nontransgenic (*nt*). Notch expression is shown (*top*) and tubulin was visualized as a loading control (*bottom*). *C,* FACS analysis was done on splenocytes from transplant recipient mice as in Fig. 1*D*. *D,* moribund tTA-N^{ic} mice present with multiple enlarged lymph nodes and an enlarged abdomen (*arrows*, day 0). The mice was placed on doxycycline drinking water and documented 3 days later. Notice the reduction in lymph nodes 3 days following doxycycline treatment (day 3) as well as noticeable reduction in thoracic and abdominal sections. Mice were continually monitored and most mice look indistinguishable from nontransgenic mice (day 17). Representative of multiple experiments.

phenotype; therefore, we examined the CD4 and CD8 status of T cells in the periphery following doxycycline administration to determine if mature single-positive T cells were able to develop. We observed a gradual increase in normally differentiated TCR-positive cells in the periphery throughout the time course, beginning as early as 24 hours (Fig. 3*B*; data not shown). The brisk appearance of normal $CD4^+$ and $CD8^+$ single-positive T cells in the periphery might indicate that following inactivation of Notch there is a dual wave of apoptosis and differentiation that occurs following inactivation of N^{ic} expression.

Regression of Notch-induced leukemia is followed by rapid relapse. An emerging concept in tumor biology is that of oncogene addiction, such that a tumor cell maintains a strict dependence on the initiating oncogenic mutation. To determine if this might be the case in N^{ic} lymphoma, we treated leukemic mice with a constant doxycycline regimen. As before, all tumors regressed immediately following administration of doxycycline; however, 100% of the mice rapidly relapsed with disease with a mean latency of 20 days and the longest latency of 40 days (Fig. 3*C*). To investigate the nature of the relapse we first analyzed expression from the transgene by doing both Western blot and reverse transcription-PCR (RT-PCR) analysis using transgene-specific primers. We observed that the majority (60%) of the relapsed tumors reactivated expression of the Top-$Notch^{ic}$ transgene (Fig. 3*D*). To show that these tumors are indeed insensitive to doxycycline treatment, we transplanted leukemic cells from relapsed mice into naive recipient mice and either treated doxycycline or left mice untreated. In all cases tested, mice that received cells from relapsed mice developed disease with the same latency whether doxycycline was given or not (Fig. 3*C*). In those cases where the transgene was reactivated in the relapsed lymphoma, the expression of the Notch transgene was insensitive to the presence of doxycycline in the drinking water (see Supplementary Fig. S1*B*). These data indicate that although tumors regress following attenuation of the Notch signal, the majority of tumors relapse by reactivating the transgene. This data suggests a strong selective pressure to reacquire Notch activity and thus showing a true addiction to the initiating oncogenic lesion.

Notch suppresses p53. To identify the molecular mechanism of apoptotic death that occurs following inactivation of the Notch transgene, we examined the status of the tumor suppressor protein p53. To address the role of p53 in regression of N^{ic}-induced lymphoma, multiple mice were transplanted with a single primary leukemia and were allowed to develop advanced disease. Doxycycline was then given and two mice were euthanized at each time point following the initiation of doxycycline treatment. At the time of treatment ($T = 0$), the tumors displayed high levels of N^{ic} expression and nearly undetectable levels of p53. Over the

course of the experiment, there was a dramatic increase in p53 protein level that began to emerge at 24 hours and peaked at 36 hours after initiation of doxycycline treatment (Fig. 4*A*). The increase in p53 protein levels is concomitant with the increase in the percentage of apoptotic cells, suggesting that p53 may be instructing the apoptotic program following inhibition of Notch signaling (Fig. 3*B*). To further characterize the relationship between Notch signaling and p53, we did Western blot analysis on a panel of primary tTA-N^{ic} tumors (Fig. 4*B*). Nearly all tumors displayed high levels of N^{ic} protein and low or undetectable levels of p53 protein. This indicates that Notch activity has a role in regulating p53 protein stability. To genetically examine the relationship between N^{ic} and p53, mice that were compound double hemizygous for both the Top-$Notch^{ic}$ and tTA transgenes were mated with mice heterozygous for *p53* (30). The resulting offspring included mice that were compound hemizygous for Top-$Notch^{ic}$ and tTA (tTA-N^{ic}) or mice compound hemizygous for Top-$Notch^{ic}$ and tTA and heterozygous for p53 (tTA-N^{ic}/$p53^{+/-}$) in an F1 hybrid FVB/129 background. Kaplan-Meier analysis of the offspring from these matings showed that onset of disease was unaffected by p53 status (Fig. 4*C*). Analysis of the expression levels of N^{ic} and p53 revealed that tumors expressed N^{ic} but did not express p53. Loss of heterozygosity (LOH) at the wild-type allele of p53 is typically associated with progression of tumorigenesis in many model systems. Although all the tTA-N^{ic} and tTA-N^{ic}/$p53^{+/-}$ mice displayed expression of p53 mRNA, we analyzed the p53 genomic locus for LOH by Southern blot analysis. We examined a tTA-N^{ic} and a tTA-N^{ic}/$p53^{+/-}$ mouse that succumbed to disease with the

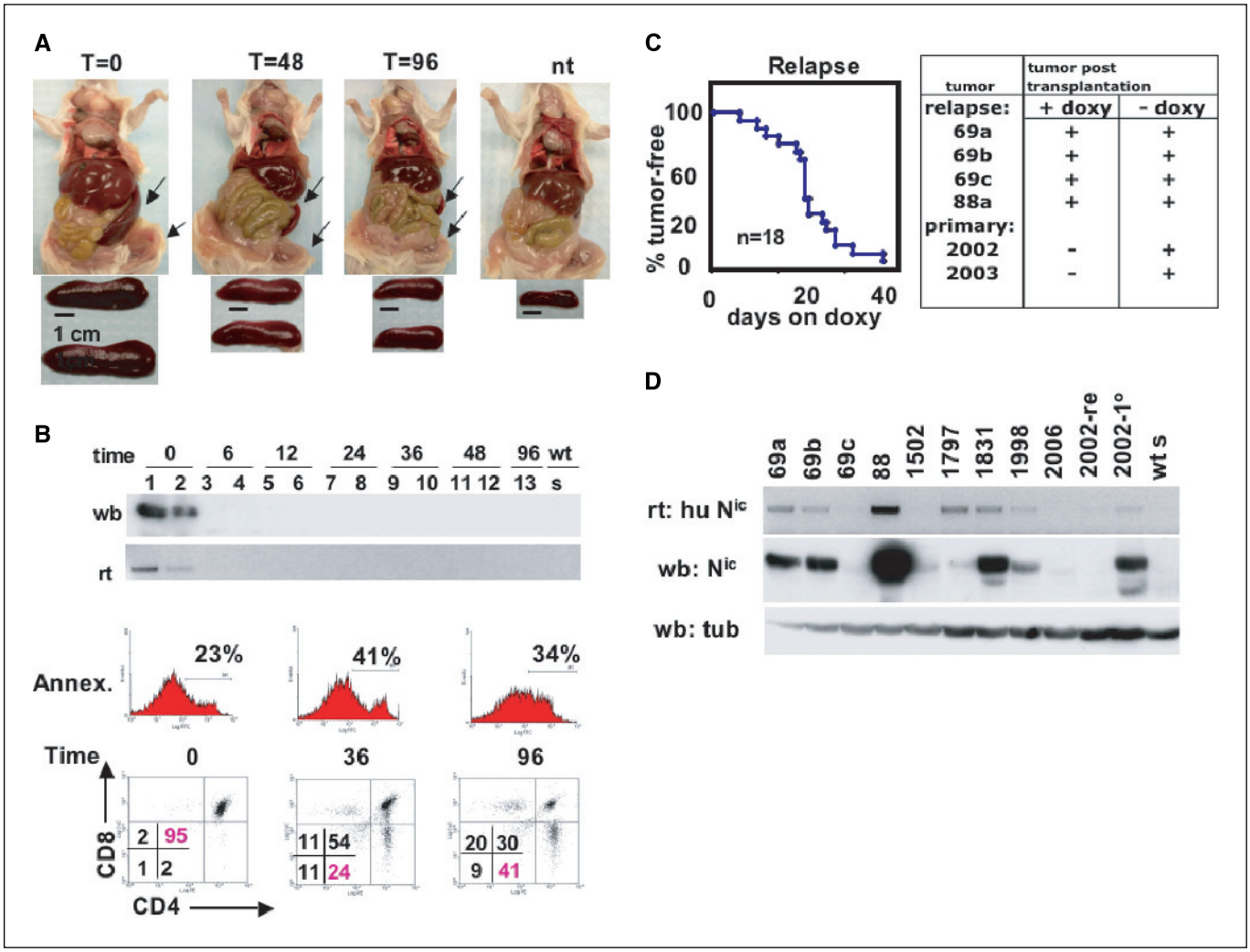

Figure 3. Doxycycline treatment of moribund tTA-N^{ic} mice results in tumor regression and rapid relapse. *A,* multiple mice were transplanted with the same lymphoma and once mice became moribund with disease they were administered doxycycline (*doxy*). Two mice were euthanized at the time of treatment ($T = 0$) and at the specified times thereafter. The regression of disease was followed by gross autopsy and compared with a nontransgenic control (*nt*). Notice the marked reduction in the size of the spleen over the period of doxycycline treatment (*bottom*). Spleen samples are shown at the same scale with the line corresponding to 1 cm. *B,* tissues from the mice above were analyzed for expression of the transgene by both Western blot analysis (*top*) and RT-PCR using transgene specific primers (*bottom*). Single-cell suspensions were prepared from the spleens of the mice and were analyzed by FACS analysis as in Fig. 1*D*. In addition, the percentage of cells undergoing apoptosis was also detected by FACS analysis by counting the number of cells that stained positive for FITC-Annexin V. Representative of at least three different primary transplants. *C,* survival curve of moribund tTA-N^{ic} mice placed on continuous doxycycline treatment ($n = 18$, *left*). Serial transplant of relapsed lymphoma cells into nontransgenic mice results in doxycycline-insensitive tumors (relapse), whereas leukemic cells transplanted from primary tumors (primary) grow only in the absence of doxycycline following transplant. *D,* expression analysis for the Top-$Notch^{ic}$ transgene was done from the splenocytes of mice that relapse with disease. RT-PCR (*top*) and protein (*middle*) was detected from relapse lymphoma. Tubulin is shown as a loading control (*bottom*). *2002-1°,* primary tTA-N^{ic} lymphoma; *2002-re,* relapse of 2002-1° following transplant and doxycycline treatment.

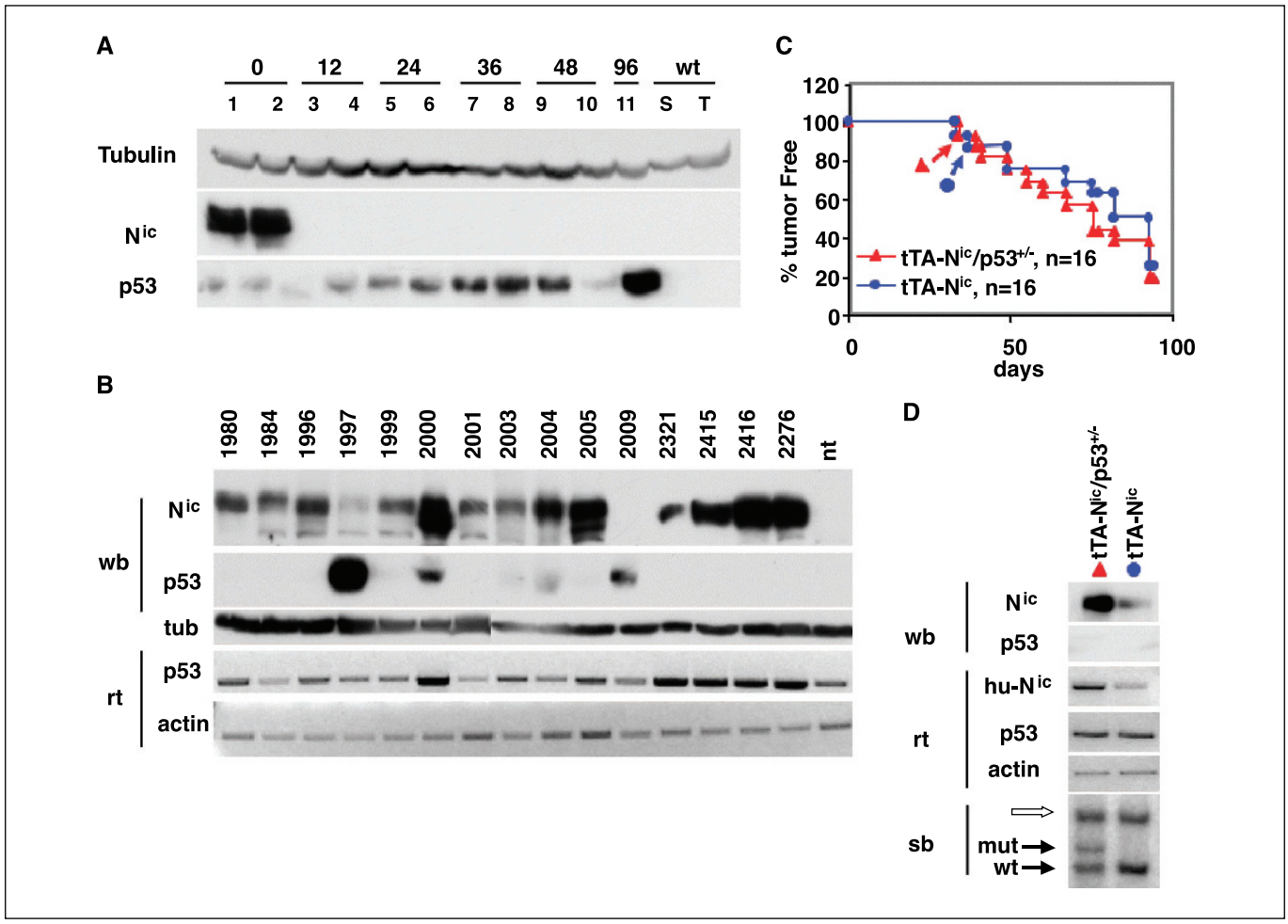

Figure 4. Notch suppresses p53 activity. *A,* multiple mice were transplanted with the same lymphoma and followed as in Fig. 3*A*. Western blot analysis was done on the mice from the time course with antibody against the Notch transgene (*middle*), p53 (*bottom*), and tubulin (*top*) as a loading control. Numbers on top indicate the time in hours following the administration of doxycycline (*doxy*). Samples were compared with the spleen (*S*) and thymus (*T*) from a nontransgenic mouse (*nt*). *B,* Western blot (*wb*) and RT-PCR (*rt*) analysis was done for the indicated proteins from a panel of primary tTA-N^{ic} tumor samples compared with a nontransgenic mouse. *C,* Kaplan-Meier analysis of tTA-N^{ic} mice mated with $p53^{+/-}$. *Arrows,* mice analyzed in (*D*). *D,* Western blot (*wb*), RT-PCR (*rt*), and Southern blot analysis was done on two mice that succumbed with disease at the earliest onset. *Open arrow,* pseudo gene (*bottom*).

quickest onset and observed no LOH, indicating that there is no selective pressure to lose the *p53* locus during N^{ic}-induced tumorigenesis (Fig. 4*D*). These data further supports the hypothesis that Notch signaling suppresses p53 function during the progression of disease.

We next assessed if p53 status affects the ability of the disease to regress following inactivation of the Notch transgene. Primary tTA-N^{ic} and tTA-$N^{ic}/p53^{+/-}$ mice were allowed to become moribund and then given doxycycline in the drinking water. Mice were then euthanized 5 days following doxycycline treatment and the status of regression was analyzed by gross autopsy (Fig. 5*A*). Compared with tTA-N^{ic} littermates, tTA-N^{ic} /$p53^{+/-}$ mice displayed a clear deficiency in tumor regression. As further support for the role for p53 in the regression of N^{ic}-induced lymphoma, the levels of both p53 protein and message were dramatically diminished in tumors that displayed incomplete regression. Reduction of p53 expression was not due to LOH at the wild-type allele of p53, as shown by Southern blot analysis, nor was this diminution of p53 expression due to reactivation of the Top-Notch^{ic} transgene, as the samples were negative for human Notch message and protein (data not shown). If p53 is responsible for regression of N^{ic}-induced lymphoma, we rationalized that the kinetics of regression would be affected by the loss of one allele of p53 in the tTA-$N^{ic}/p53^{+/-}$ mice. To examine the kinetics of regression, tTA-N^{ic} and tTA-$N^{ic}/p53^{+/-}$ mice that had lymphoma were treated with doxycycline and the percent of cells undergoing apoptosis was determined 48 hours later (Fig. 5*B*). Again, consistent with a role for p53 in the regression of N^{ic}-induced tumors, we observed that tumors from tTA-$N^{ic}/p53^{+/-}$ mice do not efficiently initiate an apoptotic response when compared with tumors from tTA-N^{ic} mice that have two copies of the *p53* gene. Thus, Notch suppresses functional p53 and inhibition of Notch expression results in the activation of a p53-dependent apoptotic program.

Inhibition of the mdm2-p53 interaction leads to activation of p53 in N^{ic} tumor cells. Results from the biochemical and genetic experiments provide strong evidence for the role of Notch in suppressing p53 activity. Because mdm2 is the major factor known to directly regulate p53 stability, we examined the role of mdm2 in the suppression of p53 by N^{ic}. We took advantage of stimuli that are known to inhibit the mdm2-p53 interaction and asked if they could lead to p53 protein accumulation and subsequent apoptosis of tumor cells. We treated cells derived from tTA-N^{ic} tumors, as well as

additional murine and human cell lines with either Nutlin or ionizing radiation. Nutlin is a small molecule that was designed to bind to mdm2 and disrupt the mdm2-p53 interaction (31). When cells derived from a primary tTA-N^{ic} tumor are exposed to either ionizing radiation or Nutin, there is a dramatic increase in p53 protein levels, which is accompanied by an increase in the number of apoptotic cells (Fig. 6*A*). Similarly, treatment of nontransgenic splenocytes with Nutlin and ionizing radiation resulted in an accumulation of p53, indicating that tTA-N^{ic} tumor cells has a normal p53 response. To further understand the mdm2-p53 interaction, we examined a number of established cell lines that express constitutive N^{ic}. The Molt-3 cell line was derived from a human T-ALL, and the NA1 cell line is a murine T-cell lymphoma line (provided by K. Georgopoulos), both of which express constitutively nuclear Notch that is in a high molecular weight complex similar to tTA-N^{ic} tumors (Fig. 1*F*; Supplementary Fig. S1*C*). In these cell lines, p53 was undetectable by Western blot analysis; however, treatment of these cells, with either Nutlin or ionizing radiation, led to a rapid accumulation of p53 protein, revealing that these cells also have a wild-type p53 response (Fig. 6*B*). Consistent with this, p53 levels accumulate upon treatment with the proteosome inhibitor MG-132 (Fig. 6*B*). In addition, accumulation of p53 in these cells is accompanied by an increase in the percentage of cells that stain positive for the apoptotic marker, Annexin V (Fig. 6*A*; Supplementary Fig. S1*D*). In contrast, Notch-expressing cell lines treated with either the phosphatidylinositol 3-kinase inhibitor, wortmanin, or the mitogen-activated protein kinase inhibitor UO126 as a control did not lead to accumulation of p53 or p53-dependent apoptosis (Fig. 6*B*; Supplementary Fig. S1*D*). Two cell lines, Jurkat and HH, which do not have constitutive Notch activity or functional p53, do not display any significant differences in cell viability following treatment with any of the inhibitors, indicating that the apoptosis observed in the NA1 and Molt-3 cells is specifically

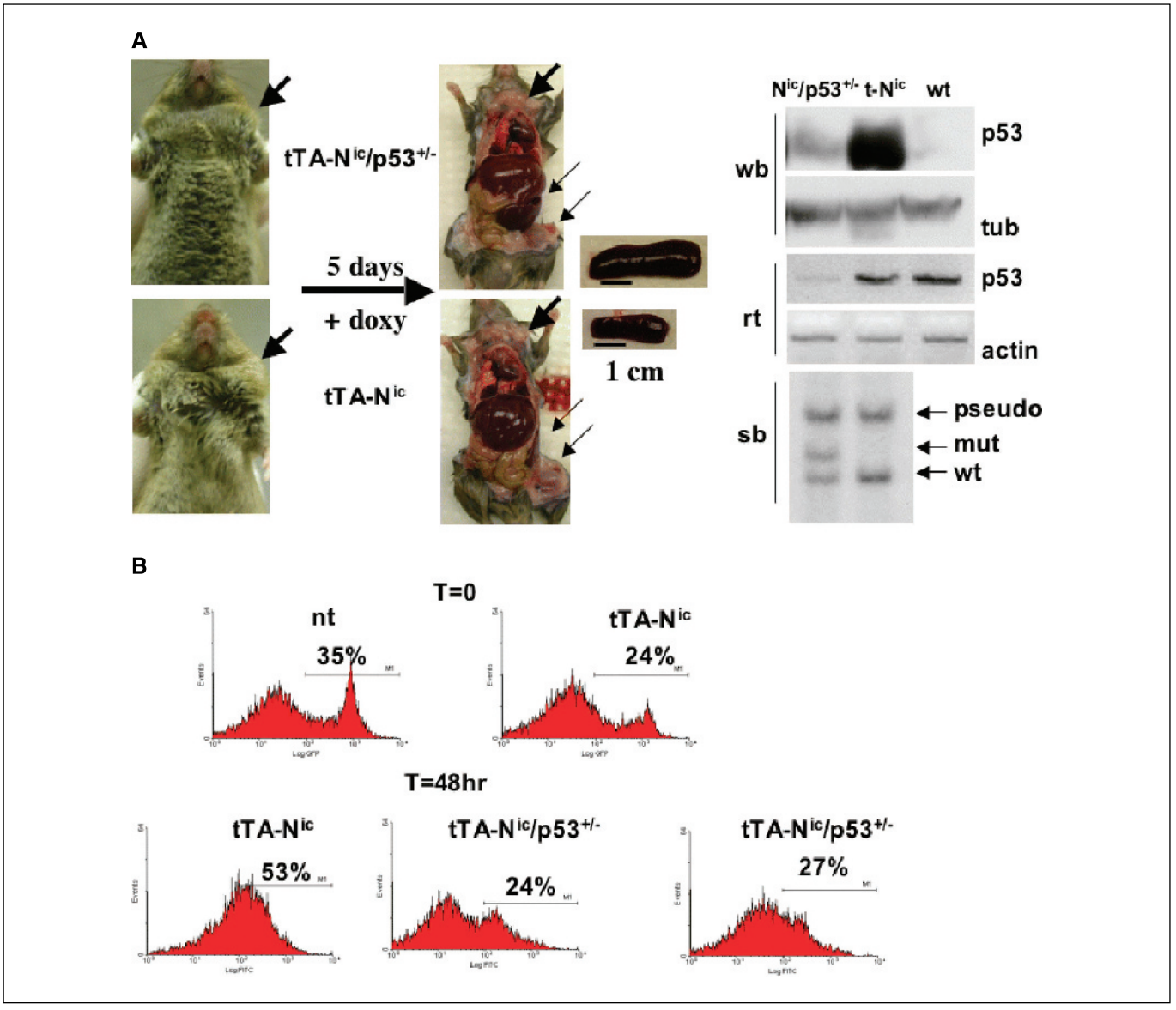

Figure 5. tTA-N^{ic}/p53$^{+/-}$ mice have altered regression kinetics following inhibition of Notch signaling. *A,* gross autopsy of tTA-N^{ic} and tTA-N^{ic}/p53$^{+/-}$ mice that were euthanized 5 days following doxycycline (*doxy*) treatment. Expression analysis of these mice shows a decrease in p53 expression in the tTA-N^{ic}/p53$^{+/-}$ (N^{ic}/p53$^{+/-}$) mouse compared with the tTA-N^{ic} (t-N^{ic}) mouse. Southern blot (*sb*) shows no loss of heterozygosity in tTA-N^{ic}/p53$^{+/-}$ mouse. *B,* tTA-N^{ic} and tTA-N^{ic}/p53$^{+/-}$ mice that were moribund were given doxycycline.

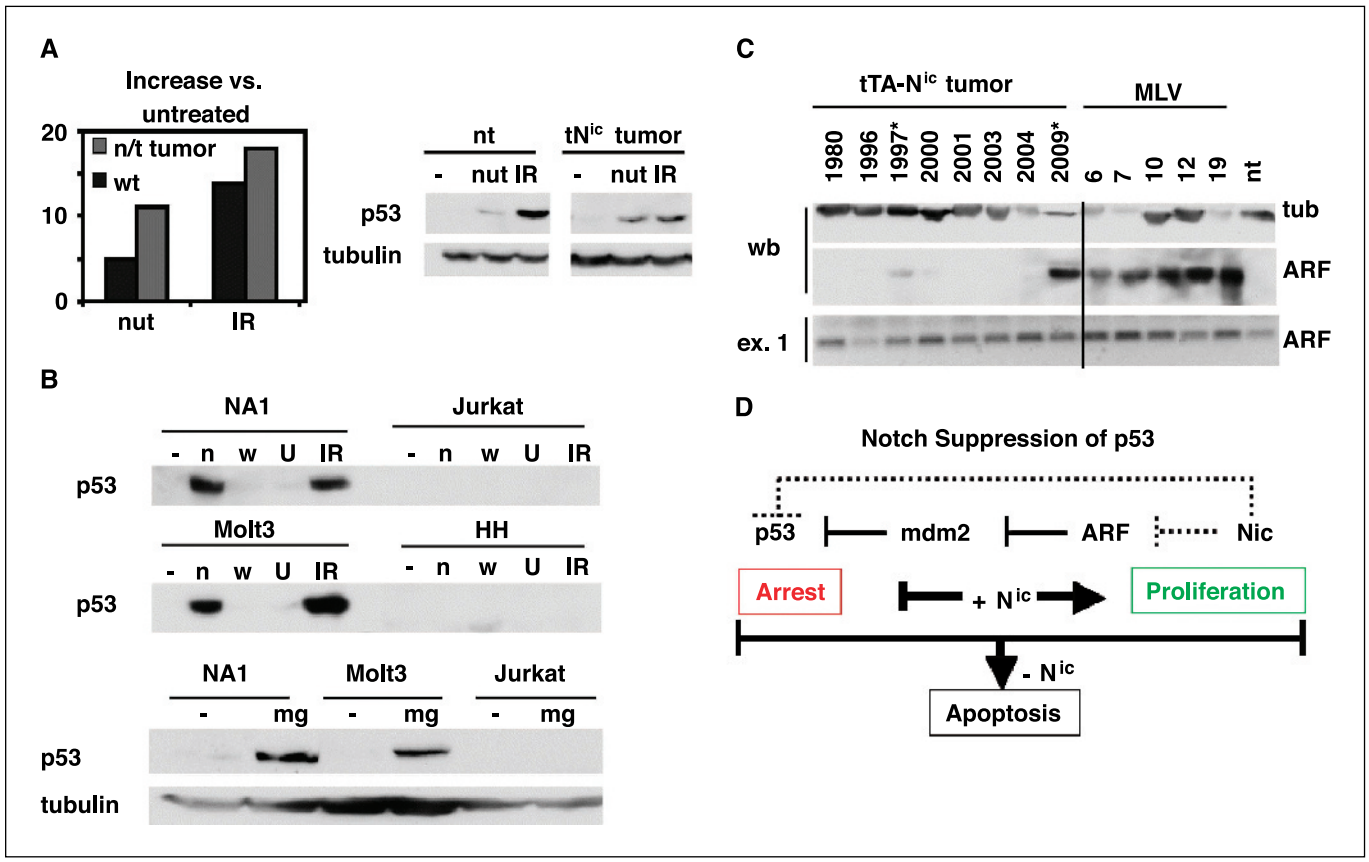

Figure 6. Inhibition of the mdm2 interaction activates p53 in N^{ic} expressing cells. *A,* single-cell suspensions were made from the spleen of either nontransgenic (*nt*) or tTA-N^{ic} (tN^{ic} tumor) leukemic spleen. Cells were treated as indicated for 4 hours. Western blot analysis for p53 protein (*top*), percent increase in Annexin-positive staining cells compared with nontreated cells (*bottom*). *B,* analysis of cell lines following treatment with (−) DMSO, (*n*) nutlin, (*w*) wortmanin, (*U*) UO126, (*IR*) irradiation. Western blot analysis for p53 4 hours following treatment (*top*), percent increase in the number of cells that stain positive for Annexin V 10 hours following treatment when compared with DMSO treatment only (*bottom*). Western blot analysis for p53 (*top*) and tubulin (*bottom*) 2 hours following treatment of indicated cells lines for 2 hours. DMSO (−) and MG-132 (*mg*). *C,* analysis of $p19^{ARF}$ from a panel of primary tTA-N^{ic} tumors compared with tumors from nontransgenic FVB mice infected with the insertional mutagen MLV. Western blot analysis (*wb*), RT-PCR from cDNA extracted from the tumor samples (*rt*), PCR from genomic DNA extracted from tumor samples (ex.1). Tubulin loading control (*tub*), antibody or primers specific to the p19ARF protein/gene (*ARF*); loading control for RT-PCR (*b-actin*). tumors 1997* and 2009* are the tumors with the least or no detectable N^{ic} expression (see Fig. 4*B*). *D,* the model representing the suppression of p53 by Notch. We provide data showing that a p53 response is suppressed in tumors expressing N^{ic} (*dotted line*). We provide direct evidence that this suppression to be caused by inactivation/repression of the ARF mdm2-p53 tumor surveillance system (*solid lines*). Constitutive Notch activity ($+N^{ic}$) leads to proliferation and inhibition of the p53 response. Inactivation of Notch activity ($-N^{ic}$) in tumors leads to activation of p53 and apoptosis.

due to the activation of p53 (Fig. 6*B*; Supplementary Fig. S1*D*). Therefore, in primary tTA-N^{ic} tumors, as well as in cell lines that have constitutive Notch activity, p53 is suppressed through an mdm2-dependent mechanism. Furthermore, overcoming the mdm2-p53 interaction drives apoptosis of N^{ic} leukemic cells, even in the presence of Notch activity.

To further understand the mechanism by which Notch is regulating the mdm2-p53 pathway, we examined the status of $p19^{ARF}$ in primary tTA-N^{ic} tumors. $p19^{ARF}$ is activated during aberrant proliferation and binds to mdm2 to inhibit its activity, leading to activation of p53 and apoptosis. In this manner, the ARF-mdm2-p53 pathway is thought to behave as a tumor surveillance mechanism against aberrantly proliferating cells. Western blot analysis using an antibody to $p19^{ARF}$ showed that primary tTA-N^{ic} tumors do not express detectable levels of $p19^{ARF}$, whereas a panel of random MLV-induced tumors express high levels of $p19^{ARF}$ (Fig. 6*D*). PCR analysis of exon 1b from the ARF locus revealed that the locus is intact in tTA-N^{ic} tumors, indicating that Notch is likely affecting ARF protein levels. The finding that tTA-N^{ic} tumors do not express $p19^{ARF}$ supports the model that p53 is constitutively degraded through an mdm2-dependent mechanism.

Discussion

The use of inducible transgenic models permits investigation of issues related to the initiation, progression, and maintenance of neoplastic diseases, in contrast to noninducible models that only allow the investigation of oncogenic potential. Herein, we describe the development of a tetracycline inducible mouse model for Notch-induced T-cell lymphoma. We show that both initiation and maintenance of lymphoma requires continued expression of N^{ic} and that this requirement on Notch activity lies, in part, in the ability of Notch to suppress p53 function. Following inactivation of the Notch transgene in mice, p53 levels accumulate in tumors, which then regress as a consequence of apoptotic death. Furthermore, p53 can be activated in tumor cells expressing active Notch by various stimuli, also resulting in the initiation of apoptosis. Taken together, these data strongly indicate that suppression of p53 by Notch is an important step in Notch-induced lymphomagenesis. In addition, regression of Notch-induced tumors by attenuation of Notch expression is likely not a state of disease remission, because tumors rapidly relapse in 100% of treated mice with a mean latency of 20 days. These findings support the importance of Notch activity in tumorigenesis

and provide new insights into the molecular mechanisms of Notch-induced tumorigenesis.

Oncogene addiction. In the Top-Notch^{ic} model, 100% of mice relapse with neoplastic disease following inactivation of Notch, the initiating oncogene. Interestingly, in a majority of the relapsed tumors Notch expression was reactivated, indicating a strong selective pressure for Notch activity (Fig. 3). In contrast, other tumor models have dramatically different relapse kinetics and characteristics. For example, the Felsher group reported that expression of c-*myc* under control of the EμSR-tTA leads to T-cell lymphoma (27). The EμSR-tTA mouse strain used in that study is the same as the one we used in our experiments. They reported in their study that inactivation of c-*myc* expression in tumors results in regression and that only 20% of mice relapse with disease after a protracted latency (32). Furthermore, none of the relapsed tumors expressed the human c-*myc* transgene, indicating that relapse is due to newly acquired mutations and not reactivation of the *myc* transgene. The contrast in the kinetics and characteristics of lymphoma relapse in the *myc* and Notch models raises two important points. First, c-*myc*-induced lymphoma have a low potential to relapse, in terms of both latency and incidence (20%), indicating the mice enter a state of disease "remission." However, Notch-induced lymphoma invariably relapse with rapid kinetics following inhibition of the transgene, indicating that these tumors do not enter a state of prolonged disease remission. Second, whereas Notch-induced tumors display a strict requirement for Notch activity, Myc-induced tumors do not seem to reactivate Myc expression in relapsed tumors. Therefore, this leads one to conclude that addiction to the initiating oncogene greatly depends on the particular oncogene. However, does the tissue type of the tumor influence the dependence on the initiating oncogene? The Chodosh group has reported that c-*myc*-induced mammary adenocarcinomas also regress following inactivation of the *myc* transgene, but the kinetics and dependence on *myc* expression is not the same as that reported for T-cell lymphoma (33). Many of these tumors incompletely regress and harbor a mutation in the *Kras2* gene that is thought to exist in the tumor before attenuation of transgene expression. These tumors never reactivate the transgene and display a resurgence in tumor growth within 40 days of *myc* inactivation. Tumors from this study that do completely regress also eventually relapse; however, with a protracted latency and incomplete penetrance, such that of mice that are monitored for >1 year on doxycycline 50% relapse with disease. Again, in contrast to the Notch model, only 20% of these relapsed tumors express the c-*myc* transgene.

What is the driving force for the relapse of neoplastic disease in these mouse models? Several issues come to mind when considering this question; the issues of genomic instability, tumor burden, and cancer stem cells, among others. Using a serial transplantation assay, we clearly showed that tumor burden is linked to relapse of disease in the Top-Notch^{ic} model. For example, if a single-cell suspension (1×10^6 cells), prepared from a N^{ic} lymphoma, is transplanted into a naive recipient mouse, the cells are not capable of tumor formation if Notch signaling is attenuated the day following transplantation (Fig. 2). In contrast, treatment of a mouse with late stage disease results in relapse with a frequency of 100%. Furthermore, after multiple (5) serial transplants of a single tTA-N^{ic} lymphoma, cells never lost sensitivity to doxycycline, indicating that even after multiple cycles of tumor formation cells never emerged that were insensitive to doxycycline, providing evidence that mutation drives relapse and not cancer stem cells in the Top-Notch^{ic} model. Therefore, we propose that in the case of Notch, the driving force in relapse is the generation of an environment that is conducive to mutations by massive cell death and large tumor burden. Moreover, Notch does not inherently induce genomic instability but that genomic instability is a consequence of the environment. In contrast, Myc drives genomic instability, which results in the accumulation of mutations throughout the initiation of neoplastic disease; this hypothesis is supported by the report from the Felsher and Chodosh groups (33).

Regulation of p53 activation by Notch. In this report, we show that Notch suppresses p53 in lymphomagenesis. We derive this conclusion from several lines of evidence. Western blot analysis of a panel of primary N^{ic}-induced lymphoma showed that nearly all N^{ic}-induced tumors have undetectable levels of p53 protein (Fig. 6*A*). Furthermore, p53 is not mutated in these tumors as shown by the fact that p53 is activated following treatment with γ-irradiation, Nutlin, or immediately following inactivation of the Notch transgene (Figs. 6 and 4). These results strongly indicate that Notch plays a direct role in regulating p53 stability.

To secure a genetic proof to the hypothesis that Notch is suppressing p53 in lymphomagenesis, we examined the effect of mutant p53 on the latency and penetrance of Notch-induced tumor formation. To do this we created a mouse line that harbors the Top-Notch^{ic} transgene on a p53$^{+/-}$ background. For most oncogenes, such as *Myc* and *Wnt-1,* a p53$^{+/-}$ background dramatically accelerates tumor formation concomitant with LOH at the p53 locus (34–36). In contrast, p53 status had no observable effect on tumor initiation or progression by Notch. Furthermore, there was no LOH at the *p53* locus in tumors from these mice (Fig. 4*D*). The clear lack of selective pressure to lose *p53* in the Notch model strongly supports our hypothesis that Notch is actively suppressing p53 in lymphomagenesis.

How does Notch suppress p53 activity in lymphomagenesis? We propose that suppression of p53 by N^{ic} is mediated through an mdm2-dependent mechanism. We draw this conclusion from several experiments. Because we did not observe any decrease in the mRNA levels of p53, we reasoned that p53 must be suppressed by a proteolytic mechanism. In support of this notion, treatment of lymphoma cells with the proteosome inhibitor MG-132 leads to a rapid accumulation of p53 (Fig. 6). Because mdm2 is the primary factor leading to destruction of p53, we sought to examine the status of the mdm2-p53 connection. Using cell lines derived from human and mouse T-cell lymphomas with activated Notch, as well as tTA-N^{ic} lymphoma, we showed that the mdm2-p53 pathway is indeed intact. We showed this by inhibiting the interaction of mdm2 and p53 with two well-characterized stimuli, γ-irradiation and Nutlin. Treatment of cells with these agents dramatically induced p53 levels and apoptosis, indicating that p53 is being held in check by mdm2. These data strongly support the hypothesis that Notch is suppressing p53 via an mdm2 mechanism. Consistent with this hypothesis, we show that tTA-N^{ic} tumors do not express ARF protein. A lack of ARF protein would lead to the constitutive degradation of p53 by mdm2. However, we cannot rule out the possibility that Notch affects p53 stability by an mdm2-independent mechanism. Because Notch suppresses p53, there is no selective pressure against components of the Arf-Mdm2-p53 pathway. Therefore, the possibility remains that Notch could inhibit p53 via a yet to be characterized pathway while leaving the Arf-mdm2-p53 pathway intact, therefore creating, in effect, two independent pools of p53. In such a scenario, we would expect to be able to activate p53 by inhibiting the mdm2-p53 interaction. Considering this possibility, we have examined the expression of

Cop1 and Pirh2 and did not observed any changes in mRNA levels (data not shown). However, we also investigated effects on mdm2 and we did not observe and significant differences. Reports in the literature have suggested that Notch affects aspects of mdm2 function, but we failed to reproduce these in our studies (37). Therefore, the precise mechanism for the suppression of p53 by Notch remains elusive, but it seems that the likely mechanism is through disruption of the Arf-mdm2-p53 tumor surveillance pathway. Regardless of the mechanism, our study reveals that inhibition of the mdm2-p53 interaction is sufficient to cause accumulation of p53 and induce apoptosis and thereby provides a rational basis for treatment of Notch-positive tumors.

Clinical implications. How does the ability of Notch to suppress p53 in lymphomagenesis pertain to human T-ALL? In a survey of pediatric T-ALL, the Hayashi group found that only 5% (3 of 57) patients harbored mutations in the *p53* gene, indicating that suppression of p53, rather than mutation of the *p53* gene might be a more common event in human T-ALL (38). However, in this same study they reported that 67% (12 of 18) of established T-ALL cell lines had mutations in p53, representing a fundamental difference between primary samples and cell lines. Consistent with this study only one (6%, 1 of 15) of primary tTA-N^{ic} lymphomas expressed a potential mutated p53 (Fig. 4*B*). We propose that the high incidence of Notch mutations in patients contributes to the low incidence of p53 mutations in T-ALL patients. Accordingly, we show that p53 is capable of being activated and cause apoptosis of Notch expressing lymphoma cells. We propose that therapeutics designed to activate p53 by specifically inhibiting its proteolytic degradation will be an effective class of treatments for patients with mutated Notch. To this end, we show that treatment of Notch expressing cell lines or Notch-expressing lymphoma with the small molecule Nutlin or ionizing radiation leads to cell death through a p53-dependent apoptotic program (Fig. 6). This line of experiments provides a proof-of-principle for this class of therapeutics and shows that if suppression of p53 is overcome, Notch expressing tumor cells can be eradicated.

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