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Inactivation of *p53* is insufficient to allow B cells and B cell lymphomas to survive without *Dicer*

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Abstract

Inactivation of p53, the master regulator of cellular stress and damage signals, often allows cells that should die or senesce to live. Loss of *Dicer*, an RNAse III-like enzyme critical in microRNA biogenesis, causes embryonic lethality and activation of the p53 pathway. Several non-hematopoietic cell types that contain inactivated p53 have been shown to survive *Dicer* deletion, suggesting p53 loss may protect cells from the negative consequences of *Dicer* deletion. However, here, we report that loss of p53 did not provide a survival advantage to B-cells, as they underwent rapid apoptosis upon *Dicer* deletion. Moreover, a deficiency in *p53* neither rescued the *Dicer* deletion-induced delay in Myc-driven B-cell lymphomagenesis, nor allowed a single B-cell lymphoma to develop with biallelic deletion of *Dicer*. A p53 deficiency did, however, restore the pre-B/B-cell phenotype and CD19 surface expression of the lymphomas that emerged in conditional *Dicer* knockout Eμ-*myc* transgenic mice. Moreover, p53 loss in transformed B-cells did not confer protection from apoptosis, as *Dicer* deletion in established *p53*-null B-cell lymphomas induced apoptosis, and all of the 1,260 B-cell lymphoma clones analyzed that survived Cre-mediated *Dicer* deletion retained at least one allele of *Dicer*. Moreover, *Dicer* deletion in lymphomas *in vivo* reduced tumor burden and prolonged survival. Therefore, inactivation of *p53* is insufficient to allow untransformed B-cells and B-cell lymphomas to survive without *Dicer*, presenting a potential therapeutic opportunity for the treatment of B-cell lymphomas.

Keywords

B cell lymphoma; Dicer; microRNA; Myc; p53

Introduction

MicroRNA (miRNA) are small non-coding RNA that regulate gene expression post-transcriptionally and have essential roles in development, proliferation, apoptosis, and transformation (1, 2). Alterations in miRNA expression are linked to tumor development, including hematopoietic malignancies (1, 2, 3). Moreover, the oncogene c-Myc, which is

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frequently overexpressed in many human malignancies and a driver of B-cell lymphomagenesis, transcriptionally regulates the expression of many miRNA (4).

miRNA are transcribed in a precursor form and processed with enzymes, such as Dicer, an RNase III enzyme with critical roles in cell differentiation, proliferation, and survival (5). Loss of one allele of *DICER* or reduced DICER expression or enzymatic activity is reported in multiple solid organ tumors (6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16). Mouse models revealed Dicer is a haploinsufficient tumor suppressor in soft tissue sarcoma, lung adenocarcinoma, and retinoblastoma (17, 18). In contrast, we showed *Dicer* heterozygosity had no effect on the rate of B-cell lymphoma development (19). Therefore, differences in the requirements for Dicer and the effects of reduced Dicer expression in different tissues remain unresolved.

The p53 tumor suppressor, which induces apoptosis or cell cycle arrest upon cellular stresses (20), responds to defects in miRNA biogenesis, and therefore, may be required to signal problems in this pathway. Specifically, in untransformed murine embryonic fibroblasts (MEFs), deletion of *Dicer* leads to p53 activation and premature senescence, which is delayed with loss of *p53* (21). We previously detected an increased frequency of *p53* inactivation in lymphomas in a mouse model of Myc-induced B-cell lymphoma (Eμ-*myc*) expressing B-cell-directed Cre and two conditional *Dicer* alleles, suggesting a connection between *p53* activation and *Dicer* deletion in B-cells (19). Moreover, data from three groups, including our own, showed expression of Cre in $Dicer^{fl/fl}$ mice in B-cell progenitors or mature B-cells results in B-cell apoptosis (19, 22, 23). This apoptosis was partially rescued by overexpressing the anti-apoptotic Bcl-2 protein or reducing the pro-apoptotic Bim protein (22). Although *p53*-null murine sarcoma cells and p53 inactivated mesenchymal stem cells can survive *Dicer* deletion (23), *p53* deletion was synthetically lethal in Dicer and Rb deficient retinal progenitor cells (24). Therefore, the role of p53 in monitoring defects in miRNA biogenesis and cell survival in the context of a *Dicer* deficiency remains unclear.

Using mouse models, we determined the contribution of p53 to B-cell survival and lymphoma development with loss of Dicer. A *p53* deficiency did not rescue the defect in B-cell development, the reduction in B-cell survival, or the delay in Myc-induced lymphomagenesis upon *Dicer* deletion. It did restore the B-cell lymphoma phenotype. However, none of the lymphomas that emerged had deleted both alleles of *Dicer*. Moreover, established B-cell lymphomas lacking *p53* underwent apoptosis when *Dicer* was deleted, significantly extending survival in mouse models. Thus, p53 loss is insufficient to allow survival and growth of B-cells and B-cell lymphomas in the absence of Dicer, and thus, targeting Dicer may have therapeutic potential for treating B-cell lymphomas.

Materials and Methods

Mice

C57Bl/6 Eμ-*myc* (25) and CD19-*cre* (26) transgenic mice, $Dicer^{fl/fl}$ mice from Dr. Steve Jones (21), and $p53^{-/-}$ mice from Dr. Guillermina Lozano (27) were intercrossed to obtain the mice needed for this study. Littermates were used in all analyses. For experiments with nude mice, 1.5×10^6 or 0.5×10^6 *p53* deleted $Dicer^{fl/fl}$/Eμ-*myc* lymphoma cells expressing a

tamoxifen-inducible form of Cre ($CreER^{T2}$) were injected (subcutaneous or intravenous, respectively) into 6-week-old *Foxn1*$^{nu/nu}$ female mice (Harlan labs). Tamoxifen (2 mg) or corn oil (vehicle control) was injected (intraperitoneal) once daily for 3 days starting the day of lymphoma injection for two cohorts (one subcutaneous and one tail vein injected cohort) or after lymphomas were 90–150mm^3 for a second subcutaneous cohort. Subcutaneous tumors were measured with calipers and tumor volume calculated. Blood was collected for flow cytometric and microscopic analyses from the mice where lymphoma was injected into the tail vein. Mice were humanely sacrificed prior to lymphoma development or for survival studies, at humane endpoints, and tumors/tissues were harvested and analyzed. Log-rank tests determined statistical significance for survival. All studies were in accordance with state and federal guidelines and were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Western and Southern blotting

Whole cell protein lysates from B-cell lymphomas and pre-B cells were generated and Western blotted as previously described (28). Antibodies against p19Arf (GeneTex), p53 (Ab-7; Calbiochem), Mdm2 (C-18; Santa Cruz), Cre (Novagen), Dicer (Cell Signaling), cleaved Caspase 3 (Cell Signaling), and β-actin (Sigma) were used. As previously described (28, 29), *p53* was sequenced and Southern blots for *p53* with genomic DNA from lymphomas was performed.

Phenotype analysis

Lymphoma cells and splenocytes from littermates prior to lymphoma development were analyzed by flow cytometry following incubation with fluorochrome-linked antibodies against surface receptors as previously reported (19, 29).

Quantitative real-time PCR

Total RNA was isolated from lymphomas with TRIzol (Invitrogen) according to the manufacturer's protocol. As previously described, cDNA was generated, and SybrGreen (SABiosciences) and TaqMan MicroRNA Assays (Applied Biosciences) were used to perform qRT-PCR, in triplicate, for mRNA and miRNA analysis, respectively (19, 30). mRNA and miRNA expression were normalized to *β-actin* and *RNU6b* expression, respectively, and the data presented as $2^{-\Delta Ct}$.

Dicer gene rearrangement analysis

Genomic DNA was isolated from frozen and cultured lymphomas, pre-B cells, and MEFs using the REDExtract-N-Amp Tissue PCR Kit (Sigma). PCR was performed with primers specific for unrearranged and Cre-lox-deleted *Dicer* alleles, as previously published (19, 21). PCR conditions allowed for 10–15% contaminating normal tissue without detecting unrearranged floxed *Dicer* alleles.

Pre-B cell and lymphoma cell survival analyses

Primary pre-B cell cultures from *p53*$^{-/-}$/*Dicer*$^{fl/fl}$, *p53*$^{+/-}$/*Dicer*$^{fl/fl}$, and *p53*$^{-/-}$/*Dicer*$^{+/fl}$ mice and primary *p53* deleted or *Arf* deleted *Dicer*$^{+/fl}$ or *Dicer*$^{fl/fl}$ Eμ-*myc* lymphoma cells

were generated as previously described (19, 28). Cells were infected with a bicistronic retrovirus (MSCV) encoding CreER^{T2} (31) and GFP or GFP alone. Cell number and viability were determined by Trypan Blue Dye exclusion assays and proliferation was measured by MTS assays (490 nm; CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega) after plating equal numbers of cells, in triplicate, and adding 1 μM 4-OHT or vehicle (ethanol) control. Apoptosis was evaluated by Western blotting for cleaved Caspase 3 and by flow cytometry following propidium iodide staining for fragmented (sub-G1) DNA and Annexin V/7-AAD staining after adding 1 μM 4-OHT or vehicle (ethanol) control in triplicate, *in vitro*, or after administering tamoxifen or vehicle (corn oil) for the nude mouse experiments. For single-cell analyses, GFP-positive lymphoma cells were placed one cell/well into 96-well plates by a flow cytometer and visually inspected. Vehicle (ethanol) control or 4-OHT (1 μM) was added to each well and surviving clones were harvested and *Dicer* gene rearrangement was determined by PCR.

Results

p53 deficiency does not rescue lymphoma latency in Myc overexpressing *Dicer$^{fl/fl}$* mice

Previously, we reported *Dicer* deletion in B-cell precursors resulted in delayed Myc-induced B-cell lymphoma development and the inability of a B-cell lymphoma to emerge with biallelic *Dicer* deletion (19). To determine whether B-cell lymphomas could develop without *Dicer* in the context of a *p53* deficiency, we generated *p53$^{+/-}$/Dicer$^{fl/fl}$*/Eμ-*myc* mice and littermate controls that were also transgenic for B lineage-restricted CD19-*cre* recombinase*; p53*-null Eμ-*myc* mice cannot be generated (26). c-Myc in Eμ-*myc* transgenic mice and Cre in CD19-*cre* transgenic mice are first expressed in B-cell precursors and continue throughout the life of the B-cell (25, 26). There was a pronounced delay in lymphomagenesis and extended survival in CD19-*cre^{+}/p53$^{+/-}$/Dicer$^{fl/fl}$*/Eμ-*myc* mice compared to their CD19-*cre^{-}/p53$^{+/-}$/Dicer$^{fl/fl}$*/Eμ-*myc* littermates (53 and 34 days mean survival, respectively; Fig. 1A, $p<0.0001$, log-rank test). All but one (DC1122) of the 23 lymphomas analyzed lacked p53 protein expression, and all overexpressed p19Arf protein, an indicator of p53 inactivation (subset of those analyzed is shown in Fig. 1B). Sequencing of *p53* in DC1122 revealed a mutation (G263R) in its DNA binding domain. Southern blots showed all lymphomas lacking p53 protein had deleted their wild-type allele of *p53* (representative data of those analyzed is shown in Fig. 1C). Therefore, all lymphomas were functionally *p53*-null. In addition, Mdm2, a negative regulator of p53, was overexpressed in 35% of the lymphomas (Fig. 1B). Thus, there was a delay in Myc-induced lymphomagenesis caused by *Dicer* deletion in *p53* heterozygous mice, and a deficiency in Dicer did not alter selection for p53 inactivation in the lymphomas that arose.

Dicer is not a haploinsufficient tumor suppressor in Myc-induced B-cell lymphoma (19). To determine whether a p53 deficiency would allow Dicer to function as a haploinsufficient tumor suppressor in B-cells, we evaluated B-cell lymphoma development in the context of *Dicer* heterozygosity. Cre-positive and Cre-negative *p53$^{+/-}$/Dicer$^{+/fl}$*/Eμ-*myc* transgenic mice had a similar rate of lymphoma development with mean survivals of 35 and 36 days, respectively (Fig. 1A). Evaluation of lymphomas that developed in CD19-*cre^{+}/p53$^{+/-}$/Dicer$^{+/fl}$*/Eμ-*myc* mice showed 100% (17 of 17 analyzed) lacked p53 protein, due to deletion

of the wild-type allele, and overexpressed Arf (subset of those analyzed is shown in Figs. 1D and 1E). These results indicate a p53 deficiency did not allow *Dicer* heterozygosity to accelerate B-cell lymphomagenesis.

Loss of *p53* rescues the type of B-cell lymphoma that develops

Previously, we determined approximately 40% of the lymphomas that emerged in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice were of very early precursor B-cell origin, B220^{+}/CD4^{+}/CD43^{+}/Sca1^{+} (19). We evaluated whether a p53 deficiency would alter the development or frequency of this phenotype by assessing lymphomas from *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice. Fourteen of 16 (88%) of the lymphomas analyzed were typical Eμ-*myc* pre-B and/or B-cell lymphomas (25) and expressed B220 and CD19, and were either IgM^{-} or IgM^{+}; none were B220^{+}/CD4^{+}/CD43^{+}/Sca1^{+} (Table 1). Unexpectedly, 2 of 16 lymphomas were CD3^{-}/CD4^{+}/CD8^{+}/CD43^{+} early T-cell lymphomas (Table 1). All lymphomas analyzed from *p53*$^{+/-}$/CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* littermate controls and from *Dicer* heterozygous *p53*$^{+/-}$/CD19-*cre*$^{+}$/Eμ-*myc* mice were typical Eμ-*myc* lymphomas (Table 1). Thus, a *p53* deficiency fully restored development of the characteristic Eμ-*myc* B-cell lymphoma in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice, but it also allowed T-cell lymphomas to develop.

A deficiency in p53 rescues CD19 expression in B-cell lymphomagenesis

CD19 expression was absent or decreased in 65% of the lymphomas from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice, resulting in reduced or absent Cre expression (19). Preventing CD19 expression was one mechanism by which lymphomas could avoid *Dicer* deletion. To assess the consequences of a *p53* deficiency on CD19 expression in the lymphomas in this study, we evaluated *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas for CD19 cell surface expression. None of the 14 pre-B/B-cell lymphomas analyzed by flow cytometry lacked or had reduced CD19 cell surface expression (Fig. 2A; $p<0.0001$, Fisher's exact test). However, 13 of 23 (57%) *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas analyzed lacked or had significantly decreased Cre protein (Fig. 2B), and 12 of the 13 (92%) had reduced *Cre* mRNA (Fig. 2C). This is an unexpected result, since all the lymphomas expressed CD19 and *Cre* expression is driven by the endogenous *CD19* promoter. Of note, Cre expression occurred significantly more frequently in *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas (43%) than was previously observed in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas [12% (19); $p=0.022$, Fisher's exact test]. Analysis of 17 heterozygous floxed *Dicer p53*$^{+/-}$/CD19-*cre*$^{+}$/Eμ-*myc* lymphomas showed they all expressed Cre protein (Fig. 2D). Therefore, a deficiency in *p53* rescued CD19 surface expression and partially restored Cre expression in B-cell lymphomas from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice.

p53 deficiency is insufficient to allow *Dicer* deletion during B-cell lymphomagenesis

We previously reported that not a single lymphoma from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice had deleted both *Dicer* alleles (19). In this study, we assessed whether the exons flanked by loxP sites in the *Dicer* gene had been deleted. Evaluation of *Dicer* heterozygous *p53*$^{+/-}$/CD19-*cre*$^{+}$/Eμ-*myc* lymphomas showed that all 17 analyzed had deleted their one floxed *Dicer* allele (Fig. 3A). However, 11 of 23 (48%) lymphomas analyzed from *Dicer*$^{fl/fl}$/*p53*$^{+/-}$/CD19-*cre*$^{+}$/Eμ-*myc* mice deleted one conditional *Dicer* allele, whereas the other 12

lymphomas retained both floxed alleles (Fig. 3B). None of the 23 *Dicer*$^{fl/fl}$/*p53*$^{+/-}$/CD19-*cre*$^{+}$/Eμ-*myc* lymphomas had deleted both floxed *Dicer* alleles.

Given that Cre protein expression was lost in half of the *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas, we evaluated whether Cre had ever been functional in these tumors. Four of the 13 lymphomas that lacked Cre protein (Fig. 2B) had rearranged one *Dicer* allele (Fig. 3B), indicating they had active Cre at some point in B-cell development. Because Cre protein was present more frequently in the lymphomas that arose in *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice compared to mice that were *p53*$^{+/+}$ (43% vs 12% (19), respectively), we expected an increased incidence of Cre-mediated deletion of at least one *Dicer* allele in the *p53*$^{+/-}$ lymphomas. However, there was no statistical difference in the frequency of deleting one allele of *Dicer* between these two groups [48% vs 38% (19), respectively; p=0.57, Fisher's exact test; Fig. 3B]. Importantly, our data indicate a p53 deficiency is insufficient to allow a lymphoma to emerge when both alleles of *Dicer* have been deleted.

To determine whether *Dicer* was functional in the lymphomas that emerged, we first assessed *Dicer* protein levels. Lymphomas with one allele of *Dicer* expressed an analogous amount of Dicer protein as lymphomas that retained both alleles of *Dicer* (Fig. 3C). Moreover, mature miRNA transcript levels of miR-20a and miR-31, Dicer-dependent miRNA, were similar regardless of *Dicer* status in all lymphomas analyzed (Fig. 3D). These data indicate all lymphomas, including those with only one *Dicer* allele, expressed wild-type levels of Dicer that was fully functional in miRNA biogenesis.

p53 loss cannot rescue B-cell development following *Dicer* deletion

In vivo, biallelic *Dicer* deletion in developing B-cells with wild-type p53 induces apoptosis, causing a developmental defect, resulting in decreased mature splenic B-cells (19, 22). Protecting B-cells from this apoptosis partially rescues B-cell development (22). Since a p53 deficiency rescued the pre-B/B-cell lymphoma phenotype in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice, we questioned whether p53 mediates the *Dicer* deletion-induced B-cell apoptosis. To address this, we evaluated splenic B-cells from pre-cancerous *p53*$^{-/-}$ and *p53*$^{+/-}$ CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$ mice and CD19-*cre*$^{-}$ littermate controls. There was a modest, but statistically significant, reduction in the percentage of B220^{+}/IgM^{+} B-cells in CD19-*cre*$^{+}$/*p53*$^{-/-}$/*Dicer*$^{fl/fl}$ mice (32.9% ± 1.42) compared to CD19-*cre*$^{-}$/*p53*$^{-/-}$/*Dicer*$^{fl/fl}$ littermates (40.9% ± 1.46; p<0.0001, paired t-test; Fig. 4A). A comparable reduction in B-cells was also observed in *p53*$^{+/-}$ littermates that were either CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$ or CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$ (31.2% ± 0.90 and 40.7% ± 0.51, respectively; p<0.0001, paired t-test; Fig. 4A). As an additional control, we assessed B-cells in *p53*$^{+/+}$/*Dicer*$^{+/fl}$ mice with or without CD19-*cre* and the percentages of B-cells were similar in both, demonstrating B-cell expression of Cre did not alter B-cell numbers in the mice (Fig. 4A). Thus, deletion of one or two alleles of *p53* could not rescue the decrease in B-cell numbers induced by *Dicer* deletion, *in vivo*.

To further test the requirement for p53 in B-cell survival in the absence of Dicer, we derived primary pre-B cells from bone marrow of *p53*$^{-/-}$/*Dicer*$^{+/fl}$, *p53*$^{-/-}$/*Dicer*$^{fl/fl}$, and *p53*$^{+/-}$/*Dicer*$^{fl/fl}$ littermates. Pre-B cells were infected with a bicistronic retrovirus encoding GFP and a 4-hydroxytamoxifen (4-OHT)-inducible CreER^{T2} (31), and GFP-positive cells were

sorted by flow cytometry. All three genotypes of pre-B cells expressed equal levels of CreER^{T2} protein (Fig. 4B). To delete *Dicer*, pre-B cells were treated with 4-OHT to activate CreER^{T2}. As expected for primary pre-B cells with functional *p53*, the *Dicer*$^{fl/fl}$/*p53*$^{+/-}$ cells grew at a slower rate and were sensitive to *Dicer* loss, as indicated by decreased cell numbers and viability (Figs. 4B and 4C). Similarly, following 4-OHT treatment, *Dicer*$^{fl/fl}$/*p53*$^{-/-}$ pre-B cells experienced a dramatic decrease in total number, viability, and growth, and an increased percentage of cells containing fragmented DNA (sub-G1) and appearance of cleaved Caspase 3, compared to vehicle-treated cells, which were unaffected (Figs. 4B–E). When 4-OHT was administered to CreER^{T2} expressing *Dicer*$^{+/fl}$/*p53*$^{-/-}$ pre-B cells, no change in cell number, viability, growth, fragmented DNA, or cleaved Caspase 3 was observed (Figs. 4B–E), as would be expected for pre-B cells with one wild-type *Dicer* allele. *Dicer* gene rearrangement was assessed in the surviving pre-B cells and showed that, regardless of genotype, only one *Dicer* allele was rearranged in the CreER^{T2} activated pre-B cells (Fig. 4F). Notably, *Dicer*$^{fl/fl}$ fibroblasts containing similar levels of CreER^{T2} protein (Fig. 4B) deleted both floxed *Dicer* alleles (Fig. 4F). Therefore, loss of p53 could not rescue the rapid apoptosis induced by biallelic *Dicer* deletion in primary untransformed pre-B cells, and only pre-B cells that had retained one allele of *Dicer* could survive.

Dicer is required for B-cell lymphoma survival

Recently, Dr. Sharp and colleagues reported that a *p53*-null murine sarcoma cell line could survive and proliferate without *Dicer* (23), suggesting cellular transformation may alter the requirements for Dicer. We tested whether transformed B-cells could survive loss of Dicer if they also lacked *p53*. B-cell lymphomas were isolated from two *p53*$^{+/-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice (DC1020 and DC1185) and as controls, two *p53*$^{+/-}$/*Dicer*$^{+/fl}$/Eμ-*myc* mice (DC2385 and DC2423). p53 protein was not detected by Western blot, and Southern blot showed deletion of the remaining wild-type allele of *p53* in all four lymphomas (Figs. 5A, 1D, and 1E). The lymphomas were infected with a bicistronic retrovirus encoding CreER^{T2} and GFP or GFP alone. CreER^{T2} activation with 4-OHT in the *p53* deleted *Dicer*$^{+/fl}$/Eμ-*myc* lymphomas had no effect on cell number compared to a *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma, which showed a significant decrease in cell number after CreER^{T2} activation (Fig. 5B and Supplemental Figure S1). CreER^{T2} activation in both *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas resulted in apoptosis, whereas there was little effect following addition of vehicle control or 4-OHT to lymphomas infected with empty retrovirus (Figs. 5C–F). Specifically, the total number and viability of CreER^{T2} *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cells decreased, while the percentage of apoptotic cells (cells with fragmented, sub-G1 DNA or that were Annexin V^{+}) increased after addition of 4-OHT (Figs. 5C–F). PCR analysis revealed the *p53* deleted *Dicer*$^{+/fl}$/Eμ-*myc* lymphoma cells deleted their one floxed *Dicer* allele, while the *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cells surviving CreER^{T2} activation had only deleted one of the conditional *Dicer* alleles (Fig. 5G). Analogous results were obtained with *Dicer*$^{fl/fl}$/Eμ-*myc* B cell lymphomas that had deleted *Arf* and retained *p53* (Supplemental Figure S2).

We postulated it was possible for preferential outgrowth of lymphoma cells possessing one allele of *Dicer*, masking the presence of a small population of lymphoma cells that had deleted both alleles of *Dicer*. To evaluate this possibility, we performed single-cell sorting

for GFP-positive cells of two independent CreER^{T2} expressing *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma lines into 96-well plates. After visually confirming the presence of a single cell per well, CreER^{T2} was activated with 4-OHT, and the surviving clones were assessed. Only 26% (328 of 1,260) of the clones survived CreER^{T2} activation, whereas 98.5% (394 of 400) of the vehicle-treated clones grew out. Analysis of all 328 lymphoma clones that survived CreER^{T2} activation revealed none had deleted both *Dicer* alleles (a subset of those analyzed is shown in Fig. 5H). Instead, 306 (93.3%) had deleted one *Dicer* allele, whereas the other 22 (6.7%) maintained both floxed alleles. Moreover, analysis of the *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma used in the single-cell analysis in our previous study [DC561, (19)] where we obtained analogous results, revealed that it had biallelic *p53* deletion (Fig. 5A). Collectively, these data illustrate that B-cell lymphomas cannot survive without *Dicer*, even when *p53* is deleted. Therefore, at least one allele of *Dicer* is required for B-cell lymphoma survival.

In vivo Dicer deletion inhibits lymphoma growth and extends survival

Given that B-cell lymphomas require Dicer for survival, *in vitro*, we tested whether inactivating *Dicer* would alter lymphoma growth *in vivo* with three different mouse experiments. Firstly, *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cells (DC1020) expressing CreER^{T2} were subcutaneously injected into nude mice and CreER^{T2} was activated by tamoxifen the same day. There was a significant delay in lymphoma progression and extended survival in the mice that received tamoxifen compared to the vehicle-treated mice (Fig. 6A; p=0.0012, log-rank test). Tumors from vehicle-treated mice grew significantly larger more quickly compared to tumors from mice that received tamoxifen to activate CreER^{T2} (Fig. 6B; *p=0.0051, **p<0.003).

To determine whether loss of Dicer would impact established lymphomas, we allowed a cohort of mice to grow subcutaneous lymphomas of 90–150mm^3 and then administered tamoxifen or vehicle control (tumor sizes were matched between groups) (Figs. 6C and 6D). While the rapid rate of tumor growth continued in the vehicle-treated mice, tumor expansion in the mice that received tamoxifen to activate CreER^{T2} to delete *Dicer* slowed dramatically (Fig. 6D; *p=0.0288, **p=0.0005). Analysis of tumors that were equivalent in size prior to tamoxifen addition, showed significant and increasing apoptosis over time following tamoxifen, as indicated by increased sub-G1 DNA content (Fig. 6E, *p=0.008), Annexin V positivity (Fig. 6F, **p<0.0001), and cleaved Caspase 3 protein (Fig. 6G). The consequence of this apoptosis was that the survival of the CreER^{T2}-activated (tamoxifen) mice was significantly increased (Fig. 6C; p=0.0035, log-rank test).

To assess whether the delayed tumor growth in both experiments and the apoptosis detected was a result of CreER^{T2}-mediated *Dicer* deletion, PCR analysis of *Dicer* gene rearrangement was performed. Surviving lymphoma cells in the mice administered tamoxifen all retained at least one *Dicer* allele (Fig. 6H and Supplemental Figure S3), and expressed Dicer protein (Fig. 6G). Therefore, targeting *Dicer* deletion, *in vivo*, induced apoptosis, delaying lymphoma progression and extending survival regardless of when *Dicer* was deleted.

As a third approach to test the effects of *Dicer* deletion in lymphomas *in vivo*, we also injected *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cells expressing CreER^{T2} and GFP into the

blood stream of nude mice; tamoxifen or vehicle control administration began on the same day. By day 17 and certainly by day 21, vehicle control-treated mice had more lymphoma cells present in their blood compared to mice that received tamoxifen to activate CreER^{T2} and delete *Dicer* (Fig. 6I, *p<0.0001 and Supplemental Figure S4). Furthermore, mice that had activated CreER^{T2} (tamoxifen) lived significantly longer than control mice (Fig. 6J; p<0.0001, log-rank test). Collectively, all three *in vivo* experiments show that deleting *Dicer* in B-cell lymphomas leads to apoptosis and decreased lymphoma cell expansion, providing evidence that targeting Dicer in B-cell lymphomas may have therapeutic potential even when lymphomas lack a functional p53 pathway.

Discussion

Previously, we detected an increase in *p53* inactivation in B-cell lymphomas from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice (19), suggesting a connection between *p53* activation and *Dicer* deletion. Moreover, we also observed *Dicer* deletion in untransformed MEFs increased p53 levels and induced a premature senescent phenotype that could be delayed by deleting either the *Ink4a/Arf* or *p53* locus (21). Others reported a fraction of a murine *p53*-null, mutant K-Ras expressing sarcoma cell line and SV40-immortalized, and thus p53 and Rb inactivated, mesenchymal stem cells could survive *Dicer* deletion (23). Although the data pointed to p53 being a critical mediator of the deleterious effects of *Dicer* deletion, we show here loss of p53 could not rescue the profound apoptosis that occurs in primary B-cells and B-cell lymphomas upon *Dicer* deletion. All approaches to obtain *p53*-null B-cells or B-cell lymphomas that had biallelic *Dicer* deletion resulted in one *Dicer* allele being retained in any surviving cells, whereas *Dicer*-null fibroblasts could be easily generated. These results indicate Dicer, and consequently miRNA, have essential functions in B-cell survival for both untransformed and malignant B-cells that cannot be overcome by loss of p53. Also, lymphomas that lacked Arf could not survive *Dicer* deletion, indicating inactivation of the p53 pathway is insufficient to allow B cell lymphoma survival. Moreover, the data show all stages of B-cell transformation from immortalized (*p53*-null) to transformed (lymphoma) require Dicer. Additionally, a deficiency in Dicer and Rb combined with p53 inactivation resulted in synthetic lethality in retinal progenitors (24). Therefore, although p53 inactivation may provide protection from the deadly effects of *Dicer* deletion in some cellular contexts when specific genetic alterations are present, Dicer loss is lethal for B-cells and B-cell lymphomas regardless of p53 status.

Our results did show a deficiency in p53 was able to rescue several aspects of Myc-induced B-cell lymphoma development in the *Dicer*$^{fl/fl}$ background. Firstly, the early precursor B-cell lymphomas previously observed in ~40% of CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice did not occur in the p53-deficient mice; instead, only typical pre-B/B-cell lymphomas developed. Secondly, CD19 cell surface expression, which was significantly reduced or absent in 65% of the lymphomas in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice, was fully restored in lymphomas from *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice. Unexpectedly, a *p53* deficiency also allowed T-cell lymphomas to emerge, albeit at a low frequency. The explanations for changes in B-cell lymphoma phenotype and the rare development of T-cell lymphomas are currently unclear, but likely involve protection from apoptosis of a lymphoid progenitor, allowing differentiation to continue along B- and T-cell lineages. In addition, although

CD19 surface expression was restored in the pre-B/B-cell lymphomas that emerged, 57% of the lymphomas lacked or had reduced Cre protein expression. This was unexpected, as all lymphomas expressed CD19 and Cre is driven from the *CD19* promoter. Although Cre expression was downregulated in half of the lymphomas, the frequency of its expression (43%) was significantly higher than that of 12% in the CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas (19), indicating the *p53* deficiency partially rescued Cre expression. However, although Cre protein expression occurred more frequently in lymphomas in *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic mice, the number of lymphomas that underwent Cre-mediated deletion of at least one *Dicer* allele was not statistically different than the number that deleted one *Dicer* allele in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice (19). These results indicate that while more lymphomas expressed Cre, the lymphomas still prevented it from deleting both *Dicer* alleles. Our data show a *p53* deficiency still resulted in a delay in lymphoma development and did not allow biallelic *Dicer* deletion, but it did restore the lymphoma phenotype and CD19 surface expression and partially restored Cre expression in the B-cell lymphomas.

Protection from apoptosis is a critical step in B-cell development and lymphomagenesis (28, 32, 33, 34). Expression of Cre in *Dicer*$^{fl/fl}$ mice results in early B-cell progenitor (Mb1-*Cre*) or mature B-cell (Aicda-*Cre*) apoptosis and a developmental block or a lack of germinal centers, respectively (22, 35). Suppressing apoptosis by overexpressing the anti-apoptotic Bcl-2 protein and/or deleting the pro-apoptotic gene *Bim* or by expressing an immunoglobulin transgene, which provides survival signals, partially rescued B-cells from apoptosis in these systems. Since neither study confirmed biallelic deletion of *Dicer* had indeed occurred in the surviving B-cells, and since our data show B-cells do not survive *Dicer* deletion, it is likely the B-cells that survived in their studies only deleted one allele of *Dicer*. Moreover, the reduction in apoptosis that allowed more B-cells to survive and differentiate likely reflects effects on the B-cell compartment rather than on the survival of *Dicer*-deleted B-cells. In addition, it is unlikely that Bcl-2 overexpression alone would protect an untransformed B-cell from apoptosis induced by *Dicer* deletion, as the B-cell lymphomas we evaluated overexpressed Bcl-2 (unpublished observations) and rapidly died when *Dicer* was deleted. However, these results could also indicate transformed B-cells rely on Dicer more than untransformed B-cells. Certainly, further studies are needed to determine the conditions, if any, under which B-cells at any maturation stage would survive complete *Dicer* ablation.

Dicer is reported to function as a haploinsufficient tumor suppressor and promote tumorigenesis in retinal, lung epithelial, and muscle cells (17, 18). However, there is a conflicting report on muscle cells (36). In contrast, the rate of Myc-induced B-cell lymphomagenesis was similar in mice that had one or two alleles of *Dicer* (19), regardless of p53 status, indicating Dicer was not a haploinsufficient tumor suppressor in B-cells. Moreover, evaluation of Dicer protein and function in *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas with one or two *Dicer* alleles revealed analogous levels of protein and mature miRNA. Therefore, loss of one allele of *Dicer* did not change the levels of Dicer protein or function in the B-cell lymphomas. Although our results reveal Dicer inhibition as a potential therapeutic opportunity for treatment of B-cell lymphomas, which are sensitive to Dicer

loss, due to its haploinsufficient tumor suppressor functions in other cell types, this may not be possible. Therefore, it will be important in future studies to determine the cell types where Dicer functions as a haploinsufficient tumor suppressor, and whether transient inactivation of Dicer could be therapeutic for lymphoma treatment without being tumor-inducing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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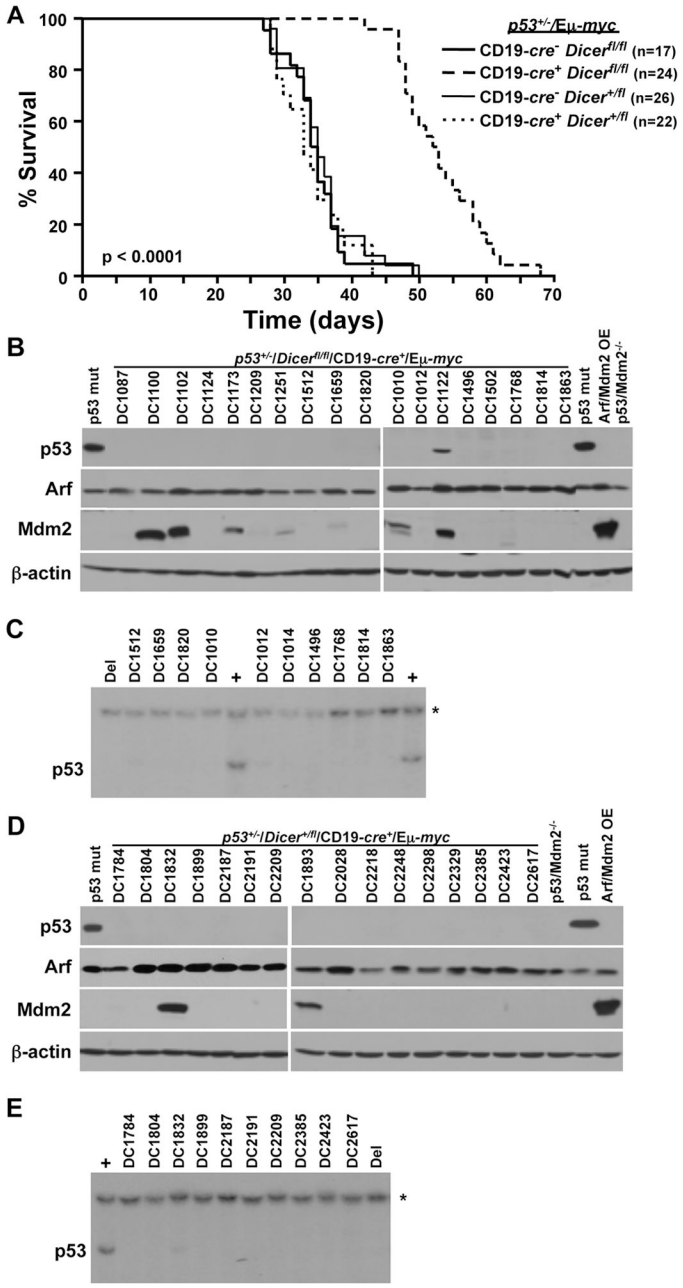

Figure 1. Delayed lymphomagenesis in *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice

(A) Kaplan-Meier survival curves of the indicated genotypes of mice ($p<0.0001$, log-rank test comparing each genotype to CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/*p53*$^{+/-}$/Eμ-*myc*). The number (n) of mice is indicated. (B, D) Western blots of lymphomas for the proteins and genotype indicated. Controls include lymphomas containing mutant (mut) p53 or overexpressing (OE) Arf and Mdm2 and *p53*$^{-/-}$/*Mdm2*$^{-/-}$ MEFs. A subset of lymphomas analyzed shown. (C, E) Representative Southern blots for *p53* of lymphomas in B and D. Lymphomas that contain (+) or have deleted (Del) *p53* were controls. Asterisk (*) denotes the DNA loading control, the p53 pseudogene.

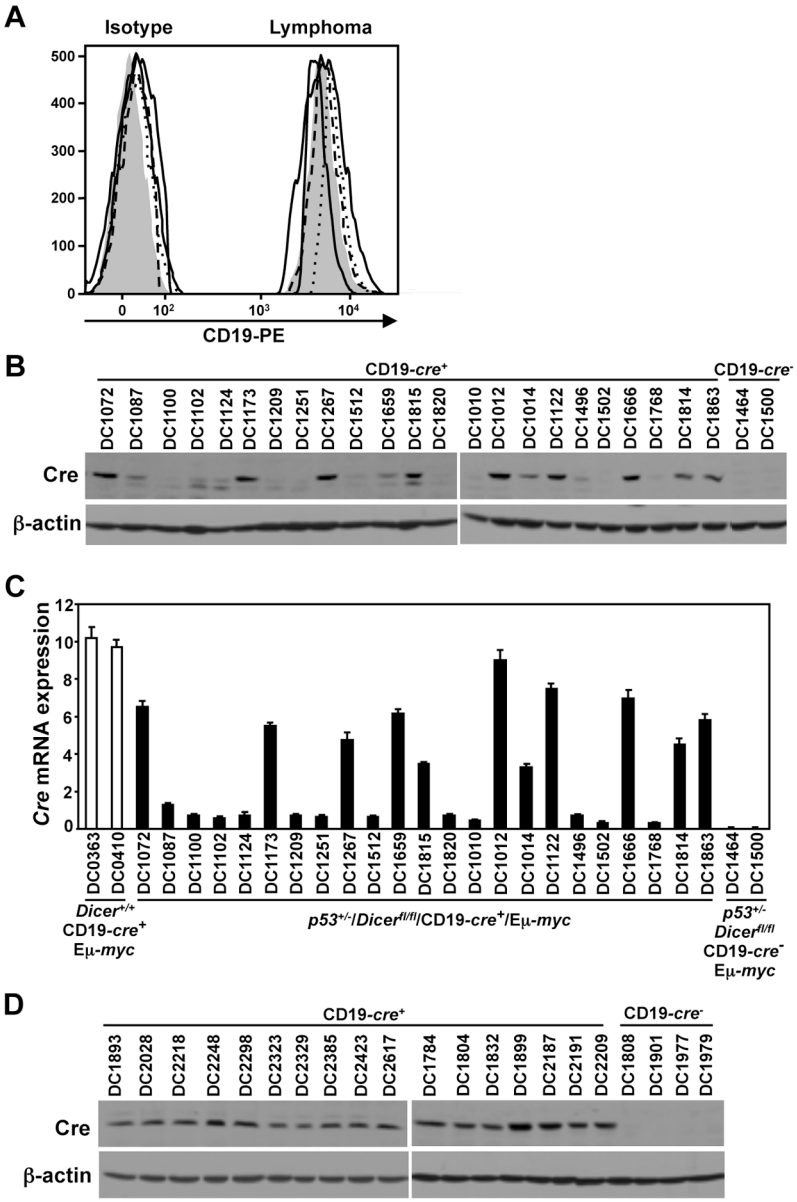

Figure 2. A deficiency in p53 rescues CD19 and Cre expression during B-cell lymphomagenesis

(A) Histograms of CD19 surface expression and corresponding isotype controls of lymphomas from four representative $p53^{+/-}$/CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* mice compared to a control $p53^{+/-}$/CD19-cre^{-}/$Dicer^{fl/fl}$/Eμ-*myc* lymphoma (shaded peaks). (B, D) Western blots for Cre and β-actin from $Dicer^{fl/fl}$ (B) and $Dicer^{+/fl}$ (D) $p53^{+/-}$/CD19-cre^{+}/Eμ-*myc* lymphomas. Lysates of lymphomas from a $Dicer^{+/+}$/CD19-cre^{+}/Eμ-*myc* mouse (B) and $Dicer^{fl/fl}$ (B) or $Dicer^{+/fl}$ (D) CD19-cre^{-}/$p53^{+/-}$/Eμ-*myc* mice were controls. (C) qRT-PCR for *Cre* expression relative to *β-actin* in lymphomas from $p53^{+/-}$/CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* mice. RNA from $Dicer^{+/+}$/CD19-cre^{+}/Eμ-*myc* and CD19-cre^{-}/$p53^{+/-}$/$Dicer^{fl/fl}$/Eμ-*myc* lymphomas were positive and negative controls, respectively.

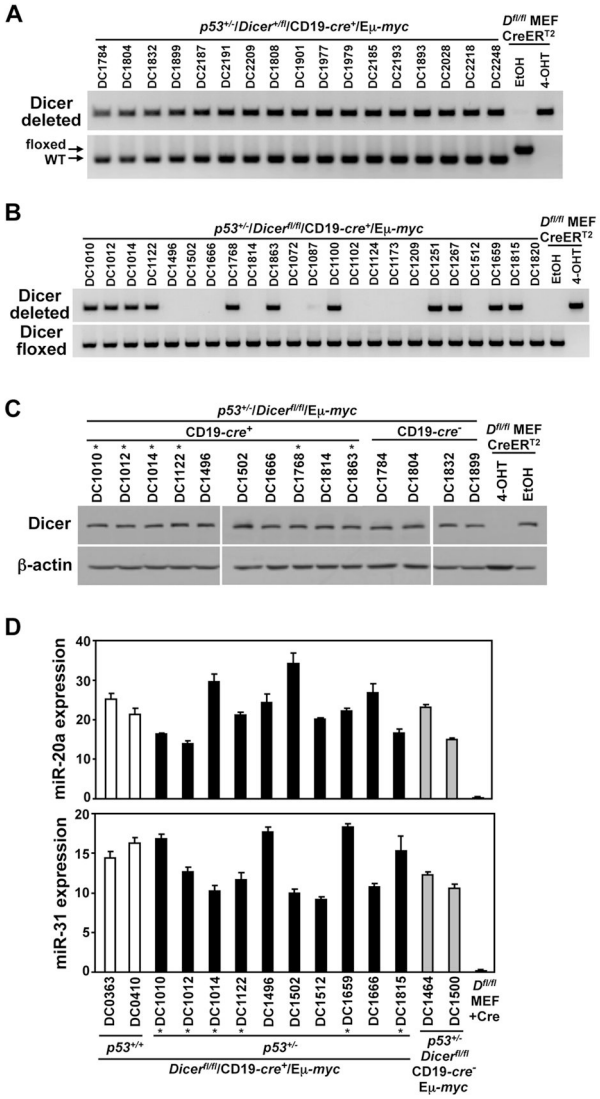

Figure 3. Biallelic *Dicer* deletion is selected against during lymphoma development

(A, B) PCR analysis for conditional deleted and floxed (not deleted) *Dicer* alleles from lymphomas of the indicated genotype. DNA from *Dicer*$^{fl/fl}$ (*D*$^{fl/fl}$) MEFs expressing an inducible CreER^{T2} treated with 4-OHT or vehicle control (EtOH) were controls. Arrows indicate unrearranged (floxed) and wild-type (WT) *Dicer* alleles. (C) Representative Western blots for Dicer and β-actin from CD19-*cre*$^{+}$ and CD19-*cre*$^{-}$ *p53*$^{+/-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas. Lysates from *Dicer*$^{fl/fl}$ (*D*$^{fl/fl}$) MEFs were controls. (D) qRT-PCR for *miR-20a* and *miR-31* relative to internal *RNU6b* small RNA in lymphomas from *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice. *p53*$^{+/+}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* and *p53*$^{+/-}$/*Dicer*$^{fl/fl}$/CD19-*cre*$^{-}$/Eμ-*myc* lymphomas and Cre-expressing *Dicer*$^{fl/fl}$ (*D*$^{fl/fl}$) MEFs served as controls. Asterisks (*) denote lymphomas that deleted one *Dicer* allele (C and D).

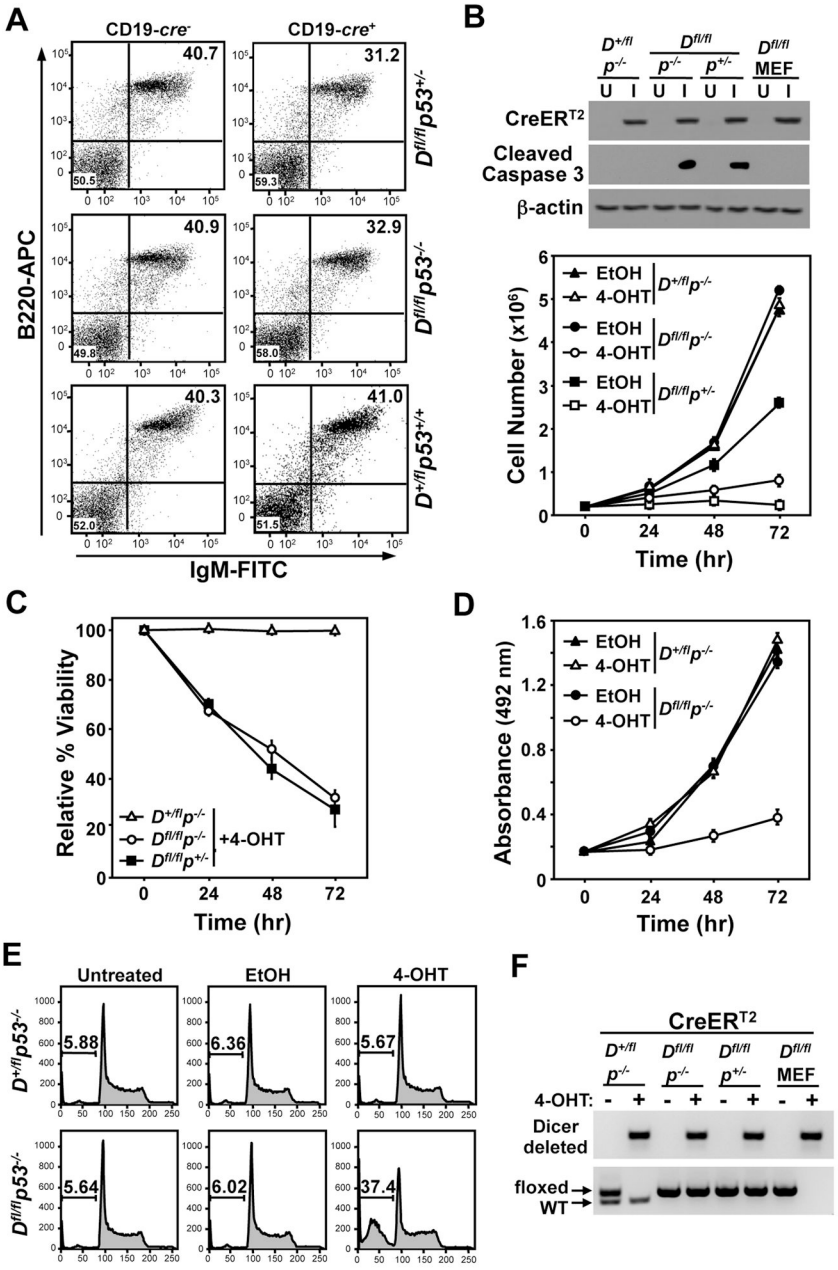

Figure 4. Loss of *p53* is insufficient for B-cell survival when *Dicer* is deleted

(A) Representative dot plots of littermate-matched splenic B-cells from CD19-*cre*$^{+}$ or CD19-*cre*$^{-}$ *Dicer*$^{+/fl}$ and *Dicer*$^{fl/fl}$ mice that were *p53*$^{+/+}$, *p53*$^{+/-}$, or *p53*$^{-/-}$. Total lymphocytes were gated and B220-APC versus IgM-FITC was assessed. (B–F) Primary pre-B cells from *p53*$^{-/-}$/*Dicer*$^{fl/fl}$, *p53*$^{+/-}$/*Dicer*$^{fl/fl}$, and *p53*$^{-/-}$/*Dicer*$^{+/fl}$ littermates were infected (I) with a retrovirus encoding CreER^{T2} or left uninfected (U). 4-OHT (+) or vehicle control (EtOH, −) was added to pre-B cell cultures at time 0 and cell number (B), viability (C), proliferation (MTS assay; D), apoptosis (cleaved Caspase 3 protein, B; sub-G1 DNA, E), and *Dicer* gene rearrangement (F) were evaluated. Western blots shown in B. Arrows

indicate unrearranged (floxed) and wild-type (WT) *Dicer* alleles in F. CreER^{T2} expressing *Dicer*$^{fl/fl}$ (*D*$^{fl/fl}$) MEFs treated with 4-OHT (+) or ethanol (−) were controls in B and F.

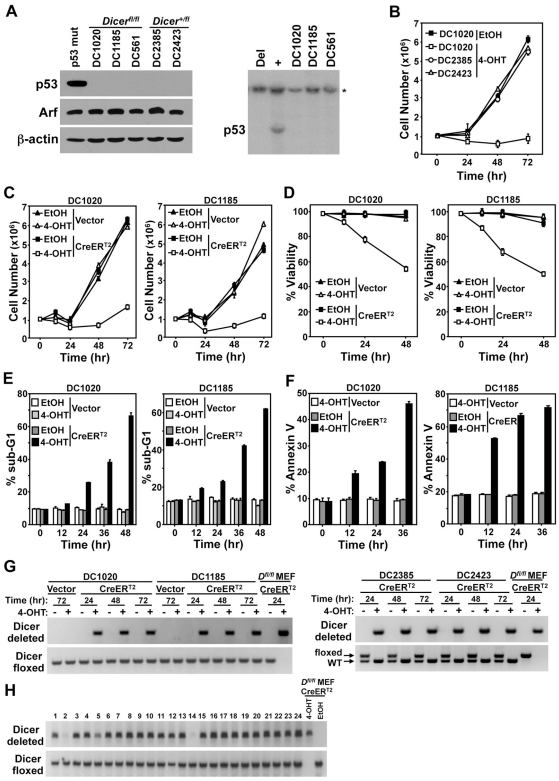

Figure 5. A deficiency in *p53* does not allow B-cell lymphomas to survive without *Dicer*

(A) $p53^{+/-}$/$Dicer^{fl/fl}$/Eμ-*myc* (DC1020 and DC1185), $p53^{+/-}$/$Dicer^{+/fl}$/Eμ-*myc* (DC2385 and DC2423) lymphoma cell lines and the $Dicer^{fl/fl}$/Eμ-*myc* lymphoma cell line (DC561) from our previous study (19) were subjected to Western blot (left) for the proteins indicated and Southern blot (right and Fig. 1E) for *p53*. A lymphoma containing mutant p53 was a control for the Western blot. Lymphomas that contain (+) or have deleted (Del) *p53* were controls for the Southern blot. Asterisk (*) denotes the DNA loading control, the p53 pseudogene. (B–F) DC2385, DC2423, DC1020, and/or DC1185 lymphoma cells were infected with a CreER^{T2}-encoding retrovirus or empty retrovirus (Vector). 4-OHT or vehicle control (EtOH) was added to the cultures at time 0 and cell number (B, C), viability (D), and apoptosis (sub-G1 DNA, E; Annexin V, F) were measured. (G) *Dicer* gene rearrangement was evaluated at the indicated intervals by PCR. (H) Representative PCR product analysis of *Dicer* gene rearrangement of GFP-positive single cell-sorted lymphoma clones that survived CreER^{T2} activation of the 328 analyzed. Conditional deleted and floxed (not deleted) *Dicer* alleles shown (G and H). CreER^{T2} expressing $Dicer^{fl/fl}$ ($D^{fl/fl}$) MEFs treated with 4-OHT or ethanol (EtOH) were controls (G and H).

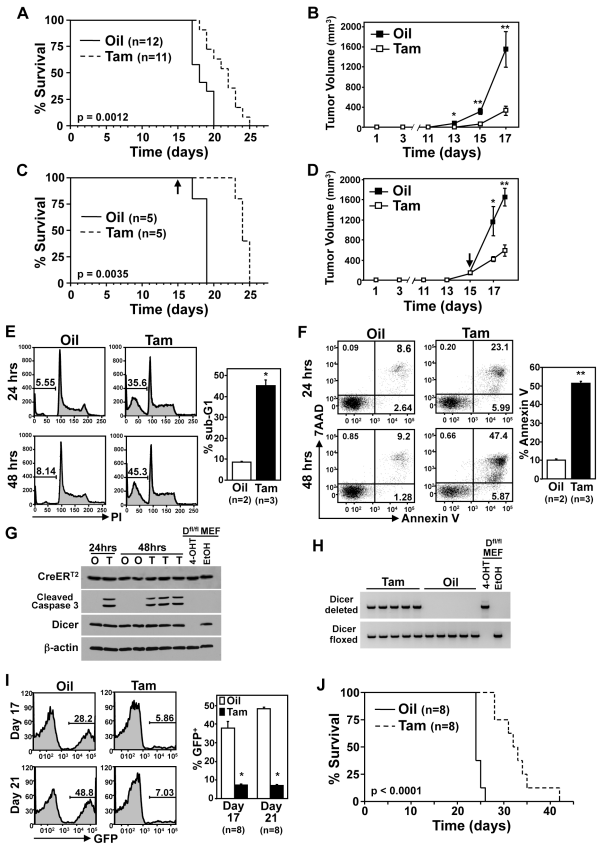

Figure 6. *Dicer* inactivation impedes tumor growth, *in vivo*

(A, C) Kaplan-Meier survival curves of nude mice injected (subcutaneously) with CreER^{T2} expressing *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cells (DC1020) and administered tamoxifen or vehicle (corn oil) control starting the day of injection (A; p=0.0012, log-rank test) or once lymphomas were 90–150mm^3 (C; p=0.0035, log-rank test). Arrow indicates the day tamoxifen administration began for C. The number (n) of mice is indicated. (B, D) Tumor volumes for mice in A and C, respectively, were measured at the indicated intervals (for B: *p=0.0051, **p<0.003; for D: *p=0.0288, **p=0.0005). In D, the arrow indicates the day tamoxifen administration began. (E–G) Apoptosis was measured at intervals following tamoxifen or vehicle control administration in matched tumor pairs by propidium iodide staining of fragmented (sub-G1) DNA (E), Annexin V/7AAD staining (F), and cleaved Caspase 3 protein detection (G). Representative data (left) and mean values at 48 hours (right) are shown for E and F; *p=0.0008, **p<0.0001, t-tests. Western blots of whole cell lysates for the proteins indicated (G). (H) PCR product analysis of *Dicer* gene rearrangement of the mice from C. Controls for G and H include protein lysates or DNA from *Dicer*$^{fl/fl}$ MEFs treated with 4-OHT or ethanol. (I, J) Nude mice were injected intravenously with CreER^{T2} expressing *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cells (DC1020) and administered tamoxifen (Tam) or corn oil (Oil) vehicle control starting the same day. Blood was assessed for GFP-positivity by flow cytometry at intervals post lymphoma injection. Representative data (left) and mean values for the indicated number of

mice are shown (I; *p<0.0001, t-test). Kaplan-Meier survival curves (J; p<0.0001, log-rank test).

Table 1

Dicer$^{fl/fl}$ Eμ-*myc* lymphoma phenotypes are rescued with a p53 deficiency

	Dicer$^{+/fl}$ *p53*$^{+/-}$ Eμ-*myc*	*Dicer*$^{fl/fl}$ *p53*$^{+/-}$ Eμ-*myc*	
Phenotype	**CD19-*cre*$^{+}$**	**CD19-*cre*$^{-}$**	**CD19-*cre*$^{+}$**
B220^{+} CD19^{+} CD43^{-} IgM^{-}	4/10 (40%)	7/10 (70%)	10/16 (63%)
B220^{+} CD19^{+} CD43^{-} IgM^{+}	6/10 (60%)	3/10 (30%)	4/16 (25%)
CD3^{-} CD4^{+} CD8^{+} CD43^{+}	0/10 (0%)	0/10 (0%)	2/16 (13%)