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 nonhematopoietic cell types that contain inactivated p53 have been shown to survive Dicer deletion, suggesting that 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. Cancer Res; 74(14); 3923–34. ©2014 AACR.
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\([\beta\beta]\) mice from Dr. Steve Jones (University of Massachusetts Medical School, Worcester, MA; ref. 21), and p53\(^{-/-}\) mice from Dr. Guillermina Lozano (University of Texas M.D. Anderson Cancer Center, Houston, TX; ref. 27) were intercrossed to obtain mice needed for this study. Littermates were used in all analyses. For experiments with nude mice, 1.5 \(\times\) 10\(^6\) or 0.5 \(\times\) 10\(^6\) p53-deleted Dicer\([\beta\beta]/E\mu\)-myc lymphoma cells expressing a tamoxifen-inducible form of Cre (CreER\(^{T2}\)) were injected (subcutaneous or intravenous, respectively) into 6-week-old Foxn1 null/male female mice (Harlan Laboratories). 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 to 150 mm\(^3\) for a second subcutaneous cohort. Subcutaneous tumors were measured with calipers and tumor volume was 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 before 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 Biotechnology), Cre (Novagen), Dicer (Cell Signaling Technology), cleaved caspase-3 (Cell Signaling Technology), and \(\beta\)-actin (Sigma) were used. As previously described (28, 29), p53 was sequenced and Southern blot analyses for p53 with genomic DNA from lymphomas were performed.

Phenotype analysis

Lymphoma cells and splenocytes from littermates before 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 SYBR Green (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 \(\beta\)-actin and RNU6B expression, respectively, and the data were presented as 2^{-ΔΔCt}.

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% to 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\([\beta\beta]\), p53\(^{-/-}\)/Dicer\([\beta\beta]\), and p53\(^{-/-}\)/Dicer\([\beta\beta]\)/E\mu\)-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 μmol/L 4-hydroxytamoxifen (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 removing 1 μmol/L 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 μmol/L) 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\([\beta\beta]\) mice

Previously, we reported that 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\([\beta\beta]\)/E\mu\)-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\([\beta\beta]\)]/E\mu\)-myc mice compared with their CD19-cre\(^{-/-}/p53\(^{-/-}/Dicer\([\beta\beta]\)]/E\mu\)-myc littermates (53 and 34 days mean survival, respectively; Fig. 1A; \(P < 0.0001\),
Figure 1. Delayed lymphomagenesis in p53+/−/CD19-cre+/Dicer+/−/Eμ-myc mice. A, Kaplan-Meier survival curves of the indicated genotypes of mice (P < 0.0001, log-rank test comparing CD19-cre+/Dicer+/−/ p53+/−/Eμ-myc to each genotype). The number (n) of mice is indicated. B and D, Western blot analyses of lymphomas for the proteins and genotype are indicated. Controls include lymphomas containing mutant (mut) p53 or overexpressing (OE) Arf and Mdm2 and p53+/−/Mdm2−/− MEFs. A subset of lymphomas analyzed is shown. C and E, representative Southern blot analyses for p53 of lymphomas in B and D are indicated. Lymphomas that contain (+) or have deleted (Del) p53 were controls. *the DNA loading control, the p53 pseudogene.
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 blot analyses showed that all lymphomas lacking p53 protein had deleted their wild-type allele of p53 (representative data of those analyzed are 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 with mean survivals of 35 and 36 days, respectively from littermate controls and from myc heterozygous mice. In addition, Mdm2, a negative regulator of p53, deficiency would alter the development or frequency of this phenotype by assessing lymphomas from p53+/− mice lacking p53 protein, due to deletion of the p53 binding domain. Southern blot analyses showed that all lymphomas lacking p53 protein expression, and all overexpressed 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+/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+/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+/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 exact test). However, 13 of 23 (57%) p53+/−/CD19-cre+/Dicer+/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, because 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+/Eμ-myc lymphomas (43%) than was previously observed in CD19-cre+/Dicer+/Eμ-myc lymphomas (12%; ref. 19; P = 0.022, Fisher exact test). Analysis of 17 heterozygous floxed Dicer p53+/−/CD19-cre+/Eμ-myc lymphomas showed that 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+/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+/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. The 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+/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

Table 1. Dicer+/− Eμ-myc lymphoma phenotypes are rescued with a p53 deficiency

<table>
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<tr>
<th>Phenotype</th>
<th>CD19-cre+</th>
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<tr>
<td>B220 / CD19 / CD43 / IgM</td>
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<td>7/10 (70%)</td>
<td>10/16 (63%)</td>
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<tr>
<td>B220 / CD19 / CD43 / IgM</td>
<td>6/10 (60%)</td>
<td>3/10 (30%)</td>
<td>4/16 (25%)</td>
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<tr>
<td>CD3 / CD4+ / CD8+ / CD43+</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
<td>2/16 (13%)</td>
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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<sup>+/−</sup>/CD19-cre<sup>+/−</sup>/Dicer<sup>fl/fl</sup>/Eμ-myc mice compared with a control p53<sup>+/−</sup>/CD19-cre<sup>/−</sup>/Dicer<sup>fl/fl</sup>/Eμ-myc lymphoma (shaded peaks). B and D, Western blot analyses for Cre and β-actin from Dicer<sup>fl/fl</sup> (B) and Dicer<sup>+/−</sup> (D) p53<sup>+/−</sup>/CD19-cre<sup>/−</sup>/Eμ-myc lymphomas. Lysates of lymphomas from Dicer<sup>fl/fl</sup> (B) or Dicer<sup>+/−</sup> (D) CD19-cre<sup>/−</sup>/p53<sup>+/−</sup>/Eμ-myc mice were controls. C, qRT-PCR for Cre mRNA expression relative to β-actin in lymphomas from p53<sup>+/−</sup>/CD19-cre<sup>/−</sup>/Dicer<sup>fl/fl</sup> Eμ-myc mice. RNA from Dicer<sup>+/−</sup>/CD19-cre<sup>/−</sup>/Eμ-myc and p53<sup>+/−</sup>/CD19-cre<sup>/−</sup>/Dicer<sup>fl/fl</sup> Eμ-myc lymphomas were positive and negative controls, respectively.

Dicer<sup>+/−</sup>/p53<sup>+/−</sup>/CD19-cre<sup>/−</sup>/Eμ-myc lymphomas had deleted both floxed Dicer alleles.

Given that Cre protein expression was lost in half of the p53<sup>+/−</sup>/CD19-cre<sup>/−</sup>/Dicer<sup>fl/fl</sup>/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<sup>+/−</sup>/CD19-cre<sup>/−</sup>/Dicer<sup>fl/fl</sup>/Eμ-myc mice compared with mice that were p53<sup>+/−</sup> (43% vs. 12%, respectively; ref. 19), we expected an increased incidence of Cre-mediated deletion of at least one Dicer allele in the p53<sup>+/−</sup> lymphomas. However, there was no statistical difference in the frequency of deleting one allele of Dicer between these two groups (48% vs. 38%, respectively; P = 0.57, Fisher exact test; Fig. 3B; ref. 19). Importantly, our data indicate that 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.
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). Because a p53 deficiency rescued the pre-B/B-cell lymphoma phenotype in CD19-cre+/Dicer0; MYC mice, we questioned whether p53 mediates the Dicer deletion-induced B-cell apoptosis. To address this, we evaluated splenic B cells from precancerous p53+/− and p53−/− CD19-cre+/Dicer0; MYC 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−/−/Dicer0; MYC mice (32.9% ± 1.42%) compared with CD19-cre−/p53−/−/Dicer0; MYC 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+/Dicer0; MYC or CD19-cre−/Dicer0; MYC (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−/− mice with or without CD19-cre and the percentages of B cells were similar in both, demonstrating that 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−/−, p53−/−/Dicer−/−, and p53−/−/Dicer0; MYC littermates. Pre-B cells were infected with a bicistronic retrovirus encoding GFP and a 4-OHT-inducible CreERT2 (31), and GFP-positive cells were sorted by flow cytometry. All three genotypes of pre-B cells expressed equal levels of CreERT2 protein (Fig. 4B). To delete Dicer, pre-B cells were treated with 4-OHT to activate CreERT2. As expected for primary pre-B cells with functional p53, the Dicer0;MYC/p53−/− cells grew at a slower rate and were sensitive to Dicer loss, as indicated by decreased cell numbers and viability (Fig. 4B and C). Similarly, following 4-OHT treatment, Dicer0;MYC/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 with vehicle-treated cells, which were unaffected (Fig. 4B–E). When 4-OHT was administered to CreERT2-expressing Dicer0;MYC/p53−/− pre-B cells, no change in cell number, viability, growth, fragmented DNA, or cleaved caspase-3 was observed (Fig. 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 CreERT2–activated pre-B cells (Fig. 4F).

Notably, Dicer0;MYC fibroblasts containing similar levels of CreERT2 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, 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+/C0/Dicerfl/fl/Eμ-myc mice (DC1020 and DC1185) and as controls from two p53+/C0/Dicer+/fl/Eμ-myc mice (DC2385 and DC2423). p53 protein was not detected by Western blot analysis, and Southern blot analysis showed deletion of the remaining wild-type allele of p53 in all four lymphomas (Figs. 1D and 5A).

The lymphomas were infected with a bicistronic retrovirus encoding CreERT2 and GFP or GFP alone. CreERT2 activation with 4-OHT in the p53-deleted Dicer+/fl/Eμ-myc lymphomas had no effect on cell number compared with a p53-deleted Dicerfl/fl/Eμ-myc lymphoma, which showed a significant decrease in cell number after CreERT2 activation (Fig. 5B and...
Supplementary Fig. S1). CreER T2 activation in both p53-deleted Dicer\(^{\beta/\beta}\)/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 (Fig. 5C–F). Specifically, the total number and viability of CreER T2 p53-deleted Dicer\(^{\beta/\beta}\)/Eμ-myC lymphoma cells decreased, whereas the percentage of apoptotic cells (cells with fragmented, sub-G1 DNA, or that were Annexin V+ increased after addition of 4-OHT (Fig. 5C–F). PCR analysis revealed that the p53-deleted Dicer\(^{\beta/\beta}\)/Eμ-myC lymphoma cells deleted their one floxed Dicer allele, whereas the p53-deleted Dicer\(^{\beta/\beta}\)/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\(^{\beta/\beta}\)/Eμ-myC B-cell lymphomas that had deleted Arf and retained p53 (Supplementary Fig. S2).

We postulated that 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 CreERT2-expressing p53-deleted Dicer\(^{\beta/\beta}\)/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 activation.

Figure 5. A deficiency in p53 does not allow B-cell lymphomas to survive without Dicer. A, p53\(^{1/1}\)/Dicer\(^{\beta/\beta}\)/Eμ-myC (DC1020 and DC1185), p53\(^{1/1}\)/Dicer\(^{\beta/\beta}\)/Eμ-myC (DC2385 and DC2423) lymphoma cell lines, and the Dicer\(^{\beta/\beta}\)/Eμ-myC lymphoma cell line (DC561) from our previous study (19) were subjected to Western blot analysis (left) for the proteins indicated and Southern blot analysis (right and Fig. 1E) for p53. A lymphoma containing mutant p53 was a control for the Western blot analysis. Lymphomas that contain (-) or have deleted (Del) p53 were controls for the Southern blot analysis. The DNA loading control, the p53 pseudogene, B-F, DC1020, DC1185, DC2385, and/or DC2423 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 and 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 CreERT2 activation of the 328 analyzed. Conditional deleted and floxed (not deleted) Dicer alleles are shown (G and H). CreERT2-expressing Dicer\(^{\beta/\beta}\)(E\(\mu\)-myC) MEFs treated with 4-OHT or ethanol (EtOH) were controls (G and H).
CreER\textsuperscript{T2} activation, whereas 98.5% (394 of 400) of the vehicle-treated clones grew out. Analysis of all 328 lymphoma clones that survived CreER\textsuperscript{T2} activation revealed that 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\textsuperscript{fl/fl}/E\textsubscript{mu}-myc lymphoma used in the single-cell analysis in our previous study (DC561; ref. 19) in which 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. First, p53-deleted Dicer\textsuperscript{fl/fl}/E\textsubscript{mu}-myc lymphoma cells (DC1020) expressing CreER\textsuperscript{T2} were subcutaneously injected into nude mice and CreER\textsuperscript{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 with the vehicle-treated mice (Fig. 6A; P = 0.0012, log-rank test). Tumors from vehicle-treated mice grew significantly larger more quickly compared with tumors from mice that received tamoxifen to activate CreER\textsuperscript{T2} (Fig. 6B; *, P = 0.0051; **, P < 0.0003).

To determine whether loss of Dicer would affect established lymphomas, we allowed a cohort of mice to grow subcutaneous lymphomas of 90 to 150 mm\textsuperscript{3} and then administered tamoxifen or vehicle control (tumor sizes were matched between groups; Fig. 6C and D). Although the rapid rate of tumor growth continued in the vehicle-treated mice, tumor expansion in the mice that received tamoxifen to activate CreER\textsuperscript{T2} to delete Dicer slowed dramatically (Fig. 6D; *, P = 0.0288; **, P = 0.0005). Analysis of tumors that were equivalent in size before tamoxifen addition, showed significant and increasing apoptosis over time following tamoxifen, as indicated by increased sub-G\textsubscript{1} DNA content (Fig. 6E; *, P = 0.0088), 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\textsuperscript{T2}-activated (tamoxifen) mice was significantly extended (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\textsuperscript{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 Supplementary Fig. S3) and expressed Dicer protein (Fig. 6G). Therefore, targeting Dicer deletion, in vitro, 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\textsuperscript{fl/fl}/E\textsubscript{mu}-myc lymphoma cells expressing CreER\textsuperscript{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 with mice that received tamoxifen to activate CreER\textsuperscript{T2} and delete Dicer (Fig. 6I; *, P < 0.0001 and Supplementary Fig. S4). Furthermore, mice that had activated CreER\textsuperscript{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\textsuperscript{+}/Dicer\textsuperscript{fl/fl}/E\textsubscript{mu}-myc mice (19), suggesting a connection between p53 activation and Dicer deletion. Moreover, we also observed that 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 that 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 that 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 that inactivation of the p53 pathway is insufficient to allow B-cell lymphoma survival. Moreover, the data show that all stages of B-cell transformation from immortalized (p53-null) to transformed (lymphoma) require Dicer. In addition, 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 that a deficiency in p53 was able to rescue several aspects of Myc-induced B-cell lymphoma development in the Dicer\textsuperscript{fl/fl}/E\textsubscript{mu}-myc background. First, the early precursor B-cell lymphomas previously observed in approximately 40% of CD19-cre\textsuperscript{+}/Dicer\textsuperscript{fl/fl}/E\textsubscript{mu}-myc mice did not occur in the p53-deficient mice; instead, only typical pre-B/B-cell lymphomas developed. Second, CD19 cell surface expression, which was significantly reduced or absent in 65% of the lymphomas in CD19-cre\textsuperscript{+}/Dicer\textsuperscript{fl/fl}/E\textsubscript{mu}-myc mice, was fully restored in lymphomas from p53\textsuperscript{fl/+}/CD19-cre\textsuperscript{+}/Dicer\textsuperscript{fl/fl}/E\textsubscript{mu}-myc mice. Unexpectedly, a p53 deficiency also allowed T-cell lymphomas to emerge, albeit at a low frequency. The explanations for changes...
Figure 6. Dicer inactivation impedes tumor growth, in vivo.
A and C, Kaplan–Meier survival curves of nude mice injected (subcutaneously) with CreERT2-expressing p53-deleted Dicer<sup>fl/fl</sup>/E<sub>myc</sub> lymphoma cells (DC1020) and administered tamoxifen (Tam) or vehicle (corn oil; Oil) control starting the day of injection (A; P = 0.0012, log-rank test) or once lymphomas were 90 to 150 mm<sup>3</sup> (C; P = 0.0035, log-rank test). Arrow, the day tamoxifen administration began for C. The number (n) of mice is indicated. B and D, tumor volumes for mice in A and C, respectively, were measured at the indicated intervals (for B: *; P < 0.0005; **; P < 0.0003; for D: *; P = 0.0288; **; P = 0.0005). D, arrow, 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 (PI) staining of fragmented (sub-G1) DNA (E), Annexin V/7-AAD 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 blot analyses 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 CreERT2-expressing Dicer<sup>fl/fl</sup> MEFs treated with 4-OHT or ethanol. I and J, nude mice were injected intravenously with CreERT2-expressing p53-deleted Dicer<sup>fl/fl</sup>/E<sub>myc</sub> lymphoma cells (DC1020) and administered tamoxifen or corn oil vehicle control starting the same day. Blood was assessed for GFP-positivity by flow cytometry at intervals after 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).
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 down-regulated in half of the lymphomas, the frequency of its expression (43%) was significantly higher than that of 12% in the CD19-cre+/Dicerfl/fl/Eμ-μcyc lymphomas (19), indicating that the p53 deficiency partially rescued Cre expression. However, although Cre protein expression occurred more frequently in lymphomas in p53+/−/CD19-cre+/Dicerfl/fl/Eμ-μcyc 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−/Dicerfl/fl/Eμ-μcyc mice (19). These results indicate that while more lymphomas expressed Cre, the lymphomas still prevented it from deleting both Dicer alleles. Our data show that 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 a B-cell development and lymphomagenesis (28, 32–34). Expression of Cre in Dicerfl/fl mice results in early B-cell progenitor (Mbl-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 ant apoptotic Bcl-2 protein and/or deleting the proapoptotic gene Bim or by expressing an immunoglobulin transgene, which provides survival signals, partially rescued B cells from apoptosis in these systems. Because neither study confirmed biallelic deletion of Dicer had indeed occurred in the surviving B cells, and because our data show that B cells do not survive Dicer deletion, it is likely that 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 data) and rapidly died when Dicer was deleted. However, these results could also indicate that 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 that Dicer was not a haploinsufficient tumor suppressor in B cells. Moreover, the evaluation of Dicer protein and function in p53+/−/CD19-cre+/Dicerfl/fl/Eμ-μcyc 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 in which Dicer functions as a haploinsufficient tumor suppressor, and whether transient inactivation of Dicer could be therapeutic for lymphoma treatment without being tumor-inducing.

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

Authors’ Contributions
Conception and design: C.M. Adams, C.M. Eischen
Development of methodology: C.M. Eischen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.M. Eischen
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Writing, review, and/or revision of the manuscript: C.M. Adams, C.M. Eischen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.M. Eischen
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Inactivation of \textit{p53} Is Insufficient to Allow B Cells and B-Cell Lymphomas to Survive Without \textit{Dicer}

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