Inactivation of p53 Is Insufficient to Allow B Cells and B-Cell Lymphomas to Survive Without Dicer

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Tumor and Stem Cell Biology

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 lymphogenesis, 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): 1–12. ©2014 AACR.

Introduction

microRNA (miRNA) are small noncoding RNA that regulate gene expression posttranscriptionally and have essential roles in development, proliferation, apoptosis, and transformation (1, 2). Alterations in miRNA expression are linked to tumor development, including hematopoietic malignancies (1–3). Moreover, the oncogene c-Myc, which is 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–like 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 (6–16). Mouse models revealed Dicer is a haploinsufficient tumor suppressor in soft tissue sarcoma, lung adenocarcinoma, and retinoblastoma (17, 18). In contrast, we showed that 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 (MEF), 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−/− 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 antiapoptotic Bcl-2 protein or reducing the proapoptotic 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 Bb-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 not 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-Mycl (25) and CD19-cre (26) transgenic mice, Dicerfl/fl 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 × 106 or 0.5 × 106 p53-deleted Dicerfl/fl/Eμ-Mycl lymphoma cells expressing a tamoxifen-inducible form of Cre (CreERT2) were injected intraperitoneally 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 mm3 for a second subcutaneous cohort. Subcutaneous tumors were measured with calipers and tumor volume was calculated.

Pre-B cell and lymphoma cell survival analyses

Primary pre-B cell cultures from p53−/−/Dicerfl/fl, p53+/−/Dicerfl/fl, and p53−/−/Dicer−/− mice and primary p53-deleted or Arf-deleted Dicer+/− or Dicer−/− Eμ-Mycl lymphoma cells were generated as previously described (19, 28). Cells were infected with a bicistronic retrovirus (MSCV) encoding CreERT2 (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 flow cytometry following propidium iodide staining for fragmented (sub-G1) DNA and Annexin V/7-AAD staining after treatment with their CD19−/−, pre-B cells, and MEFs using the REDExtract-N-Amp Tissue PCR Kit (Sigma). PCR was performed with primers specific for unarranged and Cre-lox-deleted Dicer alleles, as previously published (19, 21). PCR conditions allowed for 10% to 15% contaminating normal tissue without detecting unarranged floxed Dicer alleles.

Results

p53 deficiency does not rescue lymphoma latency in Myc-overexpressing Dicer−/− 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−/−/Eμ-Mycl mice and littermate controls that were also transgenic for B lineage-restricted CD19-cre recombinase; p53-null Eμ-Mycl mice cannot be generated (26). e-Myc in Eμ-Mycl 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−/−Eμ-Mycl mice compared with their CD19-cre−/p53+/−/Dicer−/−Eμ-Mycl 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 lymphogenesis 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 (19). To determine whether a p53 deficiency occurred signiﬁcantly more frequently in p53−/−/CD19−cre−/Dicerβ/β/Eμ-myc lymphomas than was previously observed in CD19−cre−/Dicerβ/β/Eμ-myc lymphomas (43%) than was previously observed in CD19−cre−/Dicerβ/β/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. 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−/Dicerβ/β/Eμ-myc lymphomas (43%) than was previously observed in CD19−cre−/Dicerβ/β/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−/Dicerβ/β/Eμ-myc mice.

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

CD19 expression was absent or decreased in 65% of the lymphomas from CD19−cre−/Dicerβ/β/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−/Dicerβ/β/Eμ-myc lymphomas for CD19 cell surface expression. None of the 14 pre-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−/Dicerβ/β/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−/Dicerβ/β/Eμ-myc lymphomas (43%) than was previously observed in CD19−cre−/Dicerβ/β/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−/Dicerβ/β/Eμ-myc mice.

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

Previously, we determined that approximately 40% of the lymphomas that emerged in CD19−cre−/Dicerβ/β/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−/Dicerβ/β/Eμ-myc mice. Fourteen of the 16 (88%) 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−/Dicerβ/β/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 the characteristic Eμ-myc B-cell lymphoma in CD19−cre−/Dicerβ/β/Eμ-myc mice, but it also allowed T-cell lymphomas to develop.

Table 1. Dicerβ/fl Eμ-myc lymphoma phenotypes are rescued with a p53 deficiency

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Dicerβ/fl/p53−/−</th>
<th>Eμ-myc</th>
<th>Dicerβ/fl/p53+/−</th>
<th>Eμ-myc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD19-cre−</td>
<td>CD19−cre−</td>
<td>CD19-cre−</td>
<td>CD19−cre−</td>
</tr>
<tr>
<td>B220+·CD19−·CD43−·IgM−</td>
<td>4/10 (40%)</td>
<td>7/10 (70%)</td>
<td>10/16 (63%)</td>
<td></td>
</tr>
<tr>
<td>B220−·CD19+·CD43+·IgM+</td>
<td>6/10 (60%)</td>
<td>3/10 (30%)</td>
<td>4/16 (25%)</td>
<td></td>
</tr>
<tr>
<td>CD3−·CD4−·CD8−·CD43−</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
<td>2/16 (13%)</td>
<td></td>
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</table>
23 Dicer^+/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^+/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^+/Eμ-myc mice compared with mice that were p53^+/+ (43% vs. 12%, respectively; ref. 19), 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%, 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+/Dicerfl/E-my c 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+/Dicerfl/E-my c mice and CD19-cre−/Dicerfl/E-my c controls. There was a modest, but statistically significant, reduction in the percentage of B220+/IgM+ B cells in CD19-cre+/p53−/− Dicerfl/E-my c mice (32.9% ± 1.42%) compared with CD19-cre−/p53+/−/Dicerfl/E-my c littermates (40.9% ± 1.46%; P < 0.0001, paired t test; Fig. 4A). A comparable reduction in B cells was also observed in p53+/−/Dicerfl/E-my c mice that were either CD19-cre+/Dicerfl/E-my c or CD19-cre−/Dicerfl/E-my c (31.2% ± 0.90% and 40.7% ± 0.51%, respectively; P < 0.0001, paired t test; Fig. 4A). 

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+/−/Dicerfl/E-my c, p53+/−/Dicerfl/E-my c, and p53+/−/Dicerfl/E-my c 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 Dicer+)/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, Dicer+/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 Dicer+/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, Dicer+/fl 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.

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 miR20a and miR31, Dicer-dependent miRNA, were similar regardless of Dicer status in all lymphomas analyzed (Fig. 3D). These data indicate that all lymphomas, including those with only one Dicer allele, expressed wild-type levels of Dicer that was fully functional in miRNA biogenesis.
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/Dicer+/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 E and 5A).

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−/CD19-cre−Dicer+/fl and Dicer+/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, p53−/−/Dicer+/fl, and p53−/−/Dicer−/− littersmates were infected (I) with a retrovirus encoding CreERT2 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 blot analyses are shown in B. Arrows, unrearranged (floxed) and wild-type (WT) Dicer alleles in F. CreERT2-expressing Dicer+/fl(D+/fl) MEFs treated with 4-OHT (+) or ethanol (−) were controls in B and F.
Supplementary Fig. S1). CreER<sup>T2</sup> activation in both p53-deleted Dicer<sup>fl/fl</sup>/Em-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<sup>T2</sup> p53-deleted Dicer<sup>fl/fl</sup>/Em-myc lymphoma cells decreased, whereas the percentage of apoptotic cells (cells with fragmented, sub-G<sub>1</sub> DNA, or that were Annexin V<sup>+</sup>) increased after addition of 4-OHT (Fig. 5C–F). PCR analysis revealed that the p53-deleted Dicer<sup>fl/fl</sup>/Em-myc lymphoma cells deleted their one floxed Dicer allele, whereas the p53-deleted Dicer<sup>+/+</sup>/Em-myc lymphoma cells surviving CreER<sup>T2</sup> activation had only deleted one of the conditional Dicer alleles (Fig. 5G). Analogous results were obtained with Dicer<sup>+/+</sup>/Em-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 CreER<sup>T2</sup>-expressing p53-deleted Dicer<sup>fl/fl</sup>/Em-myc lymphoma lines into 96-well plates. After visually confirming the presence of a single cell per well, CreER<sup>T2</sup> was activated with 4-OHT, and the surviving clones were assessed. Only 26% (328 of 1,260) of the clones survived
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}/Eμ-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}/Eμ-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\(^3\) 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\(_1\) 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\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. 6I). 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\textsuperscript{fl/fl}/Eμ-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 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}/Eμ-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} background. First, the early precursor B-cell lymphomas previously observed in approximately 40% of CD19-cre\textsuperscript{+/+}/Dicer\textsuperscript{fl/fl}/Eμ-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}/Eμ-myc mice, was fully restored in lymphomas from p53\textsuperscript{−/−}/CD19-cre\textsuperscript{+/+}/Dicer\textsuperscript{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...
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 Dicerfl/fl/Eμ-myc lymphoma cells (DC1020) and administered tamoxifen (Tam) or vehicle (corn oil; Oil) control starting the day of injection (A; \[P = 0.0012, \text{log-rank test}\]) or once lymphomas were 90 to 150 mm³ (C; \[P = 0.0035, \text{log-rank test}\]). 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: \[P < 0.0001, \text{log-rank test}\]; for D: \[P < 0.0001, \text{log-rank test}\]). 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; \[P < 0.0001, \text{t test}\]. 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 Dicerfl/fl MEFls treated with 4-OHT or ethanol. I and J, nude mice were injected intravenously with CreERT2-expressing p53-deleted Dicerfl/fl/Eμ-myc 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; \[P < 0.0001, \text{t test}\]). Kaplan-Meier survival curves (J; \[P < 0.0001, \text{log-rank test}\]).
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 downregulated 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-myC 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-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−/Dicerfl/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 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 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). Supressing apoptosis by overexpressing the antiapoptotic Bcl-2 protein and/or deleting the proapoptotic gene Bim or by expressing an immunoglobulin transgene, which provides B-cell 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-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 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
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.M. Adams, C.M. Eischen
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
Study supervision: C.M. Eischen

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