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MicroRNA Biogenesis is Required for Myc-Induced B cell Lymphoma Development and Survival

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Abstract

Many tumor cells express globally reduced levels of microRNAs (miRNAs), suggesting that decreased miRNA expression in pre-malignant cells contributes to their tumorigenic phenotype. In support of this, Dicer, an RNAse III-like enzyme that controls maturation of miRNA, was recently shown to function as a haploinsufficient tumor suppressor in non-hematopoietic cells. Since the Myc oncoprotein, a critical inducer of B cell lymphomas, was reported to suppress the expression of multiple miRNAs in lymphoma cells, it was presumed that a deficiency of Dicer and subsequent loss of miRNA maturation would accelerate Myc-induced lymphoma development. We report here that, surprisingly, a haploinsufficiency of Dicer in B cells failed to promote B cell malignancy or accelerate Myc-induced B cell lymphomagenesis in mice. Moreover, deletion of *Dicer* in B cells of CD19-*cre*$^{+}$/Eμ-*myc* mice significantly inhibited lymphomagenesis, and all lymphomas that did arise in these mice lacked functional Cre expression and retained at least one functional *Dicer* allele. Uncharacteristically, the lymphomas that frequently developed in the CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice were of very early precursor B cell origin, a stage of B cell development prior to Cre expression. Therefore, loss of Dicer function was not advantageous for lymphomagenesis, but rather, Dicer ablation was strongly selected against during Myc-induced B cell lymphoma development. Moreover, deletion of *Dicer* in established B cell lymphomas resulted in apoptosis, revealing that Dicer is required for B cell lymphoma survival. Thus, Dicer does not function as a haploinsufficient tumor suppressor in B cells and is required for B cell lymphoma development and survival.

Keywords

dicer; myc; lymphoma; B cell

Introduction

Dicer is an RNAse III-like enzyme that processes pre-miRNAs into mature miRNAs. miRNAs are linked to multiple biological processes, including differentiation, apoptosis, and proliferation, that are important for transformation and tumor development (1) Deletion of *Dicer* in multiple organisms, including mice, is lethal (2), highlighting the importance of Dicer in embryogenesis. However, it has been reported that a global decrease of miRNAs

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occurs in multiple tumor types (3), implying that decreased miRNA expression contributes to tumorigenesis. Moreover, Jacks and colleagues reported that suppression of *Dicer* with shRNA or deletion of *Dicer* increased the growth potential of carcinoma cell lines and oncogenic Ras-induced transformation of murine lung epithelial cells, respectively (4). However, Dicer was recently shown to be a haploinsufficient tumor suppressor in lung epithelial cells and in the retina (5,6). Heterozygous germline mutations in *DICER* were also found in a rare pediatric lung tumor (7). In contrast, deletion of *Dicer* results in growth arrest and p53-dependent senescence in primary fibroblasts and cell death of lung epithelial cells (8,9). Moreover, *Dicer* hypomorphic mice have not been reported to have an increased incidence of cancer (10). Therefore, the role Dicer has in tumorigenesis remains unclear.

The oncogene c-Myc, which is frequently overexpressed in human and murine cancers, including B cell lymphomas, was reported to suppress the expression of multiple miRNAs in B cell lymphomas (11). Yet, Myc induces the expression of the microRNA polycistron *miR-17 ~92* and the miR-106a cluster (12), and constitutive expression of the *miR-17 ~92* polycistron accelerates Myc-induced B cell lymphoma development (13). Therefore, the role of miRNAs in Myc-induced B cell lymphoma development is unresolved. Recently, deletion of *Dicer* in very early progenitor B cells by Mb1-*cre* was shown to result in precursor B cell apoptosis and B cell developmental defects (14). To determine if global loss of miRNA expression would impact B cells that are further differentiated, and more importantly whether *Dicer* deletion would contribute to Myc-induced B cell lymphoma development, we utilized conditional *Dicer* knockout mice and CD19-*cre* and Eμ-*myc* transgenic mice, which develop pre-B/B cell lymphomas. We observed a small decrease in the numbers of B cells in CD19-*cre/Dicer*$^{fl/fl}$ mice regardless of Myc status and a significant delay in B cell lymphoma development in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic mice. Interestingly, early precursor B cell lymphomas emerged in 40% of the CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic mice, and all lymphomas regardless of stage of differentiation retained one allele of *Dicer* due to loss of functional Cre. Loss of one allele of *Dicer* did not affect B cell development or Myc-induced lymphomagenesis. Deletion of both alleles of *Dicer* in established B cell lymphomas induced apoptosis. Therefore, we have demonstrated that Dicer is required for B cell lymphomagenesis and the survival of B cell lymphoma that have developed. In addition our data shows that Dicer is not a haploinsufficient tumor suppressor in B cells.

Materials and Methods

Mice

Eμ-*myc* transgenic mice (15) and CD19-*cre* (16) mice were mated and then F1s were bred to *Dicer*$^{fl/fl}$ mice that have loxP sites flanking exons 15-17 (8). F2s were bred to obtain *Dicer*$^{fl/fl}$, *Dicer*$^{+/fl}$, and *Dicer*$^{+/+}$ CD19-*cre* positive and negative and Eμ-*myc* positive and negative offspring. All analyses were performed with littermates. Only CD19-*cre* positive hemizygous mice were used and evaluated in these studies. All mice were carefully monitored and at signs of disease, tumors/tissues were collected and analyzed. Tissues/cells prior to disease were also collected and evaluated. Statistical significance of the survival between the different genotypes of Eμ-*myc* transgenic mice was determined by log-rank test. All research with mice complied with federal and state guidelines and was approved by the Vanderbilt IACUC committee.

Western and Southern blotting

B cell lymphomas were lysed and proteins Western blotted as previously described (17). Membranes were probed with antibodies specific for Cre (Novagen), Mdm2 (C18, Santa Cruz), p53 (Ab-7; Calbiochem, La Jolla, CA), p19ARF (GeneTex, San Antonio, TX), and β-

actin (Sigma). Genomic DNA from B cell lymphomas was Southern blotted for *p53* and *ARF*, and p53 was sequenced as previously described (18,19).

Phenotype Analysis

Freshly isolated lymphoma cells from CD19-*cre*$^{+}$/Eμ-*myc* mice with none, one, or two floxed *Dicer* alleles, whole spleens and bone marrow from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic mice prior to the development of lymphoma and non-Eμ-*myc* and/or non-CD19-*cre* littermates were analyzed by flow cytometry following staining with fluorescent antibodies as previously described (17,18). Flow cytometry data were evaluated with CellQuest and/or FlowJo software.

Quantitative real-time RT-PCR

Total RNA was isolated from murine cells and tissue with Trizol (InVitrogen) as per manufacture's protocol. Total RNA was further purified with an RNeasy kit (Qiagen). cDNA was generated as previously reported (20). Sequences for *β-actin*, *Dicer*, *Cre*, and *CD19*-specific primer pairs were obtained from the Primer Bank (Harvard Medical School) and synthesized by Eurofins MWG Operon. Quantitative real-time PCR was performed with SybrGreen (SABiosciences) in triplicate as previously reported (20). The data are expressed in $2^{-\text{deltaCt}}$ using *β-actin* as a reference. Taqman RT-PCR for miRNA used TaqMan microRNA Assay (Applied BioSystems) in triplicate and compared to RNU6b small RNA expression.

Dicer Gene Rearrangement Analysis

Genomic DNA was isolated using the REDExtract-N-Amp Tissue PCR Kit (Sigma) from lymphomas frozen immediately after harvesting and lymphomas grown in culture. Lymphomas from mice were greater than 85% pure. PCR analysis of DNA was performed under conditions that allow for 15% contaminating normal cells without detection of the unrearranged floxed allele. Primers for detecting unrearranged *Dicer* alleles have been previously published (8). Primers for detecting Cre-lox deleted *Dicer* alleles were CCATTGGTGCCAAGACAATG and CAGGCTCCACTCCCTAAC.

Lymphoma Cell Survival Analysis

Isolated primary *Dicer*$^{fl/fl}$/Eμ-*myc* transgenic lymphoma cells were infected with empty pBabe or CreER^{T2} encoding pBabe retrovirus (21). Infected cells were selected with puromycin. *Dicer* was deleted in the cells by activating CreER^{T2} with 1 μM 4-hydroxytamoxifen. Cell numbers and cell viability following activation of CreER^{T2} was determined by Trypan Blue Dye exclusion assay. Apoptosis as measured by fragmented (sub-G1) DNA was quantified following propidium iodide (PI) staining and flow cytometry, and further verified by Annexin V-FITC and flow cytometry.

Results

Loss of *Dicer* inhibits B cell lymphoma development

Dicer has been reported to be a haploinsufficient tumor suppressor in non-hematopoietic cells (5,6). To determine whether Dicer functions as a haploinsufficient tumor suppressor in B cells, we generated single allele conditional *Dicer* knockout mice (*Dicer*$^{+/fl}$) that were transgenic for *cre* recombinase, which was placed under the transcriptional control of one allele of the endogenous *CD19* locus (8,15,16,22). Over a year of observation, B cell malignancies did not emerge in CD19-*cre*$^{+}$/*Dicer*$^{+/fl}$ mice and their survival was similar to that of CD19-*cre*$^{-}$/*Dicer*$^{+/fl}$ and CD19-*cre*$^{+}$/*Dicer*$^{+/+}$ littermates (data not shown). CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$ mice also did not have an increased incidence of B cell cancers and had a

survival analogous to that of CD19-*cre*$^{+}$/*Dicer*$^{+/fl}$ and CD19-*cre*$^{+}$/*Dicer*$^{+/+}$ mice. Therefore, loss of one allele of *Dicer* alone is insufficient to initiate B cell lymphoma.

To test the idea that Dicer is a haploinsufficient tumor suppressor further and to determine whether loss of one allele of *Dicer* would cooperate with Myc overexpression to promote tumorigenesis in B cells in a similar manner as it did with Ras overexpression in lung epithelial cells (4,5), we generated CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$ mice transgenic for Myc (Eμ-*myc*). Eμ-*myc* transgenic mice overexpress c-Myc in B cells starting at the pre-B cell stage of development (22). CD19 expression, and thus Cre expression, in CD19-*cre* mice occurs at the pro-B cell stage and continues as B cell mature (16,23). *Dicer* heterozygous floxed CD19-*cre*$^{+}$/Eμ-*myc* mice developed lymphoma and had a survival similar to that of CD19-*cre*$^{+}$/*Dicer*$^{+/+}$/Eμ-*myc* transgenics (Fig. 1A), indicating Dicer does not have a haploinsufficiency effect on lymphoma latency or overall survival. However, evaluation of lymphoma development in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice revealed a significant delay in lymphomagenesis and an extended survival, compared to CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* (Fig. 1A), CD19-*cre*$^{+}$/*Dicer*$^{+/+}$/Eμ-*myc* (Fig. 1C), and CD19-*cre*$^{-}$/*Dicer*$^{+/fl}$/Eμ-*myc* littermates (Fig. 1B) (p=0.0001 log-rank tests). The mean survival of CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice was 351 days compared to 204 days for CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* littermates and 194 days for CD19-*cre*$^{+}$/*Dicer*$^{+/+}$/Eμ-*myc* mice. Therefore, CD19-*cre*$^{+}$/Eμ-*myc* mice with both alleles of *Dicer* floxed did not have an acceleration of lymphomagenesis; instead, they had a significantly protracted rate of lymphoma development. Our results show that a Dicer haploinsufficiency does not cooperate with Myc overexpression in B cells, and that loss of both alleles of *Dicer* in B cells inhibits Myc-induced B cell lymphoma development.

Two floxed alleles of *Dicer* alter the type of B cell lymphoma that develops

Recently it was shown that deletion of *Dicer* at a very early stage of B cell development with Mb1-*cre* led to an almost complete ablation of precursor B cells and subsequently differentiated B cells (14). We evaluated splenic B cells from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice prior to the development of lymphoma to determine whether mature B cells were present. Wild-type Eμ-*myc* transgenic mice have a reduced number (approximately 10%) of mature B cells (24), and loss of *Dicer* further decreased the number of mature B cells present. Specifically, there was a significant decrease in the percentage (and decreased total numbers) of B220^{+}/IgM^{+} B cells in pre-cancerous CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* spleens compared to spleens in CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* littermates (Fig 1C). Comparing seven littermate matched pairs, the mean percentage of B220^{+}/IgM^{+} B cells in pre-cancerous CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* spleens was 23.0% ±2.01 and in CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* littermates was 31.3% ±2.48 (p<0.0001, paired t-test). A comparable decrease in B cells due to *Dicer* deletion was detected in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$ mice in the absence of the Myc transgene (Fig. 1C). Six littermate matched pairs showed a mean of 33.8% ±4.34 B220^{+}/IgM^{+} B cells in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$ mice and 43.3% ±4.64 B220^{+}/IgM^{+} B cells in CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$ mice (p=0.0015, paired t-test). No difference in B cell numbers or development was detected in CD19-*cre*$^{+}$/*Dicer*$^{+/fl}$ or CD19-*cre*$^{+}$/*Dicer*$^{+/fl}$/Eμ-*myc* mice (data not shown). These results indicate that the CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$ mice, which express Cre later in B cell development, had only a small reduction (8.3% mean decrease) in their number of B cells, in contrast to the Mb1-*cre*$^{+}$/*Dicer*$^{fl/fl}$ mice.

Since deletion of *Dicer* in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$ and CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic mice slightly altered B cell development, we sought to determine whether loss of *Dicer* altered the typical pre-B/B cell lymphomas that arise in Eμ-*myc* transgenic mice (15,22). Flow cytometric analysis on freshly isolated tumors was performed. Fourteen of 23 (61%) of the lymphomas analyzed from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice were typical pre-B and/or B cell lymphomas that expressed B220 and were either IgM^{-} or IgM^{+}, which are characteristic of Eμ-*myc* lymphomas (22). Surprisingly, 9 of 23 (39%) of the CD19-*cre*$^{+}$/

Dicer$^{fl/fl}$/Eμ-*myc* lymphomas analyzed expressed markers characteristic of very early progenitor B cells (B220$^+$, CD4$^+$, CD43$^+$, Sca1$^+$), but lacked markers of differentiated lymphocytes IgM and CD3 (Fig. 1D), which are not observed in wild-type Eμ-*myc* transgenic mice. However, these early precursor B cell lymphomas were previously detected in Eμ-*Bcl2*/Eμ-*myc* double transgenic mice (25). Thus, a significant number of very early precursor B cell lymphomas emerged in CD19-*cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic mice, rather than the characteristic pre-B/B cell lymphomas.

Increased frequency of *p53* deletions in *Dicer*$^{fl/fl}$ CD19-*Cre*$^+$Eμ-*myc* lymphomas

Myc-induced B cell lymphomagenesis proceeds, in part, through inactivation of the ARF-Mdm2-p53 tumor suppressor pathway (17). We analyzed CD19-*cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic B cell lymphomas by Western and Southern blot to determine the frequency of alterations in ARF, Mdm2, and p53 (Fig. 2). Lymphomas that lacked the tumor suppressor ARF protein (Fig. 2A, 16 lymphomas shown out of 25 total analyzed) were subjected to ARF Southern blot analysis. *ARF* deletions were evident in 20% (5 of 25) of the lymphomas analyzed from CD19-*Cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice (Fig. 2B), similar to the percentage of *ARF* deletions in wild-type Eμ-*myc* lymphomas (17). Mdm2, a negative regulator of p53, was overexpressed in half of the CD19-*Cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas (Fig. 2A and data not shown), as is typical for wild-type Eμ-*myc* mice (17). Mutation of the tumor suppressor p53, which is usually evident as increased levels of p53 protein (Fig. 2A), occurs in a quarter of the B cell lymphomas that arise in Eμ-*myc* mice (17). Similarly, point mutations in *p53* were detected in 24% (6 of 25) of the lymphomas from CD19-*cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenics. However, *p53* deletions were detected in 16% (4 of 25) of the CD19-*cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas (Fig. 2B), whereas deletion of p53 is a rare event, occurring in 3-4% of lymphomas in Eμ-*myc* mice (17,18). Mutation or deletion of *p53* was detected in early precursor B cell lymphomas and in more mature B cell lymphomas, suggesting that inactivation of *p53* does not appear to correlate to the stage of B cell differentiation of the lymphoma in these mice. Together the *p53* mutations and deletions were present in 40% of the lymphomas in CD19-*cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice, indicating there was an increased selection for p53 inactivation in these lymphomas. An evaluation of p53 alterations in lymphomas from CD19-*cre*$^+$/Eμ-*myc* mice that were *Dicer*$^{+/fl}$ revealed a normal frequency of *p53* mutations (3 of 11, 27%) and deletions (0 of 11) (data not shown), suggesting that there was no increase in selective pressure for p53 inactivation in CD19-*cre*$^+$Eμ-*myc* lymphomas with only one floxed *Dicer* allele. Therefore, *p53* deletion occurred at an increased frequency in CD19-*cre*$^+$/Eμ-*myc* lymphomas arising in mice with two floxed *Dicer* alleles, suggesting that there is increased stress in and/or around the developing B cells in these mice.

Loss of *Dicer* is selected against during B cell lymphomagenesis

Unexpectedly, 65% (15 of 23) of the lymphomas analyzed that arose in CD19-*cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice lacked or had reduced expression of CD19, a B cell marker, at their cell surface, as determined by flow cytometry. For example, typical IgM$^+$/B220$^+$/CD19$^+$ (or IgM$^-$/B220$^+$/CD19$^+$) B cell lymphomas were detected in 35% of CD19-*cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice (Fig. 3A). In contrast, there were CD19-*cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice that had an IgM$^+$ (or IgM$^-$)/B220$^+$/CD19$^-$ (or CD19^{low}) lymphoma (Fig. 3A), which is uncharacteristic for lymphomas in Eμ-*myc* transgenic mice. Quantitative real-time PCR (qRT-PCR) showed that *CD19* mRNA levels were significantly reduced in the lymphomas where cell surface CD19 was absent or very low (Fig. 3B). Since *cre* is knocked into the *CD19* locus and cell surface CD19 was expressed at reduced levels in the majority of the CD19-*cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas, we questioned whether *cre* expression was also being affected in these lymphomas. Evaluation of *cre* by qRT-PCR revealed that 89% (17 of 19) of the CD19-*cre*$^+$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas analyzed showed significantly reduced or absent *cre* mRNA

(Fig. 3C). The lymphomas that lacked or had barely detectable *cre* mRNA did not express Cre protein (Fig. 3D). Only 12% (3 of 25) of the CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* lymphomas analyzed expressed Cre protein. There was a significant difference in Cre protein expression between CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* lymphomas and lymphomas from $Dicer^{+/fl}$/CD19-cre^{+}/Eμ-*myc* and $Dicer^{+/+}$/CD19-Cre^{+}/Eμ-*myc* mice ($p<0.0005$, chi-squared test). Interestingly, the number of floxed *Dicer* alleles appears to dictate the frequency of loss of Cre protein expression. Specifically, 64% (14 of 22) of $Dicer^{+/fl}$/CD19-cre^{+}/Eμ-*myc* lymphomas expressed Cre protein, whereas 80% (16 of 20) of the $Dicer^{+/+}$/CD19-Cre^{+}/Eμ-*myc* lymphomas expressed Cre protein (Fig. 3D). Therefore, loss of Cre expression in the lymphomas correlated to the number of floxed *Dicer* alleles and was a frequent event in CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* transgenic lymphomas.

To determine whether functional Cre protein was ever expressed in the CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* lymphomas that lacked Cre protein, and whether the Cre protein expressed in the three lymphomas (DC118, DC279, DC407) was functional, we assessed by PCR genomic DNA for the presence of the region in *Dicer* that is flanked by loxP sites. First we evaluated lymphomas from CD19-cre^{+}/$Dicer^{+/fl}$/Eμ-*myc* lymphomas and observed that 83% (20 of 24) had Cre-lox deletion of their one floxed *Dicer* allele (Fig. 4A and data not shown), consistent with Hobeika et al. reporting an 80% deletion efficiency in B cells of CD19-*cre* mice (26). However, not a single lymphoma from a CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* mouse was found to have deleted both conditional alleles of *Dicer* (Fig. 4B). Only one conditional allele of *Dicer* had been deleted in 38% (10 of 26) of the lymphomas from CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* mice, including the three lymphomas that expressed Cre protein (Figure 4B and data not shown). No deletion of either floxed *Dicer* allele was detected in 62% (16 of 26) of the lymphomas from CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* mice. Moreover, all of the CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* lymphomas analyzed expressed *Dicer* mRNA (Figure 4C) and miRNAs known to be expressed in B cells (Figure 4D and data not shown), indicating that *Dicer* was retained and functional in all of the lymphomas. The levels of miRNAs expressed varied between different CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* lymphomas and the miRNA evaluated, but were similar to controls. These data illustrate that lymphomas do not develop when both alleles of *Dicer* are deleted, but can emerge when one allele has been deleted. To test this concept further, we evaluated freshly isolated CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* lymphomas that had been placed into short-term culture and allowed to grow to derive pure populations of lymphoma cells. Again, not a single CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* lymphoma was detected that had deleted both floxed alleles of *Dicer* (Figure 4B, right panels). Only 17% (1 of 6) of the cultured lymphomas had deleted one allele of *Dicer*, whereas the rest had retained both alleles of *Dicer*. Therefore, loss of *Dicer* appears to be strongly selected against during B cell lymphoma development in CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* mice, suggesting that Dicer expression is required for B cell transformation.

Dicer expression is required for B cell lymphoma survival

Previously, Jacks and colleagues had reported that suppression of *Dicer* expression with shRNA resulted in increased proliferation and transformation potential of established epithelial cancer cell lines (4). To determine whether *Dicer* loss alters the survival or growth of already established B cell lymphomas, lymphomas were isolated from $Dicer^{fl/fl}$/Eμ-*myc* mice and infected with a 4-hydroxytamoxifen (4-OHT) inducible Cre retrovirus ($CreER^{T2}$) (21). Following selection of infected lymphoma cells with puromycin, $CreER^{T2}$ was activated with 4-OHT. For all cultures of $Dicer^{fl/fl}$/Eμ-*myc* lymphomas, the activation of $CreER^{T2}$ resulted in the induction of apoptosis, whereas addition of vehicle control (ethanol, ETOH) or addition of 4-OHT to empty retroviral infected lymphomas had little effect. The numbers of viable lymphoma cells decreased and the percentage of cells containing fragmented DNA or that were Annexin V positive increased within 24 hours post 4-OHT

addition (Fig. 5A-5C and Table 1), indicating that the lymphoma cells were undergoing apoptosis. However, in all cases, 24-48 hours following 4-OHT, surviving lymphoma cells did grow out of the cultures; these cells had similar doubling times and proliferated at a rate analogous to those treated with vehicle control (Fig. 5A and data not shown). Additionally, there were analogous percentages of CreER^{T2} or empty retroviral expressing lymphoma cells that incorporated BrdU 48 and 72 hours following 4-OHT treatment (data not shown). As expected, PCR analysis showed that the population of lymphoma cells that survived CreER^{T2} activation had only deleted one allele of *Dicer* (Fig. 5D).

To further test whether a lymphoma cell could survive without Dicer, we placed a single *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cell infected with CreER^{T2} into wells of 96 well plates, added 4-OHT to activate CreER^{T2}, and evaluated the clones that emerged. Only 22% (43 of 192) of the clones survived CreER^{T2} activation, whereas 98% of the vehicle (ETOH) treated clones survived. Of the 43 *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma clones that survived 4-OHT treatment, none had deleted both alleles of *Dicer* (Fig. 5E and data not shown). Forty of the 43 clones had deleted one allele of *Dicer*, whereas the remaining three clones had retained both alleles of *Dicer*. These data indicate that deletion of both alleles of *Dicer* is not advantageous for B cell lymphomas, but instead induces apoptosis. Therefore, one allele of *Dicer* is required for B cell lymphoma survival. In addition, loss of a single allele of *Dicer* did not confer a proliferative advantage to B cell lymphomas over those that retained both alleles of *Dicer*, but did allow cell survival.

Discussion

The role miRNAs and Dicer have in cellular processes necessary for tumorigenesis is incompletely understood. It has been reported that Myc suppresses the expression of many miRNAs, and some tumor cells express globally lower levels of miRNAs or miRNA processing enzymes (3,4,11,27). These studies contributed to the hypothesis that decreased miRNA expression enhances cellular transformation. However, our data here shows that in B cells, deletion of *Dicer*, which would block the formation of mature miRNAs, did not facilitate Myc-mediated B cell lymphomagenesis. There was no cooperation between loss of *Dicer* and Myc overexpression in B cell proliferation or tumorigenesis. In fact, in pre-cancerous CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice, there was no detectable increase in the numbers of B cells, which would be indicative of an enhanced proliferative process; instead there were reduced numbers of B cells. Moreover, CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice had a protracted rate of lymphoma development of which the decrease in B cells may have contributed. Importantly, all lymphomas that arose in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice retained at least one allele of *Dicer*. The retention of *Dicer* by a developing B cell is likely to have allowed that B cell to survive (14). The lack or loss of Cre expression would permit a B cell to retain one or both alleles of *Dicer*, resulting in an increased pool of precursor B cells that do not express Cre and consequently, an increased frequency of lymphomas that lack Cre expression and that have retained *Dicer*. In support of this idea, 39% of the lymphomas that arose in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice were early precursor B cell lymphomas that due to their stage of maturation did not yet express CD19, and therefore did not express Cre. Other lymphomas that were more differentiated and should have expressed CD19 appeared to have never expressed Cre or expressed Cre that was only able to delete one allele of *Dicer*. Consequently, all lymphomas analyzed from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice, including mature B cell lymphomas, retained at least one allele of *Dicer* and may or may not have expressed CD19. It is likely the increased stress caused by the altered B cell differentiation in the CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice accounts for the increase in p53 inactivation in the lymphomas that arise. Our results provide strong evidence that loss of *Dicer* is not advantageous, but instead strongly selected against during Myc-mediated B cell lymphoma development.

Studies with mouse models have shown that Dicer appears to be a haploinsufficient tumor suppressor in lung epithelial, muscle, and retinal cells, leading to an acceleration of cancers in these tissues when *Dicer* is heterozygous (5,6). In contrast, *Dicer* hypomorphic mice do not have an increased incidence of cancers in these or other tissues (10). Moreover, CD19-*cre*$^{+}$/*Dicer*$^{+/fl}$ mice did not have increased numbers of B cells or B cell malignancies. In addition, murine fibroblasts have normal rates of growth when only one allele of *Dicer* is deleted (8). What distinguishes the studies in non-hematopoietic tissues that show Dicer is a haploinsufficient tumor suppressor is the overexpression of oncogenic Ras and/or deletion of a strong tumor suppressor (p53 or Rb/p107) was required to observe the tumor suppressor phenotype of *Dicer* heterozygosity (5,6). However, when the Myc oncogene was overexpressed in B cells that lacked one allele of *Dicer* this did not result in an acceleration of B cell lymphomagenesis, indicating that *Dicer* heterozygosity does not cooperate with Myc overexpression in B cells. It will need to be determined whether *Dicer* heterozygosity can cooperate with Myc overexpression in non-hematopoietic tissues. Therefore in the context of *Dicer* heterozygosity alone or together with Myc overexpression, Dicer is not a haploinsufficient tumor suppressor in B cells.

Our data indicate certain miRNAs, whose maturation requires Dicer, must be necessary for Myc to induce B cell transformation, since *Dicer* is retained in all lymphomas arising in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice. Previously, it was shown that specific miRNAs are induced by Myc and facilitate B cell transformation initiated by Myc (12,13). For example, the miRNA polycistron *miR-17~92*, which is regulated by Myc, is frequently overexpressed in human B cell lymphomas, and when overexpressed in pre-cancerous Eμ-*myc* fetal liver cells, Myc-mediated lymphomagenesis was accelerated (13). Therefore, Dicer and consequently, miRNAs are required for B cell transformation initiated by Myc. Similarly, one allele of *Dicer* was retained in tumors arising in a Ras-induced mouse model of lung cancer or soft tissue sarcoma (5), indicating that Dicer is also essential for transformation of non-hematopoietic cells. It will be important in the future to identify the miRNAs required for the transformation of B cells and for non-hematopoietic cells and to determine if there are commonalities.

In addition to Dicer being required for B cell transformation, we also determined that Dicer was necessary for the survival of established B cell lymphomas. Deletion of *Dicer* led to lymphoma cell apoptosis, whereas retention of at least one allele of *Dicer* allowed lymphoma cell survival. It is postulated that non-hematopoietic tumor cell lines can survive without Dicer (5). Differences in cell type and/or pre-existing genetic alterations may account for the discrepancy. For example, hematopoietic cells, such as B cells, are poised for apoptosis, whereas fibroblasts and epithelial cells are more prone to undergo senescence. Therefore, it is conceivable that a non-hematopoietic cell could survive long enough to acquire additional genetic alterations permitting it to grow in the absence of Dicer. Consistent with this notion, loss of *p53* or the *INK4a/ARF* locus allowed fibroblasts to delay senescence induced by *Dicer* deletion (8). Similarly, deletion of *Dicer* in hepatocytes in mice led to apoptosis, but the few surviving hepatocytes developed into hepatocellular carcinoma late in life, indicting additional genetic events were necessary for hepatocyte survival (28). In contrast, cultured primary B cell lymphomas, all of which have inactivated the p53 pathway, underwent apoptosis upon *Dicer* deletion. Notably, there was a partial rescue of *Dicer* deletion-induced apoptosis of B cell precursors by overexpression of Bcl-2 (14), but it is unknown if that resulted in a B cell malignancy later in the life of those mice. Thus, complete loss of Dicer appears to be incompatible with cell viability, unless compensatory mutations occur that allow survival and growth. Therefore it will be important to define the mutations that a cell needs to acquire to live without Dicer and whether hematopoietic cells can ever survive and proliferate without Dicer.

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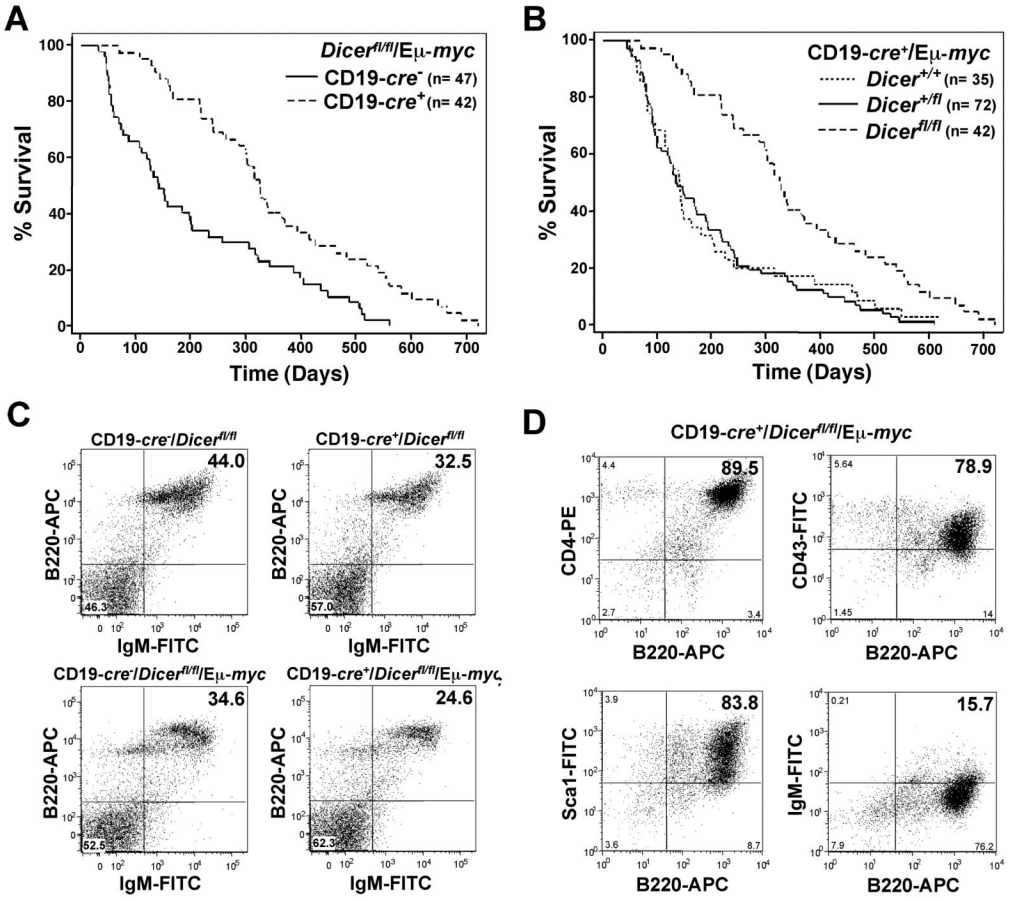

Figure 1. CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice have a protracted rate of lymphomagenesis, decreased precursor B cell numbers, and develop an altered type of lymphoma

Kaplan-Meier survival curves. (A) CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic and CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic littermates; p=0.0001 (log-rank test). (B) CD19-*cre*$^{+}$/Eμ-*myc* transgenic littermates with none, one, or two floxed *Dicer* alleles; p=0.0001 (log-rank test). The number (n) of mice in each group is denoted. (C) Representative dot plots of splenic B cells from the indicated mice (n=6 or 7 littermate matched pairs of each genotype). B220-APC versus IgM-FITC gated on total lymphocytes. (D) Dot plots of lymphoma cells from a CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic mouse expressing markers of early precursor B cells. All plots are gated on total lymphocytes. Quadrants were set with fluorochrome-linked isotype controls.

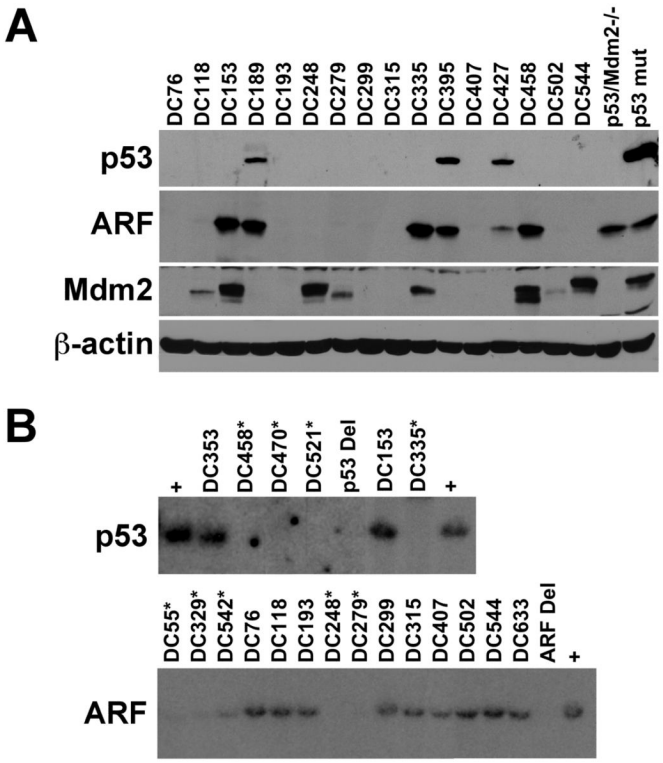

Figure 2. Increased frequency of *p53* deletions in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas

(A) Protein lysates of lymphomas from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic mice were subjected to Western blot analysis for p53, ARF, Mdm2, and β-actin. Protein lysates from *p53/Mdm2*-double null MEFs and a B cell lymphoma containing mutant p53 were used as controls. (B) Southern blots for *p53* and *ARF* of lymphomas from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic mice. Asterisks denote lymphomas that have deleted *p53* and *ARF*. Lymphomas that contain *p53* or *ARF* (plus signs) or that have deleted *p53* (p53 Del) or *ARF* (ARF Del) are denoted.

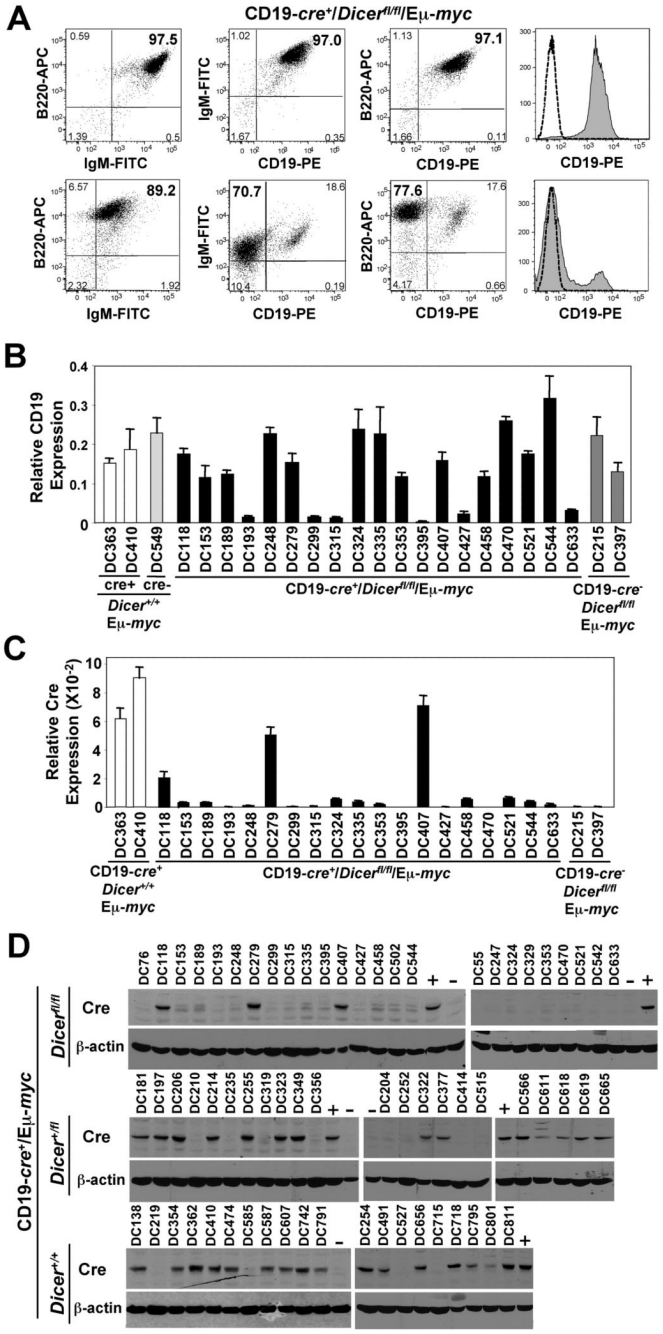

Figure 3. Decreased CD19 expression and frequent loss of Cre expression in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas

(A) Dot plots of CD19, B220, and IgM expression in two CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas. Quadrants were set with isotype controls for each lymphoma. For histograms, CD19 expression is grey and the isotype control for each is indicted with a dotted line. (B & C) qRT-PCR for CD19 (B) and Cre (C) expression relative to β-actin expression in lymphomas from the indicated genotype. (D) Western blots for Cre and β-actin in protein lysates of CD19-*cre*$^{+}$/Eμ-*myc* lymphomas with none, one, or two floxed *Dicer* alleles. Protein lysates from lymphomas from CD19-*cre*$^{+}$/*Dicer*$^{+/+}$/Eμ-*myc* lymphomas (+) and CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas (-) are indicated.

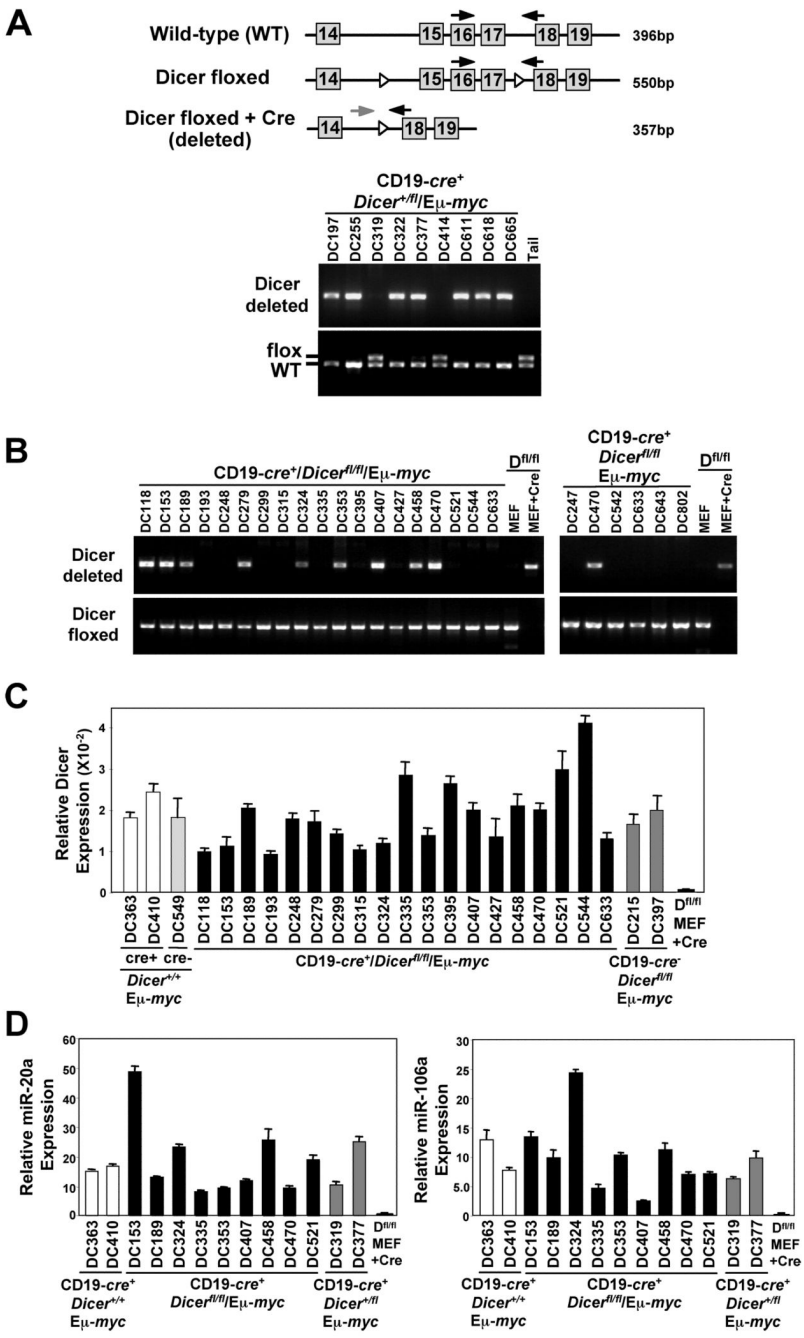

Figure 4. CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic lymphomas retain at least one *Dicer* allele

(A) Schematic of the *Dicer* locus with loxP sites denoted with open arrowheads. Location of the sites of primer pairs used to detect a region of *Dicer* by PCR are indicated with arrows. Size in base pairs (bp) of the PCR products from the specific primers is indicated. (A & B) Detection of conditional deleted and floxed *Dicer* alleles by PCR from genomic DNA from frozen CD19-*cre*$^{+}$/*Dicer*$^{+/fl}$/Eμ-*myc* lymphomas (A), frozen CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas (B, left panel), or cultured CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas (B, right panel). Genomic DNA from the tail of a *Dicer*$^{+/fl}$ mouse with a floxed *Dicer* allele is a control (A). Genomic DNA from *Dicer*$^{fl/fl}$ ($D^{fl/fl}$) mouse embryo fibroblasts (MEF) with or without Cre recombinase serves as controls for *Dicer* conditional deletion (B). (C) qRT-PCR

for *Dicer* in individual Eμ-*myc* lymphomas of the indicated genotype. A $Dicer^{fl/fl}$ ($D^{fl/fl}$) MEF expressing Cre recombinase serves as a control for *Dicer* conditional deletion. (C) Taqman qRT-PCR for miR-20a or miR-106a in the indicated Eμ-*myc* lymphomas (other miRNA also evaluated with similar results). A $Dicer^{fl/fl}$ ($D^{fl/fl}$) MEF expressing Cre recombinase serves as a control for loss of miRNA expression.

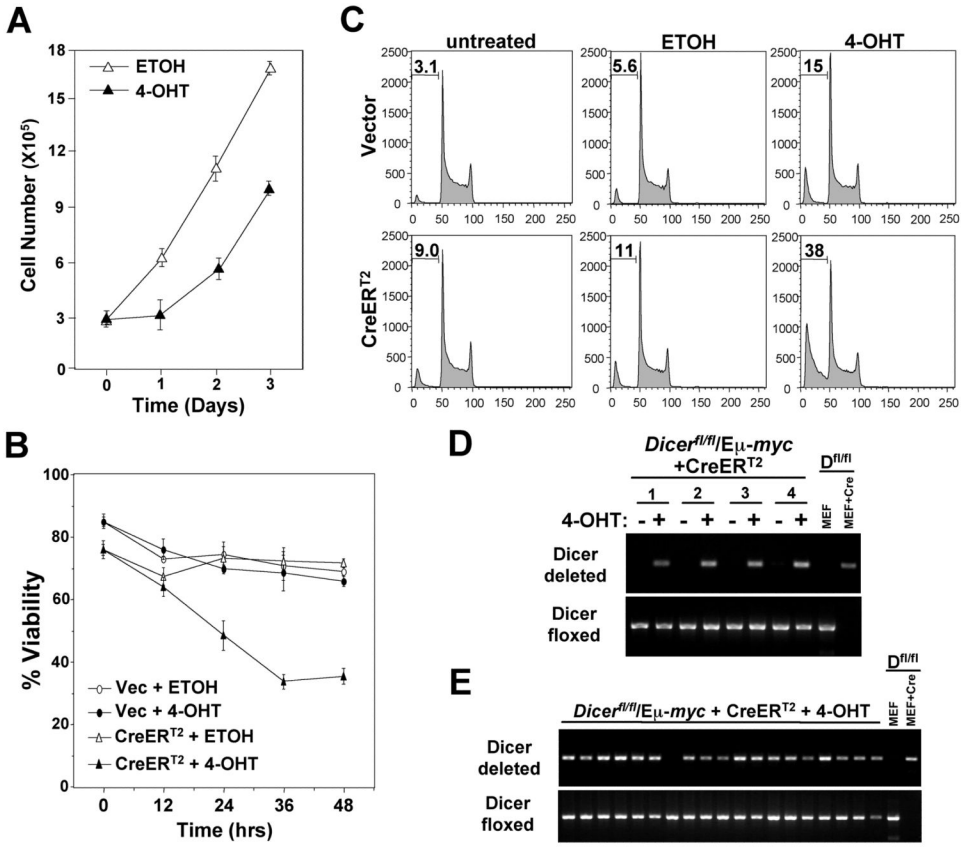

Figure 5. *Dicer* loss induces apoptosis of established B cell lymphomas

(A-E) Lymphomas from *Dicer*$^{fl/fl}$/Eμ-*myc* transgenic mice infected with CreER^{T2} encoding retrovirus or an empty retrovirus (Vec). 4-OHT or vehicle control (ETOH) was added to lymphoma cell cultures at day or time 0 and cell number (A), viability (B), apoptosis (percentage of cells in subG1 indicated, C), and *Dicer* gene rearrangement (D) were evaluated at intervals. A, B, and C contain representative data from a minimum of three independent experiments. For D, data for four independent *Dicer*$^{fl/fl}$/Eμ-*myc* CreER^{T2} expressing lymphomas are shown. (E) Dicer rearrangement PCR of FACS sorted single lymphoma clones that emerged following 4-OHT activation of CreER^{T2}. Nineteen of 43 total clones analyzed are shown. For D and E, a *Dicer*$^{fl/fl}$ (D$^{fl/fl}$) MEF with or without Cre recombinase serves as a control for *Dicer* deletion.

Table 1

Deletion of *Dicer* induces apoptosis

	untreated*	ETOH*	4-OHT*
Vector	19.9±2.2	22.1±0.6	26.0±1.1
CreER^{T2}	21.7±1.5	23.7±0.3	41.4±2.0

* percentage of Annexin V positive primary lymphoma cells