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The Transcription Factor ASCIZ and Its Target **DYNLL1 Are Essential for the Development and Expansion of MYC-Driven B Cell Lymphoma**

Graphical Abstract



Authors

David M. Wong, Lingli Li, Sabine Jurado, ..., David M. Tarlinton, Andreas Strasser, Jörg Heierhorst

Correspondence

jheierhorst@svi.edu.au

In Brief

Wong et al. show that loss of ASCIZ or its transcriptional target DYNLL1 dramatically delays the development of MYC-driven B cell lymphoma in mice. ASCIZ is essential for the MYCdependent upregulation of DYNLL1 and survival of pre-malignant cells before they can give rise to lymphoma.

Highlights

- Loss of ASCIZ or its target DYNLL1 delays lymphoma development in $E\mu$ -Myc mice
- DYNLL1 expression is hyper-activated in pre-leukemic Eµ-Myc B cell precursors
- Loss of ASCIZ or DYNLL1 is synthetic lethal with oncogenic MYC in developing B cells
- Deletion of Asciz in established Eμ-Myc tumors delays lymphoma progression



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The Transcription Factor ASCIZ and Its Target DYNLL1 Are Essential for the Development and Expansion of MYC-Driven B Cell Lymphoma

David M. Wong,¹ Lingli Li,^{1,2} Sabine Jurado,^{1,6} Ashleigh King,^{1,2} Rebecca Bamford,¹ Meaghan Wall,^{1,2,3} Mannu K. Walia,¹ Gemma L. Kelly,^{4,5} Carl R. Walkley,^{1,2} David M. Tarlinton,^{4,5} Andreas Strasser,^{4,5} and Jörg Heierhorst^{1,2,*}

¹St. Vincent's Institute of Medical Research, Fitzroy, VIC 3065, Australia

²Department of Medicine, St. Vincent's Hospital, The University of Melbourne, Fitzroy, VIC 3065, Australia

⁴Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia

⁵Department of Medical Biology, The University of Melbourne, Parkville, VIC 3052, Australia

⁶Present address: Research Institute of Molecular Pathology, 1030 Vienna, Austria

*Correspondence: jheierhorst@svi.edu.au

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SUMMARY

How MYC promotes the development of cancer remains to be fully understood. Here, we report that the Zn²⁺-finger transcription factor ASCIZ (ATMIN, ZNF822) synergizes with MYC to activate the expression of dynein light chain (DYNLL1, LC8) in the murine $E\mu$ -Myc model of lymphoma. Deletion of Asciz or Dynll1 prevented the abnormal expansion of pre-B cells in pre-cancerous $E\mu$ -Myc mice and potentiated the pro-apoptotic activity of MYC in pre-leukemic immature B cells. Constitutive loss of Asciz or Dynll1 delayed lymphoma development in $E\mu$ -Myc mice, and induced deletion of Asciz in established lymphomas extended the survival of tumor-bearing mice. We propose that ASCIZ-dependent upregulation of DYNLL1 levels is essential for the development and expansion of MYC-driven lymphomas by enabling the survival of pre-neoplastic and malignant cells.

INTRODUCTION

Members of the MYC family of helix-loop-helix transcription factors are deregulated in the majority of human cancers (Vita and Henriksson, 2006), including various B cell lymphoma subtypes (Ott et al., 2013). MYC levels are extremely low in quiescent cells, and limiting in cycling cells, but at abnormally high oncogenic levels, MYC can bind to virtually all active gene promoters (Wolf et al., 2015). In this way, MYC is believed to globally amplify the signal of other promoter-bound transcriptional activators and repressors in a manner that promotes cellular transformation and tumorigenesis. The mechanisms by which it does this remain incompletely understood (Wolf et al., 2015).

Studies of $E\mu$ -Myc mice, which carry a transgene mimicking the t(8;14) chromosomal translocation between an *lgh* enhancer

and the Myc oncogene that is characteristic of human Burkitt lymphoma, have provided substantial insight into how MYC overexpression promotes tumor development (Adams et al., 1985). In E μ -Myc mice, MYC is overexpressed in the B cell lineage and causes highly penetrant clonal B cell lymphomas (with predominantly surface-IgM [immunoglobulin M]-negative [slgM⁻] pre-B or slgM⁺ immature B cell subtypes), with a median latency of 110-120 days (Adams et al., 1985; Harris et al., 1988; Mori et al., 2008). During the pre-leukemic phase, $E\mu$ -Myc leads to elevated numbers of cycling pre-B cells in the bone marrow (Langdon et al., 1986). This is initially offset by increased apoptosis (Cory et al., 1999), which is due, in part, to direct transcriptional upregulation of the pro-apoptotic protein BIM by MYC (Lee et al., 2013; Muthalagu et al., 2014). However, random mutations that collaborate with Myc overexpression eventually tip the balance against apoptosis, which results in uncontrolled proliferation and the emergence of lymphoma (Cory et al., 1999; Egle et al., 2004; Eischen et al., 1999; Kelly et al., 2011). Consistently, the development of B cell lymphoma in $E\mu$ -Myc mice is dramatically accelerated by deletion of pro-apoptotic genes such as Bim or p53 (Egle et al., 2004; Michalak et al., 2009).

Recently, we found that the Zn²⁺-finger transcription factor ASCIZ (also known as ATMIN or ZNF822) plays an important role in normal B cell development by activating the expression of the multi-functional protein DYNLL1. DYNLL1, in turn, restrains the pro-apoptotic action of BIM at the immature B cell stage in the bone marrow. Conditional deletion of Asciz (on the C57BL/6 background) in hematopoietic stem/progenitor cells using Mx1-Cre, or in the B cell lineage using Mb1-Cre, led to the progressive loss of pre-B and immature B cells in the bone marrow and marked B lymphopenia in the periphery (Jurado et al., 2012b). Normal development of Asciz-deleted B cells could be restored by ectopic Dynll1 expression or by knockout (KO) of the pro-apoptotic DYNLL1 target BIM (Jurado et al., 2012b). A similar but milder B cell developmental defect was also reported for Cd19-Cre Asciz-deleted mice (on a mixed genetic background) (Loizou et al., 2011). Surprisingly, in contrast



³Victorian Cancer Cytogenetics Service, St. Vincent's Hospital, The University of Melbourne, Fitzroy, VIC 3065, Australia

to *Mx1-Cre* or *Mb1-Cre Asciz*-deleted mice (Jurado et al., 2012b), ~40% of these *Cd19-Cre Asciz*-deleted mice were reported to develop mature, peripheral B cell lymphomas. This was presumably due to aberrant repair of activation-induced deaminase (AID)-generated DNA breaks in germinal center B cells. The emergence of B cell lymphomas in the *Cd19-Cre Asciz*-deleted mice prompted the postulate that ASCIZ functions as a tumor suppressor (Loizou et al., 2011).

Here, we examined the roles of ASCIZ and DYNLL1 in lymphomagenesis and sought to resolve whether the discrepant lymphoma incidence in the different conditional *Asciz* KO lines is related to the timing of Cre recombinase induction during B cell development. We found that *Asciz* deletion after completion of B cell development in the bone marrow, but before peripheral B cells reach full maturity, supports normal B cell homeostasis without increased lymphoma risk. Moreover, we show that, rather than being a tumor suppressor, ASCIZ is, in fact, essential for lymphoma development originating from B cell precursors in the $E\mu$ -Myc mouse model.

RESULTS

ASCIZ Is Not Required for Mature B Cell Homeostasis and Suppression of Peripheral B Cell Lymphomas

Despite qualitatively similar B cell developmental defects, naive Cd19-Cre Asciz-deleted mice contained normal mature B cell numbers in the spleen and blood (Loizou et al., 2011), whereas Mx1-Cre or Mb1-Cre Asciz-deleted mice exhibited a marked peripheral B lymphopenia (Jurado et al., 2012b). Lower B cell numbers in Mx1-Cre or Mb1-Cre Asciz-deleted mice would be expected to stochastically reduce the risk of lymphoma development, compared to the Cd19-Cre Ascizdeleted line. To test this hypothesis and study mature B cell functions without the sequelae of an underlying developmental defect, we used Cd23-Cre to delete Asciz. Cd23-Cre is activated during the transitional B cell stage in the periphery, after completion of bone marrow B cell developmental stages, but before follicular B cells have reached sufficient maturity to activate mutagenic AID expression in germinal centers (Kwon et al., 2008). In contrast to the \sim 8-fold-lower (p < 0.0001) peripheral B cell numbers in Mb1-Cre Asciz-deleted mice, B cell numbers in the spleens of Cd23-Cre Asciz-deleted mice were indistinguishable from those in Cd23-Cre littermate controls (Figure 1A). Furthermore, we found that serum levels of the B cell survival factor BAFF were increased by ~4-fold (p < 0.0001) in *Mb1-Cre Asciz*-deleted mice (Figure 1B). This would be expected to extend the lifespan of peripheral B cells and thereby to partially compensate for lower B cell production in the bone marrow (Vincent et al., 2013). In contrast, BAFF serum concentrations were normal in Cd23-Cre Ascizdeleted mice (Figure 1B). We confirmed that Asciz was efficiently deleted by Cd23-Cre via PCR genotyping of mature splenic B cells (IgM⁺ IgD^{high}) and western blot analysis, using loss of DYNLL1 as a surrogate marker for loss of ASCIZ (Figures 1E and 1F). Thus, the combination of normal B cell numbers without increased BAFF levels indicates that ASCIZ is not intrinsically required for the homeostasis of mature peripheral B lymphocytes.

After establishing that their B cell numbers were normal, we monitored the survival of *Cd23-Cre Asciz*-deleted mice over a 90-week period. B cell lymphomagenesis in the *Cd19-Cre Asciz* model has been proposed to involve AID-dependent mutagenesis in peripheral B cells (Loizou et al., 2011), so we repeatedly immunized our mice with sheep red blood cells to increase the likelihood of "collateral" AID-induced DNA damage. However, this did not lead to any tumor-related deaths in *Cd23-Cre Asciz*-deleted mice up to 90 weeks of age (Figure 1G, blue stippled line). All mouse spleen sections at this endpoint revealed an unremarkable architecture typical of old immunized mice and similar to the *Cd23-Cre* control (Figures S1G and S1H).

To increase the sensitivity of the model, we crossed the Cd23-*Cre Asciz* mice with congenic $I\mu$ -Bc/6 knockin mice. The $I\mu$ -Bc/6 line develops diffuse large B cell lymphomas (DLBCL) with high penetrance (>60%) but long latency (15-18 months) in an AIDdependent manner (Cattoretti et al., 2005; Pasqualucci et al., 2008). If ASCIZ functioned as a tumor suppressor of peripheral AID-dependent lymphomas, we would predict that Cd23-Cre Asciz deletion should accelerate tumorigenesis in I_{μ} -Bcl6 mice. $I\mu$ -Bcl6 control mice succumbed to lymphomas with the expected incidence and long latency (Figures 1G and 1H). However, neither the time of onset of terminal disease nor the incidence of indolent lymphoma in the surviving 90-week-old mice was significantly affected by loss of ASCIZ (Figures 1G, 1H, and S1I-S1L). Collectively, these data demonstrate that AS-CIZ does not act as a cell-intrinsic tumor suppressor in mature peripheral B cells.

ATM Signaling Is Not Impaired in ASCIZ-Deficient Mature B Cells

The increased B cell lymphoma incidence in Cd19-Cre Ascizdeleted mice has been attributed to increased genome instability due to impaired ataxia telangiectasia mutated (ATM) signaling in mature B cells (Loizou et al., 2011). To monitor genome instability, we purified splenic B cells from Cd23-Cre control and Cd23-Cre Asciz-deleted mice and stimulated them for 3 days with CD40 ligand, interleukin-4 (IL4), and lipopolysaccharide (LPS). In contrast to very high levels of chromosome breaks reported in Cd19-Cre Asciz-deleted mice under similar conditions (Loizou et al., 2011), we did not observe increased genome instability in metaphase chromosome spreads of Cd23-Cre Asciz-deleted mature B cells, compared to the Cd23-Cre control (Figures S1D–S1F). We also found that canonical activation of ATM, and phosphorylation of its targets KAP1 and p53 in response to ionizing radiation, was not affected in ASCIZ-deficient B cells (Figure S1A) or fibroblasts (Figure S1B). We did not observe significant non-canonical activation of ATM by low-salt treatment in stimulated B cells-in contrast to fibroblasts treated in parallel (Figure S1B). However, phosphorylation of KAP1 was slightly increased in Asciz-deleted B cells compared to the control (Figure S1A). PCR genotyping and loss of DYNLL1 expression on western blots confirmed that Asciz was efficiently deleted in these B cell cultures (Figures S1A and S1C).

We also monitored the generation of immunoglobulin (Ig) class-switched antibodies as an in vivo marker for ATM functions in mature B cells (Reina-San-Martin et al., 2004). Serum titers of antigen-specific IgG1-switched antibodies were significantly



Figure 1. Normal B Cell Homeostasis, Functions, and Lymphoma Incidence in *Cd23-Cre Asciz*-Deleted Mice

(A) Splenic B cell numbers in *Mb1-Cre* control (n = 8) and *Asciz*-deleted mice (n = 8) and in *Cd23-Cre* control (n = 4) and *Asciz*-deleted mice (n = 6).

(B) Serum BAFF level in *Mb1-Cre* control (n = 4) and *Asciz*-deleted mice (n = 5) and in *Cd23-Cre* control (n = 4) and *Asciz*-deleted mice (n = 5).

(C and D) ELISAs of high-affinity (NP2) and total (NP14) antigen-specific serum IgG1 titers of *Mb1-Cre Asciz* (C) and *Cd23-Cre Asciz* (D) mice 13 days after immunization with NP-KLH.

(E) PCR analysis of *Asciz* deletion in FACS-sorted transitional stage 1 (T1) (IgM⁺IgD^{low}), transitional stage 2 (T2)/mature (IgM⁺IgD^{hi}), and NP2-reactive IgG1-switched B cells from *Cd23-Cre Asciz^{fi/fi}* and *Cd23-Cre* control mice compared to tail DNA from the same mice 13 days after immunization with NP-KLH. WT, wild-type.

(F) Western blot analysis of FACS-purified mature splenic B cells (IgM^{low} IgD^{high}) from a *Cd23-Cre* control and a *Cd23-Cre Asciz*-deleted mouse probed with DYNLL1 (as a surrogate marker for the presence of ASCIZ) and GAPDH (as a loading control) antibodies.

(G) Survival curves for *Cd23-Cre* control, *Cd23-Cre Asciz*-deleted, *Cd23-Cre* μ -*Bcl6* control, and *Cd23* μ -*Bcl6 Asciz*-deleted mice. Arrows indicate times of intraperitoneal injection with 10⁸ sheep red blood cells (SRBC) in 100 μ l PBS.

(H) Incidence of DLBCL (black), lymphoid hyperplasia (gray) or normal architecture in surviving mice at 90 weeks (white).

Error bars represent mean \pm SEM.

Collectively, these results indicate that ASCIZ is not directly required for ATM activation and ATM-dependent maintenance of genome stability in activated mature B cells in vitro or for the ATMdependent processing of AID-mediated DNA breaks in antigen-stimulated B cells in vivo.

Loss of ASCIZ Prevents the Development of Bone Marrow-Derived B Cell Lymphoma in $E\mu$ -Myc Mice

ASCIZ is critical for pre-B and immature B cell development stages in the bone marrow (Jurado et al., 2012b). To determine whether ASCIZ affects the develop-

reduced in immunized *Mb1-Cre Asciz*-deleted mice (Figure 1C), proportional to their reduction in mature B cell numbers. In contrast, the generation of both total and high-affinity, antigen-specific IgG1-switched antibodies was normal in *Cd23-Cre Asciz*-deleted mice (Figure 1D). PCR genotyping confirmed that *Asciz* was efficiently deleted by *Cd23-Cre* in FACS-sorted IgG1-switched, antigen-specific B cells under these conditions (Figure 1E).

ment of lymphomas originating from these stages of B lymphocyte differentiation, we intercrossed the *Mb1-Cre Asciz* line with congenic $E\mu$ -*Myc* mice. $E\mu$ -*Myc* mice are predisposed to develop pre-B and immature B cell lymphomas (Adams et al., 1985; Mori et al., 2008). As expected (Harris et al., 1988), *Mb1-Cre* $E\mu$ -*Myc* control mice developed B cell lymphomas with an average latency of 117 days (Figure 2A). Remarkably, disease onset was dramatically suppressed in the absence of ASCIZ:





Figure 2. Loss of ASCIZ Suppresses Lymphoma Development in $E\mu$ -Myc Mice

(A) Survival analysis of *Mb1-Cre Asciz*^{+/+} $E\mu$ -*Myc* mice (red) and *Mb1-Cre Asciz*^{+/+} $E\mu$ -*Myc* mice (blue).

(B) FACS immunophenotyping of four rare remaining $E\mu$ -Myc lymphomas in Mb1-Cre Asciz-deleted mice.

(C) Fractional distribution of surface IgM^+ and surface IgM^- lymphomas in control and Asciz-deleted $E\mu$ -Myc mice.

(D) Western blot analysis of the four Asciz-deleted E_{μ} -Myc lymphomas from (C) compared to normal *Mb1*-Cre control and *Mb1*-Cre Asciz-deleted splenic B cells. Asterisk denotes a cross-reacting band (that does not correspond to any possible partial Cre-lox recombination product), and $\hat{}$ denotes the pre-pro-B cell-like lymphoma from (C), which corresponds to a developmental stage before *Mb1*-Cre is normally expressed.

whereas >80% of $E\mu$ -Myc control mice died from lymphoma, <20% of Asciz-deleted Eµ-Myc mice developed lymphoma during an 8-month observation period (Figure 2A; p < 0.0001). Surface marker analysis of the rare lymphomas that still arose in the absence of ASCIZ revealed a range of B cell lymphoma subtypes, which did not differ from the $E\mu$ -Myc control cohort (Figures 2B and 2C). Western blot analysis confirmed the loss of ASCIZ in most of these lymphomas, except in one pre-pro-B cell-like lymphoma (#3 in Figures 2B and 2D), which is consistent with the notion that Mb1-Cre is not active until the subsequent pro-B cell stage (Hobeika et al., 2006). Eµ-Myc mice represent, arguably, the most widely studied animal model used to understand genetic mechanisms of B cell lymphomagenesis. Numerous mutations have been reported to accelerate the development of lymphoma in these mice; e.g., loss of Arf (Eischen et al., 1999), Bim (Egle et al., 2004), p53 (Michalak et al., 2009), or Atm (Shreeram et al., 2006). However, only relatively few mutations - e.g., loss of Bcl-xl (Kelly et al., 2011), eIF4F (Lin et al., 2012), or Moz1 (Sheikh et al., 2015)-have been reported to delay lymphoma development in $E\mu$ -Myc mice, and the protective effect of Asciz deletion that we show here is more profound than any previously published genetic intervention.

Loss of ASCIZ Is Synthetic Lethal with MYC Overexpression in Pre-leukemic B Lymphoid Progenitors

We measured the numbers of pre-leukemic B lymphoid cells in tumor-free 8-week-old mice in order to explore the underlying mechanism of the protective effect of Asciz deletion on lymphomagenesis. MYC overexpression led to the expected (Langdon et al., 1986; Strasser et al., 1996) ~3-fold increase in pre-B cell numbers in the bone marrow compared to the Mb1-Cre control (Figures 3A and 3B; p = 0.0001). Interestingly, the number of pre-B cells in Eµ-Myc $\triangle Asciz$ double mutants was reduced by \sim 7.5-fold compared to *Eµ-Myc* mice (Figures 3A and 3B; p < 0.001) and was similar to the low level observed in Mb1-Cre Asciz-deleted mice. Most strikingly, whereas immature B cell numbers were reduced by ~10-fold in Mb1-Cre Asciz-deleted mice compared to the *Mb1-Cre* and $E\mu$ -Myc controls (Figures 3A and 3B; p < 0.0001), this reduction was exacerbated to ~60-fold lower than control levels in *E* μ -*Myc* Δ *Asciz* double mutants (Figures 3A and 3B; p < 0.0001).

Loss of ASCIZ and MYC overexpression both led to increased apoptosis at the immature B cell stage (Jurado et al., 2012b; Strasser et al., 1996). Therefore, we tested whether a synergistic interaction between the two mutations could be the reason for the near-complete elimination of immature B cells in *Eµ-Myc ΔAsciz* double mutants (Figure 3B). As expected (Jurado et al., 2012b; Strasser et al., 1996), cell death was increased by ~2fold in *Mb1-Cre Asciz*-deleted (Figures 3C and 3D; p = 0.0066) or *Eµ-Myc* (Figures 3C and 3D; p = 0.0071) single-mutant immature B cells compared to control mice. However, this was increased by ~15-fold in the few remaining *Eµ-Myc ΔAsciz* double-mutant immature B lymphocytes (Figures 3C and 3D; p < 0.0001). Collectively, these data indicate that loss of *Asciz* prevents lymphoma development by potentiating the pro-apoptotic action of *Eµ-Myc* in pre-leukemic immature B cells, as well as by



antagonizing the pre-malignant expansion of pre-B cells in $E\mu$ -Myc mice.

ASCIZ Is Required for MYC-Dependent Hyper-activation of DYNLL1 Expression in Pre-leukemic B Cell Precursors

Loss of ASCIZ invariably leads to dramatically reduced DYNLL1 levels in all cell types studied to date, from Drosophila to humans (Goggolidou et al., 2014; Jurado et al., 2012a, 2012b; Zaytseva et al., 2014). Surprisingly, and in contrast to noncancerous Mb1-Cre Asciz-deleted splenic B cells, we noticed that, in all bona fide Asciz-deleted $E\mu$ -Myc lymphomas, DYNLL1 protein was maintained at high levels comparable to that of control B cells (Figure 2D). Thus, to determine how MYC overexpression affects DYNLL1 levels in the presence or absence of ASCIZ, we FACS-purified (B220⁺ CD19⁺ IgM⁻) pooled pro-B/pre-B cells from pre-malignant 8-week-old mice. As expected, DYNLL1 was essentially undetectable in Asciz-deleted pro-B/pre-B cells (Figure 4A), underscoring the efficiency of Mb1-Cre at these early stages of B cell development (Hobeika et al., 2006). Strikingly, DYNLL1 levels in Mb1-Cre $E\mu$ -Myc pro-B/pre-B cells were upregulated by >6-fold compared to Mb1-Cre control cells (Figure 4A), and this was reversed to near-normal control levels in Eµ-Myc ⊿Asciz cells (Figure 4A). This level of DYNLL1 upregulation was substantially higher than the well-established (Egle et al., 2004) ~2-fold up-

Figure 3. Synthetic-Lethal Interaction between *Asciz* Deficiency and Oncogenic MYC Deregulation in Pre-leukemic B Cell Precursors

(A) FACS plots of bone marrow cell suspensions of *Mb1-Cre* control, *Mb1-Cre* Asciz-deleted, *Mb1-Cre* $E\mu$ -Myc control, and *Mb1-Cre* $E\mu$ -Myc Asciz-deleted mice, with the immature B cell gate highlighted. Gating strategies are shown in Figure S2.

(B) Enumeration of B lymphoid fractions in the bone marrow (per leg) and B220⁺ lgM⁺ cells in the spleen. *Mb1-Cre* control (n = 13), *Mb1-Cre* $\Delta Asciz$ (n = 12 or 13), *Mb1-Cre* $E\mu$ -*Myc* (n = 12 or 13), and *Mb1-Cre* $E\mu$ -*Myc* $\Delta Asciz$ (n = 7). p values were omitted from the graph for clarity. Pro-B/pre-B cells: $E\mu$ -*Myc* versus control, p = 0.0001; $E\mu$ -*Myc* versus all others, p < 0.001; $\Delta Asciz$ versus control, p = 0.0001; Eight others, p < 0.0001; $E\mu$ -*Myc* $\Delta Asciz$ versus all others, p < 0.0001; $E\mu$ -*Myc* $\Delta Asciz$ versus all others, p < 0.0001; $E\mu$ -*Myc* $\Delta Asciz$ versus all others, p < 0.0001; $E\mu$ -*Myc* $\Delta Asciz$ versus all others, p < 0.0001; $E\mu$ -*Myc* $\Delta Asciz$ versus all others, p < 0.0001; $E\mu$ -*Myc* $\Delta Asciz$ versus all others, p < 0.0001; $E\mu$ -*Myc* $\Delta Asciz$ versus all others, p < 0.0001; $E\mu$ -*Myc* $\Delta Asciz$ versus all others, p < 0.0001.

(C) FACS plots of B220⁺ IgM^+ IgD^- -gated immature B cells stained with 7-AAD and annexin-V.

(D) Quantification of cell death (7AAD⁺ AnnV⁺ cells) in pre-B and immature B cells of *Mb1*-*Cre* control (n = 9), *Mb1*-*Cre* $\Delta Asciz$ (n = 7), *Mb1*-*Cre* $E\mu$ -*Myc* (n = 9), *Mb1*-*Cre* $E\mu$ -*Myc* $\Delta Asciz$ (n = 4) mice. p values were omitted for clarity. Immature B cells: $\Delta Asciz$ versus control, p = 0.0066; $E\mu$ -*Myc* versus control, 0.0071; $E\mu$ -*Myc* $\Delta Asciz$ versus all others, p < 0.0001.

Error bars represent mean \pm SEM. See also Figure S2.

regulation of BIM in pre-malignant E_{μ} -Myc B cell precursors (Figure 4A) or the 2- to 3-fold increased levels of ASCIZ in pre-leukemic E_{μ} -Myc pro-B/pre-B cells (Figure 4B). DYNLL1 levels in pre-malignant E_{μ} -Myc pro-B/pre-B cells were comparable to those in malignant E_{μ} -Myc lymphomas (Figure 4C). Interestingly, oncogenic MYC appeared to upregulate DYNLL1 predominantly in B cell precursors but not in mature splenic B cells (from the same mice), which contained high DYNLL1 levels already in the absence of MYC (Figure 4C). Real-time qPCR analyses confirmed that Dynll1 mRNA levels were upregulated in pre-leukemic E_{μ} -Myc pro-B/-pre-B cells (Figure 4E), albeit to a lower extent than upregulation at the protein level (Figure 4A, 4C, and 4D). Asciz mRNA levels remained unchanged (Figure 4E).

These data show that ASCIZ and MYC synergize to upregulate DYNLL1 protein levels specifically during the early stages of B cell differentiation that are vulnerable to $E\mu$ -Myc-driven lymphomagenesis. The stronger effect on protein, compared to mRNA levels, suggests that MYC regulates DYNLL1 levels in these cells by a combination of transcriptional and post-transcriptional mechanisms. Finally, it should be noted that, in four independent human Burkitt-lymphoma-derived cell lines, ASCIZ and DYNLL1 were present at high levels similar to those seen in mouse $E\mu$ -Myc pro-B/pre-B cells (Figures 4B and 4D). This suggests that high levels of ASCIZ and DYNLL1 may also be important in human MYC-driven lymphomas.



Figure 4. ASCIZ-Dependent MYC-Driven DYNLL1 Upregulation in Pre-leukemic B Cell Precursors

(A) Western blot analysis of lysates (10 μ g per sample) of FACS-sorted combined pro-B and pre-B cell fractions (B220⁺ CD19⁺ IgM⁻) from individual mice with the genotypes indicated above, probed for c-MYC, DYNLL1, BIM (EL/L), and actin.

(B) Western blot analysis of similar pro-B/pre-B cell lysates probed for ASCIZ, c-MYC, and actin.

(C) Western blot of FACS-sorted pro-B/pre-B cells (7.5 μ g per sample) of non-Cre-expressing mice and MACS-sorted splenic B cells (10 μ g per sample) from the same mice, compared to lysates of two independent *Mb1-Cre Eµ-Myc* lymphomas.

(D) Western blot of *Mb1-Cre* control, *Mb1-Cre* Eµ-*Myc*, and *Mb1-Cre* Eµ-*Myc* △*Asciz* pro-B/pre-B cells, compared to four different human Burkittlymphoma-derived cell lines.

(E) Real-time qPCR analysis of *Dynll1* and *Asciz* mRNA levels relative to *Tbp* mRNA levels in FACS-sorted pro-B/pre-B cells from control and $E\mu$ -Myc mice.

Error bars represent mean ± SEM.

ASCIZ Regulates MYC-Driven Lymphomagenesis via DYNLL1 and BIM

Deletion of Asciz reversed the highly elevated DYNLL1 levels in pre-cancerous Eµ-Myc B cell precursors to levels found in normal control cells (Figure 4A), and this correlated with a reduced lymphoma incidence in $E\mu$ -Myc $\Delta Asciz$ mice (Figure 2A). Therefore, we hypothesized that stage-specific DYNLL1 upregulation is critical for MYC-driven lymphomagenesis. To directly test this hypothesis, we generated a floxed Dynll1 allele for conditional deletion in Eµ-Myc B cell precursors. Dynll1 contains three exons, with the 89-amino-acid protein-coding sequence located on exons 2 and 3 (Figure 5A). In the Dynll1^{fl} allele, we targeted a region comprising the Dynll1 promoter and exons 1 and 2 for deletion to avoid interference with the Cog5 gene located close to the 3' end of *Dynll1* (Figure 5A). Without the promoter, and in the absence of in-frame ATG codons within exons 3, it is impossible for the recombined allele to yield any truncated DYNLL1 protein product. Homozygous Dynll1^{fl/fl} mice were born at the expected Mendelian ratios and were indistinguishable from wild-type littermates. Western blot analysis confirmed the absence of DYNLL1 protein in Dynll1^{fl/fl} B lymphoid cells of mice expressing Mb1-Cre (Figure 5B).

Mb1-Cre Dynll1-deleted mice had a B cell phenotype similar to that of *Mb1-Cre Asciz*-deleted mice, with ~2-fold lower numbers of pro-B/pre-B cells, ~8-fold fewer immature B cells (p = 0.002), and ~4-fold fewer splenic B cells (p < 0.0001) than *Mb1-Cre* control mice (Figure 5D). Similar to *Asciz* deletion (Figure 3B), loss of DYNLL1 abrogated the abnormal increase in pro-B/pre-B cell numbers in pre-malignant $E\mu$ -Myc mice and led to a synergistic >50-fold loss of immature B cells (p = 0.0032) compared to control mice (Figure 5D). Importantly, *Mb1-Cre*-mediated *Dynll1*

deletion also suppressed lymphoma development in $E\mu$ -Myc mice (Figure 5E; p = 0.0052), which is similar to the protective effect seen in $E\mu$ -Myc $\Delta Asciz$ mice (Figure 2A). Thus, these data indicate that ASCIZ exerts its essential role in MYC-driven lymphomagenesis predominantly through its molecular function as a critical *Dynll1* transcription factor.

Our data indicate that ASCIZ and DYNLL1 are critical for the survival of pre-leukemic immature B cells in $E\mu$ -Myc mice prior to the acquisition of additional oncogenic lesions and subsequent progression to malignant lymphoma (Figures 3B, 3D, and 5D). Upregulation of the pro-apoptotic protein BIM contributes to increased apoptotic cell death in premalignant Eµ-Myc B lymphoid cells (and other MYC-overexpressing cell types), and loss of Bim dramatically accelerates Eµ-Myc lymphoma development (Egle et al., 2004). As ASCIZ and DYNLL1 appear to prevent excessive BIM-mediated apoptosis of immature B cells (Jurado et al., 2012b), we hypothesized that the protective effect of Asciz (or Dynll1) deletion on lymphoma development in Eµ-Myc mice may be due to increased BIM-mediated apoptosis. To test this hypothesis, we intercrossed Mb1-Cre Asciz^{fl/fl} Eµ-Myc mice with Bim germline KO mice (Bouillet et al., 1999). Remarkably, loss of even a single allele of Bim completely reversed the protective effect of Asciz deletion on the development of $E\mu$ -Myc lymphoma (Figure 5F; p < 0.0001). In addition, loss of Asciz had no significant protective effect on the dramatically accelerated lymphoma development in E_{μ} -Myc Bim^{-/-} mice. Taken together, these data indicate that ASCIZ (and, presumably, also DYNLL1) exerts its effect on MYC-driven lymphomagenesis through inhibition of BIM-mediated apoptosis. It should be noted that BIM levels were not elevated in Mb1-Cre Asciz-deleted pro-B/pre-B cells compared



Figure 5. *Dynll1* Is Essential for MYC-Driven Lymphomagenesis and Survival of Preleukemic $E\mu$ -Myc B Cell Precursors

(A) Targeting strategy to generate a conditional $Dynl/1^{fl}$ allele. Squares indicate exons, with protein-coding regions in exons 2 and 3 indicated in black; triangles indicate LoxP sites. Dynl/1 is located close to the Coq5 gene, in converging transcriptional direction.

(B) Western blot analysis of eight independent lymphomas from *Mb1-Cre* $E\mu$ -*Myc* and *Mb1-Cre* $E\mu$ -*Myc Dynll1*^{*fl/fl*} mice probed with antibodies for DYNLL1, c-MYC, and actin. Note that the lower band in lane 8 is an uncharacterized cross-reacting band. Additional panels are shown in Figure S3B.

(C) FACS plots of B220⁺ CD43⁻-gated bone marrow cells of mice with the indicated genotypes, with immature B cells highlighted by red boxes.

(D) Enumeration of B cell subsets in bone marrow (one leg) and spleen cell suspensions of 8-weekold Mb1-Cre control (n = 7-10), Mb1-Cre Dynll1deleted (n = 9-11), Mb1-Cre $E\mu$ -Myc (n = 4), and Mb1-Cre Eµ-Myc Dynll1-deleted (n = 8) mice. p values were omitted from the graph for clarity. Pro-B/pre-B cells: $E\mu$ -Myc versus control, p = 0.0012; *Eµ-Myc* versus $\Delta Dynll1$, p < 0.0001; *Eµ-Myc* versus $E\mu$ -Myc $\Delta Dynll1$, p = 0.0009. Immature B cells: *Dynll1* versus control, p = 0.002; *Eµ-Myc* versus $\Delta Dynll1$, p = 0.003; $\Delta Dynll1$ versus $E\mu$ -Myc $\Delta Dynll1$, p = 0.0046; Eµ-Myc versus Eµ-Myc $\Delta Dynll1$, p = 0.0002; Eµ-Myc $\Delta Dynll1$ versus control, p = 0.0032. Spleen: ⊿Dynll1 versus control, p < 0.0001; *∆Dynll1* versus *Eµ-Myc ∆Dynll1*, p = 0.0001; $E\mu$ -Myc versus $E\mu$ -Myc $\Delta Dynll1$, p = 0.0002; Eµ-Myc ΔDynll1 versus control, p < 0.0001. Gating strategies are shown in Figure S3A. (E) Survival analysis of Mb1-Cre Dynll1^{+/+} Eµ-Myc mice and Mb1-Cre Dynll1^{fl/fl} Eµ-Myc mice. Note that Mb1-Cre control and Mb1-Cre Eµ-Myc mice shown in (D) and (E) are different individuals from those shown in similar graphs in Figures 2 and 3. (F) Survival curves for Mb1-Cre Eµ-Myc (red solid line; n = 21; identical to Figure 2; included here for comparison), Mb1-Cre Eµ-Myc ⊿Asciz (blue solid lines; n = 28; identical to Figure 2; included here for comparison), Mb1-Cre Eµ-Myc Bim+/- (red dashed line; n = 12), Mb1-Cre Eµ-Myc △Asciz $Bim^{+/-}$ (blue dashed line; n = 15), Mb1-Cre Eµ-Myc $Bim^{-\prime -}$ (red stippled line; n = 3), and Mb1-Cre E μ -*Myc* $\triangle Asciz Bim^{-/-}$ (blue stippled line; n = 5) mice.

p values were omitted from the graph for clarity: $E\mu$ - $Myc \ \Delta Asciz$ versus $E\mu$ - $Myc \ \Delta Asciz \ Bim^{+/-}$, p < 0.0001; $E\mu$ - $Myc \ Bim^{+/-}$ versus $E\mu$ - $Myc \ \Delta Asciz \ Bim^{+/-}$, p = 0.0003. Error bars represent mean \pm SEM.

See also Figure S3.

to the *Mb1-Cre* control (Figure 4A, lanes 1–4), in *Asciz*-deleted $E\mu$ -*Myc* pro-B/pre-B cells compared to the $E\mu$ -*Myc* control (Figure 4A, lanes 5–11), or in *Dynll1*-deleted $E\mu$ -*Myc* lymphomas compared to control $E\mu$ -*Myc* lymphomas. There were also no consistent alterations in other BH3 protein family members in *Dynll1*-deficient compared to control $E\mu$ -*Myc* lymphomas (Figure S3B). This indicates that any increased BIM activity in ASCIZ- or DYNLL1-deficient B cell precursors is due to post-translational effects, consistent with previous reports that

DYNLL1 directly binds to BIM to inhibit its pro-apoptotic activity locally at mitochondria or by sequestering BIM to microtubuli (Puthalakath et al., 1999; Zhu et al., 2004).

ASCIZ Is Required for the Sustained Proliferation of Established $E\mu$ -Myc Lymphomas

To determine whether ASCIZ is required not only for lymphoma development but also for the malignancy of established MYC-driven lymphomas, we crossed $Asciz^{fl/fl} E\mu$ -Myc mice to

Rosa26-CreERT2 mice (CreER), enabling temporally controlled Cre activation by tamoxifen treatment (Ventura et al., 2007). Cells from B cell lymphomas that had developed in these mice were then transplanted into healthy, congenic recipients. Upon emergence of circulating donor-derived lymphoma cells, recipients were treated with tamoxifen for 3 consecutive days or left untreated in the control group. In six of nine recipients transplanted with four independent CreER Asciz^{fl/fl} $E\mu$ -Myc lymphomas, tamoxifen treatment significantly delayed the time to lymphoma relapse compared to matched untreated control mice transplanted with the same lymphoma (Figures 6A and 6B). One additional tamoxifen-treated mouse survived relapse-free until the end of the observation period (Table S1) and was excluded from Figure 6 as an outlier. Tamoxifen treatment of mice transplanted with CreER Asciz^{+/+} $E\mu$ -Myc or CreER Asciz^{fl/+} $E\mu$ -Myc lymphomas (which retain at least one functional Asciz allele upon Cre activation) did not improve survival compared to lymphoma-matched untreated control recipients (Figures 6A and 6B).

Continued monitoring of circulating tumor cells in transplant recipients revealed a range of responses, including attenuated lymphoma progression (Figure 6C) and transient remission (Figure 6D) in tamoxifen-treated mice compared to untreated lymphoma-matched controls. Interestingly, we found that all relapsed CreER Asciz^{fl/fl} Eµ-Myc lymphomas from tamoxifentreated mice had recombined only one of the two Asciz^{fl} alleles and retained the other intact coding sequence (Figure 6E, left and middle panels). In control mice, the floxed Asciz allele was efficiently and completely recombined-without any survival advantage-when it was balanced by a wild-type Asciz allele in CreER Asciz^{fl/+} $E\mu$ -Myc lymphoma transplants (Figure 6C, right panel). This demonstrates that the incomplete Cre-Lox recombination in the relapsed Asciz^{fl/fl} lymphomas was not due to poor allele-specific Cre activity. These data indicate that complete loss of ASCIZ confers a substantial growth disadvantage in malignant $E\mu$ -Mvc lymphomas, which then results in the selection of Asciz^{fl/-} heterozygous cells in relapsing tumors. In this context, it is conceivable that the extended relapse-free survival of the outlier (Table S1) may be due to complete Asciz deletion in this particular transplant. In conclusion, given the rapid selection against Asciz-deleted tumor cells, the observed survival benefits (Figures 6A and 6B) are likely to significantly underestimate the true benefit of complete Asciz removal on Eµ-Myc lymphoma remission.

DISCUSSION

Here, we have shown that the ASCIZ-DYNLL1 axis plays a critical role in the development of MYC-driven lymphoma in the widely studied $E\mu$ -Myc mouse model. In addition, ASCIZ is essential for the sustained proliferation of malignant MYC-driven lymphomas, as shown by the improved survival upon deletion of Asciz in established lymphomas, with potent negative selection against Asciz null cells in relapsing tumors. ASCIZ and DYNLL1 seem to act by maintaining the survival of pre-leukemic B cell precursors by restraining the pro-apoptotic activity of BIM, akin to their role in normal B cell development. However, ASCIZ and DYNLL1 seem to be even more crucial for B cell lymphoma development than for normal B cell development, as their loss results in a specific and dramatic synthetic-lethal interaction with oncogenic MYC in pre-leukemic cells (Figures 3 and 5D).

A critical factor in the protective effect of Asciz or Dynll1 deletions may be that DYNLL1 expression is dramatically increased by oncogenic MYC levels, specifically during the B cell development stages in which most $E\mu$ -Myc lymphomas have their origin. Thus, simply reversing the elevated DYNLL1 levels to approximately physiological levels found in normal pre-B cells (Figure 4A) is sufficient to prevent lymphoma development in >80% of *Mb1-Cre Asciz*-deleted $E\mu$ -Myc mice (Figure 3A). The extent of MYC-driven upregulation of DYNLL1 in these cells appears to be due to a combination of transcriptional and posttranscriptional effects. MYC binding can be readily detected at the Dynll1 promoter (Sabò et al., 2014; Walz et al., 2014). This suggests that the ~2-fold transcriptional upregulation of Dynll1 mRNA in pre-leukemic $E\mu$ -Myc pro-B/pre-B cells may be due to a direct effect of MYC on the Dynll1 promoter. It remains to be determined how MYC leads to further upregulation of DYNLL1 at the protein level in these cells and why this is not observed in mature splenic $E\mu$ -Myc B cells (Figure 4B).

The phenotypic similarities between Mb1-Cre Asciz-deleted and Mb1-Cre Dynll1-deleted mice-in both the presence and absence of the $E\mu$ -Myc transgene – demonstrate that ASCIZ exerts its role as an essential co-factor of MYC-driven lymphomagenesis through its molecular function as a Dynll1 transcription factor. This adds to a growing body of work highlighting the importance of ASCIZ-DYNLL1 interactions in the regulation of fundamental cell functions and developmental processes (Goggolidou et al., 2014; Jurado et al., 2012a, 2012b; Rapali et al., 2011; Zaytseva et al., 2014). An alternative proposed molecular function for ASCIZ is that of an activator of the central DNA damage response kinase ATM (Cremona and Behrens, 2014). This has been invoked as an explanation for the development of peripheral B cell lymphomas in Cd19-Cre Asciz-deleted mice (Loizou et al., 2011). It should be noted that this increased B cell lymphoma risk is unique to the Cd19-Cre Asciz line and is not observed in our Cd23-Cre (Figure 1), Mb1-Cre, and Mx1-Cre Asciz-deleted mouse lines (Jurado et al., 2012b) or in Vav-Cre Asciz-deleted mice from the Behrens laboratory (Cremona and Behrens, 2014). The floxed Asciz/Atmin alleles used in the different laboratories are almost identical (resulting in deletion of exon D, encoding 601 of 818 amino acid residues). Why mature B cells from Cd19-Cre Asciz-deleted mice are prone to increased genome instability, which then drives the development of B cell lymphoma, remains enigmatic. Our results with Cd23-Cre mice clearly show that ASCIZ is not intrinsically required for ATM signaling, maintenance of genome stability, and tumor suppression in mature B cells (Figure 1; Figure S1). Cd19-Cre becomes active at an earlier stage than Cd23-Cre, so a possible explanation is that precocious deletion of Asciz somehow causes indirect effects that manifest at the mature stage. For example, the pre-B cell reductions in Cd19-Cre and Mb1-Cre Asciz-deleted mice are quantitatively comparable (Jurado et al., 2012b; Loizou et al., 2011), which suggests that, similar to our Mb1-Cre Asciz-deleted mice (Figure 1B), BAFF (B cellactivating factor from the tumor necrosis factor family) levels may also be elevated in the Cd19-Cre Asciz mice. Elevated



Figure 6. Impact of Acute Deletion of Asciz in Malignant $E\mu$ -Myc Lymphomas In Vivo

(A and B) Survival until tumor relapse (days posttransplantation) of tamoxifen-treated mice compared to matched untreated mice transplanted with the same lymphomas. Numbers indicate the total number of transplant recipients for each group, with the number of independent experiments in brackets. A tamoxifen-treated mouse that survived relapse-free until the end of the observation period was excluded from the analysis. Raw data are shown in Table S1. The p values were calculated using paired t test for matched samples (A) or unpaired t test (B).

(C and D) FACS analysis of peripheral blood leukocytes of tamoxifen-treated (top panels) and matched untreated controls (bottom panels) transplanted with the same lymphomas at the indicated times before or after initiation of tamoxifen treatment. Donor-derived tumor cells are homozygous for *Cd45.2*, and recipients are *Cd45.1*/ *Cd45.2* heterozygous.

(E) PCR analysis for *Asciz^{ff}* recombination in lymph nodes containing relapsed lymphomas dissected at the observation endpoint from matched untreated or tamoxifen-treated mice transplanted with the same tumors. The wild-type (wt) band in one of the tumor samples reflects the presence of recipient cells, which are *Asciz^{+/+}*. del, deletion. Error bars indicate mean \pm SEM. See also Table S1. BAFF extends the lifespan of peripheral B cells (Vincent et al., 2013). This could conspire with other factors from the mixed genetic background of the *Cd19-Cre Asciz* mice to allow the survival of old B cells under conditions that would otherwise activate cell death programs, for example, in response to DNA damage. Regardless of these considerations, the dramatic suppression of $E\mu$ -Myc lymphomagenesis caused by the absence of ASCIZ (Figure 2A) is diametrically opposed to the marked acceleration of $E\mu$ -Myc lymphoma development in $Atm^{-/-}$ mice (Shreeram et al., 2006), which almost certainly rules out ATM-related contributions to the phenotypes reported here.

In conclusion, our findings demonstrate that, rather than acting as a tumor suppressor in mature B cells, ASCIZ is, in fact, essential for MYC-driven development of lymphomas derived from bone marrow B cell precursors. This function of ASCIZ may be unique to MYC-driven cancers, as its deletion in $I\mu$ -Bcl6 mice (Figure 1) did not result in a comparable protective effect. ASCIZ and DYNLL1 are expressed in human Burkitt lymphoma cell lines at a level similar to the critical pre-leukemic B cell precursor stage in E_{μ} -Myc mice. In addition, the dramatic synthetic-lethal interaction between loss of ASCIZ or DYNLL1 and high MYC levels in Eµ-Myc mice suggests that the ASCIZ-DYNLL1 axis may be generally important for the pathogenesis and clinical progression of MYC-driven cancers beyond the mouse model. Inducible deletion of Asciz in hematopoietic stem and precursor cells via Mx1-Cre in adult mice has no major adverse effects on other white blood cell compartments beyond B cells (Jurado et al., 2012b), and within the B cell lineage, ASCIZ is required during development but not for maintenance of mature B cells (Figure 1). Thus, the specific synthetic-lethal effect of Asciz or Dynll1 deletion in the presence of oncogenic MYC levels indicates that the ASCIZ-DYNLL1 axis may be a potential target for the treatment of MYC-driven cancers.

EXPERIMENTAL PROCEDURES

Mice

All experimental procedures were approved by the St. Vincent's Hospital Animal Ethics Committee. All mice were maintained on a pure C57BL/6 background and housed in specific pathogen free micro-isolators. *Dynll1*^{fl} mice were generated by TaconicArtemis on C57BL/6 background. Mice in survival cohorts were culled when they were deemed to be ill by experienced animal technicians blinded to their genotypes. Lymphoma-free mice that died from other causes were excluded from analyses. Additional details are provided in the Supplemental Experimental Procedures.

Flow Cytometry, B Cell Cultures, Western Blot Analysis, and ELISAs FACS analyses were performed as described (Jurado et al., 2012b), using

conditions and antibodies indicated in the Supplemental Experimental Procedures. Gating strategies are illustrated in Figure S2B, Figure 2B, and Figure S3A for *Mb1-Cre Dynll1*-deleted mice using Hardy staining (Hardy et al., 1991).

B cell fractions were isolated using a magnetic-activated cell sorting (MACS) bead B Cell Isolation Kit (Miltenyi Biotec) or sorting of mature splenic B cells (Figure 1C; B220⁺ IgM^{Iow} IgD^{high}) and bone marrow B cell precursors (Figure 4; B220⁺ CD19⁺ IgM⁻) using a FACSAria (BD Biosciences). B cells were cultured for 3 days in the presence of CD40L, IL4, and LPS, followed by 1 hr treatment with low salt medium or 2-Gy irradiation with 30-min recovery at 37°C. Metaphase spreads were prepared from cultured cells treated with 0.1 µg/ml colcemid for 1 hr, scanned using a Zeiss Ikaros system, and scored by a clinical

cytogeneticist (M.W.). Human Burkitt-lymphoma-derived cell lines were cultured as described elsewhere (Kelly et al., 2014).

Western blots were performed as described elsewhere (Jurado et al., 2010; McNees et al., 2005), using the procedures and antibodies listed in the Supplemental Experimental Procedures. Serum was collected from 8-week-old naive mice and analyzed using a BAFF ELISA kit (R&D Systems), or at 13 days after immunization of 4- to 6-month-old mice to determine antibody titers using ELISA plates coated with NP2-BSA or NP14-BSA, and horseradish peroxidase (HRP)-labeled goat anti-mouse IgG1 (Southern Biotech 1070-05).

Real-Time qPCR

RNA was extracted from FACS-sorted pro-B/pre-B cells, and gene expression was quantified on a Stratagene Mx3000P qPCR system using Brilliant II SYBR green QPCR master mix (Agilent Technologies). Gene expression was normalized to *Tbp* levels, which are known to be unaffected by MYC overexpression in B cells (Sabò et al., 2014; Walz et al., 2014).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. The p values were calculated by two-tailed unpaired Student's t test, or paired t test for matched samples, and Mantel-Cox test for survival analyses. Grouped data represent the mean \pm SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.01.012.

AUTHOR CONTRIBUTIONS

D.M.W., L.L., S.J., A.K., M.K.W., and R.B. designed, performed and analyzed experiments; M.W. designed, analyzed, and discussed experiments; G.L.K., C.R.W., D.M.T., and A.S. discussed experiments and provided reagents; J.H. designed, performed, analyzed, and discussed experiments and wrote the paper.

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