Loss of a Single *Mcl-1* Allele Inhibits MYC-Driven Lymphomagenesis by Sensitizing Pro-B Cells to Apoptosis

**Highlights**

- Loss of one *Mcl-1* allele substantially delays lymphomagenesis

- MCL-1 antagonizes BIM in lymphoma development

- Loss of p53 reduces requirement for MCL-1 during lymphomagenesis

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**In Brief**

MCL-1 is overexpressed in various human cancers. Grabow et al. reveal the importance of MCL-1 for the survival of B cell progenitors undergoing neoplastic transformation and in lymphomagenesis. Given that non-transformed cells appear to be less dependent on MCL-1 than malignant ones, inhibitors of MCL-1 may be useful in cancer therapy.

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Loss of a Single Mcl-1 Allele Inhibits MYC-Driven Lymphomagenesis by Sensitizing Pro-B Cells to Apoptosis

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SUMMARY

MCL-1 is critical for progenitor cell survival during emergency hematopoiesis, but its role in sustaining cells undergoing transformation and in lymphomagenesis is only poorly understood. We investigated the importance of MCL-1 in the survival of B lymphoid progenitors undergoing MYC-driven transformation and its functional interactions with pro-apoptotic BIM and PUMA and the tumor suppressor p53 in lymphoma development. Loss of one Mcl-1 allele almost abrogated MYC-driven-lymphoma development owing to a reduction in lymphoma initiating pre-B cells. Although loss of the p53 target PUMA had minor impact, loss of one p53 allele substantially accelerated lymphoma development when MCL-1 was limiting, most likely because p53 loss also causes defects in non-apoptotic tumor suppressive processes. Remarkably, loss of BIM restored the survival of lymphoma initiating cells and rate of tumor development. Thus, MCL-1 has a major role in lymphoma initiating pro-B cells to oppose BIM, which is upregulated in response to oncogenic stress.

INTRODUCTION

Cells must acquire several attributes to undergo neoplastic transformation (Hanahan and Weinberg, 2011). This is driven by the activation of oncogenes, such as deregulation of MYC expression, and loss of function of tumor suppressor genes, such as mutations in p53. Many oncogenic lesions (e.g., deregulated MYC expression) drive aberrant proliferation and therefore impose a stress on nascent tumor cells that can trigger tumor suppressive processes, such as apoptosis (Delbridge et al., 2012). Accordingly, inhibition of apoptosis is required to facilitate survival of cells undergoing neoplastic transformation. Interestingly, somatically acquired gene copy-number amplifications of the pro-survival BCL-2 family members MCL1 (Myeloid Cell Leukemia-1, mouse homolog described in this study is called Mcl-1) and BCL-X (or BCL2L1; BCL-2-like 1; mouse homolog described in this study is called Bcl-x) are found in many human cancers (Beroukhim et al., 2010).

The importance of maintaining sufficient MCL-1 protein levels to sustain enhanced cell proliferation was recently demonstrated when loss of a single allele of Mcl-1 was shown to severely compromise the survival of proliferating hematopoietic progenitor cells, resulting in the failure to reconstitute a functional hematopoietic system in Mcl-1+/− mice exposed to 5-fluorouracil (5-FU) or γ-irradiation (Delbridge et al., 2015b). Activation of oncoproteins, such as MYC, causes not only enhanced proliferation but also replicative stress with concomitant DNA damage (Soucek and Evan, 2010). These stress stimuli activate the key tumor suppressor p53 (protein 53 kDa), which orchestrates downstream pathways to elicit cell-cycle arrest via p21 (protein 21 kDa, also known as cyclin-dependent kinase inhibitor 1 [CDKN1] or CDK-interacting protein 1 [Cip1] or Waf1) and/or apoptosis through PUMA (p53 upregulated modulator of apoptosis; official name of the mouse gene: Bbc3; here we used Puma) and NOXA (official gene name: Pmaip1; Phorbol-12-myristate-13-acetate-induced protein 1) (Oda et al., 2000; Nakano and Vousden, 2001; Valente et al., 2015). Defects in the p53 pathway cooperate with deregulated MYC expression in tumorigenesis. Strikingly, ~70% of human cancers present with aberrant MYC expression highlighting its importance in tumor development (Soucek and Evan, 2010). Interestingly, many of these cancers also carry p53 pathway defects (Vousden and Lu, 2002). Considerable insight into the impact of deregulated MYC expression has come from the study of Eµ-Myc transgenic mice in which MYC overexpression is driven by the Igh enhancer (Adams et al., 1985). This causes abnormally increased proliferation of B cell progenitors (Langdon et al., 1986), which upon acquisition of cooperating oncogenic lesions progress to clonal pre-B or slg+ B cell lymphoma (Harris et al., 1988). It is still unknown which pro-survival and pro-apoptotic proteins are essential for the sustained survival of B cell progenitors during neoplastic transformation following oncogene activation and what other pathways have to be deregulated to facilitate malignant transformation. Since MCL-1 is critical for the survival of proliferating hematopoietic stem and progenitor cells (Delbridge et al., 2015b), we examined the impact of limiting MCL-1 protein
levels on the survival of abnormally proliferating B cell progenitors that initiate lymphomagenesis in Eμ-Myc mice and the rate of tumor development.

RESULTS

Loss of One Allele of Mcl-1 Substantially Delays MYC-Driven Lymphoma Development

Expression from both alleles of Mcl-1 is essential for the survival of hematopoietic stem and progenitor cells during stress-induced repopulation of the hematopoietic system (Delbridge et al., 2015b). Therefore, we hypothesized that a reduction in MCL-1 protein levels might impede lymphoma development. This question is of particular interest given the ongoing efforts to develop MCL-1 inhibitors for anti-cancer therapy (Goodwin et al., 2015; Leverson et al., 2015). Hence, we generated Eμ-Myc;Mcl-1+/−/C0 mice to examine the impact of limiting levels of MCL-1 on the survival of pre-leukemic B lymphoid cells undergoing neoplastic transformation and on the rate of pre-B/B lymphoma development.

Eμ-Myc;Mcl-1+/− mice developed lymphoma with considerably reduced incidence and much longer latency compared to control Eμ-Myc mice (median survival: Eμ-Myc = 90 days versus 61% of Eμ-Myc;Mcl-1+/− lymphoma-free at 800 days; Mantel-Cox log-rank test: p < 0.0001; Figure 1A). Remarkably, at the time when almost all Eμ-Myc control mice had succumbed to lymphoma (~200 days), > 80% of Eμ-Myc;Mcl-1+/− mice still remained lymphoma-free.

The few Eμ-Myc;Mcl-1+/− mice that became sick presented with a lymphoma burden similar to sick Eμ-Myc animals, as shown by their lymphocyte counts in the blood (p = 0.6418; Figure 1B) and spleen weights (p = 0.25; Figure 1C). The lymph node weights were significantly lower in sick Eμ-Myc;Mcl-1+/− mice than sick Eμ-Myc mice (p = 0.01), but the platelet numbers were similar (p = 0.13; Figure S1). Histological examination confirmed extensive accumulation of lymphoma cells in the bone marrow and spleen of the sick Eμ-Myc;Mcl-1+/− mice, and this was comparable to the lymphoma burden seen in sick Eμ-Myc mice (Figure 1D). Flow cytometric analysis revealed that Eμ-Myc;Mcl-1+/− mice developed mostly sIg+B cell lymphoma (91%, B220+IgM+) and only rarely pre-B lymphoma (9%, B220−slg−). Conversely, 57% of Eμ-Myc mice developed sIg+B lymphoma, 36% pre-B cell lymphoma, and 7% mixed pre-B/B lymphoma (Figure S2A). Western blot
analysis showed that despite the loss of one allele of Mcl-1, the MCL-1 protein levels in many Eµ-Myc;Mcl-1-/- lymphomas were comparable to those seen in lymphomas from Eµ-Myc mice (Figure 1E). This indicates compensatory upregulation of MCL-1 expression from the remaining wild-type (WT) Mcl-1 allele. We did not observe consistent differences in the protein expression of pro-survival BCL-2 and BCL-XL or pro-apoptotic BIM and PUMA between Eµ-Myc;Mcl-1-/- and Eµ-Myc lymphomas (Figure 1E). To test whether expression from the remaining WT Mcl-1 allele is upregulated in Eµ-Myc;Mcl-1-/- lymphomas, we performed qRT-PCR analysis. We found that Mcl-1 transcript levels were not significantly different between Eµ-Myc;Mcl-1-/- and Eµ-Myc lymphomas, revealing that expression from the WT Mcl-1 allele must indeed have been upregulated in the former (Figure S2B).

Approximately 20%–30% of lymphomas arising in Eµ-Myc mice harbor defects that disable the p53 pathway (Eischen et al., 1999; Michalak et al., 2009). Western blot analysis for p53 and p19ARF (high levels of these proteins indicate defects in the p53 pathway) revealed that there was no significant difference in the frequency of p53-pathway defects between Eµ-Myc;Mcl-1-/- (38%; 6/16) versus Eµ-Myc (33%; 3/9) lymphomas (Figures 1E and S3). DNA sequence analysis failed to detect mutations in p53 that affect its DNA binding regions (the region where most mutations in p53 are found in cancers; exons 4–10) in any of the Eµ-Myc;Mcl-1-/- lymphomas (9 lymphomas tested, versus 3 WT control cell samples). These results show that loss of one Mcl-1 allele greatly delays MYC-induced lymphoma development and that this is not accompanied by an increased selection for p53 pathway defects in the few lymphomas that arise in the Eµ-Myc;Mcl-1-/- mice.

**Loss of One Allele of Mcl-1 Substantially Reduces MYC-Driven Accumulation of Pre-leukemic Cancer Initiating Cells**

Since Eµ-Myc;Mcl-1-/- mice survived considerably longer than their Eµ-Myc counterparts, we investigated the impact of loss of one Mcl-1 allele on the accumulation of pre-leukemic B lymphoid progenitors, in which neoplastic transformation proceeds to produce malignant lymphomas (Harris et al., 1988). At 4 weeks of age, Eµ-Myc mice showed no signs of malignant disease, but as previously reported (Langdon et al., 1986), they displayed a significant increase of leukocytes in the blood (p = 0.0008; Figure S4A) and spleen (p = 0.046; Figure S4B) but not the lymph nodes (Figure S4F), compared to WT mice. Eµ-Myc;Mcl-1-/- mice had markedly reduced overall leukocyte counts in these tissues compared to Eµ-Myc controls (Eµ-Myc;Mcl-1-/- versus Eµ-Myc mice: pblood < 0.0001; ppleen = 0.0472 and 48 hr ppleen = 0.0063, for comparison between Eµ-Myc and Eµ-Myc;Mcl-1-/-), n = 4-6 mice for all genotypes. Data are presented as mean ± SEM.

The blood of young Eµ-Myc;Mcl-1-/- mice had normal levels of platelets, in contrast to the platelet reduction seen in Eµ-Myc littermates (p = 0.011; Figure S4C). This is probably due to the reduced stress on the bone marrow resident hematopoietic cells as a consequence of the reduced accumulation of pre-leukemic cells, allowing normal platelet production. The numbers of erythrocytes were comparable between mice of all genotypes examined (Figure S4B).

Fluorescence-activated cell sorting (FACS) analysis revealed that 3- to 4-week-old mice of all genotypes tested (i.e., in mice with the Eµ-Myc transgene prior to appearance of malignant lymphoma) contained comparable numbers of pro-B cells (B220<sup>+</sup>sIg<sup>-</sup>c-KIT<sup>-</sup>) (Figure 2A). As reported (Langdon et al., 1986; Strasser et al., 1996), Eµ-Myc mice had increased numbers of pre-B cells (B220<sup>+</sup>c-KIT<sup>+</sup>sIg<sup>-</sup>) compared to WT controls (p = 0.0003; Figure 2B). Interestingly, the numbers of the pre-B cells and their progeny, the sIg<sup>+</sup> B cells, were significantly lower in Eµ-Myc;Mcl-1-/- mice compared to Eµ-Myc controls (p<sub>pre-B</sub> < 0.0001 and p<sub>sIg+</sub> < 0.0012; Figures 2B and 2C). In contrast, the numbers of all B cell subsets examined were comparable between WT and Mcl-1-/- mice (Figures 2A–2C).

We compared the survival of FACS sorted pro-B, pre-B, and sIg<sup>+</sup> B cells in simple medium with no added cytokines to investigate at which differentiation stage loss of one Mcl-1 allele caused increased predisposition to apoptosis in pre-leukemic lymphoma development and that this is not accompanied by an increased selection for p53 pathway defects in the few lymphomas that arise in the Eµ-Myc;Mcl-1-/- mice.
Loss of One Allele of p53 Greatly Accelerates MYC-Driven Lymphoma Development Even when MCL-1 Protein Levels Are Limiting

The tumor suppressor p53 is activated by replication stress and DNA lesions and therefore imposes a critical barrier against MYC-induced pre-B/B cell lymphoma development ([Eischen et al., 1999; Michalak et al., 2009]). Loss of p53 function precludes PUMA and NOXA induced apoptosis triggered by DNA damage, but also results in impaired DNA repair. This facilitates accumulation of additional oncogenic mutations that can drive neoplastic transformation. Accordingly, loss of one allele of p53 greatly accelerates MYC-driven lymphoma development ([Eischen et al., 1999; Michalak et al., 2009]). We generated Eµ-Myc;Mcl-1<sup>1<sup>+/<sup>C0 mice to examine whether defects in the p53-pathway could overcome the delay in MYC-driven lymphoma development imposed by loss of one allele of Mcl-1.

Consistent with previous findings ([Eischen et al., 1999; Michalak et al., 2009]), loss of one allele of p53 significantly accelerated MYC-driven lymphoma development (median survival: Eµ-Myc = 90 days, Eµ-Myc;p53<sup>+/<sup>C0 = 30 days, p < 0.0001; Figure 3A). Interestingly, Eµ-Myc;Mcl-1<sup>1<sup>+/<sup>C0 mice exhibited a median survival of 41 days. Although their survival is significantly (p < 0.0001) longer compared to Eµ-Myc;p53<sup>+/<sup>C0 mice, this delay in lymphoma onset is substantially less when comparing median survival between Eµ-Myc;Mcl-1<sup>1<sup>+/<sup>C0 and Eµ-Myc mice (Figures 1 and 3A).

Sick Eµ-Myc;Mcl-1<sup>1<sup>+/<sup>C0;p53<sup>+/<sup>C0 mice had significantly (p = 0.017) fewer lymphoma cells in their blood than sick Eµ-Myc;p53<sup>+/<sup>C0 mice; indeed the levels in the former were even below the average leukemic cell counts in the blood of sick Eµ-Myc mice (p = 0.023; Figure 3B). The spleen weights of sick Eµ-Myc;Mcl-1<sup>1<sup>+/<sup>C0;p53<sup>+/<sup>C0 mice were not significantly different from those of sick Eµ-Myc;p53<sup>+/<sup>C0 mice [Figure 3C].
and $E_{\mu}\text{-Myc};Mcl^{-/-};p53^{-/-}$ were not significantly different from those of $E_{\mu}\text{-Myc};Mcl^{-/-}$ mice (Figures 3C and S5A). This is probably due to the fact that $E_{\mu}\text{-Myc};Mcl^{-/-}$ mice become sick at a much older age and therefore have considerably higher body weights than the $E_{\mu}\text{-Myc};p53$ compound mutant mice, which develop lymphoma before they are fully grown. Histological analysis revealed similar accumulation of mutant mice, which develop lymphoma before they are fully grown. $E_{\mu}\text{-Myc};Mcl^{-/-}$ mice (Figures 3D). Although the leukemic cell counts were grown. Histological analysis revealed similar accumulation of mutant mice, which develop lymphoma before they are fully grown.

## Loss of Pro-apoptotic PUMA Accelerates Lymphoma Development in $E_{\mu}\text{-Myc};Mcl^{-/-}$ Mice Only to a Limited Extent

PUMA is essential for p53-induced apoptosis (Jeffers et al., 2003; Villunger et al., 2003) and binds with high affinity to MCL-1 (Chen et al., 2005; Kuwana et al., 2005). In rapidly proliferating hematopoietic stem and progenitor cells, PUMA is the key mediator of replicative stress-induced apoptosis, particularly when MCL-1 levels are limiting (Yu et al., 2010; Delbridge et al., 2015b). Loss of PUMA profoundly accelerates lymphoma development in $E_{\mu}\text{-Myc}$ mice, albeit to a much lesser extent than loss of even one p53 allele (Garrison et al., 2008; Michalak et al., 2009; Valente et al., 2015). This demonstrates that p53 must suppress MYC-driven lymphomagenesis through processes in addition to induction of apoptosis. Since $E_{\mu}\text{-Myc};Mcl^{-/-};p53^{-/-}$ mice develop lymphoma much more rapidly than $E_{\mu}\text{-Myc};Mcl^{-/-}$ mice, we examined how much loss of PUMA-mediated apoptosis contributes to the tumor-promoting impact of loss of p53.

Consistent with previous reports (Garrison et al., 2008; Michalak et al., 2009), $E_{\mu}\text{-Myc};Puma^{-/-}$ and $E_{\mu}\text{-Myc};Pumab^{-/-}$ mice developed lymphoma more rapidly than control $E_{\mu}\text{-Myc}$ mice (median survival: $E_{\mu}\text{-Myc} = 90$ days; $E_{\mu}\text{-Myc};Pumab = 74$ days, $p = 0.015$ versus $E_{\mu}\text{-Myc}$; $E_{\mu}\text{-Myc};Puma^{-/-} = 62$ days, $p < 0.0001$ versus $E_{\mu}\text{-Myc}$; Figure 4A). Interestingly, only loss of both alleles, but not loss of one allele, of Puma significantly increased the incidence and accelerated the onset of lymphoma in $E_{\mu}\text{-Myc};Mcl^{-/-}$ mice (median survival: $E_{\mu}\text{-Myc};Mcl^{-/-};Puma^{+/+} = 516$ days, $p = 0.62; E_{\mu}\text{-Myc};Mcl^{-/-};Pumab^{-/-} = 260$ days, $p = 0.0037$; Figure 4A).

Blood lymphocyte numbers in sick $E_{\mu}\text{-Myc};Mcl^{-/-}$, $E_{\mu}\text{-Myc};Mcl^{-/-};Puma^{+/+}$, and $E_{\mu}\text{-Myc};Mcl^{-/-};Pumab^{-/-}$ mice were comparable but all were significantly lower than those seen in $E_{\mu}\text{-Myc};Puma^{-/-}$ mice ($p = 0.0151$, $p < 0.0001$, $p < 0.0001$, respectively; Figure 4B). The spleen and lymph node weights were significantly lower in sick $E_{\mu}\text{-Myc};Mcl^{-/-}$; $Puma^{-/-}$ mice ($p_{\text{spleen}} < 0.0001$; $p_{\text{LN}} = 0.0020$) than sick $E_{\mu}\text{-Myc};Puma^{-/-}$ animals, but only the spleen weights differed significantly among sick $E_{\mu}\text{-Myc};Mcl^{-/-}$; $Puma^{-/-}$ and $E_{\mu}\text{-Myc};Puma^{-/-}$ mice ($p_{\text{spleen}} < 0.0001$; $p_{\text{LN}} = 0.43$) (Figures 4C and S5B). Platelet numbers were significantly higher in sick $E_{\mu}\text{-Myc};Mcl^{-/-}$; $Puma^{-/-}$ and $E_{\mu}\text{-Myc};Mcl^{-/-}$; $Pumab^{-/-}$ mice than sick $E_{\mu}\text{-Myc};Puma^{-/-}$ or $E_{\mu}\text{-Myc};Puma^{-/-}$ animals, respectively ($p_{\text{spleen}} = 0.0003$; $p_{\text{LN}} < 0.0001$; Figure S5B). This indicates that the stress imposed by the lymphoma burden was lower in the former. Histological and blood analyses revealed that although some $E_{\mu}\text{-Myc};Mcl^{-/-}$; $Puma^{-/-}$ and $E_{\mu}\text{-Myc};Mcl^{-/-};Pumab^{-/-}$ mice had relatively low leukemic cell counts in the blood, they still had extensive lymphoma burden in the bone marrow, spleen, and lymph nodes (Figure 4D).

FACS analysis demonstrated that 29% of $E_{\mu}\text{-Myc};Puma^{-/-}$ and 40% of $E_{\mu}\text{-Myc};Mcl^{-/-};Puma^{-/-}$ lymphomas displayed a pre-B cell phenotype. In contrast, 91% of $E_{\mu}\text{-Myc};Mcl^{-/-};Puma^{-/-}$, 83% of $E_{\mu}\text{-Myc};Puma^{-/-}$, and 86% of $E_{\mu}\text{-Myc};Mcl^{-/-};Pumab^{-/-}$ lymphomas were sig (Figure S2). Western blot analysis showed that MCL-1 protein levels were lower in several (four out of seven) $E_{\mu}\text{-Myc};Mcl^{-/-}$; $Puma^{-/-}$ lymphomas than the control $E_{\mu}\text{-Myc}$ tumors (Figures 4E and S3). This was accompanied by a slight increase in BCL-XL or BCL-2 and/or a reduction in pro-apoptotic BIM. However, a substantial fraction (three out of seven) of the $E_{\mu}\text{-Myc};Mcl^{-/-}$; $Puma^{-/-}$ lymphomas had levels of MCL-1 and BIM similar to $E_{\mu}\text{-Myc}$ lymphomas. The $E_{\mu}\text{-Myc};Mcl^{-/-};Puma^{-/-}$ lymphomas with reduced levels of MCL-1 tended to have increased levels of BCL-XL and reduced levels of BIM (Figures 4E and S3). Overall, these results show that loss of PUMA enhances lymphomagenesis in $E_{\mu}\text{-Myc};Mcl^{-/-}$ mice only to a relatively minor extent.

## Loss of BIM Substantially Accelerates Lymphomagenesis in $E_{\mu}\text{-Myc};Mcl^{-/-}$ Mice

BIM is a potent antagonist of MCL-1 and functions as a tumor suppressor in several human cancers (Tagawa et al., 2005; Bachmann et al., 2010; Richter-Larrea et al., 2010). Accordingly, loss of BIM accelerates lymphomagenesis in $E_{\mu}\text{-Myc}$ mice and reduces the pressure for acquisition of p53-pathway defects in these tumors (Egle et al., 2004). Moreover, BIM is a transcriptional target of MYC (Lee et al., 2013; Muthalagui et al., 2014). We therefore examined the role of BIM in the delay of MYC-driven lymphomagenesis exerted by loss of one Mcl-1 allele.

Consistent with previous findings (Egle et al., 2004; Delbridge et al., 2015a), $E_{\mu}\text{-Myc}$ mice lacking BIM developed lymphoma significantly faster than control $E_{\mu}\text{-Myc}$ animals (median survival: $E_{\mu}\text{-Myc};Bim^{-/-} = 67$ days, $p < 0.0001$ versus $E_{\mu}\text{-Myc}$; $E_{\mu}\text{-Myc};Bim^{+/+} = 46$ days, $p < 0.0001$ versus $E_{\mu}\text{-Myc}$). Remarkably, loss of one or both Bim allele(s) substantially increased the incidence and accelerated the rate of lymphoma development in $E_{\mu}\text{-Myc};Mcl^{-/-}$ mice (median survival: $E_{\mu}\text{-Myc};Mcl^{-/-};Bim^{-/-} = 263$ days, $E_{\mu}\text{-Myc};Mcl^{-/-};Bim^{+/+} = 60.5$ days, $p < 0.0001$ for both versus $E_{\mu}\text{-Myc};Mcl^{-/-}$; Figure S5A).

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As previously reported (Egle et al., 2004), sick $E_{\mu}$-Myc;Bim$^{-/-}$ and $E_{\mu}$-Myc;Bim$^{+/-}$ animals had significantly more leukemic cells in their blood than sick $E_{\mu}$-Myc mice ($p = 0.0005$ and $p < 0.0001$ versus $E_{\mu}$-Myc, respectively; Figure 5B). Despite their accelerated rate of lymphoma development, sick $E_{\mu}$-Myc;Mcl-1$^{+/-}$;Bim$^{+/-}$ and $E_{\mu}$-Myc;Mcl-1$^{+/-}$;Bim$^{-/-}$ mice had significantly fewer leukemic cells in their blood than sick $E_{\mu}$-Myc;Bim$^{+/-}$ and $E_{\mu}$-Myc;Bim$^{-/-}$ mice ($p < 0.0001$ and $p = 0.0003$, respectively; Figure 5B). Furthermore, sick $E_{\mu}$-Myc;Mcl-1$^{+/-}$;Bim$^{+/-}$ mice had significantly lower spleen weights ($p_{\text{spleen}} = 0.0002$) than sick $E_{\mu}$-Myc;Bim$^{+/-}$ mice, but lymph node sizes were similar (Figures 5B and S5C). Histological analysis showed that lymphoma cells were abundant in the bone marrow and spleen (Figure 5C) and had infiltrated other organs in sick mice of all genotypes (data not shown). Sick $E_{\mu}$-Myc;Mcl-1$^{+/-}$;Bim$^{+/-}$ had blood leucocyte counts comparable to sick $E_{\mu}$-Myc;Mcl-1$^{+/-}$ mice (Figure 5B), but their spleen weights were slightly heavier ($p_{\text{spleen}} = 0.047$; Figure 5B). Lymphomas from all $E_{\mu}$-Myc mice deficient for one or both Bim alleles were slg$^-$ (Figure S2).

Western blot analysis showed that $E_{\mu}$-Myc;Mcl-1$^{+/-}$;Bim$^{+/-}$ and $E_{\mu}$-Myc;Mcl-1$^{+/-}$;Bim$^{-/-}$ lymphomas tended to have MCL-1 levels comparable to control $E_{\mu}$-Myc lymphomas, and the $E_{\mu}$-Myc;Mcl-1$^{+/-}$;Bim$^{+/-}$ lymphomas also had similar levels of BIM, except for one lymphoma in which the p53-pathway was impaired (#304; Figures 5D and S3). As expected, $E_{\mu}$-Myc;Mcl-1$^{+/-}$;Bim$^{-/-}$ lymphomas lacked BIM. Interestingly, $E_{\mu}$-Myc;Mcl-1$^{+/-}$;Bim$^{-/-}$ lymphomas with lower levels of MCL-1 showed a trend toward higher expression of BCL-2 (Figures 5D and S3). These results show that loss of BIM overcomes the impairment of MYC-driven lymphoma development imposed by loss of one Mcl-1 allele.

**Reduction in BIM Overcomes the Survival Defect of Pre-leukemic $E_{\mu}$-Myc B Cell Progenitors Imposed by Loss of One Allele of Mcl-1**

The observation that loss of BIM greatly accelerates lymphomagenesis in $E_{\mu}$-Myc;Mcl-1$^{+/-}$ mice suggests that BIM-mediated apoptosis might be critical for the reduction in pre-leukemic MYC overexpressing B lymphoid cells that is caused by limiting MCL-1 levels. To test this hypothesis, we examined the consequences of BIM loss on the pre-leukemic cells in $E_{\mu}$-Myc;Mcl-1$^{+/-}$ mice. FACS sorted pre-leukemic bone-marrow derived pro-B cells (B220$^+$ ckit$^+$) from $E_{\mu}$-Myc;Mcl-1$^{+/-}$;Bim$^{+/-}$ and $E_{\mu}$-Myc;Mcl-1$^{+/-}$;Bim$^{-/-}$ mice showed significantly better
survival at 24 hr (p = 0.013 and p = 0.009, respectively), and for \( E\mu\text{-Myc;Mcl-1}^{-/-}/-\text{Bim}^{-/-} \) cells also at 48 hr (p = 0.0004), in simple tissue culture medium compared to their \( E\mu\text{-Myc;Mcl-1}^{-/-}/- \) counterparts (Figure 5E, top). This improved cell survival was reflected in significantly increased numbers of pre-leukemic pre-B cells in the bone marrow of \( E\mu\text{-Myc;Mcl-1}^{-/-}/-\text{Bim}^{-/-} \) and \( E\mu\text{-Myc;Mcl-1}^{-/-}/-\text{Bim}^-/- \) mice compared to \( E\mu\text{-Myc;Mcl-1}^{-/-}/- \) animals, with indeed their numbers rising to the levels seen in \( E\mu\text{-Myc} \) mice and even higher (Figure 5E, bottom). These results show that loss of BIM (even loss of one Bim allele) overcomes the survival defect imposed by limiting amounts of MCL-1 in \( E\mu\text{-Myc} \) pre-leukemic B lymphoid progenitors.

**DISCUSSION**

Overexpression of MCL-1 has been observed in several human cancers and is implicated in their development as well as resistance to anti-cancer therapeutics (Beroukhim et al., 2010). Here, we report on the consequences of limited MCL-1 protein levels on lymphomagenesis. Our studies reveal the importance of MCL-1 in sustaining the survival of B lymphoid cells undergoing neoplastic transformation and provide insight into the likely impact that drugs that inhibit MCL-1 may have on leukemia and lymphoma cells. Although loss of one Mcl-1 allele did not noticeably impair the survival of normal B lymphoid cells (i.e., in the absence of oncogene activation), it markedly diminished the survival of MYC overexpressing B cell progenitors. In contrast, complete loss of BCL-XL kills not only MYC-overexpressing B lymphoid cells but also their non-transformed counterparts (Kelly et al., 2011). Remarkably, loss of one Mcl-1 allele almost completely abrogated lymphoma development in \( E\mu\text{-Myc} \) mice. Similarly, acute loss of a single Mcl-1 allele kills most malignant \( E\mu\text{-Myc} \) lymphomas (Kelly et al., 2014), but not normal B lymphoid cells (Opferman et al., 2003; Delbridge et al., 2015b). This demonstrates that deregulated MYC expression imparts exquisite dependence on MCL-1, but only lesser dependence on BCL-XL (Kelly et al., 2011, 2014; Delbridge et al., 2015a) and no dependence on BCL-2 (Kelly et al., 2007).

MYC drives cell growth and proliferation, but this entails replicative stress and DNA lesions that can induce expression of pro-apoptotic BH3-only proteins (Soucek and Evan, 2010). Hence, pro-survival signaling is required to sustain the survival of MYC-overexpressing cells undergoing neoplastic transformation to allow them to acquire oncogenic lesions that are needed for full transformation. Until now little was known regarding which pro-survival BCL-2 family member(s) is/are critical for survival of nascent neoplastic B cell progenitors to facilitate lymphomagenesis. Previous studies revealed that BCL-XL (Kelly et al., 2011; Delbridge et al., 2015a), but not BCL-2 (Kelly et al., 2007), is critical. MCL-1 is, however, clearly more important than BCL-XL since loss of one allele of Mcl-1 provided similar protection as complete loss of BCL-XL (Kelly et al., 2011; Delbridge et al., 2015a). Our studies reveal that deregulated MYC expression renders lymphoma initiating pro-B cells exclusively dependent on MCL-1. Why MCL-1 is more important in these cells than its pro-survival relatives is not clear. The levels of expression of these pro-survival proteins do not readily provide an explanation; perhaps the rapid turnover of MCL-1 is needed for the efficient removal of the pro-apoptotic BH3-only proteins induced by MYC (BCL-2 and BCL-XL proteins have much longer half-lives).

Loss of BIM (even one allele) substantially accelerates lymphomagenesis in \( E\mu\text{-Myc;Mcl-1}^{-/-}/- \) mice, whereas complete loss of PUMA has much more modest impact. Thus, in MYC-overexpressing nascent neoplastic lymphoid cells, MCL-1 is mainly needed to block BIM. In contrast, the defect in emergency hematopoiesis caused by loss of one Mcl-1 allele is rescued more potently by loss of PUMA than loss of BIM (Delbridge et al., 2015b). This differential requirement for distinct BH3-only proteins for apoptosis induction may indicate cell type specific differences (i.e., non-transformed hematopoietic stem and progenitor cells versus nascent neoplastic B cell progenitors) or differences in the stresses imposed by proliferation induced by progenitor cell mobilization versus aberrant proliferation driven by deregulated MYC. MYC can activate Bim transcription directly by binding to specific sites in its promoter (Lee et al., 2013; Muthalagu et al., 2014) and indirectly by a still poorly understood process involving p53 (Happo et al., 2010).

Defects in the p53 pathway are found in \( E\mu\text{-Myc} \) lymphomas (Eischen et al., 1999; Michalak et al., 2009). Loss of a single p53 allele greatly accelerated lymphomagenesis in \( E\mu\text{-Myc;Mcl-1}^{-/-}/- \) mice. Indeed, \( E\mu\text{-Myc;Mcl-1}^{-/-}/-p53^{-/-} \) mice have a mean survival that is much shorter than that of control \( E\mu\text{-Myc} \) animals. Interestingly, complete loss of PUMA accelerates lymphomagenesis much less than loss of a single p53 allele. Thus, the tumor suppressive role of p53 in MYC-induced lymphomagenesis must involve processes in addition to PUMA-mediated apoptosis, perhaps including DNA repair or control of cellular metabolism (Vousden and Lane, 2007). This is consistent with the observation that in contrast to loss of p53, loss of p53-induced apoptosis even in combination with loss of p53-induced cell-cycle arrest and senescence (\( p53^{-/-};\text{Noxa}^{-/-};\text{p21}^{-/-} \) mice or mice with mutations in p53 that impair transcriptional induction of \( \text{Puma, Noxa, and p21} \)) does not cause spontaneous tumor development (Brady et al., 2011; Li et al., 2012; Valente et al., 2013) and that combined loss of PUMA and p21 accelerates lymphoma development in \( E\mu\text{-Myc} \) mice to a much lesser extent than loss of one p53 allele (Valente et al., 2015).

We identified BIM as the critical antagonistic of MCL-1 in lymphomagenesis as loss of BIM improved the survival of pre-leukemic \( E\mu\text{-Myc;Mcl-1}^{-/-}/- \) pro-B cells and massively accelerated lymphoma development in \( E\mu\text{-Myc;Mcl-1}^{-/-}/- \) mice. This extends previous observations that BIM is critical for apoptosis triggered by deregulated MYC expression and a potent suppressor of MYC-induced tumorigenesis (Egle et al., 2004; Muthalagu et al., 2014).

Loss of MCL-1 also delays development of acute myeloid leukemia (AML) driven by several oncogenes (Xiang et al., 2010; Glaser et al., 2012) and BCR-ABL-driven pre-B leukemia development (Koss et al., 2013). Interestingly in these models loss of both alleles of Mcl-1 was required to delay tumor development. In the case of BCR-ABL driven pre-B ALL, this may be due to the fact that this oncogenic kinase promotes BCL-XL expression, increasing overall pro-survival activity in the lymphoid cells undergoing transformation. In conclusion, MCL-1 is limiting for...
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the survival of lymphoma initiating cells, particularly those over-expressing MYC, and development of malignant lymphoma. Given that non-transformed cells appear to be less dependent on MCL-1 than malignant cells (Glaser et al., 2012; Kelly et al., 2014), inhibitors targeting MCL-1 or proteins that promote MCL-1 expression may prove efficacious in the treatment of cancer and be tolerable.

**EXPERIMENTAL PROCEDURES**

**Experimental Mice**

Experiments with mice were conducted according to the guidelines of the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee. Mcl-1<sup>-/-</sup> mice were generated from McI-1<sup>+/+</sup> mice, described previously (Vikstrom et al., 2010; Glaser et al., 2012; Kelly et al., 2014), by crossing with C57BL/6 Cre deleter mice (Schwenk et al., 1995). Bim<sup>−/−</sup> (Bouillet et al., 1999), Puma<sup>−/−</sup> (Villunger et al., 2003), p53<sup>−/−</sup> (Jacks et al., 1994), and E<sub>M</sub>-Myc mice (Adams et al., 1983) have been described previously (all maintained on a C57BL/6 background for > 20 generations).

**Genotyping**

Genotyping of mice was performed as previously reported (Grabow et al., 2012). Primer sequences will be supplied upon request.

**Blood Analysis**

Peripheral blood samples were taken at the time of sacrifice and analyzed using an ADVIA blood analyzer (Siemens).

**Histological Analysis**

Histological analysis was performed as previously reported (Delbridge et al., 2015b). Scale bars denote 200 μm.

**Flow Cytometric Analysis**

Single-cell suspensions from tissues were prepared using 100-μM sieves (BD Biosciences). Cells (5 x 10<sup>6</sup>) were stained for surface markers using fluorochrome-conjugated (FITC, APC, R-PE; Life Technologies) monoclonal antibodies to B220 (RA3-6B2), c-KIT (ACK-2 or ACK-4), IgM (5.1), and IgD (11-26C; all generated in house) for 30 min in balanced salt solution (BSS) supplemented with 2% fetal calf serum (JHR Biosciences) and 10% normal rat serum and analyzed in a FACS-Calibur (BD Biosciences).

**Immunoblotting**

Lysates were prepared in RIPA buffer complemented with COMPLETE protease inhibitors (Roche). Blots were probed using monoclonal hamster anti-mouse BCL-2 (3F11; BD Pharmingen), monoclonal mouse anti-mouse BCL-XL (2F12; BD Pharmingen), monoclonal anti-rat- mouse MCL-1 (19C4-15 [Okamoto et al., 2014]), polyclonal rabbit anti-mouse PUMA (Ab-27669, Abcam), and polyclonal rabbit anti-mouse BIM (8292; Sapphire Bioscience) antibodies. Probing with mouse monoclonal anti-HSP70 antibody (gift from Dr. R. Anderson, Peter MacCallum Cancer Institute) was used as a loading control. Polyclonal goat antibodies against mouse, rat, hamster, or rabbit IgG coupled to HRP (Southern Biotech, In Vitro Technologies) were used as secondary reagents and enhanced chemiluminescence (ECL; GE Healthcare Life Sciences) was used for detection.

**qRT-PCR Analysis**

qRT-PCR analysis was conducted as previously described (Grabow et al., 2012).

**DNA Sequence Analysis of the p53 Tumor Suppressor Gene**

Exons 4 to 10 of p53 (encoding the DNA binding domain) from 9 E<sub>M</sub>-Myc;Mcl-1<sup>−/−</sup> lymphoma samples were sequenced using the Illumina MiSeq machine (illumina) and compared to three normal WT T/B cell samples. All samples were prepared in technical duplicates using separate subsets of primers. Replicates were prepared on separate plates to ensure reproducibility. For DNA sequencing all samples were combined into one. Unique primers sets allowed differentiation between samples and replicates. Dominant reads from each primer set were aligned using MEQAlign (DNASTAR).

**Animal Survival Analysis and Statistical Analysis**

Kaplan-Meier survival curves, scatter, and bar graphs for organ weights, blood parameters, and qRT-PCR were generated and analyzed using the GraphPad Prism software (GraphPad Software). Mouse cohorts were compared by log-rank Mantel-Cox test. p values of less than 0.05 were considered to indicate significant differences. In vitro cell survival data, blood cell counts, and organ weights were plotted and analyzed with GraphPad Prism using two-tailed Student’s t test comparing two groups with each other. Data are presented as mean ± SEM.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.039.

**AUTHOR CONTRIBUTIONS**

S.G. and A.S. conceived ideas, planned experiments, and wrote the manuscript; S.G., A.R.D.D., B.J.A., and C.J.V. conducted experiments and analyzed results.

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