BET inhibition represses miR17-92 to drive BIM-initiated apoptosis of normal and transformed hematopoietic cells

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Abstract

The BET bromodomain-containing proteins, such as BRD4, are highly promising targets for treating lymphoid and myeloid malignancies. They act to modulate the expression of multiple genes that control diverse cellular processes including proliferation, survival and differentiation which are consequentially disrupted by small molecule BET bromodomain inhibitors such as JQ1. By assessing the impact of these inhibitors on normal mouse hematopoietic cells or their transformed counterparts, we establish definitively that their cytotoxic action *in vitro* and *in vivo* relies predominantly on the activation of BAX/BAK-dependent mitochondrial (intrinsic) apoptosis. In large part, this is triggered by marked up-regulation of the BH3-only protein BIM when the BET inhibitors suppress miR-17-92, a key post-transcriptional repressor of BIM expression. Thus, our study strongly suggests that mutations that permit the evasion of apoptosis (e.g. BCL2 overexpression, BIM inactivation) are likely to blunt the activity of the BET bromodomain inhibitors and should be anticipated when therapy resistance develops. Strikingly, we also found that certain normal hematopoietic cells, especially those of lymphoid origin, are as prone to apoptosis induced by the BET inhibitors as their transformed counterparts, indicating that their susceptibility to BET inhibitors did not arise from oncogenic transformation.

Introduction

There has been intense interest in targeting the BET (**b**romodomain and **e**xtra**t**erminal domain) family of proteins (BRD2, BRD3, BRD4, BRDT) for treating cancers, notably ones of hematological origin[1-8]. Other than the rare NUT midline carcinomas (NMC) in which BRD4 expression is dysregulated[9], a variety of cancers driven by other oncogenic lesions, such as deregulated c-*MYC* expression in hematological malignancies, also appear to be highly susceptible to BET inhibition[4]. Biochemically, the BET proteins act as epigenetic "readers" regulating gene expression by binding to ε-N-acetylated lysines on histones or other proteins[10, 11]. Through these interactions, the BET proteins recruit other proteins (including histone modification 'writers' and 'erasers') involved in the wider network regulating gene transcription[12]. Small molecule inhibitors of the BET proteins, such as the well-validated and extensively used tool compound JQ1, were shown to inhibit the growth of several leukemia and lymphoma derived cell lines[13].

Since the BET proteins modulate the expression of many genes, the consequences of inhibiting them are likely to be diverse, ranging from suppressing cell cycle progression driven by c-MYC[1, 4], modulation of apoptosis[2], NFκB signaling[14] to the induction of differentiation[1]. However, the relative contributions of these processes for the cytotoxic action of the BET inhibitors is unclear; likewise, whether targeting c-*MYC* expression is sufficient to account for the cytotoxic action of the BET inhibitors is unknown. Using established cell lines and well-validated mouse genetic models, we sought to establish the relative importance of apoptosis for their cytotoxic action. We focused on the mitochondrial (intrinsic) pathway to apoptosis regulated by the BCL2 protein family[15, 16] since this mechanism is implicated in the response to diverse anti-cancer agents[17-19] For example, the overexpression of BCL2 in lymphoid malignancies often accounts for their refractoriness to standard-of-care agents, such as DNA damage inducing chemotherapeutics[20]. BCL2 itself and its pro-survival relatives act to restrain

the cell death mediators BAX and BAK, thereby maintaining cellular viability. The pro-survival proteins are countered by the third sub-class of the BCL2 family, the BH3-only proteins[21]. When a cell is damaged or stressed, such as by DNA damaging cytotoxic drugs, the BH3-only proteins are activated[22]. These BH3-only proteins (e.g. PUMA, BIM) inhibit the pro-survival BCL2 proteins and/or directly activate BAX/BAK, thereby allowing the latter to drive mitochondrial outer membrane permeabilization (MOMP), the point of no return in apoptotic cell death. In the absence of both BAX and BAK, apoptosis cannot proceed[23].

By treating multiple cell lines of diverse origins or matching ones engineered to lack one or more of the key BCL2 family members, we were able to determine whether apoptosis plays any role in the killing of hematopoietic cells by the BET inhibitors. Our results from studying hematopoietic cells *in vitro* and in whole animals establish the central role played by BAX/BAK-mediated apoptosis for the cytotoxic action of the BET inhibitors. Moreover, the transcriptional up-regulation of BIM upon JQ1 treatment appears to contribute significantly. Importantly, we uncovered a novel mechanism by which the BET inhibitors prime hematopoietic cells to die: by repressing the microRNA cluster, miR17-92, which normally prevents apoptosis by reducing *BIM* mRNA levels[24-26].

Materials and methods

Cell lines and mice *MLL-ENL* AML[27] and Eμ-*Myc* lymphoma[28] cell lines were generated and cultured as previously described. Retroviral MSCV-IRES-GFP (MIG) constructs for c-MYC[29] (a gift from John Cleveland, Addgene plasmid # 18119) and (BCL2, BCLXL, MCL1, BCL2A1)[27] overexpression or BIM knock-down[30] and retroviral transduction[31] have been described. Eμ-*Myc;p53*$^{-/-}$ cell lines were derived from Eμ-*Myc* mice crossed with *p53*$^{+/-}$ mice[32]. Genome editing of *Bim* and *p53* in Eμ-*Myc* lymphoma cells has been described[33]. The vav-*BCL2* transgenic[34], *Bim*$^{-/-}$[35], *Bid*$^{-/-}$[36], *Bax*$^{-/-}$*Bak*$^{-/-}$[23], *p53*$^{-/-}$[37] and Eμ-*Myc*[38]

mice have also been described; all of these mice were maintained on a C57BL/6 background. Bone marrow chimeric (FLT) mice were generated by injecting 2×10^6 fetal liver cells (CD45.2) from E13.5 embryos by intravenous injection into lethally (2×5.5Gy, 3h apart) γ-irradiated C57BL/6 (CD45.1) recipient mice. WT, *Bax*$^{-/-}$*Bak*$^{-/-}$ reconstituted (FLT) mice or mice bearing lymphomas were administered daily doses of 50mg/kg of JQ1 by intra-peritoneal injection. Injection of 10% DMSO in the vehicle (10% 2-hydroxypropyl-β-cyclodextrin) was used as the control. Experiments with mice were conducted according to the guidelines of the Walter and Eliza Hall Institute Animal Ethics Committee.

Hematopoietic cell subsets staining, sorting and culturing LSK (Lineage-Sca-1+cKIT+) cells were isolated from lineage marker+ depleted BM cells stained with antibodies detecting Sca-1 (clone D7) and c-Kit (clone Ack4) and cultured in Dulbecco's Modified Eagle's Medium (DME) containing 10% FCS, 6 ng/ml IL-3, 100 ng/mL SCF and 50 ng/mL Flk-ligand. BM cells were lineage depleted using rat antibodies detecting CD2 (clone RM2.1), B220 (clone RA3-6B2), CD19 (clone ID3), CD3 (clone KT31.1), CD8 (clone 53-6.7), Ter119 (clone Ly-76), Mac1 (clone M1/70), and Gr-1 (clone IA8) and anti-rat IgG antibody conjugated magnetic beads (Sigma). Myeloid cells were isolated after staining with Mac-1 (clone M1/70) antibody and cultured in DMEM containing 10% FCS and 6 ng/mL IL-3. Hematopoietic subsets were stained and isolated as previously described[39, 40] and cultured on OP9 stromal cells in α-MEM medium containing 10% FCS, 2 mM L-glutamine, 10 ng/mL IL-7 and 50 μM β-mercaptoethanol. Sorted thymic T cells were cultured on OP9-DL1 stromal cells in α-MEM medium containing 10% FCS, 4 mM L-glutamine, 10 ng/mL IL-7 and 50 μM β-mercaptoethanol.

CRISPR/Cas9 gene editing The inducible lentiviral based CRSIPR/Cas9 gene editing system has been previously described[33]. Lentiviral constructs for doxycycline-inducible sgRNA expression[33] and sgRNA sequences for mouse

and human BIM[33], mouse p53[33], mouse BRD4[41], and human BAX and BAK (Gong, Huang et al. submitted) have been described. The generation of lentiviral particles, induction of sgRNA expression and sequencing of genomic DNA from clonal cell lines using the Illumina MiSeq sequencing platform have also been described[33].

Results

JQ1 triggers cell death in diverse cell lines derived from hematopoietuc malignancies

We first examined the effect of JQ1 treatment on a panel of 10 human cell lines. Using a well established assay (CellTiter-Glo) that quantifies the proliferation and survival of a population of cells, we found that JQ1 treatment reduced the viability of all the cell lines tested (Figure 1a). Since this loss in cell viability could be due to growth inhibition or to cell death, we sought to determine specifically whether these cells were undergoing cell death by assessing the ability of live cells to exclude the vital dye propidium iodide (PI). Interestingly, JQ1 induced extensive cell death in the majority of the cell lines tested (e.g. MM.1S). The inhibition of cell growth without significant cell death was only observed in two cell lines, K562 and MOLT-4 (Figure 1a).

Given the striking finding that JQ1 induced cell death in 8/10 cell lines tested, we next investigated the mechanisms of BET inhibition by utilizing the Eμ-*Myc* mouse lymphoma model which has been extensively used to test novel therapeutic approaches as well as the role of cell death induction in cancer therapy[38, 42, 43]. In this transgenic mouse strain, the immunoglobulin heavy chain gene enhancer (Eμ) drives the aberrant overexpression of *c-Myc* within the B cell compartment. From an early age, the deregulated c-MYC expression causes an abnormal polyclonal increase in pre-leukemic pro-B and pre-B cells[44, 45] and the animals invariably progress to clonal pre-B or sIg^{+ve} B cell lymphomas (mean survival of ~110 days on a C57BL/6 background)[46].

We tested multiple independently derived Eμ-*Myc* lymphomas *in vitro* and found that JQ1 reduced cell viability (EC_{50}≈40nM; Figure 1b). Like many of the human cancer cell lines (Figure 1a), significant cell death was observed (EC_{50}≈200nM). Consistent with these *in vitro* observations, mice transplanted with these Eμ-*Myc* lymphomas revealed that daily treatment with JQ1 substantially curtailed tumor growth (Figure 1c). Compared to control vehicle treated mice (mean survival 9 days), treatment with JQ1 prolonged their survival (17.5 days) (Figure 1d). JQ1 treatment also ameliorated some of the characteristic features of this aggressive disease, such as splenomegaly and hepatomegaly caused by lymphomatous infiltration into these organs (Figures 1e,f; Supplementary Figure 1). These data recapitulate and extend recent observations with a related model of high grade lymphoma, the *λ-Myc* mice[47].

We conclude from these experiments that the BET inhibitors can trigger the death of multiple hematopoietic cell lines derived from diverse models of malignancies. Next, we sought to determine the mechanism by which these compounds can kill cells.

The cytotoxic action of the BET inhibitors does not require the tumor suppressor p53

We first examined whether the cytotoxic effect of JQ1 relies on the tumor suppressor protein p53. p53 mediates the activity of many cytotoxic drugs by triggering the expression of genes that drive apoptosis or cell cycle arrest, such as *Puma* or *p21*, respectively[48]. Accordingly, Eμ-*Myc* lymphoma cells that constitutively lack p53 or have p53 expression ablated by CRISPR/Cas9 genome editing were refractory to the induction of p53-dependent apoptosis triggered by the DNA damaging drug etoposide (Supplementary Figure 2A). In sharp contrast, JQ1 killed Eμ-*Myc* lymphoma (Figure 2a) or *MLL-ENL* AML cells (Figure 2b) even when *p53* was deleted. Moreover, these Eμ-*Myc* lymphoma cells underwent G_0/G_1 cell cycle arrest induced by JQ1 whether or not they harbored *p53* (Figure 2c).

These studies reveal that the cytotoxic acton of the BET inhibitors does not require p53.

JQ1 kills Eμ-*Myc* lymphoma cells by inducing apoptosis

Next, we sought to determine the mechanism by which the BET inhibitors drive p53-independent killing of the lymphoma cells. Killing by the BET inhibitors was delayed by addition of the broad spectrum caspase inhibitor, Q-VD-OPh[49] (Supplementary Figure 2B). Cleavage of caspase-3 was apparent 24 hours after exposure to JQ1 but abolished by enforced expression of the pro-survival protein BCL2 (Supplementary Figure 2C). Enforced expression of the other pro-survival relatives, BCLXL, MCL1, BCL2A1, also protected these lymphoma cells as well as murine AML cells from killing by JQ1 (Figures 2d,e). We conclude from these studies that the BET inhibitors kill malignant hematopoietic cells by inducing the mitochondrial pathway to apoptosis.

The BET inhibitors also kill normal B lymphoid cells

To investigate if sensitivity to BET inhibition is a consequence of oncogenic transformation, we assessed the sensitivity of primary (non-transformed) B lymphoid cells isolated from wild-type C57BL/6 mice that were cultured in IL-7 supplemented medium with the stromal cell line, OP9. Using this culture system to maintain primary cells *in vitro* for a few days, we found that all the B cell subsets from bone marrow or spleen were sensitive to killing by JQ1 (EC_{50}<0.5 μM), with the less mature developmental stages being slightly more sensitive (Figures 3a,b). I-BET151, another BET inhibitor that is chemically distinct from JQ1, also killed primary B lymphoid cells (Supplementary Figure 3). Akin to the Eμ-*Myc* lymphoma cells, killing of normal B cells by JQ1 did not require p53 but was prevented by enforced expression of BCL2 (Figures 3c,d).

BET inhibition kills diverse normal hematopoietic cells by inducing BAX/BAK mediated apoptosis

The resistance imposed by the pro-survival BCL2 family proteins strongly suggested that BET inhibitors kill normal and transformed B lymphoid cells by inducing apoptosis. We therefore investigated the impact of loss of BAX and BAK on JQ1 induced cell killing, the definitive approach to establish the importance of the intrinsic apoptotic cell death pathway. *Bax*$^{-/-}$*Bak*$^{-/-}$ mice have multiple defects and most die perinatally[23]. We therefore used E13.5 *Bax*$^{-/-}$ *Bak*$^{-/-}$ embryos for fetal liver transplantation (FLT) into lethally irradiated wt recipients to generate BAX/BAK deficient bone marrow chimeric mice. BAX/BAK deficient B lymphoid cells isolated from fetal liver transplanted recipients, thereafter named *Bax*$^{-/-}$*Bak*$^{-/-}$ (FLT) B cells, were completely refractory (EC_{50}>10 μM) while WT (FLT) B cells isolated from control recipients were readily killed by JQ1 (Figure 4a) or I-BET151 (Supplementary Figure 4). Testing WT thymic T cell subsets, we found that CD4/CD8 double negative (DN) progenitors were most sensitive while the more mature double positive (DP) subtype was less sensitive and mature (CD4 or CD8 single positive) T cells were largely unaffected by JQ1 (Figure 4b). Mechanistically, and akin to the B lymphoid cells, the killing of these sensitive T cell subsets was completely prevented in absence of BAX/BAK (Figures 4b,c). Similarly, BAX/BAK-doubly deficient *MLL-ENL* AML, MM.1S or KMS-12PE cells proved resistant to being killed by JQ1 (Figures 4d,e,f).

In vivo JQ1 treatment causes significant lymphopenia driven exclusively by BAX/BAK mediated apoptosis

We next evaluated the consequences of BET inhibitor treatment *in vivo*, particularly on the hematopoietic cells. After a 10-day course, the JQ1-treated mice did not lose weight but there was a significant decrease in the numbers of circulating white blood cells (Figure 5a). Most of this decrease is accounted for by the drop in the number of B lymphoid cells (Figure 5b), which normally

comprise 40-70% of the circulating white blood cells in mice. Treatment with JQ1 did not have significant impact on hemoglobin concentration while the platelet count was modestly decreased. JQ1 did not markedly reduce the neutrophil counts or the overall myeloid cell composition in the bone marrow, spleen and blood in WT mice. In contrast, thymic T cell subsets were substantially depleted upon *in vivo* treatment with JQ1 (Supplementary Figure 5). Finally, we investigated whether BET inhibition depleted B lymphoid cells within the whole animal primarily through BAX/BAK mediated apoptosis or if induction of cell cycle arrest and differentiation contributed to the toxicity. Strikingly, the numbers of B lymphoid cells in *Bax*$^{-/-}$*Bak*$^{-/-}$ (FLT) mice were unaffected by JQ1 treatment (Figure 5c). These results show that B and T lymphoid cells are the most sensitive cell types to JQ1 treatment *in vivo* and that their killing is abrogated by combined loss of BAX and BAK.

The pro-apoptotic BH3-only protein BIM is critical for initiating JQ1 induced killing of B lymphoid cells

Given that JQ1 induced killing of lymphoid cells requires BAX and BAK, we next examined whether expression of BH3-only proteins, the initiators of the intrinsic apoptotic pathway, was altered by JQ1 treatment. Previously, BET protein inhibition has been reported to suppress *BCL2* transcription[2]. Consistent with this report we found that JQ1 treatment reduced *Bcl2* mRNA levels in Eμ-*Myc* lymphoma and *MLL-ENL* AML cell lines (Supplementary Figure 6A). However, we did not detect significant decrease in the level of BCL2 protein or its pro-survival relatives (BCLXL, MCL1) in JQ1 treated Eμ-*Myc* lymphoma (Figure 6a; Supplementary Figure 6B).

Strikingly, we found instead that levels of the BH3-only protein BIM were markedly elevated while the levels of the other BH3-only proteins assessed were unaltered in JQ1 treated cells. *BIM* mRNA levels were increased 2-6 fold 48h after JQ1 treatment in Eμ-*Myc* lymphoma (Figure 6b, Supplementary Fig.6C). *Bim* mRNA expression in Eμ-*Myc* lymphoma cells

was also increased when *Brd4* was genetically deleted by CRISPR/Cas9 genome editing; this spares *Brd2* and *Brd3* (Figure 6c, Supplementary Fig.6D). Suppressing BIM expression by RNAi mediated knock-down or targeting *Bim* using CRISPR/Cas9 genome editing significantly inhibited JQ1 induced killing of the Eμ-*Myc* lymphoma cells (Figure 6d). Moreover, normal B cells derived from *Bim*$^{-/-}$, but not those from *Bid*$^{-/-}$ mice, were significantly protected from JQ1 induced killing compared to their WT counterparts (Figure 6f). These results show that BIM plays a critical role in JQ1 induced killing of lymphoma as well as normal lymphoid cells.

In addition to these lymphoid cells, we also evaluated the role of BIM in a range of human cancer cell lines and reached the same conclusion: that JQ1 treatment increased BIM levels (Figure 6a, Supplementary Fig. 6B) by up-regulating *BIM* mRNA levels (Figure 6b, Supplementary Fig. 6C). Thus, deletion of *BIM* significantly abrogates the killing of these cells by JQ1 (Figure 6e, Supplementary Fig. 6E).

JQ1 relieves a critical brake on BIM mRNA levels

BIM is controlled at multiple levels, both transcriptional and post-transcriptional[21]. The increase in *Bim* mRNA levels 8 hours after exposure to JQ1 led us to investigate the miR17-92 cluster, a key negative regulator of *Bim* mRNA levels[24-26]. Expression of miR17-5p and miR92a-3p was rapidly suppressed by JQ1 in Eμ-*Myc* lymphoma and MM.1S cells (Figure 7a), consistent with enrichment of BRD4 at miR17-92 promoter[50, 51] (Supplementary Figure 7A). miR17-5p and miR92a-3p expression was also reduced by JQ1 treatment in the diverse human cancer cell lines (Supplementary Figure 7B). These results identify a previously unrecognized mechanism by which the BET inhibitors trigger apoptosis by repressing miR17-92 to increase *Bim* mRNA and BIM protein levels. Consistent with this observation, we also found that the genetic deletion of *Brd4* itself led to the repression of miR17-5p and miR92a-3p (Figure 7b).

The induction of apoptosis by the BET inhibitors does not rely on the depletion of c-MYC

The cytotoxic action of BET inhibition is often attributed to downregulation of c-MYC expression[4]. Consistent with previous reports, c-MYC protein and mRNA levels rapidly declined after JQ1 treatment in the human cell lines (Supplementary Figure 8A). By contrast, we could only detect a modest decline in c-MYC mRNA and protein after treating Eμ-*Myc* cells with JQ1 (Supplementary Figure 8B). To definitively establish the role of c-MYC depletion for apoptosis induction by JQ1, we engineered MM1.S, MV4;11 or HL-60 cells that overexpress c-MYC. Like the Eμ-*Myc* cells, we only observed modest reduction in c-MYC levels after JQ1 treatment (Supplementary Figure 9A,B). However, the repression of miR17-5p and miR92a-3p (Supplementary Figure 9C) and the consequent induction of BIM was unaltered compared with the parental cells (Supplementary Figure 9D). Unsurprisingly, these cells with enforced c-MYC expression died as rapidly after JQ1 treatment as the parental cells (Supplementary Figure 9E).

We conclude from these studies in diverse cell types that the depletion of c-MYC could not account for the induction of apoptosis by the BET inhibitors.

Discussion

Targeting the BET bromodomain proteins is showing great promise for treating a range of cancers including lymphoid and myeloid malignancies. Interestingly, BET inhibition appears efficacious even in tumors that are not driven by dysregulation of the BET bromodomain proteins. A compelling rationale for this is that the expression of key proto-oncogenes, such as c-*MYC*, is suppressed, thereby leading to tumor shrinkage. Indeed, the BET inhibitors have been advanced as agents to treat cancers driven by

deregulated c-*MYC* expression [4, 52]. In spite of the striking pre-clinical efficacy observed with these compounds, precisely how they act as cytotoxic agents remains unresolved. In this context, a myriad of activities have been previously implicated: apoptosis[3, 7, 53], cell cycle arrest[3, 4, 7] or differentiation[1].

Using a range of genetic models, we found that the BET inhibitors are highly efficient at inducing the mitochondrial (intrinsic) apoptotic pathway in both normal and malignant hematopoietic cells. The execution of this pathway requires BAX and BAK: in their absence apoptosis cannot proceed. Their combined absence rendered hematopoietic cells completely resistant to the BET inhibitors. The results presented here indicate that, at least in the hematopoietic cell subsets and the majority of transformed cells we investigated, apoptosis is critical for the action of these drugs. When apoptosis is impaired, the BET inhibitors induced brief cell cycle arrest but the *in vitro* growth of cells was largely unaffected. In mice, when apoptosis was prevented by the genetic deletion of *Bax* and *Bak* the BET inhibitors no longer had a significant impact on the hematopoietic system.

Having established the central role played by the induction of the intrinsic apoptotic pathway for the action of the BET inhibitors, we tackled the question of how these compounds can trigger apoptosis. We excluded any role for the tumor suppressor p53 and our data also argues against a central role for repression of *Bcl2*. Instead, the BH3-only protein BIM plays an important role, in accord with a recent study[53]. Firstly, JQ1 treatment significantly elevated BIM levels. Secondly, absence or reduction of BIM significantly blunted the action of the BET inhibitors. Thirdly, we provide compelling evidence how JQ1 treatment increases *BIM* mRNA levels, at least in part by repressing the expression of the miR17-92 cluster, a well-recognized negative regulator of BIM[24, 25]. The miR17-92 cluster is a direct transcriptional target of c-MYC[54, 55] and downregulation of c-*MYC* by BET inhibition may contribute to the reduced microRNA expression. However, our data demonstrates that BET inhibition represses miR17-92, increases BIM expression and induces apoptosis even when c-MYC expression is sustained

(by transgenic or retroviral expression), suggesting that disrupting microRNA expression is the direct consequence of BRD4 inhibition (Figure 8). Our study implicating BRD4 in directly regulating miR17-92 is consistent with its binding to the miR-17-92 promoter. Of note, we have pinpointed an important but previously unrecognized aspect of how the BET inhibitors might act: modulating the expression of critical microRNAs implicated in tumorigenesis or response to therapeutics[56].

Importantly, our findings suggest that the cytotoxic effect of BET inhibitors does not depend entirely on MYC repression. In contrast, defects in the mitochondrial (BAX/BAK mediated) apoptotic pathway thoroughly compromise the cytotoxicity of BET inhibitors and might be selected for during acquired resistance of tumor cells. For example, we would predict that tumors bearing high levels of pro-survival BCL2 proteins[57] or those that lack pro-apoptotic BIM[58] will respond poorly to the BET inhibitors. Our data also highlight a previously unappreciated sensitivity of normal hematopoietic cells, especially those of lymphoid origin, to BET bromodomain inhibition. It will be very interesting to see if our conclusions using tool compounds and model systems, as well as any impact on other tissues[59], is recapitulated in the ongoing clinical trials with this class of targeted agents.

In conclusion, our findings have significant implications for our understanding of how the BET inhibitors act, their utility and how resistance to therapy might emerge, particularly in the context of hematopoietic malignancies.

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Supplementary information is available on the Leukemia website.

References

1. Zuber J, Shi J, Wang E, Rappaport AR, Herrmann H, Sison EA, *et al.* RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* 2011 Oct 27; **478**(7370)**:** 524-528.

2. Dawson MA, Prinjha RK, Dittmann A, Giotopoulos G, Bantscheff M, Chan WI, *et al.* Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature* 2011 Oct 27; **478**(7370)**:** 529-533.

3. Mertz JA, Conery AR, Bryant BM, Sandy P, Balasubramanian S, Mele DA, *et al.* Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc Natl Acad Sci U S A* 2011 Oct 4; **108**(40)**:** 16669-16674.

4. Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, *et al.* BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* 2011 Sep 16; **146**(6)**:** 904-917.

5. Herrmann H, Blatt K, Shi J, Gleixner KV, Cerny-Reiterer S, Mullauer L, *et al.* Small-molecule inhibition of BRD4 as a new potent approach to eliminate leukemic stem- and progenitor cells in acute myeloid leukemia AML. *Oncotarget* 2012 Dec; **3**(12)**:** 1588-1599.

6. Ott CJ, Kopp N, Bird L, Paranal RM, Qi J, Bowman T, *et al.* BET bromodomain inhibition targets both c-Myc and IL7R in high-risk acute lymphoblastic leukemia. *Blood* 2012 Oct 4; **120**(14)**:** 2843-2852.

7. Roderick JE, Tesell J, Shultz LD, Brehm MA, Greiner DL, Harris MH, *et al.* c-Myc inhibition prevents leukemia initiation in mice and impairs the growth of relapsed and induction failure pediatric T-ALL cells. *Blood* 2014 Feb 13; **123**(7)**:** 1040-1050.

8. Trabucco SE, Gerstein RM, Evens AM, Bradner JE, Shultz LD, Greiner DL, *et al.* Inhibition of bromodomain proteins for the treatment of human diffuse large B-cell lymphoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2015 Jan 1; **21**(1)**:** 113-122.

9. French CA, Miyoshi I, Kubonishi I, Grier HE, Perez-Atayde AR, Fletcher JA. BRD4-NUT fusion oncogene: a novel mechanism in aggressive carcinoma. *Cancer Res* 2003 Jan 15; **63**(2)**:** 304-307.

10. Shi J, Vakoc CR. The Mechanisms behind the Therapeutic Activity of BET Bromodomain Inhibition. *Mol Cell* 2014 Jun 5; **54**(5)**:** 728-736.

11. Filippakopoulos P, Picaud S, Mangos M, Keates T, Lambert JP, Barsyte-Lovejoy D, *et al.* Histone recognition and large-scale structural analysis of the human bromodomain family. *Cell* 2012 Mar 30; **149**(1)**:** 214-231.

12. Belkina AC, Denis GV. BET domain co-regulators in obesity, inflammation and cancer. *Nat Rev Cancer* 2012 Jul; **12**(7)**:** 465-477.

13. Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, *et al.* Selective inhibition of BET bromodomains. *Nature* 2010 Dec 23; **468**(7327)**:** 1067-1073.

14. Huang B, Yang XD, Zhou MM, Ozato K, Chen LF. Brd4 coactivates transcriptional activation of NF-kappaB via specific binding to acetylated RelA. *Molecular and cellular biology* 2009 Mar; **29**(5)**:** 1375-1387.

15. Willis S, Day CL, Hinds MG, Huang DCS. The Bcl-2-regulated apoptotic pathway. *Journal of Cell Science* 2003; **116:** 4053-4056.

16. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008 Jan; **9**(1)**:** 47-59.

17. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002; **108**(2)**:** 153-164.

18. Shangary S, Johnson DE. Recent advances in the development of anticancer agents targeting cell death inhibitors in the Bcl-2 protein family. *Leukemia* 2003 Aug; **17**(8)**:** 1470-1481.

19. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* 2013 Oct; **13**(10)**:** 714-726.

20. Juin P, Geneste O, Gautier F, Depil S, Campone M. Decoding and unlocking the BCL-2 dependency of cancer cells. *Nat Rev Cancer* 2013 Jul; **13**(7)**:** 455-465.

21. Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell death and differentiation* 2002; **9**(5)**:** 505-512.

22. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nature reviews Molecular cell biology* 2014 Jan; **15**(1)**:** 49-63.

23. Lindsten T, Ross AJ, King A, Zong W, Rathmell JC, Shiels HA, *et al.* The combined functions of proapoptotic Bcl-2 family members Bak and Bax are

essential for normal development of multiple tissues. *Mol Cell* 2000; **6**(6)**:** 1389-1399.

24. Koralov SB, Muljo SA, Galler GR, Krek A, Chakraborty T, Kanellopoulou C, *et al.* Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell* 2008; **132**(5)**:** 860-874.

25. Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ, *et al.* Targeted deletion reveals essential and overlapping functions of the mIR-17~92 family of miRNA clusters. *Cell* 2008; **132**(5)**:** 875-886.

26. Mogilyansky E, Rigoutsos I. The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. *Cell death and differentiation* 2013 Dec; **20**(12)**:** 1603-1614.

27. Glaser S, Lee EF, Trounson E, Bouillet P, Wei A, Fairlie WD, *et al.* Anti-apoptotic Mcl-1 is essential for the development and sustained growth of acute myeloid leukemia. *Genes & development* 2012; **26:** 120-125.

28. Kelly GL, Grabow S, Glaser S, Fitzsimmons L, Aubrey BJ, Okamoto T, *et al.* Targeting of MCL-1 kills MYC-driven mouse and human lymphoma cells even when they bear mutations in p53. *Genes & development* 2013**:** in press.

29. Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, *et al.* Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 2008 Jan 10; **451**(7175)**:** 141-146.

30. Bouillet P, Robati M, Bath ML, Strasser A. Polycystic kidney disease prevented by transgenic RNA interference. *Cell death and differentiation* 2005 Feb 2005; **12**(7)**:** 831-833.

31. Glaser S, Metcalf D, Wu L, Hart AH, DiRago L, Mifsud S, *et al.* Enforced expression of the homeobox gene Mixl1 impairs hematopoietic differentiation and results in acute myeloid leukemia. *Proc Natl Acad Sci U S A* 2006 Oct 31; **103**(44)**:** 16460-16465.

32. Michalak EM, Jansen ES, Happo L, Cragg MS, Tai L, Smyth GK, *et al.* Puma and to a lesser extent Noxa are suppressors of Myc-induced lymphomagenesis. *Cell Death Differ* 2009 May; **16**(5)**:** 684-696.

33. Aubrey BJ, Kelly GL, Kueh AJ, Brennan MS, O'Connor L, Milla L, *et al.* An Inducible Lentiviral Guide RNA Platform Enables the Identification of Tumor-Essential Genes and Tumor-Promoting Mutations In Vivo. *Cell Rep* 2015 Mar 3; **10**(8)**:** 1422-1432.

34. Ogilvy S, Metcalf D, Print CG, Bath ML, Harris AW, Adams JM. Constitutive Bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival. *Proc Natl Acad Sci U S A* 1999 Dec 21; **96**(26)**:** 14943-14948.

35. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, *et al.* Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 1999 Nov 26; **286**(5445)**:** 1735-1738.

36. Kaufmann T, Tai L, Ekert PG, Huang DCS, Norris F, Lindemann RK, *et al.* The BH3-only protein bid is dispensable for DNA darnage- and replicative stress-induced apoptosis or cell-cycle arrest. *Cell* 2007 Apr 20; **129**(2)**:** 423-433.

37. Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT, *et al.* Tumor spectrum analysis in p53-mutant mice. *Curr Biol* 1994 Jan 1; **4**(1)**:** 1-7.

38. Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, *et al.* The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* 1985 Dec 12-18; **318**(6046)**:** 533-538.

39. Merino D, Khaw SL, Glaser SP, Anderson DJ, Belmont LD, Wong C, *et al.* Bcl-2, Bcl-x(L), and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. *Blood* 2012 Jun 14; **119**(24)**:** 5807-5816.

40. Khaw SL, Merino D, Anderson MA, Glaser SP, Bouillet P, Roberts AW, *et al.* Both leukaemic and normal peripheral B lymphoid cells are highly sensitive to the selective pharmacological inhibition of prosurvival Bcl-2 with ABT-199. *Leukemia* 2014 Jan 9.

41. Shi J, Wang E, Milazzo JP, Wang Z, Kinney JB, Vakoc CR. Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. *Nat Biotechnol* 2015 Jun; **33**(6)**:** 661-667.

42. Mason KD, Vandenberg CJ, Scott CL, Wei AH, Cory S, Huang DC, *et al.* In vivo efficacy of the Bcl-2 antagonist ABT-737 against aggressive Myc-driven lymphomas. *P Natl Acad Sci U S A* 2008 Nov 18; **105**(46)**:** 17961-17966.

43. Happo L, Cragg MS, Phipson B, Haga JM, Jansen ES, Herold MJ, *et al.* Maximal killing of lymphoma cells by DNA-damage inducing therapy requires not only the p53 targets Puma and Noxa but also Bim. *Blood* 2010 Dec 9; **116**(24)**:** 5256-5267.

44. Langdon WY, Harris AW, Cory S, Adams JM. The c-myc oncogene perturbs B lymphocyte development in E-mu-myc transgenic mice. *Cell* 1986 Oct 10; **47**(1)**:** 11-18.

45. Strasser A, Harris AW, Huang DCS, Krammer PH, Cory S. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J* 1995; **14:** 6136-6147.

46. Harris AW, Pinkert CA, Crawford M, Langdon WY, Brinster RL, Adams JM. The E mu-myc transgenic mouse. A model for high-incidence spontaneous lymphoma and leukemia of early B cells. *J Exp Med* 1988 Feb 1; **167**(2)**:** 353-371.

47. Bhadury J, Nilsson LM, Muralidharan SV, Green LC, Li Z, Gesner EM, *et al.* BET and HDAC inhibitors induce similar genes and biological effects and synergize to kill in Myc-induced murine lymphoma. *Proc Natl Acad Sci U S A* 2014 Jul 1; **111**(26)**:** E2721-2730.

48. Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer* 2002; **2**(8)**:** 594-604.

49. Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. *Cell* 1997 Nov 14; **91**(4)**:** 443-446.

50. Liu W, Ma Q, Wong K, Li W, Ohgi K, Zhang J, *et al.* Brd4 and JMJD6-associated anti-pause enhancers in regulation of transcriptional pause release. *Cell* 2013 Dec 19; **155**(7)**:** 1581-1595.

51. Loven J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR, *et al.* Selective inhibition of tumor oncogenes by disruption of super-enhancers. *Cell* 2013 Apr 11; **153**(2)**:** 320-334.

52. Bandopadhayay P, Bergthold G, Nguyen B, Schubert S, Gholamin S, Tang Y, *et al.* BET bromodomain inhibition of MYC-amplified medulloblastoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2014 Feb 15; **20**(4)**:** 912-925.

53. Patel AJ, Liao CP, Chen Z, Liu C, Wang Y, Le LQ. BET bromodomain inhibition triggers apoptosis of NF1-associated malignant peripheral nerve sheath tumors through Bim induction. *Cell Rep* 2014 Jan 16; **6**(1)**:** 81-92.

54. Barski A, Jothi R, Cuddapah S, Cui K, Roh TY, Schones DE, *et al.* Chromatin poises miRNA- and protein-coding genes for expression. *Genome research* 2009 Oct; **19**(10)**:** 1742-1751.

55. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 2005 Jun 9; **435**(7043)**:** 839-843.

56. van Haaften G, Agami R. Tumorigenicity of the miR-17-92 cluster distilled. *Genes & development* 2010 Jan 1; **24**(1)**:** 1-4.

57. Beroukhim R, Mermel C, Porter D, Wei G, Raychaudhuri S, Donovan J, *et al.* The landscape of somatic copy-number alteration across human cancers. *Nature* 2010; **463**(7283)**:** 899-905.

58. Mestre-Escorihuela C, Rubio-Moscardo F, Richter JA, Siebert R, Climent J, Fresquet V, *et al.* Homozygous deletions localize novel tumor suppressor genes in B-cell lymphomas. *Blood* 2007 Jan 1; **109**(1)**:** 271-280.

59. Bolden JE, Tasdemir N, Dow LE, van Es JH, Wilkinson JE, Zhao Z, *et al.* Inducible in vivo silencing of Brd4 identifies potential toxicities of sustained BET protein inhibition. *Cell Rep* 2014 Sep 25; **8**(6)**:** 1919-1929.

Figure legends

Figure 1 JQ1 induces cell death in a broad range of hematopoietic cancer cells.

(a) Induction of cell death by JQ1. The indicated human hematopoietic cancer cell lines were treated with JQ1 (0.01-10 μM) and the CellTiter-Glo (CTG) was used to monitor their viability. To specifically detect for cell death, the % of live cells which exclude the vital dye propidium iodide (PI) was determined by flow cytometry. The EC_{50} values represent means ± 1 S.E.M determined from at least 3 independent experiments, performed in triplicate, with viability (CellTiter-Glo assay) or survival (PI uptake) of JQ1 treated cells normalized to that of control (vehicle; DMSO) treated cells.

(b) Killing of Eμ-*Myc* lymphoma derived cell lines by JQ1. Eμ-*Myc* lymphoma derived cell lines were treated with JQ1 (0.01-5 μM) and the data from 3 independent experiments performed in triplicate done with the 5 independently derived lines were pooled together and processed as described in (a).

(c) Impact of JQI treatment on mice bearing Eμ-*Myc* lymphomas. C57BL/6-albino mice were injected i.v. with 2 x 10^6 luciferase-expressing Eμ-*Myc* lymphoma cells. Treatment was commenced 3 days after lymphoma transplantation with 50 mg/kg body weight JQ1 delivered i.p. daily for 20 days. Shown are representative bioluminescent images of 2 vehicle-treated (left) or JQ1-treated (right) mice 3 (top) or 6 days (bottom) after lymphoma injection.

(d) JQ1 treatment prolongs the survival of mice harboring Eμ-*Myc* lymphomas. As described in Figure 1c, mice bearing Eμ-*Myc* lymphomas were treated daily with either vehicle (control) or JQ1 for 20 days. The Kaplan-Meier survival curves show significantly prolonged survival of the JQ1-treated mice ($p < 0.0001$). n=12 in each

group with data pooled from experiments with three independently derived Eμ-*Myc* lymphoma.

(e) JQ1 treatment reduces the splenomegaly (left) and hepatomegaly (right) in mice bearing Eμ-*Myc* lymphomas. Quantification of spleen or liver weights from Eμ-*Myc* lymphoma bearing mice treated with vehicle or JQ1. n=6 in each group; paired groups were compared using Students' t-test and corrected for multiple comparisons using Holm-Sidak method (α=0.05).

Figure 2 The cytotoxic action of the BET inhibitors does not require the tumor suppressor p53 but is prevented by pro-survival BCL2 family proteins

(a) The killing of Eμ-*Myc* lymphoma by JQ1 is p53 independent. The viability of independently derived Eμ-*Myc* lymphoma cell lines that were $p53^{+/+}$ (WT; n=3), constitutively $p53^{-/-}$ (n=3) or engineered to be p53-deficient by CRISPR/Cas9 genome editing (n=3) was determined by flow cytometry (PI^{-ve}) 48 h after treatment with JQ1. For CRISPR/Cas9 genome editing, *Eμ-Myc* lymphoma cells were transduced with Cas9 and either a non-targeting (nt) guide RNA or one of two different guide RNAs targeting *p53*. The data are from 3 clonal cell lines transduced with *p53* or nt sgRNAs representing means ± 1 S.E.M determined from at least 3 independent experiments, performed in triplicate.

(b) The viability of *MLL-ENL* transduced AML cells lines that were $p53^{+/+}$ (WT; n=2),or constitutively $p53^{-/-}$ (n=2) was determined as described in Figure 1a.

(c) JQ1 induces p53-independent cell cycle arrest. Panels show the cell cycle distribution of WT (top) or constitutively p53-deficient Eμ-*Myc* lymphoma cell lines (bottom) after exposure to JQ1 (200 nM) in the presence of 25 μM Q-VD-OPh to block apoptosis. 5Gy γ-irradiation

inducing G_0/G_1 and G_2/M arrest in WT cells but only G_2/M arrest in *p53*$^{-/-}$ cells was used as control (right panels).

(d,e) The pro-survival BCL2 proteins protect Eμ-*Myc* lymphoma (d) or *MLL-ENL* AML cells (e) from killing by JQ1. Cells were transduced to express GFP alone or GFP plus BCL2, BCLXL, MCL1 or BCL2LA1 and cultured in the presence of JQ1 for 48 h to determine cell viability (PI^{-ve}) by flow cytometry. The data represent means of cell viability ± 1 S.E.M from three independent experiments.

Figure 3 B lymphoid cells can be readily killed by JQ1 through induction of apoptosis.

(a) Non-transformed mouse B lymphoid cells are highly sensitive to JQ1. The proportions of viable (PI^{-ve}) B lymphoid cells of the indicated subsets isolated from the bone marrow and cultured in the presence of IL-7 and OP9 feeder cells was determined by flow cytometry 48 h after treatment with JQ1.

(b) Similar analysis of *in vitro* survival of B lymphoid cell subsets purified by FACS sort from the spleen.

(c) BCL2 overexpression, but not loss of p53, protects bone marrow-derived B lymphoid cells from killing by JQ1. B lymphoid cell subsets were isolated from *p53*$^{-/-}$ (green) or vavP-*BCL2* transgenic mice (red) and cultured in the presence of JQ1 for 48 h and cell viability (PI^{-ve}) was determined by flow cytometry.

(d) Heat map summarizing the sensitivity to JQ1 of B cell subsets purified from the indicated organs (bone marrow, spleen, lymph nodes or peripheral blood) of WT, *p53*$^{-/-}$ or vavP-*BCL2* mice.

The data shown in (a-d) represent means ± 1 S.E.M determined from at least 3 mice of each genotype, each experiment performed in triplicate, and viability of JQ1 treated cells was normalized to vehicle (DMSO) treated cells.

Figure 4 JQ1 induces BAX/BAK mediated apoptosis in diverse hematopoietic cell subsets and cancer cell lines.

(a,b) Loss of BAX/BAK prevents killing of B lymphoid (a) and T lymphoid (b) cell subsets isolated from mice that had been reconstituted by fetal liver transplantation (FLT) with wild-type (green) or BAX/BAK deficient (red) donor cells and treated in culture with JQ1 to determine cell viability (PI^{-ve}) after 48 h by flow cytometry. The data shown represent means ± 1 S.E.M determined from at least 3 reconstituted mice, each experiment performed in triplicate and normalized to vehicle (DMSO) treated cells.

(c) Table summarizing the sensitivities to JQ1 of various hematopoietic cell subsets isolated from fetal liver transplanted (FLT) mice and treated in culture as described in Figure 4a,b. .

(d,e,f) Loss of BAX/BAK prevents the killing of *MLL-ENL* AML (d), MM.1S (e) or KMS-12PE cells (f) by JQ1 in culture as described in (a) and (b).

Figure 5 *In vivo* lymphopenia by JQ1 treatment is driven exclusively by BAX/BAK mediated apoptosis.

(a) Blood analysis of mice before and after 10 days of treatment with vehicle or JQ1. WT mice were treated with daily intra-peritoneal (IP) injections of vehicle (green; n=6) or 50 mg/kg body weight JQ1 (red; n=6). JQ1 treatment causes a decrease in the numbers of circulating white blood cells (WBC, $p < 0.01$).

(b) JQ1 treatment causes loss of B cell subsets. The B lymphocyte containing organs (bone marrow, spleen and peripheral blood) were isolated from WT mice that had been treated with JQ1 (as described in a) and B lymphoid subsets were enumerated by flow cytometry. JQ1 treatment caused a significant drop in most of the B cell subsets examined. The data shown represent means ± 1 S.E.M derived from

vehicle-treated (n=6) or JQ1-treated (n=6) mice; the cellularity of the JQ1 treated mice was normalized to the cellularity of the vehicle-treated mice. **p<0.01, *p<0.05.

(c) The combined absence of BAX and BAK prevents the loss of the B cell subsets caused by *in vivo* treatment with JQ1. B lymphoid subsets from mice reconstituted with a *Bax*$^{-/-}$/*Bak*$^{-/-}$ hematopoietic system and treated with vehicle (n=5) or JQ1 (n=5) were determined by flow cytometry. The data shown represent means ± 1 S.E.M.

Figure 6 JQ1 triggers apoptosis by inducing pro-apoptotic BIM.

(a) JQ1 increases BIM protein levels. Immunoblot analysis to detect BCL2, BCLXL, MCL1, BIM, BAD, BID and PUMA in Eμ-*Myc* lymphoma (left) or MM.1S cells (right) treated with 1 μM JQ1 for 2-72 h in the presence of 25 μM Q-VD-OPh to block apoptosis. Probing for HSP70 was used as loading control.

(b) JQ1 treatment increases *Bim* mRNA expression. *Bim* mRNA in Eμ-*Myc* lymphoma (left) or MM.1S cells (right) was measured by RT-PCR at the indicated time points following treatment with 1 μM JQ1 treatment in the presence of 25 μM Q-VD-OPh to block apoptosis.

(c) Loss of *Brd4* by CRISPR/Cas9 genome editing increases *Bim* mRNA expression. Eμ-*Myc* lymphoma cell were transduced with Cas9 and either one of two doxicyclin-inducible sgRNAs targeting *Brd4*. *Bim* mRNA expression was measured by RT-PCR after inducing sgRNA expression with doxycycline and normalized to vehicle-treated cells, in the presence of 25 μM Q-VD-OPh to block apoptosis.

(d) Loss or knock-down of *Bim* reduces killing of Eμ-*Myc* lymphoma cell by JQ1. BIM was either disrupted by transduction with Cas9 and *Bim* sgRNA vectors (solid red line) or knocked down by transduction with a *Bim* shRNA vector (dotted red line). As controls, WT Eμ-*Myc* lymphoma cells were either transduced with Cas9 and non-targeting

(nt) sgRNA constructs (solid green line) or an empty knock-down construct (dotted green line. Clonal cell lines were treated with increasing concentrations of JQ1 and viability was determined by flow-cytometry.

(e) Loss of BIM by CRISPR/Cas9 significantly delays apoptosis in MM.1S cells treated with JQ1.

(f) Constitutive absence of BIM, but not BID, partially protects normal B lymphoid cells from JQ1-induced killing. Dose response curves showing the JQ1 sensitivity of bone marrow derived B lymphoid cells isolated from WT (green), *Bid*$^{-/-}$ (blue) or *Bim*$^{-/-}$ (red) or mice. The data in (d,e,f) represent means ± 1 S.E.M determined from 3 independent experiments, performed in triplicate.

Figure 7 JQ1 increases BIM by inhibiting the miR17-92 cluster.

(a) JQ1 suppresses expression of the miR17-92 cluster. Real-time (RT) qPCR analysis of miR-17-5p and miR-92a-3p expression in Eμ-*Myc* lymphoma and MM.1S cells at the indicated time points following treatment with 1 μM JQ1 treatment in the presence of 25μM Q-VD-OPh to block apoptosis.

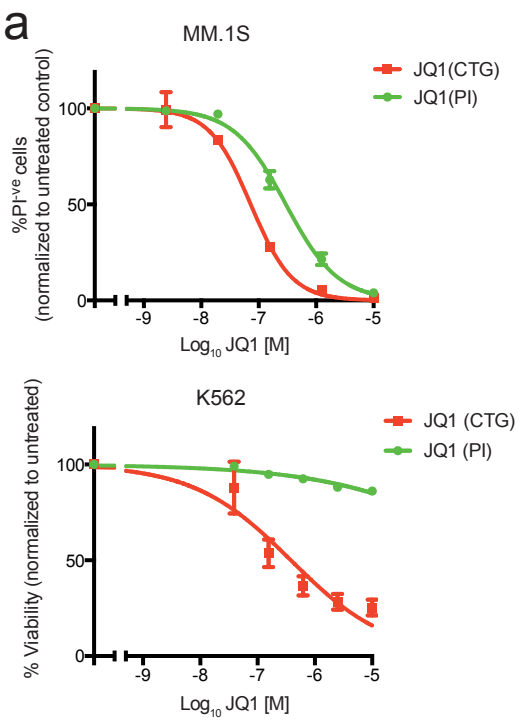
(b) Loss of BRD4 suppresses expression of the miR17-92 cluster. Real-time (RT) qPCR analysis of miR-17-5p and miR-92a-3p expression in Eμ-*Myc* lymphoma cells before and after genetic ablation of *Brd4* by CRISPR/Cas9.

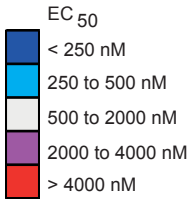
Figure 8 Model for the killing of Eμ-*Myc* pre-B-/B lymphoma cells by JQ1

Our results suggest that the downregulation of c-*MYC* (**I**) plays little or nor role in the cytotoxic effect of JQ1 on the Eμ-*Myc* pre-B-/B lymphoma cells. Instead, we found that the BH3-only protein BIM plays a central role, as it does in NF1-associated malignant peripheral nerve sheath tumors[53]. BIM may

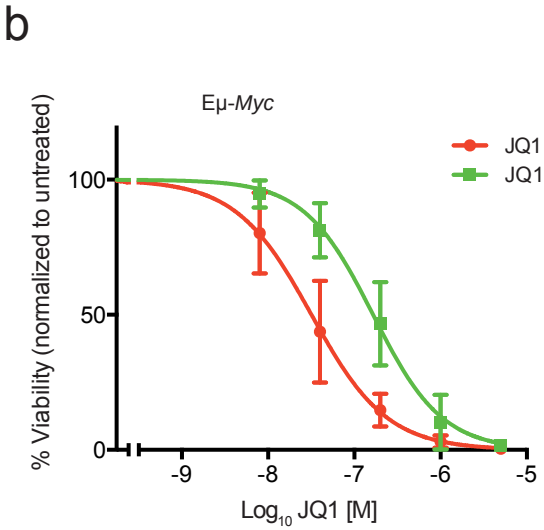
842 be upregulated transcriptionally (**II**) or post-transcriptionally, possibly by
843 inhibiting its degradation by the proteasome (**III**) We also provide compelling
844 evidence for repression of *miR-17-92* (**IV**) which is known to be an important
845 negative regulator of BIM. These processes (**II**-**IV**) all prime the cell to
846 undergo apoptosis by markedly elevating the levels of BIM.

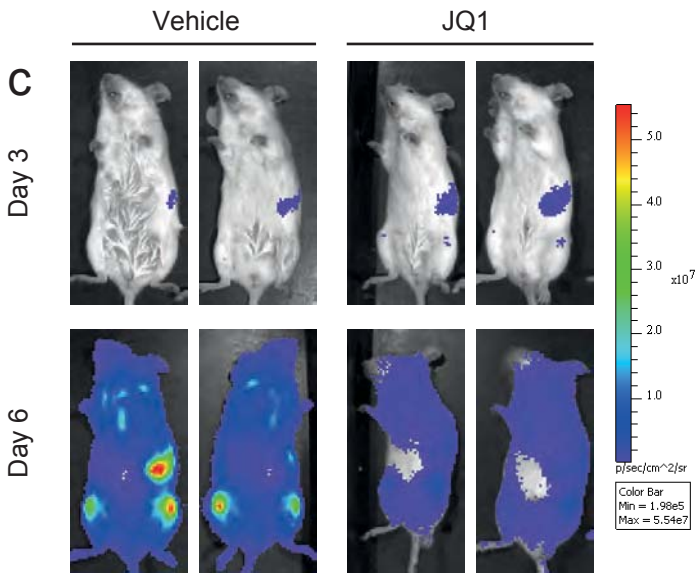
Figure 1 Xu *et al.*

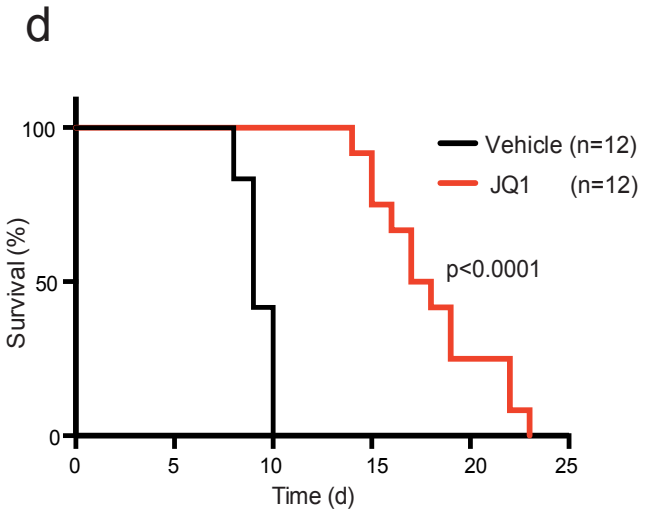




Cell line	Tumor type	Molecular lesion	CTG, IC_{50} (nM)	PI, IC_{50} (nM)
MM.1S	MM	myc amplification	73	300
KMS-12PE	MM	myc amplification	74	610
OPM-2	MM	myc amplification	64	100
RPMI-8226	MM	myc amplification	180	580
MV4;11	AML	MLL-AF4 fusion	50	270
HL60	PML	t(15;17) translocation	37	160
Raji	Burkitt's lymphoma	myc amplification	320	370
Daudi	Burkitt's lymphoma	myc amplification	57	170
K562	CML	BCR-ABL fusion	380	>4000
MOLT-4	T-ALL	Notch mutation	290	>4000







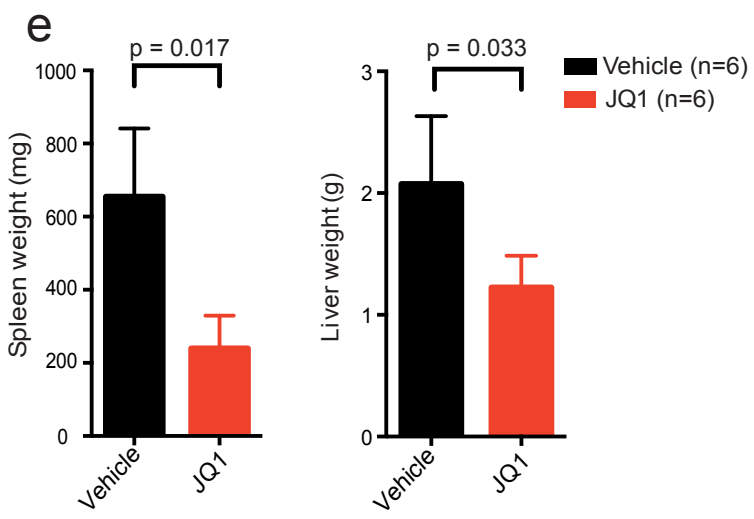


Figure 2 Xu *et al.*

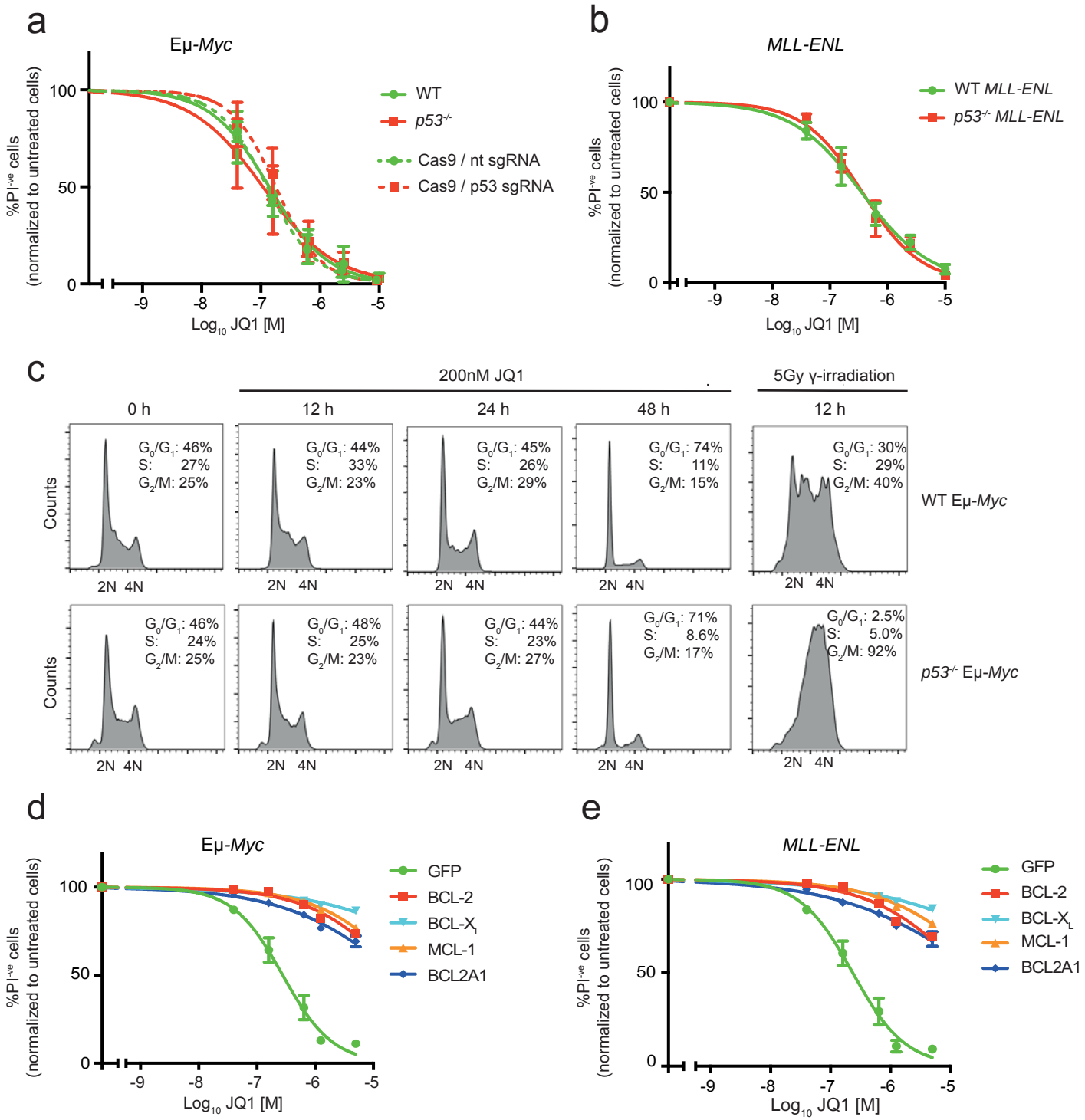


Figure 3 Xu *et al.*

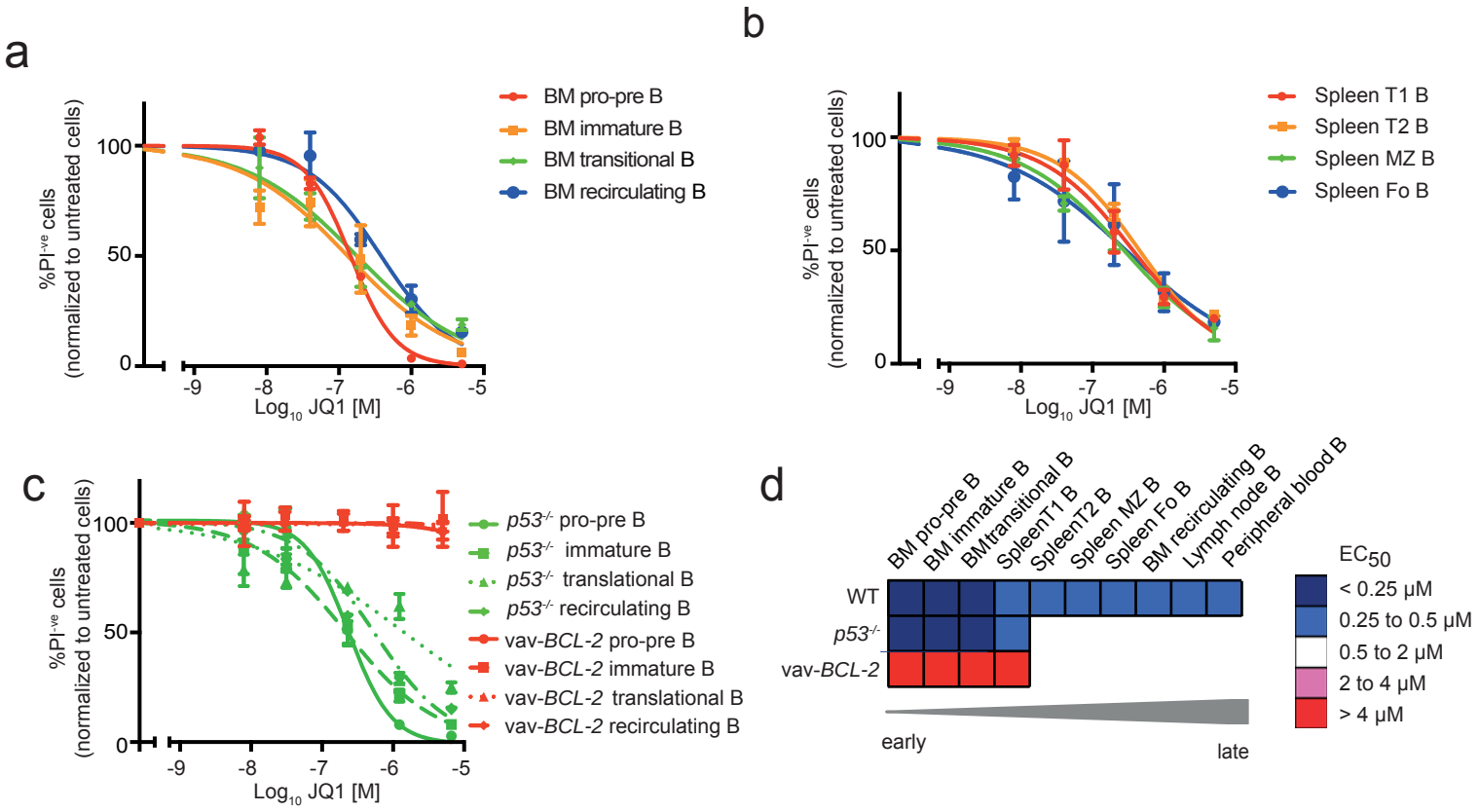
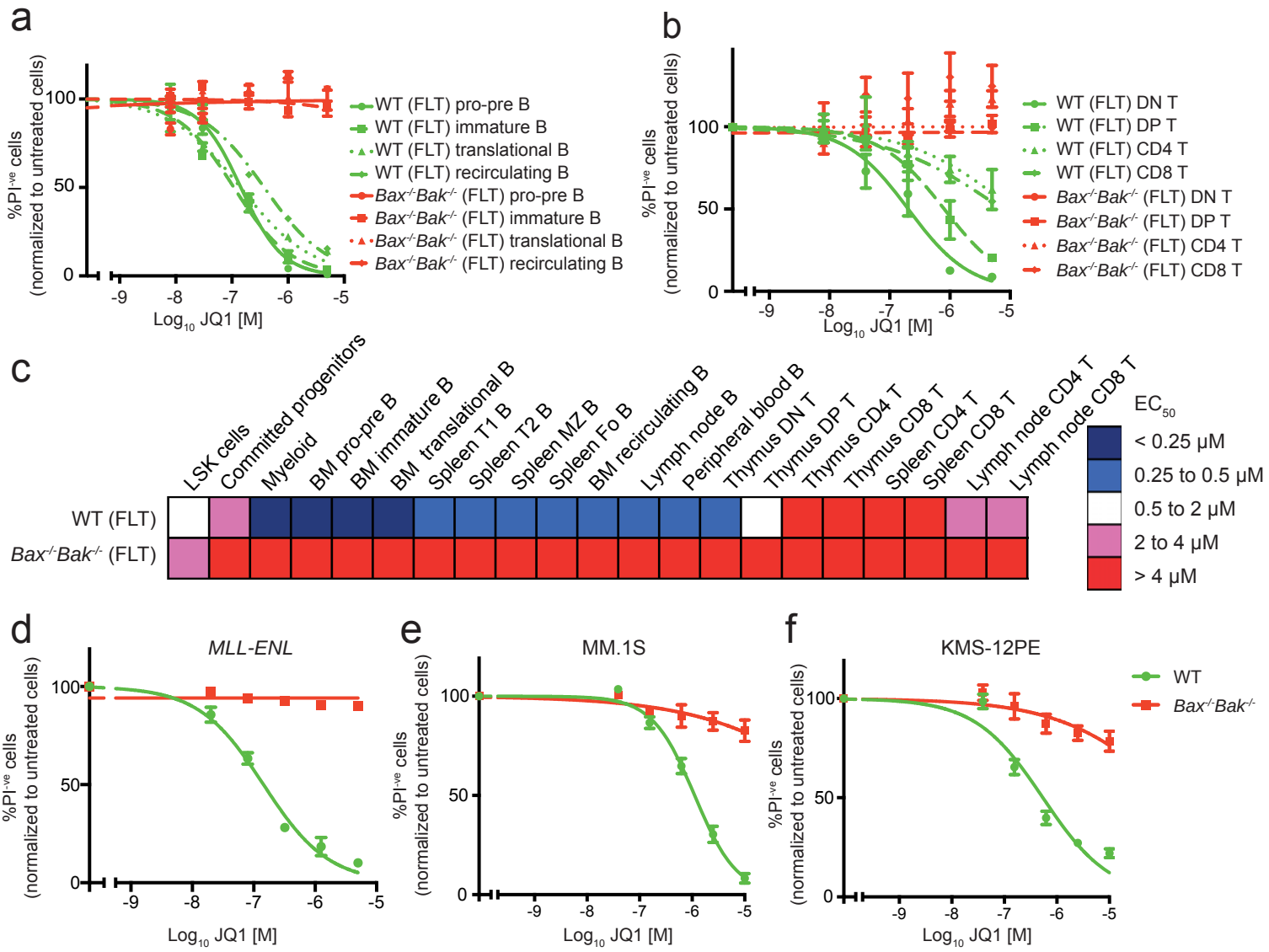


Figure 4 Xu *et al.*



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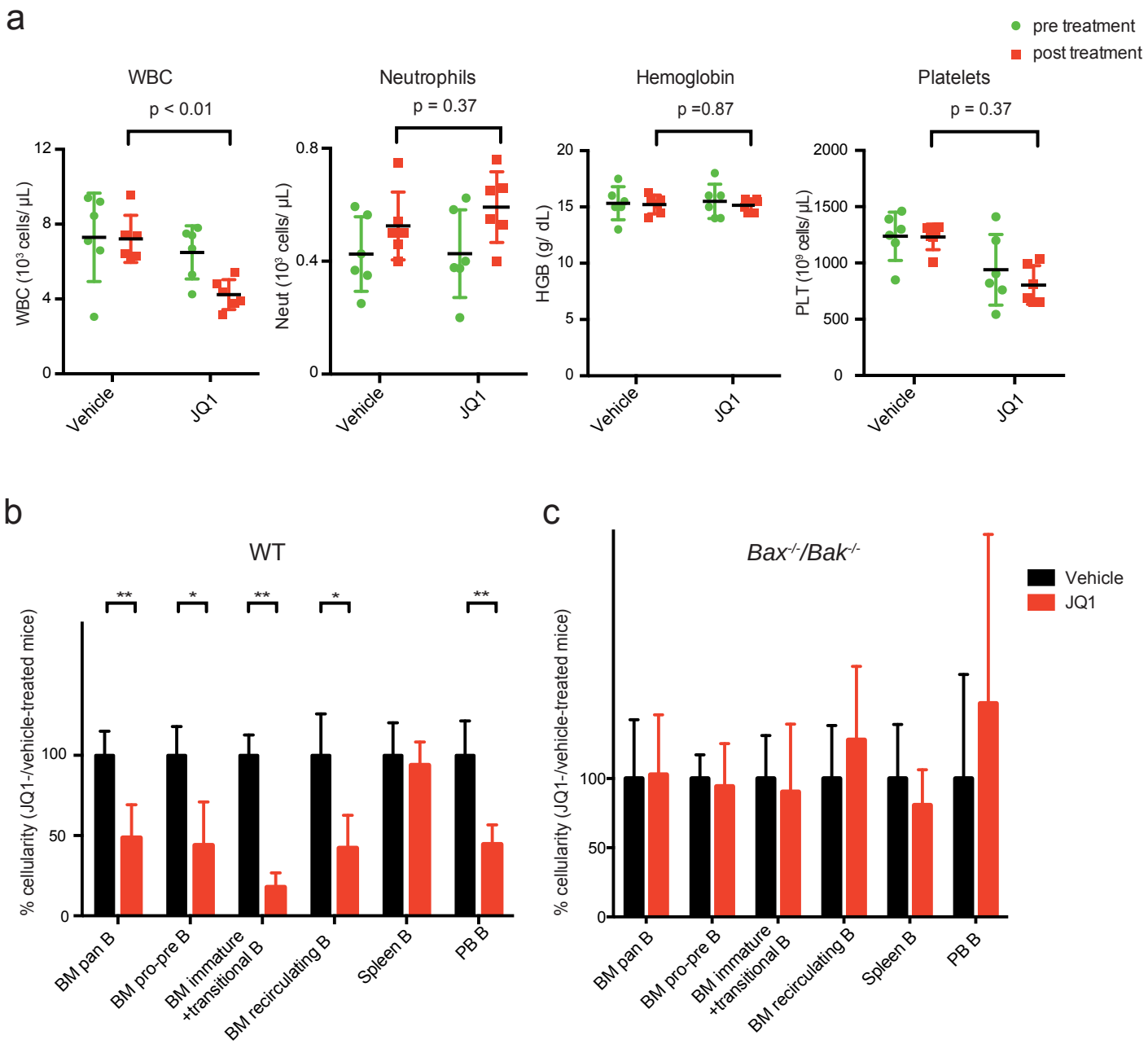

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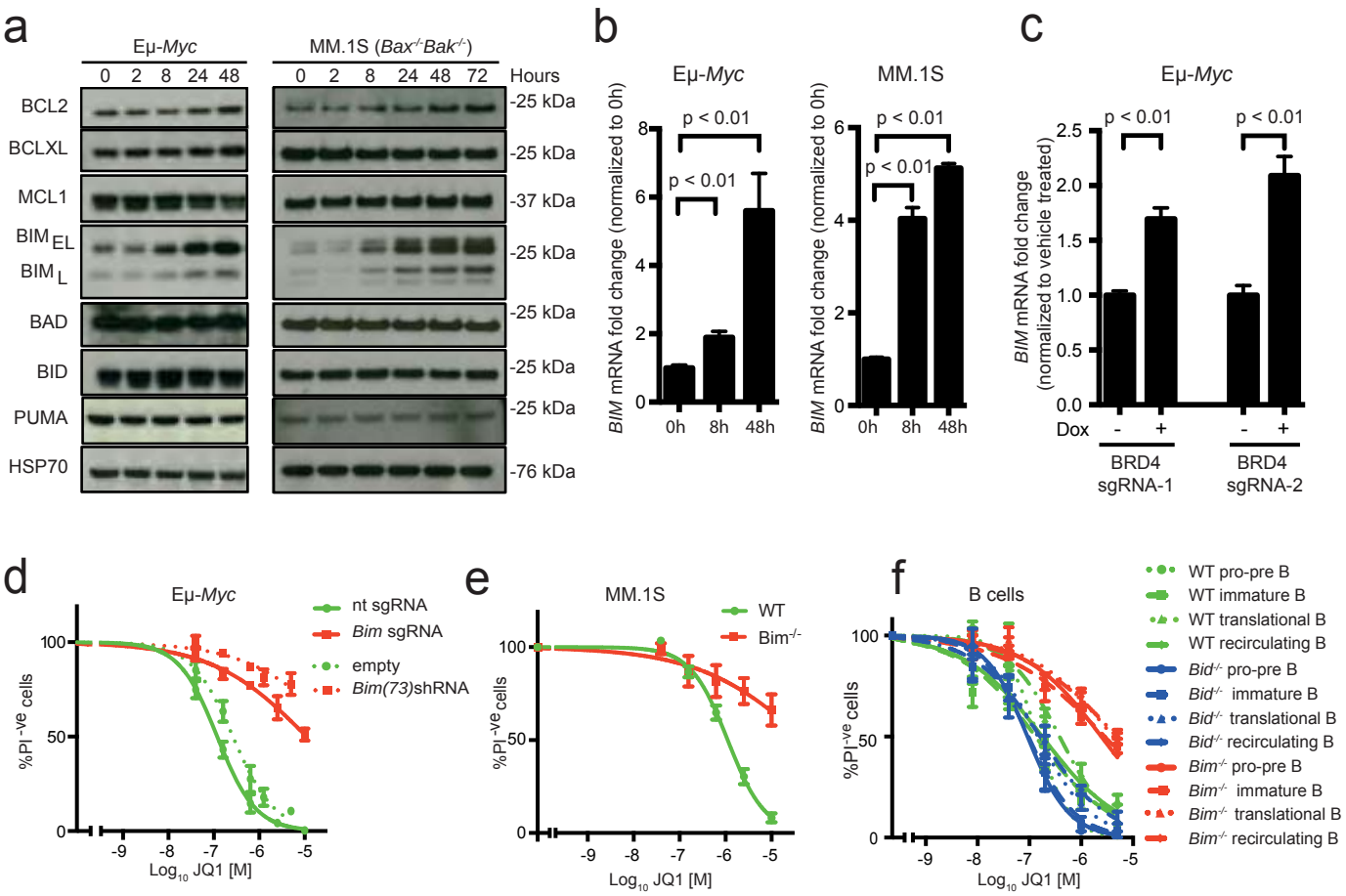

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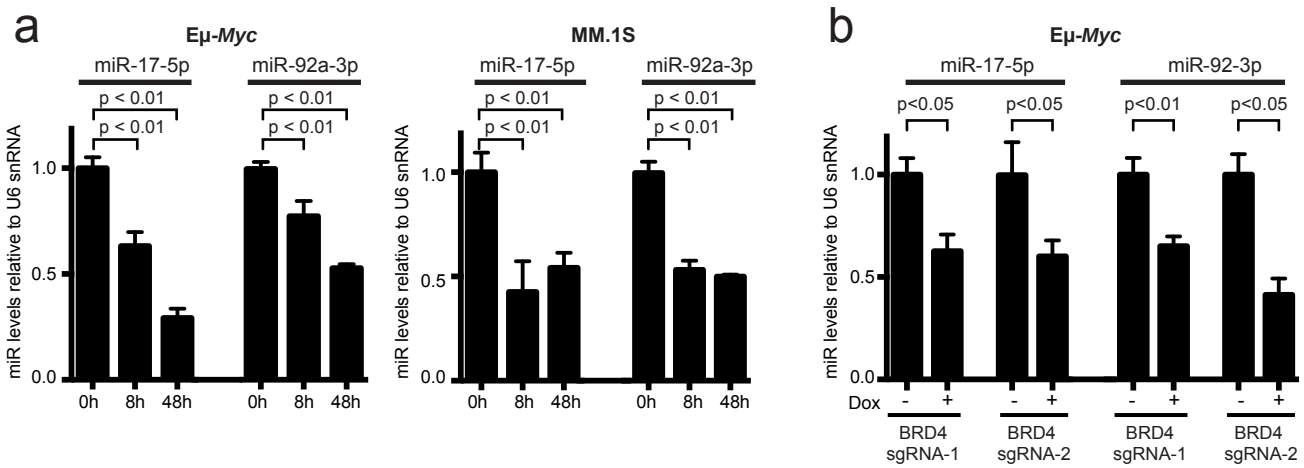
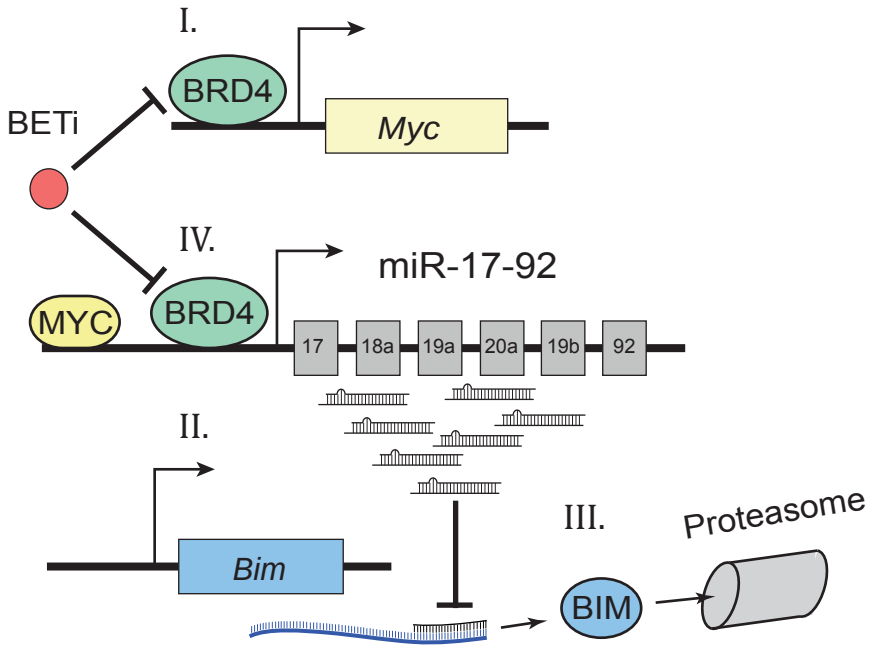
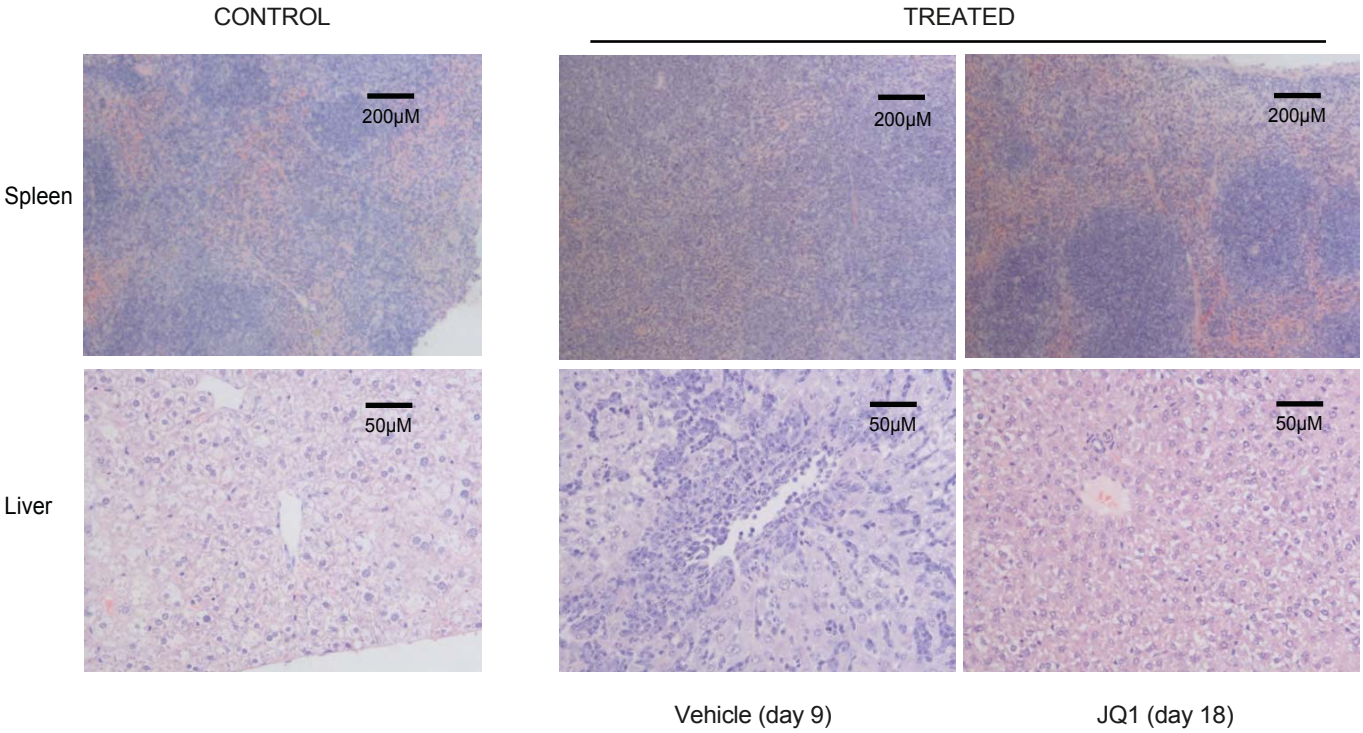


Figure 8 Xu *et al.*

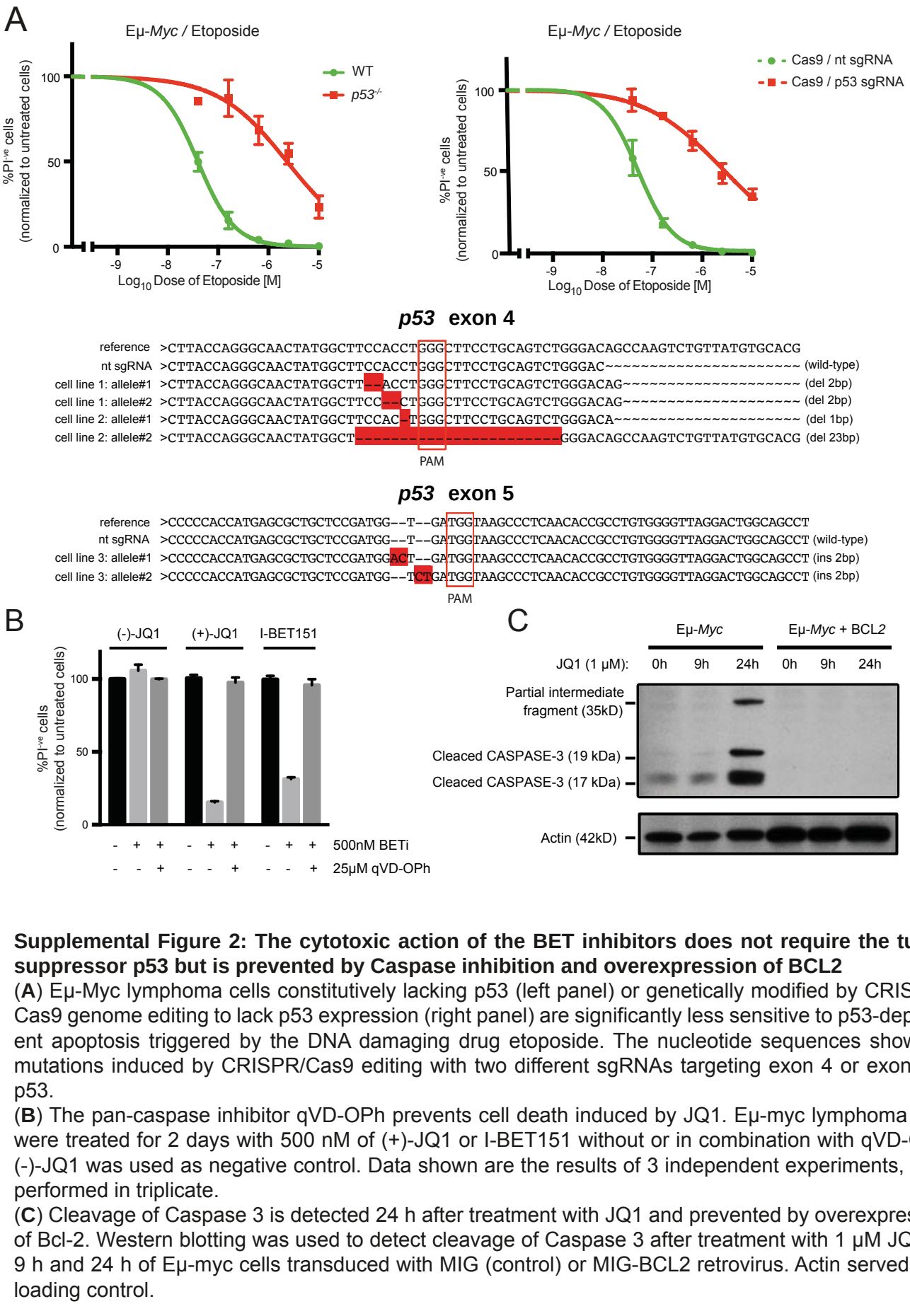


Supplementary Figure 1 Xu et al.



Supplementary Figure 1. JQ1 treatment attenuates Eμ-myc lymphoma progression.
Representative histological sections (H&E stained) show spleen (top) and liver (bottom) from untreated normal mice (left), vehicle treated (day 9, middle) or JQ1 treated (day 18, right) mice bearing Eμ-myc leukemias. JQ1 treated mice maintained normal spleen architecture and had nearly no leukemic infiltration around vessel in the liver compared to vehicle control treated mice.

Supplementary Figure 2 Xu et al.



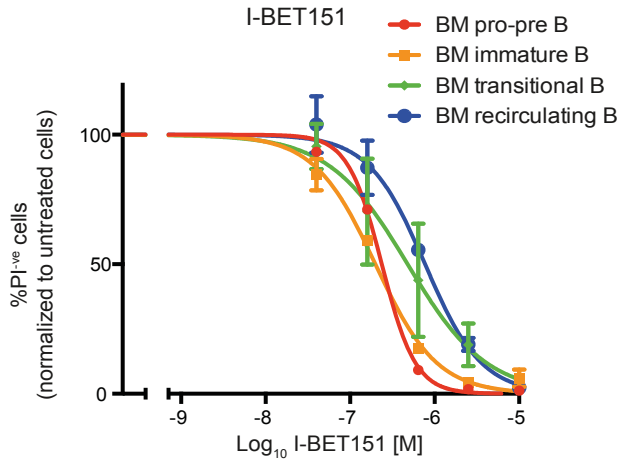
Supplemental Figure 2: The cytotoxic action of the BET inhibitors does not require the tumor suppressor p53 but is prevented by Caspase inhibition and overexpression of BCL2

(**A**) Eμ-Myc lymphoma cells constitutively lacking p53 (left panel) or genetically modified by CRISPR/-Cas9 genome editing to lack p53 expression (right panel) are significantly less sensitive to p53-dependent apoptosis triggered by the DNA damaging drug etoposide. The nucleotide sequences show the mutations induced by CRISPR/Cas9 editing with two different sgRNAs targeting exon 4 or exon 5 of p53.

(**B**) The pan-caspase inhibitor qVD-OPh prevents cell death induced by JQ1. Eμ-myc lymphoma cells were treated for 2 days with 500 nM of (+)-JQ1 or I-BET151 without or in combination with qVD-OPh. (-)-JQ1 was used as negative control. Data shown are the results of 3 independent experiments, each performed in triplicate.

(**C**) Cleavage of Caspase 3 is detected 24 h after treatment with JQ1 and prevented by overexpression of Bcl-2. Western blotting was used to detect cleavage of Caspase 3 after treatment with 1 μM JQ1 for 9 h and 24 h of Eμ-myc cells transduced with MIG (control) or MIG-BCL2 retrovirus. Actin served as a loading control.

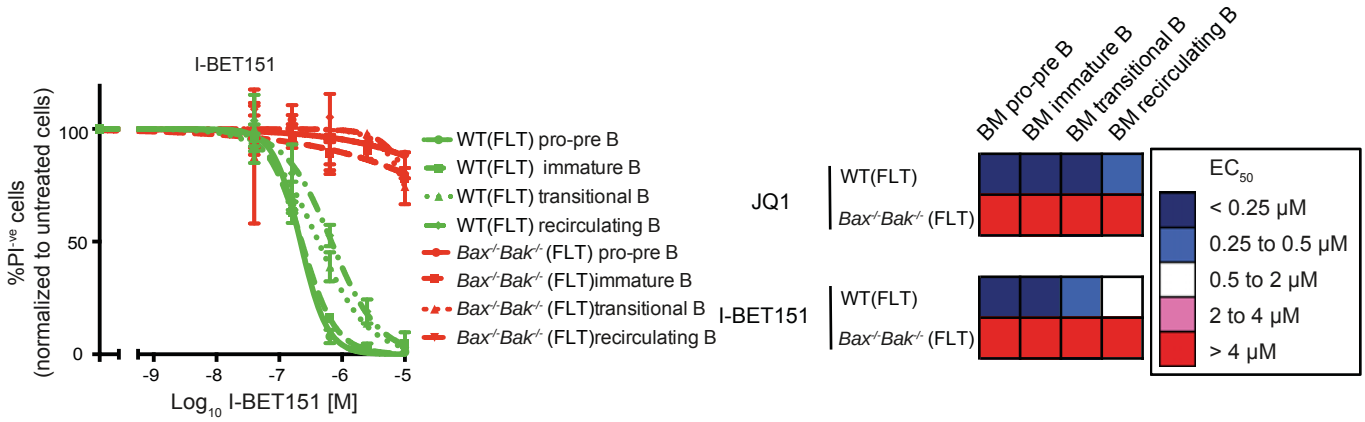
Supplementary Figure 3 Xu et al.



	JQ1 EC_{50} (nM)	I-BET151 EC_{50} (nM)
BM pro-pre B	140	240
BM immature B	130	200
BM transitional B	180	480
BM recirculating B	380	770

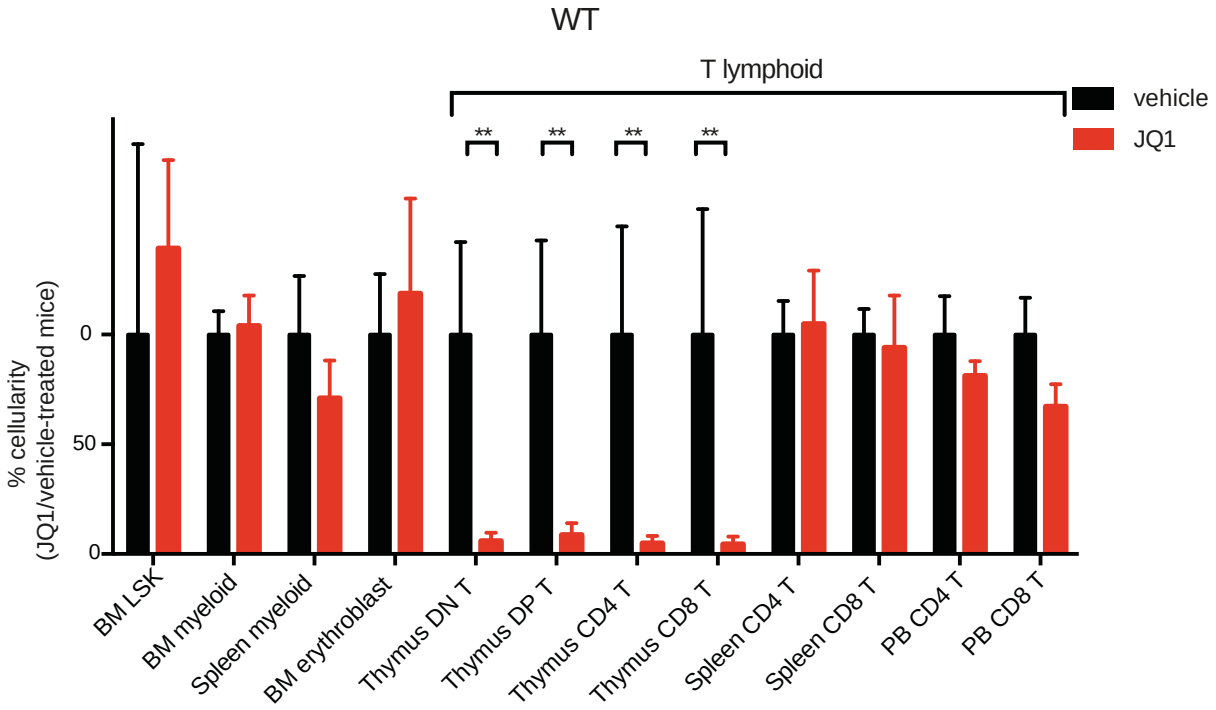
Supplementary Figure 3: B lymphoid cells can be readily killed by iBET-151 through induction of apoptosis.
WT B lymphoid cells are highly sensitive to iBET-151. The proportions of viable (PI-ve) B lymphoid cells of the indicated subsets isolated from the bone marrow and cultured in the presence of IL-7 and OP9 feeder cells was determined by flow cytometry 48 h after treatment with another BET bromodomain inhibitor, I-BET151, at indicated doses (0.05-10μM). Table on the right summarizes the EC50 for JQ1 and I-BET-151 for the tested B cell populations. The data shown in represent means ± 1 S.E.M determined from at least 3 mice, each experiment performed in triplicate, and viability of iBET-151 treated cells was normalized to vehicle (DMSO) treated cells.

Supplementary Figure 4 Xu et al.



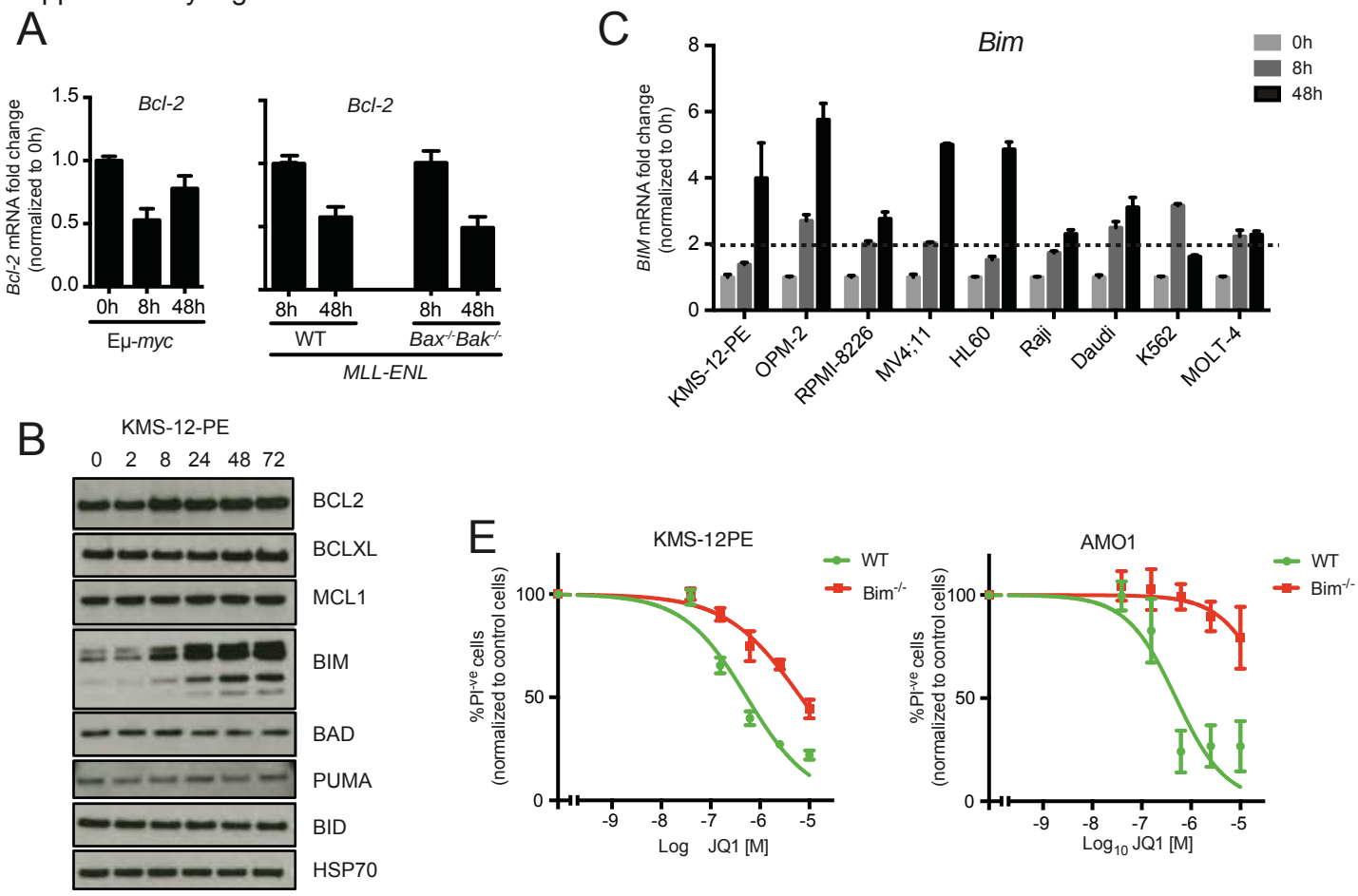
Supplementary Figure 4: Loss of BAX/BAK prevents killing of B lymphoid by I-BET-151. Lymphoid B cell subsets were isolated from mice that had been reconstituted by fetal liver transplantation (FLT) with wild-type (green) or BAX/BAK deficient (red) donor cells and treated in culture with I-BET-151 to determine cell viability (PI-ve) after 48 h. The data shown represent means ± 1 S.E.M determined from at least 3 reconstituted mice, each experiment performed in triplicate and normalized to vehicle (DMSO) treated cells.

Supplementary Figure 5 Xu et al.



Supplementary Figure 5: JQ1 treatment *in vivo* causes loss of T cell subsets. Analysis of blood and haematopoietic organs (bone marrow, thymus and spleen) after JQ1 treatment *in vivo* detected a significant drop in thymic T cell populations (DN, DP, CD4+ and CD8+). The data shown represent means ± 1 S.E.M derived from vehicle-treated (n=6) or JQ1-treated (n=6) mice after 10 days.

Supplementary Figure 6 Xu et al.



reference			counts	percentage
1	TAGAAACCTCCCAAATGTCTACAACGCAGG--CCCAGGCCCAACCCCAGCCAGCAAATGCAGCCAGCAC			
2	TAGAAACCTCCCAAATGGC----------GCCCAACCCCAGCCAGCAAATGCAGCCAGCAC	-16	3286	29.5
3	TAGAAACCTCCCAAATGTCTACAACGCAG---CCCAGGCCCAACCCCAGCCAGCAAATGCAGCCAGCAC	-1	2525	22.6
4	TAGAAACCTCCCAAATGTCTACAACGCA--------GGCCCAACCCCAGCCAGCAAATGCAGCCAGCAC	-6	2425	21.7
5	TAGAAACCTCCCAAATGTCTACAACGCAG-GCCCCAGGCCCAACCCCAGCCAGCAAATGCAGCCAGCAC	1	1341	12.0
6	TAGAAACCTCCCAAATGTCTACAACGCAGG--CCCAGGCCCAACCCCAGCCAGCAAATGCAGCCAGCAC	WT	1075	9.6
7	TAGAAACCTCCCAAATGTC----------TACAACGCAGGC--TCAATGCAGCCAGCAC	-20, 5 mutated	95	0.9
8	TAGAAACCTCCCAAATGGC----------CCAACCCCAGCCAGCAAATGCAGCCAGCAC	-18	80	0.7
9	TAGAAACCTCCCAAATGTCTACAACGCAGGGCCCCAGGCCCAACCCCAGCCAGCAAATGCAGCCAGCAC	+2	64	0.6
10	TAGAAACCTCCCAAATGTCTACAACGCA----CCCAGGCCCAACCCCAGCCAGCAAATGCAGCCAGCAC	-2	61	0.5
11	TAGAAACCTCCCAAATGTCTACAACGCAG----CCAGGCCCAACCCCAGCCAGCAAATGCAGCCAGCAC	-2	60	0.5
	********** * * *** * **********	total counts	11152	

reference			counts	percentage
1	TAAAACACCCATGGATATGGGAACAATAAAGAAGC-GCTTGGAAAACAACTATTACTGGAATGCTCAGGAAT	+1	13078	75.6
2	TAAAACACCCATGGATATGGGAA----------CAACTATTACTGGAATGCTCAGGAAT	-22	852	4.9
3	TAAAACACCCATGGATATGGGAACAATAAAGA----------AAACAACTATTACTGGAATGCTCAGGAAT	-10	729	4.2
4	TAAAACACCCATGGATATGGGAACAATAAAGAA---GCTTGGAAAACAACTATTACTGGAATGCTCAGGAAT	-2	573	3.3
5	TAAAACACCCATGG----------AATGCTCAGGAAT	-44	308	1.8
6	TAAAACACCCATGGATATGGGAACAATAAAGAAGC-GCTTGGAAAACAACTATTACTGGAATGCTCAGGAAT	WT	264	1.5
7	TAAAACACCCATGGATATGGGAACAATA----------AACAACTATTACTGGAATGCTCAGGAAT	-15	239	1.4
8	TAAAACACCCATGGATATGGGAACAATAG----------AACAACTATTACTGGAATGCTCAGGAAT	-14,1 mutated	209	1.2
9	TAAAACACCCATGGATATGGGAA----------AACAACTATTACTGGAATGCTCAGGAAT	-20	132	0.8
10	TAAAACACCCATGGATATGGGAACAATAAAGAAGCGGCTTGGAAAACAACTATTACTGGAATGCTCAGGAAT	+1	131	0.8
	********** **********	total counts	17307	

Supplementary Figure 6: JQ1 triggers apoptosis by inducing pro-apoptotic BIM

(A) JQ1 treatment decreased *Bcl2* mRNA expression. *Bcl-2* mRNA in Eμ-Myc lymphoma (left) or *MLL-ENL* AML cells (right) was measured by RT-PCR at the indicated time points following treatment with 1 μM JQ1 treatment in the presence of 25 μM Q-VD-OPh to block apoptosis.

(B) JQ1 increases BIM protein levels in KMS-12-PE cells. Immunoblot analysis to detect BCL2, BCLXL, MCL-1, BIM, BAD, BID and PUMA in KMS-12-PE cells treated with 1 μM JQ1 / 25 μM Q-VD-OPh.

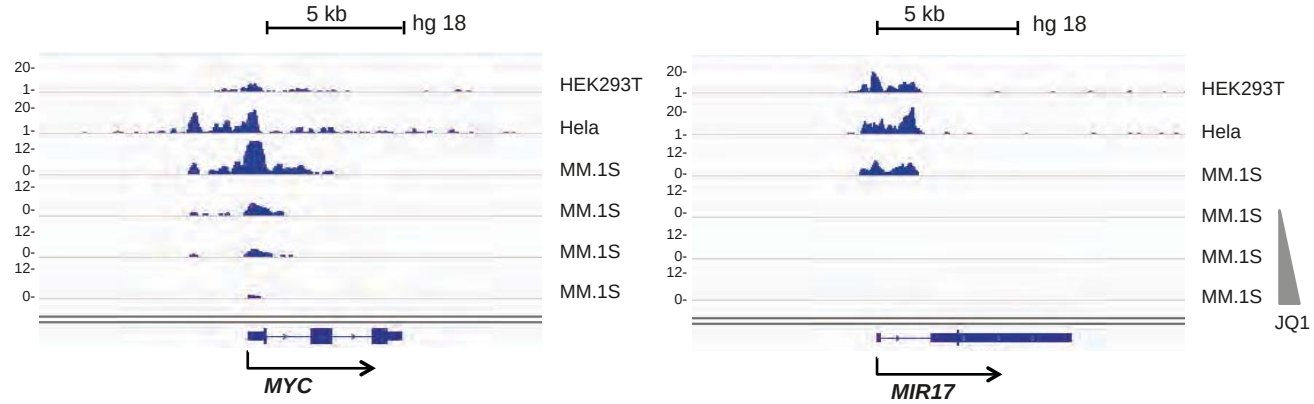
(C) JQ1 treatment increases *BIM* mRNA expression. *BIM* mRNA was measured by RT-PCR in a panel of human cell lines at the indicated time points following treatment with 1 μM JQ1 / 25 μM Q-VD-OPh.

(D) Next-generation sequencing confirmed high frequency of mutated Brd4 alleles in a non-clonal Cas9 Eμ-*Myc* lymphoma cell line after doxycycline-induced expression of BRD4 sgRNAs targeting Brd4. .

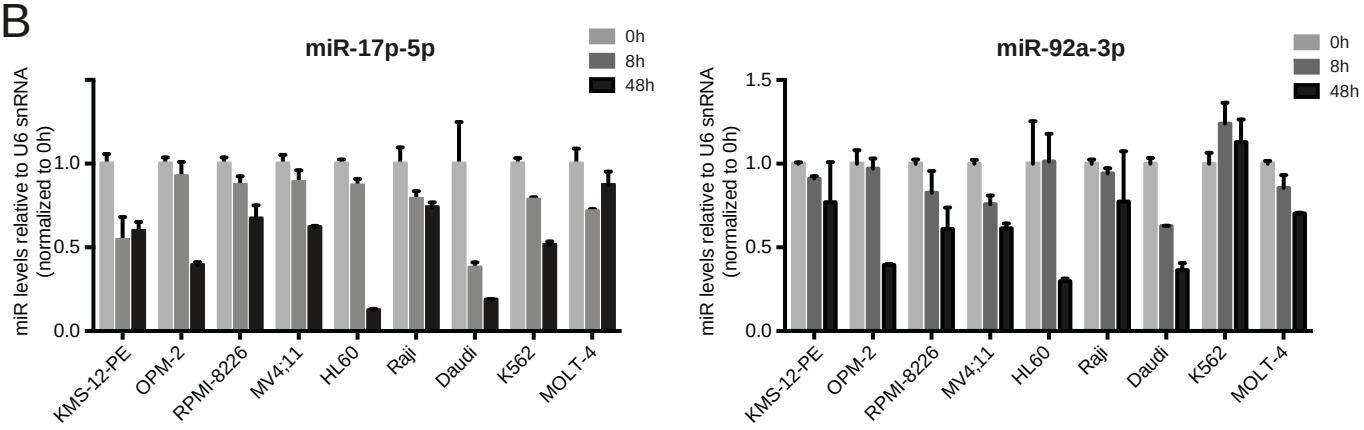
(E) Loss of BIM reduces killing of KMS-12-PE and AMO1 cell by JQ1. BIM expression was ablated by transduction with Cas9 and BIM sgRNA vectors. Clonal cell lines were treated with increasing concentrations of JQ1 and viability was determined by flow-cytometry.

Supplemental Figure 7 Xu et al.

A

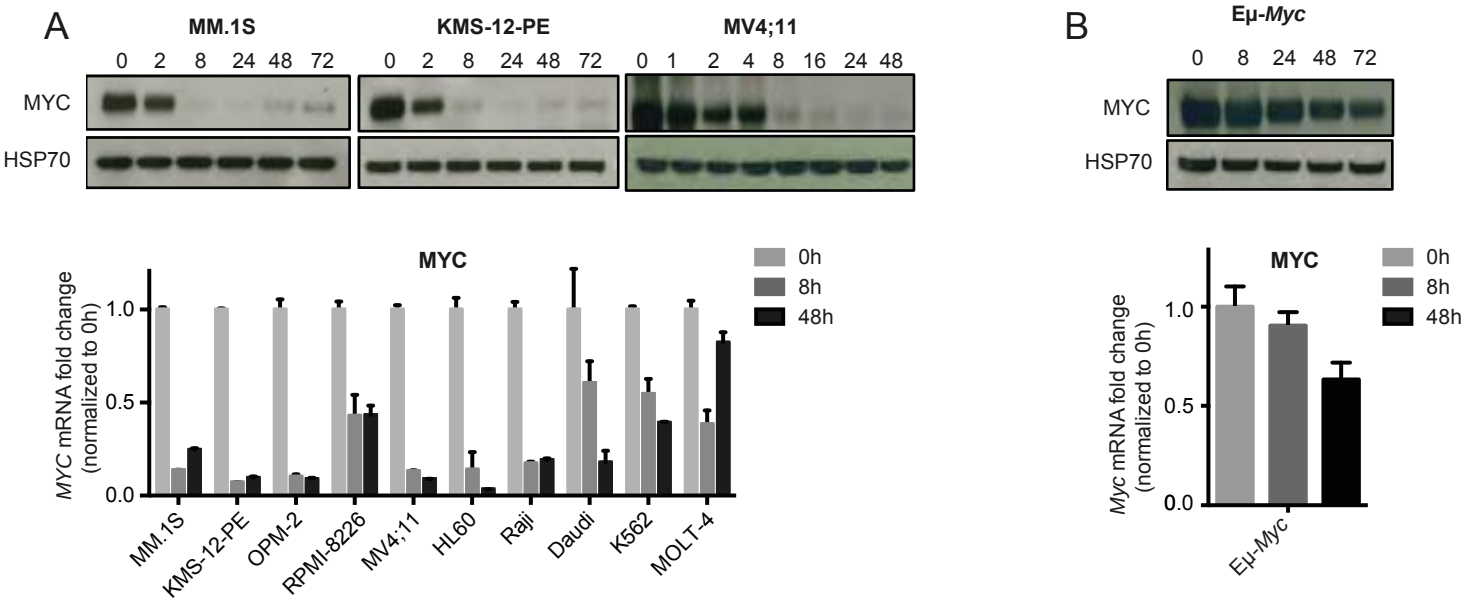


B



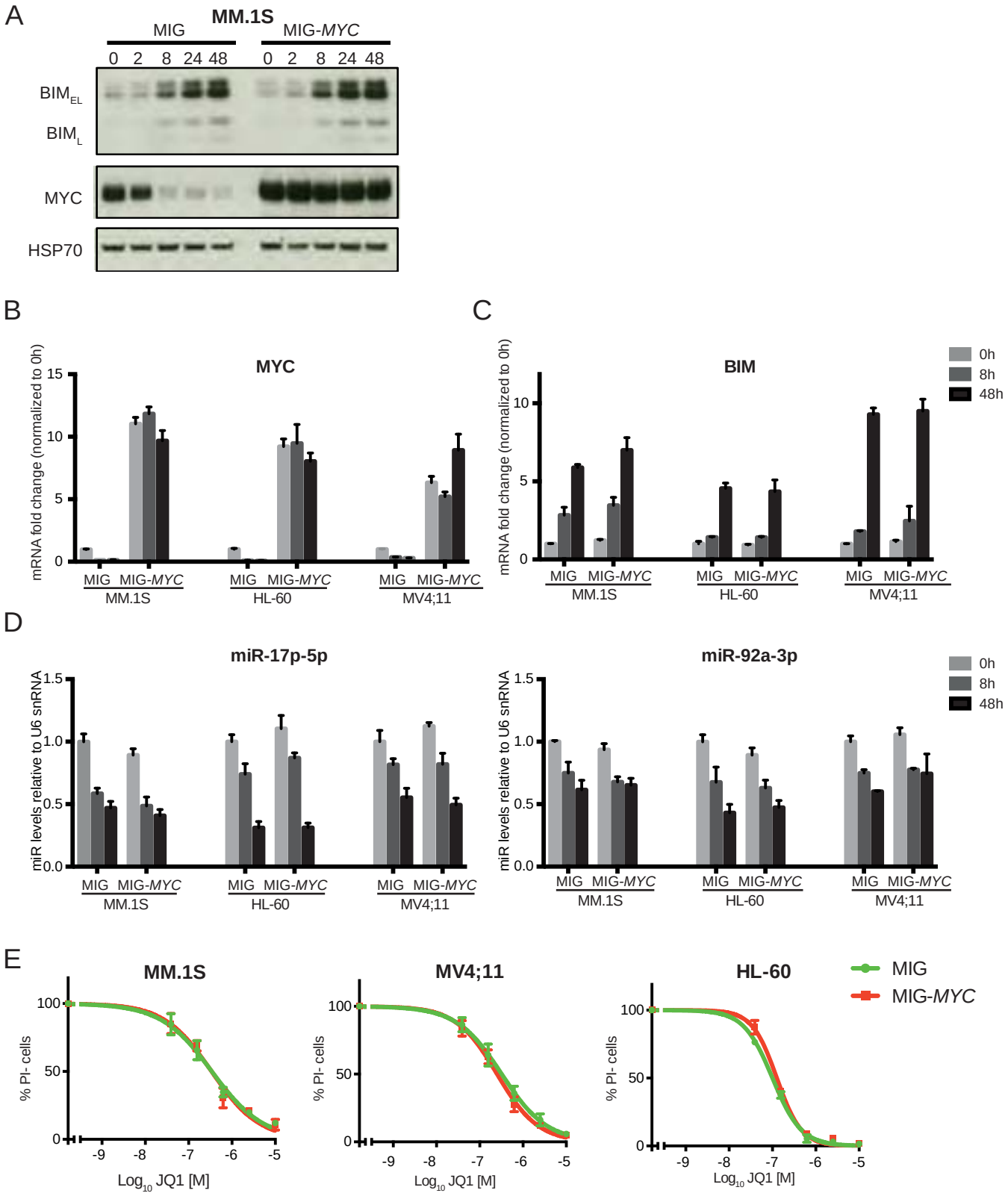
Supplementary Figure 7: BET inhibition decreases expression of miR17-92 cluster
(A) BRD4 is enriched at the miR17-92 cluster. The publicly available ChIP dataset from Liu et al. Cell 2013 Dec 19; 155(7): 1581-1595 (ref. 50) and Loven et al. Cell 2013 Apr 11; 153(2): 320-334 (ref. 51) shows binding of BRD4 at the MYC gene (left panel) and the miR17-92 cluster (right panel). **(B)** JQ1 suppresses expression of the miR17-92 cluster in human cell lines. Real-time qPCR analysis of miR-17-5p and miR-92a-3p expression in a panel of human cell lines at the indicated time points following treatment with 1 μM JQ1 treatment in the presence of 25μM Q-VD-OPh to block apoptosis.

Supplementary Figure 8



Supplementary Figure 8: Myc expression after JQ1 treatment.
(A) MYC protein (upper 3 panels) and mRNA levels (bottom panel) in human cell lines following treatment with 1 μM JQ1 and 25 μM Q-VD-OPh to block apoptosis. Note that all cell lines except MOLT-4 undergo significant loss of MYC expression
(B) MYC protein (top panel) and mRNA levels (bottom panel) in Eμ-Myc lymphoma cells treated with 1 μM JQ1 and 25 μM Q-VD-OPh. Transgenic MYC expression is less affected by BET inhibition in this model.

Supplementary Figure 9. Xu et al.



Supplementary Figure 9: The cytotoxic action of JQ1 is not prevented by sustained MYC expression
(A) Western blot to detect MYC and BIM protein in MM.1S cells overexpressing GFP alone (MIG) or GFP and MYC (MIG-MYC) at the indicated time points following treatment with 1 μM JQ1 / 25 μM Q-VD-OPh .
(B, C, D) mRNA expression of MYC (B), BIM (C), miR-17-5p and miR-92a-3p (D) was detected by RT-PCR at the indicated time points following treatment with 1 μM JQ1 / 25 μM Q-VD-OPh.
(E) The proportions of viable (PI-ve) MM.1S, MV4;11 or HL-60 overexpressing either GFP alone (MIG) or GFP and c-MYC (MIG-MYC) was determined by flow cytometry 48 h after treatment with JQ1.

Supplementary Methods

Real-Time qPCR Analysis A total of 2×10^6 lymphoma cells were harvested for RNA extraction (Qiagen). RNA was then transcribed into cDNA using TaqMan Reverse Transcriptase (Applied Biosystems). Real-time qPCR was performed by using Taqman Universal Master Mix (Applied Biosystems) on either ABI Prism 7900 (Applied Biosystems) or LightCycler 480 (Roche) using Taqman primer/MGB probe sets mouse *Bim*: Mm00437796; mouse *Bcl2*: Mm00477631; mouse *Hmbs* (control): Mm01143545; human *BIM*: Hs00708019_S1; human *c-MYC*: Hs00153408_m1; human *HMBS* (control): Hs00609296_g1 (Life Technologies). For mouse c-*Myc* mRNA quanftification, real-time qRCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) and *c-Myc* primers se:5'- CAACGTCTTGGAACGTCAGA-3' and as:5'-TCGTCTGCTTGAATGGACAG-3' and *Rplp0* (control) primers se: 5'-GCAGGTGTTTGACAACGGCAG-3' and as: 5'-GATGATGGAGTGTGGCACCGA-3'. Data were analyzed using the $\Delta\Delta$Ct method. For the analysis of the miR17-92 clusters, 10ng of total RNA was reverse transcribed using the TaqMan MicroRNA assay kit (Applied Biosystems) with microRNA specific primers. qPCR was performed using miR-17-5p (#002308 for mouse and human) or miR-92-3p (#000430 for mouse; #000431 for human) probes; the U6 snRNA (#001973 for mouse and human) probe was used as an internal control.

Immunoblotting and antibodies Cells were lysed in cell lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail; Roche) for 30 min on ice. Protein quantification was performed using the Bio-Rad Bradford assay. Protein (30 µg each lane) was then separated on pre-cast 4-12% SDS gradient gels (NuPAGE, Invitrogen) by SDS-PAGE and transferred to nitrocellulose membranes using the iBlot Gel transfer system (Life Technologies). The membranes were blocked in 5 % skim milk in PBS and 0.1 % Tween20 for 1h at room temperature. Membranes were then probed with the monoclonal antibodies: mouse anti-BCL2 (clone 7, BD Pharmingen), rat anti-MCL1 (clone

19C4), mouse anti-HSP70 (clone N6, a gift from Dr R Anderson, Peter MacCallum Cancer Centre, Melbourne), mouse anti-β-actin (clone AC-40, Sigma) or the rabbit polyclonal antibodies: rabbit anti-BCLXL (BD Biosciences), rabbit anti-BIM (Stressgen), rabbit anti-BID (Stressgen), rabbit anti-BAD (Stressgen), rabbit anti-PUMA (ProSci), rabbit anti-Caspase-3 (cleaved, Chemicon), rabbit anti-c-MYC (Abcam). HRP conjugated goat anti-Rat IgG (Southern Biotech), sheep anti-Mouse IgG and donkey anti-rabbit IgG were used as secondary antibodies.

***In vivo* imaging of Eμ-*Myc* lymphoma.** For *in vivo* imaging, lymphoma cells were transduced with pMSCV-Luc2-IRES-mCherry retrovirus, transplanted into C57BL/6-albino recipient mice that were injected with 50 mg/kg luciferase substrates (Life Technologies) for imaging on the Xenogen IVIS spectrum system (Perkin Elmer). Spleens and livers were fixed in 10% formalin, paraffin-embedded and stained with hematoxylin plus eosin (H&E) and photographs taken on a Nikon Eclipse E600 microscope with ZEISS AxioCam MRC5 camera using Axiovision (Ver4.8) software. Peripheral blood analysis was performed on ADVIA 120 blood analyzer equipped with a mouse analysis software module (Bayer).