**MLL-Rearranged Acute Lymphoblastic Leukemias Activate BCL-2 through H3K79 Methylation and Are Sensitive to the BCL-2-Specific Antagonist ABT-199**

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**In Brief**
Therapies designed to exploit specific molecular pathways in aggressive cancers are an exciting area of research. Mutations in the MLL gene cause aggressive incurable leukemias. Benito et al. show that MLL leukemias are highly sensitive to BCL-2 inhibitors, especially when combined with drugs that target mutant MLL complex activity.

**Highlights**
- *MLLr* ALL blasts express high levels of BCL-2, BAX, and BIM
- MLL/AF4 activates *BCL2* through H3K79 methylation
- *MLLr* ALL cells are exquisitely sensitive to BCL-2 antagonist ABT-199
- ABT-199 treatment synergizes with H3K79 methylation inhibitors on *MLLr* samples

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SUMMARY

Targeted therapies designed to exploit specific molecular pathways in aggressive cancers are an exciting area of current research. MLL mutations such as the t(4;11) translocation cause aggressive leukemias that are refractory to conventional treatment. The t(4;11) translocation produces an MLL/AF4 fusion protein that activates key target genes through both epigenetic and transcriptional elongation mechanisms. In this study, we show that t(4;11) patient cells express high levels of BCL-2 and are highly sensitive to treatment with the BCL-2-specific BH3 mimetic ABT-199. We demonstrate that MLL/AF4 specifically upregulates the BCL-2 gene but not other BCL-2 family members via DOT1L-mediated H3K79me2/3. We use this information to show that a t(4;11) cell line is sensitive to a combination of ABT-199 and DOT1L inhibitors. In addition, ABT-199 synergizes with standard induction-type therapy in a xenotransplant model, advocating for the introduction of ABT-199 into therapeutic regimens for MLL-rearranged leukemias.

INTRODUCTION

Mixed-lineage-leukemia (MLL) is one of the most frequently translocated genes (MLL-rearranged or MLLr) in hematologic malignancies and produces aggressive leukemias where more targeted therapeutic approaches are particularly needed. Translocation t(4;11)(q21;q23) generates MLL/AF4 and AF4/MLL fusion products, both of which function as transcriptional activators. The role of AF4/MLL in t(4;11) leukemias is controversial, as it has transformation potential (Bursen et al., 2010) but is not expressed in all t(4;11) patients (Andersson et al., 2015). Conversely, the MLL/AF4 fusion protein is expressed in all t(4;11) patients, and knockdowns of MLL/AF4, even in the presence of AF4/MLL, are sufficient to stop t(4;11) leukemias from growing (Thomas et al., 2005).

t(4;11) leukemias are diagnosed mainly as precursor B cell acute lymphoblastic leukemia (B-ALL) in both infants, children, and adults, and they predict poor long-term outcomes, even with aggressive chemotherapy or therapy combined with stem cell transplantation (Beldjord et al., 2014; Dreyer et al., 2015; Pieters et al., 2007). t(4;11) leukemias have very few cooperating mutations, especially in infants (Andersson et al., 2015), suggesting that MLL/AF4 is the primary driver of continued leukemogenesis. Therefore, understanding the function of the MLL/AF4 fusion protein and the genes that it regulates will be essential for the development of targeted t(4;11) therapies.
BCL-2 family proteins mediate an intrinsic, mitochondrial apoptosis pathway. BCL-2, BCL-X\textsubscript{L}, and MCL-1 are anti-apoptotic BCL-2 family proteins, while BCL-2 homology 3 (BH3) proteins BIM, BID, BAD, NOXA, PUMA, and HRK are pro-apoptotic proteins that trigger cell death. Previous studies demonstrated high expression of BCL-2 in MLLr pediatric ALL (Robinson et al., 2008). Using chromatin immunoprecipitation sequencing (ChIP-seq), we and others have detected direct binding of MLL/AF4 (Guenther et al., 2008; Wilkinson et al., 2013) to the BCL-2 gene. This suggests, but does not completely establish, that MLL/AF4 and other fusion proteins could be the cause of increased BCL-2 levels through direct upregulation of BCL-2 transcription. Supporting the potential importance of this observation, activity of the first-generation BCL-2 antagonist has indicated that BCL-2 inhibition could be exploited for MLLr leukemias (Robinson et al., 2008; Ur-tishak et al., 2013). ABT-199/GDC-0199 (venetoclax) is a BH3 mimic that specifically targets BCL-2 while sparing BCL-X\textsubscript{L}, thus avoiding thrombocytopenia (Chonghaile et al., 2014; Pan et al., 2014; Souers et al., 2013; Vaillant et al., 2013; Vandenberg and Cory, 2013). ABT-199 has achieved promising anti-leukemia activity in patients with chronic lymphocytic leukemia (CLL) (Molica, 2015), and it has been reported to have preclinical activities in estrogen-receptor-positive breast cancer, acute myeloid leukemia (AML), early T cell progenitor leukemia, Myc-driven B cell lymphomas, and acute lymphoblastic leukemia (Alford et al., 2015; Chonghaile et al., 2014; Pan et al., 2014; Souers et al., 2013; Vaillant et al., 2013; Vandenberg and Cory, 2013).

Recruitment of P-TEFb (a heterodimer consisting of Cyclin T1 or T2 and the CDK9 kinase) and transcription elongation factors such as ENL and AF9 (Lin et al., 2010; Mueller et al., 2007; Yokoyama et al., 2010) are thought to be major ways in which MLL/AF4 activates gene targets. Other mechanisms have been proposed, including an ENL/AF9 direct interaction with the polycomb group (PcG) protein CBX8 (Maethner et al., 2013). In addition, ENL and AF9 interact directly with DOT1L (Biswas et al., 2011; Leach et al., 2013; Mohan et al., 2010), a histone methyltransferase that specifically methylates lysine 79 on histone 3. Since ENL or AF9 and DOT1L exist in a separate, distinct complex from MLL/AF4 (Biswas et al., 2011; Leach et al., 2013), it is unclear whether or how MLL/AF4 has any direct effect on recruitment of the DOT1L protein, but increased H3K79me2/3 levels are strongly associated with MLL/AF4 binding and with high levels of gene activation (Krivtsov et al., 2008).

In this study, we explored the dependence of ALL subtypes on BCL-2 family proteins and examined the antitumor efficacy of ABT-199 in ALL, with a special focus on the MLLr types. Our findings indicate that direct transcriptional upregulation of BCL-2 by MLL/AF4 confers sensitivity to the selective BCL-2 antagonist ABT-199. We also show that MLL/AF4 promotes high levels of BCL-2 expression by binding directly to the locus and keeping it active via maintenance of H3K79me2/3 without affecting P-TEFb recruitment. This MLL/AF4 regulatory activity is specific to BCL-2 and has no effect on other BCL-2 family members. This led to the finding that the DOT1L inhibitors sensitize MLLr leukemias to BCL-2 inhibition with ABT-199. Importantly, we were also able to show that ABT-199 synergizes with standard-induction-type chemotherapeutic agents, suggesting that ABT-199 could be a useful addition to MLLr therapeutic regimens.

RESULTS

\textbf{t(4;11) ALL Is Associated with High Levels of BCL-2, BAX, and BIM}

Expression of 12 pro- and anti-apoptotic proteins was studied in 186 ALL cases by reverse-phase protein analysis (RPPA). Supervised clustering demonstrated distinct differences in acute lymphoblastic leukemia (ALL) with different cytogenetic characteristics (p < 0.005; false discovery rate [FDR], <0.2%). Patients with 8q24 (CMYC) translocation (Figure 1; n = 9) expressed low levels of BCL-2 and BAX while maintaining high expression of BIM and intermediate levels of MCL-1. No specific patterns were seen in t(9;22) (Ph+) ALL. Patients with
(A) Schematic of MLL/AF4 direct interactions and mutually exclusive complexes (dotted arrows). Gene activation could occur (1) by promoting H3K79 methylation, (2) by inhibiting CBX8 activity, or (3) AF4/AF4 direct recruitment of P-TEFb (ie. Cyclin T1 and CDK9) and serine 2 phosphorylation of RNA polymerase II. (B) ChiP-seq and ATAC-seq peaks in SEM cells across the BCL-2 locus. PCR primers (1–6) used in subsequent experiments are shown as yellow arrowheads. P, promoter; E, enhancer. (C) BCL-2 is ranked 8,381 out of 8,647 genes (top 5%) marked with H3K79me2 in SEM cells. (D) Real-time PCR for different targets in SEM cells treated with either a control (black bars) or an MLL-AF4-specific siRNA (gray bars). Signal was normalized to control treated cells and is the average of five independent knockdown experiments. Error bars indicate SD. *p < 0.02. (E) Western blots for the indicated proteins in SEM cells treated with either a control (–) or MLL-AF4 specific (+) siRNA. (F–H) ChiP experiments (the average of three to five independent knockdowns) for MLL(N), AF4(C), ENL, AF9, AFF4, or CDK9 in SEM cells treated with either control (dark colored bars) or MLL-AF4 siRNAs (light colored bars). PCR primers are as indicated in (A). A9 = a primer set in the well-known MLL-AF4 target gene HOXA9, used as a positive control for ChiP. Error bars indicate SEM. See also Figure S2.

Figure 2. MLL-AF4 Binds to the BCL-2 Gene and Keeps It Active

MLL/A4 Directly Controls Activation of the BCL-2 Gene

Confirming previously published data (Guenther et al., 2008; Wilkinson et al., 2013), ChiP-seq using an MLL N-terminal antibody (Ab) (MLL(N)) and an AF4 C-terminal Ab (AF4(C)) in the MLL/A4-positive B-ALL cell lines SEM and RS4;11 shows that MLL/A4 binds to the BCL-2 locus (Figure 2B; Figure S2A). ChiP-seq for and, therefore, that BCL-2 is a potential therapeutic target in these ALL subtypes.

MLL/A4 Directly Controls Activation of the BCL-2 Gene

Confirming previously published data (Guenther et al., 2008; Wilkinson et al., 2013), ChiP-seq using an MLL N-terminal antibody (Ab) (MLL(N)) and an AF4 C-terminal Ab (AF4(C)) in the MLL/A4-positive B-ALL cell lines SEM and RS4;11 shows that MLL/A4 binds to the BCL-2 locus (Figure 2B; Figure S2A). ChiP-seq for
BH3 Profiling Demonstrates BCL-2 Dependence of MLLr Primary ALL

ABT-199 is a BCL-2-selective inhibitor recently shown to be active in ALL (Alford et al., 2015). In bimolecular fluorescence complementation (BiFC) assays (Figure 5A; Vela et al., 2013), ABT-199 inhibited BCL-2/BIM interactions, and ABT-737 inhibited the interactions of BCL-2 and BCL-X<sub>L</sub> with BIM, according to the known specificity of these inhibitors. BH3 profiling is a technique that identifies the BCL-2 protein family addictions of cancer cells based on the selective binding of BH3 proteins to specific anti-apoptotic BCL-2 family proteins (Certo et al., 2006; Chonghaile et al., 2014; Pan et al., 2014). Using ALL blast mitochondria from 16 samples consisting of primary ALL cells or patient-derived xenografts, we found a statistically significant correlation between mitochondrial sensitivity to BAD BH3 peptide (indicating BCL-2, BCL-X<sub>L</sub>, or BCL-W dependence) and cell viability determined by half-maximal inhibitory concentration (IC<sub>50</sub>) values of ABT-199 (Figure 5B; Table S2). No correlation was found for the mitochondrial response to specific MLL/AF4 complex components (summarized in Figure 2A) shows that ENL binding closely matches the profile of MLL/AF4 binding, while H3K79me2/3 creates a broad domain across the locus (Figure 2B). Comparable to two canonical MLL/AF4 target genes, BCL-2 is a typical MLL/AF4 target gene in that it is marked with very high levels of H3K79me2 (Figure 2C). A potential downstream enhancer—identified by its enrichment for H3K4me1, H3K27Ac, and the presence of ATAC sequencing (ATAC-seq) peaks—is also bound by the MLL/AF4/ENL complex (Figure 2B, blue shaded region marked with an E). A peak of H3K4me3 and binding of the SET1 complex (CFP1) are detected at the BCL-2 promoter (Figure 2B), suggesting that the locus could also be regulated by H3K4 methyltransferase complexes.

For a functional analysis of MLL/AF4 activity, SEM and RS4;11 cells were treated with unique MLL/AF4-specific small interfering RNAs (siRNAs) (Thomas et al., 2005). MLL/AF4 siRNA knockdowns led to a reduction of BCL-2 gene expression that was comparable to that of HOXA9 and RUNX1 (Figure 2D; Figure S2B) and also resulted in reduced BCL-2 protein levels in replicate experiments (Figures 2E and S2C). MLL/AF4 siRNA treatment also reduced MLL/AF4 binding to BCL-2 (Figures 2F and 2D) without affecting wild-type MLL protein levels (Figures 2D, 2E, and S2B) or wild-type MLL or AF4 binding to BCL-2 (Figure S2E). Conversely, wild-type MLL knockdowns had no effect on BCL-2 expression (Figure S2F), indicating that MLL/AF4 directly activates BCL-2 while wild-type MLL is dispensable for BCL-2 activation in t(4;11) cells.

MLL/AF4 knockdowns were associated with reduced ENL binding to BCL-2 in both SEM and RS4;11 cells (Figures 2G and S2D), but there was only a marginal effect on AF9 binding, especially compared to HOXA9 (Figure 2G). ENL mRNA levels are unaffected by MLL/AF4 knockdowns (Figure 2D), but ENL protein levels are reduced (Figure 2E), suggesting that the direct interaction between MLL/AF4 and ENL may somehow stabilize the ENL protein. In contrast to the results observed with ENL, no effect was seen on the binding of AFF4, CDK9, or Cyclin T1 in SEM cells (Figures 2H and S2G), suggesting that MLL/AF4-mediated activation of BCL-2 does not occur through P-TEFb recruitment and stabilization. Although CBX8 binding was easily detected at HOXC8 (a known Polycomb target in SEM cells), no change in CBX8 binding was seen at BCL-2 (Figure S2H).

Instead, MLL/AF4 knockdowns were associated with a loss of DOT1L binding at BCL-2 (Figure S2I). Together, these results suggest that neither pathway 2 nor pathway 3 (see Figure 2A) is important components of MLL/AF4-mediated regulation of BCL-2 but that MLL/AF4, instead, stabilizes both ENL and DOT1L binding (pathway 1) at BCL-2.

MLL/AF4 Controls BCL-2 Gene Activation by Promoting Increased H3K79me2/3 Levels

To explore MLL/AF4 function further, we performed ChIP for several different histone marks in MLL/AF4 knockdowns. Consistent with the observed loss of DOT1L binding, both H3K79me2 and me3 levels are reduced across BCL-2 (Figure 3A). We also observed reductions of H3K27Ac, especially at the downstream enhancer region of BCL-2 (Figure 3B), whereas no significant changes in H3K4me3 levels were seen (Figure 3C).

To determine whether reduction of H3K79me2/3 levels alone could impact expression of BCL-2 or other gene targets, SEM cells were treated with either 2 μM or 5 μM of the DOT1L inhibitor EPZ5676 for 7 days. The MLL/AF4 target genes HOXA9, RUNX1, and BCL-2 (but not any other BCL-2 family members) all showed reduced expression with the 2- and 5-μM treatment at day 7 (Figure 3D), and this correlated with a loss of H3K79me2/3 globally and at the BCL-2 and HOXA9 loci (Figures 3E and 3F). Although both the 2-μM and 5-μM treatments impacted BCL-2 expression, only the 5-μM treatment produced an observable reduction in BCL-2 protein levels (Figure 3E), comparable to that seen when BCL-2 is directly targeted by siRNAs that can disrupt leukemic growth (Figures S3A and S3B). Together with the data in the previous section, these results indicate that the primary way that MLL/AF4 controls activation of BCL-2 is through maintaining H3K79Me2/3 levels (see Figure 3G for a summary).

To determine whether DOT1L activity and BCL-2 inhibition were cooperative, we examined growth-inhibitory activity of two structurally distinct DOT1L inhibitors, SGC0946 and EPZ5676, combined with the selective BCL-2 inhibitor ABT-199. Consistent with our aforementioned results using 2 μM EPZ5676, treatment with 1 μM EPZ5676 had very little detectable effect on BCL-2 family protein levels (Figure S3C). However, a combined blockade of DOT1L and BCL-2 demonstrated deeper growth-inhibitory effects in t(4;11) SEMK2 cells (Figure 3H) but not in the non-MLLr cell line Nalm-6 (Figure S3D).

In SEM cells, MLL/AF4 is bound to both MCL-1 and BIM, but not BAX or BCL-2L1 (Figures 4A and 4B), and BCL-2 family members are associated with a range of H3K79me2/3 levels (Figures 4A–4C). MLL/AF4 knockdowns have little effect on ENL or H3K79me3 levels at these loci (Figure 4B), and there is no effect on the expression levels of MCL-1, BIM, BAX, or BCL-2L1 (Figures 4D and 4E). Together, these data suggest that MLL/AF4 specifically activates BCL-2 by promoting increased H3K79me2/3 levels, while other BCL-2 family members are not dependent on MLL/AF4 or H3K79me2/3 for their expression, even though BIM and BAX are both highly expressed in t(4;11) patient samples.
BCL-Xₐ-selective HRK, MCL-1-selective NOXA, or pan-BCL-2 family BIM BH3 peptides (Figure 5B). Similar correlations were obtained in a different set of adult and pediatric B-ALL samples treated with ABT-737 (Figure S4A). Next, we plotted sensitivity to the BAD BH3 peptide against sensitivity to the HRK peptide (Figure 5C). Five of the six MLLr samples and additional four B-ALL samples showed BCL-2 dependency (shaded area).

**MLLr Cells Expressing High Levels of BCL-2 Are Susceptible to ABT-199-Induced Apoptosis In Vitro and to a Combination of ABT-199 with Chemotherapy**

Our gene transcription studies and functional BH3 profiling predict that MLLr ALL will be particularly dependent on BCL-2 for survival. In a series of genetically diverse ALL cell lines, two t(4;11)-positive cell lines, RS4;11 and SEM-K2, exhibited high sensitivity to ABT-199 (Figures 5D and S4B). BCL-2 protein expression was highest in MLLr RS4;11 and SEMK2 together with the pro-B ALL cell line REH; and BCL-2, but not MCL-1 or BCL-Xₐ, expression correlated with sensitivity to ABT-199 (r = −0.82, p = 0.008) (Figures 5E and S4C). In a panel of 24 primary ALL samples, 92% were sensitive to ABT-199 (IC₅₀ < 1 µM; Figures 5F and S4D), and 78% were sensitive to ABT-737. Interestingly, all five MLLr samples—four t(4;11) cases and one t(9;11) case—had IC₅₀ ≤ 1.0 µM. Western blot analysis of ALL blasts (n = 9) confirmed high BCL-2 protein levels (Figure S4E). High sensitivity of primary B-ALL (non-MLL) samples to BCL-2 inhibition was validated in a separate cohort of adult and pediatric samples treated with ABT-199 or dual BCL-2/BCL-Xₐ inhibitor ABT-263 for 8 hr (Figure S4F).

In all cell lines (REH, SEMK2, and RS4;11), a combination of ABT-737 or ABT-199 with chemotherapy agents (vincristine [VCR], doxorubicin [DOX], dexamethasone [DEXA], cytarabine [AraC], L-asparaginase [L-ASP]) was commonly synergistic (Table S3), with best responses observed upon combination with L-ASP (combination index values, <0.1). In six primary ALL samples, each combination produced significantly greater cytotoxic activity (Figures 6A and 6B). Consistent with the cell line data, the combination of L-ASP with ABT-199 exhibited the greatest effect. Treatment with L-ASP in ALL patients is able to induce apoptosis specifically in lymphoblast cells. We reasoned that the nature of the L-ASP/ABT-199 synergistic interaction could be due to enhancement of apoptotic pathways in MLL/AF4 cells. Consistent with this, western blot analysis of RS4;11 or SEMK2
cells treated with L-ASP, VCR, or DEXA showed that each treatment significantly reduced the levels of anti-apoptotic proteins MCL-1 and BCL-XL and the apoptotic factor FAS-associated factor 1 (FAF1; Figure 6C). Interestingly, MLL/AF4 binds and activates FAF1 expression (Figures 6D–6F), and although FAF1 does not appear to contribute to leukemic growth (Figure S5), MLL/AF4 could contribute to high levels FAF1 protein in the cell and sensitivity to apoptosis induction.

**ABT-199 Attenuates Tumor Growth of MLLr precursor-B ALL In Vivo and Enhances Anti-leukemia Effects of Standard Chemotherapy**

Next, we investigated the anti-leukemic efficacy of ABT-199 in MLLr ALL in vivo using primary ALL xenograft models. In a highly aggressive primary xenograft ICN3 model, there was a marked reduction of circulating CD19-positive cells after 4 days of treatment initiated on day 45 post-cell injection (Figure 7A). Despite high levels of blasts in bone marrow (BM) on day 10 due to rapid disease progression, half of the treated mice appeared to benefit (Figure 7A). In the ALL-236-GFP model, treatment with ABT-199 for 10 days reduced tumor burden measured by bioluminescence or circulating GFP(+) cells (Figures S6A and S6B).

Next, the anti-leukemic effects of ABT-199 in combination with an induction-type regimen, VXL, comprising VCR, L-ASP, and DEXA, were evaluated in mice injected with cells from two t(4;11)-positive ALL patients (#542 and #682). Leukemic cells from both of these patients were found to be BCL-2 dependent by BH3 profiling, with sample #682 more sensitive to BIM peptide (Figure 7B). Leukemia burden (human CD45-positive cells) was determined serially in peripheral blood for case #682. Because case #542 showed a more aggressive disease course, leukemia burden was determined only on day 42 of treatment.
In mice engrafted with #542, ABT-199 alone had minimal effects on peripheral blood leukemia burden (Figure 7C). VXL alone modestly reduced leukemia burden by 23% (p = 0.007). Unexpectedly, the combination of ABT-199 with VXL reduced leukemia burden >70% (p < 0.0001; Figure 7C), revealing striking synergy in this ALL PDX model.

In case #682 mice, both VXL and ABT-199 showed anti-leukemia effects (Figure 7D), consistent with higher sensitivity by BH3 profiling. On day 60 (Figure S6C), all six control mice carried circulating human CD45-positive cells (mean ± SD, 81.8% ± 7.1%), while one mouse from each of the six treated with ABT-199 and the six treated with VXL was leukemia free. Strikingly, circulating human CD45-positive cells were not detected in any of the mice in the combination cohort up to day 69, although positive cells were detected again on day 103 (data not shown). Two mice in the combination-treatment group died from chemotherapy-related toxicity and infection but were leukemia-free on autopsy.

**DISCUSSION**

Lymphoid malignancies utilize the anti-apoptotic BCL-2 family proteins to maintain viability under conditions of oncogenic stress. Because of this dependence, the leukemia cells are...
susceptible to inhibition of anti-apoptotic BCL-2 family proteins. It has been reported that the BCL-2/BCL-X\textsubscript{L} inhibitors ABT-737, ABT-263, and ABT-199 induce rapid and robust apoptosis in ALL cells, both in vitro (Alford et al., 2015; Del Gaizo Moore et al., 2008; High et al., 2010) and in human-derived xenografts (Suryani et al., 2014). In this context, we investigated the expression of BCL-2 family members in a large series of ALL patient samples by proteomic profiling. In accordance with molecular

Figure 6. ABT-199 in Combination with Chemotherapy Exhibits Cytotoxic Activity against Primary ALL Cells

(A and B) ALL primary samples (n = 6) and one sample from a patient with t(4;11) biphenotypic leukemia were treated with each single reagent or in combination with ABT-199 at the following concentrations: ABT-199, 0.015 \mu M; AraC, 1 \mu M; DOX, 40 ng/ml; VCR, 1 ng/ml; and L-ASP, 5 U/ml. At 24 hr, viability (A) and cell death (B) were determined by Annexin V and 7-AAD staining. Each dot represents one sample. Error bars indicate SEM. *p < 0.05.

(C) Western blot analysis of RS4:11 or SEMK2 cells left untreated (Ctrl) or treated for 48 or 24 hr, respectively, with: L-ASP (ASP), 2 U or 5 U (RS4:11 or SEMK2, respectively); VCR, 5 ng/ml; or DEXA, 1 \mu M. Representative blot of one of the three experiments that yielded similar results is shown.

(D–F) MLL/AF4 binds to the \textit{FAF1} gene (D), and MLL/AF4 siRNA treatment reduces MLL-AF4 binding to \textit{FAF1} (E) and reduces \textit{FAF1} protein levels (western blot, F). Error bars in (E) represent SEM for three independent knockdown experiments.

See also Figure S3 and Table S3.
heterogeneity of ALL, the proteomic profiles were widely dispersed but closely associated with cytogenetic abnormalities. t(4;11) ALL was closely associated with high levels of BCL-2, BAX, and BIM. BCL-2 has been previously implicated in the pathogenesis of MLLr leukemias, and disruption of MLL-fusion-driven BCL-2 expression has been proposed as a major mechanism of action for the bromodomain inhibitor I-BET151 (Dawson et al., 2011). Examination of publicly available gene expression datasets demonstrated that pediatric MLLr ALL expresses high BCL-2 mRNA levels, consistent with previous findings in a smaller subset of pediatric ALL patients (Robinson et al., 2008). In this study, we demonstrate a direct role for MLL/AF4 in maintaining BCL-2 expression, providing a plausible explanation for dependency of MLLr ALL cells on BCL-2 anti-apoptotic activity.

Interestingly, although we show that BCL-2 expression is MLL/AF4 dependent, other BCL-2 family members such as MCL-1, BIM, BAX, and BCL-2L1 show no direct dependence on MLL/AF4 for their expression. This is despite the fact that we detect low levels of MLL/AF4 binding and H3K79me2 at both the MCL1 and the BIM genes. These results highlight the fact that it is important to functionally validate ChIP-seq experiments, since the presence of binding is not necessarily functionally relevant. Importantly, we also show that wild-type MLL has no direct role in activating BCL-2 in t(4;11) cells, indicating that BCL-2 overexpression is an MLL/AF4-specific regulatory event. Therefore, treatment with BCL-2 inhibitors targets a direct pathway of the MLL/AF4 driver mutation, indicating that this could be a specific vulnerability of these poor-prognosis t(4;11) leukemias.

Although the correlation between MLL/AF4 binding and H3K79 methylation levels has been observed before (Guenther et al., 2008; Krivtsov et al., 2008; Wilkinson et al., 2013), there was previously little direct evidence that MLL/AF4 acted primarily through H3K79me2/3 levels. Instead, past work has suggested that MLL/AF4 functions primarily by recruiting a large transcription elongation complex that includes AFF4, P-TEFb, ENL, AF9, and other proteins (Lin et al., 2010; Mueller et al., 2007; Yokoyama et al., 2010). It is unknown what the exact
role of H3K79me2/3 is, but recent work has suggested that it functions, in part, by disrupting SIRT1-mediated silencing (Chen et al., 2015). DOT1L inhibitors were able to reduce BCL-2 expression, while MCL1, BIM, BAX, and BCL-2L1 show almost no sensitivity to DOT1L inhibitors. This suggests that the role of H3K79me2/3 is very gene and context specific and underscores the importance of future work designed to further explore the function of this important histone mark and the complexes that regulate it.

The AF4 protein interacts directly with ENL, but the ENL:AF4 interaction and the ENL:DOT1L interactions are mutually exclusive, so how could MLL/AF4 cause recruitment of DOT1L and increased H3K79me2 levels? Structural analysis of the AF9-DOT1L interaction has shown that AF9 (and, by extension, ENL) interacts with AF4 and DOT1L through the same intrinsically disordered domain (Kuntimaddi et al., 2015; Leach et al., 2013). Intrinsically disordered allows for rapid association kinetics and the possibility of a rapid, dynamic exchange of binding partners between ENL:AF4 and ENL:DOT1L. Thus, it is possible that MLL/AF4 has a direct impact on binding of DOT1L by increasing the local concentration of ENL and/or AF9 proteins. To fully understand this possibility, further work is needed that studies the dynamic interactions of these protein complexes in vivo.

Our observation that treatment of SEM cells with DOT1L inhibitors appears to cooperate with ABT-199 treatment provides an interesting proof of principle that DOT1L inhibitors could potentially be used to sensitize MLLr leukemias to treatment with ABT-199. DOT1L inhibition affects gene targets other than BCL-2 (e.g., HOXA9 and RUNX1); therefore, the nature of this cooperative effect could be due to a general inhibition of a range of different MLL/AF4 targets rather than one or a few MLL/AF4 targets. However, this provides an important proof of principle that combining inhibitors that target MLL/AF4 complex activity can be used in combination with inhibitors of important MLL/AF4 target gene products.

Through BH3 profiling, we demonstrate the predominant dependence of MLLr B-lineage ALL cells on BCL-2, as mitochondrial sensitivity to BCL-2-selective BAD peptide was more potent than BCL-XL-selective HRK, MCL-1-selective NOXA, and non-specific BIM peptide. Further, the robust response observed upon treatment with the BAD peptide showed excellent correlation with mitochondrial depolarization achieved in primary ALL blasts with ABT-199, confirming the on-target BCL-2-dependent activity of this agent. Notably, an additional four B-ALL and two out of five precursor T cell ALL (T-ALL) samples likewise demonstrated BCL-2 dependence. These data indicate that several phenotypically and genetically distinct ALL subtypes utilize BCL-2 as a primary pro-survival mechanism (Chonghaile et al., 2014).

Our results suggest that the presence of t(4;11) may predict response to ABT-199 in ALL. We found that the human MLLr B-ALL cell lines expressed the highest levels of BCL-2 protein and exhibited the greatest sensitivity to ABT-199 among the cell lines tested. Among the primary B-ALL samples tested, all MLLr were highly sensitive. In addition to its single-agent efficacy, ABT-199 showed beneficial results when combined with induction-type conventional chemotherapy in PDX models of B-lineage MLLr ALL established from patient-derived leukemia cells. In four separate in vivo experiments, short-term ABT-199 treatment (7–10 doses) had only transient anti-leukemia effects, yet it profoundly enhanced efficacy of the VXL chemotherapy regimen. The synergistic response between ABT-199 and chemotherapy treatment is likely to be complex and not due to a single factor. Previous studies have documented synergistic anti-leukemia efficacy of dual BCL-2/XL inhibitor ABT-737 in ALL cells, including xenograft models upon combination with VXL, the regimen used here (Kang et al., 2007); this synergy was attributed to the ability of L-ASP to downregulate MCL-1 protein levels (High et al., 2010). Further, anti-mitotic agents have been shown to synergize with BH3 mimetics and reduce MCL-1 protein levels (Chen et al., 2011; Leverson et al., 2015; Tan et al., 2011; Wong et al., 2012), at least in part through phosphorylation and proteasomal degradation of MCL-1 during mitotic arrest (Wertz et al., 2011). Our data in MLLr cell lines support these findings, demonstrating downregulation of both MCL-1 and BCL-XL by these agents. The regulation of the FAF1 locus by MLL/AF4 presents another interesting possibility. High FAF1 protein levels are able to enhance apoptosis, and FAF1 protein is degraded upon induction of apoptosis (Menges et al., 2009). This suggests that MLL/AF4-mediated FAF1 overexpression could sensitize t(4;11) cells to induction of apoptosis by factors such as L-ASP, as long as the anti-apoptotic activity of BCL-2 is also inhibited by ABT-199. This could further explain synergy between BCL-2 antagonist and standard chemotherapeutic agents used in ALL regimens seen in our studies. In summary, our findings demonstrate that BCL-2 is a direct target of the rearranged MLL in ALL cells, translating into BCL-2 dependence and vulnerability to selective, on-target BCL-2 inhibition by the clinically active agent ABT-199. These findings strongly advocate introduction of ABT-199, which recently demonstrated impressive efficacy in CLL trials, into the clinical armamentarium of ALL therapy.

**EXPERIMENTAL PROCEDURES**

**Reagents**
ABT-199 and ABT-737 were provided by AbbVie.

**Cell Lines, siRNA, Primary Samples, and Cultures**
Cell lines used for this study are detailed in the Supplemental Experimental Procedures. MLL/AF4 siRNA experiments were performed as described by Thomas et al. (2005), with differences noted in the Supplemental Experimental Procedures. All animal experiments were reviewed by institutional animal committees. All work with human samples was approved by the institutional review board at the University of Texas MD Anderson Cancer Center and the Dana-Farber Cancer Institute. Primary samples were derived from patients with ALL after informed consent was obtained in accordance with institutional guidelines set forth by the MD Anderson Cancer Center and Dana-Farber Cancer Institute. Clinical sample information is summarized in Table S1.

**Western blot Analysis**
Western blot analysis was performed as previously described (Pan et al., 2014; Wilkinson et al., 2013). Antibody sources are listed under Supplemental Experimental Procedures.

**RPPA**
Expression of pro- and anti-apoptotic BCL-2 family proteins was studied in 186 patients diagnosed with ALL by RPPA. Antibodies used are listed in Table S4. The methodology and validation of RPPA are described elsewhere (Kornblau et al., 2011).
BIFC
Interactions between anti-apoptotic proteins BCL-2, BCL-X<sub>L</sub>, and MCL-1 and pro-apoptotic proteins BIM and NOXA were studied by BIFC (Vela et al., 2013).

ChIP Assays and ChIP-Seq
ChIP and ChIP-seq experiments were performed as described in the Supplemental Experimental Procedures and as previously described (Mline et al., 2009; Wilkinson et al., 2013).

Intracellular BH3 Profiling of Primary ALL Cells
Intracellular BH3 profiling on primary ALL cells was performed as previously described (Pan et al., 2014).

In Vivo Murine Leukemia Models
ALL-236-GFP/Luc cells generated from pre-B-ALL with t(4;11) (Terzykska et al., 2012) and ICN3 xenograft cells generated from a child with relapsed MLLr pre-B-ALL (Duy et al., 2011) were injected intravenously into nonobese diabetic–severe combined immunodeficiency (NOD SCID)/IL2R<sup>−/−</sup>-KO (NSG) mice. Mice were treated with vehicle (Phosal 50 PG/polyethylene glycol [PEG]40/ethanol, 60/30/10 v/v) or ABT-199 (100 mg/kg/day) by oral gavage.

Statistical Analysis
Data were analyzed by the two-tailed Student’s t test or the Mann-Whitney test, if appropriate. Differences were considered statistically significant at p < 0.05. Unless otherwise indicated, data are expressed as mean ± SD. Additional details on experimental procedures are included in the Supplemental Experimental Procedures.

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