Myc induces miRNA-mediated apoptosis in response to HDAC inhibition in hematologic malignancies

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Abstract

Alterations in the expression or function of histone deacetylases (HDACs) contribute to the development and progression of hematologic malignancies. Consequently, the development and implementation of HDAC inhibitors has proven to be therapeutically beneficial, particularly for hematologic malignancies. However, the molecular mechanisms by which HDAC inhibition (HDACi) induces tumor cell death remain unresolved. Here, we investigated the effects of HDACi in Myc-driven B-cell lymphoma and five other hematopoietic malignancies. We determined that Myc-mediated transcriptional repression of the miR-15 and let-7 families in malignant cells was relieved upon HDACi, and Myc was required for their upregulation. The miR-15 and let-7 families then targeted and downregulated the anti-apoptotic genes Bcl-2 and Bcl-xL, respectively, to induce HDACi-mediated apoptosis. Notably, Myc also transcriptionally upregulated these miRNA in untransformed cells, indicating that this Myc-induced miRNA-mediated apoptotic pathway is suppressed in malignant cells, but becomes reactivated upon HDACi. Taken together, our results reveal a previously unknown mechanism by which Myc induces apoptosis independent of the p53 pathway and as a response to HDACi in malignant hematopoietic cells.
Introduction

Aberrant expression or function of histone deacetylases (HDACs) have been implicated in hematopoietic malignancies (1). Although HDAC inhibition (HDACi) induces tumor cell death while leaving normal cells relatively unaffected, the underlying mechanism(s) behind this remain unclear. Changes in expression of survival genes were observed following HDACi (1); however, whether these alterations resulted from hyper-acetylation of promoters or altered expression of transcriptional mediators was not determined. HDACi also affects DNA replication, likely from the inability to deacetylate histones at replication forks (2,3).

Myc, an oncogenic transcription factor, is dysregulated in most hematopoietic malignancies (4). However, untransformed cells undergo apoptosis to counter hyper-proliferative signals from Myc dysregulation. Specifically, Myc overexpression activates the p53 tumor suppressor pathway, eliciting apoptosis (5,6). Myc-induced apoptosis also occurs independent of p53 through down-regulation of anti-apoptotic Bcl-2 and Bcl-xL proteins, by an indirect and unclear mechanism (7-9). Both pathways become inactivated during tumorigenesis (10).

Myc transcriptionally activates or represses numerous genes, regulating many cellular processes (11). Myc represses protein-coding genes by recruiting HDACs and by binding and inhibiting the transcriptional activator Miz-1 (11,12). Myc also regulates the expression of non-coding RNA, including microRNA (miRNA) that bind mRNA, typically inhibiting translation (13). In malignant cells, Myc represses many miRNA while specifically up-regulating others (13,14). Although Myc-mediated transcriptional activation has been extensively studied, mechanisms of Myc-mediated repression and their contribution to tumorigenesis are less understood.

Here we describe a previously unknown miRNA-mediated mechanism of Myc-induced
apoptosis. We determined cellular transformation status dictates whether Myc transcriptionally activates or represses the miR-15 and let-7 families that target anti-apoptotic \( Bcl-2 \) and \( Bcl-x_L \), respectively. This apoptotic mechanism was inactivated in transformed hematopoietic cells, but reactivated by HDACi. Our data reveal a general mechanism underlying HDACi-mediated malignant hematopoietic cell death and provide new insight into Myc-induced apoptosis.

**Materials and Methods**

**Cell lines, transfection, infection, and vectors**

Daudi, Ramos, Raji, Su-DHL-6, and NIH3T3 cells were cultured as described by the American Type Culture Collection. OCI-Ly-19 and OCI-Ly-3 cells were cultured in RPMI-1640 containing 10% fetal bovine serum. P493-6 cells from Dr. Dirk Eick (Helmholtz-Zentrum-Muenchen) were cultured as described (15). Tetracycline (0.1\( \mu \)g/ml; Sigma) was added to cultures of P493-6 cells for 24 hours to turn off MYC expression. Cell lines were obtained between 2001 and 2007 and immediately frozen, so cells were cultured for less than six months. Primary murine pre-B cultures were generated and infected with retrovirus as previously described (5); details in Supplemental. \( E\mu-myc \) lymphoma cells were previously isolated and maintained as published (5). Wild-type or \( p53^{-/-} \) murine embryonic fibroblasts (MEFs) were generated and cultured as described (6). Fibroblasts were transfected using Lipofectamine2000 (Invitrogen). Retroviral infections of fibroblasts were performed as previously reported (6). Vector/retrovirus details are in Supplemental.

**HDAC inhibition and cell growth and apoptosis assays**

*In vitro* experiments utilized 1\( \mu \)M 4-hydroxytamoxifen (4-OHT) or vehicle (EtOH), and 5nM
Depsipeptide (Celgene), 10µM RGFP966 (Repligen), or vehicle (DMSO). Cell number and/or viability were determined by Trypan-Blue Dye exclusion (triplicate) and proliferation by MTT (Sigma; 570nm), MTS (Promega; 490nm), or Alamar Blue (Invitrogen) assays (quadruplicate). Apoptosis was evaluated by flow cytometry following propidium iodide (sub-G1 DNA) or AnnexinV/7-AAD staining.

Mice
For the in vivo lymphoma experiments, 10-12 weeks old C57Bl/6 (Jackson Laboratory) mice were subcutaneously injected (one flank) with 4x10^6 Eµ-myc lymphoma cells as previously described (16). Once tumors reached 200 mm^3, Depsipeptide (2mg/kg) or vehicle (DMSO) was intraperitoneally injected. Mice were sacrificed at intervals for tumor evaluation. Studies complied with state and federal guidelines and were approved by the Vanderbilt Institutional Animal Care and Use Committee. Normal B-cells were purified from spleens of mice with the IMag Mouse B-Lymphocyte Enrichment Set (BD-Biosciences).

Human tissue
Normal human B-cells were purified from leukoreduction filters (Red Cross) and de-identified fresh spleens using the IMag Human B-Lymphocyte Enrichment Set (BD-Biosciences). De-identified fresh spleen and frozen lymph nodes were obtained from the Cooperative Human Tissue Network, following Institutional Review Board approval as non-human subject research (#150139).
Western blotting

Whole cell protein lysates were prepared and Western blotted as reported (6,17). Fibroblasts were lysed 48 hours post-transfection.

Antibodies

Western blotting antibodies: Bcl-2, Bcl-xL (BD-Biosciences); Mcl-1 (Rockland); Bim (22-40, Calbiochem); cleaved Caspase-3 (Cell-Signaling); Myc, H3K9K14ac (Millipore); H3K56ac, H4K5ac, H3, H4 (Abcam); Bax (N-20), Miz-1 (H-190, Santa-Cruz); and β-actin (Sigma). qChIP antibodies: Myc (N-262) and isotype controls (Santa-Cruz), RNA polymerase-II (Ser2-phosphorylated, Abcam), and H3K9K14ac (Millipore).

Quantitative chromatin immunoprecipitation (qChIP)

qChIP was performed as previously described (18). Primer sequences and antibodies in Supplemental.

Quantitative real-time PCR (qRT-PCR)

RNA was isolated, cDNA was generated, and SybrGreen (SA-Biosciences) and TaqMan MicroRNA Assays (Applied Biosciences) were used for qRT-PCR (triplicate) to measure mRNA and miRNA, respectively, as previously described (19,20). mRNA and miRNA expression were normalized to β-actin and RNU6b levels, respectively, and presented as 2^−ΔΔCt. Primer sequences in Supplemental.
Luciferase assays

NIH3T3 cells were transfected with luciferase reporters, β-galactosidase control plasmid, and 50nM miR-15 or miR-195 miRIDIAN miRNA mimics or control RNA (Dharmacon/ThermoScientificBio), and/or 200nM miScript Target Protectors (Qiagen). Luciferase and β-galactosidase activity was measured as previously described (19).

Statistics

Student’s *t*-tests were used to statistically evaluate the data in Fig. 1A, 1C, 2D-G, 3D, 3E, 4B-G, 5A-E, 6E, 7A, 7C, 7E, and 7F. Wilcoxon rank-sum tests determined statistical significance for Fig. 2B, 2C, 3B, and 4A.

Results

**HDAC inhibition decreases Bcl-2 and Bcl-xL expression, inducing apoptosis in multiple hematopoietic malignancies**

To evaluate the molecular events following HDACi, B-cell lymphomas from Eμ-*myc* transgenic mice (Myc-driven B-cell lymphoma model; (21) and human Burkitt’s lymphoma lines were treated with HDAC inhibitors. Depsipeptide (Depsi, class-I HDACi), RGFP966 (HDAC3i; (22), RGFP233 (HDAC1/2i), RGFP963 (HDAC1/2/3i), and Panobinostat (pan-HDACi) all decreased cell expansion and number (Fig. 1A, Supplementary Fig. S1A, S1B). Depsi also reduced cell expansion in nine other malignant human hematopoietic lines, including acute myeloblastic leukemia (Kasumi), chronic myelogenous leukemia (K562), acute T-cell leukemia (Jurkat, Loucy), cutaneous T-cell lymphoma (Hut-78, MyLa), diffuse large B-cell lymphoma
Furthermore, HDACi decreased cell viability (Fig. 1A, Supplementary Fig. S1B) and increased Caspase-3 cleavage (Fig. 1B), characteristics of apoptosis.

To identify the molecular determinants of HDACi-mediated apoptosis, we assessed expression of crucial pro-survival proteins. Compared to vehicle control-treated cells, HDACi of murine and human B-cell lymphoma cells decreased protein expression of anti-apoptotic Bcl-2 and Bcl-x\textsubscript{L}, but not Mcl-1 (Fig. 1B, Supplementary Fig. S1D). Moreover, Depsi treatment also decreased Bcl-2 and Bcl-x\textsubscript{L} protein in nine other malignant hematopoietic cell lines (Supplementary Fig. S1D). Decreased \textit{Bcl-2} and \textit{Bcl-x\textsubscript{L}} mRNA (Fig. 1C) may explain the reduction in protein. Inhibiting Bcl-2 and/or Bcl-x\textsubscript{L} reportedly kills malignant hematopoietic cells, including lines we evaluated (23). Thus, HDACi specifically decreased Bcl-2 and Bcl-x\textsubscript{L} expression, inducing apoptosis.

**HDAC inhibition reveals post-transcriptional regulation of \textit{Bcl-2} and \textit{Bcl-x\textsubscript{L}}**

HDACi-induced effects on histone acetylation were evaluated to gain insight into the mechanism responsible for decreasing Bcl-2 and Bcl-x\textsubscript{L}. Western blotting showed increased global histone acetylation marks associated with active transcription in murine and human lymphoma cells treated with Depsi or RGFP966 (Fig. 2A, Supplementary Fig. S2A). Analogous results were obtained in the other nine malignant hematopoietic cell lines tested (Supplementary Fig. S2B).

Histone acetylation is typically associated with gene activation, yet \textit{Bcl-2} and \textit{Bcl-x\textsubscript{L}} mRNA decreased after HDACi. To investigate this, we examined precision global run-on transcription coupled with massively parallel sequencing (PRO-seq; (24) data for \textit{BCL-2} and \textit{BCL-X\textsubscript{L}} loci from Daudi cells treated for 4hrs with Depsiteptide or vehicle control (Acharya et
This analysis showed no significant expression changes (increased or decreased) at these loci (Supplementary Fig. S2C), suggesting a post-transcriptional mechanism is likely responsible for the observed changes in Bcl-2 and Bcl-xL mRNA.

Therefore, we assessed expression of the miR-15 family (miR-15a, -16-1, -195, -497) and let-7a, as these miRNA post-transcriptionally target and negatively regulate Bcl-2 and Bcl-xL expression, respectively, and induce apoptosis in multiple cell types (Supplementary Fig. S3; (25). Consistent with previous reports (14,26), miR-15 family and let-7a levels were decreased in human diffuse large B-cell lymphoma (DLBCL) and Burkitt’s lymphoma cell lines compared to purified B-cells and normal lymph nodes (Fig. 2B). Similarly, B-cell lymphomas from Eμ-myc transgenic mice also had reduced levels of these miRNA compared to levels in pre-cancerous Eμ-myc splenocytes and purified B-cells (Fig. 2C).

Notably, HDACi increased miR-15a and miR-195 (representative miR-15 family members) and let-7a in lymphoma cells (Fig. 2D, Supplementary Fig. S4A, S4B). miR-31 levels were unaffected, demonstrating not all miRNA were up-regulated with HDACi. All other malignant hematopoietic lines evaluated showed similar results (Supplementary Fig. S4C), indicating this HDACi-induced effect is not cell-type specific. Furthermore, with Depsi dose escalation, miRNA levels increased, indicating a dose response to HDACi (Supplementary Fig. S4D).

To evaluate whether the miR-15 family and let-7a were being transcribed upon HDACi, we measured primary miRNA transcripts of the miR-15a/16-1 and miR-195/497 clusters and let-7a. Following HDACi, these pri-miRNA transcripts increased (Fig. 2E). We next performed qChIP for RNA polymerase-II phosphorylated on serine 2 (RNAPII-p-Ser2), which is indicative
of active transcriptional elongation. HDACi resulted in RNAPII-p-Ser2 enrichment at miR-15 family and let-7a promoters in lymphoma cells (Fig. 2F). Enrichment was not observed at regions upstream of these promoters or in vehicle control-treated cells. Additionally, there was more RNAPII-p-Ser2 enrichment at miR-15 family and let-7a promoters in Depsi-treated lymphoma compared to normal lymphocytes (Supplementary Fig. S4E), indicating increased transcription following HDACi in lymphoma. Thus, HDACi in lymphoma activates transcription of the miR-15 family and let-7a.

To determine whether the HDACi-induced increase in the miR-15 family or let-7a was responsible for lymphoma cell death, we retrovirally expressed the miRNA in two lymphoma lines. Lymphomas expressing the miR-15a/16-1 or let-7a clusters showed reduced Bcl-2 or Bcl-x<sub>L</sub> protein, cell expansion, and viability, and had increased Caspase-3 cleavage, sub-G1 DNA, and AnnexinV-positivity (Fig. 2G, Supplementary Fig. S4F-H). Thus, increased levels of the miR-15 family or let-7a are sufficient to induce apoptosis in lymphomas.

**In vivo** HDAC inhibition increases miR-15 family and let-7a levels, inducing lymphoma cell death

To extend our investigations *in vivo*, E<sub>μ</sub>-myc lymphoma cells were subcutaneously injected into C57Bl/6 mice. Once tumors reached 200mm<sup>3</sup>, mice were administered Depsi or vehicle control, and tumors harvested at intervals. Analogous to our *in vitro* results, HDACi increased active histone acetylation marks (Fig. 3A) and levels of the miR-15 family and let-7a (Fig. 3B), and decreased Bcl-2 and Bcl-x<sub>L</sub> protein (Fig. 3C). Apoptosis was evident by Caspase-3 cleavage, AnnexinV-positivity, and sub-G1 DNA (Fig. 3C-E). These data confirm HDACi activates miR-15 family and let-7a transcription that adversely affect the expression of crucial pro-survival proteins, inducing lymphoma cell apoptosis *in vivo.*
Myc transcriptionally up-regulates the miR-15 family and let-7a in untransformed cells

Myc transcriptionally activates specific miRNA, while repressing others in cancer cells (13,14). To determine whether Myc mediated the repression of the miR-15 family and let-7a and/or their induction following HDACi, we evaluated untransformed and transformed cells with altered Myc levels. Unexpectedly, in contrast to transformed cells, Myc-overexpressing pre-cancerous Eμ-myc spleens had increased miR-15 family and let-7a transcripts compared to wild-type littermate spleens (Fig. 4A). miR-31 levels were analogous between genotypes, indicating Myc overexpression selectively increased specific miRNA in non-transformed cells.

We next assessed primary murine pre-B-cells retrovirally expressing MycER, a 4-hydroxytamoxifen (4-OHT)-inducible Myc (27). Upon MycER activation with 4-OHT, miR-15 family and let-7a levels significantly increased compared to vehicle control-treated pre-B-cells (Fig. 4B). This increase was analogous to that of miR-20a, a well-known Myc-induced miRNA (13). Levels of miR-31 were unaffected. Similar results were obtained following MycER activation in untransformed fibroblasts (Supplementary Fig. S5A), indicating this effect also occurs in non-hematopoietic cells. Addition of 4-OHT to non-MycER-expressing pre-B-cells or fibroblasts had no effect on miRNA levels (Supplementary Fig. S5B). Therefore, Myc does not repress, but instead induces the miR-15 and let-7 families in untransformed cells.

To determine whether Myc was transcriptionally activating the miR-15 and let-7 families in untransformed cells, we utilized a MycER mutant lacking the Myc-Box-II domain (MycΔMBII-ER) essential for Myc-mediated transcriptional activation (11). Levels of the miR-15 family and let-7a were not induced following MycΔMBII-ER activation in wild-type murine embryonic fibroblasts (MEFs), but were when transcriptionally competent MycER was activated (Fig. 4C). When primary transcripts of the miR-15a/16-1 and miR-195/497 clusters and let-7a
were evaluated, MycER, but not MycΔMBII-ER, induced their expression in MEFs and pre-B-cells (Fig. 4D, 4E), indicating Myc-mediated transcription was necessary to up-regulate these miRNA.

To assess whether Myc was at the miRNA promoters and whether active transcription was occurring, qChIP was performed. qChIP for Myc in MycER-expressing MEFs revealed enrichment at the promoter regions of the miR-15a/16-1 and miR-195/497 clusters and let-7a following 4-OHT-induced MycER activation (Fig. 4F). Importantly, RNAPII-p-Ser2 and H3K9K14ac, indicators of active transcription, were also enriched (Fig. 4F). No enrichment was observed at regions upstream of the miRNA promoters or in vehicle control-treated cells. Similar qChIP results were obtained in vivo when non-transformed pre-cancerous Eμ-μc transgenic spleens were compared to non-transgenic littermate-matched spleens (Fig. 4G), further demonstrating that Myc transcriptionally up-regulates these miRNA families in untransformed cells.

**Myc is required for HDACi-induced miR-15 family and let-7a transcriptional up-regulation**

To determine the role of Myc in repressing the miR-15 and let-7 families in malignant cells, qChIP was performed on Myc-overexpressing murine and human lymphoma cells. Myc was enriched at the promoters of both miR-15 family clusters and let-7a in both cell lines, but not at upstream regions (Fig. 5A). ENCODE MYC ChIP-seq data (28) also showed MYC at these promoters in hematopoietic and non-hematopoietic malignancies and non-transformed human cells (Supplementary Fig. S6A). Importantly, Myc was enriched at these same loci in both the presence and absence of HDACi (Fig. 5A). Consistent with Myc-mediated repression of these loci in lymphoma, there was significantly more enrichment of Myc at the promoter regions of the
miR-15a/16-1 and miR-195/497 clusters and let-7a in lymphoma cells compared to normal lymphocytes (Supplementary Fig. S6B).

Myc transcriptionally activates CAD and represses p21 (11), consistent with the increase in RNAPII-p-Ser2 and H3K9K14ac at CAD and the lack of enrichment at p21 we observed in the lymphoma cells (Fig. 5B). Neither RNAPII-p-Ser2 nor H3K9K14ac enrichment was detected at promoters of either miR-15 family cluster or let-7a in the lymphomas (Fig. 5B). Collectively, the data indicate Myc activates miR-15 family and let-7a transcription in untransformed cells, whereas it appears to repress their transcription in transformed cells. Moreover, HDACi of B-cell lymphoma induced the miR-15 family and let-7a to levels similar to those in non-transformed pre-cancerous B-cells overexpressing Myc (Supplementary Fig. S6C). Furthermore, the derepression of the miR-15 family and let-7a detected in lymphomas following 6 hours of HDACi did not appear to be due to changes in Myc protein, as Myc levels were similar for at least 12 hours after HDACi (Supplementary Fig. S6D). Together, our data suggest HDACi converts Myc from a transcriptional repressor into a transcriptional activator in lymphoma.

To test whether Myc was required for HDACi-induced up-regulation of the miR-15 family and let-7a, we utilized the human B-cell lymphoma line, P493-6, that expresses a tetracycline-regulatable MYC (15). With tetracycline present, MYC levels were significantly reduced and HDACi failed to increase miR-15a, miR-195, or let-7a levels (Fig. 5C). Only when MYC was expressed did HDACi increase their expression. Therefore, Myc was required to mediate the HDACi-induced up-regulation of the miR-15 family and let-7a. Additionally, irrespective of HDACi, when MYC expression was off, levels of the miR-15 and let-7 families were slightly increased compared to when MYC was expressed, providing additional evidence MYC represses these miRNA in lymphoma.
To further assess the requirements of MYC on miR-15 family and let-7a expression, we performed qChIP with P493-6 cells. When Myc was expressed, it was enriched at the miR-15 family and let-7a promoters (Fig. 5D). Upon HDACi, enrichment of RNAPII-p-Ser2 and H3K9K14ac (marks of active transcription) at miR-15 family and let-7a promoters was only observed in MYC-expressing cells (Fig. 5E). When MYC was not expressed, a slight enrichment of RNAPII-p-Ser2 and H3K9K14ac was detected at the miR-15 family and let-7a promoters, regardless of HDACi (Fig. 5E). These data show MYC mediated the repression of the miR-15 family and let-7a and was required for their HDACi-induced transcriptional up-regulation.

Myc transcriptional activity is required to suppress Bcl-2 and Bcl-xL expression

In untransformed cells, Myc suppressed Bcl-2 and Bcl-xL expression, inducing apoptosis through an indirect, unresolved mechanism (7,8), purportedly through binding Miz-1 (9). However, Bcl-2 and Bcl-xL proteins are overexpressed in the majority of Eμ-myc B-cell lymphomas and human lymphomas (8). Evaluation of Eμ-myc B-cell lymphomas compared to normal B-cells and spleens from pre-cancerous Eμ-myc mice confirmed these results (Fig. 6A). In contrast, pre-cancerous Eμ-myc spleens with increased Myc had reduced Bcl-2 and Bcl-xL protein compared to non-transgenic littermate-matched spleens (Fig. 6B). Mcl-1 and Bax (pro-apoptotic Bcl-2 family member) were unaffected. Similarly, in MycER-expressing primary pre-B-cells and MEFs, decreases in Bcl-2 and Bcl-xL were detected following MycER activation (Fig. 6C). No change in Mcl-1 or Bax expression was detected, whereas Bim, a pro-apoptotic Bcl-2 family member up-regulated by Myc (29), was increased upon MycER activation (Fig. 6C). Therefore, with elevated Myc, non-transformed cells decrease Bcl-2 and Bcl-xL expression, whereas their expression is increased in transformed cells.

Myc associates with the transcription factor Miz-1 through a motif requiring valine 394,
reportedly suppressing \( Bcl-2 \) \(^{9}\). To investigate this, MycER harboring a point mutation (V394D) disrupting the Myc:Miz-1 interaction (MycV394D-ER; Supplementary Fig. S7A) \(^{30}\) was expressed in wild-type MEFs. Following MycV394D-ER activation with 4-OHT, Bcl-2 and Bcl-x\(_L\) were suppressed equivalently to cells expressing wild-type MycER, ruling out a Miz-1-mediated mechanism (Fig. 6D). Analogous data were obtained using \( p53 \)-null MEFs (Supplementary Fig. S7B), consistent with prior results in \( p53 \)-null myeloid and B-cells \(^{7,8}\).

These data reveal a Miz-1- and \( p53 \)-independent mechanism for Myc-mediated suppression of Bcl-2 and Bcl-x\(_L\) expression. Moreover, MycV394D-ER effectively induced miR-15 family and let-7a expression (Fig. 6E), further supporting this miRNA-mediated mechanism of Myc-induced suppression of Bcl-2 and Bcl-x\(_L\). Additionally, evaluation of ChIP-seq data for Miz-1 in four human and six murine cell lines/tissues \(^{31-34}\) showed Miz-1 enrichment at promoters of known Miz-1-regulated genes (e.g., \( VAMP4 \)) (Supplementary Fig. S7C). However, Miz-1 was not enriched at the miR-15 family or let-7a promoters in any of the cell lines/tissues evaluated, indicating Miz-1 does not transcriptionally regulate these miRNA.

To determine whether Myc transcriptional activity is required to decrease Bcl-2 and Bcl-x\(_L\) expression in normal cells, wild-type MEFs expressing MycER or the transcriptionally inactive Myc\(\Delta\)MBII-ER mutant were evaluated. Following 4-OHT addition, decreased Bcl-2 and Bcl-x\(_L\) and increased Bim expression were only observed in cells expressing wild-type MycER and not Myc\(\Delta\)MBII-ER (Fig. 6F). Again, Mcl-1 and Bax expression were unaffected. Therefore, Myc-mediated transcriptional activity is necessary for Bcl-2 and Bcl-x\(_L\) down-regulation.

**Myc transcriptionally induces the miR-15 family and let-7a that then target \( Bcl-2 \) and \( Bcl-x_L \)**

We reasoned that defining the Myc-induced mechanism of Bcl-2 and Bcl-x\(_L\) down-
regulation in normal cells would provide insight into the mechanism behind their down-regulation following HDACi. To test whether the reduction in Bcl-2 and Bcl-xL was a direct consequence of Myc-induced up-regulation of the miR-15 family and let-7a in untransformed cells, luciferase assays were performed in MycER-expressing fibroblasts with reporters harboring wild-type or mutated miRNA binding sites in the Bcl-2 or Bcl-xL 3’-untranslated region (3’-UTR). Following MycER activation, luciferase activity decreased in cells containing the reporter with wild-type miR-15 family or let-7a binding sites in the Bcl-2 or Bcl-xL 3’-UTR, respectively (Fig. 7A). Luciferase activity remained unchanged in cells containing reporters with mutated miR-15 family or let-7a binding sites or cells expressing the transcriptionally impaired MycΔMBII-ER mutant (Fig. 7A). A reporter containing the miR-17 family binding site of the p21 3’-UTR served as a positive control, as p21 is a validated target of the Myc-regulated miR-17 family (13). These data indicate a novel mechanism where Myc transcriptionally up-regulates the miR-15 family and let-7a, which then target the 3’-UTR of Bcl-2 and Bcl-xL, respectively, leading to their down-regulation in untransformed cells.

To further validate this mechanism, wild-type MEFs were transfected with modified RNA molecules (Target Protectors) designed to block the miR-15 family or let-7a from binding their specific target sites in the Bcl-2 or Bcl-xL 3’-UTR, respectively. Bcl-2 and Bcl-xL protein increased in wild-type MEFs transfected with Bcl-2 or Bcl-xL Target Protectors, respectively, as endogenous miR-15 family members and let-7a were unable to bind and inhibit their expression (Fig. 7B). Combining the Bcl-2 Target Protector with miR-15a overexpression, which alone decreased Bcl-2 protein, rescued Bcl-2 protein expression (Fig. 7B). Then, MycER-expressing fibroblasts were transfected with luciferase reporters, as described above, together with the Bcl-2 or Bcl-xL Target Protectors. In the presence of Bcl-2 or Bcl-xL Target Protectors, little, if any,
decrease in luciferase activity was detected following MycER activation (Fig. 7C). Next, MEFs expressing MycV394D-ER (unable to interact with Miz-1) were transfected with Bcl-2 or Bcl-xL Target Protectors. MycV394D-ER activation decreased Bcl-2 and Bcl-xL protein expression in the absence of any Target Protector (Fig. 7D). However, levels of Bcl-2 or Bcl-xL were maintained when Target Protectors blocked the miR-15 or let-7 family binding sites, respectively (Fig. 7D). Therefore, down-regulation of Bcl-2 and Bcl-xL upon Myc activation in untransformed cells was due to induction of the miR-15 family and let-7a that bind the 3’-UTR of Bcl-2 and Bcl-xL, respectively.

We then tested whether targeting of Bcl-2 and Bcl-xL by the miR-15 family and let-7a contributes to Myc-induced apoptosis, independent of p53. MycV394D-ER was activated in p53-null MEFs under reduced serum conditions with or without Target Protectors. MycV394D-ER-activated MEFs containing Target Protectors had increased cell expansion and reduced cleaved Caspase-3 and AnnexinV-positivity (Fig. 7D-F). Thus, Myc induces the expression of miR-15 family and let-7a independent of p53, leading to apoptosis. Collectively, the data reveal a novel mechanism whereby Myc up-regulates the miR-15 family and let-7a that target Bcl-2 and Bcl-xL in untransformed cells to trigger apoptosis, and that this mechanism is re-activated in lymphomas following HDACi.

**Discussion**

Although selective killing of tumor cells by HDACi is being clinically tested and multiple effects have been noted, such as altered expression of apoptotic genes and DNA damage (1-3), the mechanism(s) for its affects remains incompletely understood. Here we show HDACi
switches Myc from a repressor to an activator of miRNA that control the expression of Bcl-2 and Bcl-\(\text{x}_L\), significantly contributing to HDACi-mediated tumor cell death. We identified a mechanism of HDACi-induced apoptosis that occurs in Myc-driven B-cell malignancies and likely contributes to other human cancers. Our data provide evidence of a novel Myc-induced miRNA-mediated mechanism of apoptosis that is present in non-transformed cells, repressed in malignant cells, and reactivated in tumor cells upon HDACi.

It was previously postulated that HDACi kills myeloid leukemia cells through changes in expression of extrinsic apoptotic pathway proteins (35). Others have reported HDACi causes global changes in gene expression that alter the apoptotic threshold in favor of cancer cell killing (36). Specifically, expression profiling showed HDACi altered mRNA levels of pro- and anti-apoptotic Bcl-2 family members, yielding a pro-apoptotic signature in malignant cells (36). However, measuring stable pools of individual or multiple mRNA as an indirect readout of transcription would miss the post-transcriptional regulation of gene expression. Our data reveal increased miRNA transcription, rather than direct transcriptional repression, leads to down-regulation of anti-apoptotic \(Bcl-2\) and \(Bcl-\text{x}_L\) gene expression. Likewise, other groups have reported HDACi-mediated changes in miRNA expression (37-40). For example, HDACi of a breast cancer line changed the expression of 27 miRNA within 5 hours, including let-7a (37). However, they reported down-regulation of let-7a, whereas we detected increased let-7a upon HDACi; this discrepancy may be due to differences in the cell types evaluated or the HDAC inhibitors used. Importantly, our results indicate HDACi-induced changes in \(Bcl-2\) and \(Bcl-\text{x}_L\) were mediated by Myc. Myc is overexpressed and/or dysregulated in most human malignancies and is essential in cancers driven by other oncogenes, such as mutant Ras (4,41). Thus, our data, providing a molecular link between Myc, miRNA, and Bcl-2 and Bcl-\(\text{x}_L\), yield new insights into
the transforming action of Myc, and suggest how this pathway can be targeted therapeutically. Moreover, our work suggests the expression of these miRNA may be useful biomarkers for HDACi sensitivity.

Due to previous reports that Myc repressed miRNA in malignant cells (14,26), we were initially surprised when Myc induced the expression of the miR-15 family and let-7a in untransformed cells. However, Myc overexpression drives cancer cell proliferation, but triggers apoptosis in untransformed cells (4). Myc induces apoptosis by activating the p53 pathway and simultaneously down-regulating Bcl-2 and Bcl-xL mRNA expression through an indirect mechanism (7,8), reportedly involving Miz-1 (9). Myc expression did not change the half-life of Bcl-2 (7); therefore, we suspected a transcriptional or post-transcriptional mechanism. The miR-15 family and let-7a were known to target Bcl-2 and Bcl-xL, respectively, contributing to apoptosis (25), so we investigated whether a connection between Myc, these miRNA, and Bcl-2 and Bcl-xL down-regulation existed. Indeed, Myc suppressed the expression of Bcl-2 and Bcl-xL independent of its interaction with Miz-1 and of p53, which can itself transcriptionally repress Bcl-2 and Bcl-xL expression (42,43). Transcriptionally competent Myc was required for the reduction in Bcl-2 and Bcl-xL and the induction of the miR-15 and let-7 families. Moreover, open chromatin and activated RNA polymerase-II were observed at the miRNA promoters in Myc-overexpressing untransformed cells. Luciferase reporter assays confirmed that Myc induced the miR-15 family and let-7a, which directly targeted Bcl-2 and Bcl-xL 3’-UTRs, respectively. By inhibiting the miR-15 family from binding Bcl-2 and let-7a from binding Bcl-xL, the decrease in Bcl-2 and Bcl-xL following Myc activation was blocked. Combined, our data provide strong evidence that in untransformed cells, Myc induces the miR-15 family and let-7a that then target Bcl-2 and Bcl-xL, respectively, triggering apoptosis. These results reveal a novel miRNA-
mediated mechanism of tumor suppression that is activated in normal cells upon Myc dysregulation.

Our data indicate Myc transcriptionally activates the miR-15 family and let-7a in untransformed cells, while transcriptionally repressing them in transformed cells. In lymphoma cells, Myc was present at miR-15 family and let-7a promoters, which were closed and transcriptionally inactive, indicating Myc was likely mediating this repression, as had been reported (14). Our data from multiple hematopoietic malignancies indicate HDACs contributed to the repression of both miR-15 family clusters and let-7a, as repression was relieved following HDACi, resulting in transcriptional up-regulation of these miRNA, which required Myc. The HDAC3-selective inhibitor RGFP966 induced miR-15a and let-7a to an equal extent as the class-I HDAC inhibitor Depsipeptide and the more specific HDAC1/2/3 inhibitor RGFP963 did, suggesting HDAC3 may be the primary HDAC involved in mediating the repression. Consistent with these results, others have reported HDAC3 is specifically involved with repression of miR-29a/b/c and miR-15a/16-1 in B-cell and mantle-cell lymphoma lines, respectively (40,44). Moreover, Myc is reported to recruit HDAC3 to the promoters of protein-coding genes and miRNA to repress their expression (12,40,44). However, RGFP233, the HDAC1/2 inhibitor, also increased levels of miR-15a and let-7a, indicating they may also contribute to the repression.

Although how Myc and HDAC interactions contribute to tumorigenesis remains unresolved, our data suggest that Myc, together with HDACs, alter the epigenome leading to repression of miRNA and possibly other genes whose expression results in cancer cell apoptosis. HDACi relieves the transcriptional repression of the miR-15 and let-7 families in malignant hematopoietic cells, resulting in transcription of these miRNA, which targeted Bcl-2 and Bcl-xL, killing the cancer cells.
Furthermore, hematopoietic cell lines with either mutant or wild-type \( p53 \) showed analogous results, indicating HDACi-induced effects are independent of \( p53 \) status. Given the \( p53 \) pathway is inactivated and Myc is dysregulated in most human cancers (4,45), our results identify a new potentially therapeutic avenue to induce apoptosis that capitalizes on Myc and is independent of \( p53 \). Of note, overexpression of Bcl-2 and/or Bcl-\( x_L \) protected from HDACi-induced cell death (46-49), supporting our conclusion that miRNA targeting these genes leads to apoptosis. Our studies have identified a novel mechanism of Myc-induced apoptosis that capitalizes on miRNA to suppress the expression of crucial pro-survival proteins. While this mechanism is inactivated in malignancies through epigenetic alterations involving HDACs, we have shown it can be reactivated by HDACi. Our current study provides a novel mechanism that underlies HDACi-mediated cell death and offers new insights that should aid in improving cancer therapies.

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Figure Legends

Figure 1. HDACi reduces Bcl-2 and Bcl-xL expression and induces apoptosis. Cells remained untreated (UT) or received Depsipeptide (Depsi), RGFP966 (966), or vehicle (DMSO) control. (A) Following drug administration, proliferation (Alamar Blue; quadruplicate), cell number (triplicate), and viability (triplicate) were determined at the indicated intervals in murine (Eμ-\textit{myc}, EM330) and human (Daudi) lymphoma cells. (B, C) Protein and mRNA levels were evaluated by Western blot (B) and qRT-PCR (triplicate) (C), respectively. Cleaved Caspase-3 (CC3). mRNA expression was normalized to \textit{β}-\textit{Actin}. Error bars are SD for A (*p<0.01) and SEM for C (*p<0.03); p-values determined by comparison to DMSO.

Figure 2. HDACi reverses Myc-mediated repression of the miR-15 family and let-7a. Lymphoma cells were treated with Depsipeptide (Depsi), RGFP966 (966), or vehicle (DMSO) control. (A) Western blots in murine (EM330) and human (Daudi) lymphoma cells were performed. (B, C) Mature miRNA levels were determined by qRT-PCR (triplicate) in (B) human diffuse large B-cell lymphoma (DLBCL, Burkitt’s lymphoma), two normal lymph nodes, and purified B-cells from peripheral blood (PB) and spleen (Sp), and (C) six murine Eμ-\textit{myc} lymphomas, two pre-cancerous Eμ-\textit{myc} spleens, and purified splenic B-cells. (D, E) Lymphoma cells were treated with Depsipeptide (Depsi), RGFP966 (966), or vehicle (DMSO) for the time indicated. Mature miRNA (D) or pri-miRNA (E) levels were determined by qRT-PCR (triplicate) in Eμ-\textit{myc} (EM330) and Daudi lymphoma cells. Small RNA, \textit{RNU6b}, was used for qRT-PCR normalization for B-E. (F) After treatment with Depsi or vehicle (DMSO) for 4hrs, ChIP with anti-RNAPII-phosphorylated-Serine2 (RNAPII-p-Ser2) or isotype control (IgG) was performed followed by qRT-PCR (triplicate) for the indicated promoters or upstream regions.
(up; negative controls) in murine (EM330) and human (Daudi) lymphoma cells. Values are relative to their respective IgG control and input DNA. (G) EM330 lymphoma cells were infected with either empty retrovirus (vector) or retrovirus encoding the miR-15a/16-1 or let-7a/7f miRNA clusters (14). Western blotting (left) was performed; U, uninfected; CC3, Cleaved Caspase-3. Viability (center; triplicate) and the percentage of cells with sub-G1 DNA content (right; triplicate) were determined at the indicated intervals. Error bars are SEM for B-F. B and C, *p<0.01, lymphoma versus mean of all normals; D-F, *p<0.001, Depsi or 966 versus DMSO. Error bars are SD for G, *p<0.03, compared to empty vector.

**Figure 3. In vivo, HDACi increases miR-15 family and let-7a levels, inducing lymphoma cell death.** C57Bl/6 mice with subcutaneous Eμ-myc lymphoma tumors (EM330) that reached 200mm³, were treated with Depsipeptide (Depsi) or vehicle (DMSO) control (n=4/group). Tumors were harvested 24hrs later and levels of the indicated proteins (A) and histone acetylation marks (C) were determined by Western blot. (B) miRNA levels were assessed by qRT-PCR (triplicate), and RNU6b was used for qRT-PCR normalization. As a positive control, cultured EM330 lymphoma cells (in vitro) were treated with vehicle (DMSO; -) or Depsi (+). Apoptosis was measured by cleaved Caspase-3 (CC3), AnnexinV-positivity (triplicate) (D), and propidium iodide staining of sub-G1 (apoptotic) DNA (triplicate) (E). Error bars are SEM for B (*p<0.001, Depsi versus mean of all DMSO controls) and SD for D and E (*p<0.03, Depsi versus DMSO).

**Figure 4. Myc transcriptional activity is necessary to induce the miR-15 family and let-7a in non-transformed cells.** Mature miRNA (A-C) and pri-miRNA (D, E) levels were determined
by qRT-PCR (triplicate) and are normalized to \( RNU6b \) levels. (A) Pre-cancerous splenocytes from \( \varepsilon\mu\text{-}myc \) mice and wild-type (WT) non-transgenic littermates were evaluated (\( n=4/\text{group} \)). (B-E) MycER or Myc\textDelta MBII-ER was activated with 4-OHT or vehicle (EtOH) control at the indicated intervals in primary pre-B-cells (B, E) and MEFs (C, D). (F, G) Following ChIP with anti-Myc, anti-RNAPII-phosphorylated-Serine2 (RNAPII-p-Ser2), anti-H3K9K14ac, or isotype controls (IgG), qRT-PCR for the indicated promoters or regions upstream (up) Myc does not bind (negative controls) was performed (triplicate). (F) MycER-expressing \( p53^{-/-} \) MEFs received vehicle (EtOH; -) or 4-OHT (+) for 4hrs to induce MycER. (G) Splenocytes from wild-type (non-transgenic; Tg-) or pre-cancerous \( \varepsilon\mu\text{-}myc \) transgenic (Tg+) littermate mice. Values for qChIP are relative to their respective IgG control and input DNA. Error bars are SEM; *\( p<0.001 \); (A) \( \varepsilon\mu\text{-}myc \) versus mean of all WT spleens; (B, E, F) 4-OHT versus EtOH; (C, D) MycER versus Myc\textDelta MBII-ER, and (G) \( \varepsilon\mu\text{-}myc \) (+) versus wild-type (-).

**Figure 5. Myc is required for HDACi-induced transcriptional up-regulation of the miR-15 family and let-7a.** (A, B, D, E) Following ChIP with anti-Myc, anti-RNAPII-phosphorylated-Serine2 (RNAPII-p-Ser2), anti-H3K9K14ac, or isotype controls (IgG), qRT-PCR for the indicated promoters or regions upstream (up) Myc does not bind (negative controls) was performed (triplicate). (A) Murine (EM330) and human (Daudi) lymphoma cells treated with Depsipeptide (Depsi) or vehicle (DMSO) for 4hrs. (B) \( p21 \) (Myc repression target) and \( CAD \) (Myc activation target) were controls (11). Values for qChIP are relative to their respective IgG control and input DNA. (C) Human P493-6 lymphoma cells containing tetracycline-regulatable MYC (P493-6) exposed to tetracycline (+; MYC-OFF) for 24hrs or not (MYC-ON) were Western blotted. These cells were also treated for 12hrs with Depsi or vehicle (DMSO) control.
miRNA were measured by qRT-PCR (triplicate) and normalized to \( RNU6b \) levels. (D, E) Before qChIP analyses, P493-6 cells received tetracycline for 24hrs (+; MYC-OFF) or not (-; MYC-ON) prior to treatment with Depsipeptide (Depsi) or vehicle (DMSO) for 4hrs. Values for qChIP are relative to their respective IgG control and input DNA. Error bars are SEM; *\( p < 0.0002 \) (B), *\( p < 0.01 \) (E), both RNAPII-p-Ser2 and H3K9K14ac versus IgG (B), Depsi versus DMSO (C).

**Figure 6.** Myc transcriptional activity regulates Bcl-2 and Bcl-xL expression independent of Miz-1 and p53. (A) \( E\mu-my\) lymphomas (n=11) and pre-cancerous \( E\mu-my\) purified B-cells (n=2) and spleens (n=3) were Western blotted. (B) Pre-cancerous splenocytes from \( E\mu-my\) mice (n=5) and wild-type (WT) non-transgenic littermates (n=5) were Western blotted. (C, D, F) At intervals following addition of 4-OHT, wild-type (WT) MycER-expressing murine embryonic fibroblasts (MEFs) and primary pre-B-cells (C) and MycV394D-ER- (D) and MycΔMBII-ER-expressing (F) WT MEFs were harvested and Western blotted. (E) miRNA levels were determined by qRT-PCR (triplicate; normalized to \( RNU6b \) levels) following MycV394D-ER activation with 4-OHT or vehicle (EtOH). Error bars are SEM; *\( p < 0.001 \), 4-OHT versus EtOH.

**Figure 7.** Myc induces the miR-15 family and let-7a that then target Bcl-2 and Bcl-xL. (A, C) Luciferase expression vectors containing the 3’-untranslated region (3’-UTR) of Bcl-2 and Bcl-xL with the wild-type (WT) or a mutated (Mut) miRNA binding site were transfected into fibroblasts expressing the 4-OHT-inducible MycER or MycΔMBII-ER. An expression vector containing the miR-17 family binding site of the wild-type (WT) \( p21 \) 3’-UTR was a positive control (13). Luciferase activity was measured (triplicate) 48hrs following vehicle (EtOH) control or 4-OHT addition to activate MycER. A \( \beta \)-galactosidase reporter plasmid was co-

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transfected for normalization. (B-F) miR-15 family and let-7a miRNA binding sites in the Bcl-2 and Bcl-x<sub>L</sub> 3'-UTR were blocked with site-specific small molecules (TP; Target Protectors). (B) Wild-type MEFs transfected with either Bcl-2 or Bcl-x<sub>L</sub> Target Protectors (TP) and/or miR-15a mimic were Western blotted. UT, untransfected cells. (D-F) p53<sup>−/−</sup> MEFs, with or without Bcl-2 and/or Bcl-x<sub>L</sub> Target Protectors (TP), expressing the 4-OHT-inducible MycV394D-ER were Western blotted (D), subjected to MTT assay (E; quadruplicate), or analyzed for AnnexinV-positivity (triplicate) by flow cytometry at intervals following addition of 4-OHT. Cleaved Caspase-3 (CC3). Error bars are SEM (A and C, *p<0.009, 4-OHT versus EtOH) and SD (E, *p<0.02; F, *p<0.0001; both TP versus control).
Adams et al. Figure 1

A

Fluorescence (x 10^3)

Cell Number (x 10^6)

% Viability

Time (hr)

UT

DMSO

Depsi

966

B

Time (hr):

0 6 12 24 6 12 24 6 12 24

CC3

Bcl-2

Bcl-xL

Mcl-1

β-Actin

EM330

C

Relative expression

Time (hr)

Bcl-2

Bcl-xL

Mcl-1

EM330

D

Time (hr):

0 6 12 24 0 6 12 24 0 6 12 24

CC3

BCL-2

BCL-xL

MCL-1

β-Actin

Daudi

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A

Eμ-myc lymphomas

pre-cancerous Eμ-myc

Bcl-2
Bcl-xL
β-Actin

B

WT Eμ-myc

Bcl-2
Bcl-xL
Mcl-1
Bax
β-Actin

C

4-OHT (hr): 0 12 24
MycER

MycER

Bcl-2
Bcl-xL
Mcl-1
Bax
Bim
MycER

β-Actin

WT MEF pre-B

D

4-OHT (hr): 0 12 24
MycER V394DER

Bcl-2
Bcl-xL
Mcl-1
Bax
Bim
MycER or MycV394DER

β-Actin

WT MEF

E

Relative expression

15a
195
let7a
EtOH
20a
31

4-OHT

Time (hr) 0 3 6

F

4-OHT (hr): 0 12 24
MycER ΔMBIIER

Bcl-2
Bcl-xL
Mcl-1
Bax
Bim
MycER or MycΔMBIIER

β-Actin

WT MEF
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Myc induces miRNA-mediated apoptosis in response to HDAC inhibition in hematologic malignancies

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