THE CONTRIBUTION OF MIRNA BIOGENESIS AND MYC-REGULATED MIRNA IN APOPTOSIS AND TUMORIGENESIS

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To my Mom and Dad, my biggest cheerleaders.

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LIST OF ABBREVIATIONS

3’-OH	3-prime hydroxyl group
3’-UTR	3-prime untranslated region
4-OHT	4-hydroxytamoxifen
7-AAD	7-amino-actinomycin D
Ac	acetylation
Ago2	argonaute-2
AIF	apoptosis inducing factor
AML-ETO	fusion protein between acute myelogenous leukemia and eight twenty one
APAF1	apoptotic protease activating factor 1
APC	allophycocyanin
Arf	alternative reading from of the Ink4a locus/p19Arf
ATCC	American Type Culture Collection
ATM	Ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	Ataxia telangiectasia and Rad 3
B220	CD45R/B220 B cell antigen
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
Bcl-w	B cell lymphoma 2-like protein 2

Bcl-x_L	B cell lymphoma extra-large/B cell lymphoma 2-like protein 1
BR-HLH	basic helix-loop-helix
Bid	BH3 interacting-domain death agonist
Bim	Bcl-2-interacting mediator of cell death
bp	base pair
C57Bl/6	C57 black-6 mouse
Cad	carbamoyl-phosphate synthetase 2
CC3	cleaved Caspase 3
CD	cluster of differentiation
CD19	B cell antigen CD19/cluster of differentiation 19
CD3	cluster of differentiation 3
CD4	cluster of differentiation 4
CD43	leukosialin/sialophorin/cluster of differentiation 43
CD8	cluster of differentiation 8
CD95	Fas
CDKN1A	cyclin-dependent kinase inhibitor 1A
cDNA	complimentary DNA
C/EBPα	CCAAT/enhancer binding protein alpha
ChIP	chromatin immunoprecipitation
CLL	chronic lymphocytic leukemia
c-Myc	v-Myc avian myelocytomatosis viral oncogene, cellular homolog
$CreER^{T2}$	4-OHT-inducible cre recombinase/estrogen receptor fusion protein
CTCL	cutaneous T cell lymphoma

DBD	DNA binding domain
Depsi	depsipeptide/Romidepsin
DGCR8	DiGeorge syndrome critical region 8
DLBCL	diffuse large B cell lymphoma
DMEM	Dulbecco's Modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DLEU2	deleted in lymphocytic leukemia 2
Eμ	immunoglobulin heavy chain enhancer
EBV	Epstein-Barr virus
ENCODE	Encyclopedia of DNA Elements
EtOH	ethanol
ER	estrogen receptor
FACS	fluorescence activated cell sorting
FDA	food and drug administration
FITC	fluorescein isothiocyanate
G1	growth phase 1
G2	growth phase 2
GEO	Gene expression omnibus
GFP	green fluorescent protein
Gr-1	myeloid differentiation antigen Gr-1
H3	histone 3

H3K56ac	histone 3 with acetylation at lysine 56
H3K9K14ac	histone 3 with acetylation at lysines 9 and 14
H4	histone 4
H4K5ac	histone 4 with acetylation at lysine 5
HAT	histone acetyl transferase
HDAC	histone deacetyl transferase
HDACi	HDAC inhibition
HGP	Human Genome Project
IAP	inhibitor of apoptosis protein
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-7	interleukin-7
IP	immunoprecipitation
IRES	internal ribosome entry site
kDa/kD	kilodalton
L-Myc	v-Myc avian myelocytomatosis viral oncogene, lung carcinoma-derived homolog
LN	lymph node
LZ	leucine zipper
M	mitosis
Mac-1	antigen CD11b
Max	Myc-associated factor X
MB	Myc box
MC29	avian myelocytomatosis virus

MCL	mantle cell lymphoma
Mcl-1	myeloid cell leukemia 1
Mdm2	murine double minute 2
Me	methylation
MEF	murine embryonic fibroblast
MM	multiple myeloma
miRNA/miR	microRNA
Miz-1	Myc-interacting zinc finger protein 1
mRNA	messenger RNA
MSCV	murine stem cell virus
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-*(*4,5-dimethyl-2-thiazolyl*)*-2,5-diphenyl-2H-tetrazolium bromide
Mut	mutant
MycER	Myc estrogen receptor fusion protein
Ncl	nucleolin
NCoR	nuclear receptor corepressor
NF-Y	Nuclear transcription factor Y
NLS	nuclear localization sequence
N-Myc	v-Myc avian myelocytomatosis viral oncogene, neuroblastoma-derived homolog
Npm	nucleophosmin
OD	optical density
ODC	ornithine decarboxylase

p21	cyclin-dependent kinase 1A
p300	E1A binding protein p300
p53	tumor protein p53
PAZ	piwi/argonaute/zwilli
PB/PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PML-RARα	fusion protein between promyelocytic leukemia and the retinoic acid receptor alpha
PRC	polycomb repressive complex
pre	precursor
pri	primary
PRO-seq	precision nuclear run-on and sequencing
P-TEFb	positive transcription factor b
PUMA	p53 upregulated modulator of apoptosis
qRT-PCR	quantitative real-time polymerase chain reaction
Ras	rat sarcoma viral oncogene homolog
Rb	retinoblastoma protein
RFP	red fluorescent protein
RGFP	compounds provided by Repligen Corporation
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference

RNAPII	RNA polymerase II
RNAPII-p-Ser2	RNA polymerase phosphorylated at serine 2
RNAPII-p-Ser5	RNA polymerase phosphorylated at serine 5
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SAHA	suberoylanilide hydroxamic acid/Vorinostat
SAGA	Spt-Ada-Gcn5-Acetyltransferase
Sca-1	stem cell antigen 1
Seq	sequencing
shRNA	short-hairpin RNA
siRNA	short-interfering RNA
SMAC	second mitochondria-derived activator of caspases
SMRT	silencing mediator for retinoid or thyroid hormone receptors
Sp	spleen
SP1	Specificity protein 1
SV40	Simian vacuolating virus 40
SWI/SNF	SWItch/Sucrose non-fermentable
SYBR	Synergy Brands, Inc.
TAD	Transactivation domain
TALL	T cell acute lymphoblastic leukemia
Tam	tamoxifen
TCGA	The Cancer Genome Atlas
TF	transcription factor

Tg	transgenic
TP	Target Protector
TRBP	human immunodeficiency virus transactivating response RNA-binding protein
TRRAP	transformation/transcription domain-associated protein
TSA	trichostatin A
WB	western blot
WCL	whole cell lysate
WDR5	WD repeat-containing protein 5
WT	wild-type
XPO5	exportin-5
YY1	Yin yang 1

CHAPTER I

INTRODUCTION

Introduction to Cancer

Defining cancer

While there are many types of cancer, all cancers arise from cells that grow uncontrollably, disregarding the rules that govern growth and division. Normally, cells are exposed to signals that dictate whether the cell should divide, differentiate, or die; however, cancer cells develop autonomy to these signals. Each subsequent cell produced by division of the first cancer cell and its progeny also display uncontrollable cell growth, leading to the formation of a tumor. Ultimately, if the cancer cells continue to proliferate and invade surrounding tissue, a process known as metastasis, it can be fatal. In fact, metastatic disease accounts for as much as 90% (Chaffer and Weinberg, 2011) of cancer-related deaths. Therefore, the development of more advanced molecular biological techniques and therapies may help diagnose and treat potential cancers sooner, long before tumors spread.

Hallmarks of cancer

Cancers arise from the acquisition of changes that disrupt the cellular balance between cell division and quiescence. These changes are often the result of mutations in the genome or are triggered by external factors in the environment. While the accumulation of deleterious aberrations typically induces cell death, they can also provide cells with a

selective advantage, allowing them to multiply more rapidly than normal cells. Even though cancer is a multi-gene, multi-step disease, almost all cancers share a common set of characteristics. Reviewed by Drs. Hanahan and Weinberg in 2000 and revised in 2011, the hallmarks of cancer (Figure 1) describe the regulatory circuits of a cell and how common defects in these networks alter cell physiology in favor of cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

Normal cells are hardwired with crucial defense mechanisms that are breached through the process of transformation into a cancer cell. The fundamental hallmarks, or characteristics used to simplify the complexities of cancer include, self-sufficiency in growth signals, insensitivity to anti-growth signals, resisting cell death, enabling limitless replicative potential, sustained angiogenesis, and activating tissue invasion and metastasis (Hanahan and Weinberg, 2000). In the last decade, two additional and potentially general hallmarks have been implicated in the pathogenesis of some, if not all, cancers. Specifically, deregulation of cellular energetics to reprogram metabolism and the ability to avoid immune destruction have been described as "emerging hallmarks" (Hanahan and Weinberg, 2011). These cancer-promoting functions, which are acquired in multiple tumor types by different mechanisms at different times, allow cancer cells to survive, divide, and spread. More recently, it has become appreciated that the acquisition of these alterations is facilitated by two "enabling characteristics," including the development of genome instability and tumor-promoting inflammation (Hanahan and Weinberg, 2011). Furthermore, aside from cancer cells, tumors are suggested to harbor a battery of normal cells recruited to facilitate the acquisition of hallmark characteristics by creating a more favorable "tumor microenvironment" (Hanahan and Weinberg, 2011). Trying to simplify

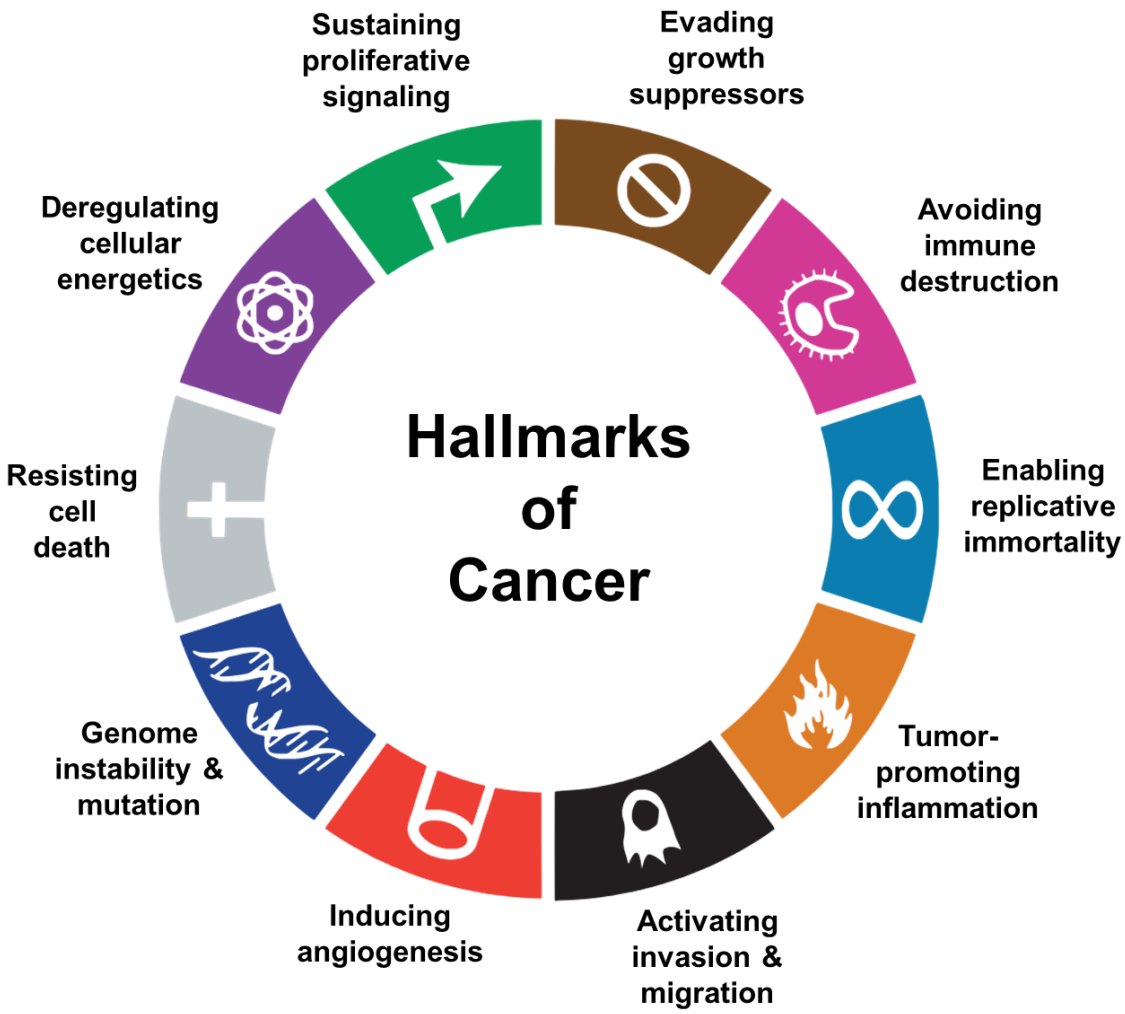

Figure 1. Hallmarks of cancer. The complexity of cancer can be reduced to key biological features ("hallmarks") acquired during the multi-step process of tumor development. Adapted from Hanahan and Weinberg, 2011.

and dissect the pathways involved in cancer initiation and progression have been vital in understanding this disease.

Cancer genetics

While the Human Genome Project (HGP) has revealed that there are about 20,500 genes in the human genome (National Human Genome Research Institute website), only a small number of genes have been associated with cancer (Watson *et al.*, 2013). Each type of cancer can likely be characterized by numerous somatic mutations, but only a subset of these mutations contribute to tumorigenesis (Watson *et al.*, 2013). As such, alterations in a single gene are often associated with multiple forms of cancer. Several of the genes that contribute to cancer development fall into three main categories: oncogenes, tumor suppressors, and DNA repair genes. Oncogenes, such as Myc and Ras, are the derivatives of normal cellular genes known as proto-oncogenes, which produce protein products that drive cell cycle progression. The aberrant forms of these genes, oncogenes, become hyperactive, resulting in excessive cellular proliferation. The conversion of proto-oncogenes into oncogenes typically occurs by mutation, chromosomal rearrangement, viral integration, and amplification (Lee and Muller, 2010). In contrast, tumor suppressor genes like p53 and Rb are essential to limit normal cell division, repair errors in the DNA, and dictate whether a cell lives or dies. Tumor suppressor genes can become functionally inactive through loss-of-function mutation, inhibition by negative regulators, or deregulation from post-translational modifications (i.e., phosphorylation status), all of which disrupt the protective role of tumor suppressors and increase the proliferative capacity of the cell (Lee and Muller, 2010). A third type of cancer-associated genes are

those that repair damaged DNA and maintain proper chromosomal architecture, including Brca1, Brca2, and Xp. DNA damage is not only the result of various environmental factors (i.e., ionizing radiation, UV light, chemicals), but can also occur as a result of errors during DNA replication. If DNA is not properly repaired, further mutations can accumulate, increasing the frequency of cancer-promoting alterations in the cell. Despite our increasing knowledge on how cancer genes function, many cancers cannot be linked to a single gene or set of genes. Cancer involves the progressive accumulation of mutations in multiple genes, adding further complexity to the disease.

Myc

History

In the late 1970s, the first *Myc* gene was identified from the avian acute oncogenic retrovirus MC29 (myelocytomatosis) (Duesberg *et al.*, 1977). The transformative retroviral oncogene responsible for causing myelocytomatosis in chickens was identified through hybridization studies and the resulting DNA sequence was termed v-*Myc* (Mellon *et al.*, 1978; Sheiness *et al.*, 1978). The same sequences were identified in DNA of non-infected cells, suggesting that viral oncogenes could be incorporated into normal cellular DNA (Roussel *et al.*, 1979; Sheiness and Bishop, 1979). In 1982, the cellular v-*Myc* homolog, c-*Myc* (hereafter referred to as Myc), was cloned and characterized (Vennstrom *et al.*, 1982). Shortly thereafter, the human *MYC* gene was reported to be deregulated in Burkitt's lymphoma, an aggressive non-Hodgkin B cell lymphoma that results from a t(8;14) chromosomal translocation placing *MYC* under the control of the immunoglobulin (Ig)

locus (Dalla-Favera *et al.*, 1982a; Neel *et al.*, 1982; Taub *et al.*, 1982). These reports revealed that an endogenous human proto-oncogene possessed transformative capacity without being mutated. Reciprocal chromosomal translocations involving one of the Ig loci and a proto-oncogene are characteristic of many types of B cell lymphoma (Kuppers and Dalla-Favera, 2001; Willis and Dyer, 2000). The Eμ-*myc* mouse model genetically recapitulates Burkitt's lymphoma, which has proven to be a crucial tool for studies of Myc-driven B cell lymphomagenesis (Adams *et al.*, 1985). To this day, MYC is one of the most important proteins in cancer biology, as its copy number, expression, stability, and/or activity is dysregulated in at least 70% of all human cancers (Tansey, 2014).

It is estimated that at least 15% of all human genes are regulated by Myc (Dang *et al.*, 2006; Zeller *et al.*, 2006). One of the earliest phenotypes associated with Myc expression was its ability to transform cells. Specifically, expression of Myc, in the presence of an additional activated oncogene (Ras), was the key factor that allowed for the transformation of embryonic fibroblasts (Land *et al.*, 1983). Studies using transgenic mouse models showed dysregulation of Myc expression is sufficient to drive tumorigenesis in different tissues (Adams *et al.*, 1985; Chesi *et al.*, 2008). In addition, cancer cells become "addicted" to continuous Myc overexpression, as turning Myc "off" or disabling Myc resulted in growth arrest, apoptosis, or differentiation (Felsher, 2010; Felsher and Bishop, 1999; Jain *et al.*, 2002; Marinkovic *et al.*, 2004; Pelengaris *et al.*, 1999; von Eyss and Eilers, 2011). Similar results were obtained when endogenous Myc was inhibited in tumors driven by other oncogenes (i.e., KRAS-G12D or SV40 viral antigen) (Sodir *et al.*, 2011; Soucek *et al.*, 2008). These results suggest that Myc is not only important for tumor initiation, but also tumor maintenance. Furthermore, Myc has a crucial role in development as evidenced

by the death of *Myc* knockout mice at embryonic day E9.5 (Davis *et al.*, 1993). This early lethality is likely due to the fact that Myc regulates diverse cellular processes, including cell growth and metabolism, proliferation, apoptosis, and differentiation (Tansey, 2014). While the multi-functional Myc protein has certainly proved fascinating, it has also added tremendous complexity to the field of cancer biology.

Structure and transcriptional regulation by Myc

In mammals, Myc (or c-*Myc*) is the most characterized member of the proto-oncogenic transcription factor family comprised of c-Myc, N-Myc, and L-Myc (Brodeur *et al.*, 1984; Nau *et al.*, 1985). Alignment of the Myc family members across multiple species has illustrated the presence of highly conserved domains within the proteins, termed Myc boxes (MB). Currently, six distinct regions of conservation have been appreciated: five Myc boxes (MBI, MBII, MBIIIa, MBIIIb, MBIV) and a basic helix-loop-helix leucine zipper (BR-HLH-LZ) region (Tansey, 2014) (Figure 2). A transcriptional activation domain (TAD) is located at the amino-terminus of Myc. When fused to a heterologous DNA binding domain, the TAD of Myc was sufficient for transcriptional activation (Kato *et al.*, 1990). The TAD of Myc is also a "degron," as it serves to signal the rapid degradation of Myc by ubiquitin-mediated proteolysis (Salghetti *et al.*, 1999).

Myc box I is located within the TAD and is required for cellular transformation in certain cellular contexts (Herbst *et al.*, 2005; Stone *et al.*, 1987). This region is also where Myc interacts with P-TEFb (positive transcription elongation factor b), a cyclin-CDK complex responsible for phosphorylating RNA polymerase II to trigger transcriptional elongation (Eberhardy and Farnham, 2001; Eberhardy and Farnham, 2002). MBI is also a

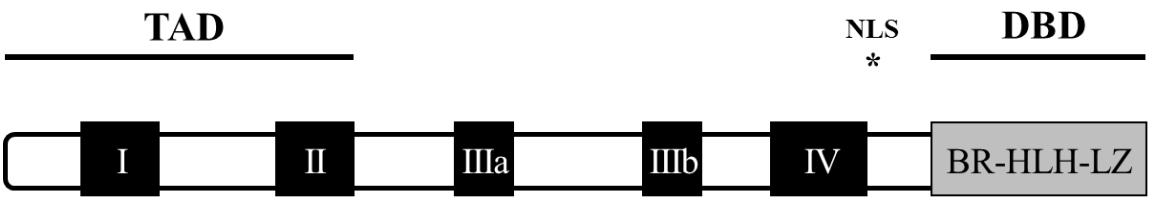

Figure 2. Conserved regions of Myc. Diagram representing the conserved amino acid sequences of Myc. The Myc Boxes I, II, IIIa, IIIb, and IV are represented by black boxes. MBI and MBII comprise the transcriptional activation domain (TAD). The C-terminal basic helix-loop-helix leucine zipper (BR-HLH-LZ; grey box) forms the DNA binding domain (DBD). The location of the nuclear localization sequence (NLS) is marked with an asterisk (*). Regions are drawn to scale.

site for a number of phosphorylation events that affect the stability of Myc (Lutterbach and Hann, 1994; Welcker *et al.*, 2004a; Welcker *et al.*, 2004b; Welcker *et al.*, 2003). Also located in the TAD is MBII, which is necessary for Myc to promote cellular transformation *in vitro* (Stone et al., 1987), promote tumor development *in vivo* (Hemann et al., 2005), and for transcriptional activation (Zhang *et al.*, 2006) and repression (Herbst *et al.*, 2005; Li *et al.*, 1994) of most Myc target genes. Association with the transcriptional coactivator TRRAP (transactivation/ transformation-associated protein) occurs through MBII (McMahon *et al.*, 1998). TRRAP binding is necessary for the subsequent recruitment of histone acetyltransferase (HAT) complexes that open chromatin to promote transcription of Myc-bound genes (Frank *et al.*, 2003; McMahon *et al.*, 2000). MBIII is required for both *in vitro* and *in vivo* transformation (Herbst *et al.*, 2005). This domain of Myc participates in destruction of Myc by the proteasome following ubiquitination of Myc (Herbst *et al.*, 2004). In addition, MBIII facilitates the recruitment of histone deacetylase 3 (HDAC3), which aids in Myc-mediated transcriptional repression (Kurland and Tansey, 2008). Recently, it was reported that recognition of Myc target genes by Myc depends on its interaction with a WD40-repeat protein, WDR5, which is mediated through the MBIII (Thomas *et al.*, 2015). Myc box IV is the least-studied of the Myc boxes. The pro-apoptotic functions of Myc require the presence of the MBIV; however, the ability of Myc to drive cell growth is not affected (Cowling *et al.*, 2006). Unfortunately, structural information for Myc outside its BR-HLH-LZ and TAD regions is limited.

At the carboxy-terminus of Myc lies a basic-helix-loop-helix leucine-zipper (BR-HLH-LZ) domain, which allows for sequence-specific binding of DNA upon hetero-dimerization with its obligate partner Max (Blackwell *et al.*, 1990; Blackwood and

Eisenman, 1991; Prendergast and Ziff, 1991). The canonical binding site is comprised of a CACGTG motif, known as an enhancer-box or E-box, or similar derivative (Blackwell *et al.*, 1993; Blackwell *et al.*, 1990). Heterodimerization between Myc and Max is widely accepted as a prerequisite for Myc function (Amati *et al.*, 1993a; Amati *et al.*, 1993b).

Since Myc exerts its biological effects as a transcription factor, much interest has been generated in identifying individual genes or collections of genes that are transcriptionally regulated by Myc (Kim *et al.*, 2008; Mao *et al.*, 2003; Seitz *et al.*, 2011). However, with the advent of next-generation sequencing technologies providing massive amounts of genome-wide DNA binding and gene expression data, the role of Myc in transcriptional control has grown in complexity. Myc was recently reported to increase cell size (Loven *et al.*, 2012), leading to amplification of transcription at every active gene in a given cell, seemingly without any specificity (Lin *et al.*, 2012; Nie *et al.*, 2012). While the amplification model provides an additional perspective about the actions of Myc in transcriptional regulation, this model stands in opposition with what has historically been accepted in the Myc field, which is that Myc can selectively regulate (either positively or negatively) gene expression (Cleveland *et al.*, 1988; Dang, 2014; Eilers and Eisenman, 2008; Herkert and Eilers, 2010; Kato *et al.*, 1990; Li *et al.*, 1994).

In 2014, two studies offered an alternative to the recently proposed amplifier hypothesis, returning to the idea that Myc can selectively regulate gene expression (Sabo *et al.*, 2014; Walz *et al.*, 2014). In both of these studies, cell lines in which Myc expression did not alter cell size were assessed so that any changes in transcription following Myc induction might highlight genes directly targeted by Myc (Sabo *et al.*, 2014; Walz *et al.*, 2014). Furthermore, the amplification model does not address whether the response of all

Myc-bound genes is a direct result of Myc transcriptional activation, or whether the widespread effects are due to indirect signaling. This model also does not account for the ability of Myc to function as a transcriptional repressor. A possible explanation of the difference between the models is how the data was normalized. For instance, in the amplifier model, normalization of the RNA-seq data was based solely on the amount of RNA per cell. However, to correctly account for relative changes in gene expression that could be due to broad effects such as RNA amplification, a combination of normalizing RNA profiles to reference house-keeping genes or average expression in addition to cell-based RNA levels are necessary to accurately evaluate transcriptional changes that occur in the face of RNA amplification (Kress *et al.*, 2015). Furthermore, it is proposed that the Myc-induced global upregulation of mRNA production is an indirect effect that could be attributed to the targets that Myc regulates, including proteins involved in nucleotide synthesis that exert cell-wide effects on RNA and DNA synthesis (Dang, 2014; Sabo *et al.*, 2014).

For transcriptional activation, Myc-Max heterodimers bind canonical (E-boxes) and non-canonical DNA sequences (Blackwell *et al.*, 1993; Blackwell *et al.*, 1990). Upon DNA binding, Myc associates with TRRAP, which allows for subsequent recruitment of histone acetylation complexes (HATs), including GCN5, TIP60, PCAF, p300/CBP, and TBP (Luscher and Vervoorts, 2012; Meyer and Penn, 2008) (Figure 3A). These protein complexes deposit acetyl groups onto lysine residues of histones, resulting in relaxed and open chromatin that favors binding of transcription initiation complexes (Cowling and Cole, 2006). Following transcription initiation, Myc recruits PTEF-b to phosphorylate the C-terminus of the RNA polymerase II, promoting transcriptional elongation (Eberhardy

and Farnham, 2001; Eberhardy and Farnham, 2002) (Figure 3B). Myc, through its ability to promote transcription by all three RNA polymerases (Gomez-Roman *et al.*, 2003; Grandori *et al.*, 2005), is hypothesized to regulate the transcription of at least 15% of the genome (Dang *et al.*, 2006; Zeller *et al.*, 2006). Therefore, it is surprising that Myc is considered to be a relatively "weak" transcription factor, as it typically elicits modest transcriptional changes on its target genes (Tansey, 2014).

Although Myc transcriptional activation has been the most studied form of Myc transcriptional regulation, Myc also functions as a transcriptional repressor (Eilers and Eisenman, 2008; Herkert and Eilers, 2010). Though less well understood, Myc represses the expression of genes that inhibit growth and tumorigenesis (Herkert and Eilers, 2010). The most well-characterized mechanism of Myc-mediated repression is through its association with Myc-interacting zinc finger 1 (Miz-1) (Herkert and Eilers, 2010; Peukert *et al.*, 1997) (Figure 3C). Miz-1 is a transcriptional activator that binds DNA at enhancer or initiator elements near transcriptional start sites of genes. However, when Myc-Max complexes interact with Miz-1, obligate activating factors such as the histone acetyltransferase p300 are displaced and repression cofactors such as the DNA methyltransferase Dnmt3a are recruited (Adhikary *et al.*, 2005; Brenner *et al.*, 2005; Mao *et al.*, 2004; Staller *et al.*, 2001). This interaction has been observed in a variety of cellular contexts (Brenner *et al.*, 2005; Gebhardt *et al.*, 2006; Patel and McMahon, 2006; Patel and McMahon, 2007; Schneider *et al.*, 1997; Staller *et al.*, 2001; van Riggelen *et al.*, 2010a). This anti-activation model through which Myc-Max heterodimers interact with Miz-1 has also been observed for SP1 (Gartel *et al.*, 2001), YY1 (Shrivastava *et al.*, 1993), NF-Y (Izumi *et al.*, 2001), and C/EBPα (Steinmann *et al.*, 2009) transactivator proteins.

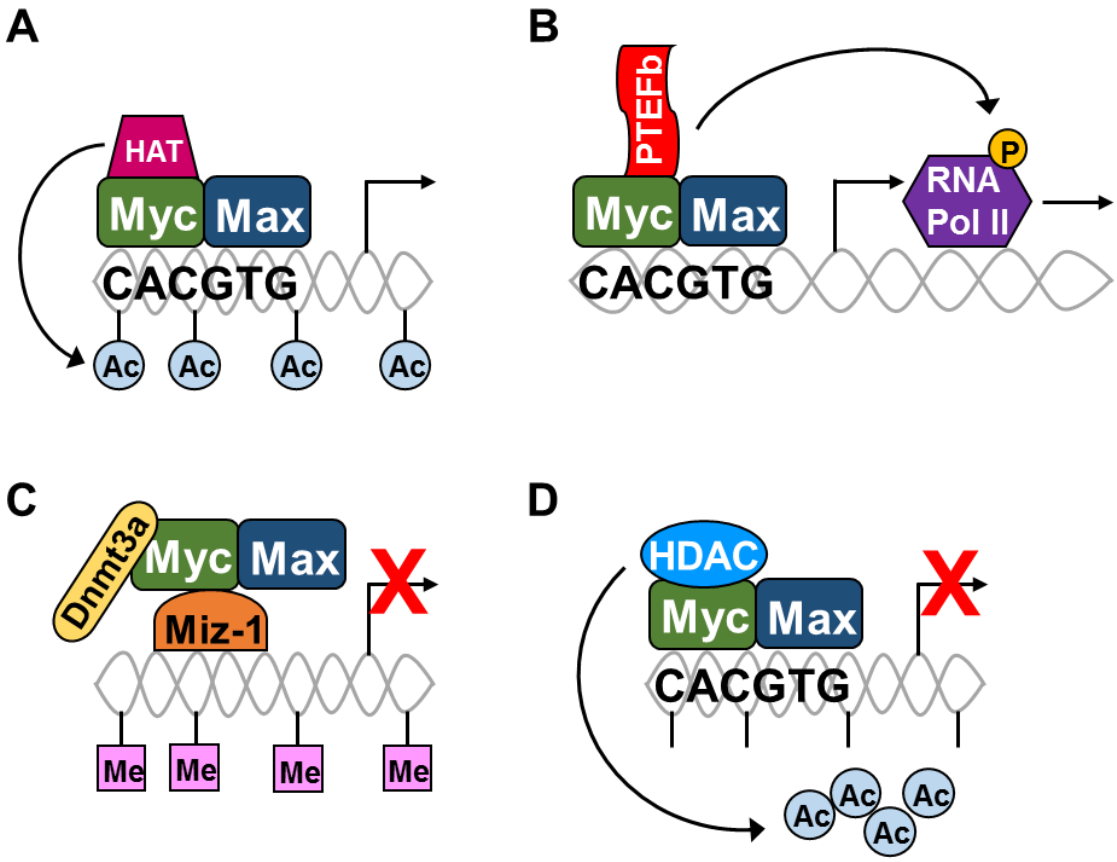

Figure 3. Myc transcriptional activation and repression. (A, B) For transcriptional activation, Myc associates with its obligate partner Max and binds DNA sequences called E-boxes (CACGTG) where it recruits transcriptional cofactors, such as histone acetyltransferase enzymes (HATs) to acetylate and open the chromatin (A) and positive elongation factor (P-TEFb), which phosphorylates paused RNA polymerase II (RNA Pol II) to promote transcriptional elongation (B). (C, D) For transcriptional repression, Myc binds transcription factors like Miz-1, displaces activating cofactors, and recruits transcriptional repressors, such as the DNA methyltransferase Dnmt3a (C). Myc also recruits histone deacetylase enzymes (HDACs) to remove acetyl groups and close chromatin to repress transcription (D). Ac, acetylation; P, phosphate; Me, methylation.

Repression by Myc can also occur through recruitment of histone deacetylase enzymes (HDACs) (Figure 3D). Specifically, Myc, through Myc box III, recruits HDAC3 to target genes, resulting in histone deacetylation and closing of the chromatin to inhibit transcription at that site (Kurland and Tansey, 2008). Repression of gene transcription though recruitment of HDAC3 has been reported for protein-coding genes and microRNA (Kurland and Tansey, 2008; Zhang *et al.*, 2012a; Zhang *et al.*, 2012b). In addition, reports of HDAC-mediated repression through Myc have also been reported for HDAC1 (Jiang *et al.*, 2007) and HDAC5 (Sun *et al.*, 2014). Furthermore, genome-wide studies have demonstrated that Myc likely represses as many genes as it activates (O'Connell *et al.*, 2003), setting Myc apart from most canonical transcription factors and adding further emphasis on the complexity of Myc.

Biological activities of Myc

Many functions of Myc have been exposed by linking its altered expression to uncontrolled cell proliferation and growth, differentiation, angiogenesis, metabolism, and apoptosis. The combination of in-depth analyses of Myc-regulated protein-coding and noncoding gene expression profiles, genome-wide DNA binding of Myc, and Myc-related DNA methylation patterns have directly linked Myc to several pathways that collectively contribute to tumorigenesis (Figure 4).

Proliferation and cell cycle

Myc is an essential gene for normal growth and development. It is expressed in numerous tissues and correlates with proliferation, as evidenced by the large number of

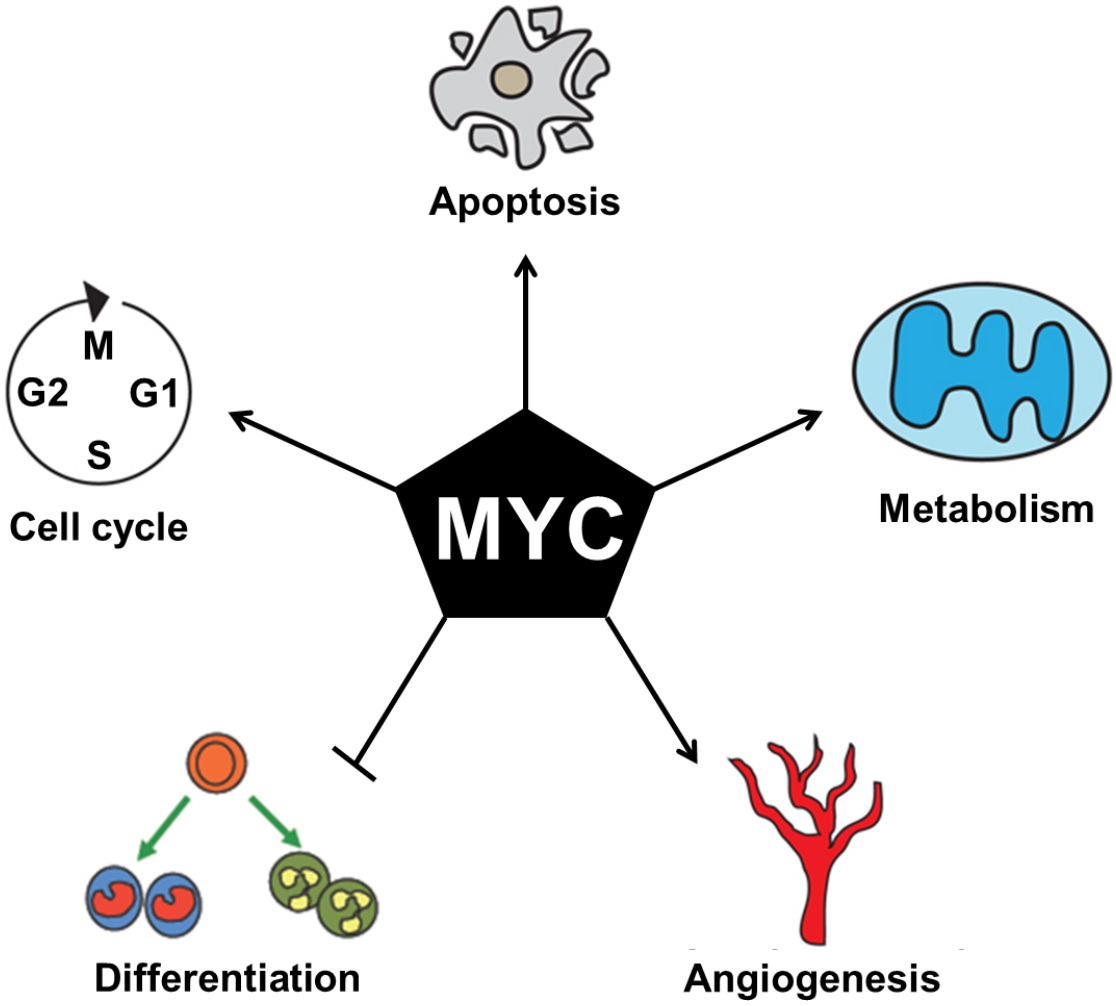

Figure 4. MYC functions in diverse cellular processes. MYC regulates the expression of many genes, including those that are involved in cellular metabolism, angiogenesis, differentiation, cell cycle control, and apoptosis. Adapted from Bui and Mendell, 2010.

growth factors and mitogens that activate its expression (Kelly *et al.*, 1983; Morrow *et al.*, 1992; Obaya *et al.*, 1999; Shibuya *et al.*, 1992). However, during development, reduced levels of Myc can induce cell cycle arrest and differentiation (Hirning *et al.*, 1989; Hirvonen *et al.*, 1990; Schmid *et al.*, 1989). Activation of Myc is sufficient to initiate DNA synthesis and, even in the absence of mitogenic stimuli, Myc is sufficient to drive cell cycle re-entry of quiescent fibroblasts (Eilers *et al.*, 1989; Eilers *et al.*, 1991; Littlewood *et al.*, 1995; Trumpp *et al.*, 2001). Moreover, deletion of *Myc* in murine embryonic stem cells resulted in mid-gestation lethality (Davis *et al.*, 1993). Furthermore, *Myc*-deficient rat fibroblasts proliferate at a reduced rate and experience a delay in phosphorylation of the retinoblastoma protein (Rb), causing defects in the cell cycle at the G1 phase (Mateyak *et al.*, 1997).

The restriction point, or the point at which the cell decides between quiescence and proliferation, occurs in the G1 phase of the cell cycle and is governed by the phosphorylation state of Rb (Blagosklonny and Pardee, 2002; Pardee, 1989). Myc expression is crucial for efficient transition from G0/G1 to S phase (de Alboran *et al.*, 2001; Obaya *et al.*, 1999; Roussel *et al.*, 1991). In order to promote cell cycle progression, Myc transcriptionally activates certain cell cycle components, such as cyclin D1, cyclin D2, cyclin E1, cyclin A2, cyclin-dependent kinase 4, and cell division cycle 25A (Cdc25a), and it also transcriptionally represses cell cycle inhibitors, including p15, p21, and p27 (Beier *et al.*, 2000; Bouchard *et al.*, 1999; Gartel *et al.*, 2001; Hermeking *et al.*, 2000; Meyer and Penn, 2008; Muller *et al.*, 1997; Obaya *et al.*, 1999; Perez-Roger *et al.*, 1999; Roussel *et al.*, 1991; Staller *et al.*, 2001). Similarly, Myc directly induces the transcription factors E2F1, E2F2, and E2F3 (Adams *et al.*, 2000; Fernandez *et al.*, 2003; Sears *et al.*, 1997).

Myc also prolongs the proliferative capacity of the cell by activating the transcription of the human telomerase gene (hTERT) (Greenberg *et al.*, 1999).

Differentiation

Early reports revealed that dysregulated Myc expression could block the differentiation of diverse precursor cells into more specialized cell types (Freytag, 1988; Miner and Wold, 1991). Similarly, Myc inactivation in hepatocellular carcinoma cells resulted in the de-differentiation of tumor cells into precursor-like hepatocytes (Shachaf *et al.*, 2004). When levels of Myc decrease, its obligate partner Max often dissociates from Myc and heterodimerizes with Mad family proteins associated with chromatin, thereby decreasing the expression of target genes with de-differentiating properties (Rottmann and Luscher, 2006; Xu *et al.*, 2001). Moreover, Myc functions in stem cell biology where it helps maintain a balance between self-renewal and differentiation (Wilson *et al.*, 2004). Notably, Myc is one of the four defined "Yamanaka factors" (Oct3/4, Sox2, Klf4, and Myc) that are sufficient to induce the reprogramming of terminally differentiated cells into an earlier, embryonic-like state (Takahashi and Yamanaka, 2006).

Angiogenesis

Myc is essential for vasculogenesis and angiogenesis during development and tumorigenesis (Baudino *et al.*, 2002). The early embryonic lethality observed in *Myc*-null mice was due, in part, to marked defects in neovascularization and erythropoiesis (Baudino *et al.*, 2002). Defects in vasculogenesis and angiogenesis are attributed to altered Myc expression, as Myc is required for the proper expression of angiogenic factors, HIF-1α,

and VEGF (Baudino *et al.*, 2002; Brandvold *et al.*, 2000; Fernandez *et al.*, 2003; Tikhonenko *et al.*, 1996). Myc signaling is a critical component of tumorigenesis, as angiogenesis must occur to ensure exponential tumor growth.

Metabolism

To fulfill the bioenergetic and biosynthetic demand that comes with increased proliferation from oncogenic stimuli, cells must undergo metabolic reprogramming (Dang, 2013). When oncogenic signaling from Myc stimulates cells to grow, glycolysis and glutaminolysis are up-regulated by increasing the expression of glucose and amino acid transporters, as well as glutamine importers (Dang, 2013; Kim *et al.*, 2007; Kim *et al.*, 2004; Lewis *et al.*, 1997; Osthus *et al.*, 2000; Shim *et al.*, 1997; Vander Heiden, 2011; Wise *et al.*, 2008). Upon mitogenic stimulation, new protein synthesis is one of the earliest events that occurs. Therefore, the assembly of new ribosomal complexes must be efficiently and quickly coordinated. Consequently, Myc transcriptionally activates the expression of ribosomal proteins and nucleolar proteins necessary for the assembly of ribosomes, including nucleophosmin (Npm) and nucleolin (Ncl) (Iritani and Eisenman, 1999; van Riggelen *et al.*, 2010b). In addition, Myc can regulate gene expression mediated by all three RNA polymerases, which increases the production of mRNA, rRNA, and tRNA (Arabi *et al.*, 2005; Kenneth *et al.*, 2007). Essential metabolic processes including the synthesis of fatty acids and pyrimidine nucleotides are also modulated by Myc levels, as it regulates the transcription of crucial genes in these processes, including fatty acid synthase (*Fasn*), carbamoyl-phosphate synthetase 2 (*Cad*), and ornithine decarboxylase (*Odc*) (Miltenberger *et al.*, 1995; Shah *et al.*, 2006; Wagner *et al.*, 1993). Myc has demonstrated

an ability to quickly and efficiently foster an environment for cells to undergo rapid expansion by enhancing new protein synthesis and reprogramming the cellular metabolic capacity.

Apoptosis

In the early 1990s, dysregulated Myc expression was reported to promote apoptosis of normal cells (Askew *et al.*, 1991; Evan *et al.*, 1992; Shi *et al.*, 1992). Apoptosis is characterized by defined morphological changes, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation, and degradation of chromosomal DNA. It was determined that with insufficient survival factors, deregulated Myc expression sensitized cells to undergo cell death as a mechanism to counter Myc hyperactivity (Askew *et al.*, 1991; Harrington *et al.*, 1994). This characteristic of Myc was later determined to be relevant for other oncogenes, including E2F1 and E1A (Meyer and Penn, 2008). Myc also sensitizes cells to apoptosis following treatments with other stimuli, including genotoxic agents (Evan *et al.*, 1992), TNF-α (Janicke *et al.*, 1994; Klefstrom *et al.*, 1994), and CD95 (Fas) (Hueber *et al.*, 1997).

Myc can induce apoptosis through a number of mechanisms. The most well-characterized mechanism is mediated by the Arf-p53-Mdm2 tumor suppressor pathway (Hermeking and Eick, 1994; Wagner *et al.*, 1994) (Figure 5). This pathway serves a critical role in monitoring and sensing various cellular stress signals, including oncogenic hyperactivity, and mediates a myriad of cellular responses to counter the increased stress. Myc induces the tumor suppressor encoded by the alternative reading frame of the *Ink4a* locus, Arf, which binds to and inhibits the E3-ligase murine double minute 2 (Mdm2), a

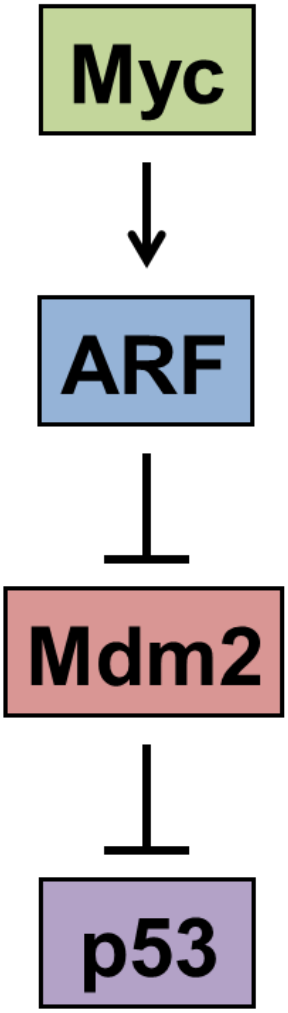

Figure 5. Arf-Mdm2-p53 tumor suppressor pathway. Sustained and increased signaling from Myc induces Arf expression. Once expressed, Arf directly binds to and interferes with the E3 ubiquitin ligase activity of Mdm2, a negative regulator of p53. This leads to p53 stabilization and activation of a complex transcriptional network of genes regulated by p53.

negative regulator of p53 (Zindy *et al.*, 1998). This, in turn, allows p53 to transcriptionally activate various target genes involved in promoting cell cycle arrest (i.e., p21) or apoptosis (i.e., Puma) (Vousden and Prives, 2009).

In normal, unstressed cells, p53 is tightly regulated and is maintained at low levels to allow for proper growth and development. p53 is rapidly degraded by the ubiquitin-dependent proteasomal pathway (Brooks and Gu, 2006) and its transcription is down-regulated through a negative feedback loop involving the Mdm2 E3-ubiquitin ligase and the related protein Mdm4 (also known as Mdmx). Increased Myc activity can also induce DNA double strand breaks (Prochownik, 2008). Double strand breaks in the DNA activate the serine/threonine kinase ataxia telangiectasia mutated (ATM), which phosphorylates and stabilizes p53 (Hong *et al.*, 2006; Lindstrom and Wiman, 2003; Pusapati *et al.*, 2006). The ability of Myc to induce the p53 pathway is a critical safeguard to prevent tumorigenesis. As such, it is not surprising that the majority of human cancers, irrespective of the cell type or underlying oncogenic stimulus, have developed mechanisms to inactivate this tumor suppression pathway (Eischen and Lozano, 2014).

Myc can also trigger cell death by disturbing the balance of proteins in the B cell lymphoma 2 (Bcl-2) family (Delbridge and Strasser, 2015). The Bcl-2 family consists of proteins that both promote and inhibit cell death. Under normal conditions, cellular stress signals, such as chemotherapy, irradiation, nutrient deprivation, and oncogenic activation, induce apoptosis through the intrinsic apoptotic pathway, which is also known as the mitochondrial or Bcl-2 apoptotic pathway (Figure 6). These stimuli activate BH3 (Bcl-2 homology 3)-only proteins Bcl-2-associated death promoter (BAD), Bcl-2-interacting mediator of cell death (BIM), and Bcl-2 homology 3-interacting domain death agonist

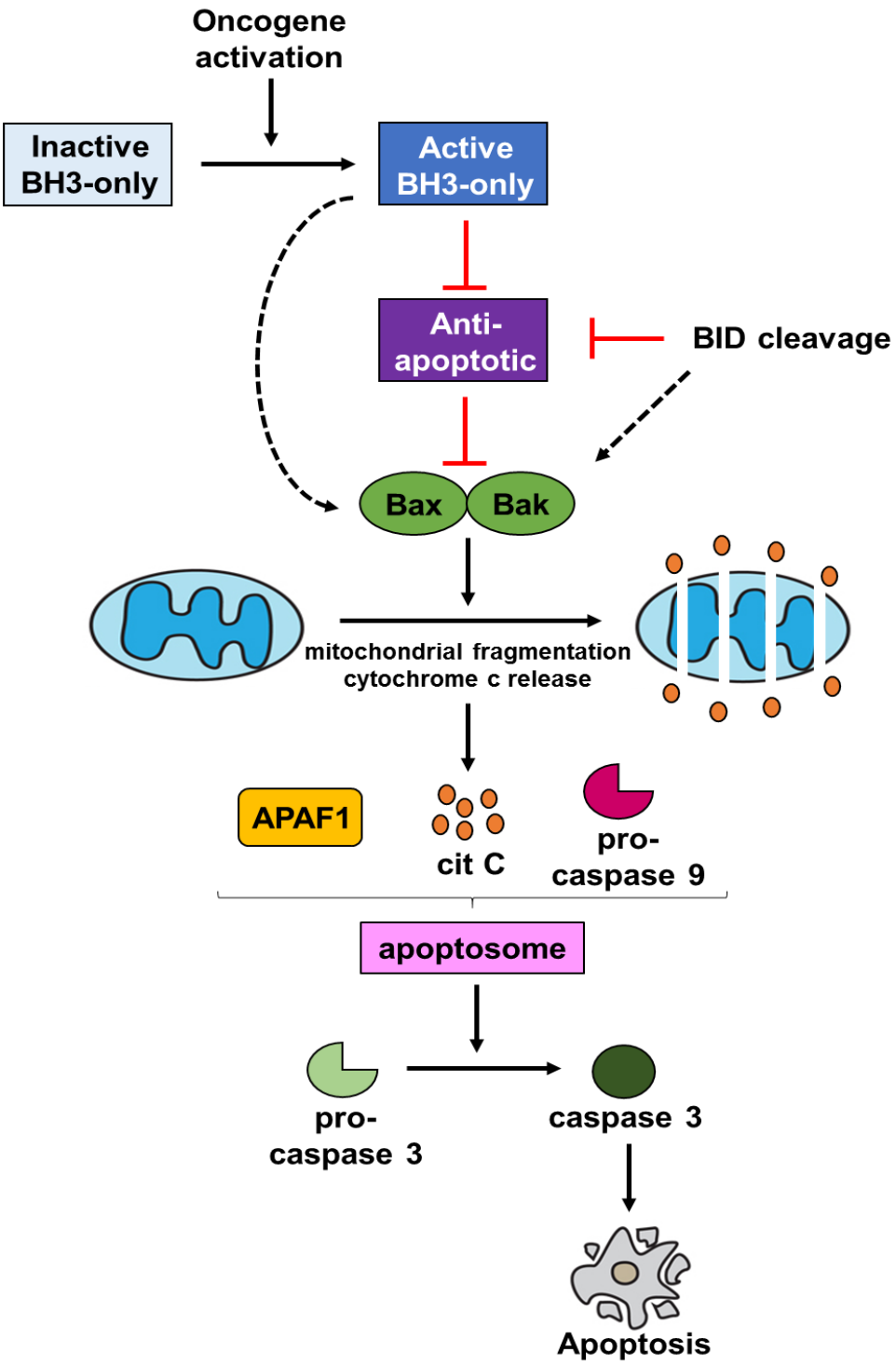

Figure 6. Bcl-2 apoptotic pathway. Oncogenic activation activates BH3-only family members, which inhibit the anti-apoptotic Bcl-2 proteins. This allows for activation of pro-apoptotic Bax and Bak, which oligomerize and form a pore in the mitochondrial membrane. The cytochrome c released from the mitochondria promotes caspase 9 activation on the APAF1 (apoptotic protease-activating factor 1) scaffold protein, forming the apoptosome. This activates effector caspases, including caspase 3, inducing apoptosis.

(BID), which are subsequently translocated to the mitochondrial membrane where they associate with and inactivate anti-apoptotic Bcl-2 family members Bcl-2, Bcl-extra-large (Bcl-x_L), Bcl-w, and myeloid leukemia cell differentiation 1 (Mcl-1). Pro-apoptotic Bcl-2 family members NOXA and p53-upregulated modulator of apoptosis (PUMA) can be transcriptionally activated by p53 and also serve to inactivate anti-apoptotic Bcl-2 family members. Inactivation of the anti-apoptotic proteins frees up the pro-apoptotic multi-domain Bcl-2 proteins Bcl-2-associated X protein (BAX) and Bcl-2 antagonist/killer (BAK), which oligomerize to form a pore in the mitochondrial membrane. The activated form of BID can also directly stimulate BAX and BAK oligomerization. Once the membrane integrity of the mitochondria is compromised, apoptotic components, including cytochrome c, second mitochondrial activator of caspases (Smac), endonuclease G, and apoptosis inducing factor (AIF) are released into the cytoplasm. Cytochrome c, together with dATP and the apoptotic protease-activating factor 1 (APAF1) scaffold protein form a complex known as the apoptosome. The apoptosome is responsible for activating the initiating caspase 9, which results in the downstream activation of effector caspases 3 and 7, the final step for apoptosis (Czabotar *et al.*, 2014).

Myc can interfere with the Bcl-2 apoptotic pathway by altering the expression and/or activity of various Bcl-2 family members. Myc was shown to suppress the expression of anti-apoptotic Bcl-2 and Bcl-x_L through indirect means (Eischen *et al.*, 2001a; Eischen *et al.*, 2001c; Maclean *et al.*, 2003). In addition, Myc-induced p53-independent cell death has been documented to occur, in part, from an indirect up-regulation of BIM, as Eμ-*myc* mice (mouse model of Myc-driven B cell lymphoma (Adams *et al.*, 1985)) with one or no *Bim* alleles rapidly developed lymphomagenesis without

inactivating the p53 pathway (Egle *et al.*, 2004; Sakamuro *et al.*, 1995). Similarly, loss of *Bax* evaded the selection of p53 inactivating mutations during Myc-driven lymphomagenesis (Eischen *et al.*, 2001b). The interplay between the pro- and anti-apoptotic Bcl-2 family members is a key determinant that governs cell survival.

MYC deregulation in cancer

The mechanisms of Myc deregulation in cancer were initially perplexing. While oncogenes such as HRAS had been identified as derivatives of normal cellular genes that had acquired activating mutations in the coding sequence (Rauen, 2013), at the time, mutations in Myc had not been reported. Rather, oncogenic activation of Myc typically occurs through chromosomal translocation, gene amplification, or insertional mutagenesis (Meyer and Penn, 2008). Dysregulation of Myc is not restricted to genetic alterations in the *Myc* loci, but through mechanisms that alter its expression and/or activity (Dang, 2012).

In light of the ability of Myc to drive tumorigenesis in mice and its role in promoting growth and proliferation, it is not surprising that Myc is dysregulated in the majority of human cancers (Tansey, 2014). Myc overexpression via chromosomal translocation was first recognized in mouse plasmacytomas and Burkitt's lymphoma (Dalla-Favera *et al.*, 1982a; Taub *et al.*, 1982). Other lymphomas and leukemia also possess *MYC* translocations, albeit at a much lower frequency. Specifically, diffuse large B cell lymphoma (DLBCL) and multiple myeloma (MM) have recurring *MYC* translocations in approximately 15% of cases (Vita and Henriksson, 2006). Fusions of *MYC* to the T cell receptor alpha (*TCR*α) locus have been reported in T cell acute lymphoblastic leukemia (TALL) (Erikson *et al.*, 1986; Shima *et al.*, 1986). In acute myeloid and lymphoid

leukemia, recurring translocations often result in oncogenic fusion proteins such as PML-RARα, AML1-ETO, and PLZF-RARα, which have all been shown to induce *MYC* expression (Muller-Tidow *et al.*, 2004; Rice *et al.*, 2009). In addition, a mutation in the FLT3 tyrosine kinase receptor in AML causes constitutive activation of FLT3, which transcriptionally upregulates *MYC* (Kim *et al.*, 2005). For *MYC*, overexpression due to translocation rather than mutation is sufficient to drive tumorigenesis.

Although *MYC* gene amplifications occur more frequently in solid tumors than hematopoietic malignancies, *MYC* is the most frequently amplified gene across all human tumors (Beroukhim *et al.*, 2010). Modest *MYC* gene amplification was reported to occur in upwards of 38% of DLBCL cases (Stasik *et al.*, 2010). In these cases, *MYC* gene amplification correlated with advanced stage disease and/or decreased survival (Rao *et al.*, 1998; Stasik *et al.*, 2010). *MYC* has also been reported on double minute chromosomes (Bruyere *et al.*, 2010; Lee *et al.*, 2009b; Mathew *et al.*, 2000; Thomas *et al.*, 2004). Likewise, multiple copies of *MYC* were present upon examination of homogenously staining regions and double-minute chromosomes in colon cancer cell lines and leukemic HL-60 cells (Alitalo *et al.*, 1983; Collins and Groudine, 1982; Dalla-Favera *et al.*, 1982b).

Retroviral promoter insertion, or insertional mutagenesis, was the first mechanism identified to lead to increased *MYC* transcription (Hayward *et al.*, 1981; Neel *et al.*, 1981; Payne *et al.*, 1981). Specifically, leukemogenesis induced by the transforming avian myelocytomatosis retrovirus (MC29) was a result of retroviral transduction that created the chimeric *v-gag-myc*. Myc was the first cellular oncogene shown to be activated via retroviral promoter insertion. Shortly thereafter, murine leukemia pro-viral sequences were identified near the *Myc* locus in mice and rats (Steffen, 1984). These results indicated that

cellular transformation could result from activation of a non-mutated oncogene. Furthermore, insertional mutagenesis became a means through which cellular oncogenes could be identified (Peters, 1990).

Changes in the coding region of *MYC* impact Myc regulation and function (Tansey, 2014). The majority of mutations cluster within the elements that regulate the stability of Myc, including the TAD/degron (Salghetti *et al.*, 2001), the "D-region" (Herbst *et al.*, 2004) and the PEST element (Gregory and Hann, 2000). The most frequently mutated residue is the Thr-58, which is the site of phosphorylation that is recognized by the E3-ligase Fbw7 to promote Myc ubiquitin-mediated degradation (Welcker *et al.*, 2004a; Welcker *et al.*, 2004b; Welcker *et al.*, 2003). Trying to tease apart the direct effects on Myc function from changes that occur as a result of increased Myc expression remains unresolved.

Cancer epigenetics

Although cancer has long been regarded to originate from the accumulation of genetic aberrations, increasing evidence demonstrates that in addition to alterations in DNA sequence, changes at the epigenetic level contribute to tumorigenesis (You and Jones, 2012). The epigenetic code is the collection of chemical and structural modifications to DNA and histones that govern the expression of genes (Turner, 2007). Unlike genetic mutations, epigenetic alterations can be reversible, and therefore, make an attractive target for cancer therapeutics (Dawson and Kouzarides, 2012).

The most frequently observed epigenetic modifications include DNA methylation, histone methylation, and histone acetylation (Turner, 2007). DNA methylation is one of the most extensively studied epigenetic mechanisms and typically suppresses the activity of genes. DNA methyltransferases (DNMTs) are responsible for the deposition of methyl groups onto the DNA, which prevent transcription factor binding and thus, gene expression. The processes of modifying histones is extremely dynamic and is responsible for regulating the architecture of the chromatin. Multiple enzymes are necessary to catalyze modification deposition ("writers"), removal ("erasers"), and recognition ("readers") (Falkenberg and Johnstone, 2014). The two best-characterized histone modifications are histone methylation and acetylation (Falkenberg and Johnstone, 2014). Histone methylation at specific residues on histone tails regulates transcriptional activation and repression (Di Croce and Helin, 2013; Slany, 2009). The acetylation of histones is also an important determinant of gene expression. Histone acetylation is primarily associated with transcriptional activation, whereas histone deacetylation is often associated with gene repression (West and Johnstone, 2014). Histone acetyltransferases (HATs) are responsible for catalyzing the acetylation of histones, which occurs on lysine residues and enhances transcription by relaxing the DNA, making it more accessible to transcriptional machinery. In contrast, histone deacetylases (HDACs) remove the acetyl marks from histones, which in turn, causes the DNA to associate more tightly, leading to transcriptional repression.

Cancer cells with a global loss of mono-acetylated (K16) and trimethylated (K20) forms of histone H4 (Fraga *et al.*, 2005) was one of the first indications that genome-wide alterations in histone acetylation contributed to cancer onset and progression (West and Johnstone, 2014). Since then, it has been appreciated that many of the enzymes responsible

for generating and translating the epigenetic code are often dysregulated in cancer through mutation, aberrant expression, or inappropriate recruitment to genetic loci (Dawson and Kouzarides, 2012). Numerous studies have linked aberrant expression of HDACs to key tumorigenic events, including the epigenetic repression of the tumor suppressor gene *CDKN1A*, which encodes the cyclin-dependent kinase inhibitor p21 (Glozak and Seto, 2007), as well as genes that encode the DNA damage repair enzymes breast cancer 1, early onset (*BRACA1*) and ataxia telangiectasia and Rad 3 related (*ATR*) (Eot-Houllier *et al.*, 2009). Reports of genetic knockdown of individual HDACs in varying tumor types demonstrated that aberrant HDAC activity influenced cell survival and tumorigenicity (West and Johnstone, 2014). Furthermore, HDACs are inappropriately recruited to gene loci by binding oncogenic fusion proteins. For example, AML1-ETO (fusion between the acute myeloid leukemia 1 [AML1] and eight twenty-one [ETO] proteins resulting from t[8;21]) and PML-RARα (fusion between promyelocytic leukemia [PML] and the retinoic acid receptor α [RARα] arising from t[15;17]) repress transcription by recruiting HDACs (Gelmetti *et al.*, 1998; Grignani *et al.*, 1998).

There are 18 HDACs in humans, eleven of which have been classified into groups (classes I, II, and IV) based on their homology to yeast proteins, subcellular localization, and enzymatic activity. Class III HDACs, also known as sirtuins, possess some overlapping functions with the classical HDACs (classes I, II, and IV), but are not affected by traditional HDAC inhibitors (HDACi) (West and Johnstone, 2014). HDACi have shown anti-cancer activity *in vitro* and *in vivo* (West and Johnstone, 2014). Four HDACi, Vorinostat, Romidepsin, Panobinostat, and Belinostat have been FDA approved for the treatment of cutaneous/peripheral T cell lymphoma and refractory multiple myeloma (Duvic *et al.*,

2007; Falkenberg and Johnstone, 2014; Ghobrial *et al.*, 2013; Odenike *et al.*, 2015; Olsen *et al.*, 2007; Piekarz *et al.*, 2009; Richardson *et al.*, 2013; San-Miguel *et al.*, 2013; Whittaker *et al.*, 2010). HDACi were initially identified based on their ability to induce tumor cell differentiation (Leder and Leder, 1975; Riggs *et al.*, 1977).

Recent studies have identified functions of HDACs in important processes such as DNA replication, DNA repair, and genome stability. For example, following targeting of HDAC3 with small molecule inhibitors or siRNA, an increase in DNA replication stress, DNA damage, and activation of dormant origins was observed in cutaneous T cell lymphomas, hematopoietic progenitors, and breast and colon carcinoma cells (Conti *et al.*, 2010; Stengel and Hiebert, 2015; Summers *et al.*, 2013; Wells *et al.*, 2013). However, the biological outcome of HDACi most often reported is apoptosis of tumor cells (Bolden *et al.*, 2006). Numerous studies have shown a correlation between HDACi-induced tumor cell death and therapeutic potential (Ellis *et al.*, 2009; Insinga *et al.*, 2005; Lindemann *et al.*, 2007; Nebbioso *et al.*, 2005; Newbold *et al.*, 2008; Vrana *et al.*, 1999). Tumor cells have demonstrated an increased sensitivity to HDACi-induced apoptosis compared to normal cells (West and Johnstone, 2014). This differential in sensitivity to HDACi has been attributed to an accumulation of reactive oxygen species (ROS) in tumor cells compared with treated normal cells (Vannini *et al.*, 2004), as well as a tumor cell-selective increase in the expression of pro-apoptotic genes (Bolden *et al.*, 2013). The combination of correlative data linking HDAC expression with patient prognosis and preclinical mouse models continue to provide insight into the roles of HDACs in tumorigenesis. These data reveal that genetic insults resulting in epigenetic dysregulation are a significant contributing factor to tumor onset and progression.

microRNA

Discovery

The existence and significance of microRNA (miRNA) is a relatively new concept to cancer biology. Until recently, researchers have primarily focused on the expression of protein-coding genes, as central dogma has always been that DNA is transcribed into RNA, which is then translated into functional protein. However, in 1993 the importance of non-coding RNA, specifically miRNA, began to be exposed. As a joint effort between the labs of Victor Ambros and Gary Ruvkun, *lin-4*, the first miRNA, was identified (Lee *et al.*, 1993; Wightman *et al.*, 1993). It wasn't until 7 years later that the second miRNA was identified. *let-7* was identified using a genetic screen approach while studying heterochronic genes in *C. elegans* (Reinhart et al., 2000). Similar to *lin-4*, *let-7* only encoded a small RNA and not a protein. Moreover, the loss of function mutations of *let-7* were partially suppressed by mutations in several genes including *lin-14*, *lin-41*, *lin-42*, and *lin-28*, all of which possess complementary sequences to *let-7* in their 3'-UTR (Reinhart *et al.*, 2000). Unlike *lin-4*, the *let-7* sequence and its temporal regulatory functions were conserved across species from flies to humans (Pasquinelli *et al.*, 2000). This sparked interest of this miRNA in other organisms because, as until then, it was considered to be a unique phenomenon in the development of *C. elegans*. Cloning and bioinformatic approaches were utilized to identify miRNA genes and to determine their evolutionary conservation (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001), ultimately showing that miRNA were evolutionarily significant and widely used

regulatory molecules to negatively regulate gene expression at the post-transcriptional level by inhibiting translation and/or degrading mRNA.

Research in the field of post-transcriptional gene silencing was developing around the same time miRNA biology began to surface. In plants, post-translational gene silencing had been documented during flower patterning following overexpression of a particular transgene (Napoli *et al.*, 1990). Furthermore, a similar strategy called RNA interference (RNAi) was being used to introduce a double-stranded RNA molecule (Fire *et al.*, 1998). Small RNAs that were about 22 nucleotides in size were derived from longer double-stranded RNAs and were determined to be the mediators of RNAi through base-pairing with perfect complementarity to target mRNA, resulting in mRNA degradation (Elbashir *et al.*, 2001b; Hamilton and Baulcombe, 1999; Hammond *et al.*, 2000; Zamore *et al.*, 2000). In support of these results, transfection of 21-nt double-stranded RNA duplexes, also known as small-interfering RNAs (siRNA), into mammalian cells silenced their predicted target genes in a sequence-specific manner (Elbashir *et al.*, 2001a). While miRNA differ from siRNA in that they are endogenous to the cell, are processed from imperfectly-paired dsRNA hairpins rather than perfectly-paired long dsRNA, have partial rather than perfect complementarity to the target mRNA, and repress translation in addition to destabilizing the target mRNA, the similarities between miRNA and siRNA caused the two fields to intersect (Bartel, 2004).

Biogenesis, target recognition, and function

miRNA are encoded as long primary transcripts (pri-miRNA) and are typically transcribed by RNA polymerase II (Bartel, 2004) (Figure 7). pri-miRNA fold into dsRNA

hairpin structures that are processed by the nuclear specific RNase III enzyme Drosha into a stem-loop precursor miRNA (pre-miRNA) of about 60-70 nucleotides in length with an overhang of 2 nucleotides at the 3' end. DiGeorge syndrome critical region 8 protein (DGCR8) is a cofactor required for Drosha processing, which, together, form the microprocessor complex (Gregory *et al.*, 2004; Han *et al.*, 2004; Landthaler *et al.*, 2004). DGCR8 measures 11 nucleotides from the base of the pri-miRNA stem and signals Drosha to cut and release the stem loop pre-miRNA structure (Han *et al.*, 2006). The 3' overhang and dsRNA stem of the pre-miRNA is recognized by Exportin 5, a RanGTP-dependent dsRNA-binding protein that actively transports the pre-miRNA into the cytoplasm (Bohnsack *et al.*, 2004; Lund *et al.*, 2004; Yi *et al.*, 2003). Once in the cytoplasm, the pre-miRNA is further processed into an RNA duplex by the RNase III endonuclease Dicer (Bernstein *et al.*, 2001; Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Ketting *et al.*, 2001). One miRNA strand of the RNA duplex is preferentially selected to be loaded into the RNA-induced silencing complex (RISC) to guide the silencing complex to the target mRNA.

The human RISC complex is primarily comprised of Dicer, the double-stranded binding protein TRBP, and Argonaute (Ago) proteins (Chendrimada *et al.*, 2005; Gregory *et al.*, 2005). Argonaute proteins are a large family of proteins that contain PAZ and PIWI domains (Hutvagner and Simard, 2008). The PAZ domain serves as a single-stranded RNA-binding domain that hold the 2 nucleotides of the 3' overhang of the miRNA duplex in place (Hutvagner and Simard, 2008). It is hypothesized that structural characteristics of the precursor RNA directs the loading of the miRNA into the appropriate silencing complex (Forstemann *et al.*, 2007; Tomari *et al.*, 2007).

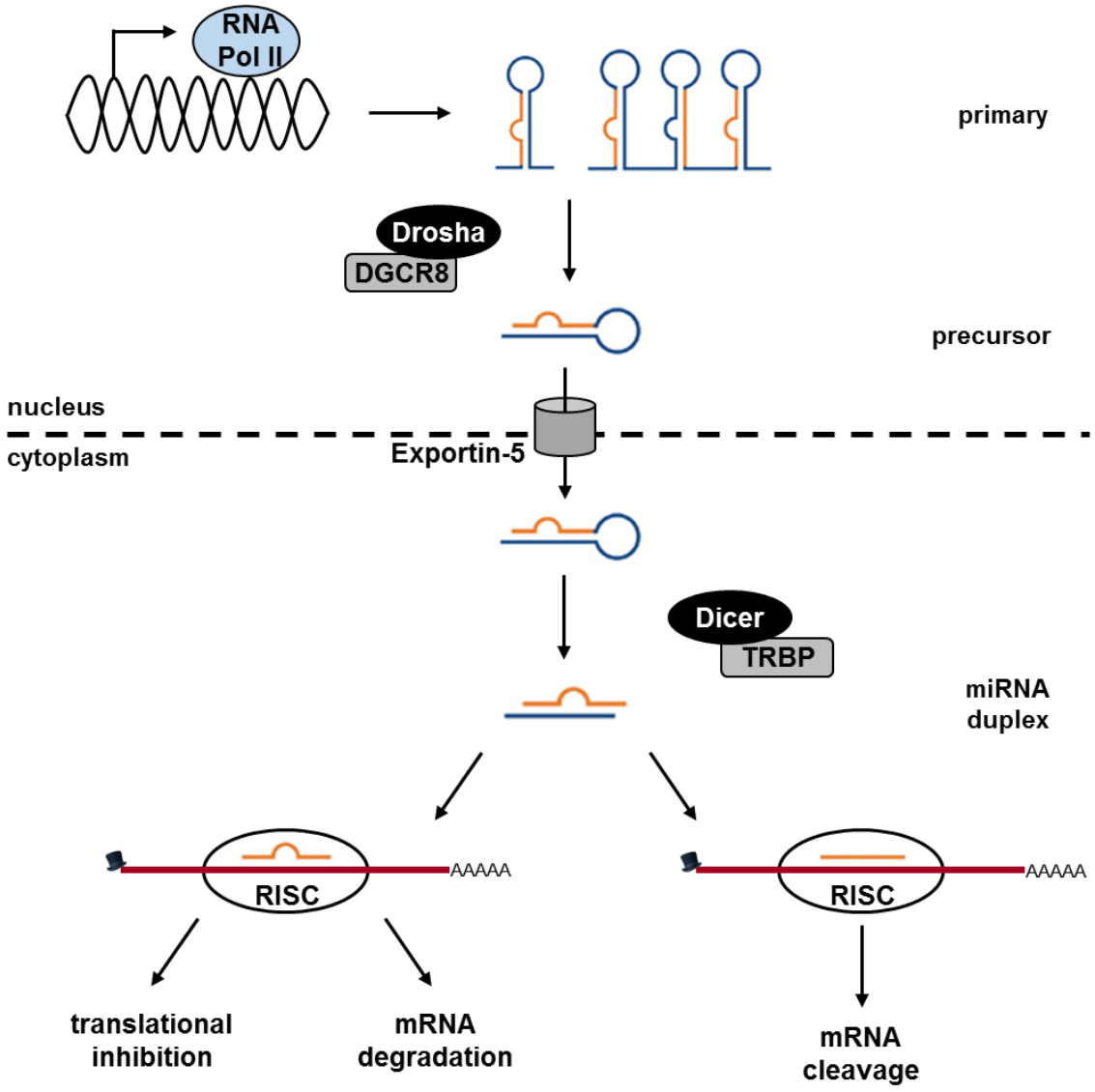

Figure 7. microRNA biogenesis. miRNA are transcribed by RNA polymerase II (pol II) into primary transcripts. The initial processing step is mediated by the Drosha-DGCR8 microprocessor complex in the nucleus. The precursor miRNA is transported out of the nucleus by Exportin-5-Ran-GTP. Once in the cytoplasm, the RNase III enzyme Dicer initiates a second processing step to produce miRNA duplexes. The duplex is separated and one strand is selected as the mature miRNA to be incorporated into the RNA-induced silencing complex (RISC) with Argonaute proteins where it guides the RISC to silence target mRNA through translational inhibition and mRNA degradation and cleavage.

The short region where the miRNA predominantly associates with the target mRNA, defined as nucleotides 2-8 on the 5' end of the mature miRNA, is known as the "seed sequence." Biochemical strategies using siRNA duplexes in place of miRNA showed that seed pairing to its target was necessary and sufficient for target repression (Doench and Sharp, 2004). Computational approaches taken to predict miRNA-mRNA interactions often result in high numbers of false positives due to the short seed sequence being the main determinant for target identification. Because of this, additional criteria beyond seed pairing have also been considered. For example, regions of pairing that occur on the 3' end of the miRNA may compensate for imperfect 5' end seed matches (Brennecke *et al.*, 2005).

The context of the miRNA binding site in the 3'-UTR is also an important determinant. For example, additional determinants, including, but not limited to, AU-rich sequences flanking miRNA binding sites, distances between multiple binding sites, bulges, pairing of miRNA nucleotides 13-16 to the binding site, and positioning the binding site at least 15 nucleotides from the stop codon influenced target identification (Bartel, 2009). Furthermore, the secondary structure of the target and the binding of additional proteins to the target also affect miRNA accessibility, and therefore function (Kedde *et al.*, 2007; Kertesz *et al.*, 2007; Long *et al.*, 2007).

The predominant function of a miRNA is to inhibit protein synthesis through translational inhibition or mRNA degradation (Figure 7). Recently, in studies using ribosomal profiling to measure simultaneous changes in protein production and mRNA, it was determined that inhibition of translation only modestly contributed to the decrease in protein levels, whereas mRNA destabilization and subsequent degradation was the primary mechanism employed by miRNA (Guo *et al.*, 2010).

While miRNA typically bind their target mRNA in the 3'-UTR, evidence of miRNA-target interactions that deviate from the canonical binding rules have more recently been reported. Artificial target sites were inserted into the 5'-UTR of an IRES luciferase reporter plasmid, which was repressed to a similar extent as those same sites in the 3'-UTR (Lytle *et al.*, 2007). miRNA binding sites have also been reported in the actual coding regions (CDS) of the stem cell pluripotency factors *Nanog*, *Oct4*, and *Sox2* (Tay *et al.*, 2008). Furthermore, miRNA seed-independent target regulation has also been reported (Lal *et al.*, 2009). As additional methods to identify miRNA-target interactions emerge, previous studies may have only accounted for a fraction of miRNA genes.

Dysregulation of miRNA in cancer

The initial indication that miRNA have important roles in human disease originated from high-throughput and functional studies in cancer cells (Hata and Lieberman, 2015). Within the past decade, miRNA have been recognized as a significant component of cancer biology. Like transcription factors, miRNA regulate diverse cellular networks and are widely accepted to regulate most biological processes (Ebert and Sharp, 2012). One of the most appreciated characteristics of miRNA in cancer is the significant difference of miRNA expression in malignant cells compared to their normal counterparts (Lu *et al.*, 2005). Commonly reported mechanisms responsible for the dysregulation of miRNA in cancer include genetic abnormalities, epigenetic and transcriptional control, and defects in miRNA biogenesis.

Genetic alterations

Chromosomal rearrangements, genomic amplifications, deletions, or mutations can alter miRNA genes, much like they affect protein-coding genes. Approximately 50% of all annotated human miRNA genes have been mapped to fragile sites or areas of the genome that are prone to breakage and rearrangement in cancer cells (Calin *et al.*, 2002; Calin *et al.*, 2004; Lagana *et al.*, 2010; Sevignani *et al.*, 2007). Indeed, 65 miRNA mapped to loss-of-heterozygosity regions (i.e., miR-15a/16-1 cluster) where tumor suppressor genes are frequently located, 61 miRNA mapped to fragile sites and breakpoint regions (i.e., let-7 family members), and 15 miRNA were mapped to amplified regions (i.e., miR-17~92 polycistron) where oncogenes are often located (Calin *et al.*, 2004).

The link between miRNA and cancer was first established with the miR-15a/16-1 cluster. The miR-15a/16-1 miRNA cluster is located in a host gene, *DLEU2* (deleted in lymphocytic leukemia 2), which is in the most frequently deleted region in chronic lymphocytic leukemia (CLL), the 13q14.3 locus (Calin *et al.*, 2002). As such, both miRNA are frequently downregulated in CLL patients (70%), demonstrating a tumor-suppressive role for these miRNA (Calin *et al.*, 2002). Genetic deletion of the miR-15a/16-1 cluster in mice recapitulated the development of human CLL (Klein *et al.*, 2010). The miRNA encoded by the let-7 family are also considered to possess tumor-suppressive characteristics. This family of miRNA were found in fragile sites associated with lung, breast, urothelial, and cervical cancers (Calin *et al.*, 2004). Transcripts of let-7 family members were downregulated in human lung cancer, which correlated with poor prognosis (Takamizawa *et al.*, 2004). In addition, loss of let-7 family members was demonstrated to result in overexpression of oncogenic RAS (Johnson *et al.*, 2005).

Some miRNA possess oncogene-like qualities and have been termed "oncomiRs." The miR-17~92 cluster is one of the most well-characterized oncogenic miRNA families (Di Leva *et al.*, 2014). The miR-17~92 cluster is located within 1kb of an intron of the *C13orf25* locus, which is a region that is frequently amplified in several types of lymphoma (Ota *et al.*, 2004) and solid cancers (Hayashita *et al.*, 2005). This miRNA cluster is often overexpressed in human cancers, including lung cancer, gastric cancer, colon cancer, neuroblastoma, medulloblastoma, and osteosarcoma (Di Leva *et al.*, 2014). Furthermore, B cell-specific miR-17~92 transgenic mice developed lymphomas with high penetrance and two intact alleles of the miR-17~92 cluster were required to drive Myc-mediated B cell lymphomagenesis (Jin *et al.*, 2013).

Epigenetic control

As mentioned earlier, aberrant epigenetic changes are a well-known feature of cancer cells. In a similar manner to protein-coding genes, miRNA are subject to the same epigenetic regulation. A large number of miRNA loci are associated with CpG islands and therefore, their expression can be modulated by methylation (Esteller, 2007; Weber *et al.*, 2007). Most reports have shown an increase in the methylation of tumor suppressor miRNA, allowing for their tumor-promoting targets to be upregulated (Di Leva *et al.*, 2014; Lujambio *et al.*, 2007). Several studies used treatment with chromatin remodeling drugs to reveal epigenetically silenced miRNA. For instance, miRNA with tumor suppressive functions that were silenced through hypermethylation (i.e., miR-127, miR-9-1, miR34b/c), were upregulated following treatment with the de-methylating agent 5-aza-2'deoxycytidine (Lehmann *et al.*, 2008; Saito *et al.*, 2006; Toyota *et al.*, 2008). Differential

miRNA expression profiles have also been observed in colorectal cancer cells lacking the DNA methyltransferase enzymes *DNMT1* and *DNMT3b* (Lujambio *et al.*, 2007). miRNA can be specifically hypermethylated in metastatic cancer, suggesting that epigenetic alterations not only facilitate tumor development, but may also promote progression to a more aggressive state (Lujambio *et al.*, 2008; Wee *et al.*, 2012; Zhang *et al.*, 2011).

miRNA can also be deregulated as a consequence of aberrant expression of histone modifiers. HDACs are overexpressed in cancers such as CLL and to mediate the repression of miR-29b, miR-15a, and miR-16-1 (Zhang *et al.*, 2012a; Zhang *et al.*, 2012b). Using a miRNA array analysis of breast cancers, repression of miR-31 was also attributed to HDACs (Cho *et al.*, 2015). When cells were treated with HDACi, miR-31 expression increased, which downregulated the Polycomb group protein BMI1 (Cho *et al.*, 2015). EZH2, a component of the Polycomb repressive complex 2 (PRC2), is recruited to the miR-26a promoter in lymphoma cells where it mediates the silencing of this tumor suppressor miRNA (Zhao *et al.*, 2013). Furthermore, the activity of epigenetic regulators can be modified by oncogenes or tumor suppressors, leading to the epigenetic dysregulation of miRNA. For example, BRCA1, a tumor suppressor in the DNA repair pathway, epigenetically represses the expression of the oncogenic miRNA miR-155 by modulating the activity of HDAC2 (Chang *et al.*, 2011).

Transcriptional control

Transcription is a major point of miRNA regulation. Many miRNA genes are located in the introns of protein-coding genes or in long non-coding RNA (Ha and Kim, 2014). miRNA can also be independent transcriptional units with their own promoter

elements and polyadenylation signals (Ha and Kim, 2014). The majority of miRNA are transcribed by RNA polymerase II (Lee *et al.*, 2004). miRNA are often in close proximity with other miRNA and are therefore transcribed as polycistronic messages into individual units or excised from mRNA. One of the first examples of transcriptional regulation of miRNA associated with cancer was the transcriptional activation of the miR-17~92 polycistron by Myc (O'Donnell *et al.*, 2005). In this study, Myc induced the expression of the miR-17~92 family, which countered the apoptotic activity of E2F1 (O'Donnell *et al.*, 2005). While Myc was responsible for the upregulation of the oncogenic miR-17~92 cluster, the predominant effect of Myc activation in human and mouse B cell lymphoma was widespread repression of miRNA expression (Chang *et al.*, 2008). Among those miRNA that were repressed by Myc, several had documented tumor suppressive qualities, including the miR-15 family, let-7 family, miR-34 family, and miR-26a (Chang *et al.*, 2008).

Defects in miRNA biogenesis

As described above, miRNA biogenesis is a multistep process involving a number of enzymes and cofactors. Mediators of the miRNA processing pathway are aberrantly expressed or mutated in tumors, resulting in inappropriate miRNA regulation (Di Leva *et al.*, 2014). Drosha mediates the cropping step of primary miRNA transcripts into precursor stem-loop structures. This step is one of the first steps in miRNA processing and is an important point of miRNA regulation. Reduced levels of *DROSHA* mRNA was shown to correlate with a poor prognosis in lung, breast, skin, endometrial, and ovarian cancers (Hata and Lieberman, 2015). The processing of pre-miRNA transcripts takes place in the

cytoplasm. As such, they must be transported from the nucleus into the cytoplasm, a process requiring the nuclear export receptor exportin-5 (XPO5). Mutations of XPO5 that trap pre-miRNA transcripts in the nucleus correlate with carcinomas characterized by microsatellite instability (Melo *et al.*, 2010). Consequently, the reduction in available pre-miRNA in the cytoplasm decreases the amount of mature miRNA produced. Tumors with microsatellite instability are also characterized by inactivating mutations of the *TARBP2* gene that encodes TRBP, a cofactor necessary for Dicer function (Melo *et al.*, 2009). Loss of TRBP destabilizes Dicer, which in turn, impairs miRNA processing (Melo *et al.*, 2009).

Loss of one allele of *DICER* or reduced DICER expression or enzymatic activity has been reported in multiple solid organ tumors (Cerami *et al.*, 2012; Forbes *et al.*, 2008; Gao *et al.*, 2013; Heravi-Moussavi *et al.*, 2012; Hill *et al.*, 2009; Karube *et al.*, 2005; Lin *et al.*, 2010; Lu *et al.*, 2005; Melo *et al.*, 2009; Pampalakis *et al.*, 2009; Torres *et al.*, 2011). Reduced activity and/or expression of Dicer can lead to miRNA downregulation (Di Leva *et al.*, 2014), which is why reduced levels of Dicer were proposed to facilitate tumorigenesis. In support of this concept, Dicer was demonstrated to function as a haploinsufficient tumor suppressor in mouse models of soft-tissue sarcoma, lung adenocarcinoma, and retinoblastoma (Kumar *et al.*, 2009; Lambertz *et al.*, 2010; Ravi *et al.*, 2012). Although heterozygous somatic mutations in *DICER1* were reported in tumor genotyping atlases, homozygous deletion have not been documented (Kumar *et al.*, 2009). Likewise, patients with heterozygous germline *DICER1* mutations are predisposed to pleuropulmonary blastomas, which retain an intact *DICER1* allele (Hill *et al.*, 2009). In contrast, Dicer hypomorphic mice, which only express 20% of normal Dicer levels, did not have an increased cancer incidence (Morita *et al.*, 2009). In addition, Dicer did not function

as a haploinsufficient tumor suppressor in B cell lymphoma, as loss of one *Dicer* allele did not affect the rate of B cell lymphomagenesis (Arrate *et al.*, 2010).

As mentioned previously, Argonaute (Ago) proteins are the critical downstream effectors of miRNA-mediated gene silencing. Mature miRNA are stabilized when bound to Ago proteins (Hata and Lieberman, 2015). Without Ago2, the most abundant Ago protein, miRNA were determined to be extremely unstable, resulting in a global reduction in miRNA abundance (Winter and Diederichs, 2011). Therefore, the amount of Ago is an important determinant in the efficiency of miRNA production. Altogether, these observations indicate that miRNA are regulated at various levels and any step along these regulatory pathways is susceptible to being altered in cancer.

CHAPTER II

INACTIVATION OF *P53* IS INSUFFICIENT TO ALLOW B CELLS AND B CELL LYMPHOMAS TO SURVIVE WITHOUT *DICER*

This chapter is from:

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Introduction

MicroRNA (miRNA) are small non-coding RNA that regulate gene expression post-transcriptionally and have essential roles in development, proliferation, apoptosis, and transformation (Almeida *et al.*, 2011; Mendell and Olson, 2012). Alterations in miRNA expression are linked to tumor development, including hematopoietic malignancies (Almeida *et al.*, 2011; Iorio and Croce, 2012; Mendell and Olson, 2012). Moreover, the oncogene c-Myc, which is frequently overexpressed in many human malignancies and a driver of B-cell lymphomagenesis, transcriptionally regulates the expression of many miRNA (Bui and Mendell, 2010).

miRNA are transcribed in a precursor form and processed with enzymes, such as Dicer, an RNase III enzyme with critical roles in cell differentiation, proliferation, and survival (Finnegan and Pasquinelli, 2013). Loss of one allele of *DICER* or reduced DICER

expression or enzymatic activity is reported in multiple solid organ tumors (Cerami *et al.*, 2012; Forbes *et al.*, 2008; Gao *et al.*, 2013; Heravi-Moussavi *et al.*, 2012; Hill *et al.*, 2009; Karube *et al.*, 2005; Lin *et al.*, 2010; Lu *et al.*, 2005; Melo *et al.*, 2009; Pampalakis *et al.*, 2009; Torres *et al.*, 2011). Mouse models revealed Dicer is a haploinsufficient tumor suppressor in soft tissue sarcoma, lung adenocarcinoma, and retinoblastoma (Kumar *et al.*, 2009; Lambertz *et al.*, 2010). In contrast, we showed *Dicer* heterozygosity had no effect on the rate of B-cell lymphoma development (Arrate *et al.*, 2010). Therefore, differences in the requirements for Dicer and the effects of reduced Dicer expression in different tissues remain unresolved.

The p53 tumor suppressor, which induces apoptosis or cell cycle arrest upon cellular stresses (Vousden and Prives, 2009), responds to defects in miRNA biogenesis, and therefore, may be required to signal problems in this pathway. Specifically, in untransformed murine embryonic fibroblasts (MEFs), deletion of *Dicer* leads to p53 activation and premature senescence, which is delayed with loss of *p53* (Mudhasani *et al.*, 2008). We previously detected an increased frequency of *p53* inactivation in lymphomas in a mouse model of Myc-induced B-cell lymphoma (Eμ-*myc*) expressing B-cell-directed Cre and two conditional *Dicer* alleles, suggesting a connection between *p53* activation and *Dicer* deletion in B-cells (Arrate *et al.*, 2010). Moreover, data from three groups, including our own, showed expression of Cre in $Dicer^{fl/fl}$ mice in B-cell progenitors or mature B-cells results in B-cell apoptosis (Arrate *et al.*, 2010; Koralov *et al.*, 2008; Xu *et al.*, 2012). This apoptosis was partially rescued by overexpressing the anti-apoptotic Bcl-2 protein or reducing the pro-apoptotic Bim protein (Koralov *et al.*, 2008). Although *p53*-null murine sarcoma cells and p53 inactivated mesenchymal stem cells can survive *Dicer* deletion

(Ravi *et al.*, 2012), *p53* deletion was synthetically lethal in Dicer and Rb deficient retinal progenitor cells (Nittner *et al.*, 2012). Therefore, the role of p53 in monitoring defects in miRNA biogenesis and cell survival in the context of a *Dicer* deficiency remains unclear.

Using mouse models, we determined the contribution of p53 to B-cell survival and lymphoma development with loss of Dicer. A *p53* deficiency did not rescue the defect in B-cell development, the reduction in B-cell survival, or the delay in Myc-induced lymphomagenesis upon *Dicer* deletion. It did restore the B-cell lymphoma phenotype. However, none of the lymphomas that emerged had deleted both alleles of *Dicer*. Moreover, established B-cell lymphomas lacking *p53* underwent apoptosis when *Dicer* was deleted, significantly extending survival in mouse models. Thus, p53 loss is insufficient to allow survival and growth of B-cells and B-cell lymphomas in the absence of Dicer, and thus, targeting Dicer may have therapeutic potential for treating B-cell lymphomas.

Materials and Methods

Mice

C57Bl/6 Eμ-*myc* (Adams *et al.*, 1985) and CD19-*cre* (Rickert *et al.*, 1997) transgenic mice, $Dicer^{fl/fl}$ mice from Dr. Steve Jones (Mudhasani *et al.*, 2008), and $p53^{-/-}$ mice from Dr. Guillermina Lozano (Montes de Oca Luna *et al.*, 1995) were intercrossed to obtain the mice needed for this study. Littermates were used in all analyses. For experiments with nude mice, $1.5x10^6$ or $0.5x10^6$ *p53* deleted $Dicer^{fl/fl}$/Eμ-*myc* lymphoma cells expressing a tamoxifen-inducible form of Cre ($CreER^{T2}$) were injected (subcutaneous or intravenous,

respectively) into 6-week-old *Foxn1*$^{nu/nu}$ female mice (Harlan labs). Tamoxifen (2 mg) or corn oil (vehicle control) was injected (intraperitoneal) once daily for 3 days starting the day of lymphoma injection for two cohorts (one subcutaneous and one tail vein injected cohort) or after lymphomas were 90-150mm^3 for a second subcutaneous cohort. Subcutaneous tumors were measured with calipers and tumor volume calculated. Blood was collected for flow cytometric and microscopic analyses from the mice where lymphoma was injected into the tail vein. Mice were humanely sacrificed prior to lymphoma development or for survival studies, at humane endpoints, and tumors/tissues were harvested and analyzed. Log-rank tests determined statistical significance for survival. All studies were in accordance with state and federal guidelines and were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Western and Southern blotting

Whole cell protein lysates from B-cell lymphomas and pre-B cells were generated and Western blotted as previously described (Eischen *et al.*, 1999). Antibodies against p19Arf (GeneTex), p53 (Ab-7; Calbiochem), Mdm2 (C-18; Santa Cruz), Cre (Novagen), Dicer (Cell Signaling), cleaved Caspase 3 (Cell Signaling), and β-actin (Sigma) were used. As previously described (Alt *et al.*, 2003; Eischen *et al.*, 1999), *p53* was sequenced and Southern blots for *p53* with genomic DNA from lymphomas was performed.

Phenotype analysis

Lymphoma cells and splenocytes from littermates prior to lymphoma development were analyzed by flow cytometry following incubation with fluorochrome-linked antibodies

against surface receptors as previously reported (Alt *et al.*, 2003; Arrate *et al.*, 2010).

Quantitative real-time PCR

Total RNA was isolated from lymphomas with TRIzol (Invitrogen) according to the manufacturer's protocol. As previously described, cDNA was generated, and SybrGreen (SABiosciences) and TaqMan MicroRNA Assays (Applied Biosciences) were used to perform qRT-PCR, in triplicate, for mRNA and miRNA analysis, respectively (Arrate *et al.*, 2010; Wang *et al.*, 2008). mRNA and miRNA expression were normalized to *β-actin* and *RNU6b* expression, respectively, and the data presented as $2^{-\Delta Ct}$.

***Dicer* gene rearrangement analysis**

Genomic DNA was isolated from frozen and cultured lymphomas, pre-B cells, and MEFs using the REDExtract-N-Amp Tissue PCR Kit (Sigma). PCR was performed with primers specific for unrearranged and Cre-lox-deleted *Dicer* alleles, as previously published (Arrate *et al.*, 2010; Mudhasani *et al.*, 2008). PCR conditions allowed for 10-15% contaminating normal tissue without detecting unrearranged floxed *Dicer* alleles.

Pre-B cell and lymphoma cell survival analyses

Primary pre-B cell cultures from $p53^{-/-}/Dicer^{fl/fl}$, $p53^{+/-}/Dicer^{fl/fl}$, and $p53^{-/-}/Dicer^{+/fl}$ mice and primary *p53* deleted or *Arf* deleted $Dicer^{+/fl}$ or $Dicer^{fl/fl}$ Eμ-*myc* lymphoma cells were generated as previously described (Arrate *et al.*, 2010; Eischen *et al.*, 1999). Cells were infected with a bicistronic retrovirus (MSCV) encoding $CreER^{T2}$ (Feil *et al.*, 1997) and GFP or GFP alone. Cell number and viability were determined by Trypan Blue Dye

exclusion assays and proliferation was measured by MTS assays (490 nm; CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega) after plating equal numbers of cells, in triplicate, and adding 1 μM 4-OHT or vehicle (ethanol) control. Apoptosis was evaluated by Western blotting for cleaved Caspase 3 and by flow cytometry following propidium iodide staining for fragmented (sub-G1) DNA and Annexin V/7-AAD staining after adding 1 μM 4-OHT or vehicle (ethanol) control in triplicate, *in vitro*, or after administering tamoxifen or vehicle (corn oil) for the nude mouse experiments. For single-cell analyses, GFP-positive lymphoma cells were placed one cell/well into 96-well plates by a flow cytometer and visually inspected. Vehicle (ethanol) control or 4-OHT (1 μM) was added to each well and surviving clones were harvested and *Dicer* gene rearrangement was determined by PCR.

Results

p53 deficiency does not rescue lymphoma latency in Myc overexpressing *Dicer*$^{fl/fl}$ mice

Previously, we reported *Dicer* deletion in B-cell precursors resulted in delayed Myc-induced B-cell lymphoma development and the inability of a B-cell lymphoma to emerge with biallelic *Dicer* deletion (Arrate *et al.*, 2010). To determine whether B-cell lymphomas could develop without *Dicer* in the context of a *p53* deficiency, we generated *p53*$^{+/-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice and littermate controls that were also transgenic for B lineage-restricted CD19-*cre* recombinase (Figure 8A)*; p53*-null Eμ-*myc* mice cannot be generated (Rickert *et al.*, 1997). c-Myc in Eμ-*myc* transgenic mice and Cre in CD19-*cre* transgenic mice are first expressed in B-cell precursors and continue throughout the life of the B-cell

(Adams *et al.*, 1985; Rickert *et al.*, 1997). There was a pronounced delay in lymphomagenesis and extended survival in CD19-*cre*$^{+}$/*p53*$^{+/-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice compared to their CD19-*cre*$^{-}$/*p53*$^{+/-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* littermates (53 and 34 days mean survival, respectively; Figure 8B, $p<0.0001$, log-rank test). All but one (DC1122) of the 23 lymphomas analyzed lacked p53 protein expression, and all overexpressed p19Arf protein, an indicator of p53 inactivation (subset of those analyzed is shown in Figure 9A). Sequencing of *p53* in DC1122 revealed a mutation (G263R) in its DNA binding domain. Southern blots showed all lymphomas lacking p53 protein had deleted their wild-type allele of *p53* (representative data of those analyzed is shown in Figure 9B). Therefore, all lymphomas were functionally *p53*-null. In addition, Mdm2, a negative regulator of p53, was overexpressed in 35% of the lymphomas (Figure 9A). Thus, there was a delay in Myc-induced lymphomagenesis caused by *Dicer* deletion in *p53* heterozygous mice, and a deficiency in Dicer did not alter selection for p53 inactivation in the lymphomas that arose.

Dicer is not a haploinsufficient tumor suppressor in Myc-induced B-cell lymphoma (Arrate *et al.*, 2010). To determine whether a p53 deficiency would allow Dicer to function as a haploinsufficient tumor suppressor in B-cells, we evaluated B-cell lymphoma development in the context of *Dicer* heterozygosity. Cre-positive and Cre-negative *p53*$^{+/-}$/*Dicer*$^{+/fl}$/Eμ-*myc* transgenic mice had a similar rate of lymphoma development with mean survivals of 35 and 36 days, respectively (Figure 8B). Evaluation of lymphomas that developed in CD19-*cre*$^{+}$/*p53*$^{+/-}$/*Dicer*$^{+/fl}$/Eμ-*myc* mice showed 100% (17 of 17 analyzed) lacked p53 protein, due to deletion of the wild-type allele, and overexpressed Arf (subset of those analyzed is shown in Figures 9C and 9D). These results indicate a p53 deficiency did not allow *Dicer* heterozygosity to accelerate B-cell lymphomagenesis.

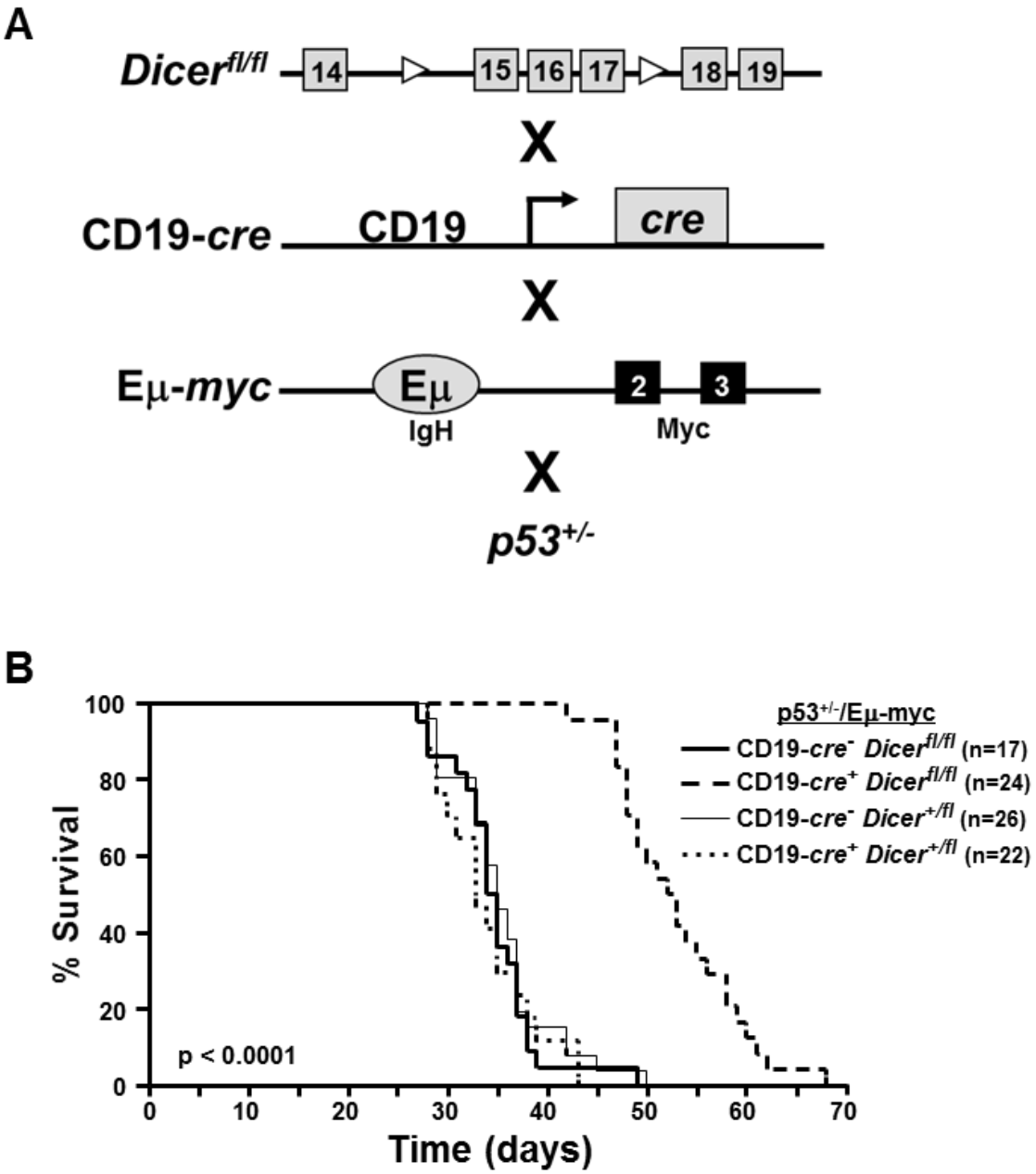

Figure 8. Delayed lymphomagenesis in *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice. (A) Schematic of mouse crosses. White triangles represent loxP sites flanking *Dicer* exons (gray boxes). Exons 2 and 3 of Myc are shown as black boxes downstream of the Eμ IgH enhancer. (B) Kaplan-Meier survival curves of the indicated genotypes of mice ($p<0.0001$, log-rank test comparing each genotype to CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/*p53*$^{+/-}$/Eμ-*myc*). The number (n) of mice is indicated.

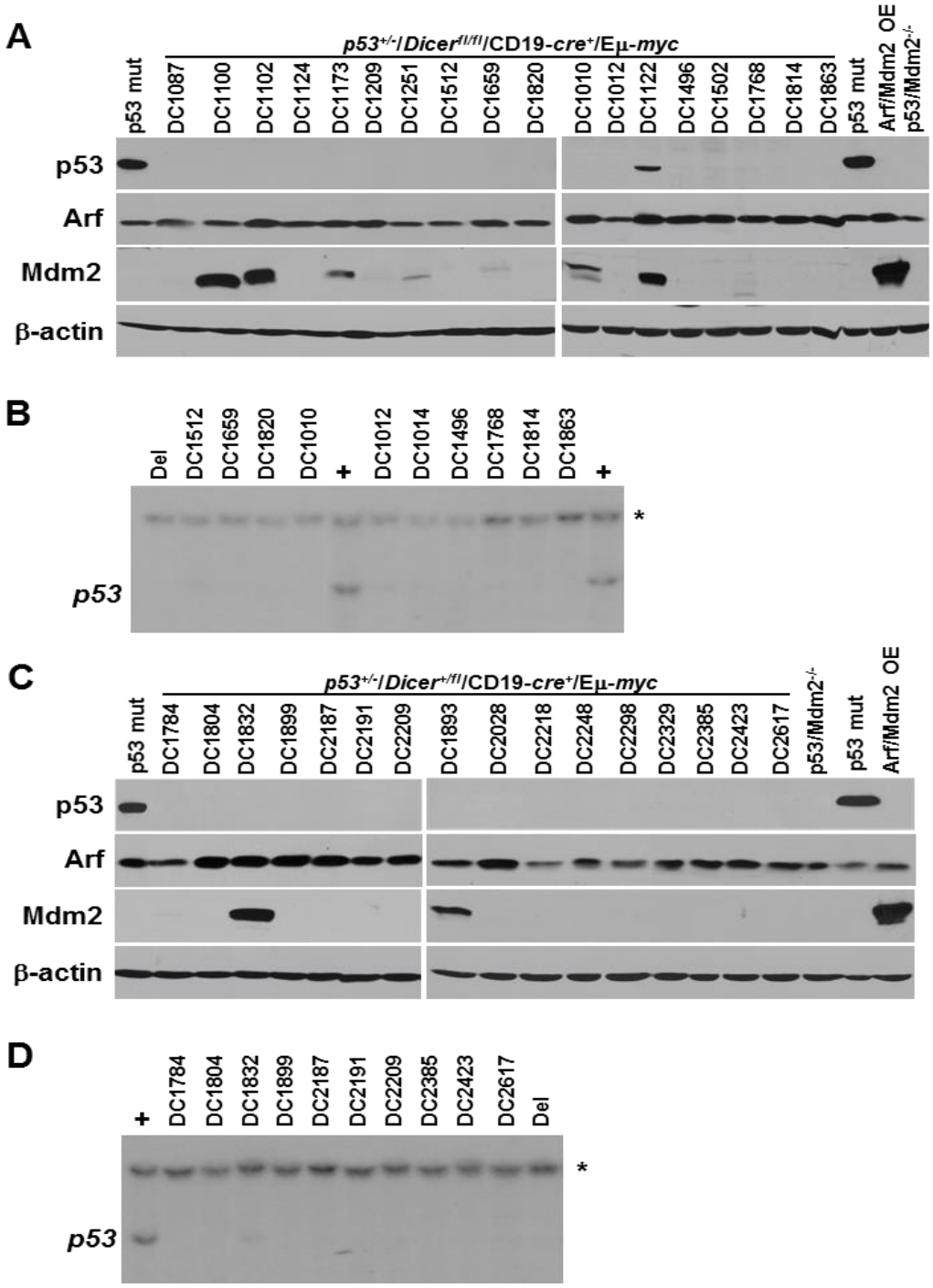

Figure 9. *Dicer* deficiency did not alter the selection for p53 inactivation. (A, C) Western blots of lymphomas for the proteins and genotype indicated. Controls include lymphomas containing mutant (mut) p53 or overexpressing (OE) Arf and Mdm2 and *p53*$^{-/-}$/*Mdm2*$^{-/-}$ murine embryonic fibroblasts (MEFs). A subset of lymphomas analyzed shown. (B, D) Representative Southern blots for *p53* of lymphomas in A and C. Lymphomas that contain (+) or have deleted (Del) *p53* were controls. Asterisk (*) denotes the DNA loading control, the p53 pseudogene. I received technical assistance with the Southern blots in B and D.

Loss of *p53* rescues the type of B-cell lymphoma that develops

Previously, we determined approximately 40% of the lymphomas that emerged in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice were of very early precursor B-cell origin, B220^{+}CD4^{+}CD43^{+}Sca1^{+} (Arrate *et al.*, 2010). We evaluated whether a p53 deficiency would alter the development or frequency of this phenotype by assessing lymphomas from *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice. Fourteen of 16 (88%) of the lymphomas analyzed were typical Eμ-*myc* pre-B and/or B-cell lymphomas (Adams *et al.*, 1985) and expressed B220 and CD19, and were either IgM^{-} or IgM^{+}; none were B220^{+}CD4^{+}CD43^{+}Sca1^{+} (Table 1). Unexpectedly, 2 of 16 lymphomas were CD3^{-}CD4^{+}CD8^{+}CD43^{+} early T-cell lymphomas (Table 1). All lymphomas analyzed from *p53*$^{+/-}$/CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* littermate controls and from *Dicer* heterozygous *p53*$^{+/-}$/CD19-*cre*$^{+}$/Eμ-*myc* mice were typical Eμ-*myc* lymphomas (Table 1). Thus, a *p53* deficiency fully restored development of the characteristic Eμ-*myc* B-cell lymphoma in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice, but it also allowed T-cell lymphomas to develop.

A deficiency in p53 rescues CD19 expression in B-cell lymphomagenesis

CD19 expression was absent or decreased in 65% of the lymphomas from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice, resulting in reduced or absent Cre expression (Arrate *et al.*, 2010). Preventing CD19 expression was one mechanism by which lymphomas could avoid *Dicer* deletion. To assess the consequences of a *p53* deficiency on CD19 expression in the lymphomas in this study, we evaluated *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas for CD19 cell surface expression. None of the 14 pre-B/B-cell lymphomas analyzed by flow cytometry lacked or had reduced CD19 cell surface expression (Figure 10A; $p<0.0001$,

Table 1. *Dicer*$^{fl/fl}$ Eμ-*myc* lymphoma phenotypes are rescued with a p53 deficiency.

	Dicer$^{+/fl}$ *p53*$^{+/-}$ Eμ-*myc*	*Dicer*$^{fl/fl}$ *p53*$^{+/-}$ Eμ-*myc*	
Phenotype	**CD19-*cre*$^{+}$**	**CD19-*cre*$^{-}$**	**CD19-*cre*$^{+}$**
B220^{+} CD19^{+} CD43^{-} IgM^{-}	4/10 (40%)	7/10 (70%)	10/16 (63%)
B220^{+} CD19^{+} CD43^{-} IgM^{+}	6/10 (60%)	3/10 (30%)	4/16 (25%)
CD3^{-} CD4^{+} CD8^{+} CD43^{+}	0/10 (0%)	0/10 (0%)	2/16 (13%)

Fisher's exact test). However, 13 of 23 (57%) *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas analyzed lacked or had significantly decreased Cre protein (Figure 10B), and 12 of the 13 (92%) had reduced *Cre* mRNA (Figure 10C). This is an unexpected result, since all the lymphomas expressed CD19 and *Cre* expression is driven by the endogenous *CD19* promoter. Of note, Cre expression occurred significantly more frequently in *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas (43%) than was previously observed in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas (12% (Arrate *et al.*, 2010); p=0.022, Fisher's exact test). Analysis of 17 heterozygous floxed *Dicer p53*$^{+/-}$/CD19-*cre*$^{+}$/Eμ-*myc* lymphomas showed they all expressed Cre protein (Figure 10D). Therefore, a deficiency in *p53* rescued CD19 surface expression and partially restored Cre expression in B-cell lymphomas from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice.

p53 deficiency is insufficient to allow *Dicer* deletion during B-cell lymphomagenesis

We previously reported that not a single lymphoma from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice had deleted both *Dicer* alleles (Arrate *et al.*, 2010). In this study, we assessed whether the exons flanked by loxP sites in the *Dicer* gene had been deleted. Evaluation of *Dicer* heterozygous *p53*$^{+/-}$/CD19-*cre*$^{+}$/Eμ-*myc* lymphomas showed that all 17 analyzed had deleted their one floxed *Dicer* allele (Figure 11A). However, 11 of 23 (48%) lymphomas analyzed from *Dicer*$^{fl/fl}$/*p53*$^{+/-}$/CD19-*cre*$^{+}$/Eμ-*myc* mice deleted one conditional *Dicer* allele, whereas the other 12 lymphomas retained both floxed alleles (Figure 11B). None of the 23 *Dicer*$^{fl/fl}$/*p53*$^{+/-}$/CD19-*cre*$^{+}$/Eμ-*myc* lymphomas had deleted both floxed *Dicer* alleles.

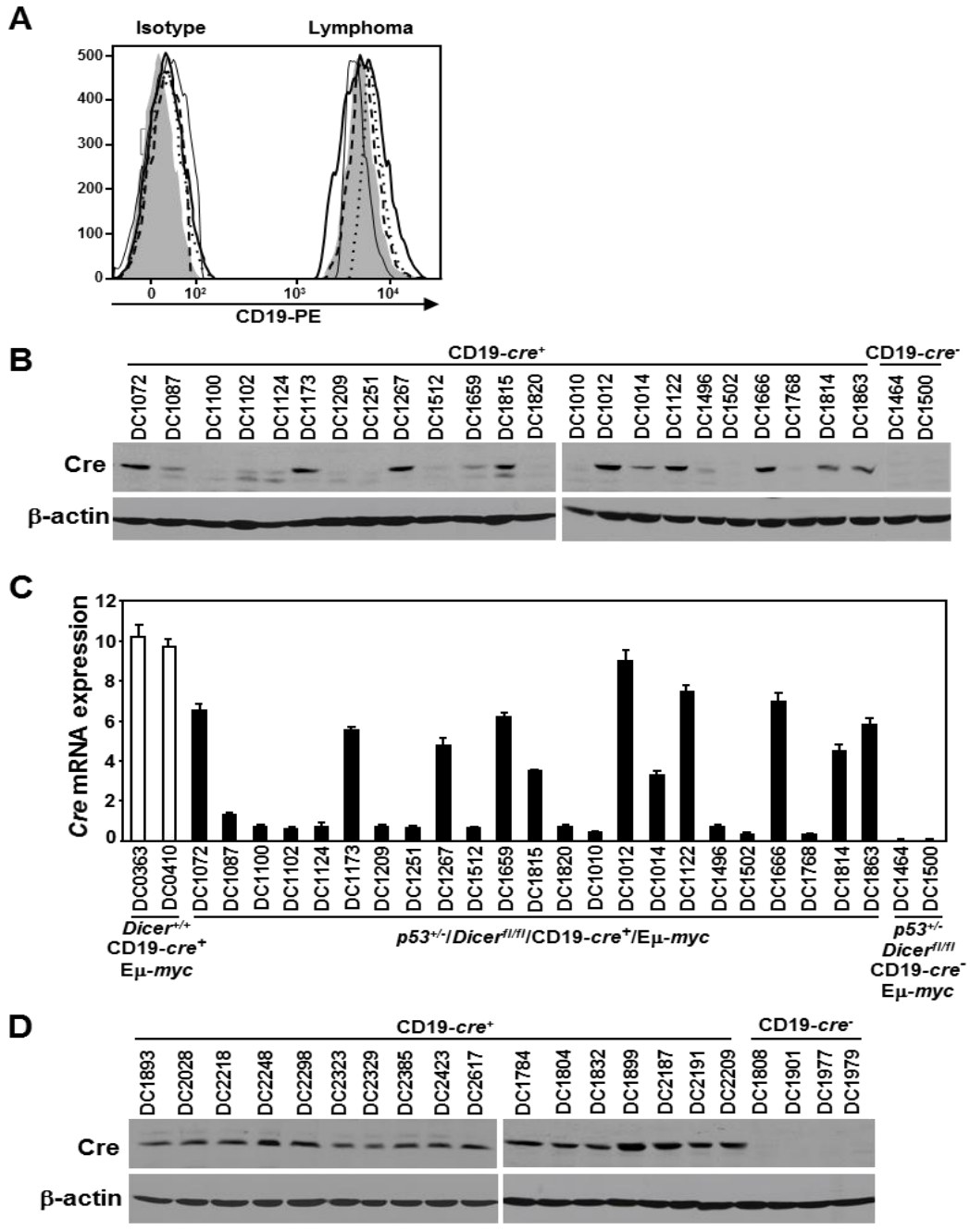

Figure 10. p53 deficiency rescues CD19 and Cre expression during B-cell lymphomagenesis. (A) Histograms of CD19 surface expression and corresponding isotype controls of lymphomas from four representative *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice compared to a control *p53*$^{+/-}$/CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma (shaded peaks). (B, D) Western blots for Cre and β-actin from *Dicer*$^{fl/fl}$ (B) and *Dicer*$^{+/fl}$ (D) *p53*$^{+/-}$/CD19-*cre*$^{+}$/Eμ-*myc* lymphomas. Lysates of lymphomas from a *Dicer*$^{+/+}$/CD19-*cre*$^{+}$/Eμ-*myc* mouse (B) and *Dicer*$^{fl/fl}$ (B) or *Dicer*$^{+/fl}$ (D) CD19-*cre*$^{-}$/*p53*$^{+/-}$/Eμ-*myc* mice were controls. (C) qRT-PCR for *Cre* expression relative to *β-actin* in lymphomas from *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice. RNA from *Dicer*$^{+/+}$/CD19-*cre*$^{+}$/Eμ-*myc* and CD19-*cre*$^{-}$/*p53*$^{+/-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas were positive and negative controls, respectively. Error bars are SEM.

Given that Cre protein expression was lost in half of the *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas, we evaluated whether Cre had ever been functional in these tumors. Four of the 13 lymphomas that lacked Cre protein (Figure 10B) had rearranged one *Dicer* allele (Figure 11B), indicating they had active Cre at some point in B-cell development. Because Cre protein was present more frequently in the lymphomas that arose in *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice compared to mice that were *p53*$^{+/+}$ (43% vs 12% (Arrate *et al.*, 2010), respectively), we expected an increased incidence of Cre-mediated deletion of at least one *Dicer* allele in the *p53*$^{+/-}$ lymphomas. However, there was no statistical difference in the frequency of deleting one allele of *Dicer* between these two groups (48% vs 38% (Arrate *et al.*, 2010), respectively; p=0.57, Fisher's exact test; Figure 11B). Importantly, our data indicate a p53 deficiency is insufficient to allow a lymphoma to emerge when both alleles of *Dicer* have been deleted.

To determine whether *Dicer* was functional in the lymphomas that emerged, we first assessed *Dicer* protein levels. Lymphomas with one allele of *Dicer* expressed an analogous amount of Dicer protein as lymphomas that retained both alleles of *Dicer* (Figure 11C). Moreover, mature miRNA transcript levels of miR-20a and miR-31, Dicer-dependent miRNA, were similar regardless of *Dicer* status in all lymphomas analyzed (Figure 11D). These data indicate all lymphomas, including those with only one *Dicer* allele, expressed wild-type levels of Dicer that was fully functional in miRNA biogenesis.

p53 loss cannot rescue B-cell development following *Dicer* deletion

In vivo, biallelic *Dicer* deletion in developing B-cells with wild-type p53 induces apoptosis, causing a developmental defect, resulting in decreased mature splenic B-cells

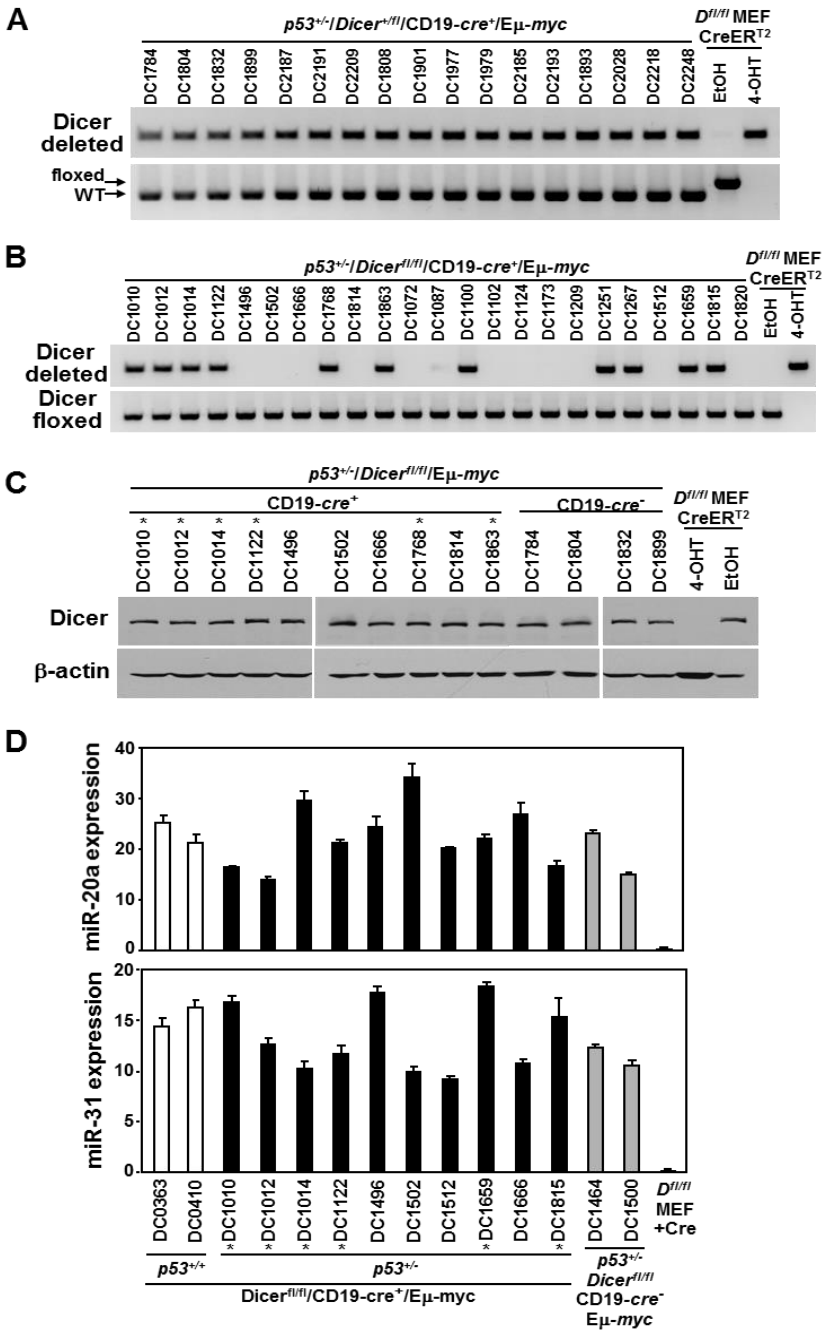

Figure 11. Biallelic *Dicer* deletion is selected against during lymphoma development. (A, B) PCR analysis for conditional deleted and floxed *Dicer* alleles from lymphomas of the indicated genotype. DNA from *Dicer*$^{fl/fl}$ (*D*$^{fl/fl}$) MEFs expressing an inducible CreER^{T2} treated with 4-OHT or vehicle control (EtOH) were controls. Arrows indicate unrearranged (floxed) and wild-type (WT) *Dicer* alleles. (C) Representative Western blots for Dicer and β-actin from CD19-*cre*$^{+}$ and CD19-*cre*$^{-}$ *p53*$^{+/-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas. Lysates from *Dicer*$^{fl/fl}$ (*D*$^{fl/fl}$) MEFs were controls. (D) qRT-PCR for *miR-20a* and *miR-31* relative to internal *RNU6b* small RNA in lymphomas from *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice. *p53*$^{+/+}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* and *p53*$^{+/-}$/*Dicer*$^{fl/fl}$/CD19-*cre*$^{-}$/Eμ-*myc* lymphomas and Cre-expressing *Dicer*$^{fl/fl}$ (*D*$^{fl/fl}$) MEFs served as controls. Asterisks (*) in C and D denote lymphomas that deleted one *Dicer* allele. Error bars are SEM.

(Arrate *et al.*, 2010; Koralov *et al.*, 2008). Protecting B-cells from this apoptosis partially rescues B-cell development (Koralov *et al.*, 2008). Since a p53 deficiency rescued the pre-B/B-cell lymphoma phenotype in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice, we questioned whether p53 mediates the *Dicer* deletion-induced B-cell apoptosis. To address this, we evaluated splenic B-cells from pre-cancerous *p53*$^{-/-}$ and *p53*$^{+/-}$ CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$ mice and CD19-*cre*$^{-}$ littermate controls. There was a modest, but statistically significant, reduction in the percentage of B220^{+}/IgM^{+} B-cells in CD19-*cre*$^{+}$/*p53*$^{-/-}$/*Dicer*$^{fl/fl}$ mice (32.9% ± 1.42) compared to CD19-*cre*$^{-}$/*p53*$^{-/-}$/*Dicer*$^{fl/fl}$ littermates (40.9% ± 1.46; $p<0.0001$, paired t-test; Figure 12). A comparable reduction in B-cells was also observed in *p53*$^{+/-}$ littermates that were either CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$ or CD19-*cre*$^{-}$/*Dicer*$^{fl/fl}$ (31.2% ± 0.90 and 40.7% ± 0.51, respectively; $p<0.0001$, paired t-test; Figure 12). As an additional control, we assessed B-cells in *p53*$^{+/+}$/*Dicer*$^{+/fl}$ mice with or without CD19-*cre* and the percentages of B-cells were similar in both, demonstrating B-cell expression of Cre did not alter B-cell numbers in the mice (Figure 12). Thus, deletion of one or two alleles of *p53* could not rescue the decrease in B-cell numbers induced by *Dicer* deletion, *in vivo*.

To further test the requirement for p53 in B-cell survival in the absence of Dicer, we derived primary pre-B cells from bone marrow of *p53*$^{-/-}$/*Dicer*$^{+/fl}$, *p53*$^{-/-}$/*Dicer*$^{fl/fl}$, and *p53*$^{+/-}$/*Dicer*$^{fl/fl}$ littermates. Pre-B cells were infected with a bicistronic retrovirus encoding GFP and a 4-hydroxytamoxifen (4-OHT)-inducible CreER^{T2} (Feil *et al.*, 1997), and GFP-positive cells were sorted by flow cytometry. All three genotypes of pre-B cells expressed equal levels of CreER^{T2} protein (Figure 13A). To delete *Dicer*, pre-B cells were treated with 4-OHT to activate CreER^{T2}. As expected for primary pre-B cells with functional *p53*, the *Dicer*$^{fl/fl}$/*p53*$^{+/-}$ cells grew at a slower rate and were sensitive to *Dicer* loss, as indicated

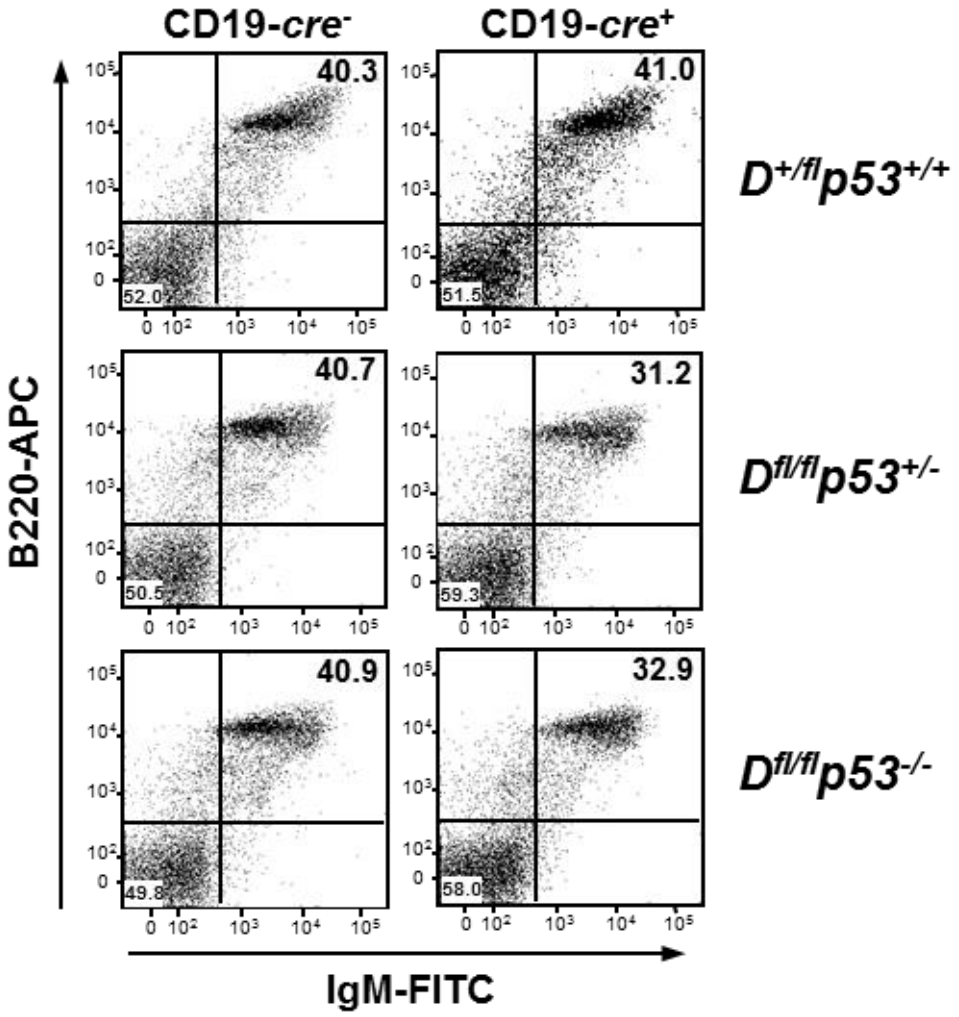

Figure 12. Loss of *p53* does not rescue the decrease in B cell numbers induced by *Dicer* deletion. Representative dot plots of littermate-matched splenic B-cells from CD19-*cre*$^{+}$ or CD19-*cre*$^{-}$ *Dicer*$^{+/fl}$ and *Dicer*$^{fl/fl}$ mice that were *p53*$^{+/+}$, *p53*$^{+/-}$, or *p53*$^{-/-}$. Total lymphocytes were gated and B220-APC versus IgM-FITC was assessed.

by decreased cell numbers and viability (Figures 13A and 13B). Similarly, following 4-OHT treatment, *Dicer*$^{fl/fl}$/*p53*$^{-/-}$ pre-B cells experienced a dramatic decrease in total number, viability, and growth, and an increased percentage of cells containing fragmented DNA (sub-G1) and appearance of cleaved Caspase 3, compared to vehicle-treated cells, which were unaffected (Figures 13A-D). When 4-OHT was administered to CreER^{T2} expressing *Dicer*$^{+/fl}$/*p53*$^{-/-}$ pre-B cells, no change in cell number, viability, growth, fragmented DNA, or cleaved Caspase 3 was observed (Figures 13A-D), as would be expected for pre-B cells with one wild-type *Dicer* allele. *Dicer* gene rearrangement was assessed in the surviving pre-B cells and showed that, regardless of genotype, only one *Dicer* allele was rearranged in the CreER^{T2} activated pre-B cells (Figure 13E). Notably, *Dicer*$^{fl/fl}$ fibroblasts containing similar levels of CreER^{T2} protein (Figure 13A) deleted both floxed *Dicer* alleles (Figure 13E). Therefore, loss of p53 could not rescue the rapid apoptosis induced by biallelic *Dicer* deletion in primary untransformed pre-B cells, and only pre-B cells that had retained one allele of *Dicer* could survive.

Dicer **is required for B-cell lymphoma survival**

Recently, Dr. Sharp and colleagues reported that a *p53*-null murine sarcoma cell line could survive and proliferate without *Dicer* (Ravi *et al.*, 2012), suggesting cellular transformation may alter the requirements for Dicer. We tested whether transformed B-cells could survive loss of Dicer if they also lacked *p53*. B-cell lymphomas were isolated from two *p53*$^{+/-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice (DC1020 and DC1185) and as controls, two *p53*$^{+/-}$/*Dicer*$^{+/fl}$/Eμ-*myc* mice (DC2385 and DC2423). p53 protein was not detected by Western blot, and Southern blot showed deletion of the remaining wild-type allele of *p53*

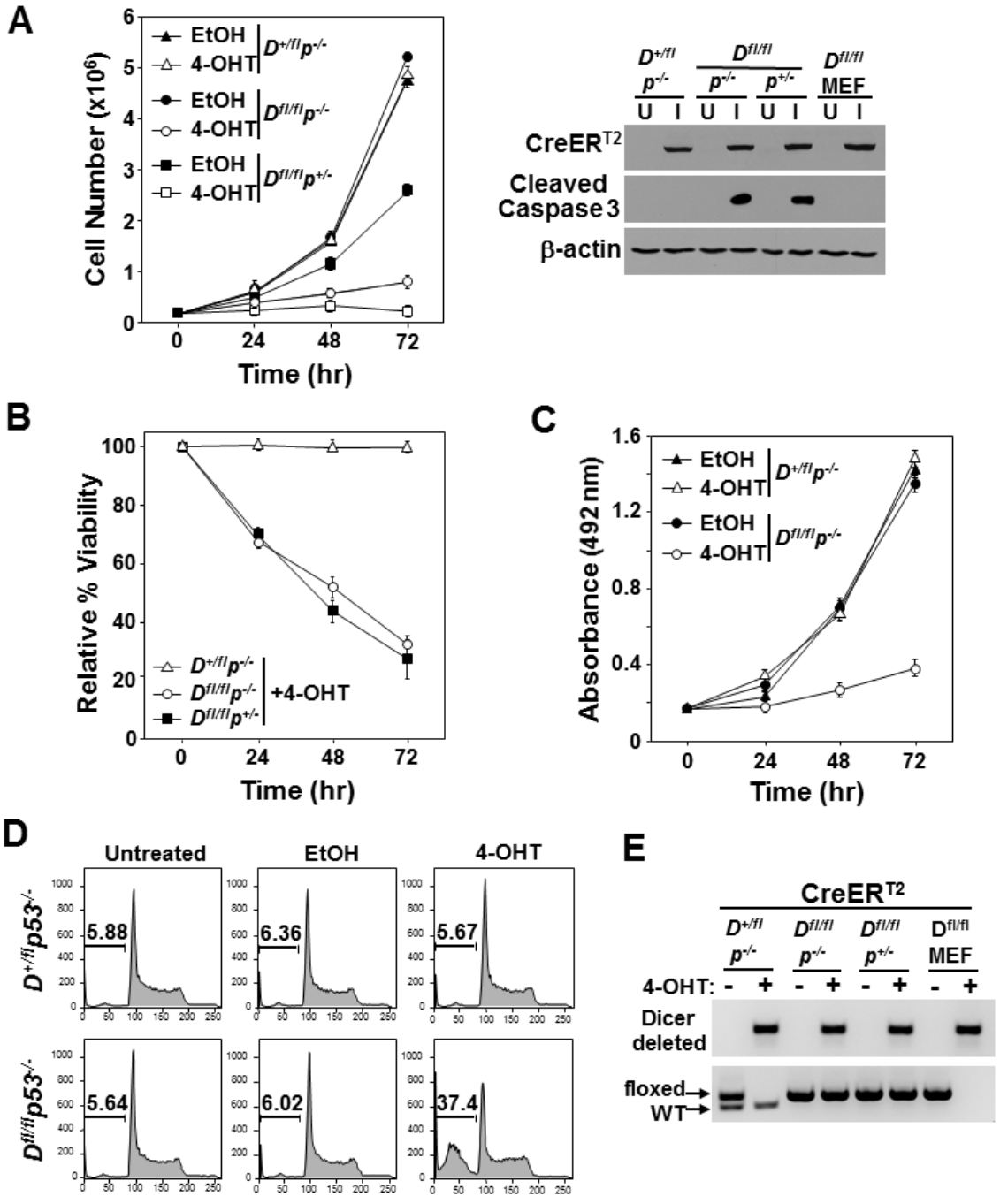

Figure 13. Loss of *p53* is insufficient for B-cell survival when *Dicer* is deleted. Primary pre-B cells from *p53*$^{-/-}$/*Dicer*$^{fl/fl}$, *p53*$^{+/-}$/*Dicer*$^{fl/fl}$, and *p53*$^{-/-}$/*Dicer*$^{+/fl}$ littermates were infected (I) with a retrovirus encoding CreER^{T2} or left uninfected (U). 4-OHT (+) or vehicle control (EtOH, -) was added to pre-B cell cultures at time 0 and cell number (A), viability (B), proliferation (MTS assay; C), apoptosis (cleaved Caspase 3 protein, A; sub-G1 DNA, D), and *Dicer* gene rearrangement (E) were evaluated. Western blots shown in A. For E, arrows indicate unrearranged (floxed) and wild-type (WT) *Dicer* alleles. CreER^{T2} expressing *Dicer*$^{fl/fl}$ (*D*$^{fl/fl}$) MEFs treated with 4-OHT (+) or ethanol (-) were controls in A and E. Error bars are SD.

in all four lymphomas (Figures 14A, 9C, and 9D). The lymphomas were infected with a bicistronic retrovirus encoding CreER^{T2} and GFP or GFP alone. CreER^{T2} activation with 4-OHT in the *p53* deleted *Dicer*$^{+/fl}$/Eμ-*myc* lymphomas had no effect on cell number or viability compared to a *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma, which showed a significant decrease in cell number and viability after CreER^{T2} activation (Figure 14B and 14C). CreER^{T2} activation in both *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas resulted in apoptosis, whereas there was little effect following addition of vehicle control or 4-OHT to lymphomas infected with empty retrovirus (Figures 15A-D). Specifically, the total number and viability of CreER^{T2} *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cells decreased, while the percentage of apoptotic cells (cells with fragmented, sub-G1 DNA or that were Annexin V^{+}) increased after addition of 4-OHT (Figures 15A-D). PCR analysis revealed the *p53* deleted *Dicer*$^{+/fl}$/Eμ-*myc* lymphoma cells deleted their one floxed *Dicer* allele, while the *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cells surviving CreER^{T2} activation had only deleted one of the conditional *Dicer* alleles (Figure 15E). Analogous results were obtained with *Dicer*$^{fl/fl}$/Eμ-*myc* B cell lymphomas that had deleted *Arf* and retained *p53* (Figure 16A-F).

We postulated it was possible for preferential outgrowth of lymphoma cells possessing one allele of *Dicer*, masking the presence of a small population of lymphoma cells that had deleted both alleles of *Dicer*. To evaluate this possibility, we performed single-cell sorting for GFP-positive cells of two independent CreER^{T2} expressing *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma lines into 96-well plates (Figure 17A). After visually confirming the presence of a single cell per well, CreER^{T2} was activated with 4-OHT, and the surviving clones were assessed (Table 2). Only 26% (328 of 1,260) of the clones

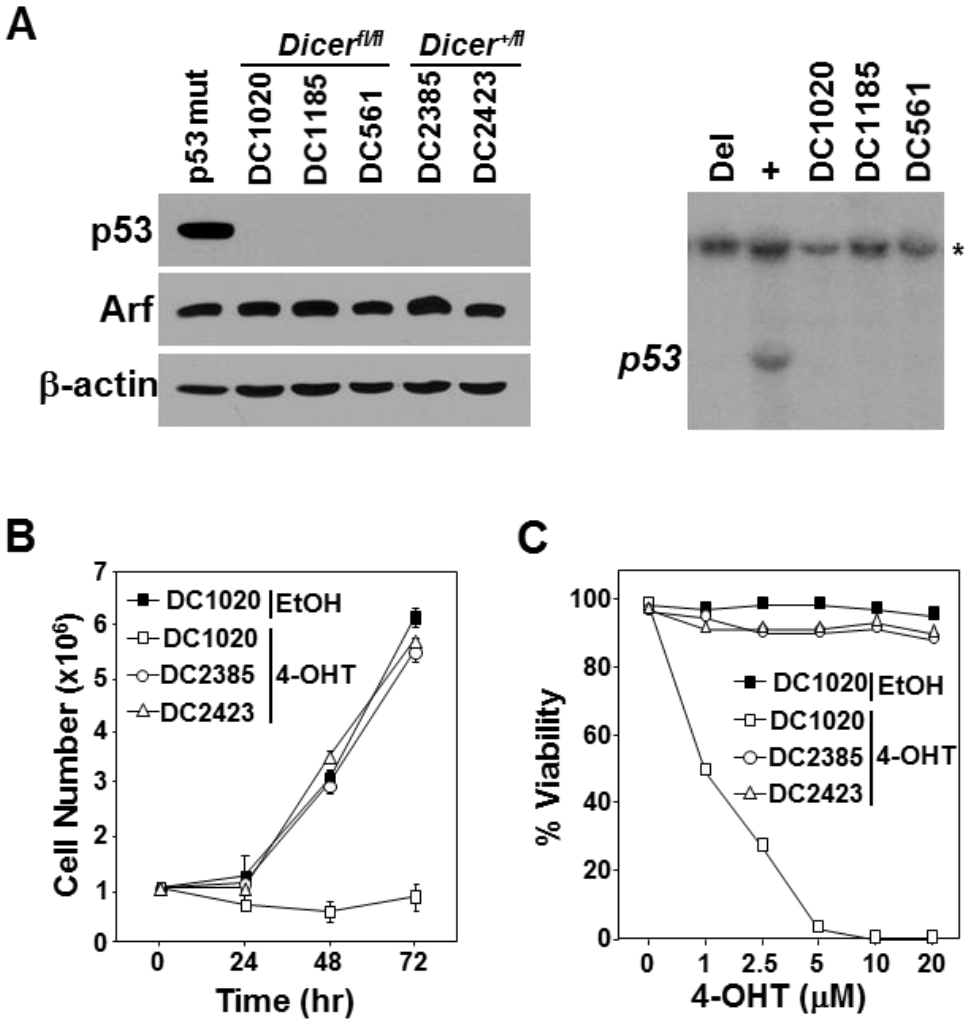

Figure 14. Decreasing viability with increasing CreER^{T2} activation only in B-cell lymphoma with two floxed *Dicer* alleles. (A) *p53*$^{+/-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* (DC1020 and DC1185), *p53*$^{+/-}$/*Dicer*$^{+/fl}$/Eμ-*myc* (DC2385 and DC2423) lymphoma cell lines and the *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cell line (DC561) from our previous study (Arrate *et al.*, 2010) were subjected to Western blot (left) for the proteins indicated and Southern blot (right and Figure 9D) for *p53*. A lymphoma containing mutant p53 was a control for the Western blot. Lymphomas that contain (+) or have deleted (Del) *p53* were controls for the Southern blot. Asterisk (*) denotes the DNA loading control, the p53 pseudogene. (B, C) DC2385, DC2423, and DC1020 lymphoma cells were infected with a CreER^{T2}-encoding retrovirus. 4-OHT or vehicle control (EtOH) was added to the cultures at time 0 and cell number (B) was measured at intervals. For C, cells were incubated with vehicle control (EtOH) or the indicated concentrations of 4-OHT to activate CreER^{T2}. After 72 hours, cells were counted in the presence of Trypan Blue dye to distinguish dead and dying cells and viability was calculated. Error bars are SD. I received technical assistance with the Southern blot in A.

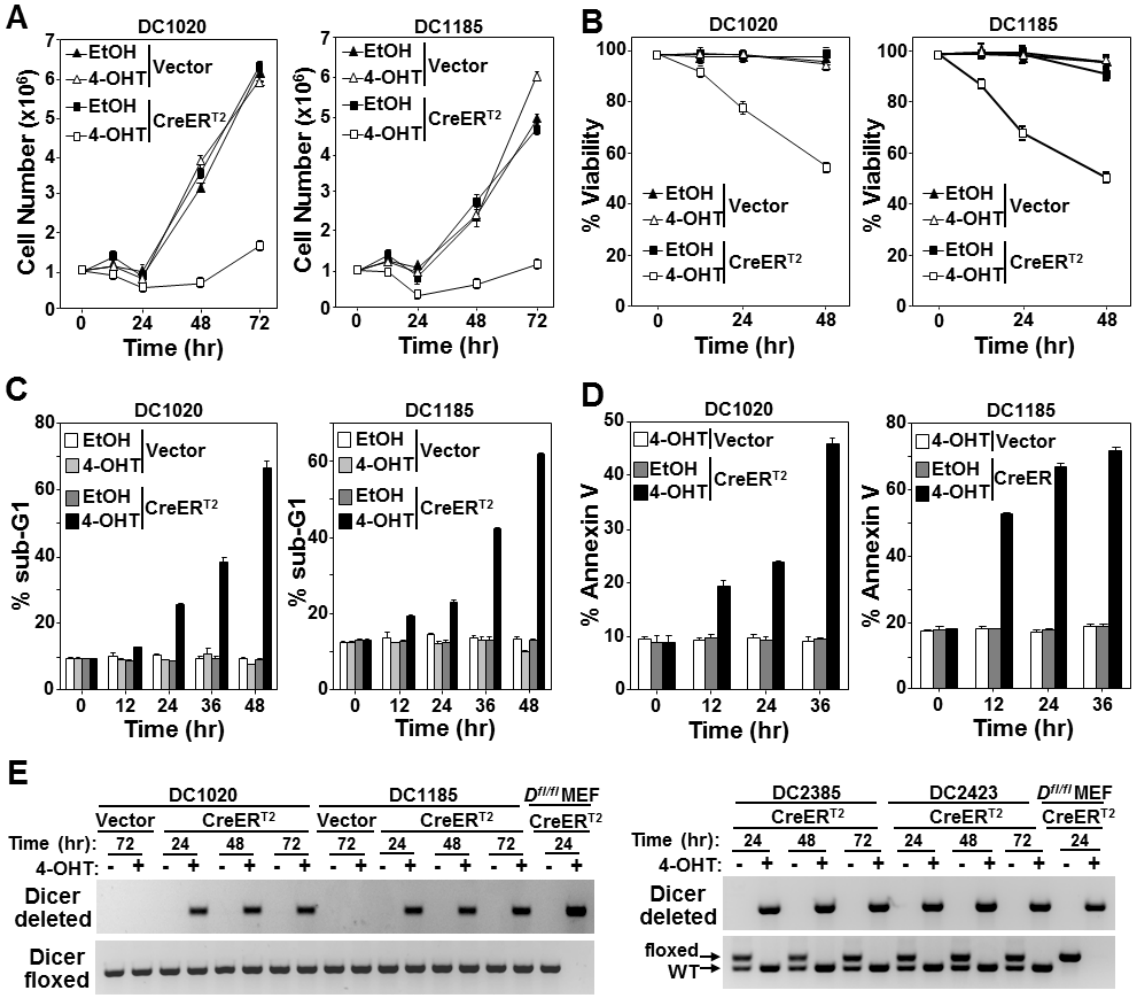

Figure 15. A deficiency in *p53* does not allow B-cell lymphomas to survive without *Dicer*. (A-D) *p53*$^{+/-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cell lines (DC1020 and DC1185) were infected with a CreER^{T2}-encoding retrovirus or empty retrovirus (Vector). 4-OHT or vehicle control (EtOH) was added to the cultures at time 0 and cell number (A), viability (B), and apoptosis (sub-G1 DNA, C; Annexin V, D) were measured at intervals. (E) *Dicer* gene rearrangement was evaluated at the indicated intervals by PCR. Conditional deleted and floxed (not deleted) *Dicer* alleles shown. CreER^{T2} expressing *Dicer*$^{fl/fl}$ (*D*$^{fl/fl}$) MEFs treated with 4-OHT or ethanol (EtOH) were controls. Error bars are SD.

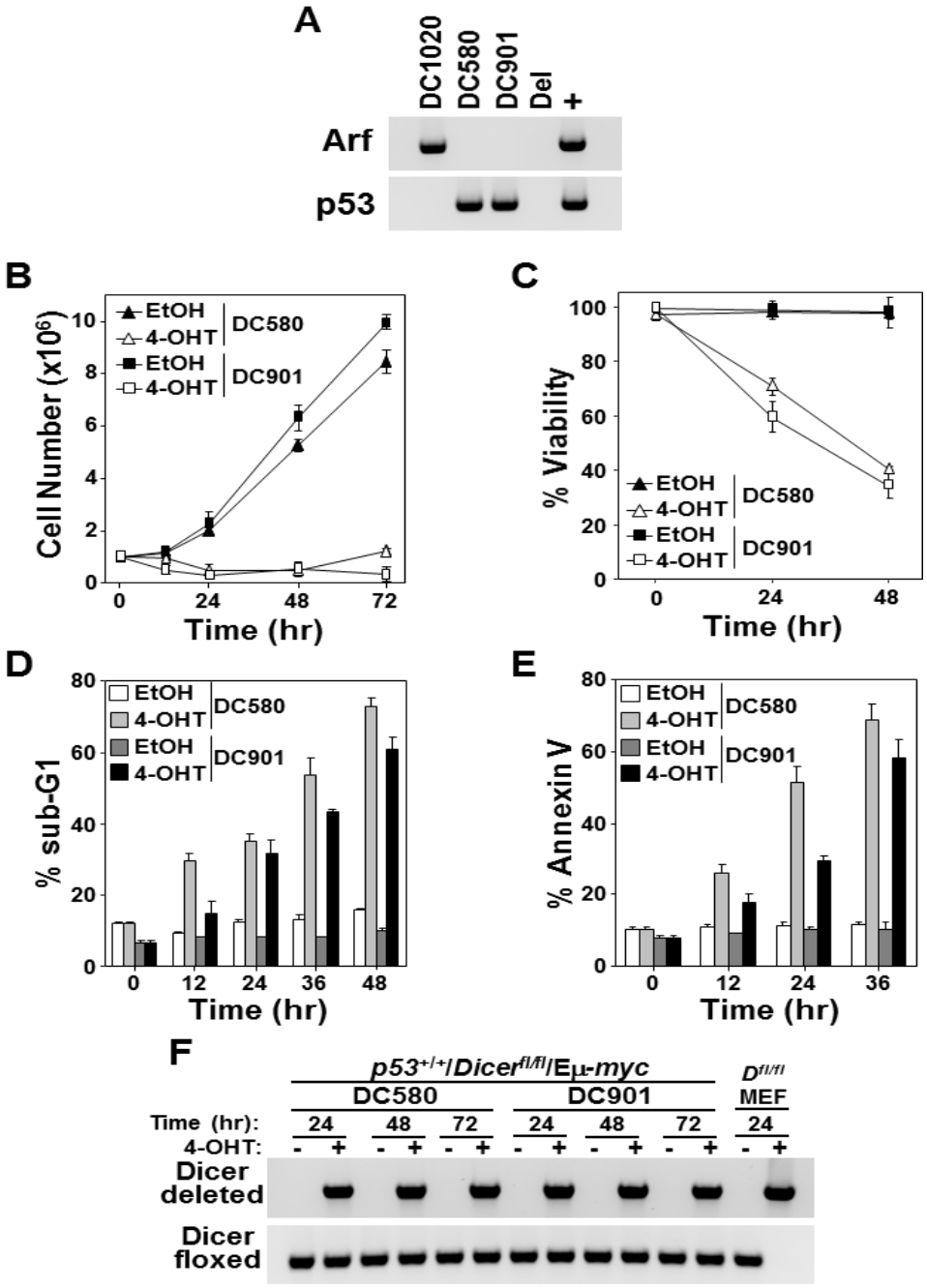

Figure 16. *Dicer* deletion in *p53* wild-type, *Arf* deleted lymphomas induces apoptosis. (A) Genomic DNA from *p53* wild-type, *Arf* deleted *Dicer*$^{fl/fl}$/Em-*myc* lymphoma cell lines (DC580 and DC901) and the *p53* deleted *Dicer*$^{fl/fl}$/Em-*myc* lymphoma cell line (DC1020) was subjected to PCR for *Arf* and *p53*. Genomic DNA isolated from tail clips from mice that contain (+) or have deleted (Del) *Arf* and *p53* were controls for the PCR. (B-E) Lymphomas were infected with a 4-OHT-inducible CreER^{T2} retrovirus. Cell number (B), viability (C), sub-G1 (apoptotic) DNA content (D), and Annexin V positivity (E) were measured at the indicated intervals following administration of vehicle control (EtOH) or 4-OHT to activate CreER^{T2}. (F) *Dicer* gene rearrangement was evaluated at the indicated intervals following vehicle control (-) or 4-OHT (+) addition by PCR. CreER^{T2} expressing *Dicer*$^{fl/fl}$ (*D*$^{fl/fl}$) MEFs were controls. Error bars are SD.

Table 2. *p53*$^{+/-}$*/Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma clone analysis.

	CreER^{T2} + EtOH	CreER^{T2} + 4-OHT
Survival	98.5% (394 of 400 clones)	26% (328 of 1260 clones)
Deletion		
0 alleles	100% (394 of 394 clones)	6.7% (22 of 328 clones)
1 allele	0% (0 of 394 clones)	93.3% (306 of 328 clones)
2 alleles	0% (0 of 394 clones)	0% (0 of 328 clones)

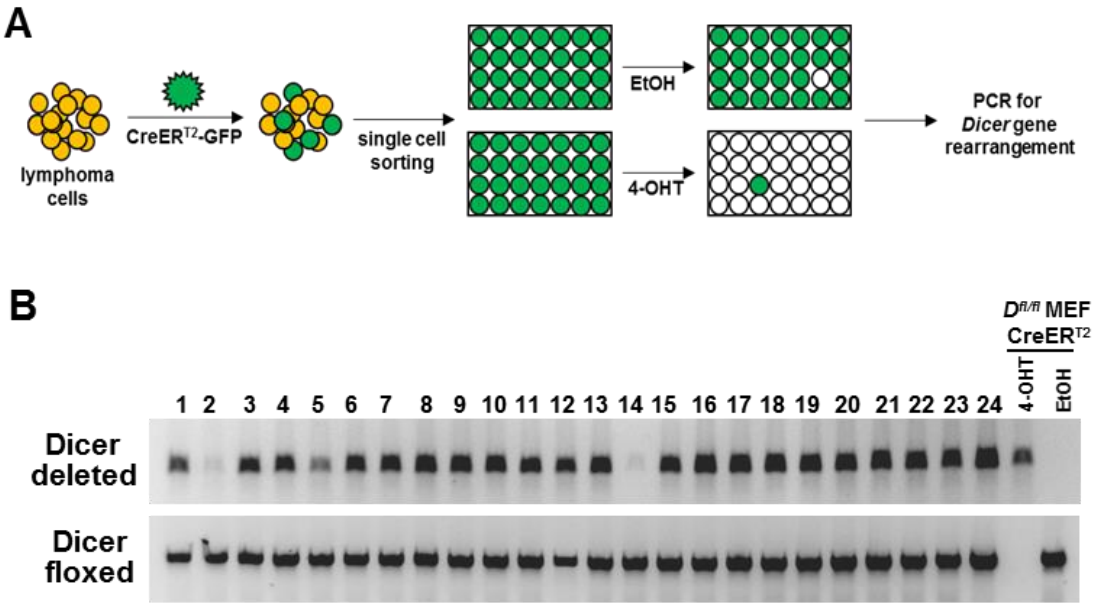

Figure 17. At least one *Dicer* allele is required for B cell lymphoma survival. (A) Experimental approach for single cell survival analysis. (B) *Dicer* gene rearrangement was evaluated by PCR. Representative PCR product analysis of *Dicer* gene rearrangement of GFP-positive single cell-sorted lymphoma clones that survived CreER^{T2} activation of the 328 analyzed. Conditional deleted and floxed (not deleted) *Dicer* alleles shown. CreER^{T2} expressing *Dicer*$^{fl/fl}$ ($D^{fl/fl}$) MEFs treated with 4-OHT or ethanol (EtOH) were controls.

survived CreER^{T2} activation. Analysis of all 328 lymphoma clones that survived CreER^{T2} activation revealed none had deleted both *Dicer* alleles (a subset of those analyzed is shown in Figure 17B). Instead, 306 (93.3%) had deleted one *Dicer* allele, whereas the other 22 (6.7%) maintained both floxed alleles. Moreover, analysis of the *Dicer$^{fl/fl}$*/Eμ-*myc* lymphoma used in the single-cell analysis in our previous study (DC561, (Arrate *et al.*, 2010)) where we obtained analogous results, revealed that it had biallelic *p53* deletion (Figure 14A). Collectively, these data illustrate that B-cell lymphomas cannot survive without *Dicer*, even when *p53* is deleted. Therefore, at least one allele of *Dicer* is required for B-cell lymphoma survival.

In vivo Dicer deletion inhibits lymphoma growth and extends survival

Given that B-cell lymphomas require Dicer for survival, *in vitro*, we tested whether inactivating *Dicer* would alter lymphoma growth *in vivo* with three different mouse experiments. Firstly, *p53* deleted *Dicer$^{fl/fl}$*/Eμ-*myc* lymphoma cells (DC1020) expressing CreER^{T2} were subcutaneously injected into nude mice and CreER^{T2} was activated by tamoxifen the same day. There was a significant delay in lymphoma progression and extended survival in the mice that received tamoxifen compared to the vehicle-treated mice (Figure 18A; p=0.0012, log-rank test). Tumors from vehicle-treated mice grew significantly larger more quickly compared to tumors from mice that received tamoxifen to activate CreER^{T2} (Figure 18B; *p=0.0051, **p<0.003).

To determine whether loss of Dicer would impact established lymphomas, we allowed a cohort of mice to grow subcutaneous lymphomas of 90-150mm^3 and then administered tamoxifen or vehicle control (tumor sizes were matched between groups)

(Figures 18C and 18D). While the rapid rate of tumor growth continued in the vehicle-treated mice, tumor expansion in the mice that received tamoxifen to activate CreER^{T2} to delete *Dicer* slowed dramatically (Figure 18D; *p=0.0288, **p=0.0005). Analysis of tumors that were equivalent in size prior to tamoxifen addition, showed significant and increasing apoptosis over time following tamoxifen, as indicated by increased sub-G1 DNA content (Figure 19A, *p=0.008), Annexin V positivity (Figure 19B, **p<0.0001), and cleaved Caspase 3 protein (Figure 19C). The consequence of this apoptosis was that the survival of the CreER^{T2}-activated (tamoxifen) mice was significantly increased (Figure 18C; p=0.0035, log-rank test).

To assess whether the delayed tumor growth in both experiments and the apoptosis detected was a result of CreER^{T2}-mediated *Dicer* deletion, PCR analysis of *Dicer* gene rearrangement was performed. Surviving lymphoma cells in the mice administered tamoxifen all retained at least one *Dicer* allele (Figure 20A-C), and expressed Dicer protein (Figure 19C). Therefore, targeting *Dicer* deletion, *in vivo*, induced apoptosis, delaying lymphoma progression and extending survival regardless of when *Dicer* was deleted.

As a third approach to test the effects of *Dicer* deletion in lymphomas *in vivo*, we also injected *p53* deleted *Dicer$^{fl/fl}$*/Eμ-*myc* lymphoma cells expressing CreER^{T2} and GFP into the blood stream of nude mice; tamoxifen or vehicle control administration began on the same day. By day 17 and certainly by day 21, vehicle control-treated mice had more lymphoma cells present in their blood compared to mice that received tamoxifen to activate CreER^{T2} and delete *Dicer* (Figure 21A and 21B, *p<0.0001). Furthermore, mice that had activated CreER^{T2} (tamoxifen) lived significantly longer than control mice (Figure 21C; p<0.0001, log-rank test). Collectively, all three *in vivo* experiments show that deleting

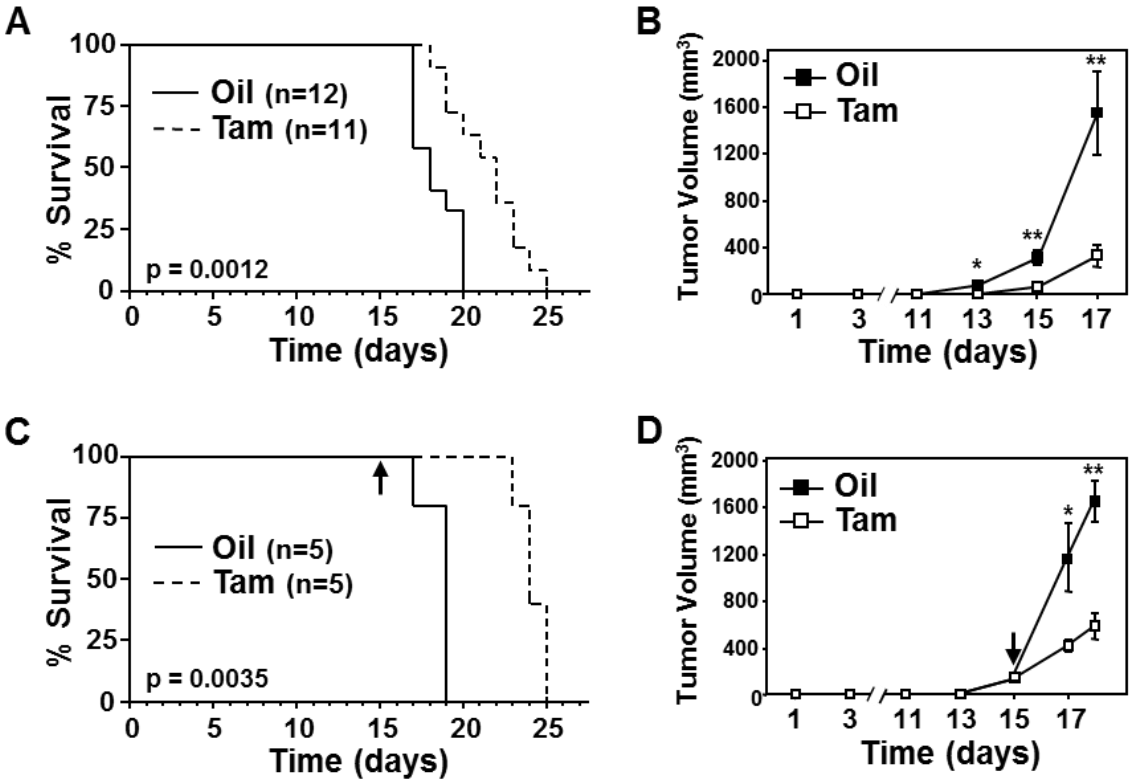

Figure 18. ***Dicer*** **inactivation impedes tumor growth,** ***in vivo*****.** (A, C) Kaplan-Meier survival curves of nude mice injected (subcutaneously) with CreER^{T2} expressing *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cells (DC1020) and administered tamoxifen or vehicle control (corn oil) starting the day of injection (A; p=0.0012, log-rank test) or once lymphomas were 90-150mm^3 (C; p=0.0035, log-rank test). Arrow indicates the day tamoxifen administration began for C. The number (n) of mice is indicated. (B, D) Tumor volumes for mice in A and C, respectively, were measured at the indicated intervals (for B: *p=0.0051, **p<0.003; for D: *p=0.0288, **p=0.0005). In D, the arrow indicates the day tamoxifen administration began. Error bars are SD. Dr. Eischen performed the lymphoma injections.

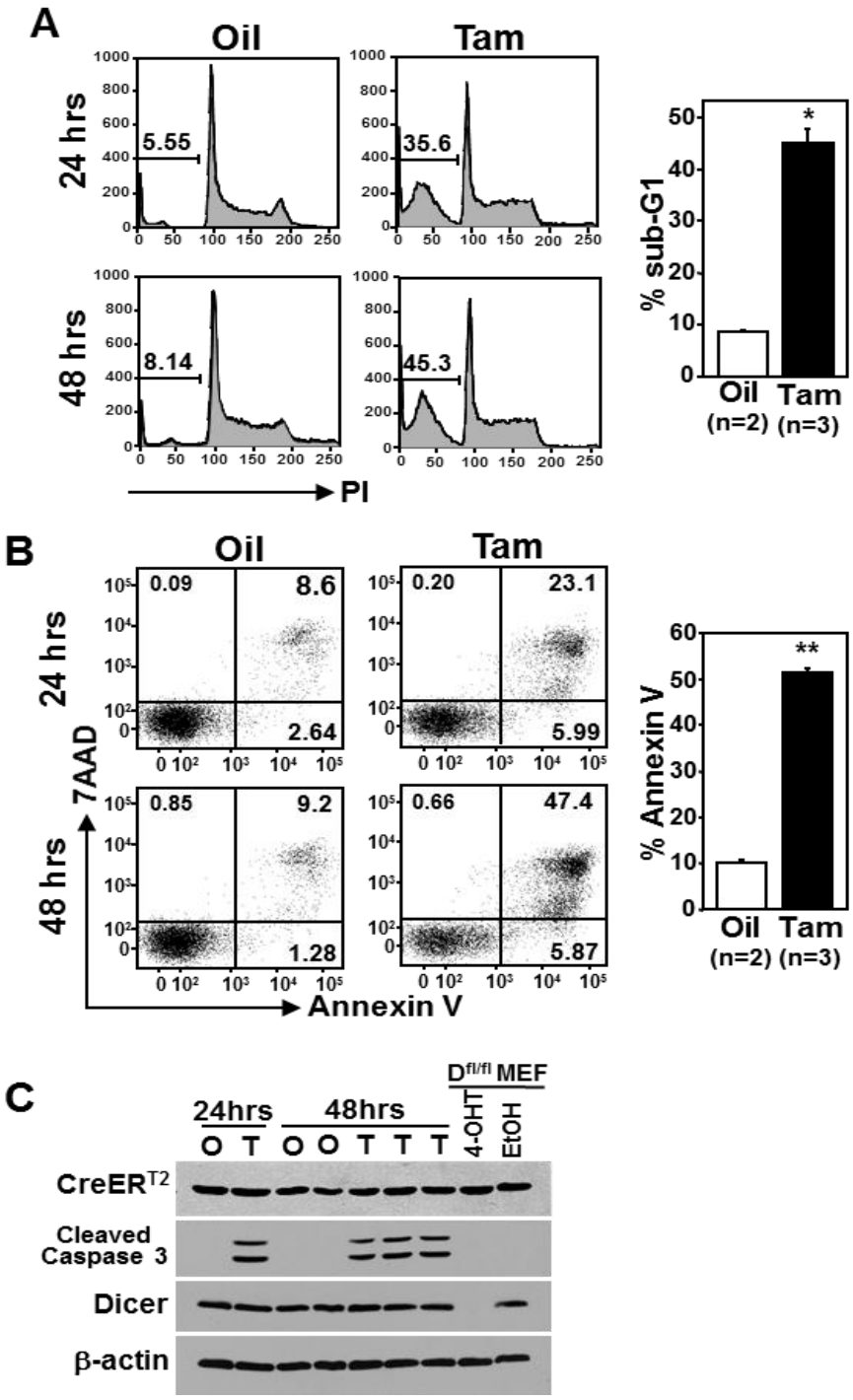

Figure 19. *Dicer* deletion induces apoptosis of lymphoma cells, *in vivo.* Apoptosis was measured at intervals following tamoxifen or vehicle control administration in matched tumor pairs from mice in Figures 18C and 18D by propidium iodide staining of fragmented (sub-G1) DNA (A), Annexin V/7AAD staining (B), and cleaved Caspase 3 protein detection (C). Representative data (left) and mean values at 48 hours (right) are shown for A and B; *p=0.0008, **p<0.0001, t-tests. Western blots of whole cell lysates for the proteins indicated (C). Controls for C include protein lysates from *Dicer*$^{fl/fl}$ MEFs treated with 4-OHT or ethanol. Error bars are SD.

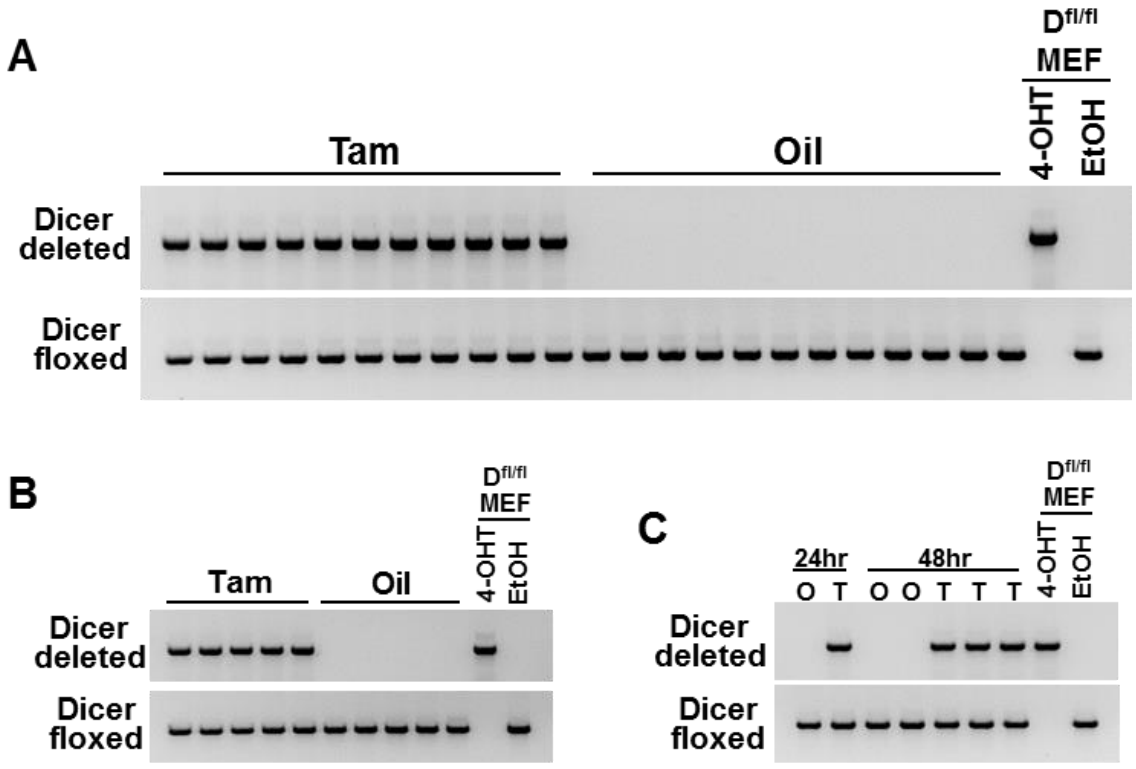

Figure 20. One allele of *Dicer* is retained in lymphomas induced to delete *Dicer, in vivo*. PCR analysis for conditional deleted and floxed (not deleted) *Dicer* alleles from *p53* deleted *Dicer*$^{fl/fl}$/Em-*myc* (DC1020) lymphoma cells expressing an inducible CreER^{T2} that were injected subcutaneously into nude mice. (A) Lymphomas isolated at humane endpoints from mice that received tamoxifen (Tam) to induce CreER^{T2} (n=11) or corn oil (Oil) vehicle control (n=12) beginning the same day as lymphoma injection (mice from Figure 18A). (B) Tamoxifen (T) or corn oil (O) vehicle control were administered once lymphomas were 90-150 mm^3 (mice from Figure 18C). Hours post tamoxifen or corn oil administration are indicated. DNA from *Dicer*$^{fl/fl}$ (*D*$^{fl/fl}$) MEFs expressing an inducible CreER^{T2} treated with 4-OHT or vehicle control (EtOH) were controls.

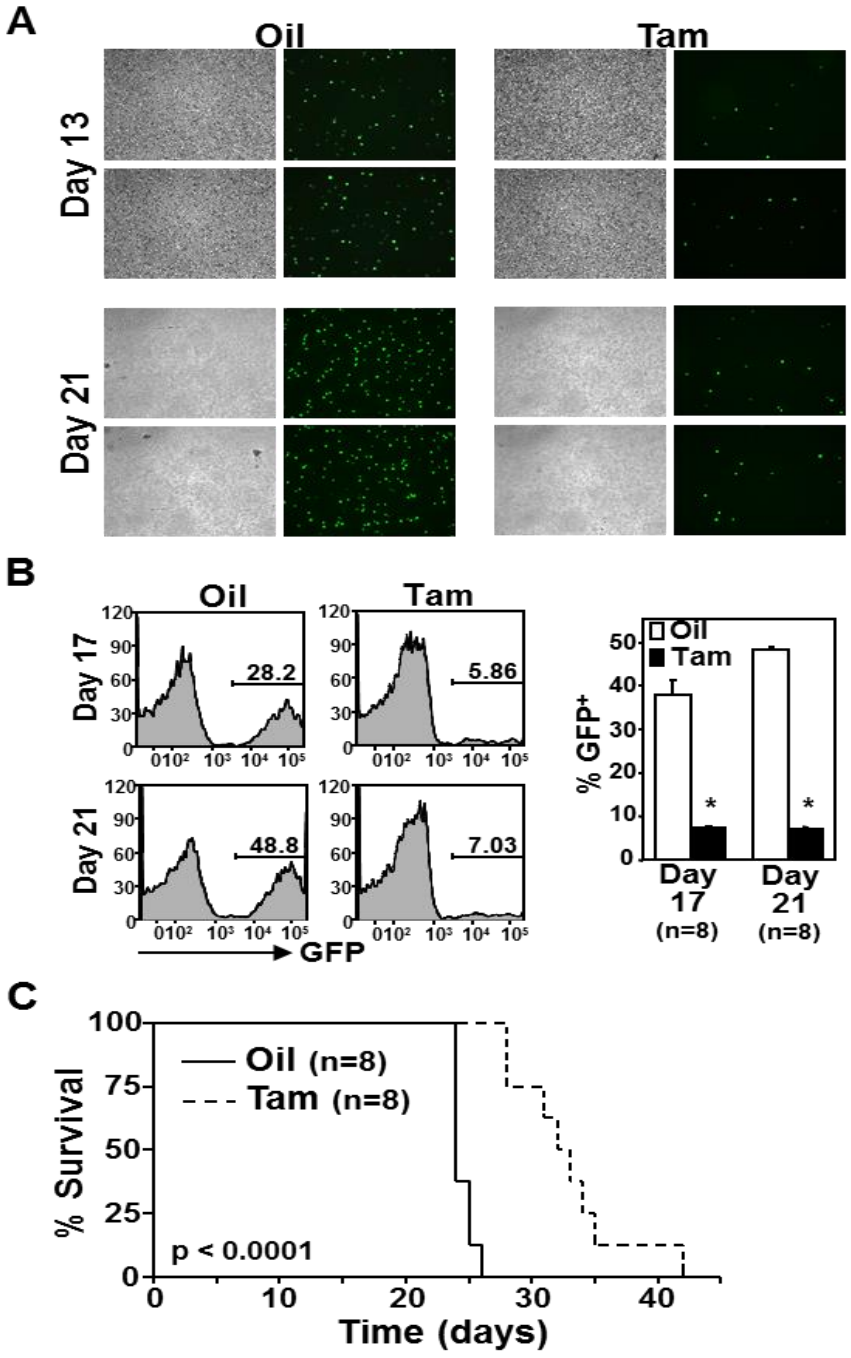

Figure 21. ***In vivo Dicer*** **deletion significantly reduces lymphoma burden in the blood of mice.** Nude mice were injected intravenously with CreER^{T2} expressing *p53* deleted *Dicer*$^{fl/fl}$/Eμ-*myc* lymphoma cells (DC1020) and administered tamoxifen (Tam) or corn oil (Oil) vehicle control starting the same day. (A) Representative microscopic images (white light, left and GFP fluorescence, right) of blood with GFP^{+} lymphoma cells in the same four mice 13 and 21 days post lymphoma injection. (B) Blood was also assessed for GFP-positivity by flow cytometry at the indicated intervals post lymphoma injection. Representative data (left) and mean values for the indicated number of mice are shown (right; *p<0.0001, t-test). Kaplan-Meier survival curves (C; p<0.0001, log-rank test). Error bars are SD. Dr. Eischen performed the lymphoma injections and I assisted Dr. Eischen with the blood collections.

Dicer in B-cell lymphomas leads to apoptosis and decreased lymphoma cell expansion, providing evidence that targeting Dicer in B-cell lymphomas may have therapeutic potential even when lymphomas lack a functional p53 pathway.

Discussion

Previously, we detected an increase in *p53* inactivation in B-cell lymphomas from CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice (Arrate *et al.*, 2010), suggesting a connection between *p53* activation and *Dicer* deletion. Moreover, we also observed *Dicer* deletion in untransformed MEFs increased p53 levels and induced a premature senescent phenotype that could be delayed by deleting either the *Ink4a/Arf* or *p53* locus (Mudhasani *et al.*, 2008). Others reported a fraction of a murine *p53*-null, mutant K-Ras expressing sarcoma cell line and SV40-immortalized, and thus p53 and Rb inactivated, mesenchymal stem cells could survive *Dicer* deletion (Ravi *et al.*, 2012). Although the data pointed to p53 being a critical mediator of the deleterious effects of *Dicer* deletion, we show here loss of p53 could not rescue the profound apoptosis that occurs in primary B-cells and B-cell lymphomas upon *Dicer* deletion. All approaches to obtain *p53*-null B-cells or B-cell lymphomas that had biallelic *Dicer* deletion resulted in one *Dicer* allele being retained in any surviving cells, whereas *Dicer*-null fibroblasts could be easily generated. These results indicate Dicer, and consequently miRNA, have essential functions in B-cell survival for both untransformed and malignant B-cells that cannot be overcome by loss of p53. Also, lymphomas that lacked Arf could not survive *Dicer* deletion, indicating inactivation of the p53 pathway is insufficient to allow B cell lymphoma survival. Moreover, the data show all stages of B-

cell transformation from immortalized (*p53*-null) to transformed (lymphoma) require Dicer. Additionally, a deficiency in Dicer and Rb combined with p53 inactivation resulted in synthetic lethality in retinal progenitors (Nittner *et al.*, 2012). Therefore, although p53 inactivation may provide protection from the deadly effects of *Dicer* deletion in some cellular contexts when specific genetic alterations are present (Figure 22), Dicer loss is lethal for B-cells and B-cell lymphomas regardless of p53 status.

Our results did show a deficiency in p53 was able to rescue several aspects of Myc-induced B-cell lymphoma development in the *Dicer*$^{fl/fl}$ background. Firstly, the early precursor B-cell lymphomas previously observed in ~40% of CD19-*cre*$^{+}$*/Dicer*$^{fl/fl}$/Eμ-*myc* mice did not occur in the p53-deficient mice; instead, only typical pre-B/B-cell lymphomas developed. Secondly, CD19 cell surface expression, which was significantly reduced or absent in 65% of the lymphomas in CD19-*cre*$^{+}$*/Dicer*$^{fl/fl}$/Eμ-*myc* mice, was fully restored in lymphomas from *p53*$^{+/-}$/CD19-*cre*$^{+}$*/Dicer*$^{fl/fl}$/Eμ-*myc* mice. Unexpectedly, a *p53* deficiency also allowed T-cell lymphomas to emerge, albeit at a low frequency. The explanations for changes in B-cell lymphoma phenotype and the rare development of T-cell lymphomas are currently unclear, but likely involve protection from apoptosis of a lymphoid progenitor, allowing differentiation to continue along B- and T-cell lineages. In addition, although CD19 surface expression was restored in the pre-B/B-cell lymphomas that emerged, 57% of the lymphomas lacked or had reduced Cre protein expression. This was unexpected, as all lymphomas expressed CD19 and Cre is driven from the *CD19* promoter. Although Cre expression was downregulated in half of the lymphomas, the frequency of its expression (43%) was significantly higher than that of 12% in the CD19-*cre*$^{+}$*/Dicer*$^{fl/fl}$/Eμ-*myc* lymphomas (Arrate *et al.*, 2010), indicating the *p53* deficiency

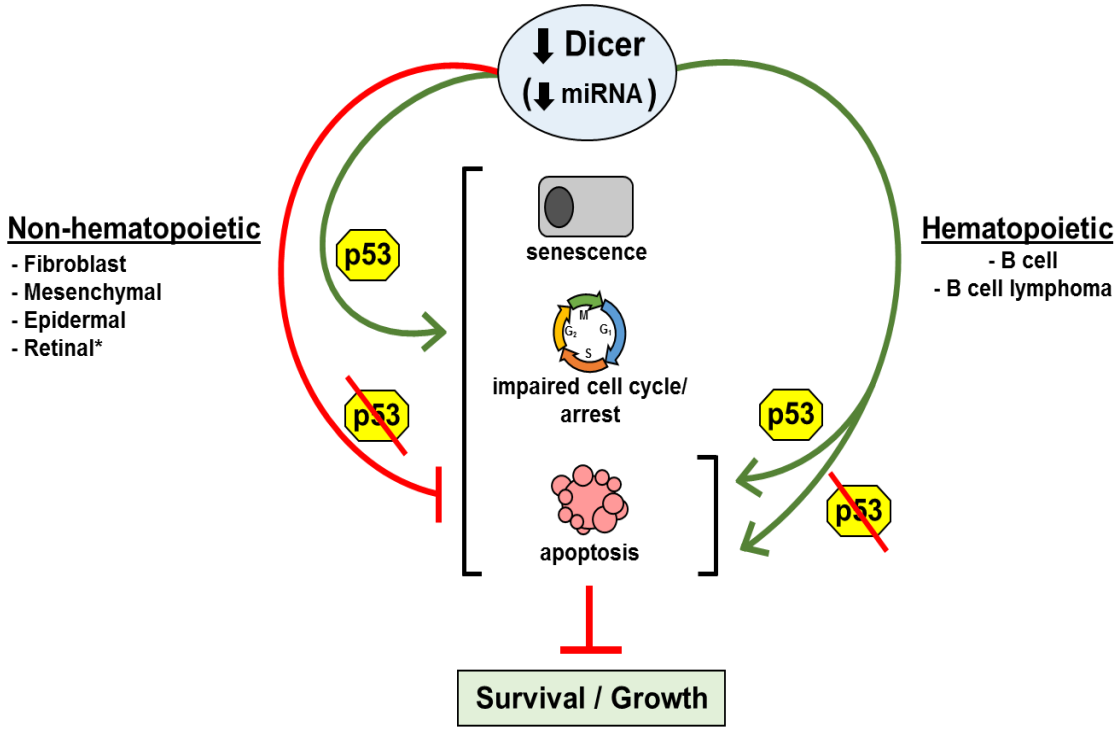

Figure 22. p53 inactivation protects some cells from the negative consequences of *Dicer* deletion. The p53 tumor suppressor responds to impaired miRNA processing by inducing senescence, cell cycle arrest, or apoptosis. However, non-hematopoietic cells that harbor inactivated p53 can survive and grow (possibly more slowly) in the absence of Dicer. However, hematopoietic cells are extremely sensitive to Dicer loss and rapidly undergo apoptosis, regardless of p53 status. *If the Rb pathway is co-inactivated with p53, there is synthetic lethality.

partially rescued Cre expression. However, although Cre protein expression occurred more frequently in lymphomas in *p53*$^{+/-}$/CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* transgenic mice, the number of lymphomas that underwent Cre-mediated deletion of at least one *Dicer* allele was not statistically different than the number that deleted one *Dicer* allele in CD19-*cre*$^{+}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice (Arrate *et al.*, 2010). These results indicate that while more lymphomas expressed Cre, the lymphomas still prevented it from deleting both *Dicer* alleles. Our data show a *p53* deficiency still resulted in a delay in lymphoma development and did not allow biallelic *Dicer* deletion, but it did restore the lymphoma phenotype and CD19 surface expression and partially restored Cre expression in the B-cell lymphomas.

Protection from apoptosis is a critical step in B-cell development and lymphomagenesis (Eischen *et al.*, 2001b; Eischen *et al.*, 1999; Eischen *et al.*, 2001c; Hardy and Hayakawa, 2001). Expression of Cre in *Dicer*$^{fl/fl}$ mice results in early B-cell progenitor (Mb1-*Cre*) or mature B-cell (Aicda-*Cre*) apoptosis and a developmental block or a lack of germinal centers, respectively (Koralov *et al.*, 2008; Xu *et al.*, 2012). Suppressing apoptosis by overexpressing the anti-apoptotic Bcl-2 protein and/or deleting the pro-apoptotic gene *Bim* or by expressing an immunoglobulin transgene, which provides survival signals, partially rescued B-cells from apoptosis in these systems. Since neither study confirmed biallelic deletion of *Dicer* had indeed occurred in the surviving B-cells, and since our data show B-cells do not survive *Dicer* deletion, it is likely the B-cells that survived in their studies only deleted one allele of *Dicer*. Moreover, the reduction in apoptosis that allowed more B-cells to survive and differentiate likely reflects effects on the B-cell compartment rather than on the survival of *Dicer*-deleted B-cells. In addition, it is unlikely that Bcl-2 overexpression alone would protect an untransformed B-cell from

apoptosis induced by *Dicer* deletion, as the B-cell lymphomas we evaluated overexpressed Bcl-2 (unpublished observations) and rapidly died when *Dicer* was deleted. However, these results could also indicate transformed B-cells rely on Dicer more than untransformed B-cells. Certainly, further studies are needed to determine the conditions, if any, under which B-cells at any maturation stage would survive complete *Dicer* ablation.

Dicer is reported to function as a haploinsufficient tumor suppressor and promote tumorigenesis in mouse models of soft-tissue sarcoma, lung adenocarcinoma, and retinoblastoma (Kumar *et al.*, 2009; Lambertz *et al.*, 2010; Ravi *et al.*, 2012). However, there is a conflicting report on muscle cells (Mito *et al.*, 2013). In contrast, Dicer hypomorphic mice, expressing 20% of normal Dicer levels, did not have an increased cancer incidence (Morita *et al.*, 2009). Moreover, we determined the rate of Myc-induced B-cell lymphomagenesis was similar in mice that had one or two alleles of *Dicer* (Arrate *et al.*, 2010), regardless of p53 status, indicating Dicer was not a haploinsufficient tumor suppressor in B-cells. Furthermore, evaluation of Dicer protein and function in $p53^{+/-}$/CD19-cre^{+}/$Dicer^{fl/fl}$/Eμ-*myc* lymphomas with one or two *Dicer* alleles revealed analogous levels of protein and mature miRNA. Therefore, loss of one allele of *Dicer* did not change the levels of Dicer protein or function in the B-cell lymphomas. Although our results reveal Dicer inhibition as a potential therapeutic opportunity for treatment of B-cell lymphomas, which are sensitive to Dicer loss, due to its haploinsufficient tumor suppressor functions in other cell types, this may not be possible. Therefore, it will be important in future studies to determine the cell types where Dicer functions as a haploinsufficient tumor suppressor, and whether transient inactivation of Dicer could be therapeutic for lymphoma treatment without being tumor-inducing.

CHAPTER III

MYC-REGULATED MIRNA-MEDIATED APOPTOTIC MECHANISM UNDERLIES THERAPEUTIC EFFECTS OF HDAC INHIBITION

This chapter is from:

Adams CM, Hiebert SW, and Eischen CM. "Myc-regulated miRNA-mediated apoptotic mechanism underlies therapeutic effects of HDAC inhibition."
In revision, 2015

Introduction

Aberrant expression or function of histone deacetylases (HDACs) have been implicated in hematopoietic malignancies (West and Johnstone, 2014). 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 (West and Johnstone, 2014); 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 (Conti *et al.*, 2010; Stengel and Hiebert, 2014).

Myc, an oncogenic transcription factor, is dysregulated in most hematopoietic malignancies (Dang, 2012). However, untransformed cells undergo apoptosis to counter hyper-proliferative signals from Myc dysregulation. Specifically, Myc overexpression activates the p53 tumor suppressor pathway, eliciting apoptosis (Eischen *et al.*, 1999; Zindy

et al., 1998). Myc-induced apoptosis also occurs independent of p53 through down-regulation of anti-apoptotic Bcl-2 and Bcl-x_L proteins, by an indirect and unclear mechanism (Eischen *et al.*, 2001a; Eischen *et al.*, 2001c; Patel and McMahon, 2007). Both pathways become inactivated during tumorigenesis (Dang *et al.*, 2005).

Myc transcriptionally activates or represses numerous genes, regulating many cellular processes (Eilers and Eisenman, 2008). Myc represses protein-coding genes by recruiting HDACs and by binding and inhibiting the transcriptional activator Miz-1 (Eilers and Eisenman, 2008; Kurland and Tansey, 2008). Myc also regulates the expression of non-coding RNA, including microRNA (miRNA) that bind mRNA, typically inhibiting translation (Bui and Mendell, 2010). In malignant cells, Myc represses many miRNA while specifically up-regulating others (Bui and Mendell, 2010; Chang *et al.*, 2008). 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, and infection

Daudi, Ramos, Raji, Su-DHL-6, Kasumi, K562, Jurkat, Loucy, Hut-78, H929, and NIH3T3 cells were cultured as described by the American Type Culture Collection. MyLa (Sigma) and OCI-Ly-19 and OCI-Ly-3 cells were cultured in RPMI-1640 containing 10% human or fetal bovine serum, respectively. P493-6 cells from Dr. Dirk Eick (Helmholtz Zentrum Muenchen) were cultured as described (Pajic *et al.*, 2000). Tetracycline (0.1μg/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 2012 and were cultured for less than six months. Primary murine pre-B cultures were generated as previously described (Eischen *et al.*, 1999). Briefly, bone marrow was harvested from 4-6 week-old wild-type mice. After hypotonic lysis of red blood cells, bone marrow cells were cultured RMPI supplemented with 10 ng/mL IL-7, 20% FBS, 55 μM β-mercaptoethanol, 2 mM glutamine, and penicillin/ streptomycin. Primary pre-B cells were infected with the bicistronic retrovirus MSCV-MycER-IRES-GFP in the presence of 8 μg/mL polybrene. GFP-positive pre-B cells were isolated by fluorescence-activated cell sorting (FACS). Eμ-*myc* lymphoma cells were previously isolated and maintained as published (Eischen *et al.*, 1999). Wild-type or *p53*$^{-/-}$ murine embryonic fibroblasts (MEFs) were cultured as described (Zindy *et al.*, 1998). Fibroblasts were transfected using Lipofectamine2000 (Invitrogen). Retroviral infections were performed as previously reported (Zindy *et al.*, 1998).

Vectors

The MSCV-MycER-IRES-GFP bicistronic retrovirus was previously described (Zindy *et al.*, 1998). Site-directed mutagenesis was used to introduce the V394D point mutation into the MSCV-MycER-IRES-GFP bicistronic retroviral vector to generate MycV394D-ER. Dr. Michael Cole (Dartmouth) kindly provided a vector containing a deletion (Δ118-152) in the Myc Box II domain of Myc (MycΔMBII). The region encoding the MycΔMBII deletion was cloned into the multiple cloning site of the MSCV-MycER-IRES-GFP bicistronic retroviral vector to generate MycΔMBII-ER. Retroviral miRNA expression vectors and empty retrovirus control (MSCV-PIG) were obtained from Dr. Josh Mendell (UT-Southwestern) (Chang *et al.*, 2008). Luciferase reporter plasmids were constructed by cloning a 60-mer of the 3'-UTR of *Bcl-2*, *Bcl-x_L*, or *p21* that contained the predicted miR-15 family, let-7 family, or miR-17 family target sequence, respectively, into the multiple cloning site of pMIR-REPORT (Invitrogen). Luciferase reporters containing mutated miRNA binding sites were similarly generated by cloning a 60-mer with a mutated (base substitutions) target sequence to prevent the miR-15 or let-7 families from binding the 3'-UTR of *Bcl-2* or *Bcl-x_L*, respectively, into the multiple cloning site of pMIR-REPORT. All plasmids were sequenced for verification prior to use. For luciferase assays, a reporter plasmid encoding β-galactosidase was used for transfection normalization (Invitrogen).

HDAC inhibition and cell survival assays

In vitro experiments utilized 1μM 4-hydroxytamoxifen (4-OHT) or vehicle (EtOH), and Depsipeptide (250pM-10nM, Celgene); RGFP233, RGFP963, RGFP966 (250nM-10μM, Repligen); Panobinostat (250pM-5nM, Novartis); 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.

Precision nuclear run-on and sequencing (PRO-seq)

Nuclei were isolated and PRO-seq was performed as previously described (Kwak *et al.*, 2013).

Mice and tissue acquisition

For the *in vivo* lymphoma experiments, 10-12 weeks old C57Bl/6 mice were subcutaneously injected (one flank) with $4x10^6$ Eμ-*myc* lymphoma cells as previously described (Adams and Eischen, 2014). 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). 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 and immunoprecipitation

Whole cell protein lysates were prepared and Western blotted as reported (Alt *et al.*, 2005; Zindy *et al.*, 1998). Fibroblasts were lysed 48 hours post-transfection. Myc was immuno-precipitated from lysates prior to Western blotting as previously reported (Alt *et al.*, 2005).

Antibodies

Western blotting antibodies: Bcl-2, Bcl-x_L (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).

ENCODE

GEO accession numbers of ENCODE datasets (Consortium, 2012) evaluated: GSM935410, GSM935643, GSM822290, GSM822286, GSM822291, GSM822298, GSM1088664, GSM1231600, GSM1181982, GSM1430924, GSM1356589, GSM1356597, GSM1386884, GSM1386876, GSM1356606, and GSM1181980.

Primers

qRT-PCR. mRNA and miRNA expression were normalized to *β-actin* and *RNU6b* levels, respectively, and presented as $2^{-\Delta\Delta Ct}$. Primer sequences for mouse and human *Bcl-2*, *Bcl-x_L*, *Mcl-1*, and *β-Actin* mRNA expression were obtained from the Harvard Medical School Primer Bank. Sequences of primers used to detect primary miRNA transcripts of the miR-

15a/16-1 cluster, miR-195/497 cluster, let-7a and miR-31 are listed below. These primers were designed such that ~100 base pairs of flanking sequence extended on either side of the stem-loop. Stem-loop sequences of each miRNA were derived from the Sanger miRBase data repository and mapped to the human and mouse genomes.

primary miRNA	Species	Forward (5' → 3')	Reverse (5' → 3')
miR-15a/16-1	mouse	CAATTATAGTATTTTAACAG	GCACATACCAGTGTTAGATT
miR-15a/16-1	human	CAATTACAGTATTTTAAGAG	GCATATTACATCAATGTTAT
miR-195/497	mouse	TCTCTCAACATGGTGCTGCC	CCTGCTAAACTATTTCCTGA
miR-195/497	human	CCTCTCAGCTTCGTGCTGTC	CCTGCTCAGCCCGTCCCTGG
let-7a	mouse	CTTTTCCCTCATACAGGAAAC	CAAAGTAAGTAAAAACTTGC
let-7a	human	CTTATCACTCACACAGGAAAC	GACAAGAAGCAAAAGGTTTC
miR-31	mouse	GCCCTACATATCATGGATGG	GGAAACAGCGTGTTTTCAAC
miR-31	human	ATCCAAGGAAGGGCGCACAT	GAAATAAGTGCGCTTTCAAT

ChIP. Primer sequences for ChIP of the promoter or upstream regions (negative controls) for the miR-15a/16-1 cluster, miR-195/497 cluster, let-7a, p21, and CAD in human cells have been previously described (Chang *et al.*, 2008; Pal *et al.*, 2003; Wu *et al.*, 2003). Primers used for performing ChIP of murine cells are listed below. Based on sequence conservation of the promoter regions between human and mouse, primers specific for mouse were designed to amplify a region near the transcription start site of the miRNA or for a negative control, approximately 500 base pairs upstream.

Transcription unit	Forward (5' → 3')	Reverse (5' → 3')
miR-15a/16-1 promoter	ACAGCTGTCCCTGCCTCC	GTAGAAGTTTCCGCATGCG
miR-15a/16-1 upstream	GTACGCATTGTTATCAAACGC	GGCAGTGAGACACCTGATCC
miR-195/497 promoter	GGGCTTTAGGCGGGAGTC	CGACTCTTCTCAACCCTTATAGGG
miR-195/497 upstream	TCTGTCTTTCTCCTGCCTCC	AAATTGGCATCGGGACAG
let-7a promoter	CGTCGCCATTTTTCCCTC	CCTCCTGGTCGCCCGCT
let-7a upstream	TCCTAGTGCATGGAAAGTTCC	TTTCAAAGCAATCTTTACGAATTC
p21 promoter	ATCGGTGAAGGAGTGGGTTGG	GACACCCACTGGGCTCAGCGC
CAD promoter	TGCCGGCTGCTTGCGCCGTCG	AGGTTAAGTAGAGTGGGGTCG

Quantitative chromatin immunoprecipitation (qChIP)

qChIP was performed as previously described (Grieb *et al.*, 2014). Primer sequences and antibodies are listed above.

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 (McGirt *et al.*, 2014; Wang *et al.*, 2008). mRNA and miRNA expression were normalized to *β-actin* and *RNU6b* levels, respectively, and presented as $2^{-\Delta\Delta Ct}$. 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 (McGirt *et al.*, 2014).

Statistics

Student's *t*-tests were used to statistically evaluate the data in Figures 23, 26A, 28, 31C, 31D, 33, 34, 35, 36B, 36C, 37A-D, 38D, 38E, 39B, 39C, 40, 43A, 43B, 44, 46D, 49B, 50C, 51A, and 51B. Wilcoxon rank-sum tests determined statistical significance for Figures 32, 38B, 39A, and 41C.

Results

HDAC inhibition decreases Bcl-2 and Bcl-x_L 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; (Adams *et al.*, 1985) and human Burkitt's lymphoma lines were treated with HDAC inhibitors. Depsipeptide (Depsi, class-I HDACi), RGFP966 (HDAC3i; (Wells *et al.*, 2013), RGFP233 (HDAC1/2i), RGFP963 (HDAC1/2/3i), and Panobinostat (pan-HDACi) all decreased cell expansion and number (Figures 23 and 24). 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 (Su-DHL-6, OCI-Ly-19), and multiple myeloma (H929) (Figure 25). Furthermore, HDACi decreased cell viability (Figure 26A) and increased Caspase-3 cleavage (Figure 26B), characteristics of apoptosis.

To identify the molecular determinants of HDACi-mediated apoptosis, we assessed expression of crucial pro-survival proteins. Compared to vehicle, HDACi of murine and human B-cell lymphoma cells decreased anti-apoptotic Bcl-2 and Bcl-x_L protein, but not Mcl-1 (Figure 27A). Moreover, Depsi treatment also decreased Bcl-2 and Bcl-x_L protein in nine other malignant hematopoietic cell lines (Figure 27B). Decreased *Bcl-2* and *Bcl-x_L* mRNA (Figure 28) may explain the reduction in protein. Inhibiting Bcl-2 and/or Bcl-x_L reportedly kills malignant hematopoietic cells, including lines we evaluated (Czabotar *et al.*, 2014). Thus, HDACi decreased Bcl-2 and Bcl-x_L expression, inducing apoptosis.

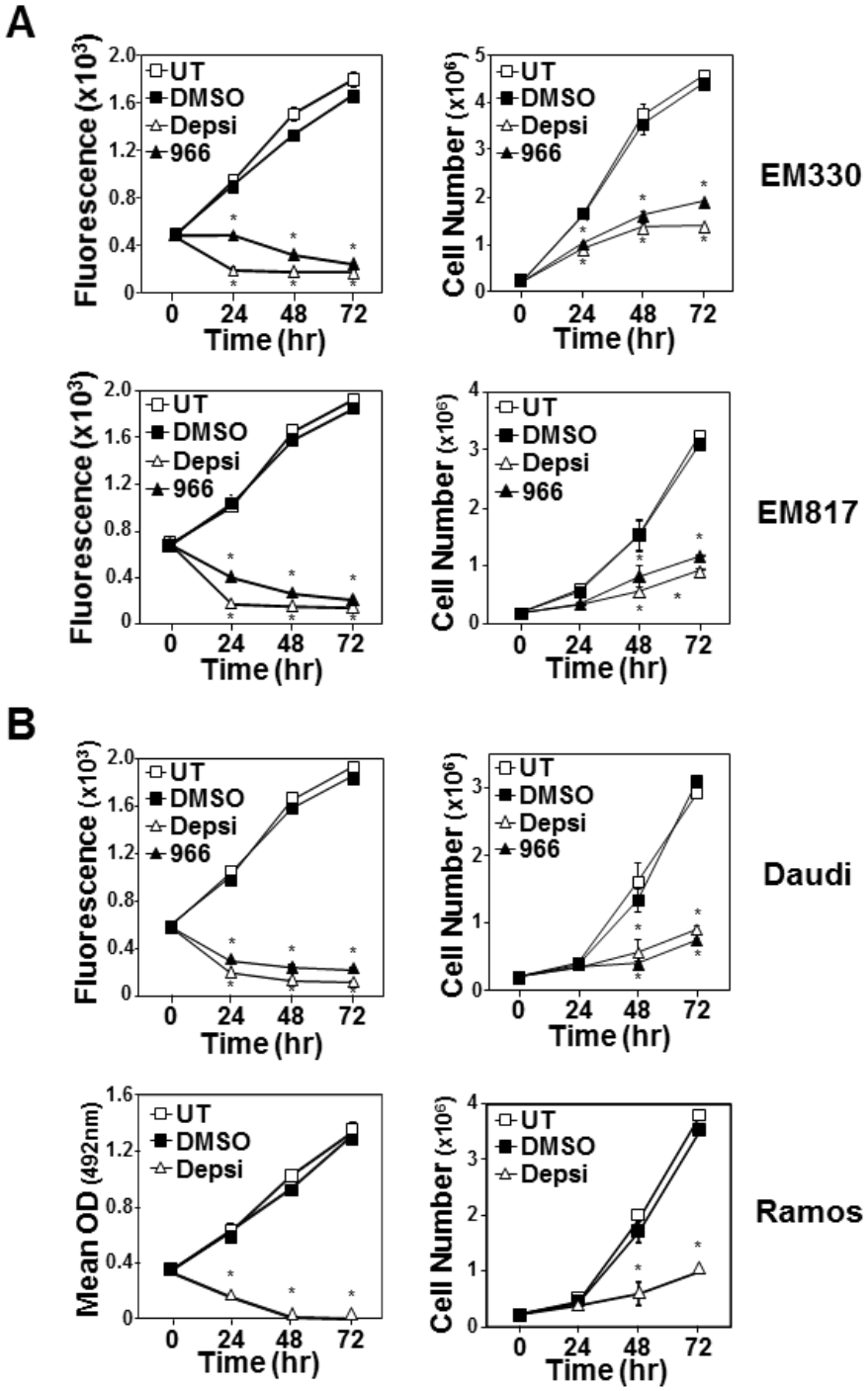

Figure 23. **HDAC inhibition decreases cell expansion and number.** Cells remained untreated (UT) or received Depsipeptide (Depsi), RGFP966 (966), or vehicle (DMSO) control. Following drug administration, proliferation (Alamar Blue or MTS; quadruplicate) and cell number (triplicate) were determined at the indicated intervals in murine (Eμ-*myc*, EM330 and EM817; A) and human (Daudi and Ramos; B) lymphoma cells. Error bars are SD (*p<0.01); p-values determined by comparison to DMSO. Technical assistance for the fluorescence assays was obtained from the Hiebert lab.

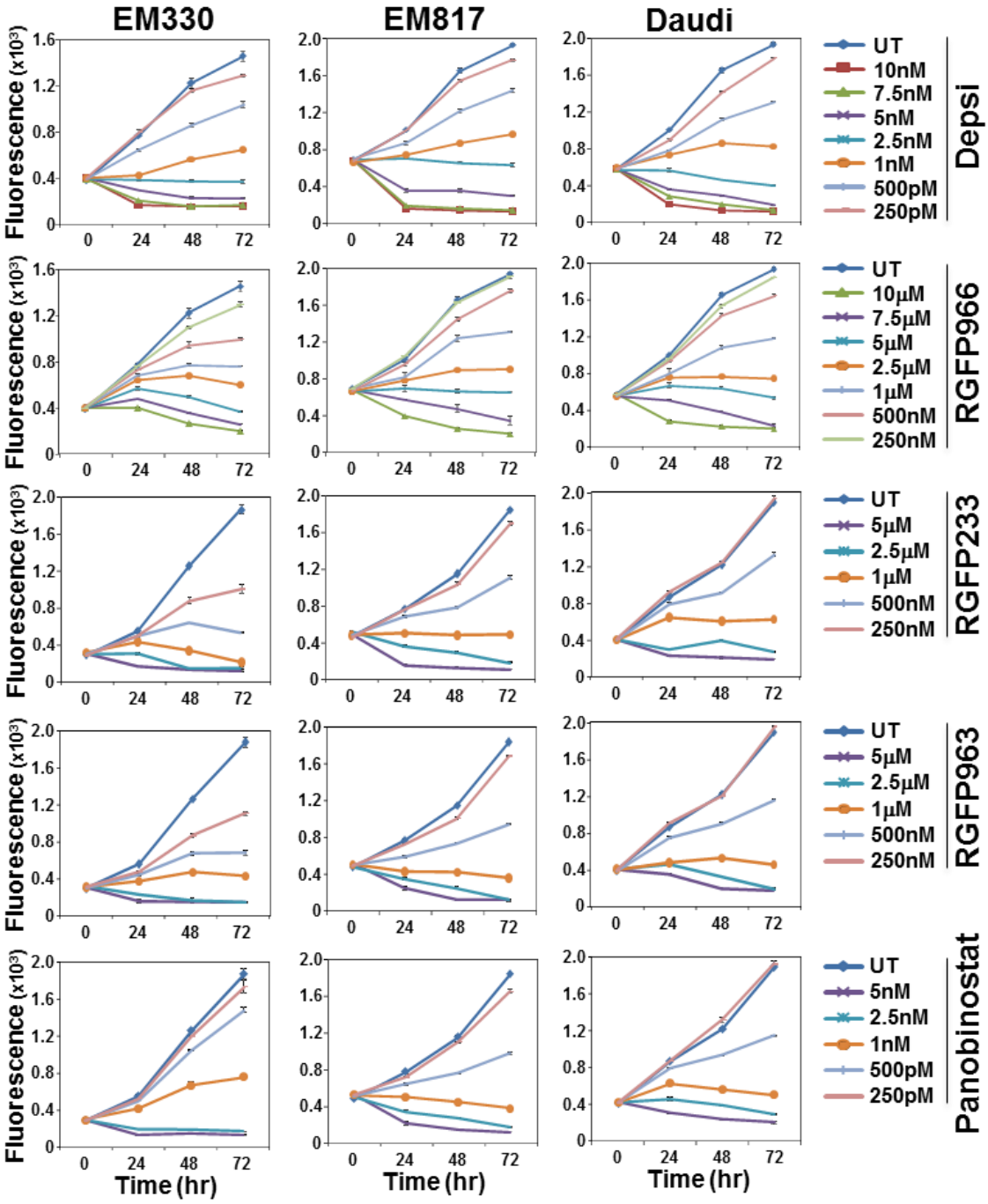

Figure 24. Lymphoma cells are sensitive to multiple HDAC inhibitors. Dose response curves (mean of quadruplicates shown) to Depsipeptide (Depsi), selective HDAC inhibitors (RGFP966, RGFP233, RGFP963), Panobinostat, or vehicle control (DMSO) were obtained by Alamar Blue assays of murine (Eμ-*myc*, EM330 and EM817) and human (Daudi) lymphoma cells. Technical assistance for the fluorescence assays was obtained from the Hiebert lab.

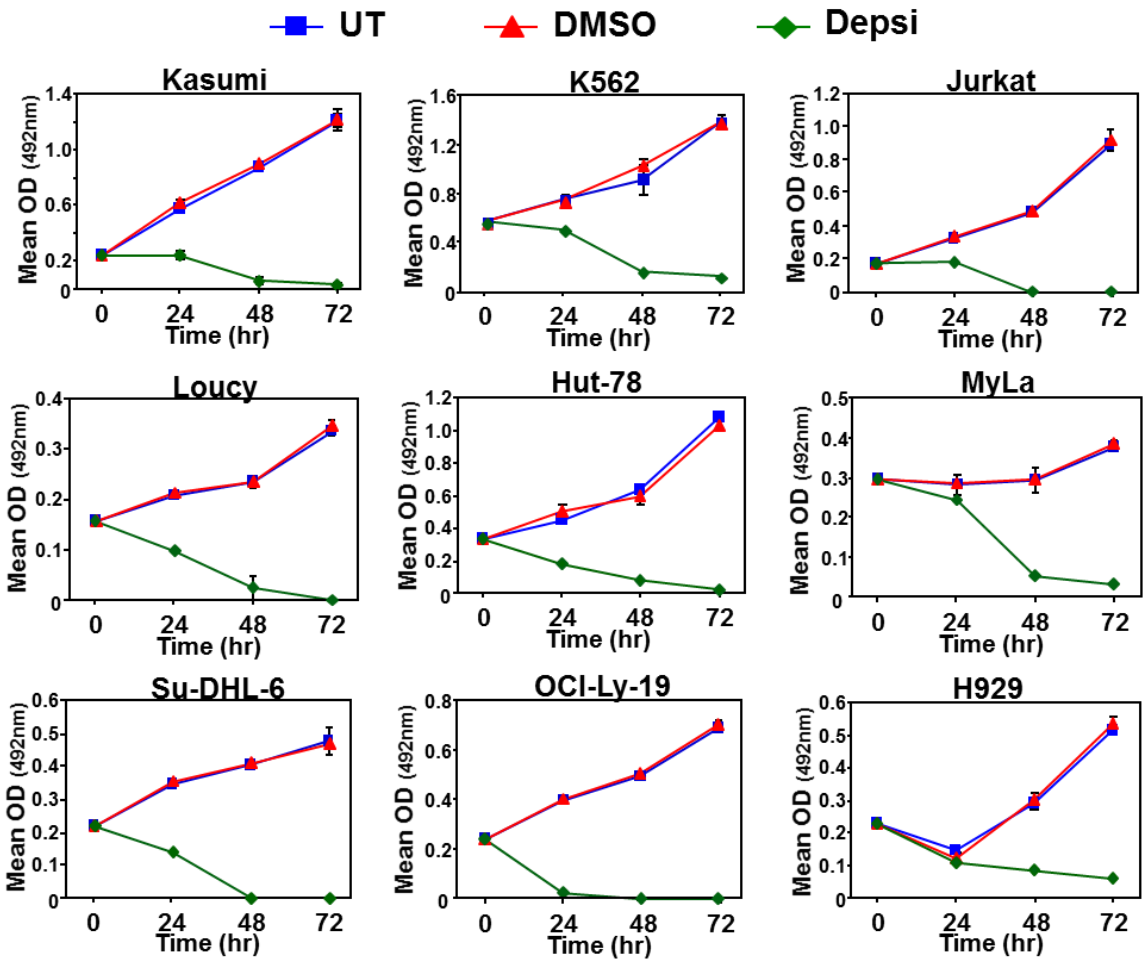

Figure 25. HDAC inhibition causes decreased cell expansion in multiple hematopoietic malignancies. Proliferation of various hematopoietic malignancies was assessed by MTS assay (quadruplicate) upon addition of Depsipeptide (Depsi) or vehicle control (DMSO) or left untreated (UT). Error bars are SD.

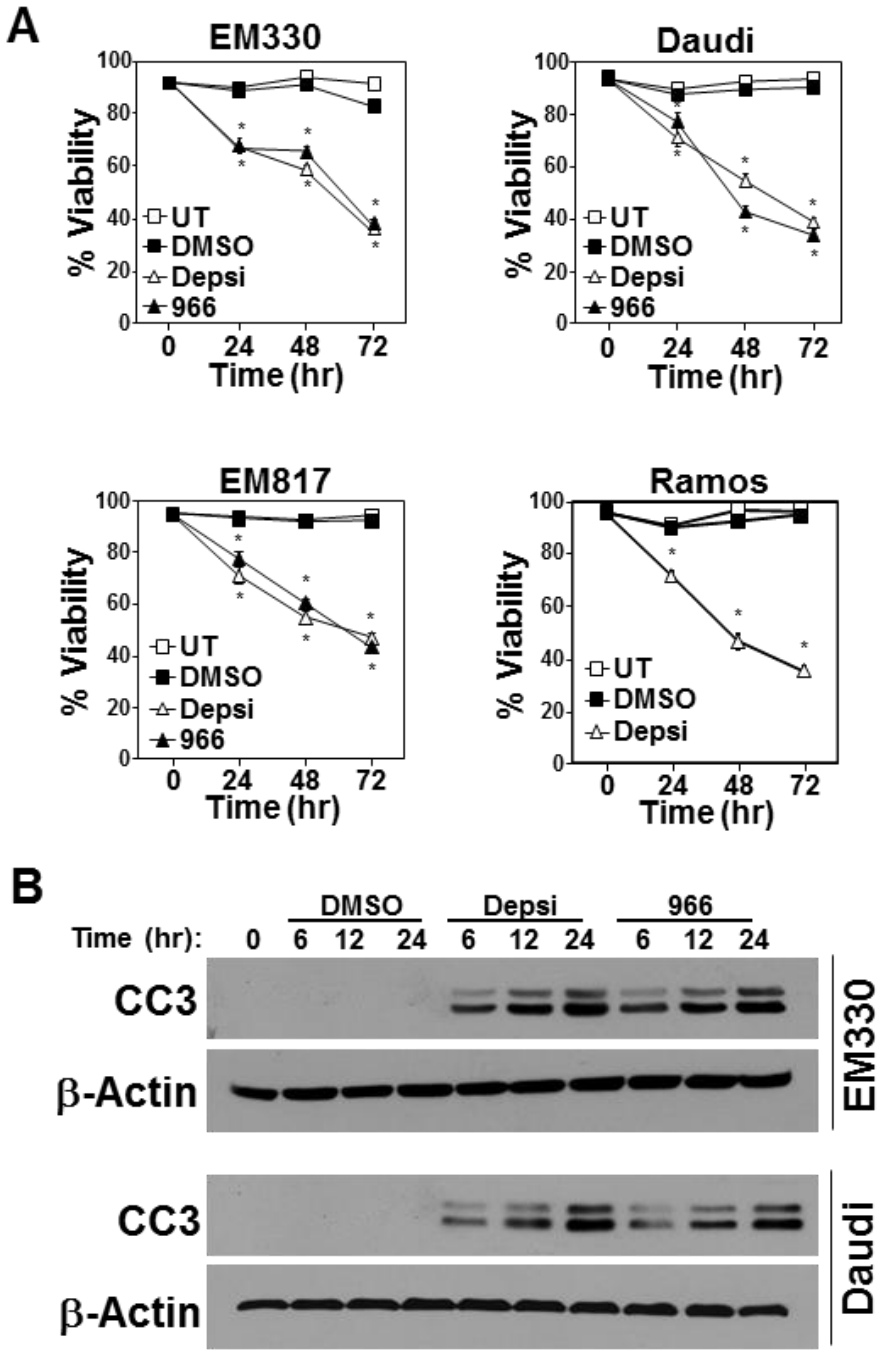

Figure 26. Apoptosis of lymphoma cells is induced by HDAC inhibition. Cells remained untreated (UT) or received Depsipeptide (Depsi), RGFP966 (966), or vehicle (DMSO) control. (A) Following drug administration, viability (triplicate) was determined at the indicated intervals in murine (Eμ-*myc*, EM330 and EM817) and human (Daudi and Ramos) lymphoma cells. (B) Western blotting was performed for the proteins indicated (CC3, Cleaved Caspase-3). Error bars are SD (*p<0.01); p-values determined by comparison to DMSO.

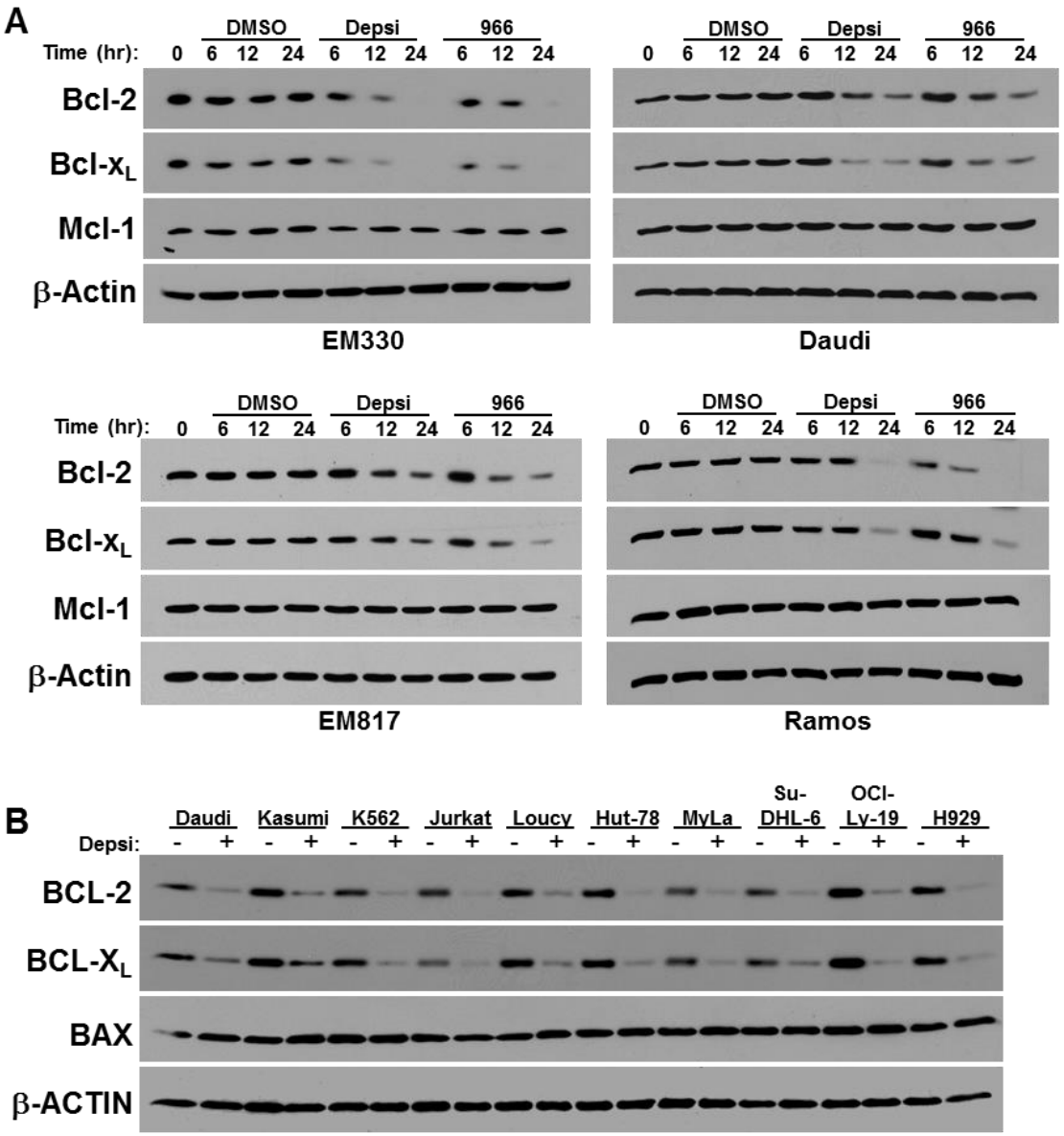

Figure 27. HDAC inhibition reduces Bcl-2 and Bcl-x_L expression in multiple hematopoietic malignancies. Cells remained untreated (UT) or received Depsipeptide (Depsi), RGFP966 (966), or vehicle (DMSO) control for the intervals indicated (A) or 24 hours (B). Levels of the indicated proteins in murine (Eμ-*myc*, EM330 and EM817) and human (Daudi and Ramos) B cell lymphoma (A) and other human hematopoietic malignancies (B) were assessed by Western blot. I received technical assistance in running the Western for B.

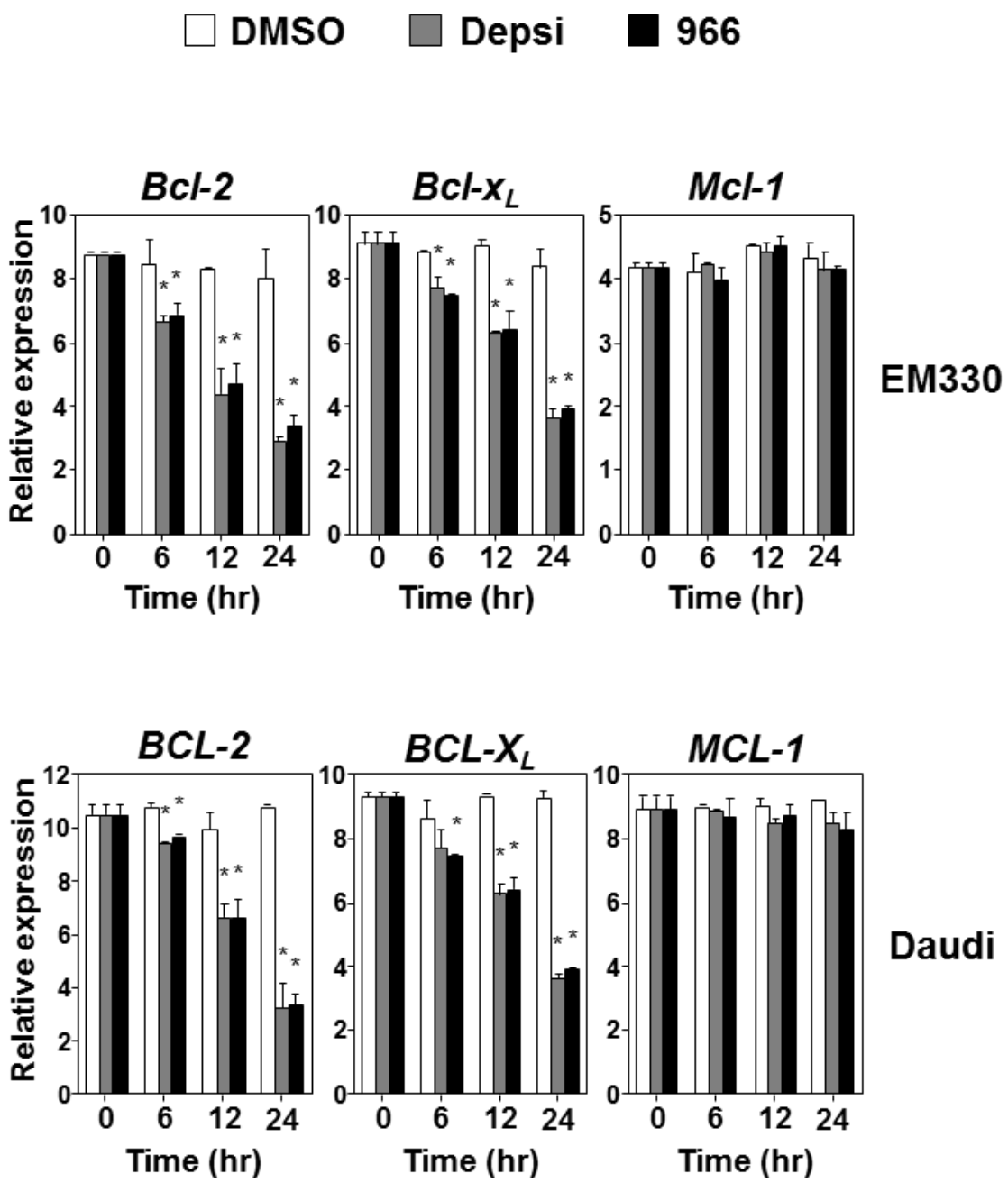

Figure 28. Decreased levels of Bcl-2 and Bcl-x_L protein is likely due to a reduction in mRNA levels. Murine (Eμ-*myc*, EM330) and human (Daudi) lymphoma cells received Depsipeptide (Depsi), RGFP966 (966), or vehicle (DMSO) control. Following drug administration, mRNA levels were evaluated by qRT-PCR (triplicate). mRNA expression was normalized to *β-Actin*. Error bars are SEM (**p*<0.03); *p*-values determined by comparison to DMSO.

HDAC inhibition reveals post-transcriptional regulation of *Bcl-2* and *Bcl-x_L*

HDACi-induced effects on histone acetylation were evaluated to gain insight into the mechanism responsible for decreasing Bcl-2 and Bcl-x_L. Western blotting showed increased global histone acetylation marks associated with active transcription in murine and human lymphoma cells treated with Depsi or 966 (Figure 29A). Analogous results were obtained in the other nine malignant hematopoietic cell lines tested (Figure 29B).

Histone acetylation is typically associated with gene activation, yet *Bcl-2* and *Bcl-x_L* mRNA decreased after HDACi. To investigate this, we examined precision global run-on transcription coupled with massively parallel sequencing (PRO-seq; (Kwak *et al.*, 2013) data for *BCL-2* and *BCL-X_L* loci from Daudi cells treated for 4hrs with Depsipeptide or vehicle control (Acharya *et al.*, manuscript in preparation). This analysis showed no significant expression changes (increased or decreased) at these loci (Figure 30), suggesting a post-transcriptional mechanism is likely responsible for the observed changes in *Bcl-2* and *Bcl-x_L* 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-x_L* expression, respectively, and induce apoptosis in multiple cell types (Figure 31) (Lima *et al.*, 2011). Consistent with previous reports (Chang *et al.*, 2008; Lu *et al.*, 2005), 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 (Figure 32A). 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 (Figure 32B).

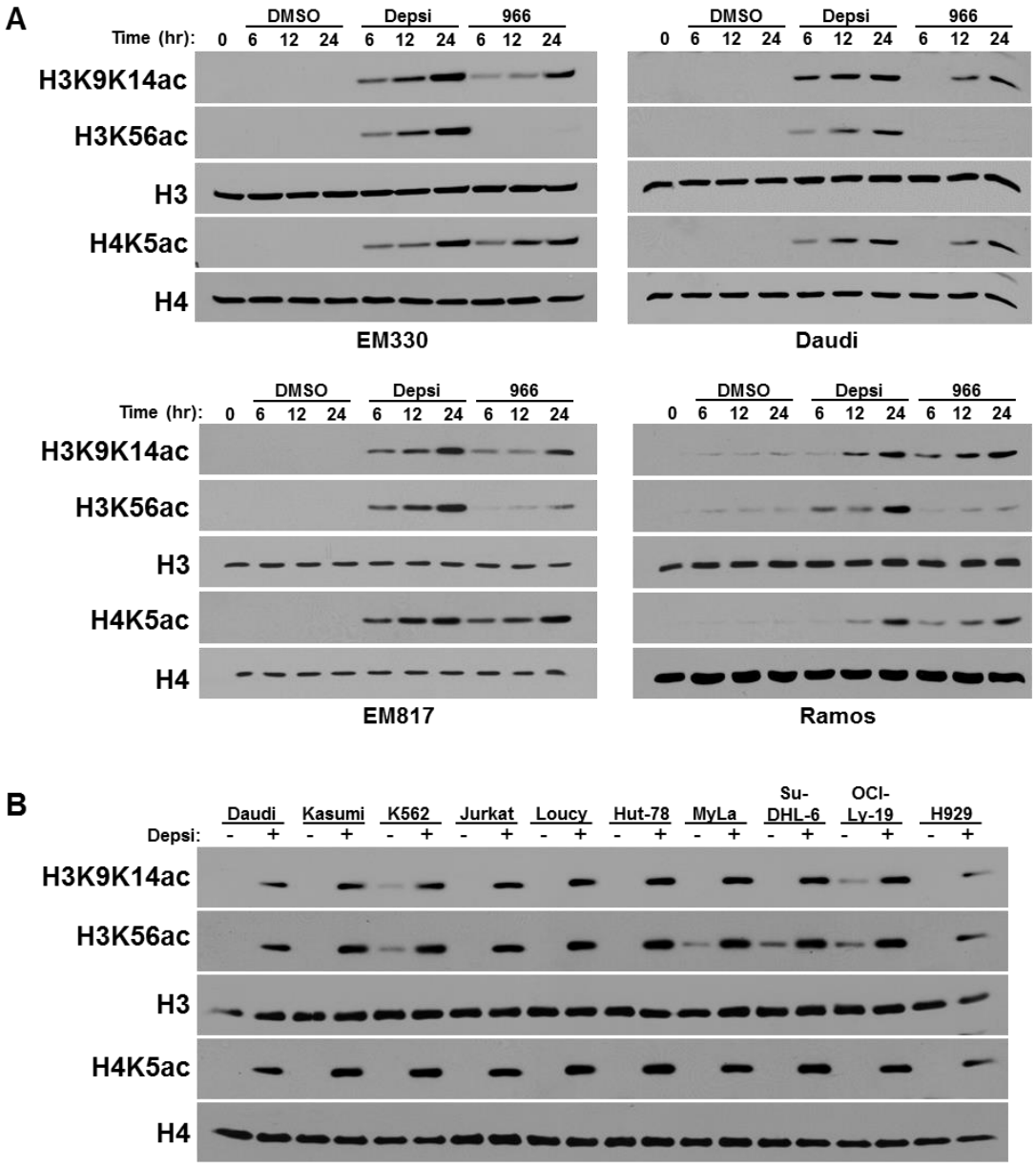

Figure 29. Global histone acetylation is increased following HDAC inhibition. Murine (EM330 and EM817) and human (Daudi and Ramos) B cell lymphomas (A) and the other indicated hematologic malignancies (B) were treated with vehicle (DMSO) control, Depsipeptide (Depsi), and/or RGFP966 (966) for the time indicated (A) or 24 hours (B). Levels of the indicated histone acetylation marks were assessed by Western blot. I received technical assistance in running the Western for B.

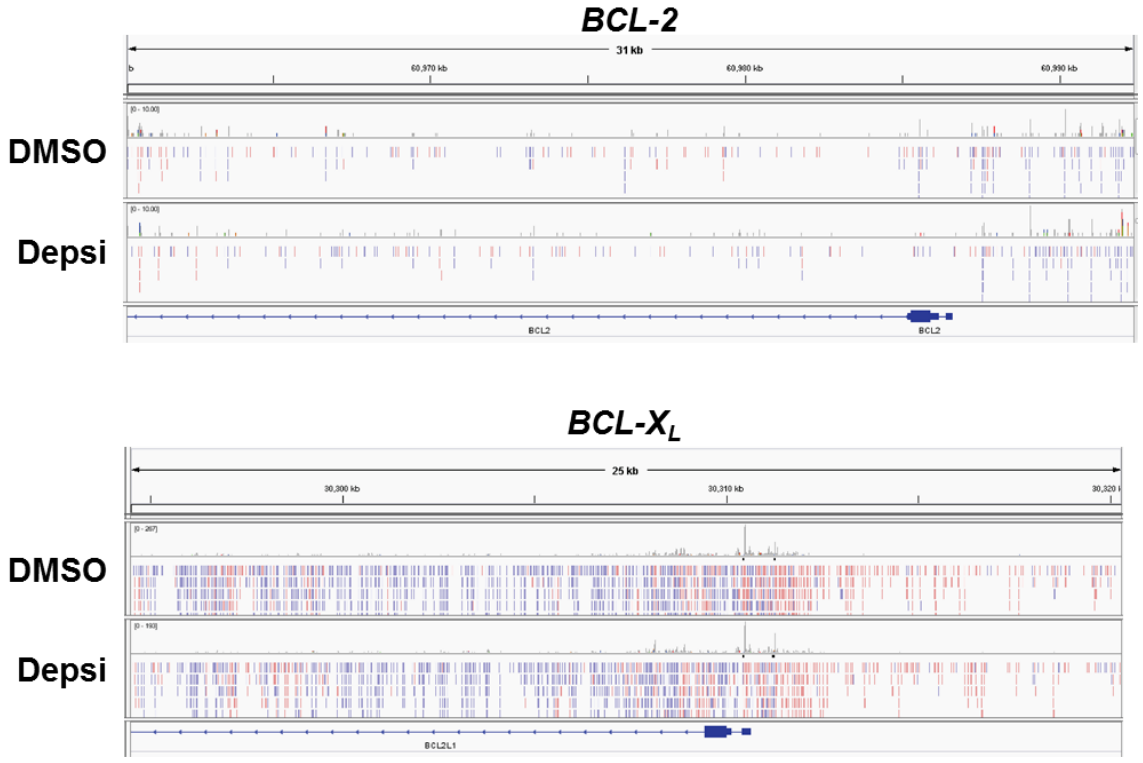

Figure 30. No change in transcription was detected at the *BCL-2* and *BCL-X_L* loci following HDAC inhibition. Precision nuclear Run-On and sequencing (PRO-seq) was used to assess RNA polymerase pausing in human lymphoma cells (Daudi) following 4 hours of treatment with Depsipeptide (Depsi) or vehicle (DMSO) control. A representative result for *BCL-2* and *BCL-X_L* is shown; red denotes reads on the "+" strand and blue are reads on the "-" strand. Technical assistance was obtained from the Hiebert lab to perform PRO-seq on nuclei I prepared.

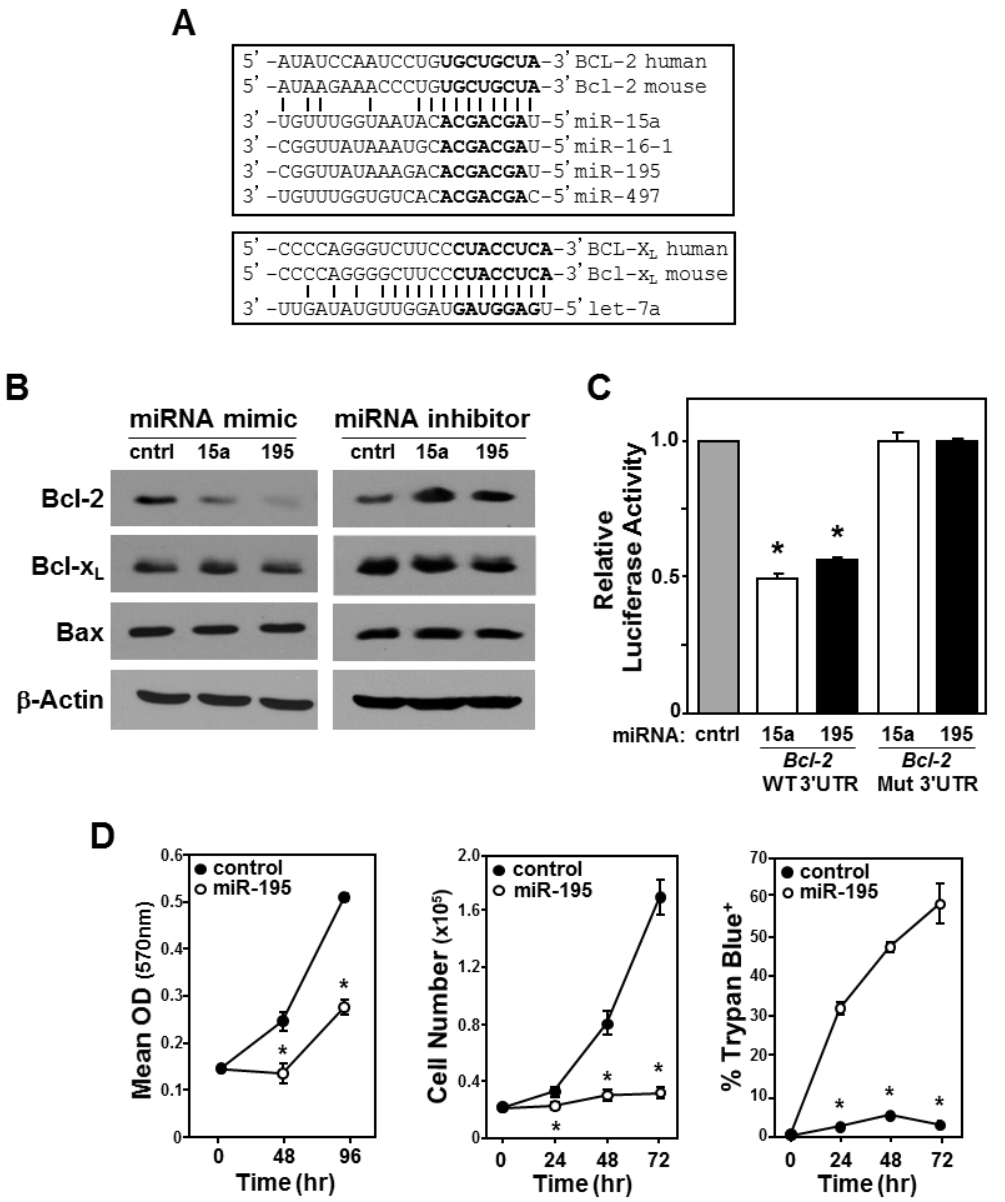

Figure 31. miR-15 family targets *Bcl-2* to induce apoptosis. (A) miRNA-15 family members with the binding site of the 3'-untranslated region (3'-UTR) of *Bcl-2* in human and mouse bolded. Let-7a with the binding site of the 3'-UTR of *Bcl-x_L* in human and mouse bolded. (B) Levels of the indicated proteins in NIH3T3 cells 48 hours after transfection with miR-15a mimic, miR-195 mimic, control RNA (cntrl), miR-15a inhibitor, miR-195 inhibitor, or control inhibitor (cntrl) were determined by Western blot. (C) NIH3T3 cells were transfected with luciferase reporter plasmids containing a portion of the 3'-UTR of *Bcl-2* with the miR-15 family seed sequence (WT) or the 3'-UTR with a mutated miR-15 family seed sequence (Mut). miR-15a or miR-195 mimic or control RNA (cntrl) were also transfected in addition to a β-galactosidase reporter plasmid for transfection normalization. Luciferase activity was measured 24 hours after transfection. (D) miR-195 mimic or control RNA was transfected, in triplicate, into NIH3T3 cells and cell growth, number, and viability were assessed at the indicated intervals. Error bars are SD; **p*<0.002 for C and **p*<0.04 for D; compared to control RNA.

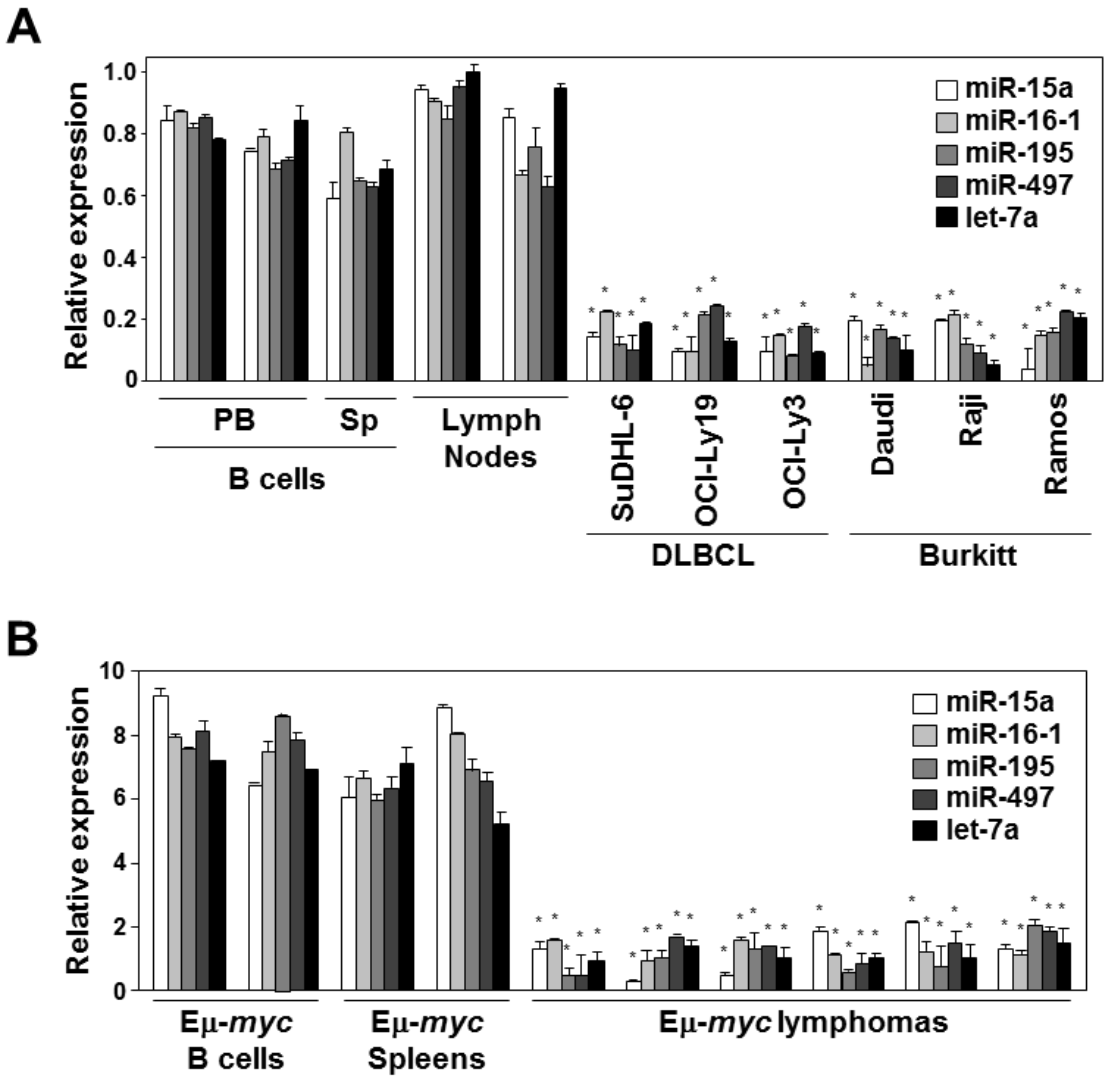

Figure 32. Levels of the miR-15 family and let-7a are decreased in lymphoma. Mature miRNA levels were determined by qRT-PCR (triplicate) in (A) human diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma, two normal lymph nodes, and purified B-cells from peripheral blood (PB) and spleen (Sp), and (B) six murine Eμ-*myc* lymphomas, two pre-cancerous Eμ-*myc* spleens, and two sets of purified splenic B-cells. Small RNA, *RNU6b*, was used for qRT-PCR normalization**.** Error bars are SEM. **p*<0.01, lymphoma versus mean of all normals I received technical assistance in isolating B cells from human peripheral blood (A) and mouse spleens (B).

Notably, HDACi increased miR-15a and miR-195 (representative miR-15 family members) and let-7a in mouse and human lymphoma cells (Figure 33). miR-31 levels were unaffected, demonstrating not all miRNA were up-regulated with HDACi. All other malignant hematopoietic lines evaluated showed similar results (Figure 34A), indicating this HDACi-induced effect is not cell-type specific. Furthermore, with Depsi dose escalation, miRNA levels increased, indicating a dose response to HDACi (Figure 34B).

To evaluate whether the miR-15 family and let-7a were being transcribed upon HDACi, we measured primary transcripts of the miR-15a/16-1 and miR-195/497 clusters and let-7a. Following HDACi, these pri-miRNA transcripts increased (Figure 35A). 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 (Figure 35B). 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 (Figure 35C), 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_L protein, cell expansion, and total cell number (Figure 36A-C). In addition, these lymphoma cells had decreased viability and an increase in sub-G1 DNA content (apoptotic), AnnexinV-positivity and Caspase 3 cleavage (Figure 37A-E). Thus,

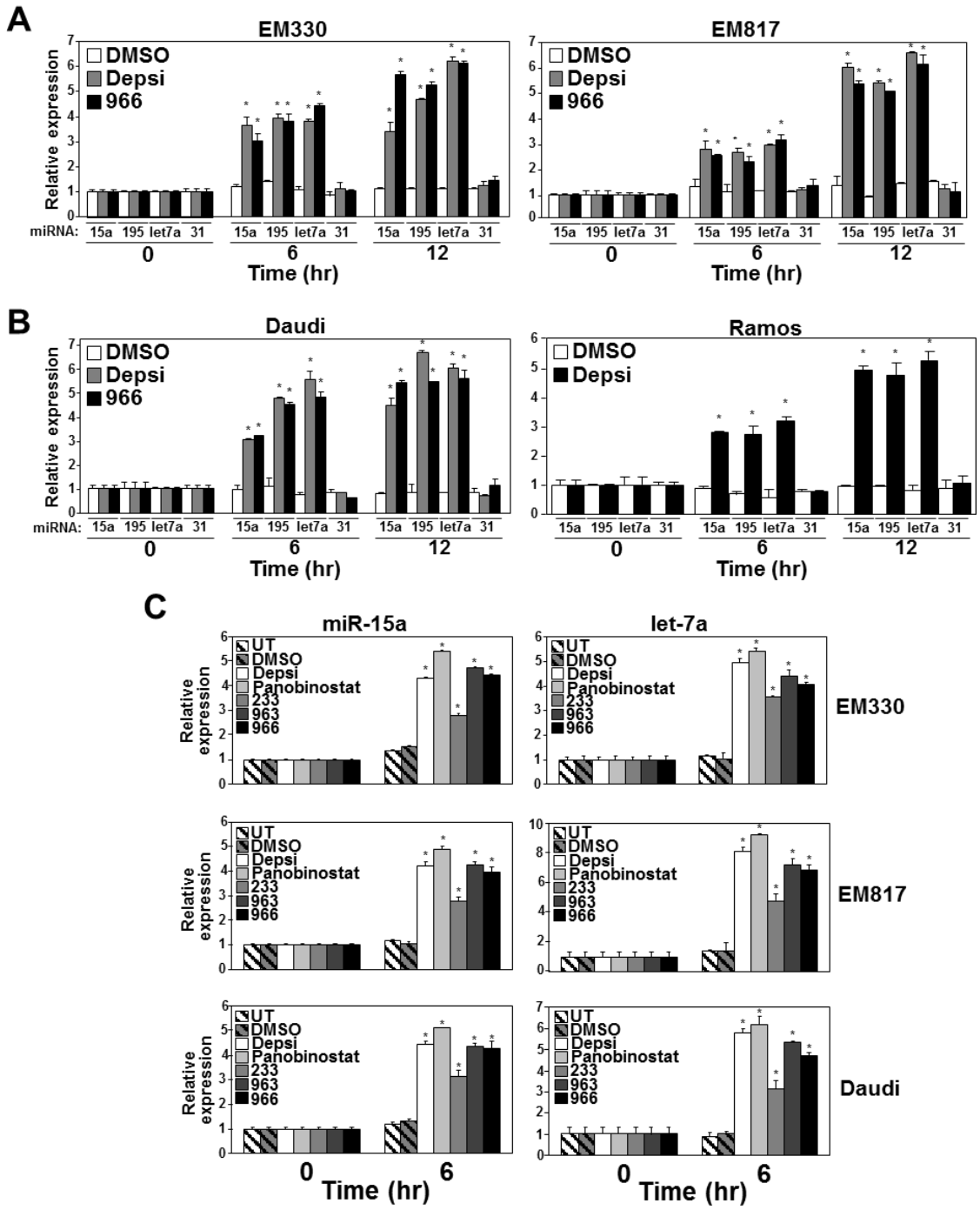

Figure 33. miR-15 family and let-7a increase in lymphomas upon HDAC inhibition. Murine (EM330 and EM817; A) and human (Daudi and Ramos, B) lymphomas were treated with Depsipeptide (Depsi), RGFP966 (966), or vehicle control (DMSO). Murine (EM330 and EM817) and human (Daudi) lymphomas were treated with Depsipeptide (Depsi), selective HDAC inhibitors (RGFP233, RGFP963, RGFP966), or vehicle control (DMSO) for the time indicated (C). Levels of the indicated miRNA were assessed by qRT-PCR (triplicate), and levels of *RNU6b* were used for normalization. Error bars are SEM. *p<0.01, HDACi versus DMSO.

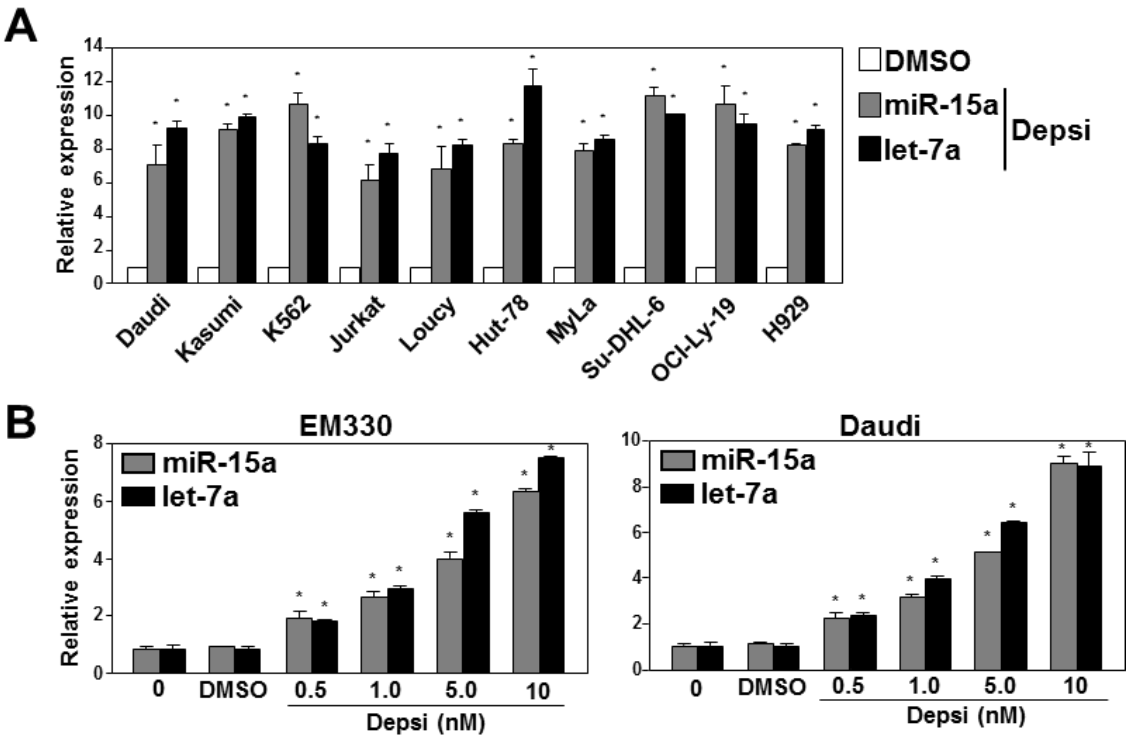

Figure 34. HDAC inhibition increases miR-15 family and let-7a repression in multiple hematopoietic malignancies. The indicated hematologic malignancies (A) or murine (EM330) and human (Daudi) B cell lymphomas (B) were treated with Depsipeptide (Depsi; 5nM for A and the indicated concentrations for B) or vehicle control (DMSO) for 12 hours. For B, the amount of DMSO added was equivalent to that for 10nM Depsi. Levels of the indicated miRNA were assessed by qRT-PCR (triplicate), and levels of *RNU6b* were used for normalization. Error bars are SEM. **p*<0.001 for A and **p*<0.03 for B. Statistical significance was determined by comparing Depsi versus DMSO**.**

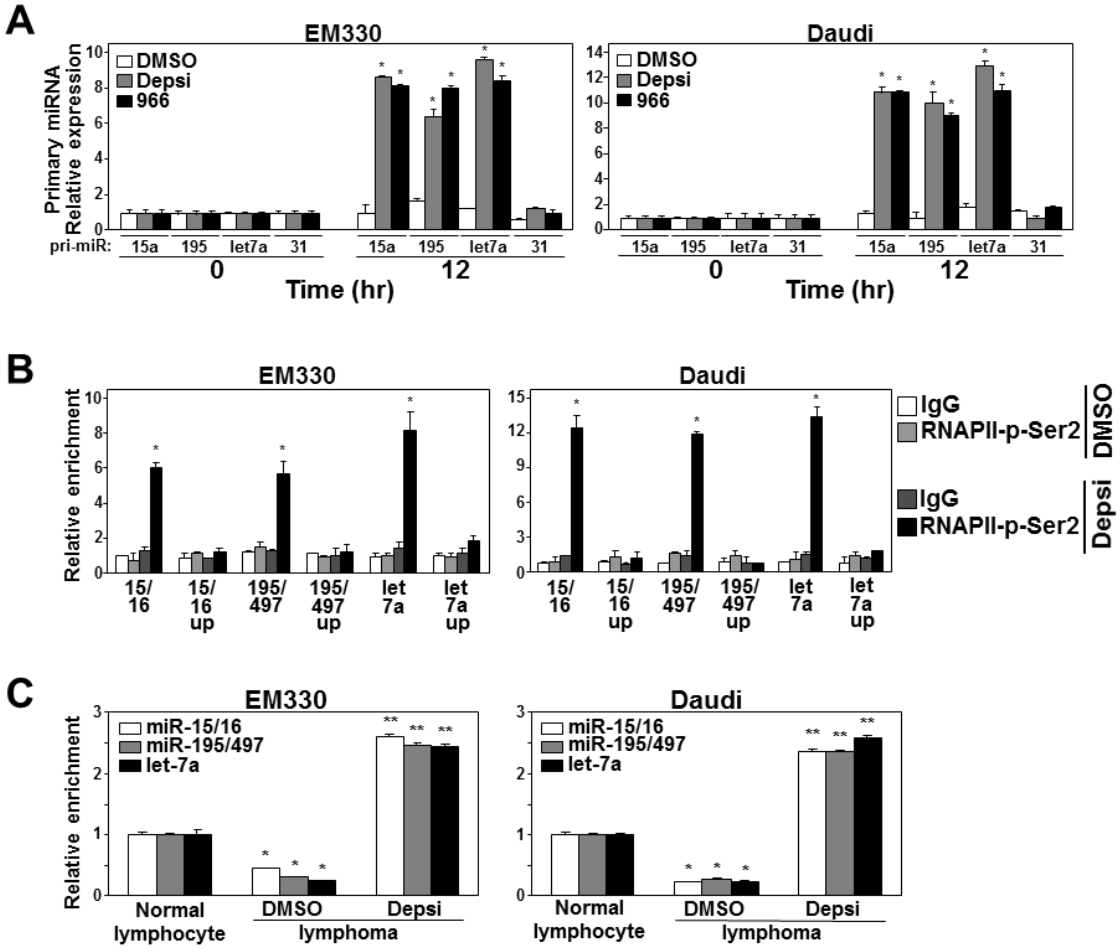

Figure 35. HDAC inhibition reverses miR-15 family and let-7a repression in lymphoma. (A-C) Murine (EM330) and human (Daudi) lymphoma cells were treated with Depsipeptide (Depsi), RGFP966 (966), or vehicle (DMSO) for the time indicated (A) or for 4 hours (B, C). (A) pri-miRNA levels were determined by qRT-PCR (triplicate). (B) 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). Values are relative to their respective IgG control and input DNA. (C) Murine (EM330) and human (Daudi) lymphoma cells were treated with Depsipeptide (Depsi) or vehicle (DMSO) control for 4 hours. ChIP for RNAPII-p-Ser2 was performed with the lymphoma cells and normal lymphocytes isolated from mouse and human spleens. qRT-PCR was then performed for the indicated regions. Values for qChIP are relative to input DNA, IgG control, and normal lymphocyte controls. Error bars are SEM. For A and B, *p<0.001, Depsi or 966 versus DMSO; and for C, *p<0.01 and **p<0.004, Depsi and DMSO versus normal lymphocyte.

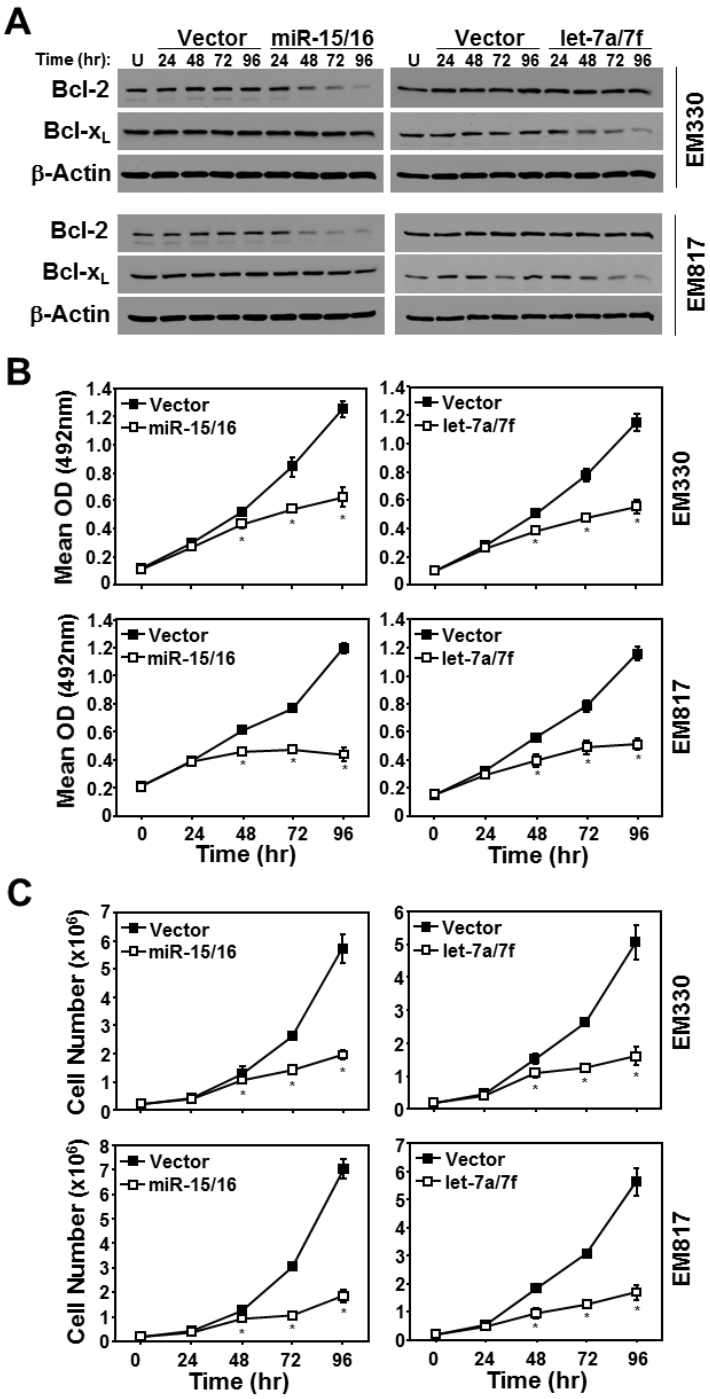

Figure 36. Overexpression of the miR-15 or let-7 families is sufficient to decrease Bcl-2 and Bcl-x_L expression, respectively and reduce cell growth. Eμ-*myc* lymphoma cell lines (EM330 and EM817) were retrovirally infected with either empty MSCV-PIG (vector) or MSCV-PIG encoding the miR-15a/16-1 or let-7a/7f miRNA clusters. (A) Western blotting was performed for the proteins indicated (U, uninfected). (B) Cell growth was measured by MTS assay (492nm; quadruplicate) and cell number was determined by Trypan Blue dye exclusion (triplicate). Error bars are SD for B and C, *p<0.03, compared to empty vector.

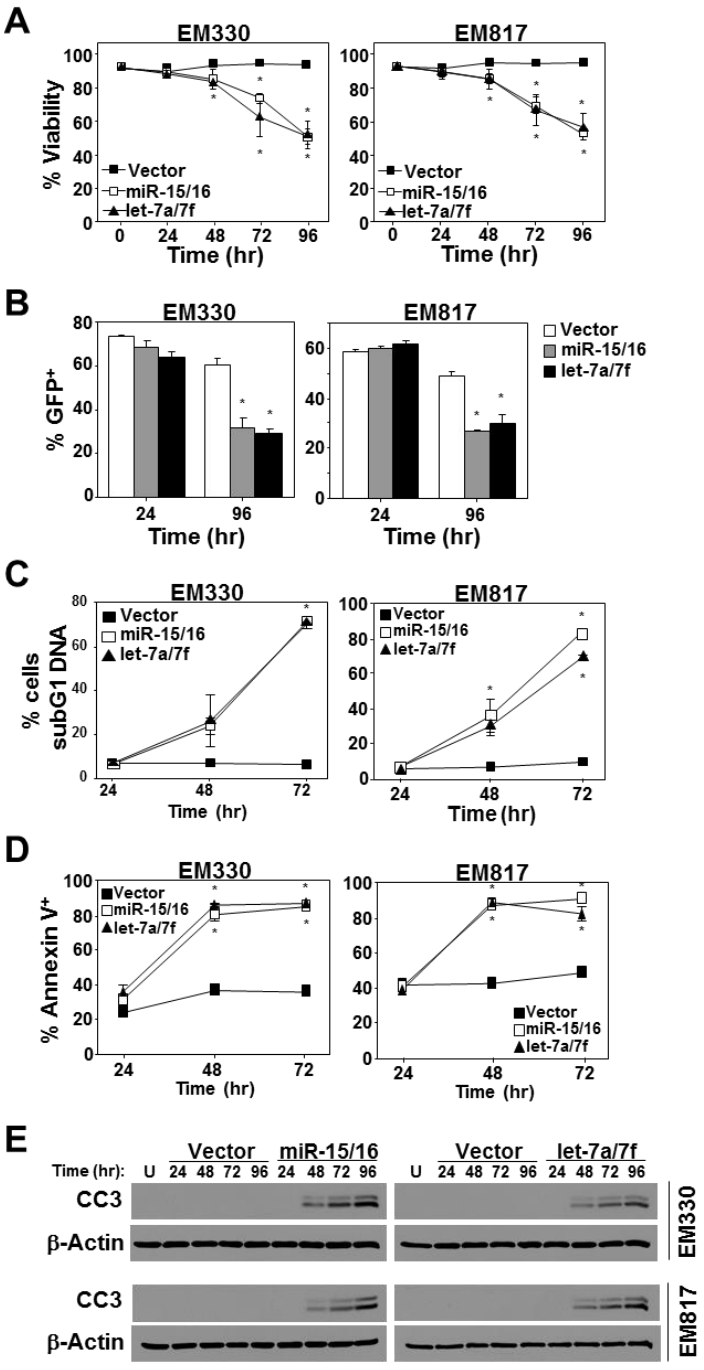

Figure 37. Overexpression of the miR-15 or let-7 families induces apoptosis. Eμ-*myc* lymphoma cell lines (EM330 and EM817) were retrovirally infected with either empty MSCV-PIG (vector) or MSCV-PIG encoding the miR-15a/16-1 or let-7a/7f miRNA clusters. (A) Viability was determined by Trypan Blue dye exclusion (triplicate). Flow cytometry was used to assess GFP-positivity (B), the percentage of cells with subG1 (apoptotic) DNA content following propidium iodide staining (C), and Annexin V-positivity (D) at the indicated intervals (each in triplicate). (E) Western blots were performed for the proteins indicated (U, uninfected; CC3, Cleaved Caspase-3). Error bars are SD. *$p<0.03$ for A and *$p<0.01$ for B-D; compared to empty vector.

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*, Eμ-*myc* lymphoma cells were subcutaneously injected into C57Bl/6 mice. Once tumors reached 200mm^3, mice were administered Depsi or vehicle control, and tumors harvested at intervals. Analogous to our *in vitro* results, HDACi increased active histone acetylation marks (Figure 38A) and levels of the miR-15 family and let-7a (Figure 38B), and decreased Bcl-2 and Bcl-x_L protein (Figure 38C). Apoptosis was evident by Caspase-3 cleavage, AnnexinV-positivity, and sub-G1 DNA (Figure 38C-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 (Bui and Mendell, 2010; Chang *et al.*, 2008). 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 (Figure 39A). miR-31 levels were analogous between genotypes, indicating Myc

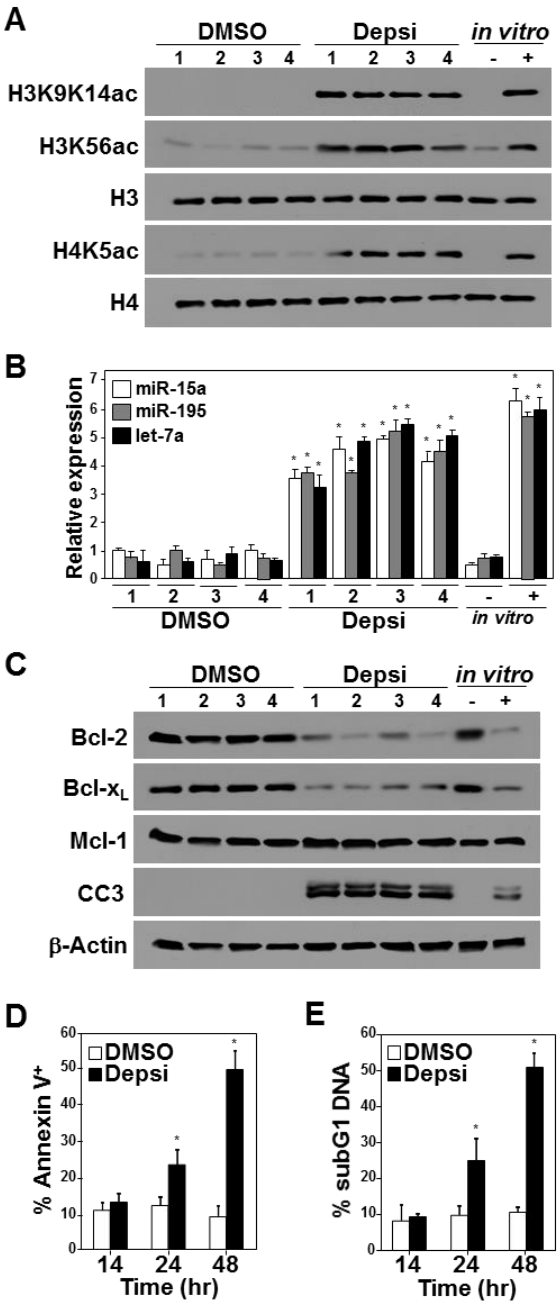

Figure 38. *In vivo*, HDACi increases miR-15 family and let-7a levels, inducing lymphoma cell death. C57Bl/6 mice with subcutaneous Eμ-*myc* lymphoma tumors (EM330) that reached 200mm^3, were treated with Depsipeptide (Depsi) or vehicle (DMSO) control (n=4/group). Tumors were harvested 24hrs later and 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).

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 (Littlewood *et al.*, 1995). Upon MycER activation with 4-OHT, miR-15 family and let-7a levels significantly increased compared to vehicle control-treated pre-B-cells (Figure 39B). This increase was analogous to that of miR-20a, a well-known Myc-induced miRNA (Bui and Mendell, 2010). Levels of miR-31 were unaffected. Similar results were obtained following MycER activation in untransformed fibroblasts (Figure 39C), 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 (Figure 39D). 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 (Eilers and Eisenman, 2008). 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 (Figure 40A). 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 (Figure 40B and 40C, respectively), 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

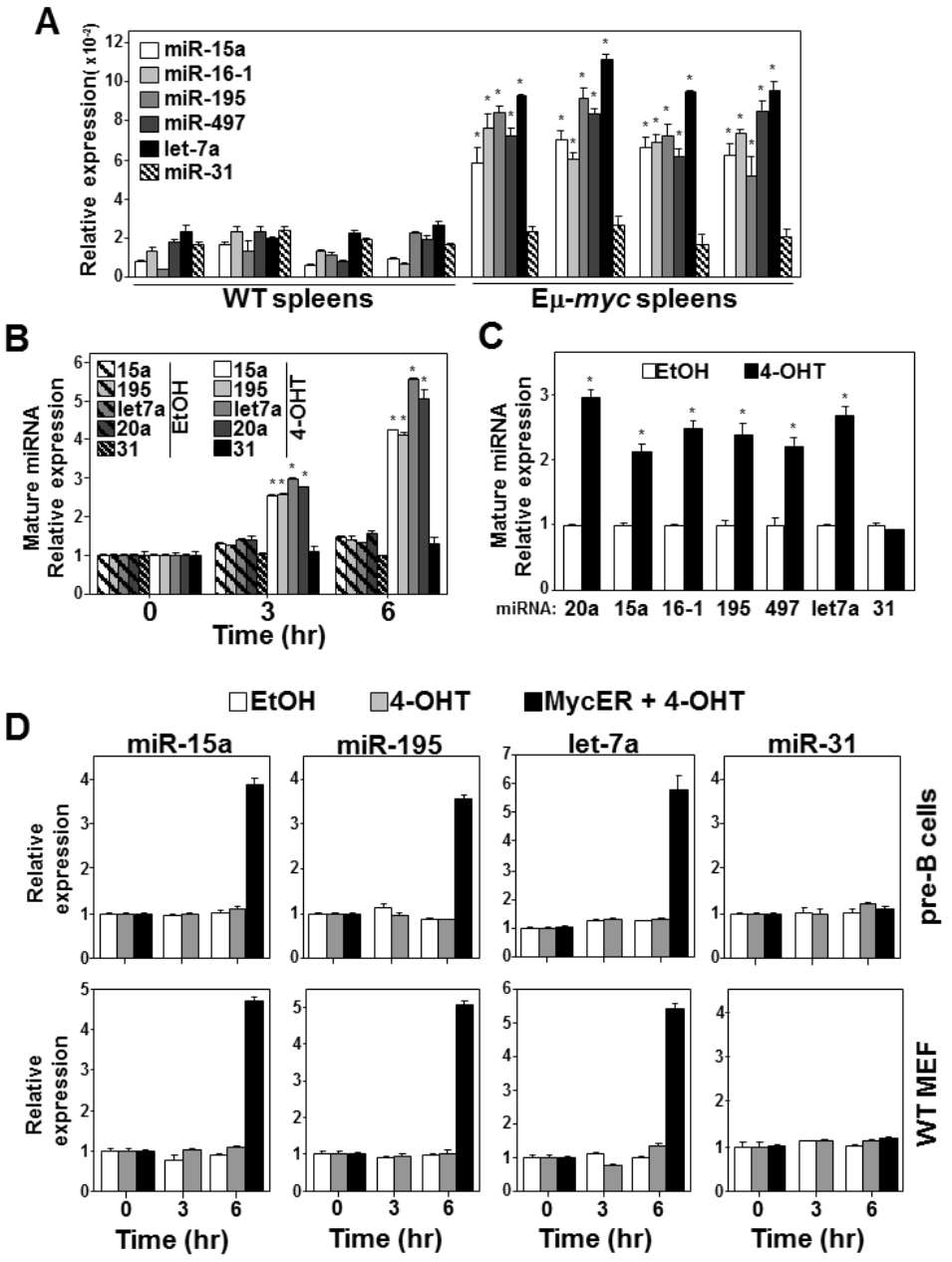

Figure 39. Myc upregulates members of the miR-15 family and let-7a in untransformed cells. Relative expression of the indicated miRNA was determined by qRT-PCR. (A) Pre-cancerous splenocytes from Eμ-*myc* mice and wild-type (WT) non-transgenic littermates were evaluated (n=4/group). (B) miRNA levels were assessed in MycER-expressing primary pre-B cells at the indicated intervals (B) and NIH3T3 fibroblasts following 8 hours (C) of MycER activation with 4-OHT or vehicle control (EtOH). (D) Relative expression of the indicated miRNA was evaluated by qRT-PCR following addition of 4-OHT or vehicle (EtOH) control to cultures of primary pre-B cells or wild-type murine embryonic fibroblasts (WT MEF) at intervals. For comparison, 4-OHT was added for 6 hours to cultures of pre-B cells or wild-type MEFs infected with a retrovirus expressing MycER. miRNA expression was normalized to the expression of small RNA *RNU6b*. Error bars are SEM. *p<0.001 for A-C, Eμ-*myc* vs. mean of all WT spleens (A) and 4-OHT vs. EtOH (B, C).

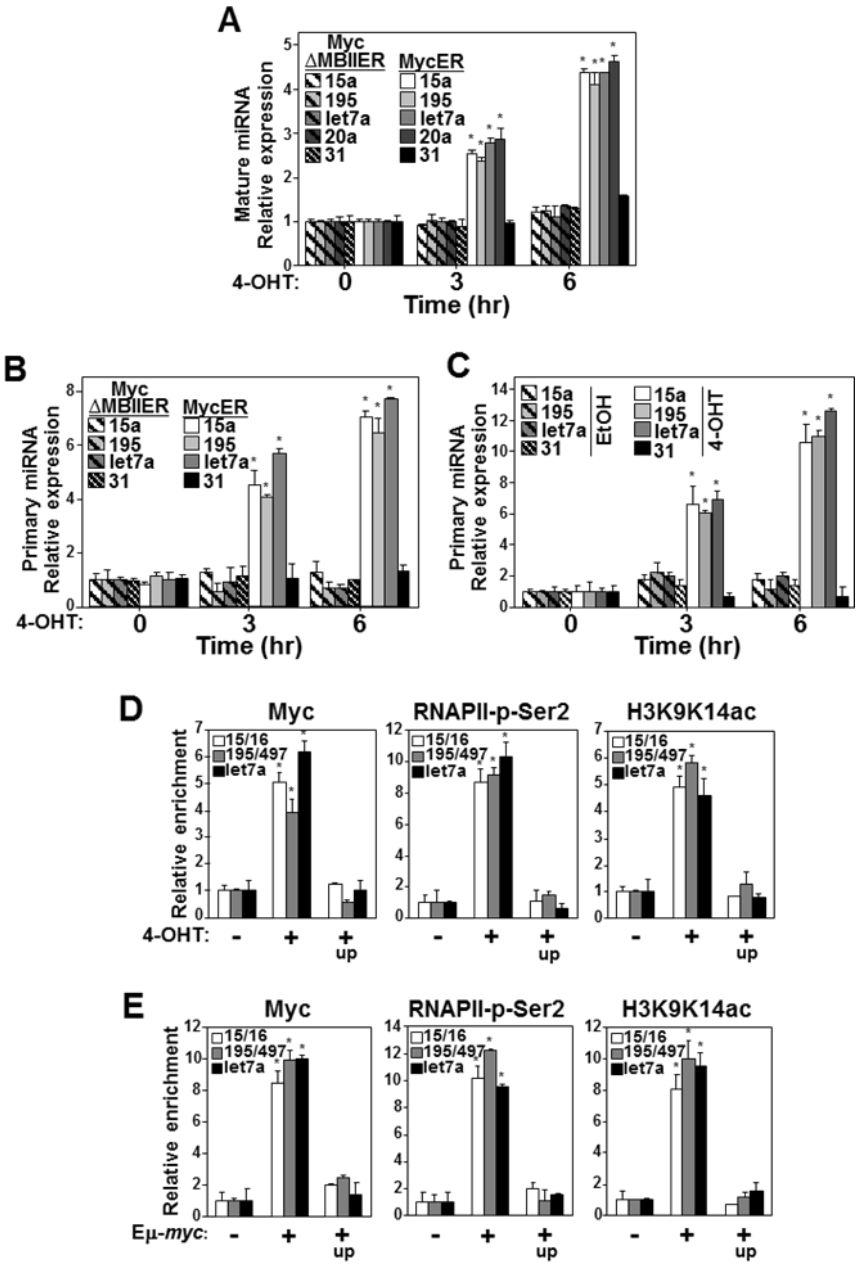

Figure 40. Myc transcriptional activity is necessary to induce the miR-15 family and let-7a in non-transformed cells. Mature miRNA (A) and pri-miRNA (B, C) levels were determined by qRT-PCR (triplicate) and are normalized to *RNU6b* levels. (A-C) MycER or MycΔMBII-ER was activated with 4-OHT or vehicle (EtOH) control at the indicated intervals in MEFs (A, B) and in primary pre-B-cells (C). (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). (D) MycER-expressing *p53*$^{-/-}$ MEFs received vehicle (EtOH; -) or 4-OHT (+) for 4hrs to induce MycER. (E) Splenocytes from wild-type (non-transgenic; Tg-) or pre-cancerous Eμ-*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 for A-E; MycER versus MycΔMBII-ER (A, B), 4-OHT versus EtOH (C, D), and Eμ-*myc* (+) versus wild-type (-) for E.

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 (Figure 40D). Importantly, RNAPII-p-Ser2 and H3K9K14ac, indicators of active transcription, were also enriched (Figure 40D). 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-*myc* transgenic spleens were compared to non-transgenic littermate-matched spleens (Figure 40E), 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 (Figure 41A and 41B). ENCODE MYC ChIP-seq data (Consortium, 2012) also showed MYC at these promoters in hematopoietic and non-hematopoietic malignancies and non-transformed human cells (Figure 42). Importantly, Myc was enriched at these same loci in both the presence and absence of HDACi (Figure 41A and 41B). 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 (Figure 41C).

Myc transcriptionally activates *CAD* and represses *p21* (Eilers and Eisenman, 2008), consistent with the increase in RNAPII-p-Ser2 and H3K9K14ac at *CAD* and the

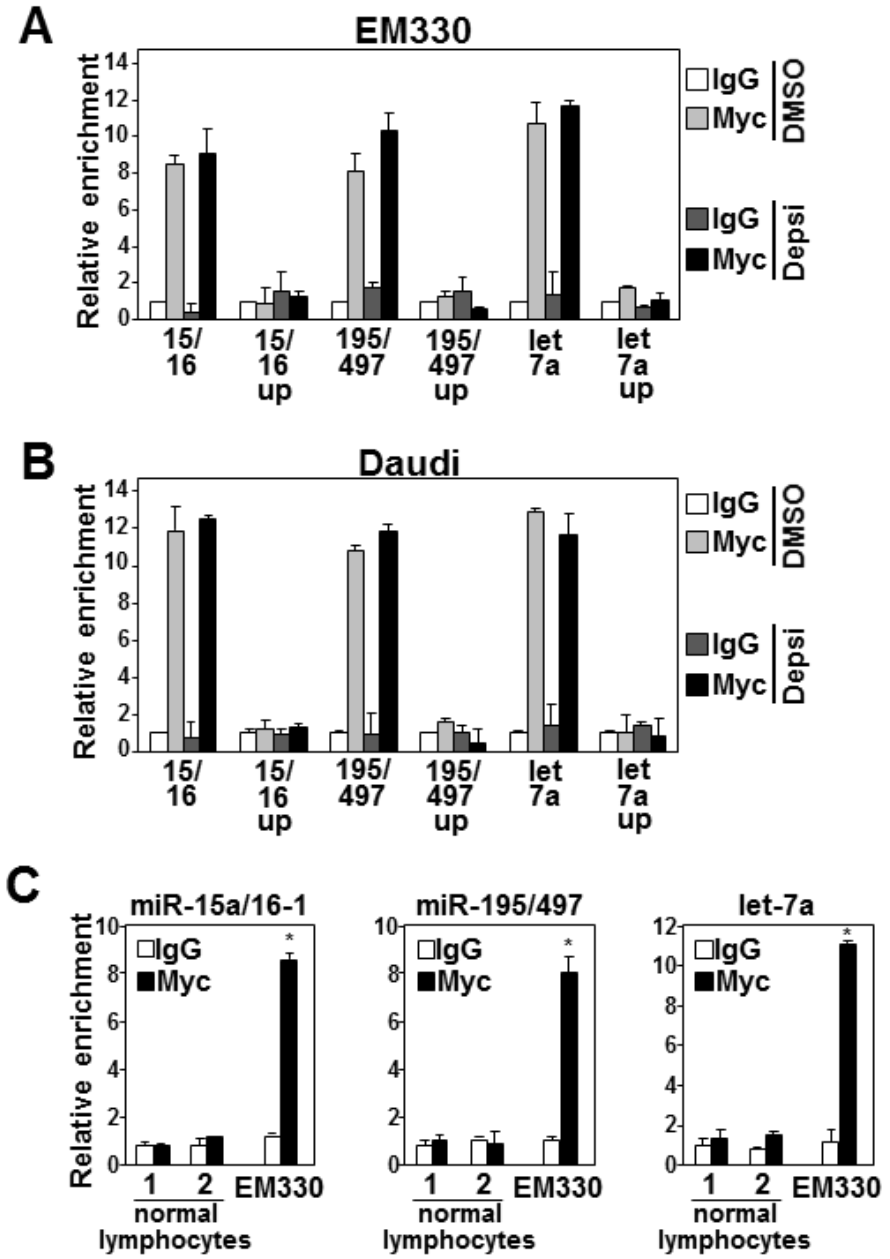

Figure 41. Myc localizes to the miR-15 family and let-7a loci in lymphoma cells regardless of HDAC inhibition. (A-C) Following ChIP with anti-Myc or isotype control (IgG), qRT-PCR for the indicated promoters or regions upstream (up) Myc does not bind (negative controls) was performed (triplicate). Murine (EM330; A) and human (Daudi; B) lymphoma cells were treated with Depsipeptide (Depsi) or vehicle (DMSO) for 4hrs. (C) ChIP for Myc was performed with murine B cell lymphoma cells (EM330) and normal lymphocytes isolated from two C57Bl/6 spleens for non-tumor controls. Following ChIP with antibodies against Myc or isotype control (IgG), qRT-PCR for the indicated promoter regions was performed in triplicate. Values for qChIP are relative to their respective IgG control and input DNA. Error bars are SEM. For C, *p<0.002, Myc of EM330 versus the mean for Myc of the normal lymphocytes.

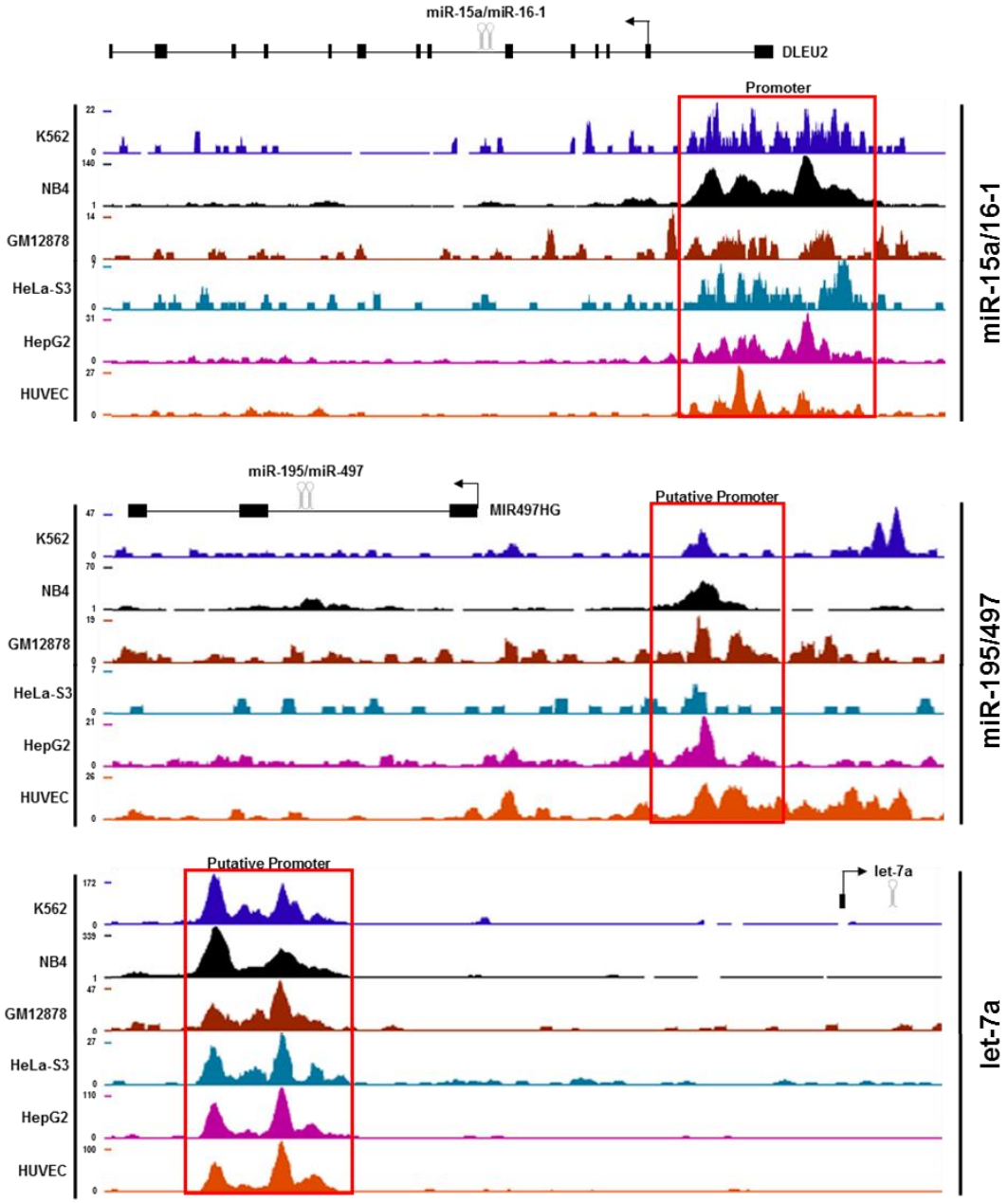

Figure 42. MYC associates with the promoters of the miR-15 family and let-7a in multiple human malignancies. Evaluation of ENCODE MYC ChIP-seq data (Consortium, 2012) demonstrates MYC enrichment at the promoter of the miR-15a/16-1 and miR-195/497 clusters and let-7a (peaks boxed in red) in chronic myelogenous leukemia (K562), acute promyelocytic leukemia (NB4), lymphoblastoid (GM12878), cervical carcinoma (HeLa-S3), hepatocellular carcinoma (HepG2), and human umbilical vein endothelial cells (HUVEC).

lack of enrichment at *p21* we observed in the lymphoma cells (Figure 43A). Neither RNAPII-p-Ser2 nor H3K9K14ac enrichment was detected at promoters of either miR-15 family cluster or let-7a in the lymphomas (Figure 43A). 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 (Figure 43B). Furthermore, the de-repression 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 (Figure 43C). 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 (Pajic *et al.*, 2000). With tetracycline present, MYC levels were significantly reduced and HDACi failed to increase miR-15a, miR-195, or let-7a levels (Figure 44A). 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 (Figure 44B). Upon HDACi, enrichment of RNAPII-

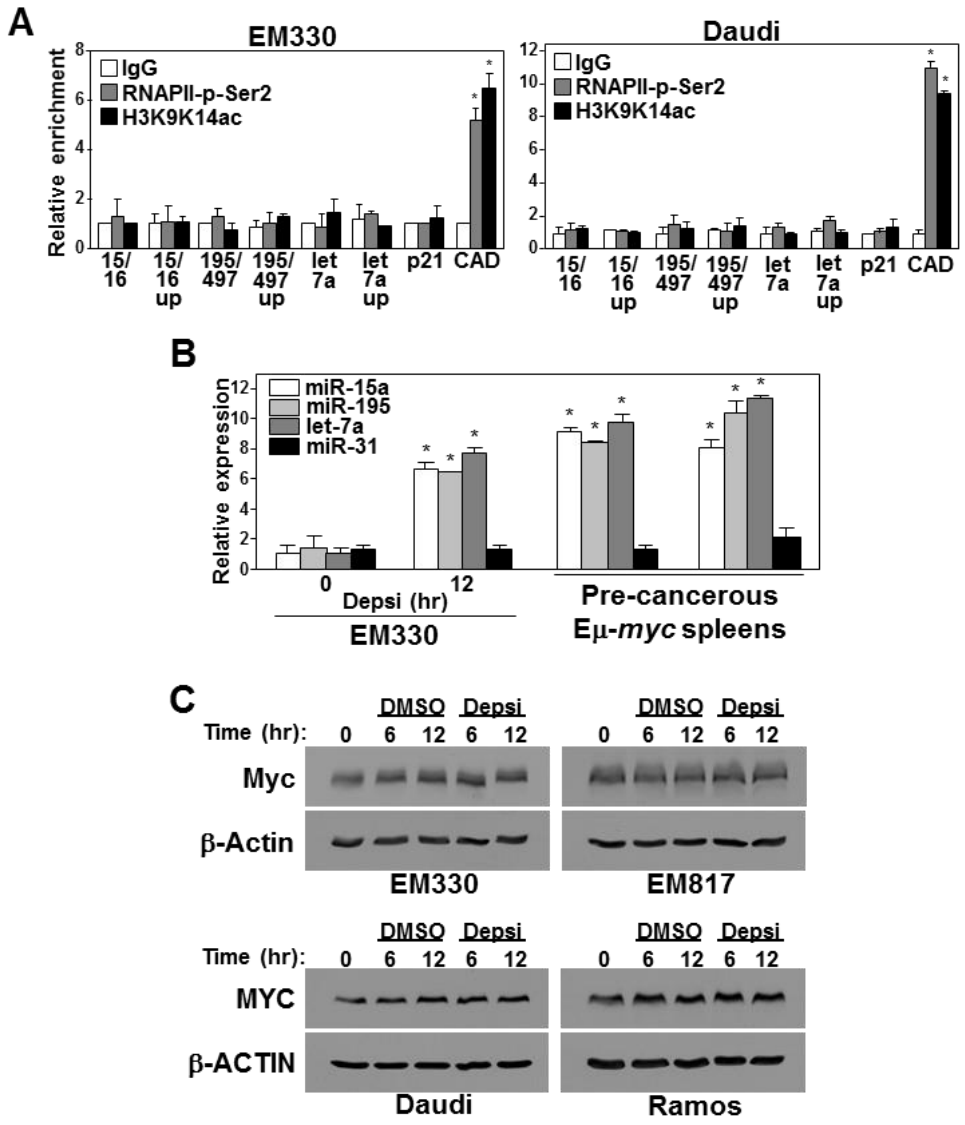

Figure 43. miR-15 family and let-7a loci are repressed in lymphoma and reactivated by HDAC inhibition. (A) Murine (EM330) and human (Daudi) lymphoma cells treated with Depsipeptide (Depsi) or vehicle (DMSO) for 4hrs. Following ChIP with anti-Myc, anti-RNAPII-phosphorylated-Serine2 (RNAPII-p-Ser2), anti-H3K9K14ac, or isotype controls (IgG), qRT-PCR for the indicated promoters or regions upstream (up) Myc does not bind (negative controls) was performed (triplicate). *p21* (Myc repression target) and *CAD* (Myc activation target) were controls (Eilers and Eisenman, 2008). Values for qChIP are relative to their respective IgG control and input DNA. (B) Relative expression of the indicated miRNA was determined by qRT-PCR (triplicate) comparing murine (Eμ-*myc*) lymphoma cells (EM330) following 12hrs of HDAC inhibition with Depsipeptide (Depsi) to that of two pre-cancerous Eμ-*myc* spleens overexpressing Myc. miRNA expression was normalized to the expression of small RNA *RNU6b*. (C) Whole cell protein lysates were Western blotted for Myc and β-Actin in murine (EM330, EM817) and human (Daudi, Ramos) lymphoma cell lines following treatment with Depsipeptide (Depsi) or vehicle (DMSO) control for the indicated time. Error bars are SEM. *$p<0.0002$ for A, RNAPII-p-Ser2 and H3K9K14ac vs. IgG and *$p<0.001$ for B, comparisons to EM330 0hr Depsi.

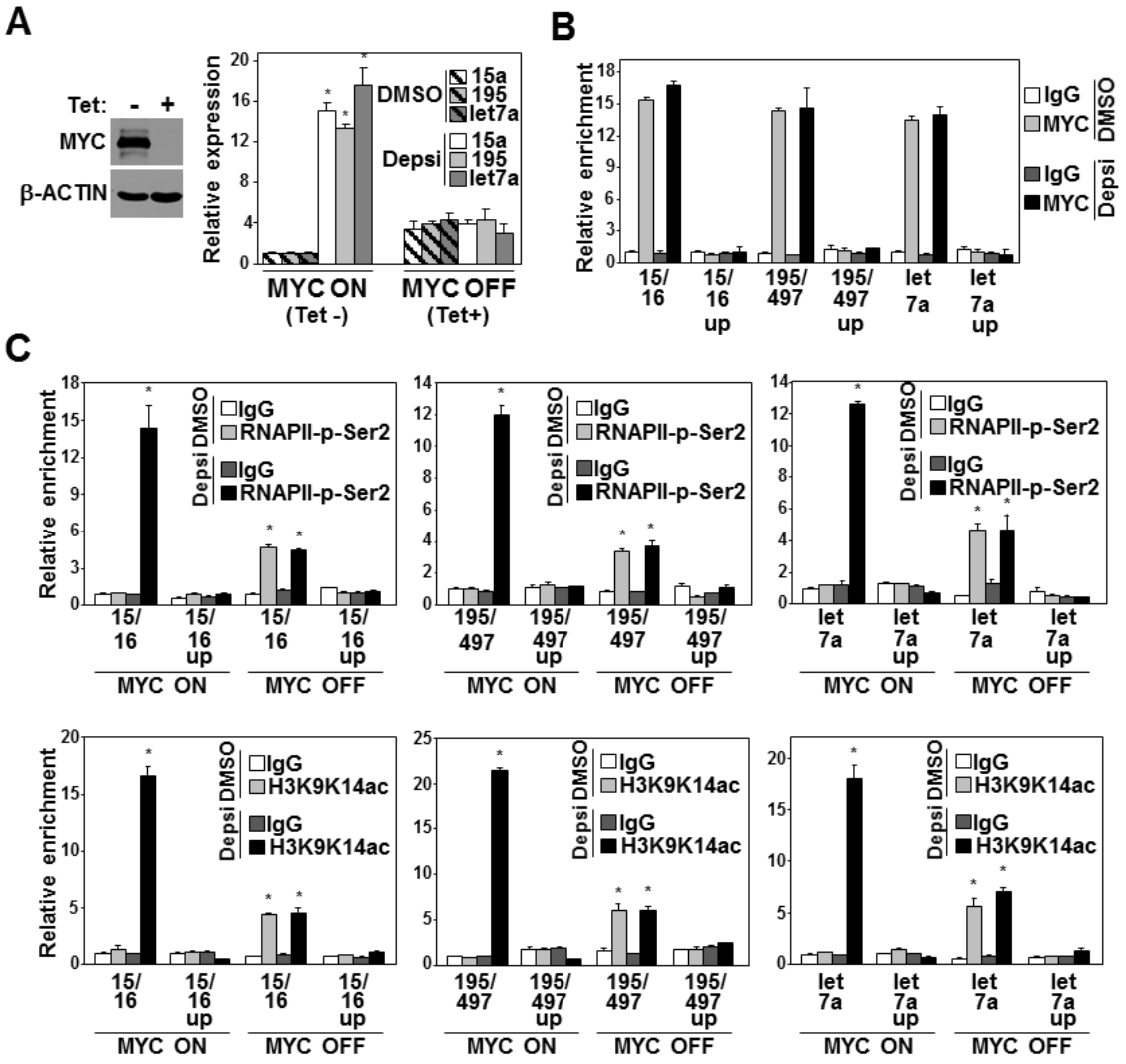

Figure 44. Myc is required for HDACi-induced transcriptional up-regulation of the miR-15 family and let-7a. (A) Human P493-6 lymphoma cells containing tetracycline-regulatable MYC (P493-6) exposed to tetracycline (+; MYC-OFF) for 24hrs or not (MYC-ON) were Western blotted. These cells were also treated for 12hrs with Depsi or vehicle (DMSO) control. miRNA were measured by qRT-PCR (triplicate) and normalized to *RNU6b* levels. (B, C) 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. 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). Values for qChIP are relative to their respective IgG control and input DNA. Error bars are SEM. *p<0.01; Depsi versus DMSO (A) or RNAPII-p-Ser2 and H3K9K14ac vs. IgG (C).

p-Ser2 and H3K9K14ac (marks of active transcription) at miR-15 family and let-7a promoters was only observed in MYC-expressing cells (Figure 44C). 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 (Figure 44C). 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-x_L expression

In untransformed cells, Myc suppressed Bcl-2 and Bcl-x_L expression, inducing apoptosis through an indirect, unresolved mechanism (Eischen *et al.*, 2001a; Eischen *et al.*, 2001c), purportedly through binding Miz-1 (Patel and McMahon, 2007). However, Bcl-2 and Bcl-x_L proteins are overexpressed in the majority of Eμ-*myc* B-cell lymphomas and human lymphomas (Eischen *et al.*, 2001c). Evaluation of Eμ-*myc* B-cell lymphomas compared to normal B-cells and spleens from pre-cancerous Eμ-*myc* mice confirmed these results (Figure 45A). In contrast, pre-cancerous Eμ-*myc* spleens with increased Myc had reduced Bcl-2 and Bcl-x_L protein compared to non-transgenic littermate-matched spleens (Figure 45B). 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-x_L were detected following MycER activation (Figure 45C). No change in Mcl-1 or Bax expression was detected, whereas Bim, a pro-apoptotic Bcl-2 family member up-regulated by Myc (Egle *et al.*, 2004), was increased upon MycER activation (Figure 45C). Therefore, with elevated Myc, non-transformed cells decrease Bcl-2 and Bcl-x_L expression, whereas their expression is increased in transformed cells.

Myc associates with the transcription factor Miz-1 through a motif requiring valine

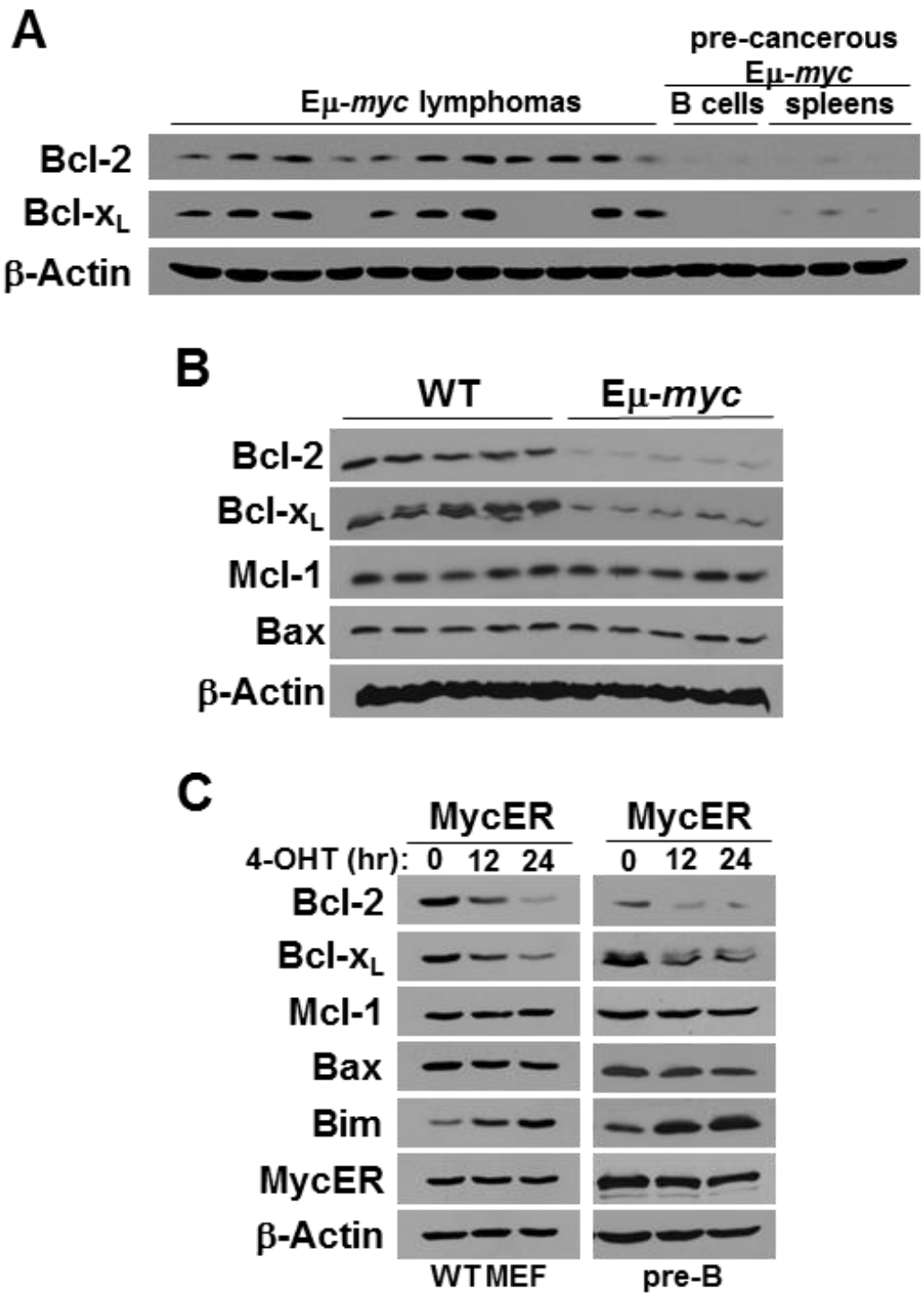

Figure 45. Expression of Bcl-2 and Bcl-x_L is regulated by Myc. Western blotting was performed for the indicated proteins in Eμ-*myc* lymphomas (n=11) and pre-cancerous Eμ-*myc* purified B-cells (n=2) and spleens (n=3) (A), pre-cancerous splenocytes from Eμ-*myc* mice (n=5) and wild-type (WT) non-transgenic littermates (n=5) (B), and at intervals following addition of 4-OHT to wild-type (WT) MycER-expressing murine embryonic fibroblasts (MEFs) and primary pre-B-cells (C).

394, reportedly suppressing *Bcl-2* (Patel and McMahon, 2007). To investigate this, MycER harboring a point mutation (V394D) disrupting the Myc:Miz-1 interaction (MycV394D-ER; Figure 46A) (Herold *et al.*, 2002) 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 (Figure 46B). Analogous data were obtained using *p53*-null MEFs (Figure 46C), consistent with prior results in *p53*-null myeloid and B-cells (Eischen *et al.*, 2001a; Eischen *et al.*, 2001c). 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 (Figure 46D), 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 (Peter *et al.*, 2014; Rashkovan *et al.*, 2014; Walz *et al.*, 2014; Wolf *et al.*, 2013) showed Miz-1 enrichment at promoters of known Miz-1-regulated genes (e.g., *VAMP4*) (Figure 47). 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 MycΔMBII-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 MycΔMBII-ER (Figure 48). Again, Mcl-1 and Bax expression were unaffected. Therefore, Myc-mediated transcriptional activity is necessary for Bcl-2 and Bcl-x_L down-regulation.

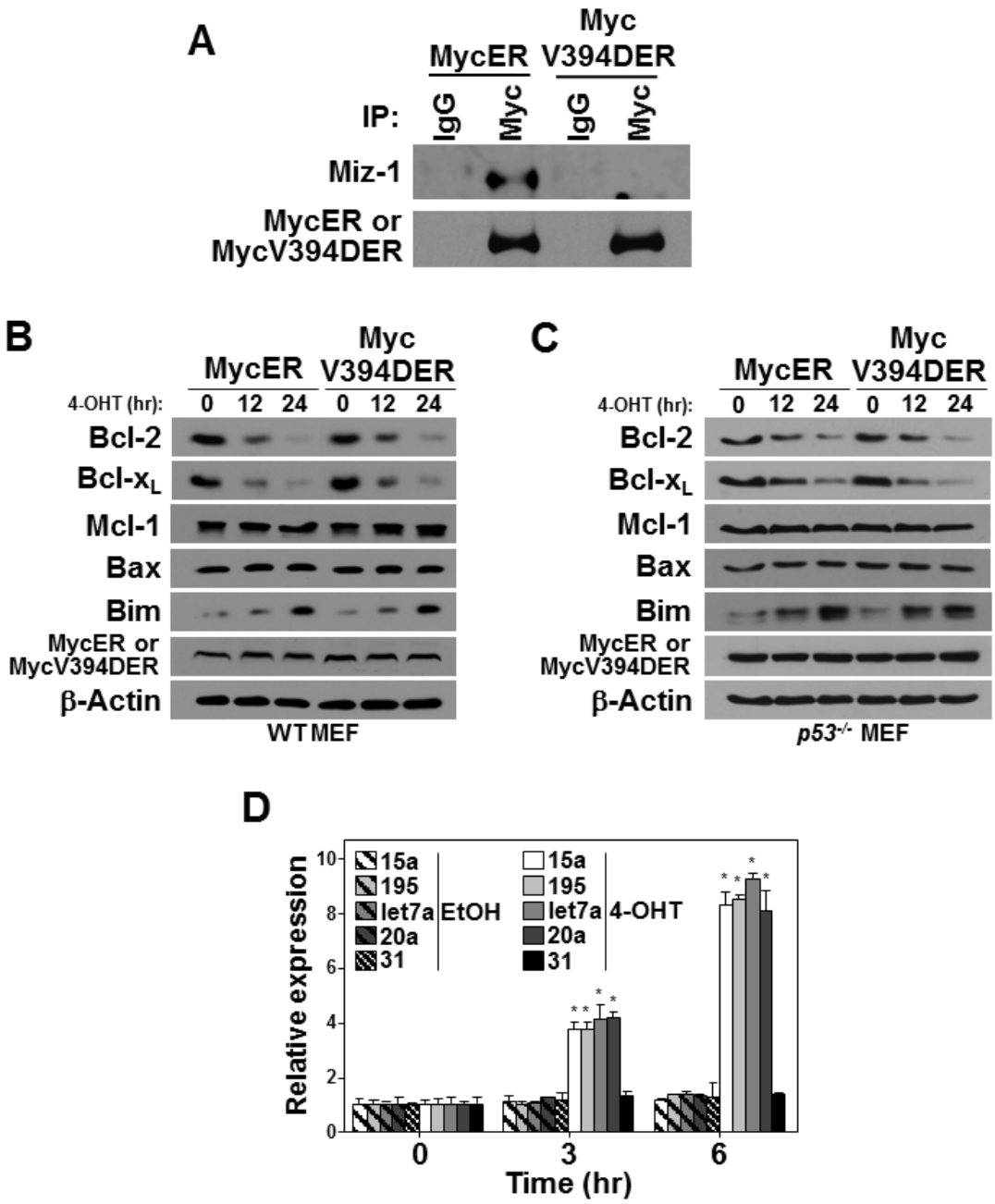

Figure 46. Myc transcriptional activity regulates Bcl-2 and Bcl-x_L expression independent of Miz-1 and p53. (A, C) *p53*-null MEFs or wild-type (WT MEF; B) expressing wild-type MycER or MycV394D-ER, a Myc mutant that cannot associate with Miz-1. MycER and MycV394D-ER were activated with 4-OHT for 4 hours (A) or the time indicated (B, C). (A) Immunoprecipitations of whole cell protein lysates with anti-Myc or isotype (IgG) control were Western blotted for Miz-1 and Myc. (B, C) Whole cell protein lysates were Western blotted for the indicated proteins in wild-type MEFs (B) and *p53*-null MEFs (C). (D) 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.

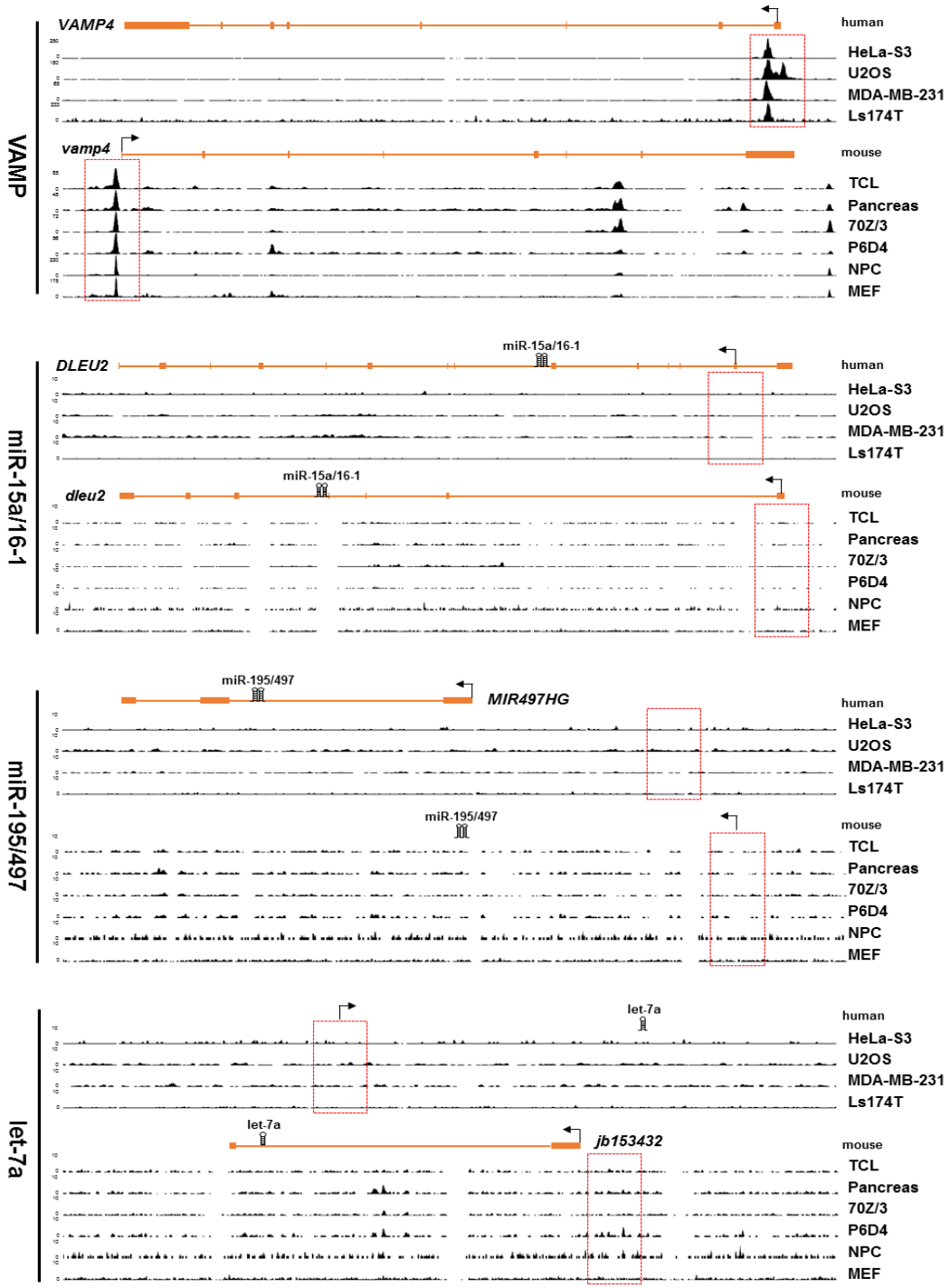

Figure 47. Miz-1 does not associate with the miR-15 and let-7 family promoters. Evaluation of Miz-1 ChIP-seq data at the promoters of *VAMP4*, a known Miz-1 regulated gene, the miR-15a/16-1 and miR-195/497 clusters, and let-7a in human cervical carcinoma (HeLa-S3), human osteosarcoma (U2OS), human breast carcinoma (MDA-MB-231), human colorectal adenocarcinoma (Ls174T), murine T cell lymphoma (TCL), murine pancreatic carcinoma, murine pre-B lymphoblasts (70Z/3), murine pre-T lymphoblasts (P6D4), murine neuronal progenitor cells (NPC), and murine embryonic fibroblasts (MEF). Promoter regions are outlined in red.

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-x_L 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-x_L* 3'-untranslated region (3'-UTR) (Figure 49A). 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-x_L* 3'-UTR, respectively (Figure 49B). 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 (Figure 49B). 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 (Bui and Mendell, 2010). 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-x_L*, 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-x_L* 3'-UTR, respectively (Figure 50A). Bcl-2 and Bcl-x_L protein increased in wild-type MEFs transfected with Bcl-2 or Bcl-x_L Target Protectors, respectively, as endogenous miR-15 family members and

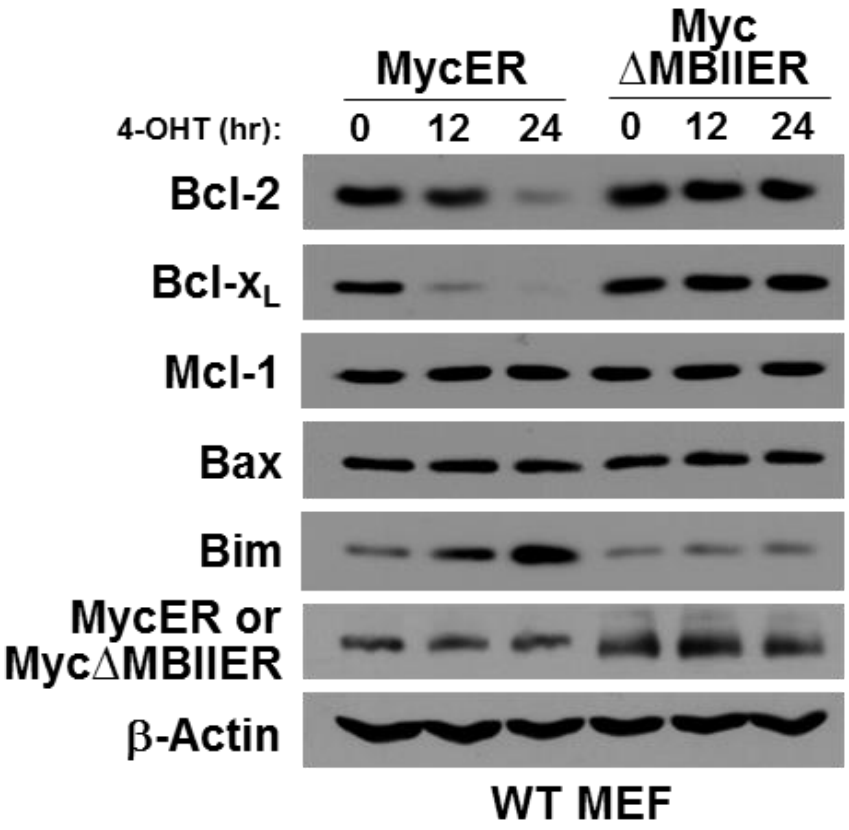

Figure 48. Myc transcriptional activity regulates Bcl-2 and Bcl-x_L expression. At intervals following addition of 4-OHT, wild-type (WT) MEFs expressing MycER or MycΔMBII-ER were harvested and Western blotted for the indicated proteins.

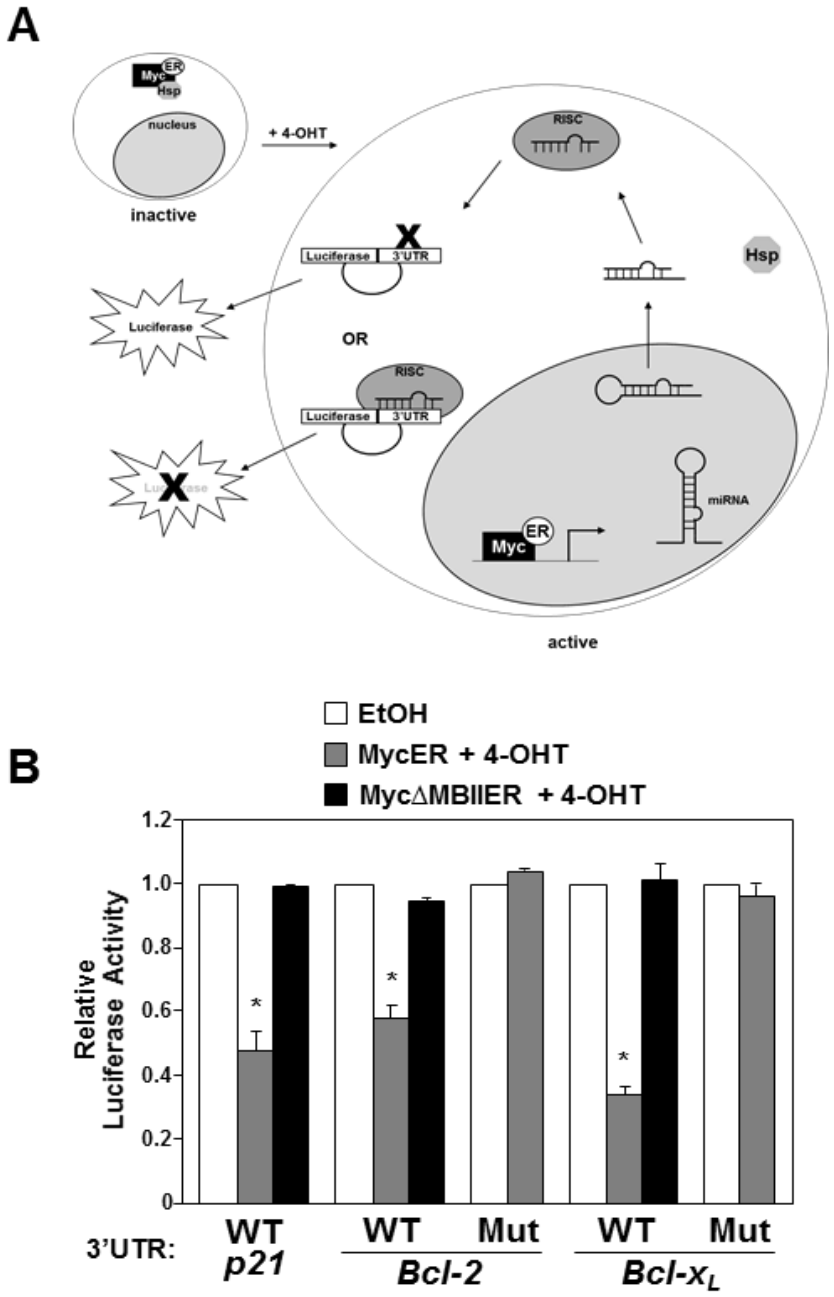

Figure 49. Myc induces the miR-15 family and let-7a that then target *Bcl-2* and *Bcl-x_L*. (A) Schematic of experimental design. (B) Luciferase expression vectors containing the 3'-untranslated region (3'-UTR) of *Bcl-2* and *Bcl-x_L* with the wild-type (WT) or a mutated (Mut) miRNA binding site were 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 (Bui and Mendell, 2010). Luciferase activity was measured (triplicate) 48hrs following vehicle (EtOH) control or 4-OHT addition to activate MycER. A β-galactosidase reporter plasmid was co-transfected for normalization.

let-7a were unable to bind and inhibit their expression (Figure 50B). Combining the Bcl-2 Target Protector with miR-15a overexpression, which alone decreased Bcl-2 protein, rescued Bcl-2 protein expression (Figure 50B). Then, MycER-expressing fibroblasts were transfected with luciferase reporters, as described above, together with the Bcl-2 or Bcl-x_L Target Protectors. In the presence of Bcl-2 or Bcl-x_L Target Protectors, little, if any, decrease in luciferase activity was detected following MycER activation (Figure 50C). Next, MEFs expressing MycV394D-ER (unable to interact with Miz-1) were transfected with Bcl-2 or Bcl-x_L Target Protectors. MycV394D-ER activation decreased Bcl-2 and Bcl-x_L protein expression in the absence of any Target Protector (Figure 50D). However, levels of Bcl-2 or Bcl-x_L were maintained when Target Protectors blocked the miR-15 or let-7 family binding sites, respectively (Figure 50D). Therefore, down-regulation of Bcl-2 and Bcl-x_L 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-x_L*, respectively.

We then tested whether targeting of *Bcl-2* and *Bcl-x_L* 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 Annexin V-positivity (Figure 51A-C). 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-x_L* in untransformed cells to trigger apoptosis, and that this mechanism is re-activated in lymphomas following HDACi (Figure 52).

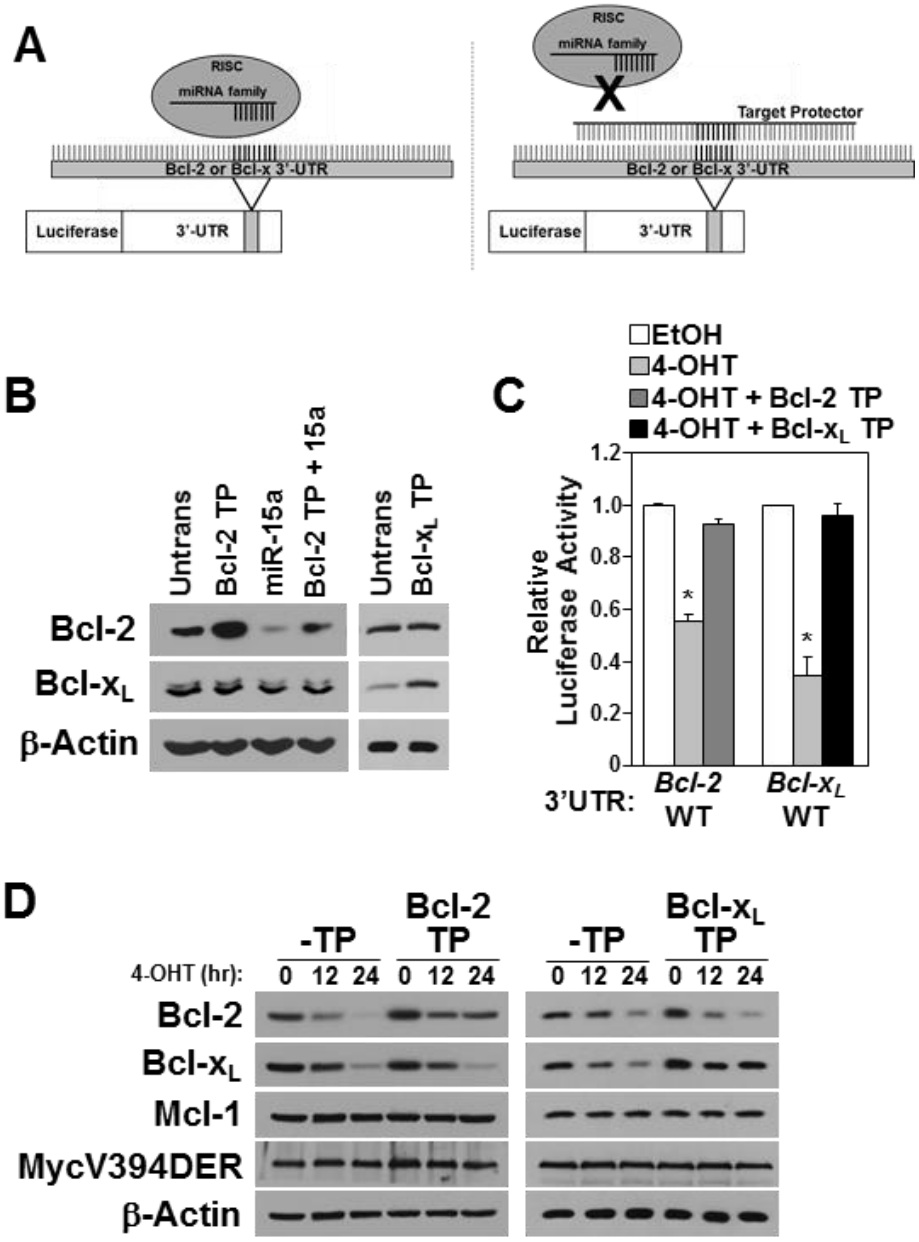

Figure 50. Blocking the miR-15 and let-7 family binding sites in the 3'-UTR of *Bcl-2* and *Bcl-x_L*, respectively, inhibits the Myc-induced decrease in their expression. (A) Target Protectors block the miR-15 family and let-7a from binding sites in the 3'-untranslated region (3'-UTR) of *Bcl-2* and *Bcl-x_L*, respectively. (B) Wild-type MEFs transfected with either *Bcl-2* or *Bcl-x_L* Target Protectors (TP) and/or miR-15a mimic were Western blotted. Untrans, untransfected cells. (C) Luciferase expression vectors containing the 3'-UTR of *Bcl-2* and *Bcl-x_L* with the miRNA binding site were transfected into fibroblasts expressing the 4-OHT-inducible MycER. Luciferase activity was measured (triplicate) 48hrs following vehicle (EtOH) control or 4-OHT addition to activate MycER. A β-galactosidase reporter plasmid was co-transfected for normalization. (D) *$p53^{-/-}$* MEFs, with or without *Bcl-2* and/or *Bcl-x_L* Target Protectors (TP), expressing the 4-OHT-inducible MycV394D-ER were Western blotted at intervals following addition of 4-OHT. Error bars are SEM. **p*<0.009, 4-OHT versus EtOH.

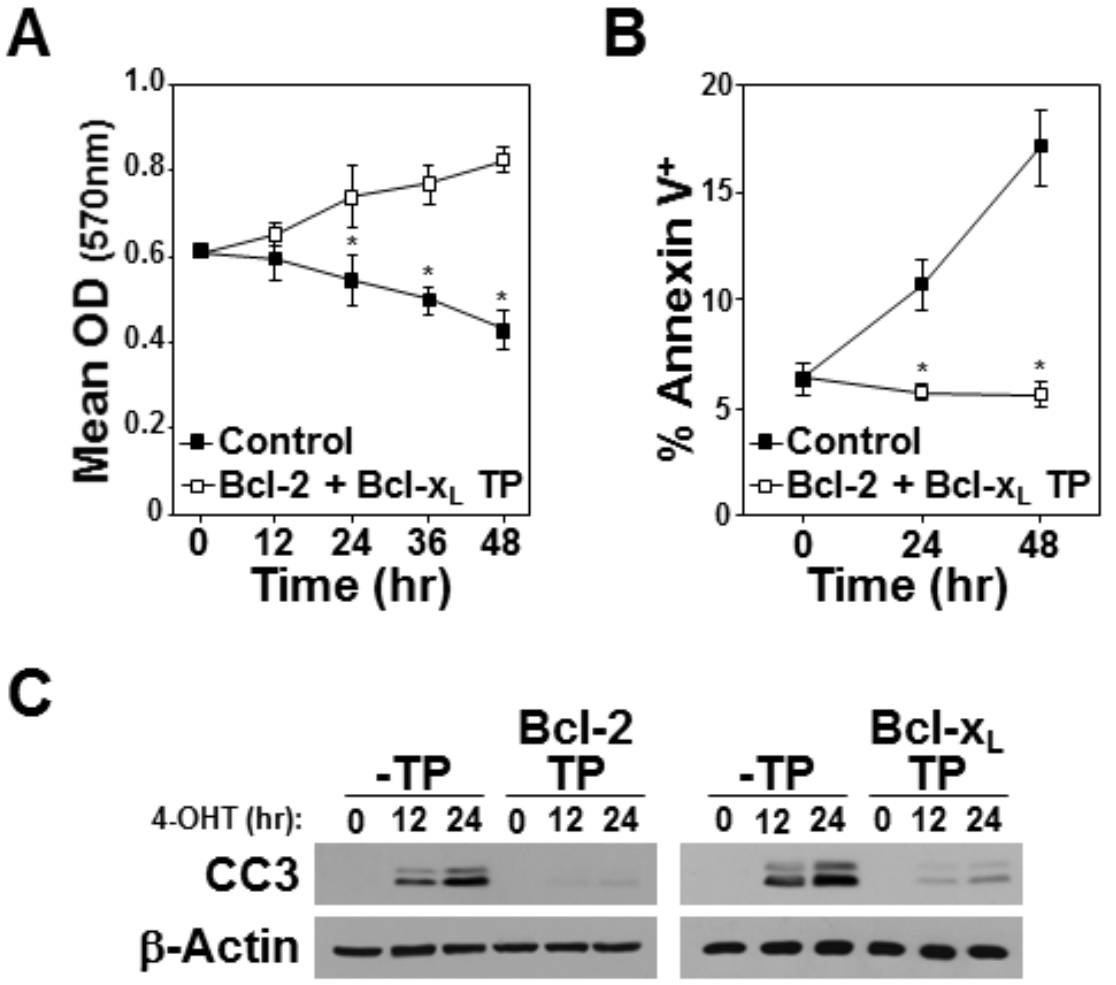

Figure 51. Blocking the miR-15 and let-7 families from binding *Bcl-2* or *Bcl-x$_L$*, respectively rescues Myc-induced apoptosis. miR-15 family and let-7a miRNA binding sites in the *Bcl-2* and *Bcl-x$_L$* 3'-UTR were blocked with site-specific small molecules (TP; Target Protectors). *p53$^{-/-}$* MEFs, with or without *Bcl-2* and/or *Bcl-x$_L$* Target Protectors (TP), expressing the 4-OHT-inducible MycV394D-ER were subjected to MTT assay (A; quadruplicate), analyzed for Annexin V-positivity (triplicate) by flow cytometry, or Western blotted (C) at intervals following addition of 4-OHT. Cleaved Caspase-3 (CC3). Error bars are SD. *p<0.02 for A and *p<0.0001 for B; both TP versus control. I received technical assistance in running the Western for C.

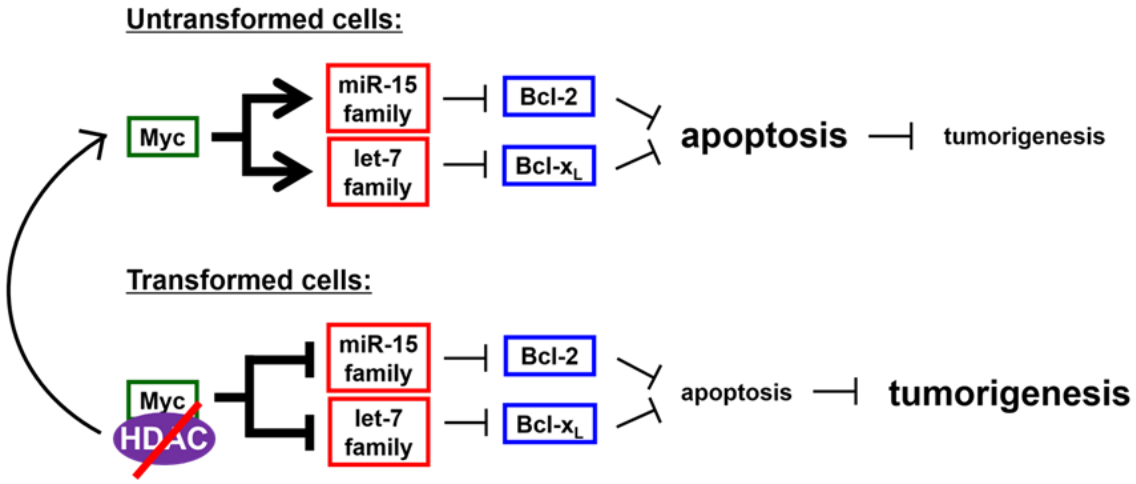

Figure 52. Myc-regulated miRNA mediate a novel mechanism of apoptosis that is re-activated by HDAC inhibition. Cellular transformation status dictates whether Myc transcriptionally activates or represses the miR-15 and let-7 families that target anti-apoptotic *Bcl-2* and *Bcl-x_L*, respectively. This apoptotic mechanism was inactivated in transformed hematopoietic cells through epigenetic alterations involving HDACs, but reactivated by HDAC inhibition.

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 (Conti *et al.*, 2010; Stengel and Hiebert, 2014; West and Johnstone, 2014), 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-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 (Insinga *et al.*, 2005). Others have reported HDACi causes global changes in gene expression that alter the apoptotic threshold in favor of cancer cell killing (Bolden *et al.*, 2013). 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 (Bolden *et al.*, 2013). 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-x_L* gene expression. Likewise, other groups have reported HDACi-mediated changes in miRNA expression (Cho *et al.*, 2015;

Majid *et al.*, 2013; Scott *et al.*, 2006; Zhang *et al.*, 2012b). For example, HDACi of a breast cancer line changed the expression of 27 miRNA within 5 hours, including let-7a (Scott *et al.*, 2006). 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-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 (Dang, 2012; Soucek *et al.*, 2008). Thus, our data, providing a molecular link between Myc, miRNA, and Bcl-2 and Bcl-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 (Chang *et al.*, 2008; Lu *et al.*, 2005), 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 (Dang, 2012). Myc induces apoptosis by activating the p53 pathway and simultaneously down-regulating *Bcl-2* and *Bcl-x_L* mRNA expression through an indirect mechanism (Eischen *et al.*, 2001a; Eischen *et al.*, 2001c), reportedly involving Miz-1 (Patel and McMahon, 2007). Myc expression did not change the half-life of Bcl-2 (Eischen *et al.*, 2001a); therefore, we suspected a transcriptional or post-transcriptional mechanism. The miR-15 family and let-7a were known to target *Bcl-2* and *Bcl-x_L*, respectively, contributing to apoptosis (Lima *et al.*, 2011), so we investigated whether a connection between Myc, these miRNA, and Bcl-

2 and Bcl-x_L down-regulation existed. Indeed, Myc suppressed the expression of Bcl-2 and Bcl-x_L independent of its interaction with Miz-1 and of p53, which can itself transcriptionally repress *Bcl-2* and *Bcl-x_L* expression (Haldar *et al.*, 1994; Sugars *et al.*, 2001). Transcriptionally competent Myc was required for the reduction in Bcl-2 and Bcl-x_L 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-x_L* 3'-UTRs, respectively. By inhibiting the miR-15 family from binding *Bcl-2* and let-7a from binding *Bcl-x_L*, the decrease in Bcl-2 and Bcl-x_L 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-x_L*, 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 (Chang *et al.*, 2008). 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 (Zhang *et al.*, 2012a; Zhang *et al.*, 2012b). Moreover, Myc is reported to recruit HDAC3 to the promoters of protein-coding genes and miRNA to repress their expression (Kurland and Tansey, 2008; Zhang *et al.*, 2012a; Zhang *et al.*, 2012b). 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-x_L*, 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 (Dang, 2012; Olivier *et al.*, 2010), 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 (Ellis *et al.*, 2009; Lindemann *et al.*, 2007; Thompson *et al.*, 2013; Whitecross *et al.*, 2009), 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.

CHAPTER IV

HISTONE DEACETYLASE INHIBITION REVEALS A TUMOR SUPPRESSIVE FUNCTION OF MYC-REGULATED MIRNA IN BREAST AND LUNG CARCINOMA

This chapter is from:

Adams CM and Eischen CM. "Histone deacetylase inhibition reveals a tumor suppressive function of Myc-regulated miRNA in breast and lung carcinoma." In revision, 2015

Introduction

Aberrant gene transcription is a defining feature of cancer, and alterations in transcription regulation often lead to cellular transformation (Hanahan and Weinberg, 2011). Complex mechanisms regulate transcription, including the addition or removal of chemical modifications, such as acetyl groups, to histone tails (Dawson and Kouzarides, 2012). Deregulation in the expression and/or activity of histone deacetylase (HDAC) enzymes, which remove acetyl groups, leads to alterations in gene expression and has been linked to the development of cancer (Dawson and Kouzarides, 2012). The predominant biological outcome following exposure of cells to inhibitors of HDACs has been the selective death of malignant cells (West and Johnstone, 2014). Although HDAC inhibitors have provided clinical benefit to the treatment of specific hematological malignancies, its impact on solid organ cancer treatment is less clear and the underlying mechanisms behind

HDAC inhibition-induced tumor cell apoptosis remain unresolved.

While the mechanism of action of HDAC inhibitors should favor chromatin decondensation and a global increase in gene transcription, only a small percentage of genes appears to be affected (Xu *et al.*, 2007). This suggests that post-transcriptional mechanisms of gene regulation are likely involved in the molecular events following HDAC inhibition. One such mechanism that has been linked to HDAC regulation includes miRNA (Chen *et al.*, 2014; Cho *et al.*, 2015; Wang *et al.*, 2013; Zhang *et al.*, 2012b). miRNA comprise a class of non-coding RNA that post-transcriptionally regulate the expression of target mRNA, typically resulting in decreased translation (Ebert and Sharp, 2012). The potential for miRNA-guided regulation of gene expression is significant, as it is predicted that the majority of all mRNAs are under miRNA control and that a single miRNA can target many mRNA (Ebert and Sharp, 2012). Therefore, HDAC inhibition-induced changes in one or more miRNA is capable of eliciting a significant downstream biological response.

Cancers often present with reduced levels of mature miRNA as compared to normal tissue of the same origin (Gaur *et al.*, 2007; Lu *et al.*, 2005). In B cell lymphomas, downregulation of miRNA expression was reported to be the result of widespread transcriptional repression by the oncogenic transcription factor MYC (Chang *et al.*, 2008). Moreover, well-known tumor suppressive miRNA, including the miR-15 and let-7 families, are repressed by MYC in human B cell lymphoma (Chang *et al.*, 2008). These miRNA have also been reported to be downregulated in breast and lung cancers (Gaur *et al.*, 2007; Lu *et al.*, 2005; Volinia *et al.*, 2006), but the involvement of MYC in their repression in these malignancies is unknown. Recently, MYC was shown to repress miR-

29 in B cell lymphomas through recruitment of HDAC3 to the miR-29 promoter (Zhang *et al.*, 2012b). Here we demonstrate that MYC repressed the miR-15 and let-7 families in breast and lung cancer, and that upon HDAC inhibition (HDACi), these miRNA were transcriptionally activated by MYC. Blocking the ability of miR-15 and let-7 families from targeting *BCL-2* and *BCL-X_L*, respectively, resulted in carcinoma cell survival. Our data reveal a previously unknown mechanism of MYC-induced apoptosis mediated by miRNA that is activated in breast and lung cancer cells by HDACi.

Materials and Methods

Cell culture, vectors, and transfection

Human breast (MDA-MD-231 and HCC1806) and lung (A549 and H1437) carcinoma cell lines were provided by Drs. Jennifer Pietenpol, Pierre Massion, and William Pao. All cells were cultured as described by the American Type Culture Collection. MDA-MB-231 cells were transfected using Lipofectamine 2000 (Life Technologies) with a SureSilencing vector encoding a *MYC* shRNA or non-targeting control shRNA (48 hours; Qiagen) or with 200 nM miScript Target Protectors (24 hours; Qiagen) designed to block miR-15 family and let-7 family binding sites in the 3'-UTR of *BCL-2* and *BCL-X_L*, respectively.

HDAC inhibition and cell survival assays

Cells were treated with 10 nM Depsipeptide (Celgene) or vehicle control (DMSO). To assess proliferation, cells were plated in quadruplicate and MTT assays were performed according to the manufacture's protocol (Sigma; 570 nm). Apoptosis was evaluated by

assessing Caspase 3 cleavage by Western blotting (see below) and by Annexin-V positivity using flow cytometry, as we previously reported (Adams and Eischen, 2014).

Western blotting

Cells were harvested 48 hours after transfection or at the indicated times following HDACi and were lysed as previously reported (Zindy *et al.*, 1998). Equal amounts of protein were resolved by SDS-PAGE and Western blotted as described (Zindy *et al.*, 1998). Antibodies against BCL-2 and BCL-X_L (BD Biosciences), cleaved Caspase 3 (Cell Signaling), MYC (Millipore); β-ACTIN (Sigma), and H3K9K14ac, H3K56ac, H4K5ac, Histone H3, Histone H4 (Abcam) were used.

RNA isolation and quantitative real-time PCR (qRT-PCR)

RNA was isolated, cDNA was generated, and TaqMan MicroRNA Assays for mature miRNA (Applied Biosciences) were used to perform qRT-PCR, in triplicate, as previously described (McGirt *et al.*, 2014). miRNA expression was normalized to small RNA *RNU6b* levels and presented as $2^{-\Delta\Delta Ct}$. Sequences of primers used to detect primary miRNA transcripts of the miR-15a/16-1, miR-195/497, and let-7a/f clusters are were designed such that ~100 base pairs of flanking sequence extended on either side of the stem-loop. Stem-loop sequences of each miRNA were derived from the Sanger miRBase data repository and mapped to the human genome. miR-15a/16-1 forward 5'-CAATTACAGTATTTT AAGAG, reverse 5'-GCATATTACATCAATGTTAT; miR-195/497 forward 5'-CCTCT CAGCTTCGTGCTGTC, reverse 5'-CCTGCTCAGCCCGTCCCTGG; let-7a/f forward 5'-CTTATCACTCACACAGGAAAC, reverse 5'-GACAAGAAGCAAAAGGTTTC.

Quantitative chromatin immunoprecipitation (qChIP)

qChIP was performed as previously described (Grieb *et al.*, 2014). The antibodies used for MYC (N-262) and the isotype controls were from Santa Cruz, and antibodies against H3K9K14ac, Histone H3, and RNA polymerase II (Ser2 phosphorylated form) were from Abcam. Primer sequences for ChIP of the promoter or upstream regions (negative controls) for the miR-15a/16-1, miR-195/497, and let-7a/f clusters were previously described (Chang *et al.*, 2008).

ENCODE data

Data from the ENCODE project was utilized (Consortium, 2012). The GEO accession numbers of the datasets evaluated are GSM1003607, GSM935441, and GSM822301.

Statistics

Student's t-tests were used to determine statistical significance.

Results

HDACi induces transcription of the miR-15 and let-7 families in breast and lung cancer cells

Cancer cells frequently select for downregulation of miRNA that inhibit growth and mediate apoptosis (Di Leva *et al.*, 2014). For example, the miR-15 and let-7 families, both of which have been shown to induce apoptosis and inhibit tumorigenesis (Lima *et al.*, 2011), are significantly downregulated in most human cancers, including breast and lung

carcinoma (Gaur *et al.*, 2007; Lu *et al.*, 2005; Volinia *et al.*, 2006). A recent mechanism of miRNA repression was reported to be mediated, in part, by the recruitment of HDACs to miRNA promoters (Zhang *et al.*, 2012b). As such, we sought to determine whether HDACs contributed to the transcriptional repression of the miR-15 and let-7 families in breast and lung cancers. Following HDACi with Depsipeptide (Depsi, pan-class I HDAC inhibitor) or vehicle control (DMSO), an increase in levels of representative members of the miR-15 and let-7 families (miR-15a and let-7a, respectively) was observed in all four breast and lung cancer cell lines evaluated (Figure 53A). Expression of other miRNA (e.g., miR-301) did not change upon HDACi, indicating that HDACi did not lead to the upregulation of all miRNA.

We postulated that the increased miRNA levels we observed were likely due to increased transcription. To determine whether the miR-15 and let-7 families were actively being transcribed following HDACi, we first assessed the expression of the primary miRNA transcripts for miR-15a and let-7a, representative members of both families. Following HDACi, we detected an increase in levels of pri-miR-15a and pri-let-7a in the breast and lung cancer cell lines (Figure 53B). Additionally, Western blotting revealed an increase in global histone acetylation marks associated with active transcription following HDACi (Figure 53C).

Global acetylation of histones is typically associated with open and active chromatin; therefore, we next evaluated the transcriptional status of the promoters of the miR-15 and let-7 families. We performed chromatin immunoprecipitation (ChIP) with the MDA-MB-231 breast cancer cell line following administration of HDACi or vehicle control. Specifically, we assessed enrichment of a phosphorylated form of RNA

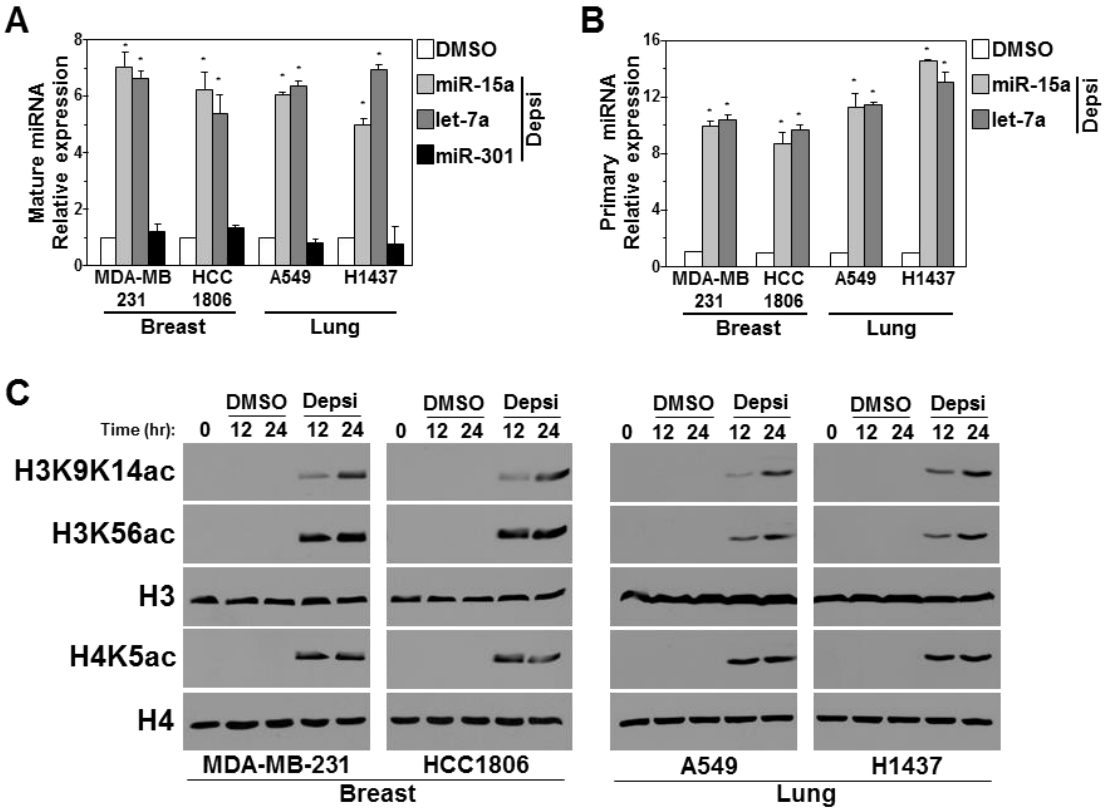

Figure 53. miR-15 and let-7 families are transcriptionally upregulated following HDACi. Human breast (MDA-MB-231, HCC1806) and lung (A549, H1437) carcinoma cell lines were treated with vehicle control (DMSO) or Depsipeptide (Depsi). (A, B) Following 12 hours of treatment with Depsi or vehicle control (DMSO), levels of the indicated mature miRNA (A) and primary miRNA (B) were determined by qRT-PCR in triplicate and normalized to small RNA *RNU6b* levels. Values for each miRNA are plotted relative to their respective DMSO sample, which was set at 1 (only one bar for the DMSO treated samples is displayed). (C) Western blot analysis of the indicated proteins at intervals following Depsi or DMSO vehicle control. Error bars are SEM. *$p<0.002$ (t-test) were determined by comparison to DMSO control.

polymerase II (serine 2 phosphorylated; RNAPII-p-Ser2), which is indicative of RNAPII engaged in transcriptional elongation. Notably, HDACi resulted in a significant enrichment of RNAPII-p-Ser2 at the promoter regions of the miR-15 family (miR-15a/16-1, miR-195/497) and the let-7a/f cluster (Figure 54A). RNAPII enrichment was not detected at regions upstream of the miRNA promoters or in cells that received DMSO vehicle control. Furthermore, H3K9K14ac, an acetylation mark associated with transcriptionally active chromatin, was also enriched at the promoter regions of miR-15a/16-1, miR-195/497, and let-7a/f following HDACi (Figure 54B). These results demonstrate that HDACi alleviates the repression of the miR-15 and let-7 families observed in malignant breast and lung cells, inducing their transcriptional upregulation.

MYC is required to mediate the HDACi-induced increase in the miR-15 family and let-7a

The oncogenic transcription factor MYC has been shown to transcriptionally activate or repress the expression of protein-coding genes and non-coding RNA, including miRNA (Dang, 2012). Previously, reduced expression of the miR-15 and let-7 families in human B cell lymphoma was reportedly due to MYC-mediated transcriptional repression at their promoter regions (Chang *et al.*, 2008). Therefore, we first investigated whether MYC was enriched at the promoter regions of the miR-15a/16-1, miR-195/497, and let-7a/f clusters in the presence and absence of HDACi using ChIP. MYC enrichment was detected in MDA-MB-231 cells that had received either DMSO vehicle control or the HDACi Depsi (Figure 55A). However, no enrichment was detected at regions upstream of the miRNA promoters (Figure 55A). ENCODE ChIP-seq data (Consortium, 2012) from

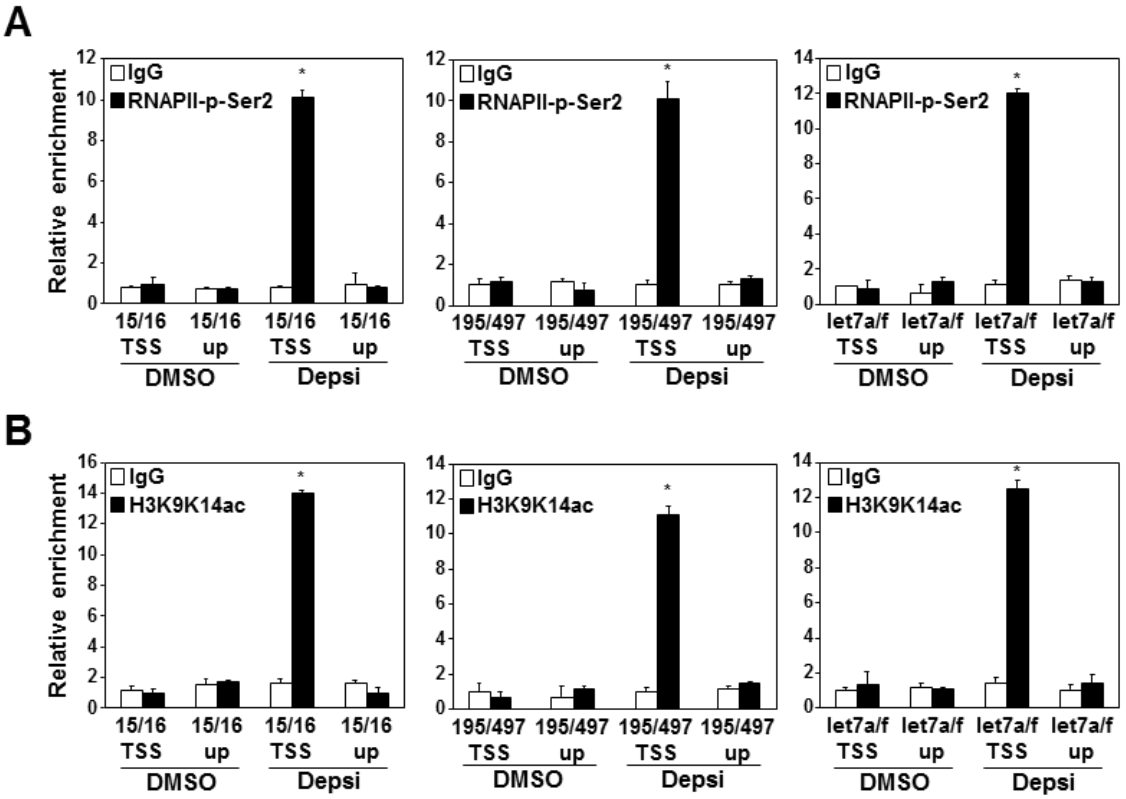

Figure 54. HDAC inhibition re-activates transcription at the miR-15 and let-7 family promoters. MDA-MB-231 breast cancer cells were treated with Depsi or vehicle control (DMSO) for 4 hours. ChIP with anti-RNAPII-phosphorylated on Serine 2 (RNAPII-p-Ser2; A) and H3K9K14ac (B) or isotype controls (IgG) was performed followed by qRT-PCR, in triplicate for the indicated promoter regions (TSS) or the upstream regions (up; negative controls). Values are relative to input DNA and their respective IgG controls and plotted relative to the first DMSO sample, which was set at 1. Error bars are SEM. *p<0.0015 (t-test) were determined by comparison to DMSO.

human breast and lung cell lines was evaluated and also showed that MYC was enriched at these same miRNA promoter regions (Figure 55B). These results suggest that MYC is bound to the promoters of the miR-15 and let-7 families regardless of their transcriptional status and HDACi.

To determine whether MYC was necessary to transcriptionally activate the expression of the miR-15 and let-7 families following HDACi, MDA-MB-231 breast cancer cells were transfected with a vector encoding a *MYC*-specific shRNA or a non-targeting shRNA. MYC protein was significantly reduced with the *MYC* shRNA (Figure 56A). Upon knockdown of *MYC* expression, HDACi did not lead to an increase in levels of miR-15a, miR-195, or let-7a (Figure 56B). These data demonstrate that MYC is required to mediate the HDACi-induced increase in miR-15 and let-7 families. Furthermore, a modest increase in miR-15a, miR-195, and let-7a levels were observed when MYC was knocked-down (Figure 56B), providing additional evidence that MYC contributes to the repression of these miRNA in malignant cells.

BCL-2 and BCL-X_L expression is reduced by HDAC inhibition

Post-transcriptional mRNA regulation is mediated in part by miRNA (Ebert and Sharp, 2012). Given the significant increase in the levels of the miR-15 and let-7 families following HDACi, we questioned whether this would impact the expression of known targets of these two miRNA families. Specifically, we evaluated the expression of crucial pro-survival proteins BCL-2 and BCL-X_L, as their 3'-UTRs are well-characterized targets of the miR-15 and let-7 families, respectively (Figure 57A) (Di Leva *et al.*, 2014; Lima *et al.*, 2011). To test this, breast and lung cancer cell lines were administered Depsipeptide or

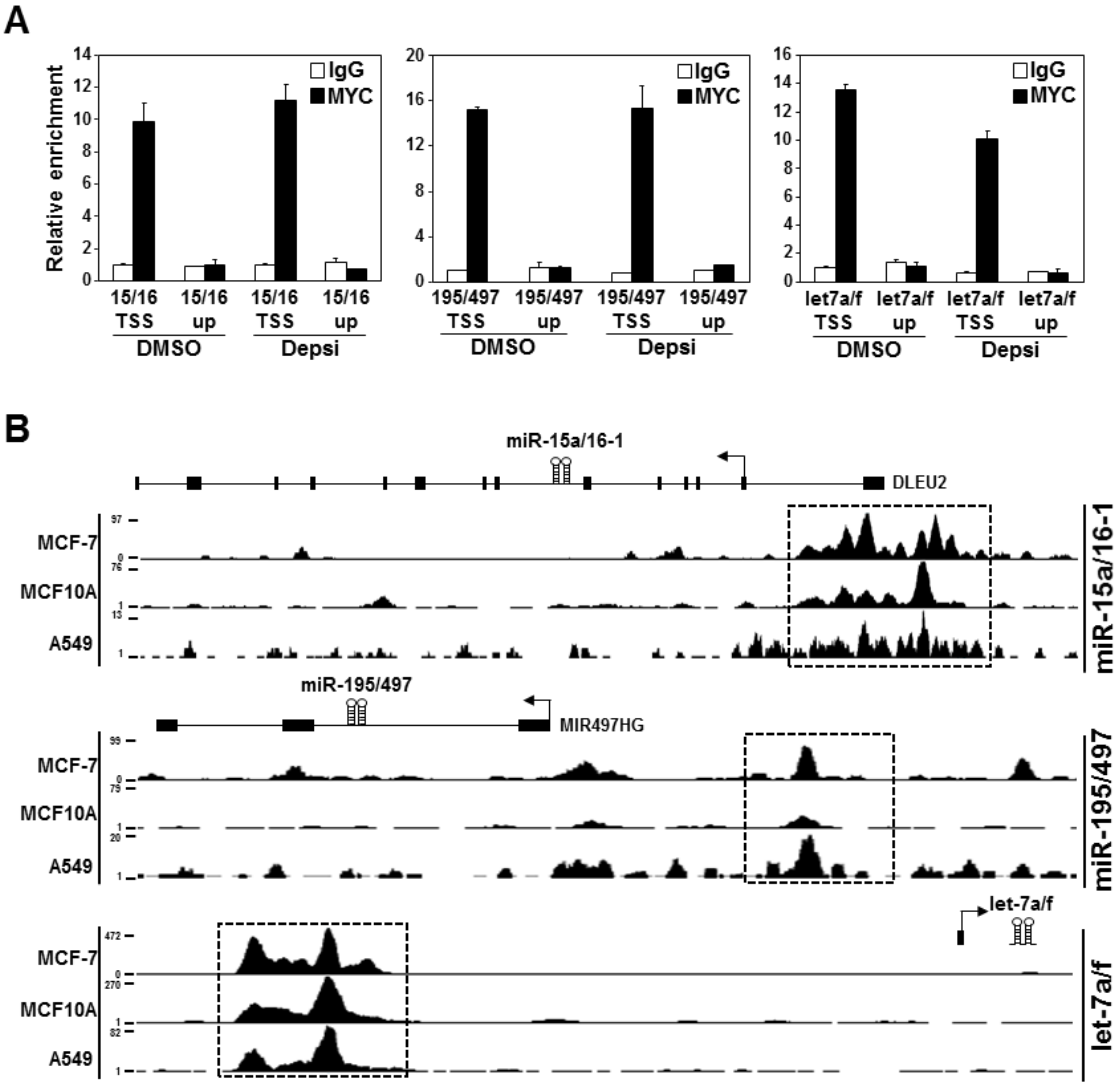

Figure 55. MYC localizes to the promoters of the miR-15 and let-7 families. (A) MDA-MB-231 breast carcinoma cells were treated for 4 hours with Depsipeptide (Depsi) or vehicle control (DMSO). Following ChIP with antibodies against MYC or isotype control (IgG), qRT-PCR for the indicated promoter regions (TSS) or the upstream (up) regions MYC does not bind (negative controls) was performed in triplicate. Values are relative to input DNA and their respective IgG controls and plotted relative to the first DMSO sample, which was set at 1. (B) Evaluation of ENCODE MYC ChIP-sequencing data.(Consortium, 2012) Peaks demonstrate MYC enrichment at the promoter of the miR-15a/16-1, miR-195/497, and let-7a/f clusters (boxed) in breast adenocarcinoma (MCF-7), immortalized mammary epithelial cells (MCF10A), and lung adenocarcinoma (A549) cells. Error bars are SEM.

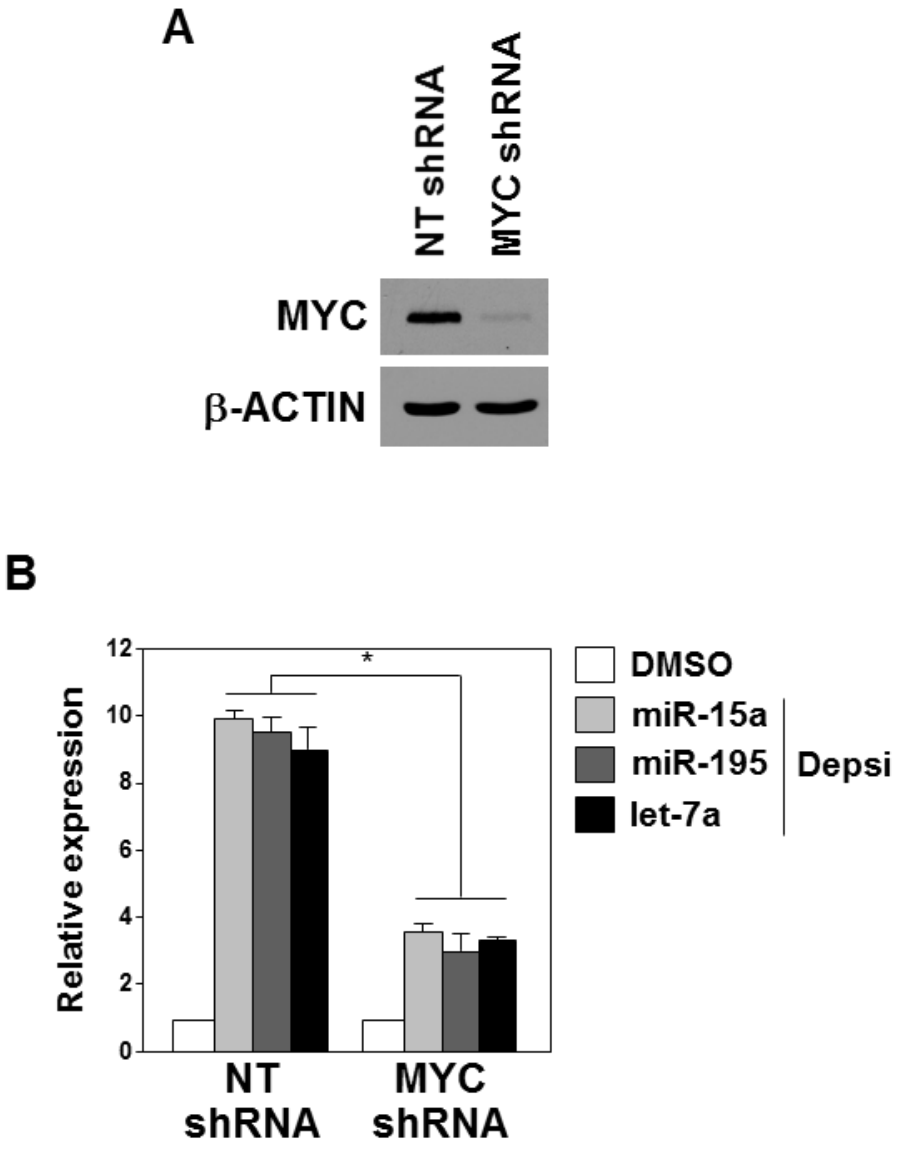

Figure 56. MYC is required to transcriptionally upregulate the miR-15 and let-7 families upon HDAC inhibition. MDA-MB-231 cells were transfected with a vector encoding either a *MYC*-specific shRNA or a non-targeting (NT) shRNA control. (A) MYC protein levels were evaluated by Western blot 48 hours after shRNA transfection. (B) Relative expression of the indicated miRNA was determined by qRT-PCR, in triplicate, following 12 hours of HDAC inhibition with Depsipeptide (Depsi) or vehicle control (DMSO). miRNA levels were normalized to the expression of small RNA *RNU6b*. Values for each miRNA are plotted relative to their respective DMSO sample, which was set at 1 (only one bar for the DMSO treated samples is displayed.) Error bars are SEM; *p<0.01 (t-test). Pia Arrate generated the data shown in A.

vehicle control (DMSO). Compared to cells exposed to vehicle control, HDACi resulted in significantly decreased BCL-2 and BCL-X_L protein levels in all four carcinoma lines (Figure 57B and 57C).

Because knockdown of *MYC* blunted the HDACi-induced increase of the miR-15 and let-7 families (Figure 56B), we tested the effects of *MYC* knockdown on their targets, BCL-2 and BCL-X_L, following HDACi. MDA-MB-231 breast cancer cells were transfected with a vector encoding a *MYC* shRNA or a non-targeting shRNA and then treated with Depsi. Levels of both BCL-2 and BCL-X_L proteins decreased within 12 hours following HDACi in the cells that had received the non-targeting shRNA (Figure 58). However, *MYC* knockdown greatly inhibited this decrease with only slightly reduced levels of BCL-2 and BCL-X_L observed after 24 hours of HDACi (Figure 58). Together the data indicate that HDACi results in reduced expression of the pro-survival proteins, BCL-2 and BCL-X_L, and that MYC is necessary to mediate the HDACi-induced decrease of BCL-2 and BCL-X_L.

HDACi induces apoptosis in breast and lung cancer cells

To test whether the decrease in expression of pro-survival proteins BCL-2 and BCL-X_L results in apoptosis, human breast and lung cancer cell lines were treated with Depsipeptide or vehicle control (DMSO) and several assays were performed. There was an obvious reduction in cell number following HDACi (Figure 59A). Moreover, in MTT assays, all four breast and lung carcinoma cell lines showed decreased cell expansion compared to cells administered vehicle control or that were left untreated (Figure 59B). Assessment of Annexin-V positivity at intervals following HDACi showed a significant

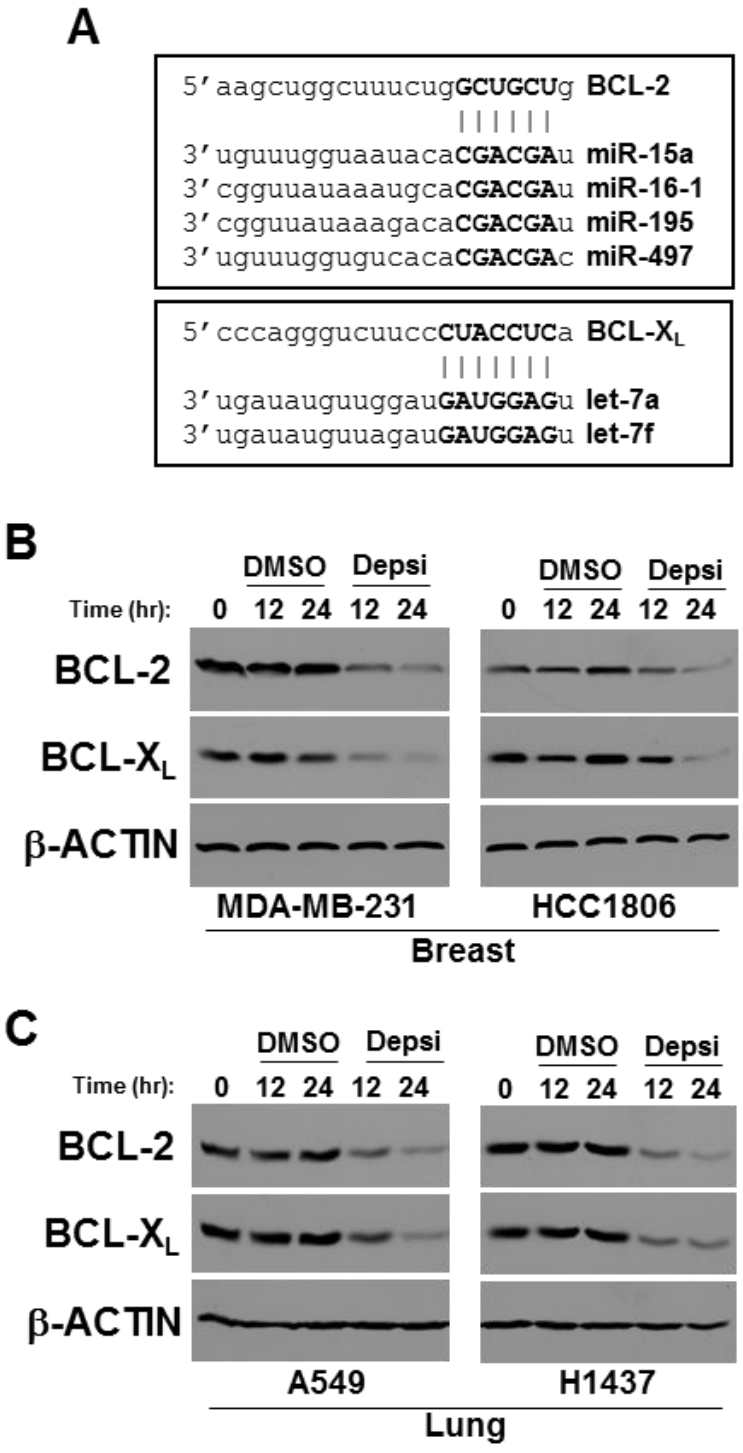

Figure 57. HDAC inhibition reduces BCL-2 and BCL-X_L expression. (A) Diagram of the miR-15 family and let-7 family binding site within the 3'-UTR of *BCL-2* and *BCL-X_L*, respectively. miRNA seed sequence is bolded. Western blots for the indicated proteins were performed with whole cell protein lysates of the indicated human breast (B) and lung (C) carcinoma cell lines following addition of vehicle control (DMSO) or Depsipeptide (Depsi) for the indicated time.

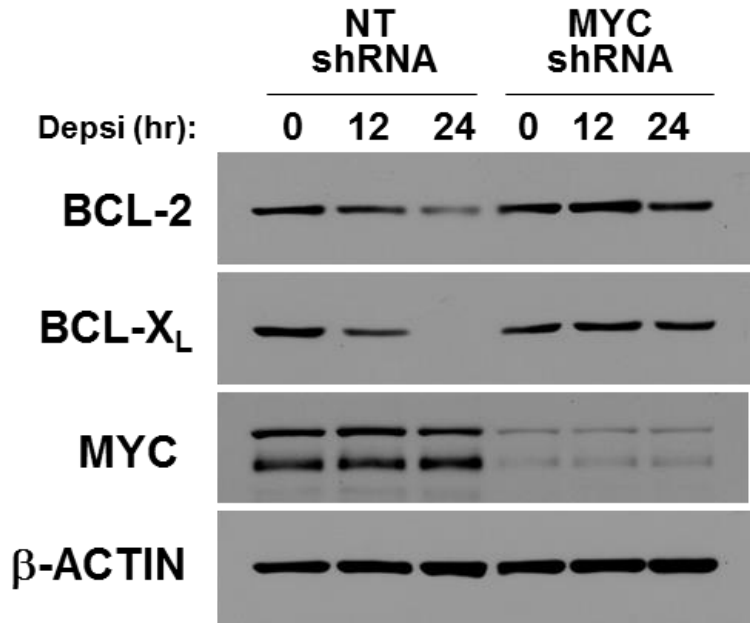

Figure 58. MYC mediates HDACi-induced decrease of BCL-2 and BCL-X_L protein expression. MDA-MB-231 cells were transfected with a vector encoding a *MYC*-specific shRNA or a non-targeting (NT) shRNA control. Forty-eight hours after transfection, cells were treated with Depsipeptide for the indicated time and Western blots for the indicated proteins were performed. I received technical assistance in running the Western.

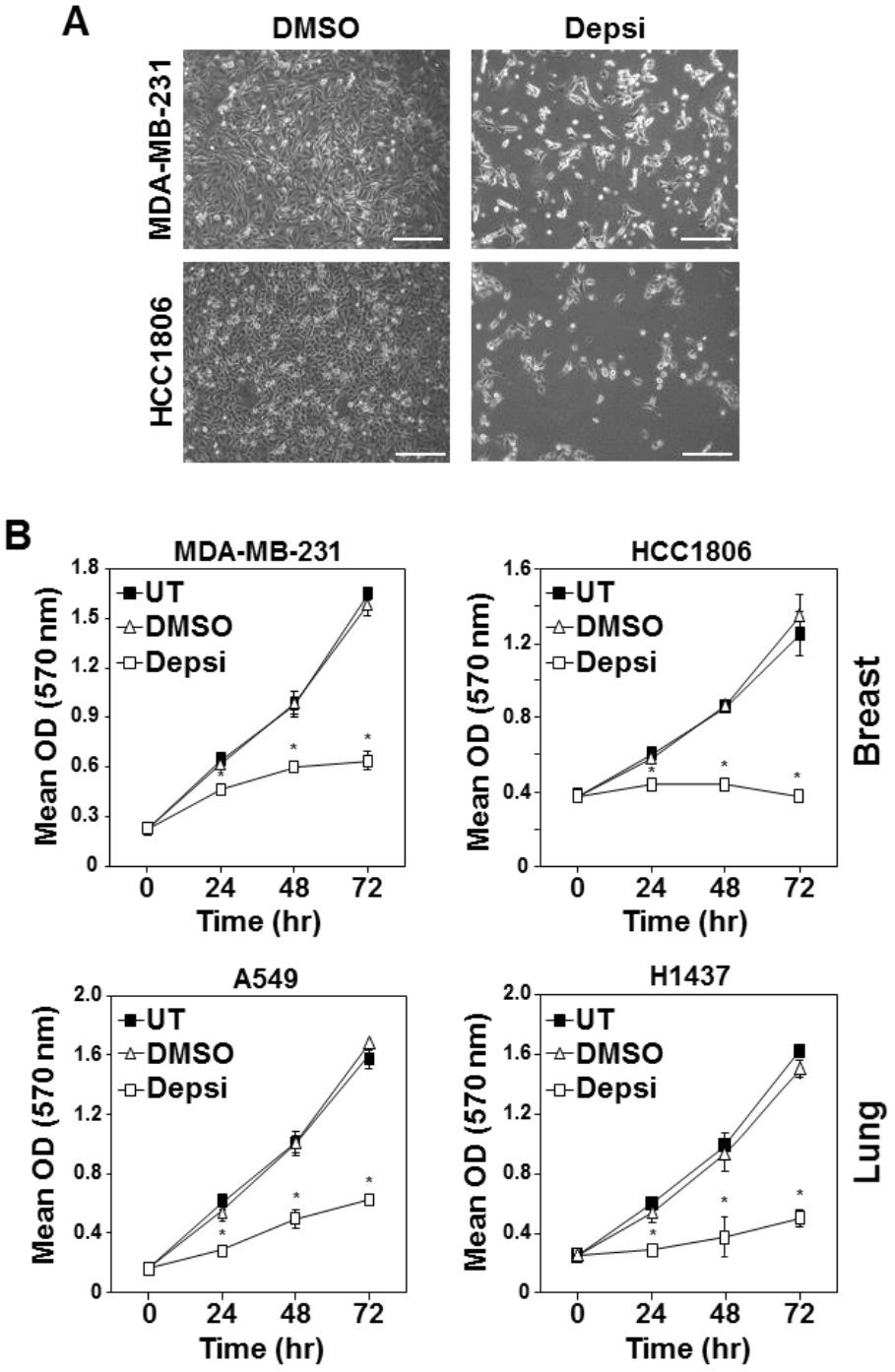

Figure 59. Cell growth is reduced by HDAC inhibition. (A) Following addition of DMSO or Depsipeptide (Depsi) to MDA-MB-231 and HCC1806 cells, representative microscopic images were taken 72 hours later (10X objective). (B) The indicated human breast and lung carcinoma cells remained untreated (UT) or were treated with vehicle control (DMSO) or Depsipeptide (Depsi). MTT assays were performed in quadruplicate every 24 hours. Error bars are SD; *p<0.01, determined by comparison to DMSO (t-test).

increase in the number of Annexin-V positive cells (Figure 60A). Furthermore, Western blotting revealed an increase in Caspase 3 cleavage after HDACi in all four carcinoma cell lines (Figure 60B and 60C). These results demonstrate that apoptosis occurs in breast and lung cancer cells with HDACi.

miR-15 and let-7 families mediate HDACi-induced apoptosis

Our data suggest that the decrease observed in BCL-2 and BCL-X_L protein following HDACi, resulting in apoptosis, was the consequence of transcriptional activation of the miR-15 and let-7 families. To directly test this, we transiently transfected MDA-MB-231 cells with modified RNA molecules termed Target Protectors that are designed to bind specific sites in the *BCL-2* or *BCL-X_L* 3'-UTR and block the binding of the miR-15 and let-7 families, respectively. In cells with the Target Protectors, the levels of BCL-2 and particularly BCL-X_L were maintained even after 24 and 48 hours of HDACi (Figure 61A). Additionally, we observed a rapid decrease in cell expansion in the MDA-MB-231 cells treated with Depsipeptide that did not receive any Target Protectors, whereas cells that had received the BCL-2 and BCL-X_L Target Protectors continued to proliferate with HDACi (Figure 61B). MDA-MB-231 cells with Target Protectors also showed a significant reduction (approximately 40%) in the percentage of Annexin-V positive apoptotic cells following HDACi (Figure 61C). In addition, there was less Caspase 3 cleavage following HDACi in cells with the Target Protectors (Figure 61A). Therefore, blocking the miR-15 and let-7 families from binding *BCL-2* or *BCL-X_L* in breast cancer cells prevented the downregulation of BCL-2 and BCL-X_L and blunted the apoptotic response resulting from HDACi. Collectively, the data reveal a novel miR-15 and let-7 family-mediated

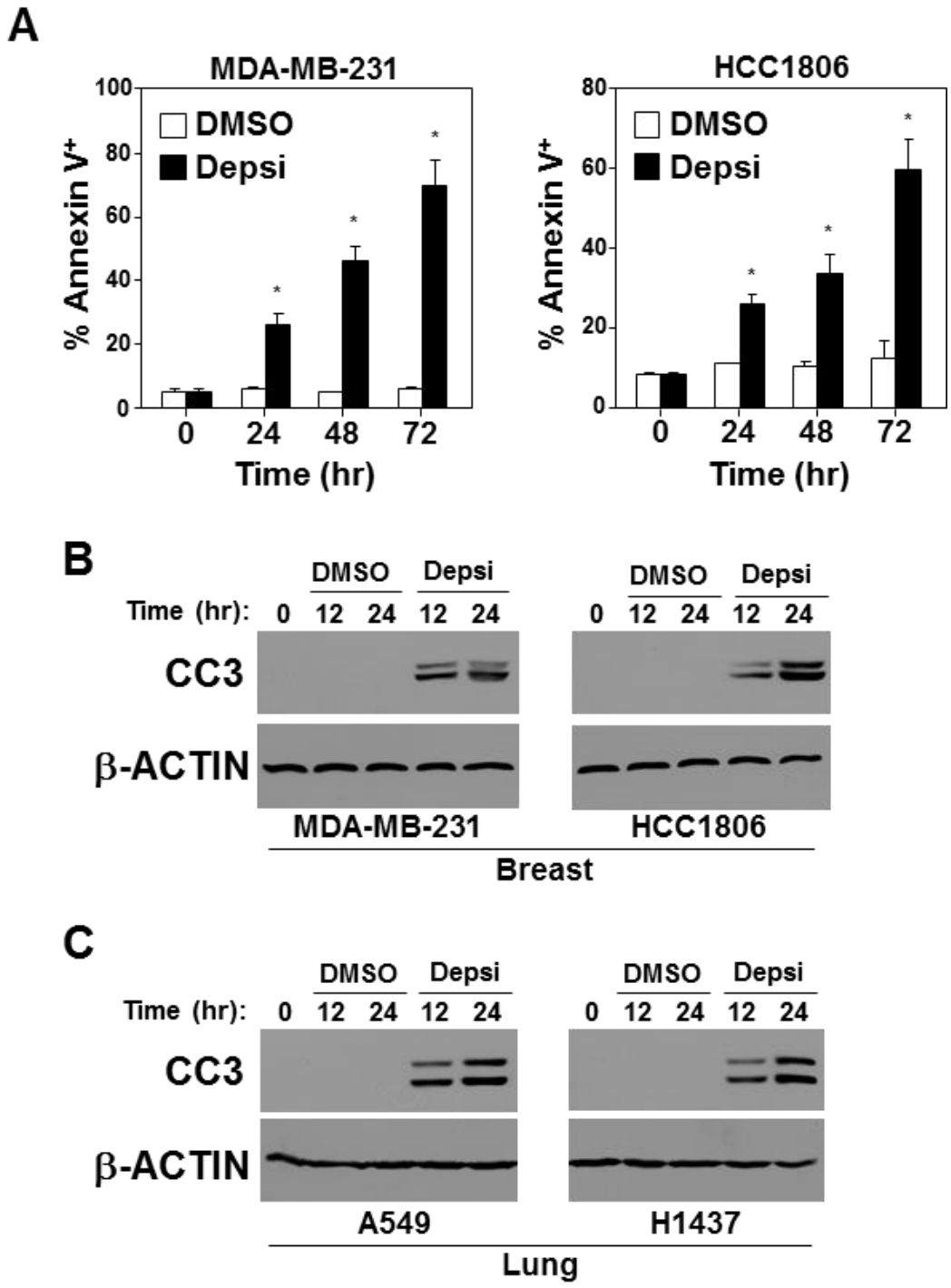

Figure 60. HDAC inhibition induces apoptosis of breast and lung cancer cells. The indicated human breast and lung carcinoma cells were treated with vehicle control (DMSO) or Depsipeptide (Depsi). (A) Annexin-V positivity (in triplicate) was measured at intervals by flow cytometry. (B, C) Cleaved Caspase 3 (CC3) was assessed by Western blot in human breast (B) and lung (C) carcinoma cell lines. Error bars are SD; *p<0.003 was determined by comparison to DMSO (t-test).

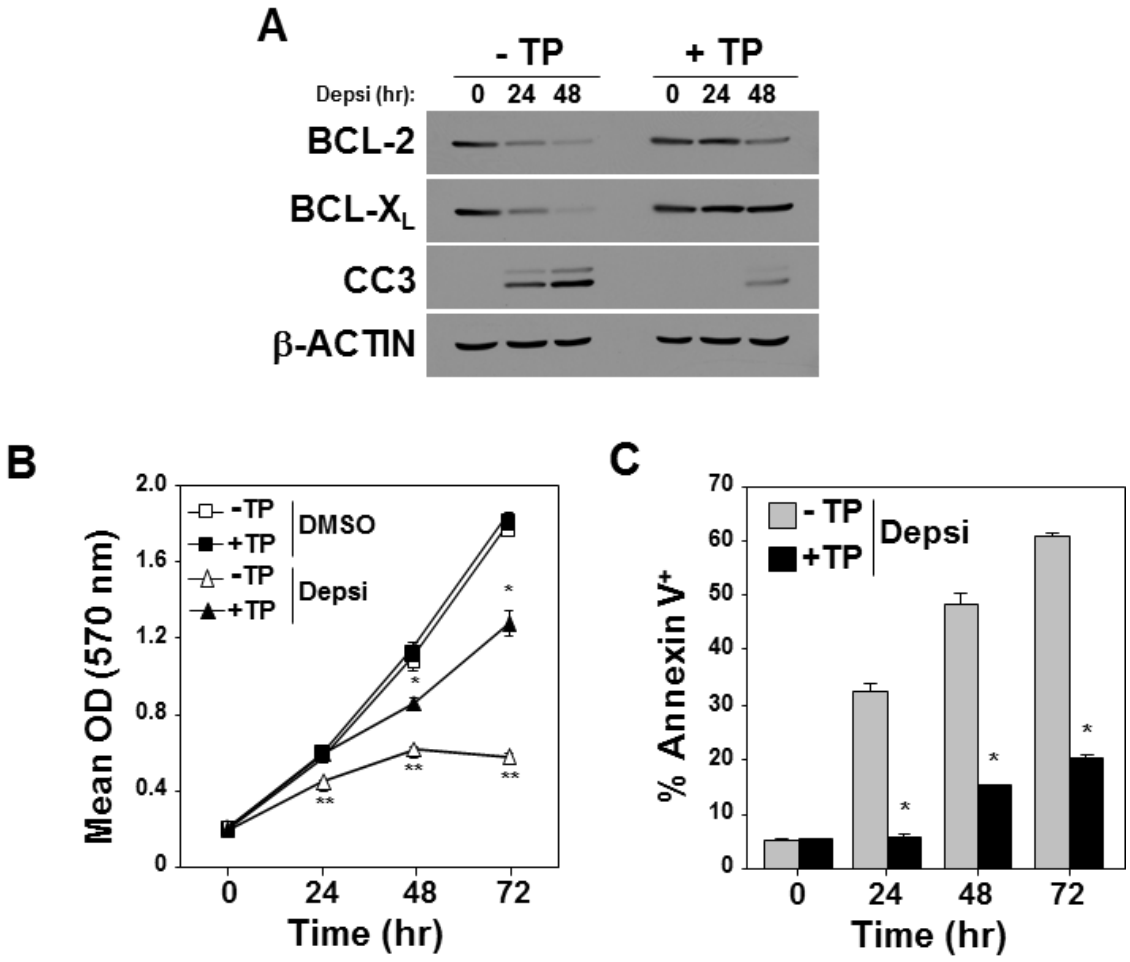

Figure 61. Blocking miR-15 and let-7 families from binding *BCL-2* and *BCL-X_L* protects from HDACi induced-apoptosis. MDA-MB-231 breast cancer cells were transiently transfected with Target Protectors that block the miR-15 family and let-7 family miRNA binding sites in the *BCL-2* and *BCL-X_L* 3'-UTR, respectively. (A) Following addition of Depsipeptide (Depsi) for the indicated intervals, total cell protein lysates from MDA-MB-231 with (+TP) or without (-TP) Target Protectors were Western blotted for the indicated proteins; cleaved Caspase 3, CC3. (B, C) Cells with (+TP) or without (-TP) the *BCL-2* and *BCL-X_L* Target Protectors were subjected to MTT assay (in quadruplicate; B) and Annexin-V analysis (in triplicate; C) by flow cytometry at intervals following the addition of Depsi or vehicle control (DMSO). Error bars are SD. For B, *p<0.01 and **p<0.001 were determined by comparison to DMSO; for C, *p<0.02 was determined by comparison to cells without Target Protectors (t-test). I received technical assistance in running the Western for A.

mechanism of apoptosis that underlies HDACi-induced breast and lung carcinoma cell death.

Discussion

In addition to acquiring genetic mutations, cancers can also arise from aberrant epigenetic alterations that modify chromatin accessibility and thus, gene expression (Dawson and Kouzarides, 2012). Development of small molecule inhibitors of HDACs, which function to compact DNA, making it inaccessible, had immediate anti-cancer application due to their potential to reactivate aberrantly silenced tumor suppressors (West and Johnstone, 2014). Although a number of HDAC inhibitors are being evaluated in preclinical cancer models and clinical trials, much remained unknown about the mechanism of cell death they induced and the molecular determinants of cancer cell sensitivity. In our current study, data demonstrate that inhibition of HDACs in human breast and lung carcinoma cells activates a miRNA-mediated mechanism of apoptosis that is induced by MYC. Our results show that, following HDAC inhibition, the MYC-regulated miR-15 and let-7 families are transcriptionally upregulated and they target and downregulate the expression of *BCL-2* or *BCL-X_L*, triggering tumor cell apoptosis.

Recently, it has been appreciated that, in addition to protein-coding genes, deregulated expression of non-coding RNA, including miRNA, is a hallmark of tumorigenesis (Hanahan and Weinberg, 2011). While miRNA expression profiling has revealed several miRNA to be upregulated in cancers relative to their normal tissue counterparts, the majority of miRNA are downregulated (Gaur *et al.*, 2007; Lu *et al.*, 2005).

These alterations in miRNA expression are beginning to be understood and some have proved to be advantageous in tumorigenesis. For example, miRNA families shown to possess tumor suppressive properties (Lima *et al.*, 2011) such as the miR-15 and let-7 families, are commonly downregulated in human cancers, including breast and lung carcinoma (Di Leva *et al.*, 2014; Gaur *et al.*, 2007; Lu *et al.*, 2005; Volinia *et al.*, 2006). Altered expression and/or recruitment of HDACs have been shown to contribute to dysregulation of specific miRNA (e.g., miR-29, miR-200b, miR-31, miR-125a, miR-125b, and miR-205) in human breast and lung cancers (Chen *et al.*, 2014; Cho *et al.*, 2015; Wang *et al.*, 2013; Zhang *et al.*, 2012b). Our data show that treatment with the pan-HDAC inhibitor Depsipeptide resulted in elevated levels of mature miRNA of the miR-15 and let-7 families. This increase in miRNA expression was selective as not all miRNA assessed were induced by HDACi. Evaluation of primary miRNA transcript levels for these miRNA indicated that the increase in mature miRNA following HDACi was a result of increased transcription of these miRNA. These data were supported by the observed enrichment of phosphorylated RNA polymerase II and the H3K9K14ac histone acetylation mark, which are both indicative of open and transcriptionally active chromatin. These results demonstrate that HDACs contribute to the downregulation of the miR-15 and let-7 families in breast and lung cancer, which can be reversed by HDACi.

Dysregulation of the oncogenic transcription factor MYC is known to dramatically alter the expression of genes to facilitate cellular proliferation and tumorigenesis (Dang, 2012). To elicit these effects, MYC coordinates the activation and repression of an extensive network of genes, including miRNA (Bui and Mendell, 2010). MYC can repress protein-coding genes and miRNA by recruiting HDACs (Kurland and Tansey, 2008; Zhang

et al., 2012b). Previously, MYC was reported to repress the miR-15 and let-7 families in B cell lymphoma, as it was enriched at the promoter regions of these miRNA (Chang *et al.*, 2008). Our results, in combination with genome-wide MYC ChIP-sequencing data (Consortium, 2012), indicate that MYC was also present at the promoter regions of the miR-15 and let-7 families in human breast and lung cancer cells. Notably, by knocking-down *MYC*, we determined that MYC was necessary for the upregulation of the miR-15 and let-7 families following HDACi. These data provide mechanistic evidence that MYC utilizes HDACs to transcriptionally silence the miR-15 and let-7 families in breast and lung cancer cells, and that HDACi converts MYC from a repressor to a transcriptional activator at these miRNA promoters.

While induction of tumor cell death has been the biological outcome most often reported following HDACi (West and Johnstone, 2014), and we observed this as well, the molecular determinants responsible for the apoptosis remained incompletely resolved. As such, we investigated known mRNA targets of the miR-15 and let-7 families that, when targeted by these miRNA, lead to cell death (Lima *et al.*, 2011). The decrease in BCL-2 and BCL-X_L protein we observed following HDACi indicated the miR-15 and let-7 families that were de-repressed and thus, transcriptionally upregulated, targeted *BCL-2* and *BCL-X_L*, respectively. Direct evidence that the cell death observed in the tumor cells following HDACi was due to the downregulation of *BCL-2* and *BCL-X_L* by the miR-15 and let-7 families, respectively, was obtained by blocking the binding sites of these miRNA families in the 3'-UTR of *BCL-2* and *BCL-X_L*. By inhibiting the miR-15 and let-7 families from binding the 3'-UTR of *BCL-2* and *BCL-X_L*, respectively, there was a significant decrease in cell death caused by HDACi. Furthermore, when *MYC* was knocked down,

HDACi had very little effect on BCL-2 and BCL-X_L protein expression. This suggested that MYC was required to mediate the HDACi-induced increase in the miR-15 and let-7 families that subsequently targeted *BCL-2* and *BCL-X_L*. These results reveal a novel Myc-regulated, miRNA-mediated mechanism of apoptosis that is inactivated in carcinomas, but that can be reactivated by HDACi.

We have demonstrated that inhibition of HDACs in human breast and lung carcinoma cells induces a previously unappreciated tumor suppressor mechanism that utilizes miRNA that decrease the expression of critical pro-survival proteins, triggering tumor cell death. Activation of this miRNA-mediated apoptotic mechanism was dependent on MYC, revealing that HDACi redirected MYC towards apoptosis, which is inactivated in transformed cells. Given that MYC is dysregulated in the majority of human cancers (Dang, 2012), our results reveal a new MYC-mediated mechanism to induce cell death that could have therapeutic potential in breast and lung cancers.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

MYC is undoubtedly one of the most extensively studied proteins to date. Close to 25,000 primary manuscripts and review articles have been written since its discovery as a *bona fide* oncogene just over 30 years ago. This motivation stems from the fact that MYC is responsible for an estimated 100,000 cancer-related deaths in the United States each year (Tansey, 2014). MYC is overexpressed in the majority of human malignancies, but is likely dysregulated in all cancers given the pathways regulating MYC are compromised during tumorigenesis. The oncogenic reputation of MYC emerges from its potential to transcriptionally regulate 15% of the genome, resulting in MYC-induced changes that impact nearly every cellular process. Although reducing Myc activity in cancer has proven beneficial (Felsher, 2010), we still don't fully understand how this occurs and what factors are involved.

During my thesis, I assessed two major forms of miRNA regulation that are frequently dysregulated in human malignancies, including transcriptional regulation and processing. Using a combination of *in vitro* strategies and genetic mouse models, I first investigated the cellular requirements of Dicer, an essential miRNA-processing enzyme, and the effects of reduced Dicer expression in different tissues, which had been unresolved. I determined that Dicer, and thus miRNA, are required for the survival and growth of B cells and B cell lymphomas, even when the p53 tumor suppressor is absent (Adams and Eischen, 2014). In this study, I also demonstrated that targeting Dicer may have therapeutic

potential for the treatment of B cell lymphomas. Furthermore, I identified a novel mechanism of Myc-induced apoptosis that is mediated by miRNA (Adams *et al.*, in revision, 2015; Adams and Eischen, in revision, 2015). In these studies, I determined that the transformation status of the cell dictated whether Myc transcriptionally activated or repressed the miR-15 and let-7 families. Previously, the miR-15 and let-7 families had been well documented to be repressed in human cancers (Bui and Mendell, 2010; Chang *et al.*, 2008; Gaur *et al.*, 2007; Lu *et al.*, 2005; Volinia *et al.*, 2006). However, no one had reported a comparison of the levels of these miRNA between cancer cells and normal cells with dysregulated Myc. I determined that Myc unexpectedly transcriptionally up-regulates the miR-15 and let-7 families in normal cells to target anti-apoptotic *Bcl-2* and *Bcl-x_L*, respectively, inducing apoptosis. This novel tumor suppressive mechanism is inactivated in multiple cancer types through transcriptional repression, but as I revealed, could be re-activated by inhibiting histone deacetylase enzymes.

As discussed below, I will highlight how data from my thesis considerably increases our understanding of Myc biology and how this new knowledge significantly moves the cancer field forward.

Tissue specific requirements for miRNA

A single miRNA can alter the expression of multiple mRNAs, resulting in the simultaneous regulation of numerous genes (Bartel, 2009). Consequently, it has been predicted that miRNA can modulate the expression of approximately 50% of all protein-coding genes (Bartel, 2009). As such, it is not surprising that miRNA dysregulation has been implicated in tumorigenesis. Although the expression of certain miRNA is elevated

in cancer, this only represents one aspect of a much larger miRNA network in cancer. Global downregulation of miRNA expression is reported to be the predominant phenotype observed across a variety of tumor types (Gaur *et al.*, 2007; Lu *et al.*, 2005; Volinia *et al.*, 2006). However, it was unclear whether the global reduction in miRNA expression was a cause or a consequence of tumorigenesis.

As mentioned in the Introduction, several studies have described possible mechanisms to account for the decrease in miRNA expression in cancer. One of these mechanisms includes reduced activity and/or expression of the miRNA-processing enzyme Dicer. This led to the hypothesis that reduced Dicer levels could facilitate tumorigenesis. In support of this concept, Kumar and colleagues reported that impaired miRNA processing via conditional *Dicer* deletion in a mouse model of lung adenocarcinoma enhanced transformation and tumorigenesis (Kumar *et al.*, 2007). Unfortunately, it was undetermined whether the tumors that emerged had indeed deleted both *Dicer* alleles. However, in a follow-up study, they demonstrated that one allele of *Dicer* was retained and functioned as a haploinsufficient tumor suppressor (Kumar *et al.*, 2009). *Dicer* was also reported to function as a haploinsufficient tumor suppressor in mouse models of soft tissue sarcoma, retinoblastoma, prostate cancer, and pancreatic ductal adenocarcinoma (Lambertz *et al.*, 2010; Ravi *et al.*, 2012; Wang *et al.*, 2014; Zhang *et al.*, 2014). In contrast, a previous investigation from Dr. Eischen's lab reported that loss of one allele of *Dicer* did not provide an advantage for B cell lymphomagenesis, and that *Dicer* deletion was strongly selected against (Arrate *et al.*, 2010). One factor that distinguished the other studies from those of Dr. Eischen's was that the haploinsufficient tumor suppressive phenotype of Dicer was observed when oncogenic Ras was overexpressed and a strong tumor suppressor (i.e., p53

or Rb) was deleted. Therefore, it was conceivable that additional genetic lesions might be sufficient to allow B cell lymphomas to survive without Dicer.

Previously, the Eischen lab detected an increased frequency of *p53* inactivation in CD19-*cre*$^{+}$/Eμ-*myc* B cell lymphomas arising in mice with two floxed *Dicer* alleles (Arrate *et al.*, 2010). This suggested that there was a connection between *p53* activation and *Dicer* deletion. In a separate study, Dr. Jones, in collaboration with Dr. Eischen, observed that deletion of *Dicer* in untransformed primary murine embryonic fibroblasts increased the levels of p53 and induced a premature senescent phenotype that could be partially rescued by deleting either the *Ink4a/Arf* or *p53* locus (Mudhasani *et al.*, 2008). Recently, Dr. Jones reported that loss of *p53* inhibited *Dicer* ablation-induced apoptosis of epidermal cells (Lyle *et al.*, 2014). Moreover, Ravi and colleagues demonstrated that a small percentage of a murine *p53*-null, mutant K-Ras-expressing sarcoma cell line and mesenchymal stem cells that had been SV40-immortalized, which inactivates p53 and Rb, could survive *Dicer* deletion (Ravi *et al.*, 2012). These studies suggested that p53 may have an essential role in mediating the effects of *Dicer* deletion. This led to the hypothesis that loss of *p53*, which often allows cells to live that should otherwise die or senesce, may be sufficient to protect B cells from the deleterious consequences of *Dicer* deletion.

Deletion of *Dicer* in B cell precursors resulted in a delay in Myc-driven B cell lymphomagenesis, and, in the lymphomas that emerged, at least one allele of *Dicer* was retained (Arrate *et al.*, 2010). I assisted in the generation of *p53*$^{+/-}$/*Dicer*$^{fl/fl}$/Eμ-*myc* mice and littermate controls that were also transgenic for the B lineage-restricted CD19-*cre* recombinase to assess the rate at which lymphomas developed and whether lymphomas could develop without *Dicer* in light of a p53 deficiency. Mice heterozygous for *p53* were

generated, as *p53*-null Eμ-*myc* mice are not viable and the lymphomas that develop lose their wild-type allele of *p53* through loss-of-heterozygosity, becoming functionally *p53*-null (Hsu *et al.*, 1995). As described in Chapter 2, and consistent with the Eischen lab's previous report, *Dicer* deletion resulted in delayed lymphoma development, which was not rescued by loss of *p53* (Adams and Eischen, 2014). This delay in lymphomagenesis is likely due to successful deletion of both *Dicer* alleles, which causes cells to undergo apoptosis, leaving behind a smaller pool of cells to undergo transformation. The cells that remain are ones that have retained an intact *Dicer* allele. I also evaluated lymphomagenesis in the context of *Dicer* heterozygosity to determine whether loss of *p53* would allow Dicer to function as a haploinsufficient tumor suppressor in B cells. For these experiments, I generated $p53^{+/-}/Dicer^{+/fl}$/Eμ-*myc* mice that were either CD19-cre^{+} or CD19-cre^{-}. Unlike the aforementioned studies demonstrating that Dicer functions as a haploinsufficient tumor suppressor, my results showed that a p53 deficiency did not allow *Dicer* heterozygosity to cooperate with Myc overexpression and accelerate lymphoma development (Adams and Eischen, 2014). Furthermore, Dicer was necessary for the survival of established B cell lymphomas, regardless of p53 status (Adams and Eischen, 2014; Arrate *et al.*, 2010). I also assessed lymphomas that had deleted *Arf*, a crucial component in the p53 tumor suppressor pathway, and determined that loss of *Arf* could not rescue B cell lymphoma-induced apoptosis following *Dicer* deletion (Adams and Eischen, 2014). Therefore, inactivation at various nodes in the p53 pathway was insufficient to allow B cell lymphoma survival.

Collectively, these results show that loss of the p53 tumor suppressor is insufficient to allow B cell lymphomas to live without *Dicer*, and therefore, a certain level of Dicer expression and thus, mature miRNA, must be required for Myc-induced B cell lymphoma

survival. These results are supported by previous reports showing certain Myc-regulated miRNA facilitate B cell lymphomagenesis (He *et al.*, 2005; O'Donnell *et al.*, 2005). In fact, the miR-17~92 polycistron have acquired an oncogenic reputation due to their ability to accelerate Myc-induced lymphomagenesis (He *et al.*, 2005). These Myc-regulated miRNA are frequently overexpressed in multiple human cancers, including B cell lymphoma (Bui and Mendell, 2010; Di Leva *et al.*, 2014; Lu *et al.*, 2005; O'Donnell *et al.*, 2005). It would be interesting to determine which specific miRNA are required for B cell transformation and whether this varies between cell types of different origin. Furthermore, there are a number of observations providing evidence that some level of miRNA expression may be necessary for tumor cell development, survival, and growth. For example, heterozygous somatic mutations in *DICER* have been reported in cancer genome copy number databases, but homozygous loss has yet to be documented (Kumar *et al.*, 2009). Heterozygous germline truncating mutations in *DICER* have been identified in families with the pleuropulmonary blastoma cancer syndrome (Hill *et al.*, 2009). In non-epithelial ovarian tumors, hypomorphic somatic missense mutations have been reported (Heravi-Moussavi *et al.*, 2012). In addition, in a mouse model of liver-specific *Dicer* deletion, the tumors that emerged all retained at least one intact *Dicer* allele (Sekine *et al.*, 2009). These studies suggest that complete loss of Dicer is selected against during tumorigenesis, which is not surprising, considering germline deletion of *Dicer* in mice fails to produce viable offspring (Bernstein *et al.*, 2003).

Previously, the Eischen lab and others have shown that biallelic deletion of *Dicer*, *in vivo*, in *p53* wild-type developing B cells induces apoptosis and causes a developmental defect that results in a decreased population of mature splenic B cells (Arrate *et al.*, 2010;

Koralov *et al.*, 2008). As described in Chapter 2, I performed a series of experiments to assess the requirement for p53 in the survival of untransformed B cells without *Dicer*. I determined that loss of p53 was unable to rescue the rapid induction of apoptosis induced by *Dicer* deletion in primary pre-B cells, and only pre-B cells that had functional Dicer could survive (Adams and Eischen, 2014). B cells were reported to be partially protected from *Dicer* deletion-induced apoptosis by overexpressing the anti-apoptotic Bcl-2 protein and/or deleting the pro-apoptotic gene *Bim*, or by expressing an immunoglobulin transgene to provide survival signals (Koralov *et al.*, 2008; Xu *et al.*, 2012). Unfortunately, neither report included an analysis of *Dicer* gene rearrangement, so we cannot conclude that *Dicer* had actually been completely deleted in the B cells that survived. In light of our studies, it is likely that the surviving B cells had retained one *Dicer* allele. Moreover, I determined that it is unlikely that Bcl-2 overexpression alone would be sufficient to protect untransformed B cells from *Dicer* deletion-induced apoptosis given that the B cell lymphomas that I utilized overexpressed Bcl-2 and underwent apoptosis immediately following *Dicer* deletion (Adams and Eischen, 2014). However, what distinguishes these studies is the transformation status of the cells. Perhaps untransformed, developing B cells can be partially protected from *Dicer* deletion when Bcl-2 is overexpressed, but established B cell lymphomas cannot, suggesting that transformed cells may be more dependent on Dicer. Additional studies would be necessary to test this hypothesis further.

When considering the current data in the field regarding whether cells can survive in the absence of *Dicer*, a common theme that emerges is the tissue-specific requirements for Dicer. It has been postulated that several different non-hematopoietic cell types, including primary and transformed cells, could survive without *Dicer*. However, we and

others have shown that hematopoietic cells are more sensitive to *Dicer* loss and undergo apoptosis. It is possible that inherent differences in the cell type, in addition to any pre-existing genetic alterations, could explain these differences. For instance, hematopoietic cells, such as B cells, are recognized for their fragility and propensity to undergo apoptosis upon encountering stress signals, unlike non-hematopoietic cells, such as fibroblasts, which are more prone to senesce in a similar situation. Therefore, upon *Dicer* deletion, it is possible that a non-hematopoietic cell could survive long enough to adapt to the stressful environment and acquire additional compensatory alterations that allow it to survive in the absence of miRNA. This concept is supported by the studies mentioned earlier that were performed in non-hematopoietic cells with loss or inactivation of *p53*. However, as my work in Chapter 2 describes, B cell and B cell lymphoma survival is incompatible with complete loss of *Dicer*, even when the strong tumor suppressor p53 is absent. Therefore, future studies are warranted to determine what alterations would allow B cells to survive without *Dicer*, if that is even possible.

Furthermore, although I demonstrated that targeting *Dicer*, *in vivo*, had potential therapeutic promise for the treatment of B cell lymphomas, this may not be a practical therapeutic approach in other cell types. For instance, we and others have shown that hematopoietic cells, such as B cells and B cell lymphomas, are highly sensitive to loss of *Dicer*. However, since *Dicer* is reported to be a haploinsufficient tumor suppressor in various non-hematopoietic cells, inhibiting *Dicer* may not be feasible due to the possibility of promoting tumorigenesis in certain cells. It remains to be determined in which cell types impaired miRNA processing can accelerate or promote tumorigenesis. Therefore, further

studies are necessary to determine the requirements of Dicer in additional tissues and whether targeting *Dicer* would be therapeutic or tumorigenic.

With that being said, there is recent evidence that adds further complexity to the concept of directly targeting *Dicer*. There is a growing body of work demonstrating that Dicer is not only essential for the processing of miRNA, but that it serves an important function in the processing of diverse double-stranded RNA molecules (Johanson *et al.*, 2013). For example, Dicer functions in the processing of endogenously-produced siRNA (Brameier *et al.*, 2011; Ender *et al.*, 2008; Langenberger *et al.*, 2010; Taft *et al.*, 2009) and viral dsRNA (Ding, 2010) in the same manner as precursor forms of miRNA. In addition, Dicer is important for the DNA damage-induced generation of non-miRNA small RNAs known as DNA-damage RNAs (DDRNAs) or double-stranded break-induced RNAs (diRNAs) (Francia *et al.*, 2012; Lee *et al.*, 2009a; Wei *et al.*, 2012). The presence of additional functions of Dicer, independent of miRNA, further demonstrate the importance of Dicer in RNA processing. The versatility of Dicer is becoming clearer, adding to its significant role in a broad range of biological processes. However, the understanding of miRNA-independent roles for Dicer, and other RNA processing machinery for that matter, is still limited; therefore, further studies are warranted to elucidate their additional functions.

Cellular transformation status dictates Myc transcriptional activity

Dysregulation of the oncogenic transcription factor Myc can induce global changes in gene expression to facilitate tumorigenesis (Tansey, 2014). To elicit these effects, Myc orchestrates the activation and repression of a large network of protein-coding genes and

non-coding RNA, such as miRNA (Bui and Mendell, 2010; Eilers and Eisenman, 2008). As a single miRNA has the potential to regulate many mRNA, it is understandable that miRNA are now appreciated as important downstream effectors of key signaling pathways (Bartel, 2009). Therefore, the regulation of miRNA by Myc can significantly alter the phenotype of the cell.

As discussed above and in Chapter 2, genetic loss of function of miRNA biogenesis is one cause of impaired miRNA expression in tumorigenesis. It is also likely that altered miRNA expression in cancer is a result of transcriptional regulation by the oncogenic transcription factor Myc. The first demonstration that miRNA contributed to the oncogenic potential of Myc came with the discovery that Myc transcriptionally activated the miR-17~92 polycistron (O'Donnell *et al.*, 2005). Following upregulation of this miRNA family by Myc, several targets were inhibited including *PTEN* and *CDKN1A*, which in turn, resulted in increased tumor-promoting properties. Despite transcriptional activation of the miR-17~92 polycistron, additional studies demonstrated that Myc hyperactivity resulted in the repression of many miRNA (Chang *et al.*, 2008). Among the list of those that were down-regulated included the miR-15 family and the let-7 family, both of which had documented tumor suppressive activity (Di Leva *et al.*, 2014). As revealed by primary transcript mapping and chromatin immunoprecipitation, Myc was demonstrated to associate directly with the promoter regions of the repressed miR-15 and let-7 families in human and mouse models of B cell lymphoma (Chang *et al.*, 2008). In Chapter 3, I also demonstrated that levels of members of the miR-15 and let-7 families were decreased in B cell lymphomas from Eμ-*myc* mice and human diffuse large B cell lymphoma and Burkitt's lymphoma cell lines as compared to control cells. I also performed chromatin

immunoprecipitation and determined that Myc was present at the promoter regions of the miR-15a/16-1 and miR-195/497 clusters and let-7a in mouse and human B cell lymphoma cells. These results validated what was described by Chang *et al.* and supported the concept that the miR-15 and let-7 families were indeed repression targets of Myc.

However, when further investigating the role of Myc-mediated transcriptional regulation of the miR-15 and let-7 families, I unexpectedly discovered that increased Myc activity did not always lead to their repression. In fact, in pre-cancerous Eμ-*myc* pre-B cells that overexpress Myc, but are not yet transformed, I observed an increase in mature miRNA transcripts of the miR-15 and let-7 families. To provide further evidence of this observation, I utilized an *in vitro* approach that allowed for induction of Myc activity using the MycER system. In non-hematopoietic cells and hematopoietic cells, I observed that MycER activation resulted in increased expression of the miR-15 family and let-7a. While these results suggested that Myc led to an increase in mature miRNA production, it was not known whether this was due to a direct impact on transcription of these miRNA or whether Myc was altering the maturation of these miRNA. Therefore, I assessed the impact of increased Myc activity on the primary transcripts (pri-miRNA) of these miRNA. As reported in Chapter 3, MycER activation increased the pri-miRNA transcripts of the miR-15 family and let-7a, indicating that transcription of these miRNA was being induced. To demonstrate that this was a direct effect of Myc transcriptional activation, I performed chromatin immunoprecipitation in pre-cancerous spleens from Eμ-*myc* mice (increased levels of Myc) and their non-transgenic littermates (wild-type levels of Myc) as well as cells expressing the inducible MycER system. In both situations, when Myc levels were increased, Myc associated with the promoter regions of the miR-15 family and let-7a.

Moreover, Myc was not enriched at sites upstream that did not contain the E-box Myc binding sequences, which provided evidence that the enrichment observed in Myc binding at the miRNA promoter regions was not simply an artifact of increased Myc expression.

Even though there was evidence that Myc bound the miRNA promoters in cancer cells and in normal cells with dysregulated Myc, which correlated with decreased and increased miRNA expression, respectively, I needed to directly assess whether the transcriptional status of the miRNA was changing. Using chromatin immunoprecipitation, I determined that increased miRNA expression correlated with an increase in marks of active transcription at the promoter regions of the miRNA. Specifically, an increase in a phosphorylated form of RNA polymerase II that is indicative of active transcription (phosphorylated at serine 2 on the C-terminus) and an increase in a histone mark characteristic of open and active chromatin were detected at the miRNA promoter regions in the untransformed cells with increased Myc activity, but were absent in the cancer cells that had decreased miRNA expression. Altogether, these exciting results demonstrated that in untransformed cells with dysregulated Myc, expression of the miR-15 and let-7 families was transcriptionally upregulated by Myc, but in cancer cells, the same miRNA were repressed by Myc. This suggested that the transformation status of the cell dictated whether Myc transcriptionally repressed or activated the miR-15 and let-7 families. Given that Myc can transcriptionally regulate non-coding RNA other than miRNA, including long non-coding RNA (Hart *et al.*, 2014), it seems plausible that Myc could differentially regulate their expression based on the transformation status of the cell, but future experiments are required to test this hypothesis.

Over the past 30 years, models describing the function of Myc as a transcriptional regulator have evolved immensely and continue to stimulate debate. At the heart of this debate are two opposing views of Myc transcriptional regulation: one, very recent view, suggests that Myc amplifies the transcription of all active genes (Lin *et al.*, 2012; Nie *et al.*, 2012), and the other view states that Myc differentially regulates the expression of genes (Eilers and Eisenman, 2008; Sabo *et al.*, 2014; Walz *et al.*, 2014). The first model states that Myc functions by associating with all active regulatory elements, and in doing so, amplifies the transcriptional program already active in the cell, leading to a global increase in signaling to drive tumorigenesis. However, this view suggests that Myc should no longer be viewed as a transcription factor with the potential to repress transcription of selected genes, which has been a fundamental mechanism of action of Myc. The work discussed above and described in more detail in Chapters 3 and 4 contributes important evidence to the longstanding concept that Myc has repressive as well as stimulatory roles in transcriptional regulation.

While the field has accumulated many mechanistic and molecular details about the role of Myc as a transcriptional regulator, some questions remain unanswered. For example, we still lack a clear picture of the precise molecular events that occur at the promoter that govern whether Myc will function as a transcriptional activator or a repressor. The current data in the field indicate that different cofactors are necessary for each transcriptional outcome, but how a switch between cofactors occurs is largely unresolved. It is possible that epigenetic modifications could serve as signals to recruit transcriptional complexes carrying select cofactors. In support of this concept, Bruno Amati's group has shown that the H3K4me3 histone modification may be a prerequisite

for Myc binding and that it is unlikely that Myc alone can initiate the change from closed to open chromatin (Guccione *et al.*, 2006). In addition, it also seems plausible that post-translational modifications to Myc could control the switch between cofactor recruitment and/or removal. For example, Myc has been shown to specify the recruitment of cofactors based on its phosphorylation status when interacting with the retinoic acid receptor α (RARα) (Uribesalgo *et al.*, 2012). Perhaps there is a certain unknown order, maybe cell type-specific or dependent on the gene(s) of interest that controls how the different cofactors are used by Myc. Does Myc employ subsets of cofactors to transcriptionally control specific gene sets and how are these "decisions" regulated?

Furthermore, the central portion of Myc, specifically Myc boxes IIIa, IIIb, and IV, is largely uncharacterized (Tansey, 2014). Since these regions are highly conserved, it seems likely that they have an important role in Myc function. Very recently, it was shown that recognition of target genes by Myc and its recruitment to chromatin largely depended on its interaction with WDR5 (Thomas *et al.*, 2015). WDR5 is a highly conserved WD40-repreat protein found in multiple chromatin regulatory complexes (Migliori *et al.*, 2012) and interacts with Myc through Myc Box IIIb (Thomas *et al.*, 2015). These results show that even after 30 years of studying Myc, significant observations are just now being exposed. Therefore, future efforts are warranted to define additional protein-protein, or even protein-DNA, interactions that occur within these regions of Myc, as these could provide mechanistic insight into what dictates whether Myc functions as a transcriptional activator or repressor. Uncovering the function of these ill-defined regions could also provide alternative routes to target Myc therapeutically. In support of this concept, mutations that disrupted the WDR5-Myc interaction reduced binding of Myc at ~80% of

its targets and decreased its ability to drive tumorigenesis (Thomas *et al.*, 2015). If additional cofactors of Myc are revealed, it will be essential to determine if the complexes function in different cancer types, as that will govern which cancers may benefit from small molecule inhibitors. And, if the novel Myc cofactors are involved in other complexes, a therapeutic window for such inhibitors would need to be established so that Myc activity can be sufficiently reduced without compromising the normal functions of the cofactor.

Aside from the fact that Myc possesses opposing transcriptional potential, we still don't know how Myc activity is different in cancer versus normal cells. As my data indicate, the transcriptional program of Myc can change and alter the expression of genes, including miRNA, to either facilitate or inhibit tumorigenesis. Another potential distinguishing feature between normal cells and cancer cells could be governed by the amount of Myc expression. A considerable amount of Myc molecules (~3000) are necessary to carry out basic biological processes. However, in tumor cells, the Myc levels can be dramatically higher (~30,000). Therefore, does increasing Myc expression (as observed in cancer cells) change Myc activity, or does an increase in Myc expression bring about new functions of Myc that are normally hidden?

Altogether, my data demonstrate that the transcriptional regulation of Myc on the miR-15 and let-7 families varies depending on the transformation status of the cell. Insight into the molecular events in which I determined could account for these observations are discussed below.

HDAC inhibition alters Myc-mediated transcription of miRNA

The most characterized mechanism of Myc-mediated transcriptional repression is an "anti-activation" model involving Miz-1. Myc interacts directly with Miz-1, a transcriptional activator, and displaces its obligate co-activator p300, which antagonizes Miz-1 transcriptional abilities. Therefore, I needed to rule in or out the possibility that interactions with Miz-1 were necessary for Myc-mediated transcriptional repression of the miR-15 and let-7 families. As such, I first evaluated publically available genome-wide chromatin immunoprecipitation data from the ENCODE project. Specifically, I assessed the binding frequency of Miz-1 at the promoter regions of the miR-15a/16-1, miR-195/497, and let-7a/f clusters in a wide variety of human and mouse, untransformed and transformed, and hematopoietic and non-hematopoietic cells. No enrichment was observed at the miRNA cluster promoter regions in any of the cells assessed, suggesting that Miz-1 was not transcriptionally repressing these miRNA.

Alternative mechanisms of Myc-mediated transcriptional repression include recruitment of HDACs to the promoters of protein-coding genes and, as more recently demonstrated, miRNA (Tansey, 2014). Utilizing HDACi, I determined that the repression of the miR-15 and let-7 families was mediated by HDACs. As described in Chapters 3 and 4, inhibition of HDACs in multiple types of human and mouse, hematopoietic and non-hematopoietic cancers resulted in increased miRNA expression. Two different approaches were used to determine whether Myc was necessary to observe the HDACi-induced increase in miRNA expression. In Chapter 3, I used a human B cell lymphoma cell line with a tetracycline-regulatable form MYC. In Chapter 4, I used an shRNA approach to target MYC in breast cancer cells. In both independent experiments, when MYC was

turned off or knocked-down, HDACi failed to increase the expression of the miR-15 and let-7 families. These data showed MYC was required to mediate the repression of the miRNA and their re-activation following HDACi. I determined that HDACi resulted in the transcriptional upregulation of these miRNA by assessing the appearance of *de novo* primary miRNA transcripts of the miR-15a/16-1 and miR-195/497 clusters and let-7a. In addition, following HDACi, chromatin immunoprecipitation revealed an enrichment of actively elongating RNA polymerase II (phosphorylated at serine 2) and the transcriptionally active H3K9K14ac mark at the miRNA promoters. The observed enrichment was larger than what was detected in normal control lymphocytes, indicating an increase in transcription.

In Chapter 3, I utilized multiple HDAC inhibitors that were either broad spectrum or HDAC-specific to gain insight into which HDACs altered the expression of the miR-15 and let-7 families. Through my analysis, I determined that the HDAC3-selective inhibitor induced the expression of these miRNA to a similar level as the class-1 (HDACs 1, 2, 3, and 8) inhibitor Depsipeptide and the inhibitor targeting HDACs 1, 2, and 3 did. This suggested that HDAC3 may be the primary HDAC involved in modulating the repression of the miR-15 and let-7 families. This result is consistent with work from Bill Tansey's lab demonstrating that Myc recruits HDAC3 to the promoters of protein-coding genes to repress their expression (Kurland and Tansey, 2008). In addition, the oncogenic fusion proteins PML-RARα, PLZF-RARα, or AML1-ETO observed in patients with t(8;21) AML have been shown to recruit HDAC3 to repress AML1 target genes (Gelmetti *et al.*, 1998; Grignani *et al.*, 1998; Hug and Lazar, 2004; Liu *et al.*, 2006). Furthermore, others have reported the specific recruitment of HDAC3 to the promoters of miR-29a/b/c in B

cell lymphoma (Zhang *et al.*, 2012b) and miR-15a/16-1 in mantle-cell lymphoma (Zhang *et al.*, 2012a) where it contributed to their repression. In addition, according to our results, it appeared as though HDACs 1 and 2 were also involved in mediating the repression of the miR-15 and let-7 families, as their inhibition also increased the transcript levels of these miRNA. These data suggest that Myc may interact with multiple HDACs to mediate the repression of the miR-15 and let-7 families. While Myc is required to mediate the vast majority of HDAC-induced repression of the miR-15 and let-7 families, there is some evidence to suggest alternative mechanisms, which could employ HDACs 1 and/or 2, may contribute. Although how the interactions between Myc and HDACs contribute to tumorigenesis are still unresolved, our data suggest that Myc, in association with HDACs, alter the epigenome in cancer cells, leading to repression of miRNA and other critical genes. However, I have shown that HDACi relieves the Myc-mediated transcriptional repression of the miR-15 and let-7 families in multiple types of human cancer, resulting in transcriptional upregulation of these miRNA.

Biological effects of HDAC inhibition

While HDAC inhibitors were initially identified based on their ability to induce differentiation of tumor cells (Leder and Leder, 1975; Riggs *et al.*, 1977), induction of tumor cell apoptosis is the biological outcome most often reported following HDACi (Bolden *et al.*, 2006). Upon HDACi with the class I HDAC inhibitor Depsipeptide (Depsi) and the HDAC3 selective inhibitor RGFP966 (966), I observed a rapid increase in apoptosis across multiple hematopoietic and non-hematopoietic cancers (described in Chapters 3 and 4). According to my analysis, the amount and rate at which apoptosis was

induced was similar between cells that received Depsi and 966, demonstrating that HDAC3 was the primary HDAC mediating the repression of the miR-15 and let-7 families. Consistent with our results, in a recent edition of *Blood*, Matthews and colleagues published that myeloid and lymphoid malignancies were more sensitive to depletion of *Hdac3* than any other class I HDAC (Matthews *et al.*, Oct. 7, 2015). Moreover, additional reports in multiple myeloma (Minami *et al.*, 2014), cutaneous T cell lymphoma (Wells *et al.*, 2013), and hematopoietic progenitor cells (Summers *et al.*, 2013) also demonstrated that inhibition of HDAC3 led to cell death. However, the recent report by Matthews *et al*. indicated that Eμ-*myc* and acute myeloid leukemia cells that were depleted of *Hdac3* were lost from culture due to decreased proliferation, not apoptosis (Matthews *et al*., Oct. 7, 2015). Likewise, in addition to apoptosis, other biological effects have been documented following inhibition of HDAC3, including DNA replication stress in cutaneous T cell lymphoma (Wells *et al*., 2013) and hematopoietic progenitor cells (Summers *et al.*, 2013). This is likely due to the role of HDAC3 in maintaining proper chromatin architecture and genome stability (Bhaskara *et al.*, 2010; Conti *et al.*, 2010; Stengel and Hiebert, 2015). Furthermore, HDAC3 is required during the development of T cells (Stengel *et al.*, 2015) and also functions in regulating crucial metabolic processes (Knutson *et al.*, 2008). The importance of HDAC3 is highlighted by the fact that conditional deletion of *Hdac3* in murine embryonic fibroblasts is incompatible with cell viability (Bhaskara *et al.*, 2008). Altogether, it is unlikely that a single mechanism of action is responsible for the anti-cancer activity observed following HDACi across multiple tumor types.

The biological effects and therapeutic outcome in response to HDAC inhibition is likely due to several factors, including cell type, pre-existing genetic and/or epigenetic

lesions, and the specific HDAC(s) being inhibited. Differences in the biological responses observed following HDACi could also be due, in part, to the potency/concentration of HDACi. For example, in the recent report from Matthews and colleagues, Eμ-*myc* lymphoma and acute promyelocytic leukemia (APL) cells were treated with low micromolar concentrations (≤ 1μM) of an HDAC3-specific inhibitor (Matthews *et al*., Oct. 7, 2015). According to their analysis described in the manuscript, apoptosis was not observed. However, in the published proceedings of the 2014 American Association for Cancer Research Meeting, they report that the same cells do indeed undergo apoptosis when treated with the same HDAC3-selective inhibitor at or above 2 μM (Matthews *et al*., Abstract In: Proceedings of the 105th Annual Meeting of the AACR, 2014). Similarly, our data (described in Chapter 3) and that of others who used 2-10 μM doses of the same compound also observed increased apoptosis (Wells *et al.*, 2013) in multiple tumor types. These data suggest that even when using the same HDACi, varying biological outcomes can be observed depending on the dosage.

The most common HDAC inhibitors target multiple HDACs simultaneously and have shown demonstrable anti-cancer activity in the clinic. For instance, the HDAC inhibitor vorinostat is approved by the FDA for the treatment of cutaneous T cell lymphoma (CTCL), romidepsin is approved for the treatment of CTCL and peripheral T cell lymphoma (PTCL), panobinostat is approved for treatment of multiple myeloma (MM), and belinostat has recently been approved for the treatment of PTCL (Falkenberg and Johnstone, 2014; West and Johnstone, 2014). While targeting multiple HDACs has therapeutic potential, genetic and pharmacological approaches are being performed to investigate whether suppression of individual HDACs or certain combinations thereof can

phenocopy broad-acting HDACi. When targeting multiple HDACs, it is difficult to decipher if the biological effects and therapeutic toxicities are the result of inhibiting a specific HDAC, the combination of inhibiting multiple HDACs, or inhibiting a larger multi-protein complex that contains one or more HDACs. For example, the catalytic domain of HDAC4 interacts with HDAC3 within the larger NCoR-SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) complex (Fischle *et al.*, 2002). Therefore, the dependence of one HDAC on another in a larger multi-protein complex suggests that small-molecule inhibitors designed to selectively target a specific HDAC may actually have broader effects than anticipated. In support of this concept, when the inhibitory activity of different HDAC inhibitors was assessed by comparing isolated HDAC enzymes to multi-protein repressor complexes containing HDACs, different effects of the HDAC inhibitors evaluated were observed depending on the physical state of the HDAC (Bantscheff *et al.*, 2011). In this same report, Bantscheff and colleagues also reported that, depending on the physical state of the HDAC, the affinities of the HDACi varied. This raises questions regarding the true specificity and selectivity of HDAC inhibitors and their biological effects when studying recombinant, purified enzymes compared to studying HDACs as part of a larger multi-protein complex, which is how HDACs are found under physiological conditions.

Currently, the success of HDAC inhibitors as single therapeutic agents for the treatment of most tumors has been marginal. Therefore, the generation of novel drug designs to enhance cell-type specific administration of HDACi is warranted. For example, a hybrid molecule was designed such that an HDACi carried an esterase-sensitive chemical motif so that when the ester molecule was hydrolyzed by monocytes and macrophages, the

active HDACi drug was trapped inside the cell (Needham *et al.*, 2011). Also, in order to target specific cancer types, it seems logical to develop hybrid molecules to inhibit specific HDACs and an additional target of a dysregulated pathway specific to that cancer. Furthermore, additional studies are necessary to determine the key HDAC-containing complexes utilized by certain cancer types, what the specific functions of the HDACs are within those complexes, and what the anticipated molecular/biological outcomes of targeting those complexes are. Another factor providing impetus to design more efficient therapies is the possibility to decrease HDACi-related side effects. For example, increasing the specificity of the inhibitor for a particular HDAC isoform could reduce toxicity, which has been an issue with the use of pan-HDAC inhibitors.

miRNA-mediated mechanism of Myc-induced apoptosis

Myc is among the most potent transforming proteins in cancer; yet, in certain situations, it is capable of inducing massive amounts of apoptosis. For example, in untransformed cells, activation of apoptosis in response to dysregulated Myc activity can occur by suppressing anti-apoptotic Bcl-2 and Bcl-x_L (Eischen *et al.*, 2001a; Eischen *et al.*, 2001c). In these reports, it was determined that Myc suppressed their expression through an unknown, but indirect mechanism. Previously, it was reported that the Miz-1 transcription factor normally activates the transcription of *Bcl-2*, which is disrupted when Myc interacts with Miz-1 and inhibits its transactivation function (Patel and McMahon, 2007). Myc interacts with Miz-1 through an amino acid (valine 394) that is located within the helix-loop-helix domain of Myc. Using a well-characterized point mutation in this amino acid (V394D), I showed that even when the Myc-Miz-1 interaction was selectively

blocked, Bcl-2 expression still decreased upon increased Myc activity. This was an essential experiment that was not performed in the aforementioned publication by Patel and colleagues. This suggested that an alternative, Miz-1-independent mechanism was indirectly regulating the expression of Bcl-2 and likely Bcl-x_L.

It had been demonstrated that miR-15a and miR-16-1 expression was inversely correlated to Bcl-2 expression in chronic lymphoid leukemia cells (Cimmino *et al.*, 2005). In this report, it was determined that miR-15a and miR-16-1 were capable of inducing apoptosis by targeting and negatively regulating the expression of *Bcl-2*. As described in Chapter 3, I revealed that Myc differentially regulated the expression of these miRNA depending on the transformation status of the cell. This led to the hypothesis that Myc was modulating the expression of these miRNA as a means to suppress Bcl-2. I demonstrated that all four members of the miR-15 family (miR-15a, miR-16-1, miR-195, and miR-497) targeted *Bcl-2*, which was supported by other studies (Yin *et al.*, 2010; Zhu *et al.*, 2011). We also extended our studies to include *Bcl-x_L*, a published target of the let-7 family of miRNA (Shimizu *et al.*, 2010), since it, too, was indirectly regulated by Myc (Eischen *et al.*, 2001c).

Preliminary evidence that supported our hypothesis stemmed from the fact that expression of the miR-15 and let-7 families were inversely correlated with the expression of their targets in the pre-malignant state and in cancer cells. We reasoned that in the untransformed cells experiencing increased Myc activity, the increase in miR-15 and let-7 expression would lead to a decrease in their targets. Through several *in vitro* and *in vivo* approaches, I determined that this was indeed the case. In addition, using the Eμ-*myc* mouse model of Myc-driven B cell lymphoma, Bcl-2 and Bcl-x_L were frequently

overexpressed in the lymphomas that developed (Eischen *et al.*, 2001c). Therefore, if the miR-15 and let-7 families were regulating the expression of Bcl-2 and Bcl-x_L, then expression of the miRNA would need to be reduced in the cancer setting to accommodate the observed increase in Bcl-2 and Bcl-x_L, which is what I observed.

I performed several experiments to directly test whether Myc induced the expression of the miR-15 and let-7 families as a means of regulating the expression of Bcl-2 and Bcl-x_L, respectively. An innovative approach that I utilized to make a connection between Myc, the miRNA, and Bcl-2 and Bcl-x_L was the use of site-specific small molecules called Target Protectors. These molecules were specifically designed to bind the exact location in the 3'-UTR of *Bcl-2* or *Bcl-x_L* where the miR-15 family and let-7 family bind, respectively. The rationale for using these molecules was that no matter how significant the induction in miRNA expression was following either Myc activation or HDACi, the binding sites of *Bcl-2* and *Bcl-x_L* were protected from only these miRNA. Also, these molecules do not interfere with the miRNA themselves, and therefore, their use did not affect the binding of the miRNA to their other biological targets. In addition, I also used these reagents in luciferase assays designed to assess luciferase activity as an indirect measure of translation. In combination with the 4-hydroxytamoxifen inducible MycER systems (using wild-type and mutant forms of Myc), these small molecules provided strong evidence that Myc directly upregulated the miR-15 and let-7 families that then targeted *Bcl-2* and *Bcl-x_L*, respectively. Furthermore, using a similar experimental approach, I showed that when the miR-15 and let-7 family binding sites were blocked, levels of Bcl-2 and Bcl-x_L protein were significantly resistant to Myc-mediated downregulation, and that Myc-induced apoptosis was attenuated.

Importantly, I generated data showing the miR-15 and let-7 families directly targeted *Bcl-2* and *Bcl-x_L*, respectively, following HDACi. When the 3'-UTR of *Bcl-2* and *Bcl-x_L* was protected from binding by the miR-15 and let-7 families, the HDACi-decrease in Bcl-2 and Bcl-x_L protein expression was almost fully blocked. In addition, as evidenced by cleaved Caspase 3 protein expression, HDACi-induced apoptosis was severely dampened, which was likely a result of minimal changes in Bcl-2 and Bcl-x_L protein expression. These results contribute important knowledge to the field regarding the mechanism of action following HDACi. Although HDAC inhibitors have shown therapeutic utility, the molecular events responsible for HDACi-mediated tumor cell death remain unresolved. Mechanistic insight into how HDACi can specifically induce apoptosis in tumor cells and why normal cells are far less sensitive could be attributed to the differential expression of miRNA observed between cancer and normal cells.

How or why HDACi leads to tumor-cell selective effects is a major unanswered question in the field. Another possible explanation that has been reported could be due to the fact that the epigenetic regulation in normal cells appears to be redundant and that, following an alteration in the epigenetic program, normal cells engage alternative pathways to compensate for the epigenetic insult, but cancer cells, which are more reliant on certain epigenetic regulators, are unable to adapt (Dawson and Kouzarides, 2012). An alternative explanation suggests that, during the epigenetic reprogramming that occurs during transformation, different epigenetic programs are established in cancer cells versus normal cells that can account for the different biological outcomes following HDACi. For example, a study comparing matched tumor and normal cells demonstrated that a pro-apoptotic transcriptional signature was induced in response to HDACi specifically in the

tumor cells (Bolden *et al.*, 2013). Induction of death receptors has also been reported to yield a tumor-cell selective response to HDACi (Insinga *et al.*, 2005; Nebbioso *et al.*, 2005). Furthermore, HDACi led to the accumulation of reactive oxygen species and caspase activation in cancer cells, but not normal cells in addition to increasing an important reducing enzyme in normal cells, but not cancer cells (Ungerstedt *et al.*, 2005).

Through several strategies, I demonstrated that Myc capitalizes on the miR-15 and let-7 families of miRNA as a novel mechanism to induce apoptosis in normal cells. Furthermore, this tumor suppressive mechanism is inactivated in tumorigenesis through an HDAC-mediated mechanism that requires Myc, but can be re-activated by HDACi. Similar results were obtained in hematopoietic and non-hematopoietic cells, suggesting that this is likely a general mechanism in multiple cell types.

Novel regulator of Myc-induced apoptosis and lymphomagenesis

While investigating the role of specific Myc-regulated miRNA in lymphomagenesis, I determined that the miR-15 family was also predicted to target the anti-apoptotic Bcl-2 family member Bcl-w, which has the highest homology with Bcl-2 in addition to Bcl-x_L (Petros *et al.*, 2004). Preliminary results from Eμ-*myc* lymphoma analysis and publically available mRNA expression analysis of human B cell lymphomas indicated that Bcl-w was frequently overexpressed in mouse and human lymphomas (Figure 62). The frequency of Bcl-w overexpression in the lymphomas that developed from Eμ-*myc* mice was similar to that of Bcl-2 and Bcl-x_L, which are known to be regulated by Myc. Furthermore, when I assessed expression of Bcl-w in pre-cancerous Eμ-*myc* spleens compared to their wild-type, non-transgenic littermates, Bcl-w expression was reduced in

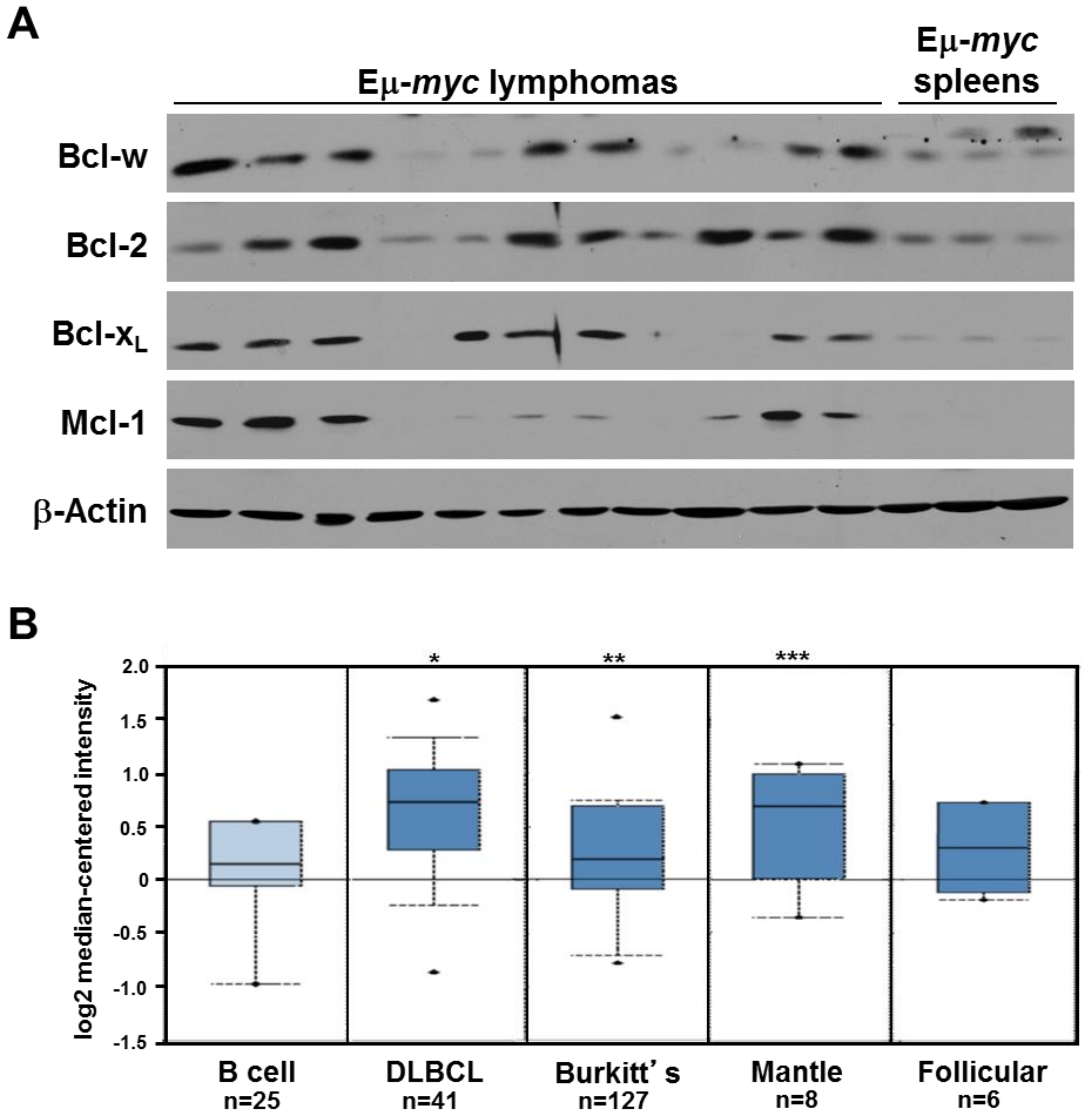

Figure 62. Bcl-w is overexpressed in mouse and human lymphomas. (A) Whole cell protein lysates of Eμ-*myc* lymphomas were Western blotted for the indicated proteins. Pre-cancerous Eμ-*myc* spleens were used as controls. (B) Publically available data (Basso *et al.*, 2005) from the Oncomine database demonstrate *BCL-W* mRNA is overexpressed in human B cell lymphomas compared to normal B cell controls. Diffuse large B cell lymphoma, DLBCL. *p=0.00000942, **p=0.05, ***p=0.027.

the transgenic spleens (Figure 63). These results were also analogous to what I observed for Bcl-2 and Bcl-x_L. This sparked the idea that perhaps Myc was regulating the expression of Bcl-w.

The lab of Suzanne Cory, an expert in Bcl-2 family proteins, discovered Bcl-w using a PCR-based cloning strategy (Gibson *et al.*, 1996). In this report, they showed that enforced expression of Bcl-w resulted in apoptotic resistance of lymphoid and myeloid cells to several cytotoxic conditions. It was concluded that Bcl-w, much like Bcl-2 and Bcl-x_L, promoted survival. They also concluded that Bcl-w protein was expressed at low levels in B and T lymphoid cell lines, but that *Bcl-w* mRNA was present in a wide variety of murine hematopoietic cell lines and tissues. A later report resulting from the collaboration between the Cory and Strasser labs surveyed a number of transformed cell lines and purified hematopoietic cells and demonstrated that Bcl-w is expressed in cells of myeloid, lymphoid, and epithelial origin (O'Reilly *et al.*, 2001). However, using gene-targeting experiments in mice, they concluded that Bcl-w was only required for spermatogenesis and did not have a significant function in other tissues. Based on these limited initial reports, it appeared as if motivation to further study the role of Bcl-w in apoptosis lost momentum. Therefore, as compared to Bcl-2 and Bcl-x_L, Bcl-w has been severely understudied.

Bcl-2 and Bcl-x_L have been implicated in Myc-driven apoptosis and lymphomagenesis (Eischen *et al.*, 2001a; Eischen *et al.*, 2001c; Kelly *et al.*, 2011; Strasser *et al.*, 1990; Vaux *et al.*, 1988). As such, we sought to determine whether loss of *Bcl-w* would affect the rate of Myc-driven lymphomagenesis. To do this, I first generated Eμ-*myc* transgenic mice expressing one, two, or no alleles of *Bcl-w* and compared the rates of lymphoma development (Figure 64). A remarkable difference in survival was observed in

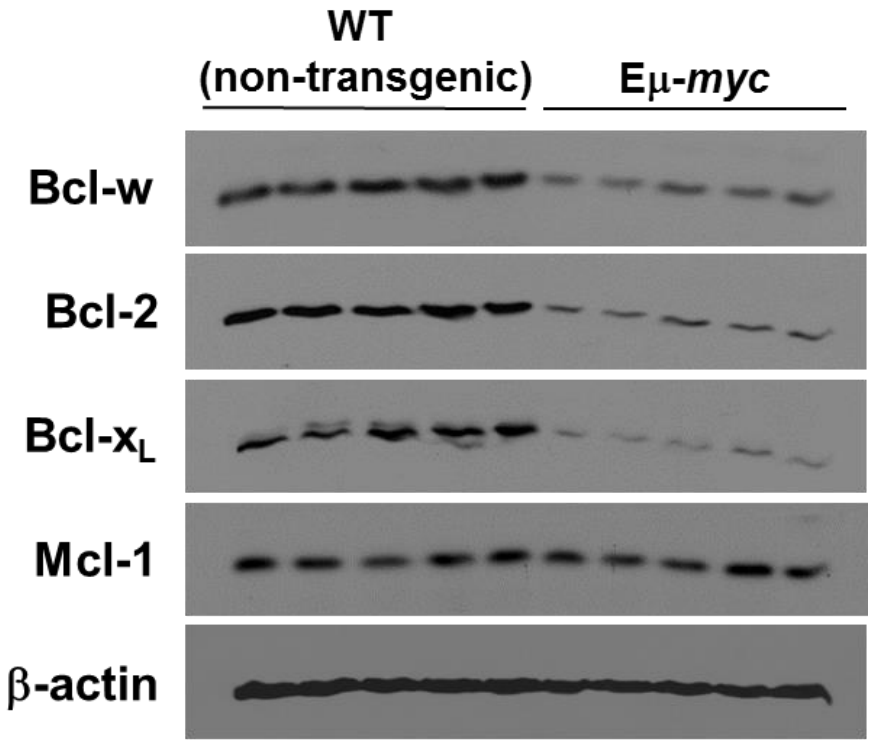

Figure 63. Bcl-w is suppressed in pre-cancerous Eμ-*myc* spleens. Whole cell protein lysates from pre-cancerous Eμ-*myc* spleens and non-transgenic wild-type (WT) littermate-matched control spleens were Western blotted for the indicated proteins.

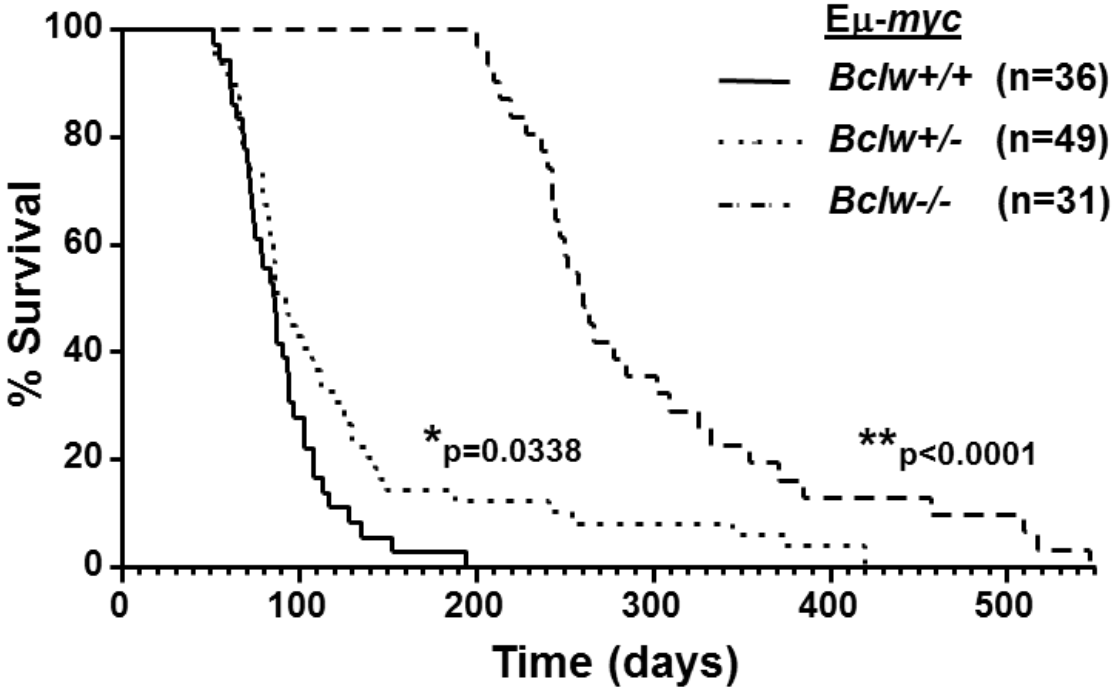

Figure 64. Loss of *Bcl-w* significantly delays Myc-driven lymphomagenesis. Kaplan-Meier survival curves of Eμ-*myc* mice with two, one, or no *Bcl-w* alleles. The number (n) of mice is indicated. Log-rank tests were performed to calculate statistical significance, which is denoted beside each survival curve.

the mice that were null for *Bcl-w* compared to their wild-type littermate-matched controls. In addition, a statistically significant delay in lymphoma development was also observed in mice that were heterozygous for *Bcl*-w. Specifically, the average survival of the Eμ-*myc* mice with two, one, or no *Bcl-w* alleles was 90, 121, and 299 days, respectively. Therefore, loss of *Bcl-w* significantly delayed Myc-driven lymphomagenesis.

A possible explanation for the delay in lymphoma development could be due to a defect in B cell development in mice deficient in *Bcl-w*. To test this possibility, immunophenotyping of the spleen and bone marrow compartments from pre-cancerous Eμ-*myc* transgenic mice expressing one, two, or no alleles of *Bcl-w* was performed and showed no difference (data not shown). Bcl-2 and Bcl-x_L are differentially expressed within the hematopoietic compartment. For example, loss of *Bcl-x_L* in mice results in high levels of apoptosis in embryonic hematopoietic cells (Motoyama *et al.*, 1995), whereas loss of *Bcl-2* in mice results in apoptosis of mature lymphocytes (Nakayama *et al.*, 1994; Veis *et al.*, 1993). Since very little is known about Bcl-w and its importance in B cell development, it might be necessary to evaluate the impact of loss of *Bcl-w* on the B cell compartment at multiple stages of B cell development.

To test the requirement of Bcl-w in normal cells and in the presence of dysregulated Myc, a number of experiments will need to be performed. Bone marrow can be isolated from mice with one, two, or no alleles of *Bcl-w* and grown under culture conditions that support the growth of pre-B cells. The role of Bcl-w in cell survival can be assessed under normal growth conditions, cytokine deprivation, and in the presence of increased Myc activity. To evaluate the effects of increased Myc activity, these cells can be infected with the retrovirus encoding the 4-hydroxytamoxifen inducible MycER. Likewise, pre-B cells

can be obtained from Eμ-*myc* transgenic mice with one, two, or no *Bcl-w* alleles. For this approach, I have already obtained preliminary results from one set of littermate-matched pre-B cells that were followed in culture to assess population doublings (Figure 65). These results show a dramatic defect in the growth of pre-B cells from *Bcl-w*-null Eμ-*myc* mice compared to wild-type, littermate-matched pre-B cells. Moreover, a reduced rate of growth was also observed for *Bcl-w*-heterozygous Eμ-*myc* pre-B cells. These growth defects could be attributed to reduced rates of proliferation and/or an increase in apoptosis. Future experiments will be necessary to test these possible explanations. Furthermore, murine embryonic fibroblasts of each genotype can also be isolated and cultured. Similar experiments can be performed in these cells to further test the role of Bcl-w in Myc-induced apoptosis and whether the effects are cell-type dependent.

Given that Bcl-w was a proposed target of the miR-15 family and that I have already reported a mechanism where Myc upregulates the miR-15 family to modulate Bcl-2 expression (Chapters 3 and 4), we hypothesized that the same mechanism may be employed to alter the expression of Bcl-w. To address this point, a similar research strategy to what was described in Chapter 3 could be utilized. Specifically, Target Protector molecules could be designed to bind the miR-15 family binding site in the 3'-UTR of *Bcl-w*, which could then be used in combination with the inducible MycER system to test the Myc-miR-15 family-Bcl-w connection.

So far, my results suggest that Bcl-w is an important factor in Myc biology. Therefore, it would be interesting to examine the role of Bcl-w in human hematopoietic malignancies, especially B cell lymphoma. As mentioned earlier, publically available data showed that *BCL-W* mRNA was upregulated in several types of human B cell lymphoma.

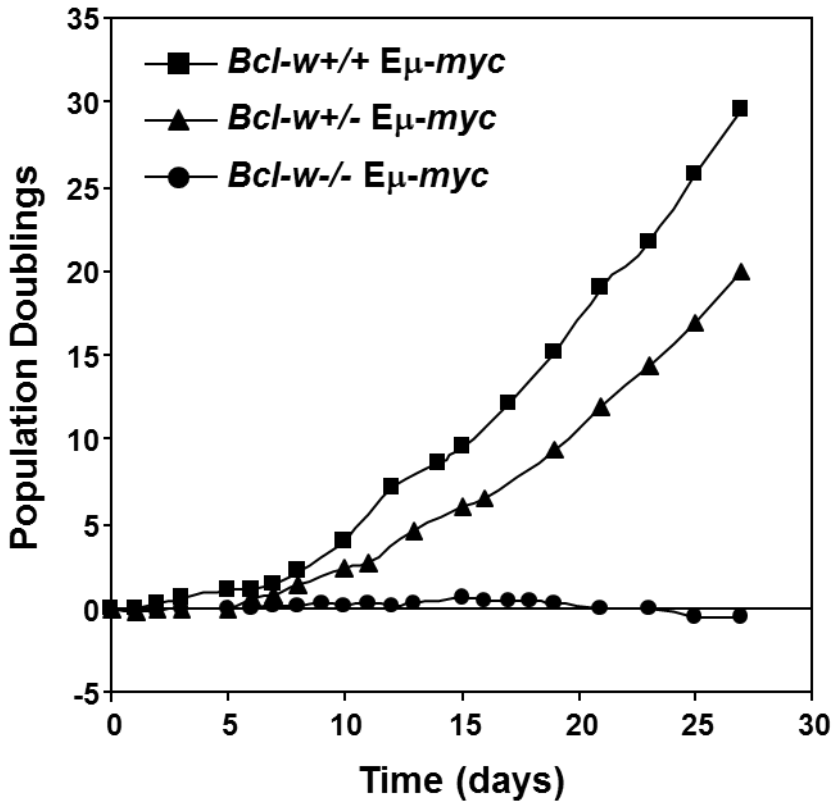

Figure 65. Reduced growth of *Bcl-w* deficient Eμ-*myc* pre-B cells. Bone marrow from littermates of the indicated genotype was placed into culture on day 0. Viable cells were counted by Trypan Blue dye exclusion at intervals, and population doublings were calculated. One experiment is shown.

To support this data, levels of BCL-W protein expression could be evaluated in patient samples of B cell lymphoma by immunohistochemistry (IHC). Expression of additional BCL-2 family members could also be assessed in the same patient samples to determine if their expression patterns are similar or different, which, depending on the result, could add significance to the role of BCL-W in human B cell lymphoma.

Collectively, my current results highlight an important and unknown role for Bcl-w in Myc-induced apoptosis and lymphomagenesis. I anticipate this new knowledge will contribute significantly to the field and will be of interest to basic scientists and clinicians alike.

Closing Remarks

In conclusion, the research described herein has substantially enhanced our understanding of Myc biology and Myc-induced tumorigenesis. I have investigated two major forms of miRNA regulation that are disrupted in cancer, including miRNA transcription and their subsequent processing. Specifically, I have demonstrated that miRNA are required for the survival of B cells and B cell lymphomas, and that, even without the strong tumor suppressor activity of p53, cells cannot survive. These studies have specifically contributed new knowledge regarding the tissue-specific requirements for the miRNA processing enzyme Dicer. Furthermore, my investigations of specific Myc-regulated miRNA have exposed a novel mechanism of Myc-induced apoptosis that functions to limit the oncogenic consequences of increased Myc activity in normal cells. This mechanism is inactivated in multiple human caner types, but can be reactivated by targeting the chromatin modifying histone deacetylase enzymes. These data provide

important information regarding the molecular events that underlie the therapeutic effects of HDAC inhibition. Furthermore, these studies have revealed an unappreciated role for the anti-apoptotic Bcl-w protein in Myc-induced apoptosis and lymphomagenesis that could result in broader scientific and clinical implications. Collectively, this research has likely stimulated as many questions as it has answered, which is the fundamental motivation behind research. I anticipate these studies will spark future scientific endeavors that will continue to move the cancer field forward.

REFERENCES

Adams, C.M., Hiebert, S.W., and Eischen, C.M. (2015). Myc-regulated miRNA-mediated apoptotic mechanism underlies therapeutic effects of HDAC inhibition. In revision.

Adams, C.M., and Eischen, C.M. (2015). Histone deacetylase inhibition reveals a tumor suppressive function of MYC-regulated miRNA in breast and lung carcinoma. In revision.

Adams, C.M., and Eischen, C.M. (2014). Cell survival is dicey without Dicer. Mol Cell Oncol 1(2): e961825.

Adams, C.M., and Eischen, C.M. (2014). Inactivation of p53 is insufficient to allow B cells and B-cell lymphomas to survive without Dicer. Cancer Res *74*, 3923-3934.

Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmiter, R.D., and Brinster, R.L. (1985). The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature *318*, 533-538.

Adams, M.R., Sears, R., Nuckolls, F., Leone, G., and Nevins, J.R. (2000). Complex transcriptional regulatory mechanisms control expression of the E2F3 locus. Mol Cell Biol *20*, 3633-3639.

Adhikary, S., Marinoni, F., Hock, A., Hulleman, E., Popov, N., Beier, R., Bernard, S., Quarto, M., Capra, M., Goettig, S., *et al.* (2005). The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. Cell *123*, 409-421.

Alitalo, K., Schwab, M., Lin, C.C., Varmus, H.E., and Bishop, J.M. (1983). Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci U S A *80*, 1707-1711.

Almeida, M.I., Reis, R.M., and Calin, G.A. (2011). MicroRNA history: discovery, recent applications, and next frontiers. Mutat Res *717*, 1-8.

Alt, J.R., Bouska, A., Fernandez, M.R., Cerny, R.L., Xiao, H., and Eischen, C.M. (2005). Mdm2 binds to Nbs1 at sites of DNA damage and regulates double strand break repair. J Biol Chem *280*, 18771-18781.

Alt, J.R., Greiner, T.C., Cleveland, J.L., and Eischen, C.M. (2003). Mdm2 haplo-insufficiency profoundly inhibits Myc-induced lymphomagenesis. EMBO J *22*, 1442-1450.

Amati, B., Brooks, M.W., Levy, N., Littlewood, T.D., Evan, G.I., and Land, H. (1993a). Oncogenic activity of the c-Myc protein requires dimerization with Max. Cell *72*, 233-245.

Amati, B., Littlewood, T.D., Evan, G.I., and Land, H. (1993b). The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. EMBO J *12*, 5083-5087.

Arabi, A., Wu, S., Ridderstrale, K., Bierhoff, H., Shiue, C., Fatyol, K., Fahlen, S., Hydbring, P., Soderberg, O., Grummt, I., *et al.* (2005). c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. Nat Cell Biol *7*, 303-310.

Arrate, M.P., Vincent, T., Odvody, J., Kar, R., Jones, S.N., and Eischen, C.M. (2010). MicroRNA biogenesis is required for Myc-induced B-cell lymphoma development and survival. Cancer Res *70*, 6083-6092.

Askew, D.S., Ashmun, R.A., Simmons, B.C., and Cleveland, J.L. (1991). Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. Oncogene *6*, 1915-1922.

Bantscheff, M., Hopf, C., Savitski, M.M., Dittmann, A., Grandi, P., Michon, A.M., Schlegl, J., Abraham, Y., Becher, I., Bergamini, G., *et al.* (2011). Chemoproteomics profiling of HDAC inhibitors reveals selective targeting of HDAC complexes. Nat Biotechnol *29*, 255-265.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281-297.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215-233.

Basso, K., Margolin, A.A., Stolovitzky, G., Klein, U., Dalla-Favera, R., and Califano, A. (2005). Reverse engineering of regulatory networks in human B cells. Nat Genet *37*, 382-390.

Baudino, T.A., McKay, C., Pendeville-Samain, H., Nilsson, J.A., Maclean, K.H., White, E.L., Davis, A.C., Ihle, J.N., and Cleveland, J.L. (2002). c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. Genes Dev *16*, 2530-2543.

Beier, R., Burgin, A., Kiermaier, A., Fero, M., Karsunky, H., Saffrich, R., Moroy, T., Ansorge, W., Roberts, J., and Eilers, M. (2000). Induction of cyclin E-cdk2 kinase activity, E2F-dependent transcription and cell growth by Myc are genetically separable events. EMBO J *19*, 5813-5823.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363-366.

Bernstein, E., Kim, S.Y., Carmell, M.A., Murchison, E.P., Alcorn, H., Li, M.Z., Mills, A.A., Elledge, S.J., Anderson, K.V., and Hannon, G.J. (2003). Dicer is essential for mouse development. Nat Genet *35*, 215-217.

Beroukhim, R., Mermel, C.H., Porter, D., Wei, G., Raychaudhuri, S., Donovan, J., Barretina, J., Boehm, J.S., Dobson, J., Urashima, M., *et al.* (2010). The landscape of somatic copy-number alteration across human cancers. Nature *463*, 899-905.

Bhaskara, S., Chyla, B.J., Amann, J.M., Knutson, S.K., Cortez, D., Sun, Z.W., and Hiebert, S.W. (2008). Deletion of histone deacetylase 3 reveals critical roles in S phase progression and DNA damage control. Mol Cell *30*, 61-72.

Bhaskara, S., Knutson, S.K., Jiang, G., Chandrasekharan, M.B., Wilson, A.J., Zheng, S., Yenamandra, A., Locke, K., Yuan, J.L., Bonine-Summers, A.R., *et al.* (2010). Hdac3 is essential for the maintenance of chromatin structure and genome stability. Cancer Cell *18*, 436-447.

Blackwell, T.K., Huang, J., Ma, A., Kretzner, L., Alt, F.W., Eisenman, R.N., and Weintraub, H. (1993). Binding of myc proteins to canonical and noncanonical DNA sequences. Mol Cell Biol *13*, 5216-5224.

Blackwell, T.K., Kretzner, L., Blackwood, E.M., Eisenman, R.N., and Weintraub, H. (1990). Sequence-specific DNA binding by the c-Myc protein. Science *250*, 1149-1151.

Blackwood, E.M., and Eisenman, R.N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science *251*, 1211-1217.

Blagosklonny, M.V., and Pardee, A.B. (2002). The restriction point of the cell cycle. Cell Cycle *1*, 103-110.

Bohnsack, M.T., Czaplinski, K., and Gorlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA *10*, 185-191.

Bolden, J.E., Peart, M.J., and Johnstone, R.W. (2006). Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov *5*, 769-784.

Bolden, J.E., Shi, W., Jankowski, K., Kan, C.Y., Cluse, L., Martin, B.P., MacKenzie, K.L., Smyth, G.K., and Johnstone, R.W. (2013). HDAC inhibitors induce tumor-cell-selective pro-apoptotic transcriptional responses. Cell Death Dis *4*, e519.

Bouchard, C., Thieke, K., Maier, A., Saffrich, R., Hanley-Hyde, J., Ansorge, W., Reed, S., Sicinski, P., Bartek, J., and Eilers, M. (1999). Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. EMBO J *18*, 5321-5333.

Brameier, M., Herwig, A., Reinhardt, R., Walter, L., and Gruber, J. (2011). Human box C/D snoRNAs with miRNA like functions: expanding the range of regulatory RNAs. Nucleic Acids Res *39*, 675-686.

Brandvold, K.A., Neiman, P., and Ruddell, A. (2000). Angiogenesis is an early event in the generation of myc-induced lymphomas. Oncogene *19*, 2780-2785.

Brennecke, J., Stark, A., Russell, R.B., and Cohen, S.M. (2005). Principles of microRNA-target recognition. PLoS Biol *3*, e85.

Brenner, C., Deplus, R., Didelot, C., Loriot, A., Vire, E., De Smet, C., Gutierrez, A., Danovi, D., Bernard, D., Boon, T., *et al.* (2005). Myc represses transcription through recruitment of DNA methyltransferase corepressor. EMBO J *24*, 336-346.

Brodeur, G.M., Seeger, R.C., Schwab, M., Varmus, H.E., and Bishop, J.M. (1984). Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science *224*, 1121-1124.

Brooks, C.L., and Gu, W. (2006). p53 ubiquitination: Mdm2 and beyond. Mol Cell *21*, 307-315.

Bruyere, H., Sutherland, H., Chipperfield, K., and Hudoba, M. (2010). Concomitant and successive amplifications of MYC in APL-like leukemia. Cancer Genet Cytogenet *197*, 75-80.

Bui, T.V., and Mendell, J.T. (2010). Myc: Maestro of MicroRNAs. Genes Cancer *1*, 568-575.

Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., *et al.* (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A *99*, 15524-15529.

Calin, G.A., Sevignani, C., Dumitru, C.D., Hyslop, T., Noch, E., Yendamuri, S., Shimizu, M., Rattan, S., Bullrich, F., Negrini, M., *et al.* (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A *101*, 2999-3004.

Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E., *et al.* (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov *2*, 401-404.

Chaffer, C.L., and Weinberg, R.A. (2011). A perspective on cancer cell metastasis. Science *331*, 1559-1564.

Chang, S., Wang, R.H., Akagi, K., Kim, K.A., Martin, B.K., Cavallone, L., Kathleen Cuningham Foundation Consortium for Research into Familial Breast, C., Haines, D.C., Basik, M., Mai, P., *et al.* (2011). Tumor suppressor BRCA1 epigenetically controls oncogenic microRNA-155. Nat Med *17*, 1275-1282.

Chang, T.C., Yu, D., Lee, Y.S., Wentzel, E.A., Arking, D.E., West, K.M., Dang, C.V., Thomas-Tikhonenko, A., and Mendell, J.T. (2008). Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet *40*, 43-50.

Chen, D.Q., Pan, B.Z., Huang, J.Y., Zhang, K., Cui, S.Y., De, W., Wang, R., and Chen, L.B. (2014). HDAC 1/4-mediated silencing of microRNA-200b promotes chemoresistance in human lung adenocarcinoma cells. Oncotarget *5*, 3333-3349.

Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., and Shiekhattar, R. (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature *436*, 740-744.

Chesi, M., Robbiani, D.F., Sebag, M., Chng, W.J., Affer, M., Tiedemann, R., Valdez, R., Palmer, S.E., Haas, S.S., Stewart, A.K., *et al.* (2008). AID-dependent activation of a MYC transgene induces multiple myeloma in a conditional mouse model of post-germinal center malignancies. Cancer Cell *13*, 167-180.

Cho, J.H., Dimri, M., and Dimri, G.P. (2015). MicroRNA-31 is a transcriptional target of histone deacetylase inhibitors and a regulator of cellular senescence. J Biol Chem *290*, 10555-10567.

Cimmino, A., Calin, G.A., Fabbri, M., Iorio, M.V., Ferracin, M., Shimizu, M., Wojcik, S.E., Aqeilan, R.I., Zupo, S., Dono, M., *et al.* (2005). miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A *102*, 13944-13949.

Cleveland, J.L., Huleihel, M., Bressler, P., Siebenlist, U., Akiyama, L., Eisenman, R.N., and Rapp, U.R. (1988). Negative regulation of c-myc transcription involves myc family proteins. Oncogene Res *3*, 357-375.

Collins, S., and Groudine, M. (1982). Amplification of endogenous myc-related DNA sequences in a human myeloid leukaemia cell line. Nature *298*, 679-681.

Consortium, E.P. (2012). An integrated encyclopedia of DNA elements in the human genome. Nature *489*, 57-74.

Conti, C., Leo, E., Eichler, G.S., Sordet, O., Martin, M.M., Fan, A., Aladjem, M.I., and Pommier, Y. (2010). Inhibition of histone deacetylase in cancer cells slows down replication forks, activates dormant origins, and induces DNA damage. Cancer Res *70*, 4470-4480.

Cowling, V.H., Chandriani, S., Whitfield, M.L., and Cole, M.D. (2006). A conserved Myc protein domain, MBIV, regulates DNA binding, apoptosis, transformation, and G2 arrest. Mol Cell Biol *26*, 4226-4239.

Cowling, V.H., and Cole, M.D. (2006). Mechanism of transcriptional activation by the Myc oncoproteins. Semin Cancer Biol *16*, 242-252.

Czabotar, P.E., Lessene, G., Strasser, A., and Adams, J.M. (2014). Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol *15*, 49-63.

Dalla-Favera, R., Bregni, M., Erikson, J., Patterson, D., Gallo, R.C., and Croce, C.M. (1982a). Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci U S A *79*, 7824-7827.

Dalla-Favera, R., Wong-Staal, F., and Gallo, R.C. (1982b). Onc gene amplification in promyelocytic leukaemia cell line HL-60 and primary leukaemic cells of the same patient. Nature *299*, 61-63.

Dang, C.V. (2012). MYC on the path to cancer. Cell *149*, 22-35.

Dang, C.V. (2013). MYC, metabolism, cell growth, and tumorigenesis. Cold Spring Harb Perspect Med *3*.

Dang, C.V. (2014). Gene regulation: fine-tuned amplification in cells. Nature *511*, 417-418.

Dang, C.V., O'Donnell K, A., and Juopperi, T. (2005). The great MYC escape in tumorigenesis. Cancer cell *8*, 177-178.

Dang, C.V., O'Donnell, K.A., Zeller, K.I., Nguyen, T., Osthus, R.C., and Li, F. (2006). The c-Myc target gene network. Semin Cancer Biol *16*, 253-264.

Davis, A.C., Wims, M., Spotts, G.D., Hann, S.R., and Bradley, A. (1993). A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes Dev *7*, 671-682.

Dawson, M.A., and Kouzarides, T. (2012). Cancer epigenetics: from mechanism to therapy. Cell *150*, 12-27.

de Alboran, I.M., O'Hagan, R.C., Gartner, F., Malynn, B., Davidson, L., Rickert, R., Rajewsky, K., DePinho, R.A., and Alt, F.W. (2001). Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. Immunity *14*, 45-55.

Delbridge, A.R., and Strasser, A. (2015). The BCL-2 protein family, BH3-mimetics and cancer therapy. Cell Death Differ *22*, 1071-1080.

Di Croce, L., and Helin, K. (2013). Transcriptional regulation by Polycomb group proteins. Nat Struct Mol Biol *20*, 1147-1155.

Di Leva, G., Garofalo, M., and Croce, C.M. (2014). MicroRNAs in cancer. Annu Rev Pathol *9*, 287-314.

Ding, S.W. (2010). RNA-based antiviral immunity. Nat Rev Immunol *10*, 632-644.

Doench, J.G., and Sharp, P.A. (2004). Specificity of microRNA target selection in translational repression. Genes Dev *18*, 504-511.

Duesberg, P.H., Bister, K., and Vogt, P.K. (1977). The RNA of avian acute leukemia virus MC29. Proc Natl Acad Sci U S A *74*, 4320-4324.

Duvic, M., Talpur, R., Ni, X., Zhang, C., Hazarika, P., Kelly, C., Chiao, J.H., Reilly, J.F., Ricker, J.L., Richon, V.M., *et al.* (2007). Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). Blood *109*, 31-39.

Eberhardy, S.R., and Farnham, P.J. (2001). c-Myc mediates activation of the cad promoter via a post-RNA polymerase II recruitment mechanism. J Biol Chem *276*, 48562-48571.

Eberhardy, S.R., and Farnham, P.J. (2002). Myc recruits P-TEFb to mediate the final step in the transcriptional activation of the cad promoter. J Biol Chem *277*, 40156-40162.

Ebert, M.S., and Sharp, P.A. (2012). Roles for microRNAs in conferring robustness to biological processes. Cell *149*, 515-524.

Egle, A., Harris, A.W., Bouillet, P., and Cory, S. (2004). Bim is a suppressor of Myc-induced mouse B cell leukemia. Proc Natl Acad Sci U S A *101*, 6164-6169.

Eilers, M., and Eisenman, R.N. (2008). Myc's broad reach. Genes Dev *22*, 2755-2766.

Eilers, M., Picard, D., Yamamoto, K.R., and Bishop, J.M. (1989). Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. Nature *340*, 66-68.

Eilers, M., Schirm, S., and Bishop, J.M. (1991). The MYC protein activates transcription of the alpha-prothymosin gene. EMBO J *10*, 133-141.

Eischen, C.M., and Lozano, G. (2014). The Mdm network and its regulation of p53 activities: a rheostat of cancer risk. Hum Mutat *35*, 728-737.

Eischen, C.M., Packham, G., Nip, J., Fee, B.E., Hiebert, S.W., Zambetti, G.P., and Cleveland, J.L. (2001a). Bcl-2 is an apoptotic target suppressed by both c-Myc and E2F-1. Oncogene *20*, 6983-6993.

Eischen, C.M., Roussel, M.F., Korsmeyer, S.J., and Cleveland, J.L. (2001b). Bax loss impairs Myc-induced apoptosis and circumvents the selection of p53 mutations during Myc-mediated lymphomagenesis. Mol Cell Biol *21*, 7653-7662.

Eischen, C.M., Weber, J.D., Roussel, M.F., Sherr, C.J., and Cleveland, J.L. (1999). Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. Genes Dev *13*, 2658-2669.

Eischen, C.M., Woo, D., Roussel, M.F., and Cleveland, J.L. (2001c). Apoptosis triggered by Myc-induced suppression of Bcl-X(L) or Bcl-2 is bypassed during lymphomagenesis. Mol Cell Biol *21*, 5063-5070.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001a). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494-498.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001b). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev *15*, 188-200.

Ellis, L., Bots, M., Lindemann, R.K., Bolden, J.E., Newbold, A., Cluse, L.A., Scott, C.L., Strasser, A., Atadja, P., Lowe, S.W., *et al.* (2009). The histone deacetylase inhibitors LAQ824 and LBH589 do not require death receptor signaling or a functional apoptosome to mediate tumor cell death or therapeutic efficacy. Blood *114*, 380-393.

Ender, C., Krek, A., Friedlander, M.R., Beitzinger, M., Weinmann, L., Chen, W., Pfeffer, S., Rajewsky, N., and Meister, G. (2008). A human snoRNA with microRNA-like functions. Mol Cell *32*, 519-528.

Eot-Houllier, G., Fulcrand, G., Magnaghi-Jaulin, L., and Jaulin, C. (2009). Histone deacetylase inhibitors and genomic instability. Cancer Lett *274*, 169-176.

Erikson, J., Finger, L., Sun, L., ar-Rushdi, A., Nishikura, K., Minowada, J., Finan, J., Emanuel, B.S., Nowell, P.C., and Croce, C.M. (1986). Deregulation of c-myc by translocation of the alpha-locus of the T-cell receptor in T-cell leukemias. Science *232*, 884-886.

Esteller, M. (2007). Epigenetic gene silencing in cancer: the DNA hypermethylome. Hum Mol Genet *16 Spec No 1*, R50-59.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z., and Hancock, D.C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell *69*, 119-128.

Falkenberg, K.J., and Johnstone, R.W. (2014). Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. Nat Rev Drug Discov *13*, 673-691.

Feil, R., Wagner, J., Metzger, D., and Chambon, P. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem Biophys Res Commun *237*, 752-757.

Felsher, D.W. (2010). MYC Inactivation Elicits Oncogene Addiction through Both Tumor Cell-Intrinsic and Host-Dependent Mechanisms. Genes Cancer *1*, 597-604.

Felsher, D.W., and Bishop, J.M. (1999). Reversible tumorigenesis by MYC in hematopoietic lineages. Mol Cell *4*, 199-207.

Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. (2003). Genomic targets of the human c-Myc protein. Genes Dev *17*, 1115-1129.

Finnegan, E.F., and Pasquinelli, A.E. (2013). MicroRNA biogenesis: regulating the regulators. Crit Rev Biochem Mol Biol *48*, 51-68.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

Fischle, W., Dequiedt, F., Hendzel, M.J., Guenther, M.G., Lazar, M.A., Voelter, W., and Verdin, E. (2002). Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. Mol Cell *9*, 45-57.

Forbes, S.A., Bhamra, G., Bamford, S., Dawson, E., Kok, C., Clements, J., Menzies, A., Teague, J.W., Futreal, P.A., and Stratton, M.R. (2008). The Catalogue of Somatic Mutations in Cancer (COSMIC). Curr Protoc Hum Genet *Chapter 10*, Unit 10 11.

Forstemann, K., Horwich, M.D., Wee, L., Tomari, Y., and Zamore, P.D. (2007). Drosophila microRNAs are sorted into functionally distinct argonaute complexes after production by dicer-1. Cell *130*, 287-297.

Fraga, M.F., Ballestar, E., Villar-Garea, A., Boix-Chornet, M., Espada, J., Schotta, G., Bonaldi, T., Haydon, C., Ropero, S., Petrie, K., *et al.* (2005). Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet *37*, 391-400.

Francia, S., Michelini, F., Saxena, A., Tang, D., de Hoon, M., Anelli, V., Mione, M., Carninci, P., and d'Adda di Fagagna, F. (2012). Site-specific DICER and DROSHA RNA products control the DNA-damage response. Nature *488*, 231-235.

Frank, S.R., Parisi, T., Taubert, S., Fernandez, P., Fuchs, M., Chan, H.M., Livingston, D.M., and Amati, B. (2003). MYC recruits the TIP60 histone acetyltransferase complex to chromatin. EMBO Rep *4*, 575-580.

Freytag, S.O. (1988). Enforced expression of the c-myc oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in G0/G1. Mol Cell Biol *8*, 1614-1624.

Gao, J., Aksoy, B.A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S.O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E., *et al.* (2013). Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal *6*, p11.

Gartel, A.L., Ye, X., Goufman, E., Shianov, P., Hay, N., Najmabadi, F., and Tyner, A.L. (2001). Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3. Proc Natl Acad Sci U S A *98*, 4510-4515.

Gaur, A., Jewell, D.A., Liang, Y., Ridzon, D., Moore, J.H., Chen, C., Ambros, V.R., and Israel, M.A. (2007). Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. Cancer Res *67*, 2456-2468.

Gebhardt, A., Frye, M., Herold, S., Benitah, S.A., Braun, K., Samans, B., Watt, F.M., Elsasser, H.P., and Eilers, M. (2006). Myc regulates keratinocyte adhesion and differentiation via complex formation with Miz1. J Cell Biol *172*, 139-149.

Gelmetti, V., Zhang, J., Fanelli, M., Minucci, S., Pelicci, P.G., and Lazar, M.A. (1998). Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. Mol Cell Biol *18*, 7185-7191.

Ghobrial, I.M., Campigotto, F., Murphy, T.J., Boswell, E.N., Banwait, R., Azab, F., Chuma, S., Kunsman, J., Donovan, A., Masood, F., *et al.* (2013). Results of a phase 2 trial of the single-agent histone deacetylase inhibitor panobinostat in patients with relapsed/refractory Waldenstrom macroglobulinemia. Blood *121*, 1296-1303.

Gibson, L., Holmgreen, S.P., Huang, D.C., Bernard, O., Copeland, N.G., Jenkins, N.A., Sutherland, G.R., Baker, E., Adams, J.M., and Cory, S. (1996). bcl-w, a novel member of the bcl-2 family, promotes cell survival. Oncogene *13*, 665-675.

Glozak, M.A., and Seto, E. (2007). Histone deacetylases and cancer. Oncogene *26*, 5420-5432.

Gomez-Roman, N., Grandori, C., Eisenman, R.N., and White, R.J. (2003). Direct activation of RNA polymerase III transcription by c-Myc. Nature *421*, 290-294.

Grandori, C., Gomez-Roman, N., Felton-Edkins, Z.A., Ngouenet, C., Galloway, D.A., Eisenman, R.N., and White, R.J. (2005). c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. Nat Cell Biol *7*, 311-318.

Greenberg, R.A., O'Hagan, R.C., Deng, H., Xiao, Q., Hann, S.R., Adams, R.R., Lichtsteiner, S., Chin, L., Morin, G.B., and DePinho, R.A. (1999). Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. Oncogene *18*, 1219-1226.

Gregory, M.A., and Hann, S.R. (2000). c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. Mol Cell Biol *20*, 2423-2435.

Gregory, R.I., Chendrimada, T.P., Cooch, N., and Shiekhattar, R. (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell *123*, 631-640.

Gregory, R.I., Yan, K.P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., and Shiekhattar, R. (2004). The Microprocessor complex mediates the genesis of microRNAs. Nature *432*, 235-240.

Grieb, B.C., Gramling, M.W., Arrate, M.P., Chen, X., Beauparlant, S.L., Haines, D.S., Xiao, H., and Eischen, C.M. (2014). Oncogenic protein MTBP interacts with MYC to promote tumorigenesis. Cancer research *74*, 3591-3602.

Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F.F., Zamir, I., *et al.* (1998). Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature *391*, 815-818.

Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., and Mello, C.C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell *106*, 23-34.

Guccione, E., Martinato, F., Finocchiaro, G., Luzi, L., Tizzoni, L., Dall' Olio, V., Zardo, G., Nervi, C., Bernard, L., and Amati, B. (2006). Myc-binding-site recognition in the human genome is determined by chromatin context. Nat Cell Biol *8*, 764-770.

Guo, H., Ingolia, N.T., Weissman, J.S., and Bartel, D.P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature *466*, 835-840.

Ha, M., and Kim, V.N. (2014). Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol *15*, 509-524.

Haldar, S., Negrini, M., Monne, M., Sabbioni, S., and Croce, C.M. (1994). Down-regulation of bcl-2 by p53 in breast cancer cells. Cancer research *54*, 2095-2097.

Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science *286*, 950-952.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature *404*, 293-296.

Han, J., Lee, Y., Yeom, K.H., Kim, Y.K., Jin, H., and Kim, V.N. (2004). The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev *18*, 3016-3027.

Han, J., Lee, Y., Yeom, K.H., Nam, J.W., Heo, I., Rhee, J.K., Sohn, S.Y., Cho, Y., Zhang, B.T., and Kim, V.N. (2006). Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell *125*, 887-901.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Hardy, R.R., and Hayakawa, K. (2001). B cell development pathways. Annu Rev Immunol *19*, 595-621.

Harrington, E.A., Bennett, M.R., Fanidi, A., and Evan, G.I. (1994). c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. EMBO J *13*, 3286-3295.

Hart, J.R., Roberts, T.C., Weinberg, M.S., Morris, K.V., and Vogt, P.K. (2014). MYC regulates the non-coding transcriptome. Oncotarget *5*, 12543-12554.

Hata, A., and Lieberman, J. (2015). Dysregulation of microRNA biogenesis and gene silencing in cancer. Sci Signal *8*, re3.

Hayashita, Y., Osada, H., Tatematsu, Y., Yamada, H., Yanagisawa, K., Tomida, S., Yatabe, Y., Kawahara, K., Sekido, Y., and Takahashi, T. (2005). A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res *65*, 9628-9632.

Hayward, W.S., Neel, B.G., and Astrin, S.M. (1981). Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature *290*, 475-480.

He, L., Thomson, J.M., Hemann, M.T., Hernando-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S.W., Hannon, G.J., *et al.* (2005). A microRNA polycistron as a potential human oncogene. Nature *435*, 828-833.

Hemann, M.T., Bric, A., Teruya-Feldstein, J., Herbst, A., Nilsson, J.A., Cordon-Cardo, C., Cleveland, J.L., Tansey, W.P., and Lowe, S.W. (2005). Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants. Nature *436*, 807-811.

Heravi-Moussavi, A., Anglesio, M.S., Cheng, S.W., Senz, J., Yang, W., Prentice, L., Fejes, A.P., Chow, C., Tone, A., Kalloger, S.E., *et al.* (2012). Recurrent somatic DICER1 mutations in nonepithelial ovarian cancers. N Engl J Med *366*, 234-242.

Herbst, A., Hemann, M.T., Tworkowski, K.A., Salghetti, S.E., Lowe, S.W., and Tansey, W.P. (2005). A conserved element in Myc that negatively regulates its proapoptotic activity. EMBO Rep *6*, 177-183.

Herbst, A., Salghetti, S.E., Kim, S.Y., and Tansey, W.P. (2004). Multiple cell-type-specific elements regulate Myc protein stability. Oncogene *23*, 3863-3871.

Herkert, B., and Eilers, M. (2010). Transcriptional repression: the dark side of myc. Genes Cancer *1*, 580-586.

Hermeking, H., and Eick, D. (1994). Mediation of c-Myc-induced apoptosis by p53. Science *265*, 2091-2093.

Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J.F., Obaya, A.J., O'Connell, B.C., Mateyak, M.K., Tam, W., Kohlhuber, F., *et al.* (2000). Identification of CDK4 as a target of c-MYC. Proc Natl Acad Sci U S A *97*, 2229-2234.

Herold, S., Wanzel, M., Beuger, V., Frohme, C., Beul, D., Hillukkala, T., Syvaoja, J., Saluz, H.P., Haenel, F., and Eilers, M. (2002). Negative regulation of the mammalian UV response by Myc through association with Miz-1. Molecular cell *10*, 509-521.

Hill, D.A., Ivanovich, J., Priest, J.R., Gurnett, C.A., Dehner, L.P., Desruisseau, D., Jarzembowski, J.A., Wikenheiser-Brokamp, K.A., Suarez, B.K., Whelan, A.J., *et al.* (2009). DICER1 mutations in familial pleuropulmonary blastoma. Science *325*, 965.

Hirning, U., Schmid, P., Schulz, W.A., Kozak, L.P., and Hameister, H. (1989). In developing brown adipose tissue c-myc protooncogene expression is restricted to early differentiation stages. Cell Differ Dev *27*, 243-248.

Hirvonen, H., Makela, T.P., Sandberg, M., Kalimo, H., Vuorio, E., and Alitalo, K. (1990). Expression of the myc proto-oncogenes in developing human fetal brain. Oncogene *5*, 1787-1797.

Hong, S., Pusapati, R.V., Powers, J.T., and Johnson, D.G. (2006). Oncogenes and the DNA damage response: Myc and E2F1 engage the ATM signaling pathway to activate p53 and induce apoptosis. Cell Cycle *5*, 801-803.

Hsu, B., Marin, M.C., el-Naggar, A.K., Stephens, L.C., Brisbay, S., and McDonnell, T.J. (1995). Evidence that c-myc mediated apoptosis does not require wild-type p53 during lymphomagenesis. Oncogene *11*, 175-179.

Hueber, A.O., Zornig, M., Lyon, D., Suda, T., Nagata, S., and Evan, G.I. (1997). Requirement for the CD95 receptor-ligand pathway in c-Myc-induced apoptosis. Science *278*, 1305-1309.

Hug, B.A., and Lazar, M.A. (2004). ETO interacting proteins. Oncogene *23*, 4270-4274.

Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T., and Zamore, P.D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science *293*, 834-838.

Hutvagner, G., and Simard, M.J. (2008). Argonaute proteins: key players in RNA silencing. Nat Rev Mol Cell Biol *9*, 22-32.

Insinga, A., Monestiroli, S., Ronzoni, S., Gelmetti, V., Marchesi, F., Viale, A., Altucci, L., Nervi, C., Minucci, S., and Pelicci, P.G. (2005). Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. Nat Med *11*, 71-76.

Iorio, M.V., and Croce, C.M. (2012). microRNA involvement in human cancer. Carcinogenesis *33*, 1126-1133.

Iritani, B.M., and Eisenman, R.N. (1999). c-Myc enhances protein synthesis and cell size during B lymphocyte development. Proc Natl Acad Sci U S A *96*, 13180-13185.

Izumi, H., Molander, C., Penn, L.Z., Ishisaki, A., Kohno, K., and Funa, K. (2001). Mechanism for the transcriptional repression by c-Myc on PDGF beta-receptor. J Cell Sci *114*, 1533-1544.

Jain, M., Arvanitis, C., Chu, K., Dewey, W., Leonhardt, E., Trinh, M., Sundberg, C.D., Bishop, J.M., and Felsher, D.W. (2002). Sustained loss of a neoplastic phenotype by brief inactivation of MYC. Science *297*, 102-104.

Janicke, R.U., Lee, F.H., and Porter, A.G. (1994). Nuclear c-Myc plays an important role in the cytotoxicity of tumor necrosis factor alpha in tumor cells. Mol Cell Biol *14*, 5661-5670.

Jiang, G., Espeseth, A., Hazuda, D.J., and Margolis, D.M. (2007). c-Myc and Sp1 contribute to proviral latency by recruiting histone deacetylase 1 to the human immunodeficiency virus type 1 promoter. J Virol *81*, 10914-10923.

Jin, H.Y., Oda, H., Lai, M., Skalsky, R.L., Bethel, K., Shepherd, J., Kang, S.G., Liu, W.H., Sabouri-Ghomi, M., Cullen, B.R., *et al.* (2013). MicroRNA-17~92 plays a

causative role in lymphomagenesis by coordinating multiple oncogenic pathways. EMBO J *32*, 2377-2391.

Johanson, T.M., Lew, A.M., and Chong, M.M. (2013). MicroRNA-independent roles of the RNase III enzymes Drosha and Dicer. Open Biol *3*, 130144.

Johnson, S.M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K.L., Brown, D., and Slack, F.J. (2005). RAS is regulated by the let-7 microRNA family. Cell *120*, 635-647.

Karube, Y., Tanaka, H., Osada, H., Tomida, S., Tatematsu, Y., Yanagisawa, K., Yatabe, Y., Takamizawa, J., Miyoshi, S., Mitsudomi, T., *et al.* (2005). Reduced expression of Dicer associated with poor prognosis in lung cancer patients. Cancer Sci *96*, 111-115.

Kato, G.J., Barrett, J., Villa-Garcia, M., and Dang, C.V. (1990). An amino-terminal c-myc domain required for neoplastic transformation activates transcription. Mol Cell Biol *10*, 5914-5920.

Kedde, M., Strasser, M.J., Boldajipour, B., Oude Vrielink, J.A., Slanchev, K., le Sage, C., Nagel, R., Voorhoeve, P.M., van Duijse, J., Orom, U.A., *et al.* (2007). RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. Cell *131*, 1273-1286.

Kelly, K., Cochran, B.H., Stiles, C.D., and Leder, P. (1983). Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell *35*, 603-610.

Kelly, P.N., Grabow, S., Delbridge, A.R., Strasser, A., and Adams, J.M. (2011). Endogenous Bcl-xL is essential for Myc-driven lymphomagenesis in mice. Blood *118*, 6380-6386.

Kenneth, N.S., Ramsbottom, B.A., Gomez-Roman, N., Marshall, L., Cole, P.A., and White, R.J. (2007). TRRAP and GCN5 are used by c-Myc to activate RNA polymerase III transcription. Proc Natl Acad Sci U S A *104*, 14917-14922.

Kertesz, M., Iovino, N., Unnerstall, U., Gaul, U., and Segal, E. (2007). The role of site accessibility in microRNA target recognition. Nat Genet *39*, 1278-1284.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev *15*, 2654-2659.

Kim, J., Lee, J.H., and Iyer, V.R. (2008). Global identification of Myc target genes reveals its direct role in mitochondrial biogenesis and its E-box usage in vivo. PLoS One *3*, e1798.

Kim, J.W., Gao, P., Liu, Y.C., Semenza, G.L., and Dang, C.V. (2007). Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. Mol Cell Biol *27*, 7381-7393.

Kim, J.W., Zeller, K.I., Wang, Y., Jegga, A.G., Aronow, B.J., O'Donnell, K.A., and Dang, C.V. (2004). Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. Mol Cell Biol *24*, 5923-5936.

Kim, K.T., Baird, K., Ahn, J.Y., Meltzer, P., Lilly, M., Levis, M., and Small, D. (2005). Pim-1 is up-regulated by constitutively activated FLT3 and plays a role in FLT3-mediated cell survival. Blood *105*, 1759-1767.

Klefstrom, J., Vastrik, I., Saksela, E., Valle, J., Eilers, M., and Alitalo, K. (1994). c-Myc induces cellular susceptibility to the cytotoxic action of TNF-alpha. EMBO J *13*, 5442-5450.

Klein, U., Lia, M., Crespo, M., Siegel, R., Shen, Q., Mo, T., Ambesi-Impiombato, A., Califano, A., Migliazza, A., Bhagat, G., *et al.* (2010). The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. Cancer Cell *17*, 28-40.

Knutson, S.K., Chyla, B.J., Amann, J.M., Bhaskara, S., Huppert, S.S., and Hiebert, S.W. (2008). Liver-specific deletion of histone deacetylase 3 disrupts metabolic transcriptional networks. EMBO J *27*, 1017-1028.

Koralov, S.B., Muljo, S.A., Galler, G.R., Krek, A., Chakraborty, T., Kanellopoulou, C., Jensen, K., Cobb, B.S., Merkenschlager, M., Rajewsky, N., *et al.* (2008). Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. Cell *132*, 860-874.

Kress, T.R., Sabo, A., and Amati, B. (2015). MYC: connecting selective transcriptional control to global RNA production. Nat Rev Cancer *15*, 593-607.

Kumar, M.S., Lu, J., Mercer, K.L., Golub, T.R., and Jacks, T. (2007). Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet *39*, 673-677.

Kumar, M.S., Pester, R.E., Chen, C.Y., Lane, K., Chin, C., Lu, J., Kirsch, D.G., Golub, T.R., and Jacks, T. (2009). Dicer1 functions as a haploinsufficient tumor suppressor. Genes Dev *23*, 2700-2704.

Kuppers, R., and Dalla-Favera, R. (2001). Mechanisms of chromosomal translocations in B cell lymphomas. Oncogene *20*, 5580-5594.

Kurland, J.F., and Tansey, W.P. (2008). Myc-mediated transcriptional repression by recruitment of histone deacetylase. Cancer research *68*, 3624-3629.

Kwak, H., Fuda, N.J., Core, L.J., and Lis, J.T. (2013). Precise maps of RNA polymerase reveal how promoters direct initiation and pausing. Science *339*, 950-953.

Lagana, A., Russo, F., Sismeiro, C., Giugno, R., Pulvirenti, A., and Ferro, A. (2010). Variability in the incidence of miRNAs and genes in fragile sites and the role of repeats and CpG islands in the distribution of genetic material. PLoS One *5*, e11166.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. Science *294*, 853-858.

Lal, A., Navarro, F., Maher, C.A., Maliszewski, L.E., Yan, N., O'Day, E., Chowdhury, D., Dykxhoorn, D.M., Tsai, P., Hofmann, O., *et al.* (2009). miR-24 Inhibits cell proliferation by targeting E2F2, MYC, and other cell-cycle genes via binding to "seedless" 3'UTR microRNA recognition elements. Mol Cell *35*, 610-625.

Lambertz, I., Nittner, D., Mestdagh, P., Denecker, G., Vandesompele, J., Dyer, M.A., and Marine, J.C. (2010). Monoallelic but not biallelic loss of Dicer1 promotes tumorigenesis in vivo. Cell Death Differ *17*, 633-641.

Land, H., Parada, L.F., and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature *304*, 596-602.

Landthaler, M., Yalcin, A., and Tuschl, T. (2004). The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. Curr Biol *14*, 2162-2167.

Langenberger, D., Bermudez-Santana, C.I., Stadler, P.F., and Hoffmann, S. (2010). Identification and classification of small RNAs in transcriptome sequence data. Pac Symp Biocomput, 80-87.

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science *294*, 858-862.

Leder, A., and Leder, P. (1975). Butyric acid, a potent inducer of erythroid differentiation in cultured erythroleukemic cells. Cell *5*, 319-322.

Lee, E.Y., and Muller, W.J. (2010). Oncogenes and tumor suppressor genes. Cold Spring Harb Perspect Biol *2*, a003236.

Lee, H.C., Chang, S.S., Choudhary, S., Aalto, A.P., Maiti, M., Bamford, D.H., and Liu, Y. (2009a). qiRNA is a new type of small interfering RNA induced by DNA damage. Nature *459*, 274-277.

Lee, R.C., and Ambros, V. (2001). An extensive class of small RNAs in Caenorhabditis elegans. Science *294*, 862-864.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843-854.

Lee, S., Kim, M., Lim, J., Kim, Y., Han, K., Kim, H.J., Cho, S.G., and Min, W.S. (2009b). Acute myeloid leukemia with MYC amplification in the homogeneous staining regions and double minutes. Cancer Genet Cytogenet *192*, 96-98.

Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., and Kim, V.N. (2004). MicroRNA genes are transcribed by RNA polymerase II. EMBO J *23*, 4051-4060.

Lehmann, U., Hasemeier, B., Christgen, M., Muller, M., Romermann, D., Langer, F., and Kreipe, H. (2008). Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. J Pathol *214*, 17-24.

Lewis, B.C., Shim, H., Li, Q., Wu, C.S., Lee, L.A., Maity, A., and Dang, C.V. (1997). Identification of putative c-Myc-responsive genes: characterization of rcl, a novel growth-related gene. Mol Cell Biol *17*, 4967-4978.

Li, L.H., Nerlov, C., Prendergast, G., MacGregor, D., and Ziff, E.B. (1994). c-Myc represses transcription in vivo by a novel mechanism dependent on the initiator element and Myc box II. EMBO J *13*, 4070-4079.

Lima, R.T., Busacca, S., Almeida, G.M., Gaudino, G., Fennell, D.A., and Vasconcelos, M.H. (2011). MicroRNA regulation of core apoptosis pathways in cancer. European journal of cancer *47*, 163-174.

Lin, C.Y., Loven, J., Rahl, P.B., Paranal, R.M., Burge, C.B., Bradner, J.E., Lee, T.I., and Young, R.A. (2012). Transcriptional amplification in tumor cells with elevated c-Myc. Cell *151*, 56-67.

Lin, R.J., Lin, Y.C., Chen, J., Kuo, H.H., Chen, Y.Y., Diccianni, M.B., London, W.B., Chang, C.H., and Yu, A.L. (2010). microRNA signature and expression of Dicer and Drosha can predict prognosis and delineate risk groups in neuroblastoma. Cancer Res *70*, 7841-7850.

Lindemann, R.K., Newbold, A., Whitecross, K.F., Cluse, L.A., Frew, A.J., Ellis, L., Williams, S., Wiegmans, A.P., Dear, A.E., Scott, C.L., *et al.* (2007). Analysis of the apoptotic and therapeutic activities of histone deacetylase inhibitors by using a mouse model of B cell lymphoma. Proc Natl Acad Sci U S A *104*, 8071-8076.

Lindstrom, M.S., and Wiman, K.G. (2003). Myc and E2F1 induce p53 through p14ARF-independent mechanisms in human fibroblasts. Oncogene *22*, 4993-5005.

Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G., and Evan, G.I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res *23*, 1686-1690.

Liu, Y., Cheney, M.D., Gaudet, J.J., Chruszcz, M., Lukasik, S.M., Sugiyama, D., Lary, J., Cole, J., Dauter, Z., Minor, W., *et al.* (2006). The tetramer structure of the Nervy homology two domain, NHR2, is critical for AML1/ETO's activity. Cancer Cell *9*, 249-260.

Long, D., Lee, R., Williams, P., Chan, C.Y., Ambros, V., and Ding, Y. (2007). Potent effect of target structure on microRNA function. Nat Struct Mol Biol *14*, 287-294.

Loven, J., Orlando, D.A., Sigova, A.A., Lin, C.Y., Rahl, P.B., Burge, C.B., Levens, D.L., Lee, T.I., and Young, R.A. (2012). Revisiting global gene expression analysis. Cell *151*, 476-482.

Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., *et al.* (2005). MicroRNA expression profiles classify human cancers. Nature *435*, 834-838.

Lujambio, A., Calin, G.A., Villanueva, A., Ropero, S., Sanchez-Cespedes, M., Blanco, D., Montuenga, L.M., Rossi, S., Nicoloso, M.S., Faller, W.J., *et al.* (2008). A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci U S A *105*, 13556-13561.

Lujambio, A., Ropero, S., Ballestar, E., Fraga, M.F., Cerrato, C., Setien, F., Casado, S., Suarez-Gauthier, A., Sanchez-Cespedes, M., Git, A., *et al.* (2007). Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res *67*, 1424-1429.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J.E., and Kutay, U. (2004). Nuclear export of microRNA precursors. Science *303*, 95-98.

Luscher, B., and Vervoorts, J. (2012). Regulation of gene transcription by the oncoprotein MYC. Gene *494*, 145-160.

Lutterbach, B., and Hann, S.R. (1994). Hierarchical phosphorylation at N-terminal transformation-sensitive sites in c-Myc protein is regulated by mitogens and in mitosis. Mol Cell Biol *14*, 5510-5522.

Lyle, S., Hoover, K., Colpan, C., Zhu, Z., Matijasevic, Z., and Jones, S.N. (2014). Dicer cooperates with p53 to suppress DNA damage and skin carcinogenesis in mice. PLoS One *9*, e100920.

Lytle, J.R., Yario, T.A., and Steitz, J.A. (2007). Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proc Natl Acad Sci U S A *104*, 9667-9672.

Maclean, K.H., Keller, U.B., Rodriguez-Galindo, C., Nilsson, J.A., and Cleveland, J.L. (2003). c-Myc augments gamma irradiation-induced apoptosis by suppressing Bcl-XL. Mol Cell Biol *23*, 7256-7270.

Majid, S., Dar, A.A., Saini, S., Shahryari, V., Arora, S., Zaman, M.S., Chang, I., Yamamura, S., Tanaka, Y., Chiyomaru, T., *et al.* (2013). miRNA-34b inhibits prostate cancer through demethylation, active chromatin modifications, and AKT pathways. Clin Cancer Res *19*, 73-84.

Mao, D.Y., Barsyte-Lovejoy, D., Ho, C.S., Watson, J.D., Stojanova, A., and Penn, L.Z. (2004). Promoter-binding and repression of PDGFRB by c-Myc are separable activities. Nucleic Acids Res *32*, 3462-3468.

Mao, D.Y., Watson, J.D., Yan, P.S., Barsyte-Lovejoy, D., Khosravi, F., Wong, W.W., Farnham, P.J., Huang, T.H., and Penn, L.Z. (2003). Analysis of Myc bound loci identified by CpG island arrays shows that Max is essential for Myc-dependent repression. Curr Biol *13*, 882-886.

Marinkovic, D., Marinkovic, T., Mahr, B., Hess, J., and Wirth, T. (2004). Reversible lymphomagenesis in conditionally c-MYC expressing mice. Int J Cancer *110*, 336-342.

Mateyak, M.K., Obaya, A.J., Adachi, S., and Sedivy, J.M. (1997). Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. Cell Growth Differ *8*, 1039-1048.

Mathew, S., Lorsbach, R.B., Shearer, P., Sandlund, J.T., and Raimondi, S.C. (2000). Double minute chromosomes and c-MYC amplification in a child with secondary myelodysplastic syndrome after treatment for acute lymphoblastic leukemia. Leukemia *14*, 1314-1315.

Matthews, G.M., Mehdipour, P., Cluse, L.A., Falkenberg, K.J., Wang, E., Roth, M., Santoro, F., Vidacs, E., Stanley, K., House, C.M., Rusche, J.R., Vakoc, C.R., Zuber, J., Minucci, S., and Johnstone, R.W. (2015). Functional-genetic dissection of HDAC dependencies in mouse lymphoid and myeloid malignancies. Blood. Epub ahead of print.

Matthews, G.M., Cluse, L.A., Wang, E., Roth, M., Vakoc, C.R., Wenyan, M., C.M., Rusche, J.R., Zuber, J., and Johnstone, R.W. (2014). RNAi-mediated depletion of histone deacetylases highlights the potential for isoform-specific inhibitors in B-cell lymphoma and acute myeloid leukemia. Proceedings of the 105th Annual Meeting of the American Association for Cancer Research. Cancer Res *74*(19 Suppl), Abstract 5533.

McGirt, L.Y., Adams, C.M., Baerenwald, D.A., Zwerner, J.P., Zic, J.A., and Eischen, C.M. (2014). miR-223 regulates cell growth and targets proto-oncogenes in mycosis fungoides/cutaneous T-cell lymphoma. The Journal of investigative dermatology *134*, 1101-1107.

McMahon, S.B., Van Buskirk, H.A., Dugan, K.A., Copeland, T.D., and Cole, M.D. (1998). The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell *94*, 363-374.

McMahon, S.B., Wood, M.A., and Cole, M.D. (2000). The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. Mol Cell Biol *20*, 556-562.

Mellon, P., Pawson, A., Bister, K., Martin, G.S., and Duesberg, P.H. (1978). Specific RNA sequences and gene products of MC29 avian acute leukemia virus. Proc Natl Acad Sci U S A *75*, 5874-5878.

Melo, S.A., Moutinho, C., Ropero, S., Calin, G.A., Rossi, S., Spizzo, R., Fernandez, A.F., Davalos, V., Villanueva, A., Montoya, G., *et al.* (2010). A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. Cancer Cell *18*, 303-315.

Melo, S.A., Ropero, S., Moutinho, C., Aaltonen, L.A., Yamamoto, H., Calin, G.A., Rossi, S., Fernandez, A.F., Carneiro, F., Oliveira, C., *et al.* (2009). A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. Nat Genet *41*, 365-370.

Mendell, J.T., and Olson, E.N. (2012). MicroRNAs in stress signaling and human disease. Cell *148*, 1172-1187.

Meyer, N., and Penn, L.Z. (2008). Reflecting on 25 years with MYC. Nat Rev Cancer *8*, 976-990.

Migliori, V., Mapelli, M., and Guccione, E. (2012). On WD40 proteins: propelling our knowledge of transcriptional control? Epigenetics *7*, 815-822.

Miltenberger, R.J., Sukow, K.A., and Farnham, P.J. (1995). An E-box-mediated increase in cad transcription at the G1/S-phase boundary is suppressed by inhibitory c-Myc mutants. Mol Cell Biol *15*, 2527-2535.

Minami, J., Suzuki, R., Mazitschek, R., Gorgun, G., Ghosh, B., Cirstea, D., Hu, Y., Mimura, N., Ohguchi, H., Cottini, F., *et al.* (2014). Histone deacetylase 3 as a novel therapeutic target in multiple myeloma. Leukemia *28*, 680-689.

Miner, J.H., and Wold, B.J. (1991). c-myc inhibition of MyoD and myogenin-initiated myogenic differentiation. Mol Cell Biol *11*, 2842-2851.

Mito, J.K., Min, H.D., Ma, Y., Carter, J.E., Brigman, B.E., Dodd, L., Dankort, D., McMahon, M., and Kirsch, D.G. (2013). Oncogene-dependent control of miRNA biogenesis and metastatic progression in a model of undifferentiated pleomorphic sarcoma. J Pathol *229*, 132-140.

Montes de Oca Luna, R., Wagner, D.S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature *378*, 203-206.

Morita, S., Hara, A., Kojima, I., Horii, T., Kimura, M., Kitamura, T., Ochiya, T., Nakanishi, K., Matoba, R., Matsubara, K., *et al.* (2009). Dicer is required for maintaining adult pancreas. PLoS One *4*, e4212.

Morrow, M.A., Lee, G., Gillis, S., Yancopoulos, G.D., and Alt, F.W. (1992). Interleukin-7 induces N-myc and c-myc expression in normal precursor B lymphocytes. Genes Dev *6*, 61-70.

Motoyama, N., Wang, F., Roth, K.A., Sawa, H., Nakayama, K., Nakayama, K., Negishi, I., Senju, S., Zhang, Q., Fujii, S., *et al.* (1995). Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science *267*, 1506-1510.

Mudhasani, R., Zhu, Z., Hutvagner, G., Eischen, C.M., Lyle, S., Hall, L.L., Lawrence, J.B., Imbalzano, A.N., and Jones, S.N. (2008). Loss of miRNA biogenesis induces p19Arf-p53 signaling and senescence in primary cells. J Cell Biol *181*, 1055-1063.

Muller-Tidow, C., Steffen, B., Cauvet, T., Tickenbrock, L., Ji, P., Diederichs, S., Sargin, B., Kohler, G., Stelljes, M., Puccetti, E., *et al.* (2004). Translocation products in acute

myeloid leukemia activate the Wnt signaling pathway in hematopoietic cells. Mol Cell Biol *24*, 2890-2904.

Muller, D., Bouchard, C., Rudolph, B., Steiner, P., Stuckmann, I., Saffrich, R., Ansorge, W., Huttner, W., and Eilers, M. (1997). Cdk2-dependent phosphorylation of p27 facilitates its Myc-induced release from cyclin E/cdk2 complexes. Oncogene *15*, 2561-2576.

Nakayama, K., Nakayama, K., Negishi, I., Kuida, K., Sawa, H., and Loh, D.Y. (1994). Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proc Natl Acad Sci U S A *91*, 3700-3704.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell *2*, 279-289.

Nau, M.M., Brooks, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., McBride, O.W., Bertness, V., Hollis, G.F., and Minna, J.D. (1985). L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature *318*, 69-73.

Nebbioso, A., Clarke, N., Voltz, E., Germain, E., Ambrosino, C., Bontempo, P., Alvarez, R., Schiavone, E.M., Ferrara, F., Bresciani, F., *et al.* (2005). Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. Nat Med *11*, 77-84.

Needham, L.A., Davidson, A.H., Bawden, L.J., Belfield, A., Bone, E.A., Brotherton, D.H., Bryant, S., Charlton, M.H., Clark, V.L., Davies, S.J., *et al.* (2011). Drug targeting to monocytes and macrophages using esterase-sensitive chemical motifs. J Pharmacol Exp Ther *339*, 132-142.

Neel, B.G., Hayward, W.S., Robinson, H.L., Fang, J., and Astrin, S.M. (1981). Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs: oncogenesis by promoter insertion. Cell *23*, 323-334.

Neel, B.G., Jhanwar, S.C., Chaganti, R.S., and Hayward, W.S. (1982). Two human c-onc genes are located on the long arm of chromosome 8. Proc Natl Acad Sci U S A *79*, 7842-7846.

Newbold, A., Lindemann, R.K., Cluse, L.A., Whitecross, K.F., Dear, A.E., and Johnstone, R.W. (2008). Characterisation of the novel apoptotic and therapeutic activities of the histone deacetylase inhibitor romidepsin. Mol Cancer Ther *7*, 1066-1079.

Nie, Z., Hu, G., Wei, G., Cui, K., Yamane, A., Resch, W., Wang, R., Green, D.R., Tessarollo, L., Casellas, R., *et al.* (2012). c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. Cell *151*, 68-79.

Nittner, D., Lambertz, I., Clermont, F., Mestdagh, P., Kohler, C., Nielsen, S.J., Jochemsen, A., Speleman, F., Vandesompele, J., Dyer, M.A., *et al.* (2012). Synthetic lethality between Rb, p53 and Dicer or miR-17-92 in retinal progenitors suppresses retinoblastoma formation. Nat Cell Biol *14*, 958-965.

O'Connell, B.C., Cheung, A.F., Simkevich, C.P., Tam, W., Ren, X., Mateyak, M.K., and Sedivy, J.M. (2003). A large scale genetic analysis of c-Myc-regulated gene expression patterns. J Biol Chem *278*, 12563-12573.

O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V., and Mendell, J.T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. Nature *435*, 839-843.

O'Reilly, L.A., Print, C., Hausmann, G., Moriishi, K., Cory, S., Huang, D.C., and Strasser, A. (2001). Tissue expression and subcellular localization of the pro-survival molecule Bcl-w. Cell Death Differ *8*, 486-494.

Obaya, A.J., Mateyak, M.K., and Sedivy, J.M. (1999). Mysterious liaisons: the relationship between c-Myc and the cell cycle. Oncogene *18*, 2934-2941.

Odenike, O., Halpern, A., Godley, L.A., Madzo, J., Karrison, T., Green, M., Fulton, N., Mattison, R.J., Yee, K.W., Bennett, M., *et al.* (2015). A phase I and pharmacodynamic study of the histone deacetylase inhibitor belinostat plus azacitidine in advanced myeloid neoplasia. Invest New Drugs *33*, 371-379.

Olivier, M., Hollstein, M., and Hainaut, P. (2010). TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harbor perspectives in biology *2*, a001008.

Olsen, E.A., Kim, Y.H., Kuzel, T.M., Pacheco, T.R., Foss, F.M., Parker, S., Frankel, S.R., Chen, C., Ricker, J.L., Arduino, J.M., *et al.* (2007). Phase IIb multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous T-cell lymphoma. J Clin Oncol *25*, 3109-3115.

Osthus, R.C., Shim, H., Kim, S., Li, Q., Reddy, R., Mukherjee, M., Xu, Y., Wonsey, D., Lee, L.A., and Dang, C.V. (2000). Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J Biol Chem *275*, 21797-21800.

Ota, A., Tagawa, H., Karnan, S., Tsuzuki, S., Karpas, A., Kira, S., Yoshida, Y., and Seto, M. (2004). Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res *64*, 3087-3095.

Pajic, A., Spitkovsky, D., Christoph, B., Kempkes, B., Schuhmacher, M., Staege, M.S., Brielmeier, M., Ellwart, J., Kohlhuber, F., Bornkamm, G.W., *et al.* (2000). Cell cycle activation by c-myc in a burkitt lymphoma model cell line. International journal of cancer Journal international du cancer *87*, 787-793.

Pal, S., Yun, R., Datta, A., Lacomis, L., Erdjument-Bromage, H., Kumar, J., Tempst, P., and Sif, S. (2003). mSin3A/histone deacetylase 2- and PRMT5-containing Brg1 complex is involved in transcriptional repression of the Myc target gene cad. Molecular and cellular biology *23*, 7475-7487.

Pampalakis, G., Diamandis, E.P., Katsaros, D., and Sotiropoulou, G. (2009). Down-regulation of dicer expression in ovarian cancer tissues. Clin Biochem *43*, 324-327.

Pardee, A.B. (1989). G1 events and regulation of cell proliferation. Science *246*, 603-608.

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Muller, P., *et al.* (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature *408*, 86-89.

Patel, J.H., and McMahon, S.B. (2006). Targeting of Miz-1 is essential for Myc-mediated apoptosis. J Biol Chem *281*, 3283-3289.

Patel, J.H., and McMahon, S.B. (2007). BCL2 is a downstream effector of MIZ-1 essential for blocking c-MYC-induced apoptosis. J Biol Chem *282*, 5-13.

Payne, G.S., Courtneidge, S.A., Crittenden, L.B., Fadly, A.M., Bishop, J.M., and Varmus, H.E. (1981). Analysis of avian leukosis virus DNA and RNA in bursal tumours: viral gene expression is not required for maintenance of the tumor state. Cell *23*, 311-322.

Pelengaris, S., Littlewood, T., Khan, M., Elia, G., and Evan, G. (1999). Reversible activation of c-Myc in skin: induction of a complex neoplastic phenotype by a single oncogenic lesion. Mol Cell *3*, 565-577.

Perez-Roger, I., Kim, S.H., Griffiths, B., Sewing, A., and Land, H. (1999). Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). EMBO J *18*, 5310-5320.

Peter, S., Bultinck, J., Myant, K., Jaenicke, L.A., Walz, S., Muller, J., Gmachl, M., Treu, M., Boehmelt, G., Ade, C.P., *et al.* (2014). Tumor cell-specific inhibition of MYC function using small molecule inhibitors of the HUWE1 ubiquitin ligase. EMBO Mol Med *6*, 1525-1541.

Peters, G. (1990). Oncogenes at viral integration sites. Cell Growth Differ *1*, 503-510.

Petros, A.M., Olejniczak, E.T., and Fesik, S.W. (2004). Structural biology of the Bcl-2 family of proteins. Biochim Biophys Acta *1644*, 83-94.

Peukert, K., Staller, P., Schneider, A., Carmichael, G., Hanel, F., and Eilers, M. (1997). An alternative pathway for gene regulation by Myc. EMBO J *16*, 5672-5686.

Piekarz, R.L., Frye, R., Turner, M., Wright, J.J., Allen, S.L., Kirschbaum, M.H., Zain, J., Prince, H.M., Leonard, J.P., Geskin, L.J., *et al.* (2009). Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous T-cell lymphoma. J Clin Oncol *27*, 5410-5417.

Prendergast, G.C., and Ziff, E.B. (1991). Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science *251*, 186-189.

Prochownik, E.V. (2008). c-Myc: linking transformation and genomic instability. Curr Mol Med *8*, 446-458.

Pusapati, R.V., Rounbehler, R.J., Hong, S., Powers, J.T., Yan, M., Kiguchi, K., McArthur, M.J., Wong, P.K., and Johnson, D.G. (2006). ATM promotes apoptosis and suppresses tumorigenesis in response to Myc. Proc Natl Acad Sci U S A *103*, 1446-1451.

Rao, P.H., Murty, V.V., Louie, D.C., and Chaganti, R.S. (1998). Nonsyntenic amplification of MYC with CDK4 and MDM2 in a malignant mixed tumor of salivary gland. Cancer Genet Cytogenet *105*, 160-163.

Rashkovan, M., Vadnais, C., Ross, J., Gigoux, M., Suh, W.K., Gu, W., Kosan, C., and Moroy, T. (2014). Miz-1 regulates translation of Trp53 via ribosomal protein L22 in cells undergoing V(D)J recombination. Proc Natl Acad Sci U S A *111*, E5411-5419.

Rauen, K.A. (2013). The RASopathies. Annu Rev Genomics Hum Genet *14*, 355-369.

Ravi, A., Gurtan, A.M., Kumar, M.S., Bhutkar, A., Chin, C., Lu, V., Lees, J.A., Jacks, T., and Sharp, P.A. (2012). Proliferation and tumorigenesis of a murine sarcoma cell line in the absence of DICER1. Cancer Cell *21*, 848-855.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature *403*, 901-906.

Rice, K.L., Hormaeche, I., Doulatov, S., Flatow, J.M., Grimwade, D., Mills, K.I., Leiva, M., Ablain, J., Ambardekar, C., McConnell, M.J., *et al.* (2009). Comprehensive genomic screens identify a role for PLZF-RARalpha as a positive regulator of cell proliferation via direct regulation of c-MYC. Blood *114*, 5499-5511.

Richardson, P.G., Schlossman, R.L., Alsina, M., Weber, D.M., Coutre, S.E., Gasparetto, C., Mukhopadhyay, S., Ondovik, M.S., Khan, M., Paley, C.S., *et al.* (2013). PANORAMA 2: panobinostat in combination with bortezomib and dexamethasone in patients with relapsed and bortezomib-refractory myeloma. Blood *122*, 2331-2337.

Rickert, R.C., Roes, J., and Rajewsky, K. (1997). B lymphocyte-specific, Cre-mediated mutagenesis in mice. Nucleic Acids Res *25*, 1317-1318.

Riggs, M.G., Whittaker, R.G., Neumann, J.R., and Ingram, V.M. (1977). n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. Nature *268*, 462-464.

Rottmann, S., and Luscher, B. (2006). The Mad side of the Max network: antagonizing the function of Myc and more. Curr Top Microbiol Immunol *302*, 63-122.

Roussel, M., Saule, S., Lagrou, C., Rommens, C., Beug, H., Graf, T., and Stehelin, D. (1979). Three new types of viral oncogene of cellular origin specific for haematopoietic cell transformation. Nature *281*, 452-455.

Roussel, M.F., Cleveland, J.L., Shurtleff, S.A., and Sherr, C.J. (1991). Myc rescue of a mutant CSF-1 receptor impaired in mitogenic signalling. Nature *353*, 361-363.

Sabo, A., Kress, T.R., Pelizzola, M., de Pretis, S., Gorski, M.M., Tesi, A., Morelli, M.J., Bora, P., Doni, M., Verrecchia, A., *et al.* (2014). Selective transcriptional regulation by Myc in cellular growth control and lymphomagenesis. Nature *511*, 488-492.

Saito, Y., Liang, G., Egger, G., Friedman, J.M., Chuang, J.C., Coetzee, G.A., and Jones, P.A. (2006). Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell *9*, 435-443.

Sakamuro, D., Eviner, V., Elliott, K.J., Showe, L., White, E., and Prendergast, G.C. (1995). c-Myc induces apoptosis in epithelial cells by both p53-dependent and p53-independent mechanisms. Oncogene *11*, 2411-2418.

Salghetti, S.E., Caudy, A.A., Chenoweth, J.G., and Tansey, W.P. (2001). Regulation of transcriptional activation domain function by ubiquitin. Science *293*, 1651-1653.

Salghetti, S.E., Kim, S.Y., and Tansey, W.P. (1999). Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. EMBO J *18*, 717-726.

San-Miguel, J.F., Richardson, P.G., Gunther, A., Sezer, O., Siegel, D., Blade, J., LeBlanc, R., Sutherland, H., Sopala, M., Mishra, K.K., *et al.* (2013). Phase Ib study of panobinostat and bortezomib in relapsed or relapsed and refractory multiple myeloma. J Clin Oncol *31*, 3696-3703.

Schmid, P., Schulz, W.A., and Hameister, H. (1989). Dynamic expression pattern of the myc protooncogene in midgestation mouse embryos. Science *243*, 226-229.

Schneider, A., Peukert, K., Eilers, M., and Hanel, F. (1997). Association of Myc with the zinc-finger protein Miz-1 defines a novel pathway for gene regulation by Myc. Curr Top Microbiol Immunol *224*, 137-146.

Scott, G.K., Mattie, M.D., Berger, C.E., Benz, S.C., and Benz, C.C. (2006). Rapid alteration of microRNA levels by histone deacetylase inhibition. Cancer Res *66*, 1277-1281.

Sears, R., Ohtani, K., and Nevins, J.R. (1997). Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals. Mol Cell Biol *17*, 5227-5235.

Seitz, V., Butzhammer, P., Hirsch, B., Hecht, J., Gutgemann, I., Ehlers, A., Lenze, D., Oker, E., Sommerfeld, A., von der Wall, E., *et al.* (2011). Deep sequencing of MYC DNA-binding sites in Burkitt lymphoma. PLoS One *6*, e26837.

Sekine, S., Ogawa, R., McManus, M.T., Kanai, Y., and Hebrok, M. (2009). Dicer is required for proper liver zonation. J Pathol *219*, 365-372.

Sevignani, C., Calin, G.A., Nnadi, S.C., Shimizu, M., Davuluri, R.V., Hyslop, T., Demant, P., Croce, C.M., and Siracusa, L.D. (2007). MicroRNA genes are frequently located near mouse cancer susceptibility loci. Proc Natl Acad Sci U S A *104*, 8017-8022.

Shachaf, C.M., Kopelman, A.M., Arvanitis, C., Karlsson, A., Beer, S., Mandl, S., Bachmann, M.H., Borowsky, A.D., Ruebner, B., Cardiff, R.D., *et al.* (2004). MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. Nature *431*, 1112-1117.

Shah, U.S., Dhir, R., Gollin, S.M., Chandran, U.R., Lewis, D., Acquafondata, M., and Pflug, B.R. (2006). Fatty acid synthase gene overexpression and copy number gain in prostate adenocarcinoma. Hum Pathol *37*, 401-409.

Sheiness, D., and Bishop, J.M. (1979). DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis virus. J Virol *31*, 514-521.

Sheiness, D., Fanshier, L., and Bishop, J.M. (1978). Identification of nucleotide sequences which may encode the oncogenic capacity of avian retrovirus MC29. J Virol *28*, 600-610.

Shi, Y., Glynn, J.M., Guilbert, L.J., Cotter, T.G., Bissonnette, R.P., and Green, D.R. (1992). Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas. Science *257*, 212-214.

Shibuya, H., Yoneyama, M., Ninomiya-Tsuji, J., Matsumoto, K., and Taniguchi, T. (1992). IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: demonstration of a novel role for c-myc. Cell *70*, 57-67.

Shim, H., Dolde, C., Lewis, B.C., Wu, C.S., Dang, G., Jungmann, R.A., Dalla-Favera, R., and Dang, C.V. (1997). c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. Proc Natl Acad Sci U S A *94*, 6658-6663.

Shima, E.A., Le Beau, M.M., McKeithan, T.W., Minowada, J., Showe, L.C., Mak, T.W., Minden, M.D., Rowley, J.D., and Diaz, M.O. (1986). Gene encoding the alpha chain of the T-cell receptor is moved immediately downstream of c-myc in a chromosomal 8;14 translocation in a cell line from a human T-cell leukemia. Proc Natl Acad Sci U S A *83*, 3439-3443.

Shimizu, S., Takehara, T., Hikita, H., Kodama, T., Miyagi, T., Hosui, A., Tatsumi, T., Ishida, H., Noda, T., Nagano, H., *et al.* (2010). The let-7 family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma. J Hepatol *52*, 698-704.

Shrivastava, A., Saleque, S., Kalpana, G.V., Artandi, S., Goff, S.P., and Calame, K. (1993). Inhibition of transcriptional regulator Yin-Yang-1 by association with c-Myc. Science *262*, 1889-1892.

Slany, R.K. (2009). The molecular biology of mixed lineage leukemia. Haematologica *94*, 984-993.

Sodir, N.M., Swigart, L.B., Karnezis, A.N., Hanahan, D., Evan, G.I., and Soucek, L. (2011). Endogenous Myc maintains the tumor microenvironment. Genes Dev *25*, 907-916.

Soucek, L., Whitfield, J., Martins, C.P., Finch, A.J., Murphy, D.J., Sodir, N.M., Karnezis, A.N., Swigart, L.B., Nasi, S., and Evan, G.I. (2008). Modelling Myc inhibition as a cancer therapy. Nature *455*, 679-683.

Staller, P., Peukert, K., Kiermaier, A., Seoane, J., Lukas, J., Karsunky, H., Moroy, T., Bartek, J., Massague, J., Hanel, F., *et al.* (2001). Repression of p15INK4b expression by Myc through association with Miz-1. Nat Cell Biol *3*, 392-399.

Stasik, C.J., Nitta, H., Zhang, W., Mosher, C.H., Cook, J.R., Tubbs, R.R., Unger, J.M., Brooks, T.A., Persky, D.O., Wilkinson, S.T., *et al.* (2010). Increased MYC gene copy number correlates with increased mRNA levels in diffuse large B-cell lymphoma. Haematologica *95*, 597-603.

Steffen, D. (1984). Proviruses are adjacent to c-myc in some murine leukemia virus-induced lymphomas. Proc Natl Acad Sci U S A *81*, 2097-2101.

Steinmann, S., Schulte, K., Beck, K., Chachra, S., Bujnicki, T., and Klempnauer, K.H. (2009). v-Myc inhibits C/EBPbeta activity by preventing C/EBPbeta-induced phosphorylation of the co-activator p300. Oncogene *28*, 2446-2455.

Stengel, K.R., and Hiebert, S.W. (2014). Class I HDACs Affect DNA Replication, Repair, and Chromatin Structure: Implications for Cancer Therapy. Antioxidants & redox signaling.

Stengel, K.R., and Hiebert, S.W. (2015). Class I HDACs Affect DNA Replication, Repair, and Chromatin Structure: Implications for Cancer Therapy. Antioxid Redox Signal *23*, 51-65.

Stengel, K.R., Zhao, Y., Klus, N.J., Kaiser, J.F., Gordy, L.E., Joyce, S., Hiebert, S.W., and Summers, A.R. (2015). Histone Deacetylase 3 Is Required for Efficient T Cell Development. Mol Cell Biol *35*, 3854-3865.

Stone, J., de Lange, T., Ramsay, G., Jakobovits, E., Bishop, J.M., Varmus, H., and Lee, W. (1987). Definition of regions in human c-myc that are involved in transformation and nuclear localization. Mol Cell Biol *7*, 1697-1709.

Strasser, A., Harris, A.W., Bath, M.L., and Cory, S. (1990). Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature *348*, 331-333.

Sugars, K.L., Budhram-Mahadeo, V., Packham, G., and Latchman, D.S. (2001). A minimal Bcl-x promoter is activated by Brn-3a and repressed by p53. Nucleic acids research *29*, 4530-4540.

Summers, A.R., Fischer, M.A., Stengel, K.R., Zhao, Y., Kaiser, J.F., Wells, C.E., Hunt, A., Bhaskara, S., Luzwick, J.W., Sampathi, S., *et al.* (2013). HDAC3 is essential for DNA replication in hematopoietic progenitor cells. J Clin Invest *123*, 3112-3123.

Sun, Y., Liu, P.Y., Scarlett, C.J., Malyukova, A., Liu, B., Marshall, G.M., MacKenzie, K.L., Biankin, A.V., and Liu, T. (2014). Histone deacetylase 5 blocks neuroblastoma cell differentiation by interacting with N-Myc. Oncogene *33*, 2987-2994.

Tansey, W.P. (2014). Mammalian MYC proteins and cancer. New Journal of Science. Article ID 757534.

Taft, R.J., Glazov, E.A., Lassmann, T., Hayashizaki, Y., Carninci, P., and Mattick, J.S. (2009). Small RNAs derived from snoRNAs. RNA *15*, 1233-1240.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663-676.

Takamizawa, J., Konishi, H., Yanagisawa, K., Tomida, S., Osada, H., Endoh, H., Harano, T., Yatabe, Y., Nagino, M., Nimura, Y., *et al.* (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res *64*, 3753-3756.

Taub, R., Kirsch, I., Morton, C., Lenoir, G., Swan, D., Tronick, S., Aaronson, S., and Leder, P. (1982). Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci U S A *79*, 7837-7841.

Tay, Y., Zhang, J., Thomson, A.M., Lim, B., and Rigoutsos, I. (2008). MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature *455*, 1124-1128.

Thomas, L., Stamberg, J., Gojo, I., Ning, Y., and Rapoport, A.P. (2004). Double minute chromosomes in monoblastic (M5) and myeloblastic (M2) acute myeloid leukemia: two case reports and a review of literature. Am J Hematol *77*, 55-61.

Thomas, L.R., Wang, Q., Grieb, B.C., Phan, J., Foshage, A.M., Sun, Q., Olejniczak, E.T., Clark, T., Dey, S., Lorey, S., *et al.* (2015). Interaction with WDR5 promotes target gene recognition and tumorigenesis by MYC. Mol Cell *58*, 440-452.

Thompson, R.C., Vardinogiannis, I., and Gilmore, T.D. (2013). The sensitivity of diffuse large B-cell lymphoma cell lines to histone deacetylase inhibitor-induced apoptosis is modulated by BCL-2 family protein activity. PloS one *8*, e62822.

Tikhonenko, A.T., Black, D.J., and Linial, M.L. (1996). Viral Myc oncoproteins in infected fibroblasts down-modulate thrombospondin-1, a possible tumor suppressor gene. J Biol Chem *271*, 30741-30747.

Tomari, Y., Du, T., and Zamore, P.D. (2007). Sorting of Drosophila small silencing RNAs. Cell *130*, 299-308.

Torres, A., Torres, K., Paszkowski, T., Jodlowska-Jedrych, B., Radomanski, T., Ksiazek, A., and Maciejewski, R. (2011). Major regulators of microRNAs biogenesis Dicer and Drosha are down-regulated in endometrial cancer. Tumour Biol *32*, 769-776.

Toyota, M., Suzuki, H., Sasaki, Y., Maruyama, R., Imai, K., Shinomura, Y., and Tokino, T. (2008). Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res *68*, 4123-4132.

Trumpp, A., Refaeli, Y., Oskarsson, T., Gasser, S., Murphy, M., Martin, G.R., and Bishop, J.M. (2001). c-Myc regulates mammalian body size by controlling cell number but not cell size. Nature *414*, 768-773.

Turner, B.M. (2007). Defining an epigenetic code. Nat Cell Biol *9*, 2-6.

Ungerstedt, J.S., Sowa, Y., Xu, W.S., Shao, Y., Dokmanovic, M., Perez, G., Ngo, L., Holmgren, A., Jiang, X., and Marks, P.A. (2005). Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors. Proc Natl Acad Sci U S A *102*, 673-678.

Uribesalgo, I., Benitah, S.A., and Di Croce, L. (2012). From oncogene to tumor suppressor: the dual role of Myc in leukemia. Cell Cycle *11*, 1757-1764.

van Riggelen, J., Muller, J., Otto, T., Beuger, V., Yetil, A., Choi, P.S., Kosan, C., Moroy, T., Felsher, D.W., and Eilers, M. (2010a). The interaction between Myc and Miz1 is required to antagonize TGFbeta-dependent autocrine signaling during lymphoma formation and maintenance. Genes Dev *24*, 1281-1294.

van Riggelen, J., Yetil, A., and Felsher, D.W. (2010b). MYC as a regulator of ribosome biogenesis and protein synthesis. Nat Rev Cancer *10*, 301-309.

Vander Heiden, M.G. (2011). Targeting cancer metabolism: a therapeutic window opens. Nat Rev Drug Discov *10*, 671-684.

Vannini, A., Volpari, C., Filocamo, G., Casavola, E.C., Brunetti, M., Renzoni, D., Chakravarty, P., Paolini, C., De Francesco, R., Gallinari, P., *et al.* (2004). Crystal structure of a eukaryotic zinc-dependent histone deacetylase, human HDAC8, complexed with a hydroxamic acid inhibitor. Proc Natl Acad Sci U S A *101*, 15064-15069.

Vaux, D.L., Cory, S., and Adams, J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature *335*, 440-442.

Veis, D.J., Sorenson, C.M., Shutter, J.R., and Korsmeyer, S.J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell *75*, 229-240.

Vennstrom, B., Sheiness, D., Zabielski, J., and Bishop, J.M. (1982). Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. J Virol *42*, 773-779.

Vita, M., and Henriksson, M. (2006). The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol *16*, 318-330.

Volinia, S., Calin, G.A., Liu, C.G., Ambs, S., Cimmino, A., Petrocca, F., Visone, R., Iorio, M., Roldo, C., Ferracin, M., *et al.* (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. Proceedings of the National Academy of Sciences of the United States of America *103*, 2257-2261.

von Eyss, B., and Eilers, M. (2011). Addicted to Myc--but why? Genes Dev *25*, 895-897.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

Vrana, J.A., Decker, R.H., Johnson, C.R., Wang, Z., Jarvis, W.D., Richon, V.M., Ehinger, M., Fisher, P.B., and Grant, S. (1999). Induction of apoptosis in U937 human leukemia cells by suberoylanilide hydroxamic acid (SAHA) proceeds through pathways that are regulated by Bcl-2/Bcl-XL, c-Jun, and p21CIP1, but independent of p53. Oncogene *18*, 7016-7025.

Wagner, A.J., Kokontis, J.M., and Hay, N. (1994). Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1. Genes Dev *8*, 2817-2830.

Wagner, A.J., Meyers, C., Laimins, L.A., and Hay, N. (1993). c-Myc induces the expression and activity of ornithine decarboxylase. Cell Growth Differ *4*, 879-883.

Walz, S., Lorenzin, F., Morton, J., Wiese, K.E., von Eyss, B., Herold, S., Rycak, L., Dumay-Odelot, H., Karim, S., Bartkuhn, M., *et al.* (2014). Activation and repression by oncogenic MYC shape tumour-specific gene expression profiles. Nature *511*, 483-487.

Wang, P., Lushnikova, T., Odvody, J., Greiner, T.C., Jones, S.N., and Eischen, C.M. (2008). Elevated Mdm2 expression induces chromosomal instability and confers a survival and growth advantage to B cells. Oncogene *27*, 1590-1598.

Wang, S., Huang, J., Lyu, H., Lee, C.K., Tan, J., Wang, J., and Liu, B. (2013). Functional cooperation of miR-125a, miR-125b, and miR-205 in entinostat-induced downregulation of erbB2/erbB3 and apoptosis in breast cancer cells. Cell death & disease *4*, e556.

Wang, Y.J., McAllister, F., Bailey, J.M., Scott, S.G., Hendley, A.M., Leach, S.D., and Ghosh, B. (2014). Dicer is required for maintenance of adult pancreatic acinar cell identity and plays a role in Kras-driven pancreatic neoplasia. PLoS One *9*, e113127.

Watson, I.R., Takahashi, K., Futreal, P.A., and Chin, L. (2013). Emerging patterns of somatic mutations in cancer. Nat Rev Genet *14*, 703-718.

Weber, B., Stresemann, C., Brueckner, B., and Lyko, F. (2007). Methylation of human microRNA genes in normal and neoplastic cells. Cell Cycle *6*, 1001-1005.

Wee, E.J., Peters, K., Nair, S.S., Hulf, T., Stein, S., Wagner, S., Bailey, P., Lee, S.Y., Qu, W.J., Brewster, B., *et al.* (2012). Mapping the regulatory sequences controlling 93 breast cancer-associated miRNA genes leads to the identification of two functional promoters of the Hsa-mir-200b cluster, methylation of which is associated with metastasis or hormone receptor status in advanced breast cancer. Oncogene *31*, 4182-4195.

Wei, W., Ba, Z., Gao, M., Wu, Y., Ma, Y., Amiard, S., White, C.I., Rendtlew Danielsen, J.M., Yang, Y.G., and Qi, Y. (2012). A role for small RNAs in DNA double-strand break repair. Cell *149*, 101-112.

Welcker, M., Orian, A., Grim, J.E., Eisenman, R.N., and Clurman, B.E. (2004a). A nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size. Curr Biol *14*, 1852-1857.

Welcker, M., Orian, A., Jin, J., Grim, J.E., Harper, J.W., Eisenman, R.N., and Clurman, B.E. (2004b). The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. Proc Natl Acad Sci U S A *101*, 9085-9090.

Welcker, M., Singer, J., Loeb, K.R., Grim, J., Bloecher, A., Gurien-West, M., Clurman, B.E., and Roberts, J.M. (2003). Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation. Mol Cell *12*, 381-392.

Wells, C.E., Bhaskara, S., Stengel, K.R., Zhao, Y., Sirbu, B., Chagot, B., Cortez, D., Khabele, D., Chazin, W.J., Cooper, A., *et al.* (2013). Inhibition of histone deacetylase 3 causes replication stress in cutaneous T cell lymphoma. PLoS One *8*, e68915.

West, A.C., and Johnstone, R.W. (2014). New and emerging HDAC inhibitors for cancer treatment. J Clin Invest *124*, 30-39.

Whitecross, K.F., Alsop, A.E., Cluse, L.A., Wiegmans, A., Banks, K.M., Coomans, C., Peart, M.J., Newbold, A., Lindemann, R.K., and Johnstone, R.W. (2009). Defining the target specificity of ABT-737 and synergistic antitumor activities in combination with histone deacetylase inhibitors. Blood *113*, 1982-1991.

Whittaker, S.J., Demierre, M.F., Kim, E.J., Rook, A.H., Lerner, A., Duvic, M., Scarisbrick, J., Reddy, S., Robak, T., Becker, J.C., *et al.* (2010). Final results from a multicenter, international, pivotal study of romidepsin in refractory cutaneous T-cell lymphoma. J Clin Oncol *28*, 4485-4491.

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell *75*, 855-862.

Willis, T.G., and Dyer, M.J. (2000). The role of immunoglobulin translocations in the pathogenesis of B-cell malignancies. Blood *96*, 808-822.

Wilson, A., Murphy, M.J., Oskarsson, T., Kaloulis, K., Bettess, M.D., Oser, G.M., Pasche, A.C., Knabenhans, C., Macdonald, H.R., and Trumpp, A. (2004). c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev *18*, 2747-2763.

Winter, J., and Diederichs, S. (2011). Argonaute proteins regulate microRNA stability: Increased microRNA abundance by Argonaute proteins is due to microRNA stabilization. RNA Biol *8*, 1149-1157.

Wise, D.R., DeBerardinis, R.J., Mancuso, A., Sayed, N., Zhang, X.Y., Pfeiffer, H.K., Nissim, I., Daikhin, E., Yudkoff, M., McMahon, S.B., *et al.* (2008). Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. Proc Natl Acad Sci U S A *105*, 18782-18787.

Wolf, E., Gebhardt, A., Kawauchi, D., Walz, S., von Eyss, B., Wagner, N., Renninger, C., Krohne, G., Asan, E., Roussel, M.F., *et al.* (2013). Miz1 is required to maintain autophagic flux. Nat Commun *4*, 2535.

Wu, S., Cetinkaya, C., Munoz-Alonso, M.J., von der Lehr, N., Bahram, F., Beuger, V., Eilers, M., Leon, J., and Larsson, L.G. (2003). Myc represses differentiation-induced p21CIP1 expression via Miz-1-dependent interaction with the p21 core promoter. Oncogene *22*, 351-360.

Xu, D., Popov, N., Hou, M., Wang, Q., Bjorkholm, M., Gruber, A., Menkel, A.R., and Henriksson, M. (2001). Switch from Myc/Max to Mad1/Max binding and decrease in histone acetylation at the telomerase reverse transcriptase promoter during differentiation of HL60 cells. Proc Natl Acad Sci U S A *98*, 3826-3831.

Xu, S., Guo, K., Zeng, Q., Huo, J., and Lam, K.P. (2012). The RNase III enzyme Dicer is essential for germinal center B-cell formation. Blood *119*, 767-776.

Xu, W.S., Parmigiani, R.B., and Marks, P.A. (2007). Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene *26*, 5541-5552.

Yi, R., Qin, Y., Macara, I.G., and Cullen, B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev *17*, 3011-3016.

Yin, K.J., Deng, Z., Huang, H., Hamblin, M., Xie, C., Zhang, J., and Chen, Y.E. (2010). miR-497 regulates neuronal death in mouse brain after transient focal cerebral ischemia. Neurobiol Dis *38*, 17-26.

You, J.S., and Jones, P.A. (2012). Cancer genetics and epigenetics: two sides of the same coin? Cancer Cell *22*, 9-20.

Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell *101*, 25-33.

Zeller, K.I., Zhao, X., Lee, C.W., Chiu, K.P., Yao, F., Yustein, J.T., Ooi, H.S., Orlov, Y.L., Shahab, A., Yong, H.C., *et al.* (2006). Global mapping of c-Myc binding sites and target gene networks in human B cells. Proc Natl Acad Sci U S A *103*, 17834-17839.

Zhang, B., Chen, H., Zhang, L., Dakhova, O., Zhang, Y., Lewis, M.T., Creighton, C.J., Ittmann, M.M., and Xin, L. (2014). A dosage-dependent pleiotropic role of Dicer in prostate cancer growth and metastasis. Oncogene *33*, 3099-3108.

Zhang, X., Chen, X., Lin, J., Lwin, T., Wright, G., Moscinski, L.C., Dalton, W.S., Seto, E., Wright, K., Sotomayor, E., *et al.* (2012a). Myc represses miR-15a/miR-16-1 expression through recruitment of HDAC3 in mantle cell and other non-Hodgkin B-cell lymphomas. Oncogene *31*, 3002-3008.

Zhang, X., Zhao, X., Fiskus, W., Lin, J., Lwin, T., Rao, R., Zhang, Y., Chan, J.C., Fu, K., Marquez, V.E., *et al.* (2012b). Coordinated silencing of MYC-mediated miR-29 by HDAC3 and EZH2 as a therapeutic target of histone modification in aggressive B-Cell lymphomas. Cancer Cell *22*, 506-523.

Zhang, X.Y., DeSalle, L.M., and McMahon, S.B. (2006). Identification of novel targets of MYC whose transcription requires the essential MbII domain. Cell Cycle *5*, 238-241.

Zhang, Z., Zhang, B., Li, W., Fu, L., Fu, L., Zhu, Z., and Dong, J.T. (2011). Epigenetic Silencing of miR-203 Upregulates SNAI2 and Contributes to the Invasiveness of Malignant Breast Cancer Cells. Genes Cancer *2*, 782-791.

Zhao, X., Lwin, T., Zhang, X., Huang, A., Wang, J., Marquez, V.E., Chen-Kiang, S., Dalton, W.S., Sotomayor, E., and Tao, J. (2013). Disruption of the MYC-miRNA-EZH2 loop to suppress aggressive B-cell lymphoma survival and clonogenicity. Leukemia *27*, 2341-2350.

Zhu, H., Yang, Y., Wang, Y., Li, J., Schiller, P.W., and Peng, T. (2011). MicroRNA-195 promotes palmitate-induced apoptosis in cardiomyocytes by down-regulating Sirt1. Cardiovasc Res *92*, 75-84.

Zindy, F., Eischen, C.M., Randle, D.H., Kamijo, T., Cleveland, J.L., Sherr, C.J., and Roussel, M.F. (1998). Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev *12*, 2424-2433.