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MYC through *miR-17-92* Suppresses Specific Target Genes to Maintain Survival, Autonomous Proliferation and a Neoplastic State

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SUMMARY

The *MYC* oncogene regulates gene expression through multiple mechanisms and its overexpression culminates in tumorigenesis. *MYC* inactivation reverses tumorigenesis through the loss of hallmark features of cancer including autonomous proliferation and survival. Here we report that *MYC* via *miR-17-92* maintains a neoplastic state through the suppression of chromatin regulatory genes *Sin3b*, *Hbp1*, *Suv420h1*, and *Btg1*, as well as the apoptosis regulator *Bim*. The enforced expression of *miR-17-92* prevents *MYC* suppression from inducing proliferative arrest, senescence, and apoptosis, and abrogates sustained tumor regression. Knockdown of the five *miR-17-92* target genes blocks senescence and apoptosis while it modestly delays proliferative arrest, thus partially recapitulating *miR-17-92* function. We conclude that *MYC*, via *miR-17-92*, maintains a neoplastic state by suppressing specific target genes.

INTRODUCTIONS

Cancers are often dependent on or addicted to the initiating oncogenes for the maintenance of the malignant phenotype (Chin et al., 1999; Felsher and Bishop, 1999; Huettner et al., 2000; Weinstein, 2002). The inactivation of a single driver oncogene can result in rapid and sustained tumor regression. Oncogene addiction has been exploited clinically in targeted therapies, such as imatinib for BCR-ABL-driven chronic myelogenous leukemia, gefitinib for lung adenocarcinoma with *EGFR* mutations, and vemurafenib for melanomas with *B-RAF* mutations (Chapman et al., 2011; Druker et al., 1996; Ladanyi and Pao, 2008). Hence,

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AUTHOR CONTRIBUTIONS

Y.L., P.S.C., and D.W.F. designed research; Y.L. and P.S.C. performed experiments; Y.L. and D.L.D. analyzed the microarray data; S.C.C. contributed to data analysis; Y.L. and D.W.F. wrote the paper.

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the targeted inactivation of oncogenes appears to be a generalizable approach for the treatment of many cancers.

The *MYC* oncogene is overexpressed in over half of human cancers (Dang, 2012). To study the role of *MYC* in the initiation and maintenance of tumorigenesis, some investigators have used the tetracycline regulatory system (Tet system) to generate reversible models of cancer (Gossen and Bujard, 1992). In these mouse models, the overexpression of a conditional *MYC* transgene initiates tumorigenesis, and its inactivation results in rapid, complete and sustained tumor regression. *MYC* inactivation is associated with the loss of many of the hallmark features of tumorigenesis and results in proliferative arrest, apoptosis, differentiation, and senescence, as well as the shutdown of angiogenesis (D'Cruz et al., 2001; Felsher and Bishop, 1999; Hanahan and Weinberg, 2011; Shachaf et al., 2004; Wu et al., 2007).

MYC is a transcriptional regulator of a multitude of genes, but it is unclear if any of these genes are responsible for *MYC* to maintain a neoplastic state (Dang, 2012). Recently, it has been shown that *MYC* may regulate gene expression as a general transcriptional amplifier (Lin et al., 2012; Nie et al., 2012). However, it has been pointed out that this would not explain how *MYC* can suppress gene expression or regulate gene expression in a specific manner (Walz et al., 2013). *MYC* has also been shown to regulate the expression of several microRNAs, including the polycistronic *miR-17-92* cluster (Bui and Mendell, 2010; O'Donnell et al., 2005; Sander et al., 2008). The *miR-17-92* cluster is overexpressed in human lymphomas (He et al., 2005). Notably, overexpression of *miR-17-92* cooperates with *MYC* to induce lymphomagenesis, while deletion of *miR-17-92* induces the death of lymphoma cells (He et al., 2005; Mu et al., 2009).

We hypothesized that *miR-17-92* is causally responsible for at least part of the mechanism by which *MYC* maintains a neoplastic state (Figure 1A). Here, we found that *MYC*, through *miR-17-92*, regulates the expression of specific chromatin regulatory genes, such as *Sin3b*, *Hbp1*, *Suv420h1*, and *Btg1*, as well as the apoptosis regulator *Bim*. Upon *MYC* inactivation, the downregulation of *miR-17-92* and the corresponding induction of these target genes is causally required for the activation of the apoptosis and senescence programs and sustained tumor regression. Hence, *MYC* suppression of these genes is one of the required mechanisms to maintain a neoplastic state.

RESULTS

Sustained *miR-17-92* Expression Rescues *MYC* Addiction *In Vitro* and *In Vivo*

We examined the level of several microRNAs known to be regulated by *MYC* using real-time quantitative PCR in three lymphoma cell lines from *E μ -tTA/tet-O-MYC* mice (O'Donnell et al., 2005; Sander et al., 2008). Upon *MYC* inactivation with doxycycline treatment, all members of the *miR-17-92* cluster were downregulated, while *miR-15/16* and *miR-26* were upregulated in a time-dependent manner (Figure S1A). Similarly, in *MYC*-driven hepatocellular carcinoma derived from *LAP-tTA/tet-O-MYC* transgenic mice, *miR-17-92*, but not *miR-15/16* and *miR-26*, was downregulated upon *MYC* inactivation

(Kota et al., 2009; Shachaf et al., 2004) (Figure S1B). Thus, MYC generally regulates the expression of *miR-17-92* in tumor cells.

We speculated that *miR-17-92* was responsible for maintaining at least some of the hallmark features of cancer in MYC-induced tumors (Figure 1A). To determine whether constitutive expression of *miR-17-92* could rescue any of the effects of MYC inactivation, we retrovirally infected MYC-induced lymphomas with Murine Stem Cell Virus containing *miR-17-92* (MSCV-*miR-17-92*), then confirmed that expression of *miR-17-92* was maintained even after MYC inactivation (Figure 1B and S1C). Retroviral *miR-17-92* expression abrogated the induction of proliferative arrest, apoptosis, and senescence, which we previously described as consequences of MYC suppression (Felsher and Bishop, 1999; Wu et al., 2007). The proliferation was sustained over a 5-day time course in *miR-17-92*-expressing cells after MYC inactivation as shown by the S/G2/M population in the flow cytometric analysis of cell cycle distributions (Figure 1C, S1D). The induction of apoptosis by MYC inactivation was blocked by *miR-17-92* as shown by the subG1 population and the 7-AAD/Annexin V double positive population (Figure 1C–D, S1D). Furthermore, retroviral *miR-17-92* expression blocked the induction of cellular senescence in a sustained manner as measured by senescence-associated β -galactosidase (SA- β -gal) staining (Figure 1E, S1E), histone H4 lysine 20 trimethylation (H4K20me3) staining (Figure 1E, S1F), and quantification of trimethylated histone H3 lysine 9 (H3K9me3) (Figure S1G). Similarly, in MYC-induced hepatocellular carcinoma and osteosarcoma (Jain et al., 2002; Shachaf et al., 2004), retroviral expression of *miR-17-92* abrogated the induction of cellular senescence upon MYC suppression as shown by SA- β -gal staining (control versus *miR-17-92*: 19-fold versus 3-fold induction in hepatocellular carcinoma, 10-fold versus 3-fold induction in osteosarcoma; Figure S1H–I). In contrast, *miR-17-92* expression in three BCR-ABL-driven B-cell leukemia cell lines failed to rescue proliferative arrest or apoptosis upon BCR-ABL inactivation (Figure S1J). Hence, *miR-17-92* specifically rescues the proliferative arrest, apoptosis, and senescence upon MYC inactivation.

Next, we examined *in vivo* whether *miR-17-92* expression rescues the phenotypes of MYC inactivation. MYC-induced lymphoma cells expressing either empty control vector or MSCV-*miR-17-92* were subcutaneously transplanted into syngeneic FVB/N hosts. Tumor cells were allowed to grow *in vivo* for about 2 weeks before MYC inactivation by doxycycline administration in the drinking water. Tumors were collected before and after MYC inactivation for examination of apoptosis, proliferation, and senescence (Fig. 2A–D). Phospho-histone H3 and Ki67 staining was used to measure mitotic and proliferative cells, respectively. Cleaved-caspase 3 staining was used for apoptotic cells and SA- β -gal and H4K20me3 staining was used for senescent cells. Upon MYC inactivation, in control versus lymphomas with *miR-17-92* expression, there was a 67% versus 15% decrease in phospho-histone H3 staining and a 85% versus 25% decrease in Ki67 staining (Figure 2A and Figure S2), a 60% increase versus no change in cleaved-caspase 3 staining (Figure 2B), and a 3-fold increase versus no change in SA- β -gal and H4K20me3 staining (Figure 2C–D). Thus, *miR-17-92* expression prevented MYC inactivation from inducing proliferative arrest, apoptosis, and senescence *in vivo*.

MYC via *miR-17-92* Regulates Specific Target Genes

We reasoned that MYC, through *miR-17-92*, regulates a specific subset of genes responsible for maintaining autonomous proliferation and survival. Since the expression of *MYC* or *miR-17-92* can be turned off independently in our conditional system, this allows for the screening of genes regulated by MYC or *miR-17-92* (Figure 1B, S3). The genes that were differentially expressed before and after MYC inactivation in the control lymphoma were categorized as MYC-regulated. The genes that were differentially expressed between control lymphoma and retroviral *miR-17-92*-expressing lymphoma when MYC was turned off in both populations were defined as *miR-17-92*-regulated (Figure S3). The specific subset of MYC target genes regulated through *miR-17-92* would appear to be co-regulated by both MYC and *miR-17-92* (Figure S3 and 3A). By comparing the microarray gene expression profiles of control lymphoma versus *miR-17-92*-expressing lymphoma upon MYC inactivation, we found that 70% of *miR-17-92*-regulated genes were also regulated by MYC (Figure 3A). The 401 overlapping genes co-regulated by MYC and *miR-17-92* were further separated into the upregulated and downregulated groups (Figure 3B). Among the genes upregulated by both MYC and *miR-17-92*, there was an enrichment of genes involved in DNA replication, repair, and cell cycle (Figure 3C). Notably, the genes downregulated by both MYC and *miR-17-92* had 4.6-fold more *miR-17-92* binding sites in their 3'UTR compared with upregulated genes (32% in downregulated versus 7% in upregulated genes, Table S1, S2). We inferred that these downregulated genes are directly regulated by *miR-17-92* binding.

Our gene list was further refined by only including genes with at least two *miR-17-92* binding sites in their 3'UTR, as predicted by each of three microRNA target scanning programs (miRanda, Targetscan, and miRWalk) (Figure 3B, 3D, and Table S3). Amongst these 15 genes were four chromatin modifiers that have not been previously reported as MYC or *miR-17-92* targets (*Sin3b*, *Hbp1*, *Suv420h1*, and *Btg1*). Also identified was the apoptosis regulator, *Bim*, that has been reported previously to be a *miR-17-92* target (Ventura et al., 2008; Xiao et al., 2008) (Figure 3D). Notably, all of these genes have been associated with proliferative control, senescence, and/or apoptosis (Berthet et al., 2002; David et al., 2008; Roninson, 2003; Swanson et al., 2004; van Oevelen et al., 2010). Thus, we focused our subsequent efforts on assessing whether these MYC/*miR-17-92* target genes could contribute to the consequences of MYC suppression in tumors.

We examined if these *miR-17-92* targets were directly regulated by MYC and *miR-17-92*. First, MYC inactivation induced the protein expression of *Sin3b*, *Hbp1*, *Suv420h1*, *Btg1*, and all three *Bim* isoforms in control but not *miR-17-92*-expressing cells as measured by Western blot analysis (Figure 4A, S4). Second, to validate whether these genes are direct targets of *miR-17-92*, a dual luciferase assay was performed by cloning 3'UTR fragments of all five genes, with either wild-type or mutant *miR-17-92* sites, downstream of the firefly luciferase coding region (Figure 4B, upper panel). Compared with mutant 3'UTRs lacking *miR-17-92* binding sites, the wild type 3'UTRs conferred significant repression as shown by the 20–60% lower firefly/renilla ratio (Figure 4B, lower panel). Hence, *Sin3b*, *Hbp1*, *Suv420h1*, *Btg1*, and *Bim* are regulated by MYC in a *miR-17-92*-dependent manner.

Next, we examined if MYC via *miR-17-92* was regulating chromatin through each of these gene products. *Sin3b* and *Hbp1* have been shown to be candidate target genes of *miR-19* (Mu et al., 2009). *Sin3b* interacts with *Hbp1* and recruits histone deacetylases (HDACs) to repress the transcription of genes related to proliferation, such as *Aurkb*, *Mybl2*, *Cdc6*, and *Bub1b* (David et al., 2008; Swanson et al., 2004; van Oevelen et al., 2010). Indeed, these genes were upregulated by *miR-17-92* and MYC (Figure 3C and Table S1). Upon MYC inactivation, there was a 3 to 8-fold increase versus a 2-fold increase in *Sin3b* binding to these promoters in control versus *miR-17-92*-expressing lymphoma according to a chromatin immunoprecipitation assay (Figure 4C). Thus, the induction of *Sin3b/Hbp1* upon MYC inactivation may contribute to proliferative arrest and cellular senescence by silencing genes related to proliferation and cell cycle.

Notably, *Suv420h1* is a histone methyltransferase that catalyzes dimethylation and trimethylation of histone H4 lysine 20 (H4K20me2 and H4K20me3) (Fraga et al., 2005; Greer and Shi, 2012). H4K20me3 is a marker of heterochromatin formation and senescence, and its loss is a common hallmark of human cancer (Fraga et al., 2005; Greer and Shi, 2012; Nelson, 2012). Upon MYC inactivation, there was an induction of both H4K20me2 and H4K20me3 in control but not *miR-17-92*-expressing lymphoma cells (Figure 4D). In contrast, the monomethylated H4K20me1, catalyzed by *Setd8* (Greer and Shi, 2012), did not increase in either control or *miR-17-92*-expressing lymphoma cells (Figure 4D), indicating that the induction of *Suv420h1* specifically increases the dimethylation and trimethylation of H4K20. Finally, *Btg1* is a tumor suppressor that can activate histone methyltransferase *Prmt1* to dimethylate histone H4 arginine 3 (H4R3me2) (Berthet et al., 2002; Lin et al., 1996). Upon MYC inactivation, there was an accumulation of H4R3me2 in control but not *miR-17-92* expressing cells (Figure 4D). Therefore, MYC inactivation via *miR-17-92* regulates the biological function of the chromatin regulatory genes *Sin3b*, *Hbp1*, *Suv420h1*, and *Btg1*.

Suppression of *Sin3b*, *Hbp1*, *Suv420h1*, *Btg1*, and *Bim* Significantly Recapitulates *miR-17-92* Function

Our results suggest that suppression of the four epigenetic regulators (*Sin3b*, *Hbp1*, *Suv420h1*, and *Btg1*, hereafter referred to as SHSB) and the pro-apoptotic protein *Bim* may contribute to MYC's ability to maintain tumorigenesis. To examine this, the *miR-30*-based retroviral short hairpin RNAs (shRNAs) were used to knock down the target genes individually or collectively in MYC-induced lymphoma cells. The target mRNA was knocked down to levels between 6–20% of the scrambled control (Figure S5A). Knocking down the expression of *Suv420h1* and *Btg1* reduced the levels of H4K20me2/3 and H4R3me2, respectively (Figure 4E–F). After 24 hours of MYC inactivation, lymphoma cells with individual knockdown of the chromatin modifiers exhibited a modest block in proliferative arrest, with 19–30% of cells still remaining in S/G2/M phases compared to only 11% for control cells (*Sin3b*: 25%; *Hbp1*: 25%; *Suv420h1*: 35%; *Btg1*: 19%; Figure S5B). Concurrently knocking down all five *miR-17-92* target genes (*Bim* and SHSB), modestly delayed the proliferative arrest after MYC inactivation as shown by flow cytometric analysis of the cell cycle distribution (Figure 5A–B). The knockdown of *Bim* alone reduced the induction of apoptosis following MYC inactivation, whereas the combined knockdown of

Bim and *SHSB* further decreased the rate of apoptosis (Figure 5C, S5C). Hence, these data suggest that *miR-17-92* regulation of its target genes *Sin3b*, *Hbp1*, *Suv420h1*, *Btg1*, and *Bim* is required for proliferative arrest and apoptosis upon MYC inactivation.

Cellular senescence is characterized by a state of permanent cell cycle arrest (Guney and Sedivy, 2006; Nardella et al., 2011). We have shown previously that even brief suppression of MYC can induce senescence and result in sustained tumor regression (Jain et al., 2002). We tested if cell cycle arrest induced by MYC inactivation is reversible (Figure 5D, ON-OFF-BackON). Upon MYC inactivation, control lymphoma cells progress from cell cycle arrest to high levels of apoptosis, with few viable cells remaining by 4–5 days after oncogene withdrawal (Figure 1C–D). To specifically examine the effect of MYC inactivation on cell cycle arrest, independently from apoptosis, we utilized lymphoma cells with the shRNA-mediated knockdown of *Bim*. Upon MYC inactivation, lymphoma cells with *Bim* knockdown persisted and underwent proliferative arrest. Moreover, they remained arrested even after reactivation of MYC (Figure 5D, upper panel). This irreversible cell cycle arrest indicated the induction of senescence by MYC inactivation. In contrast, lymphoma cells expressing *miR-17-92* or with the knockdown of *Bim* and *SHSB* resumed proliferation upon MYC reactivation (Figure 5D, middle and lower panels). Therefore, the expression of *miR-17-92* or suppression of the *miR-17-92* target genes *Sin3b*, *Hbp1*, *Sub420h1*, and *Btg1* prevents the induction of senescence upon MYC inactivation.

The shRNA-mediated suppression of *Bim* and *SHSB* also impeded the *in vivo* proliferative arrest, apoptosis, and senescence upon MYC inactivation. Tumor cells were transplanted subcutaneously into syngeneic FVB/N hosts and grown for about 2 weeks before MYC inactivation by doxycycline administration in the drinking water. In control lymphoma versus lymphomas with *Bim* and *SHSB* knockdown, there was a 4-fold decrease versus one-fold decrease in phospho-histone H3 staining, a 1.5-fold increase versus no change in cleaved-caspase 3 staining, and a 3-fold versus less than one fold increase in SA- β -gal staining (Figure 6A–C), similar to what was observed for *miR-17-92* expression (Figure 2 and 6D). Thus, *miR-17-92* target genes *Sin3b*, *Hbp1*, *Suv420h1*, *Btg1*, and *Bim* are required for the induction of proliferative arrest, apoptosis, and senescence *in vivo* upon MYC inactivation.

Suppression of *Sin3b*, *Hbp1*, *Suv420h1*, *Btg1*, and *Bim* Abrogates Sustained Tumor Regression upon MYC Inactivation

Since *miR-17-92* expression and knockdown of *miR-17-92* target genes significantly block the induction of proliferative arrest, apoptosis, and senescence both *in vitro* and *in vivo*, we examined their impact on sustained tumor regression upon MYC inactivation. MYC inactivation in lymphoma induced rapid tumor regression within 6 days (Figure 6E), without evidence of lymphoma recurrence even after six months of continuous observation (Figure 6F). In contrast, the lymphomas with enforced retroviral *miR-17-92* expression regressed only after 14 days and 80% of tumors reoccurred within 6 weeks (Figure 6E–F). Compared with the dramatic delay in tumor regression with *miR-17-92* expression, the knockdown of either *Bim* or *Bim* combined with *SHSB* modestly delayed the kinetics of tumor regression (Figure 6E). Interestingly, although *Bim* knockdown was not associated with any tumor

recurrence, the combined knockdown of *Bim* and *SHSB* was associated with the recurrence of 50% of tumors (Figure 6F). After prolonged MYC inactivation, the recurrent tumors eventually regained high levels of MYC expression, similar to what we have described previously (Figure S6) (Choi et al., 2011). Hence, the expression of *miR-17-92* or the suppression of *miR-17-92* target genes, *Sin3b*, *Hbp1*, *Suv420h1*, *Btg1*, and *Bim* prevented sustained tumor regression upon MYC inactivation.

DISCUSSION

We have found that MYC through *miR17-92* directly suppresses the expression of chromatin regulatory genes *Sin3b*, *Hbp1*, *Suv420h1*, *Btg1* and pro-apoptotic gene *Bim*. The suppression of these defined factors is causally required to maintain survival, autonomous proliferation, and self-renewal. Our results have general implications for how MYC maintains a neoplastic state.

MYC is known to globally regulate gene and protein expression (Dang, 2012). Many studies have identified hundreds of genes associated with MYC overexpression and tumorigenesis (Kim et al., 2008; Schlosser et al., 2005; Zeller et al., 2006). The expectation is that a similar multitude of genes would be required by MYC to initiate and maintain a neoplastic state. Surprisingly, we found that a single microRNA cluster, *miR-17-92*, amongst thousands of genes controlled by MYC, can maintain a neoplastic state in MYC-induced tumors by sustaining autonomous proliferation and survival. We found that this mechanism was specific to MYC and unique to *miR-17-92*. Furthermore, the function of *miR-17-92* can be partially attributed to the suppression of a small number of target genes, such as the chromatin regulatory genes *Sin3b*, *Hbp1*, *Suv420h1* and *Btg1*, as well as the pro-apoptotic gene *Bim*. Our results highlight how MYC maintains tumorigenesis through the regulation of *miR-17-92*-dependent epigenetic and survival programs.

Oncogene-induced tumorigenesis is generally suppressed through intrinsic barriers, such as apoptosis and senescence (Braig et al., 2005; Lowe et al., 2004; Nardella et al., 2011). The inactivation of a driver oncogene can restore these tumor suppressor mechanisms, even in a tumor that is genetically complex (Karlsson et al., 2003), thereby eliciting the phenomenon of oncogene addiction (Felsher, 2008; Weinstein, 2002). Hence, our results suggest that MYC via *miR-17-92* maintains autonomous proliferation, self-renewal, and survival. Correspondingly, MYC inactivation induces a loss of tumor hallmark features as a consequence of restoration of proliferative arrest, apoptosis, and senescence (Figure 7).

We have identified specific *miR-17-92* target genes that are essential for the reversal of neoplasia upon MYC inactivation. These genes drive proliferation arrest, senescence, and apoptosis by regulating chromatin modification and apoptosis. *Sin3b* interacts with *Hbp1* and recruits HDACs to silence proliferation-related genes and mediate cell cycle exit and senescence (David et al., 2008; Grandinetti et al., 2009; Swanson et al., 2004). *Suv420h1* is a histone methyltransferase that dimethylates and trimethylates H4K20 (Fraga et al., 2005; Greer and Shi, 2012). H4K20me3 is known to direct chromatin compaction and is a marker of heterochromatin formation and senescence (Greer and Shi, 2012; Lu et al., 2008; Nelson, 2012). Loss of H4K20me3 is a common feature in human cancer (Fraga et al., 2005). *Btg1*

is a tumor suppressor that is frequently lost in acute lymphoblastic leukemia (Lundin et al., 2012; Waanders et al., 2012). It is also a biomarker of chemotherapy-induced cellular senescence (Roninson, 2003). Finally, Bim is a major tumor suppressor in MYC-induced lymphomagenesis (Egle et al., 2004). Bim is also frequently lost in human B-cell lymphomas and its loss can cause chemoresistance in patients (Richter-Larrea et al., 2010). Thus, these *miR-17-92* targets are some of the critical players in the maintenance of the neoplastic state by MYC.

Recently it has been shown that MYC can function as a transcriptional amplifier of the already expressed genes within the cells without specificity (Lin et al., 2012; Nie et al., 2012). It is a remarkable finding and explains one aspect of MYC function that is consistent with prior studies (Dang, 2012; Guccione et al., 2006; Guney and Sedivy, 2006; Varlakhanova and Knoepfler, 2009). However, whether the transcriptional amplifier mechanism is required for MYC to maintain the neoplastic state is not known. Moreover, MYC's function as an amplifier is likely only one of its many functions and does not account for the ability of MYC to suppress gene expression, nor does it provide an explanation for gene-specific effects on expression (Walz et al., 2013). Our results highlight an additional mechanism by which MYC controls several essential features of a neoplastic state (Figure 7). MYC, through *miR-17-92*, controls a general ON and OFF switch of chromatin state and thereby regulates the decision between survival versus death and self-renewal versus senescence. Our findings are also complementary to MYC's role in transcriptional amplification. High levels of MYC can keep the chromatin transcriptionally accessible and allow for transcriptional amplification. When MYC is turned off, many genes are downregulated by the amplifier mechanism and this likely reduces the ability of tumor cells to grow and proliferate. However, the suppression of *miR-17-92* upon MYC inactivation allows the induction of many genes, including chromatin modifiers and apoptosis regulators involved in apoptosis and senescence (Figure 7). Hence, MYC inactivation leads to a change in the neoplastic state.

MYC has been shown before to modulate global euchromatin structure that may contribute to self-renewal and pluripotency, but the specific mechanism has been elusive (Knoepfler et al., 2006; Varlakhanova and Knoepfler, 2009). Our finding that MYC, through *miR-17-92*, regulates the heterochromatin formation may provide an explanation. Hence, MYC suppression in cancer cells results in irreversible changes in gene expression and the permanent loss of a neoplastic phenotype (Jain et al., 2002). We infer that MYC's ability to sustain autonomous proliferation, self-renewal, and survival is mediated through a *miR-17-92*-dependent chromatin regulatory and survival switch. The shut off of this epigenetic switch contributes to the mechanism of MYC-associated oncogene addiction.

EXPERIMENTAL PROCEDURES

Cell Lines, DNA Constructs, and Viruses

Conditional lymphoma and leukemia cell lines were derived from *E μ -tTA/tet-O-MYC* mice. MYC inactivation was achieved with doxycycline treatment. The *miR-17-92* was cloned into pMSCV retroviral vectors. Virus production and infection of tumor cells was performed as

previously reported (Wu et al., 2007). Construction of the 3'UTR luciferase reporters and the shRNAs can be found in Supplemental Experimental Procedures.

Tumor Transplantation

All animal experiments were approved by Stanford's Administrative Panel on Laboratory Animal Care (APLAC) and in accordance with national guidelines. The conditional MYC lymphoma cell line was transplanted into host mice and allowed to grow to 1.5cm diameter before MYC inactivation with doxycycline. Tumor diameters were measured with a caliper. Tumor volume (V) was calculated as: $V = ab^2 / 2$, where *a* indicates length (mm) and *b* indicates breadth (mm). Further details can be found in Supplemental Experimental Procedures.

Flow cytometry, microRNA quantification, Western Blot, Immunohistochemistry, SA-β-gal Staining, and Chromatin Immunoprecipitation

The microRNAs were quantified with TaqMan microRNA assay kits (Applied Biosystems). Western blotting, immunofluorescence, SA-β-gal staining, and chromatin immunoprecipitation were performed as described (van Oevelen et al., 2010; Wu et al., 2007). Details can be found in Supplemental Experimental Procedures.

Microarray Analysis

Control and *miR-17-92*-expressing cell lines were used for the microarray analysis. MYC ON and MYC OFF samples were collected at 0 hour and 48 hours after MYC inactivation. Details of the microarray analysis can be found in Supplemental Experimental Procedures.

Multiple Knockdown with shRNAs

The individual knockdown of *miR-17-92* target genes was accomplished using LMP *miR-30*-based shRNAs (OpenBiosystems). For multiple knockdown, the shRNAs were cloned into vectors with different drug selection markers to allow for simultaneous knockdown of multiple genes in the same cell. Further details can be found in Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

The *MYC* oncogene is frequently overexpressed in human cancers. *MYC* overexpression coordinates the expression of thousands of genes that could potentially contribute to its neoplastic properties. However, it was unclear which specific genes are responsible for *MYC* to maintain a neoplastic state. Here we show that *MYC* maintains a neoplastic state through the regulation of the microRNA cluster *miR-17-92*, which controls specific chromatin regulatory and survival programs. *MYC* inactivation, through the downregulation of *miR-17-92*, induces a loss of neoplastic features as a consequence of restoration of proliferative arrest, apoptosis, and senescence. Our results highlight how *MYC* maintains tumorigenesis through the regulation of *miR-17-92*-dependent epigenetic and survival programs and provide a mechanistic explanation of the phenomenon of oncogene addiction.

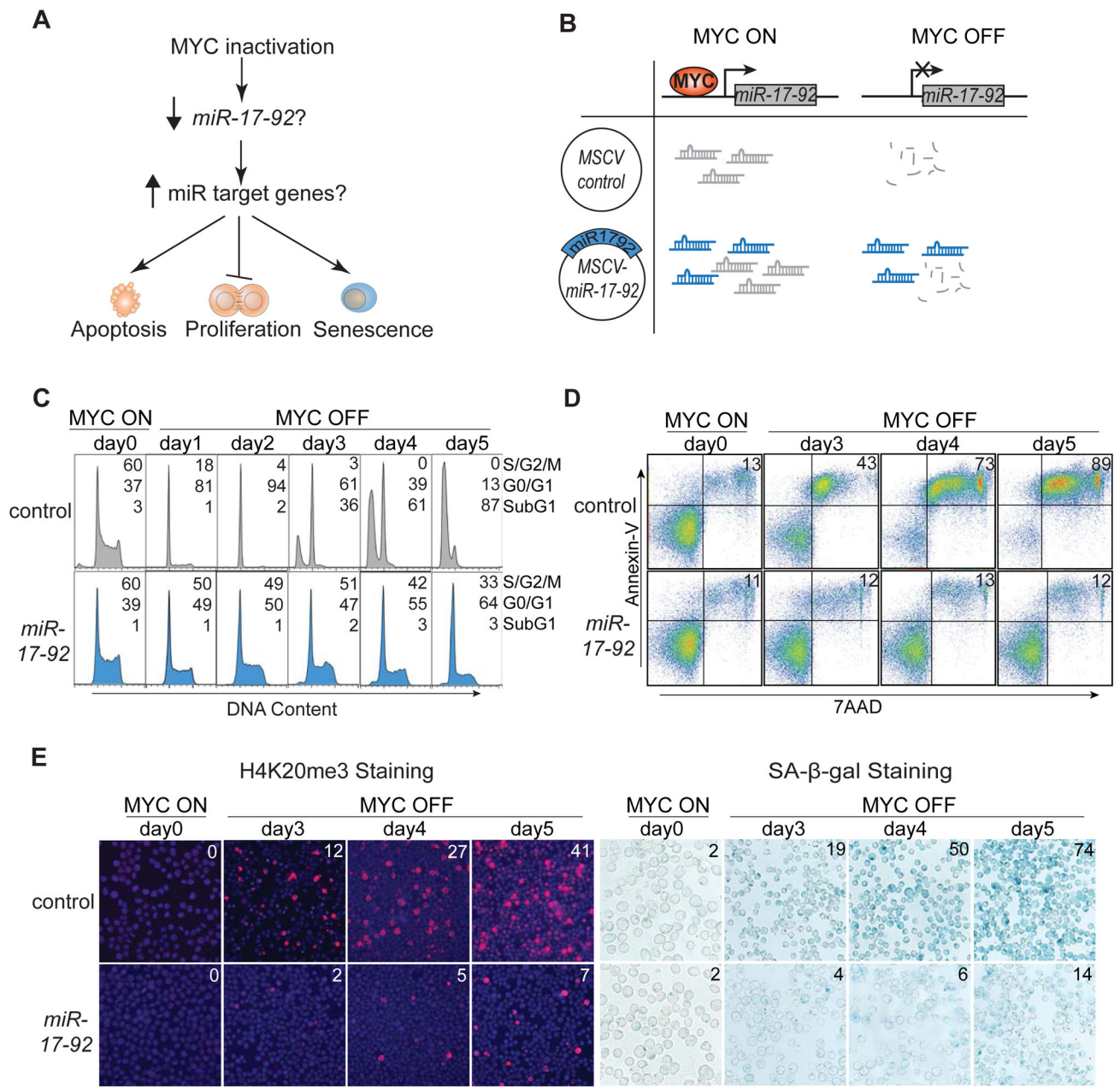


Figure 1. *miR-17-92* Expression *In Vitro* Rescues MYC Oncogene Addiction by Sustaining Proliferation and Blocking Apoptosis and Senescence

(A) Diagram of cellular changes upon MYC inactivation in MYC-driven tumors. (B) Experimental strategy to sustain *miR-17-92* with retroviral expression. The endogenous *miR-17-92* is colored grey while the exogenous MSCV-*miR-17-92* is colored blue. (C) Cell cycle analysis of tumor cells over a 5-day time course with flow cytometry after propidium iodide staining. Numbers indicate percentage of cells in different phases of cell cycle. The experiments were repeated three times with similar results. (D) Annexin V/7-AAD staining showing apoptotic cells over a 5-day time course of MYC inactivation. Numbers in upper right quadrant indicate percentage of apoptotic cells. The experiments were repeated three

times with similar results. (E) H4K20me3 and SA- β -gal staining of tumor cells after MYC inactivation for five days. The control cells are kept alive with *Bim* shRNA knockdown and *Bcl-xL* overexpression. Numbers in upper right quadrant indicate percentage of cells stained positive. Cell nuclei in the H4K20me3 panel were stained with DAPI. The experiments were repeated twice with similar results. Scale bar = 50 μ m. See also Figure S1.

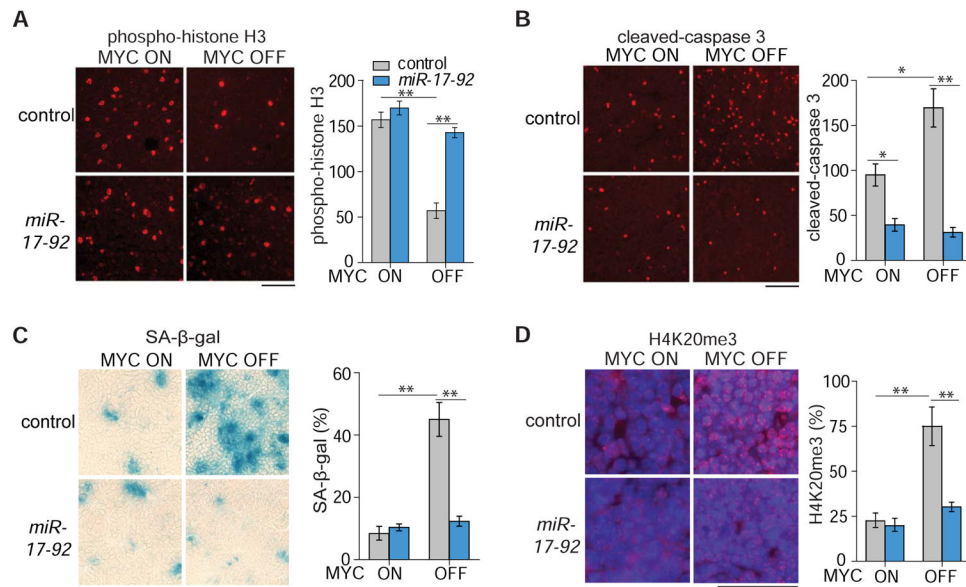


Figure 2. *miR-17-92* Expression *In Vivo* Mediates MYC Oncogene Addiction by Sustaining Proliferation and Blocking Apoptosis and Senescence

(A) Phospho-histone H3 staining showing cells in the metaphase of the cell cycle four days after MYC inactivation. The y-axis denotes number of positive staining cells per 20X magnification field. (B) Cleaved-caspase-3 showing apoptotic cells four days after MYC inactivation. The y-axis denotes number of positive staining cells per 20X magnification field. (C) SA-β-gal staining four days after MYC inactivation. The y-axis denotes percentage of area with positive SA-β-gal staining. (D) H4K20me3 staining four days after MYC inactivation. The y-axis denotes number of positive staining cells per high magnification field. Results are presented as mean \pm SEM. $n=4$ (A, B, C, D). Student's t test, $*p<0.05$. $**p<0.01$. Scale bar = 50 μ m. See also Figure S2.

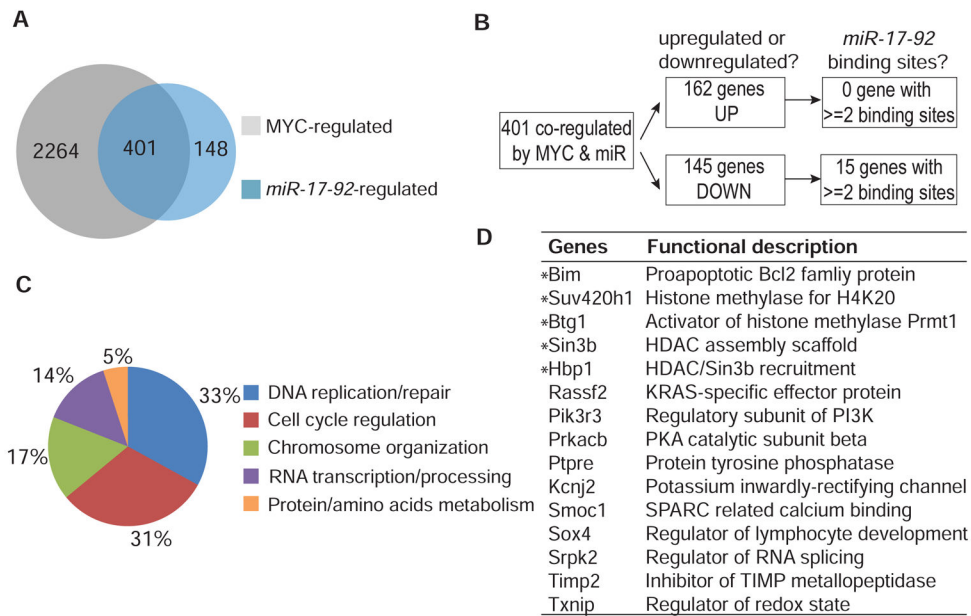


Figure 3. Identification of *miR-17-92* Target Genes by Comparative Analysis of Genes Regulated by MYC and *miR-17-92*

(A) Venn diagram of genes regulated by both MYC and *miR-17-92*. (B) Flowchart showing the analysis of the genes co-regulated by MYC and *miR-17-92*. Genes were separated into either upregulated or downregulated groups and analyzed for functional annotation and enrichment of *miR-17-92* binding sites. (C) Functional categories of genes upregulated by both MYC and *miR-17-92* according to DAVID's Bioinformatic Resource. The percentage refers to the number of genes within a particular category in relation to the total number of genes that have a GO annotation. (D) Candidate target genes with multiple *miR-17-92* binding sites. The histone modifiers and *Bim* were indicated with asterisks. See also Figure S3 and Table S1-3.

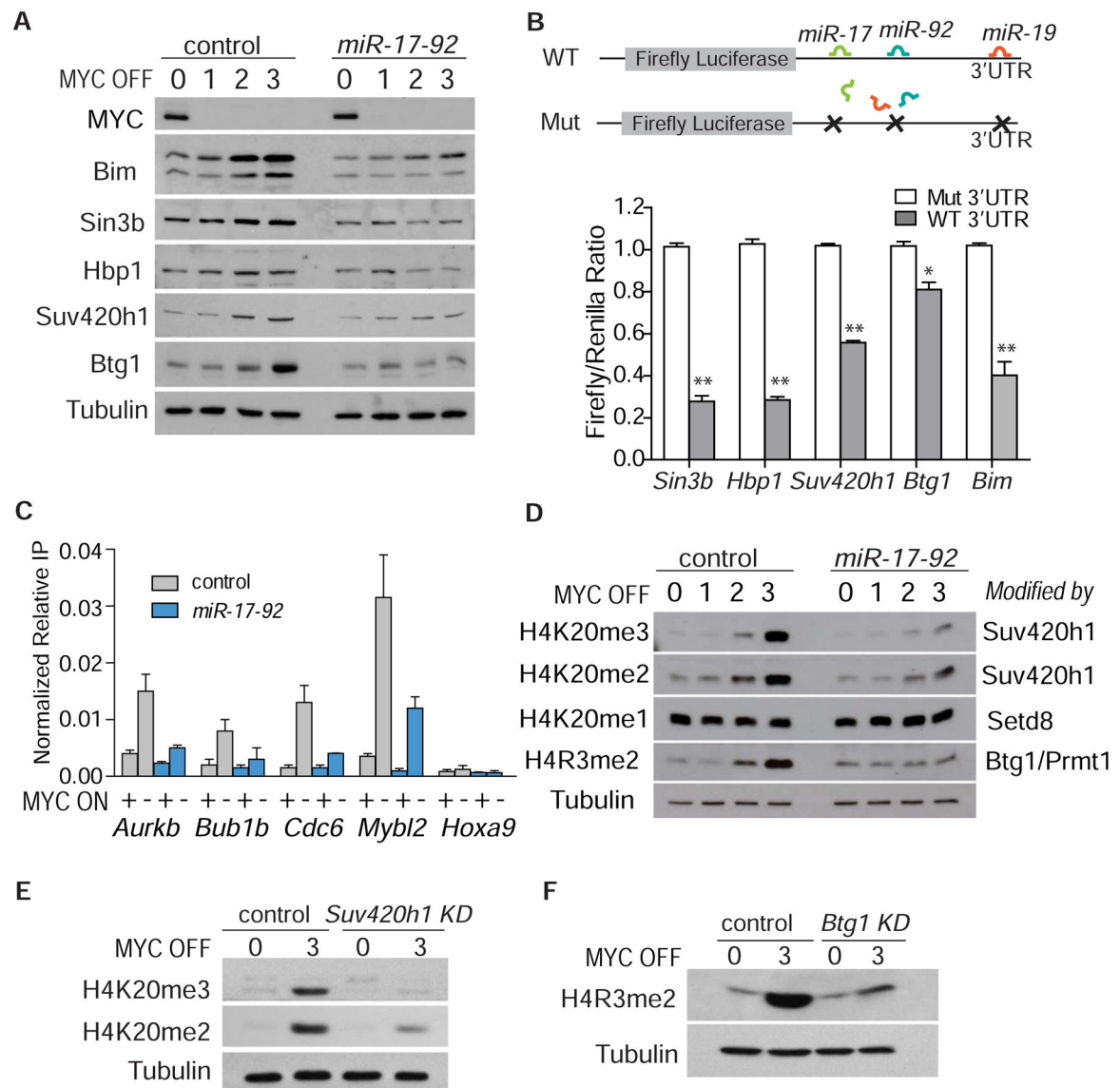


Figure 4. Validation of Target Genes of *miR-17-92*

(A) Changes in the expression of Bim and histone modifiers as detected by Western blot three days after MYC inactivation. (B) Top: Wild type (WT) and mutant (Mut) 3'UTR reporter constructs. Bottom: Dual luciferase assay using 3'UTR reporters. The firefly luciferase signals were normalized with internal control renilla luciferase. Results are presented as mean \pm SEM. Student's t test, * $p < 0.05$. ** $p < 0.01$. (C) Chromatin immunoprecipitation with Sin3b antibody showing binding of Sin3b to promoters of four proliferation-related genes upon MYC inactivation. MYC OFF samples were taken at two days after MYC inactivation. PCR with *Hoxa9* promoter specific primers was included as a negative control. Data shown are averages of two experiments and are presented as mean \pm SEM. (D) Changes in histone H4 lysine 20 and arginine 3 methylation status upon MYC inactivation. The mono-, di- and trimethylation of H4K20 and dimethylation of H4R3 are shown. MYC off time course includes day 0, 1, 2, and 3. The respective enzymes catalyzing

each of the modifications are shown on the left side of the lanes. (E) Western blot analysis of di- and trimethylation of H4K20 in the presence of *Suv420h1* knockdown. (F) Western blot analysis of dimethylation of H4R3 in the presence of *Btg1* knockdown. See also Figure S4.

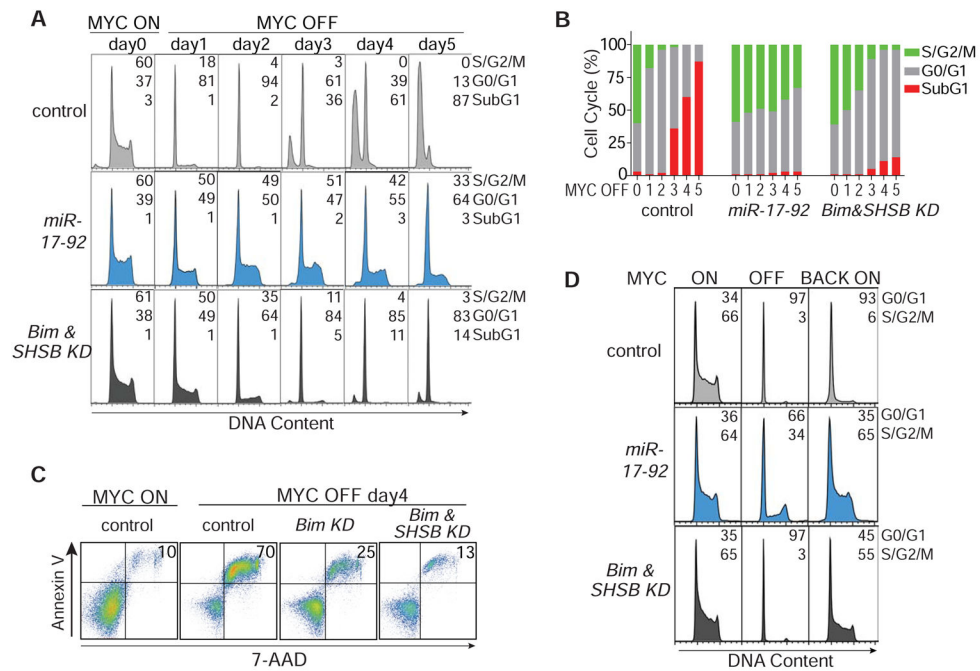


Figure 5. Suppression of *Bim* and the Chromatin Modifiers Delays Proliferative Arrest and Blocks Apoptosis and Senescence upon MYC Inactivation *In Vitro*
 (A) Cell cycle distribution of cells with combined knockdowns of *SHSB* and *Bim* upon MYC inactivation for 5 days. Numbers indicate percentage of cells in different phases of the cell cycle. (B) Bar graph summarizing data shown in Figure 5A. Different phases of the cell cycle were color-coded. (C) Apoptosis of tumor cells with knockdown of *Bim* and *SHSB* upon MYC inactivation for four days. Numbers in upper right quadrant indicate percentage of apoptotic cells. Detailed 5-day time course is shown in Figure S9. (D) Cell cycle distribution of tumor cells upon MYC inactivation and reactivation. MYC OFF samples were taken at four days of MYC inactivation with doxycycline treatment. MYC-Back ON samples were taken at three days after MYC reactivation by removing doxycycline. Events shown are gated on live cells. Numbers indicate percentage of cells in either G1/G0 or S/G2//M phase of the cell cycle. The experiments were repeated three times with similar results. See also Figure S5.

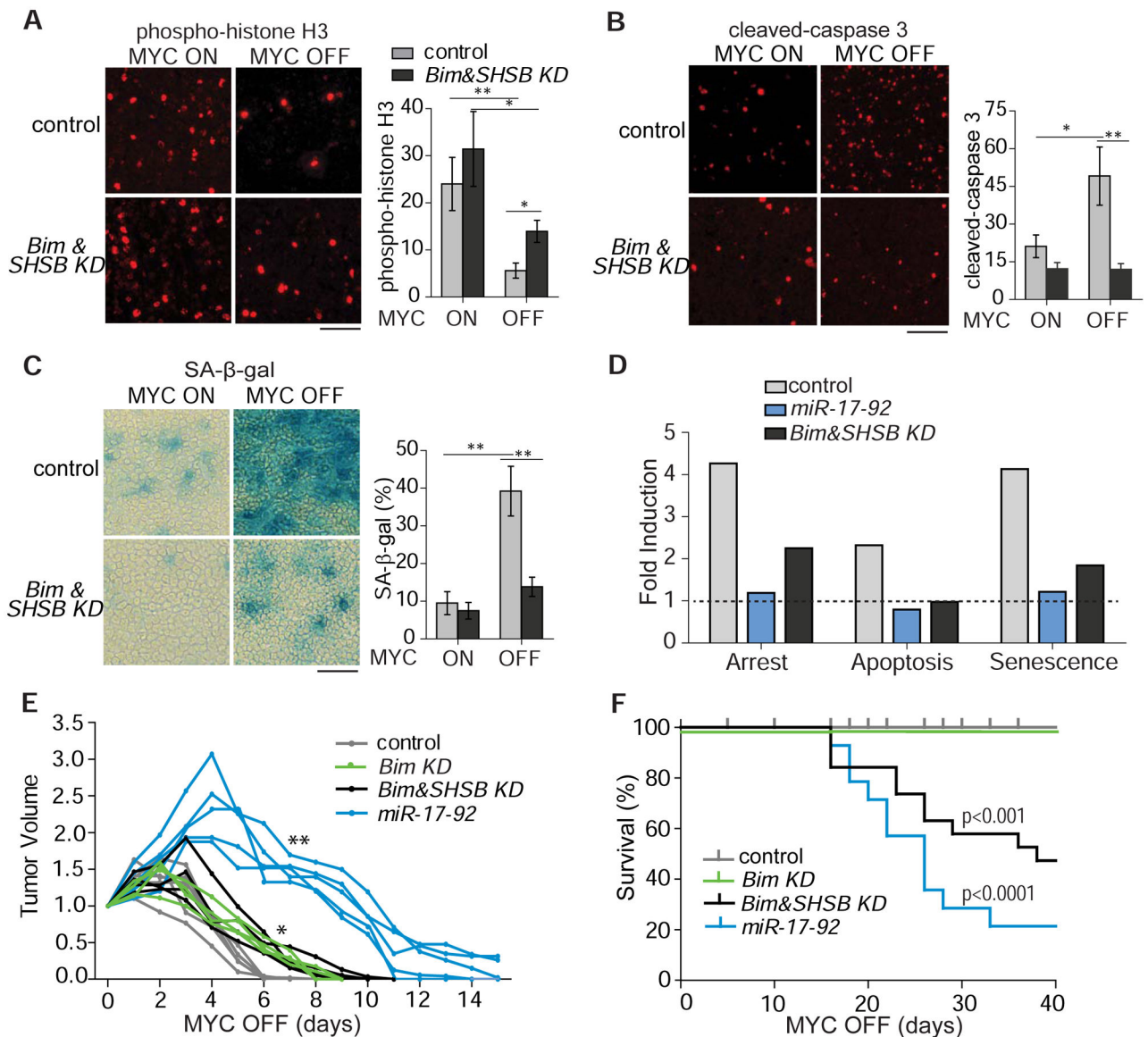


Figure 6. Suppression of *Bim* and the Chromatin Modifiers Delays Proliferative Arrest, Blocks Apoptosis and Senescence, and Abrogates Sustained Tumor Regression upon MYC Inactivation *In Vivo*

(A) Phospho-histone H3 staining showing cells in the metaphase of the cell cycle four days after MYC inactivation. The y-axis denotes number of positive staining cells per 40X magnification field. (B) Cleaved-caspase-3 showing apoptotic cells four days after MYC inactivation. The y-axis denotes number of positive staining cells per 40X magnification field. (C) SA-β-gal staining at four days after MYC inactivation. The y-axis denotes percentage of area with positive SA-β-gal staining. $n=3$. For panel 6A, 6B, and 6C, the results are presented as mean \pm SEM. Student's t test, * $p<0.05$. ** $p<0.01$. Scale bar = 50 μ m. (D) Comparison of *in vivo* induction of proliferative arrest, apoptosis, and senescence upon MYC inactivation in control lymphomas, lymphomas with retroviral *miR-17-92*, and lymphomas with knockdown of *Bim* and *SHSB*. To calculate the fold induction of

proliferative arrest, we used the MYC ON/MYC OFF ratio for phospho-histone H3 staining. To calculate the fold induction of apoptosis and senescence, we used the MYC OFF/MYC ON ratio of cleaved-caspase 3 and SA- β -gal staining, respectively. The fold induction was computed by combining data presented in Figure 2 and Figure 6A–C. The dashed line indicates no induction. (E) *In vivo* regression of transplanted tumors in SCID mouse host. $n=4-6$. For *miR-17-92* versus control, the double asterisk (**) indicates $p<0.05$ for day 3-14. For *Bim* KD or *Bim&SHSB* KD versus control, the single asterisk (*) indicates $p<0.05$ for day 5-8. The comparisons were made with the two-tailed Student's *t* test. (F) *In vivo* tumor reoccurrence upon MYC inactivation in syngeneic wild type FVB/N mice. Tumor bearing mice were monitored for six weeks of doxycycline administration in drinking water. Mice were euthanized when the diameters of the relapsed tumors reach 1 cm. $n= 10-16$. Logrank test. $p<0.001$ for tumors with *Bim&SHSB* KD. $p<0.0001$ for tumors with *miR-17-92* expression. See also Figure S6.

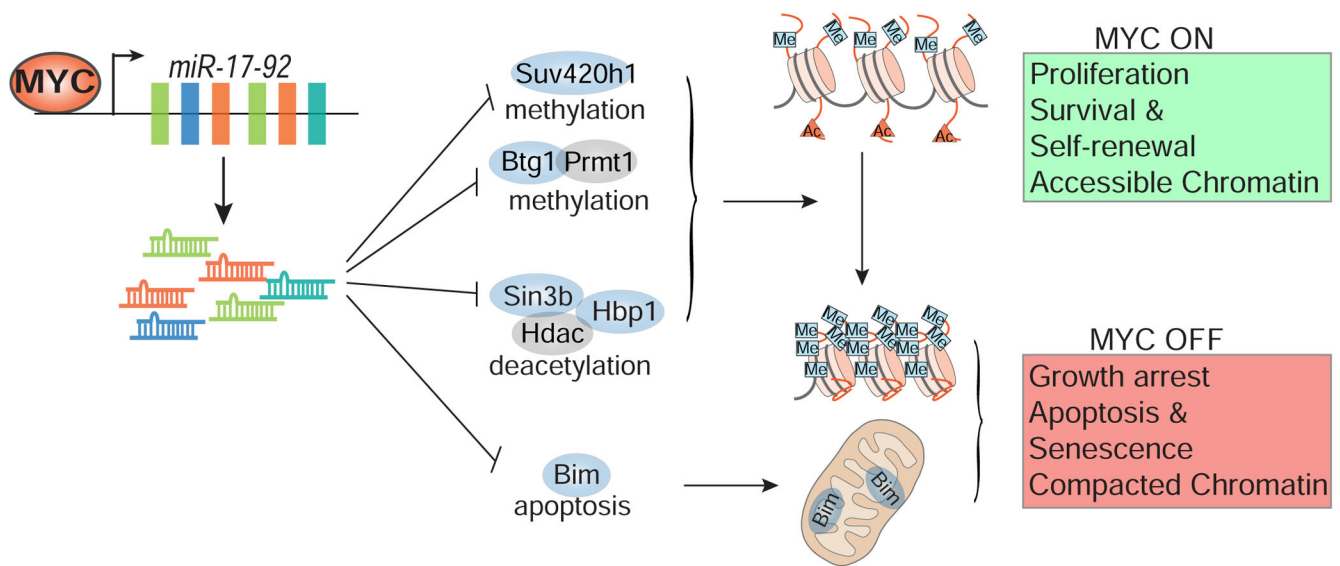


Figure 7. A Model illustrating MYC, through *miR-17-92* and its target genes, controls a chromatin regulatory and survival switch that is required to sustain a neoplastic state MYC through *miR-17-92* suppresses specific genes to maintain autonomous proliferation, self-renewal, and survival. Correspondingly, MYC inactivation induces a loss of neoplastic features as a consequence of restoration of proliferative arrest, apoptosis, and senescence.