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MYC Acts via the PTEN Tumor Suppressor to Elicit Autoregulation and Genome-Wide Gene Repression by Activation of the Ezh2 Methyltransferase

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Abstract

The control of normal cell growth is a balance between stimulatory and inhibitory signals. MYC is a pleiotropic transcription factor that both activates and represses a broad range of target genes and is indispensable for cell growth. Whereas much is known about gene activation by MYC, there is no established mechanism for the majority of MYC-repressed genes. We report that MYC transcriptionally activates the *PTEN* tumor suppressor in normal cells to inactivate the phosphoinositide 3-kinase (PI3K) pathway, thus suppressing AKT activation. Suppression of AKT enhances the activity of the EZH2 histone methyltransferase, a subunit of the epigenetic repressor Polycomb Repressive Complex 2 (PRC2), while simultaneously stabilizing the protein. MYC-mediated enhancement in EZH2 protein level and activity results in local and genome-wide elevation in the repressive H3K27me3 histone modification, leading to widespread gene repression including feedback autoregulation of the *MYC* gene itself. Depletion of either *PTEN* or *EZH2* and inhibition of the PI3K/AKT pathway leads to gene derepression. Importantly, expression of a phospho-defective EZH2 mutant is sufficient to recapitulate nearly half of all MYC-mediated gene repression. We present a novel epigenetic model for MYC-mediated gene repression and propose that PTEN and MYC exist in homeostatic balance to control normal growth, which is disrupted in cancer cells. *Cancer Res; 73(2); 695–705. ©2012 AACR.*

Introduction

Cancer is driven by the activation of oncogenes and the loss of tumor suppressors. One tumor suppressor that is frequently inactivated in diverse cancers is the phosphatase and tensin homolog gene (*PTEN*; reviewed in ref. 1). PTEN acts as a negative regulator of the phosphoinositide 3-kinase (PI3K) pathway by converting PIP3 to PIP2, which in turn prevents activation of the Akt pathway downstream of PI3K. The PI3K/Akt pathway is an important component of cell signaling that regulates a myriad of biologic processes including growth, proliferation, and apoptosis (1). Cancer cells often exhibit mutations in the PI3K/Akt pathway, including loss of PTEN, ultimately resulting in activation of the pathway and its downstream effectors (2).

The *MYC* gene is the most frequently amplified gene in human cancer, and deregulated expression of *MYC* is a hallmark of 70% of all cancers (3). Myc is a well-established pleiotropic transcription factor, and significant progress has been made in understanding the role of Myc as a transcriptional activator (4). In addition to activating target genes, Myc also represses an almost equal number of genes (3), and this repression is important for Myc-mediated cell proliferation and transformation (5, 6). However, despite major advances in the Myc field, there is no uniform mechanism of repression that satisfactorily accounts for the majority of Myc repressed genes. In fact, the first Myc-regulated gene ever described was the *MYC* gene itself, through what is generally considered an autoregulatory feedback loop (7, 8). Autoregulation is postulated to be important in fine-tuning the amount of Myc in a cell, as small changes in Myc expression are sufficient to shift the balance from normal to aberrant growth. Notably, many cancer cells have lost the ability to autoregulate (9, 10), and more Myc is advantageous for growth and proliferation of cancer.

One pathway implicated in autoregulation of the *MYC* ortholog in *Drosophila* (dmyc) is the Polycomb group of proteins (11). The Polycomb Repressive Complex 2 (PRC2) epigenetically silences genes by trimethylating lysine 27 on histone H3 (H3K27me3), a function carried out by the methyltransferase Enhancer of Zeste 2 (Ezh2), which is an integral component of the complex (12). The *Ezh2* gene is frequently amplified or overexpressed in many tumors and was described as an E2F-responsive oncogene (13).

In this study, we show that Myc suppresses the PI3K/Akt pathway via transcriptional upregulation of the *PTEN* tumor suppressor. Significantly, suppression of Akt results in Ezh2-mediated gene repression in 2 mammalian systems, including autoregulation. Activation of Ezh2 is both necessary and

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sufficient to account for nearly half of all Myc-repressed genes. We propose a general mechanism for Myc-mediated repression and autoregulation linked to an important tumor suppressor pathway.

Materials and Methods

Cell lines, drug treatments, and western blotting

The c-*myc*$^{-/-}$ (HO 15.19) and Phoenix cells were maintained in Dulbecco's Modified Eagle's Medum (DMEM) supplemented with 10% FBS. Immortalized mammary epithelial cells (IMEC; ref. 14) were cultured in DMEM:F12 50:50 media supplemented with EGF, insulin, hydrocortisone, and 5% FBS. To generate stable cell lines, retroviral vectors were used to create polyclonal populations. For LY294002 (Cayman Chemical; #70920) treatments, cells were plated subconfluently 12 to 16 hours before treatment. Treatments lasted for 2 hours followed by radioimmunoprecipitation assay (RIPA) lysis for immunoblotting. Antibody information used for immunoblotting and chromatin immunoprecipitation (ChIP) can be found in Supplementary Information.

Plasmids and RNA interference

To generate the *myc* promoter luciferase reporter, a 2.5-kb fragment containing the human *MYC* promoters (P1 and P2) and upstream regulatory elements was cloned into the pGL3 Basic vector. Ezh2/S21A and Ezh2/S21E mutants were generated using the QuikChange II Site Directed Mutagenesis Kit (Stratagene) as per manufacturer's protocol. pUSE MYR-AKT was a gift from Dr. G. Lienhard (Dartmouth, Hanover, NH). Silencer Select Pre-designed siRNAs were obtained from Ambion (Applied Biosystems) and transfected using Lipofectamine RNAi Max (Invitrogen; 10 nmol/L siRNA). At the indicated time point, cells were harvested for protein or RNA. siRNA sequences are listed in Supplementary Information (Supplementary Table S2). Silencer Select negative control siRNA was used as a transfection control and to account for nonspecific effects.

Real-time reverse transcription PCR

Total RNA was harvested from log phase cultures with TRIzol (Invitrogen), and cDNA was synthesized using the Super Script kit from Invitrogen. Two-step real-time PCR was carried out using the SYBR Green Mix (Bio-Rad) on a Bio-Rad C1000 Thermal cycler. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin was used for normalization. Primer sequences are available upon request.

Chromatin immunoprecipitation

Log phase cultures were fixed with 1% formaldehyde for 10 minutes and subjected to ChIP assays with minor modifications as previously described (15). Briefly, cells were lysed in lysis buffer (1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris, pH 8.1) and sonicated to generate DNA fragments between 200 and 1,000 bp. Cleared lysates were then diluted and incubated with the following antibodies overnight at 4°C. Immunoprecipitations were washed with RIPA buffer, and precipitated DNA was recovered. Real-time PCR amplification was then carried out using specific primers. Primer sequences are available upon request. The data are presented as percentage binding compared with input for each sample. All experiments were carried out 3 to 4 times and error bars represent SD.

Luciferase assay

Luciferase assays were conducted with the Dual luciferase Reporter Kit (Promega). The pRL vector constitutively expressing *Renilla* luciferase was used to normalize for transfection efficiency. A total of 2×10^5 IMECs were plated in 12-well dishes 24 hours before transfections. On the day of transfection, each well was transfected with pRL, pGL3 Basic (to assess basal reporter activity) or *MYC* promoter-GL3 and the indicated plasmids. Twenty-four hours later, luciferase activity was measured using the Wallac 1450 MicroBeta TriLux system (Perkin Elmer). Experiments were carried out 3 times in triplicates, and error bars represent SD.

Soft agar assay

In 6-well plates, 10,000 cells (±siRNA treatment for 48 hours) were plated in 0.3% agar, layered over a 0.6% agar base layer. Wells were re-fed with 200 μL complete media every other day. Fourteen days after plating, colonies were counted and imaged.

Results

Myc suppresses AKT through activation of PTEN expression

The *PTEN* tumor suppressor was previously shown to be a direct Myc target gene with an E-box that is occupied by Myc *in vivo* (16, 17). We analyzed *PTEN* expression and found that it is Myc-activated at both the protein and mRNA levels in human mammary epithelial cells (IMECs) and *myc*$^{-/-}$ rat fibroblast cells (Fig. 1A and B). Therefore, we decided to explore the functional consequences of *PTEN* activation by Myc.

PTEN is a dual lipid and protein phosphatase that acts as a negative regulator of the PI3K/Akt pathway (1). As PTEN dephosphorylates PIP3 to PIP2, thus attenuating Akt activation, we tested whether PTEN activation had an effect on Akt signaling. Activation of Akt involves phosphorylation of Serine 473 (pS473), so we assessed the levels of pS473 in response to exogenous Myc expression. Notably, we found that Myc inhibits Akt S473 phosphorylation in both IMECs and *myc*$^{-/-}$ rat fibroblasts (Fig. 1C). In IMECs, there was a basal level of pS473 in the parental/vector cells that becomes undetectable with Myc overexpression. In rat fibroblasts, there is no detectable pS473 in *myc*$^{+/+}$ cells, which express native levels of Myc. In contrast, *myc*$^{-/-}$ cells derived by genetic knockout have highly elevated pS473, which is subsequently suppressed to undetectable levels by reconstitution of Myc. Repression of Akt pS473 is not evident with a Myc mutant lacking the conserved Myc Box 2 transactivation domain (ΔMB2; Fig. 1C). MB2, an evolutionarily conserved motif in the Myc transactivation domain, is essential for most of the biologic activities of Myc including oncogenic transformation, transactivation, and gene repression (4). To determine whether PTEN is responsible for regulating Akt phosphorylation, we depleted *PTEN* with siRNA and analyzed the level of Akt pS473. Consistent with the established signaling pathway, depleting *PTEN* in IMECs expressing exogenous Myc restored active Akt (pS473; Fig. 1D). Taken together, these data

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show that Myc induces PTEN to inhibit AKT activation, highlighting the biologic consequence of a relationship that has only been alluded to previously.

To ensure we were not observing artifacts of overexpressing Myc beyond physiologic levels, we monitored the amount of exogenous Myc in these systems. The level of Myc overexpression in IMECs is within the range observed in breast cancer cell lines, whereas the amount of Myc reconstituted in *myc*$^{-/-}$ fibroblasts is similar to that of endogenous Myc in parental *myc*$^{+/+}$ fibroblasts (Supplementary Fig. S1A; ref. 18). Other than PTEN, several other phosphatases have been reported to inactivate Akt via dephosphorylation (19–22) but depletion had no effect on pAKT levels (Supplementary Fig, S1B and S1C).

EZH2 is activated by Myc-mediated suppression of AKT kinase activity

It was reported previously that Akt suppresses the activity of the Ezh2 histone methyltransferase by phosphorylation on Serine 21 (pS21; ref. 23). Given the previous link in *Drosophila* between Myc-mediated gene repression and Polycomb complexes (11), we decided to examine the status of this modification using phospho-specific antibodies. We observed a loss of phospho-Ezh2 (pS21) in all cells with exogenous Myc expression and suppression of pAkt (Fig. 1C) along with an increase in total Ezh2, suggesting an increase in the pool of active Ezh2 upon increased Myc expression. In addition, we found that Myc stabilizes Ezh2, without altering its mRNA levels, and that this stability is negatively correlated with its phosphorylation at Serine 21 (Supplementary Fig. S2). To test more directly whether PTEN activation was responsible for reduced pEzh2, we used 2 independent siRNAs to deplete PTEN in IMECs and observed a significant increase in phospho-Ezh2 upon loss of PTEN (Fig. 1D).

The PI3K/Akt pathway is frequently hyperactivated in a variety of tumors and thus many drugs are available to inhibit it. We used the compound LY294002 that inactivates PI3K to further investigate the role of the PI3K/Akt pathway in Ezh2 phosphorylation (24). We found that inhibiting the PI3K/Akt pathway with LY294002 in parental IMECs prevents Akt activation and phosphorylation at Serine 473 (Fig. 1E). In addition, loss of pS473 Akt induces loss of phospho-Ezh2 (pS21) and a modest increase in total Ezh2.

MYC represses genes via Ezh2 and PRC2

To test the consequence of activating Ezh2 through loss of phosphorylation, we assessed whole-cell levels of the Ezh2-associated H3K27me3 modification that is indicative of repressed chromatin. Consistent with an increase in the active fraction of Ezh2, we observed Myc-dependent elevation in

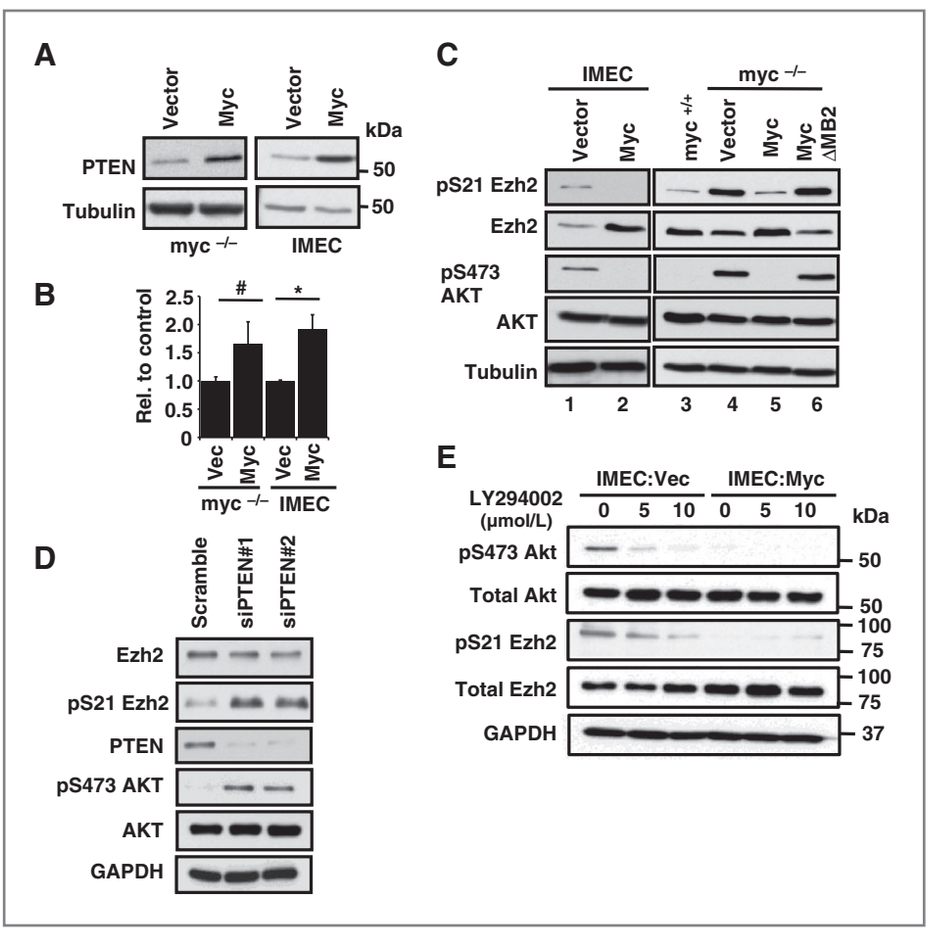

Figure 1. Myc induces PTEN expression and inhibition of the PI3K/Akt pathway, resulting in increased active Ezh2. A, immunoblot of PTEN protein levels in rat fibroblasts with or without Myc and in IMECs with or without exogenous Myc. B, reverse transcription PCR (RT-PCR) of *PTEN* RNA levels in rat fibroblasts with or without Myc and in IMECs with or without exogenous Myc. *, $P < 0.05$; #, $P < 0.01$. C, immunoblot analysis of the indicated proteins in *myc*$^{-/-}$ fibroblasts and IMECs expressing empty vector or exogenous Myc. Experiments were carried out 3 or more times and representative blots are shown. D, siRNA-mediated depletion of PTEN leads to enhanced pEzh2 and pAKT in IMECs. E, IMECs as in C were treated with LY294002 for 2 hours or left untreated. Cells were harvested and lysates were immunoblotted with the indicated antibodies. Inhibition of the PI3K/Akt axis suppresses pAkt (p-S473) and pEzh2 (p-S21 Ezh2).

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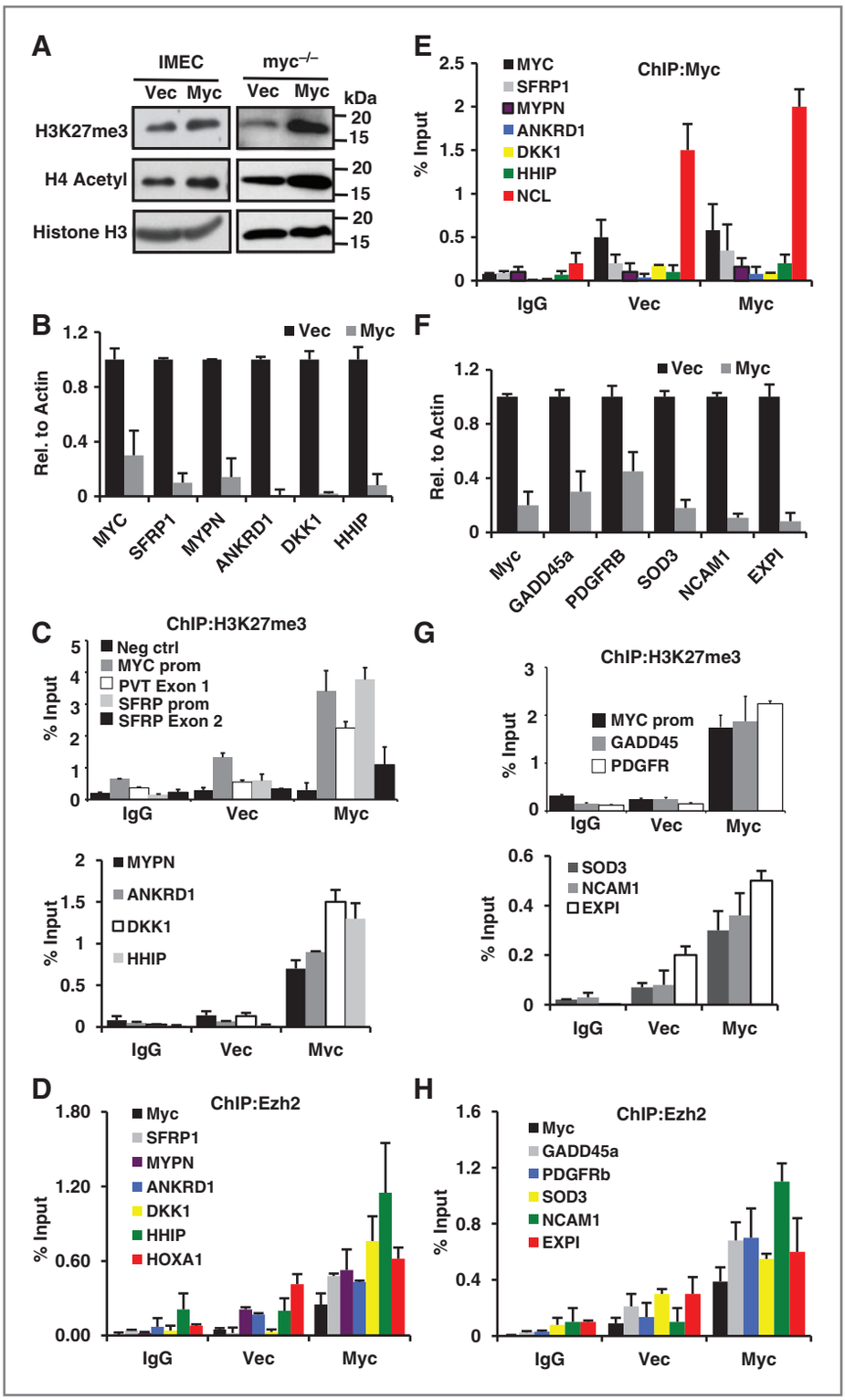

Figure 2. Overexpression of Myc enhances global H3K27 methylation and specific H3K27 methylation at the *MYC* promoter and Myc-repressed genes. A, immunoblot analysis of H3K27me3 levels in IMECs ± Myc overexpression (left) or in *myc*$^{-/-}$ rat fibroblasts reconstituted with Myc (right). Myc also enhances acetylated H4 as previously described (47). B, quantitative RT-PCR for Myc-repressed targets in IMECs transduced with empty vector or exogenous Myc. Primers specific to the indicated genes were used in analyzing transcriptional repression by Myc. C, ChIP with anti-H3K27me3 was conducted using IMECs transduced with empty vector or exogenous Myc. Immunoprecipitated DNA was assessed for H3K27me3 at the *MYC* promoter, *PVT* exon 1, *SFRP* promoter,

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global levels of H3K27me3 in both cell lines (Fig. 2A). This genome-wide increase in H3K27me3 could contribute to the large number of genes that are Myc repressed. This led us to hypothesize that Myc-mediated inhibition of the PI3K pathway via PTEN upregulation results in increased genome-wide gene repression elicited by PRC2. It is important to note that because we analyzed histones from whole-cell extracts, we cannot distinguish between chromatin-bound and non–chromatin-bound histones.

To assess a role for Ezh2 activity and H3K27me3 in gene repression, we studied several Myc-repressed genes in 2 different cell systems. As discussed earlier, the first Myc-repressed gene described was *MYC* itself so we tested whether the autoregulatory feedback loop involved repression by H3K27me3. In IMECs, we analyzed the Myc-repressed genes *MYC*, *SFRP1*, *DKK1*, *MYPN*, *ANKRD1*, and *HHIP* (Fig. 2B). It was previously shown that repression of *SFRP1* and *DKK1* is functionally important for Myc-mediated transformation (6). *MYPN*, *ANKRD1*, and *HHIP* are additional Myc-repressed genes obtained from microarray data in IMECs (25). We observed an enrichment of the H3K27me3 modification at the endogenous human *MYC* promoter in response to autorepression by ectopic Myc (Fig. 2C). A similar enrichment of H3K27me3 was also observed at exon 3 of *MYC* and at a region 30 kb downstream of the *MYC* gene encompassing the transcriptional start site of the noncoding *PVT* RNA (Fig. 2C; Supplementary Fig. S3B), which is regulated in parallel with *MYC* (26). These data suggest that the H3K27me3 mark may extend over a large area, consistent with spreading of H3K27me3 observed in *Drosophila* (27). As with the *MYC* gene itself, we found significant enrichment of the PRC2-mediated H3K27me3 modification in response to exogenous Myc expression at the promoters of all repressed genes tested (Fig. 2C). In line with the propagation of H3K27me3 along gene bodies, we observed an enrichment of H3K27me3 at exon 2 of *SFRP1,* approximately 10 kb downstream of the promoter (Fig. 2C). In contrast, 2 well-studied Myc activated target genes, nucleolin (*NCL*) and fibrillarin (*FBL*), did not show an enhancement of H3K27me3 in response to exogenous Myc (Supplementary Fig. S3B), and Myc-activated ribosomal protein targets were unaffected by overexpression of Ezh2 in $myc^{-/-}$ fibroblasts (Supplementary Table S1). We also found no binding of Myc protein itself at any of the Myc-repressed promoters (Fig. 2E).

As Ezh2 is the methyltransferase responsible for the H3K27me3 mark, we analyzed genes with enriched H3K27me3 for Ezh2 binding by ChIP. As expected, Ezh2 was detected at repressed genes with the H3K27me3 mark (Fig. 2D). Surprisingly, we did not observe enrichment of Ring1b, which is a component of PRC1, at Myc-repressed genes in IMECs (Supplementary Fig. S3A). This is consistent with a recent report showing the presence of PRC2, independent of PRC1, at bivalent domains in ES cells (28).

To further validate the role of PRC2 in Myc-mediated repression, we used the $myc^{-/-}$ rat fibroblast cell line in which 2 different selectable markers, neo and his, have been placed under control of the endogenous *MYC* promoters (29). Reconstituting Myc expression into these cells represses the endogenous *MYC* promoter similar to autorepression (30). We observed a strong induction of the H3K27me3 mark in response to reconstituted mouse Myc expression at the endogenous *MYC* promoter, as previously reported Myc-repressed genes *GADD45* and *PDGFRb*, and at additional Myc-repressed genes *SOD3*, *NCAM1*, and *EXPI* that were selected from unpublished microarrays (Figs. 2F and G). In addition, we detected an accumulation of H3K27me3 on the HOXA1 promoter, a known Polycomb-repressed target, but not at the promoter of a Myc-activated target, Nol5a (Supplementary Fig. S3C). Ezh2 was also detected in these repressed genes in accordance with the IMEC data (Fig. 2H). Thus, enhanced levels of H3K27me3 appear to be a general feature of Myc-repressed genes, including the *MYC* promoter itself.

PTEN and Ezh2 are indispensable components of Myc-mediated repression

To test whether PTEN and Ezh2 are required for Myc-mediated repression, we depleted each with specific siRNAs. *PTEN* depletion dramatically reversed the repression of the Myc-repressed genes and prevented the accumulation of the H3K27me3 modification (Fig. 3A and B; Supplementary Fig. S4B). Similarly, depletion of Ezh2 (Fig. 3C) restored expression of endogenous human *MYC*, *SFRP1*, *HHIP*, *MYPN*, *ANKRD1*, and *DKK1* genes (Fig. 3D; Supplementary Fig. S4A) and induced a loss of H3K27me3 at these promoters (Fig. 3E). Thus, both PTEN and Ezh2 are required for sustained repression and accumulation of H3K27me3. Unfortunately, $myc^{-/-}$ fibroblasts do not permit transient transfection of siRNA to carry out the same experiment.

To provide additional evidence that the PI3K pathway was linked to gene repression, we treated parental IMECs with LY294002. Chemical inhibition of the PI3K pathway (Fig. 1D) resulted in repression of Myc-repressed genes without altering the transcriptional activity of Myc-induced targets (Fig. 3F). Along with reduced expression, Myc-repressed promoters showed an accumulation of the repressive H3K27me3 mark upon treatment with LY294002 (Fig. 3G). Altogether, these data provide strong evidence for the importance of Myc-mediated inhibition of the PI3K/Akt pathway to initiate and maintain repression of genes.

SFRP exon 2, *MYPN* promoter, *ANKRD1* promoter, *DKK1* promoter, and *HHIP* promoter. IgG served as a control for nonspecific binding to beads (IgG) and NUP214 served as a negative control (Neg ctrl) for the H3K27me3 mark. D, ChIP with anti-Ezh2 antibody. In IMECs, the same gene promoters as in C were analyzed for Ezh2 binding, and increased binding of Ezh2 was observed with exogenous Myc. E, ChIP with anti-MYC was conducted in IMECs, and immunoprecipitated DNA was analyzed with primers specific for all promoters as in B. There was no significant binding over background, even with added exogenous Myc (Vec and Myc). A known Myc-binding site in the nucleolin (*NCL*) gene served as a positive control. F, quantitative RT-PCR of the indicated genes in $myc^{-/-}$ rat fibroblasts before and after reconstitution with Myc. G, ChIP with anti-H3K27me3 in $myc^{-/-}$ fibroblasts shows enhanced H3K27me3 at the endogenous *MYC* promoter and Myc-repressed genes *GADD45*, *PDGFRb SOD3*, *NCAM1*, and *EXPI*. H, repressed gene promoters as in G were analyzed for Ezh2 binding in $myc^{-/-}$ fibroblasts, showing increased binding with exogenous Myc. ChIP and quantitative RT-PCR data are presented as an average of 3 independent experiments with error bars denoting SD.

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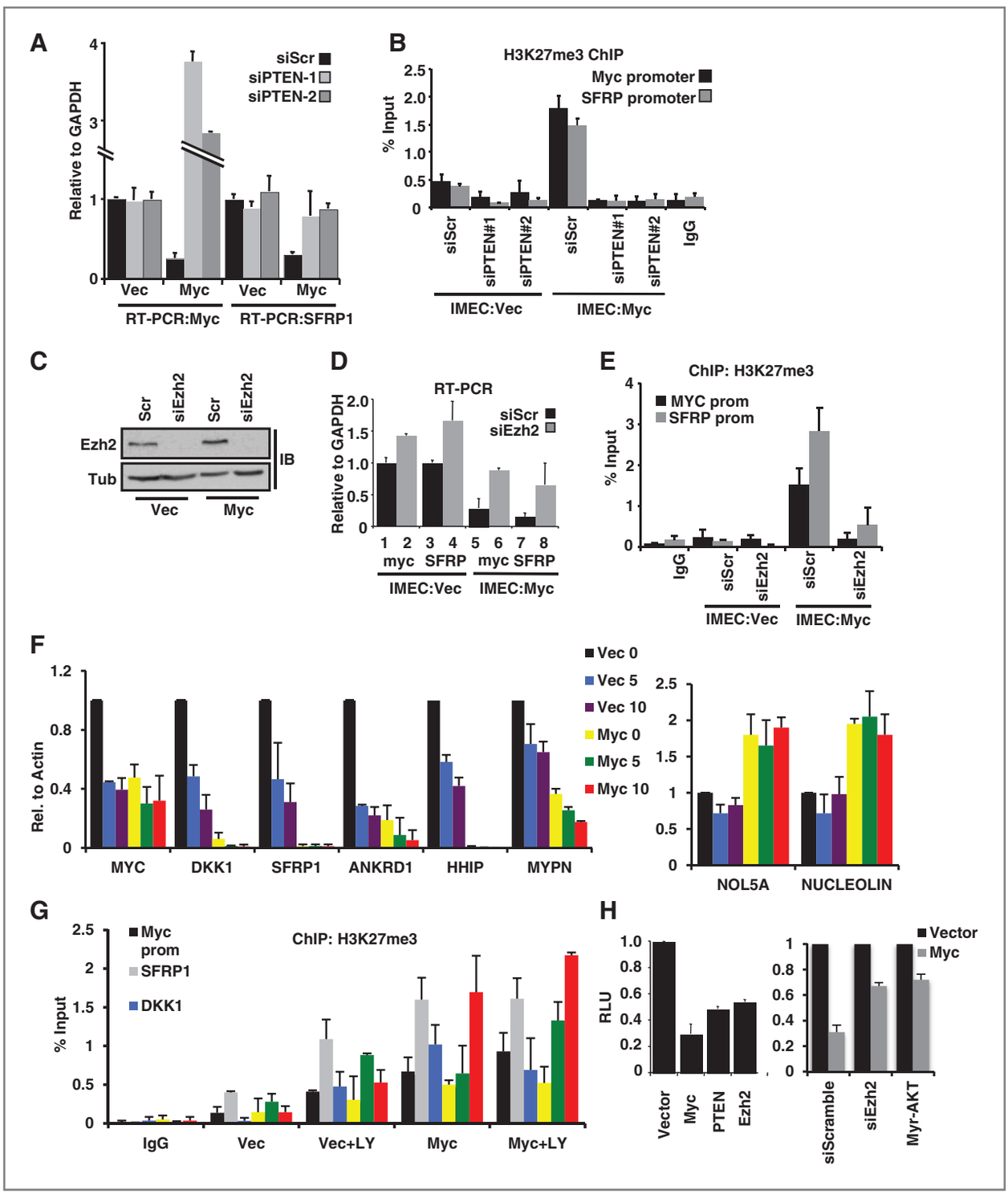

Figure 3. PTEN and Ezh2 are required for Myc-mediated gene repression. A, siRNA-mediated depletion of *PTEN* in IMECs leads to derepression of both endogenous *MYC* and *SFRP* expression. B, depletion of *PTEN* leads to loss of H3K27me3 at the *MYC* and *SFRP* promoters that are repressed by exogenous Myc. C, immunoblot analysis of Ezh2 upon siRNA depletion of *Ezh2* in IMECs transduced with either empty vector or exogenous Myc. D, quantitative RT-PCR for endogenous *MYC* and *SFRP* mRNAs. Expression of both genes is suppressed by ectopic Myc overexpression (compare lanes 1 and 5 with lanes 3 and 7). Depletion of *Ezh2* derepresses both *MYC* and *SFRP* (compare lanes 5 and 6 with lanes 7 and 8). E, ChIP with anti-H3K27me3 after siRNA treatment of *Ezh2* in IMECs. Depletion of *Ezh2* abolishes the H3K27me3 mark at the endogenous *MYC* and *SFRP1* promoters in IMEC:Myc cells. F, quantitative RT-PCR with RNA obtained from cells treated with LY294002 as in Fig. 1F. Left, inhibition of Akt activation induces repression of Myc-repressed

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To test more directly whether the levels of PTEN and Ezh2 can suppress the *MYC* promoter analogous to autoregulation, we conducted a transient assay with a *MYC* promoter luciferase reporter. Ectopic expression of Myc, PTEN, and Ezh2 all suppressed luciferase expression to a similar extent (Fig. 3H, left). Depletion of *Ezh2* with RNA interference or expression of a constitutively active form of AKT (Myr-AKT; ref. 31) is sufficient to alleviate repression of the *MYC* promoter (Fig. 3H, right). These data support a model where Myc and PTEN exist in homeostatic balance to regulate Myc-mediated gene repression and autoregulation, which is supported by a previous report that small changes in PTEN levels are sufficient to alter PI3K/Akt signaling and promote tumorigenesis (32).

Ezh2-mediated repression is independent of Miz-1

While we observed a strong dependence on Ezh2 for Myc-mediated gene repression, neither cell system exhibited a similar dependence on Miz-1 (ZBTB17), a zinc finger transcription factor previously reported to play a role in Myc-mediated repression (refs. 33, 34; Supplementary Fig. S4C–S4G). We also did not observe Miz-1 occupancy at Myc-repressed gene promoters in IMECs (Supplementary Fig. S4C) or a gain of repression in control (IMEC:Vec) cells upon loss of Miz-1 (Supplementary Fig. S4E, left). Similar conclusions could be drawn from $myc^{-/-}$ fibroblasts stably expressing a Myc mutant (V394D) defective in Miz-1 binding (35). $myc^{-/-}$ fibroblasts stably expressing exogenous mouse WT-Myc or V394E were able to repress the transcription of genes similarly (Supplementary Fig. S4F and S4G). These data suggest an alternate mechanism of Myc-mediated repression that is Miz-1 independent.

Expression of ectopic Ezh2 mimics Myc-mediated repression

As overexpression of PTEN could affect multiple signaling pathways, we wanted to determine whether altered activity of Ezh2 alone could account for Myc-mediated repression and autoregulation. To this end, we analyzed gene expression in $myc^{-/-}$ cell lines stably expressing Ezh2WT, Ezh2-S21A (phospho-defective), and Ezh2-S21E (phospho-mimetic) and compared it with repression in response to Myc overexpression. Exogenous Ezh2 expression was comparable with endogenous levels for all 3 constructs (Fig. 4A), which can be resolved because exogenous Ezh2 has a tag that alters its size. Notably, expression of Ezh2-S21A in $myc^{-/-}$ cells is sufficient to repress the *MYC* promoter similar to autorepression, and similar repression was also observed for other Myc-repressed genes (Fig. 4B). Expression of Ezh2-WT induced a modest repression, whereas the Ezh2-S21A mutant repressed as strongly as Myc. In contrast, there was no repression at all with the Ezh2-S21E mutant. Ezh2-WT enhanced H3K27me3 levels at all 3 genes (Fig. 4C). The phospho-defective mutant (S21A) induced even higher H3K27me3 levels, which correlated with stronger repression, whereas the phospho-mimetic Ezh2-S21E induced barely detectable changes (Fig. 4C). In addition, we analyzed the level of a different histone modification (H3K4me3), which is associated with actively transcribed promoters. Interestingly, we found that overexpression of either Myc or any form of Ezh2 led to a complete loss of H3K4me3 at Myc-repressed promoters, confirming the established inverse relationship between these modifications (Fig. 4D; ref. 36). In addition to Myc WT and Ezh2, we also analyzed Myc mutants with defects in either the transactivation domain (ΔMB2) or DNA-binding domain (ΔC). Neither mutant had any effect on H3K27me3 or H3K4me3 levels (Fig. 4C and D), consistent with their defect in gene repression (Fig. 4B).

We were particularly interested in determining the amount of Myc-mediated repression that can be accounted for by enhanced Ezh2 activity and elevated H3K27me3 levels. We analyzed whole-genome expression by microarray using RNA from $myc^{-/-}$ fibroblasts expressing Myc-WT or Ezh2-S21A. We chose the hyperactive, phospho-defective form of Ezh2 to avoid phosphorylation of Ezh2-WT by active Akt (Fig. 1C, lane 4) in $myc^{-/-}$ cells. Comparable levels of H3K27me3 were present in $myc^{+/+}$ parental fibroblasts, Myc-reconstituted $myc^{-/-}$ fibroblasts and $myc^{-/-}$ fibroblasts expressing ectopic Ezh2-S21A (Fig. 4E). After normalizing to empty vector, we selected all genes that were repressed 2-fold or more by Myc WT and assessed their response to Ezh2-S21A. There were 1,802 probes (1,019 unique genes) repressed more than 2-fold by Myc and 1,724 probes (999 genes) repressed by Ezh2 S21A. Of the Myc-repressed genes, 814 probes (462 genes) overlapped with Ezh2-S21A repression or a 45% overlap of repressed genes (Fig. 4F). These data strongly support an integral role for Ezh2 in Myc-mediated gene repression and autoregulation.

In addition, in our experimental setting, we observed that the transforming activity of Myc is directly tied to functional PRC2 and thus gene repression (Fig. 4G). Depletion of Ezh2 significantly impairs the ability of IMEC cells expressing exogenous Myc to form colonies in soft agar. These data corroborate previous reports that have linked Myc-mediated gene repression to its ability to transform cells (5, 37). Altogether, these data provide evidence that Myc-mediated repression is an important component of Myc biology and its function as a potent oncogene.

Discussion

This study presents a new model for the mechanism of Myc-mediated repression based on activation of *PTEN* transcription and the modulation of Ezh2 activity and repressive histone modifications. Whereas complete loss of the PTEN tumor suppressor is common in cancer, even modest changes in

genes in vector cells, mimicking the effect of exogenous Myc (compare Vec + LY294002 with Myc for each target gene). Right, Myc-activated target genes, *Nol5a* and *Nucleolin*, were unaffected by LY294002 treatment. G, Myc-repressed promoters were assessed for H3K27me3 accumulation via ChIP in samples treated with 5 μmol/L LY294002. Treatment of IMEC:Vec cells with LY294002 enhances H3K27me3 mark at Myc repressed promoters in line with reduced transcription (F). H, left, Myc, PTEN, and Ezh2 all repress a *MYC* promoter luciferase reporter in transient assays in IMECs. Right, cells were treated with RNAi against *Ezh2* or transfected with Myr-Akt 24 hours before transfection with the appropriate plasmids for the luciferase assay as stated in Materials and Methods. Graphs represent an average of at least 3 independent replicates and error bars indicate SD.

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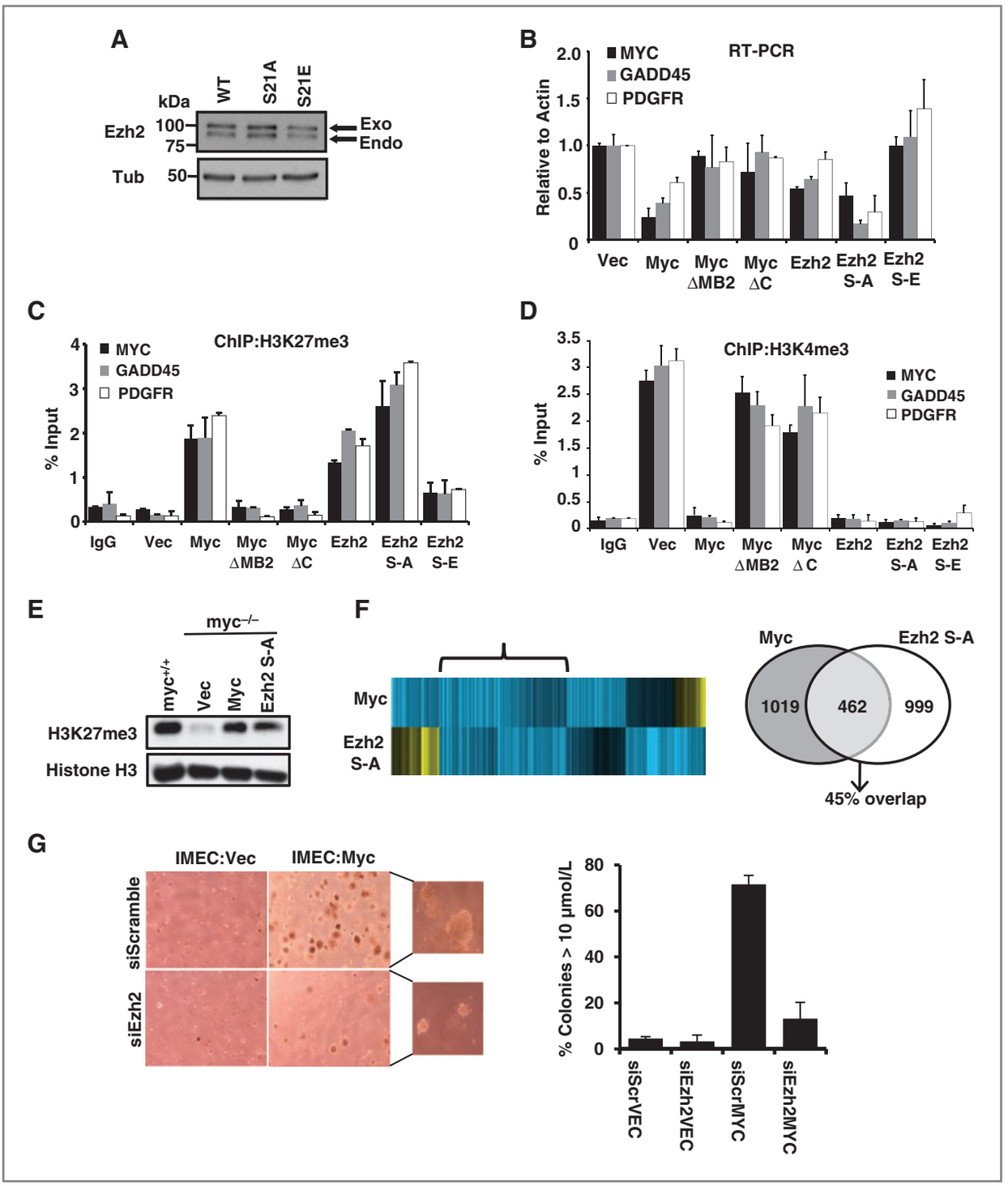

Figure 4. Ezh2 alone can repress *MYC* and Myc-repressed genes. A, Ezh2 and Ezh2 mutants were stably expressed in $myc^{-/-}$ fibroblasts. B, expression of the *MYC* promoter, *GADD45*, and *PDGFRb* was assessed by RT-PCR. C, ChIP for H3K27me3 levels at the *MYC*, *GADD45*, and *PDGFRb* promoters in the same lines from B. D, ChIP for H3K4me3 levels at the *MYC*, GADD45, and PDGFR promoters in the same lines from B. E, immunoblot analysis of total cell lysates from $myc^{+/+}$ and $myc^{-/-}$ samples expressing empty Vector (Vec), exogenous Myc (Myc), or Ezh2 S-A. Myc and Ezh2 increase global levels of H3K27me3. F, heat map of repressed genes from microarray data. Blue, downregulated; yellow, upregulated. The bracket marks genes that are repressed by both Myc and Ezh2S21A. Venn diagram shows the overlap of genes repressed by 2-fold or more in both samples. G, soft agar transformation assay with IMECs expressing empty vector (Vec) or exogenous Myc (Myc) upon depletion of *Ezh2* with RNAi. Graph represents mean number of colonies that were larger than 10 μm per 100 colonies counted (48). Data presented are average of 3 independent replicates and error bars indicate SD.

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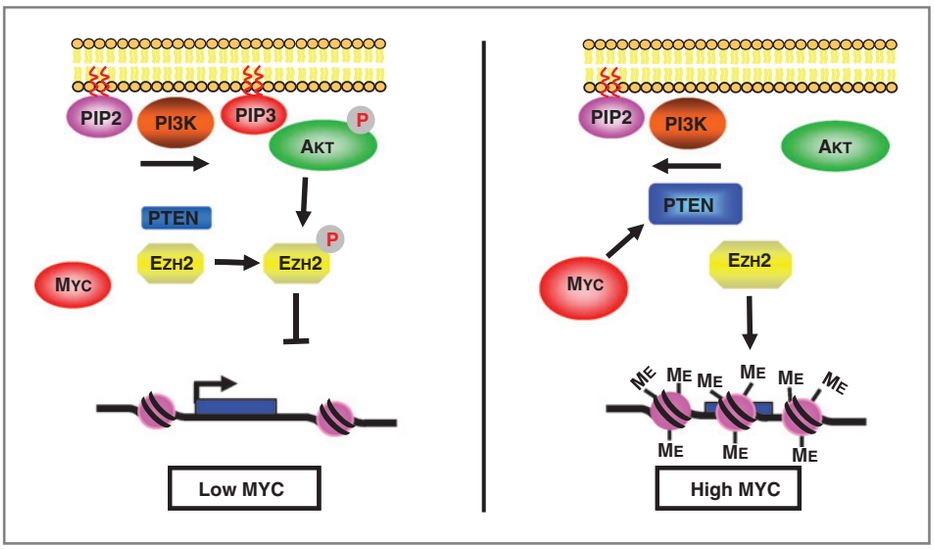

Figure 5. An epigenetic model for Myc-mediated gene repression. Left, cells with low levels of Myc have lower levels of PTEN, allowing more active AKT (pAKT), suppression of Ezh2 activity by phosphorylation (pEzh2), and reduced H3K27me3 levels in the cell. Right, elevated levels of Myc transcriptionally activate expression of the PTEN tumor suppressor that suppresses AKT activity. Suppression of AKT activity in turn suppresses phosphorylation of Ezh2, elevating its methyltransferase activity and also its protein level through reduced turnover. Higher Ezh2 activity promotes repressive H3K27me3 histone modifications on the *MYC* promoter and Myc-repressed genes.

PTEN levels are sufficient to promote oncogenic transformation (32, 38). In parallel, it has been established that incremental changes in Myc expression are a common driving force in cancer and can also contribute to inherited cancer predispositions (3, 15, 39, 40). Given these findings along with the data presented here, we propose that controlling the amount of Myc in a cell by autoregulation via modulation of PTEN expression is pivotal in maintaining the delicate balance of normal growth. Our data are consistent with recent studies showing that elevated PTEN suppresses *MYC* expression (38).

The regulation of Ezh2 by Myc occurs through the Akt pathway, which was previously shown to directly modify Ezh2 activity by phosphorylation (23). Suppression of Akt activity by PTEN reduces Ezh2 phosphorylation, increasing methyltransferase activity and simultaneously increasing Ezh2 protein levels by protein stabilization. Suppression of pAkt, pEzh2, and Ezh2 methyltransferase activity are dependent on both the MB2 and C-terminal DNA-binding domains of Myc, consistent with previous mapping of Myc domains required for gene repression and autoregulation. The dependence on the Myc transactivation domain for gene repression stems from a requirement to induce the expression of PTEN. A recent study shows consistent activation of the Akt pathway in Burkitt lymphomas (41), but it is difficult to interpret these findings in relation to the model presented here because tumors may acquire complex mutational profiles that drive oncogenic growth independent of the *MYC* pathway or gene repression.

We show that the *MYC* gene itself as well as numerous Myc-repressed genes acquire high levels of H3K27me3, which is necessary and largely sufficient to suppress transcription (Fig. 5). Repression by H3K27me3 involves a number of mechanisms such as the recruitment of PRC1 to the H3K27me3 deposited by PRC2 (42), but we find no enrichment of PRC1 components at Myc-repressed promoters. However, as PRC2 can be recruited to the histone modification that it creates (43), a small increase in Ezh2 activity could amplify the regional H3K27me3 modification through positive feedback at responsive genes (43, 44). Stable association of PRC2 complexes with repressed genes could also block the activating H3K4me3 modification as we observe. We show that Myc and Ezh2 share 45% of their repressed genes and that overexpression of Ezh2 alone can recapitulate Myc-mediated gene repression and autoregulation. These data suggest that the modulation of Ezh2 is a major mediator of Myc gene repression.

One aspect of our proposed mechanism that remains unclear is why particular promoters are responsive to Myc-mediated repression. No common motif has been associated with Myc-repressed genes other than the core initiator element in the promoter (33, 34, 45). One possibility is that certain promoters are poised to respond to a variation in H3K27me3 levels because they exist at a threshold in the balance between repressive and activating chromatin configurations. A small shift toward elevated repressive histone modifications could nucleate a localized expansion in repressive chromatin and downregulate gene expression. The balance of repressive and activating chromatin could be highly variable between cell types for individual genes, which could explain why Myc-repressed genes are so variable. An alternate possibility that is not mutually exclusive is that PRC2 complexes are guided to specific genes by noncoding RNAs that may vary in different cell types (46) or that may even be Myc induced. Nevertheless, our study presents a novel feedback pathway linking the potent tumor suppressor *PTEN* to *MYC* regulation and global changes in gene expression and chromatin modification.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Kaur, M. Cole
Development of methodology: M. Kaur, M. Cole
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Kaur, M. Cole
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Kaur, M. Cole
Writing, review, and/or revision of the manuscript: M. Kaur, M. Cole
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Cole

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MYC Acts via the PTEN Tumor Suppressor to Elicit Autoregulation and Genome-Wide Gene Repression by Activation of the Ezh2 Methyltransferase

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