Loss of SIRT3 Provides Growth Advantage for B Cell Malignancies*

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B cell malignancies comprise a diverse group of cancers that proliferate in lymph nodes, bone marrow, and peripheral blood. SIRT3 (sirtuin 3) is the major deacetylase within the mitochondrial matrix that promotes aerobic metabolism and controls reactive oxygen species (ROS) by deacetylating and activating isocitrate dehydrogenase 2 (IDH2) and superoxide dismutase 2 (SOD2). There is controversy as to whether SIRT3 acts as an oncogene or a tumor suppressor, and here we investigated its role in B cell malignancies. In mantle cell lymphoma patient samples, we found that lower SIRT3 protein expression was associated with worse overall survival. Further, SIRT3 protein expression was reduced in chronic lymphocytic leukemia primary samples and malignant B cell lines compared to primary B cells from healthy donors. This lower level of expression correlated with hyperacetylation of IDH2 and SOD2 mitochondrial proteins, lowered enzymatic activities, and higher ROS levels. Overexpression of SIRT3 decreased proliferation and diminished the Warburg-like phenotype in SIRT3-deficient cell lines, and this effect is largely dependent on deacetylation of IDH2 and SOD2. Lastly, depletion of SIRT3 from malignant B cell lines resulted in greater susceptibility to treatment with an ROS scavenger but did not result in greater sensitivity to inhibition of the hypoxia-inducible factor-1α pathway, suggesting that loss of SIRT3 increases proliferation via ROS-dependent but hypoxia-inducible factor-1α-independent mechanisms. Our study suggests that SIRT3 acts as a tumor suppressor in B cell malignancies, and activating the SIRT3 pathway might represent a novel therapeutic approach for treating B cell malignancies.

B cell malignancies represent a diverse group of diseases. Mantle cell lymphoma (MCL)† is a mature, moderately aggressive B cell non-Hodgkin lymphoma with variable course (1). Chronic lymphocytic leukemia (CLL) is the most common B cell malignancy and is characterized by the expansion and accumulation of functionally defective B cells (2–4). For many of these B cell malignancies, allogeneic hematopoietic stem cell transplantation is the only curative treatment currently available.

Sirtuins are NAD+−dependent protein deacetylases that play a major role in genome maintenance, metabolism, cell survival, and aging (5). Humans have seven sirtuins (SIRT1–7) that are localized to distinct cellular compartments and have different substrates (6). The fact that sirtuins are NAD+−dependent enzymes suggests that they are intimately linked to cellular metabolism (7, 8), and evidence shows that fluctuations of cellular NAD+ levels are closely correlated with sirtuin activity (9, 10).

Sirtuin 3 (SIRT3) is the major deacetylase within the mitochondrial matrix and regulates the acetylation state of many mitochondrial proteins (10). Moreover, our recent findings show that SIRT3 is involved in global reprogramming of the mitochondrial protein acetylome (11). SIRT3 deacetylates and thereby activates isocitrate dehydrogenase 2 (IDH2), an enzyme that catalyzes the TCA cycle redox conversion of isocitrate to α-ketoglutarate and serves as a major source of NADPH production (12–14), which is used to increase the ratio of GSH to GSGS, the major redox couple in the cell (12, 15). SIRT3 also deacetylates and activates superoxide dismutase 2 (SOD2, alias MnSOD), which also neutralizes ROS (16–18). ROS activate signaling pathways that regulate proliferation, differentiation, survival, and metabolism, including the ability of ROS to stabilize hypoxia-inducible factor-1α (HIF-1α), a major transcription factor that controls the expression of glycolytic genes (19–21). Furthermore, ROS exert effects independent of HIF-1α, such as activating MAPK/Erk and PI3K/Akt signaling and damaging proteins, lipids, and DNA, which can hamper cellular

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*** The abbreviations used are: MCL, mantle cell lymphoma; CLL, chronic lymphocytic leukemia; IDH2, isocitrate dehydrogenase 2; SOD2, superoxide dismutase 2; ROS, reactive oxygen species; DHE, dihydroxyethidium; HIF-1α, hypoxia-inducible factor-1α; Q-PCR, quantitative PCR; NAC, N-acetylcysteine; CFSE, carboxyfluorescein succinimidyl ester; VDAC, voltage-dependent anion channel.
processes and cause mutations that further promote tumor progression (19).

A number of reports suggest that SIRT3 acts as a tumor suppressor by opposing Warburg-like metabolism, which refers to the observation that cancer cells utilize aerobic glycolysis (22–24). Bypassing oxidative phosphorylation confers a growth advantage likely by providing metabolite intermediates for additional cell growth. In contrast, a number of studies demonstrate that SIRT3 expression is elevated in certain cancers and may function as an oncogene (28). The role of SIRT3 in B cell malignancies has not been fully elucidated. Furthermore, the mechanism underlying increased proliferation mediated by decreased SIRT3 expression has not been elucidated; we assess the relative contributions of the HIF-1α pathway versus other ROS-dependent pathways. Here we provide a mechanistic investigation of the role of SIRT3 in B cell malignancies using primary malignant CLL and MCL samples and B cell malignancy lines. We demonstrate that decreased SIRT3 is observed in a number of B cell malignancies and correlates with adverse clinical factors and survival. Further, we reveal that SIRT3-mediated regulation of proliferation is dependent on modulation of IDH2 and SOD2 activities. Lastly, we find that decreased SIRT3 results in increased proliferation by its effects on the ROS and HIF-1α pathways and suggest that the HIF-1α-independent ROS pathway contributes more than the HIF-1α-dependent pathway to enhancing proliferation in SIRT3-deficient cells.

**Experimental Procedures**

**Cell Culture and Assays**—Our protocol was approved by the University of Wisconsin Institutional Review Board (protocol M-2008-1011). Lymphocytes from peripheral blood of deidentified, newly diagnosed CLL patients were separated using Ficoll, viably frozen in liquid nitrogen, and thawed prior to their use in these experiments. At least 90% of the cells were positive for CD19 (data not shown). Primary B cells from healthy donors were sorted from peripheral blood using the AutoMACS Pro Separation System (Miltenyi Biotec, Auburn, CA) and anti-CD19 beads, and the resulting sorted cells are over 95% pure. The following cell lines were obtained from American Type Culture Collection (Manassas, VA): the acute lymphocytic leukemia line SUP-B15; the Burkitt’s lymphoma lines Raji and Ramos (RA-1); the MCL lines JeKo-1, Mino, Rec-1, and Z-138; and the multiple myeloma lines RPMI-8226 and U266. The MCL cell line Granta519 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). Briefly, the cells were cultured under standard conditions (in humidified incubator, 5% CO₂, 37 °C) in RPMI 1640 with 10% FBS (Cellgro, Manassas, VA), 1% nonessential amino acids (Hyclone, Logan, UT), 2 mM L-alanine-L-glutamine (Hyclone), and 1% sodium pyruvate (Hyclone). Glucose and lactate levels in the culture medium were measured using a glucose assay kit and a L-lactate assay kit (Eton Bioscience, San Diego, CA). 5 × 10⁵ cells were seeded in 1 ml of R10 medium in a 24-well plate and cultured for 2 days. Cellular ROS and was measured by staining with dihydroethidium (DHE; Sigma-Aldrich), and mitochondrial membrane potential was measured by staining with rhodamine 123 as previously described (29). A total of 50,000 events were acquired using an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI) equipped with multicolor analysis, and data were analyzed with FlowJo 7.0 (Tree Star, Ashland, OR). Unstained cells served as controls. We gated on living cells only. GSH and total glutathione levels were determined using the GSH:GSSG-Glo assay kit (Promega, Madison, WI). The cells were plated in a 96-well plate at a concentration of 3 × 10⁴ in 50 μl and analyzed 24 h after seeding. Carboxyfluorescein succinimidyl ester (CFSE) proliferation assays were performed as previously described (30). Data acquisition was performed with an Accuri C6 flow cytometer. Proliferation indexes were determined using ModFit LT (Verity Software House, Topsham, ME). For SYBR green proliferation assays, the cells were plated into 96-well plates at 5000 cells/well. After 5 days of incubation, SYBR green (Lonza) was diluted 1:600 in 10% Nonidet P-40 in PBS and added to wells at a 1:7 ratio. After an overnight incubation, fluorescence was read using a BioTek Synergy 4 plate reader. For soft agar assays, 5000 cells were resuspended in 0.3% agar and plated in triplicate in 24-well plates with a 0.6% base agar layer. The colonies were stained 14 days later with 0.005% crystal violet in 2% methanol and counted. Chemicals used in this study include N-acetyl-L-cysteine, FM19G11, and PX12 (Sigma-Aldrich).

**Immunohistochemistry**—The tissue microarray was constructed as previously described (31). Patient characteristics can be seen in Table 1. For SIRT3 immunohistochemistry, antigen retrieval was performed in a citrate buffer (pH 6). Endogenous peroxidases were quenched with 3% hydrogen peroxide. Blocking was done in 10% donkey serum, and slides were incubated with anti-SIRT3 antibody (C73E3; Cell Signaling, Beverly, MA) at 4 °C overnight, followed by biotin-conjugated anti-rabbit secondary antibody for 1 h at room temperature. ImmPACT DAB Peroxidase Substrate (Vector Labs, Burlingame, CA) was used for chromogenic detection. Slides were mounted using Perm mounting solution (Thermo Fisher, Pittsburgh, PA). Slides were scanned using a Vectra automated quantitative pathology imaging system (Perkin-Elmer Life Sciences). A spectral library was constructed using Nuance (v 3.0.1.2), and tissue images were segmented and scored using InForm (version 1.4.0).

**Mitochondrial Isolation**—To isolate mitochondria, 10–20 million cells were homogenized in a Teflon homogenizer in a sucrose buffer (150 mM sucrose, 10 mM Tris-HCl, pH 7.2). Mitochondria were purified and lysed with a 1% Nonidet P-40 lysis buffer, and the extract was used for the IDH2 and SOD2 activity assays, SOD2 immunoprecipitation assay, and Western blots for SIRT3, IDH2, and SOD2. The efficiency of mitochondrial isolation was tested by probing mitochondrial extracts for voltage-dependent anion channel (VDAC), a mitochondrial marker, and H3, a nuclear marker (see Fig. 2C).

**Enzyme Activity Assays**—Activity of SOD2 was measured using the SOD assay kit (Sigma). Activity of IDH2 was measured as previously described (14). All samples were run in duplicate, and each experiment was done from mitochondrial lysate from three independent cell culture samples. The reaction rates were calculated, and the IDH2 activity in the sample was defined as the production of 1 μmol of NADPH per second.
**SIRT3 in B Cell Malignancies**

**TABLE 1**

| Patient characteristics of 31 MCL patients in the tissue microarray | Number | Percentage |
|---------------------------------------------------------------|--------|------------|
| **Age**                                                       |        |            |
| <50                                                          | 2      | 0.65       |
| 50–59                                                        | 8      | 2.58       |
| 60–69                                                        | 5      | 1.61       |
| 70–79                                                        | 2      | 0.65       |
| ≥80                                                          | 5      | 1.61       |
| Unknown                                                      | 8      | 2.58       |
| **Sex**                                                       |        |            |
| Male                                                         | 15     | 4.84       |
| Female                                                       | 7      | 2.26       |
| Unknown                                                      | 8      | 2.58       |
| **MIPi**                                                      |        |            |
| <5                                                          | 1      | 0.32       |
| 5–6                                                         | 16     | 5.16       |
| 6–7                                                        | 11     | 3.55       |
| ≥7                                                          | 2      | 0.65       |
| **MIPi Risk**                                                 |        |            |
| Low                                                          | 10     | 3.23       |
| Int                                                          | 10     | 3.23       |
| High                                                         | 10     | 3.23       |
| **Histologic variant**                                       |        |            |
| Classic                                                      | 22     | 7.10       |
| Classic nodular                                              | 2      | 0.65       |
| Blastic                                                     | 4      | 1.29       |
| Pleomorphic                                                 | 2      | 0.65       |
| **Ann Arbor stage**                                          |        |            |
| Stage 1                                                      | 2      | 0.65       |
| Stage 2                                                      | 2      | 0.65       |
| Stage 3                                                      | 6      | 1.94       |
| Stage 4                                                      | 20     | 6.45       |

Western Blots—Equal amounts of protein were electrophoresed on 12% SDS-PAGE gels using standard conditions. The proteins were transferred to nitrocellulose membranes, which were probed with the following antibodies: SIRT3 (GeneTel Laboratories, Madison, WI), IDH2-AcK413 (GeneTel), and FLAG (Sigma-Aldrich). Anti-α-tubulin (ab4074; Abcam) was used as a loading control. HRP-conjugated goat anti-rabbit secondary antibody was used (sc-2030; Santa Cruz Biotechnology, Santa Cruz, CA). For determination of SOD2 expression, endogenous SOD2 was immunoprecipitated from mitochondrial extracts using goat anti-SOD2 antibody (sc-18503; Santa Cruz) and then probed with rabbit anti-SOD2 antibody (sc-30080; Santa Cruz).

**SIRT3, IDH2, and SOD2 Overexpression and Knockdown**—Cell lines were transfected with pcDNA3.1 containing SIRT3 or IDH2 (14) using FuGENE HD transfection reagent (Promega). The cells were plated onto a 6-well plate the day before transfection. Transfected cells were selected with 1 mg/ml G418 (Sigma) for 1 week and expanded for growth at 0.5 mg/ml G418. Depletion of SIRT3, IDH2, and SOD2 was accomplished using DharmaFECT reagent and siRNA targeting SIRT3 (1-J-004827-09; Dharmacon), IDH2 (J-004013-09), or SOD2 (J-009784-05). Scrambled siRNA was used as a control. Overexpression and depletion were assessed by Western blot.

**Quantitative Polymerase Chain Reaction (Q-PCR)**—RNA was isolated from cells using a RNeasy micro kit (Qiagen) and converted to cDNA using the Quantitect reverse transcription kit (Qiagen). Q-PCR was performed using Power SYBR green master mix (Applied Biosystems, Foster City, CA) on StepOne Plus instrument (Applied Biosystems) using standard protocol. Quantification of given genes expressed as mRNA level was normalized to the mRNA of three housekeeping genes (RRN18S, GAPDH, and ACTB). Qiagen primers were used for RRN18S, GAPDH, ACTB, IL6, and VEGFA. Primers for GLUT1, PFK1, and PDK1 were synthesized by the University of Wisconsin Biotechnology Center and have the following sequences (5′ to 3′): GLUT1 forward, GGCACAGGTGTGCT-AAAGAA; GLUT1 reverse, ACACGGTTGTAGCCAGACAG; PFK1 forward, GCTGGGGCGCCTACTACATT; PFK1 reverse, TCGAGTGCCAGTAGTCCG; PDK1 forward, CTGTTGACGGATCAAAACC; and PDK1 reverse, TCCACC-AACAAATAGAGTGCCT.

**Statistical Analyses**—Experiments with cell lines were performed using cells from three separate culture flasks. Statistical significance was assessed by Student’s t test on Microsoft Excel, and p values <0.05 were considered statistically significant. Flow cytometry histograms and plots were generated using FlowJo. Survival curves were made using the Kaplan-Meier method and compared with log rank tests using R statistical software (version 3.1.1; Vienna, Austria). Correlations were performed using Pearson correlation coefficients (GraphPad Prism, version 6.0).

**Results**

Decreased SIRT3 Is Prognostic of Worse Outcomes in Mantle Cell Lymphoma—We evaluated SIRT3 expression using immunohistochemistry on tissue microarrays containing samples from patients with mantle cell lymphoma (n = 31), follicular lymphoma (n = 6), Burkitt’s lymphoma (n = 3), and CLL (n = 6). Among the MCL cases for which survival data were collected, patients with low SIRT3 expression demonstrated worse overall survival (Fig. 1A; p = 0.10). Patients with a low MCL International Prognostic Index risk score, a prognostic score for MCL based on age, performance status, serum lactate dehydrogenase, and leukocyte count (33), had tumors with greater SIRT3 expression compared with patients with intermediate or high MCL International Prognostic Index risk scores (Fig. 1B).

Furthermore, SIRT3 expression negatively correlated with proliferation, as indicated by Ki67 score (Fig. 1C; p = 0.025). Representative immunohistochemistry images demonstrate SIRT3 expression in lymph node and tonsil compared with MCL (Fig. 1D). These initial findings suggested that SIRT3 might play a tumor suppressor role in the pathogenesis of MCL and other B cell malignancies.

Decreased SIRT3 Protein Expression and IDH2 and SOD2 Activities in CLL Primary Cells and Malignant Cell Lines—The functions of SIRT3, namely its ability to reduce ROS and mediate metabolic flexibility, oppose the Warburg effect, which is a hallmark of cancer cells (22). Utilizing cells from peripheral blood of CLL patients and healthy donors, we found that SIRT3 protein expression is decreased CLL in cells compared with primary B cells (Fig. 2, A and B). Next, we assessed the activity and acetylation status of mitochondrial IDH2, which is a *bona fide* substrate of SIRT3 (14). To assess the quality of our mitochondrial purification, we probed the mitochondrial extracts with an antibody for the mitochondrial marker VDAC (Fig. 2C). Although total IDH2 protein levels were unchanged, IDH2 was hyperacetylated on K413 in CLL samples compared with normal B cell samples (Fig. 2A), and IDH2 catalytic activity was...
significantly reduced in mitochondrial extracts from CLL cells compared with B cells from healthy donors ($p < 0.05$; Fig. 2D). These data support the idea that reduced SIRT3 expression in CLL cells has functional consequences, namely the down-regulation of IDH2 activity caused by hyperacetylation.

Because of decreased SIRT3 levels in CLL cells, we hypothesized that CLL cells would display higher mitochondrial ROS as a result of lowered capacity to generate NADPH through IDH2. We treated CLL cells and normal B cells with the ROS indicator DHE and analyzed mean fluorescence intensity by flow cytometry. After crossing the cell membrane, DHE is oxidized by in vivo ROS to generate the fluorescent molecule ethidium (34). We observed increased ethidium levels in primary CLL cells compared with primary B cells ($p < 0.01$; Fig. 2E), indicating that CLL cells exhibit increased ROS levels. In agreement with higher ROS, there were lower GSH levels in CLL samples compared with primary B cell samples ($p < 0.01$; Fig. 2F). To rule out the possibility that B cells are simply synthesizing more glutathione, we measured total glutathione levels and found no difference between CLL cells and normal B cells (data not shown), consistent with an alteration in the ratio of reduced to oxidized GSH. These observations are in agreement with previous reports that CLL cells display higher ROS compared with normal PBMCs from healthy donors and might explain the higher incidence of mtDNA mutations in CLL (35, 36).

We next hypothesized that decreased SIRT3 is common among B cell malignancies, so we assessed SIRT3 expression in a number of B cell malignancy lines: the acute lymphocytic leukemia line SUP-B15; the Burkitt’s lymphoma line Raji; and the MCL lines Granta-519, JeKo-1, Mino, Rec-1, and Z-138. We also extended our investigation to the plasma cell malignancy, multiple myeloma, using two multiple myeloma lines: RPMI-8226 and U266. Consistent with the CLL patient samples, SIRT3 expression was reduced in all of these malignant lines (except for RPMI-8226) compared with normal B cells, although the levels varied among the eight B cell malignancy lines (Fig. 2G). Interestingly, SIRT3 protein expression by Western and mRNA expression by Q-PCR demonstrated an inverse relationship (Fig. 2K). Furthermore, these B cell malignancy lines exhibited increased AcK413-IDH2 levels relative to total IDH2 and consequently displayed decreased IDH2 activity compared with primary B cells (Fig. 2, G and I). These findings were observed in all cell lines examined except for RPMI-8226, which exhibited no differences in SIRT3 expression, IDH2 acetylation, and IDH2 activity, compared with normal B cells. Among the cell lines, SIRT3
FIGURE 2. Decreased SIRT3 expression in CLL and B cell malignancy lines associated with decreased IDH2 activity.  
A, Western blots using mitochondrial extract from CLL primary cells and primary B cells from healthy donors probed for SIRT3, IDH2, and Ack413-IDH2 (the acetylated, less active form of IDH2). This was repeated for a total of 11 CLL samples and 8 normal B cell samples.  
B, relative SIRT3 expression in primary B cells (n = 8) compared with CLL cells (n = 11).  
C, Western blots of nuclear and mitochondrial extracts demonstrating the purity of mitochondrial extract, as assessed by the presence of VDAC and the lack of histone H3.  
D, IDH2 activities were determined from mitochondrial extracts.  
E, representative histogram of cells stained with DHE for ROS expression. The gray-filled peak represents CLL cells treated with NAC, a negative control, and the black-filled peak represents CLL cells treated with H2O2. The mean fluorescence intensity was determined for each sample, and then averages were calculated for all the CLL samples and all the B cell samples. The adjacent bar graph shows average mean channel fluorescence ± S.E. for CLL (n = 5) and normal B cells (n = 5).  
F, levels of GSH measured in CLL cells and B cells.  
G, Western blots using mitochondrial extract from the indicated cell lines probed for SIRT3, IDH2, and Ack413-IDH2.  
H, scatter plot showing the correlation of SIRT3 protein level with acetylated IDH2 level (both from Western blots) in the cell lines (R² = 0.692).  
I, bar graph showing IDH2 activity in primary B cells compared with the cell lines.  
J, scatter plot showing the correlation of SIRT3 protein expression (from Western blots) with SIRT3 mRNA expression (determined by Q-PCR) for the B cell malignancy lines used in G–J. * p < 0.05; ** p < 0.01.
expression inversely correlated with acetylated IDH2 level ($r = -0.832, p = 0.005$; Fig. 2H) and positively correlated with IDH2 activity ($r = 0.871, p = 0.002$; Fig. 2J). Furthermore, the cell lines expressed higher ROS levels compared with normal B cells (data not shown).

In addition to IDH2, SOD2 has been identified as a major target of SIRT3 (16–18). SIRT3 deacetylates and activates SOD2, enabling greater clearance of ROS from mitochondria. We examined whether SIRT3 expression correlated with SOD2 acetylation status and activity in the B cell malignancy lines. Western blot analysis of SIRT3 levels (Fig. 2G) revealed that Raji and Z-138 lines expressed the lowest levels of SIRT3 among the cell lines tested and that RPMI-8266 and JeKo-1 expressed the highest levels of SIRT3, so we chose these lines for SOD2-related experiments. Consistent with SIRT3 expression levels, Raji and Z-138 exhibited significantly higher levels of acetylated SOD2 and lower SOD2 activity compared with RPMI-8226, JeKo-1, and primary B cells (Fig. 3, A and B). Overall, SIRT3 expression inversely correlated with acetylated SOD2 levels ($r = -0.904, p = 0.014$; Fig. 3C) and correlated positively with SOD2 activity ($r = 0.710, p = 0.114$; Fig. 3D). Taken together, these findings suggest that a loss of SIRT3 expression in B cell malignancies impairs the ability of antioxidant pathways (IDH2 and SOD2) to mitigate ROS damage.

**SIRT3 Overexpression Reduces Warburg-like Features**—Next we investigated whether the malignant phenotypes observed in these B cell malignancy lines were direct consequences of reduced SIRT3 expression. To examine this hypothesis, we reintroduced SIRT3 expression into the Raji, Z-138, and Ramos cell lines. We verified that Ramos cells also exhibit decreased SIRT3 expression comparable to Raji and Z-138 (data not shown). The cell lines were transfected with SIRT3-containing vector and selected with G418 to establish stable expression. Subsequently, several properties of the SIRT3 transfected cells were compared with control cells (transfected with empty pcDNA3.1 vector). Western blotting was performed to demonstrate transfection efficiency (Fig. 4A). SIRT3-overexpressing cells showed decreased levels of acetylated IDH2 and SOD2 and increased IDH2 and SOD2 activities ($p < 0.05$) compared with wild type cells (Fig. 4, B and C). Furthermore, SIRT3-overexpressing cells showed decreased ROS ($p < 0.01$), increased GSH levels ($p < 0.01$), and increased mitochondrial membrane potential ($p < 0.05$) compared with control cells (Fig. 4, D–F). Mitochondrial membrane potential ($\Delta \Psi_{mt}$) was determined using the cationic dye rhodamine 123, which will accumulate within mitochondria in inverse proportion to $\Delta \Psi_{mt}$ (37). Thus, a more negative (i.e. more polarized) $\Delta \Psi_{mt}$ will accumulate more dye. SIRT3-overexpressing cells showed increased rhodamine 123 fluorescence, indicating a more polarized $\Delta \Psi_{mt}$ and enhanced oxidative metabolism (Fig. 4F).

We performed additional analyses to determine whether metabolic phenotypes associated with the Warburg effect could be reversed in the SIRT3-overexpressing cell lines. We measured glucose consumption and lactate production in the culture medium of wild type cells and SIRT3-overexpressing cells. SIRT3-overexpressing cells showed decreased glucose consumption ($p < 0.05$) and decreased lactate production ($p < 0.05$; Fig. 4, I–J), suggesting that SIRT3 overexpression suppressed these Warburg-like features. To provide additional evidence for reversal of the Warburg phenotype, we analyzed the expression of genes controlled by HIF-1α. It is well established that the HIF-1α transcription factor is stabilized...
under hypoxic conditions or when ROS levels are elevated (20). Here we demonstrate that SIRT3 overexpression decreased expression of the following genes controlled by HIF-1α: GLUT-1, PKF-1, PDK-1, VEGF-A, and IL-6 (Fig. 4K). Because the Warburg effect is associated with increased proliferation, we performed proliferation assays and observed decreased proliferation in the SIRT3-overexpressing cells compared with control cells ($p < 0.05$; Fig. 4G). Consistent with this, SIRT3-overexpressing cells grew fewer colonies in soft agar assays (Fig. 4H). Together, our data suggest
that SIRT3 overexpression in SIRT3-deficient cell lines reduces oxidative stress and confers an anti-Warburg effect.

**SIRT3 Overexpression Inhibits Proliferation Via IDH2- and SOD2-dependent Mechanisms**—We have demonstrated that overexpression of SIRT3 decreases cellular proliferation and increases IDH2 and SOD2 activity. In cells transfected with empty vector, treatment with siRNA targeting IDH2 and SOD2 increased proliferation (Fig. 5C). In cells overexpressing SIRT3, depleting IDH2 and SOD2 restored proliferation to near normal levels (i.e. vector + scrambled condition). This was observed in both Raji and Z138 cell lines.

Next we asked whether overexpression of IDH2 in the lymphoma cell lines affects proliferation. Indeed, we find that overexpression of IDH2 reduces proliferation (Fig. 5F).

To determine whether this was dependent on SIRT3, we treated IDH2-overexpressing cells with siRNA targeting SIRT3. Among these IDH2-overexpressing cells, there was no difference in proliferation based on SIRT3 depletion (Fig. 5F).

**SIRT3 Depletion Increases Sensitivity to Antioxidant Treatment**—We and others have demonstrated that decreased SIRT3 leads to increased ROS (24), and this can contribute to increased proliferation in HIF-1α-dependent and -independent pathways. HIF-1α-dependent ROS pathways include the ability of ROS to stabilize HIF-1α, resulting in increased glycolytic gene expression. In contrast, HIF-1α-independent ROS pathways include the post-translational modification of kinases and phosphatases (38, 39). To decipher the relative contribution of these two pathways to the observed proliferation, we compared the susceptibility of SIRT3 wild type and SIRT3-depleted cells to either ROS scavenging with the antioxidant N-acetylcysteine (NAC) or to HIF-1α inhibition. SIRT3 was depleted in Ramos, Rec-1, and Z-138 lines (Fig. 6A) and treated with NAC or one of two HIF-1α inhibitors: FM19G11 or PX12.

The effectiveness of NAC was assessed by DHE staining (Fig. 6B), and the effectiveness of the HIF-1α inhibitors was assessed by GLUT1 and PFK1 gene expression analysis, because these are HIF-1α target genes (Fig. 6D). Treatment with NAC decreased proliferation selectively in SIRT3-depleted cells (Fig. 6C). There was no difference in susceptibility to HIF-1α inhibition based on SIRT3 expression (Fig. 6E).

Thus, SIRT3-deficient cells sensitize to ROS scavenging but not HIF-1α inhibition. This provides evidence that the effects of decreased SIRT3 on increasing proliferation are due to ROS-dependent but HIF-1α-independent mechanisms.

**Discussion**

Mitochondrial ROS are thought to be a major contributor to neoplastic transformation and cancer progression by causing mutations of nuclear or mitochondrial DNA. The main mitochondrial deacetylase, SIRT3, regulates ROS levels by deacetylating enzymes involved in both ROS production and detoxification. In cancer, the role of SIRT3 has been controversial because studies have reported both oncogenic and tumor suppressor functions, depending on the tissue of origin. However, it is unknown whether SIRT3 plays a critical role in B cell malignancies. Here we provide evidence that a loss of SIRT3 leads to hyperacetylation of IDH2 and SOD2, thereby decreasing IDH2 and SOD2 activities in malignant CLL cells and B cell malignancy lines. Furthermore, decreased SIRT3 expression was associated with worse survival and adverse clinical features in our MCL cohort. Our findings are consistent with previous studies showing that SIRT3 deacetylates and activates IDH2 and SOD2 (14, 16–18). Notably, less activated IDH2 results in lower production of NADPH, which is required for conversion of GSSG to GSH. Therefore, our data indicate that loss of SIRT3 can lead to decreased GSH, which can result in higher ROS levels and oxidized proteins. Reintroduction of SIRT3 into the SIRT3-deficient cell lines reduced ROS via SIRT3-mediated deacetylation and activation of IDH2 and SOD2. Furthermore, SIRT3 overexpression suppressed Warburg-like features, as evidenced by decreased expression of glycolytic genes, decreased glucose uptake, and decreased lactate production. Our data suggest that reduced expression of SIRT3 leads to higher ROS, promotes a Warburg-like effect and thus could confer a growth advantage in B cell malignancies.

Interestingly, previous studies have suggested that SIRT3 can act either as a tumor suppressor or as an oncogene depending on the type of cancer and the method used to quantify expression of SIRT3 (41). SIRT3 protein level has been reported to be higher in oral squamous cell carcinoma cell lines, and knockdown of SIRT3 inhibited oral squamous cell growth (28). SIRT3 protein level is also higher in some cases of esophageal squamous cell carcinoma (42). Another study found that SIRT3 gene expression was increased in node-positive breast cancer biopsies compared with normal tissue (43). In contrast, SIRT3 protein levels are decreased in breast and hepatocellular cancers, and lower SIRT3 expression correlates with worse survival in colorectal and hepatocellular cancers (21, 23, 44–46).

Additionally, gene expression studies have shown that both RNA levels and proteins levels of SIRT3 are decreased in human breast, prostate, head, neck, and brain cancers (23). Thus, it is...
likely that SIRT3 functions are cell type- or tumor type-specific. In this study, our data suggest that SIRT3 acts as a tumor suppressor in B cell malignancies.

To our knowledge, there have been two other reports correlating the expression of SIRT3 in CLL cells with clinical data. One study showed that patients expressing lower SIRT3 mRNA had worse prognosis, although SIRT3 mRNA expression was actually higher in CLL cells compared with normal B cells (47). Another study showed that higher SIRT3 mRNA expression correlated with higher CD44 expression, and CD44 has been associated with advanced CLL (48). It is important to note that these two prior studies did not quantify SIRT3 protein levels but rather only assessed RNA transcripts. Here we analyzed protein levels in diverse cells and a tissue microarray. We found an inverse correlation between SIRT3 protein and mRNA levels in the B cell malignancy lines used in our experiments, a phenomenon that has similarly been reported for SIRT1 in AML (49). Furthermore, other reports reveal the same discrepancy and suggest that a highly regulated post-transcriptional process might be involved (50, 51). Future studies will be needed to investigate this interesting post-transcriptional regulation.

Oxidative stress is a biochemical characteristic of cancer cells attributed to multiple mechanisms, including mitochondrial

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**FIGURE 5.** SIRT3-mediated proliferation decrease is dependent on IDH2 and SOD2. A and B, IDH2 and SOD2 were knocked down in the Raji and Z-138 cell lines overexpressing SIRT3 or vector (VEC) control, as in Fig. 4. IDH2 and SOD2 Western blotting were performed to assess knockdown efficiency. C, proliferation was assessed using a SYBR green assay. After treatment with siRNA, 5000 cells/well were seeded in 96-well plates for 4 days. SYBR green was added for overnight incubation, and then SYBR content was analyzed with a plate reader. The values are normalized to the vector + scrambled condition. D, IDH2 or vector control was expressed in Raji and Z-138 cell lines. E, subsequently, cells were treated with either siSIRT3 or scrambled siRNA control. Western blots were used to demonstrate transfection efficiency. F, SYBR green proliferation assays were performed the same as in C. The bars represent means of three independent experiments ± S.E. *, p < 0.05; ns, not significant.
respiratory malfunction and oncogenic stress (19). Higher ROS levels serve to stabilize HIF-1α/HIF-1β, resulting in the up-regulation of the glucose transporters, glycolytic genes, and several critical growth factors and cytokines, such as VEGF and IL-6 (52). Expression of these glycolytic genes leads to a higher rate of glucose catabolism and lactate production, which promote glycolysis and contribute to the Warburg effect (32). In addition, ROS are known to act in HIF-1α-independent mechanisms, such as by inactivating mitochondrial metabolic enzymes such as aconitase and α-ketoglutarate dehydrogenase, leading to a suppression of the TCA cycle (40). It has been unclear whether decreased SIRT3 results in increased cellular proliferation via HIF-1α-dependent or -independent pathways. We addressed this question using an antioxidant and HIF-1α inhibitors, finding that cells depleted of SIRT3 were sensitized to antioxidant treatment but not HIF-1α inhibitors. This suggests that HIF-1α-independent ROS pathways are more important in increased proliferation in SIRT3-deficient cells. In addition, we demonstrated that the anti-Warburg-like effect of SIRT3 is dependent on IDH2 and SOD2, two bona fide substrates of SIRT3.

In summary, we demonstrate that a loss of SIRT3 contributes to cancer cell growth through mechanisms involving anti-oxidant pathways of IDH2 and SOD2 and metabolic reprogramming. Our results suggest that stimulation of the SIRT3 pathway might provide a viable therapeutic strategy for MCL, CLL, and other B cell malignancies.

**FIGURE 6. Effects of decreased SIRT3 on increasing proliferation rely more on HIF-1α-independent ROS pathways.** A, SIRT3 was knocked down in Ramos, Rec-1, and Z-138 lines using siRNA. Scrambled siRNA was used as a negative control. SIRT3 Western blotting (left panel) and Q-PCR (right panel) were performed to assess knockdown efficiency. B, NAC is capable of reducing ROS, as measured by staining with DHE and subsequent flow cytometry analysis. C, cells were stained with CFSE, rested for 12 h, and then siRNA knockdown was initiated. The cells were analyzed by flow cytometry 48 h later. Proliferation indices are plotted. The bars represent means of three independent experiments ± S.E. *, p < 0.05; ns, not significant.
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Author Contributions—W. Y. and R. A. D. designed the research, performed experiments, analyzed the results, made figures, and wrote the paper; K. A. K. processed and analyzed the tissue microarrays, analyzed the results, made figures, and wrote the paper; K. M. G. processed the patient samples; and J. M. D., P. H., F. A., and D. T. Y. designed the research, analyzed the results, and wrote the paper.

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