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Activation of p53 by SIRT1 Inhibition Enhances Elimination of CML Leukemia Stem Cells in Combination with Imatinib

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SUMMARY

BCR-ABL tyrosine kinase inhibitors (TKI) fail to eliminate quiescent leukemia stem cells (LSC) in chronic myelogenous leukemia (CML). Thus, strategies targeting LSC are required to achieve cure. We show that the NAD^+-dependent deacetylase SIRT1 is overexpressed in human CML LSC. Pharmacological inhibition of SIRT1 or SIRT1 knockdown increased apoptosis in LSC of chronic phase and blast crisis CML and reduced their growth in vitro and in vivo. SIRT1 effects were enhanced in combination with the BCR-ABL TKI imatinib. SIRT1 inhibition increased p53 acetylation and transcriptional activity in CML progenitors, and the inhibitory effects of SIRT1 targeting on CML cells depended on p53 expression and acetylation. Activation of p53 via SIRT1 inhibition represents a potential approach to target CML LSC.

INTRODUCTION

Chronic myelogenous leukemia (CML) results from malignant transformation of a hematopoietic stem cell (HSC) by the BCR-ABL oncogene. CML usually presents in a chronic phase (CP) but progresses to an accelerated phase (AP) and a terminal blast crisis (BC) (Sawyers, 1999). The BCR-ABL tyrosine kinase inhibitors (TKI) imatinib (IM), nilotinib, and dasatinib are effective in inducing remissions and prolonging survival of CP CML patients but is less effective against advanced phase CML (Eiring et al., 2011). However, even in CP CML, primitive leukemia stem cells (LSC) are retained in patients achieving remission with TKI treatment (Chu et al., 2011). Primitive, quiescent CML LSC are resistant to apoptosis following TKI treatment despite effective inhibition of BCR-ABL kinase activity (Holtz et al., 2005; Corbin et al., 2011), the mechanisms for which are not well understood. Disease recurrence is usually seen following cessation of drug treatment, even in patients with undetectable BCR-ABL expression by q-PCR (Mahon et al., 2010). These observations suggest that "cure" may be elusive for most CML patients with TKI alone. CML patients currently need to take TKI treatment indefinitely, with risks of toxicity, lack of compliance, drug resistance, relapse, and associated expense.

Recent studies from our group have shown that pan-histone deacetylase (HDAC) inhibitors in combination with IM significantly increase apoptosis in quiescent CML stem cells (Zhang et al., 2010). However, toxicity of this approach to normal stem cells remains a potential concern. Sirtuins are NAD-dependent histone deacetylases that have been linked to longevity in lower organisms and to mammalian metabolism (Bordone and Guarente, 2005; Liu et al., 2009a). Sirtuin 1 (SIRT1) is a member of the sirtuin family that regulates numerous processes, including

Significance

BCR-ABL kinase inhibitors (TKI) are effective in the treatment of CML but do not eliminate leukemia stem cells (LSC), which remain a potential source of recurrence. The NAD-dependent deacetylase SIRT1 is reported to protect stem cells against stress and functions as a tumor suppressor or tumor promoter depending on cellular context. Our studies show that SIRT1 is overexpressed in CML LSC and that SIRT1 inhibition selectively reduces CML LSC survival and growth through acetylation and activation of the p53 tumor suppressor. These results are important because they show that SIRT1-mediated p53 deacetylation contributes to CML LSC survival and resistance to TKI treatment. SIRT1 inhibition is an attractive approach to selectively target LSC that resist elimination by current treatments.

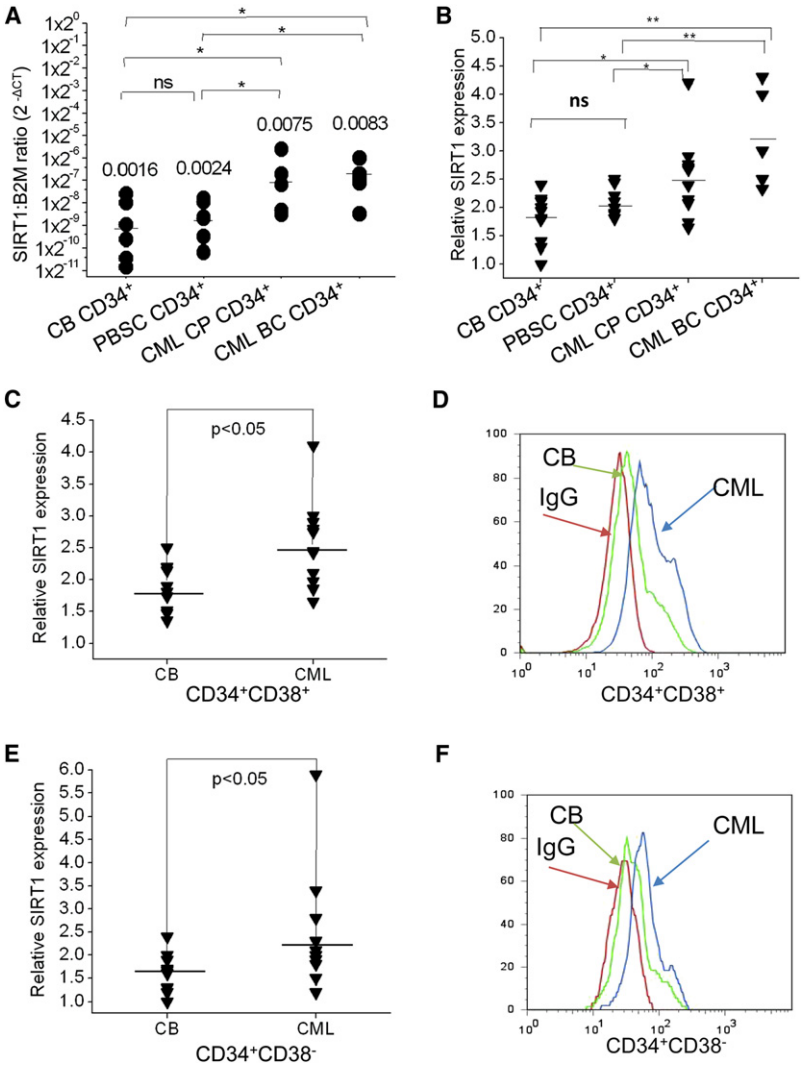


Figure 1. Increased SIRT1 Expression in CML Patients Compared with Normal Stem/Progenitor Cells

(A) Expression of *SIRT1* mRNA in CP CML (n = 5), BC CML (n = 5), cord blood (CB) (n = 6), and PBSC (n = 6) $CD34^+$ cells analyzed by Q-PCR.

(B) Expression of SIRT1 protein in CP CML (n = 11) and BC CML (n = 5) compared with CB $CD34^+$ cells (n = 10) and PBSC $CD34^+$ cells (n = 8) analyzed by intracellular labeling with anti-SIRT1 antibody. Median fluorescence intensity (MFI) of SIRT1 was expressed relative to IgG control.

(C) Expression of SIRT1 in CML (n = 11) and CB (n = 10) $CD34^+CD38^+$ committed progenitors (right panel). Representative results are shown in panel (D): CML (blue), CB (green), IgG (red).

(E) Expression of SIRT1 in CML (n = 11) and CB (n = 10) $CD34^+CD38^-$ stem cells/primitive progenitors. Representative results are shown in panel (F): CML (blue), CB (green), IgG (red). Significance: $^*p < 0.05$, $^{**}p < 0.01$ for the indicated comparisons. See also Figure S1 and Table S1.

aging, DNA repair, cell cycle, metabolism, and cell survival under stress conditions (Bordone and Guarente, 2005; Liu et al.,2009a). In contrast to Class I, II, and IV HDACs, SIRT1 activity is not inhibited by pan-HDAC inhibitors (Liu et al., 2009a). SIRT1 plays an important role in maintaining self-renewal and differentiation of murine embryonic stem cells (ESC) and HSC, especially under conditions of stress (Han et al., 2008; Narala et al., 2008; Ou et al., 2011). Importantly, SIRT1 may have a pathogenetic role in solid tumors and leukemias (Brooks and Gu, 2009; Liu et al., 2009a). SIRT1 can potentially regulate the acetylation of several transcription factors, including p53 (Luo et al., 2001), Ku70, and FoxOs (Brooks and Gu, 2009). Despite the clear inhibitory effect of increased SIRT1 expression on tumor suppressors like p53 and FoxOs, other studies suggest that SIRT1 may also have tumor-suppressive functions. In the $Apc^{+/-}$ mouse model of colon cancer, increased SIRT1 expression resulted in reduced cell proliferation and tumor formation (Firestein et al., 2008). Activation of SIRT1 by resveratrol can limit cell growth and reduce tumor formation in BRCA1-deficient tumor cells and in $Trp53^{+/-};Sirt1^{+/-}$ mice (Wang et al., 2008a, 2008b). The precise role of SIRT1 in cancer may depend on the specific cell or tumor type and the presence or absence of p53 (Brooks and Gu, 2009).

Previous studies have shown that SIRT1 expression is increased in CML blast crisis (BC) cell lines (Chen et al., 2005). Here we investigated the contribution of SIRT1 to the survival and growth of CP and BC CML LSC and progenitor cells and in LSC resistance to TKI treatment. We also investigated the role of p53 in mediating the effects of SIRT1 inhibition on CML progenitors.

RESULTS

SIRT1 Is Overexpressed in CML $CD34^+$ Cells

SIRT1 mRNA levels were significantly elevated in CP and BC CML $CD34^+$ cells (Table S1 available online) compared to $CD34^+$ cells from cord blood (CB) or normal peripheral blood stem cell collections (PBSC) (Figure 1A). SIRT1 protein levels in CML and normal $CD34^+CD38^+$ committed progenitors and $CD34^+CD38^-$ primitive progenitors were measured by intracellular labeling with anti-SIRT1 antibody and flow cytometry (Figure S1A). The ability of intracellular labeling to reliably measure SIRT1 expression was confirmed by western blotting (Figure S1B). SIRT1 protein levels were significantly elevated in CML CP and BC $CD34^+$ cells (Figure 1B), CML CP $CD34^+CD38^+$ (Figures 1C and 1D), and $CD34^+CD38^-$ cells (Figures 1E and 1F) compared to their normal counterparts.

SIRT1 Inhibition Using shRNA Reduces CML Progenitor Proliferation, Survival, and Colony Growth

To investigate the functional role of SIRT1 in CML and normal progenitors, CML and normal $CD34^+$ cells were transduced with lentivirus vectors coexpressing SIRT1 or control shRNAs together with RFP. $CD34^+RFP^+$ cells were selected using flow cytometry. Western blotting confirmed effective inhibition of SIRT1 expression, whereas the expression of the related SIRT2 was not affected (Figures 2A and 2B).

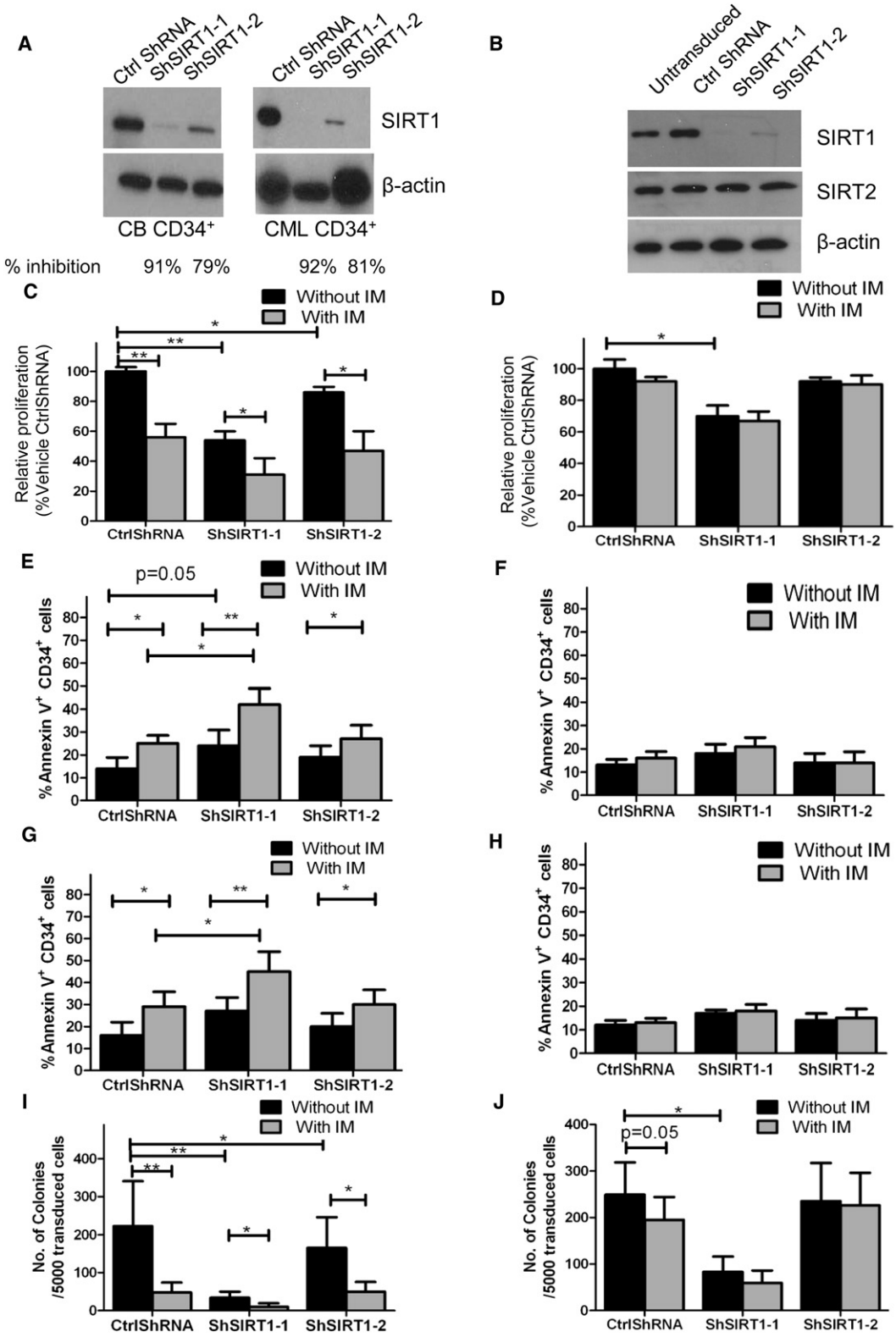


Figure 2. SIRT1 Knockdown Using Specific Anti-SIRT1 shRNA Increases Apoptosis and Inhibits Proliferation of CML Progenitors

(A) Western blotting of SIRT1 and β-actin in CB CD34$^+$ and CML CD34$^+$ cells transduced with SIRT1 shRNAs (ShSIRT1-1 and ShSIRT1-2) or with Ctrl shRNA. (B) Western blotting for SIRT1, SIRT2, and β-actin in ShSIRT1-1, ShSIRT1-2, or CtrlShRNA transduced TF-1 cells. Results are representative of 3 independent experiments.

$CD34^+$ cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) followed by culture for 72 hr in low growth factor concentrations. SIRT1 knockdown inhibited CML progenitor proliferation as measured by reduction in CFSE fluorescence. Treatment with IM resulted in further reduction of proliferation (Figure 2C). SIRT1 knockdown inhibited proliferation of CB $CD34^+$ cells to a lesser extent than CML $CD34^+$ cells (Figure 2D). Expression of ShSIRT1-1, which results in near complete inhibition of SIRT1 expression, resulted in reduced survival of CML $CD34^+$ cells (Figure 2E). IM treatment significantly increased apoptosis of SIRT1 knockdown cells, indicating that SIRT1 inhibition enhanced sensitivity of CML progenitors to IM-induced apoptosis (Figure 2E). Enhanced apoptosis of CML $CD34^+$ cells following SIRT1 knockdown, and further increase in apoptosis with IM treatment, was confirmed by Wright-Giemsa staining (Figure S2A), trypan blue staining (Figure S2B), and activated caspase-3 labeling (Figure S2C). Interestingly, SIRT1 knockdown did not affect survival of normal progenitors, with or without IM treatment (Figure 2F). Primitive, quiescent CML $CD34^+$ cells are especially resistant to IM-induced apoptosis (Holtz et al., 2005). Importantly, the combination of SIRT1 inhibition and IM enhanced apoptosis of quiescent CML progenitors identified on the basis of high CFSE fluorescence (Figure 2G). In contrast, SIRT1 inhibition did not affect survival of quiescent normal progenitors (Figure 2H). Expression of both ShSIRT1-1 and ShSIRT1-2 significantly reduced CML colony forming cell (CFC) frequency in methylcellulose progenitor assays, which was enhanced by IM (Figure 2I). Inhibition of normal CFC growth was also seen, but was significantly less than for CML progenitors (Figure 2J).

The increased effects of ShSIRT1-1, compared to ShSIRT1-2, suggest that partial inhibition of SIRT1 expression is sufficient to inhibit CML progenitor proliferation, but that near complete knockdown is required to inhibit survival. To exclude the possibility that these results were related to off-target effects, we designed a SIRT1 construct resistant to ShSIRT1-1 (SIRT1-R) (Figure S2D). Lentivirus-mediated expression of wild-type (WT) or SIRT1-R resulted in enhanced SIRT1 protein levels in TF-1/BCR-ABL cells transduced with ShSIRT1-1 (Figure S2E) and abrogated the ability of ShSIRT1-1 to induce apoptosis and inhibit growth (Figures S2F and S2G). These results indicate that ShSIRT1-1 shRNA effects are related to SIRT1 knockdown, rather than off-target effects, and confirm that near complete SIRT1 suppression is required to induce apoptosis in CML cells.

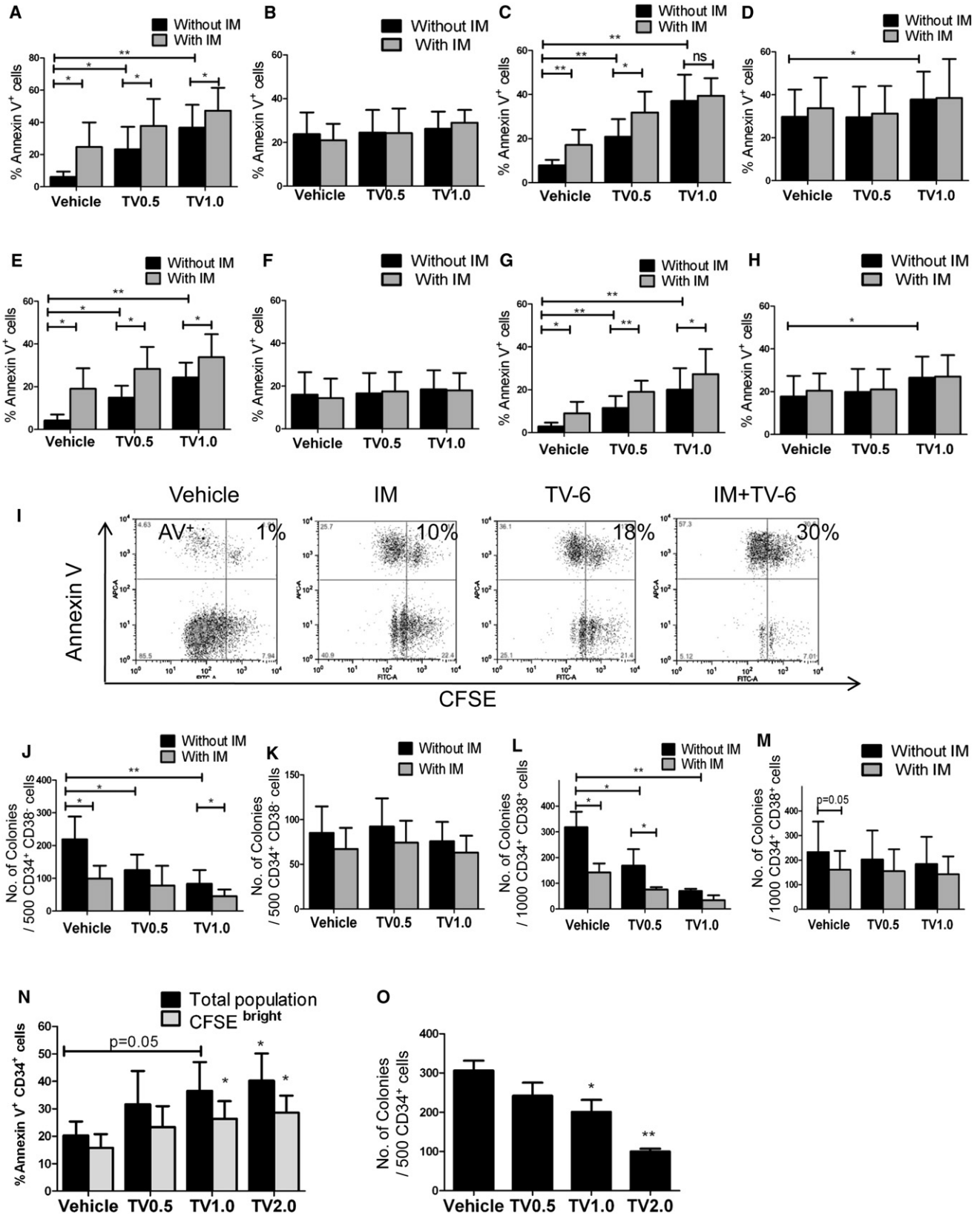
Pharmacological Inhibition of SIRT1 Induces Apoptosis and Inhibits Proliferation of CML Stem/Primitive Progenitor Cells

We tested the effects of tenovin-6 (TV-6), a small-molecule inhibitor of SIRT1, on CML and normal stem/progenitor cells (Lain et al., 2008). CFSE-labeled CML and normal $CD34^+CD38^-$ and $CD34^+CD38^+$ cells were cultured for 72 hr with TV-6, IM, or the combination. TV-6 significantly increased apoptosis of CML $CD34^+CD38^-$ cells and $CD34^+CD38^+$ cells, but not normal cells (Figures 3A–3D). A small increase in apoptosis of normal $CD34^+CD38^+$ cells was seen with higher doses of TV-6 (Figure 3D). Normal progenitor apoptosis was significantly less than for CML progenitors ($p < 0.05$). The combination of IM and TV-6 increased apoptosis in CML progenitors compared to either agent alone, and to a significantly greater extent than normal progenitors (Figures 3A–3D). Importantly, TV-6 also resulted in increased apoptosis of $CFSE^{high}$ undivided CML $CD34^+CD38^-$ and $CD34^+CD38^+$ cells, but did not increase apoptosis of undivided normal cells (Figures 3E–3I). A small increase in apoptosis of undivided normal cells was seen with higher doses of TV-6 (Figure 3H), but was significantly less than for CML cells ($p < 0.05$). Pretreatment with TV-6 for 72 hr inhibited CFC production from CML $CD34^+CD38^-$ and $CD34^+CD38^+$ cells but not normal $CD34^+CD38^-$ and $CD34^+CD38^+$ cells (Figures 3J–3M). The combination of TV-6 with IM enhanced inhibition of CML CFC growth compared to either agent alone, but did not enhance inhibition of normal CFC. Similar results were seen for cells exposed to TV-6 in methylcellulose for the 14-day duration of the CFC assay (Figures S3A and S3B). Exposure to IM for 14 days markedly inhibited CML and normal CFC growth ($p < 0.01$) compared to 14 days of exposure to TV-6 (Figures S3A and S3B). TV-6 also increased apoptosis in total and undivided $CD34^+$ cells from CML BC patients for whom IM treatment failed, and inhibited growth of $CD34^+$ cells in CFC assays (Figures 3N and 3O). In addition, TV-6 inhibited BaF3 cells expressing the IM-resistant T315I BCR-ABL mutant (BaF3/T315I) to a similar extent as BaF3 cells expressing wild-type BCR-ABL (BaF3/BA) (Figure S3C).

The effects of SIRT1 deletion on BM progenitors from young mice are apparent only when cells are grown at low O_2 tensions (Ou et al., 2011). We investigated whether selective effects of SIRT1 inhibition on CML compared to normal cells were maintained at low O_2 tensions. TV-6-induced (2 μM) apoptosis of total (Figures S3D–S3K) or undivided normal $CD34^+CD38^+$ cells was increased in hypoxic compared to normoxic conditions (Figures S3G and S3K). Inhibition of normal $CD34^+CD38^+$ cell proliferation and CFC formation was also increased in hypoxic conditions (Figures S3O and S3S). On the other hand, the effects of TV-6 on survival, proliferation, and colony formation of normal $CD34^+CD38^-$ cells (Figures S3E, S3I, S3M, and S3Q) or CML $CD34^+CD38^-$ cells (Figures S3D, S3H, S3L, and S3P) and $CD34^+CD38^+$ cells were not significantly different in hypoxic versus normoxic conditions (Figures S3F, S3J, S3N, and S3R).

TV-6 did not result in additional inhibition of proliferation of SIRT1 knockdown TF-1/BCR-ABL cells (Figure S3T). In addition, MEF from SIRT1 knockout mice (SIRT1-KO) cells were resistant to TV-6 (data not shown). Finally, ectopic expression of SIRT1

(C–J) CML (n = 5) and normal CB (n = 5) $CD34^+$ cells transduced with Ctrl ShRNA, ShSIRT1-1, or ShSIRT1-2 vectors were cultured with or without IM (2.5 μM) for 72 hr. Division of CML (C) and normal $CD34^+$ (D) cells was analyzed according to reduction in CFSE intensity, and a proliferation index was determined using ModFit software. Relative proliferation was calculated normalized to untreated controls. Apoptosis of CML (E) and normal (F) $CD34^+RFP^+$ cells was analyzed by Annexin V-Cy5 labeling. Apoptosis of undivided ($CFSE^{high}$) CML (G) and CB $CD34^+$ (H) cells was analyzed by Annexin V-Cy5 labeling. CFC assays were performed on CML (I) and normal $CD34^+$ (J) RFP^+ cells after culture with or without IM (2.5 μM) for 72 hr. Erythrocytic and granulocytic colonies were enumerated after 14 days. Results represent mean ± SEM of separate experiments. Significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with untreated cells. See also Figure S2.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decreased the sensitivity of TF-1/BCR-ABL cells to TV-6 (IC_{50} 2 μM in parental TF-1/BCR-ABL and 4.3 μM in ectopic SIRT1 expressing TF-1/BCR-ABL cells). These results suggest that TV-6 effects are indeed SIRT1 mediated.

SIRT1 Inhibition Impairs CML LSC Engraftment in Immunodeficient Mice

We evaluated the effect of ex vivo treatment with TV-6 on CML and normal $CD34^+$ stem cells capable of engraftment in NOD/SCID interleukin-2 receptor-γ chain-deficient (NSG) mice (Shultz et al., 2005) (Figure 4A). We observed reduced engraftment of CML $CD34^+$ cells treated with TV-6 (1 μM) for 72 hr at 4 weeks (short-term engraftment) ($p < 0.05$) (Figure S4A) and 12 weeks posttransplantation (longer term engraftment) (Figures S4C and S4D). Engraftment of $CD33^+$ and $CD14^+$ myeloid cells was decreased (Figures S4E and S4F). Q-PCR analysis confirmed that engrafted human cells expressed BCR-ABL (Figure S4G), and FISH analysis showed that 90% of engrafted human cells were *BCR-ABL*$^+$. These results show that SIRT1 inhibition by TV-6 selectively targets primitive human CML cells with in vivo engraftment capacity. We also compared the effect of IM (2.5 μM), TV-6 (1 μM), or the combination on CML $CD34^+$ cell engraftment in NSG mice (Figures 4B–4G). CML cells treated with IM alone ($p = 0.06$) and TV-6 alone ($p < 0.05$) demonstrated reduced engraftment in BM at 12 weeks posttransplant compared to untreated controls (Figure 4B). The combination of IM and TV-6 resulted in further inhibition of CML $CD34^+$ cell engraftment compared to IM or TV-6 alone (Figures 4B–4D), including reduced engraftment of myeloid cells (Figures 4E and 4F). Q-PCR analysis confirmed that engrafted cells expressed BCR-ABL, and that BCR-ABL expression was reduced in cells treated with IM and IM plus TV-6 (Figure 4G). Interestingly, engraftment of CB $CD34^+$ cells at both 4 weeks (Figure S4B) and 12 weeks (Figures 4H–4K) was not reduced after treatment with TV-6.

SIRT1 Inhibitor Treatment Reduces CML Stem and Progenitor Cell Growth In Vivo

The low levels of longer term engraftment of CML CP cells in NSG mice limits the use of this model to evaluate the effects of treatments administered in vivo. An inducible BCR-ABL transgenic mouse model of CML provides a representative model of CP CML that can be used for in vivo therapeutic studies (Zhang et al., 2010). These BCR-ABL mice were crossed with GFP transgenic mice, and BM cells were obtained 4 weeks after BCR-ABL induction. GFP-expressing cells selected using flow cytometry were transplanted into wild-type FVB/N mice irradiated at 900 cGy. Following engraftment, mice were treated for 3 weeks with IM (200 mg/kg/day by gavage), TV-6 (50 mg/kg/day intraperitoneally), the combination of TV-6 and IM, or vehicle (controls) (Figure 5A). TV-6 treated mice demonstrated loss of weight (data not shown) compared to control or IM-treated mice. Leukemic GFP^+ WBC and BM myeloid cells were reduced in TV-6 or IM treated mice, with further reduction with combination treatment (Figures 5B and 5C and Figure S5A). Flow cytometry analysis (Figure 5D) showed that combined TV-6 and IM treatment inhibited primitive $GFP^+Lin^-Sca\text{-}1^+Kit^+$ cells (LSK cells) (Figure S5B), LTHSC (LSK $Flt3^-CD150^+CD48^-$ cells) (Figures 5E and 5F), common myeloid progenitors (CMP) and granulocytic-macrophage progenitors (GMP) in the BM of CML mice to a greater extent than IM or TV-6 alone (Figure 5G and Figure S5C). Similar results were observed for splenic cells (Figure 5H, Figure S5D, and data not shown). In vivo administration of IM and TV-6 resulted in inhibition of LSK cell proliferation measured by EdU and DAPI labeling (Figure 5I) and enhanced LSK cells apoptosis (Figure 5J). Mice treated with the combination demonstrated significantly improved survival and maintained normal WBC counts with only small number of residual GFP^+ WBCs after discontinuation of treatment, compared to control, IM and TV-6 treated mice (Figures 5K–5M).

Consistent with previous reports, BC CML cells demonstrate robust engraftment in NSG mice with primitive $CD34^+$ cells (Figure S5E–S5I), allowing evaluation of in vivo treatment with TV-6. NSG mice engrafted with cells from an IM-resistant CML BC patient were treated with TV-6 (50 mg/kg/day intraperitoneally) or vehicle control for 3 weeks. TV-6 significantly reduced total human cells ($p < 0.01$) (Figures S5F and S5G) and human $CD34^+$ cells and myeloid cells in the BM, spleen, and peripheral blood (Figures S5H–S5K). TV-6 treatment also enhanced survival of mice transplanted with BaF3/T315I cells that are resistant to treatment with IM (Figure S5L), further demonstrating its in vivo activity against IM-resistant cells.

SIRT1 Inhibition Enhances p53 Acetylation in CML Progenitors

SIRT1 activity can be assessed by examining acetylation of p53 at K382, a known SIRT1 deacetylation site. SIRT1 knockdown using shRNA resulted in an increase in acetylated p53 protein levels in CP CML $CD34^+$ cells (Figure 6A and Figure S6A). Although increased p53 acetylation was detectable in SIRT1 knockdown cells in the absence of TSA (Figure S6A), it was more clearly seen in cells in which classic HDAC activity was

Figure 3. Pharmacological Inhibition of SIRT1 Induces Apoptosis and Inhibits Proliferation of CML Stem/Primitive Progenitor Cells

(A–D) CML $CD34^+CD38^-$ (n = 6) (A), normal $CD34^+CD38^-$ (n = 4, 2 CB and 2 PBSC) (B), CML $CD34^+CD38^+$ (n = 6) (C), and normal $CD34^+CD38^+$ cells (n = 4, 2 CB and 2 PBSC) cells (D) were exposed to TV-6 (0.5 μM [TV0.5] or 1 μM [TV1.0]), IM (2.5 μM), or the combination for 72 hr. Apoptosis was analyzed by Annexin V-Cy5 labeling.

(E–H) Apoptosis of undivided CML $CD34^+CD38^-CFSE^{high}$ (E), normal $CD34^+CD38^-CFSE^{high}$ (F), CML $CD34^+CD38^+CFSE^{high}$ (G), and normal $CD34^+CD38^+CFSE^{high}$ cells (H) analyzed by Annexin V labeling.

(I) Representative flow cytometry plots showing Annexin V^+ versus CFSE expression.

(J–M) CML (J) or normal (K) $CD34^+$ $CD38^-$ cells, and CML (L) or normal (M) $CD34^+CD38^-$ cells were cultured with TV-6 (0.5 or 1 μM), IM (2.5 μM), or the combination for 72 hr, then were plated in methylcellulose progenitor culture, and colonies were enumerated after 14 days.

(N) Apoptosis of total or undivided $CFSE^{high}$ CML BC (n = 4) $CD34^+$ cells exposed to TV-6 (0.5, 1, or 2 μM) for 72 hr analyzed by Annexin V-Cy5 labeling.

(O) CML BC (n = 4) $CD34^+$ cells exposed to TV-6 (0.5, 1, or 2 μM) were plated in methylcellulose progenitor culture and erythrocytic, and granulocytic colonies were enumerated after 14 days. Results represent the mean ± SEM of separate experiments. Significance: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with untreated controls. See also Figure S3.

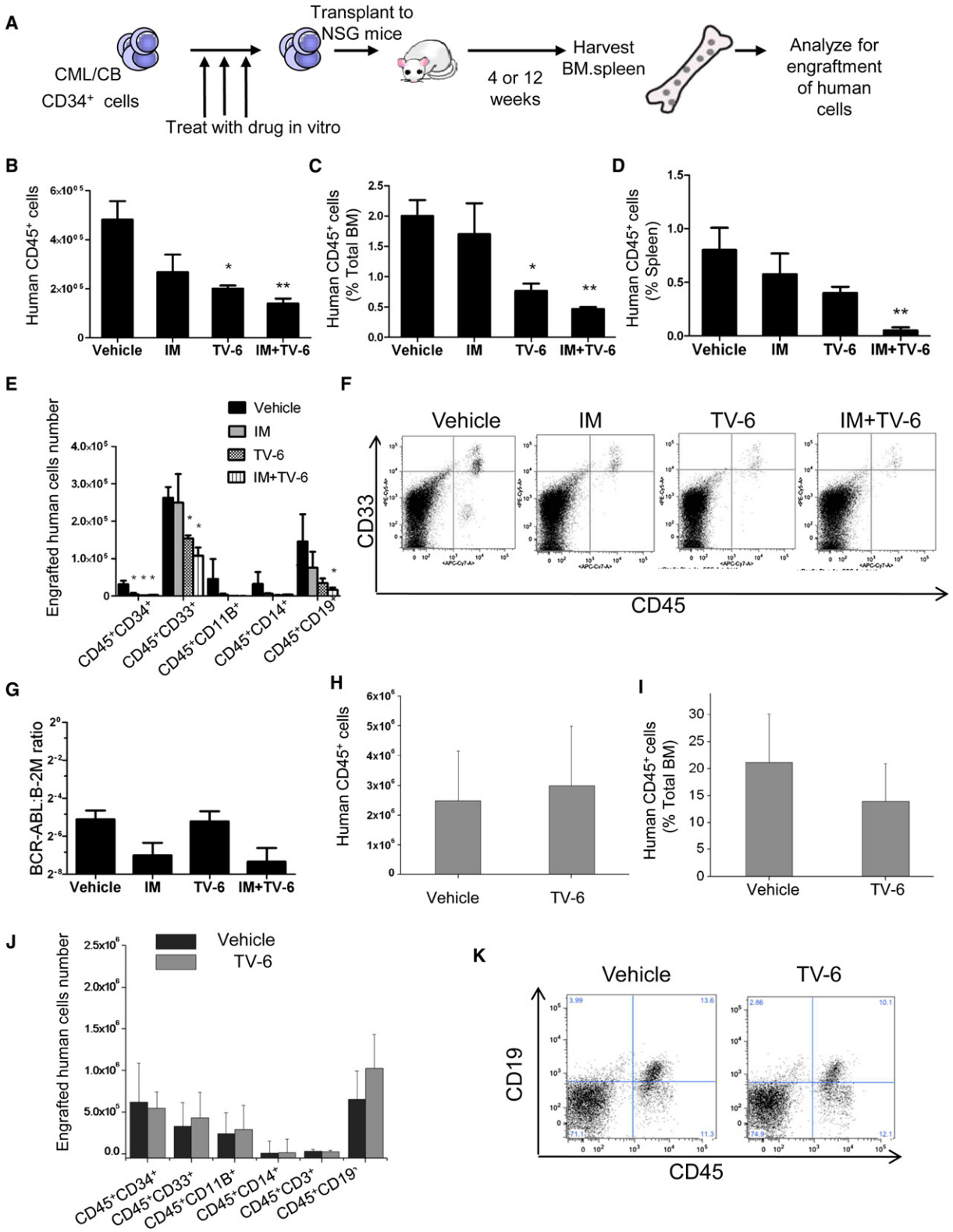


Figure 4. SIRT1 Inhibition Reduces Longer Term Engraftment of CML Stem Cells in Immunodeficient Mice

(A) CML or normal CD34$^+$ cells were treated with TV-6, IM, or combination in vitro and were injected into sublethally irradiated (300 cGy) NSG mice. After 4 or 12 weeks, human cell engraftment was analyzed by flow cytometric assessment of human CD45$^+$ cells.

(B and C) The number (B) and the percentage (C) of human CD45$^+$ cells engrafted in the BM 12 weeks after transplantation of CML CD34$^+$ cells (1 × 10^6 cells/mouse).

inhibited by TSA treatment (Luo et al., 2001; Cheng et al., 2003). Exposure of CML CP or BC CD34$^+$ cells to TV-6 also significantly enhanced acetylated p53 levels in both hypoxic and normoxic conditions (Figures 6B and 6C and Figures S6B and S6C). TV-6 treatment also increased total p53 levels, possibly by reducing degradation (Lain et al., 2008). Cells pretreated with the MDM2 antagonist Nutlin-3 to stabilize p53 levels (Vassilev et al., 2004) before the addition of TV-6 showed a rapid and marked increase in p53 acetylation without a change in total p53 levels (Figure 6B). In contrast, TV-6 did not increase acetylated p53 or total p53 levels in normal CD34$^+$ cells (Figure S6D). Acetylated p53 signals were increased and showed a nuclear distribution in SIRT1 knockdown cells (Figure 6D). A modest increase in acetylation of the SIRT2 substrate α-tubulin (Lain et al., 2008) was also seen after 48 hr, suggesting that prolonged TV-6 exposure may also modestly inhibit SIRT2 activity (Figure S6E). However, the more rapid and pronounced effect on p53 acetylation indicates more efficient inhibition of SIRT1 compared to SIRT2 activity. Ectopic expression of BCR-ABL in CB CD34$^+$ cells also resulted in increased expression of SIRT1, which was associated with reduced levels of acetylated p53 despite increased levels of total p53 (Figure S6F).

Treatment with classic HDACs can reduce BCR-ABL expression in cell lines and BC CML cells (Fiskus et al., 2006). BCR-ABL protein levels were not reduced in CP or BC CML CD34$^+$ cells after SIRT1 inhibition using either shRNA or TV-6 (Figures 6B, 6C, and 6E and Figures S6B and S6C). Treatment with IM reduced tyrosine phosphorylation of the BCR-ABL substrate CrkL in CML CD34$^+$ cells, confirming inhibition of BCR-ABL kinase activity (Figure 6A and Figure S6B). In contrast, SIRT1 inhibition did not reduce phosphorylation in CML CD34$^+$ cells (Figures 6A and 6C and Figures S6B and S6C). Therefore, the effects of SIRT1 inhibition on CML CD34$^+$ cells cannot be explained by inhibition of BCR-ABL expression or activity. Treatment with IM resulted in modest reduction in SIRT1 levels and in total p53 but not acetylated p53 levels (Figure 6A and Figure S6B).

SIRT1 Inhibition Increases p53 Transcriptional Activity

Irradiation of CML CD34$^+$ progenitors resulted in increased p53 levels (Figure 7A) and increased expression of p53 target genes, including *p21*, *Necdin* (*Ndn*), *Puma*, and *Bax* (Figure 7B). Knockdown of p53 in CML CD34$^+$ cells using lentivirus vectors expressing anti-p53 shRNA (Figure 7C) resulted in reduced expression of *p21* and *Ndn* (Figure 7D). These results indicate that p53 signaling remains subject to activation in CP CML CD34$^+$ cells. To examine the effect of SIRT1 knockdown on p53 transcriptional activity in BCR-ABL expressing cells, we cotransfected a p53 expression vector and the BP-100 p53 reporter plasmid (MDM2 promoter cloned upstream of the luciferase gene) (Dai et al., 2004) into the p53 null CML cell line K562 (Bi et al., 1992). SIRT1 knockdown resulted in increased p53 activity ($p < 0.05$) compared with controls (Figure 7E). SIRT1 knockdown also increased the activity of endogenous p53 in 293 cells (Figure S7A). Importantly, SIRT1 knockdown increased expression of p53 target genes in CML CD34$^+$ cells on Q-PCR analysis, including *Gfi-1*, *Ndn*, and *Bax* ($p < 0.05$) (Figure 7F). Although *p21* is also a p53-regulated gene in CML CD34$^+$ cells, it was not further induced by SIRT1 knockdown.

Treatment with IM resulted in a modest reduction in p53 levels in CML CD34$^+$ progenitors (Figures 6A and 7G) but did not significantly affect p53 acetylation or expression of p53 target genes (Figure S7B). Knockdown of p53 in CML CD34$^+$ cells using anti-p53 shRNA did not affect the ability of IM to induce apoptosis (Figure S7C) or inhibit proliferation (Figure S7D), indicating that IM-mediated inhibition of CML progenitors is independent of p53 expression. The combination of IM and Nutlin-3 induced significantly more apoptosis than either agent did alone (data not shown), indicating that p53 signaling can be activated in IM-treated CML CD34$^+$ cells. SIRT1 inhibition in IM-treated CML CD34$^+$ progenitors by anti-SIRT1 shRNA expression resulted in increased expression of acetylated p53 (Figure 6A). Upregulation of p53 target genes was also seen following SIRT1 knockdown in IM-treated cells (Figure 7H). These observations indicate that SIRT1 knockdown can activate p53 signaling in IM-treated CML cells.

Inhibition of CML Progenitor Survival by SIRT1 Knockdown Requires p53 Expression and Acetylation

To determine the role of p53 in mediating the effects of SIRT1 inhibition on CML progenitors, we evaluated whether shRNA-mediated p53 knockdown could mitigate the effects of SIRT1 inhibition on CML progenitors. CML CD34$^+$ cells were cotransduced with a lentivirus vector coexpressing SIRT1 shRNA and RFP and a second vector coexpressing p53 shRNA and GFP, followed by selection of cells expressing both RFP and GFP (Figure 8A). Inhibition of p53 expression significantly reduced apoptosis (Figures 8A and 8B) and enhanced growth (Figure 8C) of SIRT1-knockdown CML CD34$^+$ cells. In addition, p53 knockdown CML progenitors exposed to TV-6 showed reduced apoptosis compared with control cells exposed to TV-6 (Figure 8D). These results confirm an important role for p53 in SIRT1-mediated signaling in CML progenitors.

To determine the role of p53 acetylation in mediating SIRT1 effects, we expressed an acetylation-defective p53 mutant (p53-8KR, with all eight potential acetylation sites mutated) in

(D) The percentage of human CD45$^+$ cells engrafted in the spleen at 12 weeks. Cells from two patients were injected. The figure shows representative results from one patient (n = 3 for untreated control and TV-6 treated cells, n = 4 for IM and combination treatment).
(E) Engraftment of human CD34, CD33, CD11b, CD14, and CD19 subsets (no CD3-positive cells were seen).
(F) Representative results for CD45 and CD33 expression.
(G) *BCR-ABL* mRNA levels in CD45$^+$ cells engrafted in BM at 12 weeks were measured by Q-PCR.
(H and I) The total number (H) and percentage (I) of human CD45$^+$ cells engrafted in the BM of NSG mice receiving CB CD34$^+$ cells at 12 weeks (1×10^5 cells injected/mouse; n = 4 for untreated control; n = 7 for TV-6 treated cells).
(J) Engraftment of human CD34, CD33, CD11b, CD14, CD3, and CD19 cell subsets.
(K) Representative results for CD45 and CD19 expression. Results represent the mean ± SEM of separate experiments. Significance: $^*p < 0.05$, $^{**}p < 0.01$ compared with untreated cells. See also Figure S4.

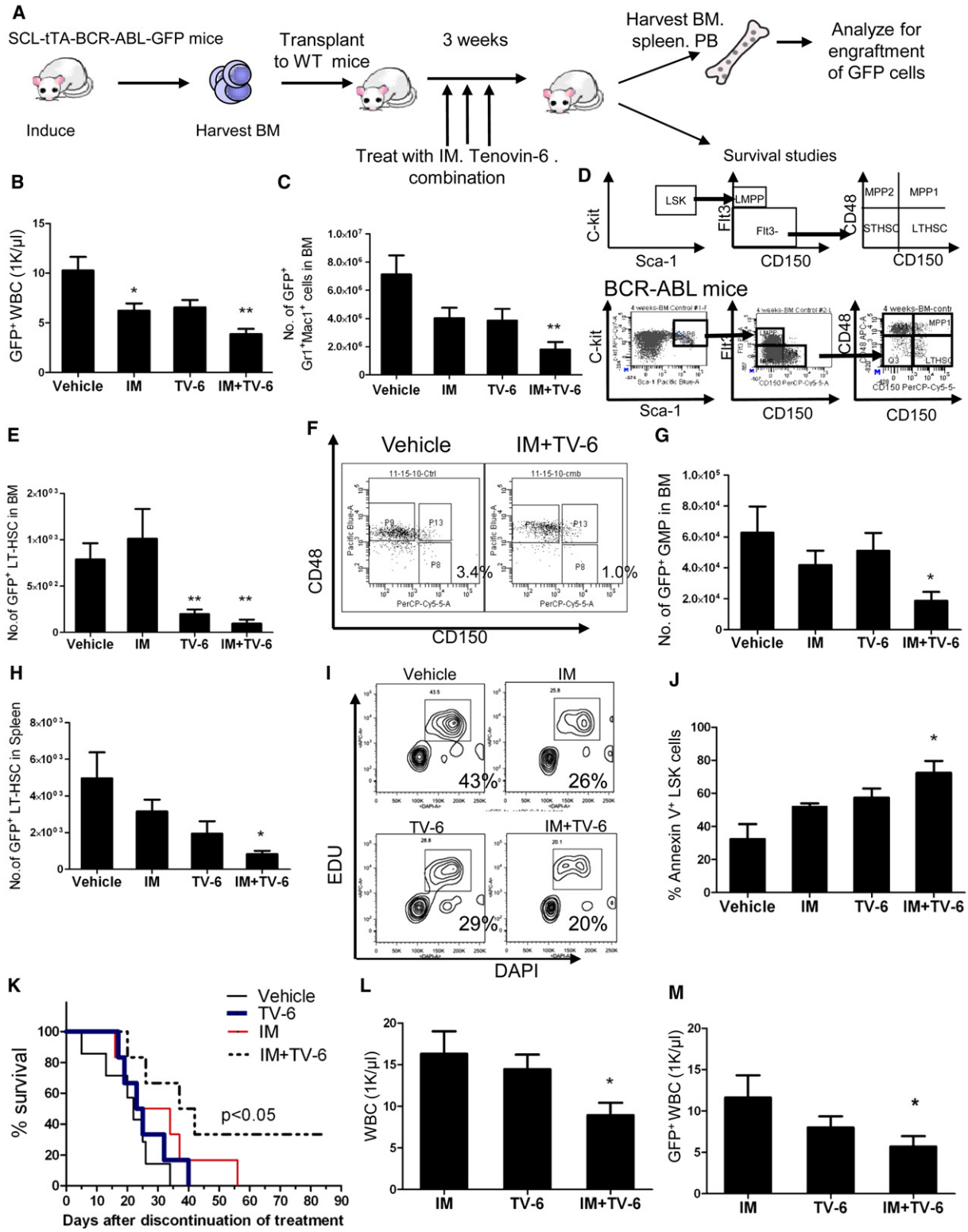


Figure 5. SIRT1 Inhibition Reduces In Vivo Growth of CML Stem Cells

(A) BCR-ABL mice were crossed with GFP transgenic mice and BM cells obtained 4 weeks after induction of BCR-ABL expression. GFP^+ cells selected by flow cytometry were transplanted into wild-type FVB/N mice irradiated at 900 cGy. Following engraftment, mice were treated for 3 weeks with IM (200 mg/kg/day by gavage), TV-6 (5 0mg/kg/day intraperitoneally), the combination of TV-6 and IM, or vehicle (controls) (n = 6 mice each).

(B) GFP^+ WBC counts 3 weeks after start of treatment.

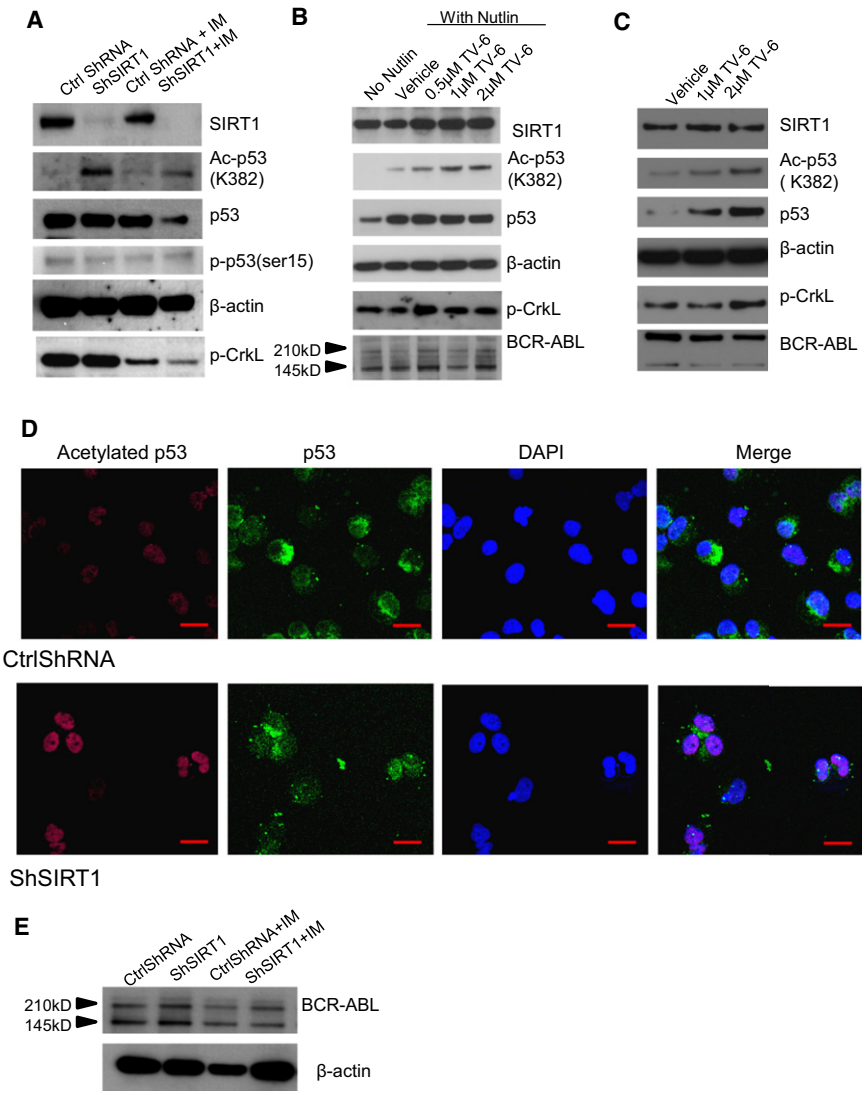


Figure 6. Increased p53 Acetylation and Nuclear Localization in SIRT1-Inhibited CML CD34$^+$ Cells

(A) Western blotting for acetylated p53 (K382), total p53, p-p53 (ser 15), β-actin, SIRT1, and p-CrkL in SIRT1 knockdown in CML CD34$^+$ cells cultured with TSA (0.1 μM) for 2 hr and exposed to IM (2.5 μM) for 8 hr.

(B) Western blotting for acetylated p53 (K382), total p53, β-actin, SIRT1, p-CrkL, and BCR-ABL in CML CD34$^+$ cells cultured with Nutlin-3 (10 μM) for 2 hr and exposed to TV-6 for 6 hr.

(C) Western blotting for acetylated p53 (K382), total p53, β-actin, SIRT1, p-CrkL, and BCR-ABL in CML BC CD34$^+$ cells cultured with TV-6 (1 or 2 μM) for 16 hr.

(D) Immunofluorescence analysis of Ac-p53 and p53 in CD34$^+$ cells. All scale bars represent a size of 10 μm. Results are representative of 3 independent experiments.

(E) Western blotting for BCR-ABL and β-actin in SIRT1 knockdown CML CD34$^+$ cells exposed to IM 2.5 μM for 24 hr. See also Figure S6.

p53 null K562 cells. Inhibition of SIRT1 using shRNA or TV-6 did not inhibit growth or induce apoptosis in parental K562 cells ($p > 0.05$) (Figure S8). However, SIRT1 knockdown in K562 cells that ectopically expressed WT p53 protein led to increased p53 acetylation (Figure 8E) and significant growth inhibition and apoptosis ($p < 0.05$) (Figures 8F and 8G). In contrast, ectopically expressed p53-8KR was not acetylated following SIRT1 knockdown (Figure 8H), and K562 cells transfected with p53-8KR did not show significant growth inhibition or apoptosis following SIRT1 knockdown (Figures 8I and 8J). These results indicate that p53 acetylation is required for growth inhibition and apoptosis following SIRT1 inhibition in BCR-ABL-expressing cells.

DISCUSSION

Our results show that inhibition of SIRT1 deacetylase enhances targeting of LSC from CML patients by TKI treatment via activation of p53 signaling, indicating an important role for SIRT1 in maintaining LSC growth and survival. Both BCR-ABL kinase-dependent and kinase-independent mechanisms contribute to increased SIRT1 activity in CML cells, with the latter potentially including epigenetic silencing of HIC1, a negative regulator of SIRT1, through methylation (Chen et al., 2005), or altered miRNA regulation of SIRT1 expression (Strum

(C) GFP$^+$ myeloid cells (Gr-1$^+$Mac-1$^+$) cells in the BM.
(D) Schema for analysis of LTHSC (E) GFP$^+$ LTHSC in BM.
(F) Representative plot for BM LTHSC for vehicle control and combination.
(G) GFP$^+$ GMP in BM.
(H) GFP$^+$ LTHSC in spleen. Apoptosis and cell cycling in BM LSK cells was evaluated after 5 days of treatment.
(I) Mice were injected intraperitoneally with EdU and were euthanized 2 hr later. The percentage of stem cells in S-phase was determined according to EdU incorporation in BM LSK cells. Representative results from one of two experiments are shown.
(J) Apoptosis was evaluated by Annexin-V and DAPI labeling (n = 3 mice per group). Significance: *$p < 0.05$, **$p < 0.01$, compared with vehicle control.
(K) Mice were followed for survival for 85 days after discontinuation of treatment (n = 6 per group). Survival of mice receiving combination treatment was significantly longer than that of mice receiving IM or TV-6 alone or vehicle-treated cohorts ($p < 0.05$, Wilcoxon test). Two mice receiving combination treatment remained alive until day 85, when the experiment was terminated.
(L and M) The total WBC count (L) and GFP$^+$ WBC count (M) in PB of mice 4 weeks after discontinuation of treatment. Significance: *$p < 0.05$, **$p < 0.01$, compared with IM. Results represent the mean ± SEM of separate experiments. See also Figure S5.

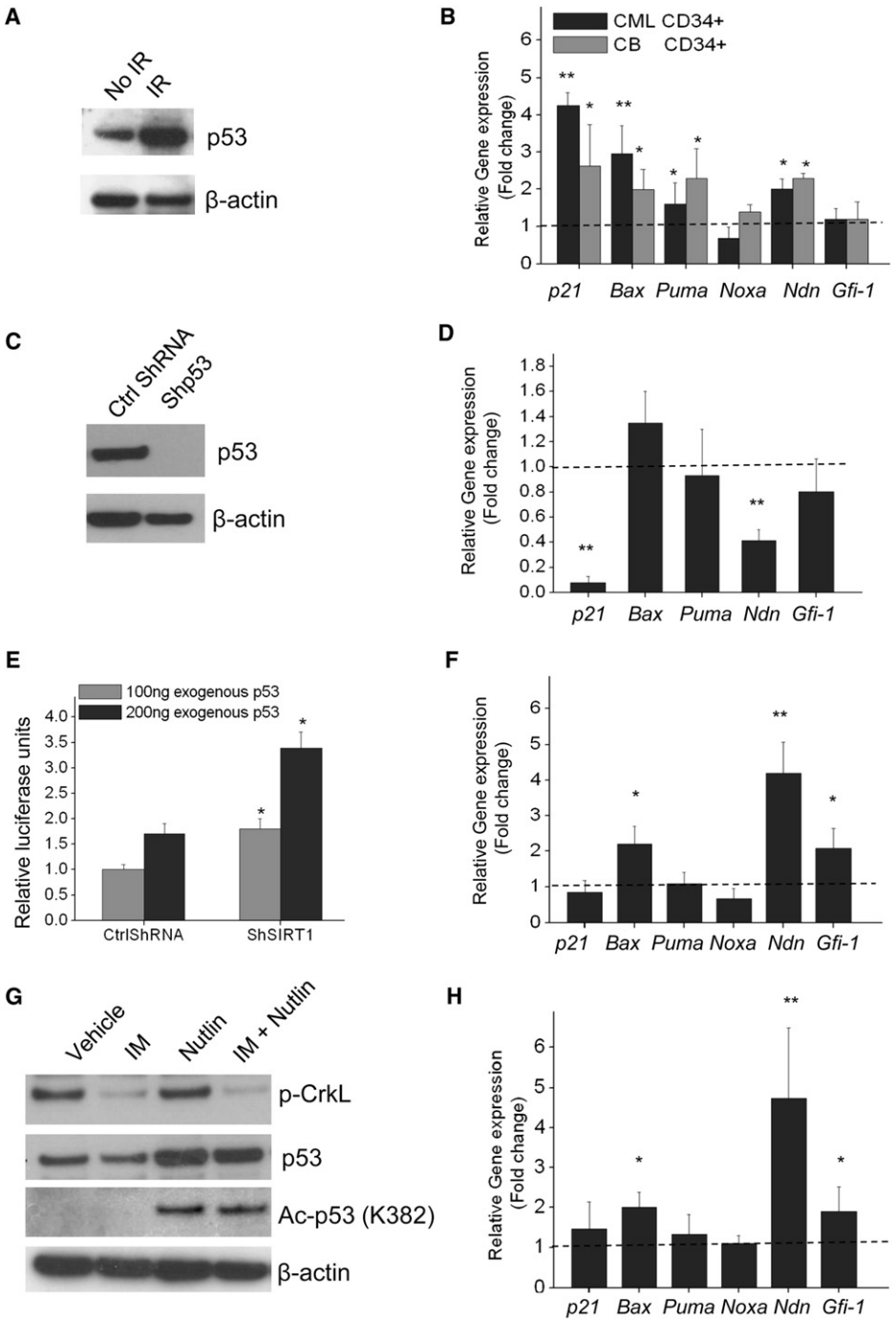


Figure 7. Signaling through p53 Is Intact in CML Progenitors, Is Enhanced by SIRT1 Knockdown, and Is Not Affected by IM

(A) Western blotting for p53 in CML CD34$^+$ cells 6 hr after exposure to irradiation (3 Gy). Results are representative of 3 independent experiments.

(B) Q-PCR analysis of p53 target genes in CP CML and normal CD34$^+$ cells 6 hr after irradiation (3 Gy) compared with nonirradiated control cells. *β-2M* was used as an internal control.

(C) Western blotting for p53 expression in CML CD34$^+$ cells transduced with an anti-p53 shRNA vector. Results are representative of 3 independent experiments.

(D) Q-PCR analysis of p53 target genes in p53 knockdown and control cells.

(E) SIRT1 knockdown or control K562 cells (n = 3) were cotransfected with plasmids expressing p53, a luciferase reporter for p53 transcription (BP100) and β-galactosidase. Relative luciferase units are normalized to β-gal expression.

(F) Q-PCR analysis of p53 target genes in shSIRT1 compared with ctrl shRNA expressing CML CD34$^+$ cells (n = 3).

(G) Western blotting for acetylated p53 (K382), total p53, p-CrkL, and β-actin in CML CD34$^+$ cells treated with IM (2.5 μM), Nutlin-3, or the combination for 8 hr.

(H) Q-PCR analysis for p53 target genes in ShSIRT1 compared with control shRNA-transduced CML CD34$^+$ cells treated with IM (2.5 μM) for 24 hr (n = 4).

Significance: *p < 0.05, **p < 0.01, compared with controls. Results shown represent the mean ± SEM of separate experiments. See also Figure S7.

et al., 2009). The selectivity of SIRT1 inhibition toward CML stem/progenitor cells is maintained in hypoxic conditions, where SIRT1 plays an important role in supporting normal hematopoiesis (Ou et al., 2011).

SIRT1 can deacetylate several lysine residues in the tumor suppressor p53 (Luo et al., 2001; Brooks and Gu, 2009). A variety of posttranslational modifications that can regulate p53 activity, including phosphorylation, acetylation, methylation, and sumoylation, have been described (Vousden and Lane, 2007). Acetylation is reported to play an important role in stabilization, nuclear localization, and transcriptional activation of p53 (Prives and Manley, 2001) and can lead to p53 activation independently of phosphorylation status (Tang et al., 2008). Although p53 mutations may occur on progression to BC CML, they are rare in CP CML (Prokocimer and Rotter, 1994). Our results indicate that p53 remains responsive to stress-induced activation in CML progenitors. SIRT1 inhibition increased p53 acetylation and expression of several p53 target genes, including *Bax*, *Necdin*, and *Gfi-1*, in CML $CD34^+$ cells. *Bax* is an important proapoptotic gene, and *Necdin* and *Gfi-1* may be important for p53-regulated quiescence of HSC (Liu et al., 2009b). Additional p53 target genes besides those identified here may also contribute to the effects of SIRT1 inhibition. Although *p21* expression was reduced in CML progenitors after p53 knockdown, SIRT1 knockdown did not increase expression of *p21* in CML progenitors, suggesting that other SIRT1-regulated pathways may counteract the effects of p53 acetylation on *p21* induction (Cheng et al., 2003). BC CML cells also demonstrated increased p53 acetylation following SIRT1 inhibition, consistent with recent reports that p53 can be activated in CML BC cells (Peterson et al., 2011). Of note, the CML BC samples studied here did not have p53 mutations (Table S1).

Although previous studies indicated that the p53 inactivation by SIRT1 promotes cell survival during stress (Luo et al., 2001), other studies have suggested that small-molecule SIRT1 inhibitors do not affect cell survival (Solomon et al., 2006) and that developmental defects in a SIRT1 knockout mouse strain are not rescued by crossing to p53 null mice (Kamel et al., 2006). The importance of p53 in mediating SIRT1 effects may depend on the cellular context. SIRT1 deacetylates several other proteins that regulate cell growth and survival besides p53. The importance of individual SIRT1 targets may depend on the cell process and cell type studied. Recent studies within our group indicate an important role for SIRT1 regulation of Ku70 in DNA repair and mutagenicity of CML cells (unpublished data). The role of other SIRT1 targets such as the FoxOs and E2F1 transcription factors in regulating quiescence and survival of CML stem and progenitor cells requires further evaluation.

There is considerable interest in restoring p53 function in cancer cells as a means of inhibiting their proliferation, or inducing senescence or apoptosis. Deacetylation of p53 via SIRT1 may play an important role in preventing p53 activation in TKI-treated CML progenitors. BCR-ABL kinase activity could also modulate p53 in CML cells by upregulation of ARF (Williams et al., 2006), phosphorylation and inactivation of MDM2 and MDMX (Zuckerman et al., 2009), or increased translation of MDM2 (Trotta et al., 2003). Despite the complex regulation of p53 in CML cells, our studies show that enhanced p53 acetylation following SIRT1 inhibition is sufficient to increase p53 transcriptional activity in CML progenitor cells and that p53 deacetylation is an important protective mechanism for CML LSC following TKI treatment. Therefore, p53 activation is a potential strategy to enhance targeting of CML LSC, especially in combination with TKI.

SIRT1 inhibitors are being investigated as potential anticancer treatments. We observed weight loss in mice during the course of three-week TV-6 treatment, but it is unclear whether this was related to SIRT1 inhibition or an off-target effect of this agent. Although TV-6 itself may not be a candidate for drug development, our observations support further investigation of SIRT1 inhibition as an approach for targeting of CML stem/progenitor cells in combination with TKI treatment. The potential tumor suppressive effects of SIRT1 need to be kept in mind when considering SIRT1 inhibitors for cancer treatment. Improved understanding of mechanisms underlying the anticancer versus tumor-promoting effects of SIRT1 inhibition in specific cell types will aid the development of more selective, nontoxic approaches for targeting LSCs in future. The results of the current studies have broader implication to other leukemias, such as AML, where SIRT1 overexpression is also observed and p53 mutations are rare (Kojima et al., 2005).

EXPERIMENTAL PROCEDURES

Samples and Materials

CB samples were provided by StemCyte (Arcadia, CA). CP CML samples were obtained from previously untreated patients at the City of Hope (COH) and the University of Glasgow. CML BC samples were obtained from patients at COH (Table S1). $CD34^+$ cell isolation and $CD3^+$ cell depletion were performed using magnetic beads (StemCell Technologies, Vancouver, BC, Canada). Leukopheresis samples were processed for $CD34^+$ cell selection with CliniMACS (Miltenyi Biotech, Germany). $CD34^+CD38^-$ and $CD34^+CD38^+$ cells were obtained by flow cytometry sorting. All subjects signed an informed consent form. Sample acquisition was approved by the Institutional Review Boards at the City of Hope, in accordance with an assurance filed with and approved by the Department of Health and Human Services, and the North Glasgow University Hospital Division of NHS Greater Glasgow and Clyde, and met all requirements of the Declaration of Helsinki. Details of cell lines, drugs, and DNA constructs are provided in the Supplemental Experimental Procedures.

Cell Transduction and Transfection

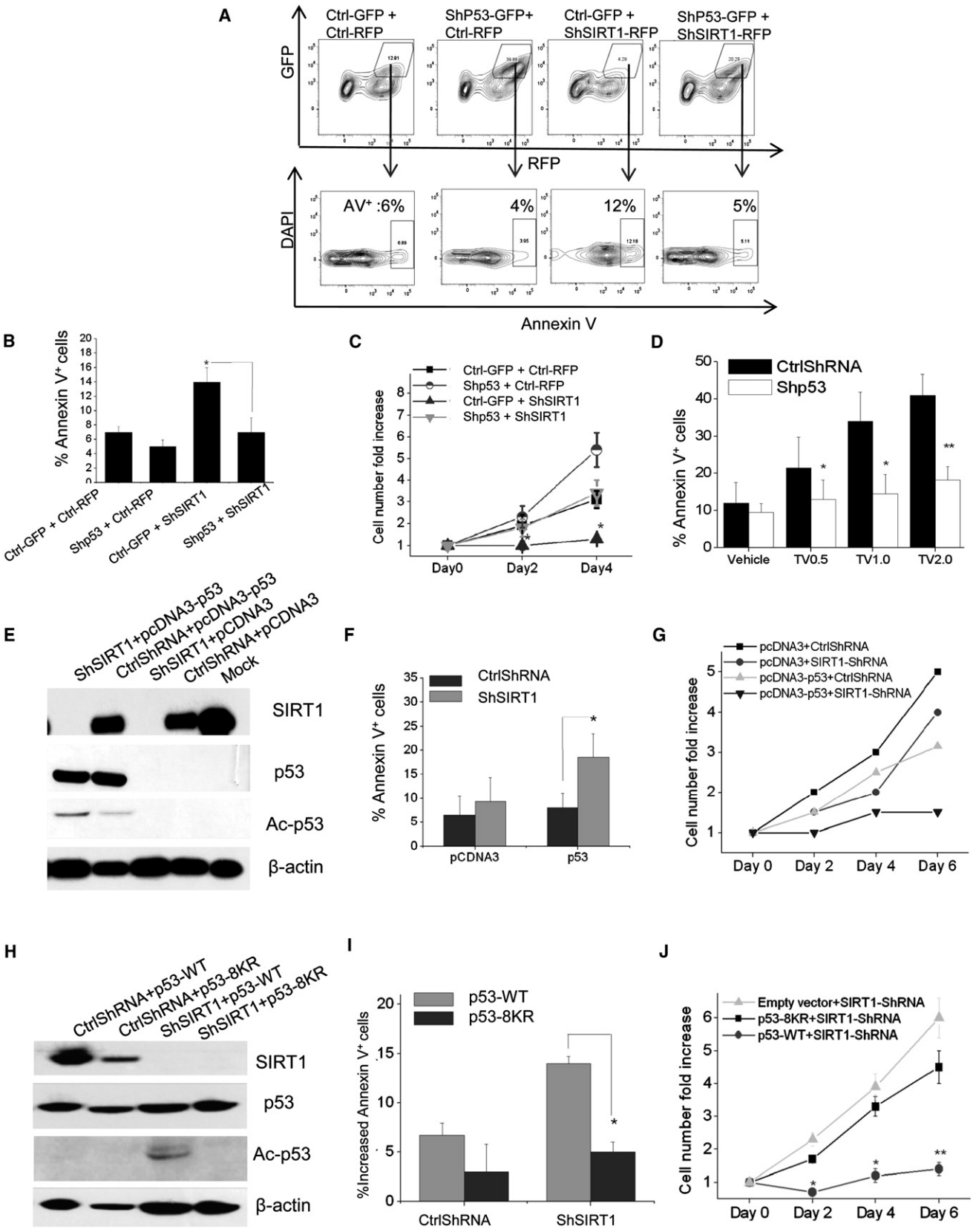
$CD34^+$ cells were transduced with lentivirus vectors expressing SIRT1 shRNA or p53 shRNA. TF-1/BCR-ABL (TF-1/BA) cells were transduced with PITA-SIRT1-R, PITA-SIRT1-WT, and vector controls (PITA). Details of the transduction procedure are provided in the Supplemental Experimental Procedures.

Intracellular Staining for SIRT1

$CD34^+$ cells were labeled with antibodies to CD34-PeCy7, Lin-APC-Cy7 (including CD2, CD7, CD10, CD11B, CD19, CD33, and CD235a), and CD38-APC (e-Bioscience), followed by fixation and permeabilization (Cytofix/Cytoperm Kit, Beckman Coulter, Fullerton, CA). Cells were then labeled with rabbit anti-human SIRT1 (Epitomics) followed by Alexa 488-conjugated goat anti-rabbit antibodies (Molecular Probes) and were analyzed by flow cytometry. Data were analyzed using FlowJo software (version 8.5.2; TreeStar, Ashland, OR). For immunofluorescence analysis, cells were labeled with anti-p53-FITC (DO-7, BD) and antiacetylated p53-K382-Alex647 (BD), as described in the Supplemental Experimental Procedures.

Analysis of Proliferation, Apoptosis, and Colony Growth

$CD34^+$ cells were labeled with CFSE (Molecular Probes, Eugene, OR), labeled with CD34-PE-Cy7 and CD38-APC, and $CD34^+CD38^-$ and $CD34^+CD38^+$ cells with uniform CFSE labeling were selected by flow cytometry (MoFlo; Cytomation, Fort Collins, CO). Cells were cultured with low


A
Ctrl-GFP + Ctrl-RFP
ShP53-GFP+ Ctrl-RFP
Ctrl-GFP + ShSIRT1-RFP
ShP53-GFP + ShSIRT1-RFP
GFP
RFP
DAPI
AV+ :6%
4%
12%
5%
Annexin V
B
% Annexin V+ cells
Ctrl-GFP + Ctrl-RFP
Shp53 + Ctrl-RFP
Ctrl-GFP + ShSIRT1
Shp53 + ShSIRT1
C
Ctrl-GFP + Ctrl-RFP
Shp53 + Ctrl-RFP
Ctrl-GFP + ShSIRT1
Shp53 + ShSIRT1
Cell number fold increase
Day0
Day2
Day4
D
CtrlShRNA
Shp53
% Annexin V+ cells
Vehicle
TV0.5
TV1.0
TV2.0
E
ShSIRT1+pcDNA3-p53
CtrlShRNA+pcDNA3-p53
ShSIRT1+pCDNA3
CtrlShRNA+pCDNA3
Mock
SIRT1
p53
Ac-p53
β-actin
F
CtrlShRNA
ShSIRT1
% Annexin V+ cells
pCDNA3
p53
G
pcDNA3+CtrlShRNA
pcDNA3+SIRT1-ShRNA
pcDNA3-p53+CtrlShRNA
pcDNA3-p53+SIRT1-ShRNA
Cell number fold increase
Day 0
Day 2
Day 4
Day 6
H
CtrlShRNA+p53-WT
CtrlShRNA+p53-8KR
ShSIRT1+p53-WT
ShSIRT1+p53-8KR
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CtrlShRNA
ShSIRT1
J
Empty vector+SIRT1-ShRNA
p53-8KR+SIRT1-ShRNA
p53-WT+SIRT1-ShRNA
Cell number fold increase
Day 0
Day 2
Day 4
Day 6

concentrations of growth factors at 37°C for up to 72 hr in normoxic conditions (21% O_2). For specific experiments, cells were cultured hypoxic conditions (5% O_2) as specifically indicated in the Results. Cells were analyzed by flow cytometry for apoptosis by Annexin V labeling and for proliferation by reduction in CFSE labeling. Committed progenitors or colony forming cells (CFC) were evaluated in methylcellulose progenitor assays as previously described. Details are provided in Supplemental Experimental Procedures.

Engraftment of Human Cells in Immunodeficient Mice

CML $CD34^+$ cells (1×10^6 cells/ mouse) or CB $CD34^+$ cells (1×10^5 cells/ mouse) were cultured for 72 hr with TV-6 (1 μM), IM (2.5 μM), or the combination or without drug (control) and were transplanted via tail vein injection into sublethally irradiated (300 cGy) 8-week-old NOD.Cg-Prkdcscid IL2rgtm1Wjl /SzJ mice (NSG mice, Jackson Laboratory, Bar Harbor, ME). Mice were euthanized after 4 or 12 weeks, and marrow contents of femurs, spleen cells, and blood cells were obtained. For CML BC samples, MNC depleted of $CD3^+$ cells were transplanted via tail vein injection (5×10^6 cells/mouse). Blood samples were obtained 4 weeks after transplantation to confirm human $CD45^+$ cells engraftment. Mice were treated with TV-6 (50 mg/kg intraperitoneally daily for 21 days) (Lain et al., 2008) or vehicle (control) for 3 weeks and were euthanized, and marrow and spleen cells were obtained and analyzed as described in Supplemental Experimental Procedures. Mouse care and experimental procedures were performed in accordance with established institutional guidance and approved protocols from the Institutional Animal Care and Use Committee at COHNMC.

In Vivo Treatment of Transgenic BCR-ABL Mice

These experiments were performed using inducible, transgenic GFP-Scl-tTa-BCR-ABL mice in the FVB/N background crossed with transgenic GFP-expressing mice (FVB.Cg-Tg [ACTB-EGFP] B5Nagy/J, Jackson Laboratories) (Zhang et al., 2010). Mice were treated with IM (200 mg/kg daily by gavage for 21 days), TV-6 (50 mg/kg body weight intraperitoneally daily for 21 days), the combination, or vehicle alone (control). After 3 weeks of treatment, animals were euthanized, and marrow and spleen cells were obtained. The number of total nucleated cells, GFP-expressing cells, and GFP^+ myeloid, progenitor, and stem cell populations were measured by flow cytometry. The effect of drug administration on apoptosis and cycling of stem cells in vivo was evaluated. Another subset of mice was followed after discontinuation of treatment, and survival was monitored for 85 days, PB counts were monitored for 28 days. Details are provided in the Supplemental Experimental Procedures. Mouse care and experimental procedures were performed in accordance with established institutional guidance and approved protocols from the Institutional Animal Care and Use Committee at COHNMC.

Luciferase Reporter Assays

K562 or 293 cells were transfected with reporter and internal control (β-gal or Renilla-CMV) plasmids. Luciferase assays were performed after 48 hr in triplicate using the luciferase reporter assay system (Promega).

Real-Time Q-PCR Analysis

Q-PCR analysis performed with primers and probes for *p21*, *Bax*, *Puma*, *Noxa*, *Necdin*, *Gfi-1*, and *Sirt1*, and *BCR-ABL* (B3A2) transcripts were measured using a real-time TaqMan assay as previously described (Chu et al., 2011). Details are provided in the Supplemental Experimental Procedures.

Western Blotting

Western blotting was performed for p53, acetylated p53, phospho-p53, SIRT1, CrkL, phopho-CrkL, Bax, ABL, tubulin, and actin. Details are provided in the Supplemental Experimental Procedures.

Statistics

Data from independent experiments were reported as the mean ± SEM. Student's t test analysis was performed to determine statistical significance.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table, eight figures, Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.12.020.

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Figure 8. Effect of SIRT1 Inhibition in CML Progenitors Is Dependent on p53 Expression and Acetylation

(A–C) CML $CD34^+$ cells were cotransduced with PLKO-GFP vectors expressing anti-p53 or control shRNA and PHIV7-RFP vectors expressing anti-SIRT1 or control shRNA.

(A) $CD34^+GFP^+RFP^+$ cells were analyzed for apoptosis by Annexin V labeling. A representative plot is shown.

(B) Cumulative results for apoptosis (n = 3).

(C) The total number of $CD34^+GFP^+RFP^+$ cells normalized to ctrl ShRNA expressing cells (n = 3).

(D) p53 knockdown or control CML progenitors were exposed to TV-6 for 48 hr, and apoptosis was analyzed by Annexin V labeling (n = 3). Significance values: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with untreated cells.

(E–G) K562 cells transduced with HIV7-RFP vectors expressing anti-SIRT1 or control shRNA were transfected with p53 expressing plasmids.

(E) Western blotting for acetylated p53 (K382), total p53, SIRT1, and β-actin.

(F) Apoptosis was assessed after 48 hr by Annexin V labeling.

(G) The fold change in cell numbers was calculated at day 2, day 4, and day 6 relative to day 0.

(H–J) K562 cells expressing a *tet* transactivator gene (K562-TTA) were generated and transduced with HIV7-RFP vectors expressing ShSIRT1 or Ctrl ShRNA. RFP^+ cells were selected and transfected with acetylation-defective (p53-8KR) and wild-type p53 constructs (n = 3). Similar transfection efficiency was confirmed by cotransfection with a β-gal plasmid.

(H) Western blotting for acetylated p53 (K382), total p53, SIRT1, and β-actin.

(I) Apoptosis was evaluated after 48 hrs by Annexin V labeling. Results are normalized to K562-RFP cells transfected with empty vector.

(J) The fold change in cell numbers at day 2, day 4, and day 6 was calculated relative to day 0. Results represent mean ± SEM for separate experiments. Significance: $^*p < 0.05$, $^{**}p < 0.01$, compared with controls. See also Figure S8.

manuscript. Liang Li designed and performed experiments and reviewed the manuscript. Z.W. designed and performed experiments and reviewed the manuscript. Y.H. performed experiments and reviewed the manuscript. T.M. performed experiments and reviewed the manuscript. T.H. provided material, interpreted data, and reviewed the manuscript. W.Y.C. designed the study, analyzed and interpreted data, and wrote the manuscript. R.B. designed the study, analyzed and interpreted data, and wrote the manuscript.

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