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The lysine methyltransferase SMYD2 is required for normal lymphocyte development and survival of hematopoietic leukemias.

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Abstract

The 5 membered SET and MYND Domain-containing lysine methyltransferase (SMYD) family plays pivotal roles in development and proliferation. Initially characterized within the cardiovascular system, one such member, SMYD2, has been implicated as an oncogene in leukemias deriving from flawed hematopoietic stem cell (HSC) differentiation. We show here that conditional SMYD2 loss disrupts hematopoiesis at and downstream of the HSC via both apoptotic loss and transcriptional deregulation of HSC proliferation and disruption of Wnt-β-Catenin signaling. Yet previously documented SMYD2 cell cycle targets were unscathed. Turning our analysis to human leukemias, we observed that SMYD2 is highly expressed in CML, MLLr-B-ALL, AML, T-ALL and B-ALL leukemias and its levels in B-ALL correlate with poor survival. SMYD2 knockdown results in apoptotic death and loss of anchorage-independent transformation of each of these hematopoietic leukemias. These data provide an underlying mechanism by which SMYD2 acts during normal hematopoiesis and as a proto-oncogene in leukemia.

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ARTHUR CONTRIBUTIONS

MAB and HOT designed research; MAE, MAB, IA, HG and JDD performed research; MAB, MAE, JDD, IA and HOT analyzed data; MAB, MAE, and HOT wrote the manuscript.

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The authors declare no competing financial interests

Introduction

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy (1). Based principally on immunophenotyping, ~80% of the pediatric cases are classified as B cell precursor (BCP) ALL, 15% as T-cell ALL and ~5% as mature B-ALL (1, 2). T-ALL differs from BCP-ALL in that onset occurs at a later age with higher leukocyte counts, significant mediastinal mass, CNS involvement and poorer prognosis (1,2). Until recently, treatment of BCP-ALL and T-ALL were comparable, but recent trials have relied on more intensive CNS therapeutics (1). Immunophenotypic and molecular features of mature B-cell ALL are similar to those of mature B-cell lymphomas, such as Burkitt's and are typically treated with chemotherapy (3).

Approximately 5% of pediatric ALL patients express the (9;22)(q34;q11) "Philadelphia (*Ph*) chromosome" fusion, which leads to expression of the B Cell Receptor (BCR)-activating, non-receptor tyrosine kinase, (BCR-ABL1). Although the breakpoints differ, *Ph* was originally considered a hallmark of Chronic Myeloid Leukemia (CML) (4). MLL (mixed-lineage-leukemia) gene rearrangements at 11q23 are present in 80% of all infant B-ALL cases and 10% of all childhood B-ALL (5, 6). MLL is a particularly aggressive subtype with a dismal prognosis (7,8). The MLL-AF4 subtype is associated predominantly with pro-B-ALL, which typically lack expression of the pre-B-cell receptor (9). Alternatively, MLL-AF9 is found in Acute Myeloid Leukemia (AML), whereas MLL-ENL is found both in MLLr-B-ALL and T-ALL of afflicted children (10). AML primarily afflicts older adults and, with the exception of Chronic Lymphocytic Leukemia (CLL), is the most common cause of leukemia-related deaths in the USA (11, 12).

A common thread among each of these leukemias is their likely cell of origin. As with B-ALL (13–19), CML appears to arise via transformation of the Hematopoietic Stem Cell (HSC) (20, 21). There is strong evidence for a HSC origin of AML (22–26), but the evidence is not without controversy (26). Prior studies in T-ALL revealed the existence of genetically diverse subclones phenotyped as HSC at diagnosis (27,28). Finally, MLL-fusion proteins can induce leukemia from either HSC or Granulocyte Macrophage Progenitors (GMP) (26). However, the molecular basis underlying stem cell progression to the above leukemias has yet to be firmly established.

Initially characterized in cardiomyocytes (30, 31), SET and MYND Domain-containing Lysine Methyltransferase 2 (SMYD2) was initially shown to di-methylate histone H3 at K4 and K36 (27). SMYD2 was later shown to regulate tumor suppression via mono-methylation at p53K370 or RB1K860 and K810 (31–33) by induction of apoptosis or cell cycle arrest, respectively. AHNAK and AHNAK2, proteins important in cell migration and invasion, were recently identified as SMYD2 targets by proteomics (34). Additional SMYD2 methylation targets include HSP90AB1, ERα, PARP1, PTEN, BMPR2 and β-Catenin (35–39).

During murine hematopoiesis, *Smyd2* is expressed at highest levels within Pluripotent (HSC, MPP) and Multipotent (CMP, PGMP, and GMLP) Progenitors and modestly among Committed Precursors with the exception of high expression levels during T cell

development at stages (DN3, DN4) in which T-cell receptor rearrangement occurs (S-Fig. 1) (40). SMYD2 is overexpressed in CLL, ALL and CML (41–43) and in a multitude of additional malignancies (44). This has led to small molecule inhibitors that target *Smyd2* enzymatic activity in mice (45, 46).

Surprisingly, given these implications, the role of SMYD2 in hematopoiesis and hematologic malignancies has remained poorly understood—prompting the informatics, genetic and biochemical effort reported here. Conditional knockout (CKO) of *Smyd2* in mice led to blocks at and downstream of the HSC—via induction of apoptosis and at least in part, by disruption of WNT signaling. While neither CKO antibody (Ab) production nor expression of previously documented SMYD2 cell cycle targets were compromised. SMYD2 levels correlated highly with B-ALL severity, and its overexpression was observed in clinical analyses of CML, MLLr-B-ALL, B-ALL, and T-ALL as well as in other leukemias. SMYD2 loss resulted in apoptotic death and loss of transformation of each of the above leukemias. Our findings are consistent with and discussed in the context of a proto-oncogenic function for SMYD2 in leukemia.

RESULTS

Conditional SMYD2 loss in mice disrupts hematopoiesis at and downstream of the HSC.

Human B-ALL is thought to develop from transformation of the hematopoietic stem cell (HSC) (7–10). In an effort to address the function of SMYD2 at this earliest and most sensitive hematopoietic lineage, we crossed *Rosa26-Lox-Stop-Lox-YFP* (YFP^{LSL}) reporter mice (47) with the well-characterized deleter strain *Mx-1cre* in which targeting initiates within the HSC lineage following induction of its recombination activity by IFN-α (48).

The resulting compound heterozygotes were then crossed with a $Smyd2^{floxflox(F/F)}$ strain described previously (30). Deletion efficiencies were monitored in both bone marrow (BM) and spleen via both FACS analyses of YFP^{LSL} (49–54) as well as by RT-qPCR and Western analysis (S-Figs. 2 and 3). Hematopoietic population definitions and antibody conjugates employed for their detection by flow cytometry are provided as S-Fig. 4.

FACS analysis using standard lymphocyte differentiation markers for cell populations are depicted as percentage distributions in Figure 1; ie, the percentage of observations that exist for each data point. $Mx\text{-}1Cre/Smyd2^{flox/flox}$ (CKO) mice displayed a significant reduction in $Lin^{-}Sca1^{+}ckit^{+}Flt3^{-}$ HSC in the BM (Fig. 1A; S-Fig. 5). As expected from these results, progression to the $Lin^{-}Sca1^{+}cKit^{+}Flt3^{int}$ Multipotent Progenitor (MPP) and on to the $Lin^{-}Sca1^{+}cKit^{+}Flt3^{hi}$ Lymphoid Multipotent Progenitor (LMPP) was significantly impaired (Fig. 1A; S-Fig. 5). We readdress this result below in the context of mechanism.

Downstream of these earliest progenitors, the hematopoietic pathway diverges into myeloid or lymphoid lineages via the committed Common Myeloid Progenitor (CMP) or the Common Lymphoid Progenitor (CLP; S-Fig. 1). As shown in Figs. 1B and S-Fig. 5, *Smyd2* CKOs displayed a significant decrease in $Lin^{-}Flt3^{+}IL\text{-}7^{+}Sca1^{low}cKit^{low}$ CLPs and a more modest decrease in $Lin^{-}Sca1^{-}cKit^{+}FcyR^{int}$ $CD34^{int}$ CMPs.

Both CMP and CLP can give rise to plasmacytoid dendritic cells (pDC), which are characterized by their high-level production of Type I interferons (50). Unexpectedly, we observed significant increases in $CD11b^{-}CD11c^{low}B220^{+}PDCA1^{+}$ pDC in CKO BM and spleen (Fig. 1C; S-Fig. 5). These results were confirmed in a *Smyd2*$^{F/F}$ cross with *Vav1-Cre,* which initiates recombination within fetal liver HSC and HSC-proximal endothelial cells (51) (S-Fig. 6). One explanation for this unanticipated result is that SMYD2 expression stalls shortly after entering the myeloid pathway at the CMP stage. An alternative explanation is proposed and tested below.

Gene expression data in the mouse (40) (S-Fig. 1) indicated that SMYD2, while expressed modestly through much of T cell differentiation, is expressed abundantly in immature double negative (DN2 and DN3) phases during which TcR rearrangement takes place. Accordingly, mutant CKO $CD4^{-}CD8^{-}$ DN splenocytes and thymocytes were significantly reduced in DN T cells as well as the downstream CD4 SP lineage, but not in other mature T cell subsets (Fig. 1D; S-Fig. 5). Reduction in $CD4^{+}$ T cells is consistent with previous observations that such decreases are often associated with human CML (52, 53).

SMYD2-deficiency modestly disrupts B cell differentiation but not antibody titers nor isotype production.

B cells progress through a series of intermediate VDJ-joining stages until they ultimately mature and are activated to antibody (Ab) secreting plasma cells (PC). SMYD2 CKOs exhibited no significant loss of $B220^{+}CD19^{+}CD43^{-}IgM\text{-}CD2^{+}$ small pre-B cells (Fig. 1E; S-Fig. 5). This was unexpected given that small pre-B1 is the stage at which transformation is observed in the majority of childhood pre-B-ALL (13–19). Similarly, SMYD2 loss either did not or only modestly reduced all downstream B cell populations, including $B220^{+}CD19^{+}IgM^{hi}IgD^{-}$ immature B cells, $B220^{+}CD19^{+}IgM^{hi}IgD^{-}CD21^{-}CD23^{-}$ Transitional (T)1 and $B220^{+}CD19^{+}IgM^{hi}IgD^{+}$ and $CD21^{+}CD23^{+}$ T2 B cells. While $B220^{+}CD19^{+}CD21^{hi}CD23^{low}$ Marginal Zone B cells were modestly reduced, $B220^{+}CD19^{+}IgM^{hi}IgD^{hi}$ splenic follicular/BM recirculating B cells accumulated at normal levels (Fig. 1F; S-Fig. 5).

These somewhat complexing results led us to determine whether CKO mice were impaired in Ab production. We injected C57BL6 mice with 4-hydroxy Nitrophenyl acetic acid; Keyhole-Limpet Hemocyanin (NP-KLH) delivered intraperitoneally as detailed in Methods and Materials. ELISAs were developed with horseradish peroxidase (HRP)-conjugated goat Ab specific for each mouse IgG isotype and concentrations were calculated using standard curves generated with corresponding, purified mouse antibodies. Consistent with the above observation of normal follicular B cells—the population from which humoral responses are primarily generated—no significant alteration in titers or isotype production were observed in three independent measurements (Fig. 2).

Loss of SMYD2 results in apoptosis of HSCs and in some, but not all, downstream lineages.

The conditional knockout results indicated that loss of SMYD2 resulted, most significantly, in loss of HSC (Fig. 1). Smaller, yet significant, losses were observed in more mature T cell

stages, and yet a number of progenitors and mature populations were unperturbed or perturbed differentially (Fig. 1E). Further complexing, if SMYD2 expression was developmentally protracted or "stalled" upon entry into the myeloid stage, why would SMYD2 loss have the opposite effect on pDC than on their CMP and CLP precursors (Fig. 1C)?

These data raised the alternative possibility that the observed decreases in the sizes of lymphoid progenitor pools reflect decreased cell survival rather than impaired development. To test this hypothesis, we employed micro-bead enrichment of $Sca1^{+}c\text{-}Kit^{+}$ HSCs from the BM of and aged-matched controls as previously described (54). We then determined HSC apoptosis by direct counting cells labeled by dual terminal UDP-nick end labeled (TUNEL). As shown in Figure 3A, $TUNEL^{+}$ nuclei showed characteristic condensation and halos within the CKO HSC, whereas WT controls showed negligible staining. Quantitation of 3 independent experiments (Fig. 3B) indicated there were ~0.50 TUNEL+ nuclei per high power field compared <0.1 in controls (*$p<0.05$). Acridine orange and Giemsa staining (Figs. 3C, D) further identified apoptotic HSCs as brightly condensed CKO chromatin and shrunken or absent cytoplasm (arrows).

Apoptosis is often linked to cell proliferation via cell-cycle regulators and apoptotic stimuli that affect both processes (55). Thus, we assessed levels of Ki-67, a conventional marker for proliferation, by flow cytometry. We observed that both the percentage of Ki-67 negative HSC cells analyzed within the live gate as well as lineage-negative (Lin−) population were significantly (n=5, *$p<0.05$) reduced in the CKO (Fig. 3E).

To further validate and extend the TUNEL data, apoptosis was quantified using FACS-sorted hematopoietic populations generated in *Mx-1Cre/Smyd2*$^{flox/flox}$ (CKO) mice and controls. We focused this analysis on developmental stages that showed significant reduction in cellularity as determined by the FACS analyses of Figure 1. Sorted cell populations were incubated with Annexin V for 15 min at room temperature followed by addition of 7-AAD, and then immediately analyzed by flow cytometry. As shown in Figure 3F, significant levels of apoptosis were confirmed in CKO HSC, as well as in MPP, LMPP and CLP progenitors. Consistent with the observations of Figure 1, we observed the opposite effect—reduced apoptosis—in pDCs and none in pre-B cells. Levels of CKO apoptosis in CD4 and CD8 SP and CD4CD8 DP T cells paralleled their reduced cellularity revealed by flow cytometry (Fig. 1D; S-Fig. 5) even though B cell levels were unphased (Fig. 3F and readdressed in Discussion).

Taken together, the data indicate that SMYD2 controls programmed cell death at multiple stages during normal hematopoietic differentiation.

SMYD2 associates with Frizzled Receptor 2 to modulate genes critical to WNT control of HSC proliferation.

As an initial search for novel pathways, we carried out yeast two-hybrid assays to search for novel SMYD2 interacting proteins. We employed SMYD2 as bait to screen a mouse late stage (E17.5) embryonic cDNA library (56).

Among the positive candidates, which interacted specifically with SMYD2 but not with other SMYD paralogues, we identified Frizzled Receptor 2 (FZD2; Fig. 4A). FZD2, as with most Frizzled receptors couples with the β-catenin canonical signaling pathway to activate WNT5A and WNT3B homodimers (57). WNTs have been shown to modulate HSC function by promoting self-renewal via a number of autocrine and paracrine based mechanisms (57). Further relevance stems from the observation that SMYD2 methylation of β-catenin is required for its nuclear localization and ultimate action as a transcriptional coactivator of the WNT pathway (58, 59).

These findings suggested that HSC development in SMYD2-deficient mice might be compromised by flawed WNT signaling. CKO and WT $Sca1^{+}c\text{-}Kit^{+}$ repopulating BM HSCs, as described above and previously (54), were analyzed by RT-qPCR for deregulated WNT transcripts using primers listed in S-Table 1. As shown in Fig. 4B, we observed ~10-fold reduction in the level of *Wnt5a* and a more modest, yet significant, reduction in *Wnt3a* following loss of SMYD2.

FZD2 was previously shown to enhance cell migration via STAT3 (60, 61). SMYD2 loss resulted in ~10-fold downregulation of STAT3 as well as significant reduction in several STAT3 target genes, including *CD166, CD34* and *Pprg* (Fig. 4B). Two additional STAT3 targets essential for the maintenance of pluripotent stem cells, *Oct-4* and *Nanog*, were significantly repressed ($p \leq 0.05$) by SMYD2. While the values of two additional targets, *Cd34* and *c-Kit,* did not meet this probability criterion, their statistical trends ($p \leq 0.07$ and $p \leq 0.10$, respectively) suggested that they, too, were influenced by SMYD2 loss. Alternatively, three central regulators of HSC (*Runx-1*, *Gata2* and *p53*) (61) were lowly expressed and statistically unaffected by SMYD2 loss (Fig. 4B). Similarly, expression of addition transcripts previously implicated (62) in HSC self−renewal (*FoxO3a, Smad4, Angpt1, Tie2, p16, Notch1, Bmi-1* and HoxB4) showed no significant differences among CKO and controls (Fig. 4B).

Together with the apoptosis data of Figure 4, SMYD2 activation of *Wnt5a/3b, Stat3* and several *Stat3* target genes suggests a specific role for *Smyd2* in self-renewal of mouse HSC.

High SMYD2 levels in B-ALL correlate with poor survival.

Next, we turned our attention to the potential role of SMYD2 in leukemia. As a first approach toward establishing a relationship between SMYD2 expression and severity of disease, we performed meta-analysis of global microarray and epigenetic data assembled in searches for biomarkers of adult B-ALL—a human neoplasm that likely develops via dysregulation of HSC development (7–10). We identified SMYD2 upregulation in a large number of B-ALL patients (both *Ph+* and *Ph-*) enrolled in 4 separate phase III clinical trials conducted by Eastern Cooperative Oncology Group (ECOG) (63, 64). As shown in Fig. 5A, high SMYD2 levels correlated with statistically significant poor survival in both *Ph-* and *Ph+* groups.

Overexpression of SMYD2 in B-ALL, CML, MLLr-B-ALL. T-ALL and other human malignancies.

To broaden the B-ALL search as well as to determine if SMYD2 overexpression is observed in other hematopoietic malignancies, we compiled gene expression data from publicly accessible microarray studies, including Gene Expression Omnibus (GEO) (65), Mile/Bloodspot (66) and Amazonia (67).

Shown in Figure 5B are our meta-analyses of these data sets in which SMYD2 expression is globally normalized to 100 (e.g., a number of 1000 is a 10x increase in SMYD2 expression). These data indicate that the highest malignant expression of SMYD2 (at least 10x) occurs in B-ALL and T-ALL. Chronic Myelogenous Leukemia (CML) data sets range from 7X normal expression down to equivalent to normal expression, whereas MLL-rearranged (MLLr)-B-ALL values range from 2- to 12-fold normal levels. We also observed modest SMYD2 overexpression in Chronic Lymphocytic Leukemia (CLL) and Diffuse Large B Cell Lymphoma (DLBCL).

Reduction of SMYD2 leads to apoptosis in leukemias.

The results of Figures 3 and 4 prompted us to determine whether SMYD2 is required for survival of leukemias in which it is overexpressed. To test this, we performed si-RNA-mediated transient knockdown (KD) of SMYD2 in a panel of CML, T-ALL, MLLr-B-ALL and B-ALL tumors. Each of these leukemias contains genomic mutations in one or more genes, but not in SMYD2 (59). Leukemias were transfected with either SMYD2-targeted si-RNA or control vector. KD efficiencies, determined at the RNA level for B-ALL NALM-1 (Fig. 6A, right panel) and at the protein level for all leukemias (S-Fig. 6) were estimated at 75−−80%. At 72 hr post transfection, caspase-3 activities, as assessed by DEVD-pNA hydrolysis, were measured in cytosolic extracts.

As shown in Figure 6A (left panel), statistically significant levels of apoptosis were observed for each of the leukemic types, with the exception of the CML, K562. K562 carries only a single TP53 mutation (68), and it remains unclear as to why this CML is resistant to SMYD-2-mediated apoptosis.

SMYD2-deficient leukemias lose transformation ability.

Next, we investigated the potential consequences of SMYD2 loss in leukemias. The ability of transformed cells to grow independently of a solid surface is a hallmark of carcinogenesis. Thus, we determined if reduction of SMYD2 expression results in loss of anchorage-independent colonies as detailed in S-Methods. Briefly, leukemias were infected with SMYD2 sh-RNA (*pRSMX-PG-shRNA-SMYD2*; detailed in S-Methods) and KD efficiencies were scored ~3–4 weeks later (S-Fig. 7). Approximately 10^6 cells were plated on a semi-solid methylcellulose-based medium to assay colony forming units (CFU). We observed significant reduction in colonies, ranging from an average of ~85% in MLLr-B-ALL, AML and B-ALL and ~35% in CML and AML (Fig. 6B, C).

Collectively our results suggest that SMYD2 is required, at least in tissue culture, for normal growth, survival and transformation of hematopoietic leukemias.

Loss of SMYD2 in hematopoietic leukemias does not alter expression of established methylation targets.

SMYD2 primarily functions post-translationally as a methyltransferase. As might be anticipated from this function, we have observed only modest changes in expression levels of established SMYD2 methylation targets during murine embryogenesis and early development (29, 30 and data not shown) with a notable exception: Cell cycle regulators. Documented loss in expression accompany loss of SMYD2-catalyzed methylation of RB, TP53/p53 (31–33) and several other cell cycle regulators in various tumor types, including HSP90AB1, ERα, PARP1, PTEN and β-Catenin (34–39).

Following SMYD2 sh-RNA-mediated KD (S-Fig. 7), we assessed the levels of these substrates by Western blotting of proteins isolated from the leukemia panel of Figure 7. In contrast to previous reports (31–39), neither RB, TP53 nor none of the additional SMYD2 methylation targets showed significant expression level changes in any of the leukemias tested.

These results suggest that SMYD2 functions in hematopoietic malignances through pathways other than cell cycle control. The data of Figure 4 suggest that those most likely might include flawed apoptotic regulation of hematopoietic stem cell development.

DISCUSSION

SMYD2 was previously implicated as an oncogenic contributor by virtue of its overexpression in hematopoietic and non-hematopoietic malignancies (41–44). We corroborated these results here via meta-analyses of phase III clinical trials (63, 64) (Fig. 5A) and via publicly accessible patient microarray analyses (65–67) (Fig. 5B). The latter data further revealed SMYD2 overexpression in B-ALL, T-ALL, CML, MLLr-B-ALL, AML and additional hematopoietic lesions, including CLL and DLBCL. These data collectively suggest an oncogenic function for SMYD2 underlying these malignancies. In support, we showed that loss of SMYD2 in the above leukemias resulted in suppression of colony formation (Fig. 6B-C) as well as widespread apoptosis (Figs. 3 and 6). Yet contrary to previous studies (31–39), neither p53, pRb nor the levels of several additional cell cycle regulators were impaired (Fig. 7).

The cancer stem cell hypothesis states that virtually all tumors are clonal progeny of a single cell (69). Yet, the route from which the initial somatic mutation develops eventually to cancer is complicated by the multiple distinct subclones arising among even the most predominant progenitors. It is generally accepted that B-ALL, CML, MLLr-B-ALL and AML develop from transformation of the Hematopoietic Stem Cell (HSC) (18–28). Consistent with these findings, we observed that *Smyd2*$^{F/F}$/*Mx-1cre* mice, in which robust *Smyd2* deletion is initiated within the HSC lineage following injection of IFN-α (S-Fig. 2), suffered significant reduction in HSC as well as in some, but not all, downstream myeloid and lymphoid lineages (Fig. 1). Alternatively, and as previously shown in AML (70), reduced CKO levels of *Smyd2* might contribute independently to myeloid stem cell maintenance. Thus, to a first approximation, *Smyd2* loss recapitulates this aspect of leukemic pathology.

Our hematopoiesis results conflict with a recent study by Bagislar and coworkers (43) in which no defects in B cell development were observed in *Smyd2*-deleted mice. While both CKOs were generated in isogenic C57BL/6, different *Smyd2* genomic regions were targeted. But a more probable reason underlying the disparity owes to the different Cre-deletion strategies employed. Bagislar et al. (43) utilized *CD19-cre* which mediates efficient deletion in mature B cells, partial deletion in immature B and pre-B cells, but very little to no recombination in B cell progenitors (43). Thus, their analysis by-passed the essential disruption and subsequent consequences downstream of a primary defect in HSC detected in our approach via *Mx1-Cre* deletion (Fig. 2). Also, we found it informative that Bagislar et al. (43) determined that *Smyd2* transcription is controlled by p54/c-MYC. This result may explain why the K562 CML, which lacks productive *c-Myc* expression (68), was the only one of 8 leukemias refractory to SMYD2 -mediated apoptosis, necrosis or transformation (Fig 6A, B).

At least one mechanism by which SMYD2 regulates hematopoiesis is apoptosis. Initially, we suspected that *Smyd2* CKO-mediated lineage reduction was a consequence of transcriptional reduction in stem/progenitor cells. Yet these cellular losses were inconsistent across both progenitor and mature populations (Fig. 1; S-Fig. 5). Indeed, some lineages were unperturbed at all (e.g., pro-B and mature B cells), whereas others exhibited increases (e.g., pDC). These results led us to suspect that *Mx-1*cre deletion more likely reflected direct action within each sub-population. This was buttressed by the fact that most of our analyses were performed within 2 days of pIpC induction of *Smyd2* deletion. This assumption proved correct. We observed significant levels of apoptosis not only in HSC (Fig. 3) but across developmental stages which paralleled their reduction in CKOs (Fig. 6). It is difficult to pinpoint a bottleneck in lymphoid development from steady state data. To better understand the relative contribution of SMYD2 to apoptosis and transcription at specific developmental stages, we plan to examine the kinetics of recovery from partial bone marrow ablation.

SMYD2 loss in HSC led to modulation of the WNT/β-catenin pathway (57). Whether these changes were transcriptionally-based or a result of apoptosis remains to be determined. We first discovered that SMYD2 interacts with FZD2, the receptor for both WNT5A and WNT3A (60) (Fig. 4A). Presumably through this interaction, SMYD2 not only activates, but perhaps stabilizes WNT5A/3A expression. STAT3 is an essential HSC transcription factor, and several STAT3 target genes also were downregulated (Fig. 4B). These results along with the reduction of CKO proliferation (Fig. 3E) align with recent reports by Li et al. (70) demonstrating that SMYD2 interacts with and methylates STAT3 to stimulate increased cell proliferation. They also support results by Deng et al (39) in which SMYD2 methylation of β-catenin promotes its nuclear entry and activation of WNT signaling. β-Catenin is an essential target for various cancer stem cells, including fusions derived from HSC-enriched LSK and AML, but not from myeloid progenitors (71–73). Development of embryonic and several somatic HSCs require β-catenin (74). We suggest that targeting the SMYD2-WNT-β-catenin pathway clinically at its cell of origin—the HSC—might provide a successful approach for future cancer therapies.

Typically, in B-cell precursor ALL, B cell development is arrested at the pre- or pro-B-cell stage (1, 2). As shown in Figure 1E, *Smyd2*-deficient mice, while severely compromised in

HSC, exhibited negligible loss of neither. Indeed, downstream lineages were only modestly, if at all, reduced (Fig. 1F; S-Fig. 5). Accordingly, our ELISA results showed normal Ab responses to a T-dependent antigen (Fig. 2) or to a T-independent antigen (data not shown), even though CD4 T_H2 apoptosis was elevated in the CKO (Fig. 3E). Perhaps CD4 T_H2 susceptibility to apoptosis rendered them allergic (ie, non-responsive) to provide B cell help. Thus, the observed CD4 T-dependent response reflected the lack of difference in B cell populations (Figure 3F). Alternatively, a low input of cells from the bone marrow (Figs. 1A and B) can build a peripheral B cell compartment with adverse characteristics, because long-lived, activated cells have a reduced tendency to differentiate into Ab-producing PC (75).

It will be fruitful to more fully characterize SMYD2 apoptotic and transcriptional leukemic targets in hopes of unraveling regulatory mechanisms critical to SMYD2 function in carcinogenesis. Finally, we suggest that *Smyd2* CKO mice, when crossed onto murine leukemic backgrounds, may provide a valuable tool to analyze the efficacy of new anti-tumor-specific drugs. Such a system does not suffer the drawback of using irradiated animals, as the onset of tumor formation should be relatively precise. This may alleviate the problems that complicate many drug studies in which small tumor precursors are employed as opposed to fully progressed tumors. This mode of tumor induction should allow the molecular mechanisms responsible for SMYD2-mediated tumor formation to be deduced.

MATERIALS AND METHODS

Smyd2-floxed C57BL/6 mice (30) were crossed with *Mx1*-Cre (48), *Mb1-Cre* (48) or *Vav*-Cre (51) C57/BL mice for deletion of *Smyd2*. Deletion efficiencies of *Mx1-Cre* and *Vav-Cre* were monitored by FACS analyses of YFP^{LSL} (S-Fig 2) (51) as well as by RT-qPCR in bone marrow and spleen (S-Fig. 3).

Analytical cytometry was performed on a FACS Fortessa and sorting was performed on a FACS Aria (BD Biosciences) followed by analysis using FlowJo (Tree Star) software (details of antibodies and conditions provided in S-Methods). In Figure 1, data are depicted as percentage distribution; ie, as a display of data that summarizes the percentage of observations for each data point calculated from the raw FACS data of S-Figures 5 and 6.

RT-qPCR and endpoint PCR was performed and normalized as detailed in S-Methods employing GAPDH as a housekeeping control. Immunization of mice with 4-hydroxy-5-nitrophenylacetic acid; Keyhole-Limpet Hemocyanin (NP-KLH), and ELISAs were performed as previously described (77).

Microbead enrichment of Sca1+c-kit+ HSCs (Hematopoietic Stem Cells) from BM of $Mx\text{-}1Cre/Smyd2^{flox/flox}$ CKO and aged-matched controls was performed as detailed previously (54) and is detailed in S-Methods. HSC staining with Giemsa and Acridine orange, as well as measurement of their proliferation by KI-67 staining/flow cytometry is provided in S-Methods.

Small interfering (si)-RNA knockdown and short hairpin (sh)-RNA interference were performed as previously described (30, 77) and detailed in S-Methods on the following leukemias: Acute Myeloblastic Leukemia (AML): HL-60 (ATCC CRL-2724) and THP-1

(ATCC TIB-202); Chronic Myelogenous Leukemia (CML): K-562 (ATCC CCL-243) and KAS-4 (ATCC CRL-2726); MLL-Rearranged Acute Lymphoblastic Leukemias (MLLr)-B-ALL): RS4;11 (ATCC CRL-1873) and SEM (ATCC CCL-119). T-Cell Acute Lymphoblastic Leukemia (T-ALL): MOLT4 (ATCC CRL-1873) and CCRF-CEM (ATCC CCL-119); B-Cell Acute Lymphoblastic Leukemia (B-ALL): NALM-6 (ATCC CRL-3273) and CCL-120/CCRF-SB (ATTC CCL-120). Methods for the growth and maintenance of each leukemia is provided in S-Methods.

Western blotting was performed as described (77) on 12.5% SDS-PAGE with commercial anti-human (h) antibodies as detailed in S-Methods.

Apoptosis assays were performed on CML, MLLr-B-ALL, B-ALL and AML leukemias following transfection with SMYD2 si-RNA (30). Measurements were performed by analytical cytometry followed by analysis using FlowJo (Tree Star) software.

Yeast two-hybrid screening to isolate SMYD2-interacting proteins employed the Matchmaker Gold Yeast Two-Hybrid system (PT1172–1, Cat No. 630439) on a cDNA library (Clontech) as previously described (56) and detailed in S-Methods.

Groups of three to eight mice were used for statistical analysis. P values were calculated with a Student's t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Points

Hematopoiesis, transcription factors, lymphocyte development, leukemia

- SMYD2 deficiency retards hematopoietic lineage progression.
- SMYD2 is required for survival and proliferation of hematopoietic-derived leukemias.

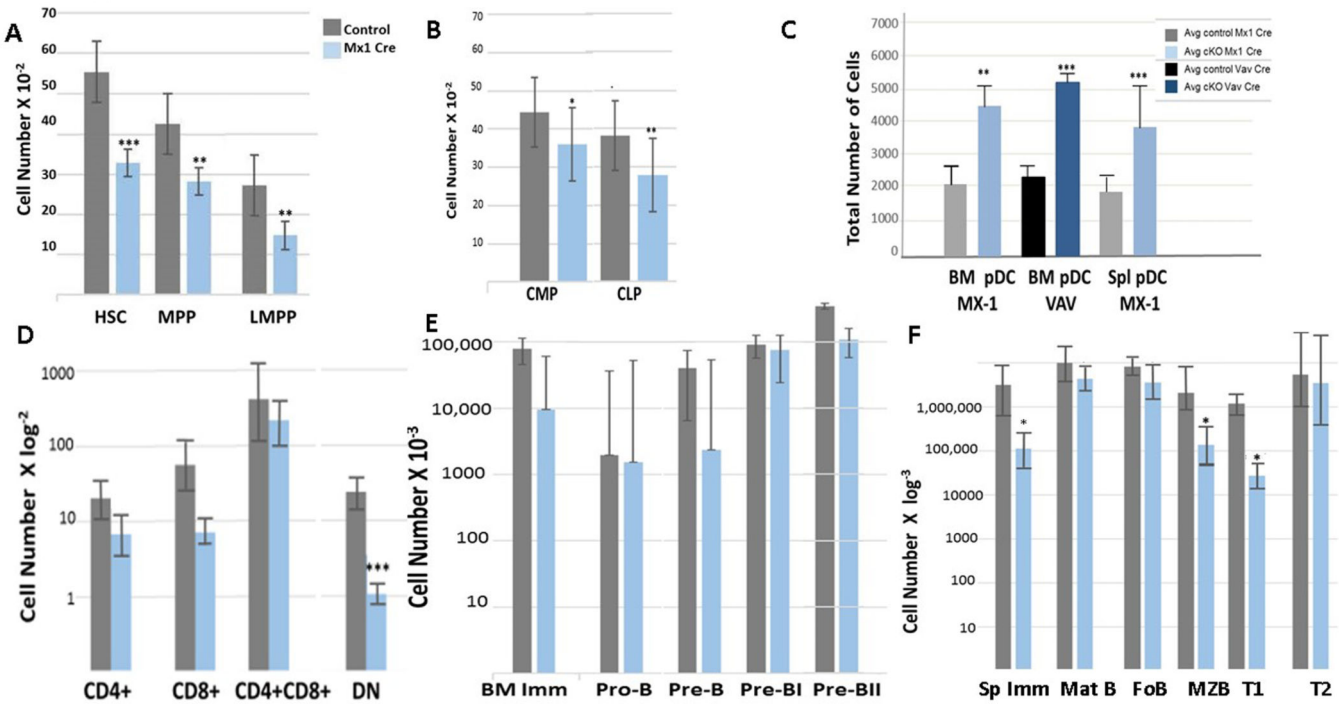

Figure 1. *Smyd2* conditional knockout (CKO) mice suffer significant losses in some, but not all, lineages downstream of the Hematopoietic Stem Cell (HSC).

Smyd2$^{F/F}$ mice were crossed with *Mx-1cre* (48), in which targeting is initiated within the HSC lineage following induction of *Cre* recombination activity by IFN-α. Shown here are flow cytometric data in tabular format; representative FACS scans are provided in S-Figs. 5 and 6, Antibodies employed are listed in S-Fig. 4. **(A)** *Mx-1Cre/Smyd2*$^{flox/flox}$ CKO mice displayed significant reduction in HSC in the bone marrow (BM) as well as in progression to Multipotent Progenitors (MPP) and further downstream to Lymphoid Multipotent Progenitors (LMPP). **(B)** Common Lymphoid Progenitors (CLP) are significantly blocked, whereas the Common Myeloid Progenitors (CMP) are modestly decreased. **(C)** Plasmacytoid Dendritic Cells (pDC) are significantly increased within both BM and spleen (SP). **(D)** Double Negative ($CD4^{-}CD8^{-}$; DN) but not CD4+CD8+ (DP), nor CD4+ or CD8+ single positive (SP) T cells are significantly reduced in BM and SP. **(E)** No significant loss of Pro-B, small pre-B1, large pre-B2 nor immature (Im) BM B cells are observed in CKOs. **(F)** Most downstream B cell lineages, including Im SP B cells, Transitional (T1) cells and Marginal Zone B (MZB) cells are unperturbed in CKO mice. Total cell numbers were determined as: Total # femur/spleen/thymus cells harvested/sample X number of cells/gate. Statistical analyses performed by student's t-test on 4–6 experimental replicates; *p≤0.05; **p≤0.005; ***p≤0.001.

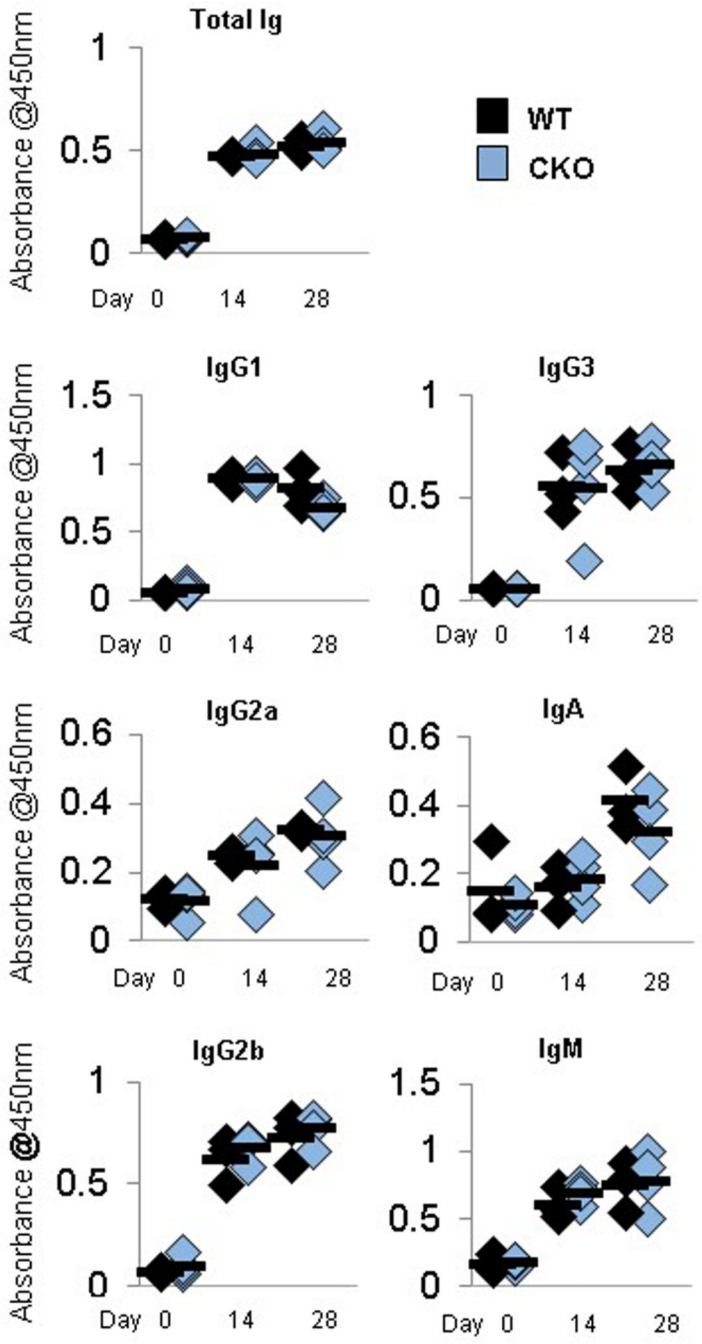

Figure 2. Antibody (Ab) titers and isotype production are unaffected in *Smyd2* CKO mice.

Mb-1Cre/Smyd2$^{flox/flox}$ CKO and age matched controls were immunized intraperitoneally with PC-KLH as detailed previously (77) and in Materials and Methods. Sera were collected at 0, 14, and 28 days after inoculation, analyzed via ELISA for HRP-conjugated goat Abs specific for IgM and IgG isotypes. Horizontal black bars indicate the means established from 3 independent immunizations.

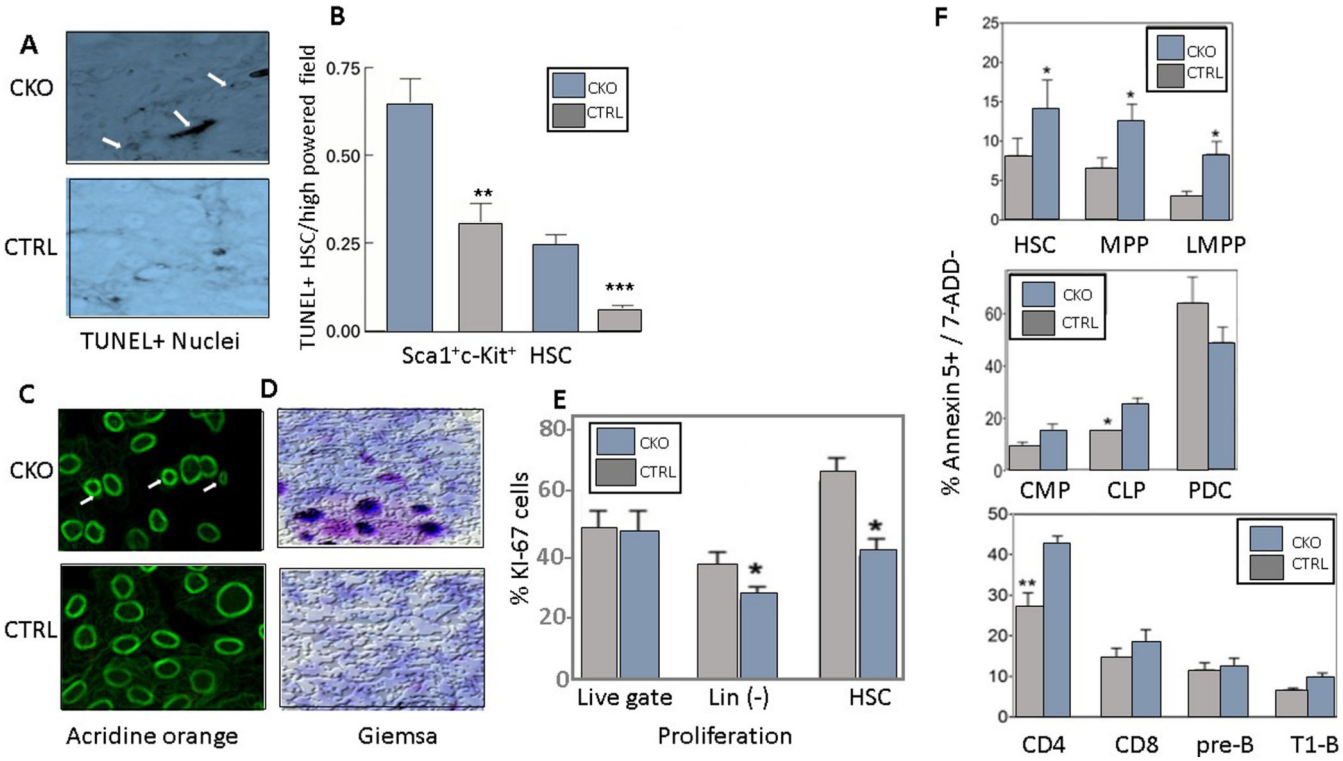

Figure 3. SMYD2-deficient HSCs and downstream lineages undergo apoptosis.

(A) Apoptotic HSCs identified by dual terminal UDP-nick end labelling (TUNEL). TUNEL + cell nuclei show significant condensation (black spots) within CKO cells. 50X magnification. **(B)** Quantification of apoptosis from TUNEL images of (A). Counting and quantitation of TUNEL+ apoptotic bodies is described in Materials and Methods and S-Methods. SMYD2. Shown are averages of 3–5 independent experiments; **p≤0.01; ***p≤0.001. **(C)** Acridine orange staining (50X) of control (CTRL) and CKO apoptotic HSCs (arrows). **(D)** Giemsa staining (60X) of cytospin preparation of condensed HSC. **(E)** CKO HSCs proliferated significantly slower than WT controls as judged by Ki-67 staining and flow cytometry. Both % of Ki-67^{-} and Lin− HSC were significantly reduced in the CKO (n=5, *p<0.05). **(F)** SMYD2-deficient, FACS-sorted CKO hematopoietic populations undergo varying levels of Annexin V^{+} apoptotic cells. Analyses were focused on developmental stages that showed significant reduction in cellularity as determined by FACS; n=5; *p≤0.05; **p≤0.005.

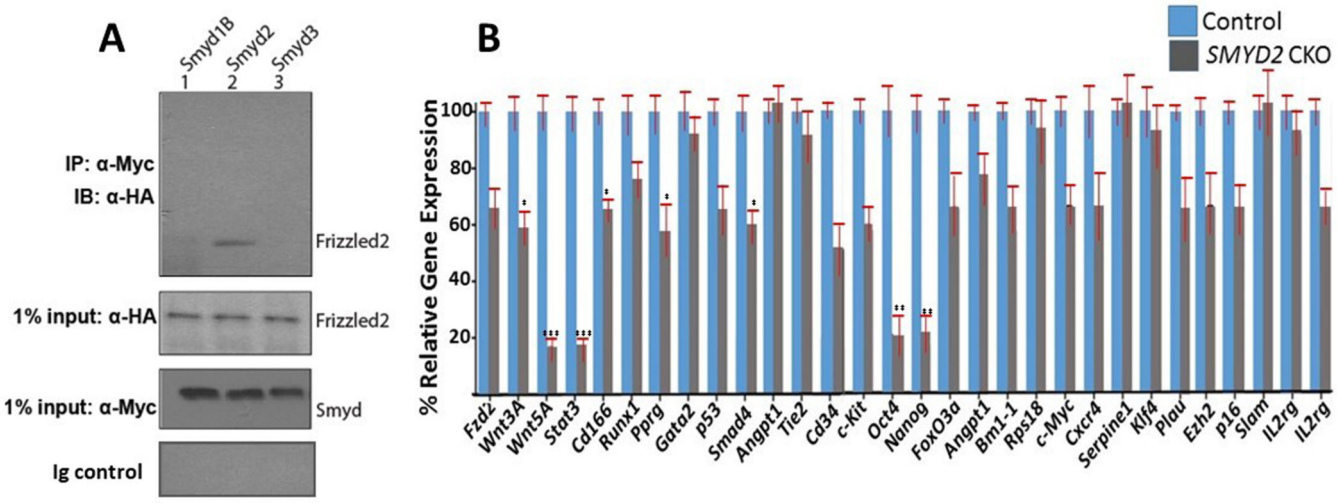

Figure 4. SMYD2 interacts with Frizzled Receptor 2 (FZD2) to regulate *Wnt5a/3a, Stat3* and several STAT3 target genes critical for self-renewal of mouse HSCs.

(A) Identification of FZD2 as an interacting partner of SMYD2 following 2-hybrid isolation as previously reported (56). Anti-HA-tagged FZD interaction with MYC-tagged SMYD2 (but not with Myc-SMYD1 nor Myc-SMYD3) was confirmed by Western blotting following transient transfection into 3T3 fibroblasts (panel 1); input controls (panels 2, 3); Ig-only negative controls (panel 4). **(B)** Knockdown of *Smyd2* in microbead enriched $Sca1^{+}c\text{-}Kit^{+}$ HSC from the BM of CKO and aged-matched CNTRs, followed by RT-QPCR analyses employing primer-pairs detailed in S-Table 1 identified *Stat3* and several *Stat3* target genes critical to HSC differentiation/proliferation. 3 independent replicates determined by students t-test as *, $p \leq 0.05$; **, $p \leq 0.01$; ***. $p \leq 0.001$.

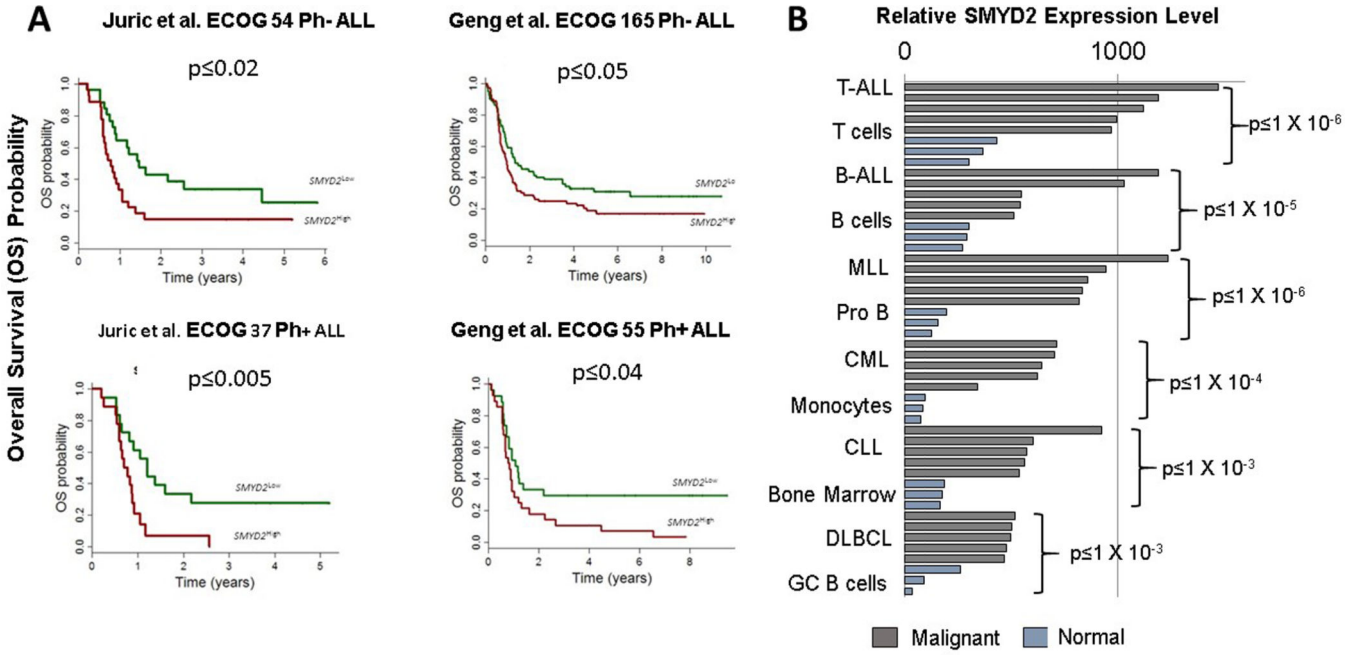

Figure 5. Meta-analyses of SMYD2 levels and survival in hematopoietic malignancies.

(A) Global microarray data indicate that SMYD2 is statistically upregulated in B-ALL patients. The numbers of both *Ph*− (upper panels) and *Ph*+ (lower panels) enrolled in 4 separate phase III clinical trials (63, 64) are indicated. **(B)** Overexpression of SMYD2 in B cell malignancies. SMYD2 (gray bars) is overexpressed ~10-fold in B-ALL, ~7-fold in CML and ranges from 2–12-fold upregulated in MLLr-B-ALL, CLL and DLBCL relative to normal, aged matched controls (blue bars). The statistical difference between normal and control subjects within each category, as calculated by students T test are shown to the right of each set. Data compiled from publicly accessible microarray studies (65–67). The vertical bars indicate the number of patients included in each analysis, in which each bar represents 50 malignant or normal patients. Accession numbers for the original data are listed in S-Methods. Expression of SMYD2 is normalized to 100 across >1000 public datasets of normal and malignant tissues (Accession numbers provided in S-Methods). Abbreviations: T-ALL; T cell Acute Lymphoblastic Leukemia; B-ALL; B Cell Acute Lymphoblastic Leukemia; MLL; Mixed Lineage Leukemia; MLLr; MLL-rearranged (MLLr) Pro B; Progenitor B cells; CML; Chronic Myeloid Leukemia; CLL; Chronic Lymphocytic Leukemia; DLBCL; Diffuse Large B Cell Lymphoma; GC B cells; Germinal Center B cells.

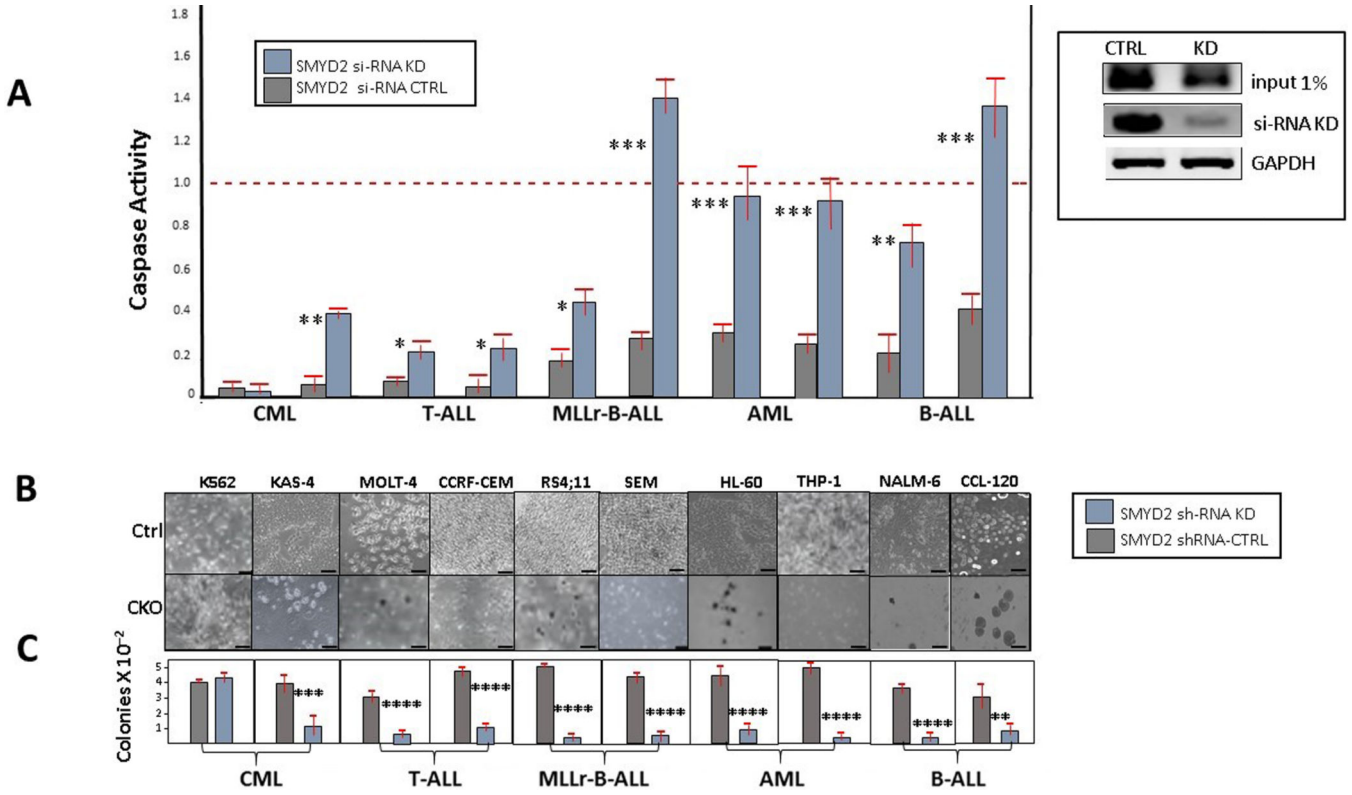

Figure 6. SMYD2 knockdown in leukemias results in apoptosis, loss of viability and reduction of growth.

(A) The indicated B-ALL, T-ALL, CML, MLLr-B-ALL and AML leukemias were transiently transfected with SMYD2 si-RNA (KD) or control si-RNA (CTRL). Left panel: Apoptosis resulting from SMYD2 si-RNA KD measured by caspase-3 colorimetric activity for CKOs (blue) and plotted relative to CTRL (gray) values set to 1.0 (red dotted line). KD efficiencies, as shown for B-ALL NALM-1 as measured by RT-PCR (right panel) and at the protein level for all leukemias (S-Fig. 7) were estimated as 15–25% relative to GAPDH control. **(B)** Leukemias were stably infected with shRNA directed at SMYD2 (*pRSMX-PG-shRNA-SMYD2*) at efficiencies calculated by Western analysis at 10–15% (S-Fig. 7). Following transduction and 21 days selection with puromycin, ~10^6 dox-induced and uninduced (CTRL) leukemia cells were assayed microscopically for colony forming units (CFU). SMYD2 KD led to reduction in viability of all leukemias except CML K562. **C.** Quantification of colonies of SMYD2 shRNA transduced leukemias of (B) scored as means of 3–5 independent measurements. Significance (students t-tests): *, p≤0.05; **, p≤0.01; ***. p≤0.001; ****, p≤0.0001. Magnification, 50X; scale bars, 50 μm.

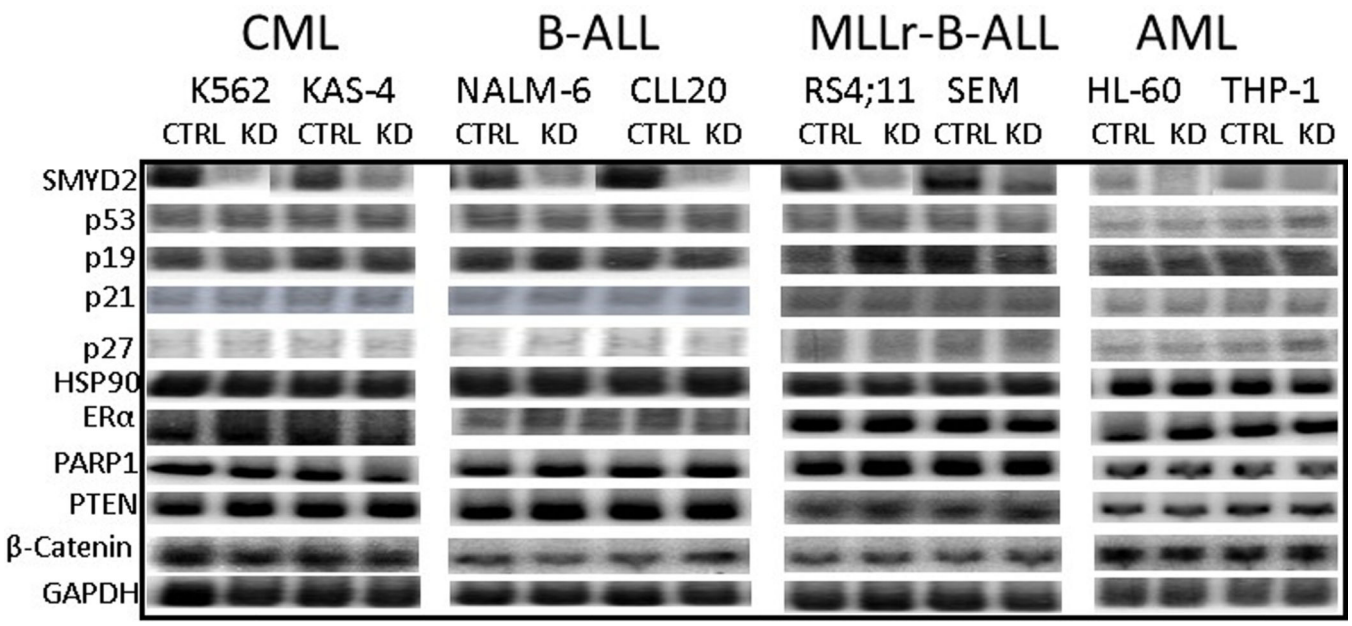

Figure 7. Expression of SMYD2 methylation targets are unaltered following loss of SMYD2 in leukemias.

Cell cycle regulators (p53, p21, ARF, and p27) and additional previously identified SMYD2 methylation targets (HSP90AB1, ERα, PARP1, PTEN and β-Catenin) were unaffected in sh-RNA transduced leukemias. Fractionation and Western blotting employing antibodies and conditions described in Materials and S-Methods. Note that autoradiographs were cut and spliced together in the SMYD2 WT and CKO row only when run on separate Western blots; measurements with no gaps were run on the same Western blots. Data was consistent within 3 independent replicas.