

An integrated approach to dissecting oncogene addiction implicates a Myb-coordinated self-renewal program as essential for leukemia maintenance

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Although human cancers have complex genotypes and are genomically unstable, they often remain dependent on the continued presence of single-driver mutations—a phenomenon dubbed “oncogene addiction.” Such dependencies have been demonstrated in mouse models, where conditional expression systems have revealed that oncogenes able to initiate cancer are often required for tumor maintenance and progression, thus validating the pathways they control as therapeutic targets. Here, we implement an integrative approach that combines genetically defined mouse models, transcriptional profiling, and a novel inducible RNAi platform to characterize cellular programs that underlie addiction to MLL-AF9—a fusion oncoprotein involved in aggressive forms of acute myeloid leukemia (AML). We show that MLL-AF9 contributes to leukemia maintenance by enforcing a Myb-coordinated program of aberrant self-renewal involving genes linked to leukemia stem cell potential and poor prognosis in human AML. Accordingly, partial and transient Myb suppression precisely phenocopies MLL-AF9 withdrawal and eradicates aggressive AML in vivo without preventing normal myelopoiesis, indicating that strategies to inhibit Myb-dependent aberrant self-renewal programs hold promise as effective and cancer-specific therapeutics. Together, our results identify Myb as a critical mediator of oncogene addiction in AML, delineate relevant Myb target genes that are amenable to pharmacologic inhibition, and establish a general approach for dissecting oncogene addiction in vivo.

[*Keywords:* oncogene addiction; MLL-AF9; AML; Myb; Smyd2; RNAi; mouse models]

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Oncogene addiction describes the dependence of cancer cells on the continued expression of a driving oncogene, such that inactivation of the oncogene product triggers the selective cell death, senescence, or differentiation of cancer cells (Weinstein 2002). The concept was initially formulated based on studies using transgenic mice engineered to conditionally express particular oncogenes, where it was observed that withdrawal of oncogene expression could produce dramatic and complete tumor regressions,

albeit with eventual disease relapse (Chin et al. 1999; Felsher and Bishop 1999; Gunther et al. 2003; Politi et al. 2006). This “addiction” of cancer cells to the driving oncogene was subsequently used to explain the remarkable efficacy and selectivity of certain molecularly targeted therapies, for example, those targeting Bcr-Abl in chronic myeloid leukemia and EGFR mutants in non-small-cell lung cancers (Druker et al. 2001; Lynch et al. 2004; Weinstein and Joe 2006). Subsequent studies have demonstrated that cancers can also become addicted to downstream mediators of driving oncogenes, providing additional entry points for therapeutic intervention (Soucek et al. 2008; Ehrenreiter et al. 2009; Hoelbl et al. 2010). Nonetheless, despite their potential importance, the precise molecular

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programs that mediate oncogene addiction remain poorly understood.

Acute myeloid leukemia (AML) exemplifies a genetically well-characterized cancer where specific lesions are linked to patient prognosis and therapy outcome (Estey and Dohner 2006). Leukemogenesis typically results from cooperating mutations that induce aberrant self-renewal in lineage-committed progenitors, often enforced by fusion proteins arising from chromosomal rearrangements, and others that increase proliferation, such as Ras pathway mutations. For example, translocations involving the *MLL* gene at 11q23 are among the most extensively characterized AML oncogenes, and are clinically associated with a dismal prognosis and poor therapy responses (Schoch et al. 2003). In patients, *MLL* rearrangements are often found in concert with activating mutations in Ras signaling pathways (Naoe et al. 1993; Liang et al. 2006; Radtke et al. 2009; Zuber et al. 2009; Chandra et al. 2010), which have been shown to cooperate with *MLL* fusion proteins to promote AML in mice (Ono et al. 2005; Stubbs et al. 2008; Zuber et al. 2009). Despite these advances in our understanding of the molecular mechanisms underlying AML pathogenesis, the majority of AML patients are not cured by the current chemotherapy regimens (Estey and Dohner 2006), underscoring the need for targeted therapeutics.

MLL encodes a histone H3K4 methyltransferase that activates transcriptional programs necessary for the establishment and maintenance of the hematopoietic system (Ernst et al. 2004). Leukemogenic fusion proteins aberrantly activate *MLL* targets, perhaps by recruiting alternative histone methyltransferases (Okada et al. 2005) and/or elongation factors (Lin et al. 2010; Yokoyama et al. 2010) to their promoters. The resulting inappropriate expression of canonical *MLL* target genes such as *HoxA9* and *Meis1* has been shown to suppress differentiation and establish aberrant self-renewal capabilities in lineage-committed progenitors, which is critical for the leukemogenic potential of *MLL* fusion proteins (Ayton and Cleary 2003; Cozzio et al. 2003; Krivtsov et al. 2006; Wong et al. 2007; Somervaille et al. 2009). In addition, transformation by *MLL* fusion proteins leads to the up-regulation of a self-renewal-associated transcriptional subprogram in "leukemia stem cells" (LSCs) that is similar to embryonic stem cells (Krivtsov et al. 2006; Somervaille et al. 2009) and overlaps with a transcriptional program controlled by the *Myc* oncoprotein (Kim et al. 2010). As self-renewal ability is likely critical for the maintenance of LSCs, targeting these programs presents a promising therapeutic opportunity in *MLL* fusion AML.

In this study, we set out to implement new RNAi technology to explore mechanisms of oncogene addiction in a manner that has not previously been possible in vivo. We chose to dissect the network's underlying addiction to an *MLL* fusion oncoprotein owing to the extensive body of work addressing its action in leukemia and the availability of tractable and well-characterized models of disease. Indeed, we demonstrated previously that a transplant-based mouse model of AML induced by the *MLL-ENL* fusion protein in cooperation with oncogenic Ras approximates

the genetics, pathology, and treatment response observed in AML patients (Schoch et al. 2003; Neubauer et al. 2008; Zuber et al. 2009; Pardee et al. 2011). We thus reasoned that these systems would provide tractable models to interrogate mechanisms that underlie oncogene addiction in a relevant setting. Through iterative rounds of hypothesis generation and testing, we demonstrate that *MLL-AF9* enforces a Myb-regulated program of aberrant self-renewal involving genes linked to LSC potential and poor prognosis in human AML, which is crucial to leukemia maintenance and is specific to cancer cells.

Results

An inducible mouse model of MLL fusion AML

To explore oncogene addiction programs in a genetically defined leukemia context, we developed an AML mouse model driven by a conditional *MLL-AF9* allele together with oncogenic Ras, which enabled us to examine the consequences of *MLL-AF9* inhibition in an established disease. In order to produce a tightly regulated system that was easy to monitor, we constructed two retroviral vectors containing dsRed-linked *MLL-AF9* under control of a tetracycline (tet) response element (TRE) promoter, and *Kras*^{G12D} linked to the "Tet-off" tet transactivator (tTA), which activates TRE expression in a doxycycline (dox)-repressible manner (Fig. 1A). Leukemias were generated by retroviral cotransduction of both vectors into hematopoietic stem and progenitor cells (HSPCs), which were transplanted into syngeneic mice. Cells harboring both constructs (dsRed⁺) induced an aggressive myelomonocytic leukemia similar to existing mouse models based on constitutive *MLL* oncogene expression, which recapitulated key features of human *MLL* fusion-driven AML (Zuber et al. 2009).

The impact of withdrawing expression of *MLL-AF9* on leukemia cell proliferation was dramatic. Dox treatment rapidly turned off dsRed, *MLL-AF9*, and *HoxA9* expression (Supplemental Fig. 1) and triggered cell cycle arrest and terminal differentiation of leukemia cells (Fig. 1B; Supplemental Fig. 2). Withdrawal of *MLL-AF9* in vivo led to disease regression and clearance of leukemia cells from all infiltrated organs (Fig. 1C; Supplemental Fig. 3) and prolonged the survival of leukemia-bearing mice (median, 16 vs. 127 d; $P < 0.0001$) (Fig. 1D). Most animals remained in remission upon discontinuing dox treatment after 45 d, and those with apparent "relapses" actually succumbed to a dsRed-negative host-derived lymphoid malignancy (Supplemental Fig. 4). These results, together with a previous report (Horton et al. 2008), demonstrate that leukemia cells require the continued expression of *MLL-AF9*.

Identifying critical mediators of leukemia maintenance

To identify molecular mechanisms underlying addiction to *MLL-AF9*, we performed genome-wide analysis of gene expression changes on leukemia cells following oncogene withdrawal. Six days following dox treatment, five independent Tet-off-regulatable *MLL-AF9*;Nras^{G12D}

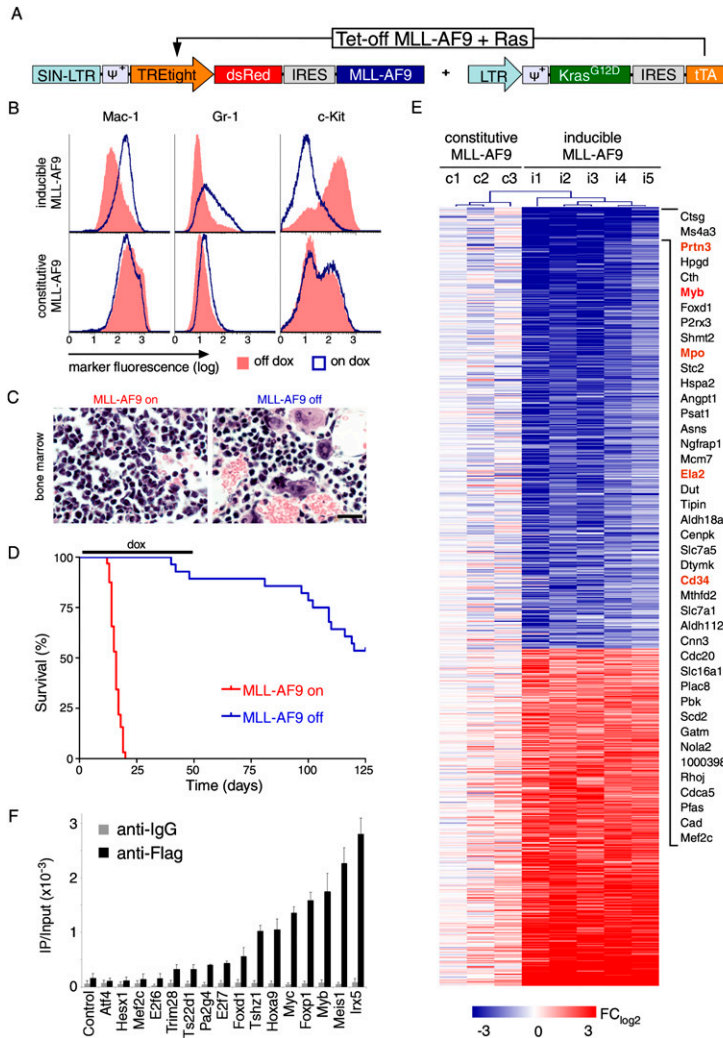


Figure 1. Withdrawal of MLL-AF9 induces differentiation and disease remission. (A) Retroviral constructs used to generate AML. (B) Representative immunophenotyping of primary Tet-off MLL-AF9;Kras^{G12D} leukemia cells harvested from mice at terminal stage and briefly grown in vitro, with or without dox (4 d). Similar results were observed in three independent primary lines. (C) Representative bone marrow histology of syngeneic recipient mice transplanted with Tet-off MLL-AF9;Kras^{G12D} AML, untreated and after dox treatment (9 d). Also see Supplemental Figure 3 for detailed histology. (D) Kaplan-Meier survival curves of mice transplanted with Tet-off MLL-AF9;Kras^{G12D} AML, which were left untreated (MLL-AF9 on; $n = 32$) or treated with dox 5 d post transplantation for 45 d (MLL-AF9 off; $n = 26$). (E) Heat map of gene expression changes in primary AML lines with either Tet-off MLL-AF9 (i1–5) or constitutive MLL-AF9 (c1–3), 6 d after dox treatment to inactivate MLL-AF9 expression. Fold change relative to untreated leukemias rendered in a blue–white–red pseudo color scheme for all genes with $P < 0.005$ and $FC_{log2} > 1.5$ or $FC_{log2} < -1.5$. The most strongly down-regulated genes are indicated and include Myb and several of its known transcriptional target genes (marked in orange). (F) ChIP to assay MLL-AF9-Flag binding to promoters of down-regulated transcription factors. Bar graph represents enrichment of immunoprecipitation relative to input. Values represent the mean \pm SE of three independent experiments.

leukemias showed a complex pattern of gene expression changes (Fig. 1E; Supplemental Table 1). Gene ontology analysis revealed a down-regulation of genes associated with cell cycle and mitosis, whereas those involved in mature myeloid cell functions were induced, suggesting that leukemia cells underwent a terminal differentiation program (Supplemental Table 2). In addition, gene set enrichment analysis (GSEA) (Subramanian et al. 2005) revealed a strong inverse correlation between global gene expression changes following MLL-AF9 withdrawal and a LSC-associated signature identified in MLL oncoprotein-expressing leukemias (normalized enrichment score [NES], 1.7 and -1.9 ; false discovery rate [FDR], 0.05 and 0) (Supplemental Fig. 5; Somervaille et al. 2009). Hence, the aberrant self-renewal and “stemness” programs induced by MLL-AF9 require continued oncogene expression.

To identify genes that might mediate these effects, we focused our subsequent analysis on differentially expressed transcription factors, as these molecules govern cell fate in normal hematopoiesis (Rosenbauer and Tenen 2007). Out of 17 strongly down-regulated transcription factors ($P < 0.005$, $FC_{log2} < -1.5$), 16 were validated by quanti-

tative RT-PCR (qRT-PCR) analysis, including established MLL-AF9 targets such as *HoxA9*, *HoxA5*, and *Meis1* (Supplemental Fig. 6). To distinguish between direct and indirect effects, we produced AMLs expressing a Flag-tagged MLL-AF9 transgene (Supplemental Fig. 7A,B) and analyzed MLL-AF9 promoter occupancy by chromatin immunoprecipitation (ChIP). In addition to binding the promoter regions of its canonical targets (*HoxA9* and *Meis1*), MLL-AF9 was also enriched at the promoters of five additional transcription factors (*Tshz1*, *Myc*, *FoxP1*, *Myb*, and *Irx5*) (Fig. 1F; Supplemental Fig. 7C), suggesting that these also may be direct MLL-AF9 targets. The most differentially expressed direct MLL-AF9 target was the transcription factor c-Myb (*Myb*), which has been shown to be induced by MLL oncoproteins through an indirect mechanism (Hess et al. 2006). However, consistent with our results in murine leukemias, ChIP analysis of human THP1 AML cells expressing a Flag-tagged MLL-AF9 also showed strong occupancy of the fusion protein on the *Myb* promoter (Supplemental Fig. 7D). Besides *Myb* itself, several of its known transcriptional targets were among the most down-regulated genes following MLL-AF9 with-

drawal (Fig. 1E; Supplemental Fig. 8; Ramsay and Gonda 2008), suggesting that Myb-dependent transcriptional programs may play a pivotal role in addiction to MLL-AF9.

Myb is required for the maintenance of leukemia

c-MYB was first identified as a proto-oncogene via its association with oncogenic retroviruses and as a target for insertional mutagenesis in mice (Klempnauer and Bishop 1984; Shen-Ong et al. 1986). More recent studies indicate that Myb is the target of somatic mutations and translocations in human cancers, and thus is an important human oncogene (Clappier et al. 2007; Persson et al. 2009). Functionally, Myb is a member of the basic helix–loop–helix family of transcription factors (Nomura et al. 1988), an important transcriptional regulator in normal stem and progenitor cell compartments (Emambokus et al. 2003; Malaterre et al. 2007), and is also a component of the LSC signature described above (Somervaille et al. 2009). Moreover, Myb inhibition is known to prevent the initiation of leukemogenesis by MLL-ENL (Hess et al. 2006) and has anti-proliferative effects in some leukemia cells (Luger et al. 2002; Somervaille et al. 2009). However, the role of Myb in leukemia maintenance has not been well characterized, in part because systems to efficiently inhibit gene expression in established disease *in vivo* were not available.

To study the role of Myb and other genes in leukemia maintenance, we took advantage of new methods to regulate gene expression using tet-responsive mir30-based shRNAs. We generated a “Tet-on RNAi-competent” model of MLL-AF9;Nras^{G12D} AML that expresses an improved reverse tTA (rtTA3) linked to MLL-AF9, as well as Nras^{G12D}-linked Luciferase to facilitate leukemia monitoring *in vivo* (Fig. 2A; Zuber et al. 2011a). These leukemia cells were then transduced with a new Tet-regulatable dual-color shRNA expression vector (TRMPV-Neo) (Zuber et al. 2011a). In this system, expression of the Venus fluorescent protein reports integration of the retrovirus, while dsRed fluorescence tracks expression of the shRNA, enabling precise fluorescent tracking and isolation of shRNA-expressing cells as the Venus⁺dsRed⁺ population.

In competitive proliferation assays, TRMPV-Neo-transduced leukemia cells were mixed with uninfected leukemia cells and treated with dox to induce shRNA expression, and the percentage of shRNA-expressing (Venus⁺dsRed⁺) cells was monitored over time using flow cytometry. As expected, TRMPV-Neo-transduced leukemia cells expressing a neutral shRNA (targeting Renilla luciferase, Ren) were maintained in the cell population; in contrast, potent Myb shRNAs were rapidly depleted relative to non-shRNA-expressing cells (dsRed⁻) (Fig. 2B,C). These results mirrored the effects of suppressing replication protein A3 (Rpa3), an essential DNA replication factor required for proliferation (McJunkin et al. 2011; Zuber et al. 2011a). In contrast to the Rpa3 shRNAs, Myb shRNAs had no effect on immortalized rtTA-expressing mouse embryonic fibroblasts (RRT-MEFs) (Fig. 2B; Fellmann et al. 2011), demonstrating that Myb shRNAs are not generally toxic.

To extend these observations to human cancer cells, we evaluated the effects of Myb inhibition in 10 human

leukemia cell lines. Suppression of Myb by two independent shRNAs impaired proliferation of all four MLL fusion-expressing cell lines tested, two of which coexpress MLL-AF9 with oncogenic Ras, as well as two other AML lines without MLL fusion proteins (Fig. 2D,E; Supplemental Fig. 9A). In four of six leukemia lines without MLL aberrations, Myb suppression had only minimal or no effect on cell proliferation, and these differences in sensitivity did not correlate with Myb expression levels (Supplemental Fig. 9B). Together, these results indicate that the sensitivity to Myb inhibition extends to human leukemia cells, particularly, but not exclusively, to MLL-rearranged contexts.

Myb suppression is tolerated in normal hematopoiesis

If Myb mediates cellular addiction to MLL-AF9, then myeloid leukemia cells should be more sensitive to Myb inhibition than their normal counterparts. To investigate the effects of RNAi-mediated Myb suppression in normal hematopoietic cells, we established a two-color competitive reconstitution assay in which HSPCs transduced with either a GFP-tagged experimental shRNA or an mCherry-tagged neutral internal control shRNA (Ren) are monitored for their ability to reconstitute the hematopoietic system of lethally irradiated recipient mice (Fig. 3A). As expected, 6 wk after transplantation, donor-derived bone marrow contained GFP- and mCherry-positive shRen-expressing cells, while cells expressing a GFP-tagged shRNA targeting Rpa3 were completely depleted (Fig. 3B,C).

In contrast, cells expressing Myb shRNAs were able to efficiently reconstitute recipient mice (Fig. 3B,C) even though Myb was suppressed to similar levels as in MLL-AF9;Nras^{G12D} leukemia cells (Fig. 3D). These results differ from the consequences of complete Myb ablation observed in knockout mice (Mucenski et al. 1991; Lieu and Reddy 2009) and suggest that hypomorphic states produced by shRNA-mediated Myb suppression are better tolerated. Lineage analysis revealed that Myb inhibition does not impede normal erythropoiesis and myelopoiesis but, consistent with previous reports (Bender et al. 2004; Fahl et al. 2009), impairs normal lymphopoiesis (Supplemental Fig. 10). Interestingly, one of the shRNAs (shMyb.2652) showed enrichment in myeloid and progenitor compartments (Supplemental Fig. 10), which, together with a previous study (Emambokus et al. 2003), suggests that hypomorphic states of Myb induce expansion rather than depletion in the myeloid compartment. Of note, cells with Myb suppression are capable of supporting long-term myeloid reconstitution, and animals remained healthy and disease-free for >12 wk (data not shown). These results indicate that the consequences of suppressing Myb are dosage- and lineage-dependent and can result in highly cancer-specific sensitivities in the myeloid lineage. They also establish a rapid and general assay for exploring the impact of gene function during normal hematopoiesis.

*Suppression of Myb eradicates AML *in vivo**

To explore the sensitivity to Myb inhibition in established AML *in vivo*, we transplanted a series of Tet-on-competent MLL-AF9;Nras^{G12D}, TRMPV-Neo-transduced leukemias

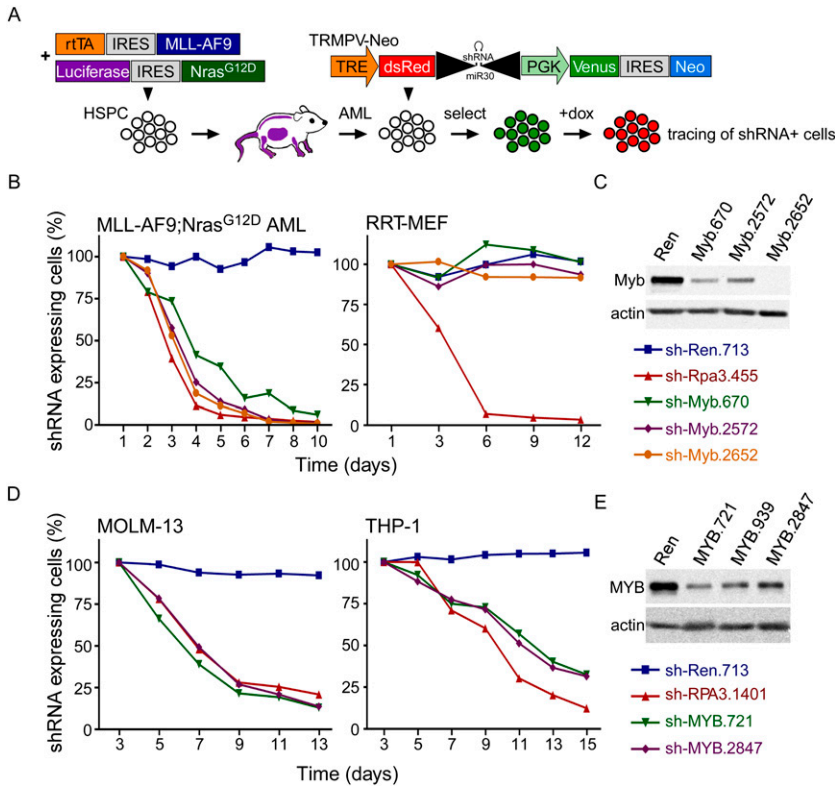


Figure 2. Expression of Myb is required for leukemia maintenance. (A) Schematic of the generation of Tet-on RNAi-competent MLL-AF9;Nras^{G12D} AML and negative selection RNAi studies using the TRMPV system. Competitive proliferation assays in indicated mouse (B) and human (D) cell types. Graphs represent the percentage of shRNA-expressing (Venus⁺dsRed⁺) cells over time, normalized to initial measurement 1–2 d after dox treatment. (C) Immunoblot for Myb in Tet-on-competent MLL-AF9;Nras^{G12D} leukemias transduced with indicated TRMPV-Neo-shRNAs, drug-selected and treated with dox for 3 d, and sorted for shRNA-expressing (Venus⁺dsRed⁺) cells to exclude cells that escape shRNA induction. (E) Immunoblot for MYB in Tet-on-competent human AML cell lines, dox-treated and sorted as described in C.

harboring shRNAs targeting Myb, the Ras effector Braf (another putative target in our model), or Ren, and induced shRNA expression with dox following leukemia detection in bioluminescence imaging (Supplemental Fig. 11A). Even using mixed populations of transduced cells, Myb suppression resulted in a delay in disease progression (Supplemental Fig. 11A) and a significant survival benefit ($P < 0.005$, Supplemental Fig. 11B). At terminal disease stage, bone marrow of mice transplanted with shRen and shBraf-transduced leukemia was completely infiltrated with shRNA-expressing cells (Venus⁺dsRed⁺), while leukemia cells expressing Myb-shRNAs were entirely gone (Supplemental Fig. 11C,D). Thus, in mice transplanted with shMyb-transduced MLL-AF9;Nras^{G12D} leukemia, progressive disease resulted from the outgrowth of cells that failed to induce Myb shRNA expression (dsRed⁻).

Even more dramatic results were observed when the experiment was repeated using single cell-derived clones that uniformly induce shRNA expression. While induction of Ren or Braf shRNAs had no effect on disease course, suppression of Myb induced the clearance of leukemia cells from all infiltrated organs, leading to complete remissions (Fig. 4A–C; Supplemental Fig. 11E). These remissions were durable, and most animals remained disease-free even after discontinuing dox treatment after 40 d (Fig. 4C). Again, those mice that subsequently developed disease harbored leukemia cells that were dsRed-negative, and thus did not express the Myb shRNA and no longer inhibited Myb expression (Supplemental Fig. 11F). This dramatic anti-leukemic response was in contrast to the minimal effects of a combined chemotherapy regimen

mimicking clinical AML therapy (Ara-C + Doxorubicin) (Zuber et al. 2009) in mice bearing the same leukemias (Fig. 4C). Thus, transient inhibition of Myb invariably induces complete remission of MLL-AF9-driven AML in vivo, leading to eradication of an otherwise aggressive and treatment refractory disease.

Myb regulates a LSC-associated program

We next performed another iteration of expression profiling and functional studies to explore the transcriptional networks and effector programs underlying the leukemia-specific dependency on Myb. Immunophenotyping and gene expression analysis revealed that suppression of Myb induces terminal differentiation into mature neutrophils as well as monocytes/macrophages (Fig. 4D; Supplemental Fig. 12), precisely phenocopying effects observed after inactivating the driving MLL-AF9 oncogene (Fig. 1B; Supplemental Fig. 12). The global gene expression changes following Myb suppression were remarkably similar to those observed after MLL-AF9 withdrawal (Spearman correlation, 0.50) (Fig. 5A,B; Supplemental Fig. 13A,B), as determined by a recently developed method to compare global expression signatures (Rank–Rank Hypergeometric Overlap [RRHO]) (Plaisier et al. 2010). Indeed, >40% of significantly altered genes in both signatures overlap (Fig. 5A; Supplemental Fig. 13C), and these influence the same biological processes (Supplemental Tables 3, 4). Such strong effects indicate that Myb is a dominant factor involved in aberrant transcription and impaired differentiation associated with MLL-AF9.

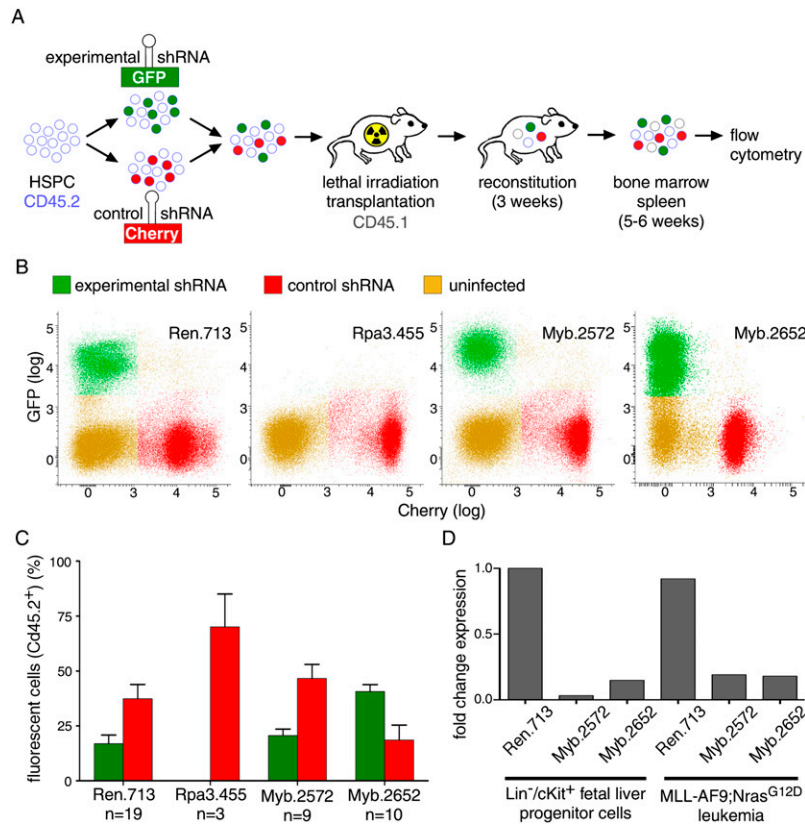


Figure 3. Suppression of Myb is tolerated in normal hematopoiesis. (A) Schematic of dual-color competitive reconstitution assay for evaluating effects of RNAi-mediated gene suppression in normal hematopoiesis. (B) Representative flow cytometry plots of donor-derived (CD45.2⁺) cells in bone marrow 4 wk after transplantation of HSPCs transduced with indicated experimental shRNAs (GFP⁺) and a neutral control shRNA (shRen, Cherry⁺). (C) Percentage of cells expressing indicated experimental shRNA (green) or the neutral control shRNA (red) in total bone marrow 4 wk after transplantation. Values represent the mean \pm SE percentage of fluorescent cells within donor-derived (CD45.1⁺) full bone marrow of multiple mice. (D) qRT-PCR analysis for *Myb* mRNA in shRNA-expressing Tet-on-competent MLL-AF9;Nras^{G12D} leukemias and fetal liver progenitor cells (FLPCs) (Lin⁻, cKit⁺). Values are normalized to actin and plotted relative to FLPc shRen.

The transcriptional module associated with Myb suppression also showed a strong inverse correlation with the LSC signature (NES, 1.5 and -1.7 ; FDR, 0.05 and 0.004) (Fig. 5C; Supplemental Fig. 13D), indicating that Myb is a master regulator of LSC-associated programs. Notably, the Myb-mediated expression profile was also significantly enriched for two gene sets associated with poor prognosis in pediatric and adult AML (NES, 1.8 and 1.5; FDR, <0.01 and 0.05) (Fig. 5D; Yagi et al. 2003; Metzeler et al. 2008), implying that this program also contributes to aggressive disease in humans. Interestingly, some canonical MLL target genes such as *HoxA9* and *Meis1* are excluded from the Myb-specific signatures (Fig. 5A; Supplemental Fig. 13C), indicating that Myb controls only a subset of MLL-AF9 targets and does not predominantly act as a cofactor in MLL complexes as proposed (Jin et al. 2010). These findings also suggest that *HoxA9* and *Meis1*, despite their known role in leukemia initiation by MLL fusion proteins (Zeisig et al. 2004), may be less important for maintaining aberrant self-renewal. Supporting this notion, multiple *HoxA9* and *Meis1* shRNAs did not affect MLL-AF9;Nras^{G12D} AML cells in competitive proliferation assays (Fig. 5E).

Components of the Myb-mediated self-renewal program are required for leukemia maintenance

Several known Myb target genes have been associated with self-renewal in different contexts, and therefore might mediate Myb-dependent aberrant self-renewal in

AML. For example, *Kit*, a target of somatic mutations in human AML (Estey and Dohner 2006), serves as a LSC marker in murine MLL-AF9-induced leukemia (Krivtsov et al. 2006). *Myc* contributes to self-renewal in normal stem cells (Smith et al. 2010) and may play a pivotal role in establishing the LSC signature described above (Kim et al. 2010). *Bcl2* promotes the self-renewal of normal hematopoietic stem cells (Milyavsky et al. 2010), and its pharmacologic inhibition is purported to target LSCs (Konopleva et al. 2006). Finally, in different analyses integrating LSC-, Myb-, and MLL-AF9-associated signatures, we consistently identified the methyltransferase *Smyd2* as a prominent LSC-associated gene suppressed upon Myb and MLL-AF9 withdrawal (Supplemental Tables 5, 6) and subsequently validated it as a Myb-regulated gene in mouse and human AML cells (Fig. 5F,G). Interestingly, *Smyd2* is amplified in esophageal squamous cell carcinoma (Komatsu et al. 2009) and methylates and inhibits the p53 and Rb tumor suppressors (Huang et al. 2006; Saddic et al. 2010), two molecules that negatively regulate self-renewal (Galderisi et al. 2006; Zhao et al. 2010). Indeed, serial replating assays indicate that enforced *Smyd2* expression enhances self-renewal of hematopoietic progenitors, similar to the known effects of *Myc* (Fig. 6A).

Our system enables the rapid evaluation of candidate tumor maintenance genes without the production of new germline transgenic mice, simply by making potent shRNAs targeting a gene of interest and assessing their impact in our transplantable model. To evaluate a possible

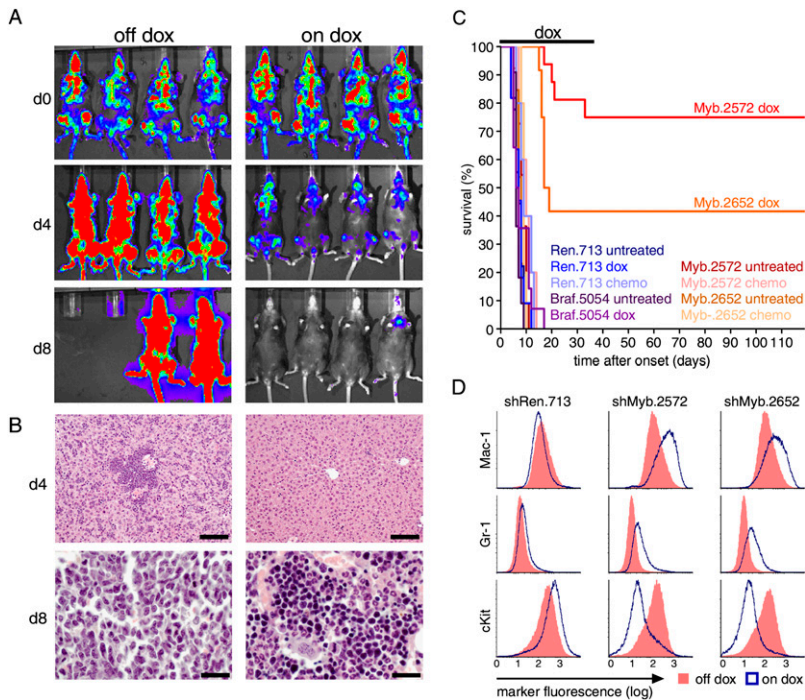


Figure 4. Suppression of Myb eradicates aggressive AML in vivo. (A) Bioluminescent imaging of recipient mice carrying clonal MLL-AF9; Nras^{G12D} AML transduced with Myb shRNAs. Mice were treated with dox at disease onset (d0) and followed up after 4 and 8 d. (B) Liver (*top*) and bone marrow (*bottom*) histology from untreated or dox-treated mice at the indicated time point. Bars: for bone marrow, 20 μ m; for liver, 100 μ m. (C) Kaplan-Meier survival curves of recipient mice untreated, treated with dox, or treated with conventional chemotherapy ($n > 4$). Dox treatment was discontinued after 40 d. (D) Representative immunophenotyping of AML cells transduced with indicated TRMPV-Neo shRNAs with or without 3 d of dox treatment.

role of the Myb-regulated genes described above in maintaining aberrant self-renewal, we analyzed the impact of their TRMPV-shRNA-mediated suppression on AML proliferation and differentiation. Suppression of Kit by multiple potent shRNAs (Supplemental Fig. 14A) had no effect on MLL-AF9;Nras^{G12D} AML (Fig. 6B). In contrast, leukemia cells expressing potent shRNAs targeting either Myc, Smyd2, or Bcl2 (Supplemental Fig. 14B–D) underwent terminal myeloid differentiation and were depleted from the population over time (Fig. 6B,D). Inhibition of Myc, but not Smyd2 or Bcl2, also impeded proliferation of RRT-MEF (Fig. 6C), indicating that strong RNAi-mediated suppression of Myc confers general anti-proliferative effects.

We next transplanted drug-selected TRMPV-Neo-transduced MLL-AF9;Nras^{G12D} leukemias, induced shRNA expression, and evaluated the bone marrow for the presence of shRNA-expressing cells at terminal disease stage (Fig. 6E,F). Suppression of Myc, Bcl2, or Smyd2 led to the depletion of leukemia cells in vivo, although in no case were the effects as dramatic or complete as observed when targeting Myb. We also performed cDNA rescue experiments using a modified competition assay in which the relative frequency of shMyb-expressing cells is monitored over time in cells that ectopically express different cDNAs (Supplemental Fig. 15A). Consistent with RNAi-based phenotypes, overexpression of Myc, Bcl2, or Smyd2 delayed Myb-dependent depletion to varying degrees, but was unable to fully rescue the inhibitory and pro-differentiation effects of suppressing Myb (Fig. 6G; Supplemental Fig. 15B). While we cannot rule out that the less-dramatic effects observed after suppressing Myc, Smyd2, or Bcl2 are due to differences in shRNA knockdown efficiency, the inability of cDNAs encoding these genes to completely rescue the effects of Myb suppression suggests that none

of them can completely replicate the effects of Myb. Instead, these results indicate that Myb exerts its central role in maintaining aberrant self-renewal through a coordinated program involving Myc, Bcl2, Smyd2, and, undoubtedly, additional target genes.

Discussion

Our study integrates mouse cancer models, transcriptional profiling, and inducible RNAi to systematically select and test candidate mediators of oncogene addiction in vivo. Building on a well-characterized and validated model, we implemented new and generalizable assays, enabling an unprecedented level of in vivo analysis of genetic dependencies in normal and cancer cells. These advances include a new Tet-on-inducible shRNA expression system optimized for target suppression in established tumors, as well as a new assay involving fluorescent-coupled shRNAs to study the consequences of suppressing the same genes in normal hematopoiesis. By combining these approaches, we identified Myb as a central mediator of oncogene addiction in AML and show that its suppression eradicates aggressive leukemia in vivo without impacting normal myelopoiesis. In addition, we identify key Myb effectors that coordinate this program, some of which are amenable to small molecule inhibition. Similar approaches can be used to systematically identify and validate tumor maintenance genes in other cancer models.

By comparing the expression profiles of AML cells produced by Myb suppression with those following MLL-AF9 withdrawal, we see that Myb is the dominant factor contributing to aberrant gene expression in MLL-AF9-expressing cells—with >40% overlap in differentially

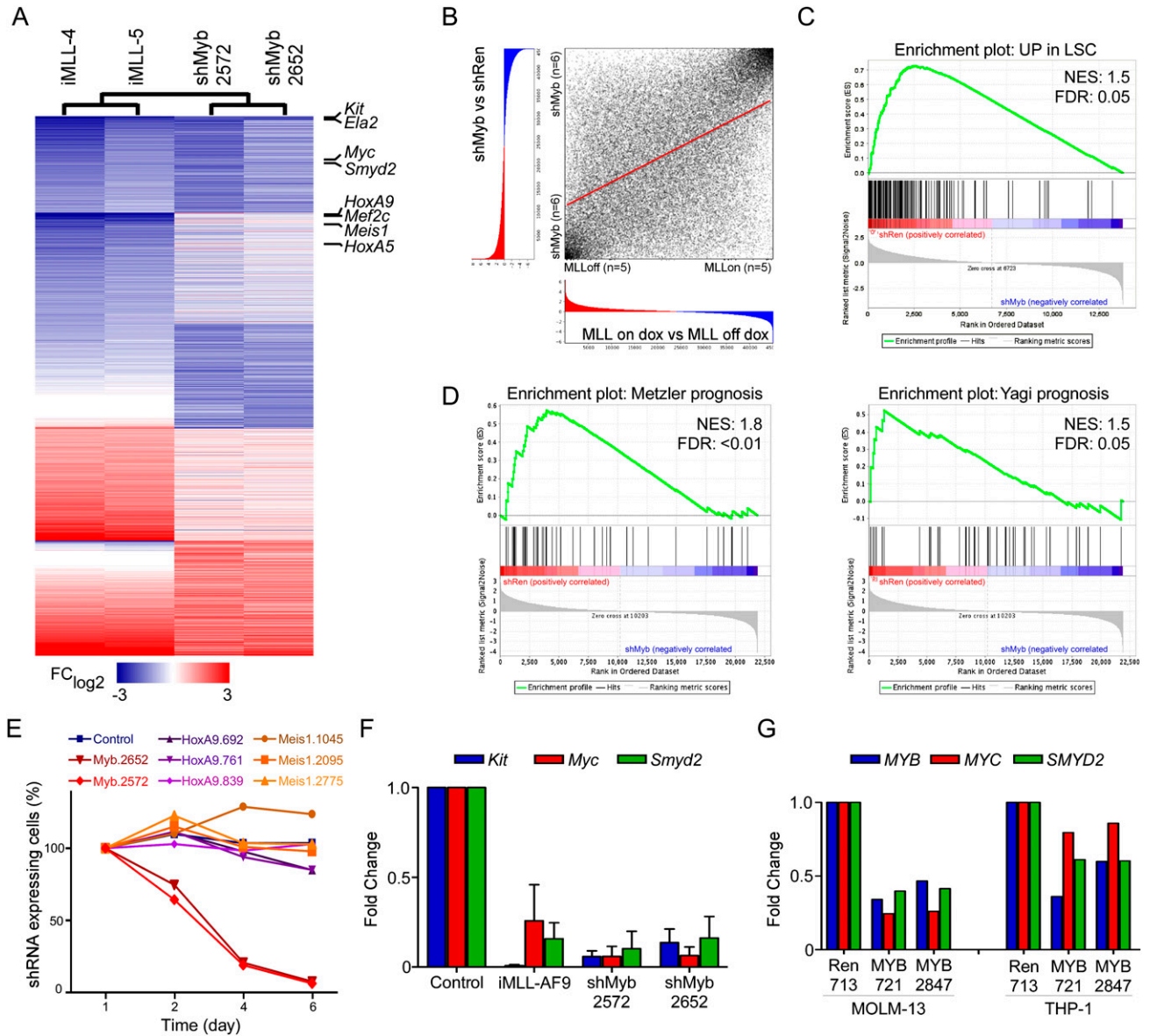


Figure 5. Myb regulates a gene expression program overlapping with MLL-AF9 and associated self-renewal. (A) Heat map of fold change in gene expression for two representative Tet-off-inducible MLL-AF9;Ras leukemia cell lines (on dox relative to off dox) and Tet-on-competent MLL-AF9;Ras leukemia transduced with the indicated Myb shRNAs (shMyb relative to shRen) for all genes with >1.5-fold change in either comparison. (B) Overlap of MLL-AF9 and Myb-regulated gene expression signatures. Using the RRHO approach, genes were rank-ordered according to their differential expression between the indicated subclasses. Spearman rank correlation coefficient = 0.50 ($P < 0.001$). (C) GSEA plot evaluating LSC-associated genes (Somerville et al. 2009) after expression of shRNAs targeting Myb (two independent shRNAs, $n = 3$) or controls (shRen or empty vector, $n = 3$). (D) GSEA plots evaluating expression of signatures associated with poor prognosis in AML patients in two different studies (Yagi et al. 2003; Metzler et al. 2008) with or without suppression of Myb. (E) Competitive proliferation assay in MLL-AF9;Nras^{G12D} leukemia. The graph represents the percentage of shRNA-expressing (Venus⁺dsRed⁺) cells over time, normalized to initial measurement after 1 d of dox treatment. (F) qRT-PCR analysis for Kit, Myc, and Smyd2 expression in Tet-off-inducible MLL-AF9;Ras leukemia treated with dox for 6 d (relative to off dox) and shRNA-expressing Tet-on-competent MLL-AF9;Nras^{G12D} leukemia transduced with the indicated shRNA, treated with dox for 3 d, and sorted for shRNA-expressing (Venus⁺dsRed⁺) cells (relative to shRen). (G) qRT-PCR analysis of MYB, MYC, and SMYD2 in human AML cell lines expressing the indicated shRNAs. Cells were treated with dox for 4 d and sorted for shRNA-expressing (Venus⁺dsRed⁺) cells prior to RNA extraction.

expressed genes. While down-regulation of Myb is required during hematopoietic differentiation (Emambokus et al. 2003), MLL-AF9 apparently “hijacks” Myb to enforce a program of aberrant self-renewal via transcriptional modules linked to LSC potential and poor prognosis in human

AML. Moreover, while this Myb-dependent program is highly enriched for genes contained within the LSC signature, it excludes some canonical MLL targets such as HoxA9 and Meis1 that are known to contribute to leukemogenesis (Ayton and Cleary 2003; Wong et al. 2007).

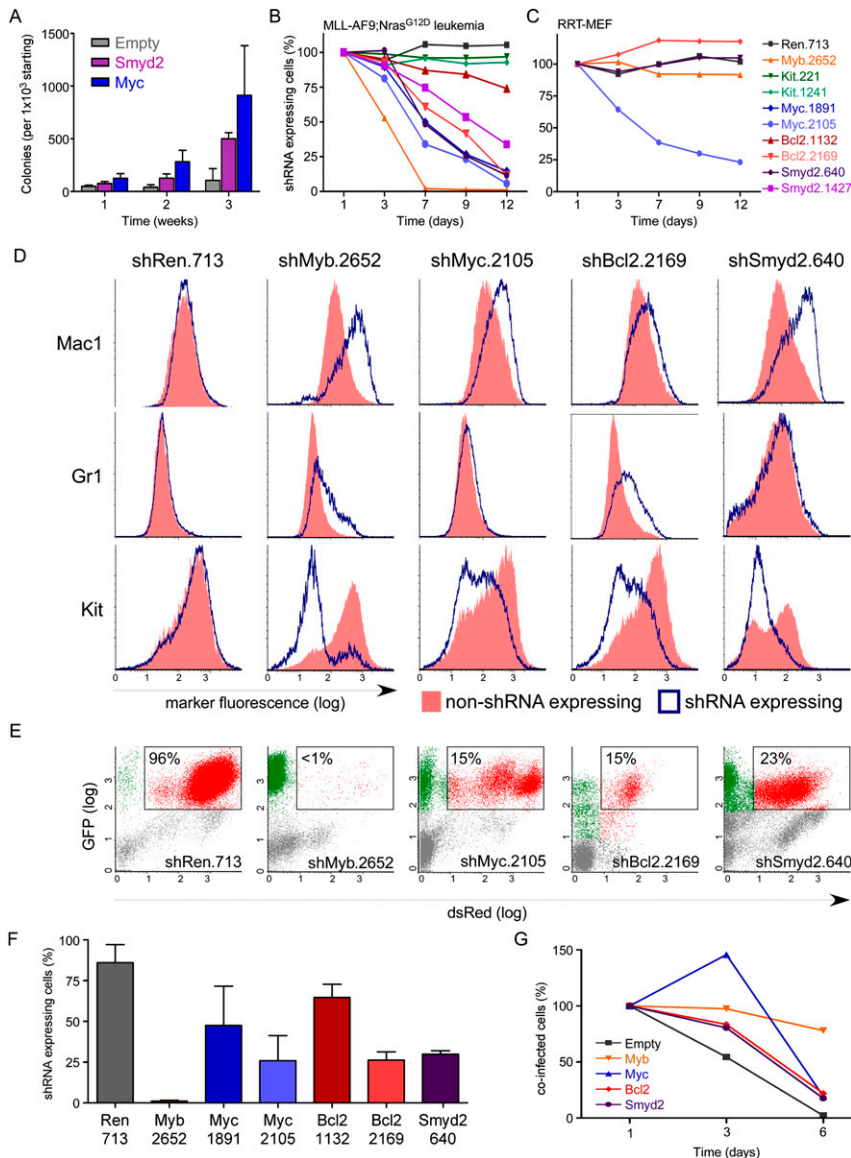


Figure 6. Suppression of Myb-regulated genes partially phenocopies effects of Myb and MLL-AF9 withdrawal. (A) Serial replating assay of fetal liver cells transduced with the indicated cDNA expression vector. Bar graphs represent the number of GFP⁺ colonies normalized to the number seeded in Methocult. Values are the mean \pm SEM of three independent experiments. Competitive proliferation assays in MLL-AF9;Nras^{G12D} leukemia (B) or immortalized RRT-MEFs (C). (D) Representative immunophenotyping of MLL-AF9;Nras^{G12D} leukemia cells transduced with the indicated TRMPV-Neo shRNAs 5 d after dox treatment, separated by shRNA-expressing (blue) and non-shRNA-expressing (red) cells within each sample. (E) Representative flow cytometry plots of donor-derived (CD45.2⁺) bone marrow cells in terminally diseased dox-treated mice. The percentage of shRNA-expressing cells (Venus⁺dsRed⁺) is indicated. (F) Percentage of shRNA-expressing cells in the bone marrow of moribund dox-treated mice in E. Values represent the mean \pm SE ($n > 4$) percentage of Venus⁺dsRed⁺ cells among all donor-derived (CD45.2⁺) cells. (G) Competitive proliferation assay of MLL-AF9;Nras^{G12D} leukemia cells cotransduced with TRN-shMyb.2572 and the indicated cDNA (tagged with GFP). See Supplemental Figure 15A for a schematic of the assay. Shown are the percentages of cells coexpressing the cDNA and shMyb.2572 (dsRed⁺GFP⁺) over time, normalized to the initial measurement after 1 d of dox treatment to induce shMyb expression.

Interestingly, the LSC signature has recently been linked to Myc activity (Kim et al. 2010), which is a direct target of Myb (Evans et al. 1990) and depends on Myb expression. Accordingly, our data suggest that Myb can act as a dominant regulator of the LSC module in AML.

Previous studies suggest that Myb might act downstream from HoxA9 and Meis1 in transformation induced by MLL-AF9 or act more directly in MLL-mediated transcription as a component of the MLL-AF9 complex (Hess et al. 2006; Jin et al. 2010). Our finding that MLL-AF9 occupies the Myb promoter as strongly as some of its canonical targets raises the possibility that MLL-AF9 directly induces Myb transcription. While it remains to be determined which mechanisms predominate, the results are consistent with the possibility that Myb acts as part of a mutually reinforcing transcription program of self-renewal, as has been described for pluripotency factors in embryonic stem cells (Pan and Thomson 2007; Young

2011). In principle, such a situation could create a positive amplification loop; consequently, transient and partial Myb suppression collapses the LSC transcription module and eradicates aggressive AML without compromising normal myelopoiesis.

In addition to identifying mechanisms of oncogene addiction, our results provide compelling *in vivo* validation of Myb as a therapeutic target. We show that partial and transient suppression of Myb using RNAi precisely phenocopies MLL-AF9 withdrawal and eradicates aggressive AML *in vivo* by restoring a program of terminal myeloid differentiation. Suppression of Myb to levels similar to those that eliminate leukemia cells does not prevent normal myelopoiesis, indicating distinct requirements for Myb in cancer and normal cells and leading to a highly cancer-specific sensitivity. Indeed, Myb suppression using RNAi enhances myelopoiesis while impairing lymphopoiesis, which is consistent with studies using a Myb hypomorph

mouse (Emambokus et al. 2003) and highlights the potential of RNAi to identify hypomorphic phenotypes of relevance to understanding human disease. Certainly, the impact of Myb inhibition on normal lymphopoiesis may present some “on-target” toxicities, but as transient Myb inhibition is sufficient to eradicate leukemia, such effects may prove manageable.

While the leukemia-specific dependency on Myb identified using a MLL-AF9;Nras^{G12D}-expressing mouse model extends to human AML, this addiction program may not be strictly genotype-specific. For example, some cell lines without MLL fusion proteins were also sensitive to Myb suppression, suggesting that other lesions may engage the same Myb-dependent self-renewal program. Regardless, the remarkable sensitivity of leukemia cells expressing MLL fusion proteins to Myb inhibition may be unique, as we have evaluated hundreds of other potential AML maintenance genes using similar methods and, to date, none has as potent effects as Myb (Zuber et al. 2011b; J Zuber, CR Vakoc, J Shi, E Wang, C Chen, and SW Lowe, unpubl.). The potency of these effects is further underscored by the generally poor response of patients harboring MLL translocations to conventional therapy in the clinic.

Although targeting of Myb itself has proven elusive (Ratajczak et al. 1992; Luger et al. 2002), several key Myb effectors such as Bcl2 and Smyd2 are established or potential drug targets (Chonghaile and Letai 2008; Spannhoff et al. 2009). Still, our RNAi-based studies as well as preliminary drug trials using the BH3-mimetic ABT-737 (S Glaser and A Strasser, pers. comm.; data not shown) suggest that targeting Myb downstream effectors individually will not be as potent as inhibiting Myb. Nonetheless, improved drugs or adroit drug combinations hold promise for more effective and less toxic therapies. Moreover, in a parallel study, we identified the bromodomain protein Brd4 as a promising therapeutic target in AML (Zuber et al. 2011b). The dramatic responses to the Brd4 inhibitor JQ1 are in large part due to a role of Brd4 in sustaining Myc expression. Remarkably, expression changes following Brd4 suppression using RNAi or a small molecule inhibitor largely overlap with those seen after suppressing Myb (Supplemental Fig. 16), suggesting that Brd4 and Myb engage a common transcriptional network and providing another pharmacological entry point for targeting aberrant self-renewal programs. Together, our results demonstrate that suppressing a single mediator of aberrant self-renewal can re-establish pre-existing cell fate programs and eliminate aggressive AML, providing hope that targeting these pathways will lead to more effective therapies for leukemia and, potentially, other cancers.

Previous efforts to target mediators of oncogene addiction have predominantly focused on components of oncogenic signaling pathways; however, in many cases, the plasticity of these programs limits drug efficacy or facilitates drug resistance and, through various feedback mechanisms, can even enhance pro-oncogenic signaling (Poulikakos et al. 2010; Pratilas and Solit 2010). Our observation that suppressing a single key mediator of aberrant self-renewal can reverse this program, re-establish terminal differen-

tiation, and thereby eliminate leukemia suggests that pre-existing cell fate programs remain hard-wired in cancer, and raises hope that agents inhibiting these programs will be less prone to resistance mechanisms. Such agents may also be effective at targeting so-called cancer stem cells, which require aberrant self-renewal programs to propagate the tumor and are considered more resistant to conventional therapies. More broadly, our study exemplifies how the combination of genetically defined mouse models and novel Tet-on RNAi technology can be used to systematically identify and evaluate new therapeutic targets for otherwise refractory cancers.

Materials and methods

Retroviral constructs

All constructs were derived from either the Murine Stem Cell Virus backbone (MSCV, Clontech) or the pQCXIX self-inactivating retroviral vector backbone (pSIN, Clontech) using standard cloning techniques. TRMPV vectors are available through Addgene (#27990, #27991, #27993, and #27996). shRNAs were designed using the Biopredsi algorithm and subcloned as described (for sequences, see Supplemental Table 7; Zuber et al. 2011a). See the Supplemental Material for more detailed vector information.

Generation and monitoring of AML

Primary leukemias were generated by cotransduction of retroviral constructs into fetal liver cells and transplantation into syngeneic recipient mice as described previously (Zuber et al. 2009). Leukemia cells were harvested from bone marrow and spleen and cultured in RPMI-1640 + 10%FBS. Peripheral blood smears and bone marrow cytopins were stained with Wright-Giemsa (Sigma); tissue specimens were fixed in 10% formalin and stained with Hematoxylin and eosin (Sigma). For immunostaining, cells were stained with specified fluorophore-conjugated antibodies (BioLegend) and analyzed by flow cytometry (LSRII, BD BioSciences). Mice were monitored and treated as described (Zuber et al. 2011a). Statistical evaluation of overall survival was based on the log-rank (Mantel-Cox) test for comparison of the Kaplan-Meier event time format. More detailed methods are provided in the Supplemental Material.

Gene expression analysis

Cultured and treated primary leukemia cells were sorted for shRNA-expressing cells when necessary using an Aria-II (BD Biosciences). Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using TaqMan reverse transcriptase reaction (Applied Biosystems). qRT-PCR analysis was performed on an iCycler mounted with an iQ5 multicolor real-time PCR detection system (Bio-Rad). All signals were quantified using the $\Delta\Delta C_t$ method and normalized to β -actin levels. Microarray experiments were performed on Mouse Genome 430A 2.0 arrays (Affymetrix). All raw microarray data files are available from the Gene Expression Omnibus (GSE30747). For Western blot analysis, the following antibodies were used: c-Myb (clone1-1, 1:500; Millipore), Flag M2 (#1804, 1:1000; Sigma), Brf1 (F-7, 1:200; Santa Cruz Biotechnology), Bcl2 (BCL/10C4, 1:1000; BioLegend), Smyd2 (#4251, Cell Signalling), and actin (HRP-conjugated, 1:10000; Sigma). ChIP was performed as described (Steger et al. 2008). Detailed methods are provided in the Supplemental Material.

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