Csnk1a1 inhibition has p53-dependent therapeutic efficacy in acute myeloid leukemia

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Despite extensive insights into the underlying genetics and biology of acute myeloid leukemia (AML), overall survival remains poor and new therapies are needed. We found that *casein kinase 1 α* (*Csnk1a1*), a serine-threonine kinase, is essential for AML cell survival in vivo. Normal hematopoietic stem and progenitor cells (HSPCs) were relatively less affected by shRNA-mediated knockdown of *Csnk1a1*. To identify downstream mediators of Csnk1a1 critical for leukemia cells, we performed an in vivo pooled shRNA screen and gene expression profiling. We found that Csnk1a1 knockdown results in decreased Rps6 phosphorylation, increased p53 activity, and myeloid differentiation. Consistent with these observations, p53-null leukemias were insensitive to Csnk1a1 knockdown. We further evaluated whether D4476, a casein kinase 1 inhibitor, would exhibit selective antileukemic effects. Treatment of leukemia stem cells (LSCs) with D4476 showed highly selective killing of LSCs over normal HSPCs. In summary, these findings demonstrate that Csnk1a1 inhibition causes reduced Rps6 phosphorylation and activation of p53, resulting in selective elimination of leukemia cells, revealing Csnk1a1 as a potential therapeutic target for the treatment of AML.

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Abbreviations used: AML, acute myeloid leukemia; GMP, granulocyte macrophage progenitor; HSPC, hematopoietic stem and progenitor cell; LSC, leukemia stem cell; LSK, $Lin^{-}Sca^{+}Kit^{+}$.

Although tremendous progress has been made in identifying recurrent somatic mutations that drive acute myeloid leukemia (AML) pathogenesis, many of these genetic lesions cause a loss of protein function and do not suggest clear therapeutic opportunities (Welch et al., 2012). Genetic screens have emerged as powerful approaches to identify vulnerabilities and therapeutic opportunities in cancer cells (Luo et al., 2009; Zuber et al., 2011). In a recent in vivo shRNA screen (Miller et al., 2013) using primary mouse MLL-AF9 leukemia cells (Krivtsov et al., 2006; Somervaille and Cleary, 2006), we found that cells expressing *Csnk1a1* shRNAs were powerfully depleted over time, indicating that Csnk1a1 is required for the survival of MLL-AF9 leukemia-propagating cells and may represent a novel therapeutic target for AML.

Csnk1a1, a serine-threonine kinase, is a central regulator of multiple pathways that are critical for normal and malignant stem cell biology, including the β catenin and p53 pathways (Liu et al., 2002; Wang et al., 2010; Zhao et al., 2010; Elyada et al., 2011; Luis et al., 2011). More precisely, Csnk1a1 suppression increases β catenin and p53 activity (Liu et al., 2002; Chen et al., 2005; Huart et al., 2009). Csnk1a1 plays a critical role in the biology of diffuse large B cell lymphoma by regulating NF-κB signaling (Bidère et al., 2009), but the role of Csnk1a1 in leukemia has not been examined. We therefore sought to explore the role of Csnk1a1 in AML.

RESULTS AND DISCUSSION

In a previous pooled in vivo shRNA screen in primary mouse MLL-AF9 leukemia cells, *Csnk1a1* scored at the top of our list of genes

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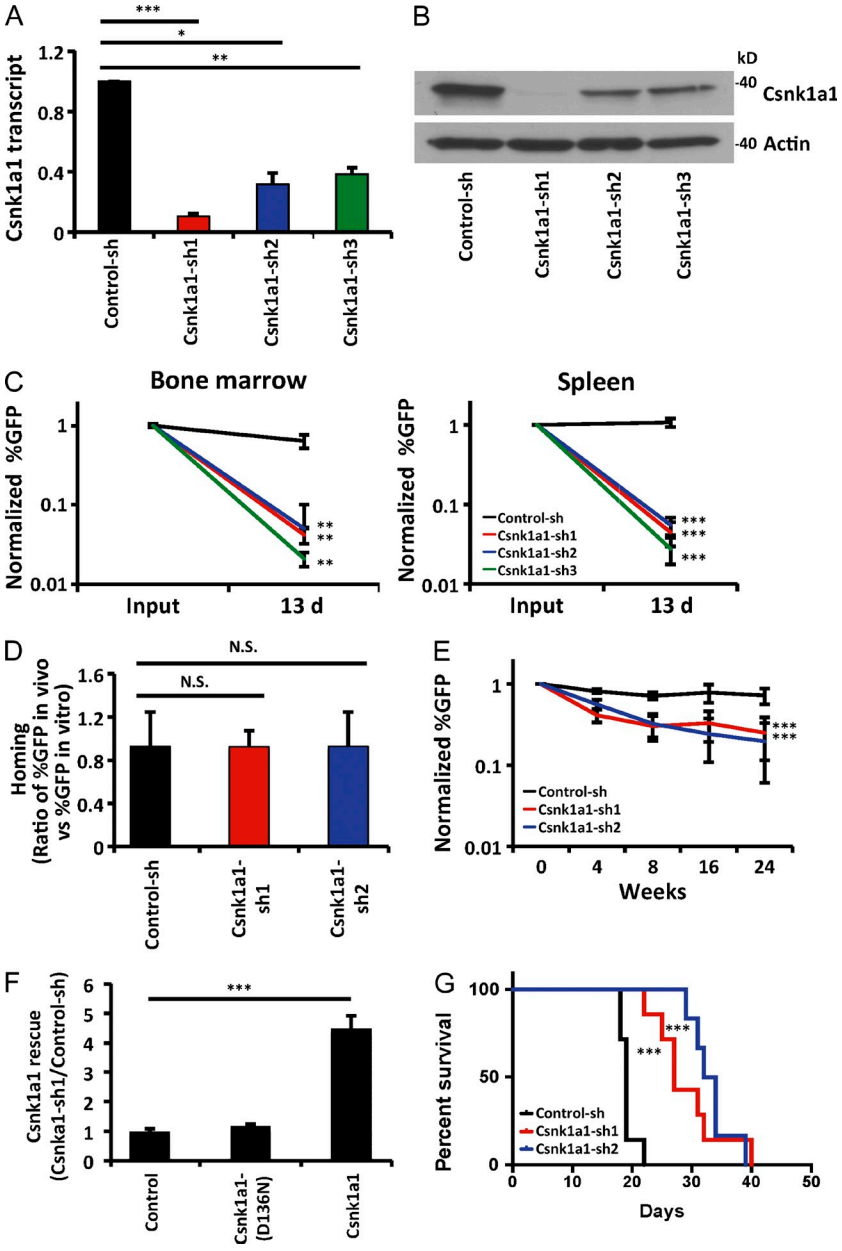

Figure 1. Silencing of Csnk1a1 selectively depletes mouse leukemia cells in a kinase-dependent manner. (A) TaqMan-PCR was used to assess *Csnk1a1* transcript levels in Csnk1a1 shRNA 1–3 (Csnk1a1-sh1-3)–expressing mouse leukemia cells. *Csnk1a1* levels are presented as the percentage of transcript remaining relative to the luciferase control shRNA (Control-sh)–expressing cells ($n = 3$). (B) Western blot demonstrating Csnk1a1 protein levels in shRNA-expressing leukemia cells together with Actin as endogenous control. (C) c-Kit^{high} dsRed^{+} leukemia cells were transduced with lentiviral vectors coexpressing GFP and shRNAs targeting *Csnk1a1* and then transplanted via the tail vein into wild-type mice. The percentage of GFP^{+} cells within dsRed^{+} population was assessed before injection (input) and in mice BM and spleen 13 d after transplant. Data are presented as the GFP percentage normalized to the input measurement (three mice per group; each mouse was injected with leukemia cells from independent transductions). (D) BM homing experiment in which the percentage of GFP^{+} leukemia cells in the BM 24 h after transplantation was compared with corresponding in vitro cultured cells (five mice per group; each mouse was injected with leukemia cells from independent transductions). (E) After being transplanted with CD45.2 LSK cells transduced with lentiviral vectors coexpressing *GFP* and shRNAs targeting *Csnk1a1*, recipient mice were assessed for the percentage of GFP^{+} cells within the CD45.2 population in the peripheral blood for 24 wk (at least five mice per group; each mouse injected with leukemia cells from independent transductions). The GFP percentage was normalized to the input measurement. (F) Csnk1a1 rescue experiment in which leukemia cells were transduced with both puromycin-resistant lentiviral shRNA vectors and retroviral vectors coexpressing *GFP* only (control), shRNA-resistant *Csnk1a1* wild-type cDNA (Csnk1a1), or a kinase-dead *Csnk1a1* cDNA (Csnk1a1(D136N)). Csnk1a1 rescue is presented as the ratio between the percentage of GFP-positive cells within Csnk1a1-sh1– versus Control-sh–expressing cells at day 6 after lentiviral transduction. (G) 100,000 sorted GFP^{+} leukemia cells carrying shRNAs were transplanted into wild-type recipient mice. Survival of the mice is shown in Kaplan–Meier curves (at least six mice per group; each mouse was injected with leukemia cells from independent transductions). Means and SD are shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

that are essential for the leukemia cells, although this finding was not validated (Miller et al., 2013). We therefore first tested the knockdown efficiency and antileukemia efficacy of individual *Csnk1a1* shRNAs. We identified three distinct shRNAs that decreased the expression of *Csnk1a1* mRNA and protein by >60% (Fig. 1, A and B). To examine whether Csnk1a1 is essential for primary mouse MLL-AF9 leukemia cells in vivo, we used lentiviruses that coexpress individual shRNAs with GFP to transduce MLL-AF9 AML cells that were enriched for leukemia stem cells (LSCs) by sorting for c-Kit^{high} cells (Krivtsov et al., 2006). After transplantation of the leukemia cells into sublethally irradiated recipient mice, we followed the percentage of GFP^{+} leukemia cells over time. Based on findings from three independent shRNAs targeting *Csnk1a1*, we found that leukemia cells with *Csnk1a1* knockdown were depleted 15- to 40-fold over a 2-wk period in both the spleen and BM, compared with cells expressing control shRNA (Fig. 1 C), without any defect in BM homing (Fig. 1 D).

To examine the effect of the same shRNAs on normal hematopoiesis, we expressed the *Csnk1a1* shRNAs in Lin^{-}Sca^{+}Kit^{+} (LSK) hematopoietic stem and progenitor cells (HSPCs) and transplanted the cells into recipient mice. In contrast to the profound depletion observed in leukemia cells after

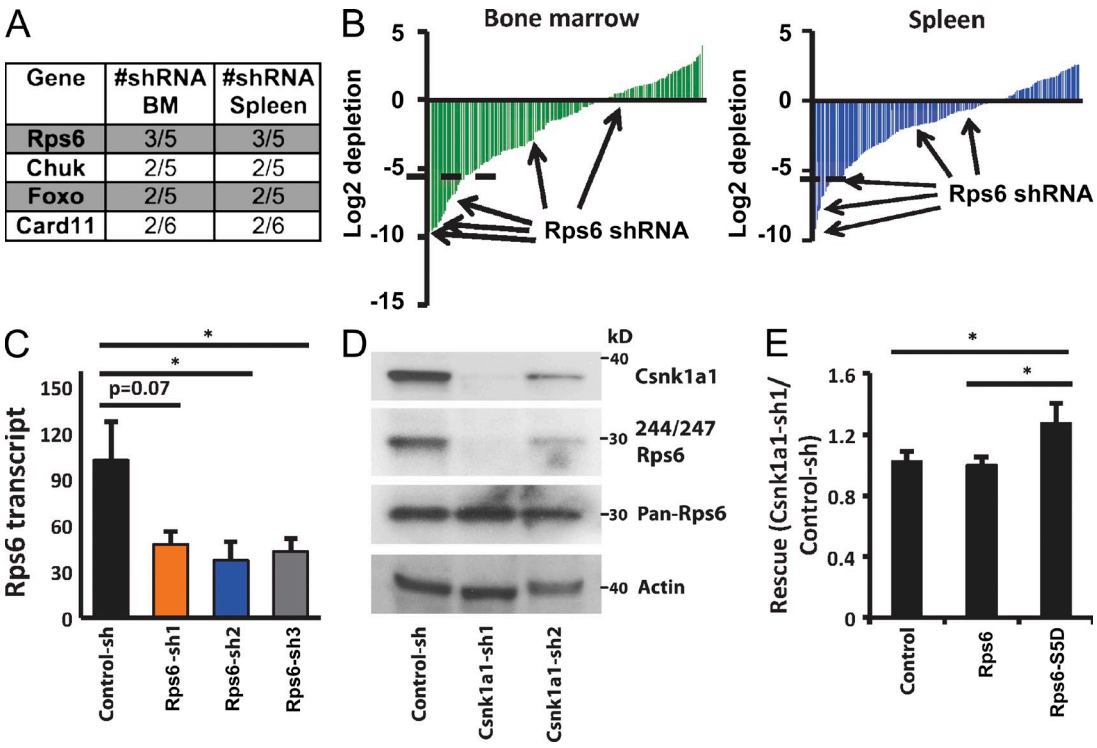

Figure 2. Csnk1a1 suppression leads to dramatic reduction in Rps6 phosphorylation. (A) Genes targeted by two or more shRNAs that depleted leukemia cells >40-fold in the BM and spleen ($n = 5$). (B) Graphs showing fold change of all shRNAs included in the screen. *Rps6* was identified as a strong hit with three separate shRNAs (Rps6-sh1–3) all depleting leukemia cells >40-fold in the BM and spleen after 14 d in vivo relative to input (same experiment as in A). Dashed lines depict 40-fold depletion. (C) TaqMan-PCR was used to assess *Rps6* transcript levels in shRNA-expressing leukemia cells. *Rps6* levels are presented as the percentage of transcript remaining relative to Control-sh–expressing cells ($n = 3$). (D) Western blot demonstrating Rps6 protein levels and Rps6 phosphorylation in shRNA-expressing mouse leukemia cells together with Actin as endogenous control. (E) Rps6 rescue experiment in which leukemia cells were transduced with both puromycin-resistant lentiviral shRNA vectors and retroviral vectors coexpressing *GFP* only (Control), Rps6 cDNA, or a Rps6-S5D (phosphomimetic mutant) cDNA ($n = 3$). Rps6 rescue is presented as the ratio between the percentage of GFP-positive cells within Csnk1a1-sh1 versus Control-sh–expressing cells. Means and SD are shown (*, $P < 0.05$).

just 2 wk, normal HSPCs expressing *Csnk1a1* shRNAs were only depleted three- to fourfold over 24 wk in a long-term reconstitution assay. These findings demonstrate that *Csnk1a1* shRNAs preferentially deplete leukemia cells (Fig. 1 E).

To address the possibility that our results were caused by off-target effects of the shRNAs, we generated an shRNA-resistant *Csnk1a1* cDNA in which multiple silent mutations were introduced at the shRNA-binding sites. Coexpression of this shRNA-resistant cDNA successfully rescued the depletion of leukemia cells expressing *Csnk1a1* shRNAs (Fig. 1 F).

Because inhibition of kinase activity is the most straightforward way to target Csnk1a1 pharmacologically, we tested whether the kinase function of Csnk1a1 is essential for leukemia cells. We introduced a known mutation that inactivates the kinase domain (Csnk1a1(D136N); Peters et al., 1999; Davidson et al., 2005; Bidère et al., 2009) into the shRNA-resistant *Csnk1a1* cDNA. We found that the kinase-dead cDNA did not rescue the effect of the *Csnk1a1* shRNAs, demonstrating that Csnk1a1 kinase function is essential for leukemia cells (Fig. 1 F).

We next examined whether suppression of *Csnk1a1* could increase the survival of leukemic mice. Leukemia cells that express GFP, and therefore *Csnk1a1* or control shRNAs expressed from the same lentiviral vector, were purified and transplanted into recipient mice. Mice injected with leukemia cells expressing *Csnk1a1* shRNAs lived significantly longer than control mice (Fig. 1 G). In aggregate, these data demonstrate that the MLL-AF9 leukemia cells are dependent on Csnk1a1 kinase function for growth and survival.

To determine the molecular mechanisms underlying the critical role of Csnk1a1 in leukemia, we performed an in vivo pooled shRNA screen on genes implicated as direct or indirect downstream targets of Csnk1a1 signaling. In this screen, 28 genes targeted by 149 shRNAs and 8 control shRNAs lacking endogenous target sequences were included (Table S1). The top hit in this screen was *ribosomal protein S6* (*Rps6*; Fig. 2 A). Suppression of *Rps6* with three independent shRNAs resulted in a dramatic depletion of leukemia cells in both spleen and BM over a 2-wk period (Fig. 2 B). All three hairpins targeting *Rps6* showed successful knockdown of the *Rps6* transcript (Fig. 2 C).

RPS6 activity is regulated by phosphorylation by CSNK1A1, which phosphorylates serine residue 247, enhancing the phosphorylation of upstream sites (Hutchinson et al., 2011), as well as by the ribosomal S6 kinases 1 and 2 (Magnuson et al., 2012).

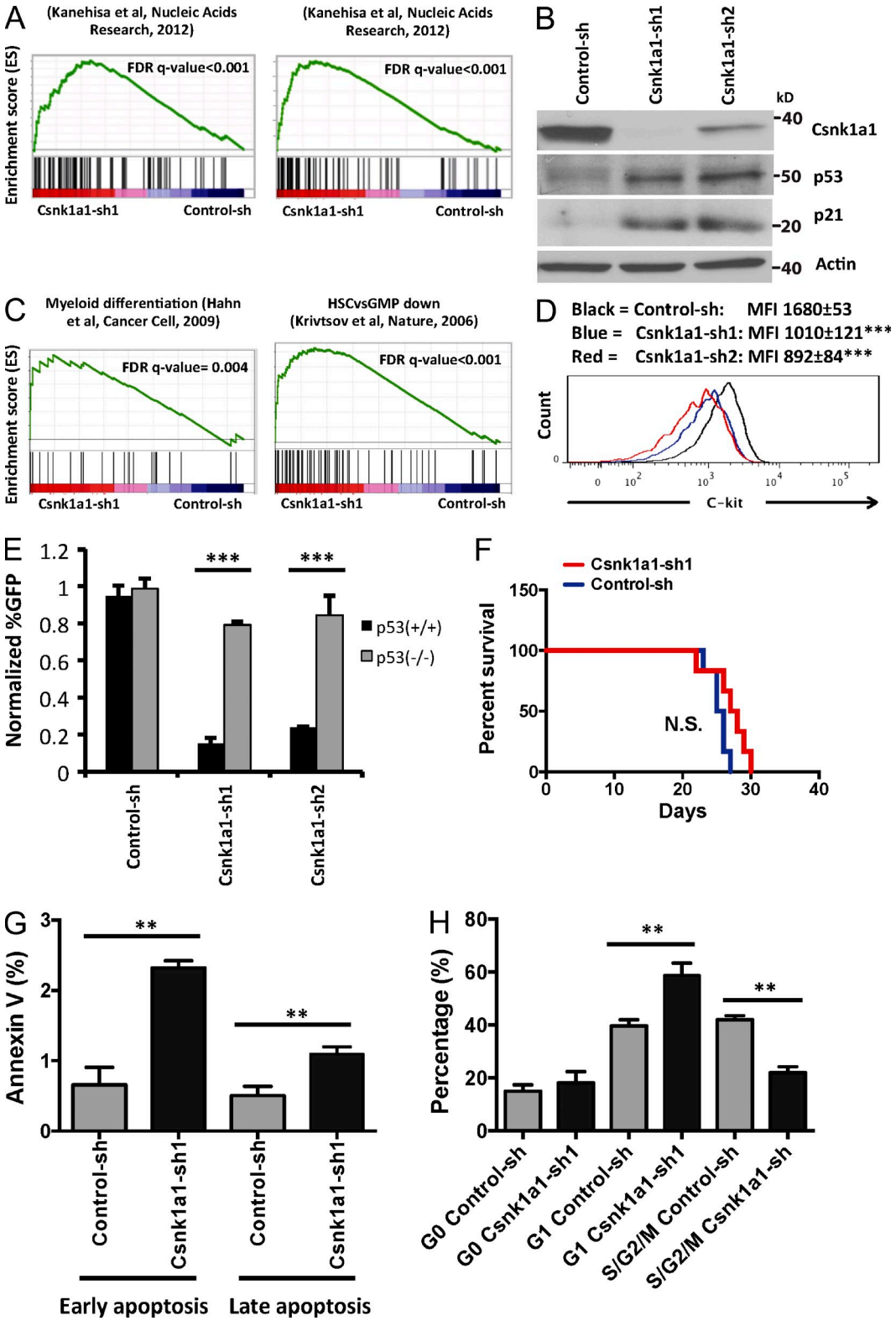

Figure 3. Csnk1a1 suppression activates a p53 response. (A) In leukemia cells with shRNA-mediated suppression of *Csnk1a1*, p53 signatures are enriched by GSEA. (B) Western blot demonstrating induced p53 and p21 expression after shRNA-mediated silencing of *Csnk1a1* in leukemia cells. Actin was used as an endogenous control. (C) In leukemia cells with *Csnk1a1* suppressed, up-regulated genes were enriched in a myeloid differentiation signature (left), and down-regulated genes were enriched in a hematopoietic stem cell (HSC) versus GMP down signature, i.e., a signature of genes down-regulated in GMPs relative to HSCs. (D) Histogram depicting c-Kit expression 72 h after transductions with shRNA-expressing lentiviral vectors. (E) MLL-AF9

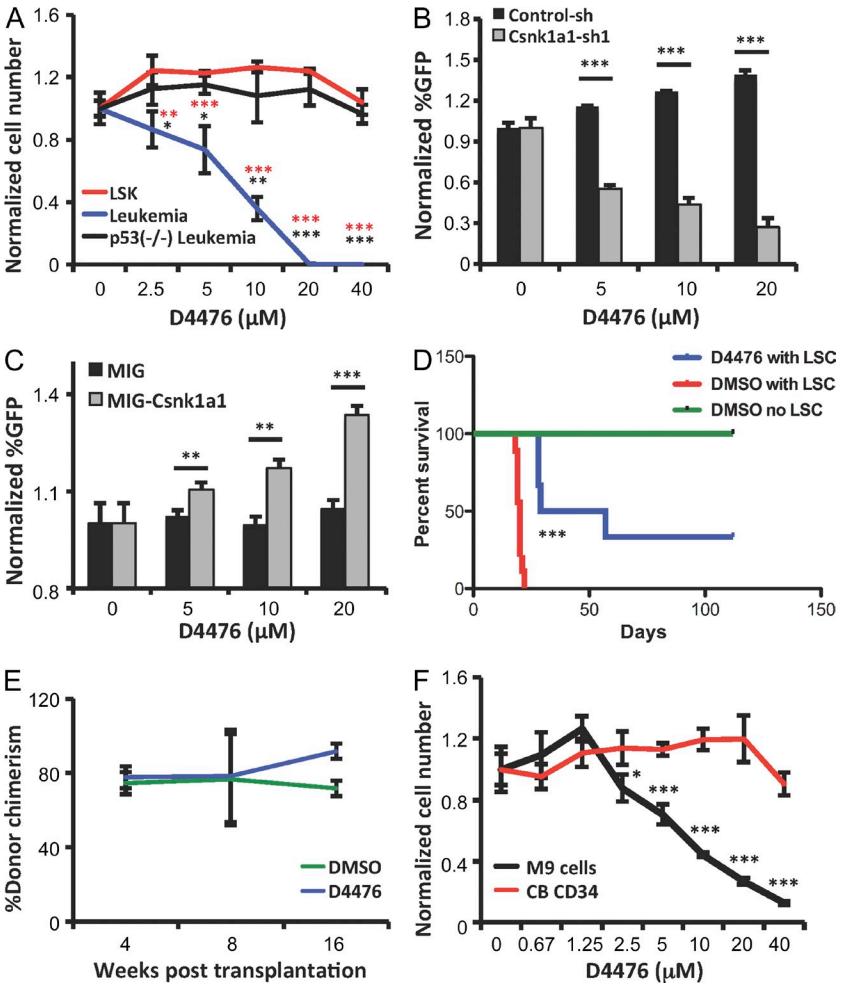

Figure 4. Small molecule inhibition of Csnk1a1 selectively kills leukemia cells. (A) Normal LSK, *Tp53*$^{-/-}$, and *Tp53*$^{+/+}$ c-Kit^{high} leukemia cells were treated in medium supplemented with increasing doses of D4476, and cell number was assessed after 4 d. Data are presented as the cell count normalized to the DMSO control ($n = 3$). Black asterisks depict significance between *Tp53*$^{+/+}$ leukemia and *Tp53*$^{-/-}$ leukemia comparison, and red asterisks depict significance between LSK and *Tp53*$^{+/+}$ leukemia cell comparison. (B) c-Kit^{high} leukemia cells were transduced with vectors coexpressing shRNAs and *GFP* and then treated with D4476 for 4 d. Data are presented as the percentage of GFP$^+$ cells after treatment normalized to the DMSO control ($n = 3$). (C) c-Kit^{high} leukemia cells were transduced with a vector coexpressing *Csnk1a1* and *GFP* and then treated with D4476 for 4 d. Data are presented as the percentage of GFP$^+$ cells after treatment normalized to the DMSO control ($n = 3$). (D) 10,000 LSK cells (CD45.1$^+$) and 10,000 c-Kit^{high} leukemia cells (dsRed$^+$) were co-cultured on GFP-positive mouse mesenchymal stroma cells for 48 h in the presence of 40 μM D4476 (6 mice; each mouse was injected with cells from independent drug treatments) or DMSO control (10 mice; each mouse was injected with cells from independent drug treatments) and then transplanted into CD45.2$^+$ recipients along with CD45.1$^+$CD45.2$^+$ competitor cells. The green line depicts the group with LSK-treated cells only, i.e., no leukemia cells were added to the wells (four mice; each mouse was injected with cells from independent drug treatments). Survival is shown in Kaplan–Meier curves. (E) Repopulation of donor cells in peripheral blood was determined as the percentage of donor (CD45.1$^+$CD45.2$^-$) cells among the donor and competitor cells after subtracting the leukemia (dsRed$^+$) cell population (same experiment as in D). The green line depicts the group with LSK-treated cells only, and the blue line depicts the group with D4476-treated mixed c-Kit^{high} leukemia and LSK cells. Flow cytometric analysis was performed at 4, 8, and 16 wk. (F) M9 leukemia cells and cord blood (CB) CD34$^+$ cells were cultured in medium supplemented with increasing doses of D4476, and cell number was assessed after 4 d. Data are presented as the cell count normalized to DMSO control ($n = 3$). Means and SD are shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

We therefore tested whether shRNA-mediated suppression of *Csnk1a1* affects the phosphorylation of Rps6 in leukemia cells. Total Rps6 protein levels were not changed after Csnk1a1 suppression, but Rps6 phosphorylation was powerfully decreased in leukemia cells (Fig. 2 D). Overexpression of a phosphomimetic mutant Rps6 cDNA (Rps6^{S5D}) that activates Rps6 partially rescued the proliferative defect induced by *Csnk1a1* knockdown (Fig. 2 E).

To obtain further insight into the molecular consequences of *Csnk1a1* suppression in leukemia, we performed gene expression profiling. We found that *Csnk1a1* knockdown significantly increased expression of a p53 signature (Fig. 3 A; Kanehisa et al., 2012). Western blot analysis confirmed increased p53 and p21 expression (Fig. 3 B). In addition, we found that knockdown of *Csnk1a1* caused changes in gene expression enriched in myeloid cell differentiation signatures (Fig. 3 C; Krivtsov et al., 2006; Hahn et al., 2009). Consistent with this result, we found decreased c-Kit expression on the surface of leukemia cells expressing *Csnk1a1* shRNAs (Fig. 3 D).

Because decreased Rps6 phosphorylation causes activation of p53 (Khalaileh et al., 2013) and Csnk1a1 may also regulate p53 through other mechanisms, we hypothesized that p53 is a

leukemia cells in *Tp53*$^{-/-}$ and *Tp53*$^{+/+}$ background were transduced with vectors coexpressing *GFP* and Control-sh or Csnk1a1-sh1. The GFP percentage was measured 2 (input) and 7 d after transduction. Data are presented as the GFP percentage normalized to the input measurement ($n = 3$). (F) 200,000 sorted GFP$^+$ *Tp53*$^{-/-}$ leukemia cells carrying Control-sh or Csnk1a1-sh1 were transplanted into wild-type recipient mice. Survival of the mice is shown in Kaplan–Meier curves (six mice per group; each mouse was injected with leukemia cells from independent transductions). (G) Leukemia cells carrying shRNAs were analyzed for early (Annexin V$^+$ Hoechst 33342$^-$) and late (Annexin V$^+$ Hoechst 33342$^+$) apoptotic cells ($n = 3$). (H) Cell cycle analysis on leukemia cells carrying shRNAs ($n = 3$). Means and SD are shown (**, $P < 0.01$ ***, $P < 0.001$;).

critical mediator of the effects of Csnk1a1 suppression in leukemia. To test this hypothesis, we generated MLL-AF9 leukemia in a *Tp53*$^{-/-}$ background. We found that *Tp53*$^{-/-}$ leukemia cells were resistant to the effects of *Csnk1a1* silencing, both in vitro and in vivo (Fig. 3, E and F). These findings demonstrate that p53 function is essential for the antileukemic effects of *Csnk1a1* knockdown. Consistent with p53 activation, *Csnk1a1* knockdown induced apoptosis and cell cycle arrest (Fig. 3, G and H; and Figs. S1 and S2).

To explore the potential therapeutic efficacy of targeting Csnk1a1, we tested whether D4476, a selective small molecule inhibitor of casein kinase 1 (Rena et al., 2004; Anastassiadis et al., 2011), would exhibit antileukemic effects. Treatment of primary c-Kit^{high} leukemia cells in vitro with D4476 killed the leukemia cells with an EC_{50} of 6.5 µM, whereas concentrations up to 40 µM had minimal effects on normal HSPCs and *Tp53*$^{-/-}$ leukemia cells under similar culture conditions (Fig. 4 A).

To address whether D4476 killed c-Kit^{high} leukemia cells by inhibiting Csnk1a1, we decreased expression of *Csnk1a1* using shRNAs and demonstrated that these cells were sensitized to D4476 in a dose-dependent manner (Fig. 4 B). Conversely, overexpression of Csnk1a1 decreased the sensitivity of leukemia cells to D4476 treatment, indicating that D4476 kills leukemia cells via on-target inhibition of Csnk1a1 (Fig. 4 C).

To examine the effect of D4476 on normal and malignant stem cells, we mixed 10,000 LSK cells with 10,000 c-Kit^{high} leukemia cells and treated them together, ex vivo, with D4476 for 48 h and then injected the cells into lethally irradiated recipient mice. We found that the mice receiving DMSO control–treated cells died rapidly from aggressive leukemia. In contrast, mice receiving D4476-treated cells survived significantly longer, and one third of these mice remained disease free for the 16-wk duration of the experiment (Fig. 4 D). Additionally, the normal HSPCs were unaffected by the inhibitor and thus contributed robustly to donor cell chimerism, indicating a selective toxicity toward the LSCs over the normal HSPCs (Fig. 4 E).

We next validated that CSNK1A1 is essential for human leukemia cells. Using MLL-ENL–transformed cord blood cells, M9 cells (Barabé et al., 2007), we found that expression of *CSNK1A1* shRNAs successfully decreased *CSNK1A1* expression and caused rapid depletion of leukemia cells (not depicted). In agreement with the mouse data, M9 cells were sensitive to D4476, whereas human cord blood CD34^{+} cells were insensitive to treatment (Fig. 4 F).

Our experiments suggest that Csnk1a1 is essential for mouse and human AML cells and that the Csnk1a1 dependence requires the Csnk1a1 kinase domain. Partial depletion of Csnk1a1 activity via shRNA knockdown or small molecule inhibition resulted in a pronounced therapeutic window with a selective loss of the leukemia population. Consistent with previous studies linking Csnk1a1 to suppression of p53 (Chen et al., 2005; Huart et al., 2009; Elyada et al., 2011), we found that lack of Rps6 phosphorylation and induction of p53 is a major consequence of Csnk1a1 loss in leukemia cells leading to enforced cell differentiation. In addition, increased expression of β catenin, downstream of Csnk1a1 inhibition, has also been shown to induce p53 (Damalas et al., 2001), and Csnk1a1 can regulate p53 by binding to MDM2 (Huart et al., 2009). Our findings suggest that targeting of Csnk1a1 provides a potential approach to the therapeutic activation of p53 in AML, a disorder predominantly associated with nonmutated *Tp53* (Patel et al., 2012).

MATERIALS AND METHODS

Leukemia model. All animal experiments were conducted with an Institutional Animal Care and Use Committee–approved protocol. Mouse MLL-AF9 leukemias in a dsRed transgenic background (6051; The Jackson Laboratory) were generated as previously described (Miller et al., 2013). To enrich for LSCs, femurs and tibias from leukemic mice were first harvested, crushed, and filtered, and red blood cells were lysed using lysis buffer (BD). Then BM cells were stained with APC-conjugated anti–c-Kit (clone 2B8; eBioscience) and dsRed^{+}c-Kit^{high} cells (20% high) were sorted (FACSAria II; BD). MLL-AF9–driven *Tp53*$^{-/-}$ leukemias were generated from c-Kit–enriched BM cells from *Tp53*$^{-/-}$ mice (002101; The Jackson Laboratory).

Cell cultures and transductions. Sorted c-Kit^{high} leukemia cells were plated in RPMI-1640 (Sigma-Aldrich) medium supplemented with 1% L-glutamine, 1% penicillin and streptomycin, and 10% fetal bovine serum (Invitrogen) in 10 ng/ml mouse IL-3 (mIL-3; PeproTech). For isolation of LSK cells, c-Kit–positive cells were first isolated using MACS beads (Miltenyi Biotec), followed by antibody staining and sorting for lineage^{low}, Sca-1^{+}, and c-Kit^{+} cells. For transductions of leukemia cells, the medium was also supplemented with 10 ng/ml mIL-6, 20 ng/ml mouse stem cell factor (mSCF), and 5 µg/ml polybrene (Sigma-Aldrich). Virus-containing medium was mixed with cells, and spinfection was performed at 2,500 rpm for 90 min at 37°C. Lentiviral vectors were pseudotyped with VSV-G envelope, whereas oncoretroviral vectors were pseudotyped with ecotropic envelope and produced using standard protocols. For LSK cells, sorted cells were put in StemSpan Serum-Free Expansion Medium (STEMCELL Technologies) supplemented with 100 ng/ml mouse thrombopoietin (mTpo; PeproTech) and 100 ng/ml mSCF (PeproTech). LSK cells were prestimulated for 24 h and transduced using spinoculation as described above. In the Csnk1a1 and Rps6 rescue experiments, leukemia cells were first transduced with the retroviral MIG vectors and then after 24 h subjected to an additional transduction with lentiviral vectors expressing shRNAs, and then 24 h later, puromycin was added to the medium at 0.5 µg/ml. M9 cells were cultured in alfa Mem (Life Technologies) medium supplemented with 10% human plasma (Sigma-Aldrich), 20% FBS, 1% L-glutamine, 1% penicillin and streptomycin, 100 ng/ml SCF (PeproTech), 10 ng/ml IL-3 (PeproTech), 5 ng/ml IL-7 (PeproTech), 5 ng/ml Flt3L (PeproTech), 50 µg/ml Gentamicin (Life Technologies), and 1 µg/ml heparin (Sigma-Aldrich).

Drug treatment using D4476. For inhibition of Csnk1a1, the small molecule D4476 (Tocris Bioscience) was used. D4476 was added to leukemia cells cultured in 96-well plates (5,000 cells per well) in medium supplemented with 10 ng/ml mIL-3. A D4476 dose titration was performed by adding 2.5 µM, 5 µM, 10 µM, 20 µM, and 40 µM D4476 to cell cultures in a final DMSO percentage of 0.4%. Similarly, D4476 was added to LSK cells cultured in SFEM medium supplemented with mTpo and mScf. The number of cells after 96 h of treatment was assessed with CountBright absolute counting beads (Invitrogen) using flow cytometry.

Microarray analysis. Global gene expression profiling of c-Kit^{high} leukemia cells was performed on sorted GFP^{+} cells, harvested 72 h after transduction with lentiviral vectors coexpressing shRNAs and GFP. RNA was extracted using TRIzol (Invitrogen), and samples were analyzed using Mouse WG-6 version 2.0 Expression BeadChip (Illumina). Enrichment of gene expression signatures was analyzed using Gene Set Enrichment Analysis (GSEA; Subramanian et al., 2005). Raw data and normalized gene expression data

are available in the Gene Expression Omnibus database under accession no. GSE52928. For detailed description of the bioinformatic analysis, see Miller et al. (2013).

Real-time PCR. Real-time PCR was performed using an ABI Prism 7500 analyzer (Applied Biosystems) using standard protocols. In brief, cells were harvested in RLT buffer, and RNA was isolated using the RNeasy kit (QIAGEN). cDNA was synthesized using SuperScript III reverse transcription enzyme (Invitrogen), followed by real-time TaqMan PCR in 384-well format. TaqMan probes used were Csnk1a1 (Mm00521593_m1), Cdkn1a (p21; Mm04205640_g1), Rps6 (Mm02342456_g1), and Gapdh (Mm99999915_g1). The relative quantity of specific transcripts was calculated using the ΔΔCT method and normalized to Gapdh as endogenous control. Triplicate samples were used.

Western blots. Western blots were performed according to standard protocols using the Criterion gel system (Bio-Rad Laboratories). In brief, cells were resuspended in IP lysis buffer (Thermo Fisher Scientific) with protease and phosphatase inhibitors and frozen. Frozen lysate was thawed and spun down, resuspended in Laemmli sample buffer, boiled at 100°C, and loaded to gradient gels (Criterion Tris-HCl gel, 8–16%). Protein transfers were done on Immobilon polyvinyl difluoride membranes. Primary antibodies used were anti-Csnk1a1 (C-19; Santa Cruz Biotechnology, Inc.), anti-p21 (Cell Signaling Technology), anti-Rps6 (Santa Cruz Biotechnology, Inc.) anti–P-Rps6 Ser244/247 (Abcam), anti–β-Actin (Cell Signaling Technology), and anti-p53 (CM-5; Leica). Stripping of membranes was performed with Restore Western blot stripping buffer (Thermo Fisher Scientific), followed by restaining with primary antibodies.

shRNA screening. The shRNA screen was performed essentially as previously described (Miller et al., 2013). In brief, 2×10^6 sorted c-Kit^{high} leukemia cells were isolated and immediately transduced with a pool of lentiviral shRNAs using spinfection as described above. Five replicates with separate transductions were performed. After 24-h incubation at 37°C, one third of the cells were harvested and frozen in PBS. Remaining cells were injected into sublethally irradiated recipient mice. 14 d after transplantation, mice were sacrificed, BM and spleens were harvested, and cells were put in PBS and frozen before genomic DNA isolation, PCR, and Illumina sequencing. The depletion of individual shRNAs was compared with the pre-injection time point and with the shRNA controls.

Viral vector cloning. Lentiviral pLKO.1 vectors coexpressing shRNAs and a puromycin resistance gene were obtained from the RNAi consortium at the Broad Institute (Moffat et al., 2006). The hairpin sequences were shuttled into a GFP expression backbone (pLKO_TRC019) using NdeI and SpeI restriction sites. Murine stem cell virus oncoretroviral vectors coexpressing Csnk1a1-sh1–resistant mouse Csnk1a1 cDNAs (Genscript; gene synthesis) and GFP connected with an internal ribosome entry site were generated. Both MIG-Csnk1a1 and MIG-Csnk1a(D136N) had the nucleotide sequence changed in the Csnk1a1-sh1 complementary region without affecting the amino acid sequence. The Rps6 wild-type cDNA and Rps6^{S5D}, a phosphomimetic mutant cDNA (all five serine phosphorylation sites mutated to aspartate [Hutchinson et al., 2011]) were synthesized at Genscript. The cDNAs were flanked by Not1 and Xho1 sites for convenient cloning into the MIG vector backbone.

Cell cycle analysis. Leukemic granulocyte macrophage progenitors (GMPs; Lin^{low}, Sca-1, c-Kit$^+$, FcγRII^{high}, CD34^{high}; dsRed$^+$), transduced with Control-sh or Csnk1a1-sh1, were harvested 72 h after transduction and washed in PBS. Then, GMPs were resuspended in fixation medium (reagent A, fix and perm kit; Invitrogen) for 10 min at room temperature in the dark. After another washing step, cells were resuspended in the permeabilization medium (reagent B, fix and perm kit; Invitrogen) for 20 min in the presence of the intracellular, directly conjugated antibody (Ki67-APC; 1:20; BioLegend). After washing, cells were stained with Hoechst 33342 (Life Technologies) for 10 min and washed before resuspension in 500 µl PBS. Cells were analyzed using the FACS LSRII.

Apoptosis. Leukemic GMPs were transduced with Control-sh or Csnk1a1-sh1, harvested 72 h later, and washed in PBS. Cells were then washed in 1× binding buffer (BD) and stained in 1× binding buffer in the presence of Annexin V–directly conjugated primary antibody for 20 min at room temperature (Annexin V–APC; BD). After another washing step, cells were stained with Hoechst 33342 according to the manufacturer's instructions and resuspended in flow buffer. Cells were subsequently analyzed using the FACS LSRII.

Homing experiment. Leukemia cells were transduced with Control-sh, Csnk1a1-sh1, or Csnk1a1-sh2 coexpressing GFP and transplanted into sublethally irradiated mice 48 h after transduction. 24 h after transplantations, BM cells were harvested, and the percentage of GFP$^+$ cells was determined using flow cytometric analysis within the dsRed$^+$ cells. The percentage of GFP$^+$ cells in corresponding in vitro cultured cells was measured 72 h after transduction.

Statistical analysis. Kaplan–Meier curves were generated using Prism 5 software (GraphPad Software). In all figures, the mean values and SDs are shown. P-values were calculated using the Student's *t* test or the Mantel–Cox (for Kaplan–Meier curves only) test. Significance is depicted with asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. For further details, see corresponding figure legends.

Ex vivo drug treatment of leukemia and LSK cells on mesenchymal stroma. LSK cells and c-Kit^{high} leukemia cells were mixed and added onto BM mesenchymal stromal cells (BMSCs) derived from actin-GFP mice (000329; The Jackson Laboratory). The mesenchymal stroma cells were prepared by plating RBC-lysed, freshly isolated BM cells in BMSC medium (400 ml α-MEM [STEMCELL Technologies], 20% FBS [HyClone], and 5 ml Pen-Strep [CellGro]). Cells were grown in flasks in a 33°C/5% CO_2 incubator for 10–17 d and split by brief trypsinization (0.25%; CellGro), filtered through a 70-µm filter, and grown another 3–4 d until nearly confluent. The cells were then trypsinized, filtered, and washed, and CD105$^+$ cells were isolated with biotin-conjugated anti–mouse CD105 antibody (eBioscience) and Dynabead M-280 streptavidin-linked magnetic beads (Invitrogen). The CD105$^+$ cell fraction was replated for 2–3 d before being used for short-term experimentation as described above. After 48 h of D4476 treatment, wells were trypsinized and cells were washed before being injected to lethally irradiated CD45.2$^+$ mice together with 1.5×10^6 freshly isolated CD45.1$^+$CD45.2$^+$ splenocytes.

Online supplemental material. Fig. S1 shows the gating strategy for Fig. 3 G. Fig. S2 shows the gating strategy for Fig. 3 H. Table S1 is a gene list showing the number of shRNAs that depleted leukemia cells >40-fold in BM and spleen. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131033/DC1.

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