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

Marcus Järås,1,2 Peter G. Miller,1 Lisa P. Chu,1 Rishi V. Puram,1 Emma C. Fink,1 Rebekka K. Schneider,1 Fatima Al-Shahrour,1 Pablo Peña,2 L. Jordan Breyfogle,1, Kimberly A. Hartwell,1,3 Marie E. McConkey,1 Glenn S. Cowley,3 David E. Root,3 Michael G. Kharas,4,5 Ann Mullally,1 and Benjamin L. Ebert1,3

1Division of Hematology, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115
2Department of Clinical Genetics, Lund University, 22184 Lund, Sweden
3Broad Institute, Cambridge, MA 02142
4Molecular Pharmacology and Chemistry Program, Sloan Kettering Institute; and 5Center for Cell Engineering; Memorial Sloan Kettering Cancer Center, New York, NY 10065

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 1α (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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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 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.

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.

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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 Csk1a1 shRNAs. We identified three distinct shRNAs that decreased the expression of Csk1a1 mRNA and protein by >60% (Fig. 1 A and B). To examine whether Csk1a1 is essential for primary mouse MLL-AF9 leukemia cells in vivo, we used lentiviruses that coexpress individual shRNAs with GFP to transduce MLL-AF9 leukemia cells that were enriched for leukemia stem cells (LSCs) by sorting for c-Kit$^{\text{high}}$ 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 Csk1a1, we found that leukemia cells with Csk1a1 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 Csk1a1 shRNAs in Lin$^{-}\text{Sca}^1\text{Kit}^+$(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 1. Silencing of Csk1a1 selectively depletes mouse leukemia cells in a kinase-dependent manner.](image-url)
 expresses 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). 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.

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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
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 percentage of GFP+ cells after treatment 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.

Figure 4. Small molecule inhibition of Csnk1a1 selectively kills leukemia cells. (A) Normal LSK, \( Tp53^{-/-} \), and \( Tp53^{-/-} \) c-Kit\(^{hi}\) 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\(^{hi}\) 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\(^{hi}\) 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\(^{hi}\) leukemia cells (dsRed\(^{+}\)) were co-cultured on GFP-positive mouse mesenchymal stroma cells for 48 h in the presence of 40 \( \mu M \) 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\(^{-}\) 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 cell competition (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\(^{hi}\) 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
critical mediator of the effects of Csnk1a1 suppression in leukemia. To test this hypothesis, we generated MLL-AF9 leukemia in a Tp53<sup>+/−</sup> background. We found that Tp53<sup>+/−</sup> 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<sup>high</sup> leukemia cells in vitro with D4476 killed the leukemia cells with an EC<sub>50</sub> of 6.5 µM, whereas concentrations up to 40 µM had minimal effects on normal HSPCs and Tp53<sup>+/−</sup> leukemia cells under similar culture conditions (Fig. 4 A).

To address whether D4476 killed c-Kit<sup>high</sup> 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<sup>high</sup> 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<sup>+</sup> 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).
are available in the Gene Expression Omnibus database under accession no. GSE52929. 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 RLIT buffer, and RNA was isolated using the RNeasy kit (QIA-GEN). cDNA was synthesized using SuperScript III reverse transcription enzyme (Invitrogen), followed by real-time TaqMan PCR in 384-well format. TaqMan probes used were Csk1a1 (Mm00521593_m1), Cdkn1a (g21; Mm04205640_g1), Rp6 (Mm02342566_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-Csk1a1 (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 retesting 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-Ki67^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, 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.

**Viral vector cloning.** Lentiviral pLKO.1 vectors coexpressing shRNAs and a puromycin resistance gene were obtained from the RNAi consortium at GMPs; Lin^+^, CD34^+^; dsRed^+^). 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.

**Cell cycle analysis.** Leukemic GMPs were transduced with Control-sh or Csk1a1-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 LSRRI.

**Homing experiment.** Leukemia cells were transduced with Control-sh, Csk1a1-sh1, or Csk1a1-sh2 coexpressing GFP and transplanted into sublethally irradiated mice 48 h after transduction. 24 h after transplants, 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.

**Ex vivo drug treatment of leukemia and LSK cells on mesenchymal stroma.** LSK cells and c-Ki67^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% CO2 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 isolated with biotin-conjugated anti–mouse CD105 antibody (Bioscience) and Dy-nabead 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.

**REFERENCES**

