The small GTPase ARF6 regulates sphingolipid homeostasis and supports proliferation in acute myeloid leukemia

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Abstract

Metabolic dependencies are emerging as promising therapeutic targets in cancer, including acute myeloid leukemia (AML). Several metabolic vulnerabilities have been identified in AML cells, including a requirement for balanced sphingolipid metabolism to maintain survival and proliferation. Here we describe a novel function of the RAS superfamily small GTPase ARF6 in maintaining sphingolipid homeostasis in AML. Genetic depletion of ARF6 inhibited the proliferation of AML cell lines and reduced colony formation of primary AML CD34$^+$ cells. Mechanistically, ARF6 promotes conversion of ceramide to sphingomyelin by enhancing sphingomyelin synthase (SGMS1/2) expression, thereby preventing accumulation of cytotoxic ceramide levels. Accordingly, higher expression of ARF6 and its effectors SGMS1/2 in AML patients' cells correlated with shorter survival in two independent AML cohorts, with ARF6 exhibiting an adverse prognostic effect independent of European LeukemiaNet genetic risk. Small molecule inhibitors of ARF6 suppressed colony formation by primary AML CD34$^+$ cells, but not cord blood CD34$^+$ cells and showed activity in xenograft models. The dependency of AML cells on ARF6 to regulate sphingolipid homeostasis may present a therapeutic opportunity.

Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous hematopoietic neoplasm with poor prognosis. Except for acute promyelocytic leukemia, standard therapy for AML had remained unchanged since the 1970s, consisting of induction chemotherapy followed by additional chemotherapy or allogeneic bone marrow transplant for consolidation. Recently the therapeutic armamentarium has been enriched by several targeted agents, including midostaurin and giltiritinib for patients with *FLT3* internal tandem duplications (*FLT3*-ITD),[1,2] and ivosidenib and enasidenib for patients with isocitrate dehydrogenase 1/2 mutations. The combination of the BCL2 inhibitor venetoclax with 5-azacytidine has improved the survival of older AML patients and is now the standard of care in this population with a poor prognosis.[3,4] In contrast to molecularly based targeted approaches, venetoclax plus 5-azacytidine activity is mostly genotype-agnostic, although efficacy in patients with *TP53* mutations or complex karyotype is lower.[4] Unfortunately, most patients eventually relapse and succumb to AML. Additionally, results in patients treated outside of clinical trials are inferior to those documented in prospective studies, indicating that venetoclax plus 5-azacytidine has mitigated but not solved the problem of refractory AML.
Aberrant metabolism is recognized as one of the cardinal features of cancer.[5] To meet the demands of rapid proliferation, cancer cells rewire metabolism to generate intermediary metabolites such as amino acids and nucleotides, sometimes creating new metabolic bottlenecks compared to their normal counterparts.[5,6] Several metabolic targets are under pre-clinical and clinical investigation in AML, including mitochondrial respiration and glutaminase.[6,7] Most reported metabolic targets in AML are genotype-agnostic.[6,7]

While early work in cancer metabolism focused on shifts in energy metabolism from oxidative phosphorylation to glycolysis in aerobic conditions (known as the Warburg effect), recent work has identified reprogramming of lipid metabolism as an equally important metabolic adaptation.[7] For example, AML cells, but not their normal counterparts, rely on very long chain fatty acid metabolism. Interestingly, fatty acid oxidation is further upregulated upon resistance to venetoclax plus 5-azacytidine.[8]

ADP ribosylation factors (ARF) are members of the RAS superfamily of small GTPases that are activated in a GTP-bound form and regulate vesicular trafficking, cytoskeletal remodeling,[9] and turnover of various cellular components.[10-12] Among the various ARF, ARF6 is recognized as a potential therapeutic target in several human diseases, such as arthritis,[13] diabetic retinopathy, sepsis, and cancer.[14-18] In addition to its role in controlling vesicular trafficking, ARF6 has been shown to regulate the phosphorylation and distribution of the phosphatidylinositide, PIP2, by mediating the activation of phosphatidylinositol kinases.[19,20] Moreover, ARF6 activates phospholipase D which generates phosphatidic acids from phosphatidylcholine. A recent study reported that ARF6-regulated retromer organization influences the distribution of cholesterol.[19]

Ceramide is a sphingolipid generated by *de novo* synthesis or conversion from sphingosine and sphingomyelin.[21,22] Ceramide functions as a pleiotropic signaling lipid that triggers apoptosis and growth arrest in various cell types,[23] including leukemia cells.[23,24] Several mechanisms have been proposed to explain the pro-apoptotic effects of ceramide, including the inactivation of AKT.[23] Since genetic deletion of an ARF6 upstream deactivating protein, Smap1, in hematopoietic cells caused myelodysplasia and AML in mice, we hypothesized that ARF6 may regulate sphingolipid metabolism in AML. Validating this notion, we show that ARF6 promotes proliferation of AML cells by maintaining sphingolipid homeostasis. Our results add to a growing body of data implicating ARF6 in cancer,[14,15,17,18,25] and suggest that targeting ARF6 in AML may hold therapeutic promise.

Methods

Immunoblot and ARF6-GTP-pulldown assay

For immunoblot assays, cells were harvested and lysed in Pierce IP Lysis Buffer (Thermo Scientific) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), and supernatant was collected. Protein concentrations in the lysates were quantified using a Micro BCA Protein Assay Kit (Thermo-Fisher). Equal amounts of proteins from different samples were added to 2x Laemmli buffer and denatured at 90°C for 10 minutes. Samples were loaded onto 10% or 15% sodium dodecylsulfate polyacrylamide gels for electrophoresis. Proteins were transferred onto methanol-activated polyvinylidene fluoride membranes for antibody detection. Specific primary antibodies (ARF6 Rb-mAb, GAPDH Rb-mAb, p-AKT-S473 Rb-mAb, pan-AKT Rb-mAb, p-ERK1/2-T202/Y204 Rb-pAb and ERK1/2 Rb-pAb, all from CST) and species-matching horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch) were used to probe for separated proteins. HRP-labeled protein bands were detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore). Chemiluminescence signals were imaged on a ChemiDoc Touch Imaging System (Bio-Rad).

ARF6-GTP–pulldown assays were performed as previously described.[13] Briefly, AML cells were treated with NAV-2729, A6-4471, or dimethylsulfoxide control overnight. After treatment, the cells were washed with phosphate-buffered saline and pelleted by centrifuge. Cell pellets were frozen at -80°C for less than 1 week before conducting pulldown. For the pulldown assay, the frozen cell pellets were lysed using Assay/Lysis Buffer (Cell Biolabs). Lysates were centrifuged, and supernatants were added to GGA3-conjugated beads (sta-40706; Cell Biolabs) and rotated at 4°C for 60 minutes. Beads were washed in lysis buffer and proteins eluted and denatured in 2× Laemmli buffer prior to immunoblot analysis. A fraction of the cell lysate was retained for measuring total ARF6.

Bioinformatics analysis

For the cohort from The Cancer Genome Atlas (TCGA), patients' survival information as well as gene expression profiles were obtained from the TCGA-NEJM-2013 database[26] of adult *de novo* AML patients using R software and its packages. Patients were separated into groups with higher expression (> median expression) and lower expression (< median expression) based on the median expression of the gene analyzed. The survival difference between these two groups was defined as the difference between the median survival times. Only genes with detectable expression were included in the analysis. Kaplan-Meier survival curves of specific genes and the linear correlation between *ARF6* and *SGMS1/2* expression were visualized using the cBioPortal engine.[27] For the MLL Münchner Leukämielabor cohort, the same analysis was independently performed by Torsten Haferlach, D Med, D Phil. Multivariant analysis was performed using the R package 'survival', with age, sex, and cytogenetic subtypes included in the analysis.

Human samples

AML patients' mononuclear cell samples and cord blood mononuclear cell samples were acquired from the Hematology Biobank at Huntsman Cancer Institute. All primary samples were separated with Ficoll, isolated and cryopreserved. The use of de-identified human samples was approved by the University of Utah Institutional Review Board under protocol number IRB00045880.

Statistics

For comparisons between two groups, if not noted oth-

erwise, a two-tailed Student *t* test was used. When variance was unequal, a Welch *t* test was applied instead. A Mann-Whitney nonparametric test was used if values did not form a normal distribution. One-way analysis of variance was used for comparing multiple groups with one independent variable. Three biological replicates were performed per group unless otherwise noted. For all statistical tests, a *P* value of less than 0.05 was used as the threshold for determining significant differences, indicated by an asterisk (*) in the figures. Standard deviations are provided in the figures.

Results

We first interrogated the function of ARF6 in THP-1 and MV4-11 AML cells by short hairpin (sh)RNA or inducible clustered regularly interspaced short palindromic repeats (CRISPR), using three different shRNA and three different CRISPR guide RNA, respectively, to disrupt ARF6. *ARF6* knockdown (KD) or CRISPR-mediated silencing reduced cell proliferation of both cell lines, evident as an increase in doubling time (*Online Supplementary Figure S1A-C*). We next assessed the effects of *ARF6* KD on colony formation by THP-1 cells, using *shARF6* and shNT (control). We noticed a modest reduction of colony formation (Figure 1A, *Online Supplementary Figure S2*). For validation, we generated a doxycycline-inducible lentiviral green fluorescence protein (GFP)-sh*ARF6* construct. We infected OCI-AML3, and OCI-AML5 AML cell lines with inducible *ARF6* and plated GFP$^+$ cells in semisolid media supplemented with cytokines with and without 100 ng/mL doxycycline. The highly effective sh*ARF6* 68 reduced colony formation by 50-65%, while the less effective sh*ARF6* 05 reduced colony formation by only 10-20% (Figure 1B, C). We next infected CD34$^+$ primary AML cells with sh*ARF6* 68. Colony formation in the presence of 100 ng/mL doxycycline was reduced by 20-50% (Figure 1D). Doxycycline alone at 100 ng/mL had no effect on colony formation by primary AML cells (*Online Supplementary Figure S1D*). For validation, we analyzed the data derived from RNA interference and CRISPR-based genome-wide screening studies available in the DepMap database (Broad Institute).[28,29] We found that CRISPR-mediated silencing inhibited the growth of nearly all AML cell lines tested, whereas RNA interference revealed ARF6 dependency in 15 out of 22 AML cells tested (*Online Supplementary Figure S3*, *Online Supplementary Tables S1* and *S2*). Given the previously reported favorable safety profile of pharmacologically inhibiting ARF6 in mice,[15,30] we hypothesized that ARF6 inhibition may have potential as a therapeutic approach to treat AML.

To evaluate whether the reliance of AML cells on ARF6 is associated with its role in regulating sphingolipid metabolism, we used high performance liquid chromatography-mass spectrometry (HPLC-MS) to comprehensively analyze the sphingolipid profiles of THP-1 cells after shRNA-mediated *ARF6* KD. Following *ARF6* KD, THP-1 cells showed a profoundly altered profile of sphingolipid abundance compared to controls transfected with non-targeting shRNA (Figure 2A). Multi-dimensional scaling revealed tight clustering of biological replicates with clear separation between ARF6-depleted cells and controls (Figure 2B). Further analysis showed that sphingomyelin species were enriched within the lipids downregulated upon ARF6 depletion, while ceramide species were enriched within the upregulated lipids; accordingly, the overall sphingomyelin concentration was reduced and the overall ceramide concentration was increased, for an increased ceramide:sphingomyelin ratio (Figure 3A, B). The reciprocal changes of sphingomyelin and ceramide abundance suggested that either ARF6 depletion leads to increased sphingomyelin hydrolysis by sphingomyelinase or reduced ceramide-to-sphingomyelin conversion by sphingomyelin synthase (Figure 3C). To distinguish between these two possibilities, we analyzed the expression of these enzymes in THP-1 cells with or without ARF6 depletion. We observed a significant reduction of sphingomyelin synthase 1 (SGMS1) and sphingomyelin synthase 2 (SGMS2), but not sphingomyelinases, upon ARF6 depletion (Figure 4A), suggesting that the increase in the ceramide:sphingomyelin ratio may be due to a reduction of sphingomyelin synthases. To substantiate this notion, we analyzed the expression of ARF6 and SGMS1/SGMS2 in the TCGA dataset of 200 AML patients.[26] We found significant positive correlations between ARF6 and SGMS1 (Figure 4B) and ARF and SGSM2 expression (Figure 4C). In contrast, expression of ARF6 and sphingomyelinases was not correlated. In addition, total ceramide concentrations significantly increased in THP-1 and MV4-11 cells upon KD of SGMS1 or SGMS2 (Figure 4D, E).

As ceramide has been shown to induce growth arrest and apoptosis in cancer cells,[23] including AML cells,[22,23] we hypothesized that increased ceramide abundance in ARF6-depleted cells may reduce proliferation. We initially confirmed that exogenous ceramide treatment inhibits proliferation of THP-1 and MV4-11 AML cells (Figure 4F). To causally implicate increased ceramide in the observed reduction of cell proliferation upon *ARF6* KD, we used GW4869, a highly specific pharmacological inhibitor of sphingomyelinase[31] to block ceramide synthesis from sphingomyelin in ARF6-depleted cells (Figure 4G). GW4869 abrogated the inhibition of cell proliferation induced by *ARF6* KD (Figure 4H). In aggregate, these observations implicate ARF6 in maintaining sphingolipid homeostasis, which in turn is required for proliferation of AML cells.

We next turned our attention to the signaling events downstream of ARF6 in AML cells. Ceramide has been shown to inhibit AKT in cancer cells through the signaling pathways of PKC, PP2A, and TXNIP.[23] To test whether the ceramide - AKT pathway is intact in AML cells, we assessed pAKT(S473) levels upon *ARF6* KD. While pAKT(S473) levels were

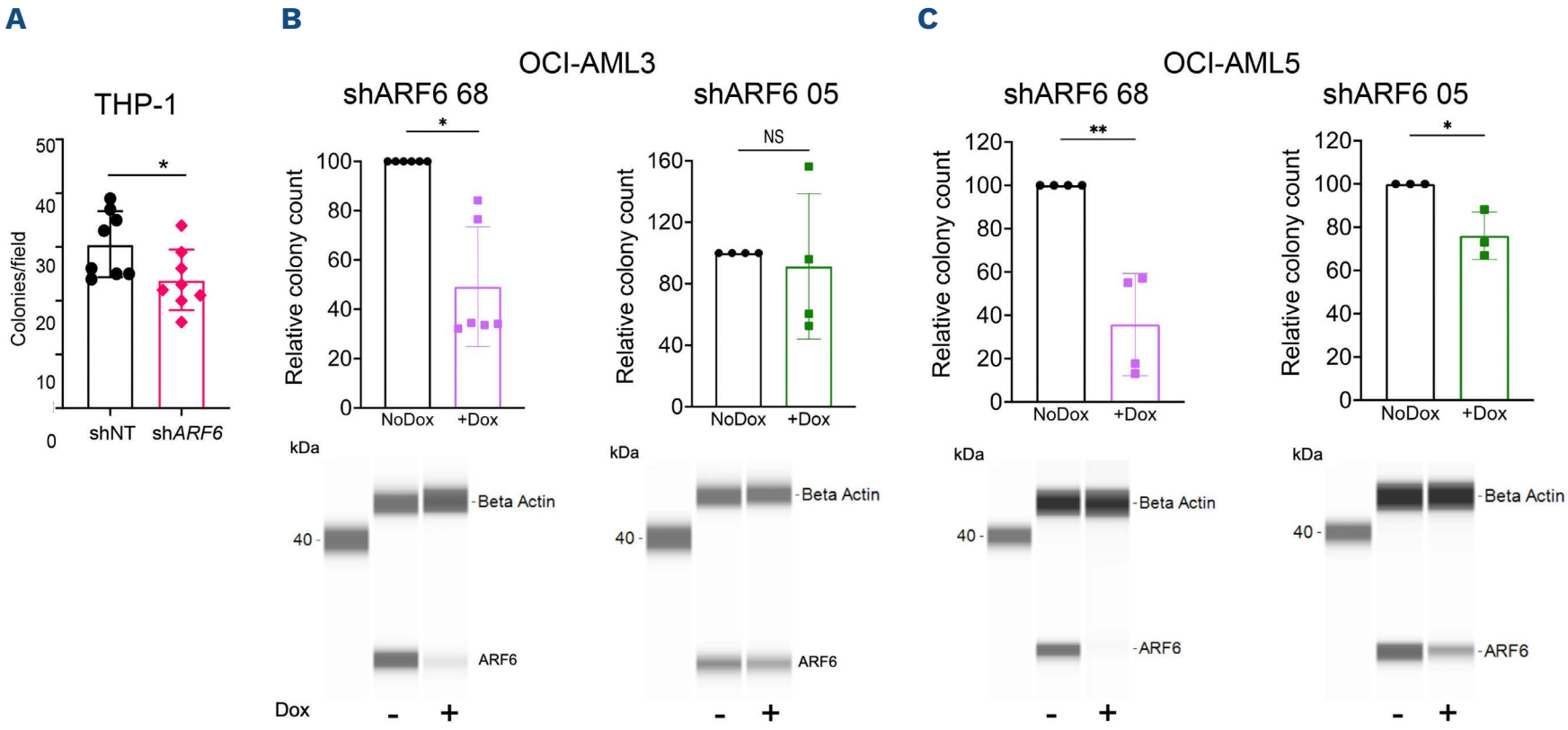

Figure 1. ARF6 depletion suppresses proliferation of acute myeloid leukemia cells. (A) Colony formation of THP-1 cells with or without *ARF6* knockdown (non-inducible). (B, C) Colony formation by OCI-AML3 and OCI-AML5 AML cells upon inducible *ARF6* knockdown. (D) Colony formation of patient-derived primary acute myeloid leukemia cells with or without doxycycline-induced *ARF6* knockdown. Somatic mutations detected by clinical next-generation sequencing are listed under each patient's sample identity. NS: not significant; **P*<0.05; ***P*<0.01. sh: short hairpin; NT: non-targeting; dox: doxycycline; AML: acute myeloid leukemia.

reduced, pERK1/2 (Thr202/Tyr204) levels were not consistently affected by *ARF6* KD (Figure 5A, B). Similar effects were seen with addition of exogenous ceramide (Figure 5C, D). When we knocked down *SGMS2*, pAKT(S473) was also reduced (Figure 5E-G). These data suggest that the reduction of cell proliferation upon *ARF6* KD is associated with accumulation of ceramide and inhibition of pAKT, thus highlighting an ARF6 - ceramide signaling axis.

After uncovering that an ARF6 - SGSM1/2 regulatory axis maintains sphingolipid homeostasis in AML cells, we next assessed its clinical significance using two independent AML cohorts of patients, the TCGA cohort of *de novo* AML cases (N=200)[26] and the Münchner Leukämielabor (MLL) AML cohort (N=579).[32] In both cohorts, patients were divided into high- and low-expression groups based on the median expression of *ARF6* and *SGMS1/2* after $\log_2$ transformation. Lower expression of *SGMS2* was associated with significantly longer survival in both cohorts, and lower expression of *ARF6* and *SGMS1* in one of the cohorts, with similar trends in the other (Figure 6A, B). This appears to be specific to the biological functions of ARF6, as the closely related ARF1 did not have an impact on survival (*Online Supplementary Figure S4*). In the TCGA cohort, the effect of high *ARF6* expression on overall and disease-free survival was similar to that of high *HOXA9* expression (Figure 6A-C, *Online Supplementary Figure S4*), a strong prognostic factor for

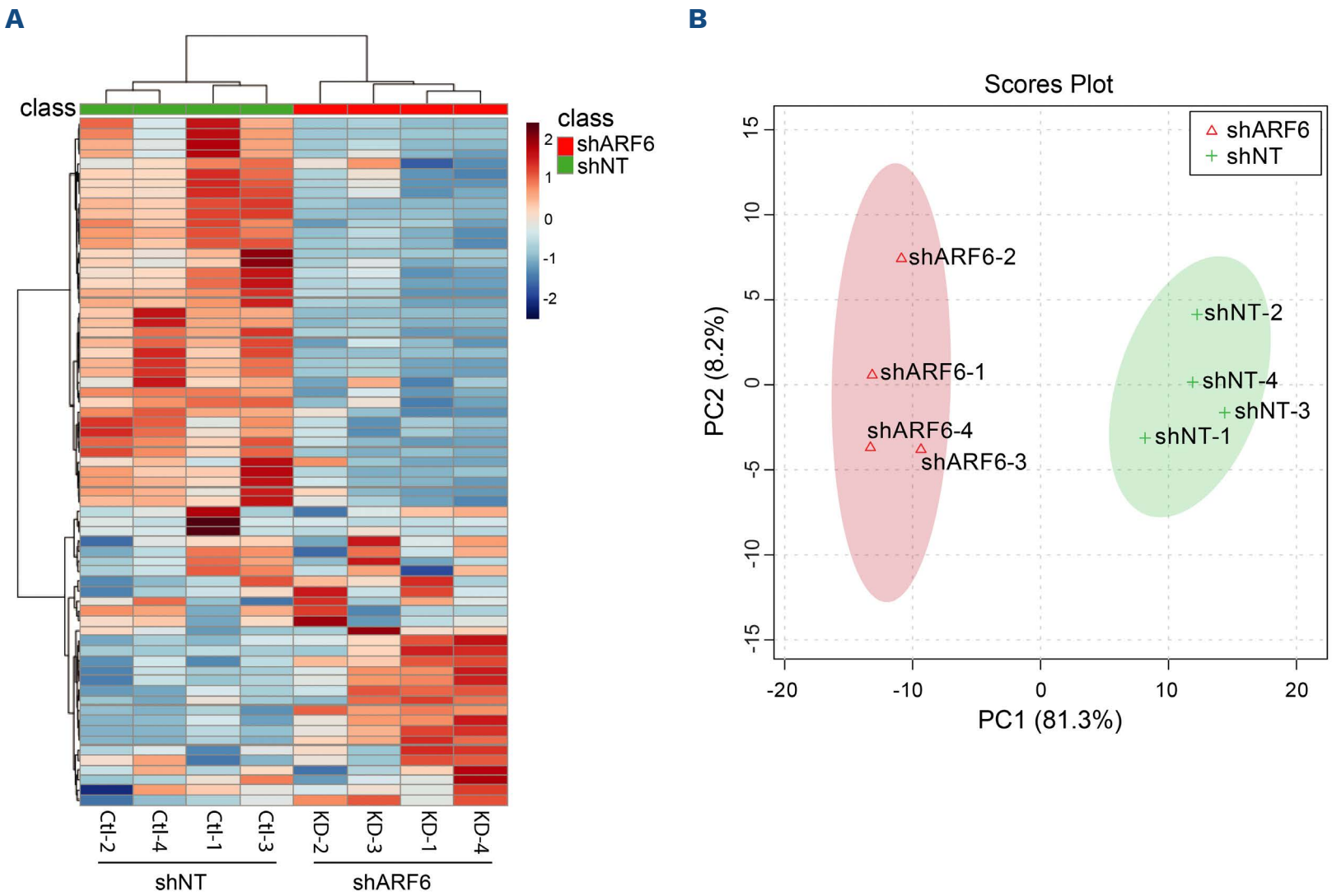

Figure 2. ARF6 maintains sphingolipid homeostasis in acute myeloid leukemia cells. (A) Clustered heat map of sphingolipid species abundance in THP-1 cells expressing non-targeting short hairpin RNA (NT-shRNA) and ARF6-shRNA. Four biological replicates of each group were analyzed by high performance liquid chromatography (HPLC) mass spectrometry (MS). Lipid abundance was quantified by area under the curve of the MS detection signals. Data were normalized to the overall sphingolipid content of the cells. Species and samples were clustered based on similarities. Ctl: NT-shRNA control. KD: ARF6-shRNA knockdown. (B) Multi-dimensional scaling plot of HPLC-MS data. Oval contours indicate grouping outcome.

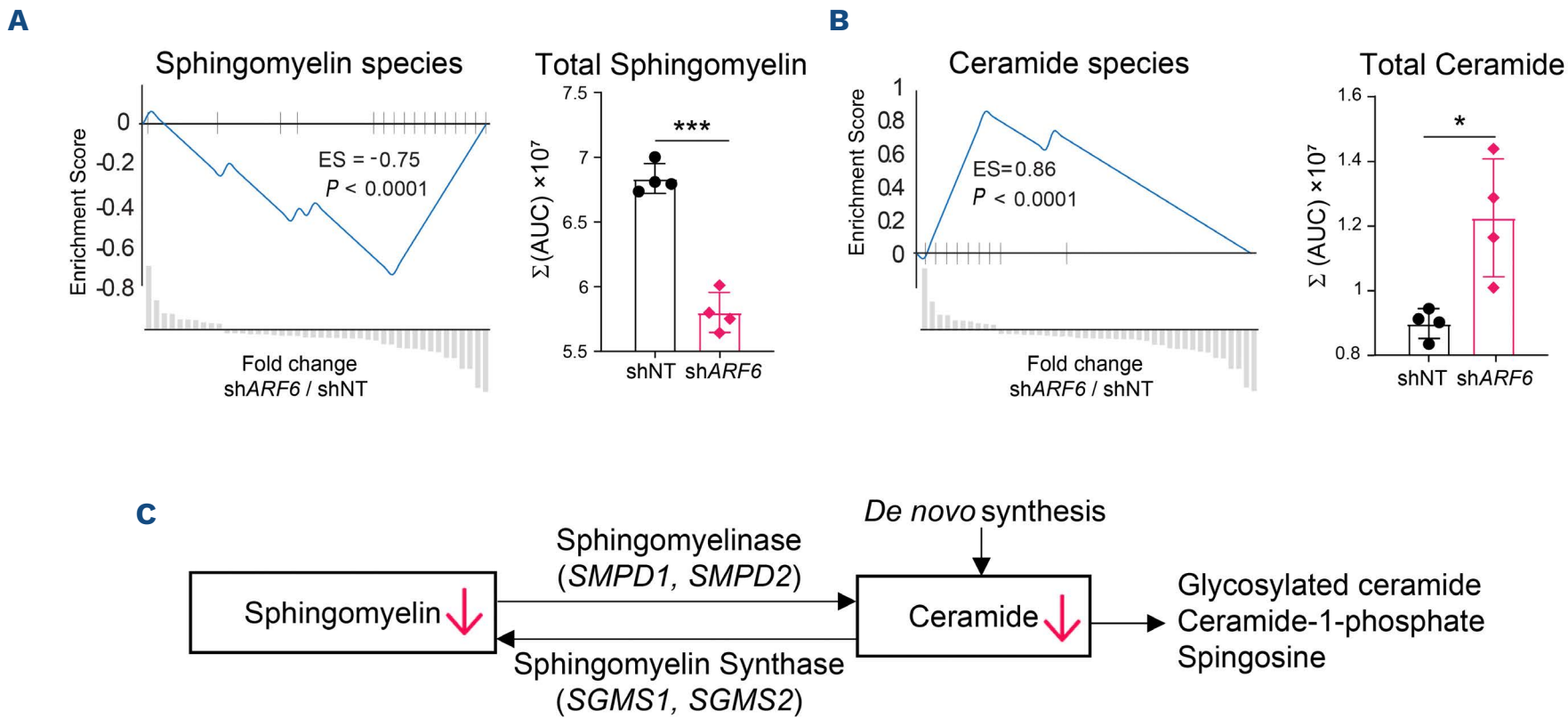

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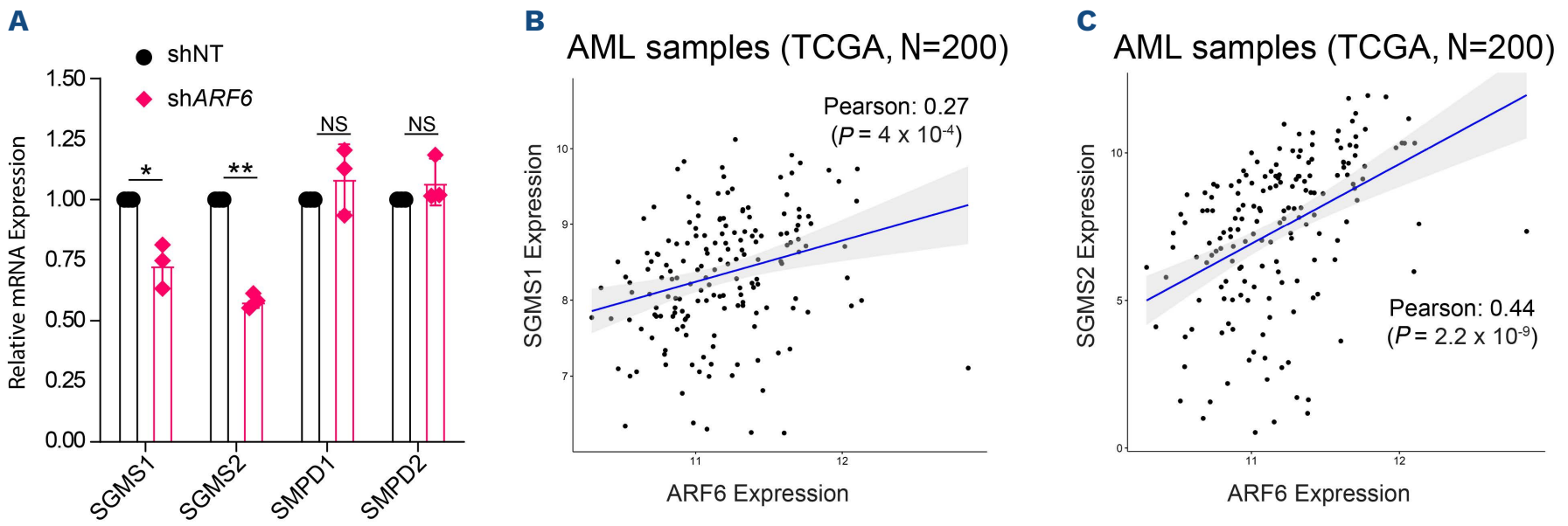
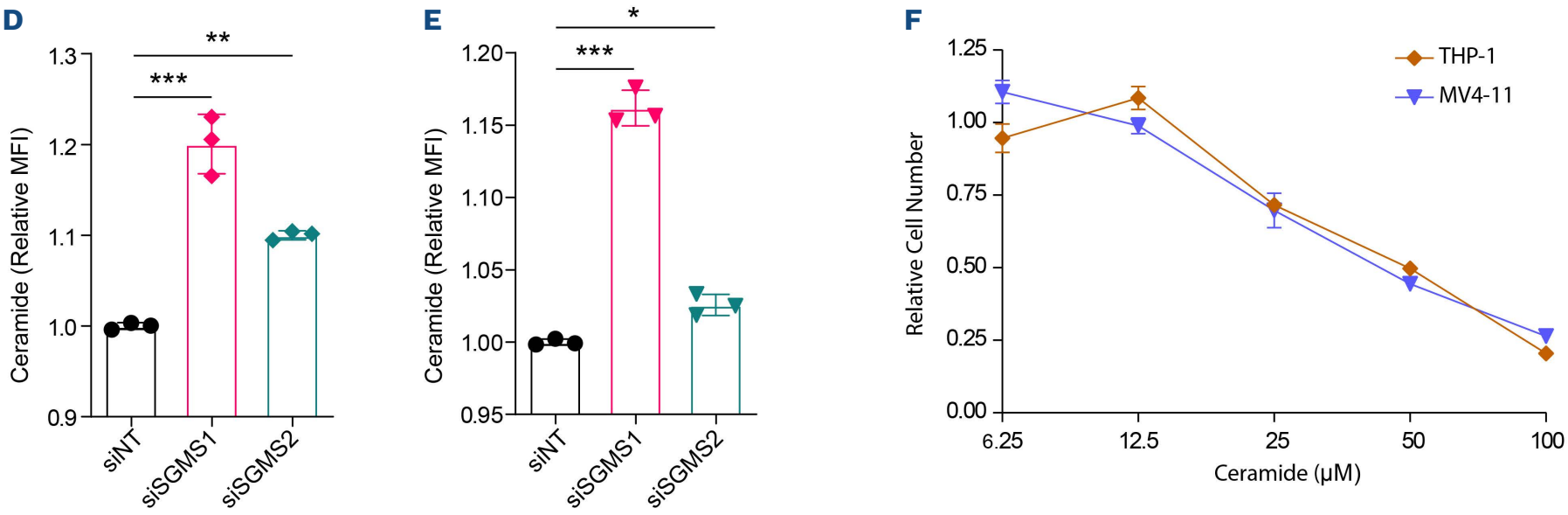
Figure 3. ARF6 depletion disrupts the ceramide/sphingomyelin balance in acute myeloid leukemia cells. (A, B) Enrichment analysis of high-performance liquid chromatography mass spectrometry data for sphingomyelin and ceramide species and quantitation of overall sphingomyelin and ceramide normalized to total sphingolipid content. (C) Schematic depicting relationships among sphingomyelin, ceramide, and ceramide metabolites. *P<0.05; ***P<0.001. ES: enrichment score; shNT: non-targeting short hairpin RNA; AUC: area under the curve.





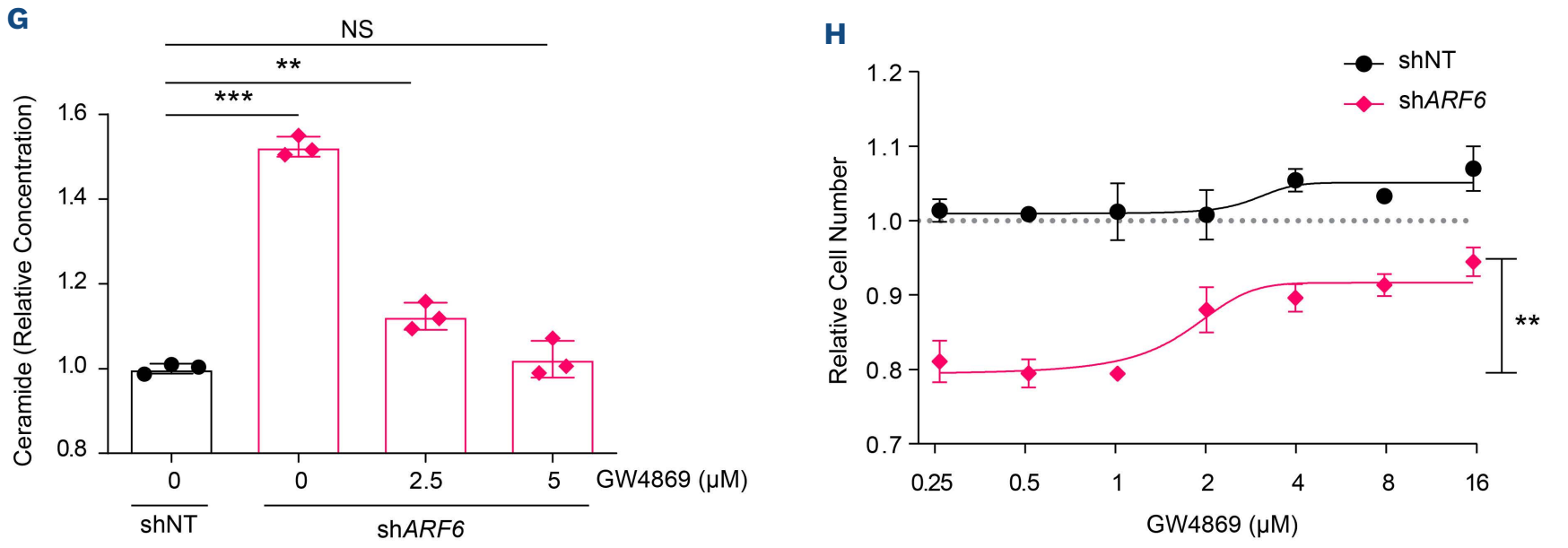


Figure 4. The ARF6-SGMS1/2 axis supports acute myeloid leukemia cell proliferation by maintaining ceramide homeostasis. (A) Quantitative real-time polymerase chain reaction analysis of the mRNA of sphingomyelin synthases and sphingomyelinases. (B, C) Positive linear correlation between the expression of *ARF6* and *SGMS1/2* in the myeloid cells from adult patients with *de novo*

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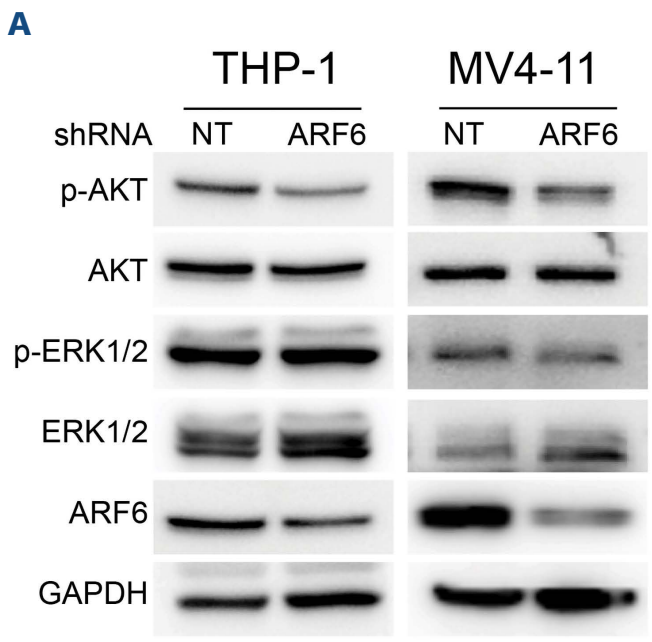
acute myeloid leukemia (AML) (TCGA-NEJM-2013 cohort). (D, E) Knockdown of *SGMS1/2* increased total cellular ceramide level, as detected by flow cytometry, in THP-1 (D) and MV4-11 (E) cells. (F) AML cell proliferation in the presence of increasing concentrations of exogenous ceramide. (G) GW4869, an inhibitor of sphingomyelinases, reduced the accumulation of ceramide in *ARF6* knockdown cells. (H) The sphingomyelinase inhibitor GW4869 (which blocks ceramide generation from sphingomyelin) rescues the *ARF6*-knockdown-induced reduction of proliferation. NS: not significant; **P*<0.05; ***P*<0.01; ****P*<0.001. shNT: non-targeting short hairpin RNA; TCGA: The Cancer Genome Atlas; siNT: non-targeting short interfering RNA.



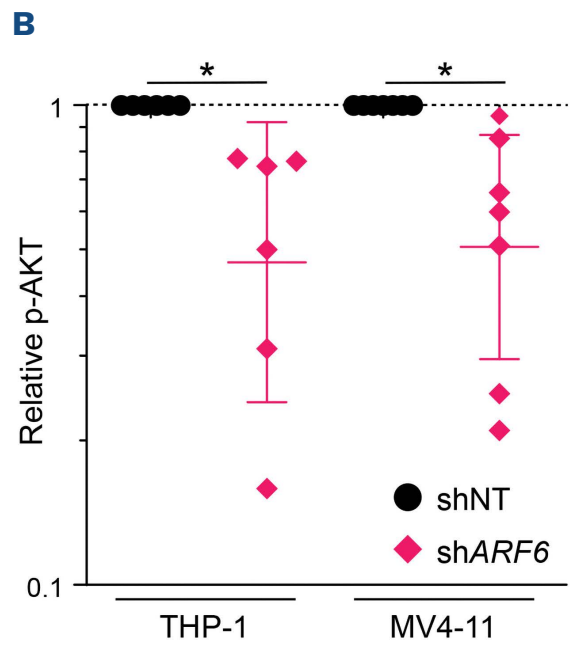
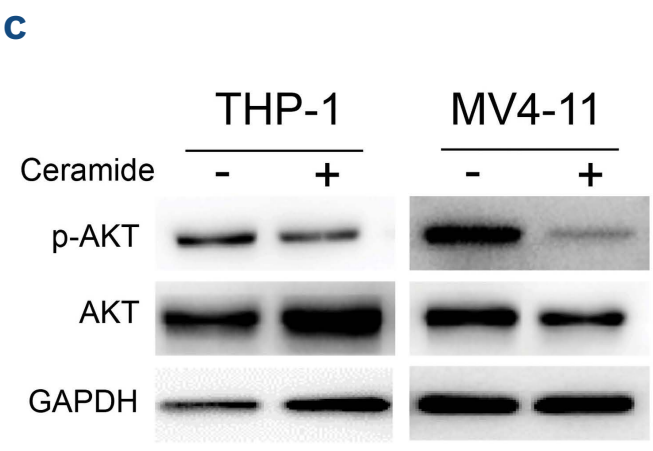
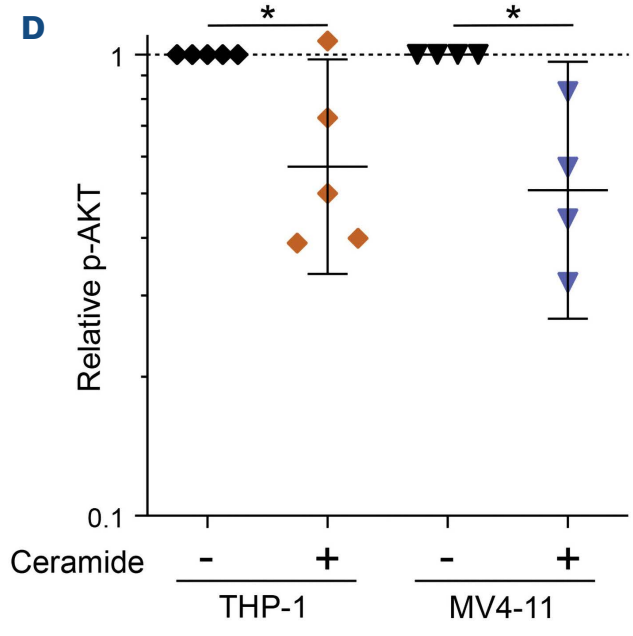
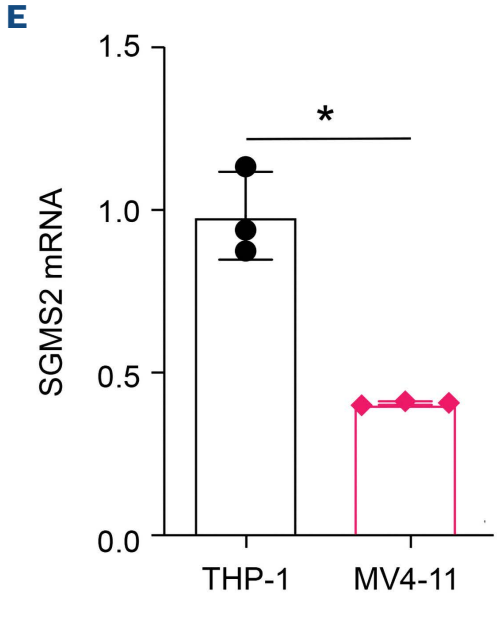
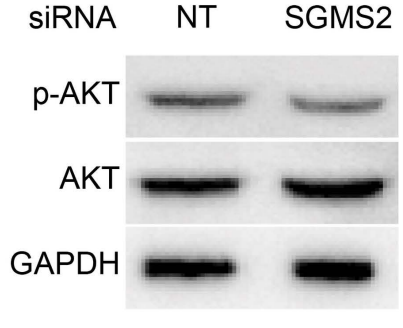


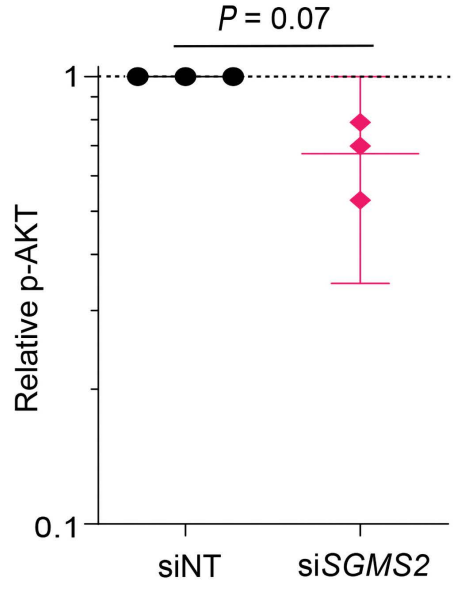
Figure 5. Perturbing ARF6 and its downstream effector inhibits pro-survival AKT activation. (A, B) Immunoblot analysis for p-AKT (S473) and pERK1/2 (Thr202/Tyr204) following *ARF6* knockdown in acute myeloid leukemia cell lines. (C, D) Immunoblot analysis of p-AKT (S473) following ceramide treatment (50 μM) on AKT activation in AML cells. (E-G) Downstream of ARF6, *SGMS2* knockdown inhibits AKT activation, as analyzed by immunoblot. **P*<0.05. NT: control.

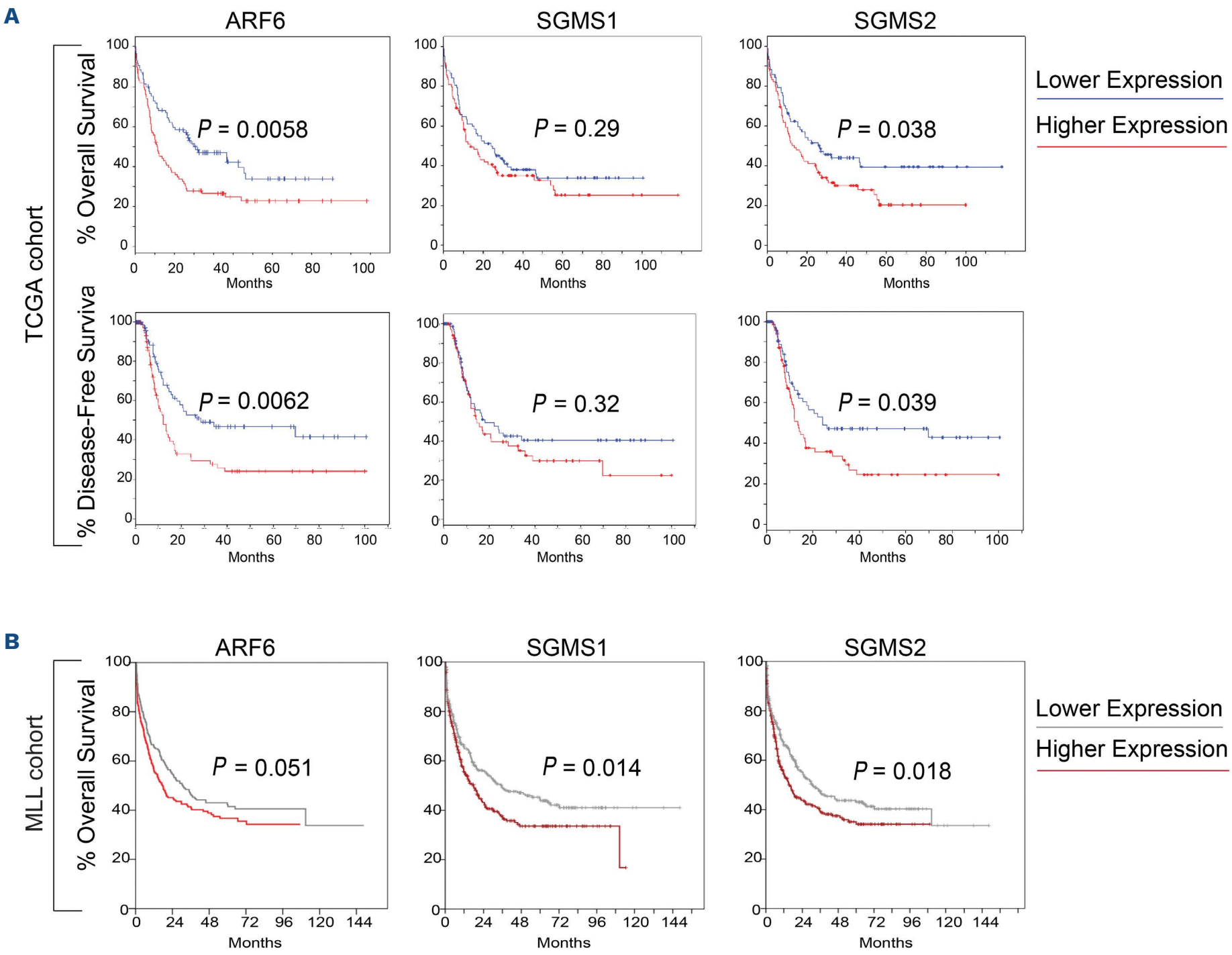












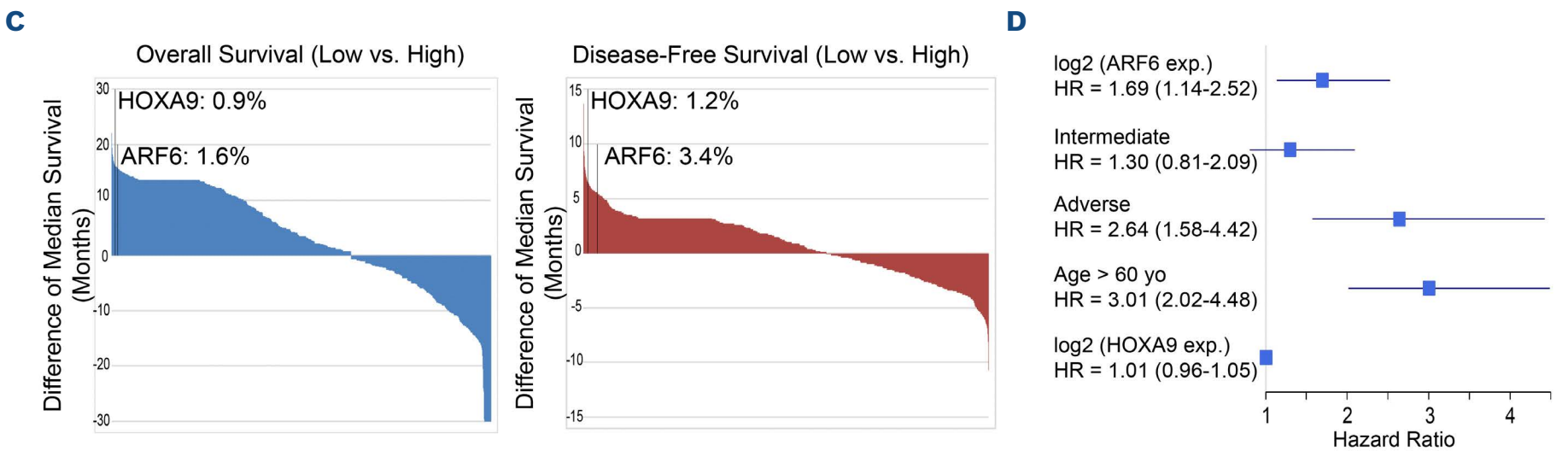


Figure 6. Lower *ARF6* or *SGMS1/2* expression in myeloid cells correlates with better survival in acute myeloid leukemia patients. (A) Kaplan–Meier survival curves of TCGA-NEJM-2013 adult *de novo* acute myeloid leukemia (AML) patients with higher (above median, red lines) and lower (below median, blue lines) expression of the indicated genes. (B) Kaplan–Meier survival curves of the Münchner Leukämielabor (MLL) AML cohort. (C) Ranking of genes according to their prognostic power for prediction of overall and disease-free survival in The Cancer Genome Atlas (TCGA) AML cohort. Only genes with detectable mRNA expression were included in the analysis. The Y axis indicates the differences of median survival time between patients with lower gene expression and higher gene expression. (D) Multivariate analysis of survival in the TCGA cohort, including European LeukemiaNet risk, *ARF6* and *HOXB9* expression. NS: not significant; *P<0.05; **P<0.01. exp: expression; HR: hazard ratio; yo: years old.

poor survival in AML.[33] We next calculated European LeukemiaNet (ELN) 2022 genetic risk of the TCGA AML cohort based on karyotype and somatic mutations. As expected, ELN risk separated low-, intermediate-, and high-risk patients (*Online Supplementary Figure S5*). Next, we tested whether *ARF6* expression added independent prognostic information, excluding additional effects from the known adverse risk factor, age >60 years. *ARF6* expression above the median remained associated with shorter survival (hazard ratio =1.69 [95% confidence interval: 1.14-2.52] for ELN high genetic risk and 1.30 [95% confidence interval: 0.81-2.09] for ELN intermediate genetic risk) (Figure 6D). Taken together, these data suggest that high *ARF6* expression contributes independently to aggressive disease biology and may constitute a therapeutic target.

As a proof-of-principle study of targeting ARF6 in AML, we tested small molecular inhibitors of ARF6 against AML cell lines and primary AML CD34$^+$ patients' cells. We previously reported the discovery of a small molecular inhibitor of ARF6, NAV-2729, which demonstrated potent efficacy in blocking ARF6 activation and its molecular functions in an *in vivo* model of uveal melanoma, which is characterized by GNAQ-driven ARF6 activation.[15] Recently, through chemical modification, we identified a second-generation ARF6 inhibitor, A6-4471, which maintained biochemical specificity and showed improved efficacy in inhibiting ARF6 activation in cell-free assays (Figure 7A). In proliferation assays of THP-1 and MV4-11 cells, the half maximal inhibitory concentration (IC_{50}) of A6-4471 was lower than that of the first-generation ARF6 inhibitor NAV-2729 (Figure 7B, C). Accordingly, treatment with A6-4471 induced apoptosis at a lower concentration compared to NAV-2729 (Figure 7D). In clonogenic assays, both NAV-2729 and A6-4471 inhibited colony formation by THP-1 cells at their respective biochemical IC_{50} concentrations (Figure 7E). In analogy to *ARF6* KD, both NAV-2729 and A6-4471 reduced p-AKT(S473) levels (Figure 7F). To confirm that the ARF6 inhibitor effects were indeed mediated through AKT, we ectopically expressed a constitutively active form of AKT (AKT1^{E17K}) in THP-1 cells.[34] AKT1^{E17K} rescued cells from the effects of ARF6 inhibitors at higher concentrations (Figure 7G). For additional validation, we co-expressed AKT1^{E17K} and doxycycline-inducible sh*ARF6* in THP-1 and OCI-AML3 cells. AKT1^{E17K} rescued colony formation and partially rescued proliferation upon *ARF6* KD in OCI-AML3 cells (*Online Supplementary Figure S6*) and THP1 cells (*data not shown*). These data demonstrate that pharmacologically inhibiting ARF6 induces apoptosis and suppresses proliferation in AML cells, and that these effects are mediated by modulation of AKT.

To test the *in vivo* therapeutic efficacy of ARF6 inhibitors, we engrafted NSG mice with THP-1 cells and treated animals for up to 5 weeks with NAV-2729 or A6-4471 (Figure 8A). Both NAV-2729 and A6-4471 reduced the number of circulating AML cells without significantly altering body weight (Figure 8B-E). We also analyzed the effects of both inhibitors in colony-forming assays of primary AML cells from four patients and umbilical cord blood CD34$^+$ cells from three donors as controls. Primary AML cells were more sensitive to ARF6 inhibitors than cord blood CD34$^+$ cells (Figure 8F, G). Lastly, we assessed whether ARF6 disruption may synergize with chemotherapeutics used in AML. THP-1 cells expressing inducible *shARF6* were treated with graded concentrations of cytarabine, daunorubicin and venetoclax. *ARF6* KD had no or minimal effects on the IC_{50} of daunorubicin and cytarabine but reduced the IC_{50} of venetoclax by ~6-fold (*Online Supplementary Figure S7*).

Discussion

As one of the hallmarks of cancer, metabolic reprogramming can render cancer cells addicted to metabolic pathways that are dispensable for their normal counterparts.[35] Like other malignancies, AML cells exhibit aberrancies in a range of metabolic pathways, including lipid metabolism.[36-38] Sphingolipid turnover is an integral component of cellular lipid metabolism. Among sphingolipids, ceramide has received considerable attention due to its unique capability to trigger pro-apoptotic signaling.[22,23] *FLT3*-ITD-positive primary AML cells and *NRAS*-mutant HL-60 cells were shown to downregulate ceramide to support cell proliferation and enhance resistance to chemotherapy.[39,40] In contrast, increased ceramide is pro-apoptotic in myeloid leukemia cells.[21,41] Efforts to develop ceramide or ceramide mimetics as therapeutic agents against AML have shown promise.[24] The small GTPase ARF6 is involved in several aspects of lipid metabolism, such as the phosphorylation and distribution of PIP2, phosphatidic acid metabolism, and cellular cholesterol distribution.[19,20,42-44] ARF6 was previously shown to promote metastasis in several solid tumors.[14-17,25,45] Deficiency of the ARF6 upstream regulator *Smap1* perturbs receptor trafficking and predisposes mice to myelodysplasia, supporting the notion that ARF6 promotes AML.[46] In accord with this, *ARF6* KD has a strong and specific anti-proliferative effect in hematopoietic cancers (DepMap DEMETER2, P=1.92x10^{-12}) and AML (DepMap DEMETER2, P=1.25x10^{-4}), while normal tissues are unaffected.[15,30]

We show that ARF6 regulates sphingolipid homeostasis in AML cells, maintaining ceramide/sphingomyelin balance to support AML survival and proliferation. The function of ARF6 as a regulator of vesicle trafficking and protein content dynamics in various cell types is well characterized.[9-12] In contrast, its role in regulating lipid metabolism is incompletely understood.[10] While previous studies have implicated ARF6 as a physiological regulator of phosphatidylinositol and cholesterol abundance and distribution in non-hematopoietic cells,[19,20,42] our study is the first to implicate ARF6 in regulating sphingolipid metabolism in cancer.

Several mechanisms were shown to contribute to the aberrant regulation of sphingolipid metabolism in AML cells.

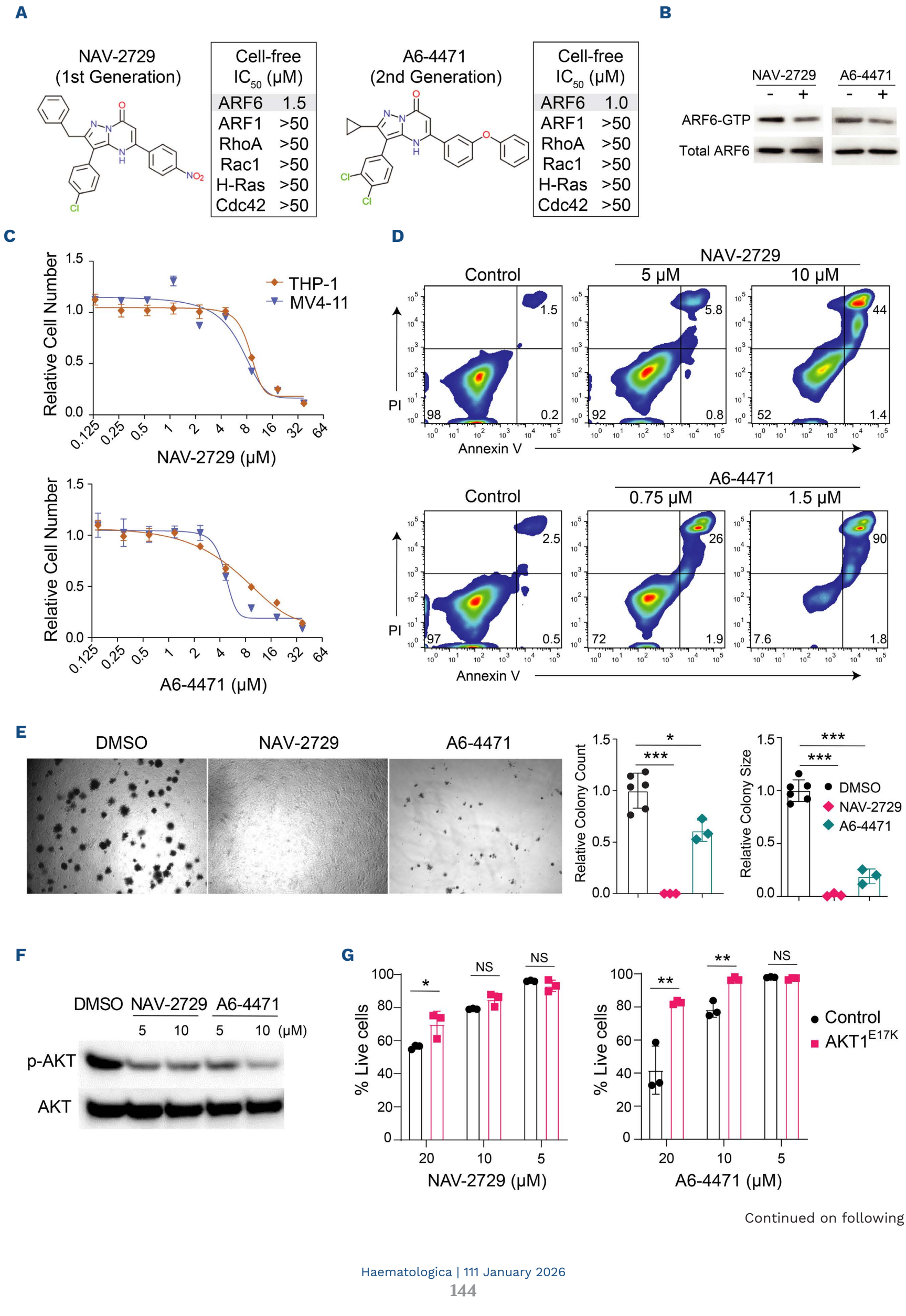


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Figure 7. ARF6 can be targeted by small molecule inhibitors to suppress acute myeloid leukemia cell proliferation. (A) Chemical structure of and specificity information for the first- and second- generation ARF6 inhibitors, NAV-2729 and A6-4471. Half maximum inhibitory concentration (IC_{50}) values were determined in cell-free biochemical analyses. (B) Immunoblot analysis of ARF6 activation (ARF6-GTP) with or without treatment of ARF6 inhibitors at their respective IC_{50} values in cell culture: 10 μM for NAV-2729, 6 μM for A6-4471. (C) Dose responses of acute myeloid leukemia cell proliferation to NAV-2729 and A6-4471. (D) THP-1 cell apoptotic response to different concentrations of NAV-2729 and A6-4471. Apoptosis was detected with annexin V + propidium iodide staining. (E) The inhibitory effect of ARF6 inhibitors on THP-1 cell colony formation. (F) Relative p-AKT level was detected by immunoblot in THP-1 cells after treatment with ARF6 inhibitor or vehicle. (G) Ectopic expression of the constitutively active $AKT1^{E17K}$ rescued ARF6 inhibitor-induced cell death at higher concentrations. NS: not significant; **P*<0.05; ***P*<0.01;****P*<0.001. PI: propidium iodide; DMSO: dimethylsulfoxide.

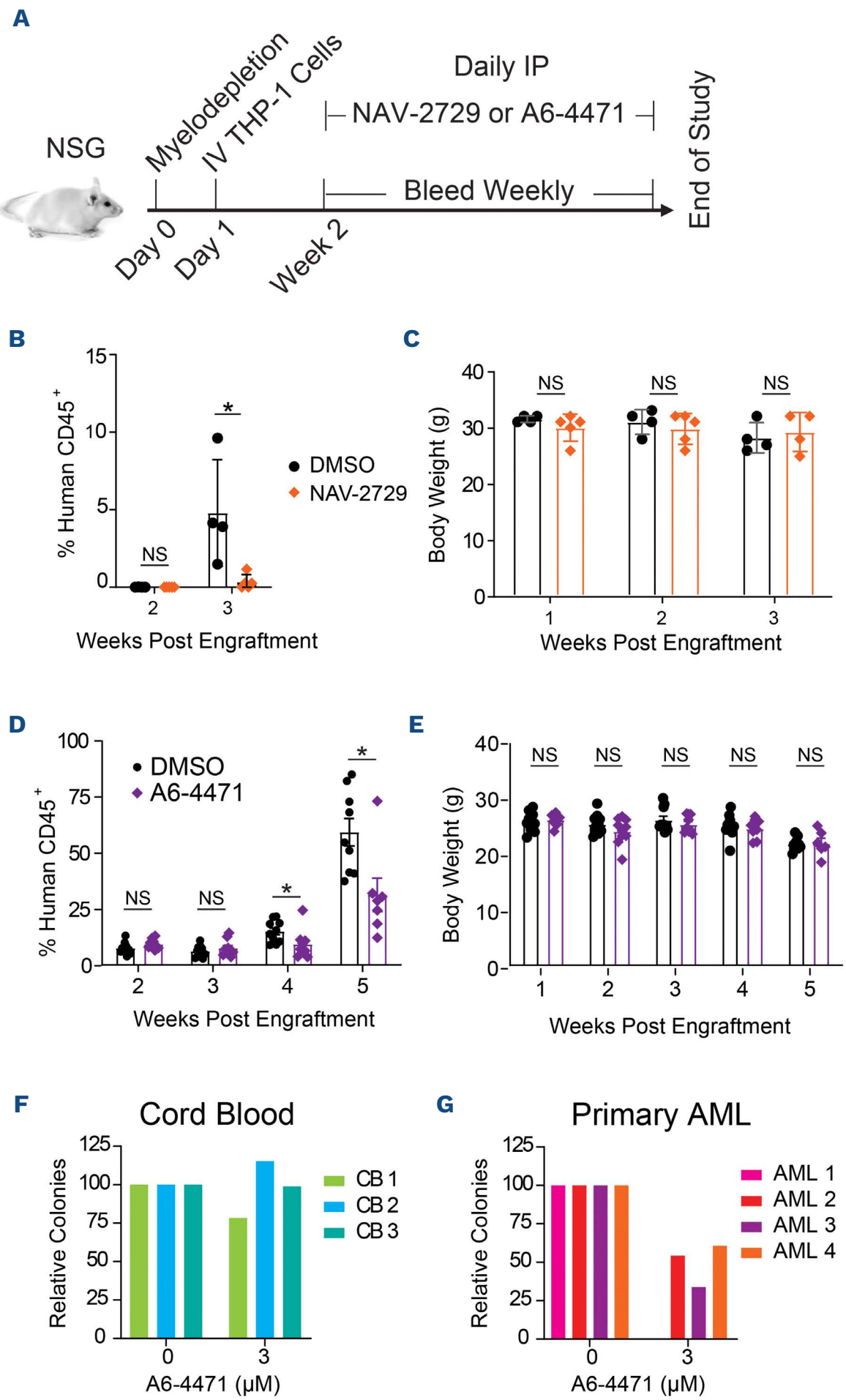

Figure 8. Efficacy and specificity of ARF6 inhibitors in an acute myeloid leukemia xenograft mouse model and in *ex vivo* primary cell cultures. (A) Schematic illustration of the experimental design. (B and D) Flow cytometry analysis of circulating acute myeloid leukemia (AML) cells in xenografted NSG mice. AML cells were identified as human $CD45^+$ and murine $CD45^-$ cells. (C and E) Body weight changes of experimental mice during the ARF6 inhibitor injections. (F) Colony formation of cord blood $CD34^+$ hematopoietic stem cells from healthy donors in response to the ARF6 inhibitor A6-4471. (G) Colony formation of patient-derived AML $CD34^+$ cells in the presence of the ARF6 inhibitor A6-4471. NS: not significant, **P*<0.05. IV: intravenous; IP: intraperitoneally; DMSO: dimethylsulfoxide.

Decreased ceramide concentrations in AML cells are associated with reduced expression of ceramide synthase[39] and/or increased expression of acid ceramidase.[38] Conversely, daunorubicin-induced cell death in U937 cells is associated with increased ceramide synthase expression and increased ceramide.[21] Another study showed that chemotherapy-resistant HL-60 cells downregulate ceramide through enhancing the activity of glucosylceramide synthase and sphingomyelin synthase.[40] However, it remained unclear in these studies which upstream mechanisms regulate the altered ceramide metabolic enzyme activities in AML. ARF6 is activated by growth factor receptors and oncogenes, resulting in activation of growth and survival signaling.[10] We now show that ARF6 maintains the ceramide/sphingomyelin balance in AML cells by enhancing expression of SGMS1/2 at the transcriptional level. Downregulation of ARF6 reduces SGMS1/2, resulting in ceramide accumulation and consequently a reduction in AML proliferation. Hence, ARF6 functions as a critical upstream regulator of sphingolipid metabolism and cell proliferation in AML, connecting oncogene signaling to sphingolipid metabolism. More experimentation will be needed to determine how ARF6 regulates SGMS1/2 transcription. In addition to the mechanism shown in this study, there may be other critical functions of ARF6 that support proliferation or survival of AML cells, such as its regulation of vesicular trafficking.

Ceramide causes apoptosis or senescence by deactivating AKT through its upstream negative regulators, PKCξ and PP2A,[23] or by ceramide-induced mitochondrial stress.[23] In AML cells, ceramide accumulation has been shown to cause lethal mitophagy, and thus a ceramide analog was proposed as a potential therapeutic agent for AML.[39] In agreement with the first mechanism, our data suggest that ARF6 inhibition reduces AML growth by decreasing pAKT, similar to its effect in cutaneous melanoma.[18] However, given the limited clinical activity of AKT inhibitors in AML, it is likely that other mechanisms contribute to the effects. For instance, mitophagy caused by ARF6 inhibition may enhance growth inhibition and apoptosis, especially as autophagy and the AKT-mTOR pathway are intertwined.[47] Regarding signaling downstream of ceramide, a prior report suggested that ceramide contributes to iron-dependent cell death (ferroptosis) in AML.[48] More work will be required to understand whether this mechanism is ARF6-dependent in AML.

Inhibiting ARF6 has been proposed as a potential therapeutic strategy for several solid tumors, given its role in promoting cancer cell survival and metastasis.[14-17,25,45] Like other small GTPases, ARF6 is a challenging drug target. We previously identified a first-generation ARF6 inhibitor, NAV-2729, and demonstrated that NAV-2729 blocks oncogenic Gαq signaling in uveal melanoma in a xenograft mouse model.[15] Based on these encouraging data we have continued the development of ARF6 inhibitors, leading to the identification of the more potent second-generation ARF6 inhibitor A6-4471. In the present study, we show that NAV-2729 and A6-4471 are active against AML cell lines, primary AML cells and mouse AML xenografts. However, the therapeutic windows of these inhibitors are relatively narrow. As such both NAV-2729 and A6-4471 should be regarded as tool compounds for inhibiting ARF6 in proof-of-principle experiments that validate the genetic disruption data. Follow-up compounds with further improved potency and preserved selectivity are under development.

Analysis of the TCGA and MLL cohorts provided evidence that activation of the ARF6/SGMS1/2 axis confers a poor prognosis, suggesting that ARF6 has an important role in AML biology. Although there was a trend toward greater ARF6 dependency in AML cell lines with wild-type *TP53* for RNA interference perturbation in DepMap (7/15 lines with DEMETER scores >0 but only 1/7 with DEMETER scores <0 were wild-type, P=0.0827, χ^2), high ARF6 expression remained a poor prognostic factor after correction for ELN genetic risk, suggesting that ARF6 impacts AML biology in a mostly genotype-agnostic manner, in accord with other metabolic vulnerabilities such as SIRT5.[49] However, as our study does not fully reflect the genetic heterogeneity of AML, it remains possible that correlations between ARF6 expression and dependency or between genotype and ARF6 dependency could become apparent upon interrogation of a larger panel of cell lines and/or primary cells.

In summary, we demonstrate that the small GTPase ARF6 regulates sphingolipid homeostasis in AML. Given the universal expression of ARF6 protein in eukaryotic cells,[10] the unique ARF6 addiction of AML cells is unexpected, but implicates ARF6 as a potential therapeutic target in AML.

Disclosures

The University of Utah has filed intellectual property rights concerning ARF6 pathways. The University of Utah has previously licensed the technology to Navigen, Inc., of which A6 Pharmaceuticals is a subsidiary. MWD's laboratory is funded by Novartis and Pfizer. MWD is a consultant/advisory board member of Novartis, Pfizer, Galena Biopharma, ARIAD Pharmaceuticals, Blueprint Medicines and Incyte.

Contributions

HGZ and NC-R designed and performed the experiments, analyzed the data and wrote the manuscript. KCJ and ADP performed and analyzed the experiments and wrote the manuscript. DY, BrB, BeB, JB and GSL performed experiments and analyzed the data. TG performed the statistical analysis and wrote the paper. TH provided key data and performed bioinformatics analysis. WZ and SJO provided funding for the study. MWD oversaw the study, provided funding, and wrote the manuscript.

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Data-sharing statement

Primary data and reagents will be made available upon reasonable request to the corresponding author.

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