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G0S2 Inhibits the Proliferation of K562 Cells by Interacting with Nucleolin in the Cytosol

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Abstract

G0/G1 switch gene 2 (G0S2) is a basic protein with ill-defined function that inhibits the proliferation of hematopoietic stem cells. Herein, we show that treatment of K562 cells with 5-azacytidine (5-Aza) resulted in a 24-fold increase in G0S2 expression and a reduction in cell growth. Conversely, gene demethylation in the presence of G0S2-specific shRNA restored proliferation, further supporting an inhibitory role for G0S2 in cell proliferation. Elevated levels of G0S2 inhibited the division of K562 cells by sequestering the nucleolar phosphoprotein nucleolin in the cytosol. G0S2 inhibited the proliferation of leukemia cells *in vivo* in xenograft models. Collectively, our data identify a new mechanism that controls proliferation in K562 cells, suggesting a possible tumor suppressor function in leukemia cells.

Keywords

K562; G0S2; nucleolin; proliferation

Introduction

A hallmark of cancer is a loss of control of cell division and the aberrant expansion of transformed cells. Therefore, genes involved in the inhibition of cellular proliferation are often inactivated during transformation to cause overt leukemia. Inactivation of tumor suppressors and cell cycle regulators by gene methylation has been reported in many types of cancers, including colon cancer, leukemia, and breast cancer [1]. The identification of novel mechanisms that control proliferation in leukemia cells will aid the development of new treatments.

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G0/G1 switch gene 2 (G0S2) is a basic protein that was identified as an early activating gene in a screen of lectin-activated human lymphocytes [2]. Therefore, it was hypothesized that G0S2 controls the G_0-to-G_1 transition. However, G0S2 was first described as inhibiting lipolysis by directly interacting with adipose triglyceride lipase in adipocytes [3, 4]. The expression of G0S2 is regulated by the peroxisome proliferator-activated receptors (PPARs) during adipogenesis, by calcineurin/nuclear factor of activated T-cells (NFAT) in blood mononuclear cells, and by all-trans-retinoic acid in acute promyelocytic leukemia cells [5–7]. In human primary fibroblasts, G0S2 interacts with Bcl-2, promoting apoptosis by preventing the formation of Bcl-2/Bax heterodimers [8]. Our group reported that G0S2 inhibits the proliferation of hematopoietic stem cells and that the G0S2 protein interacts with nucleolin in resting cells, causing cytosolic retention of nucleolin and thus preventing nucleolin's pro-proliferative functions in the nucleus/nucleolus [9]. These reports indicate that G0S2 has disparate functions that are mediated by protein-to-protein interactions in different tissues.

The finding that the G0S2 gene is epigenetically silenced in head and neck cancers, squamous lung cancer, and cisplatin-resistant cancer cells has suggested a role in carcinogenesis and chemoresistance [10, 11]. In hematological malignancies, G0S2 is part of a molecular signature in $CD34^+$ cells from patients with chronic myeloid leukemia (CML) [12, 13]. A current challenge in the treatment of CML patients is the ability to regulate the balance between quiescence and proliferation to successfully eradicate chemoresistant leukemia cells and to prevent relapses upon discontinuation of tyrosine kinase inhibitors [14, 15].

In this work, we show that the G0S2 gene is silenced by gene methylation in K562 cells and that upregulation of G0S2 expression, either by retroviral transduction or by treatment with 5-azacytidine (5-Aza), inhibits the proliferation of K562 cells both *in vitro* and *in vivo*. Elevated levels of G0S2 also correlated with a redistribution of nucleolin in the cytosol, suggesting that K562 cells downregulated G0S2 expression by promoter methylation to prevent the interaction between G0S2 and nucleolin and avoid cell cycle inhibition. Collectively, our data show that G0S2 inhibits the proliferation of K562 cells, suggesting a possible role as a tumor suppressor in leukemia cells.

Materials and Methods

Leukemic cell lines

K562, HEL, HL-60, Kasumi, Jurkat, and H9 cells were obtained from the American Type Culture Collection (ATCC). DND41 cells were obtained from Dr. Adolfo Ferrando (Columbia University, New York, NY, USA). MHH-Call-4 (Call4) and Mutz5 were obtained from the DSMZ-German collection of microorganisms and cell cultures. All of the cell lines were maintained in RPMI-1640 medium (Lonza) supplemented with 10% (vol/vol) FBS and 2 mM glutamine. Human peripheral blood cells (StemExpress) were stained with phycoerythrin-labeled anti-CD14 (BD Biosciences) for the purification of monocytes with a dual-laser MoFlo cell sorter (Cytomation, DAKO).

Retroviral transduction

V5-tagged human G0S2 was cloned into the Migr1 retroviral vector [16]. HEK 293T cells were co-transfected with a plasmid containing ψ-ampho and the retroviral vector (containing G0S2 (Migr1-G0S2) or empty (Migr1)). K562 cells were co-cultured with packaging 293T cells for two days, followed by purification of EGFP-positive cells by cell sorting using a dual-laser MoFlo cell sorter (Cytomation, DAKO). To induce G0S2 gene silencing, two G0S2-specific shRNAs (sh1 and sh2) were cloned into a silencing retroviral construct

(pSIREN-RetroQ-ZsGreen, Clontech). The G0S2 shRNA sequences were as follows: sh1, 5'-GGAAGATGGTGAAGCTGTA-3' and sh2, 5'-CGCTGACATCTAGAACTGA-3'. A luciferase-silencing retroviral construct was used as a control. shRNA retrovirus was generated as described above.

Cell cycle analysis

Cells were washed with PBS and resuspended in hypotonic buffer (0.1% sodium citrate and 0.1% Triton X-100) containing 100 μg/ml RNase A and 50 μg/ml propidium iodide. The samples were analyzed using a FACSCanto flow cytometer (BD Biosciences), and cellular DNA content was analyzed using FlowJo software (Tree Star).

Co-immunoprecipitation and immunoblotting

K562 cells transduced with either Migr1 or Migr1-G0S2-V5 were directly lysed with LDS Sample Buffer (Invitrogen) at a density of 1×10^4 cells/μl. Sonicated cell lysates (10 μl) were loaded onto NuPAGE Bis-Tris gels (Invitrogen), and proteins were transferred to polyvinylidene difluoride membranes. The proteins were detected with mouse anti-V5 (Invitrogen) or rabbit anti-β-actin (Sigma-Aldrich). Goat anti-mouse IgG or anti-rabbit IgG conjugated to peroxidase (GE Healthcare) was used as a secondary antibody. Immunoblots were developed by chemiluminescence using a detection reagent (Thermo Scientific) and analyzed using a FluorChem HD2 ChemiImager (Alpha Innotech, Santa Clara, CA). For co-immunoprecipitation, K562 cells transduced with a retroviral vector (Migr1 or Migr1-G0S2) were lysed with 50 mM Tris (pH 8), 150 mM NaCl, and 1% Triton X-100 containing a protease inhibitor cocktail (Calbiochem). The cell lysates were immunoprecipitated with Protein G-Sepharose (Invitrogen) and either mouse anti-V5 antibody (Invitrogen) or rabbit anti-nucleolin antibody (Abcam). The samples were then loaded onto NuPAGE Bis-Tris gels (Invitrogen), separated by electrophoresis, and transferred onto polyvinylidene difluoride membranes (Millipore) for immunoblot analysis. Goat anti-rabbit IgG or goat anti-mouse IgG antibody conjugated to peroxidase (GE Healthcare) was used as a secondary antibody.

Quantitative real-time PCR

Leukemia cells were cultured in the presence of 10 μM 5-Aza (Sigma) for 4–6 days, and the medium was half replenished every other day. Total RNA was extracted from 5-Aza-treated and control cells using an RNeasy Mini Kit (Qiagen). cDNA was synthesized from 100–500 ng RNA with random hexamer primers using a SuperScript III kit (Invitrogen). Quantitative real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche) as specified by the manufacturer. The primer sequences for PCR were as follows: human β-actin: forward, 5'-GCTCGTCGTCGACAACGGCTC-3' and reverse, 5'-CAAACATGATCTGGGTCATCTTCTC-3'; human G0S2: forward, 5'-CGCCGTGCCACTAAGGTC-3' and reverse, 5'-GCACACAGTCTCCATCAGGC-3'. The thermocycler conditions consisted of an initial step at 95°C for 10 min, followed by 35 cycles of a three-step PCR program consisting of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. The reactions were run on an Mx3005P instrument (Stratagene). Expression was calculated relative to β-actin expression in each sample.

Methylation analysis

An analysis of CpG island content was performed using the program MethPrimer [17]. Genomic DNA was extracted from human leukemia cells and normal monocytes using a DNeasy kit (Qiagen). Genomic DNA (5 μg) was treated with bisulfate using a Cells-to-CpG Bisulfite Conversion Kit (Applied Biosystems). The region containing the CpG island in the G0S2 gene was then amplified by PCR using EpiTaq HS (Takara). The primer sequences for

PCR were as follows: forward, 5'-TAGGTTGTTTTGGATAAGGGAAGTT-3' and reverse, 5'-TAATCTCCCACAATTCCTAAAAAAC-3'. The thermocycler conditions consisted of an initial step at 98°C for 10 sec, followed by 40 cycles of a three-step PCR program consisting of 98°C for 10 sec, 53°C for 30 sec, and 72°C for 1 min. The PCR products were loaded onto a 1% agarose gel and run at 100 V for 30 min. The specific DNA bands were purified using a QIAquick Gel Extraction Kit (Qiagen) and cloned into a TA cloning vector (pCR 2.1 TOPO, Invitrogen). Ten clones for each cell line were analyzed by DNA sequencing.

Immunofluorescence

Cells were spun onto glass slides (Thermo Scientific), fixed in 1% paraformaldehyde in PBS, and then permeabilized with 0.1% (vol/vol) Triton X-100. The slides were stained with rabbit anti-nucleolin (Abcam), mouse anti-V5 (Invitrogen), goat anti-rabbit antibody conjugated to Alexa Fluor 488 (Invitrogen), or goat anti-mouse antibody conjugated to Alexa Fluor 555 (Invitrogen). The slides were mounted with a medium containing 4'-6'-diamidino-2-phenylindole (DAPI; Invitrogen) and analyzed under an Eclipse 90i (Nikon) microscope using the imaging software NIS Elements (Nikon).

Mouse xenograft model of CML

K562 cells were transduced with Migr1-G0S2 or empty retrovirus as a control and then purified by cell sorting. To induce subcutaneous tumors, 5×10^6 GFP-positive cells/100 μl were mixed with 100 μl of high-concentration basement membrane matrix (BD Bioscience) and injected subcutaneously into the rear flank of nude mice (Jackson Laboratories). The tumor size was measured every other day, and the volume was calculated as $V=(LxW^2)x0.52$, where L is the length and W is the width of the tumor. The mice were euthanized 21 days after implantation, and the tumors were removed for gross examination and immunohistochemical analysis. The implants were fixed in 10% buffered formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin. All mice were maintained under specific pathogen-free conditions at Baylor College of Medicine (Houston, TX, USA). All experiments were performed with the approval of the Institutional Animal Care and Usage Committee of Baylor College of Medicine.

Microarray analysis

Expression of the G0S2 gene in leukemic cells from CML patients (chronic phase) was analyzed using a public dataset at GEO (GSE5550) [18]. Baseline transformation to the median of healthy volunteer samples was performed using GeneSpring software (version 12.5). The significance of changes between CML and normal bone marrow cells was evaluated by a *t*-test.

Statistical analysis

A two-tailed unpaired Student's *t*-test was performed using GraphPad software. Differences were considered significant when the P value was < 0.05. Statistics are indicated in each figure legend.

Results

G0S2 expression in leukemic cell lines

We previously reported that G0S2 expression in hematopoietic stem cells is higher than in progenitor and mature blood cells [9]. In this work, we determined the levels of G0S2 transcripts in a panel of myeloid and lymphoid leukemic cell lines, using human monocytes as a reference (Fig. 1A). We included the following cell lines in this study: HEL

(erythroleukemia), K562 (CML), HL-60 (promyelocytic leukemia), Kasumi (acute myeloid leukemia), Jurkat (acute T cell leukemia), DND41 (acute T lymphoblastic leukemia), H9 (monocytic leukemia), and Call4 and Mutz5 (B cell acute lymphoblastic leukemia). All cell lines, with the exception of K562, showed barely detectable levels of G0S2 (Fig. 1A). G0S2 expression in K562 cells was significantly lower than in normal myeloid cells (Fig. 1A).

This finding suggested that G0S2 is likely silenced in leukemic cell lines; therefore, we measured G0S2 expression after treatment with 5-Aza because epigenetic methylation is an important mechanism for suppressing gene expression in normal and cancer cells [1]. K562 cells showed a 24-fold increase in G0S2 transcripts upon 5-Aza treatment, suggesting that the G0S2 gene was inactivated by DNA methylation (Fig. 1B). The level of G0S2 expression after demethylation was higher than in human monocytes ($CD14^+$ PBMCs). G0S2 expression was also increased upon 5-Aza treatment of the HEL, HL-60, and H9 cell lines, although not to the level observed in K562 cells. In contrast, the Jurkat, Kasumi, DND41, Call4, and Mutz5 cell lines did not exhibit increased expression of G0S2 after gene demethylation.

G0S2 promoter is methylated in K562 cells

The G0S2 gene is located in chromosome 1 (1q32.2) [2, 19]. An analysis of the GC content revealed that the promoter and two exons of the G0S2 gene are embedded in a region with high CpG content (Fig. 2A) [2]. DNA methylation is an important epigenetic mechanism that cells use to control gene expression during mammalian development [20]. Cancer cells often hypermethylate genes to silence the expression of regulators of cell growth and tumor suppression [1]. Hence, we examined methylation of the G0S2 gene in leukemia cells by performing bisulfite sequencing of the proximal promoter sequence's upstream start site, exon 1, and most of the coding sequence in exon 2 (Fig. 2A). This study revealed that G0S2 regulatory sequences and exon 1 are hypermethylated in K562 cells compared with HL-60, Kasumi, and normal $CD14^+$ cells (Fig. 2A). As expected, treatment of K562 cells with 5-Aza efficiently erased the G0S2 gene methylation (Fig. 2A). Correlating with G0S2 expression, treatment with 5-Aza caused a significant reduction in the growth of K562 cells (Fig. 2B). This decreased cell growth was associated with a reduction in the number of cells in the S phase of the cell cycle and a concomitant increase in the proportion of cells in the G_0/G_1 phase (Fig. 2C). Collectively, these data indicate that the G0S2 gene is silenced by DNA methylation in K562 cells, and thus, restoration of G0S2 expression by demethylation might reduce the cells' proliferative capacity, although this effect cannot be solely attributed to G0S2.

In normal hematopoietic cells, the hydrophobic domain of G0S2 physically interacts with the arginine-glycine rich domain of nucleolin, leading to nucleolin's cytosolic retention when G0S2 levels are elevated [9]. To correlate G0S2 upregulation induced by demethylation of the G0S2 gene with nucleolin localization in leukemic cells, we performed immunofluorescence staining to detect nucleolin in K562 cells that were left untreated or were cultured in the presence of 5-Aza. As controls, we used cell lines that did not exhibit increased G0S2 expression after treatment with the demethylating agent (Fig. 1B). In contrast to Kasumi and HL-60 cells, K562 cells treated with 5-Aza showed a predominant perinuclear localization of nucleolin, whereas control cells displayed the expected nucleolar distribution (Fig. 3). Because our observations could be due to the indirect effects of global DNA demethylation, we transduced K562 cells with a retrovirus carrying G0S2-specific shRNA and then performed 5-Aza treatment. First, we confirmed efficient silencing of G0S2 expression using two different shRNAs in K562 cells treated with 5-Aza and compared with a luciferase-specific shRNA used as a control (Fig. 4A). In contrast to G0S2 reactivation (Fig. 2B), 5-Aza treatment of K562 cells with constitutive expression of G0S2 shRNA resulted in increased proliferation due to a specific silencing of the "activated" G0S2

gene (Fig. 4A). Furthermore, the cytosolic retention of nucleolin upon 5-Aza treatment was prevented in cells with retroviral expression of G0S2 shRNA, which correlated with an increased percentage of cells in the S phase of the cell cycle (Fig. 4B). These data indicate that the expression of G0S2 caused inhibition of the proliferation in K562 cells.

G0S2 inhibits proliferation in K562 cells by interacting with nucleolin

To directly demonstrate that G0S2 caused the retention of nucleolin in the cytosol and cell cycle arrest, we used a retrovirus to overexpress V5-tagged G0S2. K562 cells transduced with Migr1-G0S2-V5 showed elevated expression of the G0S2 protein compared with expression in cells transduced with empty Migr1 retrovirus as a control (Fig. 5A). The overexpression of G0S2 alone resulted in reduced cell expansion, similar to the 5-Aza treatment (Fig. 5A). Next, we examined whether ectopic G0S2 expression led to perinuclear localization of nucleolin in K562 cells. Overexpression of cytosolic G0S2, which was monitored with anti-V5 antibody, was associated with a ring-like distribution of nucleolin around the nucleus (Fig. 5B). We confirmed an interaction between overexpressed G0S2 and endogenous nucleolin in K562 cells by reciprocal co-immunoprecipitation using protein lysates from K562 cells transduced with a retrovirus carrying V5-tagged G0S2 (Fig. 5C). Nucleolin was detected in anti-V5 immunoprecipitates, and G0S2 was detected in anti-nucleolin immunoprecipitates, suggesting a direct association between G0S2 and nucleolin in K562 cells. These findings led us to propose a model in which proliferating CML K562 cells express nucleolin predominantly in nucleoli and in which induction of G0S2 results in lower proliferation and retention of nucleolin in the cytosol (Fig. 5D).

Tumor suppressor function of G0S2 in human CML?

A molecular signature of $CD34^+$ hematopoietic stem and progenitor cells in patients with CML in the chronic phase has been reported previously [18]. We compared the expression of G0S2 between $CD34^+$ bone marrow cells from CML patients and $CD34^+$ cells from normal bone marrow using this published dataset to gain insights into human disease. Global gene expression analysis revealed a significant downregulation of G0S2 expression in CML patients (n = 9) compared with normal bone marrow cells (n = 8). Therefore, this finding suggests that G0S2 likely inhibits the expansion of leukemic cells (Fig. 6A).

The role of G0S2 in the proliferation of K562 cells was further examined *in vivo* by implanting leukemic cells embedded in Matrigel into the flank of nude mice, which is a preclinical model that is widely used to evaluate the expansion of human CML cells [21]. K562 transduced with empty retrovirus as a control grew rapidly in nude mice (Fig. 6B). In contrast, K562 cells transduced with Migr1-G0S2 retrovirus resulting in slow-growing subcutaneous tumors in nude mice (Fig. 6B). The tumors were excised 21 days after tumor implantation for gross morphology and histological analysis. Gross inspection of the implanted tumors showed a significant difference in size and revealed that two of three tumors failed to develop in the K562 Migr1-G0S2 group (Fig. 6C). The hematoxilin-eosin staining of tumors collected at 13 (not shown) and 21 (endpoint) days after implantation clearly indicated increased expansion of leukemic cells in the control and fewer K562 cells in the Migr1-G0S2 group, indicating that G0S2 suppressed the proliferation of K562 cells and the establishment of tumors (Fig. 6D).

Discussion

The unifying feature of cancer is a loss of control of cell proliferation, a process that is tightly regulated in normal cells to maintain the homeostasis of different tissues. In addition to blast cancer cells, a small subset of cancer cells, known as cancer stem cells, exhibits a low proliferative rate, similar to quiescent hematopoietic stem cells. A better understanding

of the mechanisms underlying cell division in normal and transformed cells is critical for the development of targeted therapy.

Cytosine methylation plays a critical role in the regulation of gene expression in normal cells. Cancer cells hypermethylate DNA rich in CpG sequences (CpG islands) in genes associated with tumor initiation and progression; therefore, methylation landscapes can be used as biomarkers to diagnose and treat cancer [22, 23]. Gene silencing by the methylation of regulatory sequences can occur not only during cancer development but also as a secondary event to ensure the expansion of transformed cancerous cells. CML is a clonal myeloproliferative disorder caused by the chromosomal translocation of t(9;22) in a single hematopoietic stem cell, which generates the BCR-ABL oncogene [16, 24, 25]. Most patients diagnosed in the chronic phase are successfully treated with tyrosine kinase inhibitors (i.e., imatinib, dasatinib, nilotinib) and can live free of overt leukemia while in treatment. However, relapse is nearly inevitable upon discontinuation of treatment with tyrosine kinase inhibitors, which suggests the survival of a population of chemoresistant leukemic stem cells. In the absence of treatment, CML progresses from a chronic phase to an accelerated phase and finally to a blast phase or blast crisis.

Interestingly, aberrant DNA methylation is associated with blast-phase CML progression and resistance to imatinib [26]. Furthermore, G0S2 was identified as part of a signature of genes downregulated in the blast phase compared with the chronic phase [12], suggesting a possible role for G0S2 in the development of CML. It was also intriguing to find that G0S2 is significantly downregulated in chronic-phase patients compared with normal bone marrow cells. G0S2 expression is also suppressed in patients with acute myeloid leukemia with high levels of CDX2 [13]. Thus, in the future, it will be important to define whether the G0S2 gene is hypermethylated in primary samples from leukemia patients. In the current work, we found that G0S2 was silenced by gene methylation of the proximal promoter and exon 1 in BCR-ABL-positive K562 cells and that restoration of G0S2 expression, either by retroviral transduction or gene demethylation, reduced the cells' proliferative rate. Retroviral expression of G0S2 in K562 cells significantly abrogated the cells' capacity to establish tumors and to proliferate at the level of control K562 cells in a xenograft model. Collectively, activation of the G0S2 gene could be a reasonable therapeutic approach to preventing the expansion of leukemia cells.

The role of G0S2 in carcinogenesis and chemoresistance is slowly being revealed, particularly in solid tumors. A pro-apoptotic function of G0S2 was shown by direct interaction with Bcl-2 in non-small cell lung cancer and colorectal carcinoma cell lines [8]. The hydrophobic domain of G0S2 binds Bcl-2 and nucleolin, suggesting that this domain is critical for protein-to-protein interactions [8, 9]. However, we did not observe increased apoptosis in K562 cells transduced with a retrovirus carrying G0S2 (data not shown), suggesting that levels of Bcl-2 are most likely low in K562 cells. A putative tumor suppressor role is supported by several studies that showed that G0S2 is epigenetically silenced by gene methylation in several cancer cell lines [10, 11, 27]. A correlation analysis of the methylation status and histological cancer type of more than 100 patient samples revealed that G0S2 was hypermethylated in 15% of squamous lung cancer cases compared with 2.6% in non-squamous lung cancer cases [27], indicating a potential use for G0S2 methylation as a biomarker in squamous lung cancer.

G0S2 retains nucleolin in the cytosol of K562 cells, similar to normal hematopoietic cells, likely preventing the pro-proliferative functions of nucleolin, such as effects on transcription, ribosome biosynthesis, transcript stability, and translation [9, 28]. Thus, downregulation of G0S2 expression in K562 cells prevents interaction with nucleolin, releasing the proliferation brake. The cytosolic localization of nucleolin is not

unprecedented, as nucleolin has been found in the cytoplasm of chronic lymphocytic leukemia cells with high levels of Bcl-2, whereas in normal B cells, nucleolin is predominantly in nucleoli [29]. This phenomenon is likely associated with the stabilization of Bcl-2 transcripts by the binding of nucleolin to 5' ARE sites [30].

Collectively, our data support a model of downregulation of G0S2 expression to prevent cell cycle arrest induced by the interaction between G0S2 and nucleolin in K562 cells (Fig. 5D). Hence, G0S2 is emerging as a previously unrecognized inhibitor of cell division in K562 leukemic cells. Modulation of G0S2 expression may offer a therapeutic window to control the expansion of leukemia cells in patients with CML.

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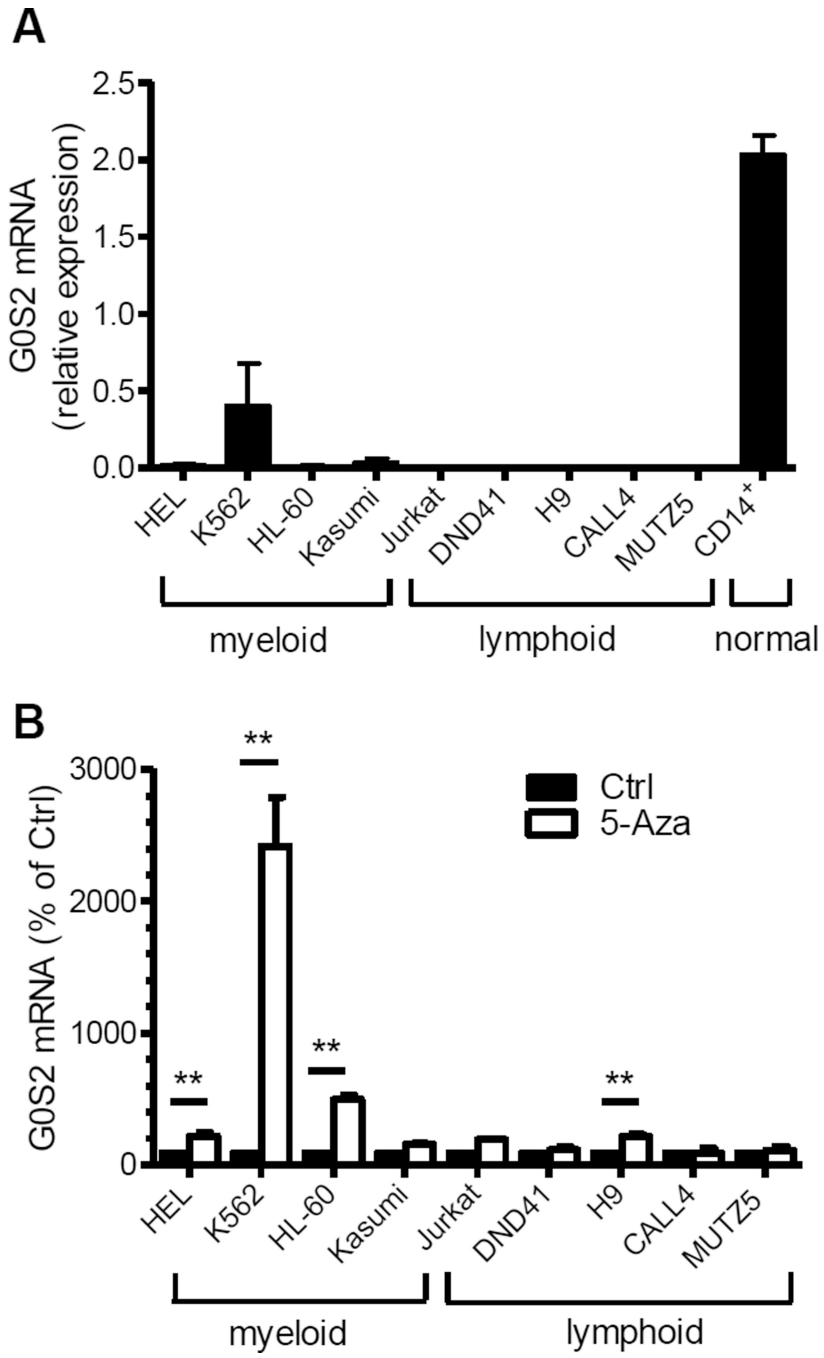

Figure 1. Expression of G0S2 in human leukemic cell lines

(A) G0S2 mRNA expression was measured by qPCR in human leukemia cell lines and normal $CD14^+$ cells. The expression of G0S2 mRNA was normalized to β-actin mRNA expression. (B) Leukemic cells were cultured in the presence of 5-Aza (10 μM) to induce gene demethylation. G0S2 mRNA expression was then measured by qPCR and is expressed as a percentage of the untreated control (Ctrl) for each cell line. Two-tailed Student's *t*-test (**, $P < 0.01$; n = 3-4).

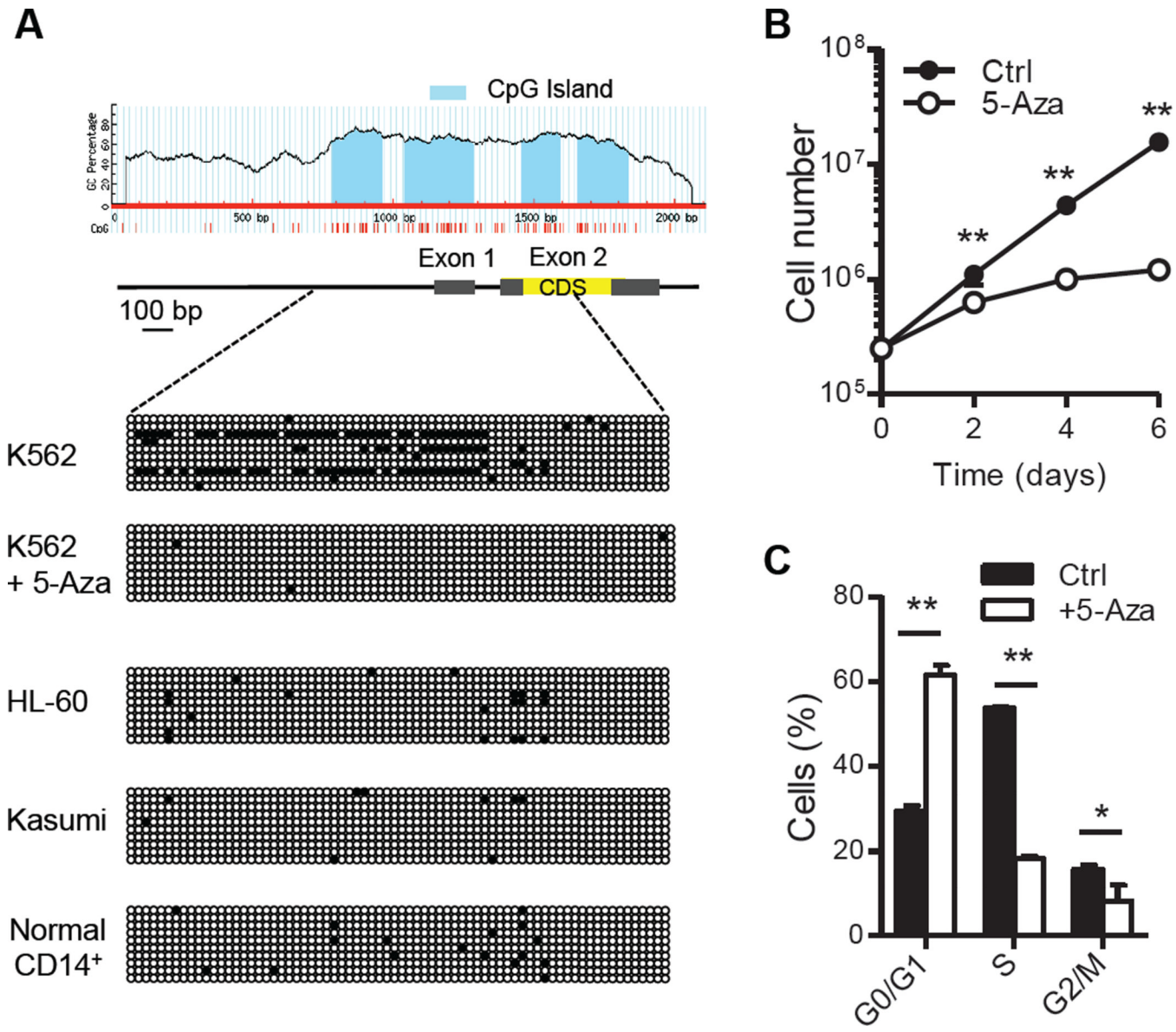

Figure 2. Methylation of the G0S2 gene correlates with the proliferation of K562 cells

(A) Schematic diagram of the GC content of the genomic region containing the G0S2 gene. Large CpG islands include the proximal promoter and two exons containing the coding sequence (CDS). The PCR products of genomic DNA treated with bisulfite were cloned and sequenced (10 clones per cell line). The open and filled circles represent unmethylated and methylated CpG, respectively. (B) Cell growth of K562 cells with or without 5-Aza treatment (n = 3). (C) DNA content was analyzed using FlowJo software (n = 3). Two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

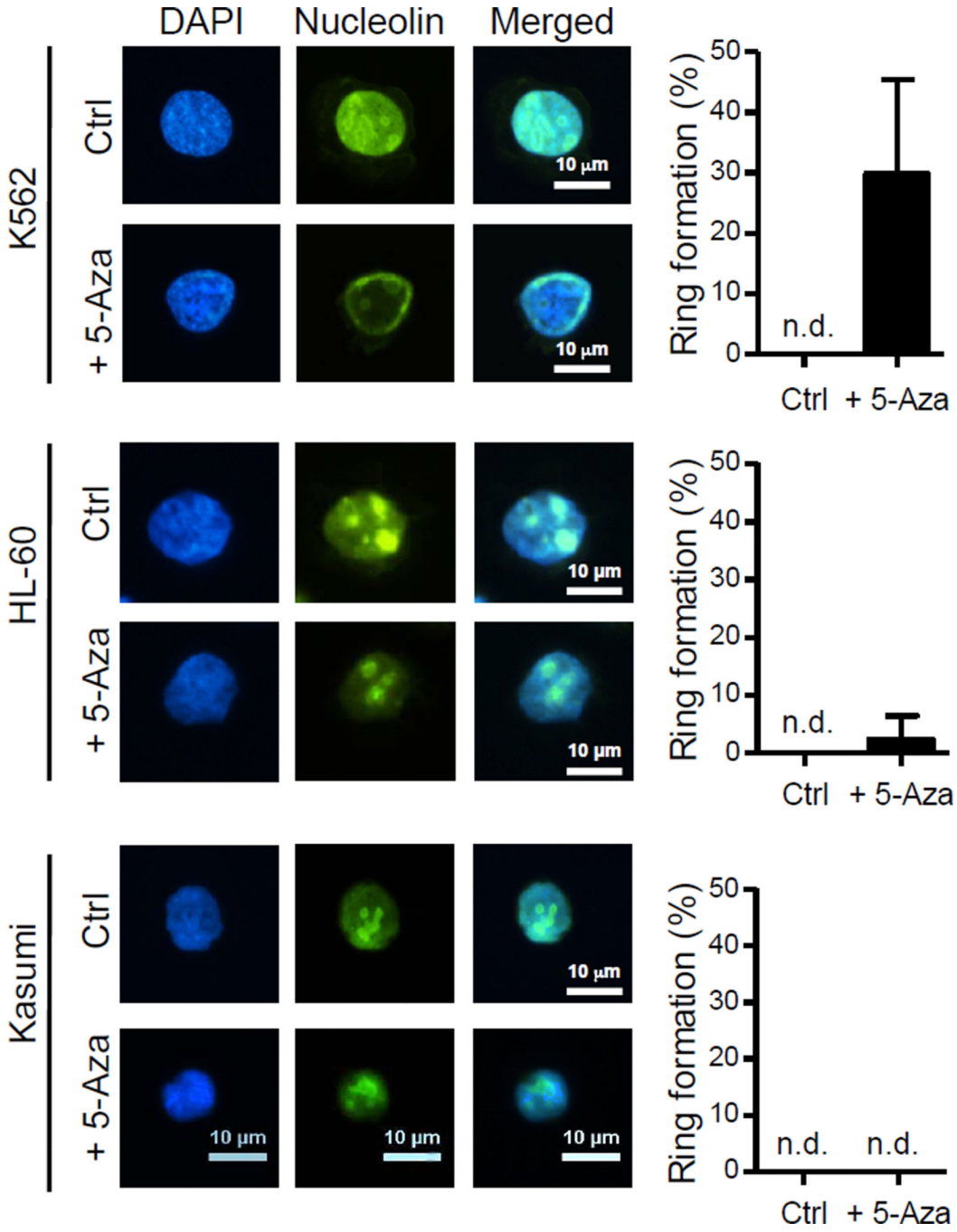

Figure 3. K562 cells treated with 5-Aza exhibit cytosolic sequestration of nucleolin

Immunofluorescence-based detection of nucleolin in leukemia cells with or without 5-Aza treatment. The expression of nucleolin is shown in green fluorescence, and nuclei (DAPI) are indicated in blue. The frequency of a ring-like distribution of nucleolin for each condition is shown on the right (n ≥ 3 per group; n.d.: not detected).

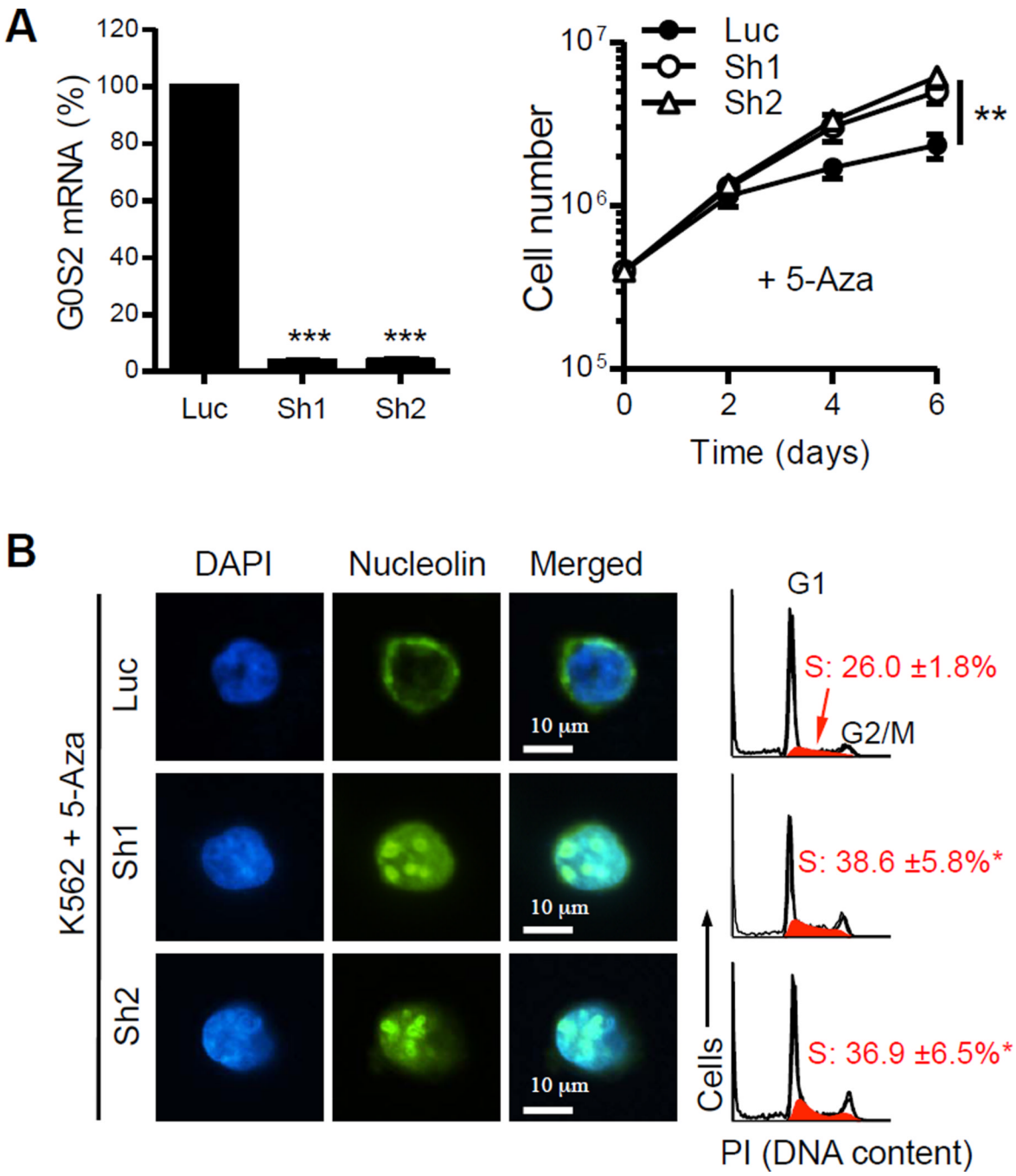

Figure 4. G0S2 gene silencing abrogates inhibition of the proliferation and cytosolic sequestration of nucleolin in K562 cells

(A) K562 cells were transduced with pSIREN-shRNA retrovirus carrying G0S2-specific (sh1 and sh2) or luciferase-specific (control) shRNA. The efficiency of gene silencing was determined by qPCR after reactivation of the G0S2 gene by 5-Aza treatment. The effect on cell proliferation is shown on the right. (B) Immunofluorescence-based detection of nucleolin in K562 cells transduced with sh1, sh2, or Luc pSIREN retrovirus cultured in the presence of 5-Aza (10 μM). The DNA content was determined by cell cycle analysis, and the percentage of cells in the S phase is indicated on the histograms. Two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; n = 3).

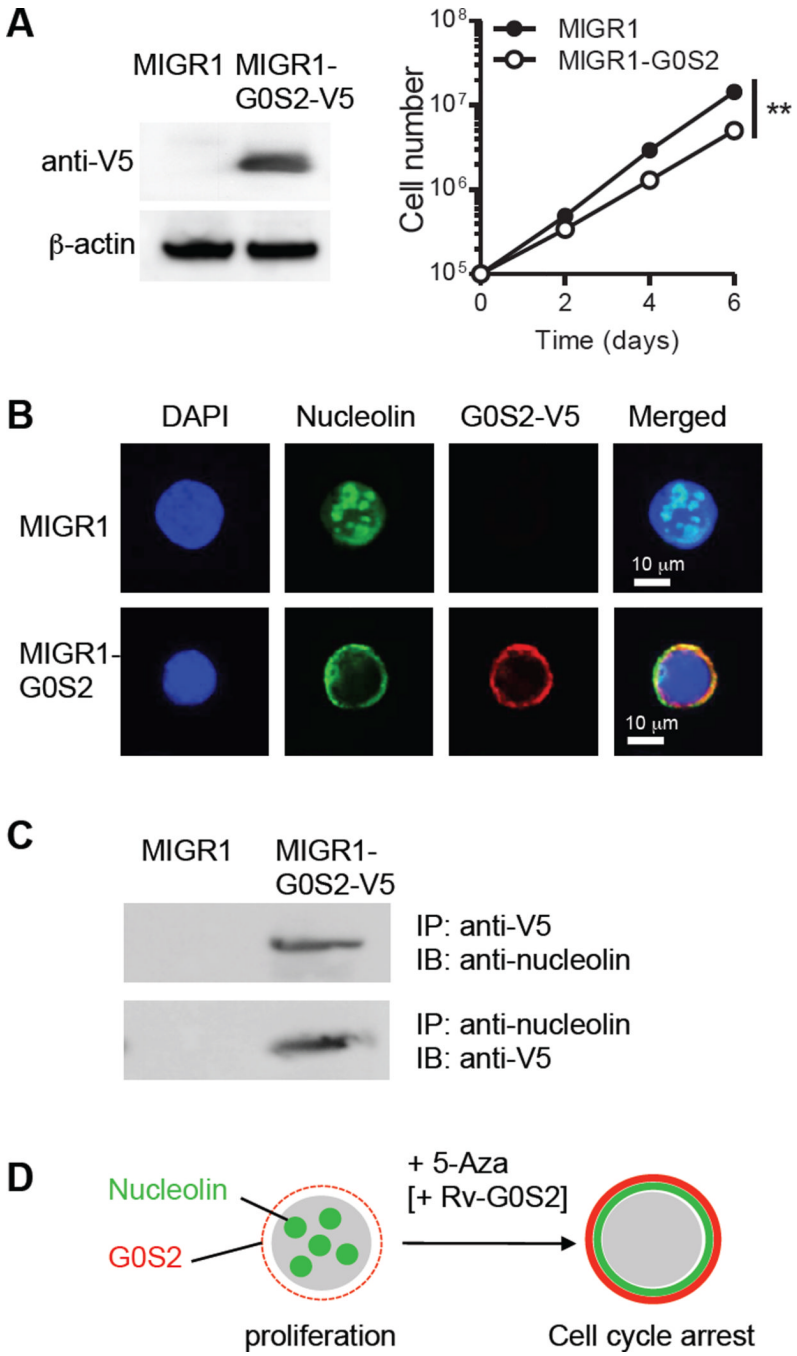

Figure 5. G0S2 inhibits proliferation in K562 cells by interacting with nucleolin

(A) K562 cells were transduced with a retrovirus containing V5-tagged G0S2 (Migr1-G0S2) or empty retrovirus as a control (Migr1). Immunoblots revealed retroviral expression of G0S2 in the K562 cells. β-actin was used as a control. (B) Detection of nucleolin (green) and V5-tagged G0S2 (red) in K562 cells by immunofluorescence staining. Staining of the nuclei with DAPI is shown in blue. (C) Reciprocal immunoprecipitation with anti-V5 and anti-nucleolin antibodies. (D) Diagram depicting the control of proliferation in CML leukemia cells by the interaction between G0S2 and nucleolin. The data are representative of three independent experiments. Two-tailed Student's *t*-test (**, $P < 0.01$; n = 3).

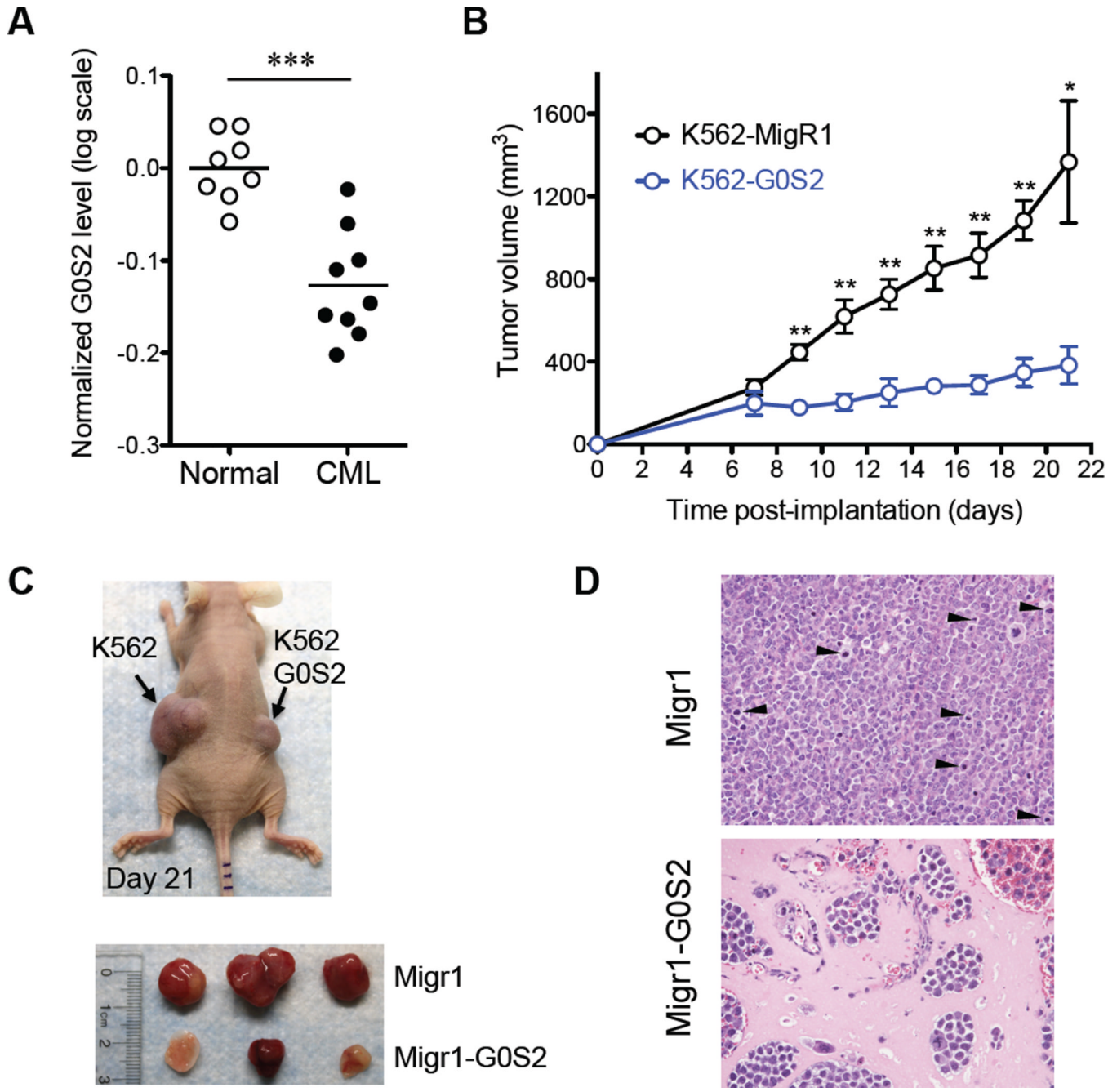

Figure 6. G0S2 expression is downregulated in chronic-phase CML patients and promotes tumor growth in a CML xenograft model

(A) Microarray analysis (GSE5550) of G0S2 expression in $CD34^+$ bone marrow cells from chronic-phase CML patients (n = 9) compared with normal bone marrow cells (n = 8). Two-tailed Student's *t*-test (***, $P < 0.001$). (B) Rate of tumor growth of K562 cells transduced with either Migr1 (empty retrovirus) or Migr1-G0S2 implanted into the flank of nude mice. The data represent the mean and SEM (n = 4, $P = 0.0057$). Two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$). (C) Representative nude mouse displaying a control tumor on the left flank and a G0S2-overexpressing tumor on the right flank. The implants were collected at

the end of the experiment. (D) Hematoxilin-eosin staining was performed on tumors harvested at the end of the experiment (day 21). Arrowheads indicate cells in mitosis.