

STAT3 β is a tumor suppressor in acute myeloid leukemia

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Key Points

- The *STAT3 β / α* mRNA expression ratio in AML patients is a favorable prognostic marker and positively correlates with overall survival.
- Transgenic *Stat3 β* expression delays disease progression and prolongs overall survival in AML mouse models.

Signal transducer and activator of transcription 3 (STAT3) exists in 2 alternatively spliced isoforms, STAT3 α and STAT3 β . Although truncated STAT3 β was originally postulated to act as a dominant-negative form of STAT3 α , it has been shown to have various STAT3 α -independent regulatory functions. Recently, STAT3 β gained attention as a powerful antitumorigenic molecule in cancer. Deregulated STAT3 signaling is often found in acute myeloid leukemia (AML); however, the role of STAT3 β in AML remains elusive. Therefore, we analyzed the *STAT3 β / α* messenger RNA (mRNA) expression ratio in AML patients, where we observed that a higher *STAT3 β / α* mRNA ratio correlated with a favorable prognosis and increased overall survival. To gain better understanding of the function of STAT3 β in AML, we engineered a transgenic mouse allowing for balanced *Stat3 β* expression. Transgenic *Stat3 β* expression resulted in decelerated disease progression and extended survival in PTEN- and MLL-AF9-dependent AML mouse models. Our findings further suggest that the antitumorigenic function of STAT3 β depends on the tumor-intrinsic regulation of a small set of significantly up- and downregulated genes, identified via RNA sequencing. In conclusion, we demonstrate that STAT3 β plays an essential tumor-suppressive role in AML.

Introduction

Signal transducer and activator of transcription 3 (STAT3) is a key transcription factor in cell proliferation, maturation, and survival and hence involved in several oncogenic pathways. Although STAT3 is most commonly described as an oncogene in cancer, evidence also shows its role as a tumor suppressor.¹⁻⁸ This opposing role of STAT3 in cancer depends considerably on its expression as different isoforms.^{1,9} Alternative splicing gives rise to full-length STAT3 α and truncated STAT3 β . STAT3 β variants lack the canonical STAT3 C-terminus and instead contain 7 unique amino acids, resulting in enhanced DNA binding affinity.¹⁰ Both isoforms are ubiquitously expressed; however, although the loss of STAT3 α leads to embryonic lethality in mice, STAT3 β is not required for viability.¹¹ Accordingly, STAT3 β was originally described as a dominant-negative regulator of STAT3.^{10,12,13} Despite that, several studies have postulated an active regulatory role for STAT3 β and identified a large number of STAT3 β -specific target genes.^{11,13-16} Recently, STAT3 β has gained attention as a powerful antitumorigenic molecule, as shown for melanoma, esophageal squamous cell carcinoma, and breast, lung, and colon cancers.¹⁷⁻²⁴

STAT3 has been shown to be constitutively active in AML cell lines and patients, causing a proliferative advantage and apoptosis protection.²⁵⁻²⁸ In addition, STAT3 activity has been associated with short disease-free survival in a subset of AML patients.²⁹⁻³² As a consequence, STAT3 became an attractive therapeutic target in AML, but results from early clinical studies with STAT3 inhibitors have shown moderate effectiveness.^{28,33-35} In contrast to these studies, Redell et al³⁶ described a protective role of increased STAT3 phosphorylation upon cytokine stimulation in AML patients, correlating with high disease-free survival. These conflicting results in AML might indicate that the impact of STAT3 inhibition in different patient subsets can vary as a result of its heterogeneous biological context.³⁷

An improved understanding of the biological functions of STAT3 isoforms in AML is therefore required. Here, we conducted an extensive study regarding the specific role of STAT3 β in AML. We found that the balance of STAT3 isoform expression in AML patients can serve as a favorable prognostic tool. Furthermore, we demonstrate that the transgenic expression of STAT3 β impairs leukemia progression. Taken together, our data identify STAT3 β as a novel tumor suppressor in AML.

Patients and methods

AML patients

This study was approved by the ethics committees of the Medical Universities of Graz and Vienna (Austria) and conducted according to the Declaration of Helsinki. Written informed consent was obtained. Diagnostic peripheral blood (PB) and bone marrow (BM) samples from AML patients were enriched for mononuclear cells using Ficoll (GE Healthcare, Milan, Italy) density-gradient centrifugation before freezing in fetal bovine serum (Gibco; Thermo Fisher Scientific, Waltham, MA) with 10% dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO), as previously described.³⁸ All samples contained >80% blast cells after enrichment. Healthy CD34⁺ hematopoietic stem and progenitor cells (HSPCs) were collected from umbilical cord blood and enriched by magnetic-activated cell sorting (Miltenyi Biotec, Gladbach, Germany).

Cytogenetic risk stratification of patients into favorable, intermediate, and adverse subgroups was applied according to the Medical Research Council classification scheme.³⁹ In a proportion of patients, information on the mutational status of the *FLT3*, *NPM1*, and *CEBPA* genes was available. Only patients receiving treatment with curative intention (chemotherapy with or without allogeneic hematopoietic stem cell transplantation) were included in the analysis of overall survival as defined by the European LeukemiaNet 2017 guidelines.⁴⁰ In accordance with literature describing an association of oncogenic *FLT3* mutations with JAK/STAT activity, primarily STAT5 and STAT3, we furthermore excluded patients with a confirmed *FLT3* mutation in survival analysis.⁴¹⁻⁴⁶

Generation of Stat3 β transgenic mice

A *Stat3 β* expression cassette containing the CAGGS promoter, a *loxP*-flanked (floxed) transcriptional/translational stop cassette, N-terminus FLAG-tagged *Stat3 β* mouse complementary DNA (cDNA), internal ribosomal entry site (*IRES*)/yellow fluorescent protein (*YFP*), and a bovine growth hormone-derived polyadenylation signal flanked by *attB* sites was assembled by conventional cloning. The *Stat3 β* expression cassette was integrated into

a bacterial artificial chromosome (BAC) containing the *Rosa26* locus (*RP24-85L15*) by PhiC31 integrase-mediated cassette exchange.⁴⁷ *Rosa26*-modified BAC DNA was purified⁴⁸ and microinjected into the pronucleus of C57BL/6 oocytes.

Animal studies

Animal experiments were approved by the Animal Ethics Committee of the Medical University of Vienna and the Austrian Federal Ministry of Education, Science and Research. Mice were bred and kept under pathogen-free conditions at the Institute of Pharmacology, Medical University of Vienna (Vienna, Austria). *Stat3 β* transgenic (*Stat3 β ^{TG}*) mice were crossed with *Mx1-Cre* recombinase transgenic (*MxCre*) floxed *Pten* mice.^{49,50} To induce *Cre*, animals were intraperitoneally injected with 100 μ L of polyinosine-polycytidine (plpC; 2 mg/mL; Sigma Aldrich) at the age of 5 to 7 weeks on 3 consecutive days.

Transplantation of FLCs

Fetal liver cells (FLCs) were isolated from *Stat3 β ^{TG}* and wild-type (wt) mice at embryonic day 13.5, genotyped, and frozen in fetal bovine serum with 10% dimethyl sulfoxide. Platinum E cells were transfected by calcium phosphate coprecipitation with the *pMSCV-MLL-AF9-IRES-Venus* vector. FLCs were thawed 1 day before infection. Cells were spinoculated (1000g for 90 minutes) with retroviral supernatant in the presence of 10 μ g/mL of polybrene (Sigma Aldrich). After 24 hours, 2×10^6 FLCs were injected into immunocompromised *NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* (The Jackson Laboratory, Bar Harbor, ME) mice via the tail vein. Infection rates for *MLL-AF9/Venus⁺* (*Venus⁺*) cells ranged from 7% to 12%. Four weeks after transplantation, mice were injected with plpC, as described in "Animal studies."

Statistics

Patient data. *STAT3 β / α* messenger RNA (mRNA) ratio values were compared between groups using the Kruskal-Wallis test followed by pairwise comparisons corrected for multiplicity by Dunn's method. To test the association between the *STAT3 β / α* mRNA ratio and survival of patients with AML, a multivariable Cox regression model was used. In addition to the log*STAT3 β / α* ratio as a continuous variable, the model included and thus corrected for established AML risk parameters: patient age at diagnosis, percentage of blasts of all white blood cells (WBCs), and cytogenetic risk group as categorical predictor. To show the effect of log*STAT3 β / α* ratio on the survival function, it was plotted once for the maximally observed ratio, once for the median, and once for the lowest observed ratio. To visualize the change in hazard ratio (HR) associated with a certain *STAT3 β / α* ratio change, it was calculated relative to the estimated survival at the median *STAT3 β / α* ratio using Exp(B) and its 95% confidence interval.

Animal and cellular data. Data were analyzed using log-rank Student *t* tests (Mantel Cox), Student *t* tests, and 1-way analyses of variance in combination with Tukey's multiple comparison post hoc test. Error bars represent means \pm standard deviations. *P* < .05 was regarded as statistically significant. *P* values are indicated as follows: **P* < .05, ***P* < .01, and ****P* < .001. The absence of a *P* value or asterisk indicates nonsignificance. Additional methods are provided in the supplemental material.

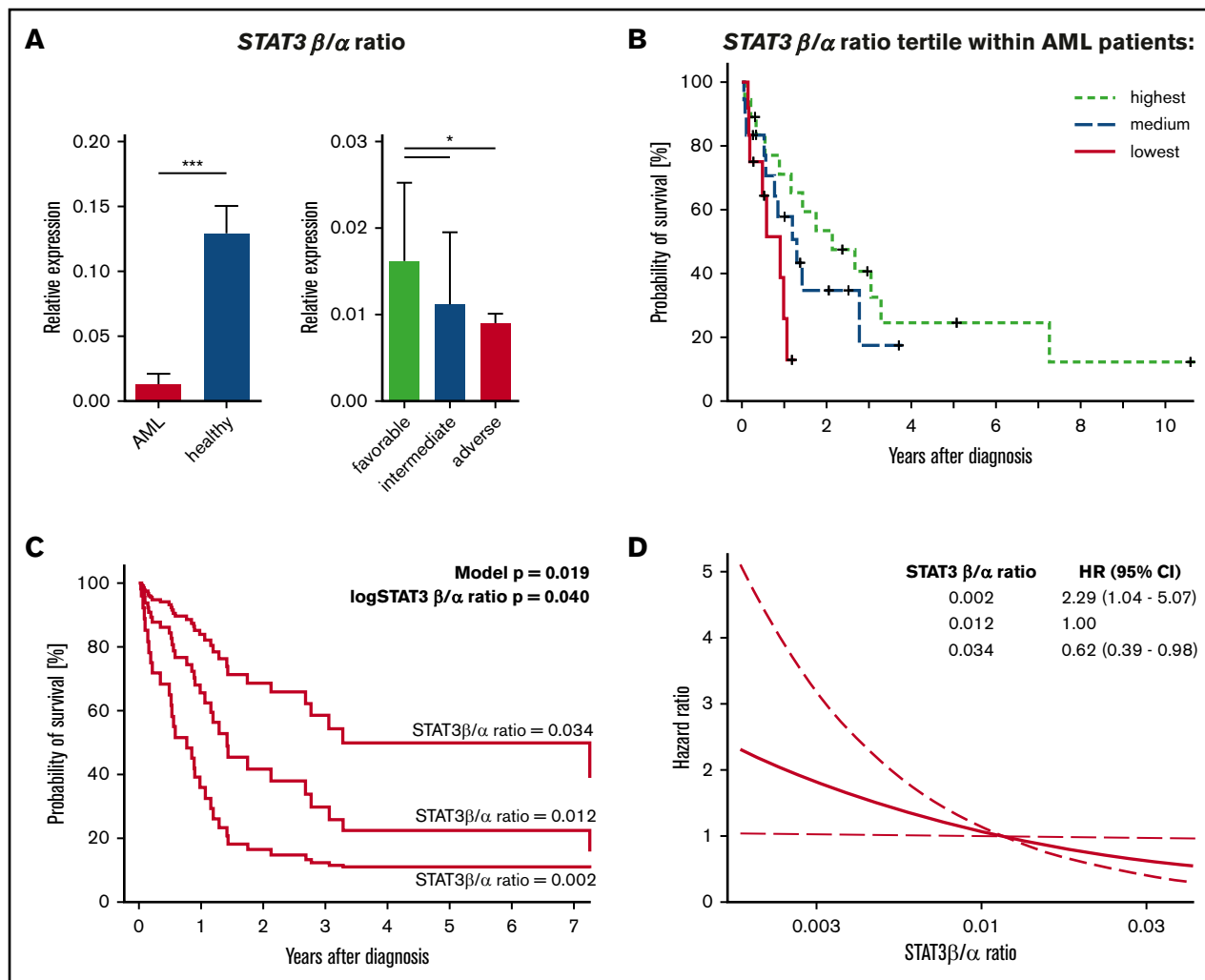


Figure 1. A higher $STAT3\beta/\alpha$ mRNA ratio correlates with clinical prognosis and survival in AML patients. (A) $STAT3\beta/\alpha$ mRNA ratio (normalized to β -ACTIN) in AML patients ($n = 94$) and HSPCs from healthy controls ($n = 8$), and $STAT3\beta/\alpha$ mRNA ratio in AML patients with a favorable ($n = 23$), intermediate ($n = 22$), or adverse ($n = 49$) prognosis. Data were compared using the Student t test and Kruskal-Wallis test. (B) Kaplan-Meier plot showing the survival of patients with a $STAT3\beta/\alpha$ mRNA ratio in the highest, medium, or lowest tertile ($n = 48$). (C) Estimated survival functions resulting from multivariable Cox regression. In addition to the $\log STAT3\beta/\alpha$ mRNA ratio in its continuous form, the model includes and thus adjusts for patient age, blast count, and cytogenetic risk category at diagnosis. For illustrative purposes, curves were plotted for the maximal, median, and minimal observed $STAT3\beta/\alpha$ mRNA ratios. The model P value refers to the hypothesis that all variables in the model collectively predict survival; the $\log STAT3\beta/\alpha$ mRNA P value tests whether the ratio predicts survival independent of other variables. Of note, the estimated survival changes continuously with the $STAT3\beta/\alpha$ mRNA ratio. Therefore, the survival can be plotted for any $STAT3\beta/\alpha$ mRNA ratio value. To visualize the magnitude of survival difference between the maximally observed (0.034) and the minimally observed (0.002), we plotted survival function for these values and for the median value as reference. Modeling the effect of $STAT3\beta/\alpha$ mRNA ratio in its continuous form allowed avoidance of arbitrary groups. (D) Change of estimated HR (thick line) with 95% confidence interval (CI; dashed lines) associated with different $STAT3\beta/\alpha$ mRNA ratio levels. The median $STAT3\beta/\alpha$ mRNA ratio was chosen as reference, not affecting the statistical analysis. The curve was plotted for a range of $STAT3\beta/\alpha$ mRNA ratios spanning from the minimum to maximum observed ratio. Estimated HRs are given for the upper and lower ends of the curve. * $P < .05$, *** $P < .001$.

Results

Higher ratio of $STAT3\beta/\alpha$ mRNA expression correlates with favorable clinical prognosis and prolonged survival in AML patients

Differential expression of $STAT3$ isoforms has been observed in AML patients, but whether their role is of oncogenic or tumor-suppressive nature is incompletely understood.²⁹⁻³² We obtained diagnostic samples from 94 AML patients (supplemental Table 1)

and analyzed mRNA expression of $STAT3$ isoforms in comparison with HSPCs from 8 healthy donors. The mRNA expression of total $STAT3$, $STAT3\alpha$, and especially $STAT3\beta$ differed between leukemic blasts and healthy HSPCs. We found significantly decreased mRNA expression levels of $STAT3\beta$ in AML samples compared with healthy HSPCs (supplemental Figure 1A). Next, patients of different cytogenetic risk groups were compared regarding $STAT3$ expression levels (supplemental Figure 1B). Strikingly, we found that the $STAT3\beta/\alpha$ mRNA expression ratio in AML samples was significantly lower

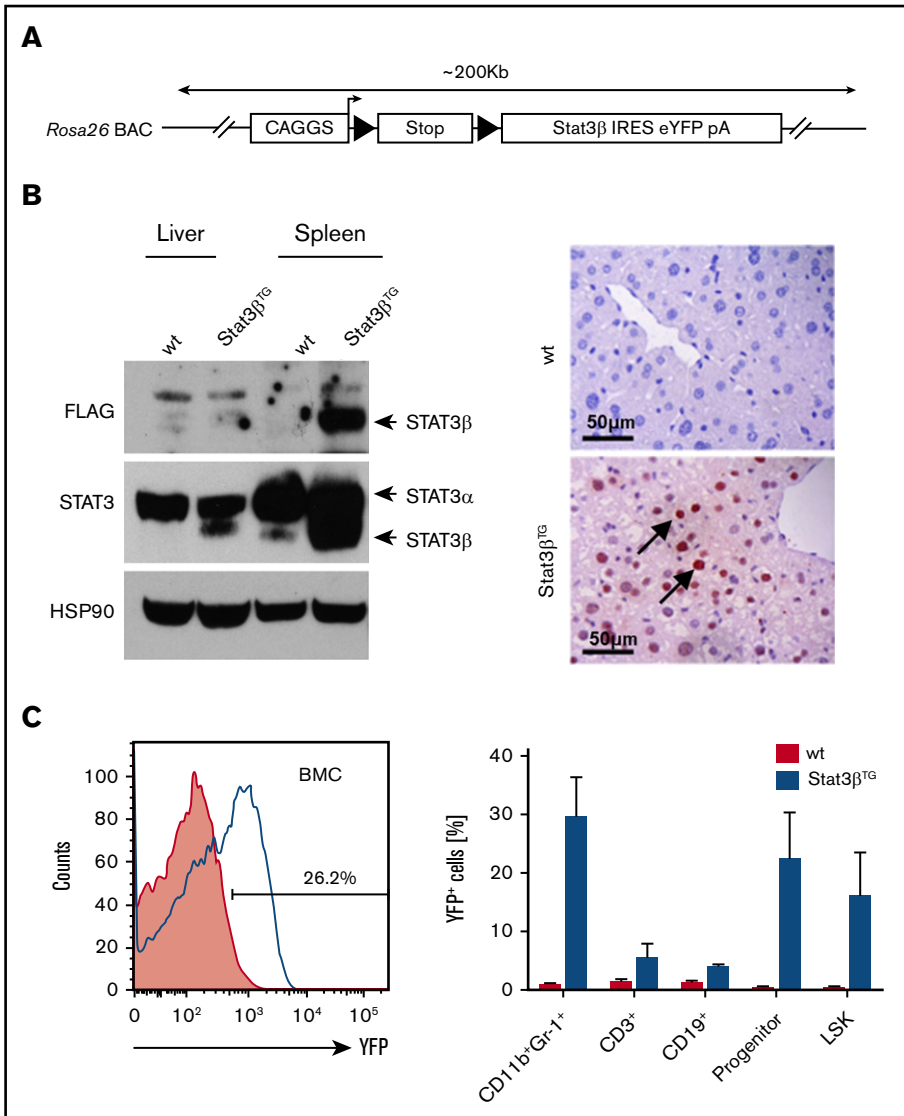


Figure 2. Generation and characterization of a *Stat3β* transgenic mouse model. (A) A schematic overview of the *Stat3β* transgene BAC construct. (B) Total cell lysates from spleen and liver were subjected to western blot analysis with the indicated antibodies (STAT3, FLAG, and HSP90). Liver sections stained with an antibody against FLAG show the expression of ectopic STAT3β. (C) A representative flow cytometry analysis of YFP⁺ BM cells (BMCs) and hematopoietic cell populations (progenitor: Lin⁻Sca-1⁺c-Kit⁻; LSK: Lin⁻Sca-1⁺c-Kit⁺) at day 20 postinduction. Data (wt vs Stat3β^{TG}) were not statistically compared.

than that in healthy HSPCs and that a high *STAT3β/α* mRNA ratio correlated with a favorable clinical prognosis (Figure 1A). Furthermore, we explored the correlation between the *STAT3β/α* mRNA ratio in AML patients ($n = 48$) and overall survival. A higher *STAT3β/α* mRNA ratio was associated with longer overall survival in AML patients (Figure 1B) after adjustment for patient age at diagnosis, percentage of blasts, and cytogenetic risk category (Figure 1C). A 10-fold increase of the *STAT3β/α* mRNA ratio was estimated to be associated with an HR of 0.34 (95% confidence interval, 0.12-0.95; Figure 1D). Taken together, the *STAT3β/α* mRNA ratio correlates with clinical prognosis and survival in AML patients, arguing for the importance of strict maintenance of balanced *STAT3β/α* mRNA expression.

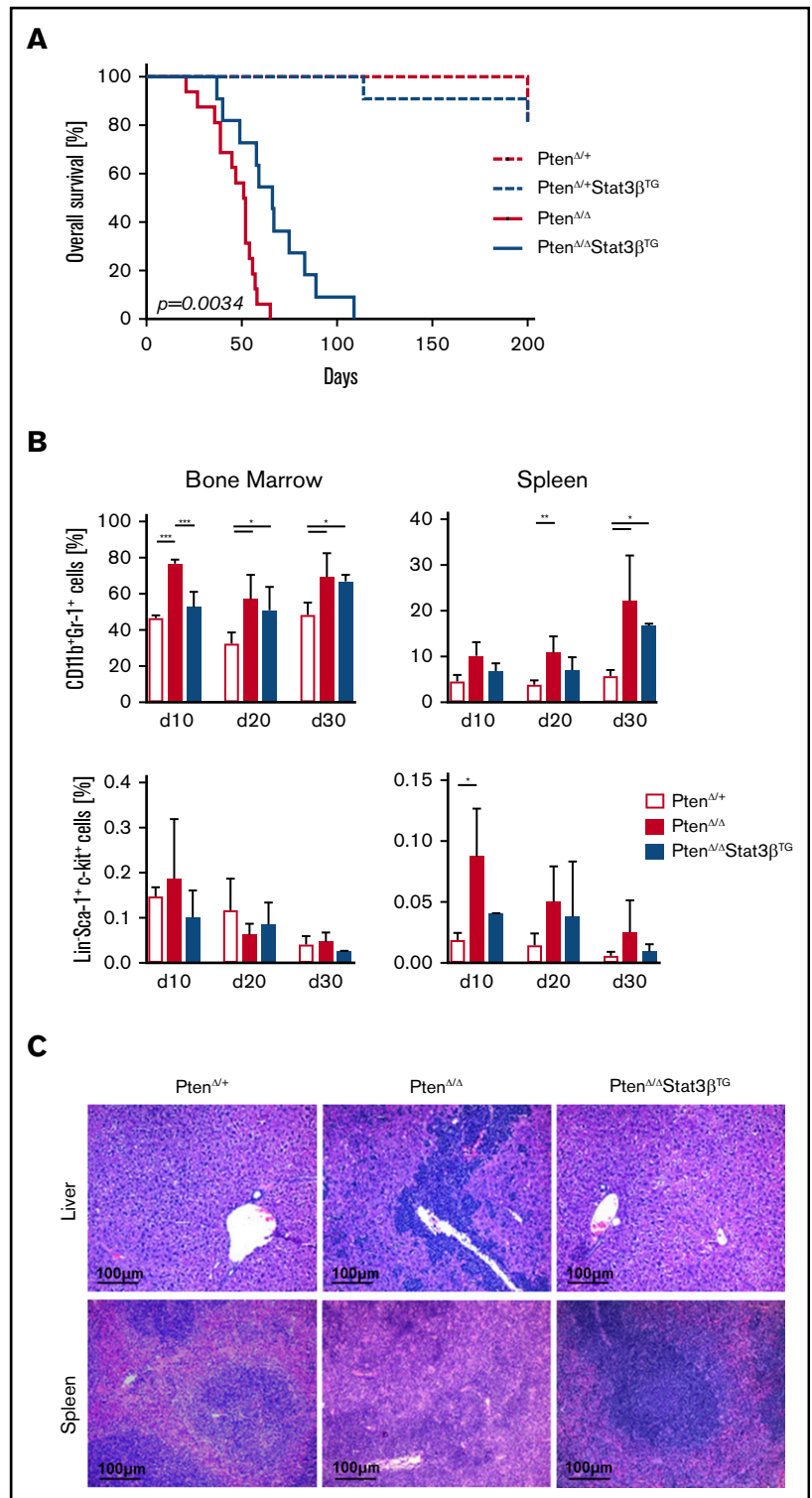
Generation and characterization of a novel *Stat3β* transgenic mouse model

To investigate the role of STAT3β, we established a novel mouse line conditionally expressing murine *Stat3β*. We chose a BAC containing the *Rosa26* locus, because this has been shown to be

open chromatin and support transgene expression. The construct for the generation of *Stat3β*^{TG} mice consisted of a CAGGS promoter followed by a floxed *Stop* cassette, FLAG-tagged murine *Stat3β* cDNA, and *IRES* and *YFP* (Figure 2A). Purified BAC DNA was microinjected into the pronucleus of C57BL/6 oocytes, and *Stat3β*^{TG} mice were identified via Southern blot and genotyping (supplemental Figure 2A). Conditional expression of the transgene was achieved by crossing *Stat3β*^{TG} mice with a plpC-inducible MxCre mouse line on a C57BL/6 background. The resulting mice, heterozygous for *Cre* and the *Stat3β* transgene (*Stat3β*^{TG}), were injected with plpC, causing the expression of CRE recombinase and the excision of the *Stop* cassette and allowing for the expression of exogenous STAT3β. Ectopic expression of STAT3β was confirmed in liver and spleen 20 days postinduction (Figure 2B). YFP was detected in >20% of BMCs as well as in hematopoietic cell subsets (Figure 2C).

Stat3β^{TG} mice were found to be phenotypically normal before and after induced *Stat3β*^{TG} expression (followed up to 12 months of life; data not shown). WBC count and relative spleen weight

Figure 3. Elevated expression of STAT3 β prolongs survival in an AML mouse model based on *Pten* deletion. (A) Kaplan-Meier plot showing the significantly different survival ($P = .0034$) of *Pten* $^{\Delta/\Delta}$ ($n = 16$) and *Pten* $^{\Delta/\Delta}$ Stat3 β^{TG} ($n = 11$) mice, with *Pten* $^{+/+}$ ($n = 9$) and *Pten* $^{+/+}$ Stat3 β^{TG} ($n = 11$) as controls. (B) Percentages of myeloid (CD11b $^{+}$ Gr1 $^{+}$) and LSK cells in the BM and spleen of control, *Pten* $^{\Delta/\Delta}$, and *Pten* $^{\Delta/\Delta}$ Stat3 β^{TG} mice at 10, 20, and 30 days postinduction ($n = 5$ per group). Data (*Pten* $^{+/+}$ vs *Pten* $^{\Delta/\Delta}$ vs *Pten* $^{\Delta/\Delta}$ Stat3 β^{TG} for each time point) were compared using 1-way analysis of variance. (C) H&E-stained liver and spleen sections of *Pten* $^{+/+}$, *Pten* $^{\Delta/\Delta}$ Stat3 β^{TG} , and control mice at day 20 postinduction. * $P < .05$, ** $P < .01$, *** $P < .001$.



(normalized to body weight) in Stat3 β^{TG} mice were comparable to those of plpC-treated wt animals (supplemental Figure 2B). Furthermore, hematopoietic cell populations in BM and spleen were unaffected by Stat3 β^{TG} expression (supplemental Figure 2C). In accordance, hematoxylin and eosin (H&E) staining of spleen and liver sections did not reveal any overt abnormalities in organ architecture or extramedullary hematopoiesis (supplemental Figure 2D).

Elevated expression of STAT3 β prolongs survival in an AML mouse model based on *Pten* deletion

The impact of STAT3 β on AML progression in vivo was investigated via the homozygous knockout of *Pten* in the hematopoietic compartment. Conditional ablation of *Pten* rapidly induces myeloproliferative neoplasms, which develop into secondary AML.^{49,50}

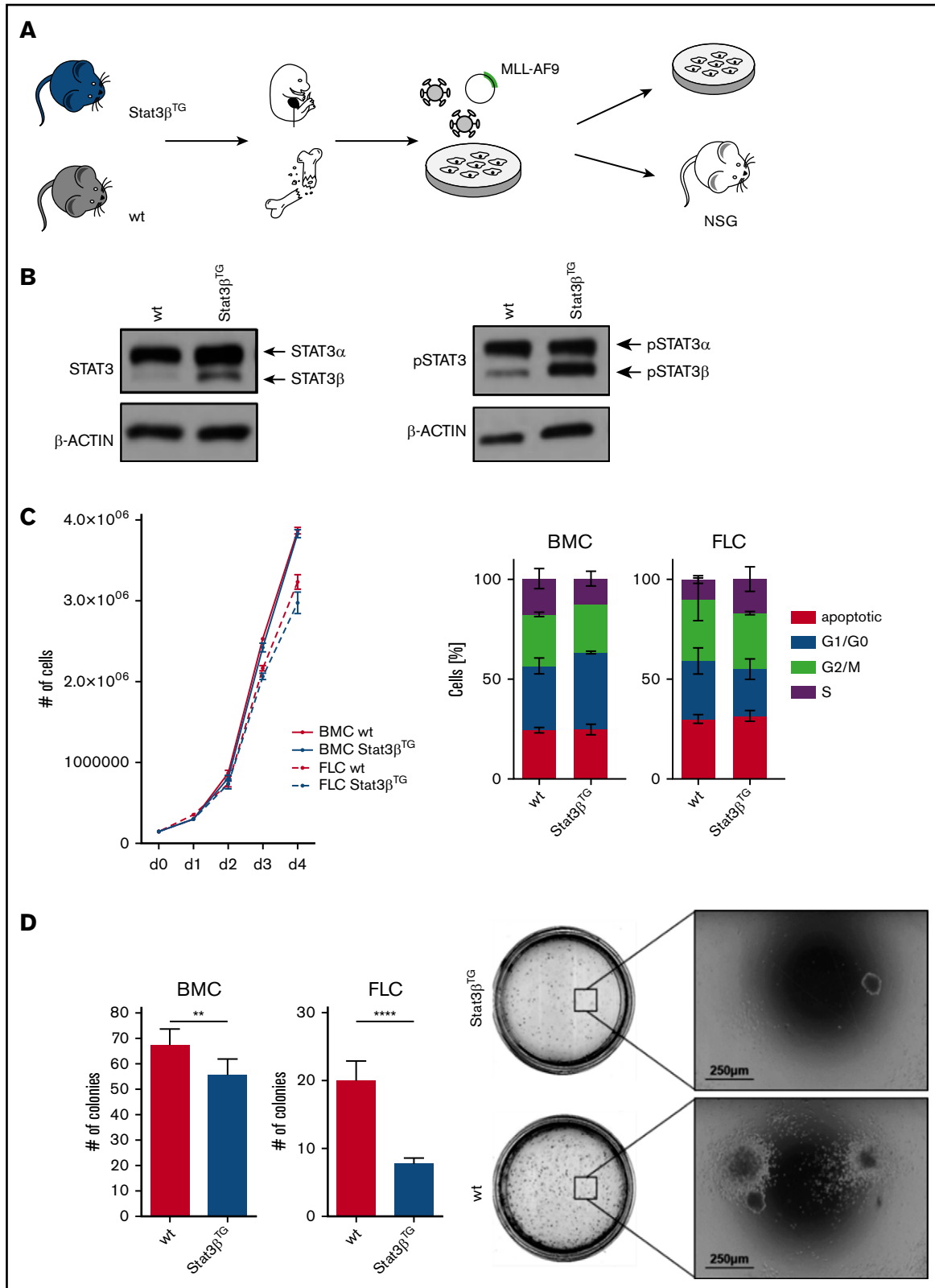


Figure 4. STAT3 β impairs colony formation capacity of MLL-AF9-transformed cells. (A) Workflow of FLCs and BMCs harvested from Stat3 β^{TG} mice and wt littermates. MLL-AF9-transformed cells were subsequently used for in vitro analysis and transplantation. (B) Western blot analysis showing STAT3 and phosphorylated STAT3 (pSTAT3) expression in FLCs transformed with MLL-AF9. Stat3 β^{TG} expression was induced via stimulation with IFN β . (C) In vitro proliferation was analyzed via growth curves and cell cycle analysis by flow cytometry. Data, indicating 3 independent experiments carried out in triplicates, were compared using the Student *t* test, and differences were

Crossbreeding and plpC treatment gave rise to MxCre:Pten^{Δ/+}, MxCre:Pten^{Δ/+}:Stat3β^{TG}, MxCre:Pten^{Δ/Δ}, and MxCre:Pten^{Δ/Δ}:Stat3β^{TG} mice (MxCre is omitted in the annotation of mice hereafter). All mice with a homozygous ablation of *Pten* included in this study developed fatal myeloid leukemia within 110 days. Strikingly, we observed a significant increase in disease latency upon Stat3β^{TG} expression in Pten^{Δ/Δ} mice (Figure 3A). We detected elevated numbers of myeloid (CD11b⁺Gr-1⁺) cells in the BM and spleen of terminally ill Pten^{Δ/Δ} and Pten^{Δ/Δ}Stat3β^{TG} mice compared with Pten^{Δ/+} control animals, consistent with an AML phenotype (supplemental Figure 3A). Although WBC count was strongly increased upon homozygous deletion of *Pten*, Pten^{Δ/Δ}Stat3β^{TG} mice exhibited a WBC count similar to that of healthy Pten^{Δ/+} controls (supplemental Figure 3B).

To address the effect of STAT3β on leukemia progression, we analyzed mice at 3 different time points after plpC injection: days 10, 20, and 30. We focused on the myeloid lineage as well as on HSPCs (defined as Lin⁻Sca-1⁺c-Kit⁺ [LSK]). Compared with Pten^{Δ/+} control mice, Pten^{Δ/Δ} mice already exhibited an increase of myeloid cells in BM and spleen at day 10, which was delayed upon Stat3β^{TG} expression (Figure 3B top). Although the number of HSPCs in the BM remained relatively unchanged, *Pten* deletion caused a significant accumulation of HSPCs in the spleen, which was less pronounced in Pten^{Δ/Δ}Stat3β^{TG} mice (Figure 3B bottom). Analysis of H&E-stained tissue sections from day 20 revealed reduced leukemic infiltration in liver and spleen of Pten^{Δ/Δ}Stat3β^{TG} compared with Pten^{Δ/Δ} mice (Figure 3C). Taken together, our data indicate that increased STAT3β levels impair leukemia progression and prolong disease latency in a *Pten* deletion-based AML mouse model.

Stat3β^{TG} expression impairs colony formation capacity of MLL-AF9-transformed cells

The human fusion oncogene *MLL-AF9* represents another widely used model for functional studies of AML.^{51,52} We harvested FLCs and BMCs from Stat3β^{TG} and wt mice and transduced them with a retrovirus encoding for *MLL-AF9*, coupled to the fluorescent protein Venus (Figure 4A). Before BM isolation, mice were treated with plpC to induce Stat3β^{TG} expression, whereas FLCs were treated with interferon β (IFNβ) in vitro to activate the *Mx1* promoter and express ectopic STAT3β (Figure 4B). As a control, all wt mice and FLCs were similarly treated with plpC or IFNβ, respectively. Of note, expression of total and phosphorylated STAT3α seemed unaffected by the expression of Stat3β^{TG} (Figure 4B). Homogeneous Venus⁺ cell populations were used for all further in vitro experiments (supplemental Figure 4A). Proliferation kinetics of MLL-AF9-transformed cells were not altered between Stat3β^{TG} and wt cells (Figure 4C). Furthermore, mRNA levels of prominent STAT3α-regulated genes involved in cell proliferation remained unchanged in Venus⁺ Stat3β^{TG} and wt FLCs (supplemental Figure 4B). However, in methylcellulose-based colony formation assays, the expression of Stat3β^{TG} led to significantly reduced numbers

of colonies for BMCs as well as FLCs (Figure 4D left) upon serial replating (supplemental Figure 4C). In addition, colony sizes were slightly reduced in Stat3β^{TG} cells (Figure 4D right). Altogether, this indicates that Stat3β^{TG} expression has no effect on STAT3α activity or proliferation but impairs the cellular capacity for self-renewal, colony formation, and tumorigenicity of MLL-AF9-transformed cells.

STAT3β has a tumor-suppressive effect in an MLL-AF9-dependent AML model

MLL-AF9-transformed Stat3β^{TG} and wt FLCs were compared in an in vivo transplantation AML model. Transduced cells were injected into immunocompromised NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice, and 4 weeks later, mice were treated with plpC to induce Stat3β^{TG} expression. We confirmed Stat3β^{TG} expression and phosphorylation with a FLAG-specific antibody and western blot (supplemental Figure 5A-B). Additionally, we investigated the mRNA expression of total *Stat3* and both alternatively spliced isoforms (supplemental Figure 5C). Taken together, these data confirm the increased expression of STAT3β resulting from the transgene in vivo posttransplantation and furthermore demonstrate the unaffected expression and activation of STAT3α. As observed in the *Pten* model, transgenic Stat3β expression caused an increase in survival of mice in comparison with mice receiving wt cells in the MLL-AF9 model (Figure 5A). Immunophenotyping of Venus⁺ blasts in PB and BM revealed high expression levels of the myeloid marker CD11b together with low to intermediate expression levels of c-kit (supplemental Figure 5D). Clear signs of leukemic infiltration in liver, spleen, and PB were found in both experimental groups at the time of euthanasia (supplemental Figure 6A).

To investigate the effect of STAT3β on MLL-AF9-driven leukemia progression, we analyzed mice 6 weeks posttransplantation. Here, the delayed disease phenotype in Stat3β^{TG} mice was evident in direct comparison; WBC count and relative spleen weight and size were reduced in the Stat3β^{TG} group (Figure 5B). Numbers of myeloid Venus⁺ blasts were notably lower in PB, BM, and spleen of Stat3β^{TG} mice (Figure 5C). H&E-stained tissue sections demonstrated significantly decreased infiltration in liver and spleen and diminished numbers of blasts in PB smears (Figure 5D). In accordance with our previous observations, these results show that the elevated expression of STAT3β delays disease progression and the leukemic infiltration of peripheral hematopoietic organs, explaining the demonstrated survival advantage in Stat3β^{TG} mice.

Next, we addressed proliferation and apoptosis in the MLL-AF9-dependent AML model. Neither in quantification of Ki67⁺ cells (supplemental Figure 6B) nor in cell cycle analysis of Venus⁺ spleen-derived blasts did we observe a significant difference in proliferation (supplemental Figure 6C). Spleen and liver sections stained for cleaved caspase 3 demonstrated similarly low levels of apoptosis in both groups (supplemental Figure 6D), which was confirmed by annexin V staining of

Figure 4. (continued) found not to be statistically significant. (D) Methylcellulose-based colony formation assays were analyzed by number of colonies upon 4 replatings (1 replating shown; n = 12 per group). Representative pictures of BMC colonies are shown. Data, indicating 2 independent experiments, were compared using the Student t test. **P < .01, ****P < .0001. NSG, NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ.

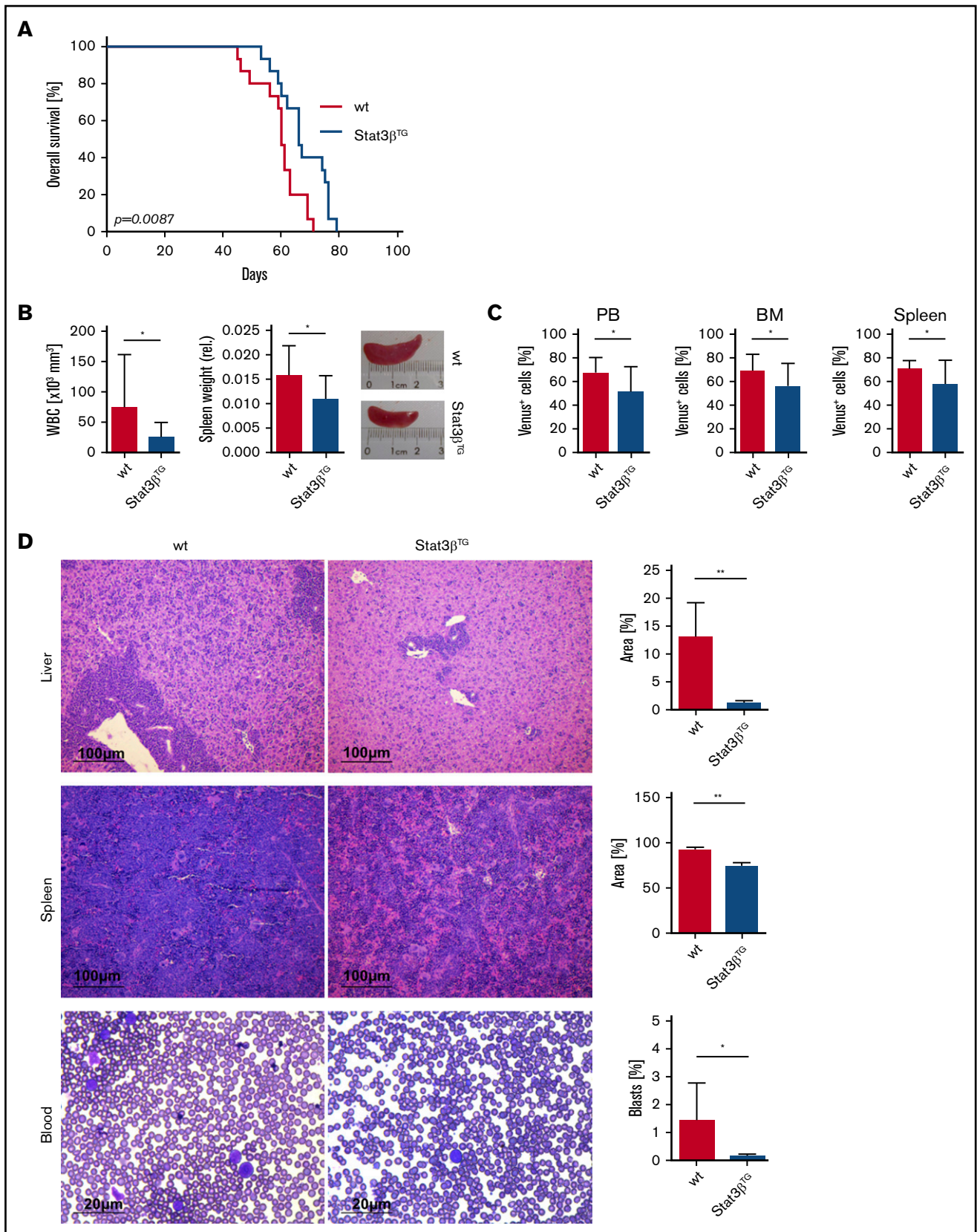


Figure 5. STAT3 β prolongs survival and has a tumor-suppressive effect in MLL-AF9-dependent AML 6 weeks posttransplantation. (A) Kaplan-Meier plot of NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice receiving transplants of Stat3 β ^{TG} and wt FLCs (n = 15 per group), showing significantly different survival ($P = .0087$). (B) WBC

blasts derived from PB, BM, liver, and spleen (supplemental Figure 6E). Thus, the antitumorigenic function of STAT3 β seems to be independent of the direct regulation of apoptosis or proliferation.

Gene expression patterns of migratory target genes change upon increased STAT3 β expression

To identify *Stat3* β ^{TG}-induced global changes in gene expression in MLL-AF9-driven leukemia, we performed RNA sequencing of sorted Venus⁺ BMCs harvested from mice 6 weeks posttransplantation. We analyzed 3 animals per group and found 70 genes that were significantly up- (32 genes) or downregulated (38 genes) upon *Stat3* β ^{TG} expression (Figure 6A). Gene set enrichment analysis revealed enriched expression of genes in the interleukin 6 (IL6)/JAK/STAT3 signaling pathway in *Stat3* β ^{TG} blasts (supplemental Figure 7A). Furthermore, genes allocated to Reactome pathways of cell surface interactions at the vascular wall were significantly upregulated, including *Sell*, *Itgax*, and *Cd177* (supplemental Figure 7B). The surface molecule SELL (L-SELECTIN, CD62L) represents an interesting target due to its role in lymphocyte migration through vasculature, because a downregulation of SELL on lymphocytes or leukemic blasts can initiate cell migration from the BM into the PB, where it is shed.⁵³⁻⁵⁶ Expression of *Sell* was significantly upregulated in *Stat3* β ^{TG} blasts at the mRNA level (Figure 6B top). We confirmed the *Stat3* β ^{TG}-specific upregulation of SELL in vivo on BM-derived Venus⁺ blasts via flow cytometry (Figure 6B middle). Additionally, we measured soluble, shed SELL in the plasma and detected decreased levels in mice receiving *Stat3* β ^{TG} cell transplants (Figure 6B bottom), indicating that *Stat3* β ^{TG} blasts migrate in lower numbers in comparison with wt. This is in accordance with our in vivo findings, which demonstrated a significantly reduced organ infiltration and blast count in the PB of the *Stat3* β ^{TG} group. Lastly, we repeated the methylcellulose-based colony formation assays with BMCs and FLCs that were previously treated with a blocking antibody for cell surface-bound SELL/CD62L. Indeed, the antibody-mediated blocking of SELL reversed the effect of *Stat3* β ^{TG} expression (Figure 6C) upon serial replating (supplemental Figure 7C). As a control, we analyzed the blocking efficiency via flow cytometry (supplemental Figure 7D). In addition, we used publicly available data to identify STAT3-specific binding motifs in the *Sell* promoter (supplemental Figure 8A). Analysis of published chromatin immunoprecipitation sequencing data sets from human and mouse samples confirmed multiple STAT3 binding events in regions across the gene (supplemental Figure 8B). In summary, these data show that STAT3 β can promote target gene induction and actively influence the expression of cell surface markers in MLL-AF9-expressing blasts involved in vascular interaction and migration. Among those markers, SELL might play a distinct role in the antitumorigenic effect of *Stat3* β ^{TG} in AML cells.

Increased levels of SELL correlate with favorable clinical prognosis and increased event-free survival in AML patients

Finally, we investigated *SELL* expression in our AML cohort of favorable, intermediate, and adverse prognostic groups and found elevated mRNA levels of *SELL* in patients with a favorable prognosis, which was associated with a higher *STAT3* β / α mRNA ratio (Figure 7A). Accordingly, we also found elevated levels of SELL/CD62L in samples derived from patients with a favorable prognosis measured via flow cytometry (Figure 7B). Using 3 of the identified STAT3 β -regulated gene targets involved in cell migration (*SELL*, *ITGAX*, *CD177*) as a STAT3 β gene signature and a publicly available AML patient data set ($n = 740$), we were able to show an association between high expression of the STAT3 β signature and prolonged event-free survival (HR, 0.78; $P = .033$; Figure 7C). In conclusion, these data suggest that STAT3 β -dependent elevated SELL expression correlates with a favorable outcome in AML patients.

Discussion

STAT3 is frequently found to be constitutively active in AML.²⁵⁻²⁷ We were able to demonstrate a low *STAT3* β / α mRNA ratio in AML cells in comparison with healthy HSPCs as well as a high *STAT3* β / α mRNA ratio in patients with a favorable cytogenetic prognosis. Moreover, a lower *STAT3* β / α mRNA ratio was associated with higher overall mortality. Notably, this relationship persists despite correction for established prognostic factors, such as cytogenetic risk category, age, and percentage of blasts at diagnosis. Although these results need to be verified in larger confirmatory studies, they are especially plausible in light of the mechanistic insight gained from our animal experiments, pointing to a central role of STAT3 β in AML. Intriguingly, findings are similar to the described STAT3 β function in esophageal squamous cell carcinoma patients, where it was established as a protective prognostic marker.¹⁷

Here, we describe a novel tumor-suppressive role for STAT3 β in AML. Evidence was provided from an inducible *Stat3* β transgenic mouse model in combination with 2 independent drivers for AML, either MLL-AF9 expression or PTEN deficiency. We demonstrated that *Stat3* β ^{TG} expression in AML blasts significantly extended survival by delaying infiltration of PB and hematopoietic organs, which is characteristic for AML. Our data further suggest that the antitumorigenic effect of STAT3 β depends on the tumor-intrinsic regulation of a small set of significantly regulated genes. Several genes of the IL6/JAK/STAT3 signaling pathway and the Reactome pathway for cell surface interactions at the vascular wall were upregulated in *Stat3* β ^{TG} blasts, demonstrating that STAT3 β can actively regulate gene expression.^{11,13-16} *SELL* was identified as a target gene of interest, because it was shown to be specifically upregulated in *Stat3* β ^{TG} blasts and is known to be expressed in newly diagnosed AML patients.⁵⁷⁻⁶¹ In particular,

Figure 5. (continued) count and relative spleen weight and size. (C) Quantification by flow cytometry of myeloid Venus⁺ blasts in PB, BM, and spleen of *Stat3* β ^{TG} and wt mice. (D) H&E-stained liver and spleen sections and PB smears of wt and *Stat3* β ^{TG} animals. Quantifications shown as infiltrate area/total organ area (%) and number of blasts/total cell number (%). (B-D) Data were compared using the Student *t* test. * $P < .05$, ** $P < .01$.

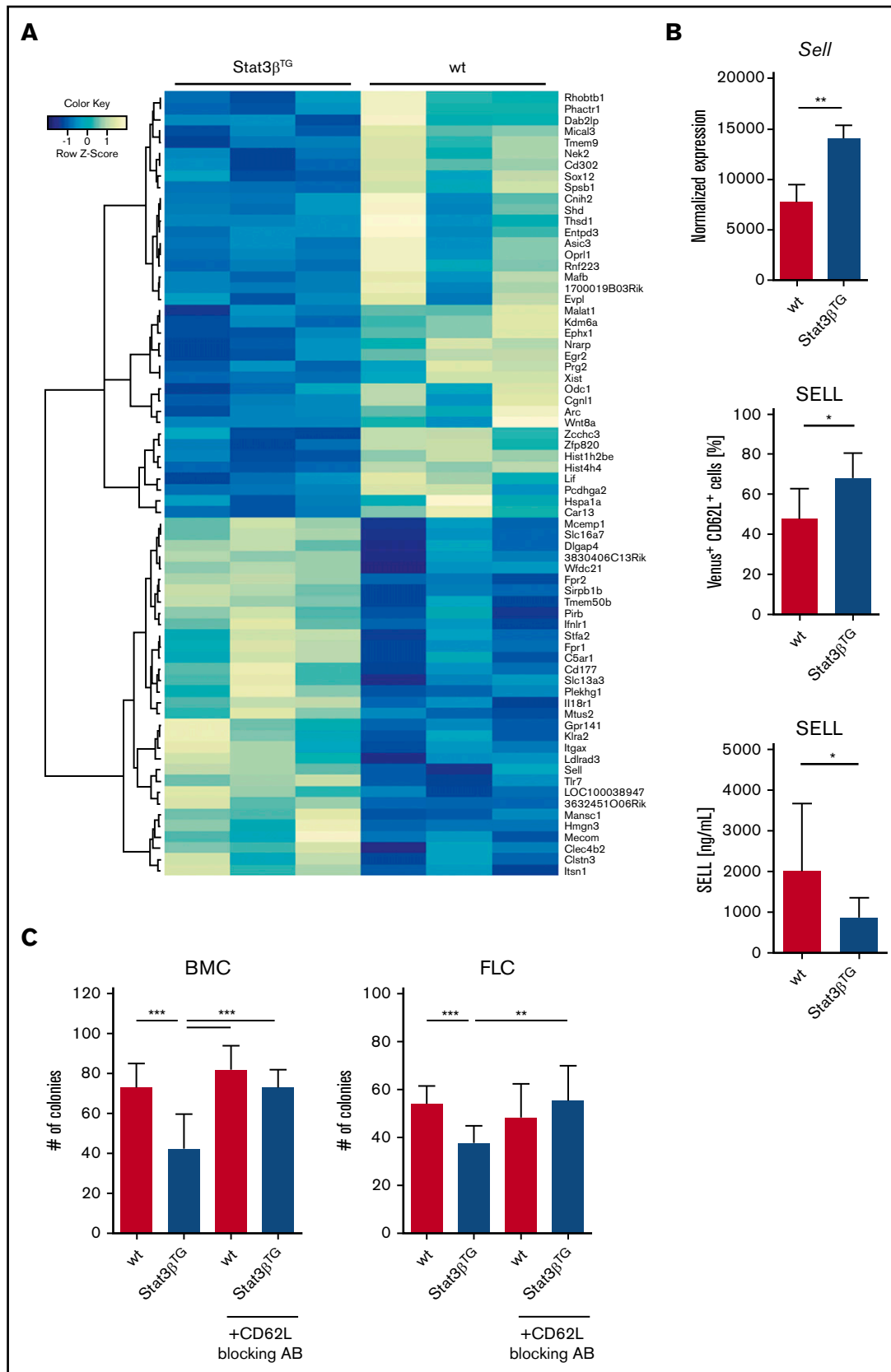


Figure 6. Gene expression patterns upon increased STAT3 β expression reveal SELL to be a potential STAT3 β target. (A) Heatmap showing significantly up-regulated (32 genes) or downregulated (38 genes) genes between murine wt and Stat3 β ^{TG} BM-derived Venus⁺ blasts (n = 3). (B) Normalized mRNA expression, displaying the

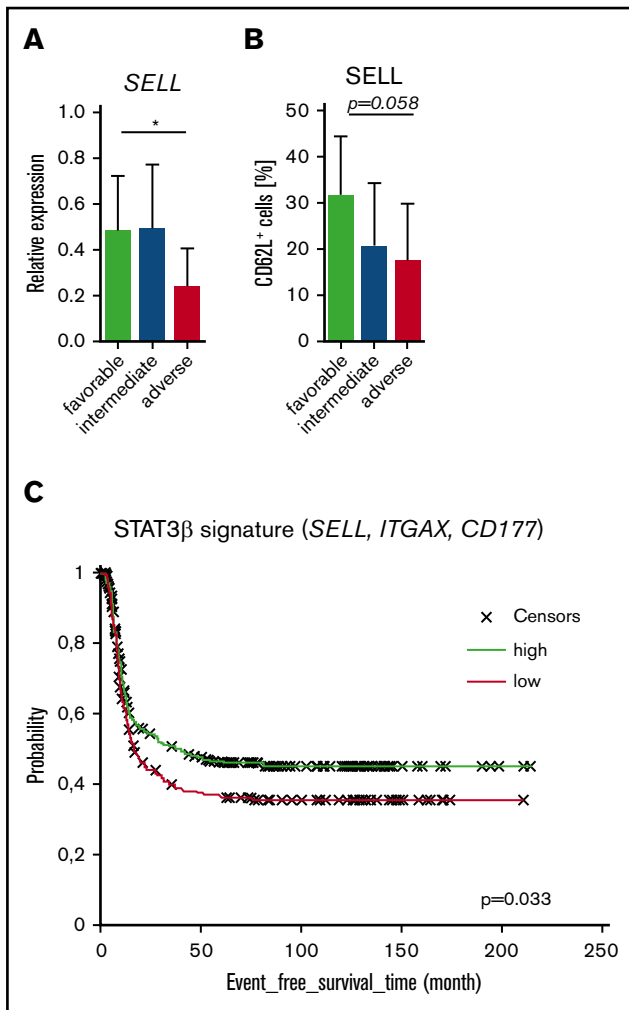


Figure 7. Increased levels of SELL correlate with favorable clinical prognosis in our AML cohort and increased event-free survival in publicly available AML patient data sets. (A) *SELL* mRNA expression levels (normalized to β -ACTIN) in AML patients with favorable (n = 9), intermediate (n = 38), or adverse (n = 8) prognosis. Data (favorable vs adverse) were compared using the Student *t* test. (B) SELL/CD62L expression levels analyzed by flow cytometry in AML blasts from patients with favorable (n = 7), intermediate (n = 38), or adverse (n = 7) prognosis. Data (favorable vs adverse) were compared using the Student *t* test, and significance is indicated by $P = .058$. (C) Gene signatures from publicly available AML patient data sets (n = 740) were compared, showing that patients with high expression of the STAT3 β signature (*SELL*, *ITGAX*, *CD177*) had better survival compared with patients with low expression in 740 AML patients (HR, 0.78; $P = .33$). * $P < .05$.

SELL is important for the homing of AML cells, because its downregulation could initiate the mobilization of AML blasts from the BM into the PB, where it is shed and remains in the PB in its

soluble form.⁵³⁻⁵⁶ In our MLL-AF9-based AML in vivo model, SELL was specifically upregulated in Stat3 β ^{TG} BM-resident AML blasts at the mRNA and protein levels in comparison with wt. This explains the delayed peripheral infiltration of Stat3 β ^{TG} blasts and the consequent suspension of disease progression. In line with this, levels of shed SELL in the PB were significantly reduced in mice receiving Stat3 β ^{TG} cells, indicating a clear reduction in AML cell mobilization.

SELL expression on hematopoietic progenitors in vitro has been described to correlate with their clonogenic potential.⁶² Likewise, we found the capacity of transformed BMCs and FLCs for in vitro colony formation to be impaired upon Stat3 β ^{TG} expression, which was preventable with antibody-mediated blocking of SELL. Together, these findings indicate that the antitumorigenic effect of Stat3 β ^{TG} expression in AML cells might be at least partially dependent on the upregulation of SELL. However, because the colony formation capacity of HSPCs and AML cells considerably depends on STAT3 activity,^{28,63} STAT3 β might also counteract STAT3 and therefore reduce colony formation. Because of its unique C-terminus, STAT3 β homodimers have been demonstrated to exhibit prolonged phosphorylation and nuclear retention⁶⁴ as well as enhanced DNA-binding affinity^{16,65} and dimer stability.⁶⁵ Thus, STAT3 β can directly compete with STAT3 α and furthermore regulate STAT3 α via the formation of heterodimers.^{15,17} Nevertheless, we failed to obtain any evidence suggesting a negative regulation of STAT3 α by STAT3 β in AML. In fact, we found the activity of STAT3 α and STAT3 α -regulated genes in AML blasts to be unaffected by the expression of Stat3 β ^{TG}, which is similar to previous findings.¹¹

In AML patients, increased *SELL* expression was reported to correlate with good-risk karyotypes.⁶⁶ Accordingly, we found SELL levels to be significantly elevated in patients with a favorable prognosis, in contrast to patients with an adverse prognosis. Furthermore, analysis of publicly available AML data sets revealed a correlation between high expressions of newly identified STAT3 β -regulated migratory genes (*SELL*, *ITGAX*, *CD177*) and superior event-free survival. In addition to *Sell*, *Itgax*, and *CD177*, we identified other novel STAT3 β -regulated genes, such as *Lif*, *Sox12*, *Maib*, and *Ifnl1*, that might also contribute to the tumor-suppressive effect of STAT3 β but whose specific role in AML remains elusive. Taken together, the exact mechanism underlying the tumor-suppressive function of STAT3 β is most likely a combination of various regulated genes, such as *Sell*, and deserves further investigation.

In summary, our study unequivocally demonstrates that STAT3 β acts as a tumor suppressor in AML and specifically regulates gene expression in AML blasts, impairing leukemia progression and extending survival.

Figure 6. (continued) upregulation of *Sell* in Venus⁺ Stat3 β ^{TG} blasts, is shown (top; n = 3 per group). Quantification of cell surface-bound SELL/CD62L in BM-derived Venus⁺ blasts, measured by flow cytometry (middle; n = 6 per group), and levels of soluble, shed SELL in plasma, assessed via enzyme-linked immunosorbent assay (bottom; n = 9 per group), demonstrate the upregulation of SELL on the cell surface of Stat3 β ^{TG} blasts and the difference in shed SELL present in PB. Data were compared using the Student *t* test. (C) Methylcellulose-based colony formation assays of wt and Stat3 β ^{TG} FLCs and BMCs, pretreated with or without a blocking antibody for SELL/CD62L (MEL-14), were analyzed by number of colonies upon 3 replatings (1 replating shown; n = 12 per group). Data were compared using 1-way analysis of variance. * $P < .05$, ** $P < .01$, *** $P < .001$.

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P.A. is a PhD candidate at the Medical University of Vienna, and this work is submitted in partial fulfillment of the requirement for a PhD.

Authorship

Contribution: P.A., T.M., E.C., and D.S. designed research; P.A. and T.M. performed experiments and analyzed data; E.C., J.H., V.J., S.H., T.E., H.P.M., A.Y., L.K., and B.G. performed and analyzed additional experiments; H.S., K.L., M.J.M.F., F.G., R.M., and E.C. provided essential material and discussion; P.A. and D.S. wrote the manuscript; and all authors approved the manuscript.

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