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MYELOID NEOPLASIA

Tumor suppressor function of *Gata2* in acute promyelocytic leukemia

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KEY POINTS

- ***Gata2* inactivation cooperates with *PML-RARA*, *RUNX1-RUNX1T1*, and *Cebpa* mutations.**
- **Ectopic *Gata2* expression suppresses *PML-RARA*–driven cell growth, even in fully transformed APL cells.**

Most patients with acute promyelocytic leukemia (APL) can be cured with combined all-trans retinoic acid (ATRA) and arsenic trioxide therapy, which induces the destruction of PML-RARA, the initiating fusion protein for this disease. However, the underlying mechanisms by which *PML-RARA* initiates and maintains APL cells are still not clear. Therefore, we identified genes that are dysregulated by *PML-RARA* in mouse and human APL cells and prioritized *GATA2* for functional studies because it is highly expressed in preleukemic cells expressing *PML-RARA*, its high expression persists in transformed APL cells, and spontaneous somatic mutations of *GATA2* occur during APL progression in mice and humans. These and other findings suggested that *GATA2* may be upregulated to thwart the proliferative signal generated by *PML-RARA* and that its inactivation by mutation (and/or epigenetic silencing) may accelerate disease progression in APL and other forms of acute myeloid leukemia (AML). Indeed, biallelic knockout of *Gata2* with CRISPR/Cas9-mediated gene editing increased the serial replating efficiency of *PML-RARA*–expressing myeloid progenitors (as well as progenitors expressing *RUNX1-RUNX1T1*, or deficient for *Cebpa*), increased mouse APL penetrance, and decreased latency. Restoration of *Gata2* expression suppressed *PML-RARA*–driven aberrant self-renewal and leukemogenesis. Conversely, addback of a mutant GATA2^{R362G} protein associated with APL and AML minimally suppressed *PML-RARA*–induced aberrant self-renewal, suggesting that it is a loss-of-function mutation. These studies reveal a potential role for *Gata2* as a tumor suppressor in AML and suggest that restoration of its function (when inactivated) may provide benefit for AML patients.

Introduction

Inherited mutations in the transcription factor *GATA2* cause monocytopenia and mycobacterial infection (MonoMAC) syndrome and Emberger syndrome, which strongly increase the risk of developing myelodysplastic syndrome or acute myeloid leukemia (AML; 80% probability by age 40).[1-3] Somatic *GATA2* mutations have been reported in ~3.4% (135/4019 cases)[4-10] of AML patients, especially in association with *CEBPA* mutations, where ~21.1% (89/422 cases)[4-6,8-12] have *GATA2* mutations. Somatic *GATA2* mutations have also been reported in AML cases initiated by mutations in *DNMT3A* and *ASXL1* and several fusion oncogenes (eg, *RUNX1-RUNX1T1*, *MLL* fusions, and *PML-RARA*).[5,9,13,14] Therefore, *GATA2* mutations are found in many AML subtypes, suggesting that they can cooperate with several initiating mutations to promote AML.[15,16] *GATA2* is highly expressed in normal CD34$^+$ cells, but not in T cells, B cells, or mature myelomonocytic cells (including promyelocytes).[6] *GATA2* is also expressed in virtually all AML subtypes (including acute promyelocytic leukemia [APL]) and in most AML samples tested to date.[6] However, there is no consensus on the mechanism by which *GATA2* contributes to AML or whether *GATA2* acts as an oncogene or a tumor suppressor.

Some reports have suggested that *GATA2* expression may promote AML by increasing cell proliferation[17,18] and that high expression correlates with poor outcomes.[19] However, other reports have suggested that *GATA2* inhibits the proliferation of hematopoietic progenitors[16,20,21] and that *GATA2* function may be disrupted in many cases of AML.[3,22-29] Finally, *GATA2* mutations in AML are nearly always heterozygous,[9,22,24] and the remaining *GATA2*^{WT} allele is often epigenetically silenced,[8,22,24,30,31] suggesting that abrogation of *GATA2* function may contribute to AML progression in some patients.

During normal hematopoiesis, *GATA2* promotes the endothelial to hematopoietic transition during embryogenesis.[32,33] Therefore, *Gata2*-knockout mice are embryonically lethal as the result of a lack of hematopoietic stem/progenitor cells (HSPCs) and anemia.[32,34] Conditional deletion of *Gata2* revealed that it is also required for the maintenance and survival of HSPCs in adult bone marrow.[35] Therefore, *Gata2* is thought to promote the self-renewal of HSPCs during normal hematopoiesis.[36]

In this study, we examined the role of *GATA2* in AML pathogenesis using the well-characterized *Ctsg-PML-RARA* mouse model, in which human *PML-RARA* is knocked into the 5′ untranslated

region of the mouse *Ctsg* gene.[37] This leads to the expression of *PML-RARA* in early myeloid progenitors,[38] aberrant self-renewal in replating assays,[39] and the development of APL with long latency (~50% penetrance at 1 year in C57BL/6 mice).[37] Using this mouse model, along with expression data from human APL samples, we were able to define genes that are canonically dysregulated in preleukemic and fully transformed APL cells. Among these genes, *GATA2* has unique properties that strongly suggest that it plays a role in the pathogenesis of this disease, as well as other AML subtypes.

Methods

Study approval

All mouse experiments were done in accordance with institutional guidelines and approved by the Animal Studies Committee at Washington University.

Mice

Details about the mouse strains, husbandry, and analysis are provided in supplemental Methods, available on the *Blood* Web site.

Exome and bulk RNA sequencing and single-cell RNA sequencing

Details are provided in supplemental Methods.

CRISPR/Cas9 gene editing

Details about *Gata2*-directed gene editing and analysis are provided in supplemental Methods.

Methylcellulose colony assays

Methylcellulose assays were performed as previously described[39] and are detailed in the supplemental Methods.

Retroviral transductions

Details about the retroviral transduction strategies are provided in supplemental Methods.

Western blotting

Western blots were performed using the Jess Western Blotting System (ProteinSimple; https://www.proteinsimple.com/jess.html), as described in supplemental Methods.

Flow cytometry and cellular purification

Flow studies were performed as previously described.[40] Mouse promyelocytes were flow purified as previously described.[38]

ATRA treatment of APL mice

Details about all-trans retinoic acid (ATRA) pellet treatment of APL-engrafted mice are presented in supplemental Methods.

Details about gene set enrichment analysis are presented in supplemental Methods.

Sex differences

Sex differences were evaluated for the donor source of mice for all assays, including colony assays, APL penetrance studies, and ATRA sensitivity. None of the sex-based differences were statistically significant.

Results

GATA2/Gata2 is dysregulated and mutated in human and mouse APL

To identify genes that are dysregulated by *PML-RARA* in human APL patients, we evaluated bulk RNA-sequencing (RNA-seq) data from the AML TCGA study, derived from 16 primary human APL cases and 3 normal promyelocyte-enriched samples.[6] Using this approach, we identified 3922 differentially expressed genes (DEGs), of which 2189 were upregulated in APL and 1733 were downregulated (fold change $\geq$2 and false discovery rate [FDR] $<$0.05 by analysis of variance [ANOVA]; Figure 1A-B; supplemental Table 1). Several of the genes that are differentially expressed in APL have previously been implicated in AML, APL, self-renewal, and/or myeloid development, including the upregulated genes *BCL2* (2.9×; previously shown to cooperate with *PML-RARA* to decrease the latency of APL[39]), *ZBTB16/PLZF1* (2.2×), *GATA2* (63.9×), *HGF* (55.7×), *PDGFRB* (9.3×), and *HDC* (64.3×) and the downregulated genes *PRTN3* (25.8×), *ELANE* (3.6×), and *CD177* (157.8×). *GATA2* is normally expressed in CD34$^+$ cells, but its expression is essentially absent in normal promyelocytes, neutrophils, and monocytes. However, *GATA2* is highly expressed in most AML French-American-British subtypes, (including M3/APL) (Figure 1C). In APL patients, somatic *GATA2* mutations have been reported in ~4.5% of cases that have been sequenced to date (6/134 cases)[4,6-8,10,14]; *GATA2* mutations cooperate with *PML-RARA*,[7] as well as with rare APL-initiating fusions[14] (Figure 1D).

To determine whether *Gata2* is mutated in APLs arising spontaneously in *Ctsg-PML-RARA* mice, we performed exome sequencing of 13 APL samples with paired normal (tail DNA) samples to define somatic mutations and copy number events (supplemental Figure 1; supplemental Table 2). Similar to previous studies, 2 of these APLs contained interstitial deletions of chromosome 2 that included the PU.1/*Spi1* and *Wt1* genes[42,43]; 7 contained amplifications of chromosome 15, where *Myc* is located[42,44,45]; and 9 had loss of 1 copy of the X chromosome, where *Kdm6a* is located.[46] Mutations in the AML-associated genes *Eppk1*, *Ptpn11*, *Kras*, *Jak1*, and *Kdm6a* were identified in 1 APL sample each,[6,46,47] and a novel somatic frameshift mutation that produces a premature in-frame stop codon was identified in *Gata2* (N363fs) in 1 case. No deletions of the *Gata2* locus were identified. To determine whether *Gata2* was recurrently mutated in this model, we sequenced all exons of the *Gata2* gene in 94 additional mouse APL samples and discovered an additional somatic R330L mutation in 1 case (data not shown). The R330L and N363fs mutations are located in zinc finger 1 and zinc finger 2 of *Gata2*, respectively.

To more precisely identify genes that may be dysregulated by *PML-RARA*, we transduced lineage-depleted wild-type (WT) mouse bone marrow cells with murine stem cell virus (MSCV)–internal ribosome entry site (IRES)-GFP–based retroviruses containing no insert (empty vector), WT *PML-RARA* complementary DNA, or a C88A mutant of *PML-RARA* (*PML-RARA*^{C88A}). The C88A mutation has previously been shown to abrogate the DNA-binding activity of *PML-RARA*,[46] and we found that *PML-RARA*^{C88A} was unable to induce aberrant self-renewal (supplemental Figure 2A-C), despite its robust expression 3 days following transduction (supplemental Figure 2B). Therefore, the *PML-RARA*^{C88A} fusion serves as a control for genes regulated by *PML-RARA*. Seven days after transduction, we sorted on GFP$^+$

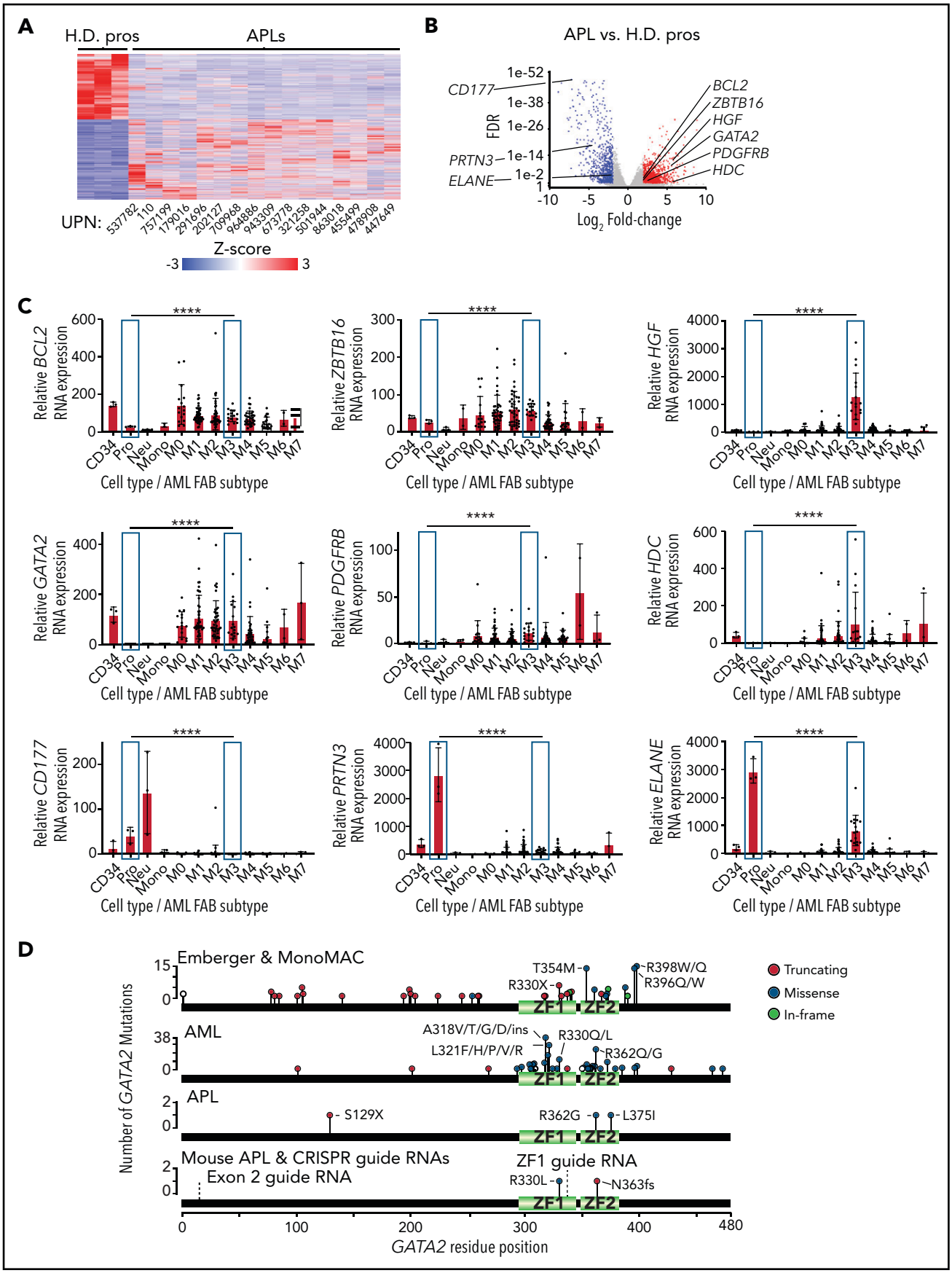

Figure 1.

cells and performed RNA-seq. This analysis led to the identification of 3299 DEGs when comparing *PML-RARA*^{WT}–transduced cells with empty vector (or *PML-RARA*^{C88A})–transduced cells (1552 upregulated, 1747 downregulated with *PML-RARA*^{WT}; fold change ≥2 and FDR <0.05 by DESeq2; Figure 2A-C; supplemental Figure 2C; supplemental Table 3). *PML-RARA*^{C88A} led to the dysregulation of a smaller number of DEGs (353 upregulated, 202 downregulated compared with empty vector; fold change ≥2 and FDR <0.05 by DESeq2), perhaps via its ability to interact with RXRA.[46] As noted in the corresponding volcano plot (Figure 2B), expression of *PML-RARA*^{WT} for 7 days caused the dysregulation of many of the same genes that were differentially expressed in human APL samples, including *Gata2* (Figure 2C).

To extend these results, we performed RNA-seq on flow-sorted preleukemic promyelocytes from *Ctsg-PML-RARA* and littermate-matched WT mouse bone marrow samples. We identified 1826 DEGs comparing *Ctsg-PML-RARA* vs WT promyelocytes (959 upregulated and 867 downregulated with *PML-RARA* expression; fold-change ≥2 and FDR <0.05 by ANOVA; Figure 2D-F; supplemental Figure 2D; supplemental Table 4). Two hundred and forty-one of these DEGs were coordinately dysregulated in the mouse and human data sets (135 upregulated and 106 downregulated; Figure 2E), including *Gata2* (Figure 2F). Among these DEGs, only 2, *Gata2* (upregulated 10.1-fold) and *Kit* (upregulated 2.93-fold), were coordinately dysregulated in preleukemic mouse and human APL samples.

To better define the effects of *PML-RARA* on bone marrow populations and gene expression, we performed single-cell RNA-seq (scRNA-seq) on bone marrow cells from young littermate-matched nonleukemic *Ctsg-PML-RARA* and WT mice. This analysis revealed an expansion of myeloid progenitors in *Ctsg-PML-RARA* marrow (Figure 3A)[37] that was restricted to the *Ctsg*-expressing (*Ctsg*$^{+}$) myeloid progenitor cell population (Figure 3B) (*PML-RARA* expression is directed by the *Ctsg* locus in this model). *Ctsg*$^{+}$ myeloid progenitors from WT and *Ctsg-PML-RARA* mice expressed several markers of myeloid precursors, including *Cd34*, *Mpo*, and *Elane* (supplemental Figure 3A). Therefore, *Ctsg* marks normal myeloid progenitors and precursors from WT and *Ctsg-PML-RARA* mice. We identified a unique cluster of myeloid progenitor cells in *Ctsg-PML-RARA* marrow and identified DEGs within this compartment: 850 genes were upregulated and 240 were downregulated in the *PML-RARA*–expressing cells compared with WT *Ctsg*$^{+}$ myeloid progenitor cells (fold-change ≥ 2 and FDR < 0.05 by ANOVA; Figure 3C-E; supplemental Table 5). Gene set enrichment analysis revealed a positive correlation with the DEGs identified in this scRNA-seq data set, the human APL RNA-seq data set, the mouse promyelocyte RNA-seq data set, and a previously published data set of APL-specific DEGs[47] (supplemental Figure 3B-D). More specifically, among these 4 data sets, 37 genes showed coordinate dysregulation (24 upregulated and 13 downregulated), including *Gata2*, which was upregulated 3.2-fold in *PML-RARA*–expressing myeloid progenitors vs WT progenitors (FDR <1E-307; *P* < 1E-303; Figure 3F).

Gata2 suppresses serial replating induced by *PML-RARA*, *RUNX1-RUNX1T1*, and *Cebpa* inactivation

To determine whether *Gata2* upregulation associated with *PML-RARA* expression was acting as a tumor promoter or a tumor suppressor, we inactivated it and assessed the consequences. We first generated *PML-RARA* × *Cas9-GFP* mice (hereafter referred to as *PML-RARA* × *Cas9* mice), and then electroporated bone marrow from these mice with CRISPR guide RNAs that targeted the 5′ end (exon 2) of *Gata2*, the zinc finger 1 domain of *Gata2*, or intron 1 of *Rosa26*[48] (as a neutral locus control; Figure 1D). Digital sequencing of polymerase chain reaction products centered around the targeted sites revealed the production of a wide "library" of insertion and deletion mutations at the appropriate target sites for each guide RNA (Figure 4A; supplemental Figure 4A,C), with an average targeting efficiency of 76.2% at the 3 target sites. Cells were then serially replated in MethoCult M3534 for 6 weeks. Targeting *Rosa26* with CRISPR-Cas9 guide RNAs did not affect the ability of *PML-RARA* × *Cas9* bone marrow cells to replate, as expected (Figure 4B). *Gata2* inactivation caused a 17.0-fold increase in the serial replating efficiency of *PML-RARA* × *Cas9* bone marrow compared with *Rosa26*-targeted cells or untransfected cells (nontargeted; Figure 4B). In addition, digital sequencing revealed that biallelic loss-of-function mutations of *Gata2* were selected for with serial replating (Figure 4C; supplemental Figure 4A-B). However, *Gata2* targeting with guide RNAs did not cause an increase in the replating efficiency within the first 3 weeks, even though *Gata2* loss-of-function mutations were selected for within 2 weeks. This suggests that myeloid progenitor cells require several weeks to overcome the transcriptional program dictated by *Gata2*. *Gata2* targeting in WT *Cas9-GFP* bone marrow cells did not induce serial replating, nor did targeting the *Rosa26* locus (Figure 4B; supplemental Figure 4C-D). *Gata2* deficiency in *PML-RARA*–expressing cells caused 2 effects: a 7.7-fold expansion in colonies (Figure 4D) and the production of larger colonies (1.43-fold increase in colony diameter) (Figure 4E). Phenotypically, the *Rosa26*- and *Gata2*-targeted *PML-RARA* × *Cas9* cells maintained myeloid lineage markers by flow cytometry (Figure 4F; supplemental Figure 4E). However, a greater proportion of *Gata2*-targeted cells expressed CD34 and coexpressed CD34 and Gr-1 (32.6- and 30.7-fold increase in these populations respectively), a distinct feature of APL in this model system.[36] Wright-Giemsa staining confirmed that the *Gata2*-targeted cells had early myeloid features with azurophilic granules (Figure 4G). Finally, *Gata2*-and *Rosa26*-targeted *PML-RARA* × *Cas9* cells were equally sensitive to ATRA, indicating that *Gata2*-deficient cells are still dependent upon *PML-RARA* expression to cause the replating phenotype (Figure 4H).

Figure 1. *GATA2* expression in normal and malignant hematopoietic cells. (A) Heat map of the 4094 DEGs between primary human APLs and healthy donor promyelocytes (H.D. pros) by RNA-seq fold change ≥2 and FDR <0.05. (B) Volcano plot of the samples from panel A. Expression changes of *BCL2*, *ZBTB16*, *HGF*, *GATA2*, *PDGFRB*, *HDC*, *CD177*, *PRTN3*, and *ELANE* are labeled. (C) *BCL2*, *ZBTB16*, *HGF*, *GATA2*, *PDGFRB*, *HDC*, *CD177*, *PRTN3*, and *ELANE* expression in flow purified healthy donor human CD34^{+} progenitors (CD34), promyelocytes (Pro), neutrophils (Neu), monocytes (Mono), and the AML French-American-British subtypes M0-M7 by RNA-seq using the AML TCGA data set.[6] Promyelocytes and M3 (APL) subtype samples are highlighted in blue. Statistical significance was determined by edgeR, which includes correction for multiple testing. *P* values are corrected for multiple testing. (D) Distribution of *GATA2* mutations from patients with Emberger syndrome, monocytopenia and mycobacterial infection (MonoMAC) syndrome, AML, or APL,[1–5,7,11,30,34,59–64] as well as those from mouse APLs identified by exome sequencing. The locations of CRISPR guide RNAs used in this study (labeled exon 2 and ZF1) are annotated with dashed lines. ****P < .0001. UPN, unique patient number; ZF1, zinc finger domain 1; ZF2, zinc finger domain 2.

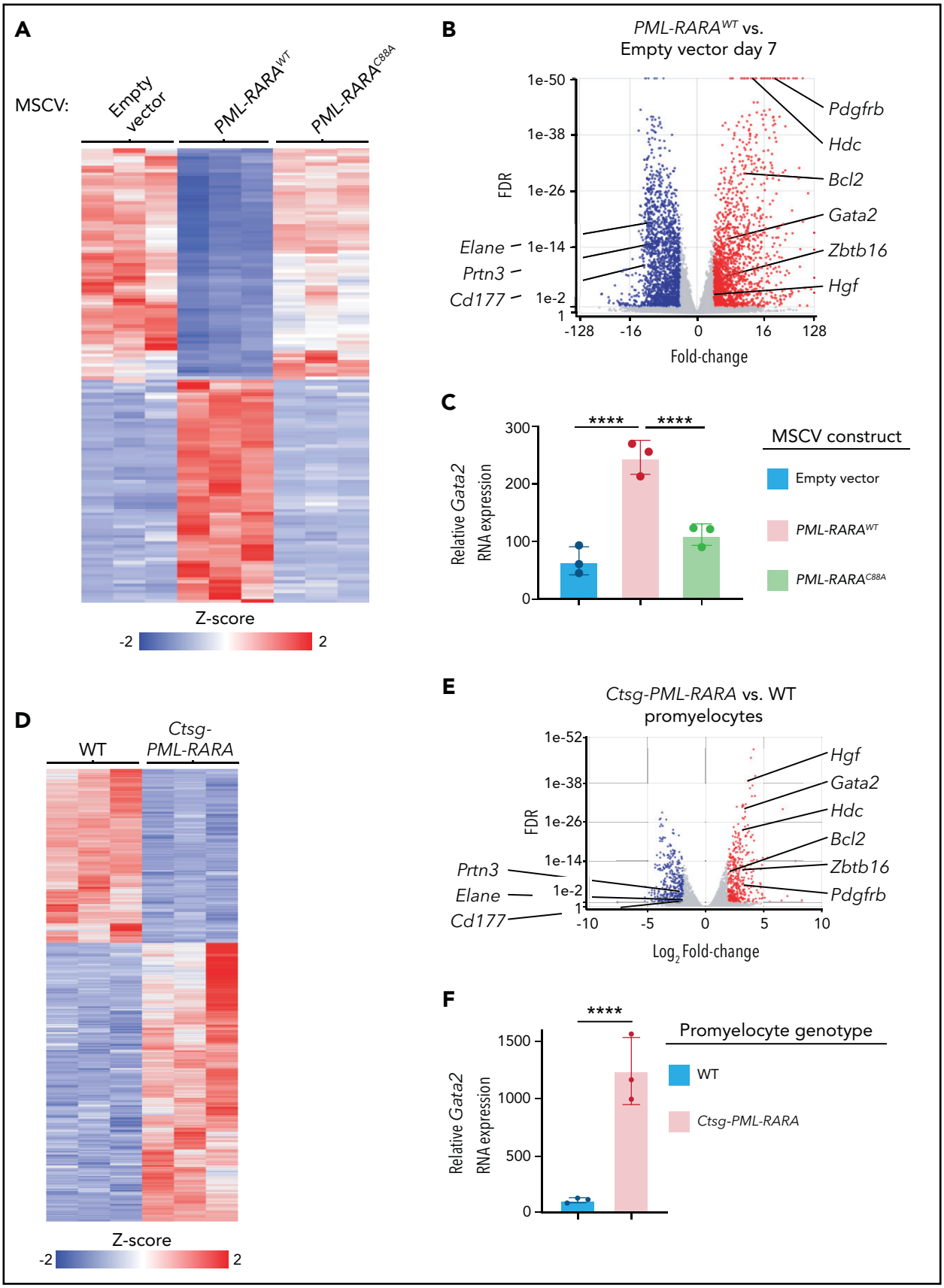

Figure 2.

We next tested whether *Gata2* suppresses the aberrant self-renewal known to be caused by *RUNX1-RUNX1T1* expression[49,50] or *Cebpa* inactivation.[51] To study *RUNX1-RUNX1T1*–induced self-renewal, we transduced WT *Cas9-GFP* bone marrow cells with *RUNX1-RUNX1T1* or empty vector MSCV retroviruses. Cells were then electroporated with CRISPR guide RNAs targeting *Gata2* or *Rosa26* and serially replated in MethoCult. We found that biallelic loss-of-function mutations in *Gata2* caused a 2.2- to 5.7-fold increase in the serial replating of *RUNX1-RUNX1T1*–expressing myeloid progenitors compared with those targeted at *Rosa26* (supplemental Figure 5). To determine whether *Gata2* inactivation affected the enhanced serial replating caused by biallelic *Cebpa* mutations, we used CRISPR guide RNAs to target the p42 isoform of *Cebpa*, which was previously shown to act as a tumor suppressor in AML.[51] The resulting biallelic *Cebpa* mutations caused aberrant serial replating and were positively selected for over time (supplemental Figure 6). The subsequent targeting of *Gata2* caused an additional 4.1-fold increase in the serial replating efficiency of *Cebpa*-deficient progenitors (compared with *Cebpa*-only or *Cebpa*- and *Rosa26*-targeted cells), whereas targeting the *Rosa26* locus had no effect.

Gata2 deficiency affects APL penetrance and latency in *Ctsg-PML-RARA* mice

Based on the replating phenotype, we predicted that *Gata2* deficiency would increase APL penetrance and decrease latency in the *Ctsg-PML-RARA* mouse model. Therefore, we electroporated *PML-RARA* × *Cas9* bone marrow cells with CRISPR guide RNAs targeting *Gata2* or *Rosa26* and transplanted the cells into sublethally irradiated mice. Mice that received *Gata2*-targeted *PML-RARA* × *Cas9* cells developed APL with 100% penetrance at 7.5 months posttransplantation compared with a concurrent cohort that received *Rosa26*-targeted *PML-RARA* × *Cas9* cells and developed APL with 20% penetrance at 15 months posttransplantation ($P < .0001$, log-rank [Mantel-Cox] test). The median leukemia-free survival of mice transplanted with *Gata2*-targeted cells was 141 days vs >361 days for *Rosa26*-targeted cells ($P < .01$; Figure 5A). Targeting of *Gata2* with guide RNAs in WT *Cas9-GFP* bone marrow cells did not lead to any leukemic deaths (Figure 5A). Digital sequencing and western blot analysis of APL samples showed that biallelic loss-of-function mutations were selected for in the APL samples in vivo, resulting in a complete absence of GATA2 protein in these tumors (Figure 5B-C; supplemental Figure 7A-C). Moreover, digital sequencing showed that 28 of 28 single-cell clones (obtained by single-cell deposition from a flow cytometer) from *Gata2*-targeted APLs contained the same biallelic loss-of-function mutations as did the bulk tumor (ie, both mutations were present in the same cells; supplemental Figure 7D). Exome sequencing of the *Gata2*-deficient APLs revealed that CRISPR/Cas9 specifically targeted *Gata2* in all of these APLs, resulting in biallelic frameshift mutations between aa 9 and 16 that caused premature stop codons in all 5 samples (supplemental Figure 8A; supplemental Table 6). In addition to the biallelic *Gata2* mutations, 1 *Gata2*-deficient APL harbored a missense mutation (F837V) in the kinase domain of *Jak1*, similar to that previously described.[44,52] *Gata2*-deficient APLs were also found to have interstitial deletions of chromosomes 2 and X (involving the *Kdm6a* gene), as well as an amplification of all or part of chromosome 15 (which contains *Myc*); all were described in APLs arising in *Gata2*-sufficient *Ctsg-PML-RARA* mice[6,40-45] (supplemental Figure 8A-B). Therefore, for 4 of 5 *Gata2*-deficient APLs sequenced, additional cooperating events probably contributed to APL development. Flow cytometry revealed that the leukemic cells derived from the *Gata2*-deficient *PML-RARA* × *Cas9* cells coexpressed CD34 and Gr-1, as described above (Figure 5D; supplemental Figure 8C-D). Morphologically, *Gata2*-deficient APLs closely resembled *Gata2*-sufficient APLs; both had abundant azurophilic granules characteristic of promyelocytes (Figure 5E). *Gata2*-deficient or -sufficient APLs were similarly sensitive to ATRA treatment in vivo (Figure 5F-G).

Tumor-suppressor activity of $Gata2^{WT}$ in APL cells

We next wished to determine whether *Gata2* overexpression could suppress the replating phenotype in *PML-RARA*–expressing cells and whether the R362G mutation (found in APL and non-APL AML patients) had gain- or loss-of-function properties. We used MSCV-based retroviruses tagged with IRES-mCherry to express $Gata2^{WT}$ or $Gata2^{R362G}$ (or an empty vector control) in *Gata2*-deficient *PML-RARA* × *Cas9* cells that had been replated in methylcellulose for 8 weeks (when *Gata2*-targeted clones with biallelic loss-of-function mutations predominate; Figure 4B-C; supplemental Figure 4A). We then serially replated unsorted mixtures of $mCherry^+$ and $mCherry^-$ cells in MethoCult and quantified mCherry-expressing cells. This strategy led to a robust expression of $GATA2^{WT}$ and $GATA2^{R362G}$ protein within 3 days (Figure 6A). Similarly, the mCherry reporter, which identified the transduced cells, was expressed in ≥25% of the cells at day 3 posttransduction (Figure 6B). $mCherry^+$ cells with $Gata2^{WT}$ were strongly selected against within 2 weeks of replating (40.7-fold reduction in the percentage of $mCherry^+$ cells, compared with day 3 post transduction; $P < .0001$ by ANOVA; Figure 6B). In contrast, cells that were transduced with an empty vector showed no change in the fraction of $mCherry^+$ cells during the same time period (1.03-fold reduction compared with day 3 posttransduction; $P = .934$, ANOVA). Cells transduced with the $Gata2^{R362G}$ vector developed a small decrease in the percentage of $mCherry^+$ cells (1.7-fold reduction; $P < .001$, ANOVA), suggesting that this mutation confers a loss-of-function phenotype. We found similar results in *Rosa26*-targeted *PML-RARA* × *Cas9* cells (Figure 6A; supplemental Figure 4F), suggesting that $Gata2^{WT}$ suppresses serial replating

Figure 2. Increased *Gata2* expression in preleukemic mouse myeloid cells expressing *PML-RARA*. (A) Heat map of the 3361 DEGs between lineage-depleted and flow-purified GFP^+ WT mouse bone marrow cells 7 days following retroviral transduction with $PML\text{-}RARA^{WT}$ compared with those transduced with an empty MSCV-IRES-GFP vector control by bulk RNA-seq (fold change ≥2 and FDR <0.05). Also plotted is the expression of the same DEGs in cells transduced with $PML\text{-}RARA^{C88A}$ MSCV-IRES-GFP vector. (B) Volcano plot of the expression changes in (A). Upregulated (red) and downregulated (blue) DEGs (fold change ≥2 and FDR <0.05) are highlighted. Expression changes in *Bcl2*, *Zbtb16* (*Plzf1*), *Hgf*, *Pdgfrb*, *Hdc*, *Elane*, *Prtn3*, and *Cd177* are labeled. (C) Relative *Gata2* RNA expression in lineage-depleted and flow-purified GFP^+ WT mouse bone marrow cells 7 days following retroviral transduction with $PML\text{-}RARA^{WT}$, $PML\text{-}RARA^{C88A}$, or an empty MSCV-IRES-GFP vector. Data in panels A-C are from 3 independent biological replicates, each from a separate 6- to 8-week-old WT mouse. (D) Heat map of the 1838 DEGs between flow-purified promyelocytes from preleukemic *Ctsg-PML-RARA* mice compared with those from WT littermates by bulk RNA-seq (fold change ≥2 and FDR <0.05). (E) Volcano plot of the expression changes in (D). Upregulated (red) and downregulated (blue) DEGs (fold change ≥2 and FDR <0.05) are highlighted. Expression changes in *Bcl2*, *Zbtb16* (*Plzf1*), *Hgf*, *Pdgfrb*, *Hdc*, *Elane*, *Prtn3*, and *Cd177* are labeled. (F) Relative *Gata2* RNA expression in flow-purified promyelocytes from preleukemic *Ctsg-PML-RARA* mice compared with those from WT mice by RNA-seq. Data in panels D-F are from 3 pairs of mice that were littermate matched and 6 to 8 weeks of age; each flow-purified promyelocyte sample was from a unique mouse. *P* values are corrected for multiple testing. ****$P < .0001$.

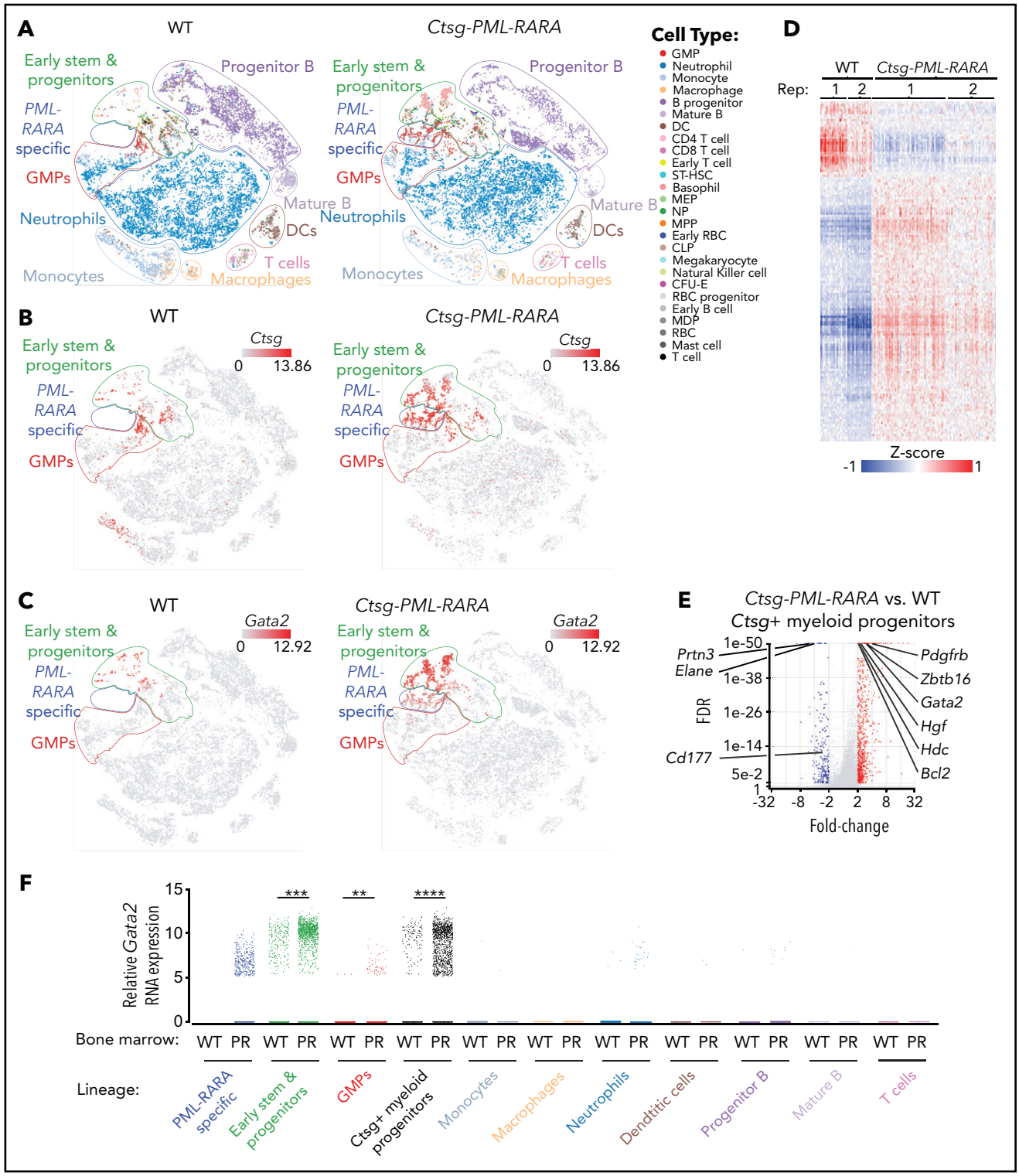

Figure 3. *PML-RARA* expression drives the formation of a unique population of preleukemic myeloid progenitors that express *Gata2*. (A) t-Distributed stochastic neighbor embedding (t-SNE) plots of scRNA-seq data from whole bone marrow cells from young nonleukemic WT mice (left panel) or *Ctsg-PML-RARA* mice (right panel) mice. Known hematopoietic cell types are labeled based on Haemopedia gene expression profiling[40,58]. Major cell populations (early stem and progenitors, GMPs, neutrophils, monocytes, macrophages, T cell, DCs, mature B, and progenitor B) are inferred, outlined, and labeled based on Haemopedia lineage assignment software and based on graph-based clustering analysis. A unique population of myeloid precursor cells that are only present in the bone marrow from *Ctsg-PML-RARA* mice is outlined in blue ("*PML-RARA* specific"). (B) t-SNE plots of the relative expression of *Ctsg* in whole bone marrow cells from WT mice (left panel) or *Ctsg-PML-RARA* mice (right panel) by scRNA-seq. (C) t-SNE plots of the relative expression of *Gata2* in whole bone marrow cells from WT mice (left panel) or *Ctsg-PML-RARA* mice (right panel) by scRNA-seq. (D) Heat map of the 1090 DEGs between *Ctsg*$^+$ myeloid precursors from *Ctsg-PML-RARA* mice compared with those from WT littermates by scRNA-seq. Replicate littermate pairs are labeled as Rep 1 and Rep 2 (fold change ≥2 and FDR <0.05 by ANOVA). (E) Volcano plot of expression changes between *Ctsg*$^+$ myeloid progenitors from *Ctsg-PML-RARA* vs WT mouse bone marrow. Expression changes in *Bcl2*, *Zbtb16* (*Plzf1*), *Hgf*, *Pdgfrb*, *Hdc*, *Elane*, *Prtn3*, and *Cd177* are labeled. (F) Relative *Gata2* expression by scRNA-seq in the various lineage populations from panel A in WT or *Ctsg-PML-RARA* (PR) bone marrow. Data in panels A-F are from 2 pairs of mice that were littermate matched and 8 to 12 weeks of age. Samples of the same genotype were concatenated for the analyses in panels A-C and E-F. ***P* = 1.37E-4, ****P* = 1.73E-142, *****P* < 1.00E-298; ANOVA. CFU-E, colony forming unit-erythroid; CLP, common lymphoid progenitor; DC, dendritic cell; GMPs, granulocyte-monocyte progenitors; MDP, monocyte dendritic cell progenitor; MEP, megakaryocyte erythroid progenitor; MPP, multipotent progenitor; NP, neutrophil progenitor; RBC, red blood cell; ST-HSC, short-term hematopoietic stem cell.

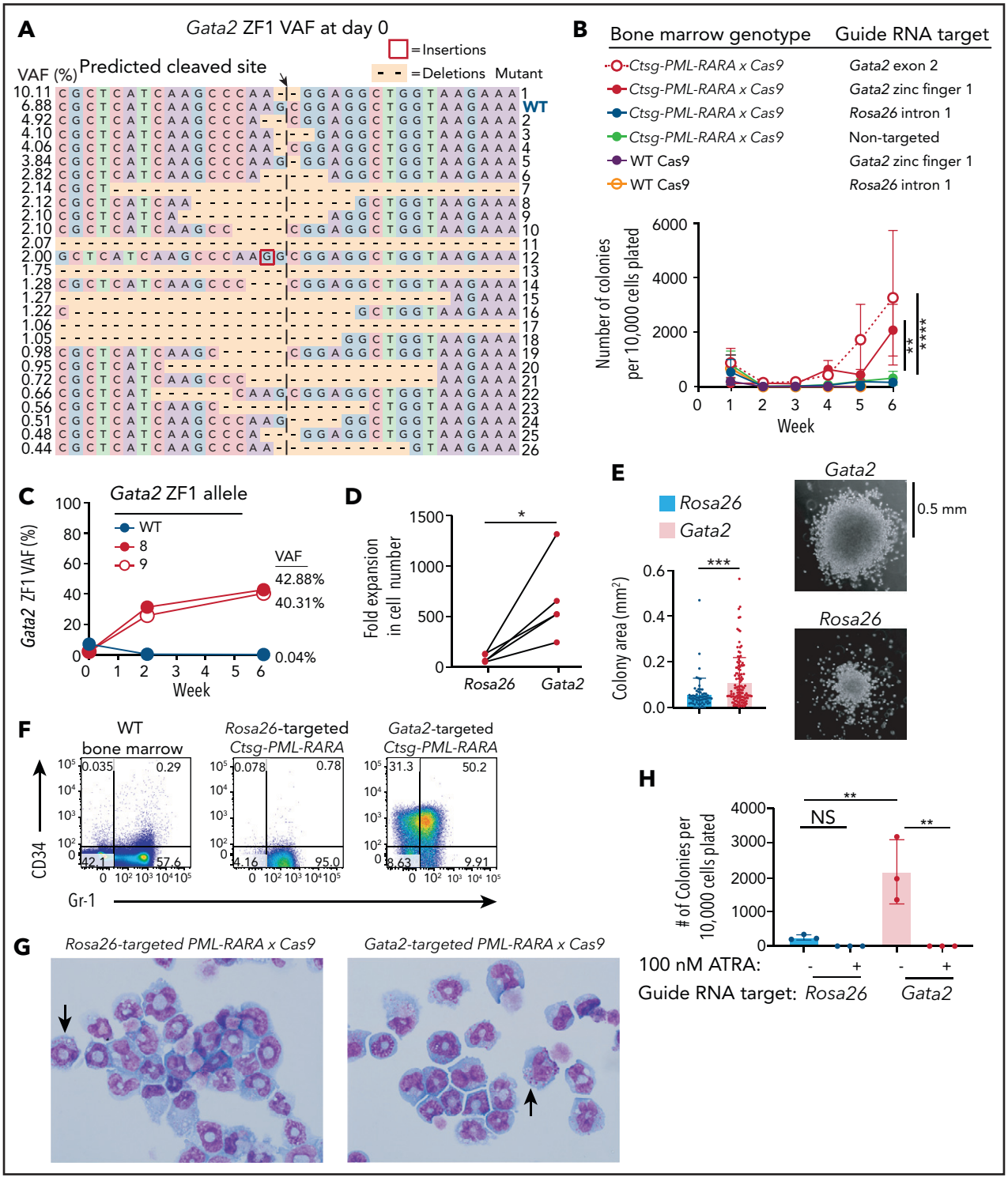

Figure 4. Effects of biallelic *Gata2* mutations on *PML-RARA*–induced serial replating. Serial replating assays using preleukemic lineage-depleted *PML-RARA* × *Cas9* or WT *Cas9* bone marrow cells that were electroporated with CRISPR guide RNAs targeting *Gata2* zinc finger domain 1 (ZF1), *Gata2* exon 2, or *Rosa26* intron 1 (neutral mutation control) prior to initial plating in MethoCult M3534. Cells were serially replated for 6 weeks. (A) The initial *Gata2* variant allele frequencies (VAF) at day 0 (day of initial plating in MethoCult) by digital sequencing following CRISPR targeting with a *Gata2* ZF1-specific guide RNA. Data are representative of 3 independent biological replicates. (B) Colony counts from serial replating assays in which preleukemic *PML-RARA* × *Cas9* or WT *Cas9* bone marrow cells were electroporated with CRISPR guide RNAs targeting *Gata2* ZF1, *Gata2* exon 2, or *Rosa26* or were left untransfected (nontargeted) prior to initial plating in MethoCult. Three or 4 independent biological replicates are plotted for each guide RNA target. $^{**}P < .01$, $^{****}P < .0001$; 2-way ANOVA at week 6. (C) VAFs of the *Gata2* ZF1 alleles WT, 8, and 9 from panel A over time. Data are representative of 3 independent biological replicates. (D) The fold expansion in the total number of live cells from the number that was plated at the beginning of week 6 compared with those at the end of week 6 for *Rosa26*- and *Gata2*-targeted *PML-RARA* × *Cas9* cells from panel B. *Gata2*-targeted samples include samples electroporated with ZF1 or exon 2 *Gata2* guide RNAs. Five independent biological replicates are plotted for each target (*Rosa26* or *Gata2*). $^{*}P < .05$; paired, 2-tailed Student *t* test. (E) The colony area of *Rosa26*- and *Gata2*-targeted *PML-RARA* × *Cas9* cells at week 6 (left panel). Data from 2 independent biological replicates are plotted for each target (*Rosa26* or *Gata2*). *Gata2*-targeted samples include those electroporated with a ZF1 or an exon 2 *Gata2* guide RNA. $^{***}P < .001$; unpaired, 2-tailed Student *t* test. Representative

regardless of whether the cells are *Gata2* sufficient or deficient. Consistent with these data, $Gata2^{R362G}$ showed a decreased capacity to suppress serial replating in *Gata2*-sufficient (*Rosa26*-targeted) *PML-RARA* × *Cas9* cells.

We next asked whether a similar strategy could suppress the growth of fully transformed APL cells deficient for *Gata2* in vivo. We transduced *Gata2*-deficient APL samples with $Gata2^{WT}$ or empty vector MSCV-IRES-mCherry–based retroviruses, cultured these cells for 3 days ex vivo, and then transplanted unsorted mixtures of $mCherry^+$ and $mCherry^-$ APL cells into sublethally irradiated mice. Western blotting showed that GATA2 protein was expressed at high levels 3 days posttransduction (the day of transplantation) in samples that were transduced with $Gata2^{WT}$ (Figure 6C-D). mCherry was similarly expressed in a large percentage of cells transfected with the empty vector or the $Gata2^{WT}$ vector on the day of transplantation (Figure 6E). As expected, mice transplanted with either vector developed APL with similar penetrance and latency (because both cohorts received a mixture of untransduced and transduced cells; $P = .6158$ by log-rank [Mantel-Cox] test; Figure 6F). However, the APL samples transduced with the *Gata2* vector contained no $mCherry^+$ cells at day 28, suggesting that these cells were eliminated because they expressed *Gata2* (Figure 6C-E,G). Conversely, APLs transduced with the empty vector continued to contain $mCherry^+$ cells. Together, these data show that addback of *Gata2* expression to *Gata2*-deficient APLs can suppress the growth of fully transformed APL cells.

Discussion

In this study we demonstrate that *Gata2* acts as a tumor suppressor in the context of *PML-RARA*–driven aberrant self-renewal and leukemogenesis. We initially showed that *Gata2* is aberrantly expressed in human and mouse models of APL, suggesting that *PML-RARA* may upregulate *Gata2* expression, perhaps promoting self-renewal and APL initiation. However, we found that biallelic loss-of-function *Gata2* mutations increased the efficiency of aberrant serial replating caused by *PML-RARA*, *RUNX1-RUNX1T1*, or *Cebpa* inactivation. *Gata2* deficiency also increased the penetrance and decreased the latency of mouse APL. In addition, retroviral addback of $Gata2^{WT}$ expression was able to suppress *PML-RARA*–driven aberrant self-renewal and APL development, whereas addback of the $Gata2^{R362G}$ mutant did not, suggesting that this recurrent AML-associated mutation has a loss-of-function phenotype.

Why does *PML-RARA* upregulate a tumor suppressor in preleukemic cells? Proliferative stress caused by *PML-RARA*[36] or other AML-initiating mutations may induce *Gata2* expression indirectly, as a physiologic "brake" to limit an aberrant proliferative response. *Gata2* has indeed been shown to inhibit the proliferation of hematopoietic progenitors in several contexts.[16,20,21] Subsequent inactivating mutations of *GATA2* may release this brake and allow for rapid AML progression. The high levels of *GATA2* expression detected in nearly all AML samples[6] strongly suggest that this may be a widespread phenomenon in this disease.

Alternatively, *GATA2* may facilitate early steps in transformation by acting as an oncogene. In this model, after transformation has occurred, high *GATA2* levels would be toxic, so that inactivation of *GATA2* would be required for the leukemic cells to survive. Consistent with this model, Saida et al[53] showed that *Gata2* haploinsufficiency increases the latency of *CBFB-MYH11*–initiated AML in mice; however, the leukemias arising in these mice are more aggressive in transplantation experiments,[53] suggesting that the residual $Gata2^{WT}$ allele may have been inactivated during tumor progression (although this was not reported in the article). In our study, only cells with biallelic *Gata2* inactivation were selected for during in vitro and in vivo expansion, suggesting that the main function of *GATA2* during AML progression is that of a tumor suppressor. This model fits with previous reports that described AML patients with heterozygous missense mutations in *GATA2*,[3,8,15,22-27] where expression of the residual $GATA2^{WT}$ allele is nearly always lost by epigenetic silencing.[8,22,24,30] Similarly, in AMLs with inv(3)/t(3;3) or atypical 3q26/*MECOM* translocations (which disrupt the −110 kb *GATA2* enhancer), 1 *GATA2* allele is silenced as a result of the translocation, and the remaining nonrearranged allele frequently acquires an inactivating mutation or its expression is lost by epigenetic silencing or deletion.[8,16,23,31] Previous data also suggest that AML progression occurs when monoallelic expression of $GATA2^{WT}$ is lost in patients with germline *GATA2* mutations.[22]

The molecular mechanisms responsible for high *GATA2* expression levels in *PML-RARA*–expressing cells are unknown. It is most likely a transcriptional mechanism, but it is not yet clear whether *GATA2* is a direct or an indirect target of PML-RARA. PML-RARA does bind near the *GATA2* gene in APL cells and cell lines,[54] but this observation does not establish cause and effect. In the *MRP8-PML-RARA* model of APL, *Gata2* expression was found to be lower in "early" promyelocytes[55]; it is not clear whether this is due to a difference in the timing of *PML-RARA* expression during myeloid development or is related to the stage of development of the tested cells. Regardless, in the different models presented in this study, all were associated with increased levels of *Gata2* expression, which corroborated the finding of persistently high *GATA2* expression in most APL patients tested to date.

Several studies have now implied that the epigenetic repression of the residual WT allele in AMLs with heterozygous missense mutations in *GATA2* is associated with methylation of the *GATA2* locus,[8,22,24] suggesting that hypomethylating

Figure 4 (continued) images of colony morphologies and sizes from *PML-RARA* × *Cas9* cells electroporated with *Gata2* or *Rosa26* guide RNAs (right panels). Images were captured using an Olympus IX-51 microscope and a QImaging QIClick camera (original magnification ×4). (F) CD34 and Gr-1 expression by flow cytometry on WT bone marrow (not replated in MethoCult) or on *Rosa26*- or *Gata2*-targeted *PML-RARA* × *Cas9* cells from panel A replated in MethoCult for 8 weeks. Data are representative of 3 or 4 independent biological replicates. (G) Wright-Giemsa staining of *Rosa26* or *Gata2* exon 2–targeted *PML-RARA* × *Cas9* cells from panel A replated in MethoCult for 8 weeks and then cultured in suspension media containing SCF, FLT3L, interleukin-3, and TPO for 2 days. Arrows point to cells containing prominent cytoplasmic azurophilic granules. Data are representative of 3 independent biological replicates. Images were captured using an Olympus BX-53 microscope and an Olympus DP-72 camera (original magnification ×100). (H) The number of colonies per 10 000 cells plated at week 6 for *Rosa26*-targeted or *Gata2*-targeted *PML-RARA* × *Cas9* cells that were cultured with 100 nM ATRA (+) or DMSO vehicle control (-). Data from 3 independent biological replicates are plotted for each target. $^{**}P < .01$; 1-way ANOVA. NS, not significant.

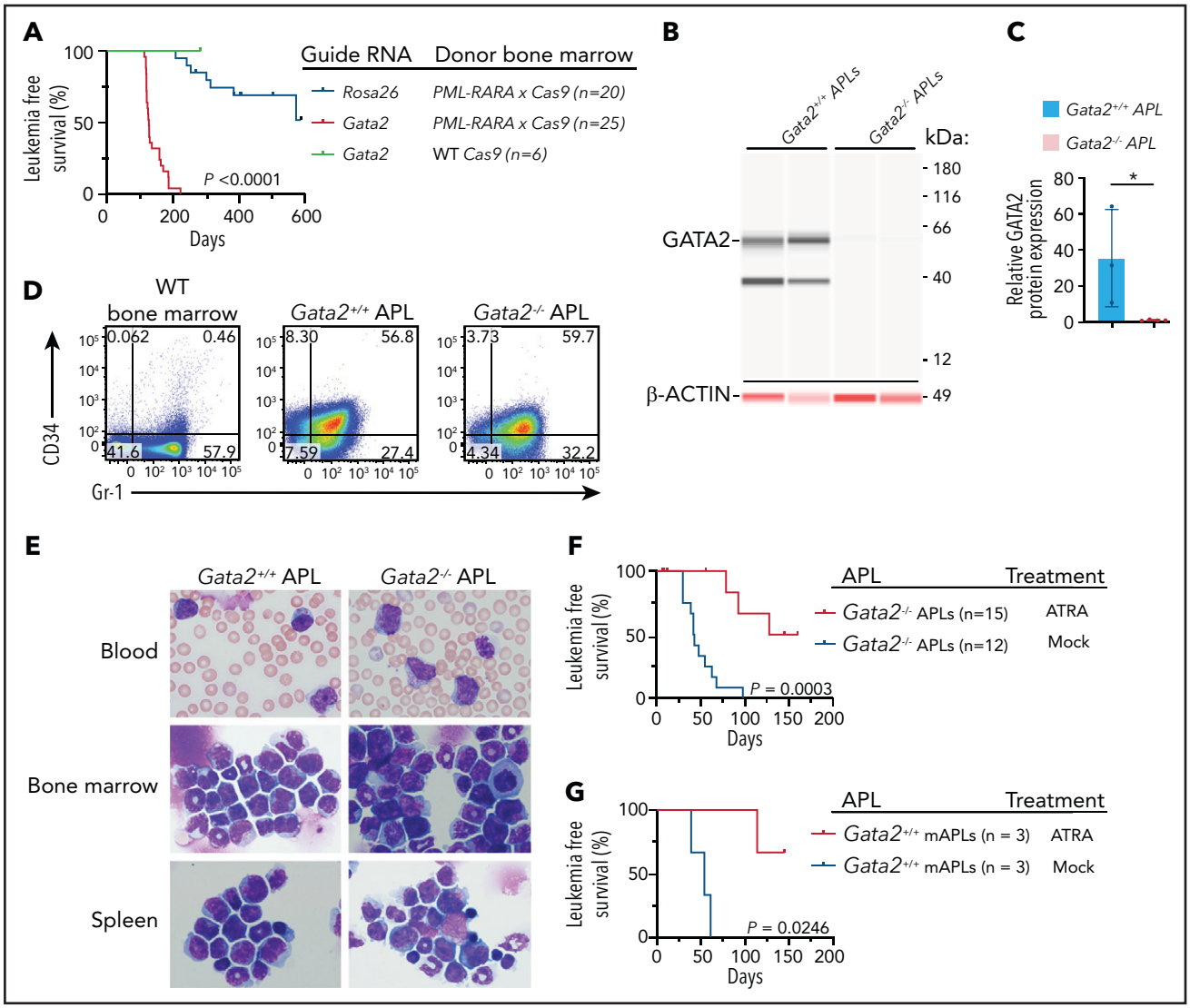

Figure 5. *Gata2* acts as a tumor suppressor in mouse APL. (A) Leukemia-free survival of recipient Ly5.1 mice transplanted with nonleukemic lineage-depleted *PML-RARA* × *Cas9* bone marrow in which *Rosa26* intron 1 or *Gata2* exon 2 was targeted with CRISPR guide RNAs prior to transplantation (n = 20 mice in *Rosa26* group from 2 cohorts of 10 mice per cohort; n = 25 mice in *Gata2*-targeted *PML-RARA* × *Cas9* bone marrow group from 3 cohorts of 5 to 10 mice per cohort; n = 6 mice in *Gata2*-targeted WT *Cas9* bone marrow group from 1 cohort of mice). $P < .0001$, log-rank (Mantel-Cox) test. (B) Protein expression, detected using a Jess by ProteinSimple apparatus, of GATA2 and β-ACTIN from the bone marrow of *Gata2*-sufficient ($Gata2^{+/+}$) or *Gata2*-targeted ($Gata2^{-/-}$) *Ctsg-PML-RARA* APL mice. Blots for GATA2 and β-ACTIN were performed on the same samples using 2 antibodies in 2 spectral channels: chemiluminescence (GATA2) and near infrared (β-ACTIN). Data are representative of 3 or 4 independent biological replicates per genotype. (C) Quantification of the relative GATA2/β-ACTIN protein expression from the bone marrow of $Gata2^{+/+}$ or $Gata2^{-/-}$ *Ctsg-PML-RARA* APL mice. Data are from 3 or 4 independent biological replicates per genotype. (D) CD34 and Gr-1 expression by flow cytometry on bone marrow from 8- to 12-week-old WT mice, $Gata2^{+/+}$ *Ctsg-PML-RARA* APL spleen cells, and $Gata2^{-/-}$ *Ctsg-PML-RARA* APL spleen cells. Data are representative of 3 or 4 independent biological replicates. (E) Wright-Giemsa staining of blood, bone marrow, and spleen from $Gata2^{+/+}$ (*Rosa26*-targeted) or $Gata2^{-/-}$ (*Gata2*-targeted) *Ctsg-PML-RARA* APL mice. Images are representative of 5 to 8 independent biological replicates for each genotype. Images were captured using an Olympus BX-53 microscope and an Olympus DP-72 camera (original magnification ×100). (F) Leukemia-free survival of recipient Ly5.1 mice transplanted with fully transformed $Gata2^{-/-}$ *Ctsg-PML-RARA* APL spleen cells and then treated with surgically implanted ATRA pellets (black lines) or control pellets (Mock; red lines). N = 12 control-treated mice; N = 15 ATRA-treated mice. $Gata2^{-/-}$ APLs were derived from 3 biologically independent mice. $P = .0003$, log-rank (Mantel-Cox) test. (G) Leukemia-free survival of recipient Ly5.1 mice transplanted with $Gata2^{+/+}$ *Ctsg-PML-RARA* APL spleen cells and then treated with surgically implanted ATRA pellets (black lines) or control pellets (Mock; blue lines). N = 3 control-treated mice, N = 3 ATRA-treated mice. $Gata2^{+/+}$ APLs were derived from 1 mouse. $P = .0246$, log-rank (Mantel-Cox) test.

agents may have a role in treating *GATA2*-mutant AML patients.[24] One caveat, however, is that *GATA2* missense mutations are nearly always progression events that occur in AML subclones.[9,11,56] In this article, we have shown that somatically acquired *Gata2* deficiency is not sufficient to directly cause AML in mice, similar to findings by other investigators.[32,35] Therefore, *GATA2* mutations are probably not initiating mutations in AML, and restoring *GATA2* expression may only diminish the growth of *GATA2*-deficient subclones. However, we also noted that *Gata2* overexpression reduced the growth of *Gata2*-sufficient hematopoietic cells, suggesting that it may have broad nonspecific effects on cell growth that could potentially affect nonmutant AML cells as well. *Gata2* overexpression also promotes myeloid differentiation in WT

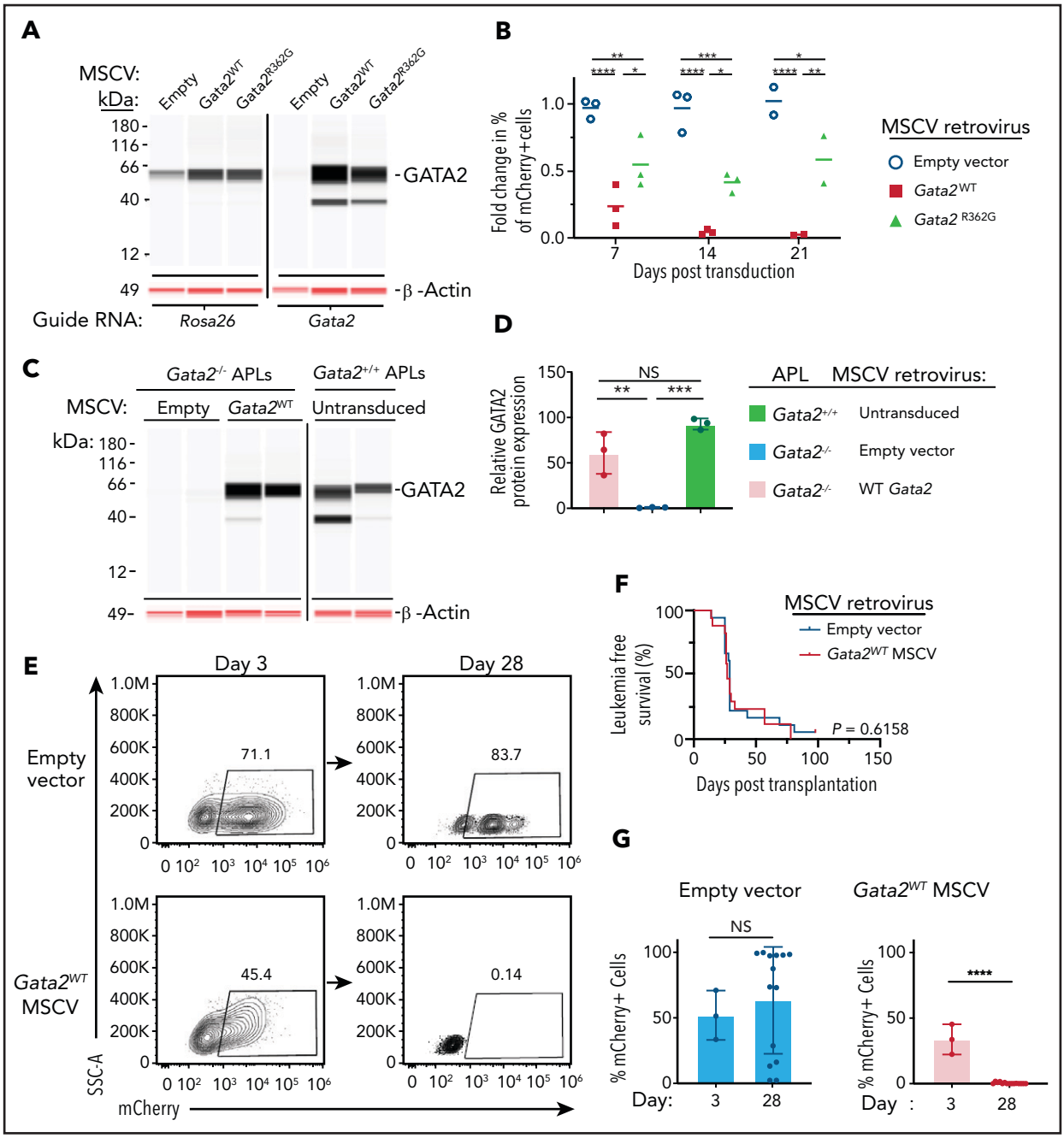

Figure 6. Restoration of *Gata2* expression inhibits *PML-RARA*-induced aberrant self-renewal and leukemogenesis. (A) Retroviral "addback" of *Gata2* in preleukemic *PML-RARA*–expressing cells. The initial protein expression detected using a *Jess* apparatus by ProteinSimple of GATA2 and β-ACTIN from preleukemic lineage-depleted *PML-RARA* × *Cas9* bone marrow cells that were electroporated with CRISPR guide RNAs targeting *Rosa26* intron 1 or *Gata2* exon 2, serially replated in MethoCult M3534 for 8 weeks, and then transduced with empty vector, $Gata2^{WT}$, or $Gata2^{R362G}$ MSCV-IRES-mCherry retroviruses. Samples are from cells that were grown in suspension culture with SCF, FLT3L, interleukin-3 (IL-3), and TPO (to minimize clonal selection pressures) for 3 days posttransduction, and then flow purified on $mCherry^+$ cells. The *Rosa26*-targeted samples were run on a separate blot from the *Gata2*-targeted samples. Blots for GATA2 and β-ACTIN were performed using the same samples with 2 antibodies in 2 spectral channels: chemiluminescence (GATA2) and near infrared (β-ACTIN). (B) Fold change in the percentage of $mCherry^+$ cells by flow cytometry in *Gata2* exon 2–targeted *PML-RARA* × *Cas9* bone marrow cells from panel A that were serially replated in MethoCult for 8 weeks, then retrovirally transduced with $Gata2^{WT}$, $Gata2^{R362G}$, or empty MSCV-IRES-mCherry vectors, and replated in MethoCult for an additional 2 to 3 weeks. Fold change is relative to the mCherry expression at day 3 posttransduction (n = 3 at days 7 and 14; n = 2 at day 21; independent biological replicates). $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$,$^{****}P < .0001$; 2-way ANOVA . (C) Retroviral "addback" of *Gata2* in $Gata2^{-/-}$ APLs. Protein expression was detected using a Jess apparatus by ProteinSimple of GATA2 and β-ACTIN at day 3 following the transduction of $Gata2^{-/-}$ APL cells with empty vector, $Gata2^{WT}$ MSCV-IRES mCherry retroviruses, or untransduced $Gata2^{+/+}$ APL cells. Cells were grown in suspension culture ex vivo with SCF, FLT3L, IL-3, and TPO (to minimize clonal selection pressures) for 3 days posttransduction. At day 3 posttransduction, APL cells were transplanted into sublethally irradiated Ly5.1 mice. Antibodies against GATA2 and β-ACTIN were used in the same samples in 2 spectral channels: chemiluminescence (GATA2) and near infrared (β-ACTIN). (D) Quantification of the relative GATA2/β-ACTIN protein expression in untransduced $Gata2^{+/+}$ APLs (green bar), empty vector–transduced $Gata2^{-/-}$ APLs (blue bar), or $Gata2^{WT}$ MSCV–transduced $Gata2^{-/-}$ APLs (red bar) from the blots in panel C. $^{**}P < .01$, $^{***}P < .001$; 2-way ANOVA. (E) mCherry expression by flow cytometry in $Gata2^{-/-}$ APL samples transduced with empty vector (upper panels) or *Gata2* MSCV-IRES-mCherry (lower panels) retroviruses. Samples are from APLs that were grown ex vivo for 3 days posttransduction with cytokines (left panels) or those that were subsequently transplanted into Ly5.1 mice and harvested on day 28 (right panels). Day-28 samples are gated on $CD45.2^+$ peripheral blood APL cells. (F) Leukemia-free survival of recipient Ly5.1 mice that were transplanted with unsorted $mCherry^-$ and $mCherry^+$ $Gata2^{-/-}$ *Ctsg-PML-RARA* APL cells following transduction with empty vector or *Gata2* MSCV-IRES-mCherry retroviruses. $P = .6158$; log-rank (Mantel-Cox) test. (G) Quantification of mCherry expression at day 3 and 28 in empty vector–transduced (left panel) and $Gata2^{WT}$ MSCV–transduced (right panel) APLs from panel D. In panels E-G, each of 3 independently derived $Gata2^{-/-}$ APLs was transduced with empty vector or *Gata2* MSCV retroviruses and then transplanted into 5-6 Ly5.1 recipient mice (18 recipient mice received empty vector–transduced $Gata2^{-/-}$ APLs; 17 recipient mice received *Gata2* MSCV-mCherry–transduced $Gata2^{-/-}$ APLs). $^{****}P < .0001$; unpaired, 2-tailed Student *t* test. NS, not significant.

hematopoietic progenitor cells[36]; therefore, enhancing its expression in AML cells may reduce growth and promote differentiation.

Our data complement other studies that have suggested that *GATA2* normally acts as a tumor suppressor in AML cells and that the loss of this activity, by genetic or epigenetic means, accelerates AML progression. In AML cells in which *GATA2* has been completely inactivated by the mutation of 1 allele and the residual WT allele has been repressed by DNA methylation, hypomethylating agents represent a rational therapeutic strategy to increase its activity. Alternative strategies will require a better understanding of the tumor-suppressive activities of this important hematopoietic transcription factor.

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Authorship

Contribution: C.D.S.K. and T.J.L. designed research ; C.D.S.K, O.R.S.R., R.B.D., M.A.C., T.P.R., N.M.H., M.H., and L.D.W. performed research; C.D.S.K, S.M.R., C.A.M., S.N.S., O.R.S.R., M.A.C., T.P.R., and T.J.L. analyzed data; and C.D.S.K. and T.J.L. wrote the manuscript.

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Footnotes

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All sequencing data for the mouse studies were deposited to the Sequence Read Archive at the National Center for Biotechnology Information database (accession PRJNA699863).

Data sharing requests should be sent to Timothy J. Ley (timley@wustl.edu).

The online version of this article contains a data supplement.

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