Tumor suppressor function of *WT1* in acute promyelocytic leukemia

Originally identified as a cancer susceptibility gene, Wilms' Tumor 1 gene (*WT1*) is overexpressed or mutated in a wide variety of malignancies, including acute myeloid leukemia (AML). WT1 is a zinc-finger transcription factor comprised of C-terminal zinc-finger DNA binding domains and an N-terminal transactivation domain thought to regulate interactions with partner proteins. Germline *WT1* mutations consist primarily of nonsense mutations that truncate the C-terminal domains, or missense mutations that disrupt DNA binding, and these mutations result in both developmental abnormalities and predisposition to Wilms' tumor.[1]

In normal human CD34+ hematopoietic stem/progenitor cells (HSPC), wild-type *WT1* is expressed at a low level, but it is highly expressed in nearly all cases of AML. Among all AML subtypes, *WT1* expression is generally highest in acute promyelocytic leukemia (APL/M3 AML), the AML subtype initiated by the *PML-RARA* fusion gene (*Online Supplementary Figure S1A and D*).[2,3] In addition, we and others[4] have identified recurrent *WT1* mutations in APL cases (11/42 [26%] in this study) (*Online Supplementary Figure S1C*). In seven of 11 of these cases, *WT1* mutations occurred at a significantly lower variant allele frequency (VAF) than *PML-RARA*, suggesting they are co-operating events in subclones (data not shown). WT1 mutations have been associated with worse prognosis in non-M3 AML, although no such association has been shown in APL. The spectrum of *WT1* mutations is similar in APL *versus* other AML cases (*Online Supplementary Figure S1B*), suggesting that *WT1* mutations may have similar biologic activities across all AML subtypes.

These observations frame a well-known - but unsolved - paradox that we attempt to address here: does a high level of wild type *WT1* expression contribute to the initiation or progression of AML/APL, or conversely, does it reflect a tumor suppressor activity, since inactivating mutations appear to contribute to disease progression?

In order to explore these questions, we first tested the ability of *Wt1* mutations to co-operate with *PML-RARA* in a well-characterized murine APL model. *Ctsg-PML-RARA* mice express the PML-RARA fusion cDNA in immature hematopoietic progenitor cells, and succumb to an APL-like disease with a latency of about 1 year in C57Bl/6J mice.[5] In order to test whether *Wt1* mutations can co-operate with *PML-RARA* in this model, we used CRISPR/Cas9 to generate indels in *Wt1* exon 8 or, as a control, the *Rosa26* locus. Since murine Wt1 is highly homologous to the human protein, these mutations should mimic those commonly found in APL patients. Despite efficient mutation generation in *Ctsg-PML-RARA* bone marrow cells (*Online Supplementary Figure S2A*), there was no survival difference between mice transplanted with *Ctsg-PML-RARA* cells with *Wt1* mutations *versus Rosa26* mutations (*Online Supplementary Figure S2C*). We therefore evaluated APL tumors arising in these mice, and observed that tumors could arise either from wild-type or mutant Wt1/Rosa26 progenitors (*Online Supplementary Figure S2B*). Surprisingly, we did not detect Wt1 protein in these tumors by western blotting (data not shown). Similarly, in a panel of 16 previously banked murine APL tumors from *Ctsg-PML-RARA* mice, RNA sequencing revealed virtually undetectable levels of *Wt1* mRNA (*Online Supplementary Figure S2D*), in contrast to the high expression seen in human APL samples. Together, these data suggest that important interspecies differences in *Wt1* regulation and function are important for the lack of a phenotype in this mouse model.

We next evaluated *WT1* expression in human CD34+ cells by transducing umbilical cord blood-derived CD34+ cells with retroviruses encoding *PML-RARA, RUNX1-RUNX1T1*, or *MYC*; *RUNX1-RUNX1T1* and *MYC* have previously been shown to confer self-renewal and expansion of human HSPCs *in vitro* and in xenograft models, and therefore act as positive controls.[6,7] Consistent with previously reported results, we found that human HSPC transduced with *RUNX1-RUNX1T1* and *MYC* expand robustly over 2 weeks in culture, while HSPC transduced with *PML-RARA* expand more slowly (data not shown). In order to test whether *WT1* expression is affected by transduction with these retroviral constructs, GFP+ cells were sorted 7 days after transduction, RNA was isolated, and *WT1* expression was measured by quantitative reverse transcription polymerase chain reaction (RT-PCR). Figure 1A shows upregulation of *WT1* mRNA in sorted human HSPC transduced with *PML-RARA, RUNX1-RUNX1T1*, and *MYC* (6 to 18-fold increase, $P<0.05$ for *PML-RARA* and *MYC*). WT1 protein abundance also increased dramatically in the same cells during this timeframe (Figure 1B). In order to identify other genes dysregulated by *PML-RARA* transduction, we transduced both mouse and human HSPC with GFP-tagged retroviruses encoding *PML-RARA* or an empty vector, as has been previously reported (n=2 and n=3 separate experiments for human and mouse cells respectively).[8,9] Seven days after transduction, GFP+ cells were sorted and bulk RNA sequencing was performed to identify differentially expressed genes (DEG, 5,347 identified for mouse samples, and 1,885 identified for human samples, Figure 1C). There was significant overlap between orthologous mouse and human DEG after transduction with *PML-RARA* (Figure 1D, $P=9.6\text{x}10^{-118}$ based on the hypergeometric test); further, of 867 overlapping orthologues, 82% were coordinately regulated. However, while *WT1* was ~13-fold upregulated in human cells transduced with *PML-RARA*, *Wt1* expression was extremely low and did not increase in murine cells transduced with *PML-RARA* (Figure 1E), validating the interspecies difference in *WT1/Wt1* regulation noted above.

GFP+ *PML-RARA*-expressing human cord blood cells expanded modestly during the first weeks of culture, but increased dramatically 3-4 weeks after transduction (*Online Supplementary Figure S3A*). After 6 weeks in culture, they resembled primary APL cells morphologically and immunophenotypically (*Online Supplementary Figure S3B and C*). In addition, they were sensitive to treatment with all-trans retinoic acid (ATRA), a hallmark of APL cells (*Online Supplementary Figure S3D*). Transduced cells were not immortalized, as they stopped proliferating around 8-9 weeks after initiation, and they failed to engraft immunodeficient mice (data not shown). Given its reported role as a tumor suppressor in other cancer types, we hypothesized that this upregulation may reflect an attempt of WT1 to suppress the proliferative response induced by *PML-RARA*, similar to the increased *TP53* activity observed in cells responding to genotoxic stressors.[10] However, as noted above, it is also possible that high *WT1* expression actively promotes the growth or survival of APL cells. In order to distinguish between these possibilities, we performed *WT1* overexpression *versus* loss-of-function experiments in *PML-RARA*-transduced cord blood cells.

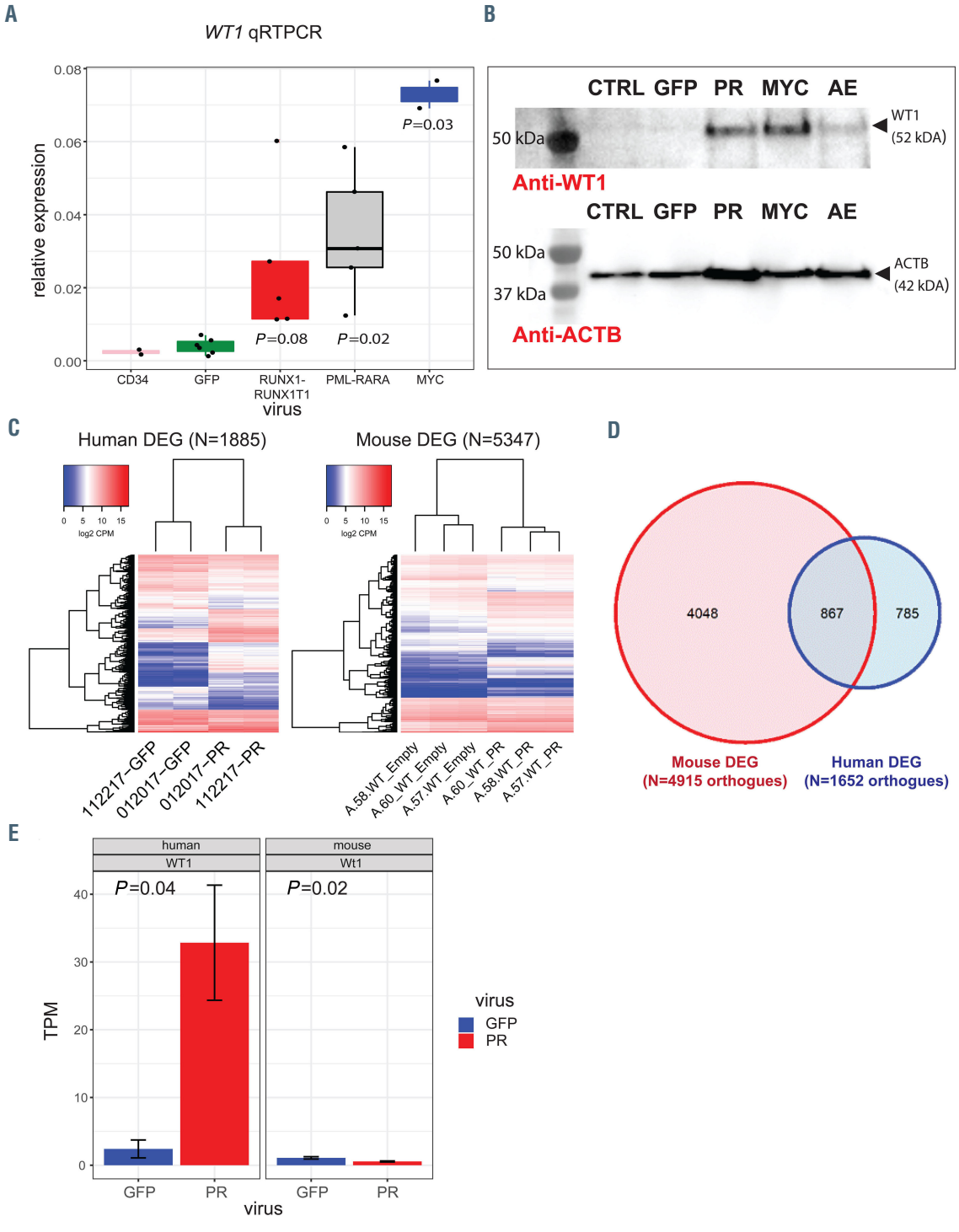

Figure 1. *WT1* expression is induced in human CD34+ cells transduced with *RUNX1-RUNX1T1*, *PML-RARA*, or *MYC*. Umbilical cord blood-derived CD34+ cells were cultured in cytokines after transduction with GFP-tagged retroviruses expressing *RUNX1-RUNX1T1*, *MYC*, *PML-RARA*, or an empty vector control. (A) *WT1* expression is induced in CD34+ cells transduced with *RUNX1-RUNX1T1*, *MYC*, or *PML-RARA* compared to controls transduced with empty vector (GFP, green) or untransduced cells (CD34, pink). CD34+ cells transduced with each vector (n=2-6 separate experiments) were cultured for 7 days, RNA was isolated from flow-sorted GFP+ cells, and *WT1* mRNA was quantified by real time polymerase chain reaction. *P*-values were calculated using Student's *t*-test. (B) Western blot showing expression of WT1 in CD34+ cord blood cells 7 days after transduction with *RUNX1-RUNX1T1* (AE), *PML-RARA* (PR), *MYC*, empty vector (GFP), or untransduced (CTRL). Lysates were made from sorted GFP+ cells except control (CTRL), which was made from equivalent cell numbers of untransduced cells cultured in parallel. Blot represents one of 3 representative experiments. (C) Heatmaps showing differentially expressed genes (DEG) in human or mouse cells transduced with a *PML-RARA*-expressing MSCV vector. Human CD34+ cells or mouse lineage-depleted bone marrow cells were transduced with IRES-GFP-tagged retroviruses containing a *PML-RARA* cDNA, or no insert (empty vector). After 7 days in culture, GFP+ cells were flow sorted and RNA was isolated for RNA sequencing. DEG were identified using a false discovery rate (FDR) cutoff of <0.05 after filtering out genes with low expression across all samples (see the *Online Supplementary Appendix*). Heatmaps show DEG in *PML-RARA vs.* empty vector-transduced human (n=2 separate experiments) and mouse (n=3 separate experiments) progenitor cells. (D) Venn diagram showing overlap in orthologous mouse and human DEG from (C). Of 4,915 mouse DEG having human orthologues, 867 are DEG in the analysis of human genes (P=9.6x10^{-118} using the hypergeometric test). (E) *WT1* expression is increased by *PML-RARA* transduction in human CD34+ cells (left panel), but not in mouse bone marrow-derived cells (right panel). WT1/Wt1 expression values from the RNA sequencing experiment described above are shown. *P*-values were calculated using Student's *t*-test. TPM: transcripts per million.

First, we used lentiviral vectors to overexpress the two most common isoforms of *WT1* (KTS+ and KTS-) in human CD34 cells in culture. In the absence of other co-operating oncogenes, *WT1* overexpression led to the rapid disappearance of transduced cells (Figure 2A), consistent with previous reports that *WT1* overexpression causes differentiation and death of CD34+ cells.[11] Next, we asked whether inactivating mutations in *WT1* could enhance expansion of CD34+ cells expressing *PML-RARA*. We transduced CD34+ cells with *PML-RARA* or empty vector, and 2 days after transduction used CRISPR/Cas9 to generate inactivating indels in *WT1*, or

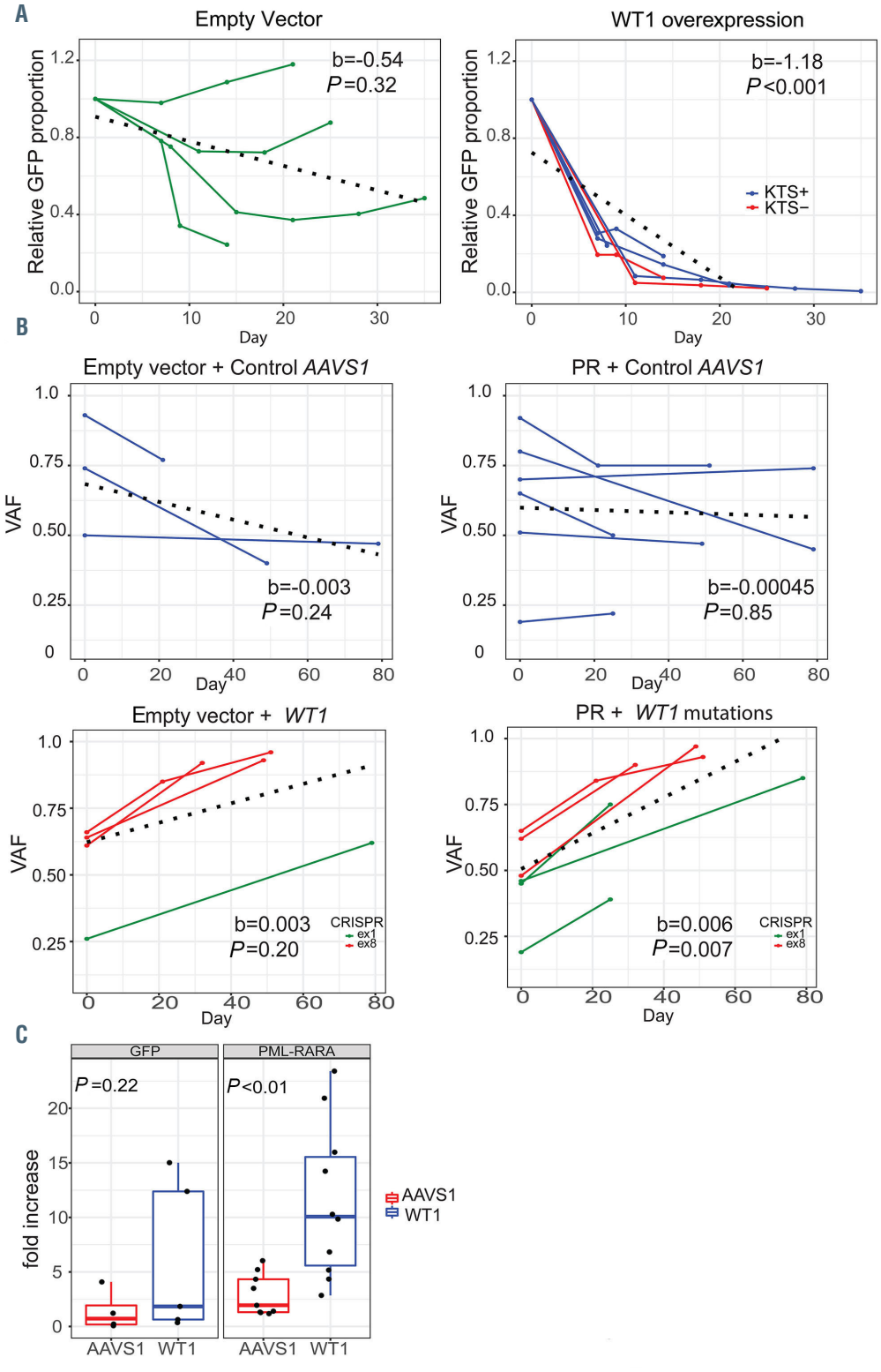

Figure 2. Legend on following page.

Figure 2. Inactivating mutations in *WT1* provide a growth advantage for *PML-RARA*-transduced CD34+ cells. (A) Umbilical cord blood-derived CD34+ cells were transduced with GFP-tagged lentiviruses encoding the two most common *WT1* isoforms (KTS+ and KTS-), or an empty vector. Cells were maintained in culture with cytokines, and GFP+ cells were quantified at different time points. Shown are percent GFP+ cells over time in *WT1* (right) or empty vector (left) transduced cultures normalized for transduction efficiency at beginning of the culture period (n=4 individual experiments). Black dotted lines show line of best fit calculated by linear regression. Transduction with *WT1* isoforms (KTS+ and KTS-) leads to loss of GFP+ cells (slope b=-1.18 per day, *P*<0.001), while empty vector-transduced cells have GFP+ cells throughout the culture period (slope b=-0.54, *P*=0.32). *P*-values were calculated using a linear regression model, and represent the probability that the slope of the best fit line equals zero. (B) Human CD34+ cord blood cells were transduced with *PML-RARA*-expressing retrovirus or empty vector, and 48 hours later CRISPR/Cas9 was used to generate mutations in *WT1* (exon 1 or exon 8) or *AAVS1* (a negative control locus). GFP+ cells were sorted at different time points from cultures that had been transduced with a vector containing *PML-RARA* (right panels) or no insert (empty vector, left panels). DNA was isolated and polymerase chain reaction products containing the guide RNA target sites were digitally sequenced to determine the precise variant allele frequencies of mutations in *WT1* (bottom panels) or *AAVS1* (top panels). Shown are change in variant allele frequency (VAF) of *AAVS1* mutations or *WT1* mutations over time (n=3-6 separate experiments). Mutations in *WT1* exon1 are shown in green, or *WT1* exon 8 in red. Black dotted lines show line of best fit calculated by linear regression. Cells containing mutations in *WT1* show a trend toward expansion in empty vector-transduced CD34+ cells (slope b=0.003 increase per day, *P*=0.20), and a statistically significant expansion in *PML-RARA*-transduced cells (slope b=0.006 increase per day, *P*=0.007). In contrast, cells with mutations in *AAVS1* do not expand over time. *P*-values were calculated using a linear regression model, and represent the probability that the slope of the best fit line equals zero. (C) Increase in overall cell numbers in cultures transduced with GFP (left) or *PML-RARA* (right). *P*-values were calculated using Student's *t*-test.

as a control, *AAVS1*. After 4-8 weeks in culture, GFP+ cells were sorted and the frequency of *WT1* or *AAVS1* indels in GFP+ cells at the end of the culture period was compared to the frequency at the beginning. *WT1* mutations were selected for over time, and were significantly increased in *PML-RARA*-transduced cells 4-8 weeks after the *WT1* mutations were introduced (Figure 2B, bottom panels). In contrast, cells containing mutations in *AAVS1* did not increase in frequency (Figure 2B, top panels). Overall cell numbers significantly increased in cells bearing both *PML-RARA* and *WT1* mutations, compared to cells with *PML-RARA* and *AAVS1* mutations, or cells transduced with an empty vector with *WT1* mutations (Figure 2C). Together, these findings suggest that *WT1* inactivation enhances the growth of *PML-RARA*-expressing hematopoietic cells, strongly suggesting that WT1 acts as a tumor suppressor in this context.

Based on the above findings, we propose a simple model to explain these paradoxical observations: *WT1* expression in HSPC is normally activated as an adaptive and inhibitory response to oncogenic mutations that cause proliferation, a response that is intended to slow their growth. The subsequent development of inactivating *WT1* mutations in some cases would then provide a further growth advantage by removing that normal inhibitory response. Supporting this hypothesis, we found that i) retroviral transduction of CD34+ cells with *PML-RARA*, *RUNX1-RUNX1T1*, or *MYC* all led to a robust induction of *WT1* expression; ii) forced expression of wild-type *WT1* by itself does not promote CD34 cell expansion; and iii) inactivation of *WT1* in *PML-RARA*-expressing CD34+ cells leads to an additional growth advantage. Although the mechanism of *WT1* gene activation by oncogenes is not yet clear, high levels of *WT1* expression are found in nearly all AML cases, regardless of subtype or mutational landscape. In addition, since the majority of AML/APL cases do not have *WT1* mutations, a corollary of this hypothesis is that leukemias with wild-type *WT1* must have developed alternative means to circumvent the inhibitory pressure that *WT1* induction may exert.

Finally, the downstream mechanisms by which *WT1* mutations lead to a growth advantage in AML cells are currently unclear, and may depend on the context of the co-operating mutations. In addition to its well-described function as a locus-specific transcription factor,[12] recent studies have suggested that *WT1* mutations may cause epigenetic changes via effects on DNA methylation and interactions with TET family methylcytosine deoxygenases.[13-16] A better understanding of how *WT1* mutations activate these pathways in AML cells will be required to fully exploit their potential therapeutic value.

Matthew J. Christopher,[1] Casey D. S. Katerndahl,[1] Hayley R. LeBlanc,[1] Tyler T. Elmendorf,[1] Vaishali Basu,[1] Margery Gang,[1] Andrew J. Menssen,[1] David H. Spencer,[1] Eric J. Duncavage,[2] Shamika Ketkar,[1°] Lukas D. Wartman,[1] Sai Mukund Ramakrishnan,[1] Christopher A. Miller[1] and Timothy J. Ley[1]

[1]Section of Stem Cell Biology, Division of Oncology, Department of Internal Medicine, Washington University in St. Louis, St. Louis, MO and [2]Department of Pathology and Immunology, Washington University in St. Louis, St. Louis, MO, USA

°Current address: Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA

Correspondence:

MATTHEW J. CHRISTOPHER- christopherm@wustl.edu

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