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A FOXO1-induced oncogenic network defines the AML1-ETO pre-leukemic program

Running title: FOXO1 is an oncogenic mediator in AML1-ETO leukemia

Scientific category: Myeloid Neoplasia

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## Key points

Increased FOXO1 is oncogenic in human CD34+ cells and promotes pre-leukemia transition.

FOXO1 is required by AML1-ETO pre-leukemia cells for the activation of a stem cell molecular program.

## Abstract

Understanding and blocking the self-renewal pathway of pre-leukemia stem cells could prevent acute myeloid leukemia (AML) relapse. In this study we show that increased FOXO1 represents a critical mechanism driving aberrant self-renewal in pre-leukemic cells expressing the t(8;21)-associated oncogene AML1-ETO (AE). Though generally considered as a tumor suppressor, FOXO1 is consistently upregulated in t(8;21) AML. Expression of FOXO1 in human CD34+ cells promotes a pre-leukemic state with enhanced self-renewal and dysregulated differentiation. The DNA binding domain of FOXO1 is essential for these functions. FOXO1 activates a stem cell molecular signature which is also present in AE pre-leukemia cells and preserved in t(8;21) patient samples. Genome-wide binding studies show that AE and FOXO1 share the majority of their binding sites, whereby FOXO1 binds to multiple crucial self-renewal genes and is required for their activation. In agreement with this observation, genetic and pharmacological ablation of FOXO1 inhibited the long-term proliferation and clonogenicity of AE cells as well as t(8;21) AML cell lines. Targeting of FOXO1 therefore provides a potential therapeutic strategy for elimination of stem cells at both pre-leukemic and leukemic stages.

## Introduction

Acute myeloid leukemia (AML) is an aggressive hematopoietic malignancy. Leukemogenesis is a hierarchical process, whereby an initiating mutation drives the development of pre-leukemic stem cells that evolve over time to overt disease through additional cooperating mutations.<sup>1</sup> Pre-leukemic stem cells can survive chemotherapy and serve as a potential reservoir of disease relapse.<sup>2,3</sup> The translocation at chromosome t(8;21) generating the AML1-ETO (RUNX1-RUNX1T1; AE) fusion protein is one of the most frequent initiating mutations, accounting for ~10% of total AML.<sup>4</sup> Although t(8;21) AML has a comparatively good prognosis and most patients enter remission, approximately half of the patients relapse and only a 60% overall survival is achieved after five years.<sup>5</sup> Pre-leukemic stem cells are evident in this AML subtype, as cells positive for AE can be detected long before disease onset or after complete remission.<sup>6-8</sup> Thus, disrupting the self-renewal program of malignant stem cells will provide therapeutic opportunities for preventing disease relapse.

We and others have shown that expression of AE in human CD34+ hematopoietic stem and progenitor cells (HSPCs) causes dysregulated differentiation and increased self-renewal of cells but without inducing AML,<sup>9,10</sup> serving as an ideal model to study the pre-leukemic stage of t(8;21) AML (AE cells). Until now, the mechanism how AE programs progenitor cells into pre-leukemia with aberrant self-renewal has not been completely understood.

The FOXO transcription factors include FOXO1, FOXO3, FOXO4 and FOXO6. Except for FOXO6 which is expressed predominantly in the brain, all other FOXOs are ubiquitously expressed and act as essential regulators controlling oxidative stress and metabolic homeostasis,<sup>11</sup> which is critical in hematopoietic stem cell (HSC) maintenance.<sup>12-14</sup> Due to their capacity to arrest the cell cycle and activate apoptosis, FOXOs are well known tumor suppressors.<sup>15</sup> However, the precise function of FOXOs in AML is unclear. The inactivation of FOXO3 in AML was reported, and restoring its activity impaired cell growth.<sup>16</sup> On the other hand, in line with its critical role in HSC maintenance,<sup>17</sup> FOXO3 was found to be

important for maintaining AML stem cells, although the molecular mechanism was not defined.<sup>18</sup> It is therefore unclear whether FOXOs serve as tumor suppressors, as oncogenes or as stem cell maintenance genes.

Different FOXO family members may have non-redundant functions.<sup>19</sup> For instance, an HSC defect has been observed only in *Foxo3*- but not *Foxo1*-deficient mice.<sup>12</sup> However, the role of FOXO1 in pre-leukemia stem cells has not been explored. Here, we show FOXO1 can function as an oncogene whose upregulation promotes self-renewal and blocks the differentiation of human CD34<sup>+</sup> HSPCs. In AE pre-leukemic cells, this oncogenic function is required for the activation of a self-renewal program. Our results demonstrate a new function of FOXO1 in AML pathogenesis, strengthen our understanding of the mechanisms that mediate the aberrant self-renewal of pre-leukemia stem cells and reveal therapeutic strategies for their elimination.

## **Methods**

### **Plasmids and reagents**

MSCV-IRES-GFP(MIG) and MSCV-AE-IRES-GFP retroviral vector was as described.<sup>20</sup> pSIN-TREtight-DsRED-IRES(tTri) and MSCV-GFP-IRES-tTA retroviral vectors were generously provided by Dr. Johannes Zuber. Insertion of AE was subcloned into tTri to build an AE-tet-off system. pSG5L-FOXO1-WT and pBabe-puro-FOXO1 DB vectors were obtained from Addgene (#10693 and #10695). Insertions of FOXO1 WT and FOXO1 DB were then subcloned into MIG. Lentiviral vector MISSION pLKO.1-shRNA-puro constructs targeting human FOXO1 (TRCN0000039578, sh-1) and non-target shRNA control (shNT-1) were obtained from Sigma, the puromycin-resistant gene in the constructs was replaced with Venus marker. GIPZ lentiviral shRNA targeting FOXO1 (V3LHS\_405827, sh-2) and non-silencing control (shNT-2) were obtained from Open Biosystems, the shRNA insertions were subcloned into SF-LV-shRNA-EGFP lentiviral vector, a gift from Dr. K. Lenhard Rudolph. FOXO1 inhibitor AS1842856 was from Millipore (344355), dissolved in DMSO.

### **Cells and culture**

Human umbilical cord blood cells (CB) were obtained by the Translational Trials Support Laboratory at CCHMC under institution-approved protocol. CD34<sup>+</sup> cells were enriched using CD34<sup>+</sup> selection kit (Miltenyi). AE pre-leukemic cells were established as described.<sup>10</sup> AE cells and MIG/FOXO1- transduced CD34<sup>+</sup> cells were cultured in IMDM with 20% BIT Serum Substitute (Stemcell Technologies #9500), supplemented with 10 ng/mL SCF, IL-3, IL-6, FLT3L, and TPO. Kasumi-1 and SKNO-1 cells were obtained from DSMZ, and cultured in RPMI 1640 with 20% fetal bovine serum (FBS) or RPMI 1640 with 10% FBS plus 10 ng/mL GM-CSF, respectively.

### **Methylcellulose colony-forming assays**

Assays were performed in MethoCult H4100 medium (Stemcell Technologies) supplemented with 20% BIT, 50  $\mu$ M  $\beta$ -Mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and the human cytokines Erythropoietin (6 U/mL), Granulocyte Colony Stimulating Factor (10 ng/mL), IL-6(20 ng/mL), IL-3 (20 ng/mL), and SCF (20 ng/mL). Colonies were scored at day 14, and then the cells were collected for replating. Some experiments were performed by using MethoCult Express medium (Stemcell Technologies), colonies were scored at day 7-10.

### **Xenograft transplantation**

Transplantation was performed on NOD/SCID/IL2RG<sup>-/-</sup> immunodeficient mice with transgenic expression of human SCF, granulocyte–macrophage colony-stimulating factor and IL-3(NSGS). 6- to 8-week-old mice were conditioned with 30mg/kg busulphan (Sigma) through intraperitoneal injection 24 h before transplantation. For shRNA knockdown experiment, 5x10<sup>5</sup> transduced cells were transplanted to each mouse via tail vein. For FOXO1 overexpression experiment, 1x10<sup>5</sup> cells were transplanted through intrafemoral injection immediately after transduction. Bone marrow was aspirated for flow cytometry at indicated time. All experiments were performed in accordance with CCHMC institutional guidelines.

### **RNA sequencing**

Three different units of CD34<sup>+</sup> CB cells were transduced with MIG, AE, FOXO1 WT and FOXO1 DB. RNA was collected from sorted GFP+CD11b<sup>-</sup> cells 5 days after transduction using RNeasy Mini Kit (Qiagen). The integrity of RNA was analyzed by Bioanalyzer (Agilent). 1 ug total RNA was used for poly(A) RNA selection, followed by cDNA synthesis using PrepX mRNA Library kit (WaferGen) and Apollo 324 NGS automatic library prep system. Sample-specific index was added to the adaptor-ligated cDNA by PCR with index-specific primers for 13 cycles. The cluster generation of indexed libraries was carried out on cBot system (Illumina) using Illumina's TruSeq SR Cluster kit v3, and then sequenced on Illumina HiSeq system using TruSeq SBS kit to generate single-end 50 cycle reads. 20-50 million reads were generated for each sample. More details about the data analyses can be found in supplemental Methods.

### **Chromatin immunoprecipitation**

ChIP-Seq assays in AE cells were performed as previously described.<sup>21</sup> More details about the protocol and the data analyses can be found in supplemental Methods.

### **Data Access**

RNASeq and ChIP-Seq data have been deposited in GEO as GSE81084 and GSE80773.

## **Results**

### **FOXO1 is upregulated in AE leukemia and pre-leukemia cells**

To identify critical regulators of self-renewal in AE AML, we examined an AML patient dataset published by Ross et al<sup>22</sup> for stem cell related genes that are significantly upregulated in t(8;21) AML cells compared to other AML subtypes. FOXO1 was one such gene (Figure 1A and supplemental Figure 1A), a result which was further validated in additional datasets (supplemental Figure 1B). Expression of other FOXO family members did not differ significantly between t(8;21) and other AML subtypes (supplemental Figure 1C). We confirmed the upregulation of FOXO1 protein in t(8;21) AML primary patient samples compared to cytogenetically normal AML samples (Figure 1B). Increased FOXO1 transcript and protein levels were also evident in AE pre-leukemia cells compared to control vector (MIT) transduced CD34<sup>+</sup> HSPCs (Figures 1C-D), at levels similar to those in t(8;21) cell lines (Kasumi-1 and SKNO-1) and primary patient samples (supplemental Figure 1D-E). In contrast, AE did not elicit upregulation of other FOXOs in the same cells (supplemental Figure 1F). The transcriptional activity of FOXOs is dependent on their nuclear localization, as they can be inactivated in AML cells by nuclear export.<sup>16</sup> Cellular fractionation and immunoblot analysis showed that FOXO1 had a primarily nuclear distribution in AE cells and Kasumi-1 cells (Figure 1E and supplemental Figure 1G). Immunostaining

confirmed this finding, suggesting that FOXO1 is active in AE pre-leukemic and leukemic cells (Figure 1F).

To test whether FOXO1 is a downstream target of AE we developed a conditional (tet-off) AE expression system, and found that the expression of FOXO1 was highly dependent on continued expression of AE (Figure 2A-B). Similarly, when analyzing our previous RNA-seq study,<sup>21,23</sup> we found that depletion of AE in Kasumi-1 cells led to a decrease of *FOXO1* transcripts (Figure 2C-D). Chromatin immunoprecipitation-sequencing (ChIP-seq) analysis in Kasumi-1 cells<sup>21,23</sup> showed that AE bound to the FOXO1 gene locus, where multiple RUNX1 binding motifs were identified (Figure 2E). We validated this binding using the anti-HA antibody to ChIP the epitope-tagged AE protein in AE cells (Figure 2F). These results suggest that FOXO1 could be a direct transcriptional target of AE.

### **FOXO1 is required to sustain the growth of AE cells**

To investigate the function of FOXO1 in AE pre-leukemia cells, we knocked down FOXO1 using two independent pairs of shRNAs. AE cells with FOXO1 depletion displayed slightly increased apoptosis and reduced cell cycle progression compared to non-targeting shRNA (shNT) transduced cells (supplemental Figure 2A-B), although the differentiation status of the cells was not altered as indicated by CD11b expression (data not shown). FOXO1 knockdown impaired growth in liquid culture and clonogenicity of AE cells in a methylcellulose assay (Figure 3A-B). Additionally, FOXO1-depleted AE cells showed a reduced long-term engraftment capacity in immunodeficient mice, as the Venus+ fraction in engrafted human cells was reduced at week 9 post transplantation, and continuously decreased at later time points (Figure 3C and supplemental Figure 2C). These results suggest that FOXO1 does not act as a tumor suppressor, instead, FOXO1 ablation impairs the self-renewal of AE pre-leukemia cells both in vitro and in vivo. In addition, depletion of FOXO1 inhibited cell growth and colony formation of t(8;21) AML cell lines (supplemental Figure 2D-E), indicating that FOXO1 is also critical for leukemia maintenance. To further test this notion, we transduced shRNAs into an AE overt leukemic clone that spontaneously evolved from pre-leukemia cells,<sup>24</sup> and evaluated its leukemogenicity in immunodeficient mice. The majority of mice receiving shNT-transduced cells succumbed to disease between 60-80 days after transplantation as expected. In contrast, recipients of FOXO1-depleted AE leukemia cells survived much longer (Figure 3D). Bone marrow examination showed that the cells with FOXO1 knockdown displayed a much lower engraftment level compared to shNT-transduced cells at a time-point when the latter were already showing overt disease (supplemental Figure 2F).

We also evaluated FOXO1's function using a FOXO1-specific inhibitor.<sup>25</sup> Inhibitor treatment dramatically reduced the growth of AE pre-leukemia cells as well as of t(8;21) cell lines (Figure 3E and supplemental Figure 2G), partially by inducing apoptosis and blocking the cell cycle (supplemental Figure 2H-I). By contrast, the inhibitor showed only minimal effects on the growth of normal human CD34+ HSPCs, with an IC50 10 times higher than those of AE-expressed cells (Figure 3E-F). Consistent with the observation in liquid culture, the colony-forming potential of normal CD34+ HSPCs was unaffected by the inhibitor. However, the clonogenic activity of AE pre-leukemia cells and AML cell lines was impaired upon FOXO inhibition (Figure 3G-H). Importantly, the inhibitor also displayed efficacy on primary cells from t(8;21) AML patients, impeding their colony formation as well (Figure 3I). These findings are in accordance with data showing that *Foxo1* knockout mice do not present with hematopoietic defects,<sup>12</sup> demonstrating that FOXO1 is specifically required in AE pre-leukemia and leukemia cells.

### **Increased FOXO1 has oncogenic activity in human CD34+ cells**

The increase in FOXO1 upon AE expression in normal CD34+HSPCs suggested that FOXO1 could play a direct role in AE pathogenesis. To test this hypothesis, we transduced CD34+ HSPCs with a retrovirus expressing wild-type FOXO1 (FOXO1 WT) or only GFP (MIG) as control. To determine whether a transforming ability of FOXO1 relied on its DNA-binding activity, we also used a FOXO1 mutant unable to bind DNA (FOXO1 DB). FOXO1 WT promoted long-term proliferation of HSPCs in liquid culture, while control and FOXO1 DB cells grew for only a limited period (Figure 4A). Immunophenotyping and morphological analyses showed that FOXO1 WT cells retained an immature morphology with very little CD11b expression while control and FOXO1 DB cells underwent terminal myeloid differentiation (Figures 4B-C). In methylcellulose clonogenic assays, FOXO1 WT cells gave rise to significantly more myeloid colonies with a dramatic loss of erythroid colonies when compared to MIG and FOXO1 DB cells (Figure 4D). In addition, FOXO1 WT cells generated substantially more colonies in both secondary and tertiary re-platings, in striking contrast to control cells, where re-plating activity was limited (Figure 4E). All these effects are similar to the phenotype elicited upon AE expression.<sup>10</sup>

To investigate the effect of FOXO1 expression *in vivo*, we transplanted transduced HSPCs into immunodeficient mice. Twelve weeks after transplantation, the proportion of GFP+ cells within human engraftment was measured. Consistent with our previous observation,<sup>26</sup> control MIG cells did not maintain long-term engraftment *in vivo* and thus the percentage of engrafted human cells expressing GFP decreased over time. FOXO1 DB cells showed a similar loss *in vivo*, but with FOXO1 WT cells, the fraction of transduced cells was maintained (Figure 4F). However, as seen for AE cells, FOXO1 WT cells were unable to initiate AML. Taken together, these data indicate that enforced expression of FOXO1 in normal CD34+ HSPCs enhances stem cell function and disrupts differentiation, thus initiating a pre-leukemic phenotype that resembles that of AE cells and highlighting an oncogenic function of FOXO1.

### **Increased FOXO1 elicits a gene expression signature shared with AE expressing cells**

The transcriptional targets of FOXOs in normal HSCs and leukemia stem cells are poorly explored. To gain a better understanding of the underlying gene network accounting for FOXO1's oncogenic role, we performed RNA-seq analysis on CD34+ HSPCs transduced with AE, FOXO1 WT, FOXO1 DB or MIG on day 5 post transduction. Hierarchical clustering analysis showed that gene expression patterns from FOXO1 DB and MIG cells clustered together, indicating that the expression of FOXO1 DB does not have a major impact on gene expression and thus was non-functional in this context. In contrast, gene expression patterns from AE and FOXO1 WT cells clustered separately from the control cells (Figure 5A and supplemental Table 1). More strikingly, a significant portion of the AE gene expression signature was also present in FOXO1 cells (Figure 5A and "Core gene" in supplemental Table 1). Pathway enrichment analysis on genes activated by FOXO1 compared to control cells showed a significant enrichment of published gene signatures characterizing AE pre-leukemia and leukemia. In addition, HSC signature genes were also significantly enriched (Figure 5B). These results suggest that FOXO1 regulates a core network associating with HSC function that is critical for AE mediated pre-leukemia stem cell programming. Accordingly, the FOXO1-activating gene signature was overrepresented in t(8;21) patient samples (supplemental Table 2), and the average expression level of FOXO1-activated genes was significantly higher in t(8;21) AML compared to other subtypes of AML (Figure 5C-D and supplemental Figure 3). Therefore, upregulation of the FOXO1 driven gene regulatory network was retained as a molecular feature in the acute leukemia stage of AE AML.

### **FOXO1 and AE share genome-wide localization and FOXO1 maintains activation of key AE target genes**

To identify the genomic targets of FOXO1 and AE we performed ChIP-Seq analysis in AE pre-leukemia cells. In total, 14723 AE- and 11835 FOXO1-bound loci were identified. Peak distributions differed slightly, with FOXO1 showing a greater localization to promoter regions than AE (Figure 6A). In agreement with our previous report showing FOXO binding motif enrichment in AE bound loci mapped in Kasumi-1 cells,<sup>21</sup> we found 61% of AE and 76% of FOXO1 bound loci were in common (Figure 6B). Unbiased motif enrichment analysis of joint peaks showed that both RUNX1 and FOXO motifs were significantly enriched, together with ERG/ETS and E-Box motifs, consistent with our previous report (Figures 6C-D).<sup>21</sup> Taken together, these results suggest that the FOXO1 and AE molecular networks are broadly interconnected.

Cross analysis with RNA-seq data showed that about 50% of AE-regulated genes have both AE and FOXO1 bound in close proximity to the gene body (Figure 6E). The shared binding of AE and FOXO1 at several target genes was validated in t(8;21) cell lines as well (supplemental Figure 4A). The genome-wide overlap of FOXO1 and AE binding sites compelled us to test whether FOXO1 was required for AE binding to its loci. Although we could detect a weak interaction between FOXO1 and AE in AE cells (supplemental Figure 4B), knockdown of FOXO1 did not affect the target binding of AE (supplemental Figure 4C), suggesting that the shared localization of the two proteins in chromatin does not require physical interaction. We identified MPL, UBASH3B and SOX4 as genomic targets for both AE and FOXO1. It has been reported that AE leukemic and pre-leukemic cells depend on MPL and UBASH3B for self-renewal and long-term proliferation.<sup>26-28</sup> In addition, SOX4 is an AML oncogene which can efficiently transform mouse HSPCs.<sup>29</sup> Histone H3 lysine 9 acetylation (H3K9Ac) and lysine 27 acetylation (H3K27Ac) are epigenetic markers associated with active promoters and enhancers.<sup>30,31</sup> The AE and FOXO1 binding peaks at these gene loci overlapped with our own published H3K9Ac enriched regions in Kasumi-1 cells as well as with H3K27Ac enriched regions identified in the ENCODE database,<sup>23,30</sup> suggesting that AE and FOXO1 bind at *bona fide* gene regulatory elements (Figure 6F). Interestingly, FOXO1 knockdown in AE cells led to the down-regulation of all three genes (Figure 6G), which may account for the impaired growth of AE cells. Similar results were observed when FOXO1 was depleted in Kasumi-1 cells, although MPL was barely expressed in these cells, possibly due to them being cytokine-independent and not relying on MPL signaling (supplemental Figure 4D). These data suggest that the activation of a substantial set of AE targets and pre-leukemic stem cell programming cannot be fulfilled by AE itself and requires the up-regulation of FOXO1.

## Discussion

Understanding the mechanisms through which leukemic oncogenes reprogram transcriptional networks and lead to enhanced self-renewal is an important prerequisite for efforts to specifically target leukemic and pre-leukemic stem cells and thus preventing relapse. The key mediators for leukemic programming of several subtypes of AML have been identified, including HOXA9/MEIS1 for MLL-translocation related AML, SOX4 for AML associated with CEBPA mutations and GATA2 for t(3;11) leukemia.<sup>29,32,33</sup> Yet how AE promotes self-renewal and whether a key mediator exists in t(8;21) AML has been less clear. In this study, we identified FOXO1 as a critical regulator of the self-renewal program in AE pre-leukemia cells that is necessary and sufficient to initiate a pre-leukemic phenotype. We show that FOXO1 is required for full activation of a set of self-renewal genes and the long-term proliferation and clonogenicity of AE cells. Importantly, enforced expression of FOXO1 in normal CD34+ HSPCs can partially recapitulate the cellular phenotype and gene signature of AE cells, revealing increased FOXO1 activity as a new molecular mechanism by which pre-leukemia stem cells establish an aberrant self-renewal program.

FOXOs have complex functions and their role in leukemia is still unclear. In AML, it has been well documented that FOXO3 acts as tumor suppressor. It was shown that active IkappaB kinase sustains

AML proliferation by phosphorylating FOXO3 and thus preventing its nuclear entry, conversely, restoring nuclear localization of FOXO3 impaired AML cell growth.<sup>16</sup> Similarly, it was demonstrated that FLT3-ITD, one frequent mutant in AML associated with poor disease outcome can promote FOXO3 phosphorylation and thus preventing FOXO3-mediated apoptosis induction.<sup>34</sup> Accordingly, a study of 511 patients showed that high levels of phosphorylated FOXO3 indicating low FOXO3 activity was an independent adverse prognostic factor in AML.<sup>35</sup> In addition, FOXO3 was shown to localize in the cytoplasm in PML-RAR $\alpha$ -expressing AML cells and becoming activated during all-trans retinoic acid (ATRA) treatment. Knockdown of FOXO3 blocked ATRA-induced granulocytic differentiation and apoptosis.<sup>36</sup> In contrast, FOXO3 was found to be important for maintaining AML stem cells in a mouse MLL-AF9 AML model. FOXO3 localized to the nucleus of AML stem cells, and depletion of FOXO3 promoted myeloid differentiation and cell death, leading to a reduction of AML stem cell frequency.<sup>18</sup> However, the molecular mechanism by which FOXO3 is required for AML stem cells was not defined, and it is therefore unclear whether FOXO3 preserves leukemia stem cell activity by coordinating metabolic balance, similar to its role in normal HSC. In the current study, we show that FOXOs can also act as oncogenic factors and actively transform normal human HSPCs cells to AML upon increased expression, highlighting a new functional aspect of FOXOs in AML. Mechanistically, our data suggest that the oncogenic function of FOXO1 in AML is through the activation of a HSC program, which is critical for AE mediated pre-leukemia stem cell programming.

Upregulation of FOXO1 is not restricted to t(8;21) AML. Interestingly, the FOXO1 core signature is also relatively enriched in inv(16) AML, correlating with high expression of FOXO1 in these samples (Figures 5C-D, supplemental Figure 1A and supplemental Figure 3). inv(16) mutations disrupt the CBFB gene, generating the CBFB-MYH11 fusion. CBFB interacts with AML1 (RUNX1) to form the core binding factor (CBF) transcription complex, a critical regulator for normal hematopoiesis.<sup>37</sup> In addition, FOXO1 upregulation was also revealed in cytogenetically normal AML with AML1 mutations.<sup>38</sup> It is therefore possible that FOXO1 is important for AML with CBF gene related mutations in general, and could play a more wide-spread role in development of other subtypes of AML as well.

By virtue of selecting and regulating a set of AE target genes, FOXO1 reinforces the AE molecular network. Our analysis showed that target binding of AE did not depend on FOXO1. Together with the fact that increased FOXO1 was sufficient to elicit part of the AE gene signature in the absence of AE, this suggests that the genomic binding of FOXO1 and AE are independent. It is possible the genomic arrangement of binding motifs could serve as the basis for the coordination of AE and FOXO1. A significant number of genes across the genome have adjacent FOXO and RUNX binding motifs.<sup>39,40</sup> It was also shown that FOXO1 and AML1 had functional interactions in breast cancer cell lines by jointly regulating a common set of genes.<sup>39</sup> Since AE also recognizes RUNX motifs for chromatin binding, AE could hijack the network of FOXO1 and AML1 and thereby cooperate with FOXO1 for promoting a pre-leukemia program. Alternatively, other co-binding factors may be involved in orchestrating the genomic binding of FOXO1 and AE. ETS transcription factors ERG and FLI1 have been reported to direct AE to specific binding regions.<sup>41</sup> Genomic co-localization of ETS factors and FOXO in non-hematopoietic tissues has been suggested.<sup>42</sup> As evidenced by our finding that ETS motifs were significantly enriched in FOXO1 bound loci, it is possible that ERG/FLI1 binding sites also demarcate targets for FOXO1, thus leading to the co-selection of targets by FOXO1 and AE.

With the understanding that FOXOs are tumor suppressors, restoration of FOXO activity is being considered as a potential cancer therapy.<sup>15</sup> Given the various functions of FOXOs in AML, caution has to be taken in applying this strategy until we fully understand the AML subtype specific role of FOXOs and evaluate the possible oncogenic effects on normal HSPCs. Considering the selective toxicity of the

FOXO1 inhibitor on AE pre-leukemic and leukemic cells, and the demonstration that an HSC defect was not seen in *Foxo1*-deleted mice,<sup>12</sup> FOXO1 may be an effective therapeutic target for specifically eliminating malignant stem cells in AE and other FOXO1-overexpressing AMLs.

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### Author Contributions

SL performed experiments, SL and JCM conceived and designed the study, analyzed data and together with CB wrote the manuscript. AP, SAA, PSC and MRI performed experiments and analyzed data. MS performed experiments, XC, BA, JZ, MTW and CB analyzed data. Conflict-of-interest disclosure: The authors declare no competing financial interests.

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## Figure legends

### Figure 1. FOXO1 is upregulated in AE leukemia and pre-leukemia cells.

(A) Microarray analysis of FOXO1 transcript levels in t(8;21)-positive compared to t(8;21)-negative AML patient samples from Ross ME et al. (B) Immunoblot showing FOXO1 protein levels in t(8;21) and cytogenetically normal (non-t(8;21)) AML patient samples. (C and D) FOXO1 mRNA levels determined by qPCR (C, n=4, error bars represent SD) and protein levels (D) in AE- or empty vector (MIT)-transduced CD34<sup>+</sup> HSPCs. (E and F) Cellular fractionation immunoblot (E) and immunofluorescence analysis (F) to determine the subcellular localization of FOXO1 in AE cells. Scale bar=20 um. All p values were calculated by unpaired two-tailed t-test.

### Figure 2. Upregulation of FOXO1 depends on the presence of AE

(A and B) qPCR and immunoblot analysis to determine FOXO1 mRNA (A, n=3, error bars represent SD) and protein (B) in AE-tet-off system following DOX addition. (C and D) RNA-seq analysis of FOXO1 expression of Kasumi-1 cells transected with control siRNA (siMM) or AE siRNA (siAE). RNA-seq tracks (C) and relative FPKM values (D) are shown. n=3, error bars represent SD. p value was calculated by unpaired two-tailed t-test. (E) ChIP-seq analysis showed AE binding patterns at FOXO1 locus in Kasumi-1 cells. The sequence of the binding peak is shown with RUNX1 binding motifs highlighted. (F) ChIP-qPCR analysis of chromatin occupancy of AE (HA-tagged) at FOXO1 locus in AE cells, with primers indicated in (E) by arrow. RPL30 locus was used as negative control. n= 3, error bars represent SD.

### Figure 3. FOXO1 inhibition impairs growth of AE cells.

(A) AE cells were transduced with two sets of non-targeting shRNA (shNT) and FOXO1 shRNA (sh-1 and sh-2) vectors that co-expressed Venus. Knockdown efficiency was confirmed by immunoblot. Growth of AE cells in liquid culture was measured as change of percentage of Venus+ cells relative to shNT by flow cytometry. One representative experiment of 3 replicates is shown. (B) CFU assay of sorted shRNA-transduced AE cells. n=3, results represent mean  $\pm$  SD. \*p<0.05, \*\*p<0.01. (C) shRNA-transduced AE cells were transplanted into immunodeficient mice. Venus+ percentage of human engrafted cells in bone marrow was examined 9 weeks later, and normalized to those before the transplantation. \*p<0.005, \*\*p<0.001. One representative experiment of 2 replicates is shown. (D) Survival curve of immunodeficient mice received fully transformed AE cells expressing shNT or FOXO1 shRNA. Two independent experiments were included, p value was calculated by log-rank test. (E) CD34+ HSPCs and AE cells were treated with the FOXO1 inhibitor AS1842856 or DMSO, cell numbers were monitored over time. n=3, results represent mean  $\pm$  SD. \*p<0.01, paired two-tailed t-test. (F) Proliferation rate of cells treated with FOXO1 inhibitor at indicated concentration for 6 days, which is determined by WST-1 assay. Data are normalized to DMSO control (0  $\mu$ M), and are shown as mean  $\pm$  SD (n=3). IC50 values for each type of cells are indicated. (G-I) CFU assay of CD34+ HSPCs, AE cells (G), t(8;21) cell lines (H) and primary patient cells (I) treated with FOXO1 inhibitor. Data are normalized to DMSO-treated cells (0  $\mu$ M). n=3, results represent mean  $\pm$  SD. \*p<0.05, \*\*p<0.005. All p values were calculated by unpaired two-tailed t-test unless noted.

### Figure 4. Increased FOXO1 promotes a pre-leukemic state in human CD34+ HSPCs .

(A) Weekly cell count of liquid culture of CD34+ HSPCs transduced with wildtype FOXO1 (WT), a FOXO1 mutant without a DNA binding domain (DB), or control (MIG) retroviral vectors. FOXO1 expression was confirmed by immunoblot. One representative experiment of 3 replicates is shown. (B) Flow cytometry analysis of the myeloid differentiation marker CD11b on cells from a week 4 culture. (C) Wright-Giemsa staining of week 4 cultured cells. Scale bar=20  $\mu$ m. (D and E) CFU assay with transduced cells. Results represent mean  $\pm$  SD of colony counts of first round (D, \*p<0.05, \*\*p<0.001) and second and third rounds (E, \*p<0.01, \*\*p<0.005), n=3. (F) Transduced cells were transplanted into immunodeficient mice. GFP+ percentage of human engrafted cells in bone marrow was examined 12 weeks later, and normalized to those before the transplantation. \*p<0.05, \*\*p<1 $\times$ 10<sup>-5</sup>. One representative experiment of 2 replicates is shown. All p values were calculated by unpaired two-tailed t-test.

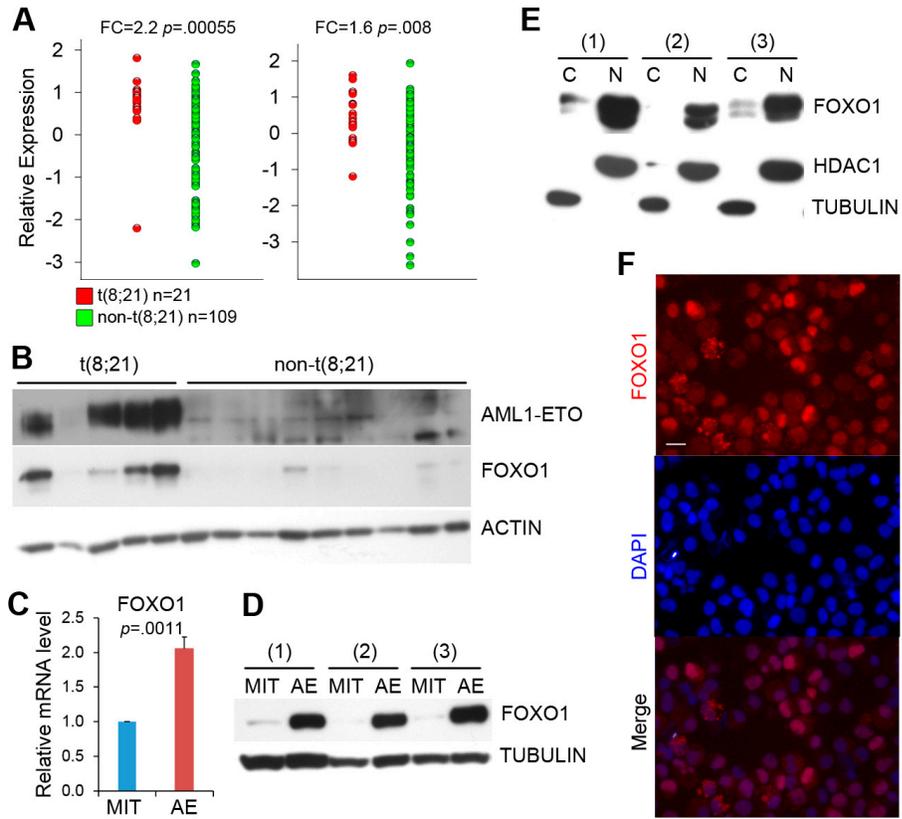
### Figure 5. FOXO1 gene signature is overrepresented in AE pre-leukemia and leukemia cells.

(A) RNAseq analysis was performed on human CD34+ HSPCs transduced with indicated genes. Heatmap depicting hierarchical clustering of 1000 significantly differentially expressed genes of AE, FOXO1 WT and FOXO1 DB cells compared to MIG cells. The detailed gene list can be found in supplemental Table 1. (B) Pathway enrichment analysis of FOXO1-activating target genes showed enrichment of AE and HSC signatures. (C) Heatmap showing the expression level of FOXO1-activating gene signatures in different subtypes of AML patient samples. The dataset was from Ross ME et al. (D) Bean-plot depicting the average of the mean-centered log<sub>2</sub> expression values of FOXO1-activating genes in different subtypes of AML patient samples. The dataset was from Ross ME et al. Black lines show the means of subgroups; each white line represents value of individual samples; polygons represent the estimated density of the data. p values were calculated by two-tailed t-test.

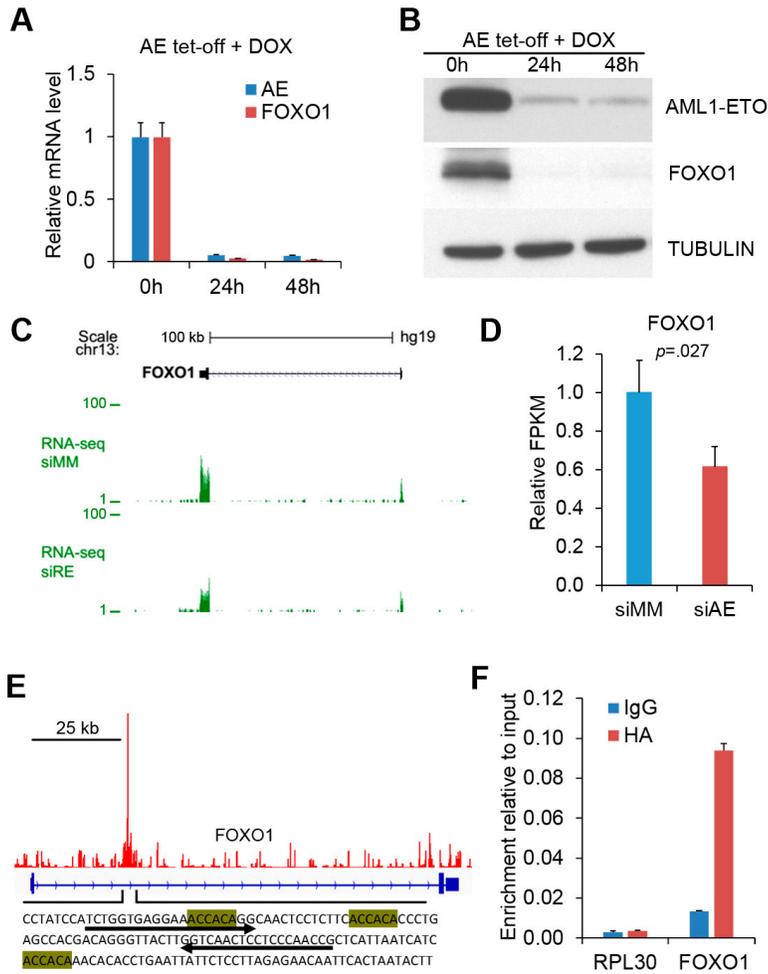
### Figure 6. FOXO1 regulates AE key target genes.

(A) Genomic location of AE and FOXO1 binding sites. (B) Venn diagram showing the overlap between AE and FOXO1 ChIP-seq peaks. (C) Enriched transcription factor binding motifs in jointly bound AE and FOXO1 ChIP-seq peaks. (D) Percentage of peaks containing predicted enriched motifs. (E) Percentage of AE-regulated genes associated with both AE and FOXO1 binding. (F) ChIP-seq tracks at *SOX4*, *MPL* and *UBASH3B* loci showing binding patterns of AE and FOXO1 in AE cells, H3K9Ac in Kasumi-1 cells, and layered H3K27Ac peaks of 7 cell lines from ENCODE. Arrows indicate jointly bound AE and FOXO1 peaks. (G) Immunoblot to determine protein levels of MPL, UBASH3B and SOX4 upon FOXO1 knockdown in AE cells.

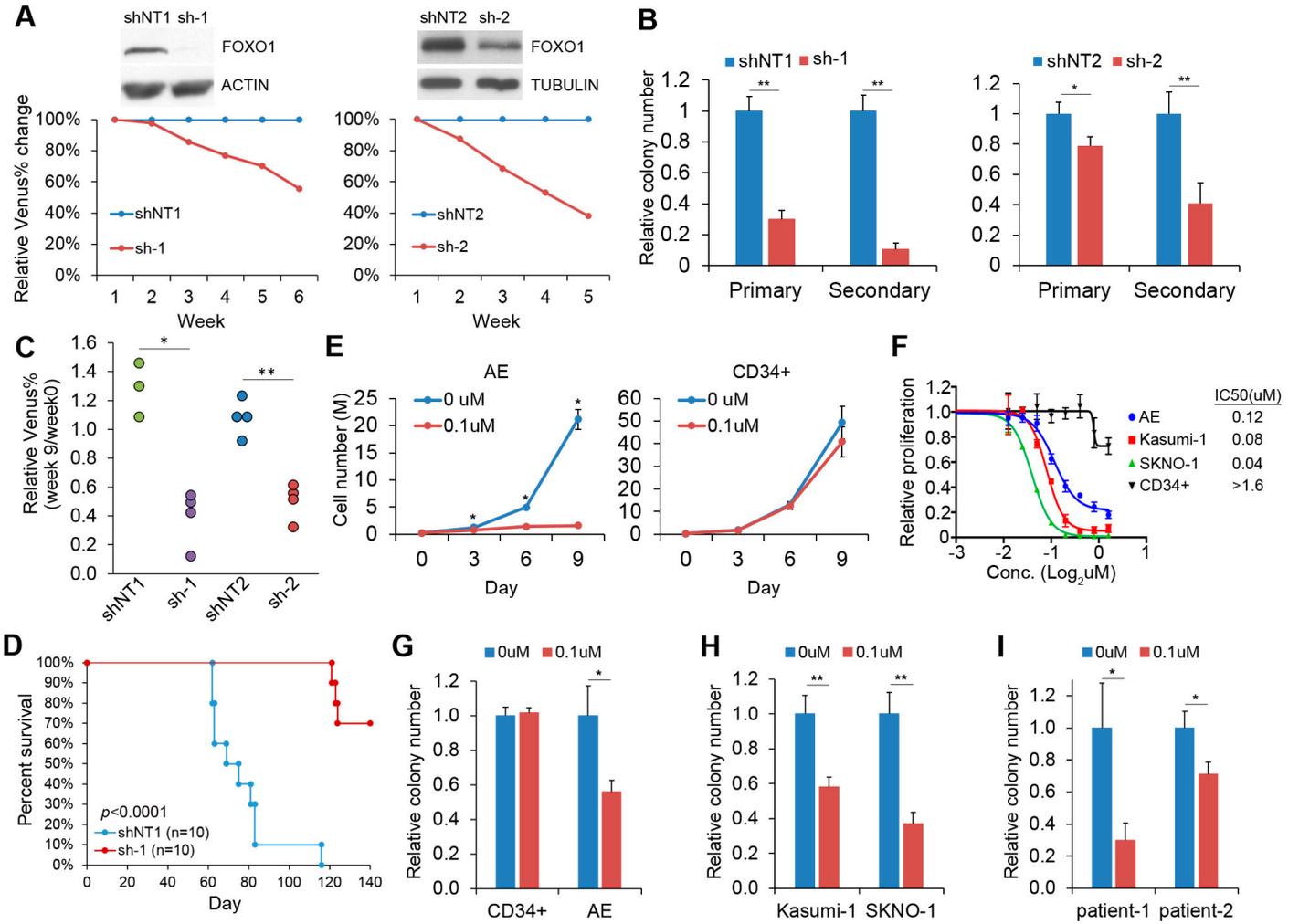
**Figure 1**



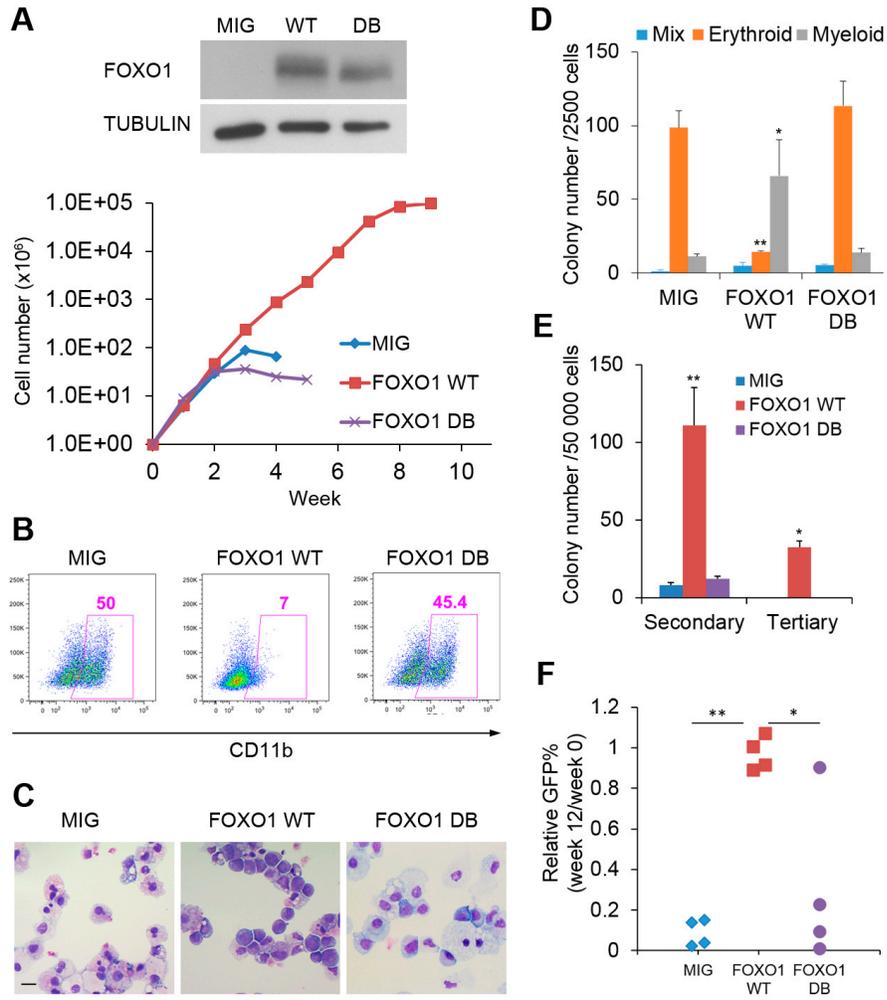
**Figure 2**



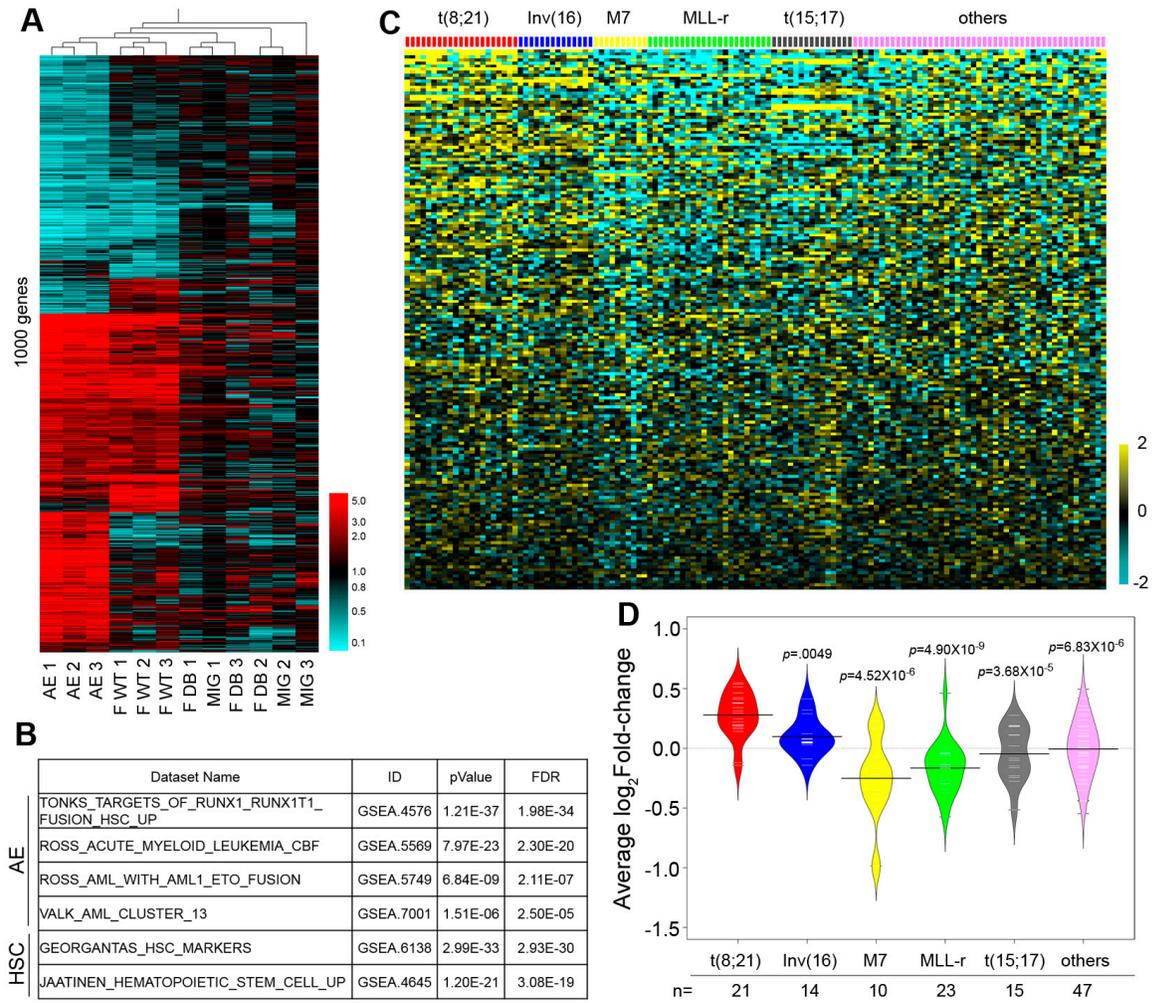
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**

