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The Role of Methylation in t(8;21) Acute Myeloid Leukemia: Potential Therapeutic Targets of Leukemic Pathogenesis

A thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

Elhaam Anvar

Committee in charge

Professor Dong-Er Zhang, Chair
Professor Clodagh O'Shea
Professor Tracy Johnson

2012

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The thesis of Elhaam Anvar is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2012

I would like to dedicate this thesis to my parents, Fereshteh Zakeri and Mahmood Anvar, and to my brother Amir Anvar, for their love, support, and tireless pushing throughout my years in college. You are my motivation. I would also like to extend my dedication to all my friends, near and far, that have always been there for me. Finally, this is also for Alex, without whom none of this would be possible.

Table of Contents

Signature Page iii

Dedication iv

Table of Contents v

List of Figures and Tables vi

Acknowledgements vii

Abstract ix

I. Introduction 1

II. Results 10

III. Discussion 52

IV. Materials and Methods 59

V. Supplemental Tables 63

References 74

List of Figures and Tables

Figure 1. Analysis of DNA methylation profiling data identifies candidate genes for study…….. 21

Table 1. Methylation status according to Figueroa et al. of genes identified from Lo et al.'s AE9a leukemia array…… 22

Figure 2. RT-qPCR screen reveals AE9a downregulated array target genes that are upregulated after decitabine treatment in murine AEtr leukemia derived cell line……... 24

Figure 3. RT-qPCR screen of AE9a downregulated target genes that are upregulated after decitabine treatment in Kasumi-1, SKNO-1, U937 and HL60 human AML cell lines…… 29

Table 2. Fold changes of methylated genes upregulated after decitabine treatment relative to untreated control…… 36

Figure 4. Toxicity of decitabine on human cells demonstrated with a titration on Kasumi-1 cell line……... 37

Figure 5. Treatment of murine AEtr cells with lower concentrations of decitabine..... 42

Figure 6. RT-qPCR screen of target genes shows slight upregulation for certain genes after low concentration decitabine treatment in Kasumi-1 cells…… 43

Figure 7. RT-qPCR screen of target genes shows slight upregulation for certain genes after low concentration decitabine treatment in AEtr cells…… 44

Figure 8. Re-expression of downregulated genes of interest in AEtr AML cells causes morphological and functional changes……... 45

Figure 9. Sodium bisulfite treatment to discern methylation levels of genes in untreated and decitabine treated AEtr AML cells…..... 51

Supplemental Table 1: AE9a leukemia microarray (downregulated) and ChIP-chip common targets.....63

Supplemental Table 2: AEtr RT-qPCR primers…….....……..….66

Supplemental Table 3: Human RT-qPCR primers…..…….....69

Supplemental Table 4: Primers for PCR amplification of genes of interest with HA tag.....72

Supplemental Table 5: Bisulfite specific PCR primers……..…….....72

Supplemental Table 6: MSP PCR primers……..……...……..……...73

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ABSTRACT OF THE THESIS

The Role of Methylation in t(8;21) Acute Myeloid Leukemia:
Potential Therapeutic Targets of Leukemic Pathogenesis

by

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Master of Science in Biology

University of California, San Diego, 2012

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The fusion protein, AML1-ETO (AE) is generated from the translocation 8;21 (q22;q22) [(t8;21)] translocation and is identified in 12-15% of de novo cases of human acute myeloid leukemia (AML). However, AML1-ETO alone is not capable of inducing leukemia in a murine transduction model, while an alternatively spliced isoform, AML1-ETO9a (AE9a), is. The resulting leukemia is a product of the effect of the AE9a transcription factor on gene expression,

including that of AML1 target genes. It has been postulated that one of the mechanisms through which AE/AE9a alter gene expression is via the recruitment of DNA methyltransferases, which it uses to methylate genes and subsequently downregulate their expression. To investigate the role of DNA methylation in gene downregulation in t(8;21) AML, I treated t(8;21) and non-t(8;21) expressing human and murine AML cells with the DNA hypomethylating drug 5-aza-2'-deoxycytidine (decitabine) to discern its effects on t(8;21) downregulated genes via RT-qPCR. From the genes that were upregulated following treatment with decitabine, I identified which are methylated based on published genome wide DNA methylation profiling study in human AML cases, and selected 4 genes of interest for study: *Cuedc1, Jdp2, Fam134b*, and *Rgs18*. To further investigate the importance of downregulation of my genes of interest, I used a retroviral vector to re-express them in a murine AML1-ETO leukemia derived cell line and observed the changes in morphology and cell survival. Finally, I attempt to confirm the methylation status of the genes of interest with bisulfite treatment.

I

Introduction

Acute myeloid leukemia abnormalities

Acute myeloid leukemia (AML) is the most common acute leukemia in adults and is characterized by leukocyte abnormalities such as excessive proliferation and poor differentiation. More than half of all cases of AML contain somatically acquired chromosomal translocations.[1] Depending on the cytogenetic cause of AML, the five year survival rate has been found to be between 15-70%, and the rate of relapse between 33-78%.[1-4]

Of the cases of AML associated with chromosomal translocations, the most common is the translocation 8;21 (q22;q22) [(t8;21)] abnormality.[5,6] The t(8;21) translocation is associated with 12-15% of de novo AML cases and approximately 40% in the AML subtype M2 of the French-American-British (FAB) classification. M2 is associated with maturation of the granulocytic family of white blood cells.[7]

Characteristics of t(8;21) associated acute myeloid leukemia

The t(8;21) translocation fuses the acute myeloid leukemia 1 (AML1) gene on chromosome 21 and the eight-twenty-one (ETO) gene on chromosome 8 to generate the AML1-ETO (AE) fusion gene.[1,7] The AE gene contains the N-terminus of the AML1 gene fused to most of the coding region of ETO and it encodes for, in its full length, a 752 amino acid protein.[5] The AML1 segment of the AE fusion protein consists of the DNA-binding runt homology domain and the ETO portion contains four of its nervy homologous regions (NHR1-4).[8,11] The AML1 gene is also known as RUNX1 or core binding factor protein-α (CBFα) and

forms a heterodimer with core binding factor protein-β (CBFβ) facilitating efficient DNA binding of AML1 to transcriptionally activate its target genes. These genes include macrophage colony stimulating factor (M-CSF) receptor, granulocyte-macrophage colony stimulating factor (GM-CSF), T-cell receptors, and the tumor suppressor p14 ARF.[8-10]

ETO, or MTG8, when fused to AML1 does not directly bind DNA but it acts as a gene repressor via interactions with histone deacetylases (HDACs), histone methyltransferases and DNA methyltransferases (DNMTs). [11-13] Gene knock-in experiments, along with others, have suggested that AE dominantly inhibits AML1 mediated transcription.[12] Through interactions with its NHR domains, ETO recruits the nuclear hormone corepressors SMRT (silencing mediator for retinoic acid receptor and thyroid hormone receptor) and N-CoR (nuclear receptor corepressor). These corepressors have been shown to associate with HDACs to negatively regulate gene expression.[8]

In addition, the DNA histone methyltransferase PRMT1 (protein arginine methyltransferase 1) has been shown to be associated with the leukemic isoforms of AE. By using PRMT1 to methylate histone H4 arginine 3 (Arg3), typically associated with transcription activation, AE is thought to activate transcription of its target genes.[14] Furthermore, it has been suggested that AE can silence target genes using DNMTs because DNMT inhibitors, such as 5-aza-2'-deoxycytidine (decitabine, DAC), help to restore AML1-ETO mediated target gene transcription.[13]

Alternately spliced leukemic isoform of AML1-ETO

Although AML1-ETO is directly involved in the pathogenesis of AML, it is not sufficient to cause the disease in mice. AML1-ETO contains AML1 exons 1-5, and ETO exons 2-11.[8] One alternatively spliced isoform of AML1-ETO, AML1-ETO9a (AE9a), encodes a C-terminally truncated AE protein of 575 amino acids and causes AML in mice rapidly.[5] AE9a includes an extra ETO gene exon, called 9a, which provides a premature stop codon after the last amino acid of ETO exon 8. This early termination results in the loss of translation of NHR3 and NHR4 from the ETO gene in AE9a. Another variant of AE, AML1-ETOtr (AEtr), encodes a C-terminally truncated AE protein of 556 amino acids and is created by a frameshift mutation due to a 1 nucleotide insertion leading to a premature stop codon. Similar to AE9a, AEtr lacks the NHR3 and NHR4 domains and strongly promotes AML development in mice [15] AEtr leukemia cells can be cultured more readily in vitro than AE9a leukemia cells and were used in this study.[26]

AML1-ETO, AML1-ETO9a, and AML1-ETOtr characteristics

The full length AE protein has been shown to act as a transcriptional repressor of AML1 target genes. This is due to the presence of the DNA-binding runt homology domain of AML1 in conjunction with the ETO NHRs, which repress gene expression by recruiting HDACs and other known repressors. As previously mentioned, repressed genes are important in AE induced leukemia and include the granulocyte-macrophage colony stimulating factor (GM-CSF), T-Cell receptors, and the tumor suppressor p14 ARF. [8-10] GM-CSF is a promoter of

myeloid cell proliferation, survival, and differentiation.[16] The tumor suppressor p14 ARF is directly inhibited by AE expression.[8]

Beyond directly inhibiting AML1 target gene expression, AE also downregulates the expression of some transcription factors such as CCAT enhancer-binding protein (C/EBPα), and PU.1 and myeloif ELF-1-like factor (MEF) of the Ets (E26) family.[17-19] PU.1 is a key transcription factor for normal myeloid development, and in cases of t(8;21) AML, AE binds to the DNA binding domain on PU.1 thus displacing its coactivator c-Jun and functionally disrupting its transactivation activity. [17] AE has also been shown to block the C/EBPα transcription factor by inhibiting autoregulation of the C/EBPα promoter thus blocking normal granulocytic (neutrophil) differentiation of myeloid cells.[18] Furthermore, MEF does not have an AML1 binding domain, but its interactions during normal hematopoiesis allow for AE to repress MEF through protein-protein interactions. This can lead to disregulation of many myeloid differentiation genes.[19] These data all suggest a strikingly harmful effect from AE on genes typically associated with hematopoiesis thereby contributing to the pathogenesis of t(8;21) AML.

Expression of AE in cells leads to not only a block in differentiation of myeloid and lymphoid lines, but also of erythroid lines in established and primary cell lines.[20-22] This is interesting because normal erythropoietic differentiation is independent of AML1 unlike maturation of megakaryocytes and differentiation of granulocytes. In fact, erythroid promoters generally lack AML1 binding sites.[20] Similar to AE's impact on PU.1, it has been shown that AE disrupts erythropoietic

lineage commitment through protein-protein interactions of the AML1 runt homology domain with GATA-1.[23-25] AE blocks GATA-1 acetylation thereby inhibiting hematopoietic precursor cells from committing to the erythroid lineage.[23]

Although the AE protein significantly impacts critical gene expression and thereby affects cell proliferation and differentiation, the fusion protein alone is not capable of leading to leukemia development.[15] In the murine retroviral transduction transplantation model, AE is unable to induce AML, while AE9a and AEtr rapidly induce AML.[1,5,15]

Analysis of gene expression microarray and ChIP-chip profiling of primary mouse leukemia cells (Lin-/Sca1-/cKit+, or LK) induced by AE9a has demonstrated that AE9a leukemia cells have both increased cell cycle and cell survival signaling, while decreasing cell differentiation compared to wildtype cells.[26]

DNA methylation and leukemogenesis in AML

The mechanisms that alter gene expression in AE9a AML cells are of particular interest because reversal of the altered expression could potentially be a therapeutic target for AML.[27] Certain promoter CpG dinucleotides, which are normally unmethylated during granulocyte maturation, have been found to be hypermethylated in AML cells such as that of C/EBPα, a late differentiation driver.[28] DNA methylation at promoter CpG dinucleotides is a commonly used marker of gene expression. Many studies have demonstrated the loss of

transcriptional activity and gene expression due to methylation, suggesting that hypermethylation of DNA silences gene expression while hypomethylation allows genes to be expressed.[29-31] Furthermore, use of the DNA hypomethylating agent 5-aza-2'deoxycytidine (decitabine, DAC) has been demonstrated to re-express previously silenced, methylated genes indicating that the dominant mode of silencing for these genes is hypermethylation.[31] An example of this is the re-expression of the human lysozyme (LZM) gene that is highly methylated in AE expressing leukemia after treatment with decitabine. [32] Another example is that of the DNA damage response (DDR) transcriptional regulator FOXO3A (forkhead box O3), whose phosphorylation is associated with poor prognosis in AML. Upon treatment with decitabine, the DDR is restored and FOXO3A becomes dephosphorylated due to the re-expression of its upstream regulator genes; this increases cellular pro-apoptotic signaling.[33] After treatment with decitabine, AML1 target genes that were previously silenced by AE have been demonstrated to be re-expressed, such as IL-3.[13] Taken together, these data suggest first that DNA methylation plays a significant role in AE gene disregulation, and second that reversal of the methylation patterns could alter the prognosis of AML.

Published data from the Melnick lab at Weill Cornell Medical College suggests a connection between DNA methylation of genes and the responsiveness of an AML patient to treatment. An examination of the methylation profiles of 344 patients with M2 AML demonstrated a unique methylation pattern for patients with t(8;21) AML (n= 21).[34] By carefully

examining the human M2 AML methylation profiling data, I identified which genes are methylated in the t(8;21) patients. Furthermore, recently published gene expression microarray and promoter occupancy (ChIP-chip) data from the Zhang lab at the University of California San Diego has identified potential AE9a direct target genes.[26] By comparing the two data sets, I identified the AE9a downregulated genes whose human counterparts are methylated in t(8;21) AML patients. To investigate whether downregulation of AE9a direct target genes is caused by DNA methylation, I treated an AEtr leukemia derived cell line with decitabine and performed RT-qPCR of those genes to determine if their expression is upregulated after treatment.[26]

Next, I confirmed the gene upregulation in the human AML cell lines Kasumi-1, SKNO-1, U937, and HL60 that have been treated with decitabine. The former two are AML cell lines which contain t(8;21), while the latter two are non-t(8;21) expressing AML cell lines. From these data, I was able to identify genes that are upregulated post decitabine treatment which are methylated in t(8;21) patients. I then expressed the selected genes in the murine AEtr AML cells. The genes were cloned into the MSCV-IRES-puromycin (MIP) vector as previously described and a human influenza hemagglutinin (HA) tag was added at the N-terminus for subsequent detection of protein expression by western blot. Next, I transfected 293T cells with the vector products and confirmed the gene expression by probing for the HA-tag on a western blot.[35] Upon confirmation of gene expression, I infected the murine AEtr AML cells and selected for the

transduced cells in puromycin. I monitored cell growth, cell cycle progression, apoptosis, and morphology of the cells to observe any changes to the AML phenotypes that may have occurred due to the re-expression of the previously downregulated genes. Lastly, I attempted to confirm the methylation state of the selected genes in untreated and decitabine treated AEtr cells via bisulfite treatment followed by PCR and sequencing. Collectively, these data demonstrate the effect of reversal of gene silencing by methylation on the pathogenesis of AE9a AML.

II

Results

Analysis of DNA methylation profiling data reveals candidate genes for study

Figueroa et al. of the Melnick lab at Weill Cornell University performed a DNA methylation profiling study to classify AML patients into different groups based on their methylation patterns. The t(8;21) patients were among the three groups that exhibited a unique methylation pattern.[34] The recently published gene expression microarray and promoter occupancy (ChIP-chip) data from the Zhang lab at the University of California San Diego identified 214 genes with AE9a binding in their regulatory regions whose expression was dysregulated in AE9a Lin-/Sca1-/cKit+ (LK) leukemia cells compared with wild-type LK cells. Of these 214 genes, 111 genes were downregulated in the AE9a leukemia cells.[26] These downregulated genes were prioritized based on the ChIP-chip data and whether they were found to be dysregulated in other human AML microarray studies. The first 53 genes were selected for further study (Supplemental Table 1).

The methylation status of the 53 downregulated AE9a target genes in human t(8;21) and non-t(8;21) AML M2 patients in the methylation profiling study from the Melnick lab[34] was examined using the GraphPad Prism V.4 software. A value of below -1 indicate that the genes are methylated (Figure 1A) while a value of above +1 suggests no methylation is present in the genes in question (Figure 1B). This analysis identified 24 of the 53 genes as methylated, 19 as unmethylated, and the remaining 10 genes were not found in the data from the Melnick group. The results are summarized in Table 1.

RT-qPCR screen reveals AE9a downregulated target genes that are upregulated after decitabine treatment in a murine AEtr leukemia derived cell line

Murine AML cells expressing the AEtr protein (AEtr cells) were generated as previously described.[26] The cells were cultured with or without 5μM decitabine for 96 hours. The RNA was extracted from the cells and then reverse transcribed to cDNA in two independent reactions. RT-qPCR was performed on the cDNAs (referred to as cDNA sample A and cDNA sample B) to determine the effect of decitabine on expression of the 53 downregulated genes from the AE9a leukemia array (Figure 2A, B). The fold differences were calculated following normalization fto GAPDH. A gene was considered to be upregulated after treatment if its expression increased more than 1.75 fold compared to the untreated cDNA. (Figure 2C, D) Each qPCR was done in duplicates, and the RT-qPCR screen was performed twice using cDNA samples A and B. The primers used are summarized in Supplemental Table 2.

Careful analysis identified twelve genes as consistently upregulated: Hmha1, Hspa1a/Hspa1b, Ptprc, Aagab, Cuedc1, Jdp2, Ly86, Itga2b, Fam134b, Rgs18, Acad11, and Cd68 (Figure 2E). Ly86 was intentionally removed from the graphs due to its extremely high fold increase for clarity of the graphs (approximately 17-fold higher expression after decitabine treatment).

RT-qPCR screen reveals AE9a downregulated target genes that are upregulated after decitabine treatment in Kasumi-1, SKNO-1-1, U937 and HL60 human AML cell lines

Of the human AML cell lines used to test the effect of a hypomethylating agent, the Kasumi-1 and SKNO-1-1 cells have the t(8;21) translocation while HL60 and U937 are non-t(8;21) cell lines. The cells were cultured with or without 8μM decitabine for 96 hours. The RNA was then harvested from the cells and cDNA was made via reverse transcription. RT-qPCR was performed in duplicates using the cDNA of each of the cell lines .The fold differences were calculated following normalization to GAPDH (Figure 3A-D). A gene was considered to be upregulated after treatment if its expression increased more than 1.75 fold compared to the untreated cDNA.

The upregulated genes were sorted according to how many cell lines they were upregulated in post decitabine treatment. 16 genes were upregulated in all four human AML cell lines (Figure 3E), 20 genes were upregulated in 3 human AML cell lines, and 9 genes were upregulated in 2 human cell lines (data not shown). Of the latter two groups, the genes of interest were the ones that were upregulated in at least both of the t(8;21) expressing AML cell lines Kasumi-1 and SKNO-1-1. 15 genes were upregulated in both t(8;21) cell lines and one non-t(8;21) cell line (Figure 3F) and 5 genes were found to be upregulated in only t(8;21) cell lines (Figure 3G).

The genes that were found to be upregulated in at least Kasumi-1 and SKNO-1-1 were then compared with the results of the RT-qPCR performed on the murine AEtr cells (data not shown). Combining that with the results from the analysis of the human AML methylation profiling data (Table 1),[34] we were able to identify which genes of the original 53 studied were upregulated post decitabine treatment in both human and murine AML cells expressing the t(8;21) gene and were found to be methylated in t(8;21) AML patients (Table 2). The methylation profile of these genes, in addition to their restored expression after treatment with a DNA hypomethylating agent, made them great candidates for further study. The human primers used are summarized in Supplemental Table 3.

Toxicity of decitabine on human cells demonstrated with a titration on Kasumi-1 cell line

Recently published articles regarding the use of the DNMT inhibitor decitabine brought to light the high concentrations of decitabine that were used to treat cells for cDNA preparation and the potential damage that may have been caused to the cells could result in genes unrelated to AML being expressed.[32,37] To test if the concentrations of decitabine used were indeed too high, Kasumi-1 cells were treated with decitabine ranging from 500nM to 8μM for up to 96 hours and the morphology changes, cell cycle progression, apoptosis, and cell growth rates were observed. The experiment was repeated 3 times. At 24 hours after

treatment, a marked change in the cell morphology was evident in all the treated samples (Figure 4A). In addition, even the lowest concentration of decitabine was found to cause a significant change in the cell cycle profile with accumulation of cells in the S+G2/M phase (Figure 4B, C). At 48 and 72 hours, a significant increase in apoptosis of the cells from 2.3% in untreated cells to 16.8% in 8μM treated cells was observed (Figure 4D, E)

Consistent with the cell cycle arrest and increased apoptosis, I observed a steady decline in the rate of growth of cells as the concentration of decitabine increased (Figure 4F). The cells were plated initially at a concentration of $1x10^5$ cells/mL and by 96 hours of treatment, the untreated samples were at an average of $24.8x10^5$ cells/mL while the 0.5μM, 1.0μM, 2.0μM, 4.0μM, and 8.0μM samples were at $16.15x10^5$, $12.3\ x10^5$, $9.75\ x10^5$, $7.6\ x10^5$, and $5.95\ x10^5$ cells/mL respectively. Taking these data and the recently published data into account, new treatments were set up with lower concentrations of decitabine as well as shorter treatment times.

Treatment of murine AEtr cells with lower concentrations of decitabine

AEtr cells were cultured at a density of $1x10^5$ cells/mL with or without decitabine (50nM, 200nM, 500nM) to test if the lower concentrations had any harmful effects on the cells. At 24 hours after treatment, cell cycle analysis indicates very little effect of decitabine on the progression of the cell cycle (Figure

5A). A 96-hour cell growth curve reveals slower growth rate (Figure 5B). This experiment must be repeated to confirm results.

RT-qPCR screen of target genes shows slight upregulation for certain genes after low concentration decitabine treatment in Kasumi-1 cells

Kasumi-1 cells were cultured for 48 hours with or without decitabine (200nM, 500nM). RNA was harvested from the cells and then reverse transcription was used to create cDNA. RT-qPCR was performed using the cDNA in duplicates for each gene. The fold differences were calculated following normalization to GAPDH (Figure 6). A gene was considered to be upregulated after treatment if its expression increased more than 1.75 fold compared to the untreated cDNA. Jdp2, Ly86, and Cd68 were among the genes that showed greatest upregulation post treatment with decitabine. A repeat of this experiment must be performed to confirm these results.

RT-qPCR screen of target genes shows slight upregulation for certain genes after low concentration decitabine treatment in AEtr cells

AEtr cells were cultured for 48 hours with or without decitabine (50nM, 200nM). RNA was harvested from the cells and then reverse transcription was used to create cDNA. RT-qPCR was performed using the cDNA in duplicates for each gene. The fold differences were calculated following normalization to

GAPDH (Figure 7). A gene was considered to be upregulated after treatment if its expression increased more than 1.75 fold compared to the untreated cDNA. Among the genes upregulated were Cuedc1, Jdp2, Rgs18, Ly86 and Cd68. A repeat of this experiment must be performed to confirm these results.

Of the six genes from the AE9a leukemia array that were demonstrated to be upregulated post decitabine treatment in both human and murine AML cell lines and methylated according to published data (Table 3), two of the genes, Ly86 and Cd68, were not proceeded with throughout the rest of the study. The reason for this is because these two genes are known to be differentiation markers and thus the upregulation shown after treatment with decitabine may not be a direct result of reversal of the methylation.[38,39]

Re-expression of downregulated genes of interest in AEtr AML cells causes morphological and functional changes

The genes Cuedc1 (CUE domain containing 1), Jdp2 (Jun dimerization protein 2), Fam134b (Family with sequence similarity 134, member B), and Rgs18 (Regulator of G-protein signaling 18) were chosen for further study to discern the effect of re-expression in murine AML cells. Full-length cDNA clones were ordered and were PCR amplified to add an HA-tag (Supplemental Table 4). Perhaps due to the high G-C content in the primer region, after many attempts Fam134b could not be amplified by PCR and thus is left for future work. The

remaining genes were ligated into the MIP vector (Figure 8A) and sequenced; the clones with no mutations were used for further study. 293T cells were transfected with MIP vectors using the Eco-Pac packaging vector. The transfection was confirmed via western blot (Figure 8B). Although its sequence was correct, Rgs18 did not appear on the western blots in any of the repeats. AEtr cells were infected in duplicates with the virus two days in a row and then selected for in puromycin (4μg/mL) for 72 hours.

Cell culture images revealed that of the three genes transduced into the AEtr cells, Jdp2 displayed the most interesting phenotype (Figure 8C). Unlike the uninfected control cells, the Jdp2 infected cells tend to form clusters. In addition, the cell growth is significantly decreased (Figure 8D). A third infection was performed using only the empty MIP vector control and the MIP-Jdp2 vector on AEtr cells (two different frozen stocks – C2090 and C3378) to confirm the phenotype seen previously (Figure 8E). In addition to the cell growth assay (Figure 8F), which confirmed the decreased cell growth in the previous infections, cell cycle analysis (Figure 8G), cytospin (Figure 8H), and apoptosis assays (Figure 8I) reveal significant changes in cell survival and self-renewal of the AEtr AML cells that express Jdp2.

Sodium bisulfite treatment to discern methylation levels of genes of interest in untreated and decitabine treated AEtr AML cells

Finally, to confirm the methylation status of the genes of interest before and after decitabine treatment, and thus infer if the morphological changes are due to hypomethylation of hypermethylated genes, bisulfite treatment was performed on genomic DNA from AEtr cells (untreated, 50nM, 200nM decitabine).[32] Using a Qiagen kit, the genomic DNA was treated with sodium bisulfite followed by PCR amplification using primers designed by the MethPrimer website (http:// www.urogene.org/methprimer/index1.html) (Supplemental Table 5). The PCR product was loaded onto agarose gels, stained with ethidium bromide, and directly visualized under UV light to confirm amplification. After many trials the only samples that were successfully amplified were the untreated and the 50nM decitabine treated AEtr-Jdp2 genomic DNA (Figure 9A). The product was cloned into the pGEM-T Easy vector and transformed into competent DH5α cells. The individual colonies thought to contain the ligated vector were selected for, but sequencing and restriction enzyme analysis showed that the vectors were empty (data not shown).

Using the bisulfite treated DNA, a different technique to show the methylation status of CpG dinucleotides was employed: methylation-specific PCR (MSP).[40] New primer pairs were ordered with one set specific to methylated sequences (therefore protected from bisulfite conversion) and the other to unmethylated sequences (susceptible to bisulfite conversion) to test if any

changes in amplification would occur between the decitabine treated DNA and the untreated DNA (Supplemental Table 6). The PCR product was loaded onto agarose gels, stained with ethidium bromide, and directly visualized under UV light (Figure 9B).

Cuedc1 did not amplify with either primer set. Rgs18 showed amplification with the methylated DNA primer pair in both the untreated and decitabine treated DNA, and no amplification with the unmethylated DNA primer pair. The primer set for Jdp2 showed that the untreated (no decitabine) DNA amplified with the only the methylated DNA primer pair. In contrast, the 50nM decitabine treated DNA no longer amplified with the methylated DNA primer pair. It also did not amplify with the unmethylated DNA primer pair.

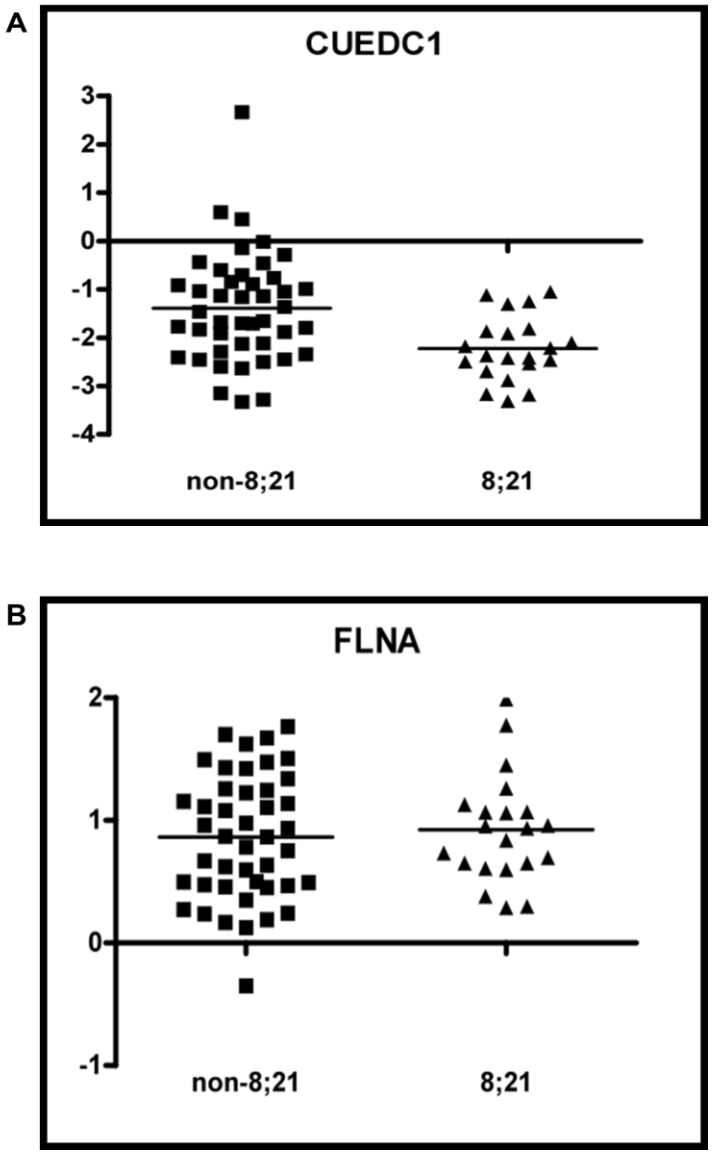


Figure 1A-B. Analysis of DNA methylation profiling data reveals candidate genes for study

t(8;21) patients: n=21 and non-t(8;21) patients: n=44. A value of below -1 as identified by the GraphPad Prism V.4 software indicates a gene is methylated. A value of above +1 indicates no methylation.

(A) GraphPad Prism V.4 analysis reveals CUEDC1 gene that is methylated, in this case in both t(8;21) and non-t(8;21) AML patients
(B) GraphPad Prism V.4 analysis reveals FLNA gene that is unmethylated, in this case in both t(8;21) and non-t(8;21) AML patients

Table 1. Methylation status according to Figueroa et al. of genes identified from Lo et al.'s AE9a leukemia array

Gene	Methylated[26]
HMHA1	
SLCO3A1	x
HSPA1A/HSPA1B	x
PARP8	x
PRKCH	x
PTPRC	
RNF144A	x
AAGAB	n.a.
FUT8	x
CUEDC1	x
JDP2	x
LY86	x
PSD4	x
SSX2IP	x
IRF2BP2	
EVL	n.a.
FLNA	
ITGA2B	n.a.
WHSC1	x
CPT1A	
FAM134B	x
H2AFY	

Gene	Methylated[26]
IDH1	
RGS18	x
CYFIP2	x
PCK2	
SATB1	
ANKRD28	
ARHGEF6	
GSPT2	n.a.
HSP90B1	
MAPKAPK2	n.a.
MYCN	
NPHP3	n.a.
PDLIM1	n.a.
SMARCD2	
SORL1	
TMEM229B	n.a.
ATP6AP1	
BCL7A	
CD68	x
GNA11	x
MEIS1	
PLXNB2	x

Table 1 continued.

Gene	Methylated[26]
SLC44A1	n.a.
SPNS3	n.a.
ATP10A	x
CCDC88C	x
CFP	x
CNKSR3	x
CPNE2	
CSAD	x
HADHA	x

x: Gene is methylated according to methylation profile data from Figueroa et al.[34]

n.a: Gene not listed in methylation profile data from Figueroa et al.[34]

Figure 2A-E. RT-qPCR screen reveals AE9a downregulated target genes that are upregulated after decitabine treatment in the murine AEtr leukemia derived cell line

RT-qPCR of murine AEtr cells cultured with or without decitabine (5μM) for 96 hours. RNA was extracted from the cells and up to 1μg of RNA was reverse transcribed on two separate occasions to cDNA creating samples A and B. SYBR green reagent was used for the RT-qPCR, and the Ct values for the genes were first averaged between duplicates and then normalized to GAPDH. The Ct values of the samples with decitabine were then compared to those without decitabine. Ly86 values intentionally not shown.

(A) Relative expression of all 53 genes (with two primer sets for genes Fam134b and Tmem229b) tested in duplicates with AEtr cDNA from RNA sample A
(B) Relative expression of all 53 genes (with two primer sets for genes Fam134b and Tmem229b) tested in duplicates with AEtr cDNA from RNA sample B
(C) Genes upregulated ≥1.75 fold from AEtr cDNA from RNA sample A
(D) Genes upregulated ≥1.75 fold from AEtr cDNA from RNA sample B Consistently upregulated genes.

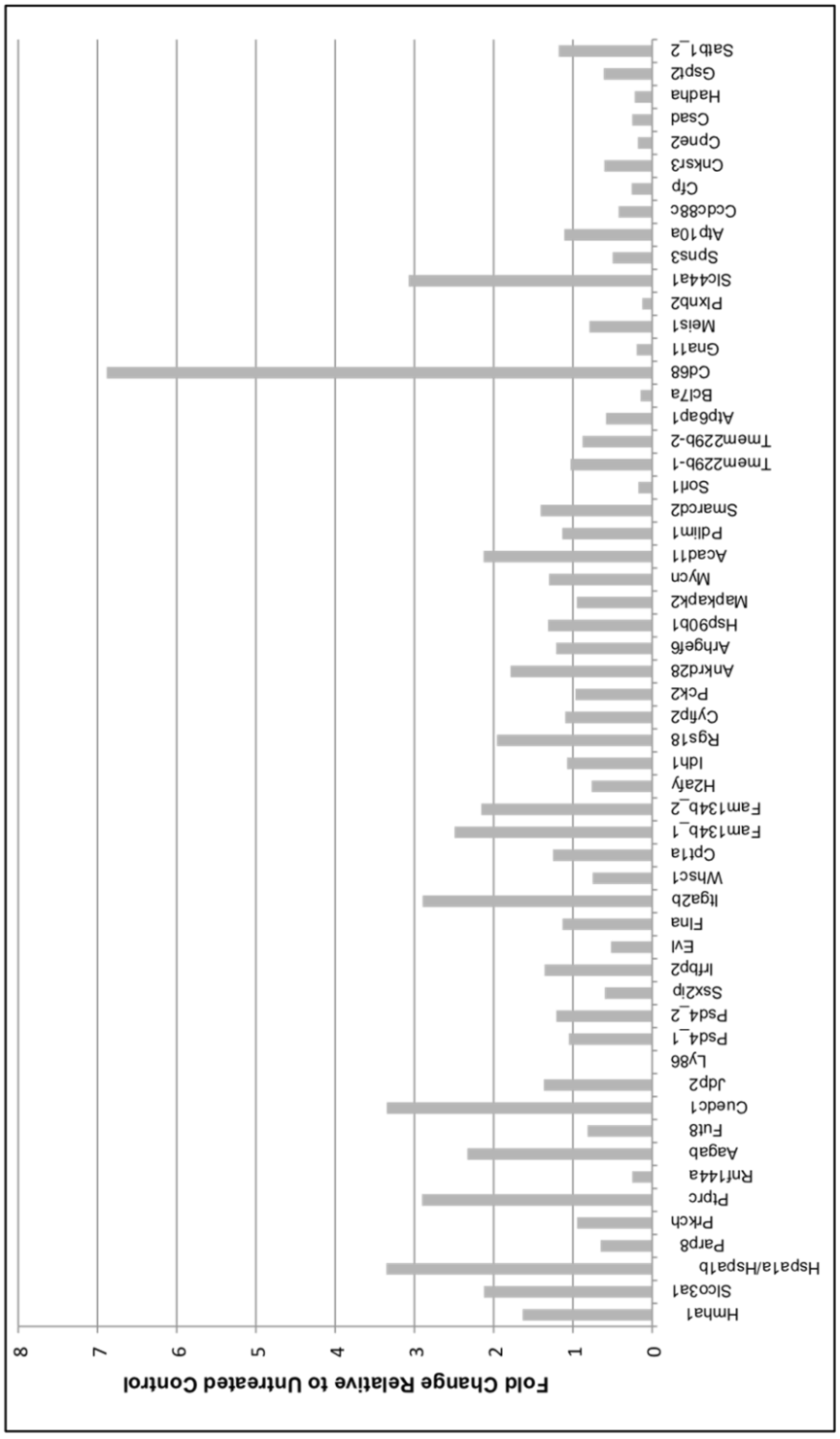

A
Fold Change Relative to Untreated Control
0
1
2
3
4
5
6
7
8
Hmha1
Slco3a1
Hspa1a/Hspa1b
Parp8
Pfkfb
Ptprc
Rnf144a
Agap
Fut8
Cuedc1
Jdp2
Ly86
Psd4_1
Psd4_2
Ssx2ip
Irfbp2
Evl
Fina
Itga2b
Whsc1
Cpt1a
Fam134b_1
Fam134b_2
H2afy
Idh1
Rgs18
Cyfip2
Pck2
Ankrd28
Arhgef6
Hsp90b1
Mapkapk2
Myo1c
Acad11
Pdlim1
Smarcd2
Sorl1
Tmem229b-1
Tmem229b-2
Atp6ap1
Bcl7a
Cd68
Gna11
Meis1
Plxnb2
Slc44a1
Spns3
Atp10a
Ccdc88c
Cfp
Cnksr3
Cpne2
Csad
Hadha
Gspl2
Satb1_2

Figure 2

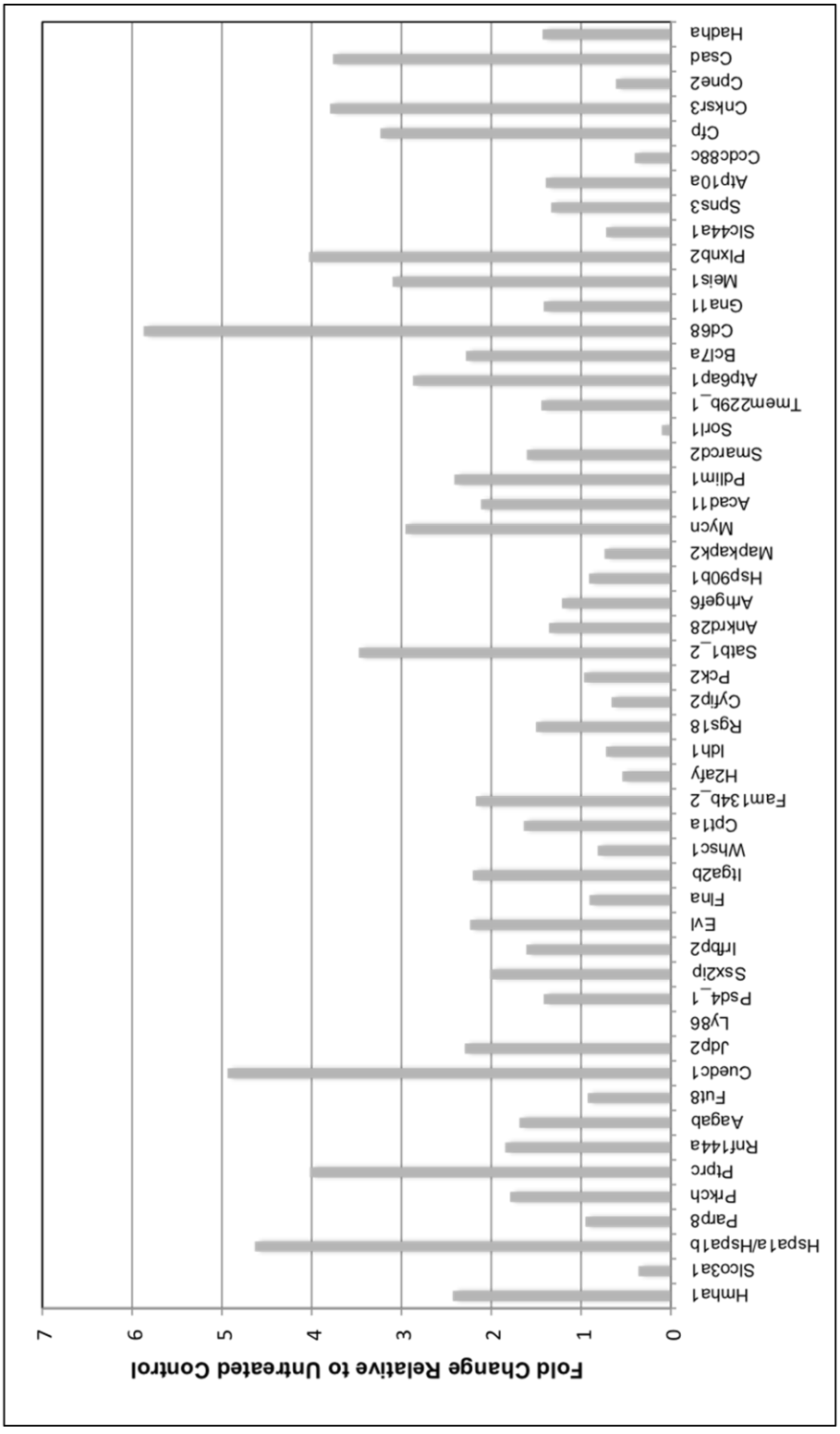
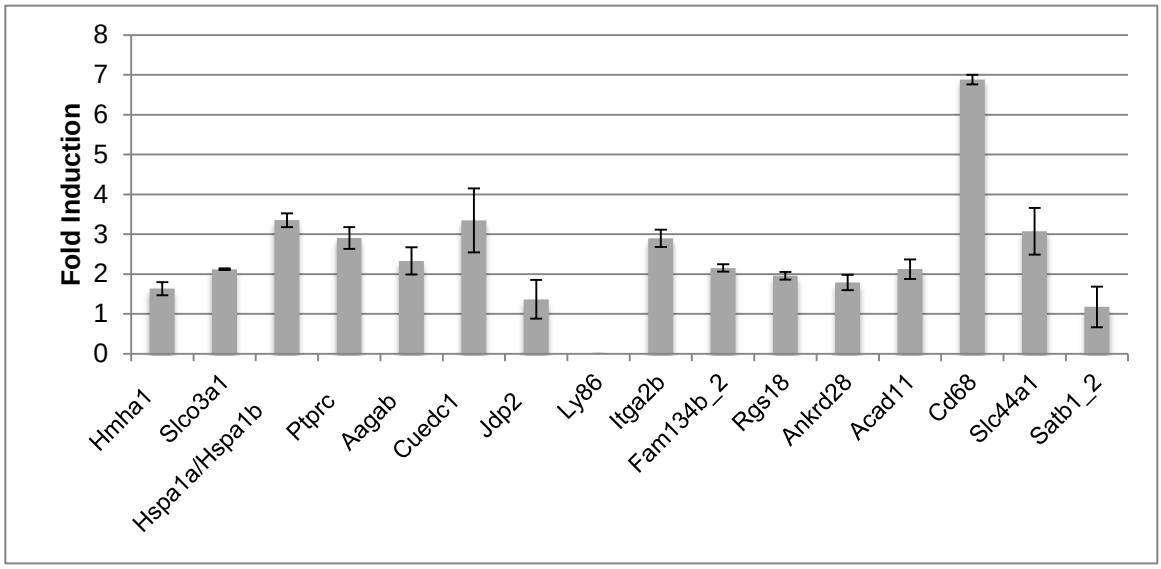


Figure 2 continued

C



D

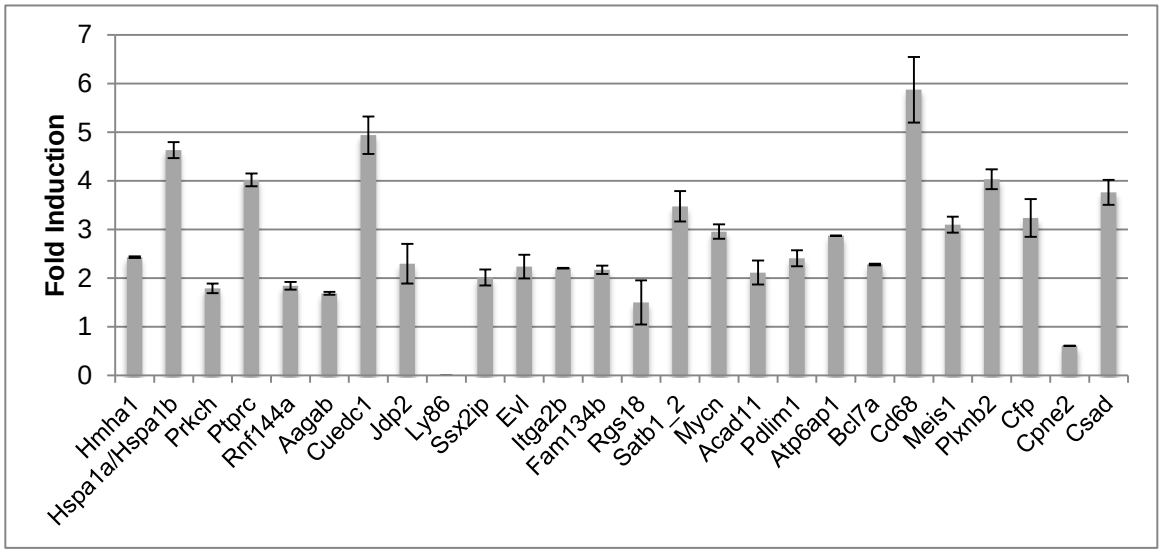


Figure 2 continued

E

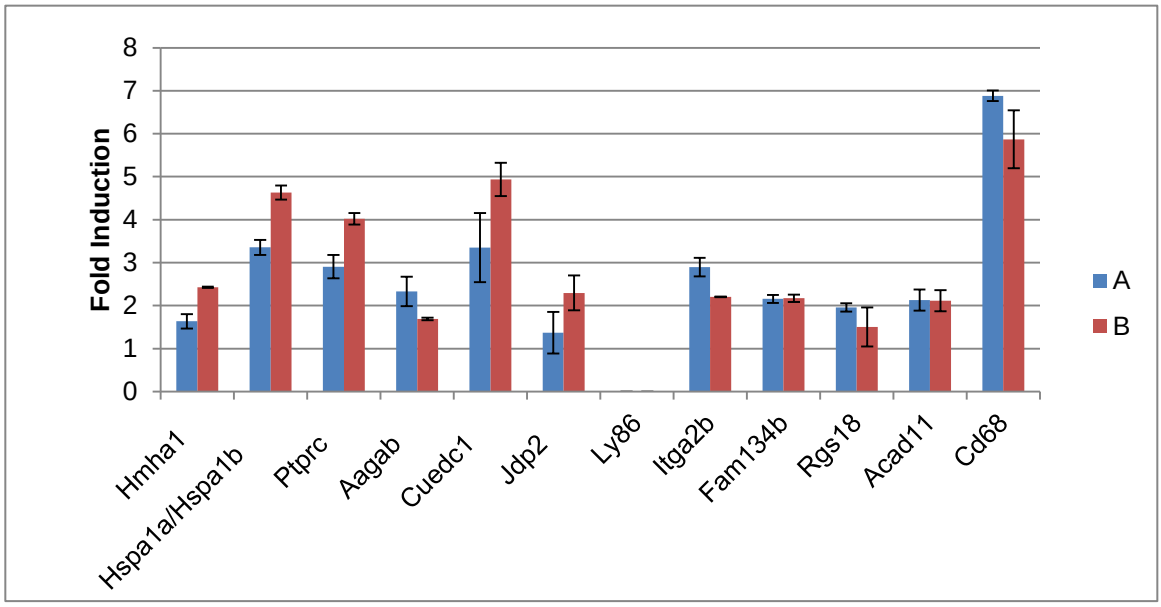


Figure 2 continued

Figure 3A-G: RT-qPCR screen of AE9a downregulated target genes that are upregulated after decitabine treatment in Kasumi-1, SKNO-1-1, U937 and HL60 human AML cell lines

RT-qPCR of human AML cells cultured with or without decitabine (8μM) for 96 hours. RNA was extracted from the cells and up to 1μg of RNA was reverse transcribed to cDNA. SYBR green reagent was used for the RT-qPCR in duplicates for each gene, and the Ct values for the genes were first averaged between duplicates and then normalized to GAPDH. The Ct values of the samples with decitabine were then compared to those without decitabine.

(A-D) Relative expression of all 53 genes tested with Kasumi-1, SKNO-1, HL60, and U937 cDNA respectively.
(E) Genes upregulated ≥1.75 fold compared to untreated samples common to all four human AML cell line cDNA.
(F) Genes upregulated ≥1.75 fold compared to untreated samples common to both Kasumi-1 and SKNO-1 cDNA, and either HL60 or U937 cDNA.
(G) Genes upregulated ≥1.75 fold compared to untreated samples common to only Kasumi-1 and SKNO-1 cDNA.

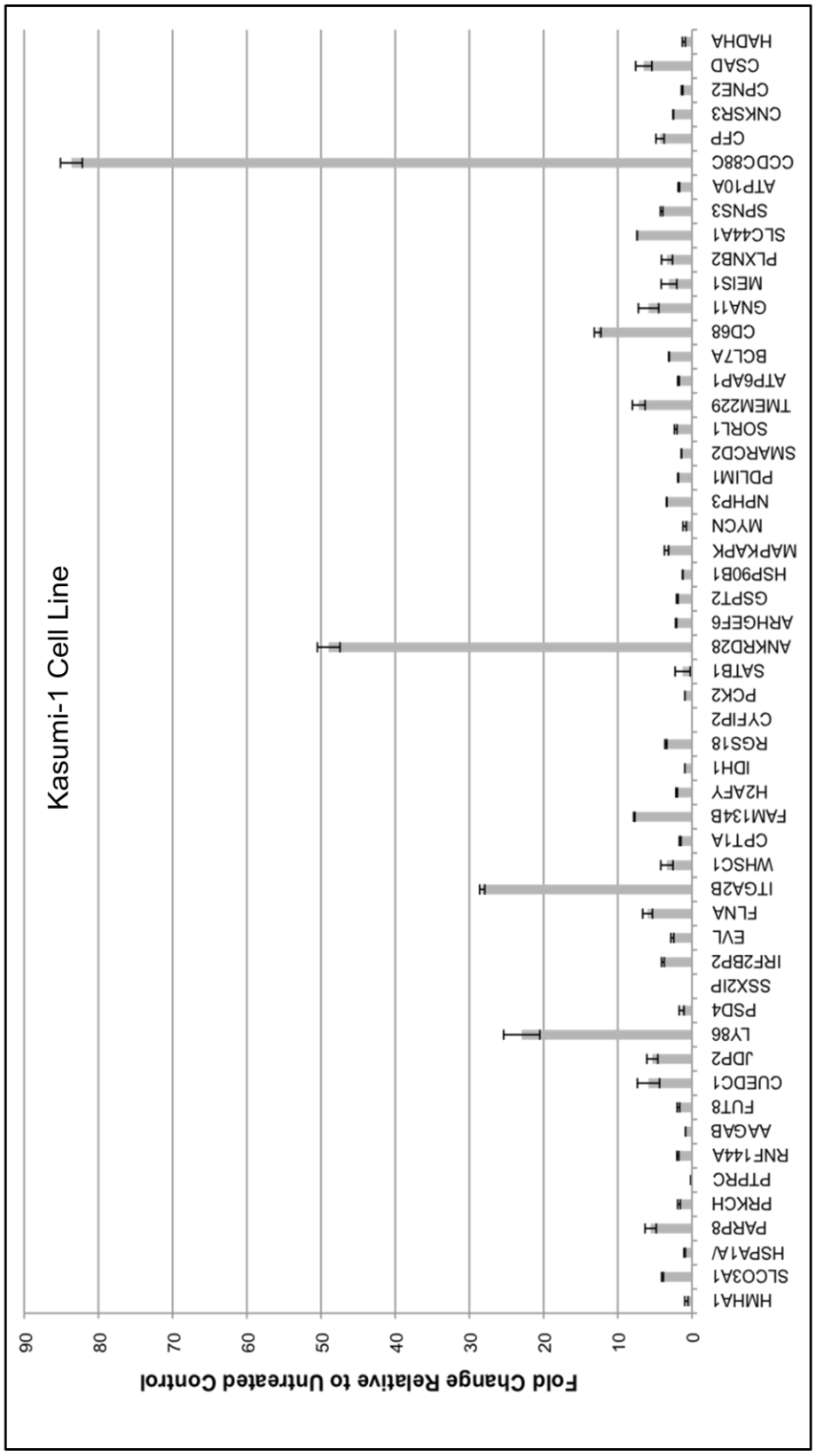

A
Kasumi-1 Cell Line
Fold Change Relative to Untreated Control
0
10
20
30
40
50
60
70
80
90
HMHA1
SLCO3A1
HSPA1A/
PARP8
PRKCH
PTPRC
RNF144A
AAGAB
FUT8
CUEDC1
JDP2
LY86
PSD4
SSX2IP
IRF2BP2
EVL
FLNA
ITGA2B
WHSC1
CPT1A
FAM134B
H2AFY
IDH1
RGS18
CYFIP2
PCK2
SATB1
ANKRD28
ARHGEF6
GSPT2
HSP90B1
MAPKAPK
MYCN
NPHP3
PDLIM1
SMARCD2
SORL1
TMEM229
ATP6AP1
BCL7A
CD68
GNA11
MEIS1
PLXNB2
SLC44A1
SPNS3
ATP10A
CCDC88C
CFP
CNKSR3
CPNE2
CSAD
HADHA

Figure 3

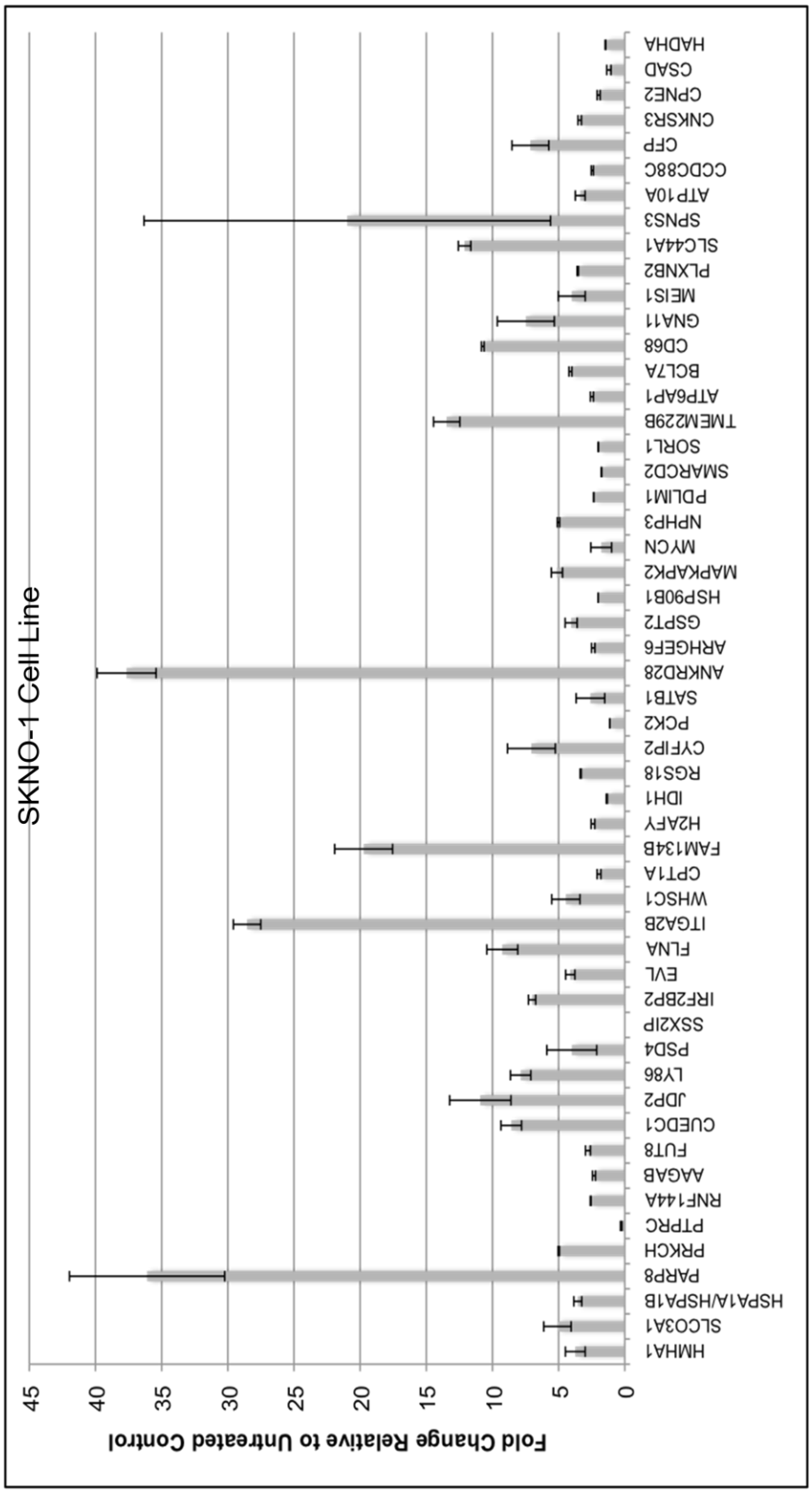


Figure 3 continued

C

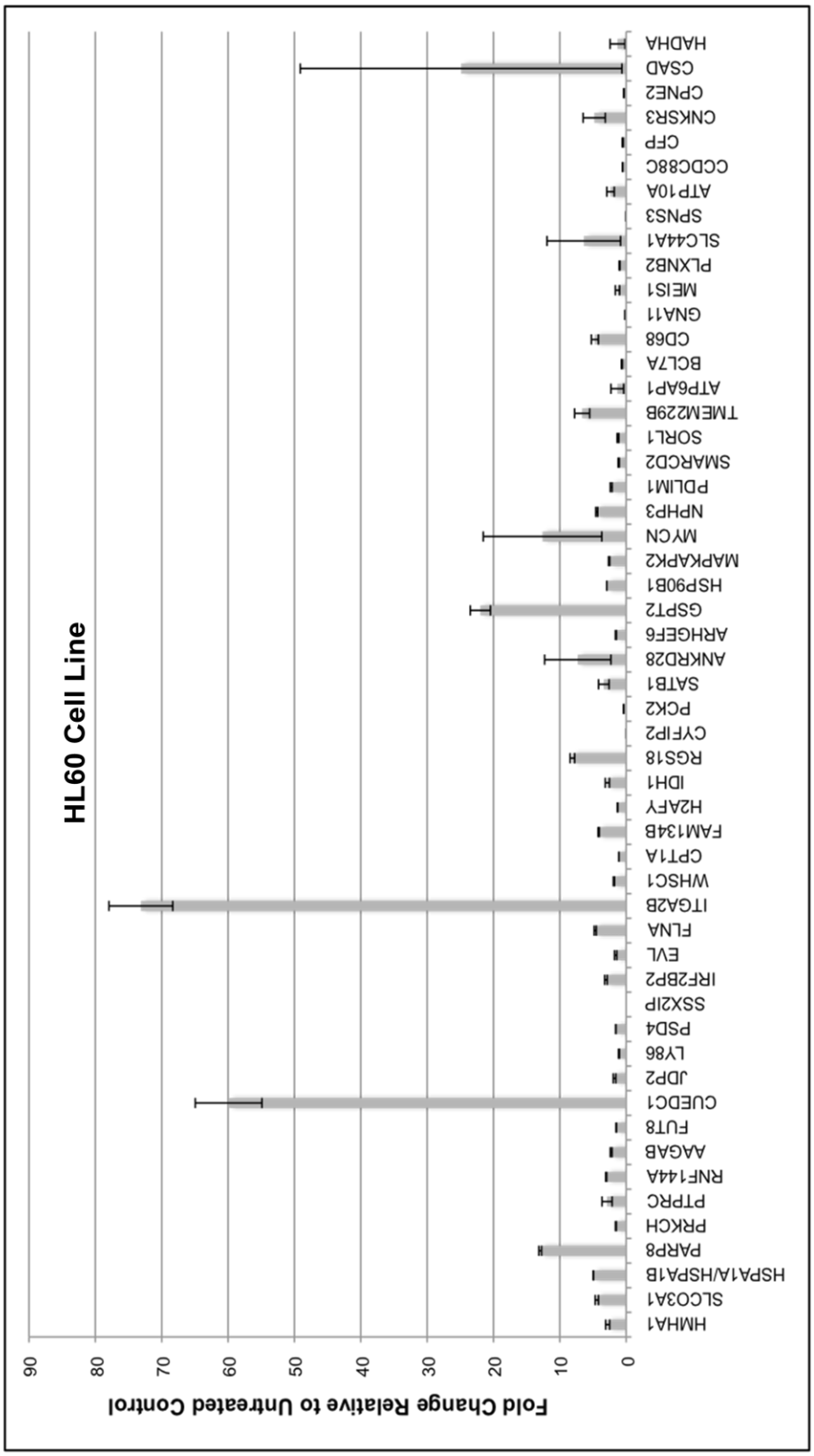


Figure 3 continued

D

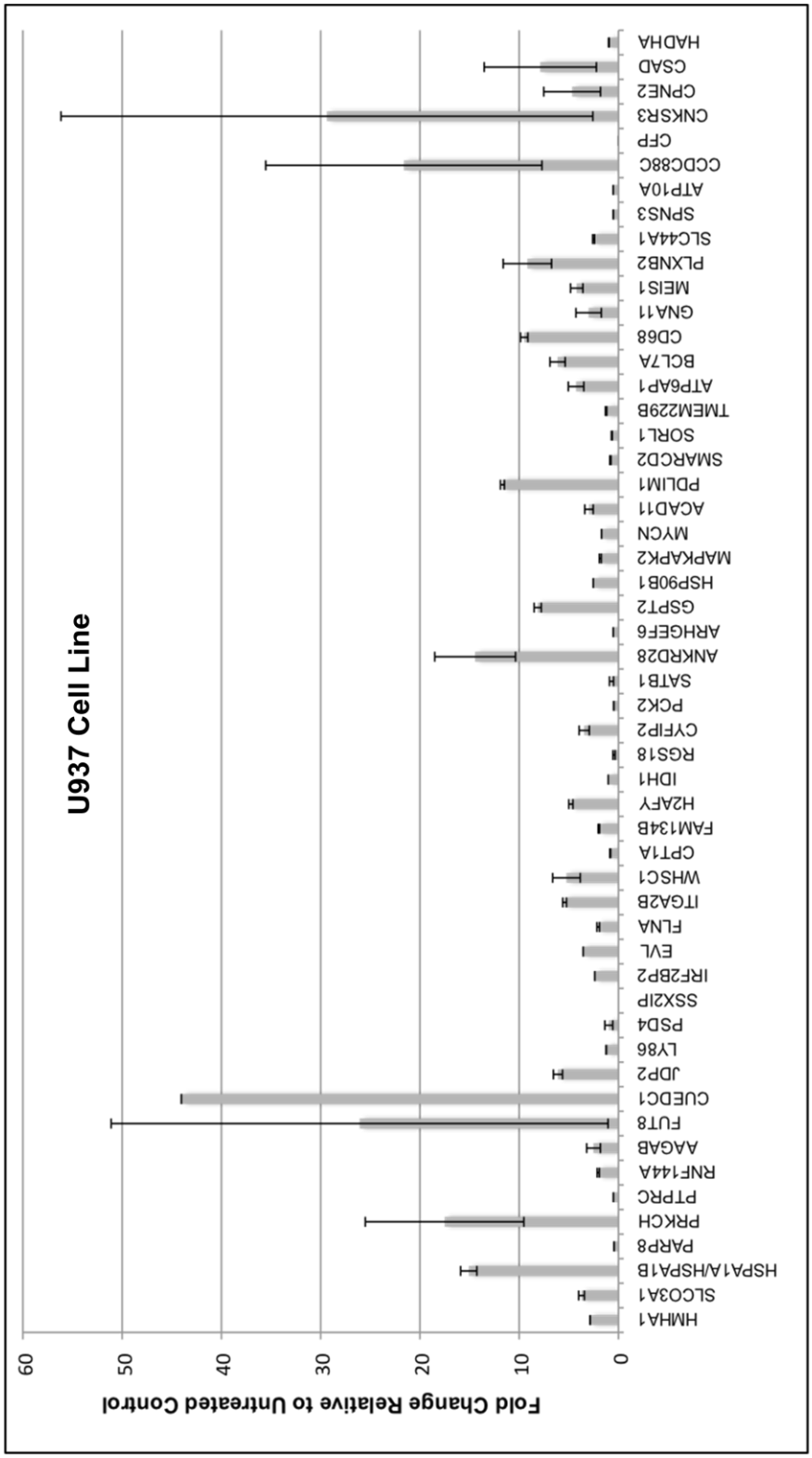
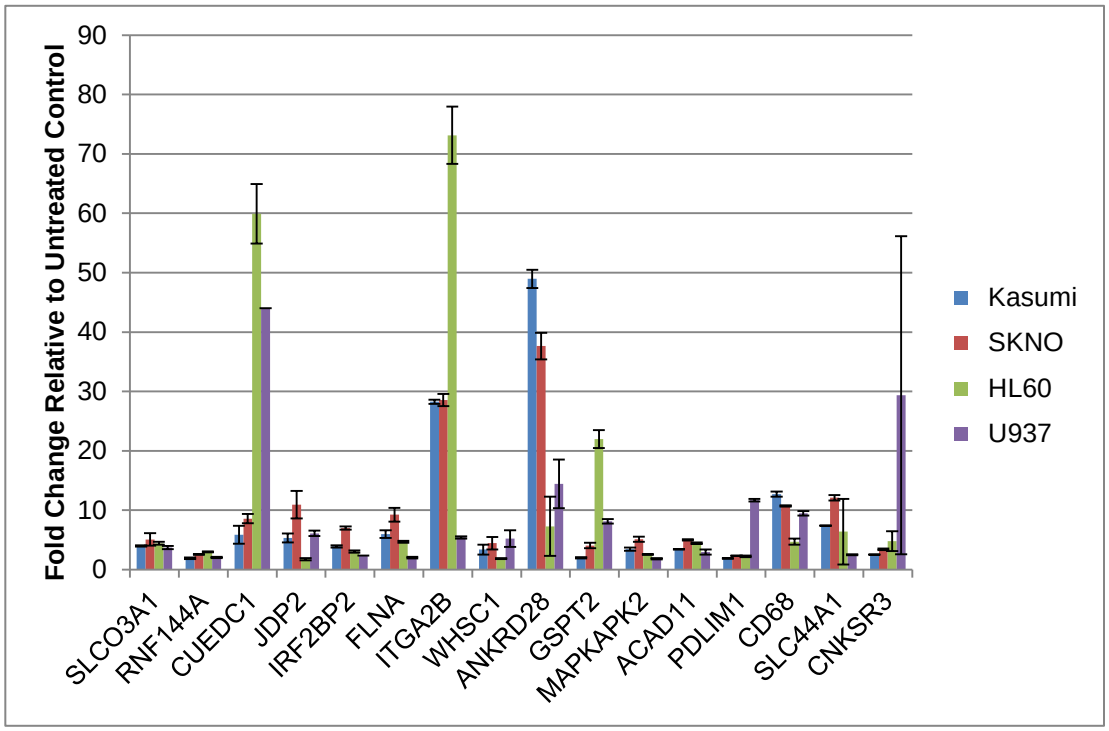


Figure 3 continued

E



F

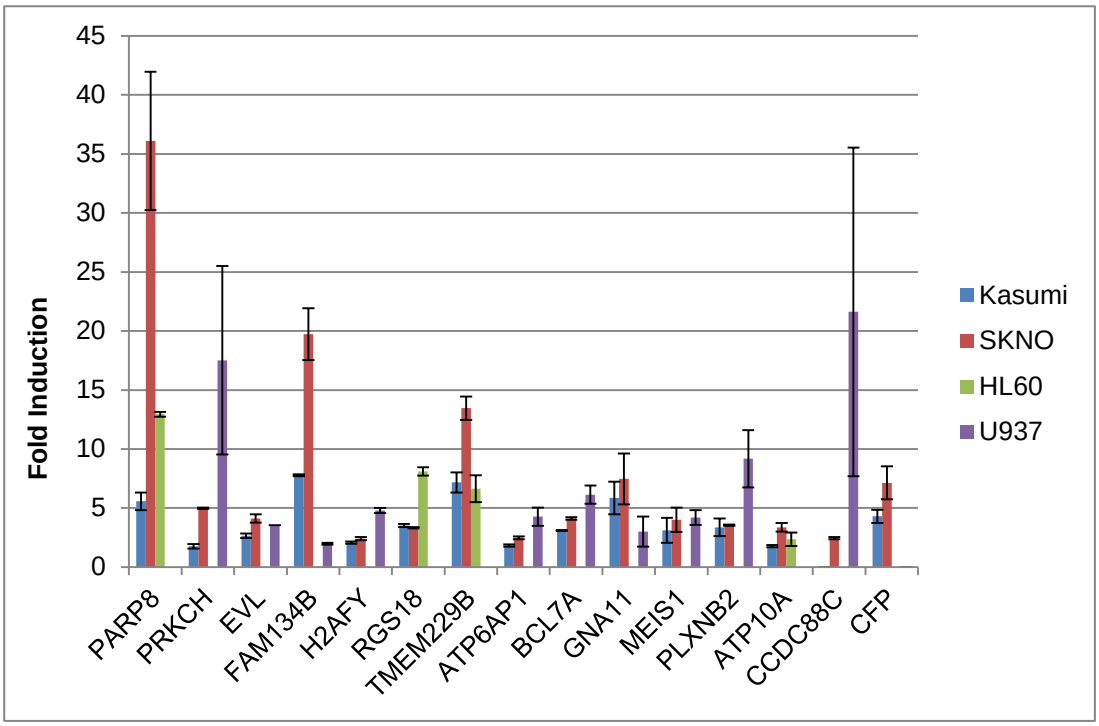


Figure 3 continued

G

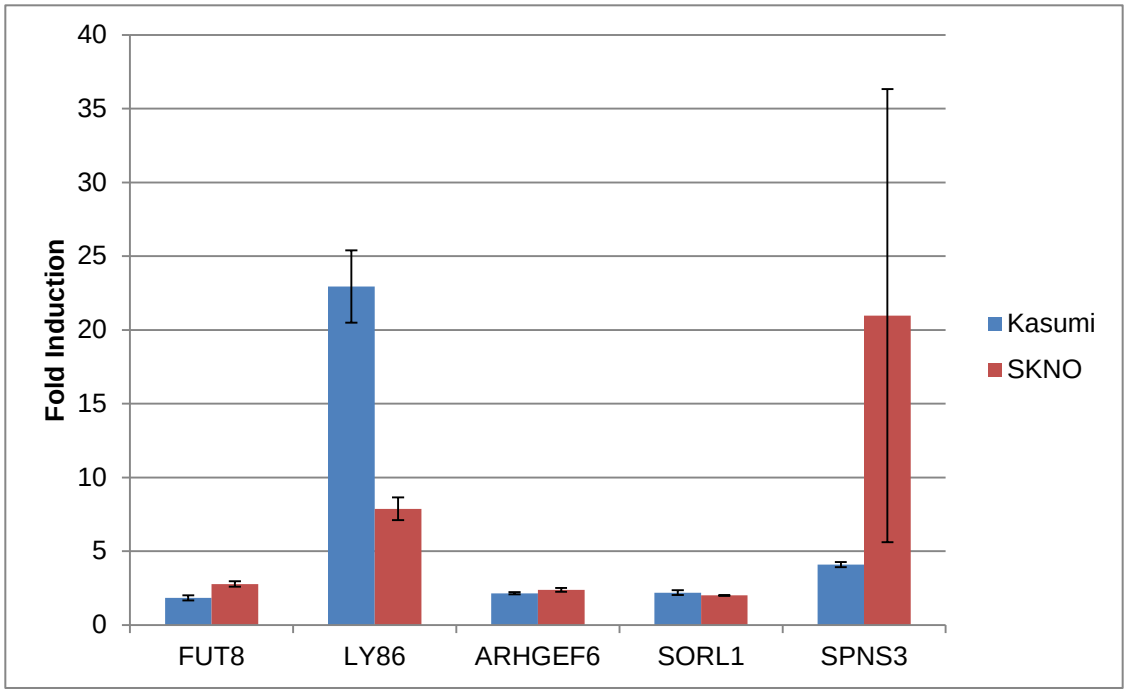


Figure 3 continued

Table 2. Fold changes of methylated genes upregulated after decitabine treatment relative to untreated control.

Gene	AEtr Cells[a]	Kasumi Cells[b]	SKNO-1 Cells[b]	Methylated[c]
CUEDC1	4.14	5.87	8.58	x
JDP2	1.83	5.34	10.92	x
LY86	17.78	22.95	7.87	x
ITGA2B	2.55	28.27	28.54	n.a.
FAM134B	2.16	7.77	19.73	x
RGS18	1.96	3.52	3.33	x
ACAD11	2.12	3.41	5.01	n.a.
CD68	6.38	12.72	10.73	x

[a] – 5μM Decitabine, 96 hour. Average fold change
[b] – 8μM Decitabine, 96 hour
[c] – According to Figueroa et al.[34]

Figure 4: Toxicity of decitabine on human cells demonstrated with a titration on Kasumi-1 cell line

A Kasumi-1 titration was set up with $1x10^5$ cells/mL treated with 0, 500nM, 1μM, 2μM. 4μM, and 8μM decitabine per well. Cell counts were taken at 24 hour intervals followed by cytospin and cell cycle analysis at 24 hours, and apoptosis assay at 48 and 72 hours. The experiment was repeated three times independently.

(A) Cytospin slides stained with Wright-Giemsa stain illustrate morphological changes to the cells with increasing decitabine concentration
(B) Representative figures from cell cycle analysis performed using propium iodide staining illustrates cells that are in G0/G1 phase (P3) or S-G2M phase (P4). Untreated, 0.5μM, and 8μM samples.
(C) Percentage of cells in G_0/G_1 phase or S-G_2M phase
(D) Representative figures from apoptosis assay – 72 hour performed using Annexin-VPE and 7-AAD staining. Top row: Q2 are dead cells, Q3 are live cells, and Q4 are early apoptosis cells. Bottom row: P2 is % of cells stained with Annexin, an indicator of apoptosis. Untreated, 0.5μM, and 8μM samples.
(E) Percentage of cells undergoing apoptosis
(F) Cell growth assay performed via cell counts.

A

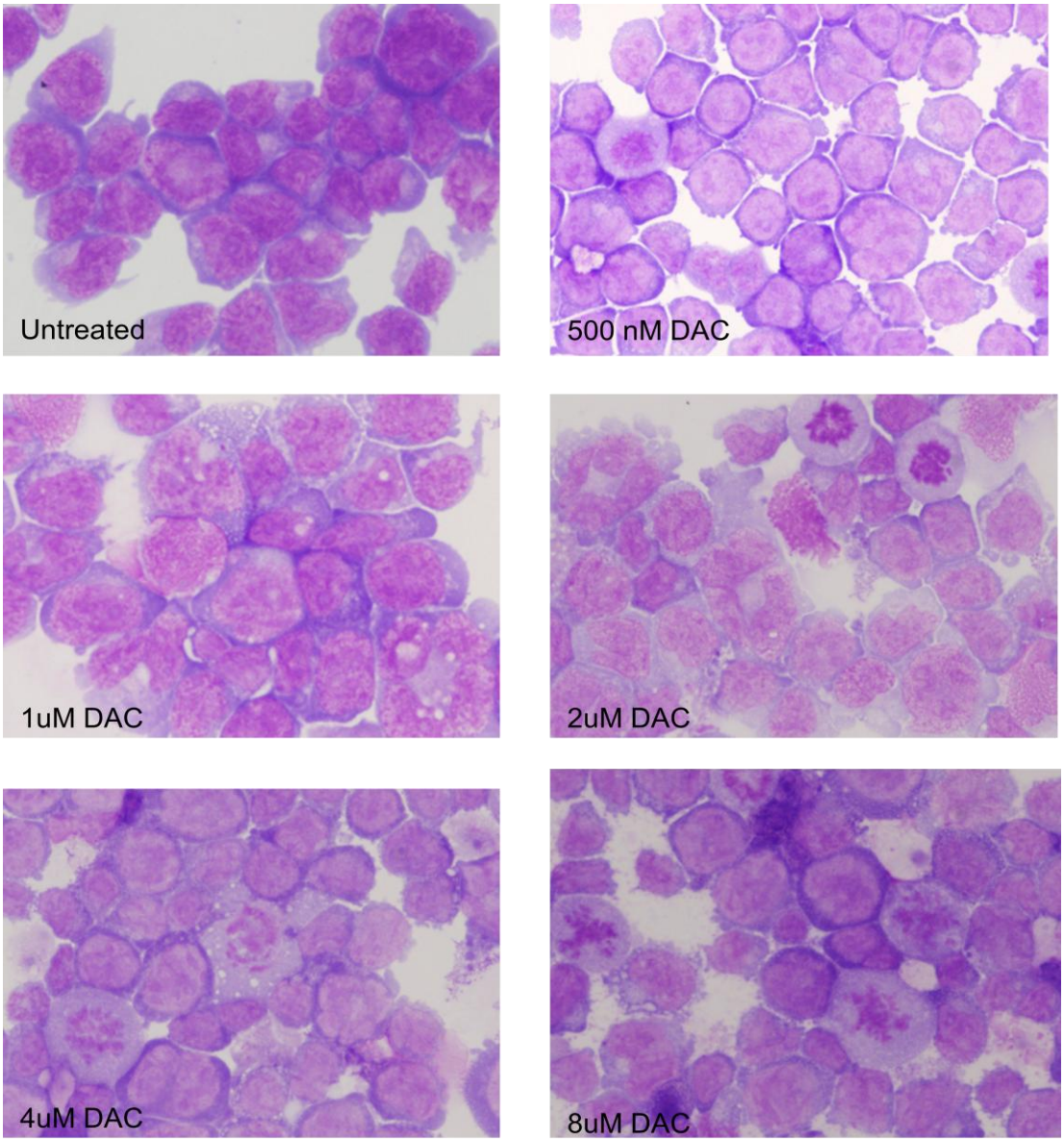
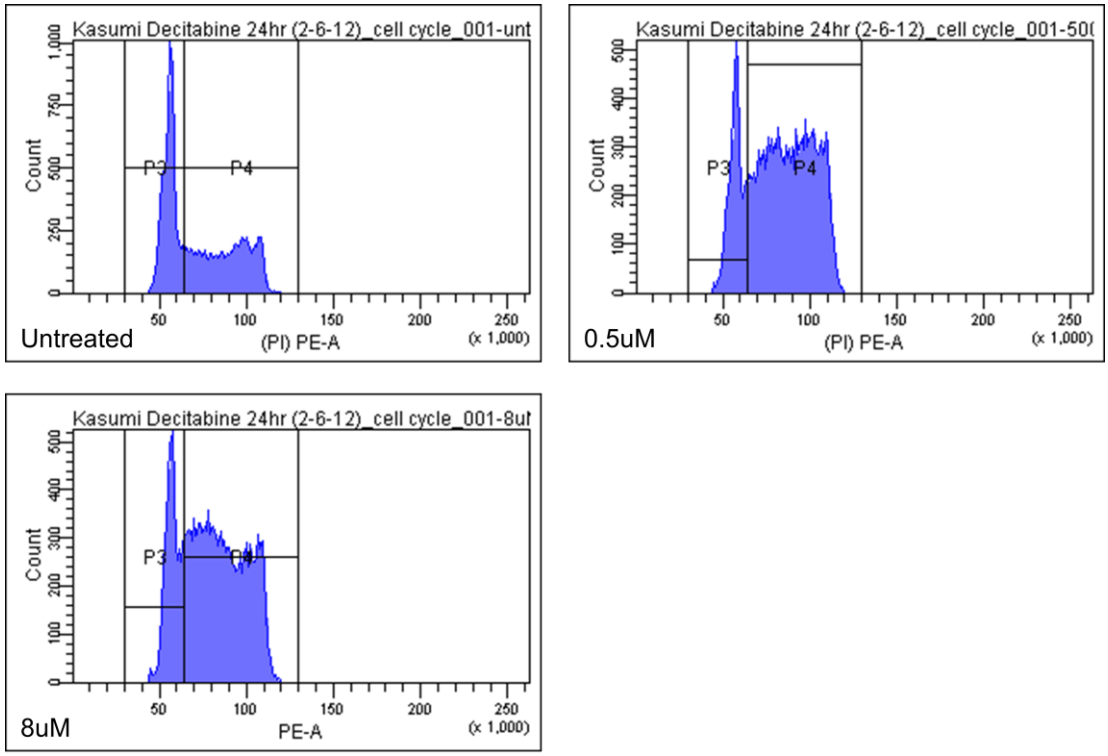


Figure 4

B



C

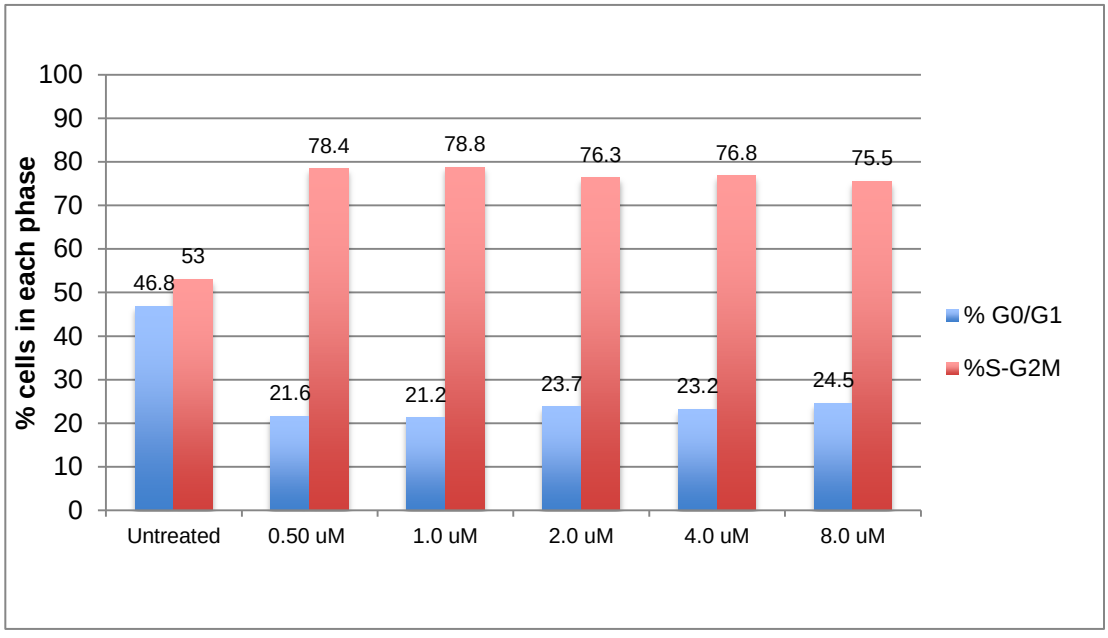
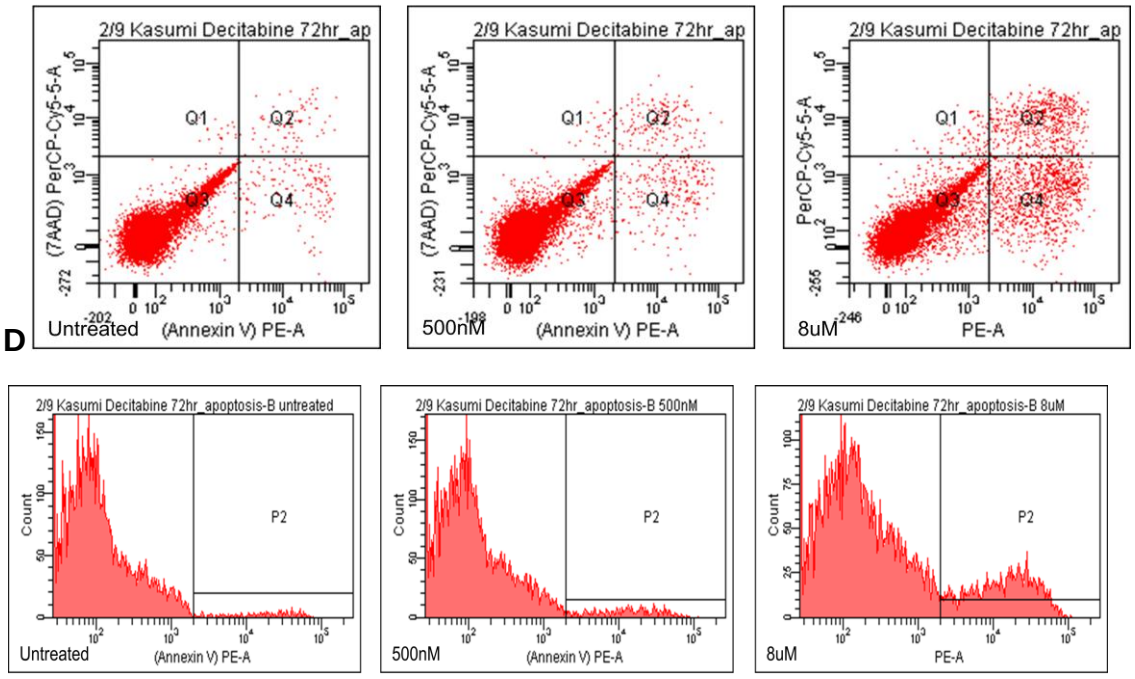


Figure 4 continued

D



E

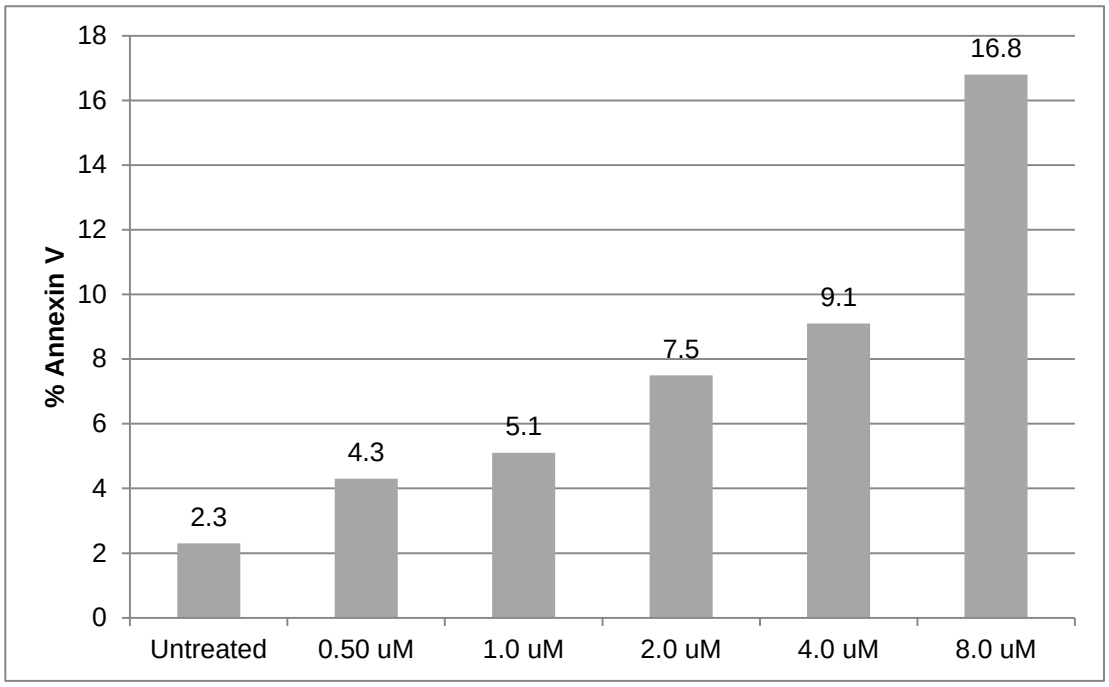


Figure 4 continued

F

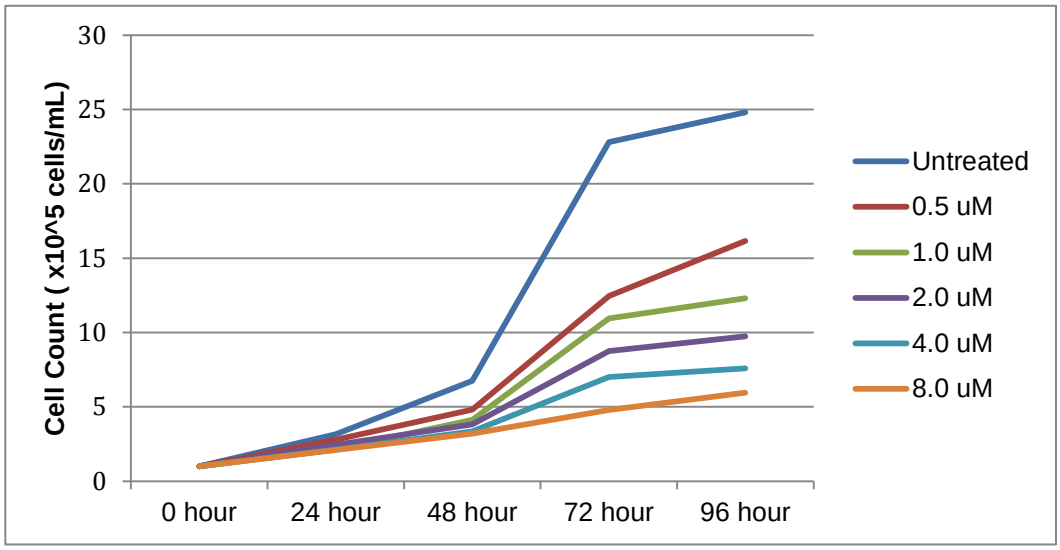
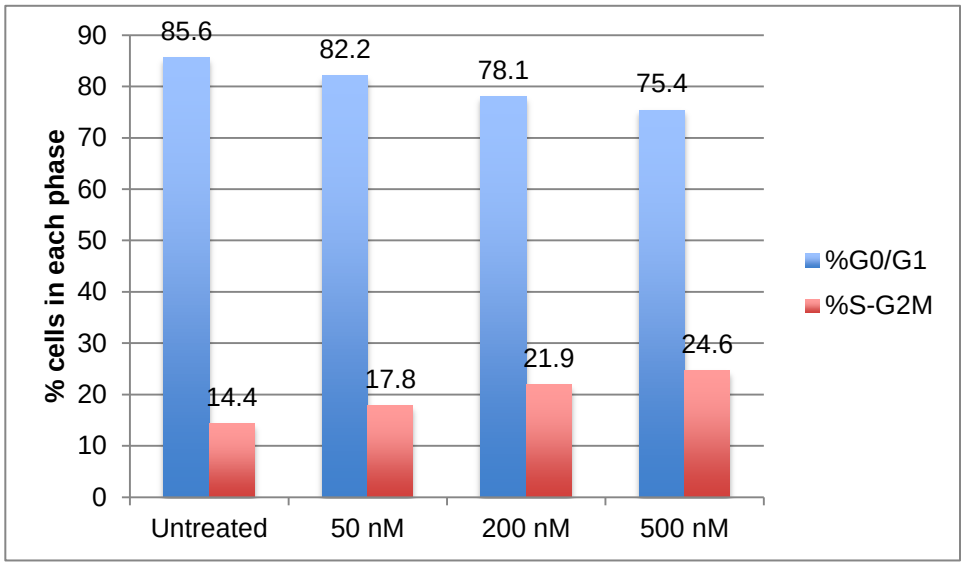


Figure 4 continued

A



B

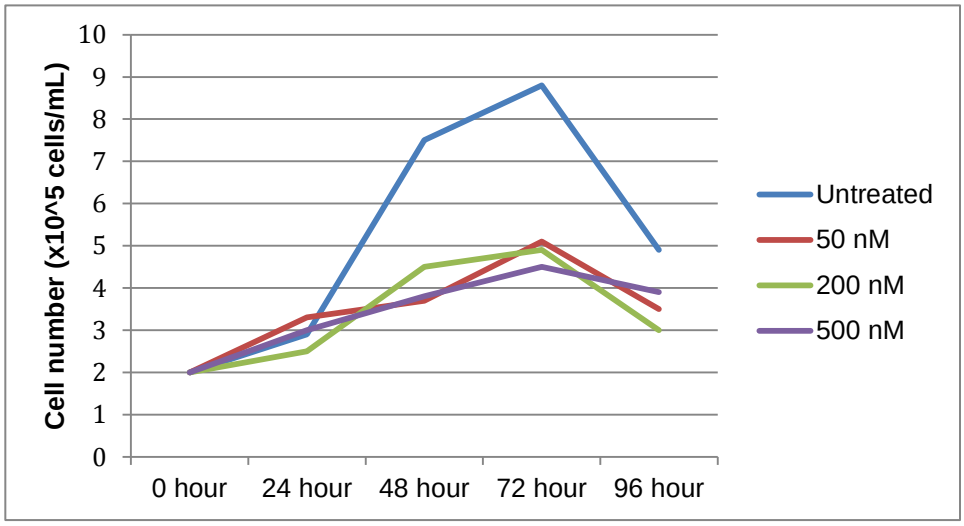


Figure 5: Treatment of murine AEtr cells with lower concentrations of decitabine

An AEtr titration was set up with $1x10^5$ cells/mL treated with 0, 50nM, 200nM and 500nM decitabine per well. Cell counts were taken at 24-hour intervals and cell cycle analysis at 24 hours with propium iodide.

(A) Percentage of cells in G0/G1 phase or S-G2M phase.
(B) Cell growth assay performed via cell counts.

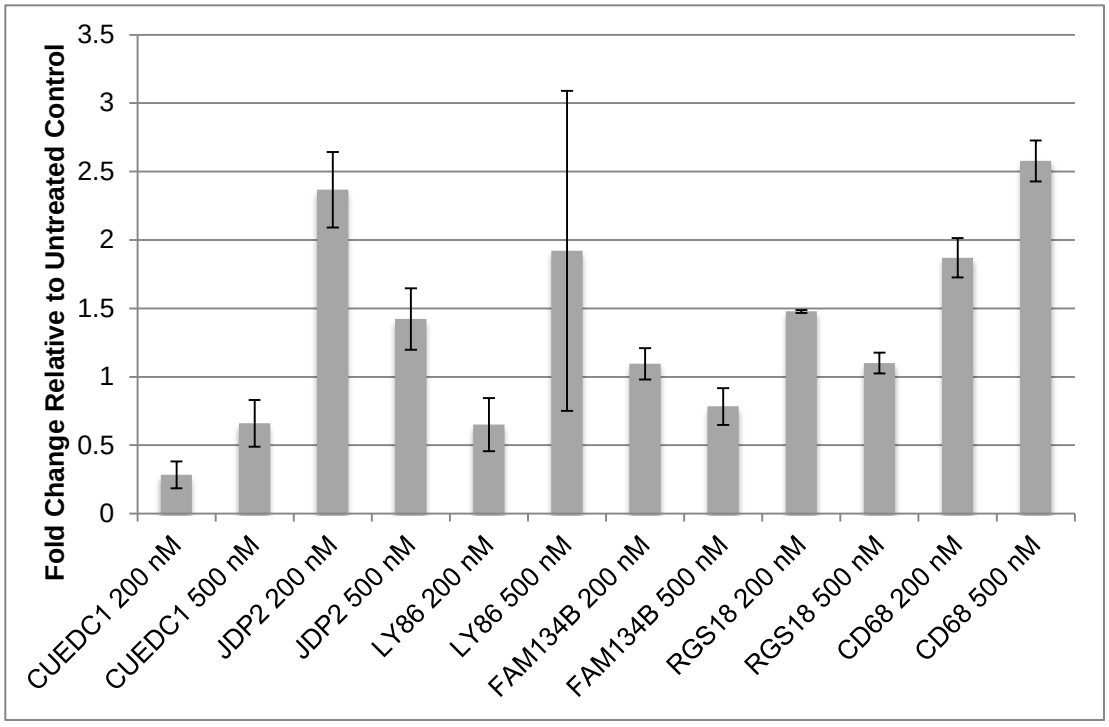


Figure 6: RT-qPCR screen of target genes shows slight upregulation for certain genes after low concentration decitabine treatment in Kasumi-1 cells

RT-qPCR of human Kasumi-1 AML cells cultured with or without decitabine (200nM, 500nM) for 48 hours. RNA was extracted from the cells and up to 1µg of RNA was reverse transcribed to cDNA. SYBR green reagent was used for the RT-qPCR, and the Ct values for the genes were first averaged between duplicates and then normalized to GAPDH. The Ct values of the samples with decitabine were then compared to those without decitabine.

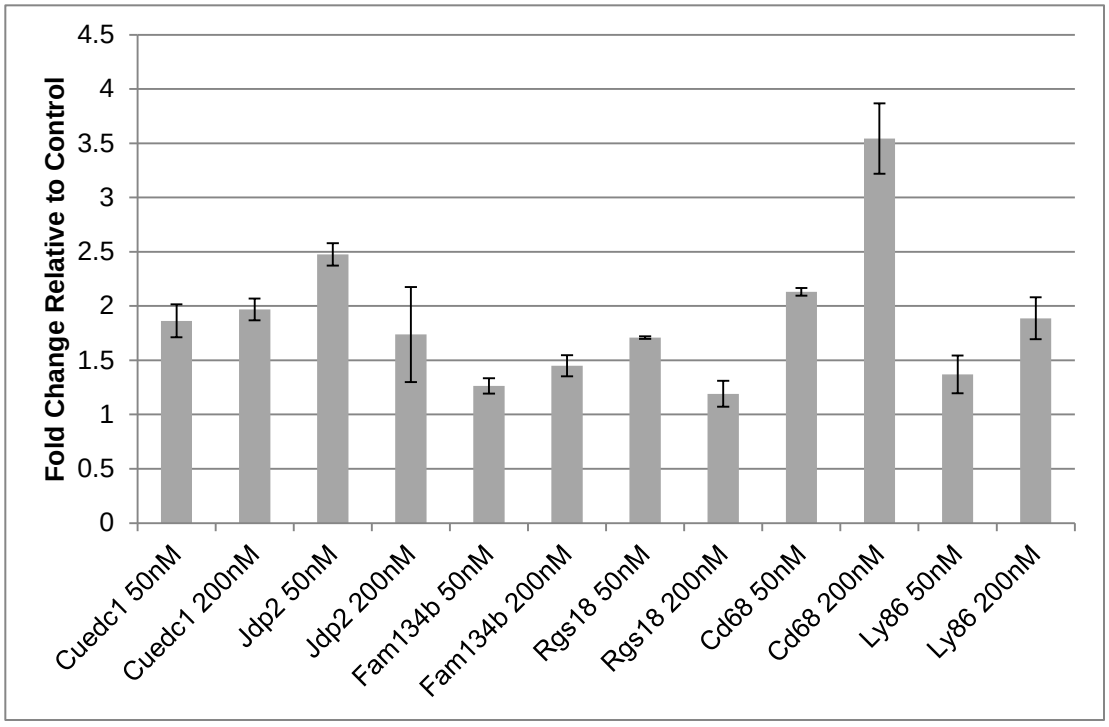


Figure 7: RT-qPCR screen of target genes shows slight upregulation for certain genes after low concentration decitabine treatment in AEtr cells

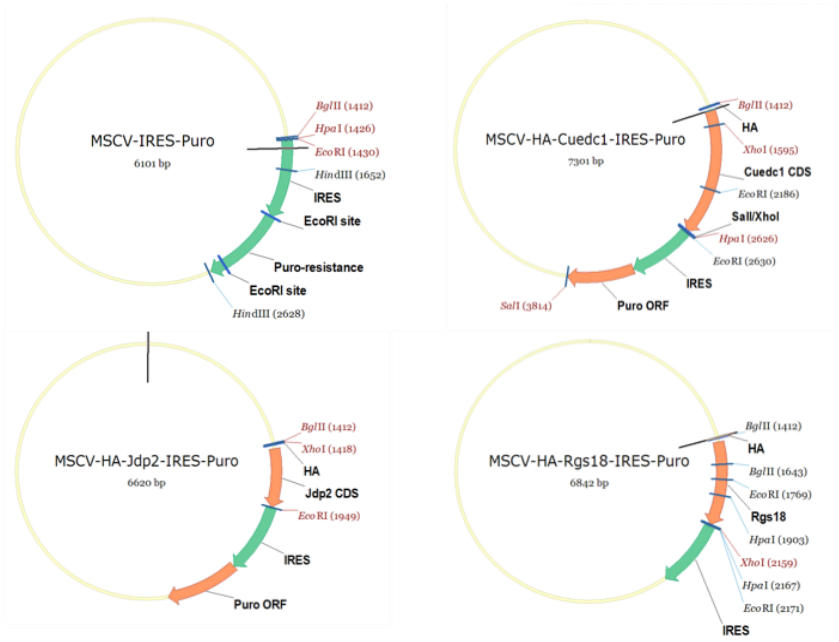
RT-qPCR of murine AEtr cells cultured with or without decitabine (50nM, 200nM) for 48 hours. RNA was extracted from the cells and up to 1µg of RNA was reverse transcribed to cDNA. SYBR green reagent was used for the RT-qPCR, and the Ct values for the genes were first averaged between duplicates and then normalized to GAPDH. The Ct values of the samples with decitabine were then compared to those without decitabine.

Figure 8: Re-expression of downregulated genes of interest in AEtr AML cells causes morphological and functional changes

The genes of interest were tagged with HA and ligated into the MIP vector and then transfected into 293T cells using the EcoPac packaging vector. The virus was then used to infect AEtr cells in duplicates in two AEtr cell stocks (frozen at different times) and the cells with the vector were selected for in puromycin (4μg/mL for 72 hours). The experiment was performed 3 times

(A) Images of the vector constructs
(B) Western blot confirmation of expression of the genes
(C) Cell culture images 24 hours after removal of puromycin
(D) Cell growth assay: average value of duplicates
(E) Cell culture images 24 hours after removal of puromycin
(F) Cell growth assay: average value of duplicates
(G) Percentage of cells in G0/G1 phase or S-G2M phase using propium iodide
(H) Cytospin slides visualized with Wright-Giemsa stain
(I) 72 hour apoptosis assay using Annexin-V

A


MSCV-IRES-Puro
6101 bp
BglII (1412)
HpaI (1426)
EcoRI (1430)
HindIII (1652)
IRES
EcoRI site
Puro-resistance
EcoRI site
HindIII (2628)
MSCV-HA-Cuedc1-IRES-Puro
7301 bp
BglII (1412)
HA
XhoI (1595)
Cuedc1 CDS
EcoRI (2186)
SalI/XhoI
HpaI (2626)
EcoRI (2630)
IRES
SalI (3814)
Puro ORF
MSCV-HA-Jdp2-IRES-Puro
6620 bp
BglII (1412)
XhoI (1418)
HA
Jdp2 CDS
EcoRI (1949)
IRES
Puro ORF
MSCV-HA-Rgs18-IRES-Puro
6842 bp
BglII (1412)
HA
BglII (1643)
EcoRI (1769)
Rgs18
HpaI (1903)
XhoI (2159)
HpaI (2167)
EcoRI (2171)
IRES

B

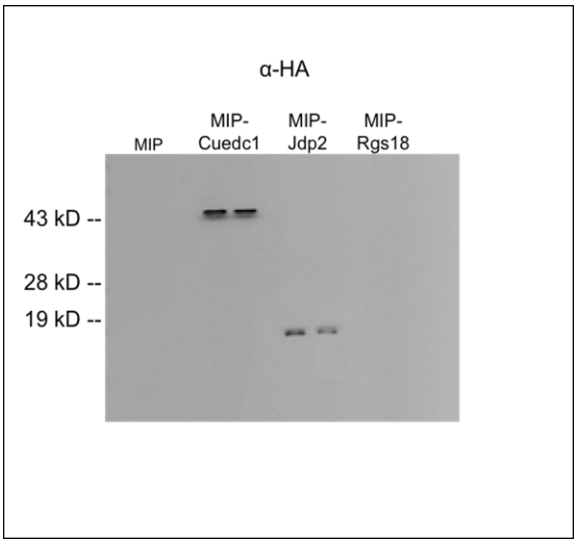
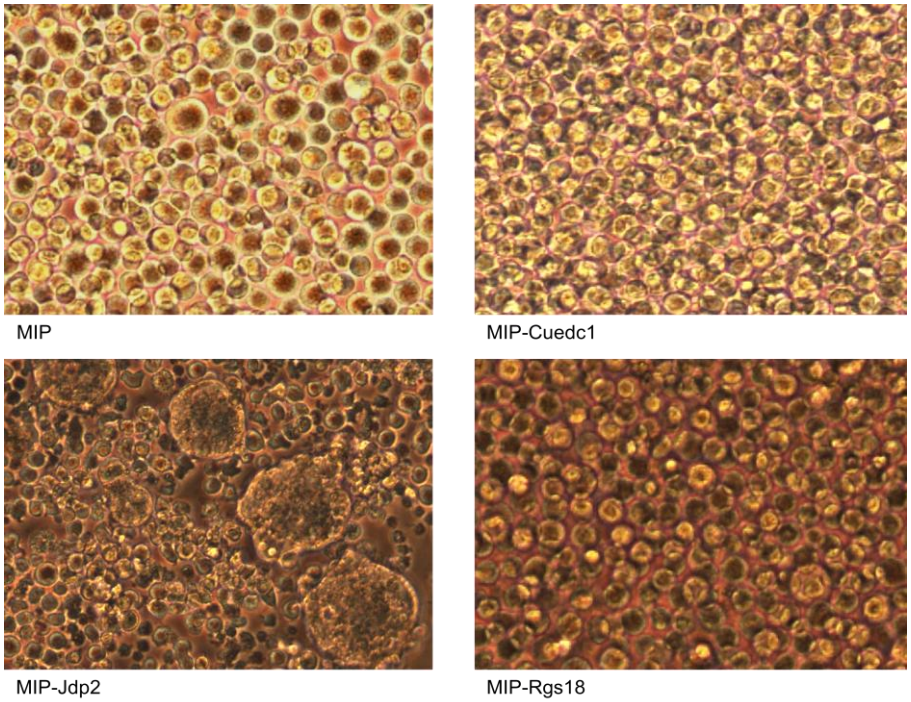

α-HA
MIP
MIP-Cuedc1
MIP-Jdp2
MIP-Rgs18
43 kD --
28 kD --
19 kD --

Figure 8.

C



D

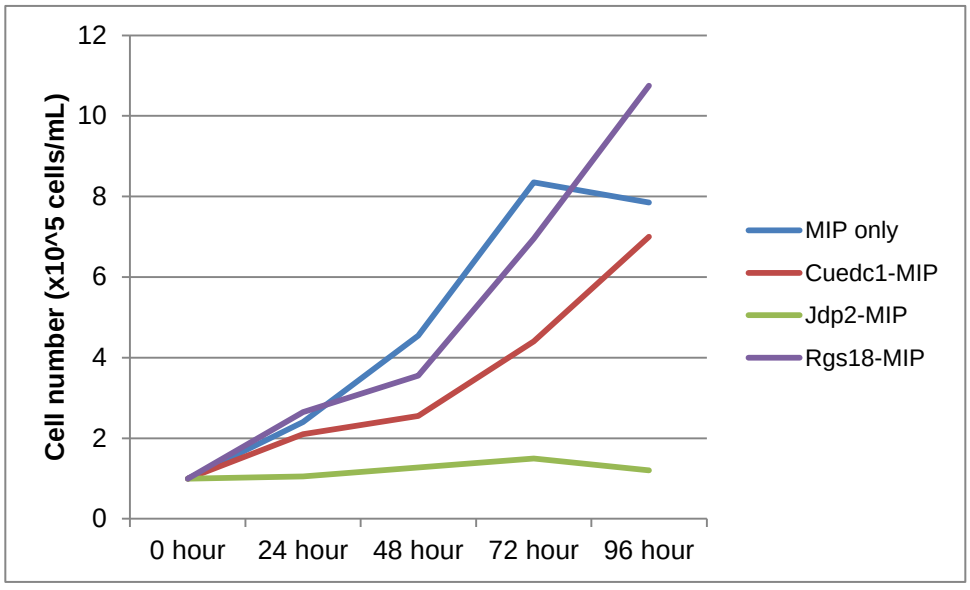
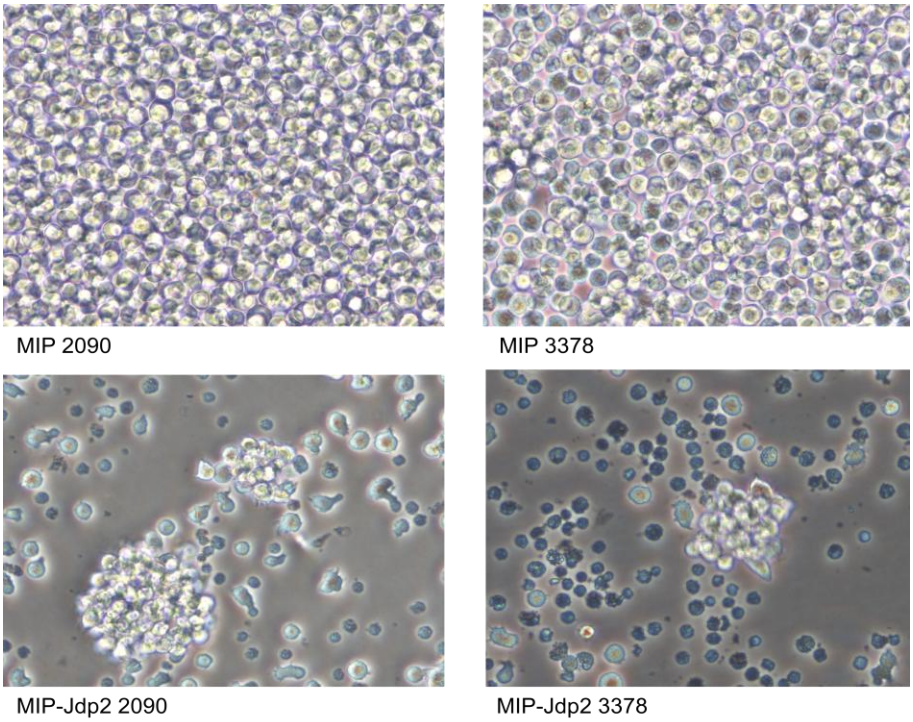


Figure 8 continued

E



F

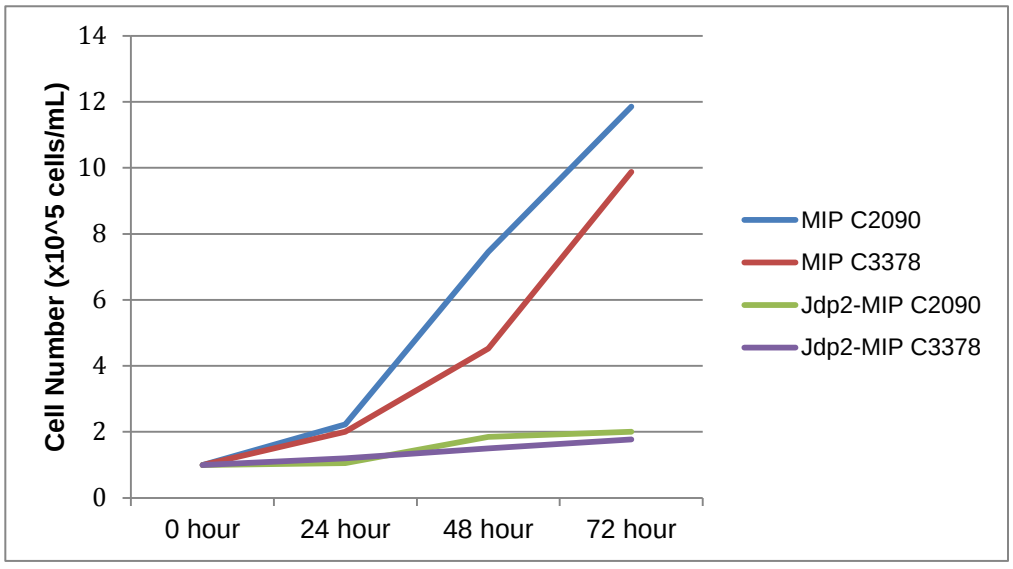
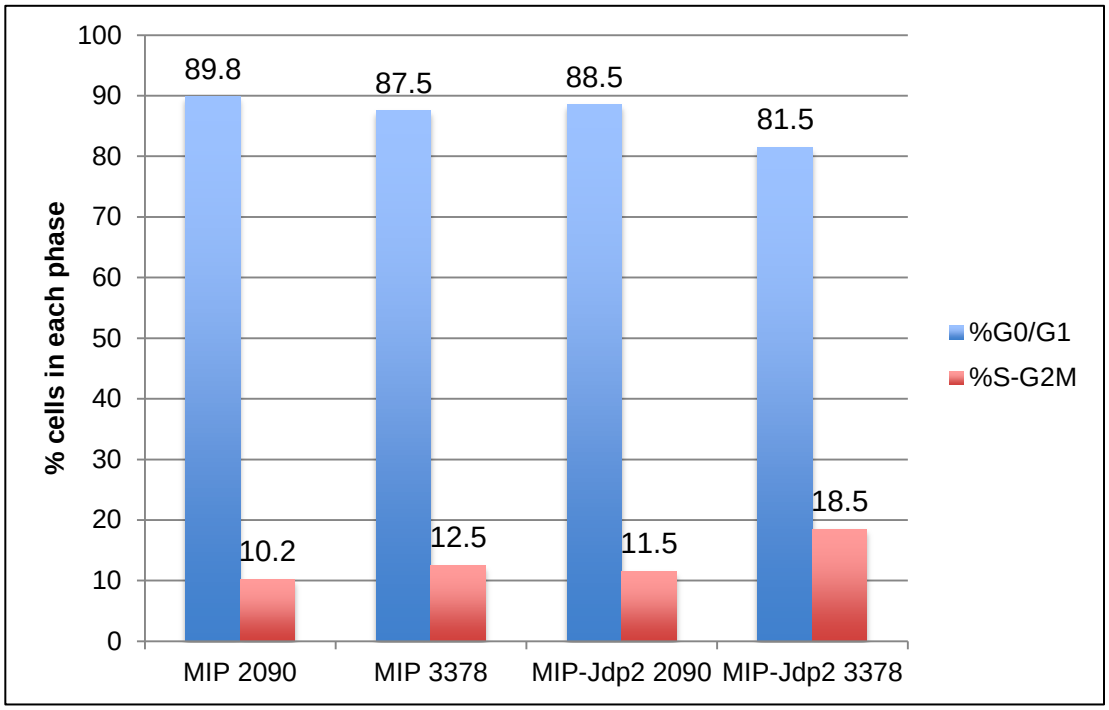


Figure 8 continued

G



H

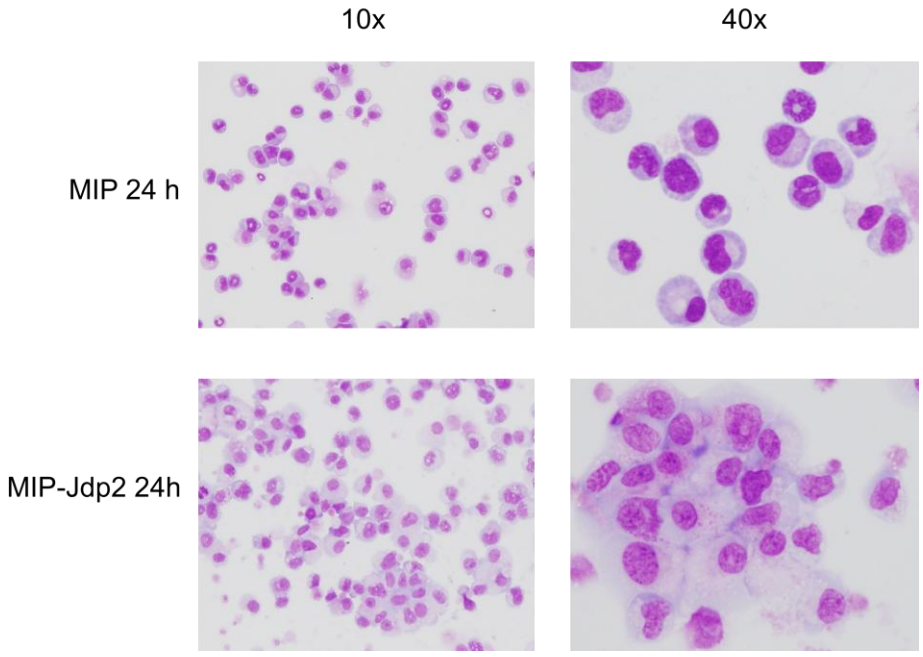


Figure 8 continued

I

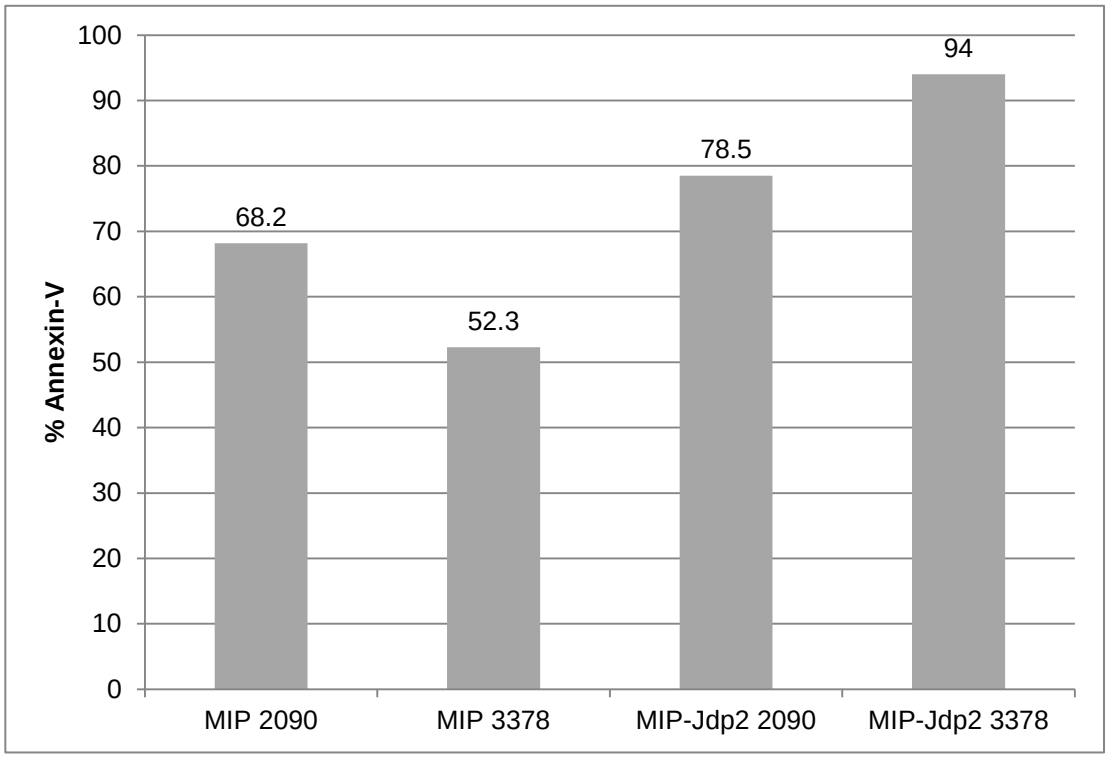


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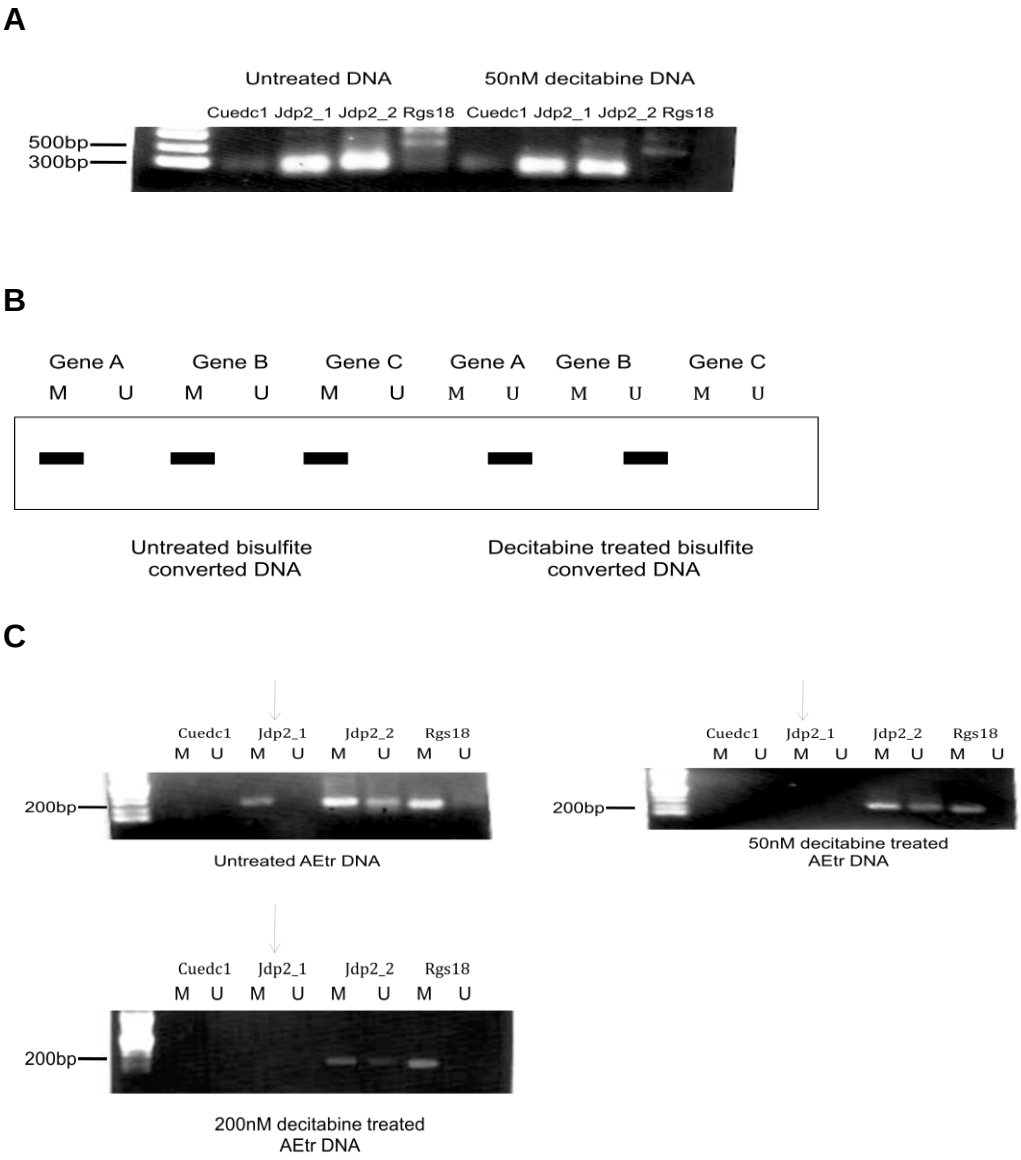


Figure 9: Sodium bisulfite treatment to discern methylation levels of genes in untreated and decitabine treated AEtr AML cells

(A) Bisulfite treated DNA was PCR amplified with bisulfite specific primers for subsequent sequencing.
(B) Schematic of MSP procedure. Genes A, B, and C are methylated in the untreated sample. After treatment with decitabine, the CpG dinucleotides of A and B are completely unmethylated, while gene C is only partially demethylated
(C) Bisulfite treated DNA was PCR amplified with methyl-specific primers. M indicates the primer set specific for DNA that is methylated (and therefore protected during bisulfite conversion). U indicates the primer set specific for unmethylated DNA (therefore unprotected during bisulfite conversion)

IV

Discussion

In this work, I have explored the role of de novo methylation as a factor contributing to the development of AML. I have used previously published data on the methylation profiles of t(8;21) AML patients in conjunction with the results of an AE9a leukemia array to identify genes that could play a crucial role in the development of AML when downregulated or silenced. Using RT-qPCR, I have determined which of those genes have upregulated expression in human and murine AML cell lines after treatment with the DNA hypomethylating agent decitabine (Table 2) and selected four target genes for further study: Cuedc1, Jdp2, Fam134b, and Rgs18. In addition, I have determined the toxic effects of decitabine on both human and murine cells. Next, I have expressed the target genes in murine AML cells to discern the effects of their re-expression, and have determined that Jdp2 leads to the most changes in the cells. Finally, I have laid the groundwork for confirmation of the methylation status of the genes of study.

Genes upregulated after decitabine treatment could affect the development of acute myeloid leukemia

The genes found to be upregulated by RT-qPCR analysis post treatment with decitabine are good candidates for study as their downregulation due to methylation, which is not present in non-leukemic cells, may provide a mechanism for leukemogenesis.[26] The two genes that were upregulated post decitabine treatment in the human t(8;21) translocation containing AML cell lines as well as the non-t(8;21) cell lines, CUEDC1 and JDP2, (Figure 3E) may not be

specifically methylated due to the increased methyltransferase activity recruited by AE, but have been identified as common targets that are dysregulated in human t(8;21) AML as well as mouse AE9a cells.[26] FAM134B and RGS18 are also upregulated by decitabine in one non-t(8;21) translocation containing human AML cell line (Figure 3F). The mode of methylation of these genes in non-t(8;21) related AML may be a promising topic for further study as it remains unclear.

According to the Genome Browser from the University of California Santa Cruz, functions of the studied genes highlight interesting characteristics that could lead to leukemogenesis if silenced. Jdp2 has been found to be involved in regulating gene expression, cell development, and cell growth/proliferation.[26] It is a member of the AP-1 transcription factor family, which regulates UV-induced apoptosis, cell differentiation, and anti-tumorgenesis.[41,42] In addition, Jdp2 acts as a repressor by recruiting HDACs to the Jun promoter.[43] The gene Rgs18 is involved in the G-protein signaling pathway. Its role is to attenuate the signaling activity of the G proteins by acting as a GAP (GTPase activating protein) increasing the rate of conversion of GTP to GDP, thus essentially terminating the signal. In most cases, G protein signaling serves to bolster cell growth and proliferation by modulating the cellular response to signals. The ability for a cell to stop this response is as critical as its ability to start it.[44] The role of Cuedc1 has been implicated in protein trafficking and degradation pathways, as well as a potential tumor marker for early stage cervical cancer.[45,46] Finally, Fam134b has

been found to be a critical gene in regulating the survival of nociceptive and autonomic ganglion.[47]

Re-expression of downregulated genes leads to morphological and functional changes in murine AEtr AML cells.

Full-length cDNA clones were ordered and PCR amplified to add an HA tag. After many trials, including use of a gradient on the annealing temperature and addition of 3%DMSO, Fam134b was still not amplified. I hypothesize that this was due to the extremely high G-C content at the 5' end of the gene. The three remaining genes were ligated into the MIP vector and transfected into 293T cells; gene expression was confirmed via western blot (Figure 8A, 8B). While the sequence was found to be correct, Rgs18 did not appear on the blot. Although a more common occurrence with the use of lentivirus vectors than retroviral vectors, it is a possibility that the MIP vector ejected the gene. Another reason for this could be that a mutation occurred in the HA tag sequence after it was sent for sequencing.

After two days of consecutive infection in AEtr cells and selection in puromycin, the cell cultures were monitored for any changes compared to the empty MIP vector control. Jdp2 caused the most interesting phenotype: cell growth rate was significantly decreased, large clusters of cells could be observed, and large multinucleated cell "balls" with no identifiable features were

also observed (Figure 8C, E). It is possible that the large “balls” are simply cells that have undergone apoptosis. On the cytospin slides, we observe cells with more differentiated morphology (Figure 8H). Apoptosis assay showed the majority of Jdp2 expressing cells were undergoing apoptosis, but the empty control vectors are also undergoing apoptosis. If the apoptosis is checked at 24 or 48 hours, there may be a more significant difference between the control and the Jdp2 expressing cells. The cell cycle analysis did not demonstrate a significant change in cell cycle progression (Figure 8G, I). Although the infection has been repeated three times, a second round of apoptosis and cell cycle analysis is required to confirm these results.

Methylation status of genes needs further work

The methylation studies performed did not provide concrete results, but gave a foundation upon which future work can be conducted. In figure 9A we see that Jdp2 did amplify in the untreated and 50nM decitabine treated samples. Continuance of the bisulfite sequencing procedure will yield useful results. However, due to time constraint and the technical difficulties encountered, I employed a different strategy to examine the methylation status of the genes of interest.

The MSP procedure, although much less definitive, does show potentially promising results for my work. The annealing temperatures were based off of a

previous study, as well as the procedure followed.[40] In figure 9B, we see that the methylated DNA specific primers amplify Jdp2 in the untreated sample, and then the band disappears in the decitabine treated samples. This indicates that the region of interest is methylated, and some level of methylation is altered upon decitabine treatment. Rgs18 shows the methylated DNA specific primer binding in both the treated and untreated samples, which perhaps indicates that the concentration of decitabine used was not sufficient to demethylate all of the CpG dinucleotides. Cuedc1 is not amplified in any of the reactions, which perhaps is due to an inappropriate annealing temperature. Interestingly, a second sequence of Jdp2 that was tested amplified with both methylated and unmethylated primers throughout all treatments.

Looking Forward

The results of a few experiments would benefit from being repeated. Among these, the first is the RT-qPCR that was conducted with both the low concentration decitabine treated Kasumi-1 cDNA, and the low concentration decitabine treated murine AEtr cDNA. The experiments were repeated 2 to 3 times each, but the results indicated that the quality of the cDNA used had degraded, most likely due to repeated freeze and thaw damage. For this reason, a freshly prepared RNA and cDNA samples would be the best way to confirm gene expression.

Next, adding an HA tag to Fam134b via PCR amplification may not be a feasible method, but other methods may be worth exploring as not many studies have been conducted regarding the role of Fam134b in leukemia/cancer. Sequencing the MIP-Rgs18 vector again may also be beneficial as it could help identify the reason why the anti-HA antibody could not detect the protein

Finally, further work in determination of the genes of interest's methylation status is required. For the bisulfite sequencing to work, it may be beneficial to run a PCR for each gene on a gradient to determine if a better annealing temperature is achievable. The temperature used, 61°C, was chosen based on a gradient ran with Jdp2 primers but that may not be the most ideal temperature for the other two primer sets. Another careful attempt at ligation and cloning the amplified products, once obtained, will allow for sequencing to be completed giving us results from which the methylation status of the genes can be confirmed.

For the MSP protocol, it is interesting to note that only Cuedc1 and Jdp2 were upregulated in the low concentration decitabine treated AEtr RT-qPCR experiment, while Rgs18 and Fam134b were not (Figure 7). If these results can be confirmed, it would suggest that the low level of decitabine is sufficient to demethylate Jdp2, but not Rgs18.

IV

Materials and Methods

Cell Culture

AEtr cells were cultured in IMDM medium containing 20% horse serum, 10% IL3 conditioned media, 10% SCF conditioned media, 1% penicillin/streptomycin, 1% glutamine, and 20 ng/mL human IL6 cytokine. The cells were cultured in a 37°C incubator with 5% carbon dioxide. The cell line was established as previously described.[26] 293T cells were cultured in DMEM medium containing 10% bovine calf serum, 1% penicillin/streptomycin, and 1% glutamine in a 37°C incubator with 5% carbon dioxide. SKNO-1, Kasumi, U93T, and HL60 cells were cultured in RPMI medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine in a 37°C incubator with 5% carbon dioxide.

Western Blot

Cell samples were washed in PBS and resuspended in RIPA buffer. Cell lysates were sonicated and protein concentrations were measured using a BioRad Protein Assay. Protein samples were diluted to 1 μg/μL in sample buffer containing 160mM Tris (pH 6.8), 6.4% sodium dodecyl sulfate (SDS), 32% glycerol, 0.032% bromophenol blue, and 20% b-mercapthenol. After being resolved by 10% SDS-PAGE, the proteins were transferred to a nitrocellulose membrane. The membranes were incubated with 5% dry milk in TBS-T (0.1% Tween 20) for 30 minutes at room temperature and then washed with TBS-T and incubated with primary mouse antibodies targeting HA overnight at 4°C in 1%

milk/TBS-T (1:2000). Membranes were then washed with TBS-T and incubated with HRP- conjugated secondary antibodies (1:4000) in 1% milk/TBS-T at room temperature for one hour. Membranes were washed with TBS-T and incubated with ECL reagent for one minute. The blots were visualized on Blue Devil film.

Quantitative RT-PCR

RNA was obtained from cell samples with Qiagen's RNeasy Mini Kit. The RNA was used for cDNA synthesis using Invitrogen's Super Script III Reverse transcriptase with random hexamers. For each PCR reaction, 1µL of the cDNA was used. The PCR was performed on a BioRad CFX Connect using KAPA 2X SYBR green qPCR mix with 0.5µM of each forward and reverse primer. The reaction cycled 40 times through 95°C - 60°C - 72°C for 30 seconds each. GAPDH was included for normalization. The size of the PCR products and specificity of the primers was confirmed on 1% agarose gels. All forward and reverse primers used are listed in Supplemental Table 2 & 3.

Plasmids

MSCV-IRES-EGFP (MigR1) and MSCV-IRES-puromycin (MIP) have been described previously.[5,35] The mouse CD45.2 ORF clones in the pCMV-SPORT6 vector (Clone ID 4976954, 4012135, 3487542, and 30846527) were purchased from Open Biosystems. Then NotI-SalI sticky end fragments containing the CD45.2 ORF were cloned into the Hpa1 site of MIP to generate MIP-CD45.2.

Retrovirus Transduction

The retrovirus production and infection were performed as described previously.[15] To infect AEtr cells, the ecotropic packaging vector pIK6.1MCV.ecopac.Utd was co-transfected with MSCV retroviral constructs into 293T cells using the PEI method. Retroviral supernatant was collected 48 hours after transfection and added to the AEtr cells ($5x10^5$ cells/well in a 6 well plate) with 4µg/mL polybrene. Cells were then centrifuged at 1400xg for 3 hours at 32°C. A second infection was conducted the next day with freshly prepared virus supernatant (made same as before). Cells were then cultured in 4µg/mL puromycin for 72-96 hours to select for the transduced cells.

DNA Methyltransferase inhibitor treatment of leukemia cell lines

U937, HL60, Kasumi-1, and SKNO-1 cell lines ($2x10^5$ cells/mL) were treated with Decitabine (50nM, 200nM, 500nM, 1µM, 2µM, 4µM, 5µM, and 8µM depending on experiment) for 24-96 hours. Controls were untreated cell samples. AEtr cells were treated with Decitabine (50nM, 200nM, 500nM) for 24-96 hours with untreated cell samples as controls.

Flow cytometric analysis

Flow cytometry was performed with data acquisition done using a BD FACSCanto flow cytometer with the FACSDiva software. Annexin V-PE was

used for apoptosis assays. Propidium iodide staining was used for cell cycle analysis.[36]

Supplemental Table 1. AE9a leukemia array (downregulated) and ChIP-chip common targets.[26]

Gene Symbol	Probeset	Fold Change[a]	Intensity (AE9a)	Intensity (WT)	ChIP-chip	Human CD34, Cell line or AML blast array data source[b]	Wouters AML[c]
*HMHA1**	1428242_at	-3.1	258.3	812.4	HA, AML1	Alcalay, Gardini	+
*SLCO3A1**	1418030_at	-19.6	22.8	444.7	HA, AML1	Alcalay, Tonks, Gardini	+
	1434537_at	-7.9	13.6	107.1			
	1448918_at	-21.7	18.1	390.2			
HSPA1A/HSPA1B	1427127_x_at	-10.5	24.7	259.2	HA, AML1	Gardini	+
PARP8	1451474_a_at	-37.0	10.7	394.2	HA, AML1		+
PRKCH	1434248_at	-8.9	62.2	554.9	HA, AML1		+
PTPRC	1422124_a_at	-200.0	8.8	1758.8	HA, AML1		+
RNF144A	1438404_at	-7.9	43.2	339.9	HA, AML1		+
AAGAB	1424016_at	-2.5	198.8	487.1	HA, AML1		+
FUT8	1460319_at	-3.4	382.3	1295.2	HA, AML1		+
CUEDC1	1435358_at	-6.3	18.8	118.5	HA, AML1		+/-
	1451447_at	-7.9	17.9	140.6			
JDP2	1450350_a_at	-8.9	13.9	124	HA, AML1	Gardini	+
*LY86**	1422903_at	-90.9	8.7	788.9	HA	Alcalay, Gardini	+
*PSD4**	1455067_at	-3.9	59.2	228.7	HA	Alcalay, Gardini	+
	1455068_at	-7.9	49.7	390.2			
*SSX2IP**	1448743_at	-23.8	12.4	296.6	HA	Ross, Gardini	+
*IRF2BP2**	1433634_at	-2.6	743.8	1970.1	AML1	Alcalay, Gardini	+/-
EVL	1434920_a_at	-43.5	7.7	333.2	HA	Alcalay, Gardini	+
FLNA	1426677_at	-4.2	237.7	1000.2	AML1	Alcalay, Lee	+

Supplemental Table 1. continued

ITGA2B	1417758_at	-5.0	130.1	649.3	AML1	Tonks	+
WHSC1	1428827_at	-2.2	176.5	384.1	HA	Alcalay	+
CPT1A	1460409_at	-2.6	297.8	786.8	HA, AML1	Alcalay	+/-
FAM134B	1424683_at	-52.6	7.7	398.8	HA	Alcalay, Gardini	+/-
	1450505_a_at	-7.6	15.5	117.7			
H2AFY	1424572_a_at	-2.6	2562.4	6775.9	AML1	Lee	+/-
IDH1	1419821_s_at	-4.2	58.5	245.8	HA	Alcalay	+/-
RGS18	1449856_at	-11.5	52.9	611.1	HA, AML1	Gardini	+/-
*CYFIP2**	1449273_at	-7.7	37	283.6	HA, AML1	Alcalay, Gardini	-
*PCK2**	1425615_a_at	-1.9	556.6	1034.2	HA	Alcalay, Gardini	-
*SATB1**	1416007_at	-22.7	9.7	218.2	AML1	Alcalay, Gardini	-
	1416008_at	-29.4	10.7	312.4			
ANKRD28	1454801_at	-2.6	200	518.1	HA, AML1	Alcalay	-
ARHGEF6	1429012_at	-14.9	35	521.9	AML1	Alcalay	-
GSPT2	1418109_at	-12.5	16.9	210	HA	Alcalay, Gardini	-
HSP90B1	1415889_a_at	-1.9	1119.9	2128.9	HA, AML1	Lee	-
MAPKAPK2	1426648_at	-8.1	128.1	1036.6	HA	Alcalay	-
MYCN	1417155_at	-12.7	9.7	123.3	HA, AML1	Tonks	-
NPHP3	1454647_at	-1.8	236.1	421.1	HA	Alcalay	-
PDLIM1	1416554_at	-3.2	557.7	1803.5	HA, AML1	Tonks	-
SMARCD2	1448401_at	-1.9	572.1	1082.2	HA	Alcalay	-
SORL1	1426258_at	-8.7	28.2	244.6	HA	Alcalay, Gardini	-
TMEM229B	1454632_at	-5.2	179	934	AML1	Alcalay	-
	1457254_x_at	-2.9	97.1	278.5			

Supplemental Table 1 continued

ATP6AP1	1449622_s_at	-1.6	728.5	1130.3	HA		+
BCL7A	1428207_at	-1.6	207.7	332.7	HA		+
CD68	1449164_at	-8.8	31.8	279.1	AML1		+
GNA11	1449144_at	-2.1	149.4	311.1	HA		+
MEIS1	1450992_a_at	-25.0	8.6	213.5	HA		+
PLXNB2	1416683_at	-13.3	17.3	230.1	HA		+
SLC44A1	1433645_at	-23.3	10.6	244.2	AML1		+
SPNS3	1429140_at	-3.4	128	440.8	HA		+
ATP10A	1447272_s_at	-16.7	10.2	171.3	HA		+/-
	1447851_x_at	-10.8	10	107.6			
CCDC88C	1427138_at	-2.2	92.9	203.8	AML1		+/-
CFP	1452279_at	-16.9	16.8	283.2	AML1		+/-
CNKSR3	1433983_at	-29.4	14	414.7	HA		+/-
CPNE2	1424831_at	-5.5	147.6	816.5	HA		+/-
CSAD	1427981_a_at	-1.8	65.1	119.3	HA		+/-
HADHA	1452173_at	-1.5	739.3	1139.6	HA		+/-

a Fold changes in the mouse AE9a leukemia array.[26]
b Genes found dysregulated in microarray studies using human CD34+ cells and cell lines transduced with AML1-ETO or t(8;21) AML blasts are indicated. Array data source: Alcalay (Alcalay et al., 2003 J. Clin. Invest. 112: 1751-1761), Ross (Ross et al., 2004 Blood 104: 3679-3687), Lee (Lee et al., 2006 PNAS 103: 1030-1035), Tonks (Tonks et al., 2007 Leukemia 21: 2495-2505), Gardini (Gardini et al., 2008 PLoS Genetics 4: e1000275).
c Gene expression patterns were examined in one large-scale AML profiling study (Wouters et al., 2009 Blood 113: 3088-3091) through Oncomine (Compendia Bioscience, Ann Arbor, MI). + indicates t(8;21)-specific up- or down-regulation (the same direction as in AE9a leukemia array) when compared to other karyotypes. - indicates no significant difference between t(8;21) and other karyotypes. +/- indicates a difference was seen in selected probesets.

* Genes that are also AML1-ETO ChIP-chip targets in U937 cells expressing the fusion protein (Gardini et al., 2008 PLoS Genetics 4: e1000275).

Supplemental Table 2: Murine AEtr RT-qPCR primers

Primer Name	Primer Sequence (5’ -> 3’)
Slco3a1_F1	TCTGCTCCTTCGTTTGTTGG
Slco3a1_R1	CGTGGTGGTGTAGAGGATGA
Hspa1a/Hspa1b_F1	TTCCAGTAGCCTGGGAAGAC
Hspa1a/Hspa1b_R1	CCACAAAACCTTAACATGGACA
Parp8_F1	GACGAGCCAGCTTCCAGTAG
Parp8_R1	TTCGGGACAACCCATATCTC
Prkch_F1	AGGTTGGACACCACGACAA
Prkch_R1	GGACTTTCCCAAAACGTGAA
Ptprc_F1	AAAAACAGGGGCATGAAAAC
Ptprc_R1	GAGTAAACAAAACAGTGGGGAAA
Rnf144a_F1	CCCTCACAATGAACCCAAAC
Rnf144a_R1	CAAGTCAGGAAGGCTGGAGA
Aagab_F1	CTGAGAGCTGGCCTGACTTA
Aagab_R1	TACCACCATCACCAGGATCA
Fut8_F1	GATAGACACGCTCCACACCA
Fut8_R1	GGGGCAAAGTAGAAAATAATCC
Cuedc1_F1	CGTGAACCCTGGAGAGAAAG
Cuedc1_R1	CACACACTGGAGGGAAAGAA
Jdp2_F1	CCAACTGCACACGGTTTTT
Jdp2_R1	CCTTAACTGGTGGGTGGATG
Ly86_F1	GACCAGCCCAAGTTCTCATT
Ly86_R1	TCAGGAGGAGGTGACAGTGG
Psd4_F1	TAGGGCTGTGTCTGTGAGGA
Psd4_R1	ATTGGAAGGGAGGATGTGTG
Psd4_F2	CCGCCTACACTTCCCTTTG
Psd4_R2	TGAGATGTTGCGTTTCACCTT
Ssx2ip_F1	GCATTGGGTTGAGATATTTGG
Ssx2ip_R1	TCTTTAGTAGAGGGATCTAGTTGAAAA
Irf2bp2_F1	TCCAAGACAAGGGAAATGTAGAC
Irf2bp2_R1	TCACCCAAAGAGTCAAACACA
Evl_F1	GGGATCAGCACCACGTAAGA
Evl_R1	TTAAGTACATCCCAGCTTCTGC
Flna_F1	GTGGTCAAGTGGGGTGATG
Flna_R1	GGGCTCAGGAAAGGAAAATG
Itga2b_F1	TACGGTGGTGGAGTGTGAG
Itga2b_R1	CCGAGTAAGGTAGGGAGGAGA
Whsc1_F1	ACCTCTGCCCCAACTCATTC
Whsc1_R1	CGCCTTTTCTTCCTCTTTCC
Cpt1a_F1	CCCACCCAGTCAGATTCCA
Fam134b_F1	GCCAAATGTAAGAGGGAGAAAG
Fam134b_R1	CCAAAATGGGCAAGAAAATG

Supplemental Table 2 continued

Fam134b_F2	CTGAGGAGGGGGATGACTTT
Fam134b_R2	TGGGTGGTTTATCTGTGCTTC
H2afy_F1	ATCCATCGCCTTCCCATC
H2afy_R1	ACCTATGCTCTCACTGTCAAAAA
Idh1_F1	AACTGTCATGTTTTCTCATGCTCT
Idh1_R1	ATTGTTCACCCTTGGAGCAC
Rgs18_F1	CTGAGACTTCACTTTGCCATC
Rgs18_R1	AGCACTGGTTGGTGTTGTTT
Cyfip2_F1	AGGTTTTTGCCATCCTGAAC
Cyfip2_R1	CTCTCCTCCTCCTCCTCCTC
Pck2_F1	CTTTGGACGCTACCTGGAAC
Pck2_R1	TTGGGCACTGTCTTCTCCTT
Satb1_F1	CAGACGTTAATGCCGACTTG
Satb1_R1	TGTCCTTCCAACTTCCTTCCT
Ankrd28_F1	TGAGCAACCCACTTCCTTT
Ankrd28_R1	ATACACAACCGACCCACCAG
Arhgef6_F1	GGAATAACAGTAGCAACCCACA
Arhgef6_R1	ATAAGGGCAAAGTCCCCAAA
Gspt2_F1	TTTCCTCAGATGGGTCGTTT
Gspt2_R1	CTGCAAAATATGGGCACAAG
Hsp90b1_F1	GGTGGAGGAAGAACCAGAAGA
Hsp90b1_R1	CACGGGATTCATAGCGAGAG
Mapkapk2_F1	CCTCTGCTTGCCCTTCTTG
Mapkapk2_R1	CGTGGTCCTGCCCTTGTC
Mycn_F1	AGTTCCAGCAAATGCCACA
Mycn_R1	ACAAAACATGCCCCTCAAAC
Acad11_F1	CGGGGCACACTCTTGACC
Acad11_R1	GGCAGTTATGGGGTTTCCT
Pdlim1_F1	GGCATCAACCTGAAACAGAAG
Pdlim1_R1	GCTGGAGGAGGGCAGTAGA
Smarcd2_F1	TCCTGGCCTTTCTAACCAAA
Smarcd2_R1	AGGAAGAAGGAGGCACCAAG
Sorl1_F1	GTGGTTCCCATCCTGTTCCT
Sorl1_R1	GCATCTTCATCGTCCTCTCC
Tmem229b_F1	CCCAAGGTAGGAGTTTCAGATAGTAAG
Tmem229b_R1	GTGATGGAGCAGGACAAAGG
Tmem229b_F2	GAACCCACCCTTACCTGGAG
Tmem229b_R2	ACCTTTCTCAAGAGCTGTCCA
Satb1_F2	TTGCTTTCCCATCCTGATTT
Atp6ap1_F1	GTCTCCCCTTTACCCTCTCTC
Atp6ap1_R1	GTCTTTCCACACCTCGCAAA
Bcl7a_F1	CCACGGTCCTCTCTCTCTCT

Supplemental Table 2 continued

Bcl7a _R1	TCAAGTGTTGGGGGAAAGTT
Cd68_F1	ACATCAGAGCCCGAGTACAG
Cd68_R1	TTGCATTTCCACAGCAGAAG
Gna11_F1	TCGTCTGTCATTCTCTTCCTCA
Gna11_R1	TCAGGGTTCAGGTCCACAAA
Meis1_F1	GGGCTTGAATTTGCATGTCT
Meis1_R1	TGCCTCCTCCTACCAGTCTC
Plxnb2_F1	TCGGGTGTTCTCTGTGTTGT
Plxnb2_R1	CCGCTTTTTCTTCTCCTTCC
Slc44a1_F1	AAAGAAGGTCCCCCAAAGAA
Slc44a1_R1	GAACAAGGCGATGGTGAGAG
Spns3_F1	GCTTCCTGTGTTGTGCCTTC
Spns3_R1	ACTCTGTTGGGGTGCTGGT
Atp10a_F1	GGCTGGGAAGTGTCTTGC
Atp10a _R1	CAGGGATTTCTTTGTCACCA
Ccdc88c_F1	TTTCAACCTCAGCCACCAC
Ccdc88c_R1	GCGTTCCTACTGCTCTCCAA
Cfp_F1	GTCACGTTCAAGGAGCTGTG
Cfp _R1	TGGGACTACATGGTGGTGTG
Cnksr3_F1	GCAACCAGAGTTAGAGTTATGAGG
Cnksr3_R1	CAGTGACGATGAGGCTTTCC
Cpne2_F1	TCATCATCGTGGGTGTGG
Cpne2_R1	TTTAGCCAGCGTTTCTTTTG
Csad_F1	CAGGCCGATATAGATTTCCTTC
Csad _R1	TGGGAGCACACACAGTCAA
Hadha_F1	CTTCCGCTTTGTGGATCTGT
Hadha _R1	GGTCTGGGTTAGTGCATGGT

Supplemental Table 3: Human RT-qPCR Primers

Primer Name	Primer Sequence (5’ -> 3’)
HMHA1_F1	TGGGCACCTTGCAGACCCAGA
HMHA1_R1	AGCCTTGTCGTGGTCCTCGC
SLCO3A1_F1	ACATCGACGACCACGTGCGG
SLCO3A1_R1	CCCCACCAGGCTCCGATCCA
HSPA1A/HSPA1B_F1	TACCAGGGTGCCGGTGGTCC
HSPA1A/HSPA1B _R1	AGCAGCAAAGTCCTTGAGTCCCA
PARP8_F1	GGAACGTGCCAGTTGATTCTGACCA
PARP8_R1	ACAGGCAGTCCTTCTCAGCTCTGG
PRKCH_F1	GGTCACGCGCCTTTTGAGGCA
PRKCH_R1	GCGTGCTCGCCTCCCTGAGT
PTPRC_F1	GCATTCTCACCCGCAAGCACC
PTPRC_R1	GTCTGCGAGTCTGCGTGCGT
RNF144A_F1	GCGCTGCCCCAAGTGCAAAG
RNF144A_R1	CGGGAGTGGCCCAGCTTGTT
AAGAB_F1	TCTCTGATCATCGGGGTGGTGC
AAGAB_R1	AGGAAGCGTCGCAGCCTTGT
FUT8_F1	GGCAGCCCTTCGGTCCACTG
FUT8_R1	GCGCTCTACATGCTGAATTTGTCCA
CUEDC1_F1	GCAGCGGCGGCGTCTATGAG
CUEDC1_R1	CGTCGATACGGGGAGGCGGA
JDP2_F1	TTCCTTTTGGTTCCCGCCCCG
JDP2_R1	CCGGGATCTGCCCAGGCATC
LY86_F1	TGTGAGGCGGCTCTGCCCAA
LY86_R1	AGGCCACGGTGGACCGTTTT
PSD4_F1	CCCCGCCACGCATTACACCA
PSD4_R1	CGCACGAATCTGCGCTGGGA
SSX2IP_F1	TCAGCGCCCCGTTGTTCGG
SSX2IP_R1	GCTGCTGTCACTCTTCTATGTAGGC
IRF2BP2_F1	TGTTGGGTTTCGAGGCCAACGG
IRF2BP2_R1	TGAGCCCCTCTGTGGATGTGGA
EVL_F1	TGGGAGCGGAGCAACTCGGT
EVL_R1	AGTCGAAGGCATCCAGGGCCA
FLNA_F1	GTGGCCTACGTGCCAGACGTG
FLNA_R1	TGAATGGTGGGGCCGATGCC
ITGA2B_F1	CCCCGAGGGGAAGCTCAGGT
ITGA2B_R1	TTCCAGGGGTGGCCGGTTCC
WHSC1_F1	GTGGTGGCACGAGCTGTCTGC
WHSC1_R1	TGCAACGCACAGGATGCCCG

Supplemental Table 3 continued

CPT1A_F1	AGTACGTGTCCAGCGGAGGGG
CPT1A_R1	CAGGTGCCTTCCAAAGCGATGA
FAM134B_F1	GGCTTCGTCGCTGCCAACCT
FAM134B_R1	GGCTTCTCCACAACTGTGCACCTC
H2AFY_F1	AGGCAAGAAAGGGGCCCGGA
H2AFY_R1	TGTCGGGTGAACGACAGCATCA
IDH1_F1	ATCCCCCGGCTTGTGAGTGGA
IDH1_R1	TCCCCATGGCAACACCACCA
RGS18_F1	AGTCTCCCCTGAAGAGGCAGTGA
RGS18_R1	GGTGAATTTGTTGAGGTCCCTTGCT
CYFIP2_F1	GGCCGTGAGGATGCGCGAAG
CYFIP2_R1	CTCCCGGGCAGAGGGTCCGT
PCK2_F1	GCTGAGACCAGTGATGGTGGCG
PCK2_R1	GGCGAGCCGGGGCACAAAAT
SATB1_F1	CACCATGGCTCGGTGCTGCTC
SATB1_R1	TGGCCGGGTCTTCTGTCGGTT
ANKRD28_F1	TCCTGCTAGAGGAAGTTACATCCCG
ANKRD28_R1	TGCGGCGTGCAATGGGGTT
ARHGEF6_F1	AGACCATCTGTGATCCGGAGGAG
ARHGEF6_R1	TGGAGGGTTGCACATCCTTTCAGG
GSPT2_F1	CGCGGTGCAGCTAAGGTTCGT
GSPT2_R1	GCTGCTGCTGCCCGAATCCAT
HSP90B1_F1	GCGTACCAAACGGGCAAGGACA
HSP90B1_R1	ACCCTGACCGAAGCGTTGCT
MAPKAPK2_F1	TGCTGAAAACAGAGCCCACCCA
MAPKAPK2_R1	CAGCCAAGTGGCCTGGTCGC
MYCN_F1	AGACACCCGCGCAGAATCGC
MYCN_R1	GTGGAGCAGCTCGGCATCGG
NPHP3_F1	GGCCAAGAGGGAGTTGGAGAGC
NPHP3_R1	ACTTCCCAAGGCTCCGCCAGT
PDLIM1_F1	TGCCATGCCCTTTACCGCCT
PDLIM1_R1	AGGCTGCTTGGAGGCTGAGCA
SMARCD2_F1	ACCTCAGCGAATCCGGGAGCTT
SMARCD2_R1	ATCGCCTTCCGCCTTGCTGG
SORL1_F1	TGGCATTGGCGAGGCCCAAG
SORL1_R1	ACCGCTTGTTGTCCGCAGGG
TMEM229B_F1	GCACTGAGGCCCAGCCAACAA
TMEM229B_R1	CCGCTGTGAACATCACCTCGCA
ATP6AP1_F1	CCCCTTGCATGTGGACCTGGC

Supplemental Table 3 continued

ATP6AP1_R1	AGGGCCGCTGTGTATGGGACA
BCL7A_F1	AGATCGGCCGGGAGCCCATT
BCL7A_R1	CGCACCCAAGAGGAATCGCTGG
CD68_F1	GCTGGACTTTGGGTGAGGCGG
CD68_R1	CGTGGGTGTCACCGTGAAGG
GNA11_F1	GCGGATCAACGCCGAGATCGAG
GNA11_R1	GAAGCCGCGCTTGTCCTCCTC
MEIS1_F1	ACGTTGCTGGAGACGTTAAGGGA
MEIS1_R1	TGCCCCCGTAATGGGGTAGATCG
PLXNB2_F1	TCGATGCTGGCCTGGAGCAGAA
PLXNB2_R1	CGCTGCGGAAGAAGTCCAGCTT
SLC44A1_F1	TGTAGCAGCGTGTCCAAGGCA
SLC44A1_R1	AGGTGCACTCGCTGGAACTGGT
SPNS3_F1	GCCGCCGTCCTCTGCTACAT
SPNS3_R1	CGGCTATGTCGGTCGCCCAG
ATP10A_F1	CAGTCGTCGTCACGTCCCCG
ATP10A_R1	TGGCTGGACTTGTTGGCGGC
CCDC88C_F1	TGGGCTCCCGGACTTGCTCA
CCDC88C_R1	AGCTACTTGGCCGCGACACG
CFP_F1	ACACCCCCACGAGGTTGACCC
CFP_R1	ACCAGAGAGGAGGTCCCGCTT
CNKSR3_F1	CGGCCTTTGTCCATGCCTGCT
CNKSR3_R1	CCAGATGCCGCTCGGTCGTG
CPNE2_F1	CGTGTGCAAGGTGGAGCTGTCA
CPNE2_R1	TTGGAGAAGGCGGGGTTGAGGT
CSAD_F1	TGGACTGCAAATGCGCTTGATGGA
CSAD_R1	CCAAACACGGCCCGGAGCAA
HADHA_F1	AGCAGTTGGTCTCAAGCAGGGGA
HADHA_R1	GTGGCGGCACCCACAGGAAA

Supplemental Table 4: Primers for PCR Amplification of cDNA clones with HA tag

Primer Name	Primer Sequence (5’ -> 3’)
Cuedc1 F_HA and BglII	AAAAGTCGACATGGCATACCCATACGACGTGCCTGACTACGCCTCCACCAGCCTGTTTCGCAGGAG
Cuedc1 R_SalI	AAAAGTCGACTTACTGTCCTTCTCTTAGGGCCTC
Jdp2 F_HA and XhoI	AAAACTCGAGATGGCATACCCATACGACGTGCCTGACTACGCCTCCATGCCTGGGCAGATCCCAGA
Jdp2 R_EcoRI	AAAAGAATTCTCACTTCTTGTCCAGCTGCTCC
Fam134b F_HA and SalI	AAAAGTCGACATGGCATACCCATACGACGTGCCTGACTACGCCTCCGCGAGCCCGGCGCCCGAG
Fam134b R_EcoRI	AAAAGAATTCTTAATGGCCTCCAAGCAGATTGG
Rgs18 F_HA and BamHI	AAAAGGATCCATGGCATACCCATACGACGTGCCTGACTACGCCTCCGATATGTCACTGGTTTTCTTCTCTC
Rgs18 R_XhoI	AAAACTCGAGTCATAACCAAATGGCAACATCTG

Supplemental Table 5: Bisulfite Sequencing PCR Primers

Primer Name	Primer Sequence (5’ -> 3’)
Cuedc1_F1	TGATGTTTTTAAGTTTAGAGAGGTT
Cuedc1_R1	ACTTATAATCTTCCCTTTATAACCC
Jdp2_F1	GTTTTTGAGGAGGGTGAGAATATT
Jdp2_R1	ATAAACTCTAACCAAACCTCCCAC
Jdp2_F2	AGAGGGAGAGTAGGGAGTTTTAAGA
Jdp2_R2	AAACAACACTACCAAACAACCTCTAA
Rgs18_F1	GAGATTGTGGGAATGGTTAATTAAT
Rgs18_R1	ATTCCTATCTATCACCCTTTTTATC

Supplemental Table 6: MSP PCR primers

Primer Name	Primer Sequence (5’ -> 3’)
Cuedc1_F1 Methylated	ATAGTTTTTATTAGTGGTTTTTCGT
Cuedc1_R1 Methylated	TATAATCTTCCCTTTATAACCCGAT
Cuedc1_F2 Unmethylated	TATAGTTTTTATTAGTGGTTTTTTGT
Cuedc1_R2 Unmethylated	TATAATCTTCCCTTTATAACCCAAT
Jdp2_F1 Methylated	GCGTTTCGGGTTATATGTAAC
Jdp2_R1 Methylated	AAACACTTATCTCTACTAAATCTCGAC
Jdp2_F2 Unmethylated	GTGTTTTGGGTTATATGTAATGT
Jdp2_R2 Unmethylated	AACACTTATCTCTACTAAATCTCAAC
Jdp2_F3 Methylated	GAGTTTAGGGTAGTTTTGTAAGAGC
Jdp2_R3 Methylated	CTTAAAACCACAAAATCAAACGAT
Jdp2_F4 Unmethylated	GTTTAGGGTAGTTTTGTAAGAGTGG
Jdp2_R4 Unmethylated	CCTTAAAACCACAAAATCAAACAAT
Rgs18_F1 Methylated	AGTAAGTTTATTAAAATTAAAGGCGG
Rgs18_R1 Methylated	CACTCATCCGAAAAAAACGTA
Rgs18_F2 Unmethylated	GTAAGTTTATTAAAATTAAAGGTGG
Rgs18_R2 Unmethylated	TATCCACTCATCCAAAAAAAACATA

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