The knock-down of Death inducer-obliterator1 gene (*DIDO1*) would promote apoptosis and inhibit proliferation of leukemia induced Endothelial cells by inhibiting the expression of *CDK6* and *CCND1*

Honghua Cao [Equal first author, 1], **Lilan Wang** [Equal first author, 1], **Chengkui Geng** [2], **Man Yang** [3], **Wenwen Mao** [4], **Linlin Yang** [5], **Yin Ma** [1], **Ming He** [1], **Yeying Zhou** [1], **Lianqing Liu** [1], **Xuejiao Hu** [1], **Jingxing Yu** [6], **Xiufen Shen** [7], **Xuezhong Gu** [8], **Liefen Yin** [Corresp., 6], **Zhenlei Shen** [Corresp. 1]

[1] Department of Hematology, The third Affiliated Hospital of Kunming Medical University, Kunming, China

[2] Department of Orthopedics, Yan'an Hospital of Kunming City, the Affiliated Hospital of Kunming Medical University, Kunming, China

[3] Department of Endocrinology, the Affiliated Hospital of Yunnan University & the Second People's Hospital of Yunnan Province , Kunming Yunnan 650021, Kunming, Yunnan, China

[4] Department of Geriatics, The second Hospital of Kunming, Kunming, China

[5] Department of Gynecology, The third Affiliated Hospital of Kunming Medical University, Kunming, China

[6] Department of Hematology, The second Affiliated Hospital of Kunming Medical University, Kunming, China

[7] Department of laboratory, The second Affiliated Hospital of Kunming Medical University, Kunming, China

[8] Department of Hematology, The First people hospital in Yunnan province, Kunming, China

Corresponding Authors: Liefen Yin, Zhenlei Shen
Email address: ylfynkm@126.com, szl1020@163.com

Background: Endothelial cells (ECs) provide a fertile niche that allows for the propagation of primitive and aggressive leukemic clones. In this study, we aimed to identify the genes function in the formation of leukemic induced ECs. Methods: HUVEC and K562 cell lines were used to perform the Genichip assay. The annotation of gene function and the interaction network analysis were performed by IPA. Then the RNAi lentiviral vectors were construct and transfected the HUVEC cell lines. Results:711 different expressed probes were identified between K562 and K562-HUVEC co-cultured cell lines. The top 30 down-regulated genes were used to construct RNAi lentiviral vector and transfected HUVEC cell lines, the proliferation of *shDIDO1*, *shZC3H18*, and *shSMURF2* cell lines was significantly inhibited. The expression of DIDO1 was inhibited both at transcript and protein level in shDIDO1 cells, which leads to the induce of cell apoptosis and inhibition of cell proliferation. The expression of cell cycle regulation genes, *CDK6* and *CCDN*, were inhibited in *shDIDO1* cell lines both at RNA and protein level. The public ChIP-seq data were used to analyze the transcription foctors or epigenetic factors that binds with *DIDO1*, the H3K4me3 and PolII (POLR2A) signals were found near Exon1 and exon2 sites. Conclusion: H3k4me3 binds with *DIDO1* and inhibit its expression, the inhibited *DIDO1* in HUVEC will inhibit the expression of *CDK6* and *CCND1*, which will inhibit the proliferation of

cells, and promote the apoptosis of the ECs.

The knock-down of Death inducer-obliterator1 gene (*DIDO1*) would promote apoptosis and inhibit proliferation of leukemia induced Endothelial cells by inhibiting the expression of *CDK6* and *CCND1*

Honghua Cao[1*], Lilan Wang[1*], Chengkui Geng[2*], Man Yang[3], Wenwen Mao[4], Linlin Yang[5], Yin Ma[1], Ming He[1], Yeying Zhou[1], Lianqing Liu[1], Xuejiao Hu[1], JingxingYu[6], Xiufen Shen[7], Xuezhong Gu[8], Liefen Yin[6#], Zhenglei Shen[1 #]

1. Department of Hematology, the third Affiliated Hospital of Kunming Medical University, Kunming 650118, China;
2. Department of Orthopedics, Yan'an Hospital of Kunming City, the Affiliated Hospital of Kunming Medical University, Kunming 650051, China;
3. Department of Endocrinology, the Affiliated Hospital of Yunnan University & the Second People's Hospital of Yunnan Province , Kunming Yunnan 650021；
4. Department of Geriatics, The second Hospital of Kunming, Kunming 650024, China;
5. Department of Gynecology, the third Affiliated Hospital of Kunming Medical University, Kunming 650118, China;
6. Department of Hematology, The second Affiliated Hospital of Kunming Medical University, Kunming 650031, China;
7. Department of laboratory, the second Affiliated Hospital of Kunming Medical University, Kunming 650031, China;
8. Department of Hematology, the First people hospital in Yunnan province, Kunming, 650032, China.

* Honghua Cao, Lilan Wang and Chengkui Geng contributed equally to this work

Corresponding authors：

1. Zhenglei Shen,
   Kunzhou Road, Kunming, Yunnan, 650118, China;
   Email: szl1020@163.com

2. Liefen Yin,
   Dianmian Street, Kunming, Yunnan, 650101, China

Email address: ylfynkm@126.com

Abstract

Background: Endothelial cells (ECs) provide a fertile niche that allows for the propagation of primitive and aggressive leukemic clones. In this study, we aimed to identify the genes function in the formation of leukemic induced ECs.

Methods: HUVEC and K562 cell lines were used to perform the Genichip assay. The annotation of gene function and the interaction network analysis were performed by IPA. Then the RNAi lentiviral vectors were construct and transfected the HUVEC cell lines.

Results:711 different expressed probes were identified between K562 and K562-HUVEC co-cultured cell lines. The top 30 down-regulated genes were used to construct RNAi lentiviral vector and transfected HUVEC cell lines, the proliferation of shDIDO1, shZC3H18, and shSMURF2 cell lines was significantly inhibited. The expression of DIDO1 was inhibited both at transcript and protein level in shDIDO1 cells, which leads to the induce of cell apoptosis and inhibition of cell proliferation. The expression of cell cycle regulation genes, CDK6 and CCDN, were inhibited in shDIDO1 cell lines both at RNA and protein level. The public ChIP-seq data were used to analyze the transcription foctors or epigenetic factors that binds with DIDO1, the H3K4me3 and PolII (POLR2A) signals were found near Exon1 and exon2 sites.

Conclusion: H3k4me3 binds with DIDO1 and inhibit its expression, the inhibited DIDO1 in HUVEC will inhibit the expression of CDK6 and CCND1, which will inhibit the proliferation of cells, and promote the apoptosis of the ECs.

Introduction

Increased angiogenesis is observed in many malignant tumors including leukemia, and it plays important roles in tumor progression. The angiogenesis may be accompanied with the increasement of circulating endothelial cells (CECs) in peripheral blood [1]. It has been reported that the higher level of CECs and endothelial precursor cells (EPCs) was associated with more aggressive disease and shorter survival [2] in chronic lymphocytic leukemia. The increasement of CECs in the peripheral blood was reported in multiple myeloma [3], myelodysplasia [4] and acute myeloid leukemia patients [5].

Endothelial cells (ECs) could originate from bone marrow-derived hemangioma blast progenitor cells [6], as it was found that the fusion BCR-ABL transcript in ECs was derived from bone marrow progenitor cells [7]. Besides, endothelial cells could derive from chronic myelocytic leukemia (CML), and the ECs are involved in sustaining the survival and proliferation of leukemic cells [8]. The ECs may interact with leukemia cells and help maintain each other's activity. The leukemic blasts could secrete numerous cytokines, which will promote the proliferation of microvascular endothelial cells in primary AML (Acute myelocytic leukemia) cells [9]. In addition, ECs could secrete of angiocrine factors, which will take part in the reconstitution of normal and malignant stem/progenitor cells [10]. The ECs could improve the survival and proliferation [11] of AML cells and reduce the chemosensitivity [12].

ECs provide a fertile niche which will promote the proliferation of primitive and aggressive leukemia cells [13]. The activated state of ECs may promote the self-renewal and lineage specific differentiation of hematopoietic stem and progenitor cells. Angiocrine factors expressed by ECs would induce the production of pro-hematopoietic cytokines through Akt and MAPK pathway, thereby balancing the self-renewal and differentiation of HSCs (haematopoietic stem cells) [14]. A better understanding of these mechanisms will help to develop an efficient strategy to therapeutically inhibit the EPCs-mediated tumoral angiogenesis.

MVD has been found to increase in many hematological malignancies, opening the way for anti-angiogenesis therapy. However, although these therapies are effective, they fail to prevent tumor progression in most patients. In future research, it is of vital importance to determine the best targets for these anti-EPC therapies and determine the patient subgroups to be treated.

In this study, we tried to investigate the mechanism of how leukemia cell induces ECs formation. Studies have shown that the human leukemia tumor cell K562 cell line or supernatant can promote the transform of HUVEC to leukemia-related ECs. In this study, human umbilical vein endothelial cells (HUVEC) and human myeloid leukemia K562 cells were used to simulate leukemia and leukemia-induced EC cells. Gene chip analysis was used to mine candidate genes, and RNAi methods were used to analyze the function of genes on leukemia-induced ECs.

Materials & Methods

Cell lines and cell culture

Human umbilical vein endothelial cells (HUVEC) and Human myeloid leukemia cell line (K562) were purchased from the American Type Culture Collection (Rockville, MD). The cell lines were maintained using RPMI (Roswell Park Memorial Institute) 1640 medium (Gibco Co, USA) supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmid constructs and transfection

For gene knockdown, the GV115 vector was used in this study, which used the green fluorescent protein as a reporter gene, and the multiple cloning sites was driven by a human U6 promoter. The *DIDO1* was targeted by the shRNA sequences of 5′- GGATGAGACTCATTCAGAA- 3′. The sequence was cloned into the multiple cloning sites by restriction enzyme of AgeI and EcoRI. Plasmid transfection was performed as a former study.[16] The cell lines were seeded into 96-well plates. After transfection for 2~3 d, the GFP was observed under a fluorescence microscope. The cells were used for further studies when the cell density in the wells reaches 70-90%.

Celigo and MTT assays

Cells were inoculated int the 96-well plates (2000 cells / well) and three repeats were taken. The cell numbers were measured by Celigo Imaging Cytometer [15] and the numbers was recorded for 5 days. The cell numbers were normalized to the cell numbers on the first day after seeding. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [16] was performed to analyze the proliferation of cells. 1 mg / mL MTT was added to each well and incubated at 37°C for 4 hours. Then the culture medium was removed and the DMSO (150 μl) was added into

each well, and then the plate was shaken for 3 minutes. The Tecan Infinite M2009PR plate reader was used to measure the absorbance at 490 nm / 570 nm.

Cell apoptosis analysis

The cell apoptosis was measured following the manufacturer's instructions of Annexin-FITC Apoptosis Detection Kit (BD Biosciences, Franklin Lake, NJ, U.S.A.). Cells were cultured in 96-well plate for 3-5 days in the 37 °C incubator, then the cells were harvested and washed in PBS. Cells were added to 0.5 ml binding buffer and Annexin V-FITC, then the cells were stained in the dark for 15 min at room temperature. Cells stained by Annexin V-FITC were considered as apoptotic cells [17] which were measured by a BD Accuri™ C6 flow cytometer (BD Biosciences). To analysis the cell apoptosis, Caspase 3/7 enzyme activity was measured by Caspase-Glo® 3/7 Assay (Promege, G8091). Caspase-Glo 3/7 reagent was added to the sample with a volume ratio of 1:1, and the cells were incubated for another 1 h at 37 °C. The Tecan Infinite M2009PR plate reader was used to detect the luminescence in each well at 490 nm/520 nm [18].

Angiogenesis analysis

The serum-free supernatants of tumor cells from different experimental groups were collected to stimulate HUVEC to form a lumen on Matrigel, and the effects of tumor cell supernatant secretions of each group on angiogenesis were performed by the Celigo instrument.

Microarray processing and data analysis

The samples were hybridized with the GeneChip microarrays (901838, Affymetrix) to determine gene expression abundance according to the manufacturer's instructions. The expression profile was preprocessed by the Limma package in Bioconductor.

A robust multiarray averaging algorithm was used to perform background correction, quantile normalization and probe summarization on the microarray data to obtain a gene expression matrix. The cut-off for the background correction was 20%, and the coefficient of variation was 25%. The Benjamini-Hochberg method was used to correct the significant difference level (FDR). The screening criteria for significantly different geneswere: |Fold Change|>1.5 and FDR<0.05 [19]. The biological pathways analysis of genes was performed by Ingenuity Pathway Analysis (IPA).

RNA extraction and qRT-PCR analysis

According to the manufacturer's protocol, the Trizol reagent (Invitrogen) was used to extract total RNA from frozen cells. For cDNA synthesis, 1 μg of total RNA was used to synthesed the cDNA by the Go Script reverse transcription system (Promega, Maddison, MA, U.S.A.). The genes were detected by the SYBR Master Mixture (DRR041B, Takara) using LightCycler480 Real-Time PCR system (Roche). For qRT-PCR, the GAPDH gene was used as endogenous control. The primers sequences and the length of the amplifications were shown in Table S1. The $2^{-\Delta\Delta Ct}$ method was used to calculate the fold change for gene expression relative to the control.

Protein extraction and Western-blot analysis

Total protein was isolated from cells using protein cell lysis buffer and extracted by centrifugation at 13000 rpm for 20 min at 4°C. The equal amount of whole cell lysate was separated by SDS-PAGE gel electrophoresis. After the proteins transferred to the PVDF

membranes (Bio-Rad, CA, U.S.A.), the membranes were blocked by 5% skimmed milk and immunoblotted with the primary antibodies at 4°C. Then the membranes were blotted with the secondary antibodies at room temperature for 1h. The following primary antibodies were used: anti-DIDO1 (1:1000, HPA049904, Sigma), anti-CCND1 (1:500, Cat2978, CST), anti-CDK6 (1:500, Cat3136, CST), anti-GAPDH (1:2000, Sc-32233, Santa Curz). The secondary antibodies were anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). The Dyne ECL STAR Western Blot Detection kit (Dyne Bio, Seoul, Korea) and a chemiluminescent image system (Fusion Solo system, Villber Lourmat) were used to analyze the protein abundance.

Statistical analysis

The data was shown as the mean ± S.D. from 3 independent replicates. The Student's t-test was performed to analyze the quantitative data. $P<0.05$ was considered statistically significant.

Results

Gene Chip microarrays analysis of HUVEC and leukemia - educated HUVEC cell lines

Human umbilical vein endothelial cells (HUVEC) are capable to differentiate into endothelial cells in vitro. In this study, HUVEC and K562 cell lines were used to perform the Gene chip. The differentially expressed genes of HUVEC cell lines and K562-educated HUVEC cell lines were analyzed, to determine the genes affecting endothelial cells in leukemia. Compared with HUVEC lines, 398 probes up-regulated expression and 323 probes down-regulated expression in K562 co-cultured HUVEC lines (Figure S1A). IPA was used to analyze the possible biological pathways the different expression genes (DEGs) involved, it was found that ERK5 Signaling was significantly inhibited (Z-score= -2.111) (Figure S1B). On the other hand, the enrichment of DEGs in the classification of diseases and functions was analyzed. It was found that, the DEGs may be involved in microtubule dynamics(Z-score=2.783), migration of brain cancer cell lines(Z-score=2.549), liver tumor (Z-score= -2.782) and cell death of mononuclear leukocytes(Z-score=-2.561) (Figure S1C).

Construction of RNAi cell lines and cell proliferation analysis

We selected the first 30 down-regulated expression genes (log2(change fold)>1, $P<0.05$) for further analysis (Table S2). RNAi lentiviral vectors for these 30 genes were constructed, and transfected into HUVEC cells. 22 transgenic cell lines were successfully obtained, including the negative control (NC) and positive control (PC). The cell count results showed that the proliferation of cells was normal in the NC group, and which was significantly inhibited in the PC group. The proliferation folds on the fifth day were 12.09 and 2.12 times higher than those on the first day in the NC and PC group, respectively. The fold change (FC) of cell count ([FC in NC group on the 5th day compared to which on the 1st day] / [FC in experiment group on the 5th day compared to which on the 1st day]) was used to evaluate the influence of gene RNAi in cell

proliferation. The proliferation of shDIDO1, shZC3H18, and shSMURF2 cell lines was significantly inhibited, and the change fold was 3.37, 2.54, 2.07, respectively (Figure S2) Among them, the proliferation of shDIDO1 cells was strongly inhibited. DIDO1 (Death inducer-obliterator 1) gene is a tumor suppressor gene, which is located on the long arm of human chromosome 20. RT-PCR found that the expression level of DIDO1 gene at the mRNA level was suppressed in shDIDO1 cell lines($p<0.05$), and the reduction efficiency reached 95.1% (Figure 1A). Western-blot detection found that the expression of DIDO1 protein in the shDIDO1 cell line decreased by 4 times, compared with the shCtrl group (Figure 1B).

The proliferation of shDIDO1 cell line is inhibited and the apoptosis is increased

The proliferation rate of shDIDO1 cell line was analyzed by Celigo (Figure 1C and D) and MTT (Figure 1E), it was found that the cell proliferation rate of the shDIDO1 gene knockdown group was significantly inhibited. This may indicate that the *DIDO1* gene is significantly related to the proliferation ability of HUVEC cells.

The number of cells in apoptotic state was detected by Annexin V-APC single staining method, and it was found that apoptosis cells in shDIDO1 group increased significantly than the HUVEC cells ($P<0.05$) after 5 days (Figure 1G). Additionally, by detecting the activity of caspase, it was found that the activity of caspase3/7 in the shDIDO1 group was significantly increased. These results indicate that the *DIDO1* gene was significantly related to the apoptosis of HUVEC cells (Figure 1H).

Due to the importantance of angiogenesis in tumor progression, we analyzed the effect of *DIDO1* gene depletion on angiogenesis. The ability of shDIDO1 HUVEC cells to form lumens was analyzed to investigate the metastasis ability of tumors. It was found that, the area of angiogenesis-related blood vessels in the shDIDO1 group was 20% less than that in the shCtrl group($P<0.05$). (Figure 1F), which indicate that the *DIDO1* gene was not significantly associated with HUVEC cell angiogenesis.

GeneChip analysis of shDIDO1 and shCtrl cell lines

In order to investigate the biological pathways *DIDO1* involved, the GeneChip expression profiles of shCtrl and shDIDO1 cell lines were analyzed. It was found that, 521 genes in shDIDO1 cell line were up-regulated and 1006 genes were down-regulated (Fold Change>1.5 and FDR<0.05), compared with shCtrl (Figure 2A, Table S3). IPA was used to analyze the biological pathways and diseases classification of the DEGs involved (Figure 2B), and it was found that the ERK/MAPK Signaling was significantly inhibited (Z-score= -2.041). Additionaly, the functions including Morbidity or mortality (Z-score = 6.734) and Organismal death (6.709), were significantly activated. The functions including Cell viability (Z-score= -5.369), Cell survival (Z-score= -5.349) were significantly suppressed (Figure 2C and Table S4).

The genes down-regulated in shDIDO1 cell lines, and which are involved in tumorigenesis and development were selected for further analysis (Table S5). First, the expression of these 30 genes were analyzed by qRT-PCR and the similar expression of these genes were identified. The genes were down-regulated in shDIDO1 cell lines and the change fold were shown in Table S5.

Besides, we found that the key genes of *CDK6* and *CCND1*, which involved in cell cycle regulation, were down-regulated in the shDIDO1 cell lines. It was found that the transcription of *CCND1* and *CDK6* in the shDIDO1 cell line were 0.364 and 0.404 times of the shCtrl group, respectively. Western-blot analysis found that the expression levels of CCND1 and CDK6 in shDIDO1 cell lines were reduced by 81.3% and 58.1%, respectively.

In addition, IPA was used to further analyze the interaction control network of *DIDO1* (Figure 2D) and those genes, it was found that the genes that directly interact with *DIDO1* are *SRSF1*, *SRPK2*, *EED* and *WWP2*.

Exploring upstream regulatory genes of *DIDO1* gene by public data

In order to analyze the genes that regulated the expression of *DIDO1*, the published ChIP-seq data of different leukemia cell lines (including K562) was used to analyze the transcription foctors of *DIDO1* gene.

There are multiple DNase-seq peaks near *DIDO1*, presuming that there is a regulatory factor binding at the corresponding position. Besides, H3K4me3 and PolII (POLR2A) may bind with DIDO1 at the transcription start site (TSS) near exon1 and exon2. Exon1 and exon2 have the binding signals of CDK7 and CDK8 near the TSS. Transcription factors of ATF1, BCLAF1, CBX3 were also found had binding peak at the TSS near exon1 and exon2 (Figure 3).

EGR1, FOS, MAX and NCOR1 may bind on exon1 of DIDOI as the binding signals were near the TSS of exon1; the exon 1 also has the peaks of H3K4me1, H3K27ac, MED1 and EP300 near the TSS. There are CHD7, SIRT6 and c-myc binding signals near the exon2 TSS. These transcription factors may be involved in the regulation of DIDO1 transcription (Figure S3). Among them, CDK7, CDK8, c-myc, FOS may be related to ERK5 Signaling signaling pathway. Previous study reported that DIDO1 has multiple isoforms.

Discussion

Although it has been well-established that CML hemangioblasts contribute to both malignant hematopoiesis and endotheliopoiesis, little is known about the exact contribution of tumor-derived endothelial cells to endotheliopoiesis in hematological malignancies. In this study, Genechip was performed to analyze DEGs between HUVEC cell lines and K562-HUVEC co-cultured cell lines, as it was reported that K562 cell culture medium, or supernatant can induce HUVEC cells to form leukemia-related endothelial cells.

Firstly, the GeneChip assay showed that expression of 323 probes was down-regulated in K562 co-cultured HUVEC lines (Figure S2A), and the ERK5 Signaling was significantly inhibited as shown by the IPA analysis. Then, we analyzed the proliferation of RNAi cell lines of the top 30 down-regulated expression genes. The proliferation of shDIDO1, shZC3H18, and shSMURF2 cell lines was significantly inhibited, and the change fold was 3.37, 2.54, 2.07, respectively.

DIDO1 localizes in the nucleus and cytosol, which is required in the early steps during the tumor progression and metastasis. As the tumors progress, the levels of *DIDO1* decrease in Esophageal Squamous Cell Carcinoma [15]. To study the functional relevance of *DIDO1* in leukemia, the

transcript and protein abundance of *DIDO1* gene was inhibited in siRNA cell lines, which resulted in an inhibition of proliferation, and an upregulation of apoptosis. There were studies reported that the overexpression of DIDO1 could translocate from the cytoplasm to the nucleus and activate the apoptotic machinery. Meanwhile, the *DIDO-1* mutant lacking nuclear localization sequences was unable to translocate to the nucleus or to trigger apoptosis. [16] This is contradictory to our research result, and the difference may be related to the different species of the cell line. The cell lines (FL5.12 and MEF) used in the previous study is of murine origin, while the cell line used in this study is of human origin. However, our result is consistent with the study performed by S Braig and A-K Bosserhoff (2013), the melanoma cells transfected with Dido1 small interfering RNAs led to an upregulation of apoptosis [17].

To investigate the genes that affected by *DIDO1*, the DEGs between shDIDO1 and shCtrl cell lines were analyzed. The ERK/MAPK Signaling was significantly inhibited in shDIDO1 lines. Besides, the morbidity or mortality and organismal death related genes were activated and the genes involved in Cell viability and Cell survival were inhibited significantly. This may indicate that the inhibition of *DIDO1* gene may promote the organismal death and inhibit the cell viability. As *DIDO1* may act as a transcription factor [18], we screened for potential *DIDO1* target genes that down regulated in shDIDO1 cell line. The IPA interaction network indicate that *DIDO1* directly interact with *WWP2*, *SRPK2* and *SRSF1*. *WWP2* (WW domain containing E3 ubiquitin protein ligase 2) gene encodes a protein that play a role in the regulation of oncogenic signaling pathways via interactions with SMAD proteins and the tumor suppressor PTEN. *WWP2* could promote the proliferation of gastric cancer cells in a PTEN-dependent manner, and its silencing will inhibit proliferation and growth of gastric cancer cells [19], suggesting a vital role of *WWP2* in cancer progression. *SRPK2* (Serine/Arginine-Rich Protein-Specific Kinase-2, SRSF protein kinase-2) is up-regulated in multiple human tumors, and plays an important role in the progression and metastasis of prostate cander[20]. *SRSF1* (serine/arginine-rich splicing factor 1) promotes proliferation and injury-induced neointima formation in vascular smooth muscle cells [21], and it could promote tumorigenesis through regulation of alternative splicing in colon cancer [22], glioblastoma [23], and other cancers. The overexpression of *SRSF1* could promote cell proliferation and delay cell apoptosis during acinar morphogenesis in breast cancer [24]. According to these previously researches, *DIDO1* may play roles by interaction with *WWP2*, *SRPK2* and *SRSF1*.

Besides, the expression of cell Cycle genes, *CDK6* and *CCND1*, were down-regulated in shDIDO1, which was verified by RT-PCR and western-blot. This may indicate that the low abundance of *DIDO1* may indirectly inhibit the expression of *CDK6* and *CCND1* to regulate the cell proliferation. DIDO1 also has 2 alternative splicing transcripts *DIDO2* and *DIDO3* [25]. It has been reported that *DIDO1* probably competes with *DIDO3* for binding to H3K4me3, *DIDO3* binds the Dido locus via H3K4me3 and RNA POL II and induces DIDO1 expression [26]. *DIDO1* binds with H3K4me3, promoting downregulation of stemness genes and *DIDO3* degradation. In this study, we found that there are H3K4me3 and Pol II (POLR2A) signals near Exon1 and exon2, suggesting that there are isoforms with Exon1 and exon2 as transcription start sites.

Conclusions

In conclusion, the apoptosis and proliferation mechanism in Leukemia Endothelial cells that may regulated by *DIDO1* was summarized in figure 4. The ERK5 signal will be inhibited by the down-regulation of *DIDO1* in shDIDO1 cell lines, and the genes in ERK5 signaling may paly roles in cell apoptosis and proliferation, and regulate the gene transcription and translation. In addition, H3k4me3 binds with *DIDO1* and inhibit its expression, the inhibited *DIDO1* in HUVEC will indirectly inhibit the expression of *CDK6* and *CCND1*, which will inhibit the proliferation of cells. The reduction of endothelial cells may inhibit the development of leukemia and inducing cell apoptosis may become a therapy to treat leukemia. The *DIDO1* gene discovered in this study provides a theoretical basis for the development of drug targets for leukemia. But it is also necessary to study the gene expression of *DIDO1* in leukemia patients.

References

[1] Wierzbowska A, Robak T, Krawczynska A, Wrzesien-Kus A, Pluta A, Cebula B, et al. Circulating endothelial cells in patients with acute myeloid leukemia. Eur J Haematol 2005; 75: 492-497, doi: 10.1111/j.1600-0609.2005.00549.x

[2] Rigolin GM, Maffei R, Rizzotto L, Ciccone M, Sofritti O, Daghia G, et al. Circulating endothelial cells in patients with chronic lymphocytic leukemia: clinical-prognostic and biologic significance. Cancer 2010; 116: 1926-1937, doi: 10.1002/cncr.24961

[3] Zhang H, Vakil V, Braunstein M, Smith EL, Maroney J, Chen L, et al. Circulating endothelial progenitor cells in multiple myeloma: implications and significance. Blood 2005; 105: 3286-3294, doi: 10.1182/blood-2004-06-2101

[4] Cortelezzi A, Fracchiolla NS, Mazzeo LM, Silvestris I, Pomati M, Somalvico F, et al. Endothelial precursors and mature endothelial cells are increased in the peripheral blood of myelodysplastic syndromes. Leuk Lymphoma 2005; 46: 1345-1351, doi: 10.1080/10428190500144235

[5] Wierzbowska A, Robak T, Krawczyńska A, et al. Circulating endothelial cells in patients with acute myeloid leukemia. Eur J Haematol. 2005;75(6):492-497. doi:10.1111/j.1600-0609.2005.00549.x

[6] Bobryshev YV, Orekhov AN, Chistiakov DA. Vascular stem/progenitor cells: current status of the problem. Cell Tissue Res. 2015;362(1):1-7. doi:10.1007/s00441-015-2231-7

[7] Gunsilius E, Duba HC, Petzer AL, et al. Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. Lancet. 2000;355(9216):1688-1691. doi:10.1016/S0140-6736(00)02241-8

[8] Shih TT, Hou HA, Liu CY, et al. Bone marrow angiogenesis magnetic resonance imaging in patients with acute myeloid leukemia: peak enhancement ratio is an independent predictor for overall survival. Blood. 2009;113(14):3161-3167. doi:10.1182/blood-2008-08-173104

353 [9] Hatfield KJ, Evensen L, Reikvam H, Lorens JB, Bruserud Ø. Soluble mediators released by
354 acute myeloid leukemia cells increase capillary-like networks. Eur J Haematol.
355 2012;89(6):478-490. doi:10.1111/ejh.12016
356 [10] Butler JM, Kobayashi H, Rafii S. Instructive role of the vascular niche in promoting
357 tumour growth and tissue repair by angiocrine factors. Nat Rev Cancer. 2010;10(2):138-146.
358 doi:10.1038/nrc2791
359 [11] Bosse RC, Wasserstrom B, Meacham A, et al. Chemosensitizing AML cells by targeting
360 bone marrow endothelial cells. Exp Hematol. 2016;44(5):363-377.e5.
361 doi:10.1016/j.exphem.2016.02.003
362 [12] Pizzo RJ, Azadniv M, Guo N, et al. Phenotypic, genotypic, and functional
363 characterization of normal and acute myeloid leukemia-derived marrow endothelial cells.
364 Exp Hematol. 2016;44(5):378-389. doi:10.1016/j.exphem.2016.01.008
365 [13] Poulos MG, Gars EJ, Gutkin MC, et al. Activation of the vascular niche supports
366 leukemic progression and resistance to chemotherapy. Exp Hematol. 2014;42(11):976-
367 986.e3. doi:10.1016/j.exphem.2014.08.003
368 [14] Moodley S, Koorbanally NA, Moodley T, Ramjugernath D, Pillay M. The 3-(4,5-
369 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is a rapid, cheap,
370 screening test for the in vitro anti-tuberculous activity of chalcones. J Microbiol Methods.
371 2014;104:72-78. doi:10.1016/j.mimet.2014.06.014
372 [15] Fütterer A, Campanero MR, Leonardo E, et al. Dido gene expression alterations are
373 implicated in the induction of hematological myeloid neoplasms. J Clin Invest.
374 2005;115(9):2351-2362. doi:10.1172/JCI24177
375 [16] García-Domingo D, Ramírez D, González de Buitrago G, Martínez-A C. Death inducer-
376 obliterator 1 triggers apoptosis after nuclear translocation and caspase upregulation. Mol Cell
377 Biol. 2003;23(9):3216-3225. doi:10.1128/mcb.23.9.3216-3225.2003
378 [17] Braig S, Bosserhoff AK. Death inducer-obliterator 1 (Dido1) is a BMP target gene and
379 promotes BMP-induced melanoma progression. Oncogene. 2013;32(7):837-848.
380 doi:10.1038/onc.2012.115
381 [18] Rojas AM, Sanchez-Pulido L, Fütterer A, van Wely KH, Martinez-A C, Valencia A.
382 Death inducer obliterator protein 1 in the context of DNA regulation. Sequence analyses of
383 distant homologues point to a novel functional role. FEBS J. 2005;272(14):3505-3511.
384 doi:10.1111/j.1742-4658.2005.04759.x
385 [19] Wang K, Liu J, Zhao X, et al. WWP2 regulates proliferation of gastric cancer cells in a
386 PTEN-dependent manner. Biochem Biophys Res Commun. 2020;521(3):652-659.
387 doi:10.1016/j.bbrc.2019.10.179
388 [20] Zhuo YJ, Liu ZZ, Wan S, et al. Enhanced expression of SRPK2 contributes to aggressive
389 progression and metastasis in prostate cancer. Biomed Pharmacother. 2018;102:531-538.
390 doi:10.1016/j.biopha.2018.03.079

[21] Xie N, Chen M, Dai R, et al. SRSF1 promotes vascular smooth muscle cell proliferation through a Δ133p53/EGR1/KLF5 pathway. Nat Commun. 2017;8:16016. Published 2017 Aug 11. doi:10.1038/ncomms16016

[22] Chen L, Luo C, Shen L, et al. SRSF1 Prevents DNA Damage and Promotes Tumorigenesis through Regulation of DBF4B Pre-mRNA Splicing. Cell Rep. 2017;21(12):3406-3413. doi:10.1016/j.celrep.2017.11.091

[23] Zhou X, Wang R, Li X, et al. Splicing factor SRSF1 promotes gliomagenesis via oncogenic splice-switching of MYO1B. J Clin Invest. 2019;129(2):676-693. doi:10.1172/JCI120279

[24] Anczuków O, Rosenberg AZ, Akerman M, et al. The splicing factor SRSF1 regulates apoptosis and proliferation to promote mammary epithelial cell transformation. Nat Struct Mol Biol. 2012;19(2):220-228. Published 2012 Jan 15. doi:10.1038/nsmb.2207

[25] Forghanifard MM, Naeimi Khorasanizadeh P, Abbaszadegan MR, Javdani Mallak A, Moghbeli M. Role of DIDO1 in Progression of Esophageal Squamous Cell Carcinoma. J Gastrointest Cancer. 2020;51(1):83-87. doi:10.1007/s12029-019-00212-1

[26] Berzoti-Coelho MG, Ferreira AF, de Souza Nunes N, et al. The expression of Death Inducer-Obliterator (DIDO) variants in Myeloproliferative Neoplasms [published correction appears in Blood Cells Mol Dis. 2018 Mar;69:123]. Blood Cells Mol Dis. 2016;59:25-30. doi:10.1016/j.bcmd.2016.03.008

Figure 1

Figure 1. The function assays of *DIDO1* gene.

A) The RNA transcript abundance of *DIDO1* gene in shDIDO1and shCtrl cell lines detected by qRT-PCR; B) Western-blot assay for the expression of DIDO1 protein; C) Cell proliferation pictures of shDIDO1and shCtrl cell lines by Celigo ;D and E) Cell count of shDIDO1and shCtrl cell lines; F) Analysis of the area of blood vessel formation; G) Cell apoptosis ratio analyzed by FACS; H) Caspase3/7 activity assays.

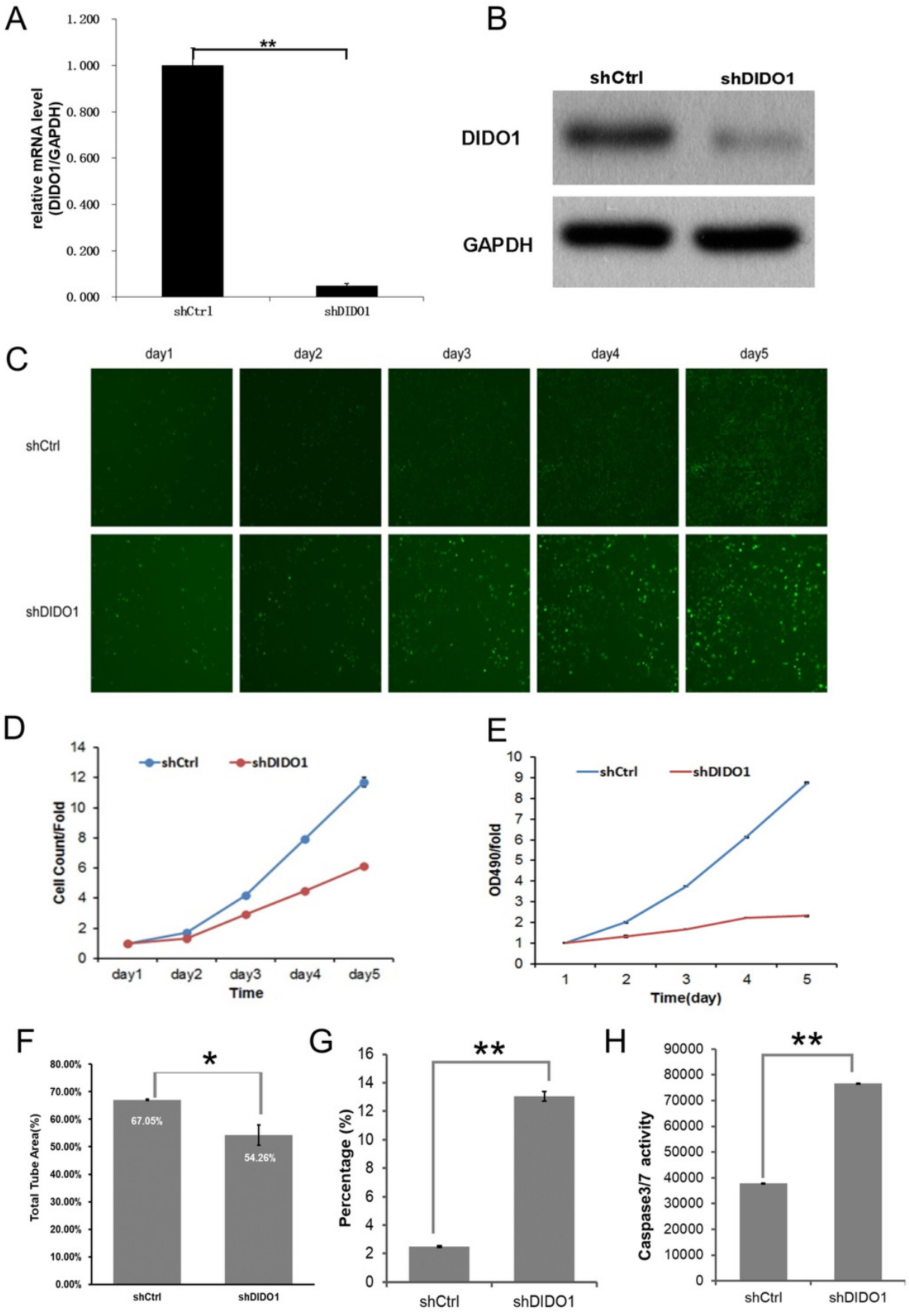

A
relative mRNA level (DIDO1/GAPDH)
1.200
1.000
0.800
0.600
0.400
0.200
0.000
**
shCtrl
shDIDO1
B
shCtrl
shDIDO1
DIDO1
GAPDH
C
day1
day2
day3
day4
day5
shCtrl
shDIDO1
D
Cell Count/Fold
shCtrl
shDIDO1
Time
E
OD490/fold
shCtrl
shDIDO1
Time(day)
F
Total Tube Area(%)
*
67.05%
54.26%
shCtrl
shDIDO1
G
Percentage (%)
**
shCtrl
shDIDO1
H
Caspase3/7 activity
**
shCtrl
shDIDO1

Figure 2

Figure2 Analysis of downstream genes regulated by *DIDO1*

A) The Volcano map of the differently expressed probes in shCtrl and shDIDO1 cell lines; B) The enrichment of DEGs in the classical signal pathway; C) Disease and function heat maps show the expression changes of DEGs indifferent diseases and functions. Orange means the disease or functional state is activated (Z-score>0), blue means the disease or functional state is inhibited (Z-score<0), and gray means the disease or functional state is not determined (Z-score cannot be calculated); The disease or function is significantly activated if Z-score>2), and significantly inhibited if Z-score<-2. Significantly activated diseases or functions include: Morbidity or mortality (Z-score= 6.734), Organic death (6.709), etc.; Significantly inhibited diseases or functions include: Cell viability (-5.369), Cell survival (-5.349); D) Gene interaction network diagram shows the interaction network between molecules; E) The expression of *CCND1* and *CDK6* analyzed by qRT-PCR and Western-blot.

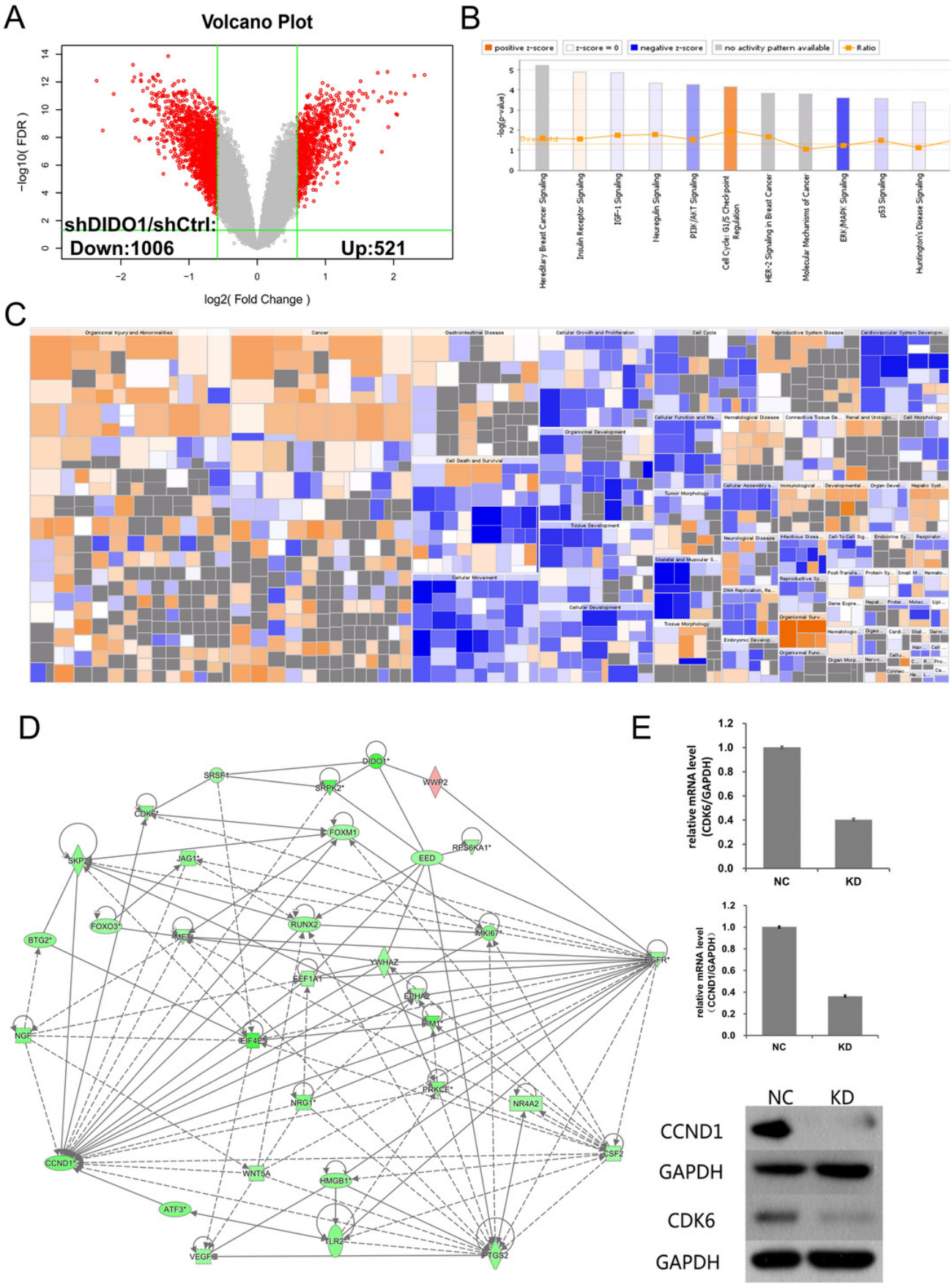

A
Volcano Plot
−log10(FDR)
log2(Fold Change)
shDIDO1/shCtrl:
Down:1006
Up:521
B
positive z-score
z-score = 0
negative z-score
no activity pattern available
Ratio
-log(p-value)
Hereditary Breast Cancer Signaling
Insulin Receptor Signaling
IGF-1 Signaling
Neuregulin Signaling
PI3K/AKT Signaling
Cell Cycle: G1/S Checkpoint Regulation
HER-2 Signaling in Breast Cancer
Molecular Mechanisms of Cancer
ERK/MAPK Signaling
p53 Signaling
Huntington's Disease Signaling
C
Organismal Injury and Abnormalities
Cancer
Gastrointestinal Disease
Cellular Growth and Proliferation
Cell Cycle
Reproductive System Disease
Cardiovascular System Develop...
Cellular Function and Ma...
Hematological Disease
Connective Tissue De...
Renal and Urologic...
Cell Morphology
Organismal Development
Cell Death and Survival
Tumor Morphology
Cellular Assembly a...
Immunological ...
Developmental ...
Organ Devel...
Hepatic Syst...
Tissue Development
Neurological Disease
Infectious Disea...
Cell-To-Cell Sig...
Endocrine Sy...
Respirator...
Cellular Movement
Skeletal and Muscular S...
Post-Translla...
Protein Sy...
Small M...
Hemato...
Reproductive Sy...
DNA Replication, Re...
Gene Expre...
Hepat...
Profei...
Molec...
Lipi...
Cellular Development
Tissue Morphology
Organismal Surv...
Hematologic...
Digest...
Cardi...
Skel...
Derm...
Embryonic Develop...
Organismal Func...
Organ Morp...
Hair...
Cell ...
Nervo...
C...
R...
Pro...
Connec...
He...
L...
Ca...
D
DIDO1*
SRSF1
SRPK2*
WWP2
CDK6*
FOXM1
RPS6KA1*
SKP2
JAG1*
EED
FOXO3*
RUNX2
BTG2*
ME2
MKI67*
EGFR*
YWHAZ
EEF1A1
EPHA2
PIM1*
NGF
EIF4E*
PRKCE*
NRG1*
NR4A2
CSF2
CCND1*
WNT5A
HMGB1*
ATF3*
TLR2
VEGFC
PTGS2
E
relative mRNA level (CDK6/GAPDH)
NC
KD
relative mRNA level (CCND1/GAPDH)
NC
KD
NC
KD
CCND1
GAPDH
CDK6
GAPDH

Figure 3

Figure3 The transcript factors and epigenetic factors bind with DIDO1 gene analyzed by public data.

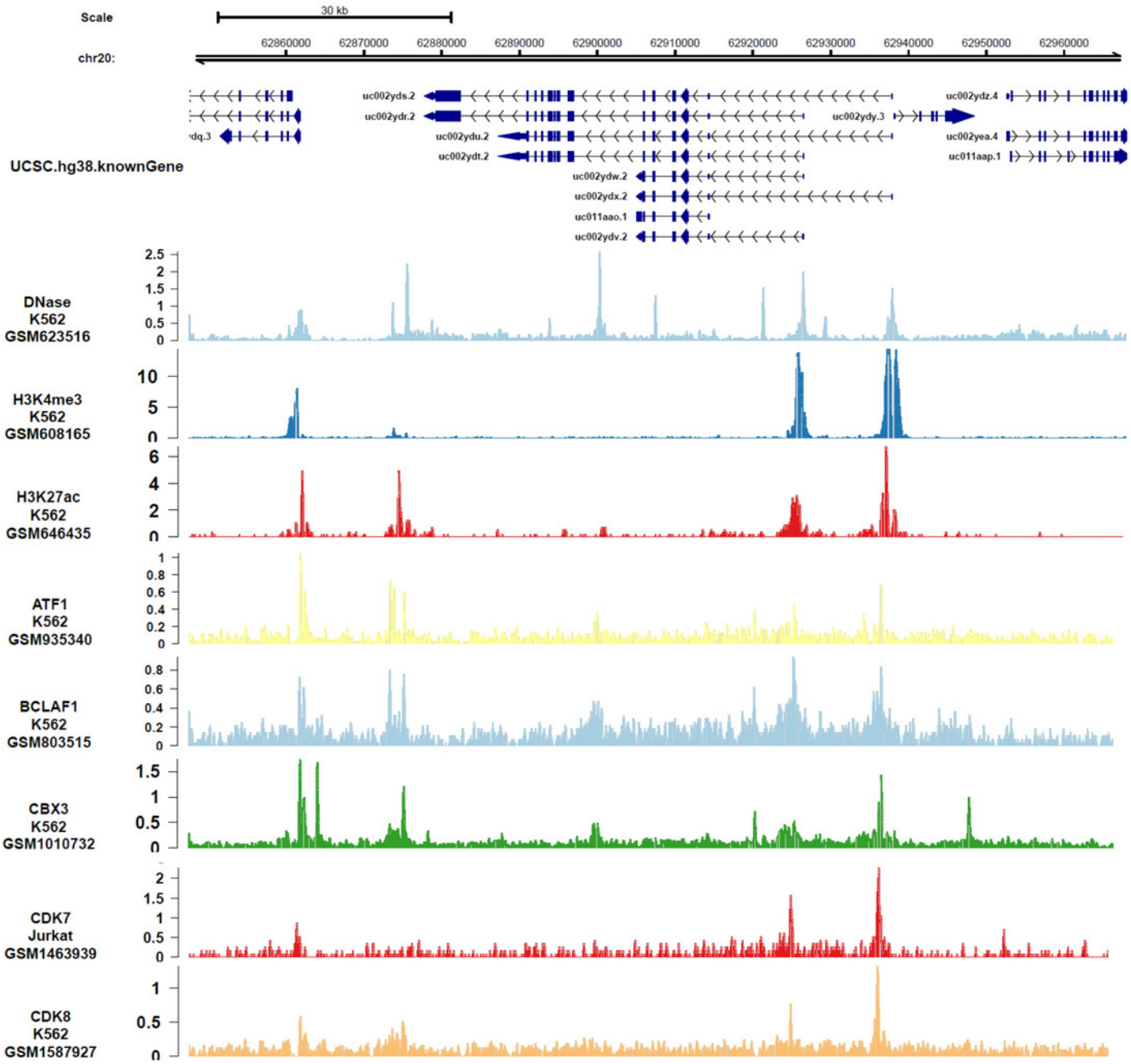
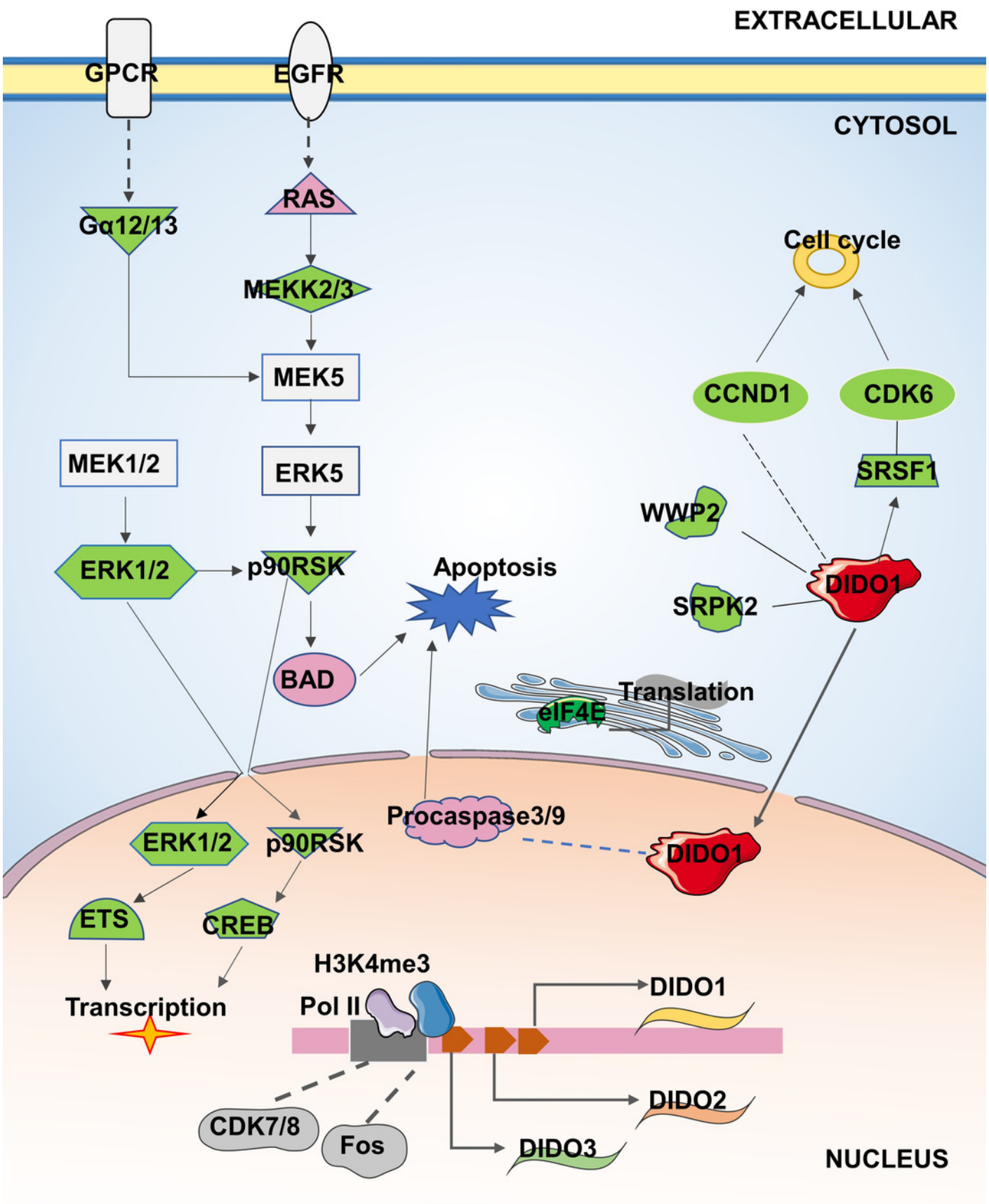


Figure 4

Figure 4 The apoptosis and proliferation mechanism in Leukemia Endothelial cells that may regulated by *DIDO1*


EXTRACELLULAR
GPCR
EGFR
CYTOSOL
RAS
Gα12/13
MEKK2/3
Cell cycle
MEK5
CCND1
CDK6
MEK1/2
ERK5
SRSF1
WWP2
ERK1/2
p90RSK
Apoptosis
DIDO1
SRPK2
BAD
Translation
eIF4E
Procaspase3/9
ERK1/2
p90RSK
DIDO1
ETS
CREB
H3K4me3
Transcription
Pol II
DIDO1
DIDO2
CDK7/8
Fos
DIDO3
NUCLEUS