博士論文

Investigation for a novel therapeutic target in acute myeloid leukemia with monosomy 7

（モノソミー7 を伴う急性骨髄性白血病に対する
新規治療標的の検討）

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Contents

Abstract

Monosomy of chromosome 7 (monosomy 7) is observed in about 9% of acute myeloid leukemia (AML) patients and related to poor prognosis. It is required to establish an effective treatment strategy based on the specific molecular pathogenesis associated with the chromosomal abnormality. This study aimed to find a novel therapeutic target in AML with monosomy 7. RNA-interference (RNAi)-based screening targeting 53 histone modification genes detected embryonic ectoderm development (EED) and bromodomain containing 4 (BRD4) as essential survival factors in AML with monosomy 7. Stable knockdown of EED markedly attenuated proliferation as well as increased cell apoptosis specifically in leukemia cells with monosomy 7. Interestingly, the polycomb repressive complex 1 (PRC1) rather than the polycomb repressive complex 2 (PRC2) activity of EED seemed to have a predominant role in the survival of monosomy 7 leukemia cells. I then explored whether the deletion of specific genes within the chromosome 7 was responsible for increased sensitivity to EED inhibition. Knockdown of general transcription factor IIi (GTF2I) increased the sensitivity to EED inhibition via increased cell apoptosis. Both EED and GTF2I inhibition suppressed expression levels of B cell leukemia/lymphoma 2 (BCL2). It is possible that synthetic lethality between EED and GTF2I induces apoptosis in leukemia

cells via suppressing expression levels of BCL2. In addition to EED, we identified BRD4 as an essential survival factor in AML with monosomy 7. Although inhibition of bromodomain and Extra-terminal motif (BET) proteins have already shown to be effective for a variety of AML, we showed that blockade of bromodomain proteins preferentially affected cellular proliferation of AML cells with monosomy 7. Mechanistically, knockout of lysine methyltransferase 2C (MLL3) or lysine methyltransferase 2E (MLL5), both encoded in the chromosome 7, resulted in increased vulnerability to JQ1-treatment. Expression levels of MYC proto-oncogene (c-Myc) and cyclin D1 (CCND1) were strongly suppressed by JQ-1 treatment especially in MLL3 and MLL5 knockout leukemia cells. It is possible that synthetic lethality between BRD4 and MLL3/MLL5 induced cell cycle arrest in leukemia cells via suppressing expression levels of c-MYC and CCND1. Our synthetic-lethality based approach identified EED and BRD4 as novel promising therapeutic targets in AML with monosomy 7.

Introduction

Acute myeloid leukemia (AML) is a clinical entity characterized by proliferation of immature myeloid cells due to various genetic or chromosomal abnormalities [1]. Although its pathogenesis has not been fully understood, previous reports revealed that various epigenetic changes including histone modification or DNA methylation caused the progression of myeloid malignancies [2-5]. Actually, epigenetic modification genes such as DNMT3A, ASXL1, TET2, or EZH2 were frequently mutated in myeloid malignancies [1]. These epigenetic abnormalities changed the gene expression patterns, and impaired cell differentiation of hematopoietic progenitor cells and caused the progression of myeloid malignancies [1-5].

Monosomy of chromosome 7 (monosomy 7) is observed in about 9% of AML patients and related to poor prognosis [6, 7]. Although the pathogenesis of monosomy 7 has still been unclear, previous reports revealed that deletion of some histone modification genes in the chromosome 7 was associated with progression of leukemia. Among them, single allele deletion of histone modification genes including EZH2 and MLL3 contributed to the pathogenesis of the monosomy 7 leukemia (Figure 1) [8].

Figure 1

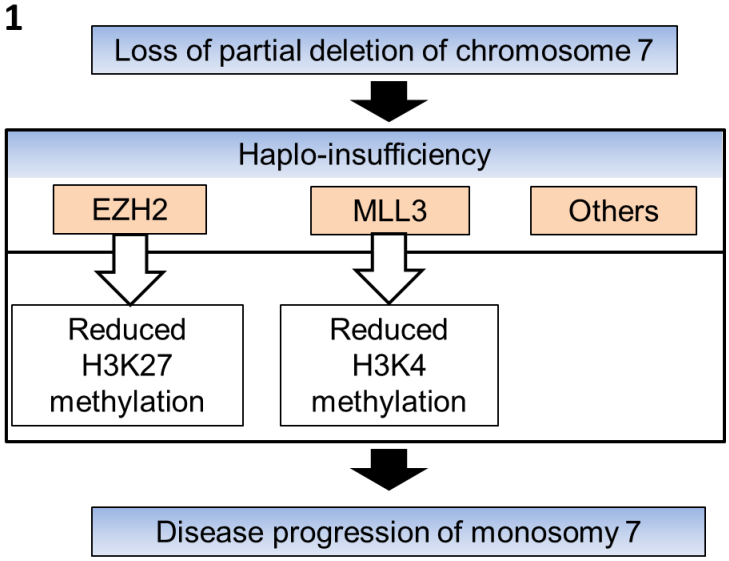


Figure 1 Pathogenesis of monosomy 7 summarized from Inaba T et al (2018).

EZH2 is a component of the polycomb repressive complex 2 (PRC2) and plays an important role for the trimethylation of lysine 27 of histone 3 (H3K27) to mediate gene silencing [9-13]. Previous reports indicated that deletion of EZH2 cooperatively caused myeloid malignancies with TET2 or RUNX1 mutations [14, 15]. MLL3 is included in an MLL protein family, and has histone methyltransferase activity for lysine 4 histone 3 (H3K4) [16, 17]. In contrast to trimethylation of H3K27, trimethylation of H3K4 generally activates gene transcription [16, 17]. As reported in EZH2, functional loss of MLL3 contributed to the pathogenesis of myeloid malignancies [18-20]. Taken together, histone modification genes including EZH2 and MLL3 were supposed to be important tumor suppressor genes (TSGs) in AML with monosomy 7. However, despite

its poor prognosis, therapeutic approach for monosomy 7 has not been well developed. One reason may be the difficulty of therapeutic approach in functional loss of TSGs. Synthetic lethality (SL) is a novel concept in which two or more genes cooperatively play an essential role for cell survival [21-23]. When SL relationship between gene A and gene B is valid, gene B inhibition specifically induces cellular death in the tumor cells which harbor the loss-of-function mutations of gene A. SL is an important concept that provides a therapeutic target in cancer cells with loss-of-function mutations of TSGs. Recently, several reports identified novel therapeutic targets in prostate cancer, lung cancer, and other malignancies based on the SL hypothesis [24-28]. However, only a few reports described the SL in hematological malignancies. One of the reasons may be the lack of TSGs commonly associated with hematological malignancies. For example, PTEN, which is a well-known tumor suppressor gene, is deleted around 90% of a certain subtype of prostate cancer [27]. However, there is no such a commonly deleted TSGs in hematological malignancies. Thus, I did not focused on the lack of TSGs but the lack of chromosome in hematological malignancies. This study aimed to identify the novel therapeutic targets in AML with monosomy 7 by synthetic lethality-based approach.

Despite its poor prognosis, there has been no previous study that evaluated the

specific therapeutic target for the monosomy 7 AML. To investigate a novel therapeutic target in malignancies, gene enrichment analysis was sometimes useful because the specific enrichment pathway was frequently important for the tumor cell survival. However, previous report showed that there was no specifically enriched or suppressed pathway in AML with monosomy 7 [29]. Thus, synthetic lethality-based approach was needed to identify the therapeutic target in AML with monosomy 7. Although loss of histone modification genes is considered to contribute to the development of AML with monosomy 7, it may also be required for some leukemia cell survivals. From the perspective of SL, I hypothesized that haplo-insufficiency of the epigenetic modifiers in the chromosome 7 is compensated by other epigenetic molecules in different chromosomes, and the AML cells with monosomy 7 are vulnerable to inhibition of those molecules. Based on this notion, this study aimed to clarify the novel therapeutic target in AML with monosomy 7.

Methods

RNA-interference-based screening

RNA-interference (RNAi)-based screening was performed using synthesized siRNAs (Silencer Select®, Thermo Fisher Scientific). Gene names and sequences of the used siRNAs were shown in Table 1.

Table 1 List of synthesized siRNAs

a.

Screening for therapeutic targets in monosomy 7 three siRNAs per each gene			
Gene name	siRNA Sequence	Gene name	siRNA Sequence
AEBP2	CAGCAUAAGCAGUACUAUAtt	ASH1L	CAAACUCCAUAAUACGGUAtt
	CACUGGAUGCGAUAAGACAtt		GGAUUGAUAAAGAGGUAAAtt
	CAUCCGUUCCAUACAUGUAtt		CCUUUGCACUACAUUCGAAtt
EZH1	CAGUUGCAUUGGUUCCCAUtt	KMT2E	GAGACGCACUUAUAGUCAAtt
	GGUAGAAGAUGAGACGGUUtt		GCCUCUACGCAUAACUACAtt
	GAUCCGUUCUGAUUAGUGAtt		CCGUAUCCCUCACAAGCUAtt
MTF2	GACAGAUUUUAUACGUUUAtt	BEND3	GUAUCACUGUGAAAGUGGAtt
	GGAUUUACCUUGUUCUAUAtt		ACAUCUCAGUGGUCAAGGUtt
	GCAUUUGUUUGGGUUGCGAtt		GCAACUACACGGAGAUCUAtt
SIRT1	GGGUCUUCCCUCAAAGUAAtt	ARID4A	GAGCGAACAUUGAGACGUAtt
	GUAAGACCAGUAGCACUAAtt		CAUUCUCAAUCUACCGGAAtt
	CAACUAUACCCAGAACAUAtt		GAUCAGUGUUUAGUUCGAUtt
SUZ12	GGACCUACGUUGCAGUUCAtt	RBBP5	CCAGUGGAGUGGUAUCUAUtt
	GGAUAGAUGUUUCUAUCAAtt		GAUUCUCCAUUUAAACCGAtt
	GGAUGUAAGUUGUCCAAUAtt		CAACCAAUAUAGAACUUCAtt
PHF19	GCCUCGUGACUUUCGAAGAtt	ZNF335	GGAACAGACAGUGACCAAUtt
	GGUCAGCAGCUCUAAGCAAtt		GGAUAGCGACUAUAAUCCAtt
	GACUUGAUGUCCAAACUGAtt		CGCAAGUACUAUUACAAGUtt
HDAC2	CUACGACGGUGAUAUUGGAtt	SETMAR	CCCAGGAACUGCUAACGAAtt
	GGGUUGUUUCAAUCUAACAtt		GAGUUCGUCGAAUCCCAAAtt
	GGCAGAUAUUUAAGCCUAUtt		CCUUGGAAUUUAUACCGAAtt
JARID2	GAAGAACGGGUGGUACGUAtt	UTX	GCAUUGUGAAAGUAAUAGAtt
	GGUUUCUAAGGUAAACGGAtt		GAAUCUACAUCGUCAGAUAtt
	GGUGGUACAAGAGAACGAAtt		CCAACUAUCUAACUCCACUtt
TRIM37	GACUAGUUAUAUCCAACAAtt	WDR82P1	AAGAGAACCCUGUACAGUAtt

	GGUAGUCUAUCACUUCGAAtt		CAAAAUAGACGAUACUAUUtt
	GGACCGGAGCAGUAUAGAAtt		GAUCCAGAAGGGUUAAUUUtt
PHF1	CAGGUUCUAUGAAUUUGAAtt	WDR61	CAAGAUCUAUGAUGUACAAtt
	GGGAGAGAGAUUAAGAAGAtt		GAUCUAUGAUGUACAACAUtt
	CCUCAGCAGUGCGCAAUCAtt		AGAGGAAAAUUCAUUCUUAtt
H2AFY2	GGCUGAUGCGUUAUCUGAAtt	KMT2B	GGGUUUUCAUUCAGAUGAAtt
	CAGCCGAAAUUGACCUCAAtt		GGUGCUAUAUGUUCCGCAUtt
	GGACAGCGAUAAAGAAGGAtt		GGCGAUUUAUGGAUGAAGAtt
RBBP4	GGAUACUCGUUCAAACAAUtt	MLL2	GAGUCGAACUUUACUGUCUtt
	GAUACUCGUUCAAACAAUAtt		GCAAAUCGCUAGCAUCAUUtt
	AAAUCAAGAUCAACCAUGAtt		CCACUCUCAUCAAAUCCGAtt
RBBP7	CUCACAUCAUUGCUACAAAtt	SETD3	CUCUCUACUUUGAAGAAGAtt
	GCUUUAUGGGAUCUGCGUAtt		CCACGAAAAUUGCUAAUGAtt
	GGAAGAAGAAUACACCGUUtt		GCAAAAAGGUCUGUCCGUUtt
EED	GGCAUAAUUAGGAUAAUAAtt	DYDC2	CCAGUGACCCAAUAGAAUAtt
	GCUUUACGAUUAUGGAAUAtt		GCUUUAUCAUUACAGGAAAtt
	CAUUAGUGUUUGCAACUGUtt		AGCAUGAAGUUGCUCAUGAtt
H2AFY	CGGUGUACUUCGUGCUUUUtt	DPY30	AGUUGUGCCUAUCUUAUUAtt
	GCAGGAACGGUUUUCCAAAtt		GCAAAGGAAAGACCACCAAtt
	GCUAAAAGGAGUCACCAUAtt		GAAGGUAGAUCUCCAGUCUtt
WDR5	CGAAAGAGAUUGUACAGAAtt	OGT	CAUUUAUGACAAUCGGAUAtt
	GAUGGGAAAUUUGAGAAAAtt		GCGUGUUCCCAAUAGUGUAtt
	AGACGAAAGAGAUUGUACAtt		GCAGUUCGCUUGUAUCGUAtt
DYDC1	CAACUGAGACAAAAGGAAAtt	ASH2L	GCCUGGUAUUUUGAAAUCAtt
	CAGUGGAUCCGAUAGAAUAtt		GCUCCUUUAGGUUAUGAUAtt
	CAGAGGAGCUCUUACUUCAtt		GGAACACCCGUUUAACAAAtt
TET3	CGAUUGCGUCGAACAAAUAtt	CTR9	GGAUAAACUUAAAAUUGCUtt
	CAGCAACUCCUAGAACUGAtt		CAAAGGAUAUUUUCGUGAAtt
	GGAUCGAGAAGGUCAUCUAtt		CACUGAAGUUGUACUCUAUtt
PAXIP1	GCAGAUGUCUGAUAAGCAAtt	SETD1A	GGACAACAACGAAUGAAAUtt
	GCAGAGUUGUUGAUGAGUAtt		CGCAGUGAGUUUGAACAGAtt
	CUACGAAAAAUUUAGAACAtt		CAACGACUCAAAGUAUAUAtt
SETD1B	CGGUGGAAAUUGUCGAAGAtt	ARID1A	GGACAAGGGAUUAAUAGUAtt
	CGUUCAAGGCUCAACCACAtt		GGAAACCUCUGGACCUCUAtt
	GGAGAUUACCUAUGACUAUtt		CGGUAUCACCGUUGAUGAAtt
RTF1	GCGUGUUCCGUUUAGAGUUtt	CDK4	UGCUGACUUUUAACCCACAtt
	CAAGAACUGUUCAAUCGCAtt		GGCUUUUGAGCAUCCCAAUtt
	GAAAAACGAAAGAACAGAAtt		CACCCGUGGUUGUUACACUtt
HIST1H1C	CGGCCACUGUAACCAAGAAtt	ARF4	CAACGAUCGUGAAAGAAUUtt
	GAAGAGCGCUAAGAAAACAtt		GGAUGUUGGUGGUCAAGAUtt
	CGGGCGGAACCAAACCUAAtt		GCAAGACAACCAUUCUGUAtt
HIST1H1D	AAACACCUGUGAAGAAAAAtt	BRD2	GUAGCAGUGUCACGCCUUAtt
	UAUCUGAGCUUAUCACCAAtt		CUGGGAGUCUUGAGCCUAAtt
	GCAUCAAAAAGACUCCUAAtt		GGUCUACCGGAUUAUCACAtt
HIST1H1E	CUUUGGCCGCUCUCAAGAAtt	BRD3	CGGCUGAUGUUCUCGAAUUtt
	CCAAGGCGGCUAAACCAAAtt		AGAGGAAGUUGAAUUAUUAtt
	CGAGCUCAUUACUAAAGCUtt		AGAUAGAAAUUGACUUUGAtt

CXXC1	ACUGCAUCCGGAUCACUGAtt	BRD4	UGAGCACAAUCAAGUCUAAtt
	GUAUAAUCCUCAGAGCAAAtt		CCUGAUUACUAUAAGAUCAtt
	CAAGCUAGAGAUUCGCUAUtt		AGAUUGAAAUCGACUUUGAtt
MLL	GGAGUGUAAUAAGUGCCGAtt	BRDT	CAAAGACCGUGAUUUAGCAtt
	CGAUUAGAGUCUACACCGAtt		GGAUGUUAAUAAUCAGUUAtt
	GGUUGCUAUAUGUUCCGAAtt		GGAUGUUUUCGAAACGCAUtt
TET2	CCCAAUCUCUCCAAUCAAAtt		
	GAGUUGUCCUGUGAGAUCAtt		
	GGCUCUUACUCUCAAAUCAtt		

b.

Screening for responsible genes in 7q

one siRNA per each gene

Gene name	siRNA Sequence	Gene name	siRNA Sequence
ACTL6B	GCCUUCUUCUUAUGCAAGAtt	BAZ1B	CCUCAUUGCAUACUACAAAtt
DNAJC2	CGAUUUGAAGGUCCAUAUAtt	FOXP2	GCAAACAAGUGGAUUGAAAtt
ING3	AGAGUCAGUGAAUUCUUUAtt	KMT2C	CCAAUGAGGUAAAAACGGAtt
TAF6	GACUACGCCUUGAAGCUAAtt	TRRAP	CUACGAUUCUGGUGGAAUAtt
SAMD9L	GGGUAAAUGAAGACCUUAAtt	KMT2E	GAGACGCACUUAUAGUCAAtt
ACTR3B	GUAGUUGACCAAGCUCAAAtt	CDK5	GCAAUGAUGUCGAUGACCAtt
EZH2	GGCACUUACUAUGACAAUUtt	GATAD1	GUUUUAUCCAGGACCAGUAtt
JHDM1D	GGAAACUUCGAGAUCAUAAtt	LRWD1	GAGCUUGACCUGUCUAACAtt
TRIM24	GUCAGUUGUUAGAACAUAAtt	UBE2H	GGCGGAGUAUGGAAAGUUAtt
LUC7L2	GCAGAGGAAGUUUAUCGGAtt	PRKAG2	CAAGUGUUAUGACAUCGUUtt
CUL1	GGAUGAGAGUGUACUGAAAtt	CUX1	GAAAGCGGCUUAUCGAACAtt
GTF2I	GAUUCUCCUUGGAUCAAUAtt	DOCK4	GCUCUUUGAUGUCCGGGAAtt
SMARCD3	AGACGAUUGAGUCCAUAAAtt		

Synthesized siRNAs were transfected through electroporation by using NEPA21®. Electroporation conditions in each cell line were shown in Table 2.

Table 2 Electroporation conditions in each cell line.

Cell lines	Voltage (V)	Pulse width (ms)	Pulse interval (ms)	number of energization	Attenuation rate (%)		
K562	125	5	50	2	10	+	Poring Pulse
	20	50	50	5	40	+/-	Transfer Pulse
F-36P	125	2.5	50	2	10	+	Poring Pulse
	20	50	50	5	40	+/-	Transfer Pulse
KG-1	150	5	50	2	10	+	Poring Pulse
	20	50	50	5	40	+/-	Transfer Pulse

Cell viability assay was then performed by color reaction using calcein (LIVE/DEAD Viability/Cytotoxicity Kit®, Thermo Fisher Scientific) 48 hours after the transfection. Intensity of color was measured by ARVO® (Perkin Elmer Japan).

Transfection of shRNAs

Plat-A packaging cells were transiently transfected with each retrovirus vector. The supernatants were used for infection. Gene-specific short hairpin RNAs (shRNAs) were designed by using pSIREN-RetroQ-ZsGreen and pSIREN-RetroQ-DsRed vectors. Empty vector was used as control shRNA. Specific shRNA targeting EED and BRD4 were designed using pSIREN-RetroQ-ZsGreen, and GTF2I was cloned into pSIREN-RetroQ-DsRed vector. Infected leukemia cells were purified by flow cytometry. The target sequences are shown in Table 3.

Table 3 Sequence of shRNAs

shRNA			Sequence
EED	1	S	GCAGTGACGAGAACAGCAATTTCAAGAGAATTGCTGTTCTCGTCACTGTTTTTTACGCGT
		A	ACGCGTAAAAAACAGTGACGAGAACAGCAATTCTCTTGAAATTGCTGTTCTCGTCACTGC
	2	S	ACGAGAACAGCAATCCAGATTCAAGAGATCTGGATTGCTGTTCTCGTTTTTTTACGCGT
		A	ACGCGTAAAAAAACGAGAACAGCAATCCAGATCTCTTGAATCTGGATTGCTGTTCTCGT
BRD4	1	S	GAGTGAAGAGGAAAGCAGATTCAAGAGATCTGCTTTCCTCTTCACTCTTTTTTACGCGT
		A	ACGCGTAAAAAAGAGTGAAGAGGAAAGCAGATCTCTTGAATCTGCTTTCCTCTTCACTC
	2	S	GCGACTACTGTGACATCATCTTCAAGAGAGATGATGTCACAGTAGTCGTTTTTTACGCGT
		A	ACGCGTAAAAAACGACTACTGTGACATCATCTCTCTTGAAGATGATGTCACAGTAGTCGC
GTF2I	1	S	GTGTAAAGAACTGGCCAAGTTCAAGAGACTTGGCCAGTTCTTTACACTTTTTTACGCGT
		A	ACGCGTAAAAAAGTGTAAAGAACTGGCCAAGTCTCTTGAACTTGGCCAGTTCTTTACAC
	2	S	GCTGAAAGAGGACGTGCTTTTTCAAGAGAAAAGCACGTCCTCTTTCAGTTTTTTACGCGT
		A	ACGCGTAAAAAACCCCGAGAACTATGATCTTTCTCTTGAAAAGATCATAGTTCTCGGGGC

S: Sense, A: Anti-sense

CRISPR/Cas9-mediated knockouts

Two single-guide (sg) RNAs were designed for each target gene using CHOPCHOP (https://chopchop.cbu.uib. no). Designed sgRNAs were cloned into the PX458 vector. Transfection was performed by using electroporation, and then 48 hours later, transfected cells were purified by flow cytometry. Target sequence of each sgRNA was shown in Table 4.

Table 4 Target sequence of sgRNAs

sgRNA	Target sequence
MLL3	ATTGACGCGACGCTCACAGGAGG
	TGTACACACCACGGTTCTGAGGG
MLL5	AGTGGTGTAGTTAAATCTGTTGG
	GTGAGTTGGTGTAGCATACGGGG

Flow cytometric analysis

Purification of transfected cells, apoptotic analysis, and cell proliferation analysis were performed using FACSAria 3 cell sorter (Becton Dickson).

Cell culture

KG-1 and F-36P were human leukemia cell lines with monosomy 7, while K562 and OCI-AML2 were human leukemia cell lines without monosomy 7. KG-1 and K562 cell lines were cultured in RPMI supplemented with 10% fatal bovine serum (FBS) and 1% penicillin (PS). OCI-AML2 cell line was cultured in MEM-alfa supplemented with 20% FBS and 1% PS. F-36P cell line was cultured in RPMI supplemented with 10% fatal bovine serum (FBS), 1% PS, and 10 ng/ml recombinant human granulocyte macrophage colony stimulating factor (hGM-CSF). Plat A was cultured in DMEM supplemented with 10% FBS, and antibiotics including PS and blasticidin.

Apoptosis and cell proliferation analysis

Apoptosis analysis was performed by using Annexin-APC (Invitrogen). Flow cytometric analysis detected the proportion of annexin-V-positive cells. Cell proliferation was evaluated by using CFSE Cell Division Assay Kit (Cayman). The change of fluorescence intensity was detected by flow cytometry.

RNA extraction, qRT-PCR

After extraction of total RNA with RNAeasy reagents (QIAGEN), reverse transcription was performed with ReverTra Ace qPCR RT Master Mix (TOYOBO). Quantitative reverse transcription PCR (qRT-PCR) was carried out in the LightCycler480 system (Roche) with THUNDERBIRD SYBR qPCR Mix according to the manufacturer's instructions (TOYOBO). Each assay was performed in triplicate and the results were normalized to 18s revels. Primer sequences are listed in Table 5.

Table 5 Primer sequence of qRT-PCR

Gene name		Sequence
18s	Fw	CCGATTGGATGGTTTAGTGAG
	Rv	AGTTCGACCGTCTTCTCAGC
EED	Fw	AATCCGGTTGTTGCAATCTT
	Rv	CAGAGGATGGCTCGTATTGC
GTF2I	Fw	TGGTCGTGTGATGGTAACAGA
	Rv	TTGATGGGGCCACAACTT
TP53	Fw	TTCTGTCCCTTCCCAGAAAA
	Rv	ACAGACTTGGCTGTCCCAGA
CASP3	Fw	TGGAATTGATGCGTGATGTT
	Rv	TCCAAAAATTATTCCTTCTTCACC
BAX	Fw	AGCAAACTGGTGCTCAAGG
	Rv	CTTGGATCCAGCCCAACA
BCL2	Fw	AGTACCTGAACCGGCACCT
	Rv	GCCGTACAGTTCCACAAAGG
BRD4	Fw	ATCTCAACCAGCACGCAGT
	Rv	ATGGCTGCTGGGGTAGTG

Antibodies and western blot analysis

For protein detection, cells were lysed with lysis buffer. Lysis buffer consisted with 10 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 0.1% Aprotinnin, 1 mM Na3NO4, 50 mM β-glycerophosphate, 2.5 mM phenylmethylsulfonylsluoride, and complete protease inhibitor cocktail (Roche Diagnostics). After 30 min at 4 degrees Celsius, lysates were centrifuged for 10 min. Supernatant was boiled with sample buffer (0.1% Tris-HCl, 4% SDS, 20% Glycerol, 7.5% bromophenol blue) at 95 degrees Celsius for 5 min. The extracted protein was subjected to sodium dodecylsulfate-polyacrylamide gel (10% for protein detection and 15% for histone detection) electrophoresis (SDS-PAGE) and analyzed by western blotting. Membranes were blocked with 5% BSA in PBS for 60 min and probed with primary antibodies at 4 degree Celsius for overnight. After washing the membranes, secondary antibodies were reacted for 3 hours at room temperature. Antibodies used in western blot analysis against beta-actin and EED were purchased from Cell Signaling, anti-rabbit IgG-HRP was purchased from Santa Cruz Biotechnology.

Inhibitors

We purchased bromodomain inhibitor JQ-1 from Sigma-Aldrich, A395 from MedChemExpress, and PTC209 from Cayman. A395 is a specific inhibitor that blocks EED binding to PRC2 complex [30]. PTC209 is a BMI-1 inhibitor and used as non-specific PRC1 inhibitor [31]. All inhibitors were dissolved in dimethyl sulfoxide.

FISH analysis

Fluorescence in situ hybridization (FISH) analysis was performed to investigate the deletion of chromosome 7 by Chromosome Science Labo (CSL), Two 7p11.2 probes and four 7q34 probes were used to check the chromosomal status.

Statistical analysis

Differences between two groups were assessed with a two-tailed unpaired t test. Statistical analyses were performed with EZR (modified R software) version 1.32 (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [32]. The level of significance was determined as two-tailed with $p<0.05$.

Results

RNA-interference-based screening detected EED, BRD4 as novel therapeutic targets in AML with monosomy 7

Based on the concept of synthetic lethality (Figure 2a), I hypothesized that AML cells with monosomy 7 are specifically vulnerable to the gene that is in synthetic lethal relationship with gene(s) within the chromosome 7. To test this, I performed an RNA-interference (RNAi)-based screening by targeting 53 genes encoding epigenetic modulator regulating histone methylation by using two AML cell lines with monosomy 7 (KG-1 and F-36P) and one control cell line (K562), shown in Figure 2b. Before screening, FISH analysis was performed to confirm the deletion of chromosome 7 in KG-1 and F-36P (Figure 2c). Deletion of 7q was observed in 87% of KG-1 cells and 97% of F-36P cells. None of K562 cells showed deletion of 7q (Figure 2c). Because EZH2 and MLL3 in chromosome 7 were related to the methylation of H3K27 and H3K4 respectively, 53 genes consisted with histone methylation genes associated with H3K27 or H3K4. Three siRNA were designed for each target gene. The first screening detected 12 genes which showed the lower cell viability of monosomy 7 cell lines compared to that of non-monosomy 7 cell lines ($p<0.25$) (Figure 2d). The secondary screening was then performed for these 12 genes with the same method. As a result,

inhibition of EED, BRD4 or BRDT significantly decreased cell viability in AML cells with monosomy 7 compared to the control. Detailed data of RNAi-based screening was shown in Table 6.

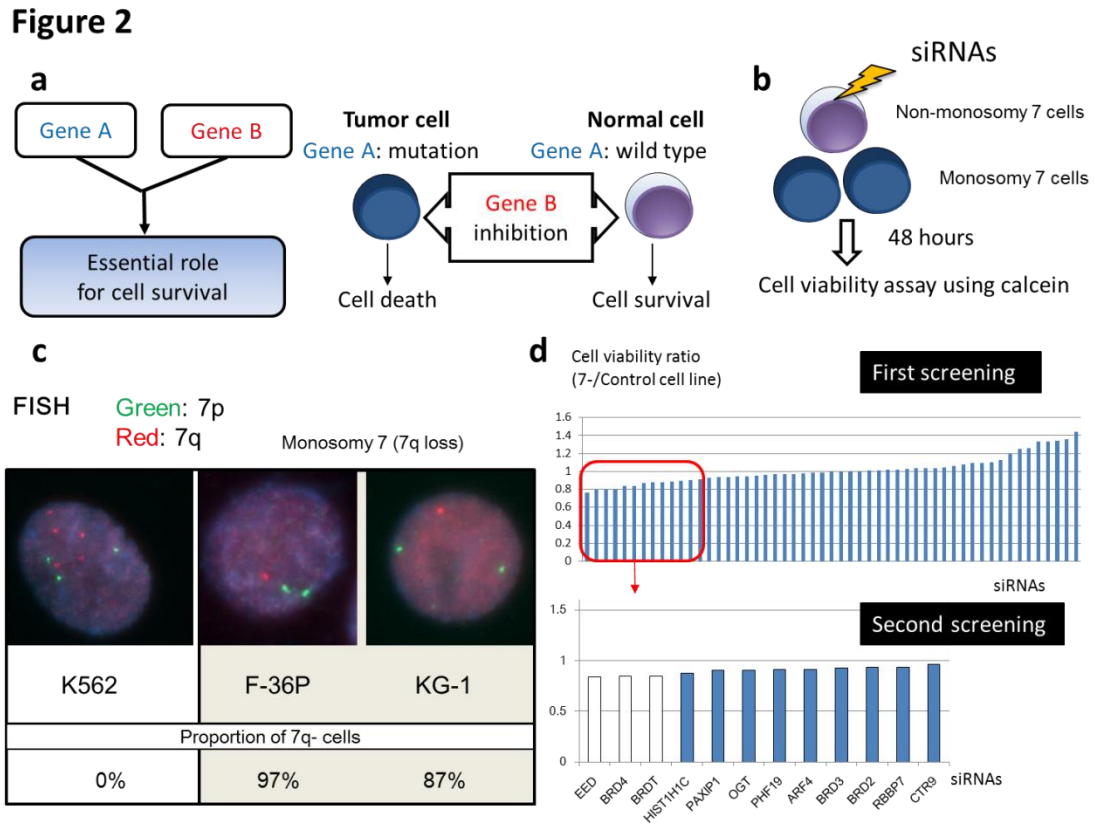


Figure 2 Synthetic lethality-based approach identified EED and BRD4 as essential survival factors in AML with monosomy 7.

(a) Concept of synthetic lethality. **(b)** Scheme of RNAi screening. **(c)** Validation of monosomy 7 in F-36P and KG-1 using FISH analysis. **(d)** Cell viability ratio in cell lines with monosomy 7 (KG-1 and F-36P) compared to control cell line (K562).

Table 6 Detailed data of RNAi-based screening

a. Viability of siRNA-transfected cells relative to that of the control siRNA-transfected cells (%) in the first screening

	Leukemia cell lines						
	Monosomy 7						
siRNAs	KG-1	F-36P	K562	siRNAs	KG-1	F-36P	K562
Control	100	100	100	TET2-1	89.67	79.83	88.5
SETD1B-1	64.84	64.44	63.85	TET2-2	75.11	81.3	78.54
SETD1B-2	64.47	63.74	55.96	TET2-3	71.11	75.26	76.63
SETD1B-3	48.11	64.04	65.77	ASH1L-1	72.08	82.5	79.65
AEBP2-1	216.09	96.9	101.57	ASH1L-2	61.54	77.94	70.21
AEBP2-2	149.89	89.43	98.5	ASH1L-3	59.47	69.47	69.14
AEBP2-3	124.42	88.81	84.56	KMT2E-1	103.91	88.52	106.47
EZH1-1	110.44	88.72	86.26	KMT2E-2	101.69	91.18	94.57
EZH1-2	160.21	85.58	95.58	KMT2E-3	99.71	87.22	102.53
EZH1-3	142.11	72.89	81.56	BEND3-1	61.22	84.43	86.23
MTF2-1	129.83	66.17	72.49	BEND3-2	92.23	81.2	87.56
MTF2-2	89.62	68.94	58.45	BEND3-3	75.55	79.28	76.28
MTF2-3	89.62	74.75	74.76	ARID4A-1	60.54	85.94	81.3
SIRT1-1	91.31	99.28	99.42	ARID4A-2	80.18	78.91	60.36
SIRT1-2	108.25	91.18	89.64	ARID4A-3	67.41	81.31	83.14
SIRT1-3	97.97	77.66	84.05	RBBP5-1	104.01	86.48	94.89
SUZ12-1	88.69	77.76	73.94	RBBP5-2	98.12	96.42	81.22
SUZ12-2	67.72	78.37	81.09	RBBP5-3	96.18	92.93	87.13
SUZ12-3	92.1	55.94	81.73	ZNF335-1	96.18	80	90.88
PHF19-1	70.2	73.11	70.91	ZNF335-2	91.53	84.79	81.17
PHF19-2	70.24	61.78	82.09	ZNF335-3	88.74	78.99	82.32
PHF19-3	74.13	55.51	74.69	SETMAR-1	75.03	71.47	80.93
HDAC2-1	97.53	87.4	88.32	SETMAR-2	65.41	76.69	69.32
HDAC2-2	102.84	71.82	83.89	SETMAR-3	60.97	69.84	73.64
HDAC2-3	102.43	64.06	86.46	UTX-1	96.85	85.55	95.52
JARID2-1	82.69	77.05	80.47	UTX-2	95.21	91.59	89.79
JARID2-2	90.29	75.67	75.15	UTX-3	96.44	86.6	90.48
JARID2-3	84.74	84.81	70.36	WDR82P1-1	85.8	93.03	95.91
TRIM37-1	88.55	45.31	68.9	WDR82P1-2	84.15	69.13	84.8
TRIM37-2	87.69	46.65	66.28	WDR82P1-3	79.57	76.7	81.72
TRIM37-3	75.34	52.91	64.97	WDR61-1	115.1	88.87	96.81
PHF1-1	118.51	91.57	99.78	WDR61-2	111.55	74.01	97.63
PHF1-2	119.65	85.96	91.57	WDR61-3	102.37	81.65	85.42
PHF1-3	101.69	76.84	96.07	KMT2B-1	111.73	72.83	94.59
H2AFY2-1	102.24	77.01	84.55	KMT2B-2	87.47	86.04	87.22
H2AFY2-3	104.72	83.66	71.92	KMT2B-3	97.67	88.11	65.47
H2AFY2-3	103.95	74.69	71.09	MLL2-1	75.67	69.94	76.35
RBBP4-1	90.36	54.03	50.15	MLL2-2	83.76	66.57	No data
RBBP4-2	88.18	72.42	56.53	MLL2-3	74.05	80.16	68.65
RBBP4-3	87.84	68.7	53.52	SETD3-1	100.27	84.72	80.07

RBBP7-1	99.46	79.88	93.66	SETD3-2	80.95	96.78	84.38
RBBP7-2	65.85	89.22	95.59	SETD3-3	97.22	91.57	79.58
RBBP7-3	79.18	89.39	99.23	DYDC2-1	87.9	77.33	79.2
EED-1	67.19	75.38	74.92	DYDC2-2	70.01	87.46	73.94
EED-2	62.62	77.89	94.15	DYDC2-3	70.35	80.95	80.82
EED-3	56.61	83.65	93.58	DPY30-1	78.98	77.08	69.21
H2AFY-1	56.08	87.2	78.79	DPY30-2	87.05	70.58	73.22
H2AFY-2	64.23	79.61	70.86	DPY30-3	87.04	58.56	74.7
H2AFY-3	40.2	85.32	69.63	OGT-1	89.91	86.18	106.34
WDR5-1	99	100.5	81.5	OGT-2	77.7	95.32	85
WDE5-2	101.63	112.8	82.56	OGT-3	80.14	92.42	105.18
WDR5-3	114.63	108.7	75.3	ASH2L-1	77.84	75.29	77.91
DYDC1-1	98.45	104	72.46	ASH2L-2	80.96	82.85	90.33
DYDC1-2	92.46	98.16	75.15	ASH2L-3	76.29	85.39	76.86
DYDC1-3	85.93	92	66.2	CTR9-1	76.81	77.95	79.84
TET3-1	87.69	96.72	63.55	CTR9-2	57.22	67.45	76.93
TET3-2	81.19	84.08	57.36	CTR9-3	74.4	74.22	78.54
TET3-3	81.15	71.78	63.81	SETD1A-1	99.23	84.22	91.46
PAXIP1-1	86.24	102.6	95.7	SETD1A-2	84.55	82.11	86.77
PAXIP1-2	46.42	96.01	104.06	SETD1A-3	96.26	80.32	87.25
PAXIP1-3	86.03	83.42	97.61	ARID1A-1	78.72	76.21	84.47
RTF1-1	36.62	77.63	84.25	ARID1A-2	67.82	78.58	81.2
RTF1-2	77.75	81.21	81.9	ARID1A-3	105.14	80.14	81.97
RTF1-3	87.62	67.79	78.66	CDK4-1	92.81	68	72.81
HIST1H1C-1	80.42	55.22	71.55	CDK4-2	98.41	67.8	74.48
HIST1H1C-2	78.41	48.03	78.12	CDK4-3	81.46	69.71	82.17
HIST1H1C-3	52.08	55.78	79.16	ARF4-1	100.84	87.31	103.12
HIST1H1D-1	83.08	87.63	97.88	ARF4-2	81.51	78.87	99.13
HIST1H1D-2	105.74	79.14	94.56	ARF4-3	86.32	83.79	107.62
HIST1H1D-3	82.62	84.81	82.19	BRD2-1	81.81	84.28	95.26
HIST1H1E-1	84.3	79.13	72.99	BRD2-2	90.18	85.22	90.4
HIST1H1E-2	98.88	88.38	77.47	BRD2-3	75.64	85.66	92.66
HIST1H1E-3	57.92	77.41	75.64	BRD3-1	75.45	73.56	84.75
CXXC1-1	79.34	75.37	73.56	BRD3-2	79.93	66.2	75.27
CXXC1-2	58.15	69.5	71.12	BRD3-3	63.58	65.82	77.05
CXXC1-3	63.87	67.36	68.05	BRD4-1	83.84	93.46	102.18
MLL-1	78.22	92.99	84.03	BRD4-2	89.33	91.2	112.59
MLL-2	86.93	90.68	86.3	BRD4-3	73.16	87.3	110.64
MLL-3	95.12	96.12	99.26	BRDT-1	73.8	84.42	106.65
				BRDT-2	77.1	88.74	105.32
				BRDT-3	82.17	78.23	105.4

b. Viability of siRNA-transfected cells relative to that of the control siRNA-transfected cells (%) in the secondary screening

	Leukemia cell lines						
	Monosomy 7						
siRNAs	KG-1	F-36P	K562	siRNAs	KG-1	F-36P	K562
Control	100	100	100	OGT-1	90.13	94.73	98.85
RBBP7-1	89.47	81.97	86	OGT-2	72.06	93.06	95.2
RBBP7-2	90.68	84.54	94.67	OGT-3	105.18	87.37	98.07
RBBP7-3	96.54	81.85	79.23	BRDT-1	75.2	89.7	90.2
EED-1	89.06	81.23	95.83	BRDT-2	83.86	83.13	94.48
EED-2	76.3	80.01	89.49	BRDT-3	95.23	86.6	85.38
EED-3	74.29	73.63	87.01	CTR9-1	84.95	76.1	79.38
PHF19-1	73.15	70.8	85.97	CTR9-2	87.54	73.11	78.84
PHF19-2	64.93	66.31	69.01	CTR9-3	74.38	72.35	72.24
PHF19-3	75.53	69.66	70.98	BRD4-1	94.76	91.7	106.32
PAXIP1-1	83.75	89.97	97.8	BRD4-2	98.72	84.32	99.56
PAXIP1-2	83.87	92.07	94.5	BRD4-3	82.09	85.13	90.53
PAXIP1-3	79.01	89.5	74.1	ARF4-1	90.14	81.34	90.68
BRD2-1	77.53	88.23	89.6	ARF4-2	92.98	71.16	87.97
BRD2-2	83.6	85.65	80.52	ARF4-3	92.57	77.13	72.68
BRD2-3	72.16	78.76	79	HIST1H1C-1	76.01	72.69	74.76
BRD3-1	78.08	81.51	78.12	HIST1H1C-2	78.82	69.01	77.49
BRD3-2	61.27	80.26	72.39	HIST1H1C-3	62.15	68.83	73.55
BRD3-3	61.03	72.72	76.64				

Inhibition of EED resulted in increased apoptotic cells in AML with monosomy 7

Based on the screening results, we focused on EED and BET bromodomain proteins for further analysis. To validate the screening results, I stably transduced leukemia cells with the shRNAs against EED and analyzed the influence on proliferation. The knockdown of EED was confirmed by qRT-PCR and western blotting (Figure 3a, b).

Figure 3

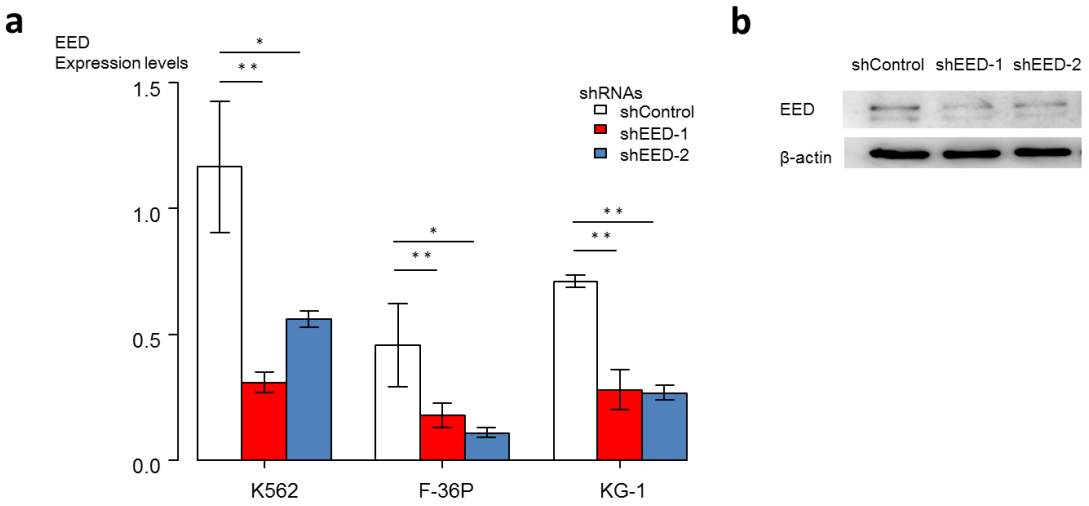


Figure 3 Confirmation of EED knockdown mediated by shRNAs.
(a) Expression levels of EED by qRT-PCR in EED-knockdown cells compared to shControl. * p<0.05, ** P<0.01 (n=3, two-sided t-test, error bars: mean±s.d.) **(b)** Western blotting analysis on knockdown of EED in KG-1 cells.

The EED knockdown significantly decreased the number of cells with monosomy 7 compared to control cells (Figure 4a). I next investigated frequency of apoptosis in leukemia cells. Knockdown of EED resulted in increased apoptotic cells specifically in monosomy 7 cells (Figure 4b). These results suggest that the inhibition of EED preferentially affects viability of monosomy 7 leukemia cells through induction of apoptosis.

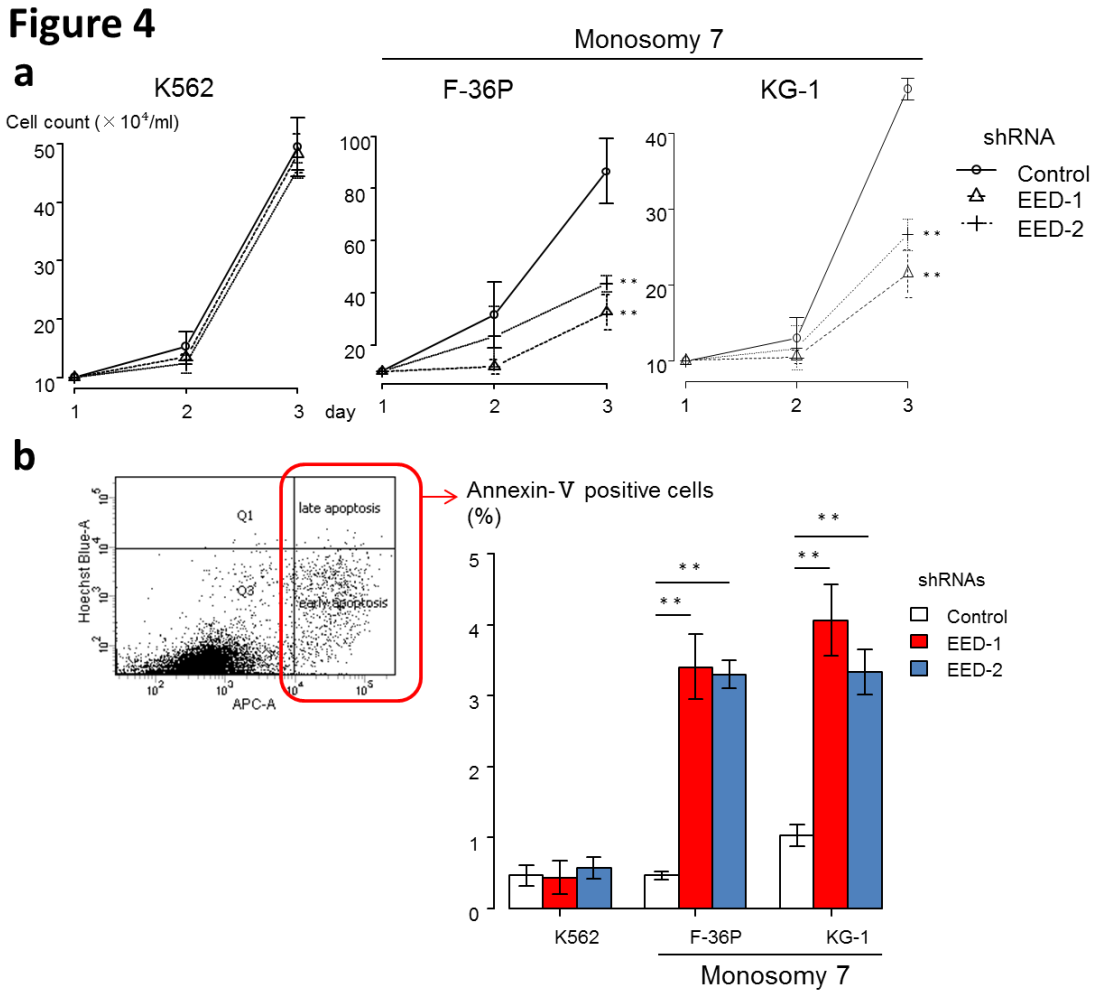


Figure 4 Stable knockdown of EED significantly decreased cell counts in AML with monosomy 7 via increased cell apoptosis.

(a) Stable knockdown of EED mediated by shRNAs and cell growth in monosomy 7 cell lines (F-36P, KG-1) and control cell line (K562). **(b)** Knockdown of EED increased cell apoptosis in leukemia cells with monosomy 7. * $p<0.05$, ** $P<0.01$ (n=3, two-sided t-test, error bars: mean±s.d.)

PRC1 rather than PRC2 activity of EED is essential for cell survival in AML with monosomy 7

The pharmacological inhibition of EED activity was then performed. Previous reports described that EED as well as EZH2 is an important component of PRC2 [33, 34]. I hypothesized that EED inhibition critically downregulates PRC2 activity specifically in monosomy 7 leukemia cells since PRC2 activity is inherently reduced in those cells due to haploinsufficient EZH2. Contrary to our notion, pharmacologic inhibition of EED by A395, which blocks the binding of EED to the PRC2 [30], was only marginally effective for the monosomy 7 leukemia cells (Figure 5a). These results suggest that EED supports survival of the monosomy 7 leukemia cells in a PRC2-independent manner. Recent studies showed that EED is not only a component of PRC2 but also interacts with the polycomb repressive complex 1 (PRC1) [35]. In contrast to modest effects of PRC2 inhibition, inhibition of PRC1 activity by PTC209 showed marked efficacy specifically in leukemia cells with monosomy 7 (Figure 5b). Similar to stable knockdown of EED, inhibition of PRC1 resulted in increased apoptotic cells in monosomy 7 cells (Figure 5c). These results collectively suggest that the PRC1 rather than PRC2 activity of EED has a predominant role in the survival of monosomy 7 leukemia cells.

Figure 5

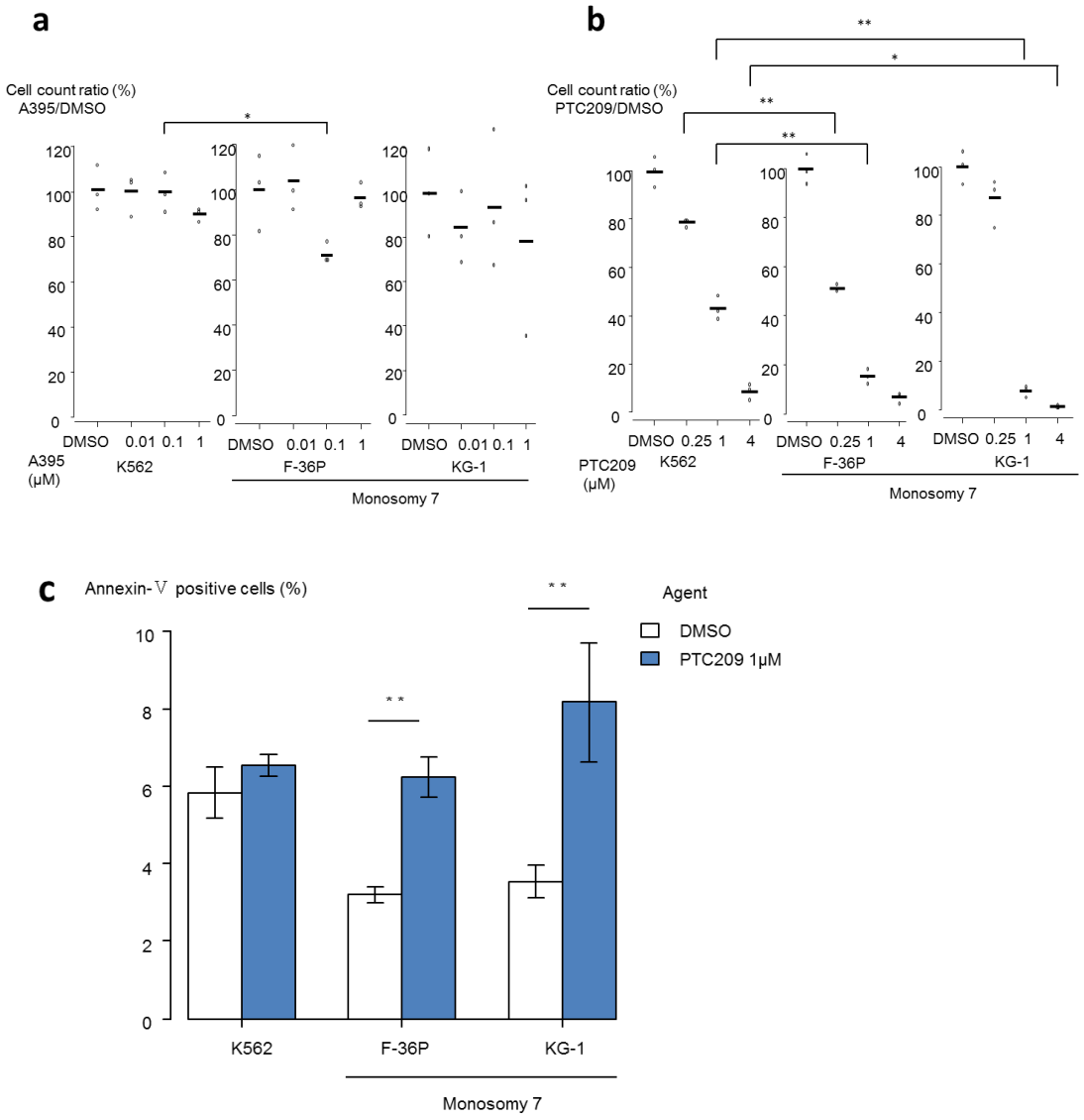


Figure 5 PRC1 rather than PRC2 activity seemed to be important for cell survival in leukemia cells with monosomy 7 cells.

(a) Cell counts ratio (%) in each concentration of A395 compared to DMSO 48 hours after administration. **(b)** Cell counts ratio (%) in each concentration of PTC209 compared to DMSO 48 hours after administration. **(c)** Proportion of Annexin-V positive cells after PTC209 treatment in leukemia cells with monosomy 7 (KG-1 and F-36P) and control cells (K562). * $p<0.05$, ** $P<0.01$ (n=3, two-sided t-test, error bars: mean± s.d.)

Haplo-insufficiency of GTF2I in chromosome 7 increased the EED dependency via increased cell apoptosis

I next explored whether the deletion of specific genes within the chromosome 7 was responsible for increased sensitivity to EED inhibition. EED was stably knocked down in K562 cells (monosomy 7-negative cells), and then siRNAs against 22 genes encoding epigenetic regulators located on the chromosome 7 were individually transfected into the cells (Figure 6a). Among the tested genes, knockdown of ACTL6B and GTF2I decreased the viability most prominently (Figure 6b). Because the expression levels of ACTL6B in leukemia cells were extremely low, GTF2I was picked-up as a candidate gene that increased EED dependency. Stable knockdown of GTF2I mediated by shRNA resulted in decreased cell viability in EED-knocked-down K562 cells compared to that in the control K562 cells. These results suggested that heterozygous deletion of GTF2I may be associated with the vulnerability to EED inhibition. Detailed data was shown in Table 7. I validated these results by using a different monosomy 7-negative cells OCI-AML2 and found that knockdown of GTF2I resulted in increased sensitivity to EED inhibition (Figure 6c). As was seen in monosomy 7 cells, GTF2I knocked-down cells showed the increased apoptosis when activity of EED was inhibited (Figure 6d).

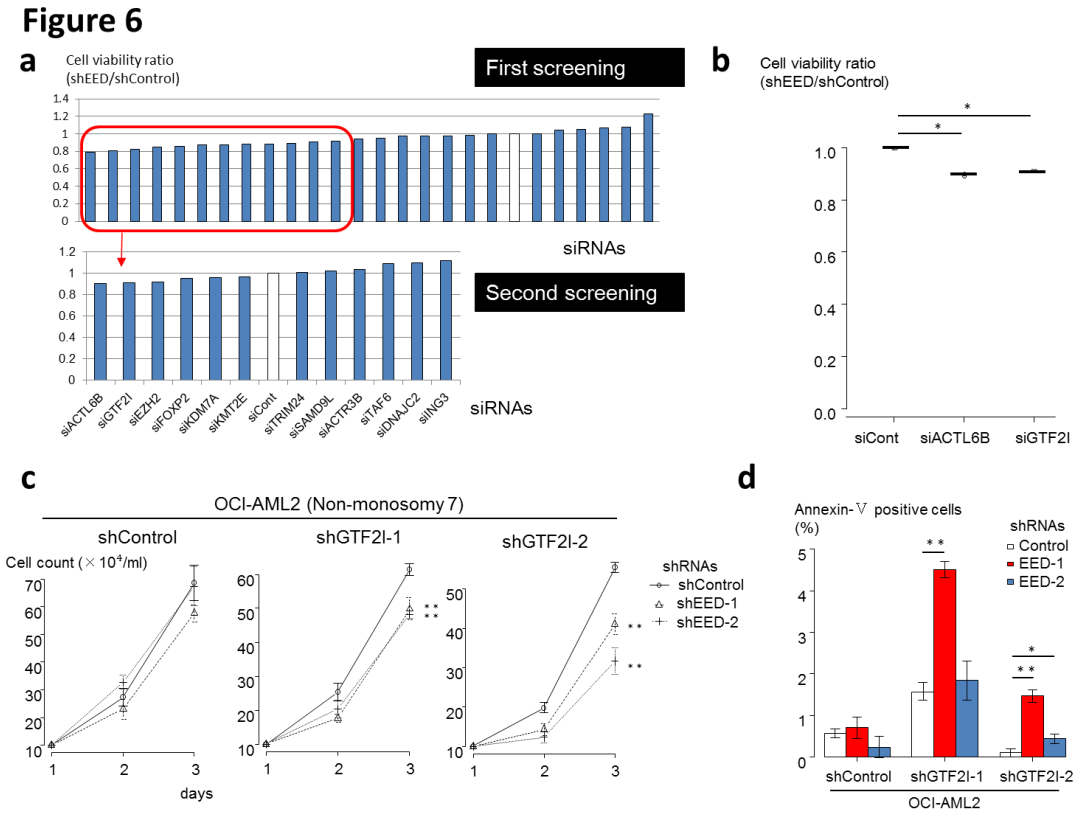


Figure 6 Investigation for responsible genes providing EED dependency.

(a) RNAi screening to detect genes that increased EED dependency. **(b)** Cell viability ratio of K562_shEED compared to K562_shControl after siRNAs transfection (n=2, two-sided t-test). **(c)** Relationships between EED dependency and stable knockdown of GTF2I (n=3, two-sided t-test, error bars: mean±s.d.). **(d)** Ratio of annexin-V positive cells after EED knockdown in GTF2I-knockdown-leukemia cells (n=3, two-sided t-test, error bars: mean±s.d.). * $p<0.05$, ** $P<0.01$.

Table 7 Individual numerical data in RNAi-based screening for candidate genes

(a) Relative cell viability (%) in first screening **(b)** Relative cell viability (%) in secondary screening

A

	K562	
siRNA	shEED	shControl
Control	100	100
ACTL6B	89.65	101.05
DNAJC2	90.12	112.27
ING3	93.39	110.06
TAF6	92.88	113.13
SAMD9L	86.45	99.04
ACTR3B	80.18	101.51
EZH2	78.42	89.5
KDM7A	70.33	79.39
TRIM24	72.12	81.46
DOCK4	93.6	87.25
CUX1	89.22	85.8
SMARCD3	88.44	83.04
GTF2I	74.43	81.45
LRWD1	78.61	74.97
LUC7L2	79.44	79.31
BAZ1B	71.17	72.29
GATAD1	72.43	74.19
PRKAG2	73.41	75.56
CDK5	108.19	87.84
KMT2E	80.65	88.79
TRPAP	80.06	82.08
CUL1	77.86	82.73
KMT2C	75.39	75.54
FOXP2	68.83	80.68
UBE2H	70.64	74.59

B

	K562	
siRNA	shEED	shControl
Control	100	100
ACTL6B	76.3	84.45
GTF2I	76.52	84.27
EZH2	87.31	95.28
FOXP2	78.6	82.62
KDM7A	84.62	88.3
KMT2E	75.37	78.17
TRIM24	82.77	82.2
SAMD9L	83.31	81.52
ACTR3B	96.02	92.93
TAF6	95.29	87.19
DNAJC2	94.78	86.57
ING3	87.47	78.21

BCL2 is a common target of the synthetic lethality between EED and GTF2I

Since inhibition of EED resulted in increased cell apoptosis in leukemia with monosomy 7, I explored whether the expression levels of genes which regulate cell apoptosis were affected by inhibition of EED. In EED-knockdown cells, expression levels of BCL2 were reduced compared to the control cells (Figure 7a). Similarly, expression levels of BCL2 were decreased in GTF2I-knockdown cells (Figure 7b). Importantly, both EED and GTF2I additively downregulated the expression levels of BCL2 (Figure 7c). Previous reports suggested that BCL2 played an anti-apoptotic effect [36, 37]. It was possible that EED keep an activity of BCL2 that were down-regulated by haplo-insufficiency of GTF2I in monosomy 7 cells. (Figure 7d). Therefore, EED may be a promising therapeutic target in AML with monosomy 7.

Figure 7

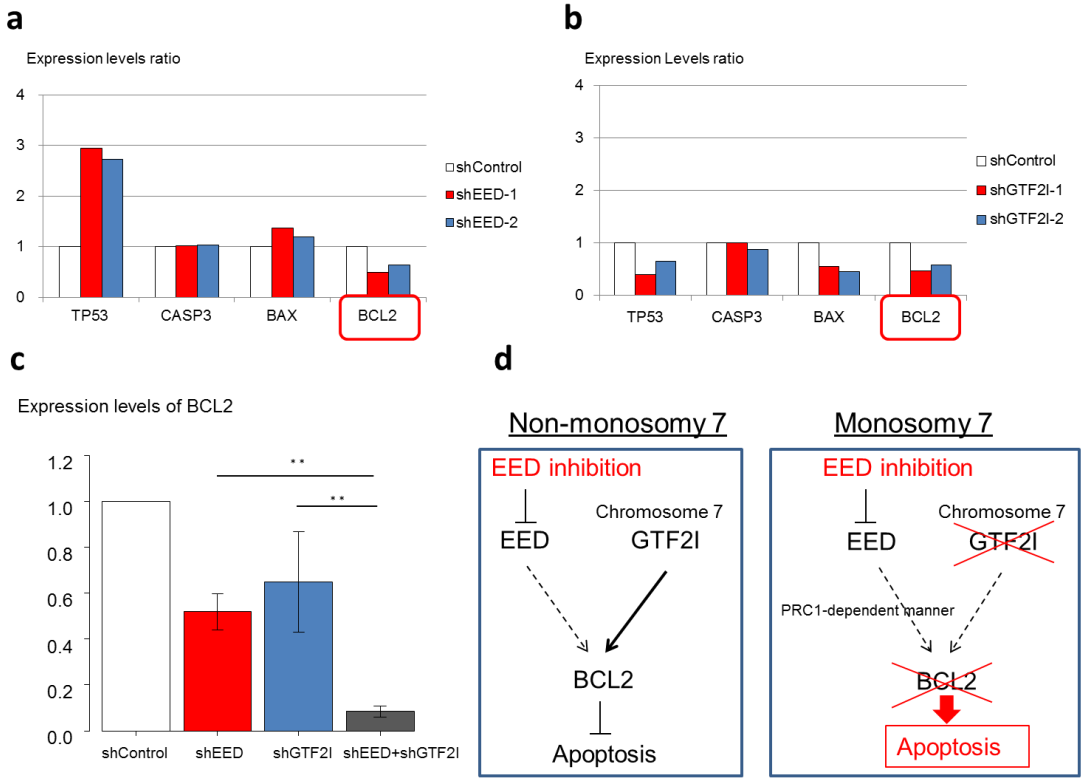


Figure 7 Both EED and GTF2I down-regulated the expression levels of BCL2. **(a)** Expression levels of apoptosis-related genes in EED-knockdown K562 cells. **(b)** Expression levels of apoptosis-related genes in GTF2I-knockdown K562 cells. **(c)** Expression levels of BCL2 in K562-shControl, K562-shEED, K562-shGTF2I, and K562-shEED-shGTF2I cells. * $p<0.05$, ** $P<0.01$ ($n=3$, two-sided t-test, error bars: mean±s.d.) **(d)** Summary of synthetic lethality between EED and GTF2I.

BRD4 is also a promising therapeutic target in AML with monosomy 7

In addition to EED, BRD4 or BRDT was identified as an essential survival factor in AML with monosomy 7 (Figure 1c). Both BRD4 and BRDT are members of bromodomain and Extra-terminal motif (BET) protein family. Because the expression levels of BRDT are extremely low in tissues other than testis [38], I first examined the efficacy of BRD4 inhibition. Stable knockdown of BRD4 was confirmed by qRT-PCR (Figure 8).

Figure 8

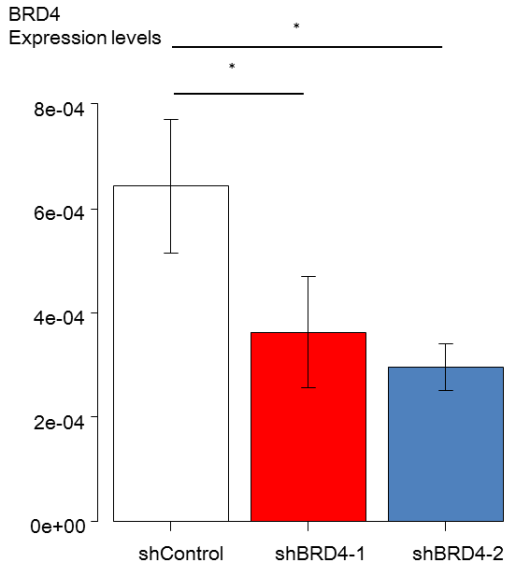


Figure 8 Expression levels of BRD4 in shRNA-mediated BRD4 knockdown cells.
Evaluation of expression levels of BRD4 in BRD4-knockdown F-36P cells. * $p<0.05$, ** $P<0.01$ (n=3, two-sided t-test, error bars: mean±s.d.)

Knockdown of BRD4 showed significant reduction in cell numbers specifically in leukemia cell lines with monosomy 7 (Figure 9a). Although inhibition of BET bromodomain proteins have already shown to be effective for a variety of AML [39-44], we showed that blockade of bromodomain proteins by JQ-1 preferentially affected cellular proliferation of AML cells with monosomy 7 (Figure 9b). In cell proliferation assay using CFSE, mean fluorescence intensity (MFI) was significantly increased by JQ-1 treatment in monosomy 7 cells (Figure 9c). In contrast to monosomy 7 cells, MFI was not increased by JQ-1 treatment in non-monosomy 7 cells (Figure 9c). These results indicated that JQ-1 induced cell cycle arrest in monosomy 7-specific manner.

Figure 9

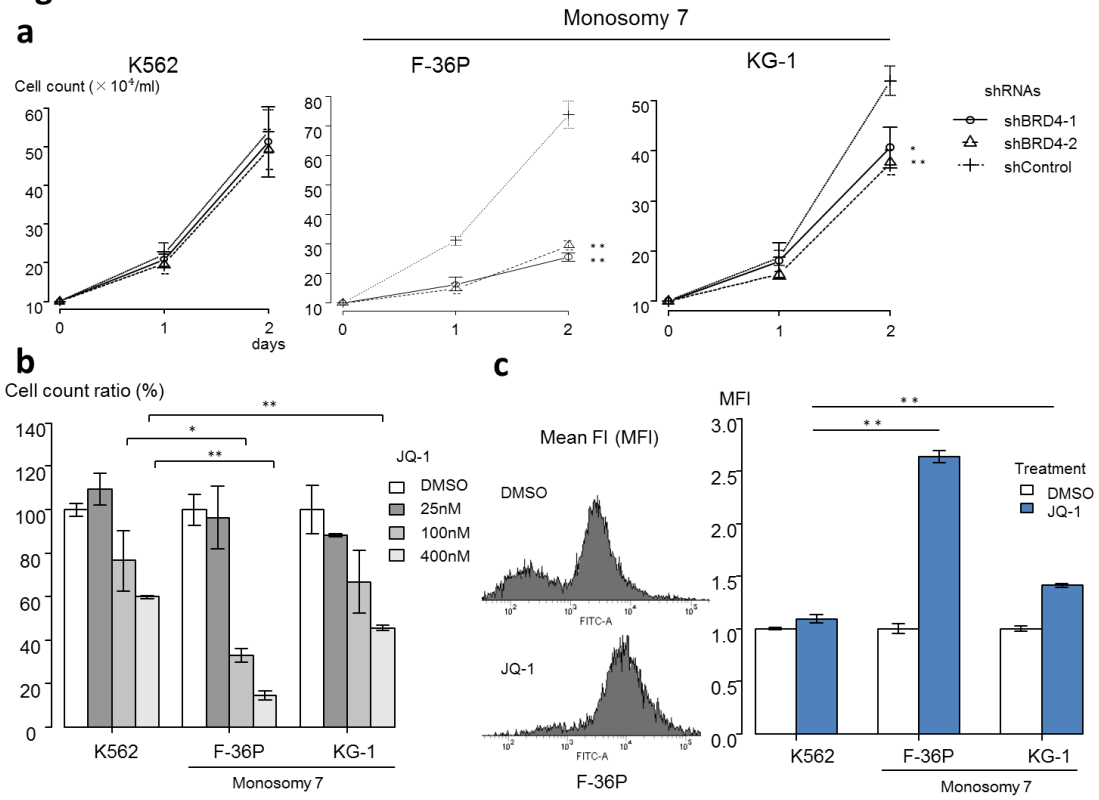


Figure 9 Inhibition of BRD4 activity decreased cell viability in leukemia cells with monosomy 7.

(a) Stable knockdown of BRD4 and cell counts in leukemia cell lines. **(b)** Cell counts ratio 72 hours after JQ-1 treatment in leukemia cell lines. **(c)** MFI after JQ-1 treatment compared to DMSO in monosomy 7 cells (F-36P and KG-1) and non-monosomy 7 cells (K562). * $p<0.05$, ** $P<0.01$ ($n=3$, two-sided t-test, error bars: mean±s.d.)

MLL3 and MLL5 were responsible genes that increased BRD4 dependency

I then explored whether the deletion of specific genes within the chromosome 7 was responsible for increased sensitivity to BET bromodomain protein inhibition. K562 cells were individually treated with the siRNAs against 22 genes in the presence of a BET bromodomain inhibitor JQ1 (Figure 10). Two independent RNAi-based screening found some candidate genes that increased JQ-1 sensitivity (Table 8). Among them, MLL3 (*KMT2C*) and MLL5 (*KMT2E*) are members of myeloid/lymphoid or mixed lineage leukemia (MLL) family and have histone methyltransferase activity for H3K4 [16-17, 45-46]. We hypothesized that inhibition of MLL activity may induced the vulnerability to JQ-1 treatment.

Figure 10

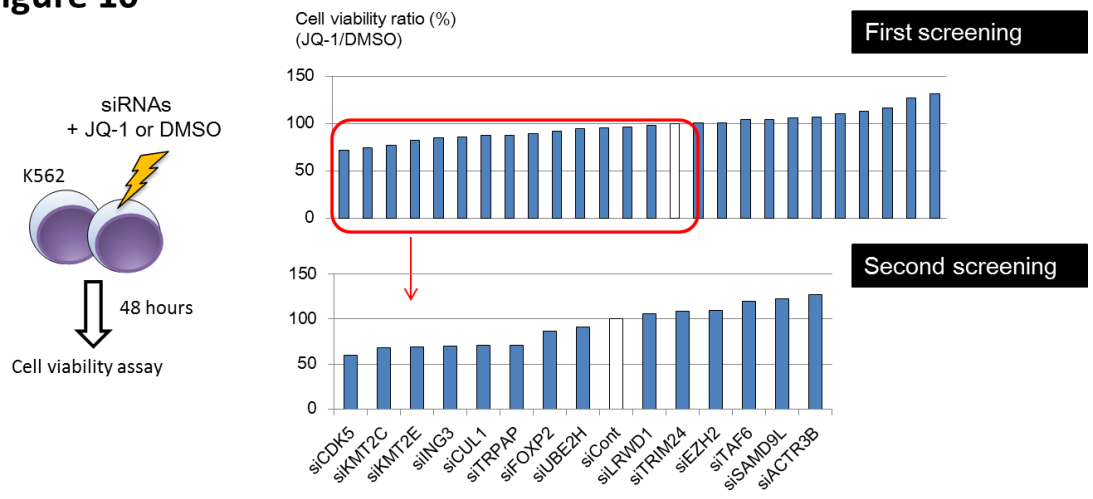


Figure 10 Investigation for responsible genes that increased JQ-1 sensitivity.

Table 8 Detailed data of RNAi-based screening for responsible gene in JQ-1 sensitivity

(a) Relative cell viability ratio (%) in first screening **(b)** Secondary screening

A

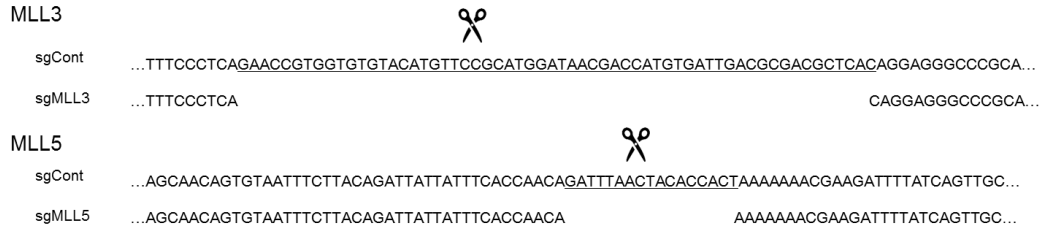
	K562	
siRNA	DMSO	JQ-1(+)
Control	100	100
ACTL6B	88.81	95.08
DNAJC2	96.33	97.17
ING3	91.31	78.17
TAF6	89.78	82.79
SAMD9L	80.72	71.73
ACTR3B	126.71	119.82
EZH2	113.72	111.64
KDM7A	104.07	104.4
TRIM24	102.12	98.11
DOCK4	91.35	119.82
CUX1	105.62	110.49
SMARCD3	88.33	97.37
GTF2I	83.53	94.33
LRWD1	92.05	87.93
LUC7L2	80.98	84.67
BAZ1B	73.11	77.48
GATAD1	62.87	79.8
PRKAG2	63.98	74.34
CDK5	108.64	89.11
KMT2E	106.63	90.61
TRPAP	114.13	84.55
CUL1	115.02	100.37
KMT2C	98.1	75.66
FOXP2	107.71	77.03
UBE2H	77.16	67.74

B

	K562	
siRNA	DMSO	JQ-1(+)
Control	100	100
FOXP2	109.48	95.19
TRPAP	133.63	95.07
KMT2C	114.46	77.77
CDK5	124.76	74.87
KMT2E	123.03	85.33
ING3	117.78	82.89
CUL1	112.91	80.19
UBE2H	92.17	84.43
SAMD9L	89.82	109.69
TAF6	87	104.09
ACTR3B	67.26	85.63
LRWD1	79.91	85.01
TRIM24	69.91	75.93
EZH2	66.14	72.77

To confirm this hypothesis, I firstly generated MLL3- and MLL5-knockout K562 (non-monosomy 7) cells using CRISPR-CAS9 system. Knockout was examined by target sequence (Figure 11).

Figure 11



Both MLL3- and MLL5-knockout K562 cells showed increased sensitivity to JQ-1 treatment (Figure 12a). Although JQ1 treatment was toxic to all the cell lines at high concentration, it affected the proliferation of MLL3 or MLL5-deficient cells when treated at 0.4 μM compared to the control (Figure 12a). In the cell proliferation assay, MFI was significantly increased by JQ-1 treatment in MLL3 or MLL5-deficient cells compared to the control cells (Figure 12b). Same as monosomy 7 cells, cell proliferation were significantly suppressed by JQ-1 treatment in MLL3 or MLL5-deficient cells (Figure 12b). It was possible that functional loss of MLL3 or MLL5 increase the JQ-1 sensitivity in monosomy 7 cells via strongly inhibiting cell proliferation.

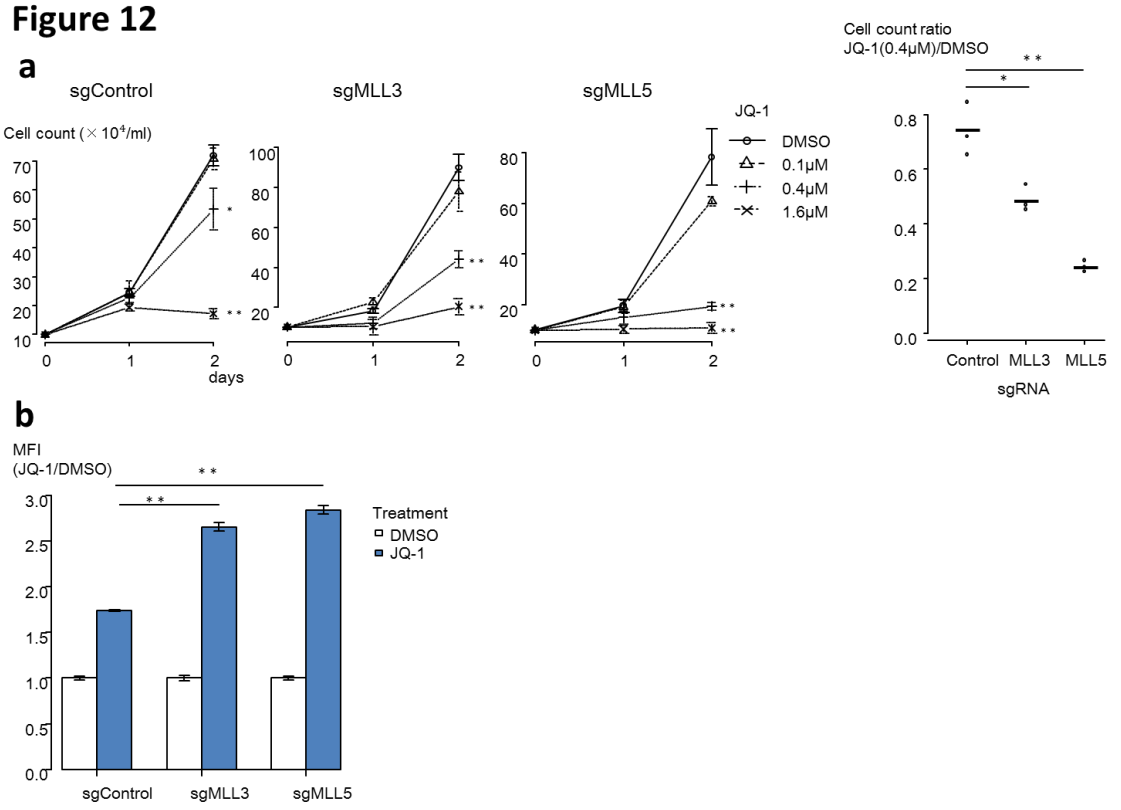


Figure 12 Knockout of MLL3 or ML5 increased BRD4 dependency.

(a) Relationships between JQ-1 sensitivity and knockout of MLL3 or MLL5 in leukemia cells. **(b)** MFI ratio (JQ-1/DMSO) 72 hours after JQ-1 treatment in K562 cells with sgControl, sgMLL3, and sgMLL5. * $p<0.05$, ** $P<0.01$ (n=3, two-sided t-test, error bars: mean±s.d.)

Based on this notion, expression levels of genes that are associated with cell cycle regulation were examined. Expression levels of c-MYC and CCND1 were significantly reduced by JQ-1 treatment especially in MLL3- and MLL5-knockout leukemia cells (Figure 13a). These results indicated that single allele deletion of MLL3 or MLL5 increased JQ-1 via abrogating CCND1 and c-MYC activity in monosomy 7 cells. Figure 13b showed the hypothesis of the synthetic lethality between BRD4 and MLL3 or MLL5.

Figure 13

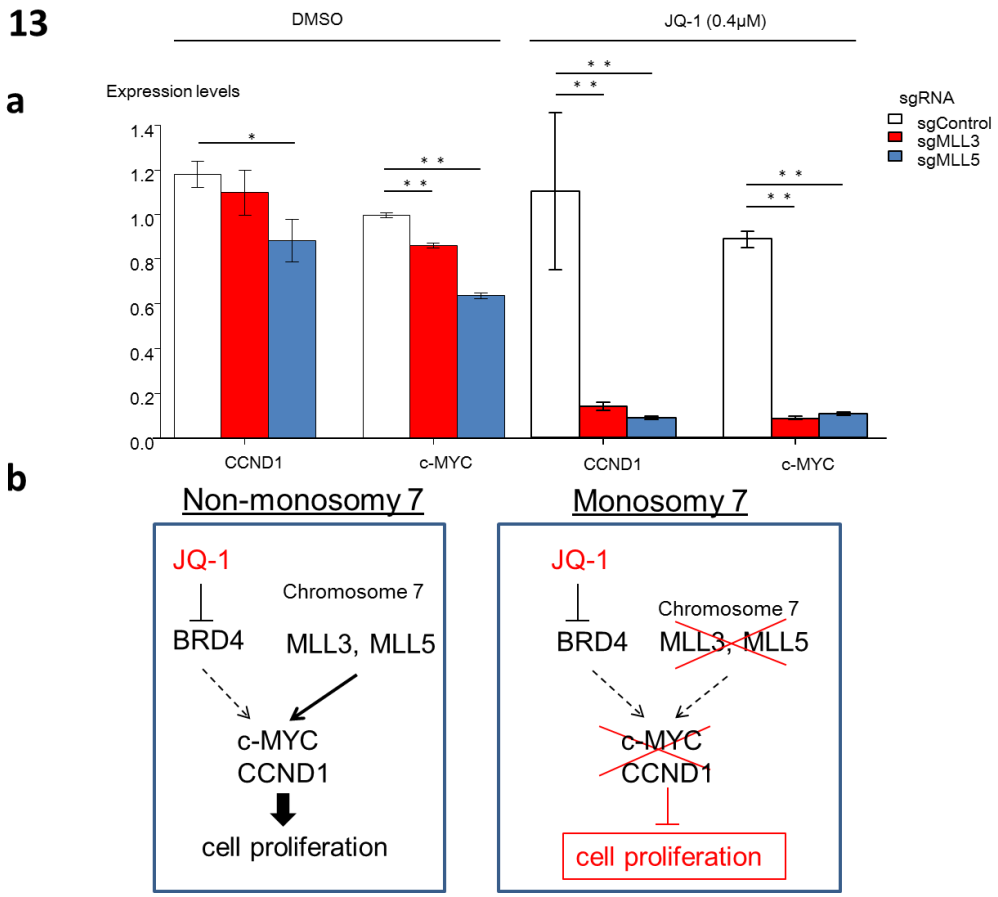


Figure 13 Knockout of MLL3 or ML5 additively decreased expression levels of c-MYC and CCND1.

(a) Expresion levels of c-MYC and CCND1 in JQ-1 treated and no-treatment K562 (non-monosomy 7) cells. **(b)** Hypothesis of synthetic lethality between BRD4 and MLL3 or MLL5. * $p<0.05$, ** $P<0.01$ (n=3, two-sided t-test, error bars: mean±s.d.)

Discussion

In this study, I demonstrated that EED and BRD4 were promising therapeutic targets in AML with monosomy 7 by the synthetic lethality-based approach. Haplo-insufficiency of GTF2I located on chromosome 7 induced EED dependencies via vulnerability to cell apoptosis. BRD4 played an essential role for cell survival in leukemia with monosomy 7 induced by lower expression levels of MLL3 or MLL5. Summary of the both synthetic lethality was shown in Table 9.

Table 9 Summary of the synthetic lethality in AML with monosomy 7

Therapeutic target	Responsible gene on 7q	Target pathway	Target gene
EED	GTF2I	Cell apoptosis	BCL2
BRD4	MLL3 or MLL5	Cell proliferation	c-MYC or CCND1

A previous report showed that inactivation of GTF2I was frequently observed in a subtype of thymic epithelial tumors and related to preferable prognosis [47]. However, the detailed function of GTF2I has not been well elucidated. Our study suggested that dysfunction of GTF2I may induce vulnerability to cell apoptosis. Both EED and GTF2I negatively regulate the anti-apoptotic molecule BCL2. Although the relationship between EED and apoptosis was not well investigated, EED may be essential for maintaining BCL2 expression and suppressing apoptosis in leukemia cells

with monosomy 7. It was possible that EED supported BCL2 expression that was impaired due to inactivation of GTF2I in monosomy 7. In this meaning, the importance of BCL2 in AML with monosomy 7 was suggested. It is still unclear whether EED directly or indirectly maintains BCL2 expression levels. One possibility is that EED suppressed TP53 activity, and suppressed TP53 maintains BCL2 expression levels. Actually, TP53 were upregulated in EED-knockdown cells (Figure 7a), and TP53 was reported to suppress the BCL2 activity [48]. It is possible that knockdown of EED upregulate the TP53 activity, and upregulated TP53 suppressed BCL2 expression levels. Further investigations are needed to elucidate the mechanism.

This study also showed that PRC2 activity of EED was not important for cell survival in AML with monosomy 7. Reduced PRC2 activity due to single allele deletion of EZH2 may be compensated by other gene such as EZH1 [49]. In addition, single allele of EZH2 may be sufficient to maintain of PRC2 activity. Actually, single allele deletion of EZH2 showed very mild progression of myeloid malignancies compared to both alleles deletion [50]. These reasons may explain that EED-PRC2 axis was not important for leukemia cell survival in monosomy 7 cells.

In contrast to GTF2I, functions of MLL3 or MLL5 were relatively well investigated in AML with monosomy 7. Especially, inactivation of MLL3 showed

progression of myeloid malignancies in mouse models [20]. JQ-1 treatment resulted in cell cycle arrest via suppressing c-MYC and CCND1 expression. Consistent with the current study, previous reports showed that both BRD4 and MLL3 upregulate the expression levels of c-MYC [20, 51]. Thus, synthetic lethality between BRD4 and MLL3 that commonly targeted c-MYC was predictable. On the contrary, synthetic lethality between BRD4 and MLL5 was newly suggested. Our results indicated that MLL3-deficient and MLL5-deficient cells showed similar characteristics including JQ-1 sensitivity. However, our results also showed that knockout of MLL5 increased JQ-1 sensitivity more drastically than knockout of MLL3. Although function of MLL5 was not well investigated in myeloid malignancies compared with MLL3, there may be some uncovered function of MLL5 that increased JQ-1 sensitivity in a different manner from MLL3. Detailed analysis was needed to elucidate the functions of MLL5.

Besides MLL3 and MLL5, other genes in chromosome 7 may also be associated with the JQ-1 sensitivity (Figure 10). Especially, CDK5 was a top candidate gene (Figure 10), and previous report suggested that CDK5 directly phosphorylated c-MYC and regulated cell cycle progression [52]. In addition, CDK5 inhibition may overcome the resistance of BET inhibitors [53]. Thus, further investigation is warranted

to evaluate the relationships between CDK5 inhibition and JQ1 sensitivity in monosomy 7.

Despite its poor prognosis, pathogenesis of monosomy 7 is still unclear. One reason may be the complicated condition of patients with monosomy 7. A majority of monosomy 7 in patients with AML appeared as a part of complex karyotypes. This complicated chromosomal abnormalities invited difficulties in investigation for therapeutic target in monosomy 7. In this sense, our synthetic lethality-based approach was effective in detecting a promising therapeutic target in malignancies with complicated background. Especially, this study is unique that providing a therapeutic target in chromosomal deletions. In patients with AML, various chromosomal abnormalities including 5q- were often observed, and many of these abnormalities did not have therapeutic strategies. Our synthetic lethality-based approach may also provide novel therapeutic target in these leukemia.

Additional studies are required to further validate my findings. First, we should evaluate the efficacy of EED or BRD4 inhibition in primary patient samples with more heterogeneous functional properties. Second, the in-vivo analysis to evaluate the efficacy of EED or BRD4 inhibition in monosomy 7 leukemia cells would strengthen the obtained data.

In conclusion, synthetic-lethality based approach identified EED or BRD4 as essential survival factors in leukemia cells with monosomy 7. These findings may be applicable for the development of a novel therapeutic approach to the chemorefractory monosomy 7 leukemia.

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