Supplementary Information

BAALC potentiates oncogenic ERK pathway through interactions with MEKK1 and KLF4.

Ken Morita, Yosuke Masamoto, Keisuke Kataoka, Junji Koya, Yuki Kagoya, Hideki Yashiroda, Tomohiko Sato, Shigeo Murata, Mineo Kurokawa.

Supplementary information includes twenty-three figures, figure legends, two tables, supplementary methods and corresponding references.
Supplementary Figure legends

Supplementary Figure S1.

(A) Relative \textit{BAALC} expressions in CD34$^+$ CD38$^-$ fraction of primary human bone marrow cells from AML patients (n = 7), control subjects (n = 7). Values are normalized to \textit{BAALC} expression in primary human whole bone marrow cells from control subjects.

(B) Relative \textit{BAALC} expressions of BAALC-overexpressed HEL and MV4-11 cells compared to AML cell lines (n = 7). Values are normalized to \textit{BAALC} expression in primary human whole bone marrow cells from control subjects.

(C and D) Cell cycle status was determined in HEL and MV4-11 cells transduced with BAALC or control vector. (C) Representative flow cytometric data showing cell cycle distributions. (D) Cumulative data of cell cycle analysis (% of S and G2/M phase) (n = 3).

(E and F) Apoptotic status was determined in HEL and MV4-11 cells transduced with BAALC or control vector. (E) Representative flow cytometric data of Annexin V and DAPI staining. (F) Cumulative data of the frequency of early apoptotic cells (Annexin
V⁺ DAPI) (n = 3).

Supplementary Figure S2.

(A) Relative BAALC expressions in Kasumi-1 and OCI-AML2 cells transduced with control or BAALC shRNA. Expression of BAALC was rescued by cotransduction of shRNA-resistant BAALC constructs. Values are normalized to BAALC expression in control shRNA-transduced cells respectively (n = 3).

(B and C) Cell cycle status was determined in Kasumi-1 and OCI-AML2 cells transduced with control or BAALC shRNA. Expression of BAALC was rescued by cotransduction of shRNA-resistant BAALC constructs. (B) Representative flow cytometric data showing cell cycle distributions. (C) Cumulative data of cell cycle analysis (% of S and G₂/M phase) (n = 3).

Supplementary Figure S3.

(A and B) Apoptotic status was determined in Kasumi-1 and OCI-AML2 cells transduced with sh_Luc. or sh_BAALC. Expression of BAALC was rescued by
cotransduction of shRNA-resistant BAALC. (A) Representative flow cytometric data for Annexin V and DAPI staining. (B) Cumulative data of the frequency of early apoptotic cells (Annexin V\(^+\) DAPI\(^-\)) (n = 3).

(C) Cumulative data showing CD11b or CD14 expressions in sh\_Luc- or sh\_BAALC- transduced Kasumi-1 and OCI-AML2 cells. Expression of BAALC was rescued by cotransducing shRNA-resistant BAALC constructs (n = 3).

Data are mean±SEM values. * P < 0.05.

**Supplementary Figure S4.**

(A) Schematic abstract of yeast two-hybrid screening. Human BAALC 1-6-8 was used as bait and a cDNA library from adult human tissues as prey.

(B and C) Interaction between BAALC and MEKK1 was examined by co-immunoprecipitation assay. HEL cells were transduced with MYC-tagged BAALC or control vector (mock). Cells were stimulated by PMA (100 ng/mL) or dimethyl sulfoxide (DMSO) for 6 hours, then immunoprecipitated by anti-MYC tag antibody followed by blotting with anti MEKK1 antibody (B), or immunoprecipitated by anti
MEKK1 antibody followed by blotting with anti anti-MYC tag antibody (C).

(D and E) Interaction between BAALC and KLF4 was examined by co-immunoprecipitation assay. HEL cells were transduced with MYC-tagged BAALC or control vector (mock). Cells stimulated by PMA were immunoprecipitated by anti-MYC tag antibody followed by blotting with anti KLF4 antibody (D) or immunoprecipitated by anti KLF4 antibody followed by blotting with anti-MYC tag antibody (E).

Supplementary Figure S5.

(A) The binding site of BAALC with MEKK1. HA-tagged MEKK1 and MYC-tagged deletion mutants of human BAALC were cotransfected to HEK293T cells. Immune complexes were precipitated by anti-MYC tag antibody and blotted by anti-HA tag antibody.

(B) The binding site of BAALC with KLF4. FLAG-tagged KLF4 and MYC-tagged deletion mutants of BAALC were cotransfected to HEK293T cells. Immune complexes were precipitated by anti-MYC tag antibody and blotted by anti-FLAG tag antibody.
(C) Growth curve of HEL cells transduced with a series of deletion mutants of BAALC. Comparison was made between BAALC-expressing cells and mock-transduced cells (n = 3).

(D and E) Cell cycle status was determined in HEL cells transduced with each deletion mutant of BAALC. (D) Representative flow cytometric data showing cell cycle status. (E) Cumulative data of cell cycle analysis (% of S and G2/M phase) (n = 3).

Data are mean ± SEM values. * P < 0.05.

Supplementary Figure S6.

(A) The binding site of MEKK1 with BAALC. MYC-tagged BAALC and FLAG-tagged deletion mutants of MEKK1 were cotransfected into HEK293T cells. Immune complexes were precipitated by anti-MYC-tag antibody and blotted by anti-FLAG tag antibody.

(B) The binding site of KLF4 with BAALC. MYC-tagged BAALC and FLAG-tagged deletion mutants of KLF4 were cotransfected into HEK293T cells. Immune complexes were precipitated by anti-MYC-tag antibody and blotted by anti-FLAG tag antibody.
Supplementary Figure S7.

(A) Immunoblotting of phosphorylated proteins of the components of MAPK, PI3K/AKT, JAK-STAT and NF-κB pathways in HEL and MV4-11 cells transduced with MYC-tagged BAALC or control vector. Cells were stimulated with 100 ng/mL PMA for 6 hours.

(B) Cumulative data of the time lapse analysis of ERK activation in HEL cells transduced with BAALC or control vector. Cells were stimulated with 100 ng/mL PMA for the indicated time periods. Values are normalized to control sample taken at 0 hours after stimulation (n = 3).

(C) Time lapse analysis of ERK activation in MV4-11 cells transduced with BAALC or control vector. Cells were stimulated with 100 ng/mL PMA for the indicated time periods. Immunobloting of phosphorylated ERK is shown.

Supplementary Figure S8.

(A) Immunoblotting of phosphorylated ERK in Kasumi-1 or OCI-AML2 cells
transduced with sh\_BAALC or sh\_Luc. BAALC was overexpressed by lentivirus in
sh\_BAALC–transduced cells. Cells were stimulated with PMA (100 ng/mL) for 6 hours.

(B) Cumulative data of the amount of MEKK1 bound to BAALC in HEL cells
transduced with BAALC or control vector. Cells were stimulated with 100 ng/mL PMA
for the indicated time periods. Values were normalized to that of BAALC
overexpressing HEL cells at 0 hours after stimulation (n = 3).

(C) Interaction between BAALC and MEKK1 in MV4-11 cells transduced with
MYC-tagged BAALC. Cells were stimulated with PMA (100 ng/mL) for the indicated
time periods. Immune complexes were precipitated by anti MYC-tag antibody and
blotted by anti MEKK1 antibody.

(D) Cumulative data of the amount of MKP3 bound to ERK in HEL cells transduced
with BAALC or control vector. Cells were stimulated with 100 ng/mL PMA for the
indicated time periods. Values were normalized to that of mock-transduced HEL cells
taken at 3 hours after stimulation (n = 3).

(E) Interaction between ERK and MKP3 in MV4-11 cells transduced with MYC-tagged
BAALC. Cells were stimulated with PMA (100 ng/mL) for the indicated time periods.
Immune complexes were precipitated by anti-ERK antibody and ERK-bound MKP3 was detected by anti MKP3 antibody.

**Supplementary Figure S9.**

(A) Immunoblotting of phosphorylated proteins of the components of MAPK, PI3K/AKT, JAK-STAT and NF-κB pathways in HEL cells transduced with MYC-tagged BAALC or control vector. Cells were stimulated with 100 ng/mL PMA for 6 hours in the presence of various pathway inhibitors U0126 (5 μM, MEK inhibitor), PD169316 (10 μM, p38 inhibitor), SP600125 (10 μM, JNK inhibitor), PI-103 (1 μM, PI3K-AKT-mTOR inhibitor), WP1066 (2 μM, JAK-STAT inhibitor) and SC-514 (10 μM, NF-κB inhibitor).

(B) Immunoblotting of phosphorylated ERK in HEL cells transduced with MYC-tagged BAALC or control vector concurrently transduced with dominant negative form of ERK1 K71R (DN-ERK).

**Supplementary Figure S10.**
(A) Growth curves of HEL and MV4-11 cells transduced with BAALC or control vector (mock) with a series of signaling pathway inhibitors. Cells were transduced with dominant negative form of ERK1 K71R (DN-ERK) or treated by U0126 (5 µM, MEK inhibitor), PD169316 (10 µM, p38 inhibitor), SP600125 (10 µM, JNK inhibitor), PI-103 (1 µM, PI3K-AKT-mTOR inhibitor), WP1066 (2 µM, JAK-STAT inhibitor) and SC-514 (10 µM, NF-κB inhibitor) (n = 3).

Data are mean±SEM values. * P < 0.05.

Supplementary Figure S11.

(A) Immunoblotting of phosphorylated ERK in HEL and MV4-11 cells cotransduced with BAALC and MKP3.

(B) Growth curve of HEL and MV4-11 cells cotransduced with BAALC and MKP3 (n =3).

(C) Cell cycle analysis (% of S and G2/M phase) of HEL and MV4-11 cells cotransduced with BAALC and MKP3 (n =3).

(D) Schematic representation of the function of BAALC in ERK pathway. Molecular
dynamics around MEKK1 were depicted. Time after ERK pathway activation was
classified into three phases, un-stimulated phase (before ERK pathway stimulation),
early phase (0 to 3 hours after ERK pathway stimulation) and late phase (6 hours after
ERK pathway stimulation and thereafter).

Data are mean±SEM values. * P < 0.05.

Supplementary Figure S12.

(A) Expression levels of ABCG2 in HEL and ABCB1, ABCG2 in MV4-11 cells
transduced with BAALC or control vector followed by treatment with AraC (1 µM) or
DMSO for 48 hours in the presence of dominant negative form of ERK1 K71R
cotransduction or various pathway inhibitors (U0126 (5 µM), PD169316 (10 µM),
SP600125 (10 µM), PI-103 (1 µM), WP1066 (2 µM) and SC-514 (10 µM)). Values are
normalized to that of control vector-transduced cells treated with DMSO (n =3).

Data are mean±SEM values. * P < 0.05.

Supplementary Figure S13.
(A) AraC sensitivity of HEL and MV4-11 cells transduced with BAALC or control vector. Cells were treated with AraC at the indicated concentrations in the presence of transduction with dominant negative form of ERK1 K71R or treatment with U0126 (5 µM), PD169316 (10 µM), SP600125 (10 µM), PI-103 (1 µM), WP1066 (2 µM) or SC-514 (10 µM). Cell viabilities were determined by trypan blue exclusion assays (n = 3). IC50 values of BAALC- or mock- overexpressing HEL and MV4-11 cells are provided respectively.

Data are mean±SEM values. * P < 0.05.

Supplementary Figure S14.

(A) Immunobloting of phosphorylated ERK in MV4-11 cells transduced with BAALC, CA-MEK1 and control vector (n = 3).

(B) Growth curve of MV4-11 cells transduced with BAALC, CA-MEK1 and control vector (n = 3).

(C) Cumulative data showing CD11b or CD14 expressions in HEL and MV4-11 cells transduced with BAALC, CA-MEK1 and control vector (n = 3).
Supplementary Figure S15.

(A) Immunoblotting of total and nuclear KLF4 in MV4-11 cells cotransduced with sh_KLF4 and CA-MEK1.

(B) Growth curve of HEL and MV4-11 cells cotransduced with sh_KLF4 and CA-MEK1. (n = 3).

(C) Cumulative data showing CD11b or CD14 expressions in HEL and MV4-11 cells cotransduced with sh_KLF4 and CA-MEK1. (n = 3)

(D) Representative images of May-Giemsa stained HEL and MV4-11 cells cotransduced with sh_KLF4 and CA-MEK1 (scale bars, 50 µm).

Data are mean ± SEM values. * P < 0.05.

Supplementary Figure S16.

(A) Immunofluorescence images of KLF4 in HEL cells transduced with MYC-tagged BAALC or control vector at low magnification (scale bars, 10 µm).

(B) Cumulative data of cytoplasm-nucleus ratio of KLF4 in HEL cells transduced with...
BAALC or control vector. (n = 3)

(C) Immunofluorescence images of KLF4 in MV4-11 cells transduced with MYC-tagged BAALC or control vector at high and low magnifications (scale bars, 10 µm).

(D) Cumulative data of cytoplasm-nucleus ratio of KLF4 in MV4-11 cells transduced with BAALC or control vector. (n = 3)

Data are mean±SEM values. * P < 0.05.

Supplementary Figure S17.

(A) Immunofluorescence images of KLF4 in Kasumi-1 cells transduced with sh_Luc. or sh_BAALC at low magnification (scale bars, 10 µm).

(B) Cumulative data of cytoplasm-nucleus ratio of KLF4 in Kasumi-1 cells transduced with sh_Luc. or sh_BAALC. (n = 3)

(C) Immunofluorescence images of KLF4 in OCI-AML2 cells transduced with sh_Luc. or sh_BAALC at high and low magnifications (scale bars, 10 µm).

(D) Cumulative data of cytoplasm-nucleus ratio of KLF4 in OCI-AML2 cells
transduced with sh_Luc. or sh_BAALC. (n = 3)

(E) Cumulative data of cytoplasm-nucleus ratio of KLF4 in CD34^+ CD38^- bone marrow cells from five AML patients with high and low BAALC expression respectively. (n = 3)

Data are mean±SEM values. * P < 0.05, ** P < 0.01.

Supplementary Figure S18.

(A) Growth curve of HEL and MV4-11 cells cotransduced with BAALC and CA-MEK1. (n = 3)

(B) Monocytic differentiation assessed by CD11b or CD14 expressions in HEL and MV4-11 cells cotransduced with BAALC and CA-MEK1. Cumulative data from three independent experiments are shown.

(C) Expression levels of monocytic differentiation-associated genes (CES1 and CSF1R) in HEL and MV4-11 cells cotransduced with BAALC and CA-MEK1. Values are normalized to the expression levels in control vector-transduced cells (n = 3).

(D) Representative images of May-Giemsa-stained HEL and MV4-11 cells
cotransduced with BAALC and CA-MEK1 (scale bars, 50 µm).

(E) Immunoblotting of nuclear KLF4 in HEL and MV4-11 cells cotransduced with BAALC and CA-MEK1.

Data are mean±SEM values. * P < 0.05, ** P < 0.01.

**Supplementary Figure S19.**

(A) Immunoblotting of nuclear KLF4 in HEL and MV4-11 cells cotransduced with BAALC and KLF4.

(B) Growth curve of HEL and MV4-11 cells cotransduced with BAALC and KLF4 (n = 3).

(C) Cumulative data of monocytic differentiation assessed by CD11b or CD14 expressions in HEL and MV4-11 cells cotransduced with BAALC and KLF4 (n = 3).

(D) Expression levels of monocytic differentiation-associated genes (CESI and CSF1R) in HEL and MV4-11 cells cotransduced with BAALC and KLF4. Values are normalized to the expression levels in control vector-transduced HEL cells (n = 3).

(E) Representative images of May-Giemsa-stained HEL and MV4-11 cells
Data are mean ± SEM values. * P < 0.05, ** P < 0.01.

**Supplementary Figure S20.**

(A) Expression levels of downstream target genes of ERK and KLF4 (CCND1, CDK6, and CDKN1A) in HEL and MV4-11 cells transduced with BAALC or control vector. Values are normalized to the expression levels in control vector-transduced cells (n = 3).

(B) Expression levels of CCND1, CDK6 and CDKN1A in Kasumi-1 and OCI-AML2 cells transduced with sh_Luc. or sh_BAALC. Expression of BAALC was rescued by cotransducing shRNA-resistant BAALC constructs. Values are normalized to the expression levels in control vector-transduced cells (n = 3).

Data are mean ± SEM values. * P < 0.05.

**Supplementary Figure S21.**

(A) Growth curve of Kasumi-1 cells transduced with tetracycline-inducible (Tet-ON)
KLF4 treated with DMSO or U0126 (5 µM) combined with PBS or doxycycline (Dox; 1 µM) (n = 3).

(B) Growth curve of BAALC- or control vector-transduced HEL cells concurrently transduced with tetracycline-inducible (Tet-ON) KLF4. Cells were treated with DMSO or U0126 (5 µM) combined with PBS or doxycycline (Dox; 1 µM) (n = 3).

(C) Cumulative data showing CD11b or CD14 expressions in Kasumi-1 cells and HEL cells cotransduced with BAALC and Tet-ON KLF4 followed by treatment with U0126 (5 µM) or Dox (1 µM) or both.

(D) U0126 sensitivity of AML cell lines. Cells were treated with U0126 at the indicated concentrations and cell viabilities were determined by trypan blue exclusion assays (n = 4). IC50 values of cell lines are provided.

Data are mean±SEM values. * P < 0.05, ** P < 0.01.

Supplementary Figure S22.

(A) Representative macroscopic pictures of livers and spleens from a human AML xenograft mouse and a normal NSG control mouse (scale bars, 1cm).
(B) Representative microscopic images of the bone marrow, the liver and the spleen from a human AML xenograft mouse and a normal NSG control mouse. Hematoxylin and eosin (H&E) staining and immunohistochemical staining with anti-human CD45 antibody were done for each slide (original magnification 40× and 600×, Scale bars, 200 µm in 40× and 20 µm in 600× images. Arrows in the spleen from a human AML xenograft mouse show hCD45+ cells.)

Supplementary Figure S23.

(A) Overall survival of NSG mice transplanted with Kasumi-1 cells transduced with sh_Luc. or sh_BAALC (n = 6).

(B) Overall survival of NSG mice transplanted with HEL cells transduced with BAALC or control vector (n = 6).

(C) Overall survival of NSG mice transplanted with HEL cells transduced with Tet-ON KLF4 followed by treatment with DMSO, U0126, Dox or U0126 plus Dox (n = 6). U0126 was administered intraperitoneally at 25 µmol/kg/week and Dox was given orally (diluted in drinking water at 1 mg/mL).
Supplementary Table S1

PCR primers used for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAALC</td>
<td>AAGGCACCAACAGATTCA</td>
<td>AAGGCCATTCTGTTCCTGGTCTG</td>
</tr>
<tr>
<td>CES1</td>
<td>GAACCACAGAGATGCTGGGAC</td>
<td>TCCCCGTGGTCTCCTATCAC</td>
</tr>
<tr>
<td>CSF1R</td>
<td>GAGCGACGTCTGGTCTATATG</td>
<td>AGGATGCCAGGGGTAGGATT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CATGTTTCGTCATGGGGTGAAACCA</td>
<td>AGTGATGGCATGGACTGTGGTCAT</td>
</tr>
<tr>
<td>ABCB1</td>
<td>CAGGAACCTGTATTTGTGGGCCACCAC</td>
<td>TGCTTCTGCCCACCACACTCAACTG</td>
</tr>
<tr>
<td>ABCG2</td>
<td>GGTGGAGGCAATCTTCGTTATAGA</td>
<td>GAGTGCCCATCACAACATCATCTT</td>
</tr>
<tr>
<td>CDK6</td>
<td>TGCACAGTGTCAACCAGAGA</td>
<td>ACCTCGGAGAAGCTGAAACA</td>
</tr>
<tr>
<td>CCND1</td>
<td>CTTCTCTCCCAAATGGCAG</td>
<td>AGAGATGGAAAGGGAAGAGA</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>GAGGCCAGGATGGAGTGAGGAGGAG</td>
<td>CAGGCCGCGTTGGAGTGGTAGAA</td>
</tr>
</tbody>
</table>
**Supplementary Table S2**

**Target sequences used for shRNA knockdown experiments.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh_BAALC #1</td>
<td>GCATGCTGGAAGATGGACT</td>
</tr>
<tr>
<td>sh_BAALC #2</td>
<td>TCACAAAGAACTGTGTCAA</td>
</tr>
<tr>
<td>sh_KLF4 #1</td>
<td>GCTCCATTACCAAGAGCTCAT</td>
</tr>
<tr>
<td>sh_KLF4 #2</td>
<td>GATCAAGCAGGAGCGGTC</td>
</tr>
<tr>
<td>sh_Luc.</td>
<td>CGTACGCGGAATTACCTCGA</td>
</tr>
</tbody>
</table>
Supplemental Methods

Cell lines

AML cell lines of THP-1 and KG1 cells were purchased from RIKEN biological resource center (BRC). Kasumi-1 and HEL cells were provided from Japanese Collection of Research Bioresources (JCRB). OCI-AML2 and MOLM13 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), and MV4-11 was from American Type Culture Collection (ATCC) respectively.

Plasmids and expression constructs

We cloned cDNA construct of CA-MEK1, MEKK1, MKP3, KLF4, ERK1 (K71R), BAALC respectively into CSII-EF-MCS-IRES2-Venus, CSII-EF-MCS-IRES2-hKO1, and CSIV-TRE-Ubc-KT vectors. Deletion mutants of BAALC, KLF4 and MEKK1 were produced by PCR-based mutagenesis from full length BAALC 1-6-8, KLF4 and MEKK1. Each mutant was cloned into MYC-tagged, FLAG-tagged, His-tagged or HA-tagged pcDNA3 expression vectors respectively. Exogenous BAALC constructs
resistant to BAALC shRNAs were also generated by target mutagenesis. Point mutation
of G168A was introduced into sh_BAALC #1 and T429C into sh_BAALC #2. All of the
PCR products were verified by DNA sequencing.

Reagents

Each cytoplasmic signaling inhibitor used in this study was as follows; U0126 (Sigma
Aldrich), PD169316 (Cayman Chemical Company), SP600125 (Sigma Aldrich), PI-103
(Santa Cruz Biotech), WP1066 (Cayman Chemical Company) and SC-514 (Santa Cruz
Biotech).

Production and transduction of retrovirus and lentivirus

Production and collection of viral supernatants and subsequent transduction was done as
previously described¹. For the production of lentivirus supernatants, HEK293T cells
were co-transfected with lentivirus vectors, psPAX2 and pMD2.G.

In vitro pull-down assay
Expressed proteins were purified by Protein mild purification kit (BML). Purification efficiency was checked by coomassie brilliant blue stains. Samples were mixed in binding buffer (50 mM Tris-HCl, 250 mM NaCl, 0.05% Nonidet P-40, 30 mM MgCl₂, pH 7.4) with protease inhibitor mixture (4 µg/mL) for 2 hours at 4 °C, then anti MYC-tag antibody was added before immunoprecipitation with sepharose G protein beads. Beads were washed twice, then proteins bound to beads were resolved by SDS-PAGE. Protein-protein interaction was analyzed with immunoblotting.

**Production and transduction of retrovirus and lentivirus**

To obtain retroviral supernatants, platinum-A (Plat-A) packaging cells were transiently transfected with each retrovirus vector by polyethylenimine (PEI, Sigma-Aldrich). Collection of viral supernatants and subsequent transduction was done as previously described ¹. For the production of lentivirus supernatants, HEK293T cells were co-transfected with lentivirus vectors, psPAX2 and pMD2.G.

**Immunoblotting**
Immunoblotting was performed as previously described. Membranes were probed with the following antibodies: anti-ERK, anti-phospho-ERK (Thr202/Tyr204), anti-MEKK1, anti-MKP3, anti-β-actin, anti-histone H3, anti-p90RSK, anti-phospho-p90RSK, anti-p38, anti-phospho-p38, anti-NF-κB, anti-phospho-NF-κB, anti-AKT, anti-phospho-AKT, anti-MYC-tag, anti-A-Raf, anti-phospho-A-Raf, anti-B-Raf, anti-phospho-B-Raf, anti-c-Raf, anti-phospho-c-Raf, anti-mTOR, anti-phospho-mTOR, anti-Raptor, anti-Rictor (Cell Signaling Technology), anti-JNK/SAPK1, anti-phospho-JNK/SAPK1, anti-JAK2, anti-phospho-JAK2, anti-STAT1, anti-phospho-STAT1, anti-STAT3, anti-phospho-STAT3, anti-STAT5, anti-phospho-STAT5, anti-STAT6, anti-phospho-STAT6 (eBioscience) and HRP-conjugated anti-FLAG-tag (Sigma Aldrich) antibodies. For secondary antibodies, anti-rabbit IgG, or anti-mouse IgG, HRP-linked antibodies (Cell Signaling Technology) were used. Blots were detected using an ImmunoStar Zeta (Wako Pure Chemical Industries) and an LAS-3000 image analyzer (Fujifilm), as recommended by the manufacturers. Protein levels were quantified with ImageJ software (NIH). To obtain nuclear and cytoplasmic extracts, an Active Motif Nuclear Extract Kit (Active Motif)
was used according to the manufacturer’s instructions.

Co-immunoprecipitation (CoIP) assay

CoIP was carried out as previously described. Immunoprecipitation was performed using anti-ERK, anti MYC-tag, anti HA-tag and anti FLAG-tag antibodies. The precipitates were analyzed by immunoblotting.

Cell culture

HEK293T and Plat-A cells were cultured in DMEM-10% heat inactivated FCS at 37 °C, 5% CO₂. Human leukemia cell lines Kasumi-1, OCI-AML2, THP-1, KG1, MOLM13, MV4-11 and HEL cells were cultured in RPMI1640 containing 10% heat inactivated FCS. Bone marrow cells of patient samples were maintained in RPMI1640 containing 20% heat inactivated FCS supplemented with cytokines of IL-3, hSCF, FLT3L and MGDF (Wako Pure Chemical Industries) at 100 ng/mL.

Cell growth curve
To assess cell proliferation, $1 \times 10^5$ cells of AML cell lines and human primary bone marrow cells were counted and transferred to 6-well plate with 4mL medium. Cell number was counted every other day.

**Real-time quantitative PCR (qRT-PCR)**

Total RNA was isolated with RNeasy mini kit (Qiagen) and reverse transcribed with Reverse script kit (TOYOBO) to generate cDNA. Real-time quantitative polymerase chain reaction (PCR) was carried out with LightCycler480 (Roche) according to the manufacturer's instructions. The results were normalized to GAPDH levels. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Primers used for qRT-PCR were listed in Supplemental Table S1.

**siRNA interference.**

Specific shRNAs targeting human BAALC were designed and cloned into pSIREN-RetroQ-ZsGreen vectors (Clontech). Control shRNA is a nonfunctional construct targeting luciferase (sh_Luc). The target sequences were provided in the
Immunofluorescence assay

Immunofluorescence assay was carried out as previously described. For immunofluorescence staining of AML cells, rabbit anti–KLF4 antibody (1:100 dilution; Cell Signaling Technology), mouse anti-MYC-tag antibody (1:100 dilution; Cell Signaling Technology), Alexa Fluor 647 goat anti-mouse IgG (1:250 dilution; Invitrogen), Alexa Fluor 594 goat anti-rabbit IgG (1:250 dilution; Invitrogen) and DAPI (1:1,000 dilution) were used. Images were acquired using an Olympus FluoView FV10i confocal microscope with a ×60 objective oil immersion lens.

Cell cycle and apoptosis assay

For cell cycle analysis, cells were fixed with fixation buffer and permeabilized with permeabilization wash buffer (BioLegend) according to the manufacturer’s instructions. For apoptosis assay, cell apoptosis was determined by Annexin V Apoptosis Detection Kit APC (eBioscience Inc.) according to the manufacturer’s instructions.
Flow cytometry

Isolation of CD34+CD38− fraction from human normal or leukemic bone cells and isolation of leukemia cell lines transduced with immunofluorescent color markers of GFP, Kusabira-Orange and Venus were performed using FACSAnia III (BD) cell sorter.

For isolation of CD34+CD38− fraction from human primary bone marrow cells, FITC-conjugated anti-human CD34, and PE-conjugated anti-human CD38 antibodies were used (eBioscience). For checking monocytic differentiation state of leukemia cells, Biotin-conjugated anti-human CD11b or CD14 and Streptavidin-APC (eBioscience) were used. Analysis was performed using FlowJo software (Tree Star Inc.).

IC50 evaluation

AML cell lines were grown in RPMI 1640 containing 10% heat inactivated FCS. For the growth inhibition assay, cells were placed at a density of 1×10^5 cells/mL. Different concentrations of U0126 were added to the media. Cell viability was assessed by counting the number of trypan blue excluding cells 4 days after starting culture. The
doses that inhibited 50% proliferation were analyzed by the median-effect method $^3$.

Statistics

Statistical significance of differences between groups was assessed with a 2-tailed unpaired Student’s t test. Equality of variances in two populations was calculated with F-test. Differences were considered statistically significant at a P value of less than 0.05.

The results were represented as the average ± SEM values from three independent experiments. In leukemia cell transplantation experiments, animals were randomly allocated to each experimental group, and the treatments were given without blinding.

The overall survival of mice is depicted by a Kaplan-Meier curve. Survival between groups was compared using the log-rank test. To measure the correlation between BAALC mRNA expression and sensitivity to MEK inhibitor (U0126) in human AML cell lines and patients samples, the Spearman’s rank correlation coefficient was used.

Study approval

BM cells derived from patients with AML were obtained from the Department of
Hematology and Oncology of the University of Tokyo Hospital. Bone marrow cells from patients diagnosed with lymphoid neoplasia without bone marrow invasion were used as normal controls. The study was approved by the ethics committee of the University of Tokyo, and written informed consents were obtained from all patients whose samples were collected.
References


Supplementary Figure S2

A

Kasumi-1

OCI-AML2

B

Kasumi-1

OCI-AML2

C

Kasumi-1

OCI-AML2
Supplementary Figure S3

A

Kasumi-1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DAPI</th>
<th>Annexin V</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh_Luc.</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1 + BAALC</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2 + BAALC</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

OCI-AML2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DAPI</th>
<th>Annexin V</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh_Luc.</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1 + BAALC</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2 + BAALC</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

B

Kasumi-1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Early apoptotic cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh_Luc.</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1 + BAALC</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2 + BAALC</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

OCI-AML2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Early apoptotic cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh_Luc.</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1 + BAALC</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2 + BAALC</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

C

Kasumi-1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD11b positive cells (%)</th>
<th>CD14 positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh_Luc.</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1 + BAALC</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2 + BAALC</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

OCI-AML2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD11b positive cells (%)</th>
<th>CD14 positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh_Luc.</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #1 + BAALC</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>sh_BAALC #2 + BAALC</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
Supplementary Figure S7

A

MAPK pathway

HEL

MV4-11

Mock

BAALC

Mock

BAALC

P-A-RAF

A-RAF

P-B-RAF

B-RAF

P-C-RAF

C-RAF

P-p38

p38

P-JNK

JNK

PI3K/AKT pathway

HEL

MV4-11

Mock

BAALC

Mock

BAALC

P-AKT

AKT

P-mTOR

mTOR

NF-κB pathway

HEL

MV4-11

Mock

BAALC

Mock

BAALC

P-NF-κB

NF-κB

JAK-STAT pathway

HEL

MV4-11

Mock

BAALC

Mock

BAALC

P-JAK2

JAK2

P-STAT1

STAT1

P-STAT3

STAT3

P-STAT5

STAT5

P-STAT6

STAT6

B

Phosphorylated ERK

0 hrs 3 hrs 6 hrs 9 hrs 12 hrs

Mock

BAALC

C

Call ysis

Mock

BAALC

P-ERK

ERK

β-actin

MYC(BAALC)
Supplementary Figure S10

A

HEL

Treatment
- Mock (-)
- Mock (+)
- BAALC (-)
- BAALC (+)

MV4-11

Treatment
- Mock (-)
- Mock (+)
- BAALC (-)
- BAALC (+)
Supplementary Figure S14

A

Cell lysate

Mock  BAALC  CA-MEK1

P-ERK  ERK  β-actin

B

Cell number (x10^6 cells)

Mock  BAALC  CA-MEK1

0  2  4  6  Days

C

HEL

CD11b positive cells (%)  CD4 positive cells (%)

Mock  BAALC  CA-MEK1  Mock  BAALC  CA-MEK1

MV4-11

CD11b positive cells (%)  CD4 positive cells (%)

Mock  BAALC  CA-MEK1  Mock  BAALC  CA-MEK1

**  *  

*  *
Supplementary Figure S21

A

Cell number (x10⁶ cells)

Days

- DMSO
- Dox
- U0126
- Dox + U0126

B

Cell number (x10⁶ cells)

Days

- Mock_DMSO
- Mock_U0126
- Mock_Dox
- Mock_Dox + U0126

- BAALC_DMSO
- BAALC_U0126
- BAALC_Dox
- BAALC_Dox + U0126

C

Kasumi-1

CD11b positive cells (%)

Dox U0126 Dox+U0126

CD14 positive cells (%)

Dox U0126 Dox+U0126

HEL

CD11b positive cells (%)

Dox U0126 Dox+U0126

CD14 positive cells (%)

Dox U0126 Dox+U0126

D

Cell viability (%)

Concentration of U0126 (μM)

Cell lines

IC50

Kasumi-1 0.77±0.05
OCI-AML2 0.822±0.36
MV4-11 12.07±0.49
HEL 20.21±0.78