
Supplemental Material

Supplemental Methods

miR-181a Expression Analyses by microRNA microarray.

Microarray microRNA expression analysis was conducted in pretreatment BM or blood mononuclear cells, as previously reported. Data are available at the MIAMExpress at EBI (http://www.ebi.ac.uk/miamexpress; accession number E-MTAB-1320).

mRNA and microRNA extraction and expression analysis by RT-PCR and Northern blot

RNA was isolated using TRIzol (Invitrogen), and 400ng of RNA was reverse transcribed into cDNA using SuperScript III First Strand Synthesis (Invitrogen) according to manufacturer’s recommendations and used for gene expression analyses. For the mature miR gene expression, 100ng of RNA was reverse transcribed using (5X) primer specific probes (Taqman) in conjunction with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed using a 7900HT Fast Real-Time System (Applied Biosystems), with the aforementioned cDNAs serving as templates in combination with TaqMan Fast Universal PCR Master Mix (2X) (Applied Biosystems) and Taqman Gene Expression Assays (Applied Biosystems). The Taqman Gene Expression Assays for miR-181a, U44, and U6 were from Applied Biosystems. For the precursor microRNA expression, 100ng of total RNA was reverse-transcribed to cDNA, amplified using the Quantitect SYBR Green RT-PCR Kit (Qiagen) according to
the manufacturer’s recommendations, and performed using a 7900HT Fast Real-Time System (Applied Biosystems). The following primers were used: GAPDH (Cat. QT00079247), 18S (Cat. QT00199367), hsa-miR-181a-1 (Cat. MP00001071), and hsa-miR-181a-2 (Cat. MP00001078).

The microRNA Northern blot was performed as previously described. Nucleic acid probes were obtained from Exiqon, using the following sequences:

miR-181a: 5’-ACTCACCGACAGCGTTGAATGTT-3’
U6: 5’-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3’.

**Transient and stable transfections, and CEBPA constructs**

K562 cells were transiently transfected using nucleofection method in Amaxa device according to manufacturer’s protocol (Lonza) for each cell line. Wild-type and patient-derived mutated CEBPA sequences (N-terminal: P23Qfs, or H24AfsX84 and the C-terminal: R300L) were cloned into EcoRI site of pMSCV expression vector (Clontech).

Generation of K562 stable lines expressing either estrogen receptor ligand binding domain (ER), or ER fused to C/EBPα-p42, or C/EBPα-p30 (in pBabe-puro vector) and stimulated with beta-estradiol (Sigma-Aldrich: E2758) was described earlier. The HA-tagged-C/EBPα plasmids were constructed and stably transfected to THP-1 cells, as previously described.

**Immunofluorescence staining and confocal microscopy**

Approximately 100,000 K562 cells transiently transfected with expression vectors containing patient-derived C/EBPα isoforms were spun onto microscope slides using cytocentrifuge. Spun cells were fixed with ice-cold acetone for 5 minutes and the slides were washed twice with washing buffer (1X PBS containing 20% FBS). Blocking and staining of cells were performed as described previously. Antibody staining was
performed according to the manufacturer’s protocol. Anti-C/EBPα rabbit polyclonal antibody (Cell Signaling Technology, Cat# 2295) was used at 1:50 dilution followed by Texas Red®-X goat anti-rabbit IgG (H+L) (diluted 1:40 in Antibody Diluent; Invitrogen). Stained cells were washed 7 times with washing buffer and mounted with VECTASHIELD® Mounting Medium containing DAPI. Images were acquired using Olympus FV1000-Filter Confocal microscope and analyzed with Olympus Fluoview software (version 2.0).

**Western blot analysis**

Western blotting was performed using lysates isolated using either RIPA buffer or NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, 78833). Protein lysates were mixed with (6X) sodium dodecyl sulfate (SDS) loading buffer (125mM Tris pH 6.8, 4% SDS, 20% glycerol, 200 mM β-mercaptoethanol, 0.2% [w/v] bromophenol blue) and boiled for 10 minutes. Alternatively, cells were collected, washed with 1X PBS, resuspended with 2X SDS loading buffer, and boiled for 10 minutes. Protein samples were loaded onto a 4-20% Criterion Tris-HCl precast gels (Bio-Rad) and transferred to 0.45µm polyvinylidene fluoride (PVDF) membrane. Membranes were blocked using 5% bovine serum albumin (BSA) or 5% milk and incubated with primary antibodies overnight at 4º C. Membranes were washed in Tris-Buffered Saline (TBS) buffer containing 0.1% Tween-20 (TBST) and probed with secondary antibodies. Following the final wash in TBST, membranes were incubated in ECL Western blotting detection reagents (GE Healthcare) and exposed to film (Denville). Antibodies used were: Actin (Santa Cruz: sc-1616), C/EBPα (Cell Signaling: #2295), UBC9 (Santa Cruz: sc-10759), HA.11 (Covance: MMS-101P), anti-FLAG M2 (Sigma, F-3165) anti-rabbit HRP-linked (GE Healthcare), anti-mouse HRP-linked (GE Healthcare).
**Electrophoretic Mobility Shift Assay (EMSA):**

FLAG-tagged p42 and p30 C/EBPα expression vectors were transiently transfected to HEK-293T cells using Transfectin reagent (BioRad, Cat.170-3350). Eighteen hours later, cells were harvested and nuclear extracts prepared as described.\textsuperscript{14,31} Briefly, cells were washed once in PBS, resuspended in an equal volume of ice-cold hypotonic buffer A [10 mM HEPES-KOH pH 7.9, 1.5 mM MgCl\textsubscript{2}, 10 mM KCl, 0.5 mM DTT, supplemented with Complete Mini Protease Inhibitor Cocktail (Roche, Cat.11836153001)], and incubated on ice for 15 min. Cells were then lysed by 7-10 passages through a 26-gauge needle and nuclei were isolated by a 20-sec. centrifugation at 14K, at 4°C. Nuclear proteins were extracted by addition of 2/3 cell pellet volume of high-salt buffer C [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 0.2 mM EDTA, 0.5 mM DTT, supplemented with Complete Mini Protease Inhibitor Cocktail]. Nuclear extracts were recovered by centrifugation at 4°C, 14K, for 5 minutes. For EMSA, double-stranded oligonucleotides spanning the predicted C/EBP-binding site (underlined):

wild-type sense: 5'-TGTAGTCTTTTGAAATGGCATA-3'

wild-type antisense: 5'-GTTTATGCCATTTCAAAAGACT-3'

mutant sense: 5'- TGTAGTCTTTTCACCAAGCATA -3'

mutant antisense: 5'- GTTTATGCTTGGTGAAAAGACT-3'

were radiolabeled by fill-in reaction using Klenow Large Fragment Polymerase I, and purified on Sephadex G-25 columns. The amount of each C/EBPα protein was quantitated by western blot and EMSAs were performed by incubating nuclear extracts containing equal amounts of p42 or p30 C/EBPα, with 50,000 cpm radiolabeled oligonucleotide in a 25-µL reaction mixture containing 10 mM HEPES-KOH buffer (pH 7.9), 50 mM KCl, 2.5 mM MgCl\textsubscript{2}, 1 mM DTT, 10% glycerol, 1 µg acetylated bovine serum albumin, and 0.5 µg poly(dI-dC) on ice for 20 min. For the supershift, 1 µl of
polyclonal anti-C/EBPα antibody (sc-61X; Santa Cruz) was added to the reaction mixture. Binding reaction products were resolved on a 4% nondenaturing polyacrylamide gel containing 1× TBE (0.089 mM Tris, 0.089 mM boric acid, and 0.002 mM EDTA) and electrophoresed at 150 V at 4°C.

**Transient transfection and luciferase assay:**
HEK-293T cells were seeded on 24-well plates at 1.5x10⁵ cells per well in 0.5 ml complete growth medium and grown for 24 hrs. Transfections were performed according to manufacturer’s instructions using Transfectin reagent (BioRad; 3 µl per reaction), 100 ng firefly luciferase vector (pGL4-11) containing 192 bps of the *miR-181a-1* proximal promoter region (wild-type, or with C/EBP site mutated; cloned between XhoI and HindIII sites), 50 ng of C/EBPα expression vector (or empty vector, pcDNA3-FLAG), and 25 ng pRL-TK Renilla luciferase vector. Cells were harvested 18 hrs later and lysed in 100 µl passive lysis buffer (PLB). Luciferase activity was measured on 20 µl lysate using Dual Luciferase Assay (Promega).

**Proliferation Assay**
THP-1 cells were transiently transfected using nucleofector kit V (Lonza: VCA-1003) with *antagomiR-181a* (Exiqon: 118066-00) or non-targeting control (Exiqon: 199002-00) and incubated at 37°C for 24 hours. After incubation, 1.5X10⁶ cells (5X10⁵ cells/mL) were treated with 3.0 μM lenalidomide or equivalent volume of 1XPBS (vehicle) every 24 hours for three days and cultured at 37°C. Next, aliquots of 1X10⁶ cells were treated with various concentrations of Ara-C (0-5 μM; Sigma-Aldrich; C1768) and cultured at 37°C for additional 72 hours. Finally, cells were incubated with MTS (Promega; G3581) and the quantity of formazan product was measured using a Thermo Multiskan Spectrum microplate reader at wavelengths 490nm and 690nm.
Supplemental Figure Legends

Supplemental Figure 1. Expression of C/EBPα-p30 isoform correlates with increased miR-181a-1 expression in AML patient bone marrow samples. Representative Western blot of young CN-AML bone marrow patient samples examining the expression of C/EBPα. The patients were previously characterized to have the following C/EBPα status: wild-type, N-terminal (I68Lfs), or C-terminal (E167GfsX3). The C/EBPα-p30 isoform is expressed in the N-terminally mutated C/EBPα patient sample. In contrast, the C/EBPα-p42 isoform is shown exclusively in wild-type and C-terminal mutated C/EBPα patient samples. Cytogenetic characteristics of the patients are provided in the table below the figure.

Supplemental Figure 2. Induction of miR-181a expression in cells co-expressing N- and C-terminal mutants of CEBPA. K562 cells were transiently transfected with empty vector (vect.), N-terminal mutant (H24Afs), C-terminal mutant (R300L), or both mutants together.

(a) Cells were stained with anti-C/EBPα antibody and DAPI, and C/EBPα protein localization was analyzed by confocal microscopy.

(b) C/EBPα p42 and p30 protein levels were determined by western blot of whole cell lysates.

(c) Total RNA was analyzed by Real Time-PCR for the expression of miR-181a. Average of three measurements and standard error mean are shown.
Supplemental Figure 3. Biological effects of long-term culture of AML patients’ blasts with lenalidomide.

(a) AML patients blasts were cultured for 5 days in the presence of vehicle (veh.), or 3 μM lenalidomide (lenalid.). Approximately 1x10⁵ cells were lysed for protein extract and analyzed by western blot for C/EBPα expression. (b) The RNA from AML patient samples was examined for the expression of miR-181a RNA by real time-PCR. The grey bars indicate vehicle treated cells and the black bars show data from lenalidomide treated cells. White bar represents RNA from untreated THP-1 cells, which were included as a reference. (c) Cell viability was determined by Trypan Blue exclusion assay (grey bars: vehicle control, black bars: lenalidomide treated cells). Patients’ cytogenetic information is provided in tabular form in panel (d) (N/A, not assessed).

Supplemental Figure 4. Correlation between C/EBPα expression and levels of miR-181a in bone marrow samples of patients treated with lenalidomide induction therapy. Quantitative real-time RT-PCR data of bone marrow samples from three patients treated with lenalidomide induction therapy (upper panel). Samples were analyzed prior to treatment (white bars) and 15 days following lenalidomide induction therapy (black bars). Increased expression of miR-181a was observed on day 15 after lenalidomide induction therapy. C/EBPα protein expression in the same samples as above was analyzed by Western blot (lower panel). Data are represented in triplicate measurements and error bars denote standard error mean.

Supplemental Figure 5. Upregulation of miR-181a by lenalidomide is C/EBPα-dependent. C/EBPα-expressing HL60 and C/EBPα-negative K562 cells were treated with 3 μM lenalidomide (lenalid.), or vehicle control (veh.) for 24 hrs. Whole cell lysates
were analyzed by western blotting for C/EBPα protein (a) and by Real Time-PCR for miR-181a expression (b). Average of three measurements and standard error mean are shown.

Supplemental Figure 6. Antitumor properties of miR-181a. (a) Relative growth and (b) colony-forming ability of THP-1 cells overexpressing miR-181a or a control vector. Each bar corresponds to average of triplicate measurements and error bars designate standard error mean. (c) Cell cycle analysis in THP-1 cells overexpressing miR-181a. (d) Flow cytometric analysis of five AML patient blasts samples overexpressing miR-181a followed by annexin V staining. Standard error mean is shown. Cytogenetic information is shown in table below the figure (N/A, not assessed; * the presence of t(15;17) was excluded based on Fluorescence In Situ Hybridization (FISH).
Supplemental Table 1. Cytogenetic and diagnostic characteristics of patients from OSU 10016 clinical trial analyzed in Fig. 5

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<th>Karyotype</th>
<th>%BM blasts</th>
<th>WBC ($\times 10^6/\mu L$)</th>
<th>% PB blasts</th>
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Supplemental Figure 1

AML Patient Blasts

N-term. wt  C-term. mutant

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<th>Mutation status</th>
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p42  p30  Actin
Supplemental Figure 2

(a) Immunofluorescence images showing DAPI, CEBPα, and Merge for different conditions: vector, H24A, R300L, and H24A+R300L.

(b) Western blots showing protein levels of p42, p30, and Actin for different conditions: vect., H24Afs, R300L, and H24Afs + R300L.

(c) Graph showing relative miR-181a-1 expression levels for different conditions: vect., H24Afs, R300L, and H24Afs + R300L.
Supplemental Figure 4

![Bar chart showing protein expression levels for Pat. 4, Pat. 5, and Pat. 6 pre and d15 samples. The chart includes a Western blot image with bands for p42, p30, and Actin.]
Supplemental Figure 5

(a) Western blot analysis showing the expression of C/EBPα-p42, C/EBPα-p30, and Actin in HL60 and K562 cells treated with vehicle (veh.) or lenalidomide (lenalid.).

(b) Graph depicting the relative expression levels of miR-181a in HL60 and K562 cells treated with vehicle (veh.) or lenalidomide (lenalid.).
Supplemental Figure 6

(a) THP-1

Relative Growth

Days

control vector

miR-181a

(b) THP-1

Colony Number

control

miR-181a

(c) control vector

Number of Events

7-AAD

sub-G₁: 1.21%

(d) AML blasts (n=5)

Annexin V

(control fold change)

control

miR-181a

Patient Karyotype Mutation status

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<th>Patient</th>
<th>Karyotype</th>
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