circASXL1-1 regulates BAP1 deubiquitinase activity in leukemia

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Materials and Methods

Patients (for both knees and AML)

Bone marrow samples from AML patients were taken from the back of the pelvic (hip) bone while bone marrow from healthy counterparts was withdrawn during Total Knee Arthroplasty as part of a standard operative procedure. All clinical samples were obtained from the National University Hospital Singapore and collected according to the requirements of the Human Biomedical Research Act. Informed consent was obtained for all clinical samples used in the study.

Isolation of $CD34^+$ hematopoietic stem cells from normal knee aspirates and AML patients

Isolation of $CD34^+$ cells was performed according to the manufacturer's instructions using CD34 MicroBead Kit UltraPure, human (Cat No. 130-100-453, Miltenyi Biotec, Germany). Purity of $CD34^+$ cells was determined by flow cytometry using BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA) at post-isolation with cell marker (CD34, clone 8G12, Cat No 348057, BD Biosciences, San Jose, CA) after exclusion of cell debris based on scatter signals and dead cells by DAPI fluorescent stain (Cat. No. 130-111-570, Miltenyi Biotec, Germany). Data analysis was performed by FACSDiva software.

An adapted protocol from the manufacturing instructions from Miltenyi Biotec was established for the isolation of $CD34^+$ cells from AML bone marrow. Percentage of $CD34^+$ cells was determined by flow cytometry at a BD LSR II Flow Cytometer at both pre- and post-isolation with cell marker after exclusion of cell debris based on scatter signals and dead cells by DAPI fluorescent stain. Data analysis was performed by FACSDiva software.

Cell culture

THP-1 leukemic cells were maintained in Roswell Park Memorial Institute medium (RPMI, Cat No. SH30027.01, Hyclone) supplemented with 10% fetal bovine serum (FBS, Cat. No. F6178, Sigma Aldrich Pte. Ltd, Merck KGaA, Darmstadt, Germany) and cultured at 37°C with 5% CO_2. HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Cat. No. D1152, Sigma Aldrich Pte. Ltd, Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS, Cat. No. F6178, Sigma Aldrich Pte. Ltd, Merck KGaA, Darmstadt, Germany) and cultured at 37°C with 5% CO_2. For functional analysis of circASXL1-1, THP-1 cells were seeded in 6-well plates at a density of 2×10^5. Transfection was carried out using X-tremeGENE siRNA transfection reagent (Cat. No. 04476093001, Sigma Aldrich Pte. Ltd, Merck KGaA, Darmstadt, Germany) following manufacturer's instructions. Antisense oligonucleotide (ASO) was designed based on the backsplice junction of circASXL1-1 and purchased from Integrated DNA Technologies (IDT, Coralville, IA) along with non-targeting negative control (scrambled: Scr). Transfection complexes were prepared in Opti-MEM medium (Cat. No. 31905070, Invitrogen, Thermo Fisher Scientific, Waltham, MA) and added to the cells at a final concentration of 20 nM.

Differentiation of human CD34$^+$ progenitors

Human $CD34^+$ progenitors obtained from knee aspirates of normal individuals were cultured in StemSpan™ SFEM II media (Cat No. 09605, STEMCELL Technologies Inc. Vancouver, Canada) supplemented with StemSpan™ CC110 cocktail of cytokines (Cat. No. 02697, STEMCELL Technologies Inc. Vancouver, Canada) as per manufacturer's instructions. After a day in culture, $CD34^+$ cells were transduced using lentiviruses generated as described

below, followed by differentiation into either granulocytes (StemSpan™ Myeloid Expansion Supplement (100X), Cat. No. 02693, STEMCELL Technologies Inc. Vancouver, Canada) or monocytes (StemSpan™ Myeloid Expansion Supplement II (100X), Cat No. 02694, STEMCELL Technologies Inc. Vancouver, Canada) for 14 days.

Generation of stable cell lines

shRNA designed against the circASXL1-1 backsplice junction was cloned into the pLKO.1 inducible vector and the GFP-pLKO (non-inducible) vector along with a corresponding non-targeting control. The shRNAs thus generated were transfected into HEK293T cells along with the lentiviral packaging plasmids pRSV-Rev, pMDLg/RRE, and pMD2.g. Lentiviruses thus generated were used to infect 2×10^6 HEK293 cells with polybrene reagent (Cat. No. 107689, Merck KGaA, Darmstadt, Germany). After 24 h, the media-containing virus was replaced with growth media followed by puromycin selection for a week until the mock cells died.

An shRNA targeting backsplice junction was cloned into a non-inducible pLKO.1 vector along with a non-targeting control. The lentiviruses generated were used to infect 1×10^6 THP-1 cells with polybrene reagent (Cat. No. 107689, Merck KGaA, Darmstadt, Germany). After 24 h, the media-containing virus was replaced with growth media followed by puromycin selection until the mock cells died. Cell morphology was captured using Axio Imager M2 microscope (Carl Zeiss, Jena, Germany).

RNA isolation and real-time PCR (Q-PCR)

Total RNA was isolated from 2.5×10^6 $CD34^+$ cells using AllPrep DNA/RNA/miRNA universal kit (Cat. No. 80204, Qiagen, Hilden, Germany) according to the manufacturer's

instructions (Qiagen, Germany). Total RNA including circRNAs and small RNAs was extracted from THP-1 and HEK293 using the miRNeasy Mini kit (Cat. No. 217004, Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA isolated was quantified using the Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA) and the quality was assessed using a Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA, USA). Twenty micrograms of total RNA were digested with 20U of RNase R (Cat. No. RNR07250, Epicentre, Lucigen Corp. WI, USA) for 2 h at 37°C followed by RNA precipitation using ethanol.

Cytoplasmic and nuclear RNA fractions were isolated using the mirVana™ PARIS™ RNA and Native Protein Isolation kit following the manufacturer's instructions (Cat. No. AM 1556, Thermo Fisher Scientific, Waltham, MA).

Complementary DNA (cDNA) conversion was carried from untreated and RNase R treated samples using High-Capacity RNA-to-cDNA™ Kit (Cat. No. 4387406, Thermo Fisher Scientific). PCR analysis was carried out using 1 µl of 1:3 diluted cDNA in a reaction mixture containing 5 µl of iTaq™ Universal SYBR® Green Supermix (Cat. No. 1725120**,** Bio-Rad, USA), 0.5 µl each of forward and reverse primer (1 µM) (**Supplemental table 1**) and the total volume adjusted to 10 µl using RNase-free water. The reaction was carried out in Applied Biosystems 7900HT Fast Real-Time PCR machine. The primer sequences are listed in **Supplemental table 1**.

Digital droplet PCR

Digital droplet PCR (ddPCR) was used to determine the copy number and subcellular localization of *ASXL1* linear and circASXL1-1 and -2 transcripts. A droplet PCR reaction (24 µl) was prepared using QX200™ ddPCR™ EvaGreen Supermix (Cat. No. 1864033, Bio-Rad, USA)

containing 5 ng of cDNA and 250 nM of primers. QX200™ automated droplet generator was used to generate PCR droplets. PCR amplification was conducted using cycling conditions: 5 min at 95°C, 40 cycles of 30 s at 95°C, and 60 s at 60°C.

RNA sequencing and bioinformatic analysis

Untreated and RNase R treated THP-1 RNA samples were used for RNA sequencing. RNA-seq libraries were prepared by using Illumina TruSeq RNA Sample Prep Kit V2 (Cat. No. FC-121-9006, Illumina Inc., San Diego, USA) and were subjected to deep sequencing with Illumina HiSeq 4000 to the depth of 150 million paired-end reads. Briefly, raw reads were assessed using FastQC version 0.10.1 and input into Mapsplice 2 algorithm for circRNA detection and annotation. The Hg19 human reference genome (Feb. 2009 human reference sequence (GRCh37)) and RefSeq annotation were used for read mapping and annotation (*1-3*).

Protein isolation and western blotting

Total protein was extracted from THP-1 and HEK293 cells using IPH lysis buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.5% Triton X100, 10% glycerol, 1 mM DTT, PMSF, PIC). The extracted protein was quantified using Bradford reagent according to manufacturer's instructions (Prod No. 500-0006, Bio-Rad). Forty micrograms of total protein from each sample were denatured at 95°C for 5 min and separated on SDS-PAGE for western blotting. The proteins were transferred to nitrocellulose transfer membranes blocked with 5% BSA and incubated with the primary antibodies, anti-H2AK119Ub (Cat. No. 8240S, Cell Signaling Technology, Danvers, MA 01923), H2A (Cat. No. ab18255, Abcam, Cambridge, MA), H3 (Cat. No. sc-10809, SantaCruz

Biotechnology, Dallas, Texas 75220), H3K27me3 (Cat. No. 9733, Cell Signaling Technology, Danvers, MA 01923), GAPDH (Cat. No. sc-20357 SantaCruz Biotechnology, Dallas, Texas 75220), BAP1 (C-4) (Cat. No. sc-28383, SantaCruz Biotechnology, Dallas, Texas 75220), overnight at 4°C. Following washing, blots were incubated with secondary HRP antibody (Cat. No. sc-2030/sc-2005, SantaCruz Biotechnology, Dallas, Texas 75220). All blots were developed with western blotting luminol reagent (Cat. No. sc-2048, SantaCruz Biotechnology, Dallas, Texas 75220) and quantified on densitometer (Bio-Rad) using Quantity One software (Bio-Rad).

Deubiquitinase (DUB) assay

Immunoprecipitation was performed using 1 mg protein isolated from HEK293 cells overexpressing either BAP1, C91A or control plasmids in the background of circASXL1-1 depletion. The lysates were incubated with FLAG-M2 beads (Cat. No. M8823, Merck KGaA, Darmstadt, Germany) overnight at 4°C with rotation. After washing the beads five times with ice-cold IP wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 1% Triton X-100, 0.5% NP-40), protein was isolated using competing FLAG peptide (Cat. No. F3290, Merck KGaA, Darmstadt, Germany). BAP1 thus obtained was incubated with 1 µg H2A ubiquityl mononucleosomes (Cat. No. 16-0020, EpiCypher, Durham, NC, USA) for 2 h in DUB buffer at 37°C followed by denaturation and western blotting.

RNA immunoprecipitation (RIP)

To study protein-circASXL1-1 interaction, RNA immunoprecipitation analysis was performed as described (*4*). Briefly, HEK293 cells were lysed in polysome extraction buffer (PEB, 10 mM HEPES pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 1 mM 0.5% NP-40, DTT, 400 mM VRC)

supplemented with protease and RNase inhibitors for 30 min on ice and the supernatant was collected by centrifugation (10000 rpm, 10 min, 4°C). The lysates were incubated with BAP1 antibody (Cat. No. A302-242A, Bethyl Laboratories Inc, TX) or control-IgG antibody (Cat. No. P120-101, Bethyl Laboratories Inc, TX) overnight at 4°C with rotation. Protein A magnetic beads (Cat. No. S1425S, New England Biolabs Inc., Ipswich, MA) were washed twice with DEPC-H_2O followed by incubation with lysate (2 h, 4°C rotation). After washing the beads five times with ice-cold 1×IP wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 1% Triton X-100, 0.5% NP-40), RNA was isolated using Trizol reagent.

Flow cytometric analysis

Human $CD34^+$ cells differentiated as described above were stained with antibodies conjugated with PE and APC. Antibodies for monocyte markers: anti-CD13 (Cat. No. MA119702, Ebioscience, Thermo Fisher Scientific, Pittsburg, USA) and anti-CD14 (Cat. No. 12-0149-42, Ebioscience, Thermo Fisher Scientific, Pittsburg, USA). Antibodies for granulocyte markers: anti-CD13 (Cat. No. MA119702, Ebioscience, Thermo Fisher Scientific, Pittsburg, USA), anti-CD15 (Cat. No. 12-8813-42, Ebioscience, Thermo Fisher Scientific, Pittsburg, USA). Flow cytometric analysis was performed using a BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA) and data analysis was performed using FACSDiva software. shControl and shcircASXL1-1 THP-1 cells were stained with CD11b antibody (Cat. No. 17-0118-42, Ebioscience, Thermo Fisher Scientific, Pittsburg, USA) conjugated with APC. FACS analysis was performed using a BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA) and data analysis was performed using FACSDiva software.

Cell growth assay

The shControl, shcircASXL1-1 THP-1 cells were seeded in 96-well plates (500 cells/well) and the experiment was performed using Cell Titer-Glo luminescent cell viability assay (Cat. No. G9242, Madison, Wisconsin USA) according to manufacturer's instruction for four days. Growth curves performed in triplicates.

Colony-forming assay for CD34$^+$ cells

After lentiviral transduction of CD34$^+$ primary cells, 1000 cells from each group (shControl and shcircASXL1-1 respectively) were seeded in duplicate in MethoCult media (Cat. No. H04535, STEMCELL Technologies Inc. Vancouver, Canada) and allowed to grow for 14 days. At the end of the experiment, granulocyte-macrophage colonies (CFU-GM) were counted and images were captured using a Zeiss Axio Observer microscope (Carl Zeiss, Jena, Germany). CFU assays were performed in triplicates.

Statistical analysis

Data are represented as mean ± SD from at least three independent experiments. Statistical significance was evaluated either by Student's t-test or ANOVA with *post hoc* Tukey test. *P* values of $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ were considered significant.

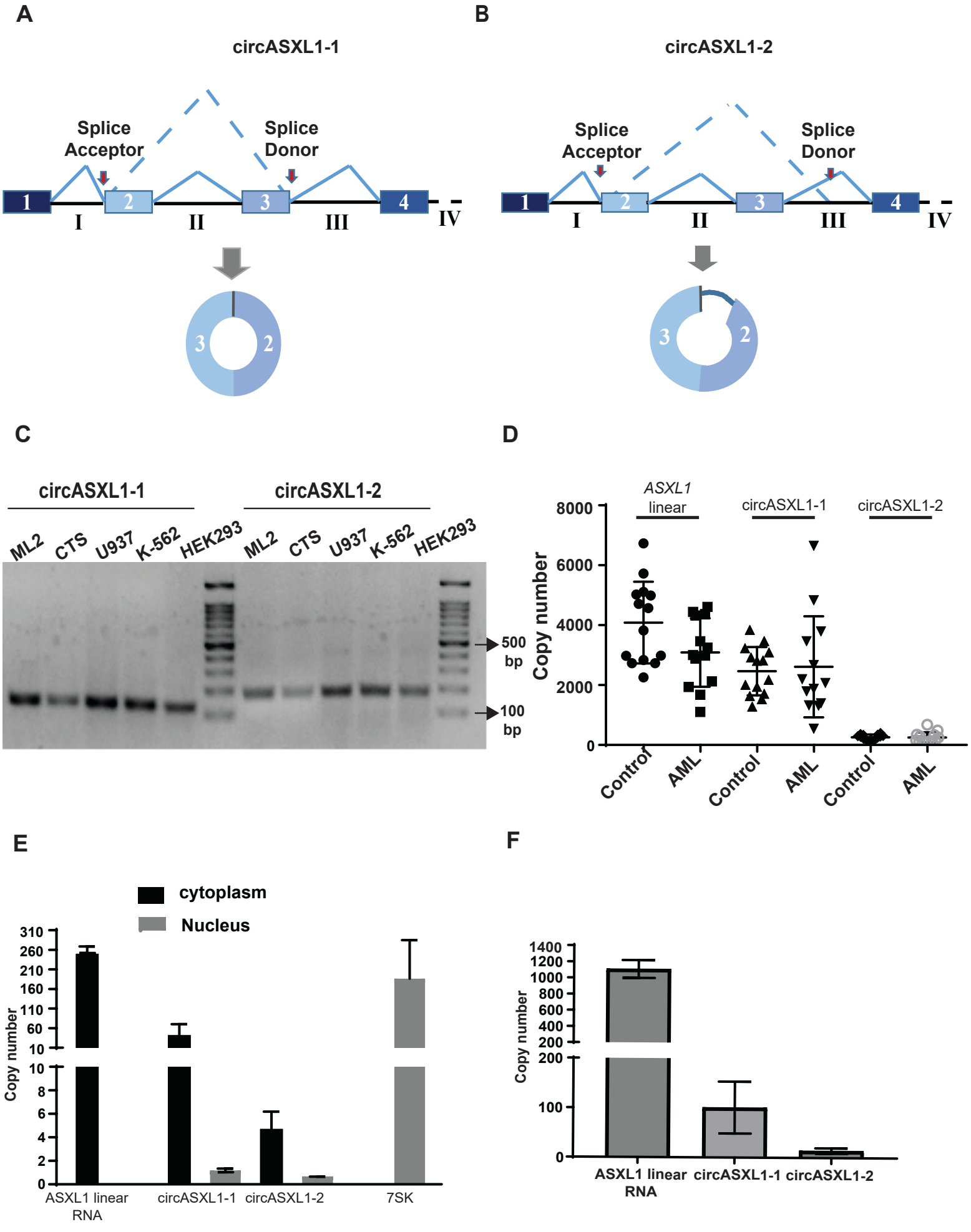
Supplemental table 1: Primer sequences used in the study

No	Gene Symbol	Sequence
1	*ASXL1* linear F	CAAGAGGAGGAGAGGGGTTG
	ASXL1 linear R	CTCTCCCTCCACTGTAGCTG
2	*GAPDH* F	CAGCCTCAAGATCATCAGCA
	GAPDH R	TGTGGTCATGAGTCCTTCCA
3	*7sk* F	AGGACCGGTCTTCGGTCAA
	7sk R	TCATTTGGATGTGTCTGCAGTCT
4	*lncFOXO1* F	CGATGTGCTGGAGTGTATGT
	lncFOXO1 R	GCAGGATGGCACTACTGATAA

Reference

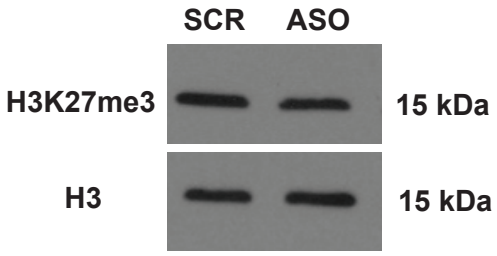
1. Y. Hu *et al.*, A probabilistic framework for aligning paired-end RNA-seq data. *Bioinformatics* **26**, 1950-1957 (2010).
2. K. Wang *et al.*, MapSplice: accurate mapping of RNA-seq reads for splice junction discovery. *Nucleic Acids Res* **38**, e178 (2010).
3. Y. Hu *et al.*, DiffSplice: the genome-wide detection of differential splicing events with RNA-seq. *Nucleic Acids Res* **41**, e39 (2013).
4. J. D. Keene, J. M. Komisarow, M. B. Friedersdorf, RIP-Chip: the isolation and identification of mRNAs, microRNAs and protein components of ribonucleoprotein complexes from cell extracts. *Nat Protoc* **1**, 302-307 (2006).

Supplementary Figure 1

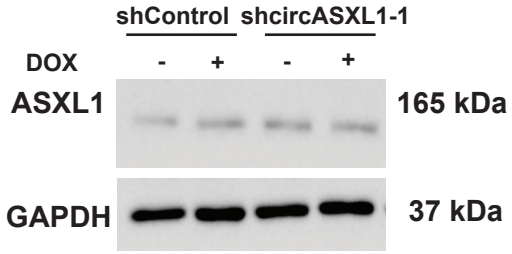


Supplementary Figure 1. circASXL1-1 and -2 are detected in multiple cell lines. (A-B) Schematic representation of circASXL1-1 **(A)** and circASXL1-2 **(B)** showing the splice donor and acceptor sites and intron-exon boundaries. **(C)** Expression of circASXL1-1 and -2 in indicated AML cell lines by Q-PCR. **(D)** Droplet digital PCR (ddPCR) analyses showing copy number of ASXL1 linear mRNA, circASXL1-1 and -2 expressions in $CD34^+$ hematopoietic stem cells from 15 healthy individuals as compared to AML patients. Each dot represents a sample. **(E)** *ASXL1* RNA copy number analysis by ddPCR. **(F)** Cytoplasmic localization of all *ASXL1* RNA isoforms demonstrated by ddPCR. 7SK small nuclear RNA serves as control for the nuclear-cytoplasmic separation. Data is represented as mean ± SD from at least three independent experiments.

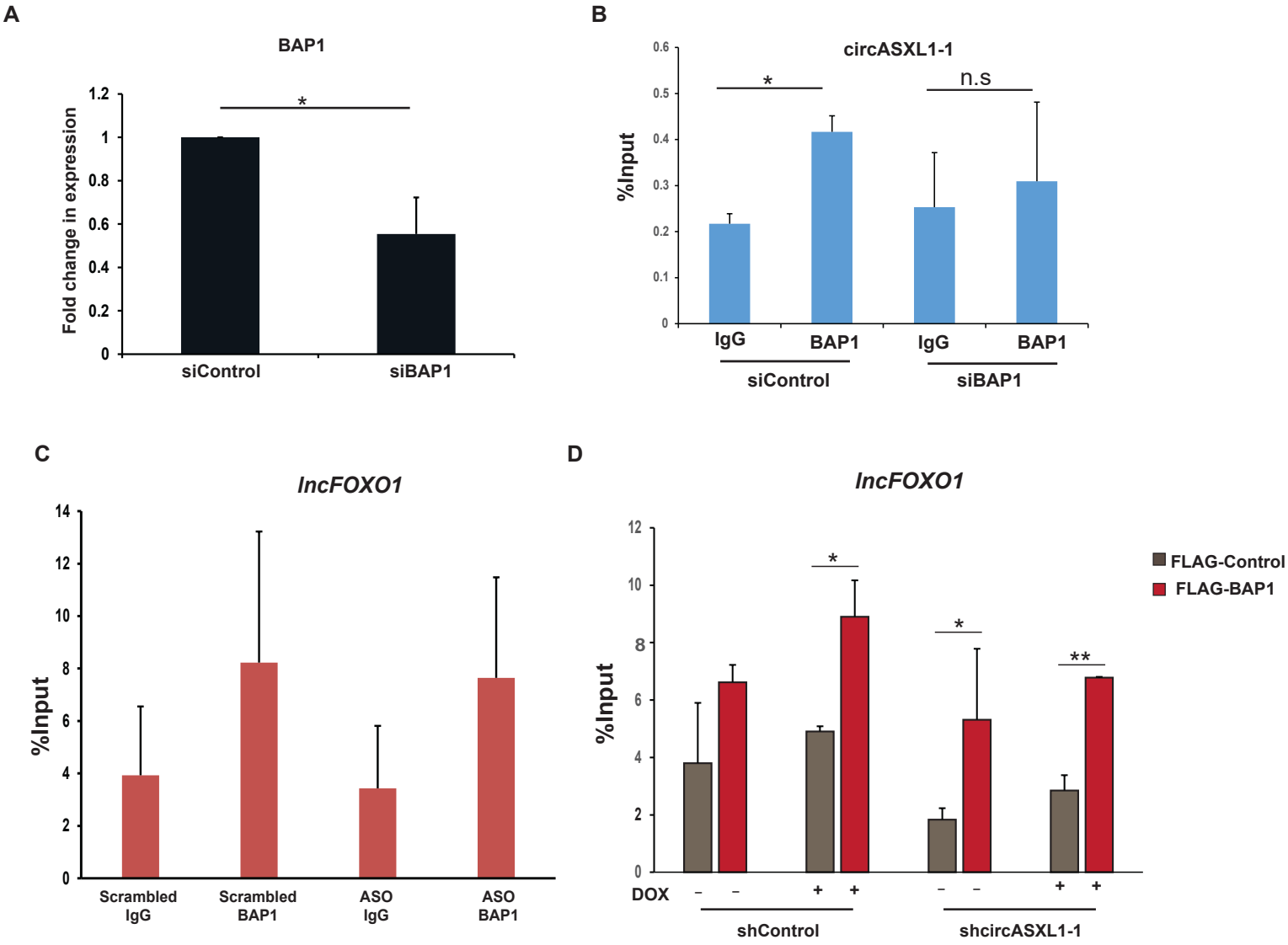
A


SCR
ASO
H3K27me3
15 kDa
H3
15 kDa

B


shControl
shcircASXL1-1
DOX
-
+
-
+
ASXL1
165 kDa
GAPDH
37 kDa

Supplementary Figure 2. circASXL1-1 depletion does not regulate PRC2 activity. (A) Western blot showing no change in H3K27me3 expression after depletion of circASXL1-1 in THP-1 cells. **(B)** Western blot showing no significant change in ASXL1 protein expression after depletion of circASXL1-1 using expression of shcircASXL1-1 induced by doxycycline for 48 h in HEK293 stable cells lines as compared to shcircASXL1-1 –dox or shControl +/- dox groups.


A
BAP1
Fold change in expression
1.2
1
0.8
0.6
0.4
0.2
0
*
siControl
siBAP1
B
circASXL1-1
%Input
0.6
0.5
0.4
0.3
0.2
0.1
0
*
n.s
IgG
BAP1
IgG
BAP1
siControl
siBAP1
C
lncFOXO1
%Input
14
12
10
8
6
4
2
0
Scrambled IgG
Scrambled BAP1
ASO IgG
ASO BAP1
D
lncFOXO1
%Input
12
10
8
6
4
2
0
*
*
**
FLAG-Control
FLAG-BAP1
DOX
– – + + – – + +
shControl
shcircASXL1-1

Supplementary Figure 3. circASXL1-1 and *lncFOXO1* bind to BAP1. (**A)** Q-PCR showing depletion of BAP1 mRNA 48 h after siBAP1 treatment as compared to siControl. **(B)** Q-PCR showing enrichment of circASXL1-1 after RNA immunoprecipitation (RIP) using antibody targeting endogenous BAP1 as compared to IgG. This enrichment is abolished followed depletion of BAP1 using siBAP1. **(C)** Q-PCR showing enrichment of *lncFOXO1* after RNA immunoprecipitation (RIP) using antibody targeting endogenous BAP1 as compared to IgG. **(D)** Q-PCR showing enrichment of *lncFOXO1* after RNA immunoprecipitation (RIP) using FLAG beads to pulldown FLAG-BAP1 as compared to HEK293 cells transfected with EV. Statistical analysis was carried out using ANOVA with *post hoc* Tukey test. Data are represented as mean ± SD from at least three independent experiments. $*p < 0.05$, $**p < 0.01$.