Signal peptide-CUB-EGF-like repeat-containing protein 1-promoted FLT3 signaling is critical for the initiation and maintenance of MLL-rearranged acute leukemia

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Supplementary Materials and Methods

Mice

The generation of *Scube1*-KO mice was as described.[1] Briefly, the floxed allele of *Scube1* (*Scube1*$^{f/f}$) was generated by the introduction of 2 loxP sites in *Scube1* gene using CRISPR-Cas9 system. Scube1 KO (Δ3) mice were generated by crossing mice carrying a new *Scube1* conditional allele (*Scube1*$^{f/f}$) with mice harboring a Cre recombinase under expression control of the mouse protamine 1 (*Prm1*) promoter. The resulting transcript is predicted to lack exons coding for NH_2-terminal EGF-like repeats, spacer region, CR motifs and COOH-terminal CUB domain. Mice homozygous for the wild-type (WT) allele or the targeted (Δ3) allele were obtained by heterozygous (Δ3/+) interbreeding and were born with the expected Mendelian ratio. These Δ3 mutant (KO) mice showed normal development, survival, and reproduction. In addition, white or red blood cell counts as well as other hematological parameters were comparable between WT and KO mice (see Supplementary Table S3). The WT allele was genotyped with F1+R1 primers and 5' and 3' loxP flanked sites were genotyped with F2+R2 and F3+R3 primers, respectively. The cre-mediated excised mutant *Scube1* was confirmed by genotyping with F2+R3 primers.

For generating tamoxifen-dependent conditional KO mice, *Scube1*$^{f/f}$ mice were bred with ROSA26 CreERT2 mice (kindly provided by Dr. Yu-Ting Yan, Institute of Biomedical Sciences, Academia Sinica, Taiwan[2] to obtain the desired genotypes as mentioned in Suppl. Fig. S8 (A).

Cell culture

THP-1 and KG-1a cells were obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). NOMO-1, and MOLM-13 cells were obtained from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). MV4-11, K562, Raji, and Daudi cells were obtained from ATCC. SEMK2 and Kasumi-1 cells were kindly provided by Dr. Shu-Ping Wang, Institute of Biomedical Sciences, Academia Sinica, Taiwan. Leukemia cell lines (THP-1, NOMO1, KG-1a, K562, MOLM-13, MV4-11, SEMK2, Kasumi-1, Raji, Daudi) were cultured in RPMI supplemented with 10% fetal bovine serum, 100 unit/ml penicillin and 100 μg/ml streptomycin with 5% CO2. HEK-293T cells were maintained in DMEM medium with

10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) with 5% CO2.

Antibodies

Anti-SCUBE1 (mAb clone #1 and #7) antibodies were produced in-house. Anti-MEIS1 (ab124686) and anti-HOXA9 (ab140631), anti-caspase 3 (ab13847), and anti-survivin (ab76424) antibodies were obtained from Abcam. Anti-phospho-tyrosine (GTX14167) polyclonal antibodies were from GeneTex (Hsinchu, Taiwan). Anti-phospho-FLT3 (Tyr591) (36461), anti-FLT3 (8F2) (3462), anti-phospho-MAPK p44/p42 (Thr202/Tyr204) (9106), anti-MAPK p44/p42 (9102), anti-phospho-AKT (Ser473) (9271), anti-AKT (9272), and cleaved caspase-3 (Asp175) (9661) antibodies were from Cell Signaling Technology. Anti-human CD45/RPE (T29/33) antibody was from Agilent. Anti-MouseCD117 (cKit)-FITC was from eBioscience.

Plasmid constructs

The inducible SCUBE1-shRNA constructs were purchased from horizondiscovery. The *SCUBE1* promoter/regulator regions were amplified with specific primer primers and genomic DNA templets from THP-1 cells and cloned into the pGL3-basic vector. HOXA9 or MEIS1 complementary DNA sequence (CDS) was amplified with primers using cDNA templates isolated from THP-1 cells and cloned into pFlag-CMV2. pMIR-FLAG-MLL-AF9 was a gift from Daisuke Nakada (Addgene plasmid # 71444; http://n2t.net/addgene:71444; RRID: Addgene_71444).[3] FLT3 cDNA (HG10445-NH), LYN cDNA (HG10829-NH), and FLT3LG cDNA (HG10315-NH) were from Sino Biological. FLT3 cDNA was further transferred to the pMyc-CMV2 vector.

Western blot analysis

Cells were lysed with cell lysis buffer (25 mM HEPES, pH 7.6, 150 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 10% glycerol and 1% Triton X-100). An amount of 5× sample buffer (250 mM Tris/HCl, pH 6.8, 10% SDS, 10% dithiothreitol, 50% glycerol and 0.5% bromophenol blue) was added to lysates for boiling at 100◦C for 10 min. Protein samples were separated by SDS/PAGE and transferred to PVDF membranes. The blots were blocked in 0.1% gelatin in phosphate buffered saline (PBS) containing 0.05% Tween 20 overnight at 4◦C, then incubated with the primary antibody and horseradish peroxidase-conjugated secondary antibody for 1 h each. Proteins were detected by using an ECL substrate and the BioSpectrum imaging system (UVP).

Quantitative RT-PCR (reverse transcription polymerase chain reaction)

Total RNA was extracted from samples by using TRIzol reagent (Life Technologies). First-strand cDNA synthesis with SuperScript II reverse transcriptase (Life Technologies) involved 5 μg RNA. One-twentieth of the first-strand cDNA reaction was used for each PCR as a template and 1 × cyber green master mix (HOT FIRE Pol, SOLIS BIODYNE, 08-24-00001) was used to run the qPCR reactions. Relative expression was calculated with the comparative CT method ($2^{-\Delta CT}$) and normalization involved the *GAPDH* gene with each PCR reaction performed in triplicate. Primers are in Supplementary Table S5.

Flow cytometry

Cells ($1x10^6$) were washed with PBS twice, FACS buffer (2% FBS in PBS) once and the primary antibody was added in 300 μl FACS buffer and kept at 4 °C for 1 h with rotation. Cells were washed with FACS buffer twice and secondary antibody (1:500) was added in 300 μl FACS buffer and kept at 4 °C for 1 h with rotation. Then cells were washed with FACS buffer twice, re-suspended in PBS and analyzed by using FACS Canto or Attune NxT-14color.

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed according to a modified protocol from.[4] Cells were crosslinked with 1% formaldehyde for 20 min at room temperature, quenched with 0.125M glycine and washed with three buffers sequentially: PBS, buffer A (0.25% Triton X 100, 10 mM EDTA, 0.5 mM EGTA, 20 mM HEPES pH 7.6), and buffer B (0.15 M NaCl, 10mM EDTA, 0.5 mM EGTA, 20 mM HEPES pH 7.6). Cells were then suspended in ChIP lysis buffer (0.15% SDS, 1% Triton X 100, 150 mM NaCl, 10 mM EDTA, 0.5 mM EGTA, 20 mM HEPES pH 7.6) and sonicated by using a Bioruptor sonicator (Diagenode) for 20 min at high power, 30 s ON, 30 s OFF. Sonicated chromatin was centrifuged at maximum speed for 10 min. The supernatant was diluted in ChIP dilution buffer (1% Triton X 100, 150 mM NaCl, 10 mM EDTA, 0.5 mM EGTA, 20 mM HEPES pH 7.6), then incubated overnight at 4°C with protein A/G mag beads (GenScript, L00277) and 2 μg anti-MEIS1 antibody (Active Motif, 39795) or IgG. Beads were washed sequentially with four different wash buffers: wash buffer 1 (0.1% SDS, 1% Triton, 150 mM NaCl, 10 mM Tris pH 8, 0.1 mM EDTA, 0.5 mM EGTA), wash buffer 2 (0.1% SDS, 1% Triton, 500 mM NaCl), wash buffer 3 (0.25 M

LiCl, 0.5% NP-40, 10 mM Tris pH 8, 0.1 mM EDTA, 0.5 mM EGTA), and wash buffer 4 (10 mM Tris pH 8, 0.1 mM EDTA, 0.5 mM EGTA) (two times each) at 4˚C for 5 min on a rotation platform. Precipitated chromatin was eluted from the beads with 400 µl elution buffer (1% SDS, 0.1 M NaHCO3) at 42°C for 30 min. Protein-DNA crosslinks were reversed at 65°C overnight in the presence of 200 mMNaCl and RNase A, followed by the addition of Proteinase K and incubated for 1 h. DNA was isolated by using the Qiagen PCR clean up kit. The input control DNA or immunoprecipitated DNA was amplified in a 20-µl reaction volume consisting of 1 µl eluted DNA template with primers (F1: TGG GGT AGT TCC CAA GTG GA, R1: AAC ATC CTG GGA AGC AGA GC; F2: CTC AAG CTC TGG TGA GAC GG, R2: AGG TAT GGA GAG CCC GAT GT). PCR involved use of Taq polymerase for 28 cycles of 94°C for 30 sec, 63.5°C for 30 sec, 72°C for 40 sec, then 5 min at 72°C. A 10-µl aliquot from each PCR reaction was separated on 1.5% agarose gel.

Luciferase reporter assay

293T cells were transiently transfected with 1 µg SCUBE1 promoter luciferase reporter plasmid and internal control (0.05 µg pRL-TK Renilla luciferase plasmid) by using Maestrofectin Transfection Reagent (Omics bio, MF002) according to the manufacturer's instructions. Cells were cultured for an additional 2 days, harvested and prepared for reporter assay with the Dual-Luciferase reporter assay system (Promega).

Lentivirus production and transduction

Lentivirus was produced in HEK-293T cells co-transfected with 2 µg pMD.G, 3 µg pCMVd2.8r and 12 µg lentiviral vector. Cells were incubated at 37 °C, 5% CO2, and lentiviral particle-containing media was collected 48 h post-transfection. Desired cells were transduced with viral supernatants and 8 µg/ml polybrene by spinoculation at 800g for 2 h. The media was discarded and cells were resuspended in fresh RPMI media and kept in normal culture conditions for 3 days.

Methylcellulose colony formation assay

Methylcellulose colony formation assay was performed according to a modified protocol from.[5] Briefly, retroviral or lentiviral supernatants were collected 48 h after transfection of HEK-293T cells and used to transduce hematopoietic progenitors and stem cells positively selected for cKit expression by FACS with an anti-cKit FITC conjugated antibody from the bone marrow of 8- to 10-week-old *Scube1 KO*, *WT*,

Scube1$^{f/f}$, or *Scube1*$^{f/f}$; *R26*^{CreERT2} C57BL/6 mice. After spinoculation by centrifugation at 800 g for 2 h at 32°C, transduced cells were cultured overnight in RPMI supplemented with 10% FCS, 20 ng/ml SCF, 10 ng/ml each IL-3 and IL-6 (all murine, GenScript). Cells were then plated in 1% methylcellulose (Sigma, M7027) in RPMI media supplemented with the same cytokines plus 10 ng/ml GM-CSF (murine, GenScript). After 5-7 days' culture, colonies were counted to calculate the transduction efficiency. Single-cell suspensions (10^4 cells) were then replated in methylcellulose media supplemented with the same cytokines. Subsequent replating was usually repeated every 5-7 days.

shRNA-mediated knockdown

To knock down *SCUBE1* we used an inducible shRNA lentiviral vector from Horizon (Clone Id: V3IHSMCG_5797472, Mature Antisense: CGCACGATGTCCTCTATGA; Clone Id: V3IHSMCG_6203207, Mature Antisense: CCGAGCTCACCACCGAAGT). The vectors are used to produce lentivirus as mentioned and transduced by spinoculation (800g for 2 h) with 8 µg/ml polybrene. Two days after transduction, stable cells were selected with 2 µg/ml puromycin. A 2-µg/ml amount of doxycycline (Sigma, D9891) was added to the stable cells with inducible shRNA to knock down SCUBE1 for 5 days.

To knockdown *MEIS1, HOXA9 or FLT3*, we used the vector-based short hairpin RNAs (shRNAs) generated by The RNAi Consortium, Academia Sinica, Taiwan. The produced lentivirus was used to transiently knock down specific genes. The target sequence is mentioned in Supplementary Table S6.

MTT assay

Cell viability was assayed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, 5 x 10^3 cells/well of 96-well plates were treated with different agents (according to the experiment) and cell viability was determined by incubating the cells with 25 µl MTT dye (Sigma, M5655) (5 mg/ml in PBS) for 2 h at 37 °C. Cells were incubated with 100 µl lysis buffer (20% SDS in 50% dimethylformamide) overnight at room temperature, and the absorbance was taken at 570 nm.[6]

Cell cycle analysis

For cell cycle analysis, cells were fixed in cold 70% ethanol overnight, washed with PBS and treated with 100 ug/ml RNAse and propidium iodide at 37℃ for 30 min. The cell cycle profiles were analyzed by using Attune NxT-14color and FlowJo.[7]

Retrovirus production and transduction

Retrovirus was produced with HEK-293T cells co-transfected with 5μg pCL-Eco and 5 μg retroviral vector (pMIR-FLAG-MLL-AF9). Cells were incubated at 37 °C, 5% CO2, and retroviral particle-containing media was collected at 48 h post-transfection. The c-Kit–positive hematopoietic progenitor cells (HPCs) were transduced with viral supernatants and 8 μg/ml polybrene by spinoculation at 800g for 2 h. The media was discarded and cells were resuspended in fresh RPMI media and kept in normal culture conditions for 3 days.

In vivo leukemogenesis study

For leukemia cell lines, 8- to 10-week-old NSG (*NOD/SCID/IL-2Rγc*$^{-/-}$) mice were sub-lethally irradiated with 200 cGy 24 h before cell transplantation, then injected intravenously with 5 × 10^5 leukemia cells (THP1or NOMO-1 with inducible shRNA#2)/mouse. After injection, mice were randomly distributed and treated with doxycycline (200 μg/ml) and sucrose (1%) or only sucrose (1%) in drinking water. The doxycycline water was changed twice a week. After 28 days, mice were sacrificed for human cell engraftment analysis, and the others were kept for overall survival evaluation. The spleen and femurs were isolated for the analysis of engraftment. The bone-marrow engraftment was analyzed by isolating cells from the femur and flow cytometry with human ant-CD45 antibody. The overall survival was analyzed by monitoring mice for symptoms of disease (ruffled coat, hunched back, weakness, reduced motility) twice a week. The animals with signs of distress were sacrificed and the death was recorded.

To generate primary transplanted mice, leukemia cells after the third-round of replating were injected via the tail vein into 6- to 10-week-old syngeneic C57BL/6 mice that received a sub-lethal dose of 550 cGy total body γ irradiation 24 h before the experiment. Mice were monitored for leukemia development and sacrificed. The leukemic cells were isolated from bone marrow, and DsRed-positive cells were sorted by using FACS. The cells were used for secondary transplantation. The secondary transplantation method used was similar to the primary transplantation. After 45 days of secondary transplantation, mice were sacrificed for leukemic cell engraftment

analysis, and the others were kept for overall survival evaluation. The engraftment was analyzed by spleen enlargement and histological analysis. Overall survival was analyzed by monitoring mice for symptoms of disease (ruffled coat, hunched back, weakness, reduced motility) twice a week. The animals with signs of distress were sacrificed and the death was recorded.

For inducible knockout study, after secondary transplantation, peripheral blood was checked for leukemic blast cells. When blast cells were more than 10% to 20% in the periphery, tamoxifen was injected. To prepare the working tamoxifen solution, tamoxifen powder was dissolved in ethanol, and vortexed for a few minutes. Sunflower oil was added at a 9:1 oil: ethanol mixture ratio. The final solution was intraperitoneally injected into the mice for 5 consecutive days at 40 mg/kg/day. At 40 days after the last injection, mice were sacrificed for leukemic cell engraftment analysis, and the others were kept for overall survival evaluation. The engraftment was analyzed by spleen enlargement and histological analysis. The overall survival was analyzed by monitoring mice for symptoms of disease (ruffled coat, hunched back, weakness, reduced motility) twice a week. The animals with signs of distress were sacrificed and the death was recorded.

Isolation of bone marrow (BM) and peripheral blood cells

BM cells were harvested from femurs and tibias of mice. Single-cell suspensions were generated by flushing and crushing bones in PBS. Mononuclear cells were isolated with gradient centrifugation by using Histopaque 1083 (Sigma). The single-cell suspension was added to the Histopaque 1083 followed by centrifugation at 260g for 30 min at room temperature. The middle white layer of mononuclear cells was isolated and washed with PBS and FACS buffer once each.

For peripheral blood cell analysis, 100 µl blood was drawn from the retro-orbital sinus/plexus from mice under anesthesia. Red blood cells were lysed with erythrocyte lysis buffer (150 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA), and cells were washed once with PBS and FACS buffer once each.

Proximity ligation assay

THP-1 or NOMO-1 cells $1x10^7$ were incubated with 10 µg/ml anti-SCUBE1 mAb (#7) or isotype control for 1 h at 4°C. Cells were washed with PBS and further incubated with HRP-conjugated secondary antibody for 30 min at 4°C. After

incubation, cells were washed with PBS and 500 µM biotin phenol (biotinyl tyramide) (Toronto Research Chemicals, B397770), and 1 mM H_2O_2 was added for 4 min. Cells were further washed and lysed with 20 mM Tris·Cl, pH 7.5, 5 mM EDTA,150 mM NaCl, 1% (v/v) Triton X-100, 0.1 M sodium thiocyanate, 10 µg/ml aprotinin, 5 µg/ml leupeptin. To check ligation efficiency, flow cytometry was used with FITC-conjugated streptavidin. After lysis, debris were cleared with centrifugation and supernatant was used for pull down of biotinylated proteins with streptavidin beads. Streptavidin beads were incubated with cell lysate for 1 h and washed with 20 mM Tris·Cl, pH 7.5, 5 mM EDTA,150 mM NaCl several times. 8M urea was added to the beads and reduced with 5 mM DTT for 25 min at 56°C and alkylated with 15 mM iodoacetamide for 45 min in the dark at room temperature. The sample solution was diluted to 1M urea with 50 mM TEABC buffer and digested with trypsin at an enzyme:protein ratio of 1:50 w/w for 16-18 h at 37 °C. The digested peptides were separated from streptavidin beads with centrifugation. Digested peptides were desalted with C18 Ziptip (Millipore) according to the manufacturer instructions, and differentially biotin-labelled peptides were analyzed by mass spectrophotometry.[8]

Immunoprecipitation

Two days after transfection, cells were lysed with 50 mM Tris, pH 8, 0.5% Triton-X 100, 150 mM NaCl, 2 mM EDTA, 10 µg/ml aprotinin, 5 µg/ml leupeptin. Lysates were clarified by centrifugation at 10,000xg for 20 min at 4 ℃. An amount of 200 µl each sample (1 mg/ml) was incubated with 1 µg of the indicated antibody and 20 µl of 50% (v/v) Protein A-agarose (Pierce) for 1 h with gentle rocking. After 3 washes with lysis buffer, precipitated complexes were solubilized with SDS-PAGE sample buffer and detected by western blot analysis. For immunoprecipitation of FLAG-tagged proteins, anti-FLAG M2-Agarose (Sigma, A2220) was used.

Generation of anti-SCUBE1 antibody

Anti-SCUBE1 monoclonal antibodies were produced by using the recombinant protein fragments containing the EGF-like domains as immunogens as described.[9] The specificity of anti-SCUBE1 monoclonal antibodies was verified by western blot analysis or flow cytometry.

Internalization assay

Anti-SCUBE1 antibody was labelled with Alexa Fluor 488 by using the commercial Antibody Labeling Kit (Thermo Fisher Scientific) according to the manufacturer's

protocol. An amount of 1 mg/mL human IgG was added to cells to reduce nonspecific binding to FcgRs at 1 h before adding anti-SCUBE1 antibody. Anti-SCUBE1 antibody was added to cells for different times as indicated at 10 μg/ml. For lysosomal trafficking, cells were first incubated with LysoView 650 (Biotium) for 2 h under growth conditions to label the acidic compartments. Images were acquired at different times by using an LSM700 confocal microscope.

Cell viability assay with drug-conjugated antibody

KG-1a, K562, THP-1, and NOMO-1 cells (5,000 cells per well) were seeded in 96-well plates. Cells were pre-blocked with human IgG at 1 mg/ml for 1 h. Then, serial dilutions of anti-SCUBE1 or anti-SCUBE1-VC-MMAE were added to the cells. Cells were incubated in normal culture condition for 5 days, then MTT assay was performed.

In vivo experiment with drug-conjugated antibody

THP-1 cells ($5x10^6$) were injected into 8-week-old NSG mice and allowed to grow. When the average tumor volume reached ~150-200mm^3, mice were randomly assigned to the IgG-Control or anti-SCUBE1-VC-MMAE treatment group. An amount of 10 mg/kg human IgG-Control or anti-SCUBE1-VC-MMAE was injected intravenously into each mouse for two times, 1 week apart. The tumor volume was measured by using a caliper and when the tumor volume in the control group reached 2000 mm^3, mice were euthanized and tumors were isolated and the weights were measured. The tumor volume was calculated as (Width (smallest length)^2 x Length (longest length)) x 0.5. The weight of mice was measured each week.

Microarray analysis of human AML samples

Data were derived from GSE68469 and GSE71014 datasets and analyzed as mentioned.[10]

In silico analysis of binding sites of MEIS1 and HOXA9

The SCUBE1 gene sequence along with its promotor and enhancers was retrieved from the UCSC genome browser. The sequence was used to find predicted binding regions of MEIS1 or HOXA9 by using PROMO.[11, 12]

Statistical analysis

Data are described with mean ± SD and were analyzed by Student *t* test (for two groups) or one-way ANOVA (for ≥3 groups) with Tukey multiple comparison analysis by using Prism 7 (GraphPad Software). $P<0.05$ was considered statistically significant.

Supplementary Table S1. Comparison of clinical manifestations for patients with acute myeloid leukemia (AML) with high and low *SCUBE1* expression

Variables	Total (n=227)	High *SCUBE1* expression (n=113)	Low *SCUBE1* expression (n=114)	*P* value
Sex				0.467
Male	118	56	62	
Female	109	57	52	
Age (year)		50 (18-84)	44 (18-88)	0.127
Lab data				
WBC (/μL)		38890 (380-341420)	13680 (470-423000)	<0.001
Hemoglobin (g/dL)		7.8 (3.7-12.4)	8.5 (3.3-13.2)	0.163
Platelet count (×1,000/μL)		53 (6-331)	42 (2-655)	0.388
Blast cell count (/μL)		18473 (0-260615)	5099 (0-348777)	<0.001
LDH (U/L)		917 (202-7734)	936 (242-13130)	0.842
FAB*				<0.001
M0	2	1 (50.0)	1 (50.0)	0.761
M1	55	21 (38.2)	34 (61.8)	0.048
M2	73	29 (39.7)	44 (60.3)	0.037
M3	26	10 (38.5)	16 (61.5)	0.220
M4	55	40 (72.7)	15 (27.3)	<0.001
M5	12	12 (100.0)	0 (80.0)	<0.001
M6	4	0 (0)	4 (100)	0.061
Karyotype	219			
t(8;21)	21	2	19	<0.001
t(15;17)	25	9	16	0.150
inv (16)	8	5	3	0.455
t(v;11q23.3)	6	5	1	0.093
t(v;11q23.3) or *MLL*-PTD mutation	15	12	3	0.015

Data are n of patients or median (range)

*Number of patients (% with high or low *SCUBE1* expression in the AML subtype)

Abbreviations: WBC, white blood cell; LDH, lactate dehydrogenase; PTD, partial tandem duplication

Supplementary Table S2. Multivariate analysis (Cox regression) of factors associated with overall survival*

Variables	Overall survival		
	HR	95% CI	*P* value
Total cohort (n=227)			
Age	1.026	1.011–1.042	0.001
WBC/1000	1.002	0.999–1.006	0.208
ELN adverse risk[$]	1.332	1.124–1.578	0.001
SCUBE1[#]	1.663	1.026–2.696	0.039

*The model was generated from a stepwise Cox regression model that included age, WBC, ELN risk category and expression level of *SCUBE1*.

Abbreviations: HR, hazard ratio; CI, confidence interval; WBC, white blood cell count; ELN, European leukemia net

[$] vs favorable and intermediate.[13]

[#]High vs low expression of *SCUBE1* according to median level.

Supplementary Table S3. Hematological parameters in wild-type (WT) and Δ3 mice

Hematological parameters	*Scube1*		
	WT (n=5)	Δ3 (n=6)	*P* value
WBC (10^9/L)	9.17±2.31	7.71±0.94	0.19
RBC (10^{12}/L)	10.99±0.31	10.95±0.15	0.80
Hb level (g/dL)	15.90±0.42	15.88±0.17	0.93
HCT (%)	55.84±2.26	56.22±0.46	0.70
MCV (fL)	50.80±0.85	51.33±0.63	0.26
MCH (pg)	14.48±0.13	14.48±0.13	0.97
MCHC (g/dL)	28.50±0.41	28.23±0.27	0.22
RDW (%)	24.66±0.53	25.47±1.33	0.24
PLT count (10^9/L)	889.20±83.54	1033.50±157.40	0.13
MPV (fL)	7.82±0.16	7.78±0.16	0.72
PCT (%)	0.48±0.06	0.65±0.24	0.15
PDW (%)	7.30±0.25	7.42±0.30	0.51

Abbreviations: WBC, white blood cell/leukocyte count; RBC, red blood cell/erythrocyte count; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin; RDW, red cell distribution width; PLT, platelet/thrombocyte; MPV, mean platelet volume count; PCT, plateletcrit; PDW, platelet distribution width

Supplementary Table S4. Membrane SCUBE1-associated protein tyrosine kinases identified by proteomic proximity labeling assay in THP-1 and NOMO-1 cells

Protein Name	TK type	Implicated in AML	Direct interaction with FLT3
FLT3	RTK	Yes[14]	-
EPHB1	RTK	Yes[15]	No
EPHB3	RTK	No	No
INSR	RTK	Yes[16]	No
LYN	Nonreceptor TK	Yes[17]	Yes[18, 19]
JAK1	Nonreceptor TK	Yes[20]	No
TYK2	Nonreceptor TK	Yes[21]	No

Abbreviations: TK, tyrosine kinase; RTK, receptor tyrosine kinase; AML, *zcute myeloid leukemia; FLT3,* Fms-like receptor tyrosine kinase 3

Supplementary Table S5. Oligonucleotide primers used in this study

Gene	Forward	Reverse	Assay
SCUBE1	TCAGACACATGCGAAGCG	CTTGCGCAGGGTCTTGAT	Q-PCR
MEIS1	GCATGAATATGGGCATGGA	CATACTCCCCTGGCATACTTTG	Q-PCR
HOXA9	AGGAGTCGCTGCTTTCTGTT	ATTAGAACGGGGAGGGGTAA	Q-PCR
GAPDH	TGAAGGTCGGAGTCAACG	AGAGTTAAAAGCCCTGGT	Q-PCR
FLT3	GGGAAGGTACTAGGATCAGGTG	GCTGGGTCATCATCTTGAGTTC	Q-PCR
Scube1	CATGATGGACACAACTGCCT	TGTGGCACTGGCATTCATAG	Q-PCR
Gapdh	CCAGAACATCATCCCTGCATC	CCTGCTTCACCACCTTCTTGA	Q-PCR
SCUBE1	TGCGGCGGCGAGCTTGGTGAC	TTTGGAGCGCAGCAGTTTGATGAA	RT-PCR
GAPDH	GCCAAAAGGGTCATCATCTC	ACCACCTGGTGCTCAGTGTA	RT-PCR
Scube1	CCCCCACCACCCCTATCAGACAGA	TTGCGGGAACGGGAGGTAAAG	RT-PCR
Gapdh	ATCATCCCTGCACCACTGGTGCTG	TGATGGCATTCAAGAGAGTAGGGAG	RT-PCR
Scube1 (F1+R1)	CCTAGGTGAGCGGTGGGTAAT	GGTTGCCTGAGGACATAAGA	Genotyping
Scube1 (F2+R2)	ACCCCAGAACCCCAGGAAACT	GAGGCAGGAGGCAAGAACCAA	Genotyping
Scube1 (F3+R3)	GGCAACCCACAAGTGACCATA	CAGGGCTGAGGGGAGTGAC	Genotyping
Rosa26 Cre1	AAAGTCGCTCTGAGTTGTTAT		Genotyping
Rosa26 Cre2		GGAGCGGGAGAAATGGATATG	Genotyping
Rosa26 Cre3		CCTGATCCTGGCAATTTCG	Genotyping

Supplementary Table S6. shRNA hairpin sequences used in this study

shRNA	**Sequence**
MEIS1 sh#1	ATGTAACCTTCATCTAGTTAA
MEIS1 sh#2	GCATGAATATGGGCATGGA
HOXA9 sh#1	CGCTGTACCCGCTGCGGTGTA
HOXA9 sh#2	TGCTGATTGTAACGGAGTTAA
FLT3 sh#1	CAAGATCTGCCTGTGATCAAG
FLT3 sh#2	CGTCTGCGTTTACTCTTGTTT

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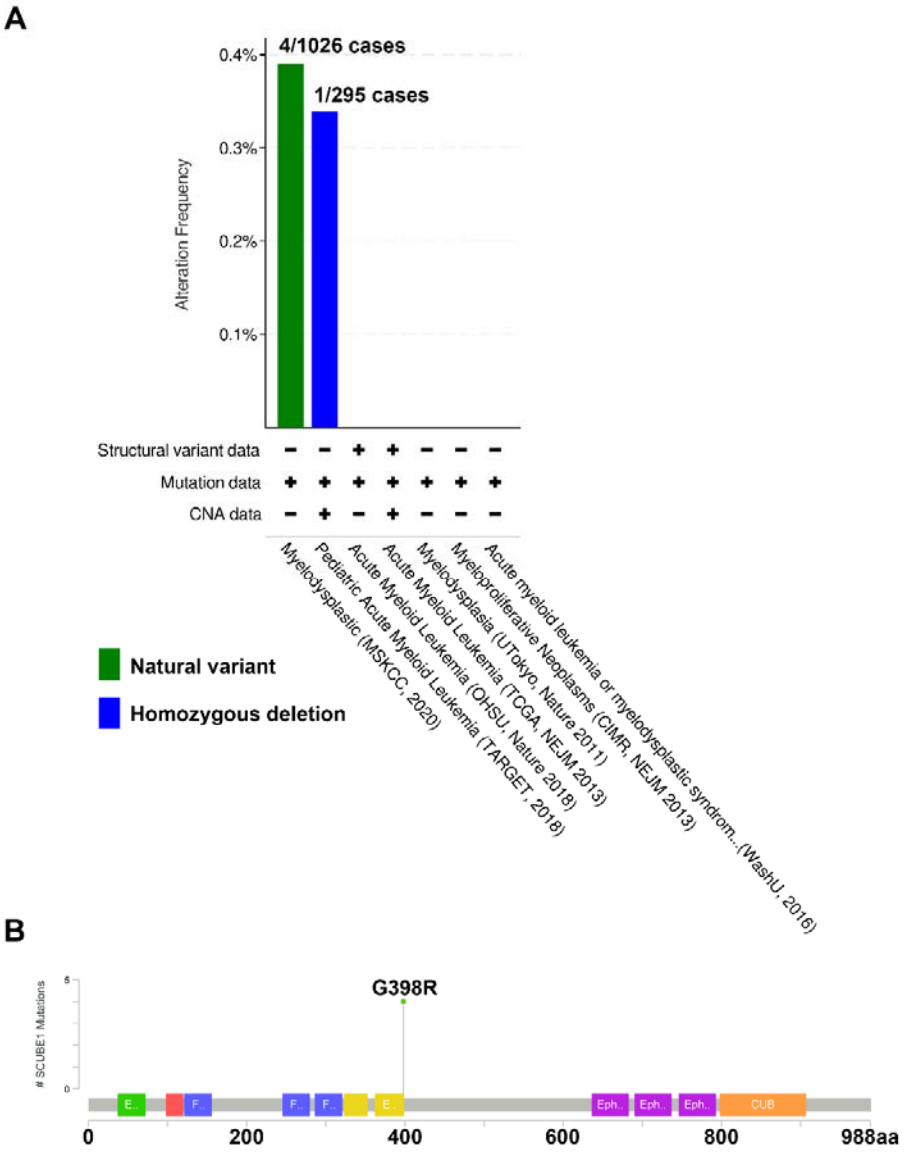
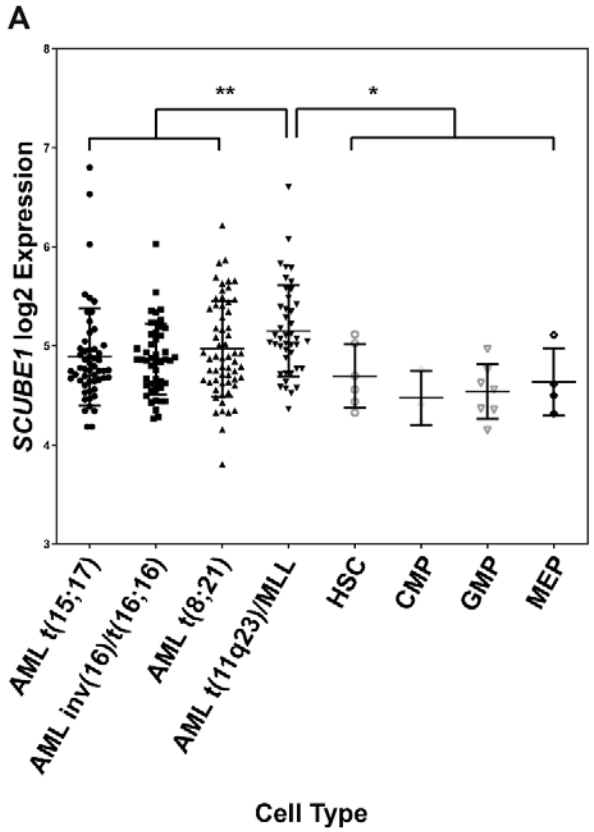
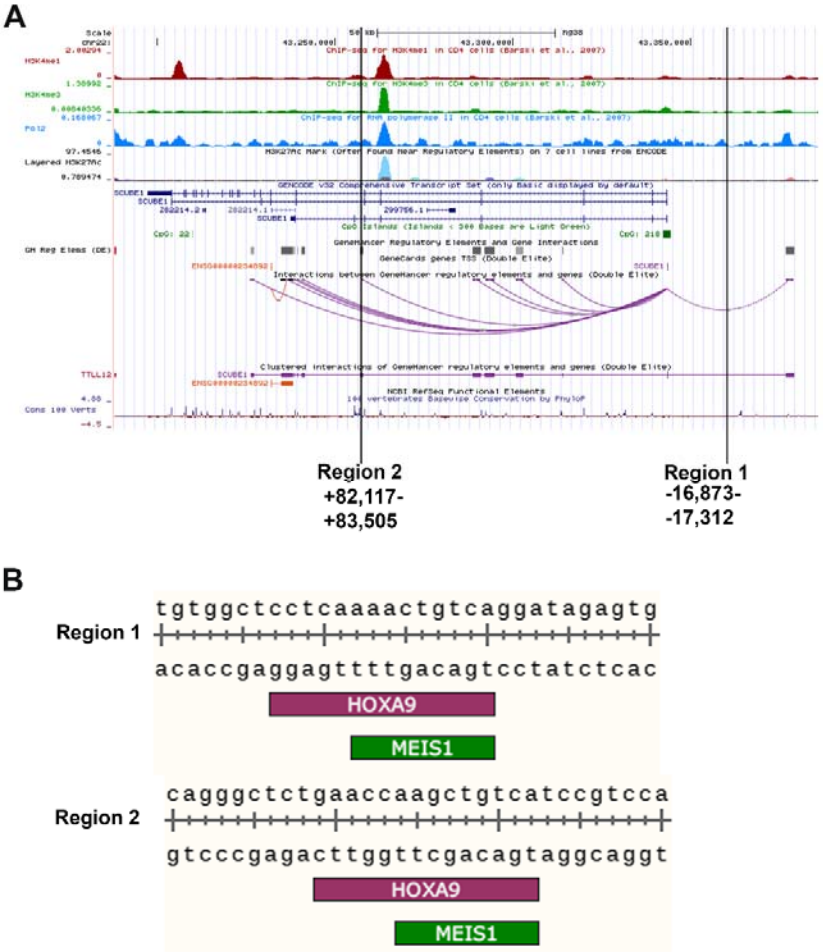


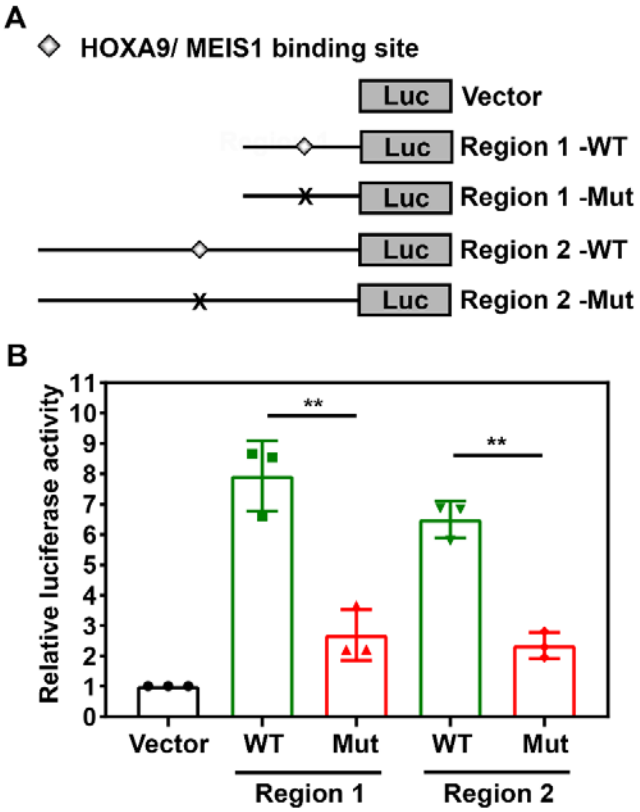
Supplementary Figure S1. Genomic alterations or genetic mutations associated with *SCUBE1* in AML or myelodysplastic syndrom (MDS) patients. (A) After surveying the public database at the cBioPortal for Cancer Genomics (http://cbioportal.org), we did not identify genomic gain or amplification of SCUBE1 gene in these AML or myelodysplastic syndromes (MDS) cohorts (see below figure). Rather, homozygous deletion of SCUBE1 was reported at extreme low frequency of 0.34 % (1 out of 295 cases) in pediatric AML (*Nature* 555: 371, 2018). (B) We only found that a natural variant G398R, a SNP (dbSNP: rs129415) but not activated mutation, was present a low frequency of 0.39% (4 of 1026 cases) in MDS. However, clinical significance of the genomic loss or this SNP of *SCUBE1* requires further investigation.



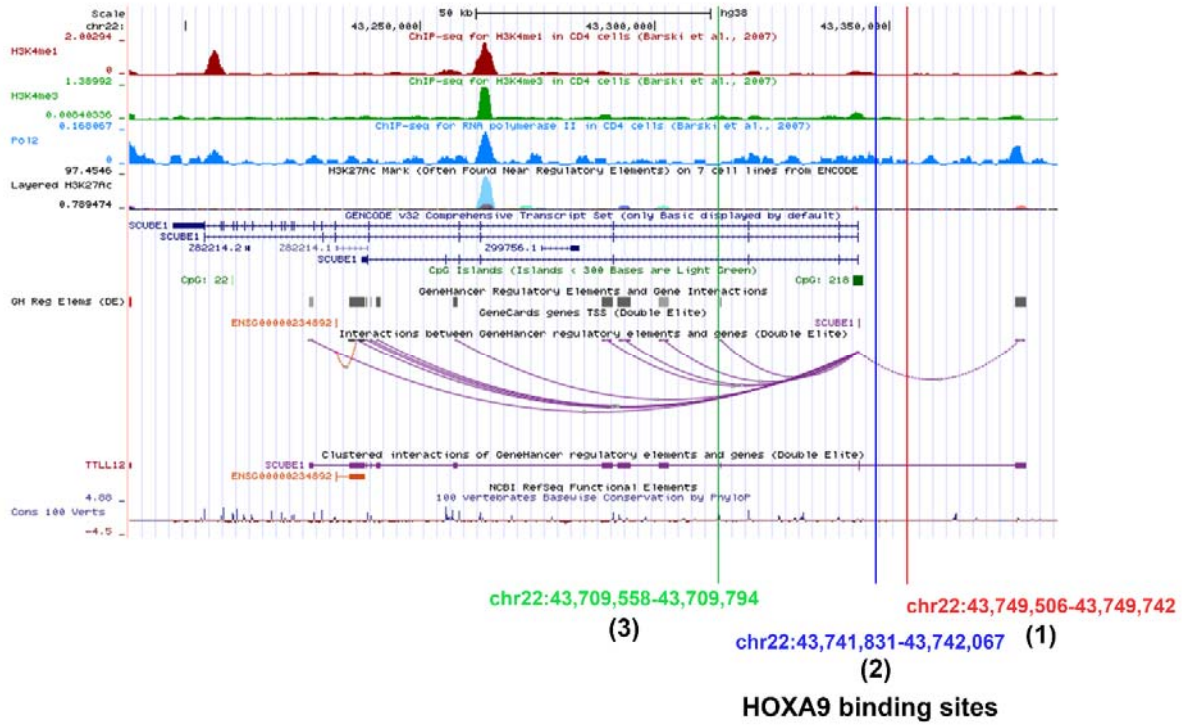
Supplementary Figure S2. *SCUBE1* expression in different AML patient samples as compared with normal hematopoietic progenitor cells. *SCUBE1* expression in MLL translocation (AML t(11q23)/MLL) is compared with normal hematopoietic stem and progenitor cells such as hematopoietic stem cells (HSCs), common myeloid progenitor cells (CMPs), granulocyte monocyte progenitors (GMPs), and megakaryocyte-erythroid progenitor cells (MEPs) and also other types of AML such as t (15;17), inv (16)/t (16;16), and t (8;21). Data were derived from the blood spot database (https:// servers.binf.ku.dk/ bloodspot). GEO accession no. for normal human hematopoietic cell is GSE42519 and human AML cells is GSE13159. inv, inversion; t, translocation. *$P < 0.05$, **$P < 0.01$.



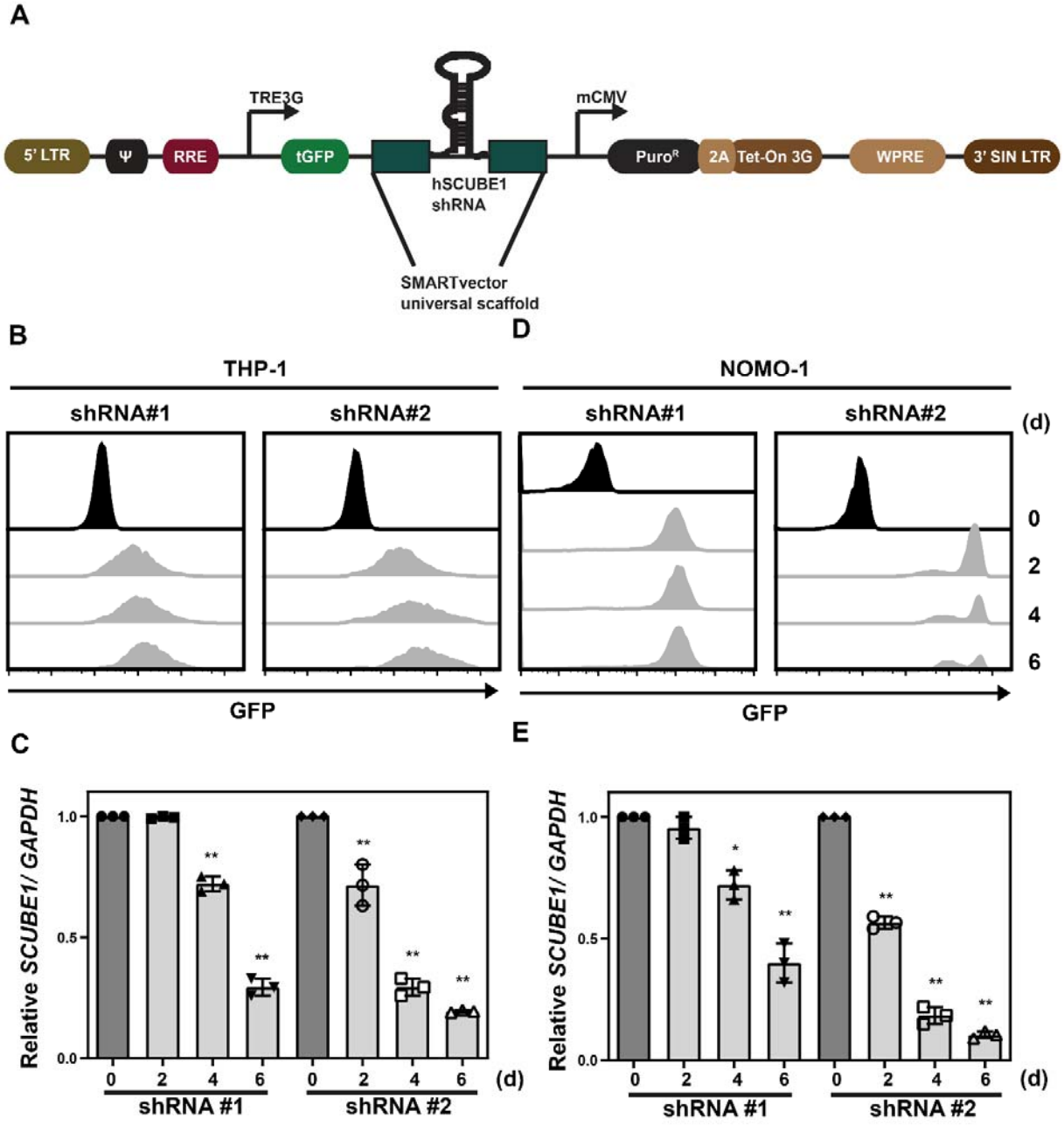
Supplementary Figure S3. MEIS1 and HOXA9 have predicted binding sites on *SCUBE1* enhancer region. (A) SCUBE1 promoter and regulator sequences were derived from the UCSC genome browser with predicted binding regions of HOXA9 and MEIS1 in region 1 (16,873-17,312 bp upstream of the *SCUBE1* transcription start site) and region 2 ((82,117-83,505 bp downstream of the *SCUBE1* transcription start site). (B) Predicted binding regions of MEIS1 or HOXA9 were identified by using PROMO.



Supplementary Figure S4. Mutations of HOXA9 / MEIS1 binding sites abolish HOXA9 / MEIS1 co-transactivation of the luciferase reporter activity. (A) Schematic representation showing the SCUBE1 region 1 and 2 reporter constructs containing the wild type (WT) or mutated (Mut) HOXA9/ MEIS1 binding sites. The HOXA9/MEIS1 binding sites are marked as diamonds, and the mutant sites are indicated as X marks. (B) Luciferase reporter assay with overexpression of HOXA9 and MEIS1 together with the region 1 or 2-WT / Mut reporter constructs in HepG2 cells. Firefly luciferase activity was normalized to Renilla luciferase activity. Data are mean ± SD from 3 independent experiments. $^{**}P < 0.01$.

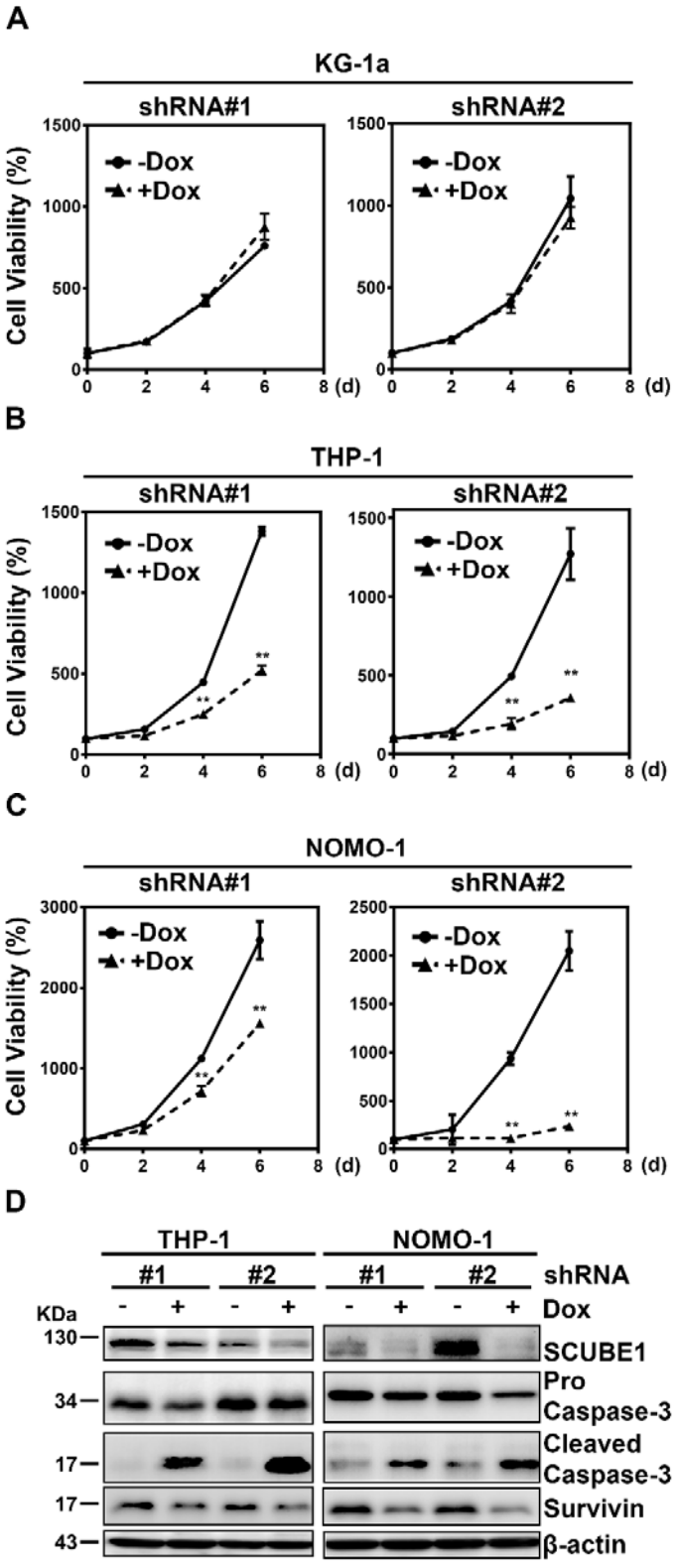


Supplementary Figure S5. Three HOXA9 binding sites at the promoter or intron of *SCUBE1* were found in a publicly available dataset. Graphical representation showing HOXA9 binding sites in *SCUBE1* gene identified by surveying ChIP-seq data. Three HOXA9 binding sites derived from a previous study (**Blood Adv. 2: 3137, 2018**) were mapped and viewed by UCSC genome browser. Locations of binding sites were indicated by chromosome number and nucleotide number. Of note, the HOXA9 binding site #1 is nearby the HOXA9/MEIS1 co-bound region #1 that we identified in Supplementary Figure S3.

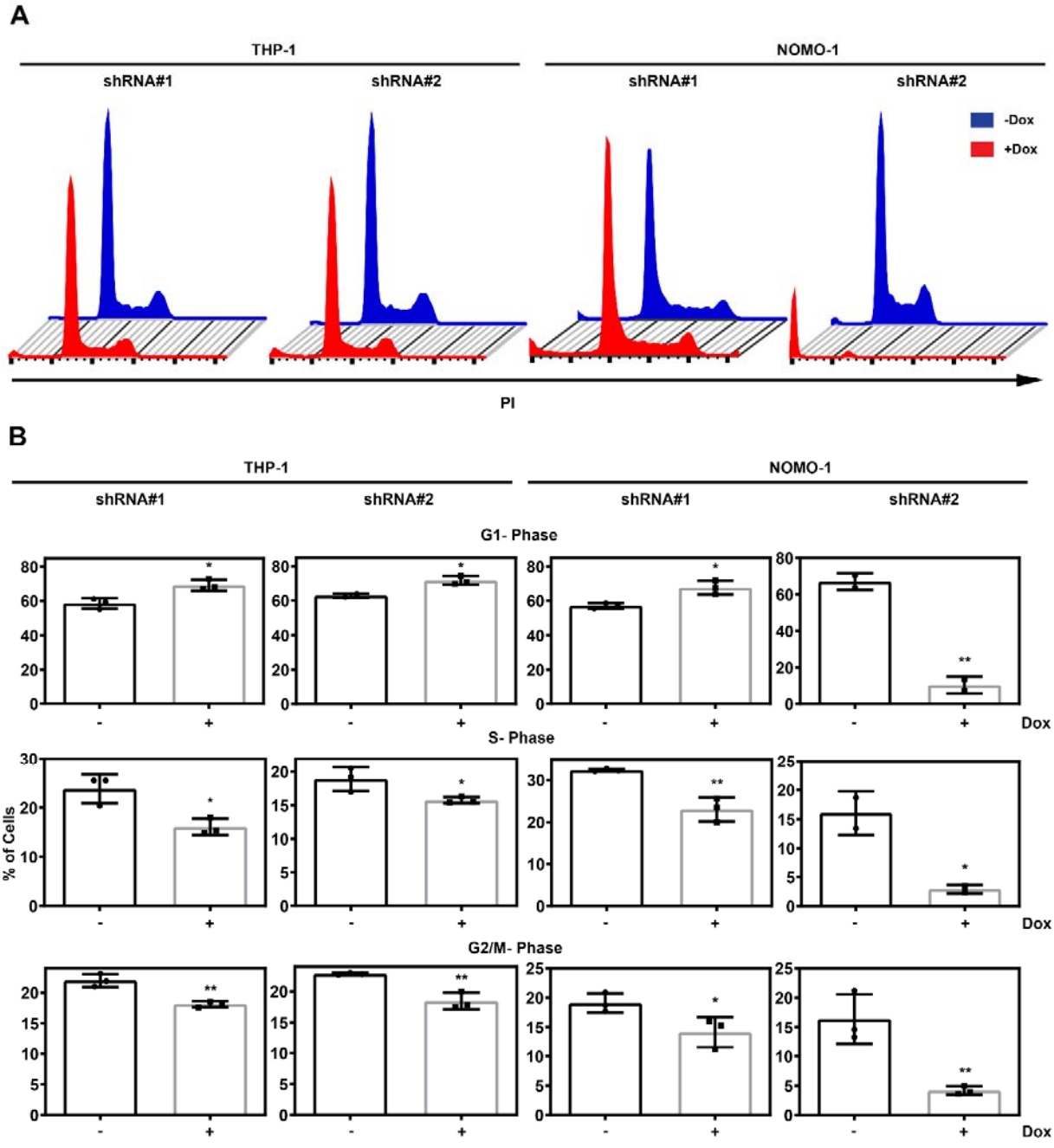


Supplementary Figure S6. Knock down of *SCUBE1* with doxycycline-inducible shRNA. (A) Graphical representation of inducible SCUBE1 shRNA construct. The construct was purchased from Horizon Discovery. The construct constitutes a tetracycline/doxycycline-inducible promoter, shRNA sequence targeting *SCUBE1*, and GFP reporter for visually tracking expression upon doxycycline induction, and puromycin resistance permits antibiotic selection of transduced cells. Upon the addition of doxycycline, the promotor is activated to produce shRNA along with GFP. 5' LTR, 5' long terminal repeat; Ψ, Psi packaging sequence; PTRE3G, inducible

promoter with tetracycline response element; tGFP, turbogreen fluorescent protein; SMARTvector universal scaffold, optimized proprietary scaffold by the manufacturer; PuroR, puromycin resistance; 2a, self-cleaving peptide; Tet-On® 3G, doxycycline-regulated transactivator protein; WPRE, Woodchuck hepatitis post-transcriptional regulatory element; 3' SIN LTR, 3' self-inactivating long terminal repeat. (B) FACS analysis of GFP expression after doxycycline treatment. THP-1 or NOMO-1 cells were transduced with lentivirus containing inducible shRNA targeted to *SCUBE1*. Two independent shRNAs were used and two clones from each cell line were selected by using puromycin resistance. Doxycycline was used to treat inducible *SCUBE1* shRNA-containing stable THP-1 or NOMO-1 cells for different times, followed by flow cytometry of expression of GFP protein. (C) qPCR analysis of *SCUBE1* mRNA after doxycycline treatment of inducible *SCUBE1* shRNA-containing stable THP-1 or NOMO-1 cells for different times. Data are mean ±SD from 3 independent experiments. *P<0.05, **P<0.01.

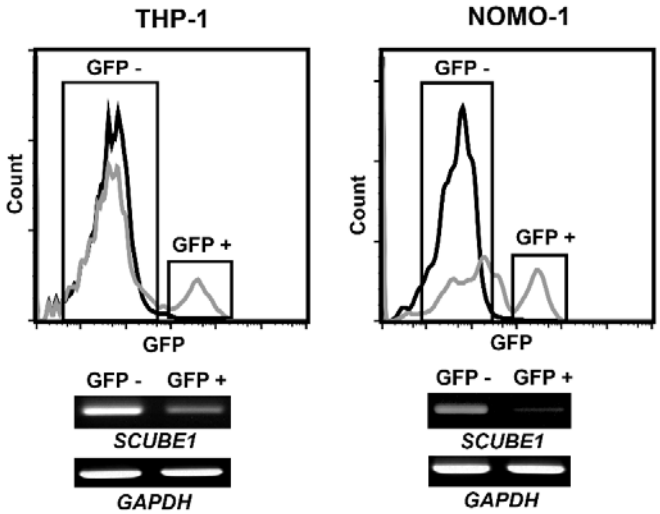

A
KG-1a
shRNA#1
shRNA#2
Cell Viability (%)
-Dox
+Dox
(d)
B
THP-1
C
NOMO-1
D
THP-1
NOMO-1
#1
#2
shRNA
KDa
Dox
130
SCUBE1
34
Pro Caspase-3
17
Cleaved Caspase-3
17
Survivin
43
β-actin

Supplementary Figure S7. Inducible knockdown of *SCUBE1* decreased cell viability and increased apoptosis in MLL-AF9–translocated AML *in vitro*. Stable cell lines with doxycycline inducible *SCUBE1*-shRNA were used to examine cell viability, cell apoptosis, and *in vivo* growth. (A-C) Doxycycline was added in the medium (dashed line) or not (solid line) for different times, and viability was assayed with MTT assay in *SCUBE1*-negative KG-1a cells or *SCUBE1*-positive THP-1 and NOMO-1 cell lines. (D) Apoptosis marker expression examined by western analysis before and after doxycycline treatment for 5 days in THP-1 or NOMO-1 cells carrying inducible *SCUBE1*-shRNA clones. Data are mean ± SD from 3 independent experiments. $^{**}P < 0.01$.

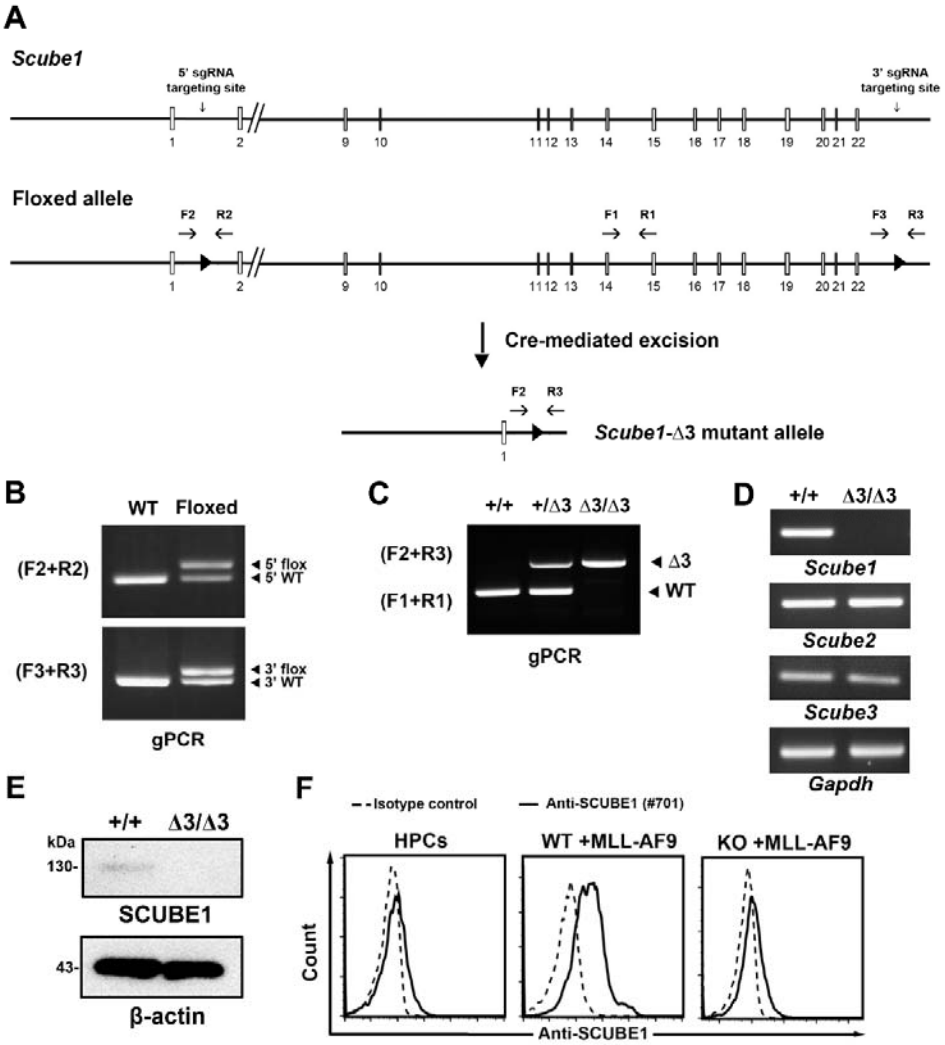


Supplementary Figure S8. Decrease in cell cycle progression after knock down of SCUBE1 in THP-1 and NOMO-1 cells using doxycycline-inducible SCUBE1 shRNA. (A) Cell cycle analysis involved using FACS. Doxycycline was used to treat inducible *SCUBE1* shRNA-containing THP-1 or NOMO-1 cells for 5 days followed by fixation and permeabilization of cells, propidium iodide staining, and flow cytometry. (B) Graphs show different stages of cell cycle corresponding to FACS. Of note, the cell cycle status of the NOMO-1 shRNA clone #2 is

markedly altered (mostly under apoptosis) probably due to high efficiency of SCUBE1 knockdown. Data are mean ±SD from 3 independent experiments. *$P < 0.05$, **$P < 0.01$.

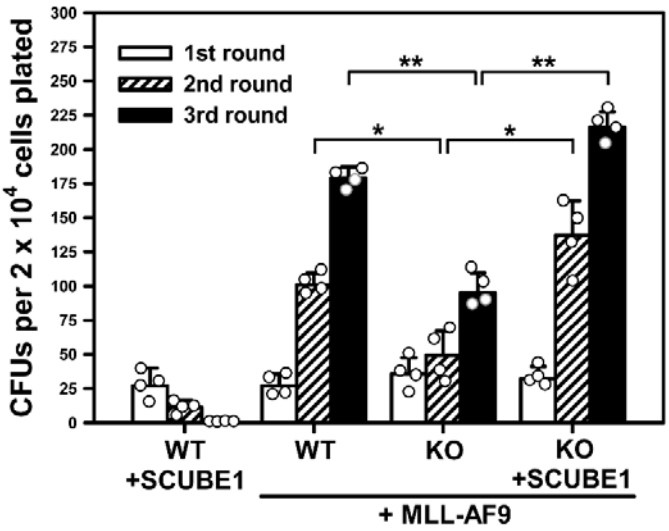


Supplementary Figure S9. Efficient knockdown of *SCUBE1* in MLL-r THP-1 or NOMO-1 cells *in vivo*. *In vivo* GFP expression of transplanted THP-1 or NOMO-1 cells having inducible shRNA from bone marrow was analyzed using flow cytometry after doxycycline treatment. RT-PCR was performed to confirm that knockdown efficiency of *SCUBE1* is high in those GFP-positive knockdown cells *in vivo*.

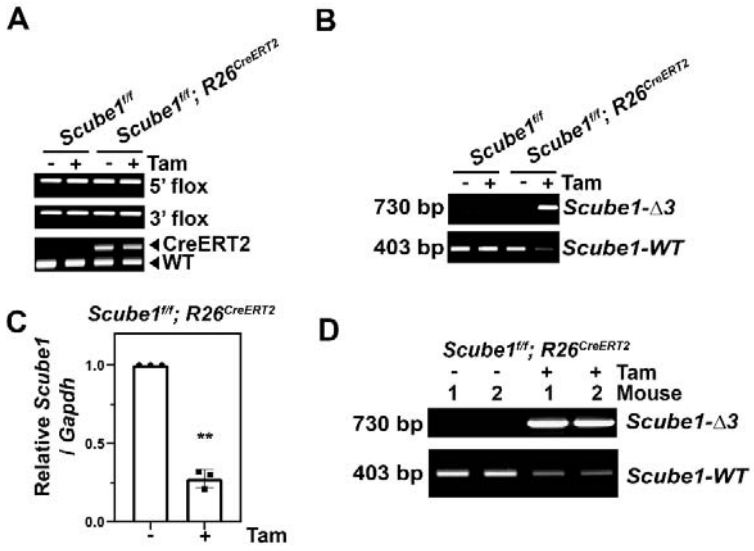


Supplementary Figure S10. Knock out of *SCUBE1* in mouse. (A) The schematic representation of *SCUBE1* knockout (KO) strategy. Scube1 KO mice were generated as described. The floxed allele (middle) of *Scube1* (*Scube1*$^{f/f}$) was generated by introducing 2 loxP sites in the *Scube1* gene with the CRISPR-Cas9 system. The loxP sites are marked as a single guide RNA (sgRNA) targeting site on wild-type (WT) *Scube1* (upper). Scube1 KO (Δ3) mice were generated by crossing mice carrying a new *Scube1* conditional allele (*Scube1*$^{f/f}$) with mice harboring a Cre recombinase under expression control of the mouse protamine 1 (*Prm1*) promoter. The resulting transcript is predicted to lack exons coding for NH_2-terminal EGF-like repeats, spacer region, CR motifs and COOH-terminal CUB domain and produce a truncated protein that contains only residues 1-37 (of a 988-amino acid wild-type SCUBE1 protein). Mice homozygous for the WT allele or the targeted (Δ3) allele were obtained by heterozygous (Δ3/+) interbreeding and were born with the expected Mendelian ratio. Δ3 mutant (KO) mice showed normal development, survival, and reproduction. In addition, white or red blood cell count as

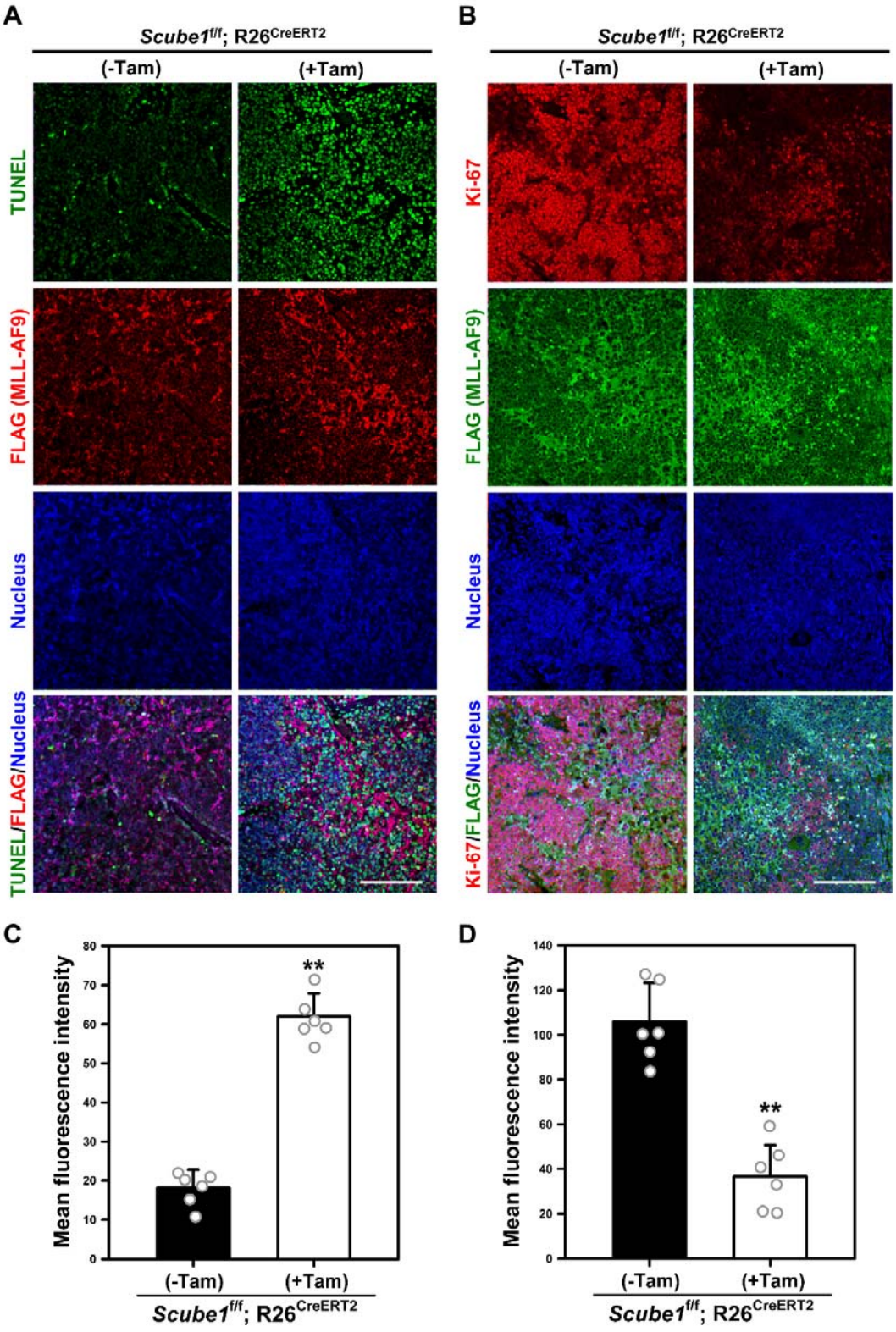
well as other hematological variables were comparable between WT and KO mice (see Supplementary Table S3). The WT allele was genotyped with F1+R1 primers, and 5' and 3' loxP flanked sites were genotyped with F2+R2 and F3+R3 primers, respectively. The cre-mediated excised mutant Sube1 (down) was confirmed by genotyping with F2+R3 primers. The primers are marked with a small arrow. The exons are marked as 1-22. Δ3/Δ3 or Scube1-Δ3 is the *Scube1* KO allele. (B) Genotyping of *Scube1* floxed allele. (C) Genotyping of *Scube1* WT and mutant allele. The WT allele with PCR primers F1+R1 has a PCR product of 403 bp and the mutant allele with the PCR primer F2+R3 has a PCR product of 730 bp. (D) RT-PCR to check *Scube1* mRNA level in MLL-AF9–transduced WT and KO hematopoietic progenitor cells (HPCs) as compared with normal SCUBE2 and SCUBE3 expression. Mutant mice completely lacked expression of *Scube1* mRNA. (E) Western blot analysis to check Scube1 protein level in MLL-AF9–transduced WT and KO HPCs. (F) Scube1 protein level on cell surface analyzed by using FACS before and after transduction of MLL-AF9 into WT and KO HPCs.



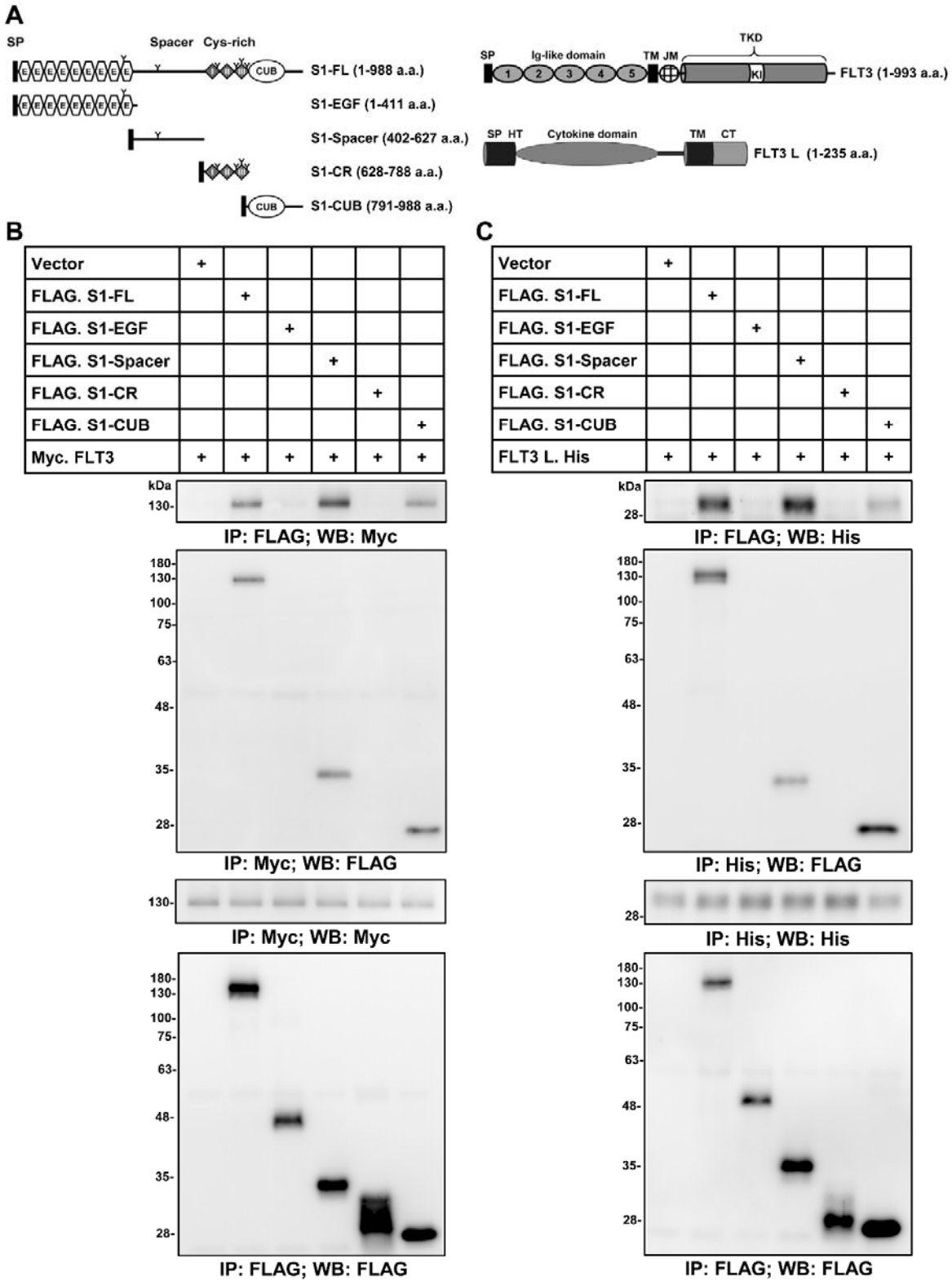
Supplementary Figure S11. *Scube1* knockout reduces and re-expression of SCUBE1 rescues colony formation ability of MLL-AF9-transduced HPCs. Three rounds of replating assay were performed after transduction of HPCs with MLL-AF9 retrovirus and colony numbers in each round of colony formation assay are presented. The figure is related to Figure 4B. Data are mean ± SD from 4 independent experiments. *$P < 0.05$, **$P < 0.01$.



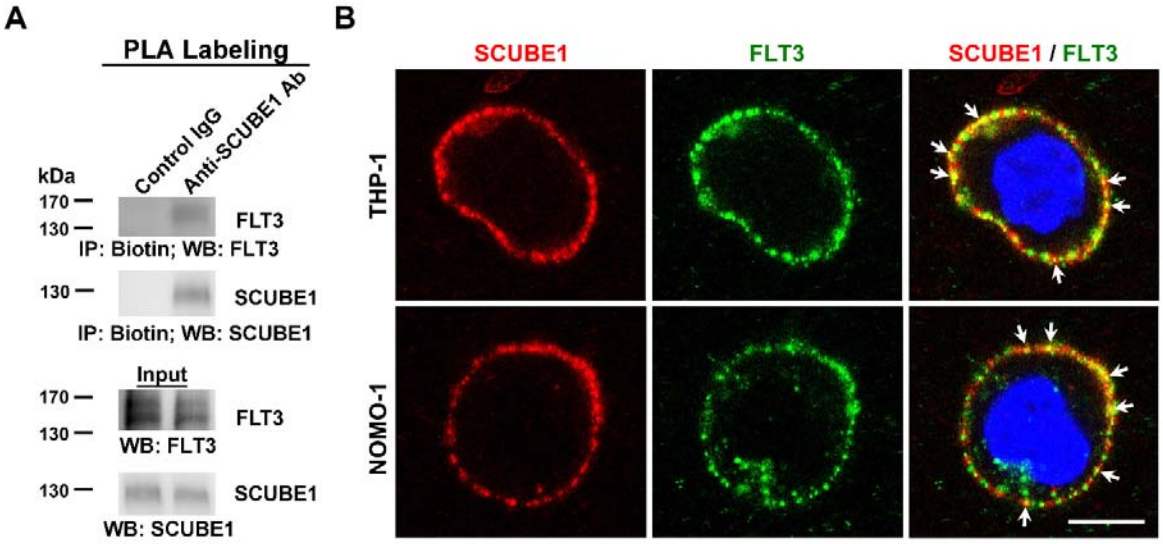
Supplementary Figure S12. Conditional knock out of *SCUBE1* in mouse. *Scube1* conditional-knockout mice (*Scube1$^{f/f}$; R26CreERT2*) by crossing the Scube1 conditional allele (*Scube1$^{f/f}$*) with a Cre-recombinase-estrogen-receptor-T2 (CreERT2) allele at the ubiquitous ROSA26 locus (*R26CreERT2*). (A) Genotyping of *Scube1* floxed allele with CreERT2 allele. (B) Genotyping of *Scube1* WT and KO allele after tamoxifen-induced knockout in vitro. HPCs from *Scube1$^{f/f}$* or *Scube1$^{f/f}$; R26^{CreERT2}* were transduced with MLL-AF9, and 3 rounds of colony formation assay was performed as mentioned. Cells were then treated with 4-OHT for 3 days, then underwent genotyping to check excision of the gene and (C) qRT-PCR to check SCUBE1 mRNA level. Data are mean ±SD from 3 independent experiments. $^{**}P < 0.01$. (D) Genotyping of *SCUBE1* WT and mutant allele after tamoxifen-induced knockout in vivo. The *Scube1$^{f/f}$* or *Scube1$^{f/f}$; R26^{CreERT2}* HPCs containing MLL-AF9 were injected in mice after the 3rd colony formation assay. After development of leukemia, mice were sacrificed, and bone marrow leukemic cells were isolated and secondary transplantation was performed. After confirming blast cells in peripheral blood, mice were treated with tamoxifen for 5 consecutive days. *Scube1* knockout efficiency was confirmed by genotyping the bone marrow cells after sacrificing mice at 40 days after last tamoxifen injection. Of note, the presence of *Scube1*-WT band at 403bp after tamoxifen-induced knockout could be because of insufficient knockout of blast cells and/or presence of host WT bone marrow cells.


A
Scube1f/f; R26CreERT2
(-Tam)
(+Tam)
TUNEL
FLAG (MLL-AF9)
Nucleus
TUNEL/FLAG/Nucleus
B
Scube1f/f; R26CreERT2
(-Tam)
(+Tam)
Ki-67
FLAG (MLL-AF9)
Nucleus
Ki-67/FLAG/Nucleus
C
Mean fluorescence intensity
80
70
60
50
40
30
20
10
0
**
(-Tam)
(+Tam)
Scube1f/f; R26CreERT2
D
Mean fluorescence intensity
140
120
100
80
60
40
20
0
**
(-Tam)
(+Tam)
Scube1f/f; R26CreERT2

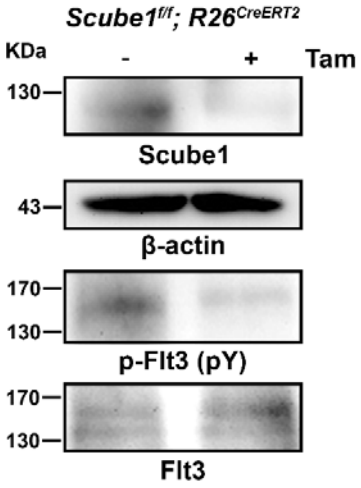
Supplementary Figure S13. *Scube1*-KO promotes apoptosis and decreased proliferation of MLL-AF9-induced leukemia stem cells (LSCs) *in vivo*. (A) TUNEL staining of representative spleen sections from WT (-Tam) and *Scube1*-KO (+Tam) mice is shown. Bar = 100 μm. Quantitative analysis of TUNEL-positive signal in WT and *Scube1*-KO spleens. Data are mean ± SD (n=6). $^{**}P < 0.01$. (B) Immunostaining of Ki-67 (a proliferation marker) in representative spleen sections from WT (-Tam) and *Scube1*-KO (+Tam) mice is shown. Bar = 100 μm. Quantification of Ki-67-positive signal in WT and *Scube1*-KO mice. Data are mean ± SD (n=6). $^{**}P < 0.01$. Anti-FLAG staining was used to confirm the MLL-AF9 (FLAG-tagged)-induced LSCs


A
SP
Spacer
Cys-rich
CUB
S1-FL (1-988 a.a.)
S1-EGF (1-411 a.a.)
S1-Spacer (402-627 a.a.)
S1-CR (628-788 a.a.)
S1-CUB (791-988 a.a.)
SP
Ig-like domain
TM
JM
TKD
KI
FLT3 (1-993 a.a.)
SP
HT
Cytokine domain
TM
CT
FLT3 L (1-235 a.a.)
B
Vector
FLAG. S1-FL
FLAG. S1-EGF
FLAG. S1-Spacer
FLAG. S1-CR
FLAG. S1-CUB
Myc. FLT3
kDa
130-
IP: FLAG; WB: Myc
180-
130-
100-
75-
63-
48-
35-
28-
IP: Myc; WB: FLAG
130-
IP: Myc; WB: Myc
IP: FLAG; WB: FLAG
C
Vector
FLAG. S1-FL
FLAG. S1-EGF
FLAG. S1-Spacer
FLAG. S1-CR
FLAG. S1-CUB
FLT3 L. His
kDa
28-
IP: FLAG; WB: His
IP: His; WB: FLAG
28-
IP: His; WB: His
IP: FLAG; WB: FLAG

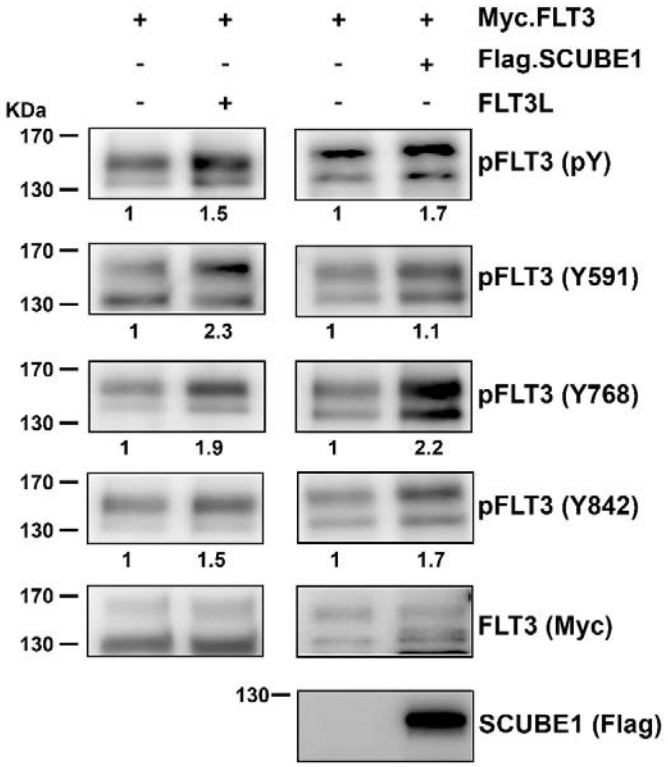
Supplementary Figure S14. Interaction of SCUBE1 with FLT3 and FLT3 ligand. (A) Schematic representation of SCUBE1 domains along with its different domain constructs (left), FLT3 domains (right up), and FLT3 ligand domains (right down). (B) Coimmunoprecipitation experiments showing interaction of SCUBE1 full length, SCUBE1-spacer, and SCUBE1-CUB domain with FLT3. SCUBE1 and its domains were tagged with FLAG-tag and FLT3 was tagged with Myc tag at the N-terminus. The SCUBE1-FL, EGF, spacer, CR, or CUB domains were transfected in HEK-293T cells along with FLT3. Two days after transfection, cells were lysed and proteins were immunoprecipitated, then underwent western blot analysis. (C) Coimmunoprecipitation experiments showing interaction of SCUBE1 full length, SCUBE1-spacer, and SCUBE1-CUB domain with FLT3 ligand. The SCUBE1-FL, EGF, spacer, CR, or CUB domains were transfected in HEK-293T cells along with N-terminal HIS-tagged FLT3 ligand. Two days after transfection, cells were lysed and proteins were immunoprecipitated and underwent western blot analysis.



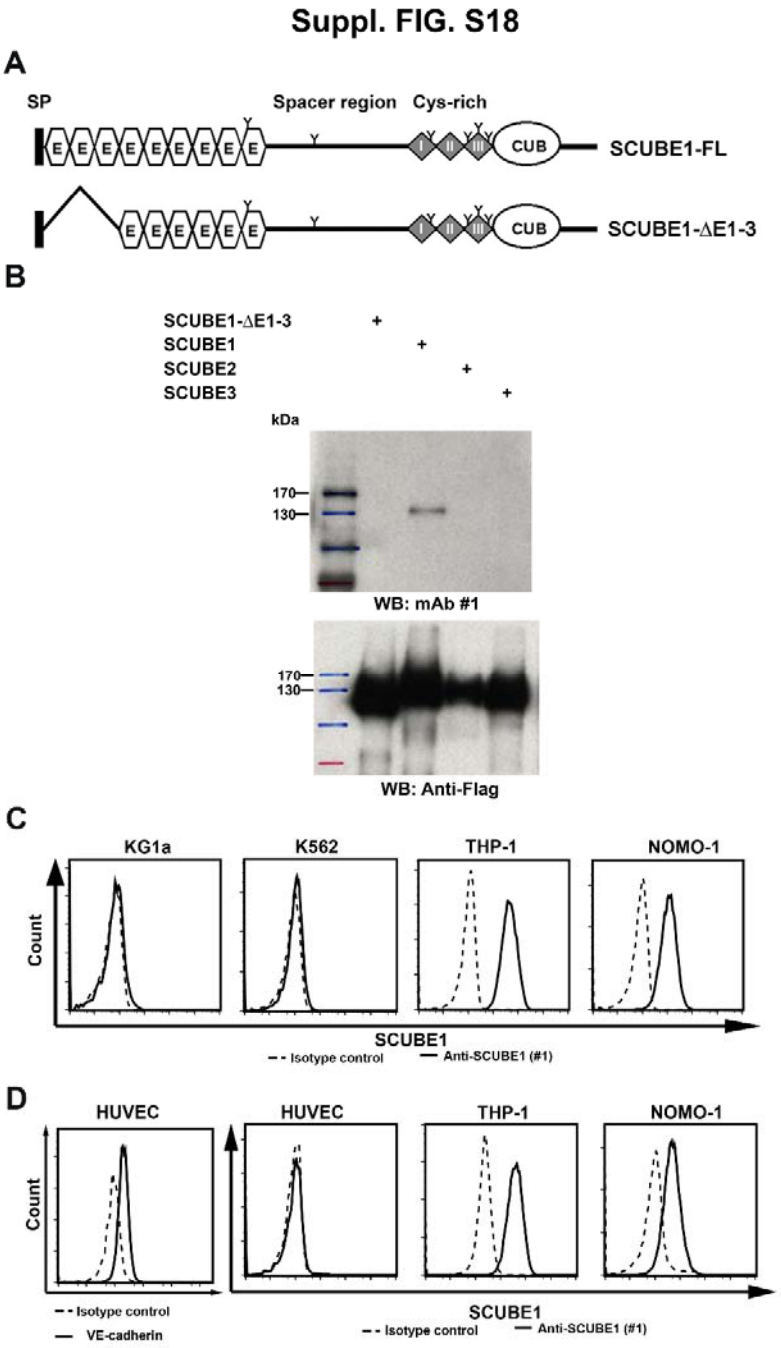
Supplementary Figure S15. SCUBE1 interacts and colocalizes with endogenous FLT3 on the plasma membranes of THP-1 or NOMO-1 cells. (A) Endogenous SCUBE1-FLT3 interaction was determined by proximal ligation assay (PLA). SCUBE1 expressed on cell-surface of THP-1 cells was probed with anti-SCUBE1 primary antibody, followed by the addition of HRP (horseradish peroxidase)-conjugated secondary antibody. The SCUBE1 proximal proteins were biotinylate by HRP with addition of biotin-phenol and H_2O_2. The biotinylated proteins were pulled down using streptavidin-agarose and western blot (anti-FLT3) was performed to conform protein-protein interaction. (B) Confocal immunofluorescence showing colocalization of SCUBE1 and FLT3 on the cell surface of THP-1 or NOMO-1 cells. SCUBE1 was detected with a mouse monoclonal antibody and Alexa-Fluor-594-conjugated goat anti-mouse IgG (red). FLT3 was localized by a rabbit antibody and Alexa-Fluor-488-conjugated goat anti-rabbit IgG (green). Arrows indicate the colocalized sites of SCUBE1 and FLT3 (yellow) on the plasma membrane of THP-1 or NOMO-1, respectively. Scale bar = 10 μm.



Supplementary Figure S16. Knockout of *Scube1* in MLL-AF9 transduced murine HPCs reduced Flt3 phosphorylation. Western blot analysis was used to determine the phosphorylation status of Flt3 with *Scube1*-knockout in MLL-AF9 transduced mouse HPCs. The MLL-AF9 transduced HPCs from the 3rd round of colony formation assay were grown in RPMI media supplemented with 10% FCS, 20 ng/ml SCF, 10 ng/ml each Flt3L, IL-3 and IL-6 (all murine, GenScript) in the absence or presence of 4-OHT (4-hydroxy tamoxifen, 10 nM) for 2 days. Then, cells were collected, lysed and performed western blot analysis.

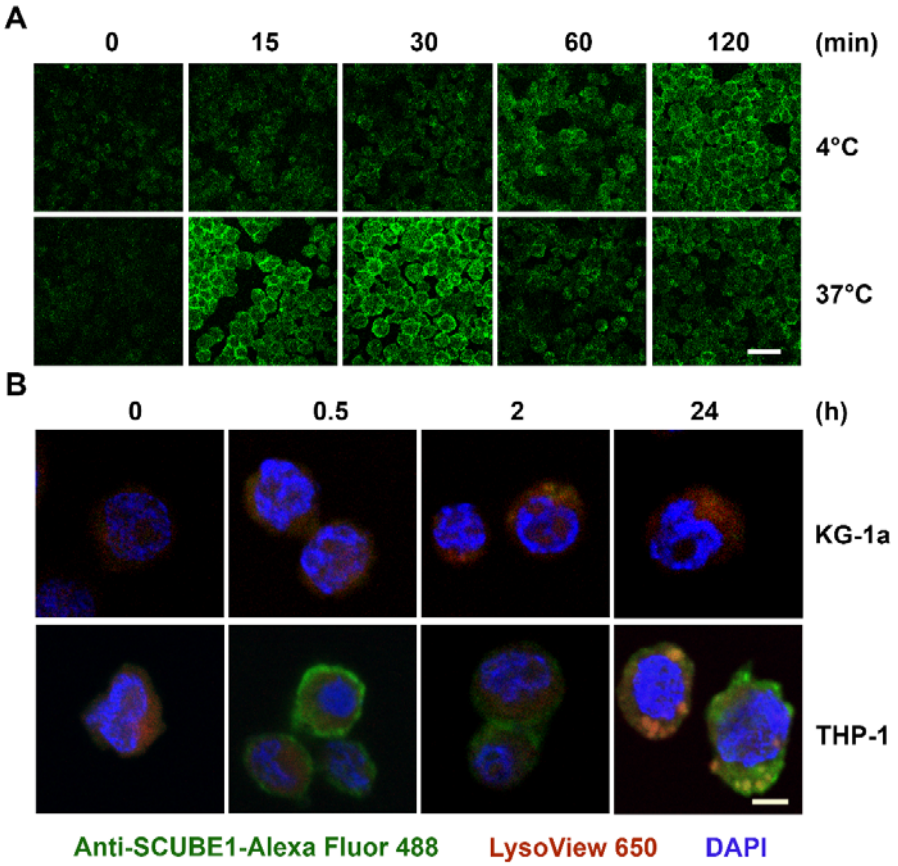


Supplementary Figure S17. SCUBE1 mediated FLT3 phosphorylation. The phosphorylation status of FLT3 with SCUBE1 interaction as compared with FLT3 ligand was checked by western blot analysis. To check the phosphorylation status of FLT3 with FLT3 ligand, HEK-293T cells underwent 2 days of Myc-tagged FLT3 transfection; 20 ng/ml human FLT3 ligand was added for 15 min. Then cells were lysed and assayed. To check the phosphorylation of FLT3 mediated by SCUBE1, Myc-tagged FLT3 was transfected in HEK-293T cells with or without Flag-tagged SCUBE1. At 2 days after transfection, cells were lysed and assayed. Besides checking total tyrosine phosphorylation (pY), site-specific phosphorylation such as Y591, Y768, and Y842 was also checked. The quantified band intensities of phospho-FLT3 bands were normalized to total FLT3 and are mentioned below the corresponding bands.

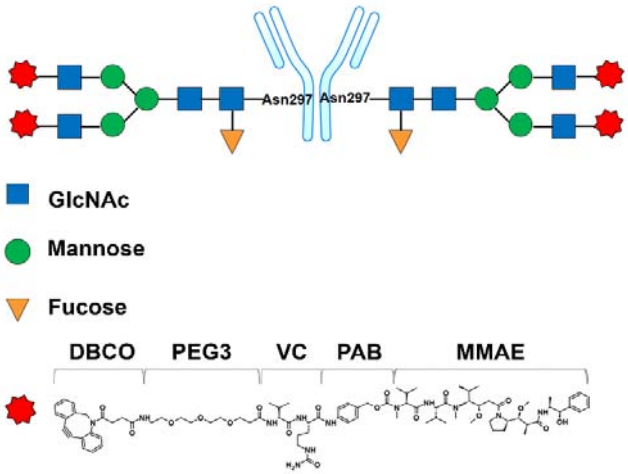


Supplementary Figure S18. Specificity of anti-SCUBE1 monoclonal antibody (mAb) (#1). (A) Schematic representation of SCUBE1 full length (SCUBE1-FL: upper) and EGF-like repeat 1-3–deleted (SCUBE1-ΔE1-3: lower) constructs. (B) Western blot analysis showing specificity of anti-SCUBE1 mAb (#1). The N-terminal flag tagged SCUBE1-FL, SCUBE1-ΔE1-3, SCUBE2, and SCUBE3 constructs were transfected in HEK-293T cells. Whole cell lysates were

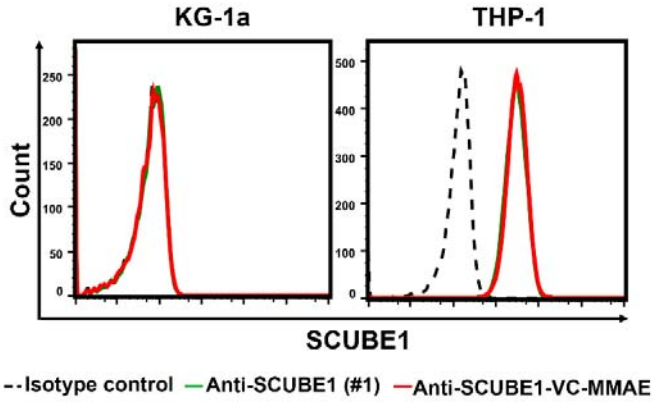
used for western blot analysis. The anti-SCUBE1 mAb (#1) detected only SCUBE1-FL. The anti-Flag antibody was used to detect the expression of proteins. (C) Flow cytometry with anti-SCUBE1 (#1) mAb to detect cell-surface expression of SCUBE1 in KG-1a, K562, THP-1, or NOMO-1 cells. (D) Flow cytometry to check the specificity of anti-SCUBE1 (#1) mAb between endothelial cells (human umbilical vein endothelial cells [HUVECs]) and human leukemic cells (THP-1 and NOMO-1). Anti-VE-cadherin antibody was a positive control for HUVEC cells. Anti-SCUBE1 (#1) mAb was not able to detect cell-surface SCUBE1 in HUVEC cells.



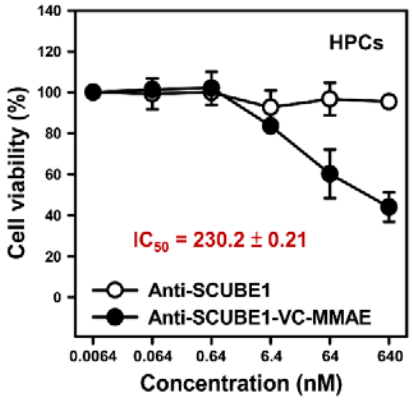
Supplementary Figure S19. Internalization of anti-SCUBE1 antibody. (A) SCUBE1 internalization assay with alexa Fluor-488–conjugated anti-SCUBE1 (#1) mAb (green) incubated with THP-1 cell sat different times at 4°C and 37°C. Images were acquired with a Zeiss LSM 510 confocal microscope, 40x (Zeiss), oil immersion lens, Zen software (Zeiss). Scale bar = 20 µM; (B) SCUBE1 internalization and localization assay with Alexa Fluor-488–conjugated chimeric anti-SCUBE1 mAb (green) incubated with THP-1 (SCUBE1 positive) and KG-1a (SCUBE1 negative) cells for different times as indicated. Human IgG 1 mg/ml was used for treatment 30 min before the addition of anti-SCUBE1 antibody to block FC to reduce nonspecific binding to FcγRs. To study localization, lysosomes were detected by using LysoView 650 (red) and nucleus with DAPI (blue). Yellow or orange fluorescence indicates co-localization of antibody to acidic lysosomal compartment. Images were acquired with a Zeiss LSM 700 confocal microscope, 63x (Zeiss), oil immersion lens, Zen software (Zeiss). Scale bar = 5 µm.



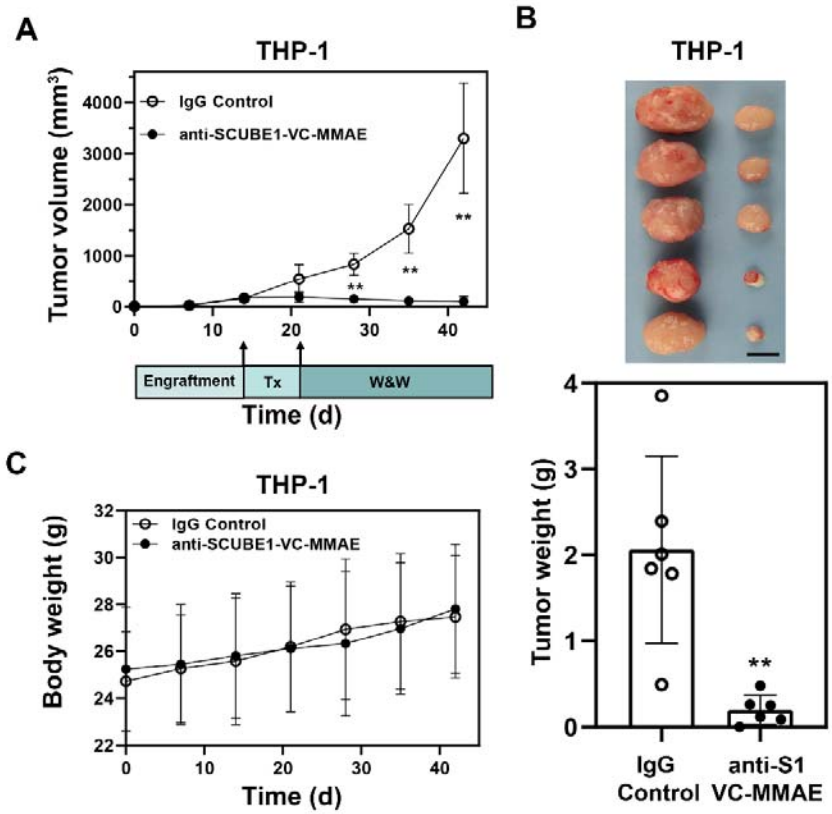
Supplementary Figure S20. Structure of anti-SCUBE1 antibody–drug conjugate (ADC). Tri-mannosyl anti-SCUBE1 antibody was conjugated to the anti-microtubule cytotoxic agent monomethyl auristatin auristatin E (MMAE) by a proteolytically cleavable DBCO-PEG3-VC-PAB linker with an average drug-to-antibody ratio of 4. The conjugated antibody was produced by the Development Center for Biotechnology, Taiwan. DBCO, dibenzocyclooctyne; PEG, polyethylene glycol; VC, valine-citrulline; PAB, para aminobenzoate; GlcNAc: N-acetylglucosamine.



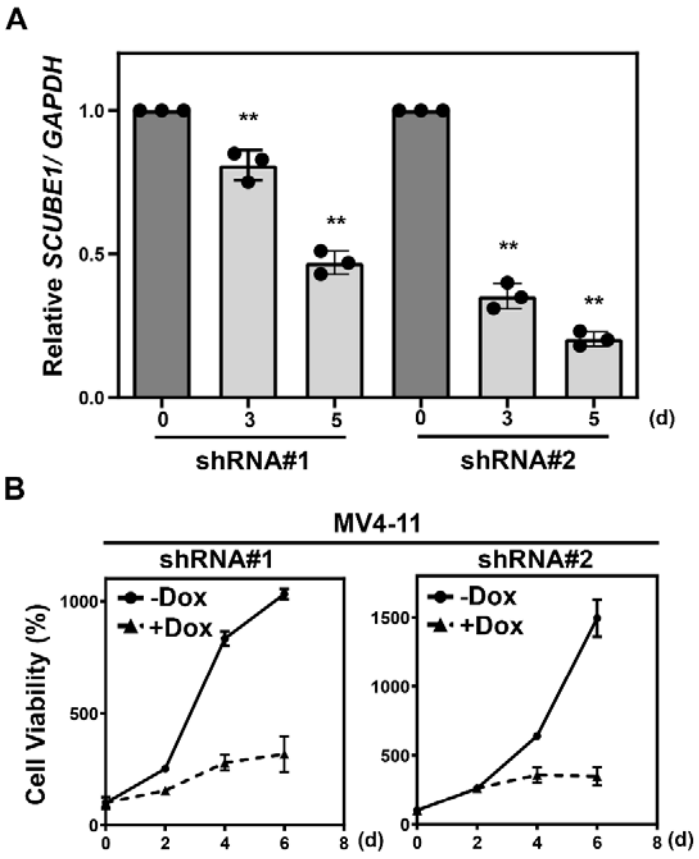
Supplementary Figure S21. Efficiency of anti-SCUBE1-VC-MMAE. The efficiency to detect cell-surface SCUBE1 by chimeric anti-SCUBE1 antibody before and after conjugation with MMAE analyzed by flow cytometry in KG-1a and THP-1 cells.



Supplementary Figure S22. Low concentration of anti-SCUBE1 ADC does not induce cytotoxicity in normal murine hematopoietic progenitor cells (HPCs). Assays were performed in the presence of the unconjugated anti-SCUBE1 antibody or anti-SCUBE1 ADC. MTT was used to measure cell viability after 5 days. The half maximal inhibitory concentration (IC_{50}, nM) of anti-SCUBE1 ADC on killing normal mouse HPCs is shown inside the graph. Note that the anti-SCUBE1 ADC is basically ineffective for its antitumor activity on normal mouse HPCs.



Supplementary Figure S23. Anti-SCUBE1 ADC reduced tumor growth of THP-1 cells in a subcutaneous model. THP-1 cells were subcutaneously injected in NSG mice. After palpable tumors were evident (tumor volume ~150-200 mm^3), mice were randomly assigned for treatment of 10 mg/kg human IgG or anti-SCUBE1 ADC. The drugs were administered intravenously for a total dose of 2 at 1 week apart as indicated by arrows (Tx). After the completion of treatment, tumor growth was monitored in a wait-and-watch (W&W) period. The tumor volume was measured by using a digital caliper. Mice were sacrificed once the tumor volume exceeded 2000 mm^3 and tumor weight was measured after isolation. (A) Anti-SCUBE1 ADC–reduced tumor volume of THP-1 cells. Data were evaluated by multiple-sample Student *t* test (n=6). (B) Collected tumors (upper panel) and their weight (lower panel). Data were evaluated with Mann-Whitney test. Scale bar= 1 cm. (C) Body weight change after transplantation until the completion of the experiment. Data are mean ±SD from 3 independent experiments. **P<0.01.



Supplementary Figure S24. Inducible knockdown of SCUBE1 reduced cell viability of MV4-11 cells. (A) qPCR analysis of *SCUBE1* mRNA after doxycycline treatment for different times of stable MV4-11 cells with doxycycline-inducible *SCUBE1*-shRNA. (B) Cell viability was assayed by MTT assay after doxycycline treatment for different times. Data are mean ±SD from 3 independent experiments. **P<0.01.