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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Abstract

A hallmark of mixed lineage leukemia gene-rearranged (MLL-r) acute myeloid leukemia that offers an opportunity for targeted therapy is addiction to protein tyrosine kinase signaling. One such signal is the receptor tyrosine kinase Fms-like receptor tyrosine kinase 3 (FLT3) upregulated by cooperation of the transcription factors homeobox A9 (HOXA9) and Meis homeobox 1 (MEIS1). Signal peptide-CUB-EGF-like repeat-containing protein (SCUBE) family proteins have previously been shown to act as a co-receptor for augmenting signaling activity of a receptor tyrosine kinase (e.g., vascular endothelial growth factor receptor). However, whether SCUBE1 is involved in the pathological activation of FLT3 during MLL-r leukemogenesis remains unknown. Here we first show that *SCUBE1* is a direct target of HOXA9/MEIS1 that is highly expressed on the MLL-r cell surface and predicts poor prognosis in *de novo* acute myeloid leukemia. We further demonstrate, by using a conditional knockout mouse model, that *Scube1* is required for both the initiation and maintenance of MLL-AF9-induced leukemogenesis *in vivo*. Further proteomic, molecular and biochemical analyses revealed that the membrane-tethered SCUBE1 binds to the FLT3 ligand and the extracellular ligand-binding domains of FLT3, thus facilitating activation of the signal axis FLT3-LYN (a non-receptor tyrosine kinase) to initiate leukemic growth and survival signals. Importantly, targeting surface SCUBE1 by an anti-SCUBE1 monomethyl auristatin E antibody-drug conjugate led to significantly decreased cell viability specifically in MLL-r leukemia. Our study indicates a novel function of SCUBE1 in leukemia and unravels the molecular mechanism of SCUBE1 in MLL-r acute myeloid leukemia. Thus, SCUBE1 is a potential therapeutic target for treating leukemia caused by MLL rearrangements.

Introduction

Signal peptide-Complement protein C1r/C1s, Uegf, and Bmp1 (CUB)-Epidermal growth factor (EGF) domain-containing protein 1 (SCUBE1) is the first member of a small secreted and membrane SCUBE protein family consisting of three members (SCUBE1 to 3).[1-3] The *SCUBE* genes are evolutionarily preserved in vertebrates from zebrafish and mice to humans.[1-7] They encode polypeptides of approximately 1,000 residues and are organized in a modular fashion, with five distinctive protein domains including an amino-terminal signal sequence, nine tandem copies of EGF-like repeats, a spacer region, three cysteine-rich motifs, and one CUB domain at the carboxy terminus.[1-7]

The function of SCUBE proteins largely depends on their subcellular distribution and cell-type-specific expression. For instance, SCUBE1 is produced and stored in the α-granules of resting platelets.[5,7] Upon pathological stimulation, it translocates from α-granules to the platelet surface where it is proteolytically released and incorporated into thrombus.[5] Our clinical study showed that plasma SCUBE1 released from activated platelets is significantly elevated and is a biomarker of platelet activation in acute coronary syndrome and acute ischemia stroke.[8] Apart from secretion, SCUBE proteins are also expressed

as peripheral membrane proteins tethered on the cell surface via the spacer and cysteine-rich repeats by two independent mechanisms (i.e., electrostatic and lectin-glycan interactions),[9] where they function as co-receptors in promoting the signaling activity of numerous growth factors mediated by receptor tyrosine kinases or receptor serine/threonine kinases including fibroblast growth factor receptor (FGFR),[6] vascular endothelial growth factor receptor (VEGFR),[10, 11] and bone morphogenetic protein receptor (BMPR).[4, 12] Moreover, our previous work showed that SCUBE2, interacting with VEGFR2 on the cell surface, could be internalized by a monoclonal anti-SCUBE2 antibody to inhibit VEGF-stimulated tumor angiogenesis, thus suppressing the pathological growth of solid tumors originating from the lung, pancreas, colon, melanoma, or Leydig cells.[11]

Rearrangement of the mixed lineage leukemia gene (*MLL*; also known as *lysine methyltransferase 2A*, *KMT2A*) on chromosomal band 11q23 accounts for 10% of all human leukemias and manifests as acute lymphoblastic leukemia or acute myeloid leukemia (AML).[13] Although conventional chemotherapy for leukemia has been improved, patients with MLL-rearranged (MLL-r) leukemia generally exhibit relatively poor responses to treatment and have a poor prognosis.[14] Expression of the *SCUBE1* gene is highly upregulated in MLL-r leukemia.[15-17] In addition, zebrafish Scube1 is implicated in primitive hematopoiesis by modulating BMP signal activity during embryogenesis.[4] However, whether SCUBE1 is actively involved in the initiation and maintenance of MLL-r leukemogenesis and, if so, whether SCUBE1 represents a potential target to treat MLL-r leukemia remain largely unknown.

In this study, we first show that SCUBE1 is cooperatively upregulated by homeobox A9 (HOXA9) and Meis homeobox 1 (MEIS1) in MLL-r leukemia. Through molecular, genetic, proteomic and biochemical studies we further demonstrated that the membrane-tethered SCUBE1 is essential for the initiation and maintenance of MLL-r leukemia by augmenting the proliferative and survival signaling axis mediated by Fms-like receptor tyrosine kinase 3 (FLT3)-Lck/Yes-related novel protein tyrosine kinase (LYN). In addition, we demonstrated that an anti-SCUBE1 monomethyl auristatin E (an anti-microtubule cytotoxin) antibody-drug conjugate (ADC) shows specific and enhanced anti-leukemic effects in SCUBE1-positive MLL-r AML cells. These results suggest that targeting cell-surface SCUBE1 might be an efficient and promising strategy for treating MLL-r AML.

Methods

Patients

The microarray data of patients' samples were derived from a previous study[18] approved by the Research Ethics Committee of National Taiwan University Hospital, Taiwan.

Mice

All experimental procedures were performed according to a protocol approved by the Institutional Animal Care and Utilization Committee, Academia Sinica, Taiwan (Protocol 20-12-1622).

Chromatin immunoprecipitation

A chromatin immunoprecipitation (ChIP) assay was performed as described previously[19] with some modification. The detailed protocol is described in the *Online Supplementary Methods*.

Methylcellulose colony-formation assay and bone-marrow transplantation

The methylcellulose colony-formation assay was performed as described elsewhere[20] with some modification. The detailed protocol is provided in the *Online Supplementary Methods*.

Proximity ligation assay and cell viability assay with antibody-drug conjugate

The detailed protocols of the proximity ligation assay and cell viability assay are provided in the *Online Supplementary Methods*.

Results

SCUBE1 is highly expressed in MLL-rearranged acute myeloid leukemia and predicts poor prognosis in *de novo* acute myeloid leukemia

Previous transcriptomic profiling independently and reproducibly identified *SCUBE1* as a highly overexpressed gene in MLL-r AML, including in one of the most common MLL translocations t(9;11) (p22; q23) resulting in MLL fused to AF9 (MLL-AF9).[15-17] However, whether *SCUBE1* is expressed at the protein level and whether its expression level has any prognostic value in AML remains unclear. We verified that along with mRNA expression, SCUBE1 is highly expressed on the cell surface of two MLL-AF9 AML cell lines (THP-1 and NOMO-1)[21] but not in a non-MLL-r AML cell line, KG-1a, which is prone to formation of the t(8:21)(q22;q22)-associated *AML1-ETO* fusion gene,[22] as determined by western blot analysis or flow cytometry analysis with a previously generated anti-SCUBE1 monoclonal antibody[23] (Figure 1A-D). Of note, *SCUBE1* is also highly expressed in a broader spectrum of hematologic malignancies including MLL-AF4 (MV4-11) leukemic cells as well as Burkitt lymphoma (Daudi) cells (Figure 1A, B). In addition, we did not identify genomic gain or amplification nor activated mutations of the *SCUBE1* gene in AML or myelodysplastic syndromes cohorts (*Online Supple-*

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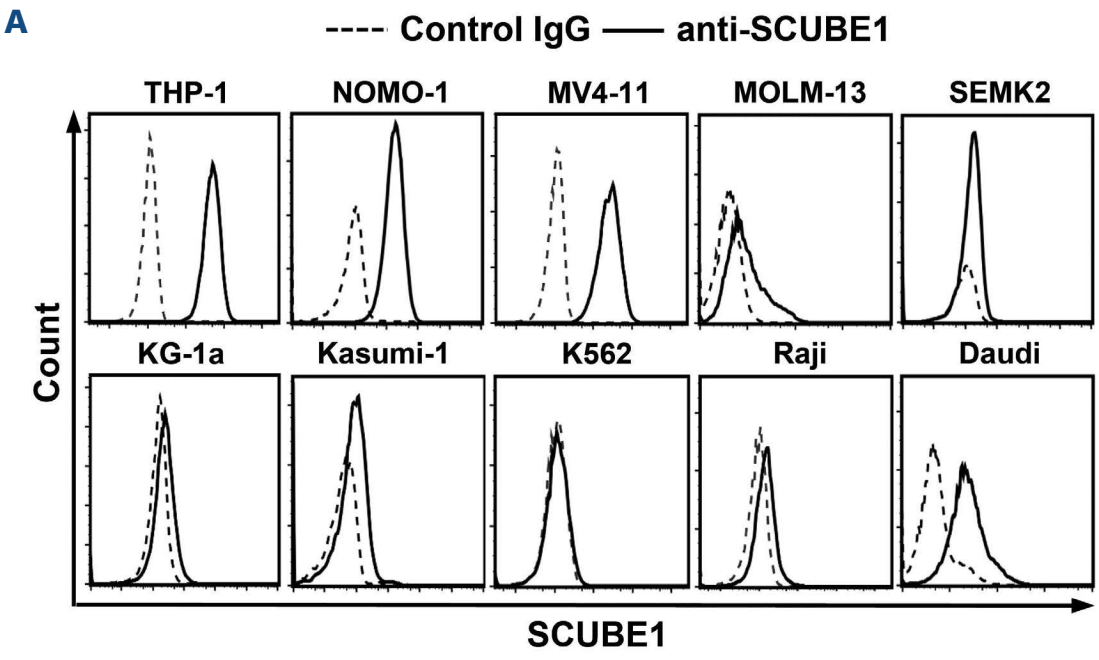

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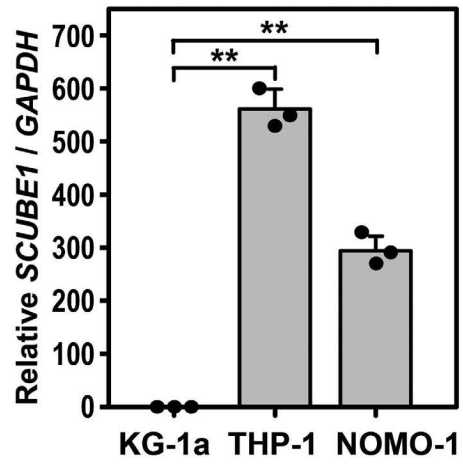

B

Cell Line	Cell Type	SCUBE1 Expression
THP-1	MLL-AF9	+++
NOMO-1	MLL-AF9	++
MOLM-13	MLL-AF9	+
MV4-11	MLL-AF4	++
SEMK2	MLL-AF4	–
KG-1a	Radiation induced AML1-ETO	–
Kasumi-1	AML1-ETO	–
K562	BCR-ABL	–
Raji	Burkitt's lymphoma	–
Daudi	Burkitt's lymphoma	+

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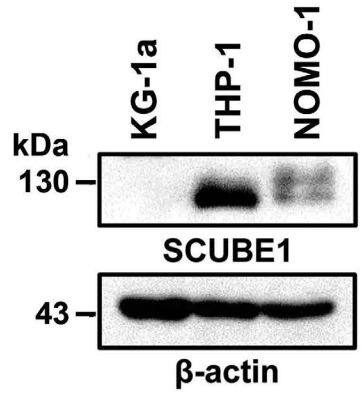

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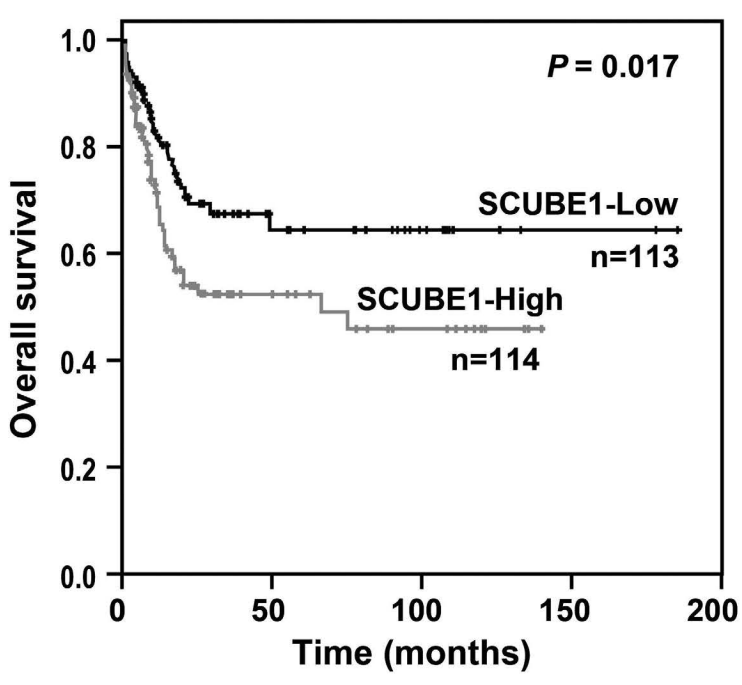

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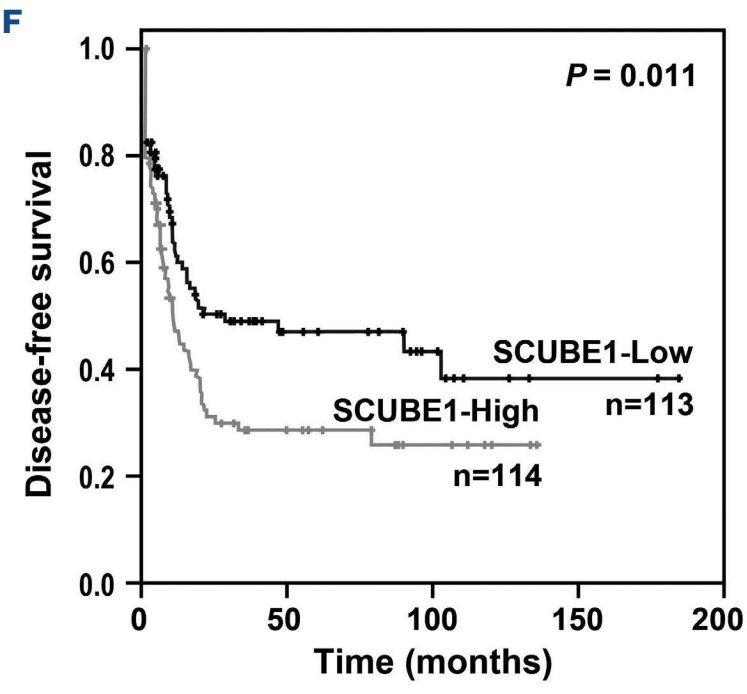


Figure 1. Expression of *SCUBE1* in MLL-rearranged acute myeloid leukemia and its association with prognosis of patients with acute myeloid leukemia. (A) Expression of SCUBE1 on the surface of leukemia or lymphoma cell lines determined by flow cytometry with anti-SCUBE1 monoclonal antibody (solid line) compared to corresponding isotype control antibody (dash line). Note that SCUBE1 is highly expressed in MLL-rearranged (MLL-AF9 or MLL-AF4) acute myeloid leukemia (AML) including THP-1, NOMO-1, MOLM-13, and MV4-11 cells as well as Daudi (Burkitt lymphoma) cells. (B) Summary of SCUBE1 expression in acute leukemia or lymphoma cell lines. (C, D) Expression of SCUBE1 in AML cell lines bearing the MLL-AF9 translocation determined at the mRNA level by quantitative polymerase chain reaction (C) and protein level by western blot analysis (D). Anti-SCUBE1 monoclonal antibody (#7), described previously, was used for western blot and flow cytometry analyses. Data are mean ± standard deviation of three independent experiments. $^{**}P<0.01$. (E) Overall survival and (F) disease-free survival of groups of patients with high SCUBE1 expression (gray line) and low expression (black line). Data were derived from GSE68469 and GSE71014 datasets.

mentary Figure S1). These data suggest that SCUBE1 upregulation might occur in AML cells at the transcriptional rather than genomic level.

We further interrogated a previously published gene expression profiling dataset of bone-marrow mononuclear cells from 227 patients with *de novo* AML.[18] High *SCUBE1* expression was associated with a high white blood cell count (P<0.001) and high blast cell count (P<0.001) (*Online Supplementary Table S1*). Patients with M4 or M5 monoblastic subtypes according to the French-American-British classification frequently have high *SCUBE1* expression (P<0.001 and P<0.001, respectively). In line with previous expression profiling studies, high *SCUBE1* expression is significantly associated with MLL abnormalities including *MLL*-r/*MLL*-partial tandem duplication (*MLL*-PTD). In addition, overall survival was shorter (median 66.1 months *vs*. not reached; log-rank P=0.017), as was disease-free survival (median 9.4 *vs*. 27.0 months; log-rank P=0.011) with high as compared to low *SCUBE1* expression after a median follow-up of 57.0 months (Figure 1E, F). On multivariate analysis, besides age or white blood cell count, we also used 2017 European LeukemiaNet risk stratification for analysis, including more comprehensive poor prognostic genetic factors,[24] and observed that high SCUBE1 expression remained an independent prognostic factor for overall survival (hazard ratio=1.663, 95% confidence interval: 1.026-2.696) (*Online Supplementary Table S2*). Our results demonstrate that *SCUBE1* is a surface protein predominantly expressed on MLL-r AML cells and high SCUBE1 expression is significantly associated with unfavorable prognosis of AML.

HOXA9 and MEIS1 cooperatively bind on distal regulatory elements and upregulate *SCUBE1* expression in MLL-rearranged acute myeloid leukemia cells

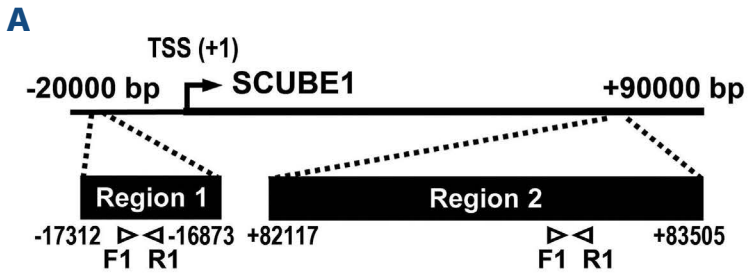
Translocations of *MLL* produce MLL oncofusion proteins that can activate transcription of downstream target genes,[19,25] including the HOXA9 and MEIS1 transcription factors that functionally collaborate to drive leukemogenesis.[26] *SCUBE1* is highly expressed in MLL-r AML cells but not in normal hematopoietic stem/progenitor cells, peripheral blood cells or in leukemia cells lacking MLL-r (*Online Supplementary Figure S2*). Hence, *SCUBE1* might be directly regulated by *MLL* fusion genes such as *MLL-AF9* or indirectly by its downstream homeodomain-containing transcription factor HOXA9 and its cofactor MEIS1, a member of the three-amino-acid-loop-extension protein family.[27]

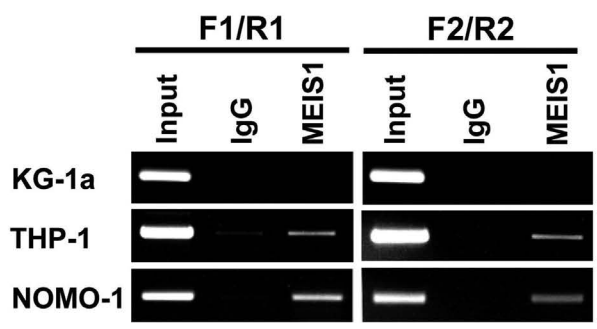
To determine whether the MLL-AF9 fusion protein directly activates the *SCUBE1* gene locus, we interrogated a previously published MLL-AF9 ChIP-sequencing dataset derived from THP-1 cells.[19] However, virtually no peaks, evidenced by coincident signals in both MLL and MLL-AF9 fusion ChIP-sequencing tracks, localized within the *SCUBE1* promoter region (*data not shown*). In addition, the gene body showed no significant enrichment of MLL-AF9-recruited epigenetic markers of H3K79me2,[19] which further supports that *SCUBE1* might not undergo transcriptional activation in MLL-r leukemia by directly targeting MLL-AF9. Rather, putative HOXA9/MEIS1 co-bound sites were found located in distal intergenic (20-kb upstream or 82-kb downstream) regulatory regions by an *in silico* bioinformatic tool PROMO (*Online Supplementary Figure S3*).[28,29] We then performed ChIP with an anti-MEIS1 antibody and confirmed that endogenous MEIS1 protein interacts with two distant regulatory DNA elements that harbor consensus HOXA9/MEIS1 co-bound sites in THP-1 and NOMO-1 cells (Figure 2A, B). In agreement with these findings, HOXA9 and MEIS1 cooperatively transactivated a regulatory DNA fragment containing the HOXA9/MEIS1 co-bound sites in a luciferase reporter assay (Figure 2C, D). Consistently, mutation of the HOXA9/MEIS1 binding site abolished HOXA9/MEIS1-mediated co-transactivation of luciferase reporter activity (*Online Supplementary Figure S4*). Furthermore, double knockdown of HOXA9 and MEIS1 by two independent combinations of lentiviral-mediated delivery of short hairpin RNA (shRNA) (Figure 2E, F) significantly decreased the expression of *SCUBE1* at both protein (Figure 2G) and mRNA (Figure 2H) levels in THP-1 or NOMO-1 cells. In agreement with previous genome-wide ChIP-sequencing experiments (*Online Supplementary Figure S5*),[30] our results suggest that SCUBE1 is likely a new target transactivated by cooperation between HOXA9 and MEIS1 at co-bound sites located in distal regulatory regions.

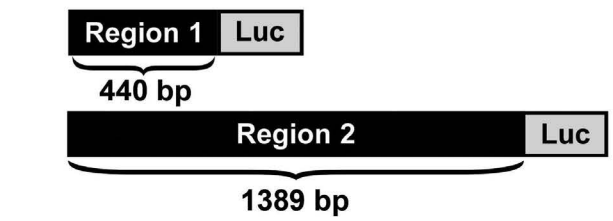
SCUBE1 is required for *in vitro* and *in vivo* MLL-rearranged leukemia cell survival

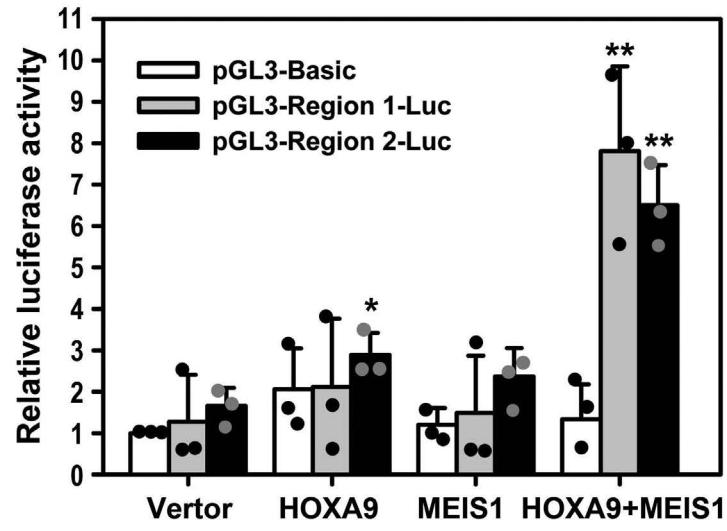
To evaluate the functional role of SCUBE1 in MLL-r leukemia, we transduced THP-1, NOMO-1, and KG-1a leukemic cell lines with inducible lentiviral (shRNA) vectors targeting *SCUBE1* (*Online Supplementary Figure S6*). After *SCUBE1* depletion, cell growth was significantly reduced in MLL-r cell lines (THP-1 and NOMO-1), but growth was unaffected in KG-1a cells (a non-MLL-r leukemic cell line) (*Online Supplementary Figure S7A-C*). Consistently, *SCUBE1* knockdown led to disruption of the G1/S and G2/M phases of cell cycle progression (*Online Supplementary Figure S8*), along with the induction of apoptosis in MLL-r leukemia cells, as revealed by a significant increase in cleaved caspase-3 and marked reduction of survivin (*Online Supplementary Figure S7D*). Both disruption of cell cycle progression and induction of apoptosis might contribute to the growth inhibitory effects of *SCUBE1* knockdown in MLL-r leukemia cells.

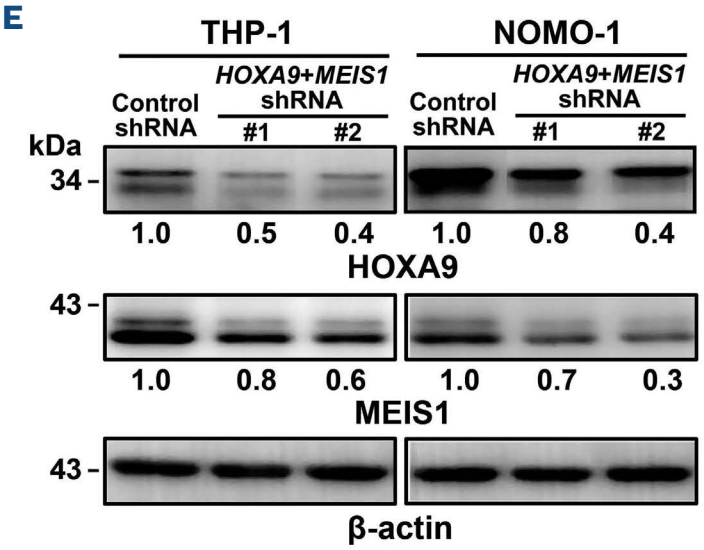
We next determined the role of SCUBE1 in leukemia propagation *in vivo*. THP-1 or NOMO-1 cells transduced with an inducible lentiviral *SCUBE1* shRNA#2 vector were

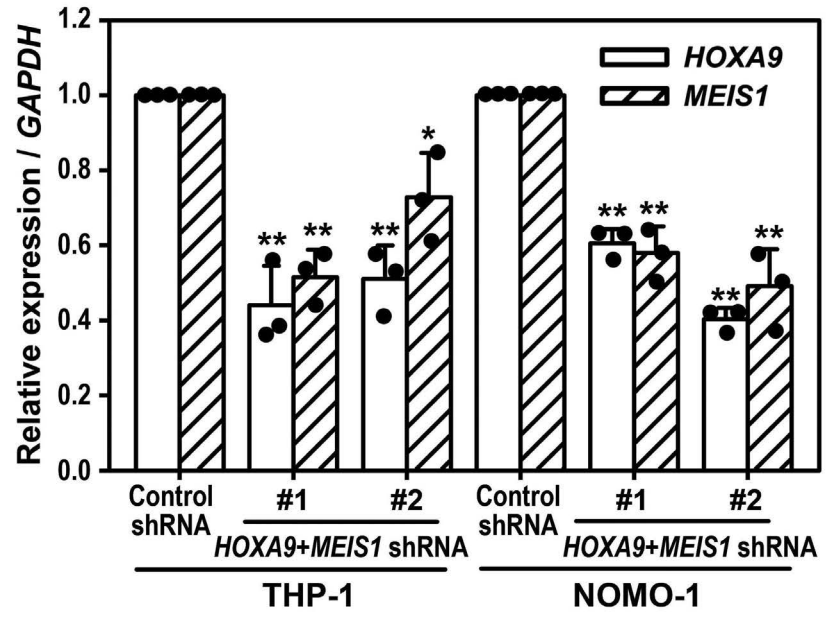


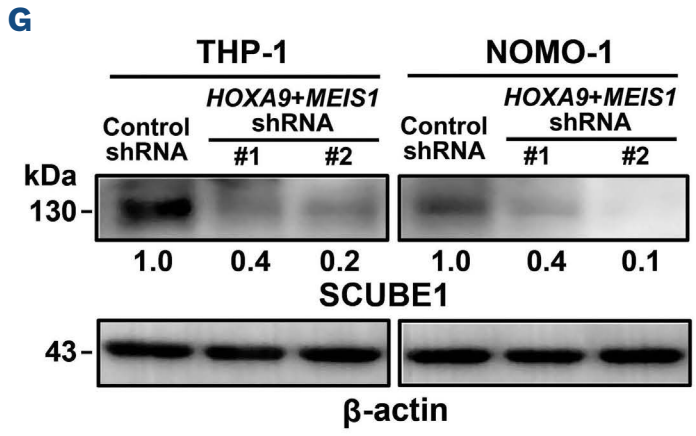












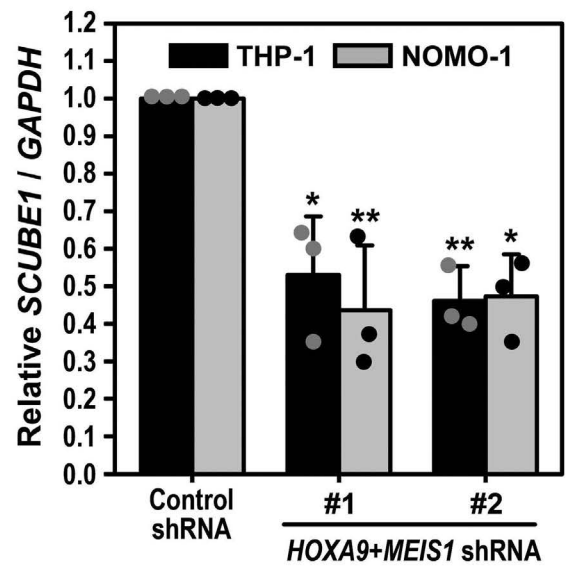


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Figure 2. HOXA9 and MEIS1 bind and transactivate the regulatory elements of *SCUBE1* in MLL-AF9 cells. (A) Graphical representation of predicted binding sites of HOXA9 and MEIS1 on human *SCUBE1* regulatory regions. The binding sites of MEIS1 and HOXA9 on *SCUBE1* enhancer or regulator regions were predicted using the PROMO database. The overlapping binding sites of HOXA9 and MEIS1 were found in two regions: region 1 (upstream of the *SCUBE1* transcription start site) and region 2 (downstream of the *SCUBE1* transcription start site). (B) Chromatin immunoprecipitation assay of KG-1a, THP-1, and NOMO-1 cells with anti-MEIS1 antibody and enriched fragments analyzed by reverse transcriptase polymerase chain reaction. Oligonucleotide F1/R1 or F2/R2 primer pairs were used to amplify ~400 bp of region 1 or 2 of enriched fragments, respectively. (C) Graphical illustration of luciferase-reporter constructs of *SCUBE1* regulatory regions. The putative regulatory 440 bp of region 1 or 1,389 bp of region 2 were cloned into the pGL3-basic vector. (D) Luciferase reporter assay with overexpression of HOXA9, MEIS1, or combined HOXA9 and MEIS1 together with the region 1 or 2 reporter constructs in HepG2 cells. *Firefly* luciferase activity was normalized to *Renilla* luciferase activity. (E, F) shRNA-mediated knockdown of transcription factors HOXA9 and MEIS1 at protein and mRNA levels in THP-1 and NOMO-1 cells. The quantified band intensities were normalized to loading controls and are mentioned below the corresponding bands. (G, H) mRNA and protein levels of *SCUBE1* with HOXA9/MEIS1 knockdown in THP-1 and NOMO-1 cells. The quantified band intensities were normalized to loading controls and are mentioned below the corresponding bands. Data are mean ± standard deviation of three independent experiments. **P*<0.05, ***P*<0.01.

transplanted into NOD-*Prkdc*^{scid} *Il2rg*^{null} (NSG) mice (Figure 3A). After treatment with doxycycline (+Dox) to induce *SCUBE1* knockdown, mice transplanted with *SCUBE1* shRNA#2 in THP-1 or NOMO-1 cells showed significant downregulation of *SCUBE1* expression (*Online Supplementary Figure S9*), reduced engraftment in bone marrow (Figure 3B) as well as reduced splenomegaly as compared with mice that did not receive doxycycline treatment (-Dox) (Figure 3C). Importantly, knockdown of *SCUBE1* (+Dox) significantly extended the survival of NSG mice as compared with that of control (-Dox) mice (Figure 3D). These data demonstrate a critical role for SCUBE1 in the growth and survival of MLL-AF9 leukemia cells both *in vitro* and *in vivo*.

SCUBE1 is important for MLL-AF9-induced transformation *in vitro* and MLL-AF9-induced leukemia progression *in vivo*

To further examine a role for SCUBE1 in leukemogenesis *in vivo*, we generated a new germline Scube1 knockout (KO) mutant mouse strain, Δ3 (*Online Supplementary Figure S10A-E*, *Online Supplementary Table S3*). We first investigated the role of SCUBE1 in MLL-AF9-mediated transformation of hematopoietic progenitor cells (HPC). c-Kit^{+} HPC isolated from bone marrow of wild-type (WT) or KO mice were transduced with lentiviruses expressing *SCUBE1* and/or retroviruses expressing MLL-AF9 as indicated (Figure 4A). Of note, similar to human MLL-AF9 AML cells, MLL-AF9-mediated transformation of murine WT HPC also markedly upregulated the cell surface expression of SCUBE1, an effect not seen in KO cells (*Online Supplementary Figure S10F*). To assess the effect of *Scube1* inactivation on MLL-AF9-mediated transformation, infected WT or KO cells were plated in methylcellulose. The number of viable colonies was reduced in the third round of methylcellulose replating in *Scube1*-KO *versus* WT HPC (Figure 4B, *Online Supplementary Figure S11*). *SCUBE1* overexpression alone did not drive the oncogenic transformation of the WT HPC, whereas re-expression of *SCUBE1* completely rescued the compromised MLL-AF9-mediated transformation by increasing the colony numbers in KO HPC, like infected WT HPC (Figure 4B and *Online Supplementary Figure S11*).

To investigate the importance of SCUBE1 in the progression of MLL-AF9-induced leukemia *in vivo*, donor MLL-AF9-transformed WT, KO, or KO HPC with restoration of *SCUBE1* expression (KO + SCUBE1) were serially transplanted into recipient C57BL/6J mice (Figure 4A). All mice receiving WT MLL-AF9 transplants died by 120 days after the second bone-marrow transplant, whereas engraftment of KO MLL-AF9 cells (deletion of *Scube1*) markedly prolonged the survival of mice for more than 200 days (Figure 4E). Consistently, re-expression of SCUBE1 (KO + SCUBE1) conferred a leukemic burden, leading to shorter survival. In agreement with the improved survival, *Scube1* inactivation (KO) prevented splenomegaly (Figure 4C) and reduced leukemia infiltration, resulting in normal spleen histology with a clear structure of red and white pulp as well as normal cell density in mice that underwent KO MLL-AF9 transplantation (Figure 4D). By contrast, mice transplanted with WT or KO+SCUBE1 MLL-AF9 cells displayed profound leukemic blast infiltration and spleen hypercellularity (Figure 4D). These results demonstrate a critical function of SCUBE1 in the initiation of MLL-AF9 leukemia *in vitro* as well as its progression *in vivo*.

SCUBE1 is critical for maintaining MLL-AF9 transformation

In addition to its function in initiating leukemia, SCUBE1 may also be required to maintain the immortalized state elicited by MLL-AF9. To test this hypothesis, we used a tamoxifen-dependent conditional KO mouse model (*Online Supplementary Figure S12A-C*). *Scube1* conditional KO (*Scube1*$^{f/f}$; *R26*^{CreERT2}) MLL-AF9-transformed HPC failed to form colonies after tamoxifen-induced deletion of *Scube1*. By contrast, a similar treatment had no effect on control (*Scube1*$^{f/f}$) MLL-AF9-immortalized cells, which indicates that treatment with tamoxifen did not cause general cell toxicity (Figure 5A, B).

To further assess the *in vivo* effect of acute inactivation of *Scube1* on leukemia maintenance, primary leukemias generated by transducing c-Kit^{+} HPC from control or inducible

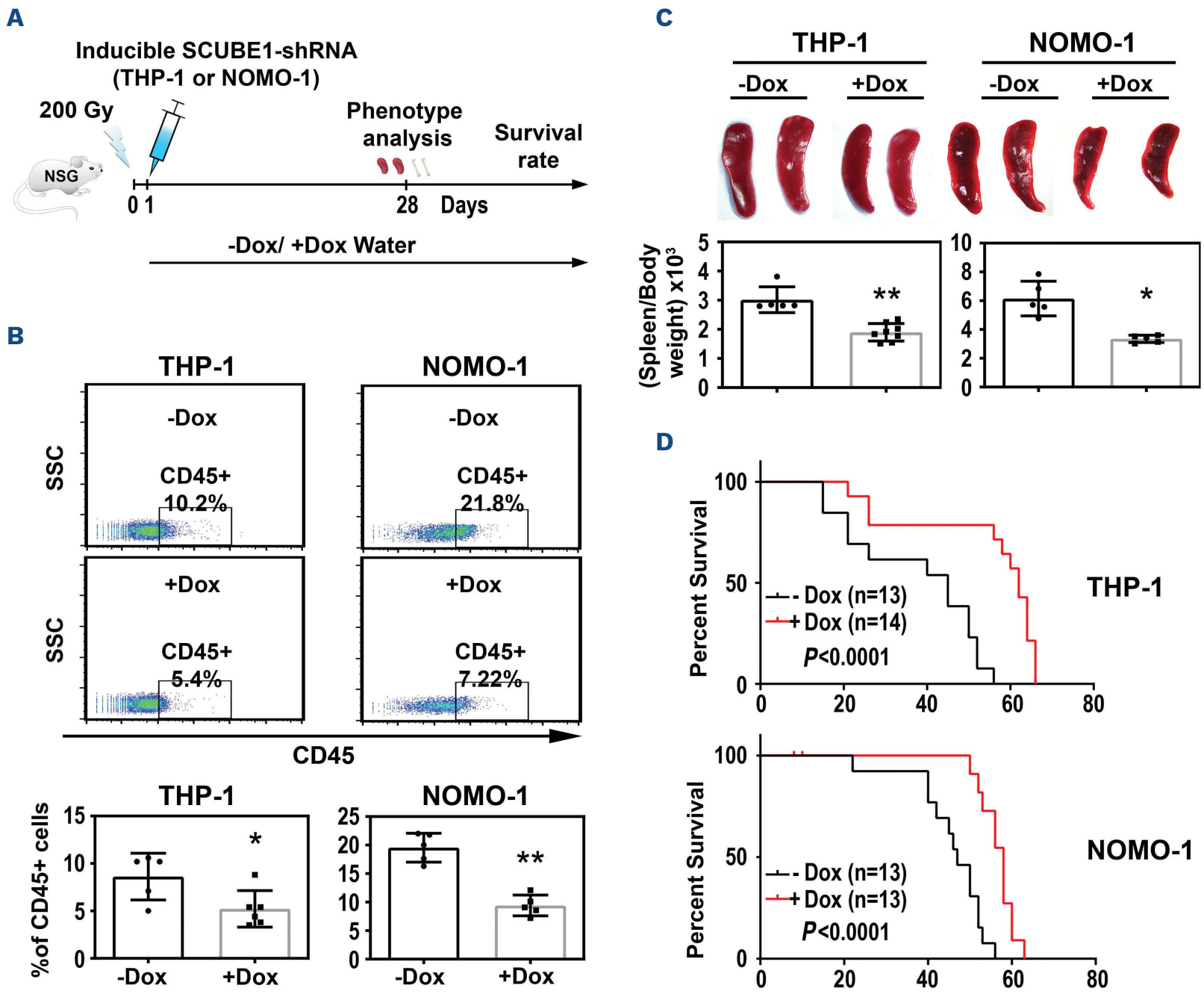

Figure 3. Inducible knockdown of *SCUBE1* in MLL-AF9-translocated acute myeloid leukemia reduced cell growth and increased the survival rate of mice. (A) Schematic representation of *in vivo* experiments to analyze the effect of *SCUBE1* knockdown on the growth of THP-1 and NOMO-1 cells. Sub-lethal irradiation of NSG mice was performed on day 0 followed by intravenous injection of THP-1 or NOMO-1 cells with inducible *SCUBE1*-shRNA #2 clone. Doxycycline (Dox) was omitted or added in the drinking water of mice on day 1. Spleen and bone-marrow infiltration was measured on day 28, and survival rate was analyzed until all mice showed disease symptoms (hunched back, lack of mobility, paralysis of hind limbs, ruffled coat). Mice were sacrificed on day 28 by cervical dislocation, and spleen and femora were isolated. (B) Bone marrow was isolated from femora and human leukemic cell infiltration was measured by flow cytometry with anti-human CD45 antibody. (C) Spleen enlargement was measured by ratio of spleen weight to body weight. (D) Kaplan-Meier curve showing survival of NSG mice engrafted with THP-1 or NOMO-1 cells bearing doxycycline-inducible *SCUBE1*-shRNA #2 clone and with (red line) or without (black line) doxycycline treatment. The median survival was 45 days (-Dox) and 65 days (+Dox) or 42 days (-Dox) and 58 days (+Dox) for THP-1 and NOMO-1 cells, respectively. **P*<0.05, ***P*<0.01.

KO mice with MLL-AF9 DsRed were transplanted into secondary recipient mice. When engraftment of leukemia cells reached 10% to 20% DsRed^{+} in peripheral blood cells, we administered tamoxifen daily for 5 days to the secondary recipient mice (Figure 5C). Effective deletion of *Scube1* was verified by genotyping peripheral blood cells at 2 weeks after tamoxifen administration (*Online Supplementary Figure S12D*). In line with a critical role of SCUBE1 in maintaining the clonogenicity of the leukemic cells, acute tamoxifen-induced *Scube1* depletion significantly prolonged survival (Figure 5D) and prevented splenomegaly (Figure 5E) of *Scube1*$^{f/f}$; *R26*^{CreERT2} mice as compared with *Scube1*$^{f/f}$ controls. In addition, we found significantly more apoptotic cells and a markedly reduced number of proliferating MLL-AF9-induced leukemia stem cells in *Scube1*-KO than WT spleens (*Online Supplementary Figure S13*). Together, these data suggest that MLL-AF9-transformed HPC require SCUBE1 to maintain clonal growth both *in vitro* and *in vivo*.

Membrane SCUBE1 binds FLT3 ligand and FLT3 receptor to facilitate activation of the FLT3-LYN signaling axis

To further elucidate the molecular mechanisms underlying the contribution of membrane SCUBE1 to leukemogenesis,

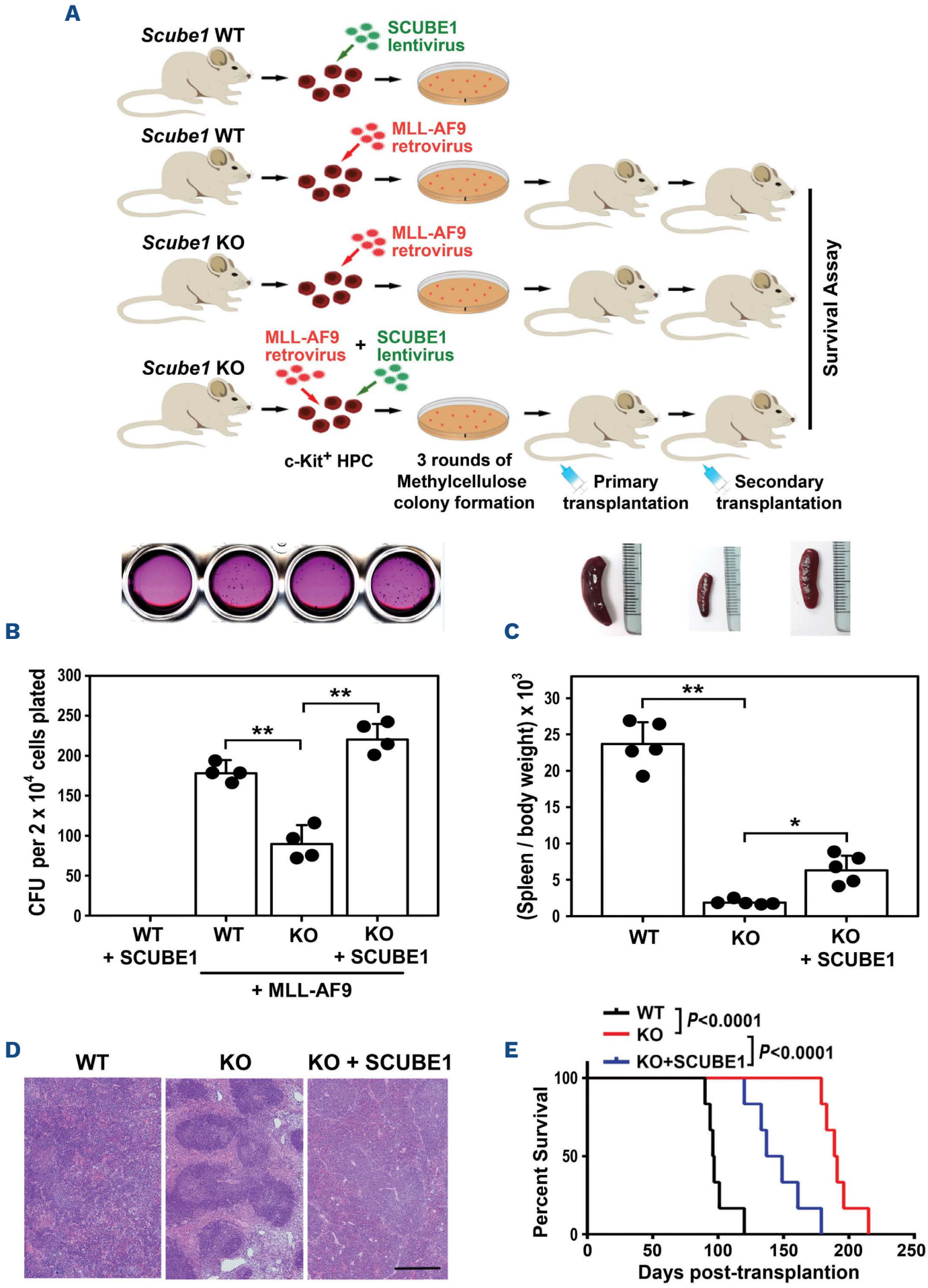

Figure 4. *Scube1* is important for initiation of MLL-AF9-induced leukemia. (A) Schematic representation of experimental procedures to evaluate the role of SCUBE1 in the initiation of leukemia. c-Kit$^+$ hematopoietic cells were isolated from *Scube1* knockout (KO) or wild-type (WT) C57BL/6 mouse bone marrow, followed by transduction of MLL-AF9 retrovirus or *SCUBE1* lentivirus and methylcellulose colony formation assay. After a third round of colony formation, cells were intravenously injected into sub-lethally irradiated C57BL/6 mice. When the primary transplanted mice showed symptoms of disease, leukemic cells were isolated from bone marrow and secondary transplantation was performed. (B) Methylcellulose colony formation assay after three rounds of replating after MLL-AF9 transduction. (C) Spleen enlargement of secondary transplanted mice. (D) Hematoxylin & eosin-stained spleen histology of secondary transplanted mice. Images were acquired with an Olympus microscope equipped with an Olympus DP70 digital camera; original magnification 10x; scale bar = 200 μm. (E) Kaplan-Meier curve showing survival of secondary transplanted mice. The median survival for WT (black line), KO (red line), and KO+SCUBE1 (blue line) cells was 96.5, 190, and 143 days, respectively. Data are mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01.

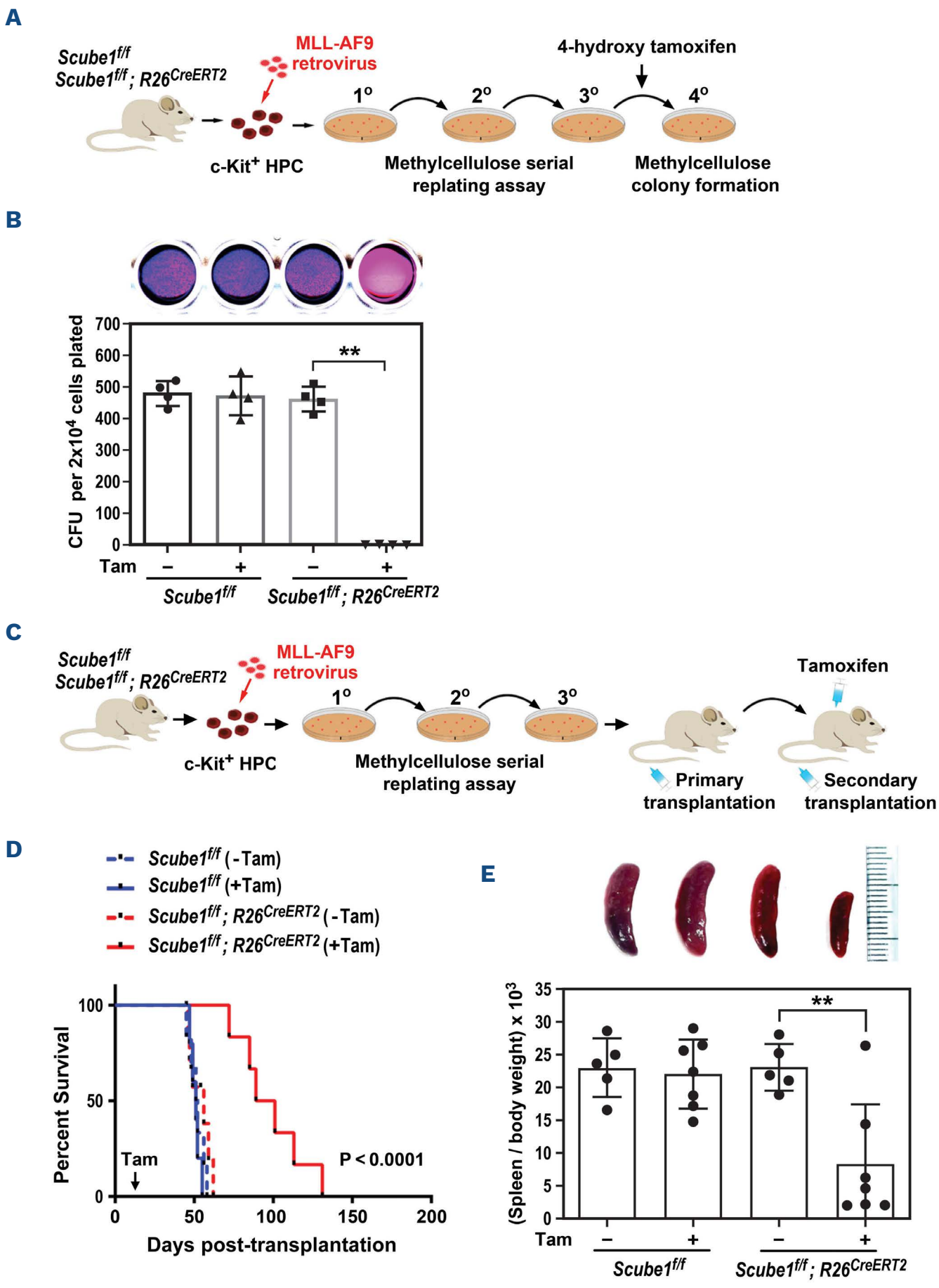

Figure 5. *Scube1* is critical for maintaining MLL-AF9-transformed leukemia stem cells. (A) Schematic representation of experimental procedures to evaluate the role of *Scube1* in maintaining leukemia stem cells *in vitro*. c-Kit$^+$ hematopoietic progenitor cells (HPC) were isolated from *Scube1*$^{f/f}$ or *Scube1*$^{f/f}$; *R26*^{CreERT2} C57BL/6 mouse bone marrow, followed by transduction of MLL-AF9 retrovirus and three rounds of methylcellulose colony formation assay. At the fourth round, 4-hydroxy tamoxifen (4-OHT) 30 nM was added for the Cre-mediated *Scube1* knockout. (B) Methylcellulose colony formation assay at the fourth round after 4-OHT treatment. (C) Schematic representation of experimental procedure to examine the role of Scube1 in the maintenance of leukemia stem cells *in vivo*. c-Kit$^+$ HPC were isolated from *Scube1*$^{f/f}$ or *Scube1*$^{f/f}$; *R26*^{CreERT2} C57BL/6 mouse bone marrow, followed by transduction of MLL-AF9 retrovirus and three rounds of methylcellulose colony formation assay. After a third round of colony formation, the cells were intravenously injected into sub-lethally irradiated C57BL/6 mice. When the primary transplanted mice showed symptoms of disease, leukemic cells were isolated from bone marrow and secondary transplantation was performed. Two weeks after transplantation, established leukemia was confirmed by blast cells in peripheral blood. After disease establishment, five doses of tamoxifen were administered to inactivate *Scube1*. (D) Kaplan-Meier curve showing survival of secondary transplanted mice. The median survival for *Scube1*$^{f/f}$ -Tam (dashed blue line), *Scube1*$^{f/f}$ +Tam (solid blue line), *Scube1*$^{f/f}$; *R26*^{CreERT2} -Tam (dashed red line), and *Scube1*$^{f/f}$; *R26*^{CreERT2} +Tam (solid red line) mice was 51.5, 51, 56, and 95 days, respectively. (E) Spleen enlargement of secondary transplanted mice. Data are mean ± standard deviation of three independent experiments. ***P*<0.01.

we used a proteomic proximity labeling assay[31] to identify membrane proteins in the immediate vicinity of surface SCUBE1. We conjugated biotin to proteins proximal to SCUBE1 and analyzed the biotin labeled proteins by mass spectrometry. After excluding non-specific proteins, we identified a total of 120 membrane proteins associated with or in close proximity to SCUBE1 commonly shared in both THP-1 and NOMO-1 cells (Figure 6A). Because SCUBE2 and SCUBE3 act as co-receptors to augment the signaling activity of receptor tyrosine kinases such as VEGFR[11,32] and FGFR[6] and because upregulation of protein-tyrosine kinase signaling is a hallmark of AML,[33,34] we paid particular attention to receptor tyrosine kinases and their downstream signaling components. Among the 120 identified proteins were four receptor tyrosine kinases - FLT3, ephrin type-B receptor 1 and 3 (EPHB1 and 3), and insulin receptor (INSR) - as well as three non-receptor tyrosine kinases - Lck/Yes-related novel protein tyrosine kinase (LYN), Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) (Figure 6A, *Online Supplementary Table S4*). FLT3, a class III receptor tyrosine kinase, consists of five extracellular ligand-binding Ig-like motifs, a member-spanning region, a juxtamembrane region followed by a tyrosine kinase domain interrupted by a kinase insert, and the carboxy terminal tail (Figure 6B).[33] FLT3 signaling is initiated by the binding of FLT3 ligand (FLT3L) to the extracellular Ig-like domains of FLT3 to induce dimerization, autophosphorylation, proximal recruitment of the Src family of non-receptor tyrosine kinases such as LYN to be activated via tyrosine phosphorylation, and subsequent activation of downstream signaling pathways including phosphatidylinositol 3-kinase/AKT or extracellular signal-regulated kinases (ERK).[33]

Because the genes encoding FLT3[33] or its direct signaling component LYN[35,36] are often over-expressed or mutated, thus leading to augmented proliferation and survival of human AML,[37,38] we further examined whether SCUBE1 biochemically interacts with FLT3L or FLT3 and, if so, whether SCUBE1 can modulate the signaling activity of the FLT3-LYN axis. To do this, we transfected HEK-293T cells with a FLAG-tagged SCUBE1 expression plasmid alone or with an expression plasmid encoding Myc-tagged FLT3 or His-tagged FLT3L. Immunoprecipitation with anti-FLAG antibody resulted in specific co-immunoprecipitation of FLT3 (Figure 6C) or FLT3L (*Online Supplementary Figure S14C*). Further deletion mapping revealed that SCUBE1 primarily interacts with the ligand-binding extracellular Ig-like domains of FLT3[39] (Figure 6C) or FLT3L via its spacer region and the CUB domain (*Online Supplementary Figure S14B, C*). Furthermore, endogenous SCUBE1 could interact and colocalize with FLT3 on the plasma membranes of THP-1 and NOMO-1 cells (*Online Supplementary Figure S15*). Together, SCUBE1 might form a complex with FLT3L and FLT3 in MLL-r AML cells.

We further evaluated the effect of SCUBE1 on activating the FLT3-LYN signaling axis by reconstituting FLT3 and LYN expression in the absence or presence of SCUBE1 in HEK-293T cells. The tyrosine phosphorylation (pY) status of FLT3 (pFLT3) or LYN (pLYN) was measured by a pan or specific anti-pLYN (pY397) antibody. As shown in Figure 6D, pFLT3 co-expressed with LYN showed a modest increase in expression, probably because of low expression of FLT3L in HEK-293T cells (*https://www.proteinatlas.org*), whereas ectopic expression of SCUBE1 markedly augmented pFLT3 as well as pLYN levels. Consistently, knockdown of SCUBE1 markedly decreased the intrinsic signaling activity of FLT3-LYN as well as the downstream activation of AKT (but not ERK), as reflected by decreased pY levels of these signaling components in THP-1 and NOMO-1 cells (Figure 6E). Likewise, downregulation of Flt3 phosphorylation was also observed in Scube1-knockout MLL-AF9 murine AML cells (*Online Supplementary Figure S16*). Furthermore, SCUBE1-mediated specific tyrosine phosphorylation/activation of FLT3 slightly differed from that of FLT3L-induced FLT3 tyrosine phosphorylation (e.g., increased pY768 and pY842 but not pY591 level) (*Online Supplementary Figure S17*). Nevertheless, additional investigation is needed to fully elucidate the molecular mechanisms underlying the SCUBE1-assisted augmentation of FLT3 activation in AML cells. Together, these data suggest that membrane SCUBE1 might be a co-receptor to facilitate FLT3L binding to FLT3, thus promoting downstream LYN and AKT signaling.

SCUBE1-targeting antibody-drug conjugate effectively inhibits cancer growth

Internalization and trafficking to lysosomes upon antibody binding to a membrane target is a key mechanism for ADC to exert their killing effect following intracellular release of cytotoxic payloads.[40] We therefore examined whether a newly generated anti-SCUBE1 monoclonal antibody clone #1 (*Online Supplementary Figure S18*) could internalize upon binding to SCUBE1 on leukemia cells. We incubated anti-SCUBE1 antibody with THP-1 cells and found that the monoclonal antibody rapidly bound (*Online Supplementary Figure S19A*), efficiently endocytosed to lysosomes and degraded after 24 h (*Online Supplementary Figure S19B*), which suggests that this monoclonal antibody can be internalized.

As a proof of concept for its potential therapeutic use, we generated an ADC combining monoclonal antibody #1 as the SCUBE1-targeting moiety with a proteolytically cleavable valine-citrulline (VC) linker and the anti-microtubule cytotoxic agent monomethyl auristatin E (MMAE) (see *Online Supplementary Figure S20*) by using the homogeneous trimannosyl glycoengineering platform.[41] The average drug-to-antibody ratio was 3.89 (Figure 7A, B). Importantly, this ADC (designated as anti-SCUBE1-VC-MMAE) retained similar binding affinity as the parental antibody (*Online Supplemen-*

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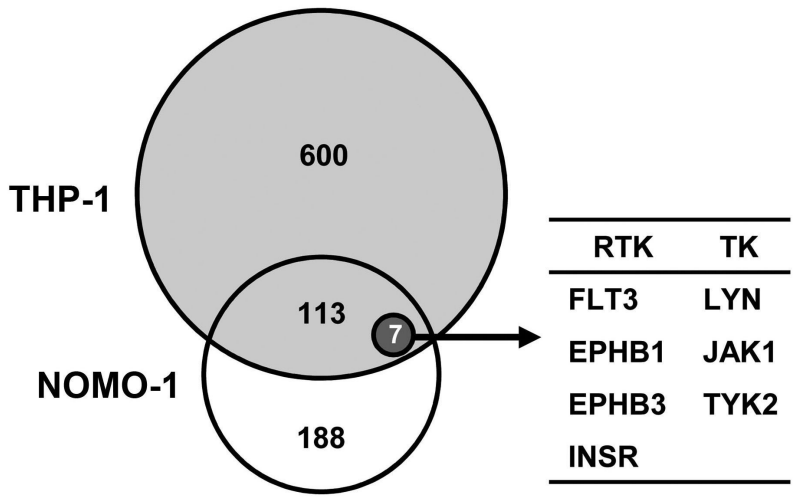

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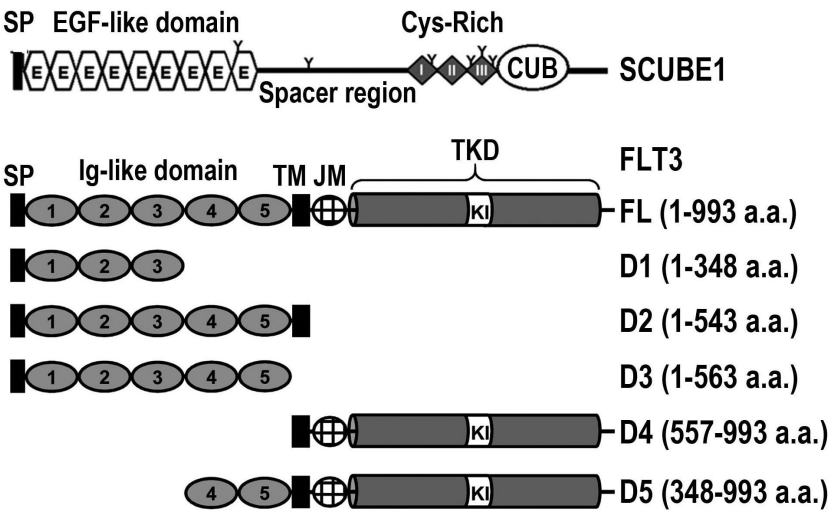
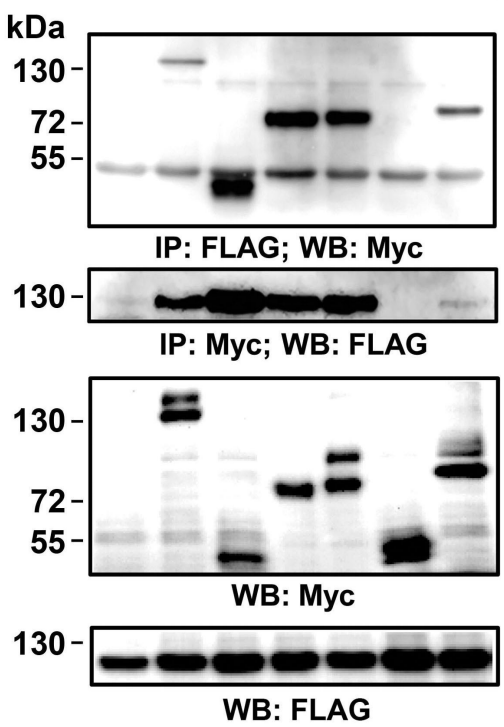


C

FLAG. SCUBE1	+	+	+	+	+	+	
Vector	+						
Myc. FLT3		FL	D1	D2	D3	D4	D5



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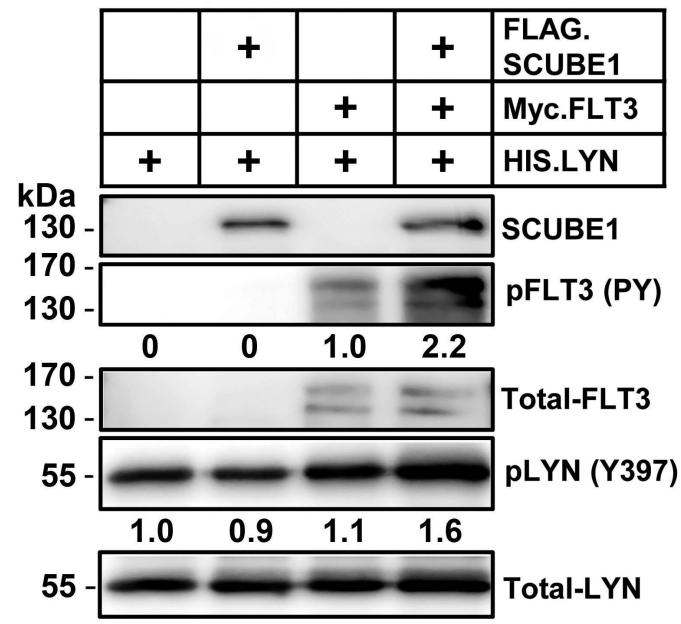

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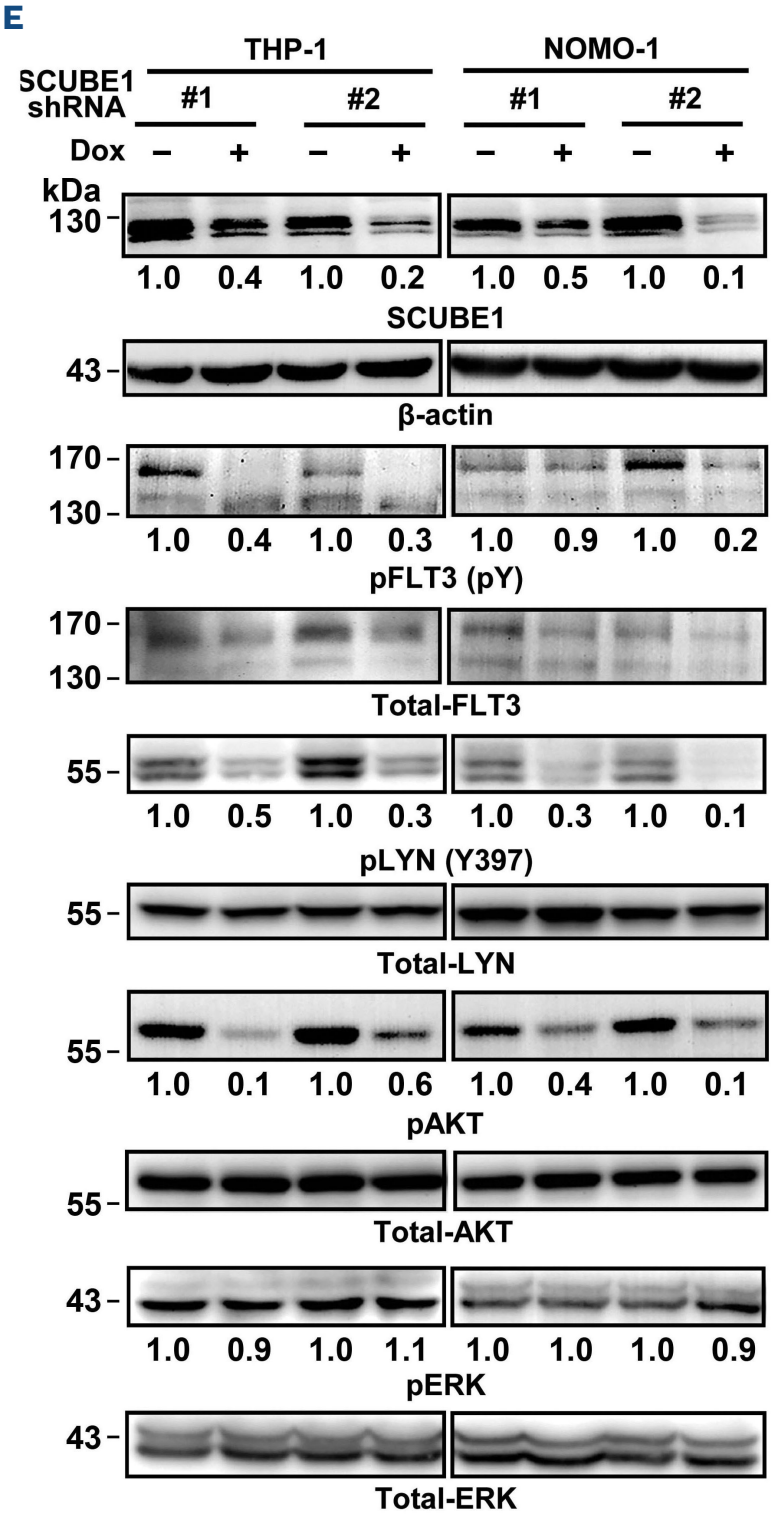

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Figure 6. SCUBE1 binds FLT3 and promotes FLT3-LYN signaling. (A) Venn diagram showing the number of membrane proteins in the immediate vicinity of surface SCUBE1 identified by proteomic proximity labeling assay in THP-1 and NOMO-1 cells. Of the 113 proteins in common, seven protein tyrosine kinases - four receptor tyrosine kinases (FLT3, EPHB1, EPHB3, and INSR) and three non-receptor tyrosine kinases (LYN, JAK1, and TYK2) - were associated with or in proximity to SCUBE1. The antibody-directed targeting of peroxidase (a combination of a primary mouse monoclonal anti-SCUBE1 antibody and an horse radish peroxidase-conjugated anti-mouse secondary antibody) to SCUBE1, followed by brief labeling with biotin-tyramide enabled proteins in the immediate vicinity of the target to be biotinylated. After cell lysis and capture by immobilized streptavidin, the biotinylated proteins were eluted with reducing agent and analyzed by liquid chromatography-mass spectrometry. The experiment was repeated with only a primary isotype control antibody to identify nonspecific proteins. Mass spectrometry analysis confirmed that SCUBE1 protein was immunoprecipitated by the anti-SCUBE1 antibody under these conditions. (B) Graphic diagrams showing the domain structure of SCUBE1 and deletion constructs of FLT3 used to map the interacting domain. FLAG epitope was added immediately after the signal peptide sequence at the NH_2-terminus of the SCUBE1 construct. Likewise, Myc epitope was tagged to the NH_2-terminus of FLT3 full-length (FL) and its deletion mutants D1, D2, D3, D4, and D5. SP: signal peptide; Cys-Rich: cysteine-rich; TM: transmembrane domain; JM: juxtamembrane domain. The tyrosine kinase domain (TKD) is separated into two parts by a short region designated the kinase insert (KI). (C) Molecular mapping of the interacting domains between SCUBE1 and FLT3. The expression plasmid encoding FLAG-tagged SCUBE1 was transfected alone or together with a series of Myc-tagged FLT3 constructs in HEK-293T cells for 2 days, then cell lysates underwent immunoprecipitation (IP), followed by western blot (WB) analysis with indicated antibodies to determine the protein-protein interactions. (D) Phosphorylation of LYN analyzed with co-expression of FLT3 and/or SCUBE1 in HEK-293T cells. NH_2-terminus HIS-tagged LYN was transfected in HEK-293T cells alone or with FLAG-tagged SCUBE1 and/or Myc-tagged FLT3. Two days after transfection, cells were lysed and western blot analysis was performed. The activation of FLT3 was detected with anti-phospho-tyrosine (pY) antibody and total FLT3 activity was detected with anti-Myc antibody. The activation of LYN was detected with a specific anti-pLYN (Y397) antibody and total LYN activity was detected with anti-HIS antibody; SCUBE1 activity was detected with anti-FLAG antibody. Of note, because of its heavy N-linked glycosylation of the extracellular domain, FLT3 displays as two higher molecular masses on western blot analysis: one corresponds to 132 kDa as a not fully processed, partially glycosylated form and the other appears at 160 kDa, representing the mature, fully glycosylated FLT3. The quantified band intensities were normalized to loading controls and are mentioned below the corresponding bands. (E) Effect of *SCUBE1* knockdown on phosphorylation/activation of the FLT3-LYN-AKT signal cascade in THP-1 and NOMO-1 cells. To knock down *SCUBE1*, stable THP-1 and NOMO-1 cell lines carrying inducible *SCUBE1*-shRNA #1 or #2 were treated without (-) or with (+) doxycycline (Dox) for 5 days. Western blot analysis was used to determine the phosphorylation status of FLT3 (pY), pLYN (Y397), pAKT (S473), or pERK1/2 (T202/Y204) or to quantify the corresponding total protein as controls. SCUBE1 was detected with anti-SCUBE1 #7 monoclonal antibody. The quantified band intensities were normalized to loading controls and are mentioned below the corresponding bands.

tary Figure S21). Five days of treatment with this ADC was effective in reducing cell viability in the SCUBE1-expressing MLL-r leukemia cell lines THP-1 and NOMO-1 (half maximal inhibitory concentration = 0.28±0.08 and 0.46±0.1 nM, respectively), with no effect seen in SCUBE1-negative KG-1a and K562 cells (Figure 7C) or normal murine HPC (*Online Supplementary Figure S22*). To further evaluate the efficacy of the ADC *in vivo*, we subcutaneously transplanted THP-1 cells into NSG mice. After treatment with anti-SCUBE1 ADC, THP-1 tumor growth was significantly reduced as compared with the IgG control (*Online Supplementary Figure S23A, B*). In addition, no antigen-independent toxicity was observed in either treatment group, as evaluated by monitoring body weight loss (*Online Supplementary Figure S23C*). These results confirm the selectivity of this ADC and suggest that surface SCUBE1 could be exploited as an MLL-r specific biomarker and could potentially be used as a therapeutic target (Figure 8).

Discussion

Our mechanistic study revealed that *SCUBE1* is a direct target of the HOXA9/MEIS1 transcriptional regulatory complex. Both HOXA9 and MEIS1 are upregulated by MLL-fusion proteins such as MLL-AF9 in AML and are essential for maintaining leukemic transformation. Our data suggest that surface SCUBE1 plays a critical pathological role in MLL-r leukemias by acting as a FLT3 co-receptor to facilitate interactions between the FLT3 ligand and FLT3, thus augmenting downstream LYN-AKT signaling to promote leukemia cell proliferation, survival and leukemogenesis. In addition, clinical association studies show that high expression of *SCUBE1* is associated with poor overall and disease-free survival in AML patients (Figure 1E, F). Furthermore, multivariate analysis confirmed that high SCUBE1 expression is an independent prognostic factor for overall survival (*Online Supplementary Table S2*). Using genetic knockdown, we first demonstrated that SCUBE1 is required for cell proliferation and survival of MLL-AF9 THP-1 and NOMO-1 leukemia cells both *in vitro* and *in vivo*. Furthermore, using global knockout and tamoxifen-inducible *Scube1* deletion mouse models, we showed that Scube1 is required for the initiation and maintenance of MLL-AF9-transformed HPC. Importantly, SCUBE1 on the plasma membrane of MLL-r leukemia cells is a potential target for immunotherapy as shown by the strong anticancer activity of an anti-SCUBE1 ADC in MLL-AF9 leukemias.

Our studies showed that the SCUBE1-FLT3 interaction is critical for leukemia survival and proliferation mediated through LYN-AKT signaling. Thus, conditional knockout and knockdown of SCUBE1 in MLL-AF9-transduced HPC and leukemia cell lines, respectively, have proven that inhibition of SCUBE1 could have therapeutic benefit for leukemia pa-

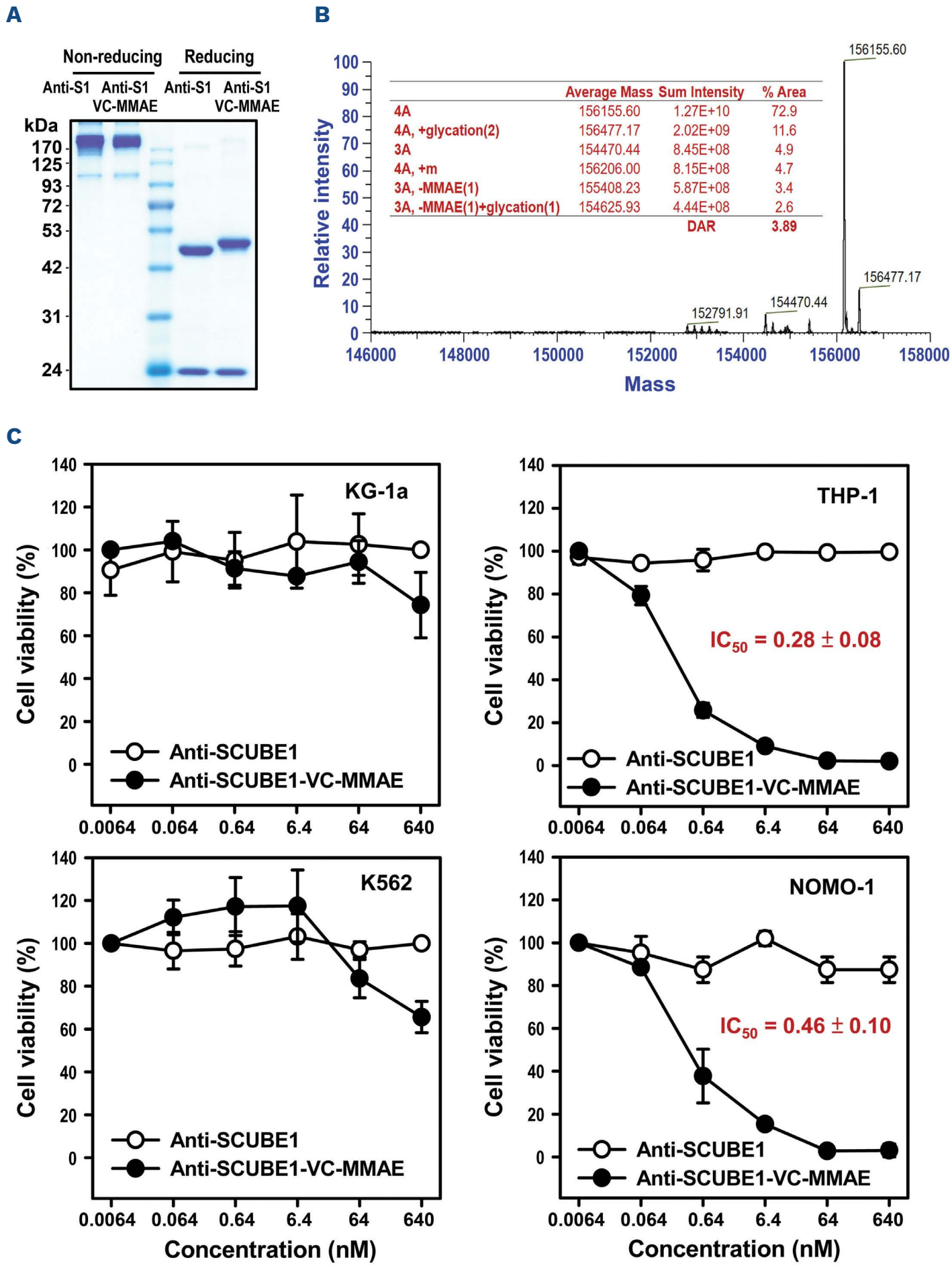

Figure 7. Production and *in vitro* characterization of an anti-SCUBE1 antibody-drug conjugate. (A) Reducing and non-reducing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) showing Coomassie blue staining of the parental anti-SCUBE1 (S1) antibody and an anti-S1 antibody-drug conjugate (ADC) (anti-S1-valine-citrulline [VC]-monomethyl auristatin [MMAE]) antibody on non-reducing and reducing SDS-PAGE. Of note, the non-reduced recombinant anti-S1 antibody was detected with a molecular mass ~180 kDa, whereas individual heavy and light chains are visible at ~55 kDa and ~25 kDa, respectively. (B) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry showed that the intact anti-SCUBE1 ADC was produced with an average drug-to-antibody ratio (DAR) of 3.89. The intact anti-S1-VC-4MMAE with two MMAE on each arm of antibody (see *Online Supplementary Figure S13*) has a peak at mass 15,6155.60 Da. "A" represents a molecular moiety of dibenzocylcooctyne (DBCO)- polyethylene glycol (PEG) 3-VC-para-aminobenzoate (PAB)-MMAE. "m" indicates uncertain modification of antibody. (C) Anti-SCUBE1 ADC induced cytotoxicity in AML cell lines. 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 the SCUBE1 expression in THP-1 and NOMO-1 cells is shown inside the graph. Note that the anti-SCUBE1 ADC did not exhibit antitumor efficacy on SCUBE1-negative KG-1a or K562 cells.

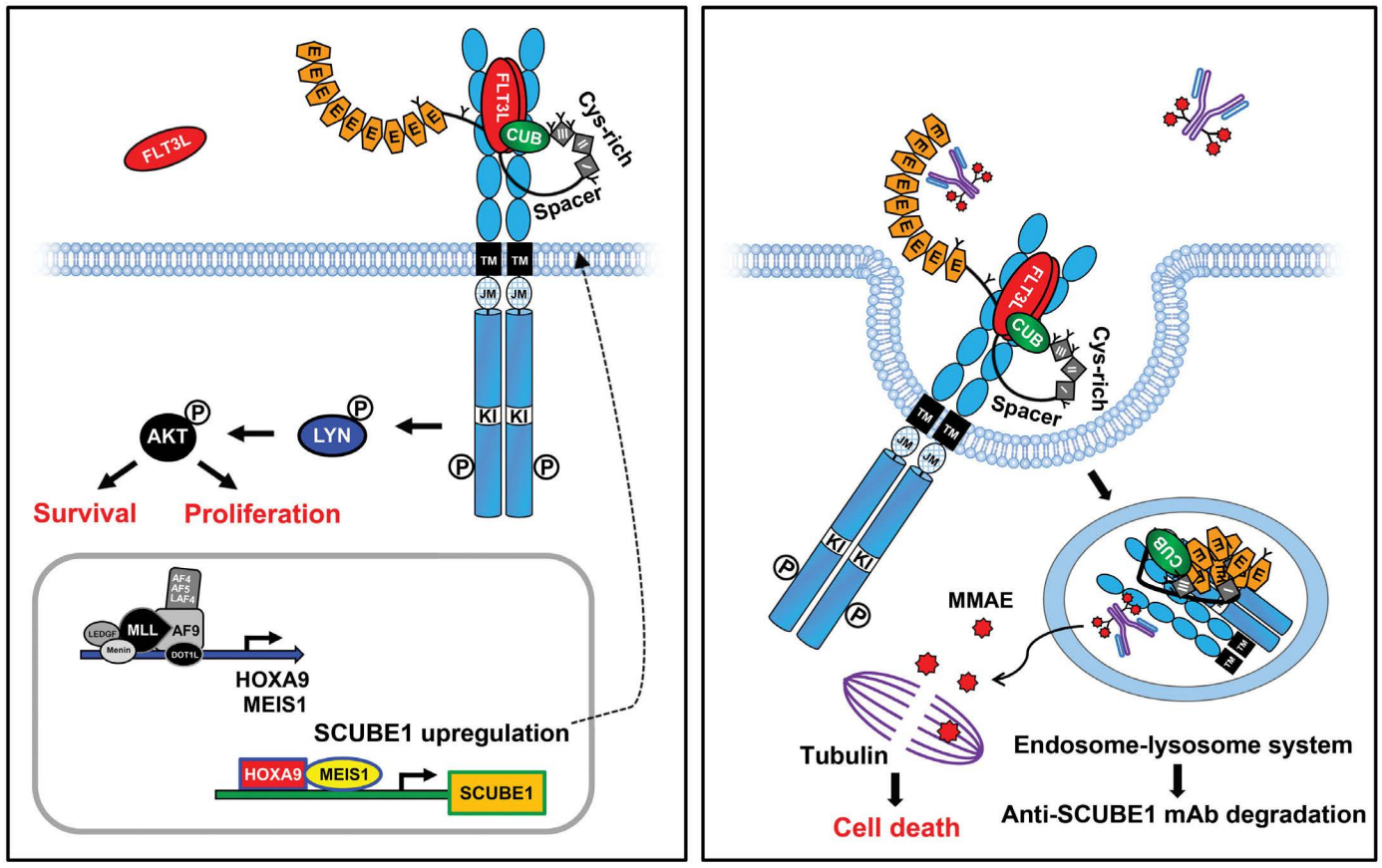

Figure 8. Working model illustrating the mechanism of action of surface SCUBE1 in MLL-rearranged leukemias and potential immunotherapy approach. Left. Our study showed that *SCUBE1* is a direct downstream target of the transcriptional regulatory complex of HOXA9/MEIS1, which are upregulated by MLL-fusion proteins such as MLL-AF9 and are essential for maintaining leukemic transformation. Surface SCUBE1 plays a critical pathogenic function in MLL-rearranged leukemias by acting as a FLT3 co-receptor via its spacer region and the COOH-terminal CUB domain in facilitating the interaction between FLT3 ligand (FLT3L) and FLT3, augmenting downstream LYN-AKT activation (tyrosine phosphorylation) for leukemic cell proliferation and survival, thus promoting leukemogenesis. Right. Surface expression of SCUBE1 on MLL-rearranged leukemia provides the opportunity for its potential use as a target for immunotherapy because an anti-SCUBE1 monoclonal antibody (mAb) conjugated to an antimitotic agent monomethyl auristatin E (MMAE) leads to significant cell killing specifically of MLL-AF9 leukemias.

tients. Our previous studies demonstrated a signaling regulatory role for SCUBE1 in BMP during mouse brain development[7] and zebrafish hematopoiesis.[4] In addition, SCUBE1 was recently implicated as a stress-inducible protein after renal ischemia-reperfusion.[12] A portion of surface-tethered SCUBE1 appears to be released into the circulation and can serve as a biomarker of platelet activation in acute coronary syndrome and acute ischemic stroke.[8] Thus, the circulating and/or surface expression levels of SCUBE1 might have diagnostic and/or prognostic value to select or stratify AML patients who might most benefit from an anti-SCUBE1 ADC regimen and/or possibly be a surrogate marker during anti-SCUBE1 therapy. However, further clinical studies are required to validate the biomarker potential of plasma SCUBE1 concentration in AML.

Although small-molecule FLT3 kinase inhibitors are clinically effective, they have drug specificity issues and the acquisition of primary and secondary resistance to treatment.[33] It will be of interest to further investigate whether SCUBE1 upregulation could be associated with refractoriness of FLT3 inhibitors and other receptor tyrosine kinase inhibitors in AML. If so, anti-SCUBE1 antibody-based drug delivery might limit the emergence of FLT3 mutants that evade small-molecule inhibitor treatment, thus minimizing the prospect of recurrent disease. Intriguingly, we also found that surface SCUBE1 is highly expressed in a FLT3-internal tandem duplication (ITD) AML cell line, MV4-11 (Figure 1A, B), and genetic knockdown of *SCUBE1* significantly inhibits the growth of MV4-11 cells (*Online Supplementary Figure S24*). FLT3-ITD is the most common constitutively active mutation in AML patients, conferring poor prognosis, with high rates of relapse even with stem cell transplantation. In addition, because *SCUBE1* is a direct target gene of HOXA9 and because HOXA9 is dysregulated in a number of leukemic genetic alterations apart from *MLL*-translocations such as NUP98-fusion,[27] surface SCUBE1 might be an ideal therapeutic target for a broader spectrum of hematologic malignancies including FLT3-ITD AML and acute lymphoblastic leukemia. In summary, we discovered that pathological SCUBE1-mediated enhancement of FLT3 signaling is essential for leukemogenesis of MLL-fusion proteins. Using MLL-AF9 as a model, we provide the first *in vivo* evidence demonstrating a role for SCUBE1 in the initiation and maintenance of MLL-r leukemias. In addition, surface SCUBE1 may represent a novel therapeutic target for delivery of an ADC, which supports the translation of this approach into the clinic in patients with SCUBE1-expressing MLL-r leukemias (Figure 8).

Disclosures

No conflicts of interest to disclose.

Contributions

BKS and R-BY designed the research and analyzed data; BKS, Y-CL, C-FT, and W-JL performed experiments; C-CL, C-TY, C-YY, H-AH, W-CC, and H-FT analyzed AML patients' data; F-AL, KYM, SRR, and S-PW provided critical material; F-AL and L-HL analyzed proteomics results; and all authors approved the final version of the manuscript.

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Data-sharing statement

Data that support the findings of this study are available from the corresponding author upon reasonable request.

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