TGFβ-activated kinase-1 knockdown in hematopoietic stem-progenitor cells causes PANoptosis and myelodysplastic syndrome-like disease in mice

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Detailed experimental procedures.

Flow cytometric analysis of PANoptosis in patient BM samples. Freshly-isolated or frozen BM cells from MDS patients or healthy donors (HDs) were processed by density gradient centrifugation using Ficoll-Paque™ PLUS to remove debris and dead cells. The mononuclear cells (MNCs) were collected and washed with PBS containing 2% FBS and then fixed and Permeabilized with Cytofix/Cytoperm™ solution (BD Bioscience) on ice for 15 min. according to the manufacturer instructions. After washing with Perm/Wash™ buffer (BD Bioscience), cells were stained with rabbit anti-active CASP3, mouse anti-p-MLKL, or rabbit polyclonal anti-ASC

for 1 h. on ice. After washing with Perm/Wash™ buffer to remove supernatant, cells were incubated on ice for 30 min. with anti-Mouse IgG-Alexa Fluor™ 594 or anti-rabbit-IgG-AlexaFluor488. Cells were then washed again with Perm/Wash™ buffer followed by flow cytometric analysis. Flow data were gated with the FlowJo. For ASC specks, single cells were analyzed by ASC area *vs.* ASC width.[1, 2] Antibodies and reagents used in this experiment are listed in **Tables S2 and S3**.

ShRNA transduction,* in vitro *expansion and transplantation of BM CD117$^+$ HSPCs. Map3k7 Mouse shRNA plasmid (Locus ID 26409) was purchased from Origene. CD117$^+$ HSPCs from *WT* mice and *Rosa26^{CreErt}Casp8$^{fx/fx}$Ripk3$^{-/-}$* mice (30 days after *Casp8* deletion was induced by 5× tamoxifen injections) were isolated by MojoSort™ Mouse CD117 Selection Kit and were cultured (1×10^6/mL) in a serum albumin-free medium for murine HSC expansion at 37°C. in a humidified 5% CO_2 and 5% O_2 incubator. This medium is composed of F12 medium, 1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X, 100X), 1% Penicillin-Streptomycin-L-Glutamine Solution (P/S/G, 100X), 10mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 0.1% polyvinyl alcohol (PVA), 100ng/ml mouse TPO and 10ng/ml mouse SCF.[3] After two days of expansion, cells were infected with *sh-Tak1-tGFP*-expressing lentivirus or luciferase shRNA (shLuc)-tGFP-expressing lentivirus by 2 × spinoculation (2,000 × g. for 4 hours at 32°C. each time, with 24 hours between centrifugations) and were incubated in HSC expansion medium for three additional days. The transduced HSPCs were then purified by FACS for GFP$^+$ cells and were further incubated in the HSC expansion medium for 15-16 additional days with medium change every other day. The efficiency of *Tak1* knockdown was verified by RT-PCR and Western blotting. Cells were collected and used for either *in vitro* analysis or *in vivo* transplantation studies.

Transplantation of the transduced HSPCs and in vivo treatments. To study *Tak1^{KD}* in MDS development, the transduced HSPCs that were collected from the above cultures and transplanted into lethally-irradiated (9.5Gy) C57Bl6/J mice by tail-vein injections. Ten recipient mice were used in each group, 5×10^5 cells per recipient. To monitor mice for hematopoietic disease development, PB samples were collected monthly from all recipients and analyzed for WBC counts, platelet counts, RBC counts, Hb concentration and RBC MCV using a Hemavet 950FS (Drew Scientific). Four months post-transplantation, all mice were sacrificed and PB, spleens,

thymuses, and BM were collected for phenotypic analysis by flow cytometry as described previously.[4, 5] All of the fluorescent antibodies used in flow cytometric analyses were purchased from either eBioscience or Biolegend (See **Table S2**). To study the responses of *Tak1*^{KD} MDS to Ripk1 inhibitor treatment *in vivo*, we transplanted another batch of mice. Both male and female mice (1:1 ratio) were used as transplantation recipients. After moderate anemia developed in mice transplanted with *Tak1*^{KD} HSPCs (hemoglobin <10g/l), we randomly divided the mice into two groups with equal distribution of males and females in each group. One group was treated with 50mg/kg GNE684 (a murine Ripk1 inhibitor) twice daily p.o. for 30 days; the mice in the other group were treated with vehicle. Mice transplanted with *scr*-HSPCs were treated and studied in parallel as controls. All mice were euthanized for hematopoietic analysis one day after the last treatment.

To study the responses of *Tak1*^{KD} HSPCs to Tak1 inhibitor and cIAP inhibitor (or Smac-mimetic) treatment *in vivo*, in addition to *Tak1*^{KD} HSPCs and *scr*-HSPCs, we also cultured c-Kit^{+} cells in parallel without infection (non-transduced HSPCs). The *Tak1*^{KD} HSPCs were mixed with non-transduced HSPCs in a 1.5:1 ratio and transplanted into recipient mice. Each mouse received 3×10^5 *Tak1*^{KD} HSPCs and 2×10^5 non-transduced HSPCs. For *scr*-control groups, *scr*-HSPCs were mixed with non-transduced HSPCs in a 1:1 ratio, each mouse receiving 2.5×10^5 *Tak1*^{KD} HSPCs and 2.5×10^5 non-transduced HSPCs. Thirty days after transplantation, the percentages of GFP^{+} cells in PB were examined to determine the contributions of the transduced HSPCs. The mice were then randomly divided into three groups. Mice in groups 1, 2 and 3 were treated with 50mg/kg HS-276, 30mg/kg Birinapant, and vehicle i.p. every other day for 14 days, respectively. Percentages of GFP^{+} cells in PB and BM were examined on the indicated days.

Mouse phenotypic analysis. Peripheral blood was analyzed at indicated time points for WBC counts, plt, RBC counts, and Hb concentration by using Hemavet 950FS (Drew Scientific Inc.). Mice were sacrificed at the indicated time points to collect peripheral blood, spleens, and BM. For HSPC analysis, after lysis of RBCs, MNCs from PB, spleens, and BM were further stained with cell surface markers and analyzed by flow cytometry as described previously[6]. Cells were stained with antibodies directed against CD3ε, B220, Gr1(Ly-6G/Ly-6C), and CD11b to detect mature hematopoietic cell types. For HSC and HPC analysis, 1×10^6 BM MNCs were incubated with BV421-conjugated antibodies against lineage^{+} cells (including CD3ε, B220, Gr-1, CD11b, and

Ter-119), anti-Sca1-PE, anti-cKit-APC, anti-CD48-PECy7, anti-CD150-BV785, anti-CD135-eFluor710, anti-CD105-BV711, anti-CD34-PE-Dazzle594, and anti-CD16/32-PE-Cy7 antibodies. For analysis of murine EBs and reticulocytes from either mouse BM or cultured cells, the cells were pre-incubated with TruStain FcX™ PLUS (anti-mouse CD16/32) antibody (Biolegend, San Diego, CA, USA, cat# 156604) at a concentration of 0.25μg/10^6 cells in a volume of 100μL for 10 min. at a temperature of 2–8°C. to block non-specific binding. The cells were then stained with Ter119 and Syto16 (nuclei) for separating $Ter119^+Syto16^+$ EBs from $Ter119^+Syto16^-$ reticulocytes and mature RBCs[7] (**Figure S3B**). The differentiation stages of EBs were further analyzed by flow cytometry for plotting of CD71 vs. Ter119 with gating the population R1, R2, R3, and R4 for Pro-EBs ($CD71^{hi}Ter119^{lo}$), Baso-EBs ($CD71^{hi}Ter119^{hi}$), Poly-EBs ($CD71^{med}Ter119^{hi}$) and Ortho-EBs ($CD71^-Ter119^{hi}$), respectively (**Figure S3B**).[8, 9] The differentiation stages of EBs were also analyzed by flow cytometry for plotting of CD44 *vs.* FSC with gating the population R1, R2, R3, and R4 for Pro-EBs ($CD44^{hi}FSC^{hi}$), Baso-EBs ($CD44^+FSC^{hi}$), Poly-EBs ($CD44^{med}FSC^{med}$) and Ortho-EBs ($CD44^{lo}FSC^{lo}$), respectively (**Figure S3B**).[8, 9] Because we found that the data from these two EB analysis assays were comparable, we only presented the data from CD71 vs. Ter119 plotting in this paper. We acquired flow cytometric data using a FACS LSR Fortessa cytometer (BD Biosciences) and analyzed them using FlowJo software. Antibodies used in this experiment are listed in **Table S2**.

In vitro erythropoietic culture for murine HSPCs. The basic culture medium for both expansion of murine erythroid progenitors and differentiation of EBs is IMDM containing L-glutamine and 25mM HEPES (Gibco), 20% fetal calf serum (Hyclone), 200μg/ml holotransferrin (Sigma, St. Louis, MO), 10μg/ml recombinant human insulin (Sigma), 1% penicillin/streptomycin (ThermoFisher Scientific) and 10^{-4} M β-Mercaptoethanol (Sigma). For erythroid progenitor expansion, Lin^-Kit^+ BM cells (at a density of 10^5 cells/ml) were cultured in basic medium supplemented with 0.5U/ml Epo (Procrit, Amgen; 1 IU/ml = 1.2ng/ml), 100ng/ml SCF, 10ng/ml IL3 (Peprotech), 10μM dexamethasone (Sigma) and 100ng/ml IGF1 (R&D Systems) with medium change every day for six days. For EB differentiation, cells were collected on day 6 and then were transferred to basic medium supplemented with 0.5U/ml Epo (Amgen) at 5×10^5cells/mL for 3 days with or without Ripk1 inhibitor GNE684 treatment. The cells were collected indicated timepoints for Western blot analysis.

Primary Human Samples. BM biopsies and/or BM aspirates from MDS patients and other hematopoietic diseases were collected at Loyola University Medical Center's clinics (IRB# 205151081313), Hematological Biobank, Jiangsu Biobank of Clinical Resources, and Lanzhou University Secondary Hospital (SUDA20221214H01) after approval of the corresponding Institutional Review Boards and in accordance with the Declaration of Helsinki. The diagnosis of MDS was assigned according to World Health Organization criteria and the risk levels of diseases were stratified using Revised International Prognostic Scoring System (IPSS-R). Baseline BM biopsies and/or aspirates were collected from patients during diagnosis before the initiation of any treatment. Genetic mutations in the patient samples were determined by a targeted amplicon-based next-generation sequencing as a standard clinical procedure. The clinical characteristics of the patients are presented in **Table S4**. BM samples from HDs were obtained from the BM transplantation section at Loyola University Medical Center, USA, and the National Clinical Research Center for Hematologic Diseases in Suzhou, China. Written informed consent was obtained from all donors. For BM biopsies, the tissues were fixed with zinc formalin and processed for paraffin sections. For BM aspirations, cells were first diluted with an equal volume of PBS containing 0.5% BSA and were subsequently separated using the standard gradient separation approach with Ficoll-Paque PLUS (Catalog number #45-001-752, Thermo Fisher Scientific). The MNCs at the interface were collected and washed once at 300 × g. for 10 min. and twice at 200 × g. for 10 min. MNCs were cryopreserved and stored under liquid nitrogen until use. For $CD34^+$ cell enrichment, MNCs were incubated with the CD34 Microbead Kit followed by magnetic-activated cell sorting (MACS) (Catalog number #130-046-702, Miltenyi Biotec). The purity of isolated $CD34^+$ cells (95% to 98%) was verified by flow cytometry.

In vitro erythroid differentiation culture and treatment for human HSPCs. We used the 3-phase erythroid culture system[10] to study MDS erythropoiesis. The basic culture medium for *in vitro* culture of erythroid progenitors is IMDM plus 2% human PB, 3% human AB serum, 200μg/mL Holo-human transferrin, 10μg/mL heparin and 10μg/mL human insulin. In the first phase, $CD34^+$ HSPCs (1×10^5/mL) were incubated in basic medium containing 10ng/mL rhSCF, 1ng/mL rhIL-3 and 1.5IU/mL rhEPO × 6 days with medium change every 2 days to expand BFU-E erythroid progenitors and CFU-E erythroid precursors. In the second phase (days 7-11), IL-3

was removed from the medium to promote the generation of ProE. In the third phase (days 11-15), the cell concentration was adjusted to 10^6/mL on day 11 and cells were cultured in basic medium containing 3IU/mL rhEPO; the concentration of transferrin was adjusted to 1mg/mL to promote the differentiation of EBs.

To study whether RIPK1 inhibition can restore normal differentiation and promote survival of MDS erythroid progenitors, $CD34^+$ HSPCs from MDS patients and HDs were cultured in the 3-phase erythroid culture system as described above and treated with 100nM of the RIPK1 inhibitor GSK3145095 or vehicle. The inhibitor and vehicle were added during medium change every two days. The numbers of phenotypic BFU-E, CFU-E, Pro-EBs, and early-Baso-EBs were analyzed on day 7 of culturing by flow cytometry. The $Syto16^+$ EBs and $Syto16^-$ reticulocytes were analyzed on day 14 of culturing by flow cytometry for Syto16 nuclear staining within the glycophorin A^+ (GPA, $CD235a)^+$ population. The differentiation stages of $Syto16^+$ EBs, including late-Baso-EBs, Poly-EBs and Ortho-EBs, were further analyzed by flow cytometry-based CD105 and CD235a expression. Death of the cultured cells was analyzed on days 7 and 14 by Annexin-V/PI staining. Antibodies and reagents used in this experiment are listed in **Tables S2 and S3**.

In vitro culture and treatment of CD34$^+$ HSPCs. $CD34^+$ HSPCs were incubated in Soluplus-based 3a medium (1% Soluplus + 1μM 740Y-P + 0.1μM butyramide + 70nM UM171) at 37°C. with 5% CO_2 and 10% O_2 for *in vitro* expansion. Medium changes were made every two days by removing half of the medium and replacing it with pre-warmed and freshly-prepared medium.[11] To study the responses of mutant HPSCs to TAK1 inhibitor treatment, HS-276 (10μM final concentration) was added on days 2 and 4 of culturing during medium changes. Cells were collected on day 6 of culturing for targeted DNA-sequencing to detect mutational variant allele frequency (VAF) of the $SF3B1^{mut}$ and other MDS gene mutations.

Colony-forming cell (CFC) assay. BM MNCs from MDS patients and mice were isolated by density-based cell separation using Lymphoprep™. Human cells were seeded in MethoCult® H4434 classic medium at a concentration at 5×10^4/ml (for human) and were incubated at 37°C. in a humidified atmosphere with 5% CO_2. CFU-E colonies were counted on day 7 and BFU-E, CFU-G/M and CFU-GMEM colonies were counted on day 14 by investigators blinded to the experimental conditions. Murine cells were seeded into MethoCult GF M3434 methylcellulose

medium at a concentration of 1×10^4 cells/ml and were incubated at 37°C. in a humidified atmosphere with 5% CO_2. CFU-E colonies were counted on day 3, BFU-E and CFU-G/M were counted on day 7, while CFU-GMEM colonies were counted on day 12 by investigators blinded to the experimental conditions.

Western blotting. Cell lysates from BM MNCs were extracted using Cell Lysis Buffer (Cell Signaling) followed by a brief sonication. The supernatants were collected after centrifugation at 4°C., 14,000 × g. for 20 min. Protein samples were separated by SDS-PAGE on 10% or 12% acrylamide gels. Proteins were transferred onto nitrocellulose membranes to examine the target proteins by antibody blotting. Antibodies used in this study are listed in Antibodies for Western blotting (**Table 3**). The band quantifications using Multi Gauge 3.0 were normalized to corresponding controls, which were set to 1.00 after being normalized to corresponding loading standards on the Western blot.

Real-time RT-PCR analysis. Total RNA was isolated from HSCs and MPP, LSK, and LK cells using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. cDNA was generated from RNA using SuperScript III reverse transcriptase (Life Technologies). The levels of mRNA of the genes of interest were examined by qRT-PCR using the TaqMan assay (Thermo Fisher Scientific) following the instructions provided by the vendor. GAPDH was used as a control. The primers for qRT-PCR used in this study are listed in **Table S1**. The threshold cycle values (CT) for each reaction were determined and averaged using TaqMan SDS analysis software (Applied Biosystems). The changes in target gene expression were calculated by the comparative CT method (fold change = $2[-\Delta\Delta CT]$), as described previously. Each sample was a mixture of LSK HSCs from three mice of the same phenotype. Triplicate RT-PCRs were performed.

Statistical analysis. Data are expressed as the means ± SD. Two-way ANOVA (multiple groups) and Student's t test (two groups) were performed to determine the statistical significance of differences among and between experimental groups. $p < 0.05$ was considered significant. All analyses were done using GraphPad Prism from GraphPad Software (San Diego, CA). The results are presented as means ± standard error (SE) of the mean. An unpaired t-test with Welch correction

was used to determine statistically significant differences between the erythroid cells under normal and hypoxic conditions.

Table S1. Primers

Primer names	Primer sequences	Product sizes
TAK1 f1	5'-GGCTTTCATTGTGGAGGTAAGCTGAGA-3'	320bp for floxed allele
TAK1 r1	5'-GGAACCCGTGGATAAGTGCACTTGAAT-3'	280bp for WT allele
TAK1 f2	5'-GCAACTTCGACAACTTGCCTTCCTGTG-3'	
TAK1 r2	5'-GCACTTGAATTAGCGGCCGCAAGCTTATAACT-3'	1000bp for mutant allele
Cre1	5'-CTAGGCCACAGAATTGAAAGATCT-3'	
Cre2	5'-GTAGGTGGAAATTCTAGCATCATC C-3'	324 bp for Cre
Cre3	5'-GCGGTCTGGCAGTAAAAACTATC-3'	
Cre4	5'-GTGAAACAGCATTGCTGTCACTT-3'	100 bp for internal control
Map3k7	Mm01191861_m1	ThermoFisher Scientific Catalog # 4351372
Gapdh	Mm99999915_g1	ThermoFisher Scientific Catalog # 4331182

Table S2. List of antibodies used in this study

Antibodies	**Venders**	**Catalogue #**
Antibodies for analysis of murine HSCs and HPCs		
Brilliant Violet 421™ anti-mouse Ly-6G/Ly-6C (Gr-1) Antibody	Biolegend	108434
Brilliant Violet 421™ anti-mouse CD3ε Antibody	Biolegend	100336
Brilliant Violet 421™ anti-mouse B220 Antibody	Biolegend	103240
Brilliant Violet 421™ anti-mouse TER-119/Erythroid Cells Antibody	Biolegend	116233
PE anti-mouse Ly-6A/E (Sca-1) Antibody	Biolegend	108108
BV605-anti-mouse Ly-6A/E (Sca1)	BioLegend	108134
APC anti-mouse CD117 (c-Kit) Antibody	Biolegend	105812
APC/Cy7 anti-mouse CD48 Antibody	Biolegend	103431
PE/Cy7 anti-mouse CD150 (SLAM) Antibody	Biolegend	115914
PE/Cyanine5 anti-mouse CD105 Antibody	Biolegend	120428
APC/Cyanine7 anti-mouse CD105 Antibody	Biolegend	120432
PE/Cy5 anti-mouse CD135 Antibody	Biolegend	135312
PerCP/Cy5.5 anti-mouse CD127 (IL-7Rα) Antibody	Biolegend	135022
CD41a Monoclonal Antibody (eBioMWReg30 (MWReg30)), PerCP-eFluor 710	Life technologies	46-0411
PE/Cyanine7 anti-mouse CD41	BioLegend	133915
PE/Cyanine7 anti-mouse CD16/32	BioLegend	101318
BV510 anti-mouse CD16/32	BioLegend	101333
PE/Dazzle594 anti-mouse CD34	BioLegend	128616
PE-Cy7 anti-mouse CD3e	BD Bioscience	552774
PE anti-mouse CD11b	Thermo Fisher Scientific	12-0112-85
APC Anti-human/mouse CD45R	Thermo Fisher Scientific	17-0452-82
PE anti-mouse CD8a	Thermo Fisher Scientific	12-0081-82
APC Anti-mouse CD4	Thermo Fisher Scientific	17-0041- 83
FITC CD3e Monoclonal Antibody (145-2C11),	Thermo Fisher Scientific	11-0031-82
Pacific Blue™ anti-mouse TER-119	Biolegend	116232
BV510 anti-mouse TER119	BioLegend	116237
PE anti-mouse/human CD44 Antibody	BioLegend	103024
Anti-mouse CD44-BV650	BioLegend	103049
APC anti-mouse CD71 Antibody	BioLegend	113820
syto-16	ThermoFisher Scientific	S7578
Anti-human antibodies for HSC and erythroblast analysis		
PE/Cyanine7 anti-human CD123 Antibody	BioLegend	306010
Brilliant Violet 421™ anti-human CD235a (Glycophorin A) Antibody	BioLegend	349132
PE anti-human CD235a (Glycophorin A) Antibody	BioLegend	349106
APC anti-human CD34 Antibody	BioLegend	378606
BD Horizon™ V450 Mouse Anti-Human CD38	BD Biosciences	561378

APC anti-human CD41	BioLegend	984504
Brilliant Violet 510™ anti-human CD41 Antibody	BioLegend	303736
Alexa Fluor® 700 anti-human CD45RA Antibody	BioLegend	304120
APC/Cyanine7 anti-human CD71 Antibody	BioLegend	334110
BD Horizon™ PE-CF594 Mouse Anti-Human CD105	BD Biosciences	562380
APC anti-human CD105 Antibody	BioLegend	323208
PerCp-conjugated anti-CD3, anti-CD4, anti-CD14, and anti-CD19		
Antibodies for Western Blotting		
TAK1 (D94D7) Rabbit mAb (human and mouse)	Cell Signaling Technology	5206
RIP (D94C12) XP® Rabbit mAb (human and mouse)	Cell Signaling Technology	3493
Phospho-RIP (Ser166) (D8I3A) Rabbit mAb (human and mouse)	Cell Signaling Technology	96323
Gasdermin D (E9S1X) Rabbit mAb (human and mouse)	Cell Signaling Technology	39754
Caspase-8 (D35G2) Rabbit mAb (human and mouse)	Cell Signaling Technology	4790
Cleaved Caspase-1 (Asp296) (E2G2I) Rabbit mAb (mouse)	Cell Signaling Technology	89332
Cleaved Caspase-1 (Asp297) (D57A2) Rabbit mAb (human)	Cell Signaling Technology	4199
β-Actin (13E5) Rabbit mAb (human and mouse)	Cell Signaling Technology	4970
NF-κB p65 (D14E12) XP® Rabbit mAb (human and mouse)	Cell Signaling Technology	8242
Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb (human and mouse)	Cell Signaling Technology	#3033
TBK1/NAK (D1B4) Rabbit mAb (human and mouse)	Cell Signaling Technology	#3504
Phospho-TBK1/NAK (Ser172) (D52C2) XP® Rabbit (human and mouse)mAb	Cell Signaling Technology	#5483
p38 MAPK (D13E1) XP® Rabbit mAb (human and mouse)	Cell Signaling Technology	#8690
Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb (human and mouse)	Cell Signaling Technology	#4511
SAPK/JNK Antibody (human and mouse)	Cell Signaling Technology	#9252
Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb (human and mouse)	Cell Signaling Technology	#4668
Phospho-MLKL (Ser358) Polyclonal Antibody (human)	ThermoFisher Scientific	PA5-105678
Phospho-MLKL (Ser358) (D6H3V) Rabbit mAb #91689 (human)	Cell Signaling Technology	91689
Phospho-MLKL (Ser345) (D6E3G) Rabbit mAb (BSA and Azide Free) (mouse)	Cell Signaling Technology	17825
MLKL (E7V4W) Mouse mAb (BSA and Azide Free) (both human and mouse)	Cell Signaling Technology	44621
Antibodies for intracellular staining and immunofluorescence staining		
Cleaved Caspase-3 (Asp175) Antibody (Rabbit)	Cell Signaling Technology	9661
Human Phospho-MLKL (T357) Antibody (mouse) Phospho-MLKL (Ser358) Polyclonal Antibody (Rabbit)	R&D system ThermoFisher Scientific	MAB9187-100 PA5-105678
Anti-ASC Antibody, clone 2EI-7 (mouse)	Millipore Sigma	04-147
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	ThermoFisher Scientific	A-11001
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	ThermoFisher Scientific	A-11005
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	ThermoFisher Scientific	A-11008
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	ThermoFisher Scientific	A-11012
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647	ThermoFisher Scientific	A-21247
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568	ThermoFisher Scientific	A-11011

Table S3. Chemical reagents

Chemicals	Venders	Catalog No.
Small molecular inhibitors		
Birinapant (xIAP inhibitor or Smac mimetic)	Medchemexpress	HY-16591
GNE684 (murine RIPK1 inhibitor)	Medchemexpress	HY-128585
GSK3145095 (Human RIPK1 inhibitor)	Medchemexpress	HY-111946
HS-276 (TAK1 inhibitor)	Medchemexpress	HY-147141E1155
Necrostatin 1S (Nec-1S)	Medchemexpress	HY-14622A
VX-765 (Belnacasan) Caspase-1/4 inhibitor	Selleckchem	No.S2228
Z-VAD-FMK (Pan-caspase)	Selleckchem	No.S7023
Z-IETD-FMK (Caspase-8)	Selleckchem	S7314
CA-074 Me (cathepsin B)	Medchemexpress	HY-103350
Cell culture reagents		
Luciferase shRNA (shLuc)-tGFP lentiviral plasmid	Origene	TR30023
Map3k7 Mouse shRNA Plasmid (Locus ID 26409)	Origene	TL516837
1× Ham's F-12 Nutrient Mix liquid media	Life Technologies (Gibco)	
Insulin-Transferrin-Selenium-Ethanolamine (ITS-X, 100X)	Thermo Fisher Scientific	51500-056
Penicillin-Streptomycin-L-Glutamine Solution (P/S/G, 100X)	Thermo Fisher Scientific	10378016
N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES)	Thermo Fisher Scientific Gibco	
Polyvinyl alcohol (PVA)	Sigma	P8136
Mouse TPO (Thrombopoietin) Recombinant Protein, PeproTech® (rmTPO)	Thermo Fisher Scientific Gibco	315-14
Mouse SCF Recombinant Protein, PeproTech® (rmSCF)	Thermo Fisher ScientificGibco	250-03
IMDM (Iscove's Modified Dulbecco's Medium)	Life Technologies (Gibco)	12440-053
100× Insulin-Transferrin-Selenium-ethanolamine (ITS-X)	Life Technologies (Gibco)	51500-056
100× Penicillin-Streptomycin-Glutamine (P/S/G)		10378-016

740Y-P	InvivoChem	V2540
Butyzamide	InvivoChem	V51526
UM171	InvivoChem	V1894
PCL-PVAc-PEG (Soluplus®)	BASF Pharma	402932-23-4
Human PB plasma	Stem Cell Technologies	70039.3
Human AB serum	Sigma-Aldrich	H4522
Holo-human transferrin	Sigma-Aldrich	T4132
heparin	Stem Cell Technologies	7980
human insulin	Sigma-Aldrich	I9278
rhSCF	PeproTech	300-07
rhIL-3	PeproTech	200-03
rhEPO	PeproTech	100-64
MojoSort™ Mouse CD117 (c-Kit) Selection Kit	Biolegend	480146
MethoCult® GF H4434 classic medium	Stem Cell Technologies, Vancouver, BC	04434
MethoCult® GF M3434 medium	Stem Cell Technologies, Vancouver, BC	03434
Reagents for immunofluorescence staining		
Citrate Buffer (pH 6.0), Concentrate	ThermoFisher Scientific	005000
eBioscience™ IHC Antigen Retrieval Solution - Low pH (10X)	ThermoFisher Scientific	00-4955-58
eBioscience™ IHC Antigen Retrieval Solution - High pH (10X)	ThermoFisher Scientific	00-4956-58
Universal antigen retrieval buffer	BiCell Scientific	BCASUB
SlowFade™ Diamond Antifade Mountant with DAPI	ThermoFisher Scientific	S36964
10X Blocking Buffer	abcom	ab126587

Table S4. Patient and control samples used for flow cytometry, immunofluorescent staining, and Western Blotting analysis of PANoptosis

Patients	Gender	Age (Y)	Mutant genes (VAF)	Pathologic Diagnosis	Experiments
P1	Male	57	*SF3B1* (42%) *TET2* (41%)	MDS-RS-MLD	Flow cytometry
P2	Male	71	*SF3B1* (40%)	MDS-RS-SLD	Flow cytometry
P3	Female	64	*SF3B1* (36%) *DNMT3A* (39%)	MDS-RS-MLD	Flow cytometry
P4	Male	72	*SF3B1* (33%)	MDS-RS-SLD	Flow cytometry
P5	Female	58	*SF3B1* (35%) *TP53* (6%)	MDS-RS-MLD	Flow cytometry
P6	Male	66	*SF3B1* (28%) ASXL1 (16%)	MDS-RS-MLD	Flow cytometry
P7	Male	75	*SF3B1* (38%)	MDS-RS-SLD	Flow cytometry
P8	Female	61	*SF3B1* (35%) *TET2* (37%)	MDS-EB1	Flow cytometry
P9	Female	55	*SF3B1* (31%) *STAG2* (11%)	MDS-EB1	Flow cytometry
P10	Male	71	*SF3B1* (39%)	MDS-RS-SLD	Flow cytometry
P11	Male	69	*SF3B1* (33%) *JAK2* (14%)	MDS-RS-MLD	Flow cytometry
P12	Male	75	*SF3B1* (44%) *TET2* (24%)	MDS-RS-MLD	Flow cytometry
P13	Male	78	*SF3B1* (37%)	MDS-RS-SLD	Flow cytometry
P14	Female	69	*SF3B1* (34%) ASXL1 (13%)	MDS-RS-MLD	Flow cytometry
P15	Female	59	*SF3B1* (36%) *BCOR* (11%)	MDS-RS-MLD	Flow cytometry
P16	Female	72	*SRSF2* (24%) *TET2* (31%) *CBL* (5%)	MDS-EB1	Flow cytometry
P17	Male	67	*SRSF2* (27%) *TET2* (31%)	MDS-MLD	Flow cytometry
P18	Female	65	*SRSF2* (23%) *TET2* (34%) *ASXL1* (15%)	MDS-EB2	Flow cytometry
P19	Female	65	*SRSF2* (35%) *NRAS* (6%)	MDS-MLD	Flow cytometry
P20	Male	54	*SRSF2* (32%) *ASXL1* (14%)	MDS-MLD	Flow cytometry
P21	Male	67	*SRSF2* (26%) *EZH2* (17%)	MDS-EB1	Flow cytometry
P22	Female	70	*TET2* (24%)	MDS-MLD	Flow cytometry
P23	Male	62	*TET2* (31%) *DNMT3A* (18%)	MDS-MLD	Flow cytometry
P24	Male	71	*TET2* (34%) *ASXL1*(15%)	MDS-MLD	Flow cytometry
P25	Female	60	*DNMT3A* (21%) *TP53* (13%)	MDS-EB1	Flow cytometry
P26	Male	71	*SF3B1* (35%) *ASXL1* (12%)	MDS-RS-MLD	IF staining *
P27	Male	68	*SF3B1* (21%) *DNMT3A* (31%)	MDS-RS-SLD	IF staining
P28	Female	59	*SSF3B1* (39%)	MDS-RS-SLD	IF staining
P29	Male	72	*SF3B1* (33%)	MDS-RS-SLD	IF staining
P30	Female	66	*SF3B1 (25%) TET2* (34%)	MDS-RS-MLD	IF staining
P31	Male	73	*SSF3B1* (41%)	MDS-RS-SLD	Western Blotting
P32	Male	64	*SF3B1* (38%)	MDS-RS-SLD	Western Blotting
P33	Female	71	*SF3B1 (35%) BCOR* (21%)	MDS-RS-MLD	Western Blotting
C1	Female	63		ID anemia [&]	Flow cytometry
C2	Female	57		ID anemia	Flow cytometry
C3	Male	68		FAD anemia [#]	Flow cytometry
C4	Female	62		ID anemia	Flow cytometry
C5	Male	69		FAD anemia	Flow cytometry

C6	Male	65		FAD anemia	IF staining
C7	Female	57		ID anemia	IF staining
C8	Female	72		FAD anemia	IF staining
C9	Female	73		ID anemia	IF staining
C10	Male	65		FAD anemia	IF staining
C11	Male	56		ID anemia	Western Blotting
C12	Female	61		FAD anemia	Western Blotting
C13	Female	55		FAD anemia	Western Blotting

*Immunofluorescence staining: IF; #Folic acid deficiency anemia: FAD anemia; &Iron-deficiency anemia: ID anemia.

Table S5. Patient and control samples used for *in vitro* culture study

Patients	Gender	Age (Y)	Mutant genes (VAF)	Pathologic Diagnosis
P34	Male	63	*SF3B1* (26%) *TET2* (34%)	MDS-RS-MLD
P35	Male	68	*SF3B1* (33%)	MDS-RS-SLD
P36	Male	66	*SF3B1* (24%) *DNMT3A* (30.5%)	MDS-RS-SLD
P37	Female	59	*SF3B1* (22%)	MDS-RS-SLD
P38	Male	68	*SF3B1* (34%) *TP53* (4%)	MDS-RS-MLD
C14	Male	61		FAD anemia
C15	Female	53		FAD anemia
C16	Female	65		ID anemia
C17	Mail	48		ID anemia
C18	Female	46		FAD anemia

Supplementary data.

Figure S1. Down-regulation of TAK1 protein in CD34$^+$ HSPCs from BM of *SF3B1^{mut}* MDS patients (associated with Figure 1). CD34$^+$ HSPCs were enriched from BM of 2 *SF3B1^{mut}* MDS patients and 2 age-matched healthy donors (HD). TAK1 protein was examined by Western blotting.

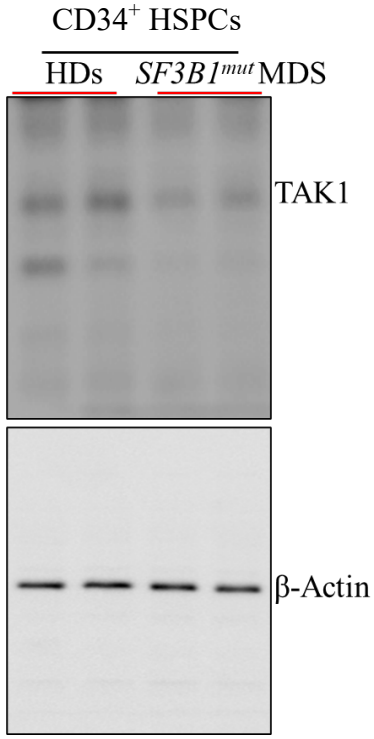


Figure S2. *Tak1*$^{-/-}$ induces PANoptosis and lysosome death in HSPCs (associated with Figure 2). c-Kit^{+} HSPCs from *Tak1*$^{fx/fx}$ *HSPCs* and *Tak1*$^{fx/fx}$*Casp8*$^{-/-}$*Ripk3*$^{-/-}$ mice were transduced with *Cre-GFP or vector-GFP*. One day post-transduction, after determining the transduction efficiency (GFP^{+} cells %), cells were treated with 20μM of pan-Casp inhibitor (Z-VAD) or 20μM of cathepsin B inhibitor (CA-074Me) daily. The relative rate of PCD of the *Tak1*^{KD} HSPCs was evaluated by detecting the GFP^{+}% every 2 days. Vehicle treatments (veh) were studied in parallel as controls. Triplicate experiments were conducted. ** indicates $p<0.01$.

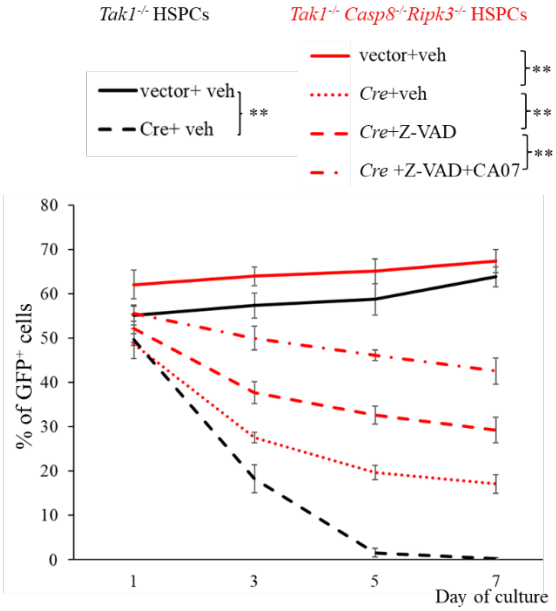


Figure S3. Gating strategies for flow cytometric analysis of HSPCs and EBs (associated with Figure 2-4). a-b. Gating strategy for flow cytometric analysis of BM HSPCs[12] (**a**) and EBs (**b**). HSCs: hematopoietic stem cells; MPP: multipotent progenitor; pre-GM: pre-granulocyte-macrophage progenitor; MkP: megakaryocytic progenitor; GMP: granulocyte-monocyte progenitor; CFU-E: colony forming unit-erythroid; EB: erythroblast; Ret: reticulocyte; RBC: red blood cells; R1: proerythroblast; R2: basophilic erythroblast; R3: polychromatophilic erythroblast, and R4: orthochromatic erythroblast. Syto16 and Ter119 staining was used to separate EBs from RBCs.[7] Two assays were used in analyzing differentiation stages of EBs in **b**.[8, 9] We found the data from two assays were comparable.

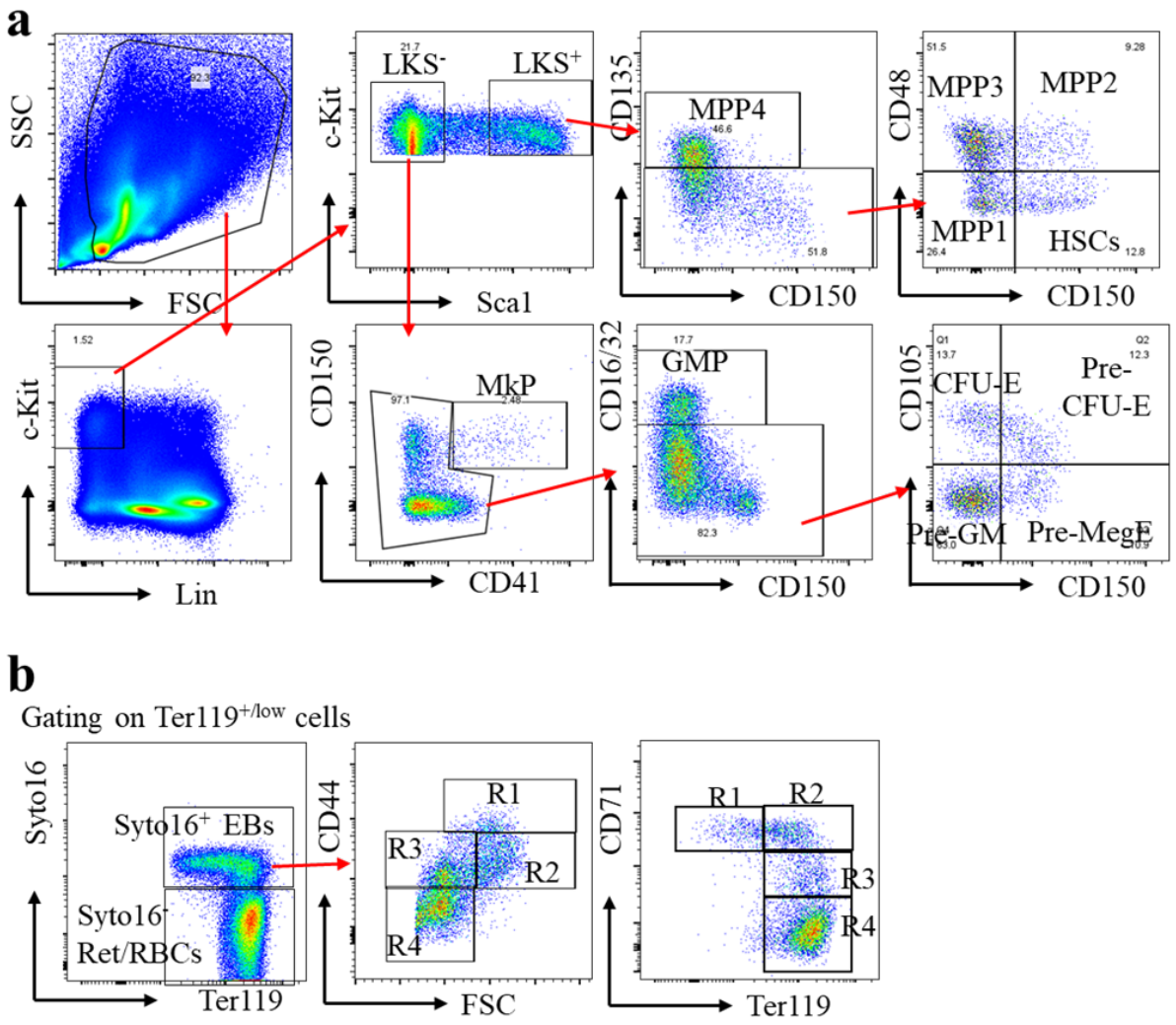


Figure S4. ***Tak1*^{KD} induces PANoptosis in HSPCs without lysosome death (associated with Figure 2).** **a**. c-Kit$^+$ HSPCs from *WT* mice were transduced with 3 shRNA for *Tak1*, respectively. Cells were collected on day two of transduction and PCD was examined by flow cytometry for Annexin V/PI staining as well as ASC speck, pMlkl and a-Casp3 staining. Scrambled shRNA (Scr) transduction was studied in parallel as a control. Triplicate experiments were conducted. ** indicates p<0.01. ns stands for not significant. **b**. c-Kit$^+$ HSPCs from *WT* and *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ mice were transduced with *sh3Tak1* or *Scr*. One day post-transduction, after determining the transduction efficiency (GFP$^+$ cells %), cells were treated with 20μM pan-Casp inhibitor (Z-VAD) or 20μM Ripk1 inhibitor (Nec1s) daily. The relative rate of PCD of the *Tak1*^{KD} HSPCs was evaluated by detecting GFP$^+$% every 2 days. Vehicle treatments (veh) were studied in parallel as controls. Triplicate experiments were conducted. ** indicates p<0.01. **c**. c-Kit$^+$ HSPCs from *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ mice were transduced with *sh2Tak1, sh3Tak1* or *Scr*. One day post-transduction, after determining the transduction efficiency (GFP$^+$ cells %), cells were treated with 20μM Z-VAD, 20μM Z-VAD + 20μM cathepsin B inhibitor CA-074Me, or 20μM CASP1/4 inhibitor VX-765. Triplicate experiments were conducted. ns indicates no-significance.

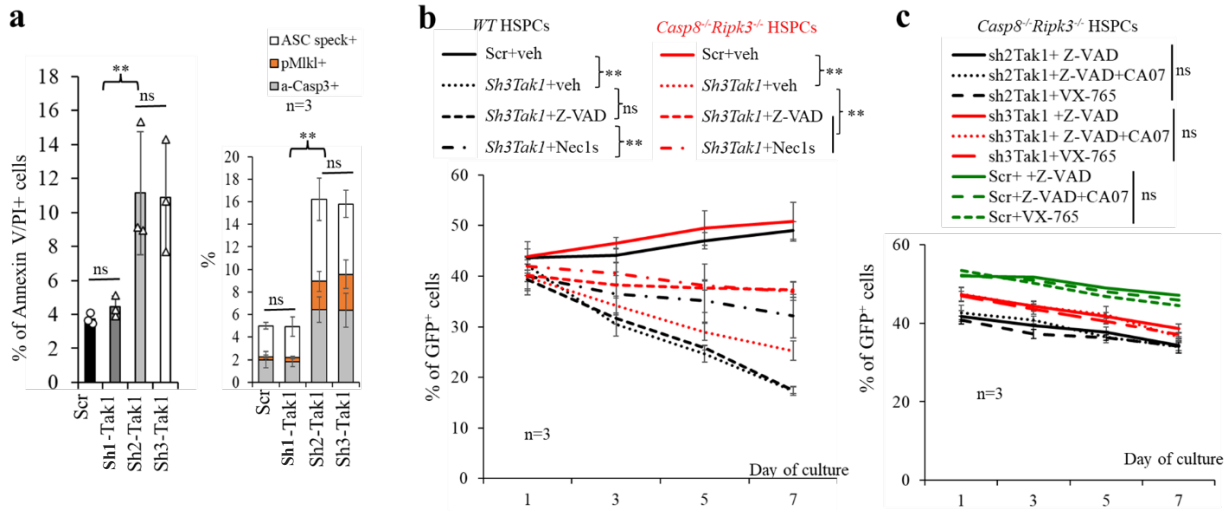


Figure S5. Mice transplanted with *Tak1*^{KD} HSPCs developed MDS-like diseases (associated with Figure 3). **a**. c-Kit$^+$ HSPCs were transduced with *Scr* or *sh3Tak1* and then expanded in HSC expansion medium for 16 days[3]. The phenotype of expanded HSCs was analyzed by flow cytometry. **b-e**. Four months post-transplantation of *Scr*-HSPCs and *Tak1*^{KD} HSPCs, PB and BM were collected from the recipient mice and analyzed by flow cytometry for neutrophils, monocytes and CD3$^+$/B220$^+$ lymphocytes in PB (**b**), GFP$^+$ cell% in PB WBCs and BM MNCs (**c**) as well as for GFP$^+$ cell% in BM Lin$^-$c-Kit$^+$ HSPCs (**d**), as well as the numbers of MPP1, MPP2, MPP3, Pre-GM, GMP and MkP in BM (**e**).

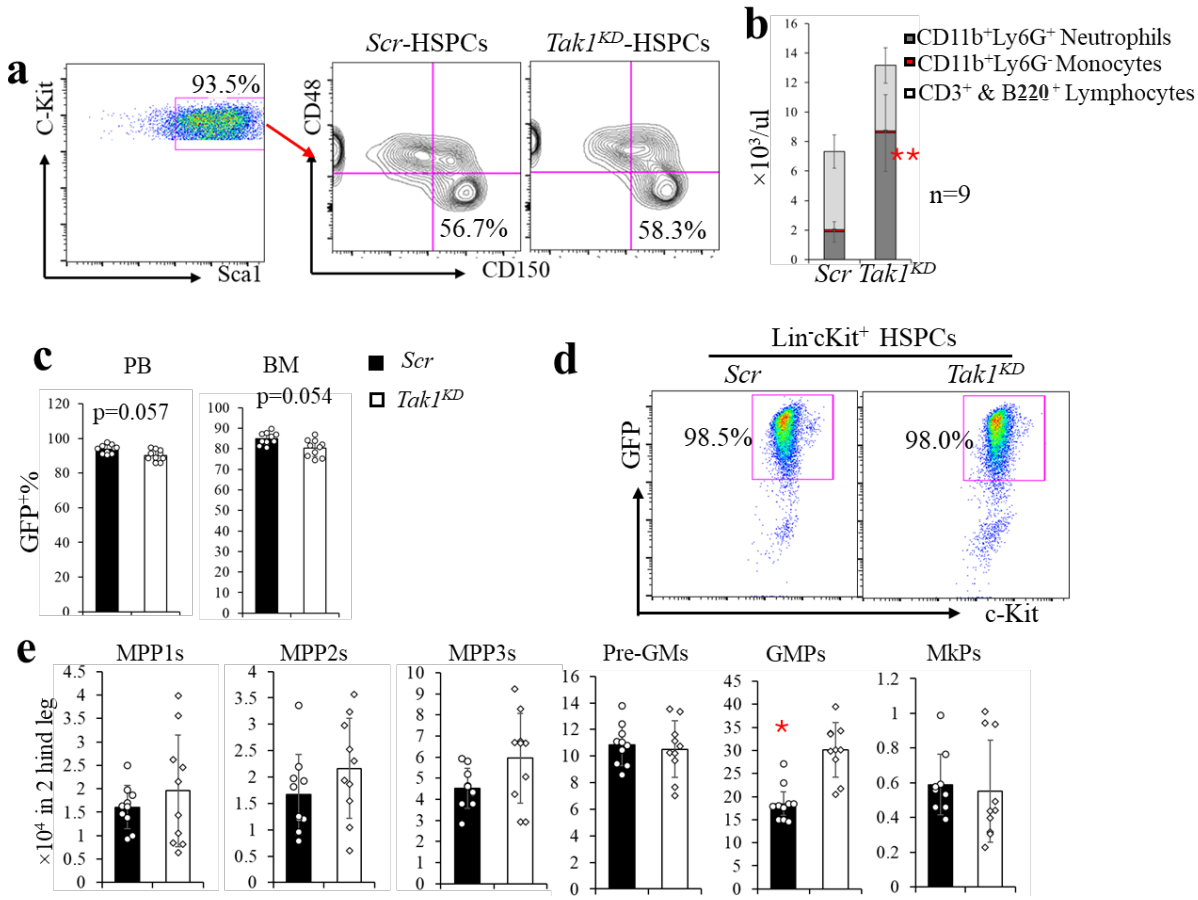


Figure S6. Ripk1 inhibition restores normal BM hematopoiesis and blood cell counts to anemic *Tak1*^{KD} mice (associated with Figure 4). a-d. After anemia developed (Hb<10g/dl) in mice transplanted with *Tak1*^{KD} HSPCs, the mice were randomly separated into two groups and treated with vehicle or GNE684, respectively, for one month. scr-HSPC transplantations were treated and studied in parallel as controls. PB WBC counts, RBC counts and MCV were analyzed on one day before the treatment (0) as well as at 1 and 2 weeks during the treatment (**a**). One day after the last treatment, mice were sacrificed for hematopoietic analysis. GFP^{+} cell% in PB WBCs and BM MNCs (**b**), as well as the number of MNCs (**c**) and MPP4 (**d**) in BM were analyzed by flow cytometry. * Indicates p<0.05. ns indicates no significance. **e-g**. *Tak1*^{KD} and *scr*-HSPCs were cultured in a two-phase *in vitro* erythropoiesis system. After 6 days of growth in expansion medium, the cells were transferred into differentiation medium for further incubation without (**e and f**) or with 30μM Nec1s, or 20μM ZIETD (CASP8 inhibitor)/GSK-872 (RIPK3 inhibitor) treatment (**g**). Cells were collected at 12, 24 and 48 hours of culturing for Gata1 protein levels by Western blotting (**e** and **g**), or at 24 hours of culturing for EB analysis by flow cytometry (**f**). **h**. *Tak1*^{KD} and *scr*-HSPCs were cultured in HSPC expansion medium with or without 30μM Nec1s or 5μM GSK8612 (TBK1 inhibitor) treatment. Cells were collected after 24 hours of culturing and p-NFκB and NFκB protein levels were compared by Western blotting.

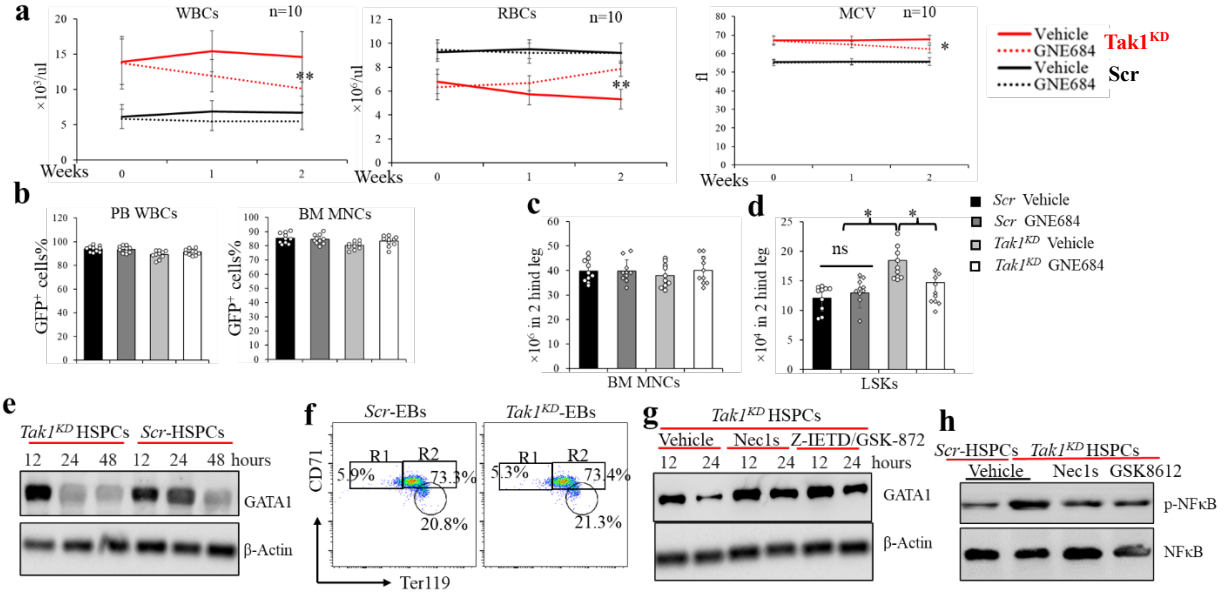


Figure S7. ***Tak1 inhibitor or cIAP inhibitor treatment selectively kills*** **Tak1^{KD}** ***HSPCs in vivo*** **(associated with Figure 5d-e)**. *sh2Tak1*-GFP-transduced HSPCs (*Tak^{KD}*) were mixed with non-transduced HSPCs (GFP$^-$) in a 1.5:1 ratio, whereas *scr*-GFP-transduced HSPCs were mixed with non-transduced HSPCs (GFP$^-$) in a 1:1 ratio. The mixed HSPCs were transplanted into lethally-irradiated mice. One-month post-transplantation, after determining the engraftment of the transduced HSPCs (GFP$^+$ cells in PB), mice were divided into three groups and treated with vehicle, HS-276 or birinapant, respectively, for two weeks. The mice were terminated 2 months later for PB analysis using Hemavet (**a**), and for examining GFP$^+$ cells within BM Lin$^-$c-Kit$^+$ HSPCs by flow cytometric analysis (**b**).

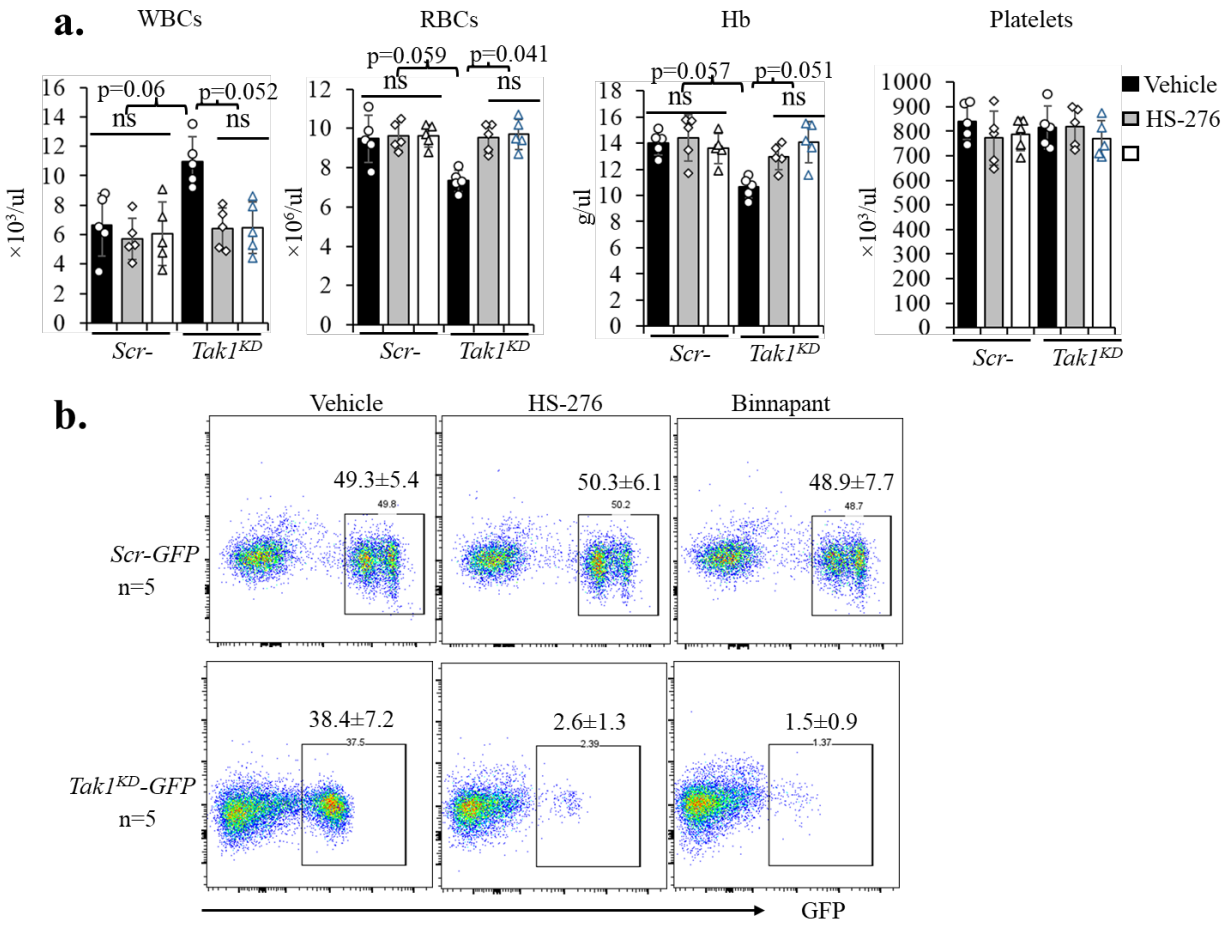


Figure S8. ***Inhibition of RIPK1 in human SF3B1^{mut} MDS samples*** **(associated with Figure 6b-g).** CD34^{+} HSPCs from HDs or MDS patients were cultured in 3-phase erythroid culture system with or without 100nM GSK3145095 treatment. Cells were collected on days 7 and 14 of culturing; BFU-E, CFU-E, Pro-EBs and early Baso-EBs were analyzed by flow cytometry on day 7 (**a**); late Baso-EBs, poly-EBs and ortho-EBs (**a**) and reticulocytes (**b**) were analyzed by flow cytometry on day 14. Representative flow cytometric data for analysis of erythroid precursors and EBs on indicated days of culture.[13]

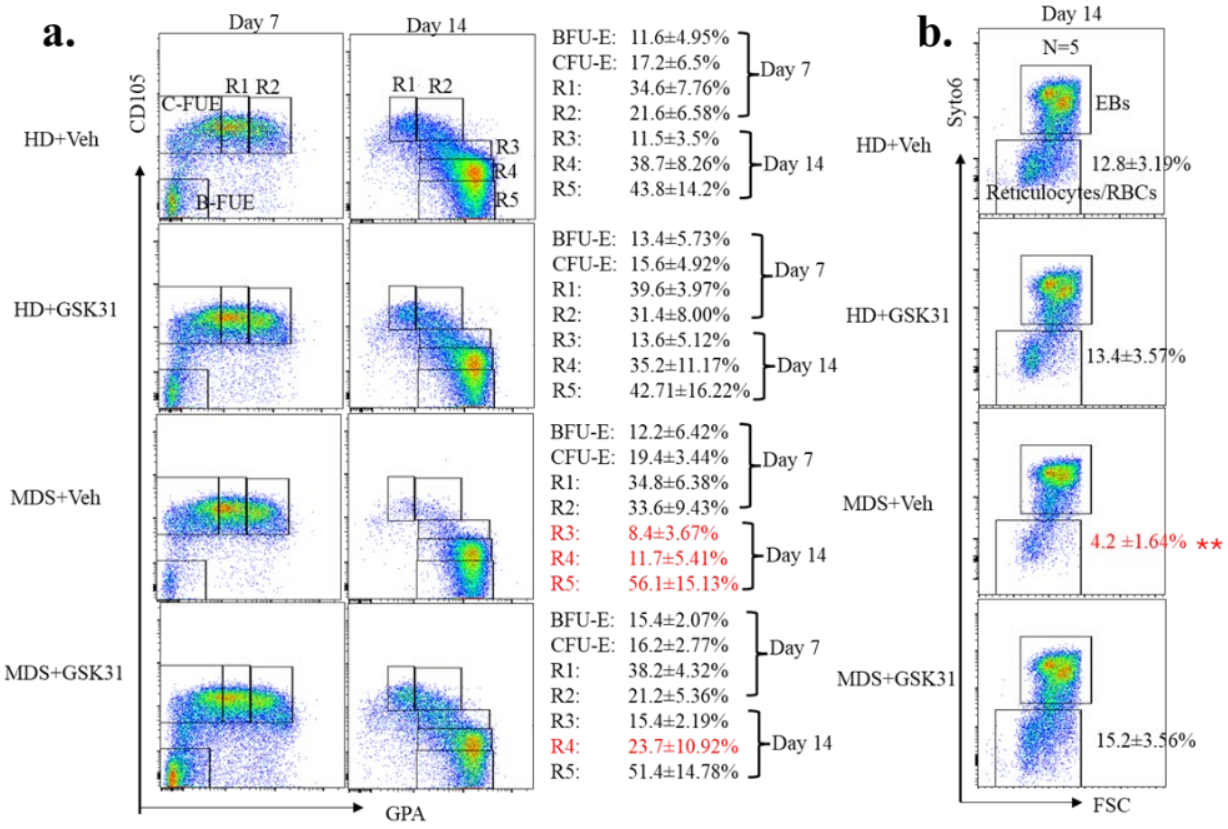
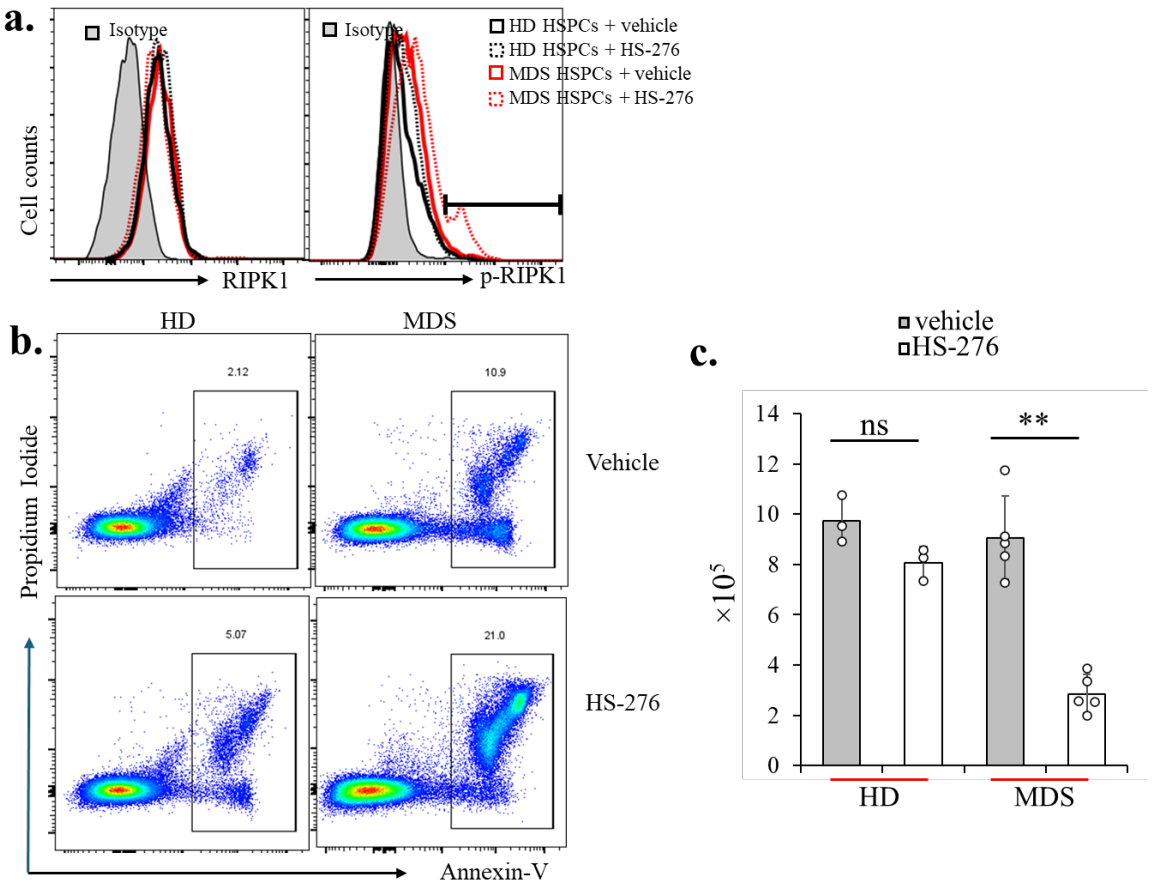


Figure S9. ***Inhibition of TAK1 in human SF3B1^{mut} MDS samples*** **(associated with Figure 6h-k).** CD34^{+} HSPCs from HDs or MDS patients were cultured in HSPC culture system. On day 2 of culturing, cells were treated with TAK1 inhibitor HS-276 or vehicle, respectively. **a.** Cells were collected on day 3 of culturing for intracellular anti-RIPK1 and anti-p-RIPK1 antibody staining followed by flow cytometric analysis for RIPK1 and p-RIPK1 protein levels. Representative flow cytometric data for analysis of RIPK1 and p-RIPK1 protein levels and cell death in HSPCs after HS-276 treatment. **b-c.** Cells were collected on day 4 of treatment for cell death analysis by Annexin-V and propidium iodide staining (**b**) and live cell counts (**c**). Isotype antibody staining was studied in parallel as a control in **a**.



Original Western blotting data.

Figure 1e.

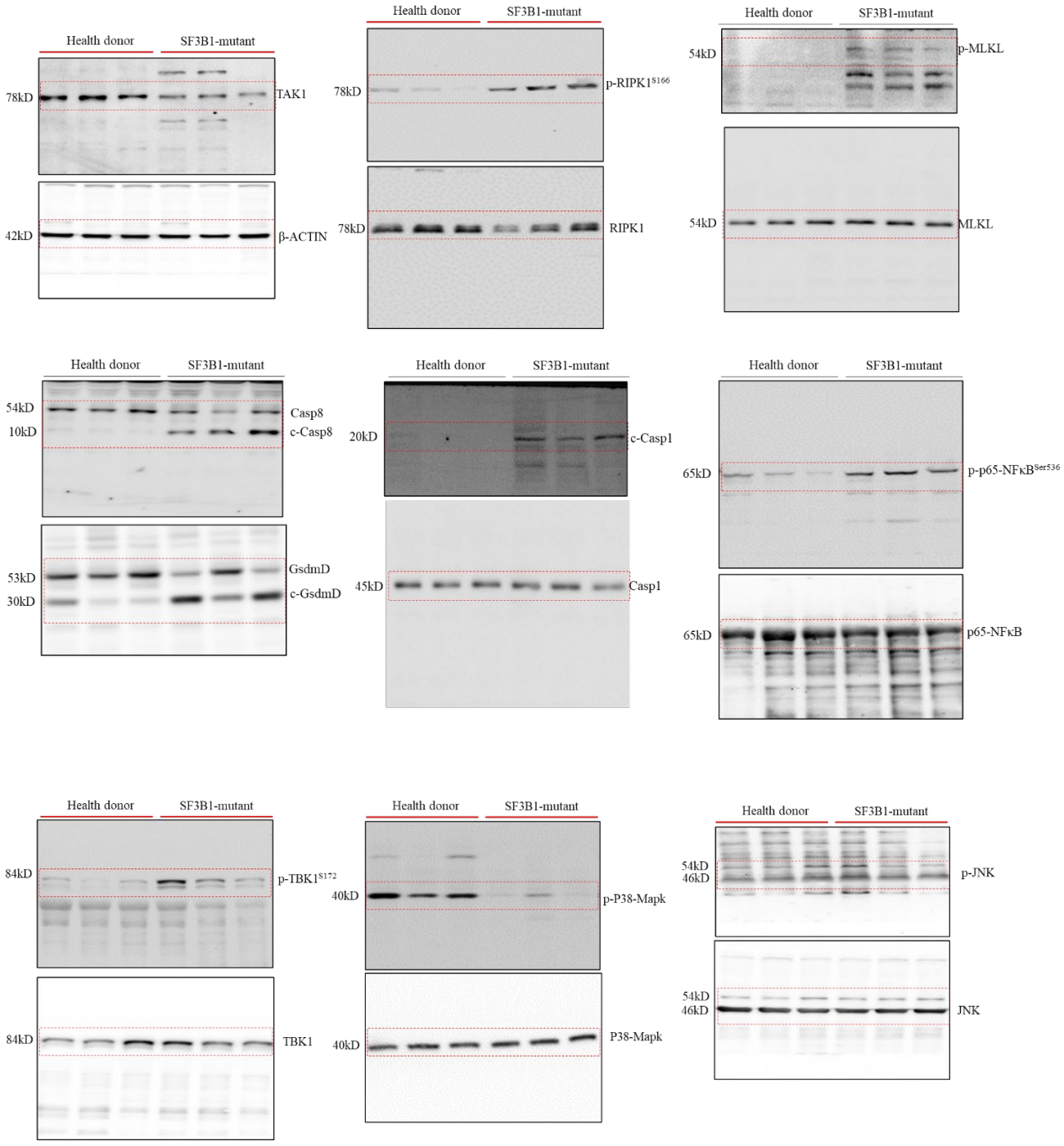


Figure 2d, f, and g.

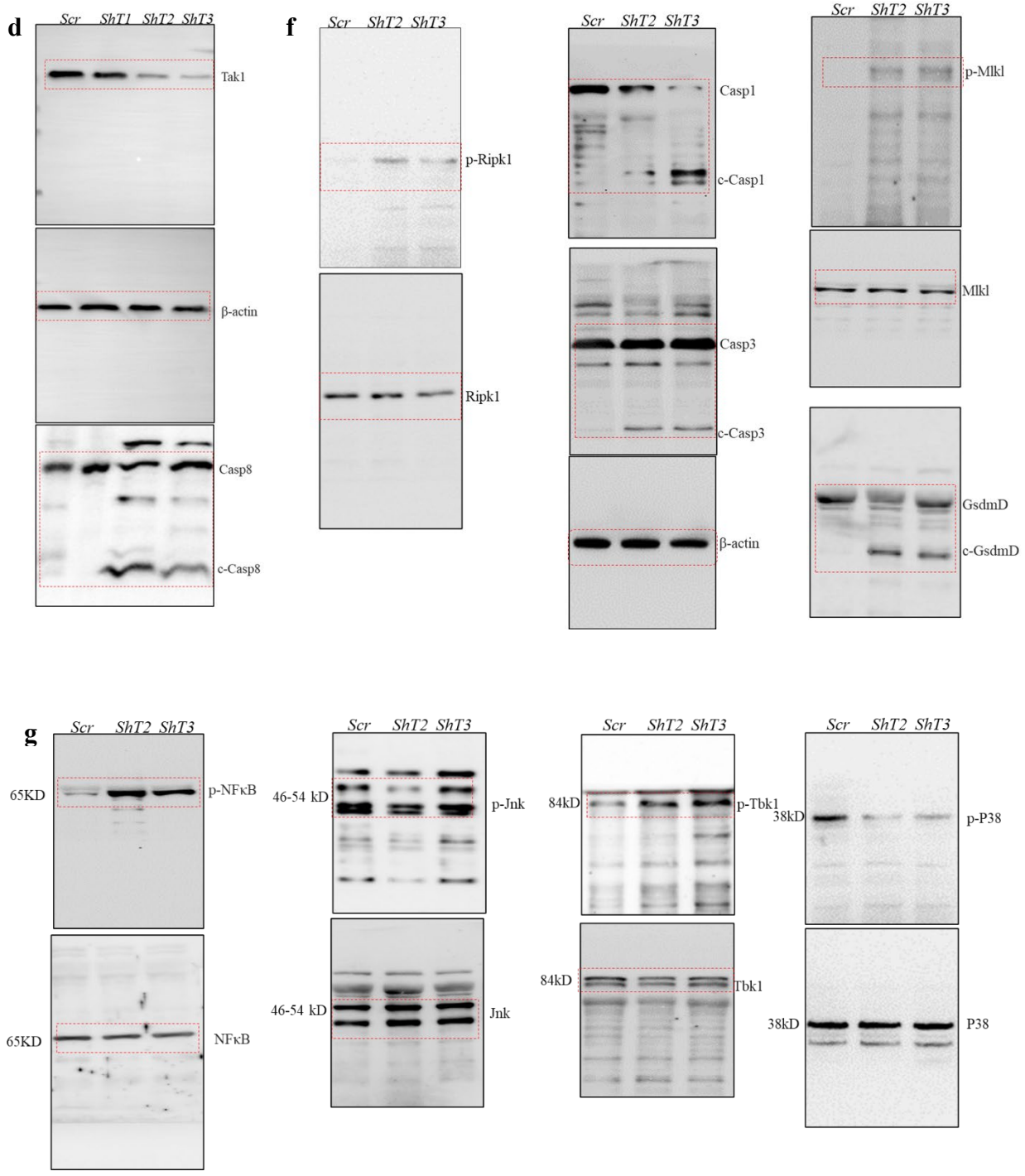
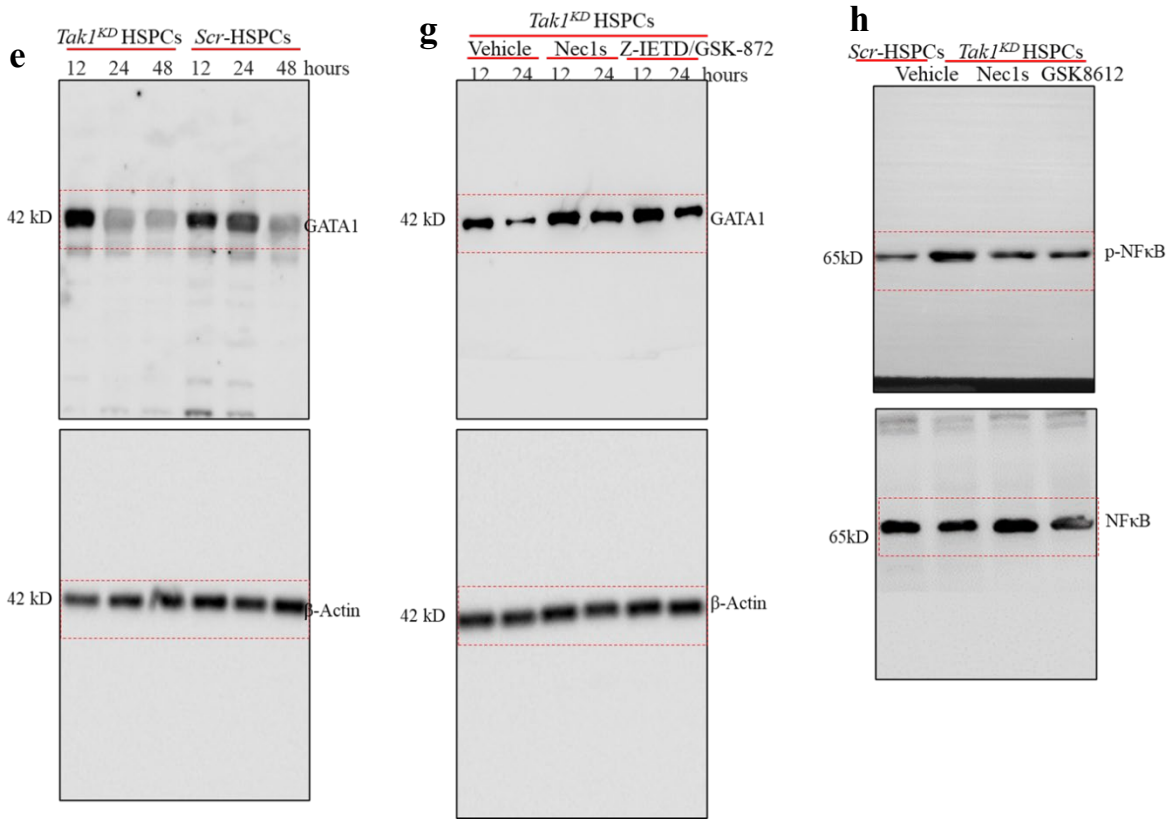


Figure S6e, g, and h.



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