Deficiency of the ribosome biogenesis gene Sbds in hematopoietic stem and progenitor cells causes neutropenia in mice by attenuating lineage progression in myelocytes

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Supplementary methods, tables and figures

Supplemental methods

Peripheral Blood Measurements

Peripheral blood was collected by submandibular bleeding in K2EDTA-coated microtainers (BD). Hematological parameters were analyzed using a Vet ABC counter (Scil Animal Care).

Flow Cytometry

Bone marrow cells were isolated as previously reported.[1] Red blood cells (RBC) from bone marrow and fetal liver were lysed with ACK lysing buffer (Lonza) before FACS staining. Peripheral blood cells were first stained for surface markers and next RBC-depleted using IOTest 3 Lysing Solution (Beckman Coulter). Bone marrow, blood and fetal liver cells were stained in PBS+0.5%FCS for 20 min on ice. To identify bone marrow lineage positivity (Lin^+), cells were co-stained with biotin-labelled antibodies against Gr1 (RB6-8C5), Mac1 (M1/70), Ter119 (TER-119), CD3e (145-2C11), CD4 (GK1.5), CD8 (53-6.7) and B220 (RA3-6B2) (all from BD), followed by incubation with Pacific Orange-conjugated streptavidin (Life Technologies). For fetal liver analysis, anti-Mac1 was excluded from the lineage cocktail as this marker is expressed in fetal hematopoietic stem cells.[2] In addition to the lineage cocktail, the following antibodies were used to identify HSPCs: Pacific Blue anti-Sca1 (D7), AF700 or PE anti-CD48 (HM48-1), PE-Cy7 anti-CD150 (TC15-12F12.2), PE anti-CD34 (HM34) (all from Biolegend), APC or PE-CF594 anti-c-Kit (2B8) and APC-Cy7 anti-CD16/32 (2.4G2) (all from BD). To analyze differentiated cells, we used APC anti-Gr1 (RB6-8C5), PE-Cy7 anti-Mac1 (M1/70) (both from Biolegend) and eFluor450 anti-B220 (RA3-6B2, eBioscience). Erythroid subsets were identified by staining with PE anti-CD71 (C2, BD) and Ter119 (TER-119, Biolegend). Dead cells were excluded based on 7-AAD staining (Biolegend). Apoptotic cells were identified with APC annexin V (BD) according to the manufacturer's protocol. For cell cycle and p53 analysis, cells were stained for surface markers, then permeabilized using Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD) following the

manufacturer's recommendations and finally stained with PE anti-Ki67 (B56, BD) and 7-AAD (cell cycle analysis) or with AF647 anti-p53 (1C12, Cell Signaling Technology). For all FACS analysis, events were recorded using a BD LSR II Flow Cytometer and analyzed with FlowJo 7.6.5 software (Tree Star). Cells were sorted with a BD FACSAria III.

Bone Marrow Morphology

For morphological studies, cytospin preparations were obtained from $5x10^5$ bone marrow cells per transplanted mouse and stained with May-Grünwald-Giemsa as previously reported.[3]

Quantitative PCR

Cells were sorted in TRIzol Reagent (Life Technologies) and RNA was extracted following the manufacturer's instructions, with addition of 25 µg linear polyacrylamide (Genelute LPA; Sigma Aldrich) as RNA carrier. Genomic DNA was eliminated by treatment with RQ1 RNase-free DNase (Promega). First strand cDNA was synthesized from poly(A)$^+$-selected RNA using SuperScript III First-Strand Synthesis System (Life Technologies). Real-Time PCR reactions were prepared with Fast SYBR Green Master Mix (Life Technologies) using the primers listed in Online Supplementary Table S1 and run on a 7500 Fast Real-Time PCR System (Life Technologies).

Table S1. Oligonucleotide primers used in this study.

Application	Target	Allele	Primer ID	Sequence	Amplicon size, bp
Genotyping	*Sbds*	Wild type	a b	CCAGGGTCACGTTAATACAAACC TGAGTTTCAATCCTCAGCATCC	329
		Floxed	a b	CCAGGGTCACGTTAATACAAACC TGAGTTTCAATCCTCAGCATCC	450
		Recombined	c d	TAAAACAAAGCTGCGGTCAAGA ATCCTCAGCATCCCGAACAA	319
	Cebpa	Wild type	e f	GCTCTAAGACCCAGCAGGC CGGCTCCACCTCGTAGAAGTC	272
		Cre	g h	CGCTAAGGATGACTCTGGT GTCTCAAGGAGAAACCACCAC	487
	Rosa 26	Wild type	i j	ACCTTTCTGGGAGTTCTCTGCTG GGAGCGGGAGAAATGGATATG	488
		EYFP	i k	ACCTTTCTGGGAGTTCTCTGCTG GCGAAGAGTTTGTCCTCAACC	200
		Recombined	i l	ACCTTTCTGGGAGTTCTCTGCTG GCTCCTCGCCCTTGCTCA	400
Quantitative RT-PCR	*Gapdh*		m n	AGGTCGGTGTGAACGGATTTG TGTAGACCATGTAGTTGAGGTCA	123
	Sbds		o p	GCGCTTCGAAATCGCCTG TCTGGTCGTCTGTCCCAAATG	167

Table S2. Complete loss of *Sbds* in *Cebpa*-expressing cells is lethal during mouse development.

Time of DNA isolation	Crossing	Genotype	Expected frequency†	No. pups
P7	**$Sbds^{f/+}$ $Cebpa^{cre/+}$ x $Sbds^{f/+}$ $Cebpa^{cre/+}$**	*$Sbds^{+/+}$ $Cebpa^{+/+}$*	1/12	7/59
	***P* = 0.0018**	*$Sbds^{+/+}$ $Cebpa^{cre/+}$*	1/6	18/59
	11 crossings	*$Sbds^{f/+}$ $Cebpa^{+/+}$*	1/6	8/59
	Average litter size = 5.36	*$Sbds^{f/+}$ $Cebpa^{cre/+}$*	1/3	23/59
		$Sbds^{f/f}$ $Cebpa^{+/+}$	1/12	3/59
		$Sbds^{f/f}$ $Cebpa^{cre/+}$	1/6	0/59
P7	**$Sbds^{f/f}$ $Cebpa^{+/+}$ x $Sbds^{f/+}$ $Cebpa^{cre/+}$**	*$Sbds^{f/+}$ $Cebpa^{+/+}$*	1/4	11/33
	***P* = 0.0043**	*$Sbds^{f/+}$ $Cebpa^{cre/+}$*	1/4	8/33
	5 crossings	*$Sbds^{f/f}$ $Cebpa^{+/+}$*	1/4	14/33
	Average litter size = 6.25	*$Sbds^{f/f}$ $Cebpa^{cre/+}$*	1/4	0/33
P7	**$Sbds^{f/+}$ $Cebpa^{cre/+}$ x $Sbds^{f/+}$ $Cebpa^{+/+}$**	*$Sbds^{+/+}$ $Cebpa^{+/+}$*	1/8	5/36
	***P* = 0.1047**	*$Sbds^{+/+}$ $Cebpa^{cre/+}$*	1/8	4/36
	5 crossings	*$Sbds^{f/+}$ $Cebpa^{+/+}$*	1/4	9/36
	Average litter size = 7.20	*$Sbds^{f/+}$ $Cebpa^{cre/+}$*	1/4	9/36
		$Sbds^{f/f}$ $Cebpa^{+/+}$	1/8	9/36
		$Sbds^{f/f}$ $Cebpa^{cre/+}$	1/8	0/36
E14.5	**$Sbds^{f/f}$ $Cebpa^{+/+}$ x $Sbds^{f/+}$ $Cebpa^{cre/+}$**	*$Sbds^{f/+}$ $Cebpa^{+/+}$*	1/4	12/49
	***P* = 0.4175**	*$Sbds^{f/+}$ $Cebpa^{cre/+}$*	1/4	9/49
	5 crossings	*$Sbds^{f/f}$ $Cebpa^{+/+}$*	1/4	17/49
	Average litter size = 9.80	*$Sbds^{f/f}$ $Cebpa^{cre/+}$*	1/4	11/49

Cumulative frequencies of litters obtained from intercrossing *$Sbds^{f/+}$* *$Cebpa^{cre/+}$* $R26^{YFP/+}$mice. The genotype of the parents and the offspring is shown. The R26 genotype is omitted for simplicity. †*$Cebpa^{cre/cre}$* mice are not viable due to the complete absence of *Cebpa* coding sequence in the cre allele[4]. *P* values refer to Pearson's chi-squared test.

Table S3. Transcriptional signatures for translation and lineage progression in *Sbds*-deficient MC-MMs.

Cellular process	Data set	Enrichment	Size	ES	NES	NOM p-val	FDR q-val
Translation	BILANGES_RAPAMYCIN_SENSITIVE_VIA_TSC1_AND_TSC2	Mutants	72	0.698	1.611	<0.001	0.2322
	BILANGES_SERUM_RESPONSE_TRANSLATION	Mutants	35	0.701	1.550	0.035	0.1657
	PENG_RAPAMYCIN_RESPONSE_DN	Mutants	243	0.613	1.511	<0.001	0.1753
	REACTOME_TRANSLATION	Mutants	140	0.788	1.477	<0.001	0.1926
	BILANGES_SERUM_AND_RAPAMYCIN_SENSITIVE_GENES	Mutants	64	0.796	1.431	0.021	0.2104
	MENSSEN_MYC_TARGETS	Mutants	52	0.676	1.397	<0.001	0.2450
	REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	Mutants	102	0.826	1.391	0.021	0.2484
	REACTOME_ACTIVATION_OF_THE_MRNA_UPON_BINDING_OF_THE_CAP_BINDING_COMPLEX_AND_EIFS_AND_SUBSEQUENT_BINDING_TO_43S	Mutants	54	0.759	1.390	<0.001	0.2458
Undifferentiated state	BHATTACHARYA_EMBRYONIC_STEM_CELL	Mutants	85	0.575	1.601	<0.001	0.2029
	JUBAN_TARGETS_OF_SPI1_AND_FLI1_DN	Mutants	90	0.582	1.572	<0.001	0.1544
	MUELLER_PLURINET	Mutants	295	0.535	1.539	<0.001	0.1572
	PARK_HSC_AND_MULTIPOTENT_PROGENITORS	Mutants	50	0.504	1.480	0.086	0.1915
	IVANOVA_HEMATOPOIESIS_INTERMEDIATE_PROGENITOR	Mutants	147	0.637	1.469	<0.001	0.1927
	BYSTRYKH_HEMATOPOIESIS_STEM_CELL_AND_BRAIN_QTL_CIS	Mutants	64	0.524	1.403	<0.001	0.2396
	SCHURINGA_STAT5A_TARGETS_DN	Mutants	15	0.478	1.397	0.026	0.2457
	RAMALHO_STEMNESS_UP	Mutants	207	0.531	1.386	<0.001	0.2487
	XU_RESPONSE_TO_TRETINOIN_AND_NSC682994_DN	Mutants	15	0.750	1.383	<0.001	0.2461
Myeloid differentiation	LIAN_NEUTROPHIL_GRANULE_CONSTITUENTS	Controls	24	-0.556	-1.719	<0.001	0.0619
	JAATINEN_HEMATOPOIETIC_STEM_CELL_DN	Controls	214	-0.561	-1.718	<0.001	0.0610
	IVANOVA_HEMATOPOIESIS_MATURE_CELL	Controls	294	-0.582	-1.644	<0.001	0.0820
	PID_AMB2_NEUTROPHILS_PATHWAY	Controls	40	-0.532	-1.612	<0.001	0.1081
	REACTOME_DEGRADATION_OF_THE_EXTRACELLULAR_MATRIX	Controls	27	-0.529	-1.601	<0.001	0.1104
	BROWN_MYELOID_CELL_DEVELOPMENT_UP	Controls	168	-0.665	-1.590	<0.001	0.1221
	TAVOR_CEBPA_TARGETS_UP	Controls	49	-0.503	-1.580	<0.001	0.1229
	PID_INTEGRIN_A9B1_PATHWAY	Controls	25	-0.575	-1.567	<0.001	0.1368
	KEGG_HEMATOPOIETIC_CELL_LINEAGE	Controls	80	-0.460	-1.557	<0.001	0.1438
	SA_MMP_CYTOKINE_CONNECTION	Controls	15	-0.629	-1.542	<0.001	0.1595
	XU_RESPONSE_TO_TRETINOIN_UP	Controls	15	-0.605	-1.478	<0.001	0.1878
	NAKAJIMA_EOSINOPHIL	Controls	27	-0.640	-1.477	0.027	0.1885
	BIOCARTA_CCR3_PATHWAY	Controls	23	-0.670	-1.467	<0.001	0.1930
	ZHOU_INFLAMMATORY_RESPONSE_LPS_UP	Controls	373	-0.380	-1.443	<0.001	0.2240
	KAMIKUBO_MYELOID_CEBPA_NETWORK	Controls	80	-0.457	-1.417	<0.001	0.2492

Curated data sets significantly enriched in *Sbds f/f* (mutants) or +/+ (controls) are shown (FDR<0.25, GSEA comparison to the C2 MSigDB collection). ES: enrichment score. NES: normalized enrichment score. NOM p-val: nominal p-value. FDR q-val: False Discovery Rate q-value.

Table S4. Enrichment of GO-terms for ribosome biogenesis in *Sbds*-deficient MC-MMs.

Data set	Enrichment	Size	ES	NES	NOM p-val	FDR q-val
REGULATION_OF_TRANSLATIONAL_INITIATION	Mutants	28	0.681	1.520	<0.001	0.1831
TRANSCRIPTION_FROM_RNA_POLYMERASE_III_PROMOTER	Mutants	17	0.703	1.523	0.032	0.1869
RIBOSOME_BIOGENESIS_AND_ASSEMBLY	Mutants	17	0.859	1.525	<0.001	0.2012
NUCLEOLUS	Mutants	119	0.700	1.551	<0.001	0.2068
RIBONUCLEOPROTEIN_COMPLEX	Mutants	141	0.672	1.589	<0.001	0.2127
RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS_AND _ASSEMBLY	Mutants	83	0.664	1.552	<0.001	0.2166
PROTEIN_RNA_COMPLEX_ASSEMBLY	Mutants	64	0.601	1.492	<0.001	0.2168
RRNA_METABOLIC_PROCESS	Mutants	15	0.845	1.589	<0.001	0.2306
TRANSLATIONAL_INITIATION	Mutants	36	0.640	1.455	<0.001	0.2368
NUCLEOLAR_PART	Mutants	17	0.783	1.467	<0.001	0.2414
RIBOSOME	Mutants	38	0.825	1.435	<0.001	0.2420

GO gene sets related to ribosome maturation and translation are significantly enriched in *Sbds f/f* (mutants) (FDR<0.25, GSEA comparison to the C5 MSigDB collection). ES: enrichment score. NES: normalized enrichment score. NOM p-val: nominal p-value. FDR q-val: False Discovery Rate q-value.

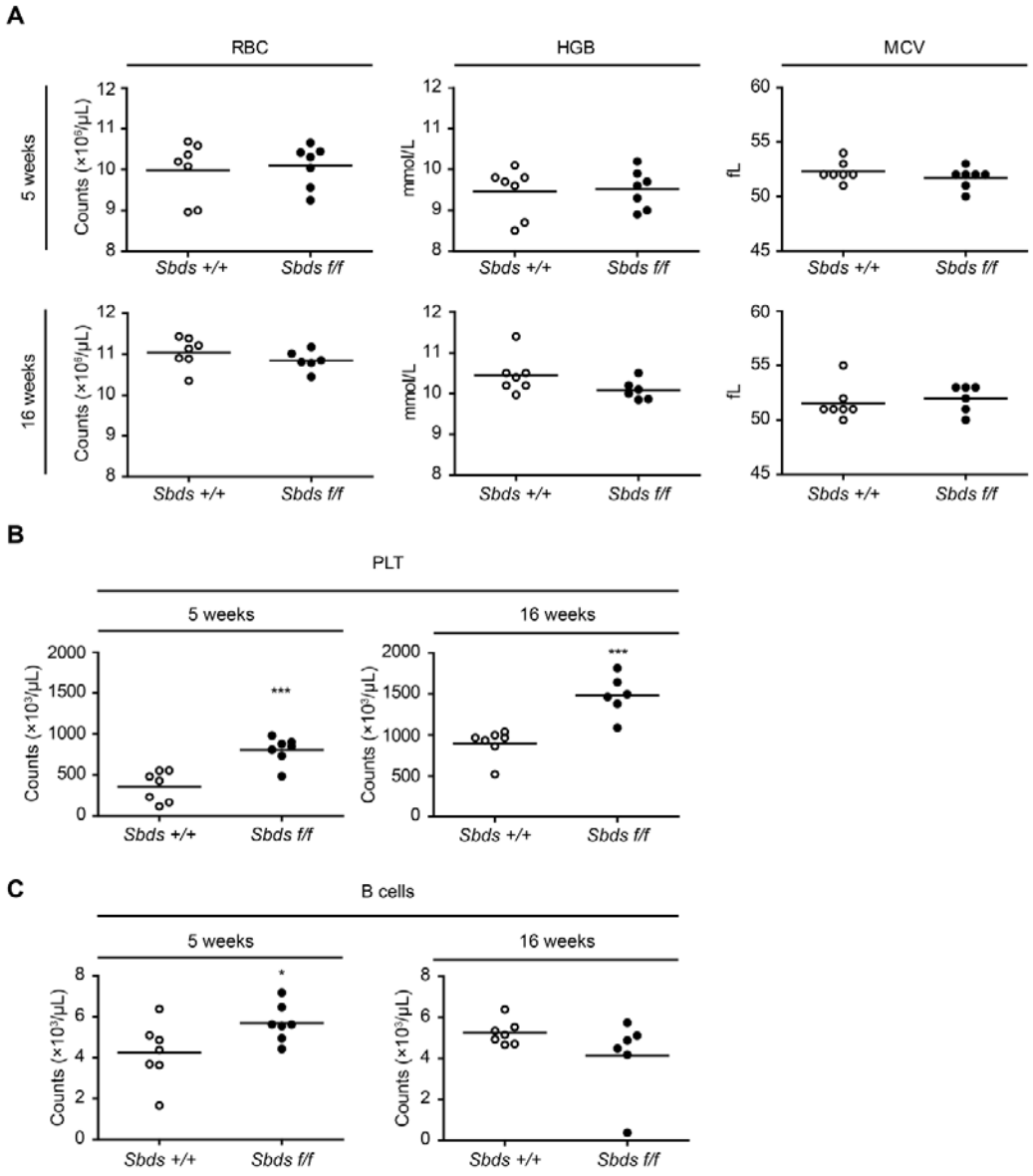

Figure S1. Effects of *Sbds* deletion from *Cebpa*-expressing hematopoietic cells on peripheral blood cell numbers.

(A) Normal levels of red blood cells counts (RBC), hemoglobin (HGB) and mean corpuscular volume (MCV) in mice injected with *Sbds* +/+ or *f/f* cells. (B) Platelet counts (PLT) in the peripheral blood. (C) Numbers of $B220^{+}$ lymphocytes (B cells) in the peripheral blood. Each circle represents one recipient mouse. Data is presented at 5 and 16 weeks after transplantation. * $P < 0.05$. *** $P < 0.001$.

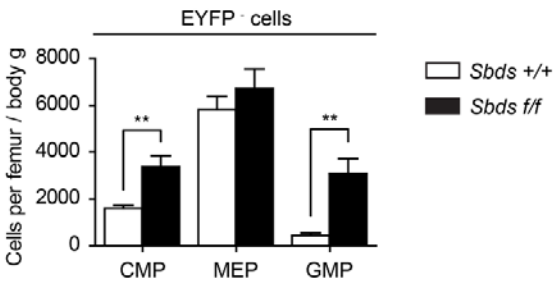

Figure S2. Expansion of EYFP⁻ progenitors in *Sbds f/f* recipients.

Absolute counts of EYFP⁻ progenitors in transplanted mice. Data is mean ± s.e.m. ** $P < 0.01$.

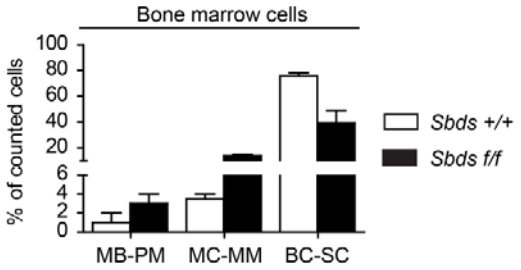

Figure S3. Increased frequency of MC-MMs and loss of mature neutrophils in *Sbds f/f* recipients. Evaluation of myeloid differentiation stages in bone marrow cytospins from *Sbds f/f* and +/+ recipients (*n* = 2). MB-PM: myeloblasts-promyelocytes. MC-MM: myelocytes-metamyelocytes. BC-SC: band cells-segmented cells. Data is mean ± s.e.m.

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