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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ABSTRACT

Shwachman-Diamond syndrome is a congenital bone marrow failure disorder characterized by debilitating neutropenia. The disease is associated with loss-of-function mutations in the *SBDS* gene, implicated in ribosome biogenesis, but the cellular and molecular events driving cell specific phenotypes in ribosomopathies remain poorly defined. Here, we established what is to our knowledge the first mammalian model of neutropenia in Shwachman-Diamond syndrome through targeted downregulation of *Sbds* in hematopoietic stem and progenitor cells expressing the myeloid transcription factor CCAAT/enhancer binding protein α (*Cebpa*). *Sbds* deficiency in the myeloid lineage specifically affected myelocytes and their downstream progeny while, unexpectedly, it was well tolerated by rapidly cycling hematopoietic progenitor cells. Molecular insights provided by massive parallel sequencing supported cellular observations of impaired cell cycle exit and formation of secondary granules associated with the defect of myeloid lineage progression in myelocytes. Mechanistically, *Sbds* deficiency activated the p53 tumor suppressor pathway and induced apoptosis in these cells. Collectively, the data reveal a previously unanticipated, selective dependency of myelocytes and downstream progeny, but not rapidly cycling progenitors, on this ubiquitous ribosome biogenesis protein, thus providing a cellular basis for the understanding of myeloid lineage biased defects in Shwachman-Diamond syndrome.

Introduction

Shwachman-Diamond syndrome (SDS; OMIM 260400) is a rare congenital multi-systemic disorder characterized by exocrine pancreatic insufficiency, skeletal defects and bone marrow failure.[1-3] The hematologic hallmark of the disease is neutropenia, which affects 88%-98% of patients[4,5] and represents, together with leukemic evolution, the main cause of morbidity and mortality in SDS.[1,6-8] Other less common manifestations are anemia, thrombocytopenia and pancytopenia.[6,7] The disease is caused by biallelic loss of function mutations in the Shwachman-Bodian-Diamond Syndrome gene (*SBDS*).[9] The two most common mutations, 258+2T→C and 183-184TA→CT, result in impaired splicing and a premature stop codon, respectively, and are associated with reduced protein levels of SBDS.[9,10]

SBDS plays an essential role in ribosome biogenesis. In particular, the concerted activity of SBDS and elongation factor-like 1 (EFL1) mediates the removal of eukaryotic initiation factor 6 (eIF6) during the cytoplasmic maturation of the pre-60S subunit, allowing the formation of the 80S ribosome.[10-12] Consistent with this notion, impaired ribosome subunit joining has been demonstrated in SDS patients[12,13] and reduced overall translation was detected in yeast models of SDS.[11] Defects in ribosome biogenesis define a group of pathologies collectively known as ribosomopathies. Causative mutations in genes linked to ribosome biogenesis have been identified in several congenital diseases including Diamond-Blackfan anemia (DBA), dyskeratosis congenita and cartilage-hair hypoplasia. While the hematopoietic system is affected in most of these conditions, leading to some degree of bone marrow failure, pronounced vulnerability of specific cell lineages discerns these disorders, with neutropenia being the specific hallmark of SDS. This reflects the central enigma in the understanding of human congenital ribosomopathies, i.e. how disruption of ribosome biogenesis, a process occurring in all tissues with a proposed generic role in protein synthesis, results in cell and tissue specific disease phenotypes.

It is thought that ribosomes, and hence the proteins related to their biogenesis, are critically important for fast cycling cells, thus leading to impaired function of hematopoietic progenitor cells, resulting in cytopenia, but this view provides no explanation for cell-type specificity (neutropenia) in SDS.[14,15]

Identification of the cell types specifically affected by dysfunction of ribosomal genes will thus be critical in deciphering the underlying molecular mechanisms driving disease pathology, ultimately enabling targeted therapies. However, progress in understanding the pathophysiology of SDS is limited by the lack of a robust mammalian model faithfully recapitulating neutropenia. Deficiency of *Sbds* leads to embryonic lethality in full knockout mice,[10,16] and transplantation of shRNA-transduced *Sbds*-deficient murine hematopoietic cells

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in wild-type recipient mice did not result in overt neutropenia, although it impaired myeloid progenitor generation.[17] Targeting *Sbds* in the hematopoietic system *via* poly(I:C) treatment of *Sbds*$^{fl/-}$*Tg:Mx1-cre* mice resulted in a severe hepatic phenotype, precluding a thorough investigation of the hematologic consequences of *Sbds* deficiency in adult hematopoietic stem cells (HSCs).[10] Thus, *in vivo* targeting of *Sbds* in postnatal mammalian hematopoiesis remains a key challenge for the field.

The basic leucine zipper transcription factor CCAAT/Enhancer-Binding Protein α (C/EBPα) is expressed in a fraction of HSCs and throughout the myeloid lineage,[18-20] thus offering an alternative approach to target hematopoietic stem and progenitor cells and their downstream myeloid lineage progeny in adult mammals.

Here, we generated a novel mouse model of genetic *Sbds* deletion through targeted downregulation of the gene in *Cebpa*-expressing cells, resulting in profound neutropenia. We show that loss of *Sbds* is well tolerated by rapidly cycling myeloid progenitor cells and identify myelocytes and their downstream progeny as the cell types within the hematopoietic hierarchy critically affected by *Sbds* deficiency through induction of cellular stress and apoptosis, thus providing a cellular and molecular basis for neutropenia in SDS.

Methods

Mice and genotyping

Cebpa$^{cre/+}$ R26 EYFP mice and *Sbds*$^{fl/+}$ mice have been previously described.[19,21] B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyCrl (B6.SJL) mice were purchased from Charles River. Mice and embryos were genotyped by PCR on DNA isolated from toes and forelimbs, respectively, using the primers listed in *Online Supplementary Table S1* (Life Technologies). Animals were maintained in specific pathogen free conditions in the Experimental Animal Center of Erasmus MC (EDC) and sacrificed by cervical dislocation. All animal work was approved by the Animal Welfare/Ethics Committee of the EDC in accordance with legislation in the Netherlands.

Fetal liver cell transplantation

Fetal livers were isolated from E14.5 embryos. Cell suspensions were centrifuged, re-suspended in a minimal volume of ACK lysing buffer (Lonza) and incubated on ice for 4 min to eliminate red blood cells. After centrifugation, cells were re-suspended in PBS+0.5% FCS. Then 7-10-week-old, lethally irradiated (8.5Gy) B6.SJL mice were transplanted with 3x10^{5} fetal liver cells by tail vein injection. Recipients received antibiotics in the drinking water for two weeks after transplantation.

RNA sequencing and GSEA analysis

cDNA was synthesized and amplified using SMARTer Ultra Low RNA kit (Clontech Laboratories) following the manufacturer's protocol. Amplified cDNA was further processed according to TruSeq Sample Preparation v.2 Guide (Illumina) and paired end-sequenced (2x75 bp) on the HiSeq 2500 (Illumina). Demultiplexing was performed using CASAVA software (Illumina) and the adaptor sequences were trimmed with Cutadapt (*http://code.google.com/p/cutadapt/*). Alignments against the mouse genome (mm10) and analysis of differential expressed genes were performed as previously described.[22] Cufflinks software was used to calculate the number of fragments per kilobase of exon per million fragments mapped (FPKM) for each gene. FPKM values of *Sbds f/f* and +/+ recipients were then compared to the curated gene sets (C2) and the Gene Ontology gene sets (C5) of the Molecular Signature Database (MSigDB) by GSEA[23] (Broad Institute), using the Signal2Noise metric and 1000 phenotype-based permutations.

Statistical analysis

Unless otherwise specified, statistical analysis was performed by an unpaired, two-tailed Student *t*-test or one-way analysis of variance. All results in bar graphs are reported as mean value ± standard error of the mean.

Additional information is provided in the *Online Supplementary Appendix*.

Results

Cebpa-*driven deletion of* Sbds *in the hematopoietic system*

To address the functional consequences of *Sbds* deficiency in early hematopoietic progenitors, we crossed *Sbds*-conditional knock-out mice, with loxP sites flanking the second exon of *Sbds*,[21] with *Cebpa*$^{cre/+}$ R26 EYFP mice[19] (Figure 1A). In this approach, Cre-mediated deletion of *Sbds* exon 2 in *Cebpa*-expressing cells results in a frameshift and consequently generates a premature stop codon, thus mimicking the effects of the 183-184TA→CT mutation in the human disease.[9] In addition, the R26 EYFP element enables the tracing of *Sbds*-depleted cells and their progeny based on enhanced yellow fluorescent protein (EYFP) expression.

Intercrossings of *Sbds*$^{f/+}$;*Cebpa*$^{cre/+}$;*R26*$^{EYFP/+}$ mice failed to generate any *Sbds*$^{f/f}$;*Cebpa*$^{cre/+}$ viable offspring (*Online Supplementary Table S2*). This, together with the small litter sizes, suggested that deficiency of *Sbds* in *Cebpa*-expressing cells is lethal in mice. Because *Cebpa* is expressed in non-hematopoietic tissues, like liver and lungs,[24] we analyzed E14.5 embryos from *Sbds*$^{f/+}$;*Cebpa*$^{cre/+}$ R26$^{EYFP/+}$ intercrosses to assess whether the lethal phenotype directly reflected hematopoietic dysfunction. Interestingly, at this gestational age, *Sbds*$^{f/f}$;*Cebpa*$^{cre/+}$;*R26*$^{EYFP/+}$ (hereafter *Sbds f/f* or mutants) offspring were found at Mendelian frequencies (*Online Supplementary Table S2*) and these embryos were morphologically indistinguishable from their littermates. Genomic PCR indicated effective deletion of exon 2 in *Sbds f/f* embryos (Figure 1A and B). We next compared hematopoiesis in *Sbds f/f* embryos with that of *Cebpa*$^{cre/+}$;*R26*$^{EYFP/+}$ controls (hence *Sbds* +/+) and found that *Sbds* recombination was associated with overall conservation of normal hematopoietic architecture in the fetal liver (Figure 1C). This suggests that embryonic lethality in this model is not caused by impaired blood cell production. The proportion of EYFP^{+}, Cre-targeted cells in each hematopoietic compartment was also similar in *Sbds f/f* and +/+ embryos, with the majority of GMPs and Gr1^{+}Mac1^{+} mature granulocytes expressing EYFP (>90% and >60% of cells, respectively) (Figure 1D). As expected,[19,20] a small fraction of immunophenotypically defined HSCs, multipotent progenitors (MPPs), megakaryocyte-erythroid progenitors (MEPs) and B220^{+} lymphocytes also expressed EYFP, indicating targeting of multilineage progenitors in this model.

Transplantation of Sbds-*deleted fetal hematopoietic cells in adult mice results in neutropenia*

To assess whether the deletion of *Sbds* in fetal hematopoietic progenitors would compromise postnatal

hematopoiesis, we transplanted fetal liver cells from of E14.5 (CD45.2$^+$) *Sbds f/f* or +/+ embryos into lethally irradiated (CD45.1$^+$) B6.SJL mice (Figure 2A) and monitored hematopoiesis by peripheral blood analysis every four weeks. In both *Sbds f/f* and +/+ recipients more than 90% of circulating blood cells were CD45.2$^+$, indicating high chimerism in reconstituted mice. Mice transplanted with *Sbds*-deficient cells developed profound neutropenia, with an average 5.9-fold reduction of Gr1$^+$ Mac1$^+$ cells in the peripheral blood five weeks after transplantation (Figure 2B). Neutropenia was stable and persisted during the entire follow up, i.e. four months after transplantation. Consistent with the hypothesis that loss of *Sbds* impairs myelopoiesis, the proportion of EYFP$^+$, Cre-targeted cells in *Sbds f/f* recipients (47.4±10.2%) was lower than that in controls (75.6±17.3%). In addition, numbers of circulating erythrocytes and B cells were unaltered in steady state hematopoiesis after transplantation, while platelet numbers were increased in transplanted mice (*Online Supplementary Figure S1*).

Overall, the data indicate that *Sbds* is essential to maintain postnatal granulopoiesis and neutrophil homeostasis.

Sbds-deficiency in the myeloid lineage arrests differentiation at the myelocyte-metamyelocyte stage

To obtain insight into the cellular events caused by *Sbds* deficiency driving the defect in neutrophil development, we sacrificed mice 17-19 weeks after transplantation. *Cebpa* is expressed in myeloid progenitor cells and in a subpopulation of early progenitors that retain multilineage differentiation capacity.[19,20] In line with this, EYFP expression was found in a small fraction of immunophenotypically defined hematopoietic stem and progenitor cells (HSPCs) and in all blood lineages (Gr1$^+$ Mac1$^+$ myeloid, Ter119$^+$ erythroid and B220$^+$ lymphoid) at varying frequencies (Table 1 and Figure 3A). The bone marrow of mice transplanted with *Sbds f/f* fetal liver cells was hypocellular, a common finding in SDS patients[7,25] (Figure 3B). Hypocellularity resulted mostly from a marked reduction in EYFP$^+$ Gr1$^+$ Mac1$^+$ neutrophils (86,437±33,601 cells per femur/body gram in *Sbds f/f* recipients and 316,442±47,984 in controls), although also the number of EYFP$^+$ lymphoid and Ter119$^+$ erythroid cells was modestly decreased in mutant mice (Figure 3C and D). Of note, myelodysplastic features were not observed in *Sbds f/f* recipients in the current model, suggesting that hematopoietic cell-extrinsic factors may contribute to myelodysplasia in SDS.[26]

Having established that *Sbds* deletion from multilineage hematopoietic progenitors results in neutropenia, we next sought to define the stages in myeloid lineage progression that were affected by loss of *Sbds*. It is generally assumed that loss of ribosome biogenesis genes affects rapidly proliferating progenitor cells with increased protein translation rates, although *in vivo* experimental support for this view has been lacking in the absence of a mammalian model of *Sbds* deficiency resulting in stable neutropenia. Efficient *Sbds* gene knockdown in EYFP$^+$ cells was first confirmed by quantitative RT-PCR throughout myeloid lineage development (Figure 3E), showing near complete deletion of *Sbds* expression at all stages of myeloid development (LKS, CMP, GMP, immature and mature neutrophils). Interestingly, *Sbds* deficiency did not result in reduced numbers of hematopoietic progenitor cells along the myeloid lineage (Figure 3C and D). Rather, a compensatory expansion of some progenitors was observed with numbers of both EYFP$^+$ and EYFP$^-$ GMPs dramatically increased in mice transplanted with *Sbds f/f* cells (average fold change, FC: 5.8 in EYFP$^+$ compartment and 5.9 in EYFP$^-$ cells) (Figure 3D and *Online Supplementary Figure S2*), suggesting the emergence of reactive granulopoiesis in this cohort, defined as adaptation of the hematopoietic system

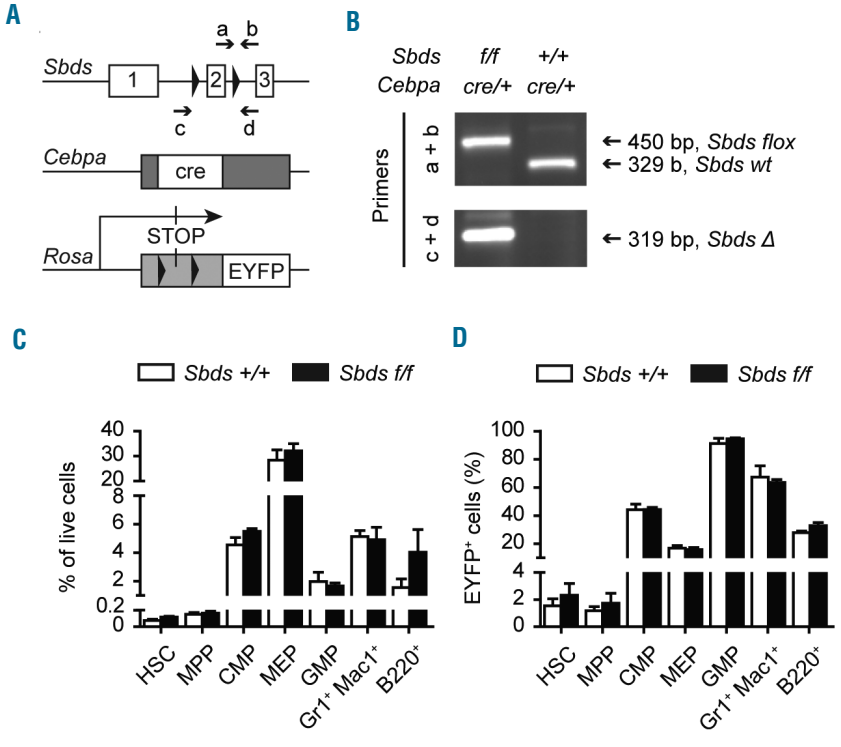


Figure 1. ***Sbds*-deletion from myeloid progenitor cells does not perturb the architecture of fetal liver hematopoiesis.** (**A**) Schematic representation of the targeting vectors and the primers used in the study. Primer sequences are listed in *Online Supplementary Table S1*. (**B**) Genomic analysis of E14.5 embryos confirmed excision of *Sbds* in f/f mice. a + b, genotyping primers. c + d, deletion primers. (**C** and **D**) Normal composition of fetal liver (*Sbds* +/+, n=4; *Sbds* f/f, n=6). (**C**) Frequency of hematopoietic subsets in live cells (7AAD-). (**D**) Proportion of EYFP$^+$ cells in each hematopoietic population. Data are mean ± s.e.m. Differences between *Sbds* +/+ and *f/f* mice are not significant. HSC, Lin$^-$ c-Kit$^+$ Sca1$^+$ (LKS) CD48$^-$ CD150$^+$ cells. Multipotent progenitors (MPP), LKS CD48$^-$ CD150$^-$ cells. Common myeloid progenitor (CMP), Lin$^-$ c-Kit$^+$ Sca1$^-$ CD34$^+$ CD16/32$^-$ cells. Granulocyte-macrophage progenitor (GMP), Lin$^-$ c-Kit$^+$ Sca1$^-$ CD34$^+$ CD16/32$^+$ cells.

to the increased demand through enhanced myeloid precursor cell proliferation in the bone marrow.[27]

We next aimed to further define the specific cell type in myeloid development that is critically dependent on *Sbds* function. FACS analysis revealed an increased frequency of c-Kit^{int} Gr1^{low} cells, previously identified as myelocytes and metamyelocytes (MC-MMs) (Figure 3F).[28] Both frequency and absolute count of more mature myeloid cells, characterized by loss of c-Kit expression and bright Gr1 staining, were lower, suggesting an arrest of lineage progression at the MC-MM stage. Morphological assessment of the bone marrow confirmed that terminal granulopoiesis is severely affected in *Sbds f/f* recipients, with accumulation of MC-MMs and reduced frequency of segmented neutrophils (Figure 3G and *Online Supplementary Figure S3*). Together, the data demonstrate that *Sbds* deletion from hematopoietic progenitor cells critically and specifically affects late stages of myeloid development, in particular the transition MC-MM to mature neutrophils.

Deficiency of Sbds *deregulates myeloid differentiation programs and prevents cell-cycle-exit in myelocytes and metamyelocytes*

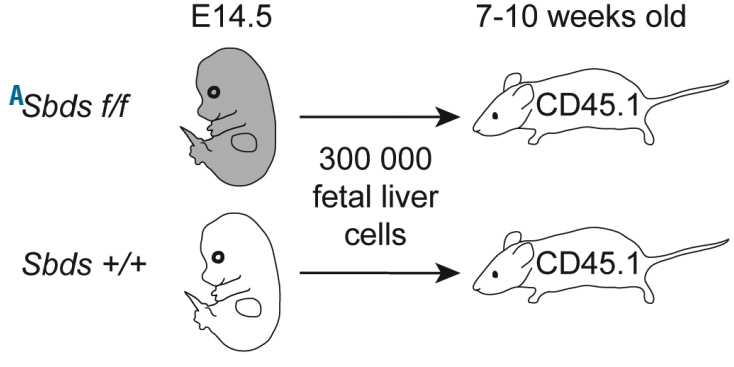
To gain insight into the molecular programs associated with the proposed block of myeloid lineage progression at the MC-MM stage, the transcriptome of prospectively FACS-isolated EYFP^{+} c-Kit^{int} Gr1^{low} (MC-MM) cells was investigated by massive parallel RNA sequencing (RNA-Seq). As expected, *Sbds* expression in this population was significantly reduced in *Sbds f/f* recipients (log2 FC = -2.39, False Discovery Rate, FDR=6.6x10^{-9}) (Figure 4A). Consistent with its postulated function in ribosome biogenesis, *Sbds* deficiency significantly (FDR<0.25) affected transcriptional signatures related to translation and ribosome biogenesis (Figure 4B and *Online Supplementary Tables S3 and S4*).

Gene set enrichment analysis (GSEA) highlighted the enrichment of relatively immature hematopoietic signatures in *Sbds f/f* MC-MMs, whereas data sets associated with cell differentiation were enriched in controls, consistent with a defect in maturation (Figure 4C and *Online Supplementary Table S3*). To specifically define the stage in which myeloid differentiation is impaired, we examined the expression of transcripts encoding constituents of

Table 1. Frequency of EYFP^{+} cells within hematopoietic subsets in the bone marrow.

	Sbds +/+[a]	*Sbds f/f*[a]
LKS[b]	3.3% (1.61% - 5.82%)	7.4% (1.67% - 11.5%)
CMP[b]	31.2% (24.0% - 41.6%)	22.5% (16.3% - 31.6%)
MEP[b]	8.2% (6.3% - 10.2%)	5.8% (4.92% - 6.79%)
GMP[b]	72.3% (63.7% - 77.6%)	67.7% (62.6% - 72.3%)
Gr1^{+} Mac1^{+}[c]	87.9% (86.2% - 89.1%)	76.1% (74.3% - 77.7%)
B220^{+}[c]	8.8% (5.4% - 13.2%)	4.4% (3.3% - 5.9%)
Ter119^{+}[d]	3.0% (2.2% - 4.1%)	1.8% (0.7% - 3.2%)

[a]*The percentage of EYFP+ cells in different hematopoietic populations is indicated as mean frequency (range).* [b]*n=7.* [c]Sbds +/+; *n=7;* Sbds f/f; *n=6;* [d]Sbds +/+; *n = 5;* Sbds f/f; *n = 4.*



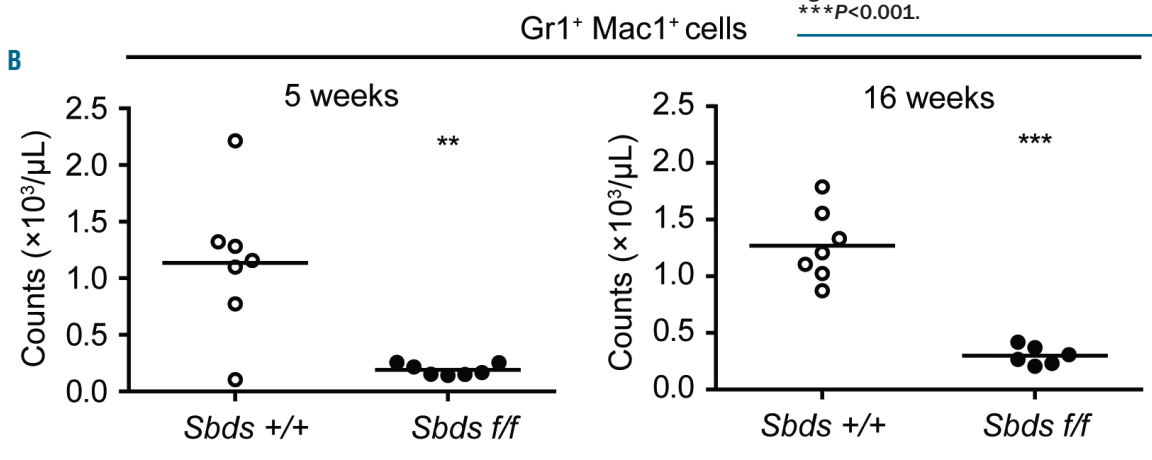


Figure 2. Loss of *Sbds* from C/EBPα-expressing cells causes neutropenia in mice. (A) Experimental design. 300,000 fetal liver cells from either *Sbds* +/+ or *f/f* embryos (E14.5) were transplanted into 7- to 10-week-old lethally irradiated CD45.1^{+} B6.SJL mice (n = 7 per group). (B) Peripheral blood analysis at five and 16 weeks after transplantation showed severe reduction of granulocytes (Gr1^{+} Mac1^{+} cells) upon *Sbds* deletion. Each circle represents one mouse. The horizontal line depicts average values. WBC: white blood cells. ***P*<0.01, ****P*<0.001.

myeloid granules. In myeloid development, primary granules are produced in promyelocytes, whereas secondary granule production starts in myelocytes and gelatinase-containing tertiary granules become apparent in metamyelocytes and in band cells.[29,30] Transcript analysis showed significantly reduced expression of secondary and tertiary granule components, whereas transcripts encoding constituents of primary granules were significantly enriched, indicating that development is specifically arrested in myelocytes, consistent with flow-cytometric findings (Figure 4D).

To definitively establish the myelocyte as the cell type in the myeloid lineage critically depending on *Sbds* expression, we next performed cell cycle analysis. Myelocytes represent the last cell in myeloid differentiation capable of mitotic division[31] and cell-cycle exit is a required process for terminal granulopoiesis.[32] Ki67 analysis by FACS demonstrated that c-Kit^{int} Gr1^{low} cells indeed failed to exit the cell cycle, congruent with the notion that *Sbds* deficiency attenuates lineage progression in myelocytes (Figure 4E and F). Molecularly, this failure to exit the cell cycle was associated with a significant reduced expression of the transcription factor retinoic acid receptor α (RARα) and its downstream transcriptional targets *Itgb2* and p27 (*Cdkn1b*),[33,34] key activators of the terminal myeloid differentiation program and cell-cycle exit (Figure 4G). Together, the data demonstrate that loss of *Sbds* from hematopoietic progenitors results in failure of lineage progression specifically in myelocytes, which is associated with attenuation of myeloid differentiation signatures and cellular events.

Loss of Sbds *results in activation of the p53 tumor suppressor pathway and apoptosis in late stage myeloid cells*

Finally, we sought to better define the underlying cellular and molecular mechanisms of the arrest in lineage progression at the stage of myelocytes. Activation of p53 has been proposed as a common mechanism in the pathogenesis of different ribosomopathies, including DBA, Treacher Collins syndrome and 5q- syndrome.[35] In SDS, overexpression of p53 in immature cells has been described in bone marrow biopsies from SDS patients[36] but the consequences of p53 activation for disease pathogenesis have not been experimentally defined. Transcriptional activation of the p53 pathway was

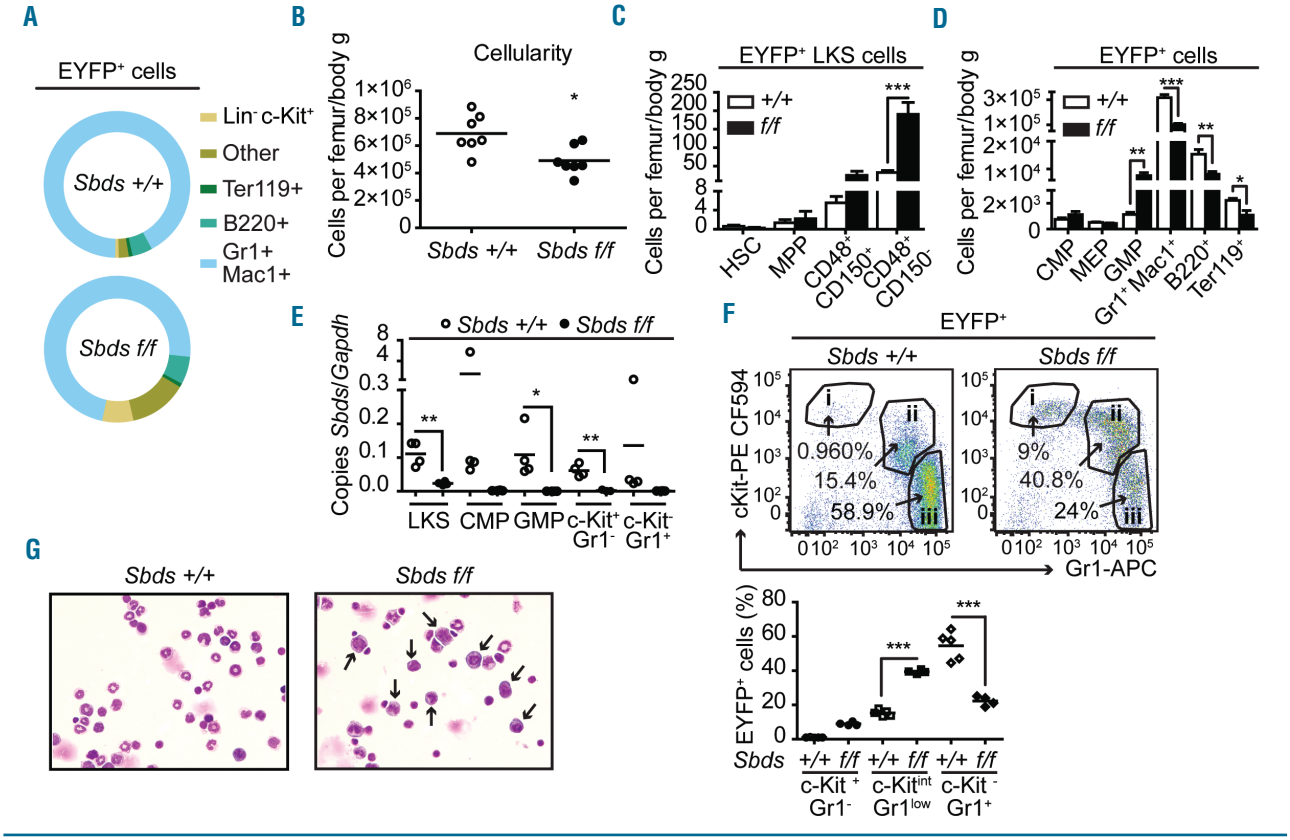


Figure 3. *Sbds*-deficiency in hematopoiesis attenuates myeloid lineage progression at the myelocyte-metamyelocyte stage. (A) Donut chart illustrating the percentage of different hematopoietic subsets within the EYFP$^+$ compartment in the bone marrow of *Sbds* +/+ and *f/f* recipients. (B) Decreased cellularity upon transplantation of *Sbds*-deficient cells. (C and D) Absolute number of EYFP$^+$ cells in different hematopoietic populations in the bone marrow (*Sbds* +/+, n=7; *Sbds f/f*, n=6, data are mean ± s.e.m). (C) LKS subsets. (D) Reduction of EYFP$^+$ mature granulocytes in *Sbds f/f*-transplanted mice is associated with an increase of granulocyte-macrophage progenitors. (E) Deletion efficacy. Expression of *Sbds* is reduced in LKS and throughout the myeloid differentiation stages in *Sbds f/f* EYFP$^+$ cells. (F) Loss of late myeloid cells in *Sbds f/f* mice. Upper panel: representative plots of EYFP$^+$ cells. Lower panel: percentage of c-Kit$^+$ Gr1$^-$ myeloblasts and promyelocytes (i), c-Kit^{int} Gr1^{low} myelocytes and metamyelocytes (ii) and c-Kit$^-$ Gr1$^+$ band and segmented cells (iii) in EYFP$^+$ cells from *Sbds* +/+ and *f/f* cohorts. (G) Accumulation of myelocytes and metamyelocytes (black arrows) in *Sbds*-deficient bone marrow (original magnification x63). * P<0.05, ** P<0.01, *** P<0.001.

observed in *Sbds f/f* MC-MMs by GSEA analysis (Figure 5A). Specifically, RNA sequencing demonstrated a significant increase in transcripts for p53 itself and many of its downstream targets, including the cell-cycle regulators *Cdkn1a* (p21) and *Zmat3* (Wig1) and the pro-apoptotic genes *Bbc3* (PUMA), *Bax*, *Tnfrsf10b* (Death Receptor 5) and *Cycs* (cytochrome c, somatic) (Figure 5B). FACS analysis confirmed intracellular accumulation of the p53 protein, specifically at late stages of myelopoiesis (MC-MM and c-Kit$^-$ EYFP$^+$ populations) in *Sbds f/f* recipients (FC = 1.78 and 1.53, respectively) (Figure 5C). In line with this activation of the p53 tumor suppressor pathway and increased expression of pro-apoptotic genes, an increased rate of apoptosis (annexin V$^+$ 7AAD- cells) was found in p53-over-expressing c-Kit^{int} and c-Kit$^-$ EYFP$^+$ populations (Figure 5D). Of note, no significant p53 accumulation or apoptosis was observed in more immature progenitors (cKit$^+$ EYFP$^+$ cells).

Collectively, the findings indicate that *Sbds* deficiency in the myeloid lineage specifically attenuates lineage progression at the myelocyte stage through activation of cellular stress pathways and induction of p53-associated apoptosis.

Discussion

Neutropenia is the principal hematologic manifestation of SDS, but the cellular and molecular mechanisms underlying this specific disease phenotype remain poorly understood. Here, we established a mammalian model of *Sbds* deficiency-induced neutropenia, revealing a critical dependency of myelocytes and their downstream progeny on the function of this ribosomal biogenesis gene through induction of p53-associated apoptosis, thus providing a cellular and molecular basis for the understanding of neutropenia in this disease.

Several findings in our study point towards myelocytes as the differentiation stage critically impaired by *Sbds* deficiency. First, the data indicate that myelocytes accumulate in *Cebpa*-cre *Sbds* mutant mice and fail to exit the cell cycle, a process typically occurring at the myelocyte stage and required for terminal differentiation towards mature neutrophils.[31,32] Secondly, massive parallel transcriptional profiling of prospectively isolated MC-MMs revealed specifically reduced expression of genes encoding constituents of secondary and tertiary granule proteins, the production of which specifically marks the transition from

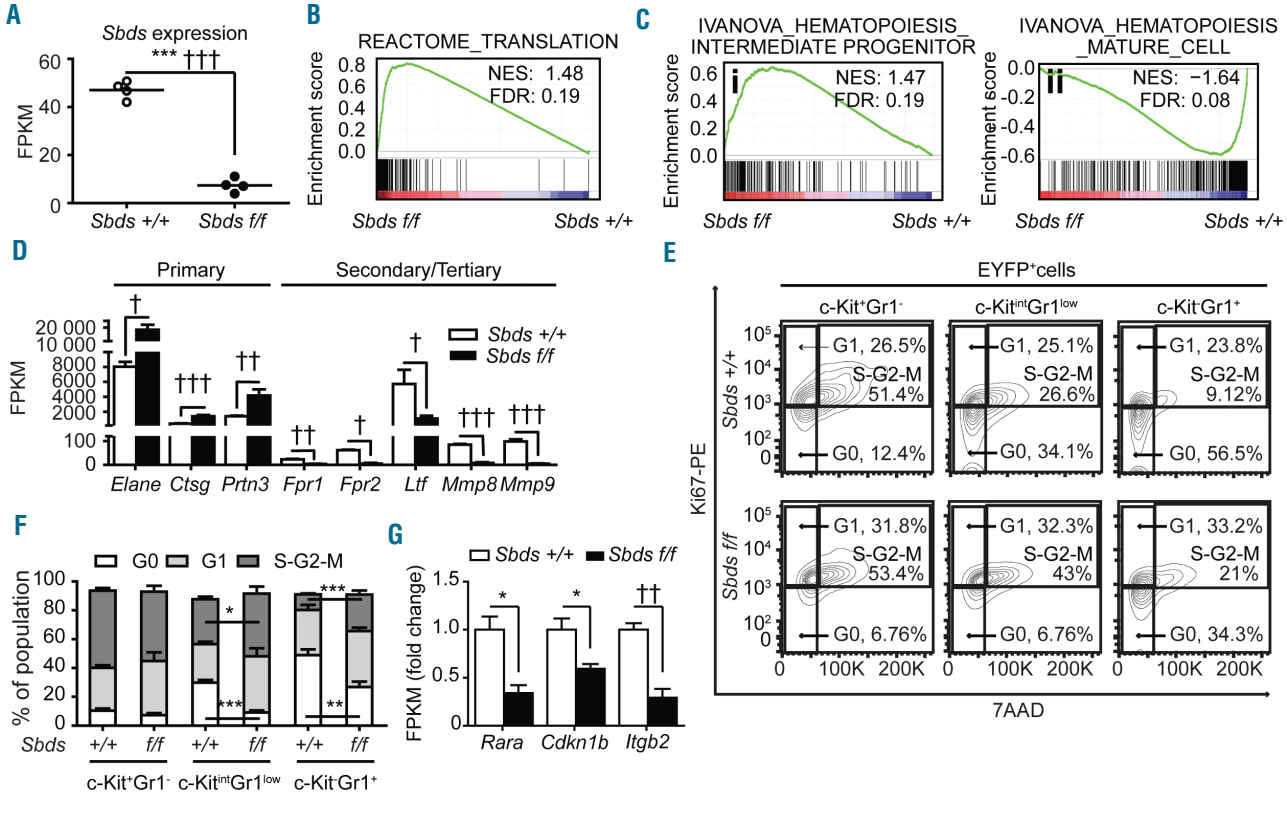


Figure 4. ***Sbds*** **deficiency results in failure of cell cycle exit and secondary granule formation in myelopoiesis. (A) Expression of *Sbds* in c-Kit^{int} Gr1^{low} EYFP$^+$ cells by RNA-Seq (n=4). Each circle represents one mouse. (B and C) GSEA plots showing enrichment of data sets relating to translation (B) and immature hematopoietic features (Ci) in c-Kit^{int} Gr1^{low} EYFP$^+$ cells from *Sbds f/f* recipients and enrichment of maturation signatures in the *Sbds* +/+ group (Cii). Genes in the datasets are represented by black bars distributed according to their differential expression between *Sbds f/f* and *Sbds* +/+ cohorts. NES: Normalized Enrichment Score. (D) Reduced expression of genes encoding secondary and tertiary granule proteins in *Sbds f/f* c-Kit^{int} Gr1^{low} EYFP$^+$ cells with enrichment of transcripts encoding primary granules components. (E and F) *Sbds* deficiency impairs cell cycle exit in myelopoiesis (*Sbds* +/+, n=5; *Sbds f/f*, n=4). (E) Gating strategy in representative FACS plots. (F) Percentage of cells in G0 (Ki67$^-$ 7AAD^{low}), G1 (Ki67$^+$ 7AAD^{low}) and S-G2-M (Ki67$^+$ 7AAD^{high}) in each EYFP$^+$ population. (G) Downregulation of *Rara*, *Cdkn1b* (p27) and *Itgb2* in recipients of *Sbds*-deficient cells (n=4). Data are mean ± s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001. †FDR<0.05, ††FDR<0.01, †††FDR<0.001.**

promyelocytes to myelocytes in myeloid development.[29,30] Finally, *Sbds* deficiency in myelocytes was associated with reduced expression of the myeloid transcription factor RARα and its downstream transcriptional targets. RARα is an important regulator of myelopoiesis with a putative role in terminal granulocyte differentiation.[37] *In vitro* differentiation studies show that RAR deficiency blocks lineage progression at the myelocyte stage[38] and *in vivo* inhibition of endogenous retinoids results in accumulation of immature myeloid cells in wild-type mice.[37]

While *Sbds* deletion in the myeloid lineage specifically attenuated lineage progression downstream of myelocytes, deficiency of *Sbds* did not functionally affect rapidly cycling hematopoietic progenitor cells (HPCs). This finding was initially unanticipated since HPCs are thought to have a relatively high rate of protein synthesis in comparison to HSCs and other cells of the hematopoietic hierarchy[39] and it seemed, therefore, reasonable to predict that rapidly cycling progenitors are more sensitive to defects of ribosome biogenesis and protein translation. In line with this view, reduced frequency or altered activity of HPCs have been previously suggested to drive cytopenia in both DBA and SDS,[40-42] even though this does not provide a satisfactory explanation for lineage specificity in these diseases. In our model, the efficacy of *Sbds* deletion was comparable throughout myeloid development (*Cebpa*-expressing LKS, CMP, GMP and the myelocyte compartment), yet the size of the progenitor pool in the bone marrow increased, while the number of mature neutrophils was reduced. This finding seems congruent with observations in zebrafish, where deletion of *Sbds* induces loss of neutrophils, but does not affect spi1 (PU.1) positive progenitors.[43]

The resulting left-shifted myelopoiesis in our model is reminiscent of that observed *in vitro* upon granulocytic differentiation of *Sbds*-knockdown hematopoietic cells, resulting in an accumulation of MC-MMs,[17] and of the maturation defect that characterizes a subset of SDS patients.[5,7,25,44] While our findings provide a basis for understanding the myeloid lineage specificity of ribosomal dysfunction in SDS, in translating these findings to human disease it is important to point out that pluripotent hematopoietic stem cells are incompletely targeted in this model. *Cebpa*-driven deletion of *Sbds* occurred in a small subset of immunophenotypically defined, multipotent HSCs, reflected in a modest decrease in erythroid and lymphoid cells in the EYFP$^+$ compartment of mutant mice. It is, therefore, reasonable to assume that *Sbds* deficiency in the hematopoietic system does not exclusively, but rather predominantly, affects the myeloid lineage. It is conceivable that, in human disease, composite effects of *Sbds* deficiency in pluripotent HSCs, potentially reducing numbers of (normally functioning) hematopoietic progenitor cells,[42,44,45] synergize with a specific impairment of lineage progression in late myeloid differentiation phases to impair granulopoiesis. Incomplete HSC targeting may also explain the apparent absence of homing defects, previously reported in a transplant model of *Sbds* downregulation in HSC.[17] This notion may help understand why we have been able to establish a stable model of neutropenia,

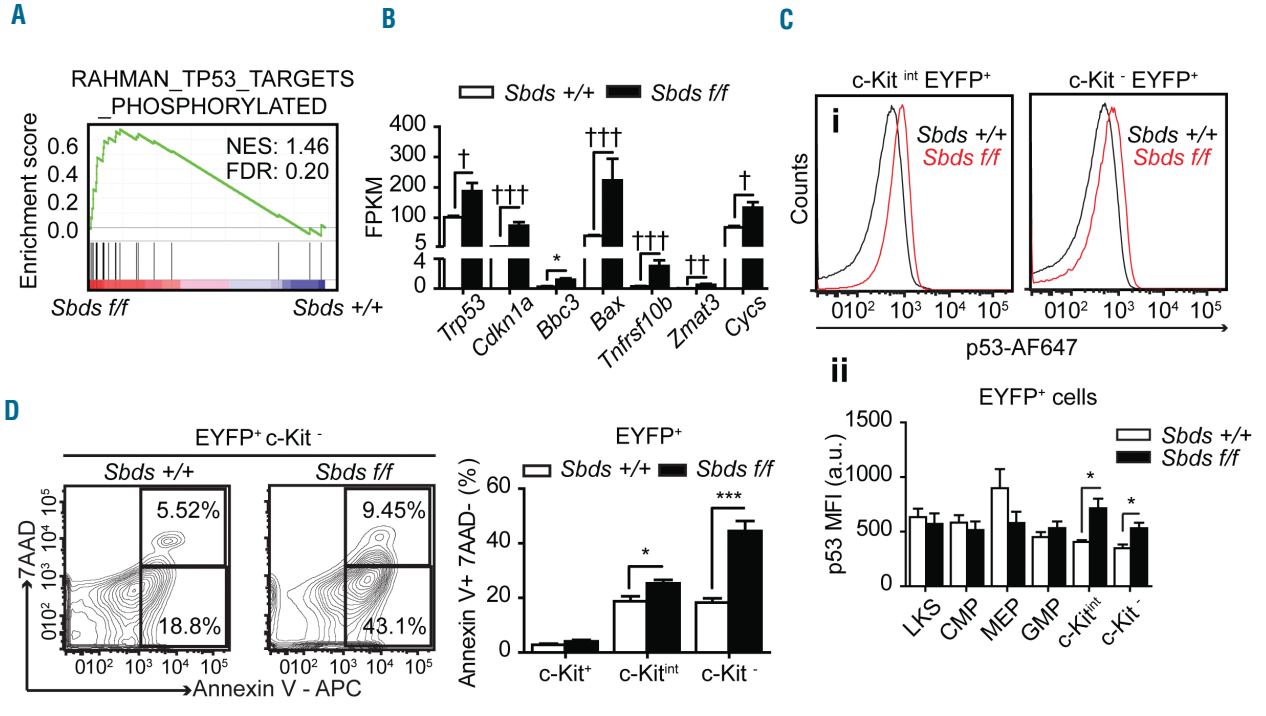


Figure 5. Activation of p53 and apoptosis in late stages of Sbds-deficient myeloid development. (A) Enrichment of p53 signatures in the transcriptome of MC-MMs from *Sbds f/f* recipients (n = 4). (B) Increased transcript levels of *Trp53* and its downstream transcriptional targets in RNA-Seq data. (C) Accumulation of p53 protein in late stages of myelopoiesis. (Ci), representative histograms. (Cii), mean fluorescence intensities in different EYFP$^+$ populations (*Sbds* +/+, n = 5; Sbds f/f, n = 4). (D) Increased rates of apoptosis in late myeloid cells upon *Sbds* deletion (n = 4). (Left) Representative plot. (Right) Frequency of annexin V$^+$ 7AAD$^-$ apoptotic cells in different stages of myelopoiesis. Data in bar graphs are mean ± s.e.m. **P*< 0.05, ****P*< 0.001. †FDR < 0.05, †††FDR < 0.001.

where incomplete targeting of HSCs allowed durable and robust engraftment of hematopoiesis, enabling the detailed analysis of long-term myeloid lineage progression in mutant mice.

Identification of the cells driving neutropenia within the *Sbds*-deficient hematopoietic hierarchy allowed us to begin defining the cellular and molecular events underlying neutropenia in SDS. Our study describes activation of the p53 pathway and an associated increase in apoptotic rates specifically in myelocytes and their downstream progeny. The data seem congruent with observations in human disease where an intrinsic propensity for apoptosis is seen in hematopoietic cells after 7-day culture of $CD34^+$ cells in medium containing G-CSF[46] with a lack of correlation between colony numbers and apoptosis rate, indicating that the number of CFU-C is not affected in SDS patients. Activation of the p53 pathway has been suggested to represent a molecular commonality in ribosomopathies and perhaps congenital neutropenias,[47] but experimental support for this view in SDS has been lacking. Our findings do not, however, exclude the possibility that other mechanisms are involved in the failure in cell-cycle progression and neutropenia in SDS.

It is conceivable that the loss of function of *Sbds* affects the translation of specific transcription factors driving terminal myeloid differentiation, including those upstream of RARα. In particular, the mRNA of specific transcription factor isoforms may be characterized by distinct 5'-UTRs, some of them predicted to have a complex secondary structure.[48] These complex 5'-UTRs are typically associated with high demands for translation initiation factors and may thus confer particular sensitivity to conditions of translational stress. Such a mechanism of reduced translation efficiency has recently been shown to affect GATA1 protein levels in the pathogenesis of DBA.[49] Alternatively, the high and specific demand of protein synthesis and co-translational assembly into secretory granules, which characterizes and defines myelocytes and their downstream progeny, may cause specific translational stress, resulting in activation of cellular alarm pathways and downstream events including impaired differentiation. In this context, it is interesting to note previous observations in induced pluripotent stem cell models of SDS, indicating granule abnormalities in pancreatic and myeloid cells,[50] and in a mouse model of pancreatic-specific *Sbds* deficiency, showing reduced *in vivo* zymogen granule formation in acinar cells,[21] perhaps pointing towards impaired secretory granule maturation as a common mechanism underlying tissue specificity in SDS.[50] Our current model will allow the interrogation of these potential mechanisms which is ultimately anticipated to result in novel, targeted therapeutic strategies for SDS.

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