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## Post-transcriptional control of C/EBP $\alpha$ and C/EBP $\beta$ proteins

Zaini, Mohamad Amr

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# CHAPTER V

## **Shwachman–Bodian–Diamond syndrome (SBDS) protein deficiency impairs translation re-initiation from C/EBP $\alpha$ and C/EBP $\beta$ mRNAs**

Kyungmin In<sup>1</sup>, Mohamad Amr Zaini<sup>1,2</sup>, Christine Muller<sup>1,2</sup>, Alan J. Warren<sup>3</sup>, Marieke von Lindern<sup>4</sup> and Cornelis F. Calkhoven<sup>1,2</sup>.

<sup>1</sup>*Leibniz Institute for Age Research - Fritz Lipmann Institute, Jena, Germany.*

<sup>2</sup>*European Research Institute for the Biology of Ageing (ERIBA), University Medical Center Groningen, the Netherlands.* <sup>3</sup>*Cambridge Institute for Medical Research, Wellcome Trust-Medical Research Council Stem Cell Institute, the Department of Haematology, University of Cambridge, Cambridge, UK.* <sup>4</sup>*Sanquin Research and Landsteiner Laboratory, Department of Hematopoiesis, Amsterdam, the Netherlands.*

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## Abstract

Mutations in the Shwachman–Bodian–Diamond Syndrome (*SBDS*) gene cause Shwachman–Diamond Syndrome (SDS), a rare congenital disease characterized by bone marrow failure with neutropenia, exocrine pancreatic dysfunction and skeletal abnormalities. The *SBDS* protein is important for ribosome maturation and therefore SDS belongs to the ribosomopathies. It is unknown, however, if loss of *SBDS* functionality affects the translation of specific mRNAs and whether this could play a role in the development of the clinical features of SDS. Here, we report that translation of the *C/EBP $\alpha$*  and *C/EBP $\beta$*  mRNAs, that are indispensable regulators of granulocytic differentiation, is altered by *SBDS* mutations or knockdown. We show that *SBDS* function is specifically required for efficient translation re-initiation into the protein isoforms *C/EBP $\alpha$ -p30* and *C/EBP $\beta$ -LIP*, which is controlled by a single *cis*-regulatory upstream open reading frame (uORF) in the 5' untranslated regions (5' UTRs) of both mRNAs. Furthermore, we show that as a consequence of the *C/EBP $\alpha$*  and *C/EBP $\beta$*  deregulation the expression of *MYC* is decreased with associated reduction in proliferation, suggesting that failure of progenitor proliferation contributes to the haematological phenotype of SDS. Therefore, our study provides the first indication that disturbance of specific translation by loss of *SBDS* function may contribute to the development of the SDS phenotype.

## Introduction

The autosomal recessive disorder Shwachman–Diamond syndrome (SDS) is caused by the expression of hypomorphic alleles carrying mutations in the Shwachman–Bodian–Diamond syndrome (*SBDS*) gene<sup>1</sup>. SDS is characterized by bone marrow failure with neutropenia, exocrine pancreatic insufficiency and skeletal abnormalities<sup>2</sup>. In mice, complete loss of SBDS function is embryonic lethal<sup>3</sup>, indicating that *SBDS* is an essential gene. Over the past decade, diverse functions for SBDS have been described, including mitotic spindle stabilization<sup>4</sup>, chemotaxis<sup>5</sup>, Fas ligand-induced apoptosis<sup>6</sup>, cellular stress response<sup>7</sup> and Rac2-mediated monocyte migration<sup>8</sup>. Nonetheless, there is now compelling evidence that SBDS functions in cytoplasmic ribosome maturation<sup>9–13</sup>. Thus, SDS should be considered a ribosomopathy caused by defective maturation of the large ribosomal subunit. Studies with eukaryotic *SBDS* and its yeast ortholog *Sdo1* showed that SBDS cooperates with the GTPase elongation factor-like 1 (EFL1) to catalyze removal of the eukaryotic initiation factor 6 (eIF6) from the 60S ribosome subunit. eIF6 is critical for biogenesis and nuclear export of pre-60S subunits and prevents ribosomal subunit association. Therefore, its release is required for ribosomal subunit association during translation initiation<sup>9,10,13–15</sup>. Currently, it is not known whether *SBDS* deficiency mainly causes a general effect on mRNA translation, or whether it results in aberrant translation of specific mRNAs that contributes to the SDS phenotype.

Neutropenia is the most prominent hematopoietic abnormality seen in almost all SDS patients<sup>16</sup>. Myeloid progenitors derived from the bone marrow of SDS patients have a reduced proliferation capacity with low frequency of CD34<sup>+</sup> cells and reduced colony forming ability<sup>17</sup>.

The CCAAT enhancer binding proteins C/EBP $\alpha$  and C/EBP $\beta$  are critical transcription factors for myelomonocytic lineage commitment, granulocyte differentiation and macrophage function<sup>18–20</sup>. Expression of C/EBP $\alpha$  and C/EBP $\beta$  proteins are strictly controlled at the mRNA-translation initiation level<sup>21–23</sup>. From consecutive initiation codons in the *C/EBP $\alpha$*  mRNA three different protein isoforms are synthesized. Extended-C/EBP $\alpha$  or full-length C/EBP $\alpha$ -p42 is expressed from a cap-proximal GUG- (CUG for rodents) or AUG-codon, respectively. A shorter N-terminally truncated C/EBP $\alpha$ -p30 isoform is translated from a distal AUG-codon.

Translation from the distal AUG into C/EBP $\alpha$ -p30 requires re-association of ribosomes following translation of a *cis*-regulatory upstream open reading frame (uORF) in the 5' untranslated region (5'UTRs) of the C/EBP $\alpha$  mRNA (**Fig. 1A**)<sup>22</sup>. Extended-C/EBP $\alpha$  is not further considered here since its expression from the non-canonical GUG codon is usually very low.

C/EBP $\alpha$ -p42 expression and induction of target genes such as the *CSF3R* (colony stimulating factor 3 receptor (granulocyte)) is essential for granulocytic differentiation<sup>24</sup>. In addition, C/EBP $\alpha$ -p42 inhibits *MYC* expression, which causes proliferating myeloid precursor cells to undergo cell cycle arrest and entry into terminal differentiation<sup>25</sup>. C/EBP $\alpha$ -p30 lacks the major part of the N-terminal transactivation sequences but retains the C-terminal DNA binding domain and therefore competes with C/EBP $\alpha$ -p42 or other C/EBPs for DNA binding<sup>20</sup>. Moreover, solitary expression of C/EBP $\alpha$ -p30 blocks granulocytic differentiation and results in an Acute Myeloid Leukemia (AML) like disease in mice<sup>26,27</sup>. Translation of the *C/EBP $\beta$*  mRNA into different protein isoforms is regulated in a similar way<sup>22,28</sup>. C/EBP $\beta$ -LAP\* and C/EBP $\beta$ -LAP proteins (LAP: liver activating protein) are translated from cap-proximal AUG-codons, while the truncated C/EBP $\beta$ -LIP (LIP: liver inhibitory protein) protein is translated from a distal AUG codon through an uORF-dependent mechanism. LAP\* expression is often very low since the LAP\*-AUG codon lacks a Kozak-consensus sequence that is required for efficient translation initiation<sup>29</sup>.

Translation of the *C/EBP $\alpha$*  and *C/EBP $\beta$*  mRNAs is highly sensitive to alterations in the translation machinery. Since both factors play such a crucial role in the development of the myelomonocytic lineage, we examined whether C/EBP $\alpha$  and C/EBP $\beta$  translation is regulated by SBDS and whether *C/EBP $\alpha$*  and *C/EBP $\beta$*  isoform expression is altered by SBDS mutations. Here, we show that SBDS is required for efficient translation of the truncated p30/LIP isoforms and that lymphoblastoid cell lines derived from SDS patients have reduced C/EBP $\beta$ -LIP levels. In addition, our study suggests that SBDS deficiency indirectly suppresses the expression of *MYC* by increasing the C/EBP $\alpha$ -p42/p30 isoform ratio. Furthermore, our study suggests that this regulatory connection between SBDS, C/EBP $\alpha/\beta$  and *MYC* is decisive for myeloid cell proliferation and differentiation. Our finding is the first evidence of specific defective mRNA translation in SDS, highlights the importance of the uORF-mediated

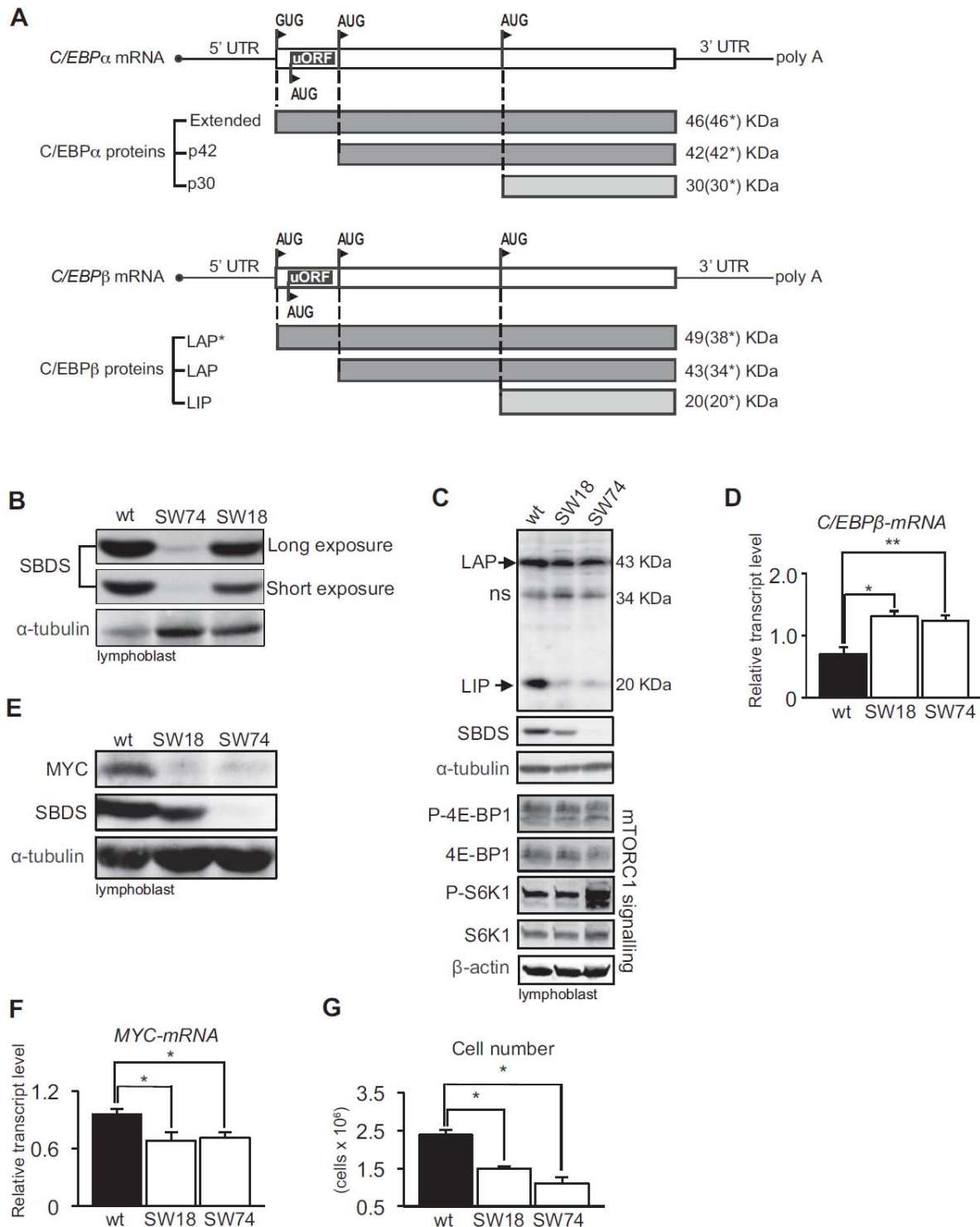
translation re-initiation mechanism that directs C/EBP $\alpha$  and C/EBP $\beta$  isoform expression for hematopoietic proliferation and differentiation control.

## Results

### **C/EBP $\beta$ -LIP expression is diminished in SDS-derived lymphoblast cells.**

To investigate whether *SBDS* mutations may affect *C/EBP*-mRNA translation, we examined C/EBP $\beta$  isoform expression in two SDS patient-derived lymphoblast cell lines compared to lymphoblasts derived from a healthy individual by immunoblotting. The SW74 cell line carries the homozygous *SBDS* mutation 258+2T>C, a common mutation in *SBDS* resulting in very low expression of wild type *SBDS* compared to the healthy control NVO12 (**Fig. 1B**); the SW18 cell line carries the heterozygous *SBDS* mutations 258+2T>C and 505C>T resulting in reduced levels of mutant *SBDS* (**Fig. 1B**)<sup>13</sup>. The C/EBP $\beta$  isoforms LAP and LIP were both expressed in the control (NVO12) cells. By contrast, LIP expression was hardly detectable in the patient-derived cells (SW18 and SW74) while LAP levels were similar to the control (**Fig. 1C**). We have shown before that expression of LIP is activated by mTORC1 signaling and that inhibition of mTORC1 results in suppression of LIP both in cell culture and in vivo<sup>22,28,31</sup>. To examine whether *SBDS* deficiency reduces mTORC1 activity and thereby regulates LIP expression we analyzed the expression and the phosphorylation status of two mTORC1 targets, the 4E-binding protein 1 (4EBP1) and the p70 ribosomal protein S6 kinase 1 (S6K1p70). *SBDS* deficiency does not significantly alter diminish the phosphorylation status of 4E-BP1 or S6K1 (**Fig. 1C, lower panels**). Thus, mTORC1 signaling and its major downstream translation control pathways through 4E-BP1 or S6K1p70 seem not to be involved in the *SBDS*-dependent regulation of LIP expression. Quantitative Real Time PCR (qRT-PCR) analysis showed that *C/EBP $\beta$*  mRNA expression was higher in the patient derived SW18 and SW74 cells (**Fig. 1D**), although this did not result in higher protein expression. Others have shown that the ratio of C/EBP $\beta$ -LAP over C/EBP $\beta$ -LIP controls expression of *Myc*<sup>32</sup>. Both *MYC* transcript 239 and protein levels were reduced in both *SBDS* mutant cell lines SW18 and SW74 compared to the NVO12 control cells (**Fig. 1E and 1F**), which was associated with a reduced proliferation capacity of the SW18 and SW74 cells (**Fig. 1E**). Hence, two different pathological

SBDS mutations correlate with reduced C/EBP $\beta$ -LIP and MYC expression and reduced proliferation.



**Figure 1. Deregulated C/EBP $\beta$  protein isoform expression in SDS.**

(A) The human C/EBP $\alpha$  and C/EBP $\beta$  mRNAs are presented with consecutive translation initiation sites (arrowheads) and each of the protein isoforms and its size (\*size of murine orthologs). Extended, p42, LAP\* or LAP proteins are expressed through regular translation initiation, omitting the uORF.



Truncated p30 or LIP proteins are expressed through translation re-initiation by post-translation ribosomes that first have translated the uORF. For detailed description of the uORFs and surrounding sequences see<sup>(21-23)</sup>. Expression of the Extended-C/EBP $\alpha$  isoform is generally weak because it uses the alternative GUG (CUG for murine) codon. Similarly, expression of the C/EBP $\beta$ -LAP\* from a non-Kozak AUG codon is mostly weak.

**(B)** SBDS protein levels were detected in SDS patient-derived lymphoblastoid cells (SW18, SW74) and wild type (wt) by immunoblotting. Long exposure shows the very low expression of wt SBDS in SW74 cells harboring the homozygous 258+2T>C mutation.

**(C)** The upper panels show immunoblots of C/EBP $\beta$  isoforms, SBDS and  $\alpha$ -tubulin as loading control in both SDS patient derived cells (SW18, SW74) and healthy control. The lower panels show immunoblots of 4E-BP1, phosphorylated-4EBP1 (P-4E-BP1), S6K1, phosphorylated-S6K1 (P-S6K1) and  $\beta$ -actin as loading control to monitor alterations in mTORC1 signaling

**(D)** qRT-PCR analysis for endogenous C/EBP $\beta$  mRNA levels in patient-derived cells (SW18, SW74) and healthy control.

**(E)** Cell multiplication assay with patient-derived cells (SW18, SW74) and healthy control. Seven days after seeding 5x10<sup>5</sup> cells, cells were harvested and counted. Immunoblots for MYC, SBDS and  $\alpha$ -tubulin (loading control) are shown underneath.

**(F)** qRT-PCR analysis of MYC transcript levels are shown. Statistical differences were determined by Student's tests. Error bars represent  $\pm$  SD (n=3), \*P<0.05, \*\*P<0.01.

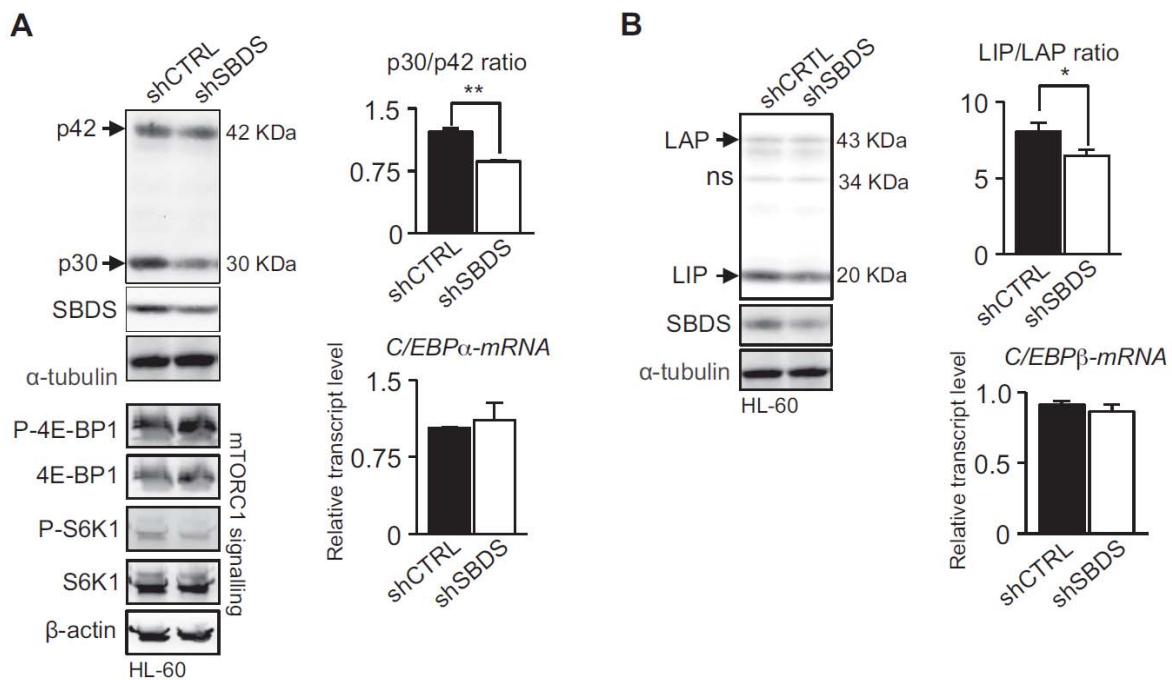
### **uORF-dependent regulation of C/EBP $\alpha$ -p30 and C/EBP $\beta$ -LIP expression by SBDS levels.**

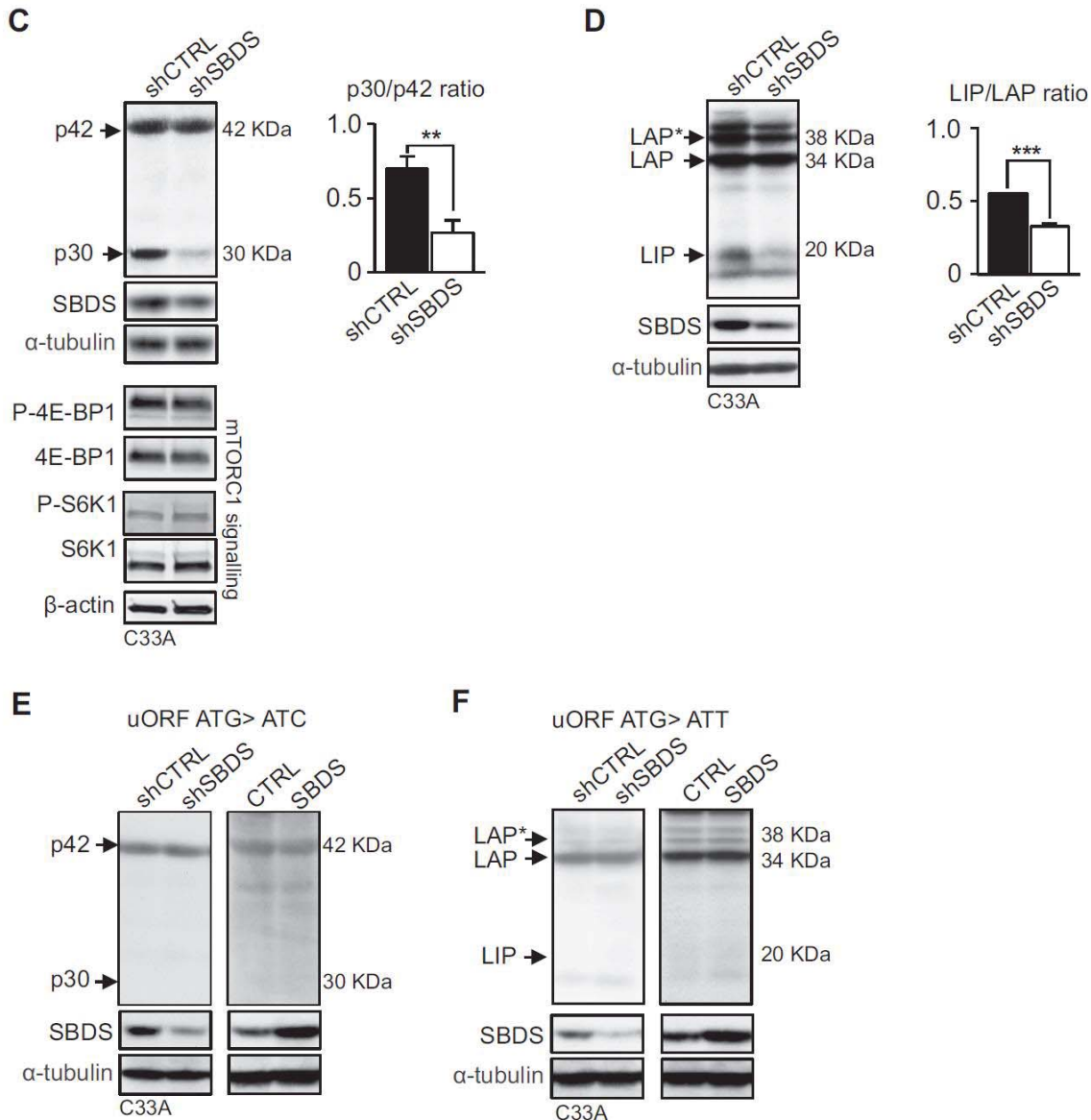
Lymphoid cells do not express C/EBP $\alpha$ . To further examine the relation between SBDS expression and C/EBP $\alpha$  and C/EBP $\beta$  expression, we performed *SBDS* knockdown in the human promyelocytic leukemia cell line HL-60 that express endogenous C/EBP $\alpha$  and C/EBP $\beta$ . *SBDS* knockdown by lentiviral *SBDS*-shRNA resulted in reduced expression of C/EBP $\alpha$ -p30 and C/EBP $\beta$ -LIP (**Fig. 2A and B**) and concomitant lower p30/p42 and LIP/LAP ratios. Similar as described for the patient derived lymphoblasts *SBDS* knockdown did not reduce phosphorylation of 4E-BP1 or S6K1, suggesting that mTORC1 signaling is not involved in the downregulation of p30/LIP upon *SBDS* knockdown (**Fig. 2A, lower panels**). No significant change was observed in C/EBP $\alpha$  and C/EBP $\beta$  mRNA levels. Overexpression of *SBDS* in HL-60 cells led to an increased expression of p30 and LIP resulting in higher LIP/LAP ratios without changing C/EBP $\alpha$  or C/EBP $\beta$  mRNA levels (**Supplementary Fig. 1A and B**). These data indicate that *SBDS* and expression of p30 or LIP might be functionally correlated.

To examine the relevance of uORF-controlled translation of the C/EBP $\alpha$  and C/EBP $\beta$  mRNAs by *SBDS* levels we transiently expressed wt cDNA constructs of rat C/*ebpa* and C/*ebpb* as well as constructs thereof carrying mutations that interfere with uORF-function in C33A human cervix carcinoma cells that expressed either

SBDS-shRNA for knockdown or SBDS-cDNA for overexpression. We used the C33A cell line for these studies because these cells tolerate high expression of p42 without inducing the usual p42-associated cell cycle arrest that would complicate analysis<sup>33</sup>. Similar to what we observed for endogenous C/EBPs, knockdown of SBDS resulted in a reduction of p30/p42 and LIP/LAP ratios through downregulation of p30 or LIP, respectively (**Fig. 2C and D**) while SBDS overexpression upregulates p30 and LIP expression (**Supplementary Fig. 1C and D**). Also in this case, SBDS knockdown did not alter mTORC1 signaling (**Fig. 2C, lower panels**).

We have shown before that the conserved uORF in the vertebrate *C/EBPα* and *C/EBPβ* mRNAs is required for translation re-initiation from the p30 or LIP translation initiation codon, respectively<sup>21,22</sup>. Mutation of the uORF-AUG into a non-AUG codon (ATC for *C/ebpα*; ATT for *C/ebpβ*) that eliminates uORF-function resulted in complete loss of p30 or LIP expression independent of *SBDS* knockdown or overexpression but did not affect p42 or LAP expression (**Fig. 2E and F**). These data indicate that regulation of p30/LIP by SBDS levels depends on the cis-regulatory function of the uORFs in the *C/EBP* mRNAs as was shown before for other translational control pathways<sup>22,28</sup>.





**Figure 2. SBDS is required for efficient C/EBP $\alpha$ -p30 and C/EBP $\beta$ -LIP expression.**

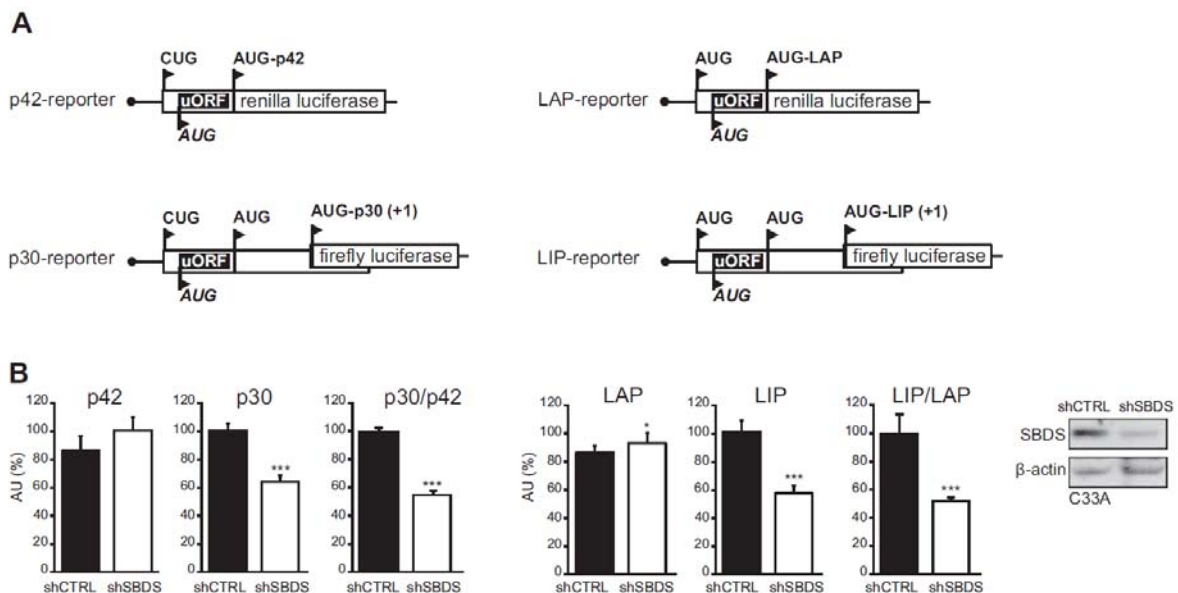
**(A)** Expression of the endogenous human C/EBP $\alpha$  (p42 and p30) or **(B)** human C/EBP $\beta$  proteins (34 kDa LAP and 20 kDa LIP) in HL-60 cells with stable SBDS knockdown (shSBDS) or control (scrambled shRNA) by immunoblotting, and quantification of p30/p42 ratio and qRT-PCR analysis of transcript levels at the right. The lower panels in (A) show immunoblots of 4E-BP1, phosphorylated-4E-BP1 (P-4E-BP1), S6K1, phosphorylated-S6K1 (P-S6K1) and  $\beta$ -actin as loading control to monitor alterations in mTORC1 signaling.

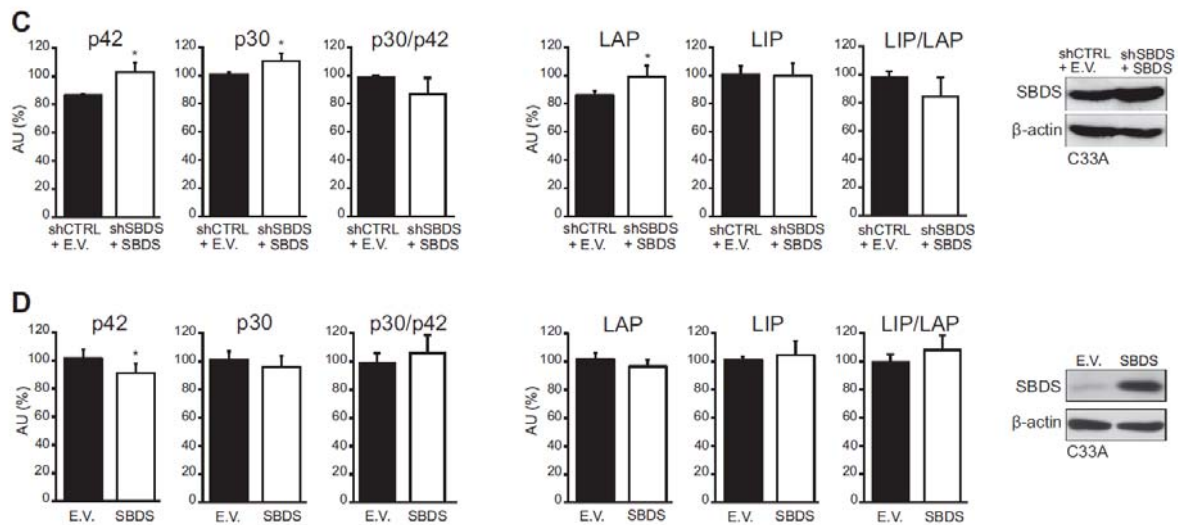
**(C)** Immunoblots of rat C/EBP $\alpha$  (p42 and p30) or **(D)** rat C/EBP $\beta$  (38 kDa LAP\*, 34 kDa LAP and 20 kDa LIP) expression in C33A cells with stable SBDS knockdown (shSBDS) and control (scrambled shRNA) transiently transfected with wild type rat *C/ebp $\alpha$*  or wild type rat *C/ebp $\beta$*  expression plasmids, respectively. Quantification of isoform ratio is shown at the top. The lower panels show immunoblots of 4E-BP1, phosphorylated-4E-BP1 (P-4E-BP1), S6K1, phosphorylated-S6K1 (P-S6K1) and  $\beta$ -actin as loading control to monitor alterations in mTORC1 signaling.

**(E)** Immunoblot of rat C/EBP $\alpha$  or **(F)** rat C/EBP $\beta$  proteins in C33A cells transiently transfected with mutated uORF-AUG (dysfunctional uORF) rat *C/ebp $\alpha$*  or *C/ebp $\beta$*  cDNA expression vectors. Statistical differences in the mRNA levels and isoform ratios were determined by student's t-tests. Error bars represent  $\pm$  SD (n=3), \*P<0.05, \*\*P<0.01, \*\*\*P<0.005.

### Loss of SBDS function specifically affects translation re-initiation

To examine the effect of SBDS function on translation initiation versus uORF mediated re-initiation we used a reporter system based on the rat *C/EBP $\alpha$* -mRNA or *C/EBP $\beta$* -mRNA structures<sup>30,34,35</sup>. Briefly, from the p42- or LAP-reporter, expression of Renilla luciferase can only be achieved by translation initiation and omission of uORF-translation, reminiscent to *C/EBP $\alpha$* -p42 or *C/EBP $\beta$* -LAP translation (**Fig. 3A**). From the p30- or LIP-reporter, expression of Firefly luciferase can only be achieved through a translation re-initiation mechanism by ribosomes that have first translated the uORF, resumed scanning and initiated at the downstream AUG codons, which is reminiscent of *C/EBP $\alpha$* -p30 or *C/EBP $\beta$* -LIP translation. In the latter case eventual translation from the *C/EBP $\alpha$* -p42 or *C/EBP $\beta$* -LAP AUG-codons do not run into the luciferase coding frames and therefore are not detected since a reading frame shift is introduced just upstream of the p30/LIP initiation sites (**Fig. 3A**)<sup>22</sup>. To normalize against effects not related to initiation/re-initiation efficiency, Renilla and Firefly luciferase expression control vectors were co-transfected along with the Renilla and Firefly reporters, respectively.





**Figure 3. Loss of SBDS function specifically affects translation re-initiation.**

**(A)** Representation of the p42- or LAP-Renilla luciferase and p30- or LIP-Firefly luciferase reporters. Renilla luciferase expression from the p42/LAP-reporters results from translation-initiation omitting the uORF, while Firefly luciferase expression from the p30/LIP reporters is achieved through uORF-765 mediated translation re-initiation<sup>30</sup>.

**(B)** C33A cells with stable *SBDS* knockdown (shSBDS) or control cells (scramble shSBDS) were transiently transfected with the p42-Renilla or p30-Firefly (graphs at the left) or LAP-Renilla or LIP-Firefly (graphs at the right) reporters, and co-transfected with respective complementary Renilla or Firefly luciferase control expression vectors. The bar graphs show luciferase values of the p42, p30 or p30/p42 ratio, and LAP, LIP or LIP/LAP ratio. Immunoblots of SBDS and  $\beta$ -actin loading control are shown at the far right.

**(C)** C33A cells with stable *SBDS* knockdown (shSBDS) were either cotransfected with empty vector control (E.V.) or SBDS expression vector and subjected to transient transfection with reporter constructs as described in (B).

**(D)** C33A cells were stably transfected with SBDS expression vector or empty vector control (E.V.) and subjected to transient transfection with reporter constructs as described in (B). Statistical differences were analyzed by Student's t-tests. Error bars represent  $\pm$  SD

C33A cell lines expressing either *SBDS*-shRNA or *SBDS*-cDNA were transiently transfected with the reporter and control constructs and used for the luciferase assay. In the SBDS knockdown cells translation initiation into p42 was not altered while translation re-initiation into p30 is strongly reduced, resulting in significant lower p30/p42 ratio (**Fig. 3B, left side**). Similarly, in the SBDS knockdown cells initiation into LAP was slightly enhanced while re-initiation was strongly reduced, resulting in significant lower LIP/LAP ratio (**Fig. 3B, right side**). Moreover, the negative effects of SBDS knockdown on p30 and LIP expression could be rescued by additional overexpression of SBDS resulting in normalized p30/p42 and LIP/LAP ratios comparable to the control (**Fig. 3C**). Contrary to the SBDS



knockdown, overexpression of SBDS did not greatly affect the levels of the individual reporters, resulting in similar p30/p42 or LIP/LAP ratios (**Fig. 3D**).

Therefore, collectively our experiments with C/EBP $\alpha$  and C/EBP $\beta$  cDNAs and the related reporter systems suggest that SBDS levels affect uORF-mediated translation re-initiation but has no significant effect on regular translation initiation.

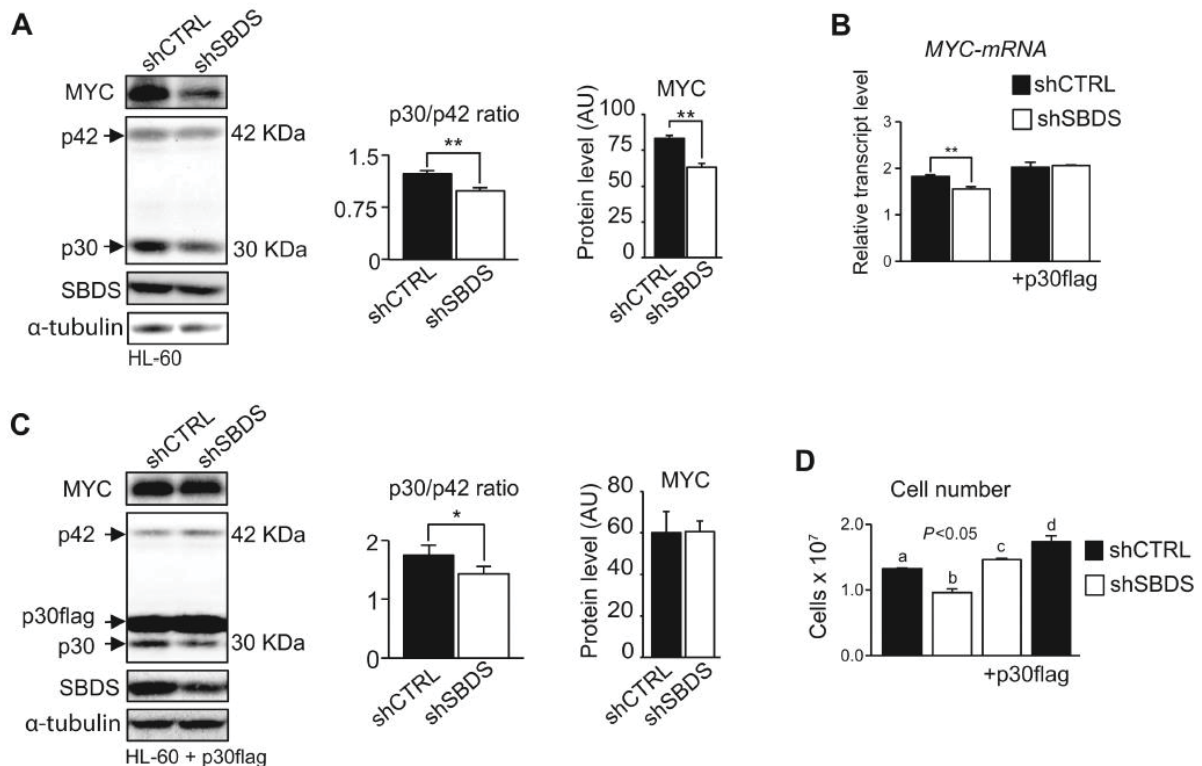
### **SBDS knockdown reduces *MYC* expression through C/EBP $\alpha$ p30/p42 ratio.**

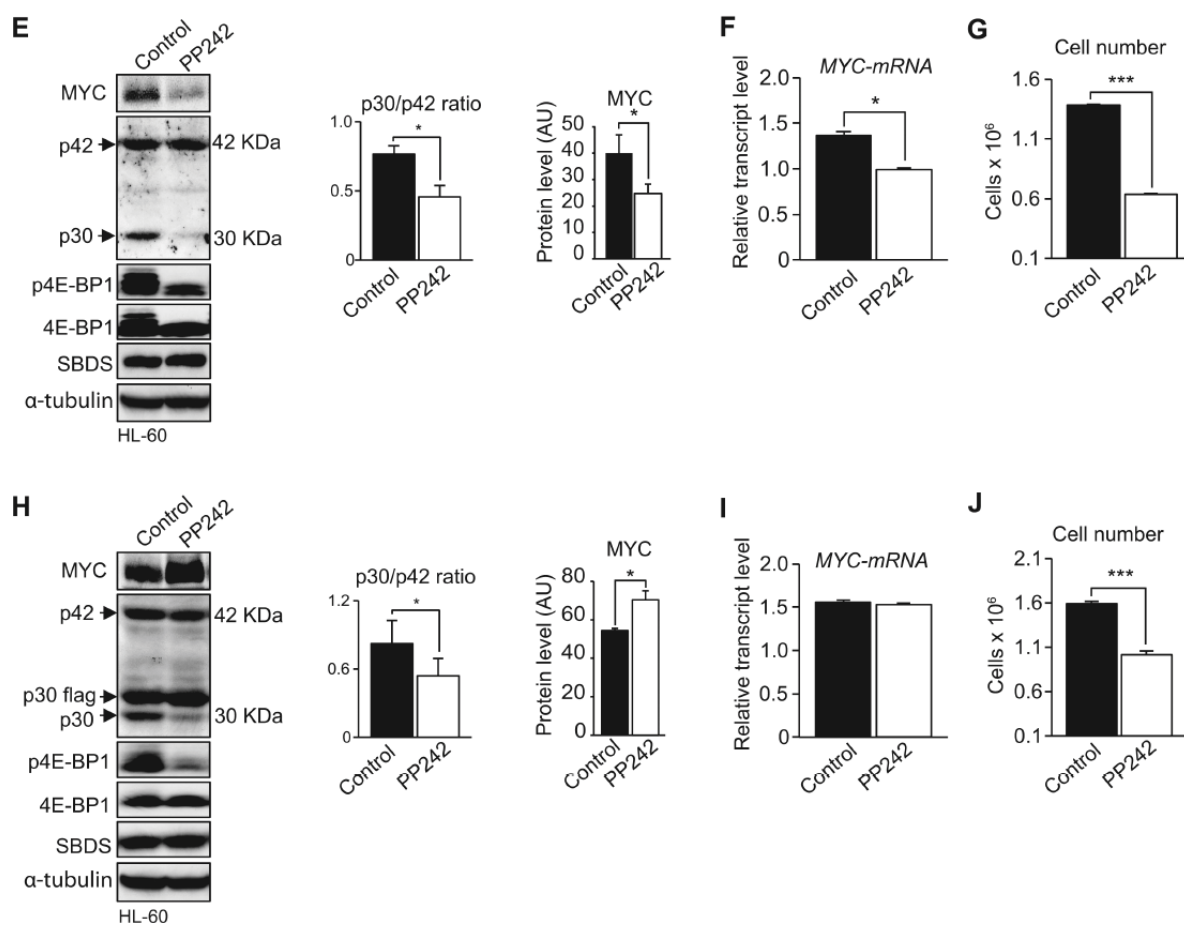
*MYC* is required for the proper balance between hematopoietic stem cell self-renewal and differentiation<sup>36</sup>. Conditional elimination of *Myc* in the bone marrow (BM) of mice results in severe cytopenia and accumulation of HSCs<sup>37</sup>. Deregulated activation of *MYC* has been found in many types of human lymphoma and leukemia<sup>38</sup>. *MYC* mRNA expression is regulated by p42 through the inhibitory interaction with E2F at the *MYC* promoter while p30 fails to do so<sup>25,39,40</sup>. For example, treatment of HL-60 cells with the growth arrest and apoptosis inducing drug CDDO (2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid) causes a transcriptional upregulation of C/EBP $\alpha$  accompanied by a translational downregulation of p30 and an increase in the p42/p30 ratio, which results in strong reduction of *MYC* mRNA levels associated with inhibition of proliferation and induction of neutrophil differentiation<sup>41</sup>. In HL-60 cells with stable SBDS knockdown and concomitant reduction of the C/EBP $\alpha$  p30/p42 and C/EBP $\beta$  LIP/LAP ratios (**Fig. 2A and B**) the expression of *MYC* protein and mRNA was decreased (**Fig. 4A and B**). To examine whether downregulation of p30 is required for *MYC* suppression we overexpressed a flag-tagged C/EBP $\alpha$ -p30 (p30flag) protein in HL-60 cells. Overexpression of p30flag rescued *MYC* protein and mRNA expression in HL-60 cells with stable SBDS knockdown (**Fig. 4C and B**), showing that *MYC* expression depends on p30. Similarly, cell multiplication was reduced upon SBDS knockdown in HL-60 cells, which could be restored by p30flag overexpression (**Fig. 4D**).

To corroborate our data and literature<sup>39</sup> suggesting that p42 inhibits *MYC* expression we examined *MYC* expression in a K562 chronic myeloid leukemia (CML) cell line stably expressing a C/EBP $\alpha$ -p42-estrogen receptor (p42ER) fusion protein<sup>39</sup>. Proteins fused to an estrogen receptor domain (ER) are sequestered in the cytoplasm through binding to heat shock proteins (HSPs). Addition of the estrogen receptor

ligand  $\beta$ -estradiol to the cell culture medium results in relocation of p42ER to the nucleus and activation of C/EBP $\alpha$ -p42-transcriptional functions. Activation of p42ER by  $\beta$ -estradiol addition resulted in strong repression of MYC mRNA and protein, which was associated with reduced proliferation capacity (**Supplementary Fig. 2A-C**). In the control experiment, activation of the unfused ER domain had no effect on MYC expression or cell proliferation (**Supplementary Fig. 2D-F**).

To substantiate the association between expression of p30 and MYC, we reduced p30 expression by inhibition of mTOR signaling<sup>22</sup>. Treatment of HL-60 cells with the mTOR inhibitor PP242 resulted in a reduced p30/p42 ratio, reduction of MYC mRNA and protein expression and reduced proliferation capacity (**Fig. 4E-G**). Overexpression of p30flag rescued both MYC mRNA and protein levels despite the inhibition of mTOR by PP242 (**Fig. 4H and I**). The proliferation capacity was partially restored by p30 overexpression (**Fig. 4J**) suggesting that the C/EBP $\alpha$  isoform ratio is decisive for MYC expression in HL-60 cells. Collectively the data suggest that MYC expression is regulated by SBDS through translational regulation of the p30/p42 C/EBP $\alpha$  isoform ratio.





**Figure 4. SBDS-dependent C/EBP $\alpha$  p30/p42 isoform ratio regulates MYC expression and cell proliferation in HL-60 cells.**

(A) Immunoblots showing endogenous MYC, C/EBP $\alpha$ , SBDS and  $\alpha$ -Tubulin (loading control) protein expression in HL-60 cells with stable knockdown of SBDS (shSBDS) or control (scrambled shRNA). Quantification of the p30/p42 isoform ratio and MYC protein levels are shown on the right panel.

(B) qRT-PCR analysis of endogenous MYC mRNA expression level in HL-60 cells and HL-60-p30flag overexpressing cells with stable knockdown of SBDS (shSBDS) or control (scrambled shRNA).

(C) HL-60 cells stably overexpressing C/EBP $\alpha$ -p30flag and knockdown of SBDS (shSBDS) or control (scrambled shRNA). Immunoblot shows endogenous MYC, C/EBP $\alpha$ , SBDS and  $\alpha$ -tubulin (loading control) protein expression. Quantification of the p30/p42 isoform ratio and MYC protein levels are shown on the right panel.

(D) Cell multiplication assay with HL-60 cells expressing scrambled shRNA (control), shSBDS (SBDS knockdown), shSBDS/C/EBP $\alpha$ -p30flag or scrambled shRNA/C/EBP $\alpha$ -p30flag. Seven days after seeding  $5 \times 10^5$  cells, cells were harvested and counted. Different letters (a, b, c, d) indicate significant differences in cell numbers determined by one way-ANOVA. Error bars represent  $\pm$  SD (n=3), P<0.05.

(E) HL-60 cells were treated with 1  $\mu$ M PP242 or DMSO as a control for 48 hours. Immunoblots show expression of MYC, p42, p30flag, p30, p4E-BP1, 4E-BP1, SBDS and  $\alpha$ -Tubulin (loading control). The phosphorylation level of 4E-BP1 indicates mTORC1 activity. Quantification of the p30/p42 isoform ratio and MYC protein levels are shown on the right panel.

(F) Endogenous MYC mRNA level by qRT-PCR.

(G) Cell multiplication assay with HL-60 cells incubated with 1  $\mu$ M PP242 or DMSO (solvent). 7 days after seeding  $5 \times 10^5$  cells, cells were harvested and counted.

(H) HL-60-p30flag overexpressing cells were treated with 1  $\mu$ M PP242 or DMSO for 48 hours and expression of MYC, p42, p30flag, p30, p4E-BP1, 4E-BP1, SBDS and  $\alpha$ -Tubulin (loading control) was



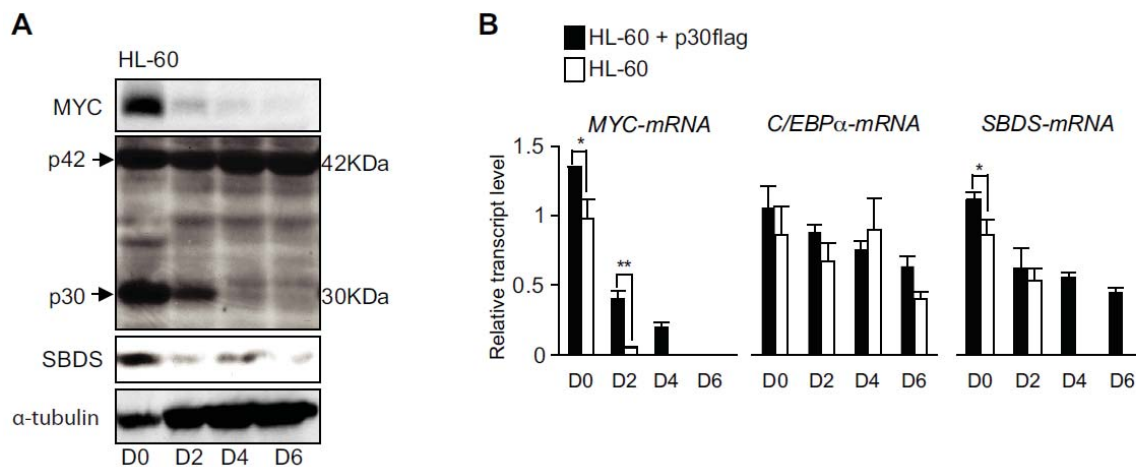
examined by immunoblotting. Quantification of the p30/p42 isoform ratio and MYC protein levels are shown on the upper panel.

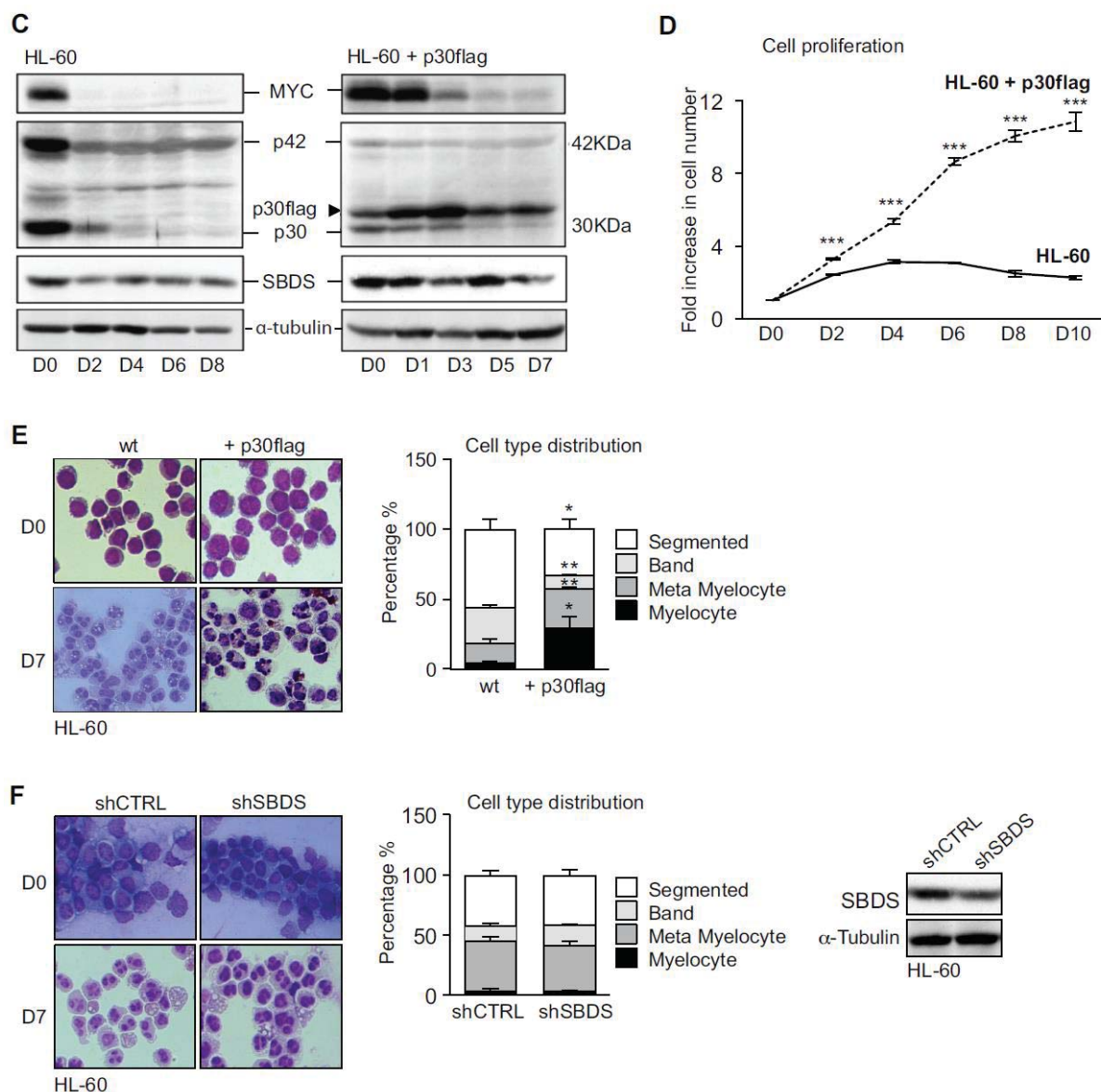
(I) and (J) Endogenous MYC mRNA transcription levels and the proliferation capacity were measured as described above. For all panels except for panel D, statistical differences were determined by Student's t-tests. Error bars represent  $\pm$  SD (n=3), \*P<0.05, \*\*P<0.01, \*\*\*P<0.005.

### Deregulation of C/EBP $\alpha$ p30 expression impedes neutrophil differentiation.

HL-60 cells differentiate into the neutrophil lineage in response to all-trans retinoic acid (ATRA) treatment. During differentiation the expression of p30, but not of p42 was reduced, resulting in a strong reduction in the p30/p42 ratio (Fig. 5A).

Transcript levels of C/EBP $\alpha$  were reduced at the final day 6 (Fig. 5B). MYC mRNA and protein levels rapidly and strongly declined during differentiation (Figure 5A and B). Intriguingly, SBDS mRNA and protein levels were also reduced during differentiation (Fig. 5A and B), as reported for other cell lines<sup>42-44</sup>. Overexpression of p30flag resulted in delayed suppression of MYC mRNA (Fig. 5A) and protein (Fig. 5C). In addition, endogenous p30 and SBDS expression are delayed (Fig. 5B and C). Furthermore, overexpression of p30flag is sufficient to sustain proliferation of HL-60 cells during ATRA treatment (Fig. 5D). Compared with control HL-60 cells, at day seven of ATRA treatment p30flag overexpression induced a significant reduction in the number of terminally differentiated, proliferation-arrested cells, with expansion of proliferative myelocytes to more than 20% (Fig. 5E). Finally, SBDS knockdown did not alter ATRA-induced differentiation of HL-60 cells (Fig. 5F).





**Figure 5. SBDS dependent C/EBP $\alpha$ -p30 expression regulates MYC expression and cell proliferation during neutrophil differentiation.**

(A) HL-60 cells undergo neutrophil differentiation with 5  $\mu$ M ATRA treatment. Immunoblots show expression of MYC, p42, p30, SBDS and  $\alpha$ -tubulin (loading control) at days 0, 2, 4 and 6 of treatment (D=days).

(B) qRT-PCR analysis of mRNA levels of MYC, C/EBP $\alpha$ , and SBDS during neutrophil differentiation.

(C) Immunoblots shows expression of MYC, p42, p30, SBDS or  $\alpha$ -tubulin (loading control) in HL-60 control cells (left) or HL-60 cells stably overexpressing C/EBP $\alpha$ -p30flag (right).

(D) Cell multiplication assay with HL-60 or HL-60 cells expressing C/EBP $\alpha$ -p30flag treated with 5  $\mu$ M ATRA at day 0. At day 0, 5x10<sup>5</sup> cells were seeded and cell numbers were determined at the indicated time points (D=day). (E) HL-60 or HL-60 expressing C/EBP $\alpha$ -p30flag treated with 5  $\mu$ M ATRA for 7 days (D7). (F) HL-60 cells expressing shSBDS for stable knockdown of SBDS or expressing control scrambled shRNA treated with 5  $\mu$ M ATRA for 7 days (D7).

(E) and (F) Giemsa staining was used for morphological analysis (magnification x200). At the right the distribution of neutrophil phenotypes is shown. All asterisks indicate significant differences between control cells and p30flag overexpressing cells or control and shSBDS expressing cells determined by Student's t-tests. Error bars represent  $\pm$  SD (n=3), \*P<0.05, \*\*P<0.01, \*\*\*P<0.005.

## Discussion

Recent studies have defined the role of SBDS in ribosome maturation<sup>9,10,13</sup>. However, so far a clear connection between reduced ribosomal SBDS function in SDS patients, deregulated translation and the disease phenotype is lacking. Our data for the first time demonstrate that translation of specific mRNAs, namely the C/EBP $\alpha$  and C/EBP $\beta$  mRNAs, is affected by loss of SBDS function. Different isoforms, full-length (p42 and LAP) and truncated (p30 and LIP), are translated from both C/EBP $\alpha$  and C/EBP $\beta$  mRNAs through the use of different translation initiation sites. Interestingly, reduced SBDS function by either shRNA mediated knockdown or by mutations in SDS patient-derived B-lymphoid cells affected in particular translation re-initiation into the truncated p30 and LIP isoforms leaving translation into the full-length isoforms by regular initiation largely unaffected. The same reduction in re-initiation but not initiation by SBDS knockdown was observed with luciferase reporters mimicking C/EBP $\alpha$ -mRNA (p42/p30 reporters) or C/EBP $\beta$ -mRNA (LAP/LIP reporters) regulation, rendering it independent of effects on protein turnover.

Moreover, re-initiation from the reporters was restored in SBDS knockdown cells by ectopic overexpression of SBDS. This suggests that reduced SBDS function does not in general affect all translation events but may specifically affect translation re-initiation. Prerequisite for translation re-initiation at the p30 and LIP initiation codons is an initial translation of a cis-regulatory small upstream open reading frame (uORF) in the C/EBP $\alpha$  and C/EBP $\beta$  mRNAs. This generates post-termination ribosomal subunits that are able to scan the mRNA further to the downstream initiation codon and re-initiate translation.

So far we do not know which step of the C/EBP $\alpha/\beta$  mRNA-specific translational event is inhibited by the reduced SBDS function. It may involve reduced recognition of the uORF initiation codon, efficiency of re-initiation at the downstream initiation codon or scanning along the mRNA following uORF translation. It seems that re-initiation after uORF translation is regulated by different or additional mechanisms compared to general cap-dependent translation initiation and we are only at the beginning of understanding the underlying mechanisms. For example, for *Drosophila* it was shown that the DENR-MCT-1 complex specifically affects re-initiation events after uORF translation of a specific set of mRNAs in proliferating cells<sup>45</sup>, and in *Arabidopsis* eIF3H is required for mTOR-mediated translation re-

initiation<sup>46</sup>. Future experiments have to address the molecular basis of the effect of SBDS depletion on *C/EBPα* and *C/EBPβ* mRNA translation. In addition it will be interesting to see whether the translation of other mRNAs harboring uORFs that promote re-initiation are sensitive to reduced SBDS function. Genome-wide ribosome profiling could reveal transcripts and specific translation modes that are affected by loss of SBDS function. SBDS promotes a conformational switch in the GTPase EFL1 that displaces eIF6 from the 60S subunit by competing for an overlapping binding site<sup>12</sup>. Displacement of eIF6 allows joining of the 60S and 40S subunits to generate actively translating 80S ribosomes. In addition, it was postulated based on in vitro reconstitution experiments that eIF6 may act in the dissociation of post-termination ribosomes and thereby stimulates ribosomal subunit recycling<sup>47</sup>. It has been shown recently that experimental reduction of eIF6 levels reduce expression levels of LIP<sup>48</sup>. The authors discuss the possibility that the anti-association activity of eIF6 is required to circumvent formation of inactive 80S after uORF translation termination and allowing post-termination 40S to re-initiate at a downstream initiation codon. If eIF6 is indeed required for keeping post-termination 40S ribosomal subunits free for re-initiation at downstream sites then SBDS would facilitate this by releasing eIF6. However, other yet to be identified mechanisms could be responsible for the regulation of p30 and LIP expression by SBDS.

Another important question addresses the biological consequence of reduced *C/EBPα* and *C/EBPβ* truncated isoform expression in the context of *SBDS* deficiency. Unfortunately, the SDS patient derived B-cells and the wt *SBDS* control cells do not express endogenous *C/EBPα*. Therefore, we examined the biological effects of reduced SBDS function in the promyelocytic leukemia cell line HL-60 in response to shRNA mediated *SBDS* knockdown because this cell line expresses both *C/EBPα* and *C/EBPβ* endogenously. Knockdown of SBDS delayed increase in viable cells counts compared to control shRNA-expressing cells, indicating that cell proliferation was attenuated in the SBDS knockdown cells. Attenuation of cell proliferation correlated with the decreased expression of the proto-oncogene MYC both at the mRNA and protein level in response to SBDS knockdown. The effect on MYC protein levels seems to be much stronger than observed for mRNA levels (**Fig. 4A, C and E**), suggesting that additional regulation at the post-transcriptional level may be involved. It has been shown earlier that *MYC* is a transcriptional target of both *C/EBPα* and *C/EBPβ*

and that the full-length isoforms act as repressors of *MYC* transcription<sup>25,32</sup>. Furthermore, alteration of the C/EBP $\alpha$  isoform ratio through treatment of HL-60 cells with the drug CDDO that downregulates p30 expression strongly reduces *MYC* mRNA levels and inhibits proliferation<sup>41</sup>, similar to what we observed in response to the reduction of p30 expression through mTOR inhibition. Therefore, we hypothesize that the altered C/EBP $\alpha$  ratio in response to SBDS knockdown leads to the inhibition of *MYC* expression, which then results in the attenuation of proliferation. This hypothesis is supported by the observation that constitutive expression of the p30flag isoform restores both *MYC* expression and proliferation in SBDS knockdown cells. Interestingly, decreased *Myc* mRNA levels were also observed in embryonic brain, liver, cartilage and bone tissue from an SDS mouse model (compound heterozygotes for *SBDS* null and *SBDS*-R126T alleles) and in the pancreas of a pancreas-specific version of this SDS mouse model. Decreased *Myc* expression in the pancreas was however shown to depend on the upregulation of p53 upon loss of SBDS function<sup>49</sup>. HL60 cells don't express functional p53 indicating that impairment of SBDS function might result in the downregulation of *Myc* and inhibition of proliferation by a different mechanism, which as we propose is the changed C/EBP isoform ratio. Both p42 and LAP have been shown to inhibit cell proliferation through repression of E2F target genes, while p30 and LIP counteract this function and thereby support proliferation<sup>31</sup> (reviewed in<sup>50</sup>). Therefore, apart from the downregulation of *Myc* the reduced expression of E2F target genes in response to the changed C/EBP $\alpha/\beta$  isoform ratio is probably involved in slowing down cell proliferation upon SBDS deficiency.

A prominent feature of SDS patients is bone marrow failure associated with neutropenia. Such a neutropenia could be caused by a failure of progenitor cells to either differentiate or to proliferate because the latter situation would strongly reduce the number of cells that undergo differentiation. Contradicting results have been published concerning the cause of neutropenia. Studies using shRNA-mediated downregulation of SBDS expression in murine hematopoietic progenitors or using an sbds deficient mouse model with deletion of SBDS predominantly in the myeloid lineage suggest that loss of SBDS interferes with terminal differentiation of neutrophils<sup>51,52</sup>. Although the exact mechanism of this differentiation block is not known, it is accompanied with reduced expression of the myeloid transcription factor



retinoid acid receptor  $\alpha$  mRNA, upregulation of p53 and induction of apoptosis<sup>52</sup>. On the other hand it was shown that hematopoietic progenitors derived from SDS patients have proliferation defects<sup>42</sup>. Similarly, SBDS knockdown in the 32D myeloblast cell line resulted in reduced proliferation without affecting differentiation<sup>44</sup>. The observation that SBDS expression is high in undifferentiated 32D cells but decreases upon differentiation<sup>44</sup> also speaks in favor for a function in proliferating cells.

*C/EBP $\alpha$*  is a decisive factor for neutrophil differentiation that at the same time is involved in proliferation control. Its deletion in mice results in a block in granulocyte differentiation and enhanced self-renewal capacity of hematopoietic stem cells<sup>53</sup>. The p42 isoform of *C/EBP $\alpha$*  induces the transcription of *CSF3R*, *C/EBP $\epsilon$* , and NE (neutrophil elastase), which are decisive for differentiation<sup>24,54</sup>. On the other hand, mutations of *C/EBP $\alpha$*  have been detected in AML patients, some of the mutations resulting in expression of only the truncated isoform and preventing expression of the p42 isoform<sup>55</sup>. Knockin mice expressing only p30 isoform develop leukemia and it has been suggested that p30 acts as a negative inhibitor of p42 by blocking the expression of differentiation related genes and by stimulating proliferation<sup>26</sup>.

Interestingly, during the differentiation of HL-60 cell we observed down regulation of SBDS expression similarly to what has been shown in 32D cells<sup>44</sup> with high levels of SBDS correlating with high levels of p30 and high levels of MYC in the early phase where the cells still proliferate. However at later stages of the differentiation process when cells stop proliferating, SBDS was downregulated concomitantly with p30 and MYC. Thus, our results support the idea that in SDS patients the reduced levels of SBDS protein preferentially affects cell proliferation and thus expansion of the early progenitor pool rather than the differentiation process *per se*.

## Materials and Methods

**Cell culture.** C33A cells were maintained in DMEM (Gibco) supplemented with 10% fetal calf serum (FCS) and 1% Penicillin/Streptomycin. HL-60 and HL-60 pCMV C/EBP $\alpha$ -p30 flag overexpressing cells were commonly maintained in RPMI (Gibco) supplemented with 10% FCS and 1% Penicillin/Streptomycin. SDS patient-derived lymphoblast cell lines including healthy wild type cell were maintained in RPMI (Gibco) with 15% FCS and 1% Penicillin/Streptomycin at 37°C with 5% CO<sub>2</sub>. For neutrophil differentiation,  $5.0 \times 10^5$  cells / ml were treated with 5  $\mu$ M ATRA (Sigma) for 7-10 days. After treatment of ATRA, cells were harvested and stained with Giemsa solution for morphologic differentiation determination. The HL-60 cells expressing C/EBP $\alpha$ -p30flag were generated by retroviral transduction with a pMSCV-neo based construct and subsequent selection of the transduced cells with G418 (1.8 mg/ml). For knockdown of SBDS in HL-60 cells the cells were transduced with the lentiviral pLKO.1 shRNA vector against human SBDS (Sigma Aldrich) or pLKO.1-scrambled shRNA as control followed by selection of the transduced cells with puromycin (1.5  $\mu$ g/ml). Cell number and viability was determined using the CASY electric field multi-channel cell counting system following the manufacturers instruction.

**Plasmid cloning and transfection.** pLKO.1 shRNA vector against human SBDS was purchased from Sigma Aldrich. shSBDS sequence: 5'-CCG AGA AAT TGA TGA GCT AAT ctc gag ATT AGC TCA TCA ATT TCT CGG-3'. For construction of the SBDS overexpression vector total mRNA was isolated from human lymphoblast cells using the RNeasy kit (Qiagen) and SBDS cDNA was synthesized using the Transcriptor First-strand cDNA Synthesis kit (Roche), which was used as a PCR template. Forward primer: 5'-GTG AAT TCA TGT CGA TCT TCA CCC CCA C-3', Reverse primer: 5'-TTT CTA GAA TCA TTC AAA TTT CTC ATC TCC TTC T-3'. The PCR product was cloned into the pLVX plasmid for lentiviral transduction using the EcoRI and XbaI restriction sites. Wild type rat C/ebp $\alpha$  and C/ebp $\beta$  cDNAs and uORF start codon mutant constructs (rat C/ebp $\alpha$ : ATG> ATC, rat C/ebp $\beta$  : ATG> ATT) were previously cloned<sup>22</sup>. C33A stable SBDS knockdown or SBDS overexpression cells were transiently transfected with wild type or mutant rat C/EBP $\alpha$  and C/EBP $\beta$  expression plasmids using FugeneHD (Roche) following the manufacturer's instructions.

**Lentiviral transduction.**  $3.5 \sim 3.8 \times 10^6$  Hek293T cells were used for co-transfection with pLKO.1 shSBDS vector (from Sigma Aldrich) or pLVX SBDS expression vector (10  $\mu\text{g}$ ) together with plasmid for viral packaging factors including pMDL-RRE (6.5 $\mu\text{g}$ ), pCMV-VSVg (3.5  $\mu\text{g}$ ) and pRSV-Rev (2.5  $\mu\text{g}$ ) were transfected using the calcium phosphate method. After 48h virus-containing medium was harvested from the transfected cells. Supernatant was collected by centrifugation and filtered using a 0.45  $\mu\text{m}$  size PVDF filter.  $5.0 \times 10^5$  cells were incubated with virus-containing supernatant with 8  $\mu\text{g}/\text{ml}$  polybrene for 24 hours. To establish stable cell lines, puromycin or G418 was added to cells in fresh medium.

**Immunoblotting.** Cells were washed with cold PBS and harvested by scraping or centrifugation. Cells were incubated with lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1mM EDTA pH 8.0, 1% TritonX-100 with Protease Inhibitors (Roche)) for 30 minutes at 4 °C or sonicated with a Bioruptor for 5 minutes at 4 °C. After centrifugation with 13000 rpm for 30 minutes at 4 °C, the supernatant was obtained and used for immunoblot analysis. The protein concentration measurement was done with the Bradford assay (BioRad). Equal amount of protein extracts were loaded to 10 or 12% SDS-PAGE and transferred to a PVDF membrane (Biorad) by semi-dry blotting for 1 hour. Membranes were washed with washing buffer (Tris buffered saline with 0.1% tween 20) and blocked for 1 hour at room temperature with blocking buffer (5% skim milk solution). The primary antibodies, SBDS (S-15), C/EBP $\alpha$  C18 (sc-9314) for human C/EBP $\alpha$ , and C/EBP $\alpha$  14AA (SC-61) for rat C/EBP $\alpha$ , C/EBP $\beta$  C19 (SC-150) for both human and rat C/EBP $\beta$ , MYC (sc-40),  $\alpha$ -Tubulin (sc-8035), 4E-BP1 (C-19) (all Santa Cruz Biotechnology) and Phosphorylated 4E-BP1 (Thr37/46) (9459), Phosphorylated p70-S6K (Thr389) (9234), p70-S6K (9202) (Cell Signaling),  $\beta$ -actin (691001) (MP Biomedicals), were diluted in blocking buffer (1:400 - 1:10000). Incubation with primary antibody was carried out at 4°C overnight using an orbital shaker. After washing with washing buffer for 10 minutes x 3 times, secondary antibody (1:5000) was added for 1 hour at RT. Protein signals were visualized using x-ray films (Amersham) with chemiluminescence detection (Perkin Elmer).

**C/EBP $\alpha$  and C/EBP $\beta$  isoform ratio measurement.** Immunoblotting was performed to visualize C/EBP $\alpha$  and C/EBP $\beta$  isoform expression as described above.



To analyze isoform ratio, band intensity in immunoblot images was measured and quantified by densitometric analysis using Alpha-imager software (Cell Bioscience) following the manufacturers instruction.

**Luciferase assay.** pcDNA3-based p42-Renilla and p30-Firefly (C/EBP $\alpha$ ) reporters were generated based on the previously described pGL3-FL-C $\alpha$  and pGL3-Tr-C $\alpha$ <sup>30</sup>: The Firefly luciferase gene was exchanged by the Renilla luciferase gene in the pGL3-FL-C $\alpha$  and both reporters were re-cloned from pGL3 into pcDNA3. For the pcDNA3-based LAP-Renilla and LIP-Firefly (C/EBP $\beta$ ) reporters, C/EBP $\beta$ -5'UTR until LAP initiation codon was cloned together with Renilla (from pGL3) in pcDNA3 and C/EBP $\beta$ - sequences spanning the 5'UTR and sequences until the LIP initiation codon with a +1frame shift (7 nt upstream of the AUG) was cloned together with Firefly in pcDNA3. The control reporters were generated by cloning Renilla (from pGL3) or Firefly (from pGL4) genes in pcDNA3. Details of the cloning strategy will be provided upon request. For the Luciferase assays 2.5 $\times$ 10<sup>3</sup>/well of C33A cells were seeded into 96 well plates. After 24 hours, cells were transfected with p42-, p30-, LAP or LIP reporters using FugeneHD (Roche). Renilla or Firefly luciferase vector were cotransfected for normalization of the reporter expression. After additional 48 hours, cells were harvested and the Stop and Glow kit (Promega) was used to measure luciferase activity.

**Quantitative Real Time PCR (qRT-PCR) of mRNA.** To detect mRNA transcription, total RNA was isolated using the RNAeasy kit (Qiagen). cDNA synthesis was performed with oligo dT primers using the Transcriptor First Strand cDNA synthesis kit (Roche). GAPDH housekeeping gene was used for normalization of gene expression. C/EBP $\alpha$ : Forward primer: 5'-GGT TTT GCT CGG ATA CTT GCC-3', Reverse primer 5'-CAC CTC ATT GGT CCC CCA G-3'; C/EBP $\beta$ : Forward primer: 5'-TTT CGA AGT TGG ATG CAA TCG-3', Reverse primer: 5'-CAA CAA GCC CGT AGG AAC AT-3'; MYC: Forward primer: 5'-TCA AGA GGT GCC ACG TCT CC-3', Reverse primer: 5'-TCT TGG CAG CAG GAT AGT CCT -3'; GAPDH: Forward primer: 5'- GTC AGT GGT GGA CCT GAC CT-3'; SBDS: Forward primer: 5'-ACG TGC TCA CAT GAG GCT T CG-3', Reverse primer: 5'-CAG CCC GGG TCA ATC AGA CAT-3'.

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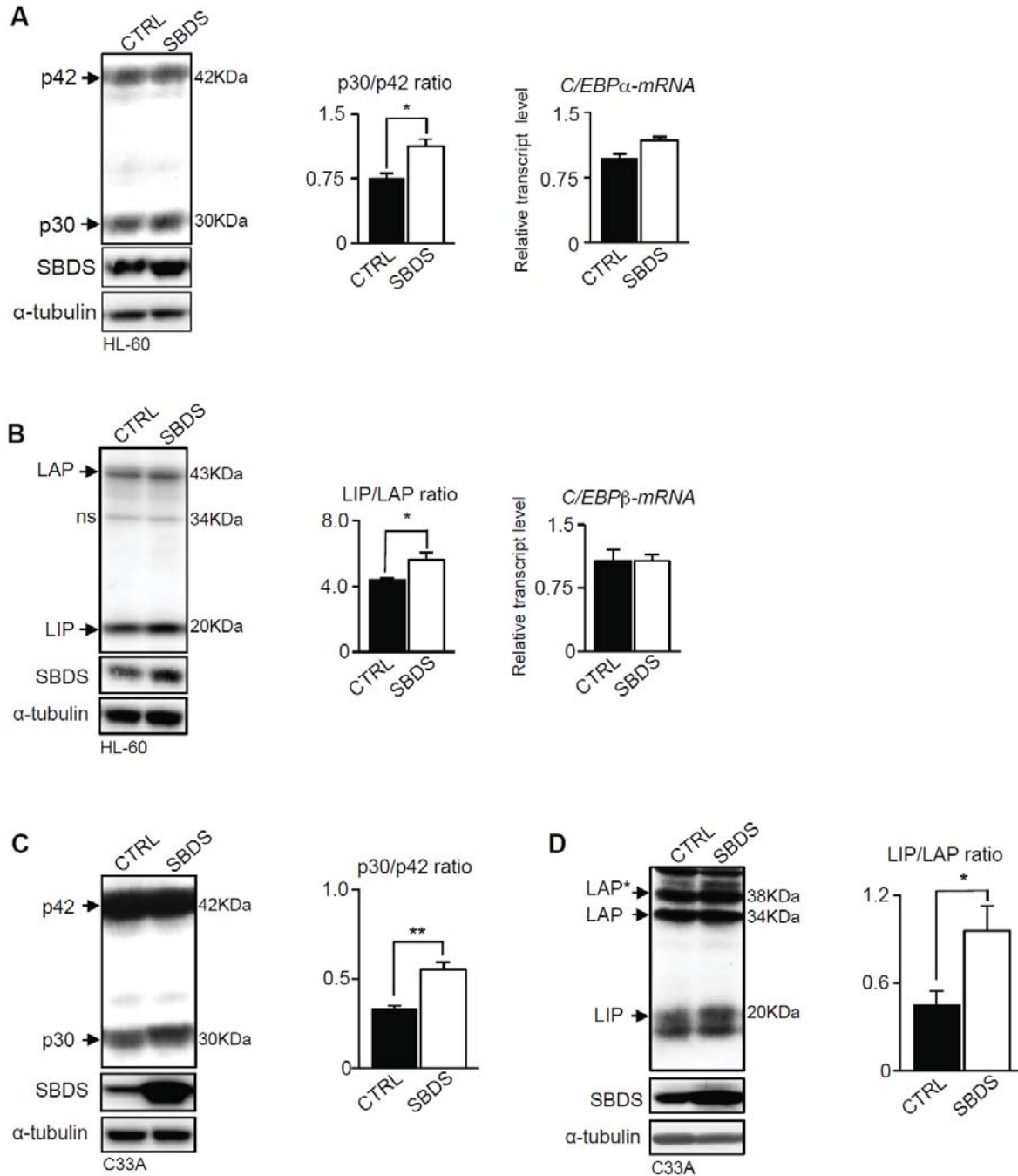
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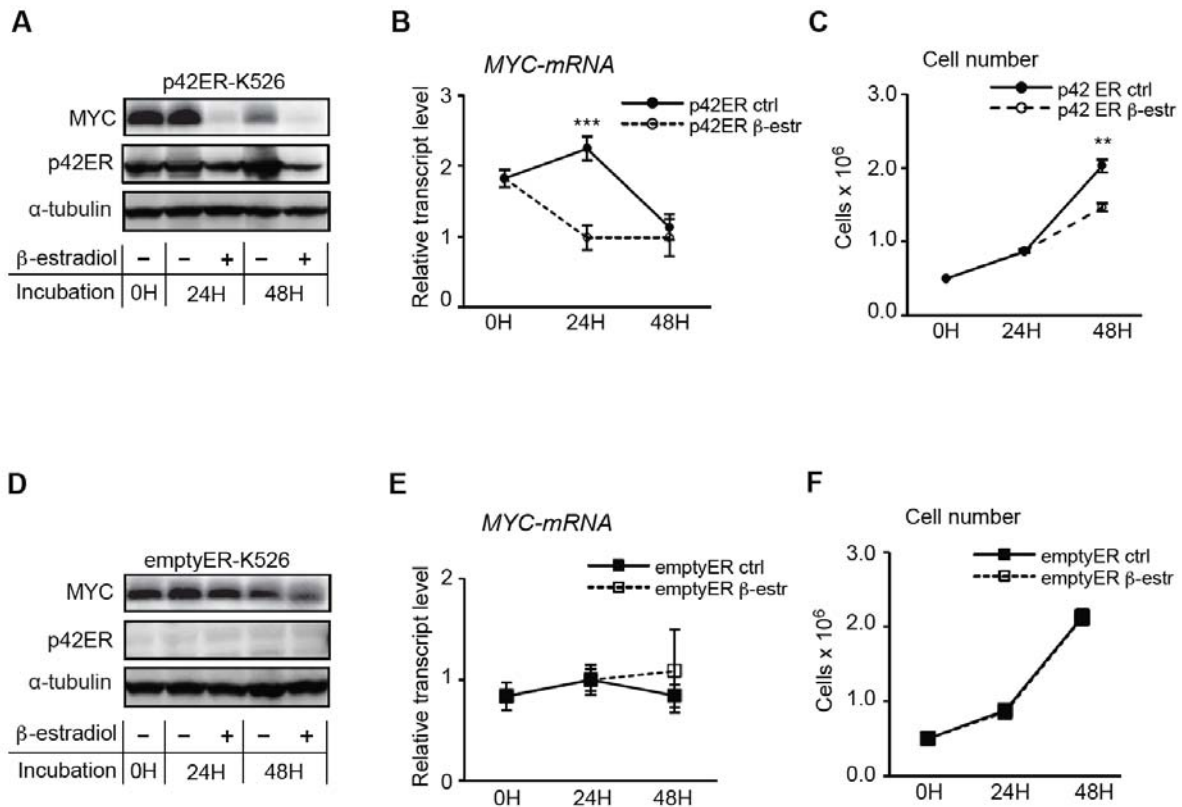
## Supplementary Figures



### Supplementary Figure 1. SBDS overexpression increases p30/p42 and LIP/LAP ratios.

(A) C/EBP $\alpha$  or (B) C/EBP $\beta$  proteins in HL-60 cells with stable SBDS overexpression (SBDS) or control (empty vector) by immunoblotting, and quantification of p30/p42 ratio and qRT-PCR analysis of transcript levels at the right.

(C) and (D) Immunoblots of C/EBP $\alpha$  or C/EBP $\beta$  expression in C33A cells with stable SBDS overexpression (SBDS) and control (empty vector) transiently transfected with wild type rat *C/ebp $\alpha$*  or wild type rat *C/ebp $\beta$*  expression plasmids, respectively. Quantification of isoform ratio is shown at the right. Statistical differences in the mRNA levels and isoform ratios were determined by Student's t-tests. Error bars represent  $\pm$  SD (n=3), \*P<0.05, \*\*P<0.01.



**Supplementary Figure 2. Activation of *C/EBP $\alpha$*  inhibits *MYC* expression, and results in downregulation of cell proliferation.**

**(A)** K562 p42ER or empty ER construct expressing cells were treated with 1  $\mu$ M  $\beta$ -estradiol, which allows p42ER or empty ER to translocate into nucleus. The expression of the MYC protein was analyzed at 24 and 48 hours after  $\beta$ -estradiol treatment by immunoblotting (24H indicates 24 hours; and 48H, 48 hours, ctrl: ethanol treated vehicle control,  $\beta$ -estr:  $\beta$ -estradiol treatment).

**(B)** The *MYC* mRNA level was determined by quantitative Real Time PCR.

**(C)** 5x10<sup>5</sup> cells were seeded in a 10 cm dish at 0H and counted at each time point after 1  $\mu$ M  $\beta$ -estradiol treatment. **(D)** The expression of the MYC protein was analyzed in empty ER construct expressing cells as described in (A).

**(E)** and **(F)** *MYC* mRNA level and cell proliferation in empty ER construct expressing cells in response to  $\beta$ -estradiol treatment were analyzed as in (B) and (C). Statistical differences in *MYC* mRNA transcription and cell proliferation (ctrl vs.  $\beta$ -estr, at the same time point) were determined by Student's t-tests. Error bars represent  $\pm$  SD (n=3), \*\*P<0.01, \*\*\*P<0.001.



