SPSB1-mediated inhibition of TGF-β receptor-II impairs myogenesis in inflammation

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Abstract

Background  Sepsis-induced intensive care unit-acquired weakness (ICUAW) features profound muscle atrophy and attenuated muscle regeneration related to malfunctioning satellite cells. Transforming growth factor beta (TGF-β) is involved in both processes. We uncovered an increased expression of the TGF-β receptor II (TβRII)-inhibitor SPRY domain-containing and SOCS-box protein 1 (SPSB1) in skeletal muscle of septic mice. We hypothesized that SPSB1-mediated inhibition of TβRII signalling impairs myogenic differentiation in response to inflammation.

Methods  We performed gene expression analyses in skeletal muscle of cecal ligation and puncture- (CLP) and sham-operated mice, as well as vastus lateralis of critically ill and control patients. Pro-inflammatory cytokines and specific pathway inhibitors were used to quantitate Spsb1 expression in myocytes. Retroviral expression plasmids were used to investigate the effects of SPSB1 on TGF-β/TβRII signalling and myogenesis in primary and immortalized myoblasts and differentiated myotubes. For mechanistical analyses we used coimmunoprecipitation, ubiquitination, protein half-life, and protein synthesis assays. Differentiation and fusion indices were determined by immunocytochemistry, and differentiation factors were quantified by qRT-PCR and Western blot analyses.

Results  SPSB1 expression was increased in skeletal muscle of ICUAW patients and septic mice. Tumour necrosis factor (TNF), interleukin-1β (IL-1β), and IL-6 increased the Spsb1 expression in C2C12 myotubes. TNF- and IL-1β-induced Spsb1 expression was mediated by NF-κB, whereas IL-6 increased the Spsb1 expression via the glycoprotein 130/JAK2/STAT3 pathway. All cytokines reduced myogenic differentiation. SPSB1 avidly interacted with TβRII, resulting in TβRII ubiquitination and destabilization. SPSB1 impaired TβRII-Akt-Myogenin signalling and diminished protein synthesis in myocytes. Overexpression of SPSB1 decreased the expression of early (Myog, Mymk, Mymx) and late (Myh1, 3, 7) differentiation-markers. As a result, myoblast fusion and myogenic differentiation were impaired. These effects were mediated by the SPRY- and SOCS-box domains of SPSB1. Co-expression of SPSB1 with Akt or Myogenin reversed the inhibitory effects of SPSB1 on protein synthesis and myogenic differentiation. Downregulation of Spsb1 by AAV9-mediated shRNA attenuated muscle weight loss and atrophy gene expression in skeletal muscle of septic mice.

Conclusions  Inflammatory cytokines via their respective signalling pathways cause an increase in SPSB1 expression in myocytes and attenuate myogenic differentiation. SPSB1-mediated inhibition of TβRII-Akt-Myogenin signalling and protein synthesis contributes to a disturbed myocyte homeostasis and myogenic differentiation that occurs during inflammation.

Keywords  Critical illness myopathy; Sepsis; Inflammation-induced muscle atrophy; SPSB1; TGFβ receptor II; Myogenic differentiation

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Introduction

Septic patients often develop intensive care unit-acquired weakness (ICUAW), which is accompanied by muscle-mass loss, increased morbidity, and mortality.\(^1,2\) ICUAW is defined as ‘clinically detected weakness in critically ill patients in whom there is no plausible aetiology other than critical illness’.\(^3\) Patients with ICUAW are classified into those with critical illness myopathy (CIM), critical illness polyneuropathy (CIP), or a combination of both. In sepsis, inflammatory cytokines, such as interleukin-6 (IL-6), IL-1\(\beta\), tumour necrosis factor (TNF), and the acute phase protein, serum amyloid A1 (SAA1), are increased in serum and skeletal muscle of patients and mice.\(^4,5\) This causes an activation of protein degradation by the ubiquitin-proteasome system (UPS) and an inhibition of protein synthesis in skeletal muscle of patients.\(^6\) We reported that by inhibiting cytokine signalling muscle atrophy in sepsis can be reduced, but not abrogated\(^4,5\) and reasoned that additional pathways must be operative, because ICU survivors still have impaired muscle function even 5 years after their illness.\(^7\) This might be due to an impaired regenerative capacity of muscle in ICU patients\(^8,9\) and septic mice\(^10\) even though a regenerative capacity via muscle residing stem cells, known as satellite cells, would be expected to be operative.\(^11\) Satellite cells are required for skeletal muscle growth and regeneration. Once activated, satellite cells undergo myogenic differentiation to form myofibers and proliferate to replenish the satellite cell pool in muscle. Orderly myogenesis requires the sequential expression of myogenic transcription factors, myoblast-fusion proteins\(^12,13\) and contractile elements.\(^14\) However, the importance of myogenesis in critical illness is not well understood. Recently, an activation of Transforming growth factor beta (TGF-\(\beta\)) signalling that is involved in myogenesis and elevated TGF-\(\beta\) receptor type II (\(\text{T}\beta\R\)) levels were reported in muscle of critically ill patients,\(^15\) suggesting their involvement in ICUAW. TGF-\(\beta\) binds to the \(\text{TN}\beta\R\) and \(\text{T}\beta\R\) complex to activate both, canonical Smad-dependent, and non-canonical signalling pathways, such as Akt that activates protein synthesis and muscle growth.\(^16\) Importantly, TGF-\(\beta\) signalling can be inhibited by the splA/ryanodine receptor (SPRY) domain and SOCS-box domain 1 (SPSB1/Spsb2) protein that targets \(\text{T}\beta\R\) for UPS-dependent degradation in neuroblastoma cells.\(^17\) Because we found SPSB1 to be strongly increased in muscle of ICUAW patients and septic mice we hypothesized that SPSB1-mediated inhibition of \(\text{T}\beta\R\)-signalling impairs protein homeostasis and subsequently myogenesis in response to inflammation, which may contribute to the impaired regenerative capacity of muscle in ICUAW patients.

Methods

Patient samples

The institutional review board of the Charité University Medicine Berlin, Germany, approved the study, and written informed consent was obtained from the patients or their legal proxy (Charité EA2/061/06; ISRCTN77569430). We analysed gene expression in vastus lateralis muscle biopsy specimens from patients prone to develop ICUAW; these patients (\(n = 7\)) were critically ill, mechanically ventilated with a SOFA-score \(\geq 8\) on three consecutive days within the first 5 days after ICU admission. Muscle biopsy specimens from healthy patients (\(n = 12\)) were used as controls (NCT 01468220). For more details on clinical data, please refer to Wollersheim et al.\(^6\)

Animal models

All animal procedures were approved by the Landesamt für Gesundheit und Soziales (Berlin, Germany; #G207/13) or the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (Rostock, Germany, #7221.3-1-074/20). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication #85-23, revised 1985), as well as the German Law on the Protection of Animals. Cecal ligation and puncture (CLP) surgery was performed in 8-week-old male B6(C)/Rj-Tyr\(^c/c\) and 12- to 16-week-old male C57BL/6 J, Nlrp3 knockout and Nlrp3 wildtype mice, as indicated and recently described\(^4,5,18\). Mice were sacrificed 24 and 96 h after surgery, and tibialis anterior (TA), gastrocnemius/plantarir (GP), extensor digitorum longus (EDL), and soleus (Soleus) muscles were harvested for analyses. For further details on experimental procedures, please refer to the supporting information.

Statistics

All experiments were performed independently and at least three times using biological triplicates each. Statistical analysis was performed using GraphPad Prism7 (GraphPad Software, Inc., USA). Differences between two groups were evaluated with unpaired two-tailed Student’s t-test. One-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test was used for comparison of more than two independent groups with only one factor. For two factors two-way ANOVA followed by Tukey’s post-hoc test was used. Data are presented as mean ± standard deviation. Photoshop (Adobe,
USA) and Illustrator (Adobe, USA) and FIJI/ImageJ software (Wayne Rasband, National Institutes of Health USA) were used for plots. $P < 0.05$ was considered statistically significant.

Results

**SPSB1 is upregulated in inflammation-induced skeletal muscle atrophy**

To identify genes involved in impaired regenerative capacity in muscle during sepsis, we performed next generation sequencing of RNAs (RNAseq) isolated from TA muscles of CLP and sham operated male wildtype (WT) mice (for details, please see Zanders et al.\textsuperscript{18}). Besides the atrophy markers Trim63/MuRF1, Fbxo32/Atrogin-1, and Fbxo30/MuSA1 (all induced $>5$-fold by sepsis, $P < 0.001$) the expression of Spsb1 was significantly increased after 24 and 96 h of sepsis (24 h: 20-fold; 96 h: 34-fold, $P < 0.001$). qRT-PCR confirmed an increased Spsb1 expression in TA of septic mice 24 and 96 h after surgery (Figure 1A). Spsb1 was also increased in GP, Soleus and EDL 24 h, and in TA and GP but not Soleus or EDL 96 h after surgery (Figure S1). Because fast twitch/type-II myofibers show the strongest atrophy response in sepsis,\textsuperscript{6} we analysed if these fibres contain higher amounts of SPSB1. Immunofluorescent staining of histological cross-sections from TA revealed that SPSB1 was enriched in type-IIa myofibers (Figure S2). Because the SPSB-protein family is composed of four well-conserved members (SPSB1, SPSB2, SPSB3, SPSB4),\textsuperscript{19} we investigated if their expression is also affected by sepsis. Spsb2 was increased in Soleus and EDL 24 and 96 h after surgery, respectively, and Spsb4 remained unchanged. Interestingly, Spsb3 expression was increased in all muscles but only 96 h after CLP surgery (Figure S1). Our data show that Spsb1 expression is increased in muscles of septic mice and shows a distinct expression pattern within the SPSB-family. We next quantitated the SPSB1 expression in muscle of ICUAW patients and found it to be increased.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Spsb1 is upregulated in inflammation-induced skeletal muscle atrophy. (A) Quantitative RT-PCR (qRT-PCR) analysis of Spsb1 from the tibialis anterior (TA) muscle in 12-week-old male C57BL/6j mice subjected to cecal ligation and puncture (CLP, $n$ = 5, 24 h; $n$ = 7, 96 h) or sham surgery (sham, $n$ = 4, 24 h; $n$ = 5, 96 h). (B) qRT-PCR of SPSB1 from the vastus lateralis muscle of patients with intensive care unit-acquired weakness (ICUAW, $n$ = 7) compared with healthy subjects (controls, $n$ = 12). (C) qRT-PCR of Spsb1 from five-day-differentiated C2C12 myotubes (MTS) treated with TNF (10 ng/mL), IL-1$\beta$ (10 ng/mL) or IL6/IL6R (100 ng/mL) for 2 h. (D) qRT-PCR of Spsb1 from MTS that were pretreated with the IKK1-inhibitor BMS-345541 (5 $\mu$M) 60 min before TNF or IL-1$\beta$ treatment. (E) qRT-PCR of Il1st and Spsb1 from MTS after Il1st-siRNA transfection and pretreatment with the JAK2 inhibitor AG490 or the STAT3 inhibitor C188–9 prior to IL-6 treatment. (F) qRT-PCR analysis of Spsb1 from the TA muscle of Nlrp3 knockout (KO) and Nlrp3 wildtype (WT) littermate control mice subjected to CLP ($n$ = 7) or sham ($n$ = 5) surgery for 96 h, as indicated. mRNA expression was normalized to Gapdh. Data in panels (A) and (D–F) were analysed with two-way ANOVA followed by Tukey’s post-hoc test; data in panel (B) were analysed with two-tailed Student’s $t$-test; data in panel (C) were analysed with one-way ANOVA followed by Tukey’s post-hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. n.s. denotes not significant.
when compared with healthy controls (Figure 1B). We also observed an increased SPSB2 and SPSB3, but not SPSB4 expression in muscle of ICUAW patients (Figure S3).

To investigate if Spsb1 is expressed in myocytes and if inflammatory cytokines regulate its expression, we treated 5-days-differentiated myotubes (MT5) with TNF, IL-1β, and IL-6/IL-6R for different time points from 1 to 72 h (Figure S4A), respectively, and measured Spsb1 expression. We also treated MT5 with SAA1 and LPS for 72 h. All inflammatory cytokines, SAA1 and LPS increased Spsb1 expression in MT5 (Figure S4A,B). The highest increase in Spsb1 expression was observed after 2 h of cytokine treatment (Figure 1C), which was used for further analyses. We treated differentiating C2C12 myoblasts with TGF-β and found it to also increase Spsb1 expression after 24 and 72 h (Figure S4C). We next investigated the signalling pathways involved in cytokine-induced Spsb1 expression. TNF- and IL-1β-induced Spsb1 expression was inhibited by the IkB kinase-inhibitor BMS-345541 (Figure 1D) indicating that this effect was mediated by NF-κB. To investigate how IL-6 increased Spsb1 expression we reduced the IL-6 receptor glycoprotein (gp) 130, encoded by Il6st, by siRNA prior to IL-6 treatment in MT5, which attenuated IL-6-induced Spsb1 expression (Figure 1E). The Jak2 inhibitor AG490 and the STAT3 inhibitor C188-9 also attenuated IL-6-induced Spsb1 expression (Figure 1E) indicating that the gp130/Jak2/STAT3 pathway mediated this effect. We previously showed that IL-1β serum levels and muscular Il6 expression were reduced in septic Nlrp3 KO mice, and that these mice are protected from muscle atrophy in sepsis.4 In accordance with these findings, Spsb1 expression increased in TA muscle of septic Nlrp3 WT but not in Nlrp3 KO mice (Figure 1F), further supporting that proinflammatory cytokines, especially IL-1β, increase muscular Spsb1 expression. In summary, our data show that inflammation in vivo and proinflammatory cytokines in vitro cause an increase in Spsb1 expression in muscle and myocytes, respectively.

**SPSB1 interacts with and increases the turnover of TGF-β receptor II**

SPSB1 is a cullin-5 E3 ubiquitin ligase adaptor20 and associates with and degrades the TβRII in non-muscle cells.17 To analyse changes in TβRII in muscle during sepsis, we performed immunofluorescent staining of histological cross sections from TA of sham- and CLP-operated mice 96 h after surgery. We found that TβRII was ubiquitously expressed in all myofibers and was localized to the sarcolemma in sham-operated mice. In contrast, membranous TβRII-staining was reduced in myofibers of CLP mice (Figure S5) suggesting an association between increased Spsb1 expression and a decrease in its putative target TβRII in myofibers during sepsis. Consistently, our RNAseq data showed a significant downregulation of genes contained in the Gene Ontology (GO) term ‘cellular response to transforming growth factor beta stimulus’ (GO:0001560, P = 8.84*10⁻⁵, FDR 0.002; Figure S6) and the Kyoto Encyclopedia of Genes and Genomes (KEGG)-pathway TGF-beta signaling pathway (mmu04350, P = 5.4*10⁻⁴, FDR 0.0038, Figure S7A,B).

We next investigated if SPSB1 regulates TβRII function in myocytes. We identified two transcript variants of TβRII, full-length TβRII and TβRII devoid of exon2 (TβRII-ΔEx2) in myocytes. Using co-immunoprecipitation analyses we found that SPSB1 physically interacted with both TβRII and TβRII-ΔEx2 to a similar degree (Figure 2A). SPSB1 and both TβRII variants were localized to the cytoplasm and the membrane of C2C12 cells (Figure S8A), where they also co-localized (Figure 2B). Endogenous TβRII and overexpressed SPSB1 colocalized in C2C12 myoblasts (Figure S8B). To investigate if the spla/ryanodine receptor (SPRY) or the SOCS-box domain in SPSB1 that are both important for protein–protein interaction17 mediate its association with TβRII we generated two SPSB1-SPRY domain mutants (Y129A and T160A/Y161A (TYAA)) and a mutant lacking the SOCS-box (ΔSOCS) (Figure 2C). The SPSB1-SPRY domain mutants have been shown to reduce the interaction between SPSB1 and its substrates.17 The SOCS-box functions as a substrate recognition component of SCF-like E3 ligases.17 Co-immunoprecipitation analyses showed that SPSB1-ΔSOCS strongly associated with TβRII in C2C12 cells, whereas the interaction between SPSB1-Y129A and SPSB1-TYAA with TβRII was reduced when compared with SPSB1 WT (Figure 2D). These data indicate that the SPRY-domain of SPSB1 is involved in its interaction with TβRII in myocytes. Ubiquitination assays showed that SPSB1 increased the ubiquitination of TβRII, which was lower in SPSB1-ΔSOCS expressing cells indicating that SPSB1 mediates ubiquitination of TβRII via its SOCS-box domain (Figure 2E). Cycloheximide (CHX) chase assays revealed that SPSB1 WT (Figure 2F) but not SPSB1-ΔSOCS reduced the half-life of endogenous TβRII in C2C12 cells (Figure S8C) indicating that SPSB1 via its SOCS-box increases the turnover of TβRII. These data suggest that SPSB1 interacts with TβRII, mediates its ubiquitination and reduces its stability.

**SPSB1 inhibits TGF-β signalling by targeting TGF-β receptor II**

We next investigated if SPSB1-mediated targeting of TβRII influences canonical Smad-dependent and non-canonical Smad-independent (i.e. Akt) TGF-β signalling pathways.21 SPSB1 caused a nuclear-to-cytoplasmic translocation of Smad3 in C2C12 cells (Figure S9A,B), which was predominantly localized in the nuclei in GFP-control cells. This was accompanied by a reduced expression of the Smad3-target gene Smad7 (Figure S9C). Likewise, and as expected,21 TGF-β treatment increased phosphorylation of Akt (Ser473),
which was attenuated by SPSB1 in C2C12 cells (Figure 3A). These data indicate that SPSB1 inhibits both canonical and non-canonical TGF-β signalling. Because Akt regulates protein synthesis and differentiation, which is decreased in muscle of ICUAW patients, and because our RNA-seq data revealed an inhibition of Akt-signalling in muscle of septic mice, we focused on this pathway for further analyses.

**SPSB1 regulates TGF-β signalling**

We next investigated if the association of SPSB1 with TβRII is necessary for inhibition of Akt-signalling. SPSB1 inhibited Akt (Ser473) phosphorylation in C2C12 cells, which was not observed for any of the SPSB1 mutants (Figure 3B). Smad3 was localized to the cytoplasm of SPSB1 transduced cells.
Figure 3 SPSB1 downregulates TGF-β signalling by its SPRY and SOCS-box domain and inhibits myogenic differentiation. (A) Five-day-differentiated C2C12 myotubes (MT5) transduced with GFP or SPSB1 were treated with TGF-β (5 ng/mL) or solvent control for 5 min. Lysates were analysed by Western blot analysis with anti-phospho Akt antibody (Ser473). Total Akt was used as control (left panel). Densitometric analysis (right panel). Data were analysed with two-way ANOVA followed by Tukey’s post-hoc test. **P < 0.01, ***P < 0.001. (B) C2C12 cells were transduced by control GFP, SPSB1 (WT) or mutants (SPSB1-Y129A, -TYAA or -ΔSOCS) retrovirus and differentiated for 5 days. Western blot analysis with anti-phospho Akt antibody (Ser473) (left panel) and densitometric analysis (right panel). Total Akt was used as control. Data were analysed with one-way ANOVA followed by Tukey’s post-hoc test. Asterisk (*) indicates significant differences between SPSB1 (wildtype or mutants as indicated) and the GFP control group, *P < 0.05, **P < 0.01, ***P < 0.001; # denotes a significant difference between indicated SPSB1 mutants and the SPSB1 wildtype group, #P < 0.05, ##P < 0.01, ###P < 0.001. (C) O-Propargyl-puromycin (OP-puro) labelling of de novo synthesized polypeptides. Scale bar, 100 μm. (D) Experimental design. (E) Protein lysates from MT5 were analysed by Western blot with anti-FLAG and anti-GAPDH antibody. (F) Immunofluorescent staining with anti-Fast MyHC antibody. Nuclei were stained with DAPI (blue). GFP (green) indicates retrovirally transduced cells. Scale bar, 50 μm.
but remained nuclear in SPSB1-mutant transduced cells (Figure S9A,B). Accordingly, SPSB1 but none of the SPSB1-mutants caused a reduction in Smad7 expression (Figure S9C). Using the O-Propargyl-puromycin (OP-puro) assay we found that protein synthesis was substantially decreased by SPSB1 overexpression in C2C12 cells (Figure 3C) when compared with controls. In contrast, SPSB1 mutants had only a minor effect on protein synthesis (Figure 3C) indicating that both the SPRY- and the SOCS-box domains are necessary to inhibit canonical and non-canonical TβRII signalling and protein synthesis.

**SPSB1 overexpression inhibits myogenic differentiation**

We consistently observed that SPSB1 overexpression inhibited myotube formation (Figure 3C,F). To investigate this phenotype, we retrovirally transduced SPSB1-IRES-GFP or IRES-GFP-control into C2C12 myoblasts and assessed myogenic differentiation (workflow in Figure 3D,E, Figure S10A), which was indeed inhibited by SPSB1 (Figure S10B). Immunocytochemistry revealed that SPSB1 overexpressing cells contained less fast myosin heavy chain protein (MyHC), a marker of terminal differentiation, were thinner and shorter, and comprised fewer nuclei when compared with GFP control cells on differentiation day 5 (Figure 3F). Quantification of differentiation and fusion indices, and the number of nuclei per myosin+-cell showed that SPSB1 transduced cells did not differentiate and remained mono-nucleated (Figure 4A,B). SPSB1 overexpression caused a reduction of the differentiation markers Myogenin/Myog, Myomaker/Mymk and Myomerger/Mymx and MyHC/Myh throughout differentiation (Figure S10C). Accordingly, Myogenin, and fast and slow MyHC were reduced in SPSB1 overexpressing cells (Figure 4B). These data show that SPSB1 inhibits both myoblast fusion and myogenic differentiation preventing myotube formation.

These effects of SPSB1 could be involved in the impaired regenerative capacity found in muscle of ICU patients and septic mice. During regeneration muscle residing stem cells, called satellite cells, provide myogenic cells that proliferate, differentiate, fuse, and form new functional myofibers. Therefore, if SPSB1 is involved in myogenic differentiation it should be contained in satellite cells. To test this hypothesis, we analysed single-cell RNAseq data from the Tabula Muris Consortium and found Spsb1 but not Spsb2, Spsb3, or Spsb4 to be enriched in satellite and mesenchymal stem cells in murine limb muscles (Figure S11A,B). Because proinflammatory cytokines increased Spsb1 expression, we reasoned that they would also inhibit myogenic differentiation. Indeed, when we treated C2C12 myoblasts with TNF, IL-1β, and IL-6/IL-6R, respectively, for 5 days myogenic differentiation was greatly perturbed (Figure 4C,D), which was also reflected by a decreased expression of late differentiation markers (Myh1, Myh3, Myh7) (Figure 4E).

In contrast to SPSB1 WT, overexpression of the SPSB1-SPRY and SPSB1-SOCS box mutants had only a minor effect of myogenesis (Figure 5A,B), which was supported by qRT-PCR (Figure S12A) and Western blot (Figure S12B,C) analyses for early (Myog, Mymk, Mymx) and late (Myh1, Myh3, Myh7) myogenic markers. SPSB1 overexpression also inhibited myogenic differentiation in commercial mouse skeletal muscle myoblasts, which was not observed for any of the SPSB1-mutants (Figure 5C,D). Our data suggest that SPSB1 attenuates myogenic differentiation by inhibition of TβRII signalling and that this effect depends on its SPRY- and SOCS-box domains.

**TGF-β signalling is essential for myogenesis**

To further investigate if TGF-β via TβRII plays a role in myogenesis, we examined the amount of TβRII protein and Tgfb1 gene expression in differentiating C2C12 cells and found them to be increased (Figure S13A,B). Immunohistochemistry revealed that TβRII was enriched in myosin+-myotubes (Figure S13C). To explore if TβRII is essential for myogenesis, we used the TβRII-specific inhibitor inducer of TβRII receptor degradation-1 (ITD-1). ITD-1 caused a dose-dependent reduction of TβRII (Figure S14A), a decreased Akt (Ser473) phosphorylation (Figure S14B) and a reduced protein synthesis (Figure S14C) in C2C12 cells when compared with vehicle-treated cells. ITD-1 impaired myogenesis as shown by shorter myosin+-myotubes with fewer nuclei (Figure S14D), inhibited myoblast fusion and differentiation (Figure S14E) and decreased the expression of early (Myog, Mymx) and late (Myh3) myogenic markers (Figure S13C,D) and Myogenin protein content in differentiating C2C12 cells (Figure S14FA). The amount of slow-twitch but not fast-twitch MyHC was reduced by ITD-1 (Figure S14F). ITD-1 also caused an increase in some myogenic markers (Myh1, Myh7) (Figure S13D). These data suggest that TGF-β/TβRII-Akt-signalling plays a role in protein synthesis and myogenesis.

**Akt restores myogenesis in SPSB1 overexpressing cells**

As SPSB1-mediated inhibition of TβRII-Akt signalling inhibited myogenesis, we tested if co-expression of myristylated, constitutively active Akt (Akt-Myr) restores myogenesis in SPSB1 overexpressing cells. Transduction of Akt-Myr resulted in an increased Akt (Ser473) phosphorylation (Figure 6A), an increased protein synthesis and an elevation of fast-twitch MyHC in C2C12 cells (Figure 6B,C). Akt-Myr reverted the inhibitory effects of SPSB1 on protein synthesis (Figure 6B),
**Figure 4** SPSB1 and proinflammatory cytokines inhibit myogenic differentiation. (A) Differentiation index, fusion index, and nuclei distribution in myosin positive (myosin+) cells were quantified from images in Figure 3 panel (F). (B) Western blot analysis of lysates from GFP and SPSB1 transduced cells that were differentiated for 5 days with indicated antibodies. GAPDH was used as loading control. Densitometric analysis is shown in the right panel. Data in panel (A; Differentiation and fusion index) and (B) were analysed with two-tailed Student’s t-test; data in panel A (myosin+ cells) were analysed with two-way ANOVA followed by Tukey post hoc test; asterisk (*) indicates a significant difference of SPSB1 (WT) or mutants compared with GFP group, *P < 0.05, **P < 0.01, ***P < 0.001; * indicates a significant difference of SPSB1 mutants compared with SPSB1 (WT) group, #P < 0.05, ##P < 0.01, ###P < 0.001. N = 3 biologically independent experiments; data are presented as mean ± standard deviation. (C) Immunofluorescent staining of 5 days differentiated C2C12 cells treated with TNF (10 ng/mL), IL-1β (10 ng/mL) or IL6/IL6R (100 ng/mL), as indicated, every 24 h throughout differentiation, with anti-fast MyHC antibody. Nuclei were stained with DAPI (blue). Scale bar, 100 μm. (D) Differentiation index, fusion index, and nuclei distribution in myosin+ cells were quantified from images in panel (C). (E) qRT-PCR analysis of Myh1, Myh3, and Myh7 mRNA expression was normalized to Gapdh. Data in panel (D; Differentiation and fusion index) were analysed with two-tailed Student’s t-test; data in panel (D; myosin+ cells) and (E) were analysed with two-way ANOVA followed by Tukey post hoc test; *P < 0.05, **P < 0.01, ***P < 0.001. N = 3 biologically independent experiments; data are presented as mean ± standard deviation.
Figure 5  SPSB1 mediated inhibition of myogenic differentiation depends on its SPRY and SOCS-box domain. (A, B) C2C12 myoblasts were transduced by control GFP, SPSB1 (WT) or mutant (SPSB1-Y129A, -TYAA or -ΔSOCS) containing retrovirus and differentiated for 5 days. (A) Immunofluorescent staining with anti-fast MyHC antibody. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. (B) Differentiation index, Fusion index, and Nuclei distribution in each myosin+ cell were quantified from images in panel (A). (C, D) Primary myoblasts were transduced by control GFP, SPSB1 (WT) or mutant (SPSB1-Y129A, -TYAA or -ΔSOCS) containing retrovirus and differentiated for 5 days. (C) Immunofluorescent staining with anti-fast MyHC antibody (red). GFP (green) indicates retrovirally transduced cells. Scale bar, 100 μm. (D) Differentiation index, Fusion index, and Nuclei distribution in each myosin+ cell were quantified from images in panel (C). Data in panels (B and C; Differentiation and Fusion index), were analysed with two-tailed Student’s t-test; data in panels (B) and (H) (Nuclei distribution in myosin+ cells) were analysed with two-way ANOVA followed by Tukey’s post-hoc test; asterisk (*) indicates significant differences between SPSB1 (wildtype or mutants as indicated) and the GFP control group, *P < 0.05, **P < 0.01, ***P < 0.001; denotes a significant difference between indicated SPSB1 mutants and the SPSB1 wildtype group, †P < 0.05, ‡P < 0.01, ††P < 0.001. N = 3 biologically independent experiments; data are presented as mean ± standard deviation.
Figure 6 Expression of Akt restores myogenesis in SPSB1 overexpressing cells. Cells were transduced by control GFP, SPSB1, Akt-Myr, respectively, or co-transduced by Akt-Myr and SPSB1 retrovirus and differentiated for 5 days. (A) Western blot analysis of anti-phospho Akt antibody (Ser473). Total Akt was used as control. Densitometric analysis is displayed in the right panel. (B) Cells described above were incubated with OP-puro labelling for 1 h. Red fluorescence (upper panel) corresponds to de novo synthesized polypeptides. (C) Immunofluorescent staining of above cells with anti-Fast MyHC as primary antibody and Alexa Fluor 555 conjugated secondary antibody (red). Scale bar, 100 μm. (D) Differentiation index, Fusion index, and Distribution of nuclei in myosin positive (myosin+) cells were quantified from images in panel (C). (E) Western blot was performed using lysates from above cells with anti-Fast MyHC and anti-Slow MyHC antibody. Overexpressed Akt-Myr and SPSB1 were detected by anti-HA and anti-FLAG antibody, respectively. GAPDH was used as loading control. Densitometric analysis of Western blot signals are displayed (right panel). Data in panels (A), (D; differentiation and fusion index) and (E) were analysed with one-way ANOVA followed by Tukey’s post-hoc test; data in panel (D; myosin+ cells) were analysed with two-way ANOVA followed by Tukey’s post-hoc test. Asterisk (*) indicates a significant difference between SPSB1-, Akt-Myr- or SPSB1 and Akt-Myr-treated groups compared with GFP control treated cells, *P < 0.05, **P < 0.01, ***P < 0.001; # indicates a significant difference between SPSB1- and SPSB1 + Akt-Myr-treated cells, #P < 0.05, ##P < 0.01, ###P < 0.001. N = 3 biologically independent experiments; data are presented as mean ± standard deviation.
Myogenin restores Akt signalling and protein synthesis in SPSB1 overexpressing cells. C2C12 cells were transduced by control GFP, SPSB1, Myogenin, respectively, or co-transduced by Myogenin and SPSB1 retrovirus and differentiated for 5 days. (A) Western blot with anti-Myc antibody. GAPDH was used as loading control. (B) qRT-PCR analysis of Myog mRNA expression normalized to Gapdh. (C) Western blot analysis with anti-phospho Akt antibody (Ser473). Total Akt was used as control. Densitometric analysis is shown in the right panel. (D) O-Propargyl-puromycin (OP-puro) assay: Red fluorescence (upper panel) corresponds to de novo synthesized polypeptides. (E) qRT-PCR analysis of Mymk, Mymx, Myh1, Myh3, and Myh7 from described cells. mRNA expression was normalized to Gapdh. Data were analysed with one-way ANOVA followed by Tukey’s post-hoc test. Asterisk (*) indicates a significant difference between SPSB1-, Myogenin- or SPSB1 + Myogenin- and GFP control groups, *P < 0.05, **P < 0.01, ***P < 0.001; # indicates a significant difference between SPSB1- and SPSB1 + Myogenin-treated cells, #P < 0.05, ##P < 0.01, ###P < 0.001. N = 3 biologically independent experiments; data are presented as mean ± standard deviation.
Role of SPSB1 in inflammatory muscle atrophy

Figure 8  Myogenin restores myogenesis in SPSB1 overexpressing cells. (A–C) C2C12 cells were transduced by control GFP, SPSB1, Myogenin, respectively, or co-transduced by Myogenin and SPSB1 retrovirus and differentiated for 5 days. (A) Immunofluorescent staining of above cells with anti-Fast MyHC as primary antibody and Alexa Fluor 555 conjugated secondary antibody (red). Scale bar, 100 μm. (B) Differentiation index, Fusion index, and Distribution of nuclei in myosin positive (myosin+) cells were quantified from images in panel (A). (C) Western blot was performed using lysates from above cells with anti-Fast MyHC and anti-Slow MyHC antibody. Overexpressed SPSB1 was detected by anti-FLAG antibody. GAPDH was used as loading control. Densitometric analysis of Western blot signals are displayed (right panel). Data in panels (B; Differentiation and Fusion index) and (C) were analysed with one-way ANOVA followed by Tukey’s post-hoc test; data in panel (B; myosin+ cells) were analysed with two-way ANOVA followed by Tukey’s post-hoc test. Asterisk (*) indicates a significant difference between SPSB1-, Myogenin- or SPSB1 + Myogenin- and GFP control groups, *P < 0.05, **P < 0.01, ***P < 0.001; # indicates a significant difference between SPSB1- and SPSB1 + Myogenin-treated cells, #P < 0.05, ##P < 0.01, ###P < 0.001. N = 3 biologically independent experiments; data are presented as mean ± standard deviation. (D) Immunofluorescent staining of above cells with anti-Fast MyHC as primary antibody and Alexa Fluor 555 conjugated secondary antibody (red). GFP (green) indicates retrovirally transduced cells. Scale bar, 100 μm. (E) Differentiation index, Fusion index, and Nuclei distribution in each myosin+ cell were quantified from images in panel (D). Data in panel (E; Differentiation and Fusion index) were analysed with one-way ANOVA followed by Tukey’s post-hoc test; data in panel (E; myosin+ cells) were analysed with two-way ANOVA followed by Tukey’s post-hoc test. Asterisk (*) indicates a significant difference between SPSB1-, Myogenin- or SPSB1 + Myogenin- and GFP control groups, *P < 0.05, **P < 0.01, ***P < 0.001; * indicates a significant difference between SPSB1- and SPSB1 + Myogenin-treated cells, #P < 0.05, ##P < 0.01, ###P < 0.001. N = 3 biologically independent experiments; data are presented as mean ± standard deviation.

Myogenin facilitates differentiation in SPSB1 transduced cells

Because Akt increases Myog expression,25 a key factor for differentiation,26 we tested if SPSB1-mediated inhibition of myogenesis involves Myogenin. Indeed, SPSB1 reduced Myogenin mRNA expression and protein content in differentiating C2C12 cells (Figure 4B, Figure S10C). Restoration of Myogenin expression in SPSB1-transduced myocytes increased Akt (Ser473) phosphorylation (Figure 7A–C), enhanced protein synthesis (Figure 7D) and elevated the expression of myogenin factors (Mymk, Mymx) and terminal differentiation markers (Myh3) but not Myh1 and Myh7 (Figure 7E). These effects were accompanied by an increase in differentiation and fusion indices (Figure 8A,B). Co-expression of Myogenin and SPSB1 improved myogenic differentiation and increased fast- and slow-twitch MyHC proteins (Figure 8C). Myogenin also reverted SPSB1-mediated inhibition of protein synthesis (Figure S17B), myogenic differentiation, and myoblast fusion (Figure 8D,E) in self-isolated and commercial primary myoblasts. In summary, these data show that Myogenin reverses the inhibitory effects of SPSB1 on protein synthesis, myoblast fusion and myogenic differentiation.

Myocyte-specific knockdown of Spsb1 reduces sepsis-induced muscle atrophy in mice

To test the hypothesis that knockdown of Spsb1 prevents muscle atrophy in septic mice, we generated short hairpin RNA (shRNA) to knock down Spsb1 directly in skeletal myocytes. We injected 1×10ⁱ² vg of AAV9 Spsb1_shRNA or AAV9-control_shRNA into the tail vein of male 8-week-old B6(C)/Rj-Tyrc/c mice. Six weeks later, we performed CLP- or sham-surgery for 96 h (Experimental design in Figure S18A). Spsb1_shRNA blunted CLP-induced expression of Spsb1 in TA and GP (Figure S18B). Sepsis caused a reduction in body (control_shRNA: −23%, P < 0.001; Spsb1_shRNA: −9%, P = 0.3), TA (−26%, P < 0.01) and GP (−21%, P < 0.01) weights, which was reduced by Spsb1 knockdown (Figure S18C). Quantification of haematoxylin and eosin stained histological cross-sections showed a reduction in MCSA of septic control_shRNA treated mice that was attenuated in TA of septic Spsb1_shRNA mice (control_shRNA: −25.4%, Spsb1_shRNA: −9.2%; P < 0.001; Figure S18D). To investigate any effects on protein degradation, we quantified the expression of the atrophy genes Trim63/MurF1 and Fbxo32/Atrogin-1. Spsb1 knockdown attenuated sepsis-induced increases in Trim63 and Fbxo32 expression (Figure S18E,F). These data indicate that inhibition of SPSB1 reduces inflammation-induced muscle atrophy in mice.

Discussion

We identified SPSB1 as a novel regulator of the TβRII-Akt-Myogenin pathway in skeletal muscle and myocytes. SPSB1 associates with, ubiquitinates and reduces the stability of TβRII and inhibits TβRII-signalling resulting in reduced protein synthesis and attenuated myogenic differentiation. As SPSB1 is significantly induced in skeletal muscle of critically ill patients, further studies are required to determine whether it is a potential therapeutic target in the treatment of muscle wasting in critical illness.
patients and in septic mice it may contribute to the observed inhibition of myogenic differentiation in muscle of septic patients and mice. In line with previously published work on non-myocytes,17 we proved that SPSB1 and TβRII physically interact and colocalize in myocytes, which facilitates ubiquitination of TβRII and reduces its stability. This in turn inhibited both canonical and non-canonical TβRII-signalling and negatively affected myocyte homeostasis. SPSB1 overexpression inhibited protein synthesis, myoblast fusion and myogenic differentiation, which lead to a decrease in early and late differentiation markers. We uncovered that the SPRY- and the SOCS-box domain of SPSB1, are involved in its interaction with TβRII and the regulation of Akt and Smad3 signalling. These data indicate that the SPRY-domain and the SOCS-box of SPSB1 are important for its inhibitory function on TβRII-induced myogenesis (Figure S19).

Myogenesis is a highly ordered process controlled by multiple factors, including MyoD and Myogenin.27,28 Besides, Myomaker/Mymk and Myomereger/Mymx that mediate myoblast fusion terminal differentiation markers especially MyHC are coordinately expressed.12–14 Our findings indicate that SPSB1 inhibits myogenesis by suppression of the TGF-β/TβRII-Akt-Myogenin axis. In line with previous work,25 we showed that Akt induces Myog expression and enhances myoblast fusion and myogenic differentiation. Furthermore, Myogenin increases the expression of Mymk and Mymx,29 which was also observed in our study. In contrast, SPSB1 inhibited myogenic differentiation, which was accompanied by a decreased expression of all myogenic factors. The SPSB1-phenotype was restored when Akt or Myogenin were co-expressed with SPSB1 in primary and immortalized myoblasts. Our data, together with previous work, thus indicate that the TGF-β/TβRII-Akt-Myogenin axis regulates myogenesis.

The strong increase in contractile and surface proteins during myogenesis suggests that protein synthesis is required for this process.12,13 This is supported by the observation that inhibition of protein synthesis leads to a decrease in C2C12 myoblast fusion.30 Here, we show that inactivation of TβRII by SPSB1 and ITD-1 reduces protein synthesis as well as myogenesis, further indicating that both processes are interconnected.

TGF-β family members and their downstream effectors Smad2 and Smad3 were reported to inhibit myogenesis.31,32 In contrast, other groups showed that Smad3 and Smad7 are essential for myogenesis.33,34 However, only few groups examined the function of TGF-β receptors on myotube formation. Our data provide evidence that TβRII promotes myogenesis. Specifically, TβRII expression was increased during differentiation and was enriched in myosin⁺-cells. Myoblasts treated with the TβRII inhibitor ITD-1 failed to form myotubes, which is in agreement with the observation that myoblasts expressing a dominant negative TβRII did not differentiate.35 We also showed that TGF-β/TβRII-Akt-Myogenin is essential for myogenesis. Our observation that ITD-1 inhibited both myoblast fusion and myogenic differentiation is in contrast to a recent report that showed that ITD-1 promotes fusion without affecting differentiation in primary myoblasts.36 In contrast to our study, the authors did not observe an upregulation of TβRII during differentiation indicative for methodological differences between our respective analyses. In addition, our observation that ITD-1 treatment caused an increase in fusion-related factors (Mymk, Mymx) during early, but not late differentiation suggests that TGF-β/TβRII signalling has distinct functions throughout differentiation. This hypothesis is supported by findings of Willems et al. who reported that ITD-1 abolished cardiogenesis in mouse embryonic stem cells when added between days 1–3 of differentiation but promoted it from days 3–5.24 Moreover, the different downstream effectors of TGF-β also seem to differ in their effects on differentiation. For example, Melendez et al. showed that TGF-β, via Smad2/3, inhibited myoblast fusion but not differentiation in chicken embryos.37 Our study shows that non-canonical TGF-β signalling via Akt-Myogenin promotes both myoblast fusion and differentiation. Collectively, these findings suggest that the effects of TGF-β/TβRII-signalling on myocyte biology vary depending on the models used.

SPSB1 shares 50% and 75% amino acid sequence identity with SPSB2 and SPSB4, respectively,19 whereas SPSB3 shares the least amino acid sequence identity with other SPSB-family members.19 SPSB proteins have some overlapping but also distinct targets and therefore functions. For example, SPSB1 and SPSB4, but not SPSB3 interact with human prostate apoptosis response protein-4 (hPar-4). SPSB2 also interacts with Par-4, but not as strong.38 Other SPSB proteins may therefore also play a role in TGF-β/TβRII-signalling and myogenesis. However, first, only SPSB1 was shown to be involved in TGF-β-signalling and second, only SPSB1 was highly expressed in satellite cells indicating a specific role in this cell type. Third, unlike Spsb2 the other SPSB-family members were either only regulated in some muscles, at different time points or not regulated during sepsis in mice. Interestingly, Spsb3 expression was increased in all muscles but only 96 h after CLP surgery in mice. By contrast, SPSB1, SPSB2, and SPSB3 were increased in muscle of ICUAW patients, indicating that also SPSB2 and SPSB3 are also involved in inflammation-induced muscle pathologies. However, further studies are needed to investigate their specific functions in muscle.

The proposed model that inflammatory cytokines induce SPSB1 to block TGF-β signalling and myogenesis could also be interpreted as a lifesaving mechanism during the early stages of sepsis, which may result in failed regeneration during the later disease course. Inflammatory cytokines and sepsis lead to a disturbed protein homeostasis with a decreased synthesis and an increased degradation.4–6,18 Others and we hypothesize that muscle serves as a reservoir for proteins that are used as fuel to assure survival during critical illness.
However, once the organism survives sepsis breakdown of muscle tissue can lead to weakness. As protein synthesis is energy demanding its inhibition could be beneficial during sepsis. In this regard, the cytokine-mediated increase in SPSB1 could inhibit TGF-β-induced protein synthesis, satellite cell proliferation and muscle repair to prioritize defence mechanisms in sepsis. However, the same mechanisms could lead to failed regeneration in ICUAW. Because, TGF-β via TβRII also increases the expression of SPSB117 that in turn inhibits TβRII signalling this regulation could facilitate a negative feed-back loop. Finally, TGF-β plays a crucial role in inflammation and immune responses and has both pro- and anti-inflammatory properties.39 TGF-β can promote inflammation by stimulating the recruitment and activation of immune cells and increase the expression of pro-inflammatory cytokines, such as TNF and IL-6, and activate the NF-κB pathway, which all cause muscle wasting.4,5,18 An increased expression of SPSB1 could therefore be beneficial to limit TGF-β-induced inflammation in sepsis. However, if SPSB1 also inhibits anti-inflammatory TGF-β-functions in muscle during sepsis warrants further investigation.

The inhibition of TGF-β/TβRII-Akt-Myogenin signalling by SPSB1 may shed light on the pathogenesis of the perturbed regenerative capacity observed in muscle of critically ill patients with sepsis (Figure S19). Protein synthesis, which is critical for maintenance of muscle size and function, is reduced in muscle of critically ill patients.5 Our data implicate that TβRII-mediated Akt activation plays a role in maintaining homeostasis in muscle and myocytes in men and mice and that protein synthesis is required for myogenesis. We show that SPSB1 overexpression inhibits protein synthesis and myogenic differentiation, and these effects were rescued by Akt and Myogenin. Because a reduced number and malfunction of satellite cells as well as an impaired muscle regeneration have been reported in ICUAW patients and septic mice,8–10 we propose that SPSB1-mediated inhibition of TβRII-Akt-Myogenin signalling and myogenesis contributes to a disturbed muscle homeostasis that occurs during inflammation.

Limitations

In our short-term sepsis mouse model, we observed a 20- to 34-fold increase in muscular Spsb1 expression 24 and 96 h after surgery, respectively. An increased muscular SPSB1 expression was confirmed in muscle of ICUAW patients; however, only twofold. This discrepancy is possibly due to differences in species and muscle tissue investigated, the time point of analyses, and the underlying causes of critical illness as well as its treatment. Additionally, data from patients and from a well-defined and standardized mouse model in which only male C57BL/6 J mice of the same age were used may differ due to the experimental design and the heterogeneity of the study subjects. Our mechanistical data on the role of SPSB1 in myogenic differentiation suggest the TβRII-Akt-Myogenin pathway contributes to defective muscle regeneration and myopathy observed in ICUAW patients. However, if SPSB1 is involved in the pathomechanisms underlying the up to 5 years persistent failure to regenerate cannot be answered by our work and warrants further investigations.

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Conflict of interest

The authors declare no conflict of interest.

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

References


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