


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

Yi Li^{1,2}, Niklas Dörmann², Björn Brinschwitz², Melanie Kny¹, Elisa Martin², Kirsten Bartels^{2,3}, Ning Li², Priyanka Vooiri Giri², Stefan Schwanz², Michael Boschmann¹, Susanne Hille^{4,5}, Britta Fielitz^{2,3}, Tobias Wollersheim⁶, Julius Grunow⁶, Stephan B. Felix^{2,3}, Steffen Weber-Carstens⁶, Friedrich C. Luft¹, Oliver J. Müller^{4,5} & Jens Fielitz^{1,2,3*} 

¹Experimental and Clinical Research Center (ECRC), Charité-Universitätsmedizin Berlin, Max Delbrück Center (MDC) for Molecular Medicine in the Helmholtz Association, Berlin, Germany; ²DZHK (German Center for Cardiovascular Research), partner site Greifswald, Greifswald, Germany; ³Department of Internal Medicine B, Cardiology, University Medicine Greifswald, Greifswald, Germany; ⁴Department of Internal Medicine III, University of Kiel, Kiel, Germany; ⁵German Centre for Cardiovascular Research (DZHK), partner site Hamburg/Kiel/Lübeck, Germany; ⁶Department of Anaesthesiology and Operative Intensive Care Medicine (CCM, CVK), Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin and Berlin Institute of Health, Berlin, Germany

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 (T β RII)-inhibitor SPRY domain-containing and SOCS-box protein 1 (SPSB1) in skeletal muscle of septic mice. We hypothesized that SPSB1-mediated inhibition of T β RII 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 /T β RII 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-1 β (IL-1 β), and IL-6 increased the *Spsb1* expression in C2C12 myotubes. TNF- and IL-1 β -induced *Spsb1* expression was mediated by NF- κ B, whereas IL-6 increased the *Spsb1* expression via the glycoprotein 130/JAK2/STAT3 pathway. All cytokines reduced myogenic differentiation. SPSB1 avidly interacted with T β RII, resulting in T β RII ubiquitination and destabilization. SPSB1 impaired T β RII-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 T β RII-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; TGF β receptor II; Myogenic differentiation

Received: 4 November 2022; Revised: 7 March 2023; Accepted: 15 April 2023

*Correspondence to: Jens Fielitz, Universitätsmedizin Greifswald, Klinik und Poliklinik für Innere Medizin B, Forschungscluster III, Fleischmannstr. 41, 17475 Greifswald, Germany. Email: jens.fielitz@uni-greifswald.de

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 β , 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 mice¹⁰ 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- β) signalling that is involved in myogenesis and elevated TGF- β receptor type II (T β RII) levels were reported in muscle of critically ill patients,¹⁵ suggesting their involvement in ICUAW. TGF- β binds to the T β RI and T β RII 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- β signalling can be inhibited by the *splA*/ryanodine receptor (SPRY) domain and SOCS-box domain 1 (*SPSB1/Spsb1*) protein that targets T β RII 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 T β RII-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 ≥ 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/plantaris* (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 \pm 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.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,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),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

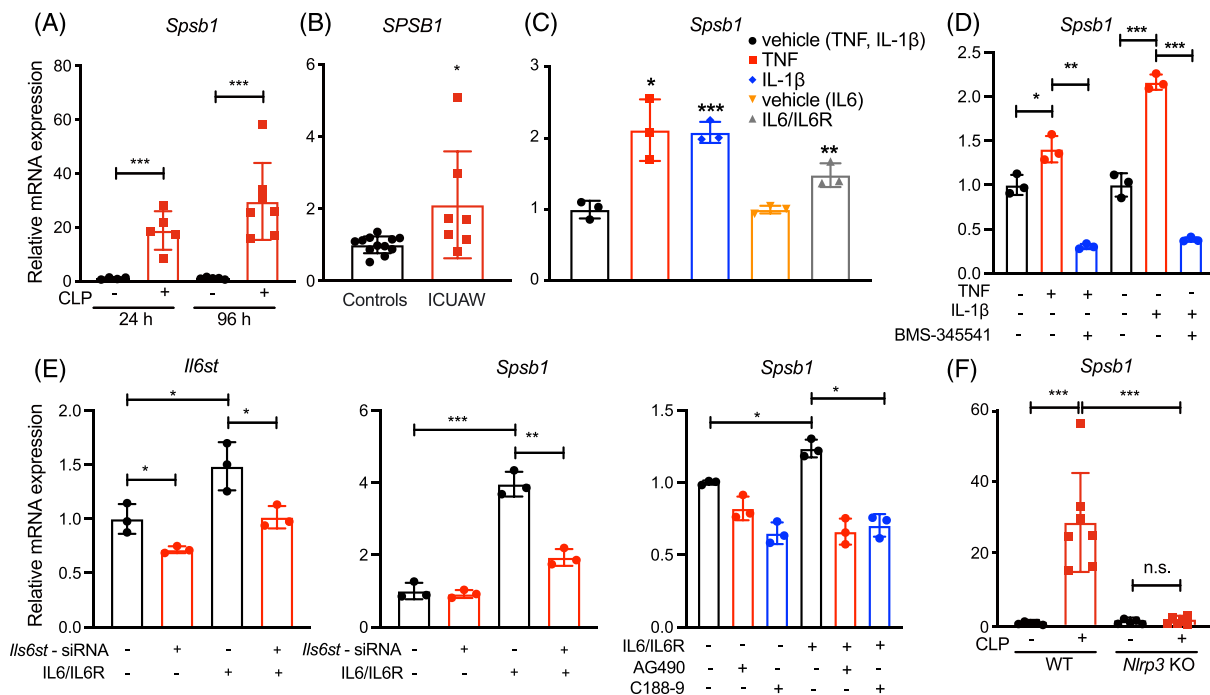


Figure 1 *Spsb1* is upregulated in inflammation-induced skeletal muscle atrophy. (A) Quantitative RT-PCR (qRT-PCR) analysis of *Spsb1* from the tibialis anterior (TA) muscle in 12-week-old male C57BL/6J mice subjected to cecal ligation and puncture (CLP, $n = 5$, 24 h; $n = 7$, 96 h) or sham surgery (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 (MT5) treated with TNF (10 ng/mL), IL-1 β (10 ng/mL) or IL6/IL6R (100 ng/mL) for 2 h. (D) qRT-PCR of *Spsb1* from MT5 that were pretreated with the IKK1-inhibitor BMS-345541 (5 μ M) 60 min before TNF or IL-1 β treatment. (E) qRT-PCR of *Il6st* and *Spsb1* from MT5 after *Il6st*-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-1 β , 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-1 β -induced *Spsb1* expression was inhibited by the I κ B kinase-inhibitor BMS-345541 (Figure 1D) indicating that this effect was mediated by NF- κ B. 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-1 β 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 those 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-1 β , 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 T β RII in non-muscle cells.¹⁷ To analyse changes in T β RII 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 T β RII was ubiquitously expressed in all myofibers and was localized to the sarcolemma in sham-operated mice. In contrast, membranous T β RII-staining was reduced in myofibers of CLP mice (Figure S5) suggesting an association between increased *Spsb1* expression and a decrease in its putative target T β RII in myofibers during sepsis. Consistently, our RNAseq data showed a significant downreg-

ulation of genes contained in the Gene Ontology (GO) term 'cellular response to transforming growth factor beta stimulus' (GO:0071560, $P = 8.84 \times 10^{-5}$, FDR 0.002; Figure S6) and the Kyoto Encyclopedia of Genes and Genomes (KEGG)-pathway 'TGF-beta signaling pathway' (mmu04350, $P = 5.4 \times 10^{-4}$, FDR 0.0038, Figure S7A,B).

We next investigated if SPSB1 regulates T β RII function in myocytes. We identified two transcript variants of T β RII, full-length T β RII and T β RII devoid of exon2 (T β RII- Δ Ex2) in myocytes. Using co-immunoprecipitation analyses we found that SPSB1 physically interacted with both T β RII and T β RII- Δ Ex2 to a similar degree (Figure 2A). SPSB1 and both T β RII variants were localized to the cytoplasm and the membrane of C2C12 cells (Figure S8A), where they also co-localized (Figure 2B). Endogenous T β RII and overexpressed SPSB1 colocalized in C2C12 myoblasts (Figure S8B). To investigate if the sprA/ryanodine receptor (SPRY) or the SOCS-box domain in SPSB1 that are both important for protein-protein interaction¹⁷ mediate its association with T β RII we generated two SPSB1-SPRY domain mutants (Y129A and T160A/Y161A (TYAA)) and a mutant lacking the SOCS-box (Δ SOCS) (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- Δ SOCS strongly associated with T β RII in C2C12 cells, whereas the interaction between SPSB1-Y129A and SPSB1-TYAA with T β RII was reduced when compared with SPSB1 WT (Figure 2D). These data indicate that the SPRY-domain of SPSB1 is involved in its interaction with T β RII in myocytes. Ubiquitination assays showed that SPSB1 increased the ubiquitination of T β RII, which was lower in SPSB1- Δ SOCS expressing cells indicating that SPSB1-mediate ubiquitination of T β RII via its SOCS-box domain (Figure 2E). Cycloheximide (CHX) chase assays revealed that SPSB1 WT (Figure 2F) but not SPSB1- Δ SOCS reduced the half-life of endogenous T β RII in C2C12 cells (Figure S8C) indicating that SPSB1 via its SOCS-box increases the turnover of T β RII. These data suggest that SPSB1 interacts with T β RII, 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 T β RII 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),

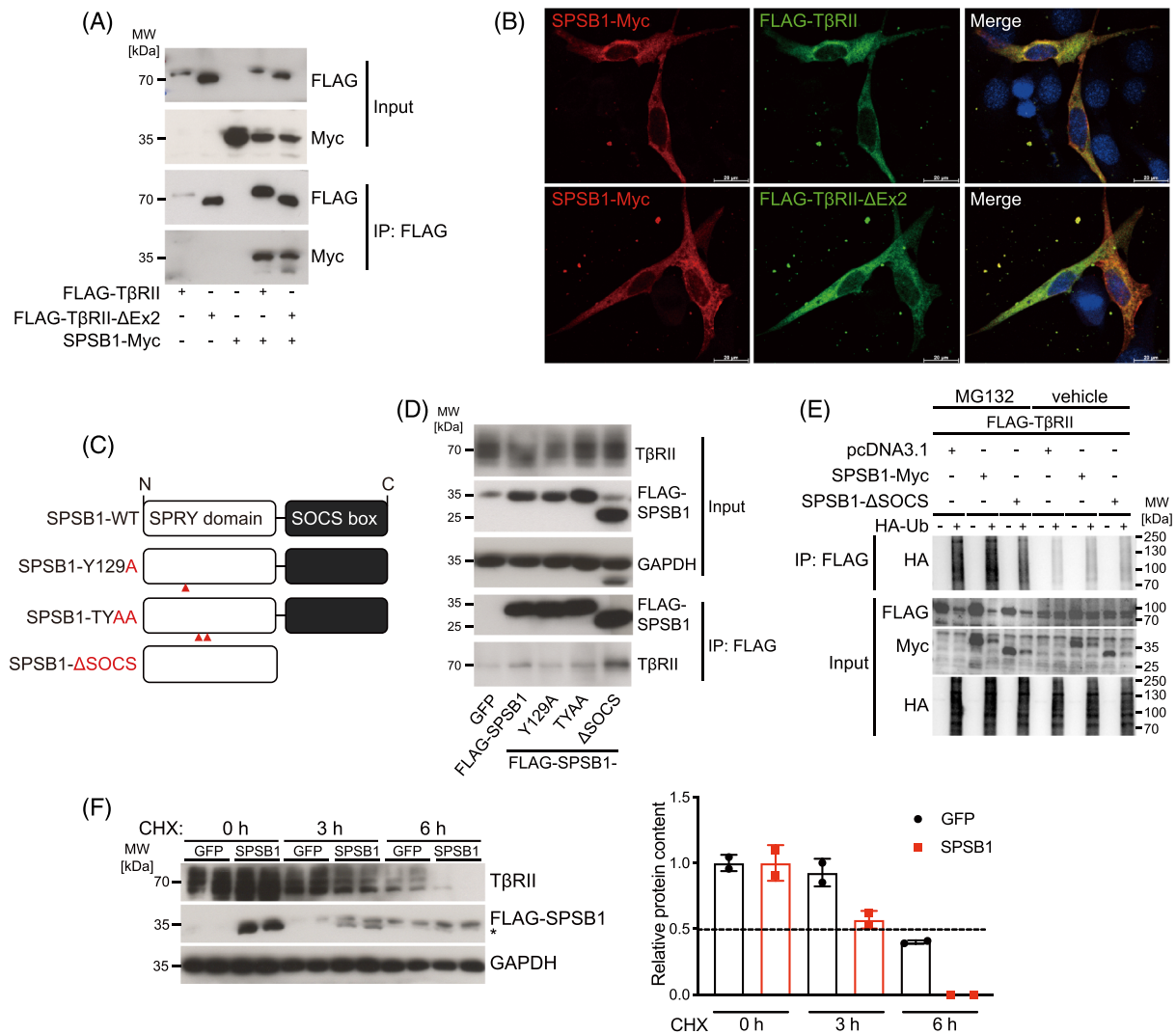


Figure 2 SPSB1 associates with and ubiquitinates TGF- β receptor II and reduces its stability. (A) Co-immunoprecipitation (Co-IP) using lysates from C2C12 cells transfected with FLAG-T β RII, FLAG-T β RII- Δ Ex2 and SPSB1-Myc. Extracts were immunoprecipitated (IP) with anti-FLAG agarose and detected with antibodies against Myc and FLAG, as indicated. Input Western blot with anti-Myc and anti-FLAG antibody as indicated. (B) Immunofluorescence using anti-FLAG antibody together with A488-coupled secondary antibody (green) and anti-Myc antibody together with A555-coupled secondary antibody (red) to detect SPSB1-Myc, FLAG-T β RII and FLAG-T β RII- Δ Ex2 in transfected C2C12 cells. Nuclei were stained with DAPI (blue). Scale bar, 20 μ m. (C) Pictogram of functional domains and mutation sites of SPSB1 constructs. (D) Co-IP of FLAG-SPSB1 (WT) and mutants (SPSB1-Y129A, -TYAA or - Δ SOCS) with endogenous T β RII from MT5 C2C12 cells overexpressing GFP, SPSB1 and SPSB1 mutants. Extracts were IP with anti-FLAG agarose and detected with anti-T β RII antibody. Input proteins were detected with anti-FLAG, T β RII and GAPDH antibodies as indicated. (E) COS-7 cells were transfected with FLAG-T β RII, SPSB1-Myc, SPSB1- Δ SOCS and HA-Ub, as indicated. Cells were treated with MG132 (25 μ M) or vehicle (DMSO 0.25%) 42 h post-transfection for a further 6 h. Cells were lysed 48 h post-transfection and lysates were immunoprecipitated (IP) with anti-FLAG affinity gel. Immunoblotting (IB) with indicated antibodies. (F) Cells were infected with a retrovirus encoding GFP or SPSB1 for 48 h and then treated with cycloheximide (CHX, 50 μ g/mL) for indicated timepoints. Anti-FLAG antibody shows overexpressed FLAG-SPSB1; specific band is indicated by asterisk. GAPDH was used as loading control. Densitometric analysis; dotted line indicates 50% abundance of T β RII. $N = 3$ biologically independent experiments; representative blots are shown. Data are presented as mean \pm standard deviation.

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,²² which is decreased in muscle of ICUAW patients,^{6,10} and because our RNA-seq data revealed an inhibition of Akt-signalling in muscle of septic mice,¹⁸ we focused on this pathway for further analyses.

SPSB1 regulates TGF- β signalling

We next investigated if the association of SPSB1 with T β RII 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

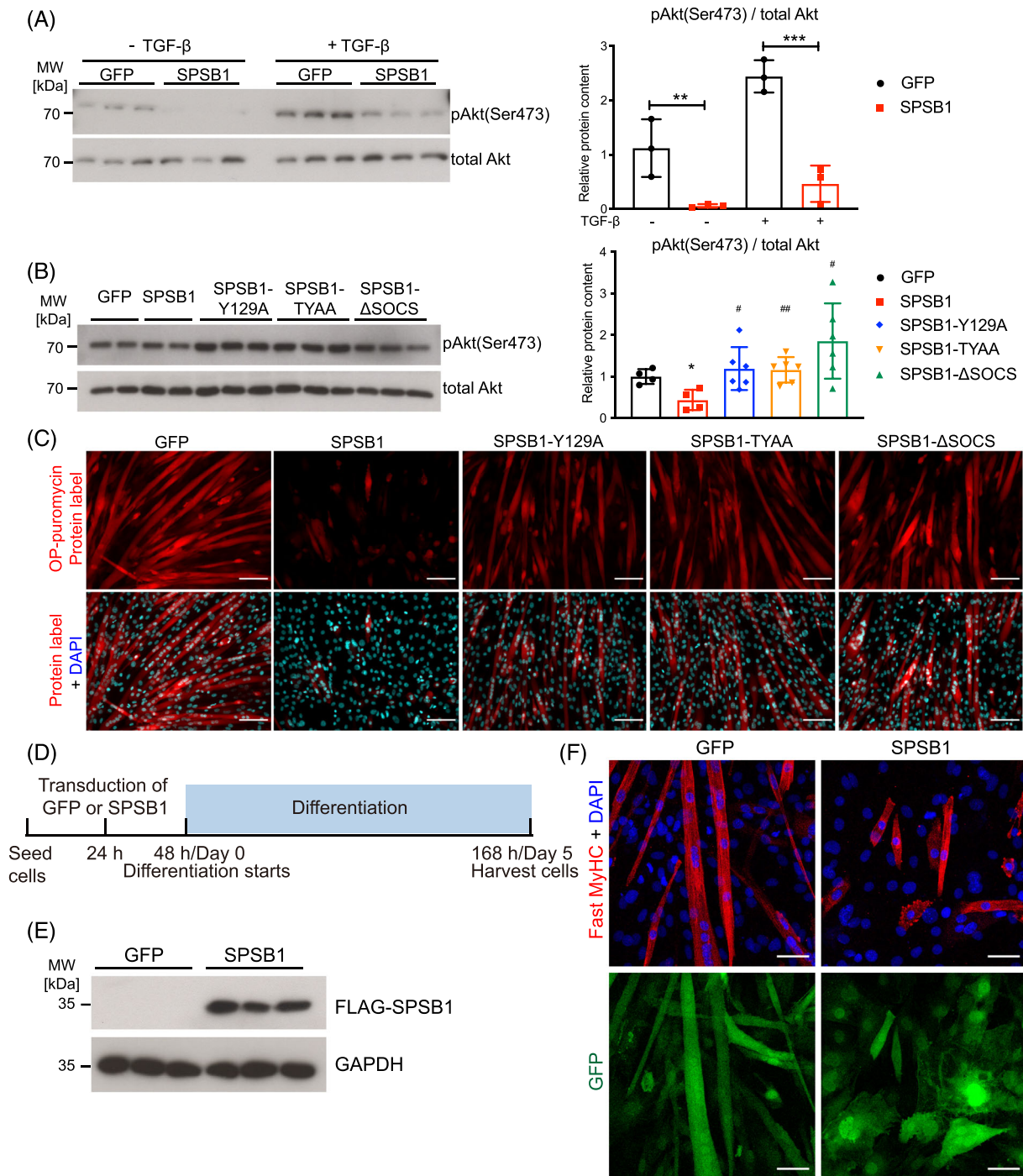


Figure 3 SPSB1 downregulates TGF- β signalling by its SPRY and SOCS-box domain and inhibits myogenic differentiation. (A) Five-day-differentiated C2C12 myotubes (MT5) transduced with GFP or SPSB1 were treated with TGF- β (5 ng/ml) or solvent control for 5 min. Lysates were analysed by Western blot analysis 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 - Δ SOCS) 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 μ m. (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 μ m.

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 T β RII 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^{8,9} and septic mice.¹⁰ During regeneration muscle residing stem cells, called satellite cells, provide myogenic cells that proliferate, differentiate, fuse, and form new functional myofibers.¹¹ 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*²³ 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-1 β , and IL-6/IL-6R, respectively, for 5 days myogenic differentiation was greatly perturbed (Figure 4C,D), which was also reflected by a de-

creased 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 T β RII 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 T β RII plays a role in myogenesis, we examined the amount of T β RII protein and *Tgfb1* gene expression in differentiating C2C12 cells and found them to be increased (Figure S13A,B). Immunohistochemistry revealed that T β RII was enriched in myosin⁺-myotubes (Figure S13C). To explore if T β RII is essential for myogenesis, we used the T β RII-specific inhibitor *inducer of T β RII receptor degradation-1* (ITD-1).²⁴ ITD-1 caused a dose-dependent reduction of T β RII (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 /T β RII-Akt-signalling plays a role in protein synthesis and myogenesis.

Akt restores myogenesis in SPSB1 overexpressing cells

As SPSB1-mediated inhibition of T β RII-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),

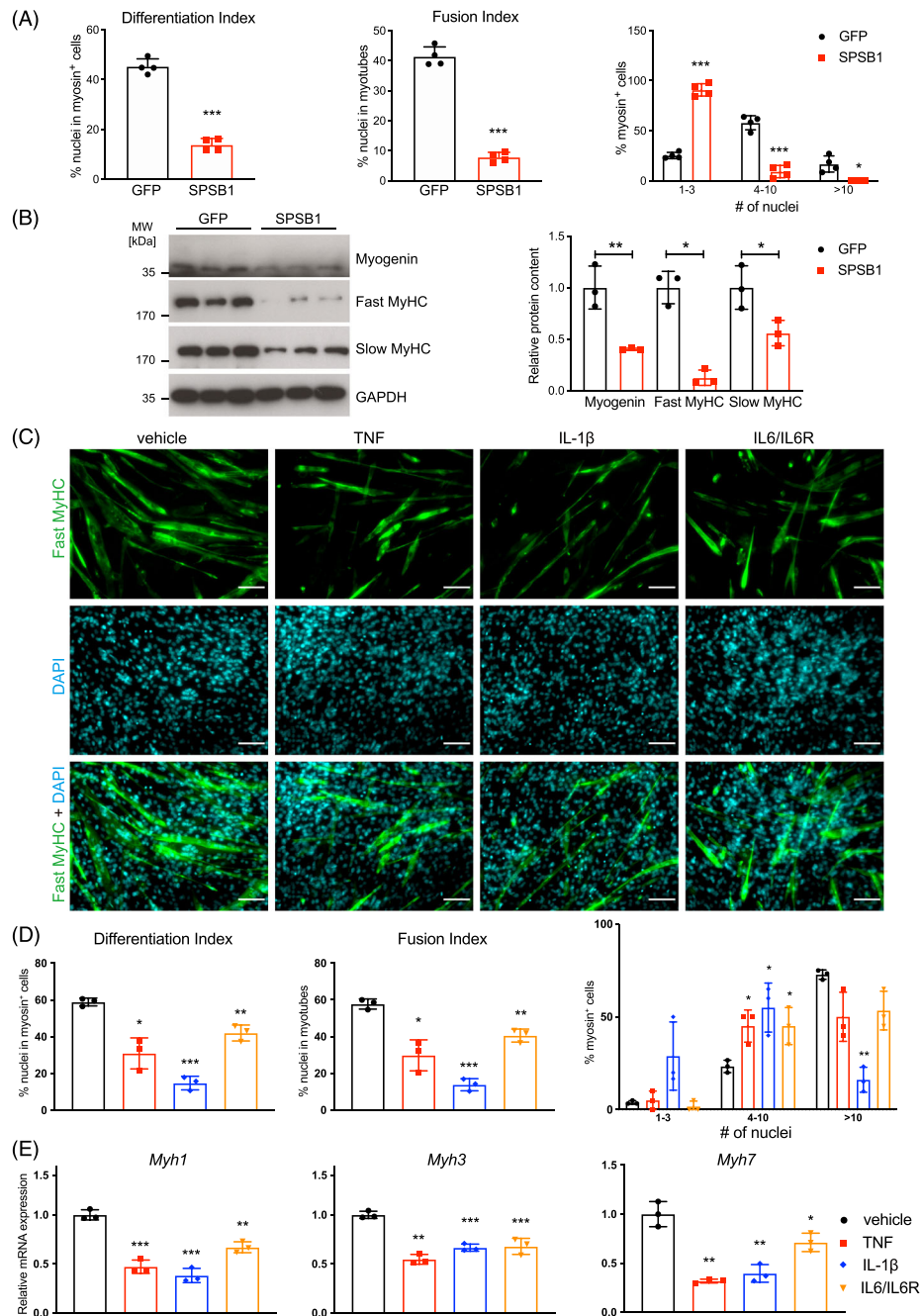


Figure 4 SPSB1 and proinflammatory cytokines inhibit myogenic differentiation. (A) Differentiation index, fusion index, and nuclei distribution in myosin positive (myosin⁺) cells were quantified from images in Figure 3 panel (F). (B) Western blot analysis of lysates from GFP and SPSB1 transduced cells 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.

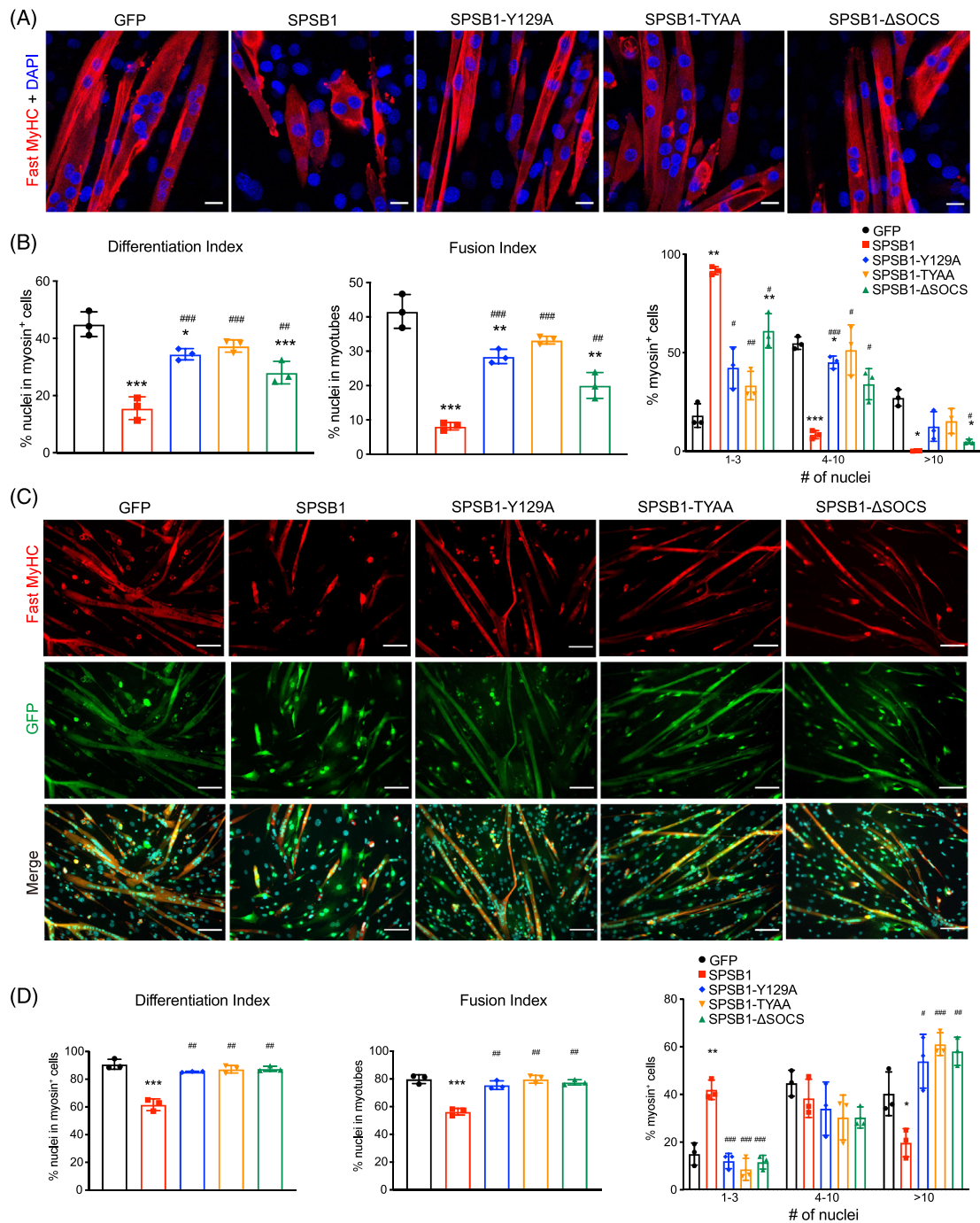


Figure 5 SPSB1 mediated inhibition of myogenic differentiation depends on its SPRY and SOCS-box domain. (A, B) C2C12 myoblasts were transduced by control GFP, SPSB1 (WT) or mutant (SPSB1-Y129A, -TYAA or -ΔSOCS) containing retrovirus and differentiated for 5 days. (A) Immunofluorescent staining with anti-fast 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.

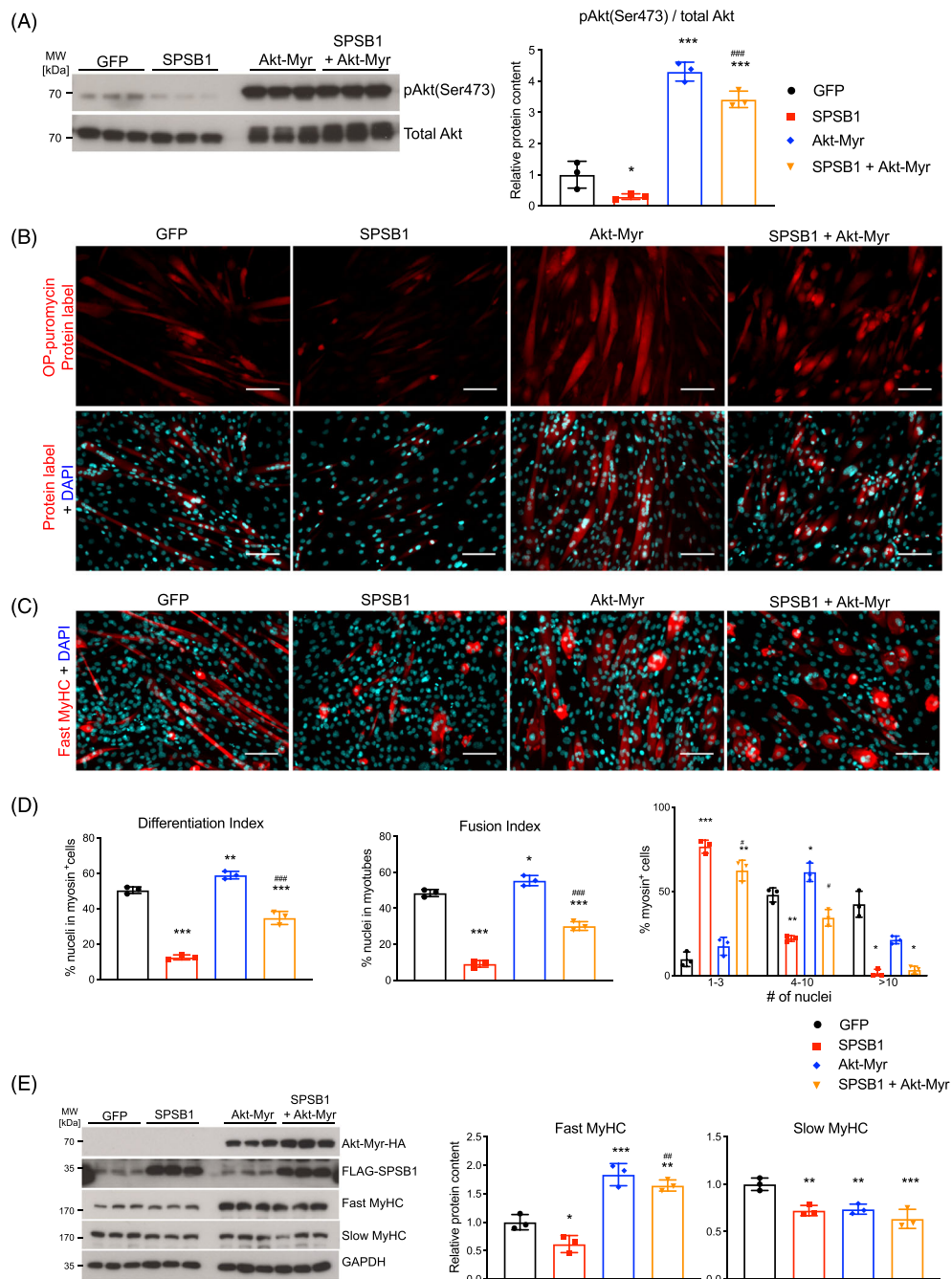


Figure 6 Expression of Akt restores myogenesis in SPSB1 overexpressing cells. Cells were transfected by control GFP, SPSB1, Akt-Myr, respectively, or co-transfected by Akt-Myr and SPSB1 retrovirus and differentiated for 5 days. (A) Western blot analysis of anti-phospho Akt antibody (Ser473). Total Akt 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 μ m. (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 \pm standard deviation.

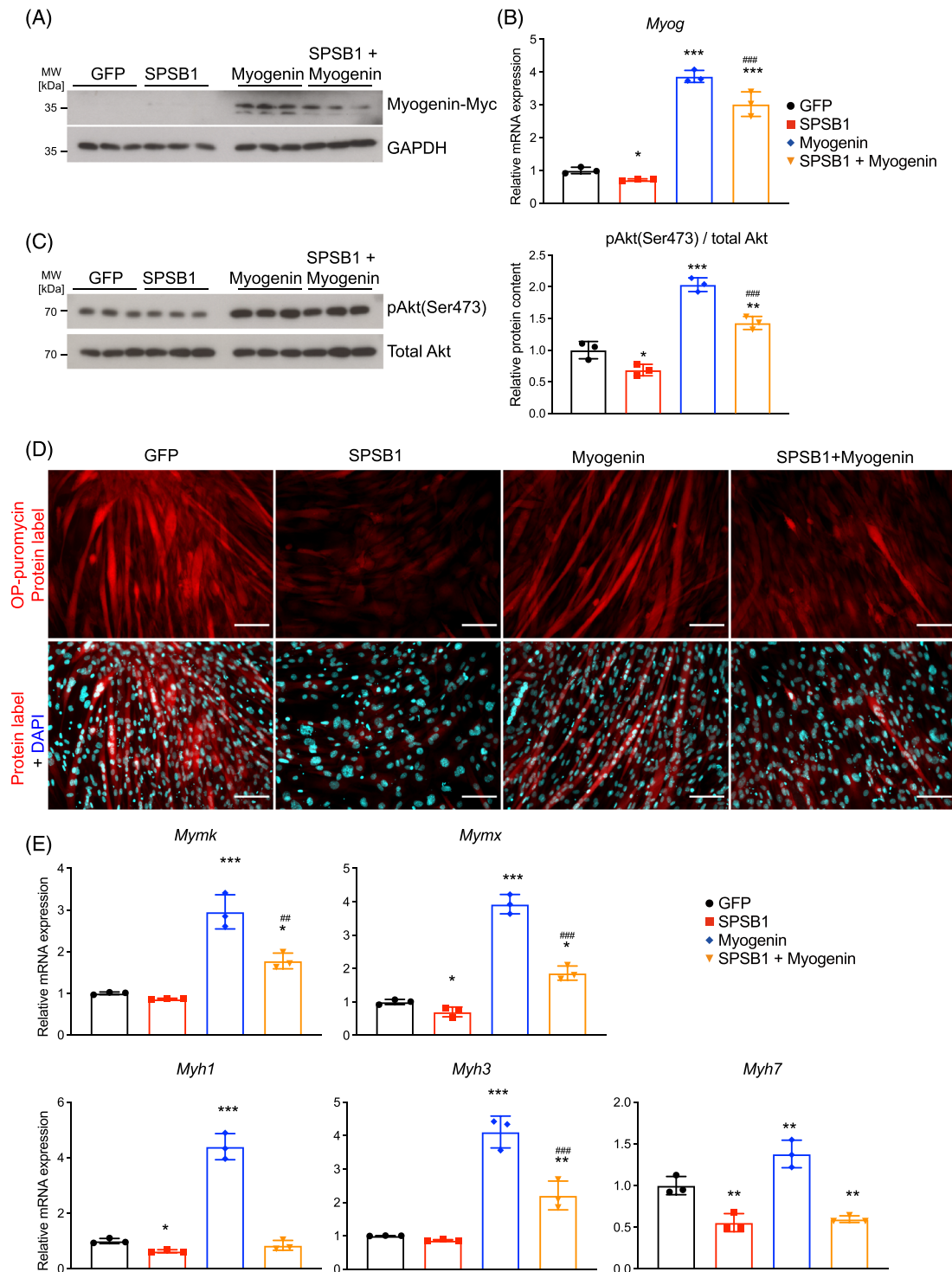


Figure 7 Myogenin restores Akt signalling and protein synthesis in SPSB1 overexpressing cells. C2C12 cells were transduced by control GFP, SPSB1, Myogenin, respectively, or co-transduced by Myogenin and SPSB1 retrovirus and differentiated for 5 days. (A) Western blot with anti-Myc antibody. GAPDH was 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 \pm standard deviation.

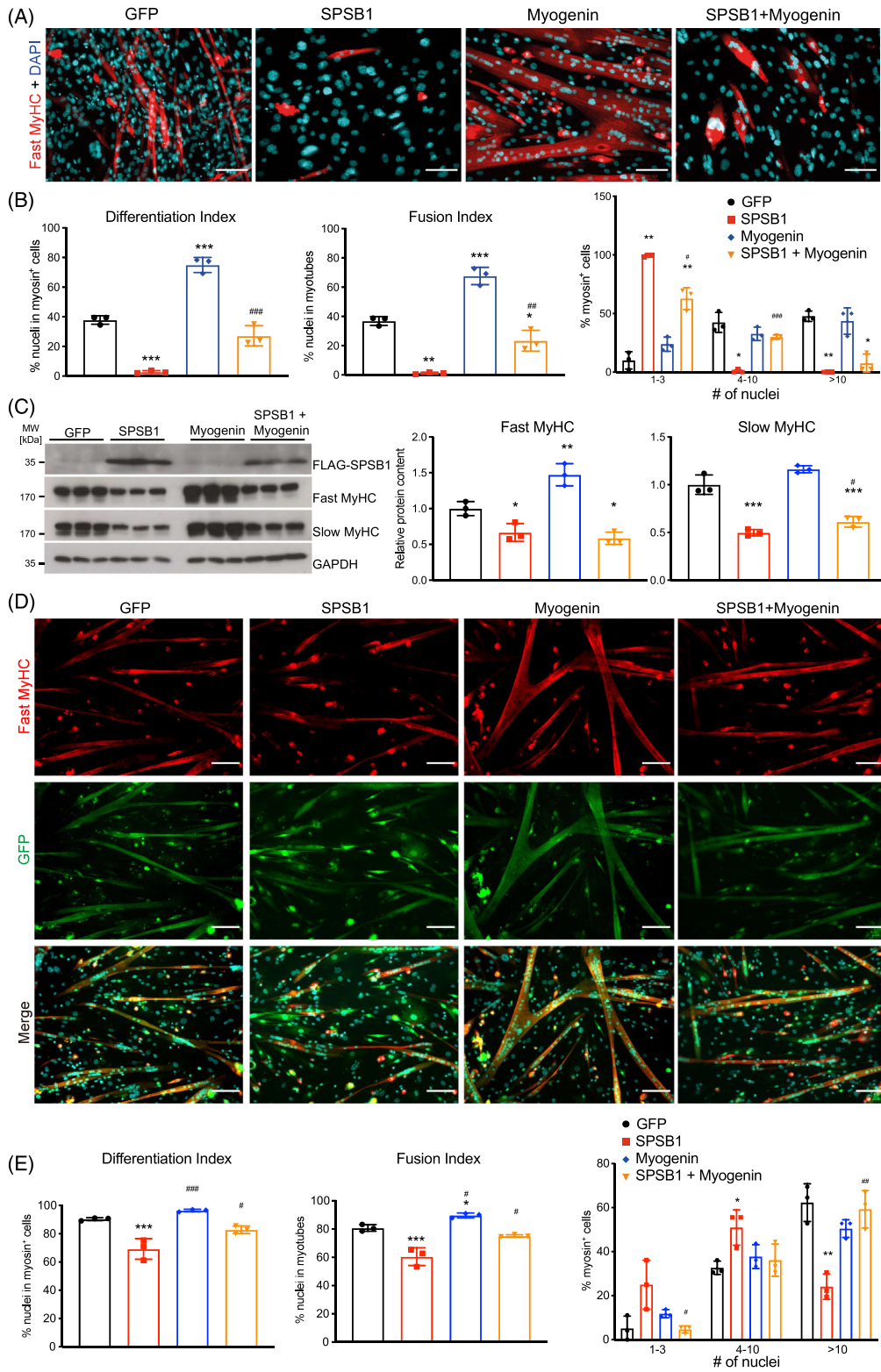


Figure 8 Myogenin restores myogenesis in SPSB1 overexpressing cells. (A–C) C2C12 cells were transduced by control GFP, SPSB1, Myogenin, respectively, or co-transduced by Myogenin and SPSB1 retrovirus and differentiated for 5 days. (A) Immunofluorescent staining of above cells with anti-Fast MyHC as primary antibody and Alexa Fluor 555 conjugated secondary antibody (red). Scale bar, 100 μ m. (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 \pm standard deviation. (D, E) Primary myoblasts were transduced by control GFP, SPSB1, Myogenin, respectively, or co-transduced by Myogenin and SPSB1 retrovirus and differentiated for 5 days. (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 μ m. (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 \pm standard deviation.

myogenic differentiation, myoblast fusion (Figure 6D) and MyHC content (Figure 6E) as well as myogenic factors (*Myog*, *Mymk*, *Mymx*) (Figure S15A) and terminal differentiation markers (*Myh1*, *Myh3*) in C2C12 cells (Figure S15B). However, *Myh7* expression (Figure S15B) and slow-twitch MyHC (Figure 6E) remained unaffected by Akt-Myr. Akt-Myr also reverted SPSB1-mediated inhibition of protein synthesis, myogenic differentiation, and myoblast fusion (Figures S16A, B and S17A) in self-isolated and commercial primary myoblasts. Together, these data show that Akt-Myr rescues the inhibitory effects of SPSB1 on protein synthesis, myoblast fusion and myogenic differentiation.

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 myogenic 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 1×10^{12} vg of AAV9 *Spsb1*_shRNA or AAV9-control_shRNA into the tail vein of male 8-week-old B6(C)/Rj-Tyr^{c/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 T β RII-Akt-Myogenin pathway in skeletal muscle and myocytes. SPSB1 associates with, ubiquitinates and reduces the stability of T β RII and inhibits T β RII-signalling resulting in reduced protein synthesis and attenuated myogenic differentiation. As SPSB1 is significantly induced in skeletal muscle of critically ill

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 T β RII physically interact and colocalize in myocytes, which facilitates ubiquitination of T β RII and reduces its stability. This in turn inhibited both canonical and non-canonical T β RII-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 T β RII 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 T β RII-induced myogenesis (Figure S19).

Myogenesis is a highly ordered process controlled by multiple factors, including MyoD and Myogenin.^{27,28} Besides, Myomaker/*Mymk* and Myomerger/*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 /T β RII-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 /T β RII-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 of C2C12 myoblast fusion.³⁰ Here, we show that inactivation of T β RII 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 T β RII promotes myogenesis. Specifically, T β RII expression was increased during differentiation and was enriched in myosin⁺-cells. Myoblasts treated with the T β RII inhibitor ITD-1 failed to form myotubes, which is in agreement with the observation that myoblasts expressing a dominant negative T β RII did not differentiate.³⁵ We also showed that TGF- β /T β RII-Akt-Myo-

genin 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 T β RII during differentiation indicative for methodological differences between our respective analyses. In addition, our observation that ITD-1 treatment caused an increase of fusion-related factors (*Mymk*, *Mymx*) during early, but not late differentiation suggests that TGF- β /T β RII 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 /T β RII-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 /T β RII-signalling and myogenesis. However, first, only SPSB1 was shown to be involved in TGF- β -signalling¹⁷ and second, only SPSB1 was highly expressed in satellite cells indicating a specific role in this cell type. Third, unlike *Spsb1* 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 T β RII also increases the expression of SPSB1¹⁷ that in turn inhibits T β RII 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- κ B 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 /T β RII-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.⁶ Our data implicate that T β RII-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 T β RII-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* ex-

pression 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 T β RII-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.

Acknowledgements

We thank Karlotta Struckmann for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation [FI 965/9-1 and FI 965/10-1 to [JF]] and the DZHK (German Center for Cardiovascular Research), partner site Greifswald [81Z5400153 to [JF], 81Z0400103, 81X2400142, and 81X2700230 to [JF and OM]]. We acknowledge support for the article processing charges from the DFG and the open access publication fund of the University of Greifswald. The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the *Journal of Cachexia, Sarcopenia and Muscle*.⁴⁰

Open Access funding enabled and organized by Projekt DEAL.

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

1. Ali NA, O'Brien JM Jr, Hoffmann SP, Phillips G, Garland A, Finley JC, et al. Acquired weakness, handgrip strength, and mortality in critically ill patients. *Am J Respir Crit Care Med* 2008;**178**: 261–268.
2. Cacciani N, Skarlen A, Wen Y, Zhang X, Addinsall AB, Llano-Diez M, et al. A prospective clinical study on the mechanisms underlying critical illness myopathy—A time-course approach. *J Cachexia Sarcopenia Muscle* 2022;**13**:2669–2682.
3. Stevens RD, Marshall SA, Cornblath DR, Hoke A, Needham DM, de Jonghe B, et al. A framework for diagnosing and classifying intensive care unit-acquired weakness. *Crit Care Med* 2009;**37**: S299–S308.

4. Huang N, Kny M, Riediger F, Busch K, Schmidt S, Luft FC, et al. Deletion of Nlrp3 protects from inflammation-induced skeletal muscle atrophy. *Intensive Care Med Exp* 2017;**5**:3.
5. Hahn A, Kny M, Pablo-Tortola C, Todiras M, Willenbrock M, Schmidt S, et al. Serum amyloid A1 mediates myotube atrophy via Toll-like receptors. *J Cachexia Sarcopenia Muscle* 2020;**11**:103–119.
6. Wollersheim T, Woehlecke J, Krebs M, Hamati J, Lodka D, Luther-Schroeder A, et al. Dynamics of myosin degradation in intensive care unit-acquired weakness during severe critical illness. *Intensive Care Med* 2014;**40**:528–538.
7. Herridge MS, Tansey CM, Matte A, Tomlinson G, Diaz-Granados N, Cooper A, et al. Functional disability 5 years after acute respiratory distress syndrome. *N Engl J Med* 2011;**364**:1293–1304.
8. Dos Santos C, Hussain SN, Mathur S, Picard M, Herridge M, Correa J, et al. Mechanisms of Chronic Muscle Wasting and Dysfunction after an Intensive Care Unit Stay. A Pilot Study. *Am J Respir Crit Care Med* 2016;**194**:821–830.
9. Walsh CJ, Batt J, Herridge MS, Mathur S, Bader GD, Hu P, et al. Transcriptomic analysis reveals abnormal muscle repair and remodeling in survivors of critical illness with sustained weakness. *Sci Rep* 2016;**6**:29334.
10. Rocheteau P, Chatre L, Briand D, Mebarki M, Jouvion G, Bardon J, et al. Sepsis induces long-term metabolic and mitochondrial muscle stem cell dysfunction amenable by mesenchymal stem cell therapy. *Nat Commun* 2015;**6**:10145.
11. Relaix F, Zammit PS. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Development* 2012;**139**:2845–2856.
12. Millay DP, O'Rourke JR, Sutherland LB, Bezprozvannaya S, Shelton JM, Bassel-Duby R, et al. Myomaker is a membrane activator of myoblast fusion and muscle formation. *Nature* 2013;**499**:301–305.
13. Quinn ME, Goh Q, Kurosaka M, Gamage DG, Petrany MJ, Prasad V, et al. Myomerger induces fusion of non-fusogenic cells and is required for skeletal muscle development. *Nat Commun* 2017;**8**:15665.
14. Schiaffino S, Rossi AC, Smerdu V, Leinwand LA, Reggiani C. Developmental myosins: expression patterns and functional significance. *Skelet Muscle* 2015;**5**:22.
15. Zhu X, Kny M, Schmidt F, Hahn A, Wollersheim T, Kleber C, et al. Secreted Frizzled-Related Protein 2 and Inflammation-Induced Skeletal Muscle Atrophy. *Crit Care Med* 2017;**45**:e169–e183.
16. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;**113**:685–700.
17. Liu S, Nheu T, Luwor R, Nicholson SE, Zhu HJ. SPSB1, a Novel Negative Regulator of the Transforming Growth Factor-beta Signaling Pathway Targeting the Type II Receptor. *J Biol Chem* 2015;**290**:17894–17908.
18. Zanders L, Kny M, Hahn A, Schmidt S, Wundersitz S, Todiras M, et al. Sepsis induces interleukin 6, gp130/JAK2/STAT3, and muscle wasting. *J Cachexia Sarcopenia Muscle* 2022;**13**:713–727.
19. Wang D, Li Z, Messing EM, Wu G. The SPRY domain-containing SOCS box protein 1 (SSB-1) interacts with MET and enhances the hepatocyte growth factor-induced Erk-Elk-1-serum response element pathway. *J Biol Chem* 2005;**280**:16393–16401.
20. Nishiya T, Matsumoto K, Maekawa S, Kajita E, Horinouchi T, Fujimuro M, et al. Regulation of inducible nitric-oxide synthase by the SPRY domain- and SOCS box-containing proteins. *J Biol Chem* 2011;**286**:9009–9019.
21. Zhang YE. Non-Smad Signaling Pathways of the TGF-beta Family. *Cold Spring Harb Perspect Biol* 2017;**9**.
22. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, et al. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* 2001;**3**:1014–1019.
23. The Tabula Muris Consortium, Overall coordination, Logistical coordination, Organ collection and processing, Library preparation and sequencing, Computational data analysis, Cell type annotation, Writing group, Supplemental text writing group & Principal investigators Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. *Nature* 2018;**562**:367–372.
24. Willems E, Cabral-Teixeira J, Schade D, Cai W, Reeves P, Bushway PJ, et al. Small molecule-mediated TGF-beta type II receptor degradation promotes cardiomyogenesis in embryonic stem cells. *Cell Stem Cell* 2012;**11**:242–252.
25. Xu Q, Wu Z. The insulin-like growth factor-phosphatidylinositol 3-kinase-Akt signaling pathway regulates myogenin expression in normal myogenic cells but not in rhabdomyosarcoma-derived RD cells. *J Biol Chem* 2000;**275**:36750–36757.
26. Zammit PS. Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. *Semin Cell Dev Biol* 2017;**72**:19–32.
27. Berkes CA, Tapscott SJ. MyoD and the transcriptional control of myogenesis. *Semin Cell Dev Biol* 2005;**16**:585–595.
28. Venuti JM, Morris JH, Vivian JL, Olson EN, Klein WH. Myogenin is required for late but not early aspects of myogenesis during mouse development. *J Cell Biol* 1995;**128**:563–576.
29. Chen B, You W, Wang Y, Shan T. The regulatory role of Myomaker and Myomixer-Myomerger-Minion in muscle development and regeneration. *Cell Mol Life Sci* 2020;**77**:1551–1569.
30. Pauw PG, Kaffer CR, Petersen RJ, Semerad SA, Williams DC. Inhibition of myogenesis by ouabain: effect on protein synthesis. *In Vitro Cell Dev Biol Anim* 2000;**36**:133–138.
31. Massague J, Cheifetz S, Endo T, Nadal-Ginard B. Type beta transforming growth factor is an inhibitor of myogenic differentiation. *Proc Natl Acad Sci U S A* 1986;**83**:8206–8210.
32. Liu D, Black BL, Derynck R. TGF-beta inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3. *Genes Dev* 2001;**15**:2950–2966.
33. Ge X, McFarlane C, Vajjala A, Lokireddy S, Ng ZH, Tan CK, et al. Smad3 signaling is required for satellite cell function and myogenic differentiation of myoblasts. *Cell Res* 2011;**21**:1591–1604.
34. Kollias HD, Perry RL, Miyake T, Aziz A, McDermott JC. Smad7 promotes and enhances skeletal muscle differentiation. *Mol Cell Biol* 2006;**26**:6248–6260.
35. Filvaroff EH, Ebner R, Derynck R. Inhibition of myogenic differentiation in myoblasts expressing a truncated type II TGF-beta receptor. *Development* 1994;**120**:1085–1095.
36. Girardi F, Taleb A, Ebrahimi M, Datye A, Gamage DG, Peccate C, et al. TGFbeta signaling curbs cell fusion and muscle regeneration. *Nat Commun* 2021;**12**:750.
37. Melendez J, Siero D, Salgado D, Morin V, Dejardin MJ, Zhou C, et al. TGFbeta signaling acts as a molecular brake of myoblast fusion. *Nat Commun* 2021;**12**:749.
38. Filippakopoulos P, Low A, Sharpe TD, Uppenberg J, Yao S, Kuang Z, et al. Structural basis for Par-4 recognition by the SPRY domain- and SOCS box-containing proteins SPSB1, SPSB2, and SPSB4. *J Mol Biol* 2010;**401**:389–402.
39. Bergmann CB, Beckmann N, Salyer CE, Hanschen M, Crisologo PA, Caldwell CC. Potential Targets to Mitigate Trauma- or Sepsis-Induced Immune Suppression. *Front Immunol* 2021;**12**:622601.
40. von Haehling S, Coats AJS, Anker SD. Ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2021. *J Cachexia Sarcopenia Muscle* 2021;**12**:2259–2261.