Dysregulation of Tweak and Fn14 in skeletal muscle of spinal muscular atrophy mice

Katharina E. Meijboom1,2, Emma R. Sutton3, Eve McCallion3, Emily McFall3, Daniel Anthony5, Benjamin Edwards1, Sabrina Kubinski6, Ines Tapken6,7, Ines Bünermann7, Gareth Hazell1, Nina Ahlskog1,8, Peter Claus6,7, Kay E. Davies1, Rashmi Kothary4, Matthew J. A. Wood1,8 and Melissa Bowerman1,3,9*

Abstract

Background: Spinal muscular atrophy (SMA) is a childhood neuromuscular disorder caused by depletion of the survival motor neuron (SMN) protein. SMA is characterized by the selective death of spinal cord motor neurons, leading to progressive muscle wasting. Loss of skeletal muscle in SMA is a combination of denervation-induced muscle atrophy and intrinsic muscle pathologies. Elucidation of the pathways involved is essential to identify the key molecules that contribute to and sustain muscle pathology. The tumor necrosis factor-like weak inducer of apoptosis (TWEAK)/TNF receptor superfamily member fibroblast growth factor-inducible 14 (Fn14) pathway has been shown to play a critical role in the regulation of denervation-induced muscle atrophy as well as muscle proliferation, differentiation, and metabolism in adults. However, it is not clear whether this pathway would be important in highly dynamic and developing muscle.

Methods: We thus investigated the potential role of the TWEAK/Fn14 pathway in SMA muscle pathology, using the severe Taiwanese Smn−/−; SMN2 and the less severe Smn2B/− SMA mice, which undergo a progressive neuromuscular decline in the first three post-natal weeks. We also used experimental models of denervation and muscle injury in pre-weaned wild-type (WT) animals and siRNA-mediated knockdown in C2C12 muscle cells to conduct additional mechanistic investigations.

Results: Here, we report significantly dysregulated expression of Tweak, Fn14, and previously proposed downstream effectors during disease progression in skeletal muscle of the two SMA mouse models. In addition, siRNA-mediated Smn knockdown in C2C12 myoblasts suggests a genetic interaction between Smn and the TWEAK/Fn14 pathway. Further analyses of SMA, Tweak−/−, and Fn14−/− mice revealed dysregulated myopathy, myogenesis, and glucose metabolism pathways as a common skeletal muscle feature, providing further evidence in support of a relationship between the TWEAK/Fn14 pathway and Smn. Finally, administration of the TWEAK/Fn14 agonist Fc-TWEAK improved disease phenotypes in the two SMA mouse models.

Conclusions: Our study provides mechanistic insights into potential molecular players that contribute to muscle pathology in SMA and into likely differential responses of the TWEAK/Fn14 pathway in developing muscle.

Keywords: Spinal muscular atrophy, Survival motor neuron, Smn, Tweak, Fn14, Glucose metabolism, Skeletal muscle, Atrophy, Denervation
neuron 1 (SMN1) gene [2]. The major pathological components of SMA pathogenesis are the selective loss of spinal cord alpha motor neurons and muscle wasting [3]. Skeletal muscle pathology is a clear contributor to SMA disease manifestation and progression and is caused by both denervation-induced muscle atrophy [4] and intrinsic defects [5, 6]. As skeletal muscle is the largest insulin-sensitive tissue in the body and is involved in glucose utilization [7], it is not surprising that muscle metabolism is also affected in SMA. Impaired metabolism has indeed been reported in SMA types 1, 2 and 3 patients [8]. A better understanding of the specific molecular effectors that contribute to SMA muscle physiopathology could provide mechanistic insights in SMA muscle pathology and help therapeutic endeavors aimed at improving muscle health in patients [9].

One pathway that plays a crucial role in chronic injury and muscle diseases is the tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its main signaling receptor, the TNF receptor superfamily member fibroblast growth factor-inducible 14 (Fn14) [10]. TWEAK is ubiquitously expressed and synthesized as a type 2 transmembrane protein but can also be cleaved by proteolytic processing and secreted as a soluble cytokine [10]. The role of the TWEAK/Fn14 pathway in skeletal muscle is conflicting as it has been demonstrated to have both beneficial and detrimental effects on muscle health and function [11, 12]. Indeed, pathologically high levels of TWEAK activate the canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which promotes myoblast proliferation and thus inhibits myogenesis and the early phases of muscle repair and regeneration [13]. Conversely, lower physiological concentrations of TWEAK activate the non-canonical NF-κB pathway that promotes myoblast fusion and myogenesis [11]. The transmembrane protein Fn14 is typically dormant or present in low levels in normal healthy muscle [14]. Atrophic-inducing conditions (e.g. casting and surgical denervation) stimulate the expression of Fn14, leading to the chronic activation of the TWEAK/Fn14 pathway and sustained skeletal muscle atrophy [15]. We have also demonstrated an increased activity of the Tweak/Fn14 pathway in skeletal muscle of a mouse model of the neurodegenerative adult disorder amyotrophic lateral sclerosis (ALS), which is characterized by a progressive and chronic denervation-induced muscle atrophy [16]. In addition, various reported downstream effectors of the TWEAK/Fn14 pathway play critical roles in the regulation of muscle metabolism such as peroxisome proliferator-activated receptor-gamma coactivator 1a (PGC-1α), glucose transporter 4 (Glut-4), myogenic transcription factor 2d (Mef2d), hexokinase II (HKII) and Krüppel-like factor 15 (Klf15) [17–20].

Although the TWEAK/Fn14 pathway has been ascribed roles in both skeletal muscle health regulation and metabolism, both of which are impacted in SMA [9, 21], this pathway has yet to be investigated in the context of SMA. Furthermore, all research on this pathway has been performed in adult mice and therefore has never been explored in early phases of muscle development. We thus investigated the potential role of TWEAK and Fn14 in SMA and in early phases of post-natal skeletal muscle development. We report significantly decreased levels of both Tweak and Fn14 during disease progression in two distinct SMA mouse models (Smmn−/−;SMN2 and Smmn2B+/−) [22, 23]. We also observed dysregulated expression of PGC-1α, Glut-4, Mef2d and HKII, previously proposed metabolic downstream effectors of TWEAK/Fn14 signaling [18, 24], in skeletal muscle of these SMA mice. In addition, more in-depth analyses revealed partial overlap of aberrantly expressed genes that regulate myopathy, myogenesis, and glucose metabolism pathways in skeletal muscle of SMA, Tweak−/− and Fn14−/− mice, further supporting potential shared functions between the TWEAK/Fn14 pathway and SMN in developing muscle. Finally, administration of Fc-TWEAK, an agonist of the TWEAK/Fn14 pathway, improved disease phenotypes in skeletal muscle of SMA, Tweak−/− and Fn14−/− mice, further supporting potential shared functions between the TWEAK/Fn14 pathway and SMN in developing muscle.

Methods

Animals and animal procedures

Wild-type mice FVB/N and C57BL/6J and the severe Smmn−/−;SMN2 mouse model (FVB.Cg-Smm1tm1Hung Tg(SMN2)2Hung/J) [22] were obtained from the Jackson Laboratories. The Smmn2B−/− mouse model [23, 25] was kindly provided by Dr. Lyndsay M. Murray (University of Edinburgh). Tweak−/− [26] and Fn14−/− mouse models [27] were generously obtained from Linda C. Burkl (Biogen).

Smmn−/−;SMN2 and Smmn+/−;SMN2 mice were generated by breeding Smmn+/− mice with Smmn−/−;SMN2/SMN2 mice as previously described [28]. Smmn2B−/− and Smmn2B+/− mice were generated by breeding Smmn+/−;SMN2/2B mice and Smmn+/− mice as previously described [23].

Experimental procedures with live animals were authorized and approved by the University of Oxford ethics committee and UK Home Office (current project license PEDFDC6F0, previous project license 30/2907) in accordance with the Animals (Scientific Procedures) Act 1986, the Keele University Animal Welfare Ethical Review Body and UK Home Office (Project License P99AB3B95) in accordance with the Animals (Scientific Procedures) Act 1986.
For nerve crush and cut experiments, post-natal day (P) 7 wild-type (WT) FVB/N mice were anesthetized with 2% isoflurane/oxygen before one of their lateral thighs was shaved and a 1 cm incision in the skin was made over the lateral femur. The muscle layers were split with blunt scissors and the sciatic nerve localized and crushed with tweezers for 15 s for the nerve crush. For the nerve cut, an ~2-mm section of the nerve was removed and the transection was confirmed under an operating microscope at ×12.8. The skin incision was closed with surgical glue, and animals were allowed to recover on a warming blanket. Ipsilateral and contralateral TA muscles were harvested at P14 and either fixed in 4% paraformaldehyde (PFA) for 24 h for histological analyses or snap frozen for molecular analyses.

Laminin staining of skeletal muscle
TA muscles were fixed in PFA overnight. Tissues were sectioned (13 μm) and incubated in blocking buffer for 2 h (0.3% Triton-X, 20% fetal bovine serum (FBS) and 20% normal goat serum in PBS). After blocking, tissues were stained overnight at 4 °C with rat anti-laminin (1:1000, Sigma L0663) in blocking buffer. The next day, tissues were washed in PBS and probed using a goat-anti-rat IgG 488 secondary antibody (1:500, Invitrogen A-11006) for 1 h. PBS-washed tissues were mounted in Fluoromount-G (Southern Biotech). Images were taken with a DM IRB microscope (Leica) with a 20× objective. Quantitative assays were performed blinded on 3–5 mice for each group and five sections per mouse. The area of muscle fiber within designated regions of the TA muscle sections was measured using Fiji (ImageJ) [29].

Hematoxylin and eosin staining of skeletal muscle
TA muscles were fixated in 4% PFA and imbedded into paraffin blocks. For staining, muscles were sectioned (13 μm) and deparaffinized in xylene and then fixed in 100% ethanol. Following a rinse in water, samples were stained in hematoxylin (Fisher) for 3 min, rinsed in water, dipped 40 times in a solution of 0.02% HCl in 70% ethanol, and rinsed in water again. The sections were next stained in a 1% eosin solution (BDH) for 1 min, dehydrated in ethanol, cleared in xylene, and mounted with Fluoromount-G (Southern Biotech).

Images were taken with a DM IRB microscope (Leica) with a 20× objective. Quantitative assays were performed blinded on 3–5 mice for each group and five sections per mouse. The area of muscle fiber within designated regions of the TA muscle sections was measured using Fiji (ImageJ) [29].

Cell culture
Both C2C12 myoblasts [30] and NSC-34 neuronal-like cells [31] were maintained in growth media consisting of Dulbecco’s Modified Eagle’s Media (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin (all Life Technologies). Cells were cultured at 37 °C with 5% CO₂. C2C12 myoblasts were differentiated in DMEM containing 2% horse serum for 7 days to form multinucleated myotubes. Cells were regularly tested for mycoplasma and remained mycoplasma-free.

In vitro siRNA knockdown
For small interfering RNA (siRNA) transfections, C2C12 myoblasts were seeded onto 12-well plates at a 50% confluence and cultured overnight in 2 mL of DMEM. Cells were washed with PBS prior to siRNA transfection, whereby 100 pmol of each siRNA (Tweak, Fn14,
and primer efficiencies were calculated with the LinReg
expression was quantified using the Pfaffl method [32],
by the value corresponding to 1/(average of all samples
malized expression of each referent sample in that group
expression values were set to 1 by multiplying the nor -
compared to a referent group, for which the normalized
the normalized expression of the experimental groups is
PolJ [33]. For all qPCR graphs,
RNA poly -
PCR software. We normalized relative expression level of
IDT and sequences for primers were either self-designed
was analyzed using the StepOne Software v2.3 (Applied
Mix Hi-ROX (PCR Biosystems). The same amplification
was analyzed on a StepOnePlus Real-Time PCR Thermocycler
Technologies as per manufacturer’s instructions.
The same RNA extraction method was employed for
similar experiments and equal RNA amounts were used
between samples within the same experiments. cDNA
was prepared with the High-Capacity cDNA Kit (Life
Technologies) or qPCRBIO cDNA Synthesis Kit (PBCR
Biosystems) according to the manufacturer’s instructions.
The same reverse transcription method was employed
for similar experiments. The cDNA template was ampli-
fied on a StepOnePlus Real-Time PCR Thermocycler
(Life Technologies) with SYBR Green Mastermix from
Applied Biosystems or with qPCRBIO SyGreen Blue
Mix Hi-ROX (PCR Biosystems). The same amplification
method was used for similar experiments. qPCR data
was analyzed using the StepOne Software v2.3 (Applied
Biosystems). Primers used for qPCR were obtained from
IDT and sequences for primers were either self-designed
or ready-made (Supplementary Table 1). Relative gene
expressions were compared to DMEM without the
siRNAs for 1 day or with a differentiation medium mix
without the siRNAs for 7 days.

**qPCR**

RNA was extracted from tissues and cells either by a
RNeasy kit from QIAGEN or by a Isolate II RNA Kit from
Bioline or by guanidinium thiocyanate-acid-
phenol-chloroform extraction using TRizol Reagent
(Life Technologies) as per manufacturer’s instructions.
The same RNA extraction method was employed for
similar experiments and equal RNA amounts were used
between samples within the same experiments. cDNA
was prepared with the High-Capacity cDNA Kit (Life
Technologies) or qPCRBIO cDNA Synthesis Kit (PBCR
Biosystems) according to the manufacturer’s instructions.
The same reverse transcription method was employed
for similar experiments. The cDNA template was ampli-
fied on a StepOnePlus Real-Time PCR Thermocycler
(Life Technologies) with SYBR Green Mastermix from
Applied Biosystems or with qPCRBIO SyGreen Blue
Mix Hi-ROX (PCR Biosystems). The same amplification
method was used for similar experiments. qPCR data
was analyzed using the StepOne Software v2.3 (Applied
Biosystems). Primers used for qPCR were obtained from
IDT and sequences for primers were either self-designed
or ready-made (Supplementary Table 1). Relative gene
expression was quantified using the Pfaffl method [32],
and primer efficiencies were calculated with the LinReg
PCR software. We normalized relative expression level of
all tested genes in mouse tissue and cells to RNA pol-
ymerase II polypeptide J (Polj) [33]. For all qPCR graphs,
the normalized expression of the experimental groups is
compared to a referent group, for which the normalized
expression values were set to 1 by multiplying the nor-
malized expression of each referent sample in that group
by the value corresponding to 1/(average of all samples
in that referent experimental group). That value was then
used to multiply the normalized relative expression of
each sample in all experimental groups.

**PCR array**

RNA was extracted using the RNeasy® Microarray Tis-
sue Kit (QIAGEN). cDNA was generated with the RT² First
Strand Kit (QIAGEN). qPCRs were performed using RT²
Profiler® PCR Array Mouse Skeletal Muscle: Myogenesis
and Myopathy Mouse (PAMM-099Z, SABiosciences) and
RT² Profiler® PCR Array Mouse Glucose Metabolism
(PAMM-006Z SABiosciences). The data were analyzed
with RT Profiler PCR Array Data Analysis (version 3.5),
and mRNA expression was normalized to the two most
stably expressed genes between all samples. We used the
publicly available database STRING (version 11.5) for
network and enrichment analysis of differently expressed
genes [34]. The minimum required interaction score was
set at 0.4, medium confidence.

**Western blot**

For westerns in Fig. 1, freshly prepared radioimmuno-
precipitation (RIPA) buffer was used to homogenize tis-
cue and cells, consisting of 50 mM Tris pH 8.8, 150 mM
NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS,
and complete mini-protease inhibitors (Roche). Equal
amounts of total protein were loaded, as measured by
Bradford assay. Protein samples were first diluted 1:1
with Laemmli sample buffer (Bio-Rad, Hemel Hemp-
stead, UK) containing 5% β-mercaptoethanol (Sigma)
and heated at 100 °C for 10 min. Next, samples were
loaded on freshly made 1.5 mm 12% polyacrylamide
separating and 5% stacking gel, and electrophoresis was
performed at 120 V for ~1.5 h in running buffer. Subse-
quently, proteins were transferred from the gel onto a
polyvinylidene fluoride membrane (Merck Millipore) via
electroblotting at 120 V for 20 min in transfer buffer con-
taining 20% methanol. Membranes were then incubated
for 2 h in Odyssey Blocking Buffer (Licor). The mem-
brane was then probed overnight at 4 °C with primary
antibodies (P105/p50, 1:1000, Abcam ab32360; Actin,
1:1000, Abcam ab3280) in Odyssey Blocking Buffer and
0.1% Tween-20. The next day, after three 10-min washing

(See figure on next page.)

**Fig. 1** Aberrant expression of Tweak and Fn14 in skeletal muscle of Smn−/−;Smn2 SMA mice. a–g qPCR analysis of parvalbumin (a), Tweak (b), Fn14 (c), Pgc-1α (d), Met2d (e), Glut-4 (f), and HKII (g) in triceps, gastrocnemius, TA, and quadriceps muscles from postnatal day (P) 0 (birth), P2 (pre-symptomatic), P5 (early symptomatic), P7 (late symptomatic), and P19 (end stage) Smn−/−;Smn2 and wild-type (WT) mice. Normalized relative expression values are compared to WT P0. Data are mean ± SEM, n = 3-4 animals per experimental group, two-way ANOVA, Sidak’s multiple comparison test between genotypes, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. h–j Quantification of NF-κB p50/actin protein levels in the TA of
pre-symptomatic (P2) (h) and late-symptomatic (P7) (i) Smn−/−;Smn2 mice and age-matched WT animals. Images are representative immunoblots. Data are mean ± SEM, n = 3-4 animals per experimental group, unpaired t-test, ns, not significant (h), p = 0.0215 (i)j. Quantification of NF-κB p50/actin and p105/actin protein levels in the quadriceps (quad) of late-symptomatic (P7) Smn−/−;Smn2 mice and age-matched WT animals. Images are representative immunoblots. Data are mean ± SEM, n = 3-4 animals per experimental group, two-way ANOVA, uncorrected Fisher’s LSD, ****p < 0.0001, ns, not significant. k qPCR analysis NF-κB-inducing kinase (NIK) in TA muscle of late-symptomatic P7 Smn−/−;Smn2 and age-matched WT animals. Data are mean ± SEM, n = 3-4 animals per experimental group, unpaired t-test, p = 0.0094
Fig. 1 (See legend on previous page.)
steps with PBS, the membrane was incubated for 1 h at room temperature with secondary antibodies (goat anti-rabbit IgG 680RD, 1:1000, LI-COR 926-68071; goat antimouse IgG 800CW, 1:1000 LI-COR, 926-32210). Lastly, the membrane was washed three times for 10 min in PBS and visualized by scanning 700 nm and 800 nm channels on the LI-COR Odyssey CLx infrared imaging system (LI-COR) for 2.5 min per channel. The background was subtracted, and signal of protein of interest was divided by signal of the housekeeping protein.

For westerns in all others figures, the same steps were followed with the following key differences. Bio-Rad TGX Stain-Free gels were used, and gels were imaged on a ChemiDoc Bio-Rad Imager before transfer to quantify total protein used for normalization. The primary antibodies used were NF-kB2 p100/p52 (Cell Signaling, no. 4882, 1:1000), NF-kB1 p105/p50 (Cell Signaling, no. 12540, 1:1000), and Tweak (Abcam, ab37170, 1:1000). The secondary antibody used was goat anti-rabbit IgG secondary Dylight 800 (Invitrogen, SA5-100036, 1:1000). Quantification was performed using the Bio-Rad Image Lab software.

Statistical analysis
All statistical analyses were done with the most up to date GraphPad Prism software. When appropriate, a Student’s unpaired two-tail t-test, a one-way ANOVA, or a two-way ANOVA was used. Post hoc analyses used are specified in figure legends. Outliers were identified via the Grubbs’ test. For the Kaplan-Meier survival analysis, the log-rank test was used and survival curves were considered significantly different at p < 0.05.

Results
Tweak and Fn14 are dysregulated in two SMA mouse models
We firstly investigated the expression of Tweak and Fn14 in skeletal muscle of the severe Taiwanese Smn−/−;SMN2 mouse model [22], using muscles with reported differential vulnerability to neuromuscular junction (NMJ) denervation (vulnerability: triceps brachii > gastrocnemius > TA > quadriceps femoris) [35]. Muscles were harvested from Smn−/−;SMN2 and WT mice at several time points during disease progression: birth (post-natal day (P) 0, pre-symptomatic (P2), early symptomatic (P5), late symptomatic (P7), and end stage (P10)). Muscle pathology in this SMA mouse model during disease progression has been well documented [36, 37].

We assessed the expression of parvalbumin, a high affinity Ca2+-binding protein, which is downregulated in denervated muscle [38, 39] and a marker of muscle atrophy in skeletal muscle of SMA patients and Smn−/−;SMN2 mice [40]. We observed a significant decreased expression of parvalbumin mRNA during disease progression (Fig. 1a) in SMA mice compared to WT animals, further confirming parvalbumin as a bona fide marker of muscle atrophy in SMA [40]. Furthermore, we noted that parvalbumin expression was downregulated at earlier time points in the two most vulnerable muscles (triceps and gastrocnemius) [35] of SMA mice compared to WT animals (Fig. 1a).

We next evaluated the expression of Tweak and Fn14 and observed significant decreased levels of Tweak mRNA in muscles of Smn−/−;SMN2 mice during disease progression, except in the quadriceps (Fig. 1b). Similarly, we found significantly lower levels of Fn14 mRNA in all muscles of Smn−/−;SMN2 mice during disease progression (Fig. 1c) compared to WT animals. Interestingly, the decreased expression of Fn14 in denervated and atrophied muscles of neonatal animals is different to previous reports in adults where denervation-induced atrophy stimulates its expression [15, 16].

As mentioned above, the TWEAK/Fn14 pathway has been reported to negatively influence the expression of metabolic effectors Klf15, Pgc-1α, Mef2d, Glut-4 and HKII [18]. Given that we have previously published a concordant increased expression of Klf15 in skeletal muscle of SMA mice during disease progression [41], we next evaluated if the additional metabolic targets proposed to be modulated by Tweak and Fn14 were similarly dysregulated in the predicted directions. We indeed observed that the mRNA expression of Pgc-1α, Mef2d, Glut-4 and HKII was significantly upregulated in muscles of Smn−/−;SMN2 mice at symptomatic time points (P5–P10) compared to WT animals (Fig. 1d–g), showing an expected opposite pattern to both Tweak and Fn14 (Fig. 1b–c) [18]. Notably, we also found that in most muscles, mRNA levels of Pgc-1α, Mef2d, Glut4 and HKII were significantly decreased in pre-symptomatic Smn−/−;SMN2 mice (P0–P5) compared to WT animals (Fig. 1d–g), independently of Tweak and Fn14 (Fig. 1b–c). TWEAK and Fn14 have also been reported to impact the canonical and non-canonical NF-κB pathways in skeletal muscle [42, 43]. In pre-symptomatic (P2) TA muscle, we observed no significant difference in the expression of NF-κB1 (p50), a component of the canonical NF-κB pathway, between Smn−/−;SMN2 mice and WT animals (Fig. 1h), consistent with normal Tweak and Fn14 levels (Fig. 1b–c). Conversely, there was a significant decreased expression of NF-κB1 (p50) in TA muscle of symptomatic Smn−/−;SMN2 mice compared to WT animals at P7 (Fig. 1i), in line with reduced levels of Tweak and Fn14 (Fig. 1b). These findings are validated in P7 quadriceps, where NF-κB1 (p50) levels are also significantly decreased in Smn−/−;SMN2 mice compared to WT animals (Fig. 1j). We found no significant
difference for the p105 NF-κB1 component. Of note, for
all NF-κB1 p50/105 westerns, the p105 component was
always more difficult to detect and sometimes even unde-
tectable such as was the case for P7 TAs. We also inves-
tigated the expression of NF-κB-inducing kinase (NIK),
involved in the non-canonical NF-κB activation pathway
[44]. We observed that mRNA levels of NIK were signifi-
cantly increased in TA muscle of P7 Smn+/−;SMN2 mice
compared to WT animals (Fig. 1k), suggesting that dys-
regulated activity of Tweak and Fn14 in skeletal muscle
of SMA mice may influence both the canonical and non-
canonical NF-κB pathways, which play key regulatory
roles in muscle health and metabolism [11, 12].

Finally, we evaluated the expression of Tweak and
Fn14 in skeletal muscle of the less severe Smn2B/−/− mouse
model of SMA [23]. TA muscles were harvested from
Smn2B/−/− mice and age-matched WT animals at P0 (birth),
P2 (early pre-symptomatic), P4 (late pre-symptomatic),
P11 (early symptomatic), and P19 (end stage). Similar to
the Smn+/−/−;SMN2 mice, muscle pathology in this SMA
mouse model during disease progression has been well
documented [36, 37]. We found a significant decreased
dexpression of parvalbumin (Fig. 2a), Tweak (Fig. 2b) and
Fn14 (Fig. 2c) in muscle from Smn2B/−/− mice during dis-
ease progression compared to WT animals, similar to
that observed in the more severe Smn+/−/−;SMN2 SMA
mouse model (Fig. 1a–c). We have previously reported
the aberrant increased expression of Klf15 in the TA
muscle of Smn2B/−/− mice during disease progression [41].
However, Pgc-1a expression was increased at P11 only
(Fig. 2d), Mef2d at P2 only (Fig. 2e), Glut-4 at P11 only
(Fig. 2f), while HKII was significantly decreased at P0 and
P19 and significantly increased at P4 (Fig. 2g), sugges-
ting that the proposed negative impact of Tweak and Fn14
activity on these metabolic effectors may be dependent
on disease severity, age, and/or genetic strain. Tweak
downregulation in triceps of P18 Smn2B/−/− mice was con-
firmed by western (Fig. 2h). Furthermore, contrary to
what was observed in the Smn+/−/−;SMN2 mice, there was
no significant difference in the NF-κB1 p50 component but
a significant decreased expression of the NF-κB1 p105 component in skeletal muscle of Smn2B/−/− mice
compared to WT animals (Fig. 2i). For the NF-κB2 path-
way, we found no significant difference for either the p52
or the p100 components (Fig. 2j). Thus, our results point
to distinct profiles of the NF-κB1 and 2 pathways in skel-
etic muscle of the two SMA mouse models, which could
be due to differential expression and/or processing of the
components and to non-Tweak/Fn14 pathways.

To determine if the dysregulated expression of Tweak,
Fn14, and the previously reported metabolic effectors in
SMA muscle is independent of disease status, we inves-
tigated the mRNA expression of Tweak, Fn14, Pgc-1a,
Mef2d, Glut-4, HKII and Klf15 in triceps of P7 WT, Smn2B/−/− and Smn+/−/− mice (Supplementary Fig. 1), a time
point at which significant changes were already observed
in the Smn+/−/−;SMN2 mice. Smn2B/−/− and Smn+/−/− mice
express ~70% and 50% of full-length functional Smn pro-
tein compared to WT animals, respectively, and do not
display a canonical SMA phenotype [23, 45]. While we
found some instances of differential expression (Glut-4:
Smn2B/−/− vs Smn+/−/−, HKII: Smn2B/−/− vs Smn+/−/− and Klf15:
WT vs Smn+/−/−), there is no clear correlation between
non-pathological Smn levels (WT vs Smn2B/−/− vs Smn+/−/−)
and expression of molecular components associated with
the Tweak/Fn14 pathway (Supplementary Fig. 1).

We have thus demonstrated that Tweak, Fn14, and
associated metabolic effectors are dysregulated during
progressive muscle atrophy in two SMA mouse models,
and that this is most likely due to pathological levels of
Smn depletion.

**Denervation does not affect Tweak and Fn14
during the early stages of muscle development**

As SMA muscle pathology is defined by both intrinsic
defects and denervation-induced events, we set out to
determine which of these may influence the dysregu-
lation of Tweak and Fn14 in SMA muscle. We firstly
addressed the denervation component by performing
nerve crush experiments in which the sciatic nerves of
P7 WT mice were crushed and the muscle harvested at
P14 [46]. Of note, the sciatic nerve was crushed in only
one hind limb, leaving the other control hindlimb intact.
Quantification of myofiber area in TA muscles showed a

![Figure 2](https://example.com/figure2)

**Figure 2** Aberrant expression of Tweak and Fn14 in skeletal muscle of Smn2B/−/−-SMA mice. a–g qPCR analysis of parvalbumin (a), Tweak (b), Fn14 (c), Pgc-1a (d), Mef2d (e), Glut-4 (f), and HKII (g) in TA muscles from P0 (birth), P2 (pre-symptomatic), P4 (pre-symptomatic), P11 (early symptomatic), and P19 (end stage) Smn2B/−/− and WT mice. Normalized relative expressions are compared to WT P0. Data are mean ± SEM, n = 3–4 animals per experimental group, two-way ANOVA, Sidak’s multiple comparison test between genotypes, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

h Quantification of Tweak protein levels normalized to total protein in the triceps of late-symptomatic (P18) Smn+/−/− mice and age-matched WT animals. Images are representative immunoblots. Data are mean ± SEM, n = 6–7 animals per experimental group, unpaired t-test, p = 0.014.

i Quantification of NF-κB1 p50 and p105 protein levels normalized to total protein in the triceps of late-symptomatic (P18) Smn+/−/− mice and age-matched WT animals. Images are representative immunoblots. Data are mean ± SEM, n = 6–7 animals per experimental group, unpaired t-test, ns, not significant (p50), p = 0.0354 (p105).

j Quantification of NF-κB2 p52 and p100 protein levels normalized to total protein in the triceps of late-symptomatic (P18) Smn+/−/− mice and age-matched WT animals. Images are representative immunoblots. Data are mean ± SEM, n = 3–4 animals per experimental group, unpaired t-test, ns, not significant (p52), p = 0.0532 (p100).
significant decrease in myofiber size in the nerve crush muscle compared to the control hind limb (Fig. 3a–c).

Expression analyses further revealed that there were no significant changes in mRNA levels of parvalbumin, Tweak, Fn14, PGC-1α, Mef2d, Glut-4 and HKII in the denervated muscle compared to the control TA muscle (Fig. 3d). Interestingly, while denervation in adult muscle has previously been reported to induce a dramatic
surge in Fn14 expression [15, 16], this did not occur in the denervated muscles of our pre-weaned mice, suggesting an age and/or development regulatory element to this response. We also investigated the expression of Klf15 and Smn and similarly observed no significant differences between the nerve crush and control muscles (Fig. 3d).

To ensure that our results were not influenced by the potential reinnervation of muscles following a nerve crush, we repeated the experiments by performing a nerve cut instead. We observed that this complete denervation of TAs in pre-weaned mice does not significantly impact the mRNA expression of Tweak, Fn14, PGC-1α, Mef2d, Glut-4, HKII and Klf15 compared to uninjured control hind limbs (Supplementary Fig. 2).

Overall, these results suggest that the dysregulation of parvalbumin, Tweak, Fn14, and the proposed metabolic effectors in SMA muscle during disease progression is most likely not denervation dependent.

Fig. 3  Tweak and Fn14 are not dysregulated in denervated (nerve crush) muscles of pre-weaned mice. A sciatic nerve crush was performed on postnatal day (P) 7 WT FVB/N mice, and both ipsilateral (nerve crush) and contralateral (control) TA muscles were harvested at P14. a Representative images of hematoxylin and eosin-stained cross sections of control and nerve crush TA muscles. Scale bars, 100 μm. b Myofiber area in control and nerve crush TA muscles. Data are mean ± SEM, n = 3–6 animals per experimental group, unpaired t-test, p = 0.0020. c Myofiber size distribution in control and nerve crush TA muscles. d qPCR analysis of parvalbumin, Tweak, Fn14, Pgc-1α, Mef2d, Glut-4, HKII, Klf15, and Smn in control and nerve crush TA muscles. Normalized relative expressions for each gene are compared to control muscle. Data are mean ± SEM, n = 4–6 animals per experimental group, two-way ANOVA, uncorrected Fisher’s LSD, ns, not significant.
Intrinsic muscle injury affects Tweak and Fn14 during the early stages of muscle development

We next investigated what impact impairing intrinsic muscle integrity would have on Tweak and Fn14. To do so, we used cardiotoxin to induce myofiber necrosis. Cardiotoxin was injected in P10 WT mice into the left TA, while the right TA was injected with equal volumes of 0.9% saline and used as a control. TAs were harvested after 6 days, a time point where muscles are still in an immature and regenerating mode [47]. Indeed, analysis of centrally located nuclei showed a significantly increased percentage of regenerating myofibers in cardiotoxin-treated muscles compared to saline-treated TAs (Fig. 4a–b).

We then proceeded with molecular analyses and observed that the atrophy marker parvalbumin was significantly downregulated in cardiotoxin-treated TA muscles compared to saline-treated TA muscles (Fig. 4c). Fn14 mRNA expression was significantly increased after cardiotoxin injury (Fig. 4c), in accordance with previous research showing that muscle damage conditions activate Fn14 [15]. Conversely, Pgc-1α, Glut-4, HKII and Klf15 mRNA levels were significantly downregulated (Fig. 4c), supporting their previously reported negative response to

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**Fig. 4** Tweak and Fn14 are dysregulated in cardiotoxin-induced muscle necrosis in pre-weaned mice. Cardiotoxin was injected in the left TA muscle of postnatal day (P) 10. The right TA muscle was injected with equal volumes of 0.9% saline. TA muscles were harvested 6 days later. **a** Representative images of hematoxylin and eosin-stained cross sections of saline- and cardiotoxin-injected TA muscles. Scale bars, 100 μm. **b** Percentage of muscle fibers with centrally-located nuclei in saline- and cardiotoxin-injected TA muscles. Data are mean ± SEM, n = 3 animals per experimental group, unpaired t-test, p = 0.0020. **c** qPCR analysis of parvalbumin, Tweak, Fn14, Pgc-1α, Mef2d, Glut-4, HKII, Klf15, and Smn in saline- and cardiotoxin-injected TA muscles. Normalized relative expressions for each gene are compared to saline-treated muscle. Data are mean ± SEM, n = 3 animals per experimental group, two-way ANOVA, uncorrected Fisher’s LSD, ns, not significant, *p < 0.05, ***p < 0.001, ****p < 0.0001
active Tweak and Fn14 [18]. Interestingly, Tweak mRNA expression remained unchanged (Fig. 4c), contrary to previous reports of upregulation following cardiotoxin injury in adult muscle [48], suggesting a differential response in early developmental stages of skeletal muscle. Notably, Smn expression was significantly increased in the regenerating muscles compared to saline-treated TA muscles (Fig. 4c), perhaps due to SMN’s reported role during muscle fiber regeneration [49].

Together, these results suggest that intrinsic muscle injury in pre-weaned mice induces a dysregulation of Tweak, Fn14 and previously reported proposed metabolic effectors. However, the changes were in the opposite direction than that observed in SMA muscles (Fig. 1b), perhaps due to the necrosis and regeneration events that occur following cardiotoxin injury [50], which are not typically found in muscles of SMA mice.

Genetic interactions between Smn, Tweak, and Fn14 in muscle

We next wanted to further understand the potential relationship between dysregulated expression of Tweak, Fn14, and Smn in skeletal muscle of SMA mice. To do so, we evaluated the impact of Tweak and Fn14 depletion in the early stages of muscle development by performing molecular analyses on P7 triceps from Fn14−/−, Tweak−/− and WT mice. In Tweak−/− mice, we observed a significant increased expression of Fn14 with a concomitant significantly decreased expression of Klf15 compared to WT animals (Fig. 5a). Notably, we found a significant decreased expression of Smn in Tweak−/− triceps compared to WT mice (Fig. 5a), suggesting a direct or indirect positive interaction between Tweak and Smn levels. For their part, Fn14−/− mice displayed a significant downregulation of parvalbumin and a significant upregulation of Pgc-1α (Fig. 5b). These analyses further support the previously reported negative influence of Fn14 on Pgc-1α and Klf15 expression as well as the absence of overt pathological muscle phenotypes in young Tweak−/− and Fn14−/− mice [15, 51].

To further dissect the relationship between Smn, Tweak, and Fn14 during myogenic differentiation, we performed siRNA-mediated knockdown of Smn, Tweak and Fn14 in C2C12 myoblasts and evaluated the effect on the expression of Tweak, Fn14, and the previously reported proposed metabolic effectors in undifferentiated (day 0) and differentiated (day 7) cells. Reduced levels of Smn, Tweak and Fn14 were significantly maintained in both proliferating and differentiated cells following transfection with siSmn, siTweak, and siFn14, respectively (Fig. 5c−e). We observed an interaction between Smn, Tweak and Fn14 specifically in differentiated C2C12s, whereby Smn expression was significantly upregulated in Fn14-depleted D7 cells (Fig. 5c), Tweak expression was significantly reduced in Smn-depleted D7 cells (Fig. 5d) and Fn14 levels were significantly decreased in Tweak- and Smn-depleted D7 cells (Fig. 5e). Similarly, the effects of siRNA-mediated knockdown of Smn, Tweak and Fn14 on the metabolic effectors were only apparent in differentiated C2C12s (Fig. 5f−j). Indeed, both knockdown of Tweak and Fn14 resulted in a significant upregulation of Pgc-1α (Fig. 5f) and Mef2d (Fig. 5g). While Glut-4 expression was neither affected by depletion of Smn, Tweak, or Fn14 (Fig. 5h), HKII mRNA levels were significantly decreased following knockdown of all three (Fig. 5i). Finally, Klf15 expression was significantly increased in siRNA-mediated knockdown of Fn14 only (Fig. 5j). The upregulation of Pgc-1α, Mef2d and Klf15 in Tweak- and/or Fn14-depleted differentiated C2C12 cells is in accordance with the previously reported downregulation of these genes when Tweak and Fn14 are active, while the unchanged Glut-4 and downregulated HKII levels were not [52].

Thus, using both in vivo and in vitro models, we have provided evidence for a potential interaction between Smn, Tweak and Fn14 and subsequent impact on the previously proposed downstream metabolic effectors (Fig. 5k). Our results suggest that the aberrant expression of Tweak and Fn14 in SMA muscle during disease progression may be due to a dynamic interplay between muscle-specific conditions and the molecular impact, individual and combined, of reduced expression of Smn, Tweak and Fn14 in the early developmental stages of skeletal muscle.
Fig. 5 (See legend on previous page.)
Overlap of dysregulated myopathy and myogenesis genes and glucose metabolism genes in SMA, Fn14−/− and Tweak−/− mice

To further decipher the potential contribution(s) of Smn, Tweak, and Fn14 depletion to SMA muscle pathology, we used commercially available mouse myopathy and myogenesis qPCR arrays (SABiosciences), which measure expression levels of a subset of 84 genes known to display and/or regulate myopathy and myogenesis. We used triceps (vulnerable) and quadriceps (resistant) from P7 Smn−/−;SMN2, Tweak−/− and Fn14−/− mice. WT FVB/N mice were compared to SMA animals and WT C57BL/6 mice were compared to Tweak−/− and Fn14−/− mice to account for differences due to genetic strains. Unsurprisingly, we observed a larger number of significantly dysregulated myopathy and myogenesis genes in triceps of Smn−/−;SMN2 mice than in the more resistant quadriceps, some of which overlapped with the subset of genes aberrantly expressed in Fn14−/− and Tweak−/− mice (Fig. 6a, Table 1, Supplementary file 1).

We also used the publicly available database STRING [34] to perform network and enrichment analysis of the shared differentially expressed genes in both triceps and quadriceps (Table 1), which revealed that there were no known protein-protein interactions between any of the dysregulated genes and Smn, Fn14, or Tweak (Fig. 6b). Interestingly, the central connectors Myod1 and Myf6 were upregulated in Tweak−/− and Fn14−/− mice and Pax7 was downregulated in the triceps of all three experimental groups (Table 1). Myod1 and Myf6 are key myogenic regulatory factors (MRFs) and are normally upregulated after skeletal muscle injury [53]. Pax7 is a canonical marker for satellite cells, the resident skeletal muscle stem cells [53], and reduced activity of Pax7 leads to cell-cycle arrest of satellite cells and dysregulation of MRFs in skeletal muscle [54]. Furthermore, Titin (Ttn) was downregulated in the quadriceps muscles of all three mouse models and plays major roles in muscle contraction and force production, highlighted by titin mutations leading to a range of skeletal muscle diseases and phenotypes [55].

Next, as SMN, TWEAK, and Fn14 have been associated with glucose metabolism abnormalities [18, 56], we performed similar gene expression analyses with commercially available qPCR arrays (SABiosciences) containing a subset of 84 genes known to display and/or regulate glucose metabolism. We found a similar large number of genes that were dysregulated in both triceps and quadriceps muscles of Smn−/−;SMN2 mice, some of which overlapped with those differentially expressed in Fn14−/− and Tweak−/− mice (Fig. 6c, Table 2, Supplementary file 2). STRING network and enrichment analysis [34] revealed that there are no known protein-protein interactions between any of the dysregulated genes and Smn, Fn14, or Tweak (Fig. 6d). Further analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways composed of the glucose metabolism genes significantly dysregulated in the same direction in triceps and quadriceps muscles of P7 Smn−/−;SMN2, Fn14−/− and Tweak−/− mice as well as the downstream effectors of the TWEAK/Fn14 pathway studied in this project (Pgc-1α, Mef2d, Glut4, Klf15 and HKII) reveals that many aspects of glucose metabolism such as insulin signaling and glycolysis are dysregulated in Smn−, Tweak−, and Fn14-depleted mice (Table 3).

We thus show a shared pattern of aberrantly expressed genes that modulate myogenesis, myopathy and glucose metabolism in SMA, Tweak-depleted, and Fn14-depleted skeletal muscle, suggesting that Smn, Tweak, and Fn14 may act synergistically on muscle pathology and metabolism defects in SMA muscle.

Fig. 6 Overlap between dysregulated genes involved in myopathy, myogenesis, and glucose metabolism in skeletal muscle of Smn−/−;SMN2, Fn14−/−, and Tweak−/− mice.

(a) Venn diagram showing overlap of genes involved in myopathy and myogenesis that are significantly dysregulated in the same direction (either up or downregulated, p < 0.05) in triceps and quadriceps muscle from postnatal day (P) 7 compared to Smn−/−;SMN2, Fn14−/−, and Tweak−/− mice to age- and genetic strain-matched wild-type (WT) mice. (b) Network and enrichment analysis of the overlap of significantly dysregulated myopathy and myogenesis genes in triceps and/or quadriceps of P7 Smn−/−;SMN2, Fn14−/−, and Tweak−/− mice using STRING software. Smn (Smn1), TWEAK (Tnfsf12), and Fn14 (Tnfrsf12a) are included in the analysis. Colored nodes represent query proteins and first shell of interactors. Filled nodes indicate that some 3D structure is known or predicted. Connection colored lines between nodes represent either known interactions (turquoise: from curated databases, magenta: experimentally determined), predicted interactions (green: gene neighborhood, yellow: textmining, black: co-expression, light blue: protein homology).

(b) Venn diagram showing overlap of genes involved in glucose metabolism that is significantly dysregulated in the same direction (either up or downregulated, p < 0.05) in triceps and quadriceps muscle from P7 compared to Smn−/−;SMN2, Fn14−/−, and Tweak−/− mice to age- and genetic strain-matched WT mice. (c) Network and enrichment analysis of the overlap of significantly dysregulated myopathy and myogenesis genes in triceps and/or quadriceps of P7 Smn−/−;SMN2, Fn14−/−, and Tweak−/− mice using STRING software. Smn (Smn1), TWEAK (Tnfsf12), and Fn14 (Tnfrsf12a), HKII (Hk2), Glut4 (Slc2a4), Pgc-1α (Ppargc1a), Klf15, and Mef2d are included in the analysis. Colored nodes represent query proteins and first shell of interactors. Filled nodes indicate that some 3D structure is known or predicted. Connection colored lines between nodes represent either known interactions (turquoise: from curated databases, magenta: experimentally determined), predicted interactions (green: gene neighborhood, red: gene fusions, dark blue: gene co-occurrence) or other interactions (yellow: textmining, black: co-expression, light blue: protein homology).
Fig. 6 (See legend on previous page.)
Table 1  Myogenesis and myopathy genes significantly dysregulated in the same direction in triceps and quadriceps of P7 Smn⁻/−;SMN2, Fn14⁻/− and Tweak⁻/− mice when compared to P7 WT mice

| Gene                                                                 | Triceps                    | Quadriceps                |
|---------------------------------------------------------------------|-----------------------------|----------------------------|
|                                                                     | Smn⁻/−;SMN2 | Fn14⁻/− | Tweak⁻/− | Smn⁻/−;SMN2 | Fn14⁻/− | Tweak⁻/− | Smn⁻/−;SMN2 | Fn14⁻/− | Tweak⁻/− |
| Crystallin alpha B (Cryab)                                          | Up 1.3245  | 1.3814  | 1.3711  | 0.010565  | 1.3711  | 0.044153  | 1.386      | 0.013894  | 1.2332  | 0.000616  | ns        |
| Dystroglycan 1 (Dag1)                                                | Down ↑ -1.1823 | -1.3607 | -1.3350 | 0.001058  | -1.3757 | 0.039065  | -1.1416  | 0.003343  | -1.4885  | 0.001277  | ns        |
| Insulin-like growth factor binding protein 5 (Igfibp5)              | Down -6.5548 | -1.3643 | -1.2758 | 0.008243  | -8.0095 | 0.000017  | -1.2813  | 0.006583  | ns       | ns        |
| Myogenic factor 6 (Myf6)                                             | Up 1.1221  | 0.000823 | 1.6485  | 0.03036   | 1.0137  | 0.831317  | ns        | 1.4051  | 0.006745  | ns        |
| Myogenic differentiation 1 (Myod1)                                   | Up 1.2595  | 0.046953 | 1.91    | 0.00036   | 1.2813  | 0.006583  | ns        | ns       | ns        | ns        |
| Paired Box 7 (Pax7)                                                  | Down -1.7035 | -1.1814 | -1.1522 | 0.014681  | -2.4268 | 0.000151  | ns        | ns       | ns        | ns        |
| Protein kinase AMP-activated non-catalytic subunit gamma 3 (Pik3cg3) | Down -13.700 | -1.7401 | -1.6191 | 0.001641  | -8.0475 | 0.000019  | -1.6521  | 0.005939  | -1.6346  | 0.006477  | ns        |
| Pyruvate dehydrogenase kinase 4 (Pdk4)                               | Up 7.7734  | 1.8326  | 2.209   | 0.004106  | 5.3978  | 0.05184  | 1.9628  | 0.006922  | 1.5978  | 0.003346  |
| Ribosomal protein S6 kinase B1 (Rps6k1b1)                           | Down -1.1105 | -1.1183 | -1.3350 | 0.006426  | -1.541  | 0.012036  | -1.1078  | 0.027897  | -1.4257  | 0.000143  |
| Titin (Ttn)                                                          | Down -1.4206 | -1.1322 | 0.023526 | ns        | -1.2847 | 0.000386  | -1.1965  | 0.01243  | -1.2861  | 0.001181  |

N* not significant, ↑ significantly upregulated, ↓ significantly downregulated. Fold change (2^(-delta-delta CT)) is the normalized gene expression (2^(-delta CT)) in the test sample divided the normalized gene expression (2^(-delta CT)) in the WT samples. Fold-change values greater than 1 indicate a positive- or an upregulation and the fold regulation is equal to the fold change. Fold-change values less than 1 indicate a negative or downregulation and the fold regulation is the negative inverse of the fold change. The p-values are calculated based on a Student’s t-test of the replicate 2^(-delta CT) values for each gene in the control group and treatment groups.
Table 2: Glucose metabolism genes significantly dysregulated in the same direction in triceps and quadriceps of P7 Smn<sup>−/−;SMN2<sup>−/−</sup>, Fn14<sup>−/−</sup> and Tweak<sup>−/−</sup> mice when compared to P7 WT mice

| Gene | Triceps | | | Quadriceps | | |
|------|---------|---------|---------|---------|---------|---------|
|      | Smn<sup>−/−;SMN2<sup>−/−</sup> | Fn14<sup>−/−</sup> | Tweak<sup>−/−</sup> | Smn<sup>−/−;SMN2<sup>−/−</sup> | Fn14<sup>−/−</sup> | Tweak<sup>−/−</sup> |
|      | Fold change | p-value | Fold change | p-value | Fold change | p-value | Fold change | p-value | Fold change | p-value | Fold change | p-value |
| 1,4-Alpha-glucan branching enzyme 1 (Gbe1) | Down | 1.2158 | 0.001826 | −1.1649 | 0.00362 | ns | ↑ | −1.3664 | 0.00073 | −1.648 | 0.002807 |
| Dihydrolipoamide S-succinyltransferase (Dlst) | Down | −1.4834 | 0.000409 | −1.1593 | 0.000992 | −1.5512 | 0.009639 | −1.7555 | 0.000001 | −1.2472 | 0.000422 | −1.4384 | 0.000389 |
| Enolase 1 (Eno1) | Down | −2.8937 | 0 | −1.2057 | 0.001592 | −1.2553 | 0.012037 | −2.8182 | 0 | −1.3798 | 0.000748 | −1.6093 | 0.000595 |
| Filamin B (Fh1) | Down | −1.2988 | 0.002491 | −1.11 | 0.011675 | −1.2603 | 0.044285 | −1.4732 | 0.000033 | −1.1362 | 0.010963 | ns |
| Fructose-bisphosphatase 2 (Fbp2) | Up | 1.3862 | 0.002522 | 1.6462 | 0.003035 | 1.4036 | 0.004574 | 1.5193 | 0.000348 | 1.4564 | 0.00028 |
| Glycogen phosphorylase muscle-associated (Pygm) | Down | −1.2185 | 0.002346 | ns | | | | −1.2577 | 0.000493 | −1.1492 | 0.034146 | −1.1388 | 0.045356 |
| Isocitrate dehydrogenase 3 (NAD(+)alpha) (Idh3a) | Down | −1.3412 | 0.014566 | −1.0994 | 0.022547 | −1.2728 | 0.000786 | −1.6021 | 0.000063 | −1.3412 | 0.002972 | −1.288 | 0.023111 |
| Isocitrate dehydrogenase 3 (NAD(+)beta) (Idh3b) | Down | −1.2006 | 0.00032 | −1.1227 | 0.017864 | ns | | −1.3376 | 0.00003 | −1.1815 | 0.001146 | −1.1462 | 0.018887 |
| Oxoglutarate dehydrogenase (Ogdh) | Down | −1.184 | 0.032659 | −1.0914 | 0.009317 | −1.3354 | 0.001844 | −1.295 | 0.00066 | −1.2062 | 0.010753 | −1.4183 | 0.011564 |
| Phosphofructokinase, liver type (Pfk1l) | Down | −1.3403 | 0.002443 | ns | | | | −1.8003 | 0.003361 | −1.2159 | 0.003577 | −1.699 | 0.00477 |
| Pyruvate dehydrogenase E1 alpha 1 subunit (Pdh1a) | Down | ns | −1.1094 | 0.005985 | −1.1421 | 0.040785 | −1.915 | 0.020901 | −1.2056 | 0.006742 | −1.2258 | 0.009299 |
| Pyruvate dehydrogenase kinase 4 (Pdk4) | Up | 7.4434 | 0.005267 | 1.6982 | 0.017988 | 1.7597 | 0.03066 | 5.3286 | 0.038343 | 1.7406 | 0.003824 | 1.556 | 0.006172 |
| Pyruvate dehydrogenase kinase 1 (Pdk1) | Down | −1.7093 | 0.000041 | −1.1129 | 0.006326 | −1.1699 | 0.049145 | −1.7605 | 0 | −1.3544 | 0.00663 | ns |
| Triosephosphate isomerase 1 (Tpi1) | Down | −1.8595 | 0.000342 | ns | | | | −1.8225 | 0.00006 | −1.2313 | 0.002462 | −1.2697 | 0.011066 |

Ns not significant, ↑ significantly upregulated. Fold change (2ΔΔCT) is the normalized gene expression (2ΔΔCT) in the test sample divided the normalized gene expression (2ΔΔCT) in the WT samples. Fold-change values greater than 1 indicate a positive or an upregulation and the fold regulation is equal to the fold change. Fold-change values less than 1 indicate an negative or downregulation and the fold regulation is the negative inverse of the fold change. The p-values are calculated based on a Student’s t-test of the replicate 2ΔΔCT values for each gene in the control group and treatment groups.
Administration of the Fc-TWEAK agonist improves a subset of disease phenotypes in two SMA mouse models

Finally, we evaluated the impact of increasing Tweak activity on disease progression and muscle pathology in SMA mice.

Of note, while the \( Smn^{+/-};SMN2 \) and \( Smn^{2B/-} \) mice are healthy littermates in terms of life span and reproductive abilities, they nevertheless have reduced levels of Smn, which in itself has been demonstrated to impact certain phenotypic features (e.g., tail and ear necrosis, metabolism, gene expression). As such, and similar to previous studies [41], comparisons were performed between untreated and treated animals of the same genotype, allowing us to determine if the effects were SMA-dependent and/or -independent, without the addition of a potential compounding factor.

Firstly, \( Smn^{-/-};SMN2 \) mice and healthy littermates received a daily subcutaneous injection of Fc-TWEAK (15.8 \( \mu \)g), a fusion protein with the murine IgG2a Fc region [26], starting at birth. We found that Fc-TWEAK did not significantly impact weight or survival of \( Smn^{-/-};SMN2 \) mice compared to untreated and IgG-treated controls (Fig. 7a–b). Additional lower (7.9 \( \mu \)g) and higher doses (23 and 31.6 \( \mu \)g) were also administered but proved to negatively impact weight and survival (Supplementary Fig. 3).

Triceps from P7-untreated and Fc-TWEAK-treated (15.8 \( \mu \)g) \( Smn^{-/-};SMN2 \) SMA mice and \( Smn^{+/-};SMN2 \) healthy littermates were further processed for molecular analyses of the Tweak/Fn14 pathway. We observed that Fc-TWEAK administration did not influence the expression of \( Tweak \) (Fig. 7c) or \( Fn14 \) (Fig. 7d) in neither \( Smn^{+/-};SMN2 \) nor \( Smn^{-/-};SMN2 \) mice compared to untreated animals. Similarly, Fc-TWEAK did not induce changes in \( Pgc-1\alpha \) expression (Fig. 7e). We did observe a significant downregulation of \( Mef2d \) in Fc-TWEAK-treated muscles of \( Smn^{-/-};SMN2 \) SMA mice compared to untreated animals (Fig. 7f). \( Glut-4 \) mRNA expression remained unchanged in both \( Smn^{+/-};SMN2 \) and \( Smn^{-/-};SMN2 \) Fc-TWEAK-treated mice (Fig. 7g). \( HKII \) was significantly upregulated in muscle of Fc-TWEAK-treated \( Smn^{+/-};SMN2 \) healthy littermates, while it was significantly downregulated in Fc-TWEAK-treated \( Smn^{-/-};SMN2 \) SMA mice compared to untreated groups (Fig. 7h). \( Klf15 \) was significantly downregulated in Fc-TWEAK-treated \( Smn^{-/-};SMN2 \) SMA mice only compared to untreated SMA animals (Fig. 7i). The absence of overt changes in the expression of Tweak, Fn14 and the previously reported proposed downstream metabolic effectors may be due to the 24-h time-lapse between the last Fc-TWEAK injection and harvest of tissues, which could have led to missing key time points at which transcriptional profiles were significantly impacted.

### Table 3

| Pathway ID | Pathway description | Count in gene set | False discovery rate (FDR) |
|------------|---------------------|-------------------|---------------------------|
| 01200      | Carbon metabolism  | 13                | 7.62e-22                  |
| 01120      | Microbial metabolism in diverse environments | 13 | 1.87e-19 |
| 00010      | Glycolysis/Gluconeogenesis | 8 | 2.09e-13 |
| 00020      | Citrate cycle (TCA cycle) | 7 | 2.09e-13 |
| 01100      | Metabolic pathways | 16 | 7.65e-13 |
| 01230      | Biosynthesis of amino acids | 7 | 8.75e-11 |
| 00051      | Fructose and mannose metabolism | 5 | 1.7e-08 |
| 04910      | Insulin signaling pathway | 6 | 3.09e-07 |
| 00500      | Starch and sucrose metabolism | 4 | 8.58e-06 |
| 04152      | AMPK signaling pathway | 5 | 8.58e-06 |
| 01210      | 2-Oxocarboxylic acid metabolism | 3 | 2.79e-05 |
| 00030      | Pentose phosphate pathway | 3 | 0.000126 |
| 04066      | HIF-1 signaling pathway | 4 | 0.000141 |
| 00052      | Galactose metabolism | 3 | 0.000145 |
| 04920      | Adipocytokine signaling pathway | 3 | 0.000138 |
| 00620      | Pyruvate metabolism | 2 | 0.0177 |
| 04973      | Carbohydrate digestion and absorption | 2 | 0.0177 |
| 04930      | Type II diabetes mellitus | 2 | 0.0227 |
| 00310      | Lysine degradation | 2 | 0.0233 |
While we did not capture the short-term molecular effects of Fc-TWEAK administration, quantification of myofiber area in TA muscles showed that daily Fc-TWEAK treatment significantly increased myofiber area in skeletal muscle of P7 Smn−/−;SMN2 mice compared to untreated SMA animals (Fig. 7l–k). Furthermore, the expression of atrophy markers parvalbumin, MuRF-1 and atrogin-1 [57] was also restored towards normal levels, whereby parvalbumin expression was significantly increased (Fig. 7i), while MuRF-1 and atrogin-1 expression was significantly downregulated (Fig. 7m–n) in triceps of Fc-TWEAK-treated Smn−/−;SMN2 SMA mice compared to untreated SMA animals, further supporting an improvement in muscle health. We did not however detect changes in MRFs Myod1 and myogenin [53] (Fig. 7o–p).

We next assessed the effect of Fc-TWEAK in Smn2B−/− mice, which are typically more responsive to Smn-dependent treatment strategies [41, 58–60]. Due to the longer treatment period in these mice (20 days) and the observed toxicity in daily injected mice (> 10 days), the Smn2B−/− and Smn2B+/− mice received subcutaneous injections of Fc-TWEAK and IgG control (15.8 μg) every 4 days, starting at birth. Both IgG and Fc-TWEAK did not significantly impact the weight of Smn2B−/− mice compared to untreated SMA animals (Fig. 7q).

However, Fc-TWEAK significantly increased the lifespan of Smn2B−/− mice compared to both IgG-treated and untreated animals (Fig. 7r). Molecular analyses of the mRNA levels of Tweak, Fn14 and the previously reported proposed molecular effectors in triceps from P15 animals only showed a significant effect of Fc-TWEAK on the expression of Glut-4, whereby it was downregulated in Fc-TWEAK-treated Smn2B−/− mice compared to untreated animals (Fig. 7s). Similar to the above, the limited impact of Fc-TWEAK on the expression of Tweak, Fn14 and the previously reported metabolic effectors in P15 animals may be due to the 72-h time-lapse between the last injection of Fc-TWEAK and tissue harvest. This experimental paradigm was chosen to follow the optimal dosing regimen and perform molecular analyses at a symptomatic time-point that was not too close to the end stage of the disease. Nevertheless, to determine if molecular changes could be captured following a shorter time-lapse between the Fc-TWEAK injection and tissue harvest and to determine if the response to Fc-TWEAK is different in WT tissues that express 100% Smn, we treated WT and Smn2B−/− with Fc-TWEAK (15.8 μg) every 4 days from birth until P16 and harvested skeletal muscle 3-h post-injection. While Fn14 mRNA expression remained unchanged in the triceps from both WT and SMA mice (Supplementary Fig. 4a), we found differential expression...
Fig. 7 (See legend on previous page.)
patterns of the other metabolic effectors proposed to be influenced by Tweak and Fn14. Indeed, following Fc-TWEAK injections, Tweak mRNA is significantly increased in WT animals and unchanged in Smn2B/− mice (Supplementary Fig. 4b). PGC-1α and Mef2d are unchanged in WT animals and significantly decreased in Smn2B/− mice (Supplementary Fig. 4c–d), while Glut-4, HKII and Klf15 are significantly increased in WT animals and significantly decreased in Smn2B/− animals (Supplementary Fig. 4e–g). Similarly, we observed a specific decrease of the NF-κB2 p100 component (all other components were unchanged) in Fc-TWEAK-treated WT animals compared to untreated controls, while it is significantly upregulated in Fc-TWEAK-treated Smn2B/− mice compared to untreated animals (Supplementary Fig. 4h).

As improvements in muscle health parameters were observed in Fc-TWEAK-treated Smn2B/−;SMN2 SMA mice, we performed similar investigations in Smn2B/− mice. Contrary to the more severe mouse model, we did not find any significant changes in expression levels of parvalbumin, MuRF-1, atrogin-1 and myod1 in either Smn2B/+/− or Smn2B/− Fc-TWEAK-treated animals (Fig. 7t–w). We did observe a significant increase in myogenin mRNA expression that was limited to Fc-TWEAK-treated healthy littermates (Fig. 7x). These results suggest that the impact of Fc-TWEAK on molecular markers associated with muscle health may be dependent on age, disease severity and/or genetic strain. Despite the lack of impact of Fc-TWEAK on muscle atrophy and health markers, quantification of myofiber area in TA muscles shows a significant increase in muscle size in Fc-TWEAK-treated Smn2B/− mice compared to untreated SMA animals (Fig. 7y–z).

While MuRF-1 and atrogin-1 are well described atrophy markers [57], whose expression has previously been well characterized in skeletal muscle of Smn−/−;SMN2 and Smn2B/− mice at various time points during disease progression [61], there is also evidence that they can be induced by the Tweak/Fn14 signaling cascade [24]. We therefore investigated their levels in quadriceps and triceps of F10 Fn14−/− mice (Supplementary Fig. 5) and find that while atrogin-1 levels are unchanged compared to WT animals (Supplementary Fig. 5a), MuRF-1 levels are significantly downregulated in both muscles of Fn14−/− mice, consistent with the previously reported positive correlation between Tweak/Fn14 activity and MuRF-1 expression (Supplementary Fig. 5b) [24]. These results suggest that the reduced levels of MuRF-1 observed in skeletal muscle of Fc-TWEAK-treated SMA mice are most likely linked to improved muscle health. Furthermore, the differential effect of Fc-TWEAK on the expression of MuRF-1 and atrogin-1 in Smn−/−;SMN2 and Smn2B/− mice is most probably due to the previously reported distinct regulatory processes that contribute to muscle atrophy in both models [61].

Taken together, our results suggest that promoting Tweak activity in SMA mice has the potential to improve weight, survival and muscle pathology, suggesting that restoring the Tweak and Fn14 signaling in SMA muscle may lead to sustainable therapeutic benefits.

**Discussion**

Motor neuron death and muscle pathology bidirectionally impact on each other in SMA. Indeed, while loss of motor neurons significantly contributes to muscle atrophy, there is also evidence for muscle-intrinsic abnormalities in SMA skeletal muscle, which could be directly or indirectly caused by SMN deficiency [5, 6, 62–64]. In this study, we attempted to address the underlying mechanisms of muscle-intrinsic abnormalities leading to muscle pathology in SMA by investigating the role of TWEAK and Fn14 in muscle atrophy in SMA. To the best of our knowledge, this is the first study to evaluate the TWEAK and Fn14 pathway in SMA and in early stages of muscle development.

Notably, we showed decreased expression of Tweak and Fn14 in skeletal muscle of two distinct SMA mouse models during disease progression, which is contrary to previous reports of increased TWEAK/Fn14 activity in experimental models of atrophy in adult muscle [52, 65, 66], suggesting that TWEAK and Fn14 may have distinct roles in skeletal muscle during development and adulthood. Indeed, Tweak mRNA expression is significantly lower in skeletal muscle of 30-day-old WT mice compared to 90-day-old animals, suggesting an age-dependent regulation [16]. Moreover, we observed that the dysregulation of TWEAK, Fn14 and the previously proposed metabolic effectors in skeletal muscle of preweaned mice appears to be influenced by intrinsic muscle impairments and not denervation, which is in contrast to what has been previously reported in experimental models of adult muscle denervation [15, 16], further suggesting distinct developmental roles for Tweak and Fn14 in skeletal muscle. Given that muscles from younger mice are more resistant to surgically-induced denervation than those from older mice [67], TWEAK and Fn14 may contribute to this age-dependent differential vulnerability of muscle to pathological insults. Thus, the role of TWEAK/Fn14 signaling in muscle pathology may be more nuanced and be influenced by a combination of factors such as absolute levels, downstream signaling cascades activated (e.g. canonical vs noncanonical NF-κB signaling pathways), developmental stage of the muscle, state of muscle atrophy (e.g. chronic vs acute) and primary origin of muscle pathology (e.g. denervation vs intrinsic insult) [11, 12].
Another key observation from our study is a potential interaction and/or overlap between Tweak, Fn14 and Smn and their downstream signaling cascades in muscle. It has previously been demonstrated that once Tweak binds to Fn14, the complex will activate several NF-kB molecular effectors, including TRAF6 and IKK [68]. Interestingly, SMN has been reported to prevent the activation of TRAF6 and IKK, thereby negatively regulating the muscle atrophy-inducing canonical NF-kB pathway [69]. These studies thus suggest converging roles for TWEAK, Fn14 and Smn in muscle, which is further supported by our findings. Indeed, we found that independent Tweak, Fn14 and Smn depletion had an impact on each other’s expression in differentiated C2C12 cells and murine muscle. Furthermore, there was an overlap of dysregulated myogenesis, myopathy and glucose metabolism genes in SMA, Fn14−/− and Tweak−/− mice. Of note, the aberrantly regulated genes in young Tweak−/− and Fn14−/− mice did not perfectly overlap, supporting previous reports of Tweak-independent roles of Fn14 during myogenesis [70]. Thus, these results suggest that aberrant expression of TWEAK and Fn14 in SMA muscle may be a consequence of combined events resulting from muscle atrophy and reduced SMN expression. However, Smn depletion most likely needs to reach pathological levels as we did not observe obvious changes in the Tweak/Fn14 signaling pathway in skeletal muscle of non-SMA hypomorphic SmnΔ7/Δ7 and SmnΔ+/− mice. Performing genome-wide RNA sequencing studies could also help elucidate the extent of shared genes and pathways regulated by TWEAK, Fn14 and SMN. Indeed, while we have focused on a subset of previously reported and proposed metabolic effectors and the NF-kB pathways, other canonical pathways such as MAPK signaling, known to have functional interactions with Tweak, Fn14 and Smn, may also display converging roles in muscle health [71, 72].

In addition, our results in developing mice do support the previously reported negative regulation of the metabolic factors Pgc-1α, Mef2d, Glut-4, Klf15 and HKII in adult muscle [18]. Further analyses of a subset of specific glucose metabolism genes showed that about 20% of these genes were dysregulated in the same direction in Fn14−/−, Tweak−/− and SMA mice. Our KEGG analysis of these shared dysregulated metabolic genes further support the potential relationships and roles of TWEAK, Fn14 and SMN involved in the regulation of glucose metabolism. Indeed, the AMPK signaling pathway, found to be aberrantly regulated in Fn14−/−, Tweak−/− and SMA, is as a master regulator of skeletal muscle function and metabolism [73]. Interestingly, a previous study in SMNΔ7 SMA mice further showed that chronic treatment with the AMPK agonist AICAR prevented skeletal muscle pathology [74]. In addition, AMPK directly phosphorylates PGC-1α [75], which is also dysregulated in Smn−/−, Tweak- and Fn14-depleted models [66, 76]. We also found that glycolysis and pyruvate metabolic pathways, which culminate in the generation of ATP, are also dysregulated in SMA, Fn14−/− and Tweak−/− mice. Interestingly, siRNA-mediated Smn knockdown in NSC-34 cells showed a significant decrease in ATP production [77]. ATP was also decreased in Smn−/−;Smn2N2 mice and in Smn morphant zebrafish [78]. These results could explain mitochondrial dysfunction in SMA patients [5]. Thus, our study strengthens the notion of metabolic dysfunctions contributing to SMA muscle pathology and suggests a potential mechanistic link with the TWEAK/Fn14 pathway.

However, it is important to note that although our findings support the idea that the aberrant expression of Pgc-1α, Mef2d, Glut-4, Klf15 and HKII is due to the dysregulated expression of Tweak and Fn14 in SMA muscle, further mechanistic insights are required to fully understand the extent of the transcriptional regulation of these key metabolic effectors by TWEAK/Fn14 signaling in developing post-natal muscle. Indeed, their differential dysregulations in Smn−/−;Smn2N2, Smn2B/2B−/−, Tweak−/− and Fn14−/− muscle as well as the varying impact that Fc-Tweak injections had on their expression levels suggest that additional regulatory mechanisms may be contributing to our observations.

Our findings also confirm that not all skeletal muscles are equally affected in SMA. Indeed, we observed that the SMA skeletal muscle atrophy marker parvalbumin was significantly decreased from an earlier time point in the vulnerable triceps and gastrocnemius muscles than in the more resistant TA and quadriceps muscles. Notably, we also found that 20% more myogenesis- and myopathy-related genes were dysregulated in the more vulnerable triceps muscles of Smn−/−;Smn2N2 mice compared to the resistant quadriceps muscles. Conversely, the number of glucose metabolism genes dysregulated in SMA triceps and quadriceps muscles was not significantly different. Previous studies have reported that muscle vulnerability is more closely associated with NMJ denervation than with location or fiber-type composition [35]. Our results further suggest that denervation events in vulnerable SMA muscles have a more prominent effect on myogenesis and myopathy than on glucose metabolism.

Finally, modulating Tweak activity via Fc-TWEAK in two SMA mouse models led to interesting observations. Firstly, Fc-TWEAK administration specifically increased life span in the milder Smn−/− mouse model, while it did not impact disease progression in the severe Smn−/−;Smn2N2 mice. This is consistent with previous studies, including ours, demonstrating that the
**Supplementary Information**

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**Additional file 1: Supplementary Figure 1.** No overt dysregulation of Tweak and Fn14 in skeletal muscle of non-SMA hypomorphic Smn-depleted mice. qPCR analysis of Tweak (a), Fn14 (b), Pgc-1α (c), Mef2d (d), Glut-4 (e), HkII (f) and Klf15 (g) in triceps and quadriceps from post-natal day (P) 7 wild-type (WT), Smn2B/− and Smn−/− mice. Normalized relative expressions for Fc-TWEAK-treated SMA mice are compared to untreated SMA mice.  

**Supplementary Figure 3.** Effect of varying Fc-TWEAK doses on disease progression in Smn−/−;SMN2 SMA mice. Fc-TWEAK (7.9, 15.8, 23.7 and 31.6 μg) was systemically administered daily to Smn−/−;SMN2 SMA mice. Data are mean ± SEM, n = 5-10 animals per experimental group, two-way ANOVA, Sidak’s multiple comparison test. a. Survival curves of untreated Smn−/−;SMN2 SMA mice and Smn−/−;SMN2 SMA mice that received daily subcutaneous injections (starting at P0) of Fc-TWEAK (7.9, 15.8, 23.7 and 31.6 μg). Data are mean ± SEM, n = 5-10 animals per experimental group, two-way ANOVA, Sidak’s multiple comparison test. b. Survival curves of untreated Smn−/−;SMN2 SMA mice and Smn−/−;SMN2 SMA mice that received daily subcutaneous injections (starting at P0) of Fc-TWEAK (7.9, 15.8, 23.7 and 31.6 μg). Data are mean ± SEM, n = 5-10 animals per experimental group, two-way ANOVA, Tukey’s multiple comparison test. c. Log-rank (Mantel-Cox).

**Supplementary Figure 5.** Decreased expression of the Tweak/Fn14 pathway in SMA muscle and defining its role in general in maintaining muscle homeostasis throughout the life course.

**Abbreviations**

ALS: Amyotrophic lateral sclerosis; ANOVA: Analysis of variance; cDNA: Complementary deoxyribonucleic acid; DEGs: Differently expressed genes; DMEM: Dulbecco’s Modified Eagle’s Media; PBS: Fetal bovine serum; FDR: False discovery rate; GO: Gene ontology; H&E: Hematoxylin and eosin; KEGG: Kyoto Encyclopedia of Genes and Genomes; miRNA: Messenger RNA; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NML: Neuromuscular junction; P: Post-natal day; p: Probability value; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; PFA: Paraformaldehyde; qPCR: Quantitative polymerase chain reaction; RIPA: Radioimmunoprecipitation; RNA: Ribonucleic acid; RNAi: RNA interference; RT-qPCR: Reverse transcriptase-quantitative PCR; SEM: Standard error of the mean; siRNA: Small interfering RNA; SMA: Spinal muscular atrophy; STRING: Search Tool for the Retrieval of Interacting Genes/Proteins; TA: Tibialis anterior; WT: Wild-type.
MuRF-1 expression in skeletal muscle of P7 Fn14^+/+ mice. qPCR analysis of Atrogin-1 (a) and MuRF-1 (b) in quadriceps and triceps from post-natal day (P) 7 wild type (WT) and P7 Fn14^−/− mice. Normalized relative expressions are compared to WT. Data are mean ± SEM, n = 4 animals per experimental group, unpaired t test, p = 0.0164 (MuRF-1 quadriceps), p = 0.0283 (MuRF-1 triceps), n.s. = not significant.

**Additional file 2: Supplementary Table 1.** Mouse primers used for quantitative real-time PCR.

**Additional file 3: Supplementary file 1.** Myopathy and myogenesis gene expression changes in quadriceps and triceps of post-natal day 7 Smn−/−;SMN2 (Tweak KO) and Fn14^−/− (Fn14 KO) compared to age- and genetic strain-matched wild type animals.

**Additional file 4: Supplementary file 2.** Glucose metabolism gene expression changes in quadriceps and triceps of post-natal day 7 Smn−/−;SMN2 (Tweak KO) and Fn14^−/− (Fn14 KO) compared to age- and genetic strain-matched wild type animals.

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Authors’ contributions

Conceptualization, MB; methodology, KEM, ERS, and MB; validation, KEM and M; formal analysis, KEM, RS, EM, EM, SK, and MB; investigations, KEM, ERS, EM, MDA, B, SK, IT, IB, GH, NA, and MB; writing—original draft preparation, KEM and MB; writing—review and editing, KEM, ERS, EM, DM, DA, BE, SK, IT, IB, GH, NA, PC, KE, RK, NJAW, and MB; visualization, KEM, ERS, EM, and MB; supervision, PC, KE, RK, NJAW, and MB; project administration, MB; funding acquisition, RK, NJAW, and MB. The authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or in the supplementary information.

Declarations

Ethics approval and consent to participate

Experimental procedures with live animals were authorized and approved by the University of Oxford ethics committee and UK Home Office (current project license P764EDGFO, previous project license 30/2907) in accordance with the Animals (Scientific Procedures) Act 1986, the Keele University Animal Welfare Ethical Review Body and UK Home Office (Project License P99AB3895) in accordance with the Animals (Scientific Procedures) Act 1986, the University of Ottawa Animal Care Committee according to procedures authorized by the Canadian Council on Animal Care and the German Animal Welfare law and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, reference numbers 15/1774 and 19/3309).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

1Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK. 2Gene Therapy Center, UMass Medical School, Worcester, USA. 3School of Medicine, Keele University, Staffordshire, UK. 4Regenerative Medicine Program and Department of Cellular and Molecular Medicine, Ottawa Hospital Research Institute and University of Ottawa, Ottawa, Canada. 5Department of Pharmacology, University of Oxford, Oxford, UK. 6Center for Systems Neuroscience and Institute of Neuroanatomy and Cell Biology, Hannover Medical School, Hannover, Germany. 7SMATHERIA — Non-Profit Bio-medical Research Institute, Hannover, Germany. 8Department of Paediatrics, University of Oxford, Oxford, UK. 9Wolfson Centre for Inherited Neuromuscular Disease, RJAH Orthopaedic Hospital, Oswestry, UK.

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