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## **Proteome-wide adaptations of mouse skeletal muscles during a full month in space**

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## **Abstract**

The safety of space flight is challenged by a severe loss of skeletal muscle mass, strength and endurance that may compromise the health and performance of astronauts. The molecular mechanisms underpinning muscle atrophy and decreased performance have been studied mostly after short duration flights, and are still not fully elucidated. By deciphering the muscle proteome changes elicited in mice after a full month aboard the BION-M1 biosatellite, we observed that the antigravity *soleus* incurred the greatest changes compared to locomotor muscles. Proteomics data notably suggested mitochondrial dysfunction, metabolic and fiber type switching toward glycolytic type II fibers, structural alterations, and calcium signaling-related defects to be the main causes for decreased muscle performance in flown mice. Alterations of the protein balance, mTOR pathway, myogenesis and apoptosis were expected to contribute to muscle atrophy. Moreover, several signs reflecting alteration of telomere maintenance, oxidative stress and insulin resistance were found as possible additional deleterious effects. Finally, eight days of recovery post flight were not sufficient to restore completely flight-induced changes. Thus, in-depth proteomics analysis unraveled the complex and multifactorial remodeling of skeletal muscle structure and function during long-term space flight, which should help defining combined sets of countermeasures before, during and after the flight.

**Keywords**

Weightlessness; Mouse; Skeletal muscle; Atrophy; Proteomics; Metabolism; Telomeres

## Introduction

The culmination of research in human space physiology over the past 40 years has demonstrated that spaceflight induces muscle atrophy, bone demineralization, cardiovascular and metabolic dysfunction, on which under-nutrition is superimposed.<sup>1</sup> None of the numerous countermeasures tested so far have proven to be fully effective,<sup>2-4</sup> but given the relatively short duration of the missions, most astronauts returning to Earth do not encounter difficulties in recovering. Human spaceflight programs have moved to address the new challenges in the next phase of space exploration towards asteroids, Moon or Mars. We have much less information regarding the physiological changes associated with long-duration missions extending from 30 days to months in orbit<sup>5</sup> and, amongst the priorities, is the development of more effective and efficient countermeasures to limit or prevent the impact of the loss of muscle mass and strength as astronauts will be expected to work and live on new planetary surfaces.<sup>2-6</sup>

Although spaceflight-induced disuse atrophy is particularly pronounced in human antigravity muscles like the *soleus* muscle, a large reduction in size and strength is also observed in locomotor skeletal muscles.<sup>2, 4, 6, 7</sup> Microgravity exposure ranging from 8 days up to 197 days in a few cases has indeed reported losses of muscle mass (6-20%) and strength (0-48%), independently of the muscle group studied.<sup>8</sup> In addition, a phenotype switch from slow oxidative (type I) to fast glycolytic (type II) muscle fibers is observed in parallel with the loss of mass and strength.<sup>2, 4, 7, 9-12</sup> This further affects performance as glycolytic fibers are more susceptible to fatigue, less sensitive to insulin and the transition from slow-oxidative towards fast-glycolytic muscle fibers is associated with metabolic syndrome and inflexibility.<sup>13</sup>

Muscle atrophy during spaceflight and simulated microgravity, in humans and rodents, is due to a drop in protein synthesis resulting in negative protein balance.<sup>2, 14-16</sup> When an energy deficit is superimposed, or when stress is present, catabolism may further worsen the negative protein balance.<sup>12, 17, 18</sup> This is associated with induction of the ubiquitin-dependent proteasome system and cathepsin L,<sup>11, 17, 19</sup> decreased expression of genes related to muscle growth (PI3-kinase/Akt/mTOR cascade), increased expression of myostatin, and alteration of the levels of a number of other mRNAs.<sup>11, 17, 20-22</sup> The changes in muscle properties during human spaceflight are complicated by the fact that every astronaut has had a rigorous exercise program in space. Therefore, further research is needed to explore the rate and magnitude of changes in muscle during long duration spaceflight without exercise, to better understand the effects of microgravity *per se* and to develop more effective and efficient countermeasures.

Recent advances in proteomic technology allow biologists to investigate global cellular responses and address mechanistic questions.<sup>23, 24</sup> Despite an increased interest in muscle physiology, there is relatively little proteomics data examining the physiological adaptations to space.<sup>12, 25</sup> Using cutting-

edge quantitative proteomics and classical cellular biology, the aim of the present study was to get an in depth view of the molecular pathways affected by space flight in mouse skeletal muscle during the 30-day flight of the BION-M1 biosatellite experiment and following an 8-day recovery period.<sup>26</sup> This is a unique dataset as it is one of the longest flights performed in rodents so far, apart from a 3-month experiment on the ISS, during which only one mouse survived.<sup>11, 27</sup> As muscles differing in their fiber type composition appear to respond differently to microgravity, we investigated the response to space flight of the *soleus*, *extensor digitorum longus* and *vastus lateralis* muscles.

## **Materials and methods**

### ***Ethical statement***

All experimental procedures of the Bion-M1 project were approved by the Biomedical Ethics Committee of the Russian Federation State Research Center-Institute for Biomedical Problems (IBMP), the Russian Academy of Sciences (protocol No. 319), and conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (8th ed., 2011).

### ***Experimental design***

A total of 5 experimental groups of mice from the Bion-M1 Space Mission<sup>26</sup> were used (Figure 1). The BION-M1 satellite (Roskosmos Program, Russia) was launched from Baikonur cosmodrome (Kazakhstan) on April 19, 2013. Male 19-20 week-old C57/BL6 mice were maintained during the 30-day high-orbit (~550 km) flight in special modules (confined housing and specific diet conditions), with three animals per module. Approximately 13-16 hours after landing, flown animals were weighed and randomly split into two groups. Hence, five mice were immediately sacrificed by cervical dislocation to constitute the Flight group (F) and four other mice were sacrificed after an 8-day recovery period (Recovery group, R). Three control groups were formed, with animals maintained on Earth during 30 days either under space confined housing and diet conditions (Ground Control group, GC, n=5) or in standard housing conditions (Vivarium Control group, VC, n=5), and animals housed in standard conditions during 38 days to form the Recovery Control group (RC; n=5).

Immediately after sacrifice, muscle tissue from three different muscles, namely *Vastus lateralis* (VL), *Extensor digitorum longus* (EDL) and *Soleus* (SOL), were dissected and stored at -80°C until analysis. We obtained the three muscle tissues from the left hindlimb of mice from all groups, except for the R and RC groups, for which only the EDL muscle was available.

### ***Fiber type composition analysis***

The fiber type composition of Bion-M1 mice muscles was determined based on proteomics data using unique peptides only to circumvent the high sequence homology of myosin heavy chains. MaxLFQ intensities (see below) of each myosin heavy chain isoform (MyH) were normalized to the protein length (i.e. molecular weight) and isoform abundance was expressed as the fraction of total MyH-intensity in each individual muscle. In addition, the size of the VL muscle was large enough to enable us to complement proteomics-based data by assessing its fiber type composition using qPCR. Total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit and concentration was determined by spectrophotometric analysis (Eppendorf AG, Hamburg, Germany). Integrity (RNA Integrity Number >7) was checked with the Agilent 2100 bioanalyzer (Agilent Technologies) using the RNA 6000 Nano kit.<sup>28</sup> The reverse transcription reaction was performed with 2µg of total RNA using the RevertAid First

Strand cDNA Synthesis kit (Thermo Scientific). qPCR analysis was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems) with 10 $\mu$ L of KAPA SYBR Fast Universal Readymix (CliniSciences), 300nM of both forward and reverse primers (Table 1), 2 $\mu$ L of diluted cDNA template and water in a final volume of 20 $\mu$ L. All PCR runs were performed in duplicate using the following cycle parameters: 20s at 95 $^{\circ}$ C, 40 cycles of 3s at 95 $^{\circ}$ C and 30s at 60 $^{\circ}$ C. Relative mRNA levels were normalized to ribosomal protein S9 (rpS9) and tubulin housekeeping gene levels, which were unaffected by the experiment. Results are expressed using the comparative cycle threshold (CT). The relative changes in the level of a specific gene were calculated with the  $\Delta\Delta$ CT formula.

### ***Skeletal muscle protein extraction***

Frozen tissue samples were weighted and grinded using a ball mill (3x 30 seconds @ 25Hz; MM400, Retsch) under liquid nitrogen and proteins were extracted using 7.5 volumes (i.e. 7.5 $\mu$ L/mg of tissue) of extraction buffer (8 M urea, 2 M thiourea, 2 % CHAPS, 2 % DTT, 30 mM Tris pH 8, protease inhibitors). After solubilisation for 30 minutes at 37 $^{\circ}$ C, samples were centrifuged for 15 minutes at 15000 x g, 4  $^{\circ}$ C to remove cell/tissue debris. Protein concentration in the supernatants was determined by a Bradford assay using BSA as standard (both from Bio-Rad).

### ***Differential proteome analysis***

50  $\mu$ g of each sample were mixed with 4x SDS sample buffer (1x: 50 mM Tris pH 6.8, 1 mM EDTA, 5 %  $\beta$ -mercaptoethanol, 2.5 % SDS and 10 % glycerol), incubated at 37  $^{\circ}$ C for 10 minutes and run for 15 minutes at 35 V into a 4 % polyacrylamide stacking gel in order to let proteins enter approximately 4 mm into the gel. Electrophoresis was stopped at this point and gels were stained by Colloidal Coomassie Blue. Both the "stacked" protein-band and the part of the gel above this band were excised with each of them being again divided into 4 pieces before in-gel digestion using an automatic pipetting device (MassPrep, Waters, Milford, MA). Following de-staining, reduction (DTT) and alkylation (iodoacetamide), proteins were digested with trypsin (300 ng/band) overnight at 37  $^{\circ}$ C. Peptides were consecutively extracted in 40 % and 60 % acetonitrile/0.1 % formic acid in water for one hour each and in 100 % acetonitrile (ACN) for 15 minutes at 450 rpm on an orbital shaker. Just before LC-MS/MS analysis, volume of peptide extracts was reduced in a speed-vac and sample volumes were adjusted to 25  $\mu$ L with 1% ACN, 0.1 % formic acid. Samples were analysed on a nanoUPLC-system (nanoAcquity, Waters) coupled to a quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive plus, Thermo Scientific, San Jose, CA). Two  $\mu$ L of each sample were concentrated/desalted on a trap column (C18, 180  $\mu$ m x 20 mm, 5  $\mu$ m; Waters) using 99 % of solvent A (0.1 % formic acid in water)/1 % solvent B (0.1 % formic acid in ACN) at a flow rate of 5  $\mu$ L/min for 3 minutes. Afterwards, peptides were transferred



and eluted from the separation column (BEH130 C18, 250 mm x 75  $\mu$ m, 1.7  $\mu$ m; Waters) maintained at 60 °C using a 85 minutes (VL) or 150 minutes (EDL and SOL) gradient from 3-40 % of B.

The Q-Exactive Plus was operated in positive ion mode with source temperature set to 250°C and spray voltage to 1.8 kV. Full scan MS spectra (300-1800 m/z) were acquired at a resolution of 140,000 at m/z 200, a maximum injection time of 50 ms and an AGC target value of  $3 \times 10^6$  charges with the lock-mass option being enabled (445.12002 m/z). Up to 10 most intense precursors per full scan were isolated using a 2 m/z window and fragmented using higher energy collisional dissociation (HCD, normalised collision energy of 27) and dynamic exclusion of already fragmented precursors was set to 60 s. MS/MS spectra were acquired with a resolution of 17,500 at m/z 200, a maximum injection time of 100 ms and an AGC target value of  $1 \times 10^5$ . The system was fully controlled by XCalibur software (v3.0.63; Thermo Fisher Scientific).

To assure good data quality by following instrument performance in “real-time” all along the experiment, we implemented several QC measures based on chromatographic separations as well as peptide/protein identification and quantification metrics. For each muscle, a sample pool comprising equal amounts of all protein extracts was made, and injected regularly during the whole experiment. A set of reference peptides (iRT kit; Biognosys AG, Schlieren, Switzerland) was added to each sample before LC-MS/MS analysis. For a given muscle, all samples were injected using a randomized and blocked injection sequence (one biological replicate of each group plus pool in each block). To minimize carry-over, a column wash (50% ACN during 20 min.) was included in between each block, in addition to a solvent blank injection, which was performed after each sample. Peak intensities and retention times of reference peptides, as well as protein identification rates, were monitored in a daily fashion. MS raw data were processed using MaxQuant (version 1.5.3.30). Peak lists were created using default parameters and searched using Andromeda search engine implemented in MaxQuant against a protein database created using the MSDA software suite.<sup>29</sup> The database contained only canonical mice protein sequences (Swissprot; Taxonomy ID: 10090; 16688 entries), which were downloaded in November 2014. Sequences of common contaminants like keratins and trypsin (247 entries) were finally added to the database (contaminants.fasta included in MaxQuant). The first search was performed using precursor mass tolerance of 20 ppm, and 4.5 ppm for the main search after recalibration. Fragment ion mass tolerance was set to 20 ppm. Carbamidomethylation of cysteine residues was considered as fixed and oxidation of methionine residues and acetylation of protein N-termini as variable modifications during the search. A maximum number of one missed cleavage and a false discovery rate (FDR) of 1% for both peptide spectrum matches (minimum length of seven amino acids) and proteins was accepted during identification. Regarding quantification, data normalisation and protein abundance estimation was performed using the MaxLFQ (label free quantification) option implemented in MaxQuant<sup>30</sup> using a “minimal ratio count” of one. “Match between runs” was enabled

using a one minute time window after retention time alignment. Both unmodified and modified (acetylation of protein N-termini and oxidation of methionine residues) peptides were considered for quantification while shared peptides were excluded. All other MaxQuant parameters were set as default. Only proteins with at least four of five valid values per group as well as the ones “absent” (i.e. 0 valid values) in samples from a given group were kept for further analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>31</sup> partner repository with the dataset identifier PXD005035.

### ***Western-blot analyses***

10 µg of each sample were separated on 8-16% Bio-Rad Mini-PROTEAN<sup>®</sup> TGX Stain-Free<sup>™</sup> Precast Gels (Bio-Rad, Hercules, CA, USA). Gels were imaged after activation using the Bio-Rad ChemiDoc<sup>™</sup> Touch Imaging System and transferred to nitrocellulose membranes (0.2 µm) using the Bio-Rad Trans-Blot<sup>®</sup> Turbo<sup>™</sup> Transfer System. Blots were immediately imaged to check for proper transfer, they were blocked for 1h at room temperature with a solution of TBS-T (Tris 25mM, NaCl 137mM, KCl 2.68 mM, 5% Tween 20) containing 4% of BSA, and they were then incubated overnight at 4°C with primary antibodies targeting telomeres regulation as it appeared a key pathway affected by microgravity from the global proteomic approach. We therefore selected antibodies against TCAB1 (ABIN873001, antibodies-online), shelterin complex subunits (TRF1: sc-5596, TRF2: sc-47693, POT1: sc-337789, and Rap1: sc-53434 from Santa Cruz Biotechnology Inc., CA, USA; TIN2: ab197894 from Abcam, Paris, France), and telomerase (TERT: ab183105, Abcam). These antibodies were used at 1/200 (POT1), 1/500 (TCAB1, TERT, TRF1, TRF2, Rap1) and 1/1000 (TIN2) dilution in the blocking solution. After 3 washings for 10 min each in TBS-T, blots were incubated for 1h at room temperature with peroxidase-conjugated secondary antibodies (sc-2004 and sc-2005; Santa Cruz Biotechnology Inc.) diluted (1/5000) in the blocking solution. After 3 washings for 10 min each in TBS-T, blots were incubated for 1 min in Luminata<sup>™</sup> Classico Western HRP substrate (Merk Millipore, Molsheim, France) and then imaged for chemiluminescence using the ChemiDoc Touch Imaging System (Bio-Rad). Image analysis was done using Bio-Rad Image Lab<sup>™</sup> software v5.1. Signals were normalized to total proteins as measured on the stain-free gel image, and intensity values were expressed relative to those in VC mice, which were assigned an arbitrary value of one.

### ***Statistical and functional annotation analysis***

All statistics were performed in R (v3.0.2), except for PCR data for which they were performed using the GraphPad Prism (6.0h version). Changes between the initial and final (i.e. after 30d) body mass for the F, GC and VC groups of mice was tested for significance ( $p < 0.05$ ) using paired student t-tests and changes between the initial, intermediary (i.e. after 30d) and final (i.e. after 38d) body mass using two-

way ANOVA ( $p < 0.05$ ) with Tukey as post-hoc test including adjustment of p-value according to Bonferroni-Holm ( $p < 0.05$ ).

Changes in the fiber type composition of the three muscles were tested using two-way ANOVA ( $p < 0.05$ ) with Tukey as post-hoc test including adjustment of p-value according to Bonferroni-Holm ( $p < 0.05$ ) for proteomics-based data. For PCR data, normality of distribution was checked with the Kolmogorov test. We used one way analysis of variance to compare group differences. If overall analysis of variance revealed significant differences, post hoc (pairwise) comparisons were performed using Fisher's LSD test. Differences were considered significant if  $p < 0.05$

Concerning western-blot analyses and the proteomics comparison of F, GC, and VC LFQ values, we performed two-way ANOVA with Tukey as post-hoc test including adjustment of p-value according to Bonferroni-Holm ( $p < 0.05$ ), after checking of normality distribution (Shapiro-Wilk test) and homoscedasticity (Bartlett test). In proteomics, only proteins with ANOVA and Tukey (F vs. GC and F vs. VC)  $p$ -values  $< 0.05$ , and with a Tukey  $p$ -value  $> 0.05$  (GC vs. VC) were considered significantly changed due to spaceflight. For recovery effects in *extensor digitorum longus*, only proteins with a Tukey  $p$ -value  $> 0.1$  (GC vs. VC) were first considered. Then GC and VC were considered as a single control group that was compared to RC using Welch Two Sample t-test, and only proteins with a  $p$ -value  $> 0.1$  were retained to minimize age-effects since animals from the RC group were one week older. ANOVA and Tukey (F vs. GC and F vs. VC;  $p$ -values  $< 0.1$ ) finally highlighted 54 proteins that were considered as changed by spaceflight and for which we checked whether recovery reverted their regulation.

Gene/protein-enrichment and functional annotation analysis of proteomics data was performed using the desktop version of DAVID (Ease v2.1) and an updated version of Gene Ontology (GO) databases (April 2016). Enriched GO terms were filtered by only considering those with an Ease score lower than 0.1, a Benjamini  $p$ -value lower than 0.05 and a fold enrichment higher than 2. Enriched GO terms were grouped together into broad functional categories, which were then considered as enriched broad functions.

## **Results and discussion**

### ***Body and skeletal muscle mass in Bion-M1 mice***

There were no differences in body mass at baseline and changes due to the 30-day spaceflight conditions or the simulated housing conditions on Earth, or following the additional 8-days for the recovery groups did not reach significance. The lack of effect of the microgravity environment was previously reported for spaceflight of shorter duration.<sup>32</sup>

A marked reduction in the activity of flown Bion-M1 mice after landing has been reported, with decreased distance moved, movement speed and rearing frequency, whereas grooming duration was much higher.<sup>26</sup> It was suggested that this could be related to an adverse impact of space flight on the sensorimotor systems of these animals. Although no significant change was found in muscle mass between groups, trends towards a reduction of the mass of the *soleus* and EDL after the 30 day-flight in comparison to the Vivarium and Ground control conditions were noticed, while the *vastus lateralis* (VL) appeared less affected (Figure 2A). Consistently, the myofiber size has recently been shown to be reduced in *soleus* and EDL of flown Bion-M1 mice.<sup>22</sup> The impact of space flight on skeletal muscles could then favour inactivity of mice. After 8 days of recovery post-flight, the EDL mass was partially restored.

### ***Proteomics data quality***

Retention times remained very stable across the three experiments with standard deviations of iRT peptides of 18, 20 and 21 seconds for EDL, VL and SOL, respectively, which corresponds to a median CV below 1% indicating decent and steady HPLC performance. Moreover, peak areas of standard peptides were very reproducible with CVs below 20% for all iRT-peptides in all samples. The data reported by MaxQuant confirmed the above-mentioned observations since peptide sequence identifications were equally consistent across all samples of the same muscle exhibiting CVs of 2, 6 and 7 % for VL, EDL and SOL, respectively. As expected, quantitative reproducibility after normalisation of technical biases (LFQ intensity) and taking into account all quantified proteins was higher in the pools than in the individual samples. Within pools, median intragroup CVs of 12, 16 and 12 % were observed for VL, EDL and SOL, while in the individual samples, the values were slightly higher (18, 20 and 16%), but still very good for a label-free proteomics experiment using a data-dependent acquisition approach.

### ***Skeletal muscle fiber type composition in Bion-M1 mice***

All myosin heavy chain isoforms (MyH) that were present in the protein database were identified and quantified in all three muscles by mass spectrometry-based analysis, except MyH6 and MyH10 which

were not detected in both the EDL and VL muscles, and MyH14 which was not seen in the VL muscle (Figure 2B-D). For all three muscles, proteomics-based fiber type composition was nicely consistent with literature data,<sup>33-36</sup> with the main detected MyH isoforms being MyH7 (type I), MyH1 (type IIX), MyH4 (type IIB) and MyH3 (embryonic) and MyH8 (neonatal). The fiber type composition of the *soleus* muscle (Figure 2B) shows that the switch from slow type I to fast type II fibers triggered by short duration spaceflight<sup>10</sup> is maintained during this long term flight, as evidenced by the changes in the proportion of MyH7 (-15%), MyH1 (+11%) and MyH4 (+16%). *Soleus* proteomics data are thus in line with previous immunohistochemistry and transcriptomic data.<sup>22</sup> Although their presence in adult skeletal muscle has been reported elsewhere,<sup>37, 38</sup> the functional significance of embryonic and neonatal MyH isoforms remains unclear.<sup>39</sup> As they exhibit properties of slow myosins,<sup>39</sup> the decreased proportion in the *soleus* muscle of flown mice (especially MyH8; -11%) is consistent with the fiber type switch triggered by spaceflight (see above). There were no significant differences in the proportion of type I and type II fibers in the EDL or VL (Figure 2C-D). The slight trend towards their increased levels in the EDL muscle of mice in the Recovery group would argue for the possibility that MyH3 and MyH8 contribute to myofibrillogenesis upon return to Earth. However, whether this is a mechanism contributing to muscle recovery would require further studies.

Using PCR analysis of the type II fiber subgroups in VL there was a trend toward increased type IIA (MyH2) in space flown mice (~3-5-fold), while the reverse was observed for MyH1 (~1.3-fold) mRNA levels (Figure 2E). Hence, mRNA level changes were not necessarily translated at the protein level (see Figure 2D). The fact that MyH2 mRNA was found at a very low concentration may explain the observed large intragroup variation. As the VL muscle in mice is predominately composed of type IIB fibers (Figure 2D) and considering the low abundance of MyH2 gene expression, these data suggest there was no change in VL fiber type during spaceflight.

### ***Overview of skeletal muscle proteome changes across Bion-M1 mice groups***

The investigation of changes induced in skeletal muscle tissue of flown mice relative to Vivarium and Ground control animals was performed using quantitative proteomics according to MS1 intensity based label-free quantification. A total of 2029, 1709, and 1413 proteins were analysed in the *soleus*, EDL and VL muscles, respectively (Figure 3A). Statistical analysis revealed 60% of differentially expressed proteins were common to all muscle groups (Figure 3B) but that the *soleus* muscle was the most affected with the abundance of 663 proteins being significantly changed across groups (see Table S-1). At the transcriptomic level, *soleus* has also been shown recently to be more strongly affected by microgravity than EDL.<sup>22</sup>

### ***Skeletal muscle proteome changes due to confinement on Earth***

There was only minimal impact of the confinement conditions on Earth as there were only 15-25 proteins differentially expressed in the three muscle groups between GC and VC groups (Figure 3C). In all three muscles there were changes in a small number of proteins involved in extracellular matrix remodelling, cytoskeleton, transport/trafficking, amino acid metabolism, differentiation/development, and purine metabolism (see Table S-1). In the EDL only, the effects of confinement at 1g were restricted to the upregulation of Pcyt1a, a protein involved in lipid metabolism, and the downregulation of pyruvate carboxylase, of proteins with an inhibitory action on proteases (Serpina1e and Pzp), and of Cbr2, which responds to oxidative stress. In the VL muscle, the effects of confinement at 1g resulted in the upregulation of proteins involved in protein synthesis (Eif3g), mitochondrial respiratory chain (Ndufs4) and lipid metabolism (Ptpmt1), and the downregulation of proteins involved in protein synthesis (Hspa8, Ppid), protein catabolism (Gsp1), and protease inhibition (Serpina1e). The *Soleus* muscle was little more affected than the two locomotor muscles. Indeed, some *soleus* proteins involved in protein translation were either up- (Rpl38, Eif3i) or downregulated (Eif1), catabolic proteins were either up- (Dcun1d2, Uchl1, Psm4) or downregulated (Capn3, Lactb). In addition, proteins in the mitochondrial respiratory chain were upregulated (ATP6v1b2, Cox6c), proteins of the carbohydrate metabolism were either up- (Taldo1) or downregulated (Pfkf1), and one protein involved in lipid metabolism was downregulated (slc27a1). Similar trends were found at the transcriptomic level in the Bion-M1 experiment<sup>22</sup>. Hence, confinement at 1g triggers isolated omics regulations in mouse skeletal muscle, which is in line with the previously reported evidence that, on Earth, cage confinement does not produce significant effects on the mouse musculoskeletal system.<sup>40</sup>

### ***Skeletal muscle proteome changes due to spaceflight in Bion-M1 mice***

In space-flown animals, the abundance of 318 proteins in the soleus, 34 in the EDL and 3 in the VL was similarly changed compared to both control conditions (Figure 3C). Additional proteins (102, 31, and 7 proteins in the *soleus*, EDL and VL muscles, respectively) were significantly regulated in F mice due to confinement according to the level of gravitational stress, i.e. only in comparison with GC animals (Figure 3C). Similarly, other proteins (103, 47, and 11 proteins in the *soleus*, EDL and VL muscles, respectively) were significantly regulated in F mice due to combined effects of confinement and microgravity, i.e. only in comparison with VC animals (Figure 3C). Similar trends were found recently at the transcriptomic level in the *soleus* and EDL muscles of Bion-M1 mice, but the authors focused only on differential genes between F and GC mice, without considering that 40% of them were unchanged between F and VC mice.<sup>22</sup> Here we considered all differential proteins in flown animals, i.e. either *versus* only one or both control groups.

**Functional annotation analysis.** Focusing first on muscle proteins that were found to exhibit differential expression in space flight compared to both control groups (GC and VC), we observed that 172 GO terms were significantly enriched in the *soleus* and 50 in EDL. When considering muscle proteins that were differential between F and either GC or VC animals, 39 GO terms were significantly enriched in the *soleus*, 28 in the EDL and 5 in the VL. Altogether, significantly enriched GO annotations were distributed into significantly enriched pathways and functions (Figure 4A-C and Table S-2), including tissue remodelling, carbohydrate and protein metabolism, and telomere function in both the *soleus* and EDL. Muscle contraction, development and adaptation, muscle structure and fiber type transition, and response to oxidative stress were only enriched in the *soleus* during microgravity, further underlying the drastic effect of weightlessness on postural muscles. Recently published transcriptomics data revealed similar space flight-induced changes in Bion-M1 muscles.<sup>22</sup> Specific effects on for example telomere function were nevertheless only highlighted by proteomics data from the current study. On the whole, these data are in line with what was previously known from assessment of muscle mass or contractile properties.<sup>4, 6, 41</sup>

**Telomere function** (Figure 5A-F, Figure 6A, Figure S-1 and Table S-4). Examining in more detail the significantly regulated proteins in response to space flight, we observed that many subunits of the TRiC/CCT chaperonin (Cct2, Cct3, Cct4, Cct5, Cct6a, Cct7, Cct8, Tcp1) were downregulated in the *soleus* and, to a lesser extent, in the EDL and VL muscles. TRiC/CCT plays a critical role in cellular protein folding, including folding of the telomerase cofactor TCAB1.<sup>42</sup> While TCAB1 levels were not changed significantly across Bion-M1 groups in all three muscles (Figure 6A and Figure S-1), telomerase trafficking to Cajal bodies in flown mice could be reduced due to downregulation of TRiC/CCT.<sup>43</sup> To further test the hypothesis of challenged telomere maintenance in flown mice, we used western-blot analysis to measure the levels of telomerase (TERT) and shelterin, a complex formed by TRF1, TRF2, POT1, TIN2, Rap1 and TPP1, which specifically associates with telomeric repeats and protects chromosome ends.<sup>44</sup> For TERT we detected bands only in the *soleus* and EDL. These bands could correspond to monomers (~130 kDa) and functional dimers (~260 kDa), the former appearing less abundant in the EDL of F *versus* GC mice and the latter in the *soleus* of F *versus* GC mice and the EDL of F *versus* VC animals. Intriguingly, TERT dimers appear more abundant than monomers in *soleus*, while the reverse was observed in EDL. Whether this could correspond to specific differences between oxidative and glycolytic muscles remains to be elucidated. The levels of *soleus* and EDL POT1 and TRF1 were significantly increased in flown *versus* both GC and VC animals (~2-2.5-fold; figure 6A). In addition, TRF2 and RAP1 levels were higher in the *soleus* of F compared to GC mice, while the reverse was observed for TIN2. In the VL muscle, only the levels of TRF1 and TIN2 were increased in flown *versus* GC mice. These changes could reflect alteration of shelterin function<sup>45</sup> in flown mice, which could favor DNA damage and defects in telomere length maintenance during spaceflight. To date, data

dealing with telomere changes after simulated or real microgravity is scarce and almost restricted to different *in vitro* models. For example, it has been reported that simulated microgravity does not alter the telomere length of human bone mesenchymal stem cells,<sup>46</sup> while a significant decrease in telomerase activity has been recorded in rat bone mesenchymal stem cells.<sup>47</sup> After simulated microgravity, the level of telomerase mRNA has been shown to be increased in human cardiovascular progenitor cells.<sup>48</sup> Quiescent human WI-38 fibroblasts flown on the STS-93 space shuttle mission (2 days) exhibited an increase in the gene expression of telomerase.<sup>49</sup> The only known study, publicized but still not published, that measured telomere length *in vivo* after a long-term space flight is that of Prof. S Bailey reporting in an n-of-1 trial from the NASA twin study of Scott and Mark Kelly, that the length of human telomeres appear lengthened after the flight. Of course such data have to be considered cautiously because the trend is counter-intuitive, measured in only one individual, and the lengthening of telomeres has not been shown in humans. Hence, data on telomere responses to microgravity appear possibly different depending on the species or the tissue considered. To date, it has been reported that space radiation and microgravity (either simulated or real) are factors that particularly cause DNA damage, which could involve oxidative stress.<sup>50-52</sup> The imbalance of telomere length in Bion-M1 flown mice would favor DNA damage and possibly defects of DNA repair. Although this was not possible here due to lack of samples, measurements of telomere length should be envisioned in future studies to definitely conclude on that point.

**Protein balance** (Figure 5A-F and Table S-4). The downregulation of ribosomal proteins (Nop56, Rplp0), t-RNA ligases (Sars, Rars, Dars, Kars, Eprs), and translation initiation (Eif4a2) and elongation (Eef1a2, Eef1b, Tsfm) factors that we found in flown animals are in line with a reduction of protein synthesis in both the *soleus* and EDL muscles. In parallel, the changes elicited in F *versus* both GC and VC mice were consistent with a decrease in protein catabolic processes in the *soleus* muscle, due to decreased expression of ubiquitin conjugating enzyme E2 (Ube2o), ubiquitin ligase E3 (Nedd4), and several proteasome subunits (Psmc2, Psmc4, Psmc1, Psmc2), while only the abundance of the calpain-1 catalytic subunit (Capn1) was increased. When examining the proteins that were significantly regulated in F *versus* GC or VC animals, we observed the downregulation of additional components of the ubiquitin proteasome system in *Soleus* and EDL muscles (Psmc5, Psmc6, Psmc8, Hctd1, Trim 72, Uba3, Uba5, Ubxn6, Usp5, and Usp47). The same observation was made for components of the COP9 signalosome subunits (Cops5, Cops8) and for proteases of the ClpP complex (Clpp, Clpx). COP9 signalosome is a regulator of the ubiquitin proteasome system,<sup>53</sup> which is probably involved in muscle cachexia.<sup>54</sup> The protein complex or some of its subunits like Cops5 are also involved in many other biological responses, including cell proliferation, cell cycle regulation, signal transduction of notably NF- $\kappa$ B, and transcriptional coactivation.<sup>53</sup> Downregulation of Cops5 and Cops8 during long-term space flight could then adversely affect protein stability, but also muscle developmental and cellular



processes in a more global manner. ClpP proteases are located in the mitochondrial matrix where they play a key role in the mitochondrial unfolded protein response, thus ensuring mitochondrial protein homeostasis in response to stress.<sup>55</sup> ClpP deficiency in C2C12 muscle cells has been shown to result in a series of mitochondrial alterations, including reduced respiration, altered morphology, blunted unfolded protein response, increased generation of reactive oxygen species, inhibition of translation, and impairment of cell proliferation.<sup>56</sup> Downregulation of ClpP proteases in flown Bion-M1 mice may thus particularly compromise muscle mitochondrial function.

Thus, proteomics data established the molecular bases of protein turnover that characterizes skeletal muscles during prolonged space flight. Regulation of all protein synthesis-related factors supports the already reported decrease in protein synthesis during long-term space travels.<sup>16</sup> On the contrary, the regulation of proteolytic-related factors in Bion-M1 mice suggests that the increase in proteolysis and expression levels of proteolytic-related factors that have been reported during short-term space flight<sup>19</sup> are not evident in the long-term. This is also supported by the transcriptomic data collected in Bion-M1 mice.<sup>22</sup>

**Carbohydrate and lipid metabolism** (Figure 5A-F and Table S-4). In space-flown mice, *soleus* proteins involved in glycogen/glucose metabolism were increased (Aldoa, Eno1 and Eno2, Fbp2, Gpd1 and Gpd2, Gpi, Pdk4, Pkg1, Phka1, Phkb, Phkg1, Pgm1, Pkm, Pygm, Stbd1, Tkt, Tpi1). Conversely, proteins associated with fatty acid synthesis, transport and oxidation were decreased (Acaa1a, Acad10, Acadm and Acads, Acasb, Acadvl, Acat1, Acox1, Acsf2, Acsf3, Acsl1, Cpt1b, Ech1, Eci1, Fabp3, Hadh, Hsd17b4, Lpl, and Mlycd). Hence, proteomics data support the metabolic shift from slow-oxidative towards fast-glycolytic muscle fibers that is observed in *soleus* upon weightlessness,<sup>2, 4, 7, 9-12</sup> and which may occur prior to muscle atrophy.<sup>57</sup> Because the generation of ATP by glycolytic processes is limited and favours acidification, this metabolic shift likely affects *soleus* muscle performance in a sustainable manner during prolonged space flight. In EDL, we observed downregulation of two proteins involved in lipid metabolism (Ces1d and Osbpl1a), thus suggesting that the type II fiber phenotype is also favoured in a mixed fiber skeletal muscle despite being already predominantly glycolytic.

**Mitochondrial function** (Figure 5A-F and Table S-4). Consistently with the above-mentioned hypothesis for impaired function of mitochondria, the abundance of proteins in the mitochondrial respiratory chain (Atp5b, Etfa, Coq6, Cox5a, Cox5b, Etfdh, Ndufa10, Nudfa9, Ndufa10, Nudfb9, Ndufb10, Ndufs1, Ndufs2, Ndufs3, Ndufv1, Ndufv2, Samm50) and of other mitochondrial proteins (Mfn1, Mtfp1) were reduced in the *soleus* of flown animals. Additionally, the level of creatine kinase S-type (Ckmt2) was downregulated as well in the *soleus* muscle of flown mice. These data may reflect a reduction in ATP production, and ultimately in the performance of this antigravity muscle during prolonged space flight. Whether this is related to a decrease in the number of mitochondria during microgravity remains to be elucidated.

**Antioxidant proteins** (Figure 5A-F and Table S-4). There was an increase in *soleus* muscle antioxidant proteins (Gpx3, Gstm1 and Gstm2, Sod1) in space-flown mice, while a few others (Cat, Gsr) were decreased. Similar regulations (Sod1 and Cat activities) have been reported after hindlimb unloading in the rat.<sup>58</sup> These data suggest that disruption of the antioxidant machinery likely contributes to oxidative stress not only in disused atrophied muscles,<sup>59</sup> but also in prolonged weightlessness. Adverse consequences of *soleus* muscle oxidative stress during space flight may be linked to its ability to depress protein synthesis and activate proteases,<sup>60</sup> thus favouring protein unfolding and degradation, as well as structural alterations and contractile dysfunction.

**Structural proteins** (Figure 5A-F and Table S-4). The abundance of many structural proteins, either cytoskeletal or belonging to the extracellular matrix, were found to change in F *versus* both GC and VC mice (see Tables S1 and S4 for a complete list). This extends previous findings in skeletal muscles of flown mice, which reported few changes in structural proteins like actin and alpha-actinin 1,<sup>61</sup> or titin and nebulin.<sup>62</sup> Although no conclusive data is presented at this stage, the importance of structural alterations is such that they could impair intracellular trafficking, signalling pathways, and contractile efficiency in the *soleus* muscle of flown Bion-M1 mice, and to a lesser extent in their EDL. Amongst structural proteins, Csrp3, also termed MLP, plays a central role in muscle differentiation and maintenance by modulating expression of myogenin and MyoD1, and by regulating the correct formation of autophagosomes.<sup>63</sup> In space-flown mice, Csrp3 was upregulated in EDL and downregulated in *Soleus*. Similar regulations have been found at the mRNA level in *soleus* of rats after 10 days of immobilization.<sup>64</sup> Hence, Csrp3 regulations at the protein level would support induction of myogenesis in EDL but could favour the more marked atrophy of *soleus* during prolonged space flight. However, transcriptomic data reveal contrasting data as increased expression of MyoD and myogenin were found in *soleus* but not in EDL of flown mice.<sup>22</sup>

**Other regulated proteins** (Figure 5A-F and Table S-4). A set of other regulations was observed in flown mice. For example, the upregulation of centromere protein V (Cenpv) and histones H1.0 (H1f0), H1.2 (Hist1h1c) and H2B type 1-P (Hist1h2bp) could be linked to chromatin reorganization in the *soleus* of Flight mice. We also observed up- (Casq1, Pvalb, Slc25a5) or downregulation (Atp2a2, Camk2g) of proteins that play a role in calcium metabolism or signalling in the *Soleus* and EDL. These changes may be linked to the metabolic and fiber type switch already discussed above, and to reduced contractility in microgravity. Few *soleus* apoptosis-related proteins were also found to be either up- (Mdm4) or downregulated (Htra2, Pdcd6ip) in F compared to control mice, which may indicate a role of apoptosis as an additional event contributing to wasting of this muscle during long-term space flight, as hypothesized already for disuse-induced muscle atrophy.<sup>65</sup> Interestingly, the abundance of regulatory-associated protein of mTOR (Rptor) was reduced in *soleus* of F mice. In response to growth factors like IGF-1, the PI3-kinase/Akt/mTOR signaling pathway is known to regulate muscle mass, notably because

Akt/mTOR stimulates protein synthesis.<sup>66</sup> Rptor, a main accessory protein associated with mTOR in the mTORC1 complex, regulates mTOR signaling.<sup>67</sup> In addition, Rptor depletion in mice muscle has been shown to inhibit mTORC1 activity and trigger a severe myopathy,<sup>68</sup> characterized by structural and functional alterations compromising skeletal muscle excitation-contraction coupling.<sup>69</sup> Hence, lowered levels of muscle Rptor in F mice could be sufficient to block activation of p70<sup>S6K</sup>, reduce protein synthesis and evoke dysfunctions of muscle dynamics during space flight. In addition, muscle Rptor KO mice have also been reported to be insulin resistant.<sup>70</sup> Downregulation of Rptor during space flight could then also constitute a mechanism favoring muscle insulin resistance and diabetogenic effects of space flight.<sup>71, 72</sup> ELKS/Rab6-interacting/CAST family member 1 (Erc1), also termed ELKS, is a regulatory subunit of the IKK complex, which is central to NFκB activation.<sup>73, 74</sup> Because NFκB mediates the catabolic effects of inflammatory cytokines like TNFα in different models of muscle-wasting through the ubiquitin proteasome system,<sup>75, 76</sup> the upregulation of Erc1 in the EDL of F mice likely constitute an additional mechanism directed toward EDL atrophy during prolonged space flight. Of the remaining changes elicited at Og, regulation of immune-related proteins (Ig kappa, upregulated in EDL and VL muscles; Tollip, downregulated in *Soleus*) would reflect some possible link between the response to microgravity and inflammation process. Such an hypothesis is supported by few regulations seen at the transcriptomic level in Bion-M1 mice.<sup>22</sup> The Na<sup>+</sup>/K<sup>+</sup> pump is crucial for skeletal muscle excitability and function, and it is downregulated during immobilisation.<sup>77</sup> The non-catalytic component of the Na<sup>+</sup>/K<sup>+</sup> pump (atp1a1) being downregulated in the *soleus* of F mice, this may reflect overall lowered consumption of ATP and diminished performance of the muscle during space flight.

### ***Skeletal muscle proteome changes due to recovery in Bion-M1 mice***

Because the return of the satellite on Earth, its landing and opening, and the procedures of sacrifice and collection of the samples required more than 10 hours (see the Materials and Methods section), the effects of the flight we observe may be impacted by the short recovery phase. Nevertheless, it is important to keep in mind that the mice in the recovery group have undergone the same conditions. Very minor changes were observed when comparing the three control groups (only 43 proteins differentially expressed; see Table S-1), which argue for minimal effects of housing conditions on Earth and the age of animals. To determine the overall effects of the 8-day recovery period post-flight in EDL, we nevertheless used a stringent criterion on statistical p-values (see above) to filter out any protein exhibiting possible abundance changes between the three control groups and we retained only 54 proteins with p-values<0.1 regarding spaceflight effects. Thus, the effects of the Recovery period considered only the fraction of the EDL proteome that is not affected by housing conditions on Earth and the age of animals. For these 54 proteins, plotting the changes due to spaceflight as a function to the changes due to recovery revealed a global negative linear correlation relationship ( $r^2=0.43$ ). This

highlights a general trend in which most of the spaceflight-induced proteome changes were reverted after 8 days of recovery on Earth, whether they were due to weightlessness *per se*, confinement at 0g or the combination of both (Figure 6B and Table S-5). The restoration of protein abundance to initial levels is highly correlated ( $r=-0.84$ ,  $p\text{-value}=3.5\times 10^{-9}$ ) for the 30 proteins previously found to be significantly regulated due to spaceflight relative to control conditions (VC and GC). The association remained significantly correlated ( $r=-0.66$ ,  $p\text{-value}=3.3\times 10^{-8}$ ) when all 54 proteins were included. More precisely, the abundance of subunits of the T-complex protein 1 (Cct3, Cct4, Cct8, Tcp1) and of TERT, TRF1 and POT1 were fully restored upon recovery, which may indicate normalization of protein folding and telomere function. The abundance of proteins regulating protein balance were mostly (Cops5, Eef1a2, Eif4a2, Rars, Rplp0, Serpinh1, Sars) or partially (Rbm3, Nop56 and Tarsl2) restored. This incomplete restoration of protein metabolism in the Recovery mice may be due to the fact that muscle mass was not completely recovered after 8 days post-flight (Figure 2A). The same may apply to humans as at least 2 months postflight are required for a full recovery of muscle mass and strength, despite inflight exercise countermeasures.<sup>78, 79</sup> With respect to lipid metabolism proteins, Ospbl1a and Acadsb were restored but the lipase Ces1D and hydrolase Hint2 were not affected by the recovery period. Ces1D has been reported to be important for basal lipolysis in adipose tissue.<sup>80</sup> Interestingly, the pharmacologic restraint of basal lipolysis by inhibition of Ces1D has been reported to elicit therapeutic benefits in mouse models of obesity-diabetes against multiple features of the metabolic syndrome, including blockage of adipocyte basal lipolysis, prevention of liver steatosis and enhancement of liver insulin sensitivity.<sup>81</sup> Thus, it is tempting to propose that the maintenance of Ces1d at low levels during the recovery period in Bion-M1 mice may help enhance muscle insulin sensitivity and/or reflect more global repression of this protein in the adipose tissue and liver, which could temper the diabetogenic effects of microgravity. Hint2 is a member of the superfamily of the histidine triad AMP-lysine hydrolase proteins, which stimulates mitochondrial lipid metabolism, respiration and glucose homeostasis, and accelerates calcium pumping into mitochondria in the liver.<sup>82</sup> It is also required for an optimal steroidogenic response in H295R cells, which could favour maintenance of mitochondrial potential.<sup>83</sup> Although there is no literature data on muscle Hint2, its levels were still elevated in EDL of mice postflight and could then be related to the need to favour mitochondrial function for the correct recovery of this muscle.

Spaceflight-induced increased levels of proteins involved in mitochondrial biogenesis and function (Stoml2),<sup>84</sup> mitochondrial fission (Mff),<sup>85</sup> catabolism of branched chain amino acids (Bckdha),<sup>86</sup> inactivation of pyruvate dehydrogenase complex thus minimizing carbohydrate oxidation (Pdk4),<sup>87</sup> and of Slc16a3, a lactate transporter primarily responsible for lactate extrusion out of muscle,<sup>88, 89</sup> tended to be incompletely restored after the recovery period. Mitochondrial function, fuel partitioning, and efflux of lactate (supposedly overproduced due to enhanced glycolysis during spaceflight) are

therefore processes that may need more than 8 days for a full restoration. Because branched chain amino acids stimulate protein synthesis in normal but not atrophied rodent muscles,<sup>90</sup> the still elevated levels of Bckdha might not be involved in restoration of protein synthesis in Recovery mice.

We finally observed that spaceflight-induced upregulation of cysteine and glycine-rich protein 3 (Csrp3) and ELKS/Rab6-interacting/CAST family member 1 (Erc1), and downregulation of carnosine N-methyltransferase (Carnmt1) were further aggravated after 8 days of recovery. For Csrp3, this may reflect the need for sustaining myogenesis (see above) more than one week after a spaceflight. For Erc1, this could indicate that the NF- $\kappa$ B signalling pathway (see above) is still activated after 8 days of recovery, which would slow down muscle recovery. Carnmt1 is an N-methyltransferase mediating the formation of anserine from carnosine.<sup>91</sup> Although the physiological role of these two dipeptides is not understood, they both seem to exert pH neutralization of muscle lactate, antiglycemic, antiglycation, and antioxidant and anti-fatigue effects. The maintenance of low abundance levels of Carnmt1 after 8 days of recovery would then mean that anserine and carnosine do not have similar properties in regard to these processes, carnosine being thus more important for recovery.

***Concluding remarks: a step further towards efficient countermeasures against adverse effects of space flight on skeletal muscles?***

Only one of the three mice who flew for 91 days aboard the International Space Station in a mouse drawer system (MDS) project was still alive after return to Earth.<sup>11, 27</sup> Although it cannot be ruled out that spaceflight *per se* was not the triggering cause for death, drastic mitigation of the adverse effects of long duration space flight is required, with skeletal muscle maintenance being one of the priority targets. In few recent papers, omics and in particular mass spectrometry-based proteomics offer very promising approaches for gaining novel insights into prolonged space flight-induced animal physiological changes.<sup>12, 18, 92</sup> However, apart from a muscle proteomics analysis performed in only one mouse exposed to weightlessness for 91 days,<sup>27</sup> large-scale data are to date restricted to a single study reporting transcriptomics changes elicited by a 30-day space-flight in mice.<sup>22</sup> To fill this gap, we performed in-depth analysis of the proteome of three different muscles of five mice flown for one month within the Bion-M1 project. It should be noted here that except for additional measurements at the telomere level, the very small amount of samples that were available did not enable us to validate proteomics-driven hypotheses. This very important point should be considered in future studies of weightlessness effects on muscle physiology. At this stage, our proteomics findings are in line with previous data showing that antigravity muscles are more affected by space flight than other skeletal muscles, and that their response is dominated by a fiber type and metabolic switch toward glycolytic type II fibers. The regulations highlighted by proteomics data would support that decreased muscle size and performance are likely the result of a series of complementary processes during space flight, especially in *soleus* (Figure 7). Importantly, most of our current findings support previous global measurements or isolated molecular regulation assessments performed in actual as well as simulated microgravity conditions (see Table S-7). Despite various discrepancies that could reflect species- or strain-specific responses to disuse and/or that could be due to the fact that some previous data have been generated from individuals who underwent exercise or nutritional countermeasure programs or who exhibited a lack of energy consumption, this underlines the complementarity and great interest both models have for deciphering disuse-induced muscle wasting and associated metabolic alterations.

Thus, prolonged weightlessness and confinement at 0g triggered changes that suggest alteration of muscle protein balance, mitochondrial function, metabolism, structure, contractility, and telomere function. Although it is obvious for weightlessness, it is also probably near impossible to circumvent confinement in spatial environments. In addition to exercise programs, new inflight countermeasures should then consider simultaneous mitigation of decreased protein synthesis, oxidative stress, energetics and structural defects, while favoring muscle insulin sensitivity, myogenesis and telomere maintenance. As observed in the EDL of Bion-M1 mice, proteomics-derived findings could reflect that

several processes, including protein synthesis, myogenesis, mitochondrial function, and possibly muscle insulin sensitivity, are not fully restored after 8 days of recovery. Postflight countermeasures should then focus on accelerating the normalization of these alterations upon return to Earth.

## Supporting Information

**Supplementary Figure S-1. Overview of *vastus lateralis* regulated proteins in response to spaceflight in Bion-M1 mice**

**Figure S-2. Annotated MS/MS spectra for all single peptide-based protein identifications in the EDL, *soleus*, and *vastus lateralis* muscles of Bion M1 mice**

**Table S-1. List of analysed proteins in the EDL, *soleus*, and *vastus lateralis* muscles of Bion M1 mice, their annotations and statistical analysis**

**Table S-2. Significantly enriched GO terms from differential muscle proteins in flown Bion-M1 mice (Flight group; F) compared to the Ground Control (GC) and Vivarium Control (VC) animals**

**Table S-3. Significantly enriched GO terms from down- and up-regulated muscle proteins in flown Bion-M1 mice (Flight group) compared to both the Ground Control and Vivarium Control animals**

**Table S-4. Significantly down- and up-regulated muscle proteins in flown Bion-M1 mice (Flight group) compared to the Ground Control (GC) and Vivarium Control (VC) groups**

**Table S-5. Overview of recovery-induced restoration of protein abundances in Bion-M1 mice**

**Table S-6. List of single peptide-based protein identifications in the EDL, *soleus*, and *vastus lateralis* muscles of Bion M1 mice and their annotations**

**Table S-7. Comparison of the effects of actual and simulated microgravity on skeletal muscles**



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**Competing interests**

The authors declare no competing financial interests.

## Figure legends

### Figure 1. Experimental design of the Bion-M1 mice experiment

From the Bion-M1 Space Mission, we obtained the muscles from 24 mice that were divided into 5 experimental groups according to gravity and housing conditions. See main text for further details. \*Only EDL samples were available for the Recovery and Recovery Control groups. Drawings by F. Bertile.

### Figure 2. Skeletal muscle mass and fiber type composition in Bion-M1 mice

Skeletal muscle masses are given as the means  $\pm$  sem (A). Fraction of total myosin heavy chains (MyH) deduced from proteomics data is given as the means  $\pm$  sem of MaxLFQ values for fiber type I (MyH7), IIX (MyH1) and IIB (MyH4) in the soleus (B), extensor digitorum longus (C) and vastus lateralis (C) muscles. For the vastus lateralis muscle, relative mRNA levels of fiber type IIA (MyH2), IIX (MyH1), and IIB (MyH4) are given in arbitrary units as fold changes  $\pm$  sem vs. the Vivarium Control (VC) condition, which was arbitrarily normalized to 1 (D). For bars that do not share the same superscript letter, values were found significantly different (ANOVA and Tukey test p-values <0.05).

### Figure 3. Overview of proteomics data in Bion-M1 mice

The total number of analysed (black bars) and of differentially expressed (white bars) proteins is given for each of the 3 muscles (A). For each muscle, percentages that are given between brackets indicate the fraction of differential proteins relative to the total number of analysed proteins. Venn diagrams show overlapping of proteomics datasets from each of the 3 muscles (B). The number of proteins for which abundance levels were significantly changed (ANOVA and post-hoc Tukey tests p-values <0.05) in the Flight (F) group compared to both or only one of the control groups (Ground Control, GC; Vivarium Control, VC) and in the GC versus the VC group is shown for the three muscles (C).

### Figure 4. Functional annotation analysis of proteomics data in Bion-M1 mice

Considering proteins that were found differential in flown mice (F) compared to Vivarium control (VC) and Ground control (GC) animals, either simultaneously (F vs. VC & F vs. GC) or independently (F vs. VC or F vs. GC), functional annotation analysis revealed significantly enriched GO terms in the *soleus* (panel A), *extensor digitorum longus* (EDL, panel B), and *vastus lateralis* (panel C) muscles of Bion-M1 mice (Table S-2 and S-3). Enriched GO terms were then grouped together into broad pathways and functions, thus highlighting that a high number of proteins involved in tissue remodeling, metabolism, muscle function, and telomere function were regulated during spaceflight.



### Figure 5. Overview of regulated proteins in response to spaceflight in Bion-M1 mice

The regulation is shown for key soleus and *extensor digitorum longus* proteins found differentially expressed during spaceflight in flown (F) versus both Vivarium control (VC) and Ground control (GC) mice (panels A and B), in F versus only VC mice (panels C and D), and in F versus only GC mice (panels E and F). Fold changes observed in F relative to VC and GC animals (either averaged for panels A and B or considered separately for other panels) are expressed as a function to their abundance (LFQ intensity) in the Flight group. Categorization of regulated proteins in broad functional categories is detailed in Table S-4. OXPHOS, oxidative phosphorylation; CK, Creatine kinase.

### Figure 6. Overview of telomere-associated protein levels and restoration of protein abundances in response to recovery in Bion-M1 mice

Representative Western blots are shown for protein levels of telomere associated proteins in the *soleus* and EDL muscles of Bion-M1 mice (A). Significance for up- and down-regulated proteins in flown mice versus VC or GC animals (p-values < 0.05; in red and green, respectively) is indicated by filled (soleus) and open (EDL) symbols. The restoration of protein abundances in response to recovery (B) is shown for *extensor digitorum longus* proteins with ANOVA and Tukey p-values < 0.1 regarding assessment of spaceflight effects (Flight [F] vs. Vivarium Control [VC] and F vs. Ground Control [GC]; see Table S-1). Proteins with ANOVA and Tukey p-values < 0.1 when comparing VC to GC LFQ intensities and then those with Welch Two Sample t-test p-values < 0.1 when comparing GC plus VC LFQ intensities to Recovery Control (RC) LFQ intensities were not considered. Fold changes (log<sub>2</sub>) of LFQ values observed in F mice relative to the mean of those measured in VC, GC, and RC animals are expressed as a function to fold changes (log<sub>2</sub>) in Recovery (R) vs. F mice. Symbols were set according to the possible differential expression of proteins previously observed as a result of weightlessness and/confined conditions (see Figure 5A). The dotted line (y=-x) represents the theoretical anti-correlation curve that would be obtained if recovery-induced restoration of protein abundances to initial levels was perfect. The closer the proteins to this line, the better the restoration of their initial levels. Categorization of regulated proteins in broad functional categories is detailed in Table S-5. OXPHOS, oxidative phosphorylation.

### Figure 7. Overall effects of long duration space flight in mice skeletal muscle

An overview of regulations induced by spaceflight in the muscles of Bion-M1 mice is illustrated, as well as their expected adverse effects on muscle size, performance and metabolism. Plain arrows and indicate regulation of proteins significantly changed in flown mice versus both GC and VC animals.

Dashed arrows indicate regulation of proteins significantly changed in flown mice *versus* only VC or GC animals. Red and orange stars indicate possible consequences of oxidative stress and depressed mTOR signalling, respectively. NSP: newly synthesized proteins; UPS: ubiquitin proteasome system; GNG: gluconeogenesis; FA: fatty acids; ECM: Extracellular matrix. Drawings by F. Bertile.

**Table 1. Real-time PCR primers**

<b>Gene</b>	<b>Forward</b>	<b>Reverse</b>	<b>Amplicon size</b>
rpS9	CGGCCCCGGGAGCTGTTGACG	CTGCTTGCGGACCCTAATGT	247 bp
tubulin	CTGGAACCCACGGTCATC	GTGGCCACGAGCATAGTTATT	115 bp
MyH7 (I)	ATAGCAGGAAAGGGGCTGA	GCCTCTCATCACGCATCTC	132 bp
MyH2 (IIA)	AGGCGGAAAGAAGCTACCAT	GTGGTGATCAGCAGCATTTC	83 bp
MyH1 (IIX)	CATGTCCAAAGCCAACAGTG	CTGAGCCAGCTTCTTCTTGG	106 bp
MyH4 (IIB)	CTGCAGGACTTGGTGGACA	CTTGGCCAGGTTGACATTG	96 bp

For TOC only

