c-Myc overexpression increases ribosome biogenesis and protein synthesis independent of mTORC1 activation in mouse skeletal muscle

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Running title: c-Myc stimulates ribosome biogenesis and protein synthesis
Abstract

**Background:** High-intensity muscle contractions (HiMC) are known to increase c-Myc expression which is known to stimulate ribosome biogenesis and protein synthesis in tumor cells. However, whether the increase in c-Myc stimulates ribosome biogenesis and protein synthesis in skeletal muscles remains unknown.

**Methods:** We investigated the effect of adeno-associated virus (AAV)-mediated c-Myc overexpression, with or without fasting or percutaneous electrical stimulation-induced HiMC, on ribosome biogenesis and protein synthesis in adult mouse skeletal muscles.

**Results:** AAV-mediated overexpression of c-Myc in mouse skeletal muscles for 2 weeks increased the DNA polymerase subunit *POLI* mRNA, 45S-pre-rRNA, total RNA, and muscle protein synthesis without altering mechanistic target of rapamycin complex 1 (mTORC1) signaling under both ad libitum and fasted conditions. RNA-seq analyses revealed that c-Myc overexpression mainly regulated ribosome biogenesis-related biological processes. The protein synthesis response to c-Myc overexpression mirrored the response with HiMC. No additional effect of combining c-Myc overexpression and HiMC was observed.

**Conclusion:** c-Myc overexpression is sufficient to stimulate skeletal muscle ribosome biogenesis and protein synthesis without activation of mTORC1. Therefore, the HiMC-induced increase in c-Myc may contribute to ribosome biogenesis and increased protein synthesis following HiMC.

**Keywords:** exercise, c-Myc, ribosome biogenesis, protein metabolism, RNA-Seq
**Background**

High-intensity muscle contraction (HiMC)-stimulated protein synthesis, such as after resistance exercise, is believed to contribute to increases in skeletal muscle mass over time. Acutely, HiMC stimulates muscle protein synthesis by facilitating translational efficiency while more chronic adaptations include an increase in basal muscle protein synthesis by increasing ribosome availability/translational capacity (1-3). Simultaneously, muscle protein degradation remains unchanged or increases slightly after resistance exercise (4, 5). Therefore, HiMC-induced net positive protein balance is thought to be mainly due to increased muscle protein synthesis rather than decreased muscle protein degradation.

Mechanistic target of rapamycin (mTOR), especially mTOR complex 1 (mTORC1), is a major regulator of protein synthesis by regulating both translational efficiency and capacity (6, 7). However, recent studies have indicated that muscle contraction-induced increase in protein synthesis is not solely dependent on mTORC1 (8-13). The transcription factor c-Myc is also known to stimulate protein synthesis in tumor cells, possibly by stimulating ribosome biogenesis (14, 15). Previous studies reported that HiMC acutely increased c-Myc expression at both mRNA and protein levels (8, 11, 16-18). The increase in c-Myc after HiMC was also observed during treatment with the mTORC1 inhibitor, rapamycin (8, 11). Moreover, an increase in c-Myc protein was observed after chronic resistance training in rats and humans (8, 19, 20). Therefore, resistance exercise-induced increase in c-Myc could contribute to ribosome biogenesis and protein synthesis. In support of this, pharmacological inhibition of c-Myc in C2C12 myotubes inhibited protein synthesis (11). Moreover, a recent study reported that c-Myc overexpression in the skeletal muscle of 1-day-old chick induced muscle hypertrophy (21). However, whether c-Myc upregulation induces ribosome biogenesis and protein synthesis in mature skeletal muscle *in vivo* remains unclear.

In addition to its role in the transcriptional regulation of ribosome biogenesis, c-Myc regulates transcription of several genes linked to cell growth and metabolism in a B lymphoid tumor model (22, 23), indicating that many of the HiMC-induced changes in skeletal muscle gene expression could be mediated by c-Myc upregulation. However, whether this is the case is presently unknown. Thus, we investigated the effect of c-Myc overexpression on gene expression, ribosome biogenesis, and protein synthesis in skeletal muscle in this study. Further, we investigated how c-Myc overexpression interacted with suppression and stimulation of mTORC1 signaling and protein synthesis during fasting and after HiMC.
Methods

Ethical approval

All experimental procedures performed in this study were approved by the Danish Animal Experimental Inspectorate (License # 2017-15-0201-01311) and carried out in accordance with the European Convention for Vertebrate Animals Used for Experiments and Other Scientific Purposes.

Animals

Female C57BL/6J mice (11 weeks old) purchased from Taconic (Lille Skensved, Denmark) were housed under 12 h light/dark cycle, with food and water ad libitum.

Adeno-associated virus vectors

Adeno-associated virus serotype 6 (AAV6) that express mouse c-Myc (NM_001177352) under the control of the CMV promoter (AAV6-c-Myc) or empty control CMV vector (AAV6-CON) were generated, and 1 × 10\(^10\) vector genomes were injected into the right and left gastrocnemius muscles of mice, respectively, under 2% isoflurane anesthesia.

Experiment 1: c-Myc overexpression during fasting

Two weeks after AAV6-c-Myc and AAV6-CON injection, mice were fasted from 8 AM. Muscle samples were obtained after cervical dislocation before, 3 h, and 9 h after food removal. The muscles were frozen rapidly in liquid nitrogen and stored at -80°C until further analyses.

Experiment 2: c-Myc overexpression on HiMC effects

Two weeks after AAV6-c-Myc and AAV6-CON injection, the mice were anesthetized with 2% isoflurane, and the right gastrocnemius muscle was contracted isometrically by fixing the sole of the foot at a 90° angle using percutaneous electrical stimulation (100 Hz, 5 sets of ten 3 s contractions, 7 s rest between contractions, 3 min rest between sets), as described previously (24). The left gastrocnemius muscle served as a control. Muscle samples were obtained after cervical dislocation 3 h after muscle contraction. Tissues were frozen rapidly in liquid nitrogen and stored at -80°C until further analyses.

Western blotting

Powdered frozen muscle samples were homogenized in 10 times the volume of lysis buffer (20 mM Tris-HCl [pH 7.5], 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, and 150 mM NaCl) containing Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). After centrifugation at 10,000 × g for 10 min at 4°C, the supernatant was collected and the protein concentration of each sample was determined using a Protein Assay Rapid Kit Wako II (FUJIFILM Wako, Osaka, Japan). Samples were diluted in lysis buffer to equal protein concentration, mixed with 3× sample buffer, and boiled at 95°C for 5 min. Equal amounts of protein
were separated electrophoretically and subsequently semi-dry transferred to ClearTrans® SP polyvinylidene difluoride (PVDF) membranes (FUJIFILM). The membranes were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and blocked with 1% dry milk for 60 min at 22-24°C. The membranes were then washed and incubated overnight at 4°C with specific primary antibodies. Antibodies against phospho-p70S6K (Thr389, cat#9205), total-p70S6K (cat#2708), phospho-4E-BP1 (Thr36/45, cat#9459), total-4E-BP1 (cat#9452), phospho-AMPK (Thr172, cat#2535), total-AMPK (cat#2532), LC3B (cat#2775), ubiquitin (cat#3933), and c-Myc (cat#5605) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against total-REDD1 (cat#10638-1-AP) was obtained from Proteintech (Rosemont, IL, USA). Antibodies against p62 (cat#PM045) were obtained from MBL (Nagoya, Japan). The membranes were washed again in TBST and incubated for 1 h at room temperature with an anti-rabbit secondary antibody (cat#7074, Cell Signaling Technology) and washed again. The membranes were treated with chemiluminescent reagents, and the protein bands were detected using the ChemiDoc XRS Plus System (Bio-Rad, Hercules, CA, USA). The membranes were stained with Coomassie blue to verify equal loading in all lanes. The band intensities were quantified using the Image Lab (Bio-Rad).

**Muscle protein synthesis**

*In vivo* rate of protein synthesis in the muscle was measured using the SUnSET method (25). Under isoflurane gas anesthesia, the mice were intraperitonally injected with 0.04 μmol puromycin/g body weight in sterile saline. The gastrocnemius muscle was removed 15 min after puromycin administration. Following homogenization as described above, and centrifugation at 2000 × g for 3 min at 4°C, the supernatant was collected and processed for western blotting. Mouse monoclonal anti-puromycin antibody (cat#MABE343, Millipore, Billerica, MA, USA) and anti-mouse IgG2a secondary antibody (#115-035-206, Jackson ImmunoResearch, West Grove, PA, USA) were used to detect puromycin incorporation.

**Real-time PCR**

Total RNA was extracted from 10 mg of powdered frozen muscle tissue using ISOGEN II (Nippon Gene, Tokyo, Japan). The purity and concentration of RNA were measured using a spectrophotometer (Synergy HT; BioTek, Winooski, VT, USA), and 500 ng of total RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio, Kusatsu, Japan). Real-time RT-qPCR was performed using the appropriate primer and TB Green® Premix Ex Taq™ II (Takara Bio) on a Thermal Cycler Dice® Real-Time System II (Takara Bio). Gene expression levels were measured by the absolute quantification method and were normalized to Gapdh. Primers used in the study were obtained from Thermo Fisher Scientific. Primer sequences are provided below: *c-Myc* F-5’-CGGACACACCCCACTCTTGGAA, R-5’-AGGATGTAGGCGGTGGCTT; 45S pre-rRNA F-5’-CTGACACGCTGTCTTTCCC-3’, R- 5’-GTGAGCCGAAATAAGGTGCC-3’; *Pol I* F- 5’-
GGACTGGCAGAAGCATGGA-3’, R- 5’-TCAAGGGAGGTATGGCCTGC-3’; Ubf F- 5’-
AACCCAAAAACTCCCCAGCAA-3’, R- 5’-CCTCCTTCGTAGTGCGATCC-3’; Gapdh F- 5’-
TGCACCACCACTGCTTAG-3’, R- 5’-GGATGCAGGGATGATGTTC-3’

Sequence library preparation, RNA-seq, and sequence data analysis

RNA-seq was performed as previously described (26). Briefly, total RNA was extracted using TRIzol reagent according to the manufacturer’s instructions. The extracted RNA was purified using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and RNA concentration was measured by spectrophotometry (Nanodrop One; Thermo Fisher Scientific). The RNA sequence library was prepared using the QuantSeq 3 mRNA-Seq Library Prep Kit (Lexogen). The prepared library was sequenced using MiniSeq (Illumina, San Diego, CA, USA) on MiniSeq system (Illumina). Quality checked and adapter sequence trimmed read count data were analyzed with integrated differential expression and pathway (iDEP) analysis (27). First, read count data were normalized using EdgeR (minimal counts per million: 0.5). Differential gene expression analyses were performed with limma-voom (c-Myc OE effect; false discovery rate (FDR) cutoff: 0.1, minimal fold change: 2) and DEseq2 (c-Myc OE × HiMC; FDR cutoff: 1, minimal fold change: 2). Pathway enrichment analyses were performed using the generally applicable gene set enrichment for pathway analysis (GAGE; FDR cutoff: 0.20) with the GO Biological Process. Raw data are available from Gene Expression Omnibus, accession#GSE161402.

Statistical analyses

Data were analyzed by two-way ANOVA. Post-hoc analyses were performed using t-tests, with Benjamini and Hochberg FDR correction for multiple comparisons. The level of significance was set at $P < 0.05$.

Data availability: Raw data of RNA-seq analysis are available from Gene Expression Omnibus, accession#GSE161402. All remaining data are contained within the article.
Results

c-Myc overexpression stimulated ribosome biogenesis and muscle protein synthesis

Two weeks of AAV6-c-Myc treatment in gastrocnemius muscle significantly increased the protein level of c-Myc about 3.5-fold (Fig. 1A and Table 1, main effect of AAV6-c-Myc treatment: P < 0.0001). The increased c-Myc expression was sufficient to increase protein synthesis by ~20% and this was not affected by up to 9h of fasting (Fig. 1B, main effect of AAV6-c-Myc treatment: P = 0.0017). Fasting decreased gastrocnemius muscle wet weight (main effect of fasting: P = 0.0037). Overexpression of c-Myc did not change the muscle wet weight within the time-frame studied (data not shown).

Total RNA and mRNA of POL1, UBTF, and 45S pre-rRNA were increased following c-Myc overexpression (Fig. 1C, main effect of AAV6-c-Myc treatment: P = 0.0228, < 0.0001, = 0.0043, and < 0.0001, respectively). These effects were not influenced by up to 9h of fasting, although 45S pre-rRNA decreased with fasting time (main effect of fasting: P = 0.0324). On the other hand, although c-Myc overexpression increased total 4E-BP1 (Fig. 1D and Table 1, main effect of AAV6-c-Myc treatment: P = 0.0408), it did not affect the decrease with fasting in phosphorylation of 4E-BP1 and p70S6K, downstream targets of mTORC1 (Fig. 1D and Table 1). Moreover, upstream regulators of mTORC1, including AMPK and REDD1, were unaffected following c-Myc overexpression, while REDD1 increased with fasting time (Fig. 1D and Table 1, main effect of fasting: P = 0.0062). This indicates that c-Myc does not regulate mTORC1 signaling at rest or during acute fasting.

Apart from c-Myc, RNA-Seq identified 17,871 genes of which 16 genes were significantly altered (Up: 11 genes, Down: 5 genes) following AAV6-c-Myc treatment (Fig. 1E, raw data are available from Gene Expression Omnibus, accession#GSE161402). GO pathway analysis revealed that c-Myc overexpression mainly upregulated genes related to ribosome biogenesis, ncRNA metabolic process, ncRNA processing, rRNA processing, rRNA metabolic process, ribosomal small subunit biogenesis, and ribonucleoprotein complex biogenesis, while it downregulated genes related to chemotaxis (Fig. 1F).

Fasting increased LC3-II (Fig. 1G, main effect of fasting: P = 0.0267), but did not affect LC3-I, p62 or general ubiquitination of proteins with no effect of c-Myc overexpression, indicating that c-Myc does not regulate resting or fasting-induced autophagy and the ubiquitin-proteasome system.

c-Myc overexpression did not potentiate HiMC-induced muscle protein synthesis but increased the number of genes that were changed by HiMC

AAV6-c-Myc treatment and HiMC individually and additively increased the c-Myc protein level (Fig. 2A and Table 2, main effect of AAV6-c-Myc treatment: P < 0.0001, main effect of HiMC: P = 0.0004). AAV6-c-Myc treatment tended to increase basal muscle protein synthesis (Fig. 2B, main effect of AAV6-c-Myc treatment: P = 0.0985) and HiMC increased muscle protein synthesis significantly (Fig. 2D and Table 2, main effect of HiMC: P = 0.037, = 0.001, < 0.0001, respectively).
2B, main effect of HiMC: $P = 0.0036$). However, although no interaction was observed (Fig. 2B, $P = 0.1701$), relative change in protein synthesis with HiMC was smaller in c-Myc overexpression group as compared with control group (Fig. 2B, $P = 0.041$).

Signaling-wise, HiMC increased the phosphorylation of p70S6K and 4E-BP1 (Fig. 2C and Table 2, main effect of HiMC: both $P < 0.0001$), with no significant changes in total proteins. However, although c-Myc overexpression did not affect HiMC-induced 4E-BP1 phosphorylation, it attenuated HiMC-induced p70S6K phosphorylation (Fig. 2C and Table 2, interaction: $P = 0.0091$). Neither HiMC nor c-Myc overexpression affected the phosphorylation of AMPK (Fig. 2C and Table 2). HiMC decreased the expression of the endogenous mTORC1 inhibitor REDD1 independent of c-Myc overexpression (Fig. 2C and Table 2, main effect of HiMC: $P < 0.0001$).

RNA-seq identified 133 and 170 genes that were changed by HiMC in the control and c-Myc overexpressed group, respectively (Fig. 2E and 2G, raw data are available from Gene Expression Omnibus, accession#GSE161402). Therefore, c-Myc overexpression increased the genes that were changed by HiMC. However, no significant differences were observed on any identified genes between the contracted muscles from the control and c-Myc overexpressing groups.
Discussion

c-Myc is known to regulate several genes and stimulate ribosome biogenesis and protein synthesis in tumor cells (14, 15, 22, 23). Therefore, c-Myc upregulation in skeletal muscle following HiMC could be one of the mechanisms promoting HiMC-induced muscle anabolism. Here, we observed, for the first time, that c-Myc overexpression was sufficient to stimulate ribosome biogenesis and protein synthesis in skeletal muscles. Further, the number of genes that were regulated by HiMC was increased by c-Myc overexpression, indicating that c-Myc interacts with other transcriptional regulators activated by HiMC. Thus, our results suggest that c-Myc upregulation in skeletal muscle after HiMC contributes to HiMC-induced muscle anabolism.

As hypothesized, c-Myc overexpression stimulated ribosome biogenesis and protein synthesis in skeletal muscles. Given that HiMC increases c-Myc in skeletal muscles (8, 11), our results suggest that the HiMC-induced increase in c-Myc contributes to an increase in ribosome biogenesis and muscle protein synthesis following HiMC. mTORC1 is one of the major regulators of ribosome biogenesis and muscle protein synthesis (8, 11, 28). In this study, c-Myc overexpression did not alter the phosphorylation of the downstream targets of mTORC1, p70S6K and 4E-BP1 in skeletal muscles. Therefore, our results indicate that c-Myc stimulates ribosome biogenesis and muscle protein synthesis independent of mTORC1. However, although c-Myc overexpression increased muscle protein synthesis, it did not increase muscle mass. In addition, neither autophagy nor ubiquitin-proteasome system was affected by c-Myc overexpression. Further studies, including detailed time-course studies, are required to identify the precise mechanism of c-Myc in the regulation of muscle mass.

While c-Myc overexpression stimulated ribosome biogenesis and muscle protein synthesis, it did not additively increase HiMC-induced muscle protein synthesis. In fact, the change in protein synthesis by HiMC was smaller in the c-Myc overexpression group. This indicated that upregulation of c-Myc genes is part of the mechanism behind HiMC-induced protein synthesis but that c-Myc-mediated ribosome biogenesis/increased translational capacity does not potentiate HiMC-induced muscle protein synthesis. Interestingly, c-Myc overexpression also inhibited HiMC-induced p70S6K phosphorylation, while no change was observed in upstream negative regulators of mTORC1, including AMPK and REDD1. The HiMC-induced increase in skeletal muscle c-Myc protein continues for a relatively long period (> 24 h) and is also observed under basal conditions after chronic HiMC (training) in rats and humans (8, 19, 20) along with an attenuated response of mTORC1/p70S6K signaling and muscle protein synthesis to HiMC following training (29-31). The mechanisms underlying the elevated resting protein synthesis and attenuated anabolic response to HiMC remain unclear. We speculate that c-Myc may mechanistically contribute to the chronically resistance exercise trained skeletal muscle phenotype, given that c-Myc overexpression mimics HiMC in terms of increased basal muscle protein synthesis.
but lowered mTORC1/p70S6K responsiveness to HiMC. Further studies are required to clarify this point.

c-Myc is a potent transcription factor that is estimated to regulate at least 15% of all genes (32). For example, c-Myc is highly expressed in cancer cells and is associated with the Warburg effect via transcriptional regulation of glycolytic enzymes (33). Therefore, we hypothesized that c-Myc overexpression would stimulate a wide range of genes, in particular those related to ribosome biogenesis- and glycolysis-related genes. However, in this study, RNA-Seq revealed that only 16 genes were altered following c-Myc overexpression, although c-Myc overexpression increased the changes in gene expression mediated by HiMC. Interestingly, a recent study identified 19,354 c-Myc-binding sites in chicken primary myoblasts, and only 1,061 in myotubes (21). These results indicate that while c-Myc is still important for ribosome biogenesis and protein synthesis, it is quantitatively not a major transcription factor in fully differentiated muscle cells (adult skeletal muscle fibers in vivo).

Conclusions

We demonstrated that c-Myc overexpression stimulates ribosome biogenesis and protein synthesis without activation of mTORC1 in mouse skeletal muscle. However, it inhibits HiMC-induced phosphorylation of p70S6K and muscle protein synthesis, an effect reminiscent of chronic training conditions. Thus, c-Myc upregulation may both contribute to the HiMC-induced increase in protein synthesis and the decreased mTORC1/protein synthesis response observed with chronic resistance exercise training.
Declarations

Ethical approval
All experimental procedures performed in this study were approved by the Danish Animal Experimental Inspectorate (License # 2017-15-0201-01311).

Consent for publication
Not applicable.

Data availability
Raw data of RNA-seq analysis are available from Gene Expression Omnibus, accession#GSE161402. All remaining data in this article are available from the corresponding author upon reasonable request.

Conflicts of interest
The authors declare that they have no conflicts of interest with the contents of this article.

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Author’s contributions
TEJ and RO conceived and designed the work. TM, SA, JRK, CHO, ZL, KW, TS, KH, YT, and RO performed experiments. TM, SA, and KW analyzed the data. TM, SA, and RO interpreted the results of the experiments. RO drafted the manuscript. JRK, CHO, ZL, KN, TEJ, and RO edited and revised the manuscript. The authors read and approved the final manuscript.
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Table 1. Effect of c-Myc overexpression and fasting on total signaling proteins.

|                  | 0h        | 3h        | 9h        |
|------------------|-----------|-----------|-----------|
|                  | CON       | Myc       | CON       | Myc       | CON       | Myc       |
| c-Myc*           | 1.00 ± 0.55 | 3.90 ± 2.24 | 0.67 ± 0.48 | 3.22 ± 1.69 | 0.78 ± 0.40 | 3.72 ± 2.13 |
| p-p70S6K#        | 1.00 ± 0.39 | 0.97 ± 0.41 | 0.17 ± 0.06 | 0.21 ± 0.09 | 0.17 ± 0.04 | 0.14 ± 0.02 |
| p70S6K           | 1.00 ± 0.20 | 0.92 ± 0.18 | 0.69 ± 0.09 | 0.89 ± 0.20 | 0.81 ± 0.13 | 0.92 ± 0.14 |
| p-4E-BP1#        | 1.00 ± 0.17 | 1.02 ± 0.10 | 0.75 ± 0.07 | 0.75 ± 0.09 | 0.67 ± 0.07 | 0.81 ± 0.10 |
| γ/total 4E-BP1#  | 1.00 ± 0.15 | 0.97 ± 0.16 | 0.76 ± 0.16 | 0.79 ± 0.16 | 0.72 ± 0.09 | 0.66 ± 0.13 |
| 4E-BP1*          | 1.00 ± 0.38 | 1.10 ± 0.34 | 0.86 ± 0.09 | 1.17 ± 0.29 | 0.94 ± 0.34 | 1.00 ± 0.40 |
| REDD1#           | 1.00 ± 0.26 | 1.19 ± 0.19 | 1.04 ± 0.32 | 1.05 ± 0.40 | 1.76 ± 0.53 | 1.81 ± 0.41 |
| p-AMPK           | 1.00 ± 0.12 | 1.02 ± 0.11 | 1.02 ± 0.09 | 1.00 ± 0.13 | 1.05 ± 0.14 | 1.00 ± 0.11 |
| AMPK             | 1.00 ± 0.27 | 0.94 ± 0.27 | 0.94 ± 0.21 | 1.05 ± 0.11 | 0.96 ± 0.26 | 0.89 ± 0.37 |
| LC3-I            | 1.00 ± 0.41 | 1.09 ± 0.35 | 1.10 ± 0.30 | 1.28 ± 0.37 | 0.99 ± 0.28 | 1.00 ± 0.27 |
| LC3-II#          | 1.00 ± 0.38 | 1.30 ± 0.76 | 2.44 ± 1.24 | 2.11 ± 0.64 | 2.59 ± 0.99 | 2.74 ± 1.08 |
| LC3-II/I#        | 1.00 ± 0.44 | 1.08 ± 0.48 | 1.95 ± 0.73 | 1.58 ± 0.50 | 2.34 ± 0.47 | 2.52 ± 0.71 |
| p62              | 1.00 ± 0.17 | 0.94 ± 0.24 | 0.71 ± 0.14 | 0.90 ± 0.24 | 0.81 ± 0.15 | 0.77 ± 0.15 |
| Ubiquitin        | 1.00 ± 0.09 | 1.03 ± 0.10 | 0.93 ± 0.08 | 0.91 ± 0.16 | 0.86 ± 0.17 | 0.92 ± 0.18 |

Data are expressed as means ± SD. *Main effect of Myc overexpression, # main effect of fasting.
Table 2. Effect of c-Myc overexpression and HiMC on total signaling proteins.

| HiMC | CON |  |  |  |  |
|------|-----|---|---|---|---|
|      | −   | + | − | + |   |
| c-Myc*" | 1.00 ± 0.41 | 1.87 ± 0.29 | 2.65 ± 0.61 | 5.17 ± 2.04 |   |
| p70S6K | 1.00 ± 0.19 | 0.99 ± 0.19 | 1.10 ± 0.18 | 1.02 ± 0.22 |   |
| γ/total 4E-BP1# | 1.00 ± 0.28 | 1.75 ± 0.39 | 0.99 ± 0.28 | 1.50 ± 0.90 |   |
| 4E-BP1 | 1.00 ± 0.28 | 0.90 ± 0.24 | 1.06 ± 0.40 | 0.90 ± 0.38 |   |
| REDD1# | 1.00 ± 0.37 | 0.52 ± 0.23 | 0.79 ± 0.29 | 0.47 ± 0.25 |   |
| p-AMPK | 1.00 ± 0.30 | 0.86 ± 0.20 | 1.00 ± 0.21 | 1.08 ± 0.54 |   |
| AMPK | 1.00 ± 0.40 | 0.93 ± 0.33 | 0.91 ± 0.29 | 0.66 ± 0.30 |   |

Data are expressed as means ± SD. *Main effect of Myc overexpression, "main effect of HiMC.
Figure 1. Effect of c-Myc overexpression and fasting. AAV6-c-Myc treatment increased c-Myc protein expression (A, n=6). c-Myc overexpression increased protein synthesis (B, n=6) and ribosome biogenesis (C, n=6), but not mTORC1 signaling (D, n=6). RNA-Seq identified 16 genes that were significantly altered by c-Myc overexpression (E, n=3) and subsequent GO pathway analysis revealed 13 biological processes that were modified by c-Myc overexpression (F). c-Myc overexpression did not affect autophagy and ubiquitin-proteasome system (G, n=6). Data are expressed as means ± standard deviation. *P < 0.05 main effect of c-Myc overexpression.
Figure 2. Effect of c-Myc overexpression and high-intensity muscle contraction (HiMC). AAV6-c-Myc treatment and HiMC additively increased c-Myc protein expression (A, n=8). HiMC increased muscle protein synthesis but relative change in protein synthesis by HiMC was greater in the control group (B, n=8). c-Myc overexpression did not affect HiMC-induced mTORC1 signaling except for p70S6K phosphorylation (C and D, n=8). RNA-seq identified 133 and 170 genes that were changed by HiMC in the control and c-Myc overexpression groups, respectively (E and G, n=3), and subsequent GO pathway analysis revealed 14 and 15 biological processes that were modified by HiMC in the control and c-Myc overexpression groups, respectively (F and H). Data are expressed as means ± standard deviation. Different characters indicate significant differences (p < 0.05), †P < 0.05 main effect of HiMC, †P < 0.05 vs. CON.