Impact of resistance exercise on ribosome biogenesis is acutely regulated by post-exercise recovery strategies

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ABSTRACT

Muscle hypertrophy occurs following increased protein synthesis, which requires activation of the ribosomal complex. Additionally, increased ribosomal capacity, with an RNA (rRNA) synthesis has also been implicated in resistance training-induced skeletal muscle hypertrophy. The time course of ribosome biogenesis following resistance exercise (RE) and the impact exerted by differing recovery strategies remains unknown. In the present study, the activation of transcriptional regulators, the expression levels of pre-rRNA and mature rRNA components were measured through 48 h after a single bout RE. In addition, the effects of either low intensity cycling (active recovery, ACT) or a cold water immersion (CWI) recovery strategy were compared. Nine male subjects performed two bouts of high-load RE randomized to be followed by either 10 min of either ACT or CWI. Muscle biopsies were collected before RE, and at 2, 24 and 48 h after RE. RE increased the phosphorylation of the p38-MNK1-eIF4E axis, an effect only evident with ACT recovery. Downstream, cyclin D1 protein, total eIF4E, Upstream Binding Factor 1 (UBF1) and c-Myc proteins were all increased only after RE with ACT. This corresponded with elevated abundance of the pre-rRNAs (45S, ITS-28S, ITS-5.8S) from 24 h after RE with ACT. In conclusion, coordinated upstream signaling and activation of transcriptional factors stimulated pre-rRNA expression after RE. CWI, as a recovery strategy, markedly blunted these events, suggesting that suppressed ribosome biogenesis may be one factor contributing to the impaired hypertrophic response observed when is used regularly CWI after exercise.
Introduction

Resistance exercise (RE) training results in marked structural and contractile adaptations with skeletal muscle. This complex tissue remodeling is dependent upon increased protein synthesis to enable muscle fiber hypertrophy. The ribosome is the cellular machinery that translates the transcriptional gene information of the messenger RNA (mRNA) into new proteins. In addition to ribosomal activation (translation initiation), recent evidence has implicated ribosome biogenesis in the dynamic regulation of skeletal muscle mass (8, 11, 35), with increased transcription of the ribosomal DNA (rDNA) into ribosomal RNA (rRNA). However, little is known of the activation of mechanisms governing ribosome biogenesis, and the time course of rRNA transcription following a bout of RE.

The first and rate-limiting step of ribosome biogenesis is transcription of the rDNA into the precursor 45S rRNA. This requires various transcriptional factors such as Upstream Binding Factor (UBF) and Transcription Initiation Factor IA (TIF-IA) to bind to the rDNA promoter and induce the synthesis of the pre-rRNA by the dedicated RNA polymerase I (Pol I) (17). After 45S pre-rRNA is transcribed, it is rapidly processed by cleavage into multiple transcripts which form the mature rRNAs, 28S, 18S and 5.8S. Together with 80+ ribosomal proteins, these mature rRNAs eventually form the large and small subunits of the ribosome (9).

The regulation of UBF protein activity is central for rRNA synthesis and cell growth (6, 33, 34). Its phosphorylation status is a key determinant for rDNA transcription (32, 34). UBF is phosphorylated by cyclin-dependent kinase 4 (CDK4) when a complex is formed between CDK4 and the cell cycle regulator, cyclin D1. We have recently proposed that high-load resistance exercise increases cyclin D1 protein expression in skeletal muscle by specifically targeting the translation of cyclin D1 mRNA (8) through activation of the MAP
kinase interacting serine/threonine kinase 1 (MNK1) and eukaryotic translation initiation factor 4E (eIF4E) (28).

In the current study, ribosome biogenesis was examined from 2 h post-exercise, and extending to 24 h and 48 h post-RE in healthy trained subjects. Differing recovery strategies following intense RE are widely used by athletes, including cold water immersion. Whilst acute cold water immersion (CWI) has been shown to provide analgesia, reduce delayed onset muscle soreness (3) and muscle oedema (38), it may also negatively affect long-term muscle adaptation, as determined by training induced muscle hypertrophy and strength gains (24, 37). Thus, it was hypothesized that CWI would suppress the transcriptional signaling and activation of rRNA synthesis during exercise recovery.
Methods

Subjects and Study design

The participant characteristics and study design are described in detail elsewhere (24). Nine recreationally active young males (familiar with resistance training) completed two single-leg resistance training sessions on separate days. These sessions comprised 45° leg press, single leg squats, knee extensions and walking lunges. These exercises involved performing 3–6 sets until failure, with loads of 8, 10 and 12 repetitions maximum (RM). In a randomized, cross-over design study, the participants completed one of two recovery treatments within 5 min after exercise. These treatments involved cold water immersion (CWI) or active recovery (ACT). For the CWI trial, the participants sat up to their waist in cold water (10.0 ± 0.3°C) contained in an inflatable bath (iBody, iCool, Miami, QLD, Australia) for 10 min. In the ACT trial, the participants cycled on a stationary bicycle (Wattbike, Nottingham, United Kingdom) at a low, self-selected intensity (59.5 ± 9.4 W) for 10 min. Muscles biopsies from the Vastus lateralis were collected before, and 2 h, 24 h and 48 h after each session. Muscle samples were snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

The phosphorylation status and abundance of proteins involved in ribosome biogenesis were assessed by Western blot. A total of 25 mg of tissue was homogenized in RIPA lysis buffer (Millipore, Temecua, CA, USA) supplemented with Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA, USA). Samples were centrifuged, and the supernatant was used to measure total protein concentration using a Pierce™ BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein were boiled in 4 × Laemmli buffer, separated by SDS-PAGE, and then transferred to PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a semi-dry Trans-Blot Turbo™
device (Bio-Rad). Following 1 h blocking with 5% bovine serum albumin solution in Tris-buffered saline with 0.1% Tween 20 (TBST), the membranes were incubated with primary antibodies (1:1,000 dilution) overnight at 4°C. The following antibodies were used in this study: total UBF, p-UBF Ser484 and Ser388, cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), total TIF-IA, p-TIF Ser649, GAPDH (Abcam, Cambridge, UK), total Akt, p-Akt Thr308, total PRAS40, p-PRAS40 Thr246, p-p38 MAPK Thr180/Tyr182, total eIF4E, p-eIF4E Ser209, p-MNK1 Thr197/202 (Cell Signaling Technology, Inc., Danvers, MA, USA), and total c-Myc - 9E10 clone (Developmental Studies Hybridoma Bank, Iowa City, IA, USA). The next day, the membranes were washed in TBST before they were incubated with the appropriate anti-rabbit or anti-mouse secondary antibodies linked to horseradish peroxidase (1:5,000) for 1 h at room temperature. The membranes were once again washed in TBST and exposed on a ChemiDoc image device (Bio-Rad) using enhanced chemiluminescence reagent (ECL Select kit, GE Healthcare Ltd., Little Chalfont, United Kingdom). Bands were quantified using ImageJ software (NIH, Bethesda, MD, US).

Total RNA was extracted from ~20 mg of muscle tissue using the AllPrep® DNA/RNA/miRNA Universal Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer’s instructions. Following cDNA synthesis using High-Capacity RNA-to-cDNA™ kit (Life Technologies, Carlsbad, CA, USA), messenger and ribosomal RNA (mRNA and rRNA) were measured by RT-PCR on a LightCycler 480 II (Roche Applied Science, Penzberg, Germany) using SYBR Green I Master Mix (Roche Applied Science). Target mRNAs were: UBF (UBTF), polymerase RNA I polypeptide B (POLR1B), TIF-IA (RRN3) and cyclin D1 (CCND1). Pre-rRNAs were measured using primers designed specifically for pre-rRNA sequences spanning the 5’ end internal transcribed spacer (ITS) region, and a specific primer for the initial external transcribed spacer (ETS) region of 5’ end of 45S rRNA, namely ITS-5.8S and ITS-28S, and 45S. These primers detect only the pre-
rRNAs, that after processing and maturation will form mature ribosomes (9). Primers for the internal region of the mature rRNAs (28S, 18S and 5.8S) were also used. Primers for rRNAs were designed by QIAGEN, using RT² Profiler PCR Arrays (QIAGEN). We have previously described these primers and some target mRNAs applied in this study (8). The sequences for the other targets not described before are shown in the Table 1. The geometric mean of three reference genes (30) was used for normalization. The recently proposed human reference genes (7), chromosome 1 open reading frame 43 (C1orf43), charged multivesicular body protein 2A (CHMP2A) and ER membrane protein complex subunit 7 (EMC7) were identified as the least variable, and were therefore used as reference genes. Standard and melting curves were performed for every target to confirm primer efficiency and single product amplification.

Statistical analysis

RT-PCR data were analyzed using the 2(−ΔΔCT) method. Western blot data are presented as fold change against the protein expression for the pre-exercise sample for each trial. Data are expressed as mean ± standard error of the mean (SEM). Main effects of time and time × trial interactions were assessed using a two-way ANOVA (time post exercise × recovery strategy) with repeated measures (SigmaPlot v12.0, Systat Software, Inc., San Jose, CA, US). Student-Newman-Keuls post-hoc tests were used to determine the significance of pair-wise comparisons of changes with time and differences between the trials. Statistical significance was accepted at p≤0.05.
Results

Effect of resistance exercise and recovery strategies on protein response

p38 MAPK: Phosphorylation of p38 at the dual site Thr180/Tyr182 (p-p38 MAPK Thr180/Tyr182) increased after exercise (time effect p<0.001) and this response tended to differ between the trials (interaction p=0.068) (Figure 1A). In the ACT trial, p-p38 MAPK Thr180/Tyr182 was increased 2 h post-exercise (p<0.001), whilst it was not statistically different at the same time point (p=0.25) in the CWI trial. Compared with the CWI trial, p-p38 MAPK Thr180/Tyr182 was higher 2 h post-exercise in the ACT trial (p=0.003).

MNK1: Phosphorylation of MNK1 at Thr197 (p-MNK1 Thr197) increased after exercise (time effect p<0.001) and was different between the trials (interaction p=0.01) (Figure 1B). p-MNK1 Thr197 was only higher than pre-exercise 2 h after exercise (p<0.001) in the ACT trial. Compared with the CWI trial, it was higher 2 h after exercise in the ACT trial (p<0.001).

eIF4E: Phosphorylation of eIF4E at Ser209 (p-eIF4E Ser209) followed a similar pattern to its upstream kinase, MNK1. p-eIF4E Ser209 increased after exercise (time effect p=0.021) and this response tended to differ between the trials (interaction p=0.074) (Figure 1C). p-eIF4E Ser209 was higher than pre-exercise 2 h after exercise (p=0.001) in the ACT trial. It was also higher at this time after the ACT trial compared with the CWI trial (p=0.009). Total eIF4E protein expression also increased after exercise (time effect p=0.043) (Figure 1D). It was higher than pre-exercise 24 h post-exercise (p=0.028), and tended to remain higher 48 h post-exercise (p=0.090) only in the ACT trial (Figure 1D).

Cyclin D1: The change in cyclin D1 total protein expression was different between the trials (interaction p<0.001) (Figure 1E). It was higher than pre-exercise 2 h (p=0.002), 24 h (p<0.001) and 48 h (p<0.001) after exercise in the ACT trial. It was also higher at all three
time points after the ACT trial compared with the CWI trial: 2 h (p=0.012), 24 h (p<0.001) and 48 h (p<0.001).

Akt: The change in phosphorylation of Thr308 on Akt was different between the trials (interaction p=0.047) (Figure 2A). Compared with the CWI trial, it was higher 48 h (p=0.005) after exercise in the ACT trial.

PRAS40: Phosphorylation of Thr246 on PRAS40 (p-PRAS40 Thr246) changed after exercise (time effect p=0.002) (Figure 2B). It was lower than pre-exercise 2 h after exercise in the ACT trial (p=0.005) and the CWI trial (p=0.005). It was higher than pre-exercise 48 h after exercise in the ACT trial (p=0.043). Compared with the CWI trial, p-PRAS40 Thr246 was higher 48 h (p=0.011) after exercise in the ACT trial.

UBF: UBF1 total protein expression increased after exercise (time effect p=0.028), and this response was different between the trials (interaction p=0.041) (Figure 3A). It was higher than pre-exercise 24 h (p=0.004) and 48 h (p=0.040) after exercise in the ACT trial. Compared with the CWI trial, UBF1 total protein was higher 24 h (p=0.022) and 48 h (p=0.015) after exercise in the ACT trial. UBF2 total protein expression did not change significantly after exercise (time effect p=0.63) in either trial (Figure 3B).

UBF phosphorylation at Ser388 (p-UBF Ser388) followed the same pattern as UBF1 total protein (Figure 3C). The change in p-UBF Ser388 was different between the trials (interaction p=0.030). It was higher than pre-exercise 24 h (p=0.018) and 48 h (p=0.034) after exercise in the ACT trial. Compared with the CWI trial, p-UBF Ser388 was higher 24 h (p=0.015) and 48 h (p=0.007) after exercise in the ACT trial. The change in p-UBF Ser484 was also different between the trials (interaction p=0.032) (Figure 3D). It was higher than pre-exercise 24 h (p=0.006) after exercise in the ACT trial. Compared with the CWI trial, p-UBF Ser484 was also higher 24 h (p=0.006) after exercise in the ACT trial.
TIF-IA: Phosphorylation of Ser649 on TIF-IA increased after exercise (time effect p=0.006) (Figure 3E). It was higher than pre-exercise 2 h after exercise in both the ACT (p=0.046) and CWI (p=0.002) trials.

c-Myc: The change in c-Myc total protein expression was different between the trials (interaction p<0.001) (Figure 3F). It was higher than pre-exercise 24 h (p<0.001) and 48 h (p<0.001) after exercise in the ACT trial. Compared with the CWI trial, c-Myc total protein was higher 24 h (p<0.001) and 48 h (p<0.001) after exercise in the ACT trial.

**Effect of resistance exercise and recovery strategies on rRNA and mRNA expression**

*rRNA:* 45S pre-rRNA (5'ETS) was increased after 24 h in the ACT trial (p=0.018), and tended to remain elevated after 48 h (p=0.089) (Figure 4A). 45S pre-rRNA did not change significantly in the CWI trial. 45S pre-rRNA was higher 24 h (p=0.035) after exercise, and tended to remain higher 48 h (p=0.065) after exercise in the ACT trial compared with the CWI trial. ITS-28S rRNA was different between the trials (interaction p=0.010) (Figure 4B). It was higher than pre-exercise 24 h (p=0.003) and 48 h (p=0.003) after exercise in the ACT trial. Compared with the CWI trial, it was higher 24 h (p=0.007) and 48 h (p=0.016) after exercise in the ACT trial. The change in ITS-5.8S rRNA was also different between the trials (interaction p=0.032) (Figure 4C). It was higher than pre-exercise 24 h (p=0.007) and 48 h (p=0.004) after exercise in the ACT trial. Compared with the CWI trial, it was higher 24 h (p=0.050) and 48 h (p=0.005) after exercise in the ACT trial. The mature 28S rRNA did not change significantly after either trial (time effect p=0.73) (Figure 4D). Further, mature 18S and 5.8S followed the same response and were unchanged at all time points (data not shown).
Cyclin D1 mRNA: There were no significance changes over time or differences between trials (time effect p=0.41, interaction p=0.80) for cyclin D1 mRNA (Figure 1F).

UBF mRNA: UBF mRNA increased after exercise (time effect p<0.001), and this response tended to differ between the trials (interaction p=0.080) (Figure 5A). It was higher than pre-exercise 48 h after exercise (p=0.006) in the ACT trial, whereas it was lower than pre-exercise 24 h after exercise (p=0.037) in the CWI trial. Compared with the CWI trial, UBF mRNA was higher 24 h (p=0.040) and 48 h (p=0.007) after exercise in the ACT trial.

TIF-IA mRNA: TIF-IA mRNA increased after exercise (time effect p<0.001) (Figure 5B). It was higher than pre-exercise 24 h after exercise in the ACT trial (p=0.004) and CWI trial (p=0.026), and remained above pre-exercise 48 h after exercise in the ACT trial (p=0.048).

PolR1B mRNA: PolR1B mRNA also increased after exercise (time effect p<0.001) (Figure 5C). It was higher than pre-exercise 24 h after exercise in both the ACT (p=0.001) and CWI (p=0.033) trials.

NIP7 mRNA: NIP7 mRNA increased after exercise (time effect p<0.001) (Figure 5D). It was also higher than pre-exercise 24 h after exercise in both the ACT trial (p<0.001) and CWI trial (p=0.015), with no significant differences between trials.
Discussion

Ribosome biogenesis has recently been implicated in muscle hypertrophy induced by RE (8). In the current study, a single bout of high-load RE activated multiple cellular signaling events that enable the activation of transcriptional factors involved in the recruitment of Pol I, the initiation of ribosome biogenesis, and increased pre-rRNA expression. The analysis performed also identified the marked effect that post-exercise recovery strategies exert on the mechanisms of ribosome biogenesis. The majority of the measured signaling and transcriptional responses required for ribosome biogenesis were markedly suppressed in response to CWI immediately after the exercise bout. Thus, it is tempting to speculate that if post-exercise ribosome biogenesis is regularly suppressed in response to CWI, then this could partly explain the smaller gains in both muscle mass and strength that accompany long term use of CWI during resistance training (24).

Ribosome biogenesis is subject to complex regulation through multiple pathways (13). Of the cellular signals for rRNA transcription, UBF is a central point of convergence. UBF possesses two splice variants, UBF1 and UBF2 (21). UBF2 is more abundant in quiescent cells. Although both UBF1 and UBF2 are responsive to a growth stimulus, UBF1 expression increases to a greater extent than UBF2 (10, 12). Functionally, UBF1 is the de facto rDNA promoter transcription factor, whereas UBF2 has a lower stimulatory effect on rDNA transcription (12). Consistent with these functions, this study demonstrated that UBF1 protein expression increased during exercise recovery, with no change observed for UBF2.

The activation of UBF is regulated by the cell cycle regulator Cyclin D1, which in turn is translationally regulated by MNK1-eIF4E signaling (15, 28). In the current study, RE strongly activated the MNK1-eIF4E-cyclin D1 axis. Phosphorylation of MNK1 Thr197 and its target eIF4E Ser209 were increased early after exercise (2 h), returning to basal levels of phosphorylation by 24 h post-exercise. Consistent with this, p38, a major MNK1 kinase (14),
was strongly phosphorylated 2 h after RE. This response was accompanied by increased expression of cyclin D1 protein (but not its mRNA) at the same time point, with the increased protein levels of cyclin D1 persisting for the duration of the post-exercise recovery period (48 h). Because cyclin D1 has a short half-life (1), the sustained protein abundance cannot be solely due to eIF4E phosphorylation. The results of this study show that total eIF4E protein was also increased at later time points (24 and 48 h) of recovery. Previously, overexpression of eIF4E protein has been shown to increase cyclin D1 protein expression (25). Thus, we speculate that the higher levels of cyclin D1 measured after 24 h and 48 h may be explained by the increased eIF4E protein abundance.

Cyclin D1 forms a dimeric active complex with CDK4, a kinase that phosphorylates and activates UBF (33). Phosphorylation of UBF is critical for rDNA transcription (22, 34), specifically at Ser388 and Ser484 (2, 33). These UBF residues were highly phosphorylated at later time points of the recovery period following RE. UBF and TIF-IA, along with a few other transcriptional factors of the preinitiation complex, recruit Pol I and promote rRNA synthesis (26). TIF-IA serves as a bridge between the Pol I and the preinitiation complex formed by UBF and SL1 (26, 36). In the present study, TIF-IA phosphorylation followed a pattern opposite to that of UBF, as has previously been described (8). Following RE, there is a rapid phosphorylation of TIF-IA at Ser649, as a result of the activation of the ERK pathway, which is the upstream kinase of TIF-1A (8, 39).

Some evidence suggests that Akt is also an important regulator of ribosome biogenesis (4, 19). This led us to measure Akt phosphorylation at the phosphosite most related to its kinase activity (Thr308) (16, 31), and phosphorylation of its downstream target, PRAS40 at Thr246 (29). Akt phosphorylation was unchanged up to 24 h after exercise. PRAS40 phosphorylation was lower than pre-exercise 2 h after exercise, returning to pre-
exercise levels by 24 h. These results suggest that ribosome biogenesis may occur independently of Akt activity following RE—at least up to 24 h after exercise.

The greater expression of UBF, c-Myc and cyclin D1, in addition to UBF phosphorylation status, was matched by increased levels of rDNA transcription. All primer sequences used to perform real-time PCR for pre-rRNA (which span different portions of the 45S rRNA, namely, 45S, ITS-5.8S and ITS-28S) yielded similar results: 45S pre-rRNA was markedly increased 24 h after exercise, and remained elevated 48 h after exercise. These data are similar to those observed previously (18, 27). Additionally, mRNA expression of Pol I factors (UBF, TIF-IA and PolR1B) and the rRNA processing factor NIP7 were increased following RE. 45S rRNA undergoes cleavage and processing to form the mature ribosomal rRNA transcripts (5.8S, 18S and 28S). However, there were no changes in the abundance of the mature rRNA transcript, thus a repeated exercise stimuli may be necessary to enable increased mature cellular rRNA (8). Collectively, these data suggest that RE promotes rDNA transcription, the expression of the Pol I regulon factors and factors associated with rRNA processing.

Repeated CWI following RE has been recently found to attenuate muscle hypertrophy and strength gains to chronic resistance training (24). In the current study, CWI after a single bout of RE substantially reduced the effect of RE on the phosphorylation levels of p38, MNK1, eIF4E and UBF. CWI also markedly attenuated the expression of eIF4, cyclin D1, UBF, and c-Myc protein (shown diagrammatically in Figure 6). These data suggest that CWI has a major negative effect on the capacity to promote ribosome biogenesis after RE. It is not known how CWI exerts its negative effect on anabolic pathways; however it should involve a modulation of a rapid mechanism triggered by exercise since CWI is applied for a relative short period. This may involve the effects of RE on stress responses such as the modulation of muscle temperature, blood flow, oxidative stress, osmotic stress and/or mechanical stress,
which may be critical for proper response and adaptation (23). Thus we hypothesize that CWI may impact on the acute stress response, and this is supported by p38 MAPK data, which is a classical stress-activated kinase (5, 20).

Our data demonstrate that resistance exercise leads to sustained activity of signaling pathways involved in ribosome biogenesis that persists for the 2 days of analysis. Importantly, we provide new insights into how these specific signaling pathways may contribute to mRNA translation of specific targets in skeletal muscle after exercise. These findings have important implications for future studies, and for understanding the mechanisms underlying muscle hypertrophy. Resistance exercise appears to modulate these signaling pathways in a bi-modal fashion. Initially, shortly after RE, there is robust activation of proteins (kinases, and transcriptional and translational factors) that induce gene expression and mRNA translation of specific proteins upstream of the ribosome biogenesis machinery. During the later stages of post-exercise recovery, ribosome biogenesis is initiated, and the muscle protein synthesis capacity increases, supporting muscle growth. Another novel finding from this study is that CWI interferes with p38-MNK1-eIF4E axis and UBF and c-Myc activation responses, which resulted in a blunted increase in pre-rRNA synthesis promoted by RE. In the long term, this interference may contribute to smaller cumulative gains in muscle mass and strength in response to chronic resistance training.
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Conflict of Interest

The authors declare no conflict of interest.
REFERENCES

1. Alao JP. The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Mol Cancer* 6: 24, 2007.

2. Ayrault O, Andrique L, Fauvin D, Eymin B, Gazzeri S and Seiè P. Human tumor suppressor p14ARF negatively regulates rRNA transcription and inhibits UBF1 transcription factor phosphorylation. *Oncogene* 25: 7577-7586, 2006.

3. Bleakley C, McDonough S, Gardner E, Baxter GD, Hopkins JT and Davison GW. Cold-water immersion (cryotherapy) for preventing and treating muscle soreness after exercise. *Cochrane Database Syst Rev* 2: CD008262, 2012.

4. Chan JC, Hannan KM, Riddell K, Ng PY, Peck A, Lee RS, Hung S, Astle MV, Bywater M, Wall M, Poortinga G, Jastrzebski K, Sheppard KE, Hemmings BA, Hall MN, Johnstone RW, McArthur GA, Hannan RD and Pearson RB. AKT promotes rRNA synthesis and cooperates with c-MYC to stimulate ribosome biogenesis in cancer. *Sci Signal* 4: ra56, 2011.

5. Coulthard LR, White DE, Jones DL, McDermott MF and Burchill SA. p38(MAPK): stress responses from molecular mechanisms to therapeutics. *Trends Mol Med* 15: 369-379, 2009.

6. Drakas R, Tu X and Baserga R. Control of cell size through phosphorylation of upstream binding factor 1 by nuclear phosphatidylinositol 3-kinase. *Proc Natl Acad Sci U S A* 101: 9272-9276, 2004.

7. Eisenberg E and Levanon EY. Human housekeeping genes, revisited. *Trends Genet* 29: 569-574, 2013.

8. Figueiredo VC, Caldow MK, Massie V, Markworth JF, Cameron-Smith D and Blazevich AJ. Ribosome biogenesis adaptation in resistance training-induced human skeletal muscle hypertrophy. *Am J Physiol Endocrinol Metab* ajpendo.00050.2015, 2015.
9. Henras AK, Plisson-Chastang C, O'Donohue MF, Chakraborty A and Gleizes PE. An overview of pre-ribosomal RNA processing in eukaryotes. *Wiley Interdiscip Rev RNA* 6: 225-242, 2015.

10. Hisatake K, Nishimura T, Maeda Y, Hanada K, Song CZ and Muramatsu M. Cloning and structural analysis of cDNA and the gene for mouse transcription factor UBF. *Nucleic Acids Res* 19: 4631-4637, 1991.

11. Kirby TJ, Lee JD, England JH, Chaillou T, Esser KA and McCarthy JJ. Blunted hypertrophic response in aged skeletal muscle is associated with decreased ribosome biogenesis. *J Appl Physiol (1985)* jap.00296.2015, 2015.

12. Kuhn A, Voit R, Stefanovsky V, Evers R, Bianchi M and Grummt I. Functional differences between the two splice variants of the nucleolar transcription factor UBF: the second HMG box determines specificity of DNA binding and transcriptional activity. *EMBO J* 13: 416-424, 1994.

13. Kusnadi EP, Hannan KM, Hicks RJ, Hannan RD, Pearson RB and Kang J. Regulation of rDNA transcription in response to growth factors, nutrients and energy. *Gene* 556: 27-34, 2015.

14. Landon AL, Muniandy PA, Shetty AC, Lehrmann E, Volpon L, Houng S, Zhang Y, Dai B, Peroutka R, Mazan-Mamczarz K, Steinhardt J, Mahurkar A, Becker KG, Borden KL and Gartenhaus RB. MNKs act as a regulatory switch for eIF4E1 and eIF4E3 driven mRNA translation in DLBCL. *Nat Commun* 5: 5413, 2014.

15. Mamane Y, Petroulakis E, Rong L, Yoshida K, Ler LW and Sonenberg N. elf4E--from translation to transformation. *Oncogene* 23: 3172-3179, 2004.

16. Moore SF, Hunter RW and Hers I. mTORC2 protein complex-mediated Akt (Protein Kinase B) Serine 473 Phosphorylation is not required for Akt1 activity in human platelets [corrected. *J Biol Chem* 286: 24553-24560, 2011.
17. Moss T, Langlois F, Gagnon-Kugler T and Stefanovsky V. A housekeeper with power of attorney: the rRNA genes in ribosome biogenesis. *Cell Mol Life Sci* 64: 29-49, 2007.

18. Nader GA, von Walden F, Liu C, Lindvall J, Gutmann L, Pistilli EE and Gordon PM. Resistance exercise training modulates acute gene expression during human skeletal muscle hypertrophy. *J Appl Physiol (1985)* 116: 693-702, 2014.

19. Nguyen le XT and Mitchell BS. Akt activation enhances ribosomal RNA synthesis through casein kinase II and TIF-IA. *Proc Natl Acad Sci U S A* 110: 20681-20686, 2013.

20. Obata T, Brown GE and Yaffe MB. MAP kinase pathways activated by stress: the p38 MAPK pathway. *Crit Care Med* 28: N67-77, 2000.

21. O'Mahony DJ and Rothblum LI. Identification of two forms of the RNA polymerase I transcription factor UBF. *Proc Natl Acad Sci U S A* 88: 3180-3184, 1991.

22. O'Mahony DJ, Xie WQ, Smith SD, Singer HA and Rothblum LI. Differential phosphorylation and localization of the transcription factor UBF in vivo in response to serum deprivation. In vitro dephosphorylation of UBF reduces its transactivation properties. *J Biol Chem* 267: 35-38, 1992.

23. Peake JM, Markworth JF, Nosaka K, Raastad T, Wadley GD and Coffey VG. Modulating exercise-induced hormesis: does less equal more? *J Appl Physiol (1985)* jap.01055.2014, 2015.

24. Roberts LA, Raastad T, Markworth JF, Figueiredo VC, Egner IM, Shield A, Cameron-Smith D, Coombes JS and Peake JM. Post-exercise cold water immersion attenuates acute anabolic signalling and long-term adaptations in muscle to strength training. *J Physiol* 593: 4285-4301, 2015.

25. Rosenwald IB, Lazaris-Karatzas A, Sonenberg N and Schmidt EV. Elevated levels of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E. *Mol Cell Biol* 13: 7358-7363, 1993.
26. **Russell J and Zomerdijk JC.** RNA-polymerase-I-directed rDNA transcription, life and works. *Trends Biochem Sci* 30: 87-96, 2005.

27. **Stec MJ, Mayhew DL and Bamman MM.** The effects of age and resistance loading on skeletal muscle ribosome biogenesis. *J Appl Physiol (1985)* jap.00489.2015, 2015.

28. **Topisirovic I, Ruiz-Gutierrez M and Borden KL.** Phosphorylation of the eukaryotic translation initiation factor eIF4E contributes to its transformation and mRNA transport activities. *Cancer Res* 64: 8639-8642, 2004.

29. **Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ and Kim DH.** Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat Cell Biol* 9: 316-323, 2007.

30. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F.** Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: RESEARCH0034, 2002.

31. **Vincent EE, Elder DJ, Thomas EC, Phillips L, Morgan C, Pawade J, Sohail M, May MT, Hetzel MR and Tavare JM.** Akt phosphorylation on Thr308 but not on Ser473 correlates with Akt protein kinase activity in human non-small cell lung cancer. *Br J Cancer* 104: 1755-1761, 2011.

32. **Voit R and Grummt I.** Phosphorylation of UBF at serine 388 is required for interaction with RNA polymerase I and activation of rDNA transcription. *Proc Natl Acad Sci U S A* 98: 13631-13636, 2001.

33. **Voit R, Hoffmann M and Grummt I.** Phosphorylation by G1-specific cdk-cyclin complexes activates the nucleolar transcription factor UBF. *EMBO J* 18: 1891-1899, 1999.
34. **Voit R, Kuhn A, Sander EE and Grummt I.** Activation of mammalian ribosomal gene transcription requires phosphorylation of the nucleolar transcription factor UBF. *Nucleic Acids Res* 23: 2593-2599, 1995.

35. **von Walden F, Casagrande V, Ostlund Farrants AK and Nader GA.** Mechanical loading induces the expression of a Pol I regulon at the onset of skeletal muscle hypertrophy. *Am J Physiol Cell Physiol* 302: C1523-30, 2012.

36. **White RJ.** RNA polymerases I and III, growth control and cancer. *Nat Rev Mol Cell Biol* 6: 69-78, 2005.

37. **Yamane M, Ohnishi N and Matsumoto T.** Does Regular Post-exercise Cold Application Attenuate Trained Muscle Adaptation? *Int J Sports Med* 36: 647-653, 2015.

38. **Yanagisawa O, Niitsu M, Takahashi H, Goto K and Itai Y.** Evaluations of cooling exercised muscle with MR imaging and 31P MR spectroscopy. *Med Sci Sports Exerc* 35: 1517-1523, 2003.

39. **Zhao J, Yuan X, Frodin M and Grummt I.** ERK-dependent phosphorylation of the transcription initiation factor TIF-IA is required for RNA polymerase I transcription and cell growth. *Mol Cell* 11: 405-413, 2003.
| Target      | Primer Sequence   |
|-------------|-------------------|
| EMC7, Forward | GGGCTGGACAGACCTTTCTAATG |
| EMC7, Reverse  | CTCCATTTCGGCTCTGTCAG |
| CHMP2A, Forward | CGCTATGTCGCAGAGTTTGT |
| CHMP2A, Reverse  | GGGGGCAACTTCAGCTGCTG |
| C1orf43, Forward | CTATGGGACAGGGCTTTGG |
| C1orf43, Reverse  | TTTGGCTGCTGACTGGGTAT |
| NIP7, Forward    | CCCGGGTGTACTATGTGAGTGAGAA |
| NIP7, Reverse    | TTGTGGGGTTTAGTAATTTCCA |
**Figure legends:**

**Fig. 1.** Effect of Resistance exercise on the p38-MNK1-eIF4E axis.

Phosphorylation levels of p38 (A), MNK1 (B), and eIF4E (C), and total levels of eIF4E (D), and total cyclin D1 protein (E) and mRNA levels (F). Representative western blot figures (G). Western blot data were normalized to GAPDH, with the exception of eIF4E Ser209, which was normalized to its respective total protein. Cyclin D1 mRNA expression was normalized to the geometric mean of 3 reference genes. Values are mean ± SEM. Symbol legends: *, different from PRE within the same trial (p<0.05), #, different between trials within the same time-point (p<0.05). Main effects and interactions are presented in the text.

**Fig. 2.** Effect of resistance exercise on Akt signaling.

Phosphorylation levels of AKT (A) and PRAS40 (B). Western blot data were normalized to their respective total proteins. Values are mean ± SEM. Symbol legends: *, different from PRE within the same trial (p<0.05), #, different between trials within the same time-point (p<0.05). Main effects and interactions are presented in the text.

**Fig. 3.** Effect of resistance exercise on rDNA transcription factors.

Protein levels of UBF1 (A), UBF2 (B), phosphorylation of UBF at Ser388 (C) and Ser484 (D), TIF-IA at Ser649 (E), and protein levels of c-Myc (F). Representative Western blots figures (G). Western blot data were normalized to GAPDH. Values are means ± SEM. Symbol legends: *, different from PRE within the same trial (p<0.05), #, different between trials within the same time-point (p<0.05). Main effects and interaction are presented in the text.

**Fig. 4.** Effect of resistance exercise on rDNA transcription.

Expression of pre-rRNA as detected by primers against: 5’ETS (A), ITS-5.8S (B), and ITS-28S (C), and mature 28S rRNA (D). rRNA expression was normalized by geometric mean of 3 reference genes. Values are means ± SEM. Symbol legends: *, different from PRE within the same trial (p<0.05), #, different between trials within the same time-point (p<0.05). Main effects and interaction are presented in the text.

**Fig. 5.** Effect of resistance exercise on mRNA levels of genes involved in rDNA transcription.

Expression of UBF (A), TIF-IA (B), PolR1B (C), NIP7 (D) mRNAs. mRNA expression was normalized by geometric mean of 3 reference genes. Values are means ± SEM. Symbol legends: *, different from PRE within the same trial (p<0.05), #, different between trials within the same time-point (p<0.05). Main effects and interaction are presented in the text.

**Fig. 6.** Schematic representation of the signaling pathway affecting rRNA synthesis following resistance exercise, and where CWI might interfere with this pathway.